DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

BIOBUTANOL PRODUCTION FROM FRUIT PROCESSING INDUSTRY ORGANIC SOLID WASTES BY DARK FERMENTATION

by
Müge DALAK

February, 2022
İZMİR

BIOBUTANOL PRODUCTION FROM FRUIT PROCESSING INDUSTRY ORGANIC SOLID WASTES BY DARK FERMENTATION

A Thesis Submitted to the
Graduate School of Natural and Applied Sciences of Dokuz Eylül University
In Partial Fulfillment of the Requirements for the Degree of Master of Science
in Environmental Engineering, Environmental Engineering Program

by
Müge DALAK

February, 2022
İZMİR

M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"BIOBUTANOL PRODUCTION FROM FRUIT PROCESSING INDUSTRY ORGANIC SOLID WASTES BY DARK FERMENTATION"** completed by **MÜGE DALAK** under supervision of **PROF. DR. İLGİ KARAPINAR** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

.....

Prof. Dr. İlgi KARAPINAR

Supervisor

.....

Assoc. Prof. Dr. Serpil ÖZMIHÇI

(Jury Member)

.....

Prof. Dr. Hidayet ARGUN

(Jury Member)

.....

Prof. Dr. Okan FISTIKOĞLU

Director

Graduate School of Natural and Applied Sciences

ACKNOWLEDGMENTS

I would like to thank my supervisor Prof. Dr. İlgi KARAPINAR for her guidance, patience, and support. Through this journey, she showed me that nothing is impossible and, there is always a possible solution for every issue if you keep trying.

I would like to thank Assoc. Prof. Dr. Serpil ÖZMIHÇI, Assoc. Prof. Dr. Serkan EKER, and Instructor Cemile YÜCEL for their advices and contributions. Also, special thanks to Gizem DOĞAN, M.Sc for her help during my laboratory work.

This study was financially supported by Dokuz Eylül University, Department of Scientific Research Projects with the project number, 2020.KB.FEN.004. I would like to thank the Department of Scientific Research Projects.

Also, I would like to thank my family for their endless love, support, and patience. Last, I would like to thank Taha KOÇ, who brings joy and hope into my life with his light.

Müge Dalak

BIOBUTANOL PRODUCTION FROM FRUIT PROCESSING INDUSTRY ORGANIC SOLID WASTES BY DARK FERMENTATION

ABSTRACT

The aim of the thesis is to optimize the microwave hydrolysis of peach pulp and to produce solvent (Acetone, Butanol, and Ethanol) by batch dark fermentation.

Optimum microwave hydrolysis conditions that maximized total sugar concentration were determined by using Box-Behnken experimental design method. Independent variables were pH, particle diameter, and substrate concentration, dependent variable was the total sugar concentration. Optimum conditions that maximize the total sugar concentration (24.27 g/L) were pH of 5.5, particle diameter of 246.88 micrometers, and substrate concentration of 39.48 g/L.

Batch cultures of *Clostridium acetobutylicum* and *Clostridium pasteurianum* were used to produce solvents by dark fermentation. The effect initial substrate concentration; nitrogen and phosphorus concentrations; levels of ions like magnesium, manganese, zinc, calcium, and iron; butyric acid, and ethanol concentrations as metabolic shifters to butanol; neutral red addition as an electron carrier on solvent were evaluated. The acetone and ethanol productions were relatively enhanced while butanol production was inhibited.

Hydrogen generation as a side valuable product in ABE fermentation was achieved simultaneously by *C. pasteurianum*. Hydrogen production yield and volumetric hydrogen production rate were enhanced by the nitrogen limited (C/N=250) and phosphorus limited (C/P=43) conditions.

Keywords: Dark fermentation, batch culture, *Clostridium acetobutylicum*, *Clostridium pasteurianum*, microwave hydrolysis, peach pulp waste

MEYVE İŞLEME ENDÜSTRİSİ ORGANİK ATIKLARINDAN KARANLIK FERMANTASYONLA BİYOBÜTANOL ÜRETİMİ

ÖZ

Tezin amacı şeftali posasının mikrodalga hidroliz şartlarını optimize etmek ve karanlık fermantasyonla solvent (Aseton, Bütanol ve Etanol) üretimi yapmaktır.

Toplam şeker konsantrasyonunu maksimize eden optimum mikrodalga hidroliz koşulları Box-Behnken deneysel tasarım metodu kullanılarak belirlenmiştir. Bağımsız değişkenler pH, partikül çapı ve substrat konsantrasyonu, bağımlı değişken ise toplam şeker derişimidir. Toplam şeker konsantrasyonunu (24,27 g/L) maksimize eden optimum koşullar 5,5 pH, 246,88 mikrometre partikül çapı ve 39,48 g/L substrat konsantrasyonudur.

Karanlık fermantasyonla biyobütanol üretimi için *Clostridium acetobutylicum* ve *Clostridium pasteurianum* kültürleri kullanılmıştır. Kesikli şartlarda yapılan deneysel çalışmalarda başlangıç substrat, azot ve fosfor konsantrasyonları ile magnezyum, manganez, çinko, kalsiyum ve demir gibi iyon seviyelerinin, etanol, bütirik asit ile elektron taşıyıcı olarak nötr kırmızı ilavesi gibi şartların solvent üretimi üzerine etkileri incelenmiştir. Besi ortamı bileşimine göre aseton ve etanol üretiminde kısmen artışlar meydana gelmiş olmakla birlikte bütanol üretimi inhibe olmuştur.

C. pasteurianum ile solvent üretiminin yanı sıra yüksek seviyelerde hidrojen gazı üretimi elde edilmiştir. Hidrojen üretim verimi hacimsel hidrojen üretim hızı azot sınırlı (C/N=250) ve fosfor sınırlı (C/P=43) şartlarda daha yüksek seviyelerde elde edilmiştir.

Anahtar kelimeler: Karanlık fermantasyon, kesikli kültür, *Clostridium acetobutylicum*, *Clostridium pasteurianum*, mikrodalga hidrolizi, şeftali posası atığı

CONTENTS

Page

M.Sc THESIS EXAMINATION RESULT FORM ii
ACKNOWLEDGMENTS iii
ABSTRACT iv
ÖZ v
LIST OF FIGURES ix
LIST OF TABLES xiii

CHAPTER 1 - INTRODUCTION 1

1.1 The Problem Statement 1
1.2 BiobutanolProduction 3
1.3 Pretreatment of Biomass 4
1.3.1 Physical Pretreatment 6
1.3.2 Chemical Pretreatment 8
1.3.3 Biological Pretreatment 9
1.4 ABE Fermentation 10
1.4.1 Microorganisms Used in ABE Fermentation 12
1.4.2 Reactor Types Used in ABE Fermentation 13
1.5 Separation of Products 14
1.6 Literature Survey 16
1.7 Objectives and Scopes 24

CHAPTER 2 - MATERIALS AND METHODS 26

2.1 Biomass 26
2.2 Experimental Design for Microwave Hydrolysis 26
2.3 ABE Fermentation 27
2.3.1 Microorganisms 27
2.3.2 Fermentation Medium 28
2.3.2.1 ABE Fermentation with Clostridium acetobutylicum 28

2.3.2.2 ABE Fermentation with Clostridium pasteurianum 28
2.3.3 Dark Fermentation 29
2.4 Analytical Methods 30
2.4.1 Sample Preparation 30
2.4.2 Total Sugar Analysis 30
2.4.3 Sugar, Organic Acid and Solvent Measurement 30
2.4.4 Total Gas Measurement 31
2.4.5 Hydrogen Gas Measurement 31
2.5 Calculations 31
2.5.1 Volume of Hydrogen Gas 31
2.5.2 Hydrogen Gas as mmol (37°C) 32
2.5.3 Cumulative Volume of Hydrogen Gas 32

CHAPTER 3 - RESULTS AND DISCUSSION 33

3.1 Microwave Hydrolysis of Peach Pulp 33
3.1.1 Effect of Independent Variables on Total Sugar Concentration 36
3.1.2 Optimization of Hydrolysis Conditions 40
3.2 Dark Fermentation with *Clostridium acetobutylicum* 41
3.2.1 Effect of $CaCO_3$ Addition 41
3.2.2 Effect of Butyric Acid Addition 47
3.2.3 Effect of Magnesium, Manganese and Zinc 53
3.3 Dark Fermentation with *Clostiridium pasteurianum* 66
3.3.1 Addition of Magnesium, Manganese, Zinc and Neutral Red 66
3.3.2 The Effects of Ion Deficiencies by Simplifying the Media 73
3.3.3 Effect of Butyric Acid Addition 79
3.3.4 Effect of Ethanol Addition 86
3.3.5 Effect of Different Carbon/Nitrogen Ratios (C/N) 92
3.3.6 Effects of Different Initial Substrate Concentrations and C/P 99

CHAPTER 4 - CONCLUSION 107

REFERENCES **111**

APPENDICES **124**

LIST OF FIGURES

Page

Figure 1.1 Lignocellulosic wastes 5
Figure 1.2 Metabolic pathways for biobutanol production 12
Figure 3.1 Interaction of total sugar concentration with particle diameter and pH (S_0=39 g/L) 37
Figure 3.2 Variation of total sugar concentration with substrate concentration and pH (D_p=250 μm) 38
Figure 3.3 Variation of total sugar concentration with substrate concentration and particle diameter (pH=5.50) 39
Figure 3.4 Hydrolysis conditions where maximum total sugar concentration is achieved 40
Figure 3.5 Variation of ethanol production over time with $CaCO_3$ addition 42
Figure 3.6 Variation of butanol production over time with $CaCO_3$ addition 42
Figure 3.7 Consumption of total sugar concentration over time with addition of $CaCO_3$ 43
Figure 3.8 Consumption of glucose concentration over time with addition of $CaCO_3$ 44
Figure 3.9 Consumption of fructose concentration over time with addition of $CaCO_3$ 44
Figure 3.10 Variation of lactic acid over time with addition of $CaCO_3$ 45
Figure 3.11 Variation of acetic acid over time with addition of $CaCO_3$ 46
Figure 3.12 Variation of propionic acid over time with addition of $CaCO_3$ 46
Figure 3.13 Variation of pH with $CaCO_3$ addition 47
Figure 3.14 Variation of acetone production over time with butyric acid addition 48
Figure 3.15 Variation of ethanol production over time with butyric acid addition 48
Figure 3.16 Consumption of total sugar concentration over time with butyric acid addition 49
Figure 3.17 Consumption of glucose concentration over time with butyric acid addition 50

Figure 3.18 Consumption of fructose concentration over time with butyric acid addition 50
Figure 3.19 Variation of acetic acid concentration over time with butyric acid addition 51
Figure 3.20 Variation of butyric acid concentration over time with butyric acid addition 52
Figure 3.21 Variation of pH with butyric acid addition 52
Figure 3.22 Variation of ethanol production with time on the addition of Mg, Mn, Zn 53
Figure 3.23 Variation of total sugar with time in addition of Mg, Mn, Zn 54
Figure 3.24 Variation of glucose with time on the addition of Mg, Mn, Zn 54
Figure 3.25 Variation of fructose with time on the addition of Mg, Mn, Zn 55
Figure 3.26 Variation of acetic acid with time on the addition of Mg, Mn, Zn 56
Figure 3.27 Variation of pH with time on the addition of Mg, Mn, Zn 56
Figure 3.28 Variation of ethanol production with time on the addition of Mg, Mn, Zn 57
Figure 3.29 Variation of acetone production with time on the addition of Mg, Mn, Zn 57
Figure 3.30 Variation of total sugar with time in addition of Mg, Mn, Zn 58
Figure 3.31 Variation of glucose with time on the addition of Mg, Mn, Zn 58
Figure 3.32 Variation of fructose with time on the addition of Mg, Mn, Zn 59
Figure 3.33 Variation of acetic acid with time on the addition of Mg, Mn, Zn 60
Figure 3.34 Variation of pH with time on the addition of Mg, Mn, Zn 60
Figure 3.35 Variation of ethanol production with time on the addition of Mg, Mn, Zn 61
Figure 3.36 Variation of acetone production with time on the addition of Mg, Mn, Zn 62
Figure 3.37 Variation of total sugar with time in addition of Mg, Mn, Zn 63
Figure 3.38 Variation of glucose with time in addition of Mg, Mn, Zn 63
Figure 3.39 Variation of fructose with time in addition of Mg, Mn, Zn 64
Figure 3.40 Variation of acetic acid with time in addition of Mg, Mn, Zn 65
Figure 3.41 Variation of pH with time on the addition of Mg, Mn, Zn 65

Figure 3.42 Variation of acetone production over time with the addition of Mg, Mn, Zn and neutral red 67
Figure 3.43 Variation of acetone production over time with the addition of Mg, Mn, Zn and neutral red 68
Figure 3.44 Consumption of total sugar over time with the addition of Mg, Mn, Zn and neutral red 70
Figure 3.45 Consumption of glucose over time with the addition of Mg, Mn, Zn and neutral red 70
Figure 3.46 Consumption of fructose over time with the addition of Mg, Mn, Zn and neutral red 71
Figure 3.47 Variation of acetic acid concentration over time with the addition of Mg, Mn, Zn and neutral red 71
Figure 3.48 Variation of butyric acid concentration over time with the addition of Mg, Mn, Zn and neutral red 72
Figure 3.49 Variation of pH with the addition of Mg, Mn, Zn and neutral red 72
Figure 3.50 Variation of acetone production over time with ion deficiencies along with medium simplification 74
Figure 3.51 Variation of ethanol production over time with ion deficiencies along with medium simplification 74
Figure 3.52 Variation of hydrogen production over time with ion deficiencies along with medium simplification 75
Figure 3.53 Consumption of total sugar over time with ion deficiencies along with medium simplification 76
Figure 3.54 Consumption of glucose over time with ion deficiencies along with medium simplification 77
Figure 3.55 Consumption of fructose over time with ion deficiencies along with medium simplification 77
Figure 3.56 Variation of acetic acid level over time with ion deficiencies along with medium simplification 78
Figure 3.57 Variation of butyric acid level over time with ion deficiencies along with medium simplification 78

Figure 3.58 Variation of pH over time with ion deficiencies along with medium simplification 79
Figure 3.59 Variation of acetone production over time with butyric acid addition ... 80
Figure 3.60 Variation of ethanol production over time with butyric acid addition ... 81
Figure 3.61 Variation of hydrogen production over time with butyric acid addition 81
Figure 3.62 Consumption of total sugar over time with butyric acid addition 83
Figure 3.63 Consumption of glucose over time with butyric acid addition 83
Figure 3.64 Consumption of fructose over time with butyric acid addition 84
Figure 3.65 Variation of acetic acid level over time with butyric acid addition..... 84
Figure 3.66 Variation of butyric acid level over time with butyric acid addition..... 85
Figure 3.67 Variation of pH over time with butyric acid addition..... 85
Figure 3.68 Variation of acetone production over time with ethanol addition 87
Figure 3.69 Variation of ethanol production over time with ethanol addition..... 87
Figure 3.70 Variation of hydrogen production over time with ethanol addition 88
Figure 3.71 Consumption of total sugar over time with ethanol addition 89
Figure 3.72 Consumption of glucose over time with ethanol addition 90
Figure 3.73 Consumption of fructose over time with ethanol addition 90
Figure 3.74 Variation of acetic acid level over time with ethanol addition 91
Figure 3.75 Variation of butyric acid level over time with ethanol addition 91
Figure 3.76 Variation of pH over time with ethanol addition 92
Figure 3.77 Variation of acetone production over time with different C/N ratios..... 93
Figure 3.78 Variation of ethanol production over time with different C/N ratios 94
Figure 3.79 Variation of hydrogen production over time with different C/N ratios.. 94
Figure 3.80 Consumption of total sugar over time with different C/N ratios 96
Figure 3.81 Consumption of glucose over time with different C/N ratios..... 96
Figure 3.82 Consumption of fructose over time with different C/N ratios 97
Figure 3.83 Variation of acetic acid level over time with different C/N ratios 97
Figure 3.84 Variation of butyric acid level over time with different C/N ratios 98
Figure 3.85 Variation of pH over time with different C/N ratios 98
Figure 3.86 Variation of acetone production over time with different initial substrate concentrations and C/P ratios 100

Figure 3.87 Variation of ethanol production over time with different initial substrate concentrations and C/P ratios 101
Figure 3.88 Variation of hydrogen production over time with different initial substrate concentrations and C/P ratios 101
Figure 3.89 Consumption of total sugar over time with different initial substrate concentrations and C/P ratios 103
Figure 3.90 Consumption of glucose over time with different initial substrate concentrations and C/P ratios 104
Figure 3.91 Consumption of fructose over time with different initial substrate concentrations and C/P ratios 104
Figure 3.92 Variation of acetic acid level over time with different initial substrate concentrations and C/P ratios 105
Figure 3.93 Variation of butyric acid level over time with different initial substrate concentrations and C/P ratios 105
Figure 3.94 Variation of pH over time with different initial substrate concentrations and C/P ratios 106

LIST OF TABLES

Page

Table 1.1 Physico-chemical properties of n-Butanol……4

Table 2.1 Coded version of Box-Behnken experimental design method for 3 factors……..…….27

Table 3.1 The experimental points determined according to the Box-Behnken experimental design method, the actual and predicted total sugar concentration values…….34

Table 3.2 Model coefficients…….. 34

Table 3.3 Analysis of variance……35

Table 3.4 Validation studies and results…….36

Table 3.5 Hydrogen production rates and yields at different C/N ratios.…….....99

Table 3.6 Hydrogen production rates and yields at different C/P ratios……...106

CHAPTER 1
INTRODUCTION

1.1 The Problem Statement

The need for energy sources is increasing every day and strategies to provide new energy sources are always on the agenda of the countries. Fossil fuels are still the most common energy sources despite to their adverse effects and their cost. Unfortunately, some of the countries are in shortage of fossil fuels, the other fossil fuel-rich countries are aware of limitations of the energy in the next decays. Overpopulation contributes to excess energy consumption which brings a global energy crisis. In addition, worldwide climate change caused by global warming or CO_2 emissions initiated environmental concerns. Therefore, economical and sustainable energy sources are urgently needed (Gottumukkala et al., 2017; Pugazhendhi et al., 2019). Biofuels like bioethanol, biobutanol or biodiesel have attracted attention as alternative energy resources in recent years (Vivek et al., 2019).

Among other biofuels, biobutanol has unique properties like similar characteristics to gasoline. Consequently, it could be used as an additive to gasoline or in pure form (Nanda et al., 2017). Moreover, biobutanol has some advantages compared to bioethanol. It has more energy density and less volatility than bioethanol. Since its flashpoint is higher than ethanol, biobutanol is safer compared to bioethanol (Trindade & Santos, 2017).

Biobutanol production is achieved by acetone/ethanol/butanol fermentation (ABE fermentation). Anaerobic fermentation consists of two stages known as acidogenesis and solventogenesis. Acidogenesis involves the organic acid production, solventogenesis is provided the production of the solvents (ABE) with the conversion of organic acids (Gottumukkala et al., 2017). Clostridium species that form spore under anaerobic conditions are widely used in ABE fermentation since they can ferment many different types of substrates (Pugazhendhi et al., 2019). The most important factor that affects biobutanol productivity is product inhibition (Z. Lin et

al., 2017). The other factors that restrict biobutanol production are the inability to provide the required environmental conditions, the formation of by-products and the production cost. In order to make biobutanol a commercial product, many studies are performed to increase the production efficiency like ion supplementation, usage of genetically modified organisms, and addition of end products to the medium as accelerator or inhibitor and cofactors.

Lignocellulosic wastes have drawn attention as substrates for biobutanol production since they are abundant and can reduce production cost. Moreover when raw materials are used as substrates for biobutanol production, ethical problems like food availability and environmental problems with deforestation may be emerged (Sikarwar et al., 2017). Usage of these wastes as feedstock could prevent ethical problems and be a beneficial way for the utilization of wastes. Nevertheless due to the complex structure of biomass, appropriate physical, chemical or biological pretreatment processes are needed to access to simple sugars (Argun et al., 2016; Zhang & Shahbazi, 2011).

In the present study, the first proposal was the determination of the optimum conditions which maximize the efficiency of microwave hydrolysis by using statistical experimental design. Due to its high fruit sugar content, the peach pulp was used as an appropriate feedstock. The second proposal was the comparison of butanol production; with the addition of various compounds at different amounts and ratios to the fermentation medium, and removal of several compounds from the medium. Two types of microorganisms were used in the experiments; *Clostridium acetobutylicum* and *Clostridium pasteurianum*. In one experiment which was performed by using *Clostridium acetobutylicum*, the hydrolyzed peach pulp was used as substrate. However, due to the problems observed in butanol production, the effect of other factors was examined by using glucose as carbon source.

1.2 Biobutanol Production

Biofuels has begun to drawn attraction instead of commercial fuels since they could be considered as green fuels. Among other biofuels, biobutanol has properties almost equal to gasoline like high energy content, low vapor pressure, less volatility, high octane number etc. (Nanda et al., 2017). Therefore, it has lots of advantages, compared to ethanol. First, it could be used in pure form or blend with gasoline in any concentration while ethanol could be blended with gasoline up to 85% (Dürre, 2007). Second, since it has higher flashpoint than ethanol, butanol is safer (Trindade & Santos, 2017). Third, it is less corrosive than ethanol. Fourth, it has higher energy content comparing to ethanol (Dürre, 2007).

Louis Pasteur (1862) concluded that butyl alcohol could be acquired from the anaerobic conversion of lactic acid and calcium lactate (Volesky & Szczesny, 1983). Weizmann (1912) discovered *Clostridium acetobutylicum* could converse grains into acetone and butanol in anaerobic conditions (Volesky & Szczesny, 1983). During World War I, acetone was used for the production of the cordite (Ndaba et al., 2015). After the war, ABE production plants were established in most of the countries, due to the increasing product demand. In 1945, Japan used butanol as an airplane fuel in World War II (T. Ezeji et al., 2010). However, because of the increase in the feedstock cost and developments in petrochemical industry, interest in ABE fermentation process decreased in almost all around the world by 1960s (Lee et al., 2008). In recent years, the process had gained attention again in response to high oil price and environmental issues (Jin et al., 2011). Molasses and corn were previously used for the production of solvents (Pugazhendhi et al., 2019). Currently, biobutanol is produced from the biomass with ABE fermentation by using Clostridium species. Lignocellulosic wastes are abundant resources as feedstock. Moreover, usage of these wastes as substrate in fermentation process could reduce the production cost and decrease the pollution caused by the wastes.

ABE fermentation is not the only process for butanol production. There are several chemical processes for butanol production like Oxo synthesis, Reppe

synthesis and crotonaldehyde hydrogenation. Oxo synthesis is addition of carbon monoxide and hydrogen to a carbon-carbon double bond by using catalysts like Co, Rh or Ru. Subsequently, aldehyde mixtures produced from first step undergo hydrogenation for the butanol production. In Reppe synthesis; propylene, carbon monoxide and water react with the assistance of a catalyst for the butanol production. The process takes place at low temperature and pressure. Crotonaldehyde hydrogenation involves three stages in order of; conversion of ethanol to acetaldehyde by dehydrogenation, aldol condensation of acetaldehyde, butanol production via hydrogenation (Bankar et al., 2013; Lee et al., 2008; Ndaba et al., 2015).

Commercial biobutanol production process involves three stages as pretreatment of feedstock, ABE fermentation and separation of products. There are different methods for every stage. A convenient production process depends on the substrate structure and the feasibility of the selected methods. Table 1.1 presents the physico-chemical properties of n-butanol.

Table 1.1 Physico-chemical properties of n-Butanol (Dürre, 2007; Nanda et al., 2017)

Properties	**n-Butanol**
Caloric value (MJ/L)	29.2
Air-fuel ratio	11.2
Motor octane number	78
Flash point (ºC)	35
Freezing temperature (ºC)	-89.5
Boiling point (ºC)	117.4
Oxygen content (%)	22

1.3 Pretreatment of Biomass

Lignocellulosic wastes are abundant and widely used for biofuel production. Nevertheless, complex structure of these wastes could constitute problems about the usage of simple sugars for further fermentation steps. Hence a convenient pretreatment process is needed before fermentation phase (Argun et al., 2016; Zhang & Shahbazi, 2011). Lignocellulosic biomass comprises three types of polymer

structures; cellulose, hemicellulose and lignin (Y. Zheng et al., 2014). Cellulose comprises of D-glucose subunits, attached by β-1,4 glycosidic bonds. In addition, it is composed of regular crystalline structures and irregular amorphous structures in a plant. Due to its complicated composition, cellulose is resistant to biological and chemical pretreatments. Hemicellulose is a complex carbohydrate compound consisting of structures such as pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) and sugar acids. Unlike cellulose, hemicellulose has unorganized, amorphous and branchy composition which provides low resistance to hydrolysis. Lignin is an amorphous polymer which consists of three dimensional phenylpropane scales. Its structure exhibits resistant nature to biological and chemical processes (Hendriks & Zeeman, 2009; Taherzadeh & Karimi, 2008).

Lignocellulosic wastes could be divided into different groups as they are shown in Figure 1.1 (Zabed et al., 2016). Among these groups, agricultural wastes are rich in essential nutrients for microorganisms which take part in fermentation step. Significant portions of these wastes constitute fruit wastes. Fruit residues like pulp, peels and seeds contain high amounts of sugars which makes them attractive feedstocks for biofuel production. While edible pulp is about 33-35% of a fruit, peel consists 7-24% of it (Bikash & Sibi, 2017; Raganati et al., 2016).

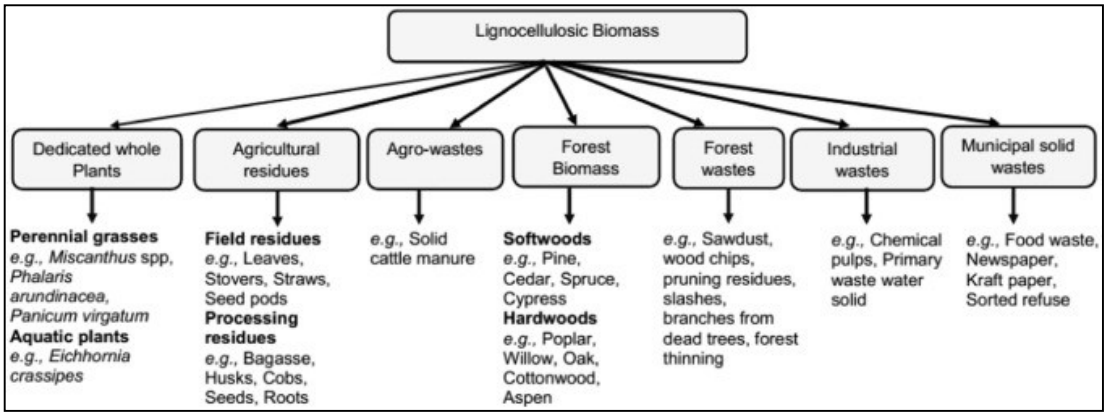


Figure 1.1 Lignocellulosic wastes (Zabed et al., 2016)

The complex structure of lignocellulosic wastes requires pretreatment processes prior to fermentation step (Trindade & Santos, 2017). There are many factors like particle size, crystallinity of cellulose and lignin content affecting the hydrolysis

efficiency of lignocellulosic biomass. An appropriate pretreatment method should have objectives like preventing the loss of sugars formed, decreasing the formation of inhibitory compounds, reducing the production cost of biofuel (Hendriks &Zeeman, 2009; Kumari & Singh, 2018). Fundamentally pretreatment methods are classified in three groups; physical, chemical and biological (Argun et al., 2016).

1.3.1 Physical Pretreatment

Physical pretreatment represents the methods that do not require any chemicals or microorganisms (Y. Zheng et al., 2014). They reduce the particle size and increase the surface area of the materials (Kumari & Singh, 2018). Physical methods that are most commonly used are communition, steam explosion, liquid hot water, extrusion, and irradiation (Argun et al., 2016).

Communition is a size reduction process in biomass material using machines like milling, two-roll milling, hammer rolling etc. It could be used prior to enzymatic hydrolysis or other pretreatment methods (Taherzadeh & Karimi, 2008). Machine selection depends on the moisture content of the biomass (Y. Zheng et al., 2014). However, the power requirement is high, and excessive size reduction could decrease the efficiency of biofuel production (Taherzadeh & Karimi, 2008).

Steam explosion is a physical process by which the biomass is treated with steam and pressure for a few minutes (Hendriks & Zeeman, 2009). Process efficiency depends on particle size, residence time, temperature, pressure and moisture content (Argun et al., 2016). The main advantage of this process is low energy requirement. However, the formation of inhibitory compounds could be occurred (Mabee et al., 2006). It is an effective process for the pretreatment of hardwood materials (Y. Zheng et al., 2014).

Liquid hot water process is a similar process with steam explosion. It requires higher retention times compared to steam explosion (Argun et al., 2016; Y. Zheng et al., 2014). Main advantages of this process are solubilization of the hemicellulose

and obtaining more accessible cellulose and decreased by-product formation (Alvira et al., 2010).

Extrusion is a process where the biomass is compressed within a driving screw. Mechanical shear procures the depolymerization of cellulose, hemicelluloses and lignin (Y. Zheng et al., 2014). Extrusion process requires lower energy than communition. Nevertheless, high pressure could induce by-product formation due to lignin degradation (Kumari & Singh, 2018).

In microwave hydrolysis, biomass is irradiated with microwaves. Molecules in the biomass cell absorb this energy and heat generation is occurred. Eventually, cell wall degrades (Argun et al., 2016). The microwave field and dielectric response are the most important parameters mainly affecting the pretreatment efficiency. This process requires shorter process time and less energy, compared to the traditional heating methods (Y. Zheng et al., 2014). Moreover, uniform dispersion of temperature could be occurred (Singh et al., 2016). Generally it is used with acid or alkaline pretreatment to provide heat assistance (Kumari & Singh, 2018).

Ultrasonication is a physical pretreatment process where biomass is subjected to ultrasound and bubble collapse takes place by cavitation, resulting in high pressure and heat formation (Argun et al., 2016). Efficiency of this process depends on the energy density and activation of cavitation sites (Luo et al., 2014). Furthermore acid or base addition could improve the process efficiency (Kumari & Singh, 2018).

In electron beam irradiation, the biomass passes through electron beams and energy from the electrons is absorbed, which changes several chemical and biological bonds within the lignocellulosic biomass (Singh et al., 2016). Main advantage of the method is that it could take place under the conditions of normal temperature and pressure (Argun et al., 2016).

1.3.2 Chemical Pretreatment

Chemicals are used for the disruption of the biomass structure (Y. Zheng et al., 2014). In principle, these chemicals break down the related chemical bonds and allow enzymatic access to fermentable sugars. Acid, alkaline, organosolv, ammonia fiber explosion, ionic liquids, wet oxidation or any combination of these processes can be used for the biomass pretreatment (Argun et al., 2016).

Acid hydrolysis could be classified as dilute (0.5-3%) and concentrated (> 30%). While dilute acid process requires high temperature and pressure, the concentrated acid hydrolysis could be performed at normal temperature and pressure conditions (Argun et al., 2016; Gashaw & Getachew, 2014). Typically, this process is applied with heat assistance (Gashaw & Getachew, 2014). Toxic by-product formation like 5-hydroxymethyl furfural or excess salt formation could be occurred (Y. Zheng et al., 2014). Moreover, reactors used in this process must be resistant to corrosion, high temperature and pressure (Argun et al., 2016).

Lignin and hemicellulose could be effectively removed in high temperature alkaline pretreatment (Argun et al., 2016). The efficiency of this process depends on lignin content of lignocellulosic biomass (Y. Zheng et al., 2014). The main disadvantages of this process are long retention time, toxic by-product formation (Kumari & Singh, 2018). However, it could be performed under normal temperature condition with H_2O_2. This method is called alkaline peroxide (ALPER) process and prevents toxic by-product formation (Argun et al., 2016; Taherzadeh & Karimi, 2008).

In organosolv pretreatment, biomass mixed with organic solvents like alcohols is treated under high temperature conditions (Kumari & Singh, 2018; Taherzadeh & Karimi, 2008). It is an effective method for the pretreatment of lignocellulosic waste with high lignin content (Taherzadeh & Karimi, 2008). Nevertheless, solvents need to be separated prior to fermentation stage due to their toxic effects on microorganisms (Alvira et al., 2010).

Ammonia fiber explosion process is a steam explosion pretreatment with liquid ammonia assistance (Alvira et al., 2010). Important parameters in this method are ammonia loading, temperature and residence time. This process does not require small particle size and prevention of inhibitory compound formation could be achieved. However, it exhibits low efficiency for materials with high lignin content (Kumari & Singh, 2018).

Ionic liquids composed of salts, which involve large organic cations or small inorganic anions could be employed in ionic liquid pretreatment to dissolve lignin in lignocellulosic biomass. Major factors affecting the pretreatment yield are retention time, temperature, anions and cations used in ionic liquids. It is an environmentally friendly process since the formation of toxic or explosive gases do not occur. Nonetheless, it is a high cost process (Alvira et al., 2010; Kumari & Singh, 2018).

In wet oxidation process, biomass is treated water and air or oxygen catalyst at high temperatures (Gashaw & Getachew, 2014). Adding oxygen at high temperatures reduces the energy requirement by making the process exothermic(Alvira et al., 2010). Important parameters in this process are oxygen pressure, water content, reaction temperature and time (Y. Zheng et al., 2014). It is an effective method for solubilization of hemicelluloses and lignin. In addition, the formation of inhibitory compounds is less compared to steam explosion or liquid hot water pretreatments (Alvira et al., 2010). However, it is a high cost process due to the equipment and catalyst used (Kumari & Singh, 2018).

1.3.3 Biological Pretreatment

Biological pretreatment methods are processes that involve the degradation of the biomass with the help of microorganisms (fungal, microbial consortium) or enzymatic pretreatment (Kumari & Singh, 2018). Main objectives are decreasing the loss of carbohydrates and increasing the removal of lignin (Y. Zheng et al., 2014).

Generally, white-, brown-, soft-rot fungi and basidiomycete fungi are used in fungal pretreatment (Y. Zheng et al., 2014). Especially many of the white-rot fungi species are highly effective for the removal of lignin from the lignocellulosic biomass (Alvira et al., 2010).

In microbial consortium treatment, organisms in natural environment (soil, dung etc.) are used for the degradation of cellulose and hemicellulose (Kumari & Singh, 2018). Sterilization of the biomass is not necessary in most cases (Y. Zheng et al., 2014).

Fermentable sugars within the lignocellulosic biomass could be released with the help of particular enzymes, which could be produced by microorganisms like fungi or bacteria. This process is known as enzymatic pretreatment. While cellulase could degrade cellulose into glucose and cellobiose, xylanase degrade hemicelluloses into xylose, arabinose or mannose (Argun et al., 2016). Enzymatic pretreatment has lower toxicity and process cost, compared to acid and alkaline hydrolysis (Gashaw & Getachew, 2014). However it is slower compared to other physical and chemical pretreatment processes (Argun et al., 2016).

1.4 ABE Fermentation

ABE fermentation is an anaerobic process, which involves the utilization of carbon source by microorganisms into solvents and by-products. The solvents produced in fermentation process are acetone, butanol and ethanol. Produced by-products consist of acids (acetic and butyric) and gases (hydrogen and carbon dioxide) (Ibrahim et al., 2017). Generally, Clostridia species are used for biobutanol production since they can ferment different types of substrates. Moreover, they form spore under anaerobic conditions (Pugazhendhi et al., 2019). *Clostridium acetobutylicum*, *Clostridium saccharobutylicum, Clostridium beijerinckii* and *Clostridium saccharoperbutylacetonicum* are most widely used for biobutanol production. Typical solvent ratio in fermentation by Clostridium species is 3:6:1

(butanol/acetone/ethanol) when the amount of produced solvents is less than 20 g/L (Ibrahim et al., 2017).

Biobutanol production occurs in two different metabolic pathways, which are cold line and hot line. Figure 1.2 presents the metabolic pathways for biobutanol production. When cold line metabolic pathway is followed, ABE fermentation consists of two stages as acidogenesis and solventogenesis (Morone & Pandey, 2014). The acidogenesis takes place in exponential growth phase. In this stage microorganisms produce cells, acids (butyric and acetic), and gases (biohydrogen and carbon dioxide) (García et al., 2011). In growth phase, pH value decreases to around 4.5, due to the acid formation (Grimmler et al., 2011). Subsequently, bioalcohol or solvent production is triggered with the acid accumulation on solventogenesis phase. Therefore, organic acid production in fermentation process is the signal for butanol production. The butyric acid formed in acidogenic phase is transformed into butanol by butyryl phosphatase enzyme. Butyryl phosphate, synthesized from Butyryl CoA, serves as a key molecule in the transition to solvent phase (Y. Wang et al., 2013; Zhao et al., 2005). Unlike cold channel, hot channel pathway avoids reassimilation of organic acids and includes only the direct reducement of acetoacetyl_CoA to butyryl-CoA, which then results in the production of butanol (Tracy, 2012).

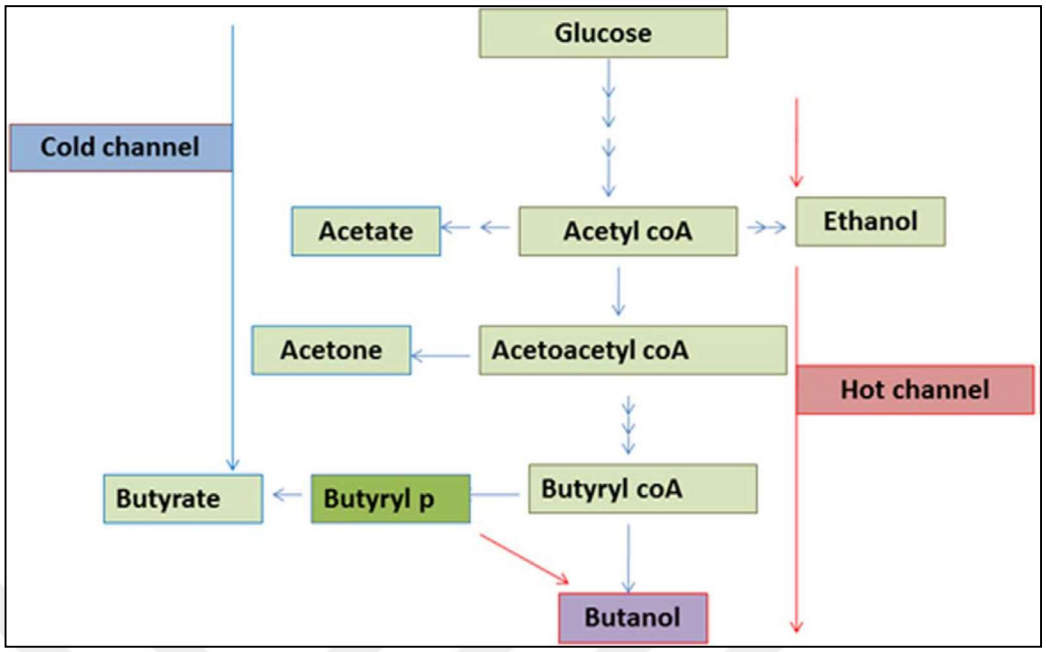


Figure 1.2 Metabolic pathways for biobutanol production (Gottumukkala et al., 2017)

1.4.1 Microorganisms Used in ABE Fermentation

Clostridial strains are most widely used for ABE fermentation under mesophilic conditions since they are capable of fermenting many different types of carbohydrates and forming spore under strict conditions (Harris et al., 2002; Poehlein et al., 2017; Pugazhendhi et al., 2019). Especially species like *C. acetobutylicum*, *C. beijerinckii*, *C. saccharoperbutylacetonicum*, *C. aurantibutyricum,* *C. saccharoacetobutylicum*, *C. pasteurianum*, *C. sporogenes*, *C. cadaveris,* and *C. tetanoorphum* are frequently investigated for their potential to produce biobutanol (Kumar & Gayen, 2011). Among these species, *C. acetobutylicum*, *C. beijerinckii*, *C. aurantibutyricum* are especially known for their capability of producing high amounts of butanol or other neutral solvents (Keis et al., 1995). Also, *C. saccharoperbutylacetonicum* and *C. saccharoacetobutylicum* have exhibited high yields for butanol production (S. Li et al., 2020).

Biobutanol at high concentrations could inhibit the growth and destroy the ability of cells to maintain pH level within the internal structures. Moreover, it may decrease the level of ATP within the cell and prevent sugar uptake (Bowles & Ellefson, 1985).

Studies in mutagenesis, evolutionary and metabolic engineering still continue for the improvement of biobutanol production with ABE fermentation (Xue et al., 2013). In addition, removal or over expression of the several genes which encode metabolic enzymes could procure an increase in butanol tolerance and yield, enhancement of substrate utilization, selectivity of butanol instead of mixed solvents/acids (Gottumukkala et al., 2017; Lee et al., 2008; Y. Li et al., 2019).

Other than *Clostridium* species, genetically modified *E. coli*, *Pseudomonas* species and *S. cerevisiae* could be used for butanol production (Rao et al., 2016). For example, *E. coli* does not naturally produce biobutanol. However, it can gain the ability to produce butanol with metabolic engineering (Y. N. Zheng et al., 2009). Moreover *Lactobacillus* spp. could naturally produce n-butanol in anaerobic fermentation of sugar (Russmayer et al., 2019).

1.4.2 Reactor Types Used in ABE Fermentation

ABE fermentation could be achieved in 3 modes which are batch, fed-batch and continuous (Holt et al., 1988; Kumar & Gayen, 2011). Batch fermentation is mainly used in biobutanol production due to its operational simplicity and low risk for contamination (Birgen et al., 2019). In this process, all of the media composition is fermented in a reactor without any interference. Main drawback of this process is low productivity of butanol (<12g/L) might be observed with an average initial substrate concentration of 60-80 g/L (Ibrahim et al., 2018; Ranjan & Moholkar, 2012). Moreover toxicity of butanol accumulation and required period for the preparation of medium could end in low productivity of butanol (Al-Shorgani et al., 2019).

Fed-batch fermentation is generally applied when substrate inhibition, due to the high concentration, might occur (S. Y. Li et al., 2011; Ranjan & Moholkar, 2012). Initially, substrate in low concentration is added to the fermentation process. After consumption of the carbon source, more substrate is added to the system while keeping the concentration under toxic level (Dolejš et al., 2014; Visioli et al., 2014).

Compared to batch culture, fed-batch system could be resulted in higher butanol concentrations (Ibrahim et al., 2018). However, simultaneous product elimination is necessary for an effective process (Lu et al., 2012).

Continuous fermentation (chemostat) process involves the substrate addition with simultaneous withdrawal of products. It has some advantages like decreasing equipment downtime, inoculation time and solvent toxicity (Ranjan & Moholkar, 2012). Nonetheless, the solvent quality and amount could be fluctuant due to the problems with dilution and harvest (Pugazhendhi et al., 2019). Hence, several methods like two-stage, immobilized culture, cell recycling are applied to chemostat system for the improvements in butanol production (Birgen et al., 2019). Immobilized cells or cell recycling could be used for the avoiding the loss of cells during harvest (Bankar et al., 2013; Gottumukkala et al., 2017). Two-stage fermentation which involves the acidogenesis and solventogenesis separately may also allow enhanced substrate degradation and efficient conversion of acids to solvent (Bankar et al., 2012).

1.5 Separation of Products

Butanol toxicity is the main problem on bacterial cells that inhibits the production and induces low yields. Consequently, an appropriate product separation is needed to overcome this problem (Z. Lin et al., 2017). An efficient separation method must be cost effective, highly selective, harmless to microorganisms, resistant to adverse interactions with broth components (García et al., 2011; Staggs & Nielsen, 2015). Several methods have been suggested like distillation, gas stripping, adsorption, liquid-liquid extraction, perstraction, pervaporation, reverse osmosis, freeze crystallization etc. (Abdehagh et al., 2014; Kujawska et al., 2015).

Distillation is a conventional method that is used for the recovery of products in ABE fermentation (Xue et al., 2017). It is based on the volatility differences of the components. Biobutanol has a higher boiling point (118ºC) than water so energy demand and process cost are usually high, especially if low biobutanol concentration

has been attained in the fermentation medium (T. C. Ezeji et al., 2004; Kamiński et al., 2011).Furthermore, this process exhibits low selectivity for butanol (Kujawska et al., 2015). These drawbacks resulted in a search for more feasible and effective separation methods. Among other researched methods; gas stripping, adsorption and pervaporation are the most common techniques that have been investigated for the butanol separation (Pugazhendhi et al., 2019).

Gas stripping is consisted of usage of gases like H_2, CO_2, N_2 to strip away the butanol from the fermentation broth. In this method, the gas is bubbled in the medium and the solvents are captured in the bubbles. Subsequently, the gas is sent to the condenser and the solvents are condensed for the separation (Abdehagh et al., 2014; T. C. Ezeji et al., 2004). Main advantages of this method are ease of operation, non-fouling nature and non-toxicity to the organisms (Kujawska et al., 2015; Xue et al., 2017). However, excessive foam formation and less butanol selectivity could be occurred (Pugazhendhi et al., 2019).

In adsorption process, butanol is adsorbed into an adsorbent which is in a packed column, followed by the recovery of the solvent with increasing temperature or replacement of the adsorbents (Xue et al., 2017). Activated charcoal, polyvinylpyridine, silicate could be used as adsorbents in butanol separation (Qureshi et al., 2005). Among these materials, silicate is the most widely used adsorbent. It is a type of silicon that has zeolite structure and hydrophobic properties (Vane, 2008).Adsorbents used in this process exhibit high selectivity for butanol and are simple to use (Pugazhendhi et al., 2019). However, this process has several disadvantages like biofouling, requirement of high solvent concentration for efficient recovery. Moreover, low capacity of adsorbent is the main drawback for the industrial usage of this method (Kamiński et al., 2011; Kujawska et al., 2015).

Pervaporation is one of the most effective recovery process where solvents are separated from the fermentation medium with the help of a selecting membrane (T. C. Ezeji et al., 2004). In this method, medium is transported selectively by diffusion through a hydrophobic membrane with the application of vacuum (Kamiński et al.,

2011; Pugazhendhi et al., 2019). Subsequently, permeate (solvents in gas phase) is recovered as a vapor and condensed in cold traps as liquid state (Abdehagh et al., 2014). Polydimethylsiloxane membranes and silicon rubber sheets are generally used for this method. Especially polydimethylsiloxane membranes are the well-studied polymeric membrane due to their high selectivity for butanol and physical stability (Pugazhendhi et al., 2019; Xue et al., 2017).
Main advantages of this process are low energy usage, not affecting organisms, to prohibit the loss of medium contents. Moreover, it requires low process temperature and has a consistent operation/cost ratio (Abdehagh et al., 2014; Qureshi et al., 2005). Nonetheless, high costs to obtain low pressure at condensation side could be required (Abdehagh et al., 2014; Kamiński et al., 2011).

1.6 Literature Survey

Biological production of butanol is provided by acetone/butanol/ethanol (ABE) fermentation which is generally performed by Clostridium sp. Especially, usage of lignocellulosic wastes as substrates for biobutanol production has drawn attention since they are abundant and can reduce the production cost (Sikarwar et al., 2017). Among these wastes, fruit materials like pulp, peels and seeds contain high levels of sugars, proteins and acids (Bikash & Sibi, 2017; Raganati et al., 2016). For instance, peach pulp is an abundant source as waste, especially since it is widely used in juice production. In addition to its high sugar content, it contains ions which could favor the solvent production like potassium, calcium, phosphorus (Argun & Dao, 2017; Raganati et al., 2016). However, there are not enough studies about hydrolysis of peach pulp or production of butanol from it.

An appropriate pretreatment step is needed prior to fermentation step, due to the complex structure of lignocellulosic biomass (Trindade & Santos, 2017). Microwave hydrolysis (MW) is a physical pretreatment method by which biomass is irradiated by microwaves (Argun et al., 2016). The process requires shorter operation time and less energy compared to traditional heating methods since it procures homogeneous energy distribution all over the material (Y. Zheng et al., 2014). Hydrolysis of

cassava pulp and tapioca flour with microwave irradiation was studied. Solutions of 2 materials (50 g/L) were conducted to MW hydrolysis at a temperature range of 140-240ºC with 4 min preheating and 5 min heating times. Highest glucose yields were 28.59% and 58.76% dry matter for cassava pulp at 230ºC and tapioca flour at 240ºC, respectively (Hermiati et al., 2011). Microwave hydrolysis could be applied with alkali or acid pretreatment to procure heat assistance (Kumari & Singh, 2018). Microwave assisted alkali pretreatment (NaOH 1%) was implemented to either sole or combined peel wastes of pineapple, orange or mango. Different power modes or operation times were evaluated. In the first step, hydrolysis yields for 5 min at different power ranges as 100-600 W were compared. Maximum reducing sugar yield was obtained from pineapple waste as 0.61 g/g at 600 W. In other step, yields of pretreatment were examined in different irradiation times as 1-6 min at 450 W and 600 W. Similar to previous step, hydrolysis of pineapple waste resulted in highest reducing sugar yield as 0.744 g/g with 2 min pretreatment at 600 W. It was reported that hydrolysis yields decreased when operation time increased to 6 min for all samples, due to the reducing water content (Tiwari et al., 2017). The effects of varied acid concentrations (1-3%) and hydrolysis time ranges (5-15 min) at 550 W to microwave assisted acid pretreatment of empty fruit bunch of palm oil (EFB) were examined. Optimum condition was 1% acid concentration with 12.5 min irradiation time. Highest reducing sugar yield at optimum conditions was 25.08% per raw EFB or 55.85% per hydrolyzed EFB. Moreover, hydrolyzed EFB at optimum condition was used for ethanol production by *Saccharomyces cerevisiae* and ethanol concentration of 1.26 g/L with 0.64 g/g yield was obtained (Fatriasari et al., 2017).

Microorganisms interact with several ions that affect the metabolic pathway for butanol production. The addition or limitation of these ions could increase or inhibit the enzymatic activity of Clostridium sp. Combined effects of zinc supplementation and magnesium deficiency could lead to enhancement in glucose conversion, reducement in ethanol concentration and early initiation of solvent production, by metabolic regulation (Mukherjee et al., 2019). Mukherjee et al. (2019) studied the individual or combined effects of zinc supplementation and magnesium deficiency on butanol production. *Clostridium acetobutylicum* ATCC824 was used to perform

batch fermentation and 89.6 g/L glucose was added to medium. $ZnSO_4.7H_2O$ and $MgSO_4.7H_2O$ were used as Zn^{2+} and Mg^{2+} sources, respectively. Lowest butanol yield was 13.72 g/L for the magnesium starved condition. However, zinc supplemented-magnesium starved medium gave the highest yield of 19 g/L. Similarly, Wu et al. (2013) concluded that zinc supplementation could enhance butanol productivity and cell growth. *Clostridium acetobutylicum* L7 was used to perform batch experiments, with 70 g/L glucose addition and $ZnSO_4.7H_2O$ was used as Zn^{2+} source. Solvent production was triggered earlier with a time of 40 h when zinc was added to medium. Without zinc supplementation, solventogenesis was initiated at the 64^{th} hour of the fermentation. Consequently, final butanol concentrations were 12.6 and 11.7 g/L for zinc supplementation and without zinc, respectively. Nonetheless, maximum cell density increased 1.2 times, when medium was supplemented with zinc.

Calcium carbonate addition as Ca^{2+} source could improve the butanol production serving as a buffering agent, which could inhibit the excessive acid accumulation in fermentation medium. Effect of $CaCO_3$ and yeast extract supplementation to enzymatic hydrolysate of dilute acid pretreated rice straw was studied. Fermentation was performed by using *Clostridium sporogenes* BE01. Total ABE concentration was 5.32 g/L with a butanol yield of approximately 64.5%. Solely $CaCO_3$ addition has been shown to increase butanol production as 4 times (Gottumukkala et al., 2013). Moreover, $CaCO_3$ supplementation may enhance the growth, glucose utilization capacity and butanol tolerance of Clostridium sp. (Richmond et al., 2011). Richmond et al. (2011) demonstrated the effect of $CaCO_3$ supplementation for two microorganisms which are *Clostridium acetobutylicum* ATCC824 and *Clostridium beijerinckii* 260. Butanol concentration increased from 11.43 to 14.78 and ABE concentration was 46% higher than control group, for *Clostridium acetobutylicum* ATCC824. Similarly, the amount of butanol titer enhanced from 11.38 to 13.89 and an increase in total solvent concentration was obtained as 31%, for *Clostridium beijerinckii* 260. Cell density has increased approximately 1.75 times for two organisms. In addition, fermentation times have decreased 33% and 20%; glucose utilization rates have increased 2 and 1.5 times for *Clostridium acetobutylicum*

ATCC824 and *Clostridium beijerinckii* 260, respectively. Furthermore, they reported that the addition of $CaCO_3$ to fermentation medium increased butanol tolerance of Clostridium sp. more than 40% which might be the reason for enhancement in ABE concentrations. Likewise, Su et al. (2020) concluded that supplementation of salts as Ca^{2+} sources could enhance sugar uptake and solvent production by up-regulating enzymatic activity. Enzymatic hydrolysate of acid pretreated corn straw was investigated for butanol production by *Clostridium acetobutylicum* CICC 8016. $CaCO_3$ and $CaCl_2$ were used as Ca^{2+} sources. Sole addition of $CaCO_3$ to medium provided complete consumption of sugar and butanol concentration was 3 times higher, compared to $CaCl_2$ added and salt-free medium. Authors concluded that especially without salt addition, non-sugary compounds in medium inhibited intracellular Ca^{2+} uptake which resulted in low ABE concentrations. Moreover, additional Ca^{+2} up-regulated the enzymatic activity by increasing protein levels which were involved in glycolysis, redox balance.

Iron limitation could lead to enhancement in butanol production. *Clostridium acetobutylicum* ATCC824 performed batch experiment under iron limited conditions with 66 g/L glucose and $FeSO_4.7H_2O$ was added to medium as Fe^{2+} source. Low amounts of acetic acid were observed while butanol was the major fermentation product. Butanol/acetone ratio was increased from 3.7 to 11.8, by iron limitation. In addition, conversion of glucose to butanol was changed from 20% to 30% (Junelles et al., 1988). Conversely, when cheese whey is used as substrate in batch experiment with *Clostridium acetobutylicum* ATCC824, iron supplementation was found to be necessary for butanol production. Initial sugar concentration was 55 g/L. $FeSO_4.7H_2O$ and $FeCl_3.6H_2O$ were used as Fe^{2+} and Fe^{3+} sources, respectively. Consequently, butanol titers were approximately 7.13 and 4.32 g/L, respectively for $FeSO_4$and $FeCl_3$. Moreover, iron deficiency led to lactic acid production instead of solvents (Durán-Padilla et al., 2014).

Butanol production could be improved by butyric acid supplementation as an initiator to steer the metabolic pathway to biobutanol production (D. S. Lin et al., 2015). Optimum butyric acid and glucose ratio was revealed with *Clostridium*

saccharoperbutylacetonicum N1-4 by Al-Shorgani et al. (2012). With a constant glucose amount of 30 g/L, the effect of different butyric acid concentrations (0-15 g/L) was studied. It was concluded that there was not any linear relationship between butanol production and initial butyric acid concentration. Highest butanol concentration of 17.76 g/L with a productivity of 0.15 g/L.h was obtained when 4 g/L butyric acid was added to the medium. Moreover, temporal slight inhibitory effects were observed with the presence of 15 g/L butyric acid, due to the adaptation of strain to high acid levels in medium. In another study, Al-Shorgani et al. (2018a) investigated the effects of butyric acid supplementation of 4 g/L to the fermentation medium under the conditions of with/without pH control. Fermentation was performed using glucose (50 g/L) as substrate by *Clostridium acetobutylicum* YM1. The optimal growth of the culture was at pH=6. Under uncontrolled pH conditions, butanol concentration was 13.5 g/L with a yield of 0.27 g/g and productivity of 0.188 g/L.h. Butyric acid addition under uncontrolled pH conditions improved butanol concentration, yield and productivity as 16.5 g/L, 0.345 g/g and 0.163 g/L.h. Controlled pH conditions did not enhance the butanol production for both with or without butyric acid addition. pH control shortened stationary phase and decreased the growth of the bacteria. Moreover, consumption of butyric acid was inhibited with pH control. Similarly, Yusof et al. (2010) studied the effect of initial butyric acid supplementation to the medium and concluded that butyric acid addition to the medium Reinforced Clostridial Medium (RCM) not only increased the butanol production at an early stage of the fermentation, but also procured protection against the devolution of Clostridial strain. The fermentation was performed with batch cultures of *Clostridium acetobutylicum* NCIMB 619, containing 0.0015 and 2 g/L butyric acid. Butyric acid supplementation during the initial stage of ABE fermentation improved the glucose conversion up to 71%. Compared to control medium, addition of 2 g/L butyric acid increased the acetone and butanol concentrations by 5 and 1.6 times, respectively. Moreover, the exponential growth phase reached up to 24 h with the presence of 2 g/L butyric acid in the medium which is resulted in higher cell density. Nonetheless, there were no significant improvements in solvent production and microbial growth with the addition of 0.0015 g/L butyric acid, compared to the medium without acid presence.

Another factor that affects butanol production is the amount of NAD(P)/NAD(P)H released during glycolysis. 4 moles of NADH are necessary to produce 1 mol of butanol from glucose. In order to overcome NADH limitation, electron carriers could be used. Thus, conversion of carbon source is directed to solvent production instead of acid formation (C. Jiang et al., 2016; Y. Jiang et al., 2009; C. Wang et al., 2018). Methyl viologen and neutral red could be used as additional electron carriers to obtain positive results. Neutral red supplementation could direct the metabolic pathway to alcohol production instead of acids by changing NAD(P) and NAD(P)H ratio (J. Zheng et al., 2015). Girbal et al. (1995) studied the effects of neutral red addition in chemostat culture of *Clostridium acetobutylicum* ATCC 824. Without neutral red presence, products were mainly acids. When neutral red was supplemented to the medium, the main products were solvents. Moreover, the amount of molecular hydrogen was lower. Butanol production rate was increased from 0.01 mmol/h.g to 2.51 mmol/h.g by 1 mM neutral red addition. Acetate and butyrate yields were decreased 11.1 and 1.6 times, respectively. Furthermore, production of dihydrogen was lower by 66.6%, which resulted in higher NAD(P)H activity. Nevertheless, neutral red addition with acid supplementation could also yield in higher butanol production. Influences of neutral red, sodium acetate and butyrate supplementations, either sole or combined on butanol production were investigated. Batch cultures of *Clostridium acetobutylicum* S10 with 20 g/L glucose addition as carbon source were used to perform ABE fermentation. In order to examine the effects of the additives, 1.5 g/L of sodium acetate, 1.5 g/L of sodium butyrate and 0.5 g/L were added to the medium. Without any acid or neutral red addition, the amount of produced butanol was 5.04 g/L for control group. Solely, neutral red supplementation improved butanol concentration up to 10%, compared to the control group. However, the addition of acids and neutral red together resulted in 18% higher butanol concentration than the control culture (C. Jiang et al., 2016). Similar to neutral red, addition of methyl viologen to fermentation medium could provide NAD(P)H formation, which is a cofactor stimulating the solvent production pathway, by preventing dihydrogen release (Peguin et al., 1994; C. Wang et al., 2018). Peguin et al. (1994) investigated the effects of methyl viologen addition at different pH values (4.5-6.5). At a pH value of 4.5, 1 mM

addition of methyl viologen to the broth remarkably increased the butanol production (0.65 mol/mol glucose) due to the enhanced NAD(P)H yield (approximately 1.1 mol/mol glucose) by prohibiting molecular hydrogen release. Lactic acid and glycerol production occurred at pH values of 5.5 and 6.5, respectively. Moreover, acetone production decreased with increasing pH values, due to decreasing acid consumption. In another study, hydA gene disrupted *Clostridium acetobutylicum* ATCC55025 was used for batch experiments to investigate the effect of methyl viologen supplementation to the medium with glucose or corn stover. Solely, gene disruption provided 0.24 g/g butanol yield with a butanol concentration of 12.9 g/L. By further 0.5 mM methyl viologen addition, 0.28 g/g butanol yield with 13.8 g/L butanol titer was obtained. Furthermore, acetic acid and butyric acid concentrations decreased by 36% and 25%, respectively. Thus, acetone concentration decreased from 3.2 g/L to 1.1 g/L. Results were similar with the experiments where carbon source was corn stover. 10.5 g/L butanol concentration and 0.24 g/g yield were obtained when corn stover was used with gene disrupted strain. Further methyl viologen addition as 0.2 mM to the medium improved butanol concentration and yield to 12 g/L and 0.28 g/g, respectively. There were no significant alterations at acid concentrations with or without methyl viologen supplementation. Consequently, acetone production was not considerably reduced (Du et al., 2021). Conversely, Hipolito et al. (2008) found out that when separator sludge hydrolysate with neutral red and methyl viologen supplementation was used in fermentation medium, there was no change in butanol production by *Clostridium saccharoperbutylacetonicum* N1-4. However, in control experiments, 5% molasses medium with 0.5 mM neutral red addition enhanced butanol titer from 4.1 g/L to 4.8 g/L and ABE concentration 1.1 times. They reported that sludge media had a really complicated structure and these additives could have formed complex compounds with other materials in sludge which might have inhibited their effects on butanol production.

In butanol production, Clostridial strains could need different important nutritional compounds like nitrogen and phosphorus to maintain biological activity. It is necessary to avoid nutrient limitations to enhance cell growth, sugar uptake and

production capacity. However, excess amounts of these nutrients could influence microbial activity and consequently inhibit butanol production. C/N and C/P ratios are varied due to employed strain type and medium composition so optimization of these ratios may improve product yield (Del Pilar Anzola-Rojas et al., 2015; Rajagopalan, 2016). Al-Shorgani et al. (2018b) studied the optimization of C/N ratio to improve butanol production by batch cultures of *C. acetobutylicum* YM1. Tryptone, yeast extract and ammonium acetate were added to fermentation medium as the nitrogen source in 6:2:3 ratio, respectively. C/N ratios varied between 5 and 120 with a constant glucose concentration of 50 g/L. Highest butanol concentration of 9.21 g/L was obtained at a C/N ratio of 12.8. Moreover, C/N=5 supported microbial growth and increased acetic acid concentration. In addition, butanol titer was low, similar to C/N=120. Gu et al. (2009) investigated the effects of ammonium acetate as nitrogen source and cassava starch as carbon source. Batch fermentations were performed by *C. acetobutylicum* EA2018. The starch concentration was kept constant while the ammonium acetate concentration was varied between 0 and 75 mM. When 30 mM ammonium acetate was added to medium, 19.4 g/L of total ABE concentration with 67% butanol yield were obtained, due to increased acid reassimilation. Higher ammonium acetate concentrations did not significantly improve butanol production. In other study, Madihah et al. (2001) investigated the effects of different C/N ratios by fixing the amounts of carbon and nitrogen sources separately. Batch experiments were performed by *C. acetobutylicum* P262. First step involved the usage of yeast extract and NH_4NO_3 as nitrogen sources in 5:2 ratio which was kept constant. Sago starch concentration ranged between 10-80 g/L. Maximum butanol concentration was obtained under relatively nitrogen limited conditions such as C/N=25.7. In other step, sago starch used as carbon source was kept at 50 g/L. Nitrogen concentrations varied between 42.8 and 514 mM with constant ratio of yeast extract and NH_4NO_3. In contrast to first step, maximum butanol yield (21.6 g/L) was accomplished in nitrogen rich environment such as C/N=3.6.

There are no detailed studies about the effects of phosphorus concentration or C/P ratio on butanol production. Effects of K_2HPO_4 as phosphorus source and $(NH_4)_2SO_4$

as nitrogen source were examined separately. Batch cultures of *C. acetobutylicum* P262 were used to carry out the experiments and carbon source was lactose. Initial K_2HPO_4 concentration varied between 20-1000 mg/L while $(NH_4)_2SO_4$ and lactose concentrations were kept constant at 2 and 60 g/L, respectively. 250 mg/L of K_2HPO_4 addition resulted in highest total solvent concentration (12 g/L) with a butanol production rate of 0.233 g/L.h. Above this level, acid production was increased and consequently, solvent production decreased. In other step, initial $(NH_4)_2SO_4$ concentration ranged between 500-2000 mg/L while K_2HPO_4 and lactose concentrations were fixed at 0.4 and 60 g/L, respectively. Highest total solvent concentration of 7.26 g/L with 0.312 g/L.h butanol production rate was obtained when 1500 mg/L $(NH_4)_2SO_4$ was added to the fermentation medium. Lower concentrations than 1500 mg/L resulted in a decrease in solvent production. Moreover, there was a linear relationship between cell growth and the amount of $(NH_4)_2SO_4$ added (Kanchanatawee & Maddox, 1990). Díez-Antolínez et al. (2016) studied the effect of KH_2PO_4 and K_2HPO_4 addition as phosphorus source on butanol production. Batch fermentations were performed by *Clostridium beijerinckii* CECT 508 and carbon source was cheese whey. Addition of both KH_2PO_4 and K_2HPO_4 had a negative impact on butanol production.

1.7 Objectives and Scopes

The scope of the thesis was to produce biobutanol from pretreated organic waste residues by fermentation. The first aim was to optimize the conditions of microwave hydrolysis of peach pulp to maximize the fermentable sugar recovery by using statistical experimental design. The second aim was to examine the butanol productivity of *C. acetobutylicum* and *C. pasteurianum* through the media composition.

The specific objectives of the thesis were to evaluate the effect of followings;

- Initial substrate concentration,
- Nitrogen and phosphorus concentrations as C/N and C/P,

- Concentrations of ions like magnesium, manganese, zinc, calcium, iron,
- Butyric acid concentration as a metabolic shifter to butanol,
- Ethanol concentration as metabolic shifter to butanol,
- Neutral red addition as electron carrier.

CHAPTER 2
MATERIALS AND METHODS

2.1 Biomass

The peach pulp was received from DIMES fruit processing industry, İzmir/TURKEY and stored at 4ºC. The residue was dried in oven at 47ºC for 3 days, and then it milled in Tungsten Carbide Mortar to the particle sizes between 180-250 μm.

2.2 Experimental Design for Microwave Hydrolysis

The hydrolysis of the peach pulp was conducted in a microwave (SAMSUNG ME711K) equipped with a stirrer. Box-Behnken experimental design, which is one of the Response Surface Method used for the optimization of process parameters, was used to optimize the microwave hydrolysis conditions of peach pulp. Independent variables and their ranges were X_1=2-5.5 as pH, X_2=180-250 μm as particle diameter and X_3=20-40 g/L as initial substrate (peach pulp) concentration. The power applied in microwave was kept constant at 600 W. Hydrolysis period was 45 min when substrate concentration was 40 g/L, and 30 min when peach pulp concentration was lower than 40 g/L. Box-Behnken design contains the factorial experimental points in which one of the factors is at center point while the other factors are at the maximum and minimum points. The number of the total experiment is 15 for the factorial points. The experiments were replicated at center point to determine the random error.

Design Expert 7 (trial version) was used to design the experiments and to evaluate the results statistically. The coded and actual experimental points of Box-Behnken design is depicted in Table 2.1. The optimal conditions were determined by using the optimization tool of the software. A quadratic response equation with the coefficient was developed. Finally, validation experimental studies were conducted at optimum

experimental conditions as well as the selected points within the ranges of independent factors.

Table 2.1 Coded version of Box-Behnken experimental design method for 3 factors

	Coded variables			Actual values of variables		
Test Number	**X_1**	**X_2**	**X_3**	**pH**	**D_p (µm)**	**S_0 (g/L)**
1	0	-1	-1	3.75	180	20
2	-1	1	0	2	250	30
3	-1	0	-1	2	215	20
4*	0	0	0	3.75	215	30
5	1	1	0	5.5	250	30
6	-1	-1	0	2	180	30
7*	0	0	0	3.75	215	30
8	0	-1	1	3.75	180	40
9	1	0	1	5.5	215	40
10	0	1	-1	3.75	250	20
11	1	0	-1	5.5	215	20
12	0	1	1	3.75	250	40
13	-1	0	1	2	215	40
14	1	-1	0	5.5	180	30
15*	0	0	0	3.75	215	30

+1: Maximum value of factor

-1: Minimum value of factor

* Central points

2.3 ABE Fermentation

2.3.1 Microorganisms

Two different types of organisms were used in experiments. *Clostridium acetobutylicum* NRRL 527 was obtained from Waste Bioprocess Laboratory of Dokuz Eylül Environmental Engineering. *Clostridium pasteurianum* DSM 525 was

obtained from The Leibniz Institute DSMZ- German Collection of microorganisms and Cell Cultures GmbH.

2.3.2 Fermentation Medium

The standard nutrient medium used in the experiments consisted of (g/L); peptone 10, meat extract 10, yeast extract 3, NaCl 1, resazurin 0.025, L-cysteine 0.2, $(NH_4)_2SO_4$ 2, KH_2PO_4 3.9, K_2HPO_4 2.8, $MgSO_4.7H_2O$ 0.25, $FeSO_4.7H_2O$ 0.1, $MnSO_4.H_2O$ 0.01, $C_2H_3NaO_2$3. Glucose was used as substrate except for $CaCO_3$ added medium and the concentration was varied depending on the experimental conditions.

2.3.2.1 ABE Fermentation with Clostridium acetobutylicum

Peach pulp and glucose were used as substrate and their concentrations were varied depending on the experimental purpose. The effects of $CaCO_3$, butyric acid, Mg, Mn, Zn on butanol production as an inhibitor of side product formation or accelerator of butanol production were investigated. Hydrolyzed peach pulp sugar concentration was 15 g/L with 10 g/L $CaCO_3$ addition to the fermentation media. Butyric acid concentration was 5 g/L in 50 g/L glucose. Magnesium was externally added into the fermentation medium at concentrations 60, 80 and 220 mg/L. The investigated manganese concentrations were 40, 60 and 200 mg/L. Finally externally added zinc concentrations were 40, 60 and 200 mg/L. The amount of glucose added was around 50 g/L for 3 experiments.

2.3.2.2 ABE Fermentation with Clostridium pasteurianum

The effects of ions; Mg, Mn, Zn on butanol production with *Clostridium pasteurianum* DSM 525 were investigated. The initial substrate concentration was kept constant at 30 g/L. Mg, Mn and Zn were added into the fermentation medium at the concentrations, 200 mg L. The electron carrier, neutral red, was added at the concentration of 289 mg/L.

Ethanol is the solvent generated in ABE fermentation. External addition of ethanol can act as an inhibitor for ethanol production and the ABE could shift to butanol generation. By considering this hypothesis ethanol was added externally in the fermentation media at a concentration of between 0.2 and 1.4 g/L. On the other hand, butyric acid could act as an accelerator of butanol generation. For this purpose, the effects of butyric acid addition between 6 and 12 g/L were investigated. Additionally, medium composition was revised (g/L); peptone 6, yeast extract 2, L-cysteine 0.2, $MgSO_4.7H_2O$ 0.3, KH_2PO_4 0.5, $FeSO_4.7H_2O$ 0.01, $C_2H_7NO_2$ 3. The initial substrate concentration was around 60 g/L for both ethanol and butyric acid added mediums.

The media composition was rich in protein, which might adversely affect butanol production. Therefore, peptone, yeast and meat extracts were removed from fermentation media to see the effect of these protein sources and to simplify the media. $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, $MnSO_4.H_2O$ compounds were also removed from the media as magnesium, iron and manganese sources, respectively. The level of the initial substrate was around 60 g/L.

The effects of C/N and C/P ratios on butanol production were investigated. For this purpose, peptone, yeast and meat extracts were removed from the medium. Instead, $(NH4)_2SO_4$ was used as nitrogen source. Carbon concentration was kept constant and nitrogen concentration in the media varied to obtain C/N ratio between 50 and 250. Similarly, C/P ratio was varied by changing carbon concentration and fixing phosphorus amount. C/P range was between 19 and 43, glucose concentration ranged between 67 and 149 g/L. Phosphorus sources were KH_2PO_4 and K_2HPO_4.

2.3.3 Dark Fermentation

Batch fermentations were performed in a shaker under anaerobic conditions at 100 rpm and 37ºC. In order to obtain anaerobic conditions, nitrogen gas (N_2) was passed through the gas phase at the beginning of the experiments. pH was measured

daily. 10 M NaOH and 60% diluted H_2SO_4 were used for pH adjustments in the experiments if required.

Experimental studies with *Clostridium acetobutylicum* were carried out in 300 mL flasks and a working volume of 200 mL. pH of the mediums was adjusted to 5.5-6.5.

Batch experiments with *Clostridium pasteurianum* were conducted in 500 mL serum bottles with 250 mL working volume. pH was monitored daily and adjusted to pH=6.0-7.0.

2.4 Analytical Methods

2.4.1 Sample Preparation

The samples removed from the liquid phase every day were centrifuged at 8000 g for 15 minutes and the clear supernatants were used for the analysis of total sugar (TS), total volatile fatty acids (TVFA) and solvents (ABE). The samples were preserved at the 4ºC fridge for further use.

2.4.2 Total Sugar Analysis

Total sugar analysis was performed daily to determine the sugar concentration in the fermentation medium. The liquid and solid phases were separated with centrifuge at 8000 rpm for 15 min by taking samples from the mediums. Phenol and sulfuric acid were added to the diluted liquid phase for total sugar analysis in spectrophotometer (Dubois et al., 1956).

2.4.3 Sugar, Organic Acid and Solvent Measurement

Measurements were made with high performance liquid chromatography (HPLC, Agilent 1100) to determine the types and concentrations of sugar, organic acid and solvents in fermentation media. 5mM H_2SO_4 was used as mobile phase. Flow rate

was 0.6 mL/min, Aminex HPX-87H Ion Exclusion Column 300 mm x 7.8 mm and UV detector at 210 nm were used. For solvent analysis, column temperature was 50ºC and refractive index detector (RID) was employed. For sugar and organic acid measurements, column temperature was 40ºC. RID for sugar analysis and diode array detector (DAD) for organic acid measurement was used. Prior to measurement, daily samples were taken from the test environments, the liquid and solid phases were separated by centrifugation at 8000 rpm for 15 minutes.

2.4.4 Total Gas Measurement

Gas measurements were made daily by liquid-gas exchange method. Liquid phase of this method was consisted of 2% H_2SO_4 and 10% NaCl.

2.4.5 Hydrogen Gas Measurement

Hydrogen gas was measured at an Agilent 6890 (Varian 3600) gas chromatograph. Alltech, Hayesep D 80/100 6" x 1/8" x 0.085" column and carrier gas (N_2) with 30 mL/min were used. Measurements were made at 35°C furnace temperature, 200°C injection temperature and 140°C filament conditions. The hydrogen gas was withdrawn from the environment with the help of a sealed glass syringe.

2.5 Calculations

2.5.1 Volume of Hydrogen Gas

$$V_{H2} = C_{H2} * V_{TG} \tag{2.1}$$

V_{H2}: The volume of hydrogen gas (mL)
C_{H2}: Hydrogen gas concentration (%H_2)
V_{TG}: The volume of total gas (mL)

2.5.2Hydrogen Gas as mmol (37°C)

$$P * V = n * R * T \quad (2.2)$$

n:mmol H_2

P: 1 atm

V_{H2}: The volume of hydrogen gas (mL)

R: 0.082 (L.atm / mol.K)

T: Temperature (K)

2.5.3 Cumulative Volume of Hydrogen Gas

$$V_{cumulative,\ i} = V_{cumulative,\ i\ -1} + V_{TG}C_{H2,i} + V_{HS,i}C_{H2,i} - V_{HS,i-1}C_{H2,\ i-1} \quad (2.3)$$

$V_{cumulative,\ i}$: The amount of H_2 produced at time t (mL)

$V_{cumulative,\ i\ -1}$: The amount of H_2 produced at time t-1 (mL)

V_{TG}: The volume of total gas (mL)

$C_{H2,i}$: The concentration of H_2 at time t (%)

$V_{HS,i}$: Headspace volume at time t (mL)

$C_{H2,\ i-1}$: The concentration of H_2 at time t-1 (%)

$V_{HS,i-1}$: Headspace volume at time t-1 (mL)

CHAPTER 3
RESULTS AND DISCUSSION

3.1 Microwave Hydrolysis of Peach Pulp

Hydrolysis conditions that maximize the total sugar concentration were optimized for biobutanol production from peach pulp. Statistical experimental design method namely Box-Behnken was used to determine the significant factors and to optimize the hydrolysis conditions. The independent variables were pH, particle diameter and initial substrate concentration, dependent variable was total sugar concentration. Effects of these independent variables on total sugar concentration were investigated.

pH (X_1), particle diameter (X_2), initial peach pulp concentration (X_3) ranges were determined as X_1=2-5.5, X_2=180-250 μm and X_3=20-40 g/L, respectively. The reproducibility of the results was tested by working on the central point of all factors with three replicates. The experimental points determined according to Box-Behnken design method, the actual and predicted values of total sugar concentration are given in Table 3.1. The quadratic polynomial model was developed as given in Equation 3.1 and the coefficients were determined depicted in Table 3.2. The similarity between observed responses from the experiments and predicted ones from the quadratic equation was tested by regression analysis. A good relationship between actual and predicted values was observed with the regression coefficient of R^2= 0.9655. Hence, model could be used to estimate total sugar concentration within specified factor limits.

$$Y_{TS} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (3.1)$$

Table 3.1 The experimental points determined according to the Box-Behnken experimental design method, the actual and predicted total sugar concentration values

Test No.	pH	Particle Diameter (µm)	Substrate Concentration (g/L)	Total Sugar Concentration (g/L)	
				Actual Value	**Predicted Value
	X_1	X_2	X_3	X_4	X_5
1	3.75	180	20	10	10.4
2	2	250	30	18	17.5
3	2	215	20	11	11.9
4*	3.75	215	30	14	15.3
5	5.5	250	30	16	17.3
6	2	180	30	16	14.8
7*	3.75	215	30	16	15.3
8	3.75	180	40	15	15.4
9	5.5	215	40	23	22.1
10	3.75	250	20	8	7.6
11	5.5	215	20	11	10.1
12	3.75	250	40	24	23.6
13	2	215	40	20	20.9
14	5.5	180	30	14	14.5
15*	3.75	215	30	16	15.3
			R^2	**0.9655**	

* Central points

** Total sugar concentrations calculated by using the surface response equation and coefficients

Table 3.2 Model coefficients

Coefficient	β_0	β_1	β_2	β_3	β_{12}	β_{13}	β_{23}	β_{11}	β_{22}	β_{33}
Value	24.121	-4.622	0.038	-1.075	-5.111×10^{-16}	0.043	0.008	0.435	-5.442×10^{-4}	-4.167×10^{-3}

Table 3.3 Analysis of variance

Source	Sum of Squares	df	Mean Square	F-value	p-value	Evaluation
Model	277.82	9	30.87	15.56	0.0038	Significant
X_1-pH	0.1250	1	0.1250	0.0630	0.8118	
X_2-Particle Diameter	15.13	1	15.13	7.63	0.0398	*Significant*
X_3-Substrate Concentration	220.50	1	220.50	111.18	0.0001	*Significant*
X_1*X_2	0	1	0	0	1	
X_1*X_3	2.25	1	2.25	1.13	0.3355	
X_2*X_3	30.25	1	30.25	15.25	0.0113	*Significant*
X_1^2	6.56	1	6.56	3.31	0.1285	
X_2^2	1.64	1	1.64	0.8274	0.4047	
X_3^2	0.6410	1	0.6410	0.3232	0.5943	
Residual	9.92	5	1.98			
Lack of Fit	7.25	3	2.42	1.81	0.3749	Not significant
Pure Error	2.67	2	1.33			
Cor Total	287.73	14				

Table 3.3 is the variance analysis of the factors. The significance of the factors and the interaction with the quadratic factors are statistically analyzed. Model F-value of 15.56 indicates that the model is significant. p-value less than 0.05 ($p<0.05$) reveals that the model term is significant or a factor that significantly affects the value of dependent variable. $p>0.1$ indicates that the factor or model term does not have a significant effect on output (total sugar concentration). According to ANOVA table, it could be concluded that particle diameter and substrate concentration significantly affect total sugar concentration. However, pH value is not a significant factor affecting the hydrolysis efficiency. In other words, performing the hydrolysis at any value in pH=2-5.5 range does not provide a significant increase or decrease in total sugar concentration. It procures an important advantage in terms of system economy and adjusting the fermentation value.

Two level or quadratic effects of the factors are also seen in the ANOVA table (Table 3.3). Interaction of peach pulp concentration and particle diameter is significant. However, the interaction between pH with the other factors is not significant. Quadratic effects show the curvature in the response. Although curvature is not significant, the 3D plots show a slight curvature. Therefore, the quadratic effects were included in the response equation. These results show that the substrate

concentration and particle diameter are the most significant factors that must be controlled at microwave hydrolysis of peach pulp for the maximum hydrolysis efficiency. Finally, insignificance of lack of fit value reveals that the model could be used to estimate total sugar concentration within specified hydrolysis conditions.

Validation studies which were shown in Table 3.4 have been carried out to prove that the model could be used to estimate the total sugar concentration within the specified conditions. Actual and predicted total sugar concentration values were compared by performing hydrolysis under different conditions within the boundary conditions. Similarity between actual and predicted sugar concentrations indicates that the model could be used to calculate estimated total sugar concentration.

Table 3.4 Validation studies and results

pH	Particle Diameter (μm)	Substrate Concentration (g/L)	Total Sugar Concentration (g/L)	
			Actual Value	Predicted Value
2.5	250	28	13	15.30
4	180	38	16	15.12
3	250	22	9	9.93
4.75	212	25	12	12.75
5.5	**250**	**39**	**23**	**24.27**

3.1.1 Effect of Independent Variables on Total Sugar Concentration

Total sugar concentrations obtained under different hydrolysis conditions were calculated by using Equation 3.1 and the coefficients in Table 3.2. The values of independent variables were varied within specified ranges. Results were presented in 3D surface plots. One of the independent variables was kept constant, while the others were changed within the range selected in experimental design in the 3D plots. The factor which was kept constants was selected according to the optimum conditions given in section 3.1.2.

Figure 3.1 shows the interaction of total sugar concentration with particle diameter (μm) and pH. Initial substrate concentration was 39 g/L. Although pH was

increased from 2 to 5.50 under conditions of 180 µm particle diameter, no significant increase in total sugar concentration was observed and an average sugar concentration of 18.93 g/L was obtained. By increasing particle diameter from 180 to 250 µm within the same pH range, a total sugar concentration of 23.79 g/L was obtained with an increase of approximately 1.3 times compared to fixed particle diameter of 180 µm within the same pH range. As seen in the ANOVA table, the two level interactions of pH did not have a significant effect on the response.Therefore, no significant changes in total sugar concentrations were observed. A slight increase in total sugar concentration is mainly, due to the particle diameter.

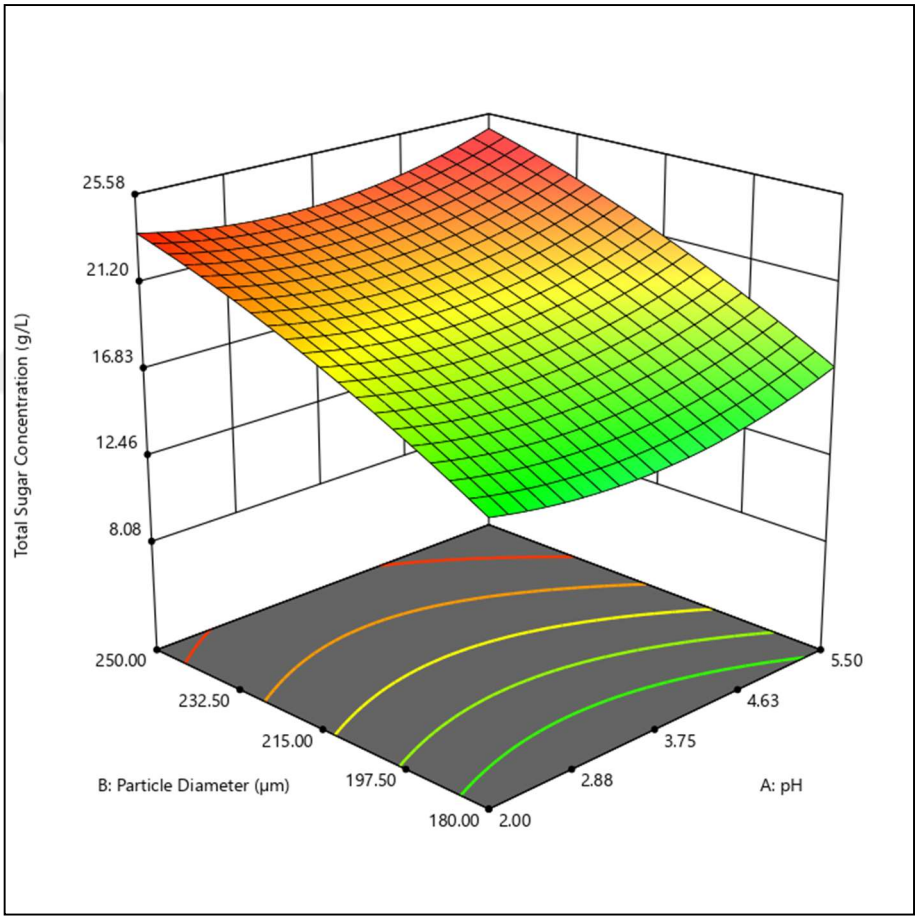


Figure 3.1 Interaction of total sugar concentration with particle diameter and pH (S_0=39 g/L)

The effect of substrate concentration and pH on total sugar concentration was shown in Figure 3.2. Particle diameter was kept constant at D_p=250 µm which was determined as optimal. The main response, total sugar concentration, increased from 8 g/L to 24.4 g/L by increasing the substrate concentration from 20 g/L to 40 g/L

under the conditions of pH 5.50. No significant changes in total sugar concentration occurs when pH is increased from 2 to 5.50 under conditions of 20 g/L substrate concentration. The hydrolysis yields sugar concentration of 9.82 g/L. Similarly, at high initial substrate concentrations, no variation in total sugar with pH is observed. The reason for the increase in the total sugar concentration was the increase in the amount of initial peach pulp. If the substrate structure is complex, more acidic conditions are needed to facilitate hydrolysis. However, due to the presence of relatively simple sugars in fruit, no direct and strong effect of pH on hydrolysis was observed.

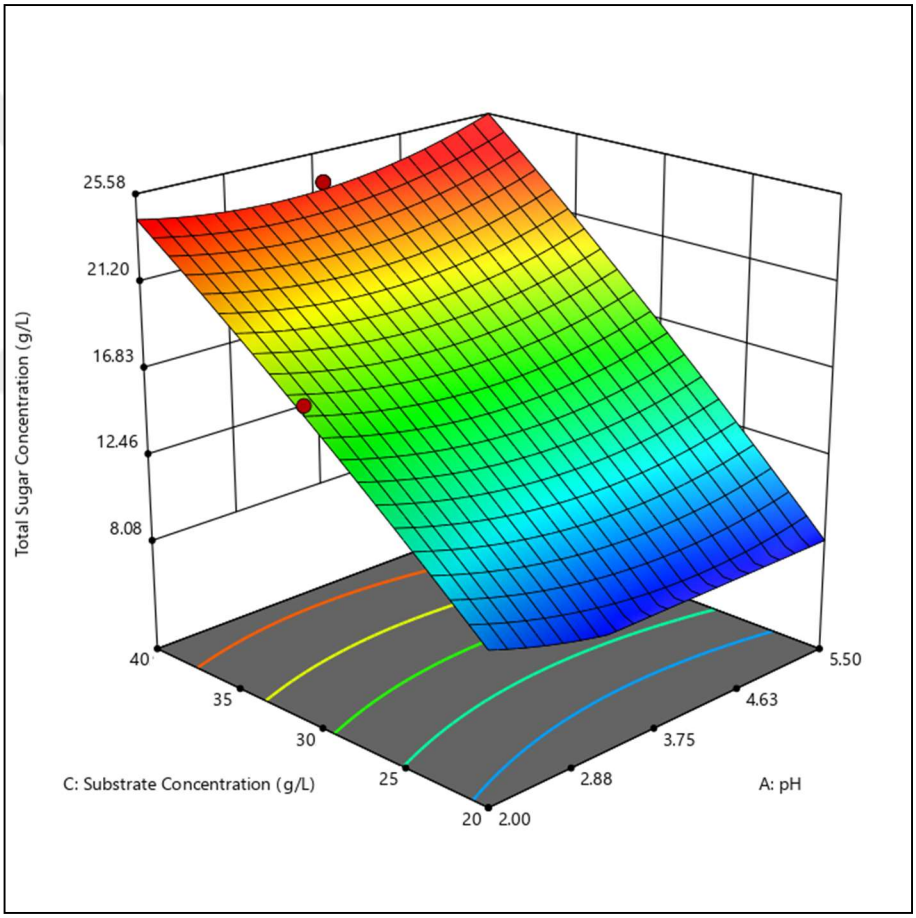


Figure 3.2 Variation of total sugar concentration with substrate concentration and pH (D_p=250 µm)

Figure 3.3 shows the effect of particle diameter and substrate concentration on total sugar concentration. Since the highest hydrolysis efficiency was observed at pH=5.50, pH value was kept constant at this level. According to the results obtained, it is observed that the particle diameter and substrate concentration have a significant

effect on the total sugar concentration at maximum pH value, and there was an interaction between these two variables. 8 g/L total sugar concentration was obtained under low particle diameter and substrate concentration conditions. By increasing the substrate concentration from 20 g/L to 40 g/L, the total sugar concentration increased to 16.85 g/L.Likewise, a total sugar concentration of 10.73 g/L was obtained by reducing the particle diameter from 250 µm to 180 µm.

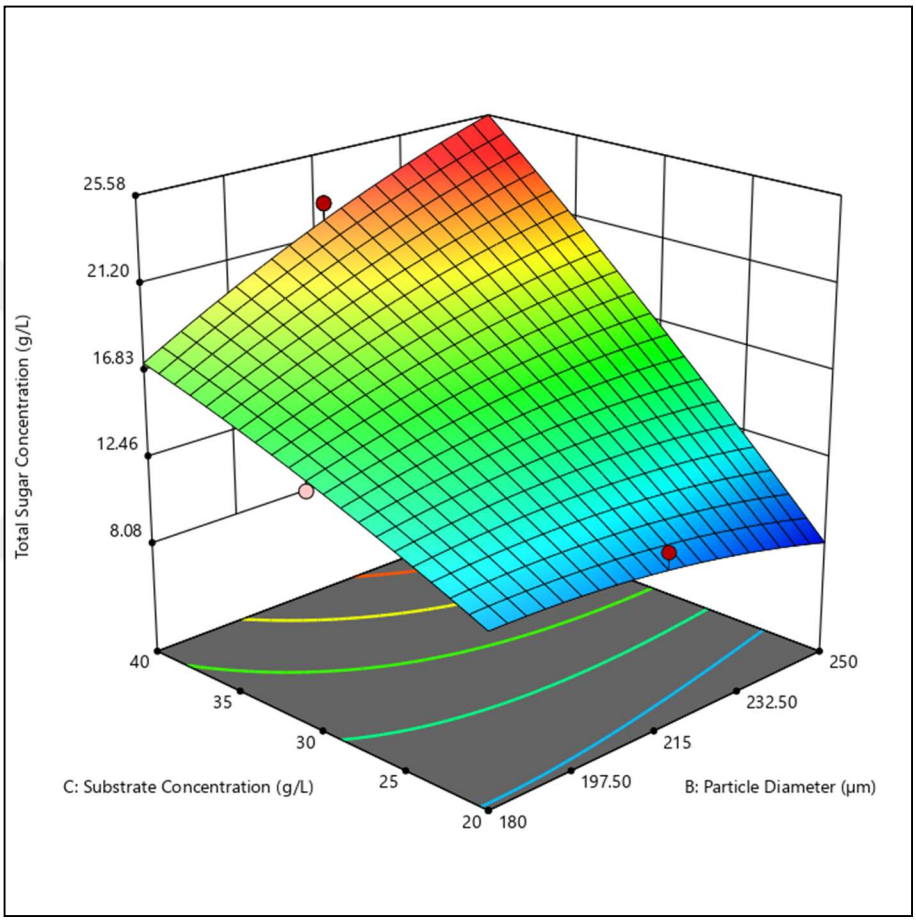


Figure 3.3 Variation of total sugar concentration with substrate concentration and particle diameter (pH= 5.50)

In summary, the maximum total sugar concentration was obtained at pH= 5.50, particle diameter D_p=246.88 µm, substrate concentration S_0=39.48 g/L, within the limits specified in the microwave hydrolysis process.

3.1.2 Optimization of Hydrolysis Conditions

The optimization module of the Design Expert software was used to determine the hydrolysis conditions with maximum total sugar concentration. Figure 3.4 shows the optimal hydrolysis conditions in microwave hydrolysis of peach pulp. pH, particle diameter (D_p), substrate concentration (S_0) were kept within ranges in the statistical experiment design model, and the total sugar concentration (TS) was maximized. According to the optimization module, a maximum total sugar concentration of 24.27 g/L was obtained under conditions at pH=5.50, D_p= 246.88 µm (approximately 250 µm), S_0=39.48 g/L. In order to validate the optimal conditions, a microwave hydrolysis was performed experimentally under the specified conditions and the observed result was TS=23 g/L which is quite close to the TS=24.27 g/L obtained from the model with an acceptable deviation. This confirms that the hydrolysis model (Equation 3.1) can be used to calculate the estimated total sugar concentration within the limits of the selected independent variable.

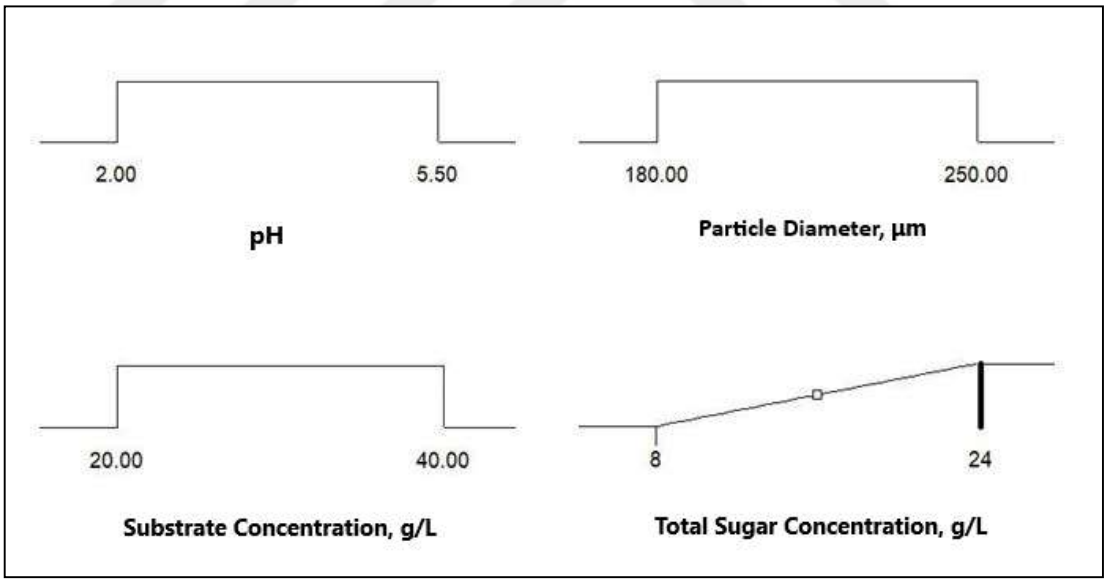


Figure 3.4 Hydrolysis conditions where maximum total sugar concentration is achieved

3.2 Dark Fermentation with *Clostridium acetobutylicum*

Microwave hydrolysis of the peach pulp was the initial stage of the fermentation for butanol production. The sugar syrup from the hydrolysis reaction was used as the substrate for the solvent production in the experiment where the effect of $CaCO_3$ addition was studied. However, due to the problems observed in butanol production, the effect of other factors was examined by using glucose as carbon source. The microbial culture was *Clostiridium acetobutylicum* for the first stage of the study. The effects of some fermentation media composition were investigated. These media components act like either an accelerator or inhibitor in fermentative solvent production.

3.2.1 Effect of $CaCO_3$ Addition

Calcium carbonate addition as Ca^{2+} source could improve the butanol production serving as a buffering agent, which could inhibit the excessive acid accumulation in fermentation medium (Gottumukkala et al., 2013). Moreover, $CaCO_3$ supplementation may enhance the growth, glucose utilization capacity and butanol tolerance of Clostridium sp (Richmond et al., 2011). Likewise, Su et al. (2020) concluded that supplementation of salts as Ca^{2+} sources could enhance sugar uptake and solvent production by up-regulating enzymatic activity. In the light of these results, the effect of $CaCO_3$ on solvent production was investigated. It is clear that Ca^{2+} act like an enhancer in solvent production. 10 g/L of $CaCO_3$ was added to the fermentation medium to increase butanol tolerance and to provide higher butanol concentration. pH was adjusted to 6.5 against pH change due to the organic acid production. A control experiment was also conducted to compare the results. Variations of ethanol and butanol concentrations over time were given in Figure 3.5 and Figure 3.6, respectively. Ethanol production increased from 0.2 g/L to 0.95 g/L for both control and $CaCO_3$-added media. Butanol concentration slightly increased to around 0.02 g/L for both control and Ca containing fermentation conditions.

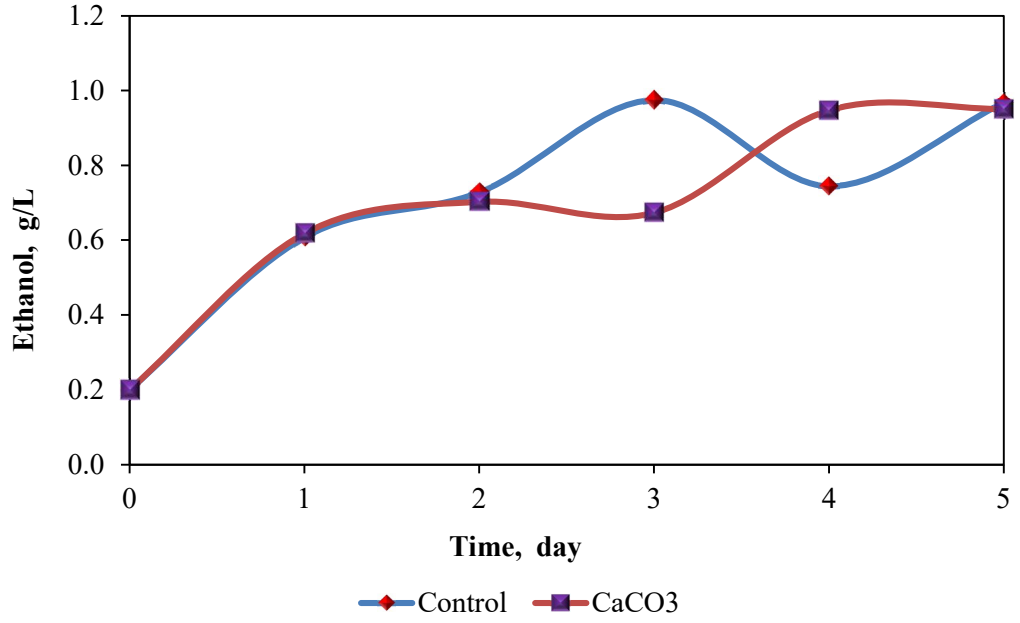


Figure 3.5 Variation of ethanol production over time with $CaCO_3$ addition

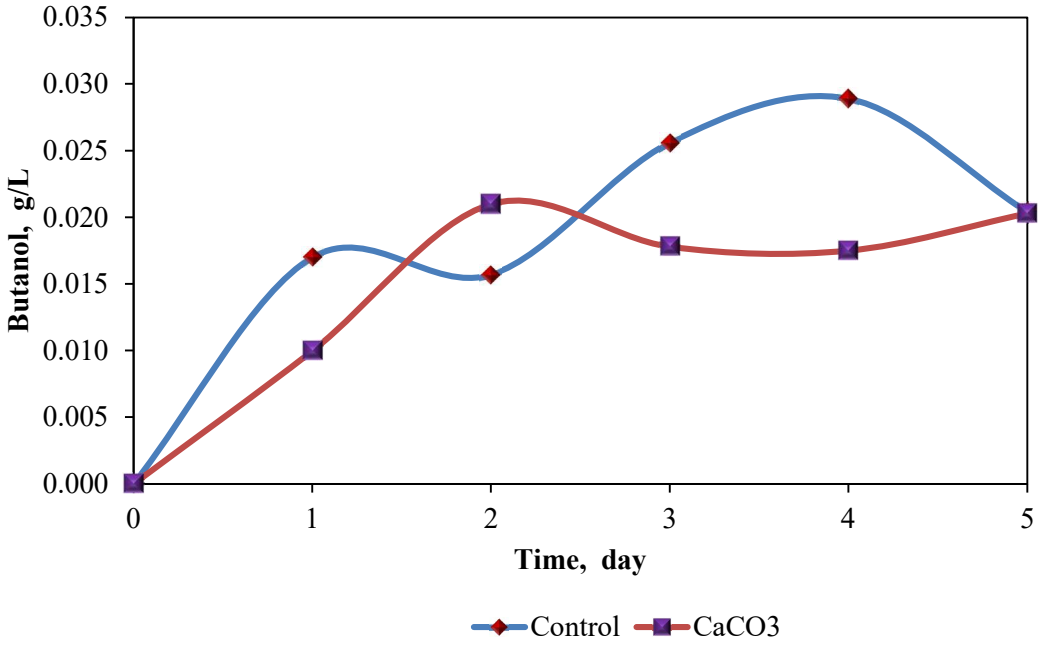


Figure 3.6 Variation of butanol production over time with $CaCO_3$ addition

Sugar consumption indicates the activity of the microbial cultures. Therefore, sugar utilization is monitored throughout the fermentation period. The sugar forms, glucose and fructose, in the hydrolysate were also analyzed to evaluate the sugar

preference of the culture for solvent production. The initial total sugar concentration was 16 g/L in this experiment. The variation of total sugar concentration over time was given in Figure 3.7 and the variations of glucose and fructose amounts were given in Figure 3.8 and Figure 3.9, respectively. 75% of total sugar was consumed in two days for two fermentation media. Glucose was consumed faster than fructose as expected.

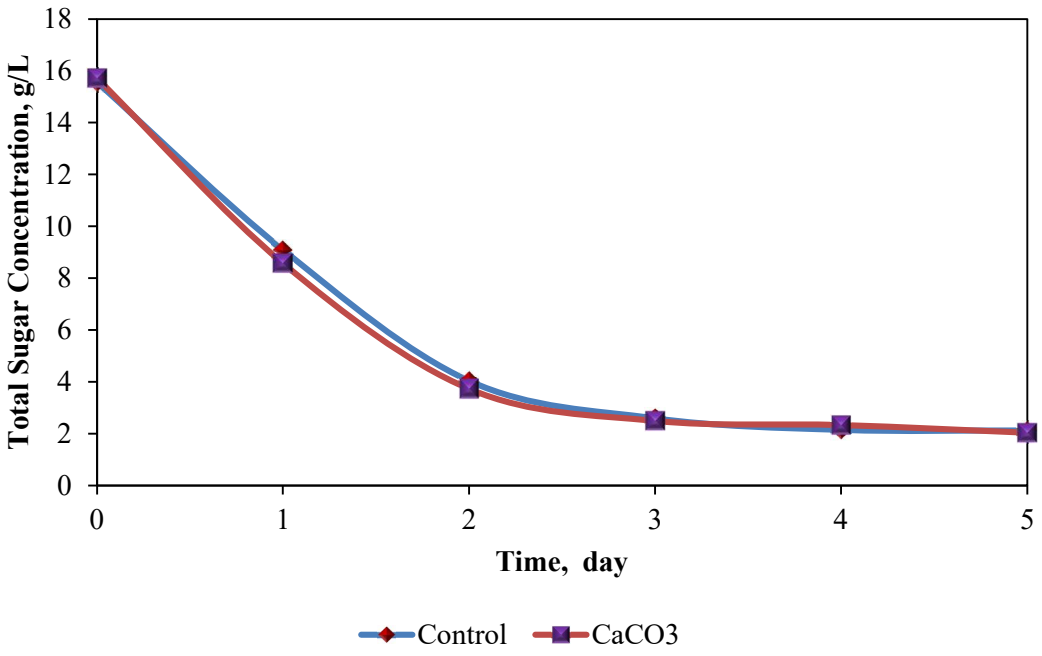


Figure 3.7 Consumption of total sugar concentration over time with addition of $CaCO_3$

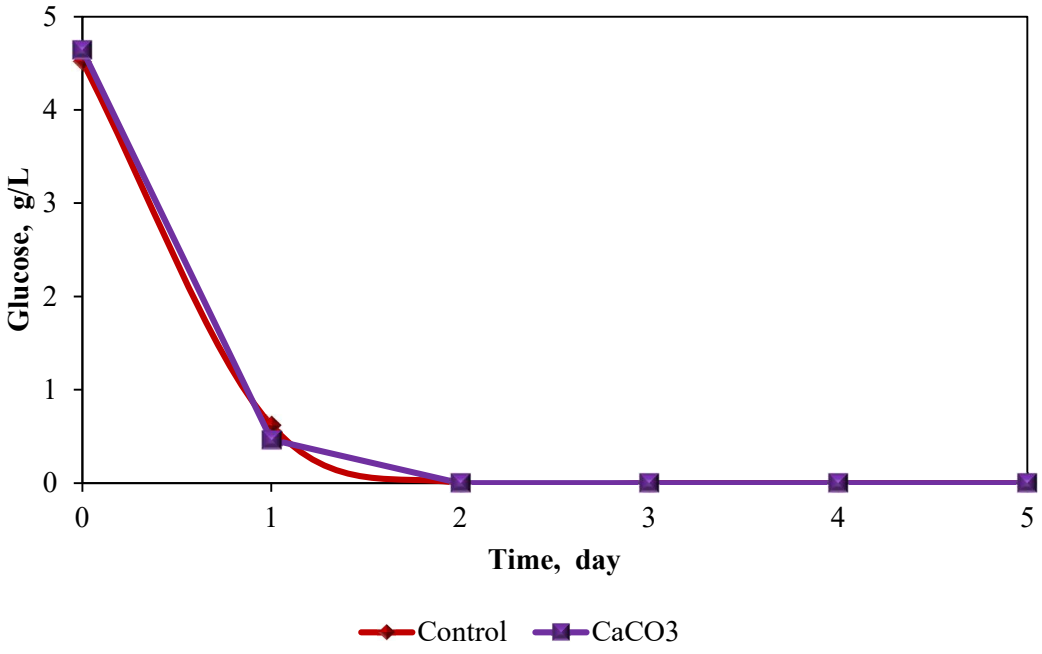


Figure 3.8 Consumption of glucose concentration over time with addition of $CaCO_3$

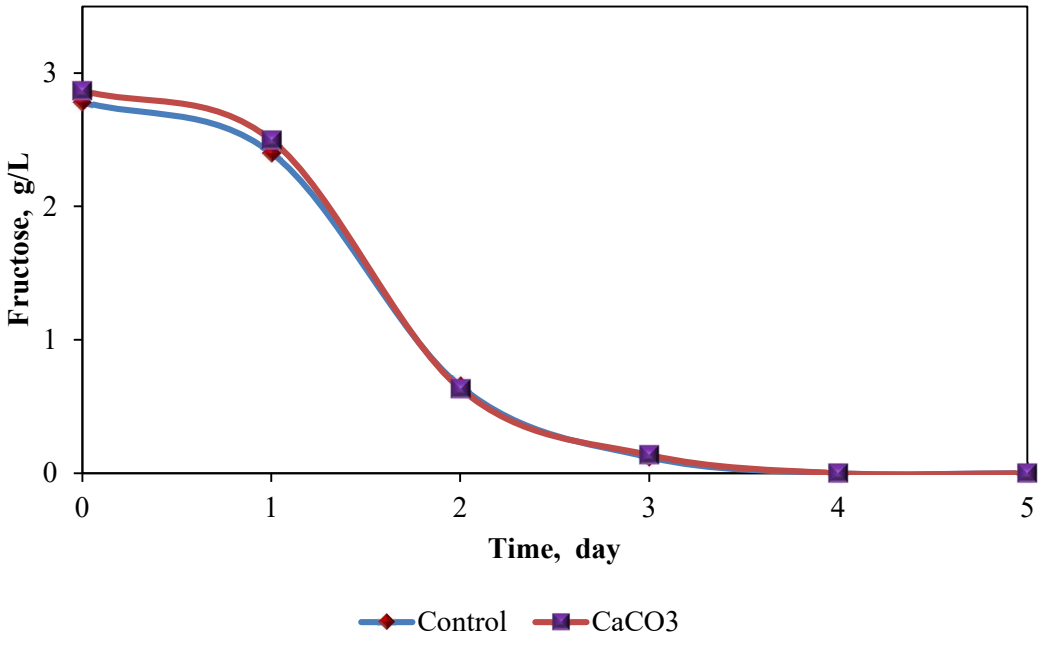


Figure 3.9 Consumption of fructose concentration over time with addition of $CaCO_3$

Lactic, acetic and propionic acids were produced as organic acids. Changes in lactic, acetic and propionic acid concentrations with time were given in Figure 3.10, Figure 3.11 and Figure 3.12, respectively. About 16 g/L sugar was consumed and the

products in terms of organic acids were 10.5 g/L lactic acid was obtained. Acetic acid production has increased up to 7 g/L and then it was consumed after this level. Propionic acid production started at 3.5 g/L and then reached up to 4.3 g/L. Variation of pH level was given in Figure 3.13. Due to the organic acid production, pH decreased to 5.7 and remained stable at 6.7 for following days. However, there was not any butyric acid production.

$CaCO_3$ was used to increase butanol tolerance and to obtain higher butanol concentration. Control and $CaCO_3$ added mediums were similar in terms of organic matter consumption and solvent production. Ethanol was produced in both media, but very small amounts of butanol (0.020 g/L) were produced. The high amount of lactic acid produced indicated that Lactobacillus species in medium dominated *Clostridium acetobutylicum* bacteria. There was no effect of $CaCO_3$ addition for neither solvent nor acid production.

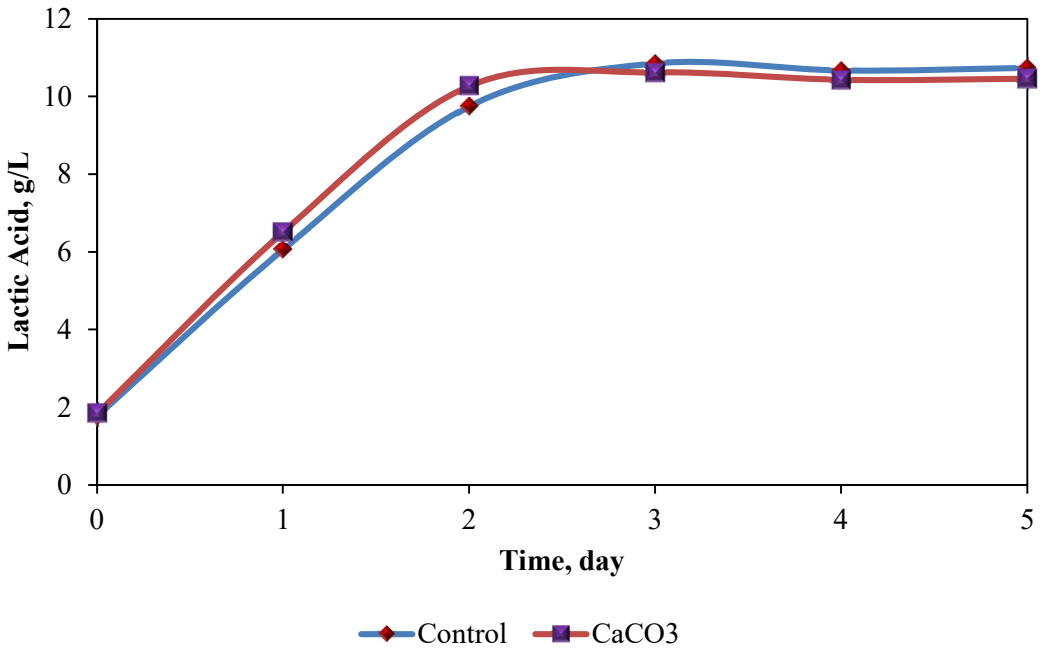


Figure 3.10 Variation of lactic acid over time with addition of $CaCO_3$

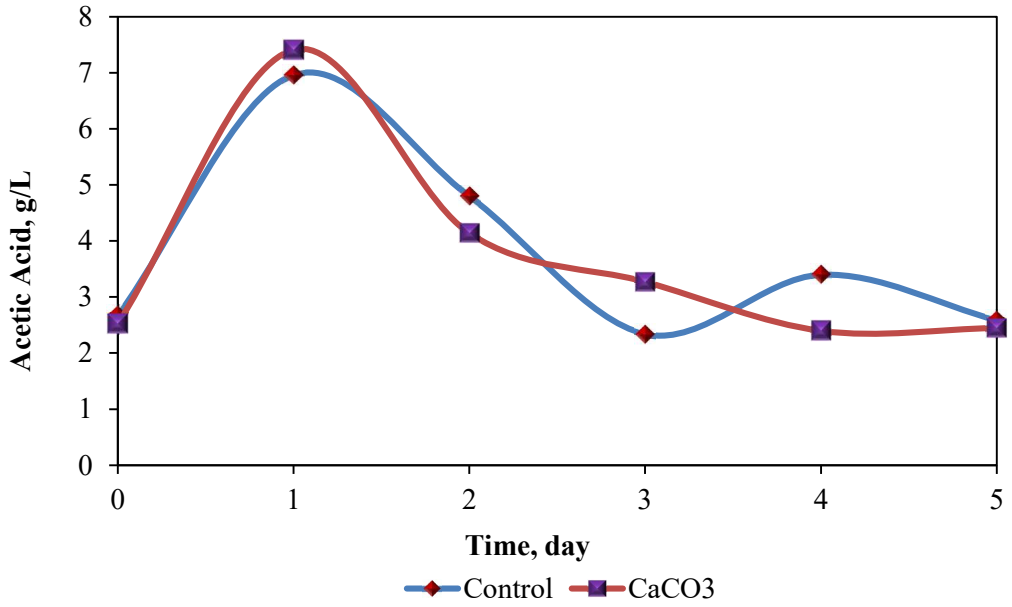


Figure 3.11 Variation of acetic acid over time with addition of $CaCO_3$

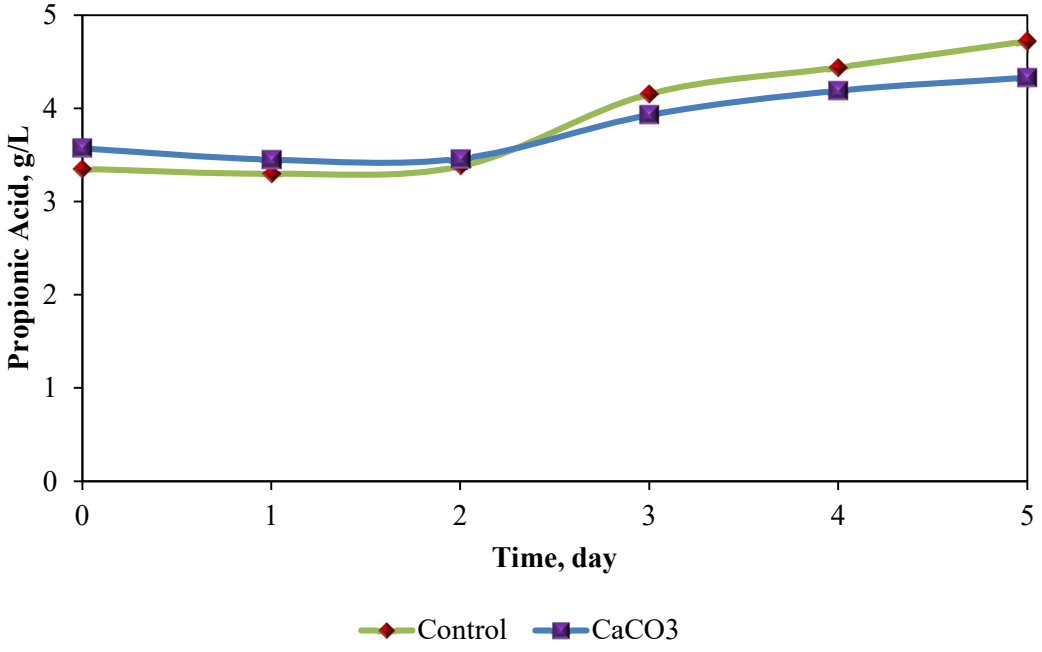


Figure 3.12 Variation of propionic acid over time with addition of $CaCO_3$

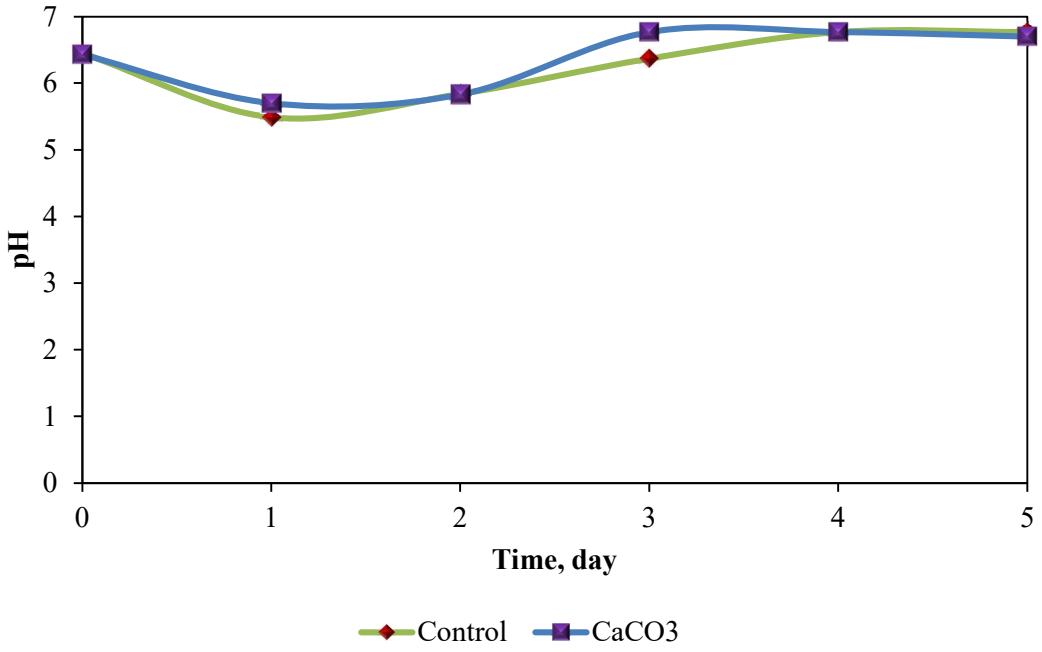


Figure 3.13 Variation of pH with $CaCO_3$ addition

3.2.2 Effect of Butyric Acid Addition

Butanol production could be improved by butyric acid supplementation as an initiator to steer the metabolic pathway to biobutanol production. (D. S. Lin et al., 2015). Optimum butyric acid and glucose ratio was revealed with *Clostridium saccharoperbutylacetonicum* N1-4 by Al-Shorgani et al. (2012). The metabolic pathway of butanol production shows that the butyric acid can be shifted to butanol production. In other words, butyric acid can act as accelerator of the butanol generation in dark fermentation. For this reason, 5 g/L of butyric acid was added to the fermentation medium as the activator of butanol production. The initial pH level was adjusted to 5.5 with dilute sulfuric acid in order to stimulate butyric acid production. The solvents as acetone, ethanol and butanol were monitored throughout the fermentation period. The initial acetone concentration was 0.12 g/L which is the concentration inherited form stock fermentation media. Although, a slight increase in the concentration occurs, no significant production was obtained (Figure 3.14). The second product in ABE fermentation was ethanol (Figure 3.15). At the beginning of fermentation, ethanol concentration was 0.89 g/L, and no significant change was observed. Unfortunately, no butanol production was obtained.

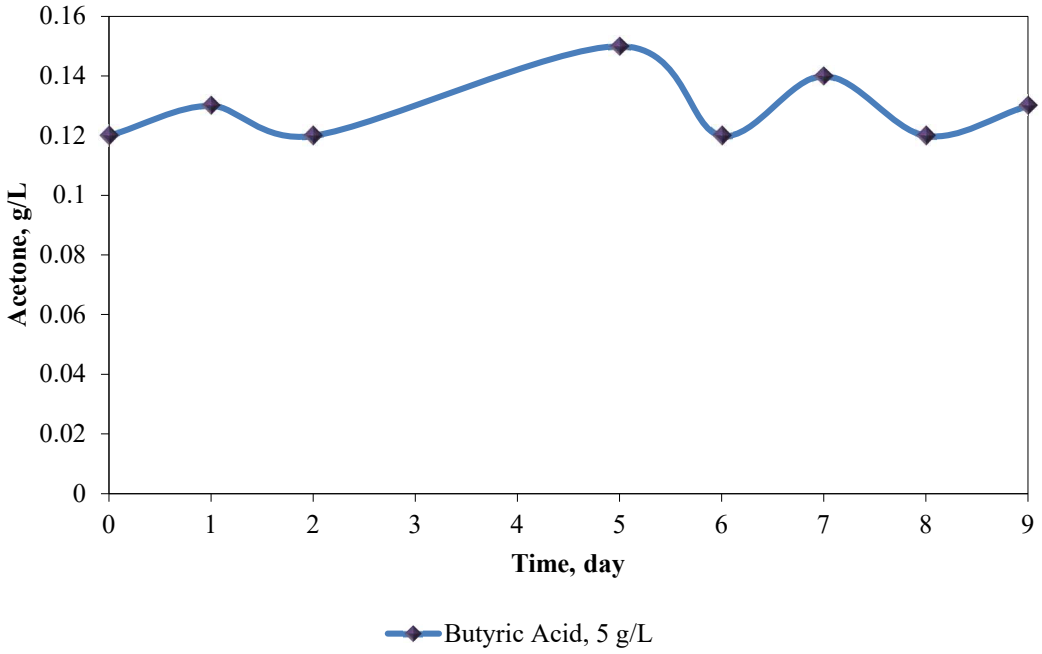


Figure 3.14 Variation of acetone production over time with butyric acid addition

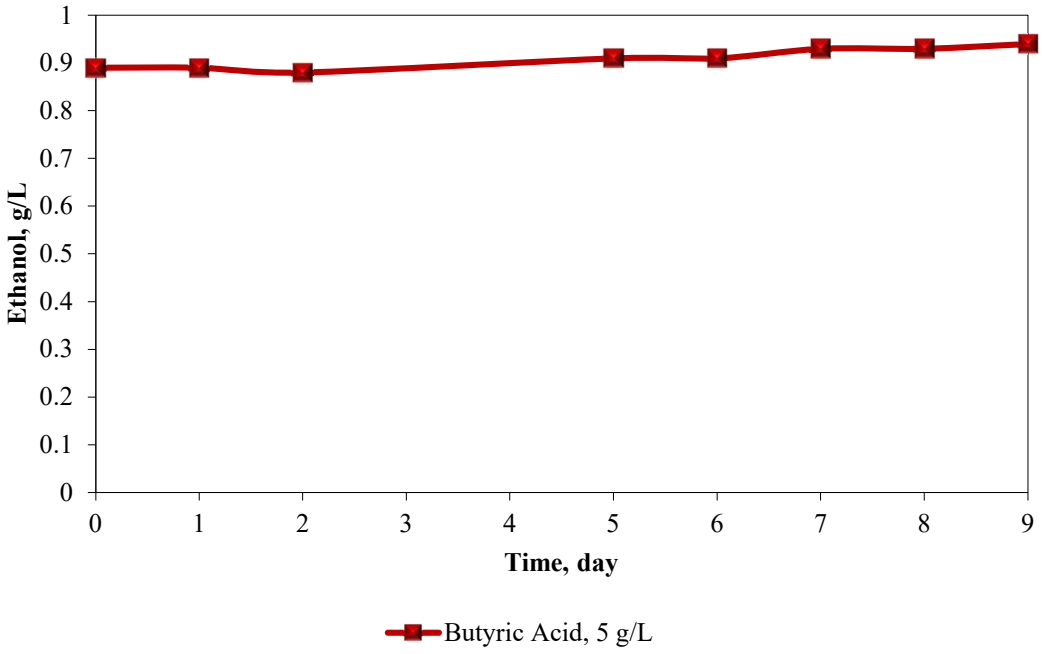


Figure 3.15 Variation of ethanol production over time with butyric acid addition

Variation of total sugar concentration with butyric acid addition was shown in Figure 3.16. Variations of glucose and fructose concentrations over time were given in Figure 3.17 and Figure 3.18, respectively. The initial total sugar concentration was

55 g/L and no reduction in concentration was obtained over time. The sum of glucose and fructose sugar concentrations was 47 g/L and sugar consumption was not observed during the fermentation process. No depletion in substrate source has been interpreted as either the organism was not viable or butyric acid concentration caused inhibition.

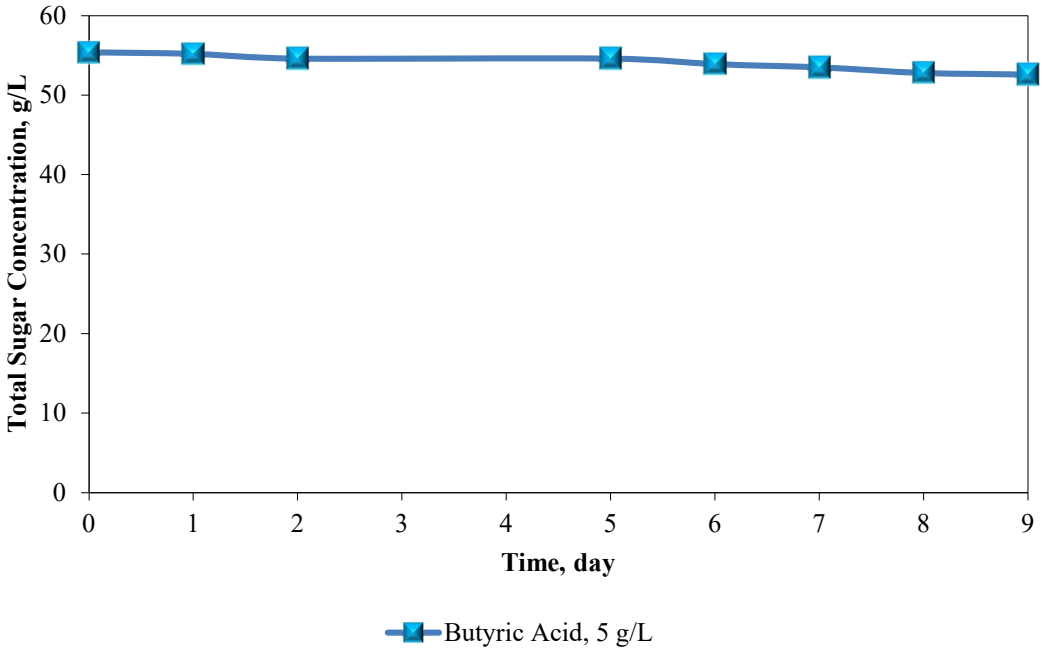


Figure 3.16 Consumption of total sugar concentration over time with butyric acid addition

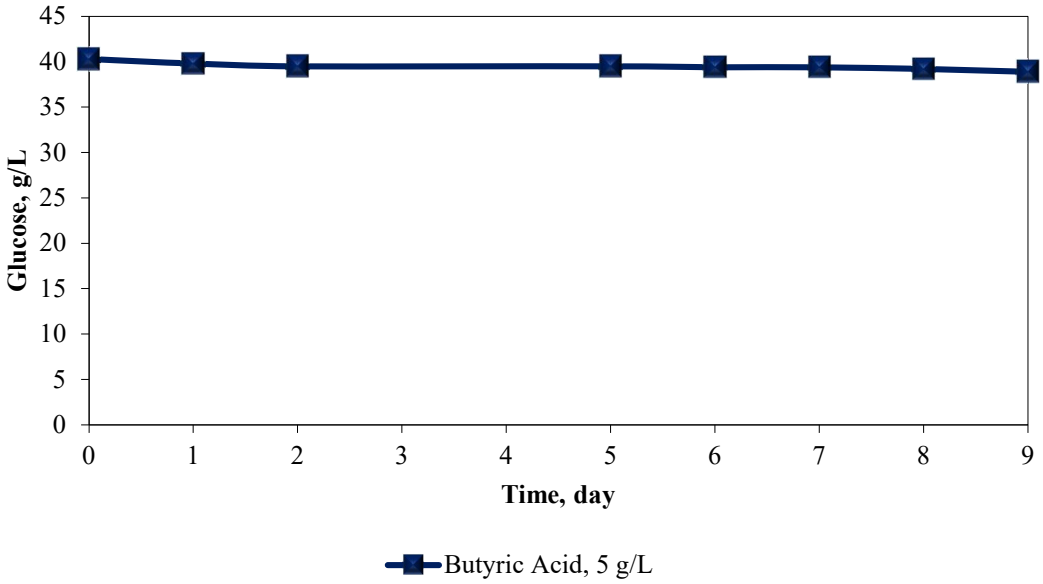


Figure 3.17 Consumption of glucose concentration over time with butyric acid addition

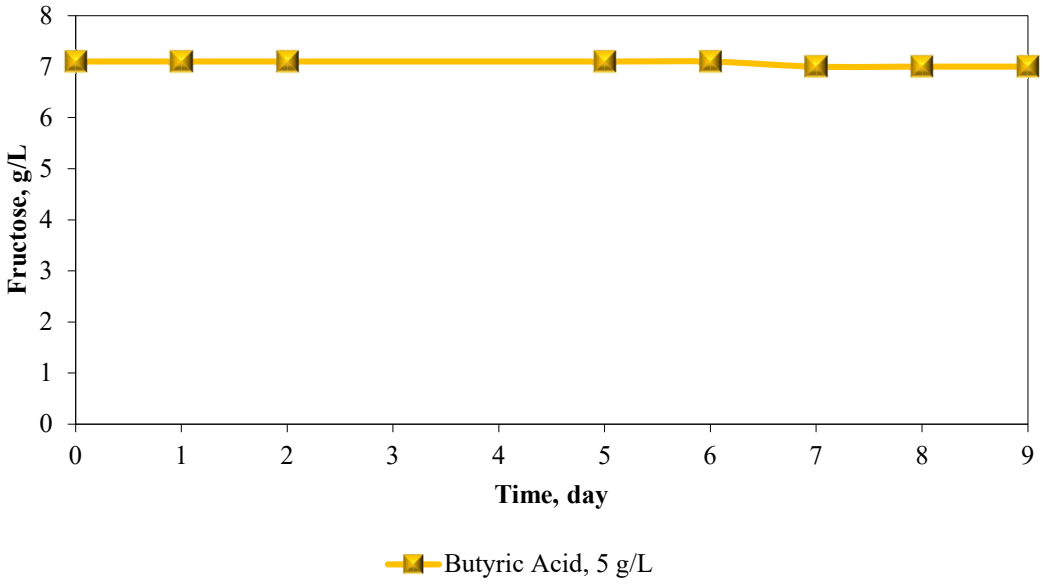


Figure 3.18 Consumption of fructose concentration over time with butyric acid addition

As seen in Figure 3.19, although there was a partial increase in acetic acid production, it only increased from 1.9 g/L to 2.1 g/L. Butyric acid concentration over time was given in Figure 3.20. Similarly, the butyric acid concentration was 4.7 g/L at the beginning, representing the added 5 g/L butyric acid concentration for activation. Additional butyric acid was not consumed. As seen in Figure 3.21, there was not any change in pH during fermentation, due to no organic acid formation.

Butyric acid was added to the experimental medium as butanol activator, but organic matter consumption and product formation were not observed. Since there was no formation of volatile fatty acids in the media, the pH remained constant throughout the experiment.It was concluded that the added butyric acid concentration (5 g/L) might have inhibited the growth of organisms, prevented organic matter consumption and product formation.

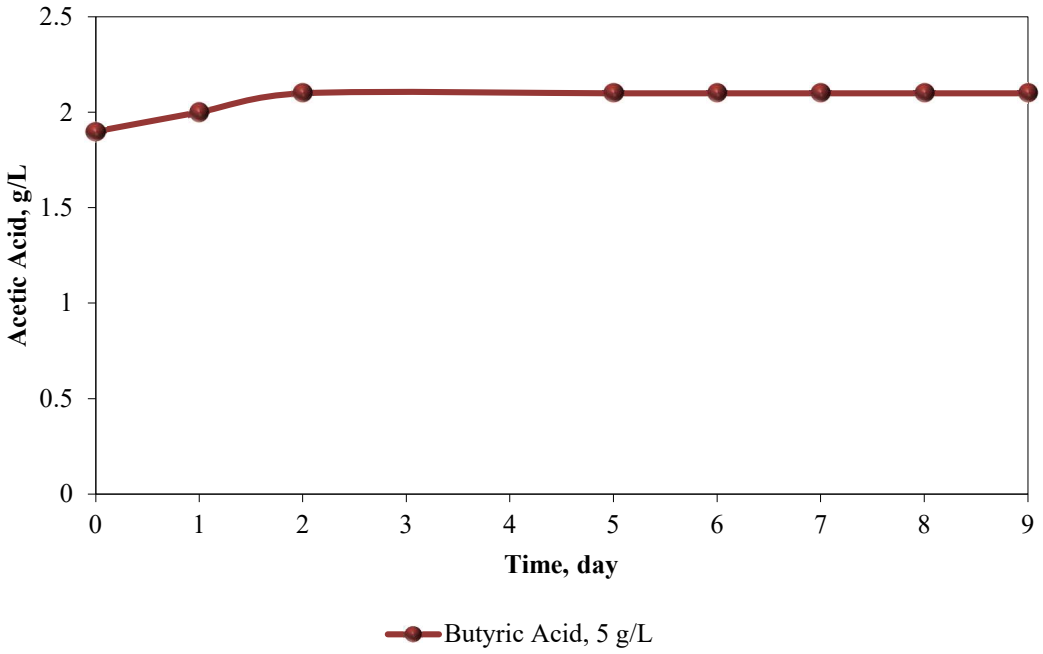


Figure 3.19 Variation of acetic acid concentration over time with butyric acid addition

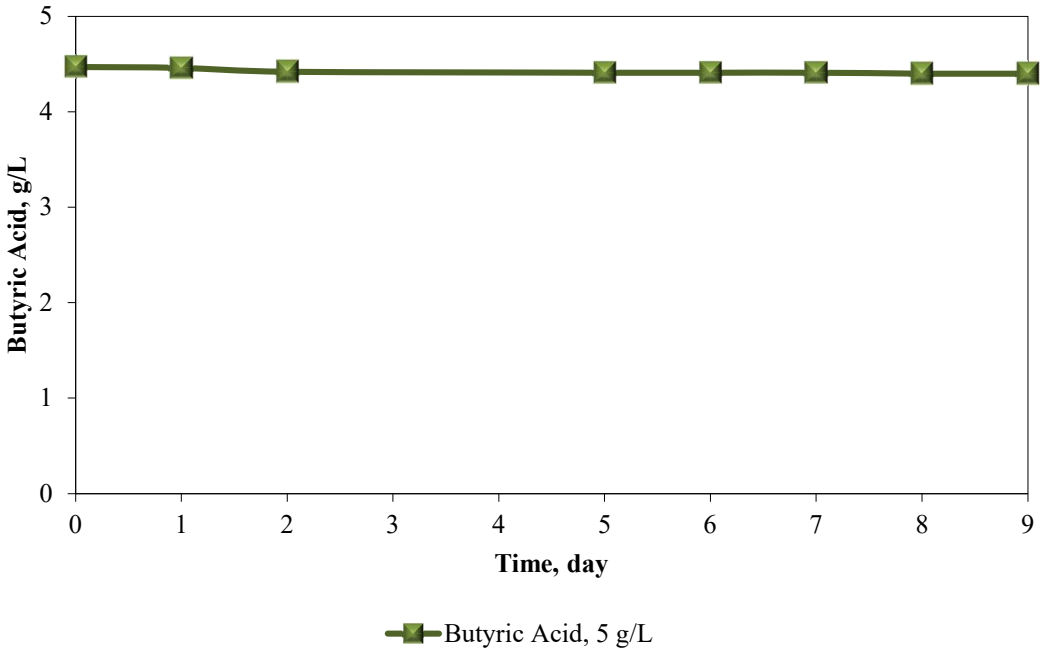


Figure 3.20 Variation of butyric acid concentration over time with butyric acid addition

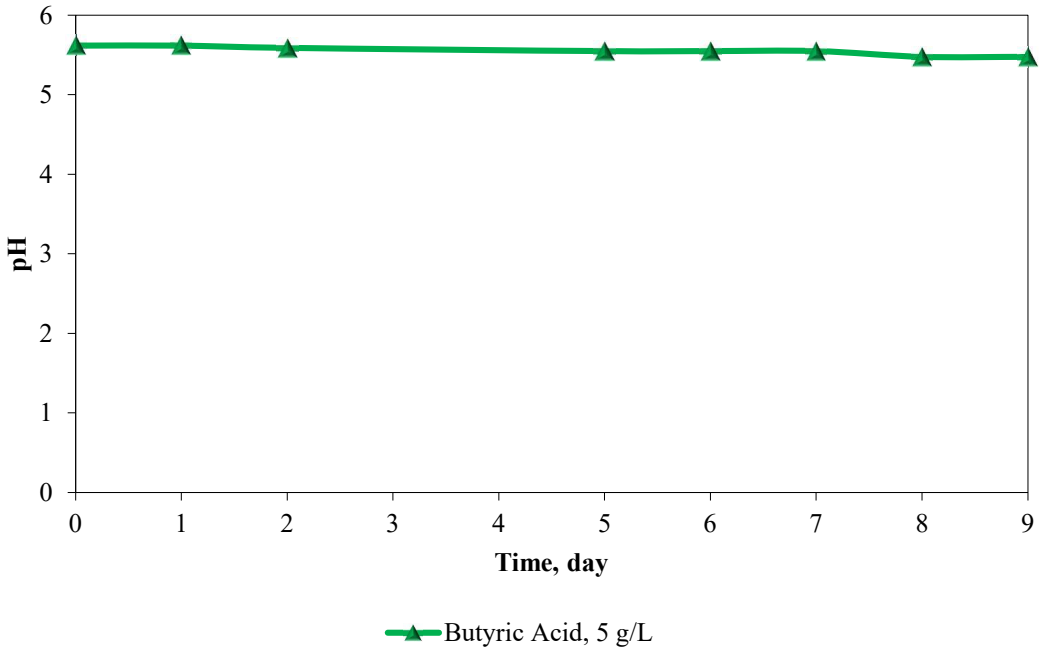


Figure 3.21 Variation of pH with butyric acid addition

3.2.3 Effect of Magnesium, Manganese and Zinc

Microorganisms interact with several ions that affect the metabolic pathway for the butanol production. Addition or limitation of these ions could increase or inhibit the enzymatic activity of Clostridium sp. Combined effects of zinc supplementation and magnesium deficiency could lead to enhancement in glucose conversion, reduction in ethanol concentration and early initiation of solvent production, by metabolic regulation (Mukherjee et al., 2019). As butanol and butyric acid activators, magnesium (Mg), manganese (Mn), zinc (Zn) were added to the fermentation media in concentrations of 60, 40, 40 mg/L, respectively. Initial pH levels were adjusted to 5.6 for each medium with dilute sulfuric acid (60% H_2SO_4) to provide the production of butyric acid. Ethanol concentrations were given in Figure 3.22. Considering the initial levels, ethanol production did not occur in the following days.

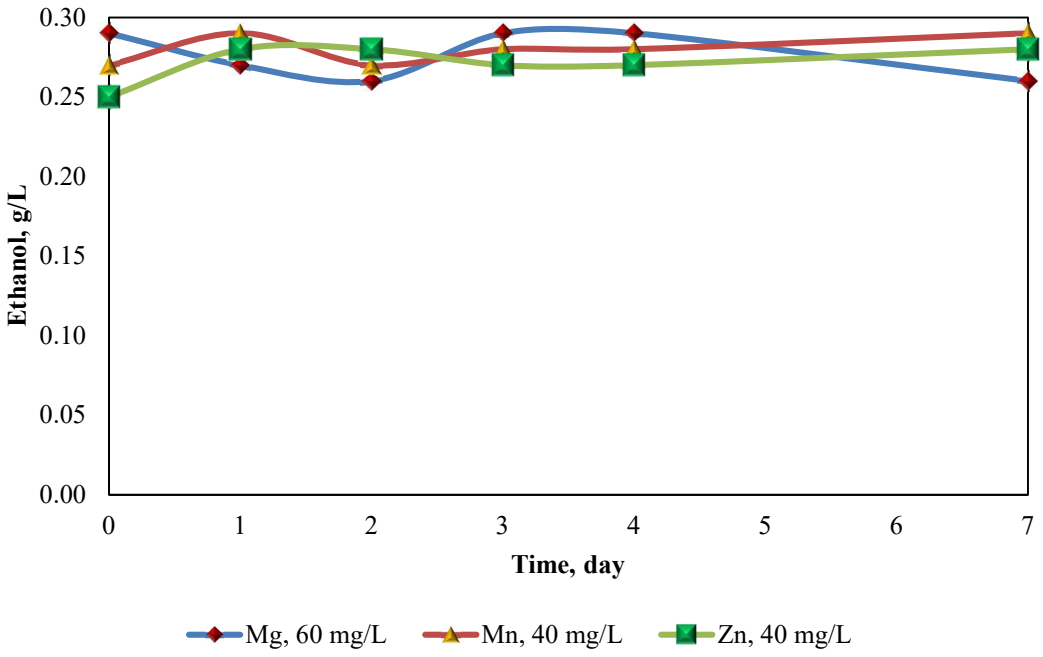


Figure 3.22 Variation of ethanol production with time on the addition of Mg, Mn, Zn

The initial total sugar concentration was 50 g/L.The sum of glucose and fructose concentrations was 52 g/L.As can be seen in Figure 3.23, total sugar was not consumed. Similarly, as in like Figure 3.24 and Figure 3.25, no significant consumption was observed in glucose and fructose concentrations.

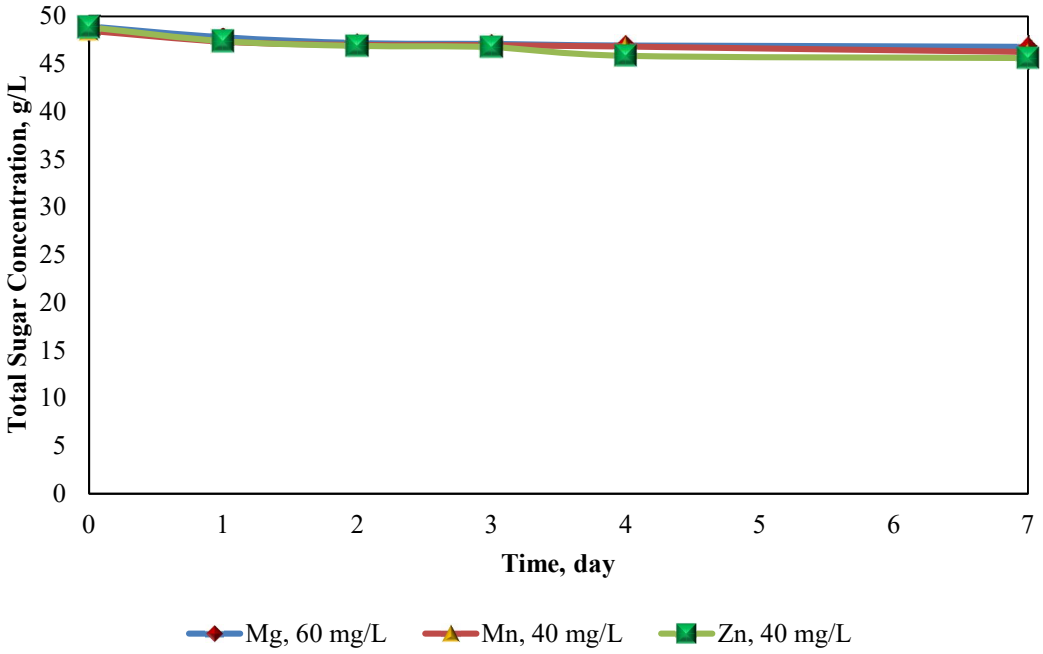


Figure 3.23 Variation of total sugar with time in addition of Mg, Mn, Zn

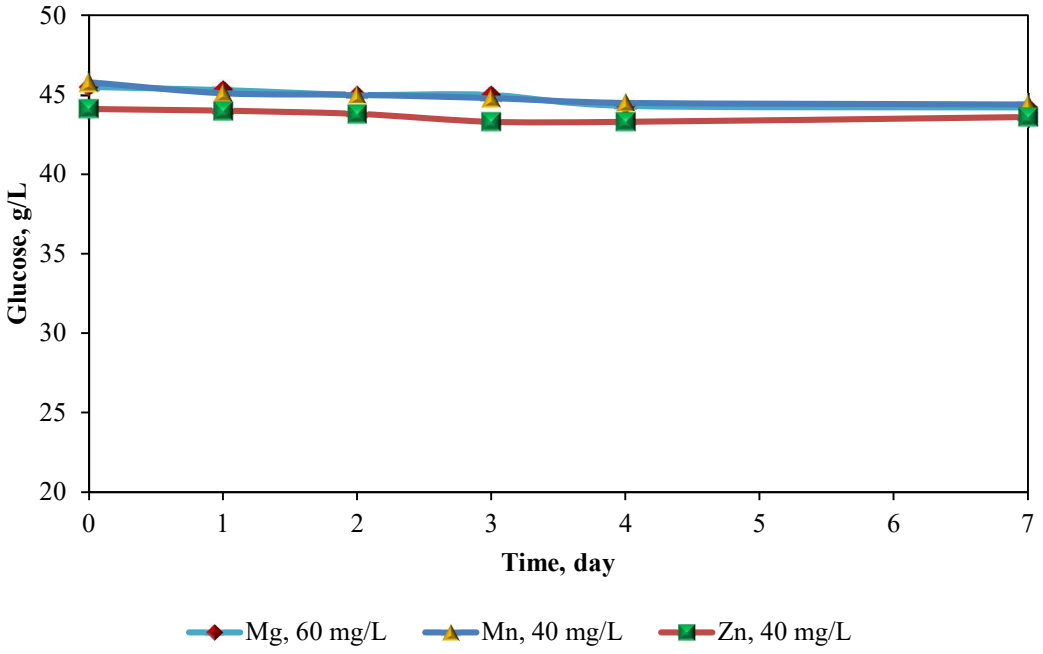


Figure 3.24 Variation of glucose with time on the addition of Mg, Mn, Zn

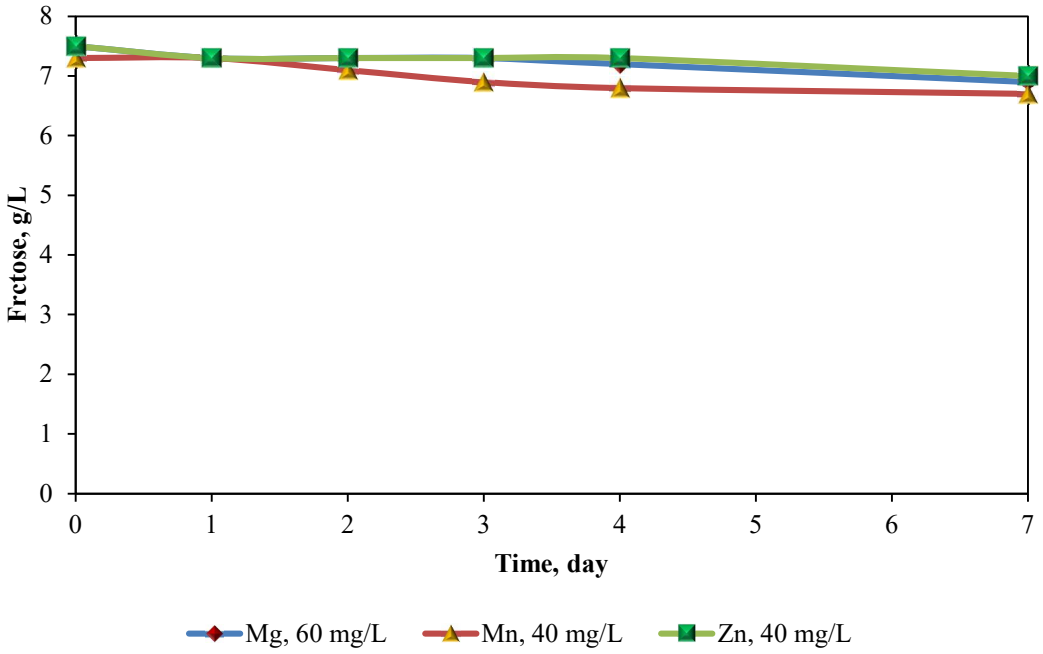


Figure 3.25 Variation of fructose with time on the addition of Mg, Mn, Zn

Acetic acid concentrations and pH levels were shown in Figure 3.26 and Figure 3.27, respectively. When the organic acid concentrations were evaluated, the acetic acid concentration remained constant at the initial conditions of 1.8 g/L. Therefore, there was no change in pH level.

Consequently, the addition of Mg, Mn, and Zn as butyric acid activators had negative effects on organisms and acted as inhibitors, instead of activators.

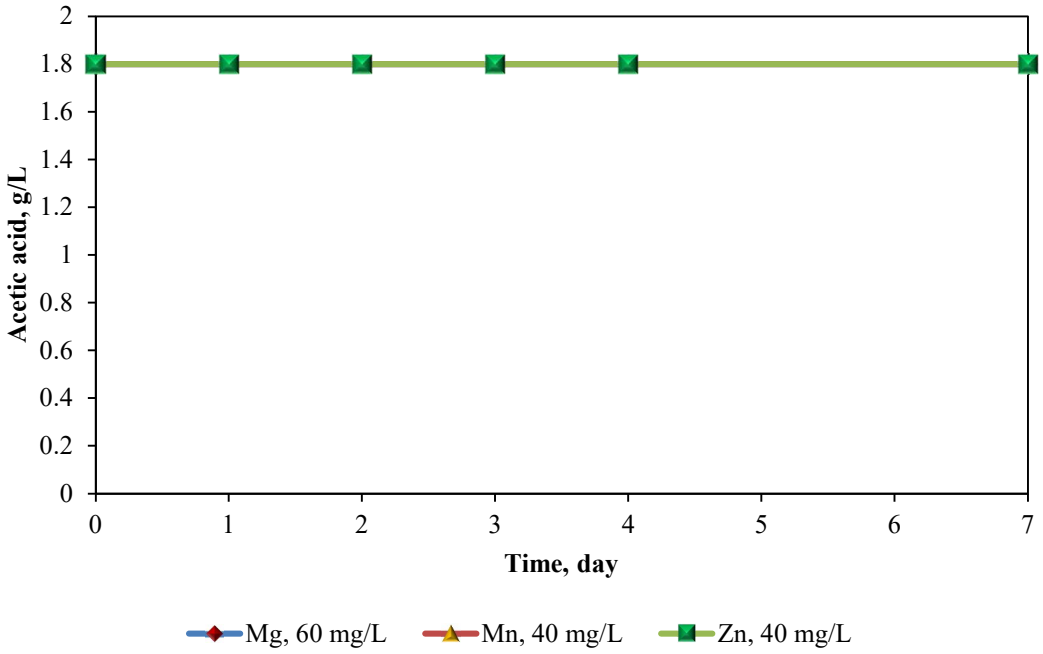


Figure 3.26 Variation of acetic acid with time on the addition of Mg, Mn, Zn

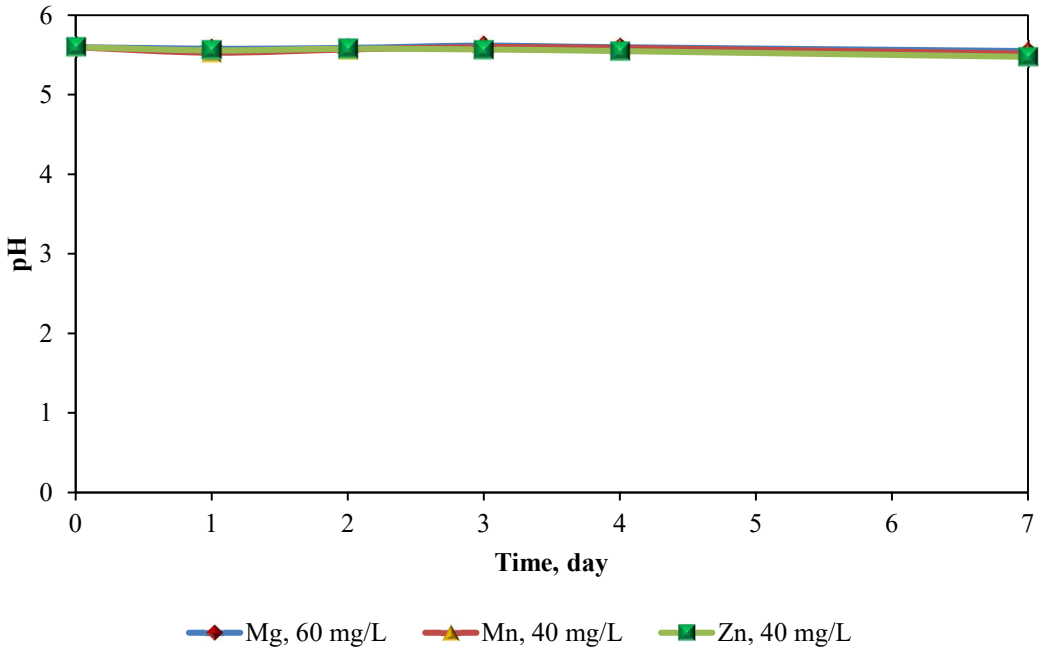


Figure 3.27 Variation of pH with time on the addition of Mg, Mn, Zn

As the second stage of the experimental studies, magnesium (Mg), manganese (Mn), zinc (Zn) in the medium were increased to 80, 60, and 60 mg/L, respectively.Initial pH levels were adjusted to 5.7 for each medium with dilute

sulfuric acid to provide the production of butyric acid.Figure 3.28 shows the change in ethanol concentration. No significant change was observed at the initial concentration of 0.75 g/L. Similarly, as in Figure 3.29, the acetone concentration remained constant at the initial condition of 0.15 g/L.

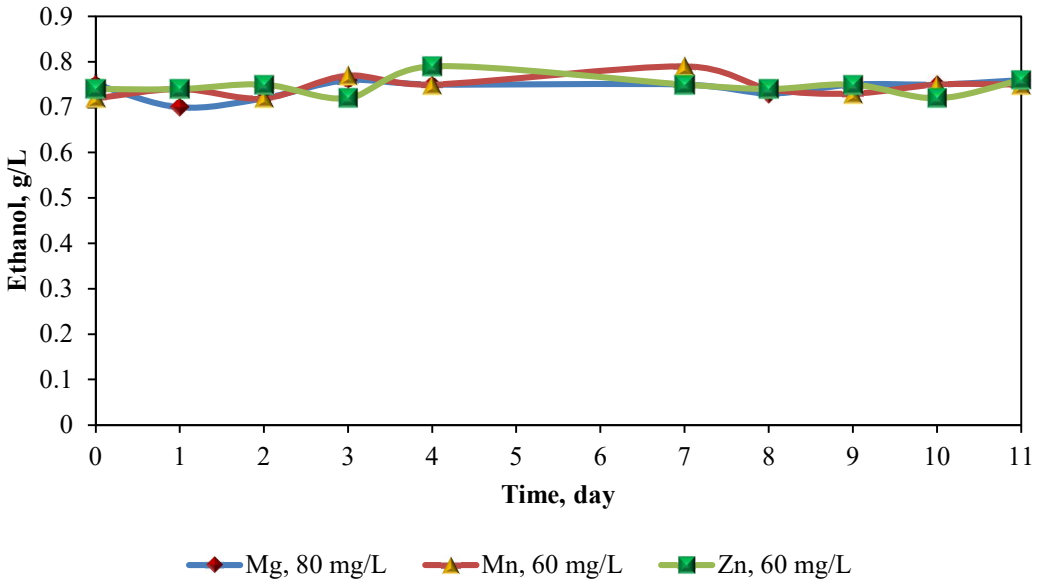


Figure 3.28 Variation of ethanol production with time on the addition of Mg, Mn, Zn

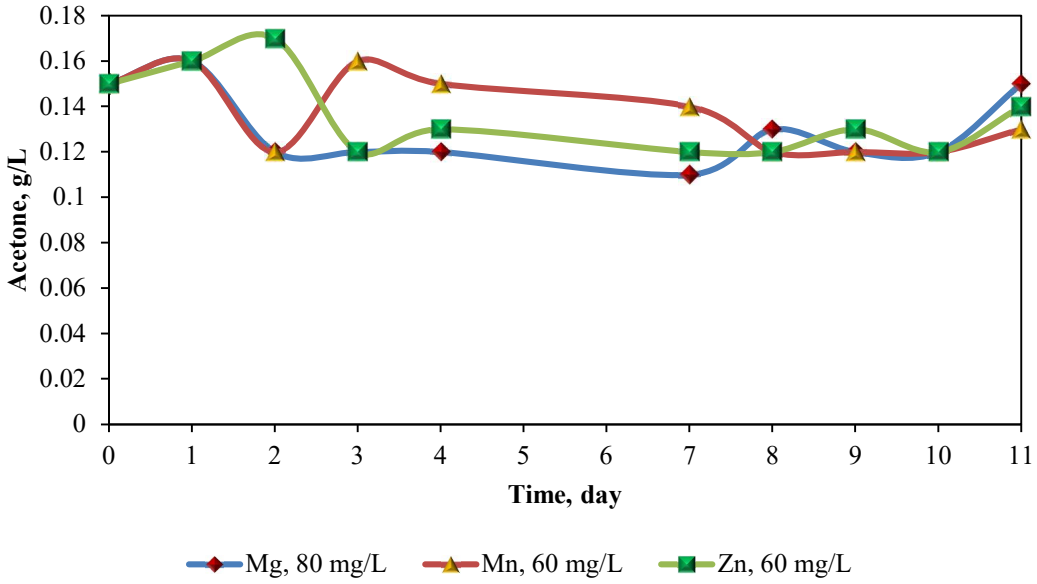


Figure 3.29 Variation of acetone production with time on the addition of Mg, Mn, Zn

The total sugar concentration was given in Figure 3.30 and the consumption of glucose and fructose concentrations were shown in Figure 3.31 and Figure 3.32, respectively. The initial total sugar concentration was 53 g/L and sugar was effectively consumed to the level of 5-6 g/L. The sum of glucose and fructose sugar concentrations was around 60 g/L. Fructose was consumed at a higher level than glucose.

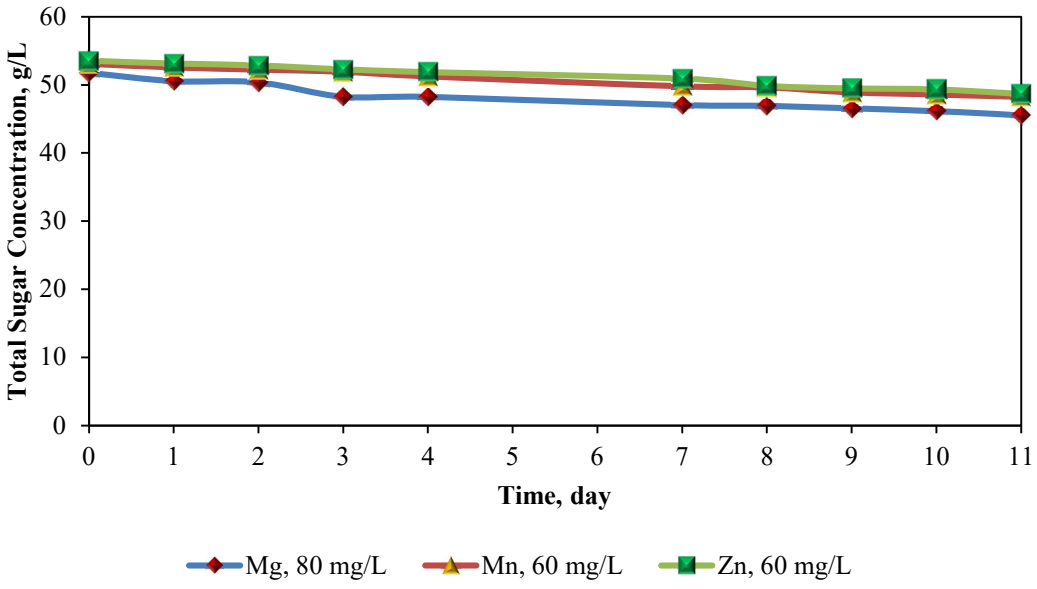


Figure 3.30 Variation of total sugar with time in addition of Mg, Mn, Zn

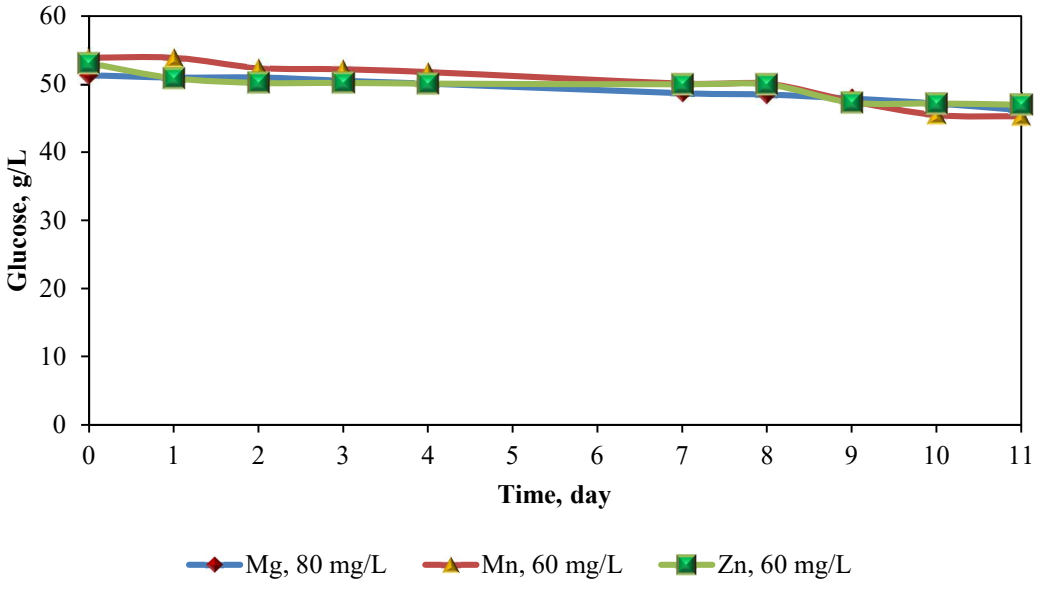


Figure 3.31 Variation of glucose with time on the addition of Mg, Mn, Zn

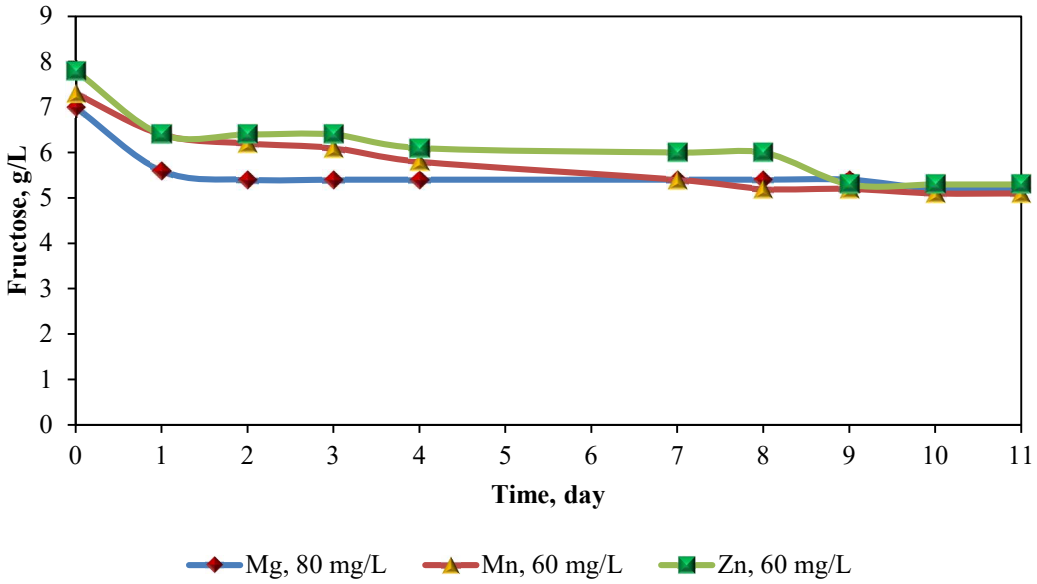


Figure 3.32 Variation of fructose with time on the addition of Mg, Mn, Zn

The change in the concentration of acetic acid and pH was given in Figure 3.33 and Figure 3.34, respectively. For the concentration of Mg at 80 mg/L, a very low amount of sugar was consumed in the fermentation medium and the total sugar was consumed up to 45 g/L. The initial concentration of acetic acid was 1.9 g/L and, the concentration remained at the same level considering the measurements of the following days. At the end of the experiment, the pH level remained constant at 5.5.

For the concentration of Mn at 60 mg/L, a very low amount of sugar was consumed in the experimental environment and, the total sugar remained around 48 g/L.The initial concentration of acetic acid was 1.9 g/L, and when the measurements of the following days were considered, no acetic acid production has occurred. The pH level remained constant at 5.4 at the end of the experiment.

For the condition of Zn concentration at 60 mg/L; likewise, a very low amount of sugar was consumed in this experimental environment and, the final total sugar was 49 g/L. The acetic acid level was at the level of 1.9 g/L according to the measurements of the first day and remained constant at this level throughout the experiment since there was no production in the following days. pH remained constant at 5.5.

In conclusion, 80 mg Mg /L, 60 mg Mn /L, 60 mg Zn /L were added to the fermentation medium as butyric acid activators and, they inhibited acetone production and ethanol production but they did not support butanol production.

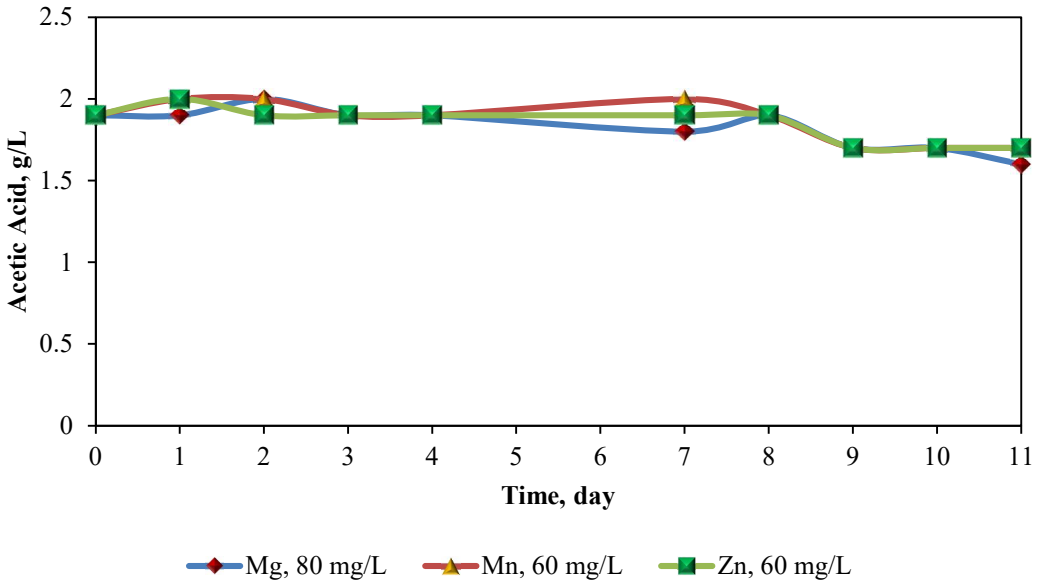


Figure 3.33 Variation of acetic acid with time on the addition of Mg, Mn, Zn

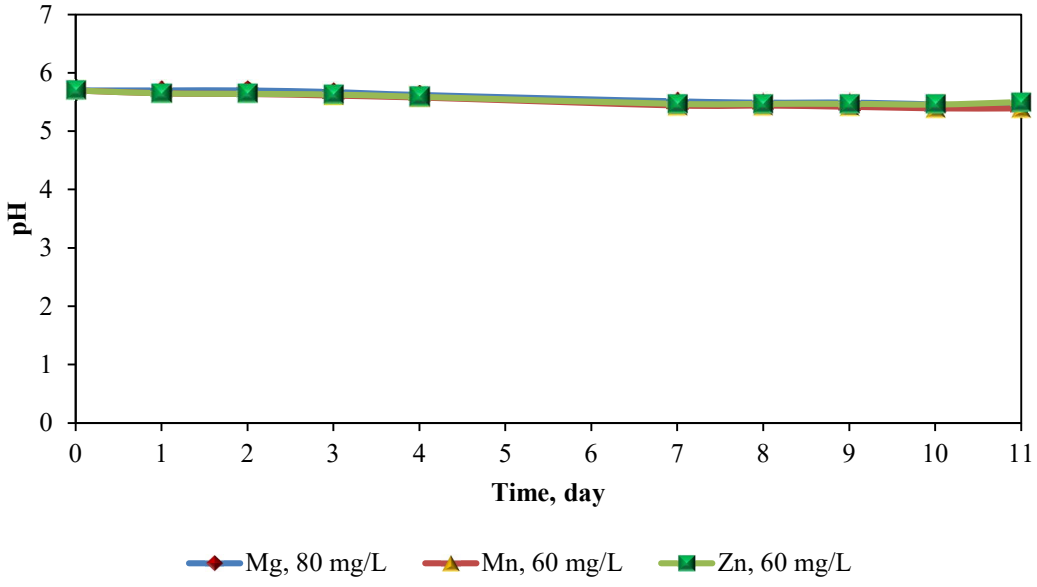


Figure 3.34 Variation of pH with time on the addition of Mg, Mn, Zn

Mg, Mn and Zn concentrations were increased to 220 mg/L, 200 g/L and 200 mg/L, respectively, in order to evaluate the effect of these trace elements, which are

stated to provide activation of butanol production in the literature. Initial pH levels were adjusted to 5.6 for each medium with dilute sulfuric acid to support the production of butyric acid. Changes in ethanol and acetone concentrations were shown in Figure 3.35 and Figure 3.36, respectively. According to the first day measurements, the initial ethanol and acetone concentrations were 0.7 and 0.15 g/L in all three media. Compared to the results of the first day, it could be concluded that there was not any solvent production.

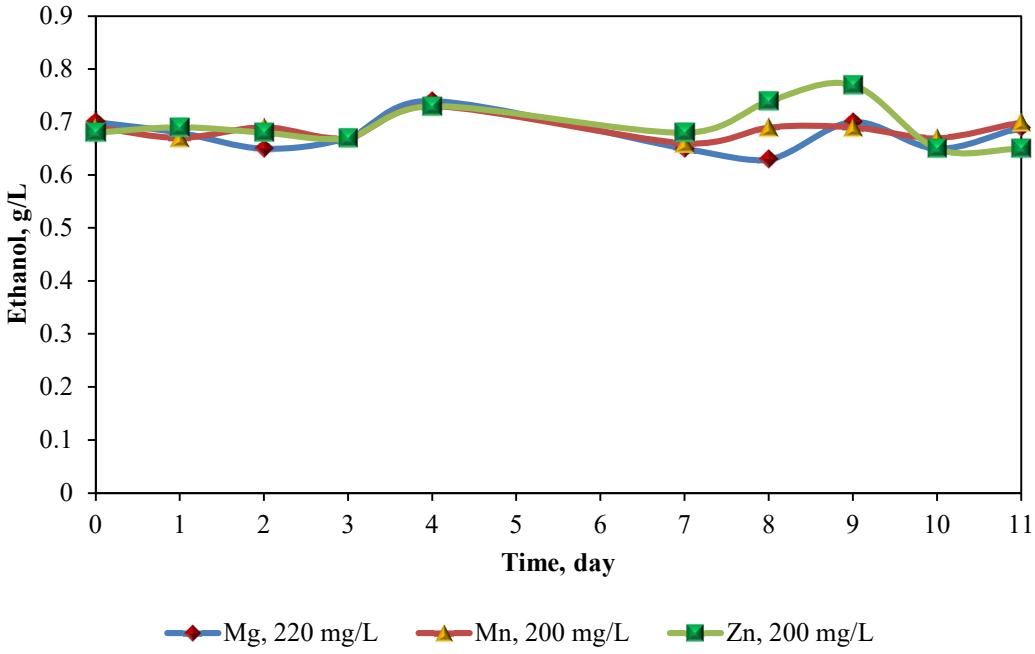


Figure 3.35 Variation of ethanol production with time on the addition of Mg, Mn, Zn

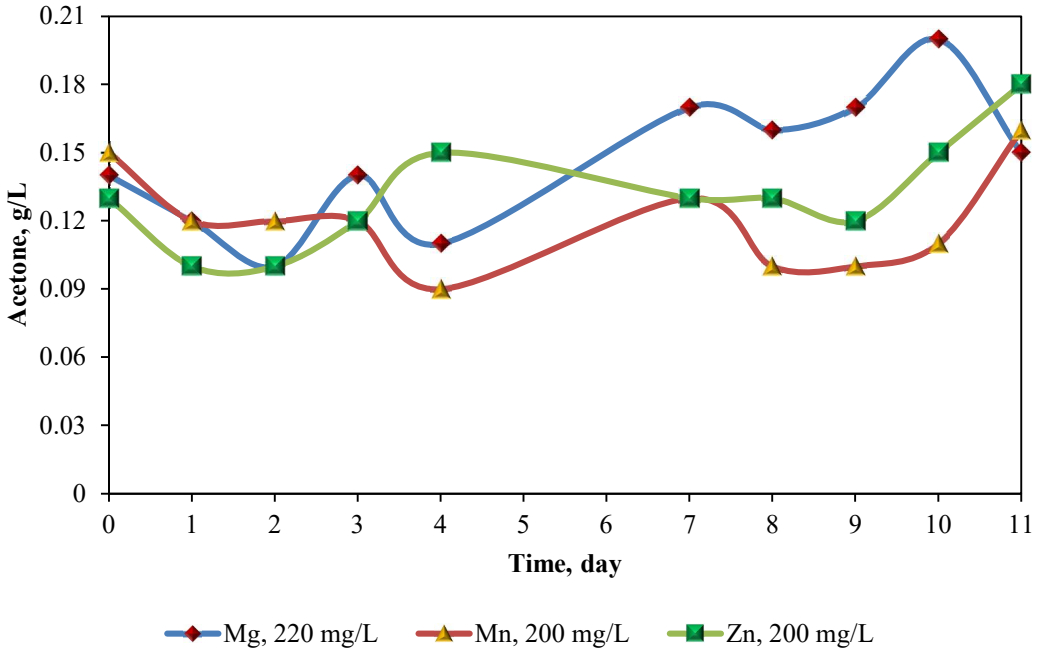


Figure 3.36 Variation of acetone production with time on the addition of Mg, Mn, Zn

Consumption of total sugar, glucose and fructose were given in Figure 3.37, Figure 3.38 and Figure 3.39, respectively. The initial total sugar concentration was 61 g/L, while the sum of glucose and fructose concentrations was 50 g/L. Total sugar consumption was approximately 10 g/L. Glucose and fructose consumptions were only 5 and 2 g/L.

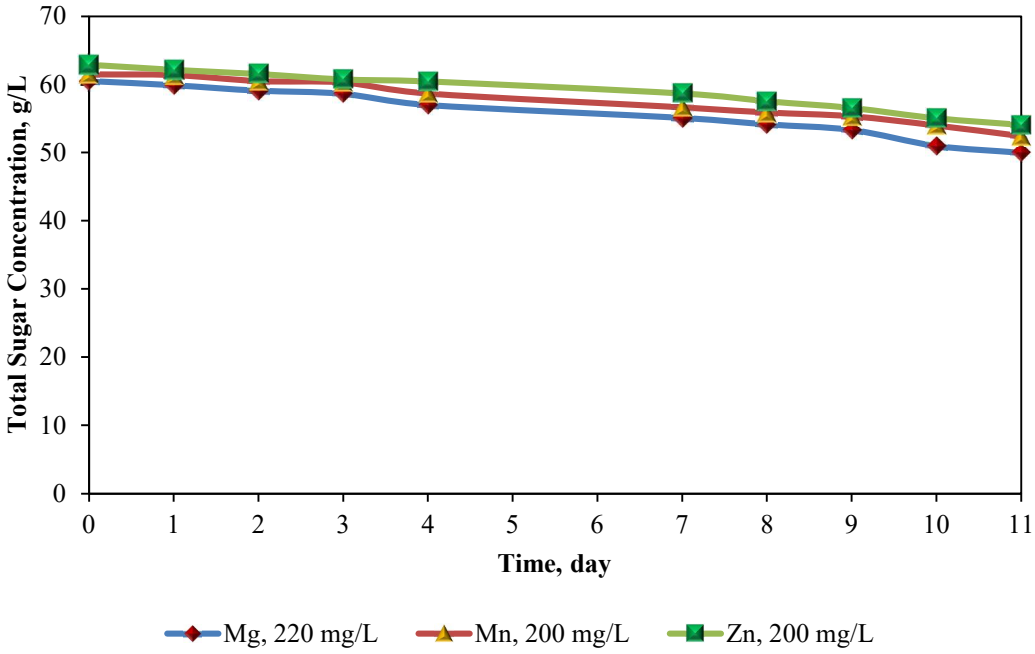


Figure 3.37 Variation of total sugar with time in addition of Mg, Mn, Zn

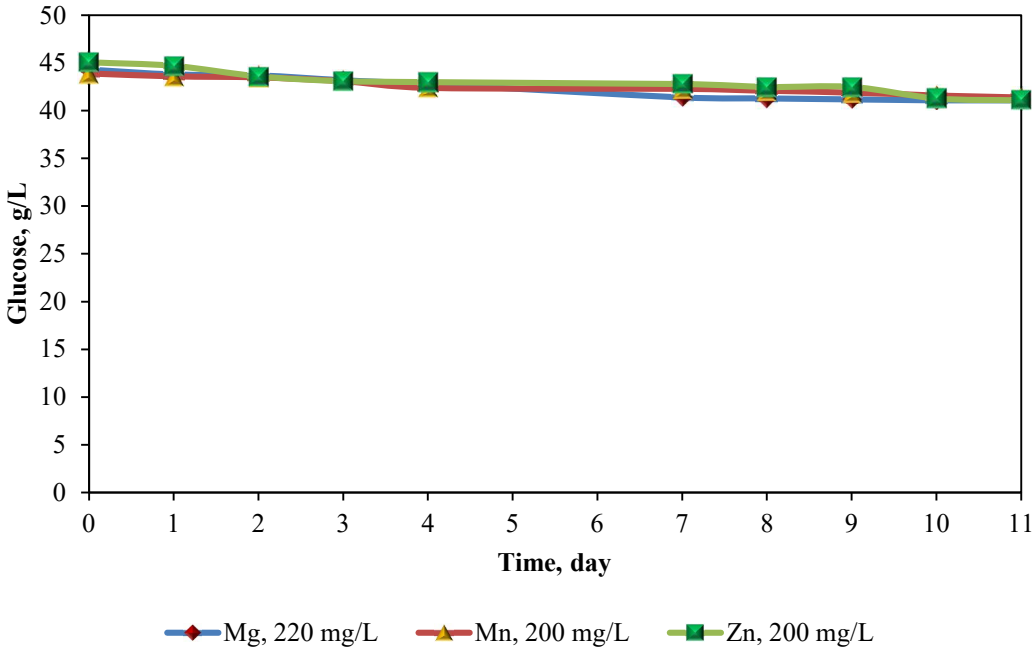


Figure 3.38 Variation of glucose with time in addition of Mg, Mn, Zn

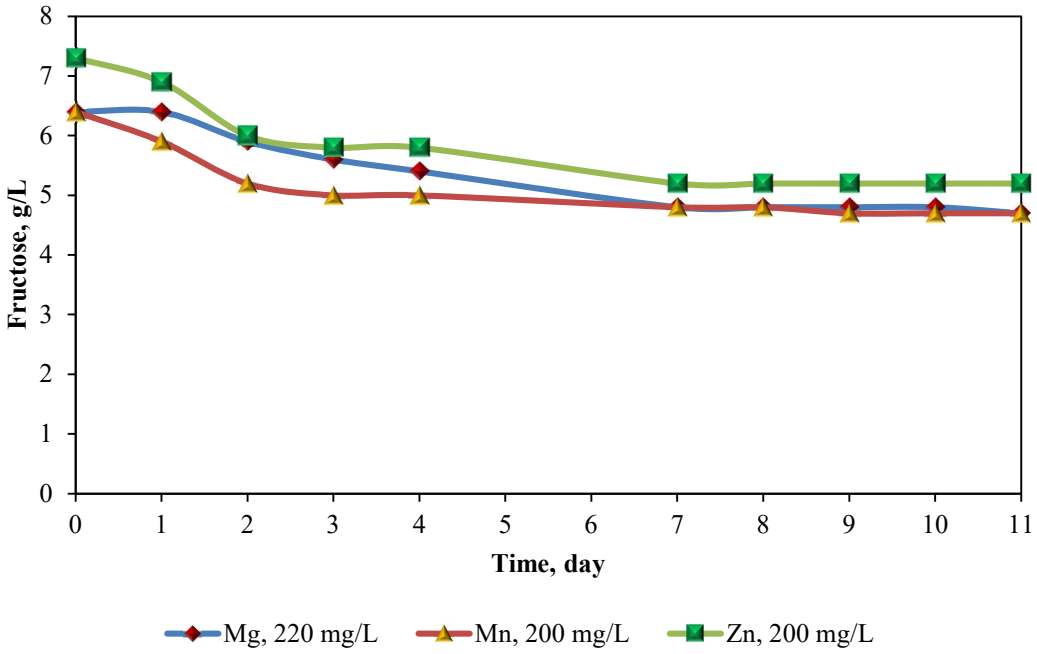


Figure 3.39 Variation of fructose with time in addition of Mg, Mn, Zn

Changes in organic acids and pH levels were given in Figure 3.40 and Figure 3.41, respectively. For the Mg concentration of 220 mg/L, initial acetic acid concentration was 1.7 g/L. Considering the measurements of the following days, a low amount of acetic acid was produced and it remained stable at 1.9 g/L. On the seventh day of the fermentation, the pH decreased to 5.3 and no substantial changes was observed for the rest of the fermentation period.

The sugar consumption at Mn=200 mg/L was very low. The initial concentration of acetic acid was 1.6 g/L, and it did not change for the fermentation period. At the end of the experiment, the pH level remained constant at 5.4 which indicates no organic acid production.

Similarly, Zn=200 mg/L did not provide any improvement in acetic acid production or sugar consumption. No changes in pH were observed.

It has been seen that the addition of Mg, Mn and Zn at high concentrations as butyric acid activators inhibited ethanol and acetone production and they did not support butanol production. Very low sugar consumption was observed in all three

mediums, but almost no product formation was obtained. Although the pH decrease in Mg added medium was slightly higher than the other two mediums, no significant effect was observed on sugar consumption and product formation.

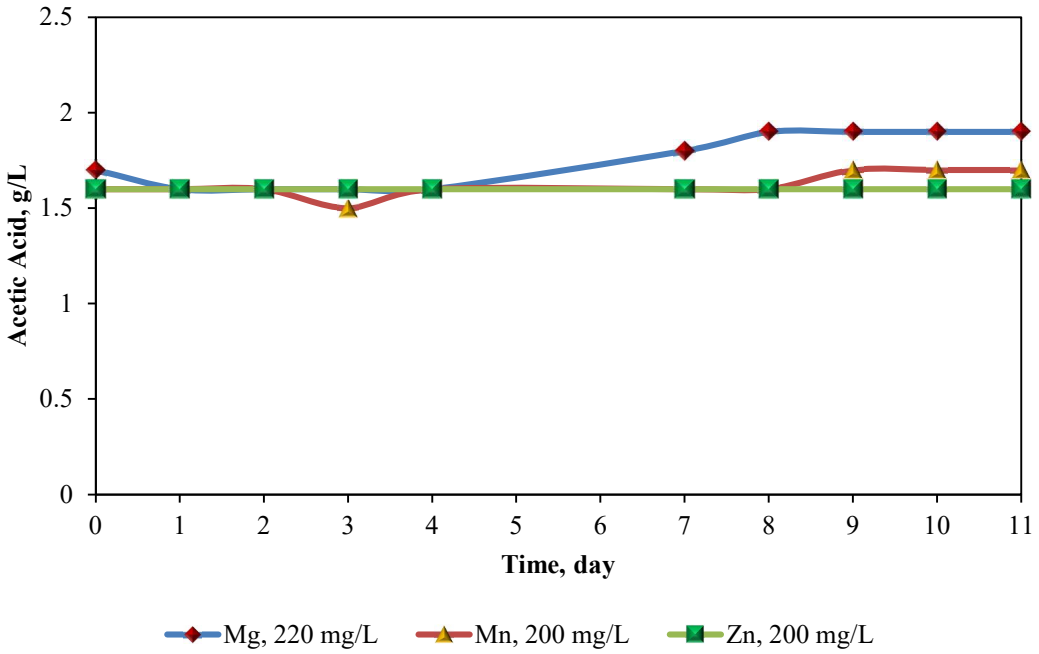


Figure 3.40 Variation of acetic acid with time in addition of Mg, Mn, Zn

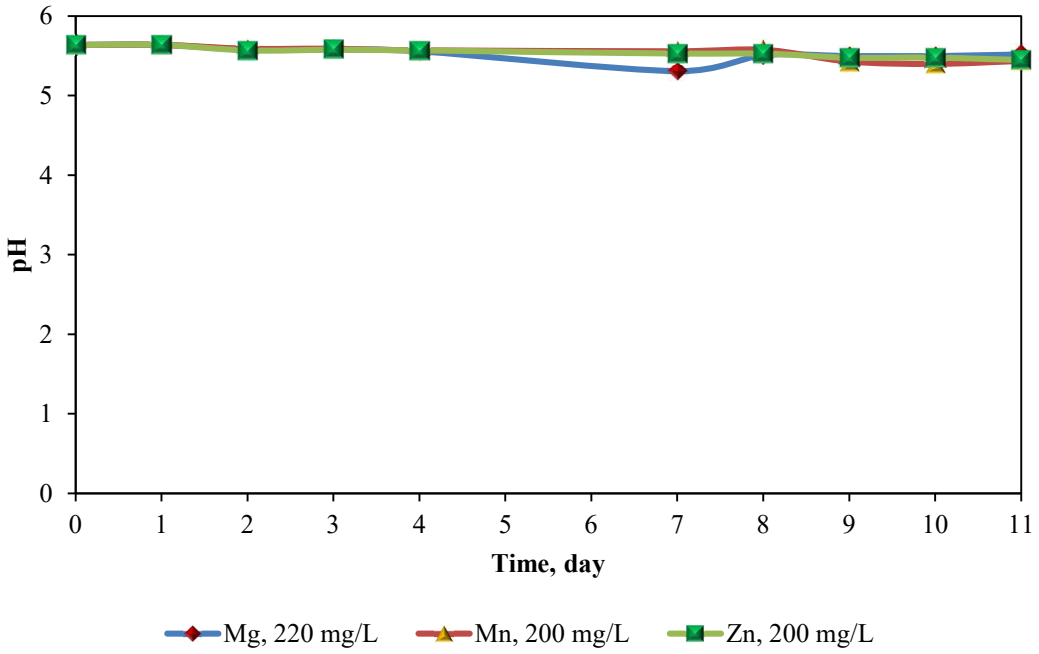


Figure 3.41 Variation of pH with time on the addition of Mg, Mn, Zn

3.3 Dark Fermentation with *Clostiridium pasteurianum*

The solvent production by *Clostiridium acetobutylicum* was not effective. Therefore a new culture which is capable of producing solvent was used. The effects of media composition in terms of inhibitors and activators were investigated.

3.3.1 Addition of Magnesium, Manganese, Zinc and Neutral Red

Microorganisms interact with several ions and addition or limitation of these ions could increase or inhibit the enzymatic activity of Clostridium sp. Another factor that affects butanol production is the amount of NAD(P)/NAD(P)H released during glycolysis. 4 moles of NADH are necessary to produce 1 mol of butanol from glucose. In order to overcome NADH limitation, neutral red as an electron carrier could be used. Thus, conversion of carbon source is directed to solvent production instead of acid formation (C. Jiang et al., 2016; Y. Jiang et al., 2009; C. Wang et al., 2018).

As a butanol and butyric acid activators on fermentation media, magnesium (Mg), manganese (Mn), zinc (Zn), neutral red were added to the medium in concentrations of 200 mg/L, 200 mg/L, 200 mg/L, 289 mg/L. The variation of acetone generation with time at different ion supplements was given in Figure 3.42. It is clearly seen that Mg and Zn enhances acetone production up to 10 g/L, but neutral red inhibits the generation. In order to observe that if excess carbon can enhance the acetone, 20 g/L sugar was added into the media externally on the 16th day. A sudden increase in acetone concentration to 12 g/L and 9 g/L was observed fro Mg and Zn added media, respectively. Neutral red was added to the media as acetone and ethanol inhibitor, and acetone production did not occur. Similarly, manganese addition did not result in acetone production. Although a lower level (5 g/L) acetone production occurred in the control experiments, the production accelerated with sugar addition and increased to 10 g/L.

Variation of ethanol concentration with time was shown in Figure 3.43. While neutral red and manganese inhibited ethanol production, magnesium and zinc did not stop ethanol production. Although the concentration reaches 0.5 g/L in the magnesium added nutrient medium, the highest production was observed in the control experiments where no butanol activator was added. However, butanol production did not occur.

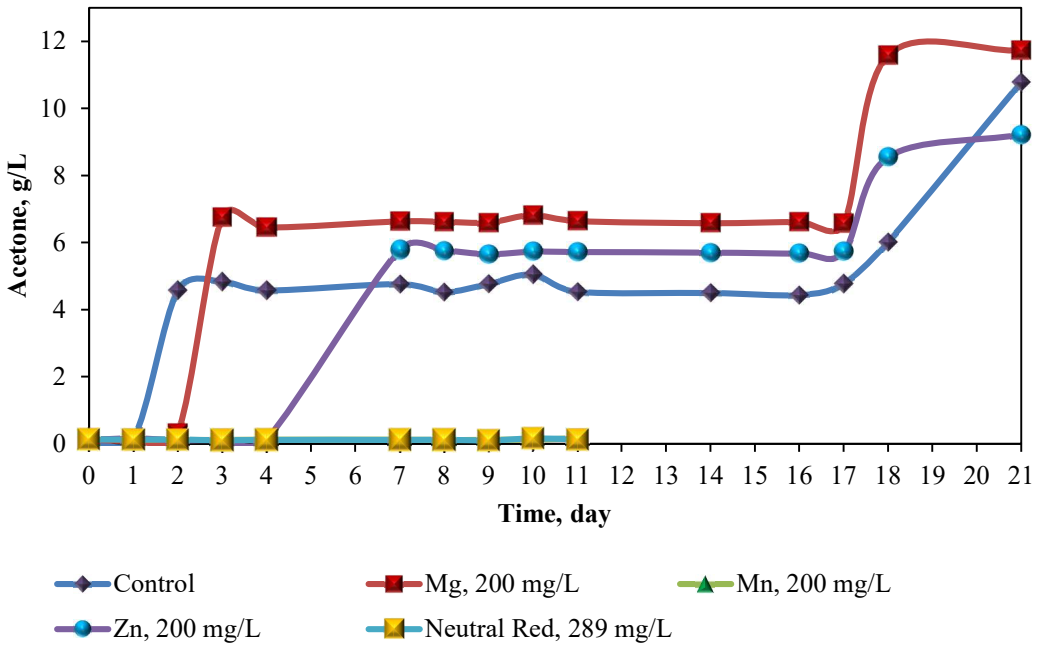


Figure 3.42 Variation of acetone production over time with the addition of Mg, Mn, Zn and neutral red

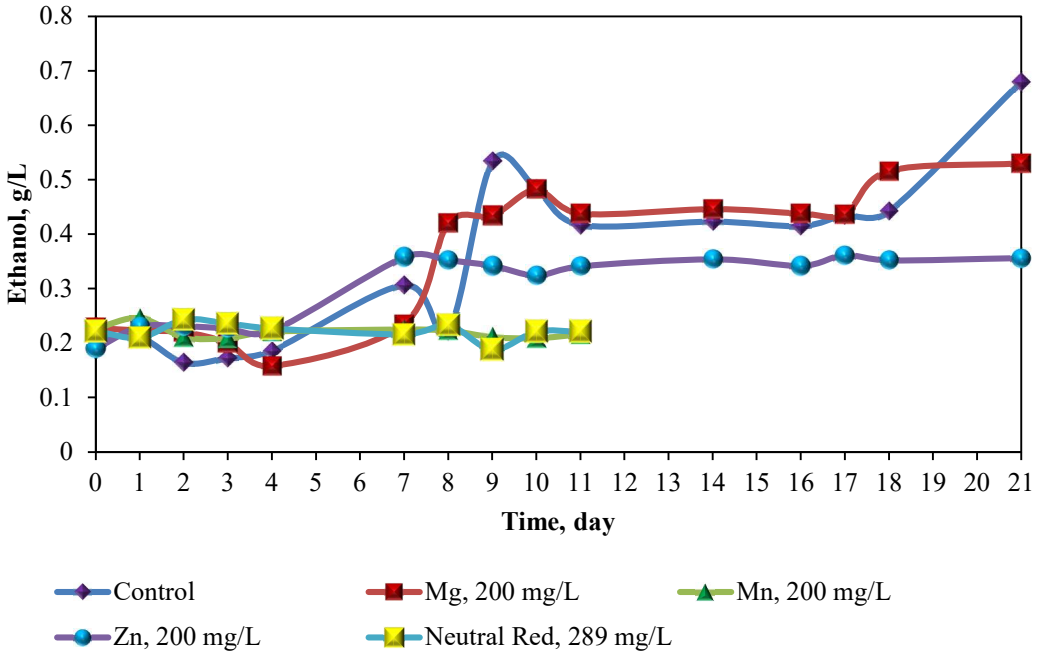


Figure 3.43 Variation of acetone production over time with the addition of Mg, Mn, Zn and neutral red

The initial total sugar concentration was 33 g/L. The variation of total sugar consumption over time was given in Figure 3.44. Consumptions of glucose and fructose were shown in Figure 3.45 and Figure 3.46, respectively. Acetic and butyric acid concentrations were given in Figure 3.47 and Figure 3.48. pH change was shown in Figure 3.49.

In the control experiments, all of the sugar was consumed. 9 g/L of acetic acid was produced, and then consumption began. Butyric acid production reached a concentration of 3 g/L. Acetone production was 5 g/L while the ethanol was 0.4 g/L.On the 16th day of the experiment, 20 g/L sugar was added to the media to achieve butanol production. After this addition, all of the sugar was consumed, resulting in acetic acid concentration of 9 g/L and butyric acid of 7 g/L. Acetone production was 10 g/L and, ethanol concentration was 0.7 g/L.

The final total sugar concentration was 1 g/L under the fermentation conditions where magnesium (Mg) was added.Glucose is completely consumed and the remaining total sugar was equivalent to fructose. Acetic acid reached the level of 9

g/L and, then consumption started. The concentration of butyric acid was 4.5 g/L. Acetone and ethanol concentrations were 6.8 g/L and 0.5 g/L, respectively. Likewise, 20 g/L sugar was added to the medium on the 16th day of the experiment to ensure butanol production. Similar to the control group, by consuming all of the sugar, the concentration of acetic acid reached 11 g/L and butyric acid reached 8 g/L. Acetone production was 11 g/L, while ethanol concentration remained constant at 0.5 g/L.

No sugar was consumed under manganese (Mn) added fermentation conditions.Acetic acid was at 1 g/L, butyric acid was not produced. The acetone concentration was 0.1 g/L while the ethanol concentration was 0.2 g/L. Compared to the first day measurements, ethanol and acetone production did not occur in the following days.

In zinc (Zn) added medium, similar to the control and magnesium (Mg) groups, all of the sugar was consumed. The remaining total sugar was the fructose equivalent. Acetic acid and butyric acid levels were 6 g/L and 4 g/L, respectively. Acetone production was 6 g/L and, ethanol level was 0.3 g/L.20 g/L of sugar was added to the medium to ensure the production of butanol on the 16th day. The acetic acid level reached 9 g/L and the butyric acid concentration increased to 8 g/L.Acetone reached up to 9 g/L, ethanol level remained constant at 0.3 g/L.The results were similar to the control and magnesium (Mg) added groups.

Sugar consumption was limited in neutral red added media. No product formation was observed.

A high amount of organic matter consumption was achieved with the addition of magnesium (Mg) and zinc (Zn). Thus acetone production was obtained.While ethanol production was limited, butanol did not generate. In manganese (Mn) and neutral red added mediums, product formation did not occur because substrate consumption was not observed. Since glucose was not consumed, it can be said that the growth of the organism was inhibited.

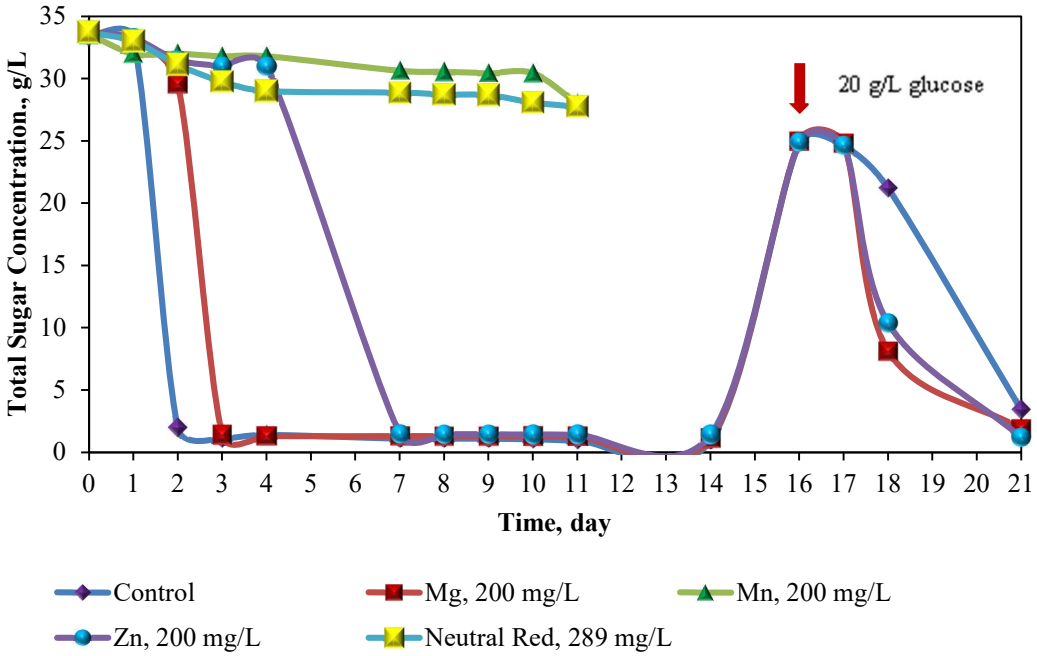


Figure 3.44 Consumption of total sugar over time with the addition of Mg, Mn, Zn and neutral red

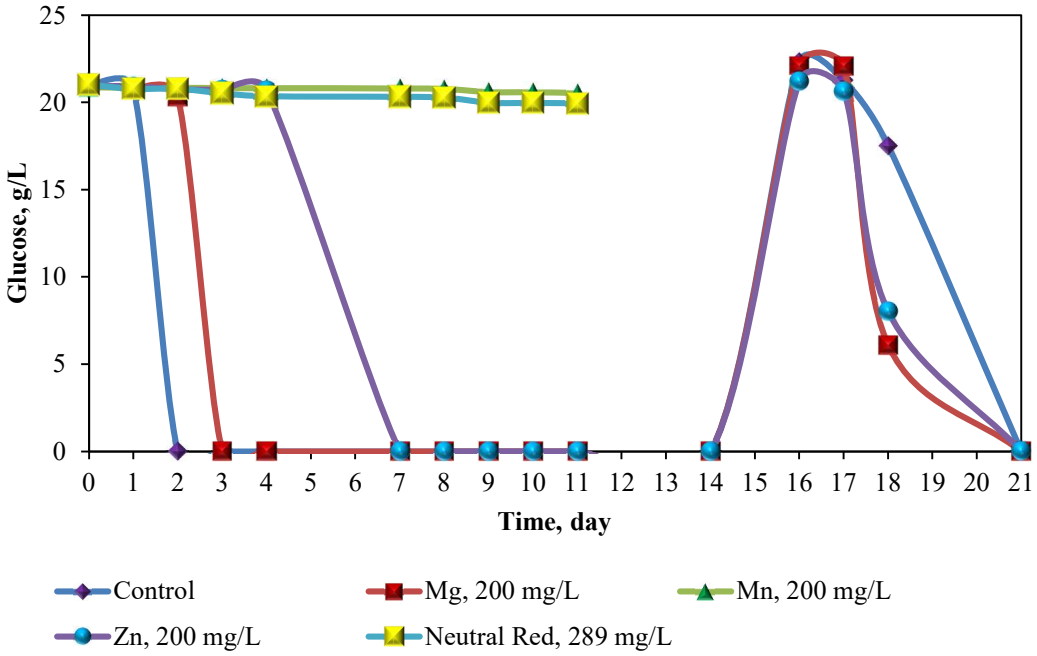


Figure 3.45 Consumption of glucose over time with the addition of Mg, Mn, Zn and neutral red

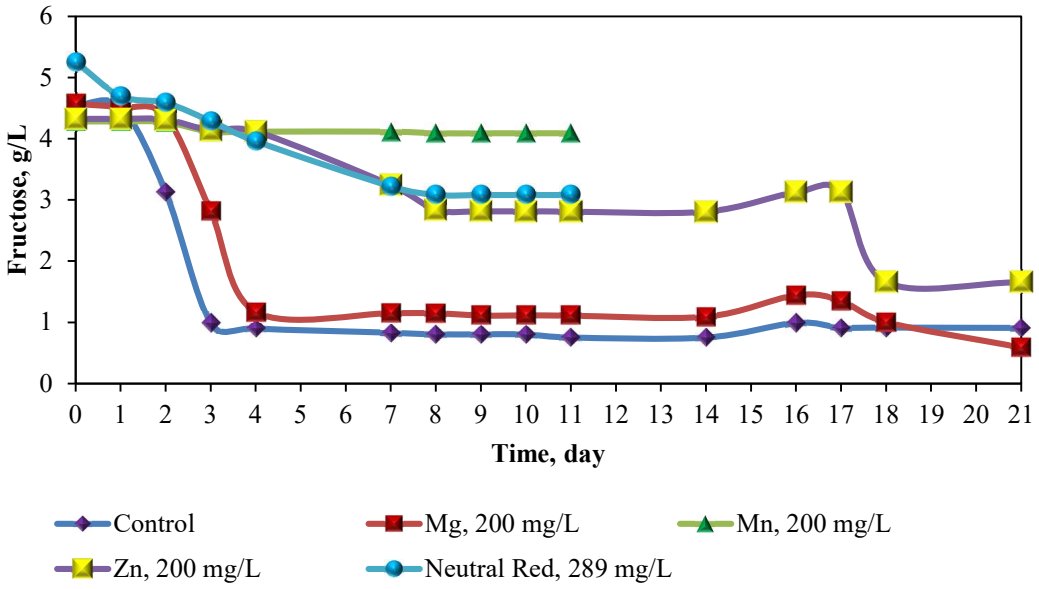


Figure 3.46 Consumption of fructose over time with the addition of Mg, Mn, Zn and neutral red

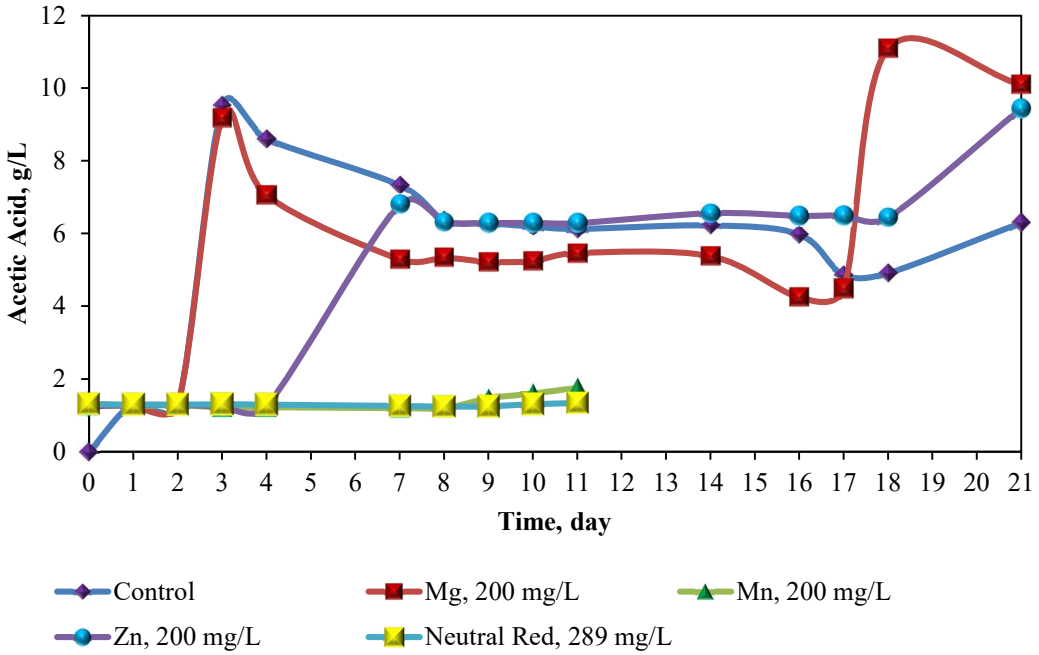


Figure 3.47 Variation of acetic acid concentration over time with the addition of Mg, Mn, Zn and neutral red

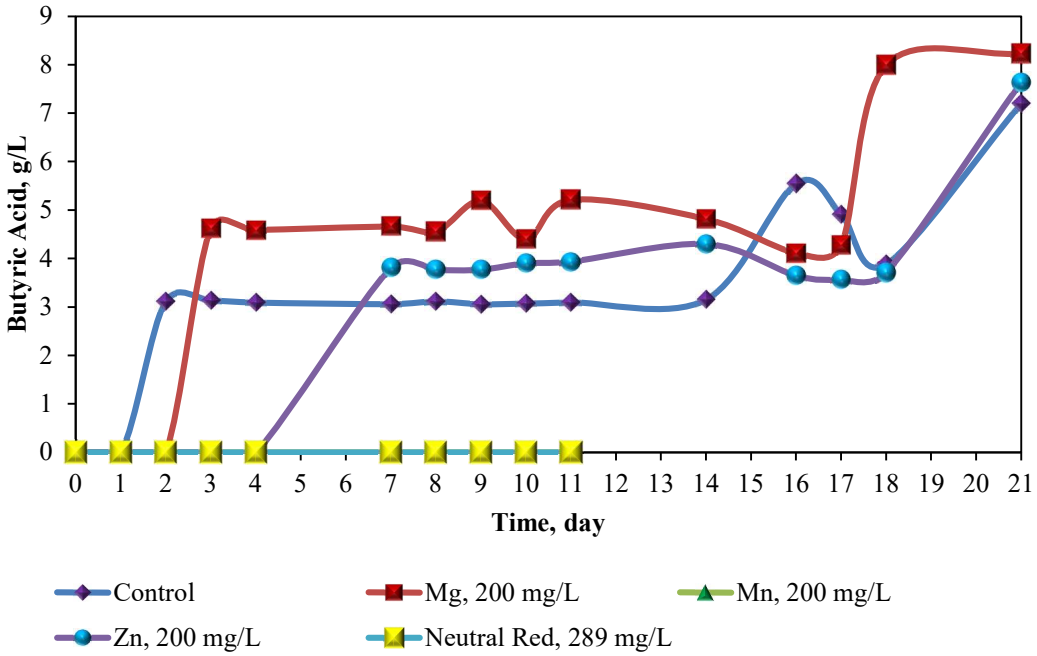


Figure 3.48 Variation of butyric acid concentration over time with the addition of Mg, Mn, Zn and neutral red

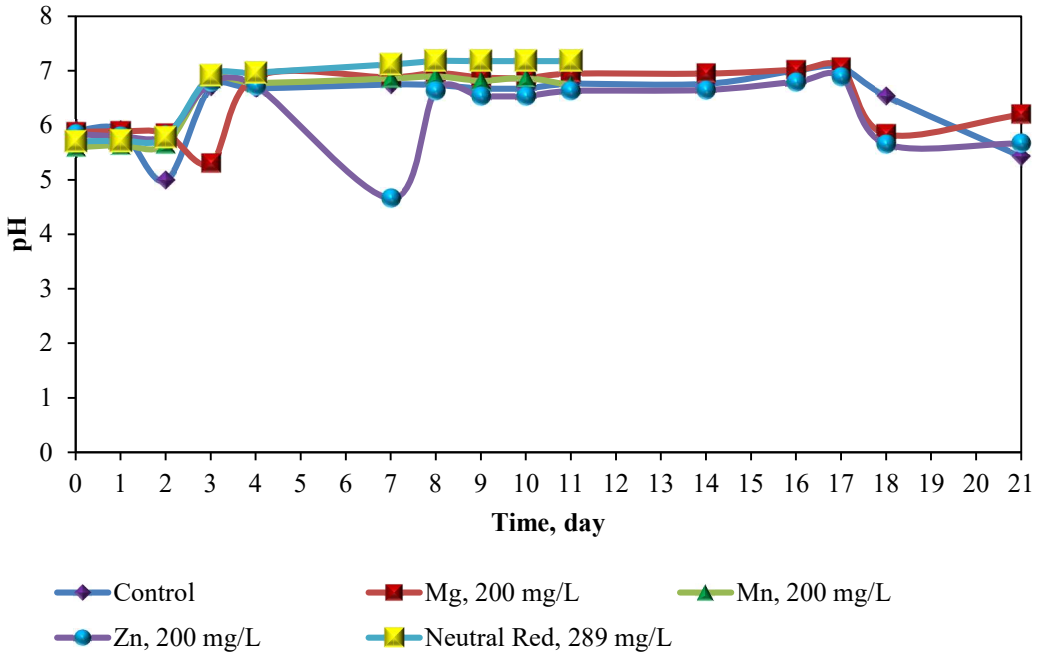


Figure 3.49 Variation of pH with the addition of Mg, Mn, Zn and neutral red

3.3.2 The Effects of Ion Deficiencies by Simplifying the Media

In addition to the effect of simplification of fermentation media, the effects of various ion deficiencies on butanol production were investigated. Apart from the control group, ion sources such as iron (Fe), magnesium (Mg), manganese (Mn) were excluded, as well as some compounds (None) from other media. Acetone and ethanol concentrations over time were shown in Figure 3.50 and Figure 3.51, respectively. In the medium where only the nutrient components were removed (None), the acetone concentration was 3 g/L at the beginning of the fermentation but increased to 17 g/L at the end of the 11th day.The iron deficiency (Fe) in the medium supported the formation of acetone, and a concentration of 18.2 g/L was obtained. Similar results were obtained for manganese (Mn) and magnesium (Mg) removed media. The acetone concentrations of 18 and 17.7 g/L were observed, respectively.Acetone production started faster in the control group containing standard media ingredients, but the product formation resulted in a lower acetone concentration of 13 g/L compared to the other groups.

Ethanol production was the lowest at 0.6 g/L in iron deficient (Fe) environment (Figure 3.51). Magnesium deficiency (Mg) supported production, resulting in an ethanol concentration of 1 g/L. Butanol production was not achieved in any experimental group.

The hydrogen generation was monitored in this experiment. Hydrogen is generated when organic acids are produced. But, solvent production should be free from hydrogen generation. Time course of hydrogen formation over time was given in Figure 3.52. The medium with the lowest hydrogen production was the control group, with a volume of 955 mL. However, manganese deficiency (Mn) promoted hydrogen production, and 1767 mL of hydrogen gas formed.

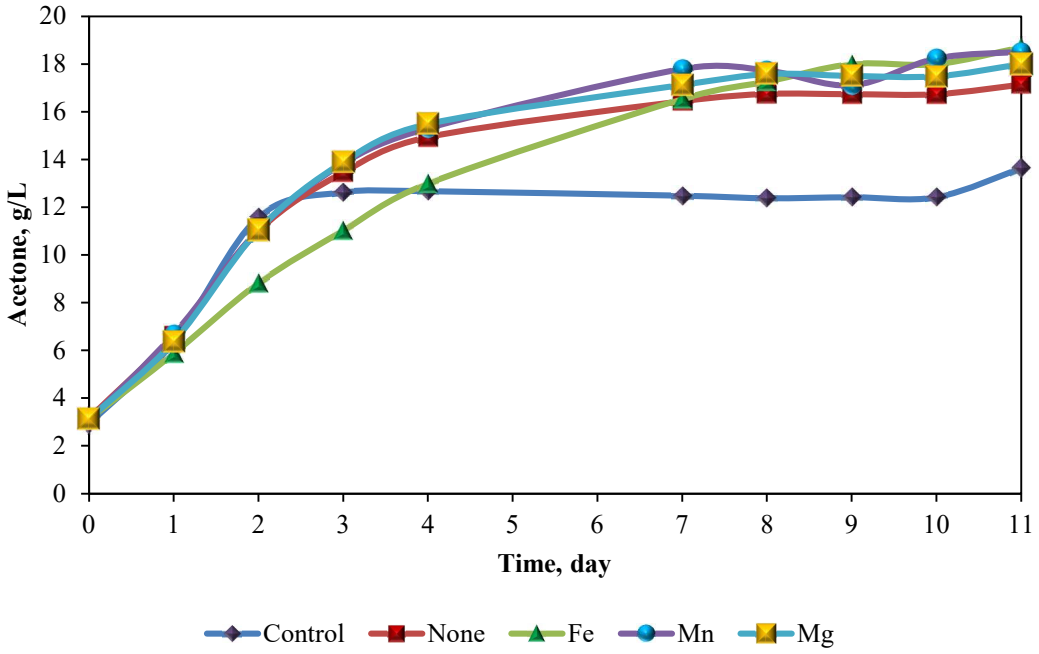


Figure 3.50 Variation of acetone production over time with ion deficiencies along with medium simplification

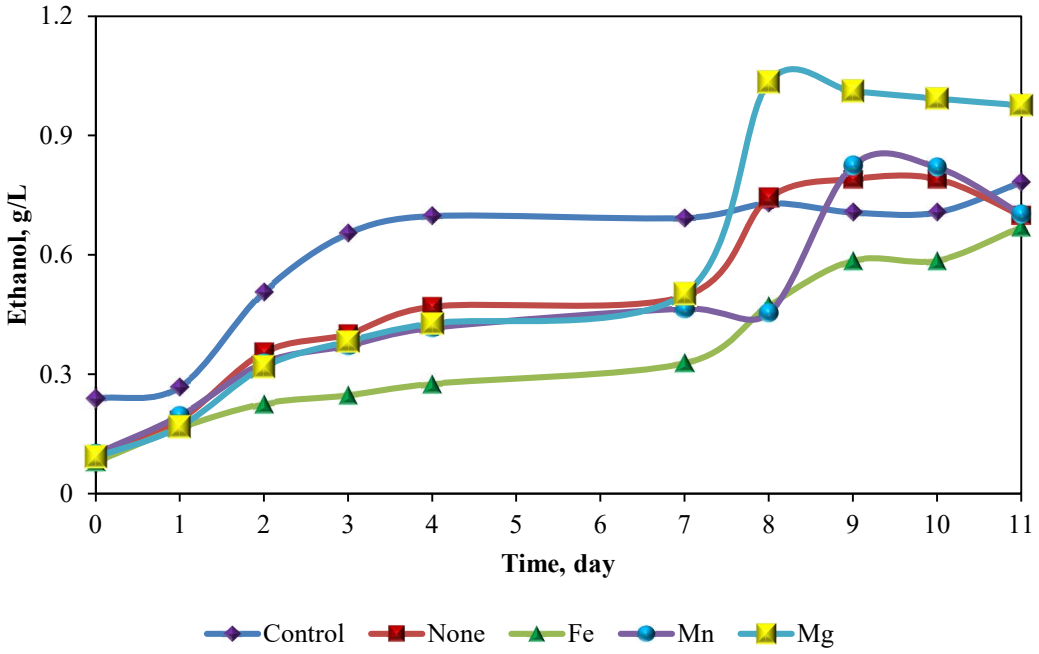


Figure 3.51 Variation of ethanol production over time with ion deficiencies along with medium simplification

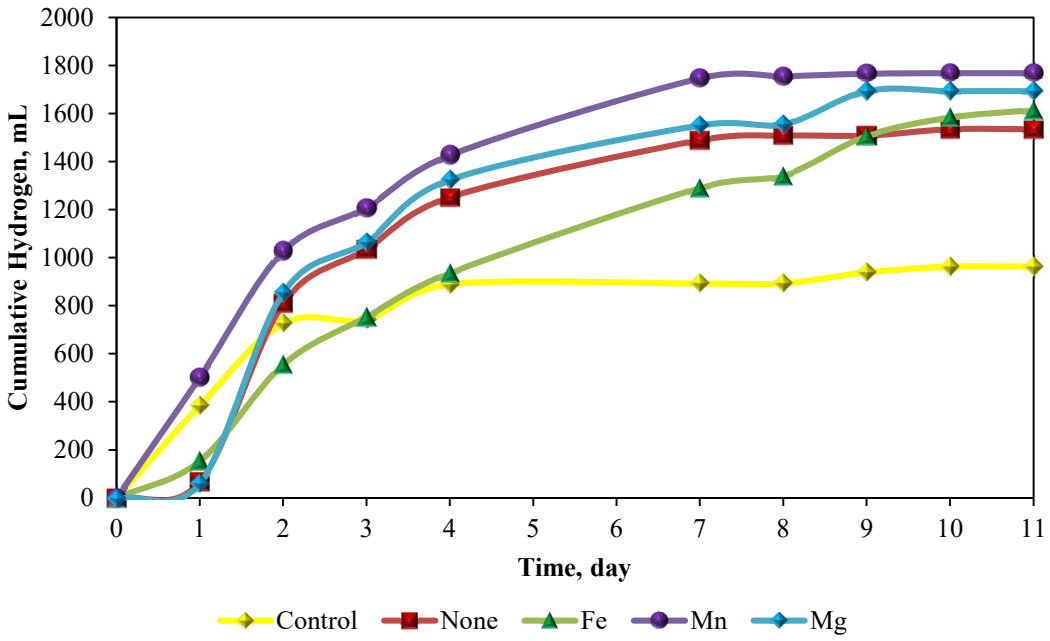


Figure 3.52 Variation of hydrogen production over time with ion deficiencies along with medium simplification

The initial total sugar concentration was 63 g/L, but the sum of glucose and fructose concentrations was around 57 g/L. Consumptions of total sugar, glucose and fructose were shown in Figure 3.53, Figure 3.54 and Figure 3.55, respectively. Acetic acid concentration over time was given in Figure 3.56 while the change of butyric acid level was shown in Figure 3.57. The pH change was given in Figure 3.58. The results were presented below.

Total sugar was consumed up to 2 g/L in control experiments.16 g/L of acetic was produced and, then consumption started. Butyric acid concentration reached 8 g/L. Acetone production was 13 g/L and ethanol was 0.7 g/L. The cumulative amount of hydrogen produced was 955 mL.

Similarly, sugar was consumed up to 2 g/L in the environment where media was simplified but ions were not excluded (None). The acetic acid level reached to 17 g/L and, then consumption started. The butyric acid level was around 15 g/L.While the acetone concentration was at 17 g/L, ethanol was 0.8 g/L. At the end of the experiment, the amount of hydrogen gas in the environment was 1526 mL.

The total sugar was consumed up to 3 g/L in the environment where the iron ion was removed (Fe) with the simplification of the environment. The acetic acid concentration was around 13 g/L and, butyric acid concentration was at 14 g/L. Acetone production was 18.2 g/L and ethanol amount was 0.6 g/L. In addition, 1526 mL of hydrogen gas was produced.

In the manganese-removed medium (Mn), sugar was consumed up to 2 g/L which is almost the same result with the other ion deficiency experiments. Acetic acid concentration reached up to 17 g/L and then consumption started. The butyric acid level was around 13 g/L. Acetone and ethanol levels reached 18 g/L and 0.8 g/L, respectively. The amount of hydrogen gas is 1767 mL.

In the magnesium-depleted environment (Mg), almost all of the sugar was consumed in the same way and the remaining sugar was equal to fructose. While the acetic acid concentration has reached 17 g/L and then consumption has begun, the butyric acid level was close to 13 g/L. Acetone concentration was 17.7 g/L and ethanol was 1 g/L. A volume of 1694 mL of H_2 gas was produced.

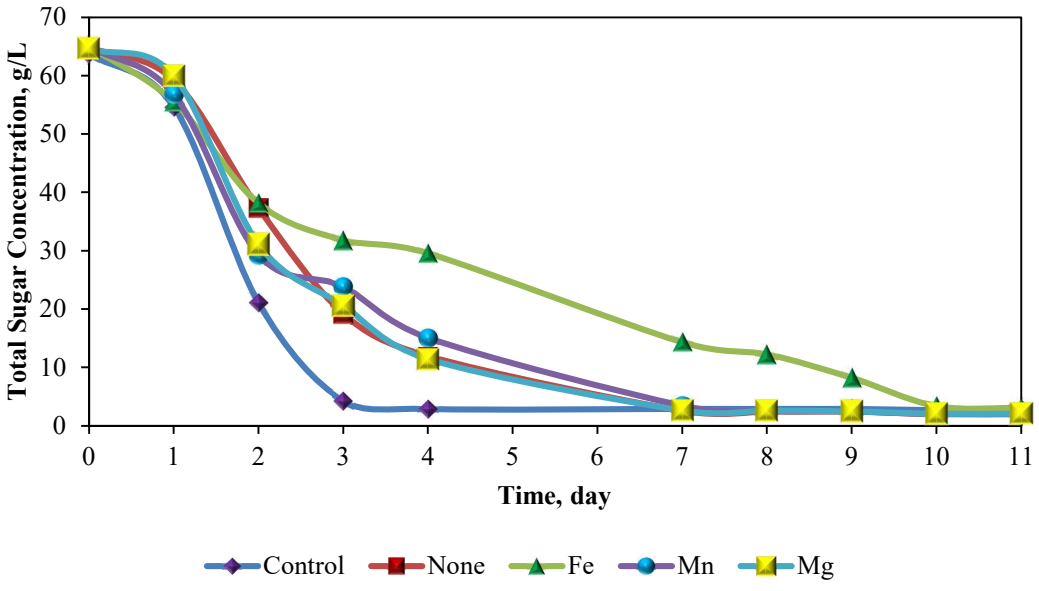


Figure 3.53 Consumption of total sugar over time with ion deficiencies along with medium simplification

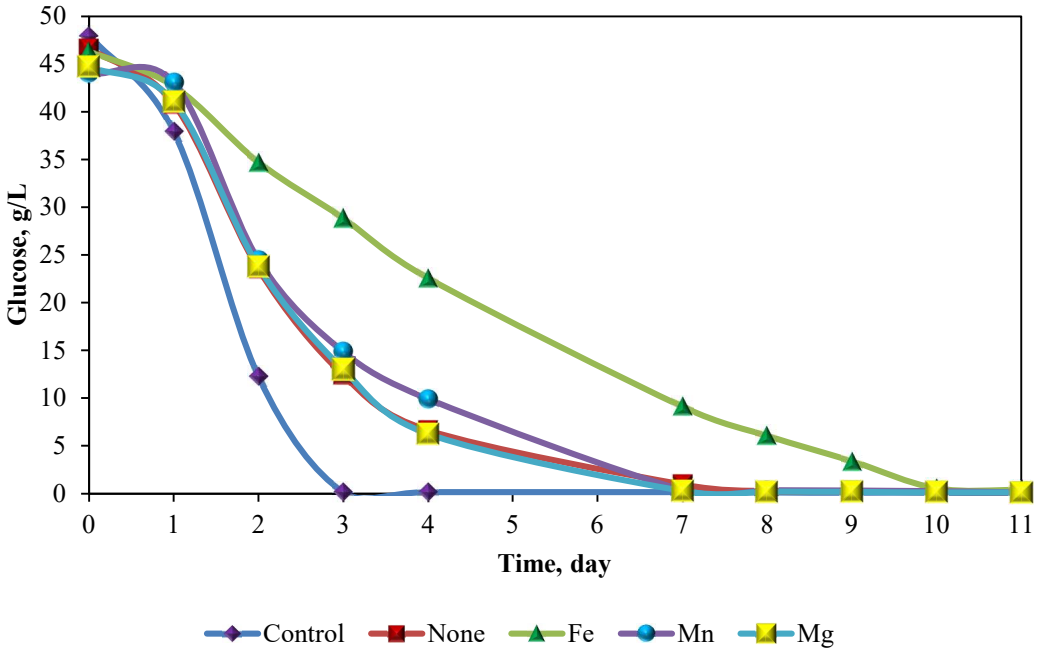


Figure 3.54 Consumption of glucose over time with ion deficiencies along with medium simplification

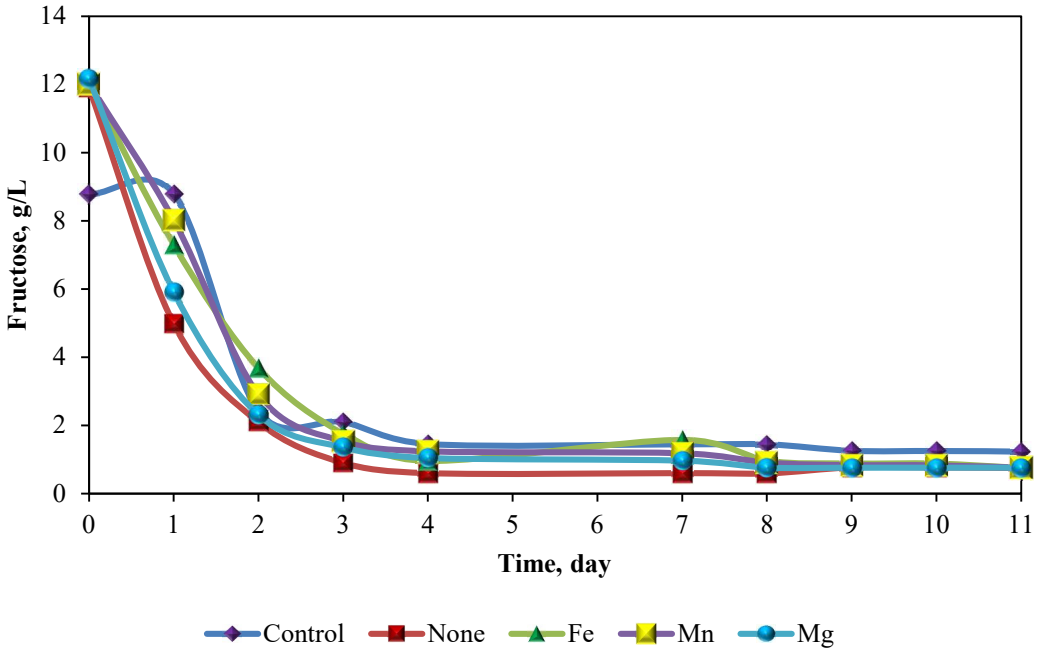


Figure 3.55 Consumption of fructose over time with ion deficiencies along with medium simplification

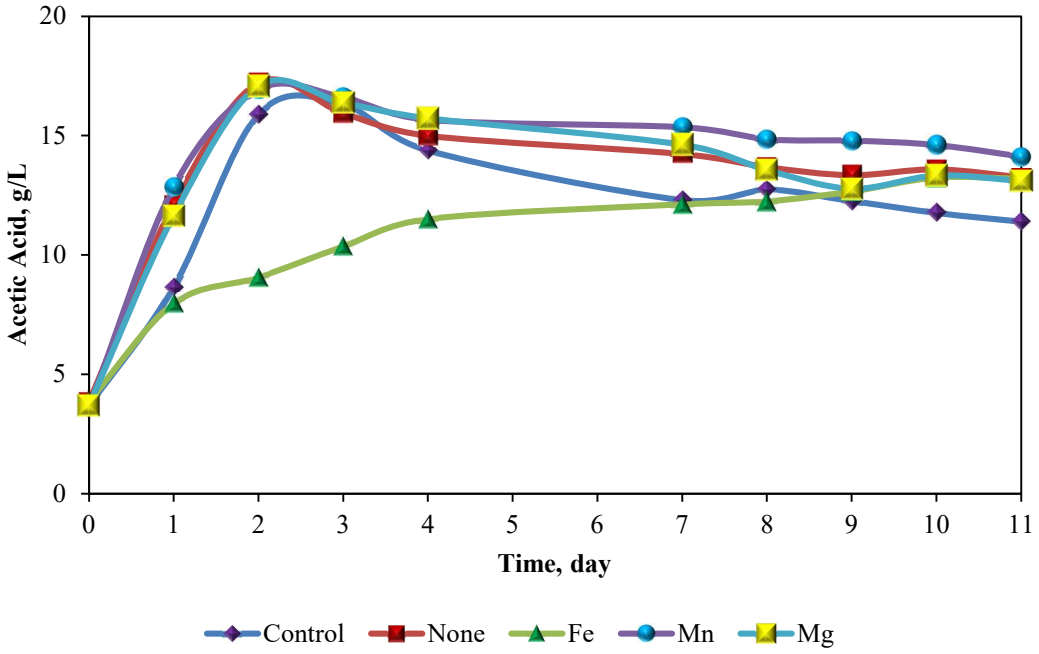


Figure 3.56 Variation of acetic acid level over time with ion deficiencies along with medium simplification

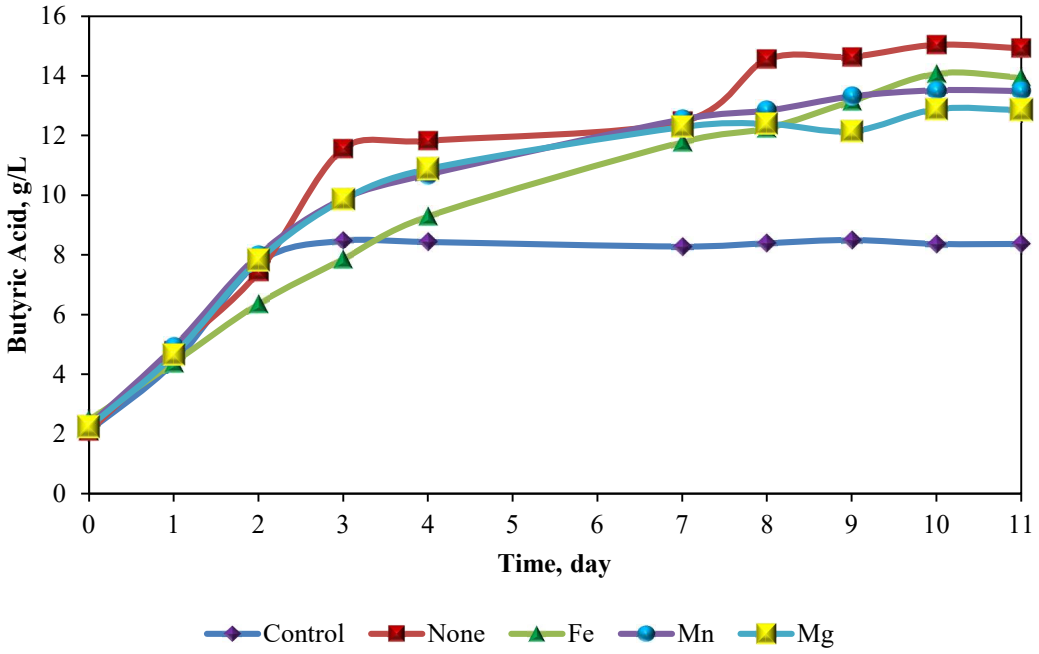


Figure 3.57 Variation of butyric acid level over time with ion deficiencies along with medium simplification

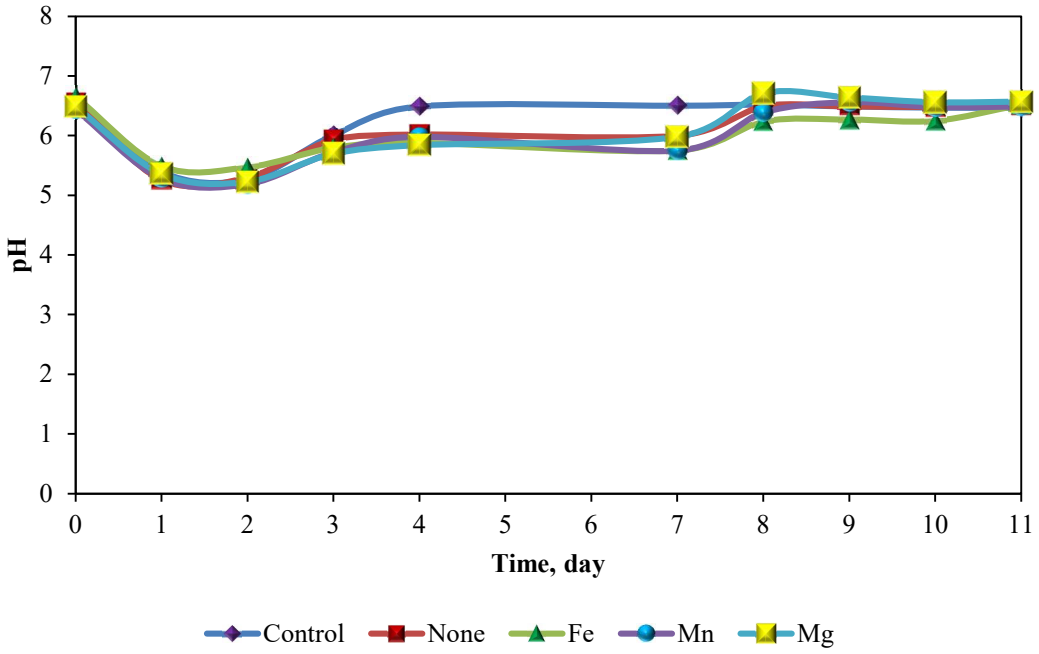


Figure 3.58 Variation of pH over time with ion deficiencies along with medium simplification

3.3.3 Effect of Butyric Acid Addition

The addition of butyric acid as an activator to the fermentation medium increases the production efficiency by directing the metabolic pathway to the butanol formation. For this reason, butyric acid was added to the nutrient media in the range of 6-12 g/L. The initial pH level was adjusted to 6 with dilute (60%) H_2SO_4 for butyric acid production. As shown in Figure 3.59, the initial level of acetone was 3 g/L which is inherited from the stock culture where fermentation occurs. The highest acetone level was observed in the control group without the addition of butyric acid and, at the end of the 10th day, acetone concentration was 14 g/L. The lowest acetone production was achieved with a concentration of 5.6 g/L in the media where 12 g/L butyric acid (B 12) was added. In the mediums where other 6, 8 and 10 g/L butyric acid (B 6, B 8, B 10) additions were present, acetone production was at the levels of 10, 9 and 6.5 g/L, respectively. As can be seen here, as the added butyric acid concentration increased, the acetone level produced decreased.

Ethanol production over time was given in Figure 3.60. The highest concentration in ethanol production was obtained in the control group with a level of 0.6 g/L. In the

medium where 6 g/L butyric acid was added, 0.5 g/L ethanol concentration was obtained, while ethanol level was around 0.4 g/L in other groups. A very slight decrease in ethanol levels was observed as the butyric acid addition increased. Butanol formation was not observed in any of the media.

As it can be seen in Figure 3.61, the highest hydrogen production was obtained in the control group as 1778 mL. The lowest production was observed as 805 mL in the medium where 10 g/L of butyric acid was added.

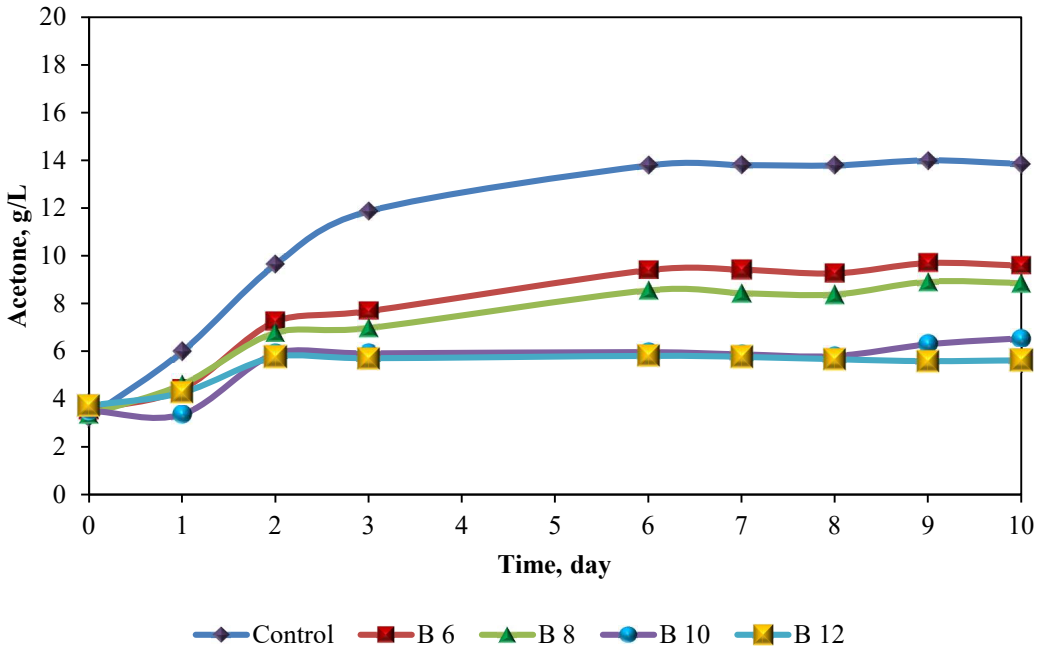


Figure 3.59 Variation of acetone production over time with butyric acid addition

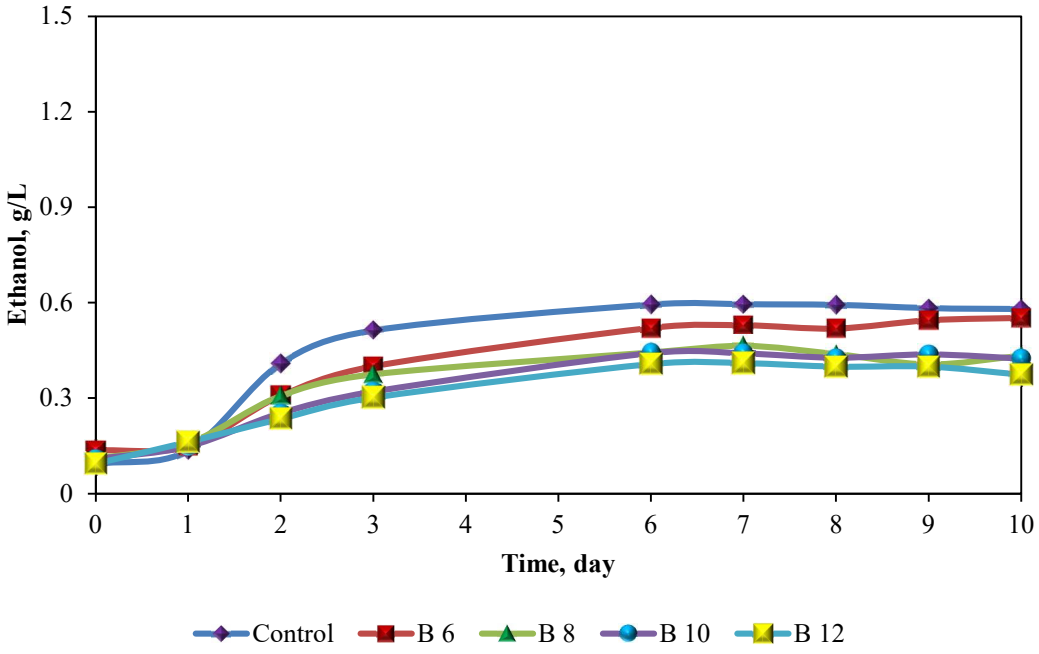


Figure 3.60 Variation of ethanol production over time with butyric acid addition

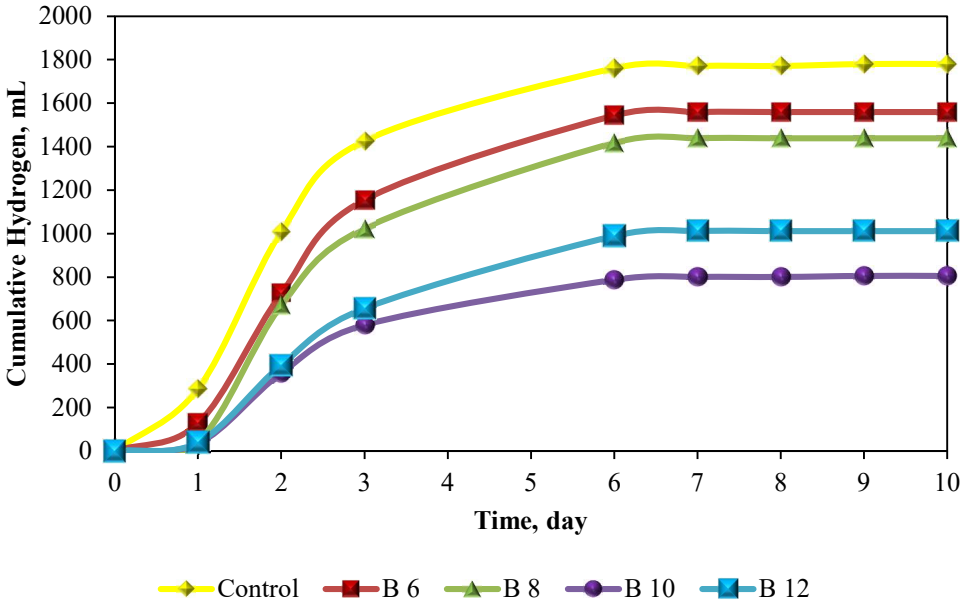


Figure 3.61 Variation of hydrogen production over time with butyric acid addition

The initial sugar concentration was 77 g/L. Total sugar, glucose and fructose consumptions over time were shown in Figure 3.62, Figure 3.63 and Figure 3.64, respectively. Changes of acetic and butyric acid concentrations with time were given

in Figure 3.65 and Figure 3.66. pH change could be seen in Figure 3.67. The results were interpreted and evaluated below.

In the control experiment without the addition of butyric acid, total sugar was consumed up to 2 g/L. Acetic acid reached the level of 14.5 g/L and then consumption started. The butyric acid level reached 9 g/L. Acetone and ethanol levels were 14 g/L and 0.6 g/L, respectively. The cumulative hydrogen level was 1778 mL.

In the media where 6 g/L (B 6) butyric acid was added, total sugar was consumed up to 12 g/L.Acetic acid reached the level of 12.7 g/L and then consumption started. The butyric acid concentration was close to 11 g/L. While the acetone concentration was at the level of 10 g/L, the ethanol concentration was 0.5 g/L. The amount of hydrogen gas was at the level of 1560 mL.

Total sugar was consumed up to 17 g/L in the environment where 8 g/L (B 8) butyric acid was added. The amount of acetic acid reached the level of 12 g/L and then consumption started. The butyric acid concentration was 12 g/L. The amounts of acetone and ethanol produced were 9 and 0.4 g/L, respectively, and 1439 mL of hydrogen gas was obtained.

In the medium where 10 g/L (B 10) butyric acid was added, total sugar was consumed up to 21 g/L. Consumption of acetic acid started after the level reached 13 g/L.The butyric acid concentration was 13 g/L. Acetone and ethanol produced reached concentration values of 6.5 and 0.4, respectively. The amount of cumulative hydrogen gas produced was 805 mL.

Total sugar was consumed up to 25 g/L in the fermentation medium where the initial butyric acid level was 12 g/L (B12). The acetic acid concentration reached 12 g/L and then consumption started. However, the butyric acid level reached 13 g/L.While the acetone level was 5.6 g/L, the amount of ethanol produced was 0.4 g/L.The amount of hydrogen gas released was at the level of 1013 mL.

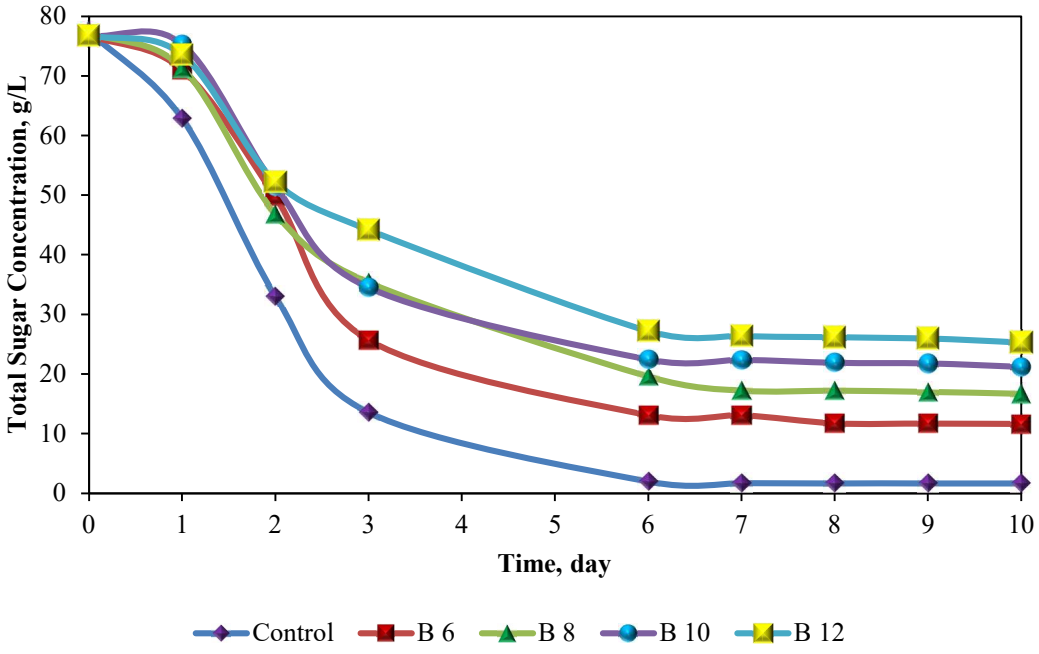


Figure 3.62 Consumption of total sugar over time with butyric acid addition

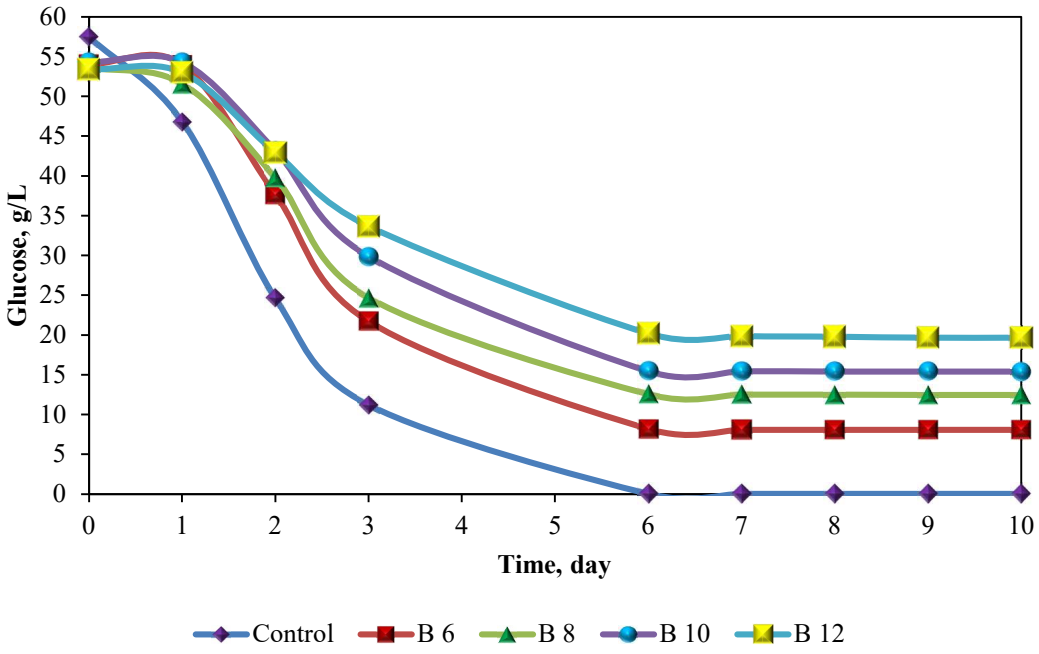


Figure 3.63 Consumption of glucose over time with butyric acid addition

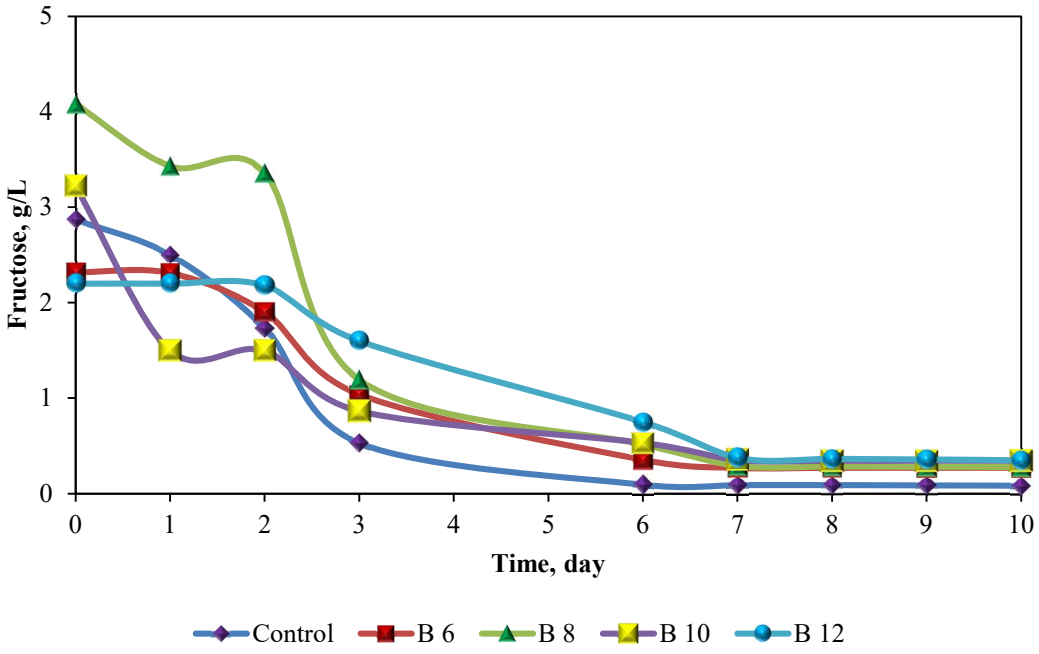


Figure 3.64 Consumption of fructose over time with butyric acid addition

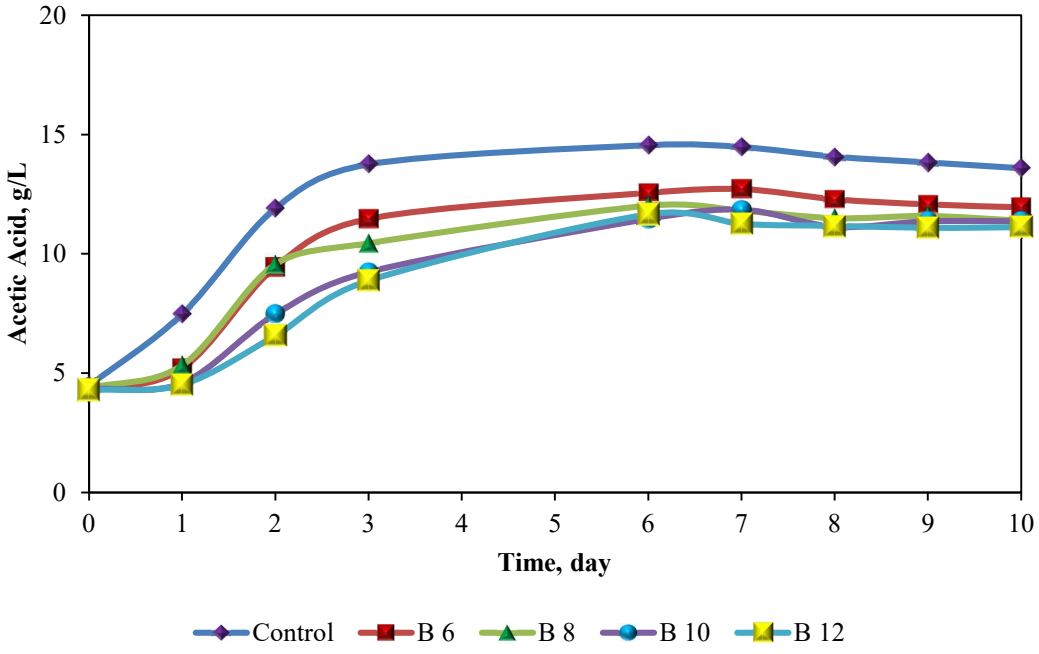


Figure 3.65 Variation of acetic acid level over time with butyric acid addition

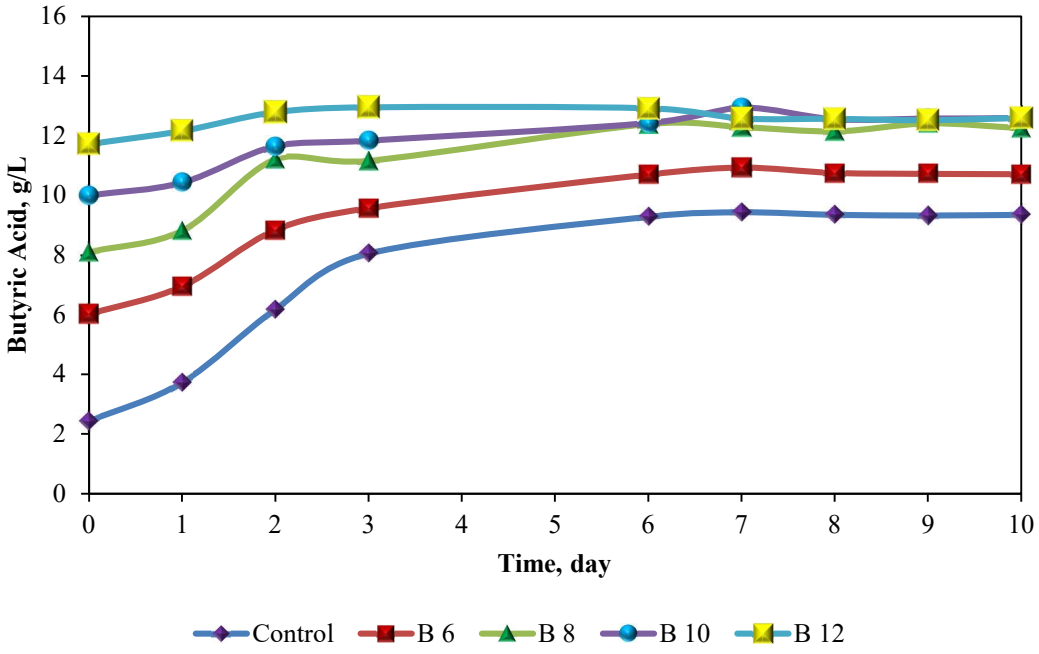


Figure 3.66 Variation of butyric acid level over time with butyric acid addition

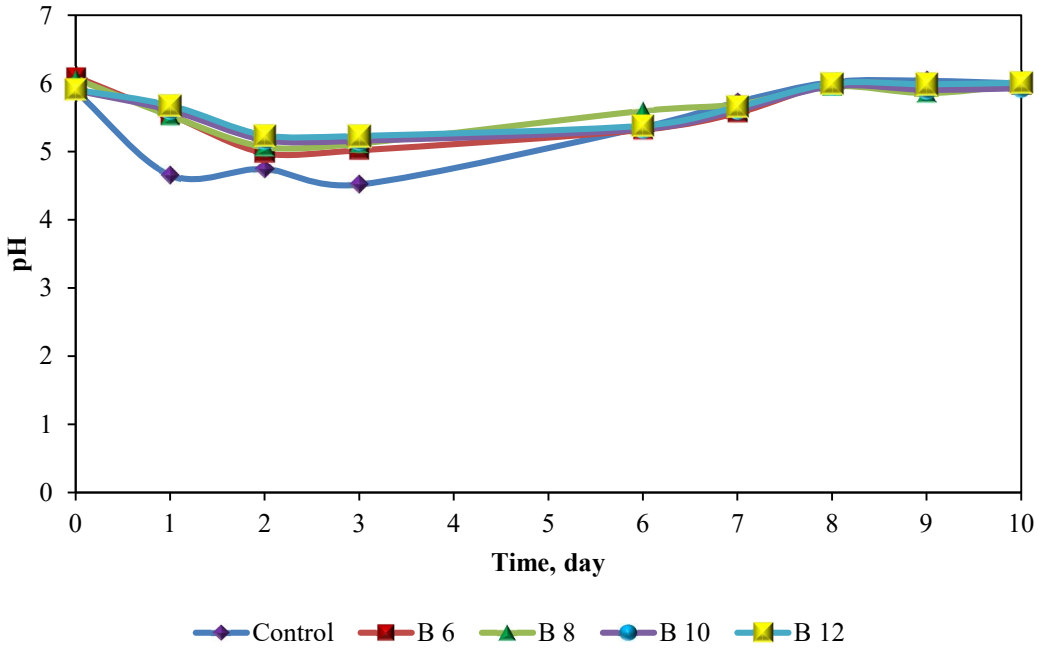


Figure 3.67 Variation of pH over time with butyric acid addition

3.3.4 Effect of Ethanol Addition

External addition of ethanol can act as inhibitor for ethanol production and the ABE could shift to butanol generation. In order to test this hypothesis, ethanol was added in the range of 0.2-1.4 g/L to the fermentation media in order to increase butanol production. The pH of the experimental media was adjusted to around 6.6 with 10 M NaOH solution. As can be seen in Figure 3.68, the initial acetone concentration was 2.5 g/L which was derived from stock culture. The highest acetone production is at the end of the 7th day, with a level of 14.3 g/L in the medium with 0.2 g/L ethanol (E 0.2). The lowest acetone production was in the control group, which involved no ethanol addition, with a 13.4 g/L level. Acetone production was 13.5 and 14 g/L in the other 1 and 1.4 g/L ethanol (E 1, E 1.4) added media, respectively. The addition of ethanol supported acetone production.

In ethanol production, the highest average production concentration was 0.7 g/L in 0.2 g/L ethanol added medium, which was shown in Figure 3.69. The lowest average production concentration was 0.3 g/L and it was observed in the medium with 1.4 g/L ethanol. The other average production concentrations were 0.5 and 0.4 g/L for the control and 1 g/L ethanol added mediums, respectively. Butanol production was not observed in any of the experimental groups.

Cumulative hydrogen production over time was given in Figure 3.70. The highest amount of hydrogen gas produced was 1640 mL and it was in the control group. The lowest gas volume was observed as 1525 mL in 1.4 g/L ethanol added medium.

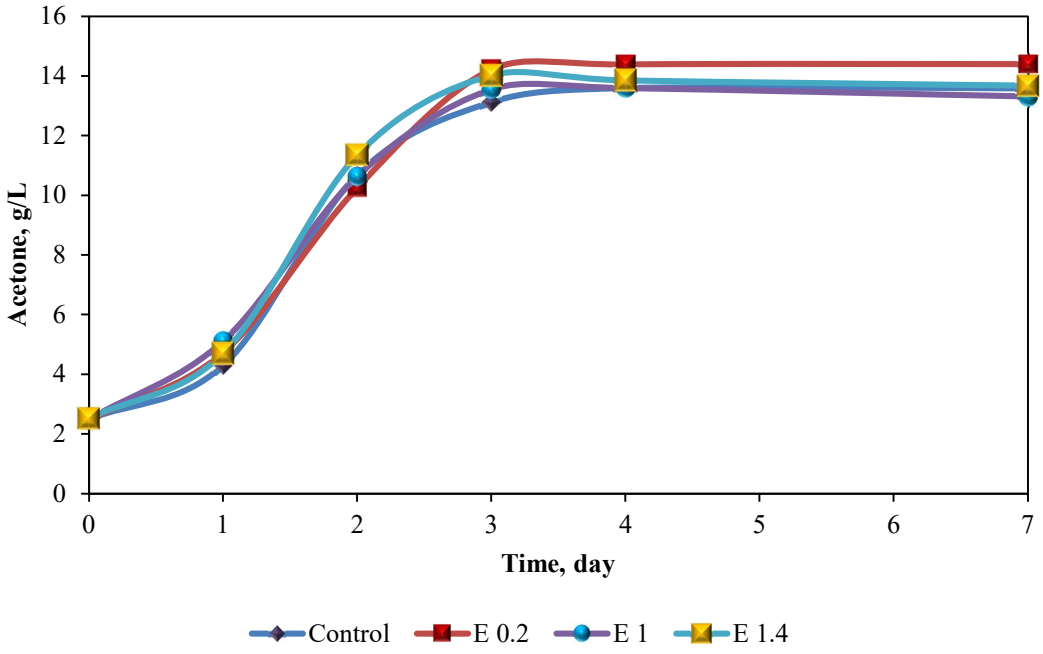


Figure 3.68 Variation of acetone production over time with ethanol addition

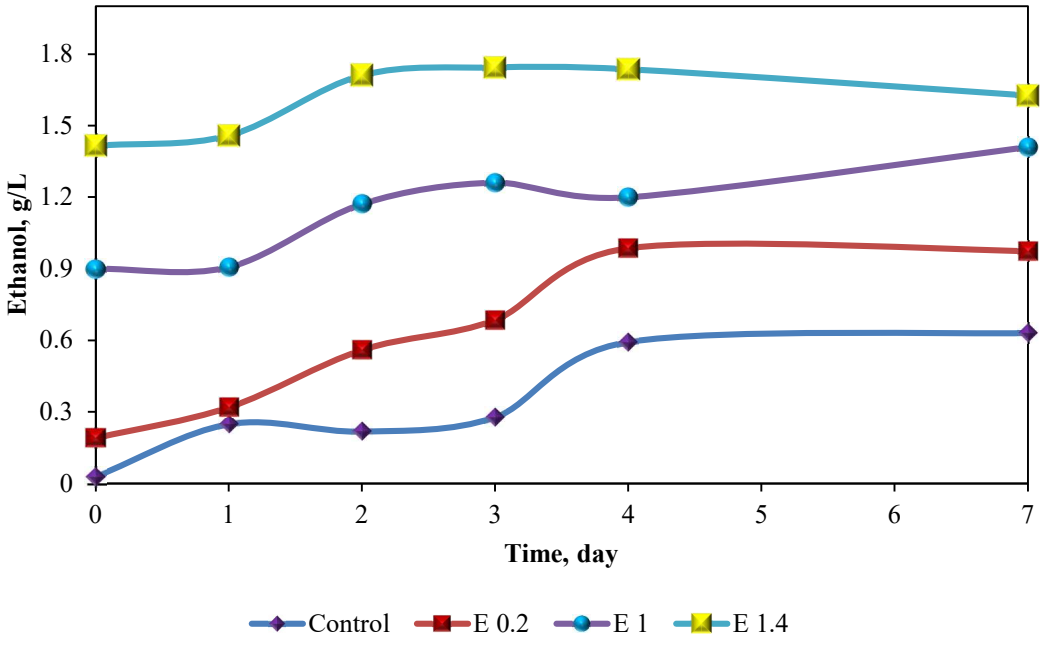


Figure 3.69 Variation of ethanol production over time with ethanol addition

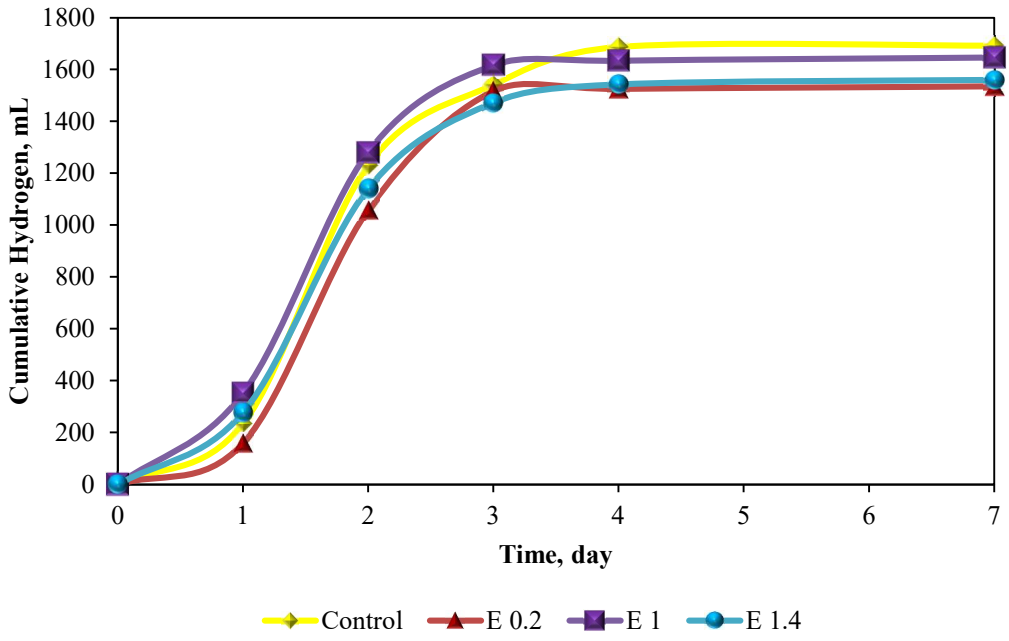


Figure 3.70 Variation of hydrogen production over time with ethanol addition

Consumptions of total sugar, glucose and fructose were presented in Figure 3.71, Figure 3.72 and Figure 3.73, respectively. Changes of acetic and butyric acids over time were shown in Figure 3.74 and Figure 3.75. pH change was given in Figure 3.76.

Total sugar was consumed up to 2 g/L in the control group. The amount of acetic acid has reached the level of 12 g/L and then consumption has started. The butyric acid level was approximately 10 g/L. The acetone concentration was 13.4 g/L and the final ethanol level was 0.5 g/L. The volume of hydrogen gas was 1640 mL.

In the fermentation medium which 0.2 g/L ethanol was added, total sugar was consumed up to 2 g/L. The amount of acetic acid reached 13 g/L and then consumption started. Butyric acid was at the level of 10 g/L. Acetone and final ethanol concentrations were 14.3 and 0.9 g/L, respectively. The cumulative hydrogen was at the level of 1526 mL.

In the media where the added ethanol level is 1 g/L, the total sugar level decreased to 2.5 g/L and, acetic acid consumption started after the amount reached 14 g/L. The

butyric acid level was 9.4 g/L. Final ethanol and acetone concentrations were 1.3 and 13.5 g/L, respectively. The amount of hydrogen gas produced was 1633 mL.

When 1.4 g/L ethanol was added to the medium, total sugar was consumed up to 2.3 g/L. Consumption started after the amount of acetic acid reached 12.5 g/L, and at the end of fermentation, the butyric acid level approached 10 g/L. Acetone and final ethanol concentrations were 14 and 1.7 g/L, respectively. The cumulative hydrogen volume was 1525 mL.

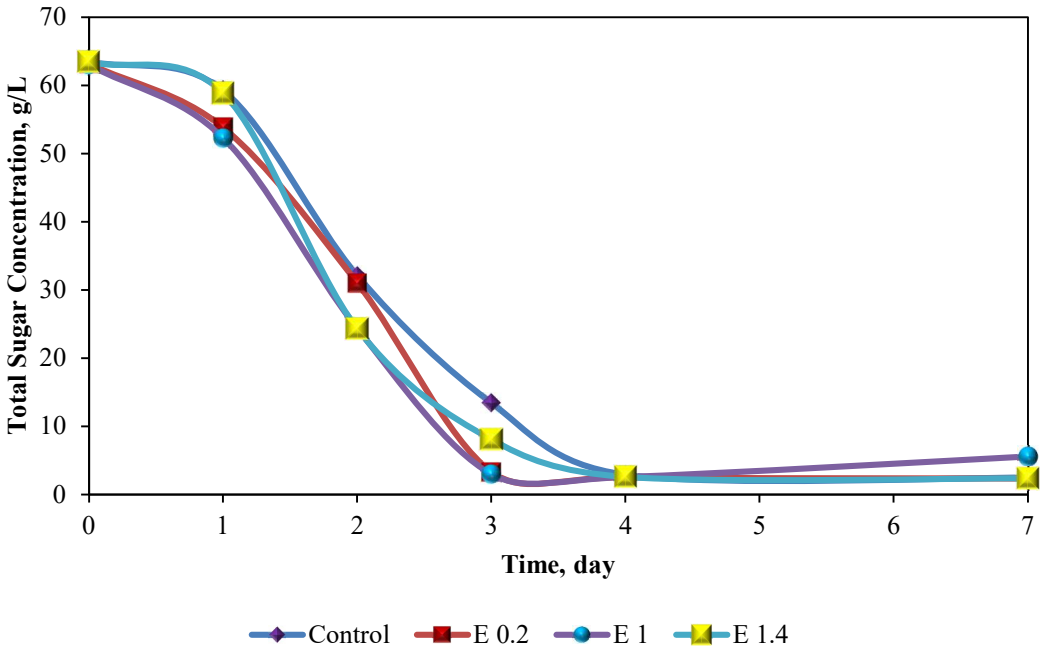


Figure 3.71 Consumption of total sugar over time with ethanol addition

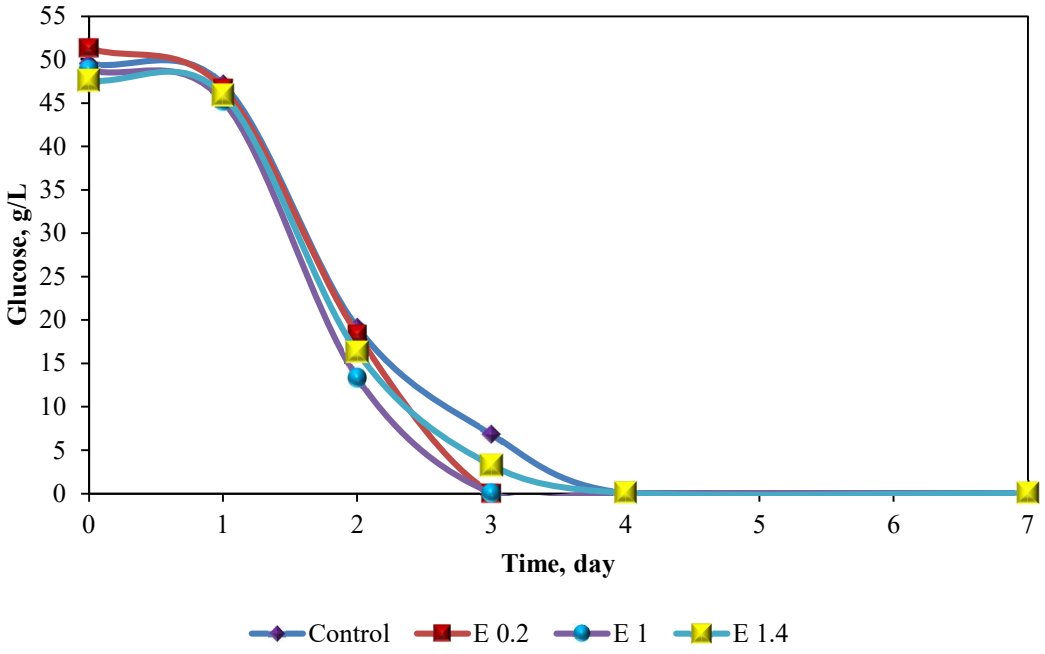


Figure 3.72 Consumption of glucose over time with ethanol addition

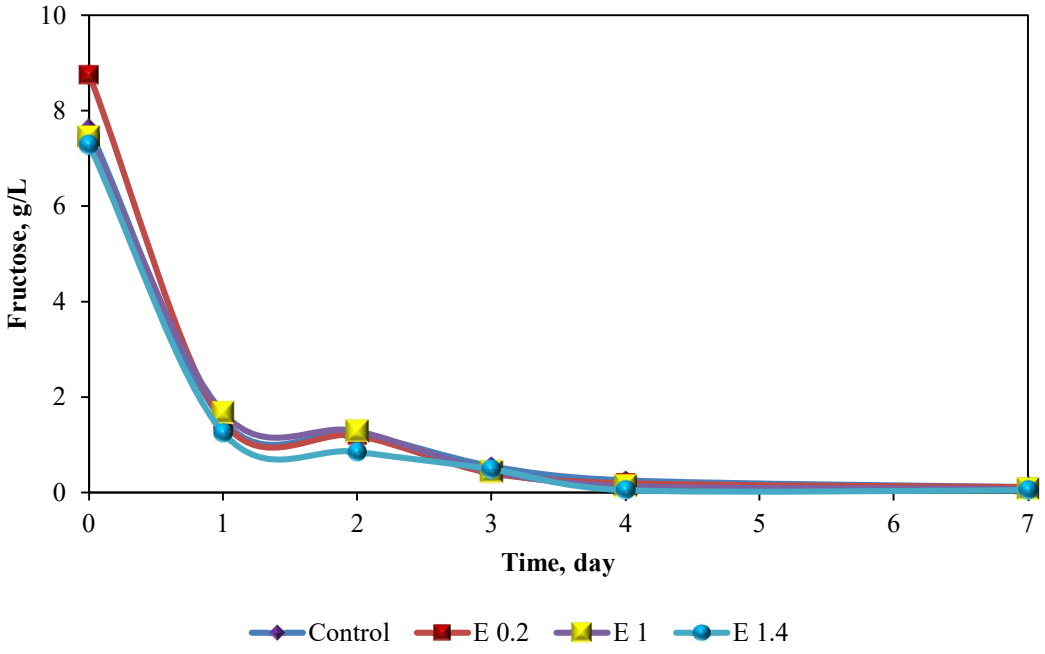


Figure 3.73 Consumption of fructose over time with ethanol addition

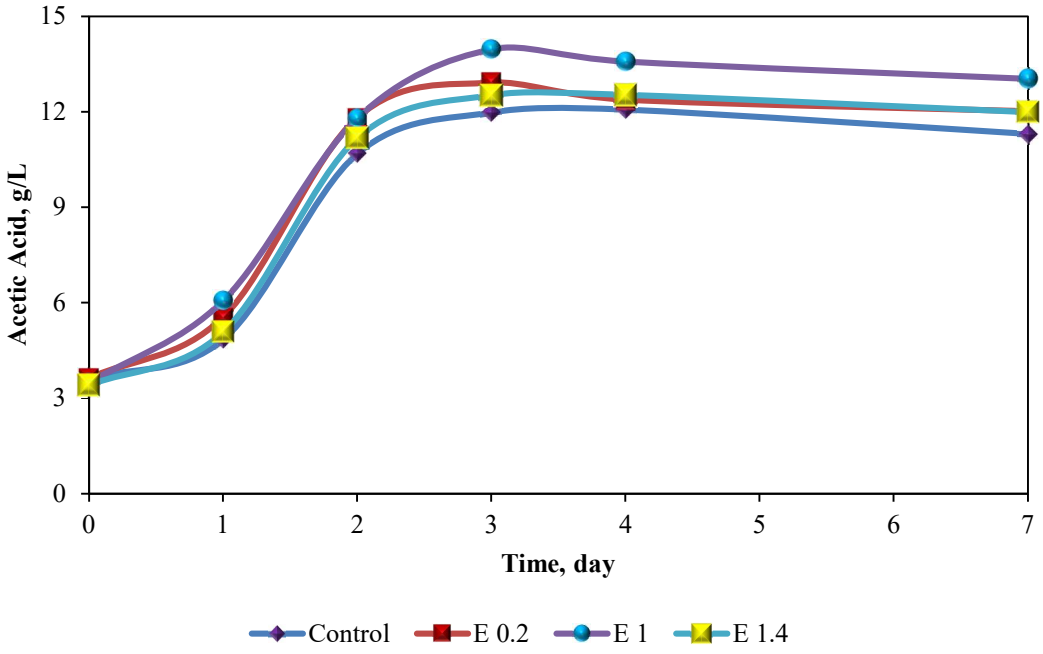


Figure 3.74 Variation of acetic acid level over time with ethanol addition

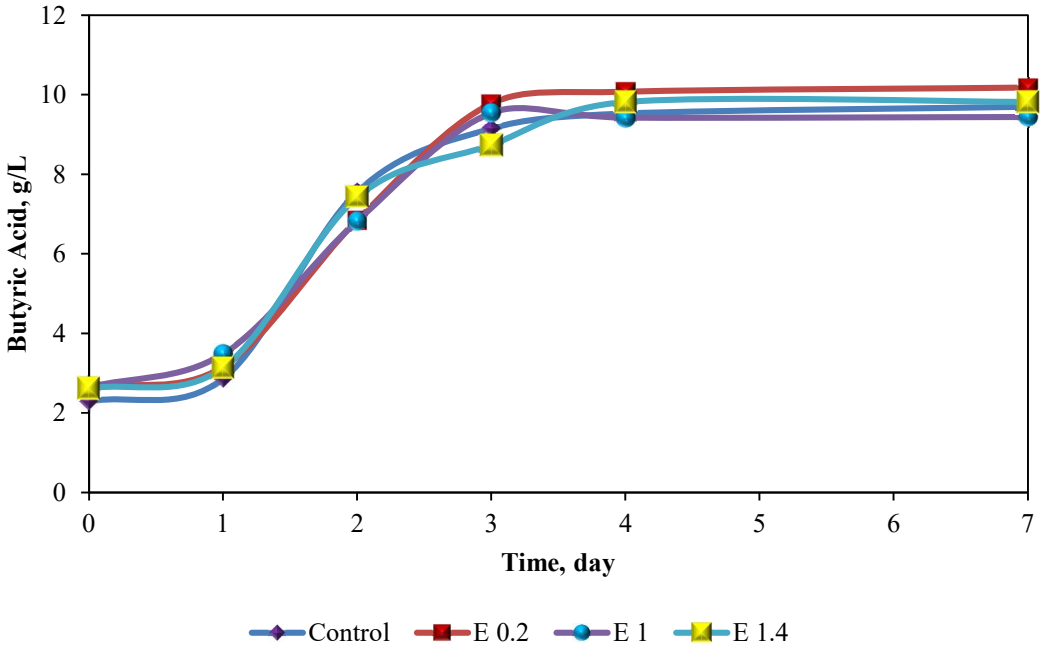


Figure 3.75 Variation of butyric acid level over time with ethanol addition

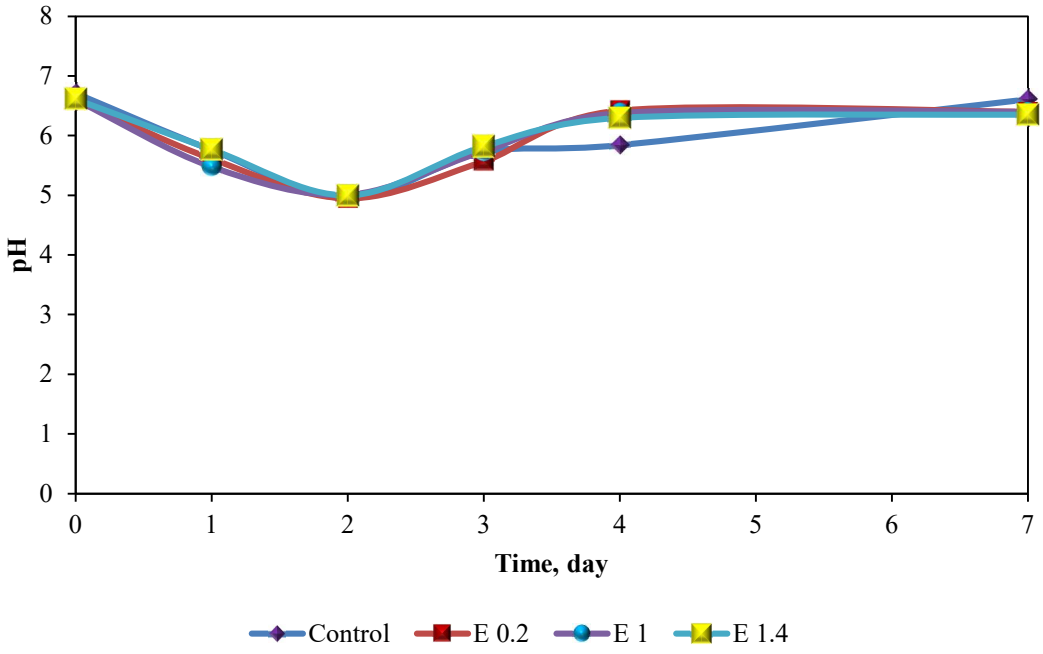


Figure 3.76 Variation of pH over time with ethanol addition

3.3.5 Effect of Different Carbon/Nitrogen Ratios (C/N)

Hydrogen, acetone, ethanol, acetic and butyric acid are valuable fermentation end products. Simultaneous fermentative production of these valuable products can be achieved by optimization of media composition and environmental conditions. Nitrogen is essential nutrient for dark fermentative bacteria and its concentration could enhance or limits one of these valuable products. Therefore, C/N ratio can be optimized for the best result (Del Pilar Anzola-Rojas et al., 2015; Rajagopalan, 2016).

The amount of glucose was kept constant and $(NH_4)_2SO_4$ was added to the nutrient media at different concentrations to provide C/N=50-250 ratios. The pH of the experimental media was adjusted to around 6.7 with 10 M NaOH solution. As can be seen in Figure 3.77, the beginning level of acetone was at a concentration of 1.6 g/L.The highest value of acetone production was 23 g/L at C/N=100. The lowest acetone level was 17 g/L and, it was observed in the medium where C/N ratio was 50. Acetone formations for media with C/N ratios of 200 and 250 were 21 and 20

g/L, respectively. The relatively nitrogen-rich environment promoted acetone production.

Ethanol production over time was presented in Figure 3.78. The highest ethanol concentration was obtained as 0.8 g/L in the medium with C/N=100.The lowest ethanol value was 0.4 and, obtained under relatively nitrogen-limited conditions in the media where C/N=200. For mediums with C/N ratios of 50 and 250, ethanol concentrations were close to 0.8 and 0.5 g/L, respectively. Butanol production was not achieved for all mediums.

Hydrogen formation over time was given in Figure 3.79. The media with the highest amount of hydrogen gas was C/N=250 with a level of 2262 mL. The lowest hydrogen yield was observed at the medium with C/N=50 and the gas volume was 1526 mL.

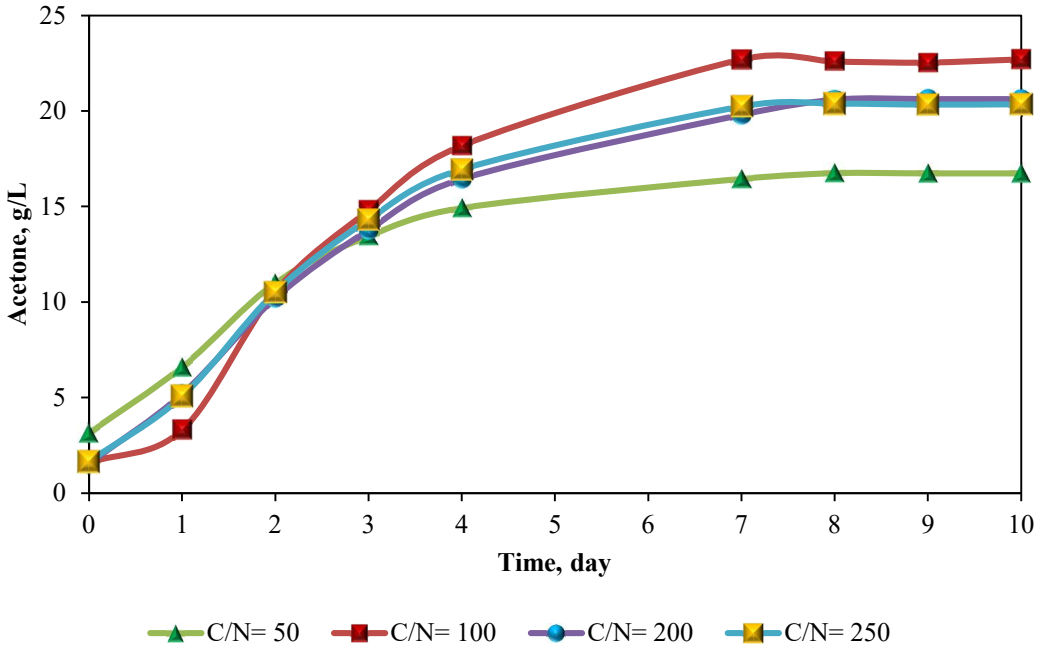


Figure 3.77 Variation of acetone production over time with different C/N ratios

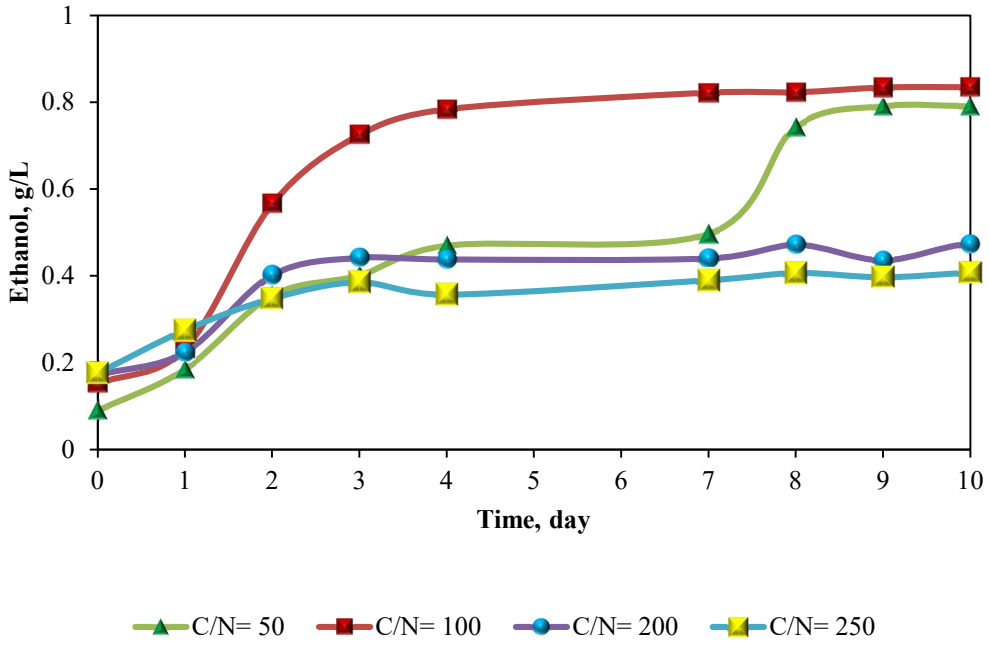


Figure 3.78 Variation of ethanol production over time with different C/N ratios

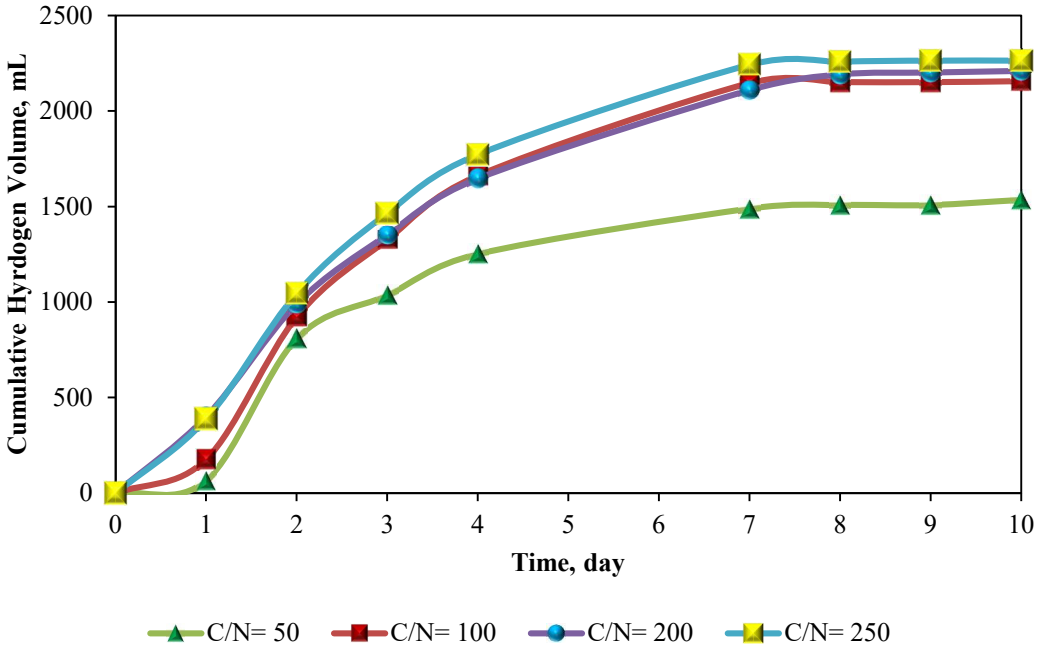


Figure 3.79 Variation of hydrogen production over time with different C/N ratios

The initial total sugar amount was 67 g/L and, the sum of glucose and fructose was around 59 g/L. The depletion of total sugar was shown in Figure 3.80. The changes in glucose and fructose levels were presented in Figure 3.81 and Figure

3.82, respectively. Variations of acetic and butyric acids were given in Figure 3.83 and Figure 3.84. Figure 3.85 shows the change in pH over time. The results were evaluated below.

In the fermentation medium with C/N=50, total sugar was depleted up to 2 g/L. The acetic acid level reached to 17 g/L and, then consumption started. The butyric acid level was around 15 g/L. The amount of acetone produced was 17 g/L and, the ethanol concentration was close to 0.8 g/L. The cumulative hydrogen volume was 1526 mL.

Total sugar was consumed up to 1.6 g/L in the experimental environment where C/N was 100. The amount of acetic acid reached to 9 g/L and, then consumption occurred.The concentration of butyric acid was 16 g/L. Acetone and ethanol concentrations were 23 and 0.8 g/L, respectively. The volume of hydrogen gas was 2153 mL.

In the media at C/N=200 ratio, the total sugar was consumed up to 2 g/L and, the levels of acetic and butyric acids were 9 and 15 g/L, respectively. The amounts of acetone and ethanol produced were 21 and 0.5 g/L, individually. The volume of hydrogen produced was 2201 mL.

Total sugar was consumed up to 2 g/L in the medium where C/N was 250. In addition, the acetic acid level reached 10 g/L, and then it is consumed. The butyric acid concentration was around 15 g/L. The amounts of acetone and ethanol produced were 20 g/L and 0.4 g/L, respectively. The cumulative volume of hydrogen gas was 2262 mL.

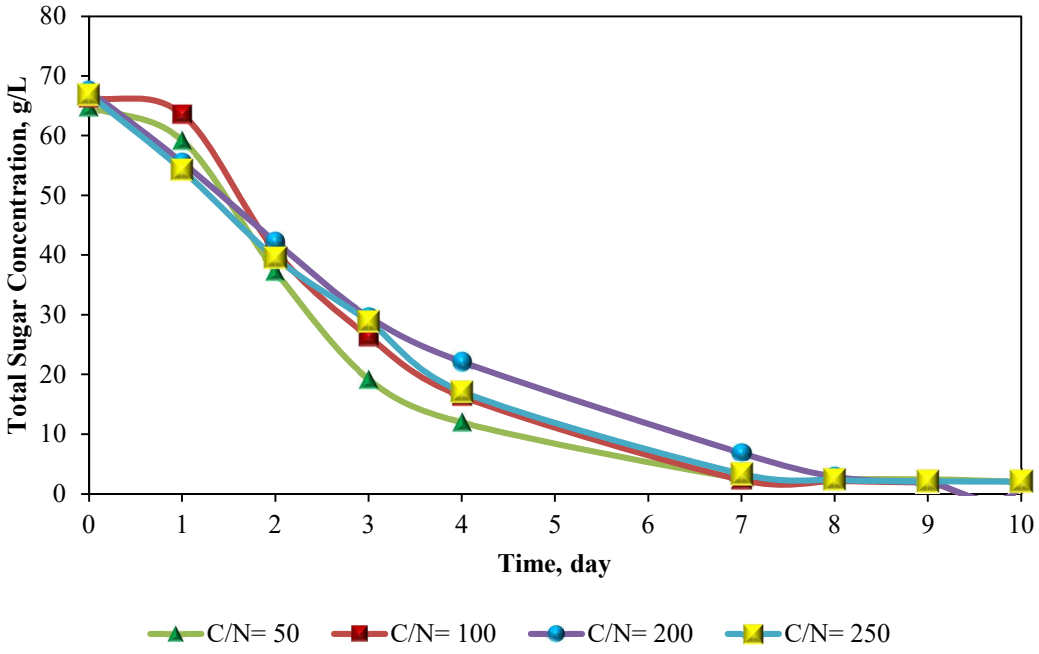


Figure 3.80 Consumption of total sugar over time with different C/N ratios

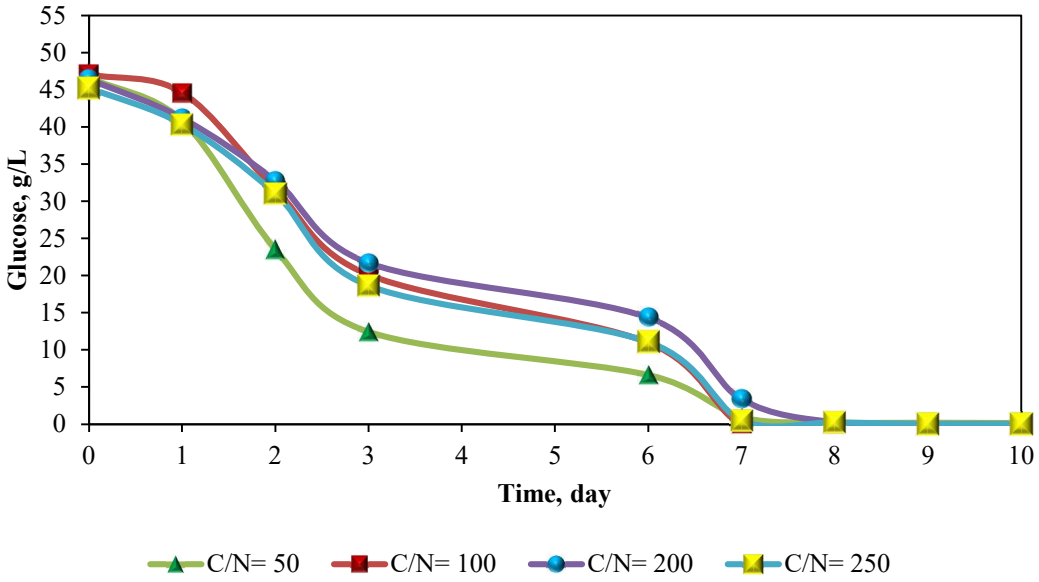


Figure 3.81 Consumption of glucose over time with different C/N ratios

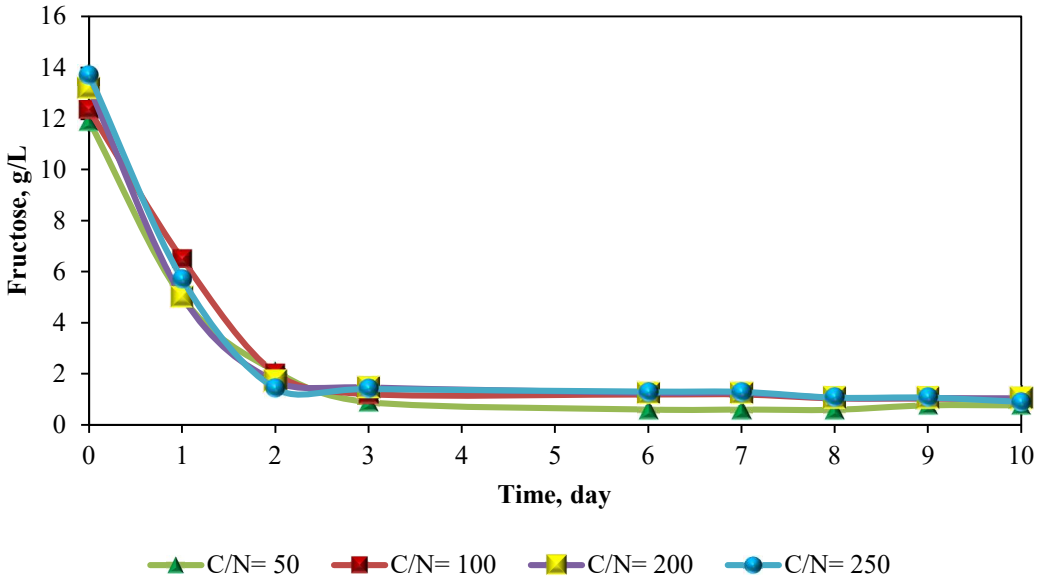


Figure 3.82 Consumption of fructose over time with different C/N ratios

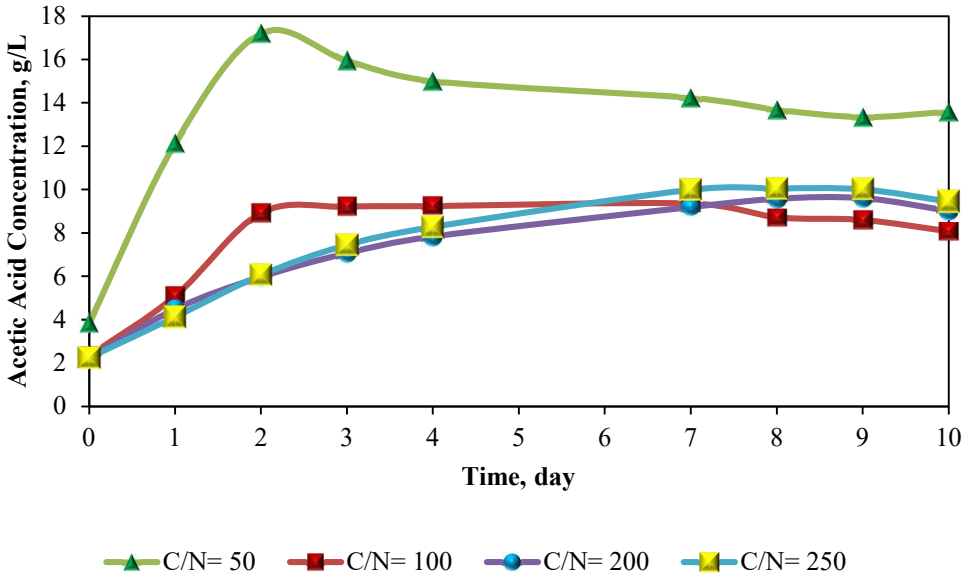


Figure 3.83 Variation of acetic acid level over time with different C/N ratios

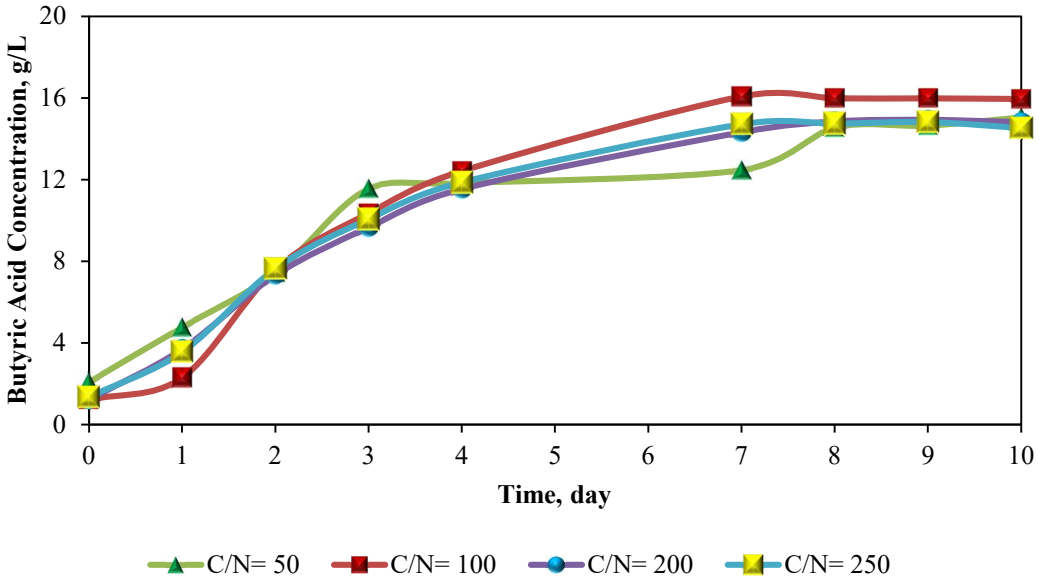


Figure 3.84 Variation of butyric acid level over time with different C/N ratios

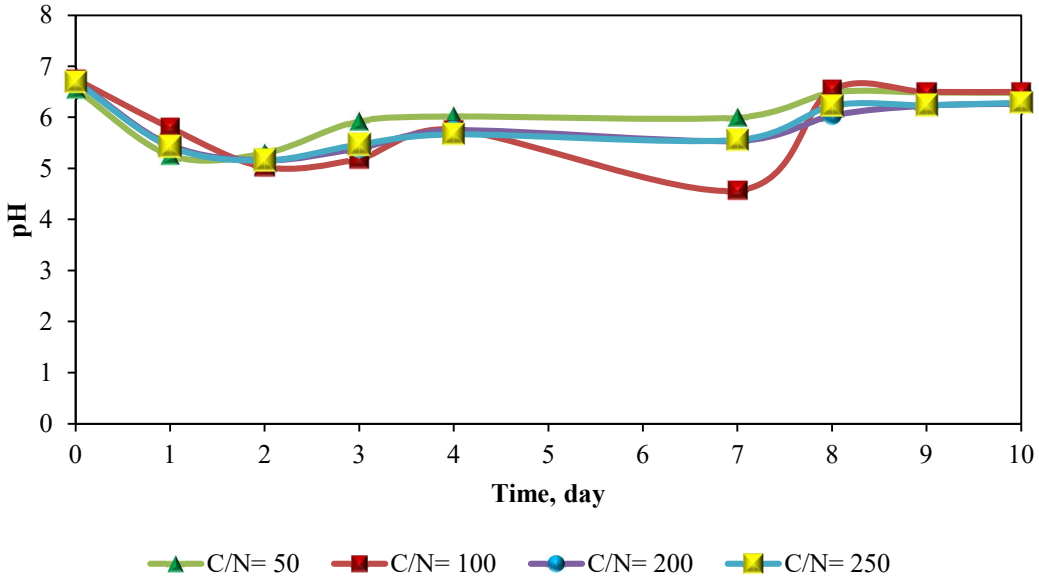


Figure 3.85Variation of pH over time with different C/N ratios

The production yields and rates were given in Table 3.5. The theoretical maximum hydrogen production yield from glucose is 4 mol/mol glucose and 2 mol/mol glucose for the end products acetic acid and butyric acid, respectively. The mixture of these organic acids is produced during fermentation; hence the average maximum yield is accepted as 2.5 mol/mol glucose consumed. The experimentally observed maximum yield is around 1 mol/mol glucose at studied C/N ratios. The

volumetric production rate (mL H_2/L) reached to its maximum value of 9057 ml H_2/L and daily volumetric production rate was between 831-1790 mL /L d. The results indicated that nitrogen limited conditions such as C/N=100-250 are more suitable to enhance the hydrogen production. But, nitrogen rich condition as C/N=50 adversely affects the hydrogen production.

Table 3.5 Hydrogen production rates and yields at different C/N ratios

C/N	Yield mol H_2/ mol glucose	Yield mL H_2/g $glucose_{consumed}$	Rate mL H_2/L	Rate mL H_2/d	Rate mL H_2/ $L_{reactor}$ d
50	0.70	24	6140	207	831
100	0.95	33.6	8627	448	1790
200	0.96	33.9	8840	304	1217
250	1.00	35.3	9057	327	1307

The results indicate that the product formation, mainly acetone and hydrogen, is enhanced for C/N=100-250. Although, the nitrogen rich environment like C/N=50 provides better ethanol, acetic and butyric acid formation, the hydrogen and acetone generations are substantially affected. The best media composition for the high product formation can be determined as C/N=100 for the sake of hydrogen generation.

3.3.6 Effects of Different Initial Substrate Concentrations and C/P

Similar to C/N ratio, C/P ratio is an important parameter to enhance the growth of microorganisms and to provide sufficient nutrient for product formation. The effect of the phosphorus concentration added to the medium could enhance or limit the product formation depending on the environmental conditions.

C/P ratio was varied between C/P=19-43 by changing the sugar concentration (67-149 g/L). Phosphorus concentration was kept constant at 1.39 g/L. The pH of the experimental media was adjusted to around 6.7 with 10 M NaOH solution. Acetone production over time was given in Figure 3.86. The initial level of acetone was 2.6 g/L and, the highest acetone concentration was 22 g/L in the medium where the initial sugar concentration was 129 g/L (C/P=37). The lowest acetone level was 15.5

g/L and, obtained in the media where the initial total sugar amount was 67 g/L (C/P= 19). The acetone concentrations obtained for the mediums, where the initial sugar amounts were 85 (C/P=25), 107 (C/P=31), and 149 g/L (C/P=43), were 16, 16.5, and 20 g/L, respectively.

As can be seen in Figure 3.87, the highest ethanol level was 2.1 g/L and obtained from the medium with 107 g/L of initial sugar concentration. The lowest ethanol level was observed in the medium with an initial substrate concentration of 149 g/L.While the amount of ethanol was 0.9 g/L for the mediums where the sugar concentrations were 67, 129 g/L, it was 1 g/L for the medium with 85 g/L of sugar concentration. Butanol was not found in any of the mediums.

Hydrogen production with time was shown in Figure 3.88. The highest hydrogen volume was 1558 mL and, observed in the medium with an initial sugar level of 107 g/L. The lowest hydrogen volume produced was 374 mL in the medium where the initial substrate level was 67 g/L.

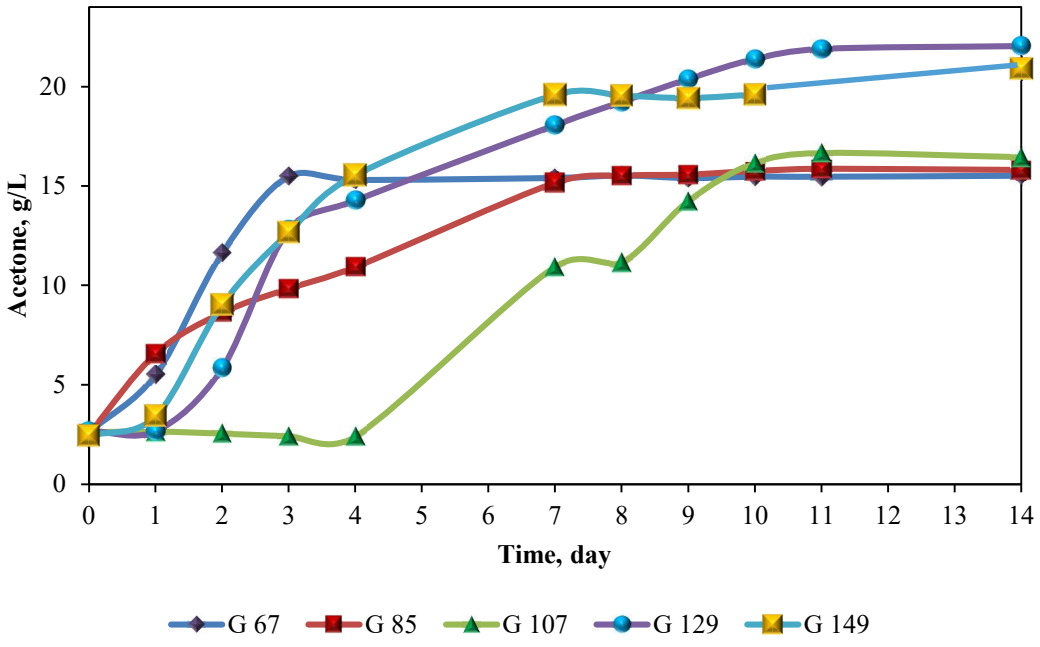


Figure 3.86 Variation of acetone production over time with different initial substrate concentrations and C/P ratios

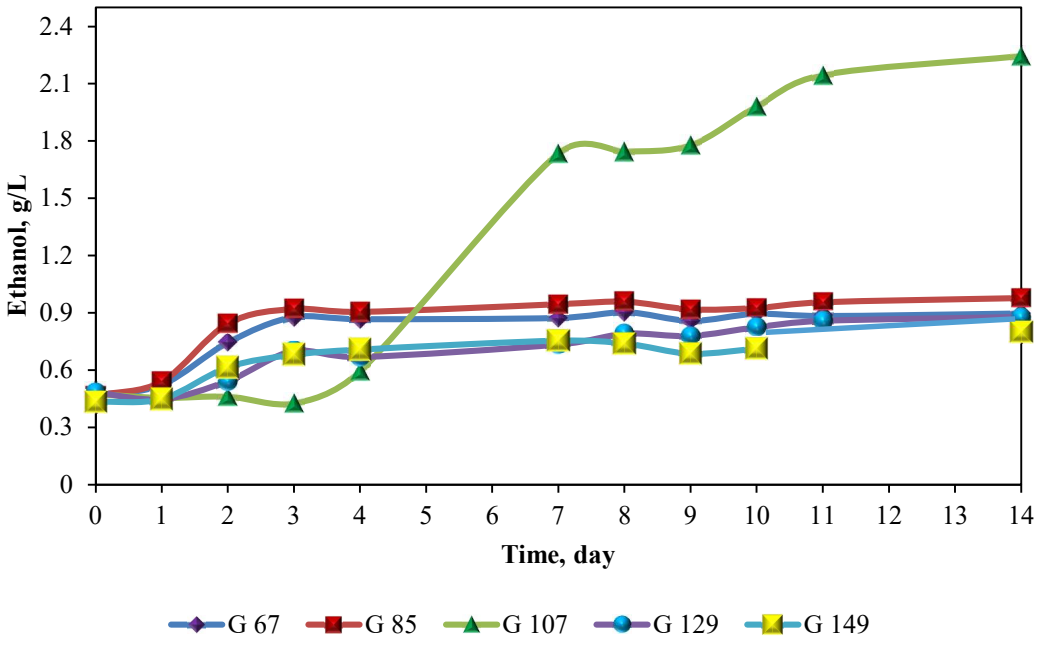


Figure 3.87 Variation of ethanol production over time with different initial substrate concentrations and C/P ratios

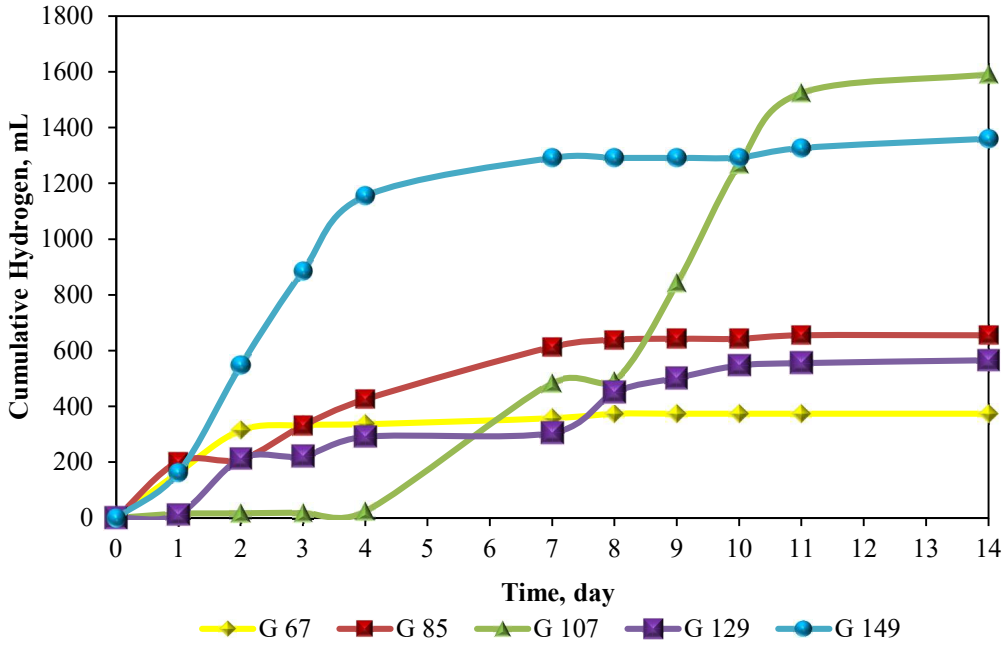


Figure 3.88 Variation of hydrogen production over time with different initial substrate concentrations and C/P ratios

Amounts of total sugar, glucose, and fructose varied according to the level of substrate added at the beginning. Consumptions of total sugar, glucose and fructose over time were presented in Figure 3.89, Figure 3.90 and Figure 3.91, respectively. Changes in acetic and butyric acid concentrations were shown in Figure 3.92 and Figure 3.93. The variation of pH level was given in Figure 3.94. The results were evaluated below.

In the medium where the initial substrate amount was 67 g/L (C/P=19), total sugar was consumed up to 3 g/L. Acetic acid concentration reached the level of 12 g/L and then consumption started. The amount of butyric acid was around 10 g/L. The concentrations of acetone and ethanol produced were 15.5 and 0.9 g/L, respectively. The hydrogen volume produced was 374 mL.

Total sugar was consumed up to 9 g/L in the medium where 85 g/L (C/P=25) sugar was added. Consumption started after the acetic acid level reached 11 g/L. The butyric acid concentration was around 11 g/L. The amounts of acetone and ethanol produced were 16 and 1 g/L, respectively. The cumulative volume of hydrogen is 655 mL.

In the media with 107 g/L (C/P=31) substrate level, total sugar was consumed up to 6 g/L. Acetic and butyric acid levels were at concentrations of 17 and 10 g/L, respectively. The amounts of ethanol and acetone produced were 2.1 and 16.5 g/L, respectively. The volume of hydrogen gas was 1558 mL.

Total sugar was consumed up to 46 g/L in the medium with 129 g/L (C/P=37) initial sugar level. Acetic and butyric acid levels were 12 and 14 g/L, respectively. While acetone concentration was 22 g/L, ethanol level reached 0.9 g/L. The volume of hydrogen gas is 561 mL.

In the medium with an initial sugar concentration of 149 g/L (C/P=43), total sugar was consumed up to 83 g/L. While the acetic acid level was 11 g/L, the amount of

butyric acid was 14 g/L. The acetone and ethanol concentrations obtained were 20 and 0.7 g/L, respectively. The volume of cumulative hydrogen was 1344 mL.

While media with 129 g/L (C/P=37) of initial sugar concentration promoted acetone formation, the production of ethanol and hydrogen was supported by the medium with a substrate concentration of 107 g/L (C/P=31).

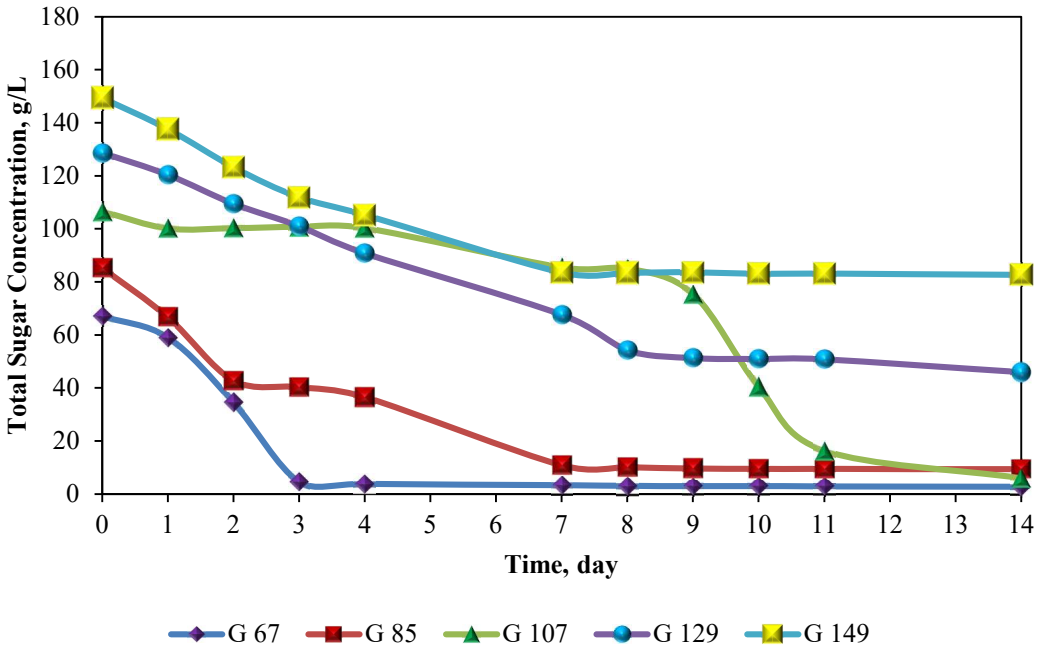


Figure 3.89 Consumption oftotal sugar over time with different initial substrate concentrations and C/P ratios

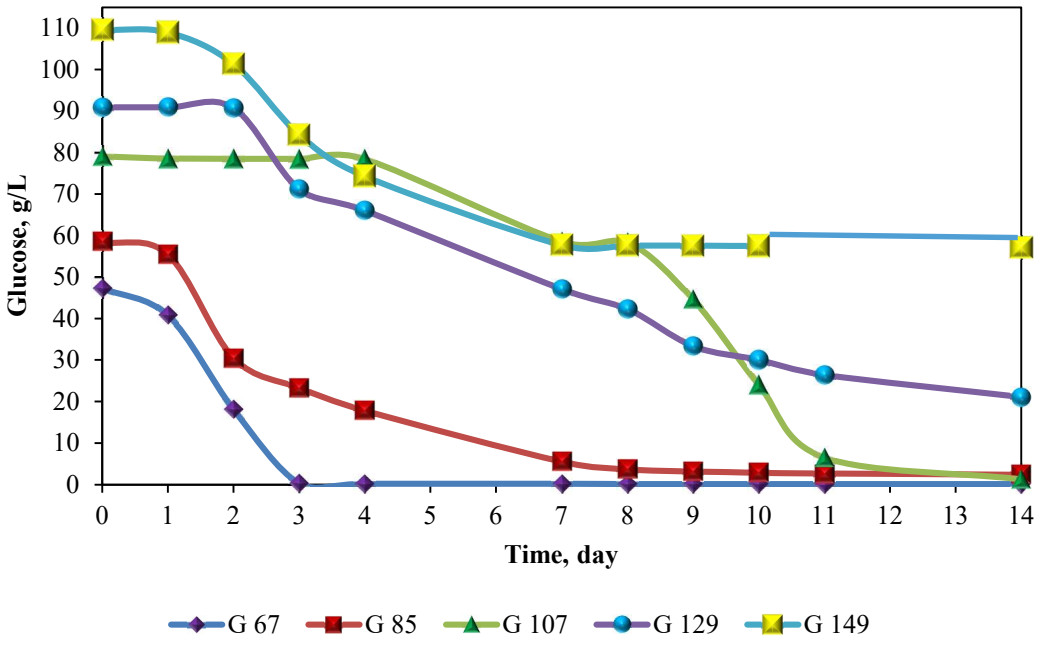


Figure 3.90 Consumption of glucose over time with different initial substrate concentrations and C/P ratios

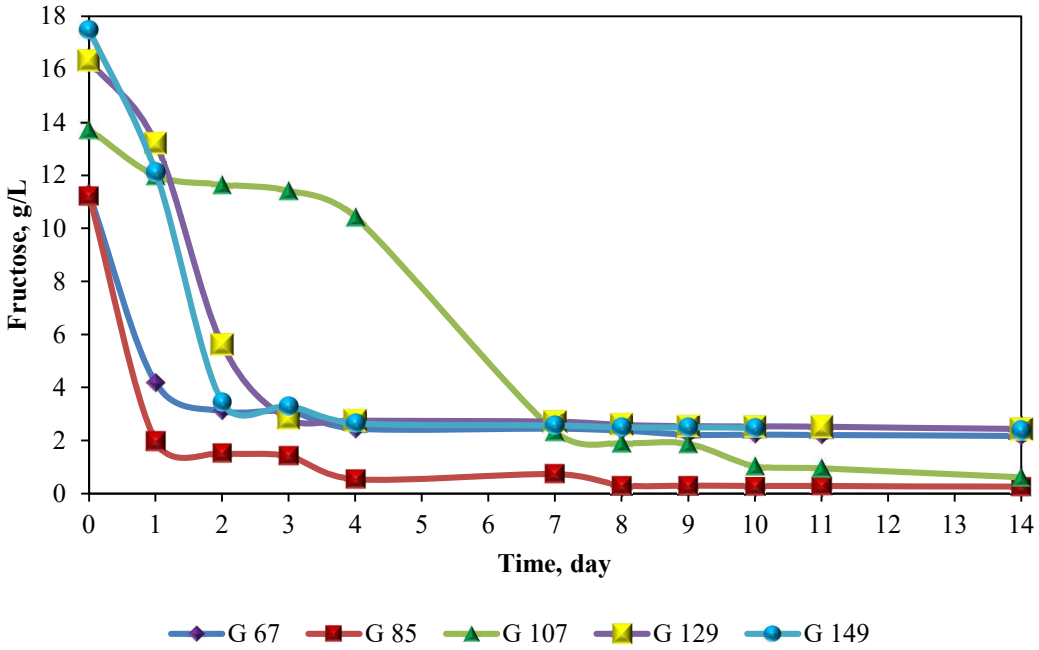


Figure 3.91 Consumption of fructose over time with different initial substrate concentrations and C/P ratios

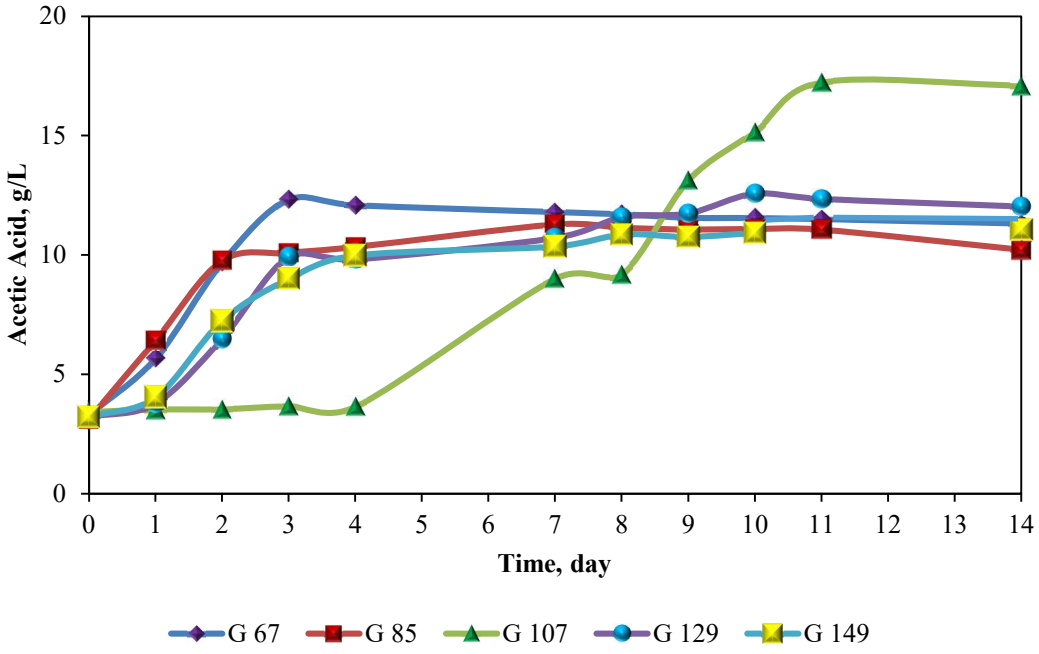


Figure 3.92 Variation of acetic acid level over time with different initial substrate concentrations and C/P ratios

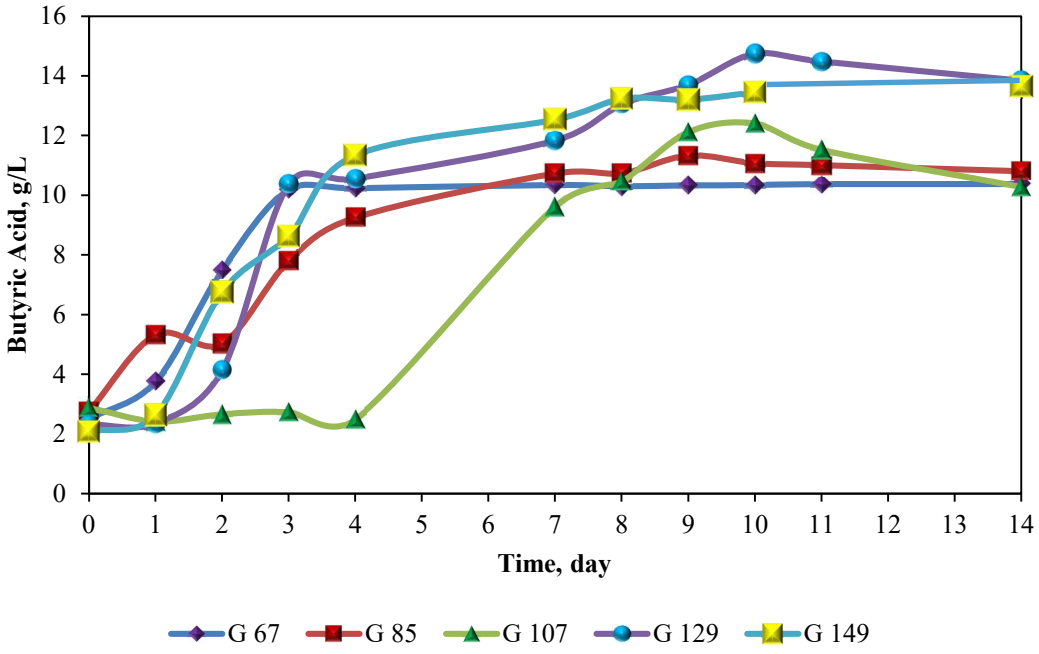


Figure 3.93 Variation of butyric acid level over time with different initial substrate concentrations and C/P ratios

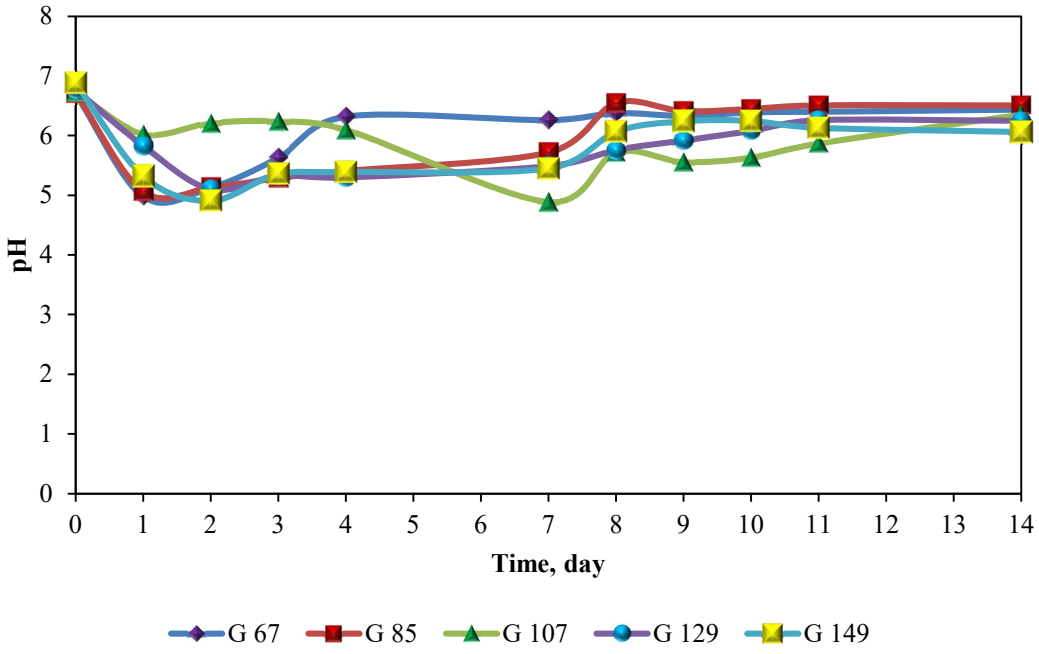


Figure 3.94 Variation of pH over time with different initial substrate concentrations and C/P ratios

The production yields and rates were given in Table 3.6. The theoretical maximum hydrogen production yield from glucose is 4 mol/mol glucose and 2 mol/mol glucose for the end products acetic acid and butyric acid, respectively. The mixture of these organic acids is produced during fermentation; hence the average maximum yield is accepted as 2.5 mol/mol glucose consumed. The experimentally observed maximum yield is around 0.57 mol/mol glucose at studied C/P ratios. The volumetric production rate (mL H_2/L) reached to its maximum value of 6367 mL H_2/L and daily volumetric production rate was between 332-794 mL /L d. The results indicated that C/P=31-43 are more suitable to enhance the hydrogen production. But, decreased C/P ratios adversely affect the hydrogen production.

Table 3.6 Hydrogen production rates and yields at different C/P ratios

C/P	Yield mol H_2/ mol glucose	Yield mL H_2/g $glucose_{consumed}$	Rate mL H_2/L	Rate mL H_2/d	Rate mL H_2/ $L_{reactor}$ d
19	0.16	5.8	1495	84	337
25	0.24	8.5	2571	83	332
31	0.44	15.4	6367	163	655
43	0.57	20.1	5444	198	794

CHAPTER 4
CONCLUSION

Hydrolysis conditions of microwave pretreatment that maximize the total sugar concentration were optimized for biobutanol production from peach pulp. Particle diameter and substrate concentration are the main parameters affecting total sugar concentration. Moreover, there are statistically significant interactions between these variables. Hydrolysis pH value and the interaction of pH with other factors do not affect the total sugar concentration. The maximum total sugar concentration was obtained at pH=5.50, particle diameter D_p=246.88 μm, substrate concentration S_0= 39.48 g/L, within the limits specified in the microwave hydrolysis process.

Butanol productivities of *C. acetobutylicum* NRLL 527 and *C. pasteurianum* DSM 525 were examined. For this purpose, the parameters were initial substrate concentration; nitrogen and phosphorus concentrations; levels of ions like magnesium, manganese, zinc, calcium, and iron; butyric acid, and ethanol concentrations as metabolic shifters to butanol; neutral red addition as an electron carrier.

In the first batch experiment of *C. acetobutylicum*, 10 g/L $CaCO_3$ was added to the medium to increase butanol tolerance and to obtain higher butanol levels. Ethanol and very small amounts of butanol (0.020 g/L) were produced. However, the high amount of lactic acid levels indicated that Lactobacillus species dominated *Clostridium acetobutylicum* bacteria. No effects of $CaCO_3$ addition for neither solvent nor acid production were observed.

In the second experiment with *C. acetobutylicum*, 5 g/L butyric acid was added to the medium as an initiator to steer the metabolic pathway to biobutanol production. Organic matter consumption and product formation were not observed. Since there was no formation of volatile fatty acids in the media, the pH remained constant throughout the experiment. The butyric acid (5 g/L) addition has inhibited the growth of organisms, prevented organic matter consumption and product formation.

In the last set of batch experiments with *C. acetobutylicum*, magnesium (Mg), manganese (Mn), zinc (Zn) were added to the fermentation media at different concentrations in order to evaluate the effect of these trace elements, which are stated to provide activation of butanol production in the literature. There was no significant depletion of substrate and pH remained still since there was not any organic acid formation. Moreover, solvent production did not occur. The addition of Mg, Mn, and Zn as butyric acid activators had negative effects on organisms and acted as inhibitors, instead of activators.

Solvent production by *Clostridium pasteurianum* was studied under the media compositions as magnesium, manganese and zinc was added as trace elements to procure activation of butanol production. Neutral red was also added as an electron carrier to overcome NADH limitation and, provide the direct conversion of carbon source to solvents. A high amount of organic matter consumption was achieved with the addition of magnesium (Mg) and zinc (Zn) and acetone production was obtained. The acetone concentrations reached 7 and 6 g/L for magnesium and zinc added mediums, respectively. Ethanol production was limited, butanol did not generate. In manganese (Mn) and neutral red added mediums, product formation did not occur because substrate consumption was not observed.

The media composition was rich in protein, which might adversely affect the butanol production. Therefore, protein sources were removed from the fermentation medium to see the effects of simplified media on the butanol production of *C. pasteurianum.* While iron deficiency (Fe) supported acetone production (18.2 g/L), magnesium deficiency (Mg) supported ethanol production, resulting in a concentration of 1 g/L. Also, hydrogen production has begun to be monitored in this experiment. However, manganese deficiency (Mn) promoted hydrogen production, and 1767 mL of hydrogen gas formed.

The addition of butyric acid to the fermentation medium could direct metabolic pathway to butanol production. With this purpose, butyric acid was added to media in the ranges of 6-12 g/L. Batch experiments were performed by *C.*

pasteurianum. The highest organic matter consumption (98%) and product formation were in the control group which has no additional butyric acid. Acetone and ethanol concentrations were 14 and 0.6 g/L, respectively. Cumulative hydrogen volume was 1178 mL. As the additional butyric acid concentration increased, sugar consumption, product formation decreased. Moreover, ethanol production was limited and butanol formation was not observed.

External addition of ethanol can act as inhibitor for ethanol production and the metabolic pathway of *C. pasteurianum* could shift to butanol generation. By this hypothesis, ethanol was added in the range of 0.2-1.4 g/L to the fermentation media in order to increase butanol production. The highest average ethanol production (0.7 g/L) and acetone (14.3 g/L) levels were accomplished with 0.2 g/L ethanol added medium. Hydrogen production was maximized in the ethanol free-control group, with a volume of 1640 mL. Ethanol addition promoted acetone production instead of butanol formation. However, this effect was not noteworthily when the acetone levels were evaluated.

Nitrogen is an important nutrient for dark fermentation and its concentration could enhance or limit product formation. C/N ratio could be optimized for the best results. Thus, C/N ratio was optimized in the range of 50-250 for the batch cultures of *C. pasteurianum*. Around 97% of the sugar was consumed in all of the environmental media. Acetone level reached to its maximum (23 g/L) in C/N=100. Hydrogen yield was around 1 mol H_2/mol glucose and the volumetric production rate was 9057 ml H_2/L for C/N=250. While nitrogen rich condition (C/N=50) negatively affected the acetone and hydrogen production, ethanol concentration was minimized under relatively nitrogen-limited conditions in the media where C/N=200. Consequently, the best media composition for the high product formation can be determined as C/N=100 for the sake of hydrogen generation.

Similar to C/N ratio, C/P ratio of the fermentation media could influence product formation. Hence, C/P ratio can be adjusted to obtain the best results. In order to achieve this goal, C/P ratio was varied in 19-43 by changing the sugar concentration

(67-149 g/L) and phosphorous concentration was kept constant at 1.39 g/L. Dark fermentation was performed by batch cultures of *C. pasteurianum*. Above 107 g/L of substrate concentration and C/P=31, organic matter consumption decreased significantly. Nevertheless, acetone levels increased conversely. Maximum acetone level was 22 g/L for 129 g/L sugar added medium (C/P=37). Ethanol concentration and hydrogen volume reached their maximum values of 2.1 g/L and 1558 mL, with 107 g/L sugar addition and C/P=31. Butanol formation was not observed.

REFERENCES

Abdehagh, N., Tezel, F. H., & Thibault, J. (2014). Separation techniques in butanol production: Challenges and developments. *Biomass and Bioenergy*, *60*, 222-246.

Al-Shorgani, N. K. N., Kalil, M. S., Ali, E., Yusofl, W. M. W., & Hamid, A. A. (2012). Enhancement of biobutanol production by butyric acid addition using *Clostridium saccharoperbutytacetonicum* N1-4 (ATCC 13564). *Biotechnology, 11*(6), 326-332.

Al-Shorgani, N. K. N., Al-Tabib, A. I., Kadier, A., Zanil, M. F., Lee, K. M., & Kalil, M. S. (2019). Continuous Butanol Fermentation of Dilute Acid-Pretreated De-oiled Rice Bran by *Clostridium acetobutylicum* YM1. *Scientific Reports*, *9*(1), 1-13.

Al-Shorgani, N. K. N, Kalil, M. S., Yusoff, W. M. W., & Hamid, A. A. (2018a). Impact of pH and butyric acid on butanol production during batch fermentation using a new local isolate of *Clostridium acetobutylicum* YM1. *Saudi Journal of Biological Sciences*, *25*(2), 339–348.

Al-Shorgani, N. K. N, Shukor, H., Abdeshahian, P., Kalil, M. S., Yusoff, W. M. W., & Hamid, A. A. (2018b). Enhanced butanol production by optimization of medium parameters using *Clostridium acetobutylicum* YM1. *Saudi Journal of Biological Sciences*, 25(7), 1308-13201.

Alvira, P., Tomás-Pejó, E., Ballesteros, M., & Negro, M. J. (2010). Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresource Technology*, *101*(13), 4851-4861.

Argun, H., & Dao, S. (2017). Bio-hydrogen production from waste peach pulp by dark fermentation: Effect of inoculum addition. *International Journal of Hydrogen Energy*, *42*(4), 2569-2574.

Argun, H., Liz, P. G., & Karapinar, I. (2016). Biohydrogen production potential of different biomass sources. In A. Singh, D. Rathore (Eds.). *Biohydrogen production: Sustainability of current technology and future perspective* (1st ed.) (11–48). New Delhi: Springer.

Bankar, S. B., Survase, S. A., Ojamo, H., & Granström, T. (2013). Biobutanol: The outlook of an academic and industrialist. *RSC Advances*, *3*(47), 24734-24757.

Bankar, S. B., Survase, S. A., Singhal, R. S., & Granström, T. (2012). Continuous two stage acetone-butanol-ethanol fermentation with integrated solvent removal using *Clostridium acetobutylicum* B 5313. *Bioresource Technology, 106*, 110-116.

Bikash, L., & Sibi, G. (2017). Fruit Wastes Hydrolysates as Feedstock: Pre-Treatment Strategies for Cost-Saving and Sustainable Microalgae Cultivation. *International Journal of Research in Environmental Science, 3*(2), 35–40.

Birgen, C., Dürre, P., Preisig, H. A., & Wentzel, A. (2019). Butanol production from lignocellulosic biomass: Revisiting fermentation performance indicators with exploratory data analysis. *Biotechnology for Biofuels 12*(1), 1-15.

Bowles, L. K., & Ellefson, W. L. (1985). Effects of butanol on *Clostridium acetobutylicum*. *Applied and Environmental Microbiology, 50*(5), 1165–1170.

Del Pilar Anzola-Rojas, M., Da Fonseca, S. G., Da Silva, C. C., De Oliveira, V. M., &Zaiat, M. (2015). The use of the carbon/nitrogen ratio and specific organic loading rate as tools for improving biohydrogen production in fixed-bed reactors. *Biotechnology Reports, 5*(1), 46-54.

Díez-Antolínez, R., Hijosa-Valsero, M., Paniagua-García, A. I., & Gómez, X. (2016). Effect of nutrient supplementation on biobutanol production from cheese

whey by ABE (acetone-butanol-ethanol) fermentation. *Chemical Engineering Transactions, 49*, 217-222.

Dolejš, I., Krasňan, V., Stloukal, R., Rosenberg, M., & Rebroš, M. (2014). Butanol production by immobilized *Clostridium acetobutylicum* in repeated batch, fed-batch, and continuous modes of fermentation. *Bioresource Technology, 169*, 723-730.

Du, G., Che, J., Wu, Y., Wang, Z., Jiang, Z., Ji, F., & Xue, C. (2021). Disruption of hydrogenase gene for enhancing butanol selectivity and production in *Clostridium acetobutylicum*. *Biochemical Engineering Journal, 171*,108014.

Durán-Padilla, V. R., Davila-Vazquez, G., Chávez-Vela, N. A., Tinoco-Valencia, J. R., & Jáuregui-Rincón, J. (2014). Iron effect on the fermentative metabolism of *Clostridium acetobutylicum* ATCC 824 using cheese whey as substrate. *Biofuel Research Journal,* 4, 129-133.

Dürre, P. (2007). Biobutanol: An attractive biofuel. *Biotechnology Journal, 2*(12), 1525-1534.

Ezeji, T., Milne, C., Price, N. D., & Blaschek, H. P. (2010). Achievements and perspectives to overcome the poor solvent resistance in acetone and butanol-producing microorganisms. *Applied Microbiology and Biotechnology, 85*, 1697-1712.

Ezeji, T. C., Qureshi, N., & Blaschek, H. P. (2004). Butanol fermentation research: Upstream and downstream manipulations. *Chemical Record, 4*(5), 305-314.

Fatriasari, W., Anita, S. H., & Risanto, L. (2017). Microwave assisted acid pretreatment of oil palm empty fruit bunches (EFB) to enhance its fermentable sugar production. *Waste and Biomass Valorization, 8*, 379-391.

García, V., Päkkilä, J., Ojamo, H., Muurinen, E., & Keiski, R. L. (2011). Challenges in biobutanol production: How to improve the efficiency*? Renewable and Sustainable Energy Reviews, 15*(2), 964-980.

Gashaw, A., & Getachew, T. (2014). Fermentation of crop residues and fruit wastes for production of ethanol and its pre-treatment methods: A review. *International Journal of Research, 1*(11), 543-556.

Girbal, L., Vasconcelos, I., Saint-Amans, S., &Soucaille, P. (1995). How neutral red modified carbon and electron flow in *Clostridium acetobutylicum* grown in chemostat culture at neutral pH. *FEMS Microbiology Reviews, 16*(2–3), 151-162.

Gottumukkala, L. D., Haigh, K., & Görgens, J. (2017). Trends and advances in conversion of lignocellulosic biomass to biobutanol: Microbes, bioprocesses and industrial viability. *Renewable and Sustainable Energy Reviews, 76*, 963-973.

Gottumukkala, L. D., Parameswaran, B., Valappil, S. K., Mathiyazhakan, K., Pandey, A., & Sukumaran, R. K. (2013). Biobutanol production from rice straw by a non acetone producing *Clostridium sporogenes* BE01. *Bioresource Technology, 145*, 182–187.

Grimmler, C., Janssen, H., Krauße, D., Fischer, R. J., Bahl, H., Dürre, P., Liebl, W., & Ehrenreich, A. (2011). Genome-wide gene expression analysis of the switch between acidogenesis and solventogenesis in continuous cultures of *Clostridium acetobutylicum*. *Journal of Molecular Microbiology and Biotechnology, 20*(1), 1-15.

Gu, Y., Hu, S., Chen, J., Shao, L., He, H., Yang, Y., Yang, S., & Jiang, W. (2009). Ammonium acetate enhances solvent production by *Clostridium acetobutylicum* EA 2018 using cassava as a fermentation medium. *Journal of Industrial Microbiology and Biotechnology, 36*(9), 1225-1232.

Harris, L. M., Welker, N. E., & Papoutsakis, E. T. (2002). Northern, morphological, and fermentation analysis of spo0A inactivation and overexpression in *Clostridium acetobutylicum* ATCC 824. *Journal of Bacteriology, 184*(13), 3586-3597.

Hendriks, A. T. W. M., & Zeeman, G. (2009). Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Technology, 100*(1), 10-18.

Hermiati, E., Azuma, J., Mangunwidjaja, D., Sunarti, T. C., Suparno, O., & Prasetya, B. (2011). Hydrolysis of carbohydrates in cassava pulp and tapioca flour under microwave irradiation. *Indonesian Journal of Chemistry, 11*(3), 238–245.

Hipolito, C. N., Crabbe, E., Badillo, C. M., Zarrabal, O. C., Mora, M. A. M., Flores, G. P., de A. Hernández Cortazar, M., & Ishizaki, A. (2008). Bioconversion of industrial wastewater from palm oil processing to butanol by *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564). *Journal of Cleaner Production, 16*(5), 632-638.

Holt, R. A., Cairns, A. J., & Morris, J. G. (1988). Production of butanol by *Clostridium puniceum* in batch and continuous culture. *Applied Microbiology and Biotechnology, 27*(4), 319-324.

Ibrahim, M. F., Kim, S. W., & Abd-Aziz, S. (2018). Advanced bioprocessing strategies for biobutanol production from biomass. *Renewable and Sustainable Energy Reviews, 91*, 1192-1204.

Ibrahim, M. F., Ramli, N., Bahrin, E. K., & Abd-Aziz, S. (2017). Cellulosic biobutanol by Clostridia: Challenges and improvements. *Renewable and Sustainable Energy Reviews, 79*, 1241-1254.

Jiang, C., Cao, G., Wang, Z., Li, Y., Song, J., Cong, H., Zhang, J., & Yang, Q. (2016). Enhanced butanol production through adding organic acids and neutral

red by newly isolated butanol-tolerant bacteria. *Applied Biochemistry and Biotechnology, 180*(7), 1416–1427.

Jiang, Y., Xu, C., Dong, F., Yang, Y., Jiang, W., & Yang, S. (2009). Disruption of the acetoacetate decarboxylase gene in solvent-producing *Clostridium acetobutylicum* increases the butanol ratio. *Metabolic Engineering, 11*(4–5), 284–291.

Jin, C., Yao, M., Liu, H., Lee, C. F. F., & Ji, J. (2011). Progress in the production and application of n-butanol as a biofuel. *Renewable and Sustainable Energy Reviews, 15*(8), 4080-4106.

Junelles, A. M., Janati-Idrissi, R., Petitdemange, H., & Gay, R. (1988). Iron effect on acetone-butanol fermentation. *Current Microbiology, 17*(5), 299-303.

Kamiński, W., Tomczak, E., & Górak, A. (2011). Biobutanol - production and purification methods. *Ecological Chemistry and Engineering S, 18*(1), 31-37.

Kanchanatawee, S., & Maddox, I. S. (1990). Effect of phosphate and ammonium ion concentrations on solvent production in defined medium by *Clostridium acetobutylicum*. *Journal of Industrial Microbiology, 5*(5), 277–282.

Keis, S., Bennett, C. F., Ward, V. K., & Jones, D. T. (1995). Taxonomy and phylogeny of industrial solvent-producing clostridia. International *Journal of Systematic Bacteriology, 45*(4), 693-705.

Kujawska, A., Kujawski, J., Bryjak, M., & Kujawski, W. (2015). ABE fermentation products recovery methods - A review. *Renewable and Sustainable Energy Reviews, 48*, 648-661.

Kumar, M., & Gayen, K. (2011). Developments in biobutanol production: New insights. *Applied Energy, 88*(6), 1992-2002.

Kumari, D., & Singh, R. (2018). Pretreatment of lignocellulosic wastes for biofuel production: A critical review. *Renewable and Sustainable Energy Reviews, 90*, 877-897.

Lee, S. Y., Park, J. H., Jang, S. H., Nielsen, L. K., Kim, J., & Jung, K. S. (2008). Fermentative butanol production by clostridia. *Biotechnology and Bioengineering, 101*(2), 209-228.

Li, S., Huang, L., Ke, C., Pang, Z., & Liu, L. (2020). Pathway dissection, regulation, engineering and application: Lessons learned from biobutanol production by solventogenic clostridia. *Biotechnology for Biofuels, 13*(1), 1-25.

Li, S. Y., Srivastava, R., Suib, S. L., Li, Y., & Parnas, R. S. (2011). Performance of batch, fed-batch, and continuous A-B-E fermentation with pH-control. *Bioresource Technology, 102*(5), 4241-4250.

Li, Y., Tang, W., Chen, Y., Liu, J., & fon F. Lee, C. (2019). Potential of acetone-butanol-ethanol (ABE) as a biofuel. *Fuel, 242*, 673-686.

Lin, D. S., Yen, H. W., Kao, W. C., Cheng, C. L., Chen, W. M., Huang, C. C., & Chang, J. S. (2015). Bio-butanol production from glycerol with *Clostridium pasteurianum* CH4: The effects of butyrate addition and in situ butanol removal via membrane distillation. *Biotechnology for Biofuels, 8*(1), 1-12.

Lin, Z., Liu, H., Yan, X., Zhou, Y., Cheng, K., & Zhang, J. (2017). High-efficiency acetone-butanol-ethanol production and recovery in non-strict anaerobic gas-stripping fed-batch fermentation. *Applied Microbiology and Biotechnology, 101*(21), 8029-8039.

Lu, C., Zhao, J., Yang, S. T., & Wei, D. (2012). Fed-batch fermentation for n-butanol production from cassava bagasse hydrolysate in a fibrous bed bioreactor with continuous gas stripping. *Bioresource Technology, 104*, 380-387.

Luo, J., Fang, Z., & Smith, R. L. (2014). Ultrasound-enhanced conversion of biomass to biofuels. *Progress in Energy and Combustion Science, 41*(1), 56-93.

Mabee, W. E., Gregg, D. J., Arato, C., Berlin, A., Bura, R., Gilkes, N., Mirochnik, O., Pan, X., Pye, E. K., & Saddler, J. N. (2006). Updates on softwood-to-ethanol process development. *Applied Biochemistry and Biotechnology, 129*(1–3), 129-132.

Madihah, M. S., Ariff, A. B., Sahaid, K. M., Suraini, A. A., & Karim, M. I. A. (2001). Direct fermentation of gelatinized sago starch to acetone-butanol-ethanol by *Clostridium acetobutylicum*. *World Journal of Microbiology and Biotechnology, 17*, 567-576.

Morone, A., & Pandey, R. A. (2014). Lignocellulosic biobutanol production: Gridlocks and potential remedies. *Renewable and Sustainable Energy Reviews, 37*, 21-35.

Mukherjee, M., Sarkar, P., Goswami, G., & Das, D. (2019). Regulation of butanol biosynthesis in *Clostridium acetobutylicum* ATCC 824 under the influence of zinc supplementation and magnesium starvation. *Enzyme and Microbial Technology, 129*, 1-9.

Nanda, S., Golemi-Kotra, D., McDermott, J. C., Dalai, A. K., Gökalp, I., & Kozinski, J. A. (2017). Fermentative production of butanol: Perspectives on synthetic biology. *New Biotechnology, 37*, 210-221.

Ndaba, B., Chiyanzu, I., & Marx, S. (2015). n-Butanol derived from biochemical and chemical routes: A review. *Biotechnology Reports, 8*, 1-9.

Peguin, S., Goma, G., Delorme, P., & Soucaille, P. (1994). Metabolic flexibility of Clostridium acetobutylicum in response to methyl viologen addition. *Applied Microbiology and Biotechnology, 42*(4), 611–616.

Poehlein, A., Solano, J. D. M., Flitsch, S. K., Krabben, P., Winzer, K., Reid, S. J., Jones, D. T., Green, E., Minton, N. P., Daniel, R., & Dürre, P. (2017). Microbial solvent formation revisited by comparative genome analysis. *Biotechnology for Biofuels, 10*, 1-15.

Pugazhendhi, A., Mathimani, T., Varjani, S., Rene, E. R., Kumar, G., Kim, S. H., Ponnusamy, V. K., & Yoon, J. J. (2019). Biobutanol as a promising liquid fuel for the future - recent updates and perspectives. *Fuel, 253*, 637-646.

Qureshi, N., Hughes, S., Maddox, I. S., & Cotta, M. A. (2005). Energy-efficient recovery of butanol from model solutions and fermentation broth by adsorption. *Bioprocess and Biosystems Engineering, 27*(4), 215-222.

Raganati, F., Procentese, A., Olivieri, G., Russo, M. E., & Marzocchella, A. (2016). Butanol production by fermentation of fruit residues. *Chemical Engineering Transactions, 49*, 229-234.

Rajagopalan, G. (2016). Effect of nitrogen salts on butanol and hydrogen production from xylan by using clostridium strain BOH3. *Journal of Applied Biotechnology & Bioengineering, 1*(2), 44-51.

Ranjan, A., & Moholkar, V. S. (2012). Biobutanol: Science, engineering, and economics. *In International Journal of Energy, 36*(3), 277-323.

Rao, A., Sathiavelu, A., & Mythili, S. (2016). Genetic engineering in biobutanol production and tolerance. *Brazilian Archives of Biology and Technology, 59*, 1-14.

Richmond, C., Han, B., & Ezeji, T.C. (2011). Stimulatory effects of calcium carbonate on butanol production by solventogenic Clostridium species. *Continental Journal of Microbiology, 5*(1), 18-28.

Russmayer, H., Marx, H., & Sauer, M. (2019). Microbial 2-butanol production with *Lactobacillus diolivorans*. *Biotechnology for Biofuels, 12*, 1-11.

Sikarwar, V. S., Zhao, M., Fennell, P. S., Shah, N., & Anthony, E. J. (2017). Progress in biofuel production from gasification. *Progress in Energy and Combustion Science, 61*, 189-248.

Singh, R., Krishna, B. B., Kumar, J., & Bhaskar, T. (2016). Opportunities for utilization of non-conventional energy sources for biomass pretreatment. *Bioresource Technology, 199*, 398-407.

Staggs, K. W., & Nielsen, D. R. (2015). Improving n-butanol production in batch and semi-continuous processes through integrated product recovery. *Process Biochemistry, 50*(10), 1487-1498.

Su, Z., Wang, F., Xie, Y., Xie, H., Mao, G., Zhang, H., Song, A., & Zhang, Z. (2020). Reassessment of the role of CaCO3 in n-butanol production from pretreated lignocellulosic biomass by *Clostridium acetobutylicum*. *Scientific Reports, 10*(1), 1-10.

Taherzadeh, M. J., & Karimi, K. (2008). Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review. *International Journal of Molecular Sciences,9*(9), 1621-1651.

Tiwari, G., Sharma, A., Dalela, M., Gupta, R., Sharma, S., & Kuhad, R. C. (2017). Microwave assisted alkali pretreatment of fruit peel wastes for enzymatic hydrolysis. *Indian Journal of Agricultural Sciences, 87*(4), 77-80.

Tracy, B. P. (2012). Improving butanol fermentation to enter the advanced biofuel market. *mBio, 3*(6), 1-3.

Trindade, W. R. S., & Santos, R. G. (2017). Review on the characteristics of butanol, its production and use as fuel in internal combustion engines. *Renewable and Sustainable Energy Reviews, 69*, 642-651.

Vane, L. M. (2008). Separation technologies for the recovery and dehydration of alcohols from fermentation broths. *Biofuels, Bioproducts and Biorefining, 2*(6), 553-588.

Visioli, L. J., Enzweiler, H., Kuhn, R. C., Schwaab, M., & Mazutti, M. A. (2014). Recent advances on biobutanol production. *Sustainable Chemical Processes, 2*(1), 1-9.

Vivek, N., Nair, L. M., Mohan, B., Nair, S. C., Sindhu, R., Pandey, A., Shurpali, N., & Binod, P. (2019). Bio-butanol production from rice straw – Recent trends, possibilities, and challenges. *Bioresource Technology Reports, 7*, 1-14.

Volesky, B., & Szczesny, T. (1983). Bacterial conversion of pentose sugars to acetone and butanol. *Advances in Biochemical Engineering/Biotechnology, 27*, 101-118.

Wang, C., Xin, F., Kong, X., Zhao, J., Dong, W., Zhang, W., Ma, J., Wu, H., & Jiang, M. (2018). Enhanced isopropanol-butanol-ethanol mixture production through manipulation of intracellular NAD(P)H level in the recombinant *Clostridium acetobutylicum* XY16. *Biotechnology for Biofuels, 11*, 1-10.

Wang, Y., Li, X., & Blaschek, H. P. (2013). Effects of supplementary butyrate on butanol production and the metabolic switch in *Clostridium beijerinckii* NCIMB 8052: Genome-wide transcriptional analysis with RNA-Seq. *Biotechnology for Biofuels, 6*(1), 1-13.

Wu, Y. D., Xue, C., Chen, L. J., & Bai, F. W. (2013). Effect of zinc supplementation on acetone-butanol-ethanol fermentation by *Clostridium acetobutylicum*. *Journal of Biotechnology, 165*(1), 18-21.

Xue, C., Zhao, J., Chen, L., Yang, S. T., & Bai, F. (2017). Recent advances and state-of-the-art strategies in strain and process engineering for biobutanol production by *Clostridium acetobutylicum*. *Biotechnology Advances, 35*(2), 310-322.

Xue, C., Zhao, X. Q., Liu, C. G., Chen, L. J., & Bai, F. W. (2013). Prospective and development of butanol as an advanced biofuel. *Biotechnology Advances, 31*(8), 1575-1584.

Yusof, S. J. H. M., Takriff, M. S., Amir, A., Kadhum, H., Mohammad, A. W., & Jahim, J. (2010). The effect of initial butyric acid addition on ABE fermentation by *C. acetobutylicum* NCIMB 619. *Journal of Applied Sciences, 10*(21), 2709-2712.

Zabed, H., Sahu, J. N., Boyce, A. N., & Faruq, G. (2016). Fuel ethanol production from lignocellulosic biomass: An overview on feedstocks and technological approaches. *Renewable and Sustainable Energy Reviews, 66*, 751-774.

Zhang, B., &Shahbazi, A. (2011). Recent developments in pretreatment technologies for production of lignocellulosic biofuels. *Journal of Petroleum & Environmental Biotechnology, 2*(2), 1-8.

Zhao, Y., Tomas, C. A., Rudolph, F. B., Papoutsakis, E. T., & Bennett, G. N. (2005). Intracellular butyryl phosphate and acetyl phosphate concentrations in *Clostridium acetobutylicum* and their implications for solvent formation. *Applied and Environmental Microbiology, 71*(1), 530-537.

Zheng, J., Tashiro, Y., Wang, Q., & Sonomoto, K. (2015). Recent advances to improve fermentative butanol production: Genetic engineering and fermentation technology. *Journal of Bioscience and Bioengineering, 119*(1), 1-9.

Zheng, Y. N., Li, L. Z., Xian, M., Ma, Y. J., Yang, J. M., Xu, X., & He, D. Z. (2009). Problems with the microbial production of butanol. *Journal of Industrial Microbiology and Biotechnology, 36*(9), 1127-1138.

Zheng, Y., Zhao, J., Xu, F., & Li, Y. (2014). Pretreatment of lignocellulosic biomass for enhanced biogas production. *Progress in Energy and Combustion Science, 42*, 35-53.

APPENDICES

APPENDIX-1: Raw Data of Batch Experiments with *C. acetobutylicum*

Table A.1.1 Raw data of control medium without $CaCO_3$ addition

Control medium without $CaCO_3$ addition											
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Lactic Acid (g/L)**	**Acetic Acid (g/L)**	**Propionic Acid (g/L)**	**Ethanol (g/L)**	**Butanol (g/L)**
0	6.45	0	15.57	0	4.52	2.785	1.78	2.67	3.35	0.200	0
1	5.49	41.55	9.10	86.26	0.62	2.403	6.06	6.96	3.30	0.608	0.017
2	5.84	74.02	4.05	100	0	0.650	9.75	4.80	3.38	0.728	0.016
3	6.37	83.30	2.60	100	0	0.120	10.85	2.34	4.15	0.975	0.026
4	6.77	86.19	2.15	100	0	0	10.67	3.40	4.44	0.745	0.029
5	6.77	86.32	2.13	100	0	0	10.74	2.59	4.72	0.968	0.020

Table A.1.2 Raw data of 10 g/L $CaCO_3$ added medium

10 g/L $CaCO_3$ added medium											
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Lactic Acid (g/L)**	**Acetic Acid (g/L)**	**Propionic Acid (g/L)**	**Ethanol (g/L)**	**Butanol (g/L)**
0	6.43	0	15.72	0	4.65	2.87	1.84	2.53	3.57	0.200	0
1	5.70	45.42	8.58	89.99	0.47	2.50	6.51	7.41	3.45	0.618	0.010
2	5.83	76.21	3.74	100	0	0.63	10.27	4.14	3.46	0.703	0.021
3	6.77	84.10	2.50	100	0	0.14	10.62	3.27	3.93	0.674	0.018
4	6.77	85.11	2.34	100	0	0	10.43	2.40	4.19	0.948	0.018
5	6.70	86.98	2.05	100	0	0	10.46	2.45	4.33	0.951	0.020

Table A.1.3 Raw data of 5 g/L butyric acid added media

5 g/L butyric acid added media										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	5.62	0	55.40	0	40.30	7.10	1.90	4.47	0.120	0.890
1	5.62	0.36	55.20	1.24	39.80	7.10	2.00	4.46	0.130	0.890
2	5.59	1.44	54.60	1.99	39.50	7.10	2.10	4.42	0.120	0.880
5	5.55	1.44	54.60	1.99	39.50	7.10	2.10	4.41	0.150	0.910
6	5.55	2.71	53.90	2.23	39.40	7.10	2.10	4.41	0.120	0.910
7	5.55	3.43	53.50	2.23	39.40	7.00	2.10	4.41	0.140	0.930
8	5.48	4.69	52.80	2.73	39.20	7.00	2.10	4.40	0.120	0.930
9	5.48	5.05	52.60	3.47	38.90	7.00	2.10	4.40	0.130	0.940

Table A.1.4 Raw data of 60 mg/L magnesium added medium

60 mg/L magnesium added medium								
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Ethanol (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**
0	5.60	0	48.95	0.29	0	45.5	7.5	1.8
1	5.58	2.35	47.80	0.27	0.44	45.3	7.3	1.8
2	5.59	3.58	47.20	0.26	1.10	45	7.3	1.8
3	5.62	3.76	47.11	0.29	1.10	45	7.3	1.8
4	5.60	4.09	46.95	0.29	2.64	44.3	7.2	1.8
7	5.55	4.35	46.82	0.26	2.86	44.2	6.9	1.8

Table A.1.5 Raw data of 40 mg/L manganese added medium

40 mg/L manganese added medium								
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Ethanol (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**
0	5.60	0	48.50	0.27	0	45.8	7.3	1.8
1	5.53	2.37	47.35	0.29	1.53	45.1	7.3	1.8
2	5.57	3.09	47.00	0.27	1.75	45	7.1	1.8
3	5.60	3.13	46.98	0.28	2.18	44.8	6.9	1.8
4	5.59	3.44	46.83	0.28	2.84	44.5	6.8	1.8
7	5.52	4.60	46.27	0.29	3.06	44.4	6.7	1.8

Table A.1.6 Raw data of 40 mg/L zinc added medium

40 mg/L zinc added medium								
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Ethanol (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**
0	5.60	0	48.87	0.25	0	44.1	7.5	1.8
1	5.56	3.01	47.40	0.28	0.23	44	7.3	1.8
2	5.58	4.03	46.90	0.28	0.68	43.8	7.3	1.8
3	5.57	4.30	46.77	0.27	1.81	43.3	7.3	1.8
4	5.55	6.14	45.87	0.27	1.81	43.3	7.3	1.8
7	5.48	6.63	45.63	0.28	1.13	43.6	7	1.8

Table A.1.7 Raw data of 80 mg/L magnesium added medium

80 mg/L magnesium added medium									
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Ethanol (g/L)**	**Acetone (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**
0	5.70	0	51.80	0.75	0.15	0	51.3	7	1.9
1	5.70	2.41	50.55	0.70	0.16	0.58	51	5.6	1.9
2	5.70	2.70	50.40	0.72	0.12	0.58	51	5.4	2
3	5.67	6.85	48.25	0.76	0.12	1.56	50.5	5.4	1.9
4	5.62	6.85	48.25	0.75	0.12	2.34	50.1	5.4	1.9
7	5.51	9.17	47.05	0.75	0.11	5.07	48.7	5.4	1.8
8	5.49	9.36	46.95	0.73	0.13	5.46	48.5	5.4	1.9
9	5.49	10.14	46.55	0.75	0.12	6.63	47.9	5.4	1.7
10	5.46	10.91	46.15	0.75	0.12	7.99	47.2	5.2	1.7
11	5.39	12.07	45.55	0.76	0.15	9.94	46.2	5.2	1.6

Table A.1.8 Raw data of 60 mg/L manganese added medium

60 mg/L manganese added medium									
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Ethanol (g/L)**	**Acetone (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**
0	5.70	0	53.10	0.72	0.15	0	53.9	7.3	1.9
1	5.65	0.94	52.60	0.74	0.16	0	53.9	6.4	2
2	5.64	1.60	52.25	0.72	0.12	2.78	52.4	6.2	2
3	5.61	2.17	51.95	0.77	0.16	3.15	52.2	6.1	1.9
4	5.58	3.48	51.25	0.75	0.15	3.90	51.8	5.8	1.9
7	5.44	6.21	49.80	0.79	0.14	7.05	50.1	5.4	2
8	5.44	6.50	49.65	0.74	0.12	7.05	50.1	5.2	1.9
9	5.42	7.91	48.90	0.73	0.12	11.87	47.5	5.2	1.7
10	5.39	8.47	48.60	0.75	0.12	15.58	45.5	5.1	1.7
11	5.39	9.13	48.25	0.75	0.13	15.96	45.3	5.1	1.7

Table A.1.9 Raw data of 60 mg/L zinc added medium

60 mg/L zinc added medium									
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Ethanol (g/L)**	**Acetone (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**
0	5.7	0.00	53.55	0.74	0.15	0	53.1	7.8	1.9
1	5.65	0.75	53.15	0.74	0.16	4.14	50.9	6.4	2
2	5.64	1.31	52.85	0.75	0.17	5.46	50.2	6.4	1.9
3	5.63	2.33	52.3	0.72	0.12	5.46	50.2	6.4	1.9
4	5.59	3.08	51.9	0.79	0.13	5.65	50.1	6.1	1.9
7	5.47	4.86	50.95	0.75	0.12	5.84	50	6	1.9
8	5.47	6.91	49.85	0.74	0.12	5.84	50	6	1.9
9	5.47	7.56	49.5	0.75	0.13	10.92	47.3	5.3	1.7
10	5.46	7.84	49.35	0.72	0.12	11.11	47.2	5.3	1.7
11	5.5	9.06	48.7	0.76	0.14	11.49	47	5.3	1.7

Table A.1.10 Raw data of 220 mg/L magnesium added medium

220 mg/L magnesium added medium									
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Ethanol (g/L)**	**Acetone (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**
0	5.64	0	60.50	0.7	0.14	0	44.3	6.4	1.7
1	5.64	0.99	59.90	0.68	0.12	1.13	43.8	6.4	1.6
2	5.58	2.31	59.10	0.65	0.1	1.35	43.7	5.9	1.6
3	5.58	3.06	58.65	0.67	0.14	2.48	43.2	5.6	1.6
4	5.56	5.79	57.00	0.74	0.11	3.39	42.8	5.4	1.6
7	5.31	8.93	55.10	0.65	0.17	6.55	41.4	4.8	1.8
8	5.51	10.50	54.15	0.63	0.16	6.77	41.3	4.8	1.9
9	5.50	11.82	53.35	0.7	0.17	7.00	41.2	4.8	1.9
10	5.50	15.70	51.00	0.65	0.2	7.22	41.1	4.8	1.9
11	5.52	17.36	50.00	0.69	0.15	7.22	41.1	4.7	1.9

Table A.1.11 Raw data of 200 mg/L manganese added medium

200 mg/L manganese added medium									
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Ethanol (g/L)**	**Acetone (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**
0	5.64	0	61.50	0.69	0.15	0	43.9	6.4	1.6
1	5.64	0.16	61.40	0.67	0.12	0.68	43.6	5.9	1.6
2	5.59	1.63	60.50	0.69	0.12	0.91	43.5	5.2	1.6
3	5.59	1.95	60.30	0.67	0.12	1.82	43.1	5	1.5
4	5.57	4.55	58.70	0.73	0.09	3.42	42.4	5	1.6
7	5.56	7.89	56.65	0.66	0.13	3.64	42.3	4.8	1.6
8	5.58	9.11	55.90	0.69	0.10	4.10	42.1	4.8	1.6
9	5.43	10.00	55.35	0.69	0.10	4.56	41.9	4.7	1.7
10	5.4	12.20	54.00	0.67	0.11	5.24	41.6	4.7	1.7
11	5.44	14.72	52.45	0.70	0.16	5.69	41.4	4.7	1.7

Table A.1.12 Raw data of 200 mg/L zinc added medium

200 mg/L zinc added medium									
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Ethanol (g/L)**	**Acetone (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**
0	5.64	0	62.90	0.68	0.13	0	45.1	7.3	1.6
1	5.64	1.19	62.15	0.69	0.10	0.89	44.7	6.9	1.6
2	5.57	2.15	61.55	0.68	0.10	2.44	43.6	6	1.6
3	5.58	3.42	60.75	0.67	0.12	1.11	43.1	5.8	1.6
4	5.57	3.90	60.45	0.73	0.15	0.22	43	5.8	1.6
7	5.53	6.68	58.70	0.68	0.13	0.44	42.8	5.2	1.6
8	5.53	8.51	57.55	0.74	0.13	0.67	42.5	5.2	1.6
9	5.48	10.10	56.55	0.77	0.12	0	42.5	5.2	1.6
10	5.48	12.48	55.05	0.65	0.15	2.66	41.3	5.2	1.6
11	5.45	13.99	54.10	0.65	0.18	0.44	41.1	5.2	1.6

APPENDIX-2: Raw Data of Batch Experiments with *C. pasteurianum*

Table A.2.1 Raw data of control medium with no addition

Control medium with no addition										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	5.91	0	33.36	0	20.99	4.55	1.32	0	0.123	0.209
1	5.91	0.48	33.20	0.94	20.79	4.52	1.30	0	0.156	0.211
2	4.99	94.00	2.00	100	0	3.13	9.52	3.12	4.576	0.164
3	6.71	96.55	1.15	100	0	0.99	8.61	3.14	4.839	0.173
4	6.68	95.79	1.40	100	0	0.90	7.33	3.09	4.578	0.186
7	6.75	96.70	1.10	100	0	0.83	6.37	3.06	4.763	0.307
8	6.74	96.70	1.10	100	0	0.81	6.29	3.12	4.529	0.222
9	6.68	96.71	1.10	100	0	0.81	6.20	3.06	4.771	0.534
10	6.68	96.81	1.06	100	0	0.81	6.13	3.07	5.054	0.483
11	6.76	97.11	0.96	100	0	0.75	6.23	3.10	4.540	0.417
14	6.76	97.12	0.96	100	0	0.75	5.98	3.15	4.502	0.423
16	7.01	0.00	24.86	0	22.34	1.00	4.86	5.55	4.441	0.415
17	7.06	1.05	24.60	4.79	21.27	0.92	4.92	4.92	4.785	0.435
18	6.54	14.68	21.21	21.49	17.54	0.92	6.30	3.89	6.024	0.442
21	5.43	86.23	3.42	99.96	0.01	0.91	9.23	7.20	10.792	0.680

Table A.2.2 Raw data of 200 mg/L magnesium added medium

200 mg/L magnesium added medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	5.88	0	33.66	0	20.96	4.57	1.31	0	0.124	0.228
1	5.89	1.72	33.08	0.69	20.81	4.51	1.27	0	0.105	0.223
2	5.85	12.12	29.58	3.01	20.33	4.37	1.29	0	0.337	0.220
3	5.31	95.83	1.40	100	0	2.82	9.18	4.63	6.765	0.200
4	6.89	96.10	1.31	100	0	1.17	7.06	4.59	6.460	0.158
7	6.88	96.10	1.31	100	0	1.15	5.29	4.66	6.633	0.235
8	6.96	96.14	1.30	100	0	1.15	5.34	4.56	6.619	0.420
9	6.88	96.14	1.30	100	0	1.11	5.23	5.20	6.602	0.434
10	6.87	96.17	1.29	100	0	1.11	5.25	4.40	6.825	0.482
11	6.95	96.24	1.27	100	0	1.11	5.47	5.21	6.644	0.438
14	6.95	96.63	1.13	100	0	1.09	5.38	4.82	6.581	0.446
16	7.02	0	24.96	0	22.10	1.45	4.25	4.12	6.620	0.438
17	7.06	0.72	24.78	0.20	22.06	1.36	4.49	4.28	6.595	0.437
18	5.84	67.41	8.14	72.39	6.10	1.00	11.09	8.00	11.593	0.516
21	6.20	92.41	1.89	100	0	0.59	10.10	8.23	11.726	0.530

Table A.2.3 Raw data of 200 mg/L manganese added medium

200 mg/L manganese added medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	5.60	0	33.54	0	20.89	4.28	1.27	0	0.139	0.227
1	5.64	4.53	32.02	0.23	20.84	4.28	1.26	0	0.141	0.246
2	5.65	4.59	32.00	0.23	20.84	4.27	1.28	0	0.118	0.211
3	6.85	5.19	31.80	0.26	20.83	4.12	1.22	0	0.120	0.209
4	6.78	5.19	31.80	0.32	20.82	4.12	1.22	0	0.128	0.221
7	6.86	8.65	30.64	0.37	20.81	4.12	1.20	0	0.130	0.225
8	6.90	8.88	30.56	0.52	20.78	4.09	1.20	0	0.114	0.223
9	6.82	9.18	30.46	1.35	20.60	4.09	1.47	0	0.124	0.211
10	6.87	9.30	30.42	1.41	20.59	4.09	1.60	0	0.132	0.209
11	6.73	16.52	28.00	1.61	20.55	4.09	1.76	0	0.176	0.216

Table A.2.4 Raw data of 200 mg/L zinc added medium

200 mg/L zinc added medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	5.84	0	33.80	0	20.94	4.33	1.25	0	0.120	0.190
1	5.80	1.66	33.24	0.03	20.93	4.33	1.28	0	0.164	0.230
2	5.82	6.80	31.50	0.83	20.77	4.31	1.29	0	0.111	0.231
3	6.79	8.17	31.04	1.03	20.72	4.14	1.25	0	0.121	0.227
4	6.73	8.28	31.00	1.12	20.71	4.12	1.25	0	0.142	0.222
7	4.66	95.65	1.47	100	0	3.25	6.82	3.81	5.796	0.358
8	6.63	95.67	1.46	100	0	2.84	6.32	3.77	5.766	0.352
9	6.54	95.70	1.45	100	0	2.82	6.29	3.77	5.656	0.342
10	6.54	95.74	1.44	100	0	2.82	6.30	3.91	5.735	0.324
11	6.63	95.78	1.43	100	0	2.81	6.29	3.94	5.724	0.341
14	6.65	95.80	1.42	100	0	2.81	6.56	4.30	5.706	0.354

Table A.2.4 continues

Time (day)	pH	Total Sugar Consumption (%)	Total Sugar Concentration (g/L)	Glucose Consumption (%)	Glucose (g/L)	Fructose (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Acetone (g/L)	Ethanol (g/L)
16	6.80	0	24.90	0	21.23	3.13	6.49	3.65	5.675	0.342
17	6.89	1.12	24.62	2.66	20.66	3.13	6.50	3.57	5.738	0.362
18	5.66	58.32	10.38	62.12	8.04	1.66	6.45	3.71	8.551	0.352
21	5.67	95.18	1.20	99.90	0.02	1.66	9.44	7.64	9.222	0.356

Table A.2.5 Raw data of 289 mg/L neutral red added medium

289 mg/L neutral red added medium										
Time (day)	pH	Total Sugar Consumption (%)	Total Sugar Concentration (g/L)	Glucose Consumption (%)	Glucose (g/L)	Fructose (g/L)	Acetic Acid (g/L)	Butyric Acid (g/L)	Acetone (g/L)	Ethanol (g/L)
0	5.71	0	33.72	0	21.02	5.26	1.31	0	0.139	0.222
1	5.72	2.14	33.00	1.11	20.79	4.70	1.30	0	0.130	0.210
2	5.78	7.71	31.12	1.11	20.79	4.59	1.30	0	0.133	0.243
3	6.92	11.74	29.76	2.34	20.53	4.29	1.31	0	0.110	0.236
4	6.97	14.00	29.00	3.17	20.36	3.96	1.30	0	0.123	0.227
7	7.12	14.35	28.88	3.28	20.33	3.22	1.26	0	0.121	0.216
8	7.18	14.77	28.74	3.51	20.29	3.08	1.24	0	0.125	0.233
9	7.18	14.95	28.68	4.88	20.00	3.08	1.25	0	0.107	0.188
10	7.18	16.73	28.08	4.97	19.98	3.08	1.31	0	0.165	0.221
11	7.18	17.67	27.76	5.08	19.96	3.08	1.34	0	0.134	0.221

Table A.2.6 Raw data of control medium in which no compound was removed

Control medium in which no compound was removed										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	6.54	0	63.72	0	47.97	8.78	3.69	2.10	2.920	0.239
1	5.39	14.22	54.66	20.93	37.93	8.78	8.66	4.39	6.382	0.268
2	5.27	66.85	21.12	74.45	12.26	2.41	15.90	7.74	11.585	0.508
3	6.01	93.22	4.32	99.69	0.15	2.11	16.33	8.48	12.630	0.654
4	6.49	95.45	2.90	99.69	0.15	1.45	14.38	8.43	12.668	0.698
7	6.50	95.46	2.90	99.69	0.15	1.45	12.30	8.28	12.482	0.693
8	6.52	95.46	2.89	99.76	0.12	1.45	12.74	8.40	12.375	0.730
9	6.49	95.47	2.88	99.79	0.10	1.26	12.26	8.51	12.426	0.707
10	6.47	95.74	2.71	99.79	0.10	1.26	11.77	8.38	12.426	0.707
11	6.47	95.77	2.70	100	0	1.23	11.40	8.37	13.642	0.783

Table A.2.7 Raw data of protein sources removed medium

Protein sources removed medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	6.56	0.00	64.86	0.00	46.66	11.90	3.85	2.09	3.160	0.090
1	5.26	8.70	59.22	12.49	40.83	4.99	12.13	4.78	6.616	0.185
2	5.30	42.46	37.32	49.54	23.54	2.10	17.21	7.43	11.006	0.355
3	5.93	70.35	19.23	73.37	12.43	0.90	15.95	11.57	13.460	0.401
4	6.02	81.56	11.96	85.77	6.64	0.60	15.00	11.82	14.935	0.470
7	6.00	96.02	2.58	97.96	0.95	0.60	14.24	12.47	16.449	0.497
8	6.49	96.21	2.46	99.45	0.26	0.60	13.68	14.56	16.750	0.744
9	6.49	96.22	2.45	99.56	0.20	0.77	13.35	14.65	16.736	0.791
10	6.48	96.85	2.04	99.64	0.17	0.77	13.59	15.05	16.736	0.791
11	6.50	96.90	2.01	99.74	0.12	0.76	13.25	14.92	17.142	0.699

Table A.2.8 Raw data of protein sources and iron removed medium

Protein sources and iron removed medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	6.65	0	64.98	0	46.35	12.04	3.88	2.46	3.120	0.080
1	5.49	14.50	55.56	7.94	42.67	7.30	7.97	4.39	5.871	0.165
2	5.47	41.34	38.12	25.09	34.72	3.68	9.06	6.37	8.828	0.226
3	5.80	51.06	31.80	37.71	28.87	1.76	10.36	7.87	11.018	0.247
4	5.88	54.51	29.56	51.31	22.57	0.96	11.51	9.30	12.998	0.276
7	5.75	77.93	14.34	80.31	9.13	1.58	12.12	11.79	16.538	0.328
8	6.24	81.13	12.26	86.90	6.07	0.97	12.24	12.24	17.265	0.473
9	6.27	87.32	8.24	92.64	3.41	0.89	12.66	13.16	17.998	0.586
10	6.25	94.80	3.38	98.81	0.55	0.88	13.25	14.08	17.998	0.586
11	6.52	95.23	3.10	99.15	0.40	0.75	13.19	13.95	18.697	0.670

Table A.2.9 Raw data of protein sources and manganese removed medium

Protein sources and manganese removed medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	6.44	0	64.92	0	44.01	12.02	3.63	2.29	3.086	0.1
1	5.28	12.48	56.82	2.20	43.04	8.03	12.86	4.91	6.676	0.196
2	5.19	54.84	29.32	44.36	24.49	2.90	16.94	7.99	11.05	0.329
3	5.72	63.22	23.88	66.22	14.87	1.53	16.61	9.89	13.847	0.372
4	5.98	76.77	15.08	77.44	9.93	1.25	15.66	10.70	15.315	0.417
7	5.75	94.67	3.46	99.13	0.38	1.19	15.36	12.54	17.798	0.465
8	6.39	96.03	2.58	99.15	0.38	0.91	14.85	12.84	17.728	0.454
9	6.56	96.22	2.46	99.27	0.32	0.84	14.79	13.32	17.109	0.825
10	6.48	96.88	2.02	99.38	0.28	0.83	14.61	13.52	18.24	0.82
11	6.49	96.89	2.02	99.69	0.14	0.76	14.12	13.49	18.516	0.702

Table A.2.10 Raw data of protein sources and magnesium removed medium

Protein sources and magnesium removed medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	6.49	0	64.62	0	44.72	12.17	3.70	2.24	3.118	0.092
1	5.36	7.15	60.00	8.12	41.09	5.92	11.65	4.65	6.376	0.168
2	5.23	51.84	31.12	46.83	23.78	2.31	17.08	7.83	11.048	0.319
3	5.70	68.11	20.61	70.89	13.02	1.36	16.38	9.86	13.898	0.383
4	5.84	82.27	11.46	86.00	6.26	1.05	15.73	10.89	15.492	0.428
7	5.98	95.91	2.64	99.36	0.29	0.97	14.63	12.29	17.122	0.503
8	6.71	95.94	2.62	99.49	0.23	0.77	13.55	12.40	17.587	1.035
9	6.64	96.16	2.48	99.58	0.19	0.76	12.78	12.15	17.505	1.012
10	6.56	96.76	2.10	99.69	0.14	0.76	13.34	12.89	17.490	0.993
11	6.57	96.77	2.09	99.72	0.13	0.76	13.10	12.86	17.999	0.976

Table A.2.11 Hydrogen production data of control medium in which no compound was removed

Control medium in which no compound was removed										
Hydrogen gas from head space									Hydrogen gas from total gas	
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**	**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0	0	
1	240	360	0.443	159.48		510	0.443	225.93	385.410	15.162
2	230	370	0.502	26.26		630	0.502	316.26	727.930	28.636
3	220	380	0.033	0		470	0.033	15.46	743.393	29.244
4	210	390	0.361	128.29		50	0.361	18.05	889.731	35.001
7	200	400	0.329	0		10	0.329	3.29	893.021	35.131
8	190	410	0.121	0		0	0.121	0	893.021	35.131
9	180	420	0.228	46.15		0	0.228	0	939.171	36.946
10	170	430	0.278	23.78		0	0.278	0	962.951	37.882
11	160	440	0.251	0		0	0.521	0	962.951	37.882

Table A.2.12 Hydrogen production data of protein sources removed medium

Protein sources removed medium										
Hydrogen gas from head space									Hydrogen gas from total gas	
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**	**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0	0	
1	240	360	0.091	32.76		370	0.091	33.67	66.430	2.613
2	230	370	0.541	167.41		1065	0.541	576.17	810.005	31.865
3	220	380	0.377	0		600	0.377	226.20	1036.205	40.763
4	210	390	0.499	51.35		330	0.499	164.67	1252.225	49.261
7	200	400	0.508	8.59		450	0.508	228.60	1489.415	58.592
8	190	410	0.478	0		40	0.478	19.12	1508.535	59.344
9	180	420	0.295	0		0	0.295	0	1508.535	59.344
10	170	430	0.350	26.60		0	0.350	0	1535.135	60.391
11	160	440	0.251	0		0	0.251	0	1535.135	60.391

Table A.2.13 Hydrogen production data of protein sources and iron removed medium

Protein sources and iron removed medium										
Hydrogen gas from head space									Hydrogen gas from total gas	
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**	**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0	0	
1	240	360	0.163	58.68		585	0.163	95.355	154.035	6.060
2	230	370	0.518	132.98		520	0.518	269.36	556.375	21.887
3	220	380	0.533	10.88		350	0.533	186.55	753.805	29.654
4	210	390	0.528	3.38		340	0.528	179.52	936.705	36.849
7	200	400	0.531	6.48		655	0.531	347.805	1290.990	50.786
8	190	410	0.316	0		160	0.316	50.56	1341.550	52.775
9	180	420	0.500	80.44		170	0.500	85.00	1506.990	59.284
10	170	430	0.503	6.29		140	0.503	70.42	1583.700	62.301
11	160	440	0.505	5.91		50	0.505	25.25	1614.860	63.527

Table A.2.14 Hydrogen production data of protein sources and manganese removed medium

Protein sources and manganese removed medium										
Hydrogen gas from head space									Hydrogen gas from total gas	
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**	**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0	0	
1	240	360	0.430	154.80		810	0.430	348.30	503.100	19.792
2	230	370	0.535	43.15		900	0.535	481.50	1027.750	40.431
3	220	380	0.376	0		480	0.376	180.48	1208.230	47.531
4	210	390	0.503	53.29		330	0.503	165.99	1427.510	56.157
7	200	400	0.537	18.63		560	0.537	300.72	1746.860	68.720
8	190	410	0.436	0		20	0.436	8.72	1755.580	69.063
9	180	420	0.451	10.66		0	0.451	0	1766.240	69.482
10	170	430	0.445	1.93		0	0.445	0	1768.170	69.558
11	160	440	0.429	0		0	0.429	0	1768.170	69.558

Table A.2.15 Hydrogen production data of protein sources and magnesium removed medium

Protein sources and magnesium removed medium										
Hydrogen gas from head space									Hydrogen gas from total gas	
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**	**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0	0	
1	240	360	0.057	20.63		705	0.057	40.40	61.025	2.401
2	230	370	0.543	180.28		1130	0.543	613.59	854.897	33.631
3	220	380	0.378	0		560	0.378	211.68	1066.577	41.958
4	210	390	0.503	52.53		410	0.503	206.23	1325.337	52.138
7	200	400	0.515	9.83		420	0.515	216.30	1551.467	61.033
8	190	410	0.141	0		20	0.141	2.82	1554.287	61.144
9	180	420	0.470	139.59		0	0.470	0	1693.877	66.636
10	170	430	0.442	0		0	0.442	0	1693.877	66.636
11	160	440	0.412	0		0	0.412	0	1693.877	66.636

Table A.2.16 Raw data of control medium without butyric acid addition

Control medium without butyric acid addition										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	5.89	0	77.46	0	57.48	2.87	4.48	2.43	3.234	0.093
1	4.66	18.90	62.82	18.51	46.84	2.50	7.49	3.73	6.000	0.136
2	4.75	57.27	33.10	57.13	24.64	1.73	11.92	6.18	9.638	0.409
3	4.52	82.51	13.55	80.42	11.25	0.53	13.76	8.05	11.873	0.513
6	5.36	97.47	1.96	99.91	0.05	0.09	14.56	9.28	13.796	0.594
7	5.73	97.82	1.69	99.91	0.05	0.09	14.48	9.43	13.807	0.595
8	6.02	97.84	1.67	99.91	0.05	0.09	14.07	9.35	13.794	0.593
9	6.04	97.84	1.67	99.91	0.05	0.09	13.83	9.32	14.006	0.583
10	6.00	97.85	1.67	99.91	0.05	0.08	13.60	9.35	13.854	0.580

Table A.2.17 Raw data of 6 g/L butyric acid added medium

6 g/L butyric acid added medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	6.1	0	76.8	0	54.06	2.32	4.25	6.02	3.547	0.136
1	5.54	7.50	71.04	0.09	54.01	2.31	5.21	6.95	4.433	0.149
2	4.98	35.22	49.75	30.24	37.71	1.90	9.46	8.84	7.25	0.308
3	5.02	66.54	25.7	59.81	21.725	1.04	11.48	9.57	7.685	0.4
6	5.32	82.93	13.11	84.83	8.202	0.35	12.57	10.70	9.402	0.521
7	5.57	82.93	13.11	85.02	8.1	0.27	12.72	10.93	9.426	0.529
8	5.99	84.74	11.72	85.04	8.086	0.27	12.29	10.75	9.266	0.519
9	5.98	84.77	11.7	85.05	8.084	0.27	12.08	10.72	9.707	0.545
10	5.97	84.86	11.63	85.06	8.078	0.27	11.95	10.70	9.595	0.552

Table A.2.18 Raw data of 8 g/L butyric acid added medium

8 g/L butyric acid added medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	6.07	0	77.28	0	53.64	4.08	4.38	8.10	3.362	0.110
1	5.53	7.69	71.34	3.82	51.59	3.43	5.34	8.82	4.619	0.159
2	5.07	39.31	46.9	25.80	39.80	3.36	9.57	11.21	6.788	0.308
3	5.12	54.26	35.35	53.94	24.71	1.19	10.45	11.16	6.978	0.375
6	5.60	74.65	19.59	76.56	12.57	0.52	12.02	12.39	8.563	0.444
7	5.69	77.72	17.22	76.65	12.53	0.29	11.81	12.29	8.440	0.465
8	5.96	77.74	17.2	76.67	12.52	0.29	11.50	12.14	8.388	0.439
9	5.86	77.99	17.01	76.70	12.50	0.28	11.59	12.41	8.928	0.406
10	5.98	78.39	16.7	76.71	12.49	0.28	11.40	12.26	8.869	0.433

Table A.2.19 Raw data of 10 g/L butyric acid added medium

10 g/L butyric acid added medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	5.9	0	76.74	0	54.30	3.22	4.34	10	3.518	0.109
1	5.61	1.88	75.30	0	54.30	1.50	4.60	10.43	3.364	0.149
2	5.17	32.83	51.55	20.48	43.18	1.50	7.48	11.65	5.907	0.253
3	5.16	55.04	34.50	44.94	29.90	0.86	9.25	11.84	5.916	0.321
6	5.33	70.76	22.44	71.51	15.47	0.53	11.47	12.42	5.967	0.442
7	5.61	70.81	22.40	71.54	15.46	0.35	11.86	12.94	5.875	0.441
8	5.96	71.46	21.90	71.57	15.44	0.34	11.14	12.55	5.806	0.427
9	5.91	71.62	21.78	71.59	15.42	0.34	11.37	12.57	6.301	0.438
10	5.93	72.37	21.20	71.63	15.40	0.34	11.37	12.59	6.549	0.424

Table A.2.20 Raw data of 12 g/L butyric acid added medium

12 g/L butyric acid added medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	5.91	0	76.80	0.00	53.41	2.20	4.30	11.72	3.727	0.094
1	5.68	4.22	73.56	0.75	53.01	2.20	4.53	12.16	4.281	0.163
2	5.24	31.97	52.25	19.60	42.94	2.18	6.58	12.79	5.774	0.235
3	5.23	42.45	44.20	36.94	33.68	1.60	8.89	12.95	5.716	0.302
6	5.38	64.57	27.21	62.16	20.21	0.75	11.66	12.92	5.816	0.407
7	5.66	65.65	26.38	62.79	19.87	0.38	11.26	12.58	5.775	0.410
8	6	65.94	26.16	62.92	19.80	0.36	11.18	12.56	5.671	0.399
9	5.99	66.20	25.96	63.17	19.67	0.36	11.10	12.52	5.596	0.399
10	6.01	67.06	25.30	63.18	19.67	0.35	11.13	12.60	5.629	0.374

Table A.2.21 Hydrogen production data of control medium without butyric acid addition

Control medium without butyric acid addition										
Hydrogen gas from head space									Hydrogen gas from total gas	
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**	**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0	0	
1	240	360	0.339	122.04		490	0.339	166.11	288.150	11.336
2	230	370	0.534	75.54		1210	0.534	646.14	1009.830	39.726
3	220	380	0.530	3.82		780	0.530	413.40	1427.050	56.139
6	210	390	0.514	0		650	0.514	334.10	1761.150	69.282
7	200	400	0.366	0		30	0.366	10.98	1772.130	69.714
8	190	410	0.346	0		0	0.346	0	1772.130	69.714
9	180	420	0.357	8.08		0	0.357	0	1780.210	70.032
10	170	430	0.323	0		0	0.323	0	1780.210	70.032

Table A.2.22 Hydrogen production data of 6 g/L butyric acid added medium

6 g/L butyric acid added medium											
Hydrogen gas from head space									Hydrogen gas from total gas		
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**		**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0		0	
1	240	360	0.226	81.36		210	0.226	47.46		128.820	5.068
2	230	370	0.506	105.86		970	0.506	490.82		725.500	28.541
3	220	380	0.506	5.06		840	0.506	425.04		1155.600	45.460
6	210	390	0.505	4.67		760	0.505	383.80		1544.070	60.742
7	200	400	0.387	0		40	0.387	15.48		1559.550	61.351
8	190	410	0.373	0		0	0.373	0		1559.550	61.351
9	180	420	0.344	0		0	0.344	0		1559.550	61.351
10	170	430	0.312	0		0	0.312	0		1559.550	61.351

Table A.2.23 Hydrogen production data of 8 g/L butyric acid added medium

8 g/L butyric acid added medium											
Hydrogen gas from head space									Hydrogen gas from total gas		
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**		**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0		0	
1	240	360	0.073	26.28		260	0.073	18.98		45.260	1.780
2	230	370	0.494	156.50		950	0.494	469.30		671.060	26.399
3	220	380	0.506	9.50		680	0.506	344.08		1024.640	40.308
6	210	390	0.512	7.40		755	0.512	386.56		1418.600	55.806
7	200	400	0.415	0		50	0.415	20.75		1439.350	56.623
8	190	410	0.392	0		0	0.392	0		1439.350	56.623
9	180	420	0.369	0		0	0.369	0		1439.350	56.623
10	170	430	0.327	0		0	0.327	0		1439.350	56.623

Table A.2.24 Hydrogen production data of 10 g/L butyric acid added medium

10 g/L butyric acid added medium										
Hydrogen gas from head space									Hydrogen gas from total gas	
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**	**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0	0	
1	240	360	0.072	25.92		130	0.072	9.36	35.280	1.388
2	230	370	0.456	142.80		400	0.456	182.40	360.480	14.181
3	220	380	0.486	15.96		420	0.486	204.12	580.560	22.839
6	210	390	0.476	0.96		435	0.476	207.06	788.580	31.022
7	200	400	0.424	0		30	0.424	12.72	801.300	31.522
8	190	410	0.341	0		0	0.341	0	801.300	31.522
9	180	420	0.345	5.09		0	0.345	0	806.390	31.723
10	170	430	0.318	0		0	0.318	0	806.390	31.723

Table A.2.25 Hydrogen production data of 12 g/L butyric acid added medium

12 g/L butyric acid added medium										
Hydrogen gas from head space									Hydrogen gas from total gas	
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**	**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0	0	
1	240	360	0.083	29.88		130	0.083	10.79	40.670	1.600
2	230	370	0.426	127.74		535	0.426	227.91	396.320	15.591
3	220	380	0.460	17.18		530	0.460	243.80	657.300	25.858
6	210	390	0.468	7.72		695	0.468	325.26	990.280	38.957
7	200	400	0.373	0		60	0.373	22.38	1012.660	39.837
8	190	410	0.353	0		0	0.353	0	1012.660	39.837
9	180	420	0.326	0		0	0.326	0	1012.660	39.837
10	170	430	0.298	0		0	0.298	0	1012.660	39.837

Table A.2.26 Raw data of control medium without ethanol addition

Control medium without ethanol addition										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	6.71	0	63.54	0	49.50	7.63	3.62	2.30	2.504	0.030
1	5.76	6.70	59.28	4.77	47.14	1.51	4.85	2.87	4.306	0.249
2	4.95	49.45	32.12	61.32	19.15	1.24	10.68	7.56	10.682	0.218
3	5.73	78.85	13.44	86.17	6.85	0.56	11.98	9.16	13.127	0.278
4	5.84	95.42	2.91	99.87	0.06	0.26	12.07	9.53	13.598	0.594
7	6.61	96.07	2.50	99.87	0.06	0.11	11.31	9.70	13.588	0.631

Table A.2.27 Raw data of 0.2 g/L ethanol added medium

0.2 g/L ethanol added medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	6.60	0	63.18	0	51.30	8.76	3.63	2.65	2.483	0.191
1	5.61	14.81	53.82	9.01	46.68	1.49	5.55	3.17	4.729	0.32
2	4.95	50.87	31.04	64.23	18.35	1.19	11.82	6.85	10.255	0.56
3	5.57	94.73	3.33	99.89	0.06	0.41	12.92	9.78	14.215	0.686
4	6.42	95.99	2.54	99.91	0.05	0.20	12.38	10.08	14.394	0.986
7	6.40	96.23	2.38	99.92	0.04	0.12	12.02	10.18	14.396	0.975

Table A.2.28 Raw data of 1 g/L ethanol added medium

1 g/L ethanol added medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	6.60	0	63.00	0	48.80	7.46	3.48	2.65	2.494	0.900
1	5.48	16.95	52.32	7.36	45.21	1.67	6.07	3.49	5.113	0.908
2	5.01	61.33	24.36	72.64	13.35	1.29	11.80	6.83	10.643	1.172
3	5.74	95.19	3.03	99.82	0.09	0.45	13.97	9.55	13.555	1.261
4	6.38	95.92	2.57	99.85	0.07	0.14	13.58	9.43	13.592	1.200
7	6.40	91.11	5.60	99.85	0.07	0.08	13.04	9.45	13.316	1.410

Table A.2.29 Raw data of 1.4 g/L ethanol added medium

1.4 g/L ethanol added medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	6.61	0	63.48	0	47.60	7.29	3.41	2.62	2.527	1.416
1	5.77	7.28	58.86	3.47	45.95	1.26	5.08	3.14	4.705	1.459
2	5.00	61.69	24.32	65.73	16.31	0.86	11.17	7.43	11.353	1.711
3	5.82	87.24	8.10	93.12	3.28	0.49	12.52	8.74	14.020	1.744
4	6.30	95.90	2.60	99.77	0.11	0.05	12.53	9.82	13.856	1.737
7	6.35	96.23	2.40	99.84	0.08	0.05	11.99	9.82	13.677	1.626

Table A.2.30 Hydrogen production data of control medium without ethanol addition

Control medium without ethanol addition											
Hydrogen gas from head space									Hydrogen gas from total gas		
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**		**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0		0	
1	240	360	0.326	117.36		360	0.326	117.36		234.720	9.234
2	230	370	0.590	100.94		1520	0.590	896.80		1232.460	48.484
3	220	380	0.533	0		580	0.533	309.14		1541.600	60.645
4	210	390	0.486	0		300	0.486	145.80		1687.400	66.381
7	200	400	0.440	0		10	0.440	4.40		1691.800	66.554

Table A.2.31 Hydrogen production data of 0.2 g/L ethanol added medium

0.2 g/L ethanol added medium											
Hydrogen gas from head space									Hydrogen gas from total gas		
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**		**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0		0	
1	240	360	0.206	74.16		410	0.206	84.46		158.620	6.240
2	230	370	0.557	131.93		1380	0.557	768.66		1059.210	41.668
3	220	380	0.539	0		850	0.539	458.15		1517.360	59.692
4	210	390	0.433	0		20	0.433	8.66		1526.020	60.032
7	200	400	0.301	0		30	0.301	9.03		1535.050	60.387

Table A.2.32 Hydrogen production data of 1 g/L ethanol added medium

1 g/L ethanol added medium											
Hydrogen gas from head space									Hydrogen gas from total gas		
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**		**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0		0	
1	240	360	0.382	137.52		560	0.382	213.92		351.440	13.825
2	230	370	0.553	67.09		1560	0.553	862.68		1281.210	50.402
3	220	380	0.517	0		650	0.517	336.05		1617.260	63.622
4	210	390	0.424	0		40	0.424	16.96		1634.220	64.289
7	200	400	0.399	0		30	0.399	11.97		1646.190	64.760

Table A.2.33 Hydrogen production data of 1.4 g/L ethanol added medium

1.4 g/L ethanol added medium											
Hydrogen gas from head space									Hydrogen gas from total gas		
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**		**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0		0	
1	240	360	0.334	120.24		470	0.334	156.98		277.220	10.906
2	230	370	0.559	86.59		1390	0.559	777.01		1140.820	44.879
3	220	380	0.510	0		650	0.510	331.50		1472.320	57.920
4	210	390	0.443	0		160	0.443	70.88		1543.200	60.708
7	200	400	0.346	0		50	0.346	17.30		1560.500	61.389

Table A.2.34 Raw data of C/N=50 medium

C/N=50 medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	6.56	0	64.86	0.00	46.66	11.90	3.85	2.09	3.160	0.090
1	5.26	8.70	59.22	12.49	40.83	4.99	12.13	4.78	6.616	0.185
2	5.30	42.46	37.32	49.54	23.54	2.10	17.21	7.43	11.006	0.355
3	5.93	70.35	19.23	73.37	12.43	0.90	15.95	11.57	13.460	0.401
4	6.02	81.56	11.96	85.77	6.64	0.60	15.00	11.82	14.935	0.470
7	6.00	96.02	2.58	97.96	0.95	0.60	14.24	12.47	16.449	0.497
8	6.49	96.21	2.46	99.45	0.26	0.60	13.68	14.56	16.750	0.744
9	6.49	96.22	2.45	99.56	0.20	0.77	13.35	14.65	16.736	0.791
10	6.48	96.85	2.04	99.64	0.17	0.77	13.59	15.05	16.736	0.791

Table A.2.35 Raw data of C/N=100 medium

C/N=100 medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	6.75	0	66.36	0	47.1	12.34	2.33	1.22	1.599	0.152
1	5.80	4.16	63.60	5.29	44.61	6.49	5.09	2.31	3.319	0.232
2	5.03	38.67	40.70	32.89	31.61	2.02	8.93	7.61	10.559	0.566
3	5.19	60.26	26.37	57.26	20.13	1.21	9.21	10.36	14.803	0.725
4	5.76	75.41	16.32	76.67	10.99	1.20	9.24	12.43	18.194	0.784
7	4.57	96.49	2.33	99.97	0.02	1.20	9.36	16.08	22.699	0.822
8	6.54	96.75	2.16	99.97	0.02	1.04	8.72	15.98	22.593	0.823
9	6.50	97.08	1.94	99.97	0.02	1.04	8.60	15.98	22.547	0.834
10	6.50	97.14	1.90	99.98	0.01	1.02	8.07	15.95	22.709	0.835

Table A.2.36 Raw data of C/N=200 medium

C/N=200 medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	6.72	0	67.56	0	46.44	13.21	2.22	1.23	1.559	0.172
1	5.46	17.76	55.56	11.33	41.18	5.03	4.49	3.71	5.192	0.224
2	5.16	37.46	42.25	29.44	32.77	1.71	5.98	7.33	10.228	0.402
3	5.4	56.08	29.67	53.29	21.69	1.47	7.08	9.67	13.779	0.442
4	5.75	67.26	22.12	69.10	14.35	1.26	7.84	11.55	16.457	0.438
7	5.54	89.89	6.83	92.77	3.36	1.25	9.20	14.33	19.816	0.44
8	6.04	95.73	2.885	99.49	0.24	1.06	9.58	14.85	20.603	0.472
9	6.23	97.05	1.995	99.96	0.02	1.05	9.61	14.93	20.63	0.436
10	6.27	97.06	1.987	99.96	0.02	1.05	9.02	14.81	20.628	0.474

Table A.2.37 Raw data of C/N=250 medium

C/N=250 medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	6.70	0	66.90	0	45.26	13.71	2.25	1.36	1.656	0.176
1	5.43	18.92	54.24	10.83	40.36	5.71	4.13	3.57	5.082	0.275
2	5.16	40.88	39.55	31.40	31.05	1.43	6.06	7.65	10.539	0.348
3	5.47	56.82	28.89	58.80	18.65	1.41	7.45	10.07	14.329	0.386
4	5.67	74.38	17.14	75.53	11.08	1.30	8.28	11.87	16.953	0.357
7	5.56	94.98	3.36	98.80	0.54	1.30	10.00	14.72	20.249	0.390
8	6.23	96.49	2.35	99.47	0.24	1.08	10.06	14.75	20.386	0.407
9	6.24	96.85	2.11	99.95	0.02	1.07	10.01	14.82	20.343	0.397
10	6.29	96.89	2.08	99.96	0.02	0.91	9.45	14.53	20.354	0.408

Table A.2.38 Hydrogen production data of C/N=50 medium

C/N=50 medium										
Hydrogen gas from head space									Hydrogen gas from total gas	
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**	**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0	0	
1	240	360	0.091	32.76		370	0.091	33.67	66.430	2.613
2	230	370	0.541	167.41		1065	0.541	576.17	810.005	31.865
3	220	380	0.377	0		600	0.377	226.20	1036.205	40.763
4	210	390	0.499	51.35		330	0.499	164.67	1252.225	49.261
7	200	400	0.508	8.59		450	0.508	228.60	1489.415	58.592
8	190	410	0.478	0		40	0.478	19.12	1508.535	59.344
9	180	420	0.295	0		0	0.295	0	1508.535	59.344
10	170	430	0.350	26.60		0	0.350	0	1535.135	60.391
11	160	440	0.251	0		0	0.251	0	1535.135	60.391

Table A.2.39 Hydrogen production data of C/N=100 medium

C/N=100 medium										
Hydrogen gas from head space									Hydrogen gas from total gas	
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**	**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0	0	
1	240	360	0.277	99.72		280	0.277	77.56	177.280	6.974
2	230	370	0.568	110.44		1120	0.568	636.16	923.880	36.345
3	220	380	0.602	18.60		640	0.602	385.28	1327.760	52.233
4	210	390	0.593	2.51		560	0.593	332.08	1662.350	65.395
7	200	400	0.619	16.33		755	0.619	467.35	2146.025	84.423
8	190	410	0.531	0		10	0.531	5.31	2151.335	84.632
9	180	420	0.508	0		0	0.508	0	2151.335	84.632
10	170	430	0.509	5.51		0	0.509	0	2156.845	84.848

Table A.2.40 Hydrogen production data of C/N=200 medium

C/N=200 medium										
Hydrogen gas from head space									Hydrogen gas from total gas	
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**	**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0	0	
1	240	360	0.397	142.92		650	0.397	258.05	400.970	15.774
2	230	370	0.573	69.09		910	0.573	521.43	991.490	39.004
3	220	380	0.585	10.29		600	0.585	351.00	1352.780	53.217
4	210	390	0.581	4.29		500	0.581	290.50	1647.570	64.814
7	200	400	0.599	13.01		750	0.599	449.25	2109.830	82.999
8	190	410	0.544	0		150	0.544	81.60	2191.430	86.209
9	180	420	0.519	0		20	0.519	10.38	2201.810	86.617
10	170	430	0.514	3.04		10	0.514	5.14	2209.990	86.939

Table A.2.41 Hydrogen production data of C/N=250 medium

C/N=250 medium										
Hydrogen gas from head space									Hydrogen gas from total gas	
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**	**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0	0	
1	240	360	0.393	141.48		630	0.393	247.59	389.070	15.306
2	230	370	0.579	72.75		1010	0.579	584.79	1046.610	41.173
3	220	380	0.587	8.83		700	0.587	410.90	1466.340	57.685
4	210	390	0.572	0.02		535	0.572	306.02	1772.380	69.724
7	200	400	0.598	16.12		760	0.598	454.48	2242.980	88.237
8	190	410	0.523	0		30	0.523	15.69	2258.670	88.854
9	180	420	0.512	0.61		10	0.512	5.12	2264.400	89.079
10	170	430	0.496	0		0	0.496	0	2264.400	89.079

Table A.2.42 Raw data of 67 g/L glucose added medium (C/P=19)

67 g/L glucose added medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	6.7	0	67.14	0	47.35	11.32	3.30	2.50	2.623	0.467
1	4.98	12.06	59.04	13.98	40.73	4.17	5.67	3.76	5.531	0.523
2	5.13	48.44	34.62	61.71	18.13	3.11	9.68	7.50	11.643	0.745
3	5.63	93.03	4.68	99.38	0.29	3.11	12.34	10.20	15.498	0.878
4	6.32	94.22	3.88	99.55	0.21	2.45	12.09	10.24	15.313	0.867
7	6.26	94.95	3.39	99.56	0.21	2.45	11.80	10.34	15.406	0.873
8	6.38	95.32	3.14	99.67	0.16	2.38	11.70	10.30	15.533	0.904
9	6.33	95.51	3.02	99.68	0.15	2.23	11.57	10.33	15.402	0.858
10	6.39	95.51	3.01	99.69	0.15	2.22	11.55	10.35	15.473	0.896
11	6.4	95.54	2.99	99.69	0.15	2.22	11.50	10.37	15.469	0.884
14	6.44	95.88	2.77	99.73	0.13	2.17	11.32	10.38	15.523	0.897

Table A.2.43 Raw data of 85 g/L glucose added medium (C/P=25)

85 g/L glucose added medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	6.71	0	85.28	0	58.55	11.24	3.12	2.76	2.541	0.466
1	5.06	21.76	66.72	5.29	55.45	1.98	6.43	5.32	6.564	0.540
2	5.13	49.72	42.88	47.89	30.51	1.53	9.76	5.03	8.635	0.844
3	5.29	52.69	40.35	60.17	23.32	1.42	10.09	7.81	9.843	0.921
4	5.41	57.08	36.60	69.57	17.82	0.54	10.34	9.26	10.935	0.905
7	5.72	87.29	10.84	90.59	5.51	0.74	11.29	10.73	15.168	0.945
8	6.55	88.09	10.16	93.66	3.71	0.31	11.14	10.74	15.512	0.959
9	6.41	88.60	9.72	94.57	3.18	0.31	11.07	11.33	15.579	0.918
10	6.45	88.84	9.52	95.07	2.88	0.29	11.09	11.07	15.753	0.926
11	6.51	88.84	9.52	95.44	2.67	0.29	11.07	11.01	15.870	0.956
14	6.51	88.93	9.44	95.87	2.42	0.26	10.21	10.80	15.813	0.977

Table A.2.44 Raw data of 107 g/L glucose added medium (C/P=31)

107 g/L glucose added medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	6.73	0	106.60	0	79.10	13.74	3.42	2.88	2.621	0.474
1	6.03	6.00	100.20	0.66	78.58	12.00	3.52	2.42	2.655	0.455
2	6.21	5.91	100.30	0.71	78.54	11.66	3.54	2.66	2.553	0.461
3	6.24	5.44	100.80	0.76	78.50	11.42	3.66	2.74	2.433	0.426
4	6.1	5.82	100.40	0.83	78.44	10.42	3.64	2.50	2.421	0.595
7	4.89	19.79	85.50	25.98	58.55	2.34	9.00	9.62	10.940	1.736
8	5.72	20.17	85.10	26.09	58.46	1.92	9.20	10.49	11.159	1.744
9	5.56	29.17	75.50	43.60	44.61	1.88	13.12	12.12	14.243	1.778
10	5.63	62.01	40.50	69.58	24.06	1.04	15.17	12.41	16.172	1.981
11	5.87	84.62	16.40	91.71	6.56	0.95	17.24	11.53	16.665	2.146
14	6.35	94.33	6.04	98.37	1.29	0.62	17.10	10.28	16.445	2.245

Table A.2.45 Raw data of 129 g/L glucose added medium (C/P=37)

129 g/L glucose added medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	6.77	0	128.70	0	90.96	16.34	3.22	2.32	2.684	0.482
1	5.83	6.45	120.40	0.04	90.92	13.22	3.78	2.36	2.681	0.447
2	5.1	14.92	109.50	0.22	90.76	5.62	6.50	4.14	5.848	0.543
3	5.33	21.45	101.10	21.81	71.12	2.82	9.92	10.38	12.803	0.701
4	5.3	29.45	90.80	27.44	66.00	2.76	9.82	10.56	14.275	0.668
7	5.49	47.40	67.70	48.23	47.09	2.72	10.72	11.84	18.070	0.734
8	5.76	57.81	54.30	53.47	42.33	2.59	11.59	13.06	19.237	0.791
9	5.92	60.14	51.30	63.31	33.38	2.54	11.74	13.70	20.387	0.779
10	6.08	60.37	51.00	66.97	30.04	2.54	12.58	14.75	21.393	0.825
11	6.26	60.45	50.90	70.93	26.44	2.52	12.35	14.49	21.901	0.858
14	6.25	64.26	46.00	76.79	21.11	2.44	12.03	13.85	22.042	0.878

Table A.2.46 Raw data of 149 g/L glucose added medium (C/P=43)

149 g/L glucose added medium										
Time (day)	**pH**	**Total Sugar Consumption (%)**	**Total Sugar Concentration (g/L)**	**Glucose Consumption (%)**	**Glucose (g/L)**	**Fructose (g/L)**	**Acetic Acid (g/L)**	**Butyric Acid (g/L)**	**Acetone (g/L)**	**Ethanol (g/L)**
0	6.88	0	149.40	0	109.56	17.50	3.22	2.08	2.435	0.434
1	5.32	7.83	137.70	0.55	108.96	12.12	4.04	2.64	3.432	0.449
2	4.91	17.47	123.30	7.48	101.36	3.44	7.24	6.76	9.035	0.615
3	5.36	25.10	111.90	23.07	84.28	3.32	9.00	8.64	12.667	0.681
4	5.39	29.52	105.30	32.18	74.30	2.66	9.98	11.34	15.550	0.708
7	5.45	44.04	83.60	47.34	57.69	2.57	10.36	12.54	19.589	0.754
8	6.07	44.11	83.50	47.39	57.64	2.50	10.86	13.24	19.541	0.740
9	6.24	43.98	83.70	47.44	57.58	2.50	10.76	13.21	19.427	0.686
10	6.25	44.38	83.10	47.50	57.52	2.48	10.93	13.46	19.607	0.713
11	6.13	44.31	83.20	100.00	-	-	-	-	-	-
14	6.06	44.65	82.70	47.96	57.01	2.39	11.07	13.66	20.921	0.799

Table A.2.47 Hydrogen production data of 67 g/L glucose added medium (C/P=19)

67 g/L glucose added medium										
Hydrogen gas from head space									**Hydrogen gas from total gas**	
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**	**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0	0	
1	240	360	0.429	154.44		20	0.429	8.58	163.020	6.413
2	230	370	0.528	40.92		210	0.528	110.88	314.820	12.385
3	220	380	0.459	0		40	0.459	18.36	333.180	13.107
4	210	390	0.357	0		10	0.357	3.57	336.750	13.247
7	200	400	0.348	0		60	0.348	20.88	357.630	14.069
8	190	410	0.379	16.19		0	0.379	0	373.820	14.706
9	180	420	0.319	0		0	0.319	0	373.820	14.706
10	170	430	0.306	0		0	0.306	0	373.820	14.706
11	160	440	0.232	0		0	0.232	0	373.820	14.706
14	150	450	0.154	0		0	0.154	0	373.820	14.706

Table A.2.48 Hydrogen production data of 85 g/L glucose added medium (C/P=25)

85 g/L glucose added medium										
Hydrogen gas from head space									Hydrogen gas from total gas	
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**	**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0	0	
1	240	360	0.530	190.80		20	0.530	10.60	201.400	7.923
2	230	370	0.453	0		20	0.453	9.06	210.460	8.279
3	220	380	0.451	3.77		260	0.451	117.26	331.490	13.041
4	210	390	0.458	7.24		190	0.458	87.02	425.750	16.749
7	200	400	0.447	0.18		420	0.447	187.74	613.670	24.141
8	190	410	0.475	15.95		20	0.475	9.50	639.120	25.142
9	180	420	0.380	0		10	0.380	3.80	642.920	25.292
10	170	430	0.319	0		0	0.319	0	642.920	25.292
11	160	440	0.340	12.43		0	0.340	0	655.350	25.781
14	150	450	0.300	0		0	0.300	0	655.350	25.781

Table A.2.49 Hydrogen production data of 107 g/L glucose added medium (C/P=31)

107 g/L glucose added medium										
Hydrogen gas from head space									Hydrogen gas from total gas	
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**	**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0	0	
1	240	360	0.042	15.23		0	0.042	0	15.228	0.599
2	230	370	0.046	1.64		0	0.046	0	16.872	0.664
3	220	380	0.052	2.96		0	0.052	0	19.836	0.780
4	210	390	0.061	3.84		0	0.061	0	23.673	0.931
7	200	400	0.433	149.53		720	0.433	311.76	484.960	19.078
8	190	410	0.402	0		20	0.402	8.04	493.000	19.394
9	180	420	0.524	55.26		560	0.524	293.44	841.700	33.112
10	170	430	0.471	0		910	0.471	428.61	1270.310	49.973
11	160	440	0.397	0		640	0.397	254.08	1524.390	59.968
14	150	450	0.337	0		200	0.337	67.40	1591.790	62.620

Table A.2.50 Hydrogen production data of 129 g/L glucose added medium (C/P=37)

129 g/L glucose added medium											
Hydrogen gas from head space									Hydrogen gas from total gas		
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**		**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0		0	
1	240	360	0.037	13.43		0	0.037	0		13.428	0.528
2	230	370	0.532	183.41		30	0.532	15.96		212.800	8.371
3	220	380	0.527	3.42		10	0.527	5.27		221.490	8.713
4	210	390	0.540	10.34		110	0.540	59.40		291.230	11.457
7	200	400	0.479	0		30	0.479	14.37		305.600	12.022
8	190	410	0.456	0		320	0.456	145.92		451.520	17.762
9	180	420	0.457	4.98		100	0.457	45.70		502.200	19.756
10	170	430	0.456	4.14		90	0.456	41.04		547.380	21.533
11	160	440	0.433	0		20	0.433	8.66		556.040	21.874
14	150	450	0.426	1.18		20	0.426	8.52		565.740	22.256

Table A.2.51 Hydrogen production data of 119 g/L glucose added medium (C/P=43)

149 g/L glucose added medium											
Hydrogen gas from head space									Hydrogen gas from total gas		
Time (day)	**VL (mL)**	**VHS (mL)**	**CH2/100**	**VH2-HS (mL)**		**VTG (mL)**	**CH2/100**	**VTG*CH2 (mL)**		**$V_{cumulative}$ (mL)**	**Cumulative H_2 (mmol)**
0	250	350	0	0		0	0	0		0	
1	240	360	0.276	99.36		230	0.276	63.48		162.840	6.406
2	230	370	0.583	116.35		460	0.583	268.18		547.370	21.533
3	220	380	0.500	0		680	0.500	340.00		887.370	34.908
4	210	390	0.486	0		550	0.486	267.30		1154.670	45.424
7	200	400	0.481	2.86		280	0.481	134.68		1292.210	50.834
8	190	410	0.392	0		0	0.392	0		1292.210	50.834
9	180	420	0.325	0		0	0.325	0		1292.210	50.834
10	170	430	0.318	0.24		0	0.318	0		1292.450	50.844
11	160	440	0.382	31.34		10	0.382	3.82		1327.610	52.227
14	150	450	0.403	13.27		50	0.403	20.15		1361.030	53.542