Current Research in Microbiology and Biotechnology
Vol. 6, No. 1 (2018): 1391-1399
Research Article
Open Access

ISSN: 2320-2246

# Bioprocess conditions for Ethanol production from Banana waste using *Bacillus subtilis* in shake flask fermentation

Ramzi Ata Abd Alsaheb[1,*], Sami D. Salman[1] and Marwa Ata Abd alsaheb[2]

[1] *Department of Biochemical Engineering, Al-Khwarizmi College of Engineering, University of Baghdad, Baghdad, Iraq.*
[2] *Ministry of Higher Education and Scientific Research, Iraq.*

* Corresponding author: Ramzi Ata Abd Alsaheb; e-mail: ramzi@ibd.utm.my

Received: 24 November 2017 Accepted: 13 December 2017 Online: 01 January 2018

## ABSTRACT

*Bacillus subtilis* strain obtained from Laboratory of Biochemical Engineering Department at the University of Baghdad, Al-Khwarizmi College of Engineering, Baghdad, Iraq. These strains have been preserved at -80°C were screened for ethanol production and were selected for investigation based on the ethanol production. This strain isolate provided the concentration of Ethanol 5.5 g/L and reducing sugar 4.0 g/L also showed the result maximum cell dry weight reach to 4.52 g/L in media respectively with pH 4.0. The present work was focused to enhance production of Ethanol by *Bacillus subtilis through* optimization of the production medium. In this study, media chosen contained of g/L$^{-1}$ peptone 5, Yeast Extracts 6, $K_2HPO_4$ 1.5, Banana waste 20, Sodium acetate 1.5, $MgSO_4.7H_2O$ 0.57, $MnSO_4.4H_2O$ 0.12, $FeSO_4.7H_2O$ 0.03, Sodium chloride (NaCl), 1.1, 0.3 potassium chloride (KCl). The process optimization was started by optimization of medium composition using one factor at time (OFAT), followed by comparison studied between growth in un-optimized and optimized medium in the shake flask level. Result showed that, the Ethanol produced in un-optimized medium and optimized medium was 3.00[g.L$^{-1}$] and 10.0[g.L$^{-1}$], respectively. Optimization of the medium components was increased the yield of Ethanol production.

**Keywords:** Ethanol, *Bacillus subtilis,* optimization medium, fermentation.

## 1. INTRODUCTION

Ethanol is one of key organic acids with wide range of industrial applications [1]. The commercial production of Ethanol is very common and it is possible either by chemical synthetic approach or by fermentation process [2]. Ethanol as a common amusement drug is produced by fermentation process using sugars as feed stock or produced by chemical and petrochemical processes [3]. For many years, Ethanol have been produced by different kinds of microorganisms and widely applied in food and chemical industries [4]. More recently, many new applications have been reported for this acid in fuel, medical and pharmaceutical field and become one of the main ingredients in wellness industries, in general [5]. The wide application of Ethanol in food industries is based its safety class according to the food and drug rules [6].

The major purpose of the using of bio-ethanol are to utilize as octane promoter in unleaded gasoline in place of the methyl tertio butyl ether and emission also limiting hazard of environment change and employs renewable energy partially replace oil and augmentation security of provide [7]. Bio-ethanol is gotten from alcoholic fermentation of sucrose which is created from biomass [8]. Total and 90% ethanol are perfect solvents and are utilized as a part of numerous mechanical items for example, paints aromas and tinctures [9]. Ethanol proposed to non-sustenance utilizes is made unfit for human ingestion by expansion of little measures of poisonous or offensive substances for example, methanol or gasoline [10]. There is an increasing concern worldwide to find out novel and inexpensive carbohydrate sources for production of bioethanol [11]. The comparison of the feed stock

involves various issues [12] such as chemical composition for biomass, energy balance, and absorption of minerals for soil. The huge expansion of Ethanol demand in a global market is driven greatly by development of more economically large-scale fermentation process. Ethanol is a simple compound of alcohol or ethyl group joined with a hydroxyl group with chemical formula $C_2H_5OH$. It's a volatile, flammable, colorless liquid with a slight characteristic odor [13]. Ethanol is produced ultimately by the fermentation by molasses from sugar; molasses is the main source of carbon in the process of the production of Ethanol [14]. Ethanol can also be obtained from the fermentation of Lactic acid bacteria (LAB) where these bacteria have two pathways for bacterial fermentation the first pathways produced lactic Acid and The second pathways produces ethanol at different rate[15]. Homofermentative bacteria in this group transform more than 85% of glucose to lactic acid through Embden–Meyerhof–Parnas (EMP) glycolytic pathway.

Ethanol and Lactic acid is the main product of this fermentation process, But at varying ratios. They ferment 1 mol of glucose to 2 mol of lactic acid and generate a net yield of 2 mol of ATP per mole of glucose metabolized [16]. Heterofermentative. Bacteria in this group product lactic acid (50%) along with ethanol and CO2 (50%) from glucose. They ferment 1 mol of glucose to 1 mol of lactic acid, 1 mol of ethanol and 1 mol of CO2 via the phosphoketolase-dependent pathway. One mol of ATP is generated per 1 mol of glucose, which consequently results in less growth per mole of glucose metabolized when contrast with homofermentative lactic acid bacteria (LAB). [17]. In general, we still have a lot of applications and theories about the Ethanol to understand and absorb its properties and its features and its important role in the chemical industry and biotechnology an important factor in the development of those industries.

In this work several parameters (particularly nutrient sources using carbon and nitrogen sources) have been investigated such as the culture medium pH, inoculum sizes and incubation temperatures in order to reach the optimum production parameters. The process optimization was started by optimization of medium composition using one factor at time (OFAT) strategy, followed by comparison studied between growth in un-optimized and optimized medium in the shake flask level. Optimization of the medium components was increased the yield of Ethanol production. Therefore, the semi-defined medium formulation developed in this work can be used for large scale production process for Ethanol in respect to yield and cost.

## 2. MATERIALS AND METHODS

### 2.1 Microorganisms and Preparation of Working Cell Culture

*Bacillus subtilis* Strain obtained from Laboratory of Biochemical Engineering Department at the University of Baghdad, Al-Khwarizmi College of Engineering, Baghdad, Iraq. This isolate was preserved in freezer at -80°C.Were selected for investigation based on the ethanol production. The strain was conveyed by in a frozen glycerol and the cells at first were propagated on Basal agar media and adjusted to pH 7.0 before sterilization. It's incubated at 35 °C for 48 hours. The colonies formed were harvested by 50% glycerol solution and aspirated to be placed in series of 2 ml sterile cryovials tubes. These tubes were then frozen at 4.0 °C for 48 h followed by further storage as working cell bank at -80 °C ultra-deep freezer for further utilization.

When the bacteria grows, the colors of medium change from purple to yellow and the clear zone around the Single colony become clear.

### 2.2 Preparation of substrate

The banana waste utilized as substrate was gained from banana fruit chopped into small bits sizes. The peel was spread on trays and oven dry at 70°C for 48 hours. The dried peel was smash in mixer grinder and stocked in polythene bag at room temperature 25°C.

### 2.3 Fermentation medium

The medium chosen for production was optimized in shake flask study and the pH have been adjusted at 7.0. The compositions of the production media were as follows [$gL^{-1}$]: peptone 5, Yeast extracts 6, $K_2HPO_4$ 1.5, Banana waste 20, Sodium acetate 1.5, $MgSO_4.7H_2O$ 0.57, $MnSO_4.4H_2O$ 0.12, $FeSO_4.7H_2O$ 0.03, Sodium chloride (NaCl), 1.1, 0.3 potassium chloride (KCl) in laminar air flow.

### 2.4 Comparison between un-optimized and optimized medium

In accordance with optimum compositions of medium have been chosen, a comparison study between the selected optimum medium and un-optimized medium. The growth kinetics of *Bacillus subtilis* will be calculated in 250 Erlemenyer flask cultures using 50 ml volume of un-optimized medium and optimized medium. The flasks were incubated in a rotary shaker at 35ºC at 150 rpm for 48 h. Samples were tested every 6h. The effect of pH, Cell dry weight, Total Reducing sugar and Ethanol concentration was studied for growth kinetics.

### 2.5 Analytical methods

#### 2.5.1 Sample preparation and Cell dry weight determination

Samples in the form of two flasks of 50 ml broth for every shake flask taken at various time periods through cultivations. For immediate biomass determination, one ml of samples were aspirated and added to 9 ml distilled water, which is done in universal bottles. Dilution repeated until 1000 dilution. By using spectrophotometer The optical density was measured. Absorbance was taken for every dilution and replicate at 600 nm. Dilution that obedience sketch in results is taken as outcomes and cell dry weight is calculated based on the standard curve, one unit $OD_{600}$ was equal to 0.3 g $L^{-1}$.

### 2.5.2 Determination pH
The initial pH of the medium was adjusted to (4.0-7.0) was measured by using pH meter (TOLEDO, Delta 320).

### 2.5.3 -Determination reducing sugar
Prior to the aggregate sugar concentration could be measured. All non-reduce sugar (sucrose) is should have been hydrolyzed to decreasing sugars .This step could be achieved by Mono- and di-saccharides analysis using High Performance Liquid Chromatography (HPLC).

### 2.5.4 Ethanol determination
After the sampling, cell will be separated from the supernatant using cooling centrifuge at 4 ºC and 4000 rpm for 10 min. The supernatant will be used for ethanol analysis ethanol will be determined using High Performance Liquid Chromatography (HPLC).

## 3. RESULTS AND DISCUSSION
### 3.1 Ethanol production by *Bacillus subtilis*
From Figure: 1. show *Bacillus subtilis* isolate provided the concentration of ethanol was 5.5. [g/L] and total Reducing sugar reached to 4.0[g/L] in media respectively.

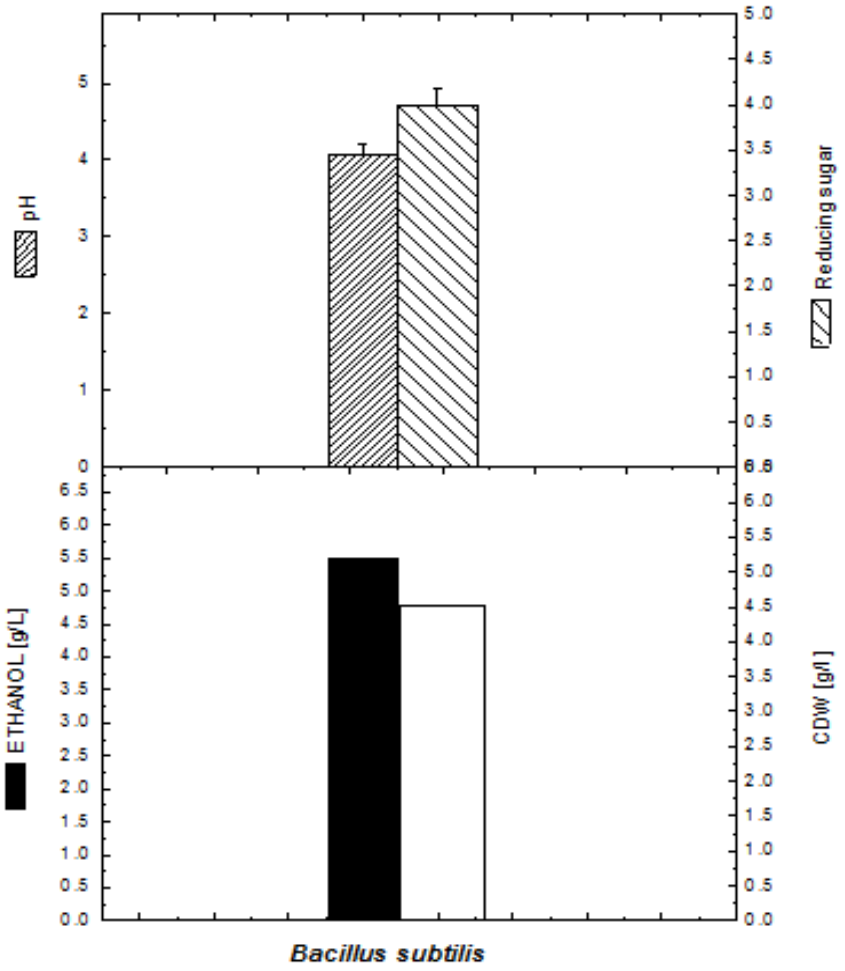



**Figure 1:** Ethanol production by *Bacillus subtilis* incubation at 35°C for 48 h under anaerobic conditions

### 3.2 Effect of different substrate concentrations on ethanol production
The appropriate concentration of substrate for ethanol production by *(Bacillus subtilis*) using medium containing 0-20g/L of Banana waste. Outcomes showed that the ethanol concentration increased with the increased with the raise in Banana waste concentration up to 15 g/L. Figure: 3. the maximum ethanol 6.50 g/L was obtained at 48 h fermentation with an initial Banana waste concentration. Ethanol concentration increased when Banana waste was higher than 15g/L. This due to inhibition by rising substrate concentration.

Therefore, 15[g/L] of Banana waste concentration was chosen to be utilized as carbon source in medium for ethanol production by the isolate *Bacillus subtilis.*

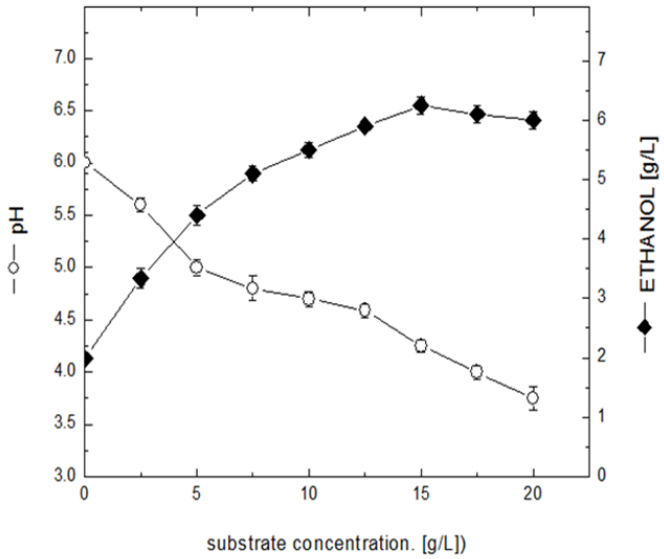



**Figure 3:** Ethanol production of bacterial isolate *Bacillus subtilis cultivation* for various concentrations of Fructose at 35°C for 48 h.

### 3.3 Effect of different peptone concentrations on ethanol production

Sources of Nitrogen cannot be ignored through *Bacillus subtilis* cultivation as it is very important in the *Bacillus subtilis* growth and metabolism during fermentation. Absorption of nitrogen is the most important component in the fermentation media beside carbon sources. However comparable to carbon sources the range of cell growth is hugely dependent on the kind and concentration of the nitrogen sources used. Effect of various nitrogen sources on Ethanol production. Varied concentrations of peptone [g/L]:0, 1, 2, 3, 4, and 5. after cultivating *Bacillus subtilis* for 48 h at 35°C under anaerobic conditions, the maximum yield of 4.25 [g/L] of ethanol was produced Figure 4. The results suggested that peptone at the concentration of 3.0 g/l was suitable for ethanol production by *Bacillus subtilis.*

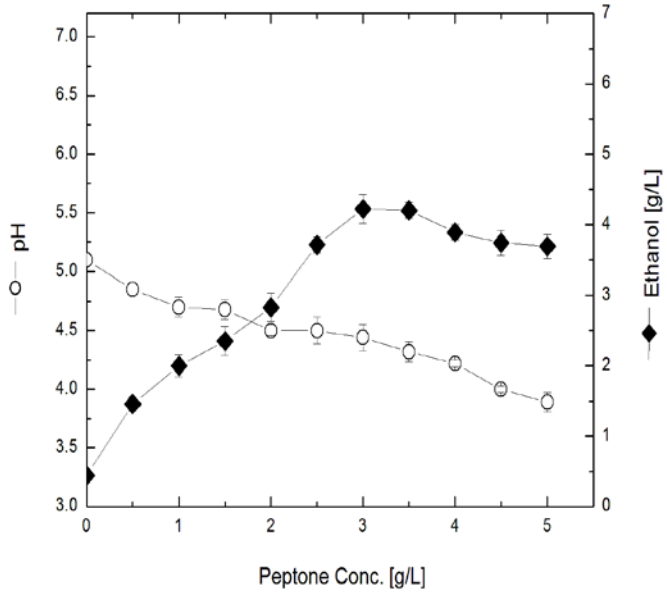



**Figure 4:** Ethanol production of bacterial isolate *Bacillus subtilis after* cultivation for various concentrations of peptone at 35°C for 48 h.

### 3.4 Effect of different yeast extract concentrations on ethanol production

One of the most important factors used for growth is yeast extract which provided complex nutrients as nitrogen source. Yeast extract is a costly material to be used in the manufacturing process, different concentrations of yeast extract [g/L]: 0, 1, 2,3,4,5 and 6, were added to medium. After cultivating *Bacillus subtilis* for 48 hours at 35°C under anaerobic conditions, the Ethanol yield was 4.5 [g/L]. Figure5. The results suggested that yeast extract at the concentration of 2.5 [g/L] was appropriate for ethanol production by *Bacillus subtilis.*

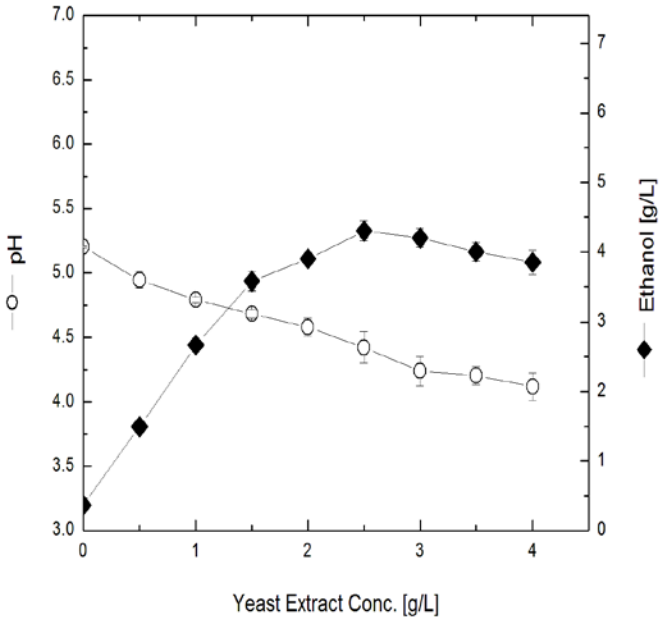



**Figure: 5** Ethanol production of bacterial isolate *Bacillus subtilis after* cultivation for various concentrations of yeast extract at 35°C for 48 h.

### 3.5 Initial pH of ethanol medium production

The influence of pH of fermentation medium on ethanol production was estimated by utilize the optimized medium at initial pH in the domain of 4-7. Results showed that pH 4.0 was found best condition than other pH Values. The maximum yield of ethanol reach to 5.25[g/L].

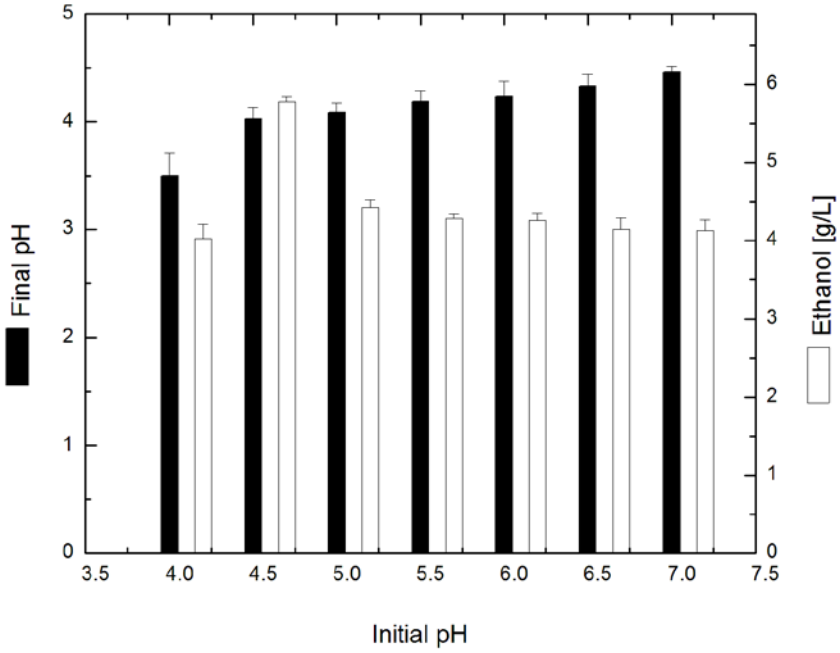



**Figure 6:** Effect of initial pH of the optimized broth on ethanol production of bacterial isolate *Bacillus subtilis when* cultivated in the medium at 35°C for 48 h.

### 3.6 Cultivation temperature

The optimal temperature for ethanol production was specified by cultivating the *Bacillus subtilis* in the optimized medium at optimum pH for 48 hours. The incubation temperatures ranging at 25.0- 40.0, and 45.0°C based the range of its growth temperatures. Results showed that the maximum Ethanol yield 5.55[g/L]. Were obtained when the cultivating medium at 35°C.

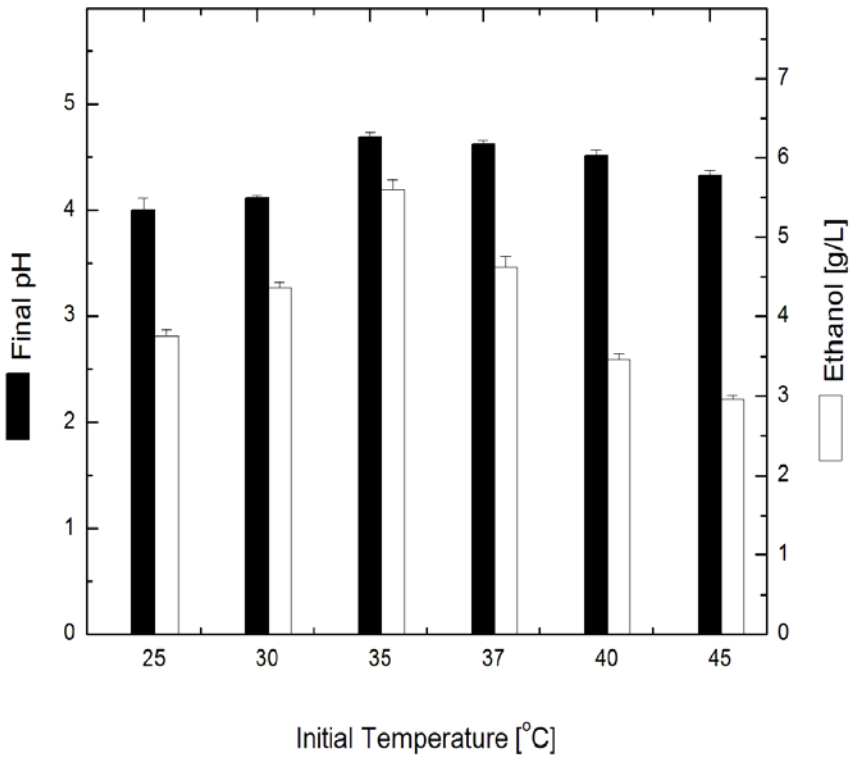



**Figure 7:** Effect of cultivation temperatures on ethanol production of bacterial isolate *Bacillus subtilis when* cultivated in the medium at 35°C for 48 h.

### 3.7 Inoculum sizes

The effect of inoculum size on Ethanol production calculated by various inoculum sizes (1-5%, v/v) were separately added to the optimized medium. Bacterial growth and ethanol production increased when inoculum size was rise up to 3% (v/v) Figure 8. And inoculum sizes at 1-5% were fiddling difference on the ethanol yields. 3% (v/v) of inoculum size could be considered to be optimal for obtain the maximum ethanol production 5.70[g/l]. Thus, 3% (v/v) of inoculum size was it adopted the best result selected for ethanol production by *Bacillus subtilis*.

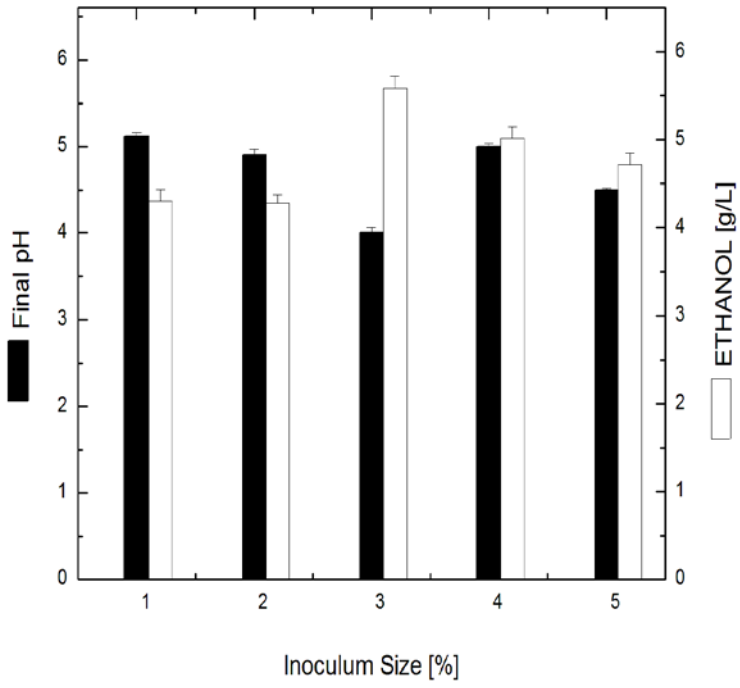
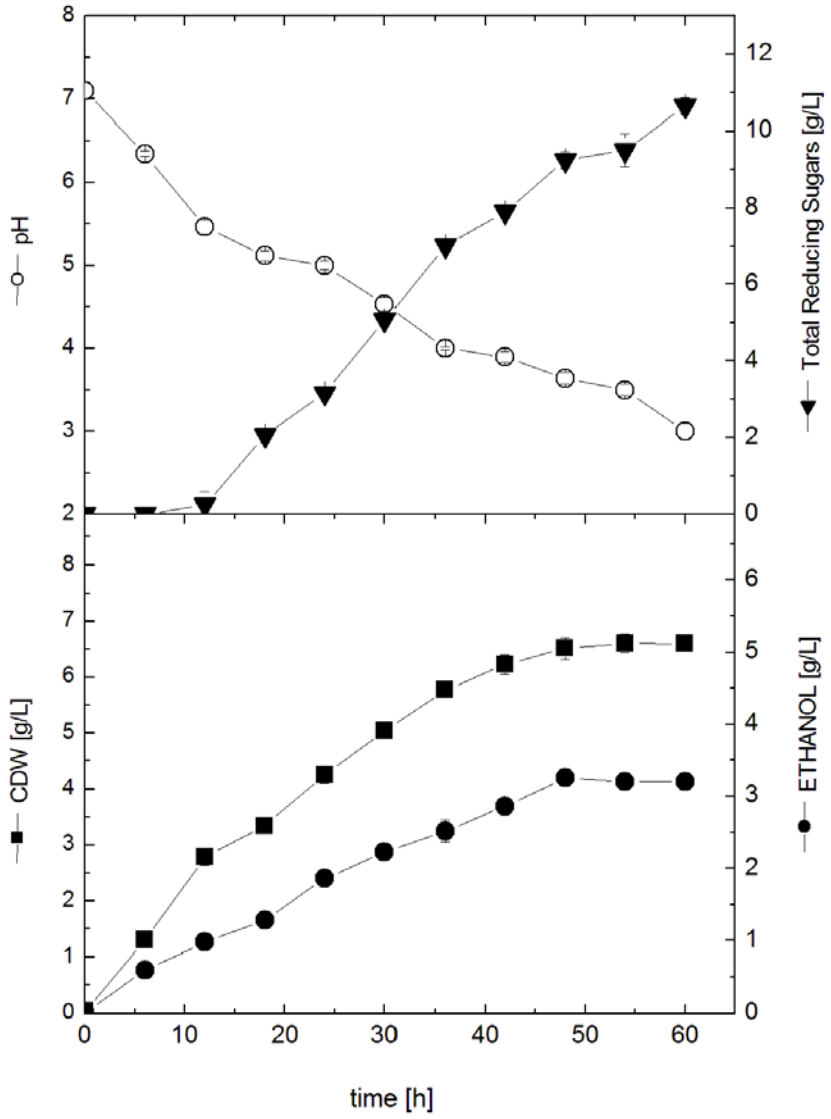



**Figure 8:** Effect of inoculum sizes on ethanol production of bacterial isolate *Bacillus subtilis when* cultivated in the medium at 35°C for 48 h.

### 3.8 Kinetics of ethanol production and cell growth by *Bacillus subtilis* cultivated in un-optimized medium

In this section of work, the growth profile, variation in the pH and ethanol production as a function of time were investigated in shake flask cultivation at 35°C for 48 hours As can be shown in Figure: 10, cells entered a lag phase for the first 6 hours of cultivation, and then the cells started to grow exponentially, where they reached a maximal cell dry weight of about 6.52 [g.L$^{-1}$] before entering the stationary phase after 48 hours of cultivation. The pH of the medium increased gradually and reached a minimal of about 3.0 .the drop of pH in cultures was due to the formation of ethanol through cultivation process.




**Figures 9**: Growth curve kinetic of *Bacillus subtilis* for un-optimized media in shake flask.

Cultivation as the production of ethanol was directly proportional to the cells growth. The specific growth rate of *Bacillus subtilis was* 0.112 [h$^{-1}$], production rate of almost 0.114[g.L$^{-1}$.h$^{-1}$] and specific ethanol production 1.038 [g.g$^{-1}$]. While, for un-optimized media The Total reducing sugar start decreasing faster from 1.2 [g/l] to 10.00 [g/l] at 48h, and it is clear the total reducing sugar increase inversely proportional to the increased production of ethanol.

### 3.9 Kinetics of cell growth and ethanol production by *Bacillus subtilis* in optimized medium

The kinetics of cell growth of *Bacillus subtilis was* calculated in shake flask level. The cells grew at 35°C for 48h in un-optimized medium, where the cell growth, ethanol production and the variation in the pH of the culture were testing every six hours Figure: 9. The growth of cells was slowly in the first 6 h, and then entered the exponential phase with a growth rate of 0.258 [g.L$^{-1}$.h$^{-1}$], and the greaterl cell mass of 9.15[g.L$^{-1}$] was obtained after 48 hours of cultivation. The Total reducing sugar for un-optimized media began to give up gradually and continuous rapidity from 1.2[g.L$^{-1}$] to 17.32 [g.L$^{-1}$] after 48 hours. The pH dropped from 7.2 to 3.5 the production of ethanol as a primary metabolite is strictly dependent on cell growth, Therefore, after 48 hours of cultivation ethanol reached to 10 [g.L$^{-1}$] and production rate 0.178 [g.L$^{-1}$.h$^{-1}$].

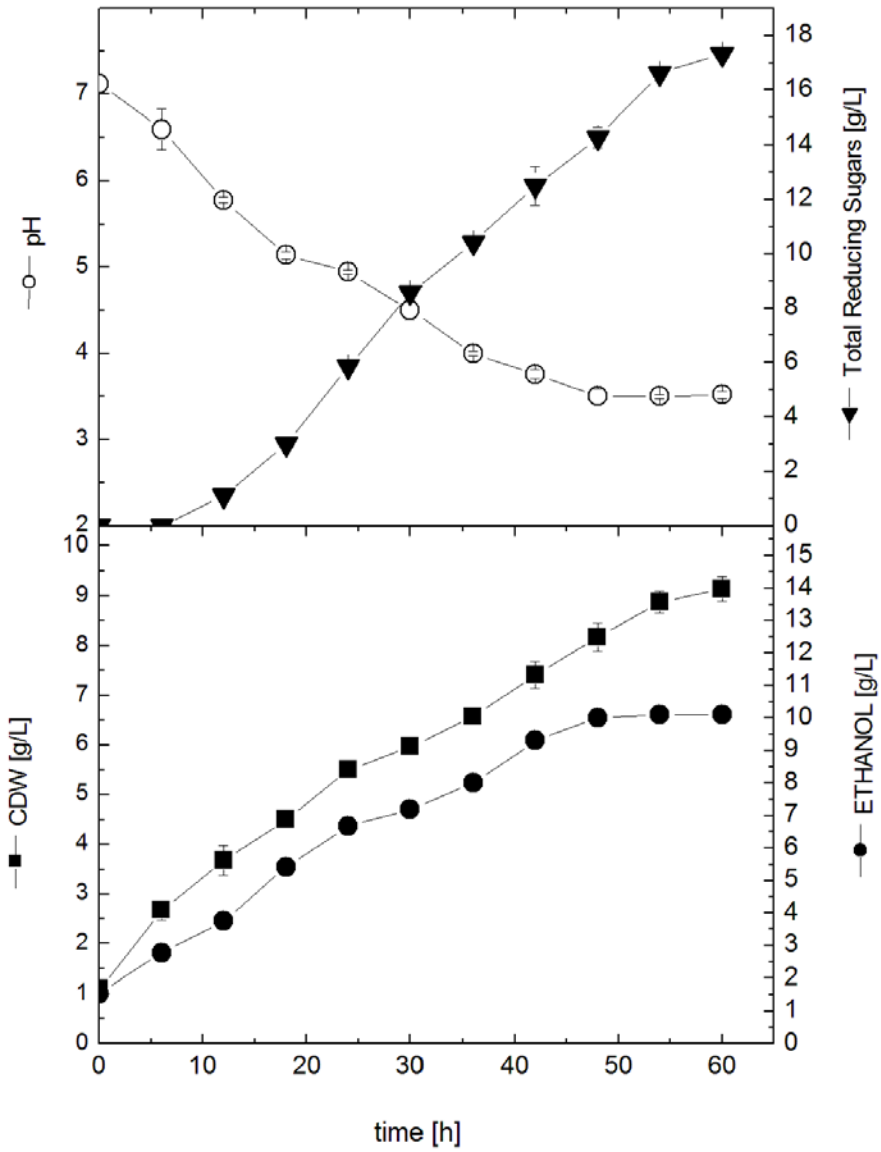



**Figure 10:** Growth curve kinetic of *Bacillus subtilis* for optimized media in shake flask.

These results showed that the optimized production medium was capable to support best growth of *Bacillus subtilis* reach to maximal cell dry weight was 9.15 [g.L$^{-1}$] with an accretion when compared to the un-optimized medium. In summary, based on data obtained from the previous experiments the overall result for shake flask fermentation is summarized in Table 1.

**Table 1:** Kinetics of Ethanol and cell growth production by *Bacillus subtilis in* shake flask cultivation conditions.

| **Parameters** | **Shake Flask** | |
|---|---|---|
| Growth Parameters | Un-optimized | Optimized |
| $X_{max}$[g.L$^{-1}$] (time) | 5.42 | 14.00 |
| dx/dt [g.L$^{-1}$.h$^{-1}$] | 0.188 | 0.258 |
| μ [h$^{-1}$] | 0.112 | 0.112 |
| **Production Parameters** | | |
| $P_{max}$[g.L$^{-1}$] (time) | 3.50 | 10.00 |
| dp/dt [g.L$^{-1}$.h$^{-1}$] | 0.114 | 0.178 |
| Final pH | 3.00 | 4.00 |

Abbreviations: $X_{max}$: maximal cell dry weight; dx/dt: growth rate; μ: specific growth rate; $P_{max}$: maximal production of Ethanol, dp/dt: Ethanol rate production

## 4. CONCLUSION

In outline, kinetic data for whole experiments proceed in this study are summarized in Table 1 was mentioned above. When compare results together, as a conclusion in shake flask fermentation level, optimized media more Ethanol production which is 10.00 [$g.L^{-1}$] compared to un-optimized media, 3.00 [$g.L^{-1}$]. For growth rate production, optimized media more which is 0.178 [$g.L^{-1}.h^{-1}$] than un-optimized media which is 0.114 [$g.L^{-1}.h^{-1}$] and maximal cell dry weight reached 6.52 [$g.L^{-1}$] for optimized media 9.15 [$g.L^{-1}$]. Shake flask experiment is for determination of the optimum conditions for the fermentation process. Therefore, optimized media produced high production and high cell growth rate result. Therefore, the semi-defined medium formulation developed in this work can be used for large scale production process for ethanol in respect to yield and cost.

## 5. REFERENCES


1. Lin, Y. and Tanaka, S., 2006. Ethanol fermentation from biomass resources: current state and prospects. *Applied microbiology and biotechnology*, *69*(6), pp.627-642.
2. Hansen, A.C., Zhang, Q. and Lyne, P.W., 2005. Ethanol–diesel fuel blends––a review. Bioresource technology, 96(3), pp.277-285.
3. Stewart, G.G., Panchal, C.J., Russell, I. and Sills, A.M., 1983. Biology of ethanol-producing microorganisms. *Critical Reviews in Biotechnology*, *1*(3), pp.161-188.
4. Y-J Wee; J-N Kim; Ryu H-W. Food Technol. Biotechnol.2006, 163-172.
5. Kirk, O., Borchert, T.V. and Fuglsang, C.C., 2002. Industrial enzyme applications. *Current opinion in biotechnology*, *13*(4), pp.345-351.
6. Zaunmuller, T., Eichert, M., Richter, H. and Unden, G., 2006. Variations in the energy metabolism of biotechnologically relevant heterofermentative lactic acid bacteria during growth on sugars and organic acids. Applied microbiology and biotechnology, 72(3), pp.421-429.
7. Weiss, J., Takhistov, P. and McClements, D.J., 2006. Functional materials in food nanotechnology. *Journal of food science*, *71*(9).
8. Balat, M. and Balat, H., 2009. Recent trends in global production and utilization of bio-ethanol fuel. *Applied energy*, *86*(11), pp.2273-2282.
9. Zabed, H., Faruq, G., Sahu, J.N. et al. 2014. Bioethanol production from fermentable sugar juice. *The Scientific World Journal*, *2014*.
10. Popovych, O., Gibofsky, A. and Berne, D.H., 1972. Medium effects for single ions in acetonitrile and ethanol-water solvents based on reference-electrolyte assumption. *Analytical chemistry*, *44*(4), pp.811-817.
11. Anderson, J.E., Kramer, U., Mueller, S.A. and Wallington, T.J., 2010. Octane numbers of ethanol– and methanol– gasoline blends estimated from molar concentrations. *Energy & Fuels*, *24*(12), pp.6576-6585.
12. Mohanty, S.K., Behera, S., Swain, M.R. and Ray, R.C., 2009. Bioethanol production from mahula (Madhuca latifolia L.) flowers by solid-state fermentation. *Applied Energy*, *86*(5), pp.640-644.
13. Gnansounou, E., Dauriat, A. and Wyman, C.E., 2005. Refining sweet sorghum to ethanol and sugar: economic trade-offs in the context of North China. *Bioresource technology*, *96*(9), pp.985-1002.
14. Dillon, H.E. and Penoncello, S.G., 2004. A fundamental equation for calculation of the thermodynamic properties of ethanol. International journal of thermophysics, 25(2), pp.321-335.
15. Van Vuuren, H.J.J. and Meyer, L., 1982. Production of ethanol from sugar cane molasses by Zymomonas mobilis. Biotechnology Letters, 4(4), pp.253-256.
16. Alsaheb, R.A.A., Aladdin, A., Othman, N.Z et al. 2015. Journal of Chemical and Pharmaceutical Research, 2015, 7 (10): 729-735. *Journal of Chemical and Pharmaceutical Research*, *7*(10), pp.729-735.
17. Zuniga, M., Pardo, I. and Ferrer, S., 1993. An improved medium for distinguishing between homofermentative and heterofermentative lactic acid bacteria. International journal of food microbiology, 18(1), pp.37-42.5
18. Zaunmuller, T., Eichert, M., Richter, H. and Unden, G., 2006. Variations in the energy metabolism of biotechnologically relevant heterofermentative lactic acid bacteria during growth on sugars and organic acids. Applied microbiology and biotechnology, 72(3), pp.421-429.




*****