

Butanol Production by *Clostridium acetobutylicum* NCIMB 13357 Utilizing Yeast Industry Wastewater as a Fermentation Medium

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Abstract. Increasing fuel costs and global environmental concerns have aggravated the search for renewable energy. Significant progress for biofuels production by microorganisms using many types of industrial and agricultural wastes has been achieved. Yeast Industry Wastewater (YIW) is one of the cost-effective and nutrients rich media that is produced in large quantities in Jordan. This study aims to investigate the ability of *Clostridium. acetobutylicum* NCIMB 13357 to utilize YIW to produce butanol. As a result, *C. acetobutylicum* was able to grow and metabolize in agar plates continuing undiluted YIW. Shake cultures of YIW supplied with glucose and ammonium acetate resulted in the production of 11.73 g L⁻¹ of butanol. Butanol production was enhanced in column bioreactor as the total carbohydrates were consumed. Nevertheless, immobilization of *C. acetobutylicum* in sodium alginates slightly enhanced the production of butanol to reach 12.47 g L⁻¹ under batch fermentation, whereas under continuous operation the maximum production of butanol recorded 18.35 g L⁻¹ and 34.85 g L⁻¹ for total solvents after 96 h of fermentation. Consequently, YIW is a promising media for butanol production with additional benefits of its use with regard to environmental and economic aspects.

Keywords: Clean energy, environmental friendly, renewable energy,
waste utilization, waste to energy

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1 Introduction

Today, more emphasis is needed to find and improve a new or even an existing process for energy production due to high energy demands. The widespread use of fossil fuels and the concerns regarding pollution problems encourages scientists to search for a new source of energy [1–4]. This issue is largely met by renewable sources of energy such as bioethanol, biodiesel, and biogas [5–9].

There is a tremendous effort being paid to solve problems related to pollution, especially wastewaters of industries [10–12]. These solutions aim to reduce the impact of anthropic processes on the environment, to produce bulk-chemicals by eco-sustainable processes, and to diversify the fuel sources [13]. Biobutanol is a valuable biofuel due to its higher energy content, higher boiling point, and reduced need for modifying combustion engines; for these reasons, it is similar to ethanol, which is already being used [14, 15]. Besides its use as a potential fuel or fuel additive, butanol is considered to be superior to ethanol due to its higher energy content, lower volatility, less corrosiveness, and lower water absorption [16].

Anaerobic bacteria of the genus *Clostridium* are capable to produce Butanol throughout Acetone–Butanol–Ethanol (ABE) metabolism [17]. The strictly anaerobic bacterium *C. acetobutylicum* has two phases of metabolism (acidogenic and solvatogenic) and naturally produces butanol along with acetone and ethanol in a mole ratio of approximately 3:6:1 [18].

The effluent of any industry is often considered as a waste and requires a net energy input for treatment without product gain or they are decomposed in the environment without capturing any benefits. These effluents though are much more economical than sugar crops to synthesize value-added products. One of the wastewaters that are being produced in large quantities (nearly 200 m³ d⁻¹) in Jordan is the Yeast Industry Wastewater (YIW). This type of wastewater has a dark brown color with a high load of COD and BOD content. Based on the elemental composition of YIW, it is considered a good source to produce many chemical feedstocks throughout fermentation as it contains significant amounts of carbon, potassium, phosphate, and nitrogen [19]. There are very few research studies conducted on the use of such wastewater for the production of chemical feedstock or even for the treatment of wastewater. Mustafa studied the utilization of YIW as a fermentation medium to produce ethanol whereas [20]. Alobaidi studied the utilization of YIW and Olive Mill Pomace (OMP) as a fermentation medium to produce hydrolytic enzymes [21]. While Saleem studied the utilization of YIW as a fermentation media to produce citric acid [22]. Therefore, this study aims to assess the ability of *C. acetobutylicum* (free or immobilized cells) to utilize YIW as a fermentation medium to produce butanol along with other solvents.

2 Materials and methods

2.1 Microorganism and inoculum preparation

C. acetobutylicum NCIMB 13357 was provided by Prof. Wan Mohtar Wan Yusoff, School of Biosciences and Biotechnology, Faculty of Science and Technology, University Kebangsaan Malaysia. Fresh inoculum suspension was prepared by transferring six loop full of *C. acetobutylicum* to a bottle containing 200 mL of Reinforced Clostridia Medium (Oxoid). The culture was incubated at 35 °C under anaerobic conditions. After 24 h, the optical density (OD₆₀₀) of the culture was measured. The culture (OD₆₀₀ = 0.5) obtained was used as inoculum.

2.2 Bacterial growth on agar plates of yeast industry wastewater

Different concentrations of YIW [(25 %, 50 %, 75 %, 100 %) v v⁻¹] were employed to prepare YIW agar plates. Each agar plate was inoculated with 0.1 mL of clostridial suspension under sterile anaerobic conditions using a biosafety cabinet (Bio Lab, Korea). Thereafter, the agar plates were incubated in anaerobic jar for 48 h at 35 °C. After incubation, the YIW agar plates were checked for bacterial growth in order to determine the best YIW concentration suitable for *C. acetobutylicum* growth by counting the colony forming unit [10].

2.3 Cultivation of *C. acetobutylicum* in YIW for butanol production

To study butanol production by *C. acetobutylicum* growing in YIW media, static cultures of the most suitable concentration of YIW were prepared. Bottles of 250 mL capacity were filled with 200 mL of YIW and sterilized in an autoclave (Hiriyama, Japan) at 121 °C for 15 min. The bottles were flushed with nitrogen prior to inoculation with 5 % (v v⁻¹) of clostridial suspension and incubated in an incubator running at 35 °C for 48 h. The contents of each bottle were then assayed for solvents concentration.

In order to improve bacterial growth in YIW shake cultures, each bottle was supplemented with different concentration of glucose. The culture showing improvement was further supplemented with different concentrations of ammonium acetate [(1 % to 4 %) w v⁻¹] as a nitrogen source.

2.4 Butanol production in column bioreactor

Amount of 1.3 L column bioreactor (borosilicate glass column, 8 cm in diameter, 50 cm in height) was loaded with 1.3 L of presterilized fresh YIW media supplemented with 25 g of glucose and 10 g of ammonium acetate. The bioreactor was flushed with nitrogen gas via sterile silicone tubing connected to an air filter for 2 min. to flush out traces of oxygen gas from the medium. The vessel was connected to a gas collector (made of silicate glass) by silicone tubing as shown in Figure 1 in order to collect the exit gases of the culture. The fermentation media was inoculated with 5 % (v v⁻¹) of fresh clostridia inoculum. The pH of the media and incubation temperature were set at 6.8 and 35°C, respectively. This experiment lasted for 48 h and samples of 5 mL were withdrawn every 24 h for analysis of ABE concentrations and total carbohydrates.



Fig. 1. A photograph of the column bioreactor connected to cylindrical glass collector

2.5 Butanol production by immobilized *C. acetobutylicum* in bottle and column bioreactor

Static bottle and column bioreactor cultures of immobilized *C. acetobutylicum* grown in YIW media to produce butanol were studied in two sets of experiment. According to Wu and Wisecarver, *C. acetobutylicum* was immobilized using sodium alginate solution (5 % w v⁻¹) [23]. The solution was freshly prepared and sterilized by autoclaving at 121 °C for 15 min. An amount of 12.5 mL of fresh inoculum *C. acetobutylicum* was added to 40 mL of sodium alginate solution. This solution was added dropwise through a syringe to 1 000 mL of sterile CaCl₂ solution containing (10 % w v⁻¹ CaCl₂ and 5 % w v⁻¹ boric acid). The resulting beads were washed with sterile CaCl₂ solution and maintained in 2 % CaCl₂ at 4 °C for 1 h before use. Thereafter, the beads were added to a pre-flushed YIW media with nitrogen gas either in bottle or bioreactor cultures at a percentage of 20 % (v v⁻¹). The pH of media and incubation temperature were set at 6.8 and 35 °C, respectively. This experiment lasted for 48 h and samples were withdrawn every 24 h for measuring solvents concentration and total carbohydrates. To improve the ABE production process, the bioreactor experiment was conducted under continuous mode of operation. The run was achieved by a continuous withdraw of 250 mL from the culture and addition of 250 mL fresh YIW media every 24 h for 5 d, the withdrawn sample was employed for analysis purposes.

2.6 Analytical methods

Bacterial growth was estimated by calculating Colony Forming Units (CFU) using standard pour plate technique. Total carbohydrates concentrations were analyzed by phenol-sulfuric acid method and detected spectrophotometrically [24]. Amount of 50 µL of 80 % phenol solution was added to 50 µL of YIW, then vortexed and 2 mL concentrated sulphuric acid were added for 10 min. at room temperature. The absorbance was read at 490 nm. A standard curve was employed to determine the total carbohydrates concentrations.

Solvents (acetone, butanol, ethanol) concentration was determined using gas chromatography (Scion 456-GC, USA) equipped with a Flame Ionization Detector (FID) and column (Rxi-1ms, Restek, 15 m * 0.25 mm ID * 0.25 mm dp) and auto linear temperature programmer. The samples were assayed under the following conditions: the column was operated by increasing the temperature from 35 °C to 200 °C at a rate of 20 °C min⁻¹; injector temperature, 200 °C; FID temperature, 280 °C; carrier gas, nitrogen with flow rate of 1 mL min⁻¹. On the other hand, butyric and acetic acid concentrations were determined by HPLC (SHIMADZU, Japan) using Inertsil column (ODS-3, C18, 5 mm, 4.6*150 mm). The mobile phase consisted of 5 g KH₂PO₄ in HPLC water adjusted to pH 2.5 with concentrated H₃PO₄. Analysis consisted of a mobile phase flow rate of 0.5 mL min⁻¹, ambient column temperature (25 °C), and injection volume of 10 µL. 2.0 mL of each supernatant sample was filtered using 0.2 µm Millipore filters prior to injection into column. Absorbance was measured at a wavelength of 210 nm.

2.7 Statistical analysis

Data was calculated as mean ± standard deviation (SD) using 2010 Microsoft Excel. Statistical analysis tests were performed with SPSS version 21.0 statistic software package using one way Analysis of Variances (ANOVA) used to determine the significant difference of each factor. $P < 0.05$ was considered statistically significant [25, 26]

3 Results and discussion

3.1 Growth of *C. acetobutylicum* on yeast industry wastewater

Agar plates of YIW were prepared and inoculated with *C. acetobutylicum* in order to determine the optimum YIW concentration suitable for bacterial growth. The results showed that *C. acetobutylicum* was able to grow best in undiluted YIW cultures as indicated by the CFU value (Table 1). The CFU count for this culture was 1 925 (2.5 folds higher) compared to the control culture (anaerobic agar) which was 715.

Table 1. Effect of YIW concentration on *C. acetobutylicum* growth

YIW concentration	CFU/mL
0 % (control)	715 ± 49.49
25 %	887 ± 89.09
50 %	1 175 ± 70.71
75 %	1 675 ± 35.35
100 %	1 925 ± 77.78

Notes: Each value represent the mean ± SD of duplicate. The association between these variables is statistically significant ($P < 0.05$).

3.2 Butanol production in static liquid cultures of YIW inoculated with *C. acetobutylicum*

Butanol production by *C. acetobutylicum* grown in liquid static cultures of undiluted YIW was studied. The results presented in Figure 2 indicated that butanol was produced in low quantities (2 g L^{-1}) and the growth of bacteria was noticeably observed after 24 h of incubation as the OD_{600} of the culture was 1.73. To enhance the production process, glucose was added to the cultures at different concentrations. The best glucose concentration for solvent production and *C. acetobutylicum* growth was 16 g L^{-1} . The total carbohydrates (T.C.) consumption was clearly observed in the culture that contains 16 g L^{-1} of glucose where the value of T.C. was 7.52 g L^{-1} . The pH value nearly kept constant without significant decrease in all cultures which supported the growth of *C. acetobutylicum* but not butanol production (Figure 2). The maximum solvent concentration achieved was about 6 g L^{-1} . When the cultures containing 16 g L^{-1} glucose were further supplemented with different concentrations of ammonium acetate as a nitrogen source, the growth activity of *C. acetobutylicum* was enhanced as indicated by the increase in T.C. consumption at different rates (Figure 3). The best ammonium acetate concentration for bacterial growth was 10 g where the total carbohydrates consumption was 7.8 g L^{-1} , and pH value was 4.9 after 24 h.

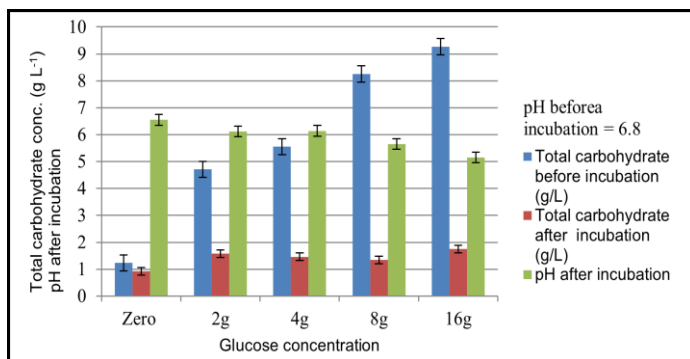


Fig. 2. Effect of glucose addition on growth of *C. acetobutylicum* that grown on YIW culture after 24 h of incubation. The association between these variables is statistically significant, ($P < 0.05$). Error bars indicate standard deviations from mean value.

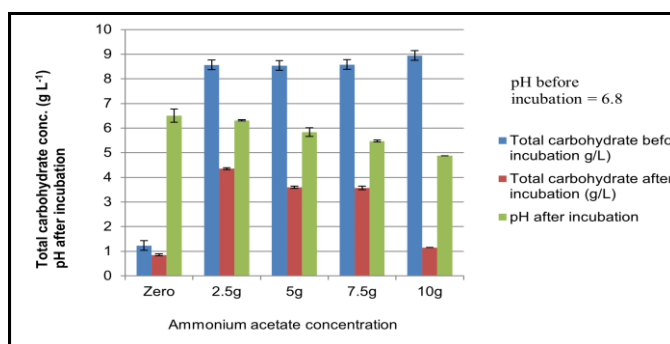


Fig. 3. Effect of ammonium acetate addition on growth of *C. acetobutylicum* that grown on YIW culture after 24 h of incubation. The association between these variables is statistically significant, ($P < 0.05$). Error bars indicate standard deviations from mean value.

To further elucidate the effect of C/N ratio on solvents production, another set of static bottle cultures were prepared with various C/N ratios. Figure 4 shows that the maximum butanol production was obtained in cultures supplied with 16 g of glucose where butanol concentration was 10.35 g L⁻¹, whereas the total ABE concentrations was 16.03 g L⁻¹, due to the total carbohydrate consumption that clearly observed in this culture. It seems that increasing glucose concentration would lead to enhance solvent production. Furthermore, the maximum acids (Butyric acid, Acetic acid) production (6.55 g L⁻¹) was obtained in cultures supplied with 16 g L⁻¹ of glucose (Table 2).

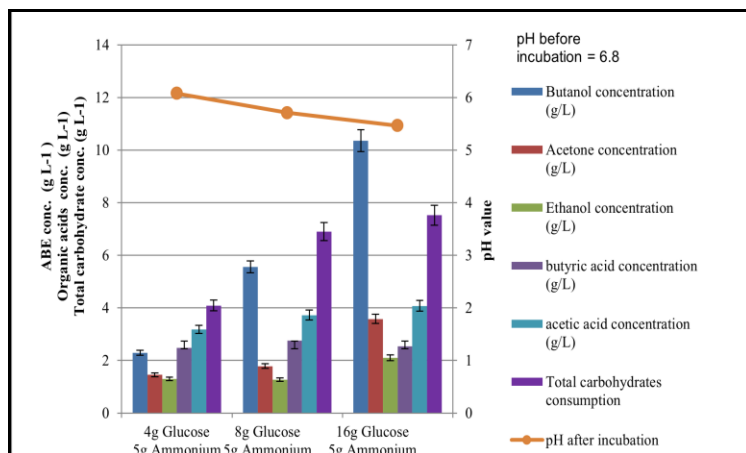


Fig. 4. Effect of glucose addition on butanol production from YIW culture after 24 h of incubation. The association between these variables is statistically significant, ($P < 0.05$). Error bars indicate standard deviations from mean value.

On the other hand, when the glucose concentration was kept constant at a concentration of 16 g L^{-1} , while ammonium acetate concentration was varied, the ABE production was slightly enhanced (Figure 5). Increasing ammonium acetate concentration from 10 g L^{-1} to 20 g L^{-1} was accompanied by a clear increase in butanol concentration from 9.99 g L^{-1} to 11.73 g L^{-1} considering that the best total carbohydrates consumption was 8.56 g L^{-1} in culture supplied with 20 g of ammonium acetate. Moreover, results indicate that the total ABE concentrations was 16.50 g L^{-1} , and the total acids (Butyric acid, Acetic acid) concentrations was 5.77 g L^{-1} as shown in Table 2.

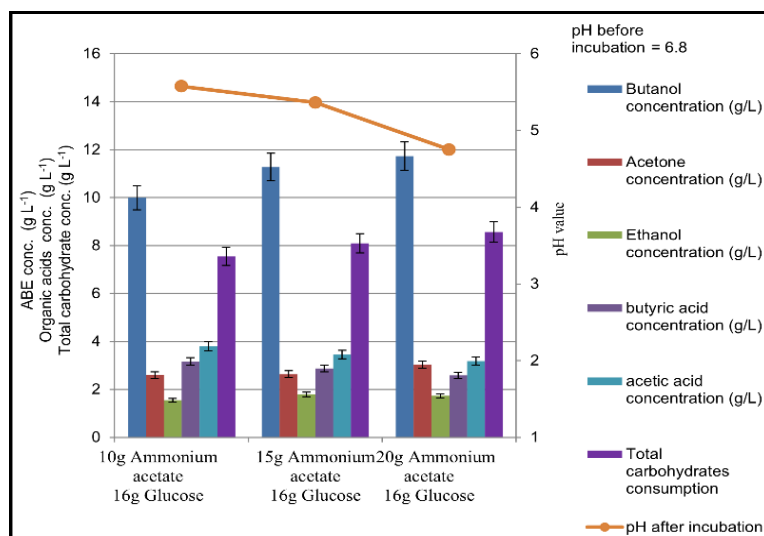


Fig. 5. Effect of ammonium acetate addition on butanol production from YIW culture after 24 h of incubation. The association between these variables is statistically significant, ($P < 0.05$). Error bars indicate standard deviations from mean value.

Table 2. Butanol production using YIW as a fermentation medium inoculated with free *C. acetobutylicum* at different concentrations of carbon and nitrogen.

Sample		T.C before incubation (g L ⁻¹)	T.C Consumption (g L ⁻¹)	Final pH	Solvent production (g L ⁻¹)			
					Acetone	Butanol	Etha-nol	ABE
Fermentation in flask scale	16 g Carbon, 5 g Nitrogen	9.27 ± 0.21	7.52 ± 0.24	5.47 ± 0.6	3.58 ± 0.14	10.35 ± 0.1	2.1 ± 0.06	16.03
	20 g Nitrogen, 16 g Carbon	9.42 ± 0.25	8.31 ± 0.17	4.750 ± 0.3	3.04 ± 0.14	11.73 ± 0.1	1.73 ± 0.08	16.5
Fermentation in column scale	After 24 h	15.31 ± 0.8	9.81 ± 0.19	5.49 ± 0.6	4.46 ± 0.15	8.38 ± 0.2	3.21 ± 0.1	16.05
	After 48 h		2.65 ± 0.17	5.91 ± 0.5	4.92 ± 0.13	6.2 ± 0.12	3.65 ± 0.2	14.77

Sample		T.C before incubation (g L ⁻¹)	T.C Consumption (g L ⁻¹)	Final pH	Acid production (g L ⁻¹)			Productivity ABE (g L ⁻¹ /h)	Yield Butanol (g L ⁻¹)
					Acetic	Butyric	T. Acids		
Fermentation in flask scale	16 g Carbon, 5 g Nitrogen	9.27 ± 0.21	7.52 ± 0.24	5.47 ± 0.6	4.01 ± 0.2	2.54 ± 0.21	6.55	0.67	0.64
	20 g Nitrogen, 16 g Carbon	9.42 ± 0.25	8.31 ± 0.17	4.750 ± 0.3	3.18 ± 0.12	2.59 ± 0.24	5.77	0.69	0.73
Fermentation in column scale	After 24 h	15.31 ± 0.8	9.81 ± 0.19	5.49 ± 0.6	2.21 ± 0.2	2.08 ± 0.21	4.29	0.69	0.33
	After 48 h		2.65 ± 0.17	5.91 ± 0.5	2.4 ± 0.12	2.3 ± 0.22	4.7	0.3	0.25

3.3 Butanol production in column bioreactor scale

Static bottle culture data was translated into column bioreactor for further enhancement of butanol production. The results showed that the maximum production of butanol and ABE in general was achieved after 24 h incubation (Figure 6). The solvents concentration was reduced by time which means that the bacteria was affected by high solvents concentration.

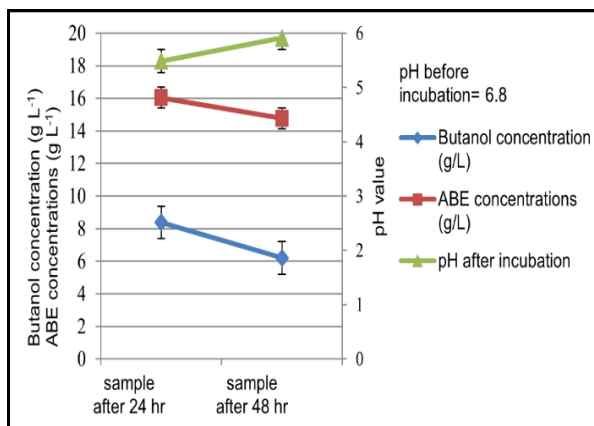


Fig. 6. Butanol production in column bioreactor scale at batch culture. Error bars represent the SD of duplicate. The association between these variables is statistically significant, ($P < 0.05$).

3.4 Butanol production by immobilized *C. acetobutylicum*

In order to reduce the stress effects of media and bacterial metabolite and hence, enhance butanol production, *C. acetobutylicum* was immobilized in sodium alginate beads to be used as inoculum. It was observed that the behavior of the cells to produce solvents was different than using free cells. The addition glucose was necessary for solvents production and *C. acetobutylicum* metabolism. There was a significant enhancement of solvents production compared to flask static culture (Table 3). The highest concentrations of butanol and ABE obtained were 11.24 g L^{-1} and 17.42 g L^{-1} , respectively. The results also showed that the total carbohydrates consumption of culture supplied with 16 g of glucose were comparable for both 8 g and 4 g. Therefore, the best total carbohydrates consumption for butanol production was 8.1 g L^{-1} (Figure 7).

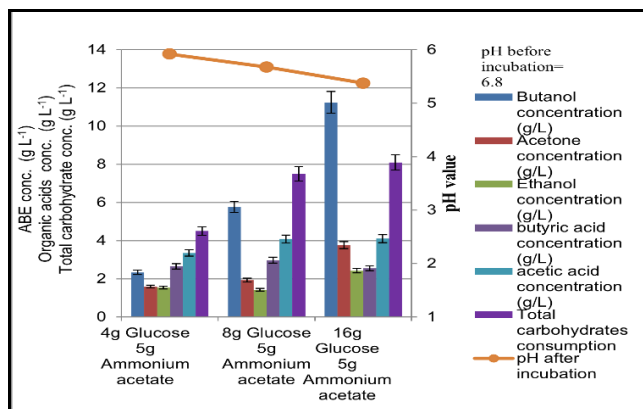


Fig. 7. Effect of glucose addition on butanol production from YIW culture that inoculated with immobilized cells after 24 h of incubation. The association between these variables is statistically significant, ($P < 0.05$). Error bars indicate standard deviations from mean value.

Furthermore, when the culture was supplied with a fixed glucose concentration of 16 g L⁻¹ and different concentrations of ammonium acetate, the maximum butanol production was obtained with 20 g L⁻¹ of ammonium acetate where butanol concentration was enhanced slight to reach 12.47 g L⁻¹ with a total ABE concentration of 17.55 g L⁻¹ (Figure 8).

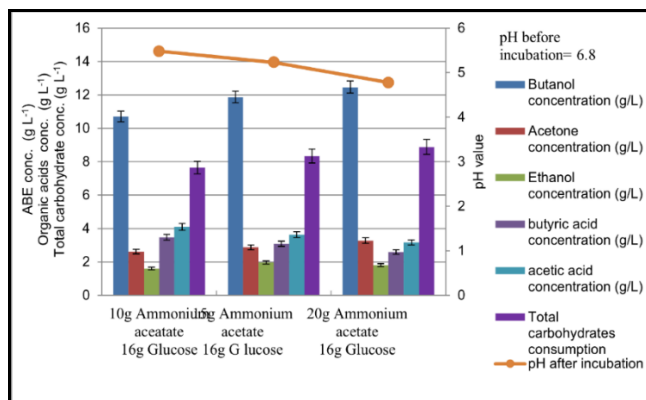


Fig. 8. Effect of ammonium acetate addition on butanol production from YIW culture after 24 h of incubation. The association between these variables is statistically significant, ($P < 0.05$). Error bars indicate standard deviations from mean value.

3.5 Butanol production by immobilized *C. acetobutylicum* in column bioreactor

The process parameters for solvents production were translated into the use of column bioreactor scale immobilized with bacteria. Figure 9 shows that there was an enhancement in solvents production as the maximum concentrations of butanol and ABE were obtained (13.75 g L⁻¹ and 22.72 g L⁻¹, respectively) after 48 h of fermentation. Solvents and organic acids production were significantly increased within 48 h due to immobilization of bacterial cells (Table 3).

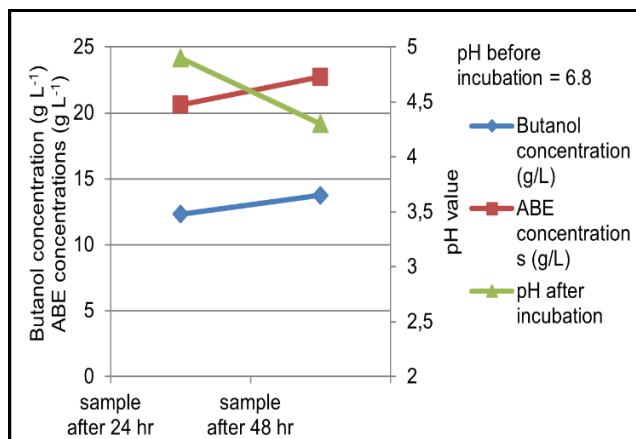


Fig. 9. Butanol production in column bioreactor scale from YIW inoculated with immobilized cells in batch culture.

Table 3. Butanol production using YIW as a fermentation medium inoculated with immobilized *C. acetobutylicum* at different concentrations of carbon and nitrogen.

Sample		T.C before incubation (g L ⁻¹)	T.C consumption (g L ⁻¹)	Final pH	Solvent production (g L ⁻¹)			
					Acetone	Butanol	Ethanol	ABE
Fermentation in flask scale	16 g Carbon, 5 g Nitrogen	9.27 ± 0.2	8.62 ± 0.08	5.38 ± 0.2	3.76 ± 0.14	11.24 ± 0.2	2.42 ± 0.1	17.42
	20 g Nitrogen 16 g Carbon	9.42 ± 0.2	9.11 ± 0.05	4.78 ± 0.2	3.27 ± 0.15	12.47 ± 0.2	1.81 ± 0.4	17.55
Fermentation in column scale	After 24 h	19.82	10.21	4.9	5.04	12.31	3.21	20.61
	After 48 h		9.31	4.3	5.32	13.75	3.65	22.72

Sample		T.C before incubation (g L ⁻¹)	T.C Consumption (g L ⁻¹)	Final pH	Acid production (g L ⁻¹)			Productivity ABE g L ⁻¹ /h	Yield butanol g L ⁻¹
					Acetic	Butyric	T. Acids		
Fermentation in flask scale	16 g Carbon, 5 g Nitrogen	9.27 ± 0.2	8.62 ± 0.08	5.38 ± 0.2	4.1 ± 0.2	2.55 ± 0.14	6.65	0.72	0.7
	20 g Nitrogen 16 g Carbon	9.42 ± 0.2	9.11 ± 0.05	4.78 ± 0.2	3.17 ± 0.1	2.6 ± 0.12	5.77	0.73	0.78
Fermentation in column scale	After 24 h	19.82	10.21	4.9	6.12	5.32	11.44	0.85	0.5
	After 48 h		9.31	4.3	6.32	5.95	12.27	0.94	0.55

When the process mode of operation was changed from batch to continuous production, the production of metabolites was enhanced and the activity of clostridial cells was noticeably improved. Solvent's production recorded a maximum concentration of 34.85 g L⁻¹ after 96 h of fermentation (Figure 10). It was observed that as the time of fermentation was increased, the production of ABE significantly increased. The recorded values of ABE were a result of clostridial cells enhancement after the addition of some metal ions of ferrous and magnesium in the form Fe₂SO₄.7H₂O and MgSO₄.7H₂O, respectively.

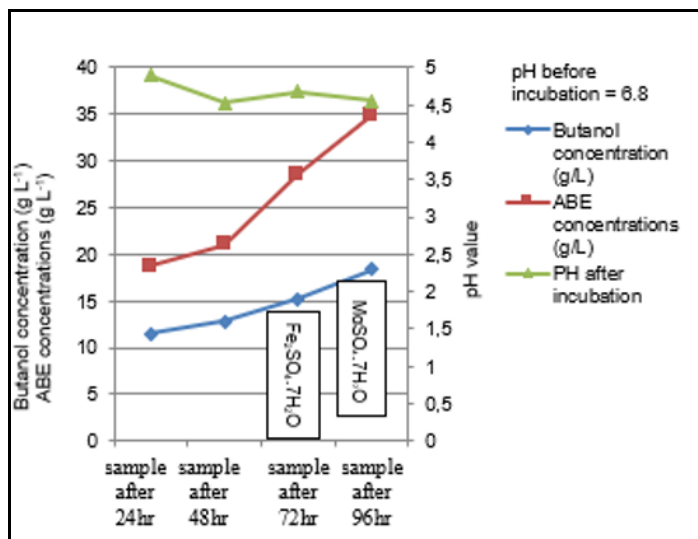


Fig. 10. Continuous butanol production by immobilized *C. acetobutylicum* grown on YIW in column bioreactor.

Yeast Industry Wastewater in Jordan is produced on a daily flowrate of 200 m³ without any valorization and treatment. The waste quantity has a high polluting power, therefore, the use of YIW as a medium for production solvents and chemicals etc. might solve part of the problems related to pollution. The utilization of YIW by *C. acetobutylicum* at various YIW concentrations in agar plates was assessed. The results revealed a significant decrease in bacterial growth in case of diluted YIW culture. *C. acetobutylicum* maximum growth was observed in agar plate containing undiluted YIW. Therefore, undiluted YIW could be appropriate for the metabolism, growth and production of bacteria. These results are in agreement with Mustafa who concluded that undiluted YIW culture was the most suitable for growth of *Saccharomyces cerevisiae* to produce ethanol [20]. Moreover, Saleem concluded that undiluted YIW was suitable for the growth of *Aspergillus niger* for the production of citric acid [22]. This could be due to the availability of high amounts of carbon, organic compounds and essential elements for growth such as: N, Fe, Ca, Cl, Mg and Na. Meanwhile dilution of YIW would reduce the organic load of materials necessary for *Clostridium* growth and metabolism.

Production of solvents, especially butanol, is manifestly influenced by C/N ratio [27]. Besides shifting the metabolic stage of the culture due to a decrease in medium pH, carbon and nitrogen is also utilized by the cells as substrate in the formation of butanol. Furthermore, the regulation of carbon and nitrogen amounts in the culture media is of great industrial importance. Therefore, in fermentation medium, the C/N ratio has a key role in butanol production using solvent-producing *Clostridium*. In this study, glucose as a carbon source was used and its concentration was varied in the range of 4 g L⁻¹ to 6 g L⁻¹. The aforementioned results indicated that a glucose concentration of 16 g L⁻¹ was optimal for the production of butanol which is in agreement with Madihah *et al.*, who reported that butanol production would increase by excess amount of carbon sources. Furthermore, the effect of C/N ratio on bacterial growth, and butanol production was investigated [28]. The results showed that the best concentration of C / N was 16 g, 20 g, where the best concentration of butanol was 10.35 g L⁻¹ and 11.73 g L⁻¹, respectively. This indicates the necessity of carbon and nitrogen addition to increase productivity of butanol during production process. Bochman *et al.*,

claimed that the butanol could be produced when the carbon and nitrogen source in media is glucose and ammonium acetate with appropriate concentration [29].

Obstacles such as low productivity and high costs of material separation have been significantly restricted in commercial conversion of biomass or waste industry effluents such as YIW into biobutanol. In this study, immobilization of *C. acetobutylicum* was used to promote biobutanol production. The strategy used here has the merit to improve total butanol production with respect to traditional culture approach by more than two folds. Additionally, this approach provided high butanol concentration in solvatogenic phase, without inhibition of bacterial growth. The technique can simultaneously improve butanol production and reduce separation cost [30]. The results of experiments conducted through the immobilized bacteria showed further development compared with free cells results. This could be due to resistance of *C. acetobutylicum* in beads toxicity of solvents, thus, enhancing in butanol production. Huang found that the bioreactor immobilized cells enhanced the yield of butanol by more than 68 %, but the technique did not couple with extraction which limited further improvement in butanol production [18]. Immobilized *C. acetobutylicum* were used in continuous fermentation. The maximum concentration of butanol in batch fermentations for *C. acetobutylicum* was 12.47 g L⁻¹ while, the continuous fermentation started the production with 11.81 g L⁻¹. This concentration was achieved at the moment when the final pH of culture after 24 h was 4.9 which could be due to increased acid production (butyric and acetic acid). After 48 h, the production of butanol reached to 12.82 g L⁻¹ by the time the culture was supplied with Fe₂SO₄.7H₂O that enhanced production. Iron is an important mineral supplement since the conversion of pyruvate to acetyl-CoA involves a ferredoxin oxidoreductase iron-sulfur protein [31]. Our results showed significant increase in butanol production where the concentration reached 15.23 g L⁻¹ after 72 h. when the culture was further supplied with MgSO₄.7H₂O, more butanol was achieved to score 18.35 g L⁻¹ for butanol and 34.85 g L⁻¹ for total ABE which further assures the importance for supplying the cultures with cofactors.

4. Conclusions

The data presented in this study shows that YIW is a suitable medium for the production of butanol through the use of *C. acetobutylicum*. Butanol production was studied in batch mode and continuous mode of operation. The maximum butanol production in batch culture with free cells was 11.23 g L⁻¹ and immobilized cells was 12.47 g L⁻¹, whilest, in continuous culture was 18.35 g L⁻¹ and could be doubled by supplying the cultures with ferrous and magnesium ions. The best glucose and ammonium acetate concentrations for butanol production were 16 g, 20 g, respectively. The main advantage of using YIW for the production of butanol is dropping its dangerous effect on the environment and improving its ecological impact.

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