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Effect of Organic Substrate on Promoting Solventogenesis in Ethanologenic Acetogene *Clostridium Ljungdahlii* ATCC 55383

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ABSTRACT

Clostridium ljungdahlii is a strictly anaerobic acetogene known for its ability to ferment a wide variety of substrates to ethanol and acetate. This bacterium presents a complex anaerobic metabolism including the acetogenic and solventogenic phases. In this study, the effect of various carbon sources on triggering the metabolic shift toward solventogenesis was considered. The bacterium was grown on fructose, glucose, acetate and ethanol in batch cultures. The fermentation results demonstrated that fructose improved ethanol production (27.1 mM) over acetate (26.3 mM), but glucose was predominantly metabolized to acetate. The bacterial cells were able to either utilize or produce ethanol (25 mM) probably through different metabolic pathways. The presence of acetate as the carbon source in the culture shifted the metabolic pathway of the cells toward solventogenic phase, but the amount ethanol formation was not considerable (3.5 mM). It was also attempted to improve ethanol production yield by varying the fructose concentration (1 to11 g/L) in the batch culture. Under the conditions of substrate depletion or high fructose concentrations, the cell growth declined and the metabolic pathway of *C. ljungdahlii* was unable to switch from acetogenesis to solventogenesis. The fructose concentration of 5 g/L was found as the suitable concentration to yield an ethanol to acetate molar ratio of 1:1.

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1. INTRODUCTION

Production of ethanol from starch, cellulose or hemicellulose through bio-chemical route is by far the most mature process for industrial production of ethanol [1]. In this conversion process, the starting plant material is degraded to hexose and pentose for further fermentation to ethanol. Two classes of microorganisms are well known for this conversion with a high ethanol production yield, very close to the theoretical value, namely *Saccharomyces cerevisiae* (yeast) and members of the genus *Zymomonas* such as *Z. mobilis* (bacteria). *S. cerevisiae* generates pyruvate through the Embden-Meyerhof- Parnas pathway (glycolysis) and *Z. mobilis* uses the Entner-Doudoroff pathway to produce pyruvate from carbohydrates which is further converted to alcohol [2].

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As a promising alternative to the bio-chemical conversion process, thermo-chemical conversion route which is the indirect fermentation process for ethanol production has pinned a lot of hope [3]. In this approach, gasification or pyrolysis of starting material which generates synthesis gas (syngas) is integrated with the fermentation process to generate various biofuels from syngas. Acetogenes which are strictly anaerobic microorganisms are usually implemented to perform this fermentation process. These obligate anaerobic bacteria are able to grow chemolithotrophically on syngas components i.e. CO and CO_2/H_2 and ferment them through the Wood-Ljungdahl pathway to volatile fatty acids and alcohols under ambient temperature and pressure [2, 4-6]. For this purpose, various *Clostridia* and *Moorella* strains have been isolated [2].

Acetogenes present a complex metabolism which involves both the acetogenic and solventogenic phases. These bacteria produce acid during their exponential

growth phase and then shift to alcohol production as the growth rate slows down and the cells enter the stationary phase [7-9]. This complex metabolic pathway of acetogenes has attracted considerable attention among researchers to determine the parameters affecting the transition between acetogenic and solventogenic phases. There is a hypothesis that a switch from acidogenesis to solventogenesis in homoacetogenic bacteria may be achieved in non-growth condition. Gaddy and Clausen [10] demonstrated that *C. ljungdahlii* produced ethanol in preference to acetate under non-growth condition of pH: 4 to 4.5 and without yeast extract, whilst optimum growth condition (pH: 5 to 7) promoted acetate production. Klasson et al. [11] studied the feasibility of producing ethanol in favor of acetate in the batch culture of *C. ljungdahlii* containing various amounts of reducing agent (30, 50 and 100 ppm). They concluded that use of reducing agents caused a slower growth of bacteria due to the reduced ATP formation; however the product ratio was promoted. They reported the highest ethanol to acetate ratio of 1:1 with benzyl viologen at the concentration of 30 ppm. In another effort to trigger the non-growth condition to improve ethanol production, Phillips et al. [12] reduced the B-vitamin concentration and eliminated yeast extract from the media, in continuous cultivation of *C. ljungdahlii*. They observed that the cell growth slightly reduced, however ethanol was the predominant product. Maximum ethanol to acetate molar ratio of 21:1 was achieved with cell recycling.

Some of the acetogenic members of the *Clostridium* genus are also capable to grow chemoorganotrophically on hexose sugars and ferment them to end products such as acetate, butyrate and ethanol. For heterotrophic growth of the bacterium, fructose and glucose are fermented to pyruvate through the glycolytic pathway. Then, the oxidative conversion of pyruvate generates acetyl-CoA which is a versatile intermediate in the metabolic pathway of acetogenes. The bacteria utilize the generated acetyl-CoA for cell material and energy production through the anabolic and catabolic pathways, respectively. In the anabolic pathway, a minor portion of acetyl-CoA is reductively carboxylated to generate pyruvate in the presence of pyruvate: ferredoxin oxidoreductase. Then, the pyruvate is converted to phosphoenolpyruvate, which is an intermediate to form the cell materials [13, 14]. The growth of *C. ljungdahlii* on syngas was compared to fructose by Cotter et al. [15]. A dense bacterial culture (1 g/L) was obtained with fructose compared to syngas (0.562 g/L). Also, a higher ethanol concentration (13 mM) was achieved with fructose in comparison to syngas (3.8 mM). They also reported that the cells pre-cultured on fructose and syngas produced a bacterial culture with the concentration of 0.850 and 0.562 g/L, during the syngas fermentation. Such difference in culture performance was probably due to the greater availability of intracellular cofactors, enzymes and maintenance energy in cells pre-adapted to fructose.

Previously, Tanner et al. [16] in the original study of *C. ljungdahlii*, while isolating the organism from natural sources, examined around 30 potential substrates for possible growth of the bacterium. However, the focus of that work was to establish the substrates capable of supporting the growth of the bacterium and culture density and product formation were not considered. Notice of little availability of data in the literature regarding the cultivation and fermentation of this acetogene which is partially due to the difficulty in cultivating this strictly anaerobic bacterium boosts the importance of research in this area. In this study, the batch cultivation of *C. ljungdahlii* was investigated using various organic substrates. The effect of fructose, glucose, ethanol and acetate as organic substrates on growth and product distribution of the bacterium in the acetogenic and solventogenic phases was assessed. The effect of fructose concentration as the best studied substrate to promote solventogenesis over acetogenesis was also considered.

2. MATERIALS AND METHODS

2. 1. Microorganism and Growth Medium *Clostridium ljungdahlii* (ATCC 55383) was grown anaerobically in a medium containing mineral salts, vitamins and trace metals from ATCC 1754 PETC medium. The basal medium contained (per 1.0 L) 5.0 g organic substrate, mineral salts, 1.0 g yeast extract, 10 mL trace elements solution, 10 mL vitamins solution and 10 mL reducing agent. Fructose, glucose, ethanol and acetate were used as organic substrates in separate experiments. The PETC salts contained (per 1.0 L): NH_4Cl (1.0 g), KCl (0.1 g), $MgSO_4.7H_2O$ (0.2 g), NaCl (0.8 g), KH_2PO_4 (0.1 g) and $CaCl_2.2H_2O$ (20.0 mg). The trace elements solution contained (per 1.0 L): nitrilotriacetic acid (2.0 g), $MnSO_4.H_2O$ (1.0 g), $Fe(SO_4)_2$ $(NH_4)_2.6H_2O$ (0.8 g), $CoCl_2.6H_2O$ (0.2 g), $ZnSO_4.7H_2O$ (0.2 mg), $CuCl_2.2H_2O$ (20.0 mg), $NiCl_2.6H_2O$ (20.0 mg), $Na_2MoO_4.$ $2H_2O$ (20.0 mg), Na_2SeO_4 (20.0 mg) and Na_2WO_4 (20.0 mg).The vitamins solution contained (per 1.0 L): biotin (2.0 mg), folic acid (2.0 mg), pyridoxine hydrochloride (10.0 mg), thiamine- HCl (5.0 mg), riboflavin (5.0 mg), nicotinic acid (5.0 mg), calcium D-(+)-pantothenate (5.0 mg), vitamin B12 (0.1 mg), p-Aminobenzoic acid (5.0 mg) and thioctic acid (5.0 mg). Reducing agent solution contained (per 100 mL): NaOH (0.9 g), L-Cysteine-HCl (4.0 g) and $Na_2S.9H_2O$ (4.0 g).

2. 2. Batch Fermentation Experiments The medium (excluding organic substrate and reducing agent) was prepared, boiled and dispensed anaerobically under nitrogen atmosphere into Wheaton serum bottles

(Borosilicate glass, Fischer Scientific, UK). Each 163 mL serum bottle contained 50 mL of liquid medium. The reducing agent, fructose, glucose, ethanol and acetate solutions were prepared in separate serum bottles. All bottles were autoclaved at 121°C for 15 min. The sterile cooled medium in each serum bottle was reduced by addition of reducing agent. The organic substrates were also added to the media in separate serum bottles. The pH of the media was adjusted to 5.9 using 1 M HCl or NaOH. Nitrogen was flushed into the bottles before the inoculation to ensure the anoxic condition. Each bottle was inoculated with 2.5 mL aliquot of a growing culture and incubated at 37°C in a shaking incubator at 150 rpm. Samples were taken from the serum bottles using a sterile syringe for 120 h and analyzed for optical density, substrate and product concentrations.

2. 3. Cell Density and Product Analysis Optical density of the samples was analyzed using a spectrophotometer (Thermospectronic, USA) at 580 nm, and the cell dry weight was determined using a pre-developed calibration curve. DNS reagent was used to determine the fructose and glucose concentration based on the spectrophotometric method [17]. Ethanol and acetate were used as organic substrates in separate bacterial cultures. They were also produced along with trace amount of acetone by the bacteria. Detection of these compounds was performed using a gas chromatograph (Agilent, 5890 series II), equipped with a flame ionization detector (FID). The samples (500 µL) were acidified with concentrated formic acid (15 µL) and 2-pentanone (15 µL) was added as internal standard. The chromatography column was 80/120 mesh Carbopack B-DA/4% Carbowax 20M (Supelco, USA). The oven temperature was initially set at 50 °C for 2 min and then ramped at a rate of 10°C/min to 175°C and hold at this temperature for 1 min. Helium was used as the carrier gas and the injector and detector temperatures were set at 150 and 225°C, respectively.

3. RESULTS AND DISCUSSION

3. 1. Effect of Organic Substrate *3.1.1. Cell growth and substrate consumption* Batch cultivation of *C. ljungdahlii* was carried out using fructose, glucose, ethanol and acetate as organic substrates. *C. ljungdahlii* has the ability to either produce ethanol or consume it for growth [16, 18]. The utilization and generation process of ethanol proceed through different mechanisms via acetyl-CoA and acetaldehyde [18]. It has been reported that no growth was observed while growing *C. ljungdahlii* on Na-acetate [19], however the result of this investigation was not in agreement with findings of others. The bacteria were able to either utilize or produce acetate. The growth profiles of *C. ljungdahlii* using fructose, glucose, ethanol and acetate and the corresponding substrate consumptions are presented in Figure 1 (A)-(D), respectively. The growth yields of *C. ljungdahlii* were 45.46, 41.82, 3.76 and 5.62 g cell per mol of fructose, glucose, ethanol and acetate, respectively. The consumption of fructose and glucose by the bacteria was very fast. A substrate conversion of 89 and 79% was achieved at 24 h for fructose and glucose. The utilization of ethanol as the substrate was slow and continued to 96 h which corresponds to the low culture density in comparison to fructose and glucose. A substrate conversion of 56% was achieved while using ethanol as the substrate. Acetate consumption initiated at the early stage of the cultivation and ceased after 24 h with 62% of substrate conversion.

3. 1. 2. Product Formation The generated acetyl-CoA follows the catabolic pathway to make ATP to provide the required energy for cell maintenance. In the catabolic pathway, acetyl-CoA is utilized as a precursor for the biosynthesis of volatile fatty acids and alcohols. During the batch growth of acetogenes, the bacteria exhibit a biphasic fermentation pattern, including acidogenic and solventogenic phases [9]. The bacteria produce volatile fatty acids such as acetate and butyrate at the initial stage of the exponential growth phase. When acid production reached a certain threshold and the culture pH decreased, the metabolic pathway of the bacteria switched to solvent production, mainly ethanol and butanol, shortly before entering the stationary phase. Utilization of ethanol as the substrate can proceed via acetaldehyde/ alcohol dehydrogenase in two steps, where ethanol is first converted to acetaldehyde and then to acetyl-CoA [18]. Acetate as the organic substrate can be either directly converted to acetyl-CoA by acetyl-CoA synthesas or reduced to acetaldehyde by aldehyde oxidoreductase with reduced ferredoxin and then to acetyl-CoA. However, the consumption of acetate was faster than ethanol; thus, it was speculated that acetate was directly converted to acetyl-CoA. The generated acetyl-CoA in the junction of catabolic and anabolic pathway is an ideal precursor for the synthesis of the cell materials, as well as ethanol and acetate. In the catabolic pathway, the acetyl-CoA (CH_3COS-CoA) is converted to acetate via formation of acetyl-phosphate (CH_3COO-P_3^{2-}) as an intermediate in the presence of phosphotransacetylase [13, 14]:

$$CH_3COS\text{-}CoA+Pi \rightarrow CH_3COO\text{-}P_3^{2-}+HS\text{-}CoA \quad (1)$$

Then, the acetyl-phosphate is transformed to acetate by acetate kinase, while a molecule of ADP is phosphorylated to ATP:

$$CH_3COO\text{-}P_3^{2-}+ADP \rightarrow CH_3COOH+ATP \quad (2)$$

For production of ethanol, NADH is utilized by the organism to form acetaldehyde (CH_3CHO) in the presence of acetaldehyde dehydrogenase:

$$CH_3COS\text{-}CoA + NADH + H^+ \rightarrow CH_3CHO + NAD^+ + HS\text{-}CoA \quad (3)$$

$$CH_3CHO + NADH + H^+ \rightarrow CH_3CH_2OH + NAD^+ \quad (4)$$

Finally, alcohol dehydrogenase catalyzes the conversion of acetaldehyde to ethanol. The conversion of acetyl-CoA to metabolic end products is a well researched pathway for most acetogenes.

The batch growth of *C. ljungdahlii* was accompanied by acetate and ethanol and a trace amount of acetone production according to the following reactions:

$$Fructose + 2ADP + 2P_i \rightarrow 2ethanol + 2ATP + 2CO_2 \quad (5)$$

$$Fructose + 3ADP + 3Pi \rightarrow 3acetate + 3ATP \quad (6)$$

The distribution of products while using fructose as the substrate is presented in Figure 1 (A). During the exponential growth phase, acetate was produced then the metabolic pathway shifted to solvent production mainly ethanol and trace amount of acetone. A sharp decrease in pH of the culture from 5.9 to 4.5 was observed after 6 h which was due to the acid production. After 24 h, the acetate concentration reached to 24.5 mM and this concentration remained almost constant throughout the batch experiment. Ethanol production initiated during the exponential growth phase and pronouncedly increased during the stationary phase of the cell growth. Due to the acetyl-CoA pathway, the production of ethanol gives rise to the ATP consumption which would not support the cell growth [11, 20]. Hence, ethanol production is promoted in non-growth condition. Maximum ethanol concentration of 27.1 mM was achieved after 120 h.

Figure 1 (B) shows the acetate, ethanol and acetone production trends of *C. ljungdahlii* grown on glucose. Although the bacterial culture was able to maintain high cell density in the non-growth phase, however ethanol production was not considerable (less than 5.6 mM). A small amount of acetone was also formed in the culture. In contrast, a high concentration of acetate (44.8 mM) was produced as the main end product. There was an indication of switch from solventogenesis to acetogenesis at 72 h, probably because of the ATP demand of the cells which increased acetate production. These results demonstrate that glucose was predominantly metabolized to acetate by *C. ljungdahlii* and there was a little flow of carbon from glucose to ethanol.

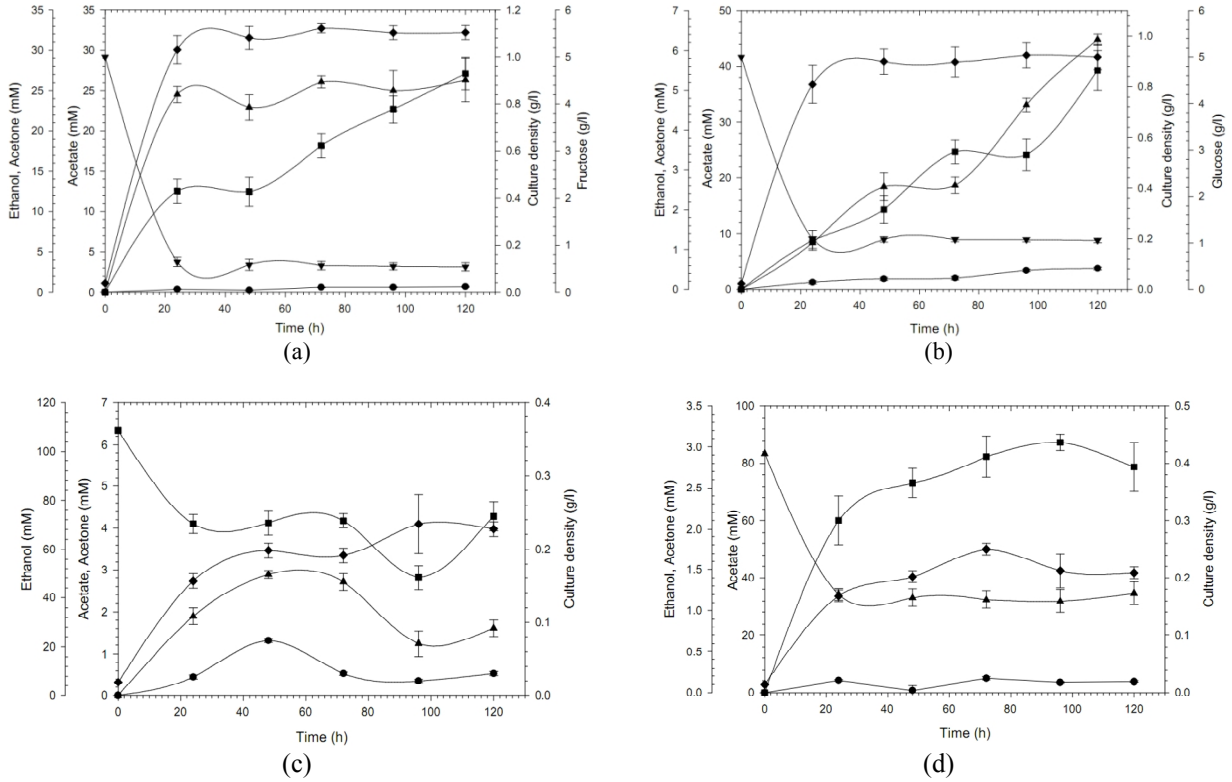

Figure1. Culture density, substrate consumption and product evolution profile of *C. ljungdahlii* using (A) fructose, (B) glucose, (C) ethanol and (D) acetate, ▲: acetate, ■: ethanol, ●: acetone, ♦: culture density, ▼: substrate consumption. The experiments were repeated three times and error bars show the standard deviation

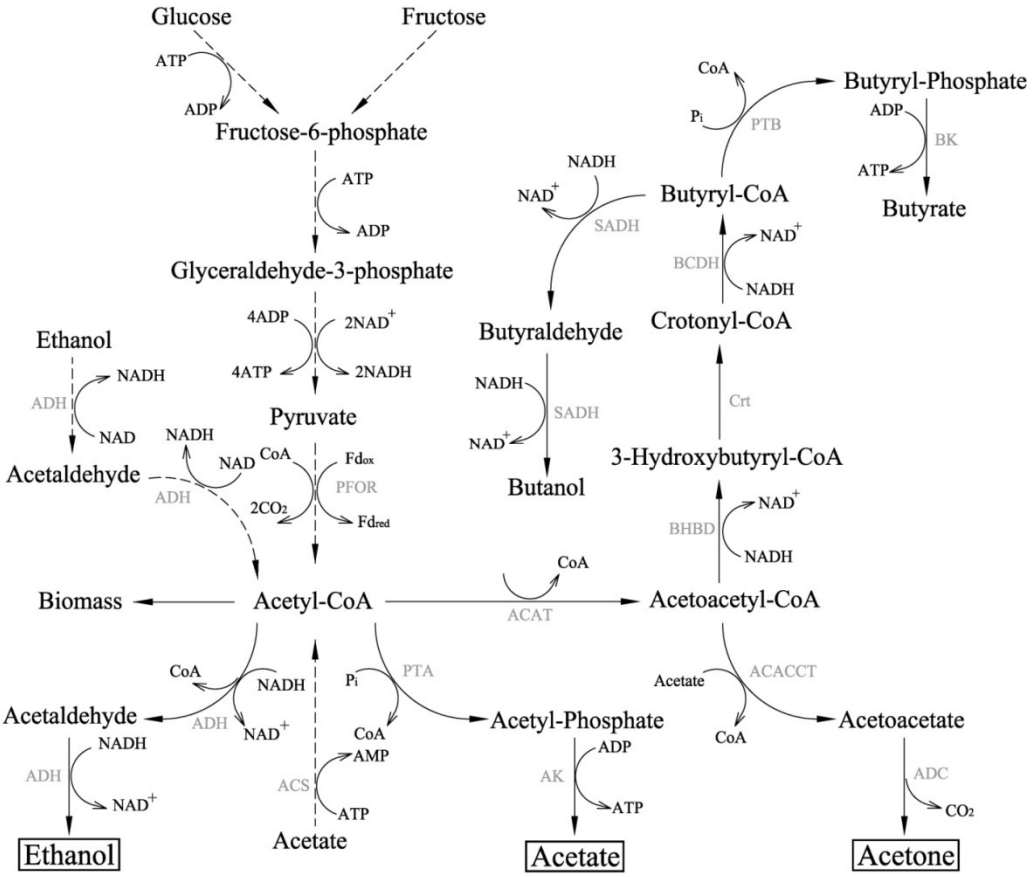

Figure 2. The proposed metabolic pathway of *C. ljungdahlii* for heterotrophic growth and product formation. Glucose and fructose are metabolized through the Embden-Meyerhof-Parnas (glycolysis) pathway. Utilization of ethanol and acetate can proceed via acetyl- CoA. The enzymes involved in reactions are summarized as: ACACCT, acetyl-CoA:acetoacetyl-CoA transferase; ACAT, acetyl-CoA acetyltransferase; ACS, acetyl-CoA synthase; ADC, acetoacetate decarboxylase; ADH, acetaldehyde/alcohol dehydrogenase; AK, acetate kinase; BCDH, butyryl-CoA dehydrogenase; BHBD,β-hydroxybutyryl-CoA dehydrogenase; BK, Butyrate kinase; Crt, crotonase; PFOR, pyruvate:ferredoxin-oxidoreductase; PTA, phosphotransacetylase; PTB, phosphotransbutyrylase; SADH, secondary alcohol dehydrogenase

The effect of ethanol as the organic substrate on product distribution of *C. ljungdahlii* was studied in the batch culture and the result of this investigation are projected in Figure 1 (C). After the initial utilization of ethanol by the cells, the consumption ceased and a small amount of acetate was formed in the culture. The amount of acetate formed in the culture was so small (2.7 mM) that could not effectively reduce the pH of the culture. Since the bacterial cells were still within their growth pH range (5 to 7), more ethanol as the substrate was consumed after 72 h of cultivation. Then, ethanol production by the bacterial cells initiated as the cells entered the stationary growth phase and 25 mM ethanol was formed in the culture at 120 h. The production of ethanol could precede using acetyl-CoA and utilization of reducing equivalent (NADH) to form acetaldehyde in the presence of acetaldehyde dehydrogenase followed by conversion of acetaldehyde to ethanol by alcohol dehydrogenase (Figure 2). The generated acetate could also be reduced to acetaldehyde by aldehyde oxidoreductase with reduced ferredoxin and result in ethanol formation [18].

The product distribution using acetate is shown in Figure 1 (D). Acetate was utilized as the organic substrate for the bacterial cell growth. However, acetate utilization stopped after 24 h and ethanol production started. Ethanol and a trace amount of acetone were the only metabolites of the supplied acetate. The most probable pathway for such observation is the conversion of acetate to acetyl-CoA in the presence of acetate kinase and phosphotransacetylase and then reduction of acetyl-CoA to ethanol by alcohol dehydrogenase [21]

$$\text{Acetate} + 2\text{NADH} + \text{ATP} \rightarrow \text{ethanol} + 2\text{NAD}^+ + \text{ADP} + \text{Pi} \quad (7)$$

Although the presence of acetate in the culture shifted the metabolic pathway of the cells to ethanol production

phase, but ethanol formation was not considerable (around 3.5 mM). Monitoring the culture pH revealed an increase in the pH from 5.9 to around 6.8 after 24 h which was not a suitable pH for ethanol production. A pH of 4.0 to 4.5 which creates non-growth condition has been reported as the appropriate pH for ethanol production using *C. ljungdahlii* [10].

Table 1 summarizes the kinetic parameters of the substrate utilization, cell growth and product formation of the bacterium using various organic substrates. The highest yield of cells from substrate ($Y_{X/S}$= 45.46 g/mol) and substrate conversion (88.69%) was achieved with fructose. It also resulted in equimolar production of ethanol and acetate (EtOH/Ac= 1.03 mol/ mol). The experimental yield of product with glucose ($Y_{P/S,\ exp}$= 2.29 mol/mol) was close to the theoretical value (2.6 mol/mol); however, acetate was the dominant end product and only a small amount of ethanol was formed in the culture (EtOH/Ac= 0.12 mol/mol). The growth of the bacterium on ethanol was slow and weak ($Y_{X/S}$= 3.76 g/mol), but the yield of product from biomass ($Y_{P/X}$= 117.89 mmol/g) was relatively considerable. Although a high ethanol to acetate ratio of (EtOH/Ac= 16.65 mol/mol) was achieved with this substrate, but the level of ethanol production (25 mM) was almost low compared to the ethanol utilization (60 mM) during the batch experiment of 120 h. Acetate was quite unsuccessful for product formation ($Y_{P/S,\ exp}$= 0.07 mol/mol) and could partially support the growth of the bacterium ($Y_{X/S}$= 5.62 g/mol). The results suggested fructose as the suitable organic substrate for further experiments.

3.2. Effect of Fructose Concentration Investigation on the effect of various organic substrates on growth and product formation of *C. ljungdahlii* showed that fructose was able to create a dense culture and successfully shifted the metabolic pathway of the bacterium toward solventogenesis in non-growing cells. It was attempted to improve the ethanol production capability of the bacterium over acetate using various concentrations of fructose. To this end, several concentrations of fructose (1, 3, 5, 7, 9 and 11 g/L) in the media were implemented and their effects on culture density and product formation constantly monitored.

3. 2. 1. Cell Growth The profile of cell growth with various substrate concentrations was described by the Volterra model which is used to predict the population growth in a batch system. This model accommodates the birth and death of the cells and considers them as the only parameters which change the population in a closed system [22, 23]. Based on this theory, the following model was developed:

$$x = \frac{x_0 e^{\mu_m t}}{1-(x_0/x_m)^2(\mu_m/(k+\mu_m))[1-e^{(k+\mu_m).t}]} \quad (8)$$

where, x_0 is the initial cell concentration (g/L), x_m the maximum cell density (g/L), μ_m the maximum specific growth rate (1/h), t the fermentation period (h) and k the cell decline or promotion constant (1/h).

Figure 3 shows the Volterra model applied to the experimental data. The model fitted to the experimental results with high regression coefficients (R^2). Sigma Plot 11 was used to calculate the coefficients of the equation. The kinetic parameters and coefficients of this model are tabulated in Table 2.

The constant k which is obtained from curve fitting is a parameter associated with the decline or promotion of the cell population. The value of k is positive if the cell population is declining by toxic by-products or depletion of the nutrient supply and the value is negative if the cell population is promoted. A positive value of k was achieved in all cases, except for fructose concentration of 5 g/L. This indicates that in the range of fructose concentrations used, except at 5 g/L, the bacterial cells were declined after reaching a maximum cell concentration. Only at fructose concentration of 5 g/L, the culture was able to maintain its viability for a long period of time.

TABLE 1: Kinetic parameters based on Volterra model for growth of *C. ljungdahlii*

Fructose concentration, (g/L)	x_0 (g/L)	x_m (g/L)	μ_m (1/h)	k (1/h)	R^2
1	0.0163	0.5034	0.2930	0.0081	0.9418
3	0.0181	0.7499	0.2903	0.0094	0.9733
5	0.0272	1.0655	0.3234	-0.0004	0.9986
7	0.0217	1.0942	0.3542	0.0032	0.9786
9	0.0262	1.1114	0.3304	0.0013	0.9914
11	0.0256	1.1455	0.3234	0.0011	0.9888

x_0, initial cell dry weight concentration; x_m, maximum cell dry weight concentration; μ_m, maximum specific growth rate; k, promotion or inhibition constant; R^2, regression coefficient

TABLE 2: Substrate consumption, cell growth and product formation of *C. Ijungdahlii* using various organic substrates

Substrate	$Y_{X/S}$ (g/mol)	$Y_{P/S,\ exp}$ (mol/mol)	$Y_{P/S,\ th}$ (mol/mol)	η %	$Y_{P/X}$ (mmol/g)	q (mmol/g/h)	EtOH/Ac (mol/mol)	Substrate conversion %
Fructose	45.46	2.17	2.60	83.46	47.69	0.39	1.03	88.69
Glucose	41.82	2.29	2.60	88.08	54.86	0.46	0.12	78.94
Ethanol	3.76	0.44	1.00	44.31	117.89	0.98	16.65	55.57
Acetate	5.62	0.07	1.00	7.42	13.52	0.11	n.d.	61.70

$Y_{X/S}$, biomass yield; $Y_{P/S,\ exp}$, experimental yield of products (ethanol and acetate); $Y_{P/S,\ th}$, theoretical yield of products; η, efficiency of substrate conversion to product ($\eta = Y_{P/S,\ exp}/Y_{P/S,\ th}$); $Y_{P/X}$, yield of product from biomass; q, specific production rate; EtOH/Ac, ratio of ethanol to acetate; n.d.: not defined

At low fructose concentrations (1 and 3 g/L) the bacterial cell concentrations exponentially increased for the first 24 h and then substantially decreased due to the fructose exhaustion. The cells started lysing and the number of viable cells decreased after 24 h, probably due to the low concentration of substrate. At high fructose concentrations (7, 9 and 11 g/L), although the bacterial cell growth was promoted and the culture reached to the maximum density at 18 h, however a brief period of decline (around 30 h) was experienced by the cells before entering the stationary phase. Even at high substrate levels, the metabolic activity of the cells declined, perhaps because the level of nutrients in the culture was insufficient to sustain the viability of the cells. Although the fructose utilization was only 72 and 85% with 11 and 9 g/L fructose, respectively, compared to 91% with 5 g/L, however, the low availability of nutrients seemed to affect the cell growth. Generally, nutrient limitations in the culture can cause some limitations in cell metabolism such as its maintenance, intracellular enzyme production and cofactor formation. Although such condition triggers the non-growing state which is an advantage for acetogenes to shift the metabolic pathway from the acetogenesis to solventogenesis, however, if the culture suffers from significant loss in cell viability and low metabolic activity, the cells may not be able to generate the correct enzymes or utilize the electron carriers to uptake the carbon substrate or metabolic intermediates [24]. Thus, it is necessary to keep the culture viable and active as any upset in cell viability will eventually affect the overall productivity.

3. 2. 2. Product Formation The acetate evolution profile in the culture using various fructose concentrations is presented in Figure 4. The acetate formation was parallel to the exponential growth of the bacterium. This trend was followed by an almost stagnant period that acetate formation ceased. The highest and lowest acetate production of 37 and 11.4 mM were devoted to the fructose concentrations of 11 and 1 g/L, respectively.

Figure 5 illustrates the ethanol production trend of the bacterium for various concentrations of fructose. At low substrate concentrations, little amount of ethanol was formed in the culture. The low metabolic activity of the cells was probably unable to shift the metabolic pathway of the bacterium from acetogenic to solventogenic phase and the overall productivity of the cells was affected by the declining cells.

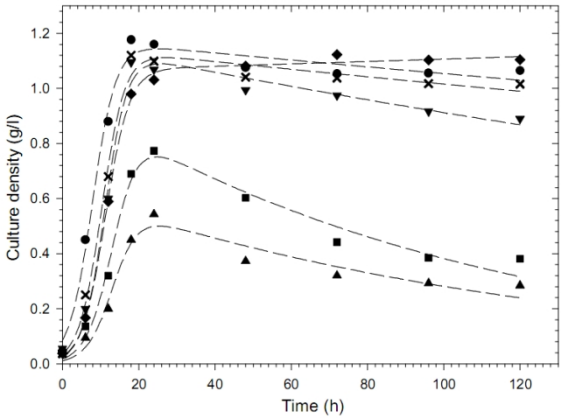

Figure 3. The Volterra model applied to the experimental data at various fructose concentrations, ▲: F1, ■: F3, ♦: F5, ▼: F7, ×: F9, ●: F11, ---: Equation (8)

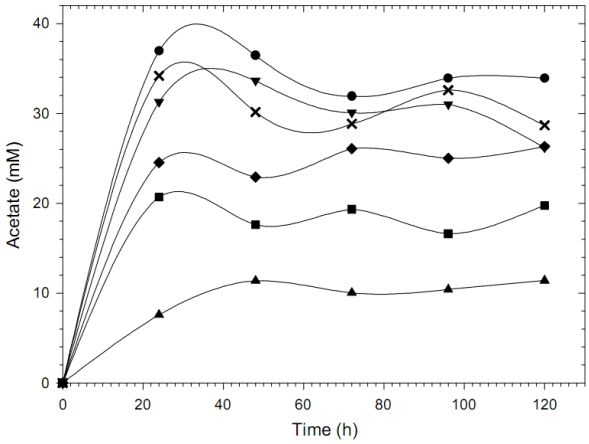

Figure 4. Acetate formation profile for various fructose concentrations, ▲: F1, ■: F3, ♦: F5, ▼: F7, ×: F9, ●: F11

TABLE 3. Substrate consumption, cell growth and product formation of *C. ljungdahlii* using various concentrations of fructose

Fructose concentration (g/L)	$Y_{X/S}$ (g/mol)	$Y_{P/S,\ exp}$ (mol/mol)	$Y_{P/S,\ th}$ (mol/mol)	η %	$Y_{P/X}$ (mmol/g)	q (mmol/g/h)	EtOH/Ac (mol/mol)	Substrate conversion %
1	43.43	2.37	2.60	91.15	54.63	0.45	0.06	91.17
3	24.83	1.365	2.60	52.51	54.98	0.46	0.06	92.07
5	45.46	2.17	2.60	83.46	47.69	0.39	1.03	88.69
7	23.86	1.05	2.60	40.38	44.06	0.37	0.41	90.57
9	23.95	0.71	2.60	27.31	29.59	0.25	0.05	84.84
11	24.18	0.79	2.60	30.38	32.76	0.27	0.03	72.02

$Y_{X/S}$, biomass yield; $Y_{P/S,exp}$, experimental yield of products (ethanol and acetate); $Y_{P/S,\ th}$, theoretical yield of products; η, efficiency of substrate conversion to product ($\eta = Y_{P/S,\ exp}/Y_{P/S,\ th}$); $Y_{P/X}$, yield of product from biomass; q, specific production rate; EtOH/Ac, ratio of ethanol to acetate.

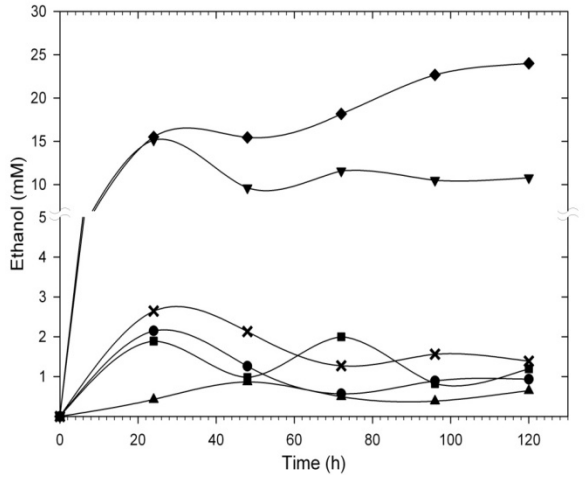

Figure 5. Ethanol production trend for various fructose concentrations, ▲: F1, ■: F3, ♦: F5, ▼: F7, ×: F9, ●: F11

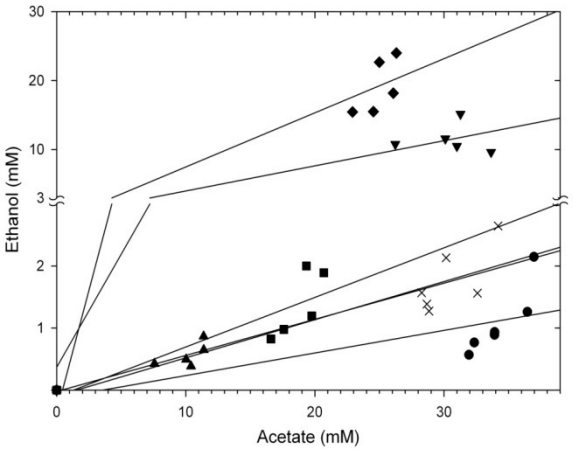

Figure 6. Product distribution for various fructose concentrations, ▲: F1, ■: F3, ♦: F5, ▼: F7, ×: F9, ●: F11, ___: Regression

At fructose concentration of 5 g/L, the ethanol production considerably improved from 12.5 mM at the onset of non-growing phase to 27.1 mM at the end of the fermentation period. A relatively high concentration of ethanol was formed with 7 g/L of fructose at 24 h; however, the ethanol production began to plateau after the cells started to decline. Very little amount of ethanol was formed at high fructose concentrations (9 and 11 g/L) due to the apparent failure of the bacterial cells to switch from acetogenic to solventogenic phase, which is a prerequisite for successful ethanol production.

Degeneration of bacterial culture is common in Clostridial strains, where the bacteria fail to switch to solventogenesis, at the late stage of the exponential phase. One possible mechanism could be the occurrence of *acid crash* which is an unwanted physiological state. This phenomenon occasionally takes place in pH-uncontrolled batch culture, where a considerable amount of acid instead of solvent is formed and the culture losses the ability to switch to the solventogenesis [9, 25, 26]. In the case of our experiments, *acid crash* might not be the actual cause for prevention of ethanol production, as the accumulation of undissociated acid in the culture was not too high; 34.5 and 33.16 mM at 24 h for fructose concentration of 9 and 11 g/L, respectively. However, they could possibly contribute to the premature termination of ethanol production.

The substrate consumption, cell growth and product formation of *C. ljungdahlii* using various concentrations of fructose are summarized in Table 3. A plot of ethanol production versus acetate formation for various fructose concentrations is depicted in Figure 6. The results suggest that use of concentrations higher or lower than 5 g/L was not effective to improve ethanol production in favor of acetate. Under the conditions of substrate depletion or high fructose concentrations, the cell growth declined and the metabolic pathway of the bacterium was unable to switch from acetogenesis to solventogenesis. The ethanol production ability of *C. ljungdahlii* (27.1 mM) was somewhat comparable to other Clostridia species. Cotter et al. [24] reported that 9.43 mM ethanol was produced in non-growing culture

of the bacterium *C. autoethanogenum* DSMZ10061 with 5 g/L of xylose as the carbon source. Liew et al. [27] utilized gelatinised sago starch (50 g/L) as the carbon source in the batch culture of *C. saccharobutylicum* DSM 13864 which was hydrolyzed to maltose and glucose during the fermentation. In this process, 136.62 mM butanol, 3.47 mM ethanol and 105.34 mM acetone was achieved.

4. CONCLUSION

The growth and product distribution of *C. ljungdahlii* was explored using various organic substrates. The results of this survey showed that fructose was the most suitable substrate to support the cell growth and improve ethanol production. Although considerable cell growth was achieved with glucose as the substrate, however the metabolic pathway of the bacterium shifted toward acetogenic phase in favor of solventogenic phase. The bacteria were able either to consume or produce ethanol probably through different metabolic pathways. Use of acetate as organic substrate supported the bacterial growth; however the culture failed to produce ethanol. Considering the effect of fructose concentration on product formation and distribution revealed that the fructose concentration of 5 g/L was the suitable concentration to improve ethanol production over acetate. At fructose concentrations higher or lower than this optimum value, the metabolic pathway of the bacteria failed to switch from acetogenesis to solventogenesis.

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Effect of Organic Substrate on Promoting Solventogenesis in Ethanologenic Acetogene *Clostridium Ljungdahlii* ATCC 55383

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چکیده

کلستریدیوم لانگالی یک باکتری استوژن به شدت بی‌هوازی است که توانایی تخمیر گروه وسیعی از سوبستراها به اتانول و استات را دارد . در طی این فرایند باکتری مسیر متابولیکی پیچیده‌ای از خود نشان می‌دهد که هر دو فاز استوژنیک (تولید اسید) و سالونتوژنیک (تولید حلال) را شامل می‌شود. در این تحقیق، تاثیر منابع مختلف کربنی روی آغاز شیفت متابولیکی به سمت فاز تولید الکل بررسی گردید. فرایند رشد باکتری در بیوراکتور ناپیوسته و در محیط‌های فروکتوز، گلوکز، استات و اتانول انجام گرفت. نتایج فرایند تخمیر ناپیوسته نشان داد که استفاده از فروکتوز به عنوان سوبسترای آلی منجر به بهبود تولید اتانول (۲۷/۱ میلی مول در لیتر) نسبت به استات (۲۶/۳ میلی مول در لیتر) شد، در حالی که گلوکز عمدتاً به استات متابولیز گردید. سلول‌ها می‌توانستند هم اتانول را مصرف و هم آن را تولید کنند (۲۵ میلی مول) که احتمالاً این دو فرایند از طریق مسیرهای متابولیکی متفاوت اتفاق می‌افتاد. وجود استات به عنوان منبع کربنی در محیط کشت مسیر متابولیکی سلول‌ها را به سمت فاز سالونتوژنیک تغییر داد، با وجود این تولید اتانول در این حالت قابل توجه نبود (۳/۵ میلی مول در لیتر). همچنین، تلاش شد تا بازده تولید اتانول با تغییر غلظت فروکتوز (۱ تا ۱۱ گرم در لیتر) بهبود پیدا کند. در شرایط کمبود سوبسترا و یا در غلظت‌های بالای سوبسترا رشد سلول کاهش یافت و مسیر متابولیکی به سمت فاز تولید حلال شیفت نکرد. غلظت فروکتوز ۵ گرم در لیتر به عنوان غلظت بهینه تعیین گردید که در آن بازده مولی تولید اتانول نسبت به استات ۱ به ۱ بود.

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