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# Enhance the Growth of *Clostridium ljungdahlii* Microbial Cells by Modifying the Medium Composition and Trace Metals

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**Keywords:** Bioethanol; Syngas fermentation; Trace metal; Growth medium; *Clostridium ljungdahlii*.

**Abstract.** Syngas fermentation is an alternative route that combines the advantages of thermochemical and biochemical processes have been proposed for biomass conversion to ethanol. One of the main obstacles to syngas fermentation is the low yield of ethanol, caused by the limited utilization of the syngas substrate due to low microbial cell concentration in the fermentation system. This research examined the modification of fermentation medium to improve microbial cell growth. The modifications were to increase the concentration of micronutrients/trace metals and macronutrients in the medium. The results showed that the maximum mass cell and maximum growth rate produced by microbial growth in the modified trace metal medium were 0.63 g/L and 0.0076 $h^{-1}$, while in a modified macronutrient medium were 0.97 g/L and 0.0298 $h^{-1}$. Modification of the macronutrient medium was able to increase the yield of biomass, but the opposite occurred in the modification of the trace metals. Meanwhile, the maximum concentration of ethanol from syngas fermentation in the modified macronutrient medium was lower than the recovery of ethanol in the standard medium.

## Introduction

The need for global fossil fuels has risen sharply along with the increase in the human population. Plant biomass is one of the world's fourth largest non-fossil renewable energy sources after geothermal, solar, and wind. One of the uses of biomass as an energy source that is more efficient than direct combustion is to convert it into fuel/biofuel such as ethanol [1].

The two main pathways for biofuel production, particularly ethanol from lignocellulose, are biochemical and thermochemical. In the biochemical pathway, biomass is pretreated with the addition of acid, alkali, or steam to break the cellulose-hemicellulose-lignin interaction. This pretreatment makes the biomass more accessible to enzymes. Pretreated biomass becomes a target for hydrolysis enzymes to obtain fermentable sugars. After that, the hydrolyzate is fermented to produce ethanol [2]. The advantages of the biochemical pathway are the high selectivity of biocatalyst products [3], the reactor is not too complex, and operating at ambient temperature and pressure [4]. However, the biochemical pathway faces several challenges such as high pretreatment and enzyme costs, relatively slow process, lignin as a by-product, soluble inhibitory compounds (e.g., acetic acid, furfural, 5-hydroxymethylfurfural, phenolic compounds [5].

The thermochemical pathway involves biomass gasification into syngas (a mixture of CO and $H_2$). Syngas then converted into biofuels using a chemical catalyst known as the Fischer-Tropsch (FT). The advantage of the FT thermochemical process is that it can process heterogeneous raw materials, including lignin, and the process is relatively fast. However, constraints in the FT thermochemical pathway include the need for extensive infrastructure, high cost of metal catalysts, high operating temperatures and pressures, requiring consistent gas quality, and stable CO:$H_2$ gas composition ratio [6].

The third pathway, namely syngas fermentation, emerged as a midpoint combining the advantages of the biochemical and thermochemical pathways. First, the biomass feedstock is

thermochemically processed to produce syngas. The produced syngas is then used as the carbon source for the microbial biocatalysts to be converted into ethanol [7].

The potential microbes for conducting the syngas fermentation process are the acetogenic bacteria that is capable of reducing organic acids to alcohol [8], including *Clostridium ljungdahlii, Clostridium autoethanogenum, Clostridium carboxidivorans P7, Acetobacterium woodii* [9]. Acetogens can grow on $H_2$ and CO, sugars, one-carbon compounds, methoxylated aromatic compounds, and alcohols. Acetogens can directly generate 1 mole of hexose to 3 moles of acetate [10], utilizing all the carbon substrates in the solution product.

The ability of microbes to convert syngas into metabolic products occurs through the reductive acetyl-CoA pathway called the Wood-Ljungdahl pathway [11]. The fermentation stage is typically divided into two stages: acidogenesis and solventogenesis [12]. The acidogenesis stage is the growth stage where cells will grow accompanied by acetate and butyrate products, while the solventogenesis stage is when the cells produce ethanol solvent compounds.

The concentration of microbial cells formed during the acetogenesis phase determines the number of fermented ethanol products. The maximum growth of microbial cells is closely related to growth media that provide all essential nutrients such as minerals, trace metals, vitamins, and reducing agents for maximum microbial growth. Therefore, the choice of growth medium depends on the species selected and the end product targeted. Reducing agents (such as sodium thioglycolate, ascorbic acid, and benzyl-viologen) cause an electron flow shift, diverting carbon flow from acid production to alcohol [13].

Two types of nutrients are required for cell growth: macronutrients and micronutrients (trace elements). The macronutrient medium is composed of carbon and magnesium compounds. Fructose is a common carbon source for *Clostridium ljungdahlii* inoculum growth. The majority of carbon substrate during anaerobic fermentation is converted into products, while a small portion is converted into cell mass. The primary source of carbon and energy for cells is carbon compounds. Magnesium is a cofactor for a number of enzymes and is found in cell membranes and cell walls. Ribosomes are dependent on $Mg^{2+}$ ions. During the exponential growth phase, magnesium and sodium play unique roles [14]. The buffer is another essential component of the microbial growth medium. $NaHCO_3$ functions as a buffer to maintain and regulate the pH of the medium. However, it can also support microbial growth as a carbon and $Na^+$ source [15]. Sodium is crucial during the exponential growth phase [16].

Trace metals are essential for the growth of acetogens, including cobalt, iron, molybdenum, nickel, selenium, and tungsten, all of which are constituents of enzymes involved in synthesizing acetate from $CO_2$. Several recent studies have shown that certain elements are essential in ethanol production [17]. The vital role of metalloenzymes in syngas fermentation makes optimization of trace metal concentrations in the growth medium important. Ethanol synthesis and production costs will be inefficient if the metalloenzyme element is deficient or excess. Several studies have reported reducing and increasing the concentration of trace metal components on microbial growth and the formation of syngas fermentation products [18]–[24].This study aims to increase the growth of *C. ljungdahlii* inoculum as an essential part of the syngas fermentation process to obtain optimal acetic acid and ethanol. So, the effect of modifying the concentration of trace metal components in the medium was examined.

## Material and Methods

**Material and culture medium.** The microorganism used in the experiment was Clostridium ljungdahlii DSM 13528 from the Deutsche Sammlung von Mikroorganismen und Zullkulturen GmbH collection center, Braunschweign, Germany. The basal culture medium used for acetogen culture was DSMZ 879, with the composition: $NH_4Cl$ 1.0 g/L, KCl 0.10 g/L, $MgSO_4$.7H2O 0.20 g/L, NaCl 0.80 g/L, $K_2HPO_4$ 0.10 g/L, $CaCl_2 \cdot 2H_2O$ 0.02 g/L, yeast extract 1.0 g/L, trace metal solution 10 mL/L, Na-resazurin solution (0.1% w/v) 0.50 mL/L, $NaHCO_3$ 1.0 g/L, D-fructose 5.0 g/L, vitamin solution 10 mL/L, L-cysteine-HCl.$H_2O$ 0.3 g/L, and $Na_2S.9H_2O$ 0.3 g/L. The trace metal solution contains: nitrilotriacetic acid 1.5 g/L, $MgSO_4.7H_2O$ 3.0 g/L, $MnSO_4.H_2O$ 0.5 g/L, NaCl 1.0 g/L,

$FeSO_4.7H_2O$ 0.1 g/L, $CoSO_4.7H_2O$ 0.18 g/L, $CaCl_2 \cdot 2H_2O$ 0.10 g/L, $ZnSO_4 \cdot 7H_2O$ 0.18 g/L, $CuSO_4 \cdot 5H_2O$ 0.01 g/L, $KAl(SO_4)_2 \cdot 12H_2O$ 0.02 g/L, $H_3BO_3$ 0.01 g/L, $Na_2MoO_4 \cdot 2H_2O$ 0.01 g/L , $NiCl_2 \cdot 6H_2O$ 0.03 g/L, $Na_2SeO_3 \cdot 5H_2O$ 0.0003 g/L, and $Na_2WO_4 \cdot 2H_2O$ 0.0004 g/L. The vitamin solution consists of: biotin 20 mg/L, folic acid 20 mg/L, pyridoxine HCl 10 mg/L, thiamine HCl 5 mg/L, riboflavin 5 mg/L, nicotinic acid 5 mg/L, D-Ca- pantothenate 5 mg/L, vitamin B12 0.1 mg/L, p-aminobenzoic acid 5 mg/L, and lipoic acid 5 mg/L. The ATCC medium procedure for anaerobic cultivation was applied in the preparation and use of the media.

**Culture Experimental Method.** After placing 225 mL of culture medium in a 500 mL vial, purging using $N_2$ gas for 15 minutes, then sealing the bottle using aluminum, the vial containing the media was autoclaved at 121ºC for 15 minutes. After that, Wolin's vitamin solution was added, sterilized by filtration, $NaHCO_3$, D-fructose, and a mixture of $Na_2S.9H_2O$-L cysteine HCl, each of which had been autoclaved in separate bottles at 121ºC for 15 minutes. Next, the precultured microbial solution was inoculated with 10% of the total media. The culture medium was then grown for 10 days at 37ºC with a stirring speed of 200 rpm.

As a modification variation, the use of trace metals $Na_2SeO_3 \cdot 5H_2O$ and $ZnSO_4 \cdot 7H_2O$ are five times higher, while $NiCl_2 \cdot 6H_2O$ and $Na_2WO_4 \cdot 2H_2O$ are ten times higher than that of the standard medium. Meanwhile, in the modified macronutrient medium, the use of fructose, and $NaHCO_3$ are double, while $MgSO_4.7H_2O$ is ten times than that of the standard medium. There are two variations of medium modification for microbial growth, namely: (1) macronutrient modification with trace element composition is standard and (2) trace element modification with macronutrient composition is standard.

When the modified macronutrient medium was used for fermentation by *C. ljungdahlii*, the carbon source of fructose was replaced by CO and $CO_2$ from syngas. As a result, gas is derived from pure gas with a composition of 25% CO, 15% $H_2$, 20% $CO_2$, and 40% $N_2$. Two variations of the medium used in syngas fermentation were: (1) standard macronutrient and trace element medium, used for inoculum growth and fermentation, and (2) modified macronutrient medium and standard trace element, used for inoculum growth and fermentation.

The fermentation process was carried out on a 1.5 L scale fermenter with a working volume of 1 L, run for 20 days, with a temperature of 37ºC and an agitation rate of 300 rpm. In the fermentation process, the pH of the fermenter is controlled so that it is not lower than 4.5 to avoid cell death, and pH control is carried out with 2M HCl and 2M NaOH solutions. Details configuration of the bioreactor used was described in Anggraini [25].

The variables reviewed in the syngas fermentation process are the yield of cell biomass, ethanol, and acetate. Hence, the analyzes carried out in this study, namely (a) analysis of the concentration of biomass cells during the fermentation process using a spectrophotometer by calculating optical density (OD) at 600nm, (b) analysis of metabolites formed in the tank medium using High Performance Liquid Chromatography (HPLC), and (c) analysis of the gas composition contained in the reservoir tank headspace using Gas Chromatography (GC).

## Result and Discussion

**The Effect of Increasing the Concentration of Trace Metals on Microbial Growth.** The modified trace metal components, namely $Ni^{2+}$ and $WO_4^{2-}$, became ten times that of the standard trace metal composition, while $Zn^{2+}$ and $SeO_3^{2-}$ increased five times that of the standard composition. The addition of trace metal components was carried out and used simultaneously on the growth medium of *C. ljungdahlii*. The results of mass production of *C. ljungdahlii* cells using standard and modified trace metals are shown in Fig.1.

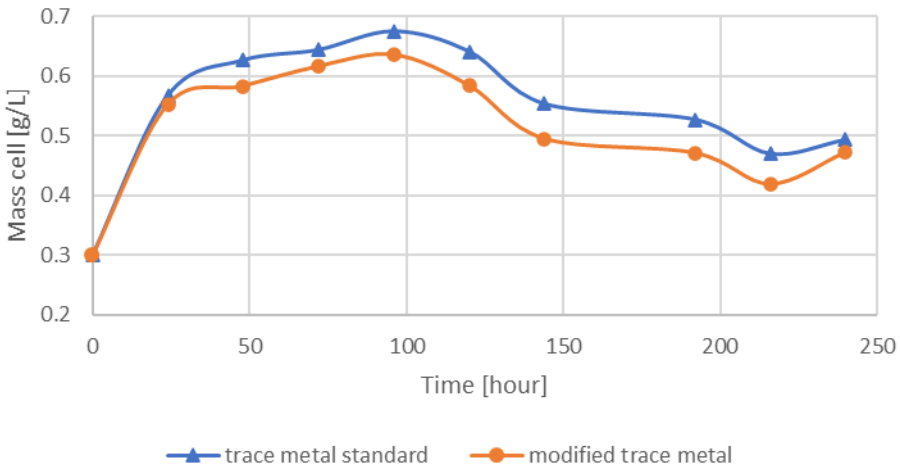


Fig. 1 Comparative curve of *C. ljungdahlii* cell growth in the medium using standard and modified trace metals.

The maximum cell mass obtained by microbial growth in the standard and modified trace metal medium was 0.67 g/L and 0.63 g/L, which was obtained at the 96th hour. Meanwhile, the maximum growth rate produced by microbial growth in medium with standard and modified trace metal was 0.0087 $h^{-1}$ and 0.0076 $h^{-1}$, respectively. The inoculum growth curve showed a decrease in the mass cell concentration when the modified trace metal was used in the medium.

Trace metal component has a different effect on the development and production of metabolites in every bacterium, which is determined mainly by the energy efficiency mechanism of the bacteria and the existence at the enzyme's active site. For example, the enzymes that participate in the Wood Ljungdahl pathway are metalloenzymes, whose activity depends on certain metals' presence in the medium.

WLP activity increased with $Ni^{2+}$ availability because an essential enzyme in WLP, CODH/ACS, which is vital for the acetyl-CoA formation, is highly nickel required. Nickel is also found in NiFe-$H_2$ase, which catalyzes hydrogen oxidation to generate an equivalent reduction for CO2 reduction. Thus, there is a high possibility of an increase in CODH/ACS activity, resulting in higher $CO_2$ reassimilation in the nickel-added medium [26]. However, nickel forms a complex with Cu in the ACS cluster. The inactive ACS (Cu-Ni) enzyme decreases carbon flow to growth and metabolites, inhibiting biomass and ethanol production [27]. Therefore, obtaining the optimum conditions for adding nickel must be balanced with a review of the concentration of Cu in the medium.

Tungsten (W) and selenium (Se) are components of FDH that function as cofactors, which catalyze the reduction of $CO_2$ to formate. For example, in *C. ragsdalei*, increasing the concentration of tungstate and selenite can optimize FDH activity [28]. Likewise, aldehyde ferredoxin oxidoreductase (AFOR) requires tungsten as a cofactor to catalyze the conversion of acids to aldehydes, utilizing the electrons supplied by reduced ferredoxin. Tungsten has a major impact on the ethanol production of *C. ljungdahlii* [24].

Zinc has shown a variable effect on various bacterial strains. The effect of Zn on growth and metabolite synthesis depends on the concentration available. A small amount of Zn, which is required for growth, is readily available by the inoculum [24].

The effect and the optimum concentration of trace metals on acetogens vary across acetogenic strains. The optimum concentration of trace metal in *C. ragsdelei* used by Saxena and Tanner [18] does not necessarily produce optimum results in *C. ljungdahlii*. The saturation level of the medium with these minor metals impacts the overall growth or metabolite production. The simultaneous increase in trace metals in a medium does not necessarily increase biomass growth. Excessive and simultaneous utilisation of trace metals can inhibit growth [23], [24]. In this study, Wolin's standard trace metal formulation gave the best results for the rapid growth of *C. ljungdahlii* microbes to be used as an inoculum in the syngas fermentation medium.

**Effect of Increasing Concentration of Macronutrient Medium on Microbial Growth.** The yield of *C. ljungdahlii* cell mass on standard and modified macronutrient medium is shown in Fig.2.

The trace element used is the standard trace element. The maximum cell mass obtained by microbial growth on standard and modified macronutrient medium was 0.67 g/L (96th hour) and 0.97 g/L (48th hour, respectively). Meanwhile, the maximum growth rate produced by microbial growth on standard and modified macronutrient medium was 0.0087 $h^{-1}$ and 0.0298 $h^{-1}$, respectively. A modified macronutrient medium was able to increase the yield of microbial cell mass.

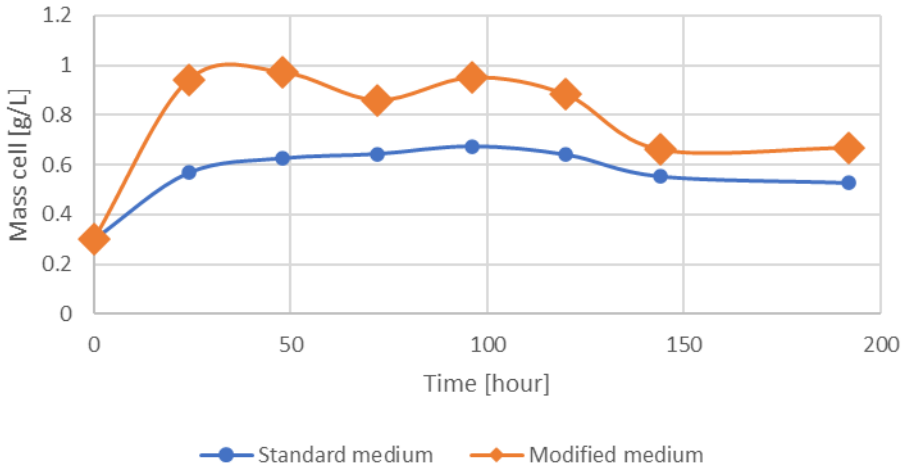


Fig. 2 Comparison curve of *C. ljungdahlii* cell growth in the medium using standard and modified macronutrient medium.

When grown in a medium, bacteria should adapt to the nutrient composition in the medium, synthesizing the essential amino acids, growth factors, and enzymes. The modified macronutrient medium allows cells to adapt to the environment so that they can go through the lag phase better and quickly enter the exponential growth phase. The rapid multiplication causes the cell mass and density to rise exponentially over time. The lag phase and the exponential growth phase of the inoculum occurred within 24 hours.

The maximum growth rate of microbes in a modified macronutrient medium is higher than the maximum growth rate in a standard medium due to fructose, magnesium in $MgSO_4.7H_2O$, and sodium in $NaHCO_3$ components added to the medium.

Carbohydrates as a carbon source provide an easily accessible and non-toxic substrate. The addition of fructose was doubled compared to the composition of the standard medium, causing an increase in biomass growth. Growth with fructose substrates allows acetogens to conserve more energy than organisms without WLP.

Rolfe [16] states that magnesium and sodium have a special role during the exponential growth phase. MgtA, corA, and mgtBC are transcriptional genes encoding for magnesium transport. MgtA was responsible for increased magnesium concentration during the exponential phase and showed high expression during the initial lag phase. The corA gene was most abundant during the stationary phase, while mgtBC was most abundant during the early lag phase. Meanwhile, the gene sequence of the sodium/proton nhaA transporter was expressed the highest during the mid-exponential growth, correlated with the highest level of sodium ions. The increase in the sodium ion level inside the cell can balance the increase in the external proton concentration due to the increased proton-motive force through exponential growth. Thus, increasing the concentration of magnesium and sodium as macronutrients in the modified medium could optimize the gene expression of mgtA, cor A, mgtBC, and proton nhaA, which affected increasing biomass growth by 1.4 times compared to the standard medium.

**Effect of Using the Modified Medium on Syngas Fermentation.**

**Maximum Mass Cell Concentration.** Inoculum transfer from a complex medium (with a carbon source of fructose) to a minimal fermentation medium (with a carbon source of synthesis gas with low solubility in liquid medium) extended the lag phase. The lag phase follows inoculation and

is a period of cell adaptation to the new environment. Microorganisms rearrange their molecular elements when relocated to a new medium. During this phase, there is only a slight increase in cell mass, with no increase in cell density. Both biomass growth curves in the synthesis gas fermentation medium show it (Fig. 3a and Fig.3b). Syngas fermentation using a standard medium was only carried out in 240 hours because biomass, acetate, and ethanol concentration had decreased.

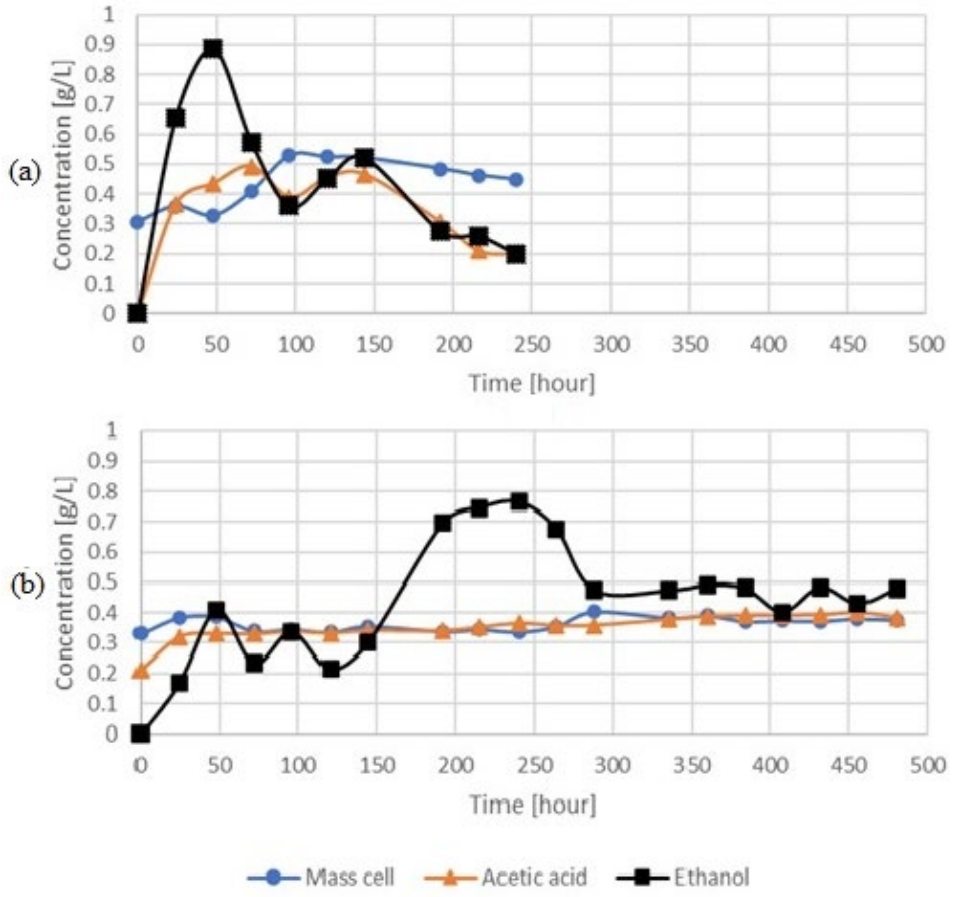


Fig. 3 Syngas fermentation product curve using (a) standard fermentation medium (b)modified fermentation medium

Fermentation in the modified medium experienced a fast exponential phase in 24 hours. However, the growth started slowing down at 48 hours, and at 72 hours, the growth became very slow (Fig.3b). As growth slowed down, nutrient depletion occurred, and ethanol began to accumulate. At this time, the cell also undergoes lysis, so the available cell mass decreases.

At 240 hours, the cell then undergoes a second growth phase. Cells can grow on the lysis products of lysed cells (occult growth). However, at that time, the ethanol concentration decreased. The possibility is caused by using ethanol as a growth substrate for growing cells. The Wood Ljungdahl Pathway allowed acetogens to access many growth substrates such as lactate or ethanol, which are products of many anaerobic fermentations. Both compounds are common in anaerobic environments and are unusable to many other organisms as carbon and energy sources [26].

Cellular regulation changes when the concentration of specific metabolites (carbon, nitrogen, phosphate) is limited. Thus, the cause of growth cessation in syngas fermentation may be due to depletion of essential nutrients or accumulation of inhibitory products. The growth rate will slow if producing inhibitory products accumulate in the medium, and the growth will stop at a certain inhibitory concentration level. In order to produce further cell growth, when the amount of ethanol has not increased, ethanol can be diluted with new media so that the high ethanol concentration does not interfere with cell growth. Before adding the new medium to the cells, harvest ethanol [14]. However, the composition of the medium used in the inoculum did not affect the initial concentration of cells in the fermenter.

**Maximum Acetic Acid Concentration.** The highest acetate yield resulted from synthesis gas fermentation on day 3 on the standard medium, which was 0.49 g/L. The maximum acetate gain on the modified medium was 0.322 g/L on day 20. Acetate concentration increases with cell concentration during the syngas fermentation process, indicating that acetate is a growth-associated product. Because acetate is the most abundant fermentative product, it can lower the pH. In the presence of $H^+$ ions, the acid produced is undissociated, can penetrate the cell wall, accumulates in the cell, and lowers the internal pH. [29]. The metabolism shifts from the acetogenic phase at neutral pH to the solventogenic phase at acidic pH due to this process. It happened in synthetic gas fermentation with a standard medium, but in the modified medium, the pH of the medium did not decrease; it remained above 6, even higher. It caused the addition of $NaHCO_3$, which acts as a buffer solution, so the pH of the medium stays at the initial conditions.

**Maximum Ethanol Concentration.** The highest yield of ethanol resulted from synthesis gas fermentation on day 2 on standard medium, amounting to 0.89 g/L, while the maximum ethanol gain on the modified medium was 0.76 g/L on day 10. On the ethanol production curve in the modified medium on days 10 to 12, the ethanol concentration decreased while the biomass concentration increased. It is probably due to the consumption of ethanol for use as a growth substrate [26].

The higher concentrations of magnesium and sodium than those used in the standard medium did not cause the production of acetic acid or ethanol to increase significantly. It is most likely due to the limited carbon source in the medium due to the use of syngas as a carbon source so that cellular regulation also changes, in contrast to when the carbon source used is fructose.

## Conclusion

This study examines the modified medium's effect on the growth of *C. ljungdahlii* inoculum. The concentration of the macronutrients: fructose, $NaHCO_3$, $MgSO_4.7H_2O$, and the micronutrients of trace metal $Ni^{2+}$, $Zn^{2+}$, $SeO_3^{2-}$, $WO_4^{2-}$ was modified. The results showed that the medium with the modification of trace metal micronutrient components did not increase mass cell gain compared to the gain in standard medium. Meanwhile, modification of macronutrient medium concentration increased mass cell gain in the inoculum. However, when the modified macronutrient medium was used as the syngas fermentation medium, the yield of biomass, acetate, and ethanol decreased compared to that of the standard medium. Therefore, further research to increase ethanol production from syngas fermentation needs to be carried out.

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