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Original Article

Optimization of different carbon, nitrogen and amino acid substrates and their effects for bioethanol production by *Escherichia coli* DH5α

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ABSTRACT: This study aims to discover the effect of different carbon, nitrogen and amino acid substrates for better ethanol production by *Escherichia coli* DH5α, where pH 7.0 ± 0.5 and 37°C is suitable for their cell growth at 12 hours incubation period during 150 rpm. The effects of different carbon sources were assessed during the exponential phase of *E. coli* DH5α, where complete consumption of different carbohydrates and amino acids were observed. Moreover, glucose with trehalose supplement induced cell growth and producing 0.15 ± 0.01 g/L ethanol at 28 hours shake flask culture measured by redox titration method. Ammonium sulphate and serin increased cell growth compared to other, while trehalose supplement with glucose (in M9 media) can increase cell growth and metabolite (ethanol) production. Therefore, this optimized and suitable condition for *E. coli* DH5α might be a base study to develop industrial energy production to fulfill partial demand in future.

KEY WORDS: *Escherichia coli* DH5α, Optimization, Culture conditions, Trehalose, Bioethanol.

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INTRODUCTION

Petroleum fuel is now widely recognized as non-renewable energy source because of their consumed supplies and the contribution to the shuffle of carbon dioxide in our environment[1]. The major concerns for developing new technologies to generate alternative fuels from renewable carbon sources have significant roles for the remedy of environmental pollution, costly use of fossil fuels[2,3]. Biofuel has drawn the attention as a sustainable energy source worldwide in recent years because they replace petroleum fuels as well as a number of environmental and economic benefits are claimed for it. Bioethanol as a biofuel has forthcoming possibilities to use as an alternative of fossil fuel. In order to make it cost and energy effective, the processes must utilize organisms to efficiently produce candidate fuels that can be optimized from a variety of feed stocks. *Escherichia coli* has become an important host organism for the microbial production of biofuels in part due to the ease at which this organism can be manipulated[4]. They can live on a wide variety of

substrates and perform mixed-acid fermentation in anaerobic conditions where subsequently can produce lactate, succinate, ethanol, acetate and carbon dioxide. When *E. coli* lives together with hydrogen-consuming organisms, such as methanogens or sulphate-reducing bacteria, the hydrogen is consumed which is produced in many pathways of mixed acid fermentation[5]. For ethanol fermentation, the technology has been advanced significantly. Promising prospects of ethanol fermentation are especially introduced including fermentation technology converting different carbon sources to ethanol by microorganism in large system[6]. In the laboratory condition, a bacterium must have an energy source consist of carbon and other required nutrients and a permissive range of physical conditions such as O_2 concentration, temperature, pH, etc. The nutritional requirements of a bacterium (as *E. coli*) are consists of C, H, O, S, Mg, Fe, Ca, Mn and traces of Zn, Co, Cu and Mo. The metabolic regulation in bacteria is maintained by a complex array of signal transduction systems where metabolic interactions and gene regulatory relationships by maintaining the balance of carbon and nitrogen metabolism[7]. The carbon and nitrogen sources are necessary for the growth and product formation in microbial cultivation [8] as *Escherichia coli* is capable of utilizing several compounds as carbon sources. However, as carbon source, glucose is preferred and its rapid utilization depends on the phosphotransferase system (PTS)[9]. So, the present study aims to investigate the culture conditions in response to different physical parameters (pH, temperature and incubation time) as well as carbon and nitrogen sources in shake flask batch culture due to optimize their cell growth and metabolite (ethanol) production. Thus understanding of *E. coli* metabolism in our study may help as a fundamental research for its subsequent industrial bioethanol production.

MATERIALS AND METHODS

Bacterial strain

In the present study, *E. coli* DH5α (*dlacZ Delta M15 Delta* (lacZYA-argF) U169 *recA1 endA1hsdR17*(rK-mK+) *supE44 thi-1 gyrA96 relA1*[10] was used as a laboratory stock.

Media compositions

Micro-aerobic batch culture was carried out by using both Luria–Bertani (LB) and Mineral (M9) medium, where LB medium contained 10.0 g/L Peptone, 5.0 g/L Yeast extract and 5.0 g/L NaCl and M9 minimal medium contained 48 mM Na_2HPO_4, 22 mM KH_2PO_4, 10 mM NaCl, 30 mM $(NH_4)_2SO_4$. The following components were filter sterilized and then added (per liter) with 1 ml of 1 M $MgSO_4$, 1 ml of 0.1 mM $CaCl_2$, 1 ml of 1 mg of Vitamin B1 and 10 ml of trace element solution containing (per liter). 0.55 g of $CaCl_2.2H_2O$, 1.67 g of $FeCl_3.6H_2O$, 0.10 g of $MnCl_2.4H_2O$, 0.17 g of $ZnCl_2$, 0.043 g of $CuCl_2.2H_2O$, 0.06 g of $CoCl_2.2H_2O$ and 0.06 g of $Na_2MoO_4.2H_2O$. The carbon source glucose (10 g/L) was used for the micro-aerobic batch culture[11] and different carbon sources as dextrose, sucrose, galactose, trehalose, lactose were used as same concentration (10 g/L) instead of glucose. Different nitrogen sources $(NH_4)_2SO_4$, potassium nitrate and peptone were used as same concentrations. Several amino acids as serin, proline, glutamate, arginine were used as a supplement (0.001g/L) during batch culture.

Culture conditions

Microaerobic batch culture was carried out in Erlenmeyer flasks at 150 rpm, where the temperature was maintained at 37°C. The pH was adjusted at 7.0 ± 0.1 with pH controller by the addition of 2.0 M HCl or 2.0 M NaOH. The inoculum was prepared by 100 µl transferring cells from a glycerol stock to 50 ml test tube containing 10 ml of LB medium. The culture was then incubated for 6 hours and 0.25 ml of culture broth was then transferred to a 125 ml Erlenmeyer conical flask containing 25 ml of LB or M9 medium.

Measurement of biomass concentrations

Cell concentration was measured by the optical density (OD) of the culture broth (at λ = 600 nm) with a spectrophotometer (Shimadzu Co, Japan). After the batch culture, *E. coli* cells from 250 ml of culture were harvested by centrifugation and dried (pellet) at 60°C for 24 hours in a laboratory oven (KOR-6L05). After confirming that the weight of the dried material was constant, the dry cell weight per litre of culture was calculated from the measured weight. It was then converted to dry cell weight (DCW) per liter based on the relationship between OD_{600} ~ DCW relationship (1 $OD_{600} \approx 0.3$ g/L)[12].

Measurement of metabolite concentrations

Glucose concentration

Glucose was measured according to Nelson's modification of Somogy's method (1944)[13], where the color intensity was measured (at λ = 500 nm) in UV Spectrophotometer (Shimadzu Co, Japan) and compared with standard D-glucose.

Ethanol concentration

The supernatant was taken after 4-5 residence times where the steady state was ascertained and centrifuged it at 12000 rpm for 10 minutes. Then the aqueous portion was taken to measure ethanol concentration. A redox titration method with sodium thiosulphate was done according to Bennett et al. (1971)[14] to measure ethanol.

Statistical analysis

Triplicate measurements were done in all cases during the observation and assessment of bacterial growth and metabolite production. Data was captured into Microsoft Excel Software, Version 2010 which used to calculate means and standard deviations. Student's t-test was applied to confirm the observed changes were statistically significant.

RESULTS

Effects of incubation time

Micro-aerobic batch culture was conducted at 150 rpm and pH 7.0 in LB and M9 medium, where temperature was kept constant at 37°C. Total batch culture was run constantly for 16 hours and 9 samples were taken after every two hours interval (where 1st sampling time was just starting time of the batch cultivation). OD (optical density) was measured (at λ= 600 nm) from those samples in a UV Spectrophotometer (Shimadzu Co, Japan). Finally, the rest of the supernatants were taken and centrifuged at 12000 rpm for 10 minutes and kept the liquid portion at 4°C for further use. After centrifugation the pellet was dried at 60°C for 24 hours to measure biomass. Then OD was converted to DCW and the result was input at excel worksheet **(Figure 1)**

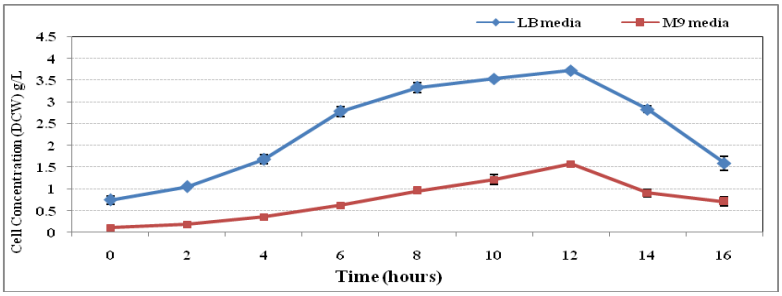

Figure 1 Effects of incubation time on their growth of *Escherichia coli* DH5α on different media. Triplicate measurements were done and compared in each case. Error bars indicate ± SD

to compare the effects of incubation time in response to different nutrient media (LB and M9). In both LB and M9 media, 12 hours incubation time show the best DCW for *Escherichia coli* DH5α.

Effects of incubation pH

Cell growth response during different culture pH (5.0, 6.0, 7.0, 7.5, 8.0 and 8.5) was studied at different culture media (LB and M9), where rpm and temperature were adjusted at 150 and 37°C, respectively. Supernatants were taken at 12 hours and measured OD (at λ=600 nm) to compare the effects of incubation pH at different media **[Figure 2(a, b)]**,

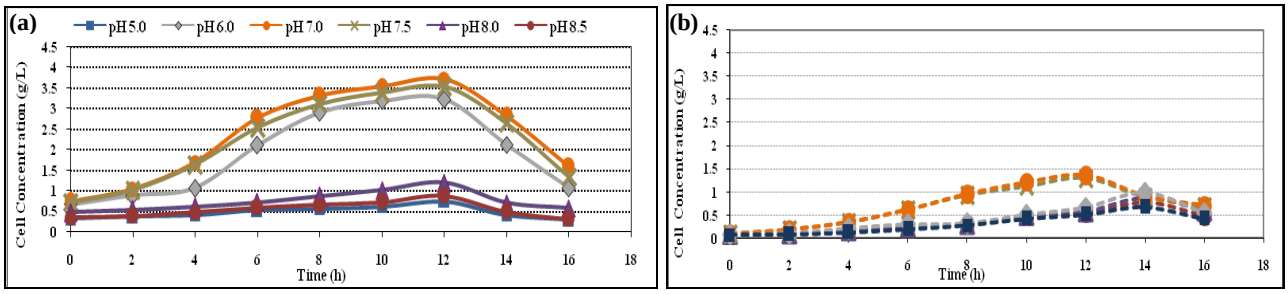

Figure 2 Effects of incubation pH on LB (a) and M9 (b) media

where the media with pH 7.0 shows the best bacterial cell growth for both LB and M9 media.

Effects of temperature

As the best incubation time and pH (12 hours and 7.0) already have been established **(Figure 1 and 2)**, so our next target was to study and compare the effects of incubation temperature. Micro-aerobic batch culture was ascertained at different temperature (28°C ± 0.5 ~ 45°C ± 0.5) in response to different culture media (LB and M9) for 12 hours, while pH and rpm was maintained at 7.0 and 150, respectively **(Figure 3)**

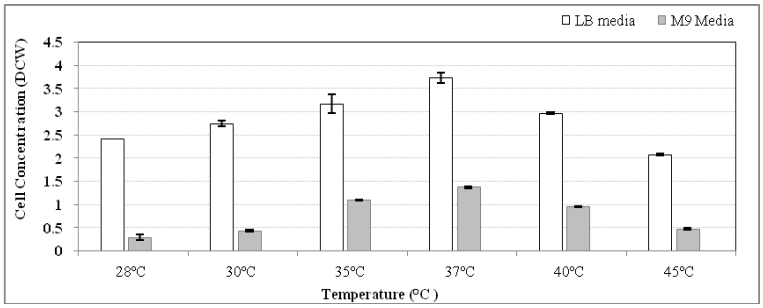

Figure 3 Effects on incubation temperature on their growth in different media

where 37°C was the optimized temperature for better cell growth of *E. coli* DH5α.

Effects of different carbon sources (M9 media)

Micro-aerobic batch culture was conducted in M9 minimal medium to study the effects of different carbon sources (glucose, dextrose, galactose, sucrose and lactose) on the growth of *Escherichia coli* DH5α at pH 7.0, and 150 rpm while incubation temperature and time were maintained at 37°C and 12 hours, respectively. Besides, effects of trehalose and lactose with glucose were also studied and compared **(Figure 4)**

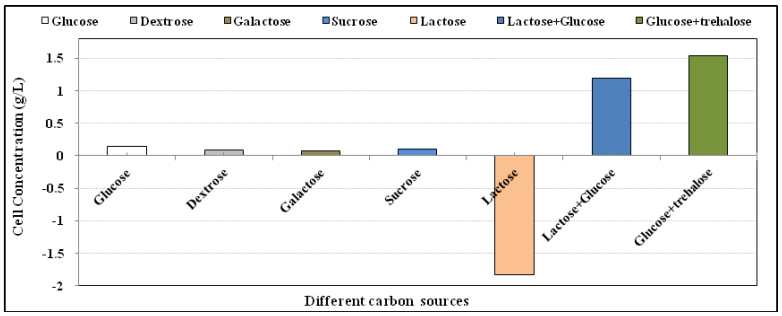

Figure 4 Effects of different carbon sources in M9 media

where trehalose shows a significant effect with glucose for better cell growth comparing to other carbon sources.

Change of culture pH in response to different carbon sources (M9 media)

The effects of different carbon sources (glucose, sucrose, dextrose and lactose) on culture pH was assessed while initial pH and rpm was 7.0 and 150, respectively at 37°C ± 0.5 **(Figure 5)**.

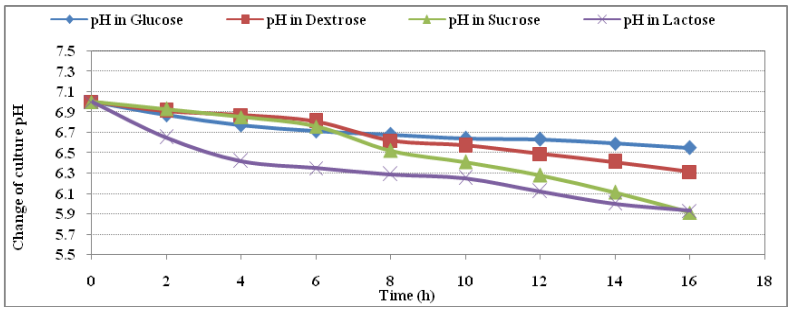

Figure 5 Change of culture pH under different carbon sources in M9 media

Batch cultures were run till 16 hours and samples were taken after every two hours interval. Finally OD was measured and data were compared.

Effects of nitrogen sources (M9 media)

Different nitrogen sources (peptone, ammonium sulphate and potassium nitrate) containing M9 medium were used to assess their effects on cell growth (DCW), where medium pH, rpm and temperature were maintained at 7.00, 150, 37°C ± 0.5, respectively. The best cell growth was observed when ammonium sulphate used in M9 media as nitrogen sources **(Figure 6)**.

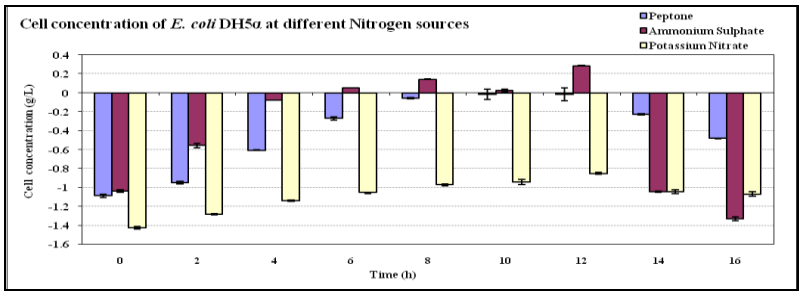

Figure 6 Effects of different nitrogen sources on cell growth

Effects of amino acids

Several amino acids (serine, proline, glutamate and arginine) were used in M9 medium to determine the effects of amino acids on their growth, while medium pH, rpm and temperature was maintained at 7.00, 150, 37°C ± 0.05, respectively and serin used in M9 media depicts better cell growth compared to others **(Figure 7)**.

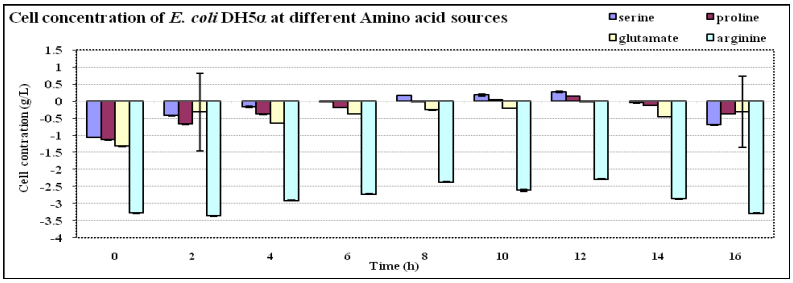

Figure 7 Effects of amino acids on the growth of *E. coli* DH5α

Determination of metabolite concentrations

Assessment of glucose concentration

Glucose concentration was measured from the supernatants during micro aerobic batch cultures at every two hours interval in M9 media (glucose containing) according to Nelson's modification of Somogy's method (1944), while medium pH, rpm and temperature was maintained at 7.00, 150, 37°C ± 0.5 respectively. OD was measured (at λ=500 nm) and data was compared.

Assessment of ethanol production by using different substrates

Redox titration method was followed after cell culture was reached at the end point of 3rd steady state (24-30 hours) to measure ethanol production.

The relationship between the moles of sodium thiosulfate and determined of ethanol was done as moles per litre by using the following equations:

$$6 \text{ mol of } S_2O_3^{2-} \equiv 1 \text{ mol of } Cr_2O_7^{2-}$$

$$2 \text{ mol of } Cr_2O_7^{2-} \equiv 3 \text{ mol of } C_2H_5OH$$

$$\text{So, } 1 \text{ mol of } S_2O_3^{2-} \equiv 0.25 \text{ mol of } C_2H_5OH$$

However, after optimization of all parameters, the best ethanol production was measured in different carbon sources by keeping the all best optimized physical parameters (pH, Temperature, Incubation time) as well as nitrogen source (ammonium sulphate) and amino acid (serin) where trehalose with glucose as carbon source show the highest ethanol concentration **(Figure 8).**

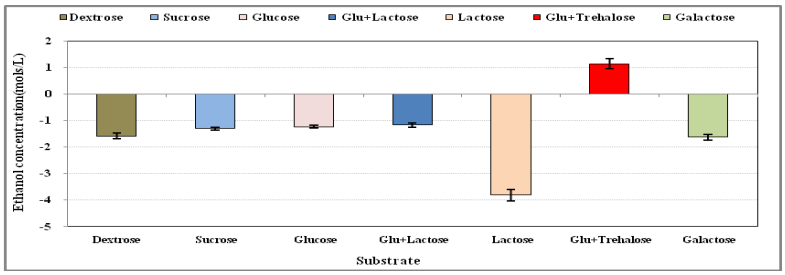

Figure 8 Assessment of ethanol production using different substrate

The possible metabolite regulation mechanism of *E. coli* DH5α is briefly illustrated in (**Figure 9)**.

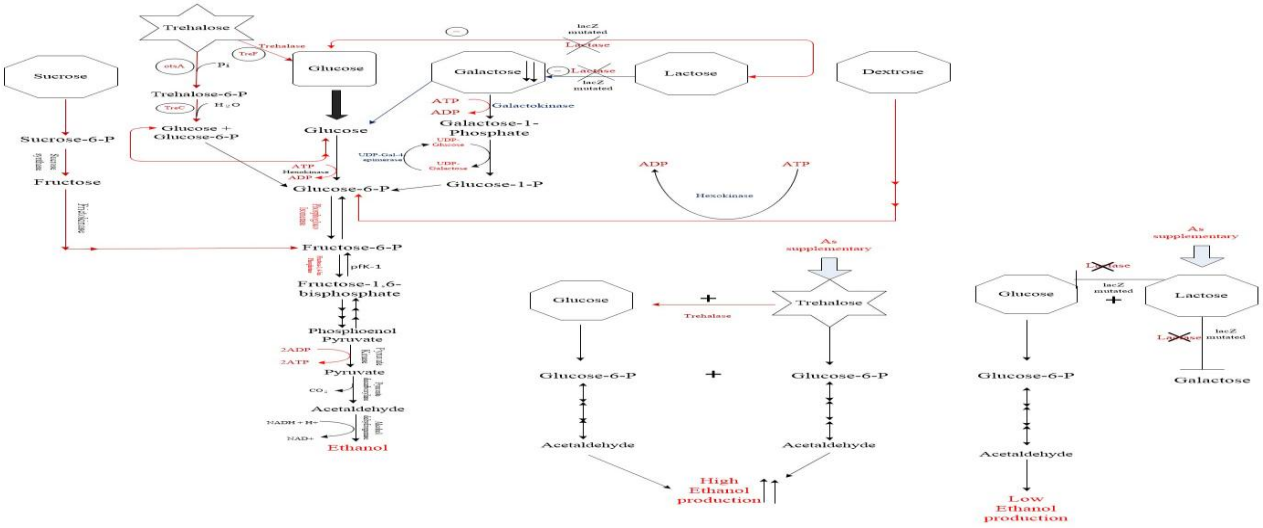

Figure 9 Metabolic pathway analysis of *E. coli* DH5α for better ethanol production in different carbon sources

DISCUSSION

Production of dry cell weight (DCW) of *E. coli* DH5α in response to different incubation parameters

(incubation time, pH, temperature, different carbon, nitrogen and amino acid sources) were examined in LB and M9 culture media, as those are the most important determining factors for bacterial growth as well as for cellular gene expression[15]. 12 hours of incubation time shows the best cell growth **(Figure 1**), as well as in response to culture pH 7.0, it produced highest cell concentration at both LB and M9 culture media **(Figure 2)** as reported by Cofre et al, 2012[16] that, culture media optimization is an important step in the design of a fermented process. pH is an important key growth regulator in bacterial cellular system where H^+ concentration in media can change the total charge of plasma membrane that affects the entrance of essential nutrients into cell[17].

Effects of temperature on cell growth are other significant characteristics of microorganisms[18] due to the relationship of microbial growth rate and culture temperature[19]. As temperature affects plasmid stability and also protein production[20], culture temperature control is also more essential that support cell growth as well as product formation. **Figure 3** shows suitable cell growth (3.72 g/L at LB and 1.37 g/L at M9 media) at 37ºC among other different culture temperatures (28ºC ~ 45ºC). Past studies showed that ethanol production is dependent on fermentation temperature and subsequently to its cell concentration (DCW)[21]. So, it is very important to optimize a suitable culture temperature as fermentation process for ethanol production regulated by some enzymes, where high temperature can deactivate that enzyme[22] and ultimately makes slow down the ethanol production.

In our study, DCW production is ~3.1 times lower ($P < 0.01$) at 28ºC and ~2.33 times lower ($P < 0.01$) at 45ºC compared to culture temperature 37ºC in M9 media, where decreased temperature is not much effective [~1.55 times lower ($P < 0.01$)] in the case of LB media. Besides, increased temperature may affect on their growth in LB media [~1.81 times lower ($P < 0.01$)] **(Figure 3)**. Previous report by sweet sorghum juice, immobilized yeast cell gave 75.79% ethanol at 28ºC but the ethanol production was growing up to maximum yield 89.89% at 37ºC[23]. Our research shows **(Figure 3)** the enhancement of cell mass at 37ºC (for both cases), can lead to higher ethanol production[24] and eventually has a relationship with elevated ethanol production. Similar results also shown by Fotadar et al., 2005[25] the optimal temperature for *E. coli* growth is 37ºC.

Glucose is a preferred carbon source[9] for rapid utilization as monosaccharide in all bacterial cells. Supplementation can enhance sugar utilization[26,27,28] is similar to our study that lactose and trehalose were used as supplements with glucose separately **(Figure 4)** showed cell growth (~6.83 times and ~8.5 times higher) compared to while glucose used as a sole source of carbon in M9 media ($P < 0.01$ for both cases). According to Gomez et al., 2012[29], when both glucose and glycerol were present as carbon sources, glucose was utilized first and glycerol was used after glucose had been completely depleted.

Interestingly, lactose (a disaccharide containing glucose and galactose) is unable to be converted into glucose inside the cell due to the lacking of *lacZ* gene. In our study this may be the reason of lowest cell growth **(Figure 4)**, also consistence with past field studies[30]. On the other hand while lactose used as a supplement with glucose, the reverse result was found **(Figure 4)**. Trehalose supplement with glucose enhanced cell concentration (1.53 ± 0.31 g/L) in *E. coli* DH5α in M9 media **(Figure 4)** may lead to higher ethanol production[24] due to increased cell growth. In this case **(Figure 8)** ethanol was produced more comparatively glucose used as a sole carbon source at 28 hours.

Metabolic system initiated mainly by glucose converted from supplemented trehalose via trehalose-6-phosphate hydrolase[31,32] as well as via phosphotransferase system where *TreS* gene is responsible for trehalose degradation[33]. Trehalose catabolism proceeds via trehalase enzyme to produce two molecules of glucose[34,35]. Phosphorolysis of trehalose produce β-Glucose-1-phosphate, glucose and trehalose 6-phosphate; trehalose 6-phosphate phosphorylase to glucose-1-phosphate and glucose-6-phosphate; trehalose-6-Phosphate hydrolase produce glucose-6-phosphate from trehalose-6-phosphate[32,36]. Metabolic flux analysis **(Figure 9)** shows the entry of trehalose into glycolytic pathway can convert glucose to continue glycolysis resulted to ethanol production from pyruvate by increasing initial glucose concentration inside the cell.

In *E. coli* the trehalose biosynthesis operon can be induced by entry into stationary phase by cell[37] as there is trehalose in the media as a supplement. Trehalose is converted to glucose may induce the production of ethanol at stationary phase. This conversion of *E. coli* to ethanolic fermentation may open new advantages for the production of recombinant products by using *E. coli* expression systems[38].

The exogenous nitrogen source supplementation also enhanced ethanol production in *S. cerevisiae*[26]. In our study different nitrogen sources **(Figure 6)** were used where ammonium sulphate supplement induced cell growth in M9 media. In the case of serine used as an amino acid source increased cell growth in M9 media also consistence with past research, where use of amino acids and sterols have demonstrated higher ethanol yield[34] **(Figure 7)**. *E. coli* DH5α showed decreased cell growth significantly **(Figure 7)** while arginine used in M9 media in our research. Biosynthesis of arginine from glutamate is carried out by a series of reactions initiated by the acetylation of glutamate by *N*-acetyl glutamate synthetase (NAGS) encoded by *argA*[39].

CONCLUSION

In this study, *Escherichia coli* DH5α was used to observe better cell growth at optimized culture condition (pH, temperature, incubation time) and metabolite (ethanol) production under microaerobic condition using different carbon sources (glucose, sucrose, dextrose and lactose). Trehalose supplementation with glucose induced cell growth which enhanced subsequent ethanol production. Finally, this research might be helpful as a base study to formulate and develop industrial production of ethanol which will solve future energy crisis.

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Conflict of interest

No conflict of interest influenced in this research.

Authors' contribution

LWM designed and supervised the project and YAR carried out the laboratory experiments. All authors have contributed in various degrees to the analytical methods used and to the manuscript preparation.

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