PROCEEDINGS OF THE YEREVAN STATE UNIVERSITY

Chemistry and Biology 2020, **54**(1), p. 55–62

Biology

THE UTILIZATION OF CARBON SOURCES MIXTURE (GLUCOSE, GLYCEROL AND FORMATE) AND GENERATION OF FERMENTATION END-PRODUCTS BY *ESCHERICHIA COLI*

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In this study anaerobic utilization of mixed carbon sources (glucose, glycerol, formate) and generation of fermentation end-products by *Escherichia coli* at slightly alkaline and slightly acidic pHs was investigated at the first time. It has been shown that *E. coli* is able to perform co-fermentation of glucose and glycerol in the presence of external formate. The latter was utilized by bacterial cells at first. Acetate is the permanent product (25–50 *mM*) during both glucose and glycerol utilization. It has been revealed that composition of fermentation end-products depends not only on external pH, but also on co-utilization of substrates.

***Keywords*:** co-fermentation, *E. coli*, carbon sources mixture, external formate, pH.

Introduction. Biological organisms acquire ways to adapt to environmental conditions, such as a mixture of different substrates, during evolution. Unicellular organisms, such as bacteria, optimally use external resources for growth. There is a lot of data on metabolic pathways during the utilization of single carbon sources, like sugars, by bacteria [1–3]. Presently, it is vital to explore mechanisms during utilization of mixed carbon sources because the substrates are accessible in the nature in the form of various carbon mixtures with different concentrations [4–6]. Nowadays, thanks to an understanding such mechanisms, bacteria can be used in different types of waste treatment, which is a critical problem [7, 8].

E. coli is a well-studied bacterium and is used for various industrial and commercial purposes [9, 10]. These bacteria are able to utilize mixed carbon sources (carbohydrates, alcohols, acids, etc.), which gives opportunity to apply them in biotechnology. *E. coli* produces molecular hydrogen (H_2) organic acids and ethanol during anaerobic growth as fermentation end products [11]. H_2 is a renewable, ecologically clean energy source and can be used in electricity generation. Bioethanol has great potential to be used as an alternative fuel in the future. Besides, genetically engineered *E. coli* is applied in various valuable chemicals production [12].

E. coli uses glucose and glycerol as a source of carbon and energy in the absence of all exogenous electron acceptors [1, 3]. Whenever glucose is present, *E. coli* metabolizes glucose first before using other carbohydrates [13]. However, bacteria can co-utilize glucose with substrates of other groups, such as glycerol [4].

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The latter is an inexpensive carbon source, because of its generation as a by-product in biodiesel production [3]. The entrance of glucose in *E. coli* cells is performed via the phosphotransferase system. Glycolysis is an almost universal central pathway of glucose catabolism, and many anaerobic microorganisms are entirely dependent on this process [14]. Glucose fermentation end products are mainly acetate, lactate, formate, and succinate as acids, ethanol as alcohol, H_2 and CO_2 as gases [14].

Glycerol metabolism is linked with glycolysis from the phase of glycerol-3-phosphate conversion to dihydroxyacetonephosphate. During glycerol fermentation, the same products were generated as in the case of glucose fermentation, but in different proportions and depending on external pH [2, 15]. CO_2 generated from formate has a regulatory effect on glycerol metabolism and could be used by bacterial cells; unlike H_2, which has a negative effect on glycerol fermentation [2]. The main distinction of mixed-acid fermentation of glucose and glycerol is the lack of lactate synthesis, low generation of acetate and high formation of succinate and ethanol from glycerol at acidic pH. This is due to bioenergetic effects [16].

Weak acids produced during fermentation have impact on intracellular pH and proton gradient. Acid transport can be electroneutral or electrogenic depending on the external pH and the fermentation substrate [15].

Pyruvate synthesis from glycerol provides more reducing equivalents (NADH + H^+) due to the cycle formation compared to utilization of glucose [2, 3]. The conversions of glycerol to ethanol and succinate are redox-balanced processes, meanwhile, ethanol generation is energetically more preferable [2]. Generally, glycerol fermentation has a bioenergetic advantage [3].

One of the substantial products of fermentation is formate, which is produced from pyruvate in non-oxidized form form and permeates to the extracellular environment. After critical reduction of growth medium pH, formate is transported back via specific channels (FocA, FocB) and further oxidized to H_2 and CO_2 through formate-hydrogenlyase (FHL) complex [17–19].

In this study utilization of carbon sources mixture (glucose, glycerol and formate) and formation of fermentation end-products by *E. coli* at slightly alkaline and slightly acidic pH was investigated. Understanding metabolic processes during co-fermentation makes it possible to obtain new approaches to regulate *E. coli* metabolism and expand its use for the production of H_2, ethanol and other chemicals.

Materials and Methods.

Bacterial Strain and Growth Medium. *E. coli* strain BW 25113 (wild type) was used in this study (genotype: *rrnB* Δ*lacZ4787 HsdR514* Δ*(araBAD)567* Δ*(rhaBAD)568 rph-1*) [20].

Overnight anaerobically cultivated bacterial cells were transferred into the high buffered peptone medium containing 20 *g*/*L* peptone, 15 *g*/*L* K_2HPO4, 1.08 *g*/*L* KH_2PO4, 5 *g*/*L* NaCl (pH 7.5) and 20 *g*/*L* peptone, 7.4 *g*/*L* K_2HPO4, 8.6 *g*/*L* KH_2PO4, 5 *g*/*L* NaCl (pH 6.5) with addition of 11 *mM* glucose, 137 *mM* glycerol and 10 *mM* sodium formate as carbon sources. Medium pH was determined by pH-meter via selective pH-electrode (HI1131, “Hanna Instruments”, Portugal) and adjusted by 0.1 *M* NaOH or HCl. Oxygen bubbles were removed from the growth medium by autoclaving (WiseClave WACS-1100, “Daihan Scientific”, South

Korea). Bacteria were grown anaerobically at 37°*C*. Sampling for HPLC was performed regularly until substrates were no longer detected.

The specific growth rate (SGR, μ) was determined spectrophotometrically (600 *nm*) by measuring of bacterial growth medium until the stationary phase using the following formula: $\mu = \ln(OD_1)–\ln(OD_2)/g$, where g is the doubling time of the optical density (OD) [6].

HPLC Setup and Samples Preparation. Separation of compounds was performed on the Agilent 1260 Infinity II LC Bio-inert system using Macherey-Nagel EC 250/4.6 NUCLEOSIL 120-5 C18 column (250×4.6 *mm*, MN720041.46, Germany). The column was cleaned with acetonitrile/water (80/20) at 60°*C* overnight and then regenerated with the experimental conditions using 5 *mM* sulfuric acid as a mobile phase in double-distilled H_2O for a few hours, which is ideally performed after each batch of analysis. For regular column maintenance, a 20 μm inlet filter (5041-2168, "Agilent", Germany) was used.

Sugars and alcohols were monitored using a refractive index detector (Agilent RID, G1362A) set to positive polarity and optical unit temperature of 55°*C* with a mobile phase in the reference cell, while organic acids were monitored using a RID and/or ultraviolet detector at 210 *nm* (Agilent DAD WR, G7115A). A sample volume of 10 μL was injected into the column using a multisampler (Agilent Bio, G5668A), and the column temperature was maintained at 60°*C* using a thermostatically controlled column compartment (Agilent MCT, G7116A).

The analytes were eluted isocratically with 5 *mM* H_2SO_4 at a rate of 0.4 *mL*/*min* for 42 *min*. Chromatograms were integrated using Agilent OpenLAB CDS. Concentrations of dissolved substances were calculated according to standard outcomes.

Samples collected during bacterial cells growth were centrifuged at 3000×g (Thermo scientific Sorvall LYNX 6000, Germany). The supernatant was pre-filtered using 0.22 μm PVDF syringe filter to exclude the presence of bacterial cells in the analytes. 5 *mM* H_2SO_4 was added into each sample.

Carbon conversion efficiency (CCE) was determined by the following formula: $CCE=C_2\times100\%/C_1$, where C_1 is the concentration of carbons in the substrates (*mM*), C_2 is the concentration of carbons in the fermentation end product (*mM*).

Results and Discussion.

Utilization of Mixed Carbon Sources and Generation of Fermentation End-products at pH 7.5. As described above, *E. coli* can utilize a mixture of glucose, glycerol and formate. The utilization of these substrates and the production of fermentation end-products were studied. SGR of *E. coli* bacteria was higher at pH 7.5 than at pH 6.5, which was previously shown during fermentation of other carbon sources [21].

Externally added formate (10 *mM*) was utilized by bacteria in the amount of 2.6 *mM* during the lag phase of bacterial growth. This fact demonstrates previous assumptions that external formate can pass into the cell and have an impact on H_2 production [22, 23]. After 2 *h*, glucose began to be utilized. As a result, one part of the synthesized formate was converted to H_2 and CO_2 by the FHL complex [24], and the second part was accumulated in the bacterial cell and exported to the external environment through specific channels [17]. In the external medium, the

concentration of formate stayed stable in the range of 9–11 *mM* until glucose disappeared. Trace amounts of glucose were found up to 72 *h* (Fig. 1). The main utilization and metabolism of glucose was performed between 2–24 *h* with utilization rate of 0.39 *mM*/*h*. During the mentioned growth period the production of formate, acetate, succinate, lactate and ethanol were detected at rates of ~0.11, ~0.42, ~0.14, ~0.073 and ~0.8 *mM*/*h*, respectively.

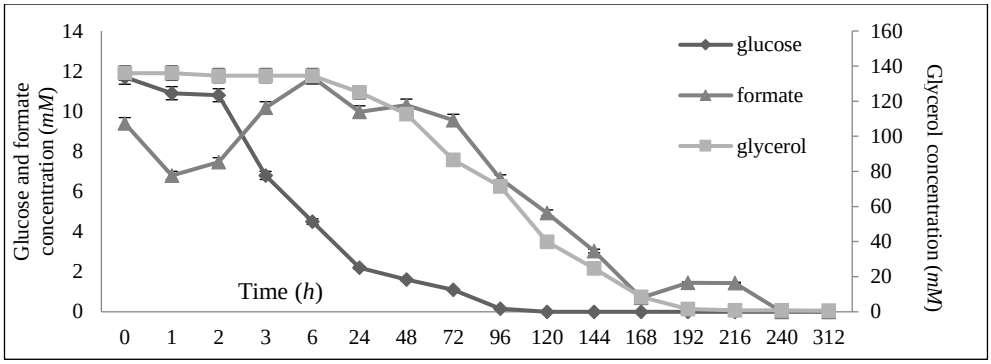

Fig. 1. Utilization of glucose, glycerol and formate by *E. coli* grown at pH 7.5. For others, see Methods.

The utilization of glycerol started after 6 *h* and lasted until 192 *h*. That is why after 192 *h* H_2 was not produced [24], and the external formate was not detected. The glycerol utilization rate was 0.712 *mM*/*h*. During the main utilization of glycerol (24–144 *h*), the formate concentration decreased down to ~5 *mM* (Fig. 1). During glycerol fermentation the following end products were generated: acetate (~0.22 *mM*/*h*), succinate (~0.03 *mM*/*h*), lactate (~0.014 *mM*/*h*), ethanol (~0.58 *mM*/*h*). The carbon conversion efficiency (CCE) of substrates to fermentation end-products during the co-utilization of glucose and glycerol at pH 7.5 is shown in the Tab. 1. The release of acids into the external medium led to external pH decreasing by 0.95 units at 192 *h* [24].

Table 1

Carbon conversion efficiency of glucose and glycerol during co-fermentation at pH 7.5. Calculations were performed according to data for 2–24 h for glucose and 24–192 h for glycerol co-fermentation (for growth condition, see Methods).

	Carbon conversion efficiency, %	
	glucose co-fermentation	glycerol co-fermentation
Formate	3.6	–
Acetate	26.3	18.7
Succinate	17.4	5.1
Lactate	6.5	1.4
Ethanol	50.1	41.7

During bacterial growth, glucose and glycerol co-fermentation was detected at pH 7.5, as was predicted in [4]. Lactate was generated with low amounts, because its high concentration affects the permeability of gram-negative bacteria by disrupting the outer membrane [25]. $NADH+H^+$ is required during the synthesis of ethanol and succinate. In the process of fermentation, the $NADH+H^+$ from glycolysis

is recycled back to NAD^+ so that glycolysis is continuing [26], and this occurs through ethanol and succinate generation. As shown, the amount of succinate produced is lower than ethanol. This is due to one ATP loss during the succinate production, which is not energetically efficient for fermenting cells. Ethanol generation from glycerol is ~4.4 fold higher than that from glucose. Glycerol generates more $NADH+H^+$ during utilization reactions, which leads to ethanol generation to balance cytoplasmic redox state.

Utilization of Mixed Carbon Sources and Generation of Fermentation End -products at pH 6.5. The fermentation process was also examined during the utilization of mixed carbon sources at pH 6.5. The amount of glucose was decreased in the growth medium starting from 2 *h* at pH 6.5. The main utilization of glucose occurred within 2–24 *h* at the rate of 0.45 *mM/h*. However, residual glucose concentrations were detected up to 48 *h*. ~1.43 *mM* of external formate imported into the cells during bacterial growth at first hours (Fig. 2). Formate transportation into the cells decreased by 1.8 fold compared with pH 7.5, which concerns not only external pH, but also intracellular pH, and is suggested to regulate H_2 production and membrane potential [22]. During the glucose fermentation formate (0.13 *mM/h*) acetate (~0.32 *mM/h*), succinate (~0.06 *mM/h*), lactate (~0.29 *mM/h*), ethanol (~0.51 *mM/h*) were produced with respective production rates.

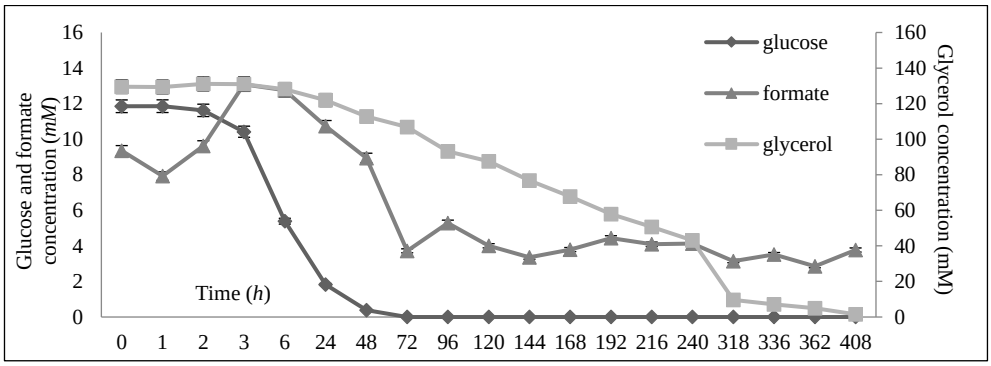

Fig. 2. Utilization of glucose, glycerol and formate by *E. coli* grown at pH 6.5. For others, see Methods.

Glycerol utilization started from 3 *h* and lasted until 408 *h* at pH 6.5. However, the utilization was mainly performed between 3–240 *h* with rate of 0.027 *mM/h* (see Fig. 2). During this period of growth, the production rates of acetate, succinate and ethanol were determined in amounts of ~0.07, ~0.014 and ~0.29 *mM/h*, respectively. As at pH 7.5, during bacterial growth, cells utilized both glucose and glycerol simultaneously at pH 6.5. The accumulation of produced acids acidified the external pH by ~0.7 units [24]. In the Tab. 2, it is shown the CCE of substrates fermented to end-products at pH 6.5 during the co-utilization of glucose and glycerol.

As the data demonstrate, acetate is the permanent end-product during fermentation at both pH 7.5 and 6.5. This is due to the formation of additional ATP and $NADH+H^+$ during acetate generation [27].

As shown, lactate concentration generated from glucose was ~4.3 fold higher at pH 6.5, compared with pH 7.5. Besides, lactate was not generated from glycerol

at pH 6.5, conversely, it was passed back by the bacterial cells at the rate of 0.01 *mM*/*h* rate. The lack of synthesis of relatively oxidized products (acetate, lactate) from glycerol (compared with glucose) reflects the highly reduced state of glycerol [2]. As mentioned, the external formate concentration from glycerol is lower at pH 6.5, compared with pH 7.5. It is due to bacterial cell requirement of CO_2 for growth at acidic pH, which is generated from formate via FHL complex [18, 19].

Table 2

Carbon conversion efficiency of glucose and glycerol during co-fermentation at pH 6.5. Calculations were performed according to data for 2–24 h for glucose and 24–192 h for glycerol co-fermentation (for growth condition, see Methods).

	CCE, %	
	glucose co-fermentation	glycerol co-fermentation
Formate	2.8	–
Acetate	20.1	12.6
Succinate	7.5	5.3
Lactate	27.6	–
Ethanol	31.6	53.4

Conclusion. Taken together, the data obtained show that *E. coli* wild type utilizes the mixture of glucose, glycerol, formate anaerobically, and metabolism is relatively stimulated at pH 7.5. Moreover, it was determined that *E. coli* is able to do co-fermentation and utilize mixed carbon sources simultaneously, regardless of external pH. Glycerol co-fermentation with glucose is more effective at pH 7.5 than at pH 6.5. Meanwhile, in the case of glucose it was shown the opposite effect. The generation of formate as fermentation end-product is little due to its conversion to H_2 and CO_2. Furthermore, the formate concentration decreases during glycerol utilization to provide more CO_2, which is required for glycerol fermentation. Mainly, acetate is generated during co-fermentation for supplying high-energy molecules. Ethanol and succinate are synthesized in relatively high amounts to balance redox state. It can be concluded that fermentation substrates force the cells to survive via balancing end products formation through choosing bioenergetically favorable pathways.

Received 18.01.2020
Reviewed 01.02.2020
Accepted 10.03.2020

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Հ. Խ. ԳԵՎՈՐԳՅԱՆ

ԱԾԽԱԾՆԻ ԱՂԲՅՈՒՐՆԵՐԻ ԽԱՌՆՈՒՐԴԻ (ԳԼՅՈՒԿՈԶ, ԳԼԻՑԵՐՈԼ, ՄՐՋՆԱԹԹՈՒ) ՅՈՒՐԱՑՈՒՄԸ ԵՎ ԽՄՈՐՄԱՆ ՎԵՐՋՆԱՆՅՈՒԹԵՐԻ ԱՌԱՋԱՑՈՒՄԸ *ESCHERICHIA COLI*-Ի ԿՈՂՄԻՑ

Ամփոփում

Այս հետազոտության մեջ առաջին անգամ ուսումնասիրվել է ածխածնի աղբյուրների խառնուրդի (գլյուկոզ, գլիցերոլ, մրջնաթթու) յուրացումն անթթվածնային պայմաններում և խմորման վերջնանյութերի առաջացումը *Escherichia coli*-ի կողմից թույլ հիմնային և թույլ թթվային pH-ի արժեքներում: Ցույց է տրվել, որ *E. coli*-ն ունակ է իրականացնել գլյուկոզի և գլիցերոլի համախմորում արտաքին մրջնաթթվի առկայության պայմաններում: Արտաբջջային մրջնաթթուն առաջինն է յուրացվում բջջի կողմից: Քացախաթթուն հանդիսանում է գլյուկոզի և գլիցերոլի խմորման ժամանակ մշտապես առաջացող արգասիք (25–50 *մՄ*): Բացահայտվել է, որ խմորման վերջնանյութերի բաղադրությունը կախված է ոչ միայն արտաբջջային pH-ից, այլ նաև համախմորվող ելանյութից:

Э. Х. ГЕВОРГЯН

УТИЛИЗАЦИЯ СМЕШАННЫХ ИСТОЧНИКОВ УГЛЕРОДА (ГЛЮКОЗА, ГЛИЦЕРИН, ФОРМИАТ) И ОБРАЗОВАНИЕ КОНЕЧНЫХ ПРОДУКТОВ ПРИ БРОЖЕНИИ У *ESCHERICHIA COLI*

Резюме

В этой работе впервые исследована анаэробная утилизация смешанных источников углерода (глюкоза, глицерин, формиат) и образование конечных продуктов сбраживания *Escherichia coli* при слабощелочном и слабокислом pH. Показано, что *E. coli* способна осуществлять смешанное сбраживание глюкозы и глицерина в присутствии экзогенного формиата. Бактериальные клетки используют экзогенный формиат в начале роста. Ацетат (25–50 *мМ*) является постоянным продуктом, образующимся при сбраживании как глюкозы, так и глицерина. Обнаружено, что состав конечных продуктов брожения зависит не только от значения pH среды, но и от смешанного сбраживания субстратов.