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Oxidative and Reductive Routes of Glycerol and Glucose Fermentation by *Escherichia coli* Batch Cultures and Their Regulation by Oxidizing and Reducing Reagents at Different pHs

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Abstract Glycerol and glucose fermentation redox routes by *Escherichia coli* and their regulation by oxidizing and reducing reagents were investigated at different pHs. Cell growth was followed by decrease of pH and redox potential (E_h). During glycerol utilization at pH 7.5 ΔpH, the difference between initial and end pH, was lower compared with glucose fermentation. After 8 h growth, during glycerol utilization E_h dropped down to negative values (−150 mV) but during glucose fermentation it was positive (+50 mV). In case of glycerol H_2 was evolved at the middle log phase while during glucose fermentation H_2 was produced during early log phase. Furthermore, upon glycerol utilization, oxidizer potassium ferricyanide (1 mM) inhibited both cell growth and H_2 formation. Reducing reagents DL-dithiothreitol (3 mM) and dithionite (1 mM) inhibited growth but stimulated H_2 production. The findings point out the importance of reductive conditions for glycerol fermentation and H_2 production by *E. coli*.

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Introduction

Glycerol is known as a cheap and abundant carbon source due to its generation as an unavoidable by-product of biodiesel and fuel production [7, 11, 35]. Crude glycerol is attractive feedstock in fermentation processes: glycerol can be used by many bacteria as a carbon source for various valuable and interesting end-products including molecular hydrogen (H_2) [7, 11, 35]. Only recently Gonzalez and co-workers [8] have discovered that glycerol dissimilation by *Escherichia coli* can take place in a fermentative manner. Study of *E. coli*-based platforms to convert low value raw glycerol to higher value reduced chemicals, fuels, or even to bacterial biomass is very attractive for biotechnology [10, 11, 16]. On the other hand, *E. coli* is the best-characterized bacterium and promising for glycerol utilization because it is one of the most commonly organisms used for metabolic engineering and industrial applications [10, 11, 16].

Glycerol metabolism in *E. coli* represents a relatively simple cluster of redox reactions leading to 3-phosphoglyceraldehyde, the entry point to the lower section of glycolytic pathway [5, 22]. These reactions result formation of different organic (acetic, succinic) acids and mostly ethanol, evolution of H_2 and CO_2. Interestingly, H_2 produced at acidic pH has negative impact on cell growth and glycerol fermentation [8, 16]. Depending on medium composition, H_2 could be also evolved at alkaline pH [29, 30]. However, the pH dependence of glycerol fermentation and H_2 production is not clear.

Besides, bioenergy is frequently stored and released by means of redox reactions. The ability of bacteria to carry out redox reactions depends on the redox state of the environment, or its redox potential (E_h) [32]. The latter itself depends on rate of redox processes. This simple relationship is hard to understand and to apply in biotechnology.

The metabolism of bacteria is known to represents a set of redox processes affecting E_h. The drop of E_h (down to −550 to 600 mV) during anaerobic growth of bacteria in the absence of external electron acceptors has been well demonstrated [1, 2, 18, 20, 26, 32, 33]. Drop of E_h might be a result of the secretion of redox active metabolites into the culture medium, leading to decrease of external pH [1, 2, 19] or might be connected with the processes on (in) the bacterial membranes [12, 15]. On the other hand, E_h is suggested to be useful for monitoring changes in the metabolic state of bacterial cultures in biotechnology and for optimizing yield of fermentation products [13, 24]. Moreover, E_h has been shown can be applied to discriminate among species of bacteria [6, 26].

In this article, absolutely novel data about oxidative and reductive processes during anaerobic utilization of glycerol by *E. coli* at a wide range of pH (5.5–7.5) are presented and compared with the fermentation of the other carbon source such as glucose. The kinetics of E_h during growth of bacteria upon glycerol fermentation was first studied: significant differences in E_h kinetics between glycerol and glucose fermentation by bacteria were observed. The effects of pH on *E. coli* growth and its link with E_h were also established. Moreover, oxidizers and reducers were used for application of positive and negative E_h values, respectively. Although bacterial growth was suppressed upon both the oxidative and reductive stresses, the reductive conditions only stimulated H_2 formation during glycerol fermentation.

Materials and Methods

Bacterial Strain and Growth, pH Determination

The *E. coli* BW25113 (*lacI*^{q} *rrnB*$_{T14}$*ΔlacZ*$_{W116}$ *hsdR514 ΔaraBAD*$_{AH33}$ *Δrha BAD*$_{LD78}$) was supplied by Prof. T.K. Wood (Department of Chemical Engineering, Texas A & M University College Station, TX, USA; presently Department of Biotechnology, Pennsylvania State University, University Park, PA, USA). Bacteria were grown in batch culture under anaerobic conditions at 37 °C in peptone medium (20 g/l peptone, 2 g/l K_2HPO_4, 5 g/l NaCl) with glycerol or glucose of different concentrations from 1 to 15 g/l at different pHs. The pH was adjusted by 0.1 M NaOH and 0.1 N HCl and measured using a pH meter with selective pH electrode (HJ1131B, HANNA Instruments, Portugal). Optical density (OD) was measured by a spectrophotometer at a wavelength of 600 nm.

E_h Measurements and H_2 Production Assays

E_h was measured by use of redox platinum (Pt) (EPB-1, Measuring Instruments State Enterprise (MISE), Gomel, Belarus, or PT42BNC, HANNA Instruments, Portugal) and titanium–silicate (Ti–Si) electrodes (EO-02, MISE, Gomel, Belarus). Ag/AgCl (saturated by KCl) electrode was as reference electrode. In contrast to Pt electrode sensitive to H_2 and oxygen, Ti–Si electrode measures the overall E_h and is not affected by the presence of H_2 (or oxygen); this is allowing H_2 detection under anaerobic conditions [22, 30]. H_2 production rate (V_{H_2}) is expressed as difference between Pt and Ti–Si electrodes readings in mV in time per unit of OD.

Before assays, E_h of two electrodes were checked in the control solution which was comprised by the mixture of 0.049 M potassium ferricyanide ($K_3[Fe(CN)_6]$) and 0.05 M potassium ferrocyanide ($K_4[Fe(CN)_6]\cdot 3H_2O$) (pH 6.86). E_h of both electrodes at 25 °C was of 245 ± 10 mV. Note that there are no significant differences between Pt and Ti–Si electrodes readings were detected during H_2 assays in bacterial suspension without carbon source added; bacterial count alteration in the suspension by ~eightfold to tenfold had no marked effect on E_h value [30]. Moreover, the determination used is closed to the method with Clark-type electrode employed by Noguchi et al. [17]. These authors had shown a strong correlation between E_h and H_2 production in liquid media.

The H_2 yield is calculated by the decrease of E_h to low negative values as described by Piskarev et al. [21]; it is expressed in mol L^{-1}. Note that the E_h decrease by H_2 evolution did not depend on salt content in water solution; pH was not affected by H_2 supplemented [21]. Moreover, H_2 yield of 0.73 mol L^{-1} (at pH 6.5) (Table 1) was close to that of 0.90 mol L^{-1} determined by using different method—gas chromatography [16].

Others, Reagents, and Data Processing

All assays were done at 37 °C. DL-dithiothreitol (DTT), sodium dithionite, meat peptone, potassium ferry and ferro cyanide were from Sigma (USA), glycerol was from Unichem (China); other reagents used were of analytical grade. Data were averaged from duplicate- or triplicate-independent measurements, for which the standard errors do not exceed 3 % (if not indicated).

Results and Discussion

The Effects of Growth Medium pH on *E. coli* Growth During Glycerol and Glucose Fermentation and E_h Drop

In this study, the lowering of medium pH and the drop of E_h was observed with increase of bacterial count (OD) in *E. coli* BW25113 batch culture at different pHs (Figs. 1, 2, 3). Interestingly, in contrast to glycerol upon increasing glucose concentration (1–15 g/l) bacterial yield was increased; maximal growth was observed for glucose concentration range of

Table 1 The effects of reducers and oxidizer on H_2 yield by *E. coli* BW25133 during log phase growth under glycerol fermentation at different pHs

Growth medium pH	Assay pH	H_2 yield (mol L^{-1})			
		Control	Reducers		Oxidizer
			DTT[c]	Dithionite[d]	Ferricyanide[d]
5.5	5.9[a], 5.8[b]	0.10 ± 0.01[a]	0.80 ± 0.04[b]	ND	0.00[c]
6.5	6.3[a], 6.4[b]	0.73 ± 0.02[a]	1.30 ± 0.04[b]	ND	0.00
7.5	7.2[a], 7.3[b]	0.70 ± 0.03[a]	1.40 ± 0.05[b]	2.00 ± 0.05[c]	0.00

ND not determined

[a] Bacterial culture was of ~8 h growth

[b] Bacterial culture was of ~3–4 h growth

[c] H_2 was not produced

[d] 3 mM DTT, 1 mM dithionite, and 1 mM ferricyanide were added into the growth medium

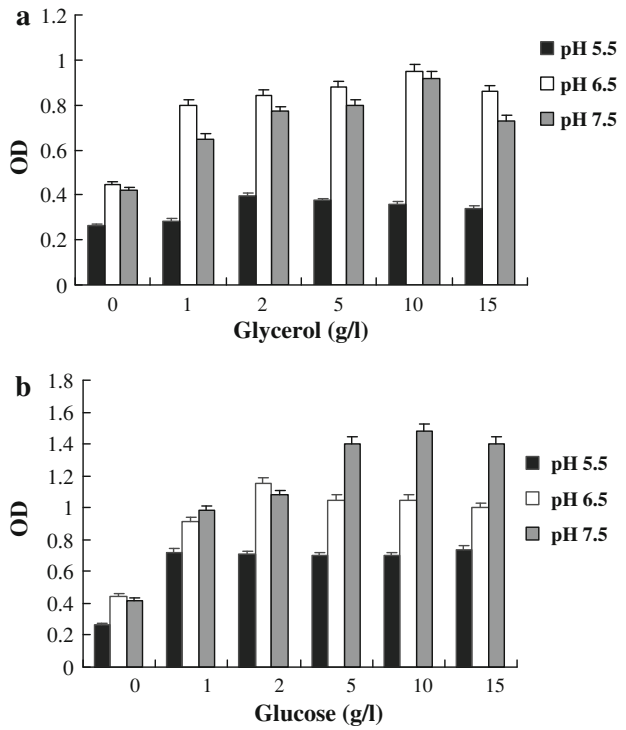

Fig. 1 *E. coli* BW25113 growth yields during fermentation of different concentrations of glycerol (**a**) or glucose (**b**) at different pHs. OD_{600} after 24 h bacterial growth was presented. Bacteria were grown as described in "Materials and methods"

5–15 g/l, especially at pH 7.5 (Fig. 1b). The results can be explained due to low concentration of protons in the external medium at a high pH when fermentation of more substrate is favored. Another explanation of this effect can be that the membrane bound transport proteins and enzymes such as proton and potassium ion translocating F_0F_1-ATPase and TrkA system, respectively, have optimal activity at pH 7.5 [27, 28] and play an essential role during glycerol and glucose fermentation [4]. This might be in favor with operation mode of F_0F_1 in association with TrkA having evidenced thermodynamic and biological consequences [27].

During glucose fermentation *E. coli* exponential growth was usually observed for period of ~5–6 h, starting when the culture was ~2 h and ending at ~7–8 h that was non-depending on pH, whereas in glycerol fermented culture exponential growth was prolonged: it was monitored for period of ~10 h, starting when the culture was of ~2 h and ending ~12 h (not shown). This seems to contradict to data that during glycerol fermentation exponential growth is more prolonged: it lasts ~24 h [9]. This contradiction might be due to differences of growth medium composition. However, maximal yield with glycerol fermented cells was observed at pH 6.5 or 7.5 with all concentrations of glycerol, especially with the concentration of 10 g/l glycerol OD was 0.95 (Fig. 1a). In spite of differences, our findings are consistent with the results obtained by different authors [8, 35] that glycerol was metabolized optimally at pH 6.3.

Effects of pH on *E. coli* growth on glycerol (see Fig. 1) can be explained by many factors: for instance, pH may affect on the activity of glycerol dehydrogenase, which is the main enzyme of glycerol metabolism [9]. The enzyme represents more oxidizing properties at pH 7.5, in contrast to pH 6.5, when it has more reductive properties. On the other hand, alkaline conditions might increase the toxicity of methylglyoxal [9]. Another factor which could contribute to the impairment of glycerol fermentation is a limited generation of CO_2, which is required for growth of bacteria and glycerol metabolism [23].

ΔpH, which is the difference between initial pH and pH value after 24 h, was 4.2- and 3.8-fold lower during glycerol utilization at pH 6.5 and 7.5, respectively, compared with glucose fermentation (Fig. 2). Besides, during bacterial glycerol fermentation at pH 5.5 was not observed medium acidification and, moreover, growth medium pH was increased compared with both glycerol and glucose

fermenting cells. This probably points out differences in fermentation end-products depending on carbon sources and medium pH [8, 14, 16, 22]. Indeed, lower amounts of acetic and less lactic acids could be produced during glycerol fermentation compared with glucose one as shown by Murarka et al. [16]. Another point is that as we have shown recently [4] H^+ extrusion through F_0F_1 and via other ways is lower compared with that under glucose fermentation.

It is worth to mention about interesting observation when bacteria were grown upon glycerol utilization at pH 5.5: in contrast to glucose fermentation during log growth phase *E. coli* has tendency to alkalize medium pH from 5.5 to 6.2 (data not shown). Probably, bacteria become well suited to acidic environment in a manner different from glucose fermented cells or involve other acid resistance mechanisms. In this respect, the intracellular pH might be different between glycerol and glucose fermentation; future determination of intracellular pH is required.

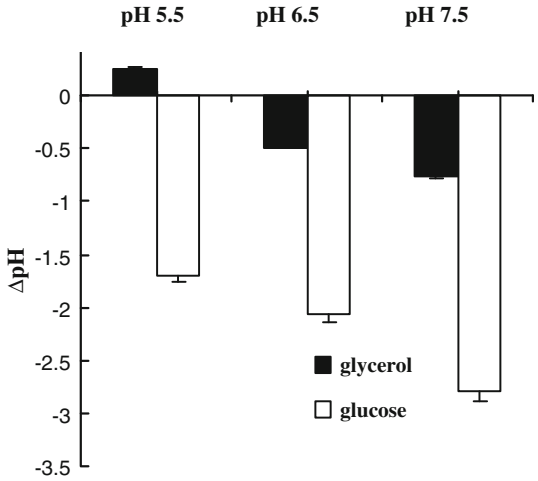

Fig. 2 pH change during *E. coli* BW25113 growth upon glycerol and glucose fermentation at different pHs. ΔpH was the difference between initial pH and after 24 h pH value. 10 g/l glucose or glycerol was added into the growth medium. For others, see legends to Fig. 1

E_h Drop and H_2 Production

E_h dependence on pH in two-based system is determined by the equation

$$E_h = E_0 + (RT/nF)\ln([\text{ox}]/[\text{red}]) + (RT/nF)\ln[H^+]$$

(E_0 is a standard ORP, R, T, and F are known parameters, [ox] and [red] are activities of oxidized and reduced compounds), following E_h decrease with increasing pH [32]. This was checked and confirmed for the assay mixture

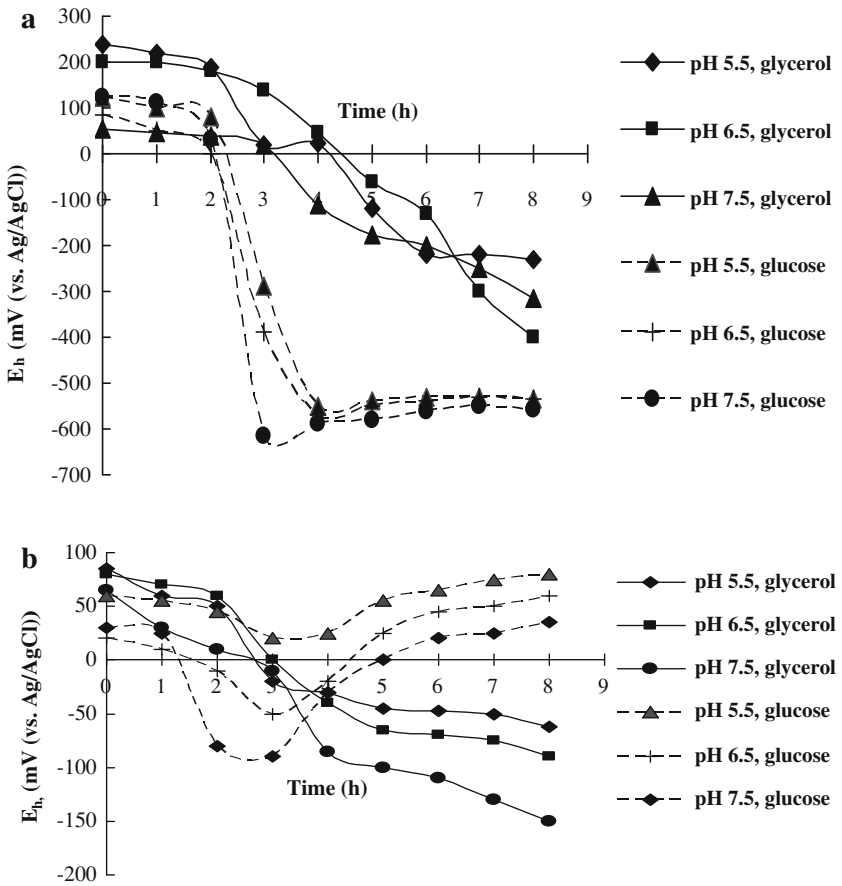

Fig. 3 The kinetics of E_h by *E. coli* BW25113 during glycerol and glucose fermentation at different pHs. E_h, measured by platinum (**a**) and titanium–silicate (**b**) electrodes were expressed in mV (vs Ag/AgCl (saturated by KCl)). Experiments were performed in triplicate, average data are shown. For others, see legends to Fig. 2

without bacteria (not shown). Though, for bacterial suspension, E_h decrease could not be described by the equation above because of complexity of processes during glucose or glycerol fermentation.

In case of glycerol fermentation by *E. coli*, E_h (measured by Pt electrode) dropped down to -400 ± 12 and -315 ± 10 mV when the culture was of 8 h growth only at pH 7.5 and 6.5, respectively (Fig. 3a). Consequently, H_2 production was observed at the middle log growth phase. In glucose fermenting cells E_h dropped to low negative values down to -600 ± 10 mV (Fig. 3b). H_2 production was observed at the beginning of log growth phase, starting when the culture was of $\sim$2–3 h, and at the stationary phase at different pHs. However, V_{H_2} was higher at acidic pH during glucose fermentation and it was decreased upon increasing pH (Fig. 4; Table 1). Interestingly, V_{H_2} was stimulated, even higher at pH 6.5 during glycerol fermentation than it was in glucose fermented culture (Fig. 4; Table 1). These results are expected due to many reasons. Among these are formate hydrogenlyase, which is responsible for H_2 production and has optimal activity at acidic pH [25, 30, 31]. On the other hand, glycerol might be metabolized more rapidly, because of its shorter metabolic pathway compared with glucose [5, 8, 22, 35]. Moreover, maximal H_2 yield was shown to occur at acidic pH [14]. It should be noted that E_h drop for 24 h of growth was less during glycerol fermentation compared with glucose one (data not shown).

Investigating the kinetics of E_h, additional remarkable differences were observed at different pHs: E_h (measured by Ti–Si electrode) with glycerol culture was negative, whereas in glucose culture at the 3 h growth E_h started to increase and after 8 h it became positive (Fig. 3b). But the E_h values for both cases depended on pH. These data confirm the effects of pH on E_h kinetics which was shown with *E. coli* and other bacteria: when growth medium pH was higher, E_h represented more negative values [18, 26, 32, 34].

The effect of substrate on E_h kinetics can be regarded by the properties of glycerol having highly reduced carbon and a higher production of reducing equivalents (NADH/NADPH/$FADH_2$) during its fermentation compared with glucose [8, 35]. The reducing equivalents should have a profound effect on the whole metabolic network, when the higher NADH availability significantly changes the end-products pattern under anaerobic conditions [3, 35].

Effects of Reducing and Oxidizing Reagents on *E. coli* Growth, E_h and H_2 Production During Glycerol Fermentation

E_h determinant role in *E. coli* growth in anaerobic conditions [1, 2, 32] means that various oxidizers and reducers affecting E_h can mediate growth of bacteria. An impermeable oxidizer $K_3[Fe(CN)_6]$ (1 mM) and reducers DTT (3 mM) and dithionite (1 mM) were used for application of E_h initial positive ($+235 \pm 10$ mV) and negative (-250 ± 10 mV) values to bacteria, respectively. In the presence of $K_3[Fe(CN)_6]$ *E. coli* growth at different pHs was inhibited (Fig. 5) resulting in decrease of E_h only to 130 ± 5 mV at the log growth phase. E_h positive or less negative values following the presence of $K_3[Fe(CN)_6]$ may oxidize thiol groups on bacterial surface and affect on membrane transport and enzymes like F_0F_1 or TrkA under anaerobic conditions, leading to low activity of key enzymes in bacterial fermentation metabolism [2].

In order to carry out the metabolic processes efficiently, it is very important to maintain the intracellular environment in the reducing state. However, membrane-permeating reducing reagents DTT (Fig. 5) and dithionite (data not shown), lowering E_h down to enough negative values (-280 ± 12 mV), suppressed the growth of *E. coli* during glycerol fermentation

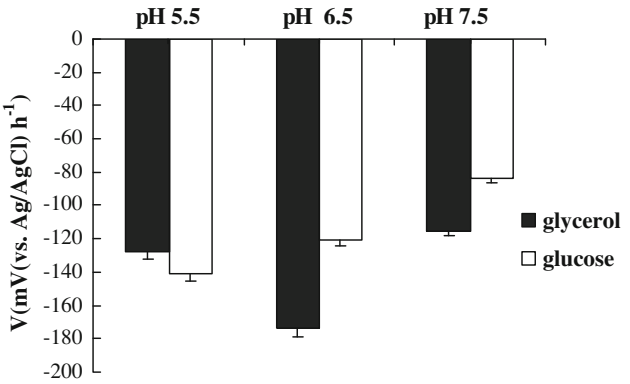

Fig. 4 H_2 production by *E. coli* BW25113 during glycerol and glucose fermentation at different pHs. H_2 production rate (V_{H_2}) was expressed as difference between Pt and Ti–Si electrodes readings per h (for 8 h of growth) and OD. For others, see legends to Fig. 2

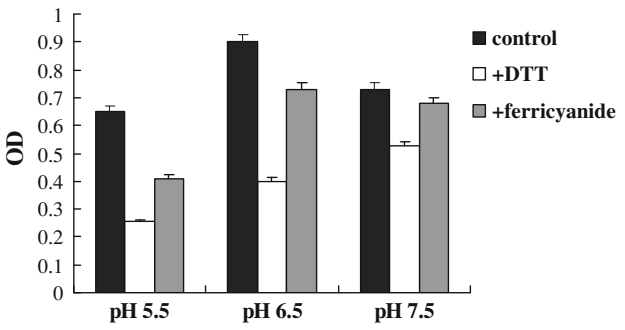

Fig. 5 The effects of ferricyanide and DTT on *E. coli* BW25113 growth during fermentation of glycerol at different pHs. Ferricyanide (1 mM) or DTT (3 mM) was added into the growth medium. OD_{600} was determined after 24 h bacterial growth. For others, see legends to Fig. 2

in a concentration-dependent manner (not shown). These results are obviously expected. When reducing conditions prevail to the extreme, they may lead to instability in the metabolism. On the other hand, it was obtained, that during glycerol fermentation DTT at different pHs and dithionite but not oxidizer at pH 7.5 stimulated ~twofold to threefold H_2 production in the log growth phase (Table 1). Similar effect with DTT was observed previously during glucose fermentation in *E. coli* [12]. This effect may be regarded to the reduced environment which might lead the increase of formate concentration [24] which then can be oxidized to H_2 and CO_2 [5, 22, 25, 31].

Note that the effect of dithionite on bacteria was investigated only at pH 7.5 because of instability of this reagent in acidic environment.

Conclusions

In this study, concentration-dependent glycerol and glucose fermentation by *E. coli* has been investigated. During bacterial growth the decrease of E_h was observed with the acidification of the medium. The kinetics of E_h by *E. coli* was first established during glycerol fermentation. The differences between *E. coli* growth on glycerol and glucose and their oxidative and reductive routes at different pHs were revealed.

The results with E_h drop indicate the strengthening of reduction processes during glycerol fermentation and suggest that appropriate concentrations of carbon sources and reductive conditions are essential for *E. coli* growth and H_2 production. The latter was observed at middle and early log growth phase during glycerol and glucose fermentation, respectively.

Moreover, reducing reagents stimulate H_2 formation during log growth phase upon glycerol fermentation. Probably, (i) this effect was regarded to properties of carbon sources, (ii) this was due to different fermentation end-products secreted to the growth medium or, at last, (iii) this might be related with modification of bacterial membrane structure, particularly of accessible thiol groups number and composition.

All this is important for bacterial cell growth and physiology.

E_h might be an additional physicochemical parameter to take into account for optimizing fermentation processes and developing H_2 production biotechnology. The coupling of H_2 production to utilization of waste materials containing high concentrations of glycerol may simultaneously provide economic and environmental benefits.

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