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Adaptive Responses in the Metabolism of *Escherichia coli* in View of Gene Expressions under Aerobic and Micro-aerobic Condition

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Abstract The major aims of biology to understanding life at a systems level. Escherichia coli is a metabolically versatile bacterium able to respond to changes in environmental factors availability. The effect of pH downshift on fermentation characteristics was investigated in a continuous culture of *Escherichia coli* at aerobic and micro-aerobic conditions. Regardless of oxygen availability, higher levels of acetate were associated with lower biomass yields and lower glucose consumption rates at pH 5.5 as compared to the observations made at pH 7.0. Observed gene expressions indicated that the down- regulation of the glucose uptake rate corresponded to the down-regulation of *ptsG* gene expression which in turn was caused by the up-regulation of *mlc* gene under the positive control of Crp. In accordance with up-regulation of *arcA* gene expression at acidic conditions, the expressions of TCA cycle-related genes such as *icdA* and *gltA*, and the respiratory chain gene *cyoA* were down-regulated, whereas *cydB* gene expression was up-regulated. Decreased activity of the TCA cycle caused more acetate formation at lower pH levels. Under micro-aerobic condition, higher levels of formate and lactate were produced at lower pH due to up-regulation of *pflA*, *yfiD* and *ldhA* genes. Meanwhile, lower levels of ethanol were produced due to the down-regulation of *adhE* gene at lower pH, as compared to the observation at neutral pH. The combined effect of pH and temperature on gene expression was also investigated and observed that decreases in the specific glucose consumption rate were associated with increases in the specific acetate production rate. This type of information is useful for the production of recombinant proteins, bio-molecules, simultaneous saccharification and fermentation (SSF) and strain improvement.

Keywords: *Escherichia coli, Gene expression, micro-aerobic condition, simultaneous saccharification and fermentation*

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1. Introduction

The ability of bacterium to respond rapidly and effectively to environmental perturbation is a distinguishing and vital aspect of their physiology. The molecular toolbox, enabling genetic engineering and studying of regulation and gene expression, is presumably the largest that exists for one particular organism [1]. Hence, Escherichia coli have been widely used by applied microbiologists to try to steer the metabolism of this organism toward the production of molecules with biotechnological value. *E. coli, a facultative anaerobe* and other related enteric bacteria show a number of genetic responses to pH changes in their growth environment by regulating gene expression [2,3,4] and protein profiles [5]. Although most acid tolerance systems are activated at the late growth phase and/or the stationary phase, acid tolerance is also observed in the exponential growth phase of *E. coli* under aerobic conditions and this is advantageous from the productivity point of view. Some of these acid resistance systems depend on the available extracellular amino acids such as glutamate, arginine and lysine. In this system, the intracellular proton is consumed by the reductive decarboxylation of the amino acid followed by the excretion of the product, such as γ-amino butyric acid (GABA) from the cytoplasm to the periplasm. This

mechanism has been shown to increase *gadAB*, which encodes glutamate decarboxylase and *gadC*, which encodes the glutamate: γ-amino butyric acid (GABA) antiporter in response to acid stress, heat shock and stationary phase signals in *E coli* [2]. Organic acids such as acetic acid, lactic acid etc. accumulate at the late growth phase or the stationary phase in typical batch cultures, and are known to increase GadA and GadB proteins at low pH [6]. The sigma factor σ^S or RpoS, which increase at the stationary phase of growth, as well as Crp (catabolite receptor protein) are involved in acid resistance [7]. It has also been shown that acid pH lowers cAMP levels in exponentially growing cells in the minimal glucose medium, potentially resulting in the elevation of RpoS. It has also been shown that the two component system of EnvZ (sensor) and OmpR (regulator) regulate protein expression. Given the role of OmpR as a possible key regulator for acid adaptation, the ompR mutant is thus sensitive to acid exposure [8]. The effects of pH on gene expression are also known to be influenced by other environmental factors, most notably the availability of oxygen. The effect of the availability of oxygen on gene expression at low pH has been investigated in a number of studies that focus on protein responses under different environmental conditions using two-dimensional gel electrophoresis (2-D gels) [9]. Under anaerobic and or micro-aerobic conditions, additional genes, such as *ackA*, *lpdA*, and *ompT* were induced [10]. It has also been shown that, at anaerobic conditions, amino acid decarboxylase is increased [11,12] whereas the oxygen-induced cytochrome *o* is repressed [13]. To avoid deleterious concentrations in the cell caused by the production at low pH, *ldhA* is induced by acid, thus producing lactate instead of the more harmful acetate plus formate [14]. Although the underlying mechanism for this acid tolerance is not yet clear, it could be activated by adapting cells at pH values ranging from 4.5 to 5.8. Despite the wealth of literatures on protein responses under different environmental conditions, only relatively few genomic studies has paid attention on the effect of pH downshift on *E. coli* metabolism. The effect of pH on metabolism is of practical interest, since culture pH is often not controlled in the industry. Meanwhile, the production of recombinant proteins and organic acids such as ethanol, lactate etc. by microbial fermentation is a widely used process in pharmaceutical, food and chemical industries. This necessitates the need to find favorable conditions (eg. temperature, pH etc.) for both the enzymatic hydrolysis and fermentation process in simultaneous saccharification and fermentation (SSF). In the present study, therefore, we investigated metabolism changes in *E. coli* at low pH with respect to gene expressions under aerobic and micro-aerobic conditions. The study also investigated the combined effects of both pH and temperature on *E. coli* metabolism.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions:

The strains used were *Escherichia coli* BW25113 ($lacI^q$ $rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$) [15]. The inoculum was prepared by transferring cells from a glycerol stock (0.1 ml) to a 50 ml L-shaped test tube containing 10 ml of Lysogeny broth (LB) medium and incubating overnight at 37° ubsequently, 1 ml of the culture broth was transferred into a 500 ml T- shaped flask containing 100 ml LB medium. Synthetic M9 minimal media ontaining 10 g/L glucose, 48 mM Na_2HPO_4, 22 mM KH_2PO_4, 10 mM NaCl, and 30 mM $(NH_4)_2SO_4$ was used as the main culture medium. Additionally, the following components were filter sterilized and then added (per liter of final medium): 1 ml of 1 M $MgSO_4$, 1 ml of 0.1 mM $CaCl_2$, 1 ml of 1 mg of Vitamin B_1 per liter, and 10 ml of trace element solution containing (per liter) 0.55 g of $CaCl_2$, 1 g of $FeCl_3$, 0.1 g of $MnCl_2.4H_2O$, 0.17 g of $ZnCl_2$, 0.043 g of $CuCl_2.2H_2O$, 0.06 g of $CoCl_2.6H_2O$ and 0.06 g $Na_2MoO_4.2H_2O$. Using a 1 L working volume, continuous cultivations were conducted at a dilution rate of 0.2 h^{-1} in a 2 L jar fermentor (M-100, Rikakikai Co. Tokyo, Japan). The culture pH was kept constant at 5.5± 0.05, 6.0± 0.5 or 7.0± 0.05 by adding either 2N NaOH or 2N HCl. Culture temperature was maintained at 37°C ± 0.5°C or 42 ± 0.5°C. Air flow rate was maintained at 1 L min^{-1} and an agitation speed of 350 rpm was selected to ensure that the dissolved oxygen level remains about 30 - 40% of air saturation in the aerobic cultivation. Micro-aerobic cultivation was initiated by aerobic cultivation for 2 h followed by a stoppage in the supply of air and reduction in the agitation speed to around 100 rpm, such that the cultivation was nearly anaerobic. CO_2 and O_2 concentrations were measured using the off-gas analyzer (DEX-2562, ABLE Co., Japan).

2.2. Determination of Biomass and Extracellular Metabolite Concentrations

Cell concentration was determined using a spectrophotometer (Ubet-30, Jasco Co., Tokyo, Japan) by converting the optical density (OD) of the culture broth at a wave length of 600 nm to dry cell weight (DCW) per liter. This approach is based on the previously reported relationship between OD and DCW. Glucose concentration was measured using enzymatic kit (Wako Co., Osaka, Japan). Acetate, formate, lactate, succinate, and ethanol concentrations were also determined using enzymatic kits (Boehringer Co., Mannheim, Germany).

2.3. RNA Isolation, cDNA Synthesis and PCR Amplification

Exactly 2.5 µl of culture broth was suspended into 5 µl RNAprotect bacteria reagent. The samples were kept on ice for 5 minutes. After centrifugation at 10,000 rpm (4°C, 10 min), the supernatant was discarded, and the pellet was stored in RNA isolation at -80°C until used. Total RNA was isolated from *E. coli* cells using Qiagen RNeasy Mini Kit (QIAGEN K.K., Japan) according to the manufacturer's recommendation. The quality of extracted RNA was determined by the optical density measurements at 260 and 280 nm, as well as from bands obtained on 1% formaldehyde agarose gel electrophoresis. The sequences of the primers used in the present study have been previously reported [16] exception the following:

mlc	5' AGCAGACCAACGCGGGCGCG 3`
	5` GACTATACGCAGGAAGGGCC 3`
gadA	5' CGGATAAACCAAACCTGGTG 3'
	5' GAATTTATCCAGCGCATCGT 3'
yfiD	5' AACTCTTTCTGGCTGCTGG 3'
	5' GATGGTCAGCTGCGGATAT 3'

Gene-specific primer pairs were designed following the criteria described by Sambrook and Russel (2001). The primers used in this study were synthesized at Hokkaido System Science Co. (Sapporo, Hokkaido, Japan). In all cases, the company confirmed the absolute specificity and purity of the primers.

RT-PCR reactions were carried out in a TaKaRa PCR Thermal Cycler (TaKaRa TP240, Japan) using Qiagen One Step RT-PCR kit (QIAGEN K.K., Japan). The 25 µl reaction mixture was incubated for 30 min at 50°C for reverse transcription (cDNA synthesis) followed by 15 min incubation at 95°C for initial PCR activation. The process was thereafter subjected to 30 cycles of amplification which consisted of a denaturing step (94°C for 1 min), annealing step (approximately 5°C below melting temperature of primers for 1 min), and an extension step (72°C for 1 min). Finally in the final extension, the reaction mixture was subjected to a temperature of 72°C for 10 min. To check for nucleic acid contamination from the reaction components, a negative control which lacks the template RNA was run concurrently in every round of RT-PCR. Thereafter, 5 µl of amplified products were run on a 1.8 % agarose gel. Gels were stained with 1 mg mL^{-1} of ethidium bromide, photographed using a Digital Image Stocker (DS-30, FAS III, Toyobo, Osaka, Japan) under UV light and analyzed using Gel-Pro Analyzer 3.1 software (Toyobo, Osaka, Japan). In order to determine the optimal amount of input RNA, two-fold diluted template RNA were amplified in RT-PCR assay under identical reaction conditions to construct a standard curve for each gene product. When the optimal amount of input RNA was determined for each gene product, RT-PCR was carried out under identical reaction conditions to detect differential transcript levels of genes. The gene *dnaA*, which encodes *E. coli* DNA polymerase, was used as an internal control for the RT-PCR determinations since it is not subject to variable expression, i.e. abundant expression at relatively constant rate. The gene expressions are thus presented as relative values to that of *dnaA*. To calculate the standard deviation, RT-PCR was independently performed three times under identical reaction conditions. To ensure that the observed changes were statistically significant, the Student's t-test was applied.

3. Results

3.1. Effect of Culture pH on Metabolism

Presented in Table 1 is the effect of culture pH on the fermentation characteristics. The results obtained indicates that a significantly higher amount of acetate was formed ($p< 0.05$), the cell yield was significantly lower ($p< 0.05$), and the specific glucose consumption rate was significantly lower ($p< 0.1$) at pH 5.5, as compared to the case at pH 7.0.

To clarify the phenomenon of *E. coli* metabolism under low pH, gene expressions were measured by RT-PCR. Figure 1 presents a comparison of gene expressions obtained at two different pH values. Results obtained indicates that significant up-regulation of the expression of *rpoS* ($p< 0.10$), *gadA* (glutamate decarboxylate gene) ($p< 0.05$) and *acs* ($p< 0.05$). These genes are known to be under the control of RpoS.

Figure 1 also shows the up-regulation of *arcA* gene expression ($p< 0.1$), where *arcA* gene product functions as a repressor of such genes as are involved in the TCA cycle under micro-aerobic condition . In accordance with the up-regulation of *arcA*, some of TCA cycle genes such as *icdA* ($p < 0.1$) and *gltA* were down-regulated ($p< 0.05$). Figure 1 also indicates observations from the expressions of the respiratory chain genes. While *cydB* was up-regulated ($p< 0.1$), *cyoA* was down regulated ($p < 0.1$). This may be due to the up-regulation of *arcA* since *cydB* operon is under the positive control of ArcA compared to *cyoA* which is under the negative control of ArcA . The *fnr* gene expression was also up-regulated, as well as *arcA*. This caused the down regulation of *lpdA* and *aceE* gene expressions. Figure 1 also indicates that the expression of *crp* gene, which codes for cAMP receptor protein Crp, was up-regulated ($p < 0.1$). Also, the expression of *sdhC*, which is known to be under the control of Crp was also up-regulated ($p < 0.1$) [17]. Moreover, *mlc* gene expression was higher ($p < 0.05$), and *ptsG* gene expression was lower ($p < 0.05$). This is in line with previously published finding that that *ptsG* is repressed by Mlc [18].

Figure 1 also shows that *cra* (catabolite repressor activator) gene expression was up-regulated ($p<0.1$) whereas glycolysis was repressed where *cra* gene product regulates the carbon flow in such a way that gluconeogenesis was activated. The down-regulations of *pfkA*, *pykA* and *zwf* gene expressions were partly due to up-regulation of *cra*. The gene expressions of *fadR* and *iclR* were also higher ($p< 0.05$, $p< 0.1$) since IclR is known to repress *aceBAK* where FadR activates *iclR*.

Table 2 shows the comparison of the fermentation data at two different pH values in the micro-aerobic condition. It was observed that higher amounts of acetate, formate, and lactate were produced, whereas lower amount of ethanol was produced at pH 5.5, as compared to the case at pH 7.0. Figure 2 compares the gene expressions at two different pH values. The gene expression patterns were similar to the observation in Figure 1 as it indicates that *gadA* ($p<0.1$) and *fnr* ($p< 0.05$) gene expression increased at pH 5.5, as compared to the case at pH 7.0. Also, *arcA* gene expression increased ($p< 0.1$) while the expressions of *icdA* ($p< 0.05$), *aceE* ($p< 0.1$) and *mdh* ($p< 0.1$) genes decreased. It was also observed that *crp* and *mlc* gene gene expression increased ($p< 0.5$) while the expressions of such genes as *ptsG* ($p< 0.05$), *ptsH* ($p< 0.1$), *lpdA* ($p< 0.1$) decreased. Under micro-aerobic conditions, additional changes were observed as *yfiD* and *pflA* gene expressions increased ($p< 0.05$). These genes are involved in formate formation. It was also observed that *ldhA* gene expression increased ($p< 0.05$) whereas *adhE* gene expression decreased ($p< 0.1$) at pH 6.0, as compared to observations made at pH 7.0.

Table 1. Comparison of fermentation parameters under aerobic condition and at different culture pH

Fermentation parameters	Culture pH		% Change
	7.0	**5.5**	
Specific glucose uptake rate (mmol/gDCW/h)	2.46 ± 0.03	2.08 ± 0.02	- 15.44
Specific acetate production rate (mmol/gDCW/h)	0.185 ± 0.02	0.302 ± 0.04	+ 67.77
Specific CER (mmol/gDCW/h)	5.83 ± 0.04	5.12 ± 0.04	- 12.17
Specific OUR (mmol/gDCW/h)	5.74 ± 0.05	5.06 ± 0.05	- 16.21
Biomass yield (gDCW/g substrate	0.421 ± 0.01	0.165 ± 0.02	- 61.92

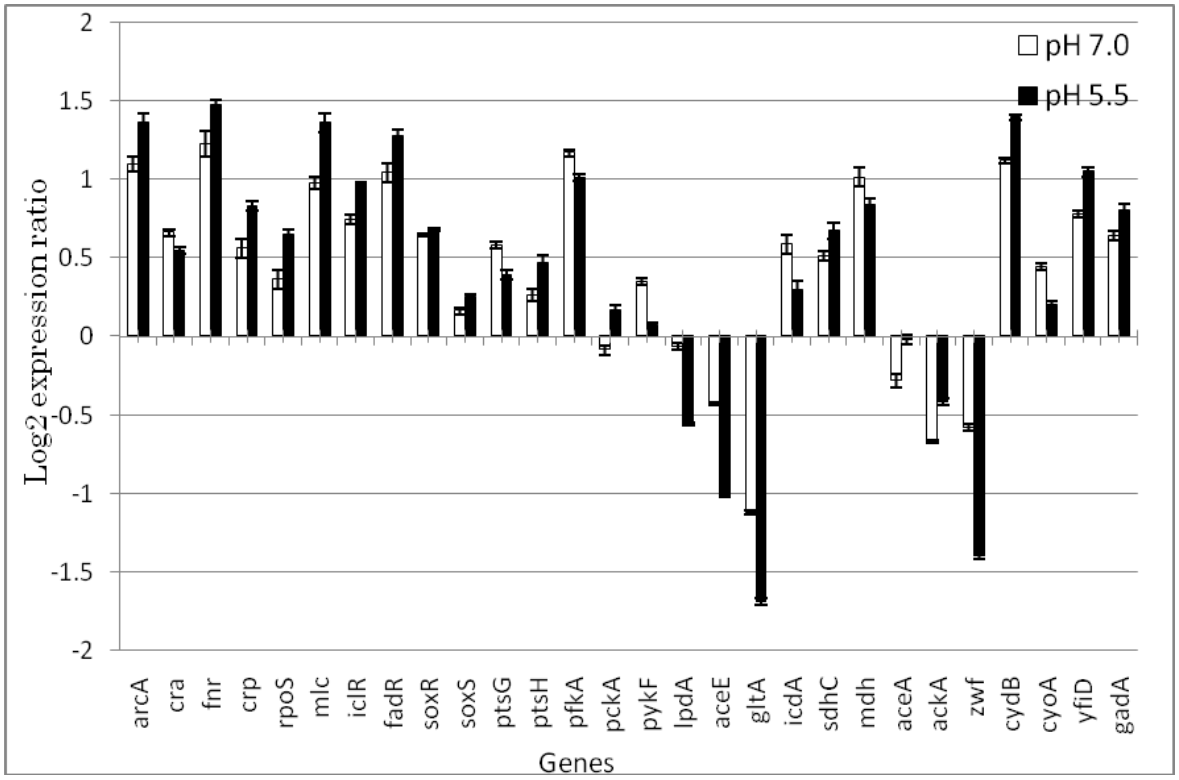

Figure 1. The effect of pH on gene expressions of the wild type *E. coli* BW25113 under aerobic condition

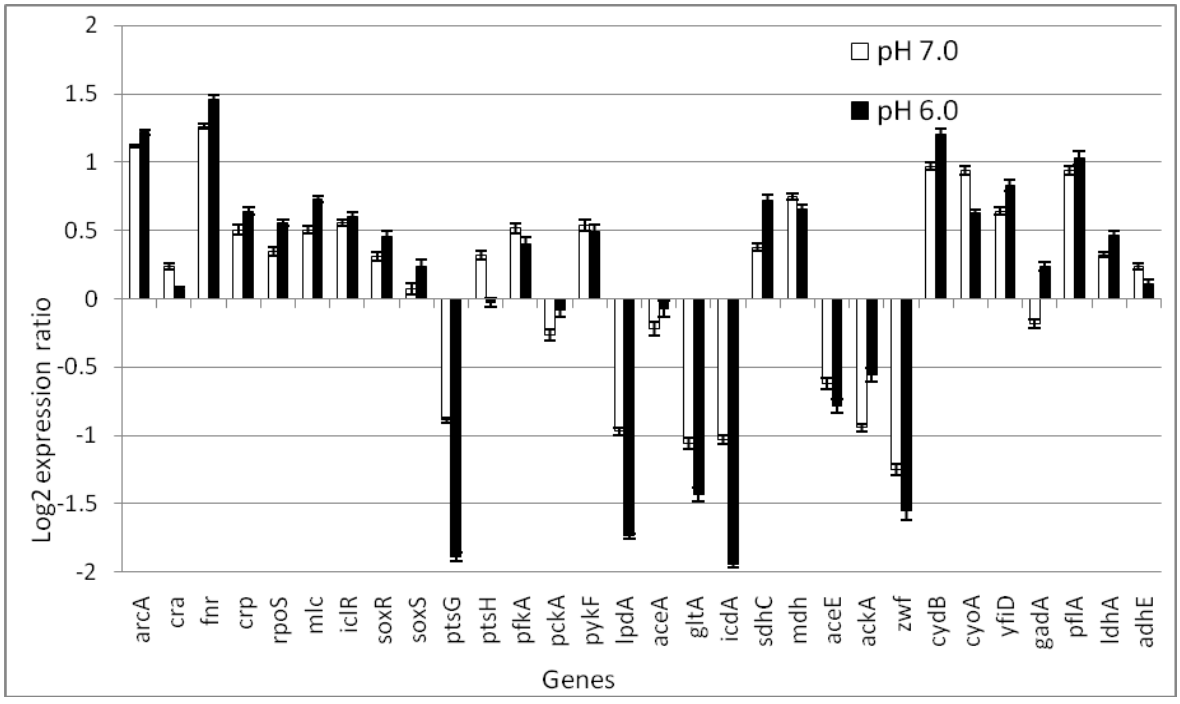

Figure 2. The effect of pH change on gene expressions of the wild type *E. coli* BW25113 cultivated under micro-aerobic condition

Table 2. Comparison of fermentation parameters under micro-aerobic condition and at different culture pH

Fermentation parameters	Culture pH		% change
	7.0	5.5	
Specific glucose uptake rate (mmol/gDCW/h)	4.19 ± 0.02	3.15 ± 0.02	-24.82
Specific acetate production rate (mmol/gDCW/h)	0.324 ± 0.02	0.481 ± 0.03	+48.45
Specific lactate production rate (mmol/gDCW/h)	2.13± 0.02	3.08± 0.02	+44.60
Specific formate production rate (mmol/gDCW/h)	1.63± 0.02	1.87± 0.03	+14.72
Specific ethanol production rate (mmol/gDCW/h)	0.456± 0.04	0.401± 0.03	-12.06
Biomass yield (gDCW/g substrate)	0.401 ± 0.02	0.201 ± 0.03	- 49.87

Table 3. Comparison of fermentation parameters under aerobic condition at pH 7 and 37° versus pH 6.0 and 42°C

Fermentation parameters	Culture pH & Temperature		% change
	7.0 & 37°	**6.0 & 42°**	
Specific glucose uptake rate (mmol/gDCW/h)	2.46 ± 0.02	2.28 ± 0.02	- 7.31
Specific acetate production rate (mmol/gDCW/h)	0.186 ± 0.02	0.307 ± 0.03	+ 65.05
Specific CER (mmol/gDCW/h)	5.83 ± 0.04	5.02± 0.04	- 13.89
Specific OUR (mmol/gDCW/h)	5.72 ± 0.03	5.18 ± 0.04	- 9.44
Biomass yield (gDCW/g substrate	0.425 ± 0.01	0.165 ± 0.02	- 61.17

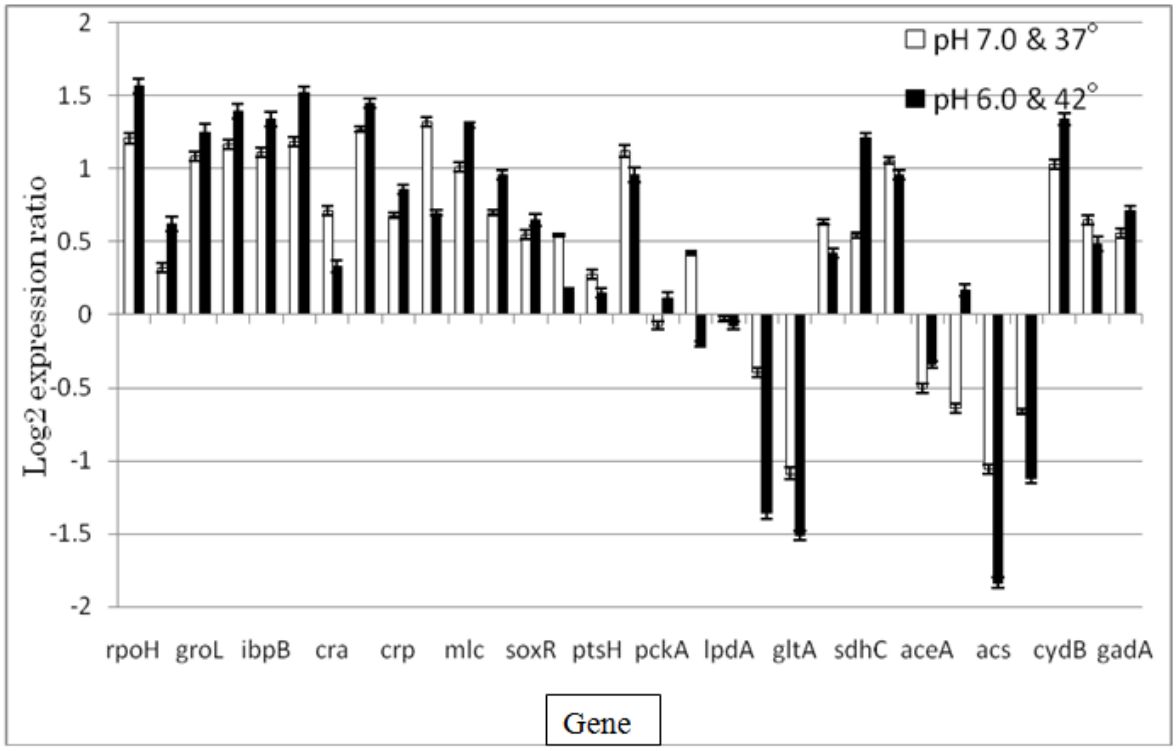

Figure 3. The combined effect of pH and temperature change on gene expressions of the wild type *E. coli* BW25113 cultivated under aerobic condition

3.2. Combined Effect of pH and Temperature on *E. coli* Metabolism

Table 3 shows the combined effects of pH and temperature on the fermentation characteristics. It was observed that decreases in the specific glucose consumption rate were associated with increases in the specific acetate production rate. The specific CO_2 production rate and the cell yield significantly decreased at pH 6.0 and 42°C, as compared to the observation made at pH 7.0 and 37°C. Since cell growth was significantly depressed at pH 5.5 and 42°C, the culture pH at 42°C was set at 6.0. Comparing gene expressions observed in the study (Figure 3), *rpoH* gene expression was found to increase, as well as the up-regulation ($p< 0.05$) of the heat shock genes *dnaK*, *groL*, *groS* and *ibpB* at pH 6.0 and 42°C unlike the observations made at pH 7.0 and 37°C. Also, *arcA* and *cydB* and *fnr* gene expression increased ($p< 0.5$, $p< 0.5$ and $p< 0.1$, respectively) along with a decrease in the expressions of *icdA* and *gltA* ($p< 0.05$ and $p< 0.1$) at pH 6.0 and 42°C. Also, *crp*, *sdhC* and *mlc* gene expression increased $p<0.05$) while *ptsG* gene expression decreased ($p< 0.05$). It was also observed that *lpdA* gene expressions increased ($p<0.1$) It was also observed that *pykF*, and *zwf* gene expressions were down-regulated ($p<0.05$, and $p< 0.1$ respectively) at pH 6.0 and 42°C, as compared to those at pH 7.0 and 37°C.

4. Discussion

It is well known that the Gad gene system is needed for survival under low pH [19]. Cells possess specific defense mechanisms against acid environments in which Gad system has been extensively studied because of its major role in the detoxification of acid-induced stress in *E. coli*. Figure 1 and Figure 2 indicate that *gadA* gene expression increased at pH 5.5 as compared to the case at pH 7.0. GadA and GadB are known to be induced at lower pH as compared to the case at pH 7. Under acid stress, the product GABA is exported by GadC. Anaerobiosis amplifies the acidic induction of amino acid decarboxylases. It has been noted that the acid-induced expression of amino acid decarboxylases is enhanced under anaerobic condition. The gad system (GadA/GadBC) neutralizes acidity and enhances survival in extreme acid; its induction during anaerobic growth may help protect alkaline-grown cells from the acidification resulting from anaerobic fermentation. Figure 1 shows that gadA was also up-regulated even at aerobic conditions.

It was demonstrated in the current study that Fnr is the major activator of *yfiD* expression, but that the extent of Fnr-mediated activation could be modulated by the indirect oxygen sensor ArcA. Moreover, in proteomic analysis, the expression of *yfiD*::*lac* reporter fusion and the intracellular content of YfiD were found to be high during growth at low pH for both aerobic and anaerobic conditions. Figures 1 and 2 show higher expression of *yfiD* at both aerobic and micro-aerobic conditions.

It has been shown that YfiD, a homologue of pyruvate formate lyase, was induced to high levels at pH 4.4 and induced two-fold higher by propionate at pH 6. Both chemicals at low pH cause internal acidification. At neutral or alkaline pH, YfiD was virtually absent and YfiD is, therefore, a strong candidate for response to internal acidification. It has been reported that the expression of cytochrome *o* is repressed by acid stress. Figure 1 - Figure 3 also indicates the down-regulation of *cyo* gene expression, which may be also partly due to up-regulation of *arcA* gene expression. Therefore, there is a complex relationship between *yfiD, cyoA* pH and oxygen level.

The up-regulation of *sdhC* gene at lower pH under both aerobic and anaerobic conditions (Figure 1 and Figure 2) is subject to complex regulatory mechanisms in relation to the respiratory metabolism. The up-regulation of *sdhC* is also partly due to the up-regulation of *crp* gene. The *ptsG* gene expression was down-regulated at lower pH as compared to the case at neutral pH (Figure 1 - Figure 3). This may be due to the up-regulation of *mlc* gene expression, a phenomenon also observed at temperature up-shift [21].

Figure 1 and Figure 2 show that *aceA* is up-regulated. This may be repressed by the up-regulation of *arcA* gene expression. One of the reason why *aceA* increased may be due to the fact that isocitrate lyase (AceA) was induced and showed substantially greater induction in acid or in base condition than at pH 7.

Figure 1 and Figure 2 also indicates the up- regulation of *rpoS* at lower pH. At low pH, acetate as a permeant acid is driven into the cell down the pH gradient and will thus reach far greater cytoplasmic concentrations in acidic media [22,23]. *E. coli* cells initially produce fermentation products such as acetate and other acidic fermentation products which induce stationary-phase stress proteins [24,25]. The growth-phase dependent sigma factor RpoS regulates several components of resistance to both acetate and formate under micro-aerobic condition, which can re-enter the cell and reach deleterious concentrations at low external. At low pH, components of pyruvate dehydrogenase complex (PDHc) gene such as *lpdA* was repressed, whereas fermentative pathways gene *pflA*, as well as *ackA* genes were up-regulated thus leading to more formate and acetate production. It should be noted that *ldhA* is induced by acid in order to produce more lactate instead of acetate.

5. Conclusion

The regulation of cytoplasmic pH in bacteria has long been studied. Little is known about the pH homeostasis of *Escherichia. coli* yet *E. coli* is known to move away from areas of low external pH or weak acids. This indicates that *E. coli* are capable of sensing difference in external pH and reacting to these changes. The present research result clarified the mechanism of metabolic changes upon pH down-shift together with temperature up-shift in relation to gene expressions of heat shock genes, global regulators and the metabolic pathway genes. In particular, the pH down-shift caused the up-regulation of *rpoS* gene, which in turn caused the up-regulation of *gadA* gene expression, and finally protected the cell from intracellular acidification. It was also found that the pH down-shift caused the up-regulation of *fnr* gene expression and activated *yfiD*, as well as *pflA* gene expression leading to the production of higher amounts of formate. Moreover, it was shown that the pH down-shift caused other TCA cycle genes to be repressed due to up-regulation of *arcA* gene. This was indirectly caused by lower dissolved oxygen concentration that led to the production of higher amounts of acetate. These findings are potentially useful for a variety of applications such as temperature-induced heterologous protein productions and solid state fermentation (SSF). In particular, the use of low pH and high temperature conditions in SSF for improved lactate production is suggested since *ldhA* gene is induced upon pH down-shift.

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Conflict of Interest

The authors declare that they have no competing interests.

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