

# Transcriptional Regulation of Exopolysaccharide-related Genes in *Lactiplantibacillus Plantarum* VAL6 Under Environmental Stresses

**Trung-Son Le**

Can Tho University

**Phu-Tho Nguyen**

An Giang University, Vietnam National University of Ho Chi Minh City

**Song-Hao Nguyen-Ho**

Can Tho University

**Tang-Phu Nguyen**

Can Tho University

**Thi-Tho Nguyen**

Hutech University

**My-Ngan Thai**

An Giang University, Vietnam National University of Ho Chi Minh City

**To-Uyen Nguyen-Thi**

Can Tho Science and Technology application Center

**Minh-Chon Nguyen**

Can Tho University

**Quoc-Khanh Hoang**

Institute of Tropical Biology

**NGUYEN Huu-Thanh** (✉ [nhthanh@agu.edu.vn](mailto:nhthanh@agu.edu.vn))

An Giang University <https://orcid.org/0000-0003-0179-0705>

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## Research Article

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# Abstract

Environmental factors can alter exopolysaccharide biosynthesis in LAB. To further clarify this potential relationship, the mRNA expression of exopolysaccharide-related genes such as *glmU*, *pgmB1*, *cps4E*, *cps4F*, *cps4J* and *cps4H* genes in *Lactiplantibacillus plantarum* VAL6 under different conditions including high temperature, acidic or alkaline pH, sodium chloride (NaCl) and carbon dioxide (CO<sub>2</sub>) intensification culture was studied. The transcriptomic data revealed that the exposure of *L. plantarum* VAL6 to acid at pH 3 increased the expression level of *cps4H* but decreased the expression levels of *pgmB1* and *cps4E*. Under pH 8 stress, *cps4F* and *cps4E* were significantly upregulated, whereas *pgmB1* was downregulated. Similarly, the expression levels of *cps4F*, *cps4E* and *cps4J* increased sharply under stresses at 42 or 47°C. In the case of NaCl stress, *glmU*, *pgmB1*, *cps4J* and *cps4H* were downregulated in exposure to NaCl at 7-10% concentrations while *cps4E* and *cps4F* were upregulated at 1 h of 10% NaCl treatment and at 5 h of 4% NaCl treatment. Remarkably, CO<sub>2</sub> intensification culture stimulated the expression of all tested genes. In addition, simultaneous changes in expression of *cps4E* and *cps4F* under environmental challenges may elicit the possibility of an association between the two genes. These findings indicated that the expression level of exopolysaccharide-related genes is responsible for changes in the yield and monosaccharide composition of exopolysaccharides under environmental stresses.

## Introduction

Lactic acid bacteria (LAB) are ubiquitous appearance in food by their generally recognized as safe status (Ismail and Nampoothiri 2010). LAB are capable of producing exopolysaccharides (EPSs) which are widely used in various applications (Baruah et al. 2016). EPS production in LAB has been received a lot of attention in recent years due to the unique biological properties of these biopolymers (Silva et al. 2019). This is exemplified in *Lactiplantibacillus plantarum*, which is commonly utilized in the health and food areas owing to its ability to produce EPSs with specific functions (Silva et al. 2019).

In nature as well as in industrial applications, LAB are often exposed to adverse environmental conditions (Le Marrec 2011). Indeed, LAB have various adaptive mechanisms to protect themselves from environmental stresses including changes related to the expression of genes and proteins (Mbye et al. 2020), to the cell's envelope with enhanced synthesis of EPSs (Huu Thanh et al. 2014). Many studies demonstrated that environmental stresses such as high temperature (Huu Thanh et al. 2014), acidic or alkaline pH, NaCl (Nguyen et al. 2021) and CO<sub>2</sub> (Ninomiya et al. 2009), etc. can stimulate EPS synthesis.

In LAB, EPSs play an important role in protecting the cells from harsh environmental conditions (Nguyen et al. 2020). In addition, EPSs are also involved in the formation of biofilm and adhesion (Caggianiello et al. 2016) as well as in determining cell interaction characteristics (Lee et al. 2016). Nonetheless, the structure and biological function of EPSs may vary depending on environmental conditions (Vu et al. 2009) that related to the transcriptional levels of *eps* genes (Boels et al. 2003).

EPS synthesis in LAB is a complex process involved in the regulation of expression of EPS-related genes (Zeidan et al. 2017). Furthermore, changing environmental conditions can alter the expression level of these genes (Wu and Shah 2018). Therefore, in this study, we evaluated the impact of various environmental challenges on the expression levels of genes involved in EPS synthesis in *L. plantarum* VAL6. The overall aim of the study was to clarify the potential relationships between these regulatory systems and environmental stresses.

## Materials And Methods

### Bacterial strain and culture conditions

*L. plantarum* VAL6 was obtained from Department of Biotechnology, An Giang University, Vietnam National University Ho Chi Minh City, Vietnam. To perform microbial cell culture for this study, *L. plantarum* VAL6 was grown in Man–Rogosa–Sharpe medium (MRS) (De Man et al. 1960). *L. plantarum* VAL6 was stored at  $-80^{\circ}\text{C}$ ; it was rehydrated in MRS broth with 2% (vol/vol) inoculum, followed by incubating at  $37^{\circ}\text{C}$  for 24 h, agitation rate was set up to 250 rpm under aerobic facultative condition.

### Bioreactor operating conditions for stress treatments

The examination was carried out in 5-L bioreactors (BIOSTAT. Sartorius Stadium, Germany). Briefly, 5 L of MRS medium was inoculated with 100 mL of overnight bacterial culture ( $\text{OD}_{595} = 1.5$ ). The pH was maintained to 6.8 by regularly adding 10 M NaOH, the temperature was kept at  $37^{\circ}\text{C}$ , and agitation rate was set up to 250 rpm. After 24 h of culture, stress treatments were then performed independently:

For thermal stress, the culture was treated with high temperature either  $42$  or  $47^{\circ}\text{C}$  for 7 h. The time was calculated when the bioreactor reached the required stress temperature. The pH was maintained to 6.8 and the temperature was kept at  $37^{\circ}\text{C}$ .

For pH stress, the culture was treated with pH conditions either pH 3 or pH 8 for 7 h. The time was calculated when the bioreactor reached the required pH. The temperature was kept at  $37^{\circ}\text{C}$ .

For NaCl stress, the culture was treated by adding NaCl to reach 4–10% concentrations for 7 h. The pH was maintained to 6.8 and the temperature was kept at  $37^{\circ}\text{C}$ .

The non-stress control of these treatments was simultaneously carried out in another bioreactor, where the pH was maintained to 6.8, the temperature was maintained at  $37^{\circ}\text{C}$  for the entire time.

In the case of  $\text{CO}_2$  treatment, right after inoculation,  $\text{CO}_2$  was continuously supplied at the rate  $250\text{ cm}^3/\text{min}$  for 4 h or 8 h. After the set time, the cultures were continued until 24 h without  $\text{CO}_2$  supplement. The 24-hour  $\text{CO}_2$  treatment was supplied with  $\text{CO}_2$  ( $250\text{ cm}^3/\text{min}$ ) during the culture period. The non-stress control of this treatment was also carried out at the same time without  $\text{CO}_2$  supplement.

### Extraction of total RNA and synthesis of first-strand cDNA

Total RNA was extracted according to the instructions of TRIzol reagent (Invitrogen, UK). RNA was treated with RQ1 RNase-free DNase (Promega, Madison, USA) to remove contamination of chromosomal DNA. Qualitative test of RNA at 260 and 280 nm was found to be more than 1.8 using a NanoDrop DeNovix DS-11 Spectrophotometer (DeNovix, Wilmington, DE USA). The first-strand cDNA was synthesized according to the instructions of the GoScript™ Reverse Transcription System Kit (Promega, Madison, USA).

## Design and synthesis of primers

The reference gene was selected from the housekeeping gene, i.e. 16S rRNA. Primers of the six target genes were designed for quantitative real-time polymerase chain reaction (qPCR) analysis based on the genome sequence of *L. plantarum* WCFS1 (Genbank: AL935263.2) using Primer-BLAST software (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The primers were synthesized by The Shanghai Brilliance Biotechnology Co. Ltd.. The primers were designed with sequence length from 18–23 nucleotides, coupling temperature ( $T_m$ ) from 57–60°C and GC rate not more than 70% (Table 1).

Table 1  
The primers used for the amplification

Gene	Primer name	Primer sequence (5'→3')	$T_m$ (°C)	Product length
<i>glmU</i>	<i>glmU</i> sense	ATGCAACAACGGATCAACGC	60.1	475
	<i>glmU</i> antisense	CGACATTCGTGTGGTGCTTG	60.1	
<i>pgmB1</i>	<i>pgmB</i> sense	TCGATGGCGTTATCACGGAC	60.3	423
	<i>pgmB</i> antisense	CGGGATCAGGTTTACCGTGT	59.8	
<i>cps4E</i>	<i>epsE</i> sense	AGTTGACTTCTGACGGTCCC	59.3	449
	<i>epsE</i> antisense	AACCGTTCCCATCAGCATCT	59.4	
<i>cps4F</i>	<i>epsF</i> sense	CTGGGGCTTTACTCCTGGTG	60	452
	<i>epsF</i> antisense	CCCCAACGTCCGATTGAGAA	60	
<i>cps4J</i>	<i>wzx</i> sense	ACGGCTCGATTTTTAGGGCT	59.8	480
	<i>wzx</i> antisense	GACGCTCATTGCGATTGGTG	60.3	
<i>cps4H</i>	<i>wzy</i> sense	TTTGCTTTGGTCATGCTGGC	60	596
	<i>wzy</i> antisense	TCGGACGTTCCGAACCAAAT	60	
16s RNA	16s sense	GCATTAAGCATTCCGCCTGG	60	183
	16s antisense	ACCTGTATCCATGTCCCCGA	60	

## qPCR experiment

The qPCR experiment was performed on an Liberty16 mobile real time PCR system (Ubiquitome Limited, New Zealand), according to the instructions of the SensiFAST SYBR No-Rox Kit (Bioline, Meridian Bioscience, USA). The reaction mixture contained 10  $\mu$ L of SensiFAST SYBR No-Rox Solution (2X), 7.4  $\mu$ L of cDNA template, 0.8  $\mu$ L of forward primer (10  $\mu$ M), 0.8  $\mu$ L of reverse primer (10  $\mu$ M) and 1  $\mu$ L of molecular grade water. As a negative control for all qPCR experiments, cDNA was replaced with RNA (without the reverse transcription step as template). The reaction for each gene was carried out in triplicate in individual qPCR reactions. The PCR amplification conditions were denaturation at 95°C for 3 min, followed by 40 cycles of amplification at 95°C for 30 s, 58°C for 30 s and 72°C for 30 s. Fluorescence performance was obtained at the end of each thermal cycle. The  $2^{-\Delta\Delta Ct}$  method was used to calculate the relative changes in gene expression (Livak and Schmittgen 2001).

## Statistical analysis

The experiments were repeated three times. All the data were expressed as means  $\pm$  standard deviations. Significance of difference was evaluated with one way ANOVA, followed by Fisher's least significant difference (LSD) procedure to identify statistically significant differences at the 95.0% confidence interval. One-way analysis of variance was performed. LSD multiple-range tests were applied to the individual variables to compare means and to assess if there was a significant difference.

## Results

The expression of EPS-related genes under thermal stress

The effect of high-temperature stresses (42 and 47°C) on the expression of EPS-related gene in *L. plantarum* VAL6 was investigated via RNA sequencing. Through analysis and comparison of the gene expression profile of the stressed *L. plantarum* VAL6 and the control (37°C), we found that *cps4F*, *cps4E* and *cps4J* genes were significantly ( $p < 0.05$ ) upregulated in response to thermal stress. Also, the exposure time played an important role in the regulation of these genes (Fig. 1).

The expression level of *cps4E* (Fig. 1d) peaked at 5 h after thermal stress with 13.4 and 38.1-fold under 42 and 47°C treatments, respectively. Similar to *cps4E*, the highest expression level at the same time of *cps4F* (Fig. 1c) was 27.6-fold under 42°C treatment and 32.8-fold under 47°C treatment. However, the expression levels of two genes decreased after exposure to thermal stress for 7 h. Meanwhile, the expression level of *cps4J* was steadily increased over time of stress and reached more than 18-fold after 7 h of treatment (Fig. 1e).

The expression of EPS-related genes under acid or alkaline stress

In order to survive and adapt to acid or alkaline stress, microorganisms have developed complex mechanisms at physiological and molecular levels (Guan and Liu 2020). In this work, the response of *L. plantarum* VAL6 to acid or alkaline stress via the transcriptional analysis of EPS-related genes was also studied. The results disclosed that *cps4H* was significantly ( $p < 0.05$ ) upregulated in exposure to acid at

pH 3. Meanwhile, *cps4F* and *cps4E* were significantly ( $p < 0.05$ ) upregulated in response to alkaline stress condition (Fig. 2).

Under stress at pH 3, there was a great increase in the expression level of *cps4H* (~ 4-fold) (Fig. 2f), but the expression levels of *glmU*, *pgmB1* and *cps4E* decreased (Fig. 2a, Fig. 2b and Fig. 2e). In the case of stress at pH 8, the expression level of *cps4F* increased steadily from 2-fold at 1 h to 3.3-fold at 7 h (Fig. 2c). As a similar pattern with *cps4F*, the expression level of *cps4E* was also rose gradually from 2.8 to 3.9-fold during the time of stress (Fig. 2d).

The expression of EPS-related genes under NaCl stress

The alteration in gene expression under osmotic stress is also to adjust cellular metabolisms (Le Marrec 2011). Therefore, it is important to consider transcriptional regulation in exposure to osmotic stress. In this study, we also investigated the effect of the addition of NaCl at different concentrations on the expression of genes involved in EPS synthesis in *L. plantarum* VAL6. The overall results indicated that the expression of *glmU*, *pgmB1*, *cps4J* and *cps4H* genes were significantly ( $p < 0.05$ ) downregulated in exposure to NaCl at 7–10% concentrations (Fig. 3).

In addition, an increase in the expression level of *cps4F* was detected (~ 1.2-fold) at 1 h of 10%-NaCl treatment and at 5 h of 4%-NaCl treatment (Fig. 3c). Similarly, *cps4E* was upregulated by 1.3 and 1.4-fold at 1 h of 10%-NaCl treatment and at 5 h of 4%-NaCl treatment, respectively (Fig. 3d).

### ***The expression of EPS-related genes under CO<sub>2</sub> intensification culture***

Unlike other stress treatments, CO<sub>2</sub> intensification culture increased the expression of all tested genes between 1.2 and 3-fold. Furthermore, with the exception of *cps4E*, the other genes were upregulated with increasing time of CO<sub>2</sub> supplementation (Fig. 4). The highest expression level of *cps4E* was 1.8-fold upon exposure to 8-hour CO<sub>2</sub> treatment, while its expression returned to 1.5-fold upon exposure to 24-hour CO<sub>2</sub> treatment (Fig. 4d).

## **Discussion**

Changes in environmental conditions may alter extracellular polysaccharide production (Lloret et al. 1998) and induce the synthesis of new type of EPSs in bacteria (Nandal et al. 2005). In addition, the overexpression of a certain gene involved in EPS synthesis can increase or decrease the level of a specific sugar component in EPSs (Nguyen et al. 2021). Hence, based on gene expression profiles, it is possible to predict the alteration of monosaccharide composition in EPSs.

In this study, *glmU* was only upregulated under CO<sub>2</sub> intensification culture but downregulated under exposure to pH 3 and NaCl concentrations at 7–10%. In comparison with a previous study in *Lactobacillus vini*, *glmU* was also downregulated under acid stress conditions (Mendonca et al. 2019). The enzymes encoded by *glmU* are glucosamine-1-phosphate N-acetyltransferase converting

glucosamine-1-phosphate to N-acetylglucosamine-1-phosphate and N-acetylglucosamine-1-phosphate uridyltransferase catalyzing the formation of UDP-N-acetyl-D-glucosamine from N-acetylglucosamine-1-phosphate (Li et al. 2011). UDP-N-acetyl-D-glucosamine is one of the essential precursors of the peptidoglycan structure of cell wall (Chapot-Chartier and Kulakauskas 2014) and the synthesis of repeating units of EPSs (Zeidan et al. 2017). Altogether, we could conclude that exposing *L. plantarum* VAL6 to CO<sub>2</sub> intensification culture impacts on the regulation of *glmU*, possibly leading to an increase in the synthesis of major component of bacterial cell wall and in the content of N-acetyl-D-glucosamine in EPSs.

The *pgmB1* encodes for  $\beta$ -phosphoglucomutase which catalyzes the interconversion of D-glucose 6-phosphate and D-glucose 1-phosphate to form beta-D-glucose 1,6-(bis)phosphate. *pgmB1* plays an important role in the formation of sugar nucleotides as UDP-glucose (Li et al. 2019). Thus, the overexpression of *pgmB1* may result in increased glucose content in EPS composition. In our study, the expression of *pgmB1* increased when *L. plantarum* VAL6 was exposed to CO<sub>2</sub> intensification culture but decreased in exposure to stresses at pH 3 and at 7–10% NaCl. These results were inconsistent with the study of Li et al. (2019) in *L. plantarum* FS5-5, *pgmB* expression increased in response to high salt stress (> 6%) but remained unalterably in response to low salt stress (< 6%) (Li et al. 2019). The difference in the stress method could be the cause of this discrepancy. While Li et al. supplement NaCl right after inoculation, our study stressed after culture for 24 h.

Among the upregulated genes, *cps4E* and *cps4F* encode for glycosyltransferases which transport sugar nucleotides to a lipid carrier to form repeating units (Zeidan et al. 2017). In *Streptococcus thermophilus* Sfi6, *epsE* (*cps4E*) encodes for a priming galactosyl-1-phosphate transferase and *epsF* (*cps4F*) encodes for a galactosyltransferase which adds the branching  $\alpha$ -1,6-galactose (Stingele et al. 1996). In *L. johnsonii* FI9785, *epsE* (*cps4E*) encodes for a glycosyltransferase which adds galactose-1-phosphate to the lipid carrier (Dertli et al. 2013). In this study, with the exception of stress at pH 3, other treatment conditions could enhance the expression of these two genes. Taken together, it was suggested that EPSs isolated from *L. plantarum* VAL6 under environmental stress may be rich in galactose. In addition, simultaneous changes in expression of *cps4E* and *cps4F* under environmental challenges may elicit the possibility of an association between the two genes.

The *cps4J* encoding for flippases which are responsible for taking repeating units and transporting them across the cytoplasmic membrane (Zeidan et al. 2017) was upregulated under stresses of high temperature, and CO<sub>2</sub> intensification culture. Meanwhile, the overexpression of *cps4H* encoding for enzymes which polymerize repeating units (Zeidan et al. 2017) was detected under stress at pH 3 and CO<sub>2</sub> intensification culture. Thus, the enzymes encoded by *cps4J* and *cps4H* are exclusively involved in the secretion of EPSs, which demonstrated that the increase in the expression levels of two genes do not change the sugar composition of the obtained EPSs, but it was related to the resulting EPS yield.

Summary, our results revealed that different environmental conditions can alter the expression level of genes involved in EPS synthesis. Based on achieved results, we propose a profile for the changes in the

expression of EPS-related genes in *L. plantarum* VAL6 by applying environmental challenges (Table 2). The expression of these genes may lead to changes in the monosaccharide composition of EPSs. However, it is necessary to further study the expression levels of the respective proteins in order to better understand the potential relationships between these regulatory systems and environmental stresses.

Table 2

Effects of environmental challenges on the expression of EPS-related gene

Stress	Gene <sup>a</sup>					
	<i>glmU</i>	<i>pgmB1</i>	<i>cps4F</i>	<i>cps4E</i>	<i>cps4J</i>	<i>cps4H</i>
High temperature	–	–	↑	↑	↑	–
Acid pH	↓	↓	–	–	↓	↑
Alkaline pH	–	–	↑	↑	–	–
High-NaCl concentration	↓	↓	↑	↑	↓	↓
CO <sub>2</sub> intensification	↑	↑	↑	↑	↑	↑

<sup>a</sup>↑ upregulation; ↓ downregulation; – non-alteration.

## Declarations

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### Conflict of interest

The authors have no financial conflicts of interest to declare.

### Author Contributions

Conceptualization, T.-S.L, P.-T.N., T.-T.N., H.-T.N.; methodology, T.-S.L, P.-T.N., T.-T.N.; investigation, T.-S.L, P.-T.N., S.-H.N.-H., T.-P.N., M.-N.T., T.-T.N.; data curation, T.-S.L, P.-T.N., T.-U.N.-T., M.-C.N., Q.-K.H., H.-T.N.; writing

—original draft preparation, T.-S.L, P.-T.N., H.-T.N.; writing—review and editing, T.-S.L, P.-T.N., M.-C.N., Q.-K.H., H.-T.N.; supervision, M.-C.N., Q.-K.H., H.-T.N.; funding acquisition, H.-T.N. All authors have read and agreed to the published version of the manuscript.

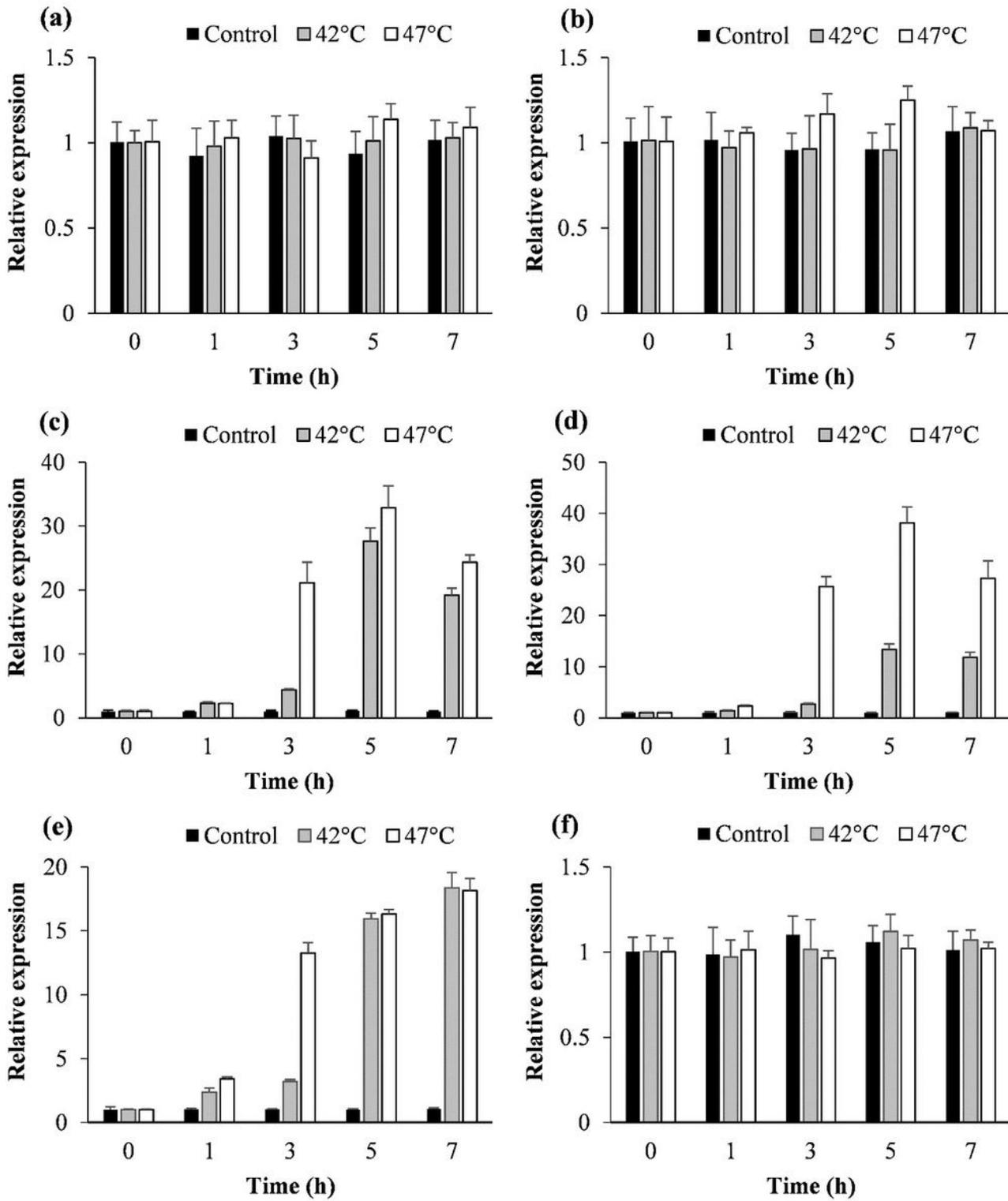
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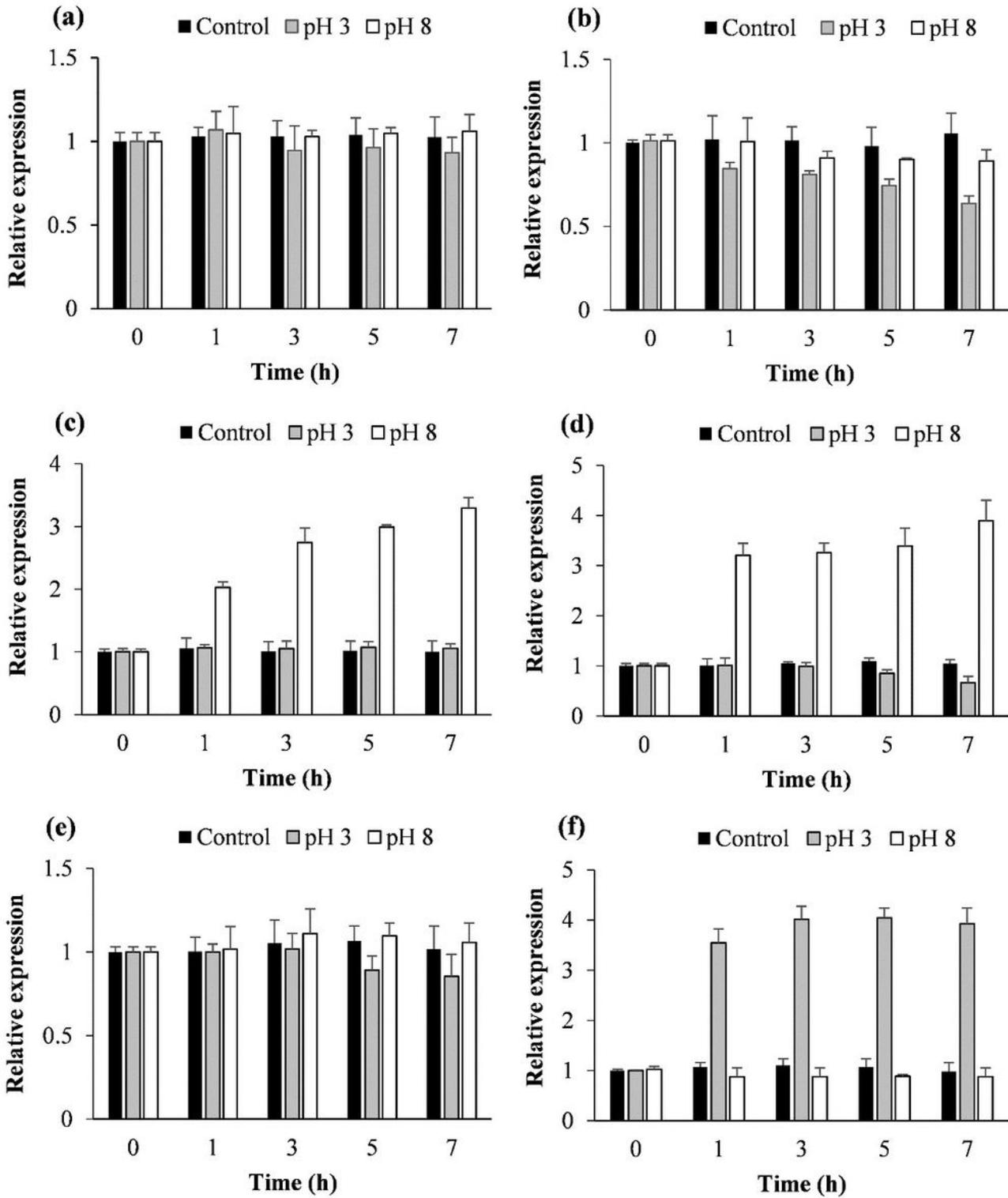
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## Figures



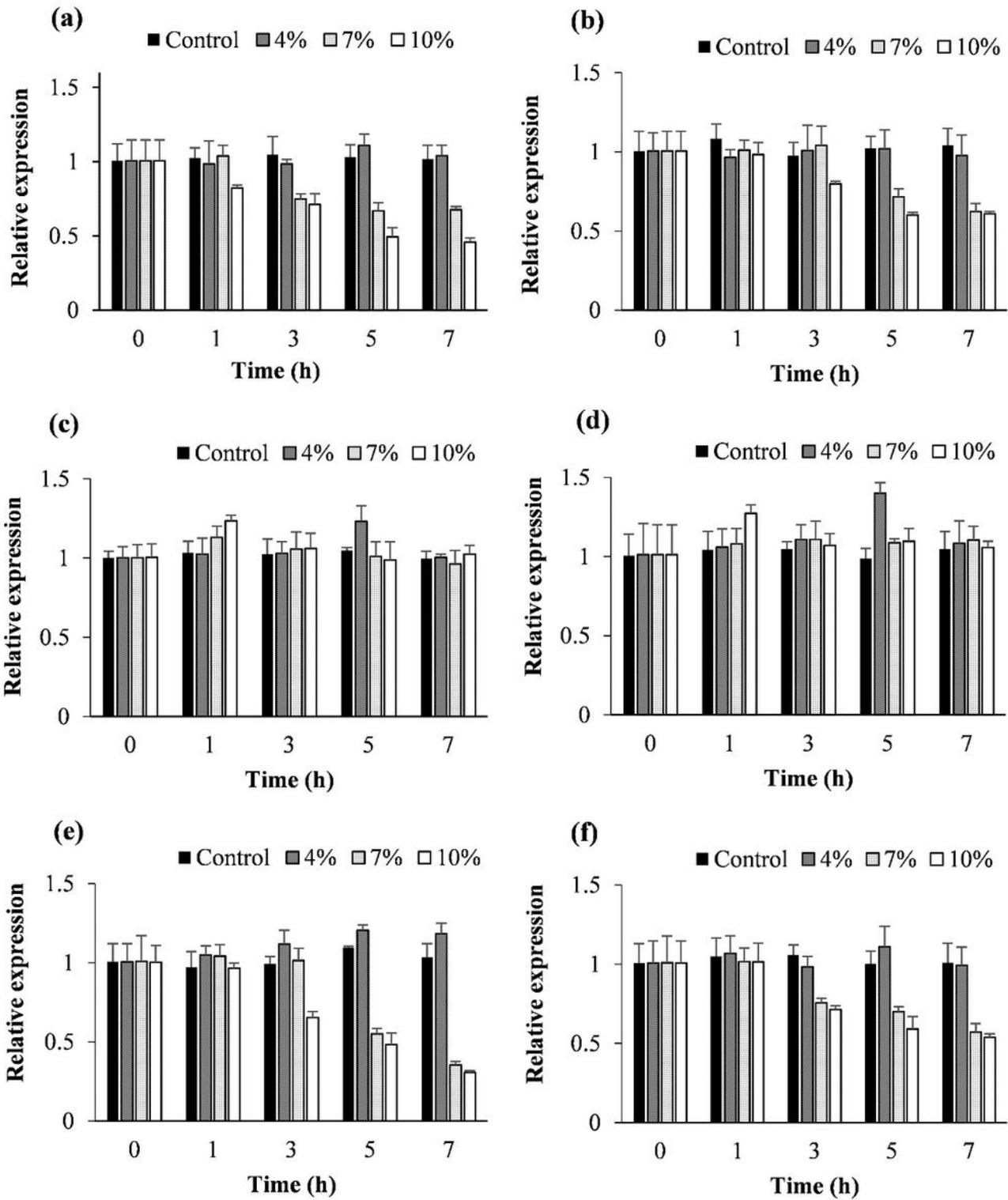
**Figure 1**

The expression of EPS-related genes under thermal stress: (a) *glmU*, (b) *pgmB1*, (c) *cps4F*, (d) *cps4E*, (e) *cps4J*, (f) *cps4H*



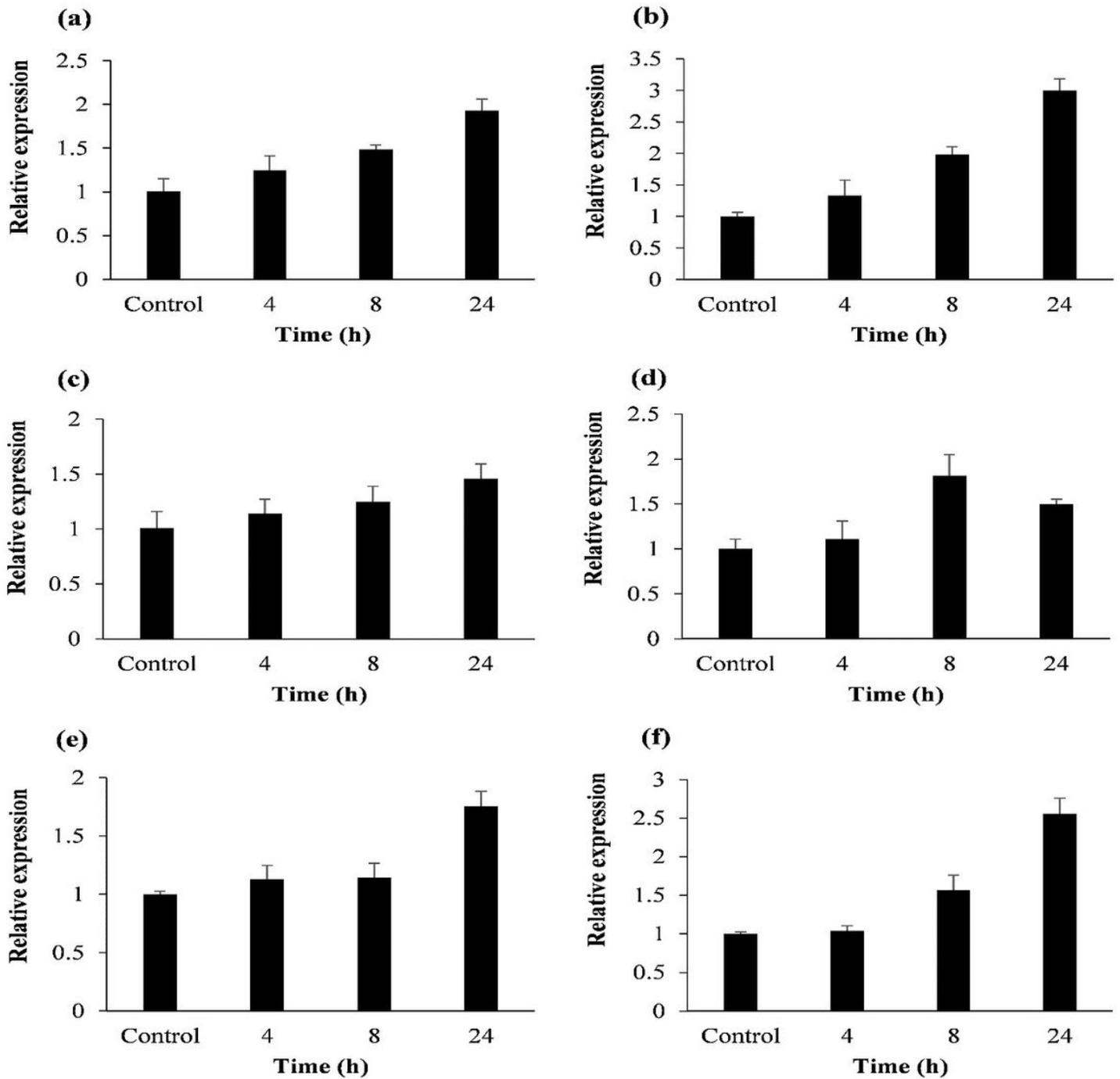
**Figure 2**

The expression of EPS-related genes under acid or alkaline stress: (a) *glmU*, (b) *pgmB1*, (c) *cps4F*, (d) *cps4E*, (e) *cps4J*, (f) *cps4H*



**Figure 3**

The expression of EPS-related genes under NaCl stress: (a) *glmU*, (b) *pgmB1*, (c) *cps4F*, (d) *cps4E*, (e) *cps4J*, (f) *cps4H*



**Figure 4**

The expression of EPS-related genes under CO<sub>2</sub> intensification culture: (a) glmU, (b) pgmB1, (c) cps4F, (d) cps4E, (e) cps4J, (f) cps4H