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Full Length Research Paper

Respective effects of sodium and chloride ion on physiological property of *Zymomonas mobilis* 232B

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Respective effects of sodium and chloride ion on growth, cell morphological changes, membrane disorganization, ion homeostasis, exoenzyme activities and fermentation performance in *Zymomonas mobilis*232B cultures were presented. In batch cultures containing 0.15 M NaCl, *Z. mobilis*232B developed filaments, and growth and ethanol production were inhibited remarkably. Chloride ion (as NH_4Cl) produced similar filaments, while sodium ion (as Na_2SO_4) had little effect on morphology changes, indicating that filament formation in NaCl-inhibited *Z. mobilis* cultures is attributable to chloride ion. Furthermore, growth and ethanol production were more strongly inhibited by chloride ion than sodium ion and the damage of cell membrane under NaCl treatment was mainly caused by chloride ion. NH_4^+ and SO_4^{2-} (as $(NH_4)_2SO_4$) had little inhibitory effects at similar concentration. It was strongly suggested that the inhibitory effects of growth, fermentation performance and morphology changes of *Z. mobilis*232B produced by NaCl were mainly due to the chloride ion.

Key words: Sodium ion, chloride ion, morphological changes, *Zymomonas mobilis*, fermentation.

INTRODUCTION

Recently, growing attention has been devoted to the conversion of biomass into fuel ethanol due to high price and environmental problems caused by fossil fuel. As anaerobic, Gram-negative bacteria, *Z. mobilis* could produce ethanol from glucose via Entner-Doudoroff (E-D) pathway effectively without biomass accumulation, combined with the pyruvate decarboxylase and alcohol dehydrogenase (Sprenger, 1996). It shows 3 to 5 fold higher ethanol productivity compared with traditional yeast fermentations with an ethanol yield of up to 97% of the theoretical maximum value from glucose (Rogers et al., 1982). *Z. mobilis* has other favorable traits such as simple growth requirements, resistance to high ethanol concentrations (up to 12%) and high sugar tolerance (Swings and De Ley, 1977; Sprenger, 1993). Despite these advantages, application of *Z. mobilis* for ethanol production is hindered by poor performances on industrial substrates such as molasses and cellulosic biomass, which often attaches certain Na^+ or Cl^- into bioreactor, producing higher Na^+ or Cl^- concentration than normal one. It has been reported that *Z. mobilis* is sensitive to salinity, just resisting to 0.1 to 0.2 M NaCl in liquid medium, while halotolerant yeast could tolerate 2 to 3 M NaCl or KCl (Vriesekoop et al., 2002; Kumar and Gummadi, 2009) and NaCl has been frequently considered as one of common salts that has effect on fermentation performance of *Z. mobilis*. In the presence of NaCl, cells expose to both specific ion toxicity and osmotic stress because high intracellular concentrations of Cl^- and Na^+ are deleterious to cellular systems (Serrano et al., 1999). Chloride ion may interfere with anionic sites involved in binding of RNA and anionic metabolites such as bicarbonate, carboxylates and sugar-phosphates. On the other hand, sodium ion may interfere with cationic sites involved in binding of K^+, Ca^{2+} or Mg^{2+} (Millar et al., 1984; Osman and Ingram, 1985).

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Abbreviations: REC, Relative electronic conductivity; **RNC,** relative nucleic acid content; **RPC,** relative protein content.

Swings and De Ley first reported that the relative low salt resistance of this bacterium, only 71% of the strains tested could grow in 1%(w/v) NaCl, whereas none could grow at 2% NaCl (Swings and De Ley, 1977), many authors have reported the reduction about biomass, substrate uptake and ethanol yield in the presence of various salts, such as $CaCl_2$, NaCl and KCl (Ranatunga et al., 2000; Bajpai and Margaritis, 1984; Kirk and Doelle, 1992). However, there is little information about the relative contributions of the Na^+ and Cl^- to the inhibitory effect. In this study, the relative contributions effects of Na^+ and Cl^- on growth, morphology changes, membrane disorganization, ion homeostasis, exoenzyme activities and fermentation performance were investigated. In lignocellulose-to-ethanol fermentations, high NaCl concentration sometimes rises when hydrochloric acid remaining from hydrolysis of the substrate is used with NaOH. In this study, it was strongly suggested that salt inhibition, especially for growth, filament formation and fermentation in *Z. mobilis* culture, was mainly attributable to chloride ion. To avoid filamentous growth and poor fermentation performance, chloride ion should be excluded from lignocellulose-to-ethanol fermentation, for example, sulphuric acid is preferable to hydrochloric acid for the hydrolysis.

MATERIALS AND METHODS

Medium, growth and fermentation conditions

The strain was anaerobically cultivated at pH 5.5 and 30°C in flasks as described by Vriesekoop et al. (2002). The salinity of the medium was adjusted with various concentrations of NaCl, $(NH_4)_2SO_4$, Na^+ (as Na_2SO_4) and Cl^- (as NH_4Cl). Batch fermentation was anaerobically performed at 30°C and 120 rpm with 100 ml of fermentation medium containing an initial glucose 150 g/l and various salinity concentrations in 250 ml Erlenmeyer flasks inoculated with 10%(v/v) pregrowing *Z. mobilis* 232B cells for 20 to 30 h.

Analysis of metabolites in culture supernatant

Ethanol was quantified by GC analysis using a porapak GDX103 mesh column. Residual glucose was determined by the dinitrosalicylic acid (DNS) technique (Lindasy, 1973).

Measurement of growth and cell viability

Cell growth was measured by monitoring the optical density of the medium at absorbance 550 nm (Loos et al., 1994). The biomass concentration was calculated from the absorbance values using predetermined correlation factor, which an optical density of 1.0 corresponds with a cell dry weight of 232 mg/l. Cell viability was determined by drop test. Aliquot (5 µl)of 10-fold serial dilutions from *Z. mobilis* 232B cultures was spotted onto solid plates containing o.15 M NaCl, $(NH_4)_2SO_4$, Na^+ or Cl^- and incubated anaerobically for 48 h at 37°C.

Photography

Cells from growing culture containing different concentrations (0 to 0.15 M) of NaCl, $(NH_4)_2SO_4$, Na^+ or Cl^- were harvested at different incubation time by centrifugation (5 min, 20°C, 12,000 rpm). Cells were washed thrice with isosmotic solution of sorbitol and resuspended in sterile water. Cells were Gram stained, then observed and photographed with a biological microscope at a magnification of 1,000.

Measurement of membrane disorganization

Salt-induced membrane disorganization was determined by evaluating the leakage of ion, nucleotide and protein of exponential phase cells harvested from culture containing a series of salt concentrations (0 to 0.15 M) of NaCl, $(NH_4)_2SO_4$, Na^+ or Cl^- and cells exposure to 0.15 M salinity at different incubation time. Ion, nucleotide and protein leakages were respectively measured by relative electronic conductivity (REC), relative nucleic acid content (RNC) and relative protein content (RPC). Cells were harvested by centrifugation and rinsed with isoosmotic solution of sorbitol thrice. Cell pellets were resuspended in 10 ml double-distilled water and centrifuged a second time at 12000 rpm for 10 min after exposure for 10 min. The upper liquid phase was separated to measure electronic conductivity (R_1), nucleotides concentration (A_1) and protein concentration (B_1), while the cell pellets were resuspended in 10 ml double-distilled water once more and then were boiled for 20 min at 100°C and centrifuged. After cell removal, electronic conductivity (R_2), nucleotides concentration (A_2) and protein concentration (B_2) were respectively measured. The REC, RNC and RPC were calculated by the following:

$$REC(\%) = \frac{R_1}{R_1 + R_2} \times 100\% \quad (1)$$

$$RNC(\%) = \frac{A_1}{A_1 + A_2} \times 100\% \quad (2)$$

$$RPC(\%) = \frac{B_1}{B_1 + B_2} \times 100\% \quad (3)$$

The electronic conductivity was measured by the electrical conductivity meter at 25°C. The nucleotide and protein concentration was respectively measured as A_{260} and A_{280}.

Exoenzyme activities analysis

The semi-quantitative assay apiZYM (bioMerieux,Marcy I Etoile, France) was used to identify the exoenzyme expression of *Z. mobilis* 232B cell induced by salt stress for a set of 19 enzymes. The exponential phase cells grown in medium containing 0.15 M salinity was diluted at similar concentration. Each well was inoculated with 60 µl of cells and incubated for 4 h at 37°C. Color development after the addition of the reagents was visually determined and ranked from ‘0’ (no activity) to ‘5’ (highest activity) according to the manufacturer instructions.

Measurement of intracellular Na^+ and K^+ content

Z. mobilis 232B cells grown in medium containing 0.15 M NaCl,

Table 1. Growth of *Z. mobilis* 232B in liquid medium containing different concentrations of $(NH_4)_2SO_4$, NaCl, Na^+ or Cl^-.

Concentration (M)	Specific growth rate, μ (h^{-1})				X_{max} (mg/l h)			
	$(NH_4)_2SO_4$	NaCl	Na^+	Cl^-	$(NH_4)_2SO_4$	NaCl	Na^+	Cl^-
0	0.451	0.451	0.451	0.451	83.06	83.06	83.06	83.06
0.05	0.443	0.319	0.402	0.338	82.43	65.28	78.33	58.06
0.10	0.412	0.288	0.343	0.203	81.03	56.11	64.72	44.72
0.15	0.398	0.162	0.216	0.124	76.13	39.17	49.44	38.33

Specific growth rate was measured during the exponential phase of the curves. X_{max} corresponds to maximum cell dry weight produced during growth.

$(NH_4)_2SO_4$, Na^+ or Cl^- were harvested at mid-log phase of its growth by centrifugation at 12,000 rpm for 10 min at 20°C. Cell pellets were resuspended and washed thrice with isoosmotic solution of sorbitol. Then cells were boiled for 20 min at 100°C to release ion and then centrifuged again. After cell removal, the upper liquid was saved to measure the intracellular Na^+ and K^+ content by ion chromatography analysis using an atomic absorption spectrometer.

RESULTS AND DISCUSSION

Growth of *Z. mobilis* 232B at different concentrations of $(NH_4)_2SO_4$, NaCl, $Na^+$and Cl^-

As shown in Table 1, $(NH_4)_2SO_4$ did not significantly impact on cells growth, suggesting that the effects of the SO_4^{2-} and NH_4^+ on *Z. mobilis* 232B growth at these concentrations could be negligible. However, when compared with control cells, specific growth rate (μ, h^{-1}) was decreased in NaCl, Na^+ and Cl^-. Furthermore, the higher when the NaCl, Na^+ and Cl^- concentration was, the less biomass was produced. Especially, when NaCl, Na^+ and Cl^- were 0.15 M in medium, X_{max} was only 47.2, 59.5 and 46.1% of X_{max} in control medium, respectively.

Growth of *Z. mobilis* 232B was relatively lowered in Cl^- compared to Na^+ and almost entirely replicated the effects of NaCl on cell growth. It was revealed that the decrease of X_{max} with increasing salt concentration could be correlated with toxic effects of high salt concentration.

In addition, the cell viability on solid medium containing 0.15 M NaCl, Na^+ Cl^- or 0.75M $(NH_4)_2SO_4$ was determined by drop test (Figure 1). Cells grew well in control and $(NH_4)_2SO_4$ medium. On the other hand, cells behaved poor growth in NaCl and Cl^- medium and cells grown in Na^+ culture exhibited a better performance. It was shown that the effects of NaCl on the growth of *Z. mobilis* 232B and cell viability was attributable to both sodium and chloride ion, with the latter having the greater effect. This, however was in contradiction to the work reported by Vriesekoop (Vriesekoop et al., 2002). They reported that sodium ion performed greater inhibitory effect on growth of *Z. mobilis* cells. *Z. mobilis*225, another *Z. mobilis* strain, was tested in the same way and performed similar growth as *Z. mobilis*232B.

Cell morphology changes in response to saline stress

As shown in Figure 2, the occurrence of filamentous growth began as early as 4 h after exposure to the 0.15 M chloride ion (a-h) and the length of cell increased gradually in chloride ion treatment time course. Cell length of *Z. mobilis* 232B grown in different concentrations of $(NH_4)_2SO_4$, NaCl, Na^+ and Cl^- for 24 h was measured (Table 2). The occurrence of filamentous growth was observed in NaCl and Cl^- culture and cell length was approximate 10 to 15 μm, which was 3 to 5 fold longer compared with normal cells. Furthermore, cell length increased with NaCl and Cl^- concentration and cells in Cl^- culture even performed longer than that grown in NaCl culture. The length of control cell was 2 to 4 μm and there was no observable difference in cell size between control cells and Na^+ treated cells. Besides, the $(NH_4)_2SO_4$ treated cells performed as normal size without filamentous performance. It was indicated that the effect of NaCl on filamentous growth was attributable to Cl^-.

Filamentous growth has been previously observed in *Z. mobilis* fermentations with the effects of osmotic pressure, increased phosphate levels, high temperature stress and ionic stress (Fein et al., 1984; Stevnsborg and Lawford, 1986; Spangler and Emert, 1986). Spangler and Emert reported that filament formation during the simultaneous saccharification and fermentation of cellulose to ethanol by *Z. mobilis* was associated with $CaCl_2$ which originated from the cellulose production medium and they assumed that the calcium ion was the key factor (Spangler and Emert, 1986), while, in this study the data indicated was attributable to chloride ion, rather than calcium ion. The increase in cell length began as early as 4 h after exposure to chloride ion, suggesting that cell filamentous growth occurred in the present of cell division. Furthermore, the filamentous cells were able to recover to normal formation when they were transferred to medium without chloride ion (unpublished data).

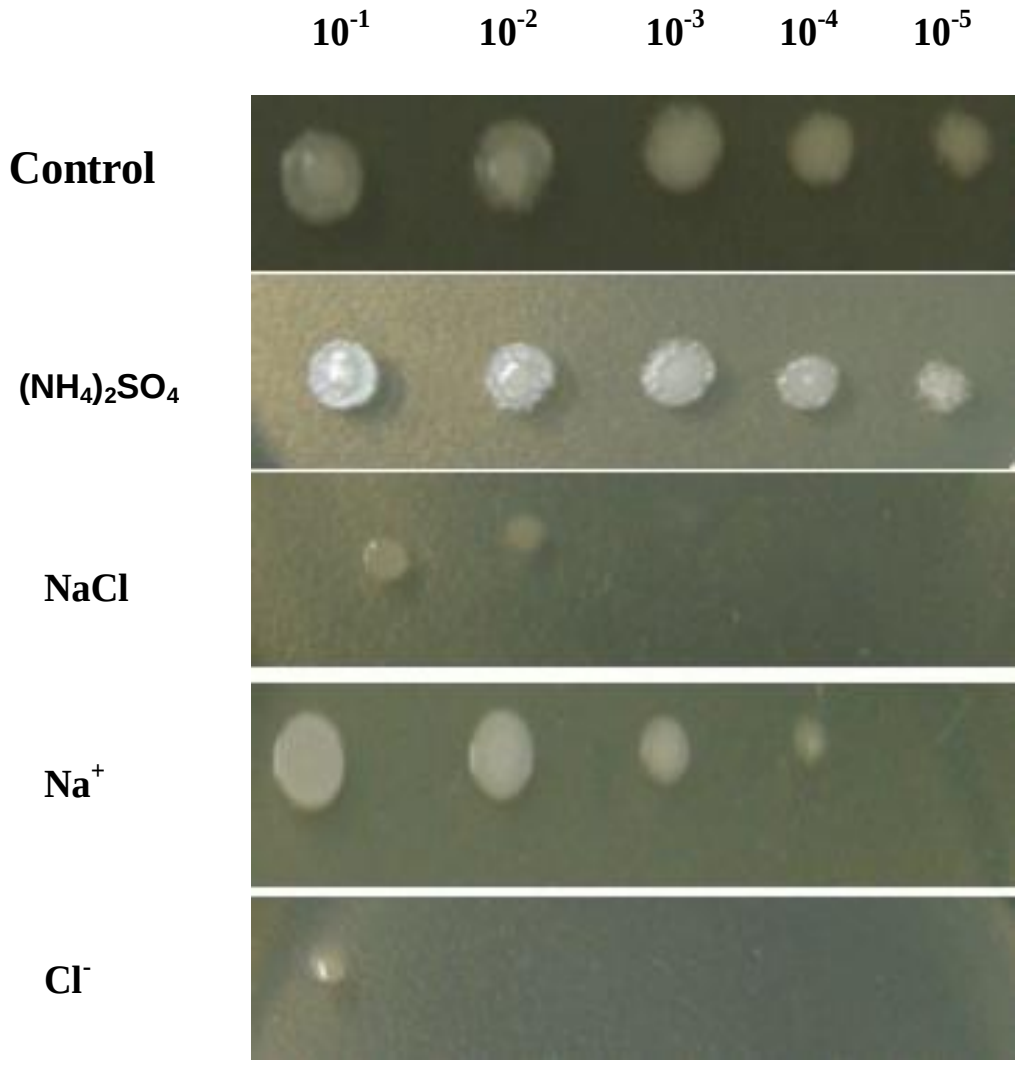

Figure 1. Viability of *Z. mobilis* 232B in solid medium containing 0.15 M NaCl, Na^+, Cl^- or 0.75 M $(NH_4)_2SO_4$

Effects of NaCl, Na^+ and Cl^- on membrane disorganization

Effects of NaCl, Na^+ and Cl^- on membrane disorganization were determined by evaluating the membrane permeability (Table 3). The REC, RNC and RPC rose markedly with the increase of salt concentration, suggesting that cell membrane disorganization was related to the degree of salt stress. As shown in Table 3, cell membrane permeability treated by Cl^- was really significantly higher than that under NaCl and Na^+ treatment, suggesting that the damage of cell membrane under NaCl treatment was mainly caused by chloride ion.

To investigate the effects of the high salinity shock on cell membrane disorganization, the cells exposed in medium containing 0.15 M NaCl, Na^+ and Cl^- for 0, 30, 60, 90, 120 and 150 min were harvested to survey the membrane permeability. As shown in Figure 3, there was no significant difference in cell membrane permeability between cell grown in 0.15 M Na^+ and control one. However, great changes have been taken place when cells were exposed to 0.15 M NaCl and Cl^- shock.

Cell membrane plays an important role in maintaining its microenvironment and metabolism. Under normal circumstances, the cell membrane has permeability to maintain ion homeostasis and restrict the movement of substance. Upon encountering the environmental stresses such as temperature shift, hyposmotic or hyperosmotic shock and salinity stress, cell membrane is easily damaged and increasing permeability enabled electrolyte, nucleotide and protein leakage, which resulted in electrical conductivity, nucleotide and protein concentration increase. The results indicated that NaCl

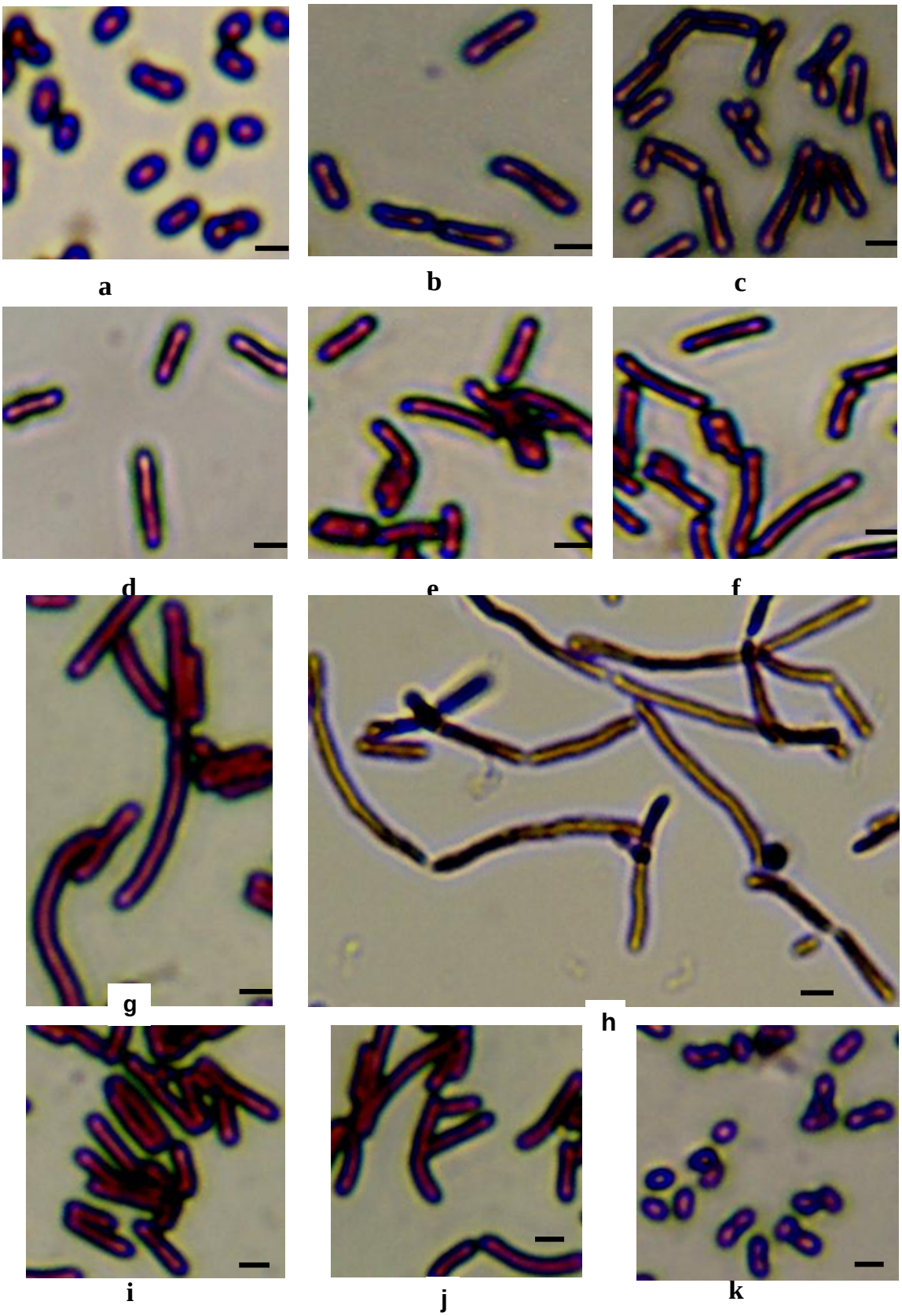

Figure 2. Time course of changes in morphology of *Z. mobilis* 232B following exposure to 0.15 M Cl^-, NaCl and Na^+. The effects of exposure to Cl^-(as NH_4Cl) for 0, 4, 8, 12, 16, 20, 24, 28 h(a-h), NaCl for 12, 28 h (i-j) and Na^+(as Na_2SO_4)(k)for 28 h are shown. The bar indicates a length of 2 µm.

Table 2. Effects of salt concentrations on size of *Z. mobilis* 232B cells grown in liquid medium with $(NH_4)_2SO_4$, NaCl, Na^+ or Cl^-.

Concentration (M)	Approximate cell length (μm)			
	$(NH_4)_2SO_4$	NaCl	Na^+	Cl^-
0	2.0-4.0	2.0-4.0	2.0-4.0	2.0-4.0
0.05	2.0-4.0	4.0-6.0	2.0-4.0	6.0-8.0
0.10	2.0-4.0	6.0-8.0	2.0-4.0	8.0-10.0
0.15	2.0-4.0	8.0-12.0	2.0-4.0	10.0-15.0

Table 3. Effects of salt stress on membrane permeability in exponential phase cells of *Z. mobilis* 232B.

Concentration (M)	REC (%)			RNC (%)			RPC (%)		
	NaCl	Na^+	Cl^-	NaCl	Na^+	Cl^-	NaCl	Na^+	Cl^-
0	55.37	55.37	55.37	14.25	14.25	14.25	16.19	16.19	16.19
0.05	64.81	61.24	98.03	23.52	20.42	23.94	30.80	31.84	37.42
0.10	81.86	74.17	99.06	35.25	31.78	45.73	58.41	44.99	79.41
0.15	95.88	86.05	99.87	46.23	41.70	57.14	63.36	46.11	84.72

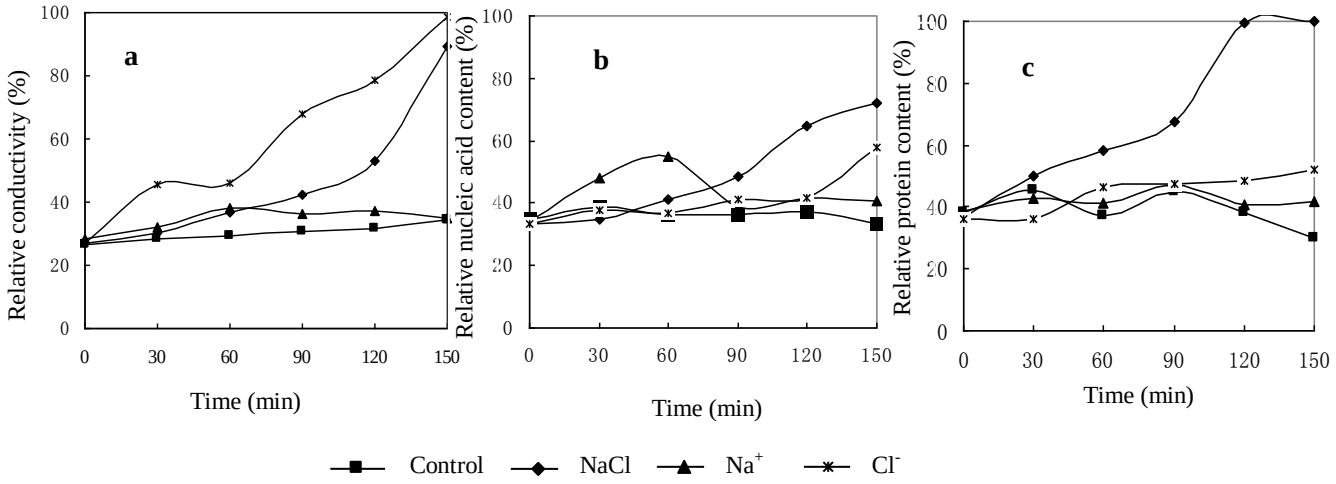

Figure 3. Time course of changes in membrane penetrability of *Z. mobilis* 232B following exposure to 0.15 M Cl^-, NaCl and Na^+. A, is relative conductivity. B, is relative nucleic acid content. C, is relative protein content.

and Cl^- caused dose-dependent leakage of ion, nucleotide and protein, while Na^+ had slight effect on cells' membrane permeability, suggesting that the effect of NaCl on cell membrane disorganization was mainly due to chloride ion and *Z. mobilis* 232B may possess certain cationic transport system for sodium extrusion to resist higher intracellular Na^+ concentration.

Furthermore, the leakage of ion may include magnesium, calcium and potassium ion which plays an important role in cell growth and fermentation. For example, a variety of enzymes in E-D glycolysis require magnesium ion as a cofactor and calcium and potassium ion are also required for fermentation, which activate pyruvate decarboxylase and pyruvate kinase in *Z. mobilis,* respectively (Hoppner and Doelle, 1983; Sols et al., 1971). Besides, membrane disorganization also causes other cofactors, enzyme and metabolites, such as intermediates of glycolysis, to outflow. It may be one of the reasons that cell in Cl^- culture grow more slowly with lower alcohol yield than that grown in culture at same Na^+ concentration.

Intracellular cationic concentration at different concentrations of NaCl, Na^+ and Cl^-

The intracellular Na^+ and K^+ concentrations of *Z. mobilis* 232B grown in different concentrations of NaCl, Na^+ and

Table 4. Changes in intracellular concentration of K^+ and Na^+ of cells grown in different concentrations of NaCl, Na^+ or Cl^-.

Concentration (M)	Intracellular concentration of K^+ (μmol/g cell dry weight)			Intracellular concentration of Na^+ (μmol/g cell dry weight)			The ratio of K^+/Na^+		
	NaCl	Na^+	Cl^-	NaCl	Na^+	Cl^-	NaCl	Na^+	Cl^-
0	256.6	256.6	256.6	29.4	29.4	29.4	15.38	15.38	15.38
0.05	155.7	166.2	56.5	101.9	71.7	48.5	1.53	2.32	1.16
0.10	70.9	73.4	48.3	106.9	86.2	70.1	0.66	0.85	0.68
0.15	56.0	60.9	22.1	153.8	109.6	90.6	0.36	0.56	0.24

Table 5. Semi-quantitative assay of exoenzyme activities from *Z. mobilis* 232B cells grown in liquid medium containing 0.15 M NaCl, Na^+or Cl^-.

Number	Enzyme	Salt stress			
		None	NaCl	Na^+	Cl^-
1	Control	0	0	0	0
2	Alkaline phosphatase	2	5	5	3
Ester-hydrolases					
3	Esterase (C4)	3	1	3	2
4	Esterase Lipase (C8)	0	0	0	0
5	Lipase (C14)	0	0	0	0
Peptide-hydrolases					
6	Leucine arylamidase	5	4	4	3
7	Valine arylamidase	0	0	0	0
8	Cystine arylamidase	3	3	3	3
9	Trypsin	0	5	5	3
10	α-Chymotrypsin	0	0	0	0
Phosphoric-hydrolases					
11	Acid phosphatase	5	3	5	4
12	Naphthol-AS-BI-phosphohydrolase	2	0	0	0
Glycosidases					
13	α-Galactosidase	0	0	0	0
14	β-Galactosidase	0	0	0	0
15	β-Glucoronidase	0	0	0	0
16	α-Glucosidase	0	0	0	0
17	β- Glucosidase	0	0	0	0
18	N-acetyl-β- glucosaminidase	0	0	0	0
19	α-Mannosidase	0	0	0	0
20	β-Fucosidase	0	0	0	0

Cl^- were measured. As shown in Table 4, cells grown in absence of salt had relative high intracellular K^+ concentration and relative low intracellular Na^+ concentration, whereas cells grown in presence of salt were found to uptake high amount of Na^+ and effuse a great deal of K^+.

In microorganism cells, maintaining high intracellular K^+ in an environment with high Na^+ concentration is a key strategy to overcome salt stress. In cytosol, K^+ is an essential activator for some enzymes and Na^+ rarely substitutes for this biochemical function. Na^+ can compete directly for K^+-binding sites on enzymes, suggesting that the intracellular K^+ to Na^+ ratio, rather than the absolute Na^+ concentration, is critical for tolerance (Carden et al., 2003). Therefore, the intracellular cationic concentration,

Table 6. Effects of salt stress on glucose uptake and ethanol production in *Z. mobilis* 232B grown in different concentrations of NaCl, Na^+ or Cl^-.

Parameter	Concentration of salt (mol/l)								
	0	0.05			0.10			0.15	
		NaCl	Na^+	Cl^-	NaCl	Na^+	Cl^-	NaCl	Na^+	Cl^-
P	70.06	53.73	66.30	49.47	44.11	57.86	40.69	36.98	51.21	33.05
X	1.53	1.15	1.42	1.18	1.08	1.24	1.12	0.87	1.16	0.73
S	1.31	42.19	15.76	50.59	52.99	30.63	61.70	69.96	41.73	79.26
$Y_{P/S}$	0.47	0.49	0.49	0.49	0.45	0.48	0.46	0.46	0.47	0.46
$Y_{X/S(10^{-2})}$	1.03	1.07	1.06	1.19	1.12	1.04	1.27	1.09	1.07	1.03
Q_P	3.51	2.69	3.32	2.47	1.84	2.41	1.70	1.23	1.71	1.10
Q_S	7.43	5.39	6.71	4.97	4.04	4.97	3.68	2.67	3.61	2.36
E	91.40	70.10	86.50	64.54	57.55	75.49	53.09	48.24	66.82	43.12
T_t	20	20	20	20	24	24	24	30	30	30

P: product of ethanol (final ethanol concentration) g/l; X: final cell mass concentration mg/g; S: residual sugar g/l; $Y_{P/S}$: ethanol yield g/g; $Y_{X/S}$: cell yield g/g; Q_P: volumetric ethanol productivity g/l/h; Q_S: volumetric substrate uptake g/l/h; E: percentage of theoretical yield (%); T_t: fermentation time h.

mostly Na^+ and K^+, are of primordial importance in understanding the response of a strain to salinity. In this study, it has been found that cells effused a great deal of K^+ and uptake high amount of Na^+ when *Z. mobilis* 232B grew both in 0.15 M Cl^- and Na^+. In addition, it was also found that the ratio of K^+/ Na^+ decreased with increase in osmotic stress and the ratio of K^+/ Na^+ of cell grown in Na^+ culture is higher than that grown in culture at same Cl^- concentration, suggesting that Cl^- is more toxic than Na^+. This observation was in agreement with our results on cell viability and fermentation performance. It has been reported that halotolerant strain accumulates high amounts of K^+ inside the cell when cells grow in high NaCl concentration (Kumar and Gummadi, 2009). Halotolerant strain's ability for extrusion of sodium to decrease the continuous inflow of Na^+ at the cost of uptake of K^+ inside the cell for survival. Our result was not in accordance with their reports. Maybe it is one of the reasons that *Z. mobilis* 232B is sensitive to NaCl, whereas halotolerant strain could grow well in high NaCl concentration.

Effects of NaCl, Na^+ and Cl^- on exoenzyme activities

The exoenzyme expression levels of test strain and control one was observed and compared using the semi-quantitative assay apiZYM (Table 5). Leucine arylamidase, cystine arylamidase and alkaline phosphatase exhibited high activity values (>3) with or without salt stress, whereas Acid phosphatase and trypsin exhibited high activity values only under salt stress, especially for NaCl and Na^+. There was little activity for most esterhydrolases, the exception for esterase (C4), which was present a lower activity after NaCl and Cl^- stress. Furthermore, the activities of most glycosidases were not detected by the method used. It was revealed that salt stress just appeared to have an effect on the activities of leucine arylamidase, cystine arylamidase and alkaline phosphatase, esterase (C4), alkaline phos-phatase and trypsin.

Effects of NaCl, Na^+ and Cl^- on fermentation

Fermentation was much inhibited by Cl^- than Na^+ (Table 5). According to the results, inhibition of 5 to 30% for activity was caused by 0.05 M NaCl, Na^+ or Cl^- and inhibition of almost 50% was observed in 0.15 M NaCl, Na^+ or Cl^-. In NaCl culture, glucose uptake and ethanol production were markedly slowed compared to cells grown in Na^+ addition medium (Table 6). Chloride ion almost entirely replicated the effects of NaCl on fermentation performance, suggesting that the inhibition of glucose consumption and ethanol production was attributable to both sodium ion and chloride ion, with the latter having the major effect.

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REFERENCES

Bajpai P, Margaritis A (1984). Effect of calcium chloride on the growth and ethanol production by free cells of *Zymomonas mobilis*. Biotechnol. Lett., 6: 673-676.

Carden DE, Walker DJ, Flowers TJ, Miller AJ (2003) Single-cell measurements of the contributions of cytosolic Na^+ and K^+ to salt

tolerance. Plant Physiol 131:676-683.
Fein JE, Barber DL, Charley RC, Beveridge TJ, Lawford HG (1984) Effect of commercial feedstocks on growth and morphology of *Zymomonas mobilis*. Biotechnol Lett 6:123-128.
Hoppner TC, Doelle HW (1983). Purification and kinetic characteristics of pyruvate decarboxylase and ethanol dehydrogenase from *Zymomonas mobilis* in relation to alcohol production. Appl. Microbiol. Biotechnol., 17: 152-157.
Kirk LA, Doelle HW (1992). The effects of potassium and chloride ions on the ethanolic fermentation of sucrose by *Zymomonas mobilis* 2716. Appl. Microbiol. Biotechnol., 37:88-93.
Kumar S, Gummadi N (2009). Osmotic adaptation in halotolerant yeast, Debaryomyces nepalensis NCYC 3413: role of osmolytes and cation transport. Extremophiles, 13: 793-805.
Lindasy H (1973). A colorimetric estimation of reducing sugars in potatoes with 3,5-dinitrosalicylic acid. Met. Potato Res., 16:176-179.
Loos H, Kramer R, Sahm H, Sprenger GA (1994). Sorbitol promotes growth of *Zymomonas mobillis* in environments with high concentrations of sugar: evidence for a physiological function of glucose-fructose oxidoreductase in osmoprotection. J. Bacteriol., 12: 7688-7693.
Millar DG, Griffiths-Smith K, Algar E, scopes RK (1982). Activity and stablity of glycolytic enzymes in the presence of ethanol. Biotechnol. Lett., 4: 601-606.
Osman YA, Ingram LO (1985). Mechanism of ethanol inhibition of fermentation in *Zymomonas* mobilisCP4. J. Bacteriol., 164: 173-180.
Ranatunga TD, Jervis J, Helm RF, Wooley RJ (2000). The effect of overliming on the toxicity of dilute acid pretreated lignocellulosics: the role of inorganics, uronic acids and ether-soluble organiscs. Enzyme Microb. Tech., 27: 240-247.
Rogers PL, Lee KJ, Skotnicki ML, Tribe DE (1982). Ethanol production by Zymomonas mobilis. Adv. Biochem. Eng., 23: 27-84.
Serrano R, Mulet JM, Rios G, Marquez JA, Larrinoa IF, Leube MP, Mendizabal I, Pascual-Ahuir A, Proft M, Ros R and Montesions C (1999). Aglimpse of the mechanisims of ion homeostasis during salt stress. J. Exp. Bot., 50: 1023-1036.
Sols A, Goncedo C, Fuente G (1971). Energy yielding metabolism in yeasts. Yeast, 2: 271-307.
Spangler DJ, Emert GH (1986). Simultaneous saccharification fermentation with Zymomonas mobilis. Biotechnol. Bioeng., 28: 115-118.
Sprenger GA (1993). Approaches to broaden the substrate and product range of the ethanologenic bacterium Zymomonas mobilis by genetic engineering. J. Bacteriol., 27: 225-237.
Sprenger GA (1996). Carbohydrate metabolism in *Zymomonas mobilis*: a catabolic highway with some scenic routes. FEMS. Microbiol. Lett., 145: 301-307.
Stevnsborg N, Lawford HG (1986). Effect of calcium on the performance and morphology of Zymomonas mobilis ATCC29191 in continous ethanol fermentations. Biotechnol. Lett., 8: 181-186.
Swings J, De Lev J (1997). The biology of Zymomonas mobilis. Bacteriol. Rev., 41: 1-46.
Vriesekoop F, Rasemusson M, Pamment NB (2002). Respective effects of sodium and chloride ions on filament formatation and growth and ethanol production in Zymomonas mobillis fermentations. Lett. Appl. Microbiol., 35: 27-31.