Understanding the NaCl-dependent behavior of hydrogen production of a marine bacterium, *Vibrio tritonius*

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Biohydrogen is one of the most suitable clean energy sources for sustaining a fossil fuel independent society. The use of both land and ocean bioresources as feedstocks show great potential in maximizing biohydrogen production, but sodium ion is one of the main obstacles in efficient bacterial biohydrogen production. *Vibrio tritonius* strain AM2 can perform efficient hydrogen production with a molar yield of 1.7 mol H_2/mol mannitol, which corresponds to 85% theoretical molar yield of H_2 production, under saline conditions. With a view to maximizing the hydrogen production using marine biomass, it is important to accumulate knowledge on the effects of salts on the hydrogen production kinetics. Here, we show the kinetics in batch hydrogen production of *V. tritonius* strain AM2 to investigate the response to various NaCl concentrations. The modified Han–Levenspiel model reveals that salt inhibition in hydrogen production using *V. tritonius* starts precisely at the point where 10.2 g/L of NaCl is added, and is critically inhibited at 46 g/L. NaCl concentration greatly affects the substrate consumption which in turn affects both growth and hydrogen production. The NaCl-dependent behavior of fermentative hydrogen production of *V. tritonius* compared to that of *Escherichia coli* JCM 1649 reveals the marine-adapted fermentative hydrogen production system in *V. tritonius*. *V. tritonius* AM2 is capable of producing hydrogen from seaweed carbohydrate under a wide range of NaCl concentrations (5 to 46 g/L). The optimal salt concentration producing the highest levels of hydrogen, optimal substrate consumption and highest molar hydrogen yield is at 10 g/L NaCl (1.0% (w/v)).

1 **Understanding the NaCl-dependent behavior of hydrogen**
2 **production of a marine bacterium, *Vibrio tritonius***

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16 **Abstract**

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18 independent society. The use of both land and ocean bioresources as feedstocks show great

19 potential in maximizing biohydrogen production, but sodium ion is one of the main obstacles in

20 efficient bacterial biohydrogen production. *Vibrio tritonius* strain AM2 can perform efficient

21 hydrogen production with a molar yield of 1.7 mol H_2/mol mannitol, which corresponds to 85%

22 theoretical molar yield of H_2 production, under saline conditions. With a view to maximizing the

23 hydrogen production using marine biomass, it is important to accumulate knowledge on the effects

24 of salts on the hydrogen production kinetics. Here, we show the kinetics in batch hydrogen

25 production of *V. tritonius* strain AM2 to investigate the response to various NaCl concentrations.

26 The modified Han–Levenspiel model reveals that salt inhibition in hydrogen production using *V.*

27 *tritonius* starts precisely at the point where 10.2 g/L of NaCl is added, and is critically inhibited at

28 46 g/L. NaCl concentration greatly affects the substrate consumption which in turn affects both

29 growth and hydrogen production. The NaCl-dependent behavior of fermentative hydrogen

30 production of *V. tritonius* compared to that of *Escherichia coli* JCM 1649 reveals the marine-

31 adapted fermentative hydrogen production system in *V. tritonius*. *V. tritonius* AM2 is capable of

32 producing hydrogen from seaweed carbohydrate under a wide range of NaCl concentrations (5 to

33 46 g/L). The optimal salt concentration producing the highest levels of hydrogen, optimal substrate

34 consumption and highest molar hydrogen yield is at 10 g/L NaCl (1.0% (w/v)).

36 **Introduction**

37 Hydrogen gas (H_2) has long been seen as an ideal alternative energy source due to its high energy

38 content of 122 KJ/g and the fact it only produces water during combustion (Atif et al., 2005;

39   Chandrasekhar et al., 2015; Hao et al., 2006; Zhao et al., 2001). Among many potential processes,

40   biological H_2 (bioH_2) production via dark fermentation offers the best option that is both renewable

41   and potentially viable for environmental and economic sustainability, allowing us to reduce our

42   dependency on fossil fuels (Akinbomi et al., 2015; Hallenbeck & Ghosh, 2009; Meinita et al.,

43   2013; Myung et al., 2014). The third generation biofuels using marine algae as feedstock provide

44   a great opportunity in maximizing biohydrogen production efficiency and could alleviate the "food

45   vs. fuel" debate that arose from the utilization of terrestrial biomasses in the first and second

46   generation biofuels (Alam et al., 2015; Goh & Lee, 2010; Singh et al., 2011). In comparison to

47   land materials, marine biomass is less affected by climate change, less limited by land availability,

48   has higher potential biomass yields per unit area and offers less competition to food supply

49   (Milledge & Harvey, 2016; Milledge & Harvey 2018). Marine macroalgae, in particular, lacks

50   lignin, contains lipids, and a significant amount of sugars (at least 50% of its total dried weight);

51   suitable attributes for bioH_2 production using biomass feedstock (Enquist-Newman et al., 2013;

52   Wargacki et al., 2012; Takeda et al., 2011). The application of macroalgae in bioH_2 production,

53   however, is potentially offset by the fact that the sugars in macroalgae, such as mannitol, are

54   seasonal (Adams et al., 2011; Suutari et al., 2015; Yanagisawa et al., 2013). Additionally, the algae

55   are also rich in salt content with relatively more sodium chloride than terrestrial biomasses

56   (Milledge & Harvey, 2016; Rupérez, 2002). High salt concentration has been reported as adversely

57   affecting H_2 productivity in some bacterial species (Alshiyab et al., 2008). Continuous exploration

58   and development of microorganisms that are capable of converting these special macroalgae

59   carbohydrates to biohydrogen, preferably under saline conditions, would resolve these challenges

60   and further enhance productivity.

61   To date, the majority of studies on fermentative H_2 production have been on strict anaerobes

62   of *Clostridium* (Ferchichi et al., 2005; Jayasinghearachchi et al., 2010; Jo et al., 2008b; Junghare

63   et al., 2012), and facultative anaerobes such as *Escherichia coli* (Maeda et al., 2012; Trchounian

64   & Trchounian, 2014; Trchounian et al., 2015) and *Enterobacter aerogenes* (Jo et al., 2008a; Zhao

65   et al., 2009; Zhao et al., 2001). These terrestrial bacteria were not suitable biocatalysts in

66   fermentative H_2 production under saline conditions due to sensitivity to salts in H_2 production

67   (Alshiyab et al., 2008). In a screening of effective H_2 producers using seaweed carbohydrates, a

68   facultative anaerobic marine bacterium, *V. tritonius* strain AM2, isolated from the gut of sea hare

69   (*Aplysia kurodai*) shows higher H_2 production than those of enterobacteria (Sawabe et al., 2013;

70   Matsumura et al., 2014). Production of gas during the fermentation of carbohydrates is an atypical

71   property in the genus *Vibrio* with only *V. aerogenes*, *V. aphrogenes*, *V. furnissii*, *V. gazogenes*, *V.*

72   *mangrovii*, *V. porteresiae*, *V. ruber*, *V. rhizosphaerae*, and *V. tritonius* among those sharing these

73   attributes out of 140 *Vibrio* species. However, a series of experiments by Matsumura et al.

74   (Matsumura et al., 2014; Matsumura et al., 2015) revealed unexpectedly high H_2 productivity by

75   *V. tritonius* cultured in glucose- and mannitol-supplemented media under saline conditions (2.25%

76   (w/v) NaCl). Surveys of the complete genome of *V. tritonius* strain AM2 shows a single 24-kb

77   gene cluster containing 21 genes that is responsible for the formation of a formate hydrogen lyase

78   (FHL) complex on the large chromosome. This vibrio FHL complex is structurally similar to the

79   *hyf* gene cluster (*hyfABCDEFGHIJ* genes) found in *E. coli* K-12 with the amino acid identity of

80   each homolog ranging from 45 to 68% (Matsumura et al., 2015). On the downstream of this vibrio

81   *hyf* gene cluster, the bacterium also carries the genes responsible for the formate dehydrogenase

82   (FDH-H), *fhlA*-type transcriptional activator, and hydrogenase maturation proteins (*hyp*) which

83   together form a "super-gene-set" of the FHL-Hyp gene cluster. The *V. tritonius* hydrogenase is

84   also classified as a [NiFe]-hydrogenase because of typical motifs coordinating the [NiFe] center

85   at the active site of its *hyfG*, the large subunit of FHL complex (Matsumura et al., 2015). The

86   fermentation product profiling and the genome surveys suggest H_2 production from glucose and

87   mannitol is via the FHL pathway (Matsumura et al., 2014; Matsumura et al., 2015).

88   *V. tritonius* AM2 is a slight halophile that is capable of growing in 0.5 to 8.0% (w/v) NaCl.

89   Optimum growth of the bacterium was observed in 3.0% NaCl (w/v) (Sawabe et al., 2013). Thus,

90   the marine bacterium potentially possesses different features relating to H_2 production compared

91   to those observed in terrestrial bacteria. Application of seawater for bacterial fermentative

92   production is still being considered as a cost-effective technology (Adessi et al., 2016; Maeda et

93   al., 2012). The idea of using marine vibrio species could be an ideal solution in intensifying further

94   progress being made for third generation biofuel production using seaweed as feedstock. *Vibrio*

95   species can also be used in bioH_2 production when domestic and food industries residual wastes

96   containing high salt concentrations are used as feedstock (Cabrol et al., 2017; Oh et al., 2003;

97   Pierra et al., 2014). Previously, *V. tritonius* was reported as successfully producing H_2 from

98   powdered seaweed using a jar fermenter under pre-optimized culture conditions (Matsumura et

99   al., 2014). These results helped the idea that the use of seaweed for fermentation can be a cost

100   effective and sustainable alternative because the fact seaweed for feedstock can be prepared using

101   simple pre-treatment such as pulverization. The halophilic and/or halotolerant natures of *Vibrio*

102   species can provide huge advantages in H_2 production and in reducing the use of freshwater for

103   feedstock pre-treatment to remove salt (Shi et al., 2013).

104   Previous research on fermentative hydrogen production by *V. tritonius* concentrated on the

105   effects of different pH levels, temperatures and substrates on higher hydrogen yields (Matsumura

106   et al., 2014). The effects of salt concentration on hydrogen production by strain AM2, however,

107 are not clearly defined and warrants further investigation. Herein, we investigate the effects of

108 different NaCl concentrations on the formate-hydrogen conversion of *V. tritonius* AM2. We used

109 a modified-Gompertz model to elucidate the kinetics of cell growth, hydrogen production and

110 substrate consumption during the fermentation process in an attempt to improve hydrogen

111 production and understand the process as NaCl levels increased. A modified Han–Levenspiel

112 model simulates the relationship of hydrogen production rates and its relative activity in response

113 to increasing NaCl concentration. We also compared the formate metabolism of *E. coli* strain JCM

114 1649, which possesses a similar FHL system in the fermentative H_2 production to those of *V.*

115 *tritonius*. We cultured the *E. coli* strain JCM 1649 in media supplemented with different NaCl

116 levels to those of the marine vibrio. This study is expected to help further the understanding of the

117 physiological impact of NaCl on formate-hydrogen bioconversion via the FHL pathway.

118

119 **Materials and methods**

120 **Bacterial strain and pre-cultivation**

121 Pure culture of *V. tritonius* strain AM2 (JCM 16456[T]) was maintained on a marine agar slant at

122 15°C and sub-cultured 3 times a month. The cells were then pre-cultured aerobically in marine

123 broth to reach a cell density of 0.8 to 1.0±0.1 OD_{620} measured by a microplate reader (Infinite

124 F200, Tecan, Switzerland), which corresponds to 1.4 to 1.7±0.1 OD_{620} using a UV/Visible

125 spectrophotometer (Ultrospec 2000, Pharmacia Biotech, USA), with shaking at 130 rpm (MMS-

126 48GR, EYELA, Tokyo, Japan) at 30°C. The pre-culture medium was a marine broth composed of

127 0.5% (w/v) polypeptone, 0.1% (w/v) yeast extract, and 75% (v/v) artificial seawater (ASW)

128 composed of 0.7 g/L KCl, 5.3 g/L $MgSO_4 \cdot 7H_2O$, 10.8 g/L $MgCl_2 \cdot 6H_2O$, 1.3 g/L $CaSO_4 \cdot 2H_2O$,

129 and 30 g/L NaCl at pH 7.5.

130   In a separate experiment, *Escherichia coli* strain JCM 1649 was used as a reference. Similar

131   to the experiment using *V. tritonius* AM2, the pure culture *E. coli* was stored on LB agar slant (1%

132   (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 1.5% (w/v) agar) at 15°C and sub-

133   cultured three times a month. Pre-culture was prepared using LB broth following a similar method

134   to that used for *V. tritonius*.

135

136   **Experimental designs**

137   Batch culture was performed using *V. tritonius* strain AM2 inoculated in a basal marine media

138   made of 0.5% (w/v) polypeptone, 0.1% (w/v) yeast extract, 100 mM MES (Dojindo, Kumamoto,

139   Japan), and 75% (v/v) basal salt solution composed of 0.7 g/L KCl, 5.3 g/L $MgSO_4 \cdot 7H_2O$ 10.8

140   g/L $MgCl_2 \cdot 6H_2O$, and 1.3 g/L $CaSO_4 \cdot 2H_2O$. To study the effects of Na^+ concentration, the

141   amount of NaCl was adjusted accordingly to constitute 0.5%, 1.0%, 2.25%, 3.0% and 5.0% (w/v)

142   of the culture media. One mL of the precultured inoculum was then added to 95 mL marine broth

143   containing 5% (w/v) mannitol in a glass bottle reactor. Batch culture was performed at 37°C and

144   pH 6.0 with gentle agitation using a magnetic stirrer (speed at 4.5, RO 10 power IKAMAG, IKA,

145   Staufen, Germany). The pH was maintained using a pH controller (DT-1023P, ABLE, Tokyo,

146   Japan) with an autoclavable electrode (FermProbe pH electrodes, Broadly-James Corp., Branford,

147   USA) with the addition of 5 N NaOH. The experiment was repeated in triplicate and samplings of

148   culture and gas were done at 12 h-intervals.

149   In batch culture experiments for *E. coli* strain JCM 1649, the bacteria was cultured on three

150   types of media namely LB broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl),

151   LB broth prepared using 60% (w/v) artificial seawater (with total NaCl equal to 2.30% and denoted

152   as LB broth with 60% ASW) and marine broth (containing 2.25% NaCl). 100 mM MES (Dojindo,

153   Kumamoto, Japan) was supplemented in all media. The strain JCM 1649 was precultured under

154   the same conditions as the strain AM2 culture, which gave a cell density of 1.3 OD_{620} after 24h

155   incubation. In these batch culture experiments, broth containing 5% (w/v) glucose was used for

156   strain JCM 1649, due to its unsuitability in utilizing mannitol. Other culture conditions were set

157   the same as those for the strain AM2. For both experiments using *V. tritonius* strain AM2 and *E.*

158   *coli* strain JCM 1649, neither external reducing agents nor sparging with inert gas is required

159   because both bacteria are capable of self-installing and maintaining anaerobiosis for hydrogen

160   production using sugars as carbohydrate (Hassan & Morsy, 2015; Matsumura et al., 2015).

161

162   **Assays**

163   The biogas produced was collected in an aluminum-bag (GL Sciences Inc, Tokyo, Japan) and

164   measured using the water displacement method. Hydrogen content in the biogas was determined

165   using gas chromatography (GC-2014, Shimadzu, Kyoto, Japan) equipped with thermal

166   conductivity detector (TCD) and ShinCarbon ST packed column (Shinwa Chemical Industries

167   Ltd., Kyoto, Japan) with Argon (Ar) as the carrier gas. The operational temperatures of the

168   injection port, oven and detector were 60ºC, 40ºC and 60ºC respectively. Extracellular ethanol was

169   measured using the same gas chromatography equipment together with a flame ionization detector

170   (FID) and HP-Blood Alcohol Capillary column (Agilent, California, USA). Helium (He) was used

171   as the carrier gas. The temperatures of the column, oven and detector were 60ºC, 40ºC and 60ºC,

172   respectively.

173   Organic acids produced, such as formate, acetate, lactate and succinate were measured using

174   high performance liquid chromatography (HPLC) with a conductivity detector and tandem

175   connected to two Shim-pack SCR-102H columns (Shimadzu, Kyoto, Japan). 5 mmol/L *p-*

176   toluenesulfonic acid was used as the mobile phase with the flow rate at 0.8 mL/min. Mannitol

177   concentration was measured by HPLC with a reflective index detector (RID) and TSKgel Amide-

178   80 column (TOSOH, Tokyo, Japan). 75% acetonitrile was used as the mobile phase with the flow

179   rate at 1.0 mL/min. The optical density (OD) of cell growth was measured using a microplate

180   reader (Infinite F200, Tecan, Switzerland) at a wavelength of 620 nm.

181

182   **Data analyses**

183   Molar yields of the fermentative products including hydrogen were analyzed using one-way

184   analysis of variance (ANOVA) with Duncan's multiple range tests. All statistical analyses were

185   performed using the Statistical Analysis System (SAS) University Edition. Probabilities of less

186   than 5% ($P<0.05$) were considered statistically significant.

187   The progress of cumulative hydrogen production, H (mL), in batch tests can also be described

188   using the following modified Gompertz model:

189    $f(X) = \alpha.Xmax.\exp\left(-\exp\left[\frac{\alpha.\mu max.e}{\alpha.Xmax}(\lambda - t) + 1\right]\right)$ eq. 1

190   Replacing $\alpha.Xmax$ with P as hydrogen production potential (mL), and $\alpha.\mu max$ by Rm, as

191   maximum hydrogen production rate (mL h^{-1}), eq. 1 can be expressed by eq. 2:

192    $H = P.\exp\left(-\exp\left[\frac{Rm.e}{P}(\lambda - t) + 1\right]\right)$ eq. 2

193   Similarly, the substrate consumption could also be expressed using the modified Gompertz

194   model (Mu et al., 2007):

195    $S_0 - S = S_{max}.\exp\left(-\exp\left[\frac{Rms.e}{S_{max}}(\lambda - t) + 1\right]\right)$ eq. 3

196   With S_0-S as the amount of substrate consumed in g/L; S_{max} as the maximum substrate

197   consumed and Rms as the maximum rate of substrate consumed (in g/L/h).

198   λ is the lag time (h) and e is equal to 2.718. All parameters in eq. 2 were non-linearly estimated

199   by converting the sum square of errors (SSE) between experimental data and estimation from the

200   models to a minimum value. This estimation was carried out using the 'Solver' function (Euler

201   integration technique) in Microsoft Excel 2010 (Kemmer and Keller, 2010). The significance of

202   the estimated parameters was tested using analysis of variance (ANOVA) as mentioned above

203   (Chen et al., 2006).

204   Hydrogen production rate, r (mL h^{-1}) of AM2 grown at various salt levels was calculated

205   following Wang and Wan (2008) in which:

206    $r = \dfrac{P}{\lambda + \left(\frac{P}{Rm}\right)}$ eq. 4

207   The hydrogen production rate was also denoted as HPR.

208   Response of the hydrogen production rate to different NaCl concentrations was calculated

209   using a modified Han–Levenspiel model as described in van Niel et al. (2003) and Zheng and Yu

210   (2005) by taking on the logarithms;

211    $ln(r) = nln\left(1 - \dfrac{C}{C_{\text{crit}}}\right) + ln\,(r\text{max})$ eq. 5

212   Where r is the hydrogen production rate (mL/h); $r$max is the maximum hydrogen production rate;

213   C is the added Na$^+$ concentration (g/L); Ccrit is the critical Na$^+$ concentration at which H$_2$

214   production ceases (g/L) and n is the degree of inhibition.

215   Using the $ln(r)$ calculated in eq. 5, the relative activity was calculated. Relative activity of

216   hydrogen production is a simple ratio between the $ln(r)$ of each 0.5, 1.0, 2.25, 3.0 and 5% NaCl

217   as X and $ln(r)$ of 0.5% NaCl as X_0 presented in percentage.

218   Relative activity $= \dfrac{X}{X_o} \cdot 100\%$ eq. 6

219   In order to describe the microbial growth in a batch culture, the Gompertz equation was

220   again modified following Zwietering et al. (1990) and Begot et al. (1996):

221     $ln(\frac{N}{N_0}) = A.exp\left(-\exp\left[\frac{\mu.e}{A}(\lambda - t) + 1\right]\right)$ eq. 7

222   where A is the logarithmic increase of bacterial population; λ is the lag time; μ is the maximal

223   growth rate; and t is the time.

224

225   **Results**

226   **Cumulative H_2 production, its kinetics and the growth of *Vibrio tritonius* at various salt levels**

227   The cumulative hydrogen production at different NaCl concentrations over time was fitted with

228   eq. 2 and is shown in Fig. 1. The experiment confirmed the significant effects of various levels of

229   NaCl on cumulative H_2 production using AM2. Substrate fermentation took place for 72 h and at

230   the end of the process, the culture in 0.5 and 1.0% (w/v) NaCl produced the highest amounts of

231   H_2, i.e. 7831 ± 295 and 8561 ± 602 mL/L reactor volume on average, respectively. On the other

232   hand, cells grown in media supplemented with 2.25% (w/v) NaCl produced slightly less hydrogen

233   at 6693 ± 443 mL/L reactor volume followed by 3.0% and 5.0% (w/v) NaCl at 1808 ± 79 and 3 ±

234   2 mL/L reactor volume, respectively. Table 1 shows the *P* as hydrogen production potential (mL),

235   *R*m, as maximum hydrogen production rate (mL h^{-1}) and λ (h) of the batch system in response to

236   various salt levels. The results imply the strain AM2 is capable of hydrogen production under a

237   wide range of NaCl concentrations, i.e. 0.5 to 3.0% (w/v). However, the amount of hydrogen

238   produced dropped as NaCl concentrations increased. Maximum H_2 production potential, *P*, was

239   observed when 0.5 and 1.0% (w/v) NaCl was supplemented in the media followed by the addition

240   of 2.25% (w/v) NaCl and showed further decreases at higher NaCl concentrations in the media.

241   The efficiency of fermentative biohydrogen is strongly connected to the presence of active

242   cells (Mu et al., 2006). Different NaCl concentrations greatly affected the growth of AM2. Cell

243   growth significantly decreased beyond 2.25% (w/v) NaCl indicating the presence of some degree

244   of inhibition with the addition of 3.0% and 5.0% (w/v) NaCl in the media. Statistical analysis of

245   the growth at the end of fermentation (72 h) for 0.5 to 2.25% (w/v) NaCl supplemented media

246   reveals that they were not significantly different ($P<0.05$) to one another. We also calculate the

247   maximum number of organisms, Xmax, the maximum growth rate, μ_{max} [$\ln(N/N_0).h^{-1}$] and the lag

248   time, λ (h) of the batch system in response to various salt levels. The maximum specific growth

249   rate, μ_{max}, was insignificant at 0.5 to 2.25% (w/v) with average $\mu_{max} = 0.8$ $\ln(N/N_0)$/h but inhibited

250   at 3.0 and 5.0% (w/v) with an average of $\mu_{max} = 0.5$ $\ln(N/N_0)$/h. *V. tritonius* showed a classical

251   growth trend under different salt concentrations and the average lag time, λ was at 2.3 h. The cells

252   enter a stationary growth phase after between approximately 14 to 23 h of fermentation and then

253   plateaued until the end of the fermentation period. Table 2 summarizes the kinetics of bacterial

254   growth at different NaCl concentrations as calculated using eq. 7. The kinetics of bacterial growth

255   and cumulative H_2 produced reveals that hydrogen production started during the exponential phase

256   in which cell growth and H_2 formation may occur simultaneously. Later, hydrogen evolution

257   continues during the stationary phases of bacterial growth and most of the hydrogen was produced

258   during this period.

259

260   **Specific H_2 production rate and yield**

261   The hydrogen production rate (HPR), r for each NaCl concentration tested was estimated using

262   eq. 4. The r values of 0.5 and 1.0% NaCl were indistinguishable (Table 1), 2.25 and 3.0% at 140

263   and 40 mL/h, respectively and 5.0% showed negative value indicating that inhibition had occurred.

264   The results, however, did not give any value to estimating the NaCl concentration, C_{50} at which a

265   50% reduction in H_2 production occurs. The logarithm of hydrogen production rate was plotted

266   against the added NaCl concentrations using eq. 5 and is depicted in Fig. 2. The curve was fitted

267   using a non-competitive inhibition model (eq. 5), ($r^2 = 0.99$) at $n = 2$ to demonstrate the relationship

268   of the hydrogen production rate to different NaCl concentrations using a modified Han–Levenspiel

269   model. The model demonstrates that the maximum hydrogen production rate was 374 mL H_2/h,

270   and C_{50} was 36 g/L NaCl. Figure 2 shows that H_2 production by AM2 is promoted with the addition

271   of 5 to 10.2 g/L (= 0.5 to 1.02% (w/v)) NaCl to the fermentation media. H_2 production is inhibited

272   under higher NaCl concentrations and is critically reduced, C_{crit} at 46 g/L (=4.6% (w/v)) NaCl.

273   Media containing 22.5 and 30 g/L NaCl resulted in 15 and 78% reduction of the original amount

274   of H_2 produced in 5 g/L NaCl containing media, respectively. The molar yields of hydrogen

275   produced at the end of fermentation (72 h) were compared and presented as mol H_2 per mol

276   mannitol. Statistical analysis by Duncan's multiple range test revealed that the H_2 yield by cells

277   grown in 5, 10 and 22.5 g/L NaCl supplemented media were not significantly different to each

278   other. The yields were greatly reduced as the NaCl concentration increases.

279

280   **Substrate consumption and effects of other fermentative products under different salt**

281   **concentrations**

282   To determine the effects of various salt levels on substrate consumption, substrate conversion

283   efficiency (SCE) were estimated by dividing the amount of mannitol consumed at a particular time

284   by the amount of initial mannitol (expressed as a percentage). Figure 3 shows the effects of NaCl

285   on mannitol consumption based on the kinetic simulation which was calculated following the

286   method described in Mu et al. (2007). At 72 h fermentation, almost all mannitol (95.6 and 98.4%)

287   was consumed in the 0.5 and 1.0% (w/v) NaCl media. Consumption was reduced as the NaCl

288   concentration increases with the highest NaCl concentration (5.0% (w/v)) showing a fluctuating

289   pattern in its substrate consumption.

290   Figure 4 depicts the molar formate concentration throughout the fermentation. For all salt

291   levels tested, formate was accumulated in the first 24 h of fermentation and was later consumed

292   until the end of experiment. Formate accumulation in 5.0% NaCl was significantly delayed and

293   most probably had caused low hydrogen production observed previously. The amount of formate

294   in the first 24h of fermentation did not show any differences ($P>0.05$) among 0.5 to 3.0% NaCl

295   tested with the highest, 2.25% NaCl had achieved 42.3 mM formate. At the end, the average of

296   10.7 mM formate was left unconsumed/imported in 0.5 to 2.25% NaCl containing media. For both

297   3.0 and 5.0% NaCl containing media, 20.8 mM formate remained.

298   Table 3 depicts the molar yields of fermentative products by AM2 in media containing 0.5

299   to 5.0% NaCl. The ANOVA of raw data shows acetate, lactate and ethanol production was

300   insignificant over the range of NaCl concentrations tested. In relation to cell growth and hydrogen

301   yield, the molar yields of succinate produced were also not significantly different from one another

302   in 0.5, 1.0 and 2.25% NaCl containing media, however, succinate production was significantly

303   reduced up to 3.0% NaCl.

304

305   **Fermentative hydrogen production behavior of *Escherichia coli* strain JCM 1649 affected by**

306   **NaCl and nutrition level of media**

307   The inverted V-shaped pattern in formate production was observed in *E. coli* strain JCM 1649.

308   Maximum formate production was observed after 6 h-cultivation in normal LB broth which

309   contains 0.5% NaCl and LB broth supplemented with 60% artificial water corresponding to 2.3%

310   NaCl. The formate produced was recorded at 12.2 mM/OD_{620} in 0.5% NaCl LB broth and 11.0

311   mM/OD_{620} in LB broth with 60% ASW (Figures 5A-B). In both cultures, most formate in the

312 supernatant then decreased until 72 h fermentation at which time all formate became undetectable.

313 On the other hand, although the maximum formate concentration in the culture using marine broth

314 reached 26.7 mM/OD$_{620}$ after 24 h-cultivation, the formate were barely consumed by the cells

315 resulting in much lower hydrogen production than those in 0.5% NaCl-LB and LB broth with 60%

316 ASW (Figure 5C). Note that, hydrogen produced from 0.5% NaCl-LB broth, LB broth with 60%

317 ASW and marine broth were 2056, 422 and 91 mL/L reactor volume, respectively (Figures 5A-

318 C). The rate of sugar consumption after 96 h-cultivation in the 0.5% NaCl-LB broth, LB broth

319 with 60% ASW, and in the marine broth were 93.2%, 93.6% and 46.0%, respectively. Growth was

320 maintained at 1.5, 1.2 and 0.9 OD$_{620}$ after 12, 14 and 48 h-cultivation in the LB broth, LB broth

321 with 60% ASW, and in the marine broth.

322

323 **Discussion**

324 Salts are essential in sustaining life on Earth, but higher salt concentrations cause various negative

325 effects in inhibiting major biochemical machineries. The use of seaweed and other marine

326 carbohydrate as alternative fuel sources to terrestrial biomass has proven to be advantageous in

327 biohydrogen production. However, most terrestrial microbial biocatalysts are likely to be less

328 effective for H$_2$ production in conditions with high salt levels that are usually observed when using

329 seaweed biomasses. In fact, the effects of salt on H$_2$ production were confirmed in *E. coli*, where

330 low H$_2$ production was recorded in the use of marine broth, which is a typical medium for marine

331 heterotrophic bacteria (Figure 5C). Using glucose as substrate, the fermentative hydrogen

332 production by the obligate anaerobe *Clostridium acetobutylicum* was also reduced by the addition

333 of salt at increasing concentrations of NaCl, and 18% reduction under 5 g/L NaCl (Alshiyab et al.,

334 2008). Therefore, the cells producing hydrogen have to be halotolerant or capable of adapting to

335   salinity in order to produce hydrogen when marine biomass is used as feedstock (Pierra et al.,

336   2014). As an initial step towards better use of seaweed and other marine carbohydrate as alternative

337   fuel sources, we studied the effect of increasing NaCl concentrations on fermentative hydrogen

338   using a marine vibrio isolated from the sea hare *A. kurodai*.

339   *V. tritonius* shows great potential in H_2 production achieving 80% yields from seaweed

340   powder even in 75% seawater-based medium (Matsumura et al., 2015). The bacterium produces

341   up to 1.7 mol H_2/mol mannitol and the molar yield of mannitol-grown cells is always higher than

342   those cultured using glucose as a substrate (Matsumura et al., 2014). Among the nine gas-

343   producing *Vibrio* spp., hydrogen production efficiency was higher in both species of Porteresiae

344   clade i.e. *V. tritonius* and *V. porteresiae*, and it was comparable to that of enterobacterial species,

345   such as *E. coli*, *Salmonella*, *Enterobacter*, and *Klebsiella* (Sawabe et al., 2013). The production

346   kinetics, however, related to salt concentration has never been characterized. Hydrogen production

347   involves complex biochemical reactions via a combination of various fermentation mechanisms

348   (Wang and Wan, 2009), thus, the use of kinetic models is the best to describe the relationships

349   between the variables and to explain the behavior of the process quantitatively (Mu et al., 2006;

350   Mullai et al., 2013). The present kinetic reports elucidate the behavior of fermentative hydrogen

351   production by *V. tritonius* AM2[T] at increasing NaCl concentrations in seawater-based media. The

352   bacterium is capable of producing hydrogen under a wide range of salt concentrations from 5 g/L

353   NaCl to 46 g/L NaCl using mannitol as substrate.

354   Vibrios are phylogenetically related to *Enterobacteriaceae* (Gomez-gil et al., 2014; Ruimy

355   et al., 1994), and previously, we showed the structure similarity between vibrio FHL complex and

356   *hyf* genes of *E. coli* (Matsumura et al., 2014; 2015). It is interesting to compare their metabolic

357   pathways and their relationship to bioH_2 production. Direct comparison of both bacteria involving

358   cell culture and fermentation, however, requires a few precautions due to the fact that *E. coli* is not

359   a halophile, the requirement of different culture conditions and the bacterium cannot ferment

360   mannitol. In this study, separate investigation on formate-hydrogen conversion by *E. coli* in three

361   different media (normal LB broth, LB broth in 60% ASW and marine broth) helped us to see that

362   hydrogen production by *V. tritonius* was much more stable than that produced from glucose by the

363   *E. coli* strain JCM 1649 in the seawater-based media. *V. tritonius* offers high hydrogen production

364   in saline environments and prolongs stable fermentation in a wide range of NaCl concentrations

365   up to 46 g/L NaCl.

366   Na^+ is one of the important factors in bacterial growth (van Niel et al., 2003; Alshiyab et

367   al., 2008), substrate intake and the yield of fermentative products (Wei et al., 1982; Casey et al.,

368   2013). Generally, growth of Gram-negative non-halophilic bacteria is inhibited under high saline

369   conditions (>12 g/L or >1.2% (w/v) NaCl) due to osmotic stress (Nakamura, 1977), which finally

370   affects their fermentative metabolism. As these organisms can experience both osmotic stress and

371   ion toxicity when exposed to high salt concentrations (Garcia et al. 1997), the efficiency of

372   fermentative $bioH_2$ is changes depending on the presence of active cells (Mu et al. 2006). When

373   compared to the LB supplemented with 0.5% NaCl (Figure 5A), it is worth to note that only hydrogen

374   production of *E. coli* was inhibited strongly under the presence of 2.30% NaCl (Figure 5B), while the

375   formate metabolism, cell growth and glucose consumption are apparently very similar in both media.

376   Later, the fermentation of glucose by *E. coli* in marine broth further shows the influence of other

377   components in the marine-based media to slightly slower cell growth, slower formate metabolism,

378   lower glucose intake and apparent decreases in hydrogen production (Figure 5C). Casey et al. (2013)

379   showed that all six pairs of anions (chloride and sulphate) and cations (sodium, potassium and

380   ammonium) in salts resulted in reduced ethanol fermentation by *Saccharomyces cerevisiae*.

381   Similarly, our study on *E. coli,* using seawater-based media adjusting at various salt levels most

382   likely suggested the effects of not only NaCl but other components in ASW that may contribute to

383   the decrease in hydrogen production. In contrast to these terrestrial microbes, our kinetics studies

384   revealed high and indiscriminate growth patterns of *V. tritonius* in seawater-based medium

385   containing 5 to 22.5 g/L NaCl with elevated and optimum hydrogen production occurring in 5 to

386   10.0 g/L NaCl (Figure 2). Though the efficiency of the fermentative system was later reduced at

387   higher NaCl levels, hydrogen production was continuously observed up to the addition of 46 g/L

388   NaCl (Figure 2). Similar decreases in hydrogen production were also observed in other studies on

389   mixed anaerobic cultures from sewage sludge, *C. acetobutylicum* and *C. butyricum* when

390   increasing concentrations of NaCl in the range of 0 to 30 g/L NaCl are introduced (Zheng et al.

391   2005; Alshiyab et al. 2008; Lee et al. 2012). In comparison with *V. tritonius*, hydrogen levels

392   produced in those studies (Zheng et al. 2005; Alshiyab et al. 2008; Lee et al. 2012) were much

393   lower and the reducing patterns occurred as early as the addition of 1 g/L NaCl. It is also worth

394   noting that NaCl or salts in general not only originated from the biomass itself but also from the

395   chemicals added during the fermentation (Casey et al., 2013; Palmqvist & Hahn-Hägerdal, 2000).

396   In our study, the potential use of *V. tritonius* as a biocatalyst for H_2 production in high saline

397   environments seems far more suitable in regard to its higher H_2 productivity, stability and ability

398   to tolerate a broad-range of salt concentrations.

399   The inverted V-shaped pattern of formate production in the fermentative systems of both

400   *V. tritonius* (Figure 4) and *E. coli* JCM 1649 (Figure 5) indicates the activities of formate being

401   exported out of and then re-imported back to the cells. Formate is not accumulated in the cells but

402   is directly oxidized or exported out of the cells to prevent acidification of the cytoplasm. The

403   transport was via a gating system named FocA (Leonhartsberger et al., 2002) which has been

404   suggested as operating bi-directionally (Falke et al., 2010; Sawers et al., 2004) and is dependent

405 on pH changes and active formate hydrogen lyase (FHL) complex (Doberenz et al., 2014; Lü et

406 al., 2011). Formate is converted to H_2 and CO_2 by the FHL complex in the absence of O_2 by its

407 substrate (formate) and increased acidity (Clark 1989). A complete genome survey suggested that

408 the strain AM2 has a single FocA homologue protein that most likely assists the re-import of

409 extracellular formate for hydrogen formation (Matsumura et al., 2015). In our current experiments,

410 increasing NaCl levels does not seem to affect formate production in *V. tritonius* over the range of

411 5 to 30 g/L NaCl in seawater-based media (Figure 4) but the formate re-uptake and hydrogen

412 produced were greatly reduced in media containing $\geq$22.5 g/L NaCl. Similarly, the formate

413 measured in the *E. coli* fermentative system grown in normal LB broth (5 g/L NaCl) and LB broth

414 prepared with 60% ASW (23 g/L NaCl) were not significantly different but the hydrogen produced

415 showed significant decreases in the latter medium. Furthermore, the formate production of *E. coli*

416 in marine broth occurs at much slower rates than those in LB broths but after 24 h fermentation,

417 more formate was detected and the amount was much higher than in those produced in the two LB

418 broths (Figure 5C). Formate re-import process by *E. coli* in marine broth was later suspended

419 (Figure 5C) resulting in only 9.1 mL hydrogen being produced. Lower nutrition levels under saline

420 conditions further affected the reduction of H_2 production of *E. coli*. Our results suggest the effects

421 of not only NaCl but also nutritional levels controlling the FHL activity through the formate re-

422 import providing the substrate of H_2.

423   Up to this point in the study, the observations suggest; 1) the strain AM2 is capable of

424 producing hydrogen under a wide range of NaCl levels, i.e. 5 to 46 g/L NaCl in the seawater-based

425 broth; 2) the amount of formate exported out of the AM2 cells within the first 24 h of incubation

426 is not affected by increasing salt levels up to 30 g/L NaCl but the formate re-import drops

427 significantly at $\geq$22.5 g/L NaCl; 3) the reduced ability of *E. coli* JCM 1649 to convert formate to

428   hydrogen in LB broth prepared with 60% ASW and almost complete shutdown that in marine

429   broth implies the influence of other ionic compounds or components in seawater on the FHL

430   complex or formate intake process; and 4) *V. tritonius* AM2 is a better candidate for fermentative

431   bioH$_2$ production in saline conditions than *E. coli*. Hypothetically, the more substrate consumed

432   (converted to formate) the more hydrogen will be produced. The actual mechanism by which the

433   hydrogen production machineries of vibrios adapt to salt is not yet understood. Careful

434   examination, however, on the *V. tritonius* system revealed that the pattern of hydrogen production

435   (Figure 1) closely resembles mannitol consumption (Figure 3). The observation supports our initial

436   hypothesis on substrate-hydrogen conversion and proposes that salt stress is heavily affecting the

437   substrate intake by cells. We later investigated the effects of increasing NaCl concentrations in

438   marine-basal media on substrate consumption by *V. tritonius* AM2. The results support the

439   hypothesis that the highest substrate conversion efficiency (SCE) of 94% and 97% were observed

440   in media containing 5 to 10 g/L NaCl, respectively, which both resulted in the highest hydrogen

441   production rate (HPR) of 240 mL/h hydrogen. The results were in agreement with

442   Jayasinghearachchi et al. (2010) who concluded that addition of 10 g/L NaCl in media resulted in

443   the optimum hydrogen production by *Clostridium amygdalinum* strain C9 in their study.

444

445

446   **Conclusions**

447   Current research is underway to understand the NaCl-dependent behavior of fermentative

448   hydrogen production of *V. tritonius* strain AM2 via FHL- complex. In conclusion, our data

449   suggests that higher NaCl levels have an overall inhibitory effect on the fermentation system yet

450   *V. tritonius* is still capable of producing hydrogen under a wide range of NaCl concentrations up

451   to 46 g/L NaCl. The fermentative system of *V. tritonius* is generally not affected in seawater-based

452   media containing 5 to 22.5 g/L NaCl, an amount that is relatively higher than common systems of

453   other bacteria. The tolerance to a wider range of NaCl concentrations is a promising attribute

454   towards a better fermentative system when seawater-based feedstocks or waste biomasses with

455   high salt concentrations are used. This behaviour could also be beneficial in developing continuous

456   batch culture for commercial-scale production. In addition to that, data presented herein (i.e. the

457   kinetics of substrate consumption, formate re-uptake data and succinate production) demonstrates

458   the need for global transcriptome studies that would provide insights in the interaction of cellular

459   reducing power or energy level on hydrogen production. As a result, our data in general suggests

460   the use of bacteria of marine origin, *V. tritonius*, for marine-based fermentative hydrogen

461   production due to its attributes being superior to those of other bacteria.

462

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655

Kinetic parameters of cumulative hydrogen production and mannitol consumption by *Vibrio tritonius* under batch fermentation at various salt levels calculated from a modified Gompertz model

NaCl concentration (%)(w/v)	Cumulative hydrogen production			Hydrogen production rate (HPR): r (mL/h)	Mannitol consumption		
	Maximum H_2 production potential: P (mL/L reactor volume)	Maximum H_2 production rate: Rm (mL/h)	Lag time: λ (h)		Maximum concentration of substrate consumption (g/L)	Maximum rate of substrate consumption (g/L/h)	Lag time: λ (h)
0.5	7879 [a]	291 [a]	5.8	240 [a]	48 [a]	1.2 [a]	0.4
1.0	8670 [a]	270 [a]	4	240 [a]	49 [a]	1.3 [a]	0.5
2.25	6968 [b]	161 [b]	6.5	140 [b]	34 [b]	0.9 [b]	0
3.0	1790 [c]	59 [c]	15.1	40 [c]	16 [c]	0.7 [c]	5.6
5.0	0 [d]	0 [d]	0	0 [d]	7 [d]	0.2 [d]	0

The kinetics of bacterial growth at different NaCl concentrations

NaCl concentration (%) (w/v)	X_{max} (ln(N/N$_0$))	μ_{max} (ln(N/N$_0$)/h)	λ (h)
0.5	5.200	0.771	2.037
1.0	5.272	0.857	2.275
2.25	5.223	0.725	2.353
3.0	4.561	0.569	2.658
5.0	3.556	0.409	2.224

The profile of fermentation products by AM2 at various salt concentrations

NaCl conc.	Molar yields (mmol/mol mannitol)			
(%)	Acetate	Lactate	Succinate	Ethanol
0.5	263 ± 26	55 ± 0	95 ± 16[a]	1610 ± 178
1.0	280 ± 6	60 ± 9	101 ± 13[a]	1469 ± 240
2.25	395 ± 30	79 ± 13	104 ± 20[a]	1664 ± 211
3.0	400 ± 121	65 ± 25	69 ± 21[b]	1517 ± 237
5.0	341 ± 112	66 ± 30	57 ± 16[b]	1330 ± 146

Figure 1

Effects of NaCl on the cumulative hydrogen production (P) of *Vibrio tritonius*.

The fermentation media containing 0.5% (◆); 1.0% (▲); 2.25% (■); 3.0% (×) and 5.0% (●) (w/v) NaCl were used. Symbols represent the experimental values and the curves were fitted with a modified Gompertz model (eq. 2) and drawn in full lines.
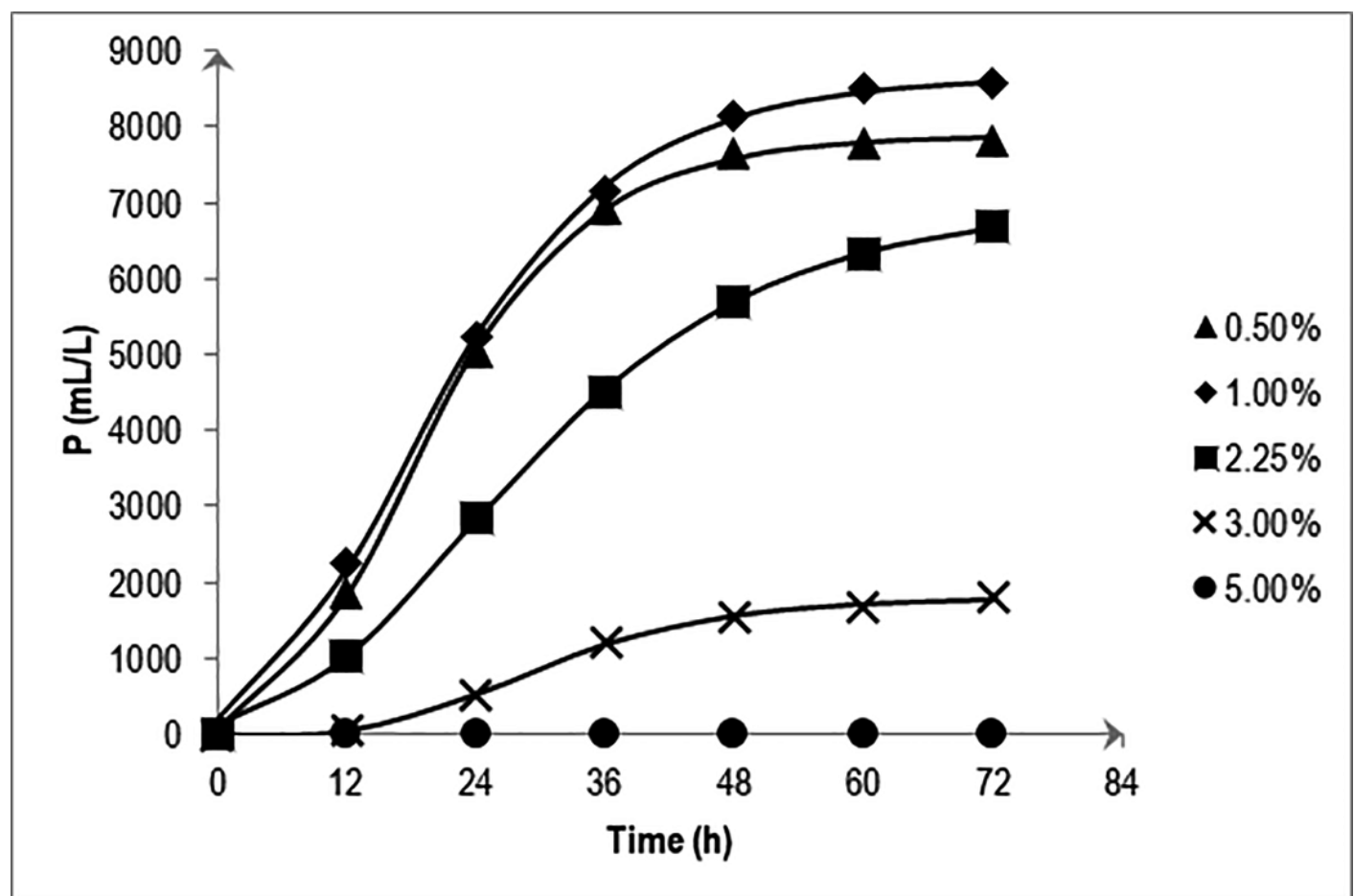
Figure 2

Effects of NaCl on the hydrogen production rate of *Vibrio tritonius*.

Logarithm of hydrogen production rate, [ln (r)] and relative activity using a non-competitive inhibition model. The fitted curve was drawn in full lines.
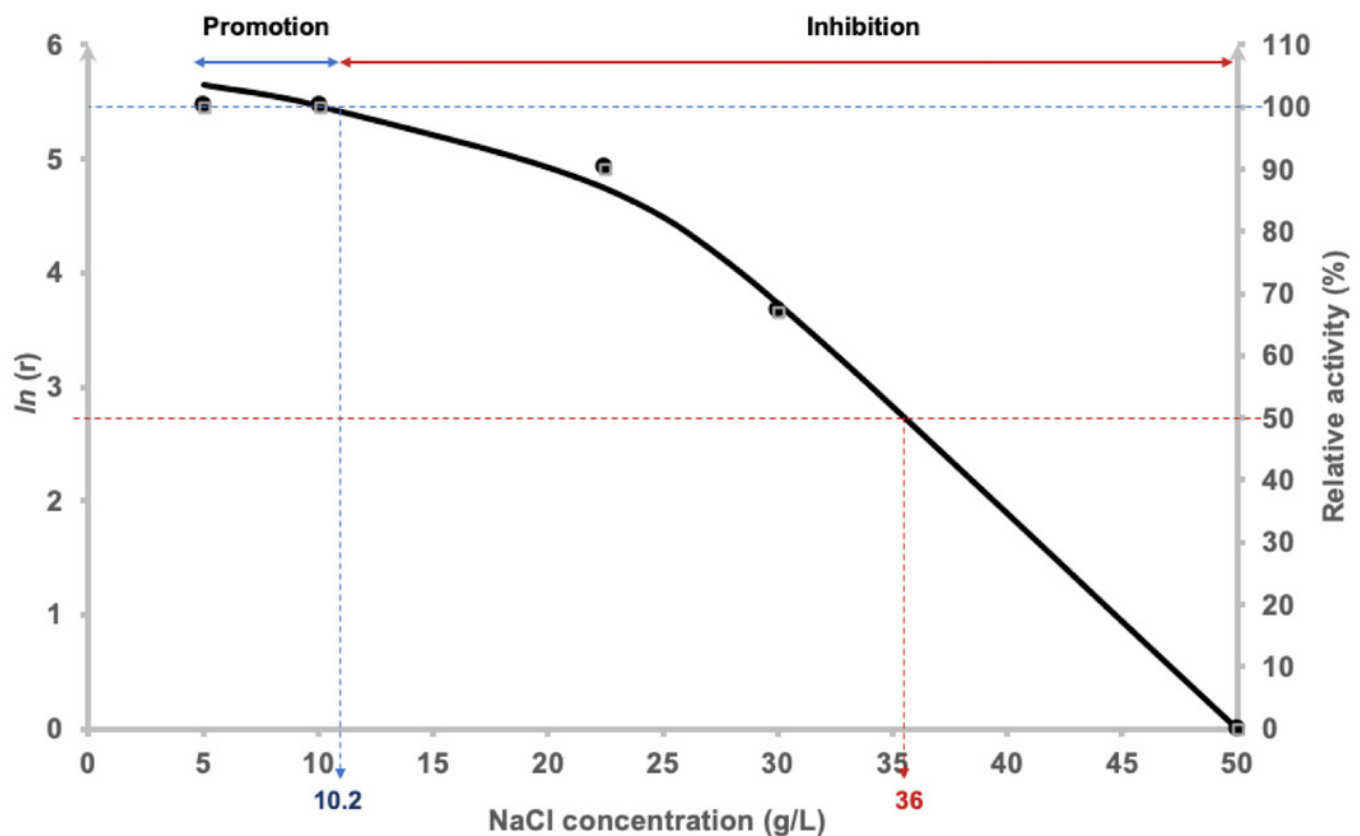
Figure 3

Effects of NaCl on the mannitol consumption by *Vibrio tritonius* AM2 based on the kinetic simulation.

The media contained 0.5% (◆); 1.0% (▲); 2.25% (■); 3.0% (×) and 5.0% (●) (w/v) of NaCl. Symbols represent the experimental values and the curves were drawn in full lines for all salt levels.
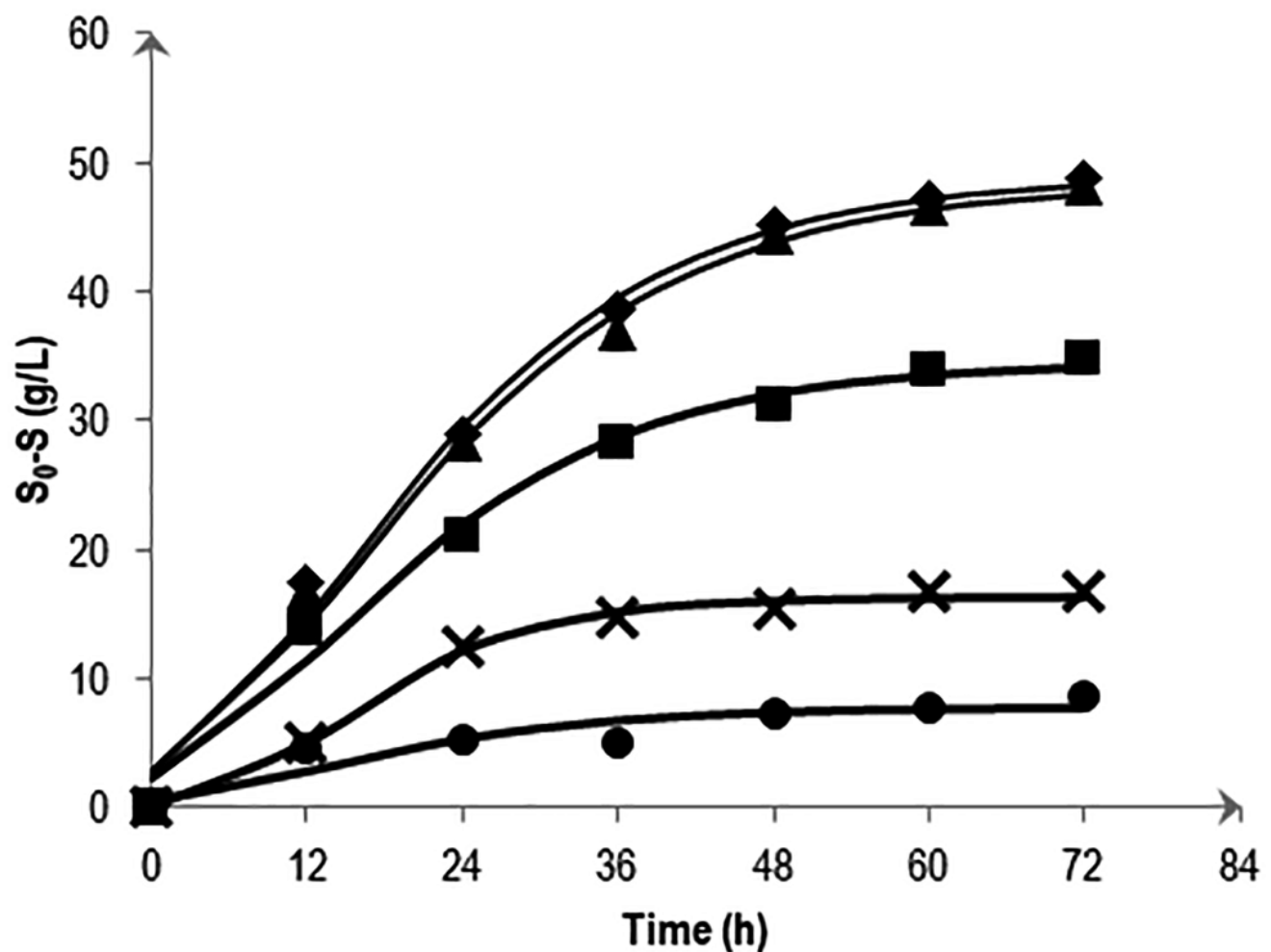
Figure 4

Effects of NaCl on extracellular formate emission and re-importation of *Vibrio tritonius*.

The fermentation media contained 0.5% (◆); 1.0% (▲); 2.25% (■); 3.0% (×) and 5.0% (●) (w/v) of NaCl. Symbols represent the experimental values and the curves were drawn in full lines for all salt levels.
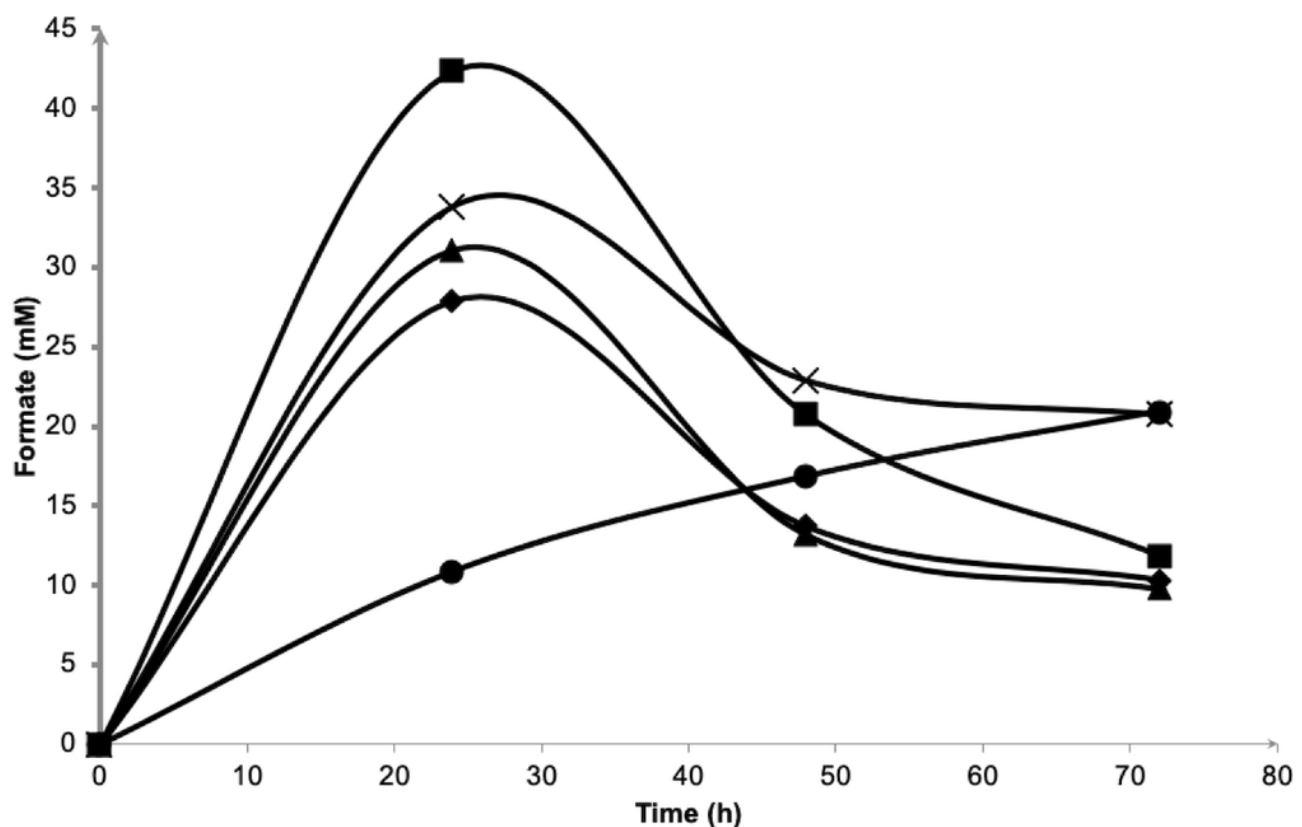
Figure 5

Time course of formate production, glucose consumption and hydrogen production of *Escherichia coli* strain JCM 1649.

Culture condition was set at pH 6.0 and 37ºC with (A) normal LB broth (with readily available 0.5% (w/v) NaCl); (B) LB broth with 60% ASW (contain 2.3% NaCl); (c) Marine broth (contain 2.25% NaCl). Symbols: ●, formate production; ■, H$_2$ production; ▲, glucose consumption; ○, growth.