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1 # Implication of exofacial thiol groups in the reducing
2 # activity of *Listeria monocytogenes*

3

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12 # Abstract

13 *Listeria monocytogenes* growing in BHI in anaerobic condition leads a decrease of
14 redox potential at pH7 (E_{h7}) from 0 to -250 mV. An investigation of mechanisms involved in
15 this reducing activity shows the implication of thiol groups located on the bacterial cell
16 surface. Indeed, after reduction of media to -250 mV, only thiol-reactive reagents could
17 restore the initial E_{h7} value. Moreover, the growth of *L. monocytogenes* in anaerobic condition
18 is characterized by a reduction then acidification phases. This suggests a sensor mechanism of
19 environmental E_{h7} to convert the metabolism way from the first phase to the second. Finally,
20 the concentration of exofacial thiol still increase strongly during the acidification phase of *L.*
21 *monocytogenes*, even after reach the minimal value of E_{h7}. These results suggest that
22 maintaining the exofacial thiol (–SH) groups in a reduced state do not depend on an active
23 mechanism. Thiol groups appear to be displayed by membrane proteins or cell wallbound
24 proteins and may participate in protecting cells against oxidative stress.
25

26 # Introduction

27 The oxido-reduction potential (E_h) was shown to have different effects on
28 microorganisms. E_h could be involved in gene expression, metabolism, physiology and so,
29 could modify their growth capacity and end product formation.
30 The E_h measurement and monitoring are increasingly used as tools for the
31 determination of bacterial activity in liquid media (Küsel and Dorsch, 2000), food products
32 (Abraham et al., 2013; Alwazeer et al., 2003; Aubert et al., 2002), soils (Fiedler et al., 2007)
33 and sediments (Schulz, 2000). Recent studies have emphasized that microbial cultures can
34 generate a reducing activity during their growth depending on the redox potential of different

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35 cellular compartments, and their ability to grow in the presence of dioxygen. (Prévost and
36 Brillet-Viel, 2013).

37 However, the mechanisms involved in bacterial reducing capacities remained poorly
38 understood. Two mechanisms have been described. The first correspond of the consumption
39 of oxidant compounds or the release of reducing end-products during the energy generation
40 metabolism. Indeed, to regenerate ATP, organic substrates are oxidized and dehydrogenated
41 during the glycolysis and citric acid cycles producing reduced electron carriers (NADH,
42 $FADH_2$). Then, these carriers are re-oxidized by electron transfer in the case of respiratory
43 metabolism to oxidants or to metabolic intermediates in fermentative metabolism. Thus, the
44 metabolic pathways of energy production determine the reducing activity of microorganism to
45 whether they are aerobic, facultative anaerobic or obligate anaerobic. Indeed, the terminal
46 electron acceptor in respiration of strict aerobic bacteria is dioxygen, which restricts the range
47 of redox potentials to values close to the oxidant values. Anaerobes meanwhile can reduce
48 external terminal electron acceptors such as NO_3^-, SO_4^{2-}, Mn (III/IV) and Fe(III) which allow
49 higher reducing capacities (until E_h = -600mV). Finally, most of anaerobes can also produce
50 strong reducing end-product such as H_2 (midpoint oxidation reduction potential, E_0' = -420
51 mV).

52 The second mechanism involved in bacterial reducing capacities which appears to be
53 not linked to metabolism (proton motive force) or production of reducing end-product have
54 been recently described in *Lactococcus lactis*. In this lactic acid bacteria, the reducing activity
55 is due to thiol groups of cell surface proteins (Michelon et al., 2010).

56 The aim of this study was to identify the mechanism involved in the reducing activity
57 of *Listeria monocytogenes*. Indeed, *L. monocytogenes* is a well-know food born pathogen
58 with a high reducing activity. It is able to decrease the E_{h7} (E_h at pH 7) to -250 mV (Ignatova
59 et al., 2008, 2010). However, the mechanism of this reducing activity was never studied

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60

61 # Materials and Methods

62 ## Bacterial strains, culture conditions

63 Three strains of *L. monocytogenes* were used in the present study. *L. monocytogenes*
64 CIP 78.35 and EGDe (CIP 107776), initially isolated from veterinary and medical case with
65 serotype 3 b and 1/2a respectively (Comi et al., 1997), were from the collection of Pasteur
66 Institute of Paris (France) and SOR100, isolated from industrial process with unkown
67 serotype, from the collection of LUBEM (Université de Bretagne Occidentale, France).
68 Concentrated stock cell suspensions were stored at -80°C in Brain Heart Infusion (BHI, AES,
69 France) supplemented with glycerol (10% v/v). Preculture of *L. monocytogenes* used as
70 inocula were grown aerobically in 10 mL BHI broth during 12h at 37°C. As need, *L.*
71 *monocytogenes* was enumerated on BHI agar plates incubated at 37°C during 24h.
72

73 ## Redox potential and pH measurement.

74 To prevent any uncontrolled E_h modification of the BHI broth due to reaction of
75 Maillard during autoclaving, BHI media was sterilized by filtration at 0.2 µm as described by
76 Ignatova et al. (2009). Anaerobic condition was reached by nitrogen bubbling (Alphagaz 1,
77 Air Liquide, France) during 5 hours. Then, BHI media was inoculated with 1 x 10^5 cells·mL^{-1}.
78 All growth and reducing activity experiments were conducted in a 400-ml water-jacketed
79 double-sidearm glass bioreactor (designed in Batailler labo, La Chapelle sur Erdre, France)
80 especially customized with butyl rubber seal caps (Laboratoires Humeau, La Chapelle sur
81 Erdre, France) to ensure air tightness. The redox potential (E_m) and pH of culture were
82 monitored at 37°C on BHI using a combined redox and pH electrode (InPro

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83 3253SG/120/PT100, Mettler Toledo, Viroflay, France). The redox electrode was referred to
84 the Ag/AgCl system. The electrode was connected on-line to a multi-channel interface
85 (Absciss interface; Absciss, Fixin, France). In order to obtain better stabilization of measured
86 redox potential, the electrode was polished with aluminum powder before each experiment
87 and treated with HCl- and pepsin-based Electrode Cleaning Solution (Grosseron, Nantes,
88 France) for 30 min once per week. A control measure was performed in tap water before each
89 experiment (Abraham et al., 2013). The measured redox values collected via the interface
90 were converted to E_h and E_{h7} values by using the software "Multi-bioreactors Central (3x E_h,
91 3xpH, 3xT°C)" (Absciss, Fixin, France) using the equation $E_h = E_m + E_{ref}$. In this equation E_h
92 is the redox potential compared to the standard hydrogen electrode, E_m the measured redox
93 potential and E_{ref} the standard potential of the reference electrode. The standard potential of
94 the reference Ag/AgCl electrode used in this study, was E_{ref} = 196.6 mV at 37°C. To
95 overcome the Nernstian effect of pH on the E_h value, the E_h was corrected to E_{h7}. The E_{h7}
96 which correspond to E_h if measured at pH 7 was calculated using the Leisner and Mirna
97 equation, $E_{h7} = E_h - [\alpha(7 - \text{pH})]$ (Leistner and Mirna, 1959) where α is the correlation
98 factor between pH and E_h. The α correlation factor for BHI at 37°C determined in this study is
99 identical to the previously published α value for this medium (35 mV·pH.U^{-1}, Ignatova et al.,
100 2010).

101 # Filtration

102 *L. monocytogenes* cells were harvested from culture by filtration using
103 polyethersulfone 0.22 µm Steritop-GP Filter Units (Millipore, Carrigtwohill, Ireland).
104 Polyethersulfone membrane filters were used because to their low protein and drug binding
105 properties. The resulting filtrate was degased using nitrogen bubbling during 3h before E_h
106 measurement.

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107 ## Neutralisation of thiol groups

108 In order to determine whether thiol-containing proteins and polypeptide chains are
109 exposed at the extracellular or cytoplasmic membrane surface, two thiol-reactive reagents, the
110 N-Ethylmaleimide (NEM) and the *4-acetamido*-4'-maleimidylstilbene-2,2'- disulfonic acid
111 (AMdiS) were used in this study. NEM is a membrane permeable compound able to react
112 with intra- and extra-cellular thiol groups. AMdiS is a high polarity water soluble compound
113 unable to cross the membrane and then can only react with the extracellular thiol groups.
114 NEM were obtained from Sigma (E3876, Sigma Aldrich, St Quentin Fallavier, France)
115 and AMdiS from Invitrogen (A-485, Invitrogen, Carlsbad, USA). A stock solution of 1M
116 NEM was prepared in methanol: water (3 : 1). The NEM was added to a final concentration of
117 25 mM in culture media. A stock solution of 65.2 mM AMdiS was prepared in water and
118 added in culture media to a 9 mM final concentration.

119 ## Titration of accessible exofacial thiol groups

120 The Ellman's method (Ellman and Lysko, 1979) using the 5,5'-dithiobis-2-
121 nitrobenzoic acid (DTNB) was used to assay the exofacial thiol groups during bacterial
122 growth. Because DTNB is a membrane impermeable compound, only the thiol groups located
123 at the bacterial cell surface could react. Thiols react with DTNB, cleaving disulfide bond to
124 give 3-thio-6-nitrobenzoate (TNB^-), ionized into a yellow colored TNB^{2-} dianion.
125 *L. monocytogenes* cells were harvested from the culture by centrifugation for 15 min
126 at 3500g then suspended in 1mL of 0,1 M potassium phosphate buffer, 60 µM DTNB, (pH 8).
127 The cell suspension was incubated 30 min at room temperature in the dark then centrifuged
128 for 15 min at 3500g. The supernatants were filtered through a 0.45 µm membrane filter
129 (Millipore). The concentration of exofacial thiol groups in the filtrate was measured by

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130 spectrophotometer at A_{412} and the concentration was calculated using N-acetyl-L-cysteine
131 standard curves (range: 5-100 µM).

132 Statistical analysis

133 The data analysis was performed using the statistical analysis software Statgraphics
134 Centurion XVI (StatPoint Technologies, Inc., Warrenton, Virginie, USA). An analysis of
135 variance (ANOVA) with $\alpha \leq 0,05$ was used to compare the results.
136

137 Results and discussion

138 Reducing activity of *L. monocytogenes*

139 The average evolutions (eight experiments) of E_{h7} and pH of *Listeria monocytogenes*
140 SOR 100, EGDe and CIP 78.35 growing at 37°C on BHI under anaerobic conditions are
141 presented figure 1. The kinetics of E_{h7} and pH during growth of *L. monocytogenes* shown two
142 consecutive phases. The first corresponds to a reduction and a constant pH phase during the 5
143 first hours of culture. This phase is characterised by the reduction of the media from initial E_{h7}
144 value (1 to 24 mV) to a minimal E_{h7} (-232 to -245 mV) depending of the strain. During the
145 reduction phase, the pH remainded stable at 7.3. After the reduction phase the E_{h7} remained
146 stable until the end of the growth of *L. monocytogenes* but the pH decreased. The second
147 phase corresponds to an acidification phase where the pH decreased from 7.3 to 5.9 between
148 the 5th hour to the 13th hour of culture, then slowly to a final pH value around 5.5 at 48h.
149 The descriptive factors of reduction and acidification phases for the three strains are
150 presented Table 1. The descriptive factors from the reduction and acidification kinetics were
151 the initial and final values of E_{h7} and pH, the lag time and duration of each phase and the

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152 mean rate of reduction and acidification. Any significant difference was shown between SOR
153 100, EGDe and CIP 78.35 by statistical analysis for each parameter.

154 To date, the reducing activity of *L. monocytogenes* has been only studied in aerobic
155 conditions (Ignatova et al., 2010). In fact, in aerobic condition, the reduction and acidification
156 are not divided into two separated phases and occur in the same time. Moreover, the final
157 value of E_{h7} is different between the two atmospheres, with a value of -179 ± 38 mV in
158 aerobic condition against -245 ± 23 mV in anaerobic condition both independently of the
159 culture medium and the initial E_{h7} value. This difference of minimal E_{h7} value is certainly due
160 to the presence for the first case of dissolved dioxygen in the media, known to be a high
161 oxidative compound. In addition, the only study on the reducing activity of another bacterium,
162 *L. lactis*, allow to compare our results to this Lactic Acid Bacteria (Michelon et al., 2010).
163 Comparatively, the reduction phase of *L. lactis* occur later than *L. monocytogenes* with a E_{h7}
164 value higher, while the acidification phase starts sooner with a pH value at 48h lower.

165 Role of the cell in the E_h decrease

166 In order to study the cell-linked reducing activity, the E_{h7} of *L. monocytogenes* cultures at the
167 end of the reducing phase (ERP), cell-free ERP filtrate and sterile BHI were compared (Figure
168 2). The E_{h7} value measured in sterile BHI were range from 10 to -40 mV. The *L.*
169 *monocytogenes* culture reduced the medium to -240 mV at ERP. The statistical analysis show
170 that for the three *L. monocytogenes* strains, E_{h7} of cell-free ERP filtrate and sterile BHI are not
171 significally different ($\alpha \leq 0{,}05$). These results shown (i) that reducing activity of *L.*
172 *monocytogenes* is not due to production of reducing end-products from metabolism released
173 in the medium (ii) that the restoration to the initial E_{h7} results from the cell removing from the
174 medium. So, the reducing activity of *L. monocytogenes* culture is mediated by the whole cells.

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175 A same observation had been made on *L. lactis* where the reducing activity was mediated by
176 the cells (Michelon et al., 2010).
177

178 Implication of Thiol groups in the decrease in E_h

179 Having refuted the hypothesis of reducing molecules excreted by the cell, we have
180 investigated the thiol cellular mechanism. To study the role of thiol compounds, two reagents,
181 N-Ethylmaleimide (NEM) and 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid
182 (AMdiS) were selected. These reagents contain maleimide, which can bind with thiol groups
183 in an irreversible reaction to suppress there activity in redox equilibrium. Due to its neutrality,
184 NEM can penetrate the cytoplasmic membrane and neutralized intra- and extra-cellular thiol
185 compounds. Contrariwise, AMdiS which is negatively charged cannot diffuse across the
186 cellular membrane. Thus, AMdiS can only react with external thiol groups. As shown in the
187 Figure 2, the addition of NEM or AMdiS to a media reduced by *L. monocytogenes* increase
188 the E_{h7} to its initial value. Therefore the experiment with NEM show that thiol groups are
189 engaged in reducing activity, while AMdiS indicate their exofacial localization.
190 Thus, we can assume that these ERP values are likely the result of an equilibrium of thiol
191 redox compounds as glutathione (E_0' = -240 mV), thioredoxin (E_0' = -195 to -270 mV) or
192 protein carrying cysteine (E_0' = -340 mV) (Mössner et al., 1999; Schafer et al., 2001; Sober
193 et al., 1968). Bioinformatics sequence analysis of *Listeria monocytogenes* EGD-e has
194 identified five proteins with a thioredoxin CXXC motif, may play a role in reducing
195 mechanisms. Among these proteins, there are four proteins with a T-X-X-C pattern and one
196 with a C-X-X-T pattern. Analysis of these proteins have identified as a thiol peroxidase, a
197 peroxiredoxin, a ribonucleotide reductase and glutathione peroxidase.

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198 **Evolution of exofacial thiol groups concentration during growth of**

199 ***L. monocytogenes***

200 The evolution of cell and exofacial thiol concentrations during the culture of *L.*
201 *monocytogenes* SOR 100, EGDe and CIP 78.35 in anaerobic BHI media are represented Fig
202 3A. The bacterial population and exofacial thiol concentration increase simultaneous
203 exponentially. Indeed, during the reduction phase, the concentration of exofacial thiols
204 increase slowly from 0 to 20 ±5 µM while during the acidification phase the concentration
205 increases to a maximum of 82 ±22 µM. Thus, the concentration of exofacial thiol still
206 increases after the end of reduction phase, unlike *L. lactis* (Michelon et al., 2010) where the
207 concentration stays stable at the end of reduction at 12 µM until the end of growth.

208 However, the ratio of thiol concentration per cell (Fig. 3B) is stable during the
209 reduction phase with a value of 1 to 5 femtomol/CFU and decrease during the acidification
210 phase to a final value of 30 attomol/CFU. Thus, the ratio is divided by a 100 factor during the
211 acidification phase.

212 The fig 3C shows the correlation between thiol concentration and E_{h7} value of the
213 media. During the reduction phase, the E_{h7} of media decrease proportionally to the increase of
214 exofacial thiol concentration. However, when the value of 20 µM of thiol is reached, the E_{h7}
215 of media stay stable at a minimum of -250mV while the thiol concentration still increases to a
216 range from 70 to 88 µM at 9h. This suggests that the Eh was stabilized when the
217 concentration of exofacial thiol molecules in the media reach a limit value (≈ 20 µM). From
218 this concentration the thiol–disulfide redox couple establish a more intense current with the Pt
219 electrode than other redox couples in the culture medium (Michelon et al., 2010; Peiffer et al.,
220 1992).

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221 In contrast with *L. lactis* (Michelon et al., 2010), the concentration of exofacial thiol of
222 *L. monocytogenes* still rises strongly until the stationary phase, while the ratio of thiol per cell
223 decrease. These results suppose a conversion of the metabolic ways to reduce the production
224 of exofacial thiol when the limit value is reached. Although the reducing activity was not
225 directly related to metabolic activity, reducing equivalents such as NADH or thioredoxin are
226 likely to be involved in the formation of exofacial thiol groups during the reducing phase.
227 These results let us suppose a sensor mechanism inducing a decrease of thiol compounds
228 production when a minimum of environmental E_{h7} value is reached. This implies a sensor
229 mechanism inducing the modification in the gene expression of the metabolism. In fact, it's
230 known that bacteria are able to sense the extra or the intracellular environmental redox state
231 with redox sensing mechanisms related to the thiol–disulfide balance and adapt their cell
232 activity (Green and Paget, 2004). Williams et al., (2005) have shown the sequence of 2 sensor
233 genes of environmental stress in *Listeria monocytogenes*, Lmo1948/Lmo1947, homolog of a
234 ResDE system of *Bacillus subtilis*. This latter is composed of a membrane sensor and a
235 cytoplasmic regulator and is involved in the regulation of aerobic and anaerobic metabolism
236 (Duport et al., 2006; Zigha et al., 2007). In addition, another study showed a gene family of
237 Crp / Fnr and their importance in resistance to oxidative stress by *Listeria* mutation targeted
238 genes (Lampidis et al., 1994; Uhlich et al., 2006). The identification of proteins located on the
239 extracellular surface and involved in the sensor mechanism and the decrease of E_h would be
240 of interest for increasing our understanding of the mechanisms involved as well as the
241 reducing activity of *L. monocytogenes*. Indeed, the systematic reduction of the extracellular
242 microenvironment may participate in protecting cells against oxidative stress and others
243 stress. Indeed, an overexpression of trxB gene encoding the thioredoxin reductase have been
244 shown in *Listeria monocytogenes* EGD-e at low temperature growth (Liu et al., 2002).
245 Another recent study demonstrated the presence and synthesis of glutathione in *Listeria*

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246 *monocytogenes* following stress caused by gallic acid and nisin, both molecules with
247 antimicrobial activities (Moro et al., 2012). Finally, it could also be able to play a role in some
248 demonstrated thiol-dependent mechanisms, as the folding of exoproteins or the virulence by
249 the action of listeriolysin (Leimeister-Wachter and Chakraborty, 1989).
250

251 ## Conclusions

252 In conclusion, the present study have shown the role of exofacial thiol in the reducing
253 activity of *L. monocytogenes* in anaerobiosis and ruled out the implication of reducing end-
254 products or the consumption of oxidizing compounds, as is mainly observed for other
255 bacterial species. The decrease of E_h during the reducing activity was directly connected to
256 the increase of cell density and concentration of thiol groups carrying on proteins on the
257 bacterial cell surface. Exofacial thiol could establish a reducing microenvironment around the
258 cell and might be used as ligands to coordinate such redox-responsive clusters (Green and
259 Paget, 2004).
260

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347 Figures legends

348 Fig. 1 : Evolution of E_{h7} (—) and pH (---) during culture of *Listeria monocytogenes* SOR 100
349 (■), EGDe (▲) and CIP 78.35 (◆) under anaerobic conditions (N_2). Average curves of 8
350 experiments are shown (SD respectively for SOR 100, EGDe and CIP 78.35 are for E_{h7} = ±
351 35 / 47 / 26 mV and pH = ± 0.17 / 0.15 / 0.10 pH units)

352

353 Fig. 2 : Histogram of E_{h7} values before inoculation, after reduction, after filtration, and after
354 addition of NEM or AMdiS in culture, for *Listeria monocytogenes SOR 100, EGDe* and *CIP*
355 *78.35*. For each treatment, the colums shown the average of a triplicate. The statistical
356 analysis was performed with ANOVA (P=0.05) and significant difference are show by O and
357 △ above the columns.

358

359 Fig. 3: Evolution in the concentration of exofacial thiol groups during reduction by *L.*
360 *monocytogenes SOR 100* (■), EGDe (▲) and CIP 78.35 (◆) under anaerobic conditions (N_2).
361 (A) Evolution of exofacial thiol concentration (histograms) and growth (curves). (B)
362 Evolution of the rate of mole of exofacial thiol per CFU. (C) E_{h7} evolution depending on the
363 exofacial thiol concentration. Phase 1 is the reduction phase while Phase 2 is the acidification
364 phase.
365

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366 Table 1 : Descriptive parameters of reduction and acidification phases of the growth of *L.*
367 *monocytogenes* and *L. lactis* in anaerobic conditions. ANOVA (P=0,05) was used to statistical
368 analysis. Each value is an average of 8 experiments. Any difference was observed between
369 SOR 100, EGDe and CIP 78.35.

	Listeria monocytogenes in BHI			*L. lactis ssp cremoris TIL 46* in MRS* (Michelon et al., 2010)
	SOR 100	EGDe	CIP 78.35	
Initial Eh_7 (mV)	1 ± 70	17 ± 92	24 ± 102	148 ± 19
Lag time of reduction (h)	0.7 ± 1.03	0.75 ±1.47	0.53 ± 0.57	1.84 ± 0.1
Rate of reduction ($mV.h^{-1}$)	-96 ± 57	-90 ± 88	-100 ± 95	-130
Time of reduction (h)	3.6 ± 1.1	4 ± 1	4.3 ± 1.5	
Final Eh_7 (mV)	-245 ± 23	-232 ± 34	-233 ± 21	-219 ± 1
initial pH	7.31 ± 0.03	7.31 ± 0.04	7.34 ± 0.05	6.37 ± 0.06
Lag time of acidification (h)	4.9 ± 1.5	5.5 ± 1.02	6.6 ± 2.9	3.43 ± 0.04
Rate of acidification (pH $units.h^{-1}$)	-0.36 ± 0.27	-0.38 ± 0.18	-0.38 ± 0.21	-0.13
Time of acidification (h)	4.31 ± 2.75	4.5 ± 2.2	4.51 ± 2.52	
Final pH (pH units)	5.88 ± 0.07	5.86 ± 0.08	5.90 ± 0.09	5.97 ± 0.06
pH at 48h (pH units)	5.54 ± 0.14	5.49 ± 0.07	5.69 ± 0.07	4.57 ± 0.06

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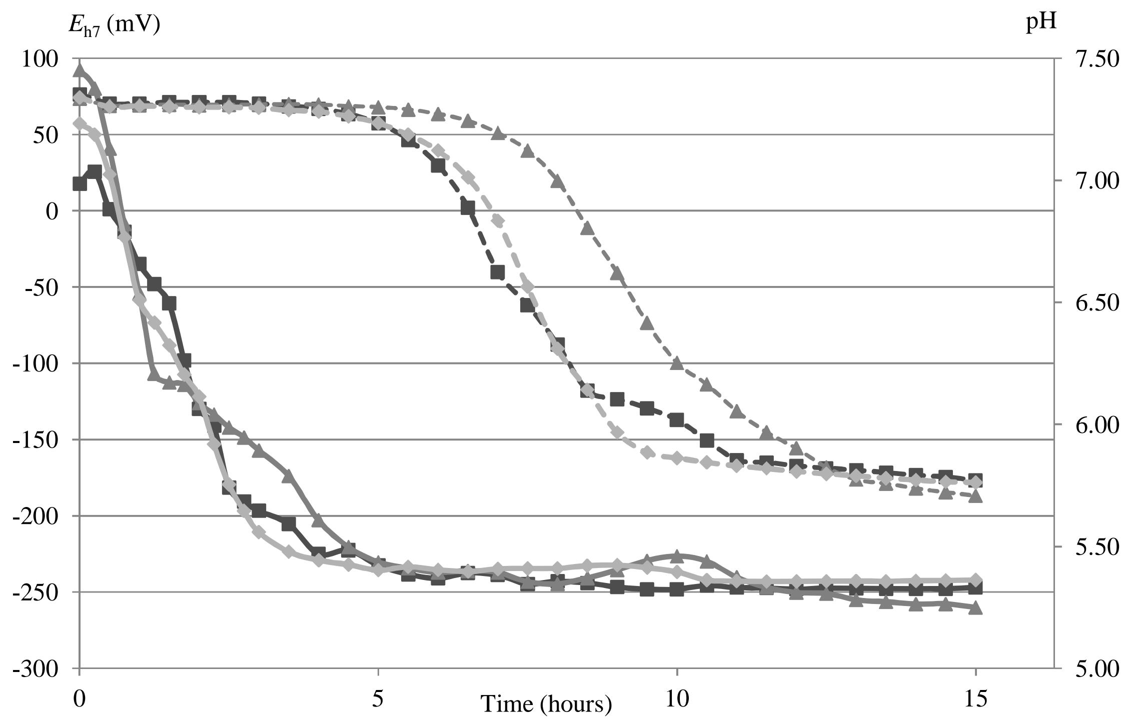

Figure 1

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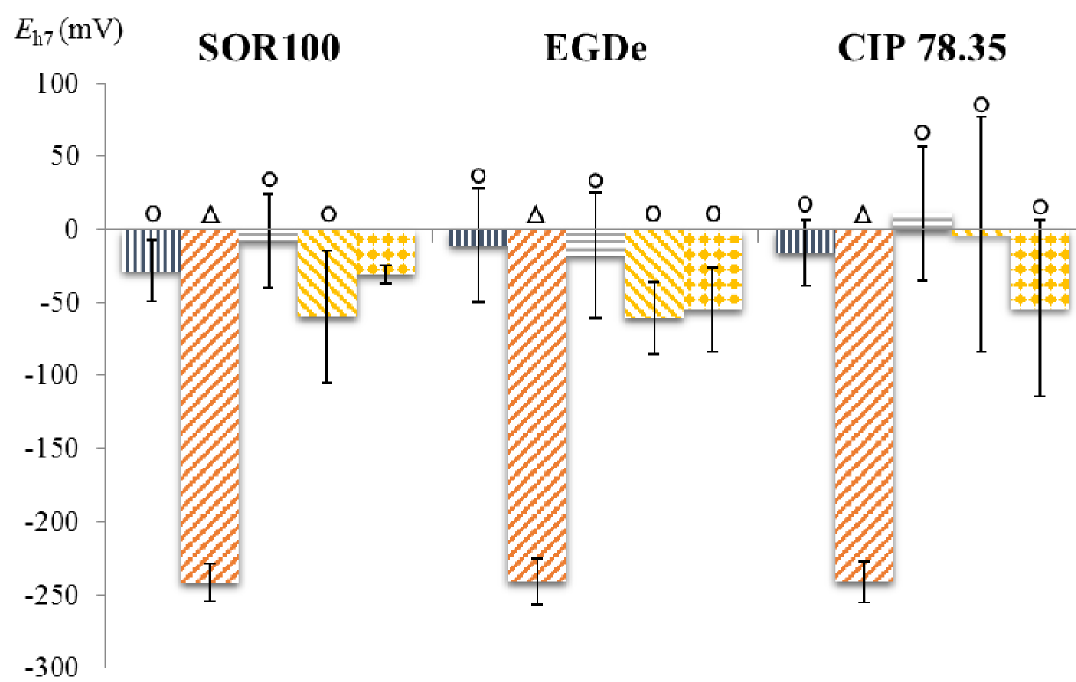

Figure 2

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