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1 RESEARCH ARTICLE

2

3 Enhanced production of natural shiga toxin by overexpressing A subunit of 4 Stx2e in Stx2e-producing *Escherichia coli* isolated in South Korea

5

6 Short Tillte : Enhanced production of shiga toxin by overexpressing A subunit of Stx2e in 7 Stx2e-producing *Escherichia coli* isolate

8

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26 **Abstract**

27 This study explored the optimal culture conditions for maximizing shiga toxin production in
28 Stx2e-producing *Escherichia coli* (STEC) 150229, isolated from porcine edema disease (ED),
29 with the goal of preparing a Stx2e toxoid vaccine candidate. High cytotoxicity was observed
30 for this strain [tissue culture cytotoxic dose 50% (10^4 $TCCD_{50}$/100 μl)] from 48 h after
31 incubation. Stx2e was overexpressed by transforming pStx2e A into STEC 150229, resulting
32 in the production of recombinant Stx2e A/B complex combined with intrinsic Stx2e B. The
33 enhanced production of Stx2e was evaluated based on the level of cytotoxicity against Vero
34 cells. The highest cytotoxicity (10^5 $TCCD_{50}$/100 μl) was observed with the samples of
35 recombinant Stx2e A/B complex eluted with 500 mM imidazole at 48 h of incubation. In
36 conclusion, the recombinant Stx2e A protein forms an active protein complex with the intrinsic
37 Stx2e B component from STEC 150229, producing high levels of shiga toxin.

38

39 **Key words:** Edema disease; recombinant protein; shiga toxin; Stx2e A subunit; Stx2e B
40 subunit

41

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42 **Introduction**

43 In pigs and wild animals, edema disease (ED) is primarily caused by Stx2e-producing
44 *Escherichia coli* [1, 2]. In humans, STEC also causes hemorrhagic colitis and/or hemolytic-
45 uremic syndrome [3]. Pigs infected with STEC, which is associated with high doses of Stx2e
46 toxin, can exhibit edema at various sites, most notably the colon, stomach, small intestine,
47 eyelids, and brain. Evidence of damage to the vascular endothelium resulting from such edema
48 includes ataxia, recumbence, convulsions, and paralysis [4], which ultimately result in a high
49 mortality rate in Stx2e-producing *E. coli* -infected pigs [5, 6].

50 Shiga toxin-producing *E. coli* (STEC) produces two major toxin types, Stx1 and Stx2,
51 which are functionally and structurally related to shiga toxin produced from *Shigella*
52 *dysenteriae* [7]. Even though there is greater than 50% genetic similarity between Stx1 and
53 Stx2 toxins [8, 9], they differ in their structural and immunological characteristics [10]. The
54 animal-related variants of STX2 in STEC, which are not found in humans, are known as Stx2e
55 [11], Stx2f [12], and Stx2g [13]. One of these, Stx2e as a component inducing enterotoxemia
56 of pigs, is mainly found in ED-affected pigs. Structurally, Stx2e toxin exhibits a
57 heterohexameric complex (1A5B type) in which a single A subunit is associated with a
58 homopentamer of B subunits [3, 14, 15]. The heterohexameric complex consists of the A
59 subunit, which plays a catalytic role, and five B subunits acting as glycolipid receptors and in
60 the binding of globotriaosylceramide (Gb_3) or globotetraosylceramide (Gb_4), on intestinal
61 epithelial cells [16-18]. In more detail, the A subunit is composed of A1 and A2 fragments,
62 which are tethered by a disulfide bridge between cysteines 241 and 260. The A1 fragment
63 contains an active site that mediates the cytotoxic effects. In contrast, the A2 fragment is
64 located at the central hole of pentameric B subunit. The A subunit exerts N-glycosidase activity
65 suspending protein synthesis within the targeted cells [15]. In this study, a cytotoxicity assay

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66 was used to demonstrate the toxin production of STEC isolates from a field case of porcine
67 ED, and enhancement of toxin production was also indicated by overexpressing recombinant
68 N-His Stx2e A/B complex with intrinsic Stx2e B in STEC 150229 (recombinant Stx2e A/B
69 complex).

70

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71 **Materials and methods**

72 **Ethics Statement**

73 This study was performed in strict accordance with the recommendations of the Guide for
74 the Care and Use of Laboratory Animals of the National Institutes of Health. All animal
75 experimental protocol was approved by the Jeonbuk National University Institutional Animal
76 Care and Use Committee (Approval Number: JBNU 2021-0136) and performed according to
77 accepted veterinary standards set by the Jeonbuk National University animal care center. Mice
78 were euthanized by CO_2 inhalation, as specified by the Jeonbuk National University
79 Institutional Animal Care and Use Committee guidelines.

80

81 **Shiga toxin producing *E. coli* strain**

82 At Chonbuk National University Diagnostic Center, edema disease-causing STEC
83 150229 was isolated from the feces of pigs from a pig farm in South Korea diagnosed with this
84 disease (CBNU-VDC) [19]. The isolated STEC 150229 strain was routinely grown in LB broth
85 (Affymetrix USB, CA, USA) at 37°C for 48 h. The cell-free supernatants of bacterial strains
86 were prepared by culturing them, centrifugation (10,000 ×g for 5 min) and filtration (0.22-µm
87 membaranefilter; Sartorius Stedim Biotech, Goettingen, Germany).

88

89 **Cytopathic effect of Shiga toxin in animal cell line**

90 The cytopathic effect (CPE) was tested in Vero cell line 76 (ATCC CRL-1587). Vero
91 cells were cultivated in Dulbecco's modified Eagle's medium (high glucose) (DMEM,
92 #LM001-05; Welgene, Korea) supplemented with 10% fetal bovine serum, 1% L-glutamine
93 200 mM (100×) (#25030-081; Gibco, NY, USA) and 1% Anti-anti (100×) (#15240-062; Gibco)
94 at 37°C and 5% CO_2. The Vero cell toxicity assay was performed in 96-well tissue culture
95 plates (#351172; Falcon, NY, USA) using 10-fold dilutions of samples. The plates were

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96 analyzed for the presence of CPE after 48 h and tissue culture cytotoxic dose 50%
97 ($TCCD_{50}$)/100 µl was calculated.
98

99 **Vector and Toxin gene cloning**

100 The gene of Stx2e A subunit (GenBank ID: CP024997.2) was amplified with specific
101 primer sets and inserted into the modified expression vector with *Nco*I and *Not*I restriction
102 enzyme (Fig. 2, pStx2eA). In order to modify an expression vector, the pelB gene, secretion
103 signal sequence, was connected to the pET30a (New England Biolabs Inc., MA, USA) vector
104 with *Nde*I and *Nco*I restriction site. A six-His tag was fused to the N-terminus of the Stx2e A
105 gene to facilitate protein purification.

106 To prepare purified Stx2e protein for antibody production in mice, two additional
107 plasmids were constructed; Stx2e A-fragment (amino acid 215-319) was inserted to pTWIN
108 vector with *Nco*I and *Bam*HI restriction enzyme (Fig. 2, pStx2e A-fusion). Stx2e B was inserted
109 to pET30a vector with *Nco*I and *Bam*HI restriction enzyme (Fig. 2, pStx2e B).
110

111 **Overexpression and purification of Stx2e and recombinant Stx2e A fusion/Stx2e B (r**
112 **Stx2e Afrag/B)**

113 The Stx2e A (Fig. 2) was overexpressed in STEC 150229 and *E. coli* BL21 (DE3) cells
114 which cultured on LB broth containing 25 µg/mL of kanamycin (Duchefa, Netherlands). The
115 protein expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG)
116 (Duchefa) at $O.D._{600}$ of 0.4 and the cells incubated for 24 h at 18°C, 170 rpm. The proteins
117 were purified from supernatant with HisTrap FF column (GE Healthcare, PA, USA) and eluted
118 using sequentially increased Imidazole concentration (25, 50, 100, 250 and 500 mM) in 10 mM
119 Tris buffer, pH7.0. The protein obtained from serial elution step was analyzed by 15% SDS-
120 PAGE.

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121 The Stx2e A-fragment and Stx2e B proteins were co-expressed on *E. coli* BL21 (DE3)
122 pLysS cells which cultured on LB broth containing 25 μg/ml of kanamycin, 50 μg/ml of
123 ampicillin (Duchefa) and 34 μg/ml of chloramphenicol (Wako, Japan). The protein expression
124 induced with 0.8 mM IPTG at $O.D._{600}$ of 0.5 and cultures were incubated for 16 h at 18°C, 150
125 rpm. Overexpressed proteins were purified with FPLC system (GE healthcare) by using
126 HisTrap FF column (buffer A : 10mM Tris-HCl, pH 8.5, 100 mM NaCl and buffer B : 10 mM
127 Tris-HCl, 100 mM NaCl, 500 mM Imidazole, pH8.5).

128

129 **Immunization of mice and challenge with recombinant Stx2e toxin**

130 Twenty C57BL/6 female mice were purchased and randomly divided into 2 groups. All
131 groups were intramuscularly primed (IM) at week 6 of age [0 week post prime immunization
132 (WPPI)] and were IM boosted at 2 WPPI. The immunized group mice were immunized with
133 the 100 μg of rStx2e A frag/B. The negative control group mice were injected with sterile
134 phosphate-buffered saline (PBS). To evaluate each the antigen-specific antibody titer, blood
135 samples were collected at 0, 2 and 4 WPPI. All samples were stored at -80°C until use.

136

137 **Results**

138 **Optimizing cultivation of Shiga toxin producing *E. coli* strain**

139 To examine the optimal cell count and cytotoxicity levels, STEC 150229 was cultivated
140 for 72 h. It showed its maximal cell count of 10^8 colony-forming units (CFU)/ml at 24 to 72 h
141 after incubation and a high cytotoxicity level of 10^4 $TCCD_{50}$/100 μl at 48 and 72 h after
142 incubation (Fig. 1). Based on the results regarding the optimal culture conditions, culture of
143 STEC150229 for 48 h was used in this experiment.

144

145 **Production of rStx2e A frag/B complex proteins and immunization of mice**

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146 To increase the production of Stx2e complex, we tried to overexpress the recombinant 6-
147 His Stx2e A protein in the STEC 150229 cell. According to our hypothesis, the recombinant
148 protein could establish an enzymatically active complex with the innate toxin proteins. During
149 the purification step, the intrinsic shiga toxin complex was removed using a column at a low
150 imidazole concentration and the recombinant complex was eluted and concentrated (Fig. 3).
151 Our findings showed that a functional Stx2e protein complex was constructed with the
152 recombinant Stx2e A-His and intrinsic Stx2e B in STEC 150229 (rSTEC 150229), the
153 cytotoxicity of which was evaluated in Vero cells.

154 To detect intrinsic and overexpressed Stx2e toxin, an antigen-specific antibody was
155 required. However, the expression level of recombinant Stx2e (6-His Stx2e A and intrinsic
156 Stx2e B) was extremely low, so it was difficult to purify and concentrate it for use as an antigen.
157 Even purified recombinant Stx2e complex concentrated 1000-fold was not detected on western
158 blotting with anti-His antibody (data not shown). In addition, rSTEC 150229 was difficult to
159 inject into mice due to the high production of shiga toxin. Thus, we constructed a new
160 recombinant protein able to produce antibodies and to detect shiga toxin of rSTEC 150229.

161 Anti-Stx2e antiserum was produced by two injections of rStx2e Afrag/B into C57BL/6
162 mice. To detect intrinsic and overexpressed recombinant Stx2e toxin, SDS-PAGE (Fig. 3A)
163 and western blotting with antiserum collected from a mouse injected with rStx2e Afrag/B were
164 performed (Fig. 3B). The culture medium harbored secreted innate Stx2e toxin and
165 recombinant toxin complex and purified proteins were concentrated 1000-fold. The estimated
166 molecular weight of Stx2e A is 32 kDa and that of intrinsic Stx2e B is 7.7 kDa (14,15). Based
167 on this estimation, Stx2e A with a size of 35 kDa was detected in STEC 150229 and rSTEC
168 150229 although the concentration was significantly higher in rSTEC 150229. In addition,
169 Stx2e A/B complex of approximately 55 kDa in size was detected only in rSTEC 150229 (Fig.
170 3B). The Stx2e A and B proteins purified from rSTEC 150229 cells were not detected in

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171 western blotting, even after 1000-fold concentration.

172

173 **Cytopathic effect of recombinant Stx2e complex protein**

174 The cytotoxicity of all samples is listed in Table 1. Deletion of the N-terminus of Stx2e
175 A, which is involved in the enzymatic reaction, was associated with the loss of cytotoxicity
176 (Table 1). The cytotoxic activities were not detected in Stx2e Afrag/B protein-overexpressing
177 *E. coli* BL21 (DE3) or in native *E. coli* BL21 (DE3), as a negative control (Table S1).

178 The cytotoxicity of unbound and eluted sample with a low imidazole concentration was
179 caused by the intrinsic toxin complex. The cytotoxic activity of recombinant 6-His Stx2e A/B
180 samples eluted with 500 mM imidazole was significantly higher than that of all other imidazole
181 elution samples of recombinant 6-His Stx2e A/B and STEC 150229 culture supernatant. The
182 cytotoxicity of eluted sample with 500 mM imidazole was measured in 10^5 $TCCD_{50}$/100 μl at
183 48 h of incubation. The other samples showed the lower cytotoxicity, 10^3 to 10^4 $TCCD_{50}$/100
184 μl at 48 h of incubation (Table 1).

185

186 **Discussion**

187 In this study, we designed recombinant 6-His Stx2e A/B plasmids based on the shiga
188 toxin sequence of STEC isolates to produce recombinant toxin proteins in a heterologous
189 expression system. We also evaluated the cytotoxicity for the toxin protein complex active
190 form of recombinant Stx2e A-His and intrinsic Stx2e B in STEC 150229. STEC 150229
191 showed that the production of shiga toxin was highest in a previous report [19]. From a total
192 of 43 isolates from diagnosed case strains, 14 edema disease-causing *E. coli* strains including
193 Stx2e and F18 genes were confirmed by toxin gene PCR. Among the edema disease-causing
194 *E. coli*, 13 strains were observed to have cytotoxicity levels of 10 to 10^2 $TCCD_{50}$/100 μl,
195 whereas STEC 150229 was observed to have a higher cytotoxicity level of 10^3 $TCCD_{50}$/100 μl

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196 in Vero cells [19] (data not shown). As it is known that these cells can synthesize and secrete
197 the intrinsic shiga toxin to the extracellular region, we hypothesized that the overexpressed
198 recombinant Stx2e A protein forms an active protein complex with the intrinsic Stx2e B
199 component from STEC 150229. The recombinant Stx2e A contained the PelB peptide, the
200 secretion leader, and it could be secreted into the periplasmic space of *E. coli*. Therefore, the
201 Stx2e complex was collected and purified from the growth medium after protein
202 overexpression. The cytotoxic activity of recombinant 6-His Stx2e A/B protein samples eluted
203 with a higher imidazole concentration was significantly higher than that of all other imidazole
204 elution samples. The cytotoxicity of recombinant 6-His Stx2e A/B from rSTEC 150229
205 samples eluted with 500 mM imidazole were measured in 10^5 $TCCD_{50}$/100 μl in Vero cells
206 upon 48 h of incubation. Higher production of shiga toxin was obtained from recombinant 6-
207 His Stx2e A/B. To detect purified recombinant Stx2e A and intrinsic B complex, we performed
208 western blotting using mouse-generated anti-Stx2e serum. The rSTEC 150229 secreted higher
209 concentrations of toxins than the wild-type STEC 150229 cells (Table 1). A nonspecific band
210 detected in the negative control appeared to represent the protein originating from *E. coli* cells.
211 However, purified Stx2e A protein was not detected. The detection limit of western blotting is
212 known to be on the order of picograms depending on the ECL sensitivity. This indicates that
213 our purified protein might have high toxicity even when present at only a low level.
214

215 **Conclusion**

216 In conclusion, the recombinant Stx2e A protein forming an active protein complex with
217 the intrinsic Stx2e B component from STEC 150229 and rSTEC 150229 produced high levels
218 of shiga toxin.
219

220 **Acknowledgements**

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227 **Author's contributions**

228 **Conceptualization:** Jung Hee Park, Won Il Kim, Jin Hur

229 **Data curation:** Byoung Joo Seo, Jeong Hee Yu, Seung Chai Kim, Byung Yong Park

230 **Supervision:** Jung Hee Park, Won Il Kim, Jin Hur

231 **Writing-original draft:** Byoung Joo Seo, Jeong Hee Yu

232 **Writing-review & editing:** Jung Hee Park, Jin Hur

233

234 **Declaration of conflicting interests**

235 The author(s) declared no potential conflicts of interest with respect to the research,

236 authorship, and/or publication of this article.

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293 Figure Legends

294 Fig. 1. The optimization and cytotoxicity levels of STEC 150229 during incubation.

295 Fig. 2. Scheme of the gene constructs of recombinant Stx2e A and B proteins. In pStx2eA
296 plasmid, the pelB signal sequence for periplasmic secretion was connected at the N-
297 terminus of the Stx2eA gene and a 6-histidine tag was inserted in the middle to
298 facilitate purification. In the pStx2eA-fusion plasmid, Stx2eA fragment (a.a. 215–319)
299 was connected to the N-terminus of the intein protein in the pTWIN1 vector. Stx2eB
300 gene was inserted into the pET30a vector, which contained a signal peptide in the N-
301 terminus.

302 Fig. 3. SDS-PAGE (A) and western blotting (B) of recombinant Stx2eA and innate Stx2eB. In
303 lanes 2 and 3, Stx2eA protein was detected (arrow). [Lane M: size marker; Lane 1: *E.*
304 *coli* BL21 (DE3) cell (negative control); Lane 2: concentrated STEC 150229 culture
305 medium; Lane 3: concentrated Stx2eA-overexpressing STEC 150229 culture medium;
306 Lane 4: flow-through; Lane 5: eluent with 250 mM imidazole; Lane 5: eluent with 500
307 mM imidazole].

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309 Table I. Cytotoxic levels of recombinant Stx2e A_6-His/B and STEC 150229 in Vero cell

Samples	Imidazole Conc. (mM)	10 fold dilutions					
		1	2	3	4	5	6
BL21		-[a)]	-	-	-	-	-
150229		+[b)]	+	+	+	-	-
BL21 + Stx2e A_6-His (pStx2eA)	flow	-	-	-	-	-	-
	20	-	-	-	-	-	-
	50	-	-	-	-	-	-
	250	-	-	-	-	-	-
	500	-	-	-	-	-	-
150229 + Stx2e A6-His (pStx2eA)	flow	+	+	+	-	-	-
	20	+	+	+	-	-	-
	50	+	+	+	-	-	-
	250	+	+	+	+	-	-
	500	+	+	+	+	+	-
Elution buffer	20	-	-	-	-	-	-
	50	-	-	-	-	-	-
	250	-	-	-	-	-	-
	500	-	-	-	-	-	-
Control		-	-	-	-	-	-

[a)] None cytotoxicity; [b)] Cytotoxicity

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312 **Supporting information**

313 **S1 Table. Cytotoxic levels of recombinant Stx2e A-fragment and Stx2e B in Vero cell.**

314 **S1 Fig. Scheme of the purification of recombinant Stx2e complex in STEC 150229 cells.**

315 The innate Stx2e complex was washed with a low imidazole concentration and the

316 recombinant Stx2e complex was eluted in an increasing imidazole concentration.

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319 Table SI. Cytotoxic levels of recombinant Stx2e A-fragment and Stx2e B in Vero cell

Samples	Imidazole Conc. (mM)	10 fold dilutions					
		1	2	3	4	5	6
BL21		-[a]	-	-	-	-	-
150229		+[b]	+	+	+	-	-
BL21 + Stx2e A-fragment (pStx2eA-fusion)	flow	-	-	-	-	-	-
	20	-	-	-	-	-	-
	50	-	-	-	-	-	-
	250	-	-	-	-	-	-
	500	-	-	-	-	-	-
BL21 + Stx2e B (pStx2eB)	flow	-	-	-	-	-	-
	20	-	-	-	-	-	-
	50	-	-	-	-	-	-
	250	-	-	-	-	-	-
	500	-	-	-	-	-	-
Elution buffer	20	-	-	-	-	-	-
	50	-	-	-	-	-	-
	250	-	-	-	-	-	-
	500	-	-	-	-	-	-
Control		-	-	-	-	-	-

[a] None cytotoxicity; [b] Cytotoxicity

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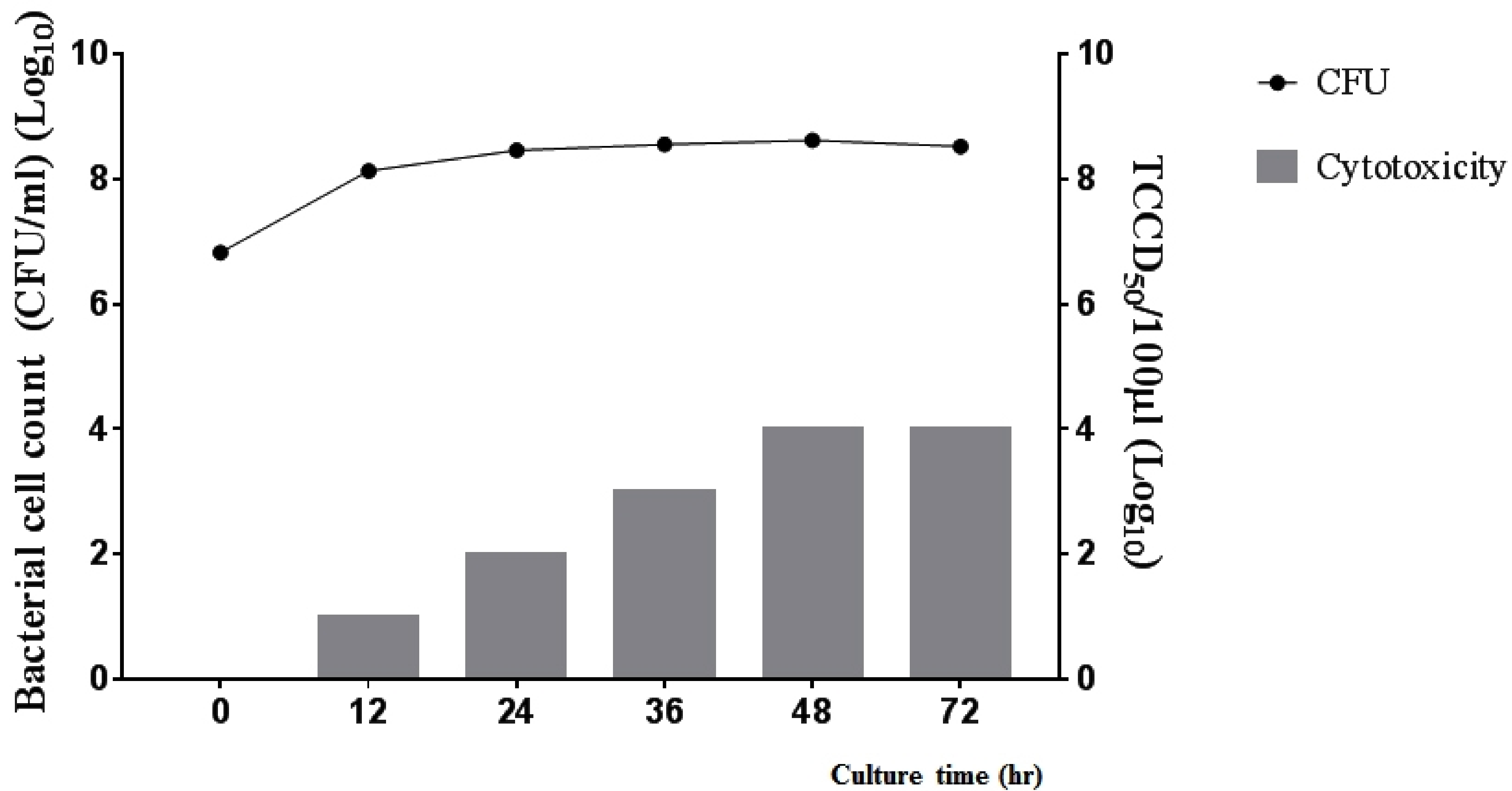
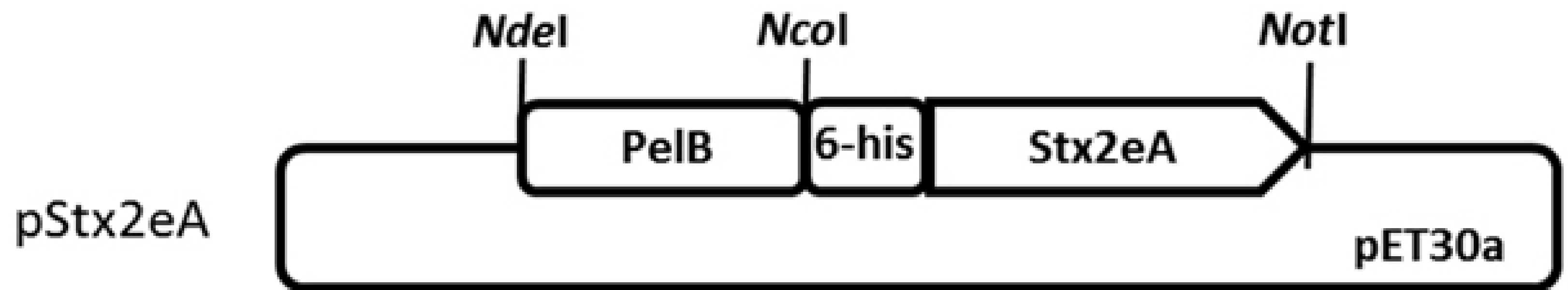
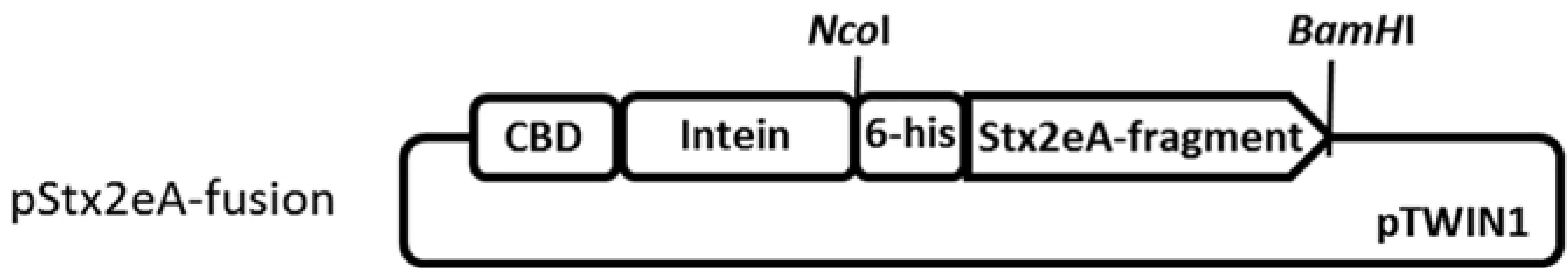
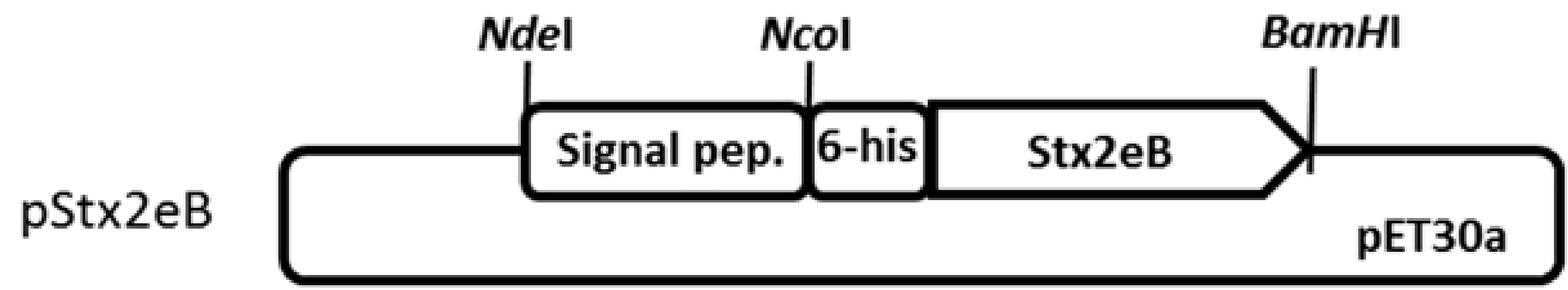


Figure







Figure

1 2 3 M 4 5 6

A.

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35

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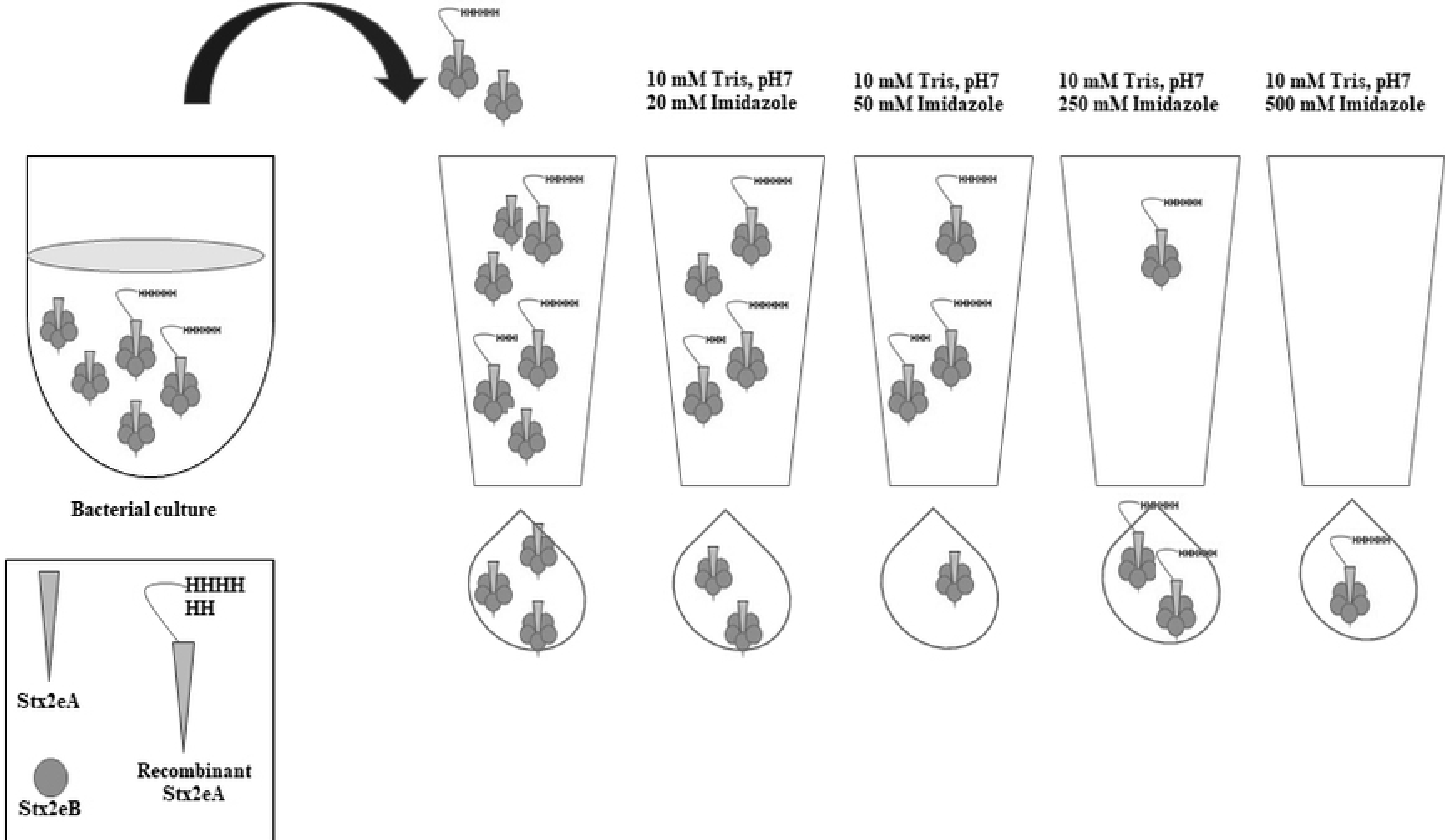
B.

Figure



Figure