INFLUENCE OF AMPICILLIN RESISTANT *CRONOBACTER SAKAZAKII* ON THE STABILITY AND EXPRESSION OFGFPUV IN MUTANT CELLS

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ABSTRACT

Construction of *Cronobacter sakazakii* with pGFPuv could facilitate the tracking of the bacteria in foodstuff. GFPuv plasmids are completed with ampicillin resistant gene for easy selection. Previous studies indicated that ampicillin resistant bacteria could not be made into GFPuv mutants, but the reason was unknown. The objectives of this study were to investigate the performance of *C. sakazakii* GFPuv mutants based on their stability and GFP expression and to understand the correlation between ampicillin resistance traits in the wild types with the success of GFPuv mutants construction. The results showed that the ampicillin resistance level of *C. sakazakii* isolates varied. Of the 12 isolates studied, 8 isolates (66.6 %) were susceptible ((FWHc3, FWHb6, FWHb15, YRw3, E2, E4, E6, and E9), two (16.66%) had intermediate resistance to ampicillin (Desb10, E7), and the other two (16.6 %) were ampicillin resistant (FWHd1, E1). The ampicillin sensitive *C. sakazakii* could be labeled with pGFPuv and their plasmid stability were most stable (E2). The ampicillin resistant *C. sakazakii* isolates also could be labeled with pGFPuv but the resulting mutants were unstable in the cell and could not express the GFPuv. The high resistance isolate could not express the GFPuv (E7 and FWHd1). Characterization of isolate resistance on antibiotic marker of plasmid is required to assure successfulness and stability of bacteria labelling process.

Keywords: *Cronobacter sakazakii,* GFPuv, Mutant,Stability, Ampicillin resistance

A. INTRODUCTION

Cronobacter sakazakii is Gram-negative bacteria considered as an emerging pathogen in foods. The bacteria are also categorized as opportunistic pathogens which cause septicemia, necrotizing enterocolitis,meningitis and could induce infant mortality (Iversen and Forsythe. 2003). Outbreak of *C. sakazakii* has not been reported in Indonesia, but successful isolation of the bacteria from dried foods such as infant formula (Estuningsih et al. 2006), weaning food (Meutia et al. 2009), fine sugar, cocoa powder, dried spices (Gitapratiwi et al. 2012), tapioca and maize flour (Hamdani 2012) has been reported. The behavior of *C. sakazakii* in food has been studied but tracking the wild type bacterium in foodstuff could be difficult. This is because it is very difficult to distinguish the target bacteria from those naturally found in the food products. Labeling of the bacterium can be useful to study the behavior the bacterium without suppressing other microorganisms (Ma et al. 2011).

A few strains of *C. sakazakii* have been successfully labeled with green fluorescent protein or GFP (Nurjanah et al. 2014) using a pGFPuv-containing plasmid completed with ampicillin resistant gene for easier selection of the mutants (Clontech 2013). The GFPuv is one of the GFP groups through DNA manipulation, having 45× greater fluorescence intensity than GFP (Crameri et al. 1996). The previous study, however, indicated that some ampicillin resistant wild type could not be transformed into mutants (Nurjanah et al. 2014), therefore further study to understand the correlation between *C. sakazakii* ampicillin resistance with the successful mutants construction is needed.

The objectives of this research were to study GFPuv expression and stability in *C. sakazakii*mutants as well as to study the correlation between *C. sakazakii* ampicillin resistant traits with the success of its transformation into mutants.

B. MATERIALS AND METHODS

Materials

Luria Bertani (LB) broth medium was obtained from Difco (Sparks, MD, USA). Druggan-Forsythe Iversen (DFI) agar medium, Buffered Peptone Water (BPW), Triptic Soy Agar (TSA) and Brain heart infusion (BHI) broth medium, were obtained from Oxoid (Hampshire, UK). Ampicillin. Super optimal broth with catabolite repression (SOC) medium was prepared by mixing 20 g/L LB medium (Difco), 3.6 g/L Glucose (Merck) and 0.95 g/L MgCl2 (Merck). Transformation (TF) solution ware prepared by mixing 10 mM Tris-HCl (Merck, Darmstadt, Germany) and 100 mM CaCl2 (Merck). The pGFPuv (3.3 kb) plasmid carrying recursive cycle mutations of GFP and containing the ampicillin resistance gene as well as the origin of replication (ori) sequence was obtained from Clontech Laboratories, Inc. (Mountain View, CA, USA).

Bacterial strains

The bacterial isolates used in this study were 12 isolates of *C. sakazakii* Desb10/JF800181 (4), YRw3/JF800185 (3) FWHc3, FWHd1/JX535018, FWHb6, FWHb15 (5) and E1, E2, E4, E6, E7, E9 (2). All bacterial isolates were obtained from Southeast Asian Food and Agricultural Science and Technology Center Bogor Agricultural University Culture Collection (Bogor, Indonesia).

Isolates Preparation and Confirmation

Stock culture of *Cronobactersakazakii* were refreshed by culturing in BHI broth medium (Oxoid) at 37^0C overnight. Fresh cultures was individually grown on DFI agar medium (Oxoid) to confirm viability and colony characteristics. *Cronobactersakazakii* isolates were visualized as green colonies on the medium (Nurjanah et al. 2014)

Ampicillin Resistant Test of *Cronobacter sakazakii*

Cronobacter sakazakii (wild type) resistance to ampicillin was tested by culturing the bacteria in ampicillin-containing media (Nurjanah et al. 2015). In brief, wild type isolates were grown in BHI broth medium (Oxoid) overnight at 37^0C and 0.1 mL of culture containing approximately 10^6 CFU was transferred onto TSA (Oxoid) with various ampicillin concentrations, i.e. 0 µg/mL, 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL and 50 µg/mL. The cultures were incubated at 37^0C for 2 days and the colonies were enumerated and compared. Based on the density of the colonies grown on ampicillin-containing TSA (Oxoid), the isolates were classified into 3 ampicillin resistant groups : susceptible, intermediate resistant and resistant.

Construction of GFP-labeled *Cronobacter sakazakii*

Labeling of *C. sakazakii* with pGFPuv (Clontech Laboratories, Inc) was done following $CaCl_2$ transformation method in competent bacterial cells (Nurjanah et al. 2014). Briefly, the competent cells were prepared by inoculation of 0.2 mL *C. sakazakii* culture in 20 mL LB broth medium (Difco) for 3 h at 37^0C with agitation in a water bath (PolyScience, Warrington, PA, USA)(120 rpm). The bacterial cells were separated from growth media by centrifugation (Hermle Z383K; Hermle Labortechnik, Wehningen, Germany) and the cell pellets were resuspended in

Statistical Analysis

Statisticalanalyses were performed using the Microsoft Excel Program 2007 (Microsoft, Berkshire, UK)

C. RESULT AND DISCUSSION

Resistance of *Cronobacter sakazakii* Isolates to Ampicillin

10 mL of TF solution. Transformation of the bacterial cells was conducted by mixing 40 µL of competent cells with 2 µL of pGFPuv (Clontech Laboratories, Inc.) and storing the mixture in ice for 30 min, followed by heat shock (42^0C, 60 sec). After heat sock, the mixture was added with 450 µL of SOC medium and incubated in a water bath (PolyScience) for 1 h at 37^0C.The final suspensions were plated on TSA (Oxoid) containing ampicillin for selection of the transformants.

Transformation was confirmed by observing bacterial colony under UV light (Desage Heidelberg Min UVIS). All of the GFP-labeledcolonies were seen as bright green when observedunder UV light.

Phenotypic Confirmation

Phenotypic confirmation was conducted by observing cells mutant under fluorescence microscopy (Olympus CH3O; Olympus Corporation, Center Valley, PA, USA) at a wavelength of 396 nm (Nurjanah et al. 2014).

PCR Analysis

Isolates not displaying fluorescent colonies after construction process were used for PCR analysis. Detection of GFP gene was performed by amplification of the GFP-sequence gene using PCR (2720 Thermal Cycler; Applied Biosystems, Foster City, CA,USA) (Nurjanah et al. 2014). Briefly, PCR was conducted using a forward primer of GFP-Start (5'- AAG CTA TCA ACT TCA AAA TTC GCC-3') and a reverse primer of (5'- TCA TCC ATG CCA TGT GTA ATC C-3') to generate a 223 bp amplicon. The PCR products wereanalyzed on 1.5% agarose gel (Merck) and visualized under a UV Gel Doc XR (Bio Rad).

Stability Testing of Plasmid GFPuv

The stability of plasmids in GFP-labeled isolates was determined using nonselective conditions (Nurjanah et al. 2014). Cultures of mutants stored at-20^0C were refreshed in media containing ampicillin overnight. Overnight cultures of GFP-labeled isolates were inoculated (1:1,000 dilution) in BHI medium (Oxoid) without ampicillin for 5 consecutive subculturing. The cultures were then diluted and spread onto agar plates without ampicillin.Plasmid stability was expressed as percentage and calculated as the ratio of the number of green fluorescent colonies with the total count obtained from the medium without ampicillin.

Ampicillin resistance of *C. sakazakii*isolateswas observed on TSA medium containing ampicillin (TSAA). The resistance of *C. sakazakii* to ampicillin varied and isolates were classified as susceptible, intermediate resistant and resistant (Al-Nabulsi et al. 2011). Susceptible isolates did not grow in ampicillin-containing

media, intermediate resistant isolates were those growing in TSA containing 10 µg/mL ampicillin , while resistant isolates were those capable of growing in TSA containing 20 µg/mL ampicillin. The range of *C. sakazakii* resistance to ampicillin is shown in Table 1. Of the 12 isolates studied, 8 isolates (66.66%) were susceptible (FWHc3, YRw3, E2, E4, E6, E7, Desb10 and E9), two (16.66%) had intermediate resistance to ampicillin (Desb10, E7) and the other two (16.66 %) were ampicillin resistant (FWHd1, E1). Previous studies reported that 53 % (Stock and Wiedemann. 2002) and 31.8% (Kim et al. 2008) of *C. sakazakii* were resistant to ampicillin. However, only 7.7 % of *Cronobacter* spp. isolated from food sources was found to be ampicillin resistant (Li et al. 2011).

Previous studies showed that some *C. sakazakii* could be viable in low concentration of ampicillin (2 µg/mL) (Kuzina et al. 2001), but other studies suggested that *C. sakazakii* could be viable until 10 µg/mL concentration of ampicillin (Noor and Poeloengan. 2008). Ampicillin resistant bacteria are resulted from the possession of a β-lactamase coding gene. The enzyme is able to hydrolyze ampicillin, thus formation of cross-linking structure in cell wall is not suppressed by ampicillin (Haddix et al. 2000). Development of resistant strains was also influenced by exposure to high concentration of antibiotics (Lee et al. 2012)

Table 1. Profile of ampicillin-resistant *C. sakazakii* (wild type)

Isolate code/GenBank	Colonies density in different ampicillin concentration						Resistance level
	0 µg/ml	10 µg/ml	20 µg/ml	30 µg/ml	40 µg/ml	50 µg/ml	
E1	+++++	+++++	++++	-	-	-	Resistant
FWHd1/JX535018	+++++	+++++	+++	-	-	-	
Desb10/JF800181	+++++	+++	-	-	-	-	intermediate resistant
E7	+++++	+	-	-	-	-	
YRw3/JF800185	+++++	-	-	-	-	-	Susceptible
FWHc3	+++++	-	-	-	-	-	
FWHb6	+++++	-	-	-	-	-	
FWHb15	+++++	-	-	-	-	-	
E2	+++++	-	-	-	-	-	
E4	+++++	-	-	-	-	-	
E6	+++++	-	-	-	-	-	
E9	+++++	-	-	-	-	-	

- = no growth
\+ = very limited (1-10 colonies)
++ = limited (11-50 colonies)
+++ = medium (51-100 colonies)
++++ = large (101-300 colonies)
+++++ = large (>300 colonies)

Resistance to ampicillin was generally plasmid-borne. Many of the bacteria resistant to ampicillin harbored plasmid carrying the antibiotic-resistant genes. These genes in the plasmid were brought by resistant (R) factor. Of the 54 plasmids isolated from Gram-negative bacteria in United States rivers tested, 38 (70%) contained the gene for ampicillin-resistant (Ash et al. 2002). The gene located in the plasmid is unstable than that located in the chromosome. Therefore, the gene in the plasmid can be easily transferred from one cell to another cell of bacteria.

Effect of Ampicillin-Resistant *Cronobacter sakazakii* on Stability and Expression of GFPuv in Mutants Cell

GFPuv-labeled colonies are resistant to ampicillin, thus colonies capable of growing on TSAA medium could be used as an initial indicator for a successful plasmid uptake. Isolates FWHb6 and FWHb15 did not produce viable colonies on TSAA, suggesting that these isolates were not capable of taking up GFPuv plasmids (Table 2). Failure of the transformation process could be caused by isolate conditions. The best condition for transformation process occurs in logarithmic phase of bacterial growth, because in this phase bacterial cell membrane is in high elasticity thus easily allows foreign DNA introduction. The different growth pattern of each isolates (Nurjanah et al. 2014), could be a factor influencing a successful transformation of the isolates. Additionally, the optimum temperature needed for heat-shock action was 42 ^{0}C. If this temperature could not disturb the membrane cell structure, the transformation process will fail (Sambrook et al. 2001). Another factor that could play a role during transformation is the difference in *C. sakazakii* heat resistance (Dewanti-Hariyadi et al. 2012, Seftiono 2012). The dissimilarity possibly accounted for various results of *C. sakazakii* transformation.

Table 2. Correlation between C. *sakazakii* resistance to ampicillin and GFPuv mutant transformation

Resistance Profile	Isolate code/GenBank	Isolates can be labeled with pGFPuv	Isolates can express GFPuv	Fluorescence stability in mutants
Resistant	E1	yes	no	not expressed
	FWHd1/JX535018	yes	no	not expressed
intermediate resistant	Desb10/JF800181	yes	yes	Unstable
	E7	yes	yes	Stable
Susceptible	YRw3/JF800185	yes	yes	Unstable
	E9	yes	yes	Unstable
	E2	yes	yes	stable
	E4	yes	yes	Stable
	E6	yes	yes	Stable
	FWHc3	yes	yes	Stable
	FWHb15	no	-	-
	FWHb6	no	-	-

- = not tested because labeling is unsuccessful

The growth of mutant colonies in TSAA medium was confirmed under UV light and an fluorescence microscope. Mutant cells showed bright fluorescence under an fluorescence microscope at 396 nm (Fig. 1). Appearance of green bacterial cells was contributed by oxidized chromophore in GFP as a result of UV wave action. The absorbed energy promoted electron excitation and generated lower energy which automatically induced fluorescent green effect (Shimomura 2005).

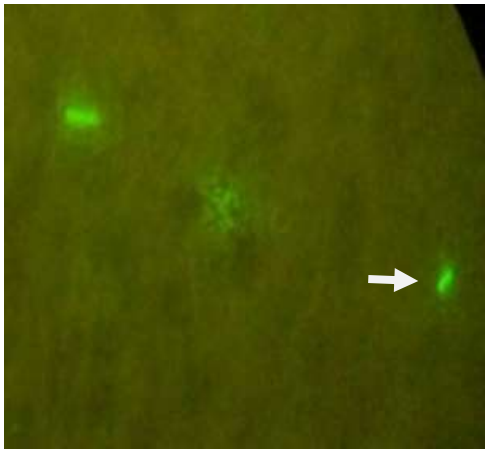
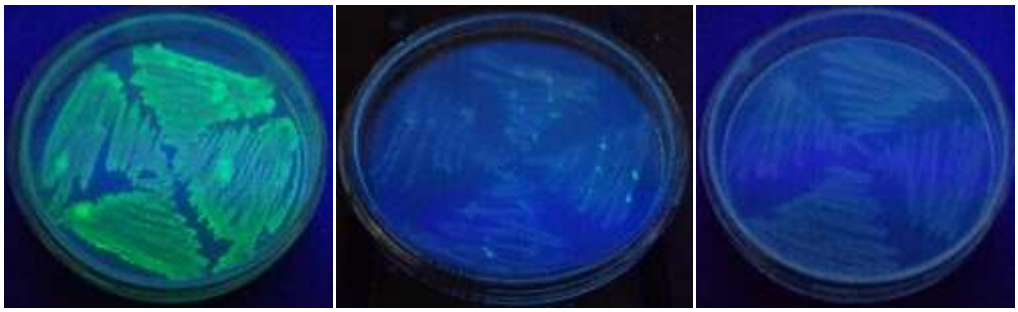


Fig. 1. GFP-labeled *C. sakazakii* (E2) cells under fluorescence microscopy (1000x)

In this study fluorescence expression of each mutants varied when it was observed under UV light. When mutant colonies gave a strong fluorescence effect (expression) they are classified as stable mutants. Unstable mutants showed weak fluorescence expression, i.e. partial fluorescence effect of the colonies. When mutants did not display fluorescent colonies they are concluded as not capable of expressing GFP in their cell (Fig. 2)



(A) **(B)** **(C)**

Fig. 2. Fluorescence stability in mutants under UV light (A) Stable (E2), (B) unstable (Desb10), (C) not expressed (E1)

This research showed that ampicillin resistant isolates produced unstable mutants and not capable of expressing GFPuv. Similarly, GFP expression was not detectable in any of the hygromycin resistant pBC-hygro-GFP transformants, even though the GFP DNA could be amplified by PCR (Zhang et al. 2013). In intermediate ampicillin resistant isolates (E7 and Desb10) we could still find fluorescent colonies, but the intensity was weaker than those of susceptible isolates. The stability and expression of genes are related to the transgene copy number present in cells (Rahman et al. 2000, Hwang et al. 2003).

The PCR analysis indicated that transformant of resistant isolates produced a thinner band (Fig. 3). The thinner band was formed because the number of copied gene was insufficient possibly due to gene silencing. Gene silencing occurred because inactive genes experienced a transcription (Hwang et al. 2003).

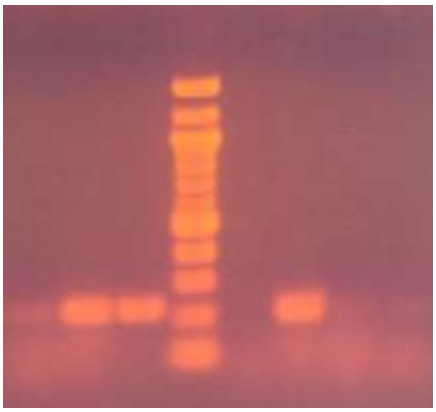


Fig. 3. Electrophoresis result of PCR product (A) Desb10 mutant, (B) YRw3 mutant, (C) control with GFP DNA,(D) 100 bp DNA ladder, (E) control without DNA, (F) E9 mutant, (G) FWHd1 mutant, (H) E1 mutant

Stability of pGFPuv in *Cronobacter sakazakii* Mutants

Plasmid stability in host cells is one key element for bacterial cell labeling. The stability of plasmids in GFP-labeled strains was determined under nonselective conditions.The plasmid stability are summarized in Table 3. All mutants from frozen storage (-20^{0}C) showed high stability (> 90%). The stability of 1st and 2nd subcultures were good, but the stability of 3rdand subsequent subculture were lower. The most stable mutant after 5 consecutive subculturing was E2 (97.20%), while E7 showed the lowest stability (73.50%). Plasmid stability or maintenance is primarilydependent on its segregational stability in which the plasmid must propagate in pace with the doubling rate of their host cells to be stably maintained in a cell population (Akasaka et al. 2015).

Table 3. Plasmid stability of mutants after frozen storage and 5 consecutive subcultures

Isolates	Plasmid stability(%)[1]					
	Storage -20^{0}C	1st subculturing	2nd subculturing	3^{td} subculturing	4th subculturing	5th subculturing
E2	99.20±0.42	98.50±0.42	98.10±0.42	98.30±0.64	97.60±1.06	97.20±0.78
E4	97.50±0.42	96.00±0.28	96.00±1.27	92.60±4.66	90.70±6.15	86.20±8.55
E6	98.70±0.00	98.30±0.28	97.70±0.00	94.80±4.38	88.60±11.95	92.00±7.92
E7	94.30±2.97	93.30±0.14	92.10±0.84	79.80±11.87	80.40±12.59	73.50±15.15
FWHc3	99.30±0.35	99.10±0.35	98.90±0.35	98.20±0.85	96.03±2.89	93.70±6.29

1)Value are means of percentage of plasmid stability±SD

In summary, the resistance of wild type *C. sakazakii* to ampicillin can be classified as susceptible, intermediate resistence and resistant. The highest resistant isolate is E1, while the lowest one is E7. Fluorescence expression of each transformants also varied when it was observed under UV light, some were stable, slighly stable, unstable and others were not expressed.The ampicillin resistant *C. sakazakii* isolates could be labeled with pGFPuv but the resulting mutants were unstable in the cell and could not express the GFPuv. Of the mutants studied, GFPuv plasmid was most stable in E2 mutant, while that in E7 was the least stable.

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REFERENCES

Akasaka N, Astuti W, Ishii Y, Hidese R, Sakoda H, Fujiwara R. 2015 Change in the plasmid copy number in acetic acid bacteria in response to growth phase and acetic acid consentration. J. Biosci. Bioeng. 119(6): 661-668.

Al-Nabulsi AA, Osaili TM, Elabedeen NOZ. Jaradat ZW, Shaker R, Khaerallah R, Tarazi YH, Holley AH. 2011. Inpact of environmental stress desiccation, acidity, alkalinity, heat or cold on antibiotic susceptibility of *Cronobacter sakazakii*. Int. J. Food Microbiol. 146: 137-143.

Ash RJ, Mauck B, Morgan M. 2002. Antibiotic resistance of Gram-negative bacteria in rivers, United States. Emerg. Infec. Dis. 8(7): 713-716.

Crameri A, Whitehorn EA, Tate E, Willem PC, Stemmer WPC. 1996. Improved green fluorescent protein by molecular evolution using DNA shuffling. Nat. Biotechnol. 14: 315-319.

Clontech. Certificate of Analysis pGFPuv. http://www.clontech.com [14 sept 2013].

Dewanti-Hariyadi R, Larasati F, Nuraida L. 2012. Survival of *Cronobacter sakazakii* in skim milk during spray drying, storage and reconstitution. J. Technol. Food Industry. 23(2): 186-192.

Estuningsih S, Kress C, Hassan AA, Akinden O, Schneider E, Usleber E. 2006. Enterobacteriaceae in dehydrated powdered infant formula manufactured in Indonesia and Malaysia. J. Food Protect. 69(12): 3013-3017.

Gitapratiwi D, Dewanti-Hariyadi R, Hidayat SH. 2012. Genetic relatedness of *Cronobacter* spp. (*Enterobacter sakazakii*) isolated from dried food products in Indonesia. Int. Food Res. J. 19(4): 1745-149.

Haddix PL, Paulsen ET, Werner TF. 2000. Measurement of mutation to antibiotic resistance: ampicillin resistance in *Serratia marcescens*. Bioscene. 26(1): 17-21.

Hamdani FW. 2012. Evaluation of genetic diversity of local isolates *Cronobacter* spp. (*Enterobacter sakazakii*) from dried foods. MS thesis, Bogor Agricultural University, Bogor, Indonesia.

Hwang GL, Rahman MA, Razak SA, Sohm F, Farahmand H, Smith A, Brooks, Maclean N. 2003. Isolation and characterisation of tilapia β-actin promoter and comparison of its activity with carp β-actin promoter. Biochim. Biophys. Acta. 1625: 11-18.

Iversen C, Forsythe S. 2003. Risk profile of *Enterobacter sakazakii,* an emergent pathogen associated with infant milk formula. Trends. Food Sci. Tech. 14: 443-454.

Kim K, Jang SS, Kim SK, Park JH, Heu S, Ryu S. 2008. Prevalence and genetic diversity of *Enterobacter sakazakii* in ingredients of infant food. Int. J. Food Microbiol. 122: 196-203.

Kuzina LV, Peloquin JJ, Vacek DC, Miller TA. 2001. Isolation and identification of bacteria associated with adult laboratory Mexican fruit flies, *Anastrepha ludens* (Diptera: Tephritidae). Curr. Microbiol. 42: 290-294.

Lee YD, Park JH, Chang H. 2012. Detection antibiotic and biofilm formation of *Cronobacter* spp. from various foods in Korea. Food Control. 24: 225-230.

Li Y, Chen Q, Zhao J, Jiang H, Lu F, Bie X, Lu Z. 2014. Isolation, identification and antimicrobial resistance of *Cronobacter* spp. isolated from various foods in China. Food Control. 37: 109-144.

Ma L, Zang G, Doyle MP. 2011. Green fluorescent protein labeling of *Listeria, Salmonella* and *Escherichia coli* O157:H7 for safety related studies. PLoS ONE 6: e18083.

Meutia YR, Dewanti-Hariyadi R, Estuningsih S. 2009. The effect of reconstitution temperature for local isolates of *Enterobacter sakazakii* (*Cronobacter* sp.) from powdered infant formula and weaning food. J. Agro-Based Industry. 26(1): 22-30.

Noor SM, Poeloengan. pp. 2008. 367-371 in: The sensitivity of *Enterobacter sakazakii* to antibiotic. National proposed prospect dairy industry to free trade *2020*. April 21, Bogor, Indonesia. Indonesian Research Center for Veterinary Science, Bogor, Indonesia.

Nurjanah S, Dewanti-Hariyadi R, Estuningsih S, Suhartono MT. 2014. Stability andgrowth characteristics of GFPuv-Labeled *Cronobacter sakazakii* isolated from foods. Food Sci. Biotechnol. 23(5): 1491-1496.

Nurjanah S, Sulistyanti ST, Dewanti-Hariyadi R. 2015. Aplication of GFPuv labeled *Cronobacter sakazakii* for evaluation of its survival during cornstarch processing. Word Eng. Technol. 3: 1-6.

Rahman MA, Hwang GL, Razak SA, Sohm F, Maclean N. 2000. Copy number related transgene expression and mosaic somatic expression in hemizygous and homozygous transgenic tilapia (*Oreochromis niloticus*). Transgenic Res. 9(6): 417-427.

Sambrook J, Russel DW. 2001. Molecular cloning: a laboratory manual. 3th ed. Cold Spring Harbor Laboratory Press, New York.

Seftiono H. 2012. Thermal inactivation kinetics of *Cronobacter* spp. *(Enterobacter sakazakii*) in infant formula and buffer system with various aw and pH. MS

thesis, Bogor Agricultural University, Bogor, Indonesia.

Shimomura O. 2005. The discovery of aequerin and green fluorescent protein. J Microscopy. 217: 3-15.

Stock I, Wiedemann B. 2002. Natural antibiotic susceptibility of *Enterobacter amnigenus*, *Enterobacter cancerogenus*, *Enterobacter gergoviae*, *Enterobacter sakazakii* strains. Clin. Microbiol. Infect. 8: 564-578.

Zhang S, Zhao B, Liu X, Gao Z, Huang X. 2013. Construction of new GFP vector and its use for *Fusarium oxysporum* transformation. Saudi J. Biol. Sci. 20: 23-27