

Application of GFPuv Labeled *Cronobacter sakazakii* for Evaluation of Its Survival during Cornstarch Processing

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Abstract

Cronobacter sakazakii is an emerging pathogen that can cause diseases for several infant groups. These bacteria were contaminated in foods, clinical utensils, and environments. In Indonesia, *C. sakazakii* has been isolated from powdered infant formulas, weaning foods, and other dried foods such as cornstarch. The objective of this research is to trace survival of *C. sakazakii* during cornstarch production step using its mutant. Mutant was constructed by inserting Green Fluorescent Protein plasmid inside to the bacterial cell that appeared green fluorescent colonies under UV observation. The presence of *C. sakazakii* during processing was conducted by artificial contamination. This research consists of three steps, *i.e.* determination of the suitable enumeration method of *C. sakazakii*'s mutant, cornstarch production from yellow corn, and survival analysis of *C. sakazakii* during endosperm soaking and cornstarch drying. The suitable enumeration method was surface plating method on TSA-ampicillin medium combining with UV light application because of ineffectiveness of ampicillin inhibition for growth of yeasts and molds. The cornstarch produced in laboratory has the same properties with commercial cornstarch in parameters of moisture content, density, and starch granule structure. The yield of cornstarch final product was 48.90% (dry whole kernel-based). *C. sakazakii* cannot survive in 48 hours soaking process at 52°C and 24 hours drying process at 50°C that is applied during cornstarch production.

Keywords

Cronobacter sakazakii, Cornstarch, Green Fluorescent Protein, Processing, Survival

1. Introduction

Cronobacter sakazakii is a gram-negative bacterium which is included into genus of *Cronobacter* spp. (previously known as *Enterobacter sakazakii*). *C. sakazakii* is an opportunistic pathogen that causes meningitis and necrotizing enterocolitis for adverse condition of infant groups. These cases are associated with infant formulae

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consumption that contaminated by this pathogen [1]. In Indonesia, *Cronobacter* spp. has been isolated [2]-[5]. All of the isolates were obtained from dried food products, *i.e.* powdered infant formula, weaning food, rice flour, tapioca, cornstarch, sugar, cocoa powder and dried spices, suggesting that they were able to survive drying. The data reported by FAO-WHO [6] showed that the ingredient added through dry mixing process was one of the sources of *Cronobacter* spp. contaminant in powder infant formulae. Current researches supported this issue, Dewanti-Hariyadi *et al.* [7] have isolated *Cronobacter* spp. from cornstarch and Fiegen [8] have isolated the pathogen from cornstarch and other ingredients (lecithin, potato starch, rice starch and wheat starch).

Cornstarch was chosen as a food model in this study due to its widespread use as ingredient for infant food products. It is important to understand survival of *Cronobacter* spp. during cornstarch production due to its presence in final products. Besides, it is important to understand the step that can reduce this microorganism and how much the reduction of this microorganism is during cornstarch production. In the cornstarch processing, corn soaking and drying step are the expected step to reduce the number of microbial contamination. Soaking the corn grits is carried out at 52°C for 48 hours and drying is carried out at a 50°C for 24 hours. Therefore, in this study, the aim is to check effectiveness of both processes in reducing *C. sakazakii* that occur in the corn grits by artificial contamination. The objective of this research was to evaluate the survival of *C. sakazakii* during cornstarch processing, especially in soaking and drying step used moderate temperature application. Contamination was conducted by artificial contamination by challenging the mutant isolates to the maize grits amongst the indigenous microflora. The suitable method for enumerating the mutant in the presence of other microorganisms (lactic, yeasts and molds) was part of this study. For specific enumeration, *C. sakazakii* is labeled by a *Green Fluorescent Protein* (GFPuv) into bacterial cells as a marker then called a *C. sakazakii* mutant. GFP is a protein from jellyfish (*Aequorea Victoria*) which can fluoresce and its gene has been inserted into a plasmid (pGFP) as a vector. The pGFPuv is a GFP variant plasmid having fluorescence signal 45-fold greater than a standard pGFP. The mutant can grow in ampicillin containing medium and is seen as a green fluorescent colony. The GFPuv labeled have been constructed for *C. sakazakii* isolates FWHc3 and YRt2a [9].

2. Methodology

2.1. Materials

Microorganisms used in this study were GFPuv labeled of *C. sakazakii* FWHc3 and YRt2a constructed from previous research [9], yeasts (*Kodamaea ohmeri*, *Candida krusei*, *Candida zeylanoides*), and molds (*Aspergillus flavus*, *Aspergillus niger*, *Fusarium oxysporum*) and lactic bacteria (*Pediococcus* sp., *Lactobacillus plantarum*, *Lactobacillus lactis*, *Lactobacillus brevis*) isolated from previous research [10]. All of isolates are collection culture of Microbiology Laboratory of SEAFast Center Bogor Agricultural University. Mutants (GFPuv labeled of *C. sakazakii* FWHc3 and YRt2a) were confirmed by growing on 100 µg/mL ampicillin containing-Tryptic Soy agar medium (TSAA), incubated overnight at 37°C and observed under UV light (Desaga Heidelberg Min UVIS). Materials used in this study were Pioneer variety corn originated from West Java, microbial medium TSA (*Tryptone Soy Agar*), BHI (*Brain Heart Infusion*), BPW (*Buffered Peptone Water*), PDA (*Potato Dextrose Agar*), MRSB (*De Man Rogosa Sharp Broth*), NB (*Nutrient Broth*), ampicillin, natrium hipoklorit (NaOCl) 2% .

2.2. Comparison of Surface and Pour Plating Technique for Mutant Enumeration

Overnight cultures of each mutant isolates from the stock were diluted and plate onto TSAA medium. Plating methods were conducted by two techniques, *i.e.* spread onto surface agar medium and pour plate method. The plates were incubated for 24 h (ca. 10^8 - 10^9 CFU/mL), and the numbers of total and green fluorescent colonies were determined. Green colonies presence was calculated as the ratio between the plate counts of green fluorescent colonies and the total counts obtained from the ampicillin medium plates and expressed in percentage. The effective plating method was chosen from two plating techniques that provide higher percentage of green colonies presence.

2.3. Evaluation of Ampicillin Inhibition to Growth of Yeasts, Molds and Lactic

The cocktail culture of molds (*A. flavus*, *A. niger*, *F. oxysporum*) was prepared by mixing at the equal proportion of each spore solution (1:1:1) that were obtained from watery agar slant of each isolates. The cocktail culture of yeasts (*K. ohmeri*, *C. krusei*, *C. zeylanoides*) was prepared by inoculating an overnight culture of each yeast iso-

lates in broth medium (NB). The cocktail culture of lactic bacteria (*Pediococcus sp.*, *L. plantarum* *L. lactis*) was prepared by the similar technique with yeast. Evaluation of ampicillin inhibition for lactic, yeasts and molds was conducted by plating each cocktail culture separately on TSAA medium using various concentrations of ampicillin (0, 25, 50, 75, and 100 µg/mL). Each mixed cultures was diluted four folds of dilution using dilute solution BPW, and plated with TSAA medium. The plates were incubated at 30°C (yeast and mold) and at 37°C (lactic bacteria) for 48 hours. The amount colonies of each plate were determined by semi qualitative expression based on the standard plate. It was expressed by negative (–) if there is no colony grown. It was expressed as 1+, 2+, 3+ 4+ or 5+, corresponding to approximately ≤ 10 , 25, 50, 75 or $\geq 90\%$ of colonies grown in plate respectively.

2.4. Cornstarch Production and Properties Analysis

Dried corn kernels were grinded by the disc mill to produce grits. Any impurities were separated from the corn grits. Corn grits were washed and cleared from the germ and pericarp (bran) [11]. The endosperms were soaked in 0.25% sodium bisulfite solution in ratio of 1:5 (1 part corn: 5 parts the solution) for 48 hours at a temperature of 52°C. After soaking, the endosperms were ground using a grinder with additional water then filtered using a screen. The suspension resulted from the filtration was precipitated for minimum 12 hours to precipitate the starch and separate the protein. The precipitated starch was dried by oven at 50°C for 24 hours. The dried starch was powdered using a blender and sieved using a 100 mesh sieve to obtain cornstarch powder. The yield of cornstarch processing was calculated based on the dry whole corn kernel. Cornstarch produced was analyzed for moisture content by oven method, whiteness by whiteness meter, density and structure of starch granule by polarized microscope. In order to compare the properties, a commercial cornstarch was also analyzed.

2.5. Artificial Contamination and Survival Analysis

Artificial contamination was conducted by challenging the *C. sakazakii* mutant FWHc3 and YRt2a separately in two step of processing during cornstarch manufacturing. The growth pattern of two mutants was compared. The first artificial contamination was conducted in endosperm grits before soaking step. One milliliter of each *C. sakazakii* mutant (ca. 10^8 CFU/ml) was inoculated into 20 g of endosperm grits placed in sterile bottle, and allowed to contact for 15 minutes. After 15 minutes, 100 ml of soaking solution (contained 0.2 g of sodium bisulfite) was added into the jar and stirred for homogenization. Soaking step was conducted at a 52°C for 48 hours. The second artificial contamination was conducted in starch before drying step. One milliliter of each mutant was inoculated into 35 g starch suspension placed in the bottles, and stirred. The suspension was transferred to a baking plate which sized 18 × 18 cm. Then covered by the filter paper that is perforated at the four corners with a size of 2 × 2 cm. Drying was carried out using oven at 50°C for 24 hours.

Enumeration the number of *C. sakazakii* was done from 10 g sample from each treatment, prepared serial dilutions appropriate and plated in the TSAA medium using surface plating method. The green fluorescence colonies were counted under UV light followed 24 hours incubation. Bacterial colonies are calculated by Standard Plate Count [12] and converted in log units. Reduction curve *C. sakazakii* is created by plotting the number of colonies (logarithmic) in sample on the Y axis and the time interval (hours) on the X axis.

3. Result and Discussion

3.1. Comparison of Surface and Pour Plating Technique for Mutant Enumeration

Surface and pour plating techniques were techniques for enumerating the number of microorganisms by growing the cell on agar medium to build visually sized colonies. Pour plating technique is commonly used for total plate count. Surface plating technique is used for enumeration aerobic obligate microorganisms or for specific purposes. In this study, these two techniques were compared and selected as a suitable method for enumerating the *C. sakazakii* mutants that are capable of remaining of fluorescence characteristics in medium. The suitable method was confirmed as the technique that provided higher percentage of green fluorescent colonies.

Pour plating technique resulted the percentage of green fluorescent colonies of *C. sakazakii* mutants FWHc3 and YRt2a were 91.48% and 89.75% respectively (based on the total colonies) (**Table 1**). It indicated that near-by one log cycle differences of number colonies did not appear the fluorescence under UV light observation, especially which colonies grown in the bottom of plate. It can lead a falls negative identification if the mutant is applied for the qualitative identification. If the mutant is applied for the quantitative application, it can allow the

Table 1. Comparison of surface and plating technique analysis.

Plating technique	Fluorescent colonies percentage of isolates (%)	
	<i>FWHc3 mutant</i>	<i>YRt2a mutant</i>
Pour Plate	91.48	89.75
Surface Plate	100	99.98

lower quantity than the actual number of these bacteria. Surface plating technique showed higher fluorescent colonies percentage is 99.98% for YRt2a mutant and 100% for FWHc3 mutant. The surface technique allowed the directly exposure of UV light to the chromophore ring protein in GFPuv resulted the fluorescence intensity of the colony. Therefore, surface plating method is suitable plating method for enumeration of GFPuv bacterial mutant.

3.2. Ampicillin Inhibition to Growth of Yeasts, Molds and Lactic

Several types of microorganisms can be found in corn kernel or grits during cornstarch processing as indigenous micro flora or as a contaminant. Several yeasts (*K. ohmeri*, *C. krusei*, *C. zeylanoides*), molds (*A. flavus*, *A. niger*, *F. oxysporum*), and lactic bacteria (*Pediococcus*, *L. plantarum*, *L. lactis*, *L. brevis*) have been found in the corn kernel [10]. For tracing the *C. sakazakii* survival or reduction during cornstarch processing, the presence of these microorganisms can offer the disorder counting result. The ampicillin supplemented into the medium used for mutant enumeration does not avoid these microorganisms to grow. Cocktail culture of yeasts (*K. ohmeri*, *C. krusei*, *C. zeylanoides*), molds (*A. flavus*, *A. niger*, *F. oxysporum*), and lactic bacteria (*Pediococcus*, *L. plantarum*, *L. lactis*, *L. brevis*) showed the different ampicillin resistance patterns (**Figure 1**). Yeasts and molds did not show the decrease of their number against the ampicillin concentration up to 100 ug/ml. It was concluded that ampicillin was ineffective to inhibit the growth of fungi and yeasts, because the mechanism of this antibiotic was to inhibit peptidoglycan synthesis in microbial cell wall during cell growth [13]. Gram Positive bacteria have a rich peptidoglycan structure in their cell wall while yeasts and mold do not have.

Unlike yeasts and molds, lactic bacteria showed slightly declined by the increasing concentration of ampicillin (**Figure 1**). Lactic bacteria are grouped to Gram Positive bacteria. However, there are some strain sensitive and some strain resistance to ampicillin. *Pediococcus* is resistant to ampicillin, whereas *Lactobacillus rhamnosus* is sensitive to ampicillin [14]. *Lactobacillus*, *S. thermophilus* and *Leuconostoc* are not the ampicillin resistance [15]. Ampicillin sensitive of lactic is caused by mechanism of ampicillin to inhibit the enzyme that functions in the formation of peptidoglycan structures in the cell walls of bacteria [16]. While the ampicillin resistant of lactic can be caused by enzymatic degradation of antibiotic compounds, changes in bacterial protein target structure or changes in the permeability properties of the membrane to the antibiotics [17]. The ampicillin resistance capability of other microorganisms is caused that ampicillin supplemented medium is not a specific method for mutant enumeration. Therefore, it is required combining by other techniques to complete as function as a specific method. The UV light application is absolutely required for completing the detection method. All of yeast, molds and lactic bacteria grown in ampicillin were not fluoresce under UV light.

3.3. Cornstarch Properties

Production of cornstarch from yellow corn yielded amount 48.90% (based on dry corn kernels) of cornstarch. The cornstarch produced in laboratory has the similar properties with commercial cornstarch in parameters of moisture content, density, and starch granule structure (**Table 2**). Whiteness properties were different due to the difference of used corn varieties. Water content of both type of cornstarch was similar and the values complied with the standard. The starch granules have the similar shape and size. Similarity of two cornstarch properties is associated with the similarity of manufacturing process. According to that, corn soaking step at temperature of 52°C for 48 hours and drying step at temperature of 50°C for 24 hours can be applied in this study for evaluation the survival or the reduction the *C. sakazakii* mutants.

3.4. Survival and Reduction of *C. sakazakii*

Survival or reduction of *C. sakazakii* during soaking and drying step were evaluated using FHWc3 and YRt2a

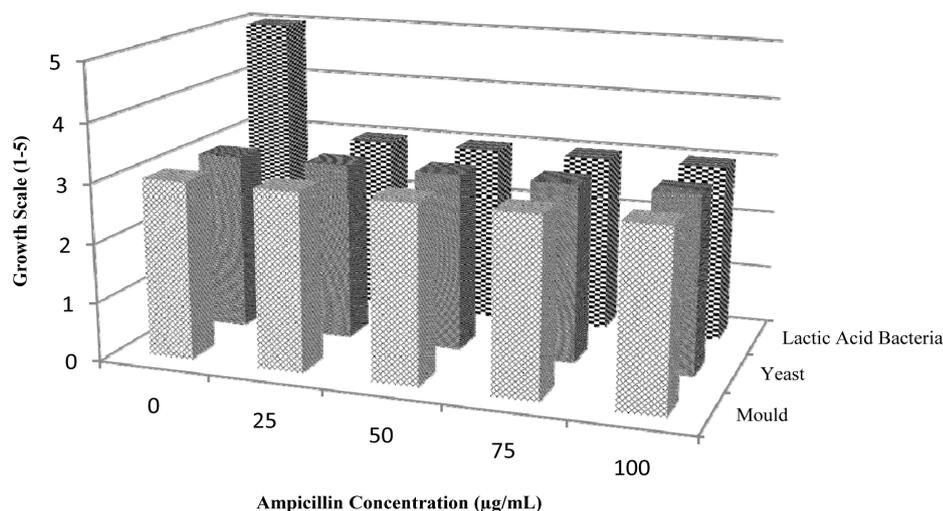


Figure 1. Effect of ampicillin to growth of yeasts, molds and lactic.

Table 2. Comparison of properties of laboratory and commercial cornstarch.

Type of cornstarch	Value			
	Moisture content (% dried based)	Density (g/mL)	Whiteness (%)	Starch granules size
Produced from laboratory	9.90	0.4410	85.54	2.5 - 15 µm
Commercial	9.02	0.4340	128.19	2.5 - 15 µm

mutant separately. Survival was determined by enumerating the mutants that remain viable after the soaking and drying step respectively. The initial number of FWHc3 and YRt2a mutants before the soaking treatment were 7.05 and 5.51 log CFU/g respectively. The result showed that after soaking step there didn't detect any mutant remain viable (LOD = 1 log CFU/g). It is concluded that soaking step for 48 hours at 52°C is effective to reduce *Cronobacter*. The growth temperature range of *Cronobacter* spp. is 6°C - 47°C [18] and the D₅₂ value of *Cronobacter* spp. is 54.8 minutes [19]. The D value indicates the time required to reduce the amount of spore or vegetative cell by 90% or one logarithm cycle [20].

The initial number of FWHc3 and YRt2a mutants before the drying treatment were 4.37 and 4.81 log CFU/g respectively. The result showed that after drying step for 6 hours, the number of FWHc3 and YRt2a mutants decreased to 2.19 and 1.45 log CFU/g respectively. The FWHc3 and YRt2a mutants survived in the short term of drying. When the drying process was continuing up to 24 hours, there was no detected any mutant remain viable. It is concluded that drying step for 24 hours at 50°C is effective to reduce *Cronobacter*.

4. Conclusion

Ampicillin is not effective to inhibit the growth of yeasts, molds and some of lactic bacteria. The suitable enumeration method for mutant enumeration in the presence of indigenous microorganisms is surface plating method on ampicillin supplemented medium combined by UV light application. The cornstarch produced in laboratory has the similar properties with the commercial cornstarch in parameters of moisture content, density, and starch granule structure. Survival analysis during cornstarch production shows that *C. sakazakii* cannot survive in 48 hours soaking at 52°C and 24 hours drying step at 50°C that is applied during cornstarch production.

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