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Use of pGFPuv Mutants to Study the Influence of Drying on the Survival of *Cronobacter sakazakii* in Corn

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Abstract:
Cronobacter sakazakii is the causative agent of meningitis and necrotizing enterocolitis in certain groups of infants. Transformation of local isolates of C. sakazakii with a Green Fluorescent Protein (GFP) plasmid has produced C.sakazakii pGFPuv mutants potential to be used for studying C. sakazakii behavior without the need to suppress other microorganisms or use of diagnostic media. This research aimsto study the effect of drying on water content and water activity of corn and to evaluate the survival of C. sakazakii pGFPuv as well as other naturally occurring microorganisms in the corn during drying. Corn with moisture content of 40% (d.b) were inoculated with C. sakazakii pGFPuv mutant to achieve an initial concentration of 10^8 *CFU/g and drying was performed at 42 ºC, 46 ºC and 50 ºC for ten days in a drying chamber. Corn samples were taken every day and water content were analysed using oven method while water activity by Aw meter.The number of C.sakazakii and total microorganisms surviving in the corn were enumerated and the survival rate of C. sakazakii was determined. The results showed that the decrease in water content occurred at a constant rate at the first day of drying followed by a falling rate decrease that correspond to the decline in Aw. Drying at all temperatures for 10 days resulted in the decrease of naturally occurring microorganisms to an undetectable level. However, at the end of the drying, C.sakazakii was still present at a concentration of 2 Log CFU/g. Scanning electron microscopy showed that C. sakazakii form colonies on the surfaces of corn and in the cavity of the tip cap. This study showed that C.sakazakiipGFPuv could be used to study the survival of C.sakazakii during corn drying and C. sakazakii displayed resistance to drying at 42, 46 and 50*^oC *for 10 days.*

Keywords: *Cronobactersakazaki, pGFPuv, corn, survival, drying.*

1. Introduction

Drying is one of the important techniques for preserving agricultural and food products. Drying takes place in the processing of many products, either as the main operation or as a consequence of other processing steps(*Chen,2009).* The objective of drying in food products is to remove the water content to a certain level, at which microbial spoilage is greatly minimized. C. sakazakii, however, was shown to have a remarkable capability to survive in a dry environment for a long time (Krokida et al., 2003). *Cronobacter*spp. (formerly *Enterobacter sakazakii*) is a Gram-negative, rod, motile, non-sporulating bacterium with peritrichous flagella. *Cronobacter*spp. was recently proposed to consist of six genomospecies (Iversen et al., 2008) and they are regarded as opportunistic human pathogens. The bacteria have been reported as the etiological agents of life-threatening bacterial infections in low birth-weight neonates and infants (Mullane et al., 2006).

Powder Infant Formula (PIF) is the only food epidemiologically linked to the cases of infant infections by *C. sakazakii*(Lou *et al*., 2014). However, *C. sakazakii* has been isolated from plant food sources and ingredients such as cereal, fruit, vegetables, legume products, herbs and spices as well as from animal food sources like milk, meat, fish and products made from corn starch as dried infant formulas (Friedemann, 2007). An international survey of dry infant formula from 35 countries found that approximately 14% of the 141 cans examined had detectable levels of *E. sakazakii* (Edelson-Mammel and Buchanan, 2004).

In general, bacteria protect themselves from increasing osmolarity by the rapid intracellular accumulation of ions, mainly K^+, followed by the accumulation of compatible solutes such as proline, glycine betaine, and trehalose. In the case of drying, which can be seen as an extreme form of osmotic stress, the bacterial cells need to preserve their biological integrity in the absence of liquid water

(Breeuwer*et al.*, 2003). *E. sakazakii* is known to survive for at least two years in powdered infant formula at water activity (Aw) as low as 0.2 (Beuchat, 2009; Adekunte*et al.*, 2010).
Iversen *et al.* (2004) isolated *Cronobacter spp* from corn flour, Restaino *et al.* (2006) isolated it from grits, while *Dewanti-Hariyadi et al.(2010) isolated it from corn starch.*Corn and corn products are commonly used as ingredients in various foods including infant formula and weaning foods. In Indonesia, *C. sakazakii* has been isolated from several PIF, weaning food and corn starch (Gitapratiwi *et al.*, 2012).
Studies on the behavior of *C. sakazakii*in food using wild type bacteria would be difficult because the target organisms are indistinguishable from the naturally occurring microorganisms. In corn for example, various fungi such as *Aspergillus, Fusarium, Penicillium, Rhizopius, Kodamea and Candida*, as well as bacteria (*Pediococcus* and *Lactobacillus*) were found (Rahmawati *et al.*, 2013). Labelling techniques become an alternative to study of the behavior of these bacteria without killing or suppressing the growth of other microbes.
This study uses *C. sakazakii* labelled with plasmid containing Green Fluorescent Protein (pGFPuv), which produces green fluorescent colonies. These *C.sakazakii* pGFPuv mutants were reported to have growth curves similar to those of the wild types (Nurjanah *et al.*, 2013).The objectives of this study were (1) to study the effect of drying on water content and water activity of corn. (2) to evaluate the survival of *C. sakazakii* pGFPuv as well as other naturally occurring microorganisms in corn during drying.

2. Materials and Methods

2.1. Materials

The main materials used in this research were *C. sakazakii* isolates and corn. *C.sakazakii* pGFPuv FWHc3 and E2 were mutants obtained by transformation of cytotoxic *C.sakazakii* using GFP labelled plasmid (Nurjanah *et al.*,2013). Cornused in this study is ahybrid from Pioneer 12planted in Bogor, Indonesia. The cornwas harvested when the water content reached 33-35% (w.b).Media and materials used include Brain Heart Infusion medium (BHI, Difco), Buffer Phosphate Water (BPW, Oxoid), Tryptic Soy Agar (TSA, Oxoid), Tryptic Soy Agar (100 µg/mL) Ampicillin (TSAA), Druggan-Forsythe-Iversen (DFI, Difco), Plate Count Agar (PCA, Oxoid), distilled water, ampicillin, NaCl 0.85%, chloramphenicol, and 0.22 µm membrane filter, glutaraldehyde solution, alcohol 95%, and tert-butanol.

2.2. Methods

2.2.1. Confirmation of *C. sakazakii* pGFPuv mutants

Confirmation of *C. sakazakii* pGFPuvmutants was carried out by streaking a loop of culture into TSAA media and incubating it at 37 °C for 24 hours. The mutants observed under UV light (Desaga 'UVIS 131100' UV Lamp UV 254nm)showed green fluorescent colonies (Nurjanah *et al.*, 2013).

2.2.2. Inoculum preparation

Two to three green fluorescent colonies on TSAA were transferred to BPW and placed in a 15 ml centrifuge tube and centrifuged (HERMLE Z 383 k) at 3500 rpm (2,600 xg) for 10 min at 4°C to separate the cell pellet. The cell pellet was washed twice using BPW and then resuspended in BPW to achieve an Optical Density (OD) of 0.4 as measured with spectrophotometer (Shimadzu UV-2450) at 590 nm.This OD corresponds to a concentration of10^8CFU/mL.

2.2.3. Inoculation

Ten ml of the above inoculum was added to a sterile plastic bag containing 200 g of corn. The corn and inoculum were mixed in a stomacher (Interscience bag mixer 400) for 60 s after which it was left for 15 minute until the corn totally absorbed the inoculum. The corn is expected to contain approximately10^8 cells/g.

2.2.4. Drying

Drying was carried out in a drying chamber at three temperatures(42 °C, 46°C, and50°C) for 10 days.

2.2.5. Data Collection and Analysis

Every day samples of corn were taken from three drying temperatures(42 °C, 46°C, and50°C) for analysis of water content, water activity and enumeration of TPC as well as *C.sakazakii*. Selected samples from the second dayof drying at 50°C were observed by scanning electron microscopy (SEM).
Water content was measured by oven (5E-MHG 6090) method at 105°C for at least 6 hours. Accurately, 2 g of the sample were weighed in covered dish previously dried at 98-100 °C and cooled in a desiccator to reach room temperature (AOAC, 2005). Water activity was measured using an Aw meter (Ro-Tronic) at 30 °C (Passot *et al.*, 2012).
The total plate count was enumerated by placing 10 g of corn in 90 mL BPW and serially diluted to achieve 25-250 colonies and incubated for 24-48 hours at 35°C. The number of colonies was calculated with the formula of Standard Plate Count (Maturin and Peeler,2001). *C. sakazakii* pGFPuv surviving in the corn after drying was enumerated by placing 10 g of corn in 90 ml of BPW and appropriately diluted and plated on the TSAA media using surface method. The plates were incubated at 37°C for 24 hours and *C. sakazakii* pGFPuv seen as green fluorescent colonies under UV light were enumerated (Maturin and Peeler, 2001). The rate of decline

of *C.sakazakii* number (log/day) during drying was indicated by plotting the number of the bacteria log CFU/g on the Y axis and the time interval (days) drying on the X axis.

Colonization of *C. sakazakii* pGFPuv on corn during drying was observed on corn samples from the second day of drying at 50° C. The corn sample was soaked in tert-butanol, frozen in the freezer and then vacuum dried (Pathan *et al.*, 2010). The samples were cut into small slices and coated with gold-Palladium using Ion Coater (Mattox and Mattox, 2003).The samples were then observed using a JEOL 5310 LV scanning electron microscope (Goldstein *et al.*,2012).

Statistical analysis

The experimental data were verified statistically with regression analysis using Microsoft Excel 2007.

3. Results and Discussion

3.1. Changes in Water Content of Corn during Drying

Corn drying under the sun is a common practice by farmers. In this research corn drying was done at three temperatures; 42 °C, 46 °C, and 50 °C, putting into consideration that these drying temperatures provides ambient environment for corn grains not to wrinkle. The results of this study confirmed other studies by Schlünder (2004) and Chen *at el.*, (2012) that suggest two phases of drying, i.e. constant rate and falling rate phase. During the initial day of drying, water was removed from the corn surface by evaporation thus causes the water content to drop accordingly. After the first day, slower removal of water occurred which was a typical trend during the falling rate phase. During this phase, water has to be removed from inside the corn, which become more and more difficult as the water percolate further through from the center to the outer part of the corn from where the evaporation took place.

The drying process is a delicate operation that achieves equilibrium between two different mass transfer process, i.e. diffusion and evaporation. During fast drying rate, evaporation rate is equal to the diffusion rate of water from the inside to the surface of corn. During slow drying rate, the rate of evaporation is greater than the rate of diffusion of water from the inside to the surface (Simpson, 2012).It was noted that the drying rate slowed down during the falling rate period and reached the state of hygroscopic equilibrium (Schlünder,2004). Eventually no more moisture can be removed from the corn and was concluded to be in equilibrium with the drying air. This result is depicted in Figure 1.

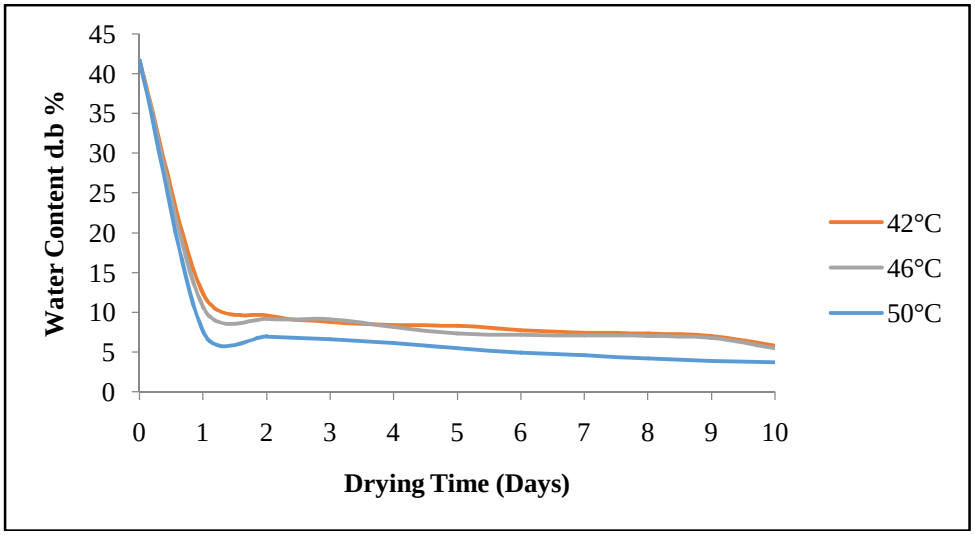

Figure 1: Changes in water content of corn during drying at 42, 46, and 50 °C.

3.2. Changes in Water Activity of Corn during Drying

During drying, the water activity (Aw) of various perishable materials generally decreases, thus enable storage at ambient temperature. Decrease in water activity is important for controlling the shelf life of foods by suppressing the growth of microorganisms (Bonazii and Dumoulin, 2011). Similar to changes in moisture content, changes in water activity during corn drying also consisted of two phases i.e. constant rate phase and falling rate phase. Constant rate phase occurred in the first day at 50 °C or in the second day of drying at 42 and 46 °C. Meanwhile the falling rate phase happened at day 3 in which water activity continued to decrease slowly until the final day of drying. Higher drying temperature had a significant effect on the decrease in water activity as observed by the slope of the linear regression. Figure 2 showed that drying at 42and 46° Cresulted in similar decrease in the water activity, while that at 50° C resulted in the lowest water activity.

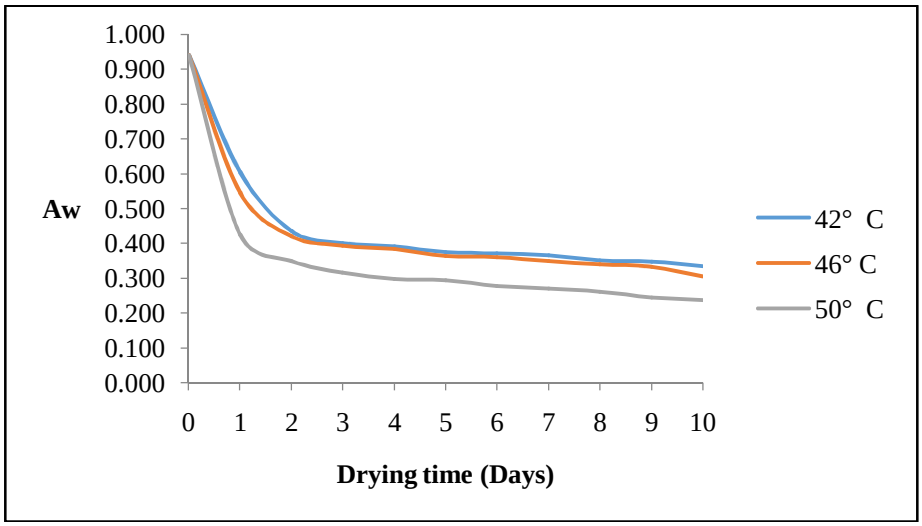

Figure 2: Changes in water activity of corn during dying at 42, 46 and 50 °C.

3.3. Changes in Total Plate Count of corn during drying

The Total Plate Count (TPC) in corn observed during drying reflects them icrobial resistance to drying. The number of microorganisms decreased during drying at the three temperatures. During the first two days of drying the number of microorganisms decreased rapidly (1.65 logCFU/g) at all drying temperatures.

The results also suggested that when the water activity was removed at a fast rate, the microorganisms had no time to adapt themselves either through genetic expression or adjustment of their metabolism (Guergolettoet *al*., 2012). After two days, the bacteria started to adapt to the high temperatures, thus the curve showed a relatively more resistant bacteria up to the eighth day. At the eighth day, in addition to temperature, the low water activity was suspected to play role in the fast decline of the number of microorganisms. At the last day of drying, microorganisms had reached the lowest microbial population regardless of the drying temperatures. Drying of corn for 10 days at 50 °C has resulted in an undetectable level of microbial counts. In general, the microorganisms were slightly more resistant during drying at 42 and 46 °C than those at 50 °C (Figure 3).

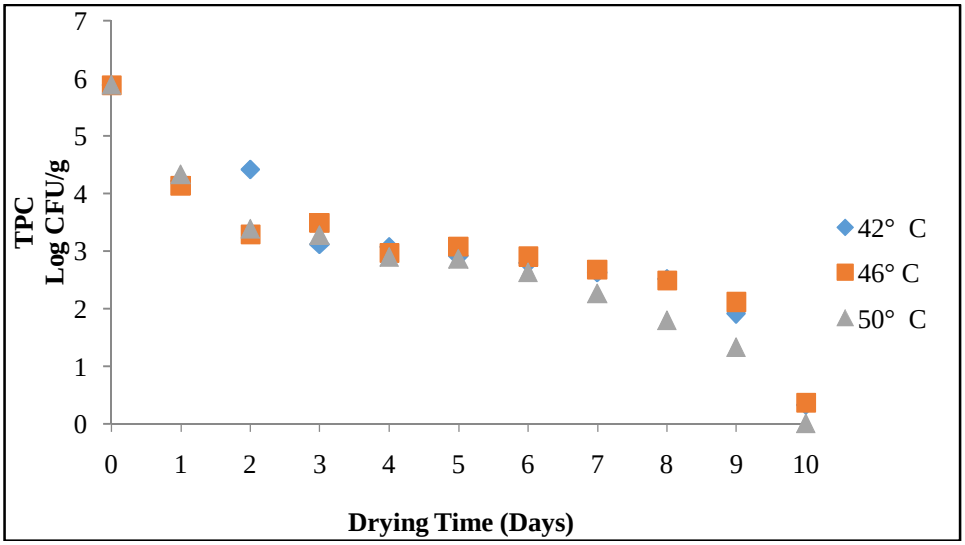

Figure 3: Changes in total plate count of corn during drying at 42, 46 and 50°C

3.4. Survival of C.sakazakii during drying

Both *C.sakazakii* pGFPuv isolates decreased in number during drying. The results also showed that the fate of decrease of *C.sakazakii* could be divided into three phases, i.e.logarithmic decrement, static phase and the final decline. For isolate FWHc3, the logarithmic decrement during drying at all temperatures occurred at day 0-3, static phase at day 3-7 and the final decline at day 7-10. Meanwhile at the same drying temperature, isolate E2experienced logarithmic decrement at day 0-5, static phase at day 5-8 and the final decline at day 8-10. Drying of E2 at 42°C and 46°C has resulted in the logarithmic decrement at day 0-6, static phase at day 6-8 and the final decline at day 8-10. *C. sakazakii* is more sensitive to drying temperature of 50°C, while the effect of drying at temperatures 42and 46°C are relatively similar. With high initial load (10^8 CFU/g), the number of surviving *C. sakazakii* after 10 day of drying at 50°C was > 10^2CFU/g.

Richardson *et al.*, (2009) reported that the infectious dose of *C.sakazakii* was 10^2CFU. The above results suggested that it was possible to have *C. sakazakii* in corn after 10-day drying at 50°C in the number that might be infective. In dry products such as PIF or weaning foods, *C. sakazakii* would not be able to grow, but after the addition of water, reconstituted PIF or weaning food is a good medium for growth. Once reconstituted, the only barriers for infection to occur are short time and low temperature storage to prevent bacterial growth (Huertas *et al.*, 2015). Seftiono (2012) reported the kinetics of inactivation of *Cronobacter spp.* during heating process of infant formula and the D values obtained ranged from 3.61-11.36 minutes at 56°C and 68.97-256.41 minutes at 50°C. Meanwhile the Z value for all isolates studied ranged from 3.54-5.69°C.Figure 4 showed the rate of decline of *C. sakazakii* number (log/day) during drying obtained by plotting the log number of the bacteria on the Y axis and the time of drying (days) on the X axis. At all drying temperatures (42, 46and 50°C), the rate of decline in the number ofFWHc3 isolates was fasterthanisolatesE2.

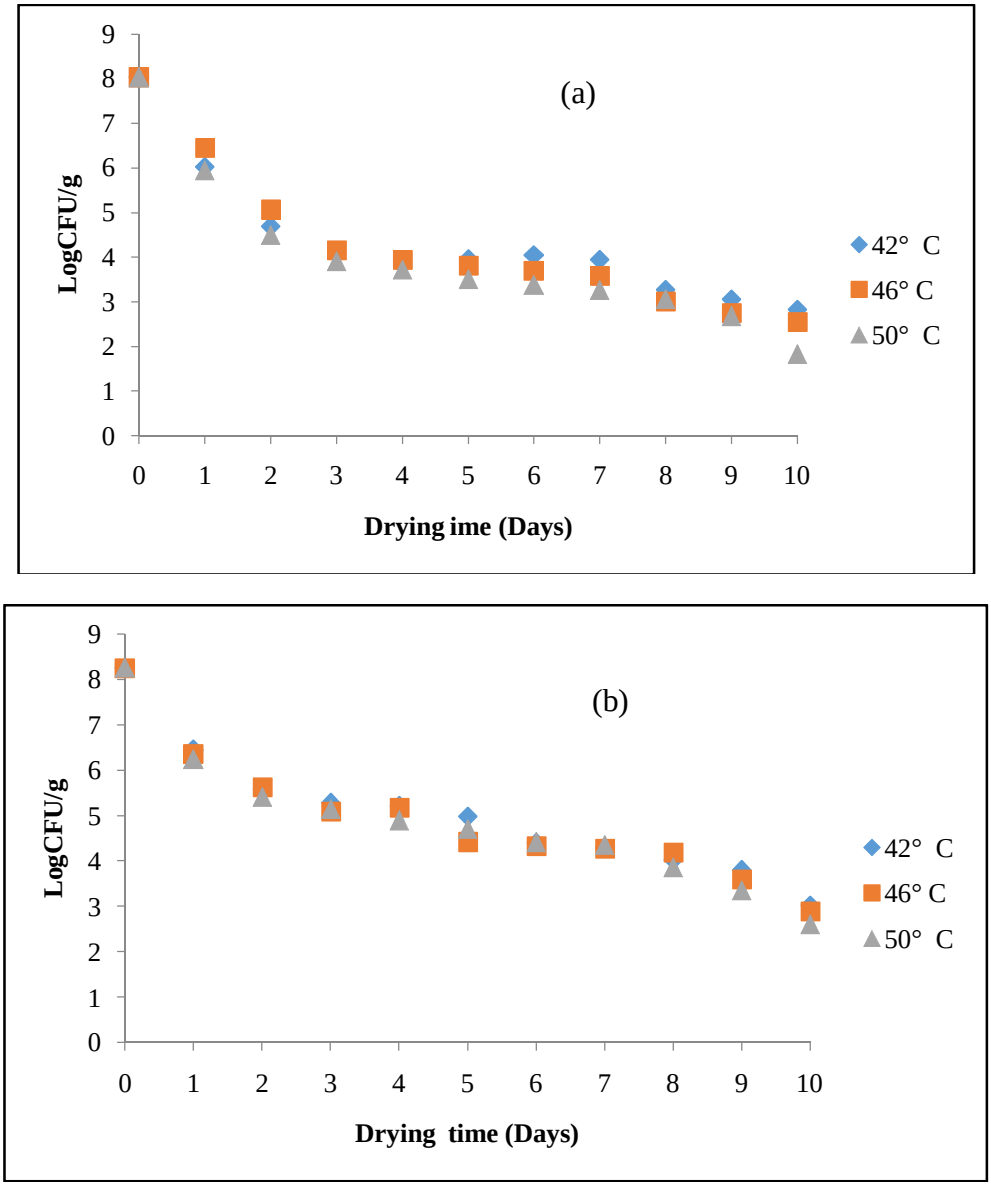

Figure 4: Survival of C. sakazakii isolates (a) FWHc3 and (b) E2 during drying at 42, 46 and 50 °C

E2 was isolated from weaning food, while FWHc3 was from tapioca. It is possible that high content of protein, vitamin, mineral as well as exposure to high temperature during the production of weaning foods, has resulted in a more resistant isolates. In contrast, FWHc3 was isolated from tapioca whose process does not involve heating thus the bacterium is less resistant to drying.

This results was similar to Nurjanah *et al.*, (2013) who showed that *C. sakazakii* was resistant to heat during corn drying at 50°C for four days. *C. sakazakii* was present in the spray drying process of formula milk (Arku *et al*., 2008). Spray drying of skimmed milk reduced 4 log of *C. sakazakii* but the survivors were more resistant to low relative humidity (Dewanti-Hariyadi *et al*., 2012). The study also concluded that drying at 40 °C was not effective to reduce *C. sakazakii* in infant formula during reconstitution. Exposure to sub-lethal temperature or a few degrees above the optimum growth temperature may increase heat resistance of the bacteria. Heatingin TSB medium at a temperature of47 °C for 15minutesincreased the survival of *C.sakazakii* agains theat, dry conditions (Chang *etal*., 2009), and spray drying of skim milk (Dewanti-Hariyadi*et al.*, 2012).

3.5. Colonization of C. sakazakiion the corn

Presence of bacteria in dried food products could be caused by their ability to carry out attachment or colonization on the surface of the food material. The results of microscopic observation with scanning electron microscopy (SEM) showed colonization of *C. sakazakii* on the surface of the corn on the second day of drying (Figure 5). At the second day of drying, the number of *C. sakazakii* was 10^5 CFU/g while the other microorganisms was 10^3 CFU/g. However, it is acknowledged that it was not possible to differentiate *C. sakazakii* from other naturally occurring bacteria through SEM. The corn water activity at the second day of drying at 50° C was recorded as 0.34; in this low water activity we assumed that appearance observed with SEM was *C. sakazakii*. This was in accordance with Beuchat *et al.,* (2009) who reported that *C. sakazakii* survive better in dried formula and cereal at low aw (0.25–0.30) than at high aw (0.69–0.82) during storage. Iversen *et al.,* (2004) studied biofilm formation of *C. sakazakii* and reported that these bacteria have the ability to stick to latex and polycarbonate and bulk in stainless steel, the biofilm formation affected by various conditions such as the composition of nutrients in the media and the relative humidity environment (Jung *et al.,* 2013). Breeuwer *et al.,* (2003) reported that *C. sakazakii* were resistant to drying process at temperatures ranging from 25° C to 45° C and it is suspected that biofilm was formed by *C. sakazakii*. The appearance of *C. sakazakii* pGFPuv on corn at second day of drying (Figure 5) is similar to biofilm observation with scanning electron microscopy by Chang *etal.,* (2009a) and Hurrell *et al.,*(2009).*C. sakazakii* is a bacterium that naturally colonizes plants, they have been found to colonize the pericarp and tip cap of corn (Nurjanah *et al.,* 2013), roots of tomato plants and roots corn (Schmid *et al*., 2009) and also as endophytic microbes from soybean. The SEM observation performed on the tip cap (Figure 6) found the bacteria in the cavity indicating that the wound became one of the entry points of this bacterium into corn. In addition, the tip cap has low water content, through which water and nutrients flow and is the only area of the kernel not covered by the pericarp (Yoshitomi and Shann., 2001), this bacterial penetration into the corn kernels is thought to have occurred through cavities contained in section of the tip cap corn.

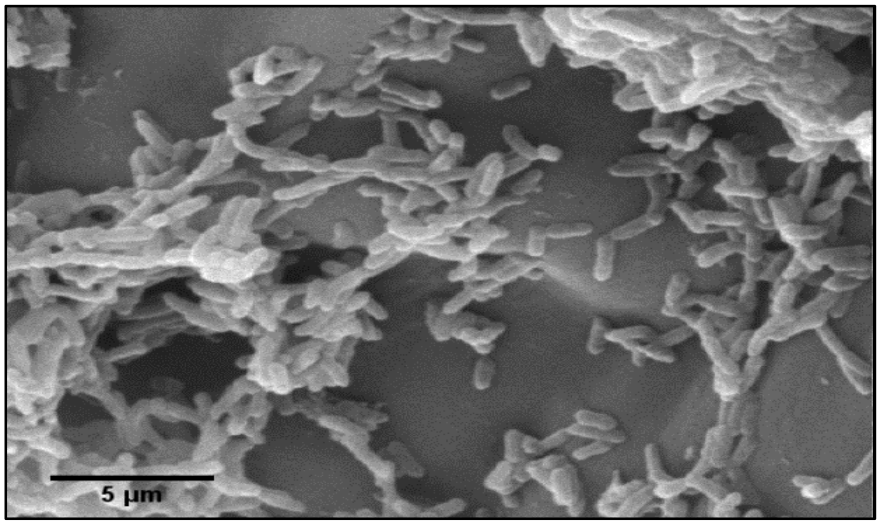

Figure 5: SEM observation of C. sakazakii colonization on the surface of corn at day 2 drying (5000X)

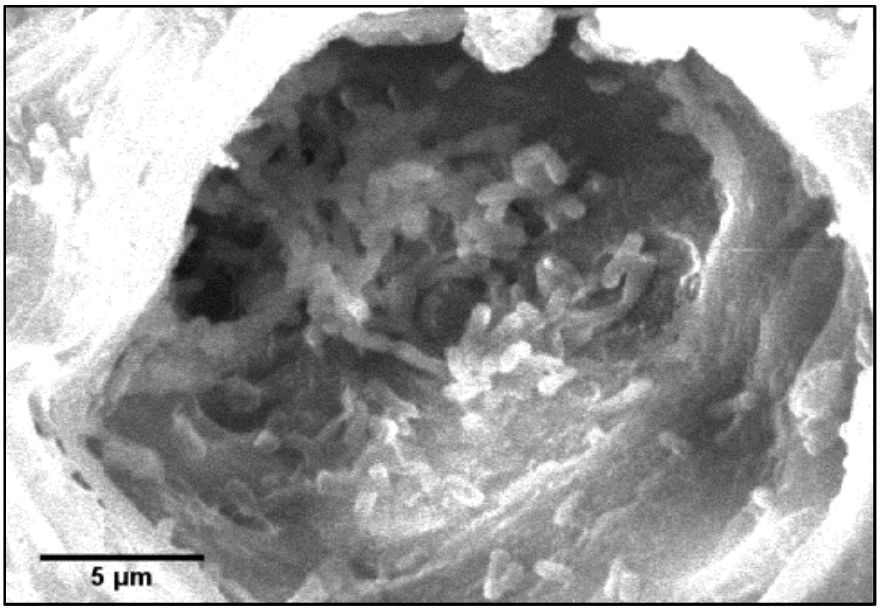

Figure 6: SEM of C. sakazakii in the cavity of corn tip cap at day 2 of drying (5000X)

4. Conclusion

Drying of corn kernel at 42° C, 46° C and 50° for 10 days resulted in decrease in the water content to 5.8,5.5,3.7%, respectively which correlate to water activities of 0.33,0.30,0.23, respectively. The decrease in Aw can be differentiated into three phases that correspond to the decrease in the number of microorganism and *C. sakazakii* pGFPuv. With an initial inoculation of *C. sakazakii* at 10^8 CFU/g, after 10-days drying at 50 °C no detectable microorganisms were observed but *C. sakazakii* pGFPuv was found at 10^2CFU /g. Isolate E2 was more resistant at three drying temperatures as compared to isolate FWHc3. The ability of *C. sakazakii* to colonize and possibly form biofilm on corn surface as well as ability to penetrate into the corn through the wound or cavities at the tip cap may have helped their survival during drying. *Cronobacter sakazakii* E2 and FWHc3 labeled with pGFPuv can be applied to study the survival of *C. sakazakii* during drying.

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6. References

i. Adekunte, A., Valdramidis, V.P., Tiwari, B.K., Slone, N., Cullen, P.J., Donnell, C.P.O. and Scannell, A. (2010). Resistance of Cronobacter sakazakii in reconstituted powdered infant formula during ultrasound at controlled temperatures: a quantitative approach on microbial responses. International Journal of Food Microbiology,142**,** 53 – 59.
ii. [AOAC]. (2005). Official Method of Analysis. Association of Official Analytical Chemist. Washington DC
iii. Arku, B., Mullane, N., Fox, E., Fanning, S. and Jordan, K. (2008). Enterobacter sakazakii survives spray drying. International Journal of Dairy Technology,61,102 – 108.
iv. Beuchat, L.R., Kim, H., Gurtler, J.B., Lin, L.C., Ryu, J.H. and Richards, G.M. (2009).Cronobactersakazakii in foods and factors affecting its survival, growth and inactivation. International Journal of Food Microbiology, 136, 204–213.
v. Breeuwer, P., Lardeau, A., Peterz, M. and Joosten, H.M. (2003). Desiccation and heat tolerance of Enterobacter sakazakii. Journal of Applied Microbiology,95, 967–973.
vi. Chang, C.H., Chiang, M.L. and Chou, C.C. (2009b).The effect of heat shock on the response of Cronobacter sakazakii to subsequent lethal stresses. Foodborne Pathogens and Disease,7, 71–76.
vii. Chen, D., Zheng, Y. and Zhu, X.(2012). Determination of effective moisture diffusivity and drying kinetics for poplar sawdust by thermogravimetric analysis under isothermal condition. Bioresource Technology, 107**,** 451–455.
viii. Chen, X.D. (2009).Food drying fundamental. In: Mujumdar, A.S. (ed.)Drying technologies in food processing. John Wiley& Sons.
ix. Dewanti-Hariyadi, R., Gitapratiwi, D., Meutia, Y.R., Estuningsih, S., Hidayat, S.H. and Nurjanah, S. (2010). Isolation of Enterobacter sakazakii (Cronobacter spp) from powdered infant formula and other dry foods obtained from Bogor area, Indonesia. Proceeding of The International Seminar Current Issues and Challenges in Food Safety: Science-Based Approach for Food Safety Management, Bogor, 2-3 December 2009.
x. Dewanti-Hariyadi R, Larasati F, Nuraida L. 2012. Survival of Cronobacter sakazakii in skim milk during spray drying. Jurnal Teknologi dan Industri Pangan, 23(2),186-192.
xi. Edelson-Mammel, S.G. and Buchanan, R.L. (2004). Thermal inactivation of Enterobacter sakazakii in rehydrated infant formula. Journal of Food Protection 67**,** 60–63.
xii. Maturin, L. and Peeler, J. T. 2001. Aerobic Plate Count. In. Bacteriology Analytical Manual,Food and Drug Administration. http:cfsan.fda.gov/ 25/09/2010 [25 Sept 2014]
xiii. Friedemann, M. (2007). Enterobacter sakazakii in food and beverages (other than infant formula and milk powder). International Journal of Food Microbiology, 116, 1–10.
xiv. Gitapratiwi, D., Dewanti-Hariyadi, R. and Hidayat, S.H. (2012). Genetic relatedness of Cronobacterspp. (Enterobactersakazakii) isolated from dried food products in Indonesia. International Food Research Journal,19,1745–1749.
xv. Goldstein, J., Newbury, D. E., Echlin, P., Joy, D. C., Romig Jr, A. D., Lyman, C. E., & Lifshin, E. (2012). Scanning electron microscopy and X-ray microanalysis: a text for biologists, materials scientists, and geologists. Springer Science & Business Media.
xvi. Guergoletto, K.B.(2012).Drying processes. In:Tsuruda, A.Y., Hirooka, E.Y., Martins, E.P., de Souza, J.C.B., Sivieri, K.and Garcia, S (eds.). Dried probiotics for use in functional food applications. INTECH Open Access Publisher.
xvii. Huertas, J.P., Álvarez-Ordóñez, A., Morrissey, R., Ros-Chumillas, M., Esteban, M.D., Maté, J.and Hill, C. (2015). Heat resistance of Cronobacter sakazakii DPC 6529 and its behavior in reconstituted powdered infant formula. Food Research International, 69, 401–409.
xviii. Hurrell, E., Kucerova, E., Loughlin, M., Caubilla-Barron, J. and Forsythe, S.J. (2009). Biofilm formation on enteral feeding tubes by Cronobacter sakazakii, Salmonella serovars and other Enterobacteriaceae. International Journal of Food Microbiology,136, 227–231.
xix. Iversen, C., Lane, M. and Forsythe, S.J. (2004). The growth profile, thermotolerance and biofilm formation of Enterobactersakazakii grown in infant formula milk. Letters in Applied Microbiology, 38**,** 378–382.

xx. Iversen, C., Mullane. N., Mc-Cardell, B., Tall, B.D., Lehner, A., Fanning, S. and Joosten, H. (2008). Cronobacter gen. nov, a new genus to accommodate the biogroups of Enterobactersakazakii, and proposal of Cronobactersakazakii gen. nov, comb. nov, Cronobactermalonaticus sp. nov, Cronobacterturicensissp. nov, Cronobacter muytjensii sp. nov, Cronobacter dublinensis sp. nov, Cronobacter genomospecies 1, and of three subspecies, Cronobacter dublinensis subsp. dublinensis subsp. nov, Cronobacter dublinensis subsp. lausannensis subsp. nov. and Cronobacter dublinensis subsp. lactaridi subsp. nov. International Journal of Systematic and Evolutionary Microbiology, 58,1442–1447.

xxi. Jung, J.H., Choi, N.Y.and Lee, S.Y. (2013). Biofilm formation and exopolysaccharide (EPS) production by Cronobacter sakazakii depending on environmental conditions. Food Microbiology, 34, 70–80.

xxii. Krokida, M.K., Karathanos, Maroulis, Z. and Marinos-Kouris, D. (2003). Drying kinetics of some vegetables. Journal of Food Engineering, 59**,** 391–403.

xxiii. Lou, X., Si, G., Yu, H., Qi, J., Liu, T. and Fang, Z. (2014). Possible reservoir and routes of transmission of Cronobacter (Enterobactersakazakii) via wheat flour. Food Control, 43, 258–262.

xxiv. Mattox, D.M. and Mattox, V.H. (2003). Vacuum coating technology.William Andrew Publishing, New York.

xxv. Mullane, N.R., Drudy, D., Whyte, P., O'mahony, M., Scannell, A.G.M., Wall, P.G. and Fanning, S. (2006). Enterobacter sakazakii: biological properties and significance in dried infant milk formula (IMF) powder. International Journal of Dairy Technology, 59,102–111.

xxvi. Nurjanah, S., Suhartono, M.T., Dewanti-Hariyadi, R., and Estuningsih, S. (2013). Application of fluorecence mutants to study survival, colonization and penetration ofCronobacter sakazakiiduring corn drying.Jurnal Teknologi dan Industri Pangan, 24(2), 184.

xxvii. Passot, S., Cenard, S., Douania, I., Tréléa, I.C. and Fonseca, F. (2012). Critical water activity and amorphous state for optimal preservation of lyophilised lactic acid bacteria. Food Chemistry,132, 1699–1705.

xxviii. Pathan, A.K., Bond, J. and Gaskin, R.E.(2010). Sample preparation for SEM of plant surfaces. Materials Today,12, 32–43.

xxix. Rahmawati, Dewanti-Hariyadi, R., Hariyadi, P., Fardiaz, D. and Richana, N. (2013). Isolation and identification of microorganisms during spontaneous fermentation of maize. Jurnal Teknologi dan Industri Pangan, 24(1), 33–43.

xxx. Restaino, L., Frampton, E.W., Lionberg, W.C. and Becker, R.J. (2006). A chromogenic plating medium for the isolation and identification of Enterobacter sakazakii from foods, food ingredients, and environmental sources. Journal of Food Protection,69, 315–322.

xxxi. Richardson, A.N., Lambert, S. and Smith, M.A. (2009). Neonatal mice as models for Cronobacter sakazakii infection in infants. Journal of Food Protection, 72, 2363–2367.

xxxii. Schlünder, E. (2004). Drying of porous material during the constant and the falling rate period: A critical review of existing hypotheses. Drying Technology,22,1517–1532.

xxxiii. Schmid, M., Iversen, C., Gontia, I., Stephan, R., Hofmann, A., Hartmann, A. and Lehner, A. (2009). Evidence for a plant-associated natural habitat for Cronobacter spp. Research in Microbiology, 60, 608–614.

xxxiv. Seftiono, H. (2012). Thermal inactivation kinetics of Cronobacterspp. (Enterobacter sakazakii) in infant formula and buffer system at various aw and pH.Thesis. Bogor Agriculture University, Bogor.

xxxv. Simpson, B.K.(2012).Food Processing.In: Nollet, L.M., Benjakul, S., Paliyath, G., and Hui, Y.H., (ed.).Food Biochemistry and Food Processing. John Wiley Sons.

xxxvi. Yoshitomi, K.J. and Shann, J.R.(2001). Corn (Zea mays.) root exudates and their impact on sup C-pyrene mineralization. Soil Biology and Biochemistry,33,1769–1776.