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SURVIVAL OF *Cronobacter sakazakii* IN SKIM MILK DURING SPRAY DRYING, STORAGE AND RECONSTITUTION

[*Ketahanan Hidup Cronobacter sakazakii dalam Susu Skim selama Proses Pengeringan Semprot, Penyimpanan dan Rekonstitusi*]

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ABSTRACT

Cronobacter sakazakii is an emerging pathogen known to survive dry conditions and its presence in powder infant formula (PIF) has been linked to several outbreaks. In Indonesia, isolation of this bacterium from various foods have been reported. The objective of this study was to determine the effect of spray drying and storage humidity on the survival of *C. sakazakii* YRc3a in skim milk and their viability upon reconstitution. The survival of *Cronobacter* during spray drying was determined by comparing the number of bacteria before and after drying. The viability of *Cronobacter* in spray dried skim milk (SDSM) during storage was observed at weeks 1 to 8 and 12. At the same intervals, SDSM containing the pathogens was reconstituted at either 27°C or 50°C and the survivors were enumerated. The data were plotted to yield survival curves. Spray drying caused 4.19 log CFU/g reduction of *Cronobacter* and the bacteria experiencing drying were less sensitive to reconstitution at 50°C. During storage, the water activity of SDSM reached equilibrium at week 2 and afterwards, they started to decrease when stored at 50% or 90% RH, but maintained its viability at 70% RH. Storage at 50% and 90% RH accelerated the death rate of *C. sakazakii* YRc3a, resulting in the decline of the viable counts for 3 log cycles. At 50% RH, *C. sakazakii* Yrc3a decreased significantly, but the survivors exhibited increased heat resistance with the lowest reduction upon reconstitution at 50°C (0.16 log CFU/ml).

Keywords: A_w, *Cronobacter sakazakii*, reconstitution, spray drying, storage

ABSTRAK

Cronobacter sakazakii adalah patogen bawaan pangan emerging yang dilaporkan mampu bertahan dalam kondisi kering dan keberadaannya dalam susu formula telah dihubungkan dengn beberapa kejadian luar biasa. Di Indonesia, isolasi bakteri ini dari beberapa jenis pangan telah dilaporkan. Tujuan penelitian ini adalah untuk mengetahui pengaruh pengeringan semprot (spray drying) *dan pengaruh kelembaban relatif terhadap sintas C. sakazakii YRc3a dalam susu skim selama penyimpanan serta viabilitasnya setelah direkonstitusi dengan air. Sintas Cronobacter selama pengeringan semprot ditentukan dengan membandingkan jumlah Cronobacter sebelum dan setelah pengeringan. Viabilitas Cronobacter dalam susu skim hasil pengeringan semprot (SSPS) selama penyimpanan diamati setiap minggu dari minggu 1-8 dan minggu ke 12. Pada interval yang sama, SSPS yang mengandung Cronobacter direkonstitusi dengan air bersuhu 27°C atau 50°C dan bakteri yang bertahan hidup dihitung. Data yang diperoleh kemudian diplot untuk menghasilkan kurva sintas. Pengeringan semprot menurunkan Cronobacter sebanyak 4,19 log CFU/g dan bakteri patogen yang telah mengalami pengeringan menjadi kurang sensitif terhadap suhu rekonstitusi 50°C. Selama penyimpanan, aktivitas air (A_w) SSPS mencapai ekuilibrium pada minggu ke 2 dan setelah itu patogen mulai menurun jumlahnya jika disimpan pada RH 50% atau 90%, tetapi dapat mempertahankan viabilitasnya jika disimpan pada RH 70%. Penyimpanan pada RH 50% dan 90% mempercepat laju kematian C. sakazakii YRc3a dan mengakibatkan jumlah bakteri turun sebesar 3 siklus log. Pada RH 50%, jumlah C. sakazakii YRc3a turun secara signifikan, tetapi bakteri yang bertahan menunjukkan peningkatan ketahanan terhadap panas dengan penurunan jumlah terendah pasca rekonstitusi pada suhu 50°C (0,16 log CFU/ml).*

***Kata kunci**: A_w, Cronobacter sakazakii, rekonstitusi, pengeringan semprot, penyimpanan*

INTRODUCTION

Cronobacter spp., previously known as *Enterobacter sakazakii,* is an opportunistic emerging foodborne pathogen reported to cause meningitis, necrotizing enterocolitis, septicemia, and death in high risk infants, with a mortality rate of 40-80% (Himelright *et al*., 2002). *Cronobacter* spp. infection may also occur in the elderly and adults with low immune system although it does not result in death (Gurtler *et al*., 2005). At present six species of *Cronobacter* spp have been identified with *C. sakazakii* as the most common isolate (Iversen *et al*., 2007).

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Infection due to *Cronobacter* spp. in high risk infants has been linked to the consumption of powdered infant formula (PIF). The bacteria have been reported to survive in dry food including PIF (Caubilla-Barron and Forsythe, 2007; Edelson-Mammel *et al*., 2005). PIF may be contaminated by *Cronobacter* spp. from the ingredients, from the unhygienic production environment (Kim *et al*., 2008) and also during the reconstitution of PIF especially when poor water quality is used, handling is insanitary and the reconstituted PIF is left at room temperature for a long period of time before consumption.

Spray drying, which is intended to reduce the water activity (A_w) of food, is generally applied during the processing of PIF. The drying occurs due to hot air (generally with an inlet temperature of 180°C) that could influence the microorganisms' viability; an outlet temperature higher than 90°C will induce cell death (Wong *et al*., 2010). *Cronobacter* spp. has a relatively higher resistance to heat than other types of *Enterobacteriaceae*, thus they have a higher chance of survival in the drying process and are carried through to the end products (Edelson-Mammel and Buchanan, 2004). Lin and Beuchat (2007) reported that *Cronobacter* spp. can survive for as long as 12 months in cereals with low A_w as compared to those with a high A_w. The bacteria are able to improve their resistance to stress conditions such as dryness by accumulating solutes that stabilize their cellular membrane so that protein and enzyme denaturation can be prevented (Breeuwer *et al*., 2003).

Cronobacter spp. has been isolated from infant follow-on formula (Estuningsih *et al*., 2006), PIF and weaning foods (Meutia *et al*., 2009) and some other dry products marketed in Indonesia (Gitapratiwi *et al*., 2012). Gitapratiwi *et al*. (2012) also reported that their genetic characteristics were closely related, however their physiological properties such as resistance to drying and dryness have not been examined. Understanding the characteristics of the isolates is important for the handling of PIF during drying and storage. The aim of this research is to evaluate the survival of a local isolate of *Cronobacter* spp. in skim milk during spray drying and storage at various relative humidities (RHs), as well as their behavior upon reconstitution before, after drying and after storage.

MATERIALS AND METHODS

Materials

Cronobacter spp used in this study was *Cronobacter sakazakii* YRc3a isolated from PIF (Gitapratiwi *et al*., 2012) which was found to be more heat resistant than the other local isolates (data not shown). The materials used in this research were skim milk, distilled water, K_2CO_3, $Na(NO_3)_2$ and $BaCl_2$ salts. The growth medium for enumeration of the pathogen was Trytipcase Soy Agar containing Yeast Extract (TSAYE). Equipment used in the study were Buchi 190 Mini spray drier, A_w meter, laminar flow, autoclave, thermometer, 3000 rpm centrifuge, water bath shaker, and incubator.

Survival of *C. sakazakii* YRc3a in skim milk during spray drying

A late log phase *C. sakazakii* YRc3a culture with an initial population of 10^8–10^9 CFU/g was inoculated into 450 mL (40% w/v) of sterile skim milk (Wan-Ling *et al*., 2010) such that the calculated number *of C. sakazakii* was ca. 10^9 CFU/g. The spray drying process was carried out in a mini spray drier with inlet and outlet temperatures of 160ºC and 82ºC, respectively. The spray dried skim milk (SDSM) samples were put in sterile bottles and placed at 4ºC (Wan-Ling *et al*., 2009). Cell viability before and after spray drying was enumerated on TSAYE after incubation at 37ºC for 48 hours (Arroyo *et al*., 2009).

Survival of *C. sakazakii* YRc3a in spray dried skim milk (SDSM) during storage at various relative humidities

The SDSM samples containing *C. sakazakii* YRc3a were stored in desiccators in which the moisture had been regulated using saturated K_2CO_3 (RH ±50%), Na $(NO_3)_2$ (RH ± 70%) and $BaCl_2$ salts (RH±90%). Samples were tested weekly for the first 8 weeks as well as at week12 for viability (Arroyo *et al*., 2009), changes in A_w (Nielsen, 2003) and water contents (AOAC, 1995).

Survival of *Cronobacter* YRc3a upon reconstitution

Reconstitution was conducted according to manufacturer instruction, in which 2.2 g of the sample was mixed with 15 mL of sterile distilled water with the temperature of 27°C or 50°C for approximately one minute (Lin and Beuchat, 2007). The number of *C. sakazakii* YRc3a in skim milk, in skim milk after spray drying and during storage that survived reconstitution was enumerated on TSAYE and the difference between the surviving *C. sakazakii* reconstituted with 27°C or 50°C was calculated as log reduction.

Statistical analysis

The viability of *C. sakazakii* YRc3a in SDSM during storage at different RH as well as their survival upon reconstitution at 27°C and 50°C were analyzed statistically using a Complete Random Factorial Design with 2 factors, i.e. the RH and duration of storage followed by *Analysis of Variance* (ANOVA) and a Duncan test (*Duncan Multiple Range Test/DMRT*).

Survival curves of *Cronobacter* YRc3a during storage and upon reconstitution

The inactivation or growth kinetics of *C. sakazakii* YRc3a during storage at various RHs follows the first order reaction. To obtain the survival rate of *C. sakazakii* YRc3a during storage, survival curves were created by plotting the log ratio of the weekly survivors with the initial numbers of *C. sakazakii* YRc3a (log Nt/No) for the Y-axis and the time interval (in weeks) for the X-axis (Teixeira, 1997). The K value can be calculated from the curve slope (y= -kt/2.303), where K is the time (in weeks) needed to change *C. sakazakii* YRc3 apopulation by a log cycle. The curve slope was calculated using the following formula :

$$\log Nt = \log No - \frac{k}{2{,}303} t$$

The slope value 2.303/k is K, i. e. a log reduction time such that:

$$\log \frac{Nt}{No} = -\frac{t}{K}$$

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RESULTS AND DISCUSSION

Survival of *Cronobacter* YRc 3a during spray drying

Table 1 shows the number of *C. sakazakii* YRc3a in skim milk before and after spray drying with inlet and outlet temperatures of 160°C and 82°C, respectively. The spray drying inactivated 4.19 log CFU/g of *Cronobacter* YRc3a. Wan-Ling *et al*. (2009) reported 4.81 log decrease of *E. sakazakii* in 40% skim milk spray dried using inlet and outlet temperatures of 180°C and 80°C, respectively. Another study reported a decrease in Bifidobacteria from $1.38x10^9$ CFU/g to $6.30x10^5$ CFU/g durings pray drying at an inlet temperature of 160°C and an outlet temperature of 85±2°C (Wong *et al*., 2010). The result suggested that the spray drying applied is similar to the spray drying commonly applied for foods and that *C. sakazakii* YRc3a thermal inactivation is similar to previous report.

Table 1. Survival of *C. sakazakii* YR c3a in skim milk during spray drying with an inlet temperature of 160°C and outlet temperature of 82°C

Average Number of *Cronobacter* Before Spray Drying (CFU/g)	Average Number of *Cronobacter* After Spray Drying (CFU/g)	Reduction of *Cronobacter* Log (CFU/g)
$3.84x10^9$	$2.51x10^5$	4.19

Survival of *Cronobacter* YRc3a in Skim milk upon reconstitution using water at 50°C before and after spray drying

Upon reconstitution with water at 50°C, the number of *C. sakazakii* YRc3a in skim milk before drying decreased by 0.64 log CFU/ml, while reconstitution of skim milk after spray drying caused a *Cronobacter* decrease of 0.35 log CFU/ml (Figure 1). Based on ANOVA and the Duncan (DMRT) test, the log reduction of *C. sakazakii* YRc3a in skim milk before and after spray drying was significantly different ($\alpha<0.05$). The result suggested that the bacterial heat resistance increased after they were spray dried. The increase in heat resistance toward 50°C water used for reconstitution might have occurred because of sub lethal injury during spray drying that induced bacterial cells' protective mechanisms toward heating. It has been known that to stabilize membrane phospolipids, bacterial cells could synthesize heat shock proteins (Hsp) and accumulate non reducing trehalose (Arroyo *et al*., 2009).

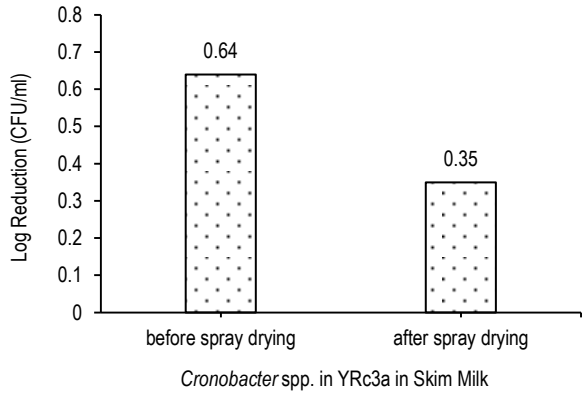

Figure 1. Reduction of *C. sakazakii* YRc3a in skim milk before and after spray drying upon reconstitution using water at 50°C

Survival of *C. sakazakii* YRc3a during storage at various RH

The relative humidity during storage influences the water activity (A_w) of the SDSM which then affects the viability of *C. sakazakii* YRc3a. Figure 2 and 3 shows changes in the A_w and water content of the SDSM during storage, respectively. The SDSM had an initial water content of 3.26% and an A_w of 0.32. During storage, A_w at various RH reached equilibrium after the second week of storage. The average equilibrium water activity of SDSM at relative humidities of 50%, 70% and 90% RH were 0.50, 0.73, and 0.85, respectively. Meanwhile the average water content at equilibrium at 50% RH storage was 7.06% and that at 70% RH was 15.09%. At 90% RH the water content continued to increase to 7 weeks of storage and an equilibrium was reached after week 7. At 90% RH, the average water content was 46.67%. Based on ANOVA and further analysis with Duncan (DMRT) test, the water activity and water content of the SDSM differred significantly ($\alpha<0.05$) due to different humidities.

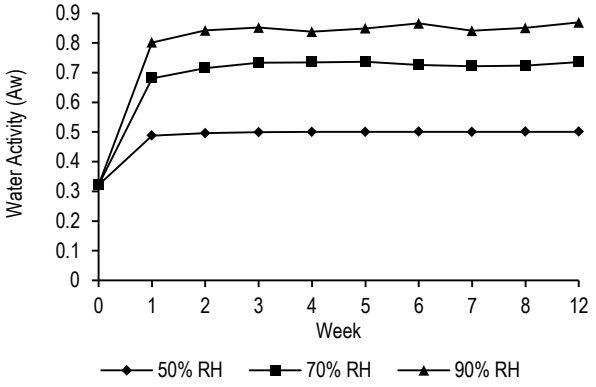

Figure 2. Changes in water activity (A_w) of spray dried skim milk (SDSM) during storage at various humidity

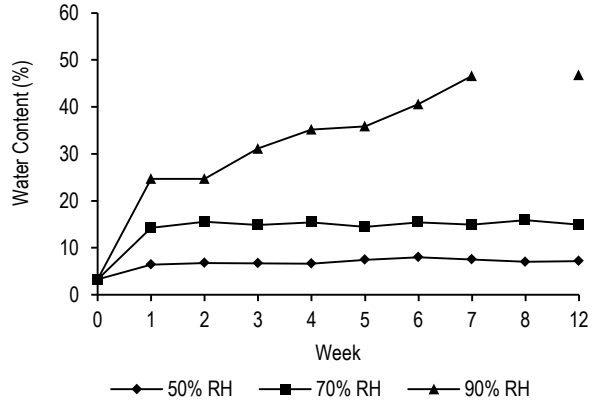

Figure 3. Changes in water content of spray dried skim milk (SDSM) during storage at various humidity

Figure 4 shows the viability of *C. sakazakii* YRc3a during storage at various RHs. It showed that storage at 70% RH could maintain the *Cronobacter* population and the bacteria would survive well. Storage at 70% RH for 12 weeks only resulted in 0.21 log CFU/g decrease in the number of *Cronobacter*. During 12 weeks of storage at 50% RH, *Cronobacter* decreased by 3.13 log CFU/g, whilst at 90% RH the population decreased the most, i.e. by 3.31 log CFU/g. The statistical analysis suggested

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that the viability of *C. sakazakii* YR c3a during storage at various RH and storage time was significantly different (α<0.05).

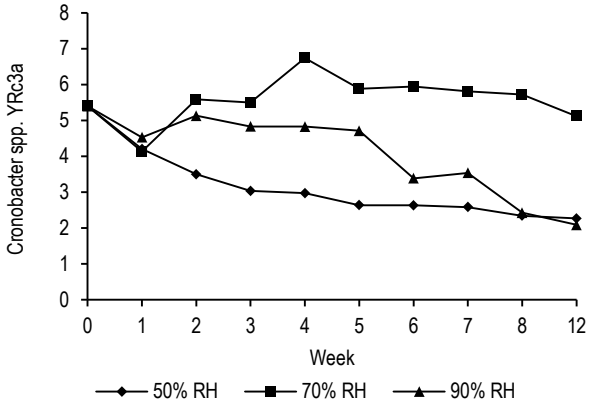

Figure 4. Fate of *C. sakazakii* YRc3a during storage at various humidity

The survival curves, plotted after 2 weeks of storage (Figure 5), suggested a very slow growth of *C. sakazakii* YRc3a during storage at 70% RH. After A_w of the SDSM reached equilibrium, the K value of the bacterium was 1000 weeks suggesting that the bacterium were just maintaining their viability (Table 2). The K values during storage at 50% and 90% RH showed a decrease or death of the pathogen. The K value during storage at 90% RH was smaller than that of 50% RH, suggesting that after two weeks the number of *Cronobacter* YRc3a decreased more rapidly during storage at 90% RH than that at 50% RH.

Table 2. The K values of *C. sakazakii* YRc3a during 12 weeks storage at various RH

RH (%)	Slope (-k/2,303)*	K=1/Slope (Week)
50	-0.031	32.26
70	0.001	1000
90	-0.045	22.22

*) positive slope indicates increase/growth; negative slope indicates decrease/death

Mattick *et al*. (2001) stated that A_w 0.12–0.46 supports the viability of bacterial cells in dry condition. During storage at 50% RH and the average A_w of SDSM of 0.5, cells started their cellular activities but the free water was not sufficient to maintain cells' viability thus the cell number decreased. On the contrary, high concentrations of free water (average A_w of 0.85 at 90% RH), also caused significant decreases in the number of *Cronobacter* because excessive concentrations of free water also inactivate bacterial cells. Day *et al*. (2009) suggested that at high A_w, bacteria in general will experience a decrease in viability. An increase in free water during the first week of the storage caused cells to undergo hypo osmotic shock. To survive this condition, bacterial cells will secrete compatible solutes such as trehalose, proline, betain, etc from their cytoplasms to decrease and equilibrate osmotic pressure within and outside the cells. This mechanism requires high energy and when this occurs continuously it will cost the bacteria ATPs and could result in cell death. The statistical analysis showed that the viability of *Cronobacter* YRc3a at different RH and time of storage were significantly different (α<0.05).

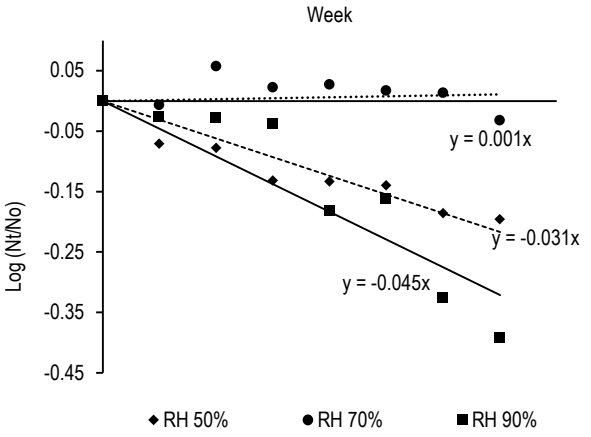

Figure 5. Survival curve of *C. sakazakii* YRc3a during storage at various humidity

Survival of *Cronobacter* spp. YRc3a in SDSM upon reconstitution after storage of SDSM at various relative humidities

Figure 6 shows the numbers of *C. Sakazakii* YRc3a after reconstitution using wate rat room temperature (27°C) while Figure 7 presents the survival curves after equilibrium was achieved at week 2. The calculated K values of *C. sakazakii* YRc3a after 12 weeks of storage at various RH are presented in Table 3. The patterns were similar to those during storage, suggesting that reconstitution with water at room temperature did not affect the pathogen.

Table 3. The K values of *C. sakazakii* YRc3a in SDSM upon reconstitution with water at room temperature (27°C) following a 12-week storage of SDSM at various RH

RH (%)	Slope (-k/2,303)*	K=1/Slope (Week)
50%	-0.044	22.727
70%	0.002	500
90%	-0.068	14.706

*) positive slope indicates increase/growth; negative slope indicates decrease/death

When reconstitution was carried out using 50°C water, the number of *C. Sakazakii* YRc3a surviving the process was lower than that reconstituted with water at room temperature (Figure 8). Figure 9 shows the survival curve after A_w equilibrium was reached. The calculated K values of *C. sakazakii* YRc3a after 12 weeks of storage at different RH swere also different from those reconstituted with 27°C water (Table 4).

Table 4. The K values of *C. sakazakii* YRc3a upon reconstitution with water at 50°C after 12 weeks of storage at various RH

RH (%)	Slope (-k/2,303)*	K=1/Slope (Week)
50	-0.035	28.571
70	0.003	333.333
90	-0.068	14.706

*) positive slope indicates increase/growth; negative slope indicates decrease/death

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Figures 6 and 8 show that upon reconstitution, *C. sakazakii* YRc3a in SDSM stored at RH 50% decreased quickly with storage time; with an average decrease of 2.43 log CFU/g in the first four weeks and 0.71 log CFU/gin in the next four weeks. The statistical analysis suggested that following storage of SDSM at various RHs, the viability of *C. Sakazakii* YRc3a in the SDSM reconstituted with water of 27°C and 50°C differred significantly (α<0.05). Figure 10 presents the combined survival curve of *C. sakazakii* YRc3a upon reconstitution with water 27°C and 50°C after the pathogen-containing SDSM underwent storage at various RHs.

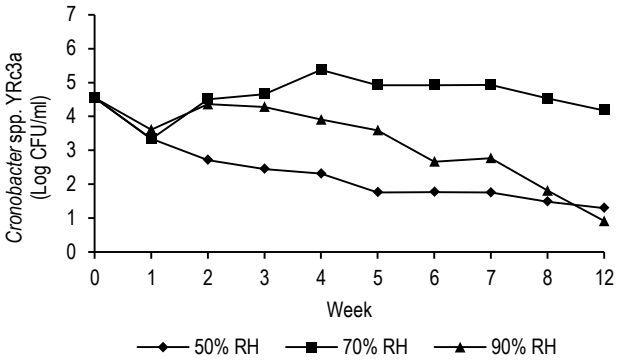

Figure 6. Survival of *C. sakazakii* YRc3a in SDSM upon reconstitution with water at 27°C following storage at various humidity

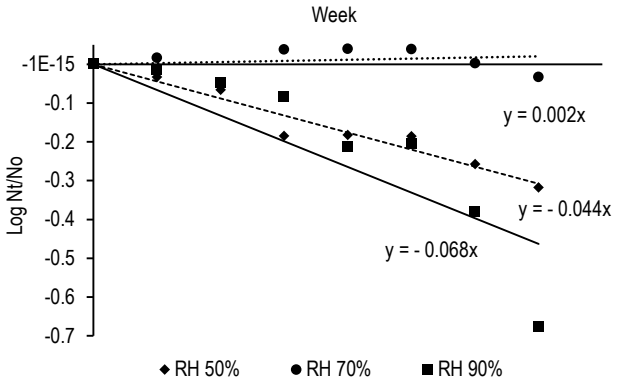

Figure 7. Survival curve of *C. sakazakii* YRc3a in SDSM upon reconstitution with water at 27°C following SDSM storage at various humidity

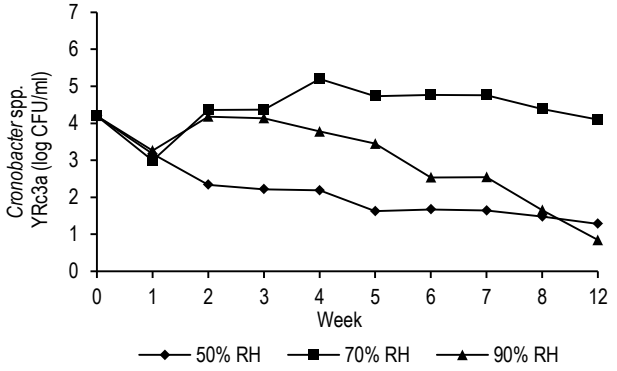

Figure 8. Survival of *C. sakazakii* YRc3a in SDSM upon reconstitution with water at 50°C following storage at various humidity

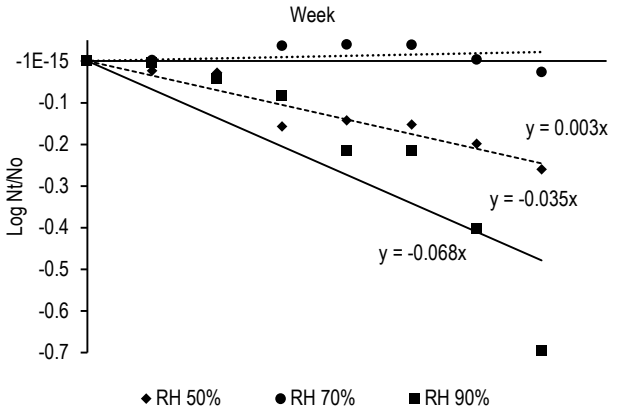

Figure 9. Survival curve of *C. sakazakii* YRc3a in SDSM upon reconstitution with water of 50°C following SDSM storage at various relative humidity

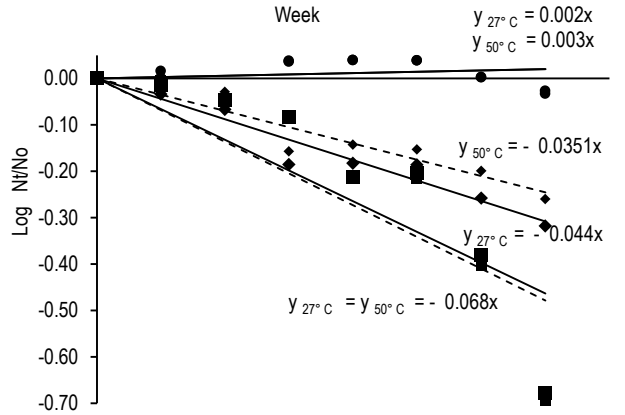

Figure 10. Survival curve of *C. sakazakii* YRc3a in SDSM upon reconstitution with water of 27°C (solid line) and 50°C (dashed line) following SDSM storage at 50% RH (◆), 70% RH (●), and 90% RH (■)

Figure 10 shows that *Cronobacter* YRc3a stored at 50% RH had increased resistance to reconstitution with 50°C water along with increase in storage time. This was shown from the increase in K values upon reconstitution at 50°C as compared to that at 27°C. *C. sakazakii* YRc3a stored at 70% RH experienceda decrease in its resistance upon reconstitution at 50°C, as seen fromthe K value upon reconstitution at 50°C which was lower than that at 27°C. Storage of *C. sakazakii* YRc3a at 90% RH did not cause any changes in bacterial resistance toward reconstitution at 50°C. Storage of SDSM at low RH caused *C. Sakazakii* YRc3a to have increased resistance to heating. The statistical analysis also suggested that the survival of *C. sakazakii* YRc3a in SDSM upon reconstitution with water at 27°C was significantly different at different storage RH (α<0.05) from that at 50°C, but it was not significantly different when storage was carried out at the same RH (α<0.05). Increased resistance to reconstitution at 50°C along with storage time was also shown by the lower average log reduction of *C. Sakazaki* YRc3a. During 12 weeks of storage, there was a decrease in the reduction of *C. sakazakii* YRc3a from 0.32 log CFU/ml (before storage) to an average of

0.16 log CFU/ml (RH 50%), 0.20 log CFU/ml (RH 70%), and 0.18 log CFU/ml (RH 90%). One of the factors influencing the heat resistance of *Cronobacter* during storage is the availability of free water (A_w). In general bacterial resistance to heating increases with a decrease in the A_w of the growth medium.

Table 5 shows the effects of various conditions, i.e. dryng and storage, on the average log reduction of *C. sakazakii* YRc3a upon reconstitution with water at 50°C. The A_w of SDSM reached an equilibrium at week-2 of the storage, and afterwards the *C. sakazakii* YRc3a stored at different storage RH were exposed to a different A_w. *C. sakazakii* YRc3a exposed to low A_w (50% RH) during storage has a higher resistance to reconstitution in water at 50°C than *C. sakazakii* YRc3a exposed to higher A_w (70% and 90% RH). Based on the statistical analysis, however, the decrease in *C. sakazakii* YRc3a upon reconstitution was not significantly different (α<0.05) during storage at various RH. On the contrary, the log reduction due to reconstitution during storage were different from that prior to storage or that not exposed to drying.

Table 5. Reduction of *C. sakazakii* YR c3a in skim milk or spray dried skim milk upon reconsitution with water at 50°C

Cronobacter Conditions	Average Reduction of *Cronobacter* in SDSM (log CFU/ml)*	Notes
Before spray drying; no storage	0.640[a]	
After spray drying; no storage	0.350[b]	
Storage at 50% RH; 12 weeks	0.161[c]	A_w= 0.500
Storage at 70% RH;12 weeks	0.203[c]	A_w= 0.729
Storage at 90% RH; 12 weeks	0.176[c]	A_w= 0.851

*) similar notation suggests that the log reduction of *Cronobacter* is not significantly different (α<0.05)

In general bacterial growth requires A_w > 0.8. When bacteria are exposed to low A_w, bacteria may undergo stress or injuries due to osmotic differences inside and outside the cells. Bacteria experiencing osmotic stress will synthesize heat shock proteins that help improve resistance to heat (Wood *et al*., 2001). During storage, *C. sakazakii* YRc3a exposed to A_w lower than their internal A_w may lose water quickly, therefore, to maintain cellular viability they accumulate compatible solute to reduce the internal A_w (osmotic effects). The lower amount of water within cells caused protein units to interact, producing a more stable protein that requires more energy to break open. This resulted in increased heat resistance at low A_w (Archer *et al*., 1998). On the contrary, when dry cells are exposed to higher A_w, the amount of water within cells will increase and solute metabolism occurs. Under this condition, enzyme and protein denaturation by heat can significantly cause cell death because water is available (Laroche *et al*., 2005).

CONCLUSION

Spray drying with inlet and outlet temperatures of 160°C and 82°C, respectively, of skim milk artificially inoculated with *C. sakazakii* YRc3a resulted in 4.19 log CFU/g decrease in the number of the pathogen. The survival of *C. sakazakii* YRc3a during reconstitution increased after spray drying, as seen by the reduction of the pathogen which decreased from 0.64 log CFU/ml (before drying) to 0.35 log CFU/ml (after drying). During 12 weeks of storage at 70% RH the number of *C. sakazakii* YRc3a in SDSM was relatively stable with a total decrease of 0.21 log CFU/g. At 70% RH storage, the pathogen had a K value of 1000 weeks based on the survival curve plotted after week-2. *C. sakazakii* YRc3a in SDSM stored at 50% experienceed a 3.13 log CFU/g decrease while those stored at 90% RH storage underwent the fastest decrease with an average log reduction of 3.31 log CFU/g an a K value of 22.22 weeks.

Survival of *C. sakazakii* YRc3a upon reconstitution at 50°C increased along with the increase in storage time and decrease in A_w. After storage at 50% RH, the log reduction of *C. sakazakii* YRc3a upon reconstitution was the lowest i.e. 0.16 log CFU/ml, while those stored at higher RH experienced higher reduction, i.e. 0.20 log CFU/ml (70% RH) and 0.18 log CFU/ml (90% RH).

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