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Research Article
Induction and Resuscitation of *Cronobacter sakazakii* into Viable but Non-culturable State at Low Temperature in Water Microcosm

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Abstract

Background and Objective: *Cronobacter sakazakii* considered to be an important human pathogen and has been increasingly linked to food-borne illness. This study aimed to investigate the fate of *C. sakazakii* in lake water under low temperature and to evaluate the potential hazard of viable but nonculturable (VBNC) cells induced under such stressful conditions. **Methodology:** A laboratory strain of *Cronobacter sakazakii* was induced into VBNC state at low temperature in water microcosm and resuscitation and virulence gene was examined. Effect of growth phase and inoculum level on induction into VBNC was determined. Results were analysed by student's t-test. **Results:** Results suggested that *C. sakazakii* strain can enter into VBNC state at low temperature. Growth phase and inoculum level has no effect on induction of VBNC. The VBNC forms of the strain examined can be resuscitated by temperature upshift and addition of catalase. The VBNC *C. sakazakii* strain showed the potential to retain virulence. Virulence gene expression and alteration of protein secretion was observed during VBNC state. **Conclusion:** It is concluded that under some stress conditions, *Cronobacter sakazakii* can not only enter into VBNC state but also retain virulence and resuscitation potential. This study will help researchers to explore mechanisms of survival and virulence of the pathogen in food value chain. Understanding these mechanisms may pave the way to device new control strategies.

Key words: *Cronobacter sakazakii*, virulence, retention, resuscitation, microcosm

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cronobacter sakazakii is a motile, non-spore forming, Gram-negative foodborne pathogen belonging to the family Enterobacteriaceae[1]. This pathogenic organism has been implicated as a cause of infant meningitis, necrotizing entercolitis (NEC) and bacteraemia and may cause death among neonates[2,3]. The infant mortality rate associated with *C. sakazakii* infections ranges from 40-80% and up to 20% of infected newborns develop severe neurological sequelae such as hydrocephalus, quadriplegia and retarded neural development[4]. *Cronobacter* has been isolated from a wide range of foods including cereals, cheese, fruits, meat, milk, vegetables, grains, herbs and spices as well as their byproducts[5]. Investigations of *Cronobacter* properties that promote environmental persistence, pathogenicity and virulence factors, identification of environmental reservoirs and methods of elimination are active areas of research[6].

Microorganisms that do not grow in culture media, but are still metabolically active and capable of causing infections in animals and plants, are said to be in a VBNC state[7]. The VBNC state in bacteria can be triggered by harsh environmental conditions such as nutrient starvation, extreme temperatures and sharp changes in pH or salinity; osmotic stress, oxygen availability. Moreover, damage to or lack of an essential cellular component including DNA; exposure to food preservatives and heavy metals; exposure to white light; activation of lysogenic phages or suicide genes such as sok/hak or autolysins may also trigger VBNC state. Decontaminating processes such as pasteurization of milk and chlorination of wastewater may also trigger bacteria to enter VBNC state[8]. Numerous bacteria, such as *Escherichia coli*, *Vibrio vulnificus*, *Salmonella enteritidis*, *Shigella sonnei*, *Shigella flexneri*, *Campylobacter jejuni* and *Legionella pneumophila*, can enter the VBNC state after exposure to adverse environmental stress. However, subsequent studies also indicated that this non-culturable state can be reversed when the stress is eliminated[9].

Though pathogens in VBNC state are unable to induce infection, they pose potential public health risk as they retain virulent properties in VBNC state and able to resume metabolic activity and virulence. Therefore, the objective of the study was to evaluate whether *C. sakazakii* can enter into VBNC state and can be resuscitated again from the state. Additionally, in VBNC state, whether *C. sakazakii* can retain their virulence potential was also an aim of the study.

MATERIALS AND METHODS

Study period: The study was carried out at Industrial Microbiology Laboratory of Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR) from January, 2012 to March, 2013.

Bacterial strains: A wild type *C. sakazakii* isolated and reported by Fakruddin *et al.*[3] from milk formula has been included in this study.

Preparation of microcosm water: Starvation media with pH 7.0 combined with different temperature were used in inducing bacterial cells to enter VBNC state. Medium was prepared from lake water and then sterilized by filtration through 0.2 μM cellulose nitrate (Sartorius) to remove autochthonous microflora and sterilized at 121°C for 20 min to destroy biological and heat sensitive antimicrobial agents that might pass through the filters.

Preparation of bacterial cells: *Cronobacter sakazakii* cells were incubated in Luria-Bertani (LB) broth (Difco, USA) at 28°C and 100 rpm overnight. The late log phase bacterial cells ($OD_{600} = 0.8$) were harvested by centrifugation at 6,000 × g for 10 min and then washed twice with sterile saline and once with sterile River Water (RW) to prevent carry-over of nutrients on inoculation of the microcosms. The bacterial concentration was adjusted with RW to 10^8-10^9 cells mL^{-1} and determined using a counting chamber and a microscope at 40X magnification (Olympus BX41, Japan).

Setting microcosm: One milliliter of cells was inoculated into 199 mL of sterile RW mediain 500 mL flasks. The microcosms were separately incubated at 4, 15 and 25°C in the dark. Microcosms were prepared in triplicate for each experiment.

Cell counts: Samples taken at the time of inoculation (initial time) and at subsequent time intervals were monitored by the plate count and direct viable count. Plate counts were obtained by spreading appropriate dilutions of cells onto VRBGA plates (HiMedia, India). Plates were incubated at 28°C and colonies were counted after 2 days. Bacterial viability was ascertained by using a Live/Dead BacLight bacterium viability kit (No. L-7007, Molecular Probes Europe BV, Leiden, Netherlands). Viable cell counts were determined by using a count chamber and a fluorescence microscope (Olympus BX41, Tokyo, Japan) and five microscopic fields were counted for each sample.

Detection of *C. sakazakii* at VBNC state by PCR: The *Esak* gene was targeted for detection of *C. sakazakii* at VBNC state using chromosomal DNA as template. The DNA was extracted from microcosm according to the method described by Haque *et al.*[10]. Primer sequence of *Esak* are *Esak*F-5′-GCTYTGCTGACGAGTGGCGG-3′; *Esak*R-5′-ATCTCTGCAGGATTCTCTGG-3′. PCR reactions were carried out with an initial denaturation step at 94°C for 3 min; followed by 35 cycles of 94°C for 1 min; 60°C for 1min 30 sec; 72°C for 1 min 30 sec, with the final extension of 5 min at 72°C. The PCR products were later electrophoresed in 1% agarose gel.

Resuscitation of non-culturable *C. sakazakii* cells: In this experiment, two methods, namely plates supplemented with H_2O_2-degrading compound (catalase) and temperature upshift, were used to perform the resuscitation of non-culturable *C. sakazakii* cells. One hundred microliter of sterile catalase (20,000 U mL^{-1}; Sigma) was spread on 1/5 VRBGA plates at a concentration of 2,000 U $plate^{-1}$. Bacterial cells (0.1 mL), which were separately collected at the time of inoculation (initial time) and at subsequent time intervals, were then spread on the agar plates and colonies were enumerated after 4 days of incubation at 28°C. Media lacking supplements were designated as non-amended controls. In the temperature upshift experiment, 0.5 mL portion of the non-culturable cell solution from water microcosm, which was collected from a sample that had entered into VBNC state for 7 days, was added to the same volume of water and then incubated at 28°C in the dark without shaking. The culturability of different samples was determined by plate count on 1/5 VRBGA plates at different time interval. If the non-culturable cells can be restored either after a temperature upshift or on plates supplemented with catalase, but not in TSB or on non-amended TSA plates, then the cells are considered to be resuscitated rather than re-grown from the remaining culturable cells.

Virulence gene expression: Expression of *C. sakazakii* virulence genes, *fliD*, *flhD*, *motA*, *motB*, *flgJ*, *ompA*, *ompX*, *uvrY*, *lpx*, *wzx* and *sod* were investigated using real-time quantitative polymerase chain reaction (RT-qPCR). Description and primer sequence of the genes are presented in Table 1. For determination of expression level of the virulence genes, total RNA was extracted from bacteria using Trizol reagent and cDNA was prepared using primescript RT reagent kit (TaKaRa Bio). Transcripts were quantified by LightCycler (Roche Diagnostics) using SYBR Premix Ex *Taq* (TaKaRa Bio) in accordance with the manufacturer's instructions. The expression levels of each gene were normalized, with the 16S rRNA gene as an internal control.

Table 1: Primers used for real time PCR

Genes	Primers	Sequences (5′-3′)
16S	Forward	CCAGGGCTACACACGTGCTA
	Reverse	TCTCGCGAGGTCGCTTCT
lpxB	Forward	GCACGACACTTTCCGTAAACTG
	Reverse	CGCCTGTTCATCGGCATT
ompA	Forward	GGCCGCATGCCGTATAAA
	Reverse	GCTGTACGCCCTGAGCTTTG
ompX	Forward	GTCTTTCAGCACTGGCTTGTGT
	Reverse	GGTGCCAGCAACAGCAGAA
flhD	Forward	CGATGTTTCGCCTGGGAAT
	Reverse	AGAGTCAGGTCGCCCAGTGT
fliD	Forward	AAAACCGCAACATGGAATTCA
	Reverse	CCGCAAACGCGGTATTG
flgJ	Forward	GACGGCGGGCAAAGG
	Reverse	GCCGCCCATCTGTTTGAC
motA	Forward	GGTGTGGGTGCGTTTATCGT
	Reverse	GCCTTCAGCGTGCCTTTG
motB	Forward	ACGGCTCGTGGAAAATCG
	Reverse	CCAGGAAGAAGGCCATCATG
sod	Forward	CGAATCTGCCGGTTGAAGA
	Reverse	CTTGTCCGCCGGAACCT
uvrY	Forward	GCGAGGACGCCATCAAAT
	Reverse	ATCCATCAGCACCACATCCA
wzx	Forward	TGCTTGGGCAGGTACAAAGTG
	Reverse	CCCTACGGGTGCAGTCACA

SDS-PAGE analysis of secreted proteins: Extracellular proteins of *C. sakazakii* before and after induction into VBNC state wereprepared according to the method described by Abdallah *et al.*[11]. Proteins were analysed by sodium dodecylsulphate (SDS)–polyacrylamide gel electrophoresis (PAGE)[12] with 15% acrylamide in the separating gel and 5% acrylamide in the stacking gel. After separation, the proteins were visualized according to standard procedures by staining with coomassie brilliant blue G250 (Sigma, Chemical Co., St Louis, MO,USA) and molecular weights were determined by means ofcommercial markers (High-Range Rainbow; Amersham, Little Chalfont, Buckinghamshire, UK).

Statistical analysis: Data were analyzed using student's t test with GraphPad Prism 6.0 and presented as Mean±Standard Error.

RESULTS

Low temperature induce VBNC state in *C. sakazakii*: Results from the study of *C. sakazakii* incubated separately in lake water microcosm at different temperatures showed that

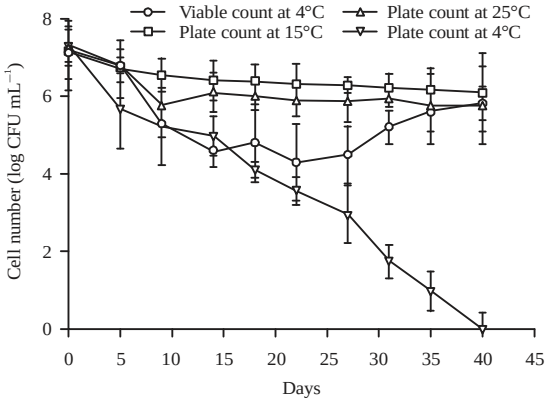

Fig. 1: Induction of viable but non-culturable (VBNC) state by *C. sakazakii* in lake water microcosm at different temperatures
Error bars represent the standard deviations

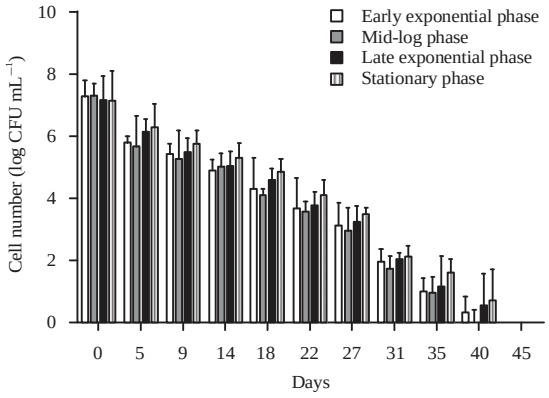

Fig. 2: Effects of growth phases on the VBNC response of *C. sakazakii* in water microcosm at 40°C
Cells were harvested in early exponential, mid log, late exponential and stationary phases of growth. One-sided error bars indicate standard error of the mean (p = 0.05)

plate counts declined to less than 1 log CFU mL^{-1} at 4°C after 40 days. The viable counts decreasedto 4.60 log CFU mL^{-1} at the end of the second week and maintained during the remaining incubation period (Fig. 1). However, while culturing at 25°C, plate counts were maintained to be in the range of 7.2~5.8 log CFU mL^{-1} during the whole cultivation period (Fig. 1). These results indicate that bacterial cells did not enter VBNC state at higher temperature but did enter into VBNC at lower temperature (4°C).

Effect of growth phase on VBNC response of *C. sakazakii*: There was little significant difference ($p<0.05$) in the rates of decline for cells from stationary, late, mid and early exponential phases of growth. Mid-log phase cells entered

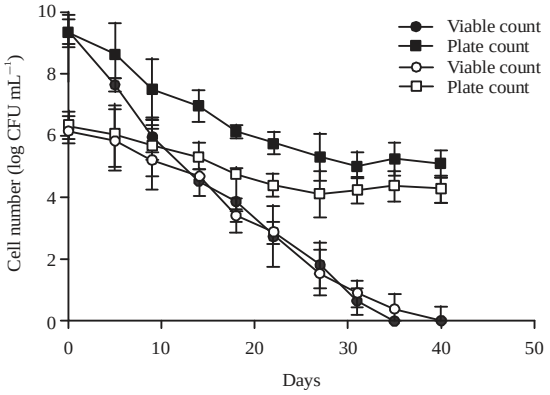

Fig. 3: Effect of inoculum size on the VBNC response of *C. sakazakii*. Lake water microcosm were inoculated with ~9 (filled symbols) and ~6 (open symbols) bacteria per mL and incubated at 4°C
Culturable populations plated on VRBGA media (box) and viable counts (circles) are indicated. Error bars represent the standard deviations

into VBNC state earlier than cells of other phases followed by early exponential phase cells (Fig. 2). Stationary phase cells entered VBNC state lately than other phases (Fig. 2).

Effect of inoculum size on VBNC response of *C. sakazakii*: In order to determine the effect of inoculum concentration on the VBNC induction of *C. sakazakii* at low temperature in lake water microcosm, two different inoculum concentrations were assessed (approximately 9 and 6 log CFU mL^{-1} culturable bacteria)[13]. At 4°C, regardless of the inoculum size, counts of culturable bacteria decreased to an undetectable level by days 30 (Fig. 3).

Detection of VBNC *C. sakazakii* by PCR: Total genomic DNA was extracted from water microcosm when bacteria entered undetectable stage and *Esak* gene was detected by PCR. All the samples showedbands of 929 bp (Fig. 4), an expected size of primers specific to *Esak* gene which correlate that the strain present in VBNC state in the water microcosm but cannot form colony due to different physico-chemical stresses.

Resuscitation of VBNC *C. sakazakii*: Two methods were used in this study to resuscitate nonculturable *C. sakazakii* from lake water microcosm-temperature upshift and catalase supplementation and both of the methods promoted resuscitation of VBNC cells. Temperature upshift from 4-28°C resuscitate VBNC cells and increase viable and detectable cell number to 6.11 log CFU mL^{-1} after 20 days (Fig. 5). The catalase treatment also showed the resuscitation of VBNC *C. sakazakii* cells (Fig. 5).

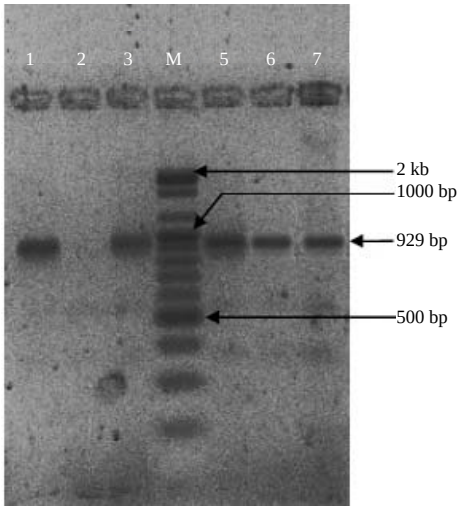

Fig. 4: Amplified product of *Esak* gene

Lane 1: DNA extracted from *C. sakazakii* grown in normal cultural condition, Lane 2: Negative control, Lane 3, 5, 6, 7: DNA extracted from lake water microcosm, Lane M: 1 kb marker

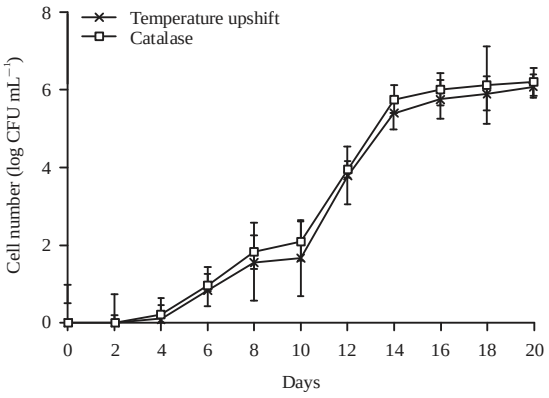

Fig. 5: Resuscitation of *C. sakazakii* from VBNC state by temperature (°C) upshift and catalase supplementation

Error bars represent the standard deviations

Retention of virulence in VBNC state: Considering the risk factor for food safety, VBNC cells should continue to harbor the potential for virulence. The constitutive and stable expression of the virulence genes analyzed in this study (*fliD*, *flhD*, *motA*, *motB*, *flgJ*, *ompA*, *ompX*, *uvrY*, *lpx*, *wzx* and *sod*) was found to be expressed in the VBNC *C. sakazakii* cells (Fig. 6).

Changes in protein expression: Protein expression of *C. sakazakii* was determined before entry and in VBNC state as well as after resuscitation from VBNC state. Protein expression

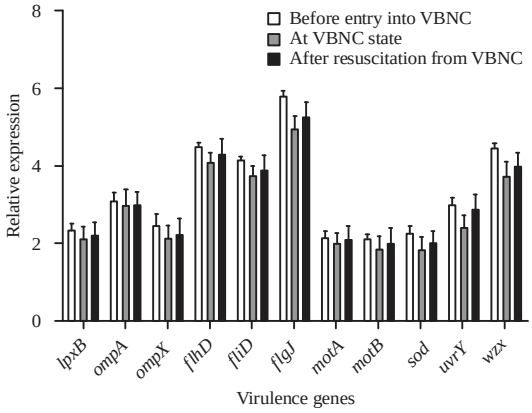

Fig. 6: Retention of virulence by *C. sakazakii* at VBNC state

Virulence gene expression was determined before entry, in VBNC state and after resuscitation. One-sided error bars indicate standard error of the mean (p = 0.05)

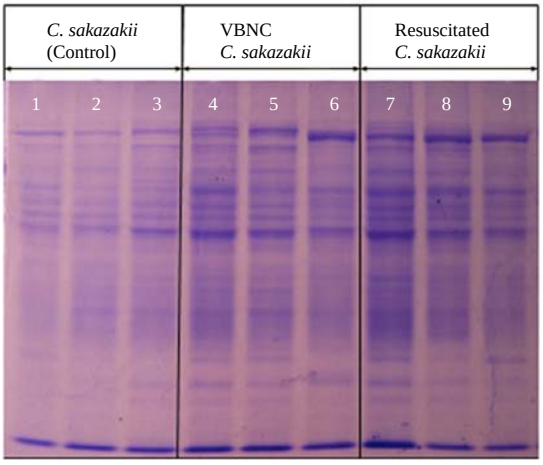

Fig. 7: Changes in protein expression during entry and exit of VBNC *C. sakazakii*

Lane-1, 2, 3: Protein expression of control *C. sakazakii*; Lane-4, 5, 6: Protein expression of *C. sakazakii* at VBNC state; Lane-7, 8, 9: Protein expression of resuscitated *C. sakazakii* cells

pattern in VBNC state was considerable different from that of *C. sakazakii* at normal condition (before entry into VBNC state) (Fig. 7). On the contrary, protein expression of *C. sakazakii* resuscitated from VBNC state was somewhat similar to that at VBNC state (Fig. 7).

DISCUSSION

Since the VBNC state was proposed, more than 60 species had been demonstrated to enter the VBNC state[14]. Environmental conditions required to enter the VBNC statediffer between bacterial species. Various bacteria can

enter the VBNC state in low temperature environments, including *Vibrio cholerae*[15], *Vibrio parahaemolyticus*[11], *E. coli*[16], *Aeromonas hydrophila*[17] and *Yersinia pestis*[18], amongst others. Several important factors had been shown to have effect on the survival of enteric bacteria in environments. These include the availability of nutrients, the changes in osmolarity and the pH and physical stresses. Many of these stresses had been shown to lead to the formation of VBNC state in bacteria[8].

In the present study, starved *C. sakazakii* was induced into VBNC state by long time incubation at 4°C in water microcosm. Figure 1 indicates that *C. sakazakii* strain capable of long-term survival in natural water, although a gradual entrance into the VBNC state was observed when cells were incubated at low (4°C) temperature. *Cronobacter sakazakii* cells showed a rapid decrease in culturability after 16 h under low nutrient conditions. Culturable cells could not be detected after incubation for 40 days, whereas viable cell counts remained above approximately 5 log CFU mL^{-1}). This finding confirms that the *C. sakazakii* strain can enter the viable but nonculturable state. Several other pathogens have been reported earlier to be induced into VBNC state at low temperature such as *Ralstonia solanacearum*[19], *Aeromonas hydrophila*[17], *Yersinia pestis*[18] and *Vibrio cholerae*[9]. Difference in growth phase and inoculum size showed no significant effect on induction of *C. sakazakii* strain to enter into VBNC state at low temperature. These results are in accordance with previous study where it is reported that pathogens enter VBNC state regardless of growth phase[20] and inoculum size[13]. To further confirm that the strain enter into VBNC state while undetectable by cultural means, PCR with specific primer was carried out and positive PCR proved that the *C. sakazakii* strain indeed entered into VBNC state (Fig. 4).

Another important characteristic of the VBNC forms was their ability to be resuscitated as a consequence of environmental changes, i.e., an increase in temperature. *Cronobacter sakazakii* strain in the VBNC state induced by low temperature were recovered by removing stress factors, such as increasing the temperature indicated with their viability (Fig. 6). The current results revealed that a temperature increase (before complete loss of culturability) recovered bacterial culturability (Fig. 5). Resuscitation of bacteria from VBNC state by temperature upshift had been reported earlier in case of *V. cholerae*[15], *E. coli*[21] and *A. hydrophila*[17]. The authors also attempted to resuscitate VBNC cells by addition of catalase to reduce oxidative stress related to long term survival at low temperature. The current study results also showed that culturability of VBNC cells of *C. sakazakii* was recovered in the presence of catalase (Fig. 5), which indicates that oxidative stress induced internally or externally may be important for induction of the VBNC state. The resuscitation of *C. sakazakii* cells by catalase (but not by autoclaved catalase) suggested hydrogen peroxide accumulation in cells exposed to low temperature stress and suppression of colony formation on regular culture medium. Resuscitation of VBNC cells by catalase had also been reported earlier for *Vibrio*[22], *E. coli*[23], *R. solanacearum*[18] and *Salmonella typhi*[24].

Several researchers had already reported that some bacteria in VBNC state can retain their pathogenic potential rendering them public health risk such as *Vibrio* spp.[9,25], *S. typhi*[24] and *A. hydrophila*[17]. In this study, it was found that the *C. sakazakii* strain retain its virulence in VBNC state as demonstrated by expression of *C. sakazakii* specific virulence associated genes (*fliD*, *flhD*, *motA*, *motB*, *flgJ*, *ompA*, *ompX*, *uvrY*, *lpx*, *wzx* and *sod*) (Fig. 6). Though expression of genes showed to be slightly lower than before entry into VBNC state. Expression of virulence genes was almost equal or increased after resuscitation from VBNC state by either temperature upshift or addition of catalase.

Secreted proteins play a major role in the virulence of bacteria. Proteins secreted by *C. sakazakii* strain were analyzed by SDS-PAGE (Fig. 7). Extracellular protein profile showed alteration in protein secretion before entry, during VBNC and after resuscitation from VBNC state. Before their incubation in water microcosm, the *C. sakazakii* strain secreted a significantnumber of proteins in the extracellular medium. Alterations of protein secretion were manifested by the appearance and/or disappearance of bands as well as in the level expression of certain proteins. Induction of *C. sakazakii* into VBNC state lead alterations in bacterial function and protein expression.

Overall, data of this study indicated that under stress conditions such as starvation and low temperature, *C. sakazakii* induces a large population of viable but nonculturable cells. The presence of stressed highly infective pathogens may be a major health concern since they cannot be detected by culturing techniques and resuscitation methods but are nonetheless ubiquitous and harbor the potential for virulence. Further investigations are required in order to better understand the risk associated with VBNC *C. sakazakii* cells that would provide a basis for risk assessment in food safety.

CONCLUSION AND FUTURE RECOMMENDATIONS

This study reported that *C. sakazakii* can enter into VBNC state at low temperature in water due to stress conditions. Cells those enter into VBNC can be resuscitated and they retain their pathogenic potential. Detailed knowledge on

mechanism of entry of *C. sakazakii* into VBNC state will pose practical applications in risk assessment and remediation efforts related to persistence of *C. sakazakii* in environment and food.

SIGNIFICANCE STATEMENT

To ensure safe food and public health safety, research should be focused on mechanisms of induction, resuscitation and virulence retention of bacteria at VBNC state. This study will help the researchers to understand the mechanisms of VBNC state induction of *Cronobacter sakazakii*.

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