IMPROVEMENT OF DETECTION METHOD AND HEAT RESISTANCE STUDY AMONG STRAINS OF *ENTEROBACTER SAKAZAKII*

By

PEI-CHUN CHEN

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Chair

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IMPROVEMENT OF DETECTION METHOD AND HEAT RESISTANCE STUDY AMONG STRAINS OF *ENTEROBACTER SAKAZAKII*

Abstract

By Pei-Chun Chen, M.S.
Washington State University
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Chair: Dong-Hyun Kang

Enterobacter sakazakii is a rare, but important cause of life-threatening neonatal meningitis and has also been associated with bacteremia and necrotizing enterocolitis. Although the natural habitat and reservoir of this pathogen remain unknown, several outbreaks had been associated with infant formula. This study was carried out to evaluate recommended enrichment broths, develop an effective quantitative analysis method, compare heat resistance among strains, and provide a guideline for infant formula preparation and handling. First, reconstituted dried infant formula (DIF), Enterobacteriaceae enrichment (EE) broth, modified tryptone soya broth supplemented with novobiocin, modified *Escherichia coli* broth with novobiocin, and buffered peptone water supplemented with vancomycin, cefixime, and cefsulodin were compared for enriching *E. sakazakii*. EE broth was the most effective of five enrichment broths, followed by reconstituted DIF. Second, a new method was developed to effectively enumerate *E. sakazakii* by fluorescence. Ninety six-well microtiter plates were used to perform a miniaturized 10-tube MPN enumeration protocol. This procedure was highly correlated ($R^2 = 0.91$) with the traditional plating method in reconstituted DIF and required much shorter time (<10 h) and less media.

Other than improving the detection method, heat resistance of *E. sakazakii* among four strains was compared in pH 7.0 phosphate buffer (PB) and reconstituted DIF. No significant (p<0.05) difference was observed at 50°C. However, as treatment temperature increased, the differences among four strains became distinguishable. The four strains were separated into two groups when 60°C was applied. Compared to PB, reconstituted DIF could protect cells from being heat damaged. At the last part, either one serving or two servings of infant formula were reconstituted by mixing with different temperatures of water including room temperature (24±1°C), 50°C, 70°C, 80°C, and 90°C. According to the survival and regrowth curves, it is proved that when hot water (70-90°C) was used, at least 1 log reduction was achieved. Therefore, *E. sakazakii* infection could be reduced by reconstituting DIF with hot water.

INTRODUCTION

Enterobacter sakazakii is an emerging foodborne pathogen and dried infant formula (DIF) had been associated with most outbreaks. Neonatal meningitis, necrotizing enterocolitis, and bacteremia are the three major illness caused by *E. sakazakii*. Worldwide, there are at least 28 outbreaks caused by *E. sakazakii*. Although the number of the outbreaks is not as many as those well known foodborne pathogens like *Escherichia coli*, the high mortality (40-80%) of neonatal meningitis caused by *E. sakazakii* made this microorganism important to be understood.

Two major problems exist for detection of *E. sakazakii*. Enrichment is an important step for *E. sakazakii* isolation since the contaminated concentration in DIF had been reported to be only 0.36-66 CFU/100 g. Although Enterobacteriaceae enrichment (EE) broth was recommended by U.S. FDA, other enrichment broths including infant formula, modified tryptone soya broth supplemented with novobiocin, modified *Escherichia coli* broth with novobiocin, and buffered peptone water supplemented with vancomycin, cefixime, and cefsulodin had also been used for enriching *E. sakazakii*. No comparison data were available to help choose an optimal enrichment broth for isolation of *E. sakazakii*. Besides, no effective method is available for quantitative analysis of *E. sakazakii*. Plating is the only method for enumeration of *E. sakazakii*. However, this method is cumbersome and at least 24 h was needed before obtaining the results. The U.S. FDA recommended protocol for *E. sakazakii* isolation even required 48-72 h for obtaining the results.

To control this pathogen and prevent the infections, more research is needed. As heat treatment is the major controlling method for reducing *E. sakazakii*

contamination from processing to consuming, its heat resistance should be further studied to gain more information among strains. In addition, water used to reconstitute DIF plays an important role for avoiding *E. sakazakii* infections. It is the last possible point to kill the microorganism. Different condition of mixing water could result in different effect to *E. sakazakii*. However, controversial recommendations was observed. According to manufacturers' preparation guidelines, powder formula was suggested to mix with cool water (2-24°C) or warm water (37-38°C) if the formula was fed or refrigerated immidiately. On the other hand, to reduce the exsisting risk, boiling water or hot water (70-90°C) had been recommended by U.S. FDA and International Food Safety Authorities Network (INFOSAN), respectively. However, they were inable to assure adequate destruction of *E. sakazakii*. U.S. FDA even removed this recommendation later on.

In this study, four parts of experiments would be accomplished. 1) Four enrichment broths along with reconstituted DIF would be compared to find out the most efficient enrichment broth for *E. sakazakii* isolation among the five media; 2) A simple and effective detection method for enumeration of *E. sakazakii* would be developed to reduce the time required for obtaining the results; 3) The survival of *E. sakazakii* among strains would be compared at different temperatures and media to gain further heat resistance information about this microorganism; 4) The survival and regrowth patterns of *E. sakazakii* in infant formula with different reconstitution conditions (temperatures and volumes) would be evaluated to provide a guildine for infant formula preparation and handling to prevent *E. sakazakii* infections.

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CHAPTER ONE

LITERATURE REVIEW

1.1 Characteristics of *Enterobacter sakazakii*

1.1.1 Biochemical Characteristics and Taxonomy

Enterobacter sakazakii, a member of the family Enterobacteriaceae, is a motile peritrichous, non-sporeforming, Gram-negative bacillus. This organism was previously known as a 'yellow pigmented *E. cloacae*' until 1980 when it was designated as a new species by Farmer *et al*. based on differences between *E. sakazakii* and *E. cloacae* in DNA-DNA hybridization, yellow pigment production, biochemical reactions, and antibiotic susceptibility. In a DNA-DNA hybridization study, Farmer and co-workers compared the DNA of a type strain of *E. sakazakii* with all the other strains previously known as *E. cloacae*. They found the type strain of *E. sakazakii* was only 31-49% related to strains of *E. cloacae*, but 83- 89% related to the other strains in this species. *E. sakazakii* was assigned to the *Enterobacter* genus because the type strain was only 41% related to *Citrobacter freundii* but 51% related to *E. cloacae* and also phenotypically closer to *E. cloacae* (Farmer *et al*., 1980).

In addition, two separate DNA hybridization groups which correlated with the presence or absence of yellow pigment were shown in strains of the species *Enterobacter* (Steigerwalt *et al*., 1976). The color of the yellow pigment produced by *E. sakazakii* is stronger at 25°C than at 36°C. Except for *E. agglomerans*, *E. sakazakii* is the only species of *Enterobacter* that may produce yellow pigments (Farmer and Kelly, 1992). In 1997, Brenner *et al*. showed that the pigmented strains could be

separated from non-pigmented strains on the basis of acid production from D-sorbitol and delayed production of DNAse on tluidine blue agar after 7 days of incubation at 36°C.

The biochemical differentiation of opportunistic *Enterobacter* species was shown in Table1 (Farmer and Kelly, 1992). *E. sakazakii* has a biochemical profile very similar to that of *E. cloacae*. Although sorbitol-fermenting *E. sakazakii* have been isolated recently (Heuvelink *et al*., 2001), the differences between these two species are that *E. sakazakii* is always D-sorbitol negative, positive for extracellular DNAse and produces yellow-pigmented colonies in general (Farmer and Kelly, 1992). Drudy *et al*. (2006) applied biochemical methods to characterize 51 environmental and food *E. sakazakii* isolates and six *E. sakazakii* type strains. According to the API 20E biochemical system (bioMerieux, Marcy l'Etoile, France), 75% of *E. sakazakii* isolates ferment inositol.

Enzymatic profiles of *E. sakazakii* and related species had also been conducted. Muytjens *et al*. (1984) studied 226 *Enterobacter* strains (containing 129 strains of *E. sakazakii)* were evaluated using the API^{TM} ZYM identification system (bioMerieux, Marcy l'Etoile, France), which is based on microbial enzyme profiles. These researchers found two major differences between *E. sakazakii* and the other *Enterobacter* species. Alpha-glucosidase was demonstrated in all strains of *E. sakazakii* but in none of the other *Enterobacter* strains, which included 19 strains of *E. aerogenes*, 60 strains of *E. cloacae* and 18 strains of *E. agglomerans*. The reproducibilities of the α-glucosidse reaction were estimated to be 89%. In another study done by Farmer *et al*. (1985), 53 of 57 strains of *E. sakazakii* were positive for

α-glucosidase activity. Not only was the presence of α-glucosidase, the absence of the enzyme phosphoamidase also unique to *E. sakazakii* isolates. In addition, Tween 80 esterase could also be a unique characteristic of E. sakazakii (Muytjens *et al*., 1984). Aldova *et al*. (1983) found that 71 of 73 strains of *E. sakazakii* contained such enzyme after 3-8 days. Postupa and Aldova (1984) also proposed that all six strains used in their study produced Tween 80 esterase after 7 days of incubation at 25°C and 37°C. Conclusively, among all the enzyme being studied, α-glucosidse is the most important one because its reaction can be used as a simple and rapid test to differentiate *E. sakazakii* form other *Enterobacter* species.

1.1.2 Morphology and Growth

Freshly isolated strains of *E. sakazakii* had two or more different morphological colony types when first streaked for purity (Farmer *et al*., 1980). One type of colony is dry or mucoid, with scallop edges, and rubbery when touched with a loop. The scallop-edged rubbery colonies reverted to typical smooth colonies on subculturing. It is not known at this time whether differences in virulence or any other phenotypic traits exist between these two colony types. All strains of *E. sakazakii* grew rapidly on trypticase soy agar at 36°C and form colonies of 2-3 mm in diameter after 24 h. At 25°C, the colonies are 1-1.5 mm at 24 h and 2-3 mm at 48 h (Farmer *et al*., 1980). These authors also found that after 24 h incubation in trypticase soy broth, all strains of *E. sakazakii* produced a large amount of sediment, which appeared to contain clumped cells and amorphous masses.

E. sakazakii can grow at a wide range of temperature and pH. Iversen *et al*.

Table 1. Biochemical differentiation of opportunistic *Enterobacter* species[a]

Test	Reaction[b]				
	E. sakazakii	*E. cloacae*	*E. aerogenes*	*E. agglomerans*	*E. gergoviae*
Lysine decarboxylase	-	-	+	-	+
Arginine dihydrolase	+	+	-	-	-
Ornithine decarboxylase	+	+	+	-	+
Growth in KCN	+	+	+	v	-
Fermentation of:					
sucrose	+	+	+	(+)	+
dulcitol	-	(-)	-	(-)	-
adonitol	-	(-)	+	-	-
raffinose	+	+	+	v	+
D-sorbitol	-	+	+	v	-
x-methyl-D-glucoside	+	(+)	-	-	-
D-arabitol	-	(-)	+	-	+
Yellow pigment	+	-	-	(+)	-

[a] Adapted from Farmer and Kelly, 1992.

[b] Where + represents 90-100% positive; (+) : 75-89% positive; v: 25-74% positive; (-): 10-24% positive; -: 0-9% positive

(2004) found this mocroorganism grew between 6 and 45°C in media including Tryptone soy broth (TSB), brain-heart infusion (BHI), Laural sulphate broth (LSB), brilliant green bile broth (BGBB), and infant formula (IF) with one strain being able to grow at 47°C but only in IF. However, Breeuwer *et al*. (2003) proposed that *E. sakazakii* can grow at 47°C in BHI. The optimum growth temperture of *E. sakazakii* was 37-43°C depending on the medium (Iversen *et al*., 2004). The lowest temperature for *E. sakazakii* to grow was in agreement with Nazarowec-White and Farber (1997b) who suggested that the lowest temperature of growth was 5.5°C for this microorganism. In other words, this microorganism has the potential to grow slowly during refregerated storage. The mean doubling time at 37°C in TSB, BHI, LSB, BGBB, and IF was 22 min ranging from 14 to 29 min. The range was less board (19-21 min) when infant formula was used. At 6°C and 21°C, the doubling time of *E. sakazakii* in infant formula were 13.7 and 1.7 h, respectively (Iversen *et al*., 2004). Skladak *et al* (1993) found ultra-high-temperature (UHT) milk cartons inoculated with 10-15 *E. sakazakii* cells/500 ml and incubated at 30°C supported good bacterial growth. The *E. sakazakii* strains can grow between pH 4.5 and 10 in BHI broth at 37°C (Breeuwer *et al*., 2003).

1.1.3 Environmental Sources and Reservoirs

The natural habitat and reservoir of *E. sakazakii* remain unknown (Iversen and Forsythe, 2003). Researchers have previously failed to isolate this organism from surface water, soil, mud, rotting wood, grain, bird dung, rodents, domestic animals, cattle and raw cow's milk (Muytjens and Kollee, 1990). However, this bacteria had

been isolated from many other sources including rice seeds, dry herbs and spices, milk powders, cheese products, baby foods, minced beef, sausage meat, fermented bread, tofu, sour tea, and vegetables (Cottyn *et al*., 2001; Iversen and Forsythe, 2003, 2004; Leclercq *et al*., 2002). In addition, the isolation of *E. sakazakii* from Mexican fruit flies and from the gut of stable fly larvae had been documented (Gakuya *et al*., 2001; Hamilton *et al*., 2003; Kuzina *et al*., 2001). *E. sakazakii* had also been found in kitchen blender, rinsed beer mug, UHT milk cartons, a dish brush, and a stirring spoon (Muytjens *et al*., 1983; Nazarowec-White *et al*., 1999; Schindler and Metz , 1990). Isolation from a wide range of hospital environment and clinical sources including cerebrospinal fluid, blood, bone marrow, sputum, urine, inflamed appendix tissue, intestinal and respiratory tracts, mouths of acute stroke patients, stomach aspirate, anal swab, vaginal tract , eye, ear, wounds stool, and feces had also been obtained (Adamson and Rodgers, 1981; Farmer *et al*., 1980; Gallagher and Ball, 1991; Gosney *et al*. 2006; Masaki *et al*., 2001; Gurtler *et al*., 2005; Ongrsdi, 2002; van Acker *et al*., 2001). In addition, *E. sakazakii* had been isolated from factories used to produce milk powder, chocolate, cereal, potato flour, spices, and pasta and household vacuum cleaners (Kandhai *et al*., 2004a), thus confirming its ubiquitous distribution. Of all the sources where *E. sakazakii* had been isolated, its presence in dried infant formula (DIF) for feeding newborn babies is of particular concerned (Clark *et al*., 1990; Himelright *et al*., 2002; van Acker *et al*., 2001). This is due to the correlation between neonate infections and infant formula (Iversen and Forsythe, 2003). The information regarding infant formula and illness associated with *E. sakazakii* would be introduced in detail later on (1.3.3).

1.1.4 Heat Resistance

Whether *E. sakazakii* is heat resistant or sensentive was controversial. Nazarowec-White and Farber (1997c) reported a *D*-value of 4.2 min at 58°C using batch methods. They concluded that *E. sakazakii* is one of the most thermotolerant members among Enterobacteriaceae found in dairy products because the *D*-value at 72°C for *E. sakazakii* was 1.3 s compared with 0.22 s, 0.16 s, and 0.07 s for *Salmonella typhimurium*, *Escherichia. coli*, and *Campylobacter jejuni*, respectively. In addition, Kindle *et al*. (1996) reported that the *E. sakazakii* viable count was reduced from 10^5 to 20 CFU/ml after exposure to microwaves (85 s, 82°C in Milumil infant formula); whereas no *E. coli*, *Pseudomonas aeruginosa* or poliovirus survived this treatment. However, Read *et al*. (1968) proposed that the heat resistance of *E. sakazakii* was similar to other Enterobacteriaceae such as *Salmonella* in rehydrated milk powder. Closer inspection of the data shows that *E. sakazakii* was not recoverable in other infant formulas with treatments from which other organisms did survive (Kindle *et al*., 1996). In addition, subsequent work (Nazarowec-White & Farber, 1999; Iversen *et al*., 2003) have clarified that the organism is less thermotolerant than *Listeria monocytogenes*. The reason for the difference in *D*-values is not clear, but could be that one of the strains in the pooled culture mixture used in the Nazarowec-White study was exceptionally heat resistant (Breeuwer *et al*., 2003).

The *D*- value and *z*-value of *E. sakazakii* had been evaluated by several researchers. The reported *D*-values at 58°C for *E. sakazakii* ranged from 0.4 to 591.9 min and the *z*-value for the organism ranged from 3.1°C to 5.8°C depending on the

different media and experimental methods used (Breeuwer *et al*., 2003; Edelson-Mammel and Buchanan 2004; Iversen *et al*., 2004, Nazarowec-White and Farber, 1997c). When pH 7.0 phosphate buffer was used (Breeuwer *et al*., 2003) with a cold shock after heat treatment, *D*-value range from 0.39 to 0.60 min. The highest *D*-value was reported by Edelson-Mammel and Buchanan (2004). The chosen strains of *E. sakazakii* in their study could be one reason, and the use of infant formula as a media, which could protect cells from being killed, could be another reason. The *D*-values at 54°C ranged from 10.2 (± 3.56) to 16.4 (± 0.67) min (Iversen *et al*., 2004). As the treatment temperature raised to 62°C, the *D*-values decreased to between 0.2 (± 0.13) and 0.4 (± 0.08) min. Although *D*-values and *z*-values varied depending on different experimental conditions, it is clear from all studies that *E. sakazakii* cannot survive a normal pasteurization process (Breeuwer*et al*., 2003).

1.1.5 Osmotic and Desiccation Resistance

E. sakazakii cells at stationary phase were found to be more resistant to osmotic and dry stress than *Escherichia coli*, *Salmonella* and other strains of Enterobacteriaceae tested (Breeuwer *et al*., 2003). The high tolerance to desiccation provides a competitive advantage for *E. sakazakii* in dry environments, as found in milk powder factories, and thereby increases the risk of post-pasteurization contamination of the finished product. High desiccation resistance also allowed *E. sakazakii* survive in dried infant formula even its water activity was as low as ca. 0.2 (Kempf and Bremer, 1998).

1.1.6 Antibiotic Resistance

The antibiotic resistance of *E. sakazakii* had been investigated in several studies. This microorganism is naturally resistant to all clindamycin, fosfomycin, fusidic acid, lincomycin, macrolides, rifampicin, and streptogramins (Stock and Wiedemann, 2002). Although the antibiotic resistance could very among strains, it has been reported that *E. sakazakii* were resistant to ampicillin, cefazolin, cefotaxamine, cephalothin, chloramphenicol, erythromycin, gentamicin, novobiocin, penicillin, sulphisoxazole, and tetracycline (Dennison and Morris, 2002; Kuzina *et al.*. 2001; Lai, 2001; Nazarowec-White and Farber, 1999). Burgos and Varella (2002) found genomic DNA from *E. sakazakii* contained multiple antibiotic resistance (mar) operons.

E. sakazakii infections have been traditionally treated with ampicillin-gentamicin or ampicillin-chloramphenicol (Lai, 2001). However, resistance to ampicillin has emerged owing to the acquisition of transferable elements and the production of β-lactamases (Girlich *et al*., 2001; Pitout, 1997). Beta-lactamases produced by *Enterobacter* species are also capable of inactivating broadspectrum penicillins and cephalosporins. This situation also appears to be increasing among isolates of *E. sakazakii*. To effectively treat *E. sakazakii* infections, Lai (2001) suggested considering the use of carbapenems or the newer cephalosporins in combination with a second agent, such as an aminoglycoside. Trimethoprirn-sulfamethoxazole may also be useful.

1.2 Current Isolation and Detection Techniques

1.2.1 U.S. FDA Recommended Method for Isolation and Identification

The U.S. Food and Drug Administration (U.S. FDA, 2002b) has a recommended method for the isolation and enumeration of *E. sakazakii* from dried infant formula (DIF). This method is based on the most probable number (MPN, detail would be introduced in 1.2.5) approach and is similar to that first used by Muytjens *et al*. (1988) except the FDA resuspend the DIF in water rather than buffered peptone water (BPW). Because of the low contamination levels typically found in DIF, sampling and sample size are critical for enumeration of *E. sakazakii*. Triplicate analysis of 100 g, 10 g, and 1 g test portions is recommended by the U.S. Food and Drug Administration (U.S. FDA, 2002b), while European Union regulation 2073/2005 (Anonymous, 2005) requires analysis of thirty 10 g test portions. The conventional steps starts with pre-enrich the DIF in nine parts of distilled water and incubate at 36°C overnight. Then, 10 ml of each suspension was removed to 90 ml EE broth for enrichment at 36°C overnight. Isolation using Violet Red Bile Glucose Agar (VRBGA) selective media were followed. Typical colonies will appear as purple colonies surrounded by a purple halo of precipitated bile acid after overnight incubation at 36°C. This protocol is selective for Enterobacteriaceae but not specific for *E. sakazakii*. Therefore, 5 presumptive *E. sakazakii* colonies are selected from VRBG and subcultured onto trptic soy agar (TSA) at 25°C for 48-72 h for yellow pigment production, followed by confirmation using API 20E biochemical identification system and oxidase test. This confirmation steps were carried out due to the reason that *E. sakazakii* is not the only species in Enterobacteriaceae that produces yellow pigment. *E. coli* and *E.*

agglomerans could also form yellow colonies, therefore had the possibility to cause false positive results (Edwards and Ewing, 1972; Farmer *et al*., 1980).

Other false results could also be obtained by using U.S. FDA recommended method. Since this method is not selective for *E. sakazakii,* it could be outgrown by other Enterobacteriaceae during the pre-enrichment and enrichment stages. Relatively few *E. sakazakii* colonies on VRBGA could cause a reduced chance of picking the organism onto TSA and therefore cause false negative results (Iversen and Forsythe, 2004). Conversely, use of yellow pigment as a defining characteristic can also result in false negatives due to the occurrence of non-pigmented *E. sakazakii* and the occasional transient nature of this trait (Iversen *et al*., 2006)

1.2.2 Enrichment Broth

Enterobacteriaceae enrichment (EE) broth and infant formula had been commonly used for *E. sakazakii* enrichment. This medium was composed of 10 g peptone, 5 g glucose, 6.45 g disodium hydrogen phosphate anhyd., 2 g potassium dihydrogen phosphate, 20 g Ox bile purified, 0.0135 g brilliant green per liter (Oxoid manual, 2007). Since infant formula was associated with several *E. sakazakii* outbreaks, it is also being used for enriching this bacterium.

Because *E. sakazakii* is a member of the Enterobacteriaceae and has physiological characteristics similar to those of *E. coli* O157:H7 (D-sorbitol fermentation negative, glucose and lactose fermentation positive, and absence of H_2S), enrichment broths recommended for *E. coli* O157:H7 may be appropriate for enrichment of *E. sakazakii*. Modified tryptone soya broth supplemented with novobiocin (mTSB + N) was

recommended by several organizations such as the International Standards Organization (ISO, 2001), U.S. Food and Drug Administration/Bacteriological Analytical Manual (FDA/BAM, 1998) and Public Health Laboratory Service (1998). The composition of this enrichment broth includes 17 g pancreatic digest of casein, 3 g papaic digest of soybean meal, 5 g sodium chloride, 4 g dipotassium hydrogen phosphate, 2.5 g glucose, 1.5 g bile salts and supplemented with 20 mg novobiocin per liter (Oxoid, 2007). FDA/BAM also suggests the use of buffered peptone water supplemented with vancomycin, cefixime and cefsulodin (BPW + VCC). This enrichment broth composed 10 g peptone, 5 g sodium chloride, 3.5 g disodium phosphate, and 1.5 g potassium dihydrogen phosphate supplemented with 7.2 mg of vancomycin, 0.045 mg of cefixime, and 9 mg of cefsulodin per liter (Oxoid, 2007). Modified *Escherichia coli* broth with reduced bile salts and novobiocin (mEC + N) is a USDA recommended enrichment medium, which is originally designed to enumerate coliforms in water and shellfish (Okrend and Rose, 1989). This broth contains 20 g tryptone, 5 g lactose, 1.12 g bile salts no. 3, 4 g di-potassium phosphate, 1.5 g mono-potassium phosphate, and 5 g sodium chloride supplemented with 20 mg of novobiocin per liter.

1.2.3 Plating Agar

Except for the time-consuming method using VRBGA together with TSA recommended by U.S. FDA, many researchers had worked hard to develop selective and differential media in order to substitute the procedures after enrichment. Several media were developed to shorten the detection time and also reduce the possibility of

false positive and false negative results. These newly developed media included OK media (Oh and Kang, 2004), Druggan-Forsythe-Iversen (DFI) media (Iversen *et al*., 2004), and Leuscher-Baird-Donald-Cox (LBDC) agar (Leuscher *et al*., 2004). The main concept of developing these media is based on the specific production of α -glucosidase by *E. sakazakii* strains (Farmer *et al*., 1985; Muytjens *et al*. 1984). All these improved methods enabled results to be obtained 2-3 days earlier than the FDA recommended method.

Oh and Kang (2004) developed a fluorogenic, differential, and selective medium, OK media. They used 4-methyl-umbelliferyl-α-D-glucoside as an indicator of the production of α-glucosidase. After 24 h of incubation at 37°C, this bacterium formed distinct, not easily diffusible, fluorogenic colonies clearly distinguishable from other non-target microorganisms under long-wave UV radiation. This medium is formulated as following: tryptone, 20.0 g; bile salts no. 3, 1.5 g; agar, 15.0 g; sodium thiosulfate, 1.0 g; ferric citrate, 1.0 g; and 4-methylumbelliferyl-α-D-glucoside, 50.0 mg per liter. Bile salts no. 3 selects for enteric bacteria. Sodium thiosulfate and ferric citrate were added as secondary selective markers for differentiation of H_2S-producing Enterobacteriaceae (*Citrobacter*, *Salmonella*, *Edwardsiella*, *and Proteus*). Incubation at 37°C for 24 h was recommended as the optimal growth condition to yield the highest fluorescent-to-total colony ratio. Further incubation (48 h) made it difficult to discriminate due to fluorescence diffusion. API 20E biochemical identification system and oxidase test were performed for confirmation of fluorescent colonies.

DFI media, developed by Iversen *et al*. (2004), added the indolyl substrate 5-bromo-4-chloro-3-indolyl-α,D-glucopyranoside (XαGlc) to a basal medium (TSA)

to differentiate *E. sakazakii* colonies from other members of the Enterobacteriaceae. Alpha-glucosidase produced by *E. sakazakii* hydrolyzed the substrate to an indigo pigment, producing blue-green colonies on this pale-yellow media. A major advantage of using the indolyl-substrates is the strong colors produced do not diffuse out from colonies and therefore even small positive colonies are visible in the presence of more numerous competitors (Iversen *et al*., 2004). Similar as OK media, a hydrogen sulphide indicator (sodium thiosulphate and ammonium iron citrate) was incorporated to differentiate weak α-glucosidase, H_2S positive organisms (such as *Proteus vulgaris*) from *E. sakazakii*. Sodium deoxycholate was the selective agent for Enterobacteriaceae. The composition of DFI media including 1 g sodium deoxycholate, 0.1 g 5-bromo-4-chloro-3-indolyl-α, D-glucopyranoside, 1 g sodium thiosulphate, and 1 g ammonium iron(III) citrate dissolved in 1 L distilled water before addition of the basal medium (TSA, 40 g). Inoculated plates were incubated at 37°C for 24 h before enumeration. Blue-green colonies were picked and confirmed by API 20 E biochemical identification system and oxidase test.

LBDC media was developed by Leuschner *et al*. (2004) for the presumptive detection of *E. sakazakii* in infant formula. Same as OK media, fluorogen 4-methyl-umbelliferyl-α-D-glucoside was used to differentiate between α-glucosidase producers and nonproducers. This is a nonselective media using nutrient agar as a basal media.

1.2.4 Other Detection Method

A detection method was developed based on two features of *E. sakazakii*: its

yellow pigmented colonies when grown on TSA and its constitutive α-glucosidase, which could be detected in a 4-h colorimetric assay (Kandhai *et al*., 2004b). Samples from environment of milk powder plants were analyzed with or without pre-enrichment in buffered peptone water followed by streaking onto violet red bile agar (VRBA). After incubated for 20-24 h at 37°C, colonies were streaked onto TSA and incubated for 24 h at 37°C or 48 h at room temperature (25°C). Yellow and oxidase negative colonies were picked for α-glucosidase detection. Paranitrophenyl-α-D-glucopyranoside was used as the substrate of α-glucosidase. The formation of the yellow colored paranitrophenyl (PNP) hydrolysate was measured using a Novaspec II spectrophotometer (Pharmacia Biotech, Cambridge, UK) at 405 nm. A minimal absorption of 0.3 at 405 nm after 4 h, equivalent to 16 μM PNP, was considered positive of α-glucosidase. None of the other coliforms were positive in the 4-h assay. After 24 h, the difference between *E. sakazakii* and the other coliforms was less clear.

1.2.5 MPN Methods

The MPN is the number which makes the observed outcome most probable. It has been applied for numerous rapid detection methods for quantitative analysis of *Salmonella* (Mohammad *et al*., 1986; Wells *et al*., 1991; Jetton *et al*., 1992; Whittemore 1993; Humbert *et al*., 1997) and *E. coli* (Bredie and Boer 1992; Chapman *et al*., 2001). The isolation and identification recommended for *E. sakazakii* by U.S. FDA (2002b) also involved the application of MPN. The theory of this method is to estimate the concentration of a target microbe in a sample by serial dilution tests. The

MPN is particularly useful for low concentrations of organisms (<100/g), especially in milk and water, and for those foods whose particulate matter may interfere with accurate colony counts. Only viable organisms would be enumerated by the MPN determination. To support the MPN method, it is assumed that the bacteria are distributed randomly within the sample. The bacteria are separate, not clustered together, and they do not repel each other. Every tube (or plate, etc.) whose inoculum contains even one viable organism will produce detectable growth or change. The individual tubes of the sample are independent (U.S. FDA, 2006).

The essence of the MPN method is to dilute the sample to such a degree that inocula in the tubes will sometimes but not always contain viable organisms (U.S. FDA, 2006). Then, the total number of tubes and the number of tubes with growth at each dilution can imply an estimate of the original, undiluted concentration of bacteria in the sample. In order to obtain estimates over a broad range of possible concentrations, microbiologists use serial dilutions incubating tubes at several dilutions. The viable cell count procedure can be improved by miniaturization (Fung, 2002). Rowe *et al*. (1977) reported miniaturization of MPN using 96-well microtiter plate, and Irwin *et al*. (2000) developed a modified Gause-Newton algorithm for calculating MPN using 96-well microtiter plates.

1.2.6 Molecular Based Detection Methods

As a means of improving the detection of *E. sakazakii*, molecular-based methods have been developed. Seo and Brackett (2005) described a quantitative real-time PCR technique in which primers and a TaqMan probe were developed to target the

macromolecular synthesis operon of *E. sakazakii*. The specificity of the assay was established using 68 *Enterobacter* and 55 non-*Enterobacter* strains. The method could detect 100 CFU/mL in reconstituted DIF without an enrichment step. More recently, Liu *et al*. (2005) developed two real-time PCR assays based on TaqMan and SYBR green technology. Both of these assays used primers that target the 16-23S rRNA spacer region and could detect 1.1 CFU of *E. sakazakii* per 100 g of infant formula after 25 h of enrichment.

1.3 Illness Associated with *E. sakazakii* by the Way of Infant Formula

1.3.1 Infant Formula Processing

To simulate breast milk, cow's milk is modified to reduce protein and mineral content, increase the amount of whey protein, the carbohydrate content, and the Ca/P ratio. The fat is modified and vitamins are added. The manufacture of dried infant formula can follow two procedures. In the 'dry procedure', skimmed milk is pasteurized and then spray dried before fat, whey, vitamins, emulsifiers, and stabilisers are added and blended. All ingredients are blended in the dry form. This method should be avoided, as it can lead to bacterial contamination (Lambert-Legace, 1982). In the second manufacturing method, dried infant formula is prepared using a 'wet procedure' where the following heat treatments are used: (1) liquid skim milk is heat treated before processing at 82°C for 20 s; (2) the pre-mix consisting of skimmed milk and fat components is heat treated at 80°C for 20 s; (3) the total mixture containing all ingredients is heat-treated at 107-110°C for 60 s; (4) the liquid mixture is concentrated using a falling film evaporator; (5) the concentrate is heat treated

again at 80°C and then immediately spray-dried. Often, these 'dry' and 'wet' procedures are combined where water soluble components are added to the milk before drying and the less soluble components are added to the blend after drying (Caric, 1993).

Pathogens can gain access from the environment, the addition of ingredients at the powder stage, and from post-processing contamination. The drying and filling are often the principle contamination site for dried products (Mettler, 1994). Therefore, care must be taken in the manufacture of infant formula to safeguard the microbiological quality of the finished product.

1.3.2 *E. sakazakii* in Infant Formula

As described in section 1.1.3, although the sources of *E. sakazakii* and its vehicles of transmission remain unclear. It had been reported that *E. sakazakii* infections had strong association with dried infant formula (DIF). Intrinsic and extrinsic contamination of DIF with *E. sakazakii* can occur. Intrinsic contamination results from the introduction of this organism to the DIF during the manufacturing process. In contrast, extrinsic contamination may result from the use of contaminated utensils, such as blenders and spoons, in the preparation of DIF (Noriega *et al*., 1990).

Unlike commercially available liquid feeds, dried infant formula is not sterile. In fact, the paper by Farmer *et al*. (1980) defining *E. sakazakii* species included a strain (NCTC 8155) which was originally isolated from an unopened can of dried milk by Thornley (1960). Therefore, *E. sakazakii* has been present in dried milk products for many decades including the period of the first meningitis case in 1958

(Nazarowec-White and Farber, 1997c) when the microorganism was still classified as the pigmented strain of *E. cloacae*. van Acker *et al*. (2001) also concluded that there was a strong relationship between intrinsic contamination of powdered milk formula with *E. sakazakii* and the development of necrotizing enterocolitis (NEC) from all the elements in their study.

Several investigations into the presence of *E. sakazakii* in DIF have been performed. Muytjens *et al*. (1988) examined 141 different powdered formulas from 35 countries. They isolated *E. sakazakii* at levels ranging from 0.36 to 66 CFU per 100 g from 20 formula samples from 13 countries. This level is similar to the value of 8 cells/100 g estimated by Simmons *et al*. (1989) for an open can of dried infant formula used during the time of an outbreak on an neonatal intensive care unit (NICU) in Memphis, Tennessee. *E. sakazakii* had also been isolated from 5 different lot numbers of unopened packages of DIF after an outbreak of neonatal meningitis in Iceland (Biering *et al*., 1989). A Canadian survey that investigated the incidence of *E. sakazakii* in DIF isolated the organism from 8 of 120 cans from 5 different manufacturers (Nazarowec-White and Farber, 1997c). Heuvelink *et al*. (2001) used a present/absence test for 25 g quantities and detected *E. sakazakii* in 1/40 dried infant formula powders and 7/170 milk powders. It has also been reported that four strains of *E. sakazakii* were cultured from dried milk and two strains were cultured from DIF in Czechoslovakia (Postupa and Aldova, 1984). This bacterium had also been isolated from ten samples (13.5%) from two Indonesian manufacturers (Estuningih *et al*., 2006). Thus, it has been clearly established that *E. sakazakii* can be found in dried infant formula and there was no particular part of the world associated with

contaminate or non-contaminated products.

E. sakazakii can grow at a temperature as low as 5.5°C and therefore potential growth of this organism could occurred during refrigerated storage. Its doubling time in infant formula at refrigeration temperature was about 13.7 h. Although the infectious dose of this organism is unknown, it is likely that with sufficient multiplication time, infections dose could be reached under refrigeration condition. Temperature abuse by repeating opening the door of the refrigerator could shorten the time of reaching infectious dose. At ambient and subambient temperatures, the growth rate of of the organism is about 75-100 min (Iversen *et al*., 2003, 2004) therefore allows the microorganism reaching infectious dose even faster. In a NICU, prolonged feeding time at ambient temperature and repeated use of enteral feed bags have been identified as risk factors (Oie and Kamiya, 2001).

1.3.3 Illness Associated with *E. sakazakii*

E. sakazakii is a rare, but important cause of life-threatening neonatal meningitis and has also been associated with bacteremia and necrotizing enterocolitis (Adamson and Rodgers, 1981; Farmer *et al*., 1980; Grimont *et al*., 1992; Jimenez and Gimenez, 1982; Joker *et al*., 1965; Kleiman *et al*., 1981; Monroe and Tift, 1979; Muytjens and Kollee, 1982; Muytjens *et al*., 1983; Nazarowec-White and Farber, 1997a; Urmenyi and Franklin, 1961). The first two cases of neonatal meningitis caused by *E. sakazakii* were reported in the United Kingdom in 1961 when both infants died (Urmenyi and Franklin, 1961). At that time, the microorganism was still considered a strain of *E. cloacea*. Subsequently, cases of *E. sakazakii* infections have been reported worldwide.

Although most documented cases involve infants, reports describe infections in adults as well.

The signs and symptoms of *E. sakazakii* meningitis at presentation, such as poor feeding, irritability, seizures, and a fever, are no different than meningitis caused by other Gram-negative organisms (Willis and Robinson, 1988). However, because of the aggressive behavior of this organism and propensity to infect the central nervous system (CNS) causing brain abscesses (Farmer *et al.*, 1980; Jimenez and Gimenez, 1982; Joker *et al.*, 1965), high mortality has been reported, ranging from 40 to 80% (Nazarowec-White and Farber, 1997a). Many of the infants died within days of infection (Adamson and Rogers, 1981; Gallagher and Ball, 1991; Joker *et al.*, 1965; Kleiman *et al.*, 198 1; Urmenyi and Franklin, 1961; Muytjens *et al.*, 1983; Willis and Robinson, 1988). Even if the patients can recover from the infection, survivors usually have severe neurological complications (Ries *et al.*, 1994). Nine of eleven patients reported by Lai (2001) with meningitis who survived the infection had follow-ups; all had hydrocephalus, some forms of developmental delay, and neurological sequelae. Premature infants are thought to be at greater risk than full-term infants, other children, or adults, and outbreaks of disease have occurred in hospital units for newborns (Arseni *et al.*, 1987; Bar-Oz *et al.*, 2001; Block *et al.*, 2002; Centers for Disease Control and Prevention, 2001; Lai, 2001; Muytjens *et al.*, 1983; Simmons, 1989; van Acker *et al.*, 2001).

Although a human vaginal tract culture yielding *E. sakazakii* has been reported, vertical transmission is unlikely because nearly half of infants with *E. sakazakii* disease in the review of Ongrsdi (2002) were delivered by cesarean section, and

symptoms developed in only one infant earlier than 3 days of age.

For the treatment of *E. sakazakii* meningitis, Willis and Robinson (1988) recommended the combined use of ampicillin and gentamicin. However, gentamicin may be unable to achieve adequate concentrations in the cerebral spinal fluid. Lai (2001) proposed the use of carbapenems or 3rd generation cephalosporins with an aminoglycoside or trimethoprim with sulfamethoxazole. This treatment regime has improved the outcome of *E. sakazakii* meningitis though the resistance of *Enterobacter* spp. to these antibiotics is increasing.

1.3.4 Novel Prevention Strategy

The use of commercial, sterilized liquid formula especially for high-risk infants can be a solution to this problem to avoid the intrinsic powder contaminants and the potential for extrinsic contamination at the time of rehydration. However, liquid formulas are generally more expensive and require larger transport and storage facilities (World Health Organization, 2004; Van Acker *et al.*, 2001).

When dried infant formula became the choice for feeding babies, several prevention strategies could be focused to prevent *E. sakazakii* infections. Initial levels of *E. sakazakii* could be controlled start from raw materials of dried infant formula. Although the source of such organism is uncertain, it is an environmental organism and therefore can be present in many raw ingredients as well as milk (Iversen and Forsythe, 2003). During the processing of DIF, heat treatment can reduce or eliminate *E. sakazakii* if they present in the raw milk or related ingredients. A 4-7D kill log reduction of microorganisms is required for process control in many thermal or

pasteurization regimes. According to the heat resistance of *E. sakazakii* calculated by Nazarowec-White and Farber (1997c), with a D_{60^oC} value of 2.5 min, heat treatment of 60°C for 17.5 min would be required to obtain a 7 log reduction. A minimum high temperature short time (HTST) pasteurization of 15 s at 71.7°C would result in >11 D kill of *E. sakazakii* in infant formula. In addition, Iversen *et al* (2004) predicted that the *D*-value at 71.2°C is 0.7 s by using decimal reduction time for the organism in infant formula at 60°C of 1.1 min and the *z*-value of 5.7°C. Therefore, the standard HTST pasteurization process (15 s at 71.7°C) will result in about a 21-log reduction of this organism. From theses two researches, it could be concluded that the organism should not survive the pasteurization process. Avoiding post-processing contamination can prevent the presence of this microorganism in the products. Drying and filling are often the principle contamination sites for dried products, which especially needed to be concerned (Iversen and Forsythe, 2003).

In addition, current application of microbiological criteria should be modified. The FAO's code of hygienic practice for foods for infants and children (Codex Alimentarus CAC/RCP 21-1979) requires a minimum of 4–5 samples with <3 coliforms/g and a maximum of 1/5 control samples with >3 but < 20 coliforms/g (Codex Alimentarius Commission, 2003). However, this criteria has not been exceeded by the numbers of *E. sakazakii* present in DIF including those associated with outbreaks reported by several researchers (Himelright *et al*., 2002; Muytjens *et al*., 1988, Nazarowec-White and Farber, 1997b, Simmons *et al*., 1989, van Acker *et al*., 2001). Therefore, these criteria must be reviewed and possibly more stringent statutes needed to be enforced (Iversen and Forsythe, 2003).

When dried infant formula comes to consumers, appropriate information and preparation instructions should be provided. Since dried infant formulas are not sterile products, they should not be fed to premature infants or infants who might have immune problems unless directed and supervised by pediatricians. Proper hygiene for preparation, use and storage are important. Once DIF was rehydrated, there is a risk of *E. sakazakii* growing over prolonged periods of storage and reaching an infectious dose for susceptible infants (Iversen and Forsythe, 2003).

Manufacturers of infant formula had made some suggestions for infant formula preparation and handling: 1) Wash hands thoroughly with soap and water before preparing formula; 2) Mix powder formula with cool water (2-24°C) or mix with warm water (37-38°C) but only if the formula was fed or refrigerated immidiatly; 3) Once infant formula was prepared, it should be fed immediately or covered and refrigerated (2-4°C) for no longer than 24 hours. If the prepared infant formula was not refrigerated, it should be fed within 2 h; 4) After feeding begins, infant formula should not be refrigerated and should be consumed within 1 h or discard; 5) Microwave heating method should not be applied to infant formula since serious burns may occur. Run warm tap water over the bottle or place the bottle in a pan of hot (not boiling) water is prefered if refrigerated formula needed to be warmed up. Warming time should be less than 15 minutes. In addition, once the bottle has been warmed, it should be used no more than 1 h or discarded. Prepared formula should not be rewarmed (warming it more than once), even if the bottle has not been fed to the baby. Rewarming formula could increase the possibility of bacterial growth in the formula as it cools off after heating (Anonymous, 2007). Prolonged feeding time at

ambient temperature and repeated use of enteral feed bags have been identified as risk factors in a NICU (Oie and Kamiya, 2001).

Recommendations other than those provided by manufacturers had also been proposed. All bottles, spoons and teats should be thoroughly cleaned and sterilized with boiling water or other suitable sanitizer between feeds. In the home settings, dried infant formula should be freshly prepared for each feed. Any milk remaining should be discarded rather than used in the following feed. Water should be brought to a rolling boil, then cooled for a few minutes to temperature of 70-90°C before reconstituting DIF. The reconstituted infant formula should be cool to body temperature before feeding and should not be kept in the heters or thermoses. In hospitals and other institutions, written guidelines should be established and their implementation should be monitored. Infant formula should be prepared by trainned personnel under aseptic technique in a designated preparation room. If infant formula needs to be prepared in advance, it should be prepared on a daily basis and kept at 4°C or below (Agostoni *et al*., 2004; Centers for Disease Control and Prevention, 2002; International food safety authorities network, 2005; U.S. FDA, 2002a).

CHAPTER TWO

MATERIALS AND METHODS

2.1. Bacterial Strains, Stock Cultures, and Culture Methods

Sixty-eight strains of *Enterobacter sakazakii* and 16 other bacterial isolates including *E. aerogenes*, *E. cloacae, Escherchia coli*, *E. coli* O157:H7, *Klebsiella pneunoniae*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *S. typhimurium* obtained from American Type Culture Collection (ATCC, Manassas, VA), U. S. Food and Drug Administration (U.S. FDA, Rockville, MD) or the Food Science and Human Nutrition culture collection at Washington State University (Pullman, WA) were used. Each of the bacterial strains was maintained in tryptic soy broth (TSB; Difco, Franklin Lakes, NJ) containing 15% glycerol at -20°C as frozen stock. For routine use, 10 μl of frozen stock were transferred into 9 ml fresh TSB using sterile disposable loops and maintained at 4°C as refrigerated stock after incubated at 37°C for 24 h.

2.2. Comparison of Enrichment Broth

2.2.1. Growth curves comparison

Four strains of *E. sakazakii* (ATCC 12868, ATCC 29004, ATCC29544, and ATCC 51329) were used. They were cultured separately in TSB at 37°C for 24 h. Each cell culture was transferred again to 9 ml fresh TSB and incubated at 37°C for another 24 h before use. These four cultures were then combined to a culture cocktail, centrifuged at 4,000 × g for 30 min and washed three times with 0.2% peptone water (Bacto, Franklin Lakes, NJ). The final pellets were resuspended in 0.2% peptone

water to approximately 10^4-10^5 CFU/ml.

Reconstituted dried infant formula (DIF) and four enrichment broths were used. Cans of commercial DIF fortified with iron for babies first year (Enfamil, Mead Johnson & Company Evansville, IN) were purchased from local grocery store and reconstituted according to instructions of manufacturer. The four enrichment broths included Enterobacteriaceae enrichment broth (EE broth; Oxoid, Cambridge, UK), modified tryptone soya broth supplemented with novobiocin (mTSB + N; Oxoid, Cambridge, UK), modified *Escherichia coli* broth with novobiocin (mEC + N; Oxoid, Cambridge, UK), and buffered peptone water supplemented with vancomycin, cefixime, and cefsulodin (BPW + VCC; Oxoid, Cambridge, UK). EE broth, mTSB, mEC, and BPW were prepared following manufacturers' directions. One vial of novobiocin selective supplement was added to either 500 ml mTSB or mEC after autoclaving to a final concentration of 20 mg/ml. BPW + VCC was prepared by adding 1 vial of VCC selective supplements into 225 ml BPW to a final concentration of 8 mg/L vancomycin, 0.05 mg/L cefixime, and 10 mg/L cefsulodin.

The growth patterns of *E. sakazakii* culture cocktail were compared in three sets of selected media. First, reconstituted DIF and four enrichment broths were evaluated. Subsequently, the four enrichment broths were supplemented with 10% reconstituted DIF as a nutrients source and compared with reconstituted DIF. Last, either 20 mg/l novobiocin or VCC (8 mg/l VCC, 0.05 mg/l cefixime, and 10 mg/l cefsulodin) was added to reconstituted DIF and were evaluated.

Ten microliters of *E. sakazakii* culture cocktail was inoculated into each media being evaluated to a concentration of 10-100 CFU/ml. the inoculated media were

incubated at 37°C. Samples were taken at 2, 4, 6, and 8 hr and 10-fold serially diluted. Appropriate diluents were surface plated onto tryptic soy agar (TSA; Difco, Franklin Lakes, NJ). Growth patterns for different enrichment media were compared after 24 h of incubation at 37°C.

2.2.2. Recovery Evaluation

A recovery test was carried out to evaluate the recovery ability of *E. sakazakii* in reconstituted DIF with or without antibiotics and four selected enrichment broths when other bacterial (see 2.1) were exist as background microflora. Culture cocktail was prepared as previously described (2.2.1) except the addition of background isolates. The culture cocktail were then inoculated into reconstituted DIF followed a U.S. FDA recommended method (2002b) to a level of 10-100 CFU/ml and incubated at 37°C for 24 h. After incubation, 10 ml of inoculated reconstituted DIF was transferred into 90 ml of each enrichment media and incubated at 37°C for additional 18 h. Samples were taken at 6, and 18 h during incubation, 10-fold serially diluted, and appropriate diluents were surface plated onto OK media. The plates were incubated at 37°C for 24 h. Typical fluorescent colonies were classified as presumptive positive. API 20E biochemical system (bioMérieux Marcy 1'Etoile, France) were used for confirmation according to the instruction of manufacturer. For positive identification of *E. sakazakii*, oxidase test was also conducted by using oxidase identification sticks (Oxoid, Cambridge, UK).

2.3. Effective Method for Quantitative Analysis

The correlation between the conventional plating and the microtiter MPN methods was conducted. Culture cocktail containing four strains of *E. sakazakii* and 16 strains of background microorganisms were prepared as previously described (2.2.2) and was inoculated into 0.2% peptone water (total of 25 samples) and reconstituted DIF (total of 30 samples) with different microbial loads ranged from 10^2-10^5. For conventional plating methods, serial 10-fold dilutions were made and appropriate diluents were surface plated on to OK media (agar). Cell numbers were enumerated after incubation for 24 h at 37°C. The fluorescent colonies were isolated and confirmed by using API 20E biochemical identification system and oxidase test. For microtiter MPN method, 10-fold serial dilutions up to 10^{-3} were made for each microbial load. Twenty-microliter of each diluent was transferred to 10 different wells containing 180 µl OK media (broth) in a 96-well microtiter plate (Greiner Bio-One, Frickenhausen, Germany) by using a multichannel pipettor equipped with 5 tips twice and well mixed before incubated at 37°C for 10 h before determination. A 10-tube MPN method was used to increase the detection limit. Fluorescent wells were enumerated by using a transilluminator (model VWR LM-20E, VWR Scientific, Bridgeport, NJ) under 365 nm wavelength (Figure 5). For confirmation of presumptive strains of *E. sakazakii*, each well was 10-fold serially diluted and appropriate diluents were surface plated onto OK media (agar). The fluorescent colonies were isolated and confirmed by using API 20E biochemical identification system and oxidase test.

For MPN computation, the method described in the Bacteriological Analytical

Manual (U.S. FDA, 2001) was used and calculated of 95% confidence intervals. An Excel spreadsheet for the 10-tube method (each 10^{-1}, 10^{-2}, and 10^{-3} dilution) was downloaded and used to compute the MPN. Since only 200-µl reaction volume was used, the numbers were multiplied by a factor of 5 to obtain the MPN in expression of 1 ml. Correlation coefficients and linear regression equation trend lines were calculated by using Microsoft Excel XP (Microsoft, Redmond, WA).

The growth curves obtained by the conventional plating method and microtiter MPN were also compared over 5 h of incubation. The culture cocktail was inoculated into 1 L of reconstituted DIF to a final microbial load of 10-100 CFU/ml. Samples were taken every hour during incubation at 37°C and enumerated by plating onto OK media or using microtiter MPN methods as previously described. The cell counts obtained from the two methods were compared.

2.4. Heat Resistance

2.4.1. Heat Resistance Screening

Heat resistances of 68 strains of *E. sakazakii* were evaluated at 55°C. Each strain was transferred in TSB and incubated at 37°C for 24 h twice before centrifuging at 4000 × g for 30 min, and resuspended with pH 7.0 phosphate buffer (PB) to original volume (cell concentration: 10^8-10^9 CFU/ml). One ml of cell suspension of each strain was inoculated in 24 ml preheated (55°C) PB in a temperature controlled water bath where the water level was maintained at least 3 cm above the sample level in tubes throughout the treatment. After heat treated for 20 min, tubes were removed from the hot water bath into ice water bath immediately and shook manually to terminate the

heating effect. Samples were 10-fold serially diluted and appropriate diluents were surface plated onto TSA. Cell numbers were enumerated after 24 hr of incubation at 37°C. Log reduction of 68 strains of *E. sakazakii* were calculated and compared.

2.4.2. Survival curves of heat resistance/heat sensitive strains

From heat resistance screening results, two heat resistant strains (FSM 293 and 2.45) and two heat sensitive strains (FSM 271 and ATCC 12868) were used to evaluate their survival curves at different temperatures. The evaluation was conducted in either PB or reconstituted DIF. Each strain was cultured in TSB at 37°C for 24 hr twice, centrifuged at 4000 × g for 30 min, and resuspended with either PB or reconstituted DIF to original volume with cell suspension concentration of 10^8-10^9 CFU/ml. Twenty-four ml of either PB or reconstituted DIF were preheated and equilibrated at 50°C, 55°C, and 60°C in a temperature controlled water bath where the water level was maintained at least 3 cm above the sample tubes throughout the whole treatment. One ml of cell suspension of each strain was inoculated in each 24 ml preheated PB or reconstituted DIF. Two ml of samples were taken at 1 h, 2 h, and 3 h from 50°C treated cell suspensions, 10 min, 20 min, and 30min from 55°C treated samples, and 1 min, 2 min, and 3 min from 60°C treated cell suspensions. The samples took from each treatment was immediately placed in sterile tubes in ice water bath to terminate the heating effect. Ten-fold serial dilutions were made and appropriate diluents were surface plated onto appropriate media. For enumeration of total cell numbers including healthy and injured cells, TSA was used. After 3 h of incubation at 37°C, which allowed recovery of injured cells, the plates were overlaid

with OK media to further select and differentiate *E. sakazakii* from background microorganisms in DIF. Additional 21-h incubation was required before enumeration. For healthy cells counts, OK media was used with incubation at 37°C for 24 h prior to enumeration. For each treatment, the $\log_{10}$ CFU/ml was plotted as a function of time.

2.5. Guideline for Dried Infant Formula Preparation and Handling

The survival curves of the four strains of *E. sakazakii* (same as heat resistance experiment) in artificially inoculated DIF were evaluated during reconstitution and storage. Two heat resistance strains (FSM 293 and 2.45) and two heat sensitive strains (FSM 271 and ATCC 12868) were used. Each strain was cultured in TSB at 37°C for 24 hr twice, centrifuged at 4000 × g for 30 min, and resuspended with pH 7.0 phosphate buffer to 2.5 × concentration of original cell suspension. Approximate 1 ml of each concentrated cell suspension was spray-inoculated onto 100 g DIF placed on paper plate by using cosmetic spryer. Inoculated DIF was collected and sealed into sterile stomach bags after air-dried for 1 h and well-mixed (10^4 – 10^6 CFU/ml after reconstitution following the guideline labeled by the manufacturer, varied among strains). In this study, sterile water with different temperatures (room temperature, 50°C, 70°C, 80°C, and 90°C) were used to reconstitute either one-serving (8.5 g DIF with 60 ml water) or two-serving (17 g DIF with 120 ml water) infant formula. The prepared infant formula were then all stored at room temperature for 10 h. Two ml of each sample were taken at 0.1, 2, 5, and 10 h. At time 0.1 h, samples were taken into sterile tubes placed in ice water bath to terminate the heating effect. Samples from each treatment were 10-fold serially diluted and appropriate diluents were surface

plated onto TSA, incubated at 37°C for 3 h prior to be overlaid with OK media as a selective and differential media. Plates were incubated at 37°C for additional 21 h before enumeration. Cell numbers (log_{10} CFU/ml) were plotted as a function of time (min).

2.6. Statistic Analysis

Triplicate data for each experiment were statistically analyzed with a completely randomized two-way ANOVA test using Minitab® student version 12 (Minitab Inc., State College, PA). Fisher's pairwise comparison with a type I error of 5% was used to assess significant differences of means.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1. Comparison of Enrichment Broth

Four enrichment broths including EE broth, mTSB + N, mEC + N, and BPW + VCC, along with reconstituted DIF were compared for the ability for enriching the growth of *E. sakazakii*. Growth curves at 37°C were monitored for a total of 8 h with samples enumeration every 2 h (Figure 1A). Among all media tested, EE broth provided the best enrichment condition, which allowed a rapid increase in cell count of *E. sakazakii*. The cell count increased 2.4 $\log_{10}$ CFU/ml after 2h enrichment and achieved to 6.2 $\log_{10}$ CFU/ml after 8 h with an initial cell count of 2.0 $\log_{10}$ CFU/ml. Although not as effective as EE broth, reconstituted DIF could also support the growth of *E. sakazakii*. The cell count in reconstituted DIF increased more obvious after 4 h (2.3 $\log_{10}$ CFU/ml) and reached an increase of 3.1 $\log_{10}$ CFU/ml after 8 h. Pagotto *et al*. (2003) reported when the bottles of reconstituted DIF containing *E. sakazakii* were stored at room temperature, levels of $>10^7$ CFU/ml were easily obtained in 10 h and potentially hazardous levels of *E. sakazakii* could be reached even sooner in formula held at 35°C to 37°C. The other three broths used in this study failed to support the growth for *E. sakazakii*. Although mEC + N had better support than Infant formula after 2 h of incubation, the cell count decreased continuously after 4 h till the end of monitoring period. The number of *E. sakazakii* increased less than 1 log after 8 h of incubation, which is similar to mTSB + N. BPW + VCC provided the worst growing condition for *E. sakazakii*. The cell number kept decreasing after

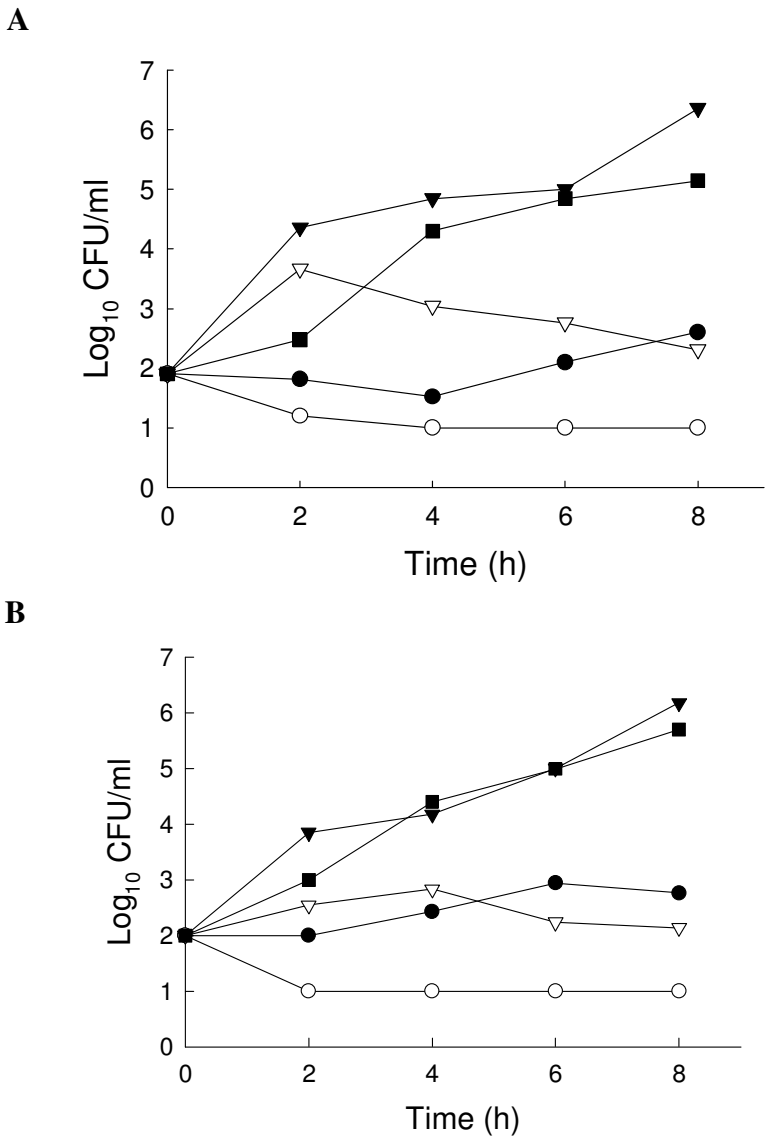

Figure 1. Growth patterns of culture cocktail containing four strains of *E. sakazakii* and 16 other isolates of Enterobacteriacea in different enrichment broths at 37°C. (A) without and (B) with 10% infant formula as a nutrient source. ●: modified tryptic soya broth supplemented with novobiocin, ○: buffered peptone water supplemented with vancomycin, cefixime and cefsulodin, ▼: Enterobacteriaceae enrichment broth, ▽: *modified Escherichia coli* broth supplemented with novobiocin, ■: infant formula.

inoculation and fell to an undetectable level (detection limit: 10 CFU/ml) before 4 h of incubation. This result is in agreement with Hepburn *et al*. (2002), who clearly indicate the poor performance of BPW + VCC, mTSB + N, and EC + N broths for *E. coli* O157:H7.

Since reconstituted DIF had associated with outbreaks of *E. sakazakii* infection, it was added to the four selected enrichment broths as a nutrient source to better understand its effect of recovering the cells (Figure 1B). When 10% (v/v) of infant formula was supplemented into the four selected media, EE broth still had the best enriching ability among the five. However, with addition of 10% reconstituted DIF, the enriching ability of EE broth slightly decreased and made it with no significant (p<0.05) difference with infant formula. The other three broths still failed to enrich *E. sakazakii*. The cell count increased less than 1 $\log_{10}$ CFU/ml in mTSB + N and mEC + N. The number of *E. sakazakii* decreased to undetectable level (detection limit: 10 CFU/ml) within 2 h in BPW + VCC. Overall, the supplementation of 10% infant formula did not help the enriching ability of all four broths for *E. sakazakii*.

Since infant formula is not a sterile product and could contain certain level of background microflora, the addition of antibiotics (VCC or novobiocin) is reasonable for enrichment. Evaluation of infant formula supplemented with or without VCC or novobiocin was carried out consequently. Enrichment broths with either novobiocin or VCC can definitely suppress the growth of *E. sakazakii* (Figure 2). Especially, when VCC was supplemented, it can not only repress the growth of *E. sakazakii* but also kill the cells to an undetectable level (detection limit: 10 CFU/ml) within 2 h.

When background microorganisms were present, only EE broth and reconstituted DIF

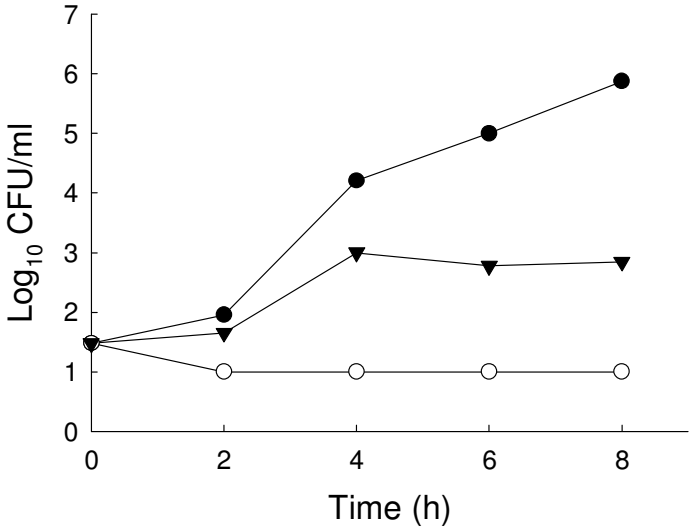

Figure 2. Growth patterns of culture cocktail containing four strains of *E. sakazakii* and 16 other isolates of Enterobacteriacea in reconstituted DIF with or without novobiocin or vancomycin, cefixime and cefsulodin at 37°C. ●: Reconstituted dried infant formula, ○: Reconstituted dried infant formula with vancomycin, cefixime, and cefsulodin, ▼: Reconstituted dried infant formula with novobiocin

could support and overgrown *E. sakazakii* cells. When EE broth was used, 100% (4/4, 4/4) of recovery was observed at both sampling time (Table 2). One sample of *E. sakazakii* failed to be recovered in reconstituted DIF at 6 h. However, with prolonged incubation time, all four samples could be recovered in reconstituted DIF after 18 h. For other enrichment broths or infant formula, as long as novobiocin or VCC were added, *E. sakazakii* could not successfully recover at either 6 h or 18 h.

Addition of bile salts in enrichment broths could affect the performance for enriching *E. sakazakii*. Bile salts no. 3 formulated in mTSB + N and mEC + N could inhibit small numbers of Enterobacteriaceae cells (Oxoid Manual, 2007). The broth with reduced bile salts (1.12 g/l) showed better recovery of *E. coli* O157 than EC + N (containing 1.5g/l of bile salts). Although the concentration of bile salts in mEC + N was reduced to 1.12 g/l, inferiority of supporting cell growth remained. In a food study, Ogden *et al*. (2001) showed that enrichment media with bile salts compared unfavorably with BPW enrichments for the recovery of stressed *E. coli* O157. Instead of bile salts no. 3, EE broth was formulated with purified ox bile. This formulation can overcome the unsatisfactory effects of inhibition on Enterobacteriaceae cells and a preliminary assay could be used to check growth by inoculating approximately one viable cell per medium unit (Mossel *et al*., 1974; Richard, 1982).

Besides bile salts no. 3, the addition of novobiocin could also be the reason of the poor enriching ability although it had been reported that most Enterobacteriaceae are resistant to novobiocin (De Smedt *et al*., 1986; Devenish *et al*., 1986; Restaino *et al*., 1977; Tate *et al*., 1990). With addition of novobiocin in enrichment broths, *Salmonella* could be successfully enriched therefore improved the isolation from fecal

Table 2. The recovery ability of enrichment broths for *E. sakazakii* in artificially inoculated infant formula at 6 h and 18 h

Enrichment broth inoculated with 10-100 CFU/ml of Four strains of *E. sakazakii* and 16 other strains of isolates	No. of positive samples / no. of total samples	
	6 h	18 h
Modified tryptone soya broth with novobiocin	0[a] /4	0/4
Buffered peptone water with vancomycin, cefixime, and cefsulodin	0/4	0/4
Enterobacteriaceae enrichment broth	4/4	4/4
modified *Escherichia coli* broth with novobiocin	0/4	0/4
Infant formula	3/4	4/4
Infant formula with vancomycin, cefixime, and cefsulodin	0/4	0/4
Infant formula with novobiocin	0/4	0/4

[a] Petri dishes having fluorescent colonies on OK medium were scored as presumptive positive and confirmed by API 20E biochemical identification systems.

samples (Zhang *et al*., 2006). Jensen *et al*. (2003) proposed that addition of Novobiocin in BPW on average increased the level of *Salmonella* 1.2 log dilution concentrations compared to the conventional non-selective BPW method. The increase of *Salmonella* in the study may be caused by a reduction in the number of competitive microorganisms. However, several reports indicated that enrichment broths with novobiocin fail to recover other members of Enterobacteriaceae such as *E. coli* (Vimont *et al*. 2006). Asakura *et al*. (1998) indicated that the enrichment broths without selective ingredients (such as novobiocin) were more effective than those in which the selective ingredients were added. They strongly suggest not adding novobiocin to the enrichment broths for the detection of *E. coli* O157:H7 and non-O157:H7 STEC from food.

Another antibiotic, VCC, used in this study could also be another undesirable inhibitor for enriching *E. sakazakii*. Its inhibition was stronger than novobiocin (Figure 2). When VCC was added to the enrichment broth, no matter BPW or reconstituted DIF, enriching ability for *E. sakazakii* was dramatically repressed in all trials. Media with the full supplement of vancomycin, cefixime and cefsulodin, also gave a significantly inferior recovery for *E. coli* O157 (Hepburn *et al.,* 2002). Foster *et al*. (2003) demonstrated that BPW without antibiotics was the superior pre-enrichment medium for the isolation of *E. coli* O157:H7. Odgen *et al*. (2001) also reported a better recovery of *E. coli* O157 when the concentration of VCC was lower. Therefore, VCC is thought to inhibit growth of Enterobacteriaceae including *E. sakazakii* and *E. coli* O157.

The main purpose of adding antibiotics in enrichment broth was to kill the

background microflora so that the target microorganisms could outgrow and be detected easier. However, depending on the different characteristics of target cells, the addition of antibiotics could have equal or stronger inhibition to target cells compared to background microflora. Target cells could be more sensitive to antibiotics when they had been stressed during processing like desiccation of infant formula. Sub-lethally damaged cells surviving in DIF can be sensitive to antimicrobial additions used to inhibit non-target bacteria.

Reducing the concentration of antibiotics could increase the enriching ability for target microorganisms. Bolton *et al.* (1995) recognized that the cefixime and cefsulodin supplements in the enrichment step were potentially inhibitory to some *E. coli* O157:H7 and tested a range of reduced concentrations, resulting in an optimum BPW enrichment containing one quarter of the normal antimicrobial strength. Comparison of the ability of 40 strains of *E. coli* O157:H7 to grow in EE broth with various levels of cefixime revealed that cefixime at 0.05 mg/l was inhibitory to outgrowth of most strains in this medium. Lowering cefixime to 0.0125 mgrl allowed uninhibited growth for 38 of 40 strains tested (Weagant, 2001).

3.2. Effective Method for Quantitative Analysis

For microbiological testing of food, effective methods that required less time for media preparation and incubation or generated less laboratory waste are of concerned. The MPN assay had been widely applied as rapid methods for quantitative analysis of *Salmonella* (Mohammad *et al*. 1986; Wells *et al*. 1991; Jetton *et al*. 1992; Whittemore 1993; Humbert *et al*. 1997) and *E. coli* (Bredie and Boer 1992; Chapman *et al*. 2001).

This method could be miniaturized using 96-well microtiter plate (Rowe *et al*., 1977). In addition, Muytjens *et al*. (1983) reported that detection of α-glucosidase allows rapid and reliable differentiation between *E. sakazakii* and other *Enterobacter* species. OK media, a selective and differential agar medium, was developed based on detecting the unique enzyme, α-glucosidase, produced by *E. sakazakii* (Oh and Kang, 2004). The formula included 4-methylumbelliferyl-α-D-glucopyranoside as a substrate of α-glucosidase to produce a fluorogenic indicator. Since bacteria could grow faster in broth media compared to solid media, this new method was proposed by incorporating microtiter MPN with OK media in broth form. Fluorescent wells under long-wave ultraviolet irradiation indicated the presence of *E. sakazakii* in such wells.

Microtiter MPN assay in this study was conducted by using 10 wells as a substitute for 10 tubes to increase the detection limit (3 tube method: <3.0, 5 tube method: <1.8, 10 tube method: <0.9 in using 0.1 g, 0.01 g, 0.001 g at 95% confidence intervals). Ten-hour incubation time was same as the study of Gray *et al*. (2002) for determination of generic *E. coli*. Although they used different fluorogenic substrate (4-methylumbelliferyl-β-D-glucuronide), same moieties (4-methylumbelliferyl) were released in both experiments by enzymatic reaction. Not only the study of Gray *et al*. (2002), fluorogenic assay for rapid detection of *E. coli* in food had also been applied by using 4-methylumbelliferyl-β-D-glucuronide (Moberg, 1985) or 4-methylumbelliferone glucuronide (Feng and Hartman, 1982) incorporated into lauryl tryptose broth. Similar as the α-glucosidase produced by *E. sakazakii*, the β-glucuronidase produced by *E. coli* could cleave those substrates to yield a

fluorescent end product. Paulsen *et al.* (2006) also used an automated MPN system to detect *E. coli* in minced meat and on carcass surface samples by the formation of fluorescent 4-methylumbilliferone.

Two samples were shown in Figure 3. The positive well numbers of these two samples were 10, 10, 5 and 10, 7, 0. These numbers could be converted to MPN values of 335.58 and 80.68. Because only 200 µl was used in each well, the values obtained needed to be multiplied by 5 to represent the MPN in 1 ml of the original sample. Thus, the cell numbers of *E. sakazakii* would be 1678 and 403 MPN per milliliter.

The correlations between the conventional plate counts and microtiter MPN with different microbial loads in 0.2% peptone water (R^2 = 0.94) or infant formula (R^2 = 0.91) were highly agreeable (Figure 4). A high correlation (R^2 = 0.99) between the automated fluorescent MPN system and the colony count methodology were also reported for the total aerobic count for carcass surfaces and minced meat in the study of Paulsen *et al*. (2006). The confirmation results from the fluorescent negative wells gave no colonies on the plates. In the contrary, presumptive *E. sakazakii* colonies were observed on those plates spread with fluorescent suspensions. All presumptive colonies were further confirmed as *E. sakazakii* by API 20E biochemical identification systems followed by oxidase tests. Conclusively, the fluorescent generation from *E. sakazakii* did not interfered by background microorganisms.

The growth pattern of *E. sakazakii* in Reconstitued DIF monitored by the conventional plating method and the microtiter MPN method were shown in Figure 5.

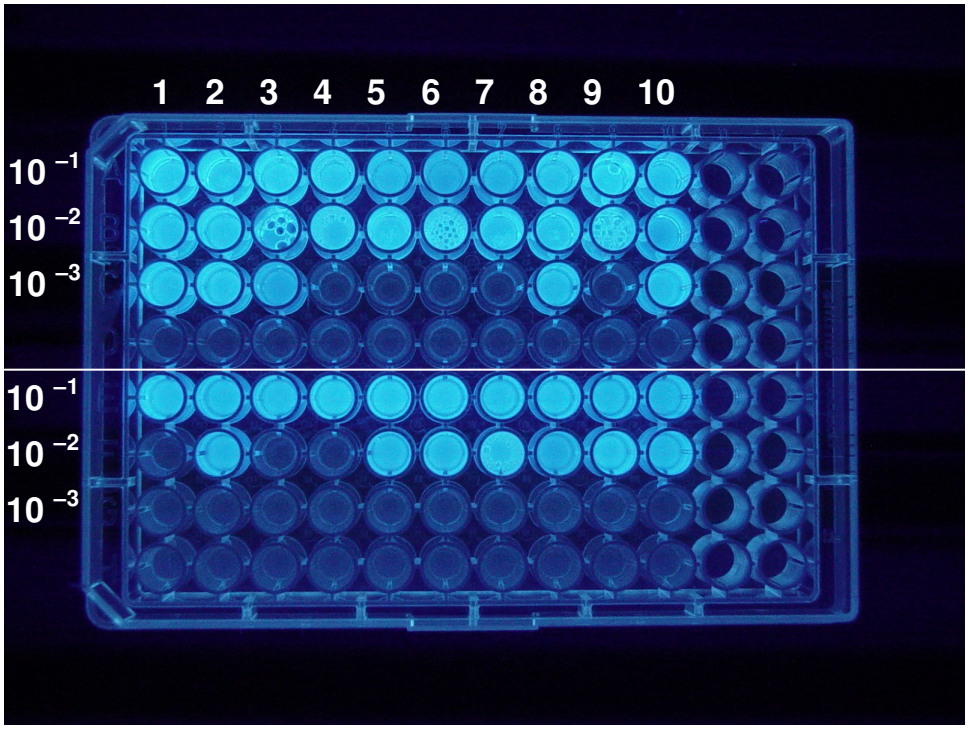

Figure 3. Ten-tube microtiter MPN assay with OK media (broth) for *E. sakazakii*. Two samples were enumerated on 96-well microtiter plate. Sample diluents (10^{-1}, 10^{-2}, and 10^{-3}) were inoculated in the plates. The numbers of positive wells were 10, 10, 5 and 10, 7, 0 for the two samples.

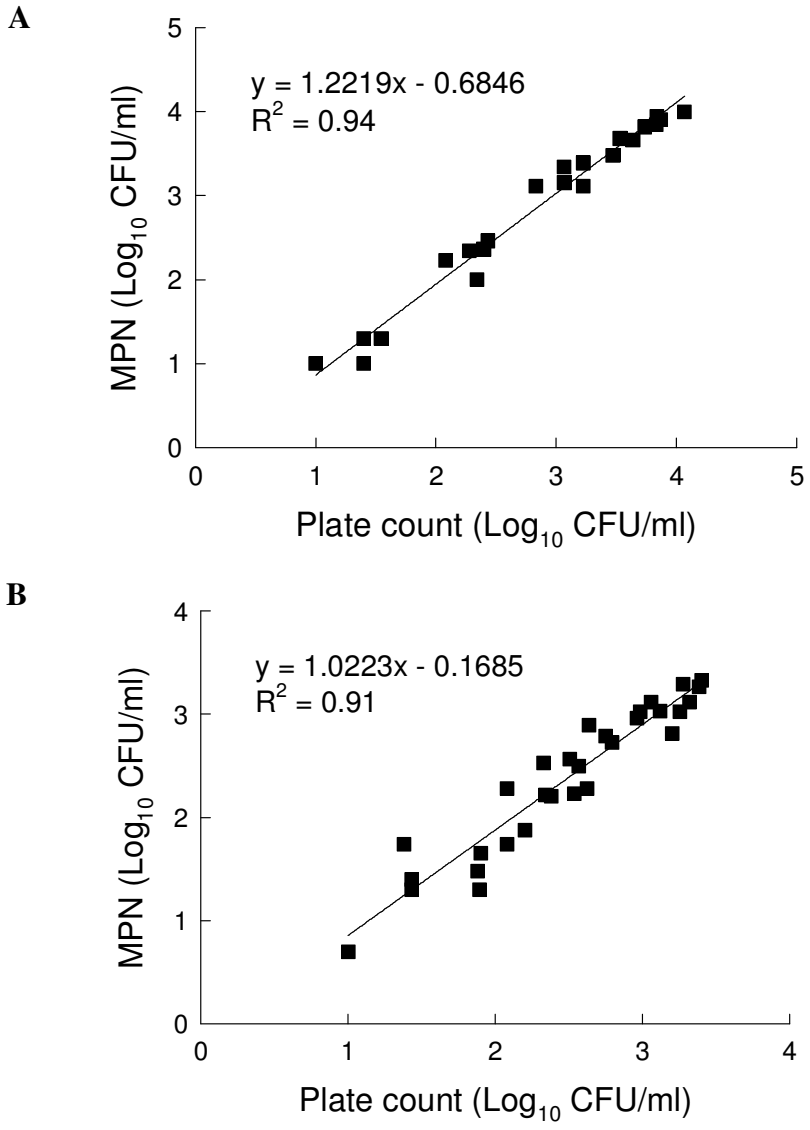

Figure 4. Correlation between the microtiter MPN method and conventional plating method for enumeration of *E. sakazakii in* (A) 0.2% peptone water and (B) infant formula inoculated with mixed culture cocktail including *E. sakazakii*.

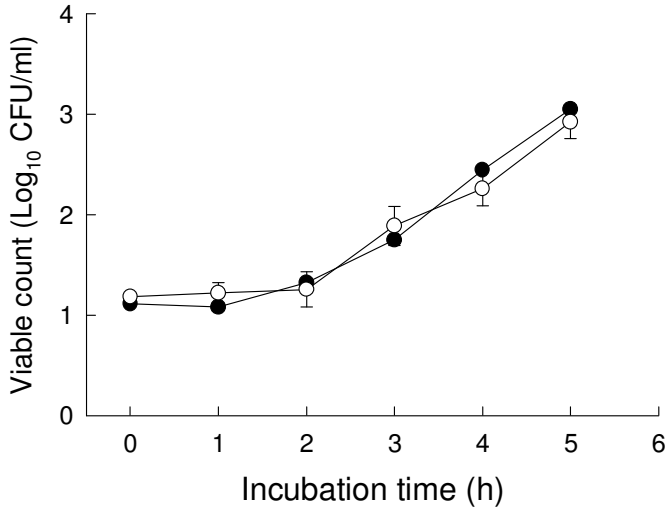

Figure 5. Growth curve comparison between the microtiter MPN method and conventional plating method for enumeration of *E. sakazakii* in artificially inoculated infant formula. ○: Microtiter MPN method, ●: conventional plating method.

The cell count obtained by the two methods were not significantly (p<0.05) different during the whole monitoring period.

The microtiter MPN method can be applied during or after enrichment steps to enumerate viable count. This rapid method has a big advantage over conventional plating methods because the growth of microorganisms is much faster in liquid medium than on solid medium. When using FDA recommended method, it requires at least 72 h culturing time using VRBG followed by TSA after enrichment (U.S. Food and Drug Administration, 2002b). Even using other newly developed fluorogenic or chromogenic plating media such as OK media (Oh and Kang, 2004) or DFI media (Iversen *et al.*, 2004) 24 h of incubation time was still required after enrichment. However, the detection time of microtiter MPN could be shorten to equal or less than 10 h, which could save at least 14 h of incubation time compared to those conventional plating methods. In addition, this method is a more economic way for *E. sakazakii* detection and generated less laboratory waste. For example, to test 10 samples, less than 60 ml of selective medium is needed for the microtiter MPN assay, whereas 500 ml of selective medium is required for the conventional plating method. Therefore, the microtiter MPN assay is a rapid, reliable and economic way to quantitatively analyze *E. sakazakii* in dried infant formula.

3.3. Heat Resistance

3.3.1. Heat Resistance Screening

Heat resistance of *E. sakazakii* varied among the 68 strains (Figure 6). The log reduction at 55°C for 20 min ranged from 0.54 ± 0.08 to 4.81 ± 0.33 $\log_{10}$ CFU/ml. According to these results, two strains each of heat resistant and heat sensitive *E.*

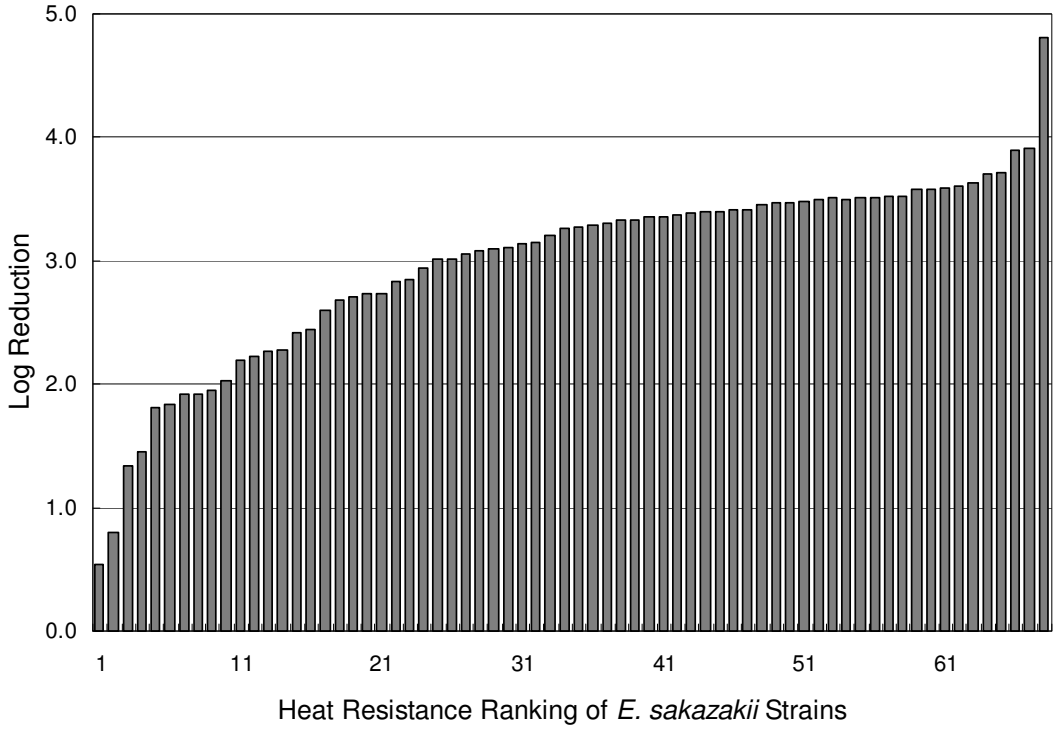

Figure 6. Log reduction of 68 strains of *E. sakazakii* after 20 min heat treatment at 55°C.

sakazakii were selected for further understanding the heat resistance characteristic.

3.3.2. Survival Curves of Heat Resistance/Heat Sensitive strains

The survival curves of all cells including injured and non-injured cells of the four selected strains of *E. sakazakii* in pH 7.0 phosphate buffer (PB) and reconstituted dried infant formula (DIF) at 50°C, 55°C, and 60°C were shown in Figures 7, 8, and 9, respectively. There were no significant difference between heat resistant strains and heat sensitive strains at 50°C for both PB and reconstituted DIF (Figure 7). However, cells suspended in PB were more vulnerable to heat compared to those in infant formula during the same treatment period. Infant formula can protect the cell surviving for at least 3 hr without significant cell reduction ($p<0.05$). When temperature was increased to 55°C, the difference between heat resistant strains and heat sensitive strains began to be distinct (Figure 8). This characteristic was clearer when cells are in PB and it took more time to separate the two groups in reconstituted DIF. At this temperature, IF provided a better protection effect than PB for *E. sakazakii* at 10 min of treatment but as time increased, the survived cells started to decrease, especially for heat sensitive strains. The numbers of survived cells dropped dramatically and similar trends as those of 55°C were observed at 60°C (Figure 9). However, at this temperature, infant formula could not provide a good protective effect as that of at lower temperatures, especially for heat sensitive strains. This result is in agreement with Breeuwer *et al*. (2003) who reported that heat treatment in reconstituted DIF did not influence the *D*-value at 58°C compared to those in pH 7.0 phosphate buffer.

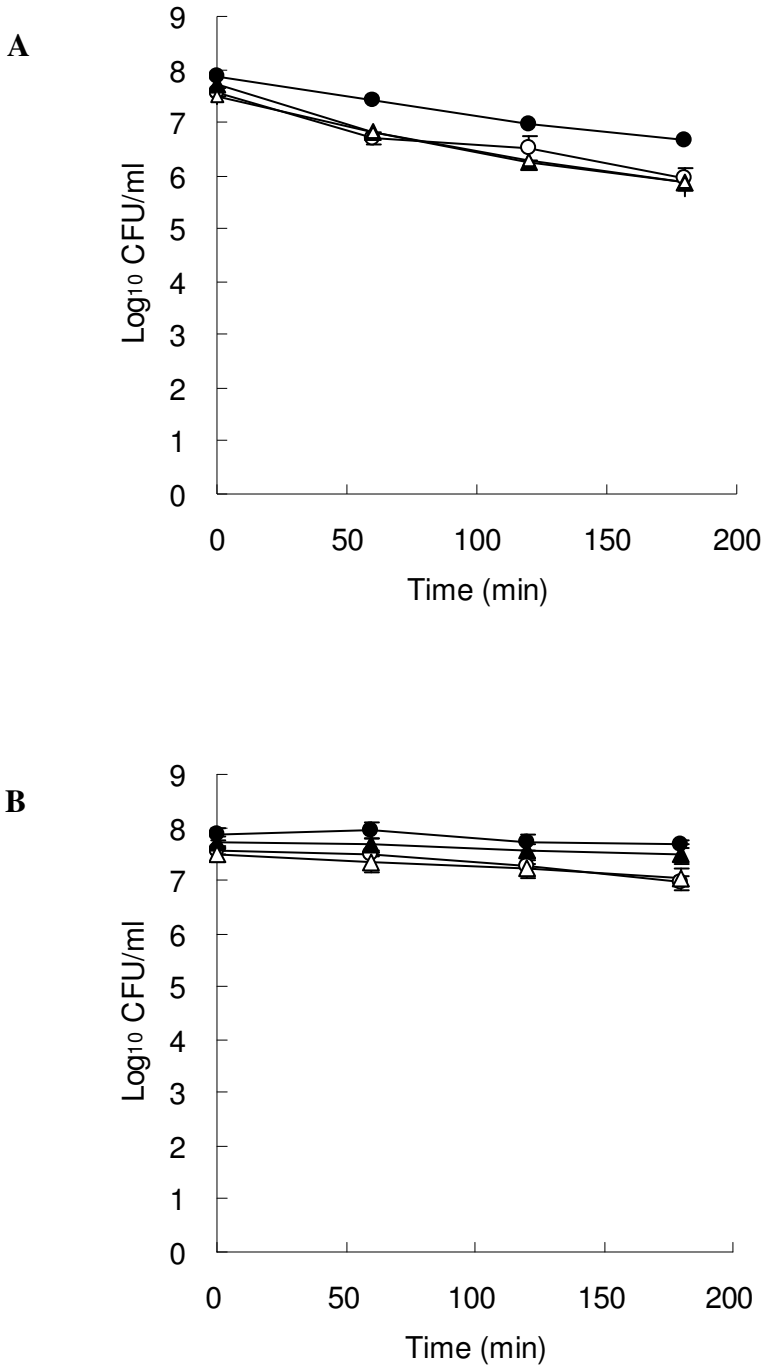

Figure 7. Survival curves of injured and non-injured cells of four selected strains of *E. sakazakii* in pH 7.0 phosphate buffer (A) and infant formula (B) at 50°C using TSA overlaid by OK media as a selective and differential media. ●: FSM 293, ○: 2. 45, ▲: FSM 271, △ : ATCC 12868.

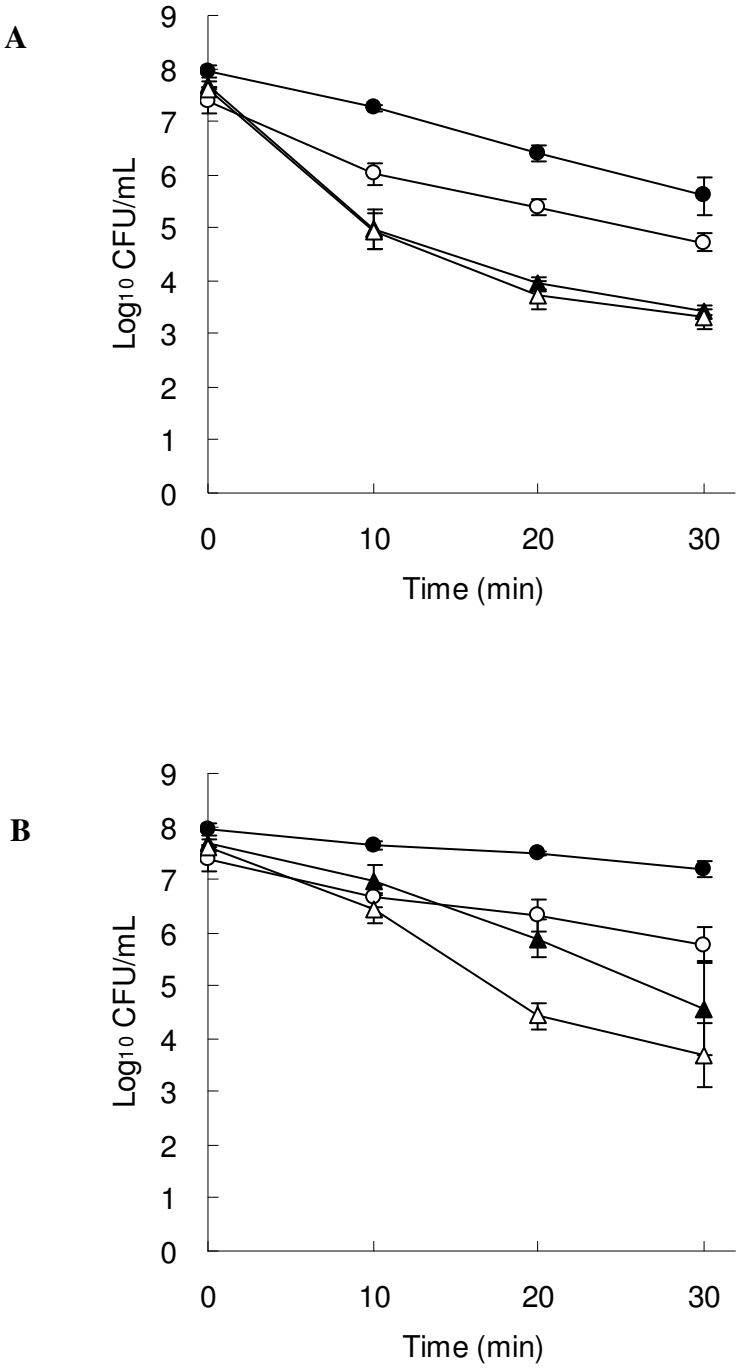

Figure 8. Survival curves of injured and non-injured cells of four selected strains of *E. sakazakii* in pH 7.0 phosphate buffer (A) and infant formula (B) at 55°C using TSA overlaid by OK media as a selective and differential media. ●: FSM 293, ○: 2. 45, ▲: FSM 271, △ : ATCC 12868.

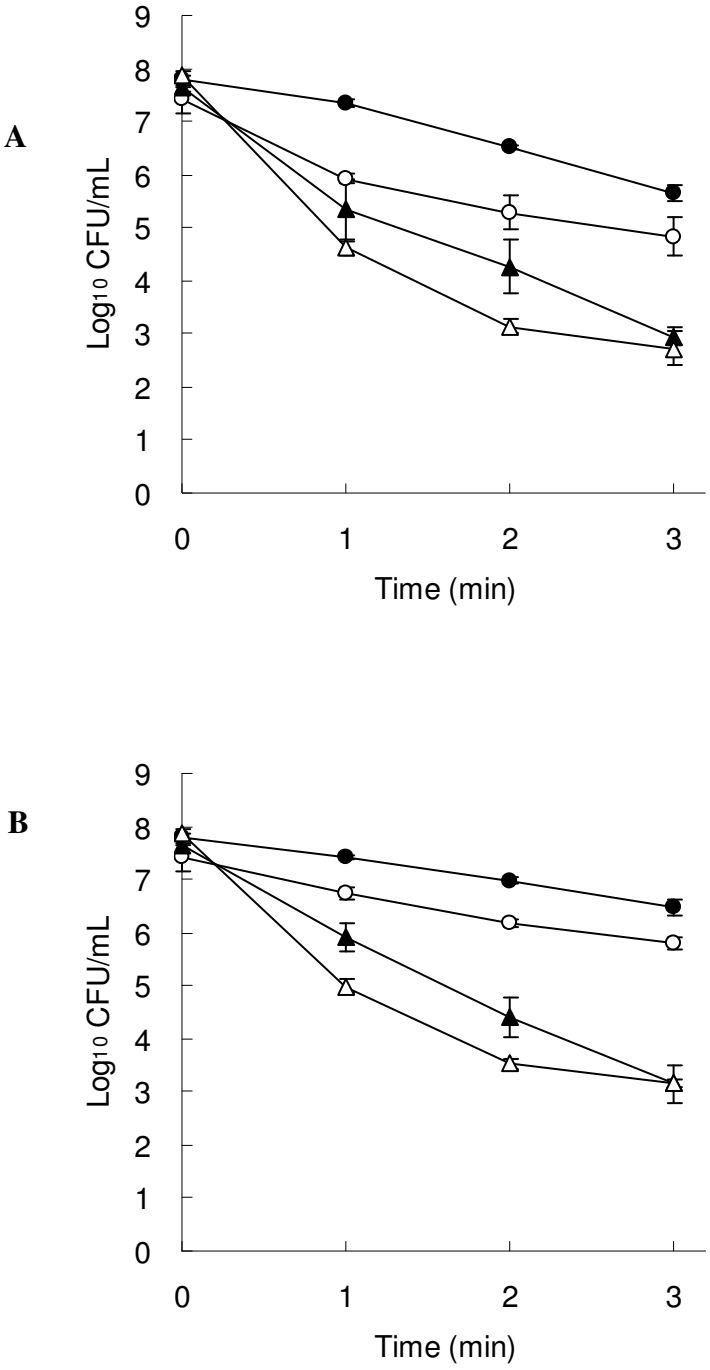

Figure 9. Survival curves of injured and non-injured cells of four selected strains of *E. sakazakii* in pH 7.0 phosphate buffer (A) and infant formula (B) at 60°C using TSA overlaid by OK media as a selective and differential media. ●: FSM 293, ○: 2. 45, ▲: FSM 271, △ : ATCC 12868.

The survival curves for only non-injured cells of the four selected strains of *E. sakazakii* in PB and reconstituted DIF at 50°C, 55°C, and 60°C were shown in Figures 10, 11, and 12, respectively. When 50°C of heat treatment was applied, no significant differences were observed within the two groups. The two strains of heat resistant strains even showed slightly more reduction at the end of the treatment. Although more cells of heat resistant strains were injured, their recovery ability was better than the heat sensitive strains, which made them to be more heat resistant. On the other hand, infant formula can significantly ($p<0.05$) protect *E. sakazakii* from being injured by 50°C of heat. It was suggested that the high content of total solids and high amount of fat in the infant formula was responsible for protecting the microorganisms from heat (Batish *et al.*, 1988). As temperature increased, the differences between two groups were more easily to be distinguished. The four strains obviously fell into two groups when 60°C of heat was applied.

The order of heat resistance of these strains was consistent with the heat resistance screening experiment. When compared the number of healthy and injured cells, each strain showed their own characteristic. Strain FSM 293 was the most heat resistant strain. It is not easy to be injured unless the temperature reached 60°C. Strain 2. 45 ranked the second among the four. Although it could be injured at 55°C, which made its non-injured cell count similar as those of heat sensitive strains, instead of being killed by the heat treatment like the heat sensitive strains, most cells of strain 2. 45 were just being injured and could be recovered once favorable condition was provided. In addition, when the temperature of heat treatment reached 60°C, its non-injured cell counts remained similar to those of 55°C. Strain ATCC 12868 was

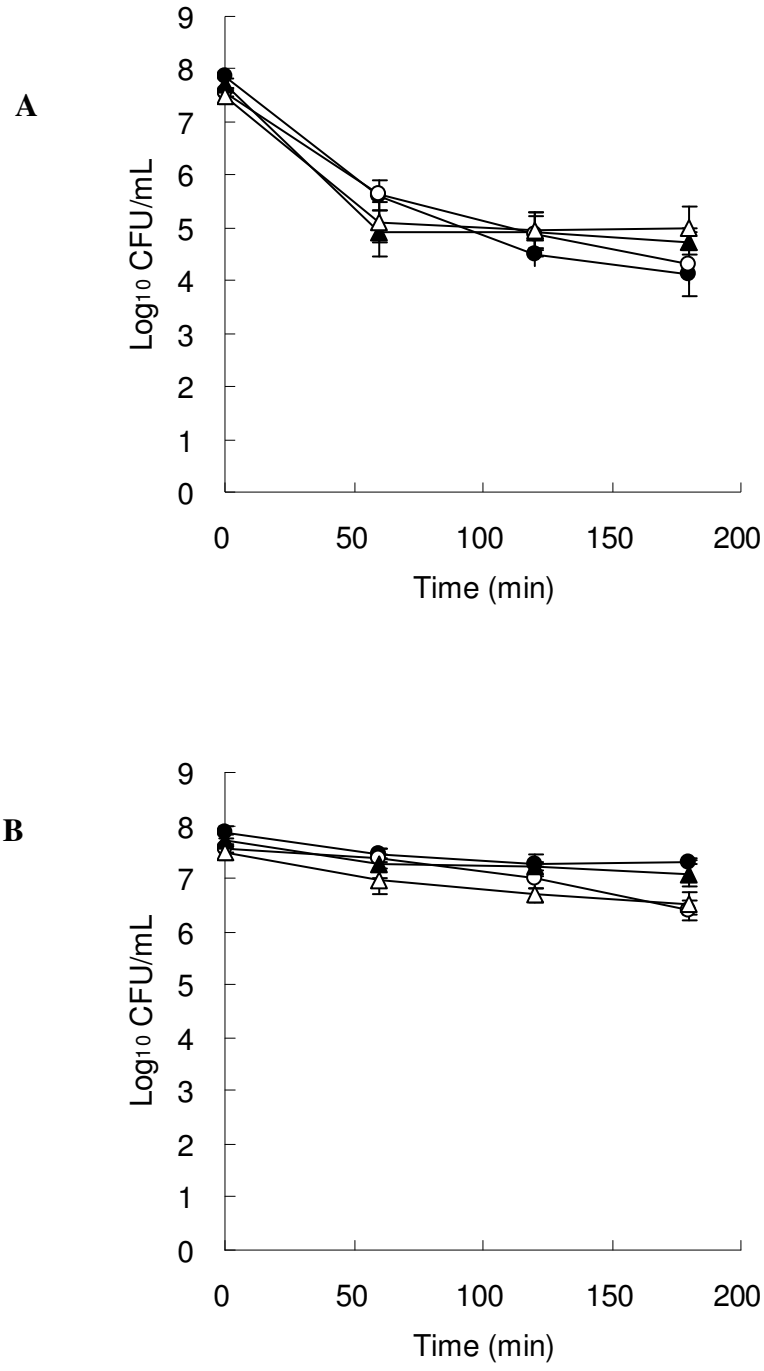

Figure 10. Survival curves of non-injured cells of four selected strains of *E. sakazakii* in pH 7.0 phosphate buffer (A) and infant formula (B) at 50°C using OK media as a selective and differential media. ●: FSM 293, ○: 2. 45, ▲: FSM 271, △ : ATCC 12868.

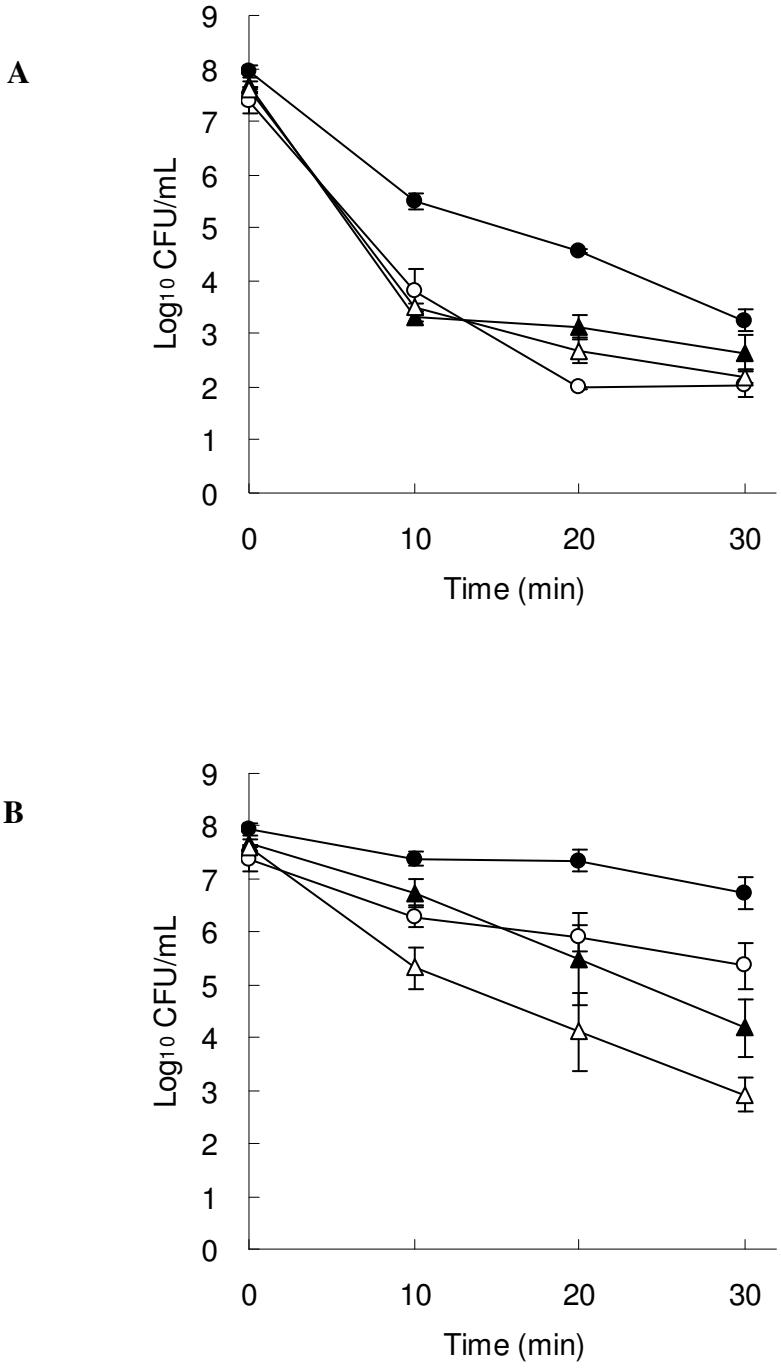

Figure 11. Survival curves of non-injured cells of four selected strains of *E. sakazakii* in pH 7.0 phosphate buffer (A) and infant formula (B) at 55°C using OK media as a selective and differential media. ●: FSM 293, ○: 2. 45, ▲: FSM 271, △ : ATCC 12868.

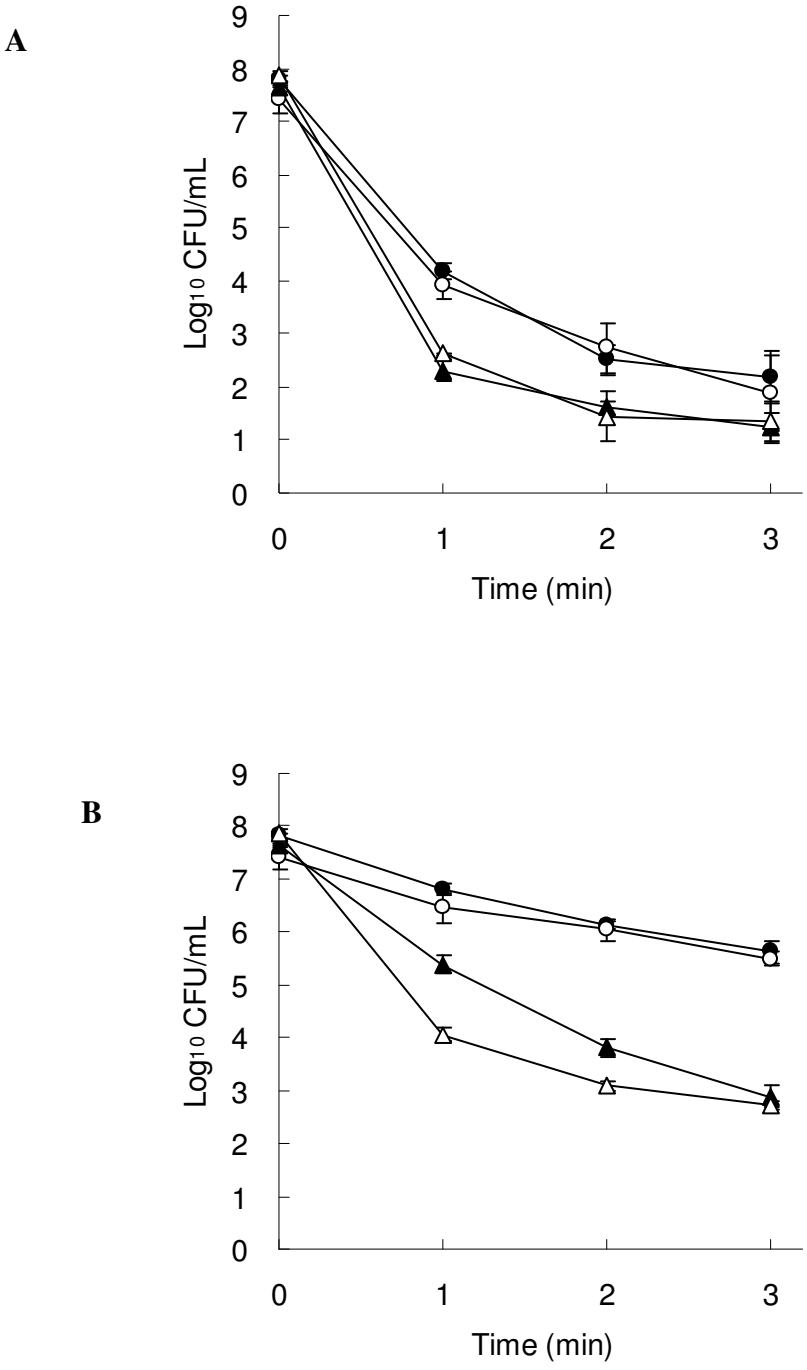

Figure 12. Survival curves of non-injured cells of four selected strains of *E. sakazakii* in pH 7.0 phosphate buffer (A) and infant formula (B) at 60°C using OK media as a selective and differential media. ●: FSM 293, ○: 2. 45, ▲: FSM 271, △ : ATCC 12868.

the most heat sensitive strains. It was hard to survive even with the protection of infant formula. The heat sensitive strains were not only more vulnerable but also had poorer recovery ability. The cells tend to die off when being heat damaged and therefore less injured cells were cultured.

3.4. Guideline for Infant Formula Preparation and Handling

The survival and regrowth curves did not significantly (p<0.05) distinguish the heat resistant strains and heat sensitive strains. In fact, as shown in Figure 14 and 15, when 120 ml of water was added, strain FSM 271 (heat sensitive strain) can survive at higher temperature than strain 2. 45 (heat resistant strain). On the contrary, significant (p<0.05) difference was observed when different volume of hot water was mixed. All four strains can survive when 60 ml of water was added (Figure 13, 14, 15, and 16); even the temperature was as high as 90°C. However, when mixed with 120 ml of hot water, *E. sakazakii* cells were killed to undetectable level (initial cell number: $10^{4\text{-}6}$; detection limit: 1 CFU/ml) at 80°C (strain 2. 45 and ATCC 12868; heat resistant and heat sensitive, respectively) or 90°C (all four strains).

Temperature decreased along with time when mixing with 60 ml or 120 ml of water (room temperature, 50°C, 70°C, 80°C, and 90°C) was monitored and showed in Figure 17. Higher temperature was achieved and maintained when larger amount of hot water (>70°C) was mixed with DIF, therefore explained why they had better killing effect for *E. sakazakii*. Although high initial temperature was chosen compared to heat resistance experiment, since the highest temperature after mixing with dried infant formula only reached to less than 70°C and did not maintained for enough time

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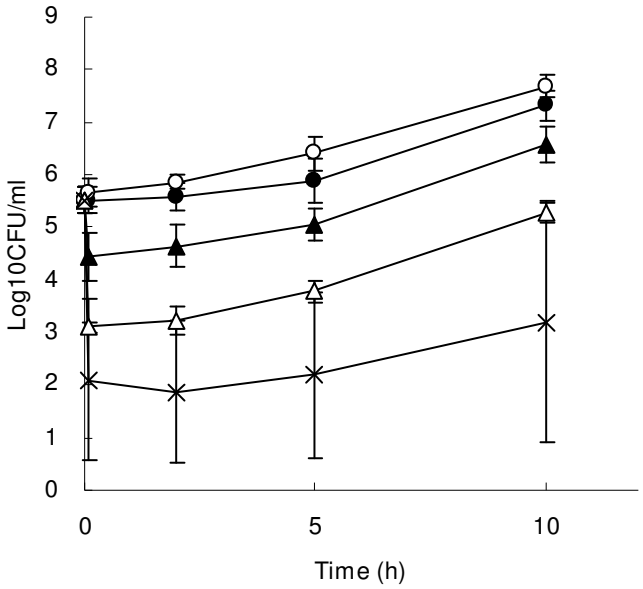

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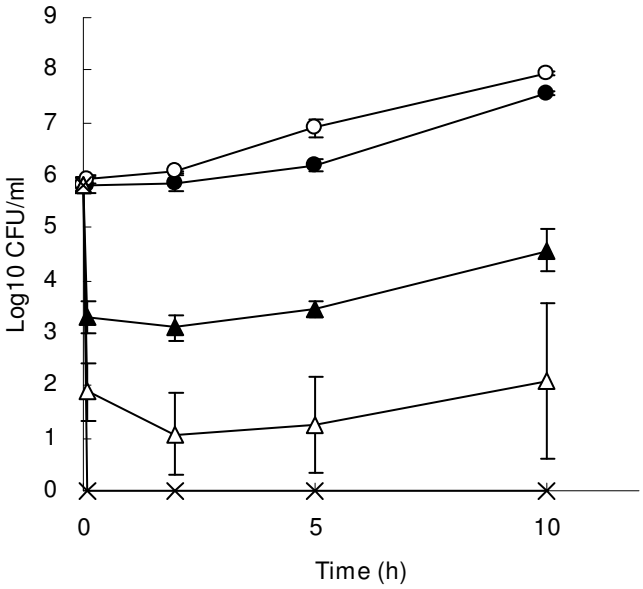
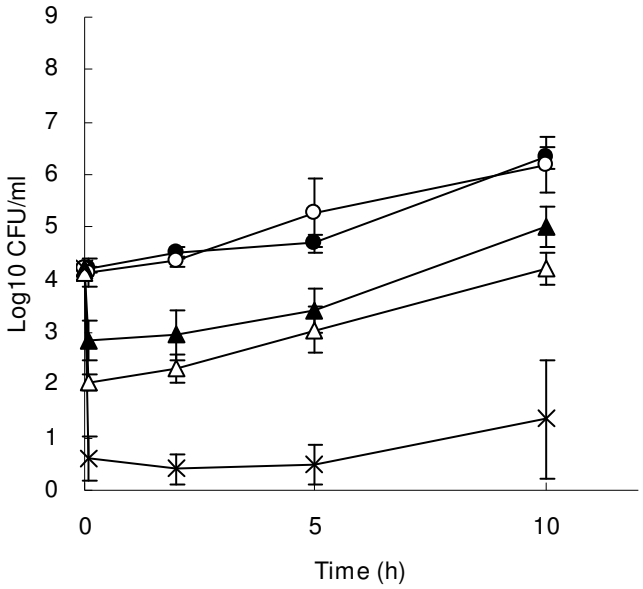


Figure 13. Survival and regrowth curves of strain FSM 293 using (A) 60 ml or (B) 120 ml water of different temperature. ●: room temperature, ○: 50°C, ▲: 70°C, △:80°C, and, ×:90°C

A



B

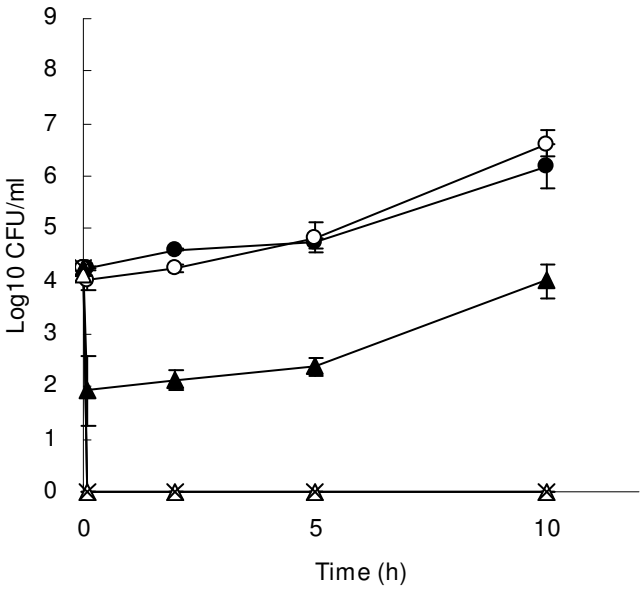
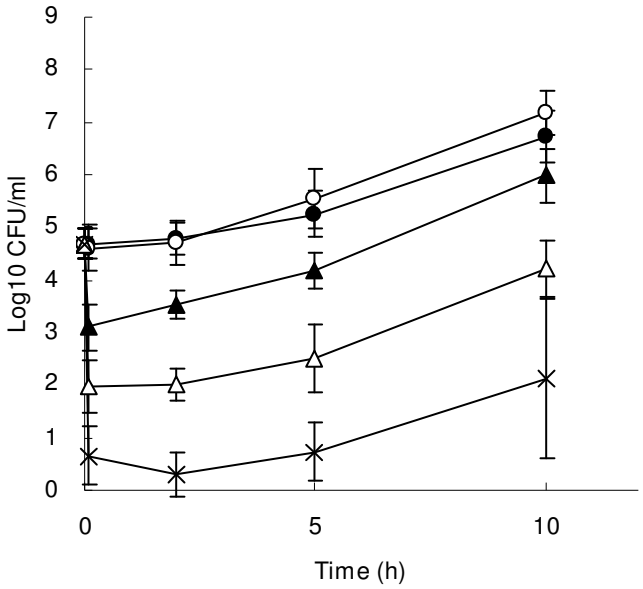


Figure 14. Survival and regrowth curves of strain 2. 45 using (A) 60 ml or (B) 120 ml water of different temperature. ●: room temperature, ○: 50°C, ▲: 70°C, △:80°C, and, ×:90°C

A



B

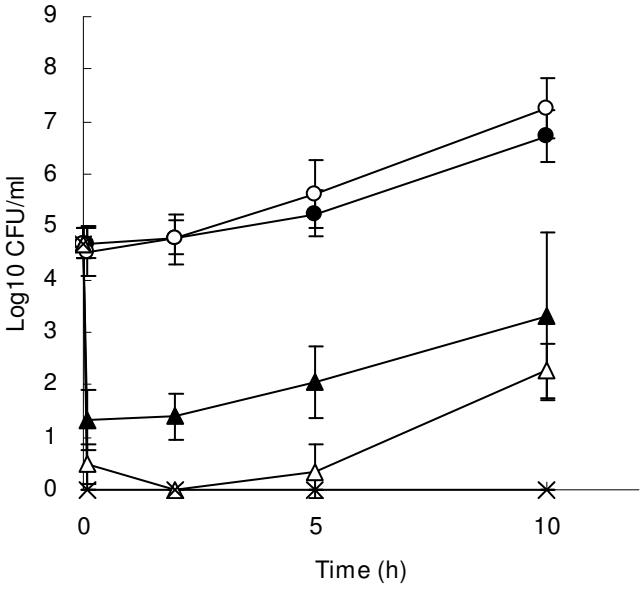
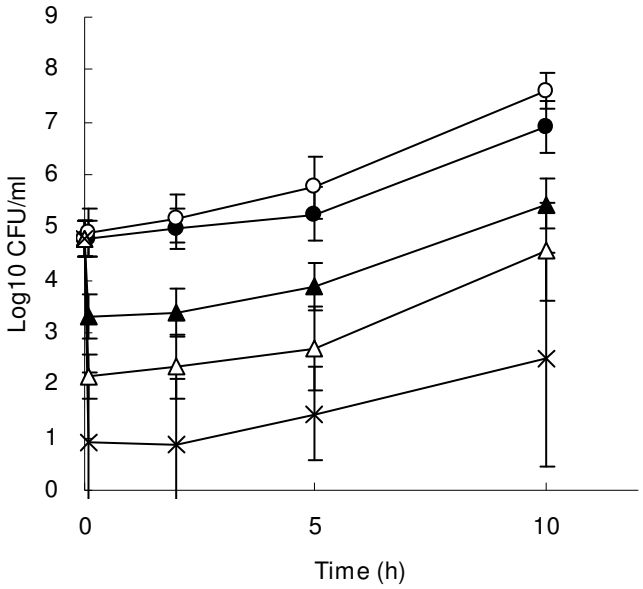


Figure 15. Survival and regrowth curves of strain FSM 271 using (A) 60 ml or (B) 120 ml water of different temperature. ●: room temperature, ○: 50°C, ▲: 70°C, △:80°C, and, ×:90°C

A



B

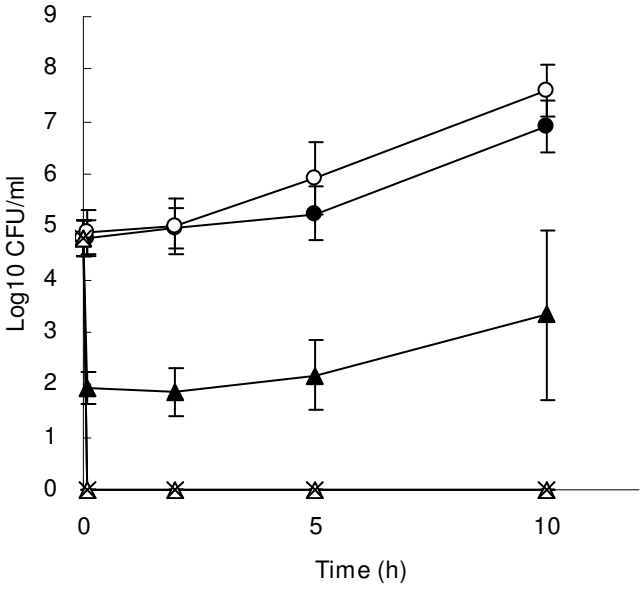


Figure 16. Survival and regrowth curves of strain ATCC 12868 using (A) 60 ml or (B) 120 ml water of different temperature. ●: room temperature, ○: 50°C, ▲: 70°C, △:80°C, and, ×:90°C

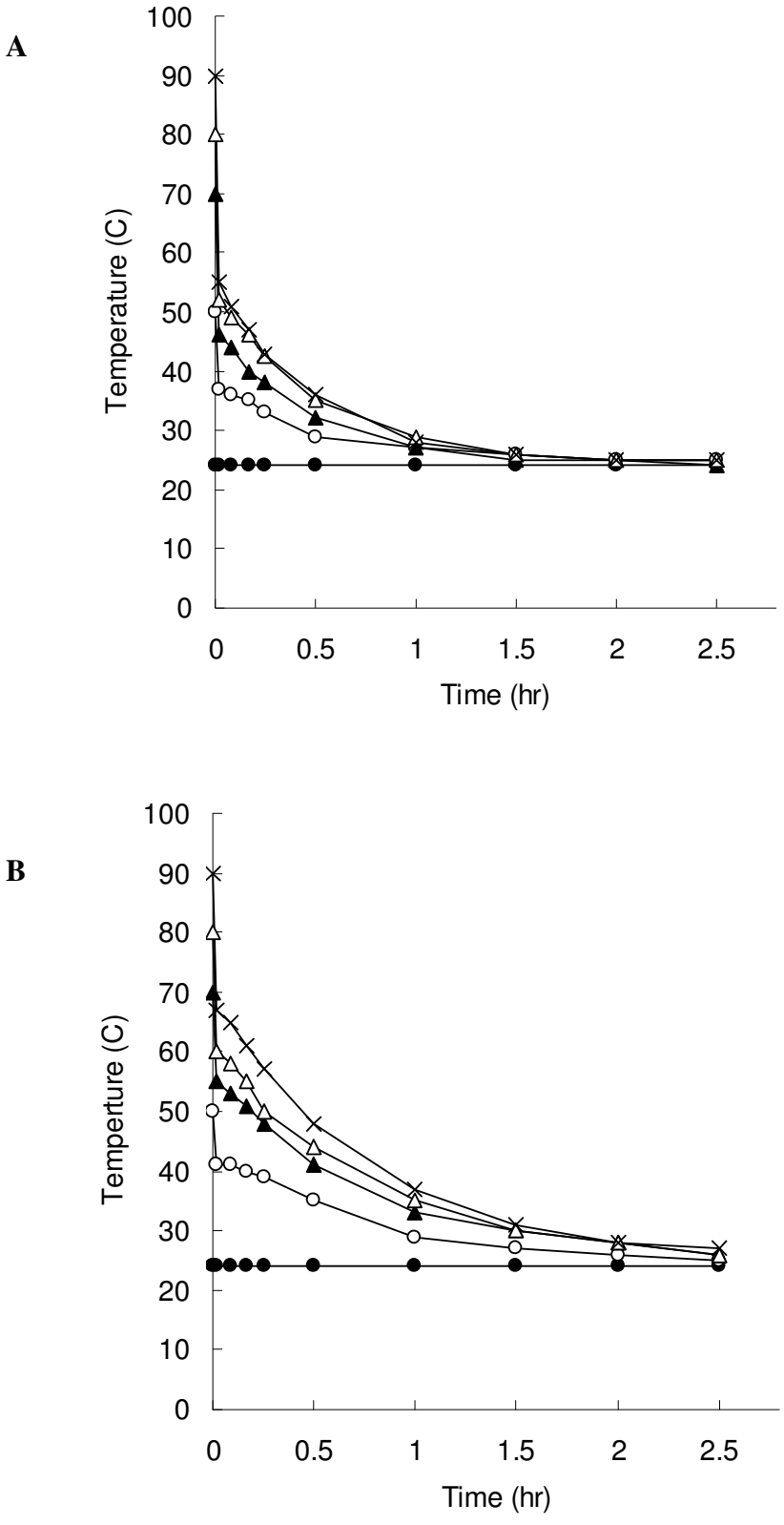


Figure 17. Temperature measurement when mixing dried infant formula with (A) 60 ml or (B) 120 ml of water. Initial temperature:●: room temperature, ○: 50°C, △: 70°C, ▲: 80°C, and, ×: 90°C

to better kill the bacteria, the heat resistant strains and heat sensitive strains could not be separated into two apparent groups like the data obtained from the experiment of survival of heat resistance/heat sensitive strains.

When 50°C of water was used, the heat could not kill the bacteria cells regardless of volume of water used or strains selected. Instead, the regrowth rate of this temperature was the highest among all treated temperatures. This is due to the temperature reached around 37°C when DIF was mixed with water or underwent this temperature zone for a period of time. This range of temperature is optimal for the growth of *E. sakazakii* (Iversen *et al*., 2004) therefore not only has no killing effect but also allowed the bacteria grew the fastest.

From the results obtained in this study, it is encouraged to use 120 ml of 90°C hot water for dried infant formula reconstitution in order to contribute a 4-6 log reduction of *E. sakazakii*. However, since *E. sakazakii* found in DIF was always with very low level of contamination ranged from 0.36 to 66.0 CFU/100 g (Muytjens *et al*., 1988; Nazarowec-White and Farber, 1997b; Simmons *et al*., 1989), 60 ml of 70°C hot water, which could cause greater than 1 log reduction for all four strains, would be enough for preventing the infection of *E. sakazakii*.

The additional advantage of preparing DIF with hot water is to at least cause cell injury, which could prolong the recovery time before cell multiplication (Figure 13, 14, 15, and 16) and potentially reduce hazardous levels of *E. sakazakii*. Nazarowec-White and Farber (1997b) reported that with an initial number of 1 CFU/ml, reconstituted formula stored at room temperature would take approximately 10 h to reach 10^7 cells in one infant feeding of 100 ml. In our study, since the cells

were injured by hot water, longer time was required to increase the microbial load.

Although heating reconstituted infant formula by microwave to the boil had been suggested to be a convenient and fast way to reduce microbial contamination of infant feed (Kindle *et al.*, 1996), uneven heating and overheating could cause hot spots in the formula and cause scalds to the mouth or throat (Dixon et al., 1997; Garland et al., 1986; Hibbard and Blevins, 1987; Sando et al., 1984; Siu and Kissoon, 1987). These cases made the heating of infant formula by microwave dangerous to infants. Therefore, it would be safer to reconstitute DIF by hot water with adequate cooling before feeding.

Since 35-37°C is optimal temperature for growth of *E. sakazakii*, it is not recommended to sit the prepared infant formula in bottle heaters, which possibly allowed the multiplication of this microorganism and increased hazardous level. It has been reported that *E. sakazakii* strains did not grow at 4°C and appeared to die-off during storage (Nazarowec-White and Farber, 1997b). Thus, if infant formula could not be consumed immediately, it should be kept in the refrigerator to minimize the possibility of cell proliferation. However, other researchers had proposed that *E. sakazakii* is able to grow in IF during storage at refrigeration temperatures for a prolonged time (Iversen *et al.*, 2004). Therefore, even the prepared infant formula were kept in the refrigerator, it is necessary to minimizing the storage time before feeding.

Several recommendations had been proposed for infant formula preparation. Obviously, use of cool water (2-24°C) or warm water (37-38°C) guided by manufacturers (Anonymous, 2007) was inappropriate in the view of reducing thr risk

of *E. sakazakii* infection. Our study assured the heat destruction to *E. sakazakii* by mixing with 70-90°C of hot water and therefore supportted the recommendation of International food safety authorities network (2005), who suggested to bring the water to a rolling boil and cool for a few minutes to temperature of 70-90°C before reconstituting DIF.

CONCLUSION

In conclusion, EE broth is the most effective broth among the five selected enrichment media, followed by reconstituted DIF. The other three media including mTSB + N, mEC + N, and BPW + VCC all failed to enrich *E. sakazakii* cells. In addition, the microtiter MPN method with the use of OK media (broth) is a simple, reliable, and economic method for enumeration of *E. sakazakii*. With this method, only 10 h was needed to obtain the results. It saved about 14 h compared to the newly developed media such as OK media (agar) or DFI media and at least 62 h compared to the U.S. FDA recommended method.

With heat resistance study, we concluded that the heat resistance varied among strains. The variation was more obvious when treated with 55°C or 60°C. From this study, we also observed that infant formula could protect *E. sakazakii* from being destructed by heat treatment. On the other hand, we confirmed that using hot water (70-90°C) to reconstite DIF could damage *E. sakazakii* cells and therefore supported the recommendation made by International Food Safety Authorities Network.

FUTURE RESEARCH

Although in this study, EE broth seems to be the most promising broth among the five being selected; many researchers had suggested that the inhibition of antibiotics against microorganisms could be influenced by concentration. Therefore, the other enrichment broths without antibiotics or with lower concentrations of antibiotics could be further evaluated to optimize an enrichment broth for *E. sakazakii* in DIF. In addition, although 37°C was used in this study for all enrichment broths, several researchers suggested that 42°C could be more optimal for the enrichment (Blais *et al*., 1997**;** Bolton *et al*., 1995; Hepburn *et al*., 2002; Ogden *et al*., 2001; Szabo *et al*., 1990). Therefore, incubation temperature could also be incorporated as a factor of influence for optimizing enrichment conditions in the future.

The factors causing the diversity of heat resistance among *E. sakazakii* strains could be further discussed. Knabel *et al.* (1990) indicated that many factors can influence the heat resistance of bacteria. Some of these include the physiological state of the organism, the heating menstruum, as well as the methodology used for bacterial recovery. However, the variation in heat resistance of *E. sakazakii* among strains could play the most important role and the correlation with the genetic diversity remained to be established.

In consideration of the adverse effects of using boiling water including the potential loss of heat sensitive nutrients (such as thiamin, vitamin B6, ascorbic acid, folate, and vitamin B12) and changes in physical characteristics of some formulas (U.S. FDA, 2002a). Further evaluation needed to be conducted to find out the optimal reconstitution temperature of water to maximize the killing effect to *E. sakazakii* and minimize the adverse effects regarding nutrients and physical characteristics of infant formula.

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