VIRULENCE POTENTIAL OF *ENTEROBACTER SAKAZAKII* AS A NEONATAL ENTEROTOXIC PATHOGEN

THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
DAIRYING
(DAIRY MICROBIOLOGY)

BY

MAMTA RAGHAV

DIVISION OF DAIRY MICROBIOLOGY
NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
KARNAL - 132001 (HARYANA), INDIA

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Regn. No. 1010305

Dedicated

To My

Beloved Parents

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This is to certify that the thesis entitled, "**VIRULENCE POTENTIAL OF *ENTEROBACTER SAKAZAKII* AS A NEONATAL ENTEROTOXIC PATHOGEN**" submitted by Ms. **Mamta Raghav** towards the partial fulfilment of the award of the degree of **Doctor of Philosophy in Dairying (Dairy Microbiology)** of the National Dairy Research Institute (Deemed University), Karnal (Haryana), India, is a bonafide research work carried out by her under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

Dated: 12 **March, 2007**

(P.K. Aggarwal)
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(Guide)

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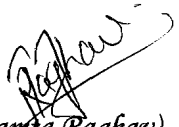

(Mamta Raghav)

VIRULENCE POTENTIAL OF *ENTEROBACTER SAKAZAKII* AS A NEONATAL ENTEROTOXIC PATHOGEN

ABSTRACT

Enterobacter sakazakii constitutes a potential health hazard particularly for the young children and is now attracting considerable attention because of its varied clinical manifestations. In this investigation from a total of 170 environmental samples 11 isolates were identified as *E. sakazakii* by biochemical and molecular characterization. The D_{55} and D_{62} values ranged from 7-12 min and 0.3-0.6 min respectively. Although showing extended lag phase the isolates could tolerate NaCl upto 5.5 %; no growth was observed in presence of 10.5 % NaCl. β-hemolytic activity, DNase, hemagglutination and serum resistance and elastase, neuraminidase, gelatinase and lipopolysachcharide production were identified as potential virulence factors in the isolates. However isolates possessed were weak collagenolytic and no aerobactin, phospholipase C and hyaluronidase activities. Plasmids were detected in six isolates and the virulence study after plasmid curing suggested that virulence might be chromatid encoded and not plasmid linked. Five isolates showed suckling mice enterotoxin activity. The enterotoxin production was maximum in casamino yeast extract medium containing 1% casamino acids at 37°C for 12 hrs. The most potential enterotoxic isolate (GF1) was selected for purification of the toxin. The toxin was purified upto 32.4 folds using ammonium sulphate precipitation, followed by DEAE-cellulose (ion exchange) and Sephadex G-100 (gel filtration) chromatography. LD_{50} of the purified enterotoxin was 56 ng. The protein and carbohydrate concentration in the enterotoxin was 1.4 mg and 0.002 mg/ml respectively. The molecular weight of the enterotoxin was about 66 kDa. The toxin had optimum activity at pH 6 and in presence of 0.05 % $ZnCl_2$. The toxin was mildly heat stable in nature as it retained activity even when heated at 90 °C for 30 min. The present study has shown occurrence of *E. sakazakii* in environment and thrown some light on possible virulene of the organism particularly in the context of enterotoxin production.

एंटीरोबेक्टर साकाजाकाई की नवजात शिशुओं के आन्त्रविष-व्याधिजन के रूप में रोगोत्पादन क्षमता

सारांश

एंटीरोबेक्टर साकाजाकाई शिशुओं के लिए एक विशेष एवं सक्षम स्वास्थ्य संबंधी संकट है तथा अपनी विविध चिकित्सा सम्बन्धी आवर्तों के कारण महत्वपूर्ण हो रहा है। पर्यावरण में से 170 नमूनों से प्रारम्भिक समृद्धि द्वारा आरम्भ में 24 व्यष्टि अलग किए गए। इनमें से जीवरासायनिको तथा आण्विक लक्षणों द्वारा 11 की ई. साकाजाकाई के रूप में पहचान की गई। इनकी डी$_{55}$ तथा डी$_{62}$ क्रमशः 7-12 तथा 0.3-0.6 मिनट आंकी गई। यद्यपि इन जांचे गए व्यष्टियों (संवर्धो) ने बढ़ी हुई मंचर अवस्था दिखाई तथापि इन्होंने 5.5 प्रतिशत तक नमक की उपस्थिति को सहा पर 10.5 प्रतिशत नमक में इनकी कोई वृद्धि नहीं हुई। जीवाणुओं के रोगात्पादन सहायक के रूप में प्राप्त बीटा-रक्तभंजन, सूत्ररक्तभंजन, रक्तकणइक्त्रण तथा रक्तर्आभरास-प्रतिरोध एवं इलास्टेज़, न्यूरामिर्नाडेज, जीलेटिनेज किण्वक और चसा महाशर्करा उत्पादन की ई.साकाजाकाई में उपस्थिति थी। परन्तु इनमें फास्फोलाइपेज़ सी, एईरोबेक्टिन तथा हाइअलयूरोनिडेज़ नहीं पाई गई। छह व्यष्टियों में प्लज्मिड मिले परन्तु इनके उपचारोप्रांत रोगोत्पादन क्षमता में अन्तर न पड़ने से पता चला कि यह क्षमता इनके गुणसूत्र में लिपिबद्ध है। इनकी आन्त्रविष क्रिया का निर्धारण दुग्ध मुंहे चूहों के प्रयोग से किया गया। एक प्रतिशत कैसामिका अम्लों से पूरित संवर्धाश में 37^0 सैल्सियस पर 12 घंटों में आन्त्रविष का अधिकतम उत्पादन हुआ। तीन-चार दिन के चूहों में आन्त्रविष क्रिया दर्शाने वाले ऐसे पांच व्यष्टियों में से सर्वाधिक क्षमतावान GF1 को विषशोधन हेतु चुना गया। अमोनिया सल्फेट द्वारा तलछट बना कर, डी ई.ए. ई.सेल्यूलोज़ पर आयन विनिमय वक्षोप्रान्त सेफाडेक्स जी-100 पर छनन क्रिया द्वारा विष को 32. 4 गुणा शोधित कर लिया गया। विष की एल.डल$_{50}$ 56 नेनोग्राम थी। शोधित विष में प्रोटीन 1.4 तथा शर्करा 0.002 मिलीग्राम प्रति मिलीलीटर थी। इसका आण्विक भार 66 सहस्त्र डालटन था। इसकी अधिकतम क्रिया पीएच 6 पर तथा 0.05 प्रतिशत में थी। यह 90^0 सैल्सियस पर 30 मिनट गर्म करने पर भी क्रियारत होने के कारण माध्यमिक उष्मा सहिष्णु स्थिर थी। प्रस्तुत अध्ययन ने इस जीवाणु की संभावित रोगोत्पादन क्षमता, विशेषतया इसके आन्त्र विष पर भी प्रकाश डाला है।

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µg	=	Microgram
µl	=	Micriliter
µM	=	Micromolar
a_w	=	Water activity
CFU	=	colony forming units
CSF	=	Cerebral spinal fluid
D	=	Decimal reduction time
d	=	Day
g	=	Gram
h	=	Hour (s)
IFM	=	Infant formulae milk
kDa	=	Kilodalton
l	=	Liter
m	=	month (s)
M	=	Molar
mA	=	Milli ampere
mg	=	Milligram
MHz	=	Megahertz
MIC	=	Minimal inhibitory concentration
min	=	Minute(s)
ml	=	Milliliter
mM	=	Millimolar
mm	=	Millimeter
N	=	Normal
ng	=	Nanogram
nM	=	Nano moles

nm	=	Nanometer
O.D.	=	Optical Density
PAGE	=	Poly Acrylamide Gel Electrophoresis
PCR	=	Polymerase Chain Reaction
pM	=	Pico moles
psi	=	Pound per Square Inch
r.p.m	=	Revolutions per Minute
Sec	=	Second
SEM	=	Standard Error of Mean
TEMED	=	N, N, N', N'- Tetra Methyl Ethylene Diamine
U	=	Unit
V	=	Volt
Vol	=	Volume
w/v	=	weight/ volume
wk	=	Week (s)
yr	=	Year (s)
α	=	Alpha
β	=	Beta
z	=	°F to decrease D by 1 log cycle

CHAPTER - 1

Introduction

1. INTRODUCTION

The unprecedented surge in technologies for detection and identification of microorganisms, particularly potential pathogens, has since been revealing the hidden mysteries of microbial domains and microorganisms. The list of such bacterial pathogens is growing quite rapidly as new species among already familiar groups are being identified. Most of these are food borne pathogens responsible for various maladies, majority of cases being fatal. One of the recently studied bacterial causes of human food borne illness is *Enterobacter sakazakii*.

Earlier *E. sakazakii* was considered as a pigmented strain of the species *Enterobacter cloacae* as their biochemical properties were very similar. Later it was distinguished from *E. cloacae* on the basis of DNA-DNA hybridization, a few biochemical properties and antibiotic susceptibility. DNA homology between the two species was less than 70% whereas to belong to same species two strains should have more than 70% DNA homology. Biochemically *E. sakazakii* is different from *E. cloacae* in fermentation of D- sorbitol, DNase production, yellow pigmentation at 25°C and enzyme profile. *Enterobacter sakazakii*, a motile peritrichous Gram negative bacterium forms yellow coloured small and smooth colonies after incubation of 24 h at 30°C. It is an opportunistic pathogen and rarely causes infection in an otherwise healthy person. There have been several outbreaks of *E. sakazakii* infection. Most of the cases are fatal and those who survive suffer from neurological and developmental abnormalities. This organism has emerged as a devastating neonatal pathogen and has been implicated most frequently in neonatal meningitis (Willis and Robinson, 1988). The first case of neonatal meningitis by the bacterium was reported by Urmenyi and Franklin (1961). Twenty cases of neonatal meningitis were reported worldwide by 1989 (Beiring *et al.*, 1989). By 2003 (FDA/CFSAN) out of 58 cases of *E. sakazakii* infections only 6 involved individuals of more than 4 yr of age. The vast majority of cases (83%) was related to infants of less than 1 yr where the fatality rate ranged from 30%- 80% despite antibiotic treatment. There are relatively fewer reports on *E. sakazakii* infections in adults. Infection with *E. sakazakii* may result in meningitis (58%),

necrotizing enterocolitis (29%) or sepsis (17%); particularly in premature and immunocompromised infants.

Recently the International Commission for Microbiological Specifications for Foods (ICMSF) ranked the organism as "severe hazard" for restricted populations (Iversen *et al.* 2004). *E. sakazakii* has been isolated from various clinical and environmental samples (Narazowec-White and Farber, 1999; Bar-Oz *et al.*, 2001; Iverson and Forsyth, 2003). Although the natural habitat of *E. sakazakii* is unknown, milk powder has been suggested as a possible mode of transmission in neonatal meningitis. *E. sakazakii* has been isolated from infant formulae obtained from 35 countries including India (Muytjens *et al.*, 1988). This highly pathogenic organism is reported to be the most heat resistant among all the members of Enterobacteriacae family and also shows dry stress resistance as it can survive in dried infant formulae which have a low water activity of *ca* 0.2 (Breeuwer *et al.*, 2003). Its heat resistance, however, is not enough to survive the organism from pasteurization treatment. This suggests that post- pasteurization contamination from environment mainly drying and filling areas could be a problem (Caric, 1993).

Latex, silicon and stainless steel are commonly used for infant feeding equipment and in manufacturing units and it is very serious that *E. sakazakii* easily forms biofilms on the surface of these materialsand hence compose a serious health problem (Iverson *et al.*, 2004). *E. sakazakii* colonizes food preparation equipment such as brushes blenders and spoons thus resulting in neonatal infections. During the last few years many new features of the organism have been discovered and explored for further understanding the physiology and pathogenesis of the organism. Work has been done on isolation and characterization of *E. sakazkaii.* Several chromogenic, selective and differential media have been proposed for specific isolation of the organism (Oh and Kang, 2004; Lehner *et al.*, 2006). Several PCR based identification methods have been proposed by various workers (Seo and Bracket, 2005; Lehner *et al.*, 2004a; Iverson *et al.*, 2006 and Lehner *et al.*, 2006a).

Although the organism has been implicated as the causal organism in several sporadic and epidemic cases of neonatal as well as adult infection and

has been fatal in most of the cases, sufficient work has not been done on the virulence factors responsible for its pathogenic nature. *E. cloacae* showed resistance to serum bactericidal activity, RBC agglutination and siderophore production but did not produce haemolysin (Keller *et al.*, 1998). *E. sakazakii,* which is very close relative of *E. cloacae,* may or may not possess these virulence properties. So this area of investigation needs to be explored. However, very little work has been carried out on enterotoxin production by the organism which is an important virulence marker of many food borne pathogens. In one such study conducted by Pagotto *et al.* (2003) four out of 18 *E. sakazakii* isolates were found to be positive for enterotoxin production.

Among the various virulence factors associated with pathogenic potential of microorganisms, toxins have been the object of investigation. In fact, the workers who studied microbial toxins were the first to propose the concept of virulence factors. Lot of work has been done on toxins produced by other pathogenic microorganisms. Biochemical properties of *E. coli* enterotoxins have been reported previously (Jacks and Wu, 1974). Speirs *et al.* (1977) assayed toxicity of *E. coli* enterotoxin with *Vero* cells. Similarly toxin from *Staphylococcus chromogenes* has been characterized (Sato *et al.*, 2004). Study of toxin, its nature, mode of action etc. can provide useful information so that some cure for the infections could be developed by restricting the activity of that particular toxin. In 2004 Sato *et al.* reported purification and characterization of *Staphylococcus chromogenes* toxin and, most importantly, therapeutic aspects of botulinum toxin studied by Johnson (1999) prompted us to investigate the toxin of *E. sakazakii* and could eventually help in deriving strategies for the cure of infection caused by this new emerging pathogenic organism as till date there is no published data pertaining either to the purification and characterization of enterotoxin or any other virulence factor. Also after Muytzens *et al.* (1988) who reported presence of the organism in Indian infant formulae samples, there is hardly any report of isolation of *E. sakazakii* from Indian environment. Also there is no information related to the role of plasmid in virulence of *E. sakazakii* thereby leaving enough scope to initiate work on this line.

The severity of the infection and high mortality rate due to *E. sakazakii* meningitis in infants, unknowm ecology and the lack of information on pathogenicity indicate that further studies on this organism are warranted. Hence in order to fill the gaps in the knowledge, this investigation has been proposed to be undertaken with the following objectives:

OBJECTIVES:

1) Isolation and characterization of *E. sakazakii* from different sources.
2) Evaluation of potential isolates for virulence with special reference to its enterotoxic potential.

CHAPTER - 2

Review of Literature

2.1 INTRODUCTION

Members of genera belonging to the Enterobacteriaceae family have earned a reputation placing them among the most pathogenic and most often encountered organisms in clinical microbiology. Most commonly encountered in the clinical laboratory include *Citrobacter, E. coli, Klebsiella, Proteus, Salmonella, Serratia, Shigell* and *Yersinia.* These large Gram- negative rods are usually associated with intestinal infections, but can be found in almost all natural habitats. They are the causative agents of such diseases as meningitis, bacillary dysentery, typhoid, and food poisoning (Willis and Robinson, 1988; Simmons *et al.*, 1989; Biering *et al.*, 1989; Noriega *et al.*, 1990; Gallagher and Ball, 1991). The aetiology of nosocomial infections has markedly changed during past few decades. Although streptococci were the major nosocomial pathogens in the pre-antibiotic era, *Enterobacter* species have been recognized as increasingly important pathogens in recent years. They have increased in incidence as nosocomial infections in general while multiple resistant strains have emerged in areas of high cephalosporin use in hospitals (Sanders and Sanders, 1997). The genus previously known as *Aerobacter* and now named *Enterobacter* contains 14 species: *E. cloacae, E. aerogenes, E. agglomerans, E. sakazakii and E. gergoviae, E. amnigenes, E. asburiae, E. cowanii, E. dissolvens, E. cancerogenus, E. hormaechei, E. cobei, E. nimipressuralis, E. pyrinus* (Kreig and Holt, 2005). The most frequently isolated in human diseases was *E. cloacae* followed by *E. aerogenes* and *E. agglomerans* (Gallagher, 1990). At that time *E. sakazakii and E. gergoviae* were not common but in recent years *E. sakazakii* has emerged as a devastating pathogen infecting mainly young ones.

2.2 SYSTEMATIC STATUS

E. sakazakii is a newly established organism that was given the status of separate species under the genus *Enterobacter* (Farmer *et al.*, 1980). Earlier it was recognized as a variant of *Enterobacter cloacae* as its characteristics were very much similar to that and was known as yellow pigmented *E. cloacae.* The first proposal for its separate position from *E. cloacae* was given by Brenner (1974). Farmer *et al.* (1980) gave detailed phenotypic explanation of the organism and proposed that the yellow pigmented organism should belong to a new species. This new organism was named *Enterobacter sakazakii* in the honour of Richii Sakazakii, a Japanese bacteriologist. To distinguish *E. sakazakii* from *E. cloacae* they studied DNA homology between them, their biochemical properties and antibiotic and enzyme profiles.

2.2.1 DNA-DNA Hybridization

DNA homology among the two species was 31-54% which suggested that the two organisms belong to two different species (Brenner *et al.*, 1973) while to belong to same species the DNA homology should be more than 70%. Also it was 53-54 % related to *Citrobacter* (Farmer *et al.*, 1980)

2.2.2 Biochemical Properties

When biochemically characterized, *E. sakazakii* and *E.cloacae* showed differences in fermentation of D- sorbitol and glycerol, gelatin hydrolysis, malonate utilization, DNase production and yellow pigmentation at 25°C.

2.2.3 Antibiotic Profile

Response of *E. sakazakii* and *E. cloacae* to various antibiotics varied significantly. Particularly, larger zone of inhibition caused in *E. sakazakii* by ampicillin and cephalosporin (Farmer *et al.*, 1980; Gaston, 1988) could be used as a means of identification of the organism. *E. sakazakii* has been reported to be more sensitive than other *Enterobacter* species. Lai (2001) reported a decrease in fatality rate of *E. sakazakii* meningitis on treatment with cephalosporin. *E sakazakii* isolated from blood and CSF of 4 wk infant patient was susceptible to aminoglycosides, chloramphenicol, nalidixic acid and tetracycline and ampicillin (Willis and Robinson, 1988). In a case study Lai (2001) found that five *E. sakazakii* strains were uniformly resistant to cefazolin, ampicillin and extended spectrum penicillins but susceptible to aminoglycosides and trimethoprim – sulphamethoxazole. *E. sakazakii* isolates showed resistance to amikacin and tobramycin but were sensitive to gentamycin (Arseni *et al.*, 1987). But during the last few years *E. sakazakii* also has been found to develop resisitance against ampicillin and gentamicin by means of transposable elements and also to β- lactam antibiotics due to the production of β- lactamase (Gurtler *et al.*, 2005). Among different *Enterobacter* species, β- lactamase seemed to be prominently produced by *E. sakazakii* as studied by Pitout *et al.* (1997) and Block *et al.* (2002). Susceptibilty of *E. sakazakii* to ampicillin, gentamycin, chloramphenicol and kanamycin was also observed by Nazarowec- White and Farber (1999), but the patients responded poorly and died. Muytzens and van de Repe (1986) studied susceptibility of 195 *E. sakazakii* isolates against 29 antibiotics against and also evaluated MICs (Table 2.1). Among eight Enterobacter species, *E. sakazakii* showed maximum susceptibility. Concentration of 24 antibiotics was ≤ 8 µg/ml that could inhibit the growth by 90%. Chloramphenicol, cefaloridin, cefsulodin, cephalothin and

sulphamethoxazole have MICs greater than 8 µg/ml. MICs of the same antibiotics against *E. cloacae* were twice that for *E. sakazakii.*

2.2.4 Enzyme Profile

The two closely related organisms can also be differentiated on the basis of their enzyme profile. Three important distinguishing enzymes are Tween 80-esterase, α- glucosidase and phosphoamidase. *E. sakazakii* produces Tween 80 esterase after 3- 8 d. It can be well differentiated from other *Enterobacter* species on the basis of presence of α- glucosidase and absence of phosphoamidase in *E. sakazakii.* The α-glucosidase reaction is more important. The reproducibility of α-glycosidase and phsphoamidase reactions was estimated to be 89 and 81%, respectively (Muytjens *et al.*, 1984). α-glucosidase hydrolyzes a number of α-D-glucopyranosides. Presence of α-glycosidase can be examined either by streaking the bacteria on nutrient agar medium supplemented with 4- methyl umbelliferyl α-D-glucoside (MUG) or by growing the culture in presence of α- nitrophenyl-α-D-glucoside. *E. sakazakii* forms golden yellow colonies on nutrient agar supplemented with α-MUG, those fluoresce under UV light. No other bacteria would show both yellow colour and fluorescence. When broth culture is centrifuged after incubation with α-nitrophenyl-α-D-glucoside, yellow colour in the supernatant indicates the presence of α-glucosidase which is produced due to the release of nitrophenol. This enzyme is encoded by two different genes. Amino acid sequences revealed that the proteins belonging to a cluster of gene products putatively responsible for the metabolism of isomaltulose (palatinose; 6-O-α-D-glucopyranosyl-D-fructose), show 4-methyl-umbelliferyl-α-D-glucoside hydrolyzing activity (Lehner *et al.*, 2006a)

Analysis of the two bacteria for acid phosphatase, esterases and glutamate-, lactate- and malate- dehydrogenases showed that enzymes of *E. sakazakii* had lower isolectric point and higher mobility. Also the enzymes within the species show high polymorphism thus facilitating their precise identification (Goullet and Picard, 1986).

2.3 PHYLOGENETIC RELATEDNESS

Based on previous reports describing about 50% genetic relationship of *E. sakazakii* with *E. cloacae* and *Citrobacter,* 16S rDNA and *hsp60* genes of these three organisms were compared, which showed that *E. sakazakii* was 97.8% close to *C. koseri,* 97.0% to *E. cloacae* and 96 % close to *C. fruendii* (Iverson *et al.*, 2004a). This phylogenetic tree revealed four distinct lineages whereas 16S rRNA genes revealed two distinct lineages within *E. sakazakii* group (Lehner *et al.*, 2004).

Table 2.1 Minimal Inhibitory concentration of antibiotics for *E. sakazakii*

Antibiotic	MIC µg/ ml		
	Range	50%	90%
Ampicillin	0.25- >128	2	4
Cefaloridin	2 - 128	8	16
Cephalothin	2- >128	64	128
Cefamandole	≤0.125 - >128	2	4
Cefoperazone	≤0.125 -16	1	2
Ceforanide	≤0.125 ->128	1	2
Cefotaxime	≤9.03- 0.5	0.125	0.125
Cefoxitin	0.5->128	8	16
Cefsulodin	2->128	32	32
Ceftazidime	≤0.03-1	0.125	0.25
Ceftizoxime	≤0.125-1	≤0.125	≤0.125
Ceftriaxope	≤.0.03- 0.5	.06	.125
Cefuroxime	0.25-32	4	8
Chloramphenicol	1->128	8	16
Ciprofloxacin	≤0.06-0.25	.0.06	0.06
Doxycyclin	1-32	4	4
Gentamicin	0.06-1	0.25	0.5
Imipenem	≤0.06-2	0.125	0.25
Moxalactam	≤0.06-1	≤.0.06	0.125
Nalidixic acid	1-16	4	4
Neomycin	0.125-32	1	2
Norfloxacin	≤0.06-1	0.125	0.125
Pipemidic acid	1-8	2	2
Piperacillin	0.125-8	2	2
Polymyxin B	0.25-64	1	1
Rifampin	2-16	8	8
Sulfamethoxazole	16->128	>128	>128
Trimethoprim	0.06-32	0.25	1
Trimethoprimsulfa	0.25-32	2	4

2.4 ISOLATION AND IDENTIFICATION

2.4.1 Cultural Method

Although *E. sakazakii* can grow normally on all media generally used for other members of Enterobacteriaceae family- MacConky agar, eosine methylene blue agar, deoxycholate agar and tergitol 7 agar, tripticase soy agar (TSA) and brain heart infusion broth are most widely used for its maintainance on which the bacterium forms yellow coloured colonies. But growth on TSA reuires 48 h incubation to produce yellow colour, which is quite longer than that needed for other enteric bacteria. Leuchner *et al.* (2004) gave an easy method for presumptive detection of *E. sakazakii* based on the presence of α- glucosidase enzyme. Isolation on α- MUG containing medium was reported by Oh and Kang (2004). They developed OK medium by modifying basal (TSA) medium in which nitrogen source and amount were optimized. This medium allowed growth of fluorescent colonies with in 24 h. Based on the same principle another method was proposed by Kandhai *et al.* (2004a). But they used colourimetric method for detection of α- glucosidase instead of nutrient agar supplemented with α-MUG used by Leuschner *et al.* (2004). Guillaume- Gentil *et al.* (2005) exploited salt tolerance property of *E. sakazakii* (Breeuwer *et al.*, 2003) and selectively enriched the organism in modified lauryl sulphate broth that is commonly used for coliforms. It was supplemented with 0.5 M NaCl and 10 mg/l vancomycin. Vancomycin was added just to inhibit Gram positive bacteria. The organism was grown even at 46°C up to 1 M NaCl. This method gave better yield than that by Leuschner *et al.* (2004). Voilet red bile glucose is one of the media currently used for isolation and enumeration (FDA, 2002) although it also shows better growth on faecal coliform agar (Leclerc *et al.*, 2001). Based on the same enzyme a chromogenic medium Druggan- Forsyth- Iverson Agar has been developed using 5- Bromo-4- chloro- 3- indolyl- α-D- glucopyranoside (Druggan *et al.*, 2004).

Yellow coloured colonies on various media as discussed above, can be preliminarily identified on the basis of biochemical tests as described earlier (Biering *et al.*, 1989; Cottyn *et al.*, 2001; Gassem, 1999; Kandhai *et al.*, 2004a; Monroe and Tift, 1979; Mosso *et al.*, 1994; Muytjens *et al.*, 1983; Nazarowec-White and Farber, 1999; No *et al.*, 2002; Seo *et al.*, 2003; Simmons *et al.*, 1989; Van Os *et al.*, 1996; Willis and Robinson, 1988). Both Enterotube II R and API 20E R have been used for presumptive confirmation. Always Gram negative, oxidase negative and catalase

positive colonies are carried further. However, API 20E biochemical strips have been found to give false results.

2.4.2 PCR Assays

To date PCR have been found to be the most reliable for preliminary identification. For identification of *E. sakazakii* PCR assays based on various genes have been developed. Lehner *et al.* (2004) developed PCR assay based on 16S rRNA gene based analysis for its identification. Seo and Brackett (2005) targeted the macromolecular synthesis (MMS) operon for identification at molecular level which can detect upto 100 CFU/ml. Iverson *et al.* (2006) applied Artificial Neural Networks (ANNs) to biochemical and 16S rDNA data and identified nucleotide sequences that gave 99.3 % discrimination of *E. sakazakii* from other loosely related species for both phenotypic and genotypic data and for other species positive for expression of the enzyme α-glucosidase the performance was 98.7 % for 16S rDNA sequence and 100% for phenotypic data. Liu *et al.* (2006) designed primers to detect 16S-23S rDNA internal transcribed spacer (ITS) of *E. sakazakii.* This PCR assay could detect upto 1.3 CFU/100 g infant formulae with the selective enrichment. Both of the PCR and oligonucleotide array procedures take only 48 h including the enrichment culture, whereas the conventional methods required at least 5 days. This study demonstrated that both of the pathogenic detections are time-saved and reliable. PCR based on α-glucosidase gene presents an excellent correlation with the 16S rRNA data (Lehner *et al.*, 2006a).

Further the isolates can be characterized phenotypically by biotyping and antibiotic profile or genotypically by ribotyping, random amplification of polymorphic DNA (RAPD) and pulsed– field gel electrophoresis (PFGE). Narazowec- White and Farber (1999) observed three ribotypes and four antibiogram patterns among *E. sakazakii* isolates. PFGE and RAPD profiles indicated minor differences between the isolates that were indistinguishable by ribotyping. The two techniques showed high degree of discrimination and enabled easy comparision of *E. sakazakii* isolates. Toxin assays, hemagglutination, serotyping and phage typing are also potential methods of analysis. Recently, Williams *et al.* (2005) have described a method to differentiate strains of *E. sakazakii* based on protein markers from the LC/MS chromatogram of bacterial cell lysates.

2.5 SOURCES OF *E. SAKAZAKII*

2.5.1 Clinical Specimens

E. sakazakii has mostly been isolated have been recovered from CSF, blood, sputum, throat, nose, stool, gut, skin, wounds, bone marrow, eye, ear and breast abscess (Farmer *et al.*, 1980; Urmenyi and Franklin., 1961; Joker *et al.*, 1965). The bacterium was isolated from respiratory tract of 17 patients in one hospital with in a period of 7 months. Bar- Oz *et al.* (2001) reported two cases in which the bacterium was isolated from blood and CSF of one patient. In other patient the bacterium was recovered from blood but CSF was sterile. The organism was also isolated from stool sample of three additional asymptomatic infants. Arseni *et al.* (1987) isolated *E. sakazakii* from throat and rectal swabs and tracheal aspirates but not from blood. Biering *et al.* (1989) isolated *E. sakazakii* from CSF of all the three patients they studied but from blood of only one patient, the other two being negative. Van Acker *et al.* (2001) isolated the organism from stomach aspirates, anal swabs and blood samples of 6 of the 12 neonates suffering from necrotizing enterocolitis. Willis and Robinson (1988) could retrieve the organism from blood and CSF samples of meningitis patients.

2.5.2 Environmental Sources

As *Enterobacter* species have been frequently isolated from soil, water, animals, sewage and human faecal samples (Sakazakii, 1974), it has been hypothesized that the primary reservoir for *E. sakazakii* might be environmental and plant materials, principle sources being water, soil and vegetables and secondary sources of contamination might be flies and rodents (Iverson and Forsyth, 2003). However, Muytjens and Kollee (1990) could not recover the organism from bovine milk, cattle, domesticated animals, rodents, birds dung, grain, rotting wood, mud, soil or surface water in the Netherlands. Kandhai *et al.* (2004) scanned samples from nine factories and 16 households. In factories the samples were collected by scrapping or sweeping surfaces in the production line environment or by sampling vacuum cleaner bags. Ten g of each sample was studied by Guillaume – Gentil *et al.* (2005) method. The organism was isolated from factories producing milk powder, cereals, chocolate, patato flour and pasta as well as domestic environment. The same group (Kandhai *et al.*, 2004) evaluated 152 dry samples from floor sweepings, spilled dry products, scrapings or vacuum cleaner bags and obtained the bacterium from 18 samples. Cruz *et al.* (2004) could isolate nine *E. sakazakii* strains from 20 dust samples and only one

from 48 water samples. Kuzina *et al.* (2001) isolated *E. sakzakii* from new and old colonies of Maxican fruit flies and Hamilton *et al.* (2003) were the first to isolate *E. sakazakii* from the midgut of the stable fly (*Stomoxys calcitrans*) larvae. These isolates formed mucoid or matt colonies. The organism has also been isolated from stethoscope and an uninoculated bottle of a bacterial culture medium (Farmer *et al.*, 1980). *E. sakazakii* has been isolated from one influent and one effluent sample of water circulated in iron pipes for 12 wks (Lee and Kim, 2003). In a survey of 26 thermal mineral water springs in Spain, 40 isolates of *Enterobacter* species including *E. sakazakii E. agglomerans, E. amnigenus* and six unidentified isolates were recovered from 13 springs (Mosso *et al.*, 1994). In the Netherlands Van Os *et al.* (1996) isolated *E. sakazakii* from grass silage. *E. sakazakii* has been isolated from floor drains, air, vacuum canister, broom bristles, a room heater and electrical control box, transition socks, clean- a- place valve, floor dryer, floor and condensate in a dry product processing environment in the United States (Gurtler *et al.*, 2005). There are many more reports of isolation of *E. sakazakii* from various environmental sources including air in hospital (Masaki *et al.*, 2001), clinical materials, soil, rhizosphere, sediments and wetlands, cutting fluids (Gurtler *et al.*, 2005) rats (Gakuya *et al.*, 2001) and crude oil (Assadi and Mathur, 1991).

2.5.3 Food Sources

Although natural habitat of the organism is not known it has been isolated from many food materials among which milk has been suggested as possible mode of transmission in many of the outbreaks (Muytjens *et al.*, 1983; Clark *et al.*, 1990; Nazarowec- White and Farber., 1997; Van Acker *et al.*, 2001; Anonymous, 2002; Leuschner *et al.*, 2004). Infact, Krieg and Holt (2005) stated that *E. sakazakii* was more prevalent in foods and the environment than in clinical settings. Out of 141 milk substitute infant formulae obtained from 35 countries, 14.2% were found positive for *E. sakazakii*; the count however was very less. Biering *et al.* (1989) isolated *E. sakazakii* from powdered- milk formula used in a hospital where neonatal infection had occurred. Van Acker *et al.* (2001) isolated *E. sakazakii* from prepared formula milk as well as unopened cans of a single batch. There was another case of isolation of *E. sakazakii* from an unopened can in 2001 (Himelright *et al.*, 2002; Wier, 2002). In another outbreak, powdered formula was negative for *E. sakazakii* but blender used in preparation was found positive (Noriega *et al.*, 1990) suggesting post-preparation contamination. The bacterium might get access to the powder from the environment or from the addition of ingredients at the powder stage

(Nazarowec- White and Farber., 1997a) but it has been proved that one strain of *E. sakazakii* (NCTC 8155) originated from dried milk only.

Six *E. sakazakii* isolates were detected in fermented bread (Gassem, 1999) and the *E. sakazakii* was most frequent even in a fermented beverage with pH ranging from 3.37- 5.53 (Gassem, 2002). Soriano *et al.* (2001) examined 370 food samples from restaurants in Spain and could isolate only one strain of *E. sakazakii*. Cottyn *et al.* (2001) recovered 20 strains of *E. sakazakii* from four seed lots of rice along with nine strains of *E. cloacae*. Nine strains of *E. sakazakii* were isolated from 50 samples of alfalfa sprouts in Maxico (Cruz *et al.*, 2004). The bacterium has also been isolated from water pipes and biofilms (Al- Hadithi and Al- Edani, 1995; Bartolucci *et al.*, 1996; Oliver, 1997) cheese, minced beef, sausage meat and vegetables (Leclercq *et al.*, 2002), mung bean sprouts (Robertson *et al.*, 2002), and also from rice starch, rice floor and eggs (Gurtler *et al.*, 2005).

2.6 SURVIVAL UNDER STRESS CONDITIONS

2.6.1 Temperature

2.6.1.1 Response to normal incubation temperature

When incubated overnight at 37 and 44°C in trypticase soy broth (TSB) the organism grows to nine logs. At 47°C shows some growth in 48 h. On TSA it forms 2-3 mm wide colonies. *E. sakazakii* has growth minimum, maximum and optimum temperature of 6, 45 and 37- 43°C depending on the growth medium. It has a generation time of 22 min at 37°C but it highly extends to 13.7 and 1.7 h at 6 and 21°C, respectively, in reconstituted infant formulae milk where the capsulated strains grow even at 47°C and produce exo-polysaccharide making the milk viscous (Iverson *et al.*, 2004). Nazarowec- white and Farber (1997b) determined the minimum growth temperature of 10 food and clinical isolates by inoculating rehydrated infant formula with 3.0 $\log_{10}$ CFU of *E. sakazakii* / ml. Minimum temperature ranged between 5.5 and 8.0°C (7°C for the ATCC 29544 type strain). Iversen *et al.* (2004) reported that six clinical and food strains grew between 6 and 45°C, with an optimum of 37–43°C, and Kandhai *et al.* (2004a) reported that the bacterium grew in reconstituted infant formula at 8–47°C. Studies have indicated that up to 20% of household refrigerator temperatures are kept at ≥ 10°C (Daniels, 1991; Harris, 1989; Rhodehamel, 1992 and Van Garde and Woodburn, 1987), thus providing temperature range at which *E. sakazakii* will grow. Nazarowec-White and Farber (1997b) reported the lag times and generation times for the ten *E. sakazakii* strains in infant formula held at 4, 10 and 23°C. No differences in behavior of these strains were noted among test infant

formulae and growth did not occur at 4°C. The lag times at 23 and 10°C were 2–3 h and 19–47 h, respectively, and mean generation time was 0.67 and 4.64 h respectively. *E. sakazakii* grew more rapidly than *Salmonella* or *E. coli.* Nazarowec-White and Farber (1997b) calculated that it would take at least 10 h for *E. sakazakii* initially at 1 CFU/ml to reach 7 $\log_{10}$ CFU/ml in rehydrated infant formula stored at room temperature, and much less time if held at 35–37°C. Iversen *et al.* (2004) reported that 70 strains grew to ca. 10^9 CFU/ml overnight in TSB at 37°C and 44°C but none grew within 24 h at 47°C. The growth phase of *E. sakazakii* inoculated into powdered infant formula had no significant effect on lag time or growth rate in reconstituted formula (Kandhai *et al.*, 2006).

2.6.1.2 Growth at high temperature

Althogh the organism cannot survive pasteurization it is among the most thermotolerant members of the Enterobacteriaceae family (Nazarowec- White and Farber, 1997b) hence may subsequently be present in desiccated products and reconstituted infant foods. However, post processing contamination cannot be ruled out in ultra high temperature processing (Skladal *et al.*, 1993). Caric (1993) observed that the presence of the organism in infant foods was due to post pasteurization contamination drying and filling area being most potent. But Breeuwer *et al.* (2003) studied the growth of *E. sakazakii* at 54, 56 and 58°C for different time intervals. For stationary phase cells the D- value at 58°C was in the range of 0.39- 0.60 min suggesting that the organism was not particularly thermoresistant. This was quite comparable to that of other Enterobacteriaceae members. However these observations were different from those given by Nazarowec- White and Farber (1997b). They reported D- value of 4.2 min at 58°C which is much higher than for most other Enterobacteriaceae. But both the studies proved that *E. sakazakii* cannot survive normal pasteurization. In a study by Iverson *et al.* (2004) all strains of *E. sakazkii* could grow at 37 and 44°C. No growth could be observed for any strain after 24 h at 47°C except one strain that grew at this temperature but only in IFM Ability to produce gas from lactose fermentation was less at 44°C than at 37°C. This was similar to that observed by Farmer *et al.* (1980) indicating that carbohydrate fermentation in *E. sakazkaii* is sensitive to temperature of 44°C and above. From 70 strains initially studied they selected six for further studies. All showed optimum growth temperature in the range of 37- 43°C. They compared D-value at 58°C to be 2.4 min compared to 4.2 min (Nazarowec- White and Farber., 1997) and 0.4 min (Breeuwer et al., 2003). Edelson-Mammal and Buchanan (2004) studied the ability of 12 strains to survive heating in rehydrated formula at 58°C and observed substantial variation among the

isolates. There was 20 fold differential between the most and least resistant strains. Half of the strains had D- value of less than 50 sec and the other half had D- value of more than 300 sec. The Z- value for the most heat resistant strain was 5.6°C (Table 2.2).

Edelson- Mammel *et al.* (2005) studied survival of clinical isolates of *E. sakazakii* in a rehydrated powdered formula for upto two year. During this period the sample was periodically rehydrated. Decrease in viable count during the initial 5 m was approximately 2.4 log cycles and in the subsequent 19 m additional 1 log decline was observed. Although a majority of cells were inactivated by storage in dehydrated powdered infant formula, a portion of cells was highly resistant to the storage conditions as concentration of *E. sakazakii* as even after 2 yr count was 300CFU/ml. Table 2.2 shows *D* and *Z* values of *E. sakazakii* studied by various workers.

Kim and Beuchat (2005) studied growth and survival of *E. sakazakii* on fresh cut fruits and vegetables when stored at 4, 12 and 25°C. The count either did not change or decreased at 4°C but grew at 12°C on all fruit except apple, strawberry, cabbage and tamato juices. At 25°C the organism grew on all fruit except strawberry. After 48 to 72 h the no of *E. sakazakii* cells decreased to < 1CFU/ml that coincided with decrease in pH and increase in lactic acid bacteria. Total count increased in all juices except strawberry at 25°C, and apple and strawberry juices at 12°C and at 4°C in cantaloupe, carrot, cucumber and lettuce juices.

2.6.2 Osmotic Pressure

Generally, bacteria protect themselves to increasing osmolarity by the rapid intracellular accumulation of ions, mainly K^+, followed by the accumulation of compatible solutes such as proline, glycine, betaine and trehalose (Kempf and Bremer, 1998). In a study of resistance of *Enterobacteriaceae* members to osmotic stress by Breeuwer *et al.* (2003), the cells were grown in 75% and 40% (w/v) sorbitol in BHI. Among all the species studied, *E. sakazakii* and *E. agglomerans* were the most resistant to osmotic stress in 40% (a_w 0.934) sorbitol which further decreased with *ca* 1 log after 2 m. However, *E. sakazakii* was sensitive to 75% (0.811) sorbitol concentration and only two strains were detectable after 4 wk. *Salmonella, E.coli, K. pneumonia, Serratia rubidea* and *C. freundii* strains decreased more than 6 log units with in two wk. Addition of compatible solutes did not improve the survival of the cells. Also, addition of glycine or betaine to *E. sakazakii* cells enhanced the growth in M9 medium in the presence of 1 mol l^{-1} which otherwise did not show any growth. Exponential phase cells were more sensitive to osmotic stress compared to stationary

pahse cells. Caubilla- Barron *et al.* (2004) studied survival of clinical and food isolates in dry infant formula milk and observed initial reduction of 2- 4 logs that increased to 6-7 logs in 6m. Compared to rehydration at ambient temperature rehydration at 60°C resulted in a 3 log reduction. Iverson and Forsyth (2003) attributed this character of survival under such conditions to capsule formation.

Table 2.2 Decimal reduction time (D-value) and Z-value for *E. sakazakii* in powdered infant formula.

D-value (min.) at various temperatures (°C)									Z-value (°C)	Reference
52	53	54	56	58	60	62	65	70		
54.8 ± 4.7		23.7 ± 2.5	10.3 ± 0.7	4.2 ± 0.6	2.5 ± 2				5.8	Nazarowec-White and Farber (1997b)
	8.3, 20.2	6.4, 7.1	1.1, 2.4	0.27, 0.34, 0.4, 0.48 0.50					3.1, 3.6	Breeuwer *et al.* (2003)
			21.1 ± 2.7	9.9 ± 0.8	4.4 ± 0.4		0.6 ± 0.3	0.07	5.6	Edelson-Mammel and Buchanan (2004)
		16.4 ± 0.67	5.1 ± 0.27	2.6 ± 0.48	1.1 ± 0.11	0.3 ± 0.12			5.8 ± 0.40	Iversen *et al.* (2004)
		11.7 ± 5.80	3.9 ± 0.06	3.8 ± 1.95	1.8 ± 0.82	0.2 ± 0.11			5.7 ± 0.12	Iversen *et al.* (2004)*

* Data for capsulated *E. sakazakii* strains

2.6.3 Dry Conditions

Breeuwer *et al.* (2003) studied the survival of stationary and exponential phase *E. sakazakii* under dry stress. After air drying the cell culture plates at 25°C for *ca* 1 h survival was studied upto 46 d. Viable count of stationary phase cells decreased to 1-1.5 log units in 46 d while *E. coli* under same conditions decreased to 4 log units. Decrease in viable count was more at 45°C and survival of cells was not improved

even after air drying in desiccator saturated with solutions of LiCl (aw 0.113), potassium acetate (a_w 0.225) or magnesium nitrate (a_w 0.529). *E. sakazakii* was most resistant among all the Enterobacteriaceae. But compared to stationary phase cells, exponential phase *E. sakazakii* was very sensitive to dry conditions showing 6 log reductions in 2 wk. However the survival was improved by addition of trehalose. Same workers (Breeuwer *et al.*, 2004) studied the genetic basis for survival of *E. sakazakii* under dry stress. Dessication results in induction of seven genes from heat shock regulon, four genes from the cyclic AMP receptor regulon, six genes involved in the stringent response and a number of genes involved in trealose synthesis and cell functions such as lipid A and lipopolysaccharide biosynthesis. This indicated that the response of *E. sakazakii* to dry conditions involves a genome- wide expression of functionally different groups of genes.

2.6.4 Effect of Other Processing Parameters

Studies have been carried to evaluate the effect of microvave heating on the destruction of microorganisms in milk which involve thermal as well as non thermal effects associated with electromagnetic radiations (Goldblith and Wang, 1967; Lechowich *et al.*, 1969; Najdovski *et al.*, 1991; Vela and Wu., 1979). Kindle *et al.* (1996) studied the effect of electromagnetic radiations (2450 MHz) on three strains. Five samples of rehydrated powdered infant formulae inoculated with five $\log_{10}$ CFU/ml were heated till boiling and then cooled. *E. sakazalii* was absent four of the five samples and that the one sample contained only 20 CFU/ml.

In recent years, there has been an increasing interest in the use of natural antimicrobial substances because of concerns regarding the safety aspects of the synthetic compounds (Abee *et al.*, 1995). The issue is especially important when selecting antimicrobials for use in infant foods. Numerous free fatty acids and their monoglycerides have been reported to possess inhibitory activities against a wide range of microorganisms, including enveloped viruses, Gram- positive and -negative bacteria and chlamydia (Bergsson *et al.*, 1998; Isaacs *et al.*, 1995; Petschow *et al.*, 1996). However the research has so far been focused on medium and long chain fatty acids. Caprylic acid is a natural eight carbon fatty acid present in breast milk and bovine milk has GRAS (Generally Recognized As Safe) status. Monocaprylin is a monoglyceride ester of caprylic acid. Nair *et al.* (2004) studied the antibacterial effect of monocaprylin on *E. sakazakii* in reconstituted infant formula. Effect of 0, 25 and 50 mM monocaprlin was determined in 10 ml samples of reconstituted infant formula

inoculated with 6.0 log CFU/ml of five strain mixture of *E. sakazakii*. The antibacterial activity of monocaprylin on *E. sakazakii* significantly increased with an increase in monocaprylin concentration from 25 to 50 mM. After one h of incubation at 37°C the 50 mM monocaprylin reduced the pathogen to undetectable level and 25 mM monocaprylin reduced the organism to < 2.0 log CFU/ml which further reduced to 1.5 log CFU/ml. Inhibition was a little less at 23°C. In 25 mM monocaprylin the pathogen had tendency to grow back, reaching back to 4.5 log CFU/ml. At 4°C 25 and 50 mM monocaprylin reduced the pathogen to undetectable level in 24 h whereas at 8°C the highest concentration of monocaprylin reduced the counts of the organism below detectable level in 24 h and 25 mM in 48 h.

Another non toxic biopolymer, Chitosan, present in crustaceans such as shrimp, lobster and crab has been found to show antibacterial activity and has been used as preservative in tofu (Chun *et al.*, 1999). *E. sakazakii* was one of the ten different bacteria isolated from tofu (No *et al.*, 2002). When antibacterial effect of six Chotisan and six Chitosan oligomers was studied in tofu immersion solutions it was observed that five of six Chitosans at a concentration of 0.1% exhibited strong inhibition and one exhibited weak inhibition, with an MIC of 400 µg/ml for all but lowest molecular weight chitosan.

2.7 PATHOGENIC POTENTIAL

2.7.1 Pathogenicity

Enterobacter are opportunistic pathogens and rarely cause infection in otherwise healthy individuals. About 25- 50% of neonatal meningitis and septicaemia cases are caused by many species of the family Enterobacteriaceae, among which members of genus *Enterobacter* have emerged as an important nosocomial pathogen. *Enterobacter* species are responsible for approximately half of all nosocomial by aquired infections and are aften implicated in co- infections (Nazarowec- White and Farber, 1997). In 1992, *Enterobacter* species were reported as being the fifth and third most common among those recovered from the urinary and respiratory tracts, respectively, of patients in intensive care units (Jarvis and Martone, 1992). Some common infective species of *Enterobacter*, particularly in newborns, include *meningitis*, *cloacae*, *aerogenes*, *agglomerans*, *hormechie*, *gargoviae* and *sakazakii* (Nazarowec-White and Farber, 1997; Weir, 2002; Willis and Robinson, 1988). Among the species of *Enterobacter*, *E. sakazakii* has usually been reported for neonates. However, to date it is not known whether this predilection for neonates reflects intrinsic

virulence or the organism's opportunity to be an early colonizer of infants (Sanders and Sanders, 1997). Little is known at the molecular level about the factors involved in the pathogenesis of *E. sakazakii*. *E. sakazakii* has been categorized as hazard A because it has been well established as a cause of illness in infants and has been found in powdered infant formulae which have epidemiologically been proved both to be the vehicle and source of infection in infants. The first report establishing the pathogenicity of illness due to *E. sakazakii* was published by Kleiman *et al.* (1981) wherein it was shown to be the causative agent in meningo-encephalitis in an otherwise healthy five wk old infant.

The specific virulence factors of *E. sakazakii* remain elusive; however, productions of exotoxins, aerobactin and hemagluttinin by *E. cloacae* have been documented (Keller *et al.*, 1998). *Enterobacter* species possess endotoxin as do the other Gram negative pathogens and therefore have all the properties imparted to this organism by this virulence factor (Biering *et al.*, 1989). However, Pagotto *et al.* (2003) studied infectivity and enterotoxin production in *E. sakazakii* using suckling mice assay and three cell lines– CHO, Vero and Y-1. Of 10 clinical isolates of *E. sakazakii* only three produced highly heat stable (boiling for 20 min) enterotoxin whereas one of the eight food isolates elaborated enterotoxin, although all the strains were lethal to suckling mice at 10^8 cfu per mouse by intraperitoneal injection but only two strains caused death by the *per os* route. In *in vitro* assays, CHO, Vero and Y-1 cells experienced both cell lysis and rounding when exposed to culture filtrates of *E. sakazakii* strain LA.

Another consistent source of many recalcitrant infections is biofilm formation. A biofilm is a population of cells growing on a surface and enclosed in an exopolysaccharide matrix (Iverson *et al.*, 2004). It is now recognized that biofilm formation is an important aspect of many bacterial diseases including native valve endocarditis, osteomyelitis, dental caries, middle ear infection and chronic lung infection in cystic fibrosis patients. Established biofilms can tolerate 10-1000 times the usual concentrations of antimicrobial agents and are also extraordinarily resistant to phagocytosis (Jefferson, 2004). *E. sakazakii* also produces viscous capsular material and therefore the organism could form a biofilm on feeding equipment and contact surfaces (Scheepe-Leberkuhne and Wagner, 1986). Following this Iverson *et al.* (2004) studied biofilm formation along with thermo- tolerance and growth profile of *E. sakazakii*. Biofilm formation was studied using sterilized 10 x 10 x 0.5-1.0 mm pieces

of latex, silicon and polycarbonate cut from infant feeding bottles and stainless steel. The organism formed biofilm on these surfaces but to a lesser extent on stainless steel. Recently Lehner *et al.* (2005) evaluated several features associated with the organism and are required for its persistence and survival. These included (i) biofilm formation and putative production of cellulose as one of the components of the extracellular matrix, (ii) adherence to hydrophilic and hydrophobic surfaces, (iii) the production of extracellular polysaccharides and (iv) the ability of *E. sakazakii* to produce cell-to-cell signaling molecules. It produces extracellular polysaccharides, composed of cellulose, capable to adhere to glass surfaces and a milky viscous mass composed of glucose, galactose, fucose, and glucuronic acid capable to adhere to both glass and air-solid interface of polyvinyl chloride microtiter wells. These workers also found two different types of acylated homoserine lactones (3-oxo-C6-HSL and 3-oxo-C8-HSL) used by *E. sakazakii* as cell-to-cell signaling molecules.

Adegbola and Old (1983) described fimbrial haemagglutinism in the genus *Enterobacter.* Two of four *E. sakazakii* strains were fimbriate, mannose sensitive, haemagglutinin positive, produced 7–8 nm thick fimbriae and tested positive with a strong antibody coating. The other two strains were non-fimbriate and haemagglutinin negative. They observed that with increasing haemagglutinating power, the proportion of fimbriate bacteria increased.

2.7.2 Epidemiology

Genus *Enterobacter* does not primarily contain human pathogens; however like other members of Enterobacteriaceae they are capable of causing opportunistic infections in hospitalized or debilitated patients. *E. sakazakii* is one such organism and is now emerging as a devastating pathogen. Among *Enterobacter* species *E. cloacae* is the most frequently isolated in human disease. Reported as uncommon human pathogen by Gallagher (1990) *E. sakazakii* organism has now been implicated as causative organism of severe form of meningitis– an acute inflammation of the membranes (meninges) of brain and sepsis.

Urmeyi and Franklin (1961) in England reported the first two known cases of neonatal meningitis caused by *E. sakazakii.* Both infants died with in 2 d of each other. It has also been reported to cause cysts in brain. Joker *et al.* (1965) reported a case of neonate in Denmark who remained well for the first 4 d of life and then began to show meningial signs, complicated by brain abscess and hydrocephalus. In 1965, the two groups together concluded that the causal organism was an uncommon *Enterobacter.*

Monoroe and Tift (1979) described the first reported case of neonatal *E. sakazakii* bacteraemia without meningitis. A case of severe meningitis, reported from Indiana, USA firmly established the pathogenicity of *E. sakazakii* (Kliemen *et al.*, 1981).

In 1987, Arseni *et al.* reported *E. sakazakii* colonization of neonates in a neonatal intensive care unit during 10 September to 17 October 1984. *E.sakazakii* was isolated from neonatal throat and rectal swabs and tracheal aspirates but not from blood. The duration of colonization ranged from 2 to 8 wk. These isolates were resistant to amikacin and tobramycin, but sensitive to gentamycin. Simmons *et al.* (1989) reported an outbreak of *E. sakazakii* infection involving four neonates, where it was suspected that infant formula was contaminated. *E. sakazakii* isolated from the formula had the same plasmid profile and enzyme electrophoresis profile as the organism isolated from the infants. In an outbreak in Iceland, strains of *E. sakazakii* isolated from three patients with neonatal meningitis were very closely related (Biering *et al.*, 1989). Clark *et al.* (1990) investigated two unrelated hospital outbreaks involving meningitis, bacteremia and colonization of patients. In 2001, Bar-Oz *et al.* described clinical aspects of *E. sakazakii* infection. In one case it was sepsis; another case sepsis and meningitis were complicated by cerebral infection and three cases had intestinal infection. Van Acker *et al.* (2001) reported an outbreak of necrotizing enterocolitis associated with *E. sakazakii* in powdered infant formulae. A cluster of infections due to nitrate-negative variants of *E. sakazakii*, occurred among premature neonates at the Hadassah Hospital, Mount Scopus, Jerusalem, in December 1999 - January 2000 (Block *et al.*, 2002). Removal of the environmental focus, a change to factory-prepared infant formulae, and segregation of affected infants controlled the event. Faecal carriage of *E. sakazakii* was observed for up to 18 wk, emphasizing the potential for cross-infection. Recent outbreaks include the death of a pre-term infant in July in New Zealand, and an outbreak in France (nine infected, four diseased and two deaths) in October-December. (International Food Safety Authorities Network, INFOSAN, 2004, http://www.whoafr.org).

Several outbreaks of *E. sakazakii* mediated meningitis have been reported worldwide (Table 2.3). Neonates and the younger children have been the most affected by the organism wherein the disease was fatal, debilitating or resulted in developmental deformities. Infants of HIV infected mothers are also at high risk because they may specifically require infant formulae and also they may be more susceptible to infection. Twenty cases of neonatal meningitis were reported globally by

1989 (Beiring *et al.*, 1989). The vast majority (83 %) of cases have been related to infants of less than one yr of age, where the fatality rate ranged from 30% –80 % despite antibiotic treatments (Muytzens *et al.*, 1983). Iverson and Forsyth (2003) reported at least 76 cases of neonatal meningitis by *E. sakazakii* worldwide in between 1958 and 2003. Infection with *E. sakazakii* may result in meningitis (58 %), necrotizing enterocolitis (29 %) or sepsis (17 %) particularly in premature and immuno-compromised infants (Adamson and Rogers, 1981; Arseni *et al.*, 1987; Biering *et al.*, 1989; Bar-Oz *et al.*, 2001; Burdette and Santos, 2000; Clark *et al.*, 1990; Gallagher and Ball, 1991; Gebremariam, 1998; Himelright *et al.*, 2002; Iversen and Forsythe, 2003; Joker *et al.*, 1965; Kleiman *et al.*, 1981; Lai, 2001; Monroe and Tift, 1979; Muytjens *et al.*, 1983; Nazarowec-White and Farber, 1997b; Noriega *et al.*, 1990; Sakata and Maruyama, 1997; Simmons *et al.*, 1989; Tekkok *et al.*, 1996; Urmenyi and Franklin, 1961; Willis and Robinson, 1988; Van Acker *et al.*, 2001). Lai (2001) reported 31 cases of *E. sakazakii* infection affecting neonates, infants, and children who were 3 d to 4 yr with half of them being one wk or less of age, and three quarters being less than one month of age. Risk of infection is more in immunocompromised, premature and underweight babies. The United States Food Net 2002 survey (htp//.www.cfsan.fao.gov) estimated that the rate of *E. sakazakii* infection among infants (based on isolation of the organism from sterile sites only) was one per 100000, whereas the rate among low-birth-weight neonates was 8.7 per 100000. Very few cases of *E. sakazakii* have been reported in adults who are considered a low risk group (Burdett and Santos, 2000; Gallagher and Ball., 1991; Kleiman *et al.*, 1981; Muytjens *et al.*, 1983 and Ries *et al.*, 1994). Mortality and morbidity of *E. sakazakii* meningitis is high and virtually all patients recovering from the central nervous system infection suffered from physical and mental abnormalities. Pathologic examinations showed evidence of meningo-encephalitis or cerebral necrotic hemorrhage and inflammation due to massive invasion by Gram negative bacilli.

Rusin *et al.* (1997) reported one case of negative effect of *E. sakazakii*, ingested in drinking water. Van Acker *et al.* (2001) reported the importance of relatively low levels of *E. sakazakii* in causing neonatal disease. Among five samples of infant formulae of the same batch four revealed less than one coliform cells/g while one sample yielded 20 coliform cells/g which caused the disease. The samples met the Codex Alimentarious Specifications. More stringent activity of the manufacturer

Table 2.3 Worldwide incidence of neonatal *E. sakazakii* infections

Location	Number of cases (deaths)	References
I Sporadic cases		
Denmark	(1)	Joker *et al.* (1965)
Georgia	1(0)	Monroe and Tift.(1979)
Macon	1	Monroe and Tift (1979)
Oklahoma	1(0)	Adamson and Rogers (1981)
Indiana	1(0)	Kleiman *et al.* (1981)
Greece	1(0)	Arseni *et al.* (1984)
Missouri	(1)	Naqvi *et al.* (1985)
Maryland	1(0)	Noreiga *et al.* (1990)
Ohio	1(0)	Gallagher and Ball (1991)
Winston Salem	1	Burdette and Santos (2000)
II Outbreaks		
England	2(2)	Urmenyi and Franklin (1961)
Netherlands	8(6)	Muytjens *et al.* (1983)
Greece	11(4)	Arseni *et al.* (1984)
Massachusetts	2(1)	Willis and Robinson (1988)
Tennessee	4(0)	Simmons *et al.* (1989)
Iceland	3(1)	Biering *et al.* (1989)
Boston	5	Lai (2001)
Belgium	12	Van Acker *et al.* (2001).
Israel	2	Bar- Oz *et al.* (2001)
Knoxville	10(1)	Himelright *et al.* (2002)

was used to control even this low incidence. Lehner and Stephan (2004) estimated that a dose having less than 10,000 cells of *E. sakazakii* would not arouse any disease symptoms in a human neonate. Pagotto *et al.* (2003) calculated an infective dose to be more than 10^7 cells.

2.7.3 Symptoms

E. sakakazakii infection results in meningitis, necrotizing enterocolitis, septicemia, desentry and osteomyelitis of the foot, pneumonia and biliary sepsis. Signs and symptoms at the onset of *E. sakazakii* infection in neonates include poor feeding, irritability, jaundice, grunting respiration, a full anterior fontanelle seizures and fever (Willis and Robinson, 1988). Meningitis leads to ventriculitis, brain abscess, infarction and cyst formation, conjunctivitis, bulging fontanelles, destruction of the frontal lobes of the brain, seizures, spastic quadriplegia, hypothermia, fever, Cheyne-Stokes respirations, bradycardia, instability of body temperature hemorrhagic cerebral

necrosis, meningoencephalitis, necrotic softened brain, cyst formation, liquifection of cerebral white matter and severe neurologic complications (Gurtlet *et al.*, 2005).

2.8 INFANT FORMULAE AS RESERVOIR

Production of dried infant formula from cow's milk has shown a steady increase since the beginning of this century. Today dried infant formula is a highly developed product and its manufacture is an important function of the dry milk industry (Knipschildt, 1986). Generally cow's milk is used in the manufacture of dried infant formula. Dried cow milk and milk products are potential sources of bacterial pathogenic to man. In one study coliforms were detected at populations of > 1 CFU/g in 3 of 124 samples of spray dried milk, 6 of 54 samples of the roller dried milk and 13 of 38 samples of infant formulae from 10 factories (Ghodker *et al.*, 1980). Populations of more than 90 CFU/ml were found in 25, 10 and 5 samples, respectively, and > 1 CFU/g in 3, 6 and 13 samples, respectively. Other than *Enterobacter*, bacterial pathogens found to be present in infant formulae included species of *Klebsiella*, *Citrobacter*, *Yersinia*, *Staphylococcus* and *Steptococcus* (Anderson *et al.*, 1984; Baldwin *et al.*, 1984; Casewell *et al.*, 1981; Fagerman, 1986; Gill and Gill, 1981; Muytjens *et al.*, 1988; Schroeder *et al.*, 1983; Simmons *et al.*, 1989). Buchanan *et al.* (2003) studied heat resistance of different strains of *E. sakazakii* and other members of Enterobacteriaceae. Powdered infant formula has an a_w of ca. 0.2 and is formulated so as to mimic the nutritional profile of human milk rather than cow milk (Breeuwer *et al.*, 2003). Nazarowec-White and Farber (1997a) noted the ways that cow milk is modified so as to achieve this goal, e.g., reducing protein and mineral content, increasing the amount of whey protein, increasing the carbohydrate content, increasing the Calcium/Phosphorus ratio, modifying the fat and adding vitamins. Production of powdered infant formula can be achieved either by 'wet' or 'dry' processing (Caric, 1993). It has been noted that chances of post processing contamination are more in dry method of preparation when dried skim milk is blended with essential ingredients (Table 2.4). A definitive statement concerning which process is inherently more or less likely to result in contamination of products with *E. sakazakii* cannot be made. This is because in-factory contamination is most likely to occur at some point between the spray drying and packaging steps.

Methods to control the enteric population in the drying and post-drying pre-packaging factory environments area is a critical criterian to prevent product contamination.

Table 2.4 **Incidence of Enterobacteriaceae and *E. sakazakii* in ingredients used in industrial/ commercial dry mixing operations for all types of powdered formula (up to 3 years)**

Ingredients Tested	No. of Samples	Number found positive for	
		Coliform or Enterobacteriaceae	*E. sakazakii*
Vitamins	793	8	0
Skim milk powder	835	1	1
Whey powder	23	3	0
Sucrose	1 691	28	0
Lactose	2 219	70	2
Banana powder/flakes	105	3	1
Orange powder/flakes	61	1	1
Lecithin	136	1	1
Starch	1 389	155	40

Factories will differ in many ways (e.g., age, building materials, design and cleanability) and those influence the level of efficiency in controlling the microbial ecology therein. Hence, risk of contamination of the final product can be expected to be a function of the particular factory environment rather than solely its manufacturing processes. *E. sakazakii* has been isolated at varying frequencies from infant formulae examined. One of the most notable surveys was conducted by Muytjens *et al.* (1988) in which the organism was detected in 14.9% of 141 samples of powdered infant formulae originating from 35 countries. Samples drawn from 13 countries were positive. *E. sakazakii* was the third most commonly isolated *Enterobacter*, just behind *E. agglomerans* and *E. cloacae;* however, none of these Enterobacteriacae members exceeded 1 CFU/g. Simmons *et al.* (1989) reported that *E. sakazakii* could out-compete *E. cloacae*, the second most common *Enterobacter*, despite *E. sakazakii* being present in products at lower populations than other bacteria. Muytjens *et al.* (1988) suggested that contamination of powdered infant formulae with Enterobacteriaceae must occur post-processing and following the final thermal treatment.

A number of workers have recovered *E. sakazakii* from powdered milk and powdered infant formula and thus confirmed or implicated powdered infant formula as a source of bacteria responsible for meningitis and associated with neonatal

necrotizing enterocolitis (Bar-Oz *et al.*, 2001; Biering *et al.*, 1989; Clark *et al.*, 1990; Muytjens and Kollee, 1990; Noriega *et al.*, 1990; Simmons *et al.*, 1989; Van Acker *et al.*, 2001). The CDC has recognized the direct link between the presence of *E. sakazakii* in powdered infant formula in an unopened can and an outbreak of *E. sakazakii* infection (Baker, 2002). An outbreak of *E. sakazakii* infection in Tennessee in 2001 was the first report from the United States that resulted in a voluntary recall of product by a manufacturer (Himelright *et al.*, 2002, U.S. Food and Drug Administration, 2002; Weir, 2002). Although epidemiologic investigation of microbial isolates from water and hospital environments failed to yield *E. sakazakii*. PFGE patterns of isolates from opened and unopened cans of infant formula were identical to those from the neonatal patient isolates. In a survey of 16 neonatal intensive care units, 25% reported using powdered infant formula as the sole food source while 31% reported using powdered formula in addition to other formulae (Himelright *et al.*, 2002).

2.9 HAZARD ANALYSIS AND RISK MANAGEMENT

It has been demonstrated that powdered infant formula is not commercially sterile and may harbour *E. sakazakii*. The issue of pathogens, particularly *Enterobacter sakazakii*, in powdered infant formula was brought to the attention of the 35th session of the Codex Committee on Food Hygiene (CCFH) by two separate processes (FAO/WHO meeting 2- 4 February 2004). The International Commission for Microbiological Specifications for Foods ranked the organism as serious hazard for restricted populations, life threatening or substantial chronic sequelae or long duration (ICMSF 2002). FAO/WHO is revising the Recommended International Code of Hygienic Practice for Foods for Infants and Children and this includes reassessing the testing of the specific pathogens associated with IFM. Richards *et al.* (2005) observed that the reconstituted infant rice cereal could support luxuriant growth of *E. sakazakii*. Therefore reconstituted cereal that is not immediately consumed should be discarded or stored at a temperature at which *E. sakazakii* and other food-borne pathogens cannot grow. Current codex advisory microbiological specifications for powdered infant formulae allow approximately 1-10 coliform bacteria/g formulae covering *E. sakazakii* of this group of coliforms. However this limit does not provide good safety as viewed by outbreaks caused by powdered infant formulae containing *E. sakazakii* even below this limit. Specific limits for *E. sakazakii* are presently being considered in Codex Committee on Food Hygiene (CCFH) deliberations. Powdered infant formula preparation interventions has been recommended as a means of preventing infections

caused by *E. sakazakii*. Suggestions included disinfecting blenders as well as boiling spoons, bottles and nipples prior to formula rehydration. Also storing the rehydrated formula at refrigeration temperatures as well as heating the preparation in a microwave oven just prior to feeding may prevent *E.sakazakii* infection (Gurtler *et al.*, 2005). However, bottle warmers may pose a risk of prolonged exposure to temperatures at which *E. sakazakii* can rapidly grow (Muytjens and Kollee, 1990). Iversen and Forsythe (2003) made recommendations focused on reducing the probability of infant infections caused by infant formulae. These include controlling the initial populations of *E. sakazakii* in raw materials on receipt, reducing populations during heat treatment of raw milk and related ingredients, preventing an increase in population of *E. sakazakii* by avoiding post-processing contamination, applying microbiological criteria and providing appropriate information and preparation instructions, e.g., labeling and consumer education. The 24th session of Codex Committee on Nutrition and Foods for Special Dietary uses (CCNFSDU) requested the CCFH to revise the Recommended International Code of Hygienic Practices for Food for Infants and Children in order to address concerns caused by pathogens those may be present in infant formulae (www.fsis.usda.gov). At the same time United States of America and Canada introduced a risk profile for *E. sakazakii* in powdered infant formulae for consideration by the committee.

FDA (2002) has alerted health care professionals about the risk of *E. sakazakii* infections in hospitalized newborns, infants, particularly premature infants or other immuno-compromised infants fed powdered infant formulae (www.fda.gov). Recently International Commission for Microbiological Specifications for Food (2002) ranked the organism as 'severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration' (Iversen *et al.* 2004).

Codex guidance also covers hygiene in manufacturing, different technological process, plant environment, consumer handling etc. Even the present technology doesnot seem to allow for the procedure of commercially sterile powders. Many concerned workers have recommended from time to time various control measures. FAO/WHO, CCFH in their 35th session (2003) also have made certain recommend-dations for global public health. The outcome of all these efforts is listed below:

1. Refrigeration to store dehydrated formulae
2. Infants should be exclusively breastfed for the first six months of life.

3. Infants who are not breastfed and who do not have access to banked donor milk, require a suitable breast milk substitute
4. Mothers and family members need to be adequately instructed for appropriate preparation (reconstitution/ rehydration) and use of the infant formulae.
5. Only the required amount of feed should be prepared at a time.
6. Use of boiled/ hot water 70-90°C for reconstitution and decreased holding time before feeding and during feeding would reduce the existing risk.
7. It should be made obligatory for the manufacturers to label that the powdered infant formula is not a sterile product.
8. Surveillance being important aspect to further reinforce risk management options it is recommended to all countries to improve surveillance system for investigating and reporting of sources and vehicles of infection by *E. sakazakii*, in order to access the level of potential underreporting. The information should be shared by all the participating laboratories.

2.10 FUTURE PROSPECTS

With in a short span of time since recognition of *E. sakazakii* as a cause of fatal neonatal meningitis much work has been done about the growth behaviour and other characteristics including methods to eliminate the organism from powdered infant formulae, thermal resistance, environmental reservoirs, pathogenicity, antibiotic resistance, exopolysaccharide production, development of rapid methods for detection, enumeration and identification and predictive modeling. However many facts of the problem are still undiscovered. Virulence factors responsible for *E. sakazakii* mediated misery are yet to be established. Potential correlations between pathogenicity and pigmentation, shape and texture of colonies DNase production and the use of other animal models and cell cultures as enterotoxin assay systems need to be investigated (Pagotto *et al.*, 2003). Although Pagotto *et al.* (2003) suggested the possibility of toxin production by the organism more work on this aspect is needed to be done. Genes involved in toxin production and other virulence factors need to be revealed. In the past toxins of many bacteria including *E. coli, Pesudomonas, Staphylococcus, Clostridium* have been isolated and purified. The successes of botulinum toxin as therapeutic agent, being used in treatment of neuromuscular disorders, have thrown some light on the way to study *E. sakazakii* toxin. Factors such as pH, aeration and growth phase on production of toxin *in vitro* also open a wide

area for study. As suggested by Lai (2001) tropism of the organism for the central nervous system in neonates and infants remains a mystery that should be deciphered. All these aspects may help in exploring advanced treatments for infections and eliminating the pathogen from the powdered milk or powdered infant formula. Baker (2002) recommended researching irradiation of powdered infant formula as an approach to control *E. sakazakii.* It was also suggested that research into protecting neonates from pathogens by using pro- or pre-biotics should be conducted. Detailed study of survival, growth and inactivation of the organism in infant formula is required. Iversen and Forsythe (2003) recommended further work to define the role of capsule production as it relates to desiccation resistance and thermal destruction, as well as characterization of virulence factors. Further investigation should also be done in the areas of phage typing, serotyping, virulence factors, tolerance to desiccation, heat and pH, lag times across a range of temperature and in an array of food matrices, biofilm formation and the use of bacteriocins, organic acids, disinfectants and other chemicals to control the growth of *E. sakazakii.*

CHAPTER - 3

Materials and Methods

The media, buffers and reagents used in the study and their compositions are given in Appendices I and II. AR or GR grade reagents were used in the study and were procured from different Indian companies like HiMedia Pvt. Ltd., S. D. Fine Chem Ltd., SRL and Qualigens. Molecular biology grade chemicals were obtained from Banglore Genei, Promega and Sigma. Taq polymerase, 10X Taq buffer, deoxyribonucleoside triphosphates, primers and DNA molecular weight markers were procured from Imperial Pvt. Ltd. (India). Double helix plasmid DNA ladder was procured from Banglore Genei Pvt. Ltd. Unless and otherwise specified water used was double glass distilled water for general purpose and HPLC grade water for molecular biology experiments.

3.1 SOURCES OF *E. SAKAZAKII*

3.1.1 Standard Culture

Standard culture of *E. sakazakii* MTCC- 659 was procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh- 160 036, India.

3.1.2 Isolation from Food and Environment Samples

Isolation of *E. sakazakii* was carried out from different environmental and food samples like river water samples from Delhi and Meerut areas, raw milk, cheese and skim milk powder from households and Experimental Dairy, NDRI and other organized dairies and infant milk formulae (IMF) and condensed milk from Karnal market. Clinical samples were obtained fron civil hospital Karnal. Two cultures suspected to be *E. sakazakii* isolated from goat faeces were obtained from a fellow worker.

3.1.2.1 Procedure for Isolation

Method described by Kandhai *et al.* (2004a) was followed with slight modifications for isolation of *E. sakazakii.* Twenty gram of sample was desolved in 225 ml buffered peptone water (pH 7.2). An inoculum of 10 ml from this pre-enriched culture was transferred into 90 ml of enterobacteriaceae enrichment broth. A loopful of culture from this enriched stock was streaked on

VRBL- agar and incubated at 37°C for 24 h. Typical coliform colonies on VRBL agar were streaked on TSA. Yellow colonies on TSA those were oxidase negative and catalase positive were selected and further confirmed by biochemical tests.

3.2 TENTATIVE IDENTIFICATION OF ISOLATES

3.2.1 Microscopic Examination

The microscopic examination included Gram staining and motility.

3.2.1.1 Gram staining

Standard Gram staining procedure was followed to perform and select the small Gram negative rods. The shape and arrangement of cells were observed.

3.2.1.2 Motility

"Hanging drop" method (Harrigan and Mc Cance, 1976) was performed to check the motility of the picked isolates. A small loopful of culture was transferred with a sterile wire loop to a clean grease free coverslip. Vaseline was applied on all the sides of the coverslip. A clean grease free cavity slide was inverted over the coverslip, so that the drop was in center, and pressed down gently to seal the coverslip in position. The slide was inverted quickly so that the droplet of culture was in the form of a hanging drop. The preparation was examined under objective.

3.2.2 Catalase

Slide method was used for catalase test. Using an inoculating needle, culture picked from a well- isolated colony was placed into a clean glass slide. A drop of 3 % hydrogen peroxide solution was added to this culture and closely observed for effervescence, caused by the liberation of oxygen, which indicated the presence of catalase enzyme.

3.2.3 Oxidase

Oxidase reaction was carried out by touching and spreading a well isolated colony on oxidase disc (HIMedia Pvt. Ltd.). Absence or presence of purple colour is observed within 5-10 sec at 25-30°C. Absence of any purple colour even after 60 sec indicated the absence of oxidase enzyme.

3.3 BIOCHEMICAL CONFIRMATION

Various morphological and biochemical tests were performed as per standard methods to identify the isolated cultures. The detail of media and reagents used has been given in Appendix I.The biochemical tests relied upon in the study were selected on the basis of previous reports regarding the identification of Gram negative *E. sakazakii* isolates (Farmer *et al.*, 1980). After incubation of plates for 24-48 h 30°C, catalase positive and oxidase negative yellow pigmented colonies were picked for activity. All the tests were performed on overnight grown cultures.Tests used are discussed as follows:

3.3.1 Methyl Red (Voges – Proskauer)

Both tests are performed on the same MR- VP broth as they are physiologically related. Medium was inoculated with the test culture. Acid production was detected by adding methyl red and acetyl methyl carbinol (VP test) was detected by addition of 0.6 ml of α- Naphthol, followed by 0.2 ml of 40% KOH with a pinch of creatine. Appearance of red colour on addition of methyl red indicated a positive VP test.

3.3.2 Indole Production

Tryptophan, an essential amino acid, is oxidized by some bacteria by the enzyme tryptophanase resulting in the formation of indole, pyruvic acid and ammonia. The test was performed by inoculating the bacterium into tryptone broth and indole production was detected by adding Kovac's reagent (dimethyl amino benzaldehyde). Appearance of cherry red colour ring on the surface indicated a positive reaction.

3.3.3 Citrate Utilization

The citrate test was performed by inoculating the bacterium into Simmon's Citrate agar where sodium citrate was the only source of carbon and energy and bromothymol blue was used as indicator. Change in colour of the medium from green to blue indicated positive reaction.

3.3.4 Hydrogen Sulfide Production

The test organisms were inoculated on Triple Sugar Iron Agar. Black precipitates after 24 h incubation indicates production of H_2S.

3.3.5 Nitrate Reduction

The ability of the selected organism to reduce nitrate to nitrite or form nitrogen gas was determined using nitrate reduction broth. Half ml of sulphanilic acid (0.8 ml in 5 N acetic acid) and α-Naphthylamine (0.5% in 5 N acetic acid) was added into the culture tubes after 24 h incubation of isolates at 37°C. The appearance of red or pink colour indicated nitrate reduction.

3.3.6 β-Galactosidase

The ability of the test isolates for β-galactosidase enzyme production was rapidly checked by using O-Nitrophenol β-D-Galactoside (ONPG) discs (HiMedia Pvt. Ltd., Mumbai). One substrate disc was put into the sterile test tube containing 100 µl of overnight active culture. The tube was incubated at 37°C, and observed for appearance of yellow colour, that indicated positive β-galactosidase activity. Yellow colour appeared within 10 min in case of fast β-galactosidase producers, while slow producers of the same required 1 h to show colour change.

3.3.7 Hydrolysis of Gelatin

The cultures were streaked on gelatine agar plates and incubated at 37°C for 24 h. Zone of clearance around the growth were observed for positive reaction, after flooding the plates with acidified mercuric chloride solution.

3.3.8 Urease Production

Urease agar slants were inoculated with the organism. After incubation at 37°C, the change in colour from yellow to pink indicated the positive test for urease production.

3.3.9 Aesculin Hydrolysis

To detect the ability of the organism to hydrolyse aesculin, the overnight grown young test culture was streaked on to esculin plates. The positive test was indicated by a dark brown to black colouration around the colonies.

3.3.10 Lysine Decarboxylase

To test for its ability to decarboxylyze lysine the culture was inoculated into lysine decarboxylase broth. Initially the colour of the medium changed from purple to yellow due to acid production. On further incubation, the change in colour again from yellow to purple indicated a positive reaction.

3.3.11 Ornithine Decarboxylase

The culture was inoculated into the ornithine broth to test the ability to decarboxylate ornithine. Initially the colour of the medium changes from purple to yellow due to acid production. On further incubation, the change in colour again from yellow to purple indicated a positive reaction.

3.3.12 Arginine Decarboxylase

To test the ability of the organism to decarboxylase arginine the culture was inoculated into arginine broth. Initially the colour of the medium changes from purple to yellow due to acid production. On further incubation, the change in colour again from yellow to purple indicates a positive reaction.

3.3.13 Phenylalanine Deaminase and Malonate Utilization

Both the tests were performed using phenylalanine malonate broth. Change in colour of the medium from light green to dark blue after overnight incubation indicated malonate utilization.

To detect phenylalanine deaminase, few drops of 10% ferric chloride solution were added to the freshly grown culture in phenylalnine malonate broth. Formation of dark green colour indicated a positive reaction.

3.3.14 Hippurate Hydrolysis

One hippurate disc was dispensed into a test tube containing 0.5 ml water to get 1.6 mg hippurate /ml (HiMedia Pvt. Ltd., Mumbai). A large loopful of inoculum was emulsified in the water and the tube was incubated at 37°C for 2 h. Five drops of ninhydrin reagent were then added and the tube was reincubated for 10 to 30 min and appearance of purple colour was observed. Sodium hippurate, when hydrolyzed, is broken down to benzoic acid and glycine. The ninhydrin reacts with glycine to form ammonia giving a purple colour.

3.3.15 Carbohydrate Fermentation

Isolates were grown overnight at 37°C in BHI broth and the cell pellet obtained by centrifugation at 10,000 x g for 5 min. The pellet was washed twice in sterile double glass distilled water and then resuspended in carbohydrate basal medium. This suspension was used as inoculum for sugar fermentation test. Determination of carbohydrate fermentation was done in phenol red broth (Ewing, 1986) containing 0.018 g phenol red dye /l as pH indicator. Three ml of

the basal medium was suspended in fermentation tubes. In each tube a specific sugar disc was added aseptically followed by inoculation of fresh and active culture @ 1%. The carbohydrates tested were adonitol, arabinose, cellobiose, dextrose, dulcitol, fructose, galactose, glycerol, inositol, inulin, lactose, mannitol, maltose mannose, mellibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose and xylose. A positive reaction was indicated by change in colour from red to yellow.

3.4 MOLECULAR CHARACTERIZATION

The biochemically identified isolates of *E. sakazakii* based on biochemical characteristics were subjected to molecular characterization for genetic confirmation of the species by 16S rRNA gene based PCR assay described by Lehner *et al.* (2004). The detailed composition of the reagents used in this study is given in appendix II.

3.4.1 Culturing of Bacteria

Biochemically identified *E. sakazakii* isolates were activated by passaging twice in TS broth at 37°C. The young cultures thus prepared were subcultured in BHI broth for genetic studies.

3.4.2 DNA Isolation and Purification

The cells from 1.5 ml of BHI broth cultures grown overnight were harvested in a microcentrifuge for 30 sec at 3,000 g. The supernatant was discarded carefully and this pellet was resuspended in 0.5 ml of SET buffer (pH 7.5) in a graduated appendorf. Lysozyme was added to the cell suspension at a concentration of 1 mg/ ml and incubated at 37°C for 1 h. Then 1/ 10 (one tenth) of its vol of 10% SDS and 0.5 mg/ ml proteinase K were added and further incubated at 55°C with continual inversions for 2 h. One third of its vol of 5M NaCl and one vol of chloroform: isoamyl alcohol (24: 1) were then added to the above mixture and incubated at room temperature for 30 min with frequent inversions. The contents were centrifuged at 4,500 rpm for 15 min and the aqueous phase was transferred to a new tube. The DNA was precipitated by adding one vol of ethanol and the tube was inverted gently. After centrifugation at 12,000 rpm for 15 min at 4°C, the DNA pellet obtained was washed with 70% ethanol and dried. The DNA pellet was finally dissolved in 200 µl of TE buffer (pH 8.0) containing 10 mg/ ml RNase A. The extracted genomic DNA

preparation along with the tracking dye was run on an agarose gel electrophoresis at 80 V for 80 min using 1 X TAE buffer and the gels were examined under UV transilluminator after staining with ethidium bromide (0.5 μg/ ml).

3.4.3 Primers

Oligonucleotide primers targeted against different regions were selected from the published information. The following specific *E. sakazakii* 16S rRNA gene targeting primers were used in PCR assay of the isolates.

Table 3.1 Oligonucleotide primers used in study

Organism and species	Primer	Target region	Primer sequence	Product size	Reference
Enterobacter sakazaki	Esak f Esak r	88-107 1017-998	GCTYTGCTGACGAGTGGCGG ATCTCTGCAGGATTCTCTGG	929	Lehner *et al.* (2004a)

3.4.4 Amplification

PCR was performed with the above primers for amplification of 16S rRNA gene. PCR amplification was carried out in a Biometra Thermocycler.

3.4.5 Preparation of Master Mix

The master mix was prepared by mixing all the reagents except the template DNA, separately in an appendorf and dispensed in PCR tubes in 23 μl quantity. Buffer (10X Taq assay containing 15 mM $MgCl_2$), dNTPs, primers and Taq polymerase were mixed and spun before dispensing to the PCR reaction tubes. Then DNA template was added (Table 3.2). The final vol was adjusted to 25 μl and the tubes were set to the PCR machine after giving a short spin. Control reaction mixture lacking DNA template was also included in each experiment.

Table 3.2 Details of PCR mix used in the study

Reagents	Vol (μl)
Sterile milli Q water	variable
Taq assay buffer	2.5
dNTPs mix	2.5
Primer mix	2.0
Taq DNA polymerase	0.3
Template DNA	2.0

3.4.6 PCR Conditions

The PCR programme used was as that given by Lehner *et al.* (2004). (Table 3.3)

Table 3.3 PCR programme for amplification of 16S rRNA gene of *E. sakazakii*

Initial denaturation	94°C/ 2 min
Denaturation	94°C/ 30 sec
Annealing	52°C/ 1 min
Extention	72°C/ 1 min 30 sec
Final extention	72°C/ 5 min
Number of cycles	30

3.4.7. Optimization of PCR Conditions

For better amplification of the template, to save time and to make the assay economic the PCR assay were optimized. The conditions that were optimized are shown in Table 3.4

Table 3.4 Optimization of different PCR conditions for better amplification

Annealing temperature	52- 64.5 °C/ 1 min
Primer concentration	9.96- 41.5 pM
dNTPs concentration	50- 300 nM
Taq polymerase concentration	0.2- 0.7 U
Amplification cycles	25, 30 and 35

a) Annealing temperature

A gradient PCR was set up wherein annealing temperature ranged from 52°C to 64.5°C. The optimal annealing temperature selected was 52.2°C for 1 min and was used for optimization of subsequent parameters. The PCR amplification parameters used included initial denaturation at 94°C for 2 min followed by denaturation at 94°C for 30 sec, annealing at 52.2°C for 1 min, extension at 72°C for 1.5 min followed by final extension at 72°C for 5 min.

b) Taq polymerase concentration

The PCR assay was performed using different concentrations of Taq polymerase ranging from as low as 0.2 units to as high as 0.7 units.

c) Primers concentration

In order to optimize the amplification of both sets of primers i.e. different concentrations ranging from 9.96 pM to 41.5 pM in different combinations, were explored for elucidating best amplification in PCR assay.

d) dNTPs concentration

In order to optimize the amplification of DNA different concentrations ranging from 50 nM to 300 nM were explored for elucidating best amplification in PCR assay.

e) Number of amplification cycles

The PCR cycling steps were repeated for 25, 30 and 35 times at 52°C for 1min.

3.4.8 Agarose Gel Electrophoresis

The DNA samples extracted from broth as well as PCR amplified products obtained with different templates were electrophoresed on the agarose gels (1.5%) following the procedure given by Sambrook *et al.* (1989).

3.4.8.1 Agarose gel

Agarose gel of 1.5% concentration was prepared by dissolving the appropriate quantities of agarose in 1X TAE buffer (pH 8.0) in microwave oven or by keeping in boiling water bath. Ethedium bromide stock solution was added directly to molten agarose solution at the rate of 0.5 μg/ ml before casting the gel (mini/ midi/ maxi).

3.4.8.2 Procedure

Molten agarose was cooled to 50°C and poured on to respective moulds of minigel (50 ml) and midi gel (100 ml) using appropriate comb (8-20). The surface was leveled before pouring the gel. After complete setting of the gel, the comb was removed carefully and the gel plate was mounted on respective electrophoresis tanks (Tarsons Products, India). The respective electrophoresis tanks were filled with 1 X TAE buffer to cover the gel to a depth of about 1 mm. The DNA samples were mixed with 5 μl of tracking dye and were loaded slowly into the slots of submarine gel using micropipette. Electrophoresis was carried out at 100 V (60 mA current) for 1 h in minipore electrophoresis apparatus and

1.5 h in case of maxi gel system. After completion of electrophoresis, the gel was taken out of the chamber and examined under UV transilluminator.

3.4.9 PCR Product Purification

To 180 µl of PCR product 440 µl of 100% ethanol was added and kept in ice for half an hr. After centrifuging at 12,000 rpm for 15 min at 4°C, ethanol was drained off. Pellet was dissolved in 300 µl of 70% ethanol and spun at 12,000 rpm for 5 min at 4°C. Ethanol was drained off and pellet was dried at 37°C for 10 min. The dried pellet was stored at -20°C.

3.4.10 BLAST Analysis

The PCR product got identified by (Banglore Genei Pvt. Ltd., India) by matching 16S rRNA sequence homology with the existing data base. The matching of sequence homology was done using BLAST analysis (http:// www.ncbi.nlm.nih.gov/BLAST/).

3.5 SUSCEPTIBILITY TO ANTIBIOTICS

Antibiotic susceptibility of the selected *E. sakazakii* isolates was carried out on BHI agar medium following disc diffusion method of Bauer *et al.* (1966) with slight modifications. The antibiotic discs (HiMedia Pvt. Ltd., Mumbai) and their concentrations used in this study included amikacin (30 mcg), amoxycillin (25 mcg), ampicillin (10 mcg), bacitracin (10 units), carbenicillin (100 mcg), cefuroxime (30 mcg), cephalothin (30 mcg), chloramphenicol (30 mcg), chlortetracycline (30 mcg), ciprofloxacin (5 mcg), clindamycin (2 mcg), erythromycin (15 mcg), gentamycin (10 mcg), kanamycin (30 mcg), methicillin (5 mcg), metronidazole (5 mcg), neomycin (30 mcg), novobiocin (30 mcg), penicillin (10 units), polymyxin B (300 units), rifampicin (5 mcg), streptomycin (10 mcg), sulphamethizole (300 mcg), tetracycline (30 mcg), tobramycin (10 mcg), vancomycin (30 mcg).

Twenty ml aliquots of molten BHI agar were poured in sterile petriplates. Actively grown each test culture of *E. sakazakii* was poured on specific plates and spread uniformly with the help of sterile cotton spreader. A set of four antibiotics was carefully placed equi-distantly over the agar surface and the plates were incubated in inverted position for 48 h at 37°C. The plates were then examined for clear zones of inhibition around each disc.The diameter of

the zone was measured with a foot rule at three different angles and recorded excluding that of the disc (6 mm). The results were interpreted as sensitive, resistant or intermediate by comparing the zones of inhibition formed around each disc on the plates with the standard table supplied by the manufacturer (HiMedia Pvt. Ltd., Mumbai).

3.6 MAINTANANCE OF THE CULTURES

All the isolates were maintained on tripticase soy agar slants and sub-cultured after every 25- 30 d. The stock of all the isolates was preserved in 20% glycerol stock medium at -70°C. The cultures were activated prior to use by sub culturing twice in tripticase soy broth.

3.7 SURVIVAL AT DIFFERENT TEMPERATURES

3.7.1 Growth at Different Temperature

The test cultures were streaked on TSA plates and incubated at 4 ± 1°C for 10 d, 37 ± 1°C, 45 ± 1°C and 55 ± 1°C for 1-2 d. The plates were observed for growth after appropriate incubation.

3.7.2 Determination of D and z value

Method of Hilton *et al.* (2001) was followed. Cells were harvested by centrifugation (3500 x g for 20 min) and suspended in 2 ml potassium phosphate buffer (0.1 M, pH 7.3). It was again centrifuged and resuspended in 2 ml phosphate buffer. It was then distributed in thin 4 mm tubes. These were placed in water baths and D and z values were studied at 55, 62 and 72°C. Samples were taken at 0, 20, 40 and 60 min at 55°C, at 0, 1, 2 and 4 min and at 0, 30 and 60 sec at 72°C. In each experiment a control was maintained at 37°C. The decimal reduction times were calculated from the slope of the best-fit line graphically determined by plotting the $\log_{10}$ of the number of viable *E. sakazakii* cells/ml versus heating time at each temperature respectively.

3.8 EFFECT OF SODIUM CHLORIDE ON GROWTH OF *E. SAKAZAKII*

Salt tolerance was studied in BHI broth (initially containing 0.5% NaCl) and this broth supplemented with additional five concentrations i.e. 0.5%, 1%, 2.5%, 5% and 10% of NaCl separately, thus making the final salt concentrations to 0.5% (normal BHI), 1%, 1.5%, 3.0%, 5.5% and 10.5%. One

hundred ml of BHI broth was dispensed in 250 ml conical flasks, inoculated with 1% overnight grown test *E. sakzakii* cultures and incubated at 37°C for up to 32 h. Aliquots were drawn at 0, 4, 8, 12, 16, 20, 24 and 28 h to measure the bacterial growth by SPC and the total cell mass as OD_{600} using PYE UV- Vis spectrophotometer.

3.9 PRELIMINARY VIRULENCE FACTORS

The potential of the isolates for their virulence was preliminary determined by testing for their ability to produce accessory virulence factors.

3.9.1 Haemolytic Activity

Biochemically confirmed *E. sakazakii* isolates were first evaluated for haemolytic activity using blood agar plates. Overnight grown cultures were streaked on agar plates and incubated at 37°C for 3-4 d (Keller *et al.*, 1998). The appearance of zone of clearance around the colony, if any, due to the breakdown of RBC by the metabolites released by organisms indicated haemolysis and the positive isolates were selected.

3.9.2 Haemagglutination Test

3.9.2.1 RBC preparation

Bovine blood was collected using anticoagulant and washed in three volumes of RBC diluent by mixing and centrifuging at 1500 rpm for 15 min and aspirating until the supernatant was completely clear. The pelleted cells were suspended to a 50% suspension in diluent and stored at 4°C and used with in 2 wk.

3.9.2.2 Procedure

The method used to test hemagglutination was that described by Adegbola and Old (1982). The cultures grown overnight were centrifuged at 2,000 x g for 15 min. The pellet was resuspended in physiological saline (0.85% NaCl). The test was performed by mixing equal volumes (50 µl) of erythrocytes and cell suspension on a glass slide. It was undisturbed for 10 min and agglutination was observed under microscope.

3.9.3 DNase Production

To test the production of DNase enzyme the isolates were streaked on DNase test agar and incubated at 37°C (Smith *et al.*, 1969). Then carefully flood the surface of the plates with 1 N hydrochloric acid. Colonies producing DNase hydrolyse the deoxyribonucleic acid (DNA) content of this medium located in their immediate vicinity. If the medium is then flooded and acidified with 1 N HCl, the DNA precipitates out (turbidity) and clear zones appear around DNase-positive colonies.

Isolates showing haemolytic, haeagglutinin and DNase activity were selected as potential virulent isolates and were subjected to more tests to determine their potentiality for virulence.

3.10 SELECTION OF POTENTIALLY VIRULENT ISOLTAES

3.10.1 Resistance to Serum Bactericidal Activity

The resistance of the bacterial strains to serum bactericidal activity was tested by the viable count assay described by Pelkonen and Finne (1987).

3.10.1.1 Serum preparation

Normal serum was obtained from a healthy bovine. Blood was allowed to clot for 30 min at room temperature. The blood was kept overnight at 4°C and then centrifuged at 4°C, pooled and stored at -70°C before use. To heat inactivate the complement, serum was incubated at 56°C for 30 min. Ethylene (glycol-bis-β-aminomethylether) tetra-acetate (EGTA) and $MgCl_2$ were added into the normal serum in the final concentration of 10 mM and 5 mM, respectively to abolish the classical pathway activity of the complement system.

3.10.1.2 Culture preparation

An overnight culture in broth was diluted 1: 10 in fresh medium and incubated at 37°C for 90 min on a rotary shaker (170 rpm). Cultures were let to cool down to 4°C slowly, centrifuged at 4°C at 1500 x g for 15 min. The pellet was suspended in cold phosphate buffered saline (PBS), pH 7.4 and kept on ice until use.

3.10.1.3 Procedure

Serum and bacteria were mixed in the same ratio and incubated at 37°C. Duplicate samples of 20 µl were drawn at 0, 45, 90 and 180 min. The samples

were diluted and plated on agar plates for viable counts. Conrol was maintained by incubating culture without addition of serum. A survival fraction of less than 40% was interpreted as sensitive, 40-90% as intermediate and more than 90% as resistant (Taylor, 1974).

3.10.2 Aerobactin Production

Aerobactin production was assayed against *E. coli* by the method of Carbonetti and Williams (1984). *E.coli* indicator strain *E.coli* LG1522 was grown to approximately 5 x 10^7 bacteria/ml in 3 ml of M9 broth supplemented with 0.4% glucose, 50 µg/ml thiamine and 200µM α,α'– dipyridyl. This culture was pour plated in M9 agar medium. Then wells were made in the plate and *E. sakazakii* test strains grown in the same broth medium were put in the wells and the plates were incubated for 48 h at 37°C.

3.10.3 Gelatinase

Gelatin hydrolysis was examined on gelatin agar plates. The cultures were streaked on the gelatin agar plates and incubated at 37°C for 24 h. Zones of clearance around the colonies were looked for, when the plates were flooded with acidified mercuric chloride solution.

3.10.4 Neuraminidase Test

Neuraminidase was determined by the thiobarbituric acid method of Warren (1959) assaying the cleavage of 1 µM of N- acetylneuraminic acid from an excess of egg white substrate.

3.10.4.1 Preparation of test organism

Overnight culture of *E. sakazakii* was centrifuged at 5,000 x g for 15 min and the pellet was resuspended in 2 ml distilled water. It is then frozen (-65°C), thawed (20°C) and centrifuged at 20,000 x g for 15 min. The supernatant was maintained.

3.10.4.2 Procedure

Neuraminidase activity of the supernatant was measured by incubation with the substrate i.e. egg albumin at 37°C for 30 min.

Reagents:

a) 0.2 M $NaIO_4$ in 9 M phosphoric acid

b) 10% sodium arsenate in 0.5 M Na_2SO_4, 0.1 N H_2SO_4

c) 0.6% thiobarbituric acid in 0.5 M Na_2SO_4, 0.1 N H_2SO_4

d) Cyclohexanone

Sample (2 ml) was mixed with 0.1 ml of per-iodate solution in a test tube shaken and kept at room temperature for 20 min. Arsenate solution (1 ml) was added and shaken until yellow brown colour disappeared. Thiobarbituic acid (3.0 ml) was added, shaken and the test tube was capped with a glass bead and heated in vigorously boiling water for 15 min. It was then placed in cold water for 5 min, where red colour faded and solution became cloudy. One ml of this solution was added to 1 ml of cyclohexane and centrifuged for 3 min. (The intensity of red colour in upper cyclohexane layer was more intense than that in the aqueous phase). Absorbance of cyclohexane phase was observed at 549 nm and concentration of neuraminic acid was calculated using the formula

$$\mu M \text{ of neuraminic acid} = \text{Vol.} \times \text{OD} / 57$$

where V = vol, OD = optical density at 549 nm and 57 = molecular extinction cofficient

3.10.5 Hyaluronidase

The ability of the isolates to produce hyaluronidase was determined by ACRA (acid-Congo red- alcohol) test (Oakley and Warrack, 1951). Sample of bovine synovial fluid (8 ml) was serially diluted and mixed with equal vol of culture filtrate and incubated for one h. It was then cooled and Congo red solution was added and mixed into it, and drops of the mixture were then allowed to fall into acid alcohol. If no hyaluronidase was present, the drop fell as a firm stable blue blob through the acid alcohol; if the hyaluronic acid in the synovial fluid was destroyed by hyaluronidase, the drop turned blue but rapidly diffused into the acid alcohol.

3.10.6 Elastase

3.10.6.1 Dying of elastin with Congo red

The staining of elastin suspension was done according to Naughton and Sanger (1961). Two g powdered elastin was suspended overnight in a saturated aqueous solution of Congo red. The elastin was washed well with water until the washings were clear of dye and it was then dried by washing successively with acetone and ether.

3.10.6.2 Procedure

The culture supernatant was added to 3.5 ml of the elastin suspension in 0.05 M- Na_2CO_3 - HCl buffer (pH 8.8) in a 15 ml centrifuge tube. The test culure was added to this elastin suspension and incubated. The amount of digestion was estimated by centrifuging down the undissolved elastin and determining A_{495} of the supernatant solution in a spectrophotometer after 24 and 48 h. A_{495} for a 0.1% solution of digested dyed elastin was about 1.0. The undissolved elastin solution was then resuspended in the supernatant and the digestion allowed continuing. All assays were done at room temperature.

3.10.7 Collagenase

The method used was that as described by Steffen and Hentges (1981). The tests cultures were inoculated in tripticase yeast extract medium supplemented with glucose (0.25%), hemin (10 µg/ml) and vitamin K1 (1 µg/ml) containing approximately 10 mg of collagen. The cultures were incubated at 37°C. The extent of collagen degradation was visually determined.

3.10.8 Phospholipase C

Egg yolk emulsion

Egg yolk emulsion was prepared by a modified method (Aggarwal and Srinivasan, 1987). After through washing rinsing and drying of fresh hen egg the shell was cracked by a gentle stroke of a sterile scalpel over the air sac area and the white poured off. The yolk sac was punctured with the tip of a 25 ml pipette and the yolk was slowly sucked in through a vacupet and transferred to a sterile reagent bottle containing sterile physiological saline in a quantity equal to the vol of yolk mixed to get 50% egg yolk emulsion. The mixture (emulsion) was tempered to 45°C in a water bath and held for 1 h. It was then cooled and maintained in a refrigerator to allow the precipitate formed to settle. The clear emulsion thus prepared lasted for more than two months. The decanted clear fluid was used to enrich the basal medium (Appendix 1).

Complete medium

The basal medium was autoclaved separately and maintained in refrigerator. Three ml of 50% egg yolk emulsion was mixed into 100 ml of it just before inoculation. After incubation for 24 h at 37°C a positive result was

indicated by an insoluble precipitate surrounding the growth. Breakdown of phosphatidylcholine (lecithin) destroys the colloidal nature of egg yolk and the resulting precipitate causes the opacity in the medium around the microbial growth.

3.10.9 Presence of Lipopolysachcharide

Overnight grown cultures were centrifuged at 10,000 rpm and the pellet was resuspended in 2 ml of 50 mM sodium phosphate buffer to which EDTA and $MgCl_2$ were added to a concentration of 0.01 M each. This bacterial suspension was placed in water bath and the temperature was raised to 70°C. An equal vol of preheated phenol (90% w/v) was added. The suspensions were cooled to 15°C. It was then centrifuged at 18,000 x g for 15 min. The aqueous layer was retained and the phenol layer was discarded. Pooled aqueous phase was dialyzed against water until there was no phenol odour (Johnson and Perry, 1976). SDS- gel electrophoresis was performed with 14 % separating gel fortified with 4 M urea (Tsai and Frasch, 1982).

3.11 ROLE OF PLASMID IN VIRULENCE

3.11.1 Isolation of Plasmid DNA

Plasmid DNA was purified by alkaline lysis method (Sambrook *et al.*, 1989). This involved following steps:

3.11.1.1 Harvesting of bacteria

A single bacterial colony was transferred into 2 ml of BHI broth and incubated at 37°C for 24 h. The culture was centrifuged at 12,000 x g for 5 min. The supernatant was discarded and the pellet was dried.

3.11.1.2 Lysis by alkali

The pellet was resuspended in 100 µl of ice cold sol. I and mixed vigorously by vortex mixer. Two hundred µl of freshly prepared sol. II was added to the cell suspension. The tubes were tightly closed and the contents were mixed rapidly by inverting the tubes 5 times. Then the tubes were stored on ice. After adding 150 µl of ice cold sol. III.The tubes were closed and vortexed gently in an inverted position for 10 sec to disperse sol. III through the viscous bacterial lysate. The tubes were further stored on ice for 5 min. The tubes were centrifuged at 12,000 x g for 5 min at 4°C and the supernatant was

transferred to a fresh tube. After adding 2 vol of ethanol at room temperature the contents of the tubes were vortexed and the mixture was allowed to stand for 2 min at room temperature. The mixture was then centrifuged at 12,000 x g for 5 min at 4°C. Supernatant was discarded and the pellet was dried. The pellet was rinsed with 70% ethanol at 4°C. The pellet was dried and redissolved in 20 µl of TE buffer and stored the extracted plasmid at -20°C. Gel electrophoresis of plasmid was carried out using 0.8% agarose gel at 80 V for 45 min.

3.11.2 Plasmid Curing

The high temperature exposure procedure of Asheshov (1966) was followed for knocking out plasmid (s). Overnight broth culture of *E. sakazakii* was diluted ¼ in fresh BHI broth and incubated at 37°C for 1 ½ h with aeration. From this 0.04 ml of culture was then added to each of two tubes containing 5.0 ml nutrient broth one pre-warmed to 37°C and the other 43-44°C, respectively. The tubes were then incubated at the appropriate temperatures. Antibiotic profile of wild type cultures and plasmid cured cultures was compared. The colonies showing loss of antibiotic resistance were picked up, cultured and studied for the presence of plasmid by gel electrophoresis. The cultures showing loss of plasmid were then studied for virlence characters that were present in wild type.

3.11.3 Virulence in Plasmid Cured Cultures

The virulence related properties were studied in plasmid cured cultures and observed if there was any loss of virulence in cultures lacking plasmid. If the cultures still showed the virulence it was inferred to be chromatid encoded and not related to plasmid.

3.12 SCREENING OF ISOLATES FOR ENTEROTOXICITY

All the isolates of *E. sakazakii* and the reference strain were screened for toxin production in casamino yeast extract medium (Evans *et al.*, 1973) after incubation at 37°C for 18 h. Culture was centrifuged at 17,000 rpm and the supernatant was tested for its toxic activity by suckling mice assay (Dean *et al.*, 1972). Suckling mice (3-4 d) were separated from their mothers immediately prior to testing and were divided into groups of four. Each mouse was

inoculated intragastrically with 0.1 ml of the crude culture centrifugate containing 1-2 drops of 2% Evans blue dye. Approximately 4 h after dosing, mice were euthanized with chloroform. The abdomens of the mice were opened and the entire intestinal tracts, were removed with sterile forceps. Upon opening the peritoneal cavity, intestines were visually examined for distention and fluid accumulation. Intestines from each group of four were pooled and weighed and the ratio of gut weight to remaining carcass weight was calculated. Results were considered positive when the ratio was ≥ 0.083. The most potentially toxic isolate was carried further for toxin purification.

3.12.1 Protein Estimation

The protein content of the culture supernatants was measured by the method of Lowry *et al.* (1951) using Bovine Serum Albumin (BSA) for standard curve (Fig3.1).

3.12.1.1 Stock reagents

a) 1% w/v copper sulphate - reagent A
b) 2% sodium potassium tartrate – reagent B
c) 0.2 M sodium hydroxide – reagent C
d) 4% w/v sodium carbonate – reagent D

These reagents were maintained at room temperature.

3.12.1.2 Lowry *et al* method of protein estimation

1. Added 1 ml of reagent A followed by 1 ml of reagent B in the mixture of 49 ml each of reagent C and D. The resultant copper - alkali solution (reagent E) was prepared fresh when needed.
2. Added 10 ml of water to 10 ml of Folin Ciocalteau reagent to prepare reagent F.
3. Added 2.5 ml of reagent E to 0.5 ml of sample.
4. Mixed and let it stand for 10 min.
5. Then added 0.25 ml of reagent F to it, mixed and let stand for 30 min.
6. Measured absorbance at 660 nm against a blank of 0.5 ml buffer in which the protein preparation has been made.

3.13 OPTIMIZATION OF ENTEROTOXIN PRODUCTION

The casamino yeast extract medium for the production of *E. coli* toxin (Evans *et al.*, 1973) was employed for supporting the maximum production of *E. sakazakii* toxin. Some nutritional and physical conditions of growth and enterotoxin production by the most potential isolate obtained previously were studied to optimize its enterotoxin production in the laboratory. The composition of the medium has been given in Table 3.5.

Table 3.5 Casamino yeast extract medium for enterotoxin production

Ingredients	Amount (g / l)
Casamino Acids	20
Yeast extract	6.0
NaCl	2.5
K_2HPO_4	8.71
Trace salts	1ml/l
pH	8.5

Trace salts: Trace salt mixture consisted of 5% $MgSO_4$, 0.5% $MnCl_2$ and 0.5% $FeCl_3$ dissolved in 0.001 N H_2SO_4.

The above medium was optimized for maximum production of toxin with respect to nitrogen sources, temperature and period of incubation. The toxicity of the culture filtrate of the most potent isolate at each variable was tested by the suckling mice assay.

3.13.1 Nitrogen Sources

In an attempt to increase enterotoxin production by *E. sakazakii* the strength of casamino acids in the medium were varied 1 and 2% or these were replced by peptone, tryptone and tryptose separately. Medium with initial pH of 7.0 was inoculated (1%) with the potent culture in 500 ml flasks, incubated at 37°C for 24 h in a shaker incubator.

3.13.2 Temperature of Incubation

Evans'medium for toxin production dispensed in different flasks was inoculated (1%) with the selected culture and incubated at 25, 37 and 45°C (Jacks and Wu, 1974).

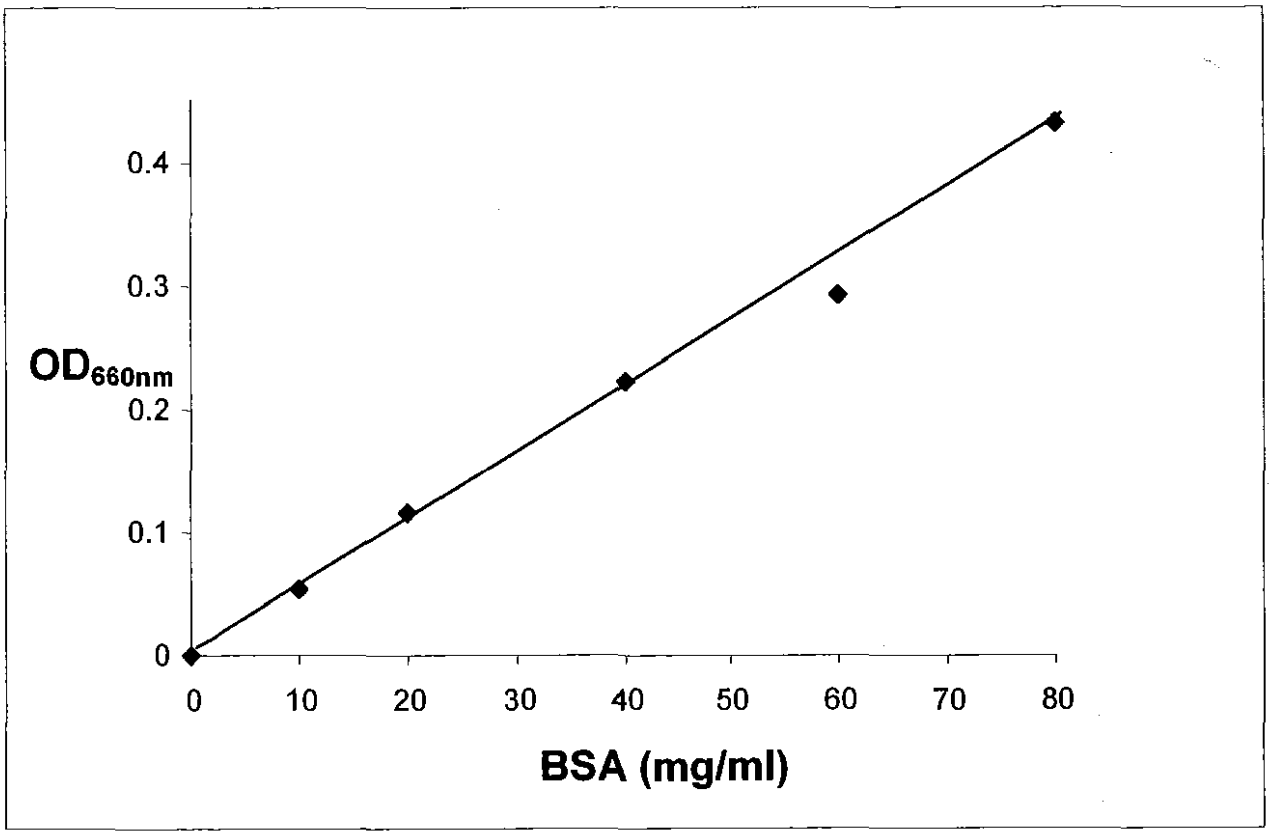


Fig 3.1 Standard curve for Bovine Serum Albumin (BSA)

3.13.3 Incubation Time

Casamino yeast extract medium for toxin production was inoculated with the selected culture, dispensed in 5 different flasks and incubated at 37°C. Samples drawn at time intervals of 6 h, 12 h, 18 h and 24 h. The culture filtrates obtained therefrom were assayed for toxin production (Jacks and Wu, 1974).

3.14 PURIFICATION OF ENTEROTOXIN

3.14.1 Precipitation with Ammonium Sulphate

Two liters of the casamino yeast extract medium finally devised for maximum toxin production was inoculated with 1% culture of the most potential toxic isolate and incubated in a shaker incubator at 150 rpm for 18 h at 37°C. Cells were removed by centrifugation at 17,000 x g for 30 min followed by membrane filtration (0.2 µm, mdi, India) of the supernatant. The toxic activity and the protein content of the supernatant were determined.

Ammonium sulphate was slowly added to the filtrate with constant stirring to achieve 80% saturation, stepwise at 4°C. After addition was complete, it was left to stand for 10 min to ensure equilibrium. The mixture was kept overnight in the cold room and then precipitates were removed by centrifugation at about 12,000 x g for 45 min at 4°C. The precipitate pellet was dissolved in 30 ml of 0.02 M tris (hydroxymethyl) aminomethane (Tris) buffer, pH 8.0. It was then dialyzed (1000 MWCO) against 5 liters of same buffer. The dialysates were assayed for protein content and toxicity.

3.14.2 Ion Exchnage Chromatography

The dialyzed sample was centrifuged at 15,000 x g for 20 min to separate insoluble particulate matter. The supernatant was subjected to ion exchange chromatography (Callahan, 1974).

3.14.2.1 Preparation of the coloumn

Anion exchange chromatography was performed using diethylaminoethyl (DEAE) cellulose. DEAE cellulose was allowed to swell overnight in double glass distilled water. It was then decolourized by washing in 0.5 N NaOH. It was then acidified with 0.5 N HCl followed by washing with dist. water to free it

from acid. Again swollen DEAE cellulose was washed in NaOH, filtered and washed free of alkali with dist water.

3.14.2.2 Charging the column and elutions

Then this thoroughly washed gel was poured in a (13 x 2.5 cm) column. The column was equilibrated with 0.05 M tris (hydroxymethyl) aminomethane buffer (pH 8.0). Then whole of the dialysed toxic protein solution was loaded on to the column at a rate of 5 ml/10 min and allowed to adsorb on to the matrix. The protein was eluted by stepwise gradient of NaCl in 0.05 M tris (hydroxymethyl) aminomethane. The fractions showing enterotoxigenicity in suckling mice were pooled together and further subjected to gel filtration.

The resultant fractions of toxin positive peak were pooled together and passed through centricon of cut off size10 kDa with a centrifugal force of 5,000 rpm at 4°C. Both retentate and filtrate were tested for toxicity. The retentate showing toxicity was further subjected to gel filtration.

3.14.3 Sephadex G-100 Gel Filtration

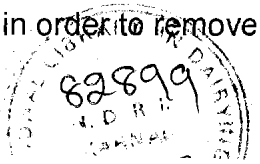
Size exclusion chromatography of the positive fraction from ion exchange chromatography was carried out on Sephadex G -100 column (fraction range 4 kDa to 150 kDa; pH range 1 – 14; workable temp. range 4-40°C; column size, 47 x 2.5 cm) with some modifications in the method described by Jacks and Wu (1974).

3.14.3.1 Preparation of the column

Phosphate buffer (0.05 M, pH 8.0) was filtered through 0.2 µm membrane and degassed. The swollen Sephadex was degassed by heating in boiling water bath for 4 h, cooled to room temperature and was poured into 47 x 2.5 cm column. The packed column was equilibrated with phosphate buffer. Blue dextran 2000 was used to measure the void vol. Fractions of 5 ml were collected at a flow rate of 38 ml/h.

3.14.3.2 Charging the column and elutions

All the fractions of the peak of ion exchange chromatography were pooled together and concentrated by dialysis against PEG 6000. The sample was centrifuged at 17,000 x g for 15 min before injecting the sample into the gel filtration column in order to remove any precipitated proteins (just to avoid



blocking of column). Two ml of this concentrated sample was injected into the column with the help of a syringe. Elution of the sample was carried out at a constant flow rate (38 ml/h) of 0.05 M NaH_2PO_4 – the elution buffer. Fractions each comprising 5 ml of the eluted sample were collected in Eppendorf. The fractions exhibiting toxicity were pooled and their protein content was determined. The column was washed with two bed vol of elution buffer and stored in 1% sodium azide prepared in the same buffer until further use.

3.15 CHARACTERIZATION OF TOXIN PRODUCED BY *E. sakazakii*

Both the crude as well as partially purified toxin preparations were further characterized.

3.15.1 Protein estimation

Protein content in the purified as well as crude protein was estimated by a combination of Lowry *et* al. (1951) method.

3.15.2 Carbohydrate estimation

Carbohydrate content of toxin preparation after each step of purification was estimated by the method proposed by Dubois *et al.* (1956).

3.15.3 Determination of molecular weight of toxin by SDS –PAGE

The molecular weight of the toxin was determined by SDS-PAGE as per the protocol of Laemmli (1970).

Reagents

Acrylamide/ bisacrylamide solution (30%)

Acrylamide	29.2 g
N, N' - Mehylene-bis-acrylamide	0.8 g

The total vol of reagent was made upto 100 ml with deionised water and the solution was stored in an amber coloured bottle at 4°C.

10% SDS

Ten g SDS was dissolved completely in 80 ml water by gentle warming and final vol was made to 100 ml with double glass dist. water. The solution was stored at room temperature.

4X Running gel buffer: (1.5 M Tris-HCl, pH 8.8)

18.15 g Tris- base was dissolved in 80 ml water. The pH was adjusted to 8.8 with 1 N HCl and the final vol was made to 100 ml with dist. water.

4X Stacking gel buffer (0.5 M Tris-HCl, pH 6.8)

Six g Tris-base was dissolved in 80 ml water and the pH was adjusted to 6.8 with 1N HCl. The final vol was made to 100 ml with dist. water.

Sample buffer

Ingredients	Quantity(ml)
SDS solution (10%)	1.6
Glycerol	0.8
2-Mercaptoethanol	0.4
1% (w/v) bromophenol blue	0.4
Tris-HCl, 1 M, pH 6.8	1.0
Deionised water	3.8

Ammonium persulphate solution (10 %)

The solution was prepared freshly each time by dissolving 100 mg ammonium persulphate in 1 ml of distilled water.

Electrode buffer 5X (pH 8.3)

Trizma Base	9.0 g
Glycine	43.2 g
SDS	3.0 g

The ingredients were dissolved in 600 ml of deionised water. The stock solution was stored at 4°C. Sixty ml of stock 5 X buffer was diluted with 240 ml of deionised water for one electrophoretic run.

Staining solution

Methanol	40 ml
Glacial acetic acid	10 ml
Brilliant blue	0.25 g

Final vol was made upto 100 ml with dist. water.

Destaining solution

Methanol	40 ml
Glacial acetic acid	10 ml

Final vol was made upto 100 ml with distilled water.

3.15.3.1 Sample preparation

The protein sample was diluted at least 1:4 with sample buffer and heated at 95°C for 4 min.

3.15.3.2 Preparation of electrophoresis gels

The separating gel and stacking gels were prepared as detailed in Table 3.6

3.15.3.3 Procedure

Glass plates were thoroughly cleaned with an acetone-swabbed tissue paper, separated by spacers and fixed onto the gel casting assembly. A 10-well comb was inserted. Subsequently, the comb was removed. The solutions were pipetted as indicated above and deaerated. The separating gel was quickly poured to the mark followed by careful overlaying with spacer gel solution. This operation was completed within 30 min. The well-forming comb was inserted into the spacer gel to make wells for sample loading.

Table 3.6 Components of separating and stacking gels

Components	Separation gel (12%) - ml	Stacking gel (4%) - (ml)
Water	1.675	3.05
Buffer	1.25	1.25
SDS (10%)	0.05	0.05
Acrylamide solution	2.0	0.665
Ammonium per sulphate (10%)	0.05	0.05
TEMED	0.005	0.005
Total	5.0 ml	5.0 ml

3.15.3.4 Electrophoresis

The gel was transferred to the electrophoresis apparatus (Tarson, India) and comb was removed. The wells were rinsed with double glass dist. water followed by running buffer. The chamber was filled with the buffer. A vol of 10 µl of molecular weight marker was loaded in Lane 1 followed by 15 µl of the treated protein samples in other lanes. The electrophoresis was carried out at a constant current of 25 mA until (approx. 4 h) the marker dye was within 0.5 cm of the end of the gel. After that, the power supply was disconnected and the gel was removed carefully. The gel was stained after fixing in the fixative.

3.15.3.5 Staining and destaining of the Gel

The gel containing protein markers was allowed to float freely in a glass tray containing staining solution and kept on an orbital shaker for 4 h at room temperature. The gel was then transferred to destaining solution in the glass tray till bands become visible against light background (approx. 2 h). The destaining solution in the tray was replaced with the fresh one at 30 min intervals. The destained gel was observed for the protein bands.

3.15.4 Effect of heat on toxicity

The Sephadex G -100 peak material was dissolved in dist. water at 200 µg/ml. After heating the enterotoxin at 50, 70, 90 and 100 °C for 30 min, the activity was studied in suckling mice. Enterotoxin without heat treatment was taken as control.

3.15.5 Effect of pH on toxicity

Sephadex second peak material was dissolved in 0.01 M phosphate buffer (pH 7.0) to a final concentration of 200 µg /ml. Then pH of the samples was adjusted to 4.5, 6, 7 and 8 with 5 N NaOH or 1 N HCl (Jacks and Wu, 1974). The samples were incubated for 4 hrs at 37°C and then each was adjusted to pH 7. Thereafter the toxicity was tested in suckling mice. Control was maintained at pH 7.0.

3.15.6 Effect of proteolytic enzymes on toxicity

The effect of pronase and trypsin was studied at a protein concentration of 200 µg/ml. Enzyme, 0.1% solution in phosphate buffer, was added to the substrate to make the final substrate-enzyme ratio 25:1. The samples were incubated at 30°C for 24 hrs. Substrate without enzyme was also included as control. After incubation the mixture was diluted to a final concentration of 200 µg/ml (Jacks and Wu, 1974).

3.15.7 Effect of metal ions on toxicity

The effect of Ca, Cu and Zn was studied on toxic peak material by adding $CaCl_2$, $CuCl_2$ and $ZnCl_2$ to a final concentration of 0.05% and incubated for 5 h.The effect of metal ions was then studied by suckling mice assay.

3.15.8 LD_{50} of purified toxin

LD_{50} was evaluated by preparing serial dilution of the Sephadex gel filtration fractions up to 10^{-8}. Ten groups containing four mice each were made. One group was used as control that was fed sterile broth without culture. Each dilution was injected in suckling mice as per Dean *et al.* (1972) and the minimum concentration that could kill 50% mice in each group was considered as the LD_{50} dose of the toxin.

CHAPTER - 4

Results and Discussion

The findings of the present study related to detection and isolation of *Enterobacter sakazakii*- a newly emerging neonatal pathogen, from various environmental sources, characterization of the isolates based on morphological, biochemical and molecular approaches, evaluation of their virulence characteristics including enterotoxicity as well as toxin production, its purification and characterization have been presented in this chapter in the form of tables and figures. The results have also been discussed with respect to the the already available reports.

4.1 ISOLATION OF *E. SAKAZAKII*

4.1.1 Selection of Colonies from Different Sources

In this investigation a total of 170 environmental samples were screened for the presence of *E. sakazakii*. The samples were collected from 9 different sources (Table 4.1). From among a large number of bacterial colonies recovered on TSA during 24-48 hr of incubation, 45 probable colonies were picked up on the basis of typical colony characteristic (golden yellow pigmentation) as was observed in reference culture MTCC 659. However a wide variation was observed in morphology of these colonies. Some colonies were comparatively bigger in size, bright lemon yellow with rough surface. These were somewhat dry, hard and sticked to the agar surface. While the others were small golden yellow colonies with smooth surface. Some were opaque and dull and others were shiny, translucent. These colonies were invariably sticky (Fig 4.1). Pre-enrichment in a non selective medium was practiced to revitalize the weak and injured cells. Thereafter, Enterobacteriaceae enrichment (EE) broth was used to increase the counts of enterobacteria. Thereafter, specific media like VRBLA was used to isolate Enterobacteriaceae. *E. sakazakii* formed typical coliform colonies on the medium as already reported (Kandhai *et al.*, 2004a). These colonies were isolated, examined visibly, microscopically and by oxidase and catalase positive.

4.1.2 Preliminary Identification of the Isolated Colonies

4.1.2.1 Microscopic examination

All 45 selected isolates were examined microscopically. Based on microscopic examination all appeared as Gram negative rods, arranged either single or in pairs (Fig 4.2) exhibited true motility by the hanging drop method.

Table 4.1 Retrival of *E. sakazakii* from environment

S. No	Samples		Sources	No. of samples		Positive samples (%)	No. of Isolate
				Screened	Yeilding Yellow colonies		
1	River water		Delhi, Meerut	20	16	5	1
2	Raw milk		Experimental Dairy	25	3	4	1
			Households	6	0	0	0
			Other organized Dairies	19	6	5.3	1
3	Pasteurized milk		Experimental Dairy	25	0	0	0
			Other organized Dairies	17	0	0	0
4	Cheese		Experimental Dairy	6	0	0	0
			Other Organized Dairies	3	4	0	0
5	Skim milk		Experimental Dairy	5	0	0	0
6	Condensed milk		Karnal Market	5	0	0	0
7	Infant formula milk		Karnal Market	16	5	31.25	5
8	Clinical samples	Blood	Civil Hospital, Karnal	6	0	0	0
		Stools		4	4	25	1
		CSF		4	1	0	0
		Throat swab		5	4	0	0
9	Goat faeces		Cattle yard	4	2	50	2
Total				170	45	6.47	11

4.1.2.2 Catalase and oxidase tests

All the 45 isolates obtained from the study were subjected to catalase and oxidase test. Of those, characteristic combination of being catalase positive and oxidase negative was exhibited by only 24 isolates (53.33%). These were

Fig 4.1 Yellow pigmented colonies of *E. sakazakii* on TSA

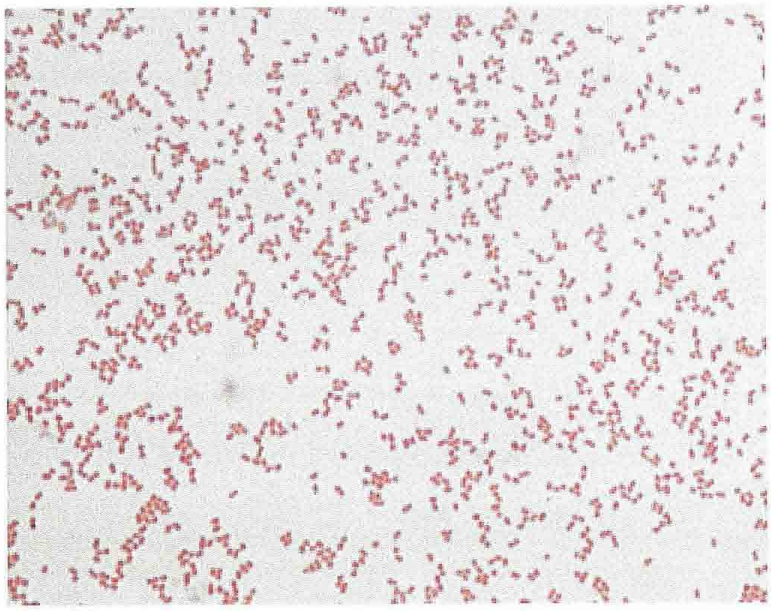


Fig 4.2 Gram stained smear of an *E. sakazakii* isolate observed under oil immersion

therefore tentatively considered to be *Enterobacter sakazakii* and were carried further for biotyping for confirmation. These 24 isolates were named as IFM1, IFM2, IFM3, IFM4, IFM5 (from infant formulae milk) RW1, RW2, RW3, RW4, RW5, RW6, RW7, RW8 (from river water) T1, T2 (from throat swab) CSF1 (from cerebral spinal fluid) S1, S2, S3 (from diarrhoeic stools), RM1, RM2 and RM3 (from raw milk) and GF1, GF2 (from goat faeces).

4.1.3 Biochemical Characterization of the Selected Isolates

All 24 short listed isolates on the basis of catalase and oxidase test and microscopic examination were further subjected to biochemical characterization. The observations were interpreted as per the criteria followed by Farmer *et al.* (1980). The related observations have been summarized in Appendix III.

Based on biochemical properties carried out in the study it was observed that all the 24 isolates (100%) as well as the reference strain *E. sakazakii* MTCC 659 produced citratase as these could utilize citrate as sole source of carbon but failed to produce indole from tryptophan breakdown. None of the *E. sakazakii* isolates produced urease. They also didnot produced H_2S from FeS. Among them 9 (36%) isolates produced acid from glucose as evidenced by methyl red reduction test, 21 (84%) positive for VP test out of which 11 (52.38%) showed delayed reaction, 21 (84%) hydrolysed esculin, 18 (72%) were nitrate reductase positive as they reduced nitrate to nitrite, 15 (60%) produced lysine decarboxylase and 13 (52%) produced ornithine decarboxylase, 9 (36 %) were positive for arginine deaminase reaction, 13 (54.2%) could hydrolyse gelatin (out of which 9 i.e. 81.81% showed delayed reaction only after 6 d), 9 (36%) were positive for phenyl alanine reaction out of which 4 (44.4%) showed delayed reaction after 3 d and 15 (60%) were positive for malonate reaction. Among the total isolates 22 (88%) showed positive reaction on ONPG discs for β-galactosidase production

Sugar fermentation of the isolates revealed more or less similar to that shown by reference culture. All the 24 isolates could ferment dextrose (except RW2, RW5 and T2 which showed varied response), fructose, galactose, maltose, mannose, mannitol, salicin, trehalose, xylose and negative for inulin. Mellibiose could be fermented by 16 (64 %) of the *E. sakazakii* strains,

arabinose by 10 (40%) and sorbitol by 6 (24%), sucrose by 22 (88%), dulcitol and adonitol by 6 (24%) isolates. Four (16%) isolates fermented cellobiose after 3 d while the remaining 21 (84%) showed positive reaction after 2 d only. Except two, all the isolates (92%) fermented lactose, 10 (40%) fermented inositol, 18 (72%) raffinose and 6 (24%) isolates fermented of glycerol. One isolate (RM3) showed varied activity for sorbitol and glycerol fermentation. Eight isolates reacted identical to the reference strain.

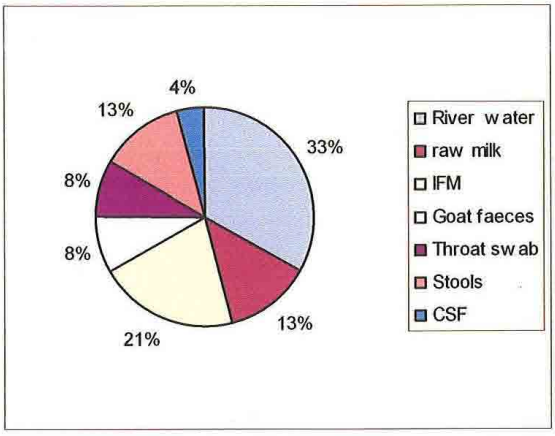
On the basis of biochemical and sugar fermentation tests 11 (45.8%) isolates *viz.* IFM1, IFM2, IFM3, IFM4 and IFM5, RW8, S2, RM2, RM3, GF1 and GF2, were identified as typical *Enterobacter sakazakii.* Among the remaining, 3 (12.5 %) were identified as *Ewingella Americana* (RW2, RW5 and T2) 6 (25.0%) as *Enterobacter cowanii* (RW1, RW6, RW7, CSF1, S3 and RM1) and 4 (16.6%) were identified as *Pantoea agglomerans* (RW3, RW4, T1 and S1) as per Kreik and Holt (2005). The data pertaining to the same are presented in Fig 4.3. The findings of the present study corroborated with those of earlier workers. The variation in ornithine, arginine and phenyl alanine tests and a few sugar fermentation tests with respect to the reference strain indicated the possible strain difference which has not so far been studied in detail.

These organisms, occurring along with *E. sakazakii* and giving yellow pigmentation, have been reported to be cause of human malaise.

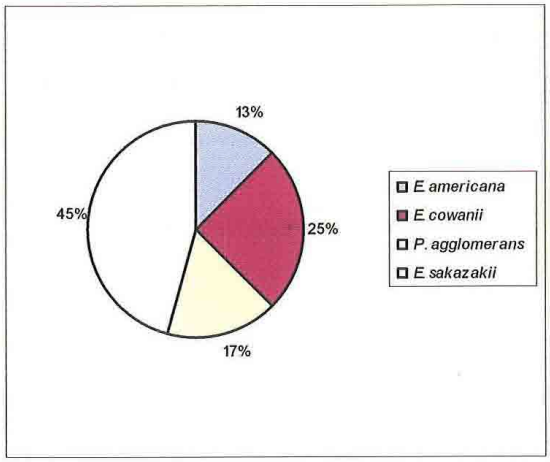
Ewingella americana has been isolated from throat swab of a diarrhoeal patient and from yamuna river water sample. It has been reported that *Ewingella americana* is usually isolated from clinical samples such as blood, urine, throat sputum and wounds. It has been isolated from outbreaks of bacterimia. It is an opportunistic pathogen with a low ability to cause extraintestinal infections in humans (McNeil *et al.*, 1987).

Pantoea agglomerans was proposed as a type species of new genus *Pantoea* to separate from genus *Enterobacter* (Gavini *et al.*, 1989). Strains of *E. agglomerans* may occur in clinical samples, blood, wounds, sputum, urine, often with dubious clinical significance (Von, 1970; Gilardi and Bottone, 1971; Pien *et al.*, 1972).This bacterium is ubicutous in environment.

Enterobacter cowanii which is also aften pigmented has been isolated from clinical specimens but its clinical significance is unknown.



A)



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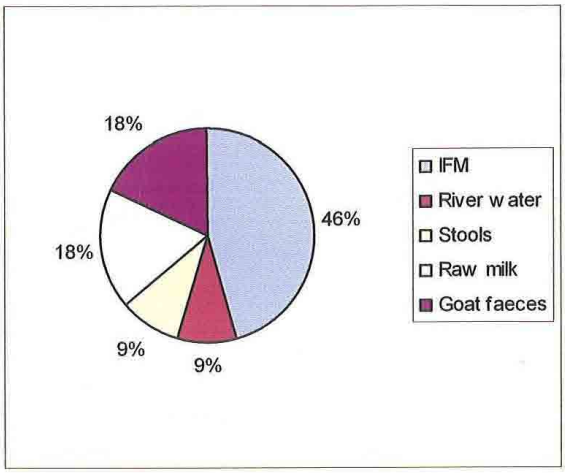


C)

Fig 4.3 A) Occurrence of yellow pigmented catalase positive & oxidase negative isolates in various samples

B) Identification of Yellow pigmented colonies on the basis of biochemical characterization

C) Occurrence of *E. sakazakii* in various samples

The occurrence these organisms along with *E. sakazakii* did not specify, there morphological, physiological or generic relatedness to *E. sakazakii.* However a detailed study about the occurrence of these organisms with *E. sakazakii* may form a separate study out of the scope of the present one.

The organism was retrieved mainly from river water, raw milk, faecal matter and IFM but was absent in other processed milk and its products or body fluids. However its presence in high heat treated IFM is alarming and may be mainly attributed to post pasteurization contamination through fortification and handling lapses. Such contamination for various other organisms is not unknown.

After tentative identification by biochemical tests the isolates were characterized at molecular level by PCR assay.

4.2 MOLECULAR CHARACTERIZATION

4.2.1 PCR Amplification of Partial 16S rRNA Gene

Gemomic DNA isolated from each *E. sakazakii* strain could be amplified by specific primer targeted against 16S rRNA partial gene. The approximate size of the PCR amplified product product was found to be 929 bp. PCR conditions like annealing temperature, number of amplification cycles and concentration of taq polymerase, primer and dNTPs were optimized to save time and make the assay more economic.

4.2.1.1 Annealing temperature

Annealing temperature was optimized by setting a gradient PCR at annealing temperature of 58°C with a gradient of 6°C and lid temperature of 105°C. PCR was carried out in 12 different tubes at annealing temperature of 52.0, 52.2, 52.8, 53.9, 55.3, 56.9, 58.5, 60.2, 61.7, 63.0, 63.0 and 64.5°C. The PCR results have been illustrated in Fig 4.4. Agarose gel electrophoresis showed that the template was amplified in all the samples subjected to various annealing temperature ranging from 52.0°C to 64.5°C. The best amplification appeared to be at 52.0°C both in terms of intensity and sharpness and hence was selected as the best annealing temperature for further studies.

4.2.1.2 Number of amplification cycles

In order to reduce the total time for PCR assay the effect of number of amplification cycles was studied. The PCR was carried out with 25, 30 and 35 amplification cycles at 52°C/ 1min. The results have been represented in Fig 4.5. It was interpreted from the electrophoresis gel pattern that the amplification of 929 bp PCR product was there in all the cases but 30 amplification cycles resulted in the best PCR amplification.

4.2.1.3 Effect of Taq polymerase concentration

The effect of Taq polymerase strength was studied by carrying out PCR with different Taq polymerase concentrations *viz* 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 U. The results of the study have been depicted in Fig 4.6. The amplification was observed at all the concentrations. However, 0.3 U of Taq polymerase was used for further studies to cut down the running cost of assay.

4.2.1.4 Effect of primer concentration

To study the effect of primer strength on amplification, the reaction was carried out at different primer concentrations of 9.96, 13.28, 16.6, 19.95, 24.93, 33.25 and 41.5 pM. The related results thus obtained have been shown in Fig 4.7. Although amplification was there in all the cases, higher concentrations showed nonspecific binding. The optimum primer concentration for further studies was 9.96 pM.

4.2.1.5 Effect of dNTPs concentration

The effect of different concentration of dNTPs (50 nM to 300 nM) on amplification 929 bp target product of *E. sakazakii* is shown in Fig 4.8. It is clear from the gel picture that amplification of specific was detected at all concentrations. However the best amplification was observed at 300 nM in terms of band density.

4.3 SEQUENCING OF PURIFIED PRODUCT

The standardized PCR programme was used for amplification of 929 bp target product of *E. sakazakii*. The picture of the gel containing the purified PCR product of the isolate IFM5 has been presented in Fig 4.9. The purified PCR product was sent to Banglore Geneii for sequencing. The concentration and purity of the sample was found to be fairly high. The sequence of the

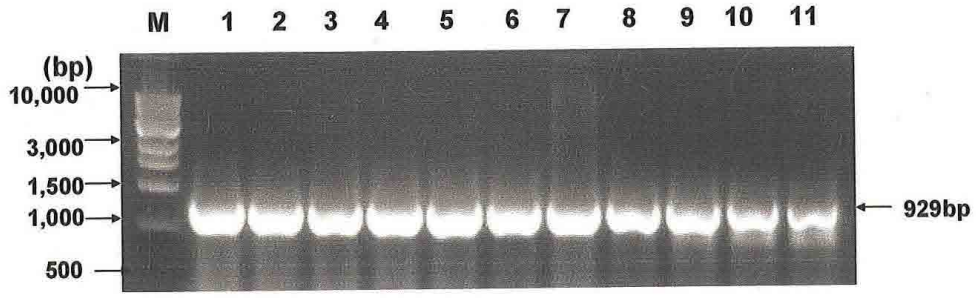


Fig 4.4 Effect of annealing temperature on PCR amplification for identification of *E. sakazakii*
Lanes : M, Marker (500bp); 1,52.0; 2, 52.2; 3, 52.8; 4, 53.9; 5, 55.3; 6, 56.9;7, 58.5; 8, 60.2; 9, 61.7; 10, 63.0; 11, 64.5

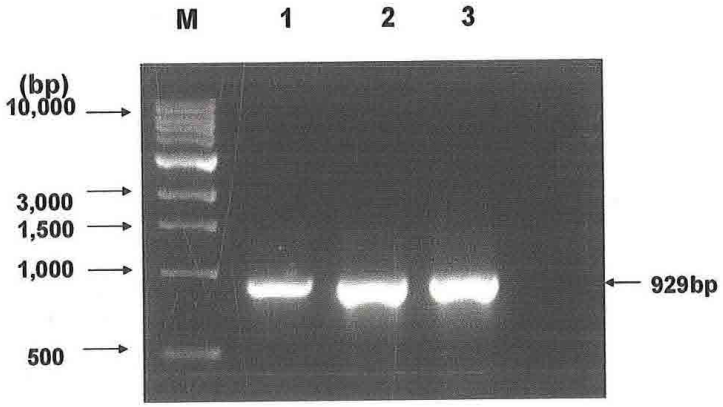


Fig 4.5 Effect of number of cycles on PCR amplification for identification of *E. sakazakii*
Lanes : M, Marker (500bp); 1, 25 cycles; 2, 30cycles; 3, 35 cycles

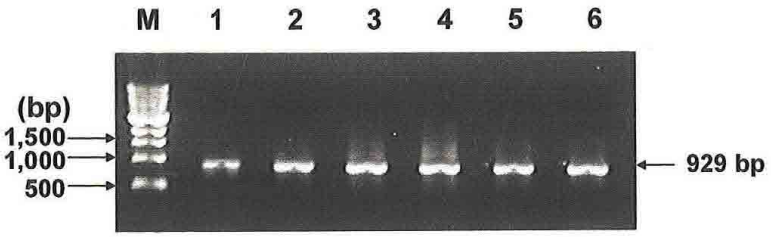


Fig. 4.6 Effect of concentration of Taq DNA polymerase on PCR amplification for identification of *E. sakazakii*

Lanes : M, Marker (500bp) 1, 0.2U; 2, 0.3U; 3, 0.4U; 4,0.5U; 5, 0.6U; 6, 0.7U a

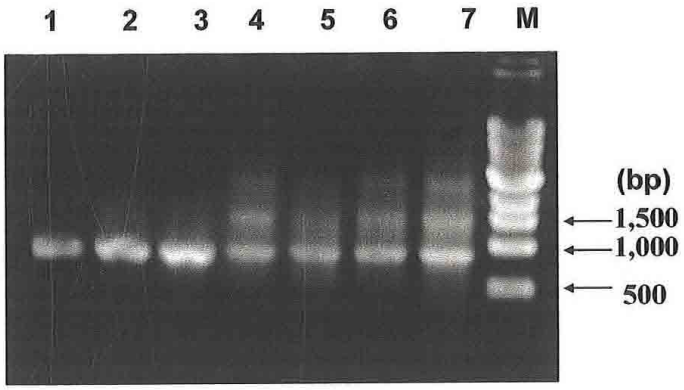


Fig. 4.7 Effect of primer concentration on PCR amplification for identification of *E. sakazakii*
Lanes : M, Marker (500bp); 1, 9.96 pM; 2, 13.28 pM; 3, 16.6pM; 4, 19.95 pM; 5, 24.93pM; 6, 33.25 pM;7, 41.5pM

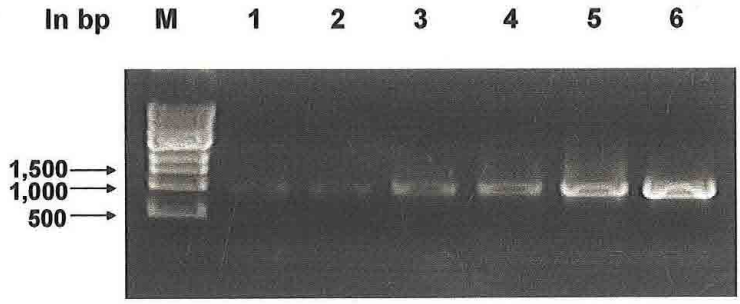


Fig 4.8 Effect of dNTP concentration on PCR amplification for identification of *E. sakazakii*
Lanes : M, Marker (500bp)1, 50nM; 2,100nM; 3, 150nM; 4, 200nM; 5, 250nM; 6, 300nM

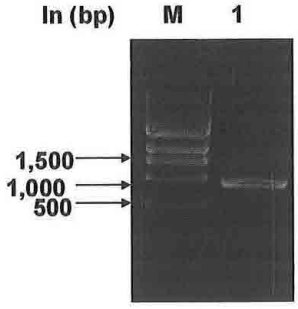


Fig 4.9 Agarose gel electrophoresis of purified PCR product of *E. sakazakii*

Lanes: M, Marker; 1, purified PCR product

specific target region thus obtained was matched with other 16S rRNA sequences in NCBI using BLAST software available at http://www.ncbi.nlm.nih.gov/BLAST/. The sequence of 16SrRNA gene of *E. sakazakii* was aligned with other available sequence in NCBI gene bank with accession numbers AY803187, AY803186, AY803190, AY752940, AY752938 and AY624073 all showing 98% homology except AY752938 which showed 97% homology (APPENDIX IV). The sequence also had homology with other related organisms *viz. Citrobacter diversus* (AF025372), *Erwinia tolentana* (AF130885), *Klebsiella plentinum* (AB004755), *Pantoea agglomerans* (AF130962) and *Salmonella paratyphii* (U88546) to the extent of 97%, 97%, 98%, 97% and 98% respectively. The sequence has been submitted to NCBI database with accession number EF031327.

After confirmation of the identity of 11 isolates as the *E. sakazakii* their other potential attributes related to possible health concerns were also investigated in the study.

4.4 ANTIBIOGRAM OF SELECTED ISOLATES

The antibiotic profile of above selected *E. sakazakii* isolates along with the reference strain was studied with respect to 26 antibiotics. The antibiogram of the standard culture (MTCC 659) and the 11 isolates *viz.* IFM1, IFM2, IFM3, IFM4 and IFM5, RW8, S2, RM2, RM3, GF1 and GF2 has been shown in Fig 4.10.

All the isolates were resistant to penicillin G, clindamycin, rifampicin, erythromycin, novobiocin, vancomycin and metronidazole but sensitive to chloramphenicol as earlier reported by Willis and Robinson (1988) also, chlorotetracycline and amoxycilin. Among those 41% isolates showed resistance and 59% showed intermediate response to kanamycin, 8% were resistant and 76% were sensitive to carbenicillin and 16 % showed intermediate response. About half of the isolates (48%) were resistant to tobramycin and the rest 52% were susceptible. 48% and 52% showed resistance and susceptibility respectively. Cefuroxime could inhibit 68% of the isolates but was resisted by 16% whereas the other 16% showed intermediate response. Polymyxin B could inhibit 84% but 16% isolates showed intermediate effect. Contrary to earlier reports Farmer *et al.* (1980), Lai (2001)

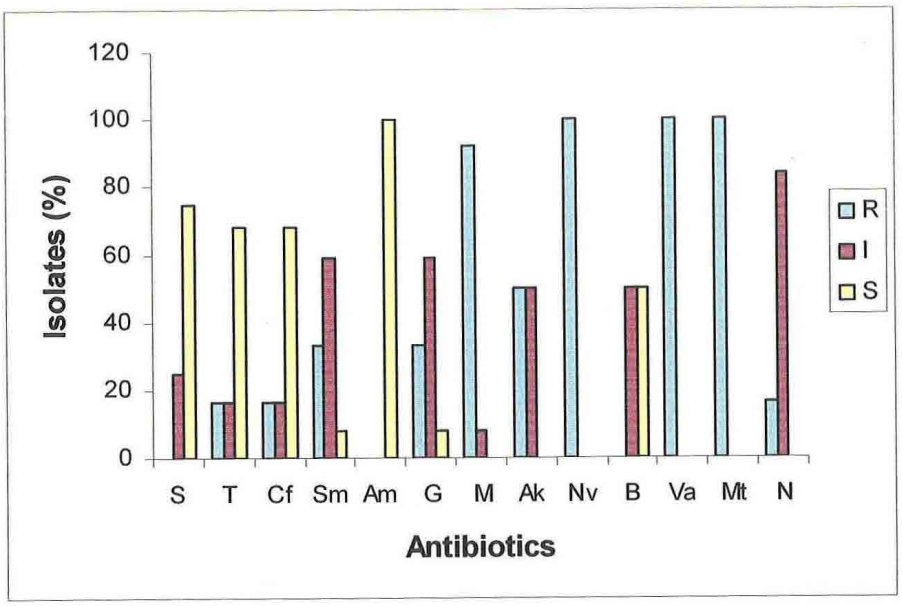
and Gaston (1988), in present study only 8% *E. sakazakii* isolates were susceptible to ampicillin. The drug was not effective against 75% and had intermediary effect against 17% isolates. Streptomycin could inhibit 75% isolates but 25% isolates had intermediate response. 16% were resistant, 68% sensitive and 84% intermediate against both tetracycline and ciprofloxacin. Only 8% were susceptible to both sulphamethazole and gentamycin while 33% were resistant and 59% showed intermediate response. Not even a single isolate was susceptible to methicillin and arnikacin but showed intermediate response, 8% and 50% respectively. 92% isolates were resistant to methicillin and 50% isolates resistance to amikacin. Bacitracin could inhibit 50% isolates and the remaining 50 % showed intermediate effect. Neomycin showed resistance and intermediate response against 16 and 84% of the isolates respectively. Although all the isolates were resistant to Penicillin G as reported by Arseni *et al.* (1987) they showed varied response to gentamycin contrary to the same worker. These isolates were then maintained in TSA slants.

Antibiotic resistance has been considered as one of the world's most pressing public health problems. The number of bacteria resistant to antibiotics has been on the increase during the recent past. Nearly all significant bacterial infections in the world are becoming resistant to the most commonly prescribed antibiotic treatments. Antibiotic resistance can cause significant danger and suffering for people who have common infections that once were easily treatable with antibiotics. The consequences of antibiotic treatment failure are longer-lasting illness and the need for more expensive and toxic medications.

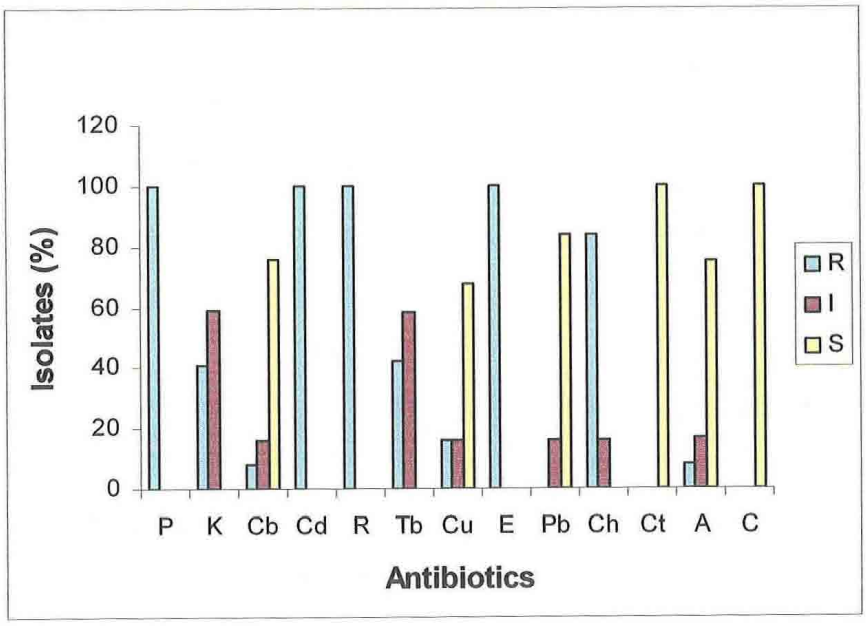
4.5 EFFECT OF TEMPETRATURE ON GROWTH OF *E. SAKAZAKII*

4.5.1 Growth at different temperature

The results pertaining to effect of different temperatures on growth profile of 11 *E. sakazakii* isolates and the standard culture, on TSA plates, are shown in table 4.2. As quiete evident from the results the best growth of all the cultures occurred at 37°C. However, only scanty growth in isolates IFM1, IFM5 and MTCC 659 was observed at 55°C while isolates IFM4, IFM5 and RM3 showed sufficient growth only after 10 d at 4°C. Compared to isolates IFM1, S2, GF1 and GF2 those grew poorly at 45°C, MTCC 659 and IFM2 showed good growth. But rest of the isolates failed to grow at this temperature.



(A)



(B)

Fig 4.10 Antibiogram of *E. sakazakii* strains

Table 4.2 Growth of *E. sakazakii* isolates at different temperature

Isolates	Temperature			
	4°C*	37°C **	45°C**	55°C**
MTCC 659	-	++++	+++	+
IFM1	-	++++	+	+
IFM2	-	++++	+++	-
IFM3	-	++++	-	-
IFM4	++	++++	-	-
IFM5	++	++++	-	+
RW8	-	++++	-	-
S2	-	++++	+	-
RM2	-	++++	-	-
RM3	++	++++	-	-
GF1	-	++++	+	-
GF2	-	++++	+	-

** 10 d , ** 48 h, - no growth; + , ++, +++ intermediate growth; ++++ maximum growth*

The scanty growth of three strains (MTCC 659, IFM1 and IFM5) indicated the particular strain resistanceto high heat treatment as was noticed from the next experiment.

4.5.2 D and Z value

The results of the study on heat resistance of bacteria at higher temperature in terms of D and Z values are presented in Fig 4.11 and Fig 4.12. With respect to their tolerance to high temperature the isolates showed D_{55} in the range of 7-12 min and D_{62} in the range of 0.3-0.6 min but the bacteria could not survive even for 30 sec at 72°C showing D_{72} value of 4 sec. The overall Z value was calculated to be 1°C. *E. sakazakii* strains in the present study were a little more heat resistant than another Enterobacteriaceae member *viz. E. coli* with D_{65} of the order of 0.1 min; Salmonella species- D_{65} 0.02- 0.25 min; *Bacillus steareothermophilus*- D_{121} is 4-5 min.. In milk chocolate D_{70} of S. *senftenberg* (the most heat resistant species of *Salmonella)* has been measured as 6-8 hr compared with only a few seconds in milk (Adam and Moss, 1996). While Murphy *et al.* (2002) reported that at 55 to 70°C, D-values for the *Salmonella* serotypes and *Listeria innocua* were 26.97 to 0.25 min and 191.94 to 0.18 min, respectively, and their *Z*- values were 7.60 to 9.83°C and 4.86 to 8.67°C, respectively. This low D- value indicated heat lability of the

organisms. This study will better enable processors to determine the process lethality of pathogens in commercial meat products. Commercial pasteurization of milk and milk products is therefore sufficient to remove this organism from the products.

The extent of the pasteurization treatment required is determined by the heat resistance of the most heat-resistant enzyme or microorganism in the food. For example, milk pasteurization historically is based on *Coxiella burnetti*, but with the recognition of each new pathogen, the required time temperature relationships are continuously being examined

4.6 SALT TOLERANCE

The response of the 11 identified *E. sakazakii* isolates and the reference strain MTCC 659 to various concentrations of common salt is shown in Fig 4.13. The growth was initially fastest in presence of 0.5% NaCl closely followed by that in 1%. It was inhibited a bit in 1.5% NaCl followed by much retarded growth in 3% NaCl. At the same time 5.5% salt concentration though initially suppressed the growth yet subsequently by 24 h the organism adapted itself to this high concentration and tended to pick up, but it was still less than that in any other lower salt concentration. However at 10.5% salt level no appreciable growth was noticed. The rate of growth in the presence of 0.5%, 1%, 1.5%, 3%, 5.5% and 10.5% was 45, 49, 64, 142.2, 367 and 1524 min respectively (Table 4.3).

Table 4.3 Log count of viable cells in presence of increasing salt concentration

Time (h)	Salt Concentration				
	0.5 %	1 %	1.5 %	3.0 %	5.5 %
0	6.54	6.65	6.77	6.69	6.39
4	7.72	7.74	7.62	7.07	6.30
8	8.39	8.19	7.95	7.13	6.30
12	8.82	8.56	8.19	7.68	6.00
16	8.86	8.90	8.65	8.41	6.62
20	8.82	8.92	8.71	8.54	6.83
24	8.92	9.03	8.78	8.67	7.57
28	8.90	8.98	8.87	8.64	7.88

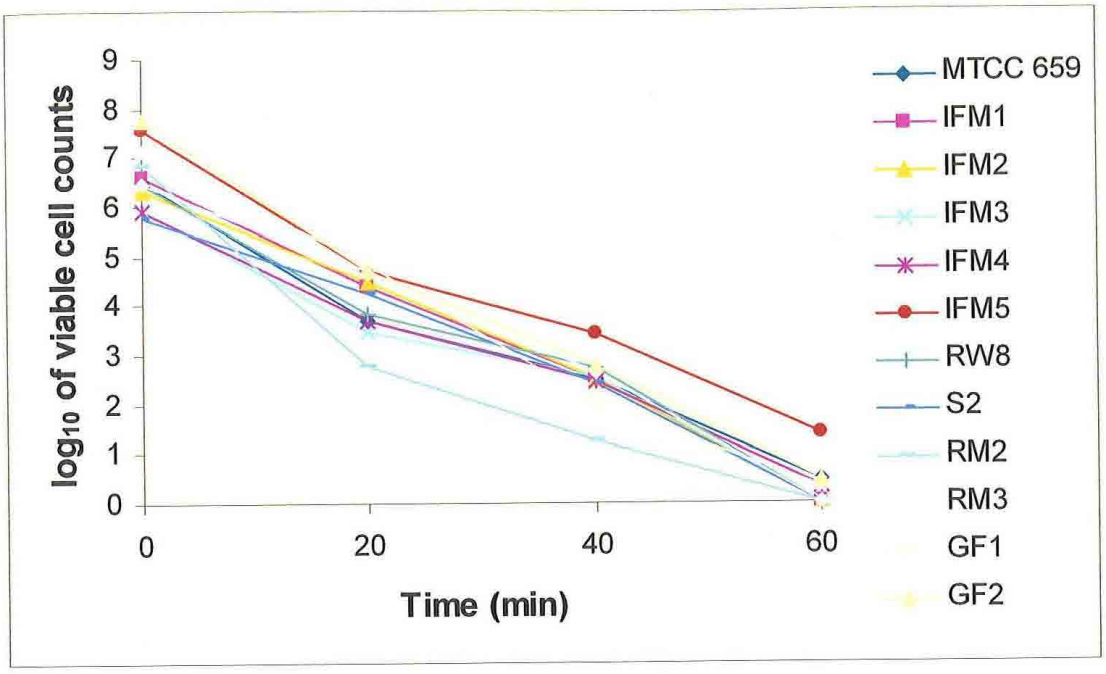


Fig 4.11 Thermoviability of *E. sakazakii* strains at 55°C

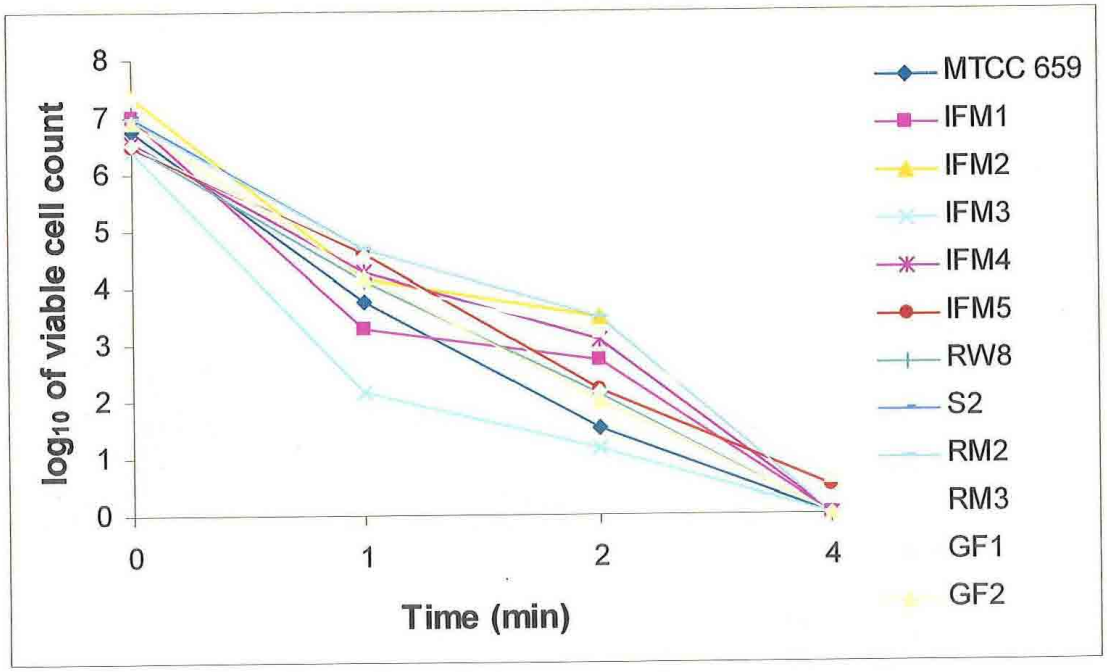
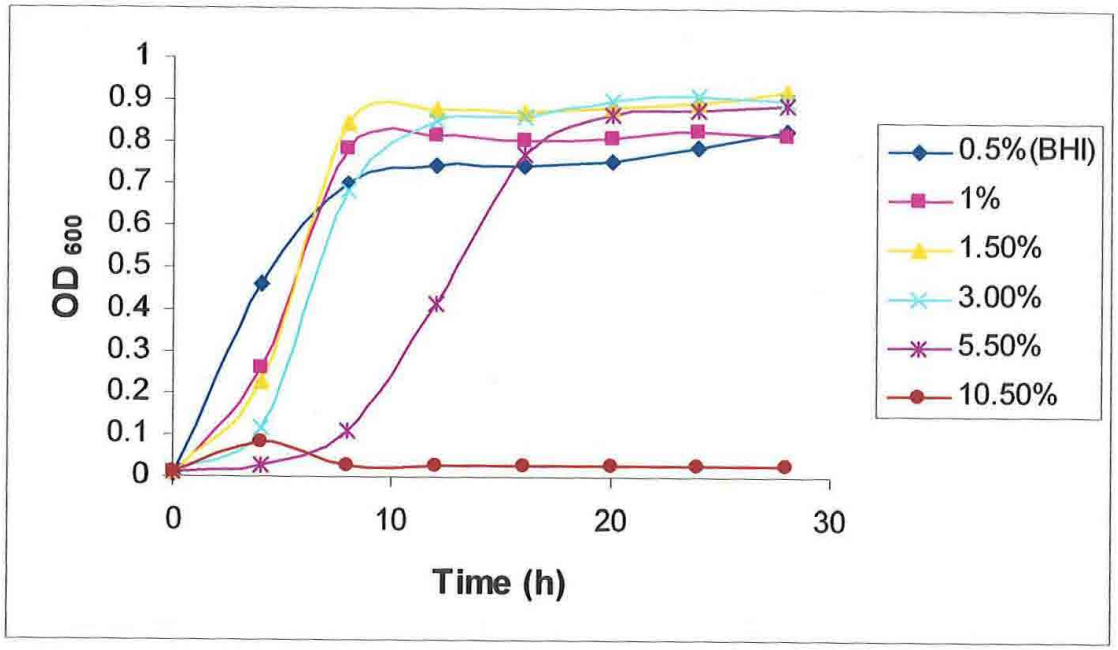
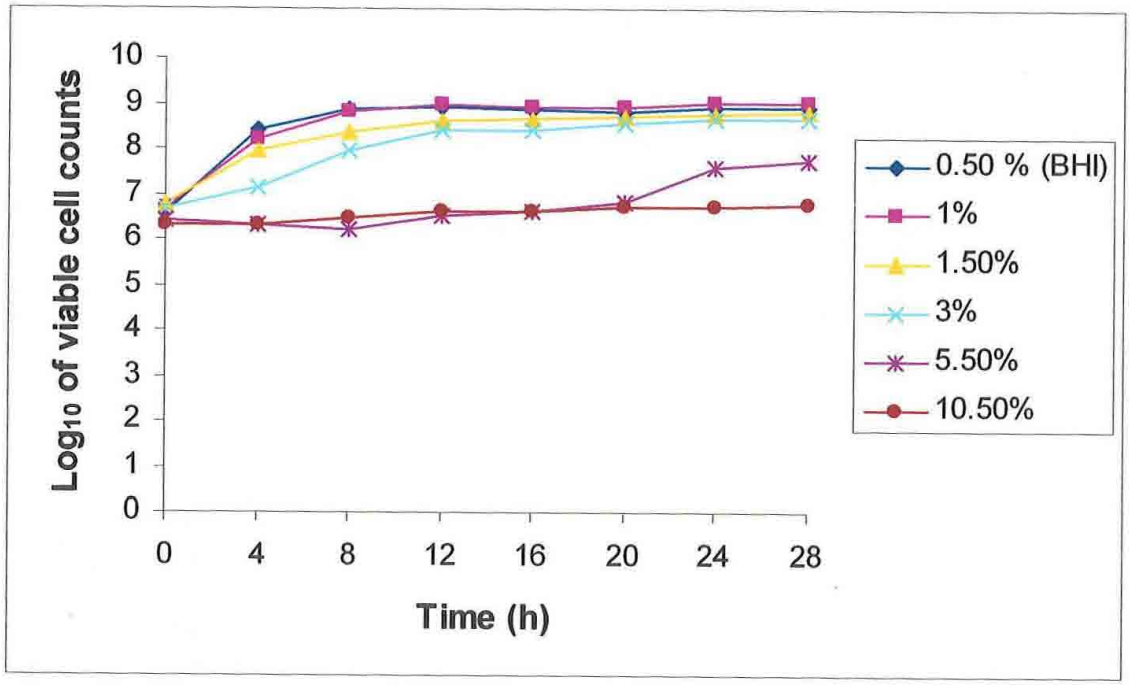


Fig 4.12 Thermoviability of *E. sakazakii* strains at 62°C



(A)



(B)

Fig 4.13 Effect of NaCl concentration on growth of *E. sakazakii* estimated by
(A) Spectrophotometry
(B) Standard plate count

This study is also the first ever report about the salt tolerance of this particular bacterium. The difference between the patterns of the curve for viable cell counts and the overall cell mass (OD_{600}) observed during the study of salt tolerance was probably due to the total cells and the metabolites etc. present in the latter case. However, the sudden increase in OD_{600} at 3% salt concentration after 16 h as compared to viable counts is inexplicable at this stage. It might be that some exo- metabolites were accumulated at this salt concentration. This aspect needs further detailed exploration.

The results of this study coincide fully with the earlier lone study made by Breeuwer *et al.* (2003) wherein they have reported the organism's salt tolerance to the rate of 1 M NaCl.The property has been utilized by Guillaume-Gentil *et al.* (2005) for selective enrichment of the organism even at 46°C. No other reports on similar lines could be found in the resting literature that could explain this effect of common salt on the growth of *E. sakazakii.* However, parallel reports on other organisms do not completely corroborate with the present findings. Growth of lactic acid bacteria was enhanced by 1-2% (w/v) of added NaCl, while NaCl concentrations above 3% (w/v) had a clear inhibitory effect (Korkeala *et al.*, 1992). The maximal tolerance factor for the CIP 69.29 strain of *Shewanella putrefaciens* was obtained when cells were incubated for 1 h in the presence of 1.5% NaCl, (Leblanc *et al.*, 2003). In *Lactobacillus casei* sodium chloride dramatically influenced bacteriocin production by decreasing both biomass production and specific bacteriocin production (Leroy and Vuyst, 1999).

4.7 SELECTION OF POTENTIAL VIRULENT STRAINS

Many individual criteria have been shown to contribute to virulence of various pathogens that may also be applicable to *E. sakazakii.* Previous reports stongly support that the tests performed in the present study are strong determinants of virulence in many pathogenic bacteria. Presence of haemolysin and collagenase in *B. cereus* (Beecher *et al.*, 2000), numerous extracellular toxins including phospholipase C, perfringolysin O, collagenase, as well as a sporulation-associated enterotoxin (Rood, 1998) as additional virulent factors are some recent findings. In the present study following tests were done to determine virulence in selected *E. sakazakii* isolates and also the reference culture.

4.7.1 Hemolysis

In the present study all the *E. sakazakii* isolates as well as the reference culture (MTCC 659) were β-haemolytic showing clear zone of hemolysis on bovine blood agar plates. The zones were comparatively clearer in IFM1 and IFM2 while RW3 was hemolytically very weak (Fig 4.14). There are several reports about the role of hemolysin in virulence of pathogens e.g. *Actinobacillus pleuropneumoniae* (Inzana *et al.*, 1991) and *E. coli* (Opal *et al.*, 1990). Hemolysins (α and β) play an important role in bacterial virulence and the pathogenesis of *A. pleuropneumoniae*. The sulfhydryl-activated hemolysin of *Listeria monocytogenes* has been implicated in its virulence. Although loss of hemolytic activity by means of mutation in transposon was accompanied by loss of virulence in the mouse infection model, a direct relationship between *in vitro* production of hemolysin and virulence was not observed (Kathariou *et al.*, 1988). On the contrary, introduction of the hemolysin-encoding plasmid pAM714 into *E. faecalis* OG1RF, a well-studied plasmid-free strain, caused a 35-fold lower LD_{50} and a much shorter survival (Singh *et al.*, 1998).

4.7.2 Hemagglutination

Agglutination of erythrocytes has been considered as an important virulence characteristic (Hagberg *et al.*, 1981). Hemagglutinin production and its involvement in virulence by *E. coli*, *Salmonella* (Duguid and Old, 1980), *Klebsiella and Serratia* (Adegbola and Old, 1982) has already been reported. Also there are reports about *E. amnigenus*, *E. cloacae* and *E. sakazakii* producing mannose sensitive hemagglutinins. In the present study all the isolates were positive for hemagglutination thus having corronance to earlier reports. Among the various isolates and the reference strain, RW3 caused maximum agglutination in bovine RBCs.

4.7.3 DNase production

DNase secretion is an important virulence characteristic of most of the pathogens usually involved in invasive infections. All the strains of Group A streptococci produce at least one extracellular DNase, and most strains make several distinct enzymes, DNase is a important virulence factor (Sumby *et al.*, 2005; Buchanan *et al.*, 2006). However Sriskandan *et al.* (2000) observed that despite loss of DNase B activity, the virulence of *S. pyogenes* was unaffected in

a mouse model of necrotizing fasciitis and myositis, contrary to Podbielski *et al.* (1996) who suggested the significance of GAS DNase as a bacterial virulence factor to be determined. Sumru *et al.* (2003) reported DNase activity was positive in 42.9% of *S. haemolyticus*, 20.7% of *S. hominis*, 17.9% of *S. xylosus* and 2.1% of *S. cohnii* isolates and concluded that DNase activity was an important virulence factor in identifying pathogenic staphylococci. All the isolates in the present study showed delayed (6 d) DNase production (Fig 4.15). This is also an important biochemical characteristic that makes the organism different from *E. cloacae* and is also one of its important virulence characteristic.

4.7.4 Response to Antibacterial Activity of Serum

Percentage survival of *E. sakazakii* isolates in presence of bovine serum has been shown in Fig 4.16. Thus reference strain (MTCC 659), IFM1, IFM2, IFM4, IFM5, S2, RM3, GF1 and GF2 were resistant, two isolatesIFM3 and RW8 were intermediate and one strain RM2 was sensitive to normal bovine serum. The growth was unaffected in control and in presence of heat inactivated serum.

Serum resistance is an important determinant in conferring pathogenicity to the bacterium. The membrane attack complex of complement disrupts the bacterial outer membrane and permits serum lysozyme to degrade the peptidoglycan which finally causes bacteriolysis (Pelkonen and Finne, 1987). Usually all the virulent strains are serum resistant and avirulent strains were serum sensitive thus clearly indicating the serum resistance as a pathogenicity characteristic (Ellis *et al.*, 1988; Morishita *et al.*, 1990). Also it has been reported that serum resistance was increased by the presence of lipopolysaccharide (Mellata *et al.*, 2003). In *Y. enterocolitica* also serum resistance has been shown to be an important virulent factor (Pai and DeStephano, 1982) and is expressed even in the absence of other phenotypic virulence-associated markers, such as the presence of V antigen, autoagglutination, and calcium dependence after growth at 37°C (Chiesa and Bottone, 1983). However, it has been well eastablished that serum susceptibility of Gram negative bacteria was influenced by the growth conditions (Taylor, 1978) e.g. *E. coli* O111:B4 was less susceptible if grown in

presence of glycerol or acetate as carbon source (Melching and Vas, 1971) and *S. typhimurium* was more susceptible to human serum when grown in diluted broth than when grown in undiluted broth (Maaloe, 1948).

4.7.5 Aerobactin

Mammals make considerable adjustments during infection that deprive the pathogen of iron. So only the microorganisms capable of securing host iron are able to survive in the host (Mokracka *et al.*, 2004) and hence iron aquisition is considered as a pathogenicity related factor eg. Yersiniabactin produced by *Yersinia* spp. is strongly connected with its virulence (Weinberg, 1978; Heeseman, 1987). All enteric bacteria synthesize siderophores which may be phenolate or hydroxamate compound. The hydroxamate synthesized by *E. aerogenes* and *E. coli* has been identified as aerobact. (Van Teil-Menkveld *et al.*, 1982). Also in a study by Opal *et al.* (1990) it was found that the majority of blood isolates that lacked aerobactin were hemolytic, whereas only a less number of blood isolates that expressed aerobactin were hemolytic. Aerobactin may be the principal mechanism of iron acquisition in extra-intestinal isolates of *E. coli*, and hemolysin may serve as an alternative mechanism in the absence of aerobactin genes. Being in line with Mokracka *et al.* (2004) *E. sakazakii* isolates in the present study were unable to produce aerobactin.

4.7.6 Gelatinase

Gelatinase is a protease that is capable of hydrolyzing gelatin, collagen, casein, haemoglobin and other peptides. Gelatinase, a virulence factor of *Enterococcus faecalis*, was found to be regulated in a cell density-dependent fashion in which it was actively produced is active in late log to early stationary phase (Nakayama *et al.*, 2001). Gelatinase producing strains of *E. faecalis* have been shown to be more virulenct causing endocarditis in an animal model (Vergis *et al.*, 2002). Singh *et al.* (1998) found that gelatinase was produced by 54% of endocarditis *E. faecalis* isolates, 58% of nosocomial clinical isolates, 62% of nosocomial fecal isolates, and 27% of faecal isolates from healthy community volunteers and also that gelatinase might worsen the disease, while Sedgley *et al.* (2005) found that gelatinase was produced by all virulent strains of *E. faecalis*. Presence of *gelE* gene (coding for gelatinase) has been reported in clinical isolates of *E. faecalis* and *E. faecium* (Dupre *et al.*, 2003). In the

present study all the *E. sakazakii* isolates along with the reference culture showed delayed gelatinase hydrolysis. However there are no reports about the presence of this gene in *E. sakazakii*.

4.7.7 Neuraminidase

All the strains of *E. sakazakii* isolates used in the present study produced neuraminidase. Similar to the reference culture MTCC 659, the isolates IFM2 and GF1 produced maximum amount of neuraminic acid from egg yolk, IFM1, IFM5 and GF2 were intermediate and IFM3, IFM4, RW8, S2, RM2 and RM3 were low producers. RM3 produced minimum andGF1 produced maximum quantity of neuraminic acid (Fig 4.17). Enzyme neuraminidase cleaves *N*-acetylneuraminic acid from mucin, glycolipids, glycoproteins and oligosaccharides on host cell surfaces. It helps the pathogen to spread to other cells of the body. The enzyme, which facilitates the penetration of cholera toxin into the epithelial cells of the intestine, is always present in virulent *V. cholerae* strains and may be absent in avirulent strains. All clinical *S. pneumoniae* isolates examined to date have been shown to produce neuraminidase. Production of neuraminidase is believed to contribute to the poor prognosis for pneumococcal meningitis. Moreover, *S. pneumoniae* neuraminidase has been detected in 78% of culture-positive middle ear effusions from patients with acute Otitis Media (OM) and in 96% of *S. pneumoniae*-positive middle ear effusions from patients with chronic OM (Tong *et al.*, 2000). A significant correlation was found between the average neuraminidase production by *Erysipelothrix rhusiopathiae* in different media and virulence. In *Haemophilus persuis* producton of neuraminidase has been reported as an important virulence factor that is needed by the bacterium for colonization in the host cell (www.life.uiuc.edu).

4.7.8 Hyaluronidase

Hyaluronidase which catalyses depolymerization of hyaluronic acid has been considered as a virulence determinat and is produced by a number of pathogenic bacteria (Netherwood *et al.*, 1998). All the *Paracoccidioides brasiliensis* isolates obtained by Assis *et al.* (2003) revealed chondroitinase and hyaluronidase activity when grown on chondroitin agar or hyaluronic acid agar, respectively. On the contrary, Wellmer *et al.* (2002) observed that

hyaluronidase deficiency had no impact on the virulence of *Streptococcus pneumoniae*. However, isolates of *E. sakazakii* in the present study did not produce hyaluronidase. However their virulence was also dependent on other auxiliary factors reported in the study.

4.7.9 Elastase

All the *E. sakazakii* isolates of the present study produced elastase during 48 hr study as observed by increased absorbance due to release of soluble Congo red into the solution from elastin-congo redcomplex. The maximum enzyme was produced by IFM2 and GF1 (Fig 4.18). It was therefore inferred that *E. sakazakii* possessed virulence and that isolates IFM1 and GF1 should otherwise be more virulent. Human neutrophil elastase (NE) is a key host defence protein as it degrades *Shigella* virulence factors at its 1,000-fold lower concentration than that needed to degrade other bacterial proteins. In neutrophils in which NE is inactivated pharmacologically or genetically, *Shigella* escapes from phagosomes thus resulting in increased bacterial survival (Yvette *et al.*, 2002). However it has been observed that elastase production is an important virulence factor and is of particular interest in *Pseudomonas aeruginosa* as it causes ulcers, necrotic skin lesions and pulmonary haemorrhages. It has been observed that *P. aeruginosa* strains deficient in elastase were less virulent (Woods *et al.*, 1982; Blackwood *et al.*, 1983).

4.7.10 Collagenase

In the present study two isolates IFM4 and RM2 were weakly positive for collagenase as observed after 48 hrs (Fig 4.19). Although Rood (1998) found phospholipase C, perfringolysin O, collagenase and enterotoxin as important virulent factors of *C. perfringens*, Awad *et al.* (2000) demonstrated that loss of the ability to produce collagenase does not alter the ability of *C. perfringens* mutant to establish a virulent infection. Collagenolytic enzymes can readily damage the vitreal and retinal architecture and have been reported to contribute to the virulence of *Pseudomonas aeruginosa* ocular infections (http://www.w3. ouhsc.edu). Expression of collagenase activity has also been reported as an important virulence factor in *Porphyromonas gingivalis* that causes periodontitis by degrading type I collagen in the supporting tissue of teeth (Kuramitsu *et al.*, 1995). Seddon *et al.* (1990) observed that all the five

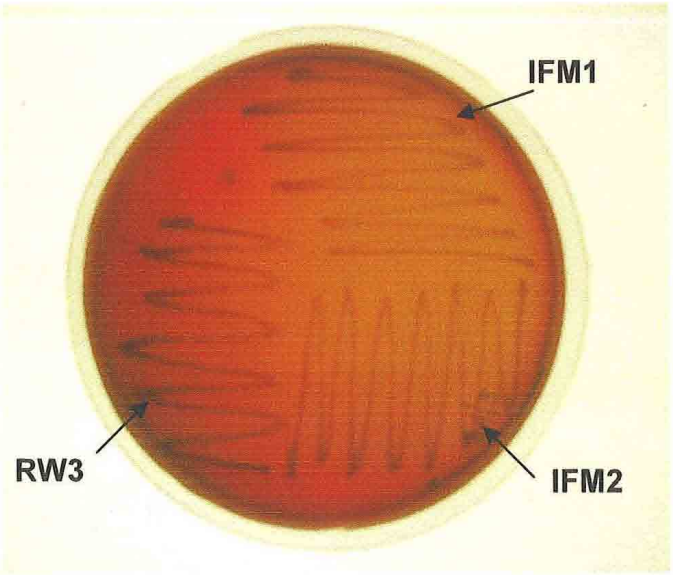


Fig 4.14 β-hemolysis by *E. sakazakii* isolates on bovine blood agar

Fig 4.15 DNase production by *E. sakazakii* strain (IFM1)

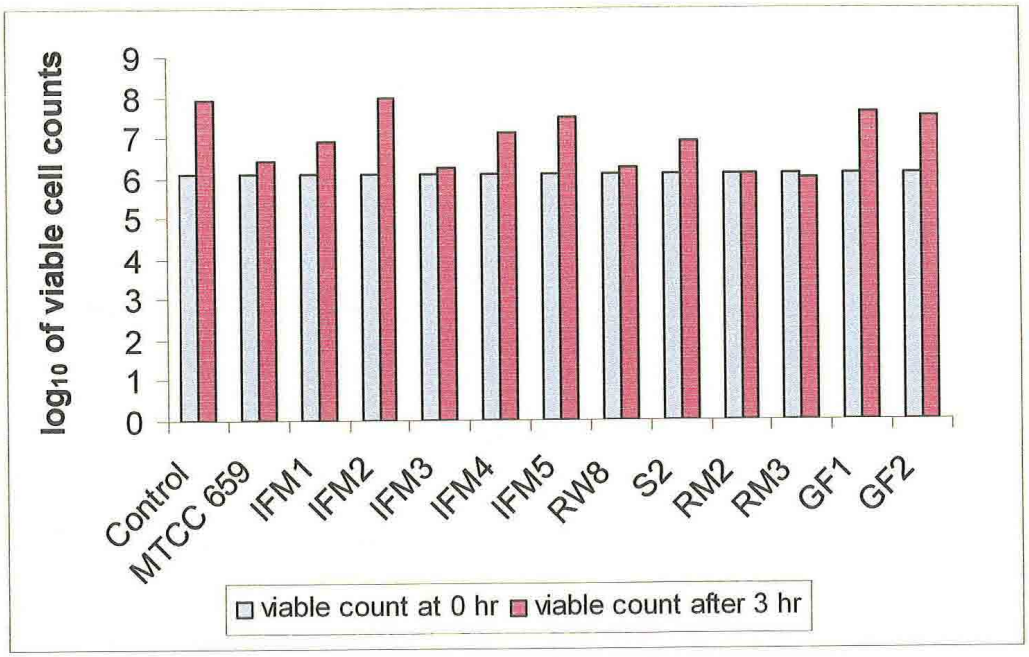


Fig 4.16 Response of *E. sakazakii* isolates to antibacterial activity of bovine serum

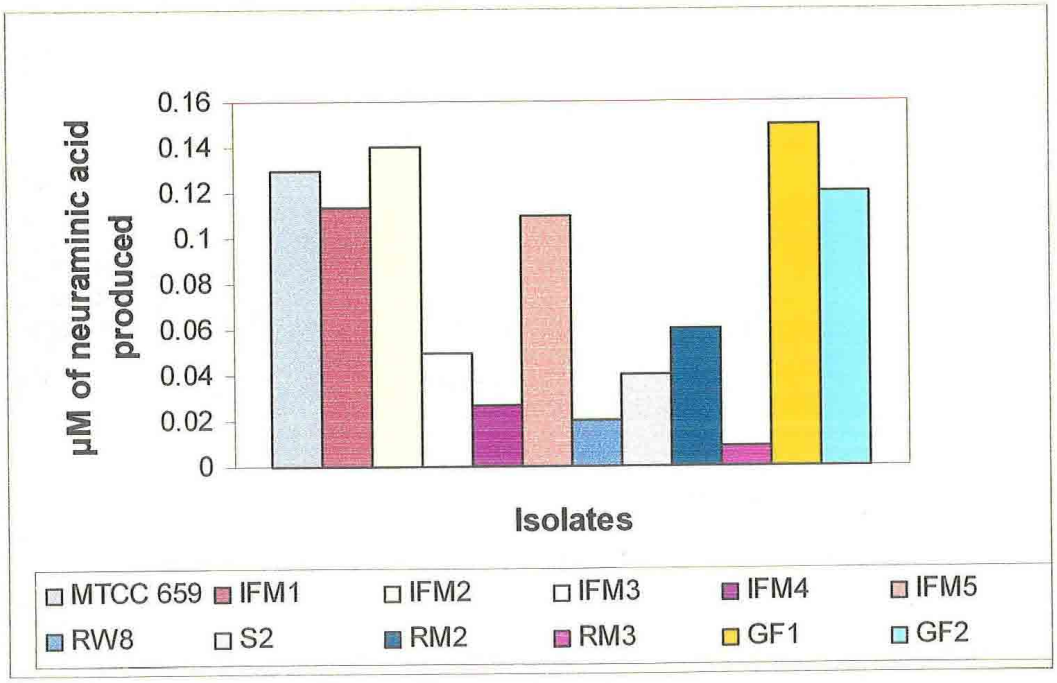


Fig 4.17 Neuraminidase activity of *E. sakazakii* strains

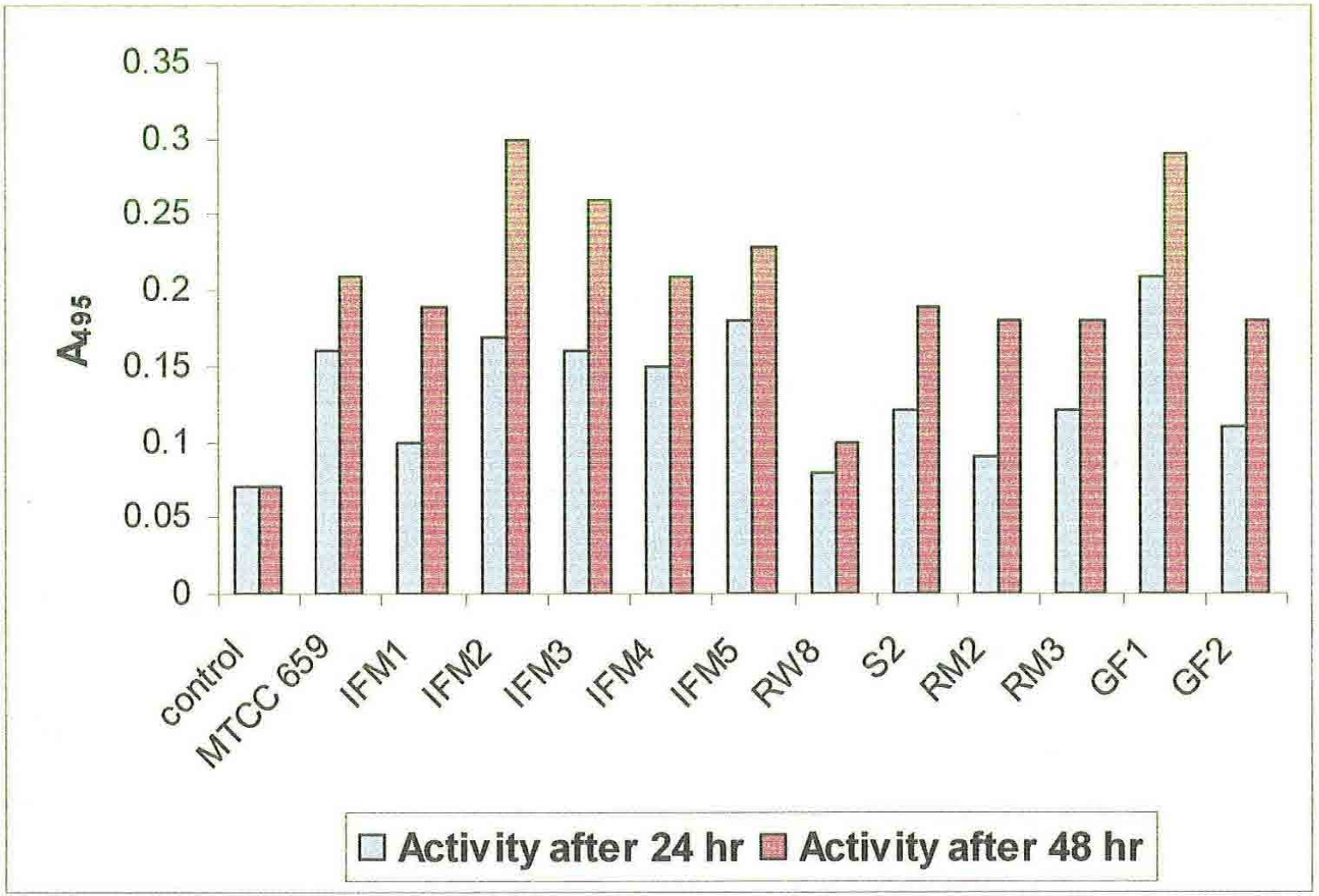


Fig 4.18 Elastase production by *E. sakazakii* strains

strains of *Clostridium difficile* known to be highly virulent in the hamster model had hyaluronidase, chondroitin-4-sulphatase and collagenase activity whereas only three of five toxigenic but poorly virulent strains had these activities, the collagenase activity being weak in all three cases thus indicating that these enzymes may or may not accessory virulence factors.

4.7.11 Phospholipase C

Phospholipase C (lecithinase) is an important virulence factor involved in cell to cell spread. In the present study none of the strains of *E. sakazakii* produced lecithinase. At present it will however be premature to delink the enterotoxicity or pathogenicity of *E. sakazakii* from the enzyme production activity. Pinyon *et al.* (1996) suggested that although lecithinase was a virulence factor produced by *Xenorhabdus bovienii*, lecithinase activity alone is not sufficient for killing of *Galleria mellonella* larvae. In lecithinase positive strains of *L. monocytogenes* it has been found that a protease was responsible for maturation of lecithinase (Coffey *et al.*, 2000). Merrino *et al.* (1999) suggested that lecithinase was an important virulence factor for pathogenesis of mesophilic *Aeromonas* (serogroup O: 34). *B. cereus* toxicity has been shown to be associated with lecithinase (Beecher *et al.*, 2000).

4.7.12 Presence of lipopolysaccharide

Lipopolysaccharide is a complex surface molecule located in the outer membrane of Gram negative bacteria. It consists of three moieties- O antigen, core oligosachharide and O side chain. The O antigen consisting of a linear polymer of oligosachharide repeating units is the most external LPS component (Valvano and Crosa, 1989). A number of Gram negative bacteria produce rough type LPS with high molecular weight because the carbohydrate portion of the LPS consists of only an oligosaccharide of approximately nine monosaccharide residues (Tsai and Frasch, 1982) and the length of LPS polymer is an important determinant of susceptibility to serum lysis (Waedlaw, 1963). It has been well eastablished that capsular polysaccharides impart serum resistance to *E. coli* (Cross *et al.*, 1984). LPS production has been reported in many pathogenic organisms like *S. abortus equi, S. minnesota, S. typhosa, Serratia marcescens* and *Shigella flexneri* and many strains of *E. coli* (Coleman *et al.*, 1979). All the *E. sakazaii* isolates in the present study

produced lipopolysaccharide and were also resistant to antibacterial activity of bovine serum keeping in line with Mellata *et al.* (2003) that LPS increased serum resistance. The LPS bands obtained from *E. sakazakii* were revealed by a highly sensitive and cost effective technique- silver staining. The bands as shown in Fig 4.20 were almost identical to that obtained from *S. dysenteriae* and two bands of low molecular weight corresponds to two repeating units of O-specific oligosaccharide (Kido *et al.*, 1990). The polysaccharide portion of LPS has been suggested to be the reactive component in silver staining. Fatty acids in the lipid component might be the binding site of silver ions through the formation of *pi* complexes.

Among the 12 various acessory virulent factors studied above, eight tested positive for the *E. sakazakii* isolates. The four tests comprising of aerobactin, collagenase (very weakly positive by two isolates), hyaluronidase and lecithinase were found to be negative. As majority of the tests were positive it gave weightage to the possible virulence of the isolates.

4.8 ROLE OF PLASMID IN VIRULENCE

Plasmids usually carry genes or cassettes of genes coding for antibiotic resistance and virulence. In members of Enterobacteriaceae plasmid has been strongly linked to virulence of the pathogen. Non-typhoid *Salmonella* strains have been reported to be associated with the organism and disseminated infection in humans. The virulence of the organism has been significantly linked to plasmids (Guiney *et al.*, 1995). In *C. jejuni* strain 81-176 virulence has been linked to two plasmid of 35 kb each (Bacon *et al.*, 2000). Plasmids have also been reported to be associated with virulence of *S. dysenteriae* (Haider *et al.*, 1990) and *Yersinia* species (Cornelis *et al.*, 1998). Studies on similar lines for *E. sakazakii* have been lacking. An attempt was therefore made to investigate the relation of the organism's pathogenicity with plasmid, if any.

4.8.1 Plasmid Detection

Alkali lysis method of Sambrook *et al.* (1989) was used to isolate plasmid from *E. sakazakii* strains. This easy method has been frequently used for isolation of plasmid (Liou *et al.*, 1999) from Gram negative bacteria like *Vibrio* species and *E. coli* (Akinbowale *et al.*, 2006; Feliciello and Chinali, 1993), *Shigella* spp. (Taneja *et al.*, 2005). It may be seen from Fig 4.21 that number of

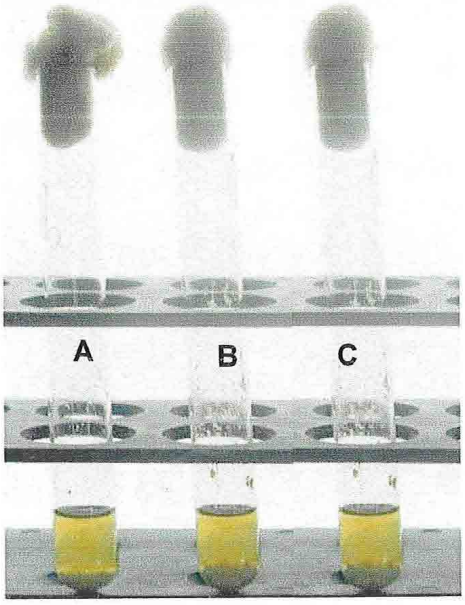


Fig 4.19 Collagenase activity of *E. sakazakii* isolates
A Positive (IFM4)
B Negative (MTCC 659)
C Control

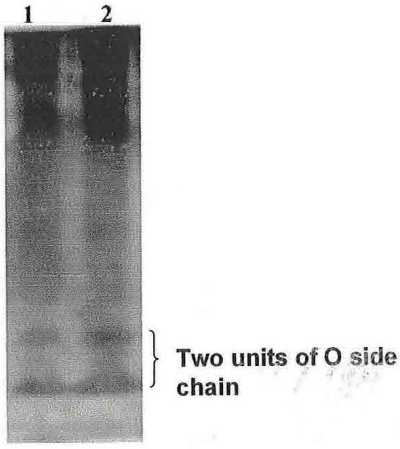


Fig 4.20 Detection of lipopolysaccharide by silver staining
Lanes:1, MTCC 659; 2, IFM1

plasmids in *E. sakazakii* cultures ranged from zero in MTCC 659, IFM1, IFM3, RM2, GF1and GF2, one in IFM2 and RM3 to two in IFM4, IFM5, RW8 and S2. The size of the plasmid was approximately 20 kbp (Fig 4.21).

4.8.2 Plasmid Curing

Removal of plasmid was attempted to acertain whether the virulence potential of *E. sakazakii* cultures was plasmid limited. High temperature exposure (Asheshov, 1966) was chosen for plasmid curing experiments and absence of plasmid was observed by electrophoretic gel profile (Fig 4.22). Antibiotic profile of wild type cultures and plasmid cured cultures was compared (Fig 4.23) and a wide difference in inhibition zone size was observed. Clindamycin, erythromycin and vancomycin to which the isolates were highly resistant produced inhibition zone of 4, 12 and 13 mm respectively (Fig 4.24). There are several reports supporting that plasmids carry antibiotic resistance genes (Soge *et al.*, 2006) hence loss of plasmids results in loss of antibiotic resistance.Colonies of cultures IFM2, IFM4, IFM5, RW8, S2 and RM3 showing loss of antibiotic resistance were picked up and studied for virlence characters that that were present in wild type.

4.8.3 Virulence in Cured Cultures

All wild type cultures of *E. sakazakii* were positive for serum resistance, gelatinase, neuraminidase, elastase and LPS whereas only 16.6 % of these were positive for collagenase activity. To determine if these properties were plasmid related the cured were again studied for the same properties. It was observed that there was no change in virulence properties exhibited by the cured cultures (lacking plasmid) from that of the wild type. This suggested that the virulence in *E. sakazakii* was chromatid related. Several reports lend support to the present observations. Awad *et al.* (2000) reported the loss of collagenase activity in chromatid colA mutants of *C. perfringens*, while the genes encoding the alpha-toxin and theta-toxin were located on the chromatid, the genes encoding many of the other extracellular toxins were located on large plasmids Rood (1998). The enterotoxin gene could be either chromatid or plasmid determined. Several of these toxin genes were associated with insertion sequences. Production of many of the extracellular toxins was regulated at the transcriptional level by the products of the *virR* and *virS* genes,

which together comprise a two-component signal transduction system. Also, there are reports that genes coding for lipopolysaccharide (LPS) are located on chromatid (Valvano and Crosa, 1989). While investigating the effect of chromosomal mutation on the synthesis of *rfe*-dependent E. coli O9 LPS, Sugiyama *et al.* (1991) confirmed that the biosynthesis of *E. coli* O9-specific polysaccharide does not require *rfc* but requires *rfe* gene. They also suggested that the *rfaL* product of *S. typhimurium* and *rfbT* product of *E. coli* O9 cooperated to synthesize *E. coli* O9 LPS in *S. typhimurium.* Conjugation and hybridization studies indicated that the genes for the synthesis and transport of aerobactin were linked and were also found on the chromatid of *S. flexneri*, *S. boydii*, and *S. sonnei* but not in *S. dysenteriae*. A number of aerobactin synthesis mutants and transport mutants have been isolated. The most common mutations are deletions of the biosynthesis or biosynthesis and transport genes. *Shigella* aerobactin genes share considerable homology with *E. coli* ColV aerobactin genes. On the ColV plasmid and in *Shigella* chromatid, the aerobactin genes are associated with a repetitive sequence which has been identified as IS1 (Lawlor and Payne, 1984). Vokes *et al.* (1999) reported the presence of the aerobactin genes on plasmids in *E. coli* pColV and *Salmonella*, on a pathogenicity island in *S. flexneri* and *S. sonnei* and in a different chromatid location in *S. boydii* and some *E. coli*. This suggests that these virulence-enhancing genes are mobile, and they may constitute an island within an island in *S. flexneri*. In *E. coli, A. aerogenes* and *Salmonella species* also aerobactin genes may be chromatid or plasmid borne (Bindereif and Neilands, 1985; Colonna *et al.*, 1992; McDougall and Neilands, 1984). Colonna *et al.* (1992) reported that in *E. coli* strains hemolysin determinants were located on the chromatid and their genetic organization was well conserved and closely resembled that of the reference haemolytic plasmid pHly152.

Interestingly, the factors responsible for resistance to complement differ in *E. coli* isolates from mammalian and avian hosts, which may reflect differences in the nature of avian and mammalian colibacillosis. In some cases, genetic determinants for serum complement resistance in avian *E. coli* are found on aerobactin- or colicin V-encoding plasmids. One such gene, *iss*, first described for its role in the serum resistance associated with a ColV plasmid from a human *E. coli* isolate, occurs much more frequently in isolates from birds with

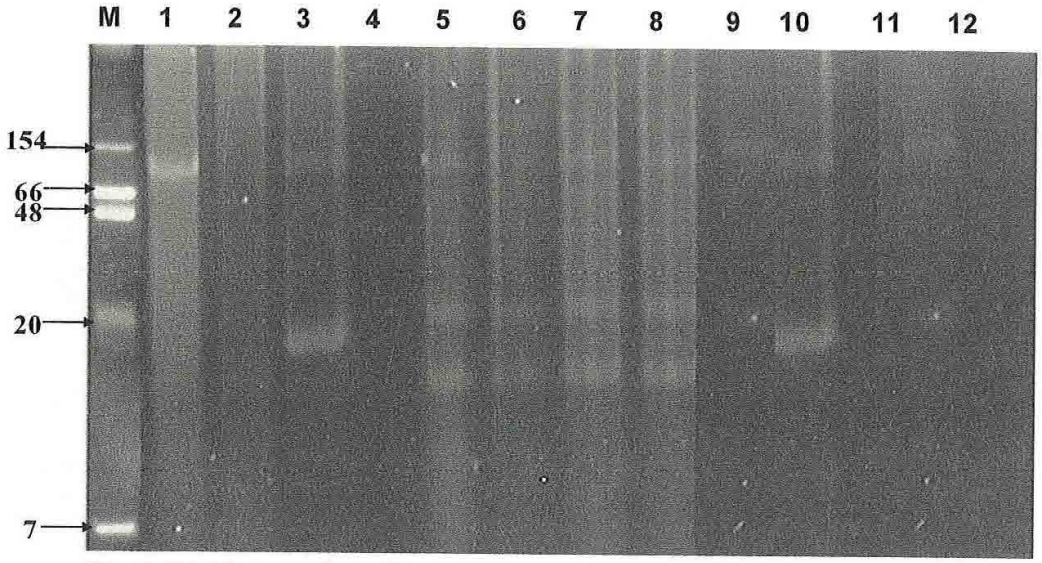


Fig 4.21 Plasmid profile of *E. sakazakii* isolates on agaorse gel

Lane: M, Marker; 1, MTCC 659; 2, IFM1; 3, IFM2; 4, IFM3; 5, IFM4; 6, IFM5; 7, RW8; 8, S2; 9, RM2;10, RM3; 11, GF1; 12, GF2

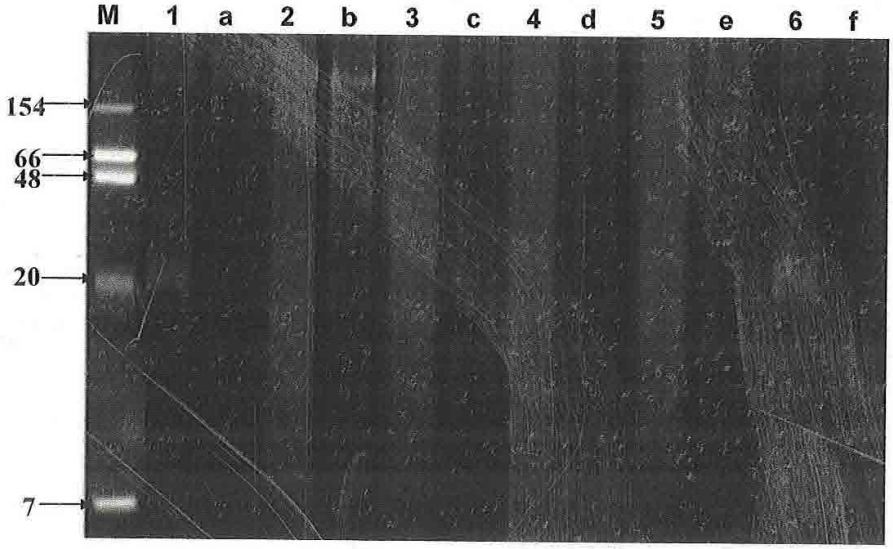
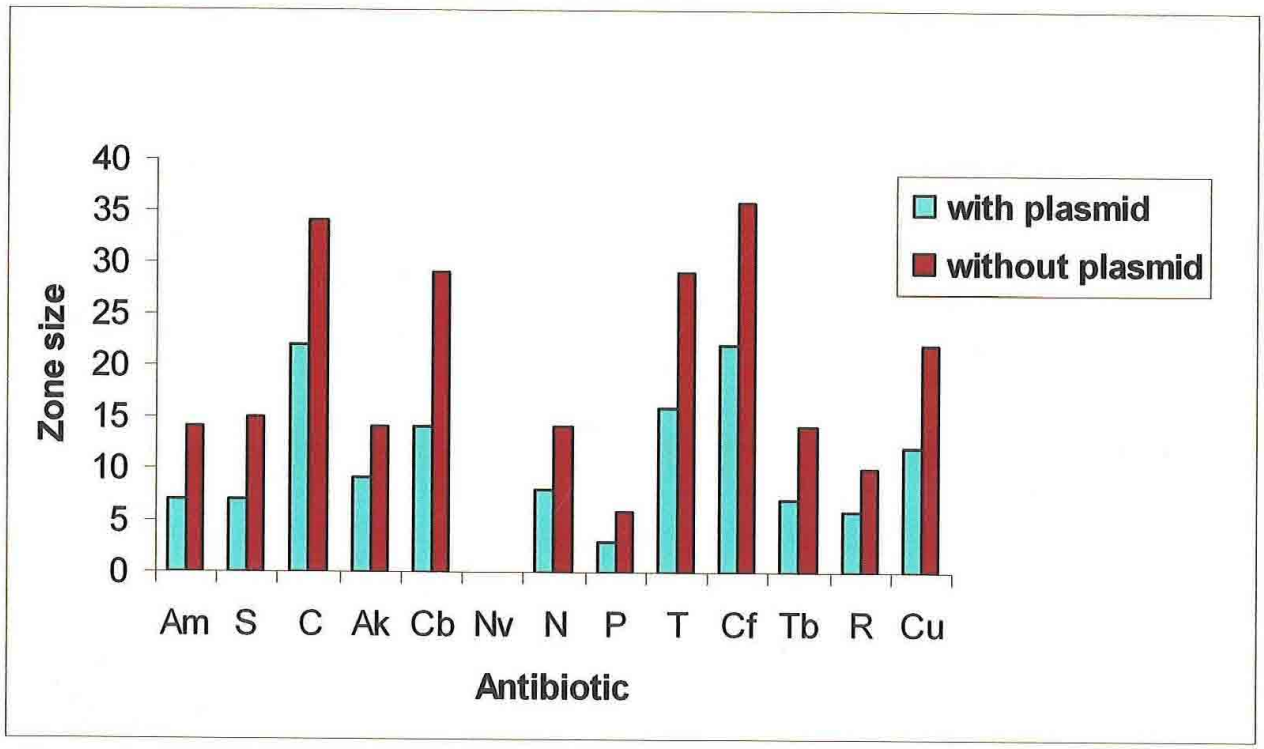
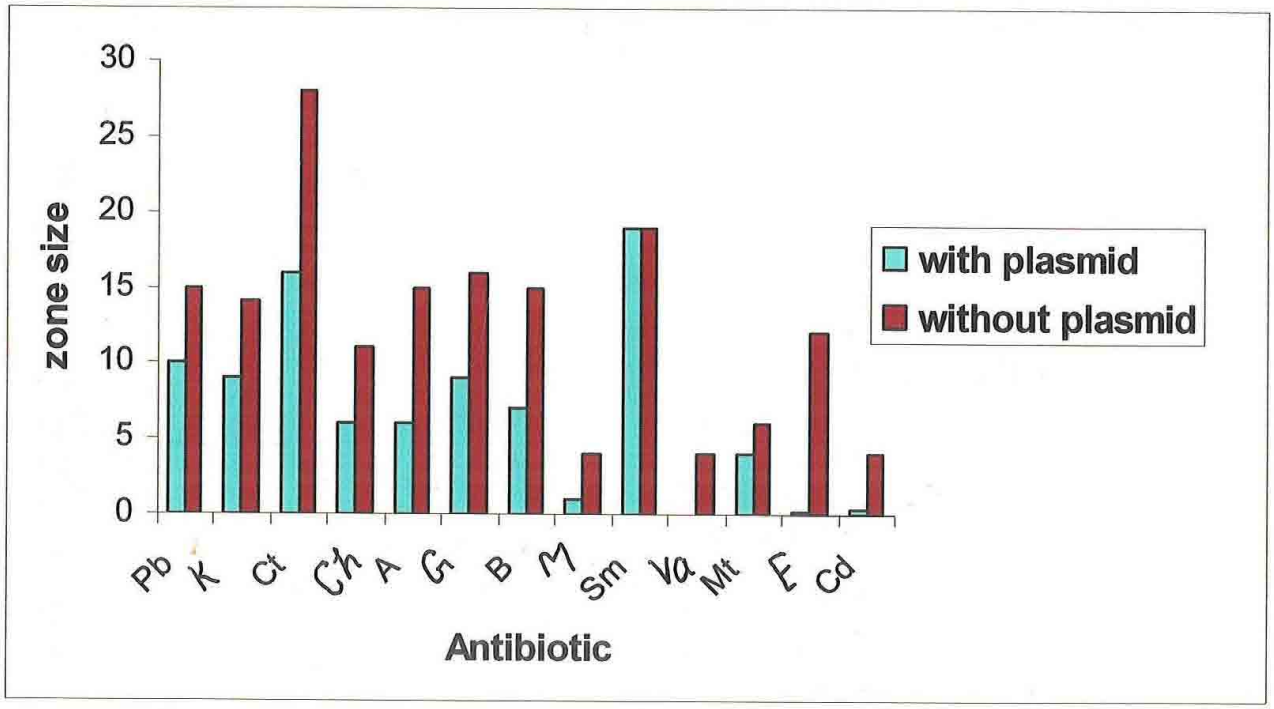


Fig 4.22 Agarose gel showing loss of plasmid after curing
Lanes: 1, IFM2; 2, IFM4; 3, IFM5; 4,RW8; 5,S2; 6, RM3- Parent strains; a- f corresponding lanes of cured cells



(A)



(B)

Fig 4.23 (A) and (B) Effect of plasmid curing on antibiotic response

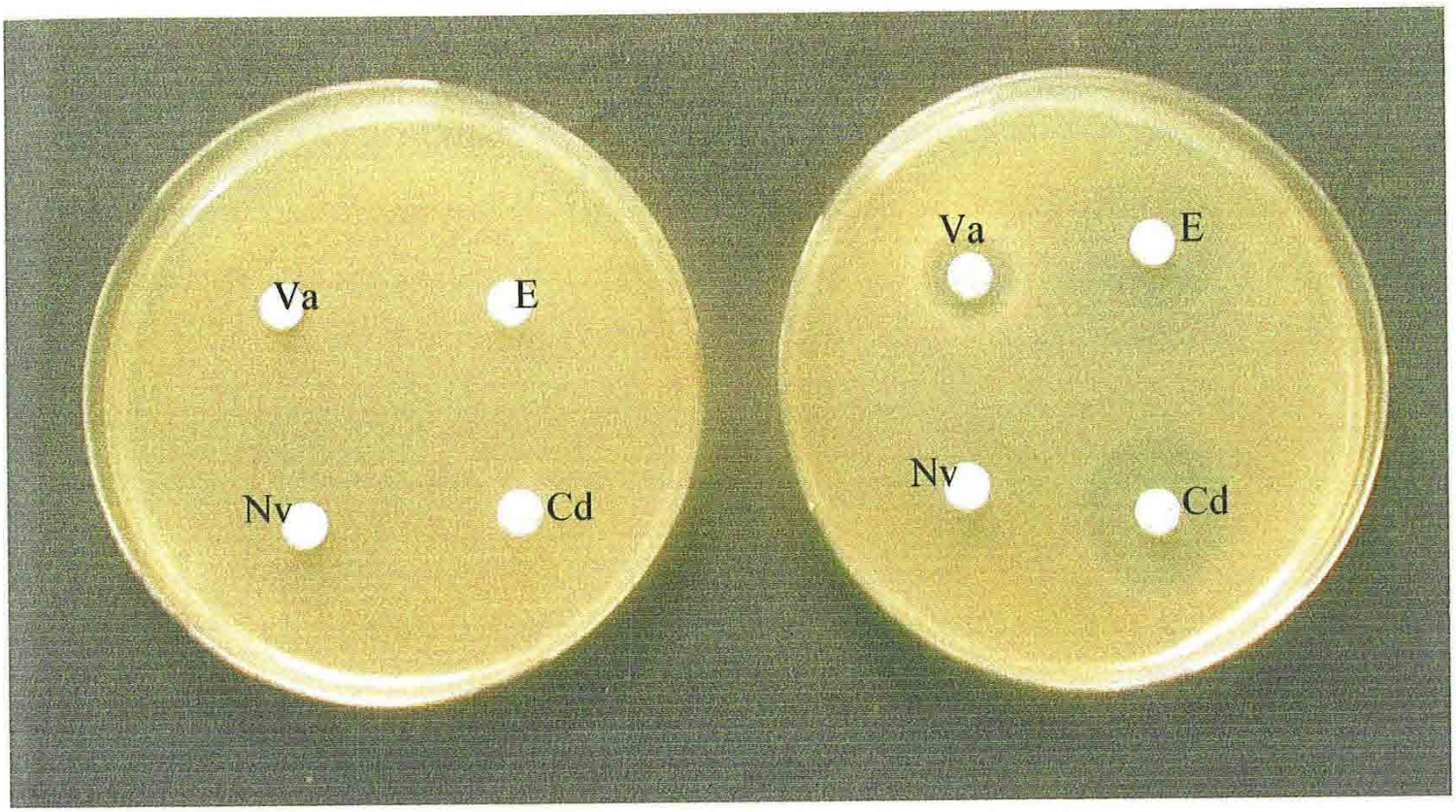


Fig 4.24 Loss of vancomycin (Va) erythromycin (E) and clindamycin (Cd) resistance in *E. sakazakii* (A) Before plasmid curing (B) After plasmid curing

colibacillosis than in faecal isolates from healthy birds. At this time, it is not known whether *iss* merely marks the presence of a larger pathogenicity unit or is itself a contributor to virulence (Nolan *et al.*, 2003). The gene *iss*, whether detected through hybridization or amplification, was found in more of the disease-associated isolates than in those of healthy birds. This difference was highly significant. Further, *iss* sequences were widely distributed among isolates of different serotypes from various avian host species and sites within these hosts. Such results suggest that possession of the *iss* sequence by an avian *E. coli* isolate may be a good indicator of that isolate's potential to cause disease. This association warrants further study because *iss* and the protein it encodes may be useful targets of future colibacillosis control efforts (Pfaff-McDonough, 2000). The sequences of the gene and the predicted protein product were compared with those of *iss* from a human *E. coli* isolate and lambda *bor*. The *iss* gene from the avian *E. coli* isolate had 96.8% identity with the *iss* gene from the human *E. coli* isolate. But Iss protein from the avian isolate had only 87% identity with *iss* from the human isolate because of a frameshift mutation in the coding sequence of *iss* from the human *E. coli* isolate (Horne *et al.*, 2000). Nishio *et al.* (2005) reported that An outer membrane protein was reportedly required for the serum resistance and this protein was also coded by a phage protein.

There are two genes *nanA* and *nanB* those coded for neuraminidase. These genes can be distinguished from each other on the basis of restriction mapping and DNA hybridization studies (Camara *et al.*, 1991). The role of neuraminidase and hyaluronidase was studied by (Berry and Paton, 2000) in *S. pneumoniae* by mutation in respective genes in pneumococcal chromatid. However, the mutants had no significant change in virulence and might have been compensated by *nanB*. A 3.0 kbp DNA fragment of *S. pneumoniae* when cloned in *E. coli* was able to degrade neuraminidase substrate 2'-(4-methylumbelliferyl)-α-D-*N*-acetyl-neuraminic acid (Berry *et al.*, 1988). Another virulence causing enzyme elastase is encoded by *lasB* gene the expression of which is regulated by quorum sensing (Whiteley *et al.*, 1999). A gene bank constructed by Schad *et al.* (1987) from *Pseudomonas aeruginosa* PAO1 was used to complement three *P. aeruginosa* elastase-deficient strains. When cloned two different genes were found to express elastase activity. In 1988 the

same group (Schad and Iglewski) studied nucleotide sequence of another elastase gene *lasA*. When expressed in *E. coli* it activated the extracellular elastase produced by *P. aeruginosa* mutant, PA O-E64 that was otherwise inactive. Su *et al.* (1991) reported the presence of 6 Kb fragment in chromosomal DNA of *E. faecalis* showing gelatinase activity.

Such genetic studies on *E. sakazakii* are further warranted for better understanding of its virulence phenomenon.

4.9 SCREENING OF ENTEROTOXIGENIC CULTURES

Production of enterotoxin by *E. sakazakii* cultures was determined by suckling mice assay. In mice injected with test culture, gut: carcass (I: C) ratio ≥0.083 was taken to be positive for toxigenicity (Dean et al., 1972). The results of suckling mice assay of *E. sakazakii* cultures is presented in Table 4.4.

Table 4.4 Suckling mice test for enterotoxic activity of *E. sakazakii*

Sr. No.	Isolates No.	Avg. intestinal weight (gm)	Avg. carcass weight (gm)	Ratio (G / C)	Inference
1	Control	0.206	2.65	0.075	-ve
2	MTCC 659	0.512	2.55	0.071	-ve
3	IFM1	0.309	2.52	0.078	-ve
4	IFM2	0.330	2.46	0.086	+ve
5	IFM3	0.435	2.546	0.08	-ve
6	IFM4	0.51	2.65	0.122	+ve
7	IFM5	0.341	2.75	0.077	-ve
8	RW8	0.263	2.61	0.070	-ve
9	S2	0.266	2.72	0.068	-ve
10	RM2	0.291	2.58	0.073	-ve
11	RM3	0.582	2.71	0.102	+ve
12	GF1	0.635	2.738	0.125	+ve
13	GF2	0.279	2.61	0.085	+ve

Avg - Average, I– Intestine, C - carcass

Among the 11 isolates and reference strain MTCC 659, only three (IFM4, RM3 and GF1) aroused strong positive suckling mice reaction and two isolates (IFM2 and GF2) tended to be enterotoxigenic. Among these an isolate GF1 from goat faeces being most toxigenic was selected for the present investigation on its enterotoxin.Table 4.5 shows distribution of enterotoxic *E. sakazakii* strains in various samples.

Table 4.5 Incidence of enterotoxic *E. sakazakii* strains in environment

Samples	No. of samples screened	Positive for *E. sakazakii*	Toxin production
Clinical samples	19	1	0
Infant formulae milk	16	5	1 + 1*
Raw milk	25	2	1
River water	20	1	0
Goat faeces	2	2	1 + 1*

* tended to give positive suckling mice reaction

4.10 STANDARDIZATION OF MEDIUM FOR ENTEROTOXIN PRODUCTION

The growth medium (casamino yeast extract medium) was standardized with respect to concentration of nitrogen source, temperature of incubation and period of incubation for maximum production of toxin. The effect of alterations made was studied in suckling mice.

4.10.1 Nitrogen source

Medium containing 1% casamino acids yielded more enterotoxin as compared to 2% in original medium (control) which is in line with Jacks and Wu (1974). Also enterotoxicity was more in presence of tryptone and tryptose substitute of casamino acids. However, no significant change could be observed when casaminoacids were substituted by peptone (Fig 4.25).

4.10.2 Temperature of Incubation

Toxicity of *E. sakazakii* (ES24) was highest at 37°C and less beyond this temperature. At 25 and 45°C the toxicity was almost similar (Fig 4.26). The findings at lower than optimum temperature were in coincidence with those of Trachmann and Maas (1998) who reported that quantity of *E. coli* enterotoxin decreased with decrease in temperature of incubation.

4.10.3 Period of incubation

When incubated at 37°C for 6, 12, 18 and 24 hr, maximum enterotoxin was produced after 12 hrs incubation as indicated by suckling mice assay. The organism started elaborating the enterotoxin by 6 hr of growth (Fig 4.27) which again subsided by 24 hr. The possible reason may be the production of various proteolytic proteins coupled with some environmental factors. This was contrary

to Jacks and Wu (1974) who reported that in *E. coli* toxin production started afer 12 hr incubation and was optimum at 18 hr. Also, contrary to Evans *et al.* (1973), it was observed that after 18 hrs period of incubation the activity was reduced by 20.2%. After 24 hrs no enterotoxicity was observed as compared to blank.

4.11 PURIFICATION OF ENTEROTOXIN

Since there was no published report available on *E. sakazakii* toxin prearation and its characterization, the general methods applicable to protein toxins of Enterobacteriaceae were used. The toxin purification procedure consisted of ammonium sulfate precipitation, ion exchange chromatography on DEAE cellulose and finally Sephadex G-100 gel filtration chromatography.

4.11.1 Ammonium Sulfate Precipitation

Two litres of 12 h grown culture (GF1) was centrifuged and the centrifugate contained 660 mg protein. This protein was precipitated from the supernatant by 80% saturatation with ammonium sulfate and the resulting precipitates were collected by centrifugation and dissolved in 30 ml of 0.02 M tris (hydroxymethyl) aminomethane buffer. This contained 61.7 mg protein. No protein could be recovered above 80% saturation with ammonium sulfate. The protein tested positive with suckling mice reaction. Carbohydrate concentration in the filtrate was 0.76 mg/ ml that was reduced to 0.35 mg/ml after ammonium sulfate precipitation.

4.11.2 Anion Exchange Chromatography

The protein after dialysis was loaded slowly eluted with same buffer to remove unbound sample that tested negative for toxicity in suckling mice. From this it could be interpreted that the toxin of interest was anionic in nature and got bound to the matrix completely.

Manual elution by step wise increase in NaCl concentration in the buffer was done to stepwise increase the ionicity: 0.1 M, 0.25 M, 0.5 M and 1 M NaCl respectively in 0. 01 M tris- hydrochloride (pH 8.0). The toxicity was detectable only in the material eluted with 0.5 M NaCl. The majority of the remaining pigment was strongly bound to the DEAE cellulose matrix and could not be eluted even with 1.25 M. The elution profile has been shown in Fig 4.28. The

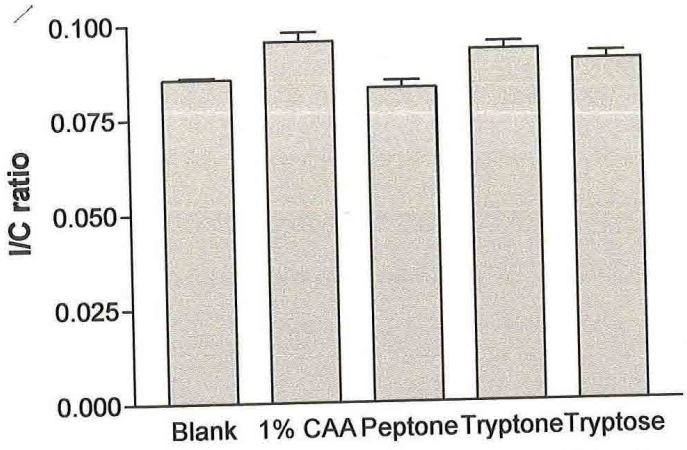


Fig 4.25 Effect of nitrogen sources on *E. sakazakii* enterotoxin production

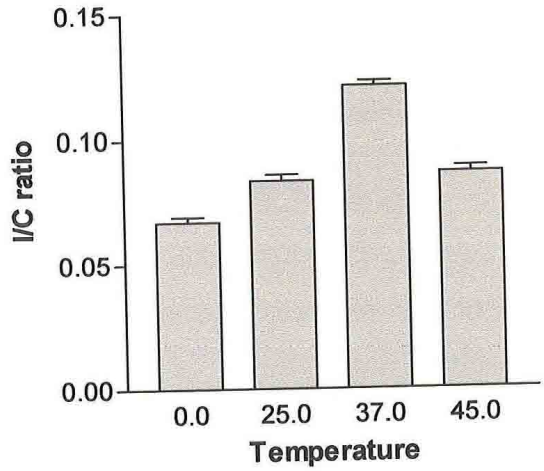


Fig 4.26 Effect of incubation temperature on *E. sakazakii* enterotoxin production

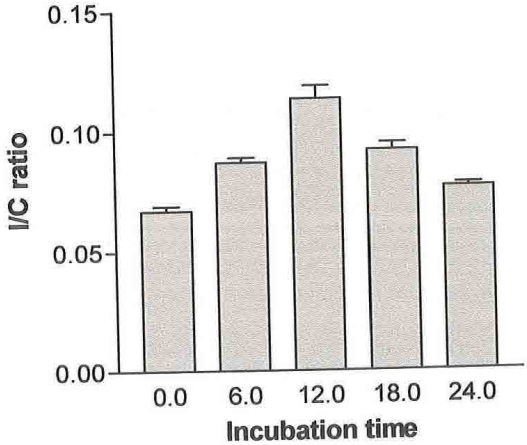


Fig 4.27 Effect of period of incubation on *E. sakazakii* enterotoxin production

enterotoxin was 31.1 folds purified and contained 9 mg protein. The carbohydrate concentration reduced to 0.058 mg/ ml.

4.11.3 Gel Filtration Chromatography

Further purification of the toxic protein was achieved by gel filtration after concentration by dialysis. The concentrated protein contained 1.5 mg protein/ ml of dialysate. The elution profile from Sephadex G- 100 is presented in Fig 4.29. A single major peak was observed with in the bed vol of the column along with two minor peaks that were discarded. The fractions of major peak were pooled together and evaluated for toxin. The toxin was 32.4 folds purified.

4.12 CHARACTERIZATION OF PURIFIED ENTEROTOXIN

4.12.1 Protein Estimation

The amount of protein in each sample of *E. sakazakii* enterotoxin was estimated after each step and has been shown in Table 4.6. Protein content per ml decreased with each step of purification but the activity increased.

4.12.2 Carbohydrate Concentration

Carbohydrate concentration also decreased at each step of protein purification. The toxin contained very less carbohydrate after ion exchange chromatography (Table 4.6).

Table 4.6 Purification profile of *E. sakazakii* enterotoxin

Purification step	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Carbohydrate Concen. (mg/ml)	1 Unit of protein (µg)	Purification fold
Culture filtrate	2,000	0.33	660	0.76	165	1
Clarification	1950	0.32	624	0.71	161	1
$(NH4)_2SO_4$ precipitation	30	2.05	61.5	0.35	14.8	11.1
DEAE cellulose	6	1.5	9	0.058	5.3	31.1
Sephadex G-100	4	1.4	5.6	0.002	5.1	32.4

4.12.3 Molecular Weight of the Purified Protein

Molecular weight of the purified toxin of *E. sakazakii* was determined to be 66 kDa on 12.5 % gel for SDS PAGE (Fig 4.30). This value was close to that of

Shigella dysenteriae toxin1- 62 kDa (Yutsudo *et al.*, 1987) and *P. aeruginosa*- 65 kDa (Challahan, 1974). The enterotoxin of these organisms effect human hosts differently. The latter toxins restrain protein synthesis by inhibiting Elongation factor 1 (EF-1)-dependent GTPase activity (Obridge *et al.*, 1987). While *E. sakazakii* causes meningitis *S. dysenteriae* causes dysentery although both are most common in children of few months to 5 yrs of age. Like *E. sakazakii, P. aerugenosa* is a nosocomial pathogen and also found in a biofilm, but it is generally associated with various infections involving urinary tract, respiratory system, dermatitis, soft tissue, bacteremia, bone and joint, gastrointestinal tract and other systemic infections, particularly in patients with severe burns and in cancer and AIDS patients who are immunosuppressed. Different characteristic of *E. sakazakii* enterotoxin having similar molecular weight is apparently due to structural differences and needs detailed study. The mode of action of E. sakazakii toxin has not yet been studied hence its activity with neural tissues cannot be well established.

4.12.4 Effect of Heat on Activity of Toxin

Fluid accumulation in terms of increased I: C ratio was observed with the samples heated at 50 and 70°C for 30 min but it was less by approximately 20% at 90°C and completely absent at 100°C. This suggested that the enterotoxin was heat stable (Fig 4.31). Although there have been no reports available in the existing literature on the heat stability of *E. sakazakii* toxin, that from *E. coli* also shown complete loss of activity at 100°C (Jacks and Wu, 1974; Whip *et al.*, 1975). In contrast, activity of *P. aeruginosa* toxin was reduced to 20% when heated at 56°C (Callahan, 1974). Findings of the present study have thus indicated that the heat stability of *E. sakazakii* enterotoxin was equivalent to that of *E. coli* but more than that of *P. aeruginosa.*

4.12.5 Effect of pH On Activity of Enterotoxin

Purified *E. sakazakii* enterotoxin exhibited optimum activity at slightly acidic pH 6. Its slight reduction at pH 7 increased to its loss at pH 8.0 (Fig 4.32). The result was thus different from those of *E. coli* heat stable enterotoxin that was more active at pH 9 than at lower pH 8 or 7 (Jacks and Wu, 1974) and *P. aeruginosa* enterotoxin that showed acivity only at pH 5.0 (Callahan, 1974). However, Mullan *et al.* (1978) that *E. coli* heat stable enterotoxin activity was

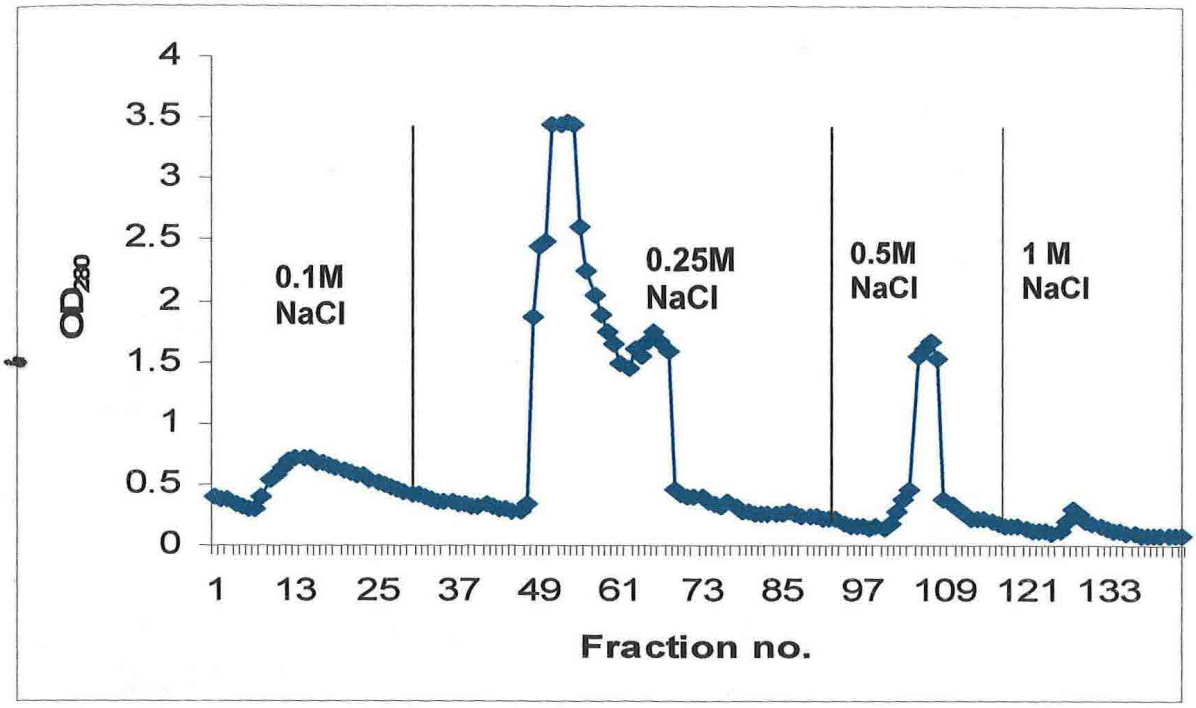


Fig 4.28 Elution profile of enterotoxin on DEAE Cellulose chromatography. 0.5 M peak was showing toxicity

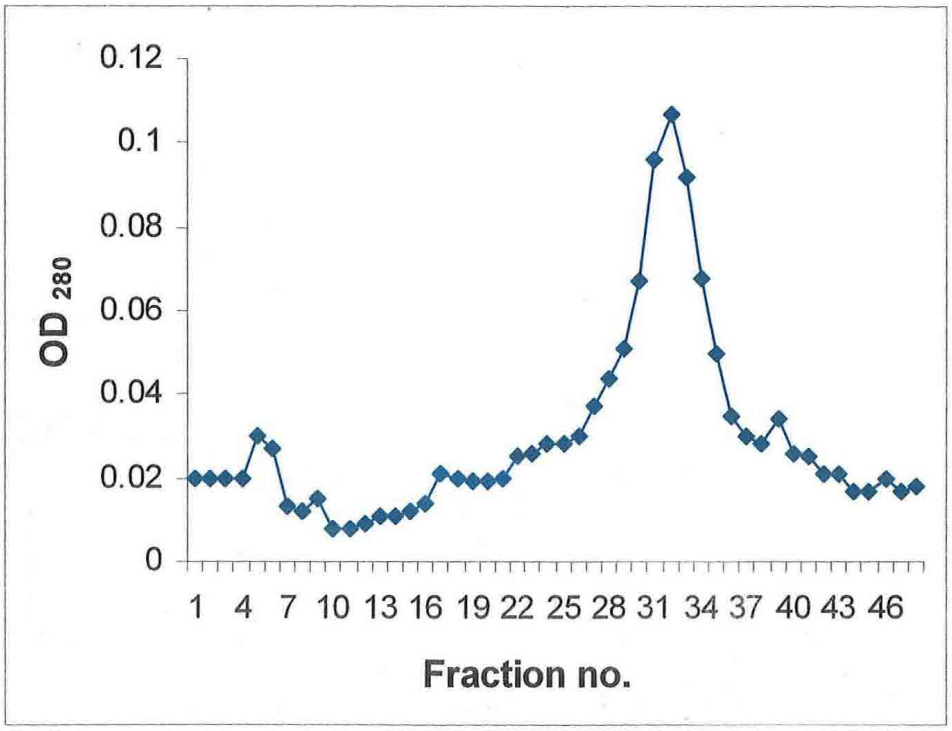


Fig 4.29 Elution profile of 0.5 M peak fractions on Sephadex G-100 gel filtration chromatography

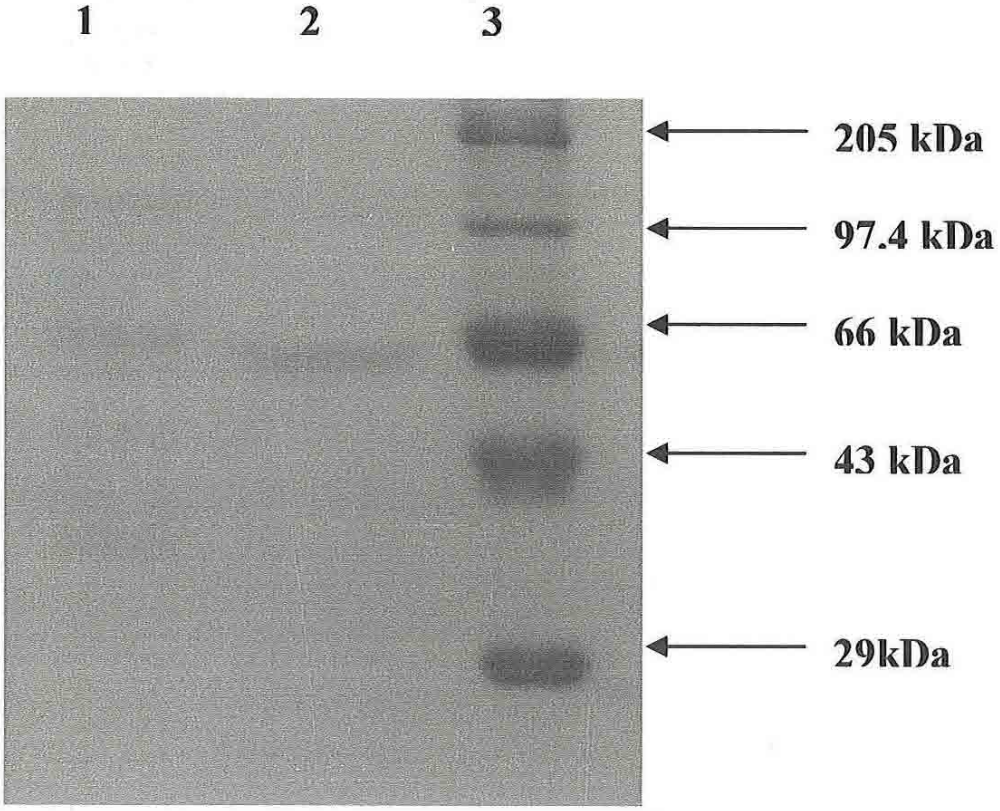


Fig 4. 30 SDS PAGE profile of *E. sakazakii* enterotoxin.
Lane 1- Crude protein, Lane 2- purified protein, Lane 3- molecular weight marker

not affected by acid but was significantly reduced at pH 10 and pH 11. Thus the present investigation has shown the importance of E. sakazakii in our normal foods which are slightly acidic and a caution may be excercised while reconstituting dry foods.

4.12.6 Effect of Proteolytic Enzymes on Activity of Enterotoxin

The results of tha experiments to determine the action of proteolytic enzymes on enterotoxin of *E. sakazakii* have been compiled in Table 4.7. Purified *E. sakazakii* enterotoxin showed decreased activity in presence of pronase. Its activity was unaffected by trypsin. It has been reported that heat stable enterotoxin of *E. coli* was resistant to proteolytic activity of pronase (Jacks and Wu., 1974) but heat labile enterotoxin of the same was destroyed by pronase (Jacks *et al.*, 1973). Thus it was followed that *E. sakazakii* toxin behaved differently in presence of different proteolytic enzymes and that it had different structure than both HT and ST of *E. coli* as was derived from different its response to heat, medium pH and proteolytic enzymes.

Table 4.7 Effect of proteolytic enzyme on activity of enterotoxin

Enzymes	I: C	Inference
Control	0. 068 ± 0.001	- ve
Enterotoxin	0.091 ± 0.003	+ ve
Enterotoxin + Pronase	0.081 ± 0.006	± ve
Enterotoxin + Typsin	0.088 ± 0.003	+ ve

4.12.7 Effect of Metal Ions

The experimental results regarding the effect of metal ions *E. sakazakii* enterotoxin activity have been presented in Fig 4.33. While toxic suspension containing $CaCl_2$ showed I: C ratio comparable to that of control, $ZnCl_2$ effected and increased the I:C ratio. This suggested that fluid accumulation in GI tract might have got stimulated by Zn^{2+}. Zn^{2+} has been reported to stimulate enterotoxin activity of Enterobactereaceae. Papageorgious *et al.* (2004) studied secondary zinc binding site in *S. aureus* and concluded that varying the concentration of zinc ions present affected the activity of the toxin. $CuSO_4$ tended to lower the I:C ratio and the result was in line with earlier report of

Singh and Mcfeters (1986) that when treated with subinhibitory concentration of copper, ST production in *E. coli* decreased in the initiation of logarithmic phase. The toxin synthesis was continuous but did not rise as rapidly as in the corresponding uninjured cells. No increase in synthesis of heat stable enterotoxin by *E. coli*, was observed in presence of Mg^{+2}, Mn^{+2} and Fe^{+3} ions (Alderete and Robertson, 1977).

4.12.8 LD_{50} of purified toxin

The minimum concentration of purified enterotoxin that could cause death in 50% mice in a group was 56 ng. At higher concentration more than 50 % death was observed. No death was observed in control. LD_{50} was still more than that of Vero toxin (Yutsudo *et al.*, 1987) and Shiga like toxin of *E. coli* (Noda *et al.*, 1987) that were 1 ng and 30 ng respectively when injected intraperitoneally. The lethal dose was much less than that for other members of Enterobacteriaceae. LD_{50} for stx 1 and stx 2 toxins of E.coli O157:H7 has been reported to be 200 ng/mouse and 1 ng/mouse respectively (Ishikawa *et al.*, 2003). Toxins of *S. dysenteriae* cytotoxin and *P. aeruginosa* are comparatively less lethal with LD_{50} 450 ng/mouse (Eiklid and Olsnes, 1983) and 280 µg/mouse (Callahan III , L.T., 1974) respectively.

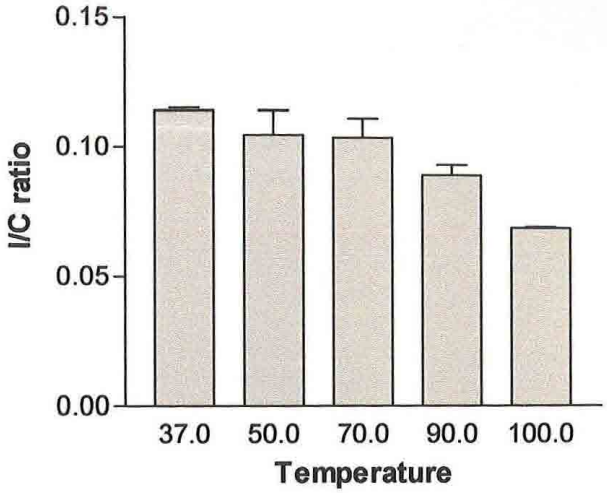


Fig 4.31 Effect of temperature (°C) on activity of *E. sakazakii* enterotoxin

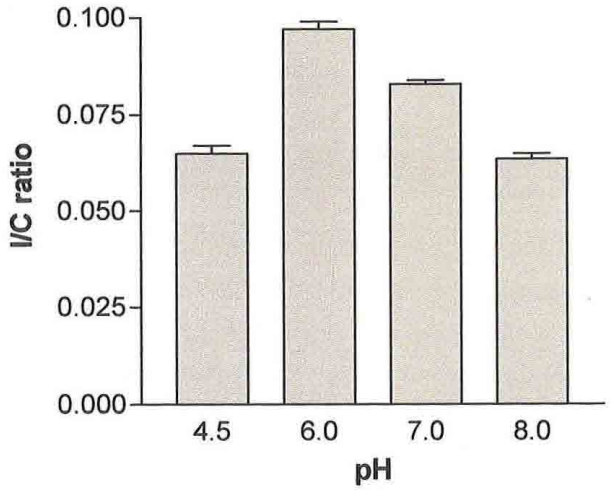


Fig 4.32 Effect of pH on activity of *E. sakazakii* enterotoxin

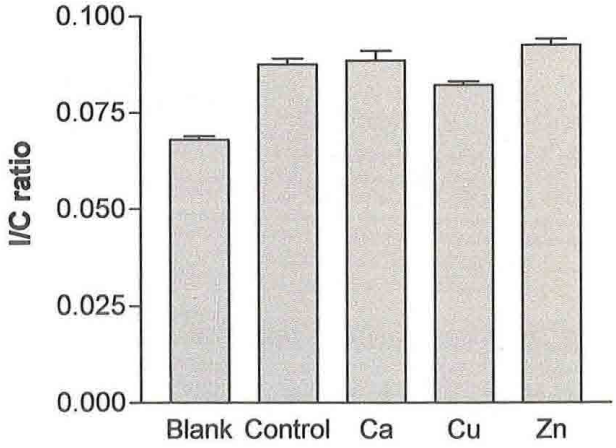


Fig 4.33 Effect of metal ions on activity of *E. sakazakii* enterotoxin

CHAPTER - 5

Summary and Conclusions

SUMMARY

1. A total of 24 isolates obtained from 170 food and environmental samples collected from nine different sources, were tentatively selected. Eleven of these were identified as *E. sakazakii* on the basis of biochemical characterization.
2. Infant milk formulae were most contaminated with *E. sakazakii* where 5 out of 16 (31.25 %) samples contained the pathogen. Among others 50 % goat faecal, 25 % diarrhoeal patient stools, 5.3 % commercial dairy milk, 4 % experimental dairy, and 5 % river water samples revealed the presence of pathogen.
3. The pathogen could not be isolated from any sample of pasteurized milk, cheese and skim milk procured from Experimental Dairy, NDRI, Karnal, India.
4. The above 11 *E. sakazakii* isolates were confirmed by species-specific PCR targeting 16S rRNA region. A PCR product of 929 bp was obtained. Amplification conditions were optimized with respect to annealing temperature (52.0 °C), concentration of Taq polymerase (0.3 U), primer (9.96 pM), dNTPs (200 nM) and number of amplification cycles (30).
5. The sequence of PCR product was got custom synthesized and analysed with respect to the available sequences at NCBI database.
6. All the 11 isolates were resistant to clindamycin, erythromycin, metronidazole, novobiocin, penicillin G, rifampicin, vancomycin; sensitive to amoxycilin, chloramphenicol and chlorotetracycline but showed varied response to ampicillin, amikacin, bacitracin, cabenicillin, cefuroxime, cephalothin, ciprofloxacin, gentamycin, kanamycin, methicillin, neomycin, polymyxin B, streptomycin, sulphamethazole, tetracycline and tobramycin.
7. All the isolates grew well at 37 °C. Isolates IFM4 and RW3 could survive low temperature of 4 °C but not higher temperature of 45 °C and 55 °C. MTCC 659 did not grow at low temperatue but showed sufficient growth at 45 °C and survived at 55 °C also.IFM2, S2, GF1and GF2showed growth only at

45 °C. Although IFM1 and IFM5 did not grow at 45 °C but showed very scanty growth at 55 °C.

8. All the isolates showed D_{55} of 7-12 min and D_{62} of 0.3-0.6 min. The overall Z value was calculated to be 1°C.
9. The organism showed extended lag phase with increasing medium NaCl concentration upto 5.5 %. No growth was observed in presence of 10.5 % NaCl.
10. All the 11 isolates of *E. sakazakii* of were hemolytic, produced DNase and agglutinated bovine RBCs.
11. The isolates did not produce areobactin, phospholipase C and hyaluronidase, but all exhibited some other virulence properties *viz.* resistance to serum bactericidal activity, gelatinase, neuraminidase elastase and presence of lipopolysaccharide. Isolates IFM4 and RM2 were collagenolytic after 48 hrs.
12. Plasmid could not be isolated from MTCC 659, IFM1, IFM3, RM2, ES24 and GF2. Gel profile showed presence of one plasmid in IFM2 and RM3 and two in IFM4, IFM5, RW8, S2 and RM2 each.
13. No change in virulence properties could be observed after plasmid curing. This suggested that virulence in *E. sakazakii* was chromatid encoded and not plasmid linked.
14. Among five isolates showing enterotoxicity in suckling mice, the most potential one (GF1) was used for purification of toxin.
15. Enterotoxin was purified after 24 hrs growth in casamino yeast extract broth. It was purified upto 32 folds using ammonium sulphate precipitation, followed by DEAE-cellulose (ion exchange) and Sephadex G-150 (gel filtration) chromatography.
16. Toxin production was more in presence of 1 % casamino acids than in 2 % in normal medium (control). Toxin production was maximum at 37 °C after 12 hrs.
17. Protein and carbohydrate concentration in purified enterotoxin was 1.4 mg and 0.002 mg/ml respectively.

18. The purified enterotoxin was characterized as having molecular weight of 66 kDa, with optimum activity at pH 6.0 and in the presence of $ZnCl_2$. The toxin with LD_{50} 56 ng retained activity even when heated at 90°C for 30 min.

CONCLUSION

The present study has revealed the presence of *E. sakazakii* in infant formulae milk, raw milk, diarrhoea patient's stools and goat faeces. The occurrence of the organism in the infant milk powder indicates its likely post processing contamination from wash water, equipments, utensils or possibly from the infected handlers. Presence of the organism in infant milk powder makes us cautiousabout their use for the most vulnerable section of society i.e. the neonates and the young ones. In the study goat – the poor man's cow in India, has been found to shed the organism in its faeces indicating the possibility of the economically weaker section of the society to contract an easy infection. Although as per Indian traditions milk is always boiled before consumption but still infection may occur during handling. The recovery of organism from goat otherwise showing healthy conditions and without any history of such disease in the recent past also indicates the possibility of the organism being a commensal and a normal inhabitant of goat intestine. The occurrence of the organism in the stools of neonate and raw milk also causes concern about the overall sanitary conditions prevailing at various levels of production, processing, transport, storage and consumption. Such incidences have so far not been reported in India. The present study is likely to have a long range bearing in quality assurance measures for foods, particularly of the dairy origin, in India.

The organism has already been considered as an opportunistic pathogen. It has been observed in the present study that. *E sakazakii* possesses some virulence properties that are recognized important in causing and establishing infection. However a detailed study about the interaction among various virulence determinants is needed. This would be beneficial in understanding the mechanism of the organism's pathogenesis particularly its enterotoxic and meningotoxic effects and hence in the prevention, prophylaxis and control.

The toxin has been found to be fairly stable at 90°C indicating that it is quite resistant to commercial pasteurization treatments. The organism has already been isolated from highly heat treated milk formulae (Nazarowec-White and Farber, 1997). This has far reaching implications for the neonates who are fed milk foods sometimes reconstituted under unhygienic conditions as might have been the case of post processing contamination.

Further molecular characterization of the enterotoxin and study of its structural subunits, if any, is therefore suggested. In our preliminary studies we could not isolate plasmid from the most toxic culture showing maximum toxicity; however detailed studies on this aspect are also warranted. The knowledge of all these together may be helpful in lessening the increasing reports of *E. sakazakii* infection and its deleterious effects.

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APPENDIX I

Composition of Media Used in the Study

Ingredients	Amount
Arginine Dihydrolase Broth	
Tryptone	5.0 g
Yeast extract	5.0 g
Di potassium hydrogenphosphate	5.0 g
Glucose	0.5 g
Arginine	3.0 g
Distilled water	1L
pH	7.0
Basal Medium for Lecithinase Test	
Tryptone	10.0 g
Di Sodium hydrogen phosphate	5.0 g
Di hydrogen potassium phosphate	1.0 g
Sodium chloride	2.0 g
Magnisium sulphate	0.1 g
Glucose	2.0 g
Distilled water	1 L
pH	7.6
Egg yolk is mixed with saline water in 1: 1 ratio and heated at 45 °C 1hr. Three milliliters of this egg yolk is added to the basal medium. This forms complete lecithinase medium.	
Blood Agar Base	
Pancreatic digest of casein	15.0g
Papaic digest of soya bean meal	5.0 g
Sodium chloride	5.0 g
Distilled water	1 L
Buffered Peptone Water	
Sodium chloride	5.0 g
Di-Sodium hydrogen phosphate	3.5 g
Di- Hydrogen potassium phosphate	1.5 g
Distilled water	1 L
pH	7.2
Carbohydrate Fermentation Broth	
Peptone	10.0 g
Sodium chloride	5.0 g
Phenol red	0.018 g
Distilled water	1L
Autoclave the medium and dispense 3 ml in fermentation tubes. Inoculate the tubes and to each fermentation tube add a single sugar disc aseptically.	

Ingredients	Amount
DNase Test Agar	
Tryptose	20.0 g
Sodium chloride	5.0 g
Deoxyribonucleic acid	2.0 g
Toluidine blue	0.10 g
Agar	15.0 g
Distilled water	1L
pH	7.3
Enterobacteriaceae Enrichment Agar	
Ox bile	20.0 g
Peptone	10.0 g
Di-Sodium hydrogen phosphate	6.45 g
Glucose	5.0 g
Di hydrogen potassium phosphate	2.0 g
Brilliant Green	0.0135 g
Distilled water	1L
pH	7.2
Esculin Agar	
Pancreatic digest of casein	13.0 g
Sodium chloride	5.0 g
Yeast extract	5.0 g
Heart infusion powder	2.0 g
Esculin	1.0 g
Ferric citrate	0.5 g
Agar	15.0 g
Gelatin Hydrolysis Agar	
Peptone	5.0 g
Beef extract	3.0 g
Gelatin	3.0 g
Agar	15.0 g
Distilled water	1L
pH	7.0
Lysine Decarboxylase Agar	
Peptone	5.0 g
Yeast extract	3.0 g
Glucose	1.0 g
L- Lysine hydrochloride	5.0 g
BCP	0.02 g
Distilled water	1L
MRVP Medium	
Buffered peptone	7.0 g
Dextrose	5.0 g
Dipotassium phosphate	5.0 g
pH	6.9

Ingredients	Amount
Nitrate Broth	
Peptone	5.0 g
Beef extract	3.0 g
KNO3	1.0 g
Distilled water	1L
pH 7.0	
Nutrient Agar	
Sodium chloride	5.0 g
Beef extract	3.0 g
Peptone	5.0 g
Agar	15 g
Distilled water	1L
Ornithine Decarboxylase Broth	
L- Ornithine	5.0 g
Yeast extract	3.0 g
Glucose	1.0 g
Bromo Cresol Purple	0.015 g
Phenylalanine Malonate Agar	
Yeast extract	1.0 g
Sodium malonate	3.0 g
Phenylalanine	2.0 g
Ammonium sulphate	2.0 g
Dipotassium phosphate	0.6 g
Monopotassium phosphate	0.4 g
Sodium chloride	2.0 g
Bromo Thymol Blue	0.025 g
Distilled water	1L
Phenylalanine Malonate Broth	
Yeast extract	1 g
Sodium malonate	3 g
DL- phenylalanine	2 g
Ammonium sulphate	2 g
Dipotassium phosphate	0.6 g
Monopotassium phosphate	0.4 g
Sodium chloride	2 g
Bromo thymol blue	1 L
Simmon Citrate Agar	
Magnesium sulphate	0.20 g
Ammonium Dihydrogen Phosphate	1.0 g
Dipotassium phosphate	1.0 g
Sodium citrate	2.0 g
Sodium chloride	5.0 g
Bromothymol blue	0.08 g
Agar	15.0 g
Distilled water	1L
pH	6.8

Ingredients	Amount
Triple Sugar Iron Agar	
Peptic digest of animal tissue	20.0 g
Yeast extract	3.0 g
Meat extract	10.0 g
Lactose	10.0 g
Dextrose	1.0 g
NaCl	5.0 g
Ferrous sulphate heptahydrate	0.02 g
Sodium thiosulphate pentahydrate	0.30 g
Phenol red	0.024g
Agar	12.0 g
Distilled water	1L
Tripticase Soy Agar	
Tripticase	15.0 g
Phytone	5.0 g
Sodium chloride	5.0g
Agar	15.0 g
Distilled water	1L
pH	7.3
Urea Agar Medium	
Peptone	1.0 g
Sodium chloride	5.0 g
Di hydrogen potassium phosphate	2.0 g
Agar	20.0 g
Distilled water	1 L
pH	
Autoclave the medium. Then add 1 g glucose and 6.0 ml phenol red medium (0.2 % solution). Add 100 ml urea solution (20 %).	

Reagents and Buffers Used in Molecular Biology Aspects

SET Buffer

NaCl : 0.435 g
EDTA : 0.930 g
Tris Base : 0.242 g
The volume was made upto 100 ml and then autoclaved under 15 psi at 121°C for 15 min.

TE Buffer (5 X)

1M Tris : 1 ml
0.5 EDTA (pH 8.0) : 200 µl
The volume was made up to 100 ml and then autoclave under 15 psi pressure at 121 °C for 15 min.

TAE Buffer (50 X)

Tris base : 242 g
Glacial Acetic Acid : 57.1 ml
EDTA (0.5 M), pH 8.0 : 100 ml
Make up the volume to 1000 ml with distilled water. Make working solution by diluting to 1 X with autoclaved water.

Ethedium Bromide

1 g of ethidium bromide was dissolved in 100 ml of H_2O. It was stirred on a magnetic stirrer to ensure that the dye has dissolved and then the container was wrapped in aluminium foil and store at 4 °C.

Loading Dye (6 X)

Bromophenol blue (0.25 %) : 0.25 g
Xylene cynol (0.25 %) : 0.125 g
Ficol (Type 400) 15% : 15 g
Distilled water : up to 100 ml

Solutions Used in Plasmid Isolation

Solution-I	Solution-II	Solution-III
50 mM glucose	0.2 N NaOH (freshly diluted from a 10 N stock)	5 M potassium acetate – 60 ml
25 mM Tris. Cl (pH 8.0)	1% SDS	Glacial acetic acid – 11.5 ml
10 mM EDTA (pH 8.0)		H_2O – 28.5 ml
Autoclaved and stored at 4°C		The resulting solution is 3 M with respect to potassium and 5 m with respect to acetate.

Biochemical Characteristics of the Isolates

S.No	Isolates	Oxidase	Catalase	Indole	MR	VP	Citrate	Urease	Gelatinase	H_2S
1	MTCC 659	-	+	-	-	$+^4$	+	-	$+^6$	-
2	IFM1	-	+	-	-	$+^4$	+	-	-	-
3	IFM2	-	+	-	-	$+^4$	+	-	$+^6$	-
4	IFM3	-	+	-	-	$+^4$	+	-	$+^6$	-
5	IFM4	-	+	-	-	$+^4$	+	-	$+^6$	-
6	IFM5	-	+	-	-	$+^4$	+	-	$+^6$	-
7	RW1	-	+	-	+	+	+	-	-	-
8	RW2	-	+	-	+	-	+	-	-	-
9	RW3	-	+	-	-	+	+	-	$+^2$	-
10	RW4	-	+	-	-	+	+	-	$+^2$	-
11	RW5	-	+	-	+	-	+	-	-	-
12	RW6	-	+	-	+	+	+	-	-	-
13	RW7	-	+	-	+	+	+	-	-	-
14	RW8	-	+	-	-	$+^4$	+	-	$+^6$	-
15	T1	-	+	-	-	+	+	-	$+^2$	-
16	T2	-	+	-	+	-	+	-	-	-
17	CSF1	-	+	-	+	+	+	-	-	-
18	S1	-	+	-	-	+	+	-	$+^2$	-
19	S2	-	+	-	-	$+^4$	+	-	$+^6$	-
20	S3	-	+	-	+	+	+	-	-	-
21	RM1	-	+	-	+	+	+	-	-	-
22	RM2	-	+	-	-	$+^4$	+	-	$+^6$	-
23	RM3	-	+	-	-	$+^4$	+	-	$+^6$	-
24	GF1	-	+	-	-	$+^4$	+	-	$+^6$	-
25	GF2	-	+	-	-	$+^4$	+	-	$+^6$	-

Contd……..

S.No	Isolates	H_2S	Esculin	NO_3	Lysine	Ornithine	Arginine	Phenyl Alanine	Malonate	Hippurate	ONPG
1	MTCC 659	-	+	+	+	+	+	-	+	-	+
2	IFM1	-	+	+	+	+	+	-	+	-	+
3	IFM2	-	+	+	+	+	+	-	+	-	+
4	IFM3	-	+	+	+	+	+	-	+	-	+
5	IFM4	-	+	+	+	-	-	-	+	-	+
6	IFM5	-	+	+	+	-	-	-	+	-	+
7	RW1	-	+	-	-	-	-	-	-	+	+
8	RW2	-	-	+	-	-	-	-	-	-	+
9	RW3	-	+	+	+	+	-	+[3]	+	-	+
10	RW4	-	+	+	+	+	-	+[3]	+	-	+
11	RW5	-	-	+	-	-	-	-	-	-	+
12	RW6	-	+	-	-	-	-	-	-	+	+
13	RW7	-	+	-	-	-	-	-	-	+	+
14	RW8	-	+	+	+	+	+	+	+	-	+
15	T1	-	+	+			-	+[3]	+	-	+
16	T2	-	-	+	-	-	-	-	-	-	+
17	CSF1	-	+	-	-	-	-	-	-	+	+
18	S1	-	+	+	+	+	-	+[3]	+	-	+
19	S2	-	+	+	+	+	+	+	+	-	+
20	S3	-	+	-	-	-	-	-	-	+	+
21	RM1	-	+	-	-	-	-	-	-	+	+
22	RM2	-	+	+	+	+	+	-	+	-	+
23	RM3	-	+	+	+	+	+	+	+	-	+
24	GF1	-	+	+	+	+	+	+	+	-	+
25	GF2	-	+	+	+	+	+	+	+	-	+

Contd...

Sugar Fermentation Tests

S.No	Isolates	Mb	Sa	Ar	Ga	Mo	In	Fc	Sb	Mn	Su	Du	Rh	De	Ad	Ce	Th	La	Is	Xy	Ma	Rf	Gy
1	MTCC 659	+	+	-	+	+	-	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	-
2	IFM1	+	+	-	+	+	-	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	-
3	IFM2	+	+	-	+	+	-	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	-
4	IFM3	+	+	-	+	+	-	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	-
5	IFM4	-	+	-	+	+	-	+	-	+	+	-	+	+	-	+	+	-	-	+	+	+	-
6	IFM5	-	+	-	+	+	-	+	-	+	+	-	+	+	-	-	+	-	-	+	+	+	-
7	RW1	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
8	RW2	-	+	-	+	+	-	+	-	+	-	-	+	+/-	-	+	+	+	-	+	+	-	-
9	RW3	-	+	+	+	+	-	+	-	+	+	-	+	+	-	+[3]	+	+	-	+	+	-	-
10	RW4	-	+	+	+	+	-	+	-	+	+	-	+	+	-	+[3]	+	+	-	+	+	-	-
11	RW5	-	+	-	+	+	-	+	-	+	-	-	+	+/-	-	+	+	+	-	+	+	-	-
12	RW6	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
13	RW7	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
14	RW8	+	+	-	+	+	-	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	-
15	T1	-	+	+	+	+	-	+	-	+	+	-	+	+	-	+[3]	+	+	-	+	+	-	-
16	T2	-	+	-	+	+	-	+	-	+	-	-	+	+/-	-	+	+	+	-	+	+	-	-
17	CSF1	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
18	S1	-	+	+	+	+	-	+	-	+	+	-	+	+	-	+[3]	+	+	-	+	+	-	-
19	S2	+	+	-	+	+	-	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	-
20	S3	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
21	RM1	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
22	RM2	+	+	-	+	+	-	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	-
23	RM3	+	+	-	+	+	-	+	+/-	+	+	-	-	+	-	+	+	+	+	+	+	+	+/-
24	GF1	+	+	-	+	+	-	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	-
25	GF2	+	+	-	+	+	-	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	-

ES1, Standard Culture; IFM1-IFM5, Isolates from Infant Formula Milk; RW1-RW8, isolates from river water; T1-T2, isolates from throat swabs; CSF1, isolate from CSF; S1-S3, isolates from diarrhoeic stools; RM1-RM3, isolates from raw milk; GF1-GF2, isolates from goat faeces; superscript, no. of days

+, positive ; -, negative; +/-, varied response

Alighnment of 16srRNA Sequence of *E. sakazakii* IFM5 With the Already Available Sequences

Partial sequence of *E. sakazakii* IFM5 16s rRNA gene PCR product

TGTCAAGACCAGGTAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCAC
CGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTC
CCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCAC
AACCTCCAAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGT
TTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTCGTCCAGGGGGCCGCCT
TCGCCACCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATT
CTACCCCCCTCTACGAGACTCAGGCCTGCCAGTTTCAAATGCAGTTCCCAGGTT
GAGCCCGGGAATTTCACATCTGACTTAACAGACCGCCTGCGTGCGCTTTACGC
CCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCAC
GGAGTTAGCAGGTGCTTCTTCTGCGGGTAACGTGAATTGCTGCGGTTAAAACCA
CAGCTCCTTCCTCCCCGAGGAGAGTACTTAACCCCGTGAAGCGTTCTATGACAA
GAGACATGGATGATCCCGGTGGCGACGGTGCGCCCTATCCACAATGAGGCTCC
CGGAGAAACTGGGGCGTGAAACAGAAT

Alignment of partilal sequence of *E. sakazakii* IFM5 16s rRNA with available sequences of other *E.sakazakii* strains

```
>  gi|55416231|gb|AY803187.1|  Enterobacter sakazakii isolate ES 11 16S
ribosomal RNA gene,
partial sequence
Length=1539

 Score = 1007 bits (508),  Expect = 0.0
 Identities = 544/554 (98%), Gaps = 2/554 (0%)
 Strand=Plus/Minus

Query  1   TGTCAAGACCAGGTA-GGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTT 59
           ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct 990 TGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTT 931

Query 60  GTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC 119
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 930 GTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC 871

Query 120 GACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCG 179
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 870 GACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCG 811

Query 180 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA 239
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 810 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA 751

Query 240 GCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA 299
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 750 GCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA 691
```

```
Query 300 TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAGGCCTGCCAGTTTCAA 359
          |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct 690 TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAAGCCTGCCAGTTTCAA 631

Query 360 ATGCAGTTCCCAGGTTGAGCCCGGGAATTTCACATCTGACTTAACAGACCGCCTGCGTGC 419
          |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct 630 ATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAACAGACCGCCTGCGTGC 571

Query 420 GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC 479
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 570 GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC 511

Query 480 ACGGAGTTAGCAGGTGCTTCTTCTGCGGGTAACGTGAATTGCTGCGGTTA-AAACCACAG 538
          |||||||||||| |||||||||||||||||||||| |||||||| |||||  |||||||
Sbjct 510 ACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATTGCTGTGGTTATTAACCACAR 451

Query 539 CTCCTTCCTCCCCG 552
          | ||||||||||||
Sbjct 450 CRCCTTCCTCCCCG 437
```

> gi|55416230|gb|AY803186.1| *Enterobacter sakazakii* isolate ES 4 16S ribosomal RNA gene, partial sequence
Length=1540

Score = 1007 bits (508), Expect = 0.0
Identities = 544/554 (98%), Gaps = 2/554 (0%)
Strand=Plus/Minus

```
Query  1   TGTCAAGACCAGGTA-GGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTT 59
           |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct 990 TGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTT 931

Query 60  GTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC 119
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 930 GTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC 871

Query 120 GACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCG 179
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 870 GACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCG 811

Query 180 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA 239
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 810 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA 751

Query 240 GCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA 299
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 750 GCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA 691

Query 300 TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAGGCCTGCCAGTTTCAA 359
          |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct 690 TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAAGCCTGCCAGTTTCAA 631

Query 360 ATGCAGTTCCCAGGTTGAGCCCGGGAATTTCACATCTGACTTAACAGACCGCCTGCGTGC 419
          |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct 630 ATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAACAGACCGCCTGCGTGC 571
```

```
Query 420 GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC 479
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 570 GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC 511

Query 480 ACGGAGTTAGCAGGTGCTTCTTCTGCGGGTAACGTGAATTGCTGCGGTTA-AAACCACAG 538
          |||||||||||| |||||||||||||||||||||| |||||||| |||||  |||||||
Sbjct 510 ACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATTGCTGTGGTTATTAACCACAR  451

Query 539 CTCCTTCCTCCCCG 552
          | ||||||||||||
Sbjct 450 CACCTTCCTCCCCG 437
```

> gi|55416234|gb|AY803190.1| *Enterobacter sakazakii* isolate FSM 266 16S ribosomal RNA gene, partial sequence
Length=1540

Score = 1003 bits (506), Expect = 0.0
Identities = 544/554 (98%), Gaps = 2/554 (0%)
Strand=Plus/Minus

```
Query 1   TGTCAAGACCAGGTA-GGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTT 59
          |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct 990 TGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTT 931

Query 60  GTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC 119
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 930 GTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC 871

Query 120 GACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCG 179
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 870 GACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCG 811

Query 180 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA 239
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 810 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA 751

Query 240 GCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA 299
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 750 GCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA 691

Query 300 TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAGGCCTGCCAGTTTCAA 359
          |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct 690 TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAAGCCTGCCAGTTTCAA 631

Query 360 ATGCAGTTCCCAGGTTGAGCCCGGGAATTTCACATCTGACTTAACAGACCGCCTGCGTGC 419
          |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct 630 ATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAACAGACCGCCTGCGTGC 571

Query 420 GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC 479
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 570 GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC 511

Query 480 ACGGAGTTAGCAGGTGCTTCTTCTGCGGGTAACGTGAATTGCTGCGGTTA-AAACCACAG 538
          |||||||||||| |||||||||||||||||||||| ||| |||||||||  |||||||
Sbjct 510 ACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCGCTGCGGTTATTAACCACAA 451
```

```
Query 539 CTCCTTCCTCCCCG 552
          | ||||||||||||
Sbjct 450 CGCCTTCCTCCCCG 437
```

> gi|541247141|gb|AY752940.1| *Enterobacter sakazakii* isolate 33 16S ribosomal RNA gene, partial sequence
Length=1408

Score = 1003 bits (506), Expect = 0.0
Identities = 544/554 (98%), Gaps = 2/554 (0%)
Strand=Plus/Plus

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Query 1   TGTCAAGACCAGGTA-GGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTT 59
          |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct 494 TGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTT 553

Query 60  GTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC 119
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 554 GTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC 613

Query 120 GACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCG 179
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 614 GACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCG 673

Query 180 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA 239
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 674 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA 733

Query 240 GCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA 299
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 734 GCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA 793

Query 300 TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAGGCCTGCCAGTTTCAA 359
          |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct 794 TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAAGCCTGCCAGTTTCAA 853

Query 360 ATGCAGTTCCCAGGTTGAGCCCGGGAATTTCACATCTGACTTAACAGACCGCCTGCGTGC 419
          |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct 854 ATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAACAGACCGCCTGCGTGC 913

Query 420 GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC 479
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 914 GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC 973

Query 480 ACGGAGTTAGCAGGTGCTTCTTCTGCGGGTAACGTGAATTGCTGCGGTTA-AAACCACAG 538
          |||||||||||| |||||||||||||||||||||| |||||||| |||||  |||||||
Sbjct 974 ACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATTGCTGTGGTTATTAACCACAA 1033

Query 539  CTCCTTCCTCCCCG 552
           | ||||||||||||
Sbjct 1034 CACCTTCCTCCCCG 1047
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>gi|54124712|gb|AY752938.1| *Enterobacter sakazakii* isolate 954 16S ribosomal RNA gene, partial sequence
Length=1451

Score = 999 bits (504), Expect = 0.0
Identities = 542/554 (97%), Gaps = 2/554 (0%)
Strand=Plus/Minus

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Query 1   TGTCAAGACCAGGTA-GGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTT 59
          ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct 965 TGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTT 906

Query 60  GTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC 119
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 905 GTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC 846

Query 120 GACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCG 179
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 845 GACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCG 786

Query 180 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA 239
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 785 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA 726

Query 240 GCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA 299
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 725 GCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA 666

Query 300 TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAGGCCTGCCAGTTTCAA 359
          |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct 665 TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAAGCCTGCCAGTTTCAA 606

Query 360 ATGCAGTTCCCAGGTTGAGCCCGGGAATTTCACATCTGACTTAACAGACCGCCTGCGTGC 419
          |||||||||||||||||||||||| |||||||||||||||||| ||||||||||||||||
Sbjct 605 ATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAACARACCGCCTGCGTGC 546

Query 420 GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC 479
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 545 GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC 486

Query 480 ACGGAGTTAGCAGGTGCTTCTTCTGCGGGTAACGTGAATTGCTGCGGTTA-AAACCACAG 538
          |||||||||||| |||||||||||||||||||||||| ||| |||| ||||| |||||||
Sbjct 485 ACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATYGCTGTGGTTATTAACCACAR 426

Query 539 CTCCTTCCTCCCCG 552
          | ||||||||||||
Sbjct 425 CRCCTTCCTCCCCG 412
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>gi|48094262|gb|AY624073.1| *Enterobacter sakazakii* strain 39d 16S ribosomal RNA gene, partial sequence
Length=944

Score = 997 bits (503), Expect = 0.0
Identities = 531/539 (98%), Gaps = 1/539 (0%)
Strand=Plus/Minus

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Query 15  AGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTC 74
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 925 AGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTC 866

Query 75  AATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTAACGCGTTAG 134
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 865 AATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTAACGCGTTAG 806

Query 135 CTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCGTTTACGGCGTGGACT 194
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 805 CTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCGTTTACGGCGTGGACT 746

Query 195 ACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTCGTC 254
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 745 ACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTCGTC 686

Query 255 CAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACC 314
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 685 CAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACC 626

Query 315 TGGAATTCTACCCCCCTCTACGAGACTCAGGCCTGCCAGTTTCAAATGCAGTTCCCAGGT 374
          |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct 625 TGGAATTCTACCCCCCTCTACGAGACTCAAGCCTGCCAGTTTCAAATGCAGTTCCCAGGT 566

Query 375 TGAGCCCGGGAATTTCACATCTGACTTAACAGACCGCCTGCGTGCGCTTTACGCCCAGTA 434
          |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 565 TGAGCCCGGGGATTTCACATCTGACTTAACAGACCGCCTGCGTGCGCTTTACGCCCAGTA 506

Query 435 ATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCAGGT 494
          |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct 505 ATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGT 446

Query 495 GCTTCTTCTGCGGGTAACGTGAATTGCTGCGGTTA-AAACCACAGCTCCTTCCTCCCCG 552
          |||||||||||||||||| |||||||||||||  ||||||| | ||||||||||||
Sbjct 445 GCTTCTTCTGCGGGTAACGTCAATTGCTGCGGTTATTAACCACAACACCTTCCTCCCCG 387
```

Alignment of partial sequence of *E. sakazakii* IFM5 16SrRNA with available sequences of other genus

```
>  gi|2209047|dbj|AB004755.1|  Klebsiella planticola gene for 16S
ribosomal RNA, partial sequence
Length=1451

 Score =  989 bits (499),  Expect = 0.0
 Identities = 542/554 (97%), Gaps = 2/554 (0%)
 Strand=Plus/Minus

Query 1   TGTCAAGACCAGGTA-GGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTT 59
          ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct 964 TGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTT 905

Query 60  GTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC 119
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 904 GTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC 845

Query 120 GACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCG 179
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 844 GACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCG 785
```

```
Query 180 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA 239
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 784 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA 725

Query 240 GCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA 299
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 724 GCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA 665

Query 300 TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAGGCCTGCCAGTTTCAA 359
          |||||||||||||||||||||||||||||||||||||||||| |||||||||| |||
Sbjct 664 TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAAGCCTGCCAGTATCAG 605

Query 360 ATGCAGTTCCCAGGTTGAGCCCGGGAATTTCACATCTGACTTAACAGACCGCCTGCGTGC 419
          |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct 604 ATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAACAGACCGCCTGCGTGC 545

Query 420 GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC 479
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 544 GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC 485

Query 480 ACGGAGTTAGCAGGTGCTTCTTCTGCGGGTAACGTGAATTGCTGCGGTTA-AAACCACAG 538
          |||||||||||| |||||||||||||||||||||| |||||||| |||||  |||||||
Sbjct 484 ACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATTGCTGTGGTTATTAACCACAN 425

Query 539 CTCCTTCCTCCCCG 552
          | ||||||||||||
Sbjct 424 CACCTTCCTCCCCG 411
```

>gi|3169782|gb|AF025372.1|AF025372 *Citrobacter diversus* strain CDC 3613-63 16S ribosomal RNA gene, partial sequence
Length=1512

Score = 987 bits (498), Expect = 0.0
Identities = 542/554 (97%), Gaps = 2/554 (0%)
Strand=Plus/Minus

```
Query 1   TGTCAAGACCAGGTA-GGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTT 59
          ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct 990 TGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTT 931

Query 60  GTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC 119
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 930 GTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC 871

Query 120 GACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCG 179
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 870 GACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCG 811

Query 180 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA 239
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 810 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA 751

Query 240 GCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA 299
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 750 GCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA 691
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Query 300 TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAGGCCTGCCAGTTTCAA 359
          ||||||||||||||||||||||||||||||||||||||||||| |||||||||| |||
Sbjct 690 TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAAGCCTGCCAGTATCAG 631

Query 360 ATGCAGTTCCCAGGTTGAGCCCGGGAATTTCACATCTGACTTAACAGACCGCCTGCGTGC 419
          |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct 630 ATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAACAGACCGCCTGCGTGC 571

Query 420 GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC 479
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 570 GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC 511

Query 480 ACGGAGTTAGCAGGTGCTTCTTCTGCGGGTAACGTGAATTGCTGCGGTTA-AAACCACAG 538
          |||||||||| |||||||||||||||||||| |||||||| |||||  |||||||
Sbjct 510 ACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATTGCTGTGGTTATTAACCACAA 451

Query 539 CTCCTTCCTCCCCG 552
          | ||||||||||||
Sbjct 450 CACCTTCCTCCCCG 437
```

> qi|4754817|gb|AF130885.1| *Erwinia toletana* strain A4 16S ribosomal RNA gene, partial sequence
Length=1493

Score = 965 bits (487), Expect = 0.0
Identities = 509/515 (98%), Gaps = 1/515 (0%)
Strand=Plus/Minus

```
Query 1   TGTCAAGACCAGGTA-GGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTT 59
          ||||||| ||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct 966 TGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTT 907

Query 60  GTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC 119
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 906 GTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC 847

Query 120 GACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCG 179
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 846 GACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCG 787

Query 180 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA 239
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 786 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA 727

Query 240 GCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA 299
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 726 GCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA 667

Query 300 TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAGGCCTGCCAGTTTCAA 359
          |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct 666 TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCTAGCCTGCCAGTTTCAA 607

Query 360 ATGCAGTTCCCAGGTTGAGCCCGGGAATTTCACATCTGACTTAACAGACCGCCTGCGTGC 419
          |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct 606 ATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAACAGACCGCCTGCGTGC 547

Query 420 GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC 479
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 546 GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC 487
```

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Query 480 ACGGAGTTAGCAGGTGCTTCTTCTGCGGGTAACGT 514
          |||||||||||| ||||||||||||||||||||||
Sbjct 486 ACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGT 452
```

> gi|1857862|gb|U88546.1|SPU88546 *Salmonella paratyphi* A 16S ribosomal RNA gene, complete sequence
Length=1541

```
 Score =  963 bits (486),  Expect = 0.0
 Identities = 539/554 (97%), Gaps = 2/554 (0%)
 Strand=Plus/Minus

Query 1   TGTCAAGACCAGGTA-GGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTT 59
          ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct 995 TGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTT 936

Query 60  GTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC 119
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 935 GTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC 876

Query 120 GACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCG 179
           |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct 875 TACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTAGACATCG 816

Query 180 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA 239
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 815 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA 756

Query 240 GCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA 299
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 755 GCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA 696

Query 300 TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAGGCCTGCCAGTTTCAA 359
          |||||||||||||||||||||||||||||||||||||||| || ||||||| |||
Sbjct 695 TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAAGCTTGCCAGTATCAG 636

Query 360 ATGCAGTTCCCAGGTTGAGCCCGGGAATTTCACATCTGACTTAACAGACCGCCTGCGTGC 419
          |||||||||||||||||||||| |||||||||||||||||| ||||||||||||
Sbjct 635 ATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAACAAACCGCCTGCGTGC 576

Query 420 GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC 479
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 575 GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC 516

Query 480 ACGGAGTTAGCAGGTGCTTCTTCTGCGGGTAACGTGAATTGCTGCGGTTA-AAACCACAG 538
          |||||||||||| |||||||||||||||||||| |||||||||||||  |||||||
Sbjct 515 ACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATTGCTGCGGTTATTAACCACAA 456

Query 539 CTCCTTCCTCCCCG 552
          | ||||||||||||
Sbjct 455 CACCTTCCTCCCCG 442
```

>gi|4754894|gb|AF130962.1| *Pantoea agglomerans* strain A43 16S ribosomal RNA gene, partial sequence
Length=1218

Score = 961 bits (485), Expect = 0.0
Identities = 508/515 (98%), Gaps = 1/515 (0%)
Strand=Plus/Minus

```
Query 1   TGTCAAGACCAGGTA-GGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTT 59
          ||||||| ||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct 745 TGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTT 686

Query 60  GTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC 119
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 685 GTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTC 626

Query 120 GACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCG 179
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 625 GACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCG 566

Query 180 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA 239
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 565 TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA 506

Query 240 GCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA 299
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 505 GCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCA 446

Query 300 TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAGGCCTGCCAGTTTCAA 359
          |||||||||||||||||||||||||||||||||||||||  ||||||||||||||||||
Sbjct 445 TTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCTAGCCTGCCAGTTTCAA 386

Query 360 ATGCAGTTCCCAGGTTGAGCCCGGGAATTTCACATCTGACTTAACAGACCGCCTGCGTGC 419
          |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct 385 ATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAACAGACCGCCTGCGTGC 326

Query 420 GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC 479
          |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct 325 GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATWACCGCGGCTGCTGGC 266

Query 480 ACGGAGTTAGCAGGTGCTTCTTCTGCGGGTAACGT 514
          |||||||||| ||||||||||||||||||||||||
Sbjct 265 ACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGT 231
```