CHARACTERIZATION OF QUORUM SENSING MEDIATED BIOFILM FORMATION AND ITS INHIBITION IN *Cronobacter sakazakii* STRAINS

A Thesis submitted in fulfillment for the requirement of the Degree of

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In

Biotechnology

by

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Under the supervision of

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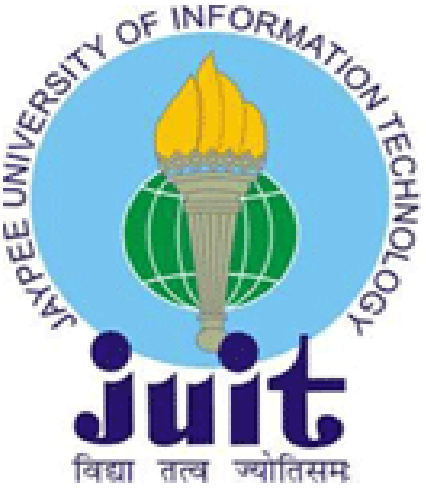

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CERTIFICATE

This is to certify that the thesis entitled, **"Characterization of Quorum Sensing mediated biofilm formation and its inhibition in *Cronobacter sakazakii* strains"** which is being submitted by **Niharika Singh (Enrollment No. 106563)** in fulfillment for the award of degree of **Doctor of Philosophy** in **Biotechnology** at **Jaypee University of Information Technology, Waknaghat, India** is the record of candidate's own work carried out by her under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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Niharika Singh

DECLARATION

I certify that:

a. The work contained in this thesis is original and has been done by me under the guidance of my supervisor.

b. The work has not been submitted to any other organization for any degree or diploma.

c. Wherever, I have used materials (data, analysis, figures or text), I have given due credit by citing them in the text of the thesis.

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LIST OF ABBREVIATIONS

µg	microgram
µl	micriliter
µM	micromolar
mg	miligram
M	molar
l	litre
w/v	weight/volume
v/v	volume/volume
g/L	gram per litre
α	alpha
β	beta
AI	Autoinducers
ANOVA	Analysis of Variance
BPW	Buffered peptone water
CDC	Centre of Disease Control and Prevention
CFU	Colony Forming Units
CSF	Cerebrospinal fluid
CV	Crystal Violet
EPS	Extra polymeric substances
ESA	*Enterobacter Sakazakii Agar*
FDA	Food and Drug Administration
FTIR	Fourier transform infra-red
h	hour
HPLC	High Performance Liquid Chromatography
ISO	International Standards Organization
ITS	Internal Transcribed Spacer
LB	Luria Broth
LC-HRMS	Liquid Chromatography-High Resolution Mass Spectrometry

Min	minutes
MLST	Multi Locus Sequence Typing
MTP	Microtiter plate
NB	Nutrient Broth
NCL	National Chemical Laboratory
NEC	Necrotizing entercolitis
O.D.	Optical Density
OMP	Outer membrane proteins
PCR	Polymerase Chain Reaction
PIF	Powdered Infant Formula
QQ	Quorum Quenching
QS	Quorum sensing
QSIs	Quorum Sensing Inhibitors
SBF	Specific Biofilm Formation index
sec	second
SEM	Scanning Electron Microscopy
TC	Trans-cinnamldehyde
TLC	Thin Layer Chromatography
TSB	Tryptone SoyBroth

CHAPTER 1

1.1 Introduction

The genus *Cronobacter* (previously known as *Enterobacter sakazakii*) is recognized as an emerging opportunistic pathogen and the etiological representative life-threatening infections in neonates and immune-compromised infants [ISO⁄TS 22964 ISO⁄TS 22964, 2006(E); Bar-Oz et al. 2001; CDC 2002; Block et al. 2002; Mullane et al. 2007]. Urmenyi and Franklin [1961] firstly, reported the case of severe *C. sakazakii* induced systemic infection in neonates in England. Since then, there have been around 150 reported cases of this contagion with 26 deaths worldwide [Gurtler et al. 2005; Friedemann 2009; Yan et al. 2013]. This pathogen received world-wide attention after an outbreak of meningitis in Tennessee in 2001 [Iversen & Forsythe 2003]. It was Farmer et al. [1980] who foremost established the taxonomic position of novel species named *C. sakazakii* which was initially referred to as Gram negative, facultative anaerobe yellow-pigmented *Enterobacter cloacae*. Till the date, the *Cronobacter* consists of seven species that include *Cronobacter dublinensis*, *Cronobacter malonaticus*, *Cronobacter muytjensii*, *Cronobacter sakazakii*, *Cronobacter turicensis*, *Cronobacter universalis* and *Cronobacter condimenti* [Joseph et al. 2012a; Brady et al. 2013; Oren & Garrity 2013; Masood et al. 2014]. Only three species, C. *sakazakii*, C. *turicensis* and C. *malonaticus* have been reported to cause neonatal infections [Joseph et al. 2012a]. Although the incidence of disease is very low, the fatality rates range between 40 to 80% and survivors often had severe neurological and developmental disorders [Friedemann 2009; Van Acker et al. 2001; Bowen & Braden 2006; Forsythe 2005]. In addition to the high fatality rate of *C. sakazakii* infections, it may consequence in severe neurological sequelae such as hydrocephalus, quadriplegia and retarded neural development in survivors (Forsythe, 2005). It is worth mentioning that premature birth and/or low birth weight are often cited as highest risk individuals for *Cronobacter* infection because they lack normal gut microflora and established gut epithelial lining which makes them more susceptible to increased mucosal permeability [Kandhai et al. 2004]. Reconstituted powdered infant formula (PIF) is reported to be the most associated vehicle for transmission of pathogen (Fig. 1.1). Voluntary recalls of PIF contaminated with *Cronobacter* in the United States, Europe and Asia-Pacific region suggested the need of a collective effort among PIF manufacturers, health-care facilities and governing bodies to develop hygienic practices and maintain higher microbiological standards [FDA. 2002].

The application of genome sequencing data and multilocus sequence typing (MLST) validated the revision of the taxonomic position of the seven identified *Cronobacter* species [Iversen et al. 2008; Yan et al. 2012; Kucerova et al. 2010; Chen et al. 2011; Baldwin et al. 2009; Shin et al. 2012; Yan et al. 2013; Grim et al. 2013; Masood et al. 2013; Zhao et al. 2014; Pightling & Pagotto 2014]. Kucervo et al. (2010) reported the sequence of 4.4 Mb (57% GC) genome of a strain of C. *sakazakii* ATCC BAA-894 coding for 4392 genes of which 223 genes were annotated as relevant to virulence and disease. The strain also harbours two plasmids, denoted as pESA2 and pESA3 (31-kb, 51% GC and 131-kb, 56% GC, respectively). Of the 21genes unique to C. *sakazakii*, these genes are reported to play a significant role in phosphotransferase systems (PTS), pilus assembly, acid transport, N-acetylneuraminate lyase and toxin/antitoxin transport systems (Yan et al. 2012). The MLST utilizing seven (*atpD, fusA, glnS, gltB, gyrB, infB* and *ppsA*) is a more successful typing method for the *Cronobacter* genus and has exhibited a high caliber of discrimination between the isolates [Joseph & Forsythe 2012; Joseph et al. 2012b; Joseph et al. 2012c; Joseph et al. 2013a; Sonbol et al. 2013]. The *Cronobacter* PubMLST database curated by Stephen Forsythe comprised the enteries for 1671 *Cronobacter* isolates reported worldwide. The MLST database of C. *sakazakii* isolates indicates clonal complex 4 (CC4) as stable and predominantly coupled with neonatal meningitis. The ribosomal-MLST (53-loci) and Clusters of Orthologous Groups–core genome (COG-cg) MLST (1865 loci) has also confirmed CC4 as dominant lineage [Forsythe et al. 2014]. However, due to limited information on the virulence characteristics of CC4, its association with neonatal meningitis is unclear [Joseph et al. 2012c]. Therefore, unveiling the virulence characteristics of this pathogen would contribute towards underpinning the association of the pathogen to infant foods and to develop mitigation strategies.

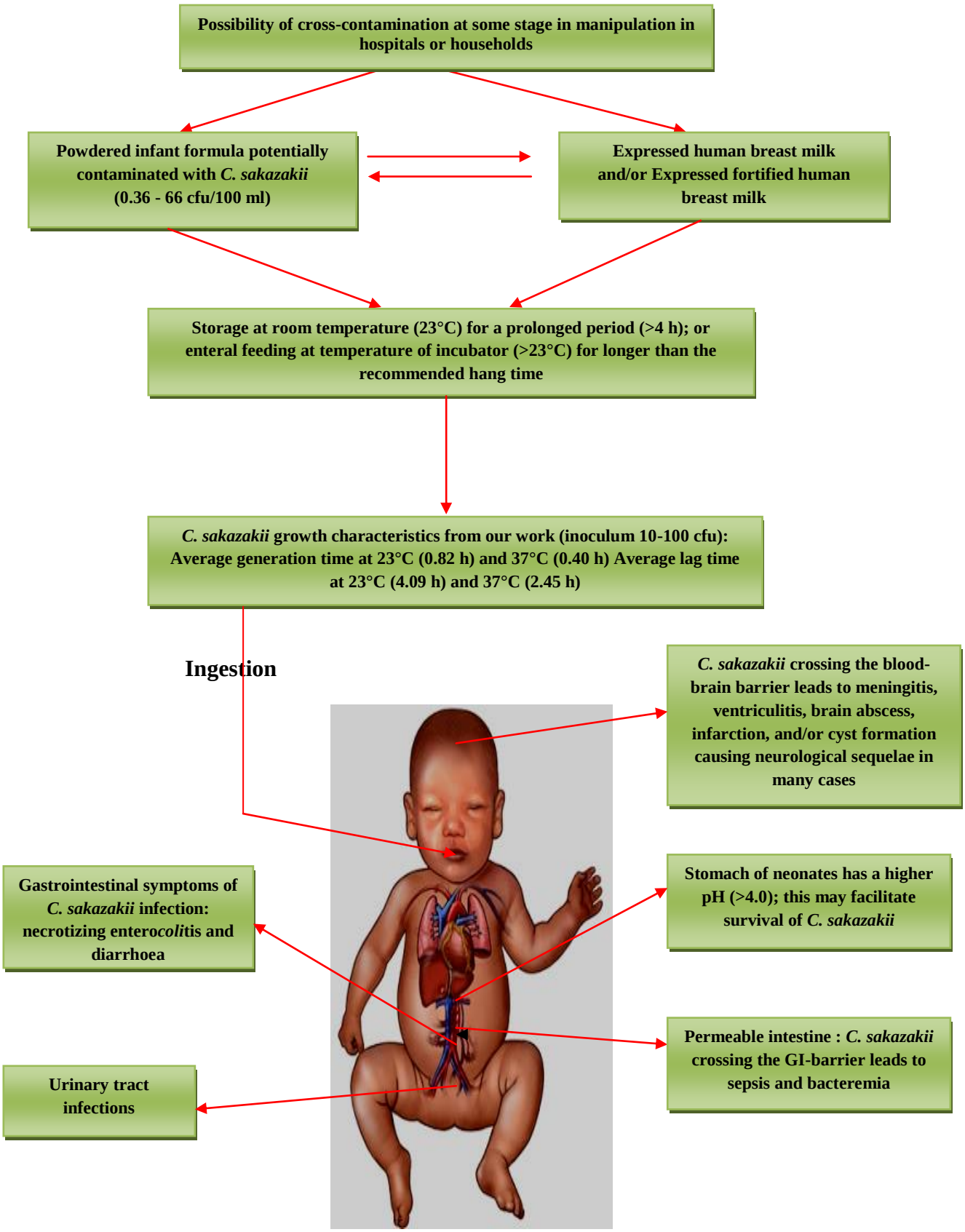

Figure 1.1: Mode of Transmission of *Cronobacter sakazakii* at risk population: neonates pre-term infants and very low-birth-weight infants

The little information on the ecology, pathogenesis and virulence of C. *sakazakii* warrants an update on this enteric pathogen with special emphasis on virulence factors associated with the pathogenesis of C. *sakazakii*.

1.2 Reservoir and mode of transmission

The bacterium is ubiquitous and has been isolated from a wide variety of foods, including cheese products, infant cereal, dried foods, fruits, vegetables, meats, water, medicinal plants, herbs and spices, bread, rice and PIF [Biering et al. 1989; Kim et al. 2005; Estuningsih et al. 2006; Friedemann 2007; Lin & Beuchat 2007; Baumgartner et al. 2009; Jaradat et al. 2009; Belal et al. 2013; Dong et al. 2013; Mozrová et al. 2013; Muller et al. 2013; Singh et al. 2013; Siqueira et al. 2013]. Moreover, the *Cronobacter* spp. has also been reported from clinical sources, including cerebrospinal fluid, blood, intestinal and respiratory tracts, bone marrow and skin wounds [Gallaher & Ball 1991]. The pathogen has also been detected from domestic vacuum cleaner bags, river water, the gut of a Mexican fruit fly and stable fly and faecal sample of animals [Kandhai et al. 2004; Hamilton et al. 2003; Raghav & Aggarwal 2007a; Goel et al. 2011]. Reconstituted PIF is the most associated vehicle for transmission of the pathogen, being an intrinsic or extrinsic contaminant during manufacturing under poor good manufacturing practices (GMP) or reconstitution of PIF (Table 1.1) [Bar-Oz et al. 2001; Van Acker et al. 2001; Biering et al. 1989; CDC 2009; http://www.fsai.ie/alerts/archive/fa 20050120.asp 2006; FAO/WHO 2008; Jarvis 2005; Himelright et al. 2002; Ray et al. 2007; Noriega et al. 1990; Simmons et al. 1989; Nazarowec-White & Farber 1997; Muytjens et al. 1983]. The presence of the pathogen as vaginal microflora has been neglected by several studies however, babies delivered through birth canal or Caesarean section (C-section) have been contracted with the pathogen few days after birth [Block et al. 2002; Yan et al. 2012; Hunter & Bean 2013].

1.3 Epidemiology

The epidemiology of *Cronobacter* species is incomplete and poorly described because of its rare infections and often underreported cases due to missing or different reporting criteria in developed and some developing countries CDC 2011]. Feeding with reconstituted PIF has been epidemiologically implicated in numerous clinical cases (Table 1.1). Cases are somewhat sporadic, but epidemics are not unusual; the utmost-risk group is neonates (<28

days old) that have low birth weights (<2,000 to 2,500 g) or the premature (<37 weeks of gestation stage). Friedemann [2009] reported the lethality of *Cronobacter* meningitis, bacteraemia and NEC to be 41.9% ($P<0.0001$), <10% and 19.0% ($P<0.05$), respectively, for 120-150 microbiologically *Cronobacter* confirmed neonatal infections occurred between 2000 and 2008. The annual occurrence rate among the premature and underweight infant is reported to a figure of 8.7 per 100,000 low-birth weight neonates in the USA, and one *Cronobacter* infection per 10,660 every low-birth neonates [Himelright et al. 2002; Stoll et al. 2004]. Hunter & Bean [2013] demonstrated the worldwide distribution of reported cases; the majority of them are within the developed countries (approx. 45%). This distribution may be underestimated since not all clinical analysis laboratories carry out research on the pathogen and not all countries have a system for reporting diseases. The Food and Drug Administration (FDA) has accounted a series of neonatal disease outbreaks in Florida, Missouri Illinois and Oklahoma in December 2011 [FDA 2012]. The limited information on its epidemiology necessitates that the researchers should record consistent and sufficiently informative data of invasive neonatal *Cronobacter* infections as developed under PubMLST database.

Table 1.1: Powdered Infant Formula (PIF) implicated in worldwide outbreaks of *Cronobacter* infection

Country	Year	No. of cases / No. of deaths	Reference(s)
New Mexico	2008	2/2	U.S. Center for Disease Control and Prevention [CDC 2008]
France	2004	4/2	RASFF (2004); Coignard et al. [2006]; Food Safety Authority of Ireland [2006]
USA	2004	1/0	FAO/WHO [2008]
New Zealand	2004	1/1	Jarvis [2005]
Tennessee	2001	10/1	CDC [2008]
Israel	1999-2000	2/0	Bar-Oz et al. [2001]
Belgium	1998	12/2	Van Acker et al. [2001]
India	1992	1/1	Ray et al. [2007]
Maryland	1990	1/0	Noriega et al. [1997]
Tennessee	1988	4/0	Himelright [2002]; Nazarowec-White et al. [1997]
Iceland	1986-1987	3/1	Biering et al. [1989]
Denmark	1983	1/1	Muytjens et al. [1983]

1.4 Pathogenicity and Virulence factors

The high mortality and fatality rate caused by C. *sakazakii* is still poorly understood, and the list of virulence factors (Table 1.2) is probably far from complete. The use of molecular approaches to identifying and studying putative virulence factors in combination with the application of animal cell line models has allowed to define some of the mechanisms of the pathogenesis. The specific virulence factors associated with the pathogenesis are discussed in this section.

1.4.1 Outer membrane proteins (OMPs)

Outer membrane proteins (OMPs) are of peculiar interest, owing to their cell-surface exposure and contribution in export of extracellular virulence factors, and in anchoring the structures that mediate adhesion and motility.

The genus *Cronobacter* has a potential to actively invade various epithelial and endothelial cells of human and animal origin (Nair & Venkitanarayanan, 2007; Kim & Loessner, 2008; Singamsetty et al., 2008). It was originally speculated that the organism can attach to human intestinal cells and replicate internally in macrophages. Several studies ever since have been put forwarded that outer membrane protein A (ompA), a multifunctional protein contributes significantly to the virulence potential of *Cronobacter* spp. (Singamsetty et al., 2008; Mittal et al., 2009; Nair et al., 2009). The previous literatures comparatively clearly demonstrated that invasion of C.*sakazakii* to INT407 cells involves contribution of both microfilaments and microtubules from host and bacterial OmpA (Nair & Venkitanarayan, 2007; Townsend et al., 2007; Singamsetty et al., 2008; Nair et al., 2009). Mittal et al. (2009) has validated that the OmpA-positive isolates have a capacity to survive in blood and the subsequent invasion of central nervous system by breaching the blood brain barrier, whereas OmpA-negative isolates could not attach to intestinal epithelial cells. In addition to Omp A, Omp X also plays vital roles in invasion not only the apical side, but also the basolateral side of the host cells and can translocate into the deeper organs (spleen and liver) (Kim et al., 2010a) (Fig. 1.2).The Omp A encoding gene is considered to be present in all *Cronobacter* strains tested, and that the same marker has also been associated with invasive *Escherichia coli*, which causes neonatal meningitis (Kim, 2000; Kim et al. 2010a,b).

The attachment and invasion properties of *Cronobacter* strains have been investigated by in vitro studies using epithelial and mammalian cell lines. Studies reported that the invasion

efficiency of *C. sakazakii* was extensively enhanced in the absence of cellular tight junctions and confirmed that there is no relationship between adhesive capacities of C.*sakazakii* and production of specific fimbriae (Mange et al., 2006; Kim & Loessner, 2008).

1.4.2 Enterotoxin

Pagotto et al. [2003] were the first to study putative virulence factors and the dose-response of *C. sakazakii* in suckling mouse. The authors successfully determined a minimum lethal dose of 10^8 colony-forming units (cfu) in neonatal mouse suggesting the possibility of enterotoxin analogue in infections. The function of this toxin may act in a parallel fashion to lipopolysaccaride (LPS), mediating toll-like receptor (TLRs) 4 activation and stimulating a host inflammatory response [Pagotto et al. 2003]. However, later it was Raghav & Aggarwal [2007b] who identified a thermostable putative toxin with molecular mass of 66 kDa. The potent activity of the toxin (LD_{50}=56 pg) emphasizes the emerging risk to neonates fed reconstituted PIF contaminated with C. *sakazakii*. The implication of the enterotoxin is still blurred as the genes encoding the putative toxin and the protein itself remain unidentified. Further studies using functional genomics and system biology might help in characterization of toxin-related genes.

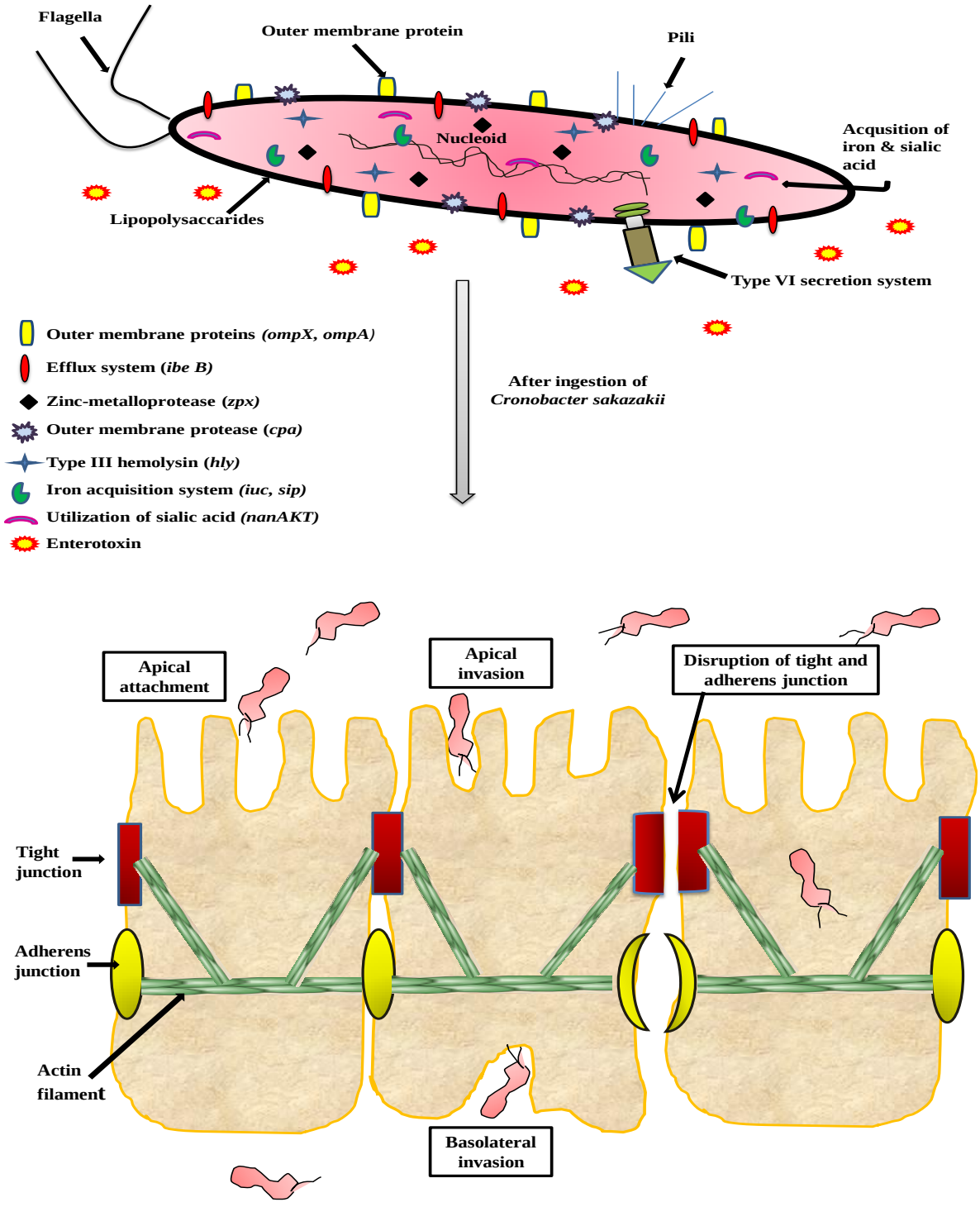

Figure 1.2: Proposed model for *Cronobacter sakazakii* infection and pathogenesis. The pathogen encodes several illustrated pathogenicity-associated factors engaged in imperative processes including adhere to host surfaces, transmigration across, invasion into and disrupt the intestinal barrier within intestinal epithelial cells [Singh et al. 2015a].

1.4.3 *Cronobacter* plasminogen activator (cpa)

Recently, the study on *C. sakazakii* BAA-894 reported presence of a plasmid (pESA3) which encodes outer membrane proteases (Cpa) that has significant identity to proteins that belong to Pla subfamily of omptins. This protease has an ability to render serum resistance to *C. sakazakii* BAA-894 by proteolytically cleaving complement components, activating plasminogen and inactivating the plasmin inhibitor α2-AP [Franco et al. 2011a]. Franco et al. [2011b] and Cruz et al. [2011] also portray the prevalence and distribution of plasmid-encoded virulence genes *cpa*, a type six secretion system (T6SS, also encoded on pESA3) and a filamentous haemaggltunin/adhesion (FHA) gene locus (located on pCTU1) among 231 *Cronobacter* strains.

1.4.4 Sialic acid utilization

Sialic acid is found in human milk in the form of sialyloligosaccharides which are highest in colostrums and as well as in infant formulas (Wang, 2009). These oligosaccharides remain undigested in neonates and infants, therefore the intestinal microvilli of neonates have increased sialic acid and N-acetylglucosamine residues leading to proliferation of gut microbiota [Lewis & Lewis 2012; Sprenger & Duncan 2012]. Recently, Joseph et al. [2013b] explained a plausible linkage between sialic acid metabolism and the pathogenicity of C. *sakazakii*. Based on the latest whole genome studies they have revealed that *C.sakazakii* is the only *Cronobacter* species that has the *nanAKT* gene cluster encoding for sialic acid utilization (Kucerova *et al.*, 2010; Joseph *et al.*, 2012). They further confirmed that only *C.sakazakii*, and not the other six *Cronobacter* species, is able to use sialic acid as a carbon source for growth. The unique utilization of sialic acid from breast milk, infant formula, milk (oligosaccarides), mucins lining the intestinal wall, and even gangliosides in the brain after passing through the blood–brain barrier could have a possible role in additional virulence factors contributing to acute life-threatening infections in neonates.

1.4.5 Iron acquisition gene system

Iron is an essential micro element for bacterial growth and metabolism and a vital factor for bacterial pathogenesis [Negre et al. 2004]. In *Cronobacter*, Franco et al. [2011b] reported that plasmid pESA3 contain two clusters of genes, a homologue of an ABC transport-mediated iron uptake siderophore system (*eitCBAD* operon) and a siderophore-mediated iron acquisition system (*iucABCD/iutA* operon). This characteristic may contribute to the systemic

survival of *C. sakazakii* and subsequent invasion of the CNS to cause diseases. It was later, Grim et al. [2012] who identified both the *feo* and *efe* systems for acquisition of ferrous iron. They confirmed that 98% of the plasmid-harboring *Cronobacter* strains have the aerobactin-like siderophore, cronobactin, for transport of ferric iron in *Cronobacter*. Cruz et al. [2011] have also revealed that *C. sakazakii* isolates harbour siderophore-interacting protein (*sip*) gene. The *sip* gene has a ferrodoxin-reductase domain with binding sites to FAD and NAD(P), capable of transfer an electron from reduced ferrodoxin to FAD and then convert NADP to NADPH [Miethke & Marahiel 2007].

1.4.6 Efflux system

Active efflux system is a recognized virulence mechanism contributing to survival of members of Enterobacteriaceae in the host's gastrointestinal tract [Touze et al. 2004]. Interestingly, *ibeB* (a gene synonymous with *cusC*) in *C. sakazakii* has been reported, belonging to constellate of genes encoding a copper and silver resistance cation efflux system, ultimately allowing the invasion to brain microvascular endothelial cells (BMEC) cells [Kucerova et al. 2010; Franke et al. 2003]. When assessed, it was discovered that the entire cation efflux operon (*cusA*, *cusB* and *cusC*) and its regulatory gene *cusR* were present in isolates colligated with neonatal infections (including C. *sakazakii* ATCC 29544^{T}, 696, 701, 767, *C. malonaticus* and *C. turicensis*) and absent in the other strains evaluated (C. *sakazakii* B894, ATCC 12868, 20, C. *dublinensis* and C. *muytjensii*) [Kucerova et al. 2010].

1.4.7 Biofilm formation

Biofilms are interface-associated consortia of microorganisms that are typically embedded in an endogenous slimy matrix referred to as extracellular polysaccharides (EPS). The attachment of bacteria to environmental exteriors and the formation of biofilms are well-known to contribute to survival and increased resistance to antimicrobial treatments [Donlan & Costerton 2002; Kim 2006; Beuchat et al. 2009]. Temperature and nutrient availability are the noteworthy factors affecting biofim formation by *Cronobacter* spp. (Kim et al. 2006; Beuchat et al. 2009]. Two hypothetical proteins have been newly described as possible adhesins engaged in biofilm formation in *Cronobacter* (ESA_00281 and ESA_00282) [Hartmann et al. 2010]. Iverson et al. [2004] reported that *Cronobacter* was able to adhere to silicon, stainless steel, polycarbonate and latex with apparently greater attachment occurring with EPS producing bacteria. Colanic acid (CA) was earlier identified as an EPS component

in *Cronobacter* spp. contributing to adherence to various surfaces and increased resistance to environmental stresses thermal, desiccation and pH [Scheepe-Leberkuhne 1986].

1.4.8 Other potential factors

Among the minor but important virulent factors, the proteolytic enzymes of strains have been found to cause deformation of the tissue cells in mice [Pagotto et al. 2003]. Kothary et al. [2007] isolated and characterized a cell-bound zinc-containing metalloprotease which is encoded by a nucleotide sequence (*zpx*), unique among all the 135 *Cronobacter* strains tested. The protease was active in against azocasein, caused rounding of Chinese's hamster ovarian cells. It is hypothesised that proteolytic enzymes may permit the organism to cross the blood–brain barrier or cause extensive cellular destruction in neonates with NEC.

Recently, Hamby et al. [2011] revealed a potential association between pathogenicity and inositol fermentation. They investigated the genomes of *C. sakazakii* and *C. turicensis* and reported that the gene for inositol monophosphatase is also associated with virulence of this pathogen.

Current studies revealed that the plasmid-encoded methyl-accepting chemotaxis protein (MCP) sequences present in C. *sakazakii* sequence type 8 (ST8) lineage are involved in virulence, invasion/adhesion, motility and biofilm formation [Choi et al. 2015]. It was also observed that this sequence was not found in any other lineages, implying that the MCP association with virulence is probably specific to the ST8 lineage.

LysR-type transcriptional regulator (LTTRs) are known to regulate a range of regulons involved in quorum sensing and virulence of bacteria [Maddocks et al. 2008; MacLean et al. 2008]. Recently, Choi et al. [2012] characterized LysR-type transcriptional regulator (LTTR) gene (ESA_01081 homolog) as a potential regulator for *C. sakazakii* ATCC 29544 pathogenesis. They reported that the putative LysR-type protein plays a role in regulating genes involved in a host cell invasion, but not in adhesion.

In another study, Cruz et al. [2011] in addition to sip and cpa identified putative virulence genes, including type III haemolysin (*hly*) in *Cronobacter* isolated from human and non-human sources. The type III hemolysin, a virulence factor in numerous pathogenesis, is an integral outer membrane protein with haemolytic activity [Baida & Kuzmin 1996; Chen et al. 2004].

Lipopolysaccarides **(**LPS**)** is an outer membrane virulence factor of *C. sakazakii*, which interacts with enterocytes through LPS mediated binding to TLR4 inducing NEC in animals [Hunter et al. 2008; Hotta et al. 1986; Feng et al. 2005; Feng et al. 2007]. In the NEC patients, the elevated level of LPS in serum and stools has been reported [Kruis et al. 1984; Caradonna et al. 2000; Noerr 2003; Sharma et al. 2007; Duffy et al. 1997; Leaphart et al. 2007]. Altogether, these findings raise the intriguing possibility that LPS may engage in the pathogenesis of NEC and the role of TLR4 within the intestinal epithelium seeks detailed consideration. It has been also reported that PIF is frequently contaminated with elevated levels of LPS, which disrupts tight junctions thereby increasing the permeability of the host cell membrane [Townsend et al. 2007a; Moriez et al. 2005].

Table 1. 2: Characteristics of major known virulence factors of *Cronobacter sakazakii*

Factors	Genes	Potential role	Reference(s)
Outer membrane proteins (OMPs)	*ompX* *ompA*	Involved in the basolateral invasion of enterocyte-like human epithelial cells	Singamsetty et al. 2008; Kim and Loessner 2008; Nair and Venkitanarayanan 2009; Kim et al. 2010
Enterotoxin	Not known yet	Heat stable toxin elaborated by the pathogen	Hunter et al. 2008; Pagotto at al. (2003); Raghav and Aggarwal 2007b
Outer membrane protease	*cpa*	Provides resistance against bactericidal activity of serum; activates plasminogen and inactivates α2-AP	Franco et al. 2011a; Cruz et al. 2011
Sialic acid utilization	*nanAKT*	Confers in pathogenesis	Joseph et al. 2013
Iron acquisition system	*iuc*	Encodes an iron-uptake system mediated by the active siderophore that plays a role in iron transport and regulation	Franco et al. 2011b; Grim et al. 2012
Efflux system	*ibeB*	Encodes copper and silver resistance cation efflux system facilitating invasion of brain microvascular endothelial cells (BMEC)	Kucervo et al. 2010
Proteolytic enzymes	*zpx*	Cause cell deformation and rounding of cells	Kothary et al. 2007
Lipopoysaccarides	Chromosomal encoded genes	Disrupt epithelial tight junctions	Moriez et al. 2005; Hunter et al. 2008
Type III hemolysin	*hly*	Hemolytic activity	Chen et al. 2004; Cruz et al. 2011

The genome study of *Cronobacter* has revealed the presence of gene for type IV pili in addition to a P pilus homologous to other pathogens such as *E. coli* [Grim et al. 2013]. Additionally the role of fibronectin, a glycoprotein in an extracellular matrix of *Cronobacter* has been postulated in the adherence to intestinal epithelial or endothelial cells [Nair & Venkitanarayanan 2007; Mittal et al. 2009; Mange et al. 2006]. However, the implications from these findings in pathogenesis and virulence have not been fully understood.

Recently, the role of *hfq* in pathogenesis of *C. sakazakii* ATCC 29544 has been demonstrated by generating the mutants using lambda red recombination where the mutants indicated defects in survival and invasion within host cells and exhibited low resistance to hydrogen peroxide [Kim et al. 2015]. *Hfq*, identified as RNA chaperone, is considered as a post-transcriptional regulator engaged in the biogenesis of quorum sensing, OMPs and various stress responses [Guillier et al. 2006; Lenz et al. 2004; Repoila et al. 2003]. The studies in other Gram negative pathogens i.e. *Escherichia coli, Listeria monocytogenes, Salmonella typhimurium, Yersinia pseudotuberculosis, and Francisella tularensis* have also expressed the importance of *Hfq* in the pathogenesis [Christiansen et al. 2004; Schiano et al. 2010; Meibom et al. 2009; Sittka et al. 2007; Kulesus et al. 2008].

Limited studies regarding the effect of *Cronobacter* invasion on immune response have been done. The pathogen is reported to persist within human macrophages indicating that the *Cronobacter* possessed virulence properties that make it allows to tolerate the intracellular environment of macrophages [Townsend et al. 2007b; Emami et al. 2012].

1.5 Quorum sensing

1.5.1 Bacterial cell-cell communication

Quorum sensing (QS), a term coined by Fuqua et al. [1994], is a fundamental process of cell-to-cell communication, yet very complex signaling process existing in most bacterial species [Alberts et al. 2002]. Basically, QS act as a regulatory circuit of three components i.e. a small diffusible signal molecule called autoinducers (AI), the gene coding for the autoinducer synthase protein and the gene for a response regulator protein. The bacteria synchronously modulate gene expression in response to density of a bacterial population and species complexity through QS (Fig. 1.3). At low cell-population densities, individual bacterial cells make the AI at a basal rate, which then diffuses or is pumped out of the cell. As the population rises, the concentration of the AI increases. At a threshold concentration, it binds

to and activates a response regulator which in turn directly or indirectly activates or represses target gene expression [Diggle et al. 2007]. The gene which encodes for the autoinducer synthase is itself activated by the autoinducer-activated response regulator and this creates a positive feedback loop that causes the entire population to switch into "quorum-sensing mode". When AIs produced by single bacterium cross the membrane of another, they bind to receptors in the cytoplasm. This autoinducer/receptor complex is then capable to attach to DNA promoters and activate the transcription of QS-controlled genes. Thus, QS enables the cells within a bacterial colony to act cooperatively, facilitating population-dependent adaptive behavior [Boyer & Wisniewski-Dye 2009].

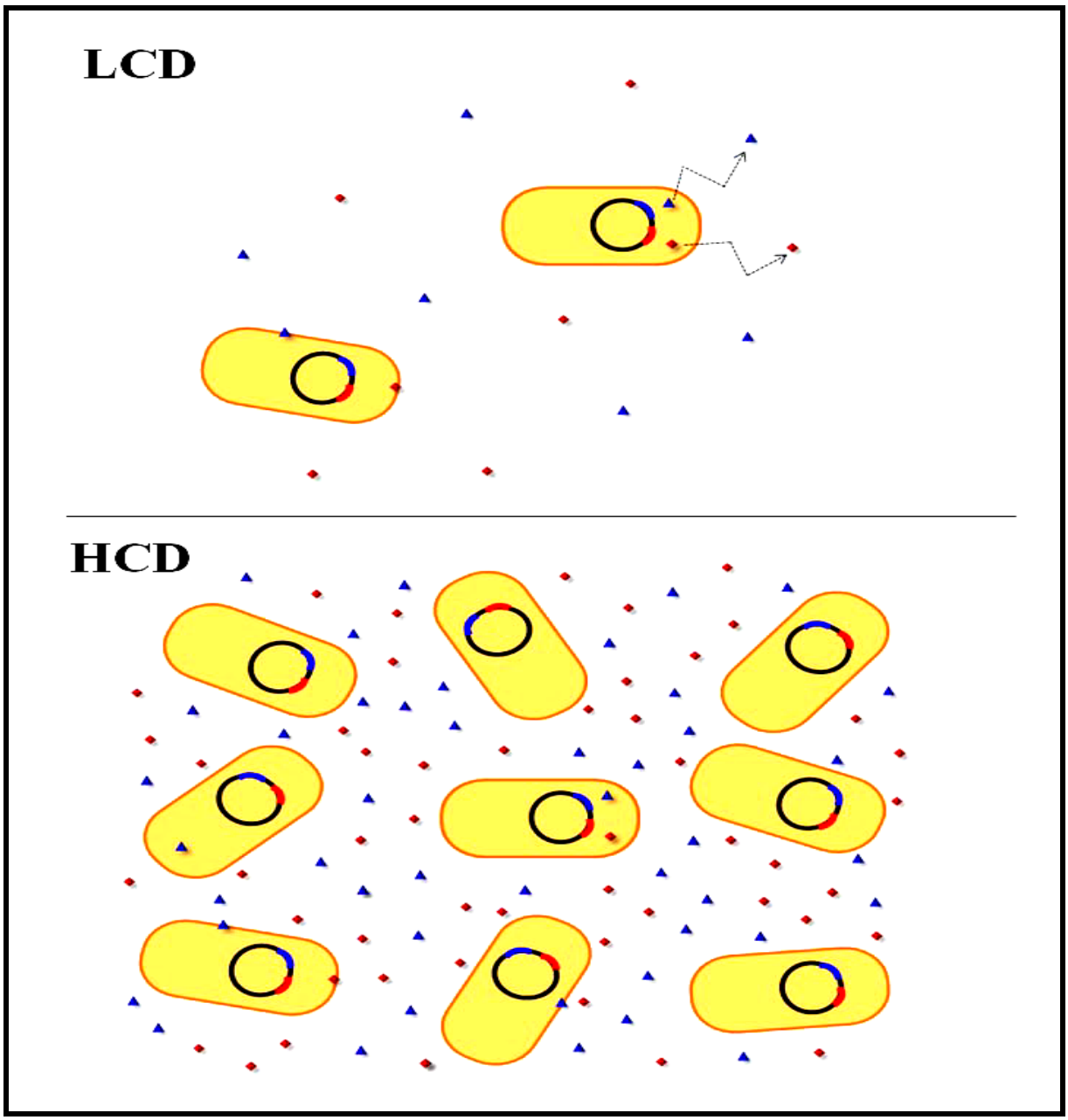

Figure 1.3: The concentration of autoinducer offers as a proxy of population cell density in bacterial quorum sensing [Adapted from Long 2010].

1.5.2 AHLs Structure

The first AI identified was 3-oxo-C6-HSL produced by *Vibrio fischeri* in the early 1990s [Eberhard et al. 1981]. AHLs consist of a common homoserine lactone moiety derived from amino acids, linked to a N-acyl side-chain (Fig. 1.4). The acyl side chain can vary in length from 4 and 14 carbon atoms, with or without either an oxo- or a hydroxy- moiety at the C3 position [Chhabra et al. 2004]. Depending on their structural proprieties, length and degree of substitution, AHLs can freely diffuse through the cell membrane or be actively secreted via an efflux pump system.

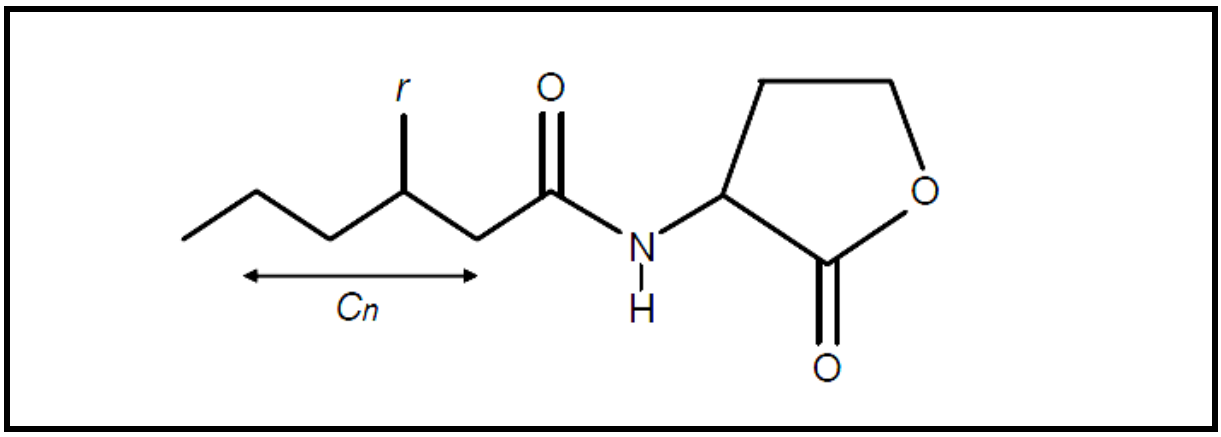

Figure 1.4: The structurs of AHLs structure sharing a common lactone ring moiety.

The AIs are grouped into various families based on their chemical structures. The best studied signals are acylated homoserine lactones (AHLs) found in Gram-negative bacteria, peptide-based signals used by Gram-positive bacteria and furanosyl borate diester (FBD), that have been identified in both Gram-negative and Gram-positive bacteria (Fig. 1.5). Many variations are found in each of this family of compounds, expanding the possibilities of diversification. This diversity allows specific recognition of intra-species signalling molecules and ensures to given bacterial species that it responds only to its own signals.

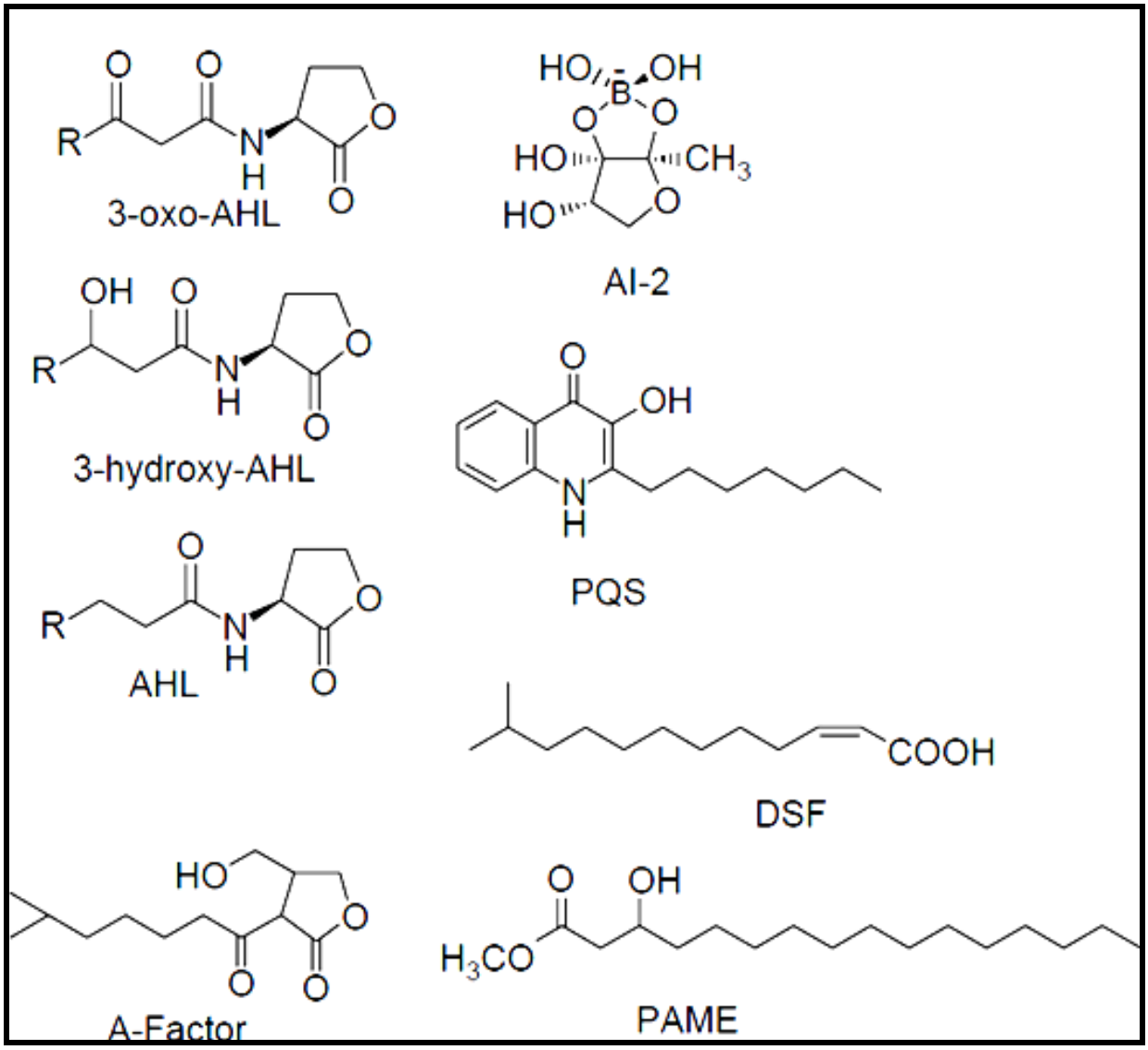

Figure 1.5: Structures of some representative quorum sensing signalling molecules [Adapted from Williams et al. 2007]. 3-oxo-AHL, N-(3-oxoacyl) homoserine lactone; 3-hydroxy-AHL, N-(3-hydroxyacyl) homoserine lactone and AHL, N-acylhomoserine lactone where R ranges from C1 to C15. The acyl side chains may also include one or more double bonds: A-factor, 2-isocapryloyl-3-hydroxymethyl-g-butyrolactone; AI-2, autoinducer-2, furanosyl borate ester form; PQS, *Pseudomonas* quinolone signal, 2-heptyl-3-hydroxy-4(1H)-quinolone; DSF,diffusible factor‘, methyl dodecenoic acid; PAME, hydroxyl-palmitic acid methyl ester

1.6 Quorum sensing signalling in Gram-negative bacteria

The signaling molecules which are commonly found in vast majority of Gram negative bacteria are derivatives of N-acyl homoserine lactones [Eberl 1999; Swift et al. 2001]. Modulation of the physiological processes controlled by AHLs occurs in a cell density- and growth phase-dependent manner. Therefore, this cell-to-cell communication system describes the ability of bacteria to monitor cell density before expressing specific genes for phenotypic traits.

There are several bacteria that are known to regulate a specific phenotype via QS for which one or more of the regulatory components have thus far eluded identification. In this thesis, we give examples of two common Gram-negative bacteria, focusing on their QS systems.

1.6.1 Quorum sensing in *Vibrio fischeri*

The model of LuxI/LuxR represents a paradigm of the quorum sensing control of gene expression in Gram-negative bacteria. Even though the basic mechanisms of LuxI/LuxR quorum sensing system are conserved, LuxR/LuxI systems have evolved and adapted to different types of regulatory networks based on the selective pressure imposed by their respective habitats.

The LuxI/LuxR QS system was first originated by the studies on the density dependent production of bioluminescence in the marine symbiotic bacterium *V. fischeri* [Nealson & Hastings 1979], which lives in a specialized light organ of the squid *Euprymna scolopes* [Ruby 1996]. Inside this organ, *V. fischeri* cells grow to very high cell density (10^{11} cells/ml) and genes encoding enzymes for bioluminescence are induced. In these partnerships, the host uses the light production by *V. fischeri* for specific purpose such as to attract mate and prey or in anti-predation approaches [Ruby 1996; Visick & McFall-Ngai, 2000]

The light production in *V. fischeri* is controlled by two regulatory components named LuxI and LuxR. LuxI gene encodes the AHL synthase that catalyzes the synthesis of AHL i.e. N-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C6 AHL). LuxR is a transcriptional regulator that responds to the AHL signal. LuxR can become active only when bound to AHL and thus promote transcription of the operon luxCDABE encoding luciferase enzymes responsible for production of light [Engebrecht et al. 1983; Eengebrecht & Silverman 1984]. The AHL freely diffuse in and out of the cell and concentration of these signaling molecules increase with increasing cell density [Kaplan & Greenberg EP 1985]. The concentration of AHLs increases

only when the bacterial population increases in cell numbers When a AI reaches critical threshold concentration, it binds to LuxR and activates it to function as a transcriptional regulator [Stevens et al. 1994]. The AHL-activated LuxR dimerises and activates the luxCDABE operon by binding to a promoter region, inducing luciferase enzymes for luminescence. This regulatory mechanism creates a positive feedback loop that floods the environment with both AHL and receptors and causes the whole population to switch into "quorum sensing mode" and committee to coordinated behaviors, such as to produce light (Fig. 1.6).

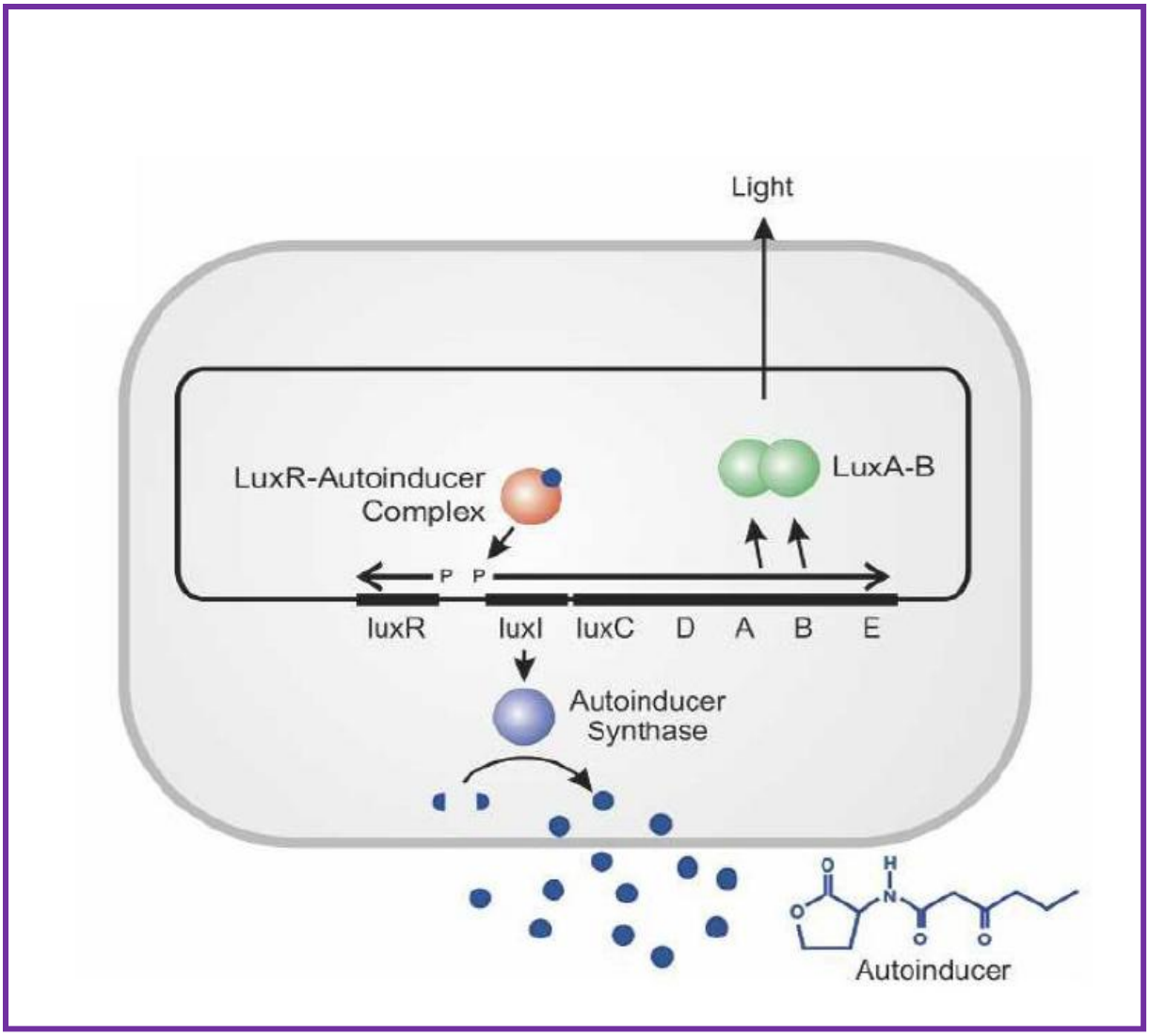

Figure 1.6: Digrammatic representation of N-acyl homoserine lactone (AHL) quorum sensing in *V. fischeri*. (Adapted from Ng & Bassler 2009].

1.6.2 Quorum sensing in *Pseudomonas aeruginosa*

P. aeruginosa contains three interconnected QS systems that regulate almost more than 500 genes, and at least 6% of the genome [Arevalo-Ferro et al. 2003; Schuster et al. 2003; Schuster & Greenberg 2006].

The first signaling system was found to drive the expression of lasB gene (encoding for elastase) and was therefore named the las system [Passador et al., 1993]. This consisted of N-(3- oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), produced by the LasI synthase, and the LasR transcriptional activator protein [Gambello & Iglewski 1991; Pearson et al. 1994).

The discovery of a second signaling system revealed that QS in P. aeruginosa is more multifaceted than initially believed [Winson et al. 1995]. The rhl QS consists of the transcriptional activator RhlR and the autoinducer synthase RhlI which directs the synthesis of N-butanoyl-L-homoserine lactone (C4-HSL) [Pearson al. 1995]. The RhlR–C4-HSL complex regulates the expression of rhlAB, required for production of rhamnolipid. These two QS circuits have been found to regulate the genes for a number of virulence determinants in *P. aeruginosa* to varying degrees.

The third QS system employs the *Pseudomonas* Quinolone Signal (PQS) that is structurally very different from the other two AHL autoinducers produced by this organism in that it is a 2-heptyl-3-hydroxy-4-quinolone [Pesci et al. 1999]. The products of the pqsABCD operon drive the production of the signal precursor 2-heptyl-4-quinolone (HHQ) which is converted to PQS via the action of PqsH (Fig. 1.7). The PqsR-PQS and PqsR-HHQ receptor signal complexes both up regulate the expression of the pqsABCD operon as well as the effector PqsE which, although an enzyme has a regulatory influence on a host of genes including virulence factors [Diggle et al. 2003].

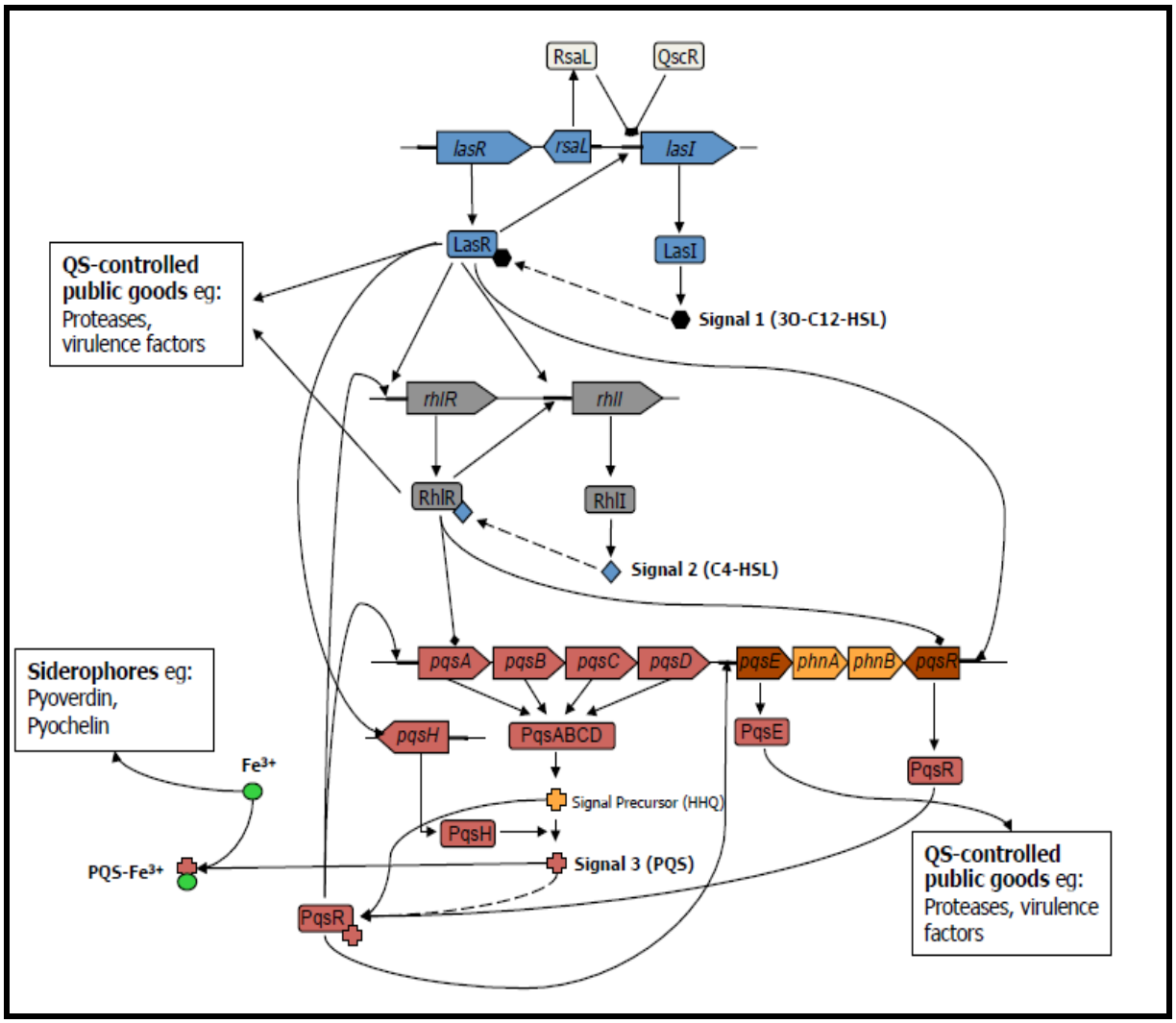

Figure 1.7: Schematic figure of quorum sensing in *P. aeruginosa*. [Adapted from Popat 2012].

Earlier, Lehner et al. [2005] illustrated the ability to produce cell-to-cell signaling molecules in *C. sakazakii*. They reported the presence of two different types of AHLs (3-oxo-C6-AHL and 3-oxo-C8-AHL) with ethyl acetate extracts of cell-free performed of strains of *C. sakazakii* using thin layer chromatography. Nevertheless, isolation and full chemical characterization of these molecules were escaping. Further, a chemical study by da Silva Araujo et al. [2011] identified three acyl-AHL molecules: (S)-N-heptanoyl-AHL, (S)-N-dodecanoyl-AHL and (S)-N-tetradecanoyl- produced by *C. sakazakii*. The identification and characterization of these molecules was carried by Gas Chromatography−Mass Spectrometer analysis.

1.7 Quorum sensing signaling in Gram-positive bacteria

A number of gram-positive bacteria are well-known to utilize QS systems. The nature of the signal molecules used in these systems differs from those of gram-negative organisms, and thus far, no gram-positive bacteria have been shown to produce AHLs. Gram-positive bacteria make use of modified oligopeptides as signals for specific interspecies communication and interact with the sensor element of a histidine kinase two-component signal transduction system [De Kievit & Iglewski 2000]. Generally, the peptide used as autoinducers is secreted outside the cell via ATP binding cassette transporter (ABC) and increase in concentration as a function of cell density. The secreted oligopeptides are detected by membrane-bound two-component sensor kinases. The binding of the peptide ligand to the kinase receptor triggers the signal transduction through phosphorylation cascade that results in the phosphorylation of a cognate response regulator protein [Kleerebezem et al. 1997; Lazazzera & Grossman 1998]. This activates it and permits it to bind DNA and hence regulate the transcription of the QS-controlled target genes as shown in Figure 1.8.

Numerous gram-positive bacteria utilize several peptides in combination with other types of QS signals for communication. Signaling of QS is often used to regulate the development of virulence in *Staphylococcus aureus*, bacterial competence for DNA uptake and sporulation in *Bacillus subtilis*, competence in *Streptococcus pneumoniae*, conjugation in *Enterococcus faecalis*, and virulence in *Staphylococcus aureus* [Dunny et al. 1999; Kleerebezem et al. 1997].

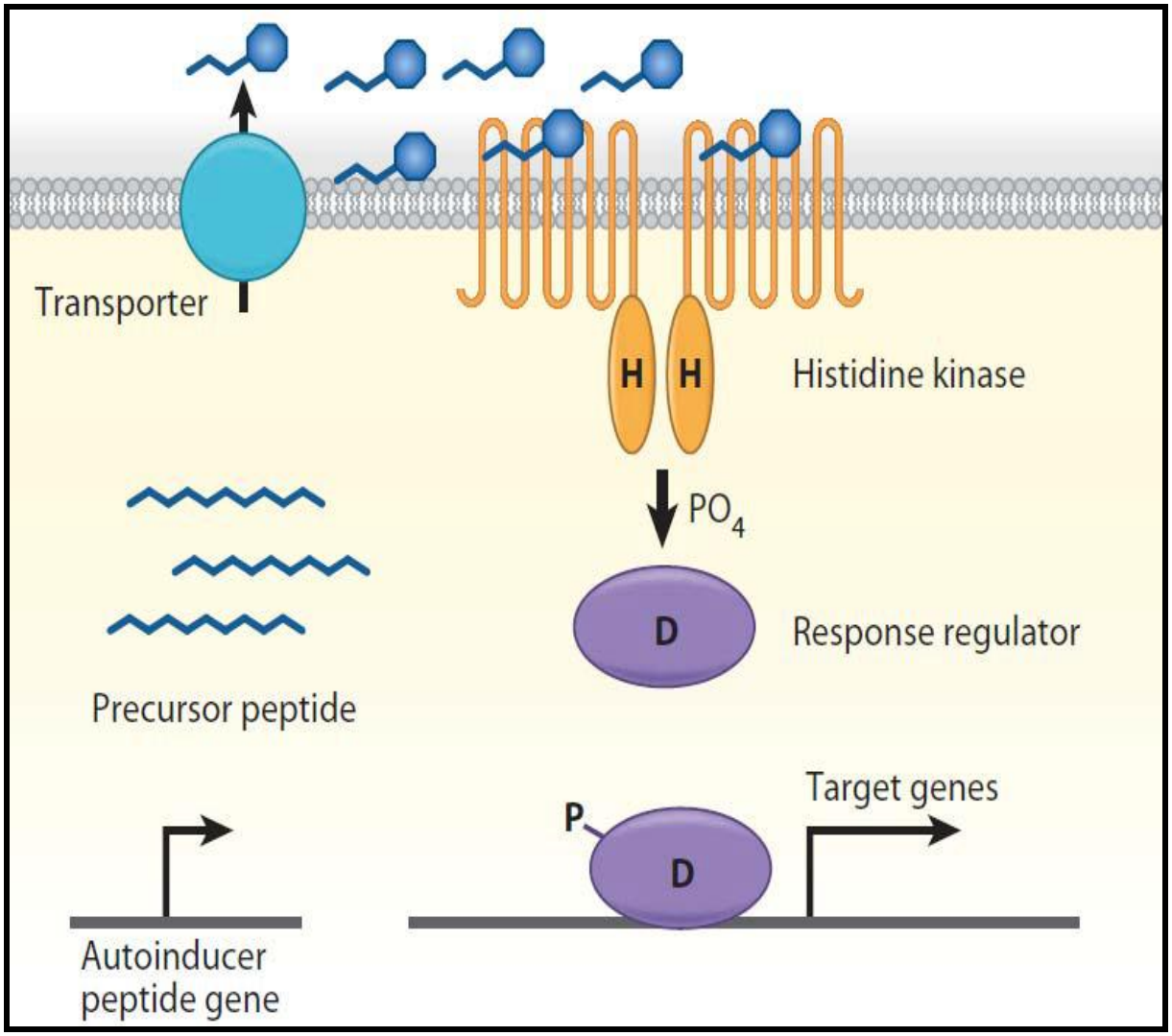

Figure 1.8: A canonical peptide-mediated two-component representation for quorum sensing in Gram-positive bacteria. Blue octagons indicate processed/modified peptide autoinducers. **[**Adapted from Ng & Bassler 2009].

1.8 Quorum sensing in Biofilm formation

Bacteria can exist as planktonic cells or as biofilms that account for over 99% of microbial life. A biofilm consists of surface-attached (sessile) communities that attach to a substratum and surrounded by an EPS matrix [Hall-Stoodley et al. 2004; Stoodley et al. 2002]. The EPS is self-generated by the bacteria and consists predominantly of exopolysaccharides, with smaller amounts of nucleic acids and proteins [Davey and O'Toole 2000; Donlan and Costerton 2002]. The increased cell density in turn accelerates signaling molecule to communicate with the responding cells for collective interactions in biofilms, possible including a different level of complexity to biofilms [Webb et al. 2003; Kreft 2004]. The biofilm mode of growth offers number of advantages over the planktonic state. These advantages include metabolic cooperation, protection from environmental stresses such as desiccation and enables resistance toan antimicrobials, increased genetic diversity via horizontal gene transfer, and cell-to-cell communication [Davey & O'Toole 2000; Donlan & Costerton 2002; Hall-Stoodley et al. 2004].

A mature biofilm is a highly dense bacterial population in which suitable conditions exist for communication in a cell. The development of biofilm can be categorized in a five-stage process [Stoodley et al. 2002] (Fig. 1.9):

(i) initial reversible adsorption of motile cells to the surface,

(ii) irreversible attachment of bacteria through the production of EPS,

(iii) microcolony formation, with multi-layering cells resulting in early development of biofilm architecture,

(iv) maturation of biofilm via multiplication and metabolism of attached microorganisms,

(v) dispersion of single cells from the biofilm and reversion to planktonic growth.

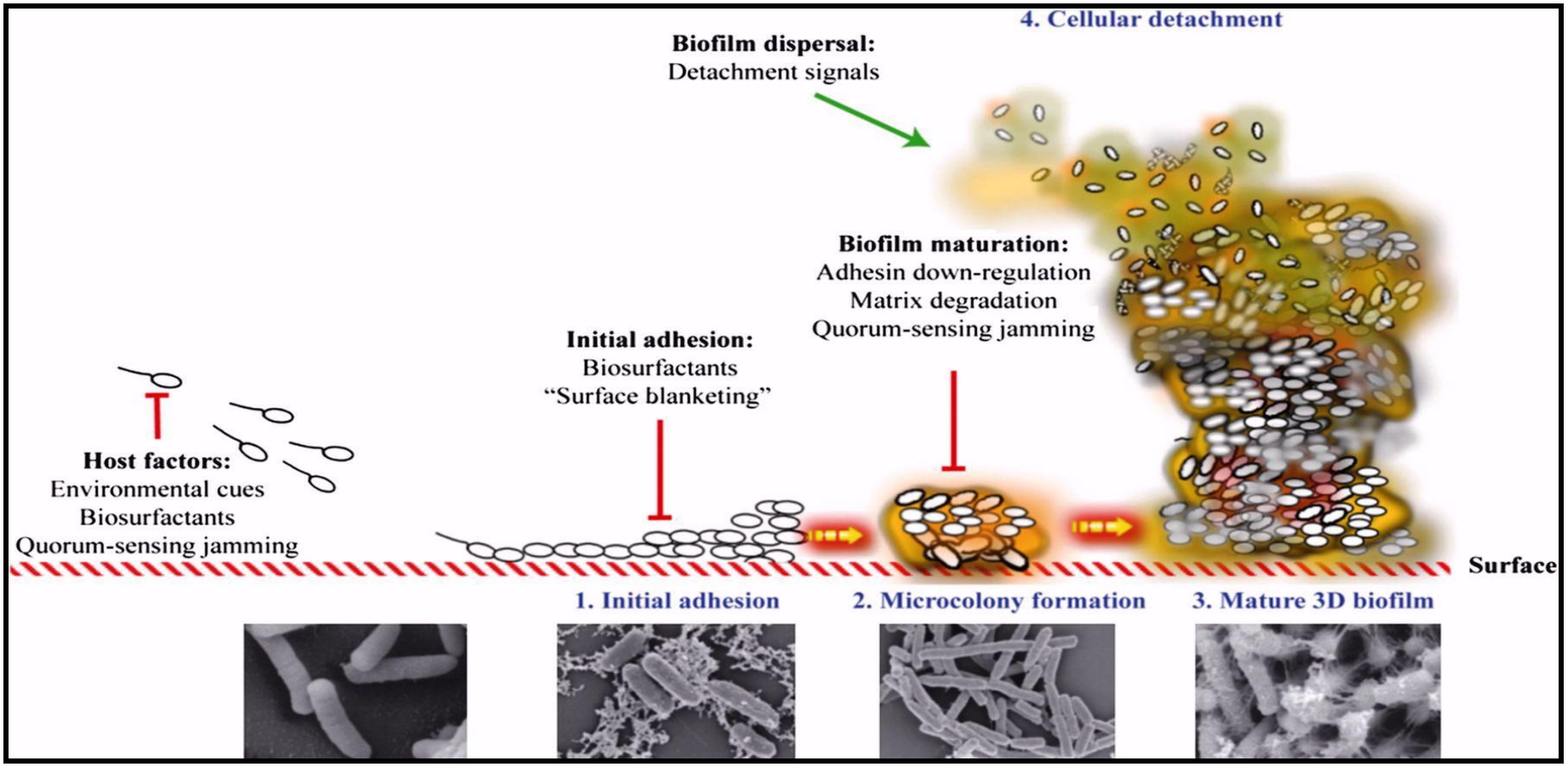

Figure 1.9: Stages of the biofilm formation process (Flemming & Wingender, 2010a).

Studies on microbial biofilms have led to realize that microbes present in biofilms have different phenotypes from those of the planktonic (free-living) counterparts, including a significantly increased resistance to antimicrobial agents and the host immune response [Lewis 2001]. Biofilms are a naturally-occurring phenomenon and form on virtually any surface that is intermittently exposed to water. These surfaces include pipe linings, plant leaves and roots, body surfaces, and medical devices such as pacemakers and catheters (Donlan and Costerton 2002; Hall-Stoodley et al. 2004).

Each step of biofilm cycle entails the expression of specific genes. In many bacteria, QS represents a central mechanism to regulate and coordinate gene expression between microcolonies, allowing bacteria to reap benefits that would be unattainable to them as individual cells. Particularly, the genes responsible for attachment of cell with surface, in addition to the synthesis of matrix elements are under regulation of QS (Mack et al. 2004 and references therein). These studies provide significant insights into the social biology of microbes in biofilms and in bacterial infections. Escalating evidence shows that QS-mediated physiological processes favor microbial interactions and are believed as major mechanisms to regulate population-level virulence of bacteria. The mutant strains of *P. aeruginosa* lacking AHL synthesis are incapable to form the proper biofilm structure [Davies et al. 1998]. Besides to AI production, the expression of various other QS-regulated genes such as adhesion proteins is also needed for biofilm formation and biofilm dispersion [Mack et al. 2004; O'Toole & Kolter 1998]. Usually, in Gram-negative bacteria such as *Vibrio* spp. and *Pseudomonas* spp., biofilm formation encouraged by the induction of QS systems [Aswathanarayan & Rai 2015]. However, in Gram-positive bacteria, this association is more complex; for *Staphyloccocus aureus*, repression of the agr QS system is actually required for biofilm formation [Ganin et al. 2015] where as in *Bacillus subtilis*, activation of QS-associated genes activates surface attachment and consequent development of a biofilm [Singh et al. 2015].

Numerous studies on biofilm formation of *C. sakazakii* was done so far which established that *C. sakazakii* has an capacity to form biofilm on silicon, glass, stainless steel, latex and polyvinylchloride [Lehner et al. 2005]. *C. sakazakii* colonized on the surface of utensils, blenders and brushes have been associated to cause neonatal infections [Bar-Oz et al. 2001]. Kim et al. [2006] demonstrated the ability of *C. sakazakii* to form biofilm on stainless steel and enteral feeding tubes. Grimm et al. [2008] have depicted the presence of cellulose as a

major component of *C. sakazakii* extracellular matrix. Later on, the study by Dancer et al. [2009b] indicated that milk constituents (casein and whey protein) are the main determinant during the formation of biofilm in *C. sakazakii*. The capability of *C. sakazakii* to attach to and form biofilms on foods and food contact surfaces may influence their persistence during manufacturing and retail storage of food products will encourage the threat of food borne diseases [Iversen & Forsythe 2003]. *C. sakazakii* has also been reported to form biofilms on glass and stainless steel, thus increasing the risk to infants, neonates and immuno compromised individuals [Iversen and Forsythe 2004a]. The biofilm formation has been reported in *C. sakazakii* to impart protection against disinfectants. [Beuchat et al. 2009]. Various studies also revealed significant variation in biofilm formation among different isolates of *C. sakazakii* and the variation in the biofilm forming abilities is generally strain specific [Lehner et al. 2005; Lee et al. 2012; Ye et al. 2015].

1.9 Quorum Quenching

Ever since the mechanism of QS was discovered, novel approaches have been targeted to find AI mimics to capably block QS signals as a possible solution to the biofilm problem. Therefore, anti-QS compounds that inhibit cell-to-cell communication, called quorum sensing inhibitors (QSI) can be of great interest in the treatment of bacterial infections [Fuqua et al. 2001; Rice et al. 2005]. QSIs may also effectively diminish microbial virulence by interfering quorum communication thus preventing microbes to attack the host, by reducing or preventing the development of biofilm formation and by prevent the expression of pathogenic and virulence gene expression. Various compounds have been evolved that can efficiently interfere with bacterial QS regulation [Manefield et al. 2002] through competitive inhibition, thus disrupting biofilm formation [Hentzer et al. 2002].

Mechanisms of QS inhibition

The mechanism of QS can be disrupted at different stages:

(i) reducing or modulating the activity of AHL cognate receptor protein or AHL synthase,

(ii) obstructing the pathway involve in the production of QS signal molecules,

(iii) degradation or disrupting the structure of the AHL, and

(iv) mimicking the signal molecules primarily by using synthetic compounds as analogues of signal molecules.

Several AHL-degrading enzymes detected in different bacteria have the prospective to be used as quorum quenchers. Several bacteria comprising *Bacillus* sp., *Variovorax paradoxus, Arthrobacter* sp., and *A. tumefaciens* produce AHL-lactonases enzymes that hydrolyze and deactivate the lactone bond in the AHLs [Dong et al. 2001; Leadbetter & Greenberg 2000; Park et al. 2003].

Anti-QS compounds were first characterized in the marine red alga, *Delisea pulchura* shown to produce halogenated furanones that disrupt QS in bacterial species by mimicking the AHL signal [Givskov et al. 1996; Raffa et al. 2005; Shiner et al. 2005]. However, this compound has limited or no therapeutic application due to high reactivity and their toxicity on the host. Jakobsen et al. (2012) concluded that ajoene is the key QSI present in garlic, and the investigation regarding toxicity revealed that ajoene has very low cytotoxicity consequences on human epithelium cells. Hence, the invention of non-toxic, broad spectrum QSIs is still needed for successful exploitation in inhibiting bacterial communication [Choo et al. 2006].

The plants were explored initially because of the therapeutic roles of natural products and continue to contribute significantly to the development of today's pharmaceuticals for treatment of numerous diseases and source of new-fangled bioactive compounds [Cragg et al. 1997]. Amongst terrestrial plants, soybean, pea, clove, rice, tomato, vanilla, grapefruit, and garlic also produce molecules that interrupt with QS in many Gram-negative species [Bjarnsholt et al. 2005; Choo et al. 2006; Girennavar et al. 2008; Khan et al. 2009; Mathesius et al. 2003; Gao et al. 2003; Singh et al. 2009; Teplitski et al. 2000]. In the recent times, various fruits and herbs were demonstrated to possess anti-QS activity in a *C. violaceum* biosensor strain and on the swarming motility of *E. coli* and *P. aeruginosa* [Vattem et al., 2007]. Fruits including raspberry, blueberry, blackberry, cranberry, and grape, and herbs such as thyme, ginger, basil, kale, oregano and turmeric exhibited moderate inhibition of these QS-controlled processes.

1.10 Objectives of the study

Cronobacter spp. are emerging pathogens that have the ability to survive in a variety of different environmental conditions such as those encountered in PIF processing facilities. As the *Cronobacter* spp. is a newly classified genus and needs more investigations for better understanding this unique group of organism. As a virulent species, it causes high mortalities in the neonates; therefore, it is important to highlight the presence and to understand which gene products are responsible for the pathogenicity of the bacteria. Further, the present study also investigates the biofilm formation, its composition and identification of QS molecules in *C. sakazakii*. The study also investigates the quorum quenching activities of the plant extracts in *C. sakazakii* and their role in inhibition of biofilm formation.

The objectives of this study are:

- **Isolation and characterization of *C. sakazakii* from different sources and detection of selected virulence traits in the *C. sakazakii.***
- **Characterization of biofilm in *C. sakazakii* and determination of key molecules involved in quorum sensing.**
- **Evaluation of quorum sensing inhibitory activity of plant extracts against biofilm forming *C. sakazakii.***

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CHAPTER 2

Prevalence and characterization of *Cronobacter* spp. from various foods, medicinal plants and environmental samples

2.1 Abstract

Dairy or non-dairy based products were explored to determine the prevalence, molecular characterization and antibiotic susceptibility of *Cronobacter* spp. The isolation was done as per ISO 22964:2006 on chromogenic media followed by further confirmation by biochemical and 16S rRNA based identification. From 219 samples, the chromogenic agar assay and biochemical tests yielded presumptive 45 isolates. Among them, only 38 isolates showed 282bp band amplified from ITS-G gene which confirming them to be as *C. sakazakii*. The *Cronobacter* spp. prevalence was highest in herbs and spices (34%) while environmental samples had contamination rates of 23% indicating plants as a possible reservoir of this pathogen. All the isolates were resistant to β-lactam derivatives (68%), macrolides (88.6%) and aminoglycosides (79.9%) but susceptible to phenicoles (31.6%) and tetracyclines (15%) derivatives. The results emphasize the screening of plant materials before their incorporation in food matrices.

2.2 Introduction

Cronobacter spp. is Gram-negative facultative anaerobe, motile, rod-shaped, non spore forming pathogenic bacterium earlier known as *Enterobacter sakazakii* [16]. *Cronobacter* spp. is recognized world-wide as emerging opportunistic food-borne pathogens and includes seven species [Iversen et al. 2008; Girlich et al. 2001; Joseph et al. 2012; Brady et al. 2013; Oren & Garrity 2013; Masood et al. 2014]. The species *C. sakazakii* is reported to cause meningitis, bacteremia and necrotizing entero*coli*tis in infants and neonates [Nazarowec-White & Farber 1997; Stephan et al. 2007; Van Acker et al. 2001] and also in elderly or immunocompromised hosts [Gosney et al. 2006; See et al. 2007]. Neonates are at the utmost risk and suffer a high mortality rates ranging between 40 to 80% [Forsythe 2005; Kothary et al. 2007; Mullane et al 2007].

The organism is ubiquitous being detected from a wide spectrum of food and food ingredients of animal and plant origin. Furthermore, it should be noted that fresh, ready-to-eat, frozen, fermented and cooked food products and water used for the preparation of food have been found to be contaminated by *Cronobacter* spp. [Iversen & Forsythe 2003; Friedemann 2007]. The pathogen has also been reported to be inhabitant of various environmental samples such as water [Friedemann 2007], insects [Kuzina et al. 2001; Hamilton et al. 2003; Butler et al. 2010] and spoon and blenders used to mix infant formula [27, 3]. Contamination of powdered infant formula (PIF) has been associated with severe systemic neonatal infections by *Cronobacter* spp. Kandhai *et al*. reported *Cronobacter* spp. in factories producing milk powder, chocolate, cereals, legumes, pasta and potato flour, as well as in domestic environments [Kandhai et al. 2004]. The human clinical samples such as cerebrospinal fluid, urine, respiratory secretions, and digestive tract and skin wounds samples are also infected by this pathogen [Corti et al. 2007; Barron & Forsythe 2007; Lai 2001].

Identifying foods that may contain *Cronobacter* is imperative to discover the possible ways for transmission of infection. With an indication that *Cronobacter* spp. infects both infants and vulnerable adults it is significant that an extensive range of foods should now be evaluated. In Indian context, there has not been a far-reaching report on association of this pathogen with the different food commodities. However, single clinical case study was reported by Ray et al. [2007] which described two cases of *C. sakazakii* infections; one preterm very low birth weight neonate with meningitis and a two month infant with bacteraemia for the first time in India.

Therefore, the purpose of this study was to investigate the occurrence of *Cronobacter* spp. in an extensive range of foods including milk powder, infant foods, herbs and spices and environmental samples in an attempt to find the reservoir for this pathogen. The isolates were further confirmed by an array of biochemical, cultural and molecular methods.

2.3 Methodology

2.3.1 Collection of samples

A total of 219 samples of different food products (raw and processed): Powdered Infant Formulae (Nestle and Amul), infant food formula (Nestle), medicinal plants, herbs, and spices were purchased from local outlets along with environmental samples (soil, water) were tested for the presence of *Cronobacter* spp (Table 2.1). The standard strains of *C. sakazakii* ATCC 12868 and E604 (kindly gifted by Dr. Ben Davies Tall, FDA, USA) were used for the comparison.

2.3.2 Enrichment and isolation

A 25 g of each sample was added to 225 ml buffered peptone water (BPW) and mixed well for pre-enrichment (ISO 22964:2006) (Fig. 2.1). Following an overnight incubation at 37°C, 10 ml of the pre-enrichment culture was inoculated into 90 ml of Enterobacteriacae Enrichment (EE) broth and incubated overnight at 37°C. A 10 µl volume of the selective enrichment was then streaked onto a tryptone soya agar (TSA). All colonies that appeared yellow on TSA were picked and subjected to further characterization using chromogenic assays.

2.3.3 Chromogenic assay for *Cronobacter* spp. positive isolates

The colonies picked from TSA were further streaked on *Enterobacter sakazakii* agar (Himedia laboratories, India) containing 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside, a chromogenic substrate which upon hydrolysis of the substrates gives blue/green colonies typical for *Cronobacter* spp. The presumptive isolates were further carried out for biochemical tests.

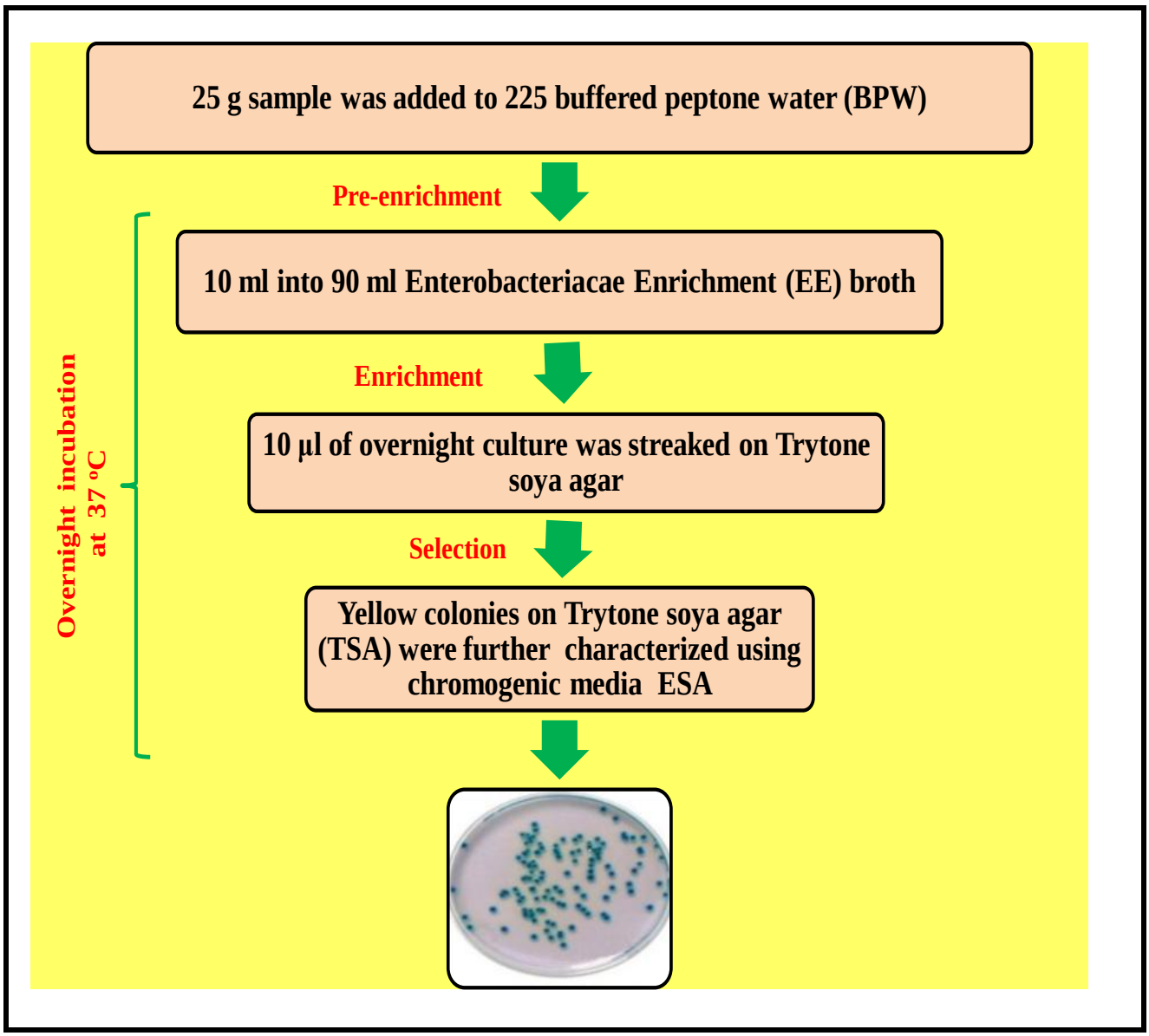

Figure 2.1: Isolation and detection of *Cronobacter* spp.

Table 2.1: Number of samples analysed for presence of *Cronobacter* spp.

Origin	Sample category	No. of samples	Number of *Cronobacter* spp. isolates	% of total samples in the category	% of total *Cronobacter* spp.
Dairy products	Raw milk Pasteurized milk Curd Yoghurt	7 9 5 2	2 (Raw milk)	8.7	4.5
Cereals and cereal products	Gram flour Wheat flour Corn flour Legume	4 3 1 1	1 (Gram flour)	11.2	2.3
Reconstituted Infant formula and milk powder	Powdered infant formula (0-6 months) Follow-on formulae (6-12 months) Growing-up milk (12-36 months)	5 3 3	2 (Opened packet)	18.2	4.4
Medicinal plants	*Saraca indica,Rauwolfia serpentine,Tribulus terrestris, Plantago ovate, Amomum subulatum,Coriandrium sativum, Carum carvi,Cuminum cymirium,Cassia augustifolia,Nux vomica,Urtica dioivia,Roylea cinerea,Citrus,Tylophora indica,Withania somnifera,Stevia,Trachyspermum ammi, Syzygium aromaticum,Nicotiana tabacum,Picrorhiza kurroa,Andrographis paniculata,Aconitum,Jatropha, V.jatamansi,Notha podhytes, Ocimum tenuiflorum, Malus domestica ,Lilium,Hypericum perforatum,Orchid, Camellia sinensis*	1each	3 (*Coriandrium sativum, Cuminum cymirium, Syzygium aromaticum*)	9.7	6.7

Herbs and Spices	Cumin Fenugreek Coriander Black pepper Clove Cinnamon Chilli powder Poppy seeds Large cardamom Nutmeg Ginger powder Turmeric powder Bishop's weed Mango powder	4 3 3 4 2 2 3 1 2 2 4 2 2 1	12 (Cumin, Fenugreek, Coriander, Black pepper, Clove, Chilli powder, Large cardamom, Ginger, Turmeric and Mango powder)	34.3	26.7
Environmental samples	Soil Water Vaccum dust	9 10 7	3 2 1	23.0	13.3
Vegetables and fruits	Vegetables Fruits	12 15	3 4	26	15.4
Clinical sample	Stool samples	15	2	13.3	4.5
Miscellaneous food products		42	10	23.8	22.2
Total		219	45		100

2.3.4 Biochemical differentiation of *Cronobacter* spp.

The isolates were identified by biochemical tests for the differentiation of *Cronobacter* spp., including Indole production, Methyl red, Voges-Proskauer, Citrate utilisation test as per the Standard Microbiological protocol. All the results were compared with the standard strains.

2.3.5 DNA extraction

The DNA was extracted from overnight grown culture using phenol:chloroform:iso-amyl alcohol method [Sambrook et al. 1989]. A single colony was inoculated in TSB and incubated at 37^{o}C for overnight. One mL of cell suspension was harvested and centrifuged at 10,000×g for 10 min at 4^{o}C. The pellet was suspended in 1 mL of sterile distilled water and the supernatants were removed after centrifugation at same conditions. The pellet was re-suspended in 500 µL sterile distilled water and then boiled in water for 10 min. After centrifugation at 13,000×g for 10 min at 4^{o}C, the supernatants were recovered in 100 µL of sterilized water. The DNA concentration of each supernatant was determined using a spectrophotometer Go scan microplate reader (Thermo, USA) at 260 nm and diluted to reach the DNA concentration of 1 µg/mL with sterilized water. The supernatants were stored at -20^{o}C for used as templates for PCR.

2.3.6 16S rRNA target PCR

The isolates were confirmed by genus-specific and species-specific PCR (Table 2.2). Isolated DNA was assessed for PCR amplification of the 16S rRNA gene and ITS region according to Jaradat et al. [2009]. The PCR reaction for genus-specific was achieved by mixing 1 µl of extracted DNA with a 49 µl of PCR mixture containing the following: 1× PCR buffer, 5 units Taq DNA polymerase (Intron), 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 1 pmol from primers P_f (5'-ACAGGGAGCCAGCTTGCTGC-3') and P_r (5'-TCCCGCATCTCTGCAGGA-3'). PCR amplification was performed as follows: 10 min at 95°C; 30 cycles of 30 sec at 95°C, 30 sec at 56°C, 2 min at 72°C; 5 min at 72°C. For the species-specific PCR, the partial nucleotide internal transcribed spacer (ITS) region was amplified using the ITS-G forward primer (5'-GGGTTGTCTGCGAAAGCGAA-3') and ITS-G reverse primers (5'-GTCTTCGTGCTGCGAGTTTG-3') with following reaction conditions: 10 min at 94^{o}C for denaturation, 30 cycles each of 30 seconds at 94^{o}C for denaturation, 1 min at 57^{o}C for annealing, 1 min at 72^{o}C for extension followed by the final extension at 72^{o}C for 5 min. The PCR products were then analyzed by electrophoresis in 1.5% (w/v) agarose gel in 1X Tris-

acetate-EDTA buffer with 0.5 mg/ml of ethidium bromide at a constant voltage of 80 V for 45 min, then visualized under UV light to confirm the presence of the amplified DNA. A non-template control was included in each run. ITS sequences of our isolates and standard sequences were analyzed by drawing the phylogenetic tree (Neighbour-joining method with 1000 bootstrap replication) using MEGA 5.1 (http://www.megasoftware.net).

Table 2.2: Details of primer pairs and PCR running conditions used in this study.

S.No	Primer	Sequence 5' to 3'	Region	Amplicon size	PCR running conditions
1.	Saka 1a Saka 1b	ACAGGGAGCAGCTTGCTGC TCCCGCATCTCTGCAGGA	V1 V3	952 bp	95°C for 4 min; 30 cycles of 95°C for 60 sec each; 50°C for 1 min; 72°C for 90 sec; 4 min at 72°C for 4 min.
2.	SG-F SG-R	GGGTTGTCTGCGAAAGCGAA GTCTTCGTGCTGCGAGTTTG	ITS-G	282 bp	94°C for 10 min; 30 cycles of 94°C for 30 sec each; 57°C for 1 min; 72°C for 1 min; 72°C for 5 min.

2.3.7 Antibiotic susceptibility test

The disc diffusion method for antimicrobial susceptibility testing as described by the National Committee for Clinical Laboratory Standards [2003] was performed on TSA using commercial antibiotic disks (Himedia Laboratories, India). *Cronobacter* spp. isolates were tested against eight antibiotic derivatives which includes β-lactams (ampicilln, cephalothin, amoxyclav, and penicillin-G), macrolides (rifampicin, clindamycin and erythromycin-G), aminoglycosides (gentamicin, amikacin, tobramycin, neomycin, kanamycin and streptomycin), coumarin-glycoside (novobiocin), tetracyclines (tetracycline), glycopeptides (vancomycin), peptide (bacitracin and carbencillin) and phenicoles (chloramphenicol). The zone of inhibition was measured and interpreted according to NCCLS guidelines for *Enterobacteriaceae* [2003].

2.4 Results and Discussion

The present study reported the isolation of *Cronobacter* spp. from a wide variety of food and environmental samples in an attempt to pinpoint their reservoir. The prevalence of *Cronobacter* spp. isolated from different commodities examined is summarized in Table 2.1. From 219 samples, among 85 presumptive isolates on TSA, only 58 isolates resulted in blue-green coloration on chromogenic ESA. Although, the chromogenic substrate is a diagnostic tool for the detection of *Cronobacter* spp., it is not consistent because these studies have given different results [Iversen et al. 2007; Drudy et al. 2006; Fanjat et al. 2007].

The biochemical tests carried out in the study revealed that all the 58 isolates as well as the reference strain ATCC 12868 and E604 were positive for citrate utilisation. All *Cronobacter* spp. isolates were negative for the production of Indole and Methyl red. However, two isolates out of 58 isolates were negative for VP test (Table 2.3).

Table 2.3: Characterization of *Cronobacter sakazakii* from different sources using biochemical testing and PCR analysis

Isolate		IMVIC Test				PCR Primers	
ID	Source	Indole production	Methyl red	Vogus Proskauer	Citrate utilisation	16S rRNA	ITS-G
E604	Standard strain	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
ATCC 12868	Standard strain	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N1	Raw milk	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N2	Vegetable	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N3	Vegetable	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N4	Raw milk	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N5	Milk powder	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N6	Stool sample	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N7	*Coriandrium sativum*	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N8	*Syzygium aromaticum*	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N9	Milk powder	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N10	*Cuminum cymirium*	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N11	Stool sample	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N12	Fruit	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N13	Fruit	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N14	Turmeric powder	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N15	Soil	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N19	Legumes	-	-	+	+	-	-
N24	Market Snacks	-	-	+	+	*Cronobacter spp.*	-
N27	Biscuits	-	-	+	+	-	-
N31	Citrus fruit	-	-	+	+	*Cronobacter spp.*	-
N35	Baked bread	-	-	+	+	-	-
N41	Pasteurized milk	-	-	+	+	-	-
N46	Coriander	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N48	Gram flour	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N50	Black pepper	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N51	Large cardamom	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N52	Nutmeg	-	-	+	+	-	-
N53	Cumin	-	-	+	+	-	-
N54	Clove	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*

N56	Mango powder	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N57	Ginger	-	-	+	+	-	-
N61	Soil	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N63	Fenugreek	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N64	Cardamom	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N65	Fenugreek	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N69	Market Soup powder	-	-	+	+	-	-
N74	Vacuum Dust	-	-	-	+	*Cronobacter spp.*	*C. sakazakii*
N75	Soil	-	-	-	+	*Cronobacter spp.*	*C. sakazakii*
N77	Chilli powder	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N79	Cumin	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N81	Pond water	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N83	Sugar	-	-	+	+	*Cronobacter spp.*	-
N85	Rain water	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N87	Vegetable	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N88	Fruit	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N92	Chilli powder	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N96	Vegetable	-	-	+	+	-	-
N101	Flattened rice	-	-	+	+	-	-
N106	Fruit	-	-	+	+	*Cronobacter spp.*	-
N108	Coriander	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N112	Fruit	-	-	+	+	*Cronobacter spp.*	*C. sakazakii*
N118	Fruit	-	-	+	+	-	-
N119	Water	-	-	+	+	-	-
N121	Biscuit	-	-	+	+	*Cronobacter spp.*	-
N134	Chocolate	-	-	+	+	*Cronobacter spp.*	-
N148	Chips	-	-	+	+	-	-
N156	Fruit	-	-	+	+	*Cronobacter spp.*	-
N162	Salt	-	-	+	+	*Cronobacter spp.*	-
N174	Fruit	-	-	+	+	*Cronobacter spp.*	-

Further, the molecular characterization targeting 16S rRNA region resulted in 408 bp amplified product in the 45 isolates in addition to standard strains. The other 13 isolates did not give the expected PCR product although they were identified as *Cronobacter* spp. by chromogenic assay which imposes the relevance of more than one technique in precise identification of the *Cronobacter* spp. The studies by Iversen *et al.* and Barron *et al*. have also suggested a new system for categorizing *Cronobacter* spp. isolates based on combination of DNA-DNA hybridization, f-AFLP and 16S rRNA gene sequences and phenotypic characteristics [Iversen et al. 2007; Iversen et al. 2008; Barron & Forsythe 2007]. The species-specific PCR based on the ITS-G sequences showed in Fig. 2.2 confirmed 38 numbers of isolates as *C. sakazakii* (Table 2.3). The other isolates may belong to other species of *Cronobacter* which might play a role mostly as environmental commensals and are probably of modest clinical significance.

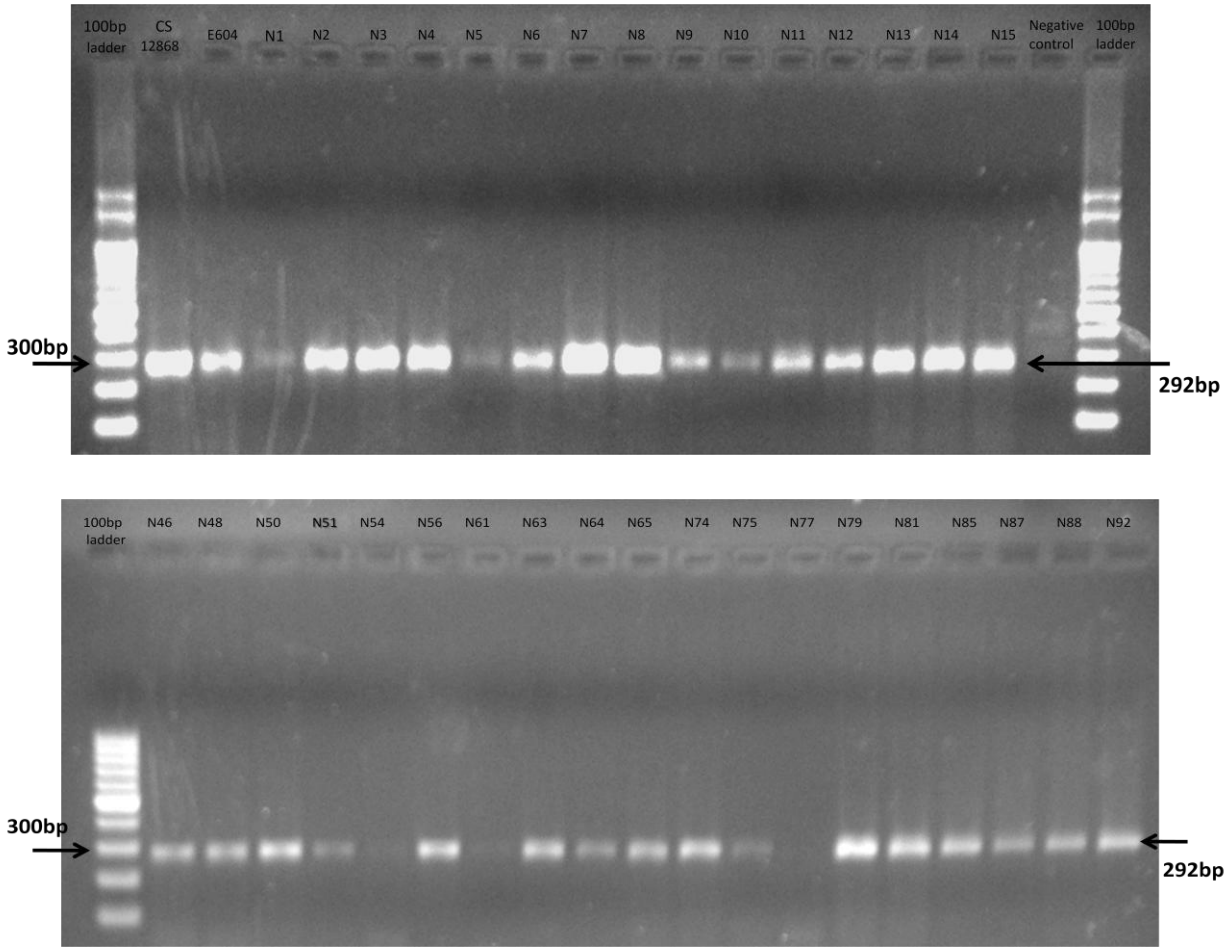

Figure 2.2: A 1.5 % agarose gel showing the PCR products derived from ITS-G.

The plant based products were the most frequently contaminated with *Cronobacter* spp. (n=102, 22%) with herbs and spices (34%) as a potential reservoir of this pathogen. The high prevalence of this pathogen with herbs and spices suggests that more precautions should be taken when home remedies containing herbs or herbal beverages are given to infants to alleviate gastrointestinal discomfort. The pervasiveness on plant based materials could be due to the resistance of the pathogen to various environmental stresses which might be due to the organism possibly colonizes plant material, and the yellow carotenoid-based pigmentation may shield it from sunlight-generated oxygen radicals [Forsythe 2005]. Studies by Forsythe [2005] and Friedemann [2007] also reported that plant origin samples are most repeatedly contaminated with *Cronobacter* spp. irrespective of the world region of analysis. *Cronobacter* spp. were also detected in environmental samples (23%) which supports the hypothesis of the role played by environmental contamination. Among dairy based products, only 12% raw milk samples were found to be contaminated with *Cronobacter* spp. with no occurrence in PIF, milk powder and pasteurized milk samples indicating that proper hygiene and healthy practices were implemented in the industrial units manufacturing these products. These findings are in agreement with reports by Iverson and Forsythe and Nazarowec-White and Farber who put forwarded that application of pasteurisation at final treatment stage eliminates all pathogens from such products [Iversen & Forsythe 2003; Nazarowec-White & Faber 1999]. We classified 38 *Cronobacter* spp. isolates by comparing the ITS sequence. The phylogenetic analysis provided two major clades as larger clade containing 27 isolates and smaller clade containing 8 isolates including E604 and ATCC 12868. The neighbour-joining tree reveals that numbers of eight isolates are closely identical to standard strains (Fig. 2.3).

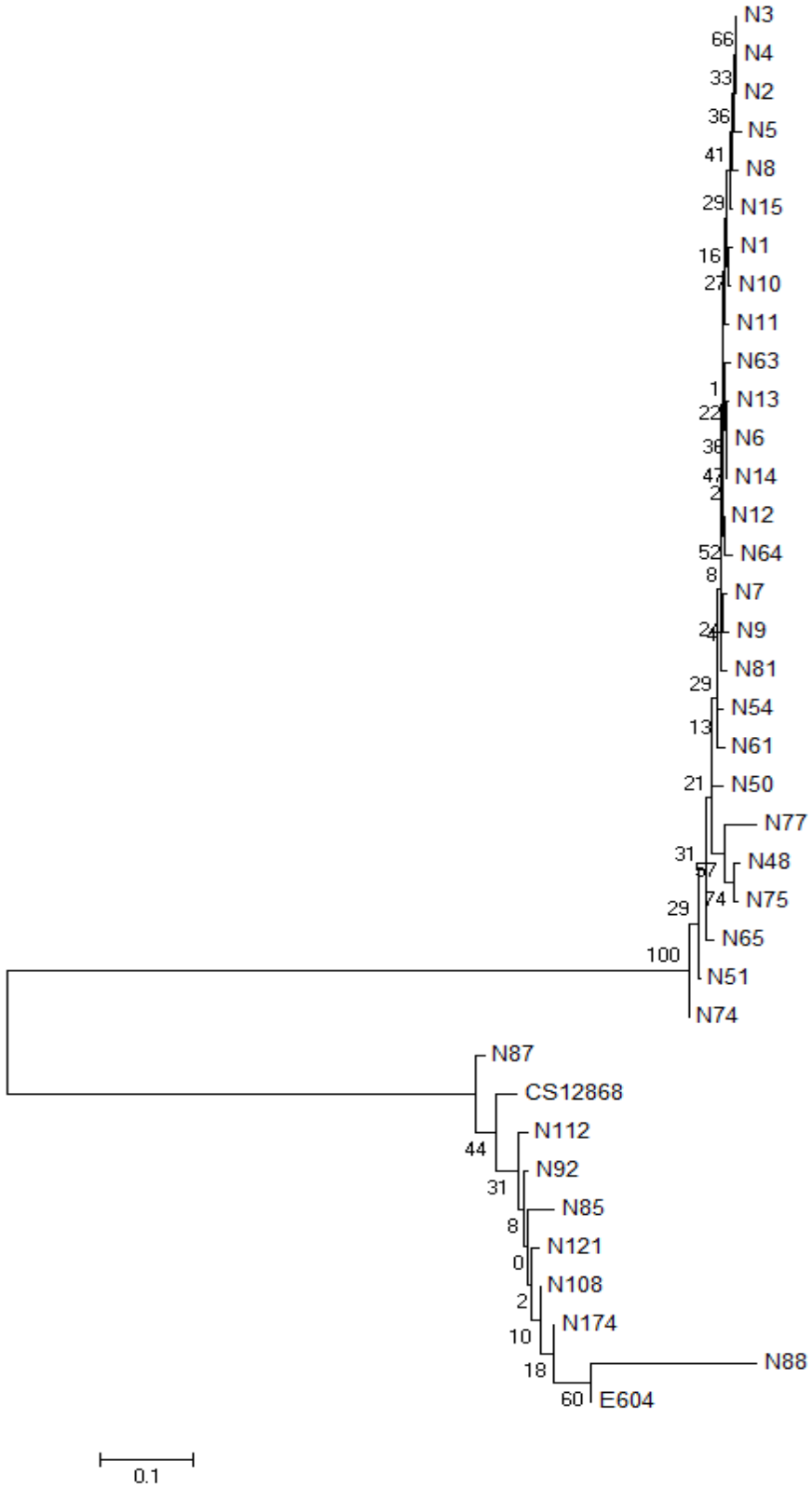

Figure 2.3: Phylogenetic tree inferred using Neighbour-Joining method for query and control ITS sequence.

The antibiotic susceptibility of *Cronobacter* spp. isolates to eight antibiotic derivatives is shown in Figure 2.4. All the 45 *Cronobacter* isolates obtained in this study were resistant to bacitracin, clindamycin, kanamycin, penicillin-G, tobramycin and vancomycin. Al-Nabulsi et al. [2009] also reported the resistance of *Cronobacter* spp. to glycopeptides (vancomycin) and coumarin-glycosides (novobiocin) derivatives. Majority of the *Cronobacter* isolates were resistant to β-lactam derivatives (68%), macrolides (88.6%) and aminoglycosides (79.9%). Stock and Wiedemann [2002] also reported the similar trend of resistance for *Cronobacter* spp. towards these antibiotics. Terragno et al. [2009] outlined the susceptibility of *Cronobacter* spp. to amoxicillin, ampicillin, chloramphenicol, gentamicin and tetracycline. Kim et al. [2008] summarized the susceptibility of *Cronobacter* spp. to tetracycline and resistance towards ampicillin or cephalothin. Our results also indicate that *Cronobacter* spp. exhibited moderate susceptible response towards phenicoles including chloramphenicol (31.6%). *Cronobacter* spp. showing tetracycline and chloramphenicol susceptibility were reported to be found at a higher ratio by other investigators [Kim et al. 2008; Nazarowec-White & Faber 1999; Stock & Wiedemann 2002]. *Cronobacter* spp. infections are conventionally treated with ampicillin-chloramphenicol or ampicillin-gentamicin [Drudy et al. 2006; Lai 2001]. However, ampicillin-chloramphenicol or gentamicin-resistant *Cronobacter* spp. have appeared because of the production of β-lactamases, acquisition of transposable elements and presence of multiple antibiotic resistance operons [Girlich et al. 2001; Pitout et al. 1997].

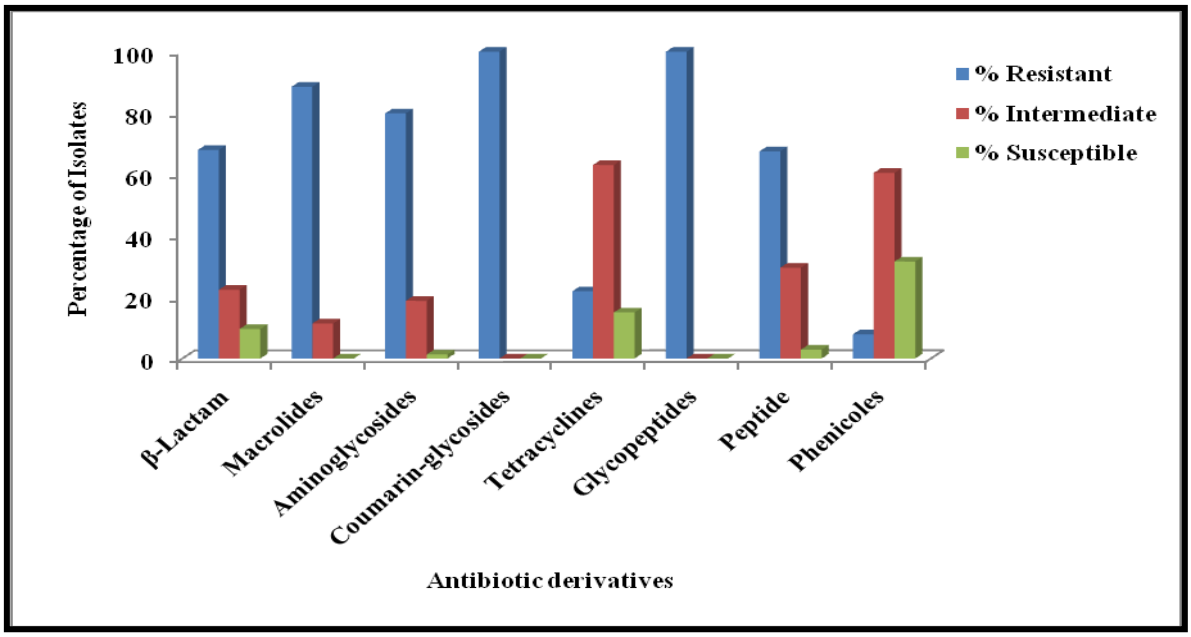

Figure 2.4: Antibiotic susceptibility of *Cronobacter* spp. by agar disc diffusion method

2.5 Conclusions

Cronobacter spp. is ubiquitous in nature, and foods of plant origin appear to be one of the most possible natural reservoirs of this pathogen. From our findings, a single method of identification of this pathogen is not sufficient to confirm its presence. Therefore, a combination of confirmation methods is necessary to completely eliminate false positives and false negatives. The antibiogram studies indicate emergence of resistance towards commonly used drugs emphasising the need on preventing the resistance and to track the resistance pattern.

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CHAPTER 3

Prevalence and profiling of virulence determinants in *Cronobacter sakazakii* isolates from different plant and environmental commodities

3.1 Abstract

Cronobacter sakazakii is an emerging pathogen causing meningitis, sepsis and necrotizing entero*coli*tis in neonates. The present study describes the profiling of nine virulence associated genes in different isolates of *C. sakazakii* derived from different plant based materials and environmental commodities. Of all the studied strains, the majority of the isolates were able to utilize inositol and sialic acid as determined by biochemical assays. Moreover, the strains exhibited β-hemolysis and displayed siderophore production and chitinase activity. Among the virulence associated genes, *hly* gene coding for hemolysin was detected in all the isolates followed by *ompA* (outer membrane protein); however, plasmid-borne genes were detected at a level of 60% for both *cpa* (Crononacter plasminogen activator) and *eitA* (Ferric ion transporter protein) gene, respectively. Furthermore, the strain *C. sakazakii* N81 showed cytotoxic effect with human Caco-2 cells. These results strongly suggest that *hly* and *ompA* could be probably one of the most prevalent virulence markers of *C. sakazakii*. The bearing of these virulence determinants indicates the pathogenic potential of C. *sakazakii* with their plausible connection with clinical manifestations.

3.2 Introduction

Cronobacter sakazakii is an extremely virulent food-borne pathogen that has been implicated in outbreaks of neonatal infections through the consumption of powdered infant formula (PIF) [Iversen et al. 2008; Singh et al. 2015a]. The genus *Cronobacter* consists of seven species, only *C. sakazakii*, *C. malonaticus*, and *C. turicensis* were related with neonatal infections and *C. sakazakii* was the species isolated/occurred more frequently in neonates [Jackson et al. O Holý & Forsythe 2014; Joseph et al. 2012; Kucerova et al. 2010; Muller et al. 2013]. Although the incidence rate is quite low, the mortality rate is high ranging from 50-80% [Healy et al. 2010]. Among different biotypes, *C. sakazakii* clonal complex 4 (CC4) is predominantly linked with neonatal meningitis, but specific virulence traits have not been established till date compared to other sequence types [Forsythe et al. 2014]. Numerous studies explored the mechanism of contagion and the virulence factors of this pathogen after realizing the threat associated with *C. sakazakii*. Outer membrane proteins (*OmpA* and *OmpX*) are one of the best characterized virulence factors and are described to play a substantial role in the adhesion / invasion of C. *sakazakii* to Caco-2 and INT-407 cells [Townsend et al. 2007; Nair et al. 2009; Mittal et al. 2009]. Further studies have shown that *C. sakazakii* exploits both microfilaments and microtubules to invade and translocate across human intestinal epithelial cells as well as endothelial cells mimicking the potential path of meningitis [Giri et al. 2011]. The role of different putative virulence factors such as surface structures (lipopolysaccharides (LPS) and exopolysaccharide (EPS) and flagella) in addition to enterotoxin production, proteolytic enzymes, have been extensively reviewed by Singh et al. [2015a].

A diverse assortment of virulence factor genes, including those for antibiotic resistance, toxins, adherence factors, and secretion systems (types 3, 4, and 6) have been found to be encoded on bacterial plasmids [Johnson et al. 2009; Juhas et al. 2008; Nazarowec-White & Farber 1997]. A plasmid-borne outer membrane protease *cpa* (*Cronobacter* plasminogen activator) is described to be entailed in *C. sakazakii* defense against the complement-dependent serum killing and efficient invasion by triggering the conversion of plasminogen to plasmin [Franco et al. 2011a; Schwizer et al. 2013]. Furthermore, plasmid-borne iron-acquisition systems, such as ABC ferric-iron transporter *eitABCE* and aerobacterin-like siderophore (cronobactin), might facilitate the pathogen in obtaining iron in an extremely iron-restricted environment, such as that of a host.

Cronobacter spp. has been portrayed as an emergent pathogen; therefore it is significant to scrutinize the genes involved in the pathogenesis of this bacterium. In order to understand the pathogenic process of *C. sakazakii* infections, this study was designed to identify the putative virulence factors produced by this pathogen. So far the data on the virulence properties of *C. sakazakii* isolated from Indian commodities is still not known.

3.3 Materials and Methods

3.3.1 Cultures and culture conditions

The multi locus sequence typing (MLST) analysis confirmed five *C. sakazakii* isolates (N13: *fus*A 8; N14:*fus*A 8; N15:*fus*A 18; N81:*fus*A 141 and N112:*fus*A 1) out of 38 *Cronobacter* spp. which were previously identified by 16S rRNA were further confirmed by rpoB species specific PCR assay [Singh et al. 2015b; Stoop et al. 2009] (Table 3.1). The standard strains, *C. sakazakii* ATCC 12868 and E604 (kindly gifted by Dr. Ben Davies Tall, FDA, USA) were used. All the isolates were maintained in Tryptone Soy Broth (TSB) supplemented with 40% glycerol and stored at -80°C. To determine the cytotoxicity of the strain, Caco-2 cells were obtained from National Centre for Cell Science, Pune, India. The Caco-2 cells (ATCC 7348406) were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Gibco, USA) supplemented with 10% of fetal bovine serum and 100 U/ml of streptomycin and penicillin [Dupre et al. 2003]. The cells were maintained at 37°C in a 5% CO_2 /95% air atmosphere.

3.3.2 Virulence determinants through biochemical assays

3.3.2.1 Inositol Fermentation test

Overnight grown *C. sakazakii* isolates in TSB was centrifuged and the cell pellet was suspended in a carbohydrate basal medium (Peptone 10%; Sodium chloride 5%; Phenol red 0.018%) with inositol (1mg/ml) and phenol red as indicator. A positive response was shown by a change in color from reddish to yellowish.

3.3.2.2 DNase activity

DNase test was performed by culturing the isolates on DNase agar for 24 h at 37°C and pouring 2 ml of 1 N HCl after incubation [Smith et al. 1969]. Only strong DNase activities displaying clear zone around growth were taken as positive.

3.3.2.3 Hemolytic activity

All the overnight grown isolates in TSB were tested for hemolytic activity on sheep blood agar plates (Himedia Laboratories, India). Overnight grown cultures were streaked on agar plates and incubated at 37^{o}C for 24 h [Keller et al. 1988]. Hemolysis was recorded as α-hemolysis, β-hemolysis, and negative (no hemolysis).

3.3.2.4 Siderophore detection

Siderophore production was determined using the chrome azurol S (CAS) agar (Himedia Laboratories, India) well diffusion assay [Schwyn & Neilands1987; Shinet al. 2001]. The wells in CAS agar plate were filled with overnight grown test culture. After incubation at 37°C for 48 h, the appearance of an orange halo around a well indicated siderophore production.

3.3.2.5 Chitinase test

Preparation of colloidal chitin: Colloidal chitin was prepared from the chitin (Himedia Laboratories, India) by the modified method of Hsu and Lockwood [1975]. In brief, chitin powder (40 g) was slowly added with 600 ml of concentrated HCl and kept for 60 min at 30^{o}C with vigorous stirring. The chitin was precipitated as a colloidal suspension by adding it slowly to 2 L of distilled water at 4-10 ^{o}C. The suspension was collected by filtration with suction on a coarse filter paper and washed by suspending it in about 5 l of distilled water. The washing was repeated 3 times until the pH of the suspension reached 3.5. After the above treatment, the colloidal chitin was used as a substrate. For screening of chitinase producing bacteria, the agar medium amended with colloidal chitin was used. The medium consisted of (g /L): Na_2HPO_4, 6; KH_2PO_4, 3; NH_4Cl, 1; NaCl, 0.5; yeast extract, 0.05; agar, 15 and colloidal chitin 1% (w/v). The screening was performed with streaking of bacterial isolates on the colloidal chitin agar medium incubated at 37^{o}C [Kaur et al. 2012]. Bacterial isolates were selected on the basis of hydrolysis zone on a creamish background after 96 h of incubation.

3.3.2.6 Utilization of sialic acid

All overnight *C. sakazakii* cultures were cultivated at 37^{o}C in M9 minimal media supplemented with 1 mg/ml of *N*-acetylneuraminic acid (Sialic acid) [Joseph et al. 2013]. The growth of each isolates was monitored by measuring the absorbance at 595 nm using a 96-well plate reader (Thermo Scientific, USA)

3.3.3 DNA extraction and Plasmid isolation

The isolates were retrieved from frozen culture stocks and cultured overnight at 37°C in TSB. An aliquot of 2 ml of each bacterial culture was used for DNA extraction using phenol:chloroform:iso-amyl alcohol method as described by Sambrook et al. [1989]. The DNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific, USA). In parallel, plasmids were isolated from a 3 ml aliquot from each broth culture by the method of Sambrook et al. [1989]. The genomic and plasmid DNA were visualized in 0.8 and 1.2% agarose gels by electrophoresis run at 90 V for 1 h. The detection of plasmids was confirmed using the *repA* targeted PCR assay as described by Franco et al. [2011b] (Table 3.1).

Table 3.1: Details of primer pairs and PCR running conditions used in this study.

S. No	Primer	Sequence 5' to 3'	Region	Amplicon	PCR running conditions
For Molecular Identification					
1.	*Csak*F *Csak*R	ACGCCAAGCCTATCTCCGCG ACGGTTGGCGTCATCGTG	rpoB	514 bp	96°C for 1 min; 30 cycles of 96°C for 1 min, 58°C for 1 min, 72°C for 2 min, 72°C for 5 min.
2.	*fus*A	GCTGGATGCGGTAATTGA CCCATACCAGCGATGATG	*fus*A	438 bp	96°C for 1 min; 30 cycles of 96°C for 1 min, 58°C for 1 min, 72°C for 2 min, 72°C for 5 min.
For Detection of Plasmids					
1.	InF2F InF2R	GAGACTGGCAGACCCGGCATA TGCGTTCACGAGCTGCCTGG	*repA*	398 bp	94°C for 3 min, 30 cycles 59°C for 30 s/72°C for 40s, 72°C for 5 min
2.	H1F H1R	CGCCTCAGTGTGTTCACTCCG TAGCTCCCATAGACTCGGATC	*repA*	551 bp	94°C for 3 min, 30 cycles 52°C for 30 s/70°C for 60s, 72°C for 5 min
3.	F1B2F F1BR	GATGCGCCTTGGCCTGTTTGT GCACAGCTTCACGAACTCCAC	IncF1B	264 bp	94°C for 3 min, 30 cycles 56°C for 30 s/72°C for 30s, 72°C for 5 min

3.3.4 Detection of virulence associated genes by PCR

The PCR was conducted in individual reactions using primers for the following nine genes: *omp* (outer membrane protein), *hly* (hemolysin), *cpa* (*Cronobacter* plasminogen activator), *chiF* (chitinaseF), *eitA* (iron acquisition gene), BAM (zinc containing metalloprotease), *sip* (siderophore), *fhaB* (filamentous hemagglutinin) and *iucC* (iron acquisition gene) that are reported to be associated with the pathogenesis [Jaradat et al. 2009; Franco et al. 2011b; Cruz et al. 2011; Yan et al. 2011]. The PCR was performed with 1 µl of DNA, 2.5 µl of 10× PCR buffer, 0.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTP (Promega) mix, 1 µl each of 10 pM primers, 0.25 µl of 5 U Taq DNA polymerase (Intron) and the final volume was made up to 25 µl using nuclease free water. The PCR mixture without DNA was used as negative control. The amplified products were analyzed in 1.5% agarose gel along with 1 kb DNA marker (Gene Ruler–Fermentas) by staining with ethidium bromide. The amplified product was visualized under UV transilluminator. The details of the primers and PCR conditions used the study are listed in Table 3.2.

Table 3.2: Details of virulence genes primer pairs and PCR running conditions used in this study.

S.No	Primer name	Sequence 5' to 3'	Targeted site	Amplicon size	PCR running conditions
1.	*ESSF* *ESSR*	GGATTTAACCGTGAACTTTTCC CGCCAGCGATGTTAGAAGA	*ompA*	469 bp	94 °C for 2 min; 30 cycles 94°C for 15 s each; 58°C for 15 s; 72°C for 30 s; 72°C min for 5 min
2.	*hly F* *hlyR*	CTAGGGTAACGGACTGTCACAGAT CTAGGAAGAAGCGTAAGCGTCTGA	ESA_00432 409290–410170	880 bp	94 °C for 3 min; 30 cycles at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min; 72 °C for 10 min
3.	*cpaF* *cpaR*	GACAACCCTGAGTTCTGGTAAC ATGCGTATTTCTGCTGGTAA	*cpa*	306 bp	94 °C for 2 min; 30 cycles 94°C for 30 s each; 56°C for 30 s; 72°C for 30 s; 72°C min for 5 min
4.	*sak_Chi_F* *sak_Chi_R*	ATGGCTACMAGYAAAYTRATYCAGGG CACCTGRTAGTTRTGVCCTTTCCAGC	*chi*	2.2 kb	95 °Cfor 2 min; 30 cycles at 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 2 min; 72 °C for 10 min
5.	*EitAF* *EitAR*	CCTTTTTCACGGCGTCGAGCTG TCTCTTCTGGTTCTCCAGCGCG	*eitA*	280 bp	94 °C for 3; 30 cycles 94°C for 60 sec each; 60°C for 30 s; 72°C for 30 s; 72°C min for 5 min
6.	*BAM122* *BAM123*	AWATCTATGACGCGCAGAACCG AAAATAGATAAGCCCGGCTTCG	*zpx*	350 bp	The hot start polymerase was activated by incubation for 15 min at 95°C; 30 cycles 95°C for 60 s each; 51°C for 60 s; 72°C for 60 s; 72°C min for 7 min
7.	*sip F* *sip R*	CTAGGCAAAAGAATCGACAAAGGG CTAGGTTGTTGTCTTTATCCGTTC	ESA_03485 c3431208–3432142	934 bp	94 °C for 3 min; 30 cycles at 94 °C for 30 s, 57 °C, for 30 s, and 72 °C for 1 min; 72 °C for 10 min
8.	*fhaF* *fhaR*	GTGGCGAACCCGTATGGCATTAC TGTCGCCTGCTATCTGCGCGTTG	*fhaB*	804 bp	94 °C for 3 min; 30 cycles 94°C for 15 sec each; 60°C for 15 s; 72°C for 30 s; 72°C min for 5 min
9.	*IucCF* *IucCR*	TGCAGTGCCTGATGTCAGGCCAT ACGCCAAACATCTCCTGATAGCG	*iucC*	660 bp	94 °C for 2; 30 cycles 94°C for 15 sec each; 60°C for 15 s; 72°C for 30 s; 72°C min for 5 min

3.3.5 Cell infection assay

Among all the *C. sakazakii* isolates, isolate N81 demonstrated maximum association with virulence genes and was used for cell infection assay. The differentiated Caco-2 cells at late post-confluence were used for infection assays (15 days post-seeding). When confluent growth was achieved, adhered cells were trypsinized, transferred to 24-well plates containing round glass slides and re-incubated. After 24 hours, the DMEM with antibiotics was replaced by DMEM supplemented with 2% of fetal bovine serum. The *C. sakazakii* isolate N81 was previously grown overnight at 37°C in TSB, diluted in DMEM containing 2% of fetal bovine serum and added to each well (ca. 10^6 cfu/ml) containing the Caco-2 cells and incubation was done at 37°C. After 3 h of incubation, the infected monolayers were washed with PBS and incubated with 1 mL DMEM. The well plates were washed with Phosphate buffered saline (PBS), fixed with methanol, and stained with Hoescht dye 33258. The immunofluorescent staining and cell morphology were examined by fluorescence with filters for UV excitation and phase-contrast microscopy using an Olympus microscope BX53.

3.4 Results

3.4.1 Virulence determinants through biochemical assays

Our results revealed that among the tested isolates, all the isolates were capable to utilise inositol however, sialic acid utilization and DNase activity was observed in only three isolates, respectively (Table 3.3).

All the isolates were hemolytic on blood agar plates and possessed chitinase activity. Expression of siderophores using the CAS assay was seen in two of the five isolated strains. However, iron depletion studies were not performed with the two CAS-negative strains to determine if siderophore activity was inducible in these strains.

3.4.2 Profiling of virulence associated genes

The carriage of nine virulence associated genes coding for *ompA*, *hly*, *cpa*, *chiF*, *eitA*, *zpx*, *sip*, *fhaB* and *iucC* was studied on genomic as well as plasmid DNA in *C. sakazakii* isolates. Table 3.4 depicts the distribution and frequency of different virulence gene used in this study. All the *C. sakazakii* strains (n=5) in this research were confirmed positive for *hly* gene, while 80% strains were able to encode *ompA*. The *chiF* gene and BAM (*zpx*) were less frequent (40%) detected in the isolates.

The isolates were screen for the presence of plasmid-borne virulence gene using PCR probing. All the seven isolates possessed the pESA3 virulence plasmid, however, only the five Indian isolates possessed pESA2. The presence of gene for *eitA* and *cpa* was detected in 60% of isolates, respectively with the maximum presence in plant based products. It was observed that all the *C. sakazakii* strains possessed *iucC* gene and lack the presence of *sip* and *fhaB* virulence genes. In general, different isolates exhibited different virulence factors, but virulence showed no obvious correlations with the source of the isolates. The strain N81 exhibited the highest virulence, therefore, was selected for cell infection assay.

3.4.3 Cell infection assay

The N81 isolate resulted in massive disruption of the host cell with the release of cell contents (Figure 3.1). The Hoechst dye 33258 staining indicated nuclear condensation and fragmentation in Caco-2 cells. The phase contrast micrograph indicated the damage to the cell membrane and cell lysis.

Table 3.3: Virulence traits identified in *C. sakazakii* isolates

Isolate	Virulence determinants through biochemical assays					
	Inositol Fermentation	DNase activity	Hemolytic activity	Siderphore detection	Chitinase activity	Sialic acid utilization
E604	+	+	+	+	+	+
ATCC 12868	+	+	+	+	+	+
N13	+	+	+	-	+	+
N14	+	-	+	-	+	-
N15	+	-	+	-	+	-
N81	+	+	+	+	+	+
N112	+	+	+	+	+	+

Table 3.4: Profiling patterns of virulence genes observed for *C. sakazakii* isolates

Isolate	Plasmid			Detection of virulence associated genes by PCR								
	pESA3/ pCTU1	pESA2/ pCTU2	pCTU3	*omp*	*hly*	*cpa*	*chi*F	*eit*A	BAM (*zpx*)	*iuc*C	*sip*	*fhaB*
E604	+	-	-	+	+	+	+	+	+	+	-	-
ATCC 12868	+	-	+	+	+	+	-	+	+	+	-	-
N13	+	+	-	+	+	+	-	-	-	+	-	-
N14	+	+	-	+	+	-	-	+	+	+	-	-
N15	+	+	-	-	+	+	-	+	-	+	-	-
N81	+	+	-	+	+	+	+	-	+	+	-	-
N112	+	+	-	+	+	-	+	+	-	+	-	-

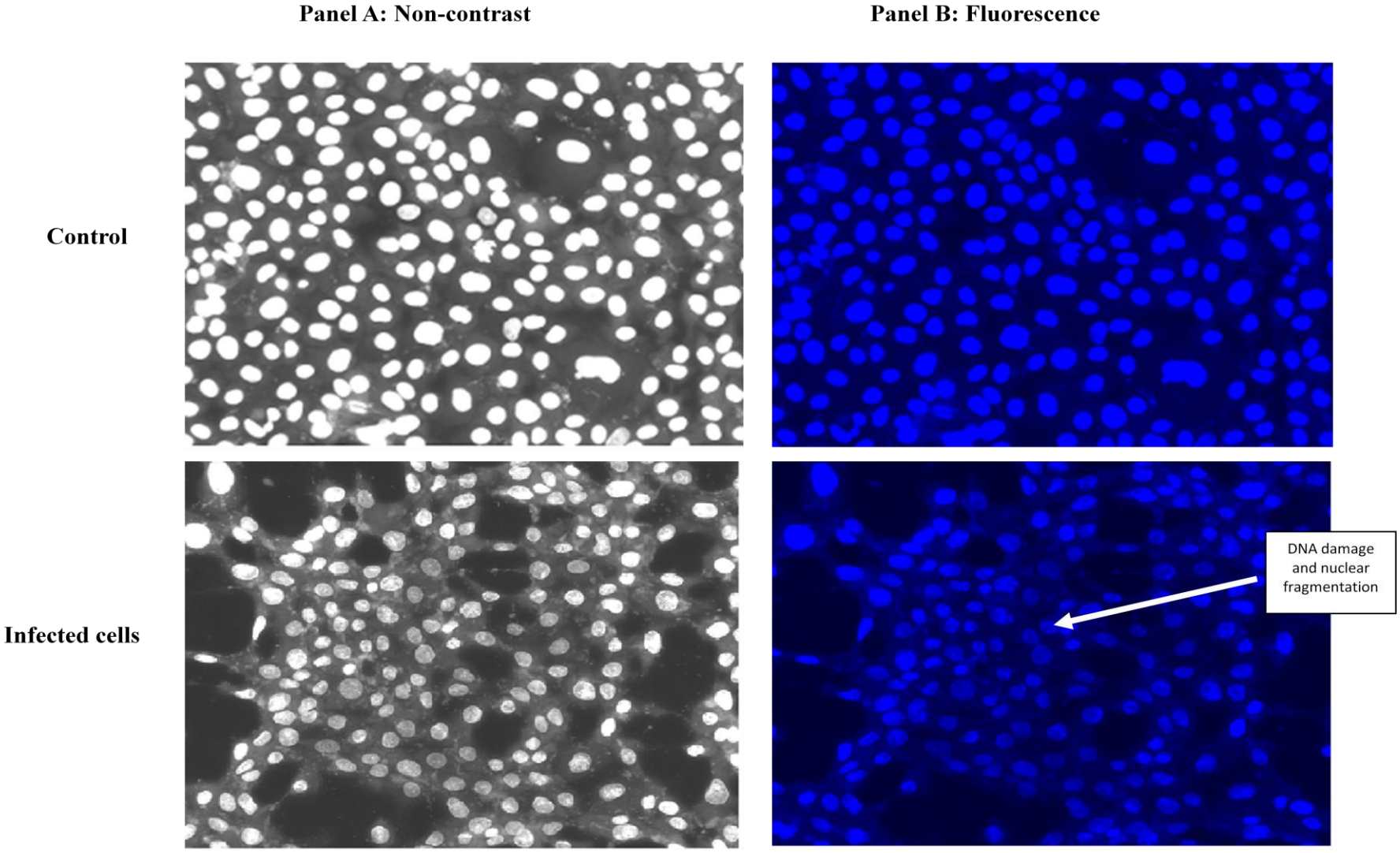

Figure 3.1: Cytotoxicity of *C. sakazakii* N81 in cells stained with Hoescht dye 33258 at 20X.

3.5 Discussion

The investigation of virulence factors and their interactions with the host is essential to understand the pathogenesis of any bacterial system. The virulence properties may also assist to decide on novel targets in drug and vaccine development [Wu et al. 2008]. To detect the virulence marker genes, almost all the established virulence associated genes were screened in the present investigation to obtain comprehensive data on presence of these genes among *C. sakazakii* isolates of Indian origin. The varying degree of virulence properties can be used to assess intra-species diversity and to establish epidemiological relationships [Davies et al. 2004].

Our results revealed that most of the isolates were positive for inositol fermentation. The presence of genes for inositol fermentation has been detected in two other meningitic strains of *C. sakazakii* (strains 701, 767) belonging to ST4 therefore recommended recently as a marker of pathogenicity for *C. sakazakii* based on the presence of the inositol monophosphatase gene (*suhB*) in various pathogenic strains [Hamby et al. 2011]. The inositol utilization operon GR29 has also been reported in *C. sakazakii* strains isolated from the environment [Grim et al. 2013]. The importance of hemolysins in virulence of bacterial pathogens has been extensively characterized. Reports about the production of hemolytic toxins by *C. sakazakii* are very scarce. In our study, the tested *C. sakazakii* isolates demonstrated both alpha and beta hemolysis.

The DNase activity which is an important criterion used to identify virulence characteristic of most of the pathogens usually involved in invasive infections [Sumby et al 2005; Buchanan et al. 2006]. The DNase is reported to interfere with the antimicrobial activity of neutrophil-produced extracellular traps (NETs) through the breakdown of the chromatin backbone as studied in infection of *Staphylococcus aureus* in a murine model [Latimer et al. 2012]. However, DNase is not entirely reliable as an indicator of pathogenicity.

Sialic acid is present in breast milk, infant formula, the mucin lining of the intestinal tract and is also a constituent of the brain ganglioside complex [Siqueira Santos et al. 2013]. Among the different species of *Cronobacter*, *C. sakazakii* is the only species that has the *nanAKT* gene cluster encoding utilization of exogenous sialic acid as a carbon source [Joseph et al. 2013]. It is likely that the ability of *C. sakazakii* to utilize sialic acid enhances its pathogenicity for neonates and young infants by producing EPS which makes it to survive against host immune system. This behaviour is probably exacerbated in the presence of different milk sources and their products, which also contain sialic acid or its precursors. In

addition, sialic acid may also help in colonizing the intestinal tract via interactions with host mucins [Joseph et al. 2012]. Grim et al. revealed the presence of two genomic regions (GR127 and GR129) that are involved in the utilization of sialic acid in the genome of *C. sakazakii* BAA-894 [Grim et al. 2013]. In our study, *C. sakazakii* isolates from plant-based products were found to utilize sialic acid emphasizing that powdered infant formula (PIF) manufacturers need to develop hygienic practices and maintain higher microbiological standards before incorporating plant based material in the food products.

The ability of the opportunistic pathogen *Cronobacter* spp. to cause meningitis is dependent upon its capacity to survive in blood and the subsequent encroachment of the cardinal neural system by breaking the blood-brain barrier. The majority of the *C. sakazakii* isolates were found positive for the *ompA* gene. Several research studies proposed that *ompA*, contributes extensively to the virulence potential of *C. sakazakii* by invasion of various epithelial and endothelial cells of human and animal origin [Mittal et al. 2009; Singamsetty et al. 2008; Nair & Venkitanarayanan 2007; Kim & Loessner 2008; Kim et al. 2010]. Mittal et al. [2009] reported that o*mpA*$^{+}$ *C. sakazakii* isolates breach blood-brain barrier and invade central nervous system (CNS) therefore it is imperative for the commencement of meningitis in a neonatal mice model. In addition to *ompA*, Kim et al. reported that *ompX* of *C. sakazakii* also played vital roles in the both apical and basolateral invasion of the host cells and can translocate into the deeper organs (spleen and liver) [Kim et al. 2010]. It was expected that this gene would be detected in all of the isolates due to its importance in cell invasion but few isolates were negative for *omp* gene. Nevertheless, not all *C. sakazakii* were reported to encode *ompA* using the primers described by Nair & Venkitanarayanan [2007], as observed by Jaradat et al. [2009] and Giri et al. [2011]. Now days, proteomics approach has also garnering attention to screen virulence factors and to recognize new diagnostic markers and therapeutic targets in pathogenic bacteria. To evaluate *Cronobacter*, Alzahrani et al. [2015] identified 18 outer membrane vesicles (OMV)-associated proteins by mass spectrometry in *C. sakazakii* strain 767 which may function in the activation of cytopathogenic and host cell responses on human intestinal epithelial cells. Another study by Ye et al. [2016] showed identification of potential virulence factors such as Dps, OmpA, and LuxS in virulent isolate (G362) and attenuated isolate (L3101) by using 2-DE technology coupled with MALDI/TOP/TOF mass spectrometry. The authors suggested that these proteins might be engrossed in pathogenicity of *C. sakazakii* through enhancing adherence/invasion to targeting tissues, resistance or tolerance to environmental stresses and host immunologic system.

The type III hemolysin, an integral outer membrane protein with hemolytic activity is reported to a potential virulence factor in several pathogens [Baida & Kuzmin 1996; Chen et al. 2004]. In our study, a majority of the isolates from plant-based products carried hemolysin genes, therefore it is suggested that individuals in the food production and food service industries, as well as those working in hospital settings should therefore be adequately informed on the risks of *Cronobacter* contamination and proliferation to enable the provision of products safe for consumption. Earlier, Cruz et al. [2011] reported the prevalence of *hly* gene in only *Cronobacter* spp. isolates mainly from clinical sources.

A plasmid borne putative gene for the plasminogen activator (*cpa*) was identified in the isolates from plant based products and environmental samples. The plasminogen activators are serine proteases, proposing that this gene could be engaged in exhibiting the maximum invasiveness of *Cronobacter* spp. Franco et al. [2011a] showed that *cpa*, expressed by *C. sakazakii* BAA-894, slowly cleaves plasminogen and increases survival in serum in comparison with a *cpa* deletion mutant. The low proteolytic activity of BAA-894 may be due to *cpa* inhibition by BAA-894 smooth lipopolysaccarides. Cruz et al. [2011] also identified this gene principally in *Cronobacter* spp. from human sources. Further research should be more focussed on establishment of the function of these genes in pathogenicity and host colonization. Although the previous literatures suggest that the serum resistance was linked to the presence of *cpa* gene, 40% of the strains used in the present study were negative for this gene. The results indicated that the resistance to serum is a property that not exclusively reliant on *cpa* gene, there might be other factors that may contribute to the resistance.

Chitinase is a putative virulence factor; and its expression has proven to be important not only for nutrient acquisition and environmental survival but also for infection in humans and animals [2011]. In our study, the isolates derived from plant-based products and environmental samples indicated the presence of chitinase activity. The role of chitinase has been already defined in infections caused by *Legionella pneumophila* [DebRoy et al 2006], *Listeria monocytogenes* [Larsen et al. 2010] and *Salmonella enterica* serovar *typhimurium* [Larsen et al. 2011].

The capacity of iron acquisition is mostly believed to be a requirement for a pathogen to establish infections when entering a host [Crosa & Walsh 2002]. In our study, *C. sakazakii* strains were tested for iron acquisition genes including *eitA* and *iucC* that could help in iron acquisition. The *eitCBAD* operon is found in several enteric pathogens, both chromosomal and plasmid borne [Johnson & Nolan 2009] which mediates the translocation of iron,

siderophores and heme [Koster 2001]. Franco et al. [2011b] demonstrated that plasmids pESA3 and pCTU1 in *C. sakazakii* contain two clusters of genes, a homologue of an ABC transport-mediated iron uptake and siderophore system (*eitCBAD* operon) and a siderophore-mediated iron acquisition system (*iucABCD/iutA* operon). The plasmid PCR profile showed that although the majority of strains harboured the *eitA* gene, however, the entire strains lack *iucC* gene. N81 was negative for both *eitA* and *iucC* whereas it was capable to produce iron siderophores with CAS agar demonstrating orange halo around the wells. This might be ascribed to the presence other siderophore-related genes that were not investigated in this work.

Bacterial metalloproteases are thought to play a role in disease by enhancing the dissemination of bacteria from local sites of infection into the systemic circulation [Miyoshi & Shinoda 2000]. The least presence of *zpx* in our isolates indicates that there must be some other mechanisms in Enterobacteriaceae family which might play role in systemic circulation. Adhesion and invasion to the host cell is a property that is associated with bacterial pathogenesis, especially of intracellular pathogens (e.g. *Salmonella* spp., *Shigella flexneri* and *Yersinia enterocolitica*) and non-intracellular pathogens (e.g., enterohemorrhagic *Escherichia coli* and uropathogenic *E. coli*) [Pizarro-Cerdá & Cossart 2006; Nicholson et al. 2009; Xicohtencatl-Cortes et al. 2009]. Our results indicated that the isolate N81 demonstrated the utmost ability to both adhere to and invade Caco-2 cells. The adhesion and invasion of *Cronobacter* spp. adhered to Caco2, HEp-2 and brain microvascular endothelial cells has been reported; however, the mechanism of binding to mammalian cells is not well understood [Mange et al. 2006]. Later on, Townsend et al. [2007] suggested that gene associated with superoxide dismutase might influence the intracellular persistence of *Cronobacter* spp.

3.6 Conclusion

The present work reports the virulence determinants in C. *sakazakii* isolates from different plant based and environmental sources in an attempt to determine the infective differences occurring at the source level**.** Our results revealed the maximum presence of *hly* followed by *ompA* and *eitA* which could be critical in the pathogenesis of enteric infections and in its systemic dissemination. A larger sample size of each source would be necessary to further address differences observed. These findings may help in explaining the diversity of the virulence traits in *C. sakazakii* which might contribute to the opportunistic nature of this pathogen.

3.7 References

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CHAPTER 4

Diverse profiles of N-acyl-homoserine lactones and biofilm formation in *Cronobacter sakazakii*

4.1 Abstract

This study was aimed to investigate biofilm formation and EPS composition by *C. sakazakii* isolates in order to elucidate the correlation between biofilm formation and EPS production. Further, the study also investigates and characterise the QS signalling molecules in *C. sakazakii*. The biofilm study among *C. sakazakii* isolates was conducted using standard microtitre plate assay and the isolates were ranked based in specific biofilm forming ability. The EPS was extracted and characterised using FT-IR. The key molecules involved in quorum sensing were extracted and characterized using FT-IR, HPLC and LC-HRMS. A higher Specific Biofilm Formation (SBF) index (p <0.05) with the presence of genes associated with cellulose biosynthesis (*bcsA*, *bcsC* and *bcsG*) was observed in the strains. The presence of long chain AHLs as quorum sensing molecules was determined by bioassay using *A. tumefaciens* NTL4(pZLR4) biosensor strain. The FT-IR and HPLC analysis of extracted AHLs indicated the presence of lactone ring, N-H bond and C-O bonds in the AHL molecules. Further, the LC- HRMS analysis established the presence of N-undecanoyl-L-AHL, N-dodecanoyl-L-AHL, N-tetradecanoyl-L-AHL, N-pentadecanoyl-L-AHL, N-(β-ketocaproyl)-L-AHL, N-octanoyl-L-AHL, N-3-oxo-octanoyl-L-AHL, N-octadecanoyl-L-AHL as key signaling molecules among *C. sakazakii* isolates. The study demonstrated that the production of AHLs as QS signal molecules involved in biofilm formation and may act as a new virulence marker of *C. sakazakii* and can be considered as futuristic potential drug targets towards eradication of biofilms of *C. sakazakii* from food processing environments.

4.2 Introduction

Cronobacter sakazakii is a wide-spread opportunistic pathogen that can form biofilms on different substrates, creating food safety risk, consequently achieving the interest of public health authorities and researchers in different countries [Singh et al. 2015a]. The International Commission on Microbiological Specifications for Foods [ICMSF 2002] established *C. sakazakii* as a ‘‘severe hazard for restricted populations, life-threatening or substantial chronic sequelae of long duration’’. The organism is ubiquitous being detected from a broad spectrum of food and food ingredients of animal and plant origin reviewed by [Singh et al. 2015b]. Contamination of powdered infant formula (PIF) has been linked with severe systemic neonatal infections by *C. sakazakii* [Healy et al. 2010]. Most of the pathogenic bacteria form multicellular biofilm communities on biotic and abiotic surfaces has been suggested as a way of survival for numerous food-associated organisms such as *Listeria monocytogenes* and *Escherichia coli* [Kumar & Anand 1998; Norwood & Gilmour 2000; Frank 2000; Williams & Braun-Howland 2003; Ryu & Beuchat 2005]. Formation of these sessile communities and their inherent resistance to antimicrobial agents is the root of many persistent and chronic bacterial infections. According to a public announcement from the US National Institutes of Health more than 80% of all microbial infections involve biofilms [Ren et al. 2005; Rasmussen & Givskov 2006]. Various factors such as flagella, fimbriae, outer membrane proteins, curli (proteinaceous surface structures), and extracellular polymeric substances (EPS) are involved in biofilm formation [Pratt & Kolter 1998]. The EPS plays a substantial part in determining the architecture of a biofilm with cellulose frequently. It has been distinguished as one of the components of EPS in biofilms formed by members of the Enterobacteriaceae [Zogaj et al. 2003; Solano et al. 2002].

Bacterial processes involved in formation of biofilms, production of EPS, virulence and bioluminescence, are mediated by quorum sensing (QS). QS is a cell-to-cell signalling often mediated by the production, release and detection of signaling molecules, the autoinducers (AI). The detection and identification of QS molecules can bestow an idea about the density of population, type of community and expression of virulence components of the infecting pathogen. Besides, these QS regulatory mechanisms are also being suggested as a novel target for developing advanced schemes to prevent infections [Rasmussen & Givskov 2006]. Earlier, Lehner et al. reported the ability of C. *sakazakii* to produce acyl homoserine lactone (AHL) and reported 3-oxo-hexanoyl-AHL and 3-oxooctanoyl-AHL as AHL molecules using

thin layer chromatography (TLC) [Lehner et al. 2005]. Nevertheless, isolation and full chemical characterization of these molecules are escaping. Therefore, this study was directed to investigate biofilm formation and EPS composition by *C. sakazakii* isolates in order to elucidate the correlation between biofilm formation and EPS production. Further, the study also investigates and characterise the QS signalling molecules in *C. sakazakii*.

4.3 Materials and Methods

4.3.1 Bacterial strains

The confirmed *C. sakazakii* isolates derived from 219 samples from food, medicinal plants, herbs and spices, environmental sources and clinical samples were investigated for the present study [Singh et al. 2015b]. The standard strains used were *C. sakazakii* ATCC 12868 and E604 (kindly gifted by Dr. Davis Tall Ben, FDA, USA). All the *C. sakazakii* isolates were cultured aerobically at 37°C in tryptic soy broth medium (TSB) which consisted of following ingredients (g/L) of casein peptone 15.0, soy peptone 5.0, sodium chloride 5.0, and agar 15.0. The two biosensors strains of *Chromobacterium violaceum* CV026 (kindly gifted by Dr. Paul Williams, University of Nottingham) and *Agrobacteruim tumefaciens* NTL4(pZLR4) (kindly gifted by Dr. Stephen K Farrand, University of Illinois, US), were used for the detection of AHLs. The *C. violaceum* CV026 was grown in Luria Bertani broth (LB) supplemented with 100 μg/mL ampicillin and 30μg/mL kanamycin. *C. violaceum* CV026 served as AHL biosensor with formation of purple violacein pigment in presence of short chain exogenous AHLs molecules [McLean et al. 1997]. The *A. tumefaciens* NTL4 (pZLR4) was utilized to detect AHLs with long acyl chains. This strain carries the plasmid pZLR4, which contains a *traG::lacZ* fusion and *traR*. In the presence of AHLs with long acyl chains the TraR protein is activated, transcription of the *traG::lacZ* fusion is turned on, and LacZ (β-galactosidase) activity can be used as a reporter of *traG* transcription [Cha et al. 1998]. *A. tumefaciens* NTL4(pZLR4) was kept in NB medium containing gentamicin (50 µg/mL) at 28°C for 24 h. Culture was preserved in the form of glycerol stock and was revived whenever required.

4.3.2 Quantification of Biofilm Formation

Biofilm formation was determined by the ability of cells to adhere to the base of 96-well polystyrene plate using the method of Boddey et al. [2006] with slight modifications. All isolates were grown overnight in TSB medium at 37^{o}C. Briefly, 230 µl of TSB medium was

added into each well of sterile 96-well polystyrene plate followed by the addition of 20 µl of overnight bacterial culture. After incubation at 37^{o}C for 48 h, the 96-cell plates were rinsed 3 times with deionized water and the adherent bacteria cells were stained with crystal violet (1.0%, w/v) for 15 min. Then, the crystal violet was liberated by glacial acetic acid (33%, v/v) following 30 min incubation. The sterile TSB was used as negative control and the OD values of each well (negative control and tested strains wells) were measured at 570 nm using Go skan microplate reader (Thermo, USA). The average value of the optical density of the negative control wells was subtracted from the values of each test well, and this difference was referred as OD_{570} nm for the biofilm-forming ability of *C. sakazakii* strains.

The extent of biofilm formation was measured by applying three different formulas: (i) BF = AB) CW, where BF is the biofilm formation, AB is the OD_{540nm} of stained attached bacteria and CW is the OD_{540nm} of stained control wells containing bacteria-free medium only (unspecific or abiotic factors) [Kadurugamuwa et al. 2003]; (ii) BF = AB ⁄CW [Soto et al. 2006]; and (iii) SBF = (AB) CW)⁄G in which SBF is the Specific Biofilm Formation index and G is the OD_{620nm} of cells growth in suspended culture [Niu et al. 2004].

4.3.3 Calcofluor binding assay

Calcofluor binding assays were used for the expression of cellulose. In brief, colonies were cultured on LB agar plate overnight at 37^{o}C, and inoculated onto LB agar supplemented with 200 mg/ml calcofluor (fluorescence brightener 28; Sigma, St. Louis, MO), and incubating at 37^{o}C for 48 h [Hu et al. 2015]]. The fluorescence was checked under a UV light. All strains were tested in duplicate in each assay, and the assay was repeated at least three times. The images of colonies on plates were taken by a Bio-Rad digital camera.

4.3.4 Detection of genes involved in biofilm formation by PCR method

The DNA was extracted using phenol:chloroform:iso-amyl alcohol method as described by Sambrook et al. [1989]. The PCR was performed with 1 µl of DNA, 2.5 µl of 10× PCR buffer, 0.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTP (Promega) mix, 1 µl each of 10 pM primers, 0.25 µl of 5 U Taq DNA polymerase (Intron) and the final volume was made up to 25 µl using nuclease free water. The amplified products were analyzed in 1.5% agarose gel along with 100 bp DNA marker (Gene Ruler–Fermentas) by staining with ethidium bromide. The amplified product was visualized under UV transilluminator. The details of the biofilm forming genes and sequences of the oligonucleotide primers are listed in Table 4.1 [Hartmann et al. 2010].

Table 4.1: PCR primers used in this study.

S. No.	Primer name	Sequence 5' to 3'	Amplicon size	PCR running conditions
1.	*bcsa*-F *bcsa*-R	AAGAAGAGTACGTGGACTGGGTGA CGCCGAGGATAATCAGGTTGTAG	171	94^{o}C/3min, 30 cycles 94^{o}C/2min, 59.5^{o}C/30 sec,72^{o}C /45sec,72^{o}C /5min
2.	*bcsg*-F *bcsg*-R	GACGGGCTATCTGAATTTCCAC GCCAGGTATCATGCCAGAACA	146	94^{o}C/3min, 30 cycles 94^{o}C/2min 58.7^{o}C/30 sec,72^{o}C /45sec,72^{o}C /5min
3.	*bcsc*-F *bcsc*-R	AGATTTGAGCGGTTATTCTTTAGGC TCGGTCTTCGTGCGGAGTG	156	94^{o}C/3min, 30 cycles 94^{o}C/2min 58.7^{o}C/30 sec,72^{o}C /45sec,72^{o}C /5min
4.	*flgj*-F *flgj*-R	TCAGGTGCCGATGAAGTTTG GCCCTTTCCAGGACGATGT	312	94^{o}C/3min, 30 cycles 94^{o}C/2min 58.7^{o}C/40 sec,72^{o}C /45sec,72^{o}C /5min
5.	*flhe*-F *flhe*-R	CATTACTGACGCTGCCTGTCC GTAGTGCCCGTCTGGTCTTCC	256	94^{o}C/3min, 30 cycles 94^{o}C/2min 58.2^{o}C/30 sec,72^{o}C /45sec,72^{o}C /5min
6.	*fild*-F *fild*-R	ATCGAGATCGAGCGTTCCAC CGCCCTTATCAACTTTGACGTATT	211	94^{o}C/3min, 30 cycles 94^{o}C/2min 58.7^{o}C/30 sec,72^{o}C /45sec,72^{o}C /5min

4.3.5 Characterization of Extra polymeric substances (EPS)

4.3.5.1 Extraction of EPS

The EPS was extracted by the modified procedure of Onbasli and Aslim [2009]. Five ml of cultured cells were subjected to centrifugation at 15,000 g for 5 min and boiled for 15 min at 100^{o}C. After keeping at room temperature for 20 min, 100 ml of trichloroacetic acid solution (TCA) was added and then kept for incubation at 37^{o}C for 1 h. The mixture was kept in ice water for 30 min and centrifuged at 15,000 g for 20 min. The supernatant containing EPS was pooled, after which an equal volume of ethanol was added. The mixture was kept at -20^{o}C for 1 h and then centrifuged at 15,000 g for 20 min again. The precipitate was then washed two times using 95% ethanol and centrifuged at 15,000 g for 20 min. The final precipitate was dissolved in 1 mL of deionized distilled water and stored at -20 ^{o}C.

4.3.5.2 EPS analysis

Once the EPS was extracted, the resulting EPS suspension (resuspended in 1 mL deionised water) was evaluated for the total amounts of carbohydrate content and protein using Phenol-Sulfuric Acid (PSA) method [Dubois et al. 1956] and Bradford method [1976], respectively. For Fourier transform infrared (FTIR) spectroscopy of EPS, the ethyl acetate extracts (100 µl) of culture supernatant were evaporated in vacuum. The infrared absorption spectra of the powder were measured using a FT-IR ((Agilent Cary 630 FTIR Spectrometer) in the 400-4000 cm^{-1} frequency range, using KBr as the reference for the detection of C=O bonds and -OH bonds [Mancuso et al. 2004].

4.3.6 Screening and characterization of AHL

4.3.6.1 Well-diffusion assay

The isolates to be tested for quorum signal production were overlaid with CV026 on a LB agar plate in parallel to the monitor strain [Mukherji & Prabhune 2015; Ravn et al. 2001]. The testing for AHL production against *A. tumefaciens* NTL4(pZLR4) was done in a similar assay supplementing the agar with 50 mg/ml X-gal. Plates were incubated for 24 h at 28 ^{o}C and violacein production and blue halo around colony read as an AHL-positive response of *C. violaceum* and *A. tumefaciens* NTL4(pZLR4) respectively.

4.3.6.2 Test tube assay

A 500 µl of overnight broth culture of *A. tumefaciens* NTL4(pZLR4) was added to 10 ml of NB medium along with 1ml of *C. sakazakii* isolates and kept for overnight incubation at 28^{o}C. Then, 2 ml of culture medium from each test tube was centrifuged at 15,000 g for 10 min to pellet down the cells. The cell pellet was solubilised in 2 ml of dimethyl sulfoxide (DMSO), vortexed and centrifuged at 15,000 g for 10 min to separate the cells. Absorbance of the supernatant containing blue pigment was measured at a wavelength of 630 nm using DMSO as the blank.

4.3.6.3 Kinetics of AHL production in *C. sakazakii* isolates

For studying the AHL production and kinetics of *C. sakazakii* strains, cultures were grown in TSB medium at 37°C with aeration. The cultures were taken at regular intervals from 0 h to 24 after inoculation. Growth was monitored by optical density at 600 nm and the AHL production was indicated with *A. tumefaciens* NTL4(pZLR4).

4.3.7 Preparation of AHL extracts

The extraction of AHLs was done as described by Shaw et al [1997]. The isolates were cultured in 500 mL of TSB with continuous shaking at 200 rpm at 37 ^{o}C for overnight. The supernatant of early stationary-phase culture was extracted twice with equal volumes of acidified ethyl acetate (0.1% formic acid). The organic phases were combined, dried over anhydrous magnesium sulfate, and evaporated to dryness by rotary evaporation at 37 ^{o}C. The residue was resuspended in 500 µl of acidified ethyl acetate and stored at -20 ^{o}C before use. Aliquots (100 µL) of the extract were withdrawn from the top layer and placed in sample vials for HPLC and mass spectrometry analysis.

4.3.8 Fourier transform infra-red (FTIR) spectroscopic analysis of AHL

In order to confirm the functional groups, FTIR spectroscopic analysis of extracted AHL was performed [Tagadoshi et al. 2015]. Analysis was done using Agilent Cary 630 FTIR spectrometer over the spectral range of 4,000.00 - 650.00. Sample was prepared by making a pellet in potassium bromide (KBr). FTIR Spectral data obtained was plotted on a graph of transmittance (%) vs. wave number (cm-1).

4.3.9 High Performance Liquid Chromatography of AHL

Synthetic 3-oxo-C8-AHL was considered as standard and initially it was analysed on Hitachi Chromeline HPLC system using Thermo Scientific C18 reverse phase HPLC column 250 X 4.6 mm [Teplitski et al. 2003]. The mobile phase used was mixture of acetonitrile (ACN) and deionized water in the ratio 1: 99,30: 70, 50:50,70:30 for 15 min each followed by ratio 99:1 for 30 min. The flow rate was maintained at 0.5 ml per minute, and column temperature was maintained at 37 °C. Above method was used to analyse extracted AHL and was detected using UV-detector system at 219 nm.

4.3.10 Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) Analysis of AHL

LC-HRMS analysis of AHL extracted was performed on Thermo Scientific, Hybrid Quadrapole Q-Exactive orbitrap mass spectrometer for identification of type of signal molecule. Chromatographic separation was carried out using LC (Accela 1250 pump), Extracted AHL sample was prepared in acetonitrile. Thermo Scientific Hypersil ODS C18 column of length 5cm and internal diameter 2.1 mm was used. Particle size of column was 1.9 μm [Fekete et al. 2007].

The mass spectrometer was operated in positive electrospray ionization mode in 70,000 full widths at half-height maximum resolution with mass range *m/z* 300 to 800. The operation conditions were as follows: spray voltage at 3.6 kV, capillary temperature at 320 °C, S-lens RF level at 50, automatic gain control (AGC) at 1×10^6, and maximum injection time at 120ms. Nitrogen was used as the sheath gas, auxiliary gas, and sweep gas, set at 45, 10, and 2, respectively (arbitrary units). The isocratic solvent system of acetonitrile and water was used in the ratio 99:1 holding for 5 mins. Flow rate was adjusted as 350μL/min. A volume of 1.5 μL of sample was injected and full LC-HRMS scan was performed using positive polarity. Data were analyzed with Thermo Scientific Xcalibur software. The exact mass-to-charge ratios of AHL were found out. Thus based on the m/z (mass to charge ratio) type of AHL was detected.

4.4 Results

4.4.1 Quantification of Biofilm Formation

For the quantification of biofilm, specific biofilm formation (SBF) index was calculated as SBF= (AB-CW)/G in which AB is the OD_{540nm} of stained attached bacteria and CW is the OD_{540nm} of stained control wells containing bacteria-free medium only and G is the OD_{600nm} of cells growth in suspended culture. The obtained results were studied by quartiles to classify semi-quantitatively the biofilm production in four categories for each of the formula used: strong (S), moderate (M) and weak (W) (Table 4.2).

Table 4.2: Semi-quantitative classification of biofilm production using three different formulas

Formula	Strong	Moderate	Weak
BF = AB-CW	>3.0	2.0-3.0	<2.0
BF = AB⁄CW	> 8.0	6.0-8.0	<6.0
SBF = (AB-CW)⁄G	>2.5	1.5-2.5	<1.5

BF: biofilm formation; AB: stained attached bacteria; CW: stained control wells;
SBF: specific biofilm formation; G: growth in suspended culture.
All values are OD_{540nm}, except G = OD_{600nm}

A strong SBF index was observed for all the *C. sakazakii* isolates at the interface of polystyrene microtiter well except isolate N13 which exhibited moderate biofilm formation (*p* value <0.05) (Table 4.3). The strong index indicates that the tested strains significantly formed biofilm under prolonged period of incubation of 48 h. The prevalence of cellulose biosynthesis genes was further analyzed among *C. sakazakii* isolates. As shown in Table 4.3, 100%, 85% and 71% strains were positive for *bcsC*, *bcsA* and *bcsG* respectively. All isolates possessed peptidoglycan hydrolase (*flgJ*) whereas the genes coding for flagellar synthesis were absent in a majority of all the isolates.

Table 4.3: Biofilm formation, composition and prevalence of biofilm forming genes among *C. sakazakii* isolates

C. sakazakii strains	Biofilm Formation and Quantification			EPS Composition		Genes involved in biofilm formation					
	BF=AB-CW	BF=AB/CW	SBF=(AB-CW)/G	Total Carbohydrate (µg/ml)	Total Protein (µg/ml)	*bcsC*	*bcsG*	*bcsA*	*flgJ*	*fliD*	*flhE*
ATCC 12868	3.2	8.06	2.95^{f}	79.21^{d}	0.58^{c}	+	+	+	+	+	+
E604	3.7	9.32	4.73^{g}	87.06^{f}	7.11^{g}	+	+	+	+	+	-
N13	1.87	4.71	1.90^{c}	55.31^{c}	5.06^{f}	+	+	-	+	-	+
N14	1.21	3.05	1.02^{a}	45.51^{b}	0.10^{b}	-	+	+	-	-	-
N15	2.98	7.51	2.27^{d}	82.76^{e}	4.70^{e}	+	-	+	+	+	-
N81	1.35	3.40	1.39^{b}	33.6^{a}	0.08^{a}	-	+	-	+	-	-
N112	2.95	7.43	2.48^{e}	121.67^{g}	1.93^{d}	+	+	+	+	-	-

+ indicates the presence of respective trait; - indicates the absence of respective trait

a-g = Different letters in the same column indicate that the values are significantly different ($p < 0.05$) as measured by 2 sided Tukey's – post-hoc range test between replications

4.4.2 EPS production and characterization

The composition of EPS surrounding the biofilm forming *C. sakazakii* consisted mainly of carbohydrate, proteins (Table 4.3). All isolates revealed significant changes in EPS composition. The composition of EPS in *C. sakazakii* possessed very low protein:carbohydrate content which differs among all the isolates ($p < 0.05$). The extracted EPS was also analyzed using FTIR spectroscopy for study of its components. The FTIR spectroscopy of the EPS revealed the presence of COOH groups (1600 cm^{-1} to 1725 cm^{-1}) and -OH (2800 cm^{-1} to 3600 cm^{-1}) groups, indicating that the samples were exopolysaccharide (Fig. 4.1). The presence of -CH- vibrations in lipids, amide I in proteins, amide II in protein and -COC- group vibration in carbohydrates is indicated by the peaks obtained at wave no. range 3,200-2,800, 1,800-1,600 and 1,600-1,500, respectively.

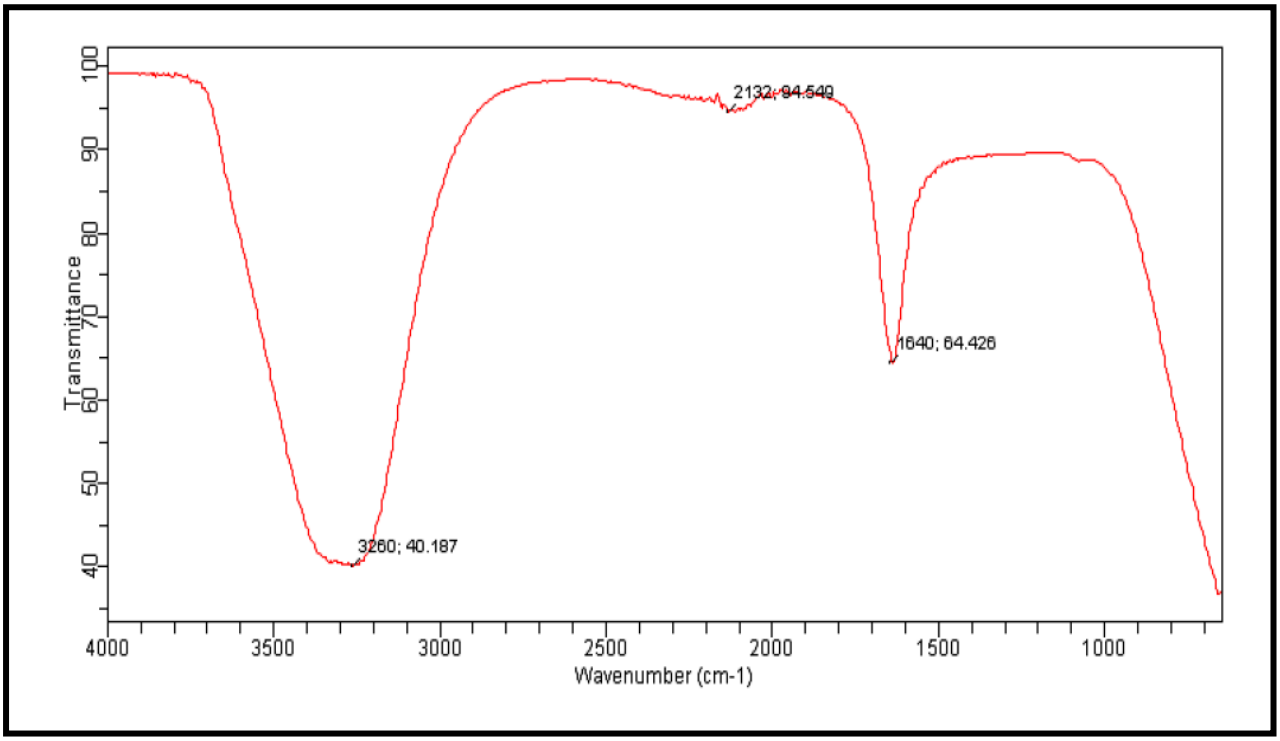
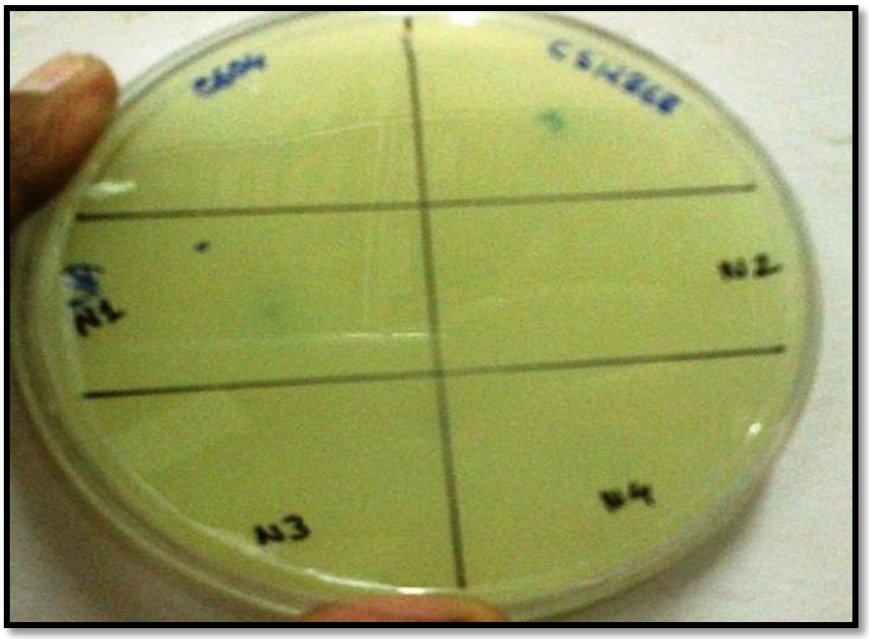


Figure 4.1: FT-IR spectra of EPS

4.4.3 AHL screening and characterization

AHL molecules in *C .sakazakii* isolates were detected using the biosensor strains *A. tumefaciens* NTL4(pZLR4) and *C. violaceum* CV026. When CV026 bioassay was carried out for the detection of short chain AHLs, all the isolates as well as reference strains produced colourless colonies, indicating that the strains were unable to synthesize AHL molecules having short acyl chains. This implicated that none of these *C. sakazakii* isolates produced short chain AHL molecules. The CV026 bioassay was done and the positive colonies producing the AHLs were used for further for production, extraction and characterization of AHL molecules.

On the other hand, resulted of the *A. tumefaciens* NTL4(pZLR4) bioassay revealed that all the strains were able to produce AHL molecules with long acyl chains (Fig. 4.2). A positive result consisted of the presence of a blue halo around a colony on the plate (Fig. 4.2) and blue suspension of the solution in test tube, resulting from hydrolysis of X-Gal (Fig. 4.3). The growth kinetic studies revealed that the first signal of AHL appeared after 6 h of incubation. The production of AHL is directly proportional to the developmental phase of the tested strain (Fig. 4.4).



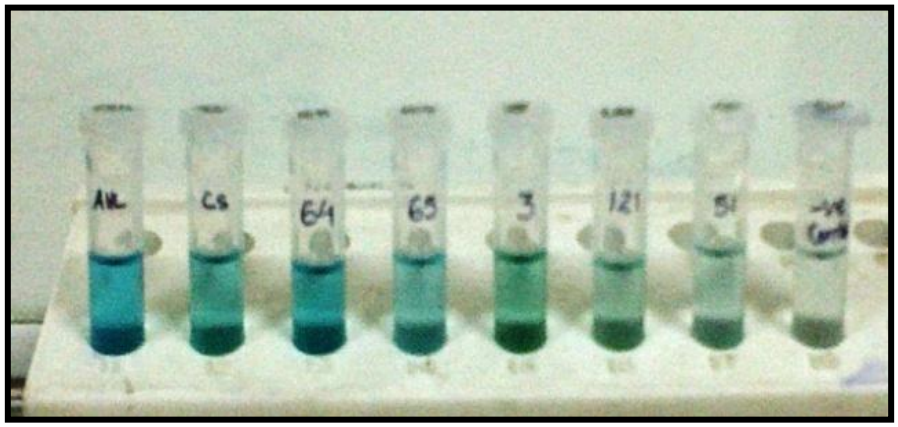


Figure 4.2: The production of blue-green chromogenic reaction in the *A. tumefaciens* NTL4(pZLR4) indicated the presence of long chain AHLs as signalling molecules in *C. sakazakii* strains.

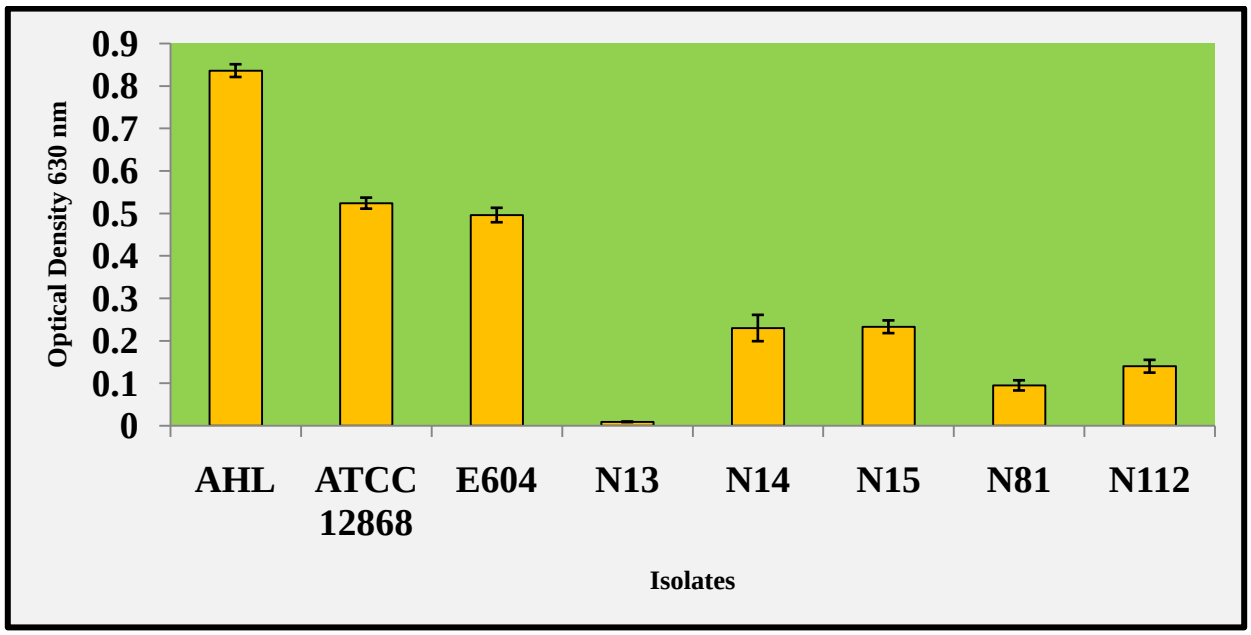


Figure 4.3: Measurement for AHL production in *C. sakazakii* isolates using *A. tumefaciens* NTL4(pZLR4) biosensor strain

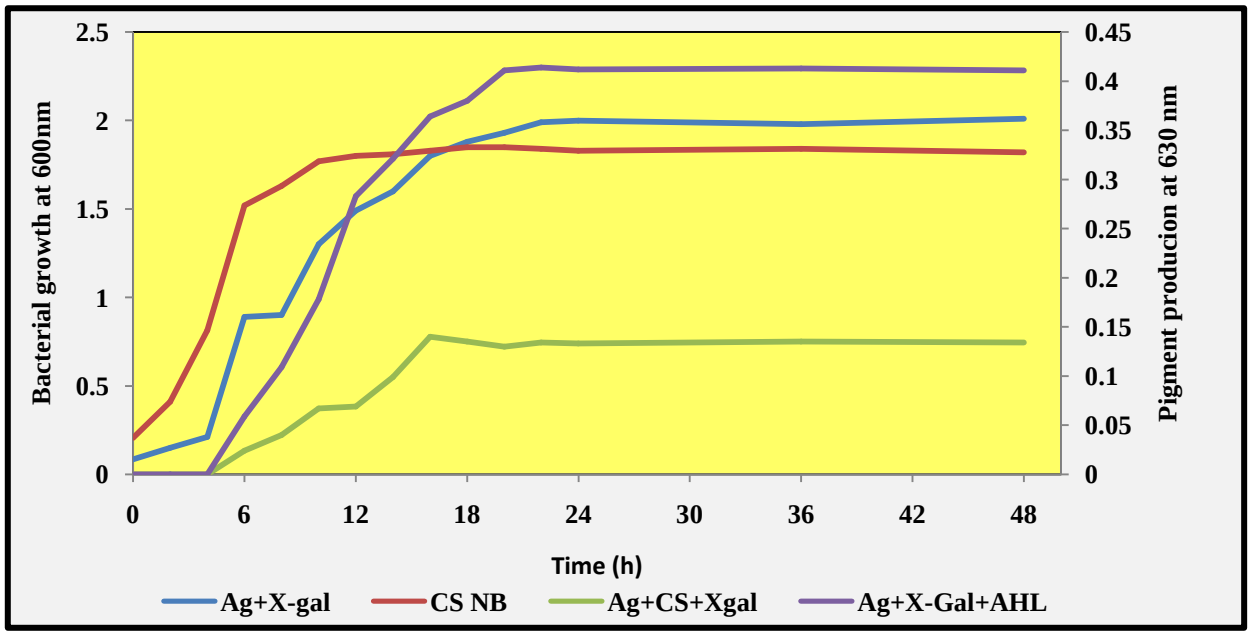


Figure 4.4: Growth kinetic studies of AHL Production

4.4.4 FTIR of extracted AHL

The FT-IR analysis of the extracted AHL showed the presence of lactone ring, N-H bond, C-O bond by peak at 1764.33 cm^{-1}, 1377.99 $cm^{-1}$and 1242.90 cm^{-1}respectively (Fig. 4.5). From FT-IR data, it could be concluded that selected strain is producing AHL.

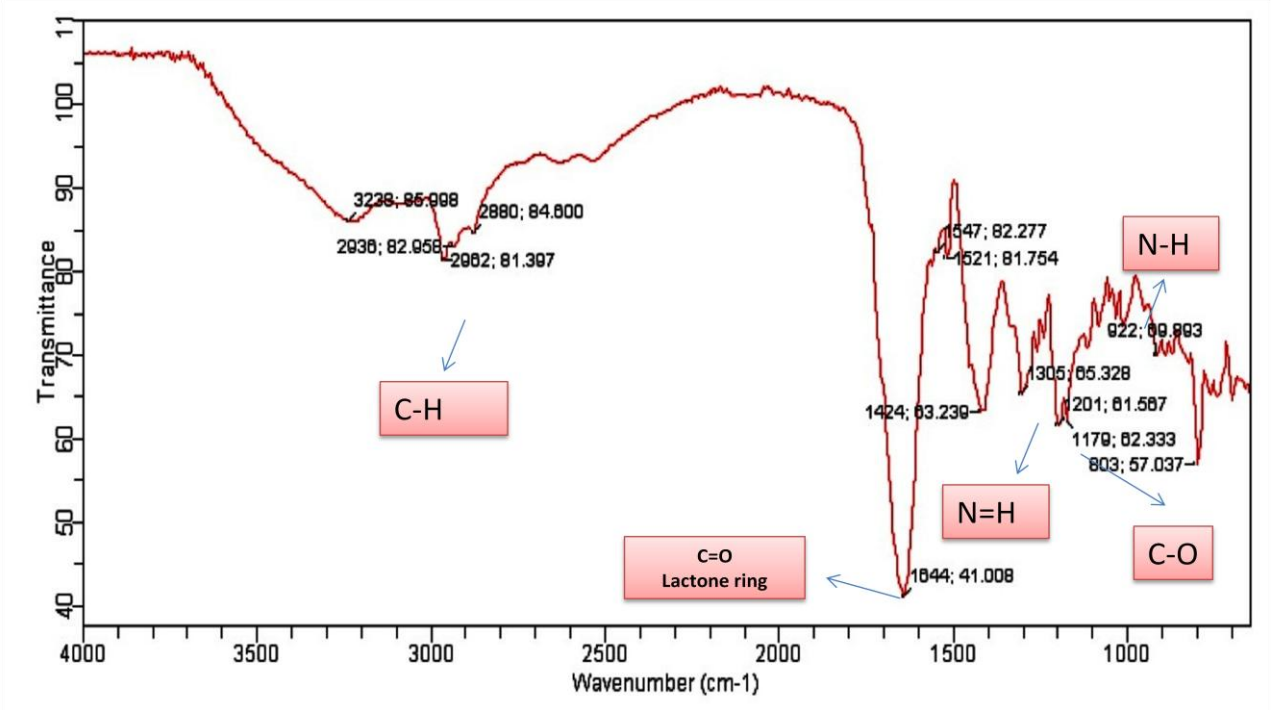


Figure 4.5: FT-IR spectra of extracted AHL

4.4.5 HPLC analysis

HPLC analysis was done for AHL extracted from CS and E604 strains, considering 3-oxo-C8 AHL (Cayman chemicals) as standard AHL. In chromatogram of 3-oxo-C8 AHL (standard) two peaks were observed at 8.92 min. and at 85.11 min. indicating elution of solvent (DMSO) and 3-oxo-C8 respectively (Fig. 4.6). In samples CS and E604, single peak was observed at 84.74 min and 84.6 min respectively. Hence the samples showed presence of AHL (Fig. 4.6).

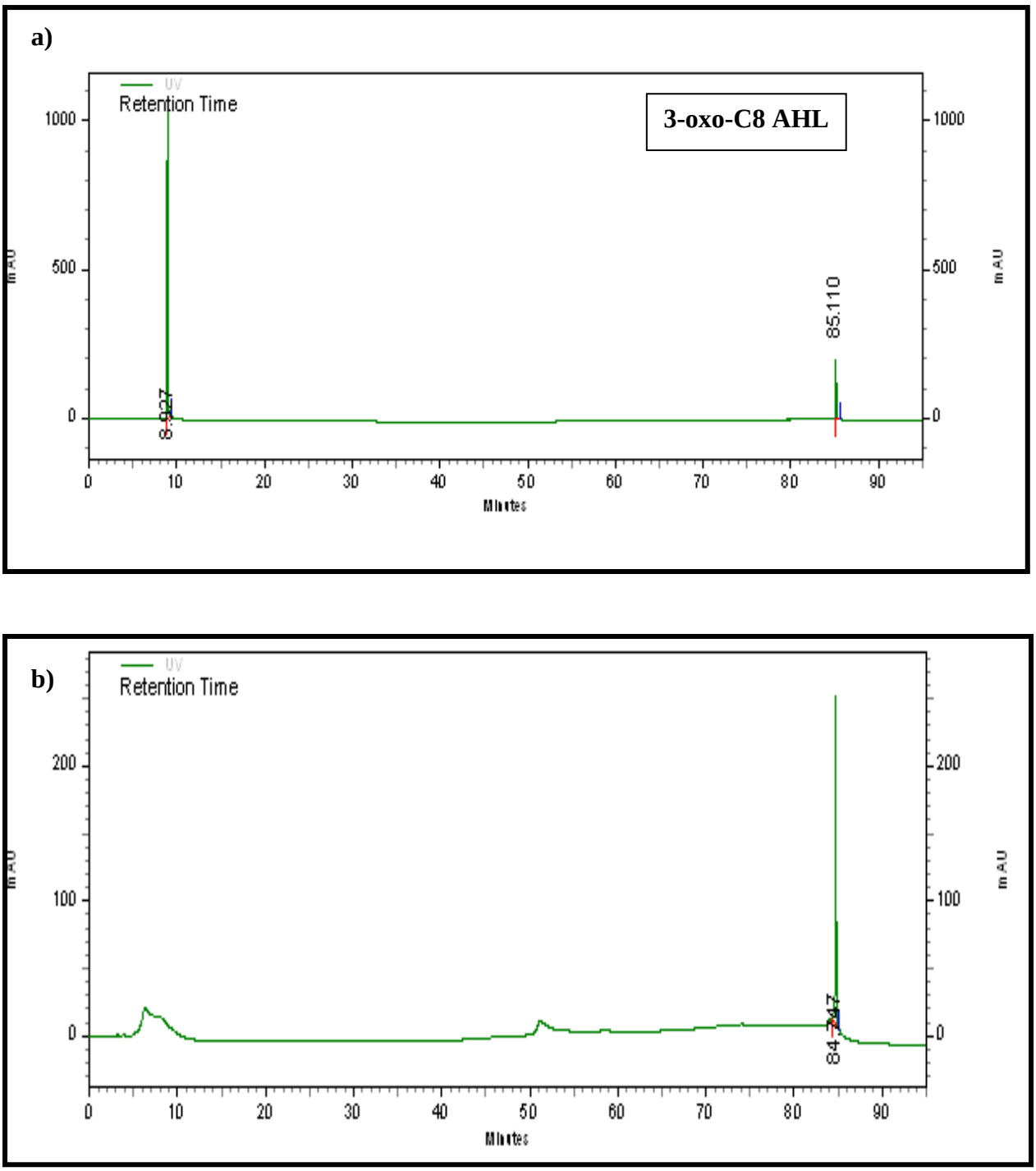
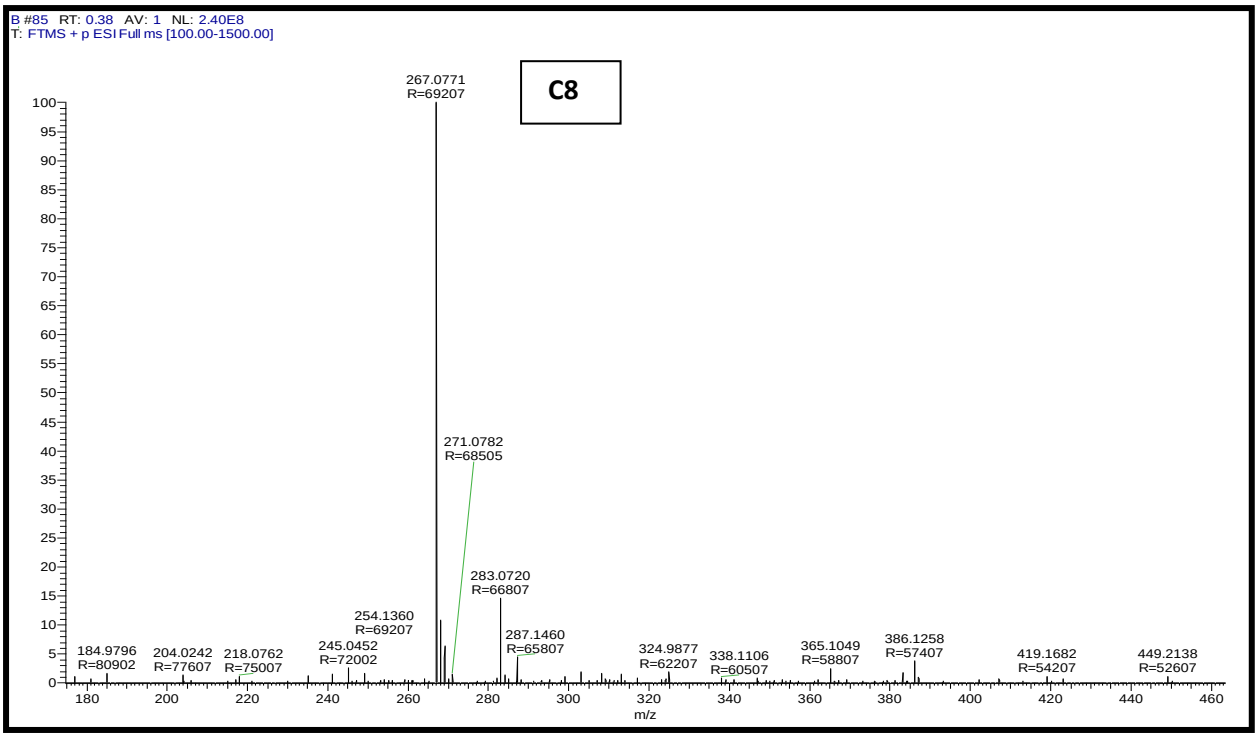


Figure 4.6: Identification of the AHL extracted from C. *sakazakii* by HPLC.

a) Chromatogram showing the retention time of standard 3-oxo- C8 AHL b) The extracted AHL were identified by comparing appeared peak retention time with the respective standard AHL.

4.4.6 LC-HRMS analysis of the AHLs

Major AHLs produced by strain CS and E604 were found out by LC-HRMS analysis. LC-HRMS analysis of sample CS showed presence of N-undecanoyl-L-AHL, N-dodecanoyl-L-AHL, N-tetradecanoyl-L-AHL, N-pentadecanoyl-L-AHL, N-(β-ketocaproyl)-L-AHL. AHL sample extracted from E604 was found to contain N-octanoyl-L-AHL, N-3-oxo-octanoyl-L-AHL, N-octadecanoyl-L-AHL (Fig. 4.7).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267.0771
R=69207
271.0782
R=68505
283.0720
R=66807
254.1360
R=69207
287.1460
R=65807
245.0452
R=72002
184.9796
R=80902
204.0242
R=77607
218.0762
R=75007
324.9877
R=62207
338.1106
R=60507
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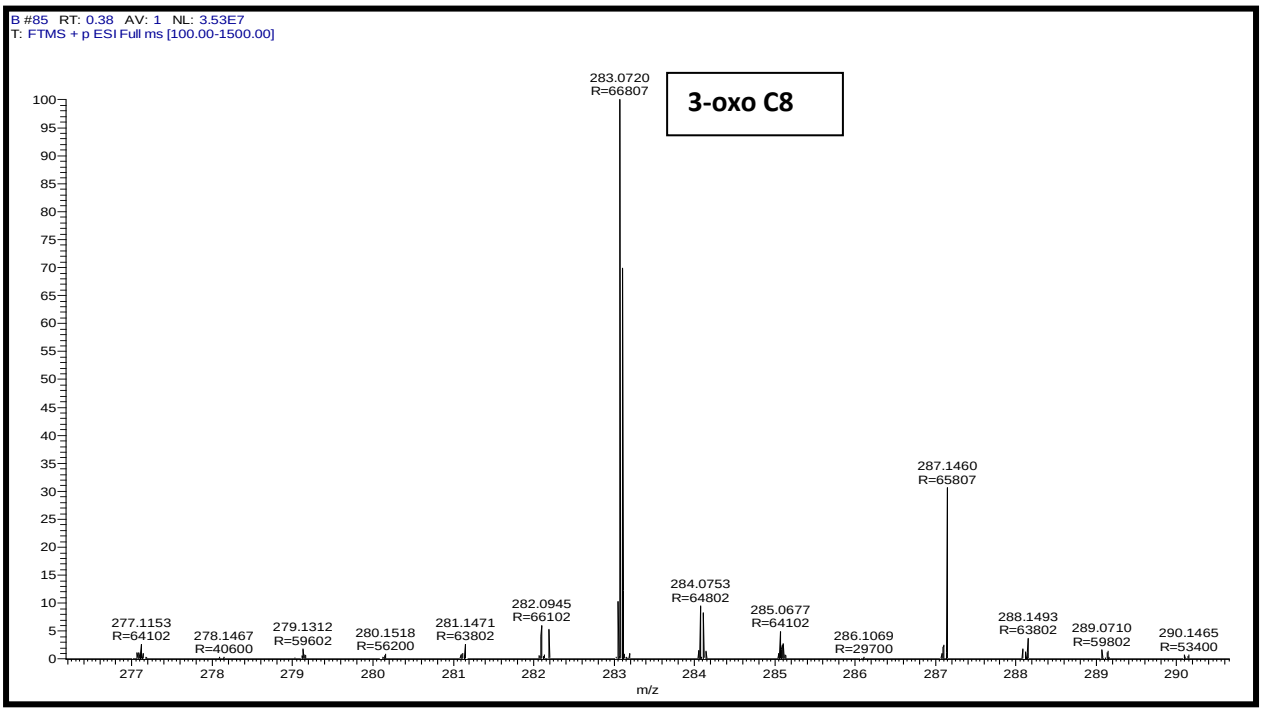
419.1682
R=54207
449.2138
R=52607
m/z


B #85 RT: 0.38 AV: 1 NL: 3.53E7
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3-oxo C8
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R=65807
284.0753
R=64802
285.0677
R=64102
282.0945
R=66102
277.1153
R=64102
278.1467
R=40600
279.1312
R=59602
280.1518
R=56200
281.1471
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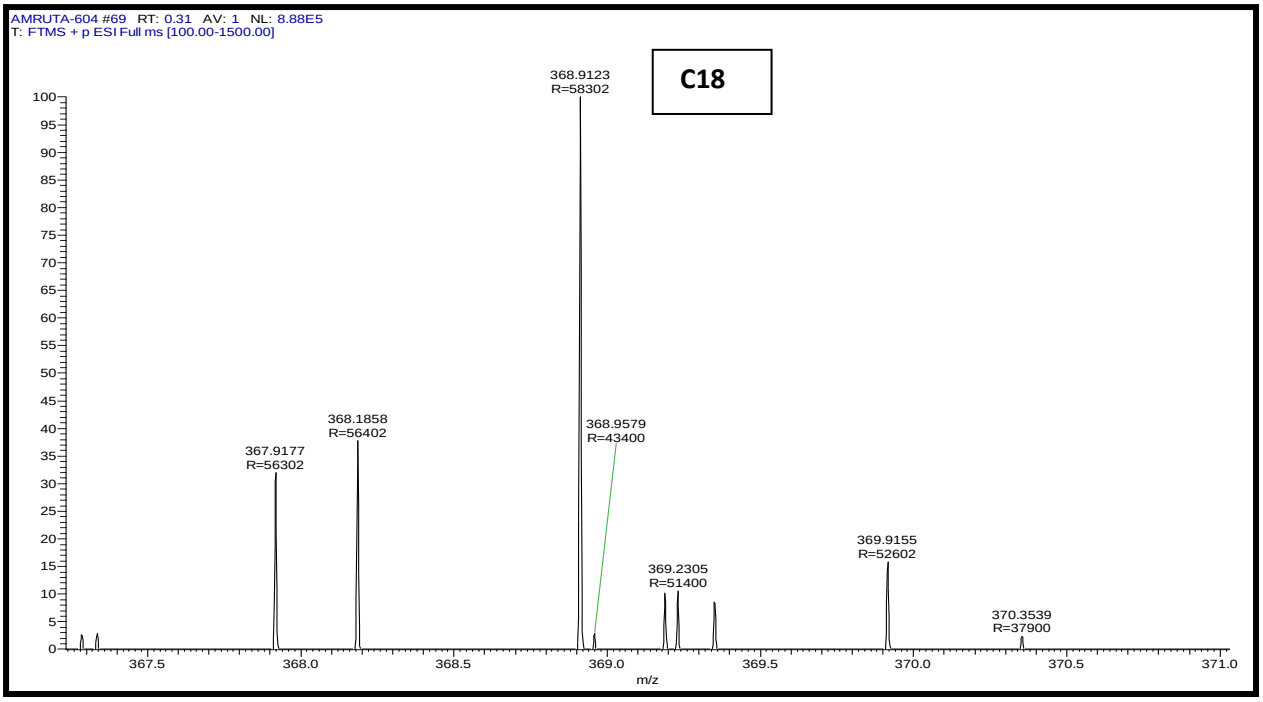
m/z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368.1858
R=56402
367.9177
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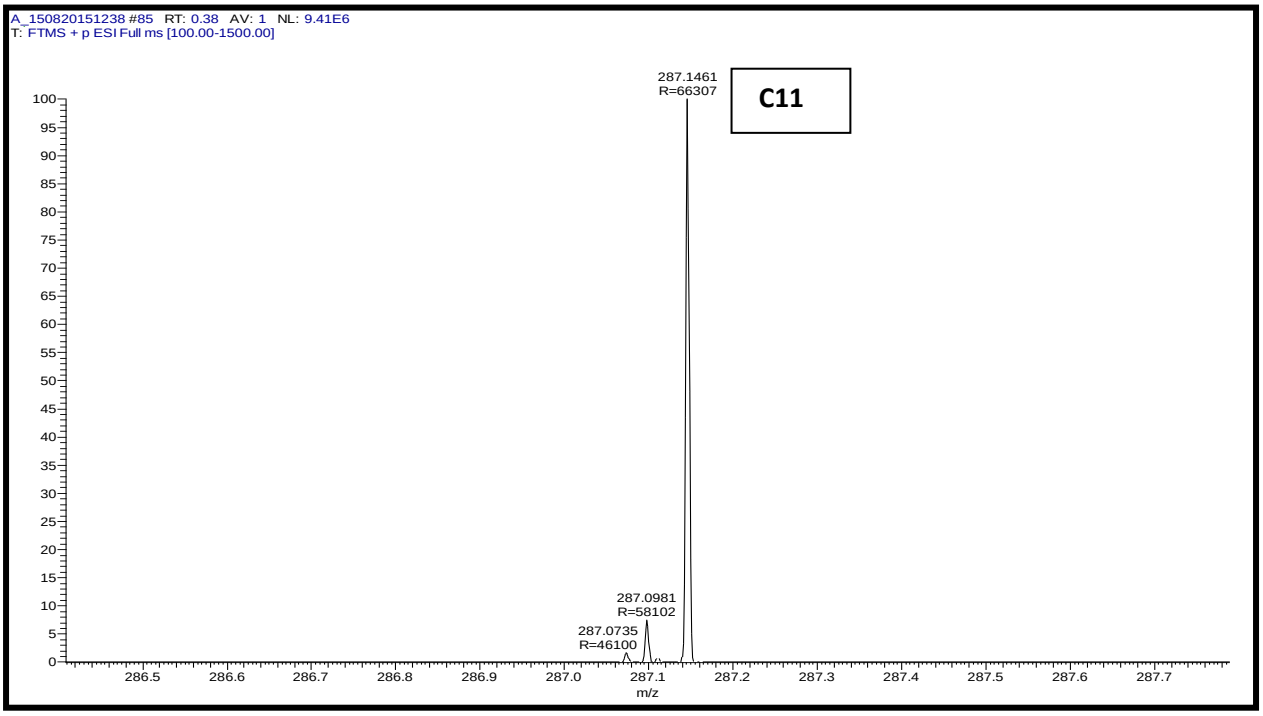
R=52602
369.2305
R=51400
370.3539
R=37900
m/z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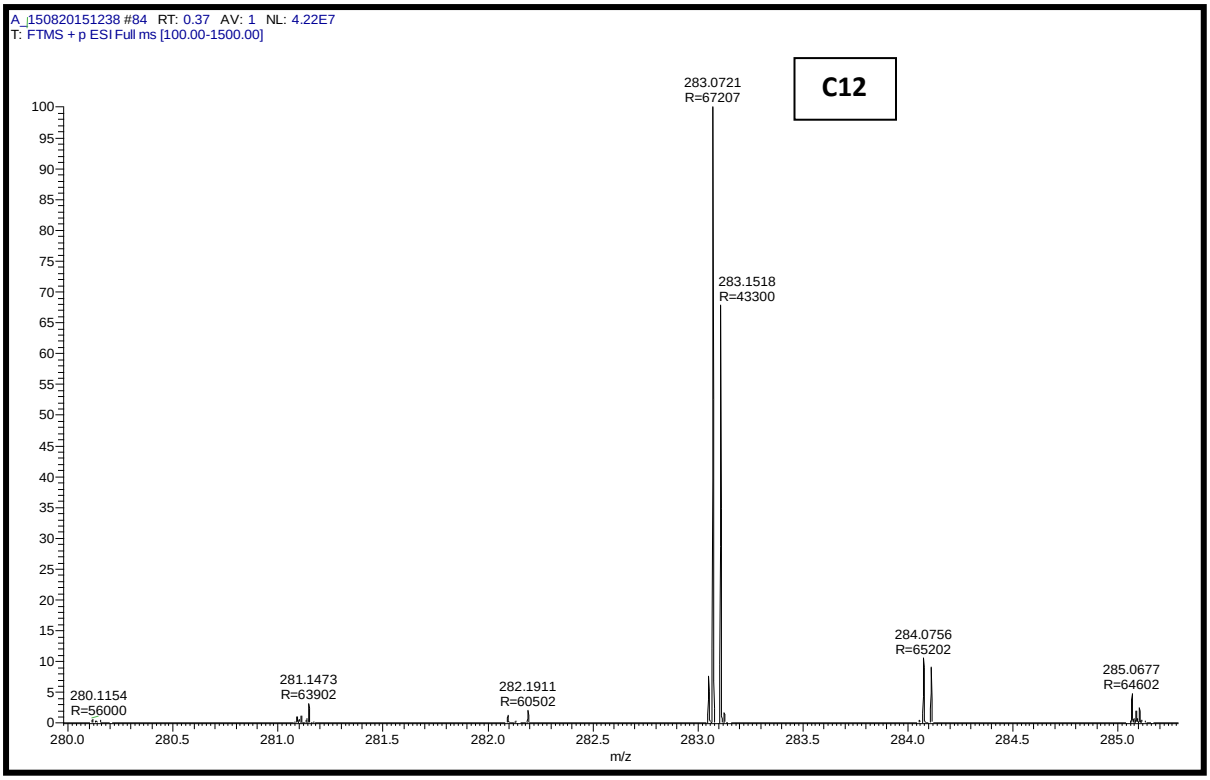
m/z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C12
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283.1518
R=43300
284.0756
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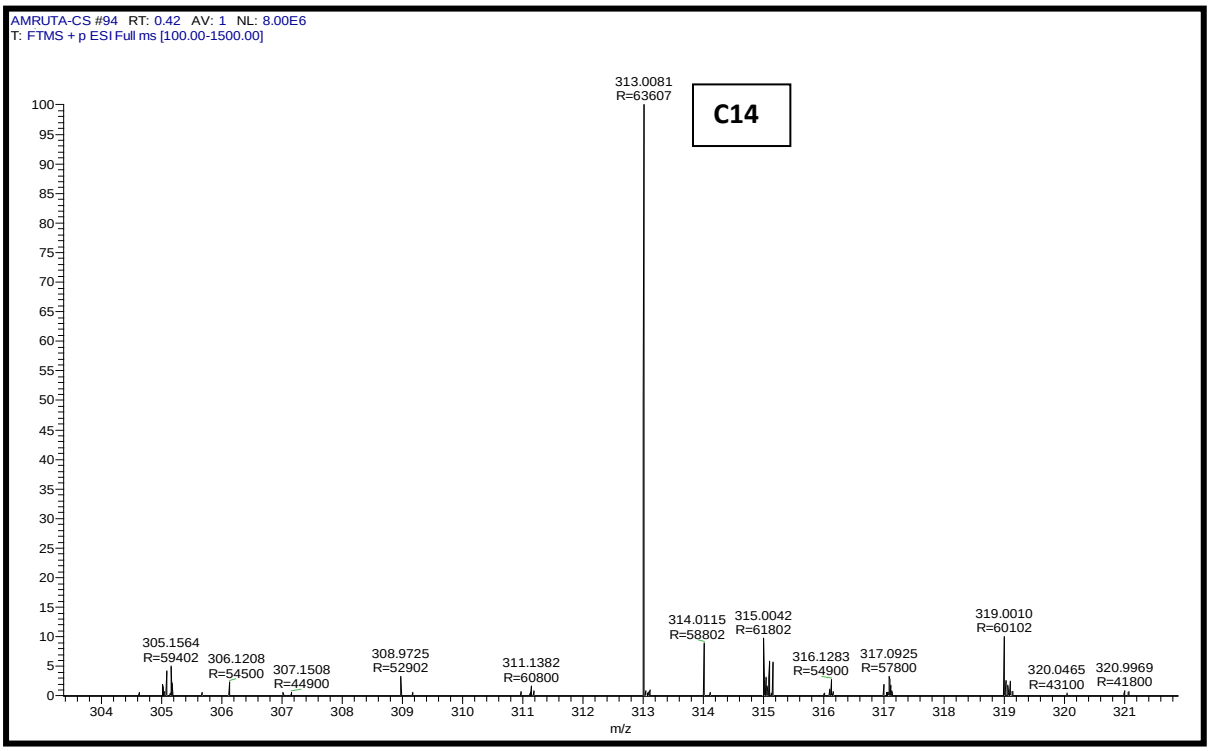
R=56000
m/z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C14
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R=58802
315.0042
R=61802
319.0010
R=60102
305.1564
R=59402
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R=54500
307.1508
R=44900
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m/z

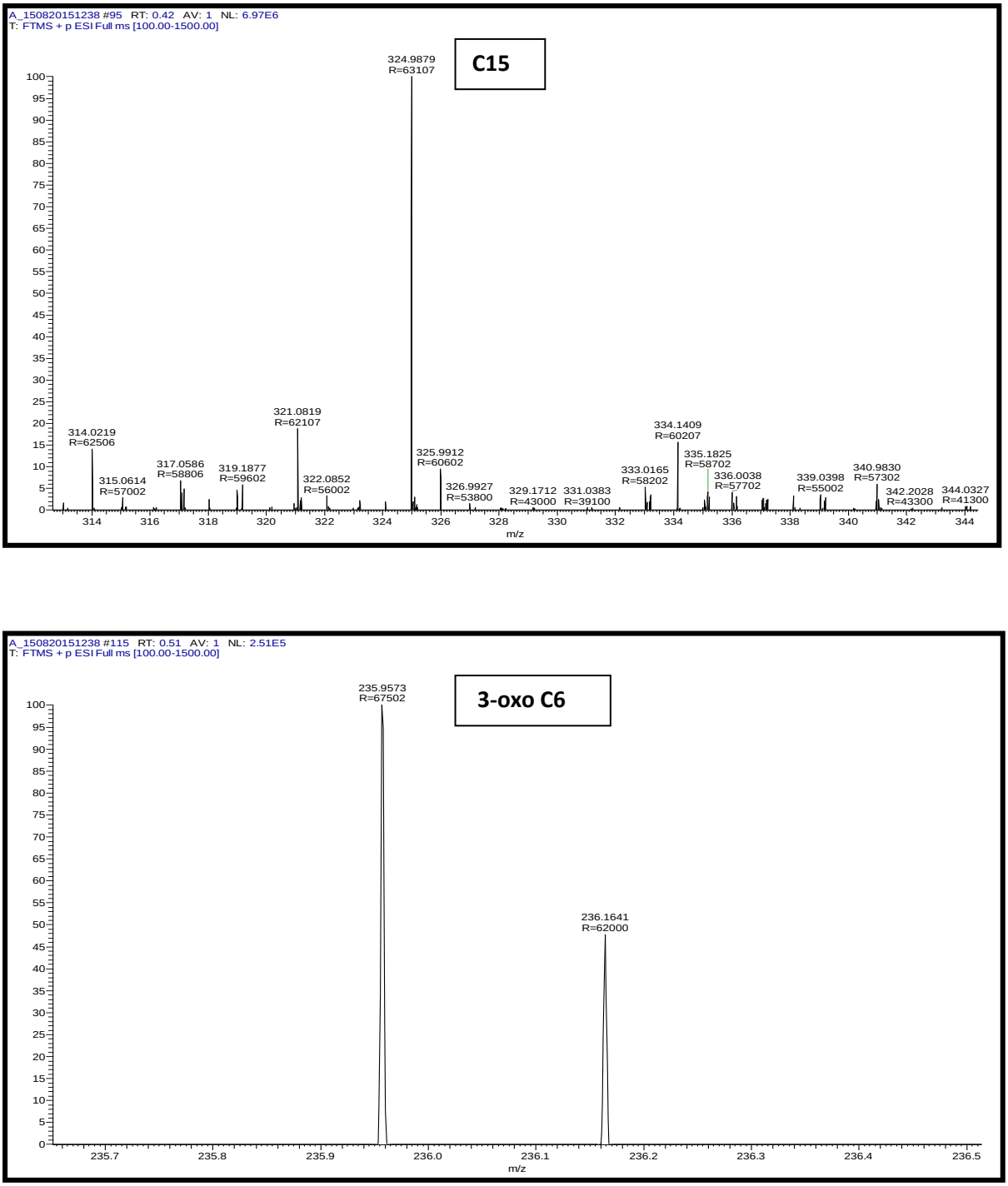

Figure 4.7: LC-HRMS profiles of AHL signals: Detection of AHLs by mass spectrometry analysis of supernatants extracts of *C. sakazakii*. A. C8 AHL (Retention time: 22.8 sec; m/z: 267.07) B. 3-oxo-C8 AHL (Retention time: 22.8 sec; m/z: 283.07) C. C18 ASL (Retention time: 18.6 sec; m/z: 368.91) D. C11 AHL (Retention time: 22.8 sec; m/z: 287.14) E. C12 AHL (Retention time: 22.2 sec; m/z: 283.07) F. C14 ASL (Retention time: 25.2 sec; m/z: 313.008) G. C15 AHL (Retention time: 25.2; m/z: 324.98) H. 3-oxo-C6 AHL (Retention time: 30.6 sec; m/z: 235.95).

4.5 Discussion

Biofilm formation in food manufacturing environments is of utmost importance because these biofilms can serve as a potential reservoir for microbial contamination leading to economic loss of food in terms of food spoilage or food borne infections. The biofilm formation provides a way of protection to the pathogens from variety of environmental stresses usually encountered in food processing units such as pH, osmotic stress, desiccation and thermal treatments [Kumar & Anand 1998; Frank 2000]. It has been approximated that over 99% of all bacterial activity in natural ecosystems is associated with bacteria organized in biofilms [Potera 1996].

The *C. sakazakii* is recently recognised as potential food borne pathogen with an ability to display remarkable resistance to desiccation and thermal treatments [Nazarowec-White & Farber 1997]. The biofilm formation by this pathogen is reported to be specific to the strain, abiotic surface and the environmental conditions. Our earlier study reported the prevalence of *C. sakazakii* among the different plant based ingredients and environmental samples [Singh et al. 2015b]. The *C. sakazakii* isolates obtained were further investigated for their potential to form biofilms on microtitre plates and biofilm associated genes. For this study, the cells in early stationary phase were selected to minimize the potential influence of different physiological states on attachment and biofilm formation. An incubation time of 48 h was used for our assays when *C. sakazakii* was grown in TSB as observed by Kim et al. [2006]. To quantify the biofilm formation, SBF index was used in present study. The SBF represents the most convenient mathematical formula to quantify bacterial biofilm as it deals with bacterial growth rate and present less variations in categorization of bacterial biofilm as suggested by Naves et al. [2007]. The strain N112 from plant based material demonstrated a strong SBF as compared to the standard strains. However, Lehner et al. [2005] did not find any significant differences among the *C. sakazakii* isolates from human, environmental and foods. Lee et al. found significant variation in biofilm formation by microtiter plate assay among different foods isolates of *Cronobacter* spp. [Lee et al. 2012]. The variation in the biofilm forming abilities is generally strain specific which was consistent with other studies in *C. sakazakii* [Ye et al. 2015]. The compositions of biofilm by *C. sakazakii* are reported to be influenced by the type of media, available nutrients, surface type and environmental conditions [Jung et al. 2013].

The outcomes of the experiments suggested that the genes for biosynthesis of cellulose are mostly present in C. *sakazakii* isolates studied. The genes coding for *bcsC*, *bcsA* were reported for cellulose synthesis in *C. sakazakii* BAA-894 whereas *bcsA* is also reported for cellulose synthesis in *C. sakazakii* ES5 [Hartmann et al. 2010]. The role of bacterial cellulose is forming the structural bridges in the biofilm by conferring chemical, mechanical, or biological protection inside the natural surroundings or facilitating cell adhesion is well documented [Ross et al. 1991]. The least prevalence of genes *flhE* and *flhD* which codes for flagellar protein synthesis in *C. turicensis* and *flgJ* coding for peptidoglycan hydrolase in *C. sakazakii* BAA-894 indicate that the flagellar moiety do not play a major role in biofilm formation by our isolates.

The high tolerance to environmental stress in *C. sakazakii* has been reported due to presence of remarkable production of EPS [Ryu & Beuchat 2005]. We also obtained a varying levels of water soluble EPS among the different isolates. Since the composition of the EPS can influence biofilm architecture [Yun et al. 2006], we investigated the total carbohydrates, concentrations of proteins, and e-DNA in the EPS of the isolates. The protein:carbohydrate ratio was very low for all the isolates (0.001-0.35). The low protein content in the EPS of the *C. sakazakii* biofilms perhaps can be attributed to enzymatic digestion by extracellular proteases and protein degradation under the prolonged incubation conditions. Although e-DNA has been reported as a component of biofilms for *P. aeruginosa*, however, we did not get any e-DNA in the EPS content of our isolates. The e-DNA is commonly considered a remnant of lysed cells [Flemming et al. 2007]. A correlation was observed between the EPS production and biofilm formation.

QS is a regulatory mechanism which enables bacteria to make collective decisions that governs various aspects of biofilm development, including adhesion, motility, development, and dispersal [Dong et al. 2008]. It is well known that biofilm-forming ability is mainly due to production of AHL mediating cell-to-cell communication system [Surette & Bassler 1998]. Therefore, we investigated the detection and quantification of AHL to analyze the relationship between AHL production and biofilm development in *C. sakazakii* isolates using *C. violaceum* CV026 and *A. tumifaciens* NTL4(pZLR4) as biosensor strains. The AHL biosensor practically relies on the LuxR protein and displays a specific attraction towards the cognate AHL and positively regulates the transcriptional of targeted gene. CV026 biosensor is most sensitive to C6-AHL; however it can also detect short-chain AHLs ranging from 4 to 8 carbon side-chain AHLs with or without C-3 substitution whereas *A. tumifaciens*

NTL4(pZLR4) detects C8 to C12-AHLs including oxo-C6-AHL and is quite sensitive to even low level of longer acyl side chain AHLs [Farrand et al. 2002].

In our study, we did not observe any QS signal molecule with *C. sakazakii* isolates using the CV026 biosensor which indicates the low production levels of AHLs with longer acyl side chains (C4-AHL to C8-AHL) which can only be detected at higher concentrations only by CV026 [McLean et al. 1997]. The negative results from *P. aeruginosa* isolates for AHLs with *C. violaceum* CV026 has been also described earlier and it was proposed that the isolates either failed to produce short chain AHLs or the level of signals was very low [Boşgelmez-Tınaz et al. 2005]. To determine the presence of long chain AHL molecules, *A. tumifaciens* NTL4(pZLR4) containing *traR* promoter and *traG*::*lacZ* transcriptional fusion was used. The *traG*::*lacZ* gets activated in the presence of exogenous AHL and results in appearance of blue colour. All the isolates of *C. sakazakii* isolates furnished a blue-green solution in the presence *A. tumefaciens* NTL4(pZLR4) and X-gal indicating the presence of long chain AHLs as signalling molecules in *C. sakazakii* (Fig. 4.2). The data represented that AHL-production is more common among bacteria isolated from herbs and spices and environmental. In the recent past, chemical study of AHL produced by *C. sakazakii* confirmed the identification of three molecules: N-heptanoyl-AHL, N-dodecanoyl-AHL and N-tetradecanoyl-AHL [da Silva et al. 2012]. Previously, Lehner et al. [2005] tentatively indicated the presence of two different types of AHLs (3-oxo- C6-AHL and 3-oxo-C8-AHL) by TLC which predicted the ability of *C. sakazakii* to produce cell-to-cell signaling molecules. However, there are no other reports on the QS molecules in *C. sakazakii*. Several isolates of *P. aeruginosa* were found to be producers of QS signal molecules belonging to C4-AHL, C6-AHL, 3-oxo-C6-AHL, C8-AHL, C10-AHL and C12-AHL [Kumar et al. 2011].

Our results reflected that the isolates which have significant levels of AHLs also showed good biofilm formation capacity. These results are in accordance with the findings of Taghadosi et al. [2015] who reported that *E. coli* isolates with the highest AHL levels also exhibited strong adherence to microplate wells. This may be due to the fact that biofilm formation is a multifactorial event and QS signals are one of such factors. Genetic analysis of biofilm formation has led to the proposal that extracellular signals and quorum sensing regulatory systems are essential for differentiated biofilms. Hence, differentiated biofilms may also be the net result of many independent interactions, rather than being determined by a particular global QS system. Khajanchi et al. [2009] proposed that AHL-mediated QS signalling system modulates the virulence of *Aeromonas hydrophila* by regulating the T6SS, metalloprotease production and biofilm formation. The high density of bacteria within

biofilms has led to the speculation that quorum-sensing genes and AHL production may be fundamentally associated with biofilm physiology. Various literatures have addressed the regulatory consequence of cell-signaling mechanisms arbitrated by AHL on the process of biofilm formation in different Gram-negative bacteria, including *P. aeruginosa* [Heydorn et al. 2002], *Burkholderia cepacia* [Huber et al. 2001] and *Serratia liquefaciens* [Labbate et al. 2004].

Further LC-HRMS analysis confirmed the presence of AHLs with a chain length equal to C6 and 3-oxo-C8 in both the reference strains produced. Of these detected AHLs, 3-oxo-C8-AHL was found to be the most abundant AHL produced by *C. sakazakii*. Taken the biosensor and mass spectra data together, it is unequivocal confirming the presence ASL in the *C. sakazakii.*

To our best knowledge, this is the first confirmed report on production of C-6 AHLs by *C. sakazakii* and confirmed the presence of 3-oxo-C8-AHL, results not before reported in the literature. Since QS regulates many important phenotypes including virulence production, hence study on QS in food-borne pathogen may lead to better understanding of how these bacteria contribute to its contamination of foods, and survival in the environment and host. Natural products isolated from plants that show anti-QS activity may provide a solution to prevent infection caused by QS pathogens [Chong et al. 2011].

4.6 Conclusion

To our best knowledge, this is the first report describing the expression of long chain AHLs in *C. sakazakii* and confirmed the presence of 3-oxo-C8-AHL. Since QS regulates the expression of many important phenotypes including virulence production, therefore the study of QS in *C. sakazakii* may lead to better understanding its survival in the different environments and hosts. Further research is warranted to determine the importance and functions of these signaling molecules and elucidating the mechanisms of QS-regulation. Natural products isolated from plants that show anti-QS activity may provide a solution to prevent infection caused by QS pathogens.

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CHAPTER 5

Inhibition of quorum sensing mediated biofilm formation in *Cronobacter sakazakii* strains

5.1 Abstract

The objective of this was to evaluated plant extracts (*Piper nigrum, Coriandrum sativum, Cuminum cyminum, Syzygium aromaticum, Trigonella foenum-graecum, Myristica fragrans, Zingiber officinale, Allium sativum and Cinnamomum verum*) for their anti-quorum sensing potential to inhibit the biofilm formation in *Cronobacter sakazakii* strains. The bioassay based on loss of pigment production by *Chromobacterium violaceum* 026 and *Agrobacterium tumefaciens* NTL4(pZLR4) was used for initial screening of the extracts. Further, the effect of extracts on the inhibition of QS-mediated biofilm in *Cronobacter sakazakii* strains was evaluated using standard crystal violet assay. The effect on biofilm texture was studies using SYTO9 staining, Light and Scanning electron microscopy. Among the tested extracts, *Piper nigrum* and *Cinnamomum verum* at 100 ppm resulted in 78% and 68% reduction in production of violacein as well as blue-green color in both biosensor strains. A higher inhibitory activity (>50%) on biofilm formation in *C. sakazakii* was observed for *P. nigrum* and *Cin. verum* whereas the other extracts possessed moderate (25-50%) and minimal (<25%) inhibitory activities. Further, the fluorescent and Scanning Electron Microscopic images indicated a major disruption in the architecture of biofilms of tested strains by *P. nigrum*. This study points to the possibility of using *P. nigrum* and *Cin. verum* as inhibitor of quorum sensing mediated biofilm formation by *C. sakazakii* which could be further explored for novel bioactive molecules to limit the emerging infections of *C. sakazakii*,

5.2 Introduction

Cronobacter sakazakii is a bacterial pathogen associated with invasive infections, with a fatality rate up to 80% and infant's cases have been epidemiologically-related to the ingestion of contaminated powdered infant formula (PIF) [Joseph et al. 2012; Kalyantanda et al. 2015; Singh et al. 2015a]. The organism is reported to form biofilms on different substrates ranging from silicon, latex, polycarbonate, stainless steel, glass, and polyvinyl chloride [Iversen et al. 2004; Lehner et al. 2005; Kim et al. 2006] and is able to produce extracellular polysaccharides (Lehner *et al*. 2005). Furthermore, there are also reports that *Cronobacter* spp. have the ability to survive in desiccated conditions for several weeks and are resistant to osmotic stresses that may be due to formation of biofilms (Iversen and Forsythe 2003). Various literature studies have indicated that the ability to form biofilm among different isolates of *Cronobacter* spp. is generally strain specific (Lehner *et al*., 2005; Kim *et al*. 2006; Hartmann *et al*. 2010; Du *et al*. 2012; Lee *et al*. 2012; Jung *et al*. 2013; Hu *et al*. 2015). The studies from Lehner *et al*. (2005) demonstrated the ability of biofilm-producing strains of *C. sakazakii* to synthesize the cell signaling molecules acyl homoserine lactones (AHLs), which mediate quorum sensing (QS). This cell-to-cell signaling along with other genetic and environmental factors have been reported to be involved in biofilm formation in bacteria (Waters and Bassler 2005; Shrout et al., 2011). Therefore, the interference with this phenomenon by means of quorum sensing inhibitors (QSI) could be an attractive approach to prevent or to reduce biofilm based infections. Several natural and synthetic anti-QS compounds have been reported where extracts of various natural products (e.g., bean sprout, chamomile, carrot and garlic) (Rasmussen *et al*. 2005) and essential oils of several plants (e.g., lavender, eucalyptus and citrus) (Szabo *et al*. 2010) have shown anti-QS effects, however, the studies on effect of these plant extract against biofilm formation and inhibition of QS in *C. sakazakii* have not been reported. Earlier reports on anti-biofilm ability of trans-cinnamaldehyde (TC) from cinnamon indicated that the compound at sub-inhibitory concentration of TC was able to inhibit biofilm synthesis and inactivate mature biofilms of *C. sakazakii* on different abiotic substrates (Amalaradjou and Venkitanarayanan, 2011); however no investigation was conducted on QS mechanisms.

The plant derived extracts are widely considered due to their safety and are being used traditionally for prevention and treatment of infections. Therefore, the present study was

undertaken to investigate for the first time the anti-QS potential of the plant extracts in *C. sakazakii* and their role in inhibition of biofilm formation.

5.3 Materials and Methods

5.3.1 Strains, media and culture conditions

In the previous study, 38 isolates of tentatively identified *Cronobacter* spp. from different commodities were identified based on 16S rRNA (Singh *et al*. 2015b). Confirmed *C. sakazakii* isolates along with the standard strains, *C. sakazakii* ATCC 12868 and E604 (kindly gifted by Dr. Ben Davies Tall, FDA, USA) were used in the present study. The two biosensors strains of *Chromobacterium violaceum* CV026 (kindly gifted by Dr. Paul Williams, University of Nottingham) and *Agrobacterium tumefaciens* NTL4(pZLR4) (kindly gifted by Dr. Stephen K Farrand, University of Illinois, US), were used for the detection of AHLs. The *C. violaceum* CV026 was cultured in Luria Bertani broth (LB) supplemented with 100 μg/mL ampicillin and 30 μg/mL kanamycin whereas *A. tumefaciens* NTL4(pZLR4) was cultured in nutrient broth (NB) medium containing gentamicin (50 µg/mL) at 28 °C for 24 h. For all the experiments, bacteria were grown in 10 mL Trytone Soy Broth (TSB) medium under shaking (130 rpm) for 24 h.

5.3.2 Plant products and extract preparation

The nine plant products (*Piper nigrum, Trigonella foenum-graecum, Coriandrum sativum, Cuminum cyminum, Syzygium aromaticum, Myristica fragrans, Zingiber officinale, Allium sativum and Cinnamomum verum*) used in this study were purchased from local outlets in the Solan province of Himachal Pradesh (Table 5.1), India and the extractions were made by following the method of Choo *et al*. (2006), with slight modifications. Each of the plant products was washed in sterile water, dried, and powdered using a mixer grinder. For the preparation of methanolic extract, powdered samples (50 g) were soaked in 300 mL of methanol for overnight under shaking at 100 rpm at 30 °C (Fig. 5.1). The methanol phase was collected and concentrated by rotary evaporator to obtain the dried residue. The residues were re-dissolved in 10% dimethyl sulfoxide (DMSO) and stored at -20 °C until further use.

Table 5.1: Plants screened for anti-Quorum Sensing and inhibition of biofilm activities

S. No	Species	Family	Common name	Part tested
1.	*Piper nigrum*	Piperaceae	Blackpepper	Fruit
2.	*Trigonella foenum-graecum*	Fabaceae	Fenugreek	Seeds
3.	*Coriandrum sativum*	Apiaceae	Coriander	Seeds
4.	*Cuminum cyminum*	Apiaceae	Cumin	Seeds
5.	*Syzygium aromaticum*	Myrtaceae	Clove	Bud
6.	*Myristica fragrans*	Myristicaceae	Nutmeg	Fruit
7.	*Zingiber officinale*	Zingiberaceae	Zinger	Rhizome
8.	*Allium sativum*	Amaryllidaceae	Garlic	Bulb
9.	*Cinnamomum verum*	Lauraceae	Cinnamon	Bark

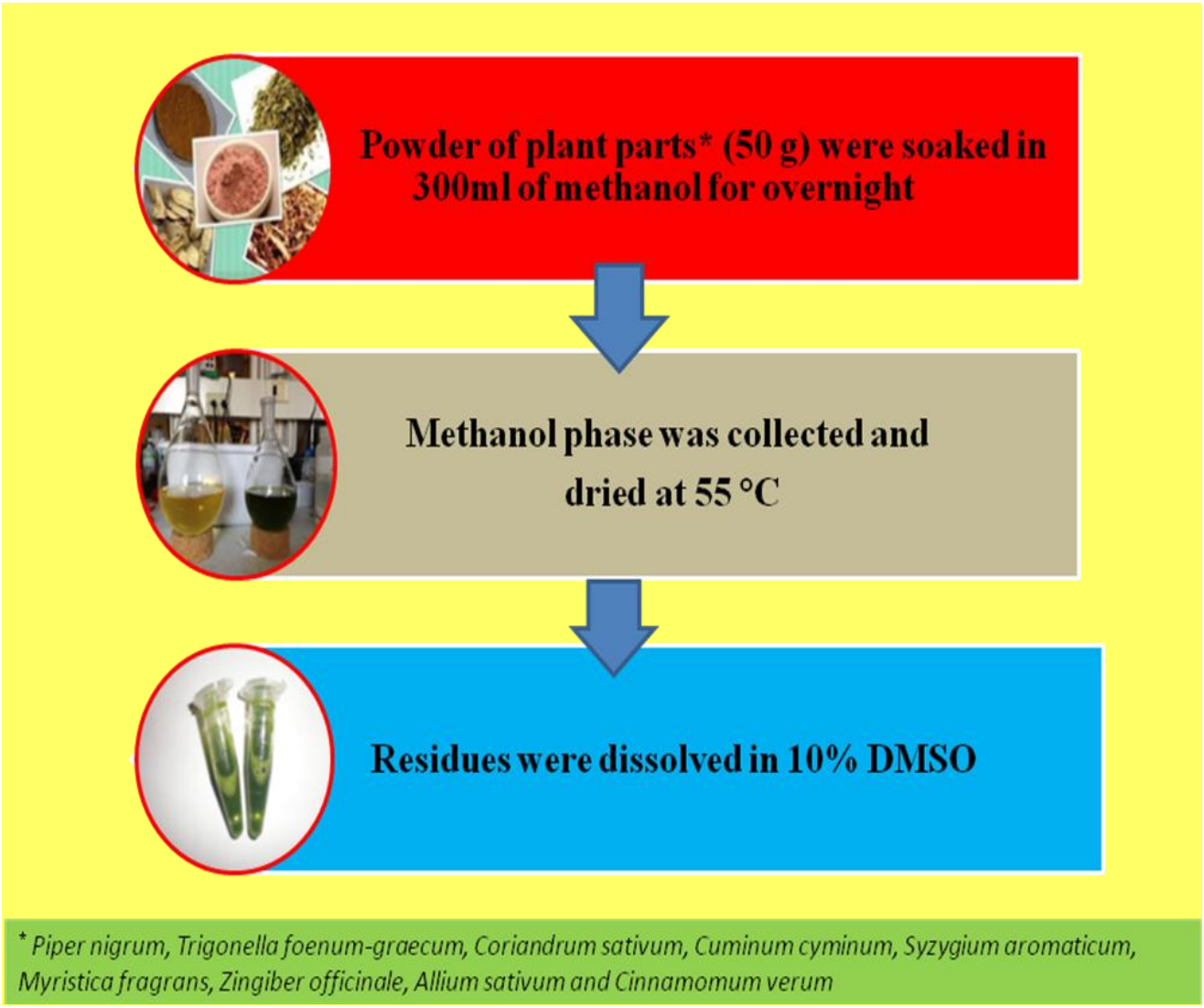

Figure 5.1: Sequential steps of preparing the methanolic plant extracts

5.3.3 Anti-quorum sensing activity of plant extracts

The anti-quorum sensing activity of the extracts was evaluated using indicator strains with well-diffusion as well as test tube assay.

5.3.3.1 Well-diffusion method

The methanolic plant extracts were screened for anti-QS activity using an AHL-based *in vitro* QS assay using two biosensor strains, *C. violaceum* CV026 and *A. tumefaciens* NTL4(pZLR4) by the agar-well diffusion test. A 50 µL sample of overnight broth culture of *C. violaceum* CV026 (10^5 cfu/mL) was added to 10 mL of LB soft agar along with 1.25 µM of C6-AHL (99%, Cayman Chemicals, USA) as QS standard molecule and the mixture was overlaid onto LB agar plates. After solidification of overlay layer, wells of 6 mm diameter were dug using a sterile corkborer. The methanolic plant extract (50 µL) was added to respective wells and the plates were incubated overnight at 28 ^{o}C in upright position (McLean *et al*. 2004; Mukherji and Prabhune 2015). The testing for QS inhibition against *A. tumefaciens* NTL4(pZLR4) was done in a similar way supplementing the agar with 50 mg/mL X-gal along with 1.25 µM of 3-oxo-C8 AHL (99%, Cayman Chemicals) as standard AHL. The inhibition of QS was detected by the presence of colorless zone with viable cells around the well.

5.3.3.2 Test tube method

The qualitative effect of extracts on the QS-controlled production in *C. violaceum* CV026 and *A. tumefaciens* NTL4(pZLR4) was determined as described previously with some modification (Choo *et al*. 2006). Briefly, 10 mL LB and NB medium containing 50 µL of each plant extracts was inoculated with *C. violaceum* CV026 and *A. tumefaciens* NTL4(pZLR4) along with 1.25 µM of C6 AHL and 3-oxo-C8 AHL, respectively. The tubes were incubated for 24 h at 28 ^{o}C in an shaking incubator (130 rpm). Next, violacein was extracted in DMSO from the cells and was quantified at optical density 580 nm in a UV-Vis spectrophotometer. Simultaneously, absorbance of the supernatant containing blue-green colouration was measured at a wavelength of 630 nm using DMSO as the blank. The percentage of inhibition was calculated by following the formula: percentage of inhibition = (control $_{OD}$ − test $_{OD}$ / control $_{OD}$) × 100.

5.3.4 Anti-bacterial activity of plant extracts

The non-antibacterial activity of all plant extracts was also determined to confirm that the halos produced on lawns of the biosensor strains resulted from QSI rather than the

antibacterial activity of the plant extracts. The well-diffusion assay was performed in tryptone soy agar (TSA) by adapting the method specified by the Clinical and Laboratory Standards Institute (CLSI 2006). Briefly, 100 µL of overnight grown *C. sakazakii* strains were uniformly spread over the surface of TSA plate. The wells were punctured and filled with 50 µL of each plant extract. The plates were incubated at 37 ^{o}C for 24 h and observed for growth inhibition.

5.3.5 Inhibition of *C. sakazakii* biofilm formation

The effect of extracts on the biofilm formation of *C. sakazakii* isolates was determined by quantifying the biofilm biomass through microtiter plate (MTP) assay (Thenmozhi *et al*., 2009). In brief, 20 µL overnight grown *C. sakazakii* isolates (10^6 cfu/mL) and extracts were added in the dose-dependent manner (50, 100 and 150 ppm) into 230 µL of fresh TSB medium. The plates were incubated without agitation for 48 h at 37 ^{o}C. After incubation, the planktonic cells in MTPs were removed by washing the wells twice with sterile water. The surface-adhered cells in the MTP wells were stained with 200 µl of 0.2% crystal violet (CV) solution. After 15 min, the excess CV solution was removed and CV in the stained cells was solubilized with 250 µl of 33% glacial acetic acid. The biofilm biomass was then quantified by measuring the intensity of CV at OD_{570nm} using UV-Vis spectrophotometer. The sterile TSB was used as negative control. The percentage of biofilm inhibition was calculated by following the formula: percentage of biofilm inhibition = (control $_{OD570\ nm}$ − test $_{OD570\ nm}$ / control $_{OD570\ nm}$) × 100. The dose of 100 ppm illustrating strong anti-biofilm activity was taken as minimum inhibitory dose and used for further experiments.

5.3.6 Microscopic analysis of *C. sakazakii* biofilms

5.3.6.1 Light microscopic analysis

The light microscopy analysis of bacterial biofilm was carried out following the method of Musthafa *et al*. (2010). A 50 µL overnight grown *C. sakazakii* isolates (10^6 cfu/mL) were added into 2 mL of fresh TSB medium containing cover slips 1 x 1 cm in 24-well MTP along with a control well without extracts. After static incubation for 48 hours at 37 ^{o}C, the cover slips were removed, rinsed with phosphate buffer (pH-6.5) and were stained with 0.2% CV. The stained cover slips with the biofilm were visualized under light microscopy at

magnification of 40X at a numerical aperture of 0.65 (Labomed CxL Monocular, CxLMONO, USA) with ToupView(x86) as imaging system.

5.3.6.2 Scanning Electron Microscopy

For SEM analysis, the biofilms of *C. sakazakii* isolates were obtained on glass cover slips as described earlier. After 48 h, the dehydrated biofilms were coated with the thin layer of gold and examined under SEM (Hitachi S-3400N, Tokyo, Japan) using an accelerating voltage of 10kV.

5.3.6.3 Fluorescent Microscopy

The biofilms on the cover slips were also visualized by fluorescence microscopy, by means of the LIVE/DEAD *Bac* Light bacterial viability kit (L10316, Invitrogen-Molecular Probes, USA) to stain cells over a 15 min period in the dark as per manufacturer's instructions. The bacteria were observed at 400X magnification using a fluorescence microscope BX53 (Olympus Microscopy, USA) equipped with imaging system Qiclick^{TM} ((Olympus, USA). The kit is composed of green-fluorescent nucleic acid stain (SYTO 9) and the red-fluorescent nucleic acid stain (Propidium iodide). The green stain can label bacteria with intact membranes and with damage membranes. In contrast, the red stain penetrates only bacteria with damaged membranes and has a diminution in the green fluorescence when both dyes are present. The excitation/emission range of the green stain is 470/510-540 nm and 470/620-650 nm for the red stain.

5.3.7 Statistical Analysis

All the experiments were run in triplicates on a single plate on three different days. The mean values were calculated for biofilm formation and percent biofilm inhibition and the comparison between the means was done by ANOVA and Tukey's multiple comparison test ($P < 0.05$) by SPSS software.

5.4 Results

5.4.1 Inhibition of quorum sensing by plant extracts

The present study investigates the anti-quorum sensing activity of methanolic extracts of nine plant products (*Piper nigrum, Trigonella foenum-graecum, Coriandrum sativum, Cuminum cyminum, Syzygium aromaticum, Myristica fragrans, Zingiber officinale, Allium sativum and Cinnamomum verum*) for their potential to inhibit biofilm formation in *C. sakazakii* strains.

The qualitative analysis of the extracts (dissolved in 10% DMSO) against biosensor strains exhibited QS inhibitory activity. Minimum Inhibitory Concentration (MIC) was determined for the plant extract (50-300 ppm) against *C. sakazakii* strains. The test extract showed growth inhibition against all the test strains beyond 150 ppm, the MIC was found to be 200 ppm for garlic and clove extract and 250 ppm for rest of the extracts. Hence, in the present study, sub-MIC concentrations (50-150 ppm) of test extract were used for further experiments. The formation of the halo zone around the well was observed for *P. nigrum* and *Cor. sativum* with 15 and 13 mm zone of violacein inhibition against *C. violaceum* CV026. Similar results were observed for the QSI using *A. tumefaciens* NTL4(pZLR4) in which production of blue-green color was inhibited in the presence of plant extract. These results demonstrated that extracts might have obstructed the interaction between the added AHLs (e.g., C-6 and3-oxo-C8 AHL) and their receptors (e.g. CviR for *C. violaceum* CV026 and TraR for *A. tumefaciens* NTL4(pZLR4). The loss of blue-green color in *A. tumefaciens* NTL4(pZLR4) (with added AHL) was detected mainly in *Cin. verum* and *Cum. cyminum* with a zone of pigment inhibition ranging from 10-12 mm. However, the other plant extracts did not show anti-QS activity. No inhibition was observed with 10% DMSO used as control.

The plant extracts resulted in decrease in violacein production in *C. violaceum* CV026. A maximum of 78% inhibition in violacein production was observed with *P. nigrum* alone (Fig. 5.2). However, a 49.0 and 34.5 % reduction in violacein production was observed with extracts from *Cor. sativum* and *A. sativum*, respectively. However, significant loss (68%) of blue-green color was detected in *A. tumefaciens* NTL4(pZLR4) with extracts from *Cin. verum* while *Cum. cyminum* and *P. nigrum* resulted in a reduction of 21.5 and 16.2%, respectively (Fig. 5.2). Negligible effect on inhibition was noted by other plant extracts.

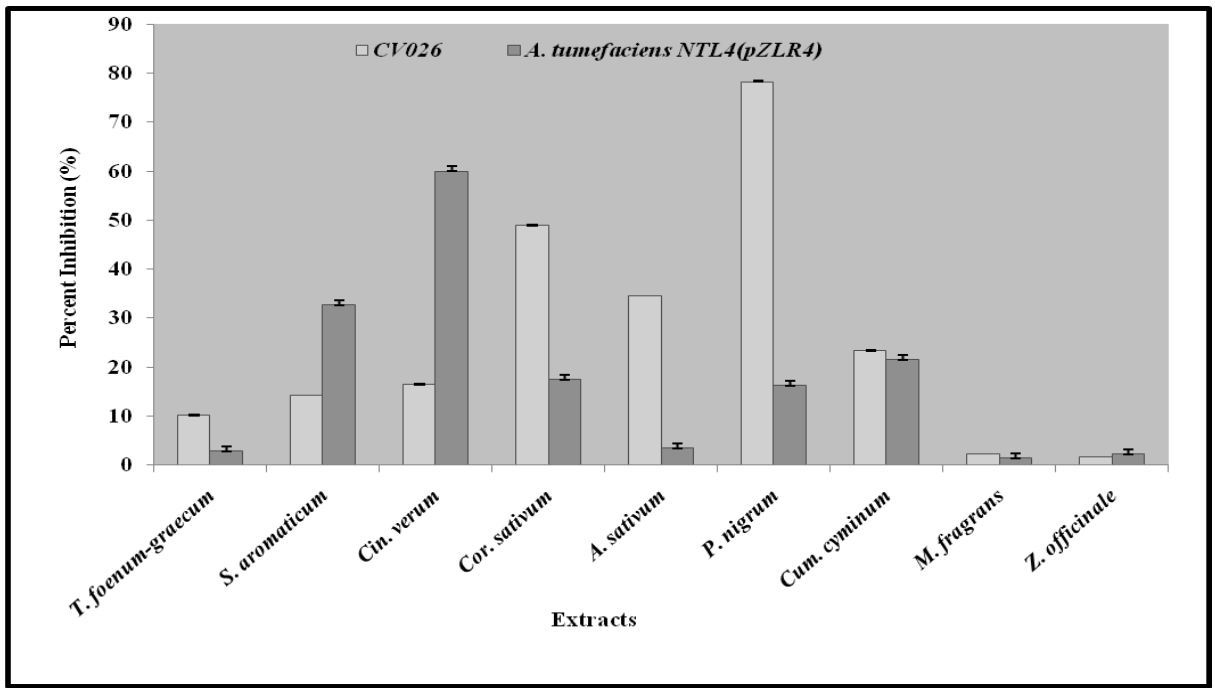

Figure. 5.2: Quantitative analysis of inhibition of violacein and blue-green pigment CV026 and in *A. tumefaciens* NTL4(pZLR4) by plant extracts.

No zone of growth inhibition was observed for plant extracts against the test strains of *C. sakazakii* which confirmed that the halo-effect created in bioassays was due to non-pigmentation (QS mediated process) in cells adjacent to the well indicating that the inhibitions caused by the plant extracts were solely due to anti-QS activity.

5.4.2 Plant extracts influence *C. sakazakii* biofilm formation

Confirmation of the biofilm-inhibitory activity of the nine plant extracts was determined at at a dose of 100 ppm of plant extracts. A significant biofilm inhibition was observed with the plant extract as compared to the negative control, when the extracts were used at a dose of 100 ppm ($P < 0.05$). The extracts were divided into three groups depending on percent biofilm inhibition (high, > 50%; moderate, 50-25% and minimal, < 25%) against *C. sakazakii* isolates. Four (*P. nigrum, S. aromaticum, T. foenum-graecum and Cin. verum*) of the nine extracts investigated, possessed higher anti-biofilm activity against the strains (Table 5.2). While the other extracts shows moderate or minimal inhibitory effect on the pathogens. The *P. nigrum* and *Cin. verum* showed a maximum reduction in biofilm biomass ranging from 55-75% against all the tested strains. Moderate inhibitory activity was exhibited by *Cor. sativum* (36-53 %) and *A. sativum* (23-56 %) while *Cum. cyminum, M. fragrans* and *Z. officinale* showed minimal activity against biofilm forming *C. sakazakii* isolates.

Table 5.2: Effect of plant extracts on inhibition of biofilms (%) for *C. sakazakii* strains.

Isolates	Total Biofilm (OD 570nm)	Biofilm inhibition (%)								
		T. foenum-graecum	*S. aromaticum*	*Cin. verum*	*Cor. sativum*	*A. sativum*	*P. nigrum*	*Cum. cyminum*	*M. fragrans*	*Z. officinale*
E604	1.79	47.16^{e}	25.47^{b}	73.85^{h}	48.83^{e}	56.61^{f}	68.85^{g}	31.6^{c}	36.54^{d}	11.67^{a}
ATCC 12868	1.65	35.15^{a}	55.09^{de}	56.36^{e}	50.9^{cd}	56.36^{e}	72.12^{f}	40.6^{b}	49.81^{c}	44.84^{b}
N13	1.11	58.34^{f}	23.24^{c}	41.65^{e}	36.17^{d}	23.69^{c}	65.88^{g}	27.2^{c}	2.87^{a}	14.18^{b}
N15	2.33	48.67^{b}	53.42^{c}	59.79^{d}	54.4^{c}	47.39^{b}	60.65^{d}	39.26^{a}	41.57^{a}	40.5^{a}
N112	1.76	59.38^{d}	65.34^{ef}	69.93^{f}	53.03^{c}	45.54^{b}	61.99^{de}	47.36^{b}	43.44^{b}	38.06^{a}

Data bearing same superscript letters are not significantly different ($p < 0.05$) among tested methnolic plant extracts as determined by 2 sided Tukey's – post-hoc range test between replication.

The results of light and SEM microscopic images revealed clear differences in biofilm structure between biofilms treated with *P. nigrum* extract and the untreated control (Fig. 5.3). The control slides showed a well developed biofilm growth of the test strain, whereas, on treatment with plant extract, scattered cell growth was observed on the glass slide. Further, fluorescent microscope images indicated well developed biofilm in control whereas the strains were treated with extract developed poor biofilm (Fig. 5.3). These observations clearly reveal the ability of *P. nigrum* extract to disturb the mature biofilms.

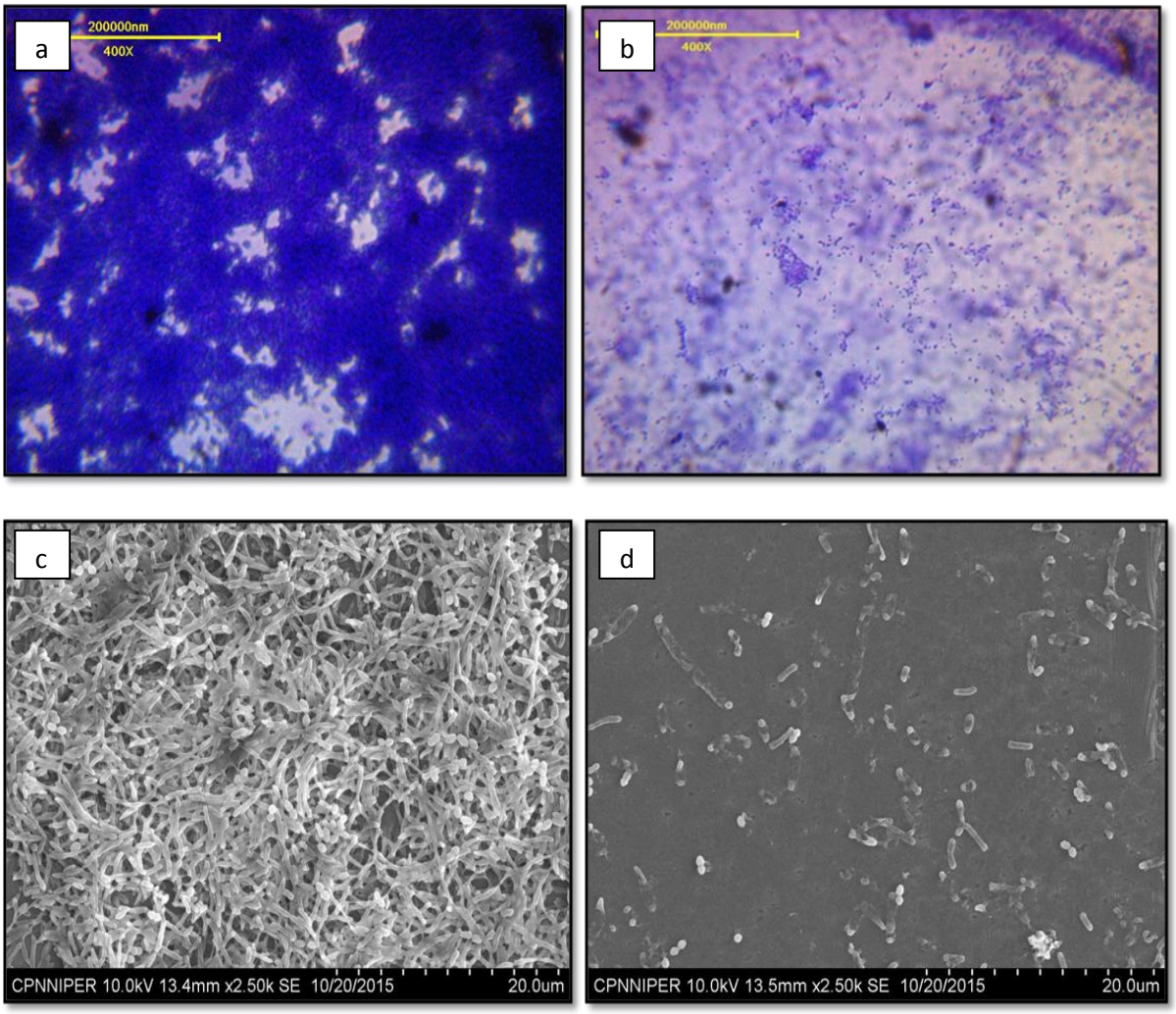

Figure 5.3: Light and Scanning electron microscope images of biofilms of *C. sakazakii* isolate grown in the absence (a, c) and presence of methanolic extract of *P. nigrum* (100 ppm) (b, d), respectively

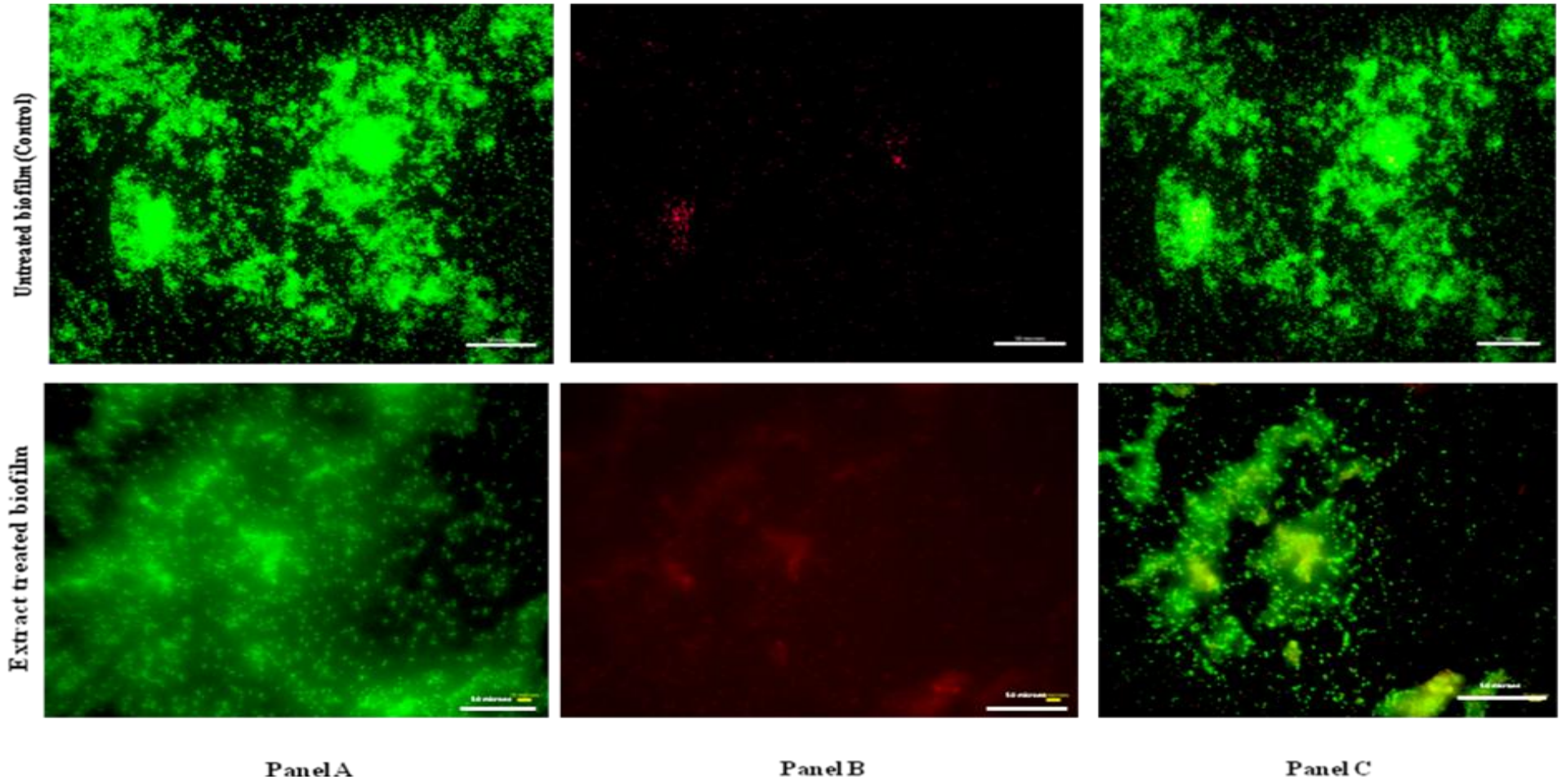

Figure 5.4: Fluorescent microscopic images of biofilms of *C. sakazakii* grown in the absence and presence of methanolic extract of *P. nigrum* (100 ppm). Panel A (left) is the image obtained from the green channel, panel B (center) from the red channel and panel C (right) is a merged image.

5.5 Discussion

The present study investigated the anti-QS activity and biofilm inhibitory effect of plant extracts in *C. sakazakii* strains. The screening of anti-QS potential of natural plant extracts was determined based on their ability to inhibit QS based biofilm formation in *C. sakazakii*. Firstly, the anti-QS potential of plant extracts was analyzed using C. *violaceum* CV026 and *A. tumefaciens* NTL4(pZLR4) as biosensor strains. The preliminary screening among the different plant extracts indicated a strong quorum quenching activity in the methanolic crude extract of *P. nigrum* and *Cin. verum* by inhibiting pigment production in both biosensor strains. Interestingly, the extracts illustrated a pigment less zone around the wells with both the biosensor strain. The lawn of bacteria in this zone indicated viable cells with loss in their QS ability. The results also signified that the extracts used in the study did not have any anti-bacterial activity against all isolates. The present data supports well with the findings of Tan *et al*. (2013) who reported the anti-QS activity of methanolic extracts of *P. nigrum* against *C. violaceum* CV026. The eugenol, a major component of *P.nigrum* has been previously reported for its QSI activity at the concentration of 150 and 200 μM which inhibited violacein production in *C. violaceum* CV026 by up to 48 and 56.5 %, respectively (Zhou *et al*. 2013). Niu *et al*. (2006) reported that cinnamaldehyde (compound of *Cin. verum*) is a potential inhibitor of 3-oxo C6 AHL in the biosensor strain *E. coli* and was also found to inhibit QS in *Vibrio harveyi* by inhibiting the 3-hydroxy C4 AHL QS signaling molecule at sub-inhibitory concentrations. The spices such as thyme, *Z. officinale* and turmeric have been described to reduce 41% violacein pigment production in *C. violaceum* (Vattem *et al*. 2007). The outcome of the present study are analogous with those of Khan *et al*. (2009), who reported 92% inhibition of violacein production in *C. violaceum* CV026 by *S. aromaticum* oil.

Screening for anti-QS activity using multiple biosensors strains removes artifact effects. If a plant extracts showed activity with a *C. violaceum* CV026 strain and not *A. tumefaciens* NTL4(pZLR4), this activity might be limited to an aspect of violacein production. In contrast, if a plant extract sample had activity against *A. tumefaciens* NTL4 (pZLR4) only, it might be circumscribed to an issue on long chain signaling molecules. The data represented that four out of nine extracts were efficiently effective at inhibiting QS in both the biosensor strains. However, moderate or negligible QS inhibitory effect was observed upon exposure to *Cum. cyminum*, *M. fragrans* and *Z. officinale* extracts. This may be due to either low concentration

of crude extracts used in the present study or the mechanism by which the compounds affect the QS system.

Some known mechanisms of QS inhibition consist of competitive binding of a signal molecules to cognate receptors, as in the subject of furanones (Manefield *et al*. 1999), and enzymatic degradation of QS signal molecule, as in the matter of AHL acylases (Dong and Zhang 2005).Various other mechanisms have been suggested for the anti-QS activity of the extracts, such as disruption of competition of the AHLs binding to the receptors by degradation of AHLs; blocking AHLs from forming AHL-receptor complex; changing the structures of the enzymes that are involved AHLs synthesis (Manefield *et al*. 1999; Dong and Zhang 2005).

The biofilm formation plays a significant part in the pathogenesis of C. *sakazakii* and developments of these biofilms are based on the signal-mediated QS system. Therefore, an interference with QS may prevent the development of bacterial biofilms and further infections. Biofilm inhibition experiments showed that all the extracts inhibited the biofilm formation of *C. sakazakii* isolates, in a dose-dependent manner. From the data obtained, it is evident that *P. nigrum* and *Cin. verum* were able to inhibit the biofilm formation in *C. sakazakii* isolates (Table 5.2). Very few studies have been conducted to inhibit the biofilms by *C. sakazakii* using various physico-chemical and biological approaches. Earlier study by Amalaradjou and Venkitanarayanan (2011) investigated the efficacy of trans-cinnamaldehyde (TC, a principal component of bark extract obtained from *Cin. verum*). They reported the efficacy of sub-inhibitory concentration of TC for inhibiting biofilm synthesis (560 and 750 µM) and inactivating mature biofilms (23 and 38 mM TC) of *C. sakazakii* at 24 and 12 ^{o}C in the presence and absence of reconstituted PIF on different abiotic surfaces such as polystyrene plates, feeding bottle coupons, stainless steel coupons and enteral feeding tube coupons. *C. sakazakii* was reduced by > 4.0 and 3.0 log cfu/mL after 96 h of exposure to 38 mM and 750 µM TC, respectively. Another study reported that when cell-free culture supernatant (CFCS, 100 μL/mL) of *Paenibacillus polymyxa* was introduced to *Cronobacter* spp. adhered to stainless steel strips at 25 ^{o}C for up to 72 h, the CFCS inhibited *Cronobacter* biofilm formation (Yang *et al*. 2013). Among the chemical approaches, quaternary ammonium and phenolic disinfectants and a combination of peroxyacetic acid and hydrogen peroxide were used and reported to exhibit different levels of lethality towards *Cronobacter*, depending on time of exposure and whether the bacterium is present in a food matrix or as biofilm (Kim *et al*. 2007). The results indicated that the population of planktonic cells of *C. sakazakii* suspended in water (7.22 to 7.40 log cfu/ml) was reduced to undetectable levels (<

0.30 log cfu/mL) within 1 to 5 min upon treatment with disinfectants, whereas numbers of cells in reconstituted PIF were decreased by only 0.02 to 3.69 log cfu/mL after treating them for 10 min. Recently, the effectiveness of a chlorine sanitizer solution against *Cronobacter* biofilms was reported to be affected by the concentration of chlorine solution (100-5000 ppm), its pH (7.0-9.0), and type of surface of conveyer belt (Buna-N or PVC) (Song *et al*. 2014). They observed that the number of viable cells in the biofilm on a Buna-N chip decreased by 3 log and for PVC by 2.21 log after 10 min of exposure to 100 ppm and 400 ppm of pH-adjusted (pH 7) chlorine solution, respectively. However, disinfectants that are regularly used in the hospital, day-care and food service kitchens are reported to be ineffective in eradicating biofilms comprised of *Cronobacter* spp. (Kim *et al*. 2007).

The results obtained in this present investigation indicated that the extracts not only reduced the biofilm biomass (as quantified by CV staining), but also reduced the microcolony formation, which was more evident from the light microscopic (Fig. 5.3) and fluorescent microscopic images (Fig. 5.4). Herein, we observed that when using SYTO9, a diminution in fluorescence was viewed in treated biofilms. The fluorescence of dead cells was more in comparison to that of living cells, this reduction proposes that treatment with QSI resulted in lower cell numbers through reduced attachment and/or increased detachment. These data suggest that the QSI may exert their effect during initial stages of attachment, or, promote detachment at later stages with reduction in AHL activity which indicates interference with the bacterial QS system.

5.6 Conclusion

In conclusion, the tested plant extracts reported in the present investigation efficiently inhibited AHL based QS mechanisms in *C. sakazakii* along with their ability to disrupt the biofilms of the pathogen. In the interest of food safety, this study introduces the QSI and anti-biofilm potential of plant extracts which can be easily incorporated as food ingredient to limit the biofilm forming ability in *C. sakazakii* from different processing environments.

5.7 References

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CHAPTER 6

SUMMARY

The thesis provides an insight into the characterization of biofilm formation, mechanism of quorum sensing in Indian isolates of *Cronobacter sakazakii.* A total of 219 samples of food and environmental commodities collected from different locations of Himachal Pradesh, India were investigated for the presence of *Cronobacter* spp. The products included 23 samples of dairy products, 35 samples of herbs and spices, 11 samples of reconstituted Infant formula and milk powder, 31 samples of medicinal plants, 26 samples of vegetables and fruits, 15 sample of stool sample and 42 samples of miscellaneous food products. The isolates on Tryptone soy broth were subjected to biochemical characterization with *Enterobacter sakazakii* agar α and tentatively identified 58 isolates belonging to *Cronobacter* genera. The 58 tentativelly identified isolates were selected for 16S rRNA and rpoB gene based identification. The results showed that 45 strains were identified by 16S PCR as *Cronobacter* spp. The other 13 isolates did not give the expected PCR product although they were identified as *Cronobacter* spp. by chromogenic assay which imposes the relevance of more than one technique in precise identification of the *Cronobacter* spp. Of these, only 38 strains were identified as *C. sakazakii* by rpoB PCR assay. These were further confirmed by *fusA* DNA (elongation factor), one of the loci in MLST database for difference of *Cronobacter* spp. sequencing analysis which revealed 5 strains as *C. sakazakii*.

These studies further reported the comprehensive data on presence nine putative virulence genes: *omp* (outer membrane protein), *hly* (hemolysin), *cpa* (*Cronobacter* plasminogen activator), *chiF* (chitinaseF), *eitA* (iron acquisition gene), BAM (zinc containing metalloprotease), *sip* (siderophore), *fhaB* (filamentous hemagglutinin) and *iucC* (iron acquisition gene) in well characterized *C. sakazakii* isolates. Our results revealed that among the tested isolates, all the isolates were capable to utilise inositol however, sialic acid utilization and DNase activity was observed in only two isolates, respectively. All the isolates exhibited haemolytic zone on blood agar plate and chitinase activity. With regard to other determinants, different expressions were noted among the isolates. In our study, *C. sakazakii* isolates from plant-based products were found to utilize sialic acid emphasizing that the food product manufacturers need to maintain microbiological standards before incorporating plant based material in the food products. This work also elucidated the presence of the putative

virulence genes on genomic as well as plasmid-borne DNA which plays pleiotropic roles in *C. sakazakii* isolates. We determined the plasmid profiles of *C. sakazakii* strains with a *repA*-targeted PCR assay and found that all the strains were positive for pESA3/pCTU1 and pESA2/pCTU2 but not pCTU3. All the *C. sakazakii* strains in this research were confirmed for the presence of *hly* gene, with one isolate observed negative for *ompA*. The ompA is probably one of the best characterized virulence markers of *C. sakazakii*. The gene *eitA* was frequently present in the isolates as well as in standard strains. It was observed that all the *C. sakazakii* strains lack genes namely *sip*, *fhaB* and *iucC*. The *chiF* gene and BAM were least commonly detected in the isolates. The least presence of *zpx* in our isolates indicates that there must be some other mechanisms in *Enterobacteriaceae* family which might play role in system.

C. sakazakii is a wide-spread foodborne pathogen that can form biofilms on a number of different substances, creating food safety risk. However, there is little information about biofilm characteristics for this species. The present study investigated the biofilm formation, its composition and frequency of biofilm associated genes in *C. sakazakii* isolates. The results showed that all the strains had a capacity to form biofilm and formed extracellular polymeric substances (EPSs) but different contents of EPS. We also observed the higher frequency of genes (*bcsA*, *bcsC* and *flgJ*) associated with cellulose biosynthesis in *C. sakazakii* isolates, which may have a role in attachment and adhesion to the surfaces. cellulose production and biofilm formation.

The bacterial processes such as the formation of biofilms, virulence and bioluminescence, are mediated by quorum sensing (QS) which is a cell-to-cell communication system often mediated by the production of signaling molecules, the autoinducers (AI). Many gram negative bacteria use N-acyl-homoserine lactones as quorum sensing signal molecules. In this study, we sought to find out if the biofilm formation among isolates of *C. sakazakii* is under the control of autoinducing quorum sensing molecules. Biofilm formation among isolates of *C. sakazakii* was assessed and the production of signal molecules were detected using *Chromobacterium violaceum* 026 and *A. tumefaciens* NTL4(pZLR4) biosensor system. Characterization of autoinducers was carried out by FT-IR, HPLC and mass spectrometric analysis. Further detection with the biosensor strain showed that all the isolates produced long chain signal molecules. The LC-HRMS analysis indicated N-undecanoyl-L-Homoserine lactone, N-dodecanoyl-L-Homoserine lactone, N-tetradecanoyl-L-Homoserine lactone, N-

pentadecanoyl-L-Homoserine lactoneN-(β-ketocaproyl)-L-Homoserine lactone, N-octanoyl-L-Homoserine lactone, N-3-oxo-octanoyl-L-Homoserine lactone, N-octadecanoyl-L-Homoserine lactone as novel signaling molecules among *C. sakazakii* isolates. These data are of great significance as the signal molecules aid in biofilm formation which in turn confer various properties of pathogenicity to the isolates including drug resistance. The use of quorum sensing signal blockers to attenuate bacterial pathogenicity is therefore highly attractive, can be considered as futuristic potential drug targets towards eradication of biofilms of *C. sakazakii* from food processing environments.

After establishing the role of QS in biofilm formation, this study was extended with a objective to determine the to quorum sensing inhibition or quorum quenching potential of the common plant products. In the present study, Quorum sensing inhibitory (QSI) activity of plant extracts products (*Piper nigrum, Trigonella foenum-graecum, Coriandrum sativum, Cuminum cyminum, Syzygium aromaticum, Myristica fragrans, Zingiber officinale, Allium sativum and Cinnamomum verum*) was evaluated using the biosensor strain CV026 and *A. tumefaciens* NTL4(pZLR4) for anti-QS and anti-biofilm properties. The methanolic extract of *P. nigrum* and *Cin. verum* cinnamon at 100 ppm showed a higher degree of anti-QS activity without affecting the bacterial growth of *C. sakazakii* isolates as was found to reduce the AHL dependent production of violacein as well as blue-green color bioluminescence and biofilm formation. Moreover, we observed major disruption in the architecture of biofilms of tested bacterial pathogen by the extract as it is more evident from SEM and fluorescent images.

CHAPTER 7

CONCLUSIONS AND FUTURE PROSPECTS

- The present study showed that *C. sakazakii* are highly diverse and share many phenotypic traits with other *Enterobacteriaceae* members highlighting the need to use several methods to confirm the identity of this pathogen. Our results revealed information on frequency of virulence gene i.e., the very high presence of *hly* followed by *ompA* and *eitA* gene which elucidates the role in the pathogenesis of enteric infections and in its systemic dissemination. A larger sample size of each source would be necessary to further address differences observed.

- Biofilm formation by microtiter plate assay varied greatly between different *C. sakazakii* isolates. This study was also elucidated the correlation between biofilm formation and EPS production. To better understand biofilm formation by *C. sakazakii*, further research should investigate the correlation between EPS production and resistance of biofilm cells on the surface against various stresses. The advances in our perceptive of the diverse factors and mechanisms involved in biofilm formation of different Gram-negative bacteria might endow with clues and stimulate expansions in the search for (natural) compounds for prevention and control of pathogenic bacteria in food-processing settings.

- This study has revealed that QS regulation is important for biofilm development. This is the first report describing the expression of long chain AHLs in *C. sakazakii* and confirmed the presence of 3-oxo-C8-AHL. Since QS regulates the expression of many important phenotypes including virulence production, therefore the study of QS in *C. sakazakii* may lead to better understanding its survival in the different environment and host. The tested plant extracts reported in the current investigation efficiently inhibited AHL based QS mechanisms in *C. sakazakii* along with their ability to disrupt the biofilms of the pathogen. In the interest of food safety, this study introduces the QSI and anti-biofilm potential of plant extracts which can be easily

incorporated as food ingredient to limit the biofilm forming ability in *C. sakazakii* from different processing environments.

- Further work should focus on characterization and identification of QS operon model in AHL synthesis and molecular insight into the expression of genes involve in AHL synthesis. The exploration of active compounds from the potential plant extract could be used for inhibition of QS signaling system, which could offer an opportunity to develop new drugs to combat pathogens. The findings reported in the present study are likely to boost the morale of the scientific community to develop effective strategies for the eradication of biofilm formation by *C. sakazakii*.

LIST OF PUBLICATIONS

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- Singh N, Patil A, Prabhune A, Raghav M and Goel G. (2017). Diverse profiles of N-acyl-homoserine lactones in biofilm forming strains of *Cronobacter sakazakii.* Virulence 8:275-281. (IF- 5.418).
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BOOK CHAPTER

Niharika Singh, Sampan Attri, Kavita Sharma and Gunjan Goel (2016). Indigenous Fermented Foods and Beverages. In Frontiers in Food Biotechnology. NOVA Publisher, pp 47-74 [ISBN: 978-1-63484-679-0].

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APPENDIX

APPENDIX

Ingredients	Amount
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
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
Carbohydrate Fermentation Broth	
Peptone	10.0 g
Sodium chloride	5.0 g
Phenol red	0.018 g
Distilled water	1L
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
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
Distilled water	1L
MRVP Medium	
Buffered peptone	7.0 g
Dextrose	5.0 g
Dipotassium phosphate	5.0 g
pH	6.9
Nutrient Agar	
Sodium chloride	5.0 g
Beef extract	3.0 g
Peptone	5.0 g
Agar	15 g
Distilled water	1L
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