IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF SIALIDASE (NEURAMINIDASE) GENE AND ENZYME FROM *Listeria monocytogenes*

BY

Christiana OKERE

(BSc. BIOCHEMISTRY, Unimaid. 2007)
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FACULTY OF SCIENCE
AHMADU BELLO UNIVERSITY, ZARIA

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DECLARATION

I hereby declare that the work in this thesis entitled *"*Identification and molecular characterization of sialidase (neuraminidase) gene and enzyme from *Listeria monocytogenes"* has been carried out by me in the Department of Biochemistry, Ahmadu Bello University, under the supervision of Prof. A.J. Nok (MFR) and Dr. A.B. Sallau. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

OKERE, Christiana

| --- | --- | --- |
| Name | Signature | Date |

CERTIFICATION

This thesis titled "Identification and molecular characterization of sialidase (neuraminidase) gene and enzyme from *Listeria monocytogenes*" by Christiana OKERE, meets the regulations governing the Award of the Degree of Master of Science degree in Biochemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

---------- Date: ----------

Prof. A. J. Nok (MFR)
(Chairman, Supervisory Committee)

---------- Date: ----------

Dr. A.B. Sallau
(Member, Supervisory Committee)

---------- Date: ----------

Prof I. A. Umar
(Head of Department)

---------- Date: ----------

Prof. A.A. Joshua
(Dean, School of Postgraduate Studies)

DEDICATION

To the glory of God, this work is dedicated to,

What I have enjoyed

What I love very much

What I hoped to be larger than.

I dedicate this work to life.

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I would like to express my intense gratitude to my supervisors, Professor A.J. Nok (MFR) and Dr. A.B. Sallau for their excellent guidance, patience, motivation, and continuous support throughout the period of this research work. I could not have imagined having better supervisors and I will never forget your labour upon me in order to make me a better person. It is my earnest prayer that God almighty in his sufficiency would reward you abundantly.

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ABSTRACT

A putative Trans-sialidase gene was identified in *Listeria monocytogenes*, a human and animal pathogen and its product was characterized. The identification was carried out using Basic Local Alignment Search Tool (BLAST), Polymerase Chain Reaction (PCR), sequencing and gene analysis/predictions. The enzyme was partially purified from the supernatant of the cultured organism by ammonium sulphate precipitation and gel filtration, the enzyme eluted as a single peak with an activity of 1.6 μM /min. The enzyme was active between pH 4.0 and 4.5, a 45.8% loss of enzyme activity was found at pH 10.0. and an optimum temperature of 30°C, activity gradually decreased with increasing temperature which was reduced to 26% at 70°C and was almost completely lost at 80°C. Effect of cations on the enzyme activity showed that Hg^{2+} and Al^{3+} at 3mM concentration are potent inhibitors while Mn^{2+}, Zn^{2+} and Ca^{2+} have no effect on the enzyme activity at same concentration compared to the control. The release of the enzyme from the *Listeria monocytogenes* was time dependent, increasing to 44×10^{-3}μM within 60 minutes of incubation. The Trans-sialidase gene was amplified and ligated into pJET1.2 blunt cloning vector to obtain a recombinant DNA which was used to transform *E.coli* DH5α. The transformed DH5α were selected, the recombinant DNA re-extracted was digested with HindIII and subcloned into pET-28b (+). The sequence analysis revealed a 342bp encoding a 114 translated amino acids protein with a calculated molecular mass of 12.132 kDa and isoelectric point of 8.4. A BLASTP search versus a non-redundant protein sequence database revealed that this protein shows the highest sequence identity to TS2 gene of *Trypanosome congolense* (100%). 59.6% for *T. vivax*, *T. brucei*, and *T. evansi*, while *T.cruzi* showed 50% and Sialidase gene of *T. rangeli* 55.1%. All together, these results suggest that *Listeria monocytogenes* Trans sialidase might be an important virulence factor and may be involved in the pathogenesis of this organism.

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ABBREVIATION, GLOSSARRIES AND SYMBOLS

% = Percentage

µl = Microlitre

β = Beta

α = Alpha

Sia = Sialic acid

LM = Listeria Monocytogenes

mg = Milligram

Neu5Ac = N-acetylneuraminic acid

mM = Millimolar

^{0}C = Degree Celsius

hr = Hour

bp = Base pair

ml = milliliter

TLC = Thin Layer Chromatography

PCR = Polymerase Chain Reaction

CHAPTER ONE

1.0 INTRODUCTION

Despite the break through and advances in medical research and treatments, infectious diseases still remain among the leading causes of death worldwide for many reasons which include: emergence of new infectious pathogens; re-emergence of old infectious pathogens and persistence of intractable infectious pathogens (WHO, 2007). These have revealed a number of pathogens such as *Listeria monocytogenes* the causative agent of listeriosis. Listeriosis is a disease of worldwide occurrence that affects all ruminants as well as other animal species and humans. It is characterized by septicaemia, encephalitis and abortion or stillbirth in some cases (Liu, 2006; Guillet *et al*., 201 `0). However, only a fraction of the infected persons manifest symptoms of the disease while others remain asymptomatic carrier but may harbor the organism in their gastrointestinal tracts. Individuals prone to symptomatic listeriosis include infants, the elderly, pregnant women, and those with underlying conditions that impair their immune functions, such as malnutrition, major surgery, low gastric acidity, and lack of physical fitness (Indrawattana *et al*., 2011). Listeriosis is acquired by eating food contaminated with bacteria called *Listeria monocytogenes* (*L. monocytogenes*) or eating infected animals or their products. Pregnant women infected with *L. monocytogenes* may have spontaneous abortions, stillborn foetuses or newborns with meningitis and death in some cases (Mandell *et al.,* 2009; Kliegman *et al.,* 2011). Recently it was found to cause acute, self-limiting febrile gastroenteritis in healthy individuals. If infection spreads to the nervous system, symptoms such as headache, stiff neck, confusion, loss of balance or convulsions can occur (Robin *et al*., 2006; Schuppler and Loessner, 2010; Siegrist, 2010).

Host cell invasion is one of the stages involved in listeriosis pathology (Seveaua *et al*., 2007), and adhesion of *L. monocytogenes* to host cells employs mechanisms which are complex and not well understood. Although infection begins with the successful entry and survival of the organism within macrophages and signaling by innate immune system is crucial for detection to initial response to microbial pathogens, it has been shown that interaction between the host cell and the intracellular pathogen after cell entry, marked disruptions of host cell functions in other to be survive (Pollpeter *et al*., 2011; Villanueva *et al*., 1994; Randhawa 2008).

Sialic acid is predominantly found as the terminal carbohydrate occuring naturally in prokaryotes as nine-carbon keto sugar acid derived from *N*-acetylneuraminic acid (Neu5Ac) (Hai *et al*., 2006). Many commensal and pathogenic bacteria use environmental (host) sialic acids as sources of carbon, nitrogen, energy, and amino sugars for cell wall synthesis (Ito *et al*., 2012). The involvement of sialic acids in cell-cell and cell-extracellular matrix interactions is now well established. The localization of these monosaccharides at exposed positions of molecules and cells, their structural diversity, and their strong electronegative charge provide the basis for sialic acid function as signaling molecules (Schauer, 2000). Sialic acid has been proven to be crucial during the life cycle and survival of pathogens in the host.

The breakdown or transfer of sialic acid residues and sialoconjugates by sialidases/trans-sialidases contributes to a wide range of important biological functions such as cellular interaction and conformational stabilization of glycoproteins in the cell membrane that could expose or mask receptors for ligand binding and other enzymatic interactions (Jihong *et al*., 2011). While the role of sialidases/trans-sialidases in sialic acid metabolism has been known in other pathogens like *Tannerella forsythia*, is yet to be explored in *L. monocytogenes*.

Sialidases or neuraminidases (EC 3.2.1.18; N-acylneuraminyl glycohydrolases) are a family of exo-glycosidases that catalyze the hydrolytic cleavage of non-reducing sialic acid that are ketosidic linked to the saccharide chains of glycoproteins and glycolipids (gangliosides) as well as to oligo- and poly-saccharides (Eugenio *et al*., 2010). They are tetrameric glycoprotein that consists of identical subunit and are widely distributed in nature, from viruses and microorganisms (such as bacteria, protozoa, and fungi) to vertebrates, but are absent in plants, insects, and yeast (Schauer, 2004).

A different type of sialidase is the "trans-sialidase" expressed by certain pathogenic protozoa (e.g., trypanosomes). These novel enzymes remove sialic acid from mammalian cell surfaces and transfer the sugar directly onto the parasite's own cell-surface acceptor, apparently providing protection from the host immune system. Microbial sialidases and trans-sialidases are powerful virulence factors that may assist invasion, unmask potential binding sites and in addition provides nutrients for some bacteria (Varki and Schauer 2009).

In bacteria, sialidase seems to be of great pathogenic significance, enhancing virulence by cleaving terminal sialic acid residues from carbohydrate polymers such as glycoproteins, gangliosides, and other sialoglycoconjugates found throughout the body, making them available as nutrients to sialidase-producing bacteria and thus increase bacterial attachment or toxin binding to host cells. After the loss of sialic acid from the host cell membrane, glycoconjugates are more vulnerable to the action of other glycosidases and proteases secreted by the bacteria, since the presence of terminal sialic acids on glycoconjugate and red blood cell, and are known to hinder the action of these enzymes (Jihong *et al*., 2011).

The action of this enzyme also contributes to the liquification of mucin barriers lining the endothelia and promotes bacterial survival in mucosal niche in several ways, including,

unmasking of cryptic host ligands used for adherence, participation in biofilm formation and modulation of immune function (Lewis and Lewis, 2012). Furthermore, it has been demonstrated by Coustou *et al*., (2012) that sialidase play a key role in infection and anaemia in animal trypanosomiasis caused by *Trypanosoma congolense*. Sialic acid stimulates biofilm growth of *Tannerella forsythia* as described by Roy *et al*., (2011). Aberrant sialylation in cancer cells is thought to be a characteristic feature associated with malignant properties including invasiveness and metastatic potential, involving sialidase which catalyzes the removal of sialic acid residues from glycoproteins and glycolipids, and has been suggested to play important roles in many biological processes through regulation of cellular sialic acid contents. The altered expression of sialidase observed in cancer would therefore suggest its involvement in the malignant process (Miyagi *et al*., 2004).

To date, however, extensive research has revealed significant insights regarding the molecular mechanisms of *L. monocytogenes* infection but the role of its sialidase/Trans- sialidase gene has not been studied as an input in fighting the high mortality rates associated with listeriosis.

1.1 Statement of Research Problem:

- ✓ Food safety is important, in that effect of unsafe food affects every man, woman, child and the nation at large. *Listeria monocytogenes* constitutes of the major sources of food contamination (WHO 2008).
- ✓ The high mortality associated with infection of *Listeria monocytogenes* and its spread is a major health concern. (Todar, 2003; Allerberger and Wager 2010).
- ✓ Food safety is an important growing public health problem in Nigeria and food-borne disease is a contributor to the huge burden of sickness and death caused by diarrhea as a

result of eating conterminated food by *L.monocytogenes*. This could be due to high adaptability of *L.monocytogenes* which leads to high incidence rate (WHO 2007).

1.2 Justification of Study:

- ✓ Bacterial infections including that of *L. monocytogenes* have been a major health and socioeconomic setback
- ✓ The need to investigate the presence of sialidase gene in the genome of *L. monocytogenes,* so as to understand more about the biochemistry of the disease and the mechanism of its pathogenesis
- ✓ This idea is to exploit possible potential that can be used in drug development against the organism and other listeriosis control measures.
- ✓ There is need to investigate the presence of sialidase gene in the genome of *L. monocytogenes,* to understand more about the biochemistry of the disease and the mechanism of its pathogenesis
- ✓ The identification and characterization of this enzyme will contribute to the reduction in listeriosis by developing potent inhibitors specifically against sialidase and also synthezies sialic acid analogue to target *Listeria* pathogens.
- ✓ In addition, sequencing sialidase gene of *L. monocytogenes* will assist in examining the adaptative changes of *L. monocytogenes* to growth in different environments.

1.3 Aim and Objective:

- Identify Sequence and Characterize sialidase gene / protein of *Listeria monocytogenes*..

Specific Objectves:

The specific objectives of this study include:

i. Identify sialidase gene in *L. monocytogenes* using Bioinformatics tool.

ii. Amplify and clone *L. monocytogenes* sialidase gene

iii. Sequence Trans-sialidase gene of *L. monocytogenes*.

iv. Characterize the gene and make comparison with other known sialidase genes.

v. Partial purification and characterization of the enzyme.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History of Listeriosis

The genus *Listeria* unlike *Salmonellae, Yersinae, Shigellae* and other pathogenic organisms was not named after its discoverer. *Listeria*, which causes listeriosis, was discovered by Murray and his colleagues in 1926, after a careful investigation of an outbreak of the disease among laboratory animals in Cambridge. They called the organism Bacterium monocytogenes. Other generic names such as *Erysipelothrix* and *Listerella* were also suggested. *Listeria* was proposed by Harvey Pirie in 1940. This name was given in order to honour Lord Dr Lister who first observed antisepsis. Thereafter, the disease was known as listerellosis for many years until the present name Listeriosis came into general use. The disease was first reported in sheep amongst the large animals in New Zealand and Western Australia in 1925 while in humans the first report was in 1929 (Gill, 1925 and Nyfeldt, 1929). However, Seeliger (1990) reported that listeriosis may have occurred before the advent of Murray *et al*., (1926) and were discussed under names like pseudotuberculosis and argentophilic septicaemia which were completely unknown in laboratory animals.

The genus *Listeria* could have seven distinct species based on the biochemical and physiological properties. The genus is closely related to the genera Brochothrix and Bacillus (Collin *et al*., 1991). The seven species are considered to represent two closely related, but distinct lines of descent, with *L. monocytogenes, L. ivanovii, L. innocua, L. seeligeri* and *L. welshimeri* forming one group known as true Listeria organisms while *L. grayi* and *L. murrayi* represents the other line (Collins *et al*., 1991; Jones, 1992). However, only *L. monocytogenes* and *L. ivanovii* are

known to be pathogenic for man and animals. Unlike *L. monocytogenes* which causes listeriosis in humans and animals, *L. ivanovii* is exclusively an animal pathogen and accounts for about 10% *Listeria* infections in animals (Jones, 1992). The organism could be rod- shaped, coccoid or filamentous. The shape depends on nutrients, environmental and cultural conditions (Hass and Kreft, 1988). Among pathogenic bacteria, there are three distinctive properties that are peculiar to the genus *Listeria* which are of interest. These properties include the ability of the genus to survive and grow within a broad temperature range (0°C – 44°C), wide pH range (5.0 – 9.5), ability to tolerate sodium chloride concentration of 5 – 25% and a variety of other toxic chemical such as tellurite, acridine dyes, lithium chloride, Nalidixic acid and cycloheximide. On blood agar plates, pathogenic *Listeria* exhibits zones of haemolysis indicating destruction of the erythrocytes by the toxic products of the organism. More so, it has the ability to survive in cells of the host‘s immune system or macrophages (Hass and Kreft, 1988; Schuchat *et al*., 1991b).

2.1.1 Types of Listeriosis

2.1.1.1 *Listeriosis in pregnancy:*

Listeriosis can be passed to an unborn baby through the placenta even if the mother is not showing signs of illness, this occurs mostly during the third trimester, and is characterised by a “flu like” illness with symptoms such as fever, back pain, and diarrhea (Acha *et al*., 2003), many cases of the infection is subclinical or inapparent; however, intrauterine infection of the foetus can lead to foetal death, spontaneous abortion, premature delivery or the birth of a foetus that dies shortly after birth (Doganay *et al*., 2003). Surviving of newborns with listeriosis is often classified as “early onset” or “late onset”. Early onset neonatal listeriosis is due to transplacental infection often presents as pneumonia and/or sepsis. Severe disease can result in widespread granulomas (granulomatosis infantisepticum). Late onset neonatal listeriosis is said to occur from infection during birth, with neonates showing symptoms of meningitis one to several weeks after

birth. In both early and late onset neonatal listeriosis, the mortality rate ranges from 20 to 30% (Krauss *et al*., 2003).

2.1.1.2 *Listeriosis of the CNS:*

Meningitis and encephlitics is the most frequently recognised listerial infection of the CNS. Common symptoms includes high fever, nuchal rigidity, tremor and/or ataxia, and seizures. The most common form of non-meningitic CNS listeriosis is encephalitis involving the brainstem (rhombencephalitis) (Doganay *et al*., 2003).

2.1.1.3 *Febrile gastroenteritis:*

A non-invasive form of listeriosis that manifests as symptoms typical of gastroenteritis, for example, fever, diarrhoea, and vomiting (Roberts *et al*., 2003).

2.1.1.4 *Glandular listeriosis*:

This resembles infectious mononucleosis with swelling of the salivary glands and nuchal lymph nodes (Krauss *et al*., 2003).

2.1.1.5 *Local listeriosis*:

Can manifest as papules and pustules on the hands and arms following direct contact with infectious material, and can be accompanied by constitutional symptoms (fever, myalgia, and/or headache) (Regan *et al*., 2003).

2.1.1.6 *Typhoid listeriosis*:

Characterised by high fever and is particularly frequent in immunocompromised individuals (Krauss *et al*., 2003).

2.1.2 Route of transmission

The predominant mode of *L. monocytogenes* transmission is by ingestion of contaminated food. *L. monocytogenes* can also be transmitted transplacentally from mother to child during pregnancy and via the birth canal during birth (Mylonakis *et al*., 1998).

Direct contact with diseased animals may lead to transmission to farmers and veterinarians during the birth of domestic farm animals (Regan *et al*., 1997).

Non monomial infections and person-to-person transmission (excluding vertical) are recognised but rare (Low *et al*., 1997).

2.2 Occurrence of *Listeria Monocytogenes*

The emergence of *L. monocytogenes* as a food-borne bacterial pathogen has been traditionally regarded as opportunistic infection which affects especially individuals with low immunity. It is of worldwide occurrence affecting all ruminants, humans as well as other animal species such as horses, swine, rabbits, and poultry making it of zoonotic importance (Todar, 2003; Andrea, 2006). The organism has the ability to infect many cell lines due to its capacity to cross the blood-brain, intestinal and placental barriers the three human barriers and is also challenging due to its resistance to antibiotics (Mohammed *et al*., 2009).

Listeria monocytogenes and other *Listeria* species are highly adaptable asporogenous motile organisms and this has made the ecological niche of *L. monocytogenes* difficult to define. The organism is found in the soil, vegetation, plants, mud, water, sludge and in the intestinal tract of man, animals and birds (Gahan & Hill 2005). Their wide distribution in the environment may probably be due to their ability to grow at temperature as low as 4°C. This feature makes them

very difficult to eliminate from food and dairy industries (Griffiths, 1989; Schuchat *et al*., 1991). The listerial biofilms formation and attachment on objects or substrates is said to involve factors such as motility organelles, specific attachment structures, long- and –short range attraction forces, hydrophobic forces and polysaccharides production (Regan *et al*., 2005) Swaminathan and Gerner-Smidt (2007) reported that *L. monocytogenes* could radically alter its metabolism in order to utilize complex substrates by converting them into useable metabolites with the aid of bio emulsifiers in their biofilm colonies. These bioemulsifiers assist the organism in attaching themselves to surfaces in order to survive in nutrient – poor and otherwise hostile environments. The attachment of the bacterium *L. monocytogenes* to a solid substrate is usually followed by microcolony formation. This microcolony then surrounds itself with extra-cellular polysaccharides (a glycocalyx) (Sutherland, 2003). The glycocalyx, besides being a physical barrier, is strongly anionic and serves as protection to the microcolony. These microcolonies ultimately become a firmly attached biofilm (Sutherland, 2003; Marshall, 1986).

The biofilm formation or surface attachment of *L. monocytogenes* and other *Listeria* species has been investigated, Archer (1990) reported that attachment and microcolony formation or biofilm formation by *L. monocytogenes* could be very difficult to eradicate once formed. It was also observed that studies with biocidal agents conducted on any non biofilm formation could lead to false sense of security (Acher, 1990). Thus *Listeria* could disappear for a time after the application of a biocide, but will certainly reappear.

L. monocytogenes has also been reported in processed food as reported by Borucki *et al* (2003). The contamination of food by *L. monocytogenes* is thought to occur most frequently in food-processing environments, where the organism persist due to their ability to attach to stainless steel and other surfaces, mediated by the production of multicellular biofilms that are resistant to

disinfection and from which cells can become detached and contaminate food products. However, they have the properties that permit them to survive effectively in food processing environments. This is due to their ability to grow under anaerobic conditions (without oxygen) and a wide variety of conditions such as high salt, acid environment, freezing and refrigerator temperatures (Kornacki 2008).

The importance of the underlying diseases was illustrated in a series of 165 adults with culture-proven *Listeria* infection: 69 percent of cases in nonpregnant adults occurred in patients with cancer, AIDS, organ transplant recipients, or corticosteroid therapy (Donnelly, 2001). The persons affected by Listeriosis are a well-defined high-risk group that includes: pregnant women, neonates and immunocompromised adults. The mortality rate associated with listeriosis is 20% (Bille *et al*., 2003)

2.3 Incubation Period of *L. Monocytogenes:*

In foodborne diseases, the incubation period is the delay between the consumption of a contaminated product and the onset of first symptoms of the disease. Unlike common food borne diseases such as salmonellosis, the incubation period of listeriosis can be long (Goulet *et al*., 2013). Linnan *et al*., (1988) calculated incubation period of *L. moncytogenes* median of 31 days (range, 11 to 70) in four patients with single exposure. However, the incubation period for *Listeria* gastroenteritis is substantially shorter than the incubation period for invasive disease (Dalton, 1997). For gastroenteritis, the mean incubation period is 24 hours (range 6 hours to 10 days) (Dalton, 1997), whereas for invasive listeriosis the median incubation period is 35 days (range 1 to 91 days).

Bjørn *et al*., (2010) reported that incubation period in the outbreak of *L. monocytogenes* was significantly shorter than previously reported, and that the fact may be due to the high number of ingested bacteria and that multiple aspects of listeriosis make the determination of a precise incubation period difficult. Incubation period can vary depending on the mode of transmission and dose received, but typically ranges from 1 to 4 weeks, and can be as high as several months. Febrile gastroenteritis as a result of *L. monocytogenes* has a short incubation period, typically 18 to 20 hours (Bille *et al*., 2003; Krauss *et al*., 2003).

2.4 Microbiology of *Listeria monocytogenes*

Listeria monocytogenes is the only *Listeria* species that regularly infects humans, although rare cases of human infections with *Listeria ivanovii* (a pathogen of ruminants) and *Listeria grayi* have been reported. It is an aerobic and facultatively anaerobic, motile, beta-hemolytic, non-spore-forming, short, gram-positive rod that exhibits characteristic tumbling motility by light microscopy (Lorber, 2010). The organism occurs singly or in short chains. On Gram stain, it may resemble pneumococci (diplococci), enterococci or diphtheroids (Corynebacteria), or be gram-variable and be confused with Haemophilus species (Lorber, 2010). In particular, when a positive blood or cerebrospinal fluid culture is preliminarily identified as diphtheroids, the clinician should consider the possibility that the isolate represents *Listeria* (Lorber, 2010).

Listeria produces a characteristic appearance on blood agar with small zones of clear beta-hemolysis around each colony. It grows well at refrigeration temperatures (4° to 10°C), rarely the "cold enrichment" technique and selective media are used when attempting to isolate *Listeria* from mixed cultures, such as stool (Bille, 2007).

2.5 Taxonomy and General Characteristics of Listeria Species

Taxonomically, *Listeriae* may be confused with member of other genera such as Brochothrix, Erysipelothrix, Lactobacillus, Kurthia and Jonesia, however, Brochothrix is non-motile; grow at 30°C but not at 35°C, while listeria intracellular parasites, able to enter, survive and multiply inside phagocytic and nonphagocytic cells (Bille, 2007).

. It is motile and grows at 0-37°C, also spreads directly from cell to cell by actin-based intracellular movements. Erysipelothrix is non-motile and catalase-negative. Kurthia is strictly an aerobe and does not produce acid from glucose. Jonsia denitrificans is Voges-Proskaüer - negative and reduces nitrate. Lactobacillus is largely non-motile; however, some strains are motile and catalase – negative with few exceptions (Jones, 1992).

2.6 Physiology and Metabolism in Listeria Monocytogenes

2.6.1 Physiology of *Listeria monocytogenes*

The physiology and metabolism of *L. monocytogenes* affect the pathogenicity of the organism These parameters include the facultative anaerobic growth in the presence of respiratory enzymes and co-factors, catalase and super oxide dismutase production which circumvent the macrophagic intracellular oxidative bursts. Others are the production of haemolysin phosphatase C while specific factors that aid in the attachment to the intestinal peyers patches and the crossing of placental and brain membrane barriers. These physiological properties have relevance to the organism‘s virulence and growth as well as infectivity. *L. monocytogenes* catalase positive, oxidase negative, fermentation of carbohydrates to acid but not to gas, hydrolysis of esculin and sodium hippurate, methyl red positive, ammonia production from

arginine, negative reaction for hydrogen sulfide production, indole negative, nitrate reductase negative, no gelatin liquefaction, no hydrolysis of starch and no urea hydrolysis. *L. monocytogenes* is partially inhibited by 0.02% azide and cyanide, and does not reduce nitrates to nitrite or produces hydrogen sulphide. On enzyme production, it is negative for urease, coagulase, arginine decarboxylase and the decarboxylases of glutaminase and arginine dihydrogenase. Moreso, the organism has no cytochromic C oxidase activity and is negative for tribietyrinase (Lianou and Sofos 2007). The biotypical and serological reactions of *L. monocytogenes* and other *Listeria* species like other organisms occur with substances at the cell-wall level. Based on cell wall composition, they are classified by the structure and composition as Gram-positive organisms, but are similar to Gram-negative pathogens in virulence characteristics (Fiedler, 1988).

2.6.2 Metabolism in Listeria monocytogenes

The metabolism of sugars by *L. monocytogenes* and other *Listeria* species for growth is homolactic. This is, however, dependent on the redox potential of the medium. Thus, the aerobic or facultatively anaerobic nature of the growth medium influences the type of acid produced (Pine *et al*., 1988). Lactic acid is the only product formed when grown anaerobically. When grown aerobically, Lactic acid and acetic acid or only acetic acid will be produced. Acetic acid accounts for 80-100% of products by sugar metabolized by *L. monocytogenes* and other *Listeria* species aerobically (Pine *et al*., 1988). Based on temperature, at 37°C glucose is fermented by the classical Embden – Meyerhoff anaerobic glycolysis pathway to yield pyruvate and lactate with the production of up to 6mol of ATP per mole of glucose (Atlas, 1984). The growth of *L. monocytogenes* and other *Listeria* species is proportional to the amount of sugar but dependent on the nature of the sugar being utilized. Studies by Pine (1988) indicated that *L. monocytogenes*

with glucose as a substrate under aerobic conditions would have a generation time of approximately forty (40) to sixty (60) minutes 5°C and approximately 24 hours at the same temperature. Using the same substrate, the growth of *L. ivanovii* and *L. seeligeri* at 37°C is slower with a generation time of seventy-five (75) minutes and thus growth is delayed compared with *L. monocytogenes* at 5°C. It was also reported that in aerobic and semi-anaerobic conditions, *L. monocytogenes*, *L. innocua, L. welshimeri, L. grayi* and *L. murrayi* would exhibit excellent growth on a variety of common organically complex laboratory media, while *L. seeligeri* and *L. ivanovii* would show less growth (Pine *et al*., 1988).

2.7 Isolation and Identification of Listeria Monocytogenes

Most *Listeria* species grow well in common bacteriological media, making it easy to cultivate. A variety of selective and differential media are available into which appropriate antibiotics are incorporated to inhibit growth of bacteria other than *Listeria* (Chen *et al*., 2006). However, the isolation of the organisms from non-sterile samples like soil, water, faeces, food and other environmental sources rely mostly on cultural procedures. These procedures are cold enrichment in non-selective and selective enrichment broths followed by plating on selective agar plates. Nevertheless, the organisms are often isolated from blood, cerebrospinal fluid, amniotic fluid, placental tissue and foetal tissue biopsy materials. These latter specimens can be directly cultured on agar containing 5% sheep, horse or rabbit blood and Nalidixic acid, Palcam agar and Oxford Listeria selective agar (Schuchat *et al*., 1992; Swaminathan and Gerner-Smidt, 2007). Other agents readily utilized to enhance the isolation of *L. monocytogenes* include aesculin. This is a glycoside found in the leaf and bark of horse chemsul trees. Hydrolysis of aesculin or salicin, a glycoside found in willow branches, releases the glucose for metabolism and leaves the aglucone. This gives rise to O – diphermols or catechols. Catechols can form co-ordinate

compounds with transition metals such as iron. This reaction is used by many bacteria in the formation of iron – binding siderophores. In the case of *Listeria* species, the released catechol is utilized as an indicator of aesculin hydrolysis to aesculitine forming dark precipitates with the iron salts in the medium (Curtis *et al*., 2006).

Cold enrichment at 4°C in non- selective broth medium has been utilized for the isolation of the *Listeria* species since Lado and Yousef *et al*., (2007) reported the psychrotropic characteristics of *L. monocytogenes*. In view of the disadvantages of cold enrichment in non-selective broth, enriched medium containing some inhibitory chemicals and antibiotics have been developed for the isolation of *Listeria* species from specimens (Severino *et al*., 2007; Swaminathan and Gerner-Smmidt 2007). The reagents mostly used in liquid enrichment media to render them selective for *Listeria* species include nalidixic acid, Lithium chloride, acriflavine, glycine, anhydride, nitrofurazone and potassium thocyanate and potassium tellurate. However, in recent times, the commonly used selective enrichment broths are: (a) United States Food and Drug Administration Medium (USFDA) – prepared from tryptic soy broth, acriflavine and nalidixic acid (Lovett, *et al*., 1987), (b) United States Department of Agriculture – Food Safety and Inspection Service (USDA – FSIS) broth – containing asculin, acriflavine and nalidixic acid, (c) Oxford Listeria selective agar and (d) Palcam broth agar (Curtis *et al*., 1989; Van-Netten *et al*., 1989). McBride and Girrad (1960) developed the first selective plating agar medium for the cultivation of *L. monocytogenes* and other *Listeria* species. Their formulation contained blood, phenylethanol agar-base, lithium chloride and glycine. However, in 1988, Lovett modified this medium by the removal of the blood component. This modification also favoured isolation of *L. monocytogenes* from foods.

In addition to the above, Lee and McClain (1986) modified the selectivity of McBride medium by increasing the concentration of the constituents and substituted glycine anhydride, moxalactam, for glycine, and called the improved, medium Lithium chloride – phenyethanol – moxalactam (LPM) agar. This is highly selective for the isolation of *L. monocytogenes*, even from heavily contaminated specimens. Nevertheless, neither the former or improved McBride had any *Listeria* differential property except by examination using Henry‘s illumination which gives blue-grey colouration on *Listeria* organisms. In order to overcome the disadvantages of the earlier formulated growth media, some workers sought to develop differential media for presumptive identification of *Listeria* species on solid culture media. Thus Curtis *et al*., (1989) and Van-Natten *et al*., (1989) developed separately two differential *Listeria* media (Oxford Listeria agar and Palcam agar) respectively by incorporating differential agents into the media. On the Palcam agar, *Listeria* colonies appear grey-green, approximately 2mm in diameter and have black – sunken centers with a black – halo against cherry red background (Van-Natten, *et al*., 1989). *Listeria monocytogenes* and other *Listeria* species colonies appear black on Oxford agar having 1mm – 3mm in diameter after 24-48 hours.and are surrounded by black halo against grey background of the medium (Curtis *et al*., 1989).

A number of cell surface proteins and virulence factors, critical for invasion of enterocytes, subsequent escape into cytoplasm, intracellular proliferation, and cell-to-cell spread have been described in *L. monocytogenes.* These includes internalin A and B (InlA and InlB) which bind to host cell receptors and facilitate entry, together with listeriolysin O (LLO) which breaks down the host cell phagosome once the organism is internalized (Condotta *et al*., 2011; Upadhyay *et al*., 2012) and Hexose phosphate transporter (Hpt) which helps *L. monocytogenes* obtain hexose sugars for metabolism while Actin A molecule helps in the polymerization of actin as a means to

inducing motility whereas phosphatidylcholine phopholipase C, which acts in concert with LLO to help release organisms that are incorporated into vacuoles during cell-to-cell spread (David, 2012). However, the role of sialidase as a potential virulence factors in *L. monocytogenes* is yet to be exploited. Because of their importance in the breaking down of glycoprotein conjugates, these enzymes could facilitate attachment and enhance pathogenesis (Aruni *et al*., 2011).

Intracellular pathogens have evolved diverse mechanisms for successful survival in host cells in which they invade the glycoconjugates that decorates the surfaces of many cells, both prokaryotic and eukaryotic. They play an important role in a wide range of biological processes such as cell–cell and small molecule–cell recognition, sialic acid is one of the prominent glycoconjugates that play such roles (Severi *et al*., 2007). However, it appears that microbes have evolved multiple routes to capture sialic acid either by *de novo* biosynthesis or acquisition from the environment through their sialidase gene or from other sialidase-expressing bacteria living in the same niche or by host sialidases that are activated in the course of inflammation (Sohanpal *et al*., 2004, 2007).

2.8 Disease Mechanisms

Listeria monocytogenes has long been used as a lowgrade intracellular pathogen to study cellular immunity (Kaufmann, 1993), and the more recent advances in molecular biology have further assisted in the understanding of listeriosis at the cellular level (Vazquez- Boland *et al.,* 2001). However, much remains obscure as to how this disease occurs. In addition to the following brief account, there may be as yet uncharacterized differences in mechanisms: between specific organs; between different species of host; and between different strains of *L. monocytogenes*. Furthermore, *L. monocytogenes* is a soil organism, which has evolved the ability to invade and

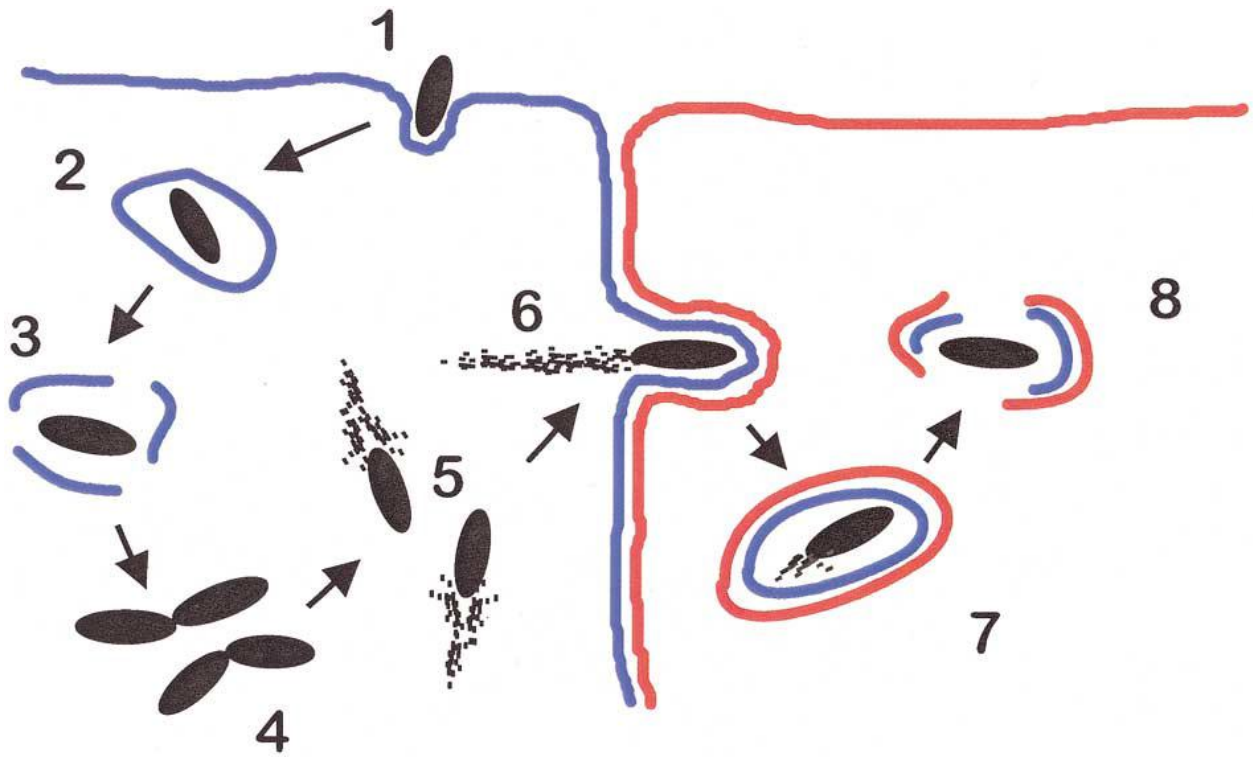
mobilise within eukaryotic cells, probably to some lower eukaryotic multi-cellular soil organism (McLauchlin, 1997).

Hence, this bacterium is not host adapted to man, rather a marginal and opportunistic pathogen, probably with multiple routes of infection and disease presentations, and is therefore unlikely to have a single infective dose. Ingestion of, usually heavily contaminated, foods is the vehicle and route of infection (McLauchlin, 1996a). In the oral route invasion may take place from sites in the nasopharyngeal or upper part of the alimentary tract. There is evidence to suggest that in sheep listerial encephalitis arises via tooth root scars and other sites in the oral mucosa where an ascending neuritis along the cranial nerves (particularly the trigeminal nerve) results in lesions in the base of the brain (Low and Donachie, 1997). However, it is not clear if this route occurs in man, although encephalitis very similar to that in sheep occur in humans in terms of site of inflammation in the brain and histological presentation (Mylonakis *et al.,* 1998). Contaminated food passes to the stomach where the acid environment may kill many of the *L. monocytogenes*. There is evidence supporting the role of antacid therapy in increasing susceptibility of some groups of patients (Fleming *et al.,* 1995) and in experimental animal infection (Schlech et al., 1993). The buffering capacity of some food types may also be important in facilitating the survival of the organism, which may then invade at sites further along the gastrointestinal tract. Other routes of infection may occur, and, in experimental animals, septicaemia can be achieved with contaminated aerosols via the respiratory route (Bracegirdle *et al*., 1994). Evidence supporting the possibility of infection via the respiratory route comes from one of the cases in a food-borne outbreak in Canada in 1981 (Schlech *et al*., 1983) in which septicaemia and aspiration pneumonia developed after eating contaminated coleslaw salad. Analysis of orally inoculated animals suggests that the small intestine is the primary site of invasion, although the

exact anatomical site and mode of infection are not well understood (Vazquez-Boland *et al*., 2001). The mechanism of the diarrhoeal illness, which presumably occurs at this stage, is not known, however following oral inoculation of animals, the bacteria can be observed, together with an inflammatory reaction, in phagocytic cells in the underlying lamina propria of the caecum and colon (Vazquez-Boland *et al.,* 2001). The invasion of the uterine or central nervous system (for patients with shorter incubation periods) may occur via the circulatory system.

Intra-uterine infection of the fetus results from haematogenous spread from the mother. Abscess formation takes place in the placenta, and this may spread via the umbilical vein or the amniotic fluid to the foetal internal organs. The series of pyrexial episodes observed in the mother may result from reinvasion of maternal blood stream from placental sites. *Listeria monocytogenes* is unusual in that it is able to survive and grow in amniotic fluid, and aspiration from this leads to the pathological changes in the foetal respiratory tracts. The organism is present in high numbers in amniotic fluid (Courcol *et al.,* 1982) and these results in widespread contamination of newborn infant and maternal sites at delivery, as well as the postnatal environment, and may result in additional neonatal cases due to cross-infection (McLauchlin *et al.,* 1986; Bortolussi and Schlech, 1995). The multiple routes of infection may be a contributory factor to the lack of a single (or simple) dose–response relationship for human listeriosis. Other routes of transmission for human listeriosis occur including direct contact with the environment (O'Driscoll *et al.,* 1999), contact with naturally infected animals (McLauchlin and Low, 1994), and via cross infection between newborn infants (McLauchlin *et al.,* 1986). However these routes are rare, and as stated above, the majority of cases are believed to be food-borne.

2.9 Mode of Entry:



Adapted and modified from José *et al*., (2001).

Figure 2.1: Representation of the intracellular life cycle of pathogenic *Listeria*:

1. Entry into host cells; 2. Survival within the phagocytic vacuole; 3. Disruption of phagosomal membranes and escape into the cytosol; 4. Replication in the cytosol; 5. Actin-based motility; 6. Direct cell-to-cell spread; 7. Survival in secondary (double membrane) phagosomes; 8. Escape from secondary phagosomes and reinitiation of the cycle.

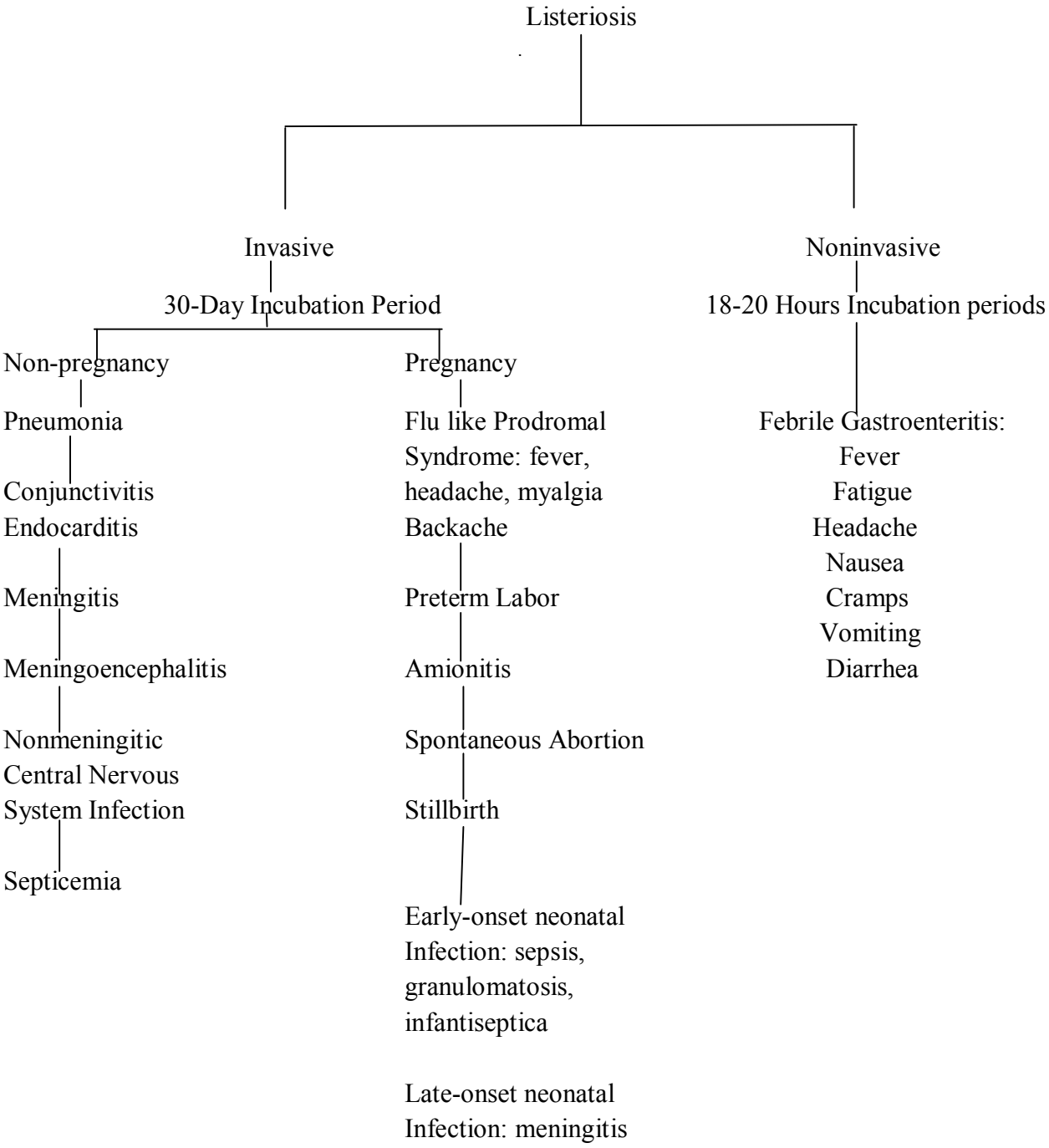
There are several properties and functions that contribute to the success of *L. monocytogenes* as an intracellular pathogen. The entry of this bacterium is due to its capacity to trigger uptake into cells that normally lack phagocytic capacities making it simple infect a large variety of cell types (Hai Yu *et al*., 2006). Following entry into host cells, bacteria initially reside within a membrane-bound vacuole, however *L. monocytogenes* rapidly escapes this compartment to enter the host cell cytosol where replication occurs. Cytosolic bacteria use actin-based motility to move and spread from cell-to-cell without contacting the extracellular environment. Normally *L. monocytogenes* is a saprophyte that undergoes a transition to an intracellular parasite in a physiological state that promotes bacterial survival and replication in host cells (Freitag *et al*., 2010). Once inside the host, the bacteria's products facilitate this process; some of the most important include internalin A and B (InlA and InlB) which bind to host cell receptors and facilitate entry. it also produces listeriolysin O (LLO) which breaks down the host cell phagosome once the organism is internalized (Condotta *et al*., 2011). Hexose phosphate transporter (Hpt) helps obtain hexose sugars for bacterial metabolism, Actin A, which polymerizes actin as a means to induce motility; and phosphatidylcholine phopholipase C, which acts in concert with LLO to help release organisms that are incorporated into vacuoles during cell-to-cell spread. Expression of these genes is controlled by PrfA (Positive regulatory factor A) which is in turn is regulated in part by environmental conditions (David, 2012).

2.10 Characteristic of Listeriosis:

There are two forms of Listeriosis, invasive and non-invasive. Invasive Listeriosis has a fatality rate of approximately 20-30% with a hospitalization rate nearing 90%. Non-invasive Listeriosis has been termed "febrile gastroenteritis". *Listeria* does not produce a lethal toxin (Lyautey *et al*., 2006)

2.10.1 Invasive Listeriosis: For invasive infections, organisms enter via the oral route and are believed to be taken up by specialized epithelial cells (M cells) in the small intestine and transported to the underlying lymphoid tissue, where they are taken up by macrophages, it invases these cells and can spread internally from cell to cell, thus avoiding exposure to antibodies (Pagotto *et al*., 2006)

2.10.2 Non-invasive Listeriosis: Non-invasive listeriosis is the mild form of disease, this type of infections is limited to the digestive system and cause mild symptoms which include: fever, diarrhoea, muscle aches, nausea, vomiting, drowsiness and fatigue (Painter and Slutsker 2007).



Adapted from Donnelly, 2001

Figure 2.2: Characterization of the invasive and noninvasive syndromes associated with adult Listeriosis.

2.11 Spread of *L. Monocytogenes:*

The reason that *L. monocytogenes* causes severe illness can be traced to its ability to induce its own phagocytosis (uptake) by host cells, followed by replication within those cells and direct transfer to another cell. Since *L. monocytogenes* remains active and unaffected within the host cells, it can spread through the body while protected from many host defenses, including antibodies and complement. Listeriae ingested with food are taken up by enterocytes or M cells near Peyer's patches in the small intestinal lining and then multiply in underlying phagocytic cells (Bille *et al*., 2003). Bacteria are carried from the intestine in macrophage in blood or lymph to the liver and spleen where most of them are killed by neutrophils acting with Küpffer cells. If the host's T cell–mediated immune response is inadequate, listeriae multiply in hepatocytes and macrophages and are carried in the blood to various organs, particularly the brain and/or uterus where they penetrate the blood–brain barrier and the placental barrier. An intricate series of virulence factors are produced by *L. monocytogenes* to facilitate each step of this invasion process. The genes coding for many of these factors are clustered together on the chromosome and regulated by the *prfA* gene (Kuhn, 1999).

2.11.1 Virulence factors

Internalins

Internalin A was first identified as a listerial surface protein that is required for the penetration of *L. monocytogenes* into non-phagocytic cells, such as epithelial cells (Gaillard *et al.,* 1991). A related protein, internalin B, plays a role in invasion of hepatocytes in the liver (Gaillard *et al.,* 1991). There is still some controversy in the literature as to whether one or the other of these proteins is more important in the invasion of different cell types and whether either is necessary

for entry into macrophages. Internalin A on the surface of listerial cells binds to a surface protein, E-cadherin, on the surface of host epithelial cells. This interaction apparently stimulates the phagocytosis of *L. monocytogenes* cells (Kuhn, 1999).

2.11.2 Surface protein p104

In addition to the internalins, another surface protein on *L. monocytogenes*, p104, has been identified and shown to play a role in adhesion to intestinal cells (Pandiripally, 1999).

2.11.3 Listeriolysin O

As listeriae are engulfed, they are enclosed within a vacuole that is surrounded by a membrane. Professional phagocytic cells begin almost immediately to kill the listeriae within the vacuoles, and survival of *L. monocytogenes* depends on escaping from the vacuole. Listeriolysin O (LLO), a bacterial poreforming toxin, is essential for lysing the vacuolar membrane and allowing *L. monocytogenes* to escape into the cytoplasm of the cell. LLO is necessary for establishing infection in mice, and its activity is enhanced by the acidic pH in the vacuole (Smith *et al.,* 1999). Mutant *L. monocytogenes* that do not produce listeriolysin can survive within vacuoles of non-professional phagocytes for a while, but they do not multiply and go on to infect other cells because they cannot escape from the vacuoles. If LLO lyses the membrane surrounding the vacuole, why does it not also attack the cell membrane, causing destruction of the cell and release of listeriae from the cell? This would be dangerous for *L. monocytogenes* because the bacteria would no longer be protected within the host cell from attack by antibodies. Analysis of the LLO molecule revealed that it contains a series of 27 amino acids at one end and that this sequence is very similar to (P, Pro; E, Glu; S, Ser; T, Thr) PEST sequences often found on proteins in humans and other animals. In these organisms, the PEST sequence is a starting place

for protein–protein interactions and, as such, often indicates proteins slated for degradation. It appears that once LLO has done its job of perforating the vacuolar membrane, it is then recognized by enzymes in the cytoplasm of the cells and is destroyed before it can damage the cell membrane (Decatur, 1995). In addition to its pore-forming function, LLO participates in other reactions related to pathogenesis of *L. monocytogenes*. Infection of murine spleen and bone marrow dendritic cells by *L. monocytogenes* results in cell death by apoptosis. Mutant bacteria that do not produce LLO do not induce apoptosis whereas purified LLO can induce this programmed cell death. LLO also can act as an inflammatory stimulus by inducing endothelial cell activation and neutrophil activation (Sibelius, 1999).

2.11.4 ActA protein

Once *L. monocytogenes* has escaped from the vacuole into the cytoplasm, it replicates. In order for these bacteria to move directly to another cell, a surface protein, ActA, induces polymerization of globular actin molecules to form polarized actin filaments. Bacterial cells move along these filaments to the cell membrane and cause portions of the membrane to bulge outwards, forming structures called listeriopods (Kocks *et al.,* 1992). These protuberances are engulfed by adjacent cells, thereby allowing dissemination of *L. monocytogenes* without exposure to antibodies or other immunoactive molecules. Other cellular factors such as are believed to be important in this actin-based motility, but they have not been well studied. ActA may also facilitate uptake of *L. monocytogenes* cells that do not produce internalins into certain types of cells (Kuhn, 1999).

2.11.5 Phospholipases

Two distinct phospholipases C synthesized by *L. monocytogenes* are phosphatidylinositol-specific phospholipase C (PI-PLC) and a broad-range or phosphatidylcholine-specific phospholipase C (PCPLC). Both apparently play a role in the invasion and spread of *L. monocytogenes* because bacteria with mutations in the genes coding for these enzymes are less virulent to mice than wild-type bacteria. Experiments with strains mutant for one or both phospholipase encoding genes demonstrate that PI-PLC aids in escape from the primary vacuole while PC-PLC is active during cell-to-cell spread of bacteria (Smith *et al.,* 1995). PC-PLC damages vacuolar membranes in some cell types, such as epithelial cells, and can therefore be a substitute for LLO (Marquis, 1995). PC-PLC also functions in the cell-to-cell spread of listeriae in the brain during cerebral listeriosis (Schluter, 1998).

PI-PLC is synthesized in an active form whereas PC-PLC is produced as an inactive precursor. A bacterial zinc-dependant metalloprotease and a host cell cysteine protease are required to cleave off part of the precursor and activate the phospholipase (Marquis, 1997).

2.11.6 Clp proteases and ATPases

In order to adapt to adverse environmental conditions (high or low pH, temperature, osmotic conditions), many bacteria have chaperone proteins that assist in the proper refolding of proteins or assembly of protein subunits and proteases which process proteins that cannot be altered conformationally. Some of the Clp (caseinolytic proteins) group of proteins, which act both as chaperones and as proteolytic enzymes, have been identified as having a role in pathogenesis of *L. monocytogenes*. ClpC ATPase is a general stress protein that aids in disruption of the vacuolar membrane and the intracellular survival of listeriae (Rouquette, 1998). ClpC also modulates

expression of the ActA protein and the internalins, apparently acting at the transcriptional level. Another ATPase, ClpE, also plays a role in listerial pathogenesis (Nair, 2000). ClpP serine protease is required for growth under stress conditions and has been shown to affect the activity of listeriolysin O and escape from vacuoles (Gaillot, 2000).

2.11.7 Protein p60

Protein p60 is murein hydrolase enzyme that catalyzes a reaction during the final stage of cell division of *L. monocytogenes*. It is normally present on the cell surface as well as being secreted into the surrounding medium. Analyses of mutations in the gene coding for this protein indicate it is important for phagocytosis of *L. monocytogenes* by some cell types (Kuhn, 1999).

All of these virulence factors participate in specific ways in the infection process, and in addition each may also affect host cell signal transduction in ways that enhance the spread of infection (Kuhn, 1999). Studies with cultures of different cell types have also demonstrated that some of these factors may be more important for infecting some cell types than others.

2.12 Adhesion of *Listeria monocytogenes*

Adherence to host cell surfaces is a necessary first step in infection by intracellular bacterial pathogens such as *L. monocytogenes*. However, it has been shown that this initial step can take place by two different mechanisms: an opsonin-dependent process, in which antibody and/or complement proteins become involved in the complex interaction between bacteria and host cell; and an opsonin-independent process, in which adhesins present on the bacterial cell surface recognize host cell receptors and attachment ensures. It is possible that these two processes result in different reactions by the host cell to the bacterium. For *L. monocytogenes*, uptake by listericidal macrophages via the complement receptor type 3 (CR3 receptor) resulted in death of

the organism, whereas uptake by nonlistericidal macrophages occurred through some other recognition factor and resulted in intracellular replication of the organism (Drevets, 1992). Adherence of *L. monocytogenes* to host cell membrane-associated structures is therefore a complex interaction.

2.13 Sialic Acid:

Studies have strongly suggested that sialic acid play an important signaling role in host defense against pathogens, However, this family of nine-carbon carbohydrates is actually scavenged from the host's glycoconjugates, through a glycosyl-transfer/ hydrolysis reaction mediated by *trans*-sialidase/sialidase, virulence factors expressed by pathogens.

Sialic acids (Sias) are a group of α-keto acids with a nine-carbon backbone, which display many types of modifications in nature (Lingquan *et al*., 2013), although sugars, which are typically found to be terminating branches of N-glycans, O-glycans, and glycosphingolipids (gangliosides) are occasionally capping side chains of GPI (glycosylphosphatidylinositols) anchors(Hendrik *et al*., 2011). Numerous pathogens exploit this sugar as an entry point to the human body, there by raising the question "Why does nature bother with sialylation". This is because the sugar is one of the most important molecules of life, since they are involve in many biological and pathological phenomena, which include cell-cell and cell extracellular matrix interactions. It plays a dual role, not only is it indispensable for their protection to and adaptation of life, but is also utilized by life threatening infectious microorganism (Jian *et al*., 2009). They not only pose a barrier to microorganisms inhabiting or invading an animal mucosal surface, but also present potential sources of carbon, nitrogen, energy, and precursors of cell wall biosynthesis (Vimr 2013). The catabolic strategies for exploiting host sialic acids as nutritional sources are as diverse as the biosynthetic mechanisms, including examples of horizontal gene transfer and

multiple transport systems. Finally, as compounds coating the surfaces of virtually every vertebrate cell, sialic acids provide information about the host environment (Vimr *et al*., 2004).

Sialic acid binding is shown to trigger a conformational switch that modulates the affinity for the acceptor substrate and concomitantly creates the conditions for efficient transglycosylation. There are two enzymes (Sialidase and Trans-sialidase) that are involve in hydrolyses or transfer of sialic acids with the mind of retenting an anomeric configuration, most times exposing a galactose residue or forming a new α-2,3-glycosidic linkage to galactose.

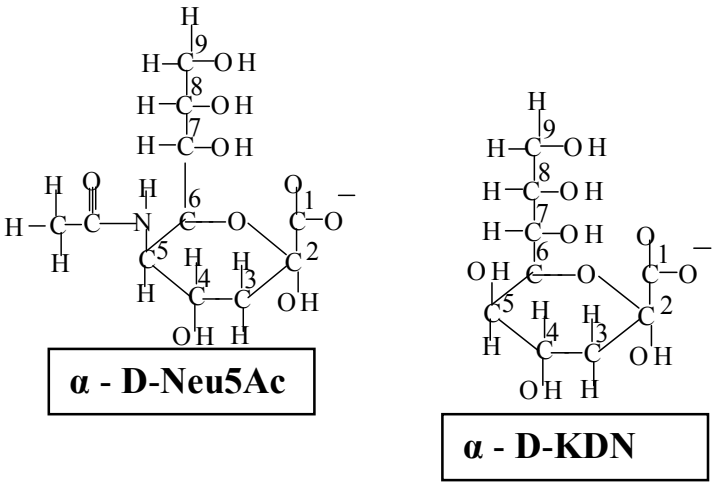


Figure 2.3: Primary Salic acid

Primary sialic acids are: 2-keto-5-acetamido-3,5-dideoxy-d-glycero-d-galactononulosonic acid (Neu5Ac) and 2-keto-3-deoxy-d-glycero-d-galactonononic acid (KDN). The only difference is the substitution at the 5-carbon position. Neu5Ac is much more common in most vertebrate cell types. All other sialic acids are thought to be metabolically derived from these two.

2.13.1 Distribution of Sialic Acid in Nature

The sialic acids is a designation given to a group of over 40 naturally occurring nine-carbon keto acids found mainly in animals of the deuterostome embryonic lineage (starfish to humans). These sugars are synthesized rarely by bacteria, and then mostly by pathogens that use sialic acids to masquerade as immunological self (Vimr *et al*., 2004).

2.13.2 Biosynthesis of Sialic Acids

Sialic acid synthesis in animals takes place primarily in the cytosol and involves four enzymes participating in a five-step process. The dual activity enzyme UDP-GlcNAc 2-epimerase/ ManNAc-6-kinase (gene symbol *GNE*) converts the starting substrate uridine 5'-diphosphate-*N*-acetyl-D-glucosamine (UDPGlcNAc) to *N*-acetyl-D-mannosamine (ManNAc) with removal of the UDP moiety and epimerization of the carbohydrate (Altheide *et al*., 2006). The kinase function of the same enzyme initiates and regulates sialic acid biosynthesis while the condensation reaction between phosphoenol pyruvate (PEP) and ManNAc-6-P initiated by euAc-9-P-synthetase (gene symbol *NANS*) results in the phosphorylated sialic acid precursor *N*-acetylneuraminic acid 9-phopshate (Neu5Ac-9-P). This precursor is dephosphorylated by Neu5Ac-9-P-phosphatase (gene symbol *NANP*) to produce the key sialic acid *N*-acetylneuraminic acid (2-ketoacetamido-3, 5-dideoxy-D-glycero-D-galactononulosonic acid; Neu5Ac) (Altheide *et al*., 2006).

2.13.3 Sialic Acids and Pathogens

Sialic acids have been shown to be important in several aspects of microbial pathogenesis; they exploit surface sialic acids for attachment and infection, many viruses bind to their host via sialic

acids on the epithelial cell surface. For example, influenza A and B viruses recognize sialic acid whereas influenza C viruses bind exclusively to 9-*O*-acetylated sialic acids (Varki, 1997). Incorporation of sialic acids into the capsules of some pathogenic bacteria aids in evading host defences by inhibiting the direct activation of the alternative complement pathway.

Sialic acid involvement in so many cellular functions has lead to many medical problems due to disturbances of their biosynthesis or degradation. Their exposed position has made them vulnerable to the action of microbial esterases, sialidases, and lyases. The actions of these enzymes can affect the amount of ligand present, masking of antigenic sites, stabilization of membranes, and immunological and other functions of Sialic acid. In this regard, microbial lectins, *sialidases*, and *trans*-sialidases are potent virulence factors (Varki and Schauer 2009).

These "trans" attachments typically have a low binding coefficient, so in order to strengthen the bonds to the host cell thereby creating a large number of attachments between the virus and the host. In pathogenic fungi, the biological function of these carbohydrates has not yet been determined but several studies have provided evidence that, as in other microbial pathogens, sialic acids may play a role in host recognition and in the evasion of host defenses (Rodrigues *et al*., 1997).

2.14 *Sialidase*

Sialidase (Neuraminidase) belong to a class of glycosyl hydrolase. Although it catalyses the removal of sialic acids from glycoconjugates, it has been implicated in diverse cellular functions (Yujin *et al*., 2013), its cluster is essential for sialic acid utilization and may perform an important function for bacterial survival in the host.

Sialidase was originally identified as a "receptor-destroying enzyme" in extracts of *Vibrio cholerae* because of its ability to release influenza virus from the surface of erythrocytes. *Sialidases* have been found in viruses, bacteria, trypanosomes, and mammalian cells (Corfield, *et al.,* 1992). In bacteria, sialidases can be used to scavenge sialic acids as a nutrient from various sialylated substrates or to recognize sialic acids exposed on the surface of the host cell. Despite the fact that bacterial sialidases share many structural features, their biochemical properties, especially their linkage and substrate specificities, vary widely. Bacterial sialidases can catalyze the hydrolysis of terminal sialic acids linked by the α(2,3)-, α(2,6)-, or α(2,8)-linkage to a diverse range of substrates. In addition, some of these enzymes can catalyze the transfer of sialic acids from sialoglycans to asialoglycoconjugates via a transglycosylation reaction mechanism. Thus, some bacterial sialidases have been applied to synthesize complex sialyloligosaccharides through chemoenzymatic approaches and to analyze the glycan structure (Kim *et al*., 2011).

Sialidase degradation of sialylated glycoconjugates mainly occurs in lysosomes, the plasma membrane and in the cytosol. Bacteria uses secreted *sialidase* to unmask penultimate sugars for binding to the host cells, or/and they consume liberated sialic acid for nutritional purposes. Influenza virus sialidases destroy the receptor for hemagglutinin (receptor-destroying enzymes), in particular after propagation and release from host cells, thus facilitating spreading (Schauer *et al*., 2011). Sialic acid liberated by sialidase action are transported from the lysosomes to the cytosol and further degraded by Sia-specific lyases yielding pyruvate and acylmannosamine (acetyl- and glycolylmannosamine). Both sialic acid and acylmannosamines can be recycled and reused for sialoglycoconjugate biosynthesis in the Golgi (Schauer *et al*., 2011)

There is evidence for two families of the bacterial enzyme, distinguished by a requirement for a divalent metal ion for maximal activity. Those not requiring metal have molecular weights of

42,000 and share some sequence similarity-e.g., the sialidases of *Clostridium perfringens*, *Clostridium sordelli*, *Salmonella typhimurium*, and *Micromonospora viridifaciens*. These enzymes also share sequence similarity with the N-terminal domain of the membrane-bound *sialidase* of *Trypanosoma cruzi* (Periera, 1991).

2.15 *Trans-Sialidase*

Trans-sialidase is an enzyme that catalyzes the transfer or hydrolysis of terminal sialic acid residues, preferentially the transference of α-2, 3-linked sialic acid to acceptors containing terminal β-galactosyl residues, instead of the typical hydrolysis reaction, found in most *sialidases* (Marcelo *et al*., 1997). In the absence of an appropriate acceptor, *Trans-Sialidases* acts as a *sialidase*, hydrolytically releasing glycosidically linked sialic acid. *Trans-Sialidases* constitute a special group of the *sialidase* family. They occur in some trypanosome species and also in a unique reversible reaction, transfer sialic acids from one glycosidic linkage with galactose (donor) to another galactose (acceptor), to form (α2–3)-sialyl linkages (Schauer &Kamerling 2011). Recently, it has been demonstrated that *trans- sialidase* is responsible of inducing transient thymic aplasia via apoptosis, this effect could allow the avoidance of the host immune system by the parasite (Laura *et al*., 2008)

Trans-sialidases strongly contribute to the pathogenicity of the trypanosomes by scavenging sialic acids from the host or blood meal to coat the parasite surface; this aids their survival strategy in the insect′s intestine, and in the blood circulation or cells of the host, and serves to compromise the immune system of the human or animal host.

Trans-Sialidase has been involved in cell invasion and its activity varies depending on the linkage specificity (Pereira *et al*., 1996), and has also been found in a few bacteria species and in

human serum. *Trans-Sialidases* are of increasing practical importance for the chemo-enzymatic synthesis of sialylated glycans (Schauer &Kamerling 2011). Hence, sialylation of parasite surfaces is believed to protect the parasites from the action of glycolytic enzymes as well as from immunocompetent substances present in the tsetse gut and blood meal respectively, as well as influencing the interaction of parasites with the gut epithelial cells. In the African trypanosomes where *trans-sialidase* (TS) is thought to be expressed only in the procyclic insect stages, TS has been shown to increase the survival, maturation and hence establishment of the parasites in the vector midgut

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials/Reagents

Sephadex G-75, Proteinase K and Mercaptoethanol were obtained from Sigma-Aldrich (USA). All other reagents were of analytical grade (98 % - 99.9 % purity). cDNA synthesis and Amplification reagents (primers, dNTP's, Taq polymerase, revertaid M-MuLV reverse transcriptase, oligo $(dT)_{18}$ primer, ribolock RNase Inhibitor and nuclease-free water) were obtained from Fermentas, South Africa. All enzyme assay reagents were of analytical grade (98 % - 99.9 % purity), Gene Amp PCR system 9700 thermal cycler, Jenway 6405 UV/ Visible Spectrophotometre, Beckman J-6B centrifuge.

The experimental organism *L. monocytogenes* was obtained from the Nigerian Institute of Medical Research (NIMR) Yaba in Lagos State Nigeria.

3.2 Experimental Design

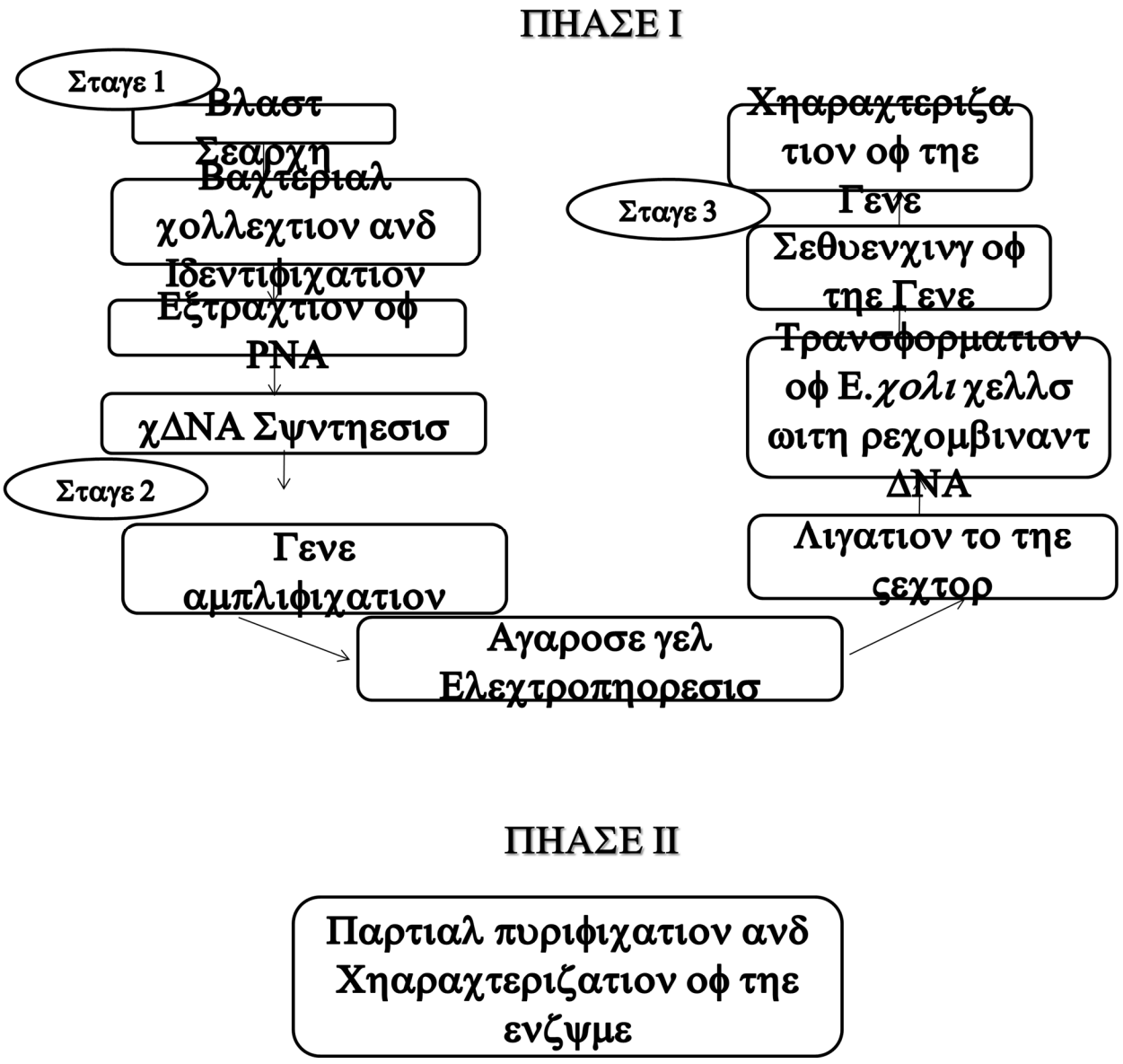


Figure 3.1 Experimental Design for the identification and molecular characterization of sialidase/Trans- sialidase gene product and protein of *L. monocytogene*.

3.3 BLAST Search of Sialidase gene in the genome of LM

BLAST (Basic Alignment Search Tool) search was conducted on the genome of *Listeria monocytogene* sequences using different known sialidase gene. The genome of LM and homologues of sialidase gene from different organisms were retrieved from NCBI gene bank (www.ncbi.nim.nih.gov) and aligned using multiple sequence alignment tool of Geneious version 6.1.

3.4 Identification and Characterization of *Listeria Monocytogenes*

3.4.1 Gram Staining

The organism was first characterized for authenticity following morphological identification by gram staining; presumptive isolates were gram stained as follows: a smear of a 24 hours colony of the presumptive isolate was made on a grease free glass slide and heat fixed over bunsen flame. It was then covered with crystal violet for 1 minute and rinsed with water. The smear was thereafter covered with Grams iodine for 1 minute and then rinsed with water. Decolorization of the smear was carried out by drop wise addition of acetone with gentle agitation for 10-30 seconds after which it was rinsed with water then counter stained with safranin for 20 seconds before finally rinsing with water and allowed to air dry. The gram stained slides were then viewed under a microscope using the oil immersion objective (x100).

3.4.2 Listeria Identification System 12L

A single colony of the presumptive isolate of a 24 hours culture was emulsified in suspended medium and mixed thoroughly to prepare a homogeneous suspension. The haemolysin reagent was warmed to room temperature, and then a sterile pasteur pipette was used to place 4 drops

(approximately 100µl) of the bacterial suspension into each well of the test strip from its foil pouch. One drop of haemolysin reagent was added to the well then covered with the lid and incubated for 24hours at 35°C.

3.5 Culturing of the Isolates

All solid and liquid culture media were prepared and sterilized according to the manufacturer's specifications. The mixture of Listeria Enrichment Agar (LEA.) was prepared with distilled water and autoclaved at 121 ºC for 15 minutes. The agar media was allowed to cool to 45 ºC and then poured into the petri dishes to solidify; it was left overnight to set properly. Then a single colony of the organism was culture on the agar, while the broth media was prepared.

3.6 Growing the Bacterial

A single colony from a 24hr streak culture of listeria monocytogenes having a green-blue colouration on the agar was then grown on Listeria enrichment media (broth) containing cycloheximide and nalidinic acid in the selective enrichment supplement (Lovett *et al*., 1987). Growth in broths with turbidity was then centrifuged and used for RNA extraction.

3.7 RNA Extraction Protocol

Principle

The RNeasy procedure represents a well-established technology for RNA purification. This technology combines the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica membrane. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate–containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is

added to provide appropriate binding conditions, and the sample is then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in 30–100 µl RNase free water.

Procedure

Bacteria cells grown to exponential phase was harvested by spinning down the broth containing the cells, and then the pellet was disrupted by adding 350µl of 70% ethanol and 350µl of RLT buffer (lysis buffer). The mixture was pipetted directly into a QIAshredder spin column and place in a 2 ml collection tube, and centrifuged for 2 min at 14,000 rpm speed, then 700 µl of the sample was transfered to an RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15 s at ≥8000rpm x g then discard the flow-through. 700 µl buffer of RW1 was added to the RNeasy spin column containing the sample and centrifuged for 15 s at 8000rpm x g to wash the spin column membrane, and then discard the flow-through. 500 µl of buffer RPE was addded to the spin column and centrifuged for 15 s at the same speed, the flow through was discarded and another 500 µl buffer RPE was added to the RNeasy spin column and centrifuged for 15 s. The flow through was discarded and the column was recentrifuged to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column. The RNeasy spin column was placed in a new 1.5 ml collection tube (supplied), add 30µl RNase-free water was added directly to the spin column membrane and allowed to stand at room temperature for 10min, finally centrifuged for 1min at 8000rpm x g to elute the RNA which was immediately used for cDNA synthesis.

3.8 cDNA Synthesis Procedure

Components of the kit was allowed to thaw then briefly centrifuged and placed on ice. 20 µl, to a nuclease-free tube was used, which was placed on ice too, 2 µl template of RNA was added plus 1 µl oligo $(dT)_{18}$ primer, then to make it up to 12 µl, 8 µlof nuclease-free water was added and 4 µl reaction buffer, 1µlribolock RNase Inhibitor, 2 µl dNTP Mix and 1µl revertaid M-MuLV reverse transcriptase was added. It was mixed well, centrifuged and incubated for 60 min at 42°C. The reaction was terminated by heating at 70°C for 5 min.

3. 9 RNA and cDNA Quality Confirmation

Agarose gel electrophoresis and Spectrophotometer were employed to confirm the quality of RNA and cDNA obtained on the nanodrop by measuring the optical density at 260/280 and 260/230 ratio. A 1 % solution of agarose was prepared by melting 1 g of agarose in 100 mL of 0.5x Tris-acetate-EDTA (TAE) buffer in a microwave oven. The mixture was then allowed to cool for a few minutes and then 3 µl of ethidium bromide was added and stirred to mix. The gel was cast using a supplied tray and comb and was then allowed to set for 20 min at room temperature on a flat surface. The wells were loaded with 11 µl of RNA/cDNA plus loading dye (10 µl of the sample + 1 µl constituted loading dye), this was allowed to run for 30 min at 100 V then exposed to UV light and documented.

3.10 Polymerase Chain Reaction (PCR)

PCR was performed using a 25 µl reaction volume containing 19 µl distilled water, 2 µl cDNA template, 0.5 µl deoxynucleotide triphosphate (dNTP's, 10mM), 0.5 µl primers (100pmol), 2.5 µl Taq buffer (10 X) and 0.5 µl Taq DNA polymerase (5U/ µl). Amplification was achieved in a thermal cycler programmed for 5 minutes at 94°C as initial denaturation, followed by 30 cycles

of 1 minute at 94°C for denaturation, 1 minute at 53°C as annealing, 30 seconds at 72°C for extension, and final extension at 72 °C for 5 min. The primers were as follows: TS2 – 5'GGC TTCA GGC TAT TA 3' for external 5'GTT TTC CAC GTC TTC 3' revs external 5'ATC TGA ACA CTC GAG 3' for internal 5'CTA TCA GAC TGC TGT 3' revs internal, TSL1 – GTG GGA TGA CAA ACT 3' for external 5'GCA ATA GAA CTC CCT 3' revs external 5'TAC TCA ACA CAC GCA T 3' for internal 5'GAG CAT CAC ACG CAT 3' revs internal and TS1- 5'TAC CGC ATA CAC TCA ATT GT 3' for external 5'GAC GAT CCC GTC GTG 3' revs external 5'CCG TTG CTG GGG TTT 3' for internal 5'ACC ACC TGG TGG CTC 3' revs internal.

Amplification products were separated alongside a molecular weight marker of Middle Range DNA Ladder (100 bp up to 5 Kb.) by electrophoresis on 1.5 % agrose gel run in 1 X Tris-acetate-EDTA (TAE) buffer, stained with Ethidium bromide and visualized under UV light. The PCR products were excised from the gel and purified using MinElute Gel Extraction system (QIAGEN) where 50µL of Buffer PB was added to the PCR product. Combined PCR product / Buffer PB were transferred to the MinElute column and centrifuged at 13,000rpm for 1 minute, the flow through was discared and an addition of 750µL of Buffer PE to the tube and centrifuged at 13,000 rpm for 1 minute and the flow through . To elute the PCR product, 20µL of Buffer EB was added into the centre of the membrane of the tube and Incubate at room temperature for 1 minute then centrifuged at 13,000rpm for 1 minute. The purified PCR product was packaged, labelled and sent for sequencing at Max Planck Institute for Marine Microbiology, University of Bremen, Germany.

3.11 Cloning Protocol

The PCR amplicon was gel purified, then the ligation mixture for 20µl reaction was set up containing 10µl of reaction buffer, 1µl of the amplicon, 1µl of the pJET cloning vector plus 1µl of T4 DNA ligase, then made up with nuclease free water.

The ligation mixture was briefly vortexed for 3-5 secs then incubated at room temperature for 5mins.

3.11.1 Competent cell Preparation

Calcium chloride method was used in competent cell preparation, where 10 µl glycerol stock of an *E.coli* DH5α containing no plasmids was allowed to thaw at room temperature and added to 40 ml of liquid S.O.C. media. The culture was incubated at 37ºC for 1 hr, then transferred to an incubator-shaker, at 37ºC, shaking at 200 rpm for 2-3 hrs until an OD_{600} of 0.2-0.4 was reached. The cells were pelleted by centrifugation at 8000 rpm for 1 min at 4ºC, then resuspended in one-half volume (20 ml) of sterile cold TB ($CaCl_2$) solution, and incubated on ice for 25 min. After another centrifugation step as above, the resulting cell pellet was resuspended in one-tenth volume (4 ml) of sterile cold TB ($CaCl_2$) solution to yield the final competent cell suspension

3.11.2 Transformation of E.coli cells

The transformation of the competent cell with the ligation mixture was carried out as follows; 2.5µl of the ligation mix was added to 50µl of the competent cell for transformation. Recombinant vector was transformed into *E.coli* DH5α, and then positive clones were selected on agar plates containing ampicillin.

3.12 Sequencing of the Trans-sialidase Gene This was done using Beckman Coulter CEQ 8000 DNA sequencer to obtain the sequence of the amplified Trans-sialidase gene obtained from the previous step.

3.13 Charaterization of the Gene:

The gene was compared with other microbial sequences of trans-sialidase gene using ClustalW version 2.0 (www.geneious) then blast (www.ncbi.nim.nih.gov) to confirm their identity and evaluate their similarity to other known Trans- sialidase. Sequence alignment was then done with the Geneious 6.1 software.

3.13 Monitoring the release of Sialidase

The progressive release of Sialidase in the culture medium of *Listeria monocytogenes* was monitored for 1hour at 10 min intervals. In this method, aliquots of the culture medium containing the enzyme was incubated with 4-methylumbelliferyl α-d-acetylneuraminic acid (MU-NANA) as substrate for 1hour and the amount of sialidase released monitored spectrophotometrically at 360nm.

3.15 Determination of Total Protein

The protein concentration was determined according to the method described by Bradford (1976). The assay is based on the principle that the maximum absorbance of an acidic solution of Coomassie blue G-250 shift from 365nm to 595nm when binding of the dye to protein occurs. Both the hydrophobic and ionic interactions stabilize the ionic form of the dye, causing a visible color change.

3.16 Sialidase Activity Assay

4-methylumbelliferyl α-d-acetylneuraminic acid (MU-NANA) was used as substrate. Sialidase activity against MU-NANA was determined by adding 100 µl of enzyme solution to 100 µl of 0.1 M phosphate buffer, pH 6.5. The reaction was started by adding 50 µl MU-NANA (1 mg/ml) and the mixture incubated at room temperature for 1 hour. The reaction was stopped at the end of the incubation period by the addition of 100 µl of 0.2 M borate buffer, pH 9.0. The absorbance was detected using the Jenway 6405 UV/Visible Spectrophotometer at 360nm.

One unit of enzyme activity was defined as the amount of enzyme which produced 1µmole of N-acetylneuraminic acid per minute under the described assay condition.

3.17 PARTIAL PURIFICATION OF *Listeria monocytogene* SIALIDASE

3.17.1 Purification with Ammonium Sulphate

The principle is based on the solubility difference between the protein and the ammonium sulphate, in which case the more soluble ammonium sulphate abstracts the water molecules thereby making the protein-protein interaction to increase but decreasing the interaction of protein with the solvent, hence precipitating the protein. Here, the solid ammonium sulphate was added slowly to the crude extract with constant stirring starting from 30% until 90% saturation was obtained. The sample was allowed to stand overnight at 4^{o}C. Thereafter, it was centrifuged at 14,000×g for 30minutes, the supernatant was discarded and the pellet was resuspended in the assay buffer and tested for Sialidase activity as described above.

3.17.2 Gel filtration on Sephadex G-75 Column

Gel filtration was carried out using Sephadex G-75. The principle is based on separating macromolecules on their various sizes., The pooled fractions of 70% saturation of ammonium

sulphate precipitation was loaded onto Sephadex G-75 column (1.6 cm ×90cm) previously equilibrated with 10mM sodium phosphate buffer, pH 8.0. The column was eluted with the same buffer, 30 fractions of 2ml each were collected at a flow rate of 2ml/26.15minutes. The Sialidase activity was determined in each of the fractions. Fractions containing the enzyme activity were pooled together and the protein concentration was estimated by the method of Bradford (1976).

3.18 CHARACTERIZATION OF THE PARTIALLY PURIFIED SIALIDASE

3.18.1 Effect of pH

The activity of the enzyme against 4-methylumbelliferyl α-d-acetylneuraminic acid (MU-NANA) as a function of pH was studied by employing the above assay procedure at room temperature. . Different pH ranges of 0.1 M sodium phosphate buffer (pH 3.0, 4.0, 5.0 and 6.0) and 0.1 M acetate buffer (7.0, 8.0, 9.0 and 10.0) were used. The reaction mixture was incubated for 1 hour, terminated with the stop buffer (0.2 M borate) and the absorbance taken at 360 nm. The substrate was used as blank, distilled water was added instead of enzyme as control.

3.18.2 Effect of Temperature

Temperature effect on the enzyme activity against 4-methylumbelliferyl α-d-acetylneuraminic acid (MU-NANA) was studied using pH optimium (pH 4.0). The reaction mixture were incubated for 1hour at different range of temperature 20 ^{0}C, 30 ^{0}C, 40 ^{0}C, 50 ^{0}C, 60 ^{0}C, 70 ^{0}Cand 80 ^{0}C. The reaction was terminated with the stop buffer (0.2 M borate) and the absorbance taken at 360 nm.

3.18.3 Determination of Kinetic Constants

The Michaelis-Menten constant (Km), maximum velocity (Vmax) were evaluated against 4-methylumbelliferyl α-d-acetylneuraminic acid (MU-NANA) as substrate at room temperature for

1 hour. The activity was assayed with varying concentrations of the substrate ranging from 0.0029mM to 0.0149mM and the absorbance was read at 360nm. The final enzyme concentration for the assay was estimated as millimolar; Km and Vmax were evaluated by plotting the data on a Lineweaver bulk plot.

3.18.4 Effect of Cations

Sialidase activity was determined in the presence of the following monovalent and trivalent metal ions: $CaCl_2$, $MgCl_2$, $AgCl_2$, $MnCl_2$, $HgCl_2$, $AlCl_2$ and $ZnCl_2$. The enzyme was pre-incubated for 10 min at room temperature with 3mM of the listed metal ions prior to substrate addition. Activity without added metal ions was taken as control.

3.19 Thin Layer Chromatography

The samples were run and developed on several small scale (i.e. 5 x 10 cm) pre-coated silica gel TLC plates (silica gel F254 nm). The mixture of enzyme and substrate incubated for 30mins, 1hour and 1hour 30 mins was performed on TLC plates. Solvent system used for the assay was chloroform/methanol/water =3/4/1 (by volume), the TLC plates were placed in developing chamber glass containers with solvent systems filled to approximately 1 cm from the bottom. The solvents moved to the top of the TLC plates as results of capillary action. The TLC pattern was observed under a UV- light.

CHAPTER FOUR

4-0 RESULTS

4.1 Molecular Analysis: To assess the existence of Sialidase/Trans-sialidase in the genome of *Listeria monocytogene,* Basic Local Alignment Search Tool (BLAST) search was conducted with other known microbial sialidase which revealed in (Figure 4.1) areas of homology/similarities.

Plate I shows the photograph of RNA revealing the ribosomal and messenger RNA (rRNA and mRNA) while Plate II shows the picture of a smeared cDNA of possible mRNA within the total RNA.

The amplification of Trans-sialidase gene from the synthesized cDNA of total RNA of *Listeria monocytogene* using primers from trypanosomes revealed 3 bands of 200, 300 and 500 base pairs respectively as shown in Plate III.

The sequenced gene from the PCR amplification shows a 342 basepair and 114 translated amino acid shown in (Figure 4.2 and Figure 4.3), which further was confirmed to show similarity with other known Trans-sialidase gene of trypanosomes as shown in Table 4.1 and Figure 4.7 repectively. *T.congolense* Trans-sialidase gene revealed highest percentage similarity of 100% while *T.rangeli* sialidase gene shows 55.1%.

Figures 4.8, 4.9 and 4.10 showed the predictions located within the sequenced gene, which includes: restriction sites prediction, Motifs and Transcription factor prediction, secondary structure prediction, antigenic prediction, signal cleavage prediction, transmembrane prediction and cyctoplasmic potentials. These predictions provide a structurally reasonable and energetically favorable way for designing drugs in further experiments.

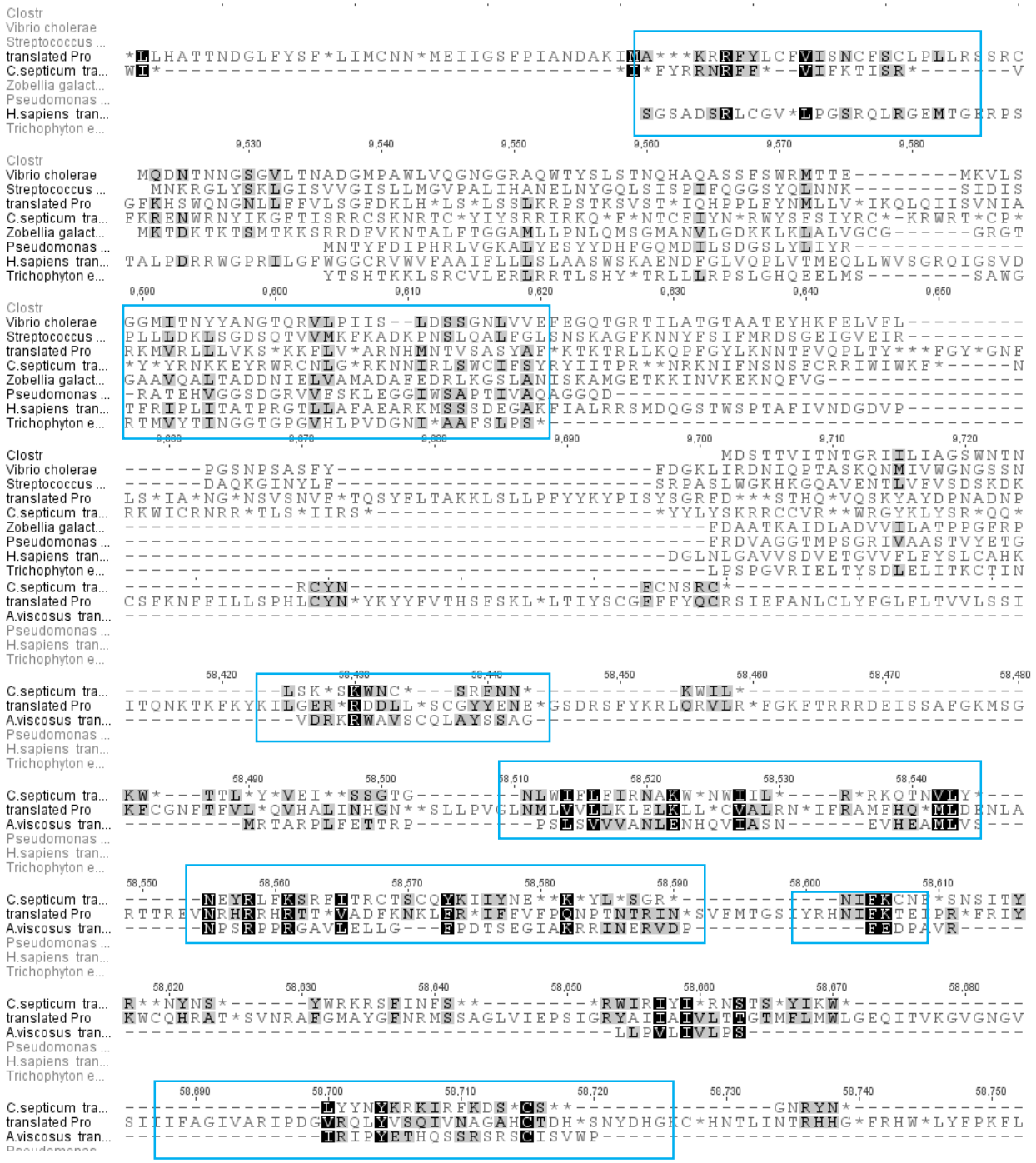


Figure 4.1: Blast search alignment: Primary sequence alighnment of some known sialidase genes within the genome of *Listeria monocytogenes*. Boxed area represents regions of identity. The listed Sialidase are from the following organisms: *C. Septicum, A.viscosus, Zobellia galactanivorans, Trichophyton equinum, H.sapiens, Vibrio cholera, Aspergillus oryzae, Streptococcus pneumonia, Pseudomonas aeruginosa and Clostridium specie.Translated protein in number 3 of first stage and line 2 of second stage represents translated protein of the genome of LM.*

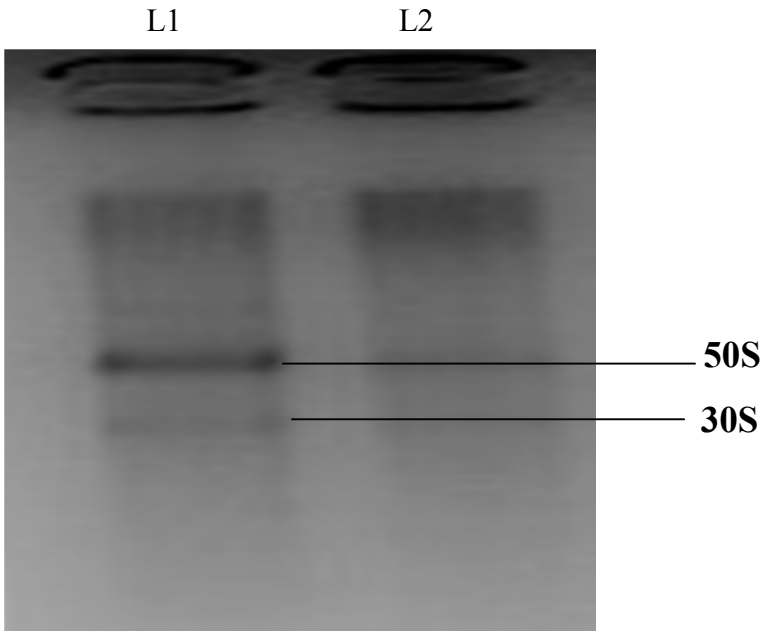


Plate I: Gel photograph showing RNA isolated from *Listeria monocytogene* both Lane 1 and Lane 2. Products were run on 1 % agarose and viewed/photographed under UV light

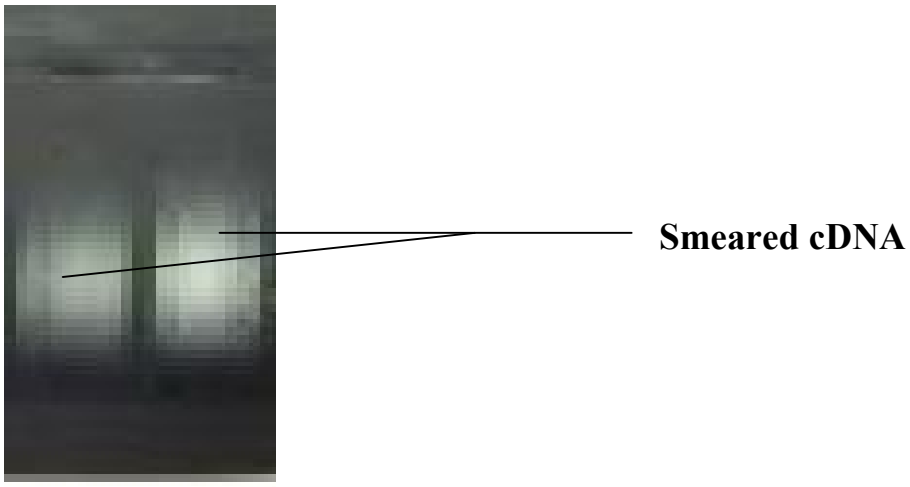


Plate II: Gel photograph showing smeared cDNA synthesized from total RNA of *Listeria monocytogene.*

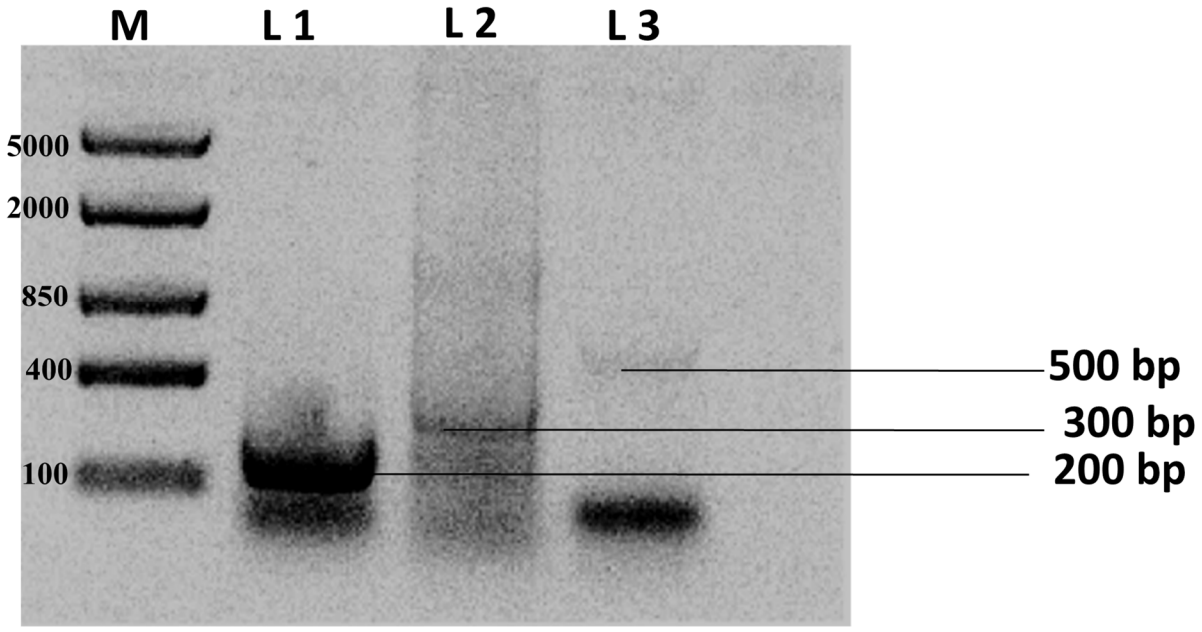


Plate III: Gel photograph showing amplified Trans-sialidase genes using PCR. Amplicons were run on 1 % agarose and viewed/photographed under UV light. M – Middle Range DNA Ladder; L1- trans-sialidase like 1 gene (TSL1); L2 - trans-sialidase 2 gene (TS2) and L3- trans-sialidase like 2 gene (TS1) from *Listeria monocytogenes*.

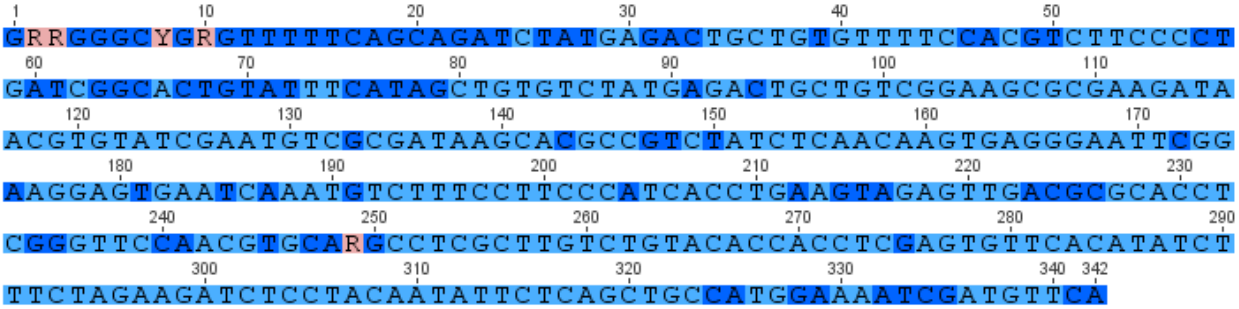


Figure 4.2: Sequenced trans-sialidase gene (342bp) of *Listeria monocytogenes*.



Figure 4.3: Translated Amino acids (114aa) of sequenced Trans-sialidase gene of LM represented as **G -** Glycine **P** – Proline, **A** – Alanine, **V** – Valine, **L** – Leucine, **I** – Isoleucine, **M** – Methionine, **C** –Cysteine, **F** – Phenylalanine,**Y** – Tyrosine, **W** – Tryptophan,**H** – Histidine, **K** - Lysine **R** – Arginine, **Q** – Glutamine, **N** – Asparagine, **E** - Glutamic Acid, **D** - Aspartic Acid **S** – Serine, **T** - Threonine

Table 4.1: **Blast Table showing summarized percentage identification of Trans-sialidase of LM with other known TS.**

E Value	U	Name	Description	Sequence ...	Topology	Organism	# Sequences	% Pairwis...	Taxonomy	% Identica...	Common N...	Genetic Code	Alignme
8.78e-03	•	T Rang SA Pre	Trypanosoma rangeli sialidase precu...	49	linear	Trypanoso...	2	55.1%	-	55.1%	-	-	-
2.38e-02	•	T Rang SA Gene	Trypanosoma rangeli sialidase gene, ...	49	linear	Trypanoso...	2	53.1%	-	53.1%	-	-	-
1.68e-02	•	T Rang LDG TS	Trypanosoma rangeli LDG trans-siali...	47	linear	Trypanoso...	2	55.3%	-	55.3%	-	-	-
8.67e-05	•	T Eva TS Gene	Trypanosoma evansi trans-sialidase ...	47	linear	Trypanoso...	2	59.6%	-	59.6%	-	-	-
9.48e-05	•	T Eva TS gene	Trypanosoma evansi trans-sialidase ...	47	linear	Trypanoso...	2	59.6%	-	59.6%	-	-	-
8.50e-02	•	T Cru TSGene	Trypanosoma cruzi TCTS-193 gene f...	56	linear	Trypanoso...	2	50.0%	-	50.0%	-	-	-
8.70e-02	•	T Cru TS cds	Trypanosoma cruzi gene for TS-193 ...	56	linear	Trypanoso...	2	50.0%	-	50.0%	-	-	-
8.53e-02	•	T Cru TS	Trypanosoma cruzi strain CL Brener ...	56	linear	Trypanoso...	2	50.0%	-	50.0%	-	-	-
5.43e-18		T Con TS2 Part Gene	Trypanosoma congolense partial TS2 gen...	45	linear	Trypanosom...	2	100.0%	-	100.0%	-	-	-
2.60e-02	•	T Bru TS Par	Trypanosoma brucei brucei strain 92...	57	linear	Trypanoso...	2	47.4%	-	47.4%	-	-	-
9.06e-05		T Bru TSL1 Gene	Trypanosoma brucei TSI1p (TSI1) gene, ...	47	linear	Trypanosom...	2	59.6%	-	59.6%	-	-	-
8.22e-05	•	T Bru TS CDS	Trypanosoma brucei putative trans-...	47	linear	Trypanoso...	2	59.6%	-	59.6%	-	-	-
6.11e-05		T Bru TS	Trypanosoma brucei brucei strain 927/4 ...	47	linear	Trypanosom...	2	59.6%	-	59.6%	-	-	-

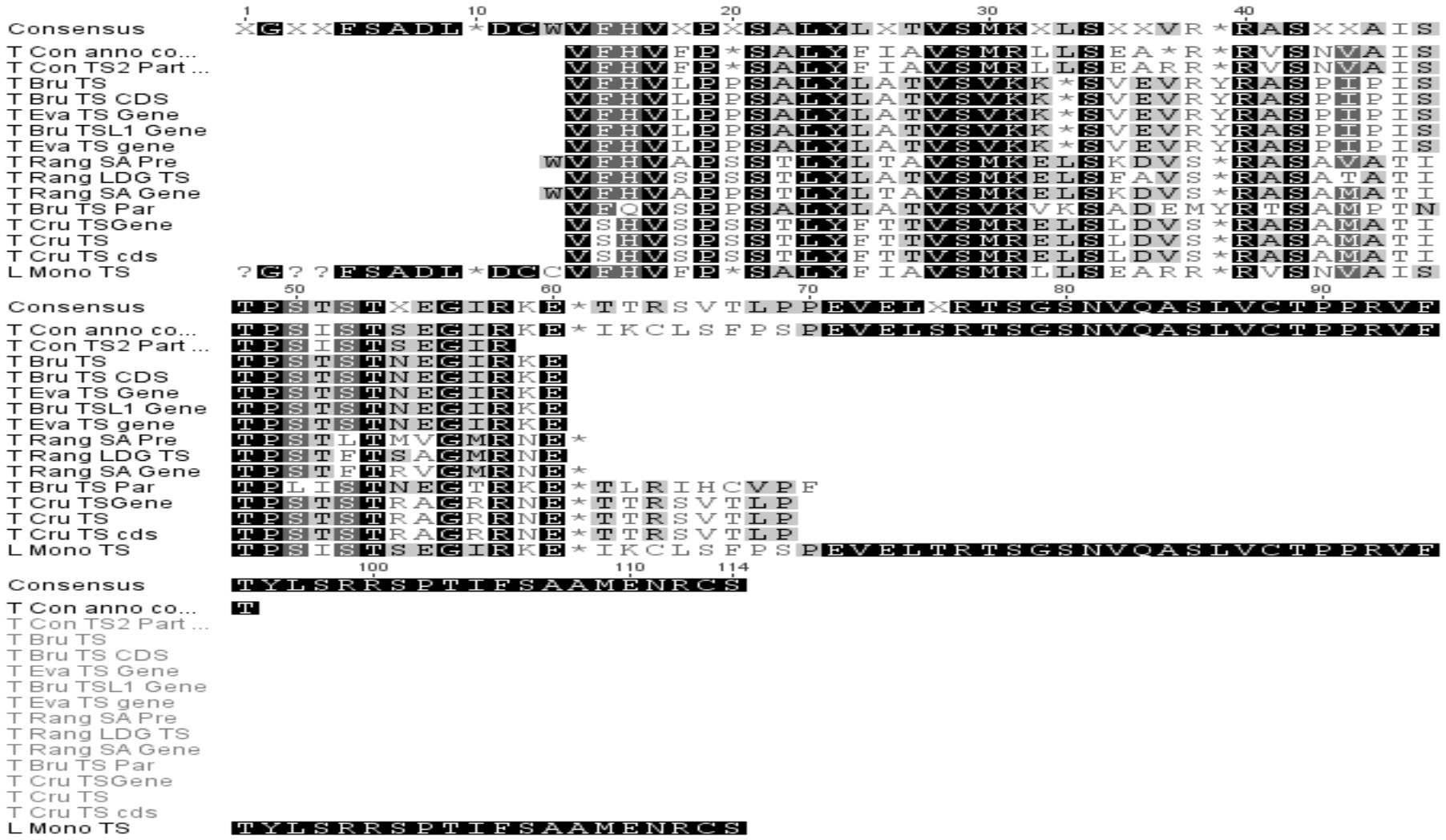


Figure 4.4: Multiple sequence alignment of LM Trans Sialidase and that of Trypanosomes.

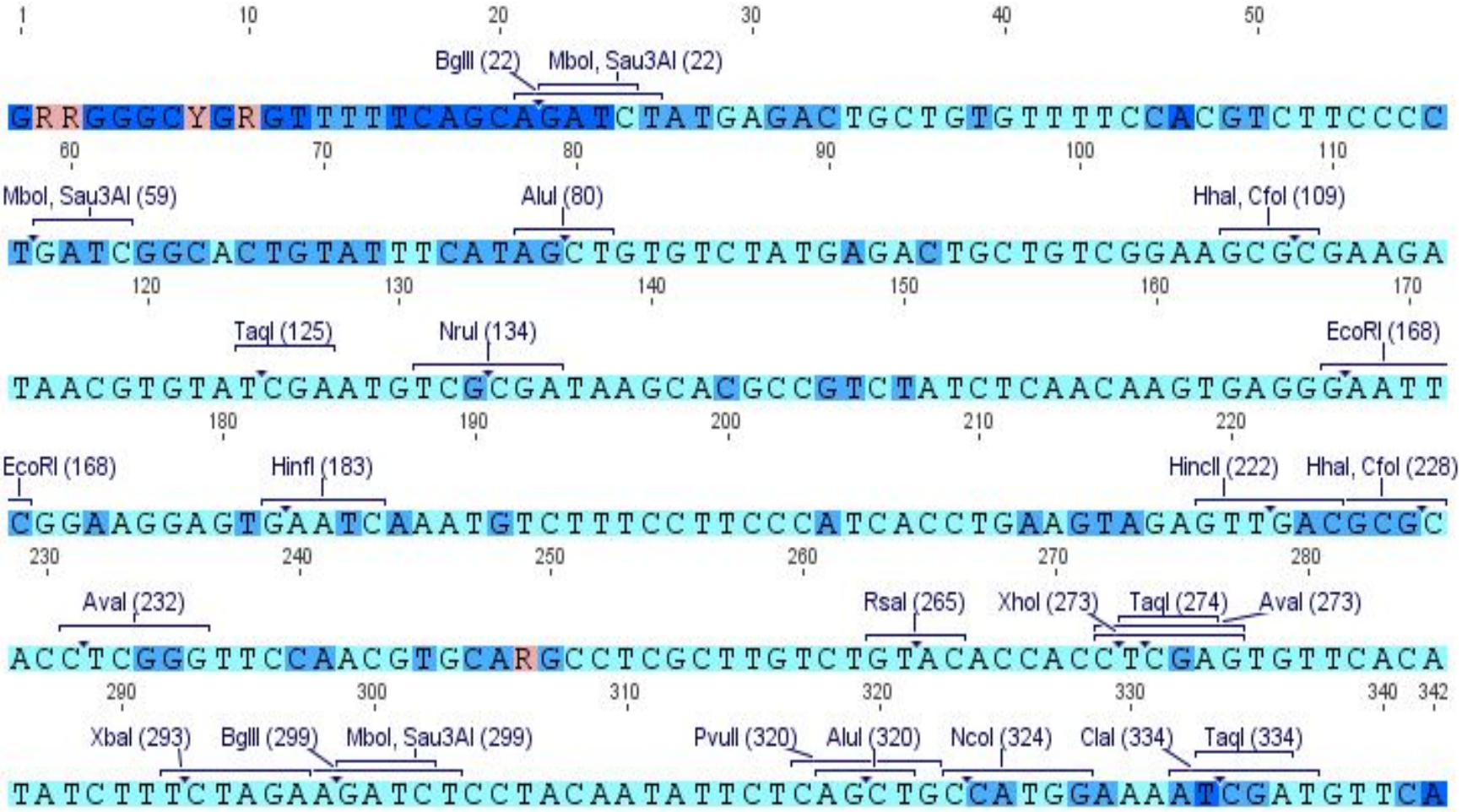


Figure 4.5: Restriction sites within Trans-sialidase gene of LM showing restriction enzyme at their restriction sites. Xbal cuts the gene at site T^CTA of position 293, Taq1 cuts the gene at site T^CGA of position 274, EcoR1 cuts the gene at site G^AAT of position 168, while Aval cuts the gene at two different site of C^TCG of position 232 and 273.

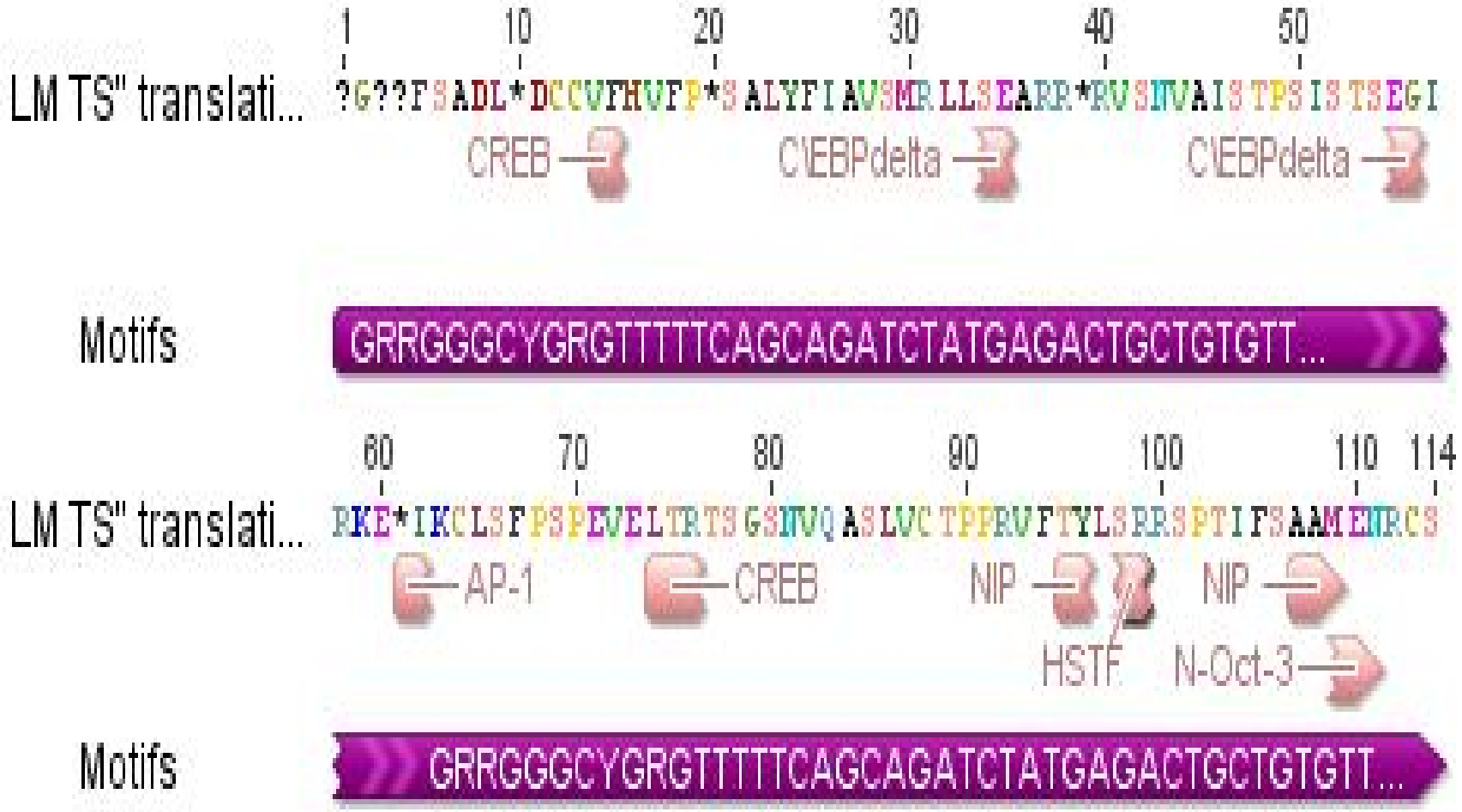


Figure 4.6: Transcription factor and motifs within translated amino acid of the sequenced gene of LM. *AP-1* (activating protein-1), *CREB* (*cAMP response element-binding* protein), *C/EBP* (CCAAT/enhancer binding protein), NIP (NF-AT-interacting protein) N-Oct-3 (octamer-binding) HSTF (*heat shock transcription factor*) and a 42 nucleotide motifs.

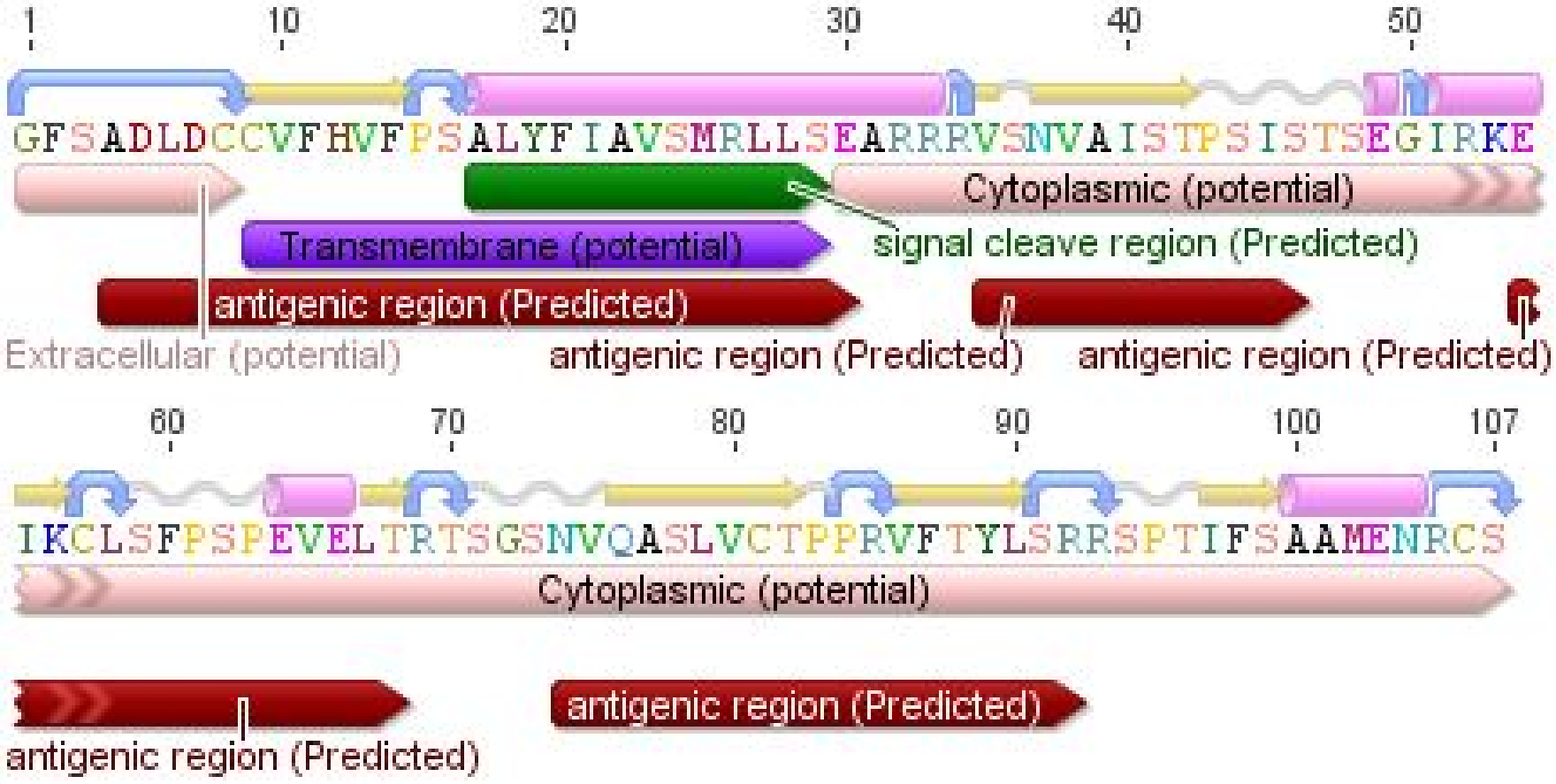


Figure 4.7: An overall view of the gene predictions showing that the signal cleavage is located within the transmembrane potential located within the sequence gene.

4.2: The progress curve for the release of Sialidase

A study of the release of sialidase from Listeria *monocytogenes* within 60 minute of incubation in broth containing about 0.02mg of 4-methylumbelliferyl α-d-acetylneuraminic acid (MU-NANA) which was left overnight showed that the release of sialidase increases to $44 \times 10^{-3}\mu M$ within the study time.

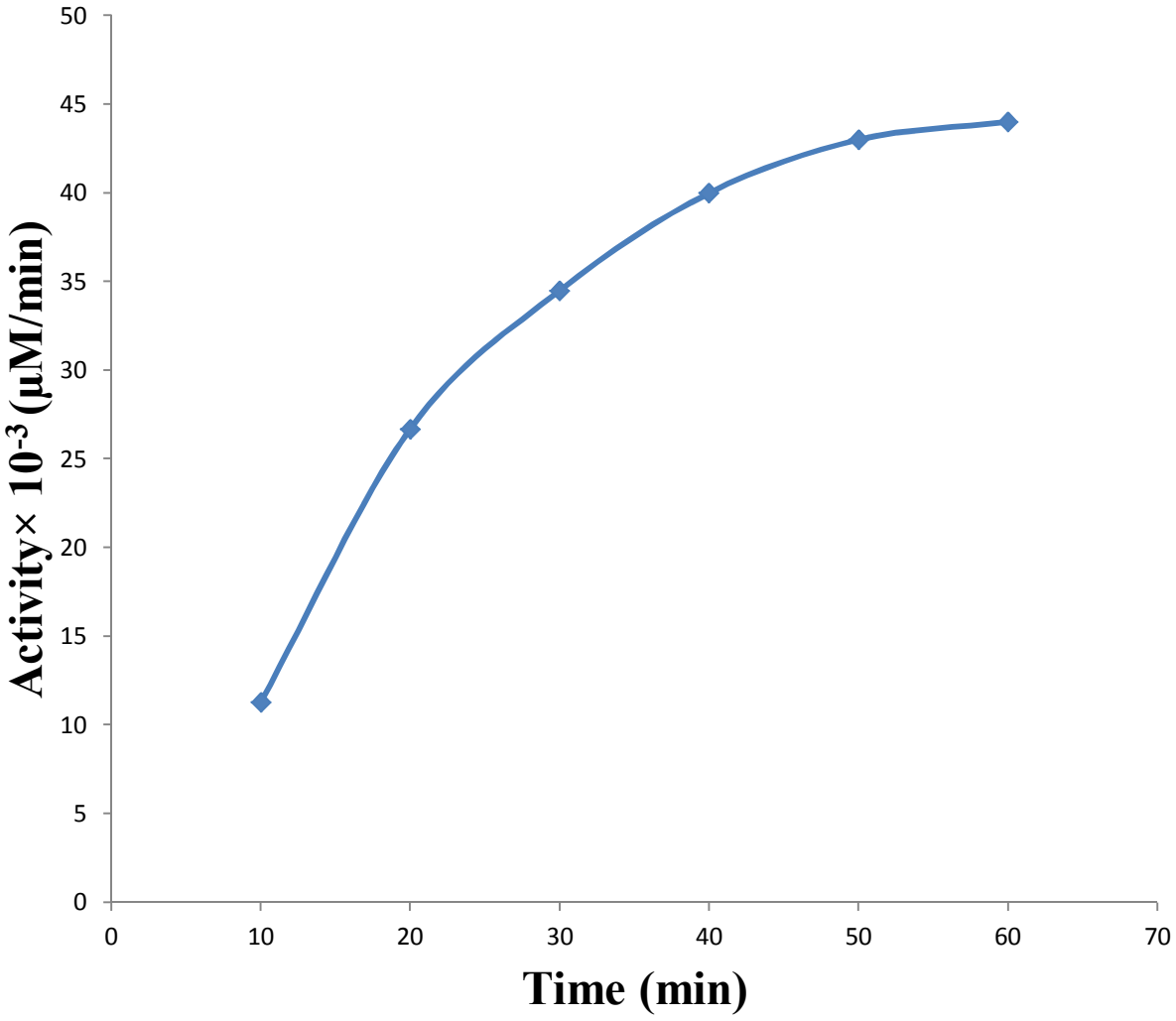


Figure 4.8: Progress curve for the release of Sialidase by *L. monocytogenes*

4.3 Partial purification of Sialidase from *Listeria monocytogenes*

In the first step of enzyme purification by ammonium sulfate precipitation (30% to 90%), specific enzyme activity of the sample gave 2.89mM/min and 39.53 % recovery for 4.25mg/ml total protein. This step served as an initial attempt to remove other proteins in the crude enzyme extract. After the final purification in column chromatography of sephadex G 75, enzyme sample showed a single peak corresponding to the fraction number 18; 6.01-fold purified of sialidase was obtained with 14.11 % recovery. The specific activity of the purified sample was 5.56 mM/min using 4-methylumbelliferyl α-d-acetylneuraminic acid (MU-NANA) as substrate.

Table 4.2: Purification profile for Sialidase isolated from L. Monocytogenes

Purification Step	Total Protein	Total Activity	Specific Activity	Purification fold	Percentage Yield
Crude Protein	4.25	12.28	2.89	1.00	100
Ammonium Sulphate Precipitation	1.68	9.34	5.56	1.96	39.53
Gel filtration on Sephadex G- 75 Column	0.60	10.43	17.38	6.01	14.11

One unit of enzyme activity was defined as the amount of enzyme which produced 1µmole of N-acetylneuraminic acid per minute under the described assay condition.

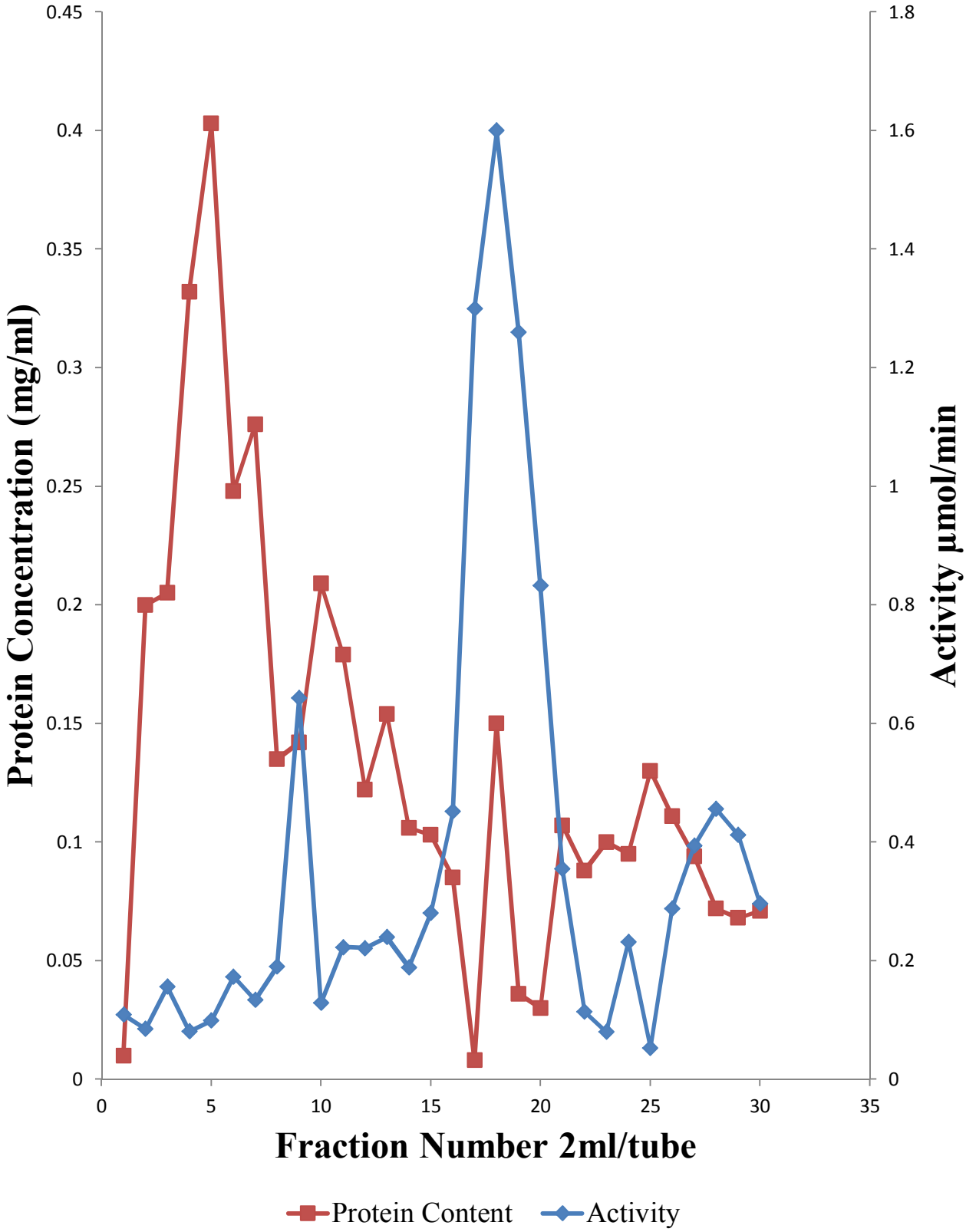


Figure 4.9: Elution profile for *L. monocytogenes* Sialidase on Sephadex G-75 Column Chromatography (1.6 x 90cm)

4.4 Characterization of Listeria Monocytogenes Sialidase

4.4.1 Optimum pH and Temperature

The effect of pH on enzyme activity was determined over a pH range of 3.0 to 10.0; the purified enzyme was active between pH 4.0 and 4.5. Some enzymes are irreversible denaturation in acidic conditions but this was not the case of sialidase of *L. monocytogenes*. A 45.8% loss of enzyme activity was found at pH 10.0; this suggested that the organism can survive slightly acidic areas.

Enzyme activity was measured at different temperatures ranging from 10°C to 80°C using MU-NANA as substrate; sialidase was optimally active at 30 ^{0}C for 8.33× 10^{-4}µmol/min (Figure 4.5). Activity gradually decreased with increasing temperature which was reduced to 26% at 70°C and was almost completely lost at 80°C.

4.4.2 Determination of Kinetic Constants

The K_M and Vmax value for the hydrolysis of 4-methylumbelliferyl α-d-acetylneuraminic acid (MU-NANA) by sialidase was 0.19mM and 0.10µmol/min respectively determined using Lineweaver-Burk plots (Figure 4.9).

4.4.3 Effects of Cations on *L. Monocytogene* Sialidase

The effect of cations on the enzyme activity studied is shown below; in Figure 4.5, it was found that the Sialidase activity was 90% inhibited by Hg^{2+}, and Al^{3+} compared to the control, This results suggested that the enzyme contain some surphur containing amino acid at the active site, which was made ineffective. Ag^{2+} and Mg^{2+} had little inhibitory effects while the enzyme activity was not affected by Mn^{2+}, Zn^{2+}, and Ca^{2+}.

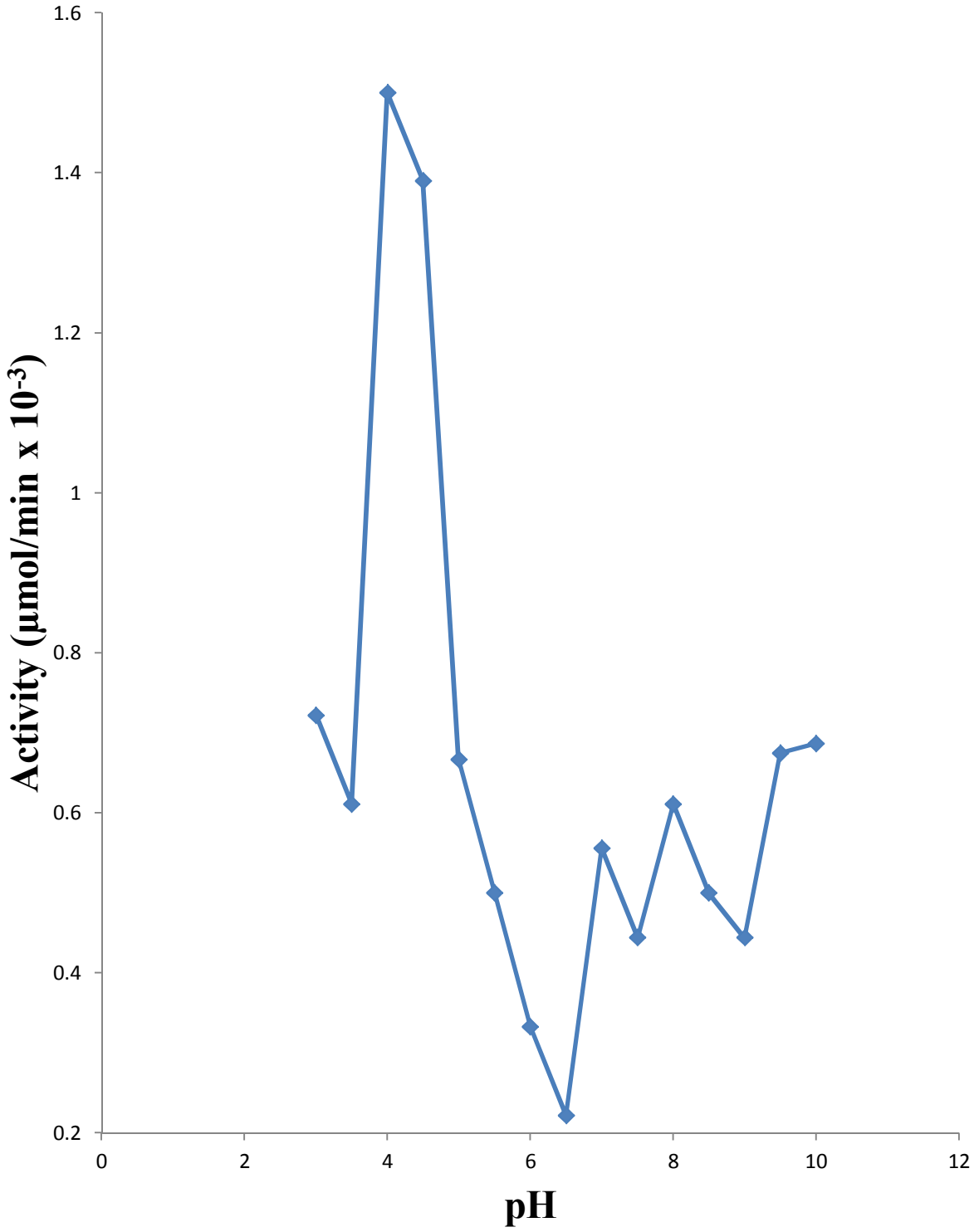


Figure 4.10: Effect of pH on enzyme activity. Activity was measured at various pH values ranging from 3.0 to i0.0 using MU-NANA as substrate at room temperature. Considering pH 4.0-4.5 with the highest activity to be the optimal pH

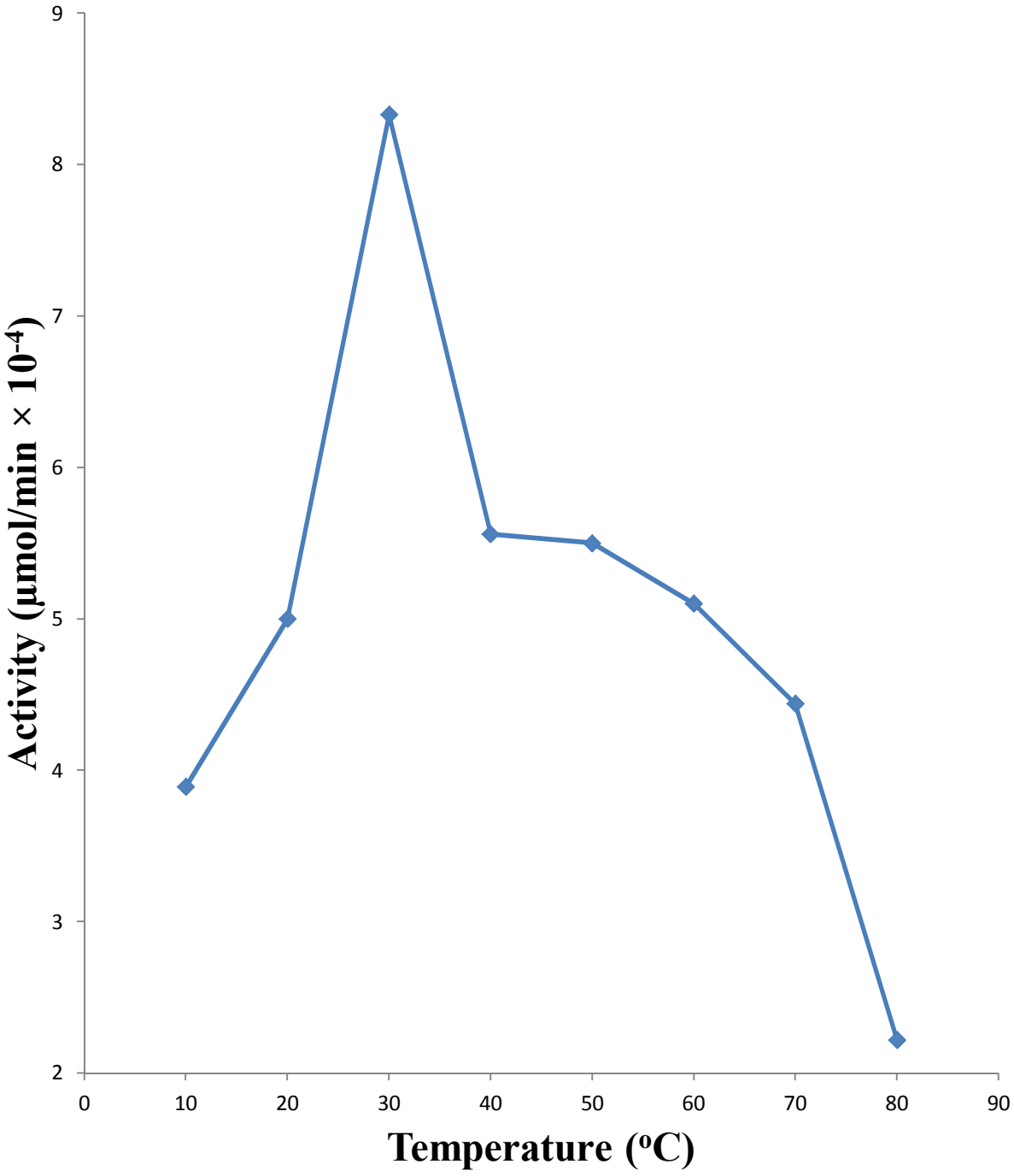


Figure 4.11: Optimum temperature for Sialidase activity: Activity was assayed at various temperatures ranging from 10°C to 80°C using MU-NANA as substrate. Enzyme activity was estimated based on the highest activity detected in the assay at 30°C to be the temperature optimal.

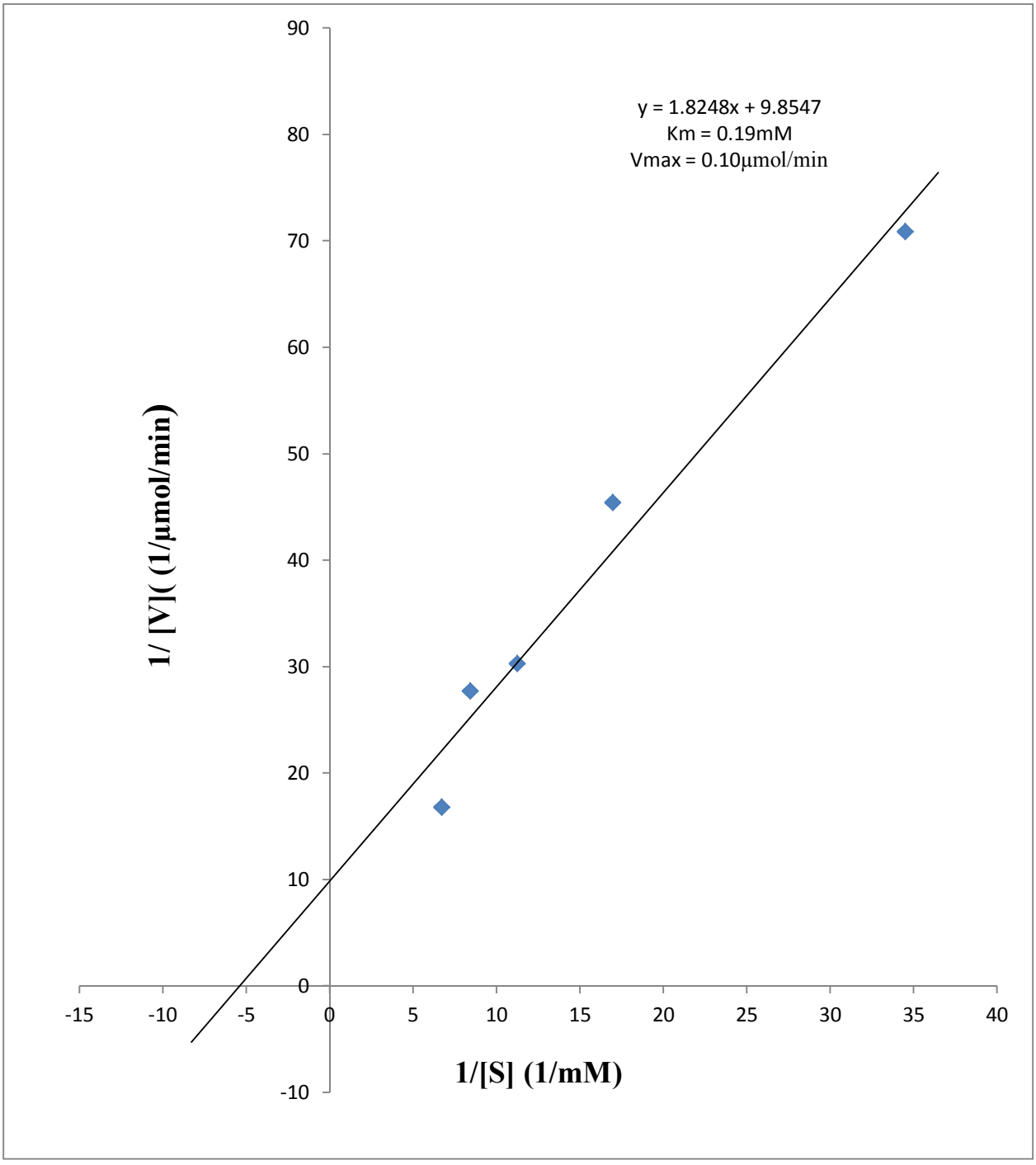


Figure 4.12: Lineweaver-Burk plot for Sialidase kinetics. 1/ [V] and 1/[S] represent the reciprocal of velocity and substrate concentration respectively.

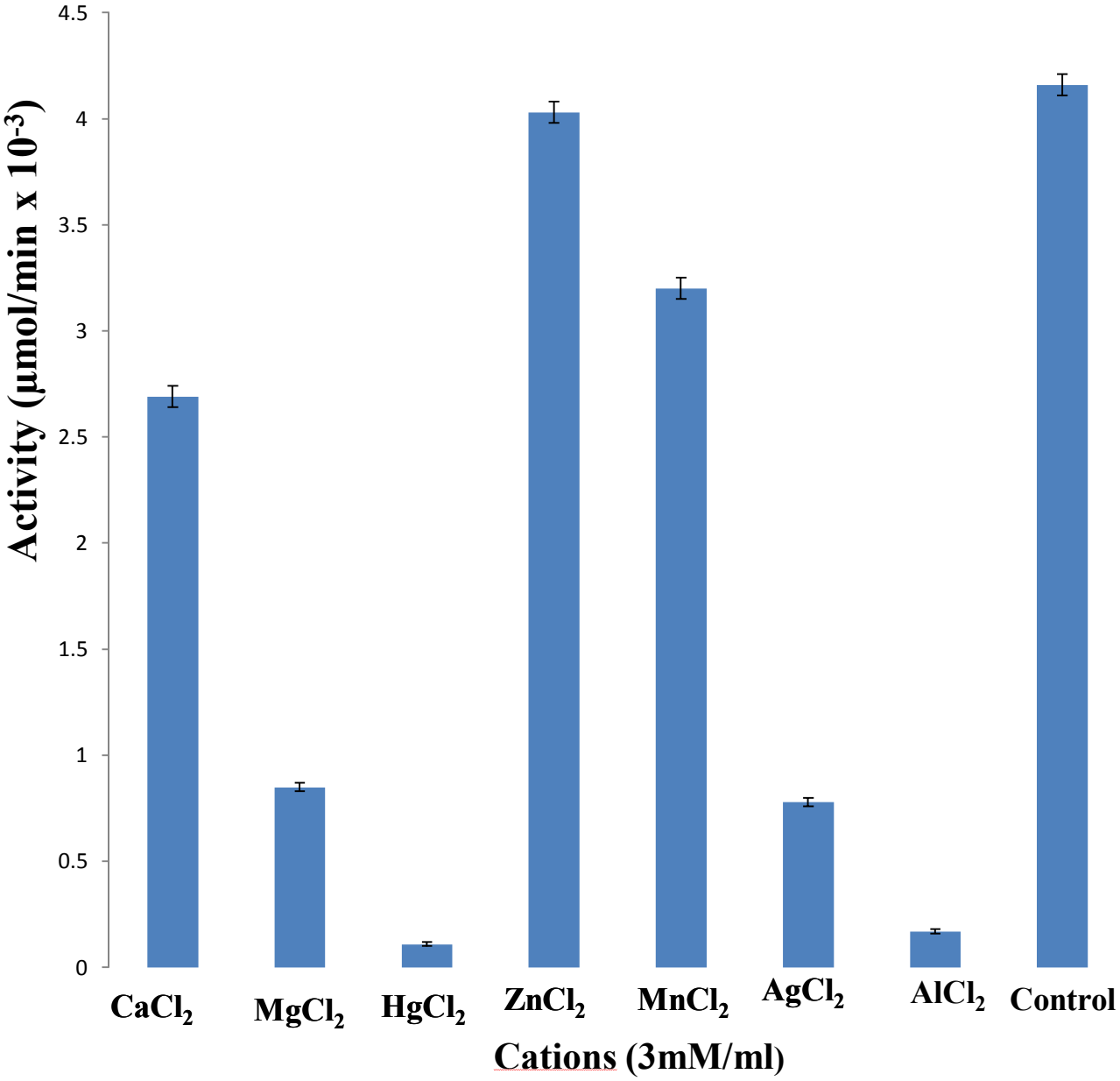


Figure 4.13 Effect of cations on the enzyme activity: The activity was measured using MU-NANA as substrate at room temperature. 90% inhibition was experienced with Hg^{2+}, and Al^{3+}

4.5 The release of neuraminic acid (sialic acid) from 4-methylumbelliferyl α-d-acetylneuraminic acid.

The release of neuraminic acid (sialic acid) from the substrate is an evidence of the ability of the enzyme to cleave off sialic acid from 4-methylumbelliferyl α-d-acetylneuraminic acid. Plate IV shows that hydrolysis occurred in lane 4 of enzyme substrate incubation releasing neuraminic acid.

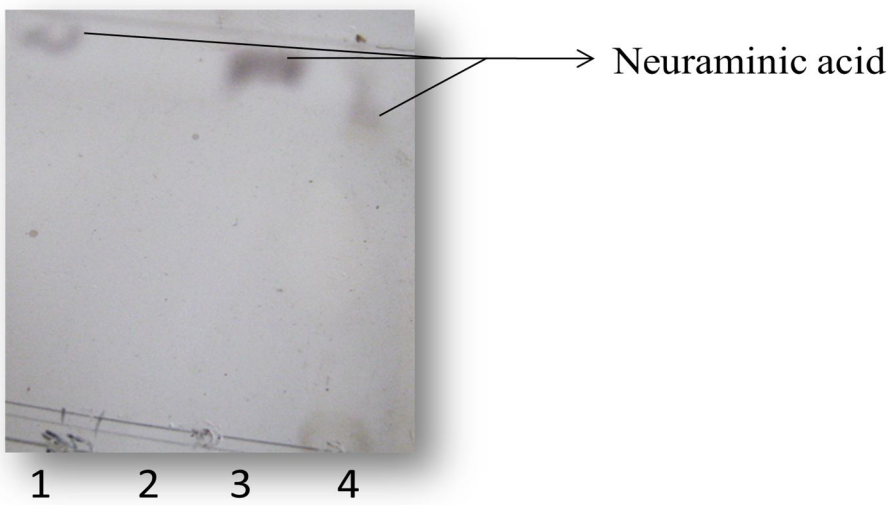


Plate IV: Hydrolysis activity of sialidase enzyme from Listeria monocytogenes on TLC plate using 4-methylumbelliferyl α-d-acetylneuraminic acid. Solvent systems used was chloroform/methanol in the ratio of 4:1

Lane 1— 4-methylumbelliferyl α-d-acetylneuraminic acid (MU-NANA)
Lane 2— 4-methylumbelliferone
Lane 3— neuraminic acid
Lane 4 — enzyme and substrate (MU-NANA) mixture

CHAPTER FIVE

5.0 DISCUSSION

Trans-sialidase/Sialidase have been known to be involved in infection by pathogens, therefore ability to check its presence in *Listeria monocytogenes* and understanding the characteristics of the enzyme raised the concern for this researcher, as it would help in the production of effective therapeutic antilisteriosis. The fairly low K_M value of 0.19 mM showed moderate affinity of the enzyme for 4-methylumbelliferyl α-d-acetylneuraminic acid (MU-NANA) which indicate specificity of the enzyme towards the hydrolysis of 4-methylumbelliferyl α-d-acetylneuraminic acid (MU-NANA) as substrate. This also further explains that the trans-sialidase might be a virulence factor used by the bacteria, in that, the V_{max} value of 0.10µmol/min obtained, implies that the enzyme at the end of every minute of post infection cleaves off 9.6 x 10^{-1}mM of free phosphate groups would have been off, leading to the infectivity. The activity of the enzyme in the presence of Ca^{2+}, Zn^{2+}, and Mn^{2+} indicates that the enzyme is not dependent on these cations for effective catalytic activity, rather there is an inhibition of about 90% with Hg^{2+} and Al^{3+} compared to the control. This could suggest that the existence of sulphur containing amino acid resides at the active site since heavy metals are known to either make inefficient the enzyme, or lower its activity.

The increase of sialidase activity with time is very important as a virulence factor indicating that pathogenicity of the organism is time dependent and this could be one of the reasons why the organism has long incubation period. However, the organism evolved other mechanism to evade elimination by host defense and probably by time saving strategy it will allow the organism for effective production of sialidase.

In relation to temperature, the optimum activity of the enzyme was similar to the body temperature at which the organism grows; hence virulence factor that functions at body temperature would be useful since pathogenic organisms are expected to grow and survive at such temperature, This might be due to greater hydrophilicity and lesser hydrophobic interactions of protein at the active site; activity was significantly reduced at a temperature of 70°C, this may be due to thermal denaturation. Similarly, the slight acidic pH for optimum activity of the enzyme could account for its ability to remain viable in vacuole of phagocytes and remain active in the presence of superoxides and peroxides after release in the vacuoles. The pH may favor the virulence ability to move into host cells by polymerization of host cell actin at one end of the bacterium, thereby helps them propel through cytoplasm since *L. monocytogenes* is an intracellular pathogen (Dussurget et al., 2004; and O'Neil 2006). The low pH is particularly important because the pathogen encounters such environments in vivo, both during passage through the stomach and within the macrophage phagosome (O'Driscoll *et al*., 1996).

This work establishes the presence of Trans-sialidase in *Listeria monocytogenes*, however, efforts to characterize the exsistence of sialidase in the genome of *L. monocytogenes* was not sucessful. The limitations involved in this work could be as a result of the absence of suitable sialidase primers required for amplification of *L. monocytogenes* and ability to optimize correctly the experimental procedures. Primers that amplified sialidase gene in *Clostridium chauvoei* and *Aspergellius fumigatus* was adapted as reported by Warwas *et al* (2007) and Vilei *et al* (2011), but were unable to amplify the gene. Futher work is therefore recommended in this area.

On the other hand, Trans-sialidase, a sub family of sialidase was amplified and characterized using primers obtained from trypanosomes. This could be an indication of similarity between *Listeria monocytogenes* mechanism of pathogenicity and that of trypanosomes as reported by Ian

et al (2002) that some intracellular pathogens such as *Trypanosoma cruzi* and *Listeria monocytogenes* use acid-activatable pore-forming proteins to mediate escape from a phagosome into the host cell cytosol.

The presence of Trans-sialidase in *Listeria monocytogenes* could also be responsible for the sialidase activity and perform the same function as sialidase as observed by Schrader and Schauer (2006) that in the absence of an appropriate acceptor this enzyme (Trans-sialidase) acts as a sialidase, hydrolytically releasing glycosidically-linked sialic acid being a virulence and pathogenic factor. The report of Srinivas *et al* (1998) showed that *N*-acetylneuraminic acid (NAcNeu), a member of the sialic acid group is involved in the attachment of *L. monocytogenes* to permissive murine macrophages but the question now will be through which mechanism. It could possibly be through the Trans-sialidase catalysed reaction.

With respect to defect in glycosylation which is as a result of reduction of free sialic acid content in host cells, it could be that Trans-sialidase and sialidase enhance infectivity in various organisms because systemic disease might have developed with a compromised immune system. Hence synthesizing cDNA from *L. monocytogenes*, we were able to amplify Trans-sialidase gene rather than sialidase gene as proposed. This finding links a hint that could be of benefit to the treatment and management of listeriosis, in that ability of the organism to cross the host barriers and survive within a large variety of phagocytic cells could be through its ability to express trans-sialidase gene, which might be that the organism masks itself with sialic acid preventing the host immune defense mechanism against it. This understanding will further facilitate effective control strategies to reduce human and animal listeriosis cases.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 SUMMARY

- *Listeria monocytogenes* is a bacterium of considerable medical and economica importance because they are the causative agents of Listeriosis, a disease which affects both livestock and human.
- In this study, we were able to identify that *Listeria monocytogenes* expresses a unique sialic acid-metabolizing enzyme, Trans-sialidase.
- The biology of this organism presents some unusual features, one of which may be the mechanism employed for the addition of sialic acid units to its own glycoproteins through Trans- sialidase mediated process, a transglycosylase for sialic acid rather than sialidase.
- The Trans-sialidase of *Listeria monocytogenes* is a potential target against Listeriosis chemotherapy in that the organism obtains sialic acid through *trans*-sialidase-mediated transfers from host sources. This makes *L. monocytogenes trans*-sialidase a potential drug target against Listeriosis.
- It could be that Trans-sialidase and sialic acid acceptors on the surface of *Listeria monocytogenes* participate in host bacteria interactions and mediate the initial stages of the Listeriosis invasion of host cells.
- The *trans*-sialidase of *Listeria monocytogenes* is thought to play an important role in the pathogenesis of Listeriosis.
- The *trans*-Sialidase from *Listeria monocytogenes* might be a key enzyme in search of new and more effective anti- Listeriosis agents.

- This work provides a reference to future studies on mechanism of infection by *L. monocytogenes*

6.2 CONCLUSION

- In this study, *Listeria monocytogenes* genome has been found to contain Trans-sialidase gene a sialic acid-metabolizing enzyme which could serve as a potential target against Listeriosis chemotherapy. The amplified genes coding for Trans-sialidase was cloned and sequenced. Furthermore, characteristic of the gene showed similarity with other known sialidase and trans- sialidase gene. The determined enzymatic characterization of Trans-sialidase from *Listeria monocytogenes* will contribute to the understanding of its role in pathogenesis in relation to other known trans-sialidase.

6.3 RECOMMENDATION

- Since infection by *Listeria monocytogenes* is of economic importance and the organism expresses trans-sialidase, qait will be essential to study the effect of inhibitors on the enzyme activity.

- Also to find out if Trans-sialidase of *Listeria monocytogenes* share a similar binding site to that of Trans-sialidase of trypanosomes.

- It will be vital to know the mechanism and structural function of *Listeria monocytogenes* Trans-sialidase activity.

- The expression pattern of this gene and their activities in the different stages of Listeriosis should be considered for futher studies.

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APPENDIX 1.0

CALCULATION FORMULA FOR PURIFICATION PROFILE

$$\text{Total Protein} = \frac{\text{Total Activity}}{\text{Specific Activity}}$$

$$\text{Total Activity} = \text{Specific Activity} \times \text{Total Protein}$$

$$\text{Specific Activity} = \frac{\text{Enzyme Activity (Total Activity)}}{\text{Total Protein}}$$

$$\text{Purification Fold} = \frac{\text{Specific Activity of Protein at a Purification Step}}{\text{Original Specific Activity (Crude)}}$$

$$\text{Percentage Yield} = \frac{\text{Total Activity of protein at a step}}{\text{Total Activity of Original Purification (Crude)}} \times 100$$

$$\text{Enzyme Activity } (\mu\text{mol/min}) = \frac{0.017 \times \text{Absorbance}}{\text{Incubation time}}$$

APPENDIX 2.0

PREPARATION OF STANDARD REAGENTS

2.1 Preparation of Phosphate Buffered Saline

The 10mM phosphate buffer (pH 8.0) used for this work was prepared dissolving 1.09 grams of Disodium hydrogen phosphate (Na_2HPO_4), 0.32 grams of Sodium Di-hydrogen Phosphate (NaH_2PO_4) and 9 grams of Sodium Chloride (NaCl) in one liter of distilled water.

2.2 Media for bacterial growth

LB medium

10 g/L Bacto -Tryptone, 5 g/L Bacto -Yeast Extract, 5 g/L NaCl, adjust the pH to 7.5 with NaOH autoclave to sterilize. Allow the auto-claved medium to cool to 55ºC and add ampicillin (final concentration 100 µg/ml). For LB plates, 1.5% Bacto-agar (15 g/L) was added prior to autoclaving.

S.O.C. medium. 2% Tryptone (bacto), 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose.

Buffer and additional solutions

Transformation Buffer ($CaCl_2$) solution 10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl. (PIPES 3.021 g/l, $CaCl_2.2H_2O$ 2.205 g/l, KCl 18.637 g/l, $MnCl_2.4H_2O$ 10.885 g/l). All the components except for $MnCl_2$ were mixed and the pH was adjusted to 6.7 with KOH. Then, $MnCl_2$ was dissolved, the solution was sterilized by filtration through a prerinsed 0.45 µm filter unit and stored at 4ºC, all salts were added as solids, always kept and used in cold.

DMSO bought from ALPHA Biotechnologies Company

2.3 Preparation Ampicillin stock solution (50 mg/ml)

2.5 g ampicillin sodium salt was dissolved in 50 ml of deionized water, filtered, sterilized and store in aliquots at 4°C.

2.4 Preparation of LB-ampicillin plates

10 g, of Bacto Tryptone®, 5g Bacto Yeast extract® and 5g NaCl was dissolved in 800 ml of water, adjusted pH to 7.0 with NaOH and add water to 1000 ml. 15 g of agar was added and

autoclave. It was allowed cool to 55°C then 2 ml of ampicillin stock solution (50 mg/ml) was added to a final concentration of 100 µg/ml.

APPENDIX 3.0

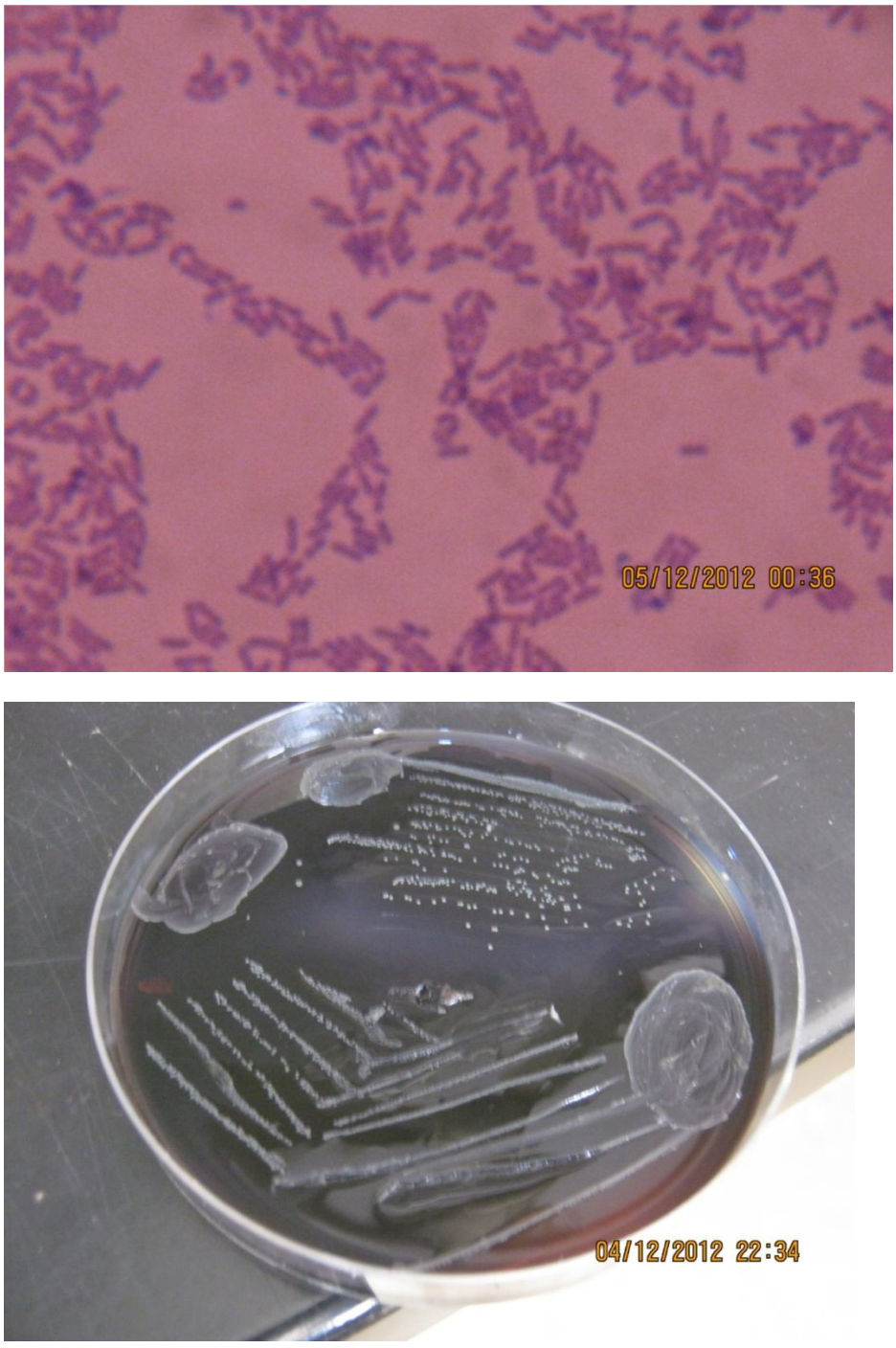


Figure 4.1: Morphology of gram stained slides and plate of *Listeria monocytogenes*.