JADAVPUR UNIVERSITY

Studies on the effect of different monosaccharides on *Vibrio cholerae*

A dissertation report submitted to Jadavpur University in partial fulfillment of the requirement for the award of the degree of

M.Sc in Bio-technology
Department of "Life science & Bio-technology"

by

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KOLKATA

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1. INTRODUCTION

1.1.Cholera :

Cholera is an infectious disease that causes severe watery diarrhea, which can lead to dehydration and even death if untreated. It is caused by eating food or drinking water contaminated with a bacterium called *Vibrio cholerae*.

1.2. Discovery:

V. cholerae was first isolated as the cause of cholera by Italian anatomist **Filippo Pacini** in 1854, but his discovery was not widely known until **Robert Koch**, working independently 30 years later, publicized the knowledge and the means of fighting the disease.

Figure 1: Pacini's discovery

Cholera came to Florence in 1854 during the Asiatic Cholera Pandemic of 1846-63. Pacini became very interested in the disease. Immediately following the death of cholera patients, he performed an autopsy and with his microscope, conducted histological examinations of the intestinal mucosa. During such studies, Pacini first discovered a comma-shaped bacillus which he described as a *Vibrio.* He published a paper in 1854 entitled, "Microscopical observations and pathological deductions on cholera" in which he described the organism and its relation to the disease. His microscopic slides of the organism were clearly labeled, identifying the date and nature of his investigations.

1.3. Morphology:

Vibrio cholerae, a gram-negative, non-sporing, non-capsulated rod-shaped bacteria with a small bend in the middle and a long tail-like flagella. Gram-negative refers to its thin cell wall, which is surrounded by a protective outer membrane. The bacteria swim quickly

about in infested water using their flagella in a whip-like manner to propel forward. *V. cholerae* can survive in warm, basic (pH > 7) and salty water for long periods of time, but it always needs a source of water. It doesn't grow well in cold and dry climates. This is one reason that cholera is endemic (remains without external input) in parts of Asia and Latin

Figure 2: A *V. cholerae* bacterium outlined in red

America. In liquid cultures they may be arranged singly, in pairs or in S-shaped spiral forms. Pleomorphism is common in old cultures.2 X 0.5 micrometer in size.

1.4. Microbiological features:

Only cholera toxin-producing (toxigenic) strains of V. cholerae are associated with cholera. While some environmental *V. cholerae* are toxigenic and capable of causing cholera, most environmental *V. cholerae* isolates are not toxigenic. Toxigenic strains harbor a filamentous bacteriophage (CTXΦ) which encodes cholera toxin (CT).

1.5. Genome:

V. cholerae has two circular chromosomes, together totaling 4 million base pairs of DNA sequence and 3,885 predicted genes. The genes for cholera toxin are carried by CTXφ, a temperate bacteriophage inserted into the V. cholerae genome. CTXφ can transmit cholera toxin genes from one V. cholerae strain to another, one form of horizontal gene transfer. The genes for toxin coregulated pilus are coded by the *Vibrio* pathogenicity island (VPI). The entire genome of the virulent strain V. cholerae El Tor N16961 has been sequenced, and contains two circular chromosomes. Chromosome 1 has 2,961,149 base pairs with 2,770 ORF's and chromosome 2 has 1,072,315 base pairs, 1,115 ORF's. The larger first chromosome contains the crucial genes for toxicity, regulation of toxicity, and important cellular functions, such as transcription and translation.

The second chromosome is determined to be different from a plasmid or mega plasmid due to the inclusion of housekeeping and other essential genes in the genome, including

essential genes for metabolism, heat-shock proteins, and 16S rRNA genes, which are ribosomal subunit genes used to track evolutionary relationships between bacteria. Also relevant in determining if the replicon is a chromosome is whether it represents a significant percentage of the genome, and chromosome 2 is 40% by size of the entire genome. And, unlike plasmids, chromosomes are not self-transmissible. However, the second chromosome may have once been a mega plasmid because it contains some genes usually found on plasmids.

Figure 3: Genome structure of *Vibrio cholerae* showing (A) chromosome1 & (B) chromosome2

1.6.Taxonomy:

Scientific Name: *Vibrio cholerae*

Classification:

Kingdom: Monera
Phylum: Proteobacteria
Class: Gamma Proteobacteria
Order: Vibrionales
Family: Vibrionaceae
Genus: *Vibrio*
Species: *V. cholerae*

1.7. Serological Classification:

Serological classification of *V. cholerae* first described by Gardner &Venkatraman (1935). Until recently, epidemic cholera was exclusively associated with *V. cholerae* strains of the O1 serogroup. All strains that were identified as *V. cholerae* on the basis of

biochemical tests but that did not agglutinate with "O" antiserum were collectively referred to as non-O1 *V. cholerae*. The non-O1 strains are occasionally isolated from cases of diarrhea (Ramamurthy et al., 1993a) and from a variety of extra intestinal infections, from wounds, and from the ear, sputum, urine, and cerebrospinal fluid (Morris & Black, 1985). They are ubiquitous in estuarine environments, and infections due to these strains are commonly of environmental origin (Morris, 1990).

The O1 serogroup exists as two biotypes, classical and El Tor; antigenic factors allow further differentiation into two major serotypes— Ogawa and Inaba. Strains of the Ogawa serotype are said to express the A and B antigens and a small amount of C antigen, whereas Inaba strains express only the A and C antigens. A third serotype (Hikojima) expresses all three antigens but is rare and unstable.

The simple distinction between V. cholerae O1 and V. choleraenon-O1 became obsolete in early 1993 with the first reports of a new epidemic of severe,cholera-like disease in Bangladesh (Albert et al., 1993) and India (Ramamurthyet al., 1993b). At first, the responsible organism was referred to as non-O1 *V.cholerae*because it did not agglutinate with O1 antiserum. However, further investigations revealed that the organism did not belong to any of the O serogroups previously described for *V. cholerae*but to a new serogroup, which was given the designation O139 Bengal after the area where the strains werefirst isolated (Shimada et al., 1993).

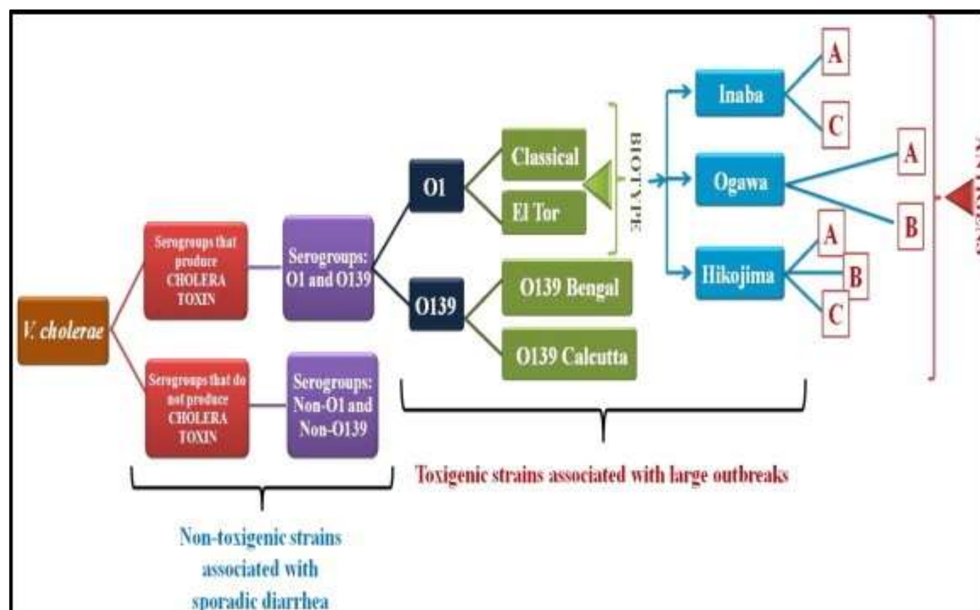


Figure 4: Classification of *V. cholerae*

1.8. Cholera pandemics:

Seven cholera pandemics have occurred in the past 200 years,

- First, 1817–24: The first cholera pandemic, though previously restricted, began in Bengal, and then spread across India by 1820.
- Second, 1829–51: A second cholera pandemic reached Russia, Hungary (about 100,000 deaths) and Germany in 1831. In 1832 it reached London and the United Kingdom and Paris. The epidemic reached Quebec, Ontario, Nova Scotia and New York in the same year, and the Pacific coast of North America by 1834.
- Third, 1852–1860: The third cholera pandemic mainly affected Russia, with over one million deaths. In 1852, cholera spread east to Indonesia, and later was carried to China and Japan in 1854. The Philippines were infected in 1858 and Korea in 1859. In 1859, an outbreak in Bengal contributed to transmission of the disease by travelers and troops to Iran, Iraq, Arabia and Russia. Japan suffered at least seven major outbreaks of cholera between 1858 and 1902.
- Fourth, 1863–1875: The fourth cholera pandemic of the century began in the Ganges Delta of the Bengal region and traveled with Muslim pilgrims to Mecca.
- Fifth, 1881–1896: The fifth cholera pandemic, according to Dr. A. J. Wall, the 1883–1887 part of the epidemic cost 250,000 lives in Europe and at least 50,000 in the Americas.
- Sixth, 1899–1923: The sixth cholera pandemic had little effect in Western Europe because of advances in public health, but major Russian cities and the Ottoman Empire were particularly hard hit by cholera deaths.
- Seventh, 1961–1975: The seventh cholera pandemic began in Indonesia, called El Tor after the strain, and reached East Pakistan (now Bangladesh) in 1963, India in 1964, and the Soviet Union in 1966. From North Africa, it spread into Italy by 1973. In the late 1970s, there were small outbreaks in Japan and in the South Pacific.

1.9. Pathogenicity & Symptoms:

V. cholerae pathogenicity genes code for proteins directly or indirectly involved in the virulence of the bacteria. During infection, *V. cholerae* secretes cholera toxin, a protein that causes profuse, watery diarrhea (known as "rice-water stool"). Colonization of the

small intestine also requires the toxin coregulated pilus (TCP), a thin, flexible, filamentous appendage on the surface of bacterial cells. *V. cholerae* can cause syndromes ranging from asymptomatic to cholera gravis. In endemic areas, 75% of cases are asymptomatic, 20% are mild to moderate, and 2-5% are severe forms such as cholera gravis. Symptoms include abrupt onset of watery diarrhea (a grey and cloudy liquid), occasional vomiting, and abdominal cramps. Dehydration ensues, with symptoms and signs such as thirst, dry mucous membranes, decreased skin turgor, sunken eyes, hypotension, weak or absent radial pulse, tachycardia, tachypnea, hoarse voice, oliguria, cramps, renal failure, seizures, somnolence, coma, and death. Death due to dehydration can occur in a few hours to days in untreated children. The disease is also particularly dangerous for pregnant women and their fetuses during late pregnancy, as it may cause premature labor and fetal death. In cases of cholera gravis involving severe dehydration, up to 60% of patients can die; however, less than 1% of cases treated with rehydration therapy are fatal. The disease typically lasts 4–6 days. Worldwide, diarrhea disease, caused by cholera and many other pathogens, is the second-leading cause of death for children under the age of 5 and at least 120,000 deaths are estimated to be caused by cholera each year. In 2002, the WHO deemed that the case fatality ratio for cholera was about 3.95%.

Why *Vibrio Cholerae* is still prevalent?

Although there is a vaccine, made from killed cholera bacteria, it is neither very effective nor does it provide immunity for long. Because of its unreliability, the World Health Organization does not recommend it. Scientists are attempting to design better vaccines; by genetic engineering, they hope to rid the cholera microbes of their toxin so that reliable, safe live vaccines can be made.

Nonpathogenic strains are also present in water ecologies. The wide variety of pathogenic and nonpathogenic strains that co-exist in aquatic environments are thought to allow for so many genetic varieties. Gene transfer is fairly common amongst bacteria, and recombination of different *V. cholerae* genes can lead to new virulent strains.

Cholera vaccines offer incomplete protection. Therefore, vaccination should never take the place of standard prevention and control measures.

1.10. Habitat:

According to WHO (2000), *V. cholerae* is often found in the aquatic environment and is part of the normal flora of brackish water and estuaries. It is often associated with algal blooms (plankton), which are influenced by the temperature of the water. Todar (2002) notes that *Vibrio* spp. are typically marine organisms, so most species require 2-3% NaCl or a seawater base for optimal growth; however, *V. cholerae* occurs in both marine and freshwater habitats in mutualistic associations with aquatic animals. *V. cholerae* is the most important *Vibrio sp*. to humans, although other species are pathogenic for invertebrates and other vertebrates. Finkelstein notes that in coastal regions it may persist in shellfish and plankton. *V. cholerae* is endemic or epidemic in areas with poor sanitation, but long-term convalescent carriers are rare.

1.11. Reservoir:

The main reservoirs of *V. cholerae* are people and aquatic sources such as brackish water and estuaries, often in association with copepods or other zooplankton, shellfish, and aquatic plants.

Cholera infections are most commonly acquired from drinking water in which *V. cholerae* is found naturally or into which it has been introduced from the feces of an infected person. Other common vehicles include contaminated fish and shellfish, produce, or leftover cooked grains that have not been properly reheated. Transmission from person to person, even to health care workers during epidemics, is rarely documented. *V. cholerae* thrives in an aquatic environment, particularly in surface water. The primary connection between humans and pathogenic strains is through water, particularly in economically reduced areas that do not have good water purification systems.

1.12. Cholera toxin:

Cholera toxin (also known as choleragen and sometimes abbreviated to CTX, Ctx or CT) is protein complex secreted by the bacterium *Vibrio cholerae*. CTX is responsible for the massive, watery diarrhea characteristic of cholera infection.

Structure:

The cholera toxin is an oligomeric complex made up of six protein subunits: a single copy of the A subunit (part A, enzymatic), and five copies of the B subunit (part B,

receptor binding), denoted as AB5. Subunit B binds while subunit A activates the G protein which activates adenylatecyclase. The three-dimensional structure of thetoxin was determined using X-ray crystallography by Zhang et al. in 1995.

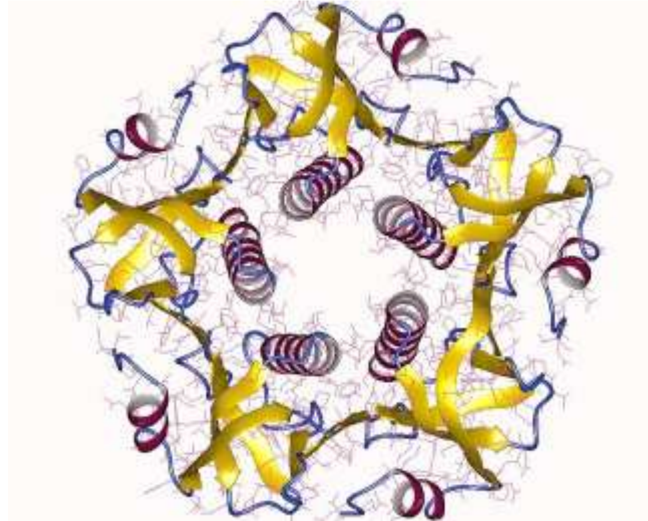


Figure 5: Structure of *V. cholerae* Cholera Toxin.

The five B subunits—each weighing 11 kDa, form a five-membered ring. The A subunit which is 28 kDa, has two important segments. The A1 portion of the chain (CTA1) is a globular enzyme payload that ADP-ribosylates G proteins, while the A2 chain (CTA2) forms an extended alpha helix which sits snugly in the central pore of the B subunit ring.

This structure is similar in shape, mechanism, and sequence to the heat-labile enterotoxin secreted by some strains of the Escherichia coli bacterium.

Mechanism:

Cholera toxin acts by the following mechanism: First, the B subunit ring of the cholera toxin binds to GM1 gangliosides on the surface of target cells. The B subunit can also bind to cells lacking GM1. The toxin then most likely binds to other types of glycans, such as Lewis Y and Lewis X, attached to proteins instead of lipids. Once bound, the entire toxin complex is endocytosed by the cell and the cholera toxin A1 (CTA1) chain is released by the reduction of a disulfide bridge. The endosome is moved to the Golgi apparatus, where the A1 protein is recognized by the endoplasmic reticulum chaperone, protein disulfide isomerase. The A1 chain is then unfolded and delivered to the membrane, where Ero1 triggers the release of the A1 protein by oxidation of protein

disulfide isomerase complex. As the A1 protein moves from the ER into the cytoplasm by the Sec61 channel, it refolds and avoids deactivation as a result of ubiquitination.

CTA1 is then free to bind with a human partner protein called ADP-ribosylation factor 6 (Arf6); binding to Arf6 drives a change in the shape of CTA1 which exposes its active site and enables its catalytic activity. The CTA1 fragment catalyses ADP-ribosylation of the Gs alpha subunit (Gαs) proteins using NAD. The ADP-ribosylation causes the Gαs subunit to lose its catalytic activity of GTP hydrolization into GDP + Pi, thus maintaining Gαs in its activated state. Increased Gαs activation leads to increased adenylatecyclase activity, which increases the intracellular concentration of 3',5'-cyclic AMP (cAMP) to more than 100-fold over normal and over-activates cytosolic PKA. These active PKA then phosphorylate the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel proteins, which leads to ATP-mediated efflux of chloride ions and leads to secretion of H2O, Na+, K+, and HCO3− into the intestinal lumen. In addition, the entry of Na+ and consequently the entry of water into enterocytes are diminished. The combined effects result in rapid fluid loss from the intestine, up to 2 liters per hour, leading to severe dehydration and other factors associated with cholera, including a rice-water stool. The pertussis toxin (also an AB5 protein) produced by Bordetella pertussis acts in a similar manner with the exception that it ADP- ribosylates the Gαi subunit, rendering it unable to inhibit cAMP production.

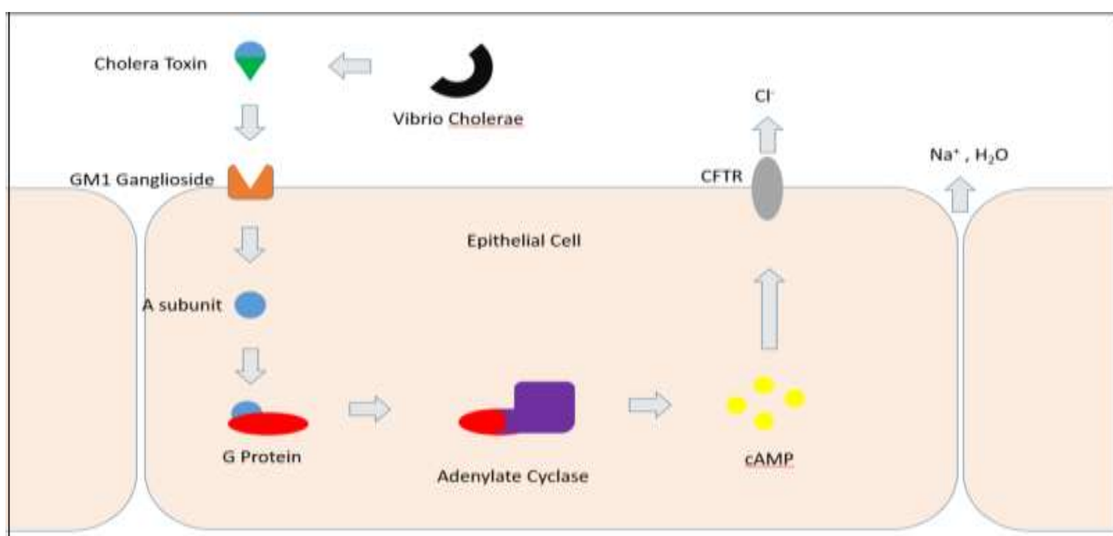


Figure 6: Mode of action of Cholera toxin

1.13.Vaccines:

Vaxchora® (lyophilized CVD 103-HgR):

Vaxchora® has been reported to reduce the chance of severe diarrhea in people by 90% at 10 days after vaccination and by 80% at 3 months after vaccination. The safety and effectiveness of Vaxchora® in pregnant or breastfeeding women is not yet known, and it is also not known how long protection lasts beyond 3 – 6 months after getting the vaccine. Side effects from Vaxchora® are uncommon and may include tiredness, headache, abdominal pain, nausea and vomiting, lack of appetite, and diarrhea.

Dukoral®, ShanChol®, and Euvichol-Plus®/Euvichol®

Three other oral inactivated, or non-live cholera vaccines are available: Dukoral (manufactured by SBL Vaccines); ShanChol (manufactured by ShanthaBiotec in India), and Euvichol-Plus/Euvichol (manufactured by Eubiologics). These cholera vaccines are World Health Organization (WHO) prequalified, but are not available in the U.S.

Cholera vaccines offer incomplete protection. Therefore, vaccination should never take the place of standard prevention and control measures.

1.14. Biofilm:

Biofilm is an association of micro-organisms in which microbial cells adhere to each other on a living or non-living surface within a self-produced matrix of extracellular polymeric substance. Biofilm formation is a multi-step process starting with attachment to a surface then formation of micro-colony that leads to the formation of three-dimensional structure and finally ending with maturation followed by detachment. During biofilm formation many species of bacteria are able to communicate with one another through specific mechanism called quorum sensing. It is a system of stimulus to co-ordinate different gene expression.

Role of Biofilm in causing disease:

Bacterial biofilm is infectious in nature and can results in nosocomial infections. According to National Institutes of Health (NIH) about 65% of all microbial infections, and 80% of all chronic infections are associated with biofilms. Bacterial biofilm is less accessible to antibiotics and human immune system and thus poses a big threat to public health because of its involvement in variety of infectious diseases.

Vibrio cholerae Biofilms and Cholera Pathogenesis:

Vibrio cholerae can switch between motile and biofilm lifestyles.Evidence has grown suggesting that *V. cholerae* can form biofilm-like aggregates during infection that could play a critical role in pathogenesis and disease transmission.This definition includes communities of *Vibrios* anchored to abiotic surfaces or to biotic substrata such as the human intestinal mucosa or the chitinous exoskeleton of crustaceans, *Vibrio* aggregates in suspension, floccules, and pellicles formed at the liquid–air interface of static cultures.

It has been established that *V. cholerae* cells in planktonic, monolayer, and mature biofilm stages differ in their global transcription profile. A major event in the transition from planktonic to biofilm lifestyle is the down-regulation of motility gene expression and induction of genes required for the biosynthesis of the biofilm extracellular matrix. In the mature biofilm microenvironment, cells are packed within a smaller volume, and nutrient accessibility and the elimination of toxic metabolic products is limited by diffusion. These conditions favor an early entry of cells into quorum sensing mode and stationary phase. As an example, the cholera autoinducer 1 was shown to accumulate to a higher concentration in biofilms compared to planktonic cells, resulting in earlier expression of the quorum sensing regulator HapR. In turn, HapR enhances the expression of the stationary phase sigma factor RpoS. Thus, the mature biofilm exhibits a gene expression pattern that favors resistance to environmental stressors.It is well established that the intracellular concentration of the second messenger cyclic diguanylic acid (c-di-GMP) controls the transition between *V. cholerae* planktonic and biofilm lifestyles. c-di-GMP is synthesized from GTP by the activity of diguanylatecyclase (DGC) exhibiting GGDEF domains and degraded to GMP by phosphodiesterases (PDE) exhibiting EAL or HD-GYP domains. The *V. cholerae* genome encodes 31 GGDEF, 22 EAL, 9 HD-GYP, and 10 combined GGDEF-EAL domain proteins. Most of these proteins display a modular architecture with added sensor, effector, and DNA binding domains.

Three major regulators sense the intracellular concentration of c-di-GMP: the σ54-dependent activator FlrA required for the expression of flagellar motility and the biofilm activators VpsR and VpsT. Five membrane-bound DGC (CdgA, H, L, K, and

M) act additively to increase the c-di-GMP pool and promote dimerization and activation of VpsT to induce biofilm formation.

1.15. Monosaccharides and its effect on *V. cholerae*:

Monosaccharide:

Monosaccharides, also called simple sugars, are the simplest form of sugar and the most basic units of carbohydrates. They cannot be further hydrolyzed to simpler chemical compounds. The general formula is CnH2nOn. They are usually colorless, water-soluble, and crystalline solids. Some monosaccharides have a sweet taste.

Decrease in Culturability of *Vibrio cholerae* Caused by Monosaccharides:

The culturability of *Vibrio cholerae* was decreased by the addition of glucose to cell suspensions in starvation media. A similar effect was observed with Mannose and N-Acetylglucosamine and fructose. This inhibitory effect is termed as“glucose shock”. It was not observed with arabinose or xylose or with carboxylates, suchas acetate and pyruvate. No acidification of the medium occurred in the presence of these carbohydrates. Glucose shock was prevented by the addition of nitrogen or phosphorus sources. In the presence of phosphate, the bacterium produced formic acid from glucose.

1.16. Mucin:

What is mucin?

Mucins are a family of high molecular weight, heavily glycosylated protein (glycoconjugates) produced by epithelial tissues in most animals.Mucins' key characteristic is their ability to form gels; therefore they are a key component in most gel-like secretions, serving functions from lubrication to cell-signalling to forming chemical barriers.

Protein structure of Mucin:

Mature mucins are composed of two distinct regions:

(1) The amino- and carboxy-terminal regions are very lightly glycosylated, but rich in cysteines. The cysteine residues participate in establishing disulfide linkages within and among mucin monomers.

(2) A large central region formed of multiple tandem repeats of 10 to 80 residue sequences in which up to half of the amino acids are serine or threonine. This area

becomes saturated with hundreds of O-linked oligosaccharides. N-linked oligosaccharides are also found on mucins, but in less abundance than O-linked sugars.

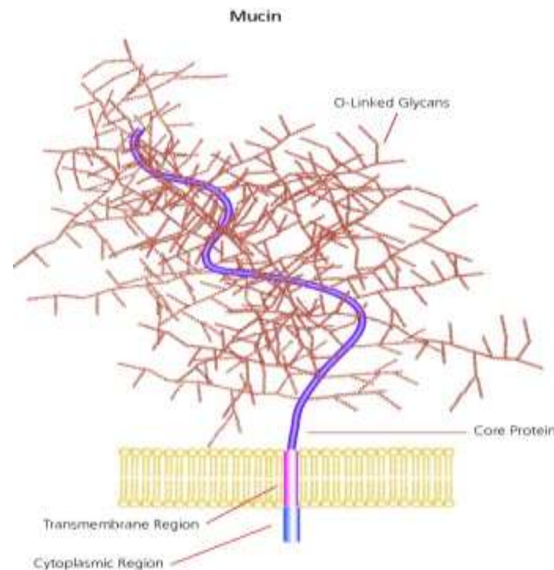


Figure 7: Structure of Mucin

***Vibrio cholerae* metalloproteinase degrades intestinal mucin and facilitates enterotoxin-induced secretion from intestine:**

To cause the diarrheal disease cholera, *Vibrio cholerae* must effectively colonize the small intestine. In order to do so, the bacterium needs to successfully travel through the stomach and withstand the presence of agents such as bile and antimicrobial peptides in the intestinal lumen and mucus. The bacterial cells penetrate the viscous mucus layer covering the epithelium and attach and proliferate on its surface.In order to reach the epithelium and deliver CT, *V. cholerae* must penetrate a highly viscous mucus layer approximately 150 µm thick, or roughly 50–75 times the body length of *V. cholera*.N-acetyl-L-cysteine, a mucolytic agent, facilitates *V. cholerae* colonization in vivo. In order to break down mucins, *V. cholerae* might rely on a mucinase complex, degrading polysaccharide and protein components of mucin in a manner analogous to known processes during *V. cholerae* departure from the intestine after infection. For example, *V.cholerae* produces a soluble mucinase, called haemagglutinin/protease (Hap), which is encoded by hapA. In a column assay, expression of hapA positively correlates with the capacity of *V. cholerae* to move through the mucus layer. As hapA is expressed late in

infection, it has been suggested that it facilitates detachment from the host epithelium and removal from the mucosa post-infection. However, because mucin induces hapA promoter activity, it is possible that Hap also facilitates initial penetration of the mucus layer. In addition, some as-yet-undiscovered mucinases might be involved in the early stages of colonization of *V. cholerae*.

While a general protease seems to be involved in initial migration through the mucus, *V. cholerae* may express specific mucinases near the location where the bacterium preferentially colonizes the intestinal epithelium. Whereas Hap is a metalloprotease that cleaves a wide variety of substrates, TagA, another metalloprotease, may specifically modify mucin glycoproteins attached to the host cell surface. TagA, which is encoded within the *Vibrio* pathogenicity island (VPI), is expressed and secreted by *V. cholerae* under virulence-inducing conditions. As the protein is positively coregulated with TCP and other virulence genes, TagA may play an important role in colonization during the later stages of movement through the intestinal mucosa. Another *V. cholerae* virulence factor, neuraminidase (NanH), is an extracellular enzyme that cleaves two sialic acid groups from the GM1 ganglioside, a sialic-acid containing oligosaccharide on the surface of epithelial cells, thereby unmasking receptors for CT. As a mucinasewith a specific role in infection, NanH may be important in aiding movement through the mucus to the specific site of infection.

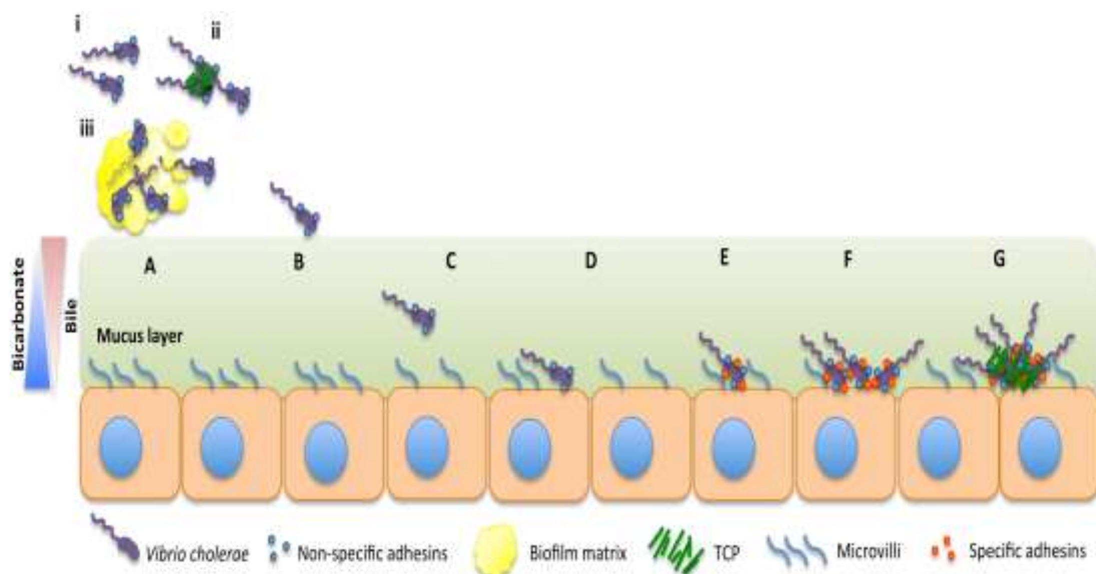


Figure 8: Adherence of *V. cholerae* on Mucin layer

1.17. Cell Culture:

What is Cell Culture?

Cell culture refers to the removal of cells from an animal or plant and their subsequent growth in a favorable artificial environment. The cells may be removed from the tissue directly and disaggregated by enzymatic or mechanical means before cultivation, or they may be derived from a cell line or cell strain that has already been established.

Primary & Secondary Cell Culture:

Primary culture refers to the stage of the culture after the cells are isolated from the tissue and proliferated under the appropriate conditions until they occupy all of the available substrate (i.e., reach confluence). At this stage, the cells have to be sub-cultured (i.e., passaged) by transferring them to a new vessel with fresh growth medium to provide more room for continued growth. This is referred to as Secondary cell culture.

To describe the maintenance of cells in culture:

Safety Equipment:

(1) Laminar flow hood (2) CO2 Incubator (3) Mechanical Pipettor (4) Inverted microscope (5) Vacuum pump and flask

Materials:

(1) Complete cell culture medium, appropriate for the cell line (2) Tissue culture flasks of appropriate sizes (3) Tissue culture plates - 96 well or 24 well. (4) Sterile pipets, assorted sizes (5) Multichannel pipet and sterile tips. (6) Pasteur pipets, (7) 70% alcohol (8) Sterile Petri dishes.

1.18. HT-29:

HT-29 is a human colon cancer cell line used extensively in biological and cancer research.

Characteristics:

Initially derived in 1964 by Jorden Fogh from a 44-year-old Caucasian female, HT-29 cells form a tight monolayer while exhibiting similarity to enterocytes from the small intestine. HT-29 cells overproduce the p53 tumor antigen, but have a mutation in the p53 gene at position 273, resulting in a histidine replacing an arginine. The cells proliferate

rapidly in media containing suramin, with corresponding high expression of the c-myc oncogene. However, c-myc is deregulated, but may have a relation with the growth factor requirements of HT-29 cells.

Applications:

In preclinical research, HT-29 cells have been studied for their ability to differentiate and thus simulate real colon tissue in vitro, a characteristic that has made HT-29 useful for epithelial cell research. The cells can also be tested in vivo via xenografts with rodents. HT-29 cells terminally differentiate into enterocytes with the replacement of glucose by galactose in cell culture, and with the addition of butyrate or acids, the differentiation pathways can be closely studied along with their dependence on surrounding conditions. Accordingly, studies of HT-29 cells have shown induced differentation as a result of forskolin, Colchicine, nocodazole, and taxol, with galactose-mediated differentiation also causing the strengthening of adherens junctions.

Culturing:

Though HT-29 cells can proliferate in cell culture lacking growth factors with a doubling time of around 4 days, the doubling time can be reduced to one day with added fetal bovine serum. The cells have high glucose consumption, and in standard medium containing 25 mM glucose and 10% serum, remain undifferentiated.

2. AIM OF THIS PROJECT:

1. The role of monosaccharide Glucose, N-acetyl D-Glucosamine, Mannose and Fructose will be elucidated in in-vitro model during infection of pathogenic *V. cholerae*.

3. MATERIALS AND METHODS:

3.1. Bacterial strains, their maintenance and growth conditions:

The *V. Cholerae* O1 El Tor strain N16961 is used in the study. These strains were obtained from NICED strain bank. Strain was maintained at -70°C in Luria broth (LB) containing 15% glycerol. The *V. cholerae* strains were streaked and incubated overnight at 37ºC on TCBS agar plates. The characteristic large, yellow, single colonies were taken for further work. Single colonies were streaked in LA plates or cultured in LB Broth (Bacto- Difco) pH 7.2 and grown overnight at 37ºC with shaking (160 rpm). This was considered as the standard laboratory culture condition.

3.2. Generation of *V. cholerae* growth curve:

First, we started with the growth curve in Nutrient rich Luria – Bertani (LB) Media and M9 minimal medium supplemented with 0.5% monosaccharides (Glucose, GlcNAc, Mannose, Fructose). Overnight culture of Wild type N16961 was centrifuged at 6000rpm for 5 minutes to pellet down the cells. The cell pellet was washed with PBS buffer and centrifuged once again in order to ensure maximum extraction of cells. The cell pellet was dissolved in PBS buffer and it was adjusted to 10^5 cells. The cultures were grown for 30 hrs and 70 hrs while growing in LB or M9 media respectively at 37°C with shaking at 150 rpm. Growth of the bacteria was determined by measuring the bacterial turbidity in spectrophotometer at 600nm.

3.3. Mucin penetration assay:

Mucin columns were prepared by adding various concentration of porcine mucin(1%, 1.5%, 2%) to M9 media in 1-ml syringes. 100µl of Mid log bacterial cultures 5x 10^8 CFU/ml were loaded on the top of the mucin columns and allowed to settle for 60 min at 37°C in static condition. Fractions (100ul) were collected from the bottom of the mucin columns. Bacteria numbers were measured by serially diluting samples, plating onto LB agar supplemented with 100µg/ml Streptomycin, and counting CFU (Liu et al, 2008).

3.4. *In vitro* adhesion assay:

Bacterial suspension of 10^7c.f.u/ml was pelleted down and dissolved in the culture medium. This suspension was added to intestinal epithelial cells HT-29 grown on 12-well plates at 80-90% confluence (Chourashi et al, 2016). After 1 h of incubation at 37°C in 5% CO2, cells were washed three times with PBS and detached using 0.1% Triton X-100. Adherent bacteria were counted after serial dilution by plating on LB agar plates supplemented with 100µg/ml Streptomycin.

3.5. Cell culture, infection and stimulation:

The human colon cancer cell line HT-29 were obtained from Laboratory Cell Culture stock, NICED. Cells were grown and maintained in DMEM (Dulbecco's Modified Eagle's Medium) (Gibco-BRL, Gaithersburg, MD, USA) at pH 7.4, supplemented with 10% fetal bovine serum (Gibco- BRL) containing penicillin ⁄ streptomycin in the presence of 5% CO2 at 37 ºC. Caco-2 cells, in addition, were supplemented with 2 mm l-glutamine and 1% nonessential amino acids (Sigma-Aldrich, St Louis, MO, USA). Cells were seeded in T-25 tissue culture flasks (Falcon, San Jose, CA, USA). Bacteria from overnight culture suspended in fresh medium without antibiotics were added at 100 MOI.

3.6. Cytokine Assay (Determination of IL-8 and TNF secretion by ELISA):

The level of IL-8 and TNF protein in the culture supernatant of infected or uninfected HT-29 cells with Wild Type N16961, supplementing with 0.5% monosaccharides was measured by ELISA. For ELISA, the OptEIA human IL-8 and TNF ELISA KITII (BD Biosciences Pharmingen, San Diego, CA, USA) was used, following the manufacturer's instructions. First Standard curve of IL-8 and TNF was obtained by serially diluting pure protein standard provided with the kit and then the amount of proteins present in the treated and untreated culture supernatant was measured extrapolating the sample value in the following standard curve.

3.7. Biofilm Assay:

Assays to quantify biofilms were performed as previously described method(Zhu, Mekalanos, 2003).In brief a 1:100 dilution of overnight grown culture of *V. cholerae*

was inoculated in LB broth into 10- by 75-mm borosilicate glass tubes and incubated for 30 hrs at 37°C. Subsequently, the tubes were rinsed three times with phosphate-buffered saline (PBS) and then filled with 1% crystal violet stain. After 5 min, excess stain was rinsed off with deionized water. The biofilm-associated crystal violet was solubilized in dimethyl sulfoxide (DMSO), and the optical density at 570 nm (OD570) of the resulting suspension was measured. All experiments were performed at least three independent times.

4. RESULTS:

4.1.Effect of monosaccharides on *V. cholerae* growth:

Our result shows that the growth rate of Wild Type (WT) *Vibrio cholerae* is almost equal in nutrient rich Luria–Bertani Media in all conditions. Bacteria overcomes the lag phase and enters into the log phase within 1 hr. of the initial innoculum.

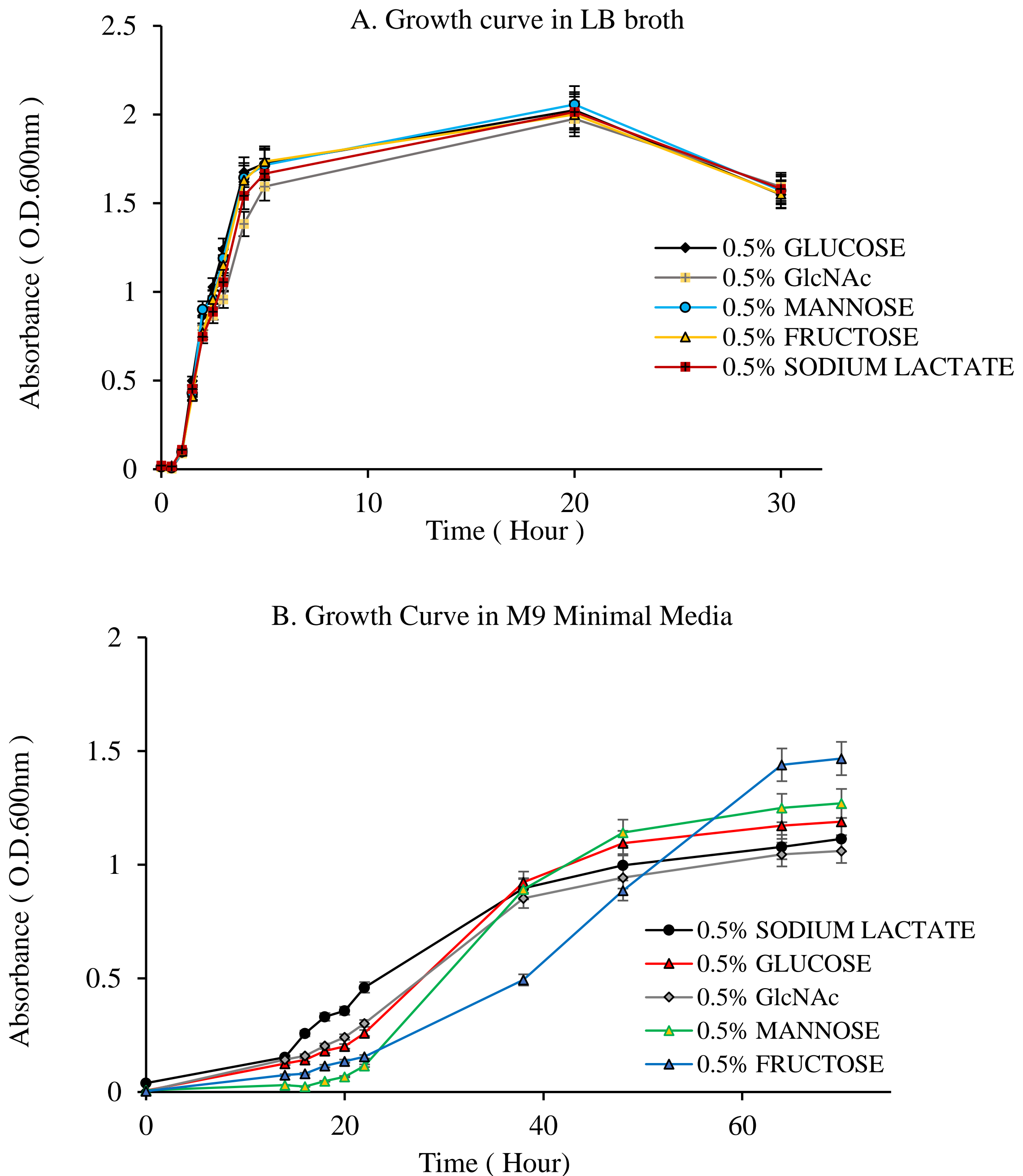
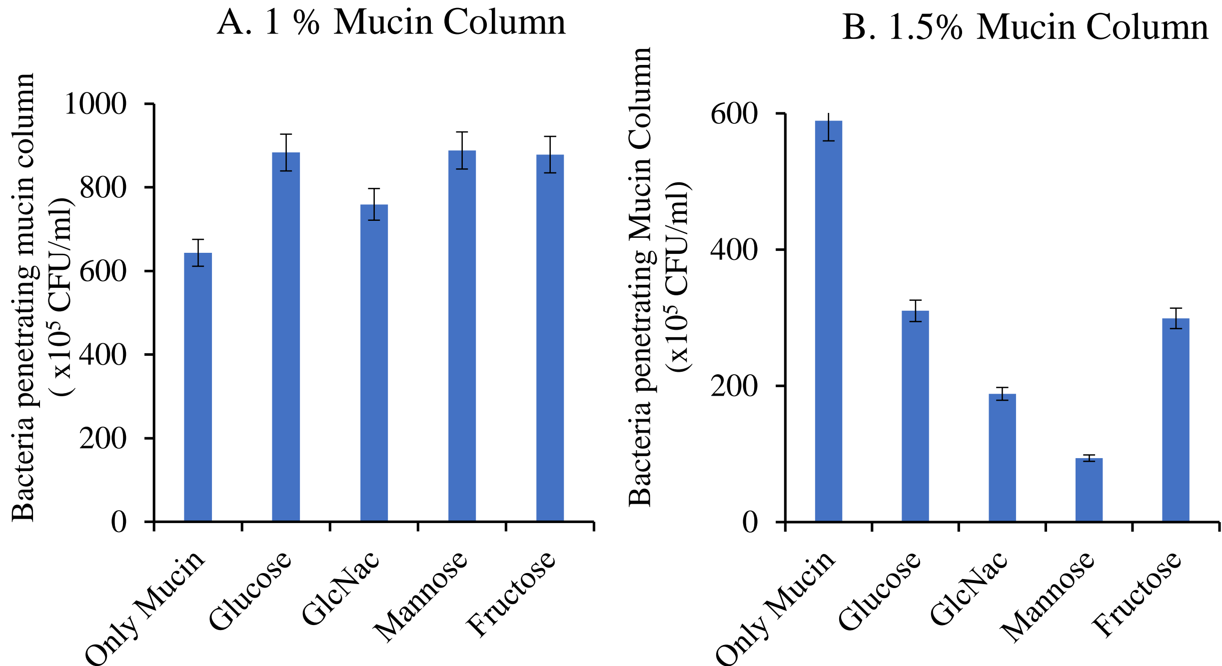


Figure 9: Growth Curve of *V. cholerae* WT N16961 in LB broth (A) and M9 minimal media (B) in presence of different monosaccharides.

Vibrio cholerae attains maximum growth at 5 hr. and thereafter it attains the stationary phase. Whereas in nutrient poor M9 media, where there is no carbon source except the supplemented monosaccharides, the lag phase is prolonged. *Vibrio* culture attains log-phase after 18 hrs. in presence of Glucose, GlcNac and Mannose. In case of Fructose supplemented media the lag phase continues upto 22 hrs of initial innoculum. All the cultures with supplemented monosaccharides attain maximum growth at 64 hrs.

4.2.Mucin Penetration Assay

V. cholerae must penetrate the intestinal mucosal layer to get access to the intestinal lumen. Then they colonize and express virulence genes. Monosaccharides could play a major role in cholera pathogenesis by retarding the penetration efficiency through the protective mucus barrier. To test this, we compared the rates of migration of WT *Vibrio cholerae* through 1%, 1.5% and 2% mucin column.



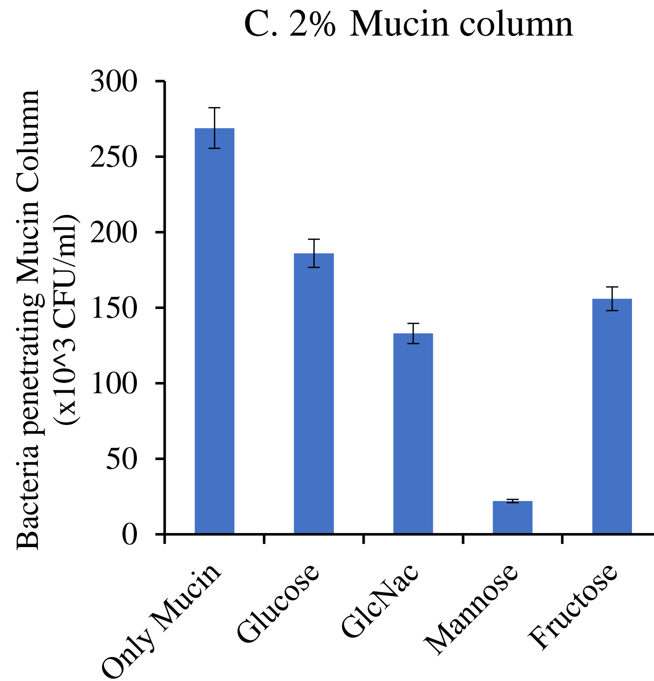


Figure 10: Mucin penetration assay of *V. cholerae* WT N16961 in presence of different monosaccharides in 1% (A), 1.5% (B) and 2% (C) mucin column.

Consistent with our hypothesis, we found that the ability of wild-type *Vibrio cholerae* to swim through the mucus layer is variable. When Monosaccharides are present in 1% Mucin column *Vibrio* attains faster motility compared to the absence of monosaccharide in the mucin. But when concentration of mucin increases the sugar residues slows down the motility of the bacteria. Glucose defect the motility by 2 fold and 1.5 fold, GlcNAc by 3.2 fold and 2 fold, Fructose by 2 fold and 1.7 fold and Mannose by 6.2 fold and 12.2 fold in 1.5% and 2% Mucin column respectively.

4.3. HT-29 Adhesion Assay

Results indicate that WT N16961 when is treated with the monosaccharides like Glucose, GlcNAc, Mannose and Fructose they are appx. 3 fold less efficient to bind to HT-29 intestinal cell lines compared to untreated wild type N16961.

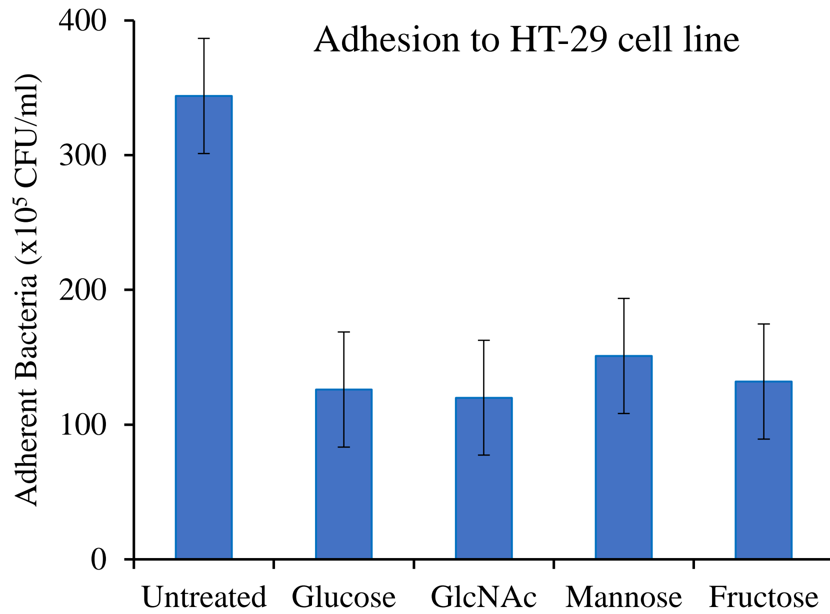
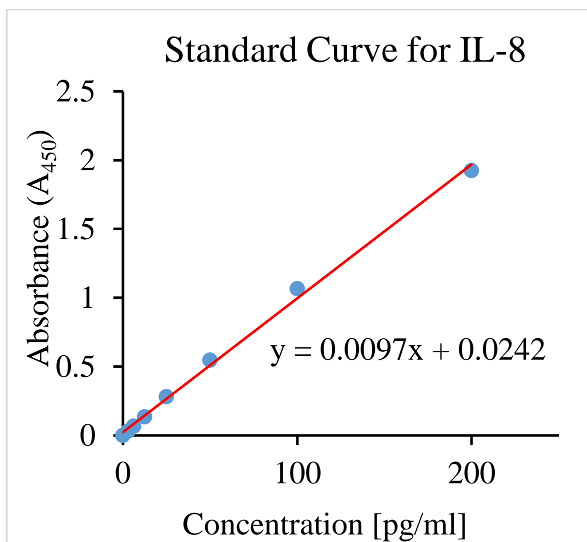
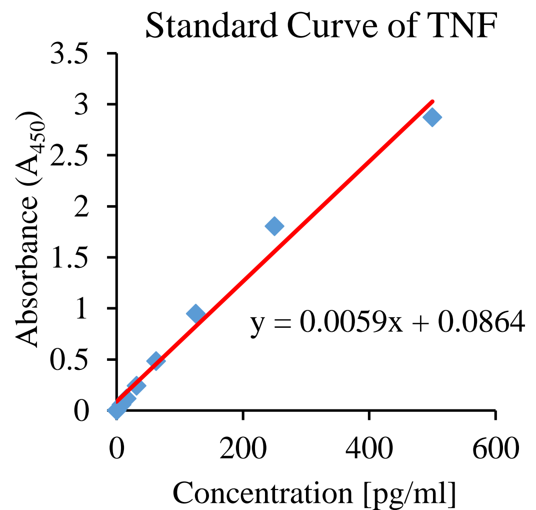


Figure 11: Effect of monosaccharides on *V. cholerae* adhesion to HT-29 cell line.

4.4. Cytokine Assays

First Standard curve of IL-8 and TNF was obtained by serially diluting the pure protein sample provided with the kit.





Standard curve equation for IL-8 is $y = .0097x + 0.0242$ and for TNF is $y = .0059x + 0.0864$, where 'y' represents Absorbance at 450nm and 'x' represents concentration in pg/ml.

Using the following equations concentration of sample cytokines was measured.

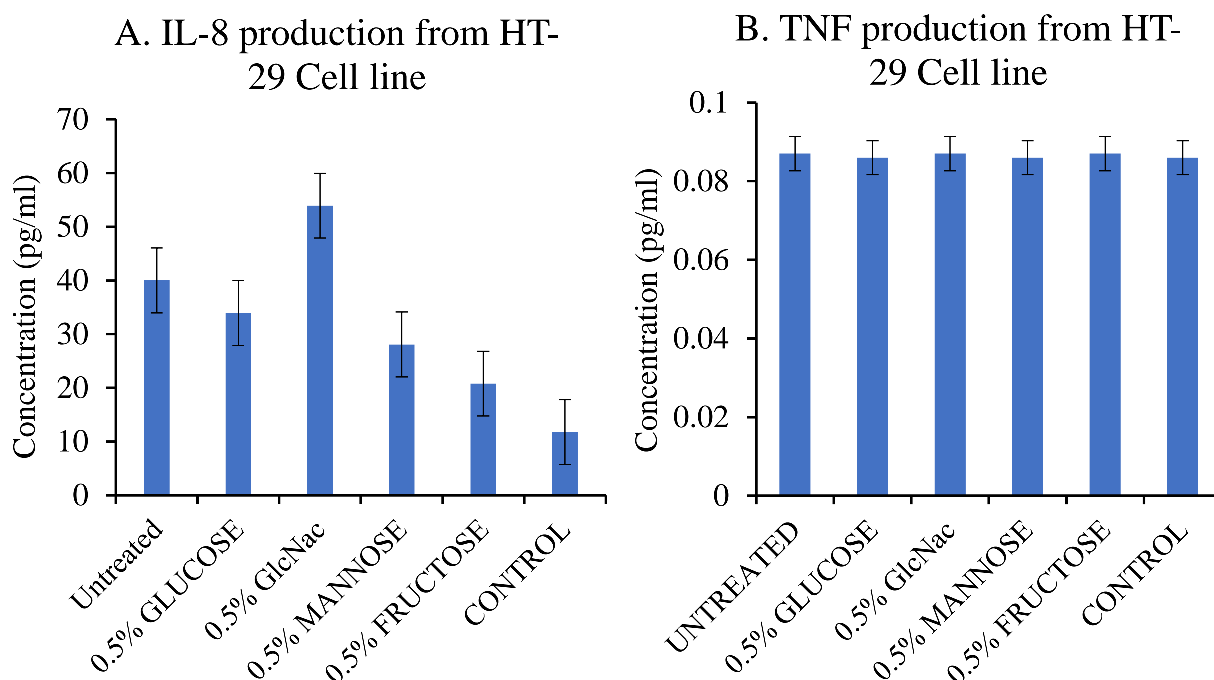


Figure 12: Effect of monosaccharides in Cytokine, IL-8 (A) and TNF (B) production by HT-29 cell line.

From our experiment it has been seen that IL-8 production is lowered in the HT-29 cell line when treated with Glucose (0.5%), Mannose (0.5%) and Fructose (0.5%). Although slight increase in IL-8 observed in GlcNAc (0.5%) treatment. On the other hand, no significant change in TNF production is observed from HT-29 cell line after 2 hours of incubation.

4.5. Biofilm Assay

Formation of *V. cholerae* biofilm is significantly reduced in all the monosaccharide treated culture tubes.

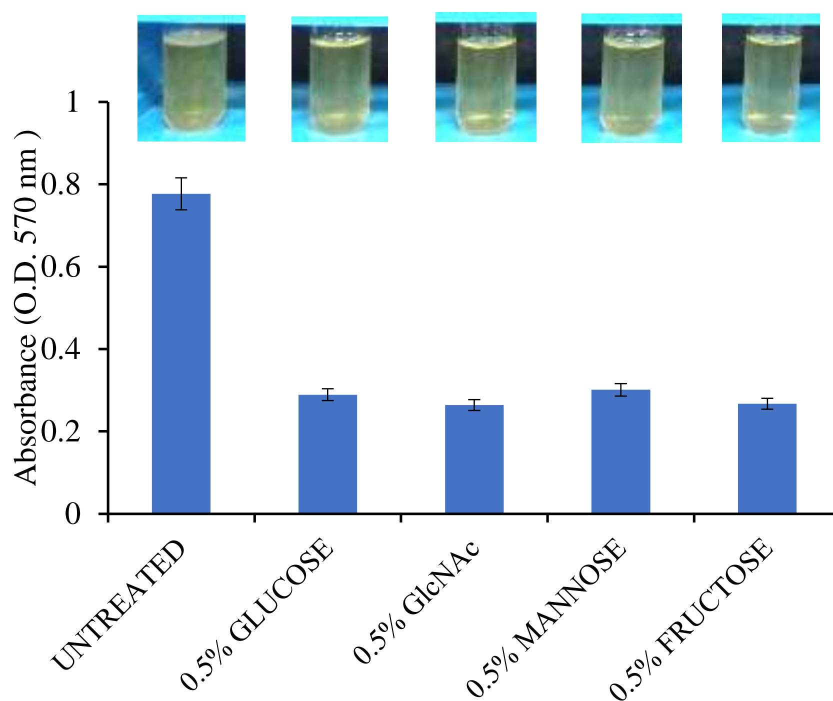


Figure 13: Biofilm formation assay by *V. cholerae* WT N16961 and effect of different monosaccharides.

5. DISCUSSION:

We have used common monosaccharides like, Glucose, N- acetyl D-Glucosamine, Mannose and Fructose in our experiment. We were intersted to see whether these monosaccharides possess any importance during entero-pathogenic infection in human. So we have done comparative studies with those saccharides that includes generation of growth curve, mucin penetrating ability, *ex-vivo* adhesion to HT-29, inflammatory cytokine assay and Biofilm formation with cholera causing bacteria *V. cholerae* N16961. Growth curve in LB showed no significant change in growth pattern of *V. cholerae.* In M9 minimal medium sugar treated *V. cholerae* shows similar growth at stationary phase, but 0.5% fructose treated culture shows prolonged lag phase upto 30 hours than the untreated control. In nutrient rich condition these sugar molecules does not possess any inhibitory function but in nutrient poor condition growth of the bacteria is delayed when the said saccharides are present. During intestinal infection *V. cholerae* needs to swim through the thick mucus barrier and then only it can bind and

adhere with the enterocytes. Our study suggests, among all the sugars, Mannose greatly reduces mucin penetrating ability of *V. cholerae* by 11 fold. And all the sugars Glucose, GlcNAc, Mannose and Fructose significantly reduced colonization to HT-29 upto 3 fold. It has long been said that cholera is a noninflammatory diarrhoeal disease, however recent study (Boris *et al*., 2001) have showed that *V. cholerae* upon binding with the intestinal epithelial cell lines produces pro-inflammatory cytokine Interlukin-8 (IL-8), which is a potent chemoattractant for polymorphonuclear leukocytes and T lymphocytes, which further causes inflammation. Our study showed that Glucose, Mannose and Fructose reduces the production of IL-8 during *ex-vivo V. cholerae* infection. Tumor necrosis factor (TNF) another regulator of inflammatory responses, is a potent lymphoid factor which is produced by wide number of cells. TNFs play a critical role in the body's resistance to infection by inducing fever and activating macrophages and in the destruction of certain tumors. However, over-production of TNF has been associated with cytotoxic effects such as cachexia. Our study showed no significant production of TNF by HT-29 cell-line. Monosaccharide treated culture also showed no reduction or induction in expression of TNF. Antimicrobial resistance is a major public health concern, particularly in hospitals and other health care settings, and have increased worldwide. In most of the cases, the limited efficacy of antibiotics in the treatment of infections is related to biofilm formation. It is estimated that biofilms contribute to over 80% of all infections in humans. Bacteria in sessile state are more protected against host defences and more resistant to antimicrobial treatment than their planktonic counterparts. Interestingly all the monosaccharides tested in this experiment significantly reduced *V. cholerae* biofilm formation. Collectively, these data suggests that these common monosaccharides play an important role in reducing adhesion, mucin penetration during pathogenesis of *V. cholerae* in host intestine.

6. CONCLUSION:

Compared with traditional chemotherapy approaches, Monosaccharides are more gentle, safe, and ecologically friendly. Thus, the addition of monosaccharides in diets has an excellent market prospect. Although antiadhesive monosaccharides have been proven to be effective in in vitro, more works are still needed in *in-vivo* animal models to demonstrate their real values.

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