# REVIEW ON GROWTH OF *NEISSERIA MENINGITIDIS* IN CHEMICALLY DEFINED MEDIAS AND ROLE OF NUTRIENTS ON GROWTH

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## ABSTRACT

*Bacterial meningitis—an infectious disease common to human beings—remains a serious global threat especially in the "meningitis belt" in the sub-Saharan Africa and the US. Neisseria meningitidis is the causative organism of this deadly disease which is a Gram-negative, aerobic, non-motile, diplococci bacteria and is characterized by its antiphagocytic polysaccharide capsule. There are 13 clinically significant serogroups of this organisms—A, B, C, D, E29, H, I, K, L, W135, X, Y, Z, which depend on the antigenic structure of their polysaccharide capsule, among which A, B, C, X, Y and W-135 are the major pathogenic strains. 10% of the entire population in the "meningitis belt" is the carrier of this organism and if the infected people are not treated on time, the mortality reaches 100%. Nutritional studies for the growth of Neisseria meningitidis were started in 1940s by a number of researchers. Protective immunity to Neisseria meningitidis bacteria involves an antibody response to polysaccharide antigen except serogroup B. Polysaccharide vaccines against* Neisseria meningitidis *prevent infection by inducing an immune response against the specific capsular polysaccharide. At present, high cost of available vaccines makes it unaffordable for the common people. The high cost of the vaccines is due to the low productivity of polysaccharide; less recovery and high cost of purification process. If the cost in producing polysaccharide is lowered down, the ultimate market cost of the vaccine can be reduced. This review aims to provide a brief account of media and process parameters for the growth of Neisseria meningitidis for better yield of polysaccharide and metabolism involved during the growth and PS production.*

***Keywords:*** *Bacterial meningitis, Neisseria meningitidis, Role of nutrients on growth*

## INTRODUCTION

Meningococcal meningitis is responsible for one-third of all bacterial meningitis cases among the human beings. Bacterial meningitis is inflammation of meninges, which are the protective membranes covering the brain and spinal cord. The highest risk of meningococcal disease occurs in the sub-Saharan Africa, that is why it is known as the "Meningitis Belt". Meningococcal disease was first reported by a Swiss physician Gaspard Vieusseux in the year 1805 during an outbreak with

33 deaths in the vicinity of Geneva, Switzerland, while *Neisseria meningitidis* organism was identified by an Austrian pathologist and bacteriologist Anton Weichselbaum in the year 1887. **[1]** *Neisseria meningitidis* has 13 clinically significant serogroups known as A, B, C, D, E29, H, I, K, L, W135, X, Y, and Z depending on the antigenic structure of their polysaccharide capsule, of which A, B, C, X, Y and W-135 are the major pathogenic strains. **[2] [3] [4]**

Nutritional studies for the growth of *Neisseria meningitidis* invitro were started with the work by Frantz in 1942. Frantz described a simple chemically defined liquid medium containing glutamic acid, cystine, glucose and inorganic salts to grow *Neisseria meningitidis.* **[5]** Following Frantz's study, a number of experiments for the cultivation of *Neisseria meningitidis* were carried out such as Watson-Scherp medium, comparative study on Frantz, Modified Frantz and Catlin 6 medium **[6] [7],** a defined agar medium for genetic transformation of *Neisseria meningitidis* **[8]** and so on. Research studies show glucose along with other sources including nitrogen (both organic and inorganic), metal ions, amino acids and others is one of the major factors in the growth of *Neisseria meningitidis*. Along with media components, physical parameters such as pH, temperature, dissolved oxygen and so on also play critical role in bacterial growth. The media components, process parameters and cultivation methods for best growth of *Neisseria meningitidis* and polysaccharide yield have been reviewed here.

**1. *Neisseria meningitidis* resist against human innate immunity**

*Neisseria meningitidis* is more resistant to complement mediate killing than other Gram-negative pathogens. Complement system is a vital part of innate immunity which plays a crucial role against pathogens. *Neisseria meningitidis* resists from complement mediated lysis through its capsular polysaccharide and lipopolysaccharide (LPS). **[9]** The main innate immune defense against microbes in humans is antimicrobial peptides and proteins. Antimicrobial molecules are classified into two structurally diverse families: (i) defensins and (ii) Cathelicidins. *Neisseria meningitidis* resists against antimicrobial peptides by the addition of phosphoethanolamine group to the lipid A of the Lipopolysaccharide (LPS) molecule, which is an integral component of the bacterial outer membrane.

Lactoferin is a key protein of the innate immune response present at the respiratory mucosal surface and in neutrophils. Even though it has multiple roles in innate immunity, its best characterized activity is scavenging and chelation of iron, which is an essential element for the growth of microorganisms, also for respiration. But *Neisseria meningitidis* expresses surface protein receptors which specifically binds

human lactoferrin. It does not only bind to lactoferrin but also removes iron from it directing into the bacterial cell. Also the *Neisseria meningitidis* polysaccharide capsule resists against the complement system. **[10]**

## 2. Epidemeology

The high-incidence of meningitis is reported in the African meningitis belt, moderate-incidence countries are from the European and African regions, and Australia, whereas low-incidence is reported from Europe and Americas (Fig-1). According to WHO (World Health Organization), more than 100 cases per 100,000 population are reported to have been affected by meningitis per year in the meningitis belt. **[11]** The outbreak coincides with the dry season, which can be a reason for low humidity and seasonal dust-wind blowing from the Sahara that damages the mucosa and produces irritant coughing that leads to transmission. *Neisseria meningitidis* serogroups B and C are the most common causes of disease in Europe, America, Australia, and New Zealand; while, serogroup A is the main cause in Africa and Asia. There happened an outbreak of W-135 serogroup in the years 2000 and 2001 among the Hajj pilgrims. Serogroup A caused the largest outbreak in 1996 which claimed more than 20,000 deaths. **[12]** According to Public Health England, MenW cases have risen since 2009. MenW was accounted for 1-2% in the period 2008-2009 which increased to 24% of cases during the period 2014-2015.

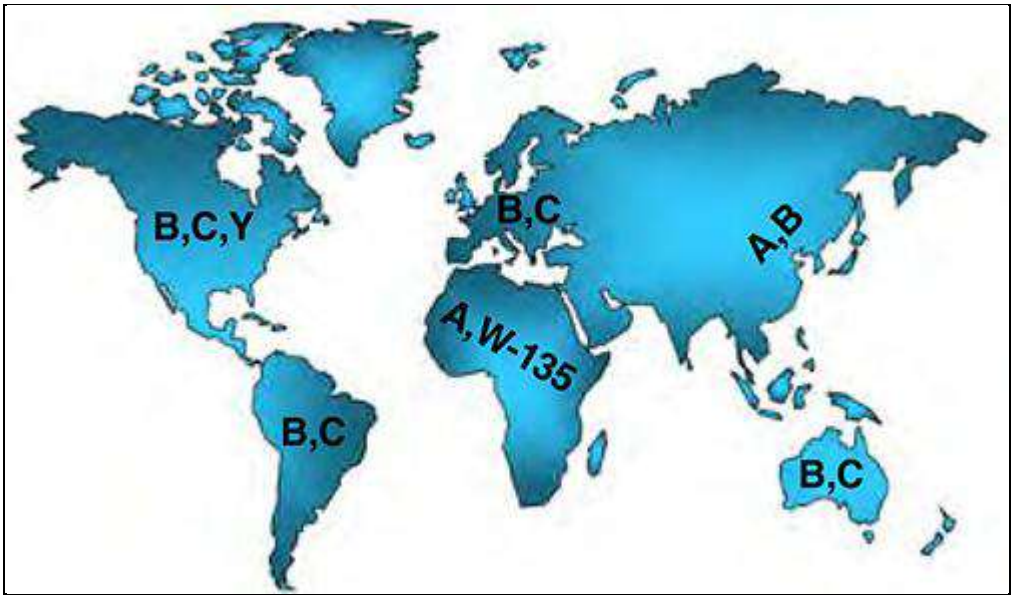


Fig-1 The distribution of *Neisseria* meningitis sero-groups vise across the globe (Source - http://www.phac-aspc.gc.ca/publicat/ccdr-rmtc/09vol35/acs-dcc-4/gfx/figure1-eng.jpg)

## 3. Growth of *Neisseria meningitidis* in various chemically defined medias

Bacteria require carbon as a source of energy to grow and this is often supplied by sugar present in the

environment. *Neisseria meningitidis* can utilize only glucose and maltose as a carbon source, even though they can use peptides as carbon source. They are usually grown in Muller-Hinton broth and Columbia agar which are peptide-based formulations. **[13]** A chemically defined simple liquid medium for growing *Neisseria meningitidis* was invented by Frantz in 1942, which contains the components Glutamic acid, Cystine, glucose and inorganic salts. By supplementing carbon dioxide along with the media components for better growth, he stated that the growth was retarded if any one of the ingredients was removed. **[5]** Following Frantz's experiments, Grossowicz in 1945 reported that glucose in Frantz medium can be substituted with lactate for meningococcal growth. **[14]** His experiments also showed that calcium is an essential ingredient for growth and the metabolic study revealed the organism is capable of growing in Frantz medium in absence of carbon dioxide resulting in large size of inoculum. This might be because of the accumulation of metabolic $CO_2$ of large inoculum. Also, the studied organism was able to grow in absence of glucose in the media after a series of subculture in Frantz medium. **[15]** In another study, using the Frantz media as the basic media, it was showed when glucose was replaced with maltose the rate of acid formation was high, and hence there was a decrease in pH as compared in glucose. Also inorganic phosphate uptake was very high or there was a rapid disappearance of inorganic phosphate from the media in presence of maltose, whereas high concentration of inorganic phosphate retarded the bacterial growth in glucose medium. **[16]**

A defined agar medium for genetic transformation of *Neisseria meningitidis* was developed in which genetic transformation of *Neisseria meningitidis* was possible by sub culturing several times and directly placing into a solution of transforming DNA without additional supplements. **[8]**

Catlin defined a medium called MCDA which supported the growth of *Neisseria meningitidis* on agar, that is on solid surface. The medium contains the following ingredients; NaCl, KCl, $NH_4Cl$, $Na_2HPO_4$, $KH_2PO_4$, $Na_3C_6H_5O_7.2H_2O$, $MgSO_4.7H_2O$, $MnSO_4.H_2O$, L-glutamic acid, L-arginine.HCl, glycine, L-serine, L-cysteine.HCl.H2O, sodium lactate of 60% syrup/mL of medium, glycerin 0.5% (v/v), washed purified agar -1% wt/vol, $CaCl_2.2H_2O$, $Fe_2(SO_4)_3$, however growth of *Neisseria meningitidis* in liquid MCDA (without agar) is not reported. Another study which reported growth with a maximum absorbance unit about 1.5 contains, Sodium L-glutamate, L-cysteine.HCl, KCl, NaCl, $MgSO_4.7H_2O$, $NH_4Cl$, Disubstituted sodium phosphate dodecahydrate, Tris substituted sodium citrate, Glucose.

A chemically defined medium named MC-6 was developed to grow *Neisseria*

*meningitidis* in which OD of culture at 600 nm reached about 10-13. This medium contains only salts, 5 amino acids and a carbon source, in the following orders: NaCl, $K_2HPO_4$, $NH_4Cl$, $K_2SO_4$, Glucose, L-glutamic acid, L-arginine, Glycine, L-serine, L-cysteine.HCl, $MgCl_2.6H_2O$. Here the harvesting of culture was done in late log or early stationary phase for maximum PS cultivation. [**17]** In Watson Scherrp medium, soy peptone was used for nitrogen source expecting amino acid supplied by soy peptone that is sufficient to sustain *Neisseria meningitidis* growth.

A comparative study on the growth and PS production of *Neisseria meningitidis* serogroup C in Frantz medium, modified Frantz medium (glucose replaced by glycerol), and Catlin medium (which contains 5 amino acids along with glucose carbon source and inorganic salts), showed Catlin media supports the growth better than the other two medias. Lower temperature (at 35°C instead of 36°C-37°C) and pH resulted in an extended lag phase after which the growth rate was high **[6].**

The final polysaccharide concentration was lower and cellular contaminants were high in the above discussed medias.

A highly enriched, phosphate free media called *Neisseria meningitidis* fastidious culture medium (NMFM) for producing capsular polysaccharides from serogroups A, C, Y and W135 was developed. **[18]** The medium contains NaCl, $K_2SO_4$, KCl, Trisodium citrate.$2H_2O$, $MgSO_4.7H_2O$, $MnSO_4.H_2O$, $MnCl_2.6H_2O$, Vitamin B12, NAD, Thiamine HCl, Soy peptone, D-Glucose, L-Glutamic acid, L-Arginine, L-Serine, L-Cysteine, Glycine, Morpholinepropanesulphonicacid (MOPS), $CaCO_3$, and $NH_4Cl$ is added in case of MenW-135 and $Fe_2(SO_4)_3$ in case of MenA serogroup. Filter sterilization of glucose and amino acid solution rather than heat allowed non-degradation of heat sensitive sugars and amino acid and increased PS production by 25%. The parameters adopted were temperature=35°C, 6% $CO_2$, 5L/min air flow, agitation frequency of 120rpm, and vessel pressure of 6 psi. Ionized calcium from $CaCO_3$ maintains the pH between 6.5-7.0 acting as buffering agent. The above experiment results in polysaccharide of about 30-40 mg/L.

An animal free and alcohol free growth medium for the cultivation of *Neisseria meningitidis* serogroups A, C, Y and W135 was developed **[19]** in which the sialic acid content in the final purified polysaccharide in serogroups C, Y and W135 was about 600-800 mg/g of PS and phosphorus in MenA was about 80 mg/g of PS. The media components include plant peptones-17.5g/L, Starch soluble-1.5 g/L, Sodium Chloride-2.0 g/L. The initial growth at 35°C for 18-20 hours was in agar plates and transferred to the growth media and the harvest hour was at 12 (+, -2) at an OD of 10 (+, -2) at 590 nm.

Pengo et al investigated and reported an optimized media components and process

parameters to cultivate capsular polysaccharide in a submerged fermentation for MenW135 serogroup. Casamino acid as nitrogen source in the medium supported the growth better than peptone and tryptose. The maximum polysaccharide yield reported under pH 7.0, temperature 36.5°C, initial DO 5% was 55mg/L. **[20]**

## 4. Nutrient sources and metabolism

### 4.1. Carbon source

Glucose, a main source of energy, plays an important role in the metabolism of majority of living things and is a good fuel because it is rich in potential energy. Also, glucose is a precursor, which can supply a huge array of metabolic intermediates for biosynthetic reactions like carbon skeletons for every amino acid, nucleotide, coenzyme, fatty acid, or other metabolic intermediates.

*Neisseria meningitidis* is capable to utilize a range of carbon sources including glucose lactate and pyruvate etc. **[21]** Lactate utilization is rapid than glucose. *lctP* mutant bacteria showed reduced growth than wild type. Glucose is converted to phosphoenol pyruvate (PEP) and then to sialic acid through ED pathway, whereas lactate is converted to sialic acid through a direct route. **[22]** Acetate cannot be used as a sole carbon source, whereas glucose is the best studied energy source which can provide energy and enhance the growth of *Neisseria meningitidis* than any other carbon sources **[23]**. *Neisseria meningitidis* can utilize propionic acid as a supplementary carbon source in which glucose is the main energy source. **[24]** In a study on *Neisseria meningitidis* serogroup C, it was reported that there exists a relationship between glucose concentration and rate of polysaccharide production, that is polysaccharide production is highest when the residual glucose concentration in the medium was lowest. Glucose can be completely catabolized through ED pathway (80% catabolism) and Pentose Phosphate (PP) pathway (20% catabolism). Oxidation via PP pathway synthesize nucleic acid from glucose for biosynthesis. **[25-27]** *Neisseria meningitidis* alters glucose metabolism in response to change of environmental pH. Glucose in chemically defined media of *Neisseria meningitidis* causes a hike in NADP linked glutamate dehydrogenase (GDH) level which in turn enhances the bacterial growth by facilitating nitrogen metabolism. **[28]** As the activity of phosphofructokinase is low, the EMP pathway does not have any contribution in the metabolism. **[29,30]** The carbon source is converted to pyruvate and to phosphoenol pyruvate which is a precursor of sialic acid, which is needed in the capsule synthesis. **[9]**

Biochemical studies revealed that in *Neisseria meningitidis* the NADP linked enzyme activities are higher than NAD linked enzyme activities. Also, the NADP linked activities were enhanced by glucose

present in the media, whereas the NAD linked activities decreased in the same conditions. Hence glucose enhances the bacterial activity and consequently the growth. **[28-31]**

### 4.2. Nitrogen sources (organic and inorganic) and metabolism

Inorganic nitrogen consumption by *Neisseria meningitidis* is significantly less whereas organic nitrogen consumption is linearly related to cell growth. **[32]**

*Neisseria meningitidis* can utilize organic nitrogen sources like casamino acid, casitone, tryptose, and peptone etc. as nitrogen source for growth, but casamino acid enhances the bacterial growth better. **[20]**

Along with organic nitrogen, inorganic nitrogen gives a positive effect to the bacterial growth. Jyssum et al reported *Neisseria meningitidis* can utilize ammonium ions ($NH_4^+$) supplemented as $NH_4Cl$ as sole source of nitrogen for growth. **[30]** Moreover, another study showed that the exclusion of $NH_4Cl$ improved the bacterial growth and polysaccharide production. **[7]**

### 4.3. Amino acids

Effect of individual amino acids on growth is not completely known. The amino acid Cysteine is a major source of sulfur in the growth of *Neisseria meningitidis* even though it can utilize $(SO_4)^{2-}$ as sulfur source. The five proteins encoded by *CysD*, *CysH*, *CysI*, *CysJ* and *CysN* are expected to boost *Neisseria meningitidis* the ability to reduce $(SO_4)^{2-}$ into $H_2S$. **[33]** Cysteine is converted to glutathione (GSH) and then to glutathione sulfide by further oxidation (GSSH). Hence it controls the cellular $H_2O_2$ level. **[34]**

Cystine along with asparagine inhibits the bacterial growth in high quantity whereas aspartate does not inhibit the growth. Gycine, tyrosine and guanidine inhibit the growth to lesser degree. Thiamine, even though it does not influence the bacterial growth, catalyzes pyruvic acid metabolism, which is the byproduct in glucose metabolism. **[14]**

*Neisseria meningitidis* lacks a functional glutamate synthase gene, hence lacks glutamine synthase enzyme for the synthesis of glutamate. **[35]** The organism takes in glutamate either from the external environment or synthesized in NADPH specific glutamate dehydrogenase (Gdh) in presence of high external $NH^{4+}$ from 2-oxoglutarate. Glutamate dehydrogenases are key enzymes that link carbohydrate and nitrogen metabolism. Glutamate is utilized in two steps: (i) degradation to succinate by dehydrogenases and (ii) transport of accumulated succinate to the enzymatic sites. Also glutamate is synthesized from α-ketoglutarate and aspartate by glutamate-oxaloacetate transaminase and utilized in a single step. **[36]** In meningococci, glutamate is converted to glutathione which is a key molecule for maintaining intracellular redox potential. This protects the bacteria from reactive oxygen species (ROS) like $H_2O_2$. Also glutamate metabolism plays a

major role against acid stress. Glutamte stimulates the citrate metabolism to a greater extent to furnish energy. Glutamate does not promote the lipo polysaccharide (LPS) sialylation in *Neisseria meningitidis.* **[35, 37-38]**

### 4.4. Growth factors / metal ions ($Fe^{2+}$, $Na^{+}$, $K^{+}$, $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, phospahate)

Iron is an essential trace element for the production of proteins involved in numerous key metabolic processes. In *Neisseria* species, there are five to six major iron regulated proteins (IRP) which are glycoproteins. Inorganic iron source facilitated preparation of medium than organic iron source. It is stated that meningococci can assimilate different iron salts supplemented in the culture media, as well as organic iron such as hemin and hemoglobin. **[39]** Iron plays a significant role in DNA replication, electron transfer in the respiratory chain, metabolism of oxygen, peroxide and superoxide. The available free iron is limited in the host, hence it processes several iron uptake system in which high-affinity receptors for iron-bound host proteins, including transferin, lactoferin (mucosal surface rich in lactoferin) and haemoglobin (blood stream has high content of haemoglobin). **[10]** Invitro grown iron-starved meningococci were found to have increased virulence in vivo, which indicated iron enhances the virulence. *Neisseria meningitidis* can utilize Ferric iron with the help of ferric binding protein (fbp) system, in fbp-deficient situation it can utilize only heme iron. **[40]**

$Mg^{2+}$ and $Ca^{2+}$ act as inorganic cellular cation and cofactors for certain enzymatic reactions. $Ca^{2+}$ plays a critical role during adherence and invasion of *Neisseria meningitidis* to host **[41]**. Grossowicz from his study on growth of *N. intracellularis* in the medium shows calcium ions ($Ca^{2+}$) and magnesium ions ($Mg^{2+}$) enhance bacterial growth. **[14]**

Manganese protects *Neisseria meningitidis* as well as *N. gonorrhoeae* against oxidative stress, whereas *Neisseria meningitidis* is more resistant to Mn than *N. gonorrhoeae,* i.e. growth of *N. gonorrhoeae* was inhibited if concentration of $MnSO_4$ in the media exceeds >100µmol/L, whereas *Neisseria meningitidis* could resist Mn effect about 50-100 times greater concentration than *N. gonorrhoea*. Also, unlike *N. gonorrhoea, Neisseria meningitidis* poses SodB (super oxide dismutaseB) and SodC (super oxide dismutaseC) activities against oxidative killing. **[34]**

Inorganic phosphates ($P_i$) play a significant role in the constituent of nucleic acid, nucleotide, phospholipids, lipopolysaccharide (LPS) or toxins and teichoic acid. **[18]** High concentration of inorganic phosphate inhibits a range of enzyme reactions in animal and plant tissues. **[16]** NaCl and KCl are required for the growth of *Neisseria meningitidis.* Several studies support that there is significant increase in growth when both NaCl and KCl are present compared to any

one of the salt and reduction in growth in absence of these ions. This study confirmed the increased growth rate with NaCl and KCl is not with the osmotic pressure variation but in requirement of these ions. **[42]**.

**5. Effect of physical parameters on growth of *Neisseria meningitidis***

Hike in ambient temperature enhances the meningococcal host immune evasion and resistance against complement. **[43]** Lower temperature (at 35°C instead of 36°C-37°C) and pH resulted in an extended lag phase which resulted a high growth rate **[6]**. The temperature fluctuation during cellular growth strictly regulates polysialic acid (PA) genesis through a molecular complex. **[44]**

Studies show change in environmental pH alters the glucose metabolism. Depending on pH fluctuations glucose channeled through glycolysis, ED or pentose-phosphate pathway. **[26]** The study on *N. gonorrhoeae* revealed the cells utilized double quantity of glucose when grown at pH 6.0 than grown at pH 8.0. **[45]** The *Neisseria meningitidis* cells grown at pH 6.5 utilized more glucose **[25]**.

The growth rate of *Neisseria meningitidis* is greatly dependent on availability of oxygen in the medium because the growth rate is directly proportional to the concentration of the DO in the medium. Limited DO concentration diminished the growth rate that favored the glucose uptake by the organism and capsular polysaccharide production. **[18]** This growth rate retardation due to oxygen limitation shows a linear behavior in the growth curve and enters into stationary phase. End of stationary phase would be better time for harvest since polysaccharide production is more. **[46]**

**6. O-acetylation of polysaccharide**

O-acetyl group attached to the bacterial polysaccharide enhances the immunogenicity. Hence O-acetyl is an important parameter which needs to be maintained.

The O-acetylation of the sialic acid (N-acetyl neuraminic acid) in serogroupW-135 influences several properties of the sialic acid molecules such as the size, net charge, biological properties like immunogenicity of antigen, activation of enzymatic activities involving sialic acid metabolism etc. **[2]**

It was reported that after the emergence of MenW-135 in the year 2000, there was an apparent increase in O-acetylation through 2000 to 2001 from 0% to 21%. Whereas for MenY, there was no change in the proportion of O-acetylation from initial examination (80%). **[47]** A study in MenA with mice showed O-acetyl groups attached polysaccharide conjugate vaccine immunization titers was 32-fold higher than de-O-acetylated (de-O-Ac) conjugate vaccine. **[48]** However, there is no published data which proves the mechanism and significance of O-acetylation on immunogenicity. The O-acetyl quantification has been done by the Hestrin method. **[49]**

## 7. CONCLUSION

The review showed growth of *Neisseria meningitides* which depends heavily on glucose and amino acid concentration. The bacteria showed better growth in recently invented chemically defined media rather than previously studied medias. It can be concluded that by optimizing the carbon source and individual amino acids and its concentration, bacterial growth and PS productivity can be increased for the development of cost effective vaccine. However individual amino acid role on growth of *Neisseria meningitidis* is not known completely.

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