ABSTRACT

Meningococcal serogroup W-135 (MenW-135) has turned out to be the reason for recent outbreak of meningitis in the sub-Saharan regions of Africa including Cameroon, Chad, Gambia, Burkina Faso, and other countries. The capsular polysaccharide (PS) of Neisseria meningitidis serogroups A, C, X, Y, and W-135 are excellent vaccine targets against meningitis caused by this bacterium. This study promotes a novel feed solution including amino acids, vitamins, and glucose along with an optimal fermentation medium; and feeding strategy to cultivate high yield polysaccharide at harvest level. The study is based on the hypothesis that glucose is one of the major growth determining factors with the combinations of nitrogen sources. In this study, fed-batch cultivation was performed in a 2L fermenter, maintaining the following conditions: (i) Temperature = 37°C (ii) pH = 7.0 (iii) Agitation frequency in the range 150 rotations per minute (rpm) to 500 rpm, and (iv) Dissolved oxygen (DO) 20%-25%. The bacterial growth and polysaccharide production were found to be enhanced when the feed flow was increased periodically from late lag phase to early decline phase. The polysaccharide production was found to be maximum in the early decline phase of the bacterial growth. The said method produced polysaccharide of about 1000mg/L, which is twice the reported method. There is limited research information available on fed-batch cultivation and feeding strategy for growing Neisseria meningitidis W-135. The major findings of this study can provide significant contribution in the field of capsular polysaccharide production from which MenW polysaccharide vaccines are manufactured.

Keywords: Neisseria meningitidis W-135, Capsular polysaccharide, Glucose feed, Fed-batch cultivation, Fermentation parameters

I. INTRODUCTION

Meningococcal disease or meningitis is a major cause of death and morbidity throughout the world. Meningitis is responsible for the inflammation of meninges, the protective membranes that covers the brain and spinal cord. Of thirteen clinically significant serogroups that depend on the antigenic structure of their polysaccharide capsule, serogroups A, B, C, X, Y, and W-135 are the major pathogenic strains. Neisseria meningitidis W-135 is the reason for the most recent outbreak of meningitis in the sub-Saharan regions of Africa [1-3]. MenW-135 polysaccharide capsule is a hetero polymer composed of α-2, 6-linked sialic acid and galactose, that is, -4)-NeuAc-α(2,6)-Gal-α-1-, in which the hydroxyl group of sialic acid residue is the site (at C-7 and C-9 positions) of O-acetylation. O-acetylation enhances the immunogenicity of the antigen [4-6]. Neisseria meningitidis is a human pathogen best known for its role in meningitis and other forms of meningococcal diseases such as meningococcal septicemia [7, 8]. Meningococcal disease is serious in nature and if not treated in time, it can progress to death over a matter of hours, in which the mortality reaches 100% [9-11].

Many attempts have been started from the beginning of the 20th century to control meningococcal disease with the administration of vaccines developed from the whole dead cells but they have been unsuccessful. Hence, isolate structures were studied for developing vaccines in which surface structures and bacterium extracellular products were most relevant [9]. The unique structure of the extracellular polysaccharide makes it a good target for vaccine design [5]. Because the bacterial capsular polysaccharides and proteins are the vaccine targets related to bacterial structure, the bacterial growth and nutrient consumption are major factors to be investigated. Although meningococcal vaccines are available in the market with different names such as Menactra for ACYW-DT, Menevo for ACYW-CRM197, and Nimenrix for ACYW-TF; they are costly due to low polysaccharide yield at fermentation harvest level. This makes the vaccines unaffordable to people of developing countries.
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Limited information is available about significance of glucose feeding along with amino acid mixture on growth of Neisseria meningitidis W-135 for improved yield of capsular polysaccharide. Major findings of this study can provide significant contribution to fed-batch mode cultivation of Neisseria meningitidis W-135. This study was performed with four experimental batches of Neisseria meningitidis W-135. The feed was supplemented periodically to enhance the growth. Polysaccharide was extracted and purified from the harvested culture by various precipitation methods and tangential flow filtration (TFF). Then the sample for polysaccharide concentration and other WHO specified parameters were analyzed. The outcome of this study can be extrapolated for the production of polysaccharide for the development of efficient and cost effective vaccine for Neisseria meningitidis W-135.

II. MATERIALS AND METHODS

2.1 Cultivation media and feed media

Fermentation media (g/L): 
- Glucose - 10.0; Sodium Chloride (NaCl) - 6.0; Dipotassium sulphate (K2SO4) - 1.0; Di potassium hydrogen phosphate (K2HPO4) - 4.0; Monosodium glutamate - 10.0; Arginine - 0.75; Serine - 0.75; Thiamine - 0.05; Cysteine - 0.4; Magnesium Chloride (MgCl2) - 0.3; Calcium Chloride (CaCl2) - 0.02; Iron sulphate (FeSO4) - 0.01; Yeast extract - 3.0; Casamino acid - 5.0; Soya - 3.0; Ammonium Chloride (NH4Cl) - 0.25

Feed media (g/L):
- Glucose - 100, Monosodium glutamate - 75, Arginine - 2, Serine - 2, MgCl2 - 0.8; CaCl2 - 0.1; Soya - 2; NH4Cl - 0.4; ready mixture of amino acids, vitamins and glucose - 3.0 to 5.0%. The ready mix is a mixture of amino acids, vitamins, and glucose. Here commercially available product named Cell Boost™ was used as ready mix.
- Fermenter (2L), New Brunswick, Bioflow-Celligen 115, New Jersey, N, USA
- Peristaltic pump (Master Flex, Cole Parmer, IL, USA)
- UV-Vis Spectrophotometer (Shimadzu)
- Biochemical analyzer (Xylem)
- Microplate reader (BioTek)
- HPLC-SEC (Waters corp., Milford, MA, USA)

2.2 Cultivation method

Fed-batch fermentation was performed to cultivate Neisseria meningitidis W-135. Single vial with 1.0 OD was taken from working cell bank and inoculated into 40 ml of growth media with pH 7.0 and allowed to grow by shake flask method at 250 rpm, 37°C. The 40 ml grown culture with OD 1.2 at 590 nm was inoculated into 760 ml of fermentation media so that the total volume was 800 ml and initial OD after inoculation was 0.05-0.08. The media in the 2L fermentation vessel was inoculated from the shake flask grown culture such that the initial OD after inoculation was 0.08-0.1. pH was adjusted to 7.00 using 0.25M alkali treatment during the course of fermentation. The fermentation parameters that were optimized for bacterial growth were:
- Temperature = 37°C, Agitation range = 150 rpm to 500 rpm, Dissolved oxygen = 20%-25%. During the fed-batch run, the feed was fed using the peristaltic pump from late lag phase that is, 4th/5th hour of the culture age.
- The feed flow rate was maintained from lesser volume in the lag phase to gradual increase to the early decline phase, according to the nutrient requirement (as per cell density measurement at OD590) At the interval of 1 hour, the growth at OD590 and glucose concentration in the media by biochemical analyzer was checked and recorded. The liquid culture was grown until the decline phase (that is, 16-18 hrs of culture age) and inactivated by adding 1.5%-2.0% (v/v) formaldehyde and was kept for 2hrs at 37°C for complete inactivation. The culture was subjected to centrifugation at 8000 rpm for 60 minutes at 2ª-8°C in which the cells got settled and supernatant was collected containing the capsular polysaccharide. The supernatant was filtered through 0.2µm membrane filter, followed by diafiltration and concentration by tangential flow filtration (TFF) using 100kDa cassette.

Experiment to determine the hourly polysaccharide production was performed to optimize the harvest hour. Culture was withdrawn (minimum volume) through the sampling port from the running batch and inactivated, centrifuged and filtered. The filtered sample was subjected to TFF using 100 kd cassette and the concentrated sample was analyzed for polysaccharide content and impurity present.

2.3 Isolation of MenW-135 polysaccharide

Isolation and purification of polysaccharide from the crude harvest was done by various precipitation methods [12].
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Crude harvest

Deoxycholate (DOC) treatment

Centrifugation and collection of supernatant and subject to diafiltration

Retentate collection and Cetyltrimethylammoniumbromide (CTAB) treatment

Centrifugation and pellet collection

Ethanol + NaCl treatment

Centrifugation and pellet collection

Pellet dissolution in water for injection (WFI) and analysis for PS and impurity

2.4 Characterization of MenW-135 polysaccharide

2.4.1. Polysaccharide content
The estimation of produced MenW-135 polysaccharide concentration in the harvested crude and purified sample was estimated by Resorcinol-HCl method [13].

2.4.2. Protein and nucleic acid impurity
The total protein was determined by Lowry method [14]. The nucleic acid content in MenW-135 sample was determined by ultraviolet (UV) spectroscopy at 260 nm assuming that a nucleic acid concentration of 50 µg/ml gives an optical density, OD$_{260}$=1.

2.4.3. Endotoxin content
The Endotoxin content was determined by Kinetic Turbidometric Limulus amoebocyte lysate (Charles River Laboratories, USA) with a microplate reader (BioTek) using Endo-Scan-V™ software (Charles River Laboratories, USA).

2.4.4. O-Acetyl content
The O-acetyl content in the produced polysaccharide was quantified by Hestrin method [15].

2.4.5. NMR spectroscopy
The structure of MenW polysaccharide was determined by NMR spectroscopy using Bruker Advance III 400 NMR spectrometer.

III. RESULTS

The current study was performed with different experimental batches of Neisseria meningitidis W-135. The effect of high glucose concentration along with amino acid mixture present in the cultivation media on bacterial growth in the course of fermentation has been shown in Fig-2 and Fig-4.

The study shows that the bacterial growth greatly depends on glucose concentration and amino acid mixture along with other nutrients in the cultivation media. In Experiment 1, feed flow rate was constant. In Experiments 2, 3, and 4, the feed flow rate increased periodically according to the glucose consumption profile determined by the Biochemical analyzer, (for example, glucose in the media was maintained constant).

Different flow rates were followed in each experiment. The periodic increase in the rate of feed optimized was 90 ml, 120 ml, 150 ml, and 180 ml respectively in the media after feeding from late lag phase to early decline phase.

Fig-1 shows the bacterial growth rate was better in Experiments 2, 3, and 4 compared to Experiment 1 which indicates that maintained glucose feeding enhanced bacterial growth.

Fig-2 shows, in Experiment 1, the media glucose approached zero that indicates the bacterial growth must have proceeded through nutrient starvation, whereas in Experiments 3 and 4, media glucose level did not
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Approach zero and maintained a concentration of 0.4-0.5g/L that showed better growth profile. Growth rate was less comparatively in Experiment 2 due to less feed flow rate and media glucose was about 0.2-0.3g/L.

Fig-1 and Fig-2 conclude that feed flow rate and glucose consumption affect bacterial growth significantly. Batches in which flow rate was maintained (Experiments 2, 3, and 4), a rhythmic increase in glucose consumption (that is, gradual decrease and maintenance of glucose present in the media) was observed, whereas in Experiment 1 in which the feed flow rate was constant, the bacterial growth diminished compared to other experiments.

Figure 1: Bacterial growth curve (OD) / hour of Neisseria meningitidis W-135 during the fed-batch cultivation

Figure 2: Glucose consumption vs bacterial growth / hour by Neisseria meningitidis W-135 during the fed-batch mode cultivation

Figure 3: Polysaccharide production with culture age

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It was found that polysaccharide production is higher in the early decline phase of bacterial growth than in the stationary phase (Fig-1 and Fig-3). From Fig-1, the decline phase started at 14 hrs to 16 hrs of culture age and from Fig-3, the polysaccharide production was maximum between 14 hrs to 16 hrs. Polysaccharide production and bacterial growth were directly proportional to each other (that is, polysaccharide production increased with culture age) until the stationary phase, but the polysaccharide concentration was found to have increased crossing the stationary phase until the early decline stage of growth.

Fig-4 shows the glucose feed supplementation rate affected bacterial growth significantly followed by capsular polysaccharide production. The polysaccharide production was maximum in the early decline phase of bacterial growth than the stationary phase. But the polysaccharide production was better in those batches (Experiments 2, 3, and 4) in which feeding strategy was maintained periodically. Fig-4 shows that the polysaccharide production increased about 45%-50% in maintained feeding strategy experiments.

**Figure 4:** Polysaccharide production and respective glucose consumption in the early decline phase of *Neisseria meningitidis* W-135 during fed-batch mode cultivation

Table-1a and Table-1b show the polysaccharide content, impurity present in the initial stage and final stage after purification. Table-1c shows the parameters specified by WHO (World Health Organization) for the final purified MenW-135 polysaccharide. The structural features of MenW polysaccharide produced was confirmed by NMR (Fig-5). The NMR spectra showed the presence of Neuraminic acid, Galactose, and O-acetylation.

<table>
<thead>
<tr>
<th>Sample stage</th>
<th>Batch Size</th>
<th>Harvest age (hrs)</th>
<th>PS (mg/L)</th>
<th>Total PS mg (2L)</th>
<th>Protein (%) *wrt PS</th>
<th>Nucleic acid (%) *wrt PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Harvest (100kDaTFF)</td>
<td>2L</td>
<td>Expt-1</td>
<td>18</td>
<td>550</td>
<td>1100</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expt-2</td>
<td>18</td>
<td>900</td>
<td>1800</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expt-3</td>
<td>18</td>
<td>950</td>
<td>1900</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expt-4</td>
<td>18</td>
<td>980</td>
<td>1960</td>
<td>92</td>
</tr>
</tbody>
</table>

| Sample stage                  |            |                  |           |                  |                     |                        |                        |
|-------------------------------|------------|------------------|-----------|------------------|---------------------|------------------------|
| Final purified PS             | 2L         | Expt-1           | <5        | <1               | 600                 | 1.2                    | 65                     |
|                               |            | Expt-2           | <5        | <1               | 686.94              | 1.54                   | <10                    |
|                               |            | Expt-3           | <5        | <1               | 628.35              | 1.36                   | <10                    |
|                               |            | Expt-4           | <5        | <1               | 667.34              | 1.38                   | 64                     |

Table-1a, Table-1b, Table-1c : Polysaccharide and impurity present in produced polysaccharide and parameter specifications as per WHO for MenW polysaccharide respectively. (*wrt - with respect to)
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IV. DISCUSSION

4.1 Improved yield of MenW-135 polysaccharide

The controlled fermentation parameters and feeding strategy resulted themselves in an improved bacterial growth that corresponds to a high yield of polysaccharide. Although Pengbo Ning et al previously showed a three-step submerged fermentation in which the polysaccharide yield was 55 µg/mL at 200L scale [8]. Glucose and amino acid mixture are the major growth determining factors in the growth of Neisseria meningitidis W-135 along with other nutrient sources. Hence, we studied the ‘feeding strategy’ with and without maintaining the feed flow rate periodically in the fed-batch mode cultivation. We had studied four experimental batches of Neisseria meningitidis W-135, in which they were performed with different feed flow rates. pH control and continuous exponential feeding strategy maintained the cells in the stationary phase for a longer time hence increasing polysaccharide yield.

4.2 Cultivation media and bacterial growth

Fed-batch mode cultivation was adopted because it gives better bacterial growth and yield of polysaccharide levels than batch fermentation [16, 17] even though pH of the media need to be controlled [18]. The fermentation media, fermentation parameters, feed media, and feeding strategy are important bacterial growth determining factors in fed-batch cultivation. Neisseria meningitidis requires mineral salts, lactate, and amino acids (cysteine and glutamic acids). Lactate enhances the growth of Neisseria meningitidis in presence of glucose and lactate metabolizes rapidly than glucose [19], but our study with different carbon sources showed lactate had no significant effect on bacterial growth (data not shown). Presence of phospho transacetylase acetate kinase pathway was indicated by the formation of acetate metabolite in the course of fermentation [20, 21], which is responsible for pH change during fermentation [9]. In this study, pH was controlled by the addition of 0.25 M alkali, which allowed the cells to survive for a longer time in the stationary phase and hence ensured better production of capsular polysaccharide.

US 7491517 disclosed a culture medium to grow Neisseria meningitidis that yielded 30-40 mg/L polysaccharide with the inclusion of NH₄Cl along with the other components for MenW [22], whereas US 7399615 disclosed a fermentation media with the substitution of NH₄Cl with soy peptone as nitrogen source, which yielded a polysaccharide concentration of 684 mg/L MenW in 2L scale and 650 mg/L of polysaccharide in 400L scale with an OD about 10–11 [23]. It was observed that bacterial growth and polysaccharide production were greatly affected by high glucose concentration and amino acid mixture concentration in the media. The glucose consumption was directly proportional to feed flow rate. The study showed, in the stationary phase even though bacterial population was not multiplied, the glucose consumption occurred that indicated the consumed glucose must have been utilized for capsular polysaccharide production. One of our findings that the initial glucose concentration in the fermentation media is directly proportional to the polysaccharide production agrees with the previously reported study [16]. In this study, the concentration of polysaccharide in the media
was estimated to be more in the stationary phase than in log phase but maximum in the early decline phase that supports the above mentioned hypothesis that glucose consumption in the stationary phase was utilized for the polysaccharide production.

Glucose present in the media is responsible for increase in NADP linked glutamate dehydrogenase (GDH) level, which in turn enhances the bacterial growth by facilitating nitrogen metabolism [24]. Glutamic acid metabolism is a major factor that enhances the growth and virulence of Neisseria meningitidis by enhancing the citrate metabolism, [25-27]. N. meningitidis lacks a functional glutamate synthase gene, hence lacks glutamine synthase enzyme for the synthesis of glutamate [28]. The organism takes in glutamate either from the external environment or synthesized in NADPH specific glutamate dehydrogenase in presence of high external NH₄⁺. We used sodium salt of glutamate at a concentration of 7-8% in the feed media for better growth. The catalytic activity of glutamate in the catabolism of succinate, fumarate, and malate was previously shown by Weiss, [29] also glutamate serves to link nitrogen and carbon metabolism and helps to maintain acid stress [30].

Iron is an essential trace element for the production of proteins involved in numerous key metabolic processes including DNA replication, and hence electron transfer in the respiratory chain, metabolism of oxygen, peroxide, and superoxide [31] was supplemented in the initial stage in a minimum concentration as iron sulphate.

In Neisseria meningitidis, principal glucose metabolic pathway is through Entner-Doudoroff (ED) pathway and Pentose Phosphate (PP) pathway. Reported studies showed the major part of pyruvate (80%) was synthesized through ED pathway and PP pathway corresponding to 20% of the metabolism. The activity of Embden-Meyerhof-Parnas (EMP) pathway in the metabolism has not been observed that indicates that phosphofructokinase gene is missing in Neisseria meningitidis [9, 32]. A NMR and enzyme study of carbon catabolism of Neisseria meningitidis showed the presence of all the citric acid cycle enzymes in the cell extracts grown on glucose and pyruvate, whereas the specific activities for most of the enzymes were significantly higher (3-5 fold) in glucose grown cells, in which the pyruvate dehydrogenase activity was more than 10 folds higher in glucose grown cells that supported our hypothesis that glucose is one of the major growth determining factors [33]. A study on metabolite production in the cultivation of Neisseria meningitidis C reported that the bacterial growth depends on acetic acid formation, whereas lactic acid metabolite production was not associated with biomass growth, on which a survey revealed that the utilization of carbon source by Neisseria meningitidis may be through different metabolic pathways depending on the cultivation conditions [34]. Cysteine, which is a major source of sulfur and hence essential for growth of N. meningitides, was fed about a concentration of 0.04%. Cysteine is converted to glutathione (GSH), then to glutathione sulfide by oxidation, hence controls the cellular H₂O₂ level [35, 36]. Transcriptome analysis suggested that cysteine depletion impairs sulfur supply for Fe-S protein assembly and cause oxidative stress [37].

In this study, glucose along with nitrogen sources and other nutrients supported bacterial growth in the initial phase, in which maintained glucose feeding played a significant role in better cultivation of Neisseria meningitidis W-135 followed by enhanced capsular polysaccharide production. Also, this study showed the polysaccharide production was maximum in the early decline phase than in the stationary phase.

4.3 Characterization of MenW polysaccharide

The structural features of MenW polysaccharide produced were confirmed by NMR, which were similar to previously reported structure [5].

V. Conclusion

Thus our experiment has proved a significant contribution to the fed-batch mode cultivation of Neisseria meningitidis W-135 that reveals the significance of maintained flow rate of glucose feed (as carbon source) in combination with nitrogen sources (soya and amino acids) on bacterial growth for better production of capsular polysaccharide. It was concluded from the study that even tough maximum glucose consumption was in the stationary phase, polysaccharide production was maximum at the early decline phase of bacterial growth. The outcome of this study could be extrapolated for the large scale cultivation of Neisseria meningitidis W-135 by fed-batch mode fermentation. The enhanced capsular polysaccharide production can help in faster development of efficient and cost effective vaccine against Neisseria meningitidis W-135.

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