Enhanced Production of *Shigella flexneri* Polysaccharide from a Newly Devised Silicate Method as a Potential Vaccine Conjugate

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Abstract

Conventional glycoconjugates are prepared with polysaccharides (PS) isolated from bacterial sources by fermentation technique. This approach has some major challenges like lower yield of PS, impurities and usage of hazardous chemicals. Reports on efficient and enhanced production of PS from *Shigella flexneri* is meager in literature. Hence, in the current study, three different types of media namely Yeast extract medium, *Shigella sonnei*-defined medium and synthetic medium were utilized for the culture of *S. flexneri*. Among the selected media it was recognized that the culture of *S. flexneri* harvested in synthetic media produced significant quantity of PS in less time when compared to the other two media. Different purification techniques such as phenol chloroform extraction, acid precipitation, detergent method, chromatographic purification and a novel silicate method were carried out to refine the harvested PS from impurities. It was observed that large impurities such as bacterial protein, debris and media components were eliminated significantly by using chromatographic and silicate methods. The final yield of purified PS was approximately 20–35% higher in silicate method which is reported for the first time in this study for purification of PS. Further, the characterization of the purified PS was done using high-performance liquid chromatography and high-performance anion-exchange chromatography with pulsed amperometric detection. Hence, the robust process developed in the present study using synthetic media and chromatographic filtration technique along with the novel silicate treatment produced significant quantities of PS from *S. flexneri* in reduced cost and time, which could be further conjugated to a suitable carrier to generate a potential *Shigella* conjugate antigen.

Introduction

Enteroinvasive gram-negative *Shigella* bacteria are the human pathogens that cause severe infection known as Shigellosis. The disease is estimated to affect approximately 165 million people annually, leading to 1.1 million deaths per year [1]. This shigellosis remains a major cause of diarrheal disease in the developing countries and causes substantial morbidity and mortality in children under the age of five years [2]. It is in the top four pathogens that cause moderate-to-severe diarrhea in African and South Asian children. Worldwide over 188 million cases of shigellosis occur annually and around 1, 64,000 associated deaths take place [3].

In view of these deaths and the severity caused by *Shigella* infections, many control measures were implemented

such as basic sanitation, usage of antibiotics and education control. But it is often difficult to control these bacterial outbreaks due to the development of multidrug-resistant *Shigella* strains [Lambert]. Hence, the development of a safe and effective vaccine against this Shigellosis is a prior strategy for the World Health Organization [3]. To develop a vaccine candidate against these *Shigella* strains, many research strategies were undertaken. Among them, from the literature, it is evident that conjugate vaccines are the most efficient and preventive approach against these bacterial infections such as caused by *Haemophilus influenzae* type b, *Neisseria meningitidis*, *Salmonella typhi* and *Streptococcus pneumoniae* [4]. All these conjugate vaccines consist of capsular polysaccharides as their main moiety along with the carrier proteins.

In *Shigella* species, the main surface antigen is polysaccharide (PS) and various attempts were made to chemically conjugate it with carrier proteins such as diphtheria toxoid and tetanus toxoid [5]. A better control of *Shigella* infections is dependent on the high immunization uptake by individuals and this disease outbreak can be prevented by developing a

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new generation of conjugated PS vaccines linked to potential carrier proteins. For this respective PS vaccine development, a very large-scale industrial production of PS from *S. flexneri* is necessary. At present, the cost of cultivation and production of PS from *S. flexneri* in a bioreactor is generally high and requires robust process [6]. And also very meager information is available from the literature on this process of improved production and purification of PS from *S. flexneri* for a pilot or industrial scale synthesis. Traditional purification processes of bacterial PS often involved hazardous solvents such as ethanol, phenol and cationic detergent

s. And experiments with enzymatic treatments did not produce required purity of bacterial PS.

Therefore, in the present study, investigations on the optimum growth rate of *S. flexneri* were done utilizing three different media, namely Yeast extract medium, *Shigella sonnei*-defined medium and synthetic media. Hestrin assay was performed to detect the quantity of PS in the cultures of *S. flexneri* grown from these three different media. To attain purified PS from *S. flexneri*, devoid of proteins and nucleic acid impurities, various purification methods were employed. Initially, traditional molecular weight cut off studies, phenol chloroform extraction, acid precipitation and detergent methods were carried out to purify the produced PS from *S. flexneri* cultures. Further, for more enhanced yield and efficient purification of *S. flexneri* PS, chromatographic purification and novel silicate methods were employed. Later, to analyze the average molecular weight distribution of the purified PS of *S. flexneri*, HPLC method was used. HPAEC-PAD was used to evaluate the different sugar composition of total PS of the *S. flexneri*. Overall, the present study is aimed to increase PS production from *S. flexneri* and to devise an efficient purification system for a potential vaccine conjugate.

Materials and Methods

Bacterial Strain

S. flexneri ATCC 120,922 (one lyophilized vial) used in the current study was procured from NCIM (National collection of Industrial microorganisms), Pune, India. Glycerol stocks of the strain was prepared and stored at -70°C [7]. All the chemicals used in the present study were purchased from Merck and Sigma, India.

Growth Media

Yeast Extract Medium (HTMC)

Cultures of *Shigella flexneri* were inoculated into 50 ml of yeast extract (HTMC) medium and incubated at 37°C at

$20\times g$ for 30 h. Seed cultures were developed by sub-culturing into 300 ml of HTMC medium and incubated in a rotary shaker at 37°C at $17\times g$ for 26 h. Composition of the medium included 30 g of yeast extract, 5.0 g of KH_2PO_4 , 20 g of K_2HPO_4 , 1.2 g of $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 1.5 ml glycerol and 0.25 ml of polypropylene glycol in a final volume of one litre distilled water. The pH was adjusted to 7.0 ± 0.2 using 0.25 M NaOH. The medium was filtered through 0.2 μm gamma irradiated capsule filter and added aseptically to the cultures of *S. flexneri*. Then, culturing was performed at 37°C , pH 7.0 and $35\times g$ of agitation speed. The different agitation speeds have been used as per the report by Scorza et al. [7] to obtain the maximal growth at 600 nm. Sterilized deoxycholate detergent of 0.5–1.0% w/v was added to the culture broth after attaining an optical density (OD_{600}) of 0.5 to extract the PS from the cell wall of *S. flexneri* [7].

Shigella sonnei Defined Medium (SSDM)

Seed cultures of *Shigella flexneri* were developed by sub-culturing into 300 ml of *Shigella sonnei*-defined medium (SSDM) and incubated in a rotary shaker at 37°C , $17\times g$ of agitation for 28 h. The composition of the medium included 30 g of glycerol, 13.3 g of KH_2PO_4 , 20 g of $(\text{NH}_4)_2\text{HPO}_4$, 1.2 g of $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 1.7 g citric acid, 0.25 ml of polypropylene glycol and trace elements in a final volume of 1 litre of distilled water. The pH was adjusted as mentioned in HTMC medium. The medium was filtered as mentioned above and added aseptically to the cultures of *S. flexneri*. Then, culturing was performed at 37°C temperature, pH 7.0 and $17\times g$ of agitation. Later, deoxycholate detergent at 0.5–1.0% w/v was added to the culture broth after attaining an optical density (OD_{600}) of 1.5 to extract the PS from the cell wall of *S. flexneri* [7].

Synthetic Medium

Seed cultures of *Shigella flexneri* were developed by sub-culturing into 300 ml of synthetic medium and incubated in a rotary shaker at 37°C , $17\times g$ of agitation for 12 h. The composition of the medium included 178 g of KH_2PO_4 , 5.3 g of $(\text{NH}_4)_2\text{HPO}_4$, 2.2 g of $\text{C}_6\text{H}_8\text{O}_7\cdot \text{H}_2\text{O}$, 1.5 g of $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 30 ml glycerol, 8.8 mg $\text{C}_{12}\text{H}_{17}\text{N}_4\text{OS}$ and trace elements in a final volume of 1 l distilled water. The pH was adjusted as mentioned above for the other two media. The medium was filtered as mentioned above and added aseptically to the cultures of *S. flexneri*. Then culturing was performed at 37°C temperature, pH 7.0 and $26\times g$ of agitation. Deoxycholate detergent at 0.5–1.0% w/v was added to the culture broth after attaining an optical density (OD_{600}) of 2.5 to extract the PS from the cell wall of *S. flexneri* [8].

Hestrin Assay for PS Detection

Acetylcholine standards were prepared (ranging from 12.5–200 $\mu\text{mol/ml}$ whose final volume was made upto 1 ml with distilled water) and then 400 μl of hydroxyl amine hydroxide of 0.2–2 M was added to the harvested *S. flexneri* samples taken in independent flasks and incubation was done at different time intervals with the addition of HCl and FeCl_3 of 0.1–0.4 M to observe the brick red color formation. The absorbance was measured at 540 nm [6] to detect the presence of PS. The protein content in samples was estimated by the BCA spectrophotometric method (using Micro BCA protein assay kit from Thermo Fischer).

Polysaccharide Purification Methods

The PS from the crude extract of *S. flexneri* were separated from the impurities such as proteins and nucleic acids based on size, charge, hydrophilicity, affinity and solubility through different techniques to find the most optimized method for highest and purest yield. Scheme 1 depicts the various strategies employed in the purification of the extracted polysaccharide.

Molecular Weight Cut Off Studies

Molecular weight cut off studies is a basic parameter to estimate the approximate size of the bacterial PS. The crude

culture *S. flexneri* was lysed by orthophosphoric acid of 0.5% w/v and incubated for 3 h at 25 °C. The 300 ml of culture was centrifuged at 11,000 $\times g$ at 4 °C for 30 min and the supernatant was collected. Selection of molecular weight cut off (MWCO) membrane amicon filters of 30, 50, 100, 500 kDa from Merck, Millipore were used to estimate the molecular size of the PS from *S. flexneri* which was spun at 3800 $\times g$ for 40–50 min to collect the supernatant [9].

Phenol Chloroform Extraction

Further, 600 ml of the crude culture of *S. flexneri* was treated with phenol–chloroform in 3:1 ratio for purification of the PS. Cell suspension was supplemented with 50 mM of EDTA. After 5 min of incubation under similar conditions, EDTA effect was stopped by adding approximately double the concentration of MgCl_2 (100 mM). At this stage cells were re-centrifuged to obtain crude PS. The supernatant having bacterial cells with PS on their surface was subjected to Cetyl Trimethyl Ammonium Bromide (CTAB) precipitation of 5%v/v and centrifugation was done at 11,000 $\times g$ for 45 min. This was followed by precipitation of PS for 3 h at – 20 °C using cold (– 20 °C) absolute ethanol with 0.5 M sodium chloride and 3 M sodium acetate (pH 5.2). Precipitated PS was collected using centrifugation and further suspended in small volume of 1X PBS (pH 7.4) buffer. This method is limited due to consumption of time and usage of hazardous chemical like phenol [10, 11].

Scheme 1 Depicts the various polysaccharide purification strategies employed

Acid Precipitation and Filtration

In this method, 600 ml of the crude culture *S. flexneri* was lysed by 0.5% w/v of orthophosphoric acid and incubated for 3 h at 25 °C. The culture was centrifuged at 11,000×g at 4 °C for 30 min. The supernatant was concentrated to 10–12 fold using 0.5 m² 100 kDa followed by CTAB precipitation (5%v/v) and centrifugation. It was collected after centrifugation and dissolved in ~ 1000 ml of distilled water followed by carbon filtration and further concentrated using a 30 kDa tangential flow ultrafiltration (TFF) cut-off membrane, followed by filtration using 0.2 µm membrane filter. The purified PS was stored at – 25 °C. The disadvantage of this method is its lower extraction rate and difficulty in purifying the PS of our interest [12, 13].

Detergent Method

In this method, 600 ml of the crude culture of *S. flexneri* was lysed by 0.2% w/v of sodium deoxycholate and incubated for 6 h at 35 °C. Then, the culture centrifuged at 11,000×g at 4 °C for 45 min. The supernatant was concentrated to 5–10-fold using 0.5 m² of 100 kDa membrane followed by 30%v/v of ethanol precipitation and then 6% w/v of AlPO₄ addition. Further, the precipitated supernatant was incubated for 8 h at 25 °C followed by centrifugation. The supernatant collected after centrifugation was once again concentrated and diafiltered using a 30 kDa tangential flow ultrafiltration cut-off membrane followed by filtration using 0.2 µm membrane filter. The purified PS was stored at – 25 °C. This method is not often suggestible for bacterial PS purification, due to the usage of ethanol step at high-scale operations [14, 15].

Chromatographic Purification

For chromatographic separation, 600 ml of the crude culture of *S. flexneri* was lysed by 0.2% w/v sodium deoxycholate and incubated for 6 h at 35 °C. Then, the culture centrifuged at 11,000×g at 4 °C for 45 min. The supernatant obtained was concentrated to 5–10-fold using 0.5 m² of 100 kDa membrane followed by CTAB precipitation of 5%v/v and then centrifuged at 11,000×g for 45 min. Then, the supernatant was passed through 0.45 µm membrane filter for filtration. The ion exchange chromatography technique is further applied to purify the PS from *S. flexneri* permeate from the proteins and nucleic acids. Resins namely Capto Q and DEAE cellulose were used to pack the column with dimensions of 1.5×25 cm. 50 ml solution (the permeate) was loaded in ion exchange column carefully and the separated fractions were collected at a flow rate of 30 ml/h. Then 0.05 M phosphate buffer (pH 7.5) was used for both the wash and equilibration. Each fraction is individually estimated for the presence of PS, proteins and nucleic acids [16, 17].

Silicate Method

In this novel method of separation, 600 ml of the crude culture of *S. flexneri* was lysed by detergent sodium deoxycholate of 0.2% w/v and incubated for 6 h at 35 °C. Then, the culture centrifuged at 11,000×g at 4 °C for 45 min. The supernatant was concentrated to 5–10-fold using 0.5 m² of 100 kDa membrane followed by CTAB (5%v/v) precipitation and centrifugation at 11,000×g for 45 min. The supernatant was then collected and passed through charcoal filters and further treated with 20 gm/l of silicate at room temperature with continuous stirring for 1 h followed by centrifugation. Then, the filtration with 0.45 µm membrane filter was done followed by concentration and diafiltration using a 30 kDa tangential flow ultrafiltration cut-off membrane followed by filtration using 0.2 µm filter.

Analytical Procedures

Protein content for all processed samples was then determined utilizing the PS samples from *S. flexneri* cultures in independent synthetic media flask of 100 µl by the spectrophotometric method using Bovine serum albumin as standard [18] and DNA content by absorption at 260 nm by spectrophotometric method [2] to assess the purity of the extracted PS. Statistical analysis for all the purifications and analytical methods were done by one-way ANOVA, using BIOSTAT software and the experimental values are mean of 20 replicates.

High-Performance Liquid Chromatography

Polysaccharide molecular weight distribution analysis was done using HPLC—size exclusion chromatography. Acid-cleaved PS samples *S. flexneri* from independent synthetic media flask of 100 µl were run on a TSK gel G3000 PWXL column (30 cm × 7.8 mm; particle size 7 µm; cat.# 808,021) with a TSK gel PWXL guard column (4.0 cm × 6.0 mm; particle size 12 µm; cat.# 808,033) (Tosoh Bioscience). The mobile phase consisted of 0.1 M NaCl, 0.1 M NaH₂PO₄, 5% CH₃CN at pH 7.2 and a flow rate of 0.5 ml/min (isocratic method) for 30 min. Void and bed volumes were calibrated with λ-DNA (λ-DNA molecular weight marker III ranging from 0.12 to 21.2 Kbp) and sodium azide, respectively. The column void volume (T_0) being 10.58 min and the total volume (T_{tot}) being 23.03 min [19].

HPAEC-PAD

The sugar composition of the *S. flexneri* PS was determined by HPAEC-PAD using a Carbo Pac PA1 column (50 mm × 250 mm) coupled to a CarboPac PA1 guard column and connected to Dionex KS5000 system. Samples

diluted at 10 µg/ml of saccharide were treated with NaOH at a final concentration of 2 M, heated at 110 °C for 6 h in a closed screwcap test tube and filtered with 0.45 µm filter before the analysis. The separation was performed with a flow rate of 1 ml/min using gradient elution of 100 mM NaOH for 32 min with increased concentration of NaNO₃ ranging from 8 to 50%, followed by a washing step for 22 min. The chromatography was monitored using the pulsed amperometric mode with a gold working electrode and an Ag/AgCl reference electrode. A quadruple-potential waveform for carbohydrates was used. The chromatographic data were processed using Dionex Chromeleon TM software. The calibration curve was set up with glucose (Fluka) in the range of 1.0–25.0 µg/ml which was treated the same way as the samples mentioned above [20]. All the above experiments have been performed in triplicates and the results are the mean of those three readings.

Results

Production of PS

It is important to reduce the cost of PS production and purification and to attain the maximum quantity from the *S. flexneri* culture. Therefore, in the current study, three important nutritive media namely HTMC, SSDM and Synthetic media were utilized to culture the experimental batches of *S. flexneri*. From the bacterial growth curve, it was observed that flask culture with synthetic media promoted maximum *S. flexneri* growth rate in a short period of time i.e., within 12 h [Fig. 1]. But, significant *S. flexneri* growth was observed only after 24 h from the HTMC and SSDM media.

Fig. 1 Mean data of growth index measurement of *S. flexneri* under three growth media conditions. Values are mean of 20 replicates $\pm$ SE; Statistical analysis by one-way ANOVA showed that there was a significant difference at $P < 0.05$ level between the different samples

Hestrin assay was performed to detect the quantity of PS produced from the cultures of *S. flexneri* that were harvested utilizing the three different media namely HTMC, SSDM and Synthetic media. From Table 1, it was evident that the produced PS of 1.26 mg/ml content was higher in the culture samples of *S. flexneri* grown using the Synthetic media. The yields of PS from *S. flexneri* grown on Synthetic medium based on the optical density (OD) at 600 nm (OD_{600} -2.4) was significantly higher than that grown on HTMC (OD_{600} -0.65) and SSDM (OD_{600} -1.4) media.

Purification of PS

PS present on the surface of the *S. flexneri* acts as a barrier for the foreign particles and carries the antigenicity. In order to extract this PS from this pathogenic bacteria, various traditional purification methods were employed namely molecular weight cut off studies, phenol–chloroform extraction, acid precipitation and detergent method. From Table 2 representing the contribution of each purification step, it is evident that the quantity of PS extracted from *S. flexneri* varied significantly from each purification process. Evaluation of PS from *S. flexneri* through molecular weight cut off studies, denoted its molecular weight to be well within the standard range of 150 to 500 KDa, important for conjugation studies. Insignificant quantities of PS from *S. flexneri* were extracted from the phenol chloroform extraction process with 0.1–0.2 mg/ml of yield. Whereas, moderate levels of PS of 0.2–0.4 mg/ml were extracted from *S. flexneri* through the acid precipitation and detergent purification methods. Due to the insignificant yields from these traditional methods, chromatographic purification and a novel silicate method for the first time was devised and employed for the purification of the PS for enhanced yield. Further, in the study, it was observed that after removal of impurities such as proteins and nucleic acids, 0.8–1 mg/l of the bacterial PS was obtained by employing chromatographic purification method. But the purified PS product recovery from *S. flexneri* is 1–1.2 mg/l which is significantly higher in the silicate purification method compared to the other

Table 1 Estimation of PS content from *S. flexneri* cultured in three different media at 12 h by Hestrin assay

S. no	Media	Absorbance at 540 nm Avg OD $\pm$ SD (of 20 replicates)	PS content (mg/ml)
1	HTMC	0.028 ± 0.005	0.608
2	SSDM	0.029 ± 0.006	0.702
3	Synthetic	0.036 ± 0.006	1.260

All parameters were expressed in mg/ml and values are mean $\pm$ SD ($n = 20$). Values followed by different letters in a column are statistically different at $P < 0.05$ [4]

Table 2 Stepwise purification of PS content from *S. flexneri* culture employing various purification techniques

	Proteins (mg/ml)	Nucleic acids	Polysaccharide (mg/ml)
<i>Molecular weight cut-off studies</i>			
30 KDa	0.85 ± 0.0002	0.55 ± 0.0004	0.72 ± 0.0006
50 KDa	0.81 ± 0.0004	0.46 ± 0.0002	0.68 ± 0.0006
100 KDa	0.67 ± 0.0006	0.39 ± 0.0002	0.65 ± 0.0003
500 KDa	0.55 ± 0.0004	0.35 ± 0.0002	0.39 ± 0.0001
<i>Phenol–chloroform extraction</i>			
Phenol–chloroform	2.7 ± 0.0007	1.8 ± 0.0006	0.2 ± 0.0013
EDTA	2.1 ± 0.0006	1.7 ± 0.0009	0.1 ± 0.0023
Filtration	1.8 ± 0.0012	1.6 ± 0.0012	0.05 ± 0.0004
<i>Acid precipitation and filtration</i>			
Acid lysis	2.3 ± 0.0133	1.8 ± 0.001	0.7 ± 0.004
CTAB precipitation	1.8 ± 0.001	1.6 ± 0.023	0.45 ± 0.001
Characoal	1.4 ± 0.001	1.4 ± 0.001	0.39 ± 0.004
Diafiltration	1.3 ± 0.023	1.4 ± 0.001	0.2 ± 0.023
Microfiltration	1.1 ± 0.043	1.2 ± 0.023	0.13 ± 0.003
<i>Detergent method</i>			
Detergent lysis	1.75 ± 0.0009	3.2 ± 0.0043	2.2 ± 0.0023
Ethanol precipitation	1.59 ± 0.0002	2.7 ± 0.004	0.8 ± 0.001
AlPO ₃ adsorption	1.4 ± 0.0433	2.3 ± 0.004	0.6 ± 0.0023
Diafiltration	1.3 ± 0.004	1.8 ± 0.003	0.4 ± 0.0023
Microfiltration	1.2 ± 0.003	1.5 ± 0.006	0.25 ± 0.0043
<i>Chromatographic purification</i>			
Detergent lysis	1.9 ± 0.093	2.8 ± 0.043	2.5 ± 0.04
Ultrafiltration	1.5 ± 0.013	2.4 ± 0.063	1.8 ± 0.023
CTAB treatment	1.1 ± 0.013	1.8 ± 0.023	1.6 ± 0.063
Charcoal filtration	0.8 ± 0.023	1.4 ± 0.04	1.4 ± 0.013
Silicate treatment	0.19 ± 0.001	0.5 ± 0.01	0.95 ± 0.006
Ultrafiltration	0.15 ± 0.001	0.75 ± 0.03	0.92 ± 0.003
Microfiltration	0.10 ± 0.063	0.1 ± 0.03	0.85 ± 0.001
<i>Silicate method</i>			
Detergent lysis	1.9 ± 0.001	2.8 ± 0.001	2.5 ± 0.043
Ultrafiltration	1.5 ± 0.002	2.4 ± 0.001	1.8 ± 0.001
CTAB treatment	1.1 ± 0.003	1.8 ± 0.001	1.6 ± 0.001
Charcoal filtration	0.8 ± 0.001	1.2 ± 0.001	1.4 ± 0.002
Silicate treatment	0.19 ± 0.033	0.14 ± 0.004	1.1 ± 0.001
Ultrafiltration	0.15 ± 0.002	0.13 ± 0.003	0.95 ± 0.004
Microfiltration	0.10 ± 0.003	0.10 ± 0.003	0.92 ± 0.001

Values are mean of 20 replicates ± SE. Statistical analysis by one-way ANOVA showed that there was a significant difference at $P < 0.05$ level between the different samples

purification methods employed. This proved the efficacy of the newly devised silicate method.

Analysis of the molecular weight distribution of the obtained purified PS from *S. flexneri* was performed using HPLC. Figure 2 shows the molecular weight of purified PS from *S. flexneri* to be varying from 176, 192 and 199 KDa for the three experimental batches of cultures which were well within the standard range of 150 to 300 KDa required for conjugation studies. Later, the cell wall sugar composition of the PS from *S. flexneri* was studied using HPEAC-PAD

chromatography. Figure 3 revealed the various identified sugars as N-acetyl glucosamine, Rhamnose and Galactose in the PS isolated from *S. flexneri* when run against their standards.

Discussion

The growth profiles and the PS production from *S. flexneri* culture are important factors, as they determine the optimal harvest time and economy of downstream processes for the

Fig. 2 HPLC chromatography profile of purified PS from three different batches of *S. flexneri* culture representing the average molecular weight distribution in KDa. The HPLC profile was detected by RI using HP-GPC column. The peak at 12.42 min represents the purified polysaccharide from different batches with molecular weights of 199, 192 and 176 KDa

Fig. 3 Representation of various sugar moieties in the PS isolated from *S. flexneri* through HPAEC-PAD chromatogram. Peak 1 corresponds to *N*-acetyl glucosamine with retention time of 7.3 min, Peak 2 corresponds to Rhamnose with the retention time of 8.5 min and Peak 3 represents to Glucose with the retention time of 11.4 min

conjugate vaccines. During the culture of *S. flexneri*, the fermentation media, physical–chemical parameters and feeding strategy are the prime most factors for the bacterial growth. An improved bacterial growth will correspond to higher yields of PS [21, 22]. In the present study, it is observed that the synthetic media enhanced the growth of the *S. flexneri* in reduced time period of within 12 h compared to HTMC and SSDM media. It is also known that the presence of glucose, amino acids and minerals are usually essential and enhance

the bacterial growth [23]. The synthetic media utilized for the culture of *S. flexneri* constituted glucose as one of its main constituents. Thus, the glucose content (a major carbon source) in the synthetic media might have enhanced the *S. flexneri* growth rates in comparison to the HTMC and SSDM media. This indicates that the PS production corresponds to the bacterial growth rates depending on the composition of the growth media [23, 24].

A purified and an economical production of PS from *S. flexneri* is very important towards the synthesis of PS-based conjugate vaccines. Hence, the aim of the present study was to develop an efficient purification method for the extraction of PS from the pathogenic *S. flexneri* bacteria that provides both higher yield and purity. PS production using conventional methods such as phenol–chloroform extraction and acid precipitation resulted in large impurities of proteins and nucleic acids [25]. From the literature, it is also evident that the usage of acid in these purification methods had reported a degradative effect on the extracted PS [26]. The detergent method employed in the present study is a traditional bacterial PS extraction method and often associated with the ethanol precipitation for better yields of purified PS [1]. Further, in order to eliminate the usage of organic solutions, the chromatographic purification method was adopted for the extraction of PS from *S. flexneri* [27]. In this study, the chromatography method is further improved with the step-up of tangential flow filtration approach for the purification of bacterial PS samples. This tangential flow filtration purification step is very efficient and the membranes were easy to clean and can be reused several times [28, 29]. Further, in the present study, an efficient silicate method was developed for the purification of PS from *S. flexneri* that greatly reduces time by eliminating the effect of phenol, solvents and chromatography resin process steps. This newly devised silicate method employed uses the advantage of the silica powder and charcoal treatments, and removes the impurities very efficiently. Silicate adsorbs nucleic acid and protein impurities very efficiently. The silicate method is cheaper than chromatographic columns for large-scale operations. Handling silica during polysaccharide recovery is less time consuming. This alternative silicate purification process significantly recovered high yields of purified PS from the pathogenic *S. flexneri* bacteria. In addition, this method is proved to be scalable and economical for the large-scale manufacturing of *S. flexneri* PS-conjugated vaccines. The average molecular distribution of the purified polysaccharide was further confirmed by HPLC to be well within the standard range of 150–300 KDa, which is optimal for conjugation studies. The sugar composition of the PS was confirmed to be N-acetyl glucosamine, rhamnose, and galactose by HPAEC-PAD based study on the retention time in comparison to their standards.

Conclusion

The present study was a successful attempt towards a cost-effective way of high-rate production of PS from the cell wall of *S. flexneri* through a newly devised technique called silicate method used for the first time in this study.

The experimental findings of the current study have shown that the synthetic media have the potential to enhance the yield of PS of *S. flexneri* to 2 folds (1.26 mg/ml) within a short time of 12 h when compared to 0.608 mg/ml in HTMC and 0.702 mg/ml in SSDM media. It was also shown that the utilization of the synthetic media is reproducible and produces debris free products that ease the process of PS purification from *S. flexneri*. In addition, the present study also provided an improved silicate method for PS extraction with enhanced yield of 20–35% (compared to traditional approaches employed in the current study) and purity which can be speculated to be applied to other Gram-negative pathogens as well. This novel silicate method employed in this study for the first time could be potentially used for the development of PS-based conjugate vaccines in industrial scale-up production. Overall, the developed method is relatively more efficient (with enhanced yield), clean (healthy extraction with reduced impurities) and cost-effective for pilot scale production.

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Compliance with Ethical Standards

Conflict of interest This paper has not been published before in any form and it is not under consideration by any other journal at the same time. All the authors approve the manuscript submission to this journal and none of the authors have any conflicting interests in the manuscript.

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