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Production and purification of Vi polysaccharide from *Salmonella enterica typhimurium* (MTCC98)

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ABSTRACT

The main objective of the research is enhanced production of Vi Antigen by the modification of fermentation media. The optimized concentration of Tryptophan is 0.5 g/100ml, Glucose concentration is 1g/100ml and the optimized Temperature is 35^0C. With decrease in concentration of Glucose, production of Vi antigen is enhanced. The quantification has been done by Single Radial Immuno Diffusion Assay. The conc. of Vi antigen is found to be 12µg in 0.6 mm diameter and 27 µg in 0.9 mm diameter ring. For the determination of purity of Vi polysaccharide OD at 215 nm is takenand the obtained values for crude broth, partially purified Vi polysaccharide, purified Vi polysaccharide are 0.853, 2.190 and 3.104 respectively. Thus, the highest optical density in purified Vi polysaccharide showed high Vi concentration. In purified Vi polysaccharide,74% recovery has been obtained by Gel filtrationchomatography. Thus,Vi polysaccharide yields good recovery by Sephadex G200 at low cost.

KEYWORDS

Glucose;
Optical density;
Typhoid fever;
Vi antigen.

INTRODUCTION

Typhoid fever is a common serious disease in many parts of the world and remain a major health problem in developing country with an estimated 21 million cases and 2,00,000 death in 2000[1]. Typhoid fever causing significant morbidity and mortality in countries that have yet achieved satisfactory control of drinking water,food and sewage disposal[2]. Control of typhoid fever may be aided by immunization, but widespread protection by this method has not been achieved for various reasons, including the limitation and insufficient use of available vaccine[3]. The two well-tolerated vaccines in use in humans provide partial and temporally limited protection and are quite distinct[4-6]. One is a live attenuated typhoid bacterium - strain Ty21a - and is administered orally. How this attenuated bacterium confers protection is not fully understood but antibody is likely to be important. This live vaccine lacks galE and so has an impaired capacity to synthesize LPS O-chain and also lacks the capsular polysaccharide (CP) Vi antigen, both considered major targets of protective antibody[7]. The importance of antibody to Vi is evi-

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denced by the use of purified Vi antigen as a standalone vaccine. Vi antigen is made of repeating units (1-4)-2-deoxy-2-N-acetyl galacturonic acid encoded within the *ViaB* locus from ST[8]. Immunization provides protection against typhoid at levels comparable to Ty21a in adults and older children in the first two years post-immunization[4,5]. The protection conferred by immunization with Vi antigen is likely to be mediated via systemic antibody as it has not been found to induce pronounced mucosal antibody responses, nor have a requirement for T cell involvement[9,10]. Therefore understanding the nature of antibody responses to Vi antigen and other vaccines based on CP is likely to be important in understanding the basis of immunity to many pathogens and improving vaccines that target them. Typhoid fever is aseptocaemic disease caused by a capsuled bacterium, Salmonella typhi,whose capsular polysaccharide is called the Vi polysaccharide. Vi is a linear homopolymer of Ţ (1-4)-D-GalpA N-acetylated at C-2 and O-acetylated at C-3[5-7]. The N and Oacetyls dominate the surface and are essential for both antigenicity and immunogenicity of Vi[7,8]. Vi antigen is a Virulence factor which by itself is capable of conferring protection from infection with S typhi and Vi is present in most isolates from blood of patients with typhoid fever. The Vi antigen is immunogenic when injected intramuscularly or subcutaneously and confers protective immunity for at least 3 years[9,11,12].

MATERIAL AND METHODS

Material

Salmonella enteric typhimurium (MTCC 98) strain used in the experiment was purchased from IMTECH, Chandigarh. All other chemicals used were of analytical grade and procured from Hi media, SD-fine chemicals and Titan Biotech ltd.

Methods

Preparation of Media

The initial media used for the cultivation of bacteria was Nutrient Broth. The Base media composition selected for flask culture contained10 g yeast extractl^{-1}, 5 g Tryptophanl^{-1}, 10 g glucosel^{-1}, 2.5 g $MgSO_4 \cdot 7H_2O l^{-1}$, 4.1 g $NaH_2PO_4 \cdot H_2O l^{-1}$ and 18.8 g $Na_2HPO_4 \cdot 7H_2O l^{-1}$[13].

Inoculum development

For the development of inoculum, lyophilized culture was inoculated in a 250 ml flask containing 100 ml Nutrient Broth in aseptic conditions. The culture was then incubated at 37°C for 12 h in shaker cabinet at 110 x g. After keeping it for 24 h culture growth of microorganism reach at stationary phase, which was then estimated by taking OD at 600 nm and refrigerated at 4^0C for further use.

Optimization of media components[13]

Optimization of tryptophan concentration

Concentration of Tryptophan was maintained at 0.25-2.0 g in each 100ml media in 5 different flasks, inoculated with 5% of the refrigerated culture of *Salmonella enterica typhimuriu*ma and incubated at 35 for 24h. Absorbance was then noted at 600nm spectrophotometrically.

Optimization of glucose concentration

Concentration of glucose was maintained at 1-5g in each 100ml media in 5 different flasks, inoculated with 5% of the refrigerated culture of *Salmonella enterica typhimuriu*ma and incubated at 35 for 24h. Absorbance was then noted at 600nm spectrophotometrically.

Optimization of temperature

Five flaskswith 100ml of optimized media inoculated with microorganism at different temperatures(25-45^0C) were taken. After 24 h of incubation at different temperature, OD was taken at 600nm and the highest OD showed better growth of microorganism.

Optimization of maximum wavelength[14]

Culture broth was taken in Vivaspin tubes and centrifuged at 4500 x g for 20 minutes. The supernatant was collected and measured at different wavelength and the highest OD was taken.

Fermentation and culture conditions at flask level

Initial shake flask cultures was performed in a 500ml shake flask containing 100 ml of base media previouslysterilized in autoclave at 15 psi, 121 and

inoculated with 5% of inoculum of bacterial culture under sterilized conditions which were then incubated in a shaker cabinet at 150 x g, 35 °C for 12 h.

Post fermentation treatment[15]

Development of vaccine for typhoid fever has been hindered because the causative agent, *S.typhimurium*cause infection and effectively pathogenic in human. Vaccine was composed of whole and inactivated *S.typhimurium* which was inactivated by heat treatment method in which microorganism was treated with 56 for 10 min.

Analysis of glucose concentration

Several reagents have been employed which assayed sugar using their reducing properties. One of such compound is 3,5-dinitrosalicyclic acid(DNS), which in alkaline solution gets reduced to 3-amino-5-nitrosalicylic acid. Six test tubes were taken and marked 1-6. Glucose solution was added in range of 0.0ml to 1ml with 0.2ml of intervals and the volume of each test tube was leveled to 1ml with distilled water and 3ml of DNS was added to each test tube whichwasthen incubated at 100°C in water bath. Absorbance was read at 540nm and a calibration graph was plotted between absorbance and glucose concentration. The unknown sample was run similarly whose absorbance was plotted in calibration graph and its concentration was determined[16].

Single radial immunodiffusion[17]

1.0 ml of culture broth was taken and centrifuged at 4500 x g for 10 min at 4 °C. The supernatant was collected and the pellet was discarded. The supernatant was then assayed for Vi antigen content by single radial immunodiffusion (SRD). Radial Immunodiffusion (RID) Assay is a specialized form of immunodiffusion in which antibody is incorporated into molten agarose, which is poured into a petridish and allowed to solidify. Small wells are cut into the agarose gel and are filled with known concentrations of antigen, which corresponds to the antibody in the agarose.

Preparation of agarose gel

17 ml of 1.0% agarose (0.17 g) was prepared in 5X Assay buffer by heating slowly till agarose get dissolved completely. Froth formation was avoided in the solution. The molten agarose was allowed to cool to about 55°C and 2 ml of molten agarose solution was kept for sealing the wells. 450 µl of antiserum was added to 15 ml of agarose solution and it was mixed by gentle swirling for uniform distribution of antibody. Agarose solution was poured containing the antiserum onto a clean petridish and was allowed to solidify for 15-20 minutes. After solidification, the gel appeared slightly opaque. The wells were punched onto gel using gel puncture by maintaining a proper distance between each well. The wells were sealed with 20µl of molten agarose solution per well and ensured that the distribution was uniform. They were allowed to solidify for 15-20 minutes.

Loading of sample

Before loading the wells were labelled 1 and 2 on plate1 and 1, 2, 3 and 4 on plate 2 with the help of a marker. On plate 1, well 1 (30and well 2 (40of Vi polysaccharide was loaded. On plate 2, well 1 (20well 2 (well 3 (40 and well 4 (50 was loaded of standard sample. The plates were placed (do not invert) inside the moist chamber (box containing wet cotton) and incubated at 37°C for overnight.

The precipitate of cetyltrimethylammonium bromide and polysaccharide were partially purified and the material resulting from this preliminary purification was referred to as intermediate product. This product was stored at 20. The method of purification had been approved by the National control authority.500ml flask was taken which contained 100ml culture broth of *Salmonellaenterica typhimurium*. Heat treatment was given at 58 for 10 min in water bath for inactivation of cell. After cooling 1% cetyltrimethyl ammonium bromide was added to the culture broth and centrifugation was carried out at 10,000 x g for 20 min. The supernatant was removed and 2 mol/100ml calcium chloride was added to the sediment for the dissociation of detergent-polysaccharide complex. The suspension was stirred for 1h. In order to remove nucleic acid and protein, 25 ml of 95% absolute ethanol was added to the suspension. After 1 h of standing, the precipitates weresedimented by centrifugation at 16,000 x

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g for 20 min and perfectly cleared supernatant was retained. For the removal of complete nucleic acid and protein 95% ethanol was added to final concentration 75 ml/100ml. After agglomeration of precipitate, the precipitated polysaccharide was collected by centrifugation at approximate 3000 x g for 10 minute. The sediment was washed with 95% absolute ethanol for removal of calcium chloride and CTAB andthe final product was called intermediate product.

The intermediate product, which contained approximate 50% by weight of the desired polysaccharide was subjected to further purification. In this purification step homogenization of an aqeous solution of the intermediate product was done with a mixture of chloroform and n-Butanol(5:1). Centrifugation was carried out at 10,000 x g for 10 minute andthe clear aqueous supernatant was decanted and homogenization of this layer was repeated until a negligible interfacial precipitate forms in centrifugation. After final purification the material was referred to as purified polysaccharide & stored at -20°C.

Gel Filtration chomatography[18]

Preparation of the column

2 g of Sephadex G -200 was dissolved in 50mM of Buffer phosphate and its pH was maintained at 7.2 and kept it for overnight. Size of column was made 10cm X 3cm. The slurry was added to the column by mixing with glass rod by keeping the funnel at the top of the column. Matrix was uniformly added to the column and the equilibration of the column was done with the equilibration buffer.

Procedure

2ml of crude *S. typhimurium* culture was added to the column. Mobile phase was 50mM of Phosphate buffer pH7.2. Fractions of 3ml were collected with the help of gravity and OD was measured at 215 nm.

RESULTS

Growth curve for*salmonella enteric typhimurium*

Growth curve (Figure 1) shows thatas the fermentation time is increasing, the growth of microorganism is also increasing, thus OD. at 600 nm is continuously increasing but after a certain time (24 h),the growth is stop and stationary phase is attained (i. e. fermentation is stopped) which is measured by

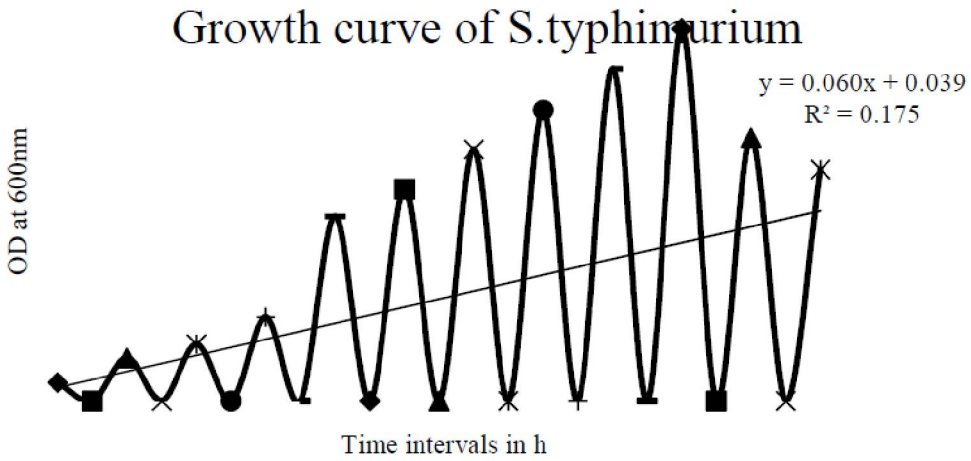

Figure 1: Growth curve of *Salmonella enteric typhimurium* MTCC 98

TABLE 1 : Optimization of tryptophan concentration

S.No.	Glucose (g/100ml)	Tryptophan (g/100ml)	Y.E (g/100ml)	Na_2HPO_4 (g/100ml)	OD_{600}
1	1.0	0.25	1.0	1.88	2.2
2	1.0	0.5	1.0	1.88	3.0
3	1.0	1.0	1.0	1.88	3.1
4	1.0	1.5	1.0	1.88	3.1
5	1.0	2.0	1.0	1.88	3.1

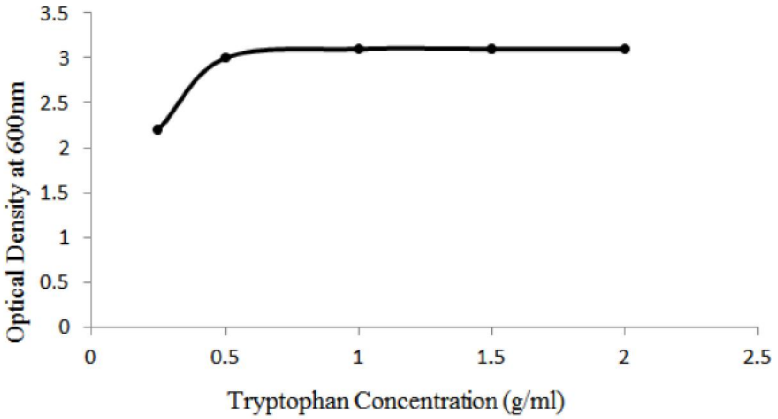

Figure 2 : Optimization of tryptophan conc.(0.25-2.0g/ml)

TABLE 2 : Optimization of glucose concentration

S.No.	Glucose (g/100ml)	Tryptophan concentration(g/100ml)	Yeast extract (g/100ml)	Na_2HPO_4 (g/100ml)	OD_{600}
1	1	0.1	1.0	1.88	3.0
2	2	0.2	1	1.88	2.2
3	3	0.3	1	1.88	2.4
4	4	0.4	1.0	1.88	1.48
5	5	0.5	1.0	1.88	0.6

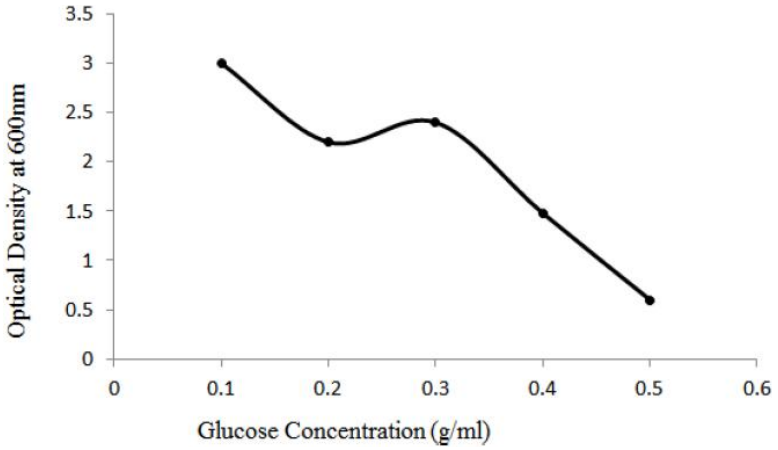

Figure 3 : Curve showing optimization of glucose conc.(1-5%)

TABLE 3 : Optimization of temperature from 25-45^{0}C

S.No.	Temperature(^{0}C)	OD.at 600 nm
1	25	0.813
2	30	1.871
3	35	3.012
4	40	2.10
5	45	1.80

the OD and the highest OD shows Vi production.

Optimization of tryptophan concentration

There was no further change in OD even when the concentration of Tryptophan was increased after 0.5 (TABLE 1, Figure 2). So the concentration of Tryptophan was fixed to 0.5g/100ml for next optimization of Glucose concentration.

Optimization of glucose concentration

In sample 1, OD was highest which was 1.0g/100ml of Glucose which showed better growth of microorganisms (TABLE 2).

Optimization of temperature

As shown in TABLE 3, at 35^{0}C, OD was found to be highest i.e. 3.012, thus better growth of microorganisms.

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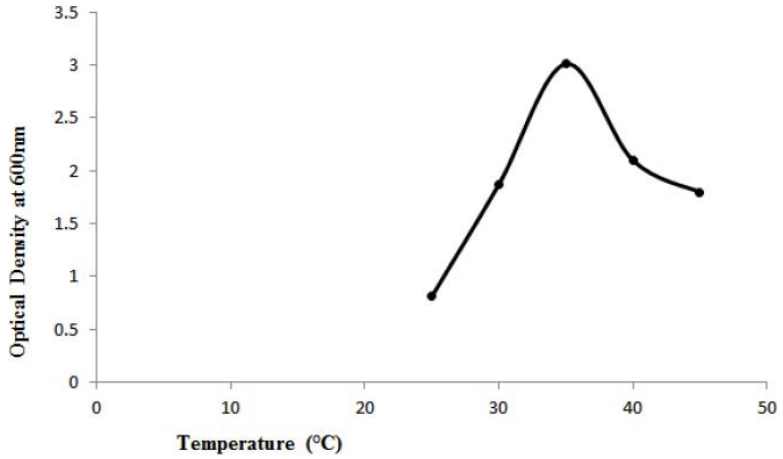

Figure 4 : Curve showing optimization of temperature

TABLE 4 : Standard glucose concentrationvs OD at 540nm by DNS method

S.No.	Glucose(ml)	Glucose (µg/ml)	D.W(ml)	DNS(ml)		OD AT 540nm
1	0	0	10	30		0.00
2	2	400	8	30		1.82
3	4	800	6	30		2.23
4	6	1200	4	30	Incubation at 100ºC for 10 min	2.50
5	8	1600	3	30		2.84
6	10	2000	0	30		3.03

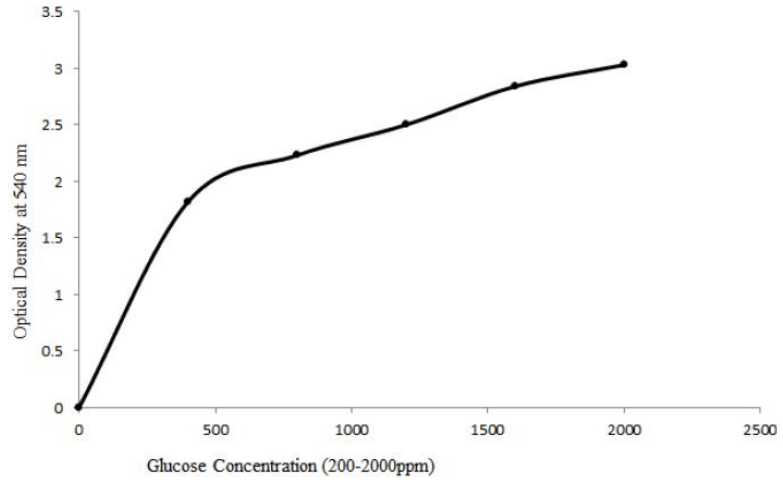

Figure 5 : Calibration graph between OD at 540nm and concentration of glucose

TABLE 5 : Fermentation media glucose conc at (0h-12h-24 h) vs OD at 540nmby DNS method

S.No.	Glucose(ml) 2ml of fermentation	D.W(ml)	DNS(ml)		OD at 540nm
1	0h	8	30		3.0
2	12 h	8	30	IncubationAt 100 ^{0}C for 10 minute	2.3
3	24 h	8	30		1.9

The concentration of Glucose in fermentation media was 2000ppm at the start of fermentation, after 12 h it was 830ppm and thereafter 24 h it dropped to 450 ppm. It showed that as the concentration of Glucose was depleting,Vi production was increasing thus, to quantify Vi concentration, SRD Assay

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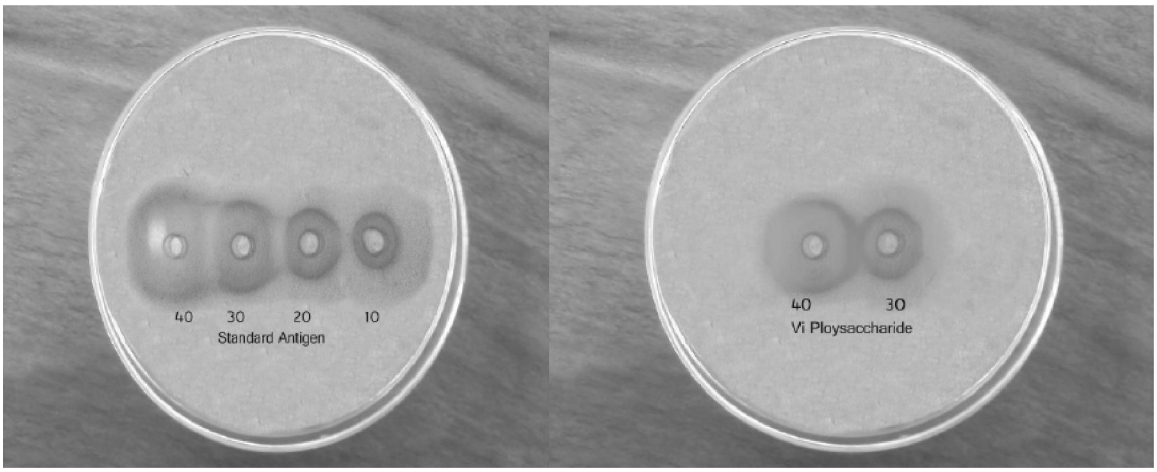

Figure 6 : SRD precipitation rings, (immuno-diffusion) between a) antibody and standard sample b) antibody and Vi

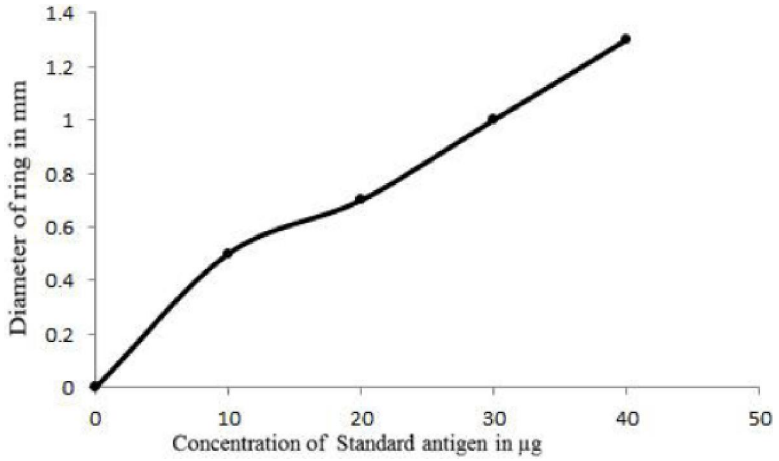

Figure 7 : Graph between diameter on Y- axis and concentration on Y axis

was performed.

Quantification of Vi concentration

A single radial immunodiffusion (SRD) assay was developed to measure Vi antigen content in liquids containing a complex mixture of nutrients, buffering components, and metabolites. The assay is an immunoassay so is specific for Vi antigen and therefore other components in the fermentation culture broth do not interfere with Quantification of the Vi. The precipitation rings werevisible after 24 hours (Figure 6). It was sure that Vi antigen was present in solution.

The diameter (though the centers of the wells) of the precipitation ring was measured in millimeters and plotted a graph of diameter of ring (on Y-axis) versus concentration of antigen (on X-axis) as shown in Figure 7 and the value of the unknown antigen concentration was calculated from the graph (TABLE 10). Diameter of test antigen was found to be 0.6mm and 0.9mm and these values when plotted in graph, the concentration of test antigen was found to be 12 µg in 0.6mm diameter ring and 27 µg in 0.9mm ring.

Optimization of maximum wavelength

As the concentration of Vi is proportional to OD at 215nm so highest OD shows highest Vi concentration (TABLE 7).

Purification of Vi polysaccharide

For the determination of purity of Vi polysaccharide OD at 215 nm was takenthice and the obtained values for crude broth, partially purified Vi polysaccharide, purified Vi polysaccharide were 0.853, 2.190 and 3.104 respectively. Thus, the highest OD in purifiedVi polysaccharide showed high

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TABLE 6 : Measured results of diameter

Concentration of standard antigen(µg)	Diameter of precipitate ring(mm) STANDARD	TEST
0	0	0
10	0.5	0
20	0.7	0.6
30	1.0	0.9
40	1.3	0

TABLE 7 : Optimized wavelength of supernatant

Wavelength	OD
200	1.941
205	1.249
210	2.546
215	3.151
220	1.543
225	0.982
230	0.584

Vi concentration.

Gel filtration chomatography

This method was used for purification of Vi polysaccharide. Different fractions of 2 ml were collected and the OD was measured at 215 nm (TABLE 8). Fraction 5-7 showed highest OD at 215 nm, thus containing higher concentration of Vi polysaccharide.

% Recovery = 8.9/12.74*100=74.1%.

CONCLUSION

Vi capsular polysaccharide is the major component of Vi polysaccharide typhoid vaccines. A purification method was developed to separate the impurities from Vi along with the optimization of glucose, tryptophan concentration and temperature optimization. Thus, it can be concluded that Vi antigen can be purified by Sephadex G200 at very low cost as it is an important vaccine for the cure of typhoid fever and mass scale production of typhoid vaccine can be done.

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TABLE 8 : OD of fractions of gel filtration chomatography at 215nm

Fractions	OD at 215 nm
1	N.D
2	N.D
3	N.D
4	N.D
5	2.951
6	3.089
7	2.876
8	N.D
9	N.D
10	N.D

ABBREVIATIONS

LPS- Lipopolysaccharide
CP- Capsular Polysaccharide
OD- Optical Density
DNS- Dinitrosalycilic acid
SRD- Single Radial Immunodiffusion
RD- Radial Immunodiffusion

REFERENCES

[1] M.I.Khan, R.I.Ochiai, J.D.Clemens; Population impact of Vi capsular polysaccharide vaccine, Vaccine, **9(5)**, 485-96 **(2010)**.
[2] R.Edelman, M.M.Levine; Summary of an international workshop on typhoid fever, Review of Infectious Disease, **8**, 329-449 **(1986)**.
[3] B.Cvjetanovic, D.Ikic, W.R.Lane, T.Manhalter, S.Tapa; Study of combined quadruple vaccine against diphtheria, Pertosis, Tetanus and Typhoid fever: reactogenecity and antigenicity, Bulletin of the World Health Organization, **2**, 43-49 **(1972)**.
[4] E.A.Engels, M.E.Falagas, J.Lau, M.L.Bennish; Typhoid fever vaccines: a meta-analysis of studies on efficacy and toxicity, BMJ, **316**, 110–116 **(1998)**.
[5] A.Fraser, M.Paul, E.Goldberg, C.J.Acosta, L.LeiboVici; Typhoid fever vaccines: Systematic review and meta-analysis of randomized controlled trials, Vaccine, **25**, 7848–7857 **(2007)**.
[6] C.A.Guzman, S.Borsutzky, Griot M.Wenk,

I.C.Metcalfe, J.Pearman, A.Collioud, D.Favre, G.Dietrich; Vaccines against typhoid fever, Vaccine, **24**, 3804–3811 **(2006)**.

[7] R.Germanier, E.Furer; Isolation and characterization of Gal-e mutant ty 21a of salmonella-typhi - candidate strain for a live, Oral Typhoid Vaccine, J.Infect.Dis., **131**, 553–558 **(1975)**.

[8] M.Raffatellu, R.P.Wilson, S.E.Winter, A.J.Baumler; Clinical pathogenesis of typhoid fever, J Infect DevCtries, **2**, 260–266 **(2008)**.

[9] J.F.Breen, M.A.Apicella; Immunogenicity of gonococcal Gc2 polysaccharide: Comparative studies with pneumococcal type III polysaccharide and Salmonella typhosa Vi antigen, Infect Immun, **22**, 195–199 **(1978)**.

[10] J.P.Lake, N.D.Reed, J.T.Ulrich, V.A.Varitek; Development of a localized hemolysis-in-gel assay for Vi antigen: Characterization of the Vi-specific PFC response of nude and normal mice, Immunol Commun, **6**, 149–165 **(1977)**.

[11] L.Jennifer, L.Adriana, A.Robert, R.H.Jessica, A.Ewan, L.Constantino, L.Jeremy, B.Laura, T.KaiMichael, A.Calman, C.Ian, R.Ian, D.Gordon, F.Adam; The capsular polysaccharide vi from salmonella typhi Is a B1b Antigen, Journal of Immunology, **189(12)**, 5527-5532 **(2012)**.

[12] T.Wangdi, C.Y.Lee, A.Spees, C.Yu, D.Kingsbury, S.Winter, C.Hastey, R.Wilson, V.Heinrich, A.Baumler; The Vi capsular polysaccharide enables salmonella enterica serovertyphi to evade microbe guided neutrophil chemotaxis, PLoSPathog, **10(8)**, 306-315 **(2014)**.

[13] S.Rondini, F.Micoli, L.Lanzilao, C.Hale, J.A.Saul, B.L.Martin; Evolution of the immunogenecity and biological activity of the citrobacterfreundiivi-CRM197 conjugate as a vaccine for salmonella enteric serovartyphi, Clinical and Vaccine Immunology, **3**: 460-468 **(2011)**.

[14] F.Rondini S.Pisoni I.Berti F.Costantino P.Sau A.Martin LB; Vi-CRM187 as a new conjugate vaccine against Salmonellatyphi, Vaccine. **22**, 712-720 **(2011)**.

[15] WHO technical report series no.840, Requirement for Vi polysaccharide typhoid vaccine WHO, Genewa, Switzerland, **8**, 14-32 **(2011)**.

[16] G.L.Miller; Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal Chem., **31**, 426-428 **(1959)**.

[17] J.M.Wood, G.C.Schild, R.W.Newman, V.Seagroatt; An improved single radial immunodiffusion technique for the assay of influenza haemagglutinin antigen: application for potency determination of inactivated whole Virus and subunit vaccine, Journal of Biological Standardization, **5**, 237-247 **(1977)**.

[18] L.Hagel; Gel filteration chomatography, Curr.Protoc.Mol.Biol., **10(9)**, 1-6 **(2001)**.