Vi capsular Polysaccharide (Typhoid Vaccine) Production from Salmonella typhi In Shake Flask and Bioreactor Fermentation process

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Abstract

Typhoid Vaccine contains Vi capsular polysaccharide, which is obtained from the salmonella typhi organism during the fermentation. Vi capsular polysaccharide production was depended on the cell growth and cell mass, greater the cell growth and cell mass higher the production of Vi capsular polysaccharide in media. The Vi capsular polysaccharide is a liner homopolymer of 1-4, 2-Dexoy -2-N-acetly galacturoinc acid. The Vi capsular polysaccharide was obtained in fed batch culture is more than 2 times of the batch fermentation with different media compositions. The Vi capsular polysaccharide is associated in from of capsule of organism. When the cell pellet of feed batch culture was processed for obtaining the Vi polysaccharide is 3 times greater than the batch fermentation. The production of the Vi polysaccharide is completely based on the media components such as SCDM, Glucose, yeast extract and magnesium sulphate and fermentation parameters and conditions. The presence of High sugar and magnesium sulphate concentration in media, the Vi polysaccharide production was inhibited. The Vi polysaccharide content was calculated from the o-acetyl estimation (as per British Pharmacopeia).

Key words: Vi Polysaccharide, SCDM, Glucose, Yeast extract and Magnesium Sulphate.

I. INTRODUCTION

Typhoid fever is a infection caused by the bacterium salmonella typhi: Vertically all strains isolated from the blood or bone morrow sample from the patients with culture typhoid fever when tested in the laboratory where found express Vi polysaccharide (Jesudasoneatl.1994; Lesmana et al., 1980). The Vi capsular polysaccharide is a liner homopolymer of α 1-4, 2 deoxy-2-N-acetyl galacuronic acid (Heyns and kissing, 1967) and it biosynthesis is reviewed in detail Virlogeux-payant and popoff, 1996. The vi capsular polysaccharide is a virulence factor and the Vi antigen has been shown to be major protective antigen against typhoid disease (Robbins and Robbins.1984) today most of the burden of typhoid disease occurs in developing countries (Crump et al.2004) particularly were sanitary conditions and poor clean drinking water is not readily available.

In the relation to the more impoverished communities improving sanitary conditions is a distant goal so the most cost-effective short-term disease against typhoid fever remains vaccination of susceptible population. The emergence of antibiotic resistant strains of salmonella typhi in recent times wain and kidgell (2004) has intensified the problems posed by treating typhoid fever cases there by elevating the importance of Vaccination. Several typhoid vaccine are licensed for the use and include Vi polysaccharide vaccine and Ty21a live oral vaccine. Both of these vaccines has good safety profile and acceptable efficiency has been demonstrated in communities where the disease was endemic. The impoverished populations living at risk of the disease remains largely unvaccinated (Acosta et al.2004).

The Vi vaccine is well suited for public health programs in countries where typhoid is endemic as it only requires one dose and it is temperature stable. This vaccine is now licensed in more than 92 countries (WHO document). The ability to

pay for the vaccine remains a major factor in the explanation of who is and who is not vaccinated so maintaining a low affordable price For the vaccine increase the likelihood of it being used in public health programs in endemic setting. One way to facilitate the supply of affordable Vi vaccine is to assist manufacturing to optimize the production of Vi during fermentation and to maximize the yield of antigen. The Vi polysaccharide content was calculated from the o-acetyl estimation (as per British pharmacopeia).

Study of Vi capsular polysaccharide production by other pathogenic bacteria such as Haemophilis influenza Type B, and Neisseria meningitides showed that production was depends up on the fermentation condition and the media components and that optimal conditions differed for each bacterium. Baroque-Ramos et al., 2005 showed that higher yields of capsular polysaccharide were obtained when N. meningitides (Serogroup C) was cultured in media when the glucose concentration was maintained below 1.0gram/L and that low oxygen tention favoured higher polysaccharide production. Takagi et al. showed that polyribosylribitol phosphate (PRP) Capsular polysaccharide production by H influenza Type B could be incensed by increasing the concentration of growth factor and controlling the pH at 7.2. Anderson et al (1976) showed that increasing H. influenza cell growth increased PRP synthesis on sold agar and liquid culture, Merritt et al., (200) extended this to culture in a bioreactor and showed the fed batch culture at 500L manufacturing scale increased the cell density and yield of PRP approximately four fold when compared to batch culture. Zhan et al (2002) Similarly Showed that pH-control and changing to fed batch fermentation increased the yield of cell and the fermentation increased the yield of cells and the production of polysialic acid by E. coli.

II. MATERIALS AND METHODS

A. Pre seed and seed media preparation

Bacterial strain and media used for cultivation and Salmonella typhi strain was used in all the experiments described in this report. The initial culture was grown on solid TSI agar media. An optimized media composition for growth salmonella typhi or for the production of Vi polysaccharide, the base media composition selected for a shake flask and bioreactor culture contained Soyabean Casein Digest Medium (Himedia)- 30gram/L. The Soyabean Casein Digest Medium was used for the pre seed media and seed activities and it supported the growth of bacterial cells. The Soyabean Casein Digest Medium was suspending 30.0 grams in 1000 ml purified water. Mix well, after dissolution of the medium completely dispense in flasks and sterilized by autoclaving at 15 lbs pressure (121°C) for 20 minutes.

B. Fermentation media preparation

An optimized media composition for growth salmonella typhi or for the production of Vi polysaccharide, the base media composition selected for bioreactor contained 30gram/L Soyabean Casein Digest Medium (Himedia), 2.5 gram/mL MgSO4. 7H20 (Qualigens) and 5 gram/L Yeast Extract (BD chemicals).The trace elements (Cobaltous Chloride-0.043% w/v, Manganese Chloride-0.26% w/v, Copper Sulphate-0.038% w/v, Boric Acid-0.053% w/v, Sodium Molybdate-0.037% w/v, Zinc Acetate-0.26% w/v) were added to the completed media preparation. Weighed the required quantity raw materials and transfer to the bottle and dissolved it completely and check the pH of the media. Make up the final volume with WFI. After complete dissolution withdraw 5 ml solution to check the initial pH. Adjust the pH to 7.2 ± 0.1 of the solution with 20% NaOH / 2% HCl. Make up the final volume with purified water.

Feed solution preparation weigh the required quantity of D-Glucose, Yeast extract and SCD M and transfer to the bottle slowly under constant stirring. Make up the final volume with purified water. Stir the solution till complete dissolution.

C. Analysis

Vi polysaccharide estimation

Fermentation culture broth 10mL was collected aseptically and centrifuging at 6000 RPM for 10 minutes at 2-8 °C. The pellet was discarded and supernatant used for the Vi polysaccharide estimation. O-acetyl group’s assay (as per B.P) was

used to measure Vi antigen content in liquid containing a complex mixture of media components. The assay is specification for Vi antigen and therefore other components in the fermentation culture broth so not interfere with quantification of the Vi polysaccharide.

Prepared 150 mg of acetyl choline in 10mL of water and stored at 2-8°C and 200µL from the stock and added 19.8mL water. It is equal to 3 mg of acetyl choline in 20ML of working stock. Also prepared 1.2 HCL and 20% ferric chloride. Prepared alkaline Hydroxylamine HCL fresh before use. The concentration of Acetylcholine were 15µg,30µg, 75µg. 150µg, 225µg.

O- Acetyl content in µmoles/ml = Sample O.D X µmoles of O-acetyl in ACC

Standard OD Volume of Sample

Vi content in mg/mL = µmoles of O-acetyl per mL X 25 X Dilution

0.085 100

The bacterial cell was added 200mL of purified water and heated at 70°Cfor 30 min by using water bath, then centrifuged at 750 rpm for 30 min. at 4°C the pellet was discarded and the supernatant measured for Vi antigen content by O-acetyl estimation. The OD_{590} was showed the same before and after heating it indicated that the cells were not lysed during heating.

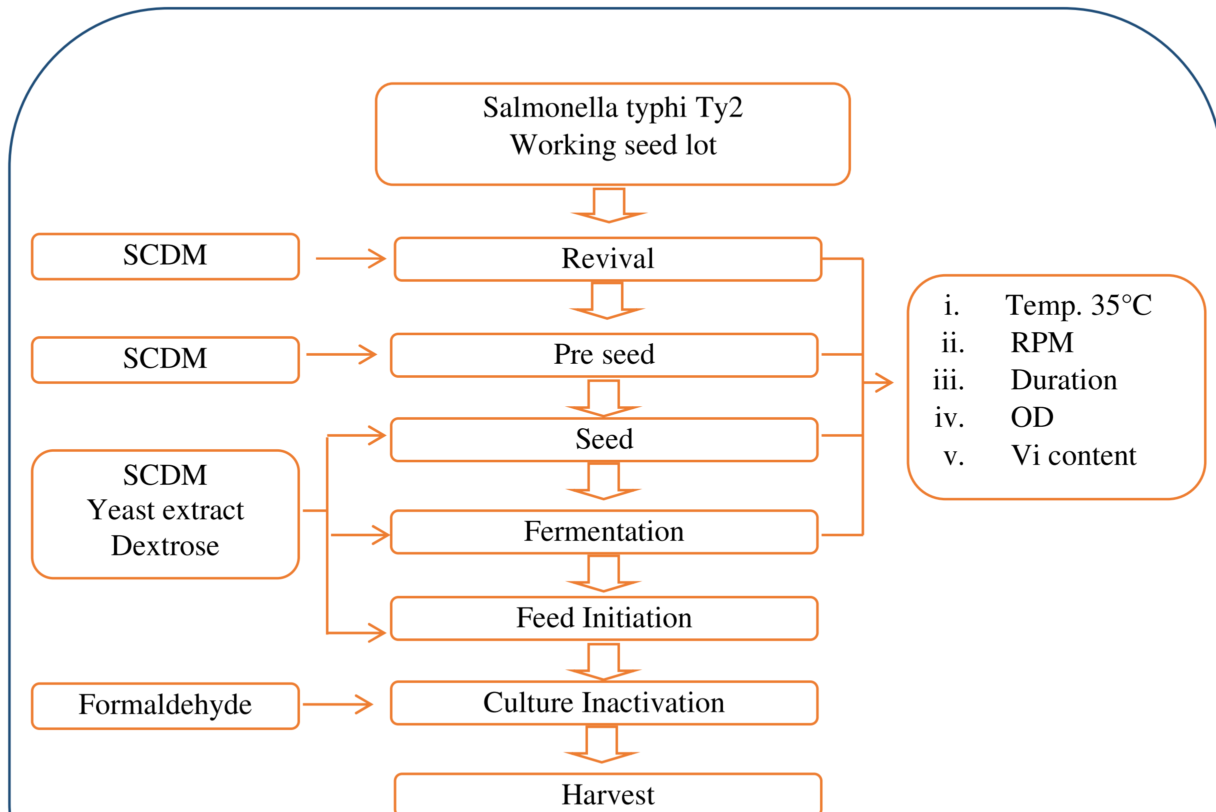
D. Culture OD estimation

During the fermentation 5 mL of culture broth was collected and used for the measuring of optical density (OD).The optical density (OD590) of the culture was measured at 590 nm using UV-Visible Spectrophotometer (Shimadzu).

E. Glucose estimation

Glucose concentration determined by UV-Visible Spectrophotometer (Shimadzu) method. Fermentation culture broth 10 mL was collected aseptically and centrifuging at 6000 RPM for 10 minutes at 2-8 °C. The pellet was discarded and supernatant used for the glucose estimation.

Flow chart:



III. RESULTS

A. Step-01: Revival

The working cell culture was reconstituted with 1mL of Soyabean casein digest medium. The reconstituted culture inoculated in 50 mL Soyabean casein digest medium. The inoculated flask labelled as “Revived culture”, Incubated the flask at 35°C for 5 hours in a shaker incubator. The OD_{590} of both the revived flask were analyzed at 2 hours interval from 0-hour onwards to till end of the incubation period. At the end of incubation, the culture was streaked on SCD agar plates for the purity and microscopic examination was performed. The results were captured in below table and illustrated in figure.

B. Step-02: Pre seed

At the end of revival incubation, the 10 mL cell culture was inoculatedinto100 mL of Soyabean casein digest medium. The inoculated flask labelled as “Pre seed culture”, Incubated the flask at 35°C for 8 hours in a shaker incubator. The OD_{590} of both the Pre seed flask were analyzed at 2 hours interval from 0-hour onwards to till end of the incubation period. At the end of incubation, the culture was streaked on SCD agar plates for the purity and microscopic examination was performed. The results were captured in below table and illustrated in figure.

C. Step-03: Seed

At the end of pre seed incubation, the 25 mL cell culture was inoculated into 500 mL of Soyabean casein digest medium. The inoculated flask labelled as “Seed culture”, Incubated the flask at 35°C for 12 hours in a shaker incubator. The OD_{590} of both the Pre seed flask were analyzed at 2 hours interval from 0-hour onwards to till end of the incubation period. At the end of incubation, the culture was streaked on SCD agar plates for the purity and microscopic examination was performed. The results were captured in below table and illustrated in figure.

D. Step-04: Fermentation

Transfer the seed flask to the BSC. In the BSC, pour the entire culture broth of the flask to a Seed transfer bottle with assembly. Bring the assembly to the fermentation area and transfer the inoculums to the fermenter aseptically. The 250 mL cell culture was inoculated into 5000 mL of Soyabean casein digest medium. During the fermenter culture broth were analyzedOD_{590}at 1 hours interval from 0-hour onwards to till end of the harvest. At the end of fermentation, the culture was streaked on SCD agar plates for the purity and microscopic examination was performed. The results were captured in below table and illustrated in figure.

Maintain the fermentation batch stirrer at 500 RPM, temp at 35±1°C and air flow at 1-4 LPM. Observe the dissolved oxygen level in media regularly after increase in OD, the DO comes almost to zero and immediately shoots up to more than 80. This is the indication of fed batch to initiate. Once DO start increasing, pH will also increase to 7.2. Connect the feed peristaltic pump by setting the dosing pump to control the pH and O2 level 20 to 40 %.

Note: Care must be taken during fed batch addition of over dose of feed will results lowering the DO level, lesser the feed will leads to increase pH levels control the pH and PO2 levels by adding feed to the culture. Observe and control the feed over 24±1 hours. Note down the Vi polysaccharide content in culture at ‘10’ hours after that every 2 hours and recorded the Vi polysaccharide content in associated documents.

Stop the air and agitation gradually when the three OD values reaches consistent or reduced OD, slowly reduce the RPM of stirrer and close the air inlet. Bring down the temperature to 10°C to 15°C by passing chilled water to the jacket, add 400 ml of formalin to the culture and stir it for 10 minutes and kept hold for 2 hours.

IV. DISCUSSION

A. Preparation of pre seed, seed and Fermentation culture:

Culture agglutination

At the end of pre seed, seed and fermentation culture broth was taken on the smear and added few drops of Vi antisera for identification of Salmonella typhi organism. An agglutination was observed visually on the smear.

Purity of the Culture

The culture was streaked on soyabean casein digest agar plates for the purity and gram staining for microscopic examination. During the entire study culture broth was observed the pure, Gram negative, coco-bacilli.

Pre seed and Seed discussion

Salmonella typhi organism was inoculated in conical flasks containing SCDM medium and kept the flask in shake incubator. After incubation period, checked the culture from agglutination with Vi Antisera, Gram staining, purity and motility. The results of these experiments are given in table.

Pre seed and Seed culture details

Experi-ments	Pre seed				Seed					
	Temp. (^{0}C)	RPM	Duration (h)	OD_{590}	Temp. (^{0}C)	RPM	Duration (h)	Seed OD	Initial pH	Final pH
Exp-01	35	150	8	1.05	32	150	12	1.85	7.2	6.51
Exp-02	35	150	8	1.12	32	150	12	1.84	7.2	6.86
Exp-03	35	150	8	1.03	32	150	12	1.72	7.2	6.91
Exp-04	35	150	8	0.95	32	150	12	1.76	7.2	6.95
Exp-05	35	150	8	1.21	32	150	12	1.73	7.2	6.87
Exp-06	35	150	8	1.08	32	150	12	1.92	7.2	6.84
Exp-07	35	150	8	1.12	32	150	12	1.92	7.2	6.82
Exp-08	35	150	8	1.11	32	150	12	2.0	7.2	6.88
Exp-09	35	150	8	0.89	32	150	12	1.89	7.2	6.78
Exp-10	35	150	8	1.21	35	200	8	2.13	7.2	6.82
Exp-11	35	150	8	0.98	35	200	8	2.21	7.2	6.97
Exp-12	35	150	8	1.11	35	200	8	2.02	7.2	6.85
Exp-13	35	150	8	1.09	35	200	8	1.92	7.2	6.68
Exp-14	35	150	8	1.06	35	200	8	2.16	7.2	6.85
Exp-15	35	150	8	1.21	35	200	8	2.34	7.2	6.91
Exp-16	35	150	8	1.02	35	200	8	2.12	7.2	6.96
Exp-17	35	150	8	1.12	35	200	8	2.23	7.2	6.94
Exp-18	35	150	8	1.09	35	200	8	2.28	7.2	6.89

The experimental batches which were performed with 8 hours and 12 hour pre seed and seed culture data the DO_{590} values observed minimum was 0.89 and maximum was 1.21 in pre seed culture and minimum was 1.72 and maximum was 2.34. For seed experiments were run at different temperature and incubation duration condition and results were detailed in above table.

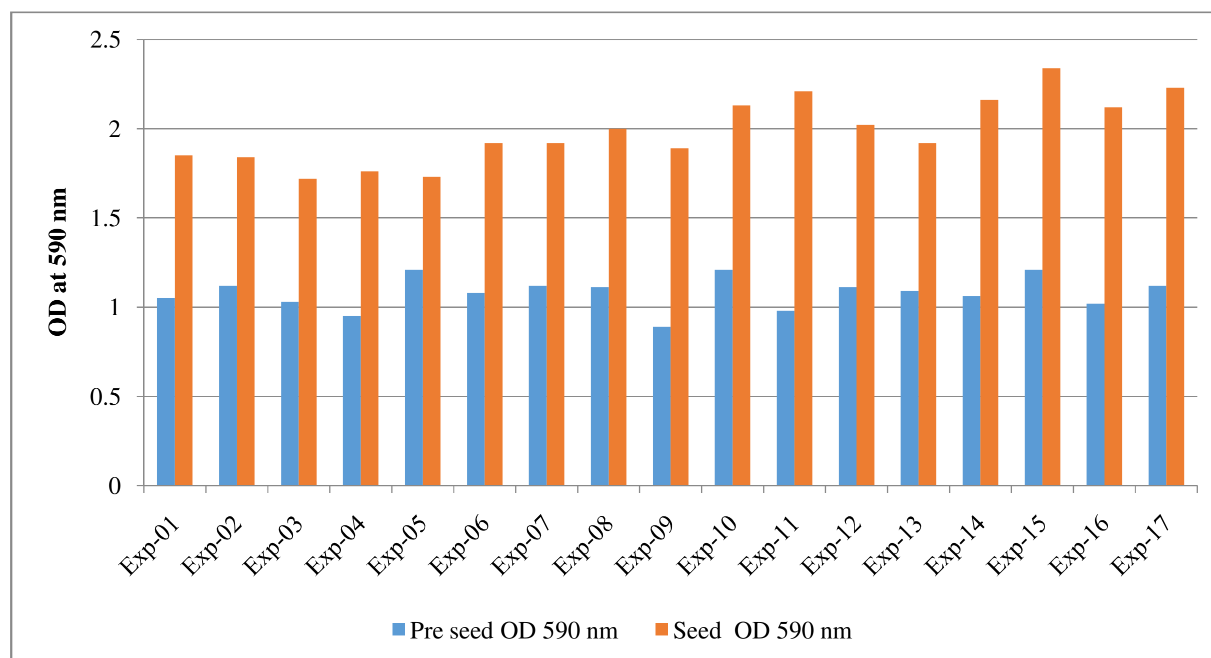


Illustration of Pre seed and Seed OD_{590} details

B. Fermentation discussion

At the end of seed incubation period checked the culture for agglutination with Vi Antisera, Gram Staining and motility. Transferred 250 mL of inoculum of cultures was aseptically added to a 10 L fermenter containing 5 L of sterilized media. The pH was controlled at 7.2 by automatic addition of NaOH 20% (w/v). The temperature was maintained at 35°C. The dissolved oxygen (DO) was controlled at 20-40% air saturation, DO was achieved by automatic variation of the agitation frequency and was controlled in the range 100-500 RPM.

Note: Care must be taken during fed batch addition of over dose of feed will results lowering the DO level, lesser the feed will leads to increase pH levels control the pH and PO2 levels by adding feed to the culture. Observe and control the feed over 24±1 hours. Note down the Vi polysaccharide content in culture at '10th ' hour and afterwards every 2 hours interval and recorded the Vi polysaccharide content in associated documents. The Vi polysaccharide content, OD_{590}nm, fermentation condition were detailed in below table.

The Vi polysaccharide content, OD_{590}nm, fermentation condition

Experiments	Culture age (h)	Feed initiation time (h)	Harvest OD_{590}	Vi polysaccharide (mg/mL)
Exp-01A	24	0.0	14.5	1.21
Exp-02B	24	0.0	15.9	1.01

The Vi polysaccharide content, OD590nm, fermentation condition

Experiments	Culture age (h)	Feed initiation time (h)	Harvest OD_{590}	Vi polysaccharide (mg/mL)
Exp-01	24	10	31.00	2.21
Exp-02	24	10	30.00	2.15
Exp-03	24	10	32.50	2.01
Exp-04	24	10	30.00	1.93
Exp-05	24	10	31.00	2.22
Exp-06	24	10	30.00	1.91
Exp-07	24	10	31.24	2.24
Exp-08	24	10	30.00	2.23
Exp-09	24	10	30.00	2.15
Exp-10	24	5.0	35.80	2.56
Exp-11	24	5.0	36.70	2.74
Exp-12	24	5.0	34.00	2.81
Exp-13	24	5.0	34.00	2.52
Exp-14	24	5.0	34.20	2.91
Exp-15	24	5.0	37.80	2.64
Exp-16	24	5.0	34.00	2.81
Exp-17	24	5.0	35.50	3.01
Exp-18	24	5.0	34.80	2.75

The Vi capsular polysaccharide content observed less in fed batch initiated fromat10^{th} hour onwards from Exp-01 to Exp-09 and fermentation with different media compositions. During the analysis OD at 590 nm and Vi polysaccharide observed less in fermentation culture broth.

The Vi capsular polysaccharide content observed more in fed batch initiated fromat 5th hour onwards from Exp-10 to Exp-18 and fermentation with different media compositions. During the analysis OD at 590 nm and Vi polysaccharide observed more in fermentation culture broth. Results were detailed in Table-03 and figure- 01 and figure-02.

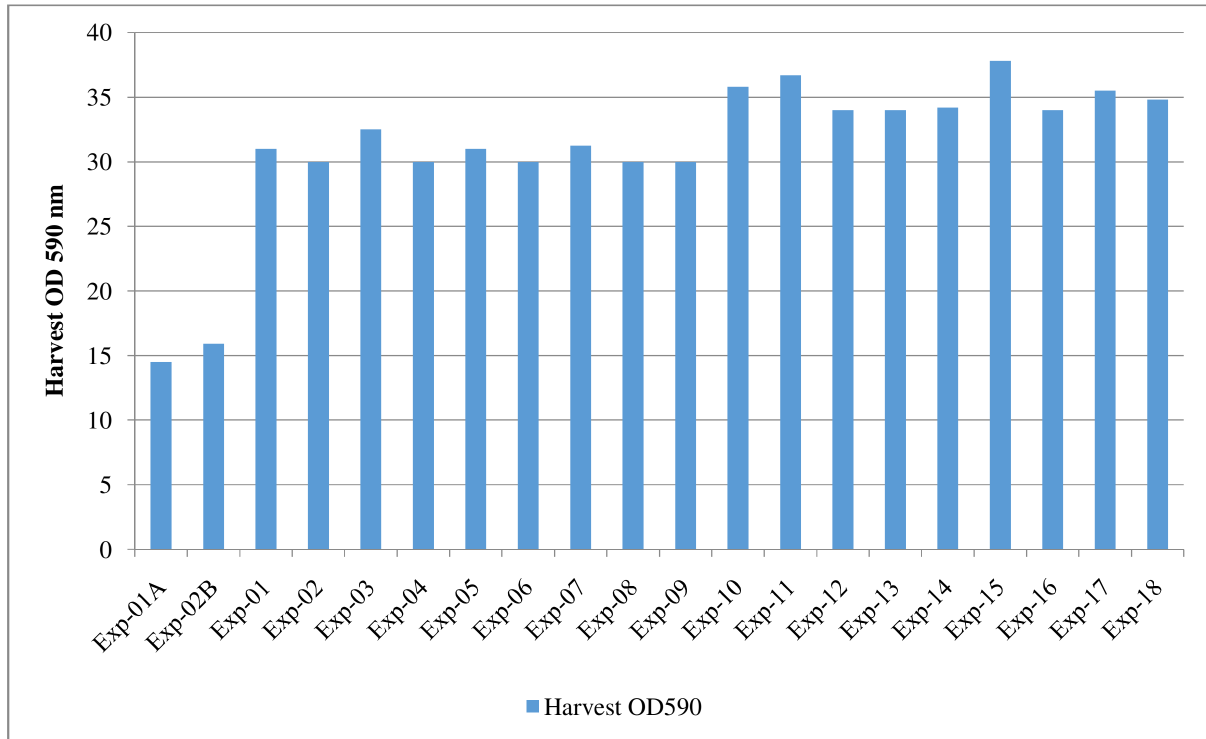


Illustration of OD_{590} fermentation harvest

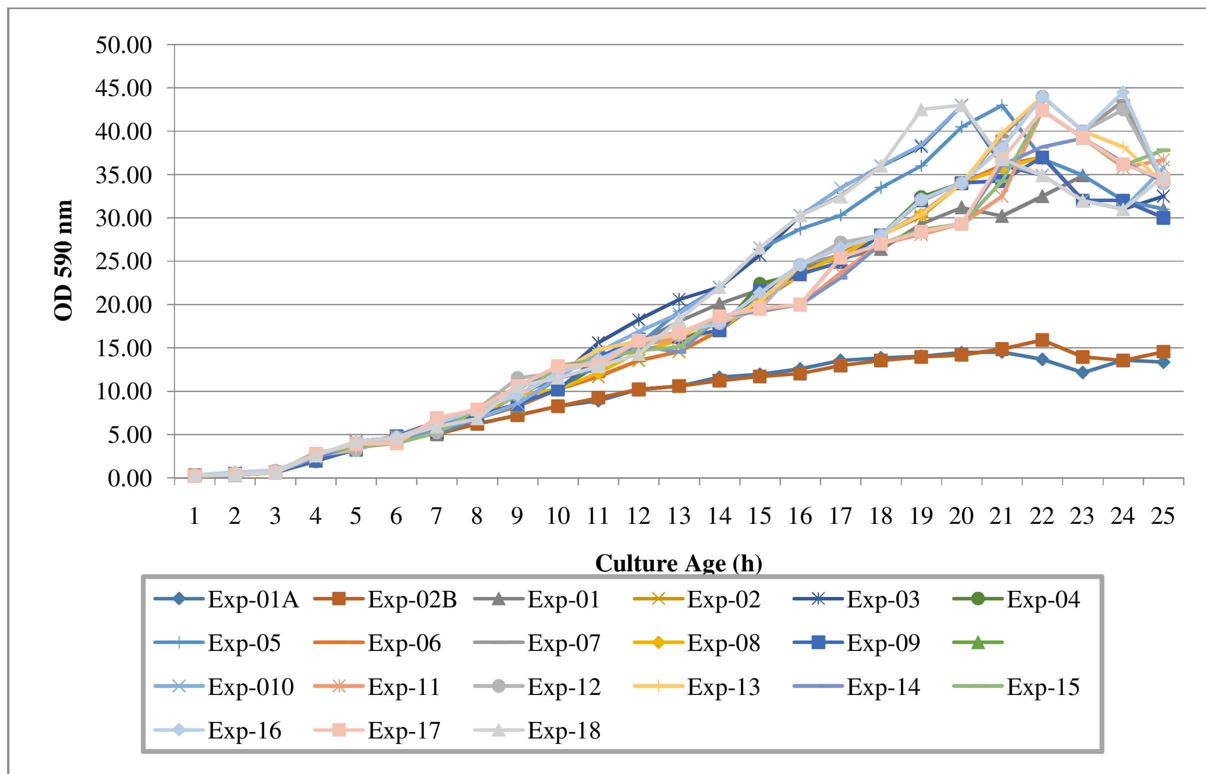


Illustration of OD_{590} during fermentation

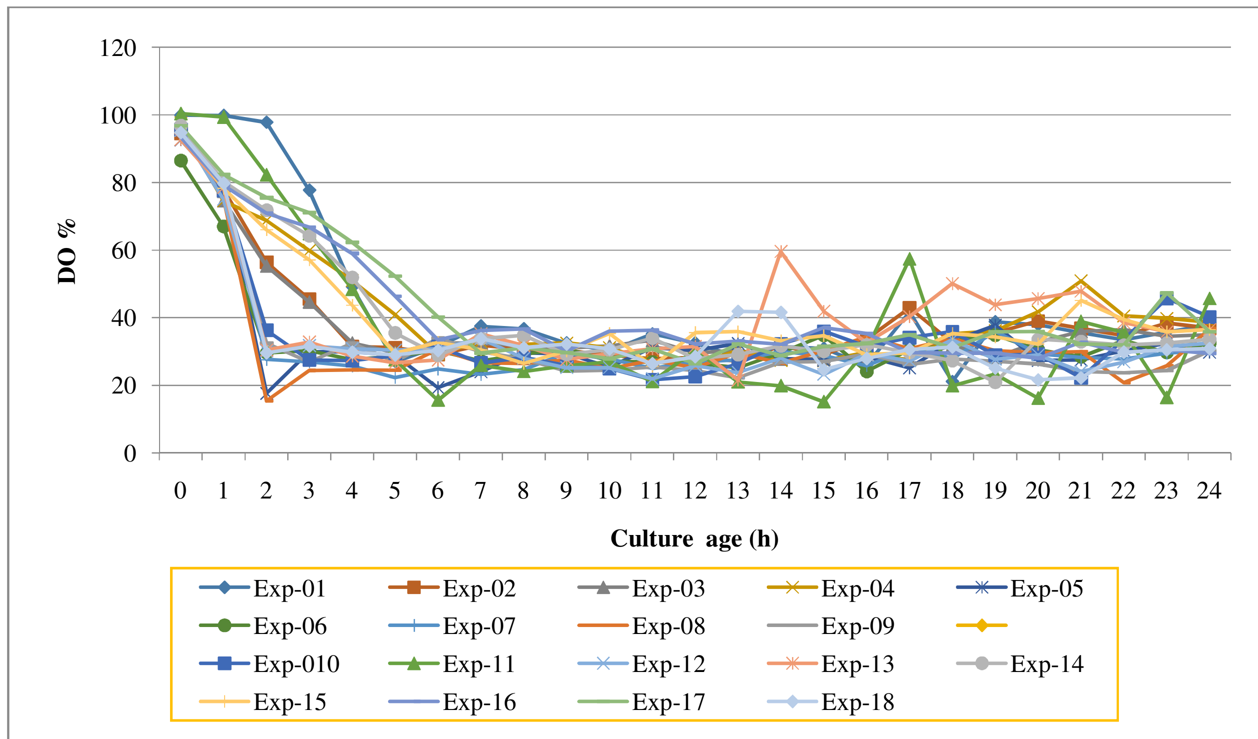


Illustration of DO during fermentation

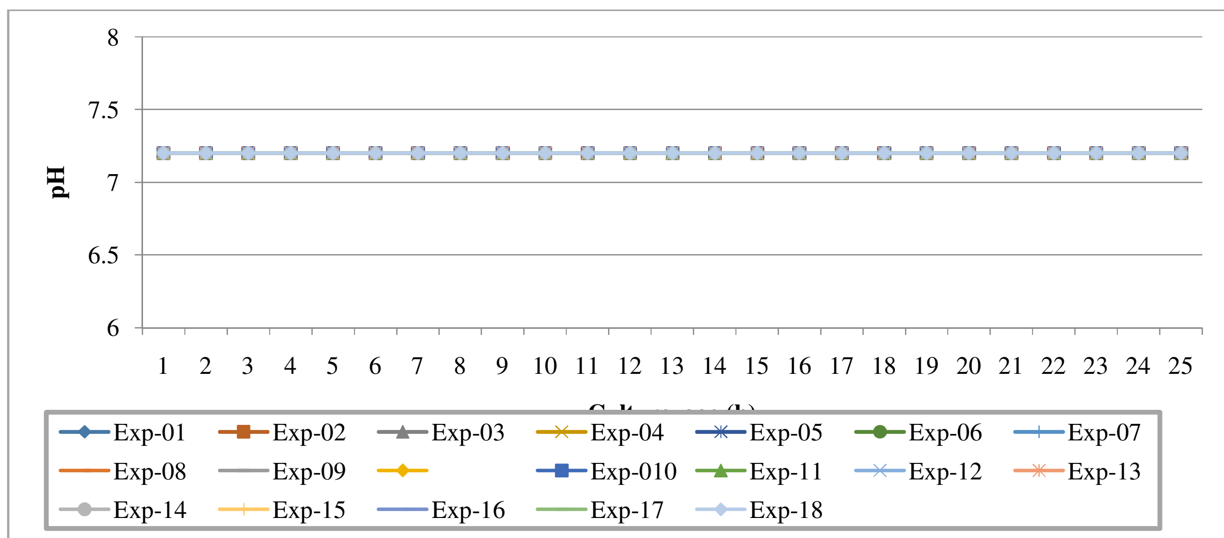


Illustration of pH during fermentation

C. Production of Vi polysaccharide content

Fermentation culture broth 10 mL was collected aseptically and centrifuging at 6000 RPM for 10 minutes at 2-8 °C. The pellet was discarded and supernatant used for the Vi polysaccharide estimation. O-acetyl group's assay (as per B.P) was used to measure Vi antigen content in liquid containing a complex mixture of media components. The assay is specification for Vi antigen and therefore other components in the fermentation culture broth so not interfere with quantification of the Vi polysaccharide. The Vi capsular polysaccharide was obtained in fed batch culture is more than 2 times of the batch fermentation with different media compositions.

The bacterial cell was added 200mL of purified water and heated at 70°Cfor 30 min by using water bath, then centrifuged at 750 RPM for 30 min. at 4°C the pellet was discarded and the supernatant measured for Vi antigen content by O-acetyl estimation. The OD_{590} was showed the same before and after heating it indicated that the cells were not lysed during heating. During the fermentation every 2 hours sample was collected and estimated the Vi polysaccharide content and details are recorded in below table.

Vi polysaccharide content and details

Experiments	Culture age (h)								
	10	12	14	16	18	20	22	24	Cut pellet
Exp-01	0.45	0.56	0.678	0.95	1.36	1.56	1.98	2.2	4.35
Exp-02	0.25	0.568	0.87	1.25	1.56	1.82	2.01	2.15	4.15
Exp-03	0.15	0.46	0.84	1.12	1.64	1.75	1.98	2.13	4.25
Exp-04	0.34	0.65	0.75	1.1	1.56	1.84	1.91	1.93	4.06
Exp-05	0.21	0.51	0.91	1.26	1.32	1.64	1.9	2.2	4.19
Exp-06	0.11	0.25	0.85	1.05	1.29	1.56	1.75	1.91	4.01
Exp-07	0.36	0.56	0.74	1.105	1.35	1.54	1.94	2.24	4.15
Exp-08	0.21	0.31	0.62	1.09	1.22	1.65	1.85	2.2	4.32
Exp-09	0.15	0.25	0.85	1.25	1.45	1.68	2.01	2.1	4.16
Exp-10	0.26	0.52	0.96	1.25	1.56	1.89	2.25	2.5	4.89
Exp-11	0.11	0.65	0.95	1.02	1.78	1.98	2.35	2.7	5.04
Exp-12	0.23	0.51	0.84	1.26	1.46	1.89	2.45	2.81	4.57
Exp-13	0.11	0.46	0.94	1.35	1.65	2.12	2.42	2.52	4.62
Exp-14	0.23	0.56	0.85	1.65	1.95	2.05	2.56	2.91	4.96
Exp-15	0.36	0.65	0.95	1.56	1.95	2.15	2.34	2.64	4.67
Exp-16	0.46	0.75	0.99	1.58	1.99	2.05	2.56	2.81	4.75
Exp-17	0.25	0.65	0.89	1.36	1.87	2.12	2.86	3.01	4.95
Exp-18	0.38	0.45	0.75	1.25	1.84	2.25	2.64	2.75	4.79

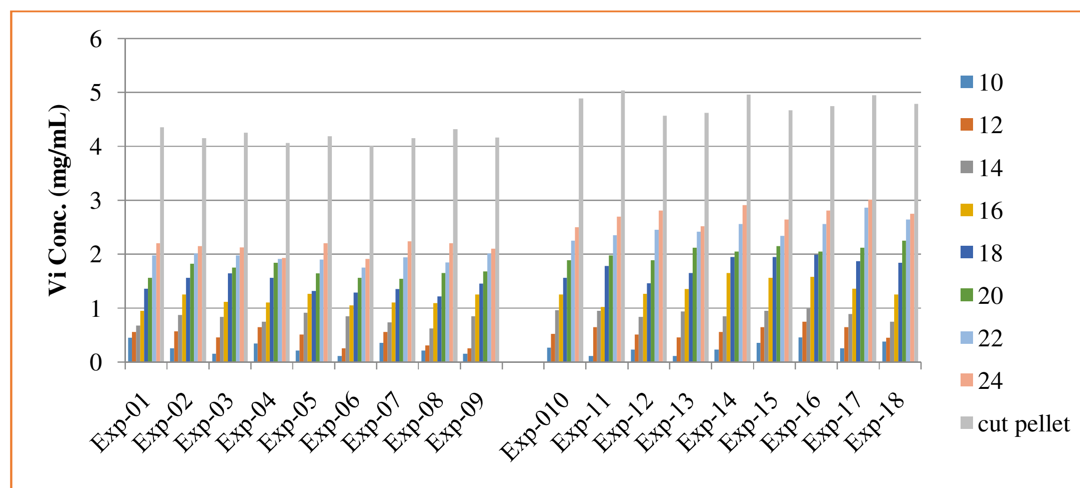


Illustration of Vi polysaccharide content

IV. CONCLUSION

Based on the optimization studies, consistency batches are performed with following conditions.

Culture Medium

The Salmonella typhi growth medium containing Soyabean Casein Digest Medium (30 g/L), yeast extract (10 g/L), and $MgSO_4.7H_20$(2.5 g/L) pH 7.2 was used as pre seed, seed and fermentation medium. The feed medium with yeast extract-(100 g/L), SCDM (30g/L) and dextrose (500 g/L) was used for fed batch. 20% (v/v) Antifoam solution was used as defoaming agent in the process. The preseed, seed, fermentation and feed medium are filter sterilized with sterile 0.2 µ filter.

Fermentation Process

The fermentation was carried out range from 1.0 to 2.5 OD_{590} (mid log phase) seed culture. The seed flask was incubated at 35°C in 200 rpm. The fermentation process parameters are temperature 35°C, pH 7.2maintained with 20% w/v NaOH, aeration 1 to 2 VVM and DO maintained 20 to 40% by oxygen cascade mode. The fermentation process with DO-RPM Cascade mode was showed in consistency in DO profile and consequent impact on fermentation kinetics. In order to avoid impact on fermentation kinetics due to inconsistent DO profile and to maintain maximum level consistency in DO profile throughout process. The feed was started at 7th hour of fermentation process and continued upto14 hours. During fed batch the glucose concentration is maintained as ≤1 g/L. After fed batch, process was maintained for two hours in stationary phase with low RPM without aeration under process temperature (35°C) and pH (7.2). After completion stationary phase inactivate the culture with formaldehyde (3.6 mL /L of culture broth) for one hour at 35°C without pH maintenance. The inactivated culture was further processed for Vi polysaccharide purification.

The Vi capsular polysaccharide was obtained in fed batch culture is more than 2 times of the batch fermentation with different media compositions and parameters. The results showed that optimizing the fermentation condition and increasing the bacterial cell members increased the capacity to produce Vi polysaccharide, The effect of the glucose concentration of Vi polysaccharide production was more difficult to understand, the result demonstrated that early exposure to a high glucose concentration (50%) resulted in an absence of Vi production during stationary phase but not during the growth phase. An optimized the media components resulted in fermenter harvest cut pellet an increase in Vi polysaccharide of 3 times that achieved in the fed batch culture mode.

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