Doehlert Matrix-assisted optimization of *Salmonella typhi* Vi polysaccharide purification parameters

Garisala Mallikarjunara Rao[1], Sathish Thadikamala[2*], Maram Chinna Eswaraiah[3]

[1]Department of Biotechnology, JNTU Hyderabad, Hyderabad, India.
[2]Department of Pharmacology, Koringa College of Pharmacy, Kakinada, India.
[3]Department of Pharmaceutical Biotechnology, Anurag Pharmacy Collage, Kodad, India.

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ABSTRACT

Typhoid fever is one of the prevalent pandemics across many developing countries caused by *Salmonella typhi*. Vi capsular polysaccharide obtained from *S. typhi* has been licensed for use as a vaccine for children aged 2 years and above to prevent typhoid fever. Production and purification of Vi polysaccharide play a vital role in the immunogenicity and cost of vaccines. Four critical parameters, cetrimide concentration (% w/v), sodium acetate concentration (% w/v), alcohol concentration (% w/v), and operational temperature (°C), were optimized by response surface methodology (RSM). Doehlert design matrix, with 22 experimental runs, was used to optimize the selected parameters. All parameters were studied at different levels. With the help of RSM, by optimizing the selected variable conditions, onefold yield increased and the final purified Vi polysaccharide has noncompliance with the World Health Organization's standards.

1. INTRODUCTION

In the worldwide history of pandemic outbreaks, typhoid fever has a significant role. At present, many countries in Africa, Latin America, and South Asia are affected by the typhoid fever and the effect shows a serious impact on the society. According to the World Health Organization (WHO), recent data depict that every year worldwide 16–33 million typhoid fever cases are reported and among those 500,000–600,000 people are dying [1,2]. In humans, *Salmonella enterica* serovar *typhi* (*Salmonella typhi*) is a causative agent for typhoid fever [3,4]. The bacteria spreads through contaminated water and food [5,6]. It was reported that due to tourism also the bacteria spreads across the various European countries and USA [3]. The rate of risk of infection is severe in countries in the continents of Asia, Africa, and Latin America where poor sanitation is observed [7]. Typhoid fever is mostly observed in underaged children and school going children.

Fatal rate was observed among children in the age group of 1–2 years. A number of antibiotics have been used to treat typhoid fever. However, emerging multidrug-resistant strains of *S. typhi* make those antibiotic treatments unsuccessful [5,6]. The low cost-effective vaccine is still the most effective way to control typhoid fever.

Currently, there are two quite distinct vaccines that are available in the market. One oral live attenuated vaccine that contain strain Ty21a and Vi polysaccharide vaccine administered parenterally [7]. The oral typhoid vaccine is given in the form of enteric capsules as three to four doses [8]. At present, the mechanism action of the oral vaccine is not fully understood [9]. Since it is administered through an oral route it activates the mucosal IgA and serum IgG and provides protection from typhoid bacteria [10,11]. The live attenuated bacteria lack the *galE* gene, are unable to synthesize the lipopolysaccharides (LPS) O-chain, and are unable to synthesis the capsular polysaccharide (CP) Vi antigen. Both LPS and CP are considered major targets for antibodies that lack these components and in the live attenuated vaccines they elicit less immunogenicity [12]. The efficacy of Ty21a enteric capsules after three doses is noted as 42%–67% at 3 years postvaccination [13,14].

CPs are composed of monosaccharides attached by glycosidic linkage. CPs have been proven to be excellent targets for bacterial vaccine development [15,16]. Vi capsular polysaccharide consist of polygalacturonic acid units linked linearly by α-1–4 linkage, and it is N-acetylated at C2 and 60%–90% O-acetylated at C3 of the galacturonic acid (Gal UA) residue [17,18]. The molecular weight and O-acetylation content play vital roles in the immunogenicity and potency of Vi polysaccharide. After 28 days of injection of single dose of 25 µg purified Vi polysaccharide given intramuscularly, it elicits 1 µg/ml anti typhoid IgG antibody in the serum [17,18]. The efficacy of Vi polysaccharide-based vaccines showed superior immunogenicity than live attenuated vaccines [19,20].

The Vi polysaccharide is produced by the fermentation of *S. typhi*. Production and purification of Vi polysaccharide play an important role in the final price fixing of the vaccine. It is necessary to produce economically viable vaccines for low-income country people. It is important to avoid costly process purification methods such as chromatography and various capital equipment to reduce the final vaccine price. It is also important to produce the vaccine to meet the WHO's standards with less impurities and high content of Vi polysaccharide [20,21]. The precipitation method was found to be an economically viable method to purify the Vi polysaccharide [20]. Various researchers have developed various methods for purification of polysaccharide, among which the cetrimide method was found to be an effective and economically viable method. The current manuscript deals with the optimization of various precipitation parameters to enhance the yield. The statistical method of optimization was employed to enhance the Vi content. To the best of our knowledge, this is the first manuscript to publish the statistical method of optimization for the enhancement of Vi content and it is purity.

2. MATERIALS AND METHODS

2.1. Bacterial Strain and Production of Vi Polysaccharide

For the production of Vi polysaccharide, *S. typhi* Tystrain was employed. The culture was stored on solid Triple sugar iron (TSI) agar slants. It was revived from the TSI slants and grown on the shake flasks. From the flask, the culture grew on the 30 l bioreactor (Sartorius). Soybean Casein Digest Medium 30 g/l supplemented with MgSO4 7H$_2$0 2.5 g/ml, yeast extract 5 g/l, and trace elements (such as cobaltous chloride 0.043% w/v, manganese chloride 0.26% w/v, copper sulfate 0.038% w/v, boric acid 0.053% w/v, sodium molybdate 0.037% w/v, and zinc acetate 0.26% w/V) were used for cultivation of *S. typhi*. The media pH was maintained at 7.2 ± 0.1 with NaOH and HCl. The prepared media were sterilized by autoclaving at 121°C for 20 minutes. The sterile media inoculated with the 2% inoculum of OD$_{590}$ 2 and were incubated for 12 and 24 hours in the shaker and bioreactor, respectively.

2.2. Clarification of Fermented Broth

The fermentation broth was clarified by using the centrifuge, and the crude Vi polysaccharide was distributed into centrifuge bottles and centrifuged at 8000 rpm for 30 minutes at 2°C–8°C. The supernatant was collected in bottle and pellets were collected in a separate bottle. The supernatant was further concentrated up

to 1.0 l by using 30 kD Tangential flow filtration (TFF) cassettes, followed by diafilteration against 10 volume change of purified water maintaining a constant retantate volume.

2.3. Purification of Vi Polysaccharide

Figure 1 shows the flow chart of the purification process. To the crude Vi polysaccharide, 10% of cetrimide was add slowly while stirring, so that the final concentration of cetrimide reached a desired concentration as per experimental conditions and was incubated in a refrigerator overnight. The centrifuged solution and pellets were collected and the supernatant was discarded. The obtained pellets were redissolved in 1M NaCl solution and ethanol was added to reach a final concentration as per experimental conditions, and the mixture was incubated in a refrigerator overnight. Then, the centrifuged solution and pellets were collected and the supernatant was discarded.

The pellets were redissolved in NaCl solution and ethanol, and to this solution a desired amount of sodium acetate was added slowly while stirring after complete dissolution of sodium acetate to make up the final volume with ethanol or water to achieve desired concentration of ethanol in the solution. The precipitate was allowed to settle down at the bottom of the container, and without disturbing the precipitate the mixture was incubated in the refrigerator for 6 hours. At the end of incubation period, the crude Vi polysaccharide mixture was distributed into centrifuge bottles and centrifuge at 8000 rpm for 30 minutes at 2°C–8°C. The supernatant was collected and the pellets were discarded.

To the collected supernatant solution a desired amount of ethanol was slowly added while stirring. The precipitate was allowed to settle down at the bottom of the container. The precipitate mixture was incubated in the refrigerator overnight. At the end of the incubation period, the Vi polysaccharide mixture was distributed into centrifuge bottles and centrifuged at 8000 rpm for 30 minutes at 2°C–8°C. The pellets contained the desired purified Vi polysaccharide. The pellets were redissolved 1M NaCl solution and additional concentrate and the NaCl was removed by diafiltration.

2.4. Diafiltration and Sterile Filtration of Vi Polysaccharide

The purified Vi polysaccharide was further concentrated by using 100 kD TFF cassettes, followed by diafiltration against 10 volume change of purified water maintaining a constant retantate volume to ensure maximal recovery of Vi polysaccharide. Finally, the sterile solution was filtered through a 0.2 µ filter and stored in a sterile container.

2.5. Optimization of Purification Conditions by Response Surface Methodology (RSM)

RSM using Doehlert design was applied for optimization of purification conditions. Four parameters, cetrimide concentration (% w/v) (C), sodium acetate concentration (% w/v) (S), final alcohol concentration (%v/v) (A), and process temperature (°C) (T), were selected as independent variables and O-acetyl content (mmol/g of Vi) was a dependent response variable. Doehlert's design for four variables was chosen from Ferreira *et al.*'s [22]
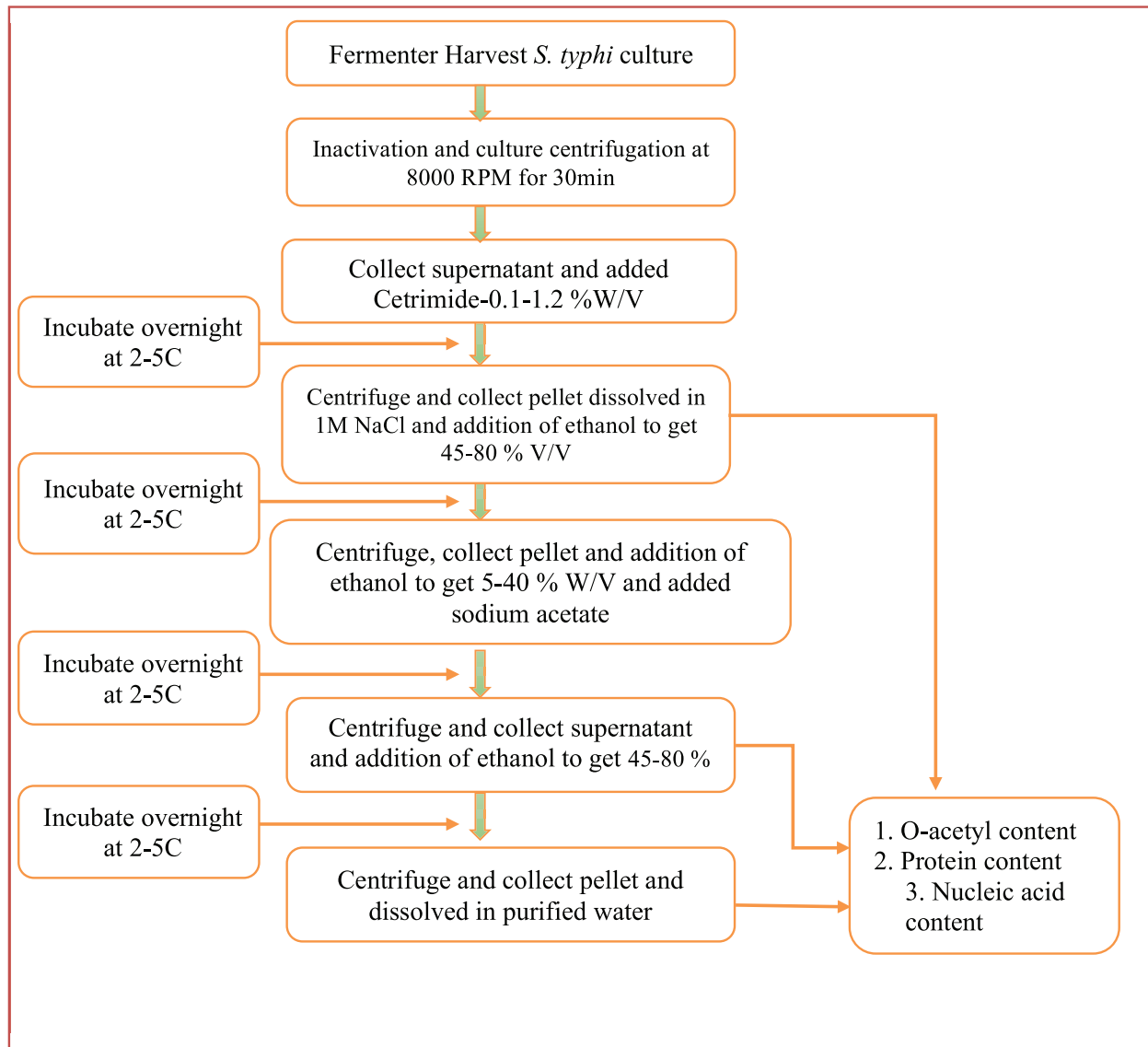
Figure 1: Flow chart of the Vi polysaccharide purification process.

study and added one additional central point. The experimental conditions were selected according to the design. Cetrimide concentration was chosen at five levels, sodium acetate and ethanol selected at seven levels, and temperature was selected at three levels with a total of 22 experimental runs. The experimental plan of RSM in both coded and real values of all independent variables is presented in Table 1.

The obtained Vi polysaccharide, in terms of O-acetyl content with respect to purification conditions, was analyzed, and the response surface model given by Equation (1) was fitted with multiple regressions through the least squares method as follows:

$$Y_i = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i}^{i} \sum_{j}^{k} \beta_{ij} x_i x_i - - - - - (1)$$

where Y_i is the predicted O-acetyl content (Y_i) taken as a response, x_i–x_j are input variables which influence the response variable Y, β_0

is the offset term, β_i is the ith linear coefficient, β_{ii} the ith quadratic coefficient, and β_{ij} is the interaction coefficients. The significance of the selected parameters is evaluated based on p-values and F-test with unequal variance ($p < 0.05$, $p < 0.01$, and $p < 0.001$). Trail version of Design-Expert-12 (State Ease Company, Minneapolis, MN) was used in this study.

2.6. Analytical Methods

Vi polysaccharide was estimated according to Hestrin [23]. The protein content was estimated by Lowry et al. [24]. UV spectroscopy method was used for estimation of nucleic content [20].

3. RESULTS AND DISCUSSION

For effective purification of the Vi polysaccharide, it is essential to optimize all the purification conditions. Initially, critical purification parameters were identified and optimized by the conventional method

(data not shown). From these studies, it was observed that cetrimide, sodium acetate concentrations, final alcohol concentration, and temperature play a vital role in the Vi polysaccharide purification. The current focus on polysaccharide purification research is oriented toward the optimization of selected parameters by statistical methods. Various statistical methods, such as the Plackett–Burman design [25], RSM with the Box–Benhan design, central composite design [26], Doehlert's design [27], and mixture designs [28], are employed in the optimization of various culture conditions. However, this is the first research employing the RSM with Doehlert's design to optimize the Vi polysaccharide purification variables.

Table 1 shows the purification of Vi polysaccharide ranged between 1.23 and 3.01 O-acetyl content (mmol/g of Vi), indicating the significance of selected factors and their concentrations on the purity of polysaccharide. Multiple regression analysis carried out by taking O-acetyl content as the response and selected parameters as independent variables. The regression coefficient (R^2) value was considered for the accuracy of the analysis, and it was preferred that R^2 value nearer to one is desirable. In the current experiment, the R^2 value obtained is 0.9963, indicating that 99.63% of the variability in the purification process can be explained by the model.

Furthermore, the observed adjusted R^2 value of 0.9889 signifies the model [26]. The coefficient of variance (CV) is a measure of the precision and accuracy of experiments. A lower CV value indicates better accuracy [26]. In the current experiment, the CV was 2.71%, which indicates good precision and reliability of the experiments conducted. The signal-to-noise ratio can be measured by adequate precision value. A value above 4 is desirable, and in the current experiment the adequate precision value of 38.377 indicated the current model can be used for further optimization.

Figure 2 shows the correlation between the experimental values and predicted values. In Figure 1, all data points near to the fitted line indicate the best correlation between the observed and predicted values [27].

Based on multiple regression analysis data, a second-order polynomial regression equation was constructed, which relates the studied parameters and purity of Vi polysaccharide empirically.

$$O-acetyl\ cont = 3.005 + 0.012*Ce - 0.0439*SA - 0.361*Al + 0.028*Te - 0.1750*Ce*Ce - 0.9851*SA*SA - 1.45*Al*Al - 1.560*Te*Te + 0.155*Ce*SA - 0.299*Ce*Al + 0.2669*Ce*Te - 0.1087*SA*Al + 0.408*SA*Te - 1.250*Al*Te - -(2)$$

Table 1: Doehlert design for optimization of Vi polysaccharide along with experimental results and predicted values.

S. No	Cetrimide concentration (% *W/V*) (Ce)	Sodium acetate conc (% *W/V*) (SA)	Alcohol conc (% *V/V*) (AL)	Temperature (°C) (Te)	O-acetyl content (mmol/g of Vi)		
					Experimental	Predicted	Error
1	0 (0.6)	0 (20)	0 (60)	0 (5)	3.00	3.01	−0.005
2	1 (1.2)	0 (20)	0 (60)	0 (5)	2.87	2.84	0.028
3	0.5 (0.9)	0.866 (40)	0 (60)	0 (5)	2.21	2.26	−0.048
4	0.5 (0.9)	0.289 (25)	0.817 (65)	0 (5)	1.45	1.48	−0.033
5	0.5 (0.9)	0.289 (25)	0.204 (80)	0.791 (15)	1.82	1.77	0.053
6	−1 (0.1)	0 (20)	0 (60)	0 (5)	2.79	2.82	−0.028
7	−0.5 (0.3)	−0.866 (5)	0 (60)	0 (5)	2.37	2.32	0.048
8	−0.5 (0.3)	0.289 (15)	−0.817 (50)	0 (5)	2.12	2.09	0.033
9	−0.5 (0.3)	0.289 (15)	−0.204 (45)	−0.791 (2)	1.83	1.88	−0.053
10	0.5 (0.9)	−0.866 (5)	0 (60)	0 (5)	2.18	2.20	−0.019
11	0.5 (0.9)	0.289 (15)	−0.817 (50)	0 (5)	2.30	2.30	0.001
12	0.5 (0.9)	0.289 (15)	−0.204 (45)	−0.791 (2)	1.69	1.70	−0.01
13	−0.5 (0.3)	0.866 (40)	0 (60)	0 (5)	2.13	2.11	0.019
14	0 (0.6)	0.577 (30)	−0.817 (50)	0 (5)	2.05	2.03	0.02
15	0 (0.6)	0.577 (30)	−0.204 (45)	−0.791 (2)	1.30	1.29	0.009
16	−0.5 (0.3)	0.289 (25)	0.817 (65)	0 (5)	1.67	1.67	−0.001
17	0 (0.6)	−0.577 (10)	0.817 (65)	0 (5)	1.47	1.49	−0.02
18	0 (0.6)	0 (20)	0.613 (70)	−0.791 (2)	1.90	1.85	0.054
19	−0.5 (0.3)	0.289 (25)	0.204 (80)	0.791 (15)	1.57	1.56	0.01
20	0 (0.6)	−0.577 (10)	0.204 (80)	0.791 (15)	1.23	1.24	−0.009
21	0 (0.6)	0 (20)	−0.613 (50)	0.791 (15)	2.28	2.33	−0.054
22	0 (0.6)	0 (20)	0 (60)	0 (5)	3.01	3.01	0.005

Values in parenthesis are real values. The error in the table is the difference between the experimental results and predicted O-acetyl content (mmol/gm of Vi)

Based on the *p*, *F*, and *t*-values, the coefficients were selected. Table 2 shows the coefficient values and their corresponding *p*, *F*, and *t*-values. The coefficients having a *p*-value below 0.05 and a high *F*-value are considered significant terms. Based on these criteria, linear terms of cetrimide, alcohol content, temperature, and interaction terms of sodium acetate with cetrimide and alcohol are insignificant. The square terms of temperature has the highest effect among all selected parameters, which indicates that temperature has a critical role in the Vi purification process. The square terms of cetrimide, alcohol content, and sodium acetate

concentration have more effect value. The parameters which have more effect value in square terms than linear terms are critical to the process, and a small variation in those parameters values has a significant effect on the final process. In this study, all selected parameters have more effect at square terms than linear terms, which indicates that all selected parameters and their levels are important for the purity of Vi polysaccharide.

3.1. Evaluation of Response Surface

The surface (3D) plots along with contour plots were (2D) generated by using the regression equation developed (Eq. 3). The 3D plots with 2D contour plots visualized the interaction influence of the selected two parameters on Vi polysaccharide purification. In these plots, two parameters were changed at different levels, whereas other parameters were kept constant at central value. The highest values of polysaccharide could be assessed with combinations of the two variables that were close to the central points. Figure 3 shows the surface plots with images of contour plots of selected variables and their influence on Vi polysaccharide recovery.

Figure 3A–C shows the cetrimide interaction with other selected three parameters for the purification of O-acety content. Figure 3A–C shows that the contours are slightly inclined toward the *y*-axis, indicating that the cetrimide concentration was slightly influenced by sodium acetate, alcohol concentration, and temperature. Figure 3D and E shows circular contours indicating that sodium acetate concentration was independent of alcohol concentration and temperature. Interaction of alcohol content with temperature depicts that alcohol content depended on process temperature (Fig. 3F).
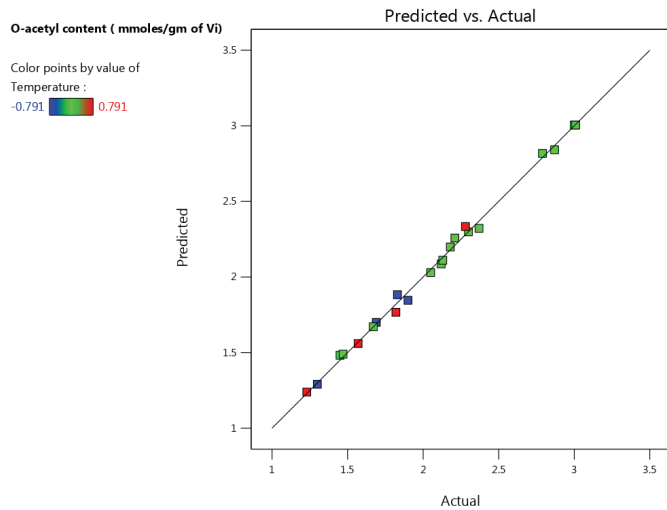

Figure 2: Correlation between experimental results (actual) and model-predicted O-acetyl content values.

Table 2: Coefficients and analysis of variance.

|	**Coefficients**	**Sum of squares**	**df**	**Mean square**	**F-value**	**p-value**
Model		5.85	14	0.418	134.6	<0.0001
Constant	3.01	–	–	–	–	<0.0001
Ce	3.005	0.0007	1	0.0007	0.2318	0.6449
SA	0.012	0.0096	1	0.0096	3.1	0.1217
AL	−0.04388	0.6541	1	0.6541	210.61	<0.0001
Te	−0.36147	0.004	1	0.004	1.29	0.2926
Ce*Ce	−1.25057	0.0306	1	0.0306	9.86	0.0164
SA*SA	−0.175	0.9702	1	0.9702	312.4	<0.0001
AL*AL	−0.98506	2.3	1	2.3	741.02	<0.0001
Te*Te	−1.45067	2.87	1	2.87	924.26	<0.0001
Ce*SA	0.028345	0.0182	1	0.0182	5.87	0.0459
Ce*AL	0.155889	0.054	1	0.054	17.4	0.0042
Ce*Te	−0.29994	0.0396	1	0.0396	12.76	0.0091
SA*AL	0.266923	0.0071	1	0.0071	2.29	0.1742
SA*Te	−0.10873	0.0928	1	0.0928	29.88	0.0009
AL*Te	0.408431	0.8707	1	0.8707	280.36	<0.0001
Residual	−1.56032	0.0217	7	0.0031		
Lack of fit		0.0217	6	0.0036	72.3	0.0898
Pure error		0	1	0		
Total		5.87	21			
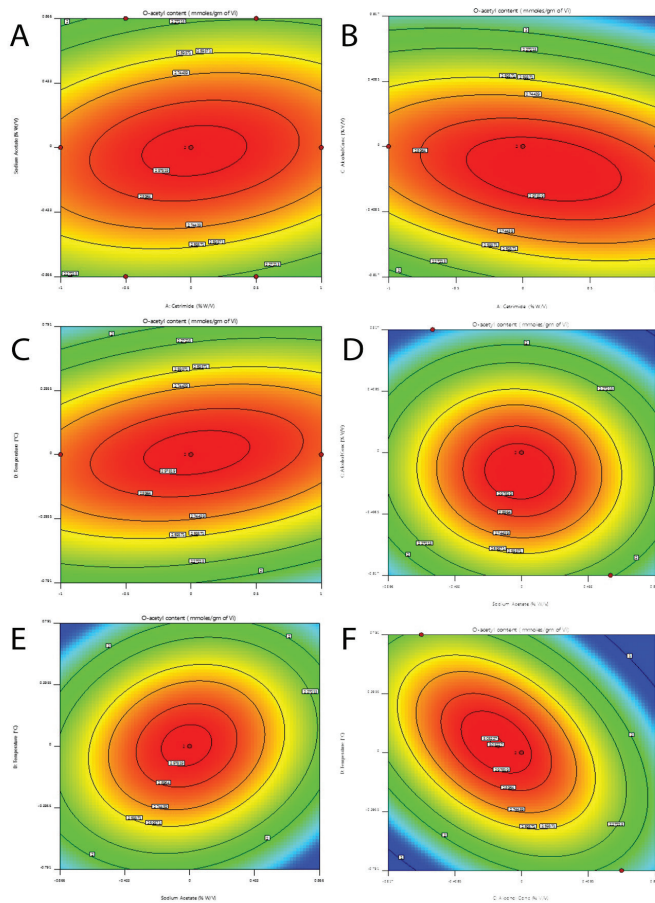
Figure 3: 3D surface plots with contours of selected parameters and their interaction influence on O-acetyal content recovery. (A) Cetrimide versus sodium acetate, (B) cetrimide versus alcohol content, (C) cetrimide versus temperature, (D) sodium acetate versus alcohol content, (E) sodium acetate versus temperature, and (F) alcohol content versus temperature.

3.2. Validation of Model

To obtain the optimum conditions, Equation (2) was used. Solving Equation (2) by numerical methods, it has given the optimum condition, i.e., cetrimide concentration 0.7% w/v, sodium acetate concentration 28% w/v, final alcohol concentration at 55% v/v, and temperature at 5.3°C. At these conditions, the predicted O-acety content was 3.1 (mmol/g of Vi); however by conducting the experiments at the predicted conditions 3.15 mmol/g of Vi O-acetyl content was observed, at these conditions 3.066 mg/g of Vi of protein and 4.135 mg/g of Vi nucleic acid content was observed. The amount of protein and nucleic acid impurities are below the WHO specifications. Overall, the study indicates that about onefold increase in Vi polysaccharide was observed compared with the conventional optimization methods.

4. CONCLUSION

The current study describes that precipitation is the best method for purification of polysaccharides. Finding the critical parameters is important for process development. In this study, four critical parameters, namely cetrimide concentration, sodium acetate concentration, alcohol concentration, and temperature, were identified for Vi polysaccharide purification. Statistical methods of optimization are effective with less number of experiments. Doehlert matrix method is diverse than the regular Box–Behnkhen and central composite design methods in RSM. This method has freedom of variables at individual levels. The total number of experiments is less with high variability in specific parameters. Overall, onefold improvement of Vi polysaccharide recovery with high purity as per the WHO's specifications was achieved.

5. ACKNOWLEDGMENTS

6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, and analysis and interpretation of data; they took part in drafting the article and revising it critically for important intellectual content; they agreed to submit the current journal; they gave their final approval of the version to be published and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors' requirements/guidelines.

7. ETHICAL APPROVAL

This study does not involve experiments on animals or human subjects.

8. CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

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