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STATISTICAL OPTIMIZATION OF FUCOIDAN PRODUCTION FROM BROWN SEAWEED SARGASSUM CINEREUM

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Abstract

Brown seaweed was collected from the Tuticorin, (latitude 8° 48’ N, longitude 78° 11’ E) Tamilnadu, India. The collected seaweed sample was identified as *Sargassum cinereum*. Fucoidan was extracted from *Sargassum cinereum*. In this present study, the optimum cultural conditions for the extraction of fucoidan from *Sargassum cinereum* were studied by Response Surface Methodology (RSM) using central composite Design with three variables pH, Time and Temperature for maximizing the production of fucoidan. The optimum level of the key variables (pH, Temperature and Incubation Time) used to determine the effect of their interactions on fucoidan yield using the statistical tool (Central composite Design (CCD) of RSM). The second order quadratic model with the optimum conditions (pH -8, Temperature - 100° C and Incubation Time – 5 hours). The nearness of the coefficient of determination (R^2 = 0.9866) to 1 ensures the satisfactory adjustment of the quadratic model to the experimental data. The fucoidan recovered from optimized medium was 9.44 ± 1.47 % of dry weight respectively.

Keywords: Central Composite Design, Fucoidan, Optimization, Response Surface Methodology, *Sargassum cinereum*, Seaweed.

INTRODUCTION

Seaweed utilization is a multi dollar industry. It is mainly based on the production of phycocolloids such as agar and carrageenan, or farming for human consumption (Fung *et al*., 2013). From the seaweeds the pharmaceutical companies extracted other compounds in need of their potential as pharmaceuticals and /or nutraceuticals (Ly *et al.,* 2005). Seaweeds are unanimously considered as a valuable source of bioactive compounds. As the secondary metabolites were produced by seaweeds in very good manner, biological activities are characterized in broad spectrum. They are also rich source of polysaccharides (Sumit, 2004). Brown seaweeds are known to contain more bioactive components than either green or red seaweeds.

Brown seaweeds are the second most abundant group of marine algae. It comprises about 2000 species. From the brown seaweeds, fucoidan can be isolated. Fucoidan is a polysaccharide that consists of sulfated fucose residues. The richest sources of fucoidan are marine organisms including brown algae. Among them, *Ascophyllum* spp., *Fucus* spp., *Laminaria* spp. and *Sargassum* spp. are the most commonly used on biotechnological aspects (Narayani *et al*., 2019; Somasundaram *et al*., 2016). However*Sargassum* is a less exploited genus in the Phaeophyceae though it grows wildly in enormous quantities almost all over the world and in Egypt (Rodríguez-Jasso *et al*., 2011; Gomaa *et al*., 2014).

Brown algae with high amount of fucoidan have been marketed as a dietary supplement or nutraceutical. Fucoidan has advantages of low toxicity, oral bioavailability and multiple mechanisms of action. Fucoidan can easily be extracted using either hot water or acidic or basic solutions (Berteau and Mulloy 2003). Algal raw material was treated with hot aqueous or acidic solutions at temperatures ranging from 70–100 ºC for several hours for the extraction of fucoidan (Ale *et al*., 2011b). Hot-water extraction of polysaccharides is usually associated with long extraction time and high temperature. Higher yields of crude fucoidan extracted from seaweed by treatment of diluted acid at ambient temperature (Ale *et al*., 2011b). But it leads to the production of undesirable product such as alginc acid. Fucose chains were degraded by diluted acid hydrolysis method. Moreover, sulphate esters were cleaved in partial manner (Pomin *et al*., 2005). The insoluble components in the fucoidan were successfully removed by the salt calcium chloride ($CaCl_2$) though $CaCl_2$lowers the yield of fucoidan (Imbs *et al*., 2015) the purity of the fucoidan can increased by using calcium chloride (Mak *et al*., 2013). More aggressive techniques involving higher temperatures and pressures, such as closed-vessel microwave-assisted extraction and autohydrolysis (Rodríguez-Jasso *et al.*, 2011; Rodríguez-Jasso *et al.*, 2013) could be expected to achieve a higher polysaccharide yield. But, they have been shown to cause a high degree of fucoidan hydrolysis, even in the absence of acid (Balboa *et al.*, 2013). Thus, it is desirable to find an economical and effective method of extraction of polysaccharides that give higher yields and preserve the native fucoidan structures in order to bring out the distinct biological properties. Citric acid is a kind of mild organic acid that is widely used in food industry as a food additive. However the information regarding the use of citric acid in the extraction of fucose containing sulfated polysaccharides from brown algae is limited (Lu *et al.*, 2013).

The present study evaluated the amount of fucoidan extracted from the brown seaweed *S.cinereum* (FSMC) using hot water at alkaline pH. As the extraction of natural compounds alters the pH of the solution over time and may influence fucoidan yield and physico-chemical characteristics. Buffering seems to be an important process. Central composite design (CCD) was applied to verify the influence of temperature, pH and incubation time in the response of FSMC yield and the conditions able to maximize the extraction yield was established. There is only limited systematic information about the influences and apparently complex interactions of extraction parameters acid, temperature and time on FSMC.

MATERIALS AND METHODS

Sample Collection

The brown seaweed was collected from the Tuticorin, (latitude 8° 48' N, longitude 78° 11' E) Tamilnadu, India and it was identified morphologically. Seaweeds from 4m depth were plucked in the hands and washed and dried in a shadow. The collected seaweed sample was identified as *Sargassum cinereum* by Dr. P. Anantharaman, Associate Professor, CAS in marine Biology, Annamalai University, Portonovo. Samples were then shade dried in till the obtaining of constant weight obtained and ground in an electric mixer. The powdered samples subsequently stored in refrigerator 4ºC

Extraction of Fucoidan

From the brown seaweed *S. cinereum* the extraction of fucoidan was performed as described by Yang *et al.* (2008). Milled seaweed of about 20 grams was treated with one litre of ethanol and stirred with a mechanical stirrer for about 12 hours at room temperature in order to remove proteins and pigments. After washing with acetone, centrifugation is done at 1800 × g for 10 minutes. Thenthe residue was left to dry at room temperature. After well drying a biomass, 5 g was taken and extracted in 100 ml of distilled water at 65°C with stirring for an hour. The extraction was done twice and the extracts were pooled. The combined extracts were centrifuged at 18500 × g for 10 minutes and the supernatant was collected. Then, the supernatant was mixed well with 1% $CaCl_2$ and the solution was kept at 4°C for overnight to precipitate alginic acid. The solution was then centrifuged at 18500 × g for 10 minutes and the supernatant was collected. Ethanol (99%) was added into the supernatant in order to arrive upon the final ethanol concentration of 30 % and the solution was placed at 4°C for 4 hours. Again, the solution was centrifuged at 18500 × g for 10 minutes and the supernatant was collected. After that ethanol (99%) was added into the supernatant in order to arrive upon the final ethanol concentration of 70% and the solution was placed at 4°C for overnight. The intact fucoidan was then obtained through filtration of the solution with a nylon membrane 0.45 µm sizes. Fucoidan extracted from *Sargassum cinereum* (FSMC) yield was estimated based on the dried biomass obtained after the treatment of the milled sample with 85% ethanol as a percentage of the algal dry weight (% dry weight).

Optimization of Physiological Factors for FSMC

Maximum yield of the FSMC obtained by subjecting the seaweed under the optimized conditions were investigated. The factors like pH, temperature and incubation time, which were expected to affect the production of FSMC.

Effect of pH, Temperature and Incubation Time in the Yield of FSMC

(a) Effect of Different Temperature

In the hot water extraction different temperatures (80, 90, 100 and 110ºC) were set in the boiling water bath then the yield of FSMC were calculated.

(b) Effect of Different Incubation Time

Different temperatures (80, 90, 100 and 110ºC) were set in the boiling water bath for different incubation time (3, 4 and 5) hours to determine the effect of incubation time in the yield of FSMC.

(c) Effect of Different pH

Different pH (2, 3, 4, 5. 6, 7, 8 and 9) were adjusted into the distilled water to determine the effect of pH on FSMC production.

Statistical Optimization

(a) Response Surface Methodology (RSM) – Statistical Analysis for FSMC

Second – order experiment was the extraction process optimization using central composite design of RSM with 3 variables: Time (x1),pH (x2) and Temperature (x3)**(Table 1)**. Optimization extraction step has 20 randomly ordered treatment with 6 replicates center point. Each condition following the extraction process Central Composite Design (CCD) of RSM are presented in **Table 2.** below. Based on experimental data, regression analysis and fit models polynomial equation second – order:

$$Y=\beta_o + \sum \beta_i \chi_i + \sum \beta_{ii} \chi_i^{\,2} + \sum \beta_{ij} \chi_i \chi_j$$

Where Y is the predicted response, β_0 the intercept term, β_i the linear coefficients, β_{ii} the quadratic coefficients, β_{ij} the interactive coefficients and x_i and x_j the coded independent variables (Song *et al*., 2007).

Statistical Analysis

Experimental designs and the polynomial coefficients were calculated and analyzed using a trial version of Design-Expert software (version 8.0.4, Stat-Ease Inc., Minneapolis, USA). Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA).

Table 1: Independent variables and their coded levels for the central composite design

Variables	-α	Low value	Coded variables	High value	+α
Time (hours)	3.31821	4	5	6	6.68179
pH	6.31821	7	8	9	9.68179
Temperature	83.1821	90	100	110	116.818

Table 2. Central composite design for extraction of FSMC

Std	Run	F1: Time	F2: pH	F3:Temperature
17	1	5	8	100
13	2	5	8	83.18207169
1	3	4	7	90
6	4	6	7	110
4	5	6	9	90
8	6	6	9	110
7	7	4	9	110
18	8	5	8	100
15	9	5	8	100
5	10	4	7	110
3	11	4	9	90
2	12	6	7	90
11	13	5	6.318207	100
19	14	5	8	100
9	15	3.31820716	8	100
16	16	5	8	100
12	17	5	9.681793	100
20	18	5	8	100
10	19	6.681792831	8	100
14	20	5	8	116.8179283

The results for each group were expressed as mean ± SD values. Data were analysed by one way ANOVA using graph pad prism software. Significant differences were determined among groups at $P < 0.05$.

RESULTS

Yield of the FSMC

The yield of FSMC was 5.651 ± 1.61 % of dry weight.

Optimization

(a) Effect of time, temperature and pH extraction on the yield of FSMC

Research effects of temperature and time extraction different on the yield of crude FSMC. Extractions were performed at 80 -110°C temperature and extraction time of 3- 5 hours. The yield of crude FSMC increased, if the extraction temperature from 80 to 100°C and 3-5 hr. The yield got decreased at the temperature of 110°C.

The results showed that the treatment of temperature at 100°C and extraction for 5hr had positive effect on the yield of FSMC. Meanwhile, extraction at 110°C within 3- 5 hr decreased the yield of FSMC. Through hot water extraction method sample boiled from 80 to 110°C for 3 hours (**Fig. 1a**). In that maximum amount of yield was observed on the 100°C. Sample boiled for 4 hours, 100° C showed maximum percent of yield (**Fig. 1b**). Maximum yield was observed in 100° C for 5 hours. (**Fig. 1c**): FSMC boiled for 5 hours at 100 °C in the pH 8 showed maximum yield (**Fig. 1d**).

Central Composite Design (CCD) and Response Surface Methodology (RSM)

The optimal level of key variables (Time, pH and Temperature) and the effect of their interactions on fucoidan production were further explored using the CCD of RSM. The design matrix and the corresponding experimental data to determine the effects of three independent variables are shown in **Table 2**. The mutual interactions between every two of the three variables which were predicted under optimized condition. The predicted maximum FSMC production was calculated (**Table 3**). By applying multiple regression analysis to the experimental data the following second order polynomial equation was established.

(a). Final Equation in Terms of Actual Factors

$$\text{Yield} = -211.65 + 7.050665 * \text{Time} + 10.53759 * \text{ph} + 3.284879 * \text{Temperature} + 0.33625 * \text{Time} * \text{ph} + 0.040375 * \text{Time} * \text{Temperature} + 0.021375 * \text{ph} * \text{Temperature} - 1.39717 * \text{Time}^2 \; 0.91987 * \text{ph}^2 - 0.01836 * \text{Temperature}^2$$

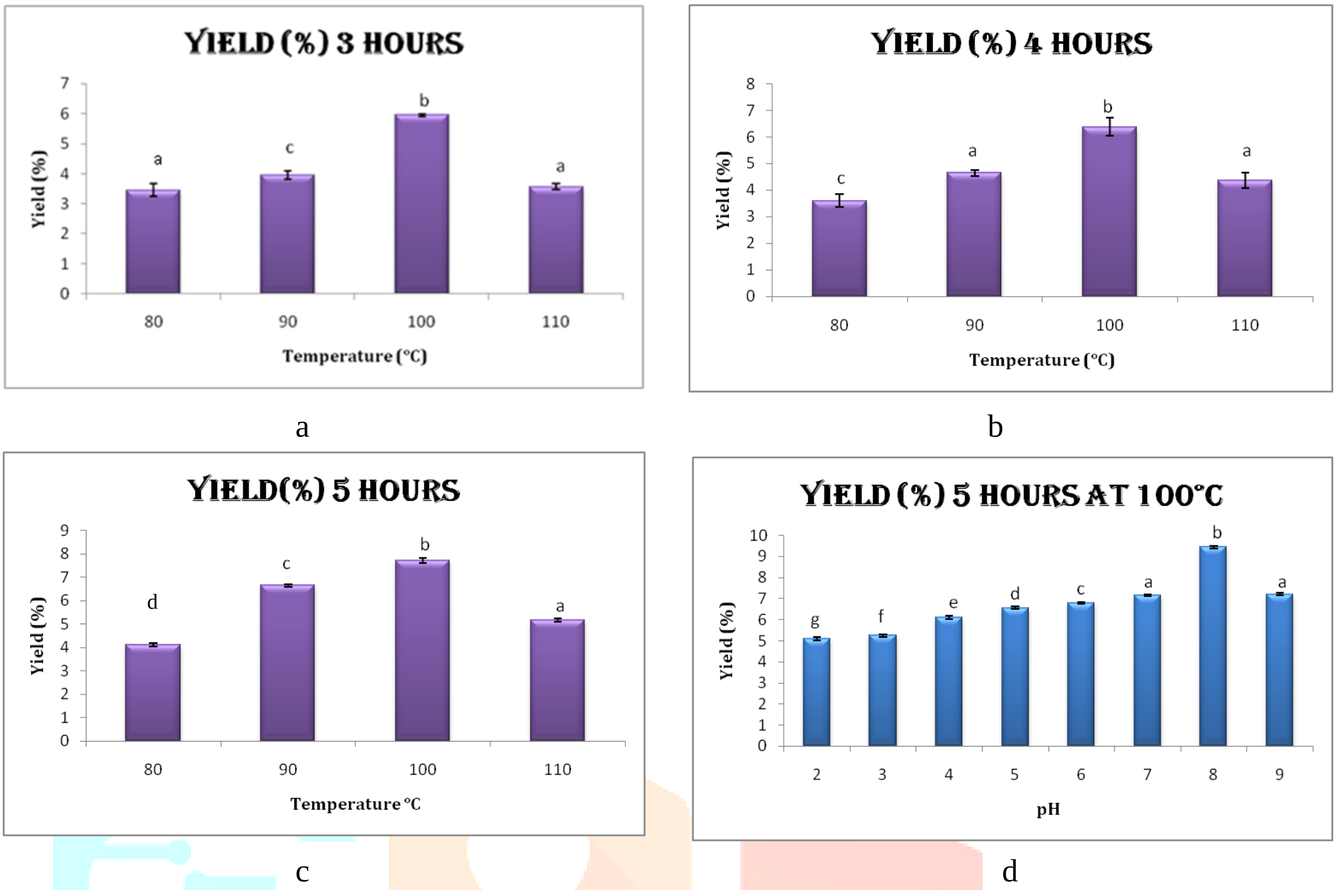


Fig. 1. Optimization of FSMC yield by correlating the temperature, time and pH. a. Effect of temperature for 3 hours on yield of FSMC, b. Effect of temperature for 4 hours on yield of FSMC, c. Effect of temperature for 5 hours on yield of FSMC, d. Effect of temperature of 100°C for 5 hours at different pH in the yield of FSMC. Note : different superscripts above of the each bar significantly different ($p < 0.05$)

Table 3. Central Composite Design for FSMC

Std	Run	F1: Time	F2: pH	F3:Temperature	R1: yield (%)	
					Actual	Predicted
17	1	5	8	100	9.4	9.77
13	2	5	8	83.18	4.71	4.81
1	3	4	7	90	7.55	7.26
6	4	6	7	110	5.66	5.51
4	5	6	9	90	5	4.92
8	6	6	9	110	5.68	5.88
7	7	4	9	110	4.95	4.79
18	8	5	8	100	9.95	9.77
15	9	5	8	100	9.54	9.77
5	10	4	7	110	5.76	5.76
3	11	4	9	90	5.37	5.44
2	12	6	7	90	5.32	5.39
11	13	5	6.31	100	7.6	7.78
19	14	5	8	100	10.47	9.77
9	15	3.31	8	100	5.96	6.15
16	16	5	8	100	9.44	9.77
12	17	5	9.68	100	6.62	6.56
20	18	5	8	100	9.85	9.77
10	19	6.68	8	100	5.56	5.49
14	20	5	8	116.81	4.33	4.35

Table 4. Variance Analysis of Response Surface Quadratic Model for FSMC Yield

Source	Sum of Squares	Df	Mean Square	F Value	*p-value* Prob > F
Model	80.9403	9	8.993366	81.65507	< 0.0001[C]
A-Time	0.511388	1	0.511388	4.643137	0.0566
B-ph	1.785579	1	1.785579	16.21213	0.0024
C-Temperature	0.244971	1	0.244971	2.224212	0.1667
AB	0.904513	1	0.904513	8.212501	0.0168
AC	1.304113	1	1.304113	11.84066	0.0063
BC	0.365512	1	0.365512	3.318663	0.0985
A^2	28.13194	1	28.13194	255.4234	< 0.0001
B^2	12.19428	1	12.19428	110.7177	< 0.0001
C^2	48.5564	1	48.5564	440.8667	< 0.0001
Residual	1.101385	10	0.110138		
Lack of Fit	0.274035	5	0.054807	0.33122	0.8747[C*]
Pure Error	0.82735	5	0.16547		
Cor Total	82.04168	19			

Where,

C model terms are significant

C* Lack of fit not significant

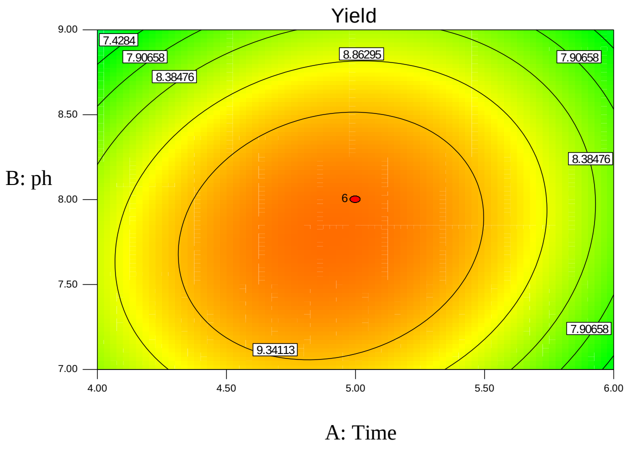
Where, Yield was the yield of the extract A the time, B the pH and C the temperature. The fit of model was checked by the coefficient of determination R^2 , which was 0.9866 for fucoidan production, indicationg that 95.0% of the variability in the response could be explained by the model. The statistical significance of the second – order model equation was determined with the F- test analysis of variance. The "Model F- value " of 81.66 indicated that the models were significant and there was only a 0.01% chance that a "Model F Value ". This large could occur due to noise (p<0.0001). The Co efficient of Variation (CV) is the ratio of the standard error of estimate to the mean value of the observed response.

Here, the low values of CV (4.78%) indicated that great reliabilities of the experiments performed. In the present study, all these results showed a good agreement between the experimental and predicted values and implied that the mathematical models were suitable for the simulation of FSMC.

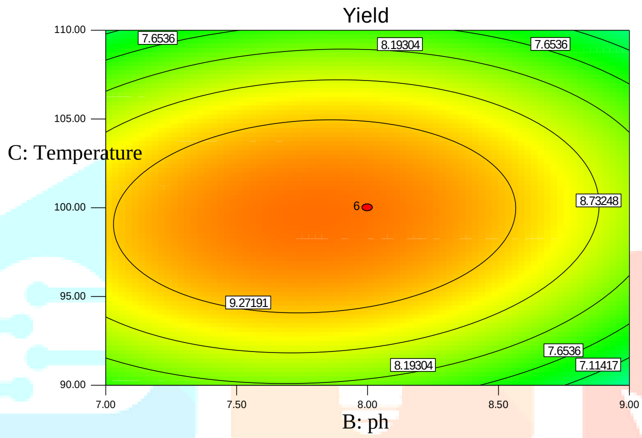
The three-dimensional response surfaces and contour plots are shown in **Fig. 2** (yield) which depicts the interactions between the two variables by keeping the other variables at their zero levels. The shapes of the contour plots, circular or elliptical, indicate whether the mutual interactions between the variables are significant or not. A circular contour plot of response surfaces indicates that the interaction between the corresponding variables can be ignored, while an elliptical or saddle nature of the contour plot suggests that the interaction between the corresponding variables is significant (Muralidhar *et al*., 2001; Xu *et al*., 2010). Under the optimum condition, the predicted maximum yield was 9.4 ± 0.41.

(b). Final Equation in Terms of Coded Factors

$$\text{Yield} = +9.77-0.19 * A-0.36 * B -0.13 * C +0.34 * A * B +0.40 * A * C +0.21 *B*C+ 1.40 *A^2-0.91* B^2 -1.83 * C^2$$



a



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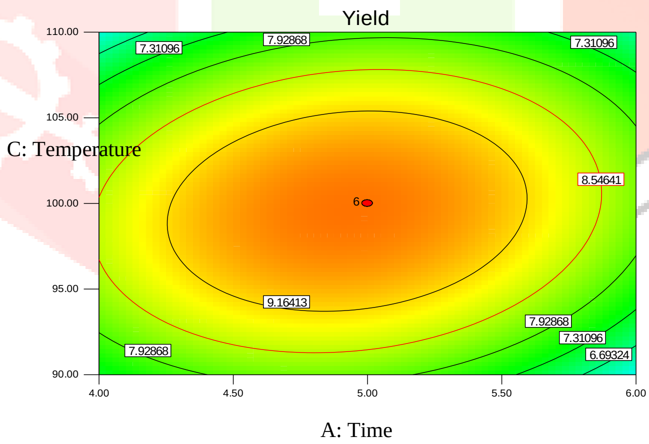
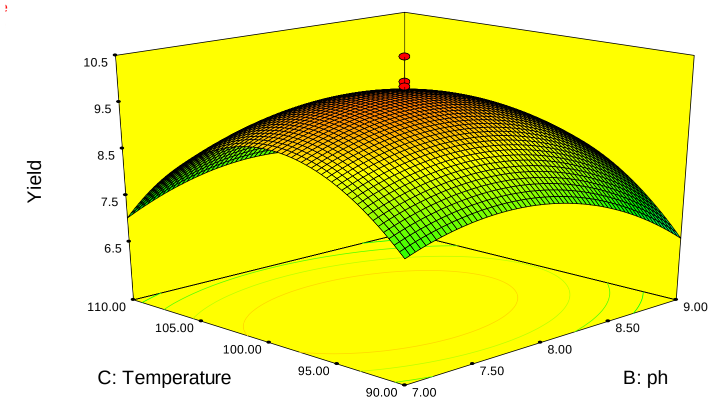


c

Fig. 2. Contour plot. a. Contour plot for time and pH, b. Contour plot for pH and temperature, c. Contour plot for time and temperature



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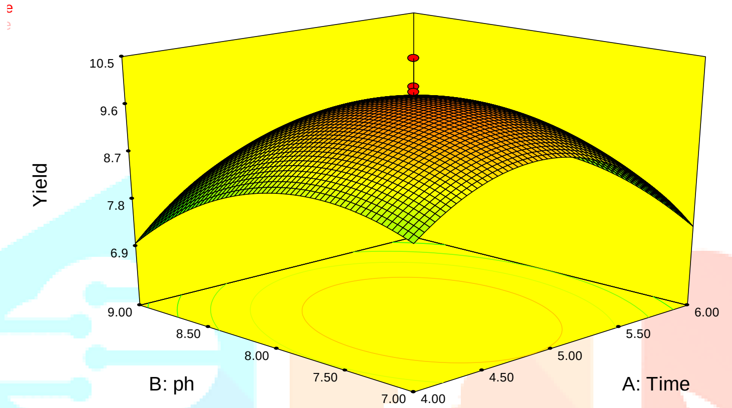

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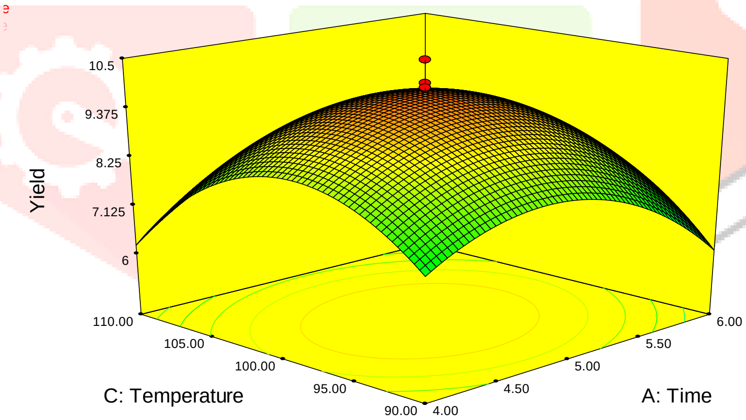
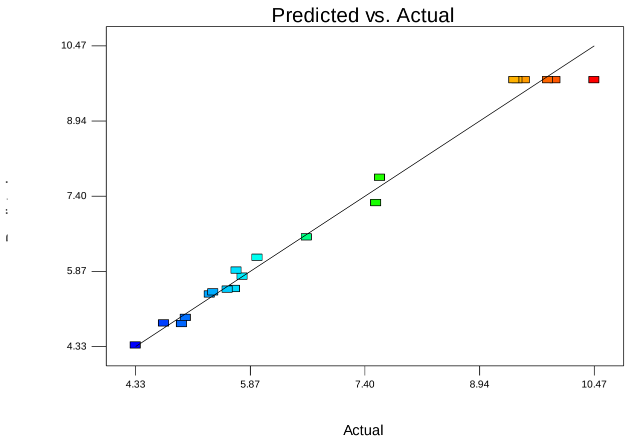
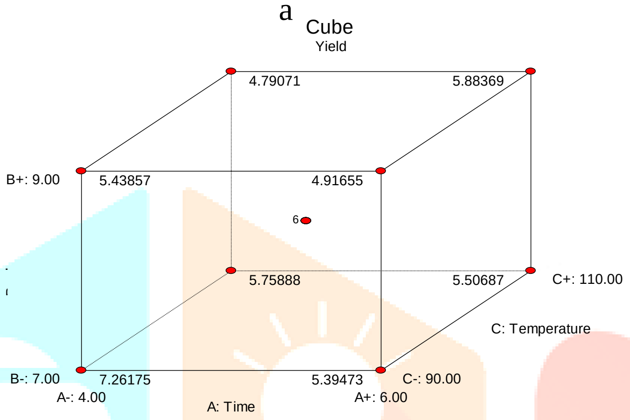


C

Fig.3. Response surface Methodology graph. a. Interaction between pH and temperature, b. Interaction between pH and time, c. Interaction between time and temperature



a



b

Fig. 4. a. Predicted vs actual, b. Cubic

Response surface and contour plot:

The 3D curve response surface and 2D contour plots representative of the regression equations of response yield. Response surface and contour plot showing the relationship between variable experiment with the response and the type of interaction between two variable tested. Circular contour plot indicates that the interaction between the corresponding variable are negligible, while elliptical contour plots indicate that the quality interaction between the corresponding variables are significant on the response (Qiao *et al*., 2009). Response surface plots and contour plots multiple response FSMC using Design - Expert software is presented in **Fig. 2** and **Fig. 3** . Effect of interaction pH and incubation time extraction is not significant on the yield (**Fig. 3a**) the shaped contour plots are circular. Interaction of temperatureand pH (**Fig. 3b**) & Temperature and incubation time (**Fig. 3c**) are significant on the yield evident from the shaped contour plots are elliptical.

Yield of the extract

The yield of fucoidan from optimized medium was 9.44 ± 1.47 % of the dry weight.

DISCUSSION

Observing a system or process while it is in operation is an important part of the learning process and is an integral part of understanding and learning about how systems and processes work. Observation on a system or process can lead to theories or hypothesis experiments demonstrate if these theories are correct. Investigators perform experiments in virtually all fields of inquiry, usually to discover something about a particular process or systems. Each experimental run is a test. More formally we can define an experiment as a test or series of runs in which purposeful changes are made to input variables of a process or system so that we may observe and identify the reasons for changes that may be observed in the output response. The investigator may want to determine which input variables are responsible for the observed changes in the response, develop a model relating the response to the important input variables and to use the model for process or system improvement or other decision making. Our focus is on experiments in engineering and science. Experimentation plays an important role in technology, commercialization's and product realization activities, which consist of new product design and formulation, manufacturing process development and process improvement (Montogomery, D.C. 1997)

The highest crude FSMC yield was 9.44 ± 1.47% of the dry weight was obtained from 100°C for 5hours at 8pH. The temperature played a key role in the FSMC yields. After 100°C the yield decreased with increase in temperature. Considering the pH, at 8 pH gave a high yield. However, incubation time at 5 hours there is considerable increase in the yield. The justification for the high yield in the alkaline pH is due to the NaOH. The hydrogen bonds in the cell wall were disrupted by NaOH which finally leads to the solubilisation of hemicellulose resulting in the hemicelluloses solubilisation. Therefore, it could effectively release insoluble polysaccharide and convert them into soluble polysaccharide. The extracted sulphated polysaccharides also contain minerals and protein which are strongly connected with them (Peasura *et al*., 2015). The yield of crude FSMC from acid hydrolysis is slightly lower than that of alkaline means of hydrolysis. During acid hydrolysis hemicelluloses, cellulose and lignin, structures were hydrolysed by acid, which was released into sulphated polysaccharide. However, sulphated polysaccharide could also be hydrolysed by acid, as they were mainly short chain polysaccharide this would reduce the final recovery (Huang *et al.*, 2010). Water in the neutral pH as solvent gave less yield of crude FSMC. Because it can dissolve only long chain soluble polysaccharides.

In previous study of Black *et al.*(1952) reported how the use of different extraction methods influenced the quantity of fucose with a disparity of 20% to 80% of total fucose of *Fucus vesiculosus* fucoidan and 20% to 55% of total fucose of *Pelvetia canaliculata* fucoidan.

The normal yield of FSMC of about 5.651 ± 1.62 % of the dry weight but 9.44 ± 1.47when extracted with alkaline pH 8 at 100°C for 5 hours. The influence of extraction method on fucose containing sulphated polysaccharides yield is further exemplified by data for the yield of *Laminaria japonica*. The yield only 1.5% of the dry weight (DW) of the seaweed when extracted with alkaline solution at 95°C for 2 hours (Sakai *et al*., 2002). But 2.3% DW when the extraction was done with water in autoclave at 120°C for 3 hours (Wang *et al*., 2008). On the other hand, the FCSPs notably fucoidan yield of a combined extract of *F. evanescens* was 12.9% DW when extracted with four times with 2% $CaCl_2$ solution at 85°C for 5 hours (Bilan *et al.*, 2002). While cold extraction with 0.4% HCl at 25°C for 5hours yielded 12.0% DW (Zvyagintseva *et al*., 2003). Typically, the maximum FCSPs yields from (dried) brown seaweeds range from 5 -7% DW. Fucoidan yields extracted from *F. vesiculosus* have thus been reported to be 7.0% DW. While the fucoidan obtained from *Sargassum horneri* and *Undaria pinnatifida* were found to be 5.2 % DW and 6.8% DW respectively (Kuda and Ikemori 2002).

Saravanan *et al*., (2016) extracted sulfated polysaccharides (fucoidan) from brown seaweed *Saccharina japonica* using conditions of temperature (80–200°C), pressure (5–100 bar), and solvents (water, 0.1% sodium hydroxide, 0.1% formic acid, 70% ethanol, 50% ethanol, and 25% ethanol).The best crude fucoidan (CF) yield was 8.23%, obtained from 140◦C and 50 bar (sodium hydroxide). In alkaline hydrolysis Saravanan *et al*. (2016) yield more amount of fucoidan than acid hydrolysis.

Conclusion

The response surface methodology proved to be useful for optimizing the extraction of fucoidan from *S. cinereum.* It is very useful and effective tool in selecting optimum extraction conditions. In various pharmaceutical formulations fucoidan is a main ingredient. Due to the potential area of application for fucoidan, utilization of fucoidan in the biomedicine and pharmaceuticals were extended. Thus RSM will be helpful to obtain the compound with desire quality and characteristics in a required quantity.

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