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Optimization of Cultural Conditions for Production of Extracellular Polysaccharide by *Halomonas xianhensis* SUR308 Using Weighted Response Surface Methodology

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Authors' contributions

This work was carried out in collaboration between all authors. Author JB conducted the experiments, compiled the results, managed the literature survey and prepared the draft manuscript. Author GD designed the statistical protocol, performed the statistical analysis and contributed in writing the manuscript. Author AKP designed the study, experimental protocol, managed the analyses of results and preparation of final manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Optimization of different physico-chemical and cultural parameters such as temperature, pH, incubation time and inoculum dose along with variation of NaCl, glucose and casein hydrolysate concentrations were carried out in the present investigation for the enhancement of extracellular polysaccharide (EPS) production by *Halomonas xianhensis* SUR308. Weighted response surface methodology (WRSM) was applied to study the interactive effects of these seven significant variables on EPS production by the isolate. A second order polynomial regression model for weighted response of the growth (O. D. at 540 nm) and production of EPS (g/L) was used to analyze the experimental data following the analysis of variance (ANOVA). Such analysis showed that the model was very significant ($p < 0.05$). Interaction among glucose, NaCl and pH was found to be most effective for growth and EPS production by *H. xianhensis* SUR308. The estimated

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optimum conditions of these variables obtained by using desirability function were 3.49% glucose, 2.5% NaCl and pH 6.8. Under such conditions the isolate produced 7.8 g/L of EPS which justify the predicted value (7.45 g/L) of EPS production in WRSM.

Keywords: Weighted response surface methodology; extracellular polysaccharide; Halomonas xianhensis; batch culture; polynomial regression model; ANOVA.

1. INTRODUCTION

Media formulation and process optimization are key steps in bioprocessing employing a rationally selected microbial strain. The influence of both nutritional components as well as environmental factors on bacterial growth and production of extracellular polysaccharides (EPS) needs to be explored for proper understanding of the fermentation process and also to maximize the yield of product. As early as 1985, Margaritas and Pace [1] have pointed out that successful designing of the fermentation process involves selection and optimization of media components, fermentation conditions, designing of fermenter as well as developing superior strains by mutation. Medium optimization by employing the "one-factor-at-a-time" method involves changing one independent variable while keeping all the others at a particular level [2]. This single-dimensional approach is laborious and time-consuming, especially for a large number of variables, and does not guarantee the determination of optimal conditions [3-4]. Such drawback of the one-factor-at-a-time method could be solved by statistical optimization techniques [5]. Factorial design and response surface methodology (RSM) are important statistical methods for optimization of number of variables by following only a few experimental trials [6].

Response surface methodology is a collection of statistical techniques for designing experiments, building models, evaluating the effects of a number of factors and searching for the optimum conditions for process optimization with higher efficiency and simplicity [7]. It is an ideal method to study and quantify the individual and combined effect of different parameters [8]. This statistical method utilizes quantitative data from appropriate experimentation to determine and simultaneously solve multivariate equations.

Currently, interest in finding the optimal experimental conditions for more than one response by using response surface methodology (RSM) is frequent in studies employing product quality improving techniques. Often in industries, it is difficult to analyze the results obtained from RSM when several dependent variables (responses) of interest, especially of opposing nature, are involved. This fact makes optimization procedure a challenging issue in industrial processes. The analysis of data from a multi-response experiment requires careful consideration of the multivariate nature of the data. Interrelationships that may exist among the responses can render univariate investigation meaningless. Several publications have presented approaches addressing multiple quality characteristics but very few published papers have focused primarily on the existence of correlation. If we desire to optimize several correlated responses simultaneously, we would end up in obtaining futile separate individual optima. Optimal condition for one response may be far from real optimum or even physically impractical for the others or may produce unsatisfactory results for the remaining responses. Thus, if correlations among quality characteristics are ignored, the designers may miss finding design variable settings that simultaneously improve the quality of all the responses, which in-turn could lead to an unrealistic solution causing model instability, over-fitting and errors of prediction.

Since the growth and production of EPS are highly correlated to each other, then we can combine these two responses into single response giving appropriate weights, called weighted response and we can use this weighted response as a representative of these responses. Then we analyze the data of weighted response using RSM. This process is called weighted response surface methodology (WRSM). Taking into account all the information mentioned above, it seemed prudent to investigate the performance of the combined approach modeling (WRSM) along with desirability function. The application of this new hybrid method is likely to improve the predictive accuracy of the model over the individual methods.

Response surface methodology has been successfully applied in the optimization of media

composition [9-10] and fermentation processes [11-12] of production of extracellular polysaccharides using microorganisms. Production of extracellular polysaccharides by microorganisms from hypersaline sources is mainly limited in halophilic bacteria and archea belonging to the genera *Halomonas, Alteromonas, Salipiger, Haloferax* and *Haloarcula* [13-14]. Optimization of conditions for EPS production by these strains was mostly accomplished by one factor at a time. Very recently, statistical experimental designs including factorial design, RSM have been implied in optimizing the cultural conditions for EPS production and found more reliable in predicting the substrate concentration with less accidental errors than the classical experiments [15] and maximum EPS production by bacterial strains through fermentation process [16].

During the survey of diversity and EPS production by halophilic bacteria from multi-pond solar salterns, a potent halophilic bacterial strain identified as *Halomonas xianhensis* SUR308 (Genbank Accession No. KJ933394) has been isolated [17-18] and the physico-chemical properties of the purified extracellular polysaccharide produced by the strain have been evaluated [19]. Here, we report the optimization of cultural conditions for production of extracellular polysaccharide by *H. xianhensis* SUR308 under batch culture using weighted surface response methodology.

2. MATERIALS AND METHODS

2.1 Bacterial Strain and Growth Condition

Halomonas xianhensis SUR308 (Genbank Accession No. KJ933394), a potent EPS producing moderately halophilic bacterium was used throughout this study. The bacterium was isolated from solar salterns of Surala, Odisha, India [17]. The isolate was maintained on slopes of medium for halophiles (MH) [20] by subculturing at a regular interval of one month and stored at 4°C as and when required. The medium contained (g/L) yeast extract (10), protease peptone (5), glucose (1), NaCl (100), $MgCl_2 \times 6H_2O$ (7), $MgSO_4 \times 7H_2O$ (9.6), $CaCl_2 \times 2H_2O$ (0.36), KCl (2), $NaHCO_3$ (0.06) and NaBr (0.026) (pH 7.2).

2.2 Production of Extracellular Polysaccharide

Growth and extracellular polysaccharide (EPS) production by the isolate SUR308 were monitored in malt extract–yeast extract (MY) medium [21] under batch culture. The MY medium contained (g/L) NaCl (100), $MgCl_2 \times 6H_2O$ (9), $MgSO_4 \times 7H_2O$, (13), $CaCl_2 \times 2H_2O$ (0.2), KCl (1.3), $NaHCO_3$ (0.05), NaBr (0.15), $FeCl_3 \times 6H_2O$ (0.005), glucose (10), yeast extract (3), malt extract (3), casein hydrolysate (5) (pH 7.2). The medium (20 ml/100 ml flask) was inoculated with freshly grown culture at 2% level and incubated at 32°C under continuous shaking. To establish conditions leading to the maximum EPS production, the isolate was grown under different cultural variables such as NaCl, temperature, pH, glucose and casein hydrolysate. Growth of the isolate SUR308 was determined by measuring optical density (O. D.) at 540 nm using an ELICO (CL 157) colorimeter. Each experiment was conducted in triplicates and the average ± SE was recorded.

2.3 Extraction and Quantification of EPS

The EPS produced by the growing culture was isolated and quantified according to the method as described by Quesada et al. [21]. To extract the soluble EPS, the culture after definite period of growth was centrifuged at 12000×g for 30 min, three volumes of chilled ethanol was added to the supernatant and kept overnight at 4°C for complete precipitation of the EPS. The precipitate was then collected by centrifugation at 12000×g for 30 min. The cell bound EPS was extracted by treating the cell mass with hot normal saline for 10 min under vigorous shaking. Following this the cell mass was separated by centrifugation at 10000×g for 15 min. Subsequently the EPS in the supernatant was recovered as per the method of soluble EPS extraction described above. The soluble and cell bound EPS were pooled, dissolved in known volume of water and quantified for total carbohydrate.

The carbohydrate content was determined by Dubois method [22]. To 1 ml of EPS solution, 0.5 ml of 5% phenol and 3.5 ml of concentrated sulfuric acid was added and incubated at 50°C for 20 min. Absorbance was recorded at 490 nm and the concentration was determined from the calibration curve prepared in the same method using glucose as standard.

2.4 Weighted Response Surface Methodology (WRSM)

Weighted response surface methodology (WRSM) was used to determine the optimum

concentration of the variables for enhancement of EPS production. Average value and standard deviation were computed for each experimental run. Systematic study of the relative contribution of the seven independent variables, namely NaCl (%), glucose (%), casein hydrolysate (%), inoculum (%), aeration (CVF ratio), temperature (°C), pH (X_1, X_2, X_3, X_4, X_5, X_6 and X_7, respectively), at -2.83, -1, 0, 1, and 2.83 levels (values) (Table 1) were followed for optimization of EPS production. The growth, O. D. at 540 nm (Y_1) and production of EPS, g/L (Y_2) was chosen as the dependent variables. As the analysis of data from a multi-response experiment requires substantial inclusion of almost the whole data set or every evaluated response, the response variables should not be investigated individually and independently of one another. Interrelationships that may exist among the responses can render such univariate investigation meaningless. Ribeiro et al. [23] stated that if the Pearson correlation coefficient (r) between two variables in a multiple response system is ≥0.6, it can be considered that the corresponding variables have good degree of linear association between them. Accordingly, a strong linkage was observed between Y_1 and Y_2 (Pearson correlation coefficient, r=0.87128). Here it was also observed that correlation between $(Y_1+Y_2)/2$ and Y_1 and between $(Y_1+Y_2)/2$ and Y_2 are very high (i.e. 0.97807 and 0.95439 respectively). Thus $(Y_1+Y_2)/2$(=W, say) is taken as a weighted response of Y_1 and Y_2 and we use this weighted response as dependent variable.

In the present investigation, RSM is being selected as it is the most currently used methodology in optimization procedures, especially when the experiment is intended to find improved or optimal process settings. Data were approximated to a second-order polynomial equation and analysis of variance (ANOVA) was generated. The significances of polynomial relations were examined statistically at a probability (*p*) of 0.05. Statistical analysis was performed in MINITAB (version 16, Minitab Inc., US) software. Weighted response is fitted to a polynomial regression equation with appropriate degree. Desirability function is used for judging the predicted weighted response under optimum condition and for determining the optimum values of the causal factors (independent variables) that maximize desirability (D).

The estimated response surface model for weighted response obtained from the polynomial regression analysis as a function of the uncoded factor levels are as follows:

$$W=-58.4972+2.5937X_2+13.4331X_7-0.0381X_1^2-0.2996X_2^2-0.8428X_7^2$$

Table 1. Results of regression analysis of the second order polynomial model for optimization of EPS production (ANOVA table for weighted response)

Term	Coefficient	P value
Constant	-58.4972	0.024
NaCl (X_1)	2.7715	0.449
Glucose (X_2)	2.5937	0.003
Casein hydrolysate (X_3)	1.6168	0.702
Inoculum (X_4)	0.2887	0.349
Aeration (X_5)	-6.7098	0.567
Temperature (X_6)	0.4896	0.546
pH (X_7)	13.4331	0.008
NaCl*NaCl (X_1^2)	-0.0381	0.007
Glucose*Glucose (X_2^2)	-0.2996	0.047
Casein hydrolysate* Casein hydrolysate (X_3^2)	-2.4585	0.505
Inoculum*Inoculum (X_4^2)	0.0252	0.549
Aeration*Aeration (X_5^2)	4.2980	0.830
Temperature*Temperature (X_6^2)	-0.0101	0.472
pH*pH (X_7^2)	-0.8428	0.008
NaCl (%)*pH (X_1X_7)	-0.3035	0.551

***The other terms cannot be estimated and were removed*

3. RESULTS

3.1 Response Analysis

Model adequacy was confirmed from the ANOVA table (Table 1), where the p-value for lack-of-fit (>0.05) and adjusted R^2 value (83.27%) show that the model describes the experimental points adequately. Furthermore, it was observed from probability plot (Fig. 1), that the weighted response (W) had the normal distribution.

The response surface plot for the weighted response scores as a function of glucose (%, w/v) and pH is shown in Fig. 2a, which indicates that the weighted response (W) are very sensitive to changes in glucose and pH of the medium. The increase in percentage of glucose and pH increased the weighted response significantly. Similarly, increase in glucose and NaCl increased the weighted response up to a particular point followed by a decrease (Fig. 2b), while increase in NaCl and pH increased the weighted response up to a particular point followed by a decrease (Fig. 2c).

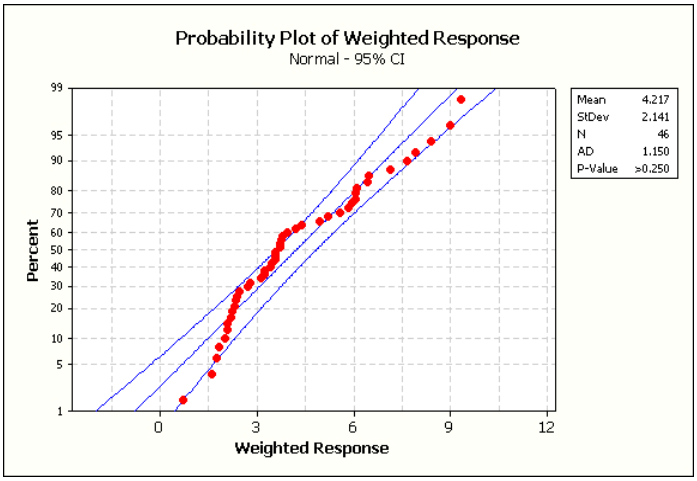

Fig. 1. Normal distribution of weighted response in probability plot

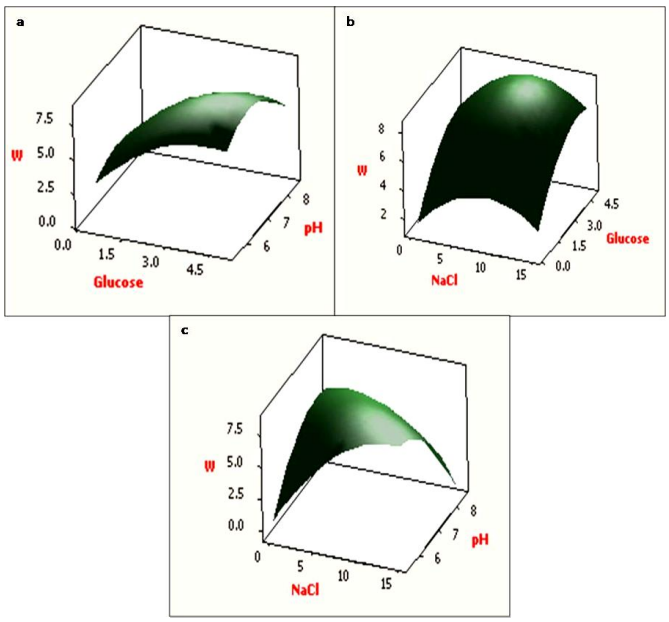

Fig. 2. Surface plot of weighted response against glucose (%) and pH (a), NaCl (%) and glucose (%) (b), NaCl (%) and pH (c) of the growth medium

3.2 Determination of Original Responses from Weighted Response Using Linear Regression Analysis

Linear regression analysis (LR) is one of the simplest statistical tools, widely used for expressing the dependence of a response variable on a predictor variable. So, for estimating the predicted value of the original response, it is plausible to consider the linear dependencies of the responses with weighted response, by using LR. The obtained linear regression equations are given below:

$$\left.\begin{array}{l}\text{Response 1} = 1.19\ W \\ \text{Response 2} = 0.810\ W\end{array}\right\}$$

All regression coefficients in the above mentioned linear equations are significant (p-value < 0.05). These empirically developed mathematical models provide a method of linking the dependent variables Y_1 and Y_2 to the explanatory variable (W). Substituting the W score of Table 2 in the above equations, the predicted values of the original responses were calculated and compared with the experimental data. Adjusted R^2 value was calculated to indicate the co-linearity between the observed and predicted results of each response. High *adj* R^2 value (95.6% for response 1 and 90.9% for response 2) well supports the use of weighted responses as the representative of the responses for correlated data in RSM.

3.3 Determination of Optimum Condition by Desirability Function

The desirability function approach is one of the most widely used methods in industry for the optimization of multiple response processes. It is based on the idea that the "quality" of a product or process that has multiple quality characteristics, with one of them outside of some "desired" limits, is completely unacceptable. The method finds operating conditions that provide the "most desirable" response values. This function transforms the estimated response variable $\hat{y}$ (in this case, $\widehat{W}$), into a desirability value *d*. The values of *d* vary in the interval $0 \leq d \leq 1$ (Eq. (1)), increasing as the desirability of the corresponding response increases.

$$d = \begin{cases} 0; & \hat{y} < y_{min} \\ \frac{\hat{y} - y_{min}}{y_{max} - y_{min}}; & y_{min} < \hat{y} < y_{max} \\ 1; & \hat{y} > y_{max} \end{cases} \quad (1)$$

Thus, in order to optimize W scores, the value of d is calculated [Eq. (1)], that was prioritized by specifying the goals and boundaries (maximum and minimum values) of W score obtained within the experimental region. Based on the proposed upper and lower bounds, the predicted W scores (= 9.25) and its desirability (= 1.000) at optimal condition is presented in Fig. 3. The behaviour of the predicted W was generated from the optimized factor of 2.5% of NaCl, 3.49% of glucose, 0.5% of casein hydrolysate, 7.88% of inoculum, 1:5 of CVF ratio (aeration) at 30°C and pH 6.8 (Fig. 3) (in order to make these parameters feasible in experimental runs, these observed optimum parameters were drawn to the nearest round figures [24].

The observed experimental values (mean of 30 measurements) were compared to the predicted values, for verifying the predicted factor settings and to check whether or not the optimal conditions derived from the experiment actually result in an improvement in product or process quality. The predicted values could realistically be achieved within a 99% confidence interval of experimental values (Table 2 and Fig. 4a, b and c).

Furthermore, it was observed from probability plot (Fig. 5a, b and c), the weighted response (W), response 1 and response 2 obtained under optimal conditions had the normal distribution. The predicted value of response 2 was much lesser than the experimental readings, but was close to the lower limit of the 99% prediction intervals. Nonetheless, in the confirmatory experiment, the experimental values are in reasonable agreement within the said confidence intervals for these optimized conditions. The closeness between the experimental and predicted values of the quality parameters also indicated the suitability of the corresponding models.

4. DISCUSSION

Seven variables such as temperature, pH, aeration, NaCl, glucose, casein hydrolysate and inoculum density were selected and values of responses (growth and EPS production) were obtained under different experimental sets as in CCD experimental plan. To determine optimal levels of the test variables for the amount of EPS production by *H. xianhensis* SUR308, the 3D response surface was described by the regression model (Table 1).

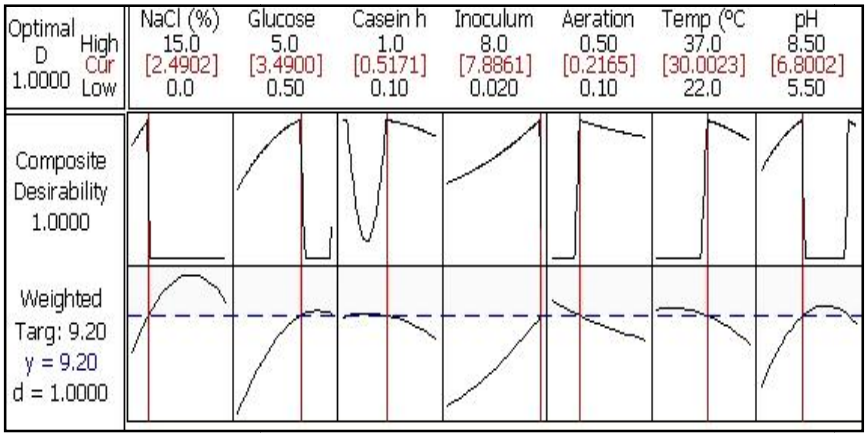

Fig. 3. Based on the proposed upper and lower bounds, the predicted W scores and its desirability under optimized conditions

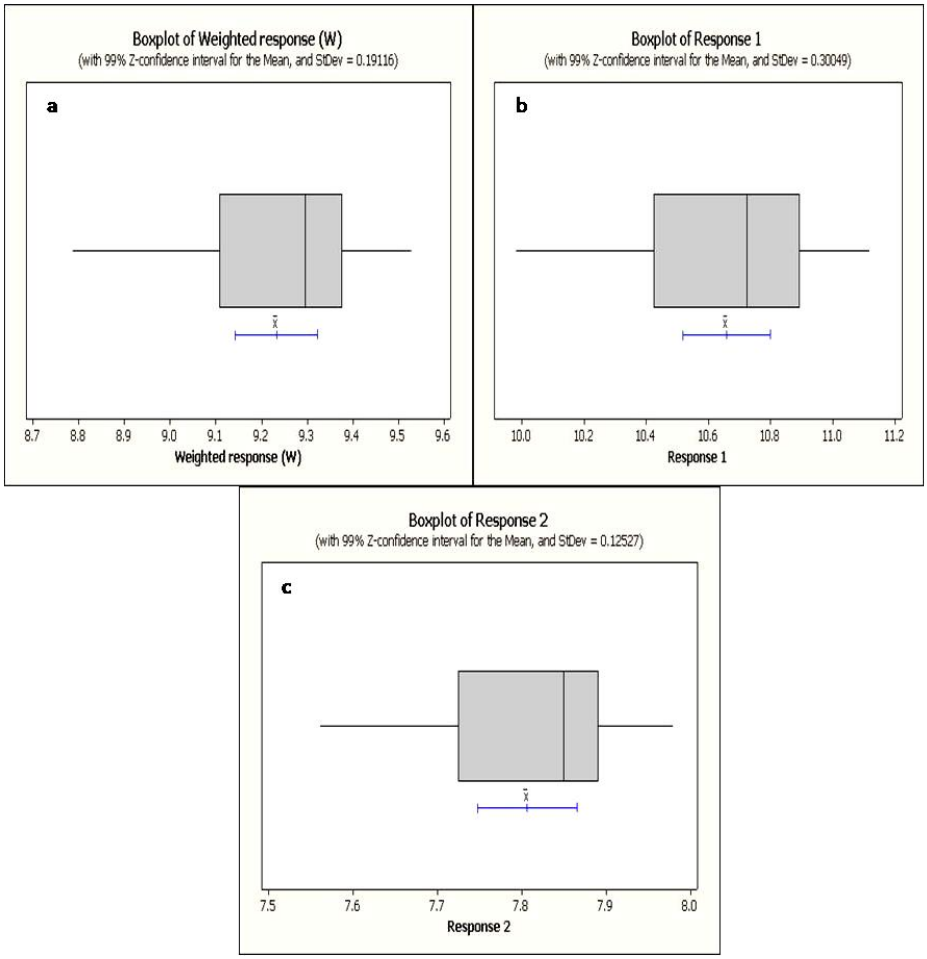

Fig. 4. Box plot of weighted response (a), response 1 (Growth, O. D. at 540 nm) (b) and response 2 (production of EPS) (c) within a 99% confidence interval of experimental values

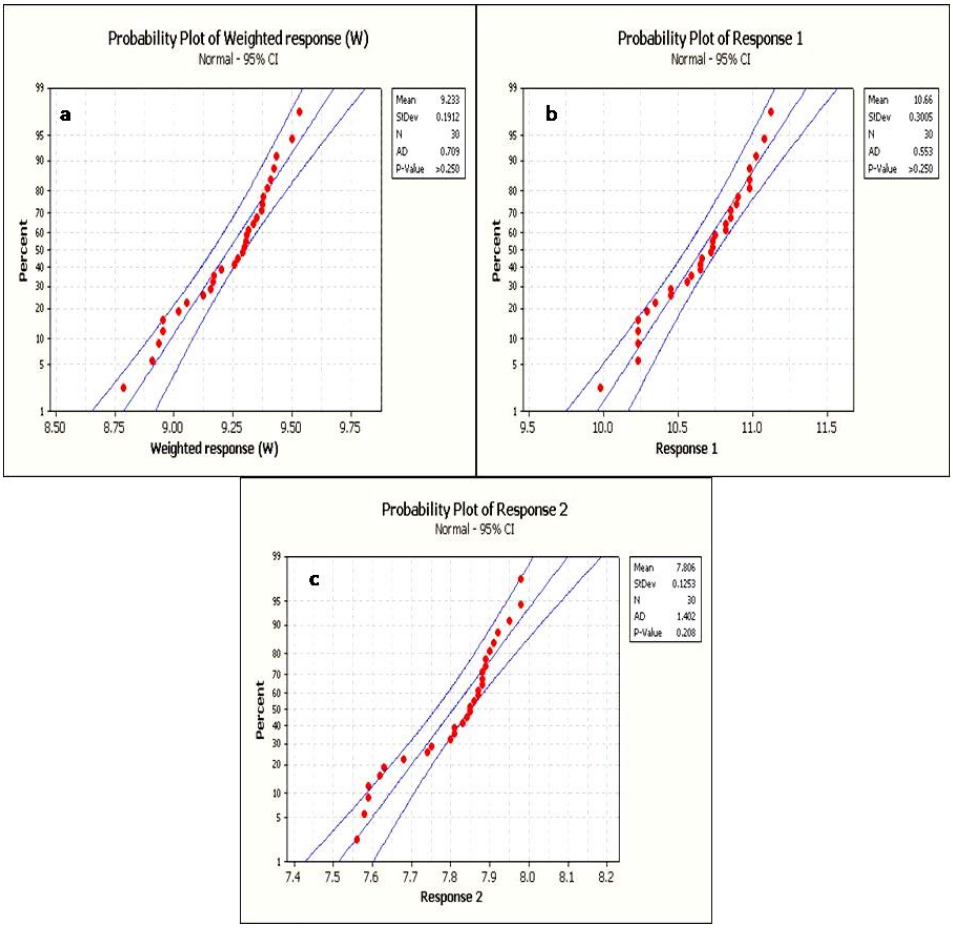

Fig. 5. Probability plot of weighted response (a), response 1 (Growth, O. D. at 540 nm) (b) and response 2 (production of EPS) (c) at 95% confidence interval of experimental values

Table 2. Confirmatory trials of the optimal conditions by comparison of experimental and predicted values

Response	No. of observation	Predicted value	Experimental value	SD**	99% confidence interval
Weighted response (W)	30	9.2	9.2327±0.1912	0.0349	(9.1428, 9.3226)
Response1 Growth, O. D.)	30	10.948	10.6590±0.3005	0.0549	(10.517, 10.8003)
Response 2 (EPS, g/L)	30	7.452	7.8063±0.1253	0.0229	(7.7474, 7.8652)

*** Each experimental value represents the means ± standard deviation from 30 replicates (n =30)*

The corresponding analysis of variance (ANOVA) is represented in three dimensional graphical response surface plots (Fig. 2, 3 and 4). The surface plots showed a significant P- values that suggested that the data was good fit with the model and was further confirmed by determination of coefficient (R^2) with R^2 of 95.6% for response 1 and 90.9% for response 2.

The response surface plots have provided a visual interpretation of interaction between variables and help in determining the optimal conditions by revealing the significance of

interactions among the variables. In this study, the interaction between the variables viz. glucose, NaCl and pH is significant. Similarly, Jeganathan et al. [25] and Manivasagam et al. [26] have demonstrated a significant interaction between glucose and NaCl using RSM in EPS production by *Halobacillus trueperi* AJSK and *Streptomyces violaceus* respectively.

Under experimental set up with these cultural variables the predicted value of EPS production by the strain *H. xianhensis* SUR308 was 7.45 g/L with 3.49% (w/v) glucose and 2.5% NaCl at pH 6.8, while the actual value was found to be 7.8 g/L under these identical conditions. The higher actual value reveals the higher accuracy of the model. A maximum EPS production of 9 g/L by a marine bacterium with glucose was reported by Nahas et al. [27] by using RSM.

5. CONCLUSION

The present study demonstrated the interactive effect of different cultural conditions on growth as well as EPS production by the isolate *H. xianhensis* SUR308 following WRSM. Statistical analysis of the experimental findings revealed that the optimum combination of cultural conditions for EPS production by the isolate comprised of 3.49% glucose, 2.5% NaCl, 0.5% casein hydrolysate, 7.8% initial inoculum, 1:5 CVF at 30°C and pH 6.8. Under these established set of combination, the actual production of EPS (7.8 g/L) was higher than the predicted value (7.45 g/L), which further justified the advantage of RSM methodology in production technology.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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