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Optimization of medium and media components for maximum biomass of Halobacterium salinarum NRC-1 using Response Surface Methodology

Divya Patil[1*], Bhoomika Vinod[1], Prof. Kavitha RV[2]

[1*]Department of Biotechnology, PES University, Bangalore-560085.

[2]Assistant Professor, Department of Biotechnology, PES University, Bangalore-560085.

Abstract

Introduction: Often, biotechnological advances are hindered by low growth rate of microorganisms utilized in the process, making optimal design of culture media a crucial aspect to consider in the biotechnology field. **Materials and Method**: Optimization of media components for growth and biomass production of *Halobacterium salinarum* NRC-1, our organism of interest, was carried out using response surface methodology, a statistical approach to optimize and improve the performance of a process by analyzing the relationships between input variables and output responses. A linear model was estimated and media components were determined based on the linear regression equation generated by the model. The variables chosen were casein enzyme hydrolysate, yeast extract, arginine, and peptone. **Results & Discussion**: An optimum result for the four variables was predicted based on the experimental response, which is 7.5 g/L of casein enzyme hydrolysate and yeast extract each, and 5.0 g/L each of peptone and arginine. The optimized medium reduced the time required for the cell culture to attain stationary phase, and showed a significant increase in the amount of biomass obtained as compared with that in standard media.

Keywords: *Halobacterium salinarum*, Response surface methodology, Optimization, Cultivation media

*Author for Correspondence.E-mail: patildiv01@gmail.com

Introduction

Oftentimes, biotechnological advances are hindered by low growth rate of microorganisms utilized in the process, making optimal design of culture media a crucial aspect to consider in the field of biotechnology.

Response Surface Methodology (RSM) is a statistical approach employed to optimize and improve the performance of a system or process by analyzing the relationships between input variables and output responses. It involves the use of statistical models and experimental design to explore the effects of multiple input variables on a response of interest, and to identify the optimal set of input variables that result in the desired response. The objective of response surface methodology is to determine the optimum levels of variables that can maximize the desired response (in this case, microbial growth). It is a useful tool for optimizing growth media, as it allows determination of the optimum levels of variables that can maximize microbial growth while minimizing the number of experiments required. [9, 20]

Halobacterium salinarum is an obligate extreme archaeon that grows in NaCl concentrations near or at saturation. A high internal salt concentration (4.0-5.0 M) is maintained in order to cope with the high osmotic pressure, therefore being iso-osmotic with the environment [4,12]. The studies and findings on the natural habitat of *H. salinarum* aided scientists in developing a growth media that provided a synthetic environment for the enrichment of the said archaeon in laboratory conditions [5, 6, 8]. According to some studies, the addition of monosaccharides in the growth media was shown to have poor biomass production due to repression of metabolic activity. Various tested nitrogen sources induced good growth of *H. salinarum*, especially partially hydrolysed casein in the form of casein enzyme hydrolysate, which is regarded as a good substrate for haloarchaeal growth.

In 1971, bacteriorhodopsin, a light-driven proton pump, was isolated from the purple membrane of *H. salinarum*, drawing significant attention to it. Bacteriorhodopsin is an outer membrane protein that converts the energy of light at an absorption maximum of 568 nm, into an electrochemical gradient which is used for ATP production by ATP synthase. It is a light-driven proton pump that transports protons in and out of the cell, exemplifying vectorial catalysis. The reversible light-triggered color change has aided in the advancement of biotechnological applications, such as in bioelectronic devices and optical information recording [13]. The latest advances are being explored in the field of Biosimilars. Mass cultures of *H. salinarum* are pink-red in color, which originates from the bacterioruberin. Its ability to tolerate high salt conditions enables its cultivation in non-sterile or minimally sterile conditions, thereby reducing costs [18,17].

Materials and Methods

Haloarcheal isolate of *Halobacterium salinarum NRC-1* was procured from the culture maintained at the National Chemical Laboratory (NCL-CSIR) of the Council of Scientific and Industrial Research in Pune, India.

The salts (Calcium chloride, Ferrous sulfate, Magnesium chloride, Magnesium sulfate, Manganous chloride, Potassium chloride, Potassium nitrate, Sodium chloride, Trisodium citrate) used during the study were all procured from S. D. Fine-Chem Limited. Yeast Extract, Casein Enzyme Hydrolysate, and Peptone were procured from HiMedia Laboratories.

Liquid cultures of the haloarchae were grown in 125mL Erlenmeyer flasks containing 25mL growth media at 37 ℃ at 100 rpm in a thermostatic orbital shaker (Ascension Innovations Pvt. Ltd., Bengaluru, India) [1, 2]. Optical density of the liquid culture was measured using the UV-spectrometer (Eppendorf Biospectrometer) at regular 24-hour intervals from the time of inoculation.

Microorganism and media

The haloarchae was grown in standard media (as defined by NCL Pune) which comprised 200 g of NaCl, 20 g of MgSO4, 2 g of KCl, 0.05 g of FeSO4, 0.04 g of MnCl2, 7.5 g of casein enzyme hydrolysate, 10 g of yeast extract, and 1 g of tri-sodium citrate dissolved in 1 L of distilled water. The archeon was grown in both solid and liquid cultures.

Basal media was used for the optimization of media components and their concentration. It comprised of 200 g of NaCl, 2 g of KCl, 1 g of KNO_3, 16 g of MgSO4, 0.02 g of FeSO4, 0.01 g of KH2PO4, 0.2 g of CaCl2, and 3 g of Sodium citrate in 1 L of distilled water. Each of the salt plays an essential role in the development of cell organelles in the archeon [2, 3, 5]. Different concentrations of the nutrients; Casein Enzyme Hydrolysate, Peptone, Arginine, and Yeast Extract, were added as obtained by the experimental runs further defined.

Liquid suspension culture for both media was performed in triplicates. The growth was observed from the initial lag phase to the final death phase during the very first trials for the standard media. Cell concentration was measured by taking the optical density of the sample using a UV-spectrometer. The growth curve obtained after plotting the OD readings against the number of days served as reference for future comparison between the optimized media and the standard media.

Screening for media and the nutrient components

As per previous studies, NaCl concentration of 20% w/v of 4M was found to be an optimal, essential, and necessary requirement for the growth of *H. Salinarum* [19]. The other salt concentrations excluding the nutrient sources; casein enzyme hydrolysate, yeast extract, peptone and arginine, were kept constant as mentioned in the basal media composition. Extensive survey of literature suggests that the four aforementioned nutrients are crucial and essential for the growth of haloarchaea. Monosaccharide sugars that are commonly found in growth media of other bacteria are not present here as this species doesn't perform glycolysis. Energy is generally produced by anaerobic respiration which requires amino acids and is provided by casein enzyme hydrolysate [2].

Peptone, although not present in the standard media, is included here due to its ability to serve as an organic nitrogen source and is known to play a vital role in multiplying cells in the initial

stages of growth cycle [6]. It was taken care that phosphates were autoclaved separately and added to the main growth media to avoid any type of precipitation.

Generating Experimental Runs

All the four nutrients subjected to analysis on their effect upon the archaeon growth were studied and an estimated range was decided after surfing research papers. The range as collected from various resources was known to provide positive growth in terms of biomass.

The fixed range was then taken as input to obtain experimental runs. Design Expert 13.0 (StatEase, Minneapolis, USA) tool was used to run the variables. Central Composite Design (CCD) was employed, which provided 30 experiments with values within the specified range. CCD produces ideal value combinations in more than 20 sets through factorial or fractional calculations. Using 4 variables (nutrients) in this case generated 30 experiments in total. Some repeated sets were observed as the model tries to cancel any uncertainty in results or human error.

		Factor 1	Factor 2	Factor 3	Factor 4
Std	Run	A:Casein Enzyme Hydrolysate	B:Yeast extract	C:Peptone	D:Arginine
		g/100 ml	g/100 ml	g/100 ml	g/100 ml
28	1	0.75	1	0.6	0.625
20	2	0.75	2	0.6	0.625
2	3	1	0.5	0.2	0.25
29	4	0.75	1	0.6	0.625
12	5	1	1.5	0.2	1
11	6	0.5	1.5	0.2	1
10	7	1	0.5	0.2	1
19	8	0.75	0	0.6	0.625
7	9	0.5	1.5	1	0.25
14	10	1	0.5	1	1
3	11	0.5	1.5	0.2	0.25
15	12	0.5	1.5	1	1
9	13	0.5	0.5	0.2	1
18	14	1.25	1	0.6	0.625
6	15	1	0.5	1	0.25
27	16	0.75	1	0.6	0.625
16	17	1	1.5	1	1
17	18	0.25	1	0.6	0.625
1	19	0.5	0.5	0.2	0.25
13	20	0.5	0.5	1	1
22	21	0.75	1	1.4	0.625
26	22	0.75	1	0.6	0.625
4	23	1	1.5	0.2	0.25
8	24	1	1.5	1	0.25
5	25	0.5	0.5	1	0.25
23	26	0.75	1	0.6	-0.125
24	27	0.75	1	0.6	1.375
25	28	0.75	1	0.6	0.625
30	29	0.75	1	0.6	0.625
21	30	0.75	1	-0.2	0.625

Figure 01: Experimental runs as generated by Design Expert software using CCD

Respective growth curves were developed for each experimental run in the batches of 10 for a total period of 2 months. These experimental runs were performed with the same physical conditions as maintained with the standard media.

Response Surface Methodology

Response Surface Methodology (RSM) tool here is used to data analyze the results after all the 30 experiments are performed. Optical density using UV-spectrometer was recorded for all the 30 experiments. The OD reading complementing the stationary phase was converted to a suitable unit reflecting the biomass concentration.

The tool comprehends the input along with the responses for further analysis. Following the analysis, the results incorporated various mathematical models generated based on the given data. The tool also suggests the best suitable model for the performed experiments with respect to its results and delivers an optimum composition for the selected nutrient components.

Results and Discussion

Standard Curve

Initial establishment of standard/reference growth curve as developed by the OD readings obtained by the standard media was successful after multiple trials in replicates. The stationary phase was observed between 5.5 to 6th day after inoculation. The graph below reflects the above information with OD readings along the y-axis and number of days in the x-axis.

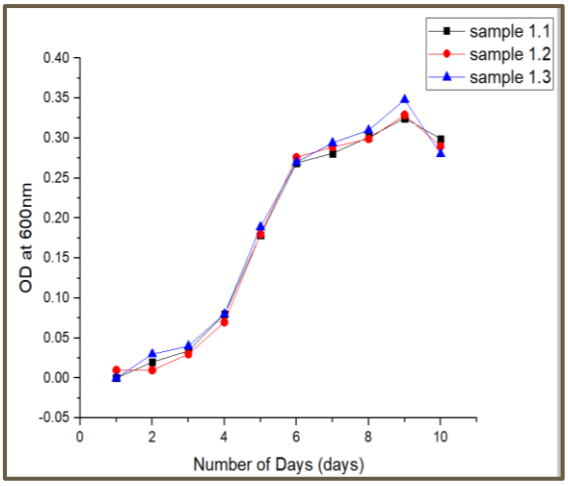

Figure 02: Growth curve plotted using standard media

This was further modified by taking the average of the replicate results for further smooth comparison with the in-house developed media.

Comparison of growth curves

The 30 experimental runs generated by the software based on CCD were divided into 3 sections and each batch consisted of 10 experiments. All the 30 experiments did not actually yield satisfying results. High salt concentration of the media also became a hindrance in some of the experiments as there was formation of salt crystals. Such low yielding and mediocre performing media composition was negated and out of the 30 combinations, six stood out the most in terms of good yield and media properties.

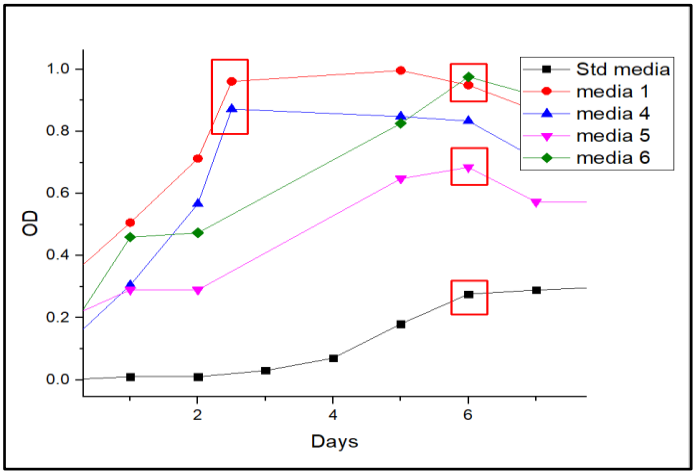

Figure 03: Comparison of growth curves with standard growth curve

The growth curves were plotted with the average OD reading against the number of days and then the results were conjugated with that of the standard growth curve. The cell culture concentrations at the stationary phase of *H. salinarum* as influenced by different concentrations of the selected nutrient sources are displayed in the graph above.

Experimental combinations 1, 4, 5, and 6 showed considerably good results among the six with respect to the biomass concentration. While combinations 1 and 4 appeared to catalyze the growth rate and reduce the time of attaining stationary phase to 2.5 days as compared with that of the standard media whose stationary phase was marked after 5.5 days.

Statistical analysis

The plotted data although served as manual comparison, further data analysis to evaluate the findings of the study was employed. RSM, a data analysis tool performed the calculations using Design Expert 13.0 (StatEase, Minneapolis, USA) software. The linear, quadratic, cubic, and 2-Interfacial effects of the independent variables were evaluated. To validate the statistical significance of the model, the Fisher's F-test for analysis of variance (ANOVA) and Student's t-test was performed on the experimental data.

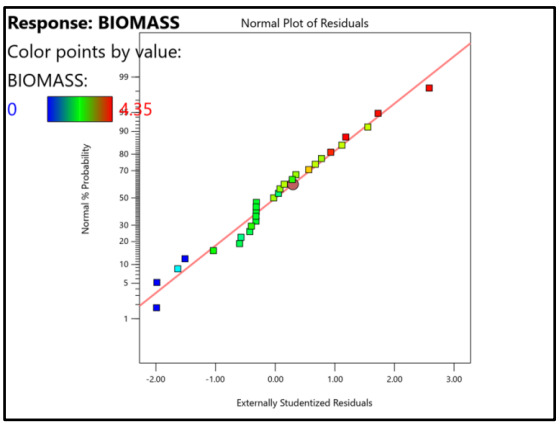

Figure 04: Studentized residual curve for the data

The response surface contour plots were observed, and the obtained linear regression equation was solved to determine the optimum concentrations of each of the selected variables.

Table 01: Biomass equation generated after data analysis

BIOMASS	=
+2.10	
+0.0897	Casein Enzyme Hydrolysate
+0.1047	Yeast Extract
+0.3138	Peptone
+0.1384	Arginine

The tool also suggested one optimum combination as shown below, that can be implemented with the in-house developed media. Solving the linear equation using the optimum combination gives the yield result as 0.0054 g/L

Confirmation Location #1

	CEH	YE	PEPTONE	ARGININE
	0.75	0.75	0.5	0.5

Figure 05: Optimum composition for the tested nutrients as suggested by the model

Conclusion

The optimized medium composition determined by response surface methodology efficiently enhanced the biomass of *Halobacterium salinarum*, achieving stationary phase at a time less than that required for standard media composition. The Design Expert software was used to determine the linear mathematical model that relates biomass of microorganisms obtained (g per L) to the concentration of the variables.

Y= 21+ 0.897*x1 + 1.047*x2 + 3.138*x3 + 1.384*x3, where Y is the predicted biomass of *H. salinarum* NRC-1 obtained and x1, x2, x3, and x4, the concentrations of casein enzyme hydrolysate, yeast extract, peptone, and arginine respectively. Considering the simplicity and inexpensive preparation of optimized media, the results of this study may be used for highly efficient production of *Halobacterium salinarum* NRC-1 on a bioreactor scale.

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Conflict of Interest

Conflict of interest declared none**.**

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