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Statistical Approach for Media Optimization of Vitamin B_{12} Production by using newly isolated *Rhizobium* species with submerged fermentation

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Abstract: *The present study deals with the improvement of the Vitamin B12 production by newly isolated Rhizobium sp. For this purpose various medium components were studied with Statistical approach to design fermentation medium. Eight variables were screened using Plackett Burman design. Further four selected variables were preceded with full factorial. Three significant variables from full factorial design such as mono sodium glutamate, cobalt nitrate and choline chloride concentration were optimized by using central composite design with response surface methodology.*

Using the optimal medium components (Choline chloride 6.25 g/l, Monosodium glutamate 1.50 g/l and Cobalt nitrate 0.38 g/l) the Vitamin B_{12} production was found five folds higher in the optimized medium as compared to the control medium.

Key words: *Full factorial, Plackett Burman, response surface methodology (Central composite design), Rhizobium sp, Submerged Fermentation, and Vitamin B_{12},*

I. Introduction

Vitamin B_{12} is also known as cobalamine is currently identified as the largest and most complex among all vitamins. A long term deficiency of Vitamin B_{12} can cause permanent damage of the brain and central nervous system (CNS). It can lead to anemia, fatigue, mania and depression when slight deficiency occurs. Vitamin B_{12} plays a key role in the normal functioning of brain and nervous system and involve in the formation of blood.

Vitamin B_{12} is required for the metabolism of every cell of the human body most importantly, DNA synthesis, regulation, fatty acid synthesis and energy production. Vitamin B_{12} can be synthesized by using bacteria by submerged fermentation [6].

For effective Vitamin B_{12} production, it is necessary to optimize culture condition and nutrient constituents of production medium. In the present study, three important statistical models Plackett Burman designs, full factorial and response surface methodology were applied to the screening and optimization of fermentation medium components in order to improve the process of Vitamin B12 production.

Method for optimization is routinely performed to achieve goals such as maximizing sensitivity and selectivity. Optimization of medium by the traditional method, involving changing one independent variable while fixing all others, is extremely time-consuming and expensive for a large number of variables. In general, the media have traditionally been optimized by the one variable -at- a time strategy, *i.e.*, Varying one factor while keeping all others constant. Although this strategy is simple and easy to apply without the need for statistical analysis, it involves a relatively large number of experiments and the interaction among factors is often ignored. To overcome this difficulty, Plackett Burman design, factorial design and response methodology can be used to optimize medium components [1]. The Plackett–Burman designs allow screening of significant factors for a large number of fermentation variables, and these designs are thus useful for selecting variables for further optimization processes [2]. Plackett-Burman experimental design assumes that there are no interactions between the different variables in the range under consideration. A linear approach is considered to be sufficient for screening. Plackett-Burman experimental design is a fractional factorial design and the main effects of such a design may be simply calculated as the difference between the average of measurements made at the high level (+1) of the factor and the average of measurements at the low level (−1). Factorial design can significantly reduce the number of experiments involved. Factorial designs were constructed to obtain maximum information from the least number of experimental runs [24].

Response surface methodology (RSM) is effectively used for designing experiments with optimum conditions for factors affecting the various bioprocesses [27]. A statistically designed optimization study is helpful in confirming previous effects and interactions of fermentation variables and in determining the optimum values of the critical factors [23]. The application of statistical experimental design techniques in fermentation process development can result in an improvement of product yield, reduce process variability, give closer confirmation of the output response to the experimental values and reduce overall cost. Prior

knowledge and understanding of the process variables is necessary for achieving a realistic model. RSM can be used to evaluate the relative significance of several factors even in the presence of complex interactions. [3][20].

Keeping in view the contribution to meet the increased demand of Vitamin B12 production at industrial level, the present study has been designed to improve the yield. Moreover, statistical approaches offer ideal ways for process optimization studies in submerged fermentation and have advantages because of the fundamental principles of statistics, randomization and replication. In contrast, mixture design and full factorial design are more efficient approaches that can deal with a large number of variables simultaneously. Moreover, the interaction among different variables can be estimated. To date, these techniques have been used by many researchers to optimize medium components, such as astaxanthin production from *Phaffia rhodozyma* [23], xylanase from halophilic eubacterium SX15 [29] , β- glucanase production from *Bacillus subtilis* ZJE-1A5 [26] avulanic acid from *Streptomyces clavuligerus* [28] micrococcin GO5 from *Micrococcus* sp. GO5 [12] and xyloketal A from *Xylaria* sp. 2508 [30].

The objective of this work was to apply a mixture design followed by a response surface methodology to investigate and optimize a medium which might positively affect Vitamin B12 production by the newly isolated *Rhizobium* sp.

II. Material and Methods

2.1. Microorganism and culture conditions

Newly isolated *Rhizobium* species was used in the present study for the Vitamin B_{12} production by submerged fermentation. The bacterial culture was maintained on a Yeast mannitol agar dehydrated medium, incubated for 48 hours at 37^{o}C then stored at 4^{o}C.

2.2 Fermentation process

The process of fermentation for Vitamin B_{12} production was comprised of three stages: lab, seed and Production medium. For the lab growth stage, grown culture from slant was inoculated into 250 ml Erlenmeyer flasks containing 35 ml of lab medium and the flasks were grown at 30 ºC with 200 rpm on shaking incubator for 48 h. Then, 10% grown lab culture was inoculated into 35 ml of seed medium in 250 ml of Erlenmeyer flasks. The flask was incubated at 30 ºC with 200 rpm for 25± 1h. Then, 10% (v/v) seed culture was transferred to the 35 ml of Production medium in 250 ml of Erlenmeyer flasks.

Seed media was composed of (g/l) Beet Molasses 50, Magnous sulphate 0.25, Zinc sulphate 0.01, Sodium molybdate 0.02, Magnesium sulphate 0.05 ,Glycerol 3.5 ,Nutrient Broth 4.5,pH 7.4 and flasks were incubated at 30ºC at 200 rpm up to 24 ± 4 h or till optimum growth appears.10 % of grown seed was transferred to production media.

The Production media composed of (g/l) Beet Molasses 20, Sucrose 8.0, Choline chloride 2.5, Magnesium sulphate1.5, Di Ammonium hydrogen Phosphate 2.0, Ammonium sulphate 1.8, Betain monohydrate 1.0 , Cobalt nitrate 0.1, Ferrous sulphate 0.02, Magnous sulphate 0.02,Monosodium glutamate3.0,Zinc sulphate 0.02,Di Methyl Benzemedazole (5,6 DMB) 0.005, Calcium-Carbonate2.0, Potassium Dihydrogen Phosphate 0.25,Glycerol 1.5 .

The flasks were incubated at 30 ºC and 200 rpm for 7days. Sucrose 50 % (pH as such) 10% (v/v) feeding was done in a production flask from log 48 to 120 h at 24 h interval Samples were analyzed by HPLC from 120 h onwards upto 168 h respectively. pH, PMV and Microscopy was checked at an alternate of 24 hours to understand the morphological features of isolated culture [18].

2.3 Quantification of Vitamin B_{12} production by HPLC analysis

Quantification of Vitamin B_{12} production was done with HPLC analysis. For the sample preparation ,12.5 g of sample was added in 5.0 ml of methanol in 25 ml of volumetric flask and was heated it at 80 ^{o}C for 15 min The volume was made with water and was centrifuged it for 10 min at 4400 rpm. After centrifugation filter the supernatant of methanol extract of culture broth was filtered and analyzed by HPLC. Hypersil BDS C18, 250 X 4.6mm, 5µl column was used, 0.1% Trifluoroacetic acid in Acetonitrile was used as mobile phase at the flow rate of 1.0ml/min.The wavelength for detection was 260nm and the total sample run time was 30mins. The column temperature was maintained 30^{o}C.Yield of Vitamin B_{12} was calculated by comparison of peak area with response to standard area. Subsequently activity of Vitamin B_{12} was calculated in all experimental Runs of statistical analysis.

2.4. Experimental Design and Data Analysis:

In the first optimization step, a Plackett-Burman (PB) design was used to determine the likely effects of medium components on Vitamin B12 production. Plackett-Burman design is an efficient screening design where main effects are considered. This is a very economical design, with the run number a multiple of four and comprises of two Level screening designs. Eight assigned variables were screened in Plackett-Burman design

with 3 dummy variables in 12 experimental runs. Eight factors consisting of major medium components prepared at two levels –1 for low level and +1 for high level [21]. The factors (gm/L) such as Betain monohydrate, Beet molasses, Choline chloride, 5-6 DMB, Glycerol, Mono sodium glutamate, Cobalt nitrate and Magnesium sulfate at the same level were studied. The actual values of the variables at low level (−1) and high level (+1) are given in (Table 1).

Various treatments in a series of experiments were developed or generated according to several standard texts and software for designing experiments [4][17][14]. To get the optimum response, we used the Plackett Burman Design and further verified the effects of the technique of Factorial experiment. These experiments were adopted to determine a suitable direction by increasing or decreasing the concentrations of variables according to the results of experiment [9-10].

2.5. Screening of medium components by using Plackett Burman

Screening of medium components was carried out by using Plackett Burman design for selection of most appropriate medium components for Vitamin B_{12} production. Selection of medium components plays a key role in Vitamin B_{12} production. Medium ingredients were taken as a variable, to check their concentration and screened them via PB design. (Table 1)

Code list:

TABLE 1: Experimental code and levels of factors in the Plackett-Burman design

Code	Variables	Low level (-)	High level (+)
A	Choline chloride	2.5	7.5
B	Betain monohydrate	2.0	10.0
C	Glycerol	5.0	15
D	Beet molasses	60	150
E	Mono sodium glutamate	3.0	5.0
F	Magnesium sulfate	2.0	5.0
G	5-6 DMB	0.20	0.50
H	Cobalt Nitrate	0.1	0.5

Eight variables were studied by Plackett Burman design in different combinations of high level (+) and low level (-) by total 11 variables including 3 dummy variables with 12 runs of experiments.

2.6. Full Factorial

The full factorial design was employed in the selection of most significant variable for Vitamin B12 production. Four independent variables, including Monosodium Glutamate., 5, 6-DMB., Choline Chloride and Cobalt Nitrate were tested and identified via the full factorial design between the average of measurements made at two levels, high setting (+) and low setting (-) respectively among above mentioned factors.

Table 2: Experimental code and levels of factors in the Full factorial design

Code	Variables	Low level (-)	High level (+)
X1	Monosodium Glutamate	1.0	3.0
X2	5, 6-DMB.	0.25	0.4
X3	Choline Chloride.	5.0	6.0
X4	Cobalt Nitrate.	0.05	0.20

The results of the above experiment were analyzed by statistical method.

2.7. Optimization of Media Components by Response Surface Method (RSM)

The Central Composite Design (CCD) under RSM [5] was employed in order to find the optimum levels of significant media components. CCD was generated using a statistical analysis package Design-Expert Software (Stat-Ease Inc., Statistic made easy, Minneapolis, MN, USA, version 8.0) and the statistical analysis of experimental data was also performed using this software. Monosodium glutamate, choline chloride and cobalt nitrate were optimized and examined at three different levels (low, middle, high) concentration coded (-1, 0, +1) as shown in Table 3.

Name of Factor	Factor Coding	Units	-1	0	+1
Monosodium Glutamate	A	g/l	1.00	1.50	2.00
Choline Chloride	B	g/l	5.00	6.25	7.50
Cobalt nitrate	C	g/l	0.25	O. 38	0.50

According to CCD for the three variables, 17 experimental runs were executed and observations were fitted to the following second order polynomial:

$Y=\beta o + \beta 1A + \beta 2B + \beta 3C + \beta 11A2 + \beta 22B2 + \beta 33C2 + \beta 12AB + \beta 13AC + \beta 23BC$

Where Y is the predicted response (vitamin B12 production in mg/gm), A, B and C are independent variable (Monosodium Glutamate, Choline Chloride and Cobalt Nitrate); βo is the regression coefficient at center point; β1, β2 and β3 are linear coefficients; β11, β22 and β33 are the quadratic coefficients; and β12, β13 and β23 are the second order interaction coefficients.

2.8. Validation of the model:

In order to verify the adequacy of the developed CCD model, confirmation runs were performed.

III. Results

3.1 Plackett-Burman design

Eight variables along with 3 dummy variables were studied by Plackett-Burman design in different combinations of high level (+) and low levels (−) by total 12 run of experiments. The effect of these variables came at different concentrations of Vitamin B12 yield with different combinations (Table 3). Out of these eight variables, including three dummy, Cobalt Nitrate, Monosodium Glutamate, Choline chloride and 5-6 DMB showed significant positive effect on product yield. The effect of medium components on Vitamin B12 production was also studied further with full factorial design.

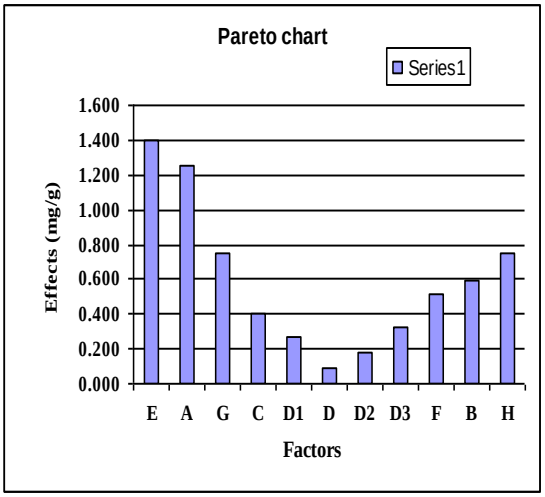
TABLE 3: Plackett-Burman experimental design for evaluation of medium components for the production of Vitamin B12 by *Rhizobium Sp* .and its response

Run	A	B	C	D	E	F	G	H	D1	D2	D3	Yield
1	+	+	-	+	+	+	-	-	-	+	-	9.23
2	-	+	+	-	+	+	+	-	-	-	+	9.56
3	+	-	+	+	-	+	+	+	-	-	-	9.45
4	-	+	-	+	+	-	+	+	+	-	-	9.52
5	-	-	+	-	+	+	-	+	+	+	-	10.05
6	-	-	-	+	-	+	+	-	+	+	+	10.58
7	+	-	-	-	+	-	+	+	-	+	+	8.54
8	+	+	-	-	-	+	-	+	+	-	+	11.33
9	+	+	+	-	-	-	+	-	+	+	-	8.78
10	-	+	+	+	-	-	-	+	-	+	+	12.03
11	+	-	+	+	+	-	-	-	+	-	+	7.59
12	-	-	-	-	-	-	-	-	-	-	-	10.68

Table 4: Statistical analysis of Plackett-Burman

Factors	SS	df	MS	F- value	p-value
A	4.688	1	4.688	24.65231	0.003
B	1.056	1	1.056	5.554374	0.057
C	0.488				
D	0.024				
E	5.824	1	5.824	30.63005	0.001
F	0.780				
G	1.673	1	1.673	8.79612	0.0251
H	1.688	1	1.688	8.874832	0.0247
D1	0.224				
D2	0.097				
D3	0.307				
Error	1.14	6	0.190144		

Fig: 1 Pareto chart of main effects



3.2. Full Factorial Design Result

Among all four variables used in this study, Choline chloride and Monosodium Glutamate showed significant effect on the Vitamin B12 production. The interactive effect of (Monosodium Glutamate: Cobalt Nitrate), (5,6 DMB and Choline Chloride), (5,6 DMB: Cobalt Nitrate) and (Monosodium Glutamate: Choline Chloride: Cobalt Nitrate) represented the significant effect on the productivity of Vitamin B12. (Figure 1)

Run No	X1	X2	X3	X4	Activity (mg/g)
1	-	-	-	-	13.0
2	+	-	-	-	17.2
3	-	+	-	-	13.2
4	+	+	-	-	17.2
5	-	-	+	-	12.4
6	+	-	+	-	13.6
7	-	+	+	-	14.5
8	+	+	+	-	17.7
9	-	-	-	+	18.0
10	+	-	-	+	16.4
11	-	+	-	+	17.1
12	+	+	-	+	14.4
13	-	-	+	+	14.7
14	+	-	+	+	15.2
15	-	+	+	+	14.9
16	+	+	+	+	15.3

Fig 2: Pareto chart for four variables and its interaction

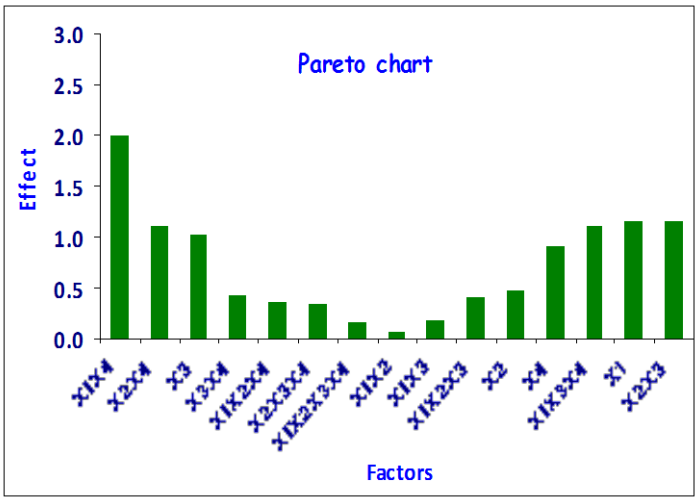


TABLE 5: Analysis of variance for Full factorial design (ANOVA)

Factors	SS	df	MS	*F-value*	*p-value*
X1**	5.290	1	5.290	12.385	9.74E-03
X2	0.902				
X3****	4.202	1	4.202	9.839	1.65E-02
X4	3.240	1	3.240	7.585	2.83E-02
X1X2	0.022				
X1X3	0.122				
X1X4*	16.000	1	16.000	37.458	4.81E-04
X2X3**	5.290	1	5.290	12.385	9.74E-03
X2X4***	5.062	1	5.062	11.852	1.08E-02
X3X4*	0.722				
X1X2X3	0.640				
X1X2X4	0.562	1	0.562	1.317	2.89E-01
X1X3X4***	5.062	1	5.062	11.852	1.08E-02
X2X3X4	0.490				
X1X2X3X4	0.090				
Error	2.990	7	0.427		

(SS- sum of square, df- degree of freedom, MS- mean square, P- p-value)

3.3. Central Composite Design (CCD)

Seventeen experiments were performed using different combinations of three variables – Monosodium Glutamate, Choline Chloride and Cobalt Nitrate. The coefficients, F-value and *p-value* was calculated and shown in table 7.

Multiple regression analysis was used to analyze the data and polynomial equation derived from a regression analysis for vitamin B12 production was shown in equation 3.

$Y= 21.69 - 0.16A + 0.36B - 0.22C + 0.37AB + 0.30AC - 0.37BC + 0.011A^2 - 0.38B^2 - 0.012C^2$…………………………….. (3)

Where, Y is response of vitamin B12 production, A is Monosodium Glutamate, B is Choline Chloride and C is Cobalt Nitrate.

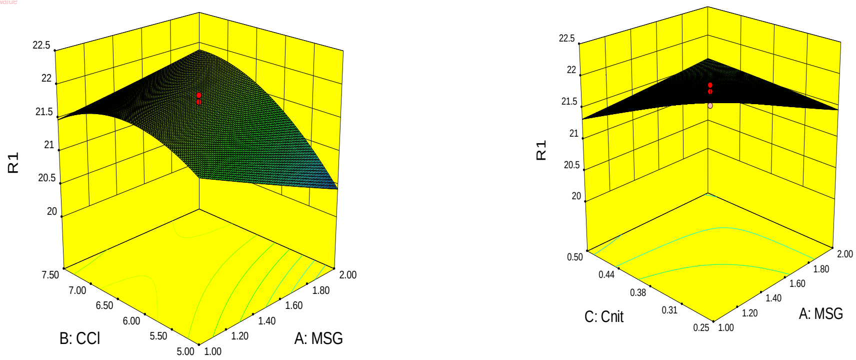
The individual effect of Choline Chloride was observed the most significant factors for vitamin B12 production. ANOVA for quadratic polynomial models was also estimated. The F-value of the model was 0.84 and p-value was 0.0009 represents the model was significant. The multiple correlation coefficient (R^2 = 0.9663) and adjusted coefficient (adjusted R^2 = 0.9159) were also high which indicated the significance of the model.

The 3D surface plots between two factors shown in fig. 3. It explained the interaction of medium constituents and the optimum concentration of each component involved in vitamin B12 production.

TABLE 7: Analysis of variance (ANNOVA) for quadratic polynomial model

Variables	Coefficient	SS	df	MS	F-value	p-value
Model	21.69	7.53	9	0.84	0.84	0.0009
A	-0,160	0.37	1	0.37	8.42	0.0272
B	0.360	1.80	1	1.80	41.07	0.0007
C	-0.220	0.66	1	0.66	15.11	0.0081
AB	0.371	1.08	1	1.08	24.71	0.0025
AC	0.300	0.72	1	0.72	16.47	0.0067
BC	0.370	1.08	1	1.08	24.71	0.0025
A^2	0.011	1.231E-003	1	1.231E-003	0.028	0.028
B^2	0.380	1.55	1	1.55	35.37	0.0010
C^2	0.012	1.666E-003	1	1.666E-003	0.038	0.8517

R^2 =0. 9663, adj R^2 = 0.9159, * = significant



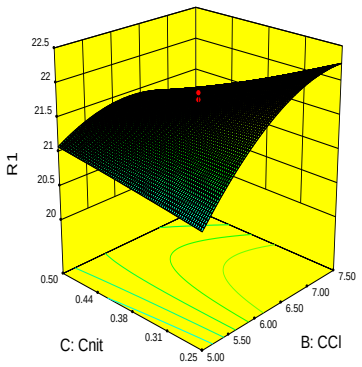


Fig 2: 3D response surface plot showing interaction between a) Monosodium Glutamate and Choline Chloride. ; b) Monosodium Glutamate and Cobalt Nitrate; c) Choline Chloride and Cobalt Nitrate

IV. Discussion

Vitamin B12 is defined as an essential micronutrient required in trace quantities that cannot be synthesized by mammals, are essential for metabolism of all living organisms and are synthesized only by microorganisms or plants. Cobalt deficiency is of major importance in several countries. The dietary Co requirement has been established at 0.1 to 0.2 ppm [19]. Vitamins are regarded as organic compounds required in the diet in small amounts to perform specific biological functions for normal maintenance, optimum growth and health of the organisms. Vitamin B12 (Cobalamin, anti-pernicious anemia factor) is a water soluble vitamin with a key role in the normal functioning of the brain and nervous system and for the formation of blood. It is normally involved in the metabolism of every cell of the human body, especially fatty acid synthesis and energy production. It is the largest and most structurally complicated vitamin and can be produced industrially only through bacterial fermentation synthesis [15].

In this present study experimental data reveal that for Vitamin B12 production there are many factors plays synchronized role for the better yield. In order to understand the above fact, we performed the experiments by using a statistical approach. In this respect various experiments were planned with different medium components as discrete variables [31].

Different statistical approaches can be used to optimize the growth medium and fermentation conditions. The conventional method of single factor optimization by maintaining other factors involved at an unspecified constant level is not only tedious, but also can lead to misinterpretation of results, especially because the interactions between different factors is overlooked [15].

In the present study, the significant increament in Vitamin B12 productivity was observed from 4.92 mg/gm to 22.55 mg/gm with the newly isolated *Rhizobium* species by using a statistical approach.

4.1 Screening of medium components by using Plackett Burman

4.1.1 Plackett-Burman

Plackett-Burman experimental design was used to screen the significant medium components which were further screened via 2^4 full factorial designs. Among eight variables mono sod glutamate, choline chloride, DMB and Cobalt nitrate were selected by PB design on the basis of effect and Pareto chart. These factors were further confirmed by full factorial design and screened variables were mono sodium glutamate, interactions of mono sodium glutamate * choline chloride and choline chloride * Cobalt nitrate showed major components for vitamin B12 production. ANOVA was applied to determine the significance level of individual factors and interactions between these factors. Remarkable difference was observed in yield of Vitamin B12 with different medium design as per the Plackett Burman.

Among all defined eight variables Monosodium glutamate, Choline chloride, Cobalt nitrate and 5-6-DMB., was selected as the most appropriate variables which were further studied with the full factorial design to get the optimum production media for better yield.

4.2. Full Factorial Design Result

Four variables were selected on the basis of effect from Placket Burman and further studied with full factorial design. The effect of these variables was obtained in different concentration of Vitamin B12. Out of these four variables, Choline chloride and Monosodium Glutamate showed significant effect on the Vitamin B12 production. The interactive effect of (Monosodium Glutamate: Cobalt Nitrate), (5,6 DMB and Choline

Chloride), (5,6 DMB: Cobalt Nitrate) and (Monosodium Glutamate: Choline Chloride: Cobalt Nitrate) represented the significant effect on the productivity of Vitamin B12.

4.3. Central Composite Design (CCD)

CCD was used to identify the critical factor. It consists of factorial, axial to recognize the quadratic effects and central trials to calculate the process variability. The p-values were used as a tool to validate the significance level of each of the coefficients and also important to recognize the model of the mutual interactions between the variables. The small p-value (less than 0.0007) indicates model terms are significant. Here choline chloride was found significant. The regression model's goodness of fit was checked by multiple correlation coefficients (R^2) [21]. The R^2 is always lies between 0 to1. The model is stronger as R^2 value close to 1. The determination coefficient was estimated as 0.9663 which shows that the statistical model can explain 96.63 % of variability in the response. The adjusted R^2 was 0.9159 which indicates the model is significant. The determination coefficient and correlation coefficient is the major tool to determine the goodness of a model.

Response surface plots were employed to understand the main variables and the interaction between two factors while other factors were held at the middle level. Interacting effects of Monosodium glutamate and cobalt nitrate and choline chloride illustrated maximum yield in vitamin B12. The optimum levels of media constituent (Choline chloride 6.25 g/l, Monosodium glutamate 1.50 g/l and Cobalt nitrate 0.38g/l) with other components were taken. Under such optimal condition, 22.55 mg/g. production of vitamin B12 was achieved.

4.4. Validation of final concentration

To verify the final concentration three sets of experiments were performed with the obtained optimal combination from the CCD for vitamin B12 production by *Rhizobium Sp*. The 21.39 mg/gm mean value for vitamin B12 production was achieved.

V. Conclusion:

Placket-Burman, full factorial and central composite design with response surface methodology were employed for the optimization of fermentation medium components for the production of vitamin B12 by newly isolated *Rhizobium Sp.* Monosodium glutamate cobalt nitrate and choline chloride were the main components involved to enhance the productivity. The optimal conditions for the enhanced productivity of vitamin B12 were Choline chloride 6.25g/l; Monosodium glutamate 1.50 g/l and Cobalt nitrate 0.38 g/l. Under such conditions the 22.55 mg/g maximum productivity was achieved. Overall, it can be concluded that the statistical technique is an imperative approach for the optimization fermentation medium components for the enhancement of productivity. Validation experiments were also carried out to verify the accuracy of the model.

The more economic production of this Vitamin B_{12} can be achieved in future by optimization of media component and process parameter by statistical tool. Mutation and genetic modification approach was proved fruitful for Vitamin B_{12} productivity enhancement. Statistical experiments help to understand the hidden complexity of the medium ingredients role in the production of Vitamin B_{12}.

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