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Optimization of Fermentation Parameters for Vitamin B_{12} Production using *Propionibacterium freudenreichii* sub sp. shermanii OLP-5 by Taguchi Method

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Vitamin B_{12} production by *Propionibacterium freudenreichii sub sp. Shermanii* was optimized using Taguchi orthogonal array (OA) design of experiment (DOE). An OA layout of L18 (2^1 x 3^7) was constructed with eight most influencing factors on vitamin B_{12} production, six factors like carbon, nitrogen, phosphate source, metal ion (Fe, Co) and inoculum size were selected based on ''one variable at a time approach'' along with two other factors such as glucose (substrate) and DMBI (precursor). All the above factors except $FeSO_4$. $7H_2O$ (two level) were taken at three levels for proposed experimental design. Analysis was done by using Qualitek-4 software at bigger is better as quality character and obtained a predicted vitamin B_{12} yield of 24.662 mg/L with a specific combination of factors. The optimal levels of factors ($FeSO_4$.$7H_2O$,12.5 mg/L; Inoculum size,10%; $(NH_4)_2HPO_4$, 0.1%; Glucose, 10%; DMBI, 0.025 g/L; Yeast extract, 1%, Sodium lactate, 1%; $CoCl_2$.$6H_2O$, 10 mg/L) obtained from designed methodology was further validated by confirmation experiment and the enhanced vitamin B_{12} (16.2 mg/L to 23.1 mg/L) was obtained from its unoptimized condition. Taguchi approach of DOE methodology proved to be efficient in evaluating the interaction of each factor individually and in combination.

Key words: Orthogonal array, *Propionibacterium freudenreichii sub sp. Shermanii*, Taguchi DOE, Vitamin B_{12}.

Vitamin B_{12} is a water soluble vitamin having very complex structure consisting of four pyrrole units. The vitamin B_{12} present in the diet is found in bound form with proteins i.e. it combines with a substance called gastric intrinsic factor (IF) and then it can be absorbed in the stomach. Intrinsic factor is secreted by the stomach lining and it tightly binds with B_{12} and helps in transport through the stomach lining and into the blood [1, 2]. Any abnormal production of this intrinsic factor results vitamin B_{12} deficiency. The most important disease associated with vitamin B_{12} deficiency is pernicious anemia [2, 3]. It is characterized by low hemoglobin levels, decreased number of erythrocytes and neurological manifestations. Vitamin B_{12} is an important cofactor for the metabolism of carbohydrate, lipids, aminoacids, and nuceic acids [4]. Dietary deficiency seen among the strict vegetarians of low socioeconomic group in the developing countries (India, Srilanka, etc.). In humans the vitamin B_{12} is required in trace amounts (1 µg/day) to assist the actions of two enzymes, methionine synthase and (R)-methy malonyl CoA mutase[2].

Vitamin B_{12} is produced by several different microorganisms such as *Streptomyces, Bacillus, Aerobacter, Propionibacterium, Alkaligens, Pseudomonas, Salmonella, Serratia, Rhizobium, Mycobacterium, Xanthomonas,* etc [5].

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Among these vitamin B_{12} producers, mainly *Propionibacterium shermanii* and *Pseudomonas denitrificans* strains are employed for industrial production due to their high vitamin B_{12} productivity and their rapid growth [2, 6]. Though *Pseudomonas denitrificans* has been employed in industrial production, *Propionibacterium shermanii* should be preferred by industry, because, it has obtained the GRAS (generally regarded as safe) status from USFDA.

Vitamin B_{12} biosynthesis occurs in two routes: (1) an aerobic (oxygen dependent pathway) that is found in organisms like *Pseudomonas dentrificans* and (2) an anaerobic, oxygen independent pathway investigated in organisms like *Bacillus megatherium, P.shermanii* and *Salmonella typhimurium* [2, 3]. In anaerobic phase *propionibacteria* synthesize cobamide complex of vitamin B_{12} and in aerobic phase it will synthesize DMBI (Dimethyl Benzimidazole), a lower ligand of vitamin B_{12} which is necessary to form complete structure of vitamin B_{12} [2, 7, 8]. In *Propionibacteium freudenreichii subsp. Shermanii*, vitamin B_{12} yield will be more when the fermentation was carried out anaerobically followed by aerobic fermentation, where as *Pseudomonas* needs only aerobic fermentation.

In the present study effect of various parameters on vitamin B_{12} production by probiotic strain of *Propionibacterium freudenreichii* sub sp. *Shermanii* OLP-5 [9] was optimized initially by 'one variable at a time approach'. Based on these experimental results an L-18 orthogonal array was constructed by selecting eight variables *viz* carbon (sodium lactate), nitrogen (yeast extract), phosphate [$(NH_4)_2HPO_4$], metal ion (Fe, Co), inoculum size, glucose (substrate) and DMBI (precursor) using Qualitek - 4 software.

MATERIAL AND METHODS

Microorganisms, and Culture Conditions

Probiotic strain of *Propionibacterium freudenreichii sub sp. Shermanii* OLP-5 used for vitamin B_{12} production is maintained on NLA slants at 4^0C. Inoculum was prepared in Sodium Lactate Broth (NLB) medium (Yeast extract - 1%, Sodium lactate - 1%, Trypticase soya - 1%, KH_2PO_4 - 0.025%, $MnSO_4$ - 0.005%), fermentation was carried out in 100 ml broth containing 8 g glucose, 1 g yeast extract, 1 g trypticase soya, 1g sodium lactate, 0.1 g KH_2PO_4, 0.2 g $(NH_4)_2HPO_4$, 0.5 mg $FeSO_4.7H_2O$, $MgSO_4.7H_2O$, 0.25 mg $MnSO_4.H_2O$, 1 mg $CaCl_2.6H_2O$, 1 mg NaCl, 1mg $CoCl_2.6H_2O$, 2.5 mg DMBI. The pH was adjusted to 6.5 with NaOH for every 12 hrs during the fermentation. All the combination experiments using the assigned parameter values were conducted using this broth and incubated at 30^0C for 72 hrs anaerobically followed by 48 hrs aerobically. After incubation, the samples were assayed for vitamin B_{12} production.

Optimization of Fermentation parameters for Vitamin B_{12} Production by "one variable at a time" Approach.

Carbon Source

The effect of carbon source on production of vitamin B_{12} was studied by incorporating various carbon sources like glucose, xylose, arabinose, lactose, sucrose, sodium lactate, mannitol, glycerol and cellobiose at 0.5 & 1% level.

Nitrogen Source

To determine the best nitrogen source for the production of vitamin B_{12}, the fermentation was carried out in the medium containing the various nitrogen sources such as urea, casein, beef extract, soya meal, yeast extract, peptone, CSL, KNO_3, NH_4Cl, NH_4SO_4 at 0.5 & 1% level.

Phosphate Source

Various phosphates like KH_2PO_4, K_2HPO_4 and $(NH_4)_2HPO_4$ at 0.05% & 0.1% concentration have been used to study their buffering effect on vitamin B_{12} production.

Mineral Source

The effect of mineral source on production of vitamin B_{12} was studied by incorporating various mineral salts ($MnSO_4.7H_2O$, $MgSO_4.7H_2O$, KCl, $CaCl_2$, $CuCl_2.2H_2O$, $CoCl_2.6H_2O$, NaCl and $FeSO_4.7H_2O$) at 2.5, 5, 10 mg/L concentration.

Temperature

The optimum temperature for vitamin B_{12} production was determined by incubating the fermentation flasks at a temperature range of 25^0 to 40^0 C.

Inoculum Density

Effect of inoculum density on vitamin B_{12} prodcution was determined with various levels of inoculum range of 2.5 to 10%.

Analysis of Vitamin B_{12}

Vitamin B_{12} analysis was done as recommended by Quesada-Chanto *et al.*[7,10]. Cells were washed with potassium phosphate buffer of pH 5.5 containing 0.1% KCN and autoclaved at 121^0C for 15 min for lysis of cells. The lysate was then centrifuged at 10,000 rpm for 15 min and supernatant was collected. The vitamin B_{12} was purified and concentrated by extraction with benzyl alcohol and chloroform as reported by Fischer[11].Vitamin B_{12} in the culture broth was assayed by High Performance Liquid Chromatography (HPLC) with an UV detector (371 nm) using reverse phase C-18 column, with a flow rate of 0.7 ml/min at 40^0C with 3:1 ratio of 0.25M NaH_2PO_4 of pH 3.5, and methanol as a mobile phase.

Taguchi Methodology

Taguchi has given an excellent methodology for quality control in the manufacturing industries[12,13]. Steps involved in Taguchi methodology are as follows:

Steps in Taguchi method

1. Identify the main function, side effects and failure mode
2. Identify the noise factors, testing conditions, and quality characteristics.
3. Identify the objective function to be optimized.
4. Identify the control factors and their lives.
5. Select the orthogonal array matrix experiment.
6. Conduct the matrix experiment.
7. Analyze the data; predict the optimum levels and performance.
8. Perform the verification experiment and plan the future action.

Design of Experiments

The first phase determines the various factors to be optimized in the culture medium that have critical effect on the vitamin B_{12} yield. Eight factors [carbon, nitrogen, and phosphate source, metal ion (Co, Fe), inoculum size, glucose and DMBI] and their levels were selected, which had significant impact on vitamin B_{12} biosynthesis as per our previous experimental results. In the next step, matrix was designed with the appropriate OA's for the selected parameters and their levels. Taguchi provides many standard OA's and corresponding linear graphs for this purpose. In the present study, three levels of eight factors were considered and the size of experimentation was represented by symbolic array of L-18 (which indicated 18 experiments trials). The three levels of all the factors have been assigned except $FeSO_4.7H_2O$ (Two levels) with a layout of L-18 (2^1 X 3^7). All the combination experiments using the assigned parameter values conducted, using Vitamin B_{12} fermentation medium and incubated at 30^0 C for five days. After fermentation the culture broth was separated and analyzed for vitamin B_{12} (Table 1).

RESULTS AND DISCUSSION

Optimization of Fermentation Parameters for Vitamin B_{12} Production by "one variable at a time approach" followed by Taguchi Methodology.

Effect of Carbon Source

Propionibacterium freudenreichii sub sp. *shermanii* – OLP-5 was grown with two levels (0.5%, 1%) of carbon sources such as glucose, xylose, arabinose, lactose, sucrose, sodium lactate, mannitol, glycerol and cellobiose. As shown in the Fig. 1, maximum yield of vitamin B_{12} (18.2 mg/L, 17.5 mg/L) was obtained with sodium lactate at 1% and 0.5% concentration respectively, whereas glycerol and lactose has given moderate production of vitamin B_{12} and others have shown less yield of vitamin B_{12}. As the sodium lactate being a neutralized organic acid, there was a small variation in pH (6.2 – 6.4), hence sodium lactate has given maximum production of vitamin B_{12} because cells did not undergo inhibition by pH when compared to other carbon sources, where the final pH is around 5 [14-16], hence less production of vitamin B_{12} occurred due to less growth of the organism. Coral *et al.* [17] obtained similar results using *Propionibacterium shermanii* for propionic acid synthesis.

Effect of Nitrogen Source

Yeast extract is the best nitrogen source for the biosynthesis of vitamin B_{12} at 1% concentration (18 mg/L), followed by casein, soya meal and corn steep liquor. But in comparision to above nitrogen sources, other sources such as beef extract, peptone, NH_4Cl, NH_4SO_4 showed less production of vitamin B_{12} (Fig. 2). However Atta *et al.*[18] obtained maximum yield of vitamin B_{12} at

0.025% of yeast extract using mixed cultures of *Streptomyces halstedii* and *Bacillus firmus*. In the studies of Bullerman *et al.*[19], the effect of yeast extract on vitamin B_{12} production with cheese whey using *Propionibacterium shermanii* resulted that various levels (0.5, 1, 1.5%) of yeast extract has given varying results with various amounts of whey.

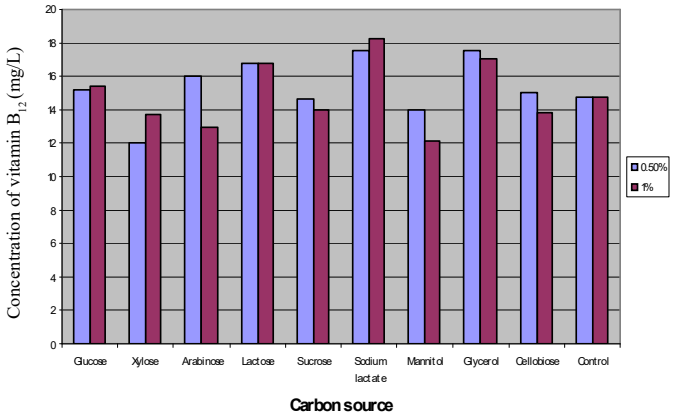

Fig. 1. Bar graph showing effect of carbon source on production of vitamin B_{12}

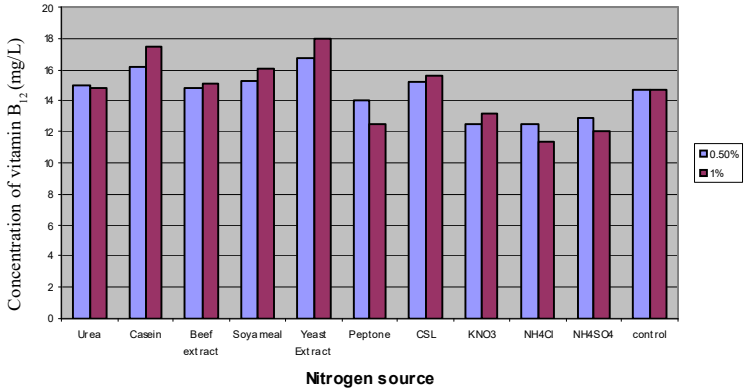

Fig. 2. Bar graph showing effect of Nitrogen source on production of vitamin B_{12}

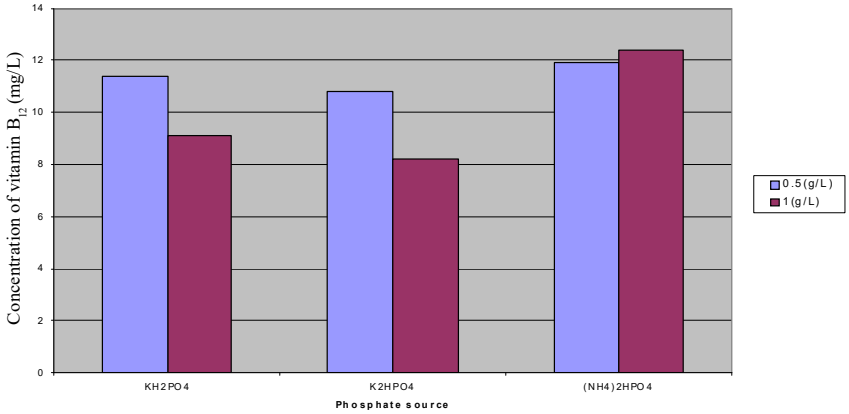

Fig. 3. Bar graph showing effect of phosphate source on production of vitamin B_{12}

Effect of Phosphate Source

Vitamin B_{12} level was studied by different phosphate sources such as KH_2PO_4, K_2HPO_4 and $(NH_4)_2HPO_4$. The maximum yield of vitamin B_{12} (12.4 mg/L) was obtained with $(NH_4)_2HPO_4$ at 1% level (Fig. 3). The reason explained that it might be due to both nitrogen source and buffering agent of $(NH_4)_2HPO_4$, whereas KH_2PO_4 and K_2HPO_4 acts as only buffering agents. Riaz *et al.* [20] showed increased vitamin B_{12} production with the increase of $(NH_4)_2HPO_4$ from 19mM to 38mM but when NH_4^+ and PO_4^{+3} ions were used separately, it resulted in one fold decrease of vitamin B_{12} synthesis.

Effect of Mineral Salts

Among various mineral salts, maximum vitamin B_{12} was produced with $CoCl_2.6H_2O$ (10.8 mg/L) and $FeSO_4.7H_2O$ (9.2 mg/L) at 10 mg/L concentration (Fig. 4). Since vitamin B_{12} contains central Co^{+2} ion in corrinoids present in all

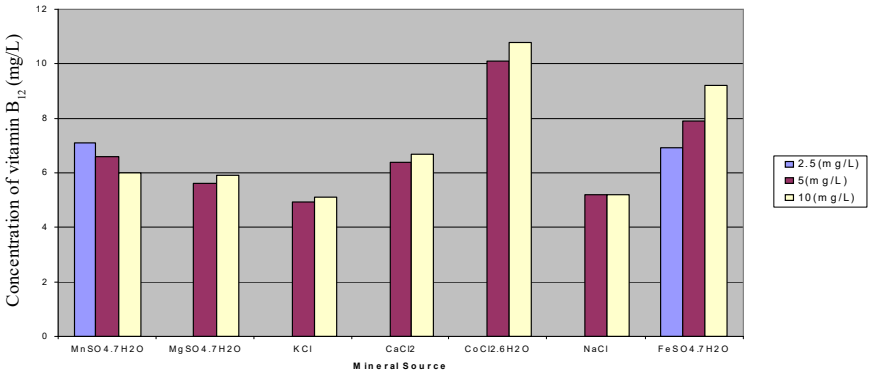

Fig. 4. Bar graph showing effect of mineral source on production of vitamin B_{12}

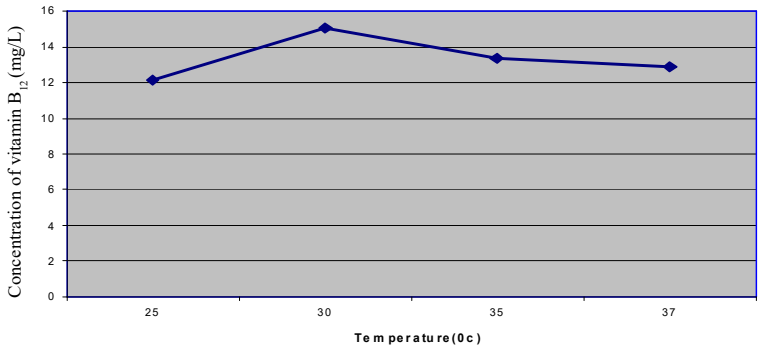

Fig. 5. Line graph showing effect of temperature on production of vitamin B_{12}

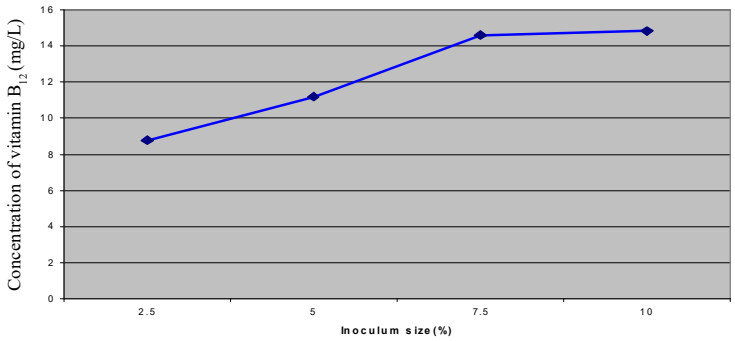

Fig. 6. Line graph showing effect of inoculum size on production of vitamin B_{12}

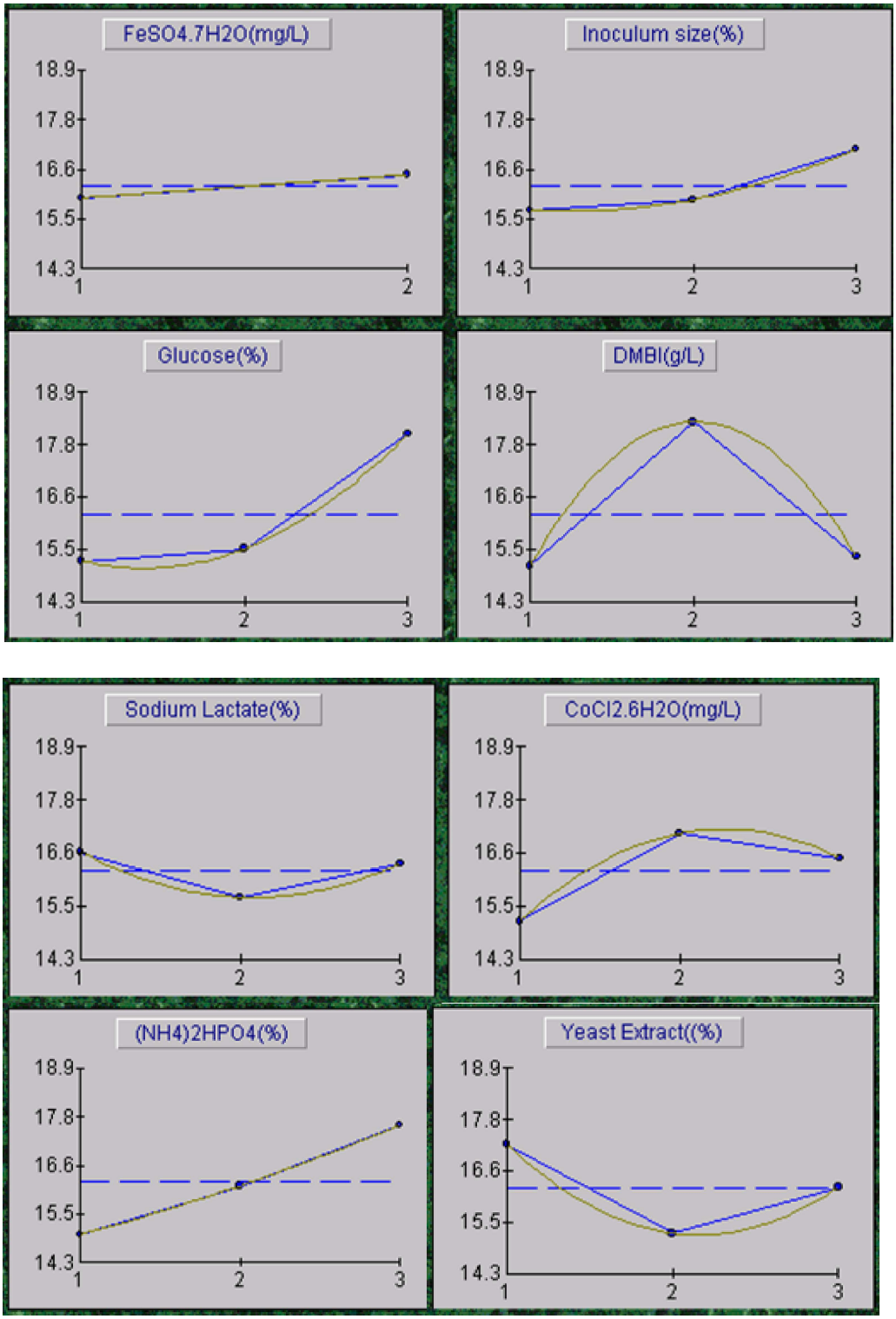

Fig. 7. Multiple Graphs of Main Effects

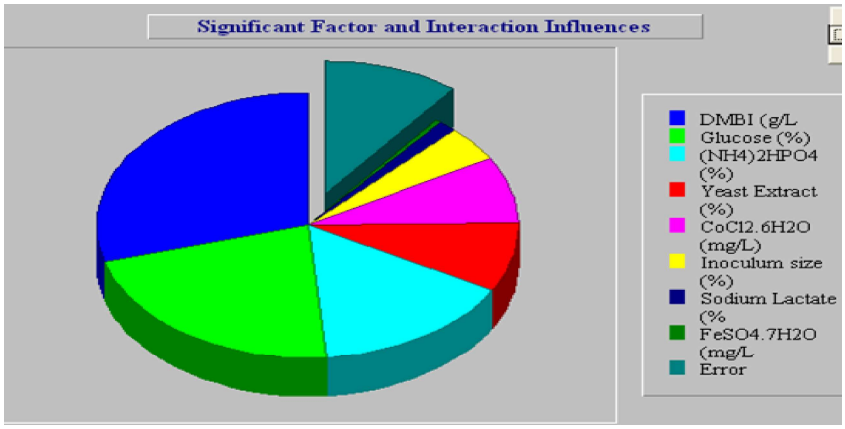

Fig. 8. Significant factor and interaction influences: Pie Chart.

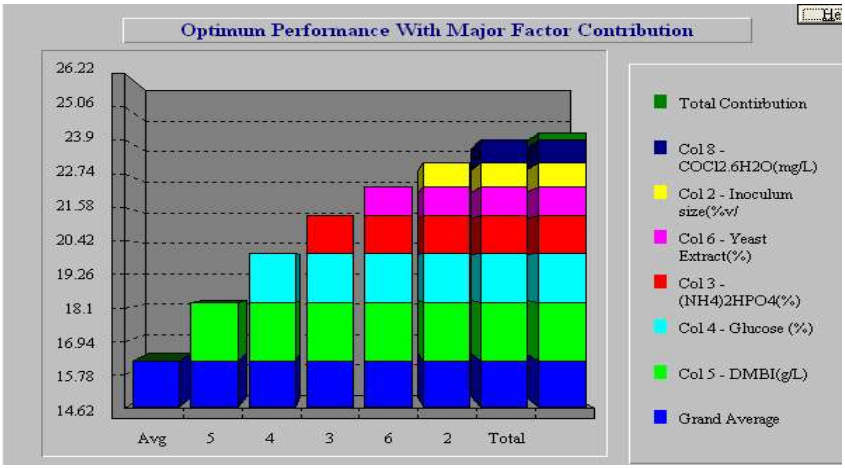

Fig. 9. Optimum Performance with Major factor Contributions

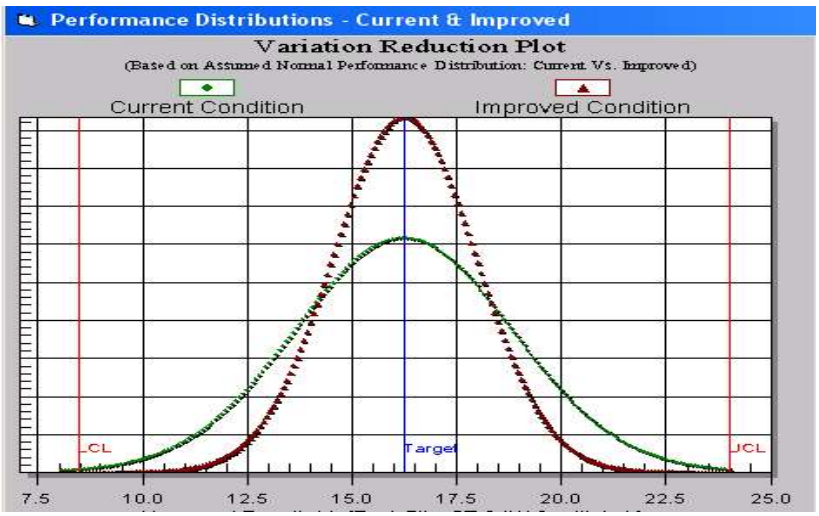

Fig.10. Variation Reduction Plot

methanogens and acetogens and number of corrinoid dependent reactions are known to take place in the intermediary metabolism of substrates by methanogens and acetogens, so it is necessary to add required amounts for the maximum biosynthesis of vitamin B_{12} [5, 21]. Several authors have reported maximum yield of vitamin B_{12} at 10 mg/L concentration of cobalt[1,4]. However some reports have shown optimum vitamin B_{12} production at 1mg/L of cobalt ion concentration [5].

Effect of Temperature

Vitamin B_{12} production was found to be optimum at incubation temperature of 30^0C. However the production decreased with the increase of incubation temperature i.e. 35-40^0C [4, 22, 23] (Fig. 5).

Effect of Inoculum Density

Highest yield of vitamin B_{12} was obtained at 10% inoculum. However there is negligible production of vitamin B_{12} from 7.5% to 10% inoculum density (Fig. 6).

Optimization of Parameters by Taguchi Methodology

In the present study, we have designed a L-18 orthogonal array by taking six influencing factors from the above results such as sodium lactate, yeast extract, $(NH_4)_2HPO_4$, $CoCl_2.6H_2O$, $FeSO_4.7H_2O$, inoculum size along with substrate (glucose) concentration and precursor (5, 6 Dimethyl Benzimidazole), since the substrate concentration and precursor 5, 6 DMBI are critical factors for the production of vitamin B_{12} [2, 24]. 5, 6 dimethyl benzimidazole, a lower alpha ligand of vitamin B_{12} which was believed to be a precursor of vitamin B_{12} biosynthesis [23]. The addition of DMBI has an advantage, as it bypasses several biosynthetic reactions required for its formation for efficient biosynthesis of vitamin B_{12} [5]. Addition of 5, 6 DMBI stimulates the production of vitamin B_{12} only by the biosynthesis of true vitamin B_{12}, whereas the biosynthesis of analogues of higher molecular weight was inhibited [20].

Taguchi experiments are analyzed to achieve either to establish the best or the optimum condition for a product or a process or to estimate the contribution of individual factors or to estimate the response under the optimum conditions. The optimum condition is identified by studying the main effect of each factor. The process involves minor arithmetic manipulation of the numerical results and usually can be done with the help of a simple calculator. The knowledge of the contribution of individual factors is a key to deciding the nature of the control to be established on a production process. The analysis of variance (ANOVA) is the statistical treatment most commonly applied to the results of the experiment to determine the percent contribution of each factor. Study of the ANOVA table for a given analysis helps to determine which of the factors need to control and which do not. Taguchi suggests two different routes to carry out the complete analysis. First the standard approach, where the result of a single run or the average of repetitive runs, are processed through main effect and ANOVA analysis. The second approach, which strongly recommends for multiple runs, is to use signal to noise ratio (S/N) for the same steps in the analysis. S/N analysis determines the most robust set of operating conditions from variations within the results.

Three levels of eight factors were considered and the size of experimentation was represented by symbolic orthogonal array of L-18 (which indicated 18 experiments trials) (Table 2). The average effects of the factors and interactions at the assigned levels on vitamin B_{12} production are depicted in Table 3 and Table 4. The difference between the average value of each factor at level 2 and 1 indicates the relative influence of the effect. The larger the difference, the stronger will be the influence. The sign of the difference (+ or -) indicates whether the change from the level 1 to 2 or 1 to 3 increased or decreased the result (Table 3). Based on these data it can be seen that DMBI, $CoCl_2.6H_2O$, $(NH_4)_2HPO_4$ showed a stronger influence to that of other factors and the least influence was noticed with yeast extract and sodium lactate with the assigned levels (Fig. 7; Table 3).However, when interactions of different factors were calculated, interaction of $FeSO_4.7H_2O$ vs sodium lactate showed highest severity index percentage 57.32%, inoculum size vs sodium lactate severity index percentage was 56.25 % and inoculum size vs $CoCl_2.6H_2O$ was only 5.38%. These results suggest that the influence of one factor on vitamin B_{12} production was dependent on the condition of other factors.

The percentage contribution of each

factor and their interactions are shown in Table 5 (Fig. 8). The last column of the ANOVA indicates the influence of each factor. DMBI was the most significant factor for vitamin B_{12} production. DMBI, Glucose and $(NH_4)_2HPO_4$ were significant at 90% confidence limit and yeast extract, $CoCl_2.6H_2O$, inoculum size and $FeSO_4.7H_2O$ were significant at 70% confidence limit and sodium

Table 1. Factors and their levels assigned to different columns

S.No	Column# / Factors	Level 1	Level 2	Level 3
1	$FeSO_4.7H_2O$ (mg/L)	10	12.5	……
2	Inoculum size (%)	5	7.5	10
3	$(NH_4)_2HPO_4$ (%)	0.05	0.075	0.1
4	Glucose (%)	6	8	10
5	DMBI (g/L)	0.0125	0.025	0.0375
6	Yeast Extract (%)	1	1.5	2
7	Sodium Lactate (%)	1	1.5	2
8	$CoCl_2.6H_2O$ (mg/L)	8	10	12

Table 2. L-18(2^1 x 3^7) Orthogonal Array

Experiment	Column								Vit. B_{12}
No	1	2	3	4	5	6	7	8	yield(mg/L)
1	1	1	1	1	1	1	1	1	12.4
2	1	1	2	2	2	2	2	2	15.9
3	1	1	3	3	3	3	3	3	18.1
4	1	2	1	1	2	2	3	3	14.5
5	1	2	2	2	3	3	1	1	12.9
6	1	2	3	3	1	1	2	2	18.5
7	1	3	1	2	1	3	2	3	13.8
8	1	3	2	3	2	1	3	1	20.9
9	1	3	3	1	3	2	1	2	16.8
10	2	1	1	3	3	2	2	1	12.9
11	2	1	2	1	1	3	3	2	14.7
12	2	1	3	2	2	1	1	3	20.2
13	2	2	1	2	3	1	3	2	15.6
14	2	2	2	3	1	2	1	3	16.8
15	2	2	3	1	2	3	2	1	17.3
16	2	3	1	3	2	3	1	2	20.8
17	2	3	2	1	3	1	2	3	15.7
18	2	3	3	2	1	2	3	1	14.6

Table 3. Main Effects

S.No	Column# / Factors	Level 1	Level 2	Level 3	L2 – L1
1	$FeSO_4.7H_2O$ (mg/L)	15.977	16.511	-	0.533
2	Inoculum size (%)	15.699	15.933	17.1	0.233
3	$(NH_4)_2HPO_4$ (%)	15	16.149	17.583	1.149
4	Glucose (%)	15.233	15.5	18	0.266
5	DMBI (g/L)	15.133	18.266	15.333	3.132
6	Yeast Extract (%)	17.216	15.249	16.266	-1.968
7	Sodium Lactate (%)	16.649	15.683	16.399	-0.967
8	$CoCl_2.6H_2O$ (mg/L)	15.166	17.05	16.516	1.884

lactate is insignificant at 70% confidence limit for Vitamin B_{12} production, respectively.

Optimum conditions and their performance in terms of contribution for achieving maximum production of vitamin B_{12} are given in Table 6 (Fig. 9). DMBI and glucose contributed a major significant role in vitamin B_{12} production than other selected parameters and their levels. The expected result at optimum condition was 24.662 mg/L with total contributions from all factors 8.418 and the current grand average performance was 16.244 mg/L (Fig. 10).

Validation

To validate the proposed experimental methodology, fermentation was performed for vitamin B_{12} production by employing the optimum level for each factor such as $FeSO_4.7H_2O$ (12.5 mg/L); inoculum size (10%); $(NH_4)_2HPO_4$ (0.1%); glucose (10%); DMBI (0.025 g/L); Yeast extract (1%), Sodium lactate (1%); $CoCl_2.6H_2O$ (10 mg/L). After application of Taguchi method, vitamin B_{12} yield was enhanced to 23.1 mg/L which was higher to the yield before optimization (16.244 mg/L). The reason explained due to strong interaction among the factors. This experimental result was very close to the predicted value (24.662 mg/L) that was obtained from the statistical evaluation.

Table 4. Estimated interaction of severity index for different parameters

S.No	Factors	Columns[@]	SI(%)[$]	Col*	Levels[&]
1	$FeSO_4.7H_2O$ x sodium lactate	1 × 7	57.32	6	(2,1)
2	Inoculum size x sodium lactate	2 × 7	56.25	5	(3,1)
3	$(NH_4)_2HPO_4$ x $CoCl_2.6H_2O$	3 × 8	54.99	11	(3,3)
4	DMBI x Yeast extract	5 × 6	44.44	3	(2,1)
5	DMBI x sodium lactate	5 × 7	43.95	2	(2,1)
6	$FeSO_4.7H_2O$ x $(NH_4)_2HPO_4$	1 × 3	43.7	2	(1,3)
7	Inoculum size x DMBI	2 × 5	42.8	7	(3,2)
8	Inoculum size x glucose	2 × 4	42.12	6	(3,3)
9	Yeast extract x sodium lactate	6 × 7	41.55	1	(1,3)
10	$(NH_4)_2HPO_4$ x sodium lactate	3 × 7	40.77	4	(3,1)
11	Glucose x sodium lactate	4 × 7	36.73	3	(3,3)
12	Glucose x Yeast extract	4 × 6	33.46	2	(3,1)
13	Sodium lactate x $CoCl_2.6H_2O$	7 × 8	32.92	15	(1,2)
14	DMBI x $CoCl_2.6H_2O$	5 × 8	31.04	13	(2,1)
15	Yeast extract x $CoCl_2.6H_2O$	6 × 8	26.19	14	(1,3)
16	Inoculum size x $(NH_4)_2HPO_4$	2 × 3	21.92	1	(1,3)
17	$FeSO_4.7H_2O$ x Inoculum size	1 × 2	21.42	3	(1,3)
18	$FeSO_4.7H_2O$ x DMBI	1 × 5	19.85	4	(2,2)
19	$(NH_4)_2HPO_4$ x Glucose	3 × 4	18.98	7	(2,3)
20	$(NH_4)_2HPO_4$ x DMBI	3 × 5	16.81	6	(3,2)
21	$FeSO_4.7H_2O$ x Yeast extract	1 × 6	15.29	7	(2,3)
22	$(NH_4)_2HPO_4$ x Yeast extract	3 × 6	14.6	5	(3,1)
23	$FeSO_4.7H_2O$ x Glucose	1 × 4	12.75	5	(1,3)
24	Glucose x DMBI	4 × 5	10.27	1	(3,2)
25	Glucose x $CoCl_2.6H_2O$	4 × 8	9.32	12	(3,2)
26	$FeSO_4.7H_2O$ x $CoCl_2.6H_2O$	1 × 8	8.22	9	(2,3)
27	Inoculum size x Yeast extract	2 × 6	6.41	4	(3,1)
28	Inoculum size x $CoCl_2.6H_2O$	2 × 8	5.38	10	(1,3)

@columns-represent the column locations to which the interacting factors are assigned.

$ si – interaction severity index (100% for 90 degrees angle between the lines, 0% for parallel lines.

* col – shows column that should be reserved if this interaction effect were to be studied (2-l factors only)

& opt – indicates the factor levels desirable for the optimum conditions (based strictly on the first 2 levels). if an interaction is included in the study and found significant (in anova), the indicated levels must replace the factor levels identified for the optimum condition without considerations of any interaction effects.

Table 5. ANOVA

S. No	Column# / Factors	DOF (f)	Sum of Sqrs (S)	Variance (V)	F- Ratio (F)	Pure Sum (S')	Percent P (%)
1	$FeSO_4.7H_2O$ (mg/L)	1	1.28	1.28	1.73	0.54	0.448
2	Inoculum size (%)	2	6.751	3.375	4.561	5.271	4.377
3	$(NH_4)_2HPO_4$ (%)	2	20.1	10.05	13.581	18.62	15.462
4	Glucose (%)	2	27.951	13.975	18.885	26.471	21.981
5	DMBI (g/L)	2	36.924	18.462	24.947	35.444	29.432
6	Yeast Extract (%)	2	11.607	5.803	7.842	10.127	8.409
7	Sodium Lactate (%)	2	3.02	1.51	2.04	1.54	1.279
8	$CoCl_2.6H_2O$ (mg/L)	2	11.307	5.653	7.639	9.827	8.16
Other/Error		2	1.479	0.739			10.452
Total		17	120.424				100.00%

Table 6. Optimum conditions and performance

S.No	Column# / Factors	Level Description	Level	Contribution
1	$FeSO_4.7H_2O$ (mg/L)	12.5	2	.266
2	Inoculum size (%)	10	3	.855
3	$(NH_4)_2HPO_4$ (%)	0.1	3	1.338
4	Glucose (%)	10	3	1.755
5	DMBI (g/L)	0.025	2	2.022
6	Yeast Extract (%)	1	1	.972
7	Sodium Lactate (%)	1	1	.405
8	$CoCl_2.6H_2O$ (mg/L)	10	2	.805

Total Contrbution From All Factors…	8.418
Current Grand Average of Performance…	16.244
Expected Result At Optimun Condition….	24.662

CONCLUSIONS

In conclusion, this study demonstrated that culture media and fermentation conditions for vitamin B_{12} production using probiotic strain of *Propionibacterium freudenreichii* sub sp. *shermanii* were optimized by a conventional "one variable method" followed by Taguchi approach. The effect of physical parameters like temperature, inoculum size and chemical parameters like carbon, nitrogen, phosphate and mineral salts were studied. These results have shown significant impact on production of vitamin B_{12}. Based on these results some selected parameters [carbon, nitrogen, and phosphate source, metal ion (Co, Fe), inoculum size, glucose and DMBI] were used for optimization using Taguchi methodology. DMBI, glucose and $(NH_4)_2HPO_4$ had more significant role, whereas $FeSO_4.7H_2O$ and sodium lactate had least significant role on vitamin B_{12} production. Vitamin B_{12} production was found to be 23.1 mg/L using *Propionibacterium freudenreichii* sub sp. *Shermanii*-OLP-5**.**

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REFERENCES

1. Gardner, N., Champagne, C.P. Production of Propionibacterium shermanii biomass and vitamin B_{12} on spent media. *J. Appl. Microbiol*., 2005; **99**: 1236–1245.
2. Martens, J.H., Barg, H., Wareen, M.J., Jahn, D. Microbial production of vitamin B_{12}. *Appl. Microbial. Biotechnol*., 2002; **58**: 275-285.

3. Scott, A.I., Roessner, C.A. Biosynthesis of cobalamin (vitamin B_{12}). *Biochemical Society Transactions*, 2002; **30**(4): 613-620.
4. Quesada-Chanto, A., Afschar, A.S., Wagner, F. Microbial production of propionic acid and vitamin B_{12} using molasses or sugar. *Appl. Microbiol. Biotechnol*., 1994; **41**: 378-383.
5. Riaz, M., Ansari, Z.A., Iqbal, F., Akram, M. Microbial production of vitamin B_{12} by methanol utilizing strain of *Pseudomonas spcie*. *Pak. J. Biochem. Mol. Biol*., 2007a; **40**(1): 5-10.
6. Krusong, W., Yongsmith, B., Sanchez, P.C. Influenced of *Lactobacillus casei* in production of high vitamin B_{12}-Tempeh. *Kasetsart J*., 1991; **25**(4): 458-462.
7. Quesada-Chanto, A., Schmid-Meyer, A.C., Schroeder, A.G., Carvalho-Jonas, M.F., Blanco, I., Jonas, R. Effect of oxygen supply on biomass, organic acids and vitamin B_{12} production by *P. shermanii*. *World J. Microbiol. Biotechnol*., 1998; **14**: 843-846.
8. Rehm, H.J. Industrielle Microbiologie. Berlin, Heidelberg, Springer Verlag, New York, 1980; ISBN 3-540-09642-6.
9. Thirupathaiah, Y., Swarupa, R.C.H., Sudhakara, R., Manikyam, A., Venkateswar, L.R. Development of efficient probiotic *P. freudenreichii* subspecies *shermanii* to combat vitamin B_{12} deficiency. *Int. J. Probiotics and Prebiotics*, 2009; **4**: 271-276.
10. Quesada-Chanto, A., Schroeder, A., Scmidt-Meyer, A., Lopez, J.A., Silveira, M.M., Jonas, R. Organic acids production by *P. shermanii*: Effect of pH, temperature, and vitamin-nitrogen source. *Zeitscrift fur Naturforschung.* 1997; **52c:** 193-196.
11. Fischer, R.A. Rapid spectrophotometric determination of vitamin B_{12} in microbial material. *Agri. Food Chem*., 1953; **1**: 951-953.
12. Taguchi, G. Introduction to quality engineering. UNIPUB/Kraus International, White Plains, 1986.
13. Rao, R.S., Kumar, G.C., Prakasham, S.R., Hobbs, P.J. The Taguchi methodology as a statistical tool for biotechnological applications: A critical appraisal. *Biotechnol. J*., 2008; **3**: 510-523.
14. Hsu, S-T., Yang, S-T. Propionic acid fermentation of lactose by *Propionibacterium acidipropionici*: Effects of pH. *Biotechnol. Bioeng*., 1991; **38**: 571-578.
15. Nanba, A., Nukada, R., Nagai, S. Inhibition by acitic acids and propionic acids of the growth of the *Propionibacterium shermanii*. *J. Fermentation Technol*., 1983; **61**: 551-556.
16. Playne, M.J., Moo-Young, M. Propionic and Butyric Acids. *Comprehensive Biotechnol*., 1985; **3**: 731-759.
17. Coral, J., Karp, S.G., Vanderberghe, l.P.S., Parada, J.L., Pandey, A., Soccol, C.R. Batch fermentation model of propionic acid production by *P. acidipropionici* in different carbon sources. *Appl. Biochem. Biotechnol*., 2008; **151**(2-3): 333-34.
18. Atta, H.M., Arafa, R.A., Salem, M.S., El-Meleigy, M.A. Microbiological studies on production of vitamin B_{12} from two mixed cultures under solid state fermentation condition. *J. Appl. Sci. Res*., 2008; **4**(11): 1463-1477.
19. Bullerman, L.B., Berry, E.C. Use of cheese whey for vitamin B_{12} production. *Appl. Microbiol*., 1966; **14**(3): 353-355.
20. Riaz, M., Ansari, Z.A., Iqbal, F., Ali, I., Ahmad, T., Akram, M. Influence of medium components and physical factors on vitamin B_{12} production by *Pseudomonas sp*. PCSIR 99. *Pak. J. Sci. Ind. Res*., 2007b; 50(6): 401-407.
21. Wood, H.G., Ragsdale, S.W., Pezacka, E. The acetyl-Co A pathway of autotrophic growth. *FEMS Microbiol. Rev*., 1986; **39**: 345 – 362**.**
22. Halbrook, E.R., Cords, F., Winter, A.R., Sutton, T.S. Vitamin B_{12} production by microorganisms isolated from poultry house litter and droppings. *J. Nutr*., 1950; **41**(4): 555-563.
23. Yongsmith, B., Sonomoto, K., Tanaka, A., Fukui, S. Production of vitamin B_{12} by immobilized cells of a propionic acid bacterium. *Eur. J. Appl. Microbial. Biotechnol*., 1982; **16**: 70-74.
24. Wang, Ze.J., Wang, H.Y., Li, Y.L., Chu, J., Huang, M.Z., Zhuang, Y.P., Zhang, Si.L. Improved vitamin B_{12} production by step wise reduction of oxygen uptake rate and dissolved oxygen limiting label during fermentation process. *Biores. Technol*., 2010; **101**(8): 2845-2852.