International Journal of Advances in Engineering and Management (IJAEM)
Volume 3, Issue 7 July 2021, pp: 2863-2873 www.ijaem.net ISSN: 2395-5252

Optimization of Vitamin B_{12} Synthesis Using Microorganisms Utilizing Dairy Wastewater.

Asra anjum[1*], Gayathri Gururajan[2] and Kathireshan A K[3]
1- Research scholar, Dept of Microbiology, VISTAS, Chennai-117.
2-Associate Professor, Dept of Microbiology, VISTAS, Chennai-117
*3-*Professor and Corresponding author - Dept of Microbiology, VISTAS, Chennai-117*

Submitted: 10-07-2021 Revised: 20-07-2021 Accepted: 23-07-2021

ABSTRACT:
Vitamin B_{12} is an essential vitamin which has been used in medical and food industries for years. Since the production of vitamin B_{12} by chemical synthesis is too expensive and are labor intensive, time consuming and costly, a number of bacterial strains such as Propionibacterium freudenreichii, Propionibacterium shermani and Pseudomonas denitrificans have been successfully used in commercial production of vitamin B_{12} industrially. Identification of vitamin B_{12} producing microbial strains and synthesis of vitamin B_{12} from Industrial wastewater especially dairy waste water was the main aim of this study. Isolation, Identification and characterization of Propionibacterium species were carried out using molecular level methods, specifically polymerase chain reaction (PCR). The aim of this research work was to examine the optimization of vitamin B12 production using RSM methodology to produce high yield from Propionibacterium species. Purification of vitamin B_{12} was carried out by using HPLC method. More recently advances in biotechnology have led to the development of methods that minimize manipulation which provide results in less time and reduce cost.

Keywords: vitamin B_{12}, microbial strains, dairy wastewater, Propionibacterium species, PCR, RSM methodology.

I. INTRODUCTION:

Traditionally, milk has been considered the most complete food provided by nature which is an important source of essential nutrients needed for the growth and development. It is also highly recommended in the diet of humans due to its high nutritional value [1]. It is mainly composed of water, proteins, lipids, carbohydrates, vitamins and minerals. Lactose is the principal carbohydrate found in milk. It has been reported that lactose can stimulate the absorption of minerals including calcium and magnesium [2]. Moreover, it has been demonstrated that milk not only contains nutritive compounds but also biologically active ones, like caseins and whey proteins. Caseins constitute 80% of the total protein present in milk; the other 20% is comprised of the whey proteins including α-lactalbumin, β-lactoglobulin and immunoglobulins [3].

Because of its high nutritional content, milk can be used to produce a large variety of dairy products such as whole, skim, lactose-free and fat free milk; or even whole or skimmed milk powder. Additional products include condensed and evaporated milk, which can be pasteurized or ultra pasteurized. Milk is also used to produce the fermented dairy products cheese, yogurt, kefir and bulgaro. Butter, milk whey, sweet/sour cream, casein acid type, lacticorrenin, caseinates, lactose, enzymatically modified milk ingredients, cream, ice cream and other dairy beverages, are the main products we obtain from milk [4] [5]

The manufacturing of dairy products represents a great concern because of pollution and damage to the environment. This problem is due to the generation of waste from livestock producers and the by-products from dairy foods [6]-[7].

Milk whey is a liquid by-product produced during the manufacturing process of cheese. It represents 85% - 90% of the original milk volume and retains 55% of the nutrients. Milk whey has proteins, lipids, soluble vitamins, minerals and carbohydrates. Of the total whey solids, 75% is lactose. Lactose is one of the most polluting by-products because of its high biochemical oxygen demand (BOD) < 35,000 ppm and its chemical oxygen demand (COD) < 60,000 ppm [8]. If this industrial effluent is discarded in the soil without prior treatment, it can modify the soil's physical and chemical composition, reducing crop yields and the availability of oxygen in water [9] [10]. It has been estimated that 40,000 liters of untreated milk whey produces contamination equivalent to that produced daily by a population of 250,000

International Journal of Advances in Engineering and Management (IJAEM)
Volume 3, Issue 7 July 2021, pp: 2863-2873 www.ijaem.net ISSN: 2395-5252

people [11]. Another by-product from the dairy industry is clarified butter sediment waste derived from the manufacturing of clarified or ghee butter. This butter waste is mainly composed of fats in the form of fatty acids and small amount of proteins [12]. It has been found that discarding milk whey in the water of rivers and lakes causes enormous pollution problems because of the large amounts of nitrogen, and phosphorus and high BOD (40 - 48,000 mg/L) and COD (89 - 95,000 mg/L) contained in this by-product [13].

The conditions mentioned above promote the process of eutrophication, causing an excessive growth of microorganisms and aquatic plants [14] [15]. However, in this present analysis it has been emphasized that the manufacturing processes of many dairy products produce large amounts of by-products. Dairy by-products can be used to obtain valuable chemical compounds that are useful in the fields of health, pharmaceuticals, food, plastics and fuels. The manufacturing industries of food, beverages and pharmaceuticals demand a variety of chemical compounds, which are used as additives for the production of many products. These additives can act as acidifying agents, stabilizers, flavor enhancers or preservatives. Some examples of additives are the organic acids (citric, lactic, succinic and propionic acid) [16]-[18]

Vitamin B_{12} is one of the most essential vitamin which is widely used in the medical and food industries.[19] Vitamin B_{12} also known as cyanocobalamin belongs to the family of cobalamin, synthesized by prokaryotes and inhibits in the development of pernicious anemia in animals. Since the production of vitamin B_{12} by chemical synthesis is too expensive, a number of bacterial strains such as Propionibacterium freudenreichi, Propionibacterium shermani and Pseudomonas denitrificans have been successfully used in commercial production of vitamin B_{12} industrially. Whereas Pseudomonas denitificans has been successfully and exclusively used in industries for its rapid growth and high productivity. It has been demonstrated that Propionibacterium species has the high potential to accumulate vitamin B_{12} and are considered as GRAS(Generally Recognized As Safe).In general a high yield of Vitamin B_{12} has been achieved by further treating the organisms with mutagenic agents such as UV light or chemical agents and selecting the strains with practical advantages such as genetic stability, productivity, reasonable growth rates and resistance to high concentrations of toxic substances present in the medium. [20]

Vitamin B_{12}, which is one of the water-soluble vitamins and naturally occurring cobalt organometallic compounds containing substances which involves a range of biochemical process such as DNA synthesis, regulation of fatty acids, amino acid metabolism as well as energy production. Vitamin B_{12} deficiency causes mitotic disorder, neuropathy, nervous system disease and pernicious anaemia. [21] To prevent from such fatal diseases, daily intake of 2.4 µg vitamin B_{12} is recommended. [22]. The aim of this study was to isolate and identify the vitaminB_{12} producing microorganism from diary wastes. To screen and characterize the vitaminB_{12} producing capability of organisms using HPLC Method.

II. MATERIALS AND METHODS:

Sample Collection and isolation of strains:

The samples were collected from different locations of Dairy wastes from industries in Chennai, Tamilnadu, India. The samples were stored in sterile vials at 4℃ for experimental purpose. The samples were homogenized and serially diluted using saline (0.9%) and plating techniques were carried out by three different mediums viz., Yeast extract medium (YEM), MRS agar, Sodium Lactate Medium.[23] The plates were incubated at 37℃ for 48hrs. The strains were stored in 50% glycerol stock at -20℃ and also working cultures were prepared by using sodium lactate medium stabs stored at 4℃.

Screening of vitamin B_{12} producing organism:

Screening by plating method

Screening of vitamin B_{12} producing organism helps in selecting the organism responsible for Vitamin B_{12} production. The colonies, which showed zone of clearance in starch agar plates, were maintained on to MRS agar plate for further experiments Selected from high dilution in the MRS agar plates, Strains were grown in MRS broth at 25°C. Purity was checked by streaking on suitable agar medium.

Molecular identification of strains by using PCR:

Frozen isolates were revived from the glycerol stock, thawed and reinoculated into freshly prepared sterile YEM and Sodium lactate broth and incubated at 37°C for 24 h. After confirmation of purity, 10 µl of active broth culture was reinoculated into 10 ml sterile YEM broth and incubated at 37°C for 10 h. Two milli litre aliquots of active log phase cultures from this broth were then used to isolate genomic DNA. To the 2 ml culture, 2–3 µl of a sodium salt of ampicillin solution (50 mg/ml) was added and the mixture incubated at 37°C for 1 h. Post incubation, the bacteria were harvested by centrifugation at

International Journal of Advances in Engineering and Management (IJAEM)
Volume 3, Issue 7 July 2021, pp: 2863-2873 www.ijaem.net ISSN: 2395-5252

5000 rpm for 5 min in a refrigerated centrifuge. The DNA was extracted by using extraction kit by Himedia laboratories. Qualitative and Quantitative Assessment of DNA: All samples of the preliminary study were analyzed by spectrophotometer. DNA concentration was determined by recording the absorbance at 260 nm (A260) using a Nanodrop spectrophotometer (Bio-Tek instruments, inc.). The purity of the DNA was determined from the A260/A280 ratio. The quality of the isolated DNA was also evaluated by (0.9% agarose) gel electrophoresis using 2 µl of isolated DNA. The type of band pattern indicates the quality of the DNA isolated.

PCR for 16s amplification:

The 16srRNA amplification was carried out by using universal primers 27F and 1492R. The PCR product was taken for sequenced and analyzed using a BLAST search algorithm to find the higher similarity sequences obtained from the Genbank in NCBI. The amplification was done by using 16s primers, Forward primer: 27F (AGAGTTTGATCMTGGCTCAG), Reverse primer: 1492 (RCGGTTACCTTGTTACGACTT).The PCR master mix was as follows: 10 µl of 10× reaction buffer (100 mM Tris [pH 8.3], 500 mM KCl, 9 mM MgCl2), PCR primers (final concentration, 40 µM each), and deoxynucleoside triphosphates (final concentration, 200 µM) in a final volume of 100 µl with 2.5 U Taq DNA polymerase. Water was then added to bring the volume to 100 µl. The PCR reaction was performed with 25 µl of the reaction mix containing ~100 ng of isolated genomic DNA, in a hot lead thermocycler (Eppendorf). The reaction mixture was subjected to an initial heating at 95°C for 2 min. The temperature was cycled through 94°C for 1 min, 55°C for 1 min, extension at 72°C for 1 min and then a final extension at 72°C for 2 min. The cycle was repeated 30 times.

PCR for specific gene identification:

For identification of Vitamin B_{12} producing organism species specific amplification was carried out by Cbi gene. Forward primer: (CAAGCATGCGGTGAGCCTA) Reverse primer: (GTCGCGCCACATCTTCTTCT). The PCR master mix was as follows: 10 µl of 10× reaction buffer (100 mM Tris [pH 8.3], 500 mM KCl, 9 mM MgCl2), PCR primers (final concentration, 40 µM each), and deoxynucleoside triphosphates (final concentration, 200 µM) in a final volume of 100 µl with 2.5 U Taq DNA polymerase. Water was then added to bring the volume to 100 µl. The PCR reaction was performed with 25 µl of the reaction mix containing ~100 ng of isolated genomic DNA, in a hot lead thermocycler (Eppendorf). The reaction mixture was subjected to an initial heating at 95°C for 2 min. The temperature was cycled through 94°C for 1 min, 53.2°C for 50secs, extension at 72°C for 1 min and then a final extension at 72°C for 2 min. The cycle was repeated 30 times.[24]

Optimization of Vitamin B_{12} using RSM methodology:

Inoculum preparation:

One milliliter of stored culture was transferred into 9 mL of fresh medium containing glucose and the culture was incubated at 30℃ for 48 h. This culture was added to 200 mL of preculture medium containing glucose and incubated for 48 h at 30C. An aliquot (10% v/v) was used to inoculate 200 mL of various culture media with crude glycerol for vitamin B_{12} fermentation. [25] The culture was maintained at 30 ℃ and the pH was adjusted daily to 6.8 with 25% ammonia solution. After 96 h, the vitamin B_{12} precursor, DMB, was added, and the vitamin B_{12} content of cultures was analyzed after 168 h. All fermentation experiments were run in duplicate.

Experimental design:

A two-step optimization strategy was employed to optimize vitamin B_{12} production by P. freudenreichii, P. shermanii. In the first step, Plackett–Burman design was used to identify which variables have significant effects on vitamin B_{12} biosynthesis. Seven variables (A–G) K3PO4, NaH2PO4.2H2O, casein Hydrolysate (acid), tryptone, biotin, Ca pantothenate, $FeSO_4$.$7H_2O$ was chosen as factors, two dummy factors were employed to evaluate the standard errors of the experiment. Low (-1) and high (+1) levels were appointed for each factor (Table 1). Average values of vitamin B_{12} concentration after 168 h of fermentation were used as response in this design.[26]

In the second step, response surface methodology was used to optimize the levels of significant variables selected by the Plackett–Burman design. A five level (-a, -1, 0, +1, +a) two-factor

Central composite design was used twice to facilitate statistical analysis of possible interactions between components in a smaller number of experiments than would be necessary for factor design. The experimental designs and responses are shown in Tables 3 and 4, for the first and the second phase of the experiment respectively.

International Journal of Advances in Engineering and Management (IJAEM)
Volume 3, Issue 7 July 2021, pp: 2863-2873 www.ijaem.net ISSN: 2395-5252

Table 1: Experimental ranges and levels of the Seven factors tested in the Plackett–Burman design.

Factor	Symbol	Ranges and levels	
		-1	+1
K_3PO_4 (g/L)	A	1	5
$NaH_2PO_4.2H_2O$ (g/L)	B	1	5
Casein Hydrolysate (acid) (g/L)	C	5	20
Tryptone (g/L)	D	10	30
Biotin (mg/L)	E	0.2	1
Ca pantothenate (mg/L)	F	2	1
$FeSO_4 .7H_2O$ (mg/L)	G	10	30

In both cases nine experiments were performed. Among them, five replications were at center points (0), four of them were axial (-a, +a) and determined to be p2. Each response obtained was used to develop the empirical model of the response surface in which each dependent variable was shown as a sum of the contributions of these two investigated factors. For the two-factor design the model Eq. (1) is:

$$Y=b_0+b_1A+b_2B+b_{12}AB+b_{11}A^2+b_{22}B^2$$

Where y: predicted yield of the response; b0: intercept; b1, b2: linear coefficients; b11, b22 quadratic coefficients, and b12: interaction coefficient. Average vitamin B_{12} concentrations after 168 h fermentation were used as response in this design.

Analytical Methods

The samples were centrifuged at 12,000 rpm for 10 min at 4℃, and then cells were boiled at 15 min in 0.01% potassium cyanide at pH 6.0 and 0.1 M phosphate buffer containing vials taken for filtration (0.45 lm syringe filters) vitamin B_{12} was quantified by using HPLC method. A mixture of 0.25 M NaH_2PO_4 pH 3.5 (phosphoric acid): methanol (75:25) at 1 mL/min was the mobile phase. Injection volume was 16µl. The detector wavelength was set at 362 nm. A standard of vitamin B_{12} (Sigma Chemicals, St. Louis, MO, USA) was used. Quantitation was based on peak area. Data acquisition and analysis were done with Agilent ChemStation software for LC 3D systems.

Dry weight:

Dry weight of biomass was estimated after centrifugation and drying to constant weight at 105℃.

Statistical analysis:

STATISTICA 6.0 software was used for all statistical analyses. The statistical tests were performed at the significance level $\alpha = 0.05$.

III. RESULTS AND DISCUSSION:

Isolation and screening of Vitamin B_{12} producing bacteria

Isolated strains of total 55 was carried out for the study, out of that 4 strains showed maximum clear zone in screening plate compared with standard strain. The zone index compared with Lactobacillus plantarum (MTCC 1325) already known efficiency of vitamin B_{12} in dairy products. Dairy products alone not only the source of isolating as P. freudenreichii, P. shermanii these strains. Other source also can existence of above mentioned organisms such as human skin, gastrointestinal tract, spoiled citrus fruits etc. The vitamin B_{12} production existed greater amount in Lactobacillus sp.has been reported in previous work (Rossi, F., et al., 1999).

International Journal of Advances in Engineering and Management (IJAEM)
Volume 3, Issue 7 July 2021, pp: 2863-2873 www.ijaem.net ISSN: 2395-5252

Fig 1: A- Sodium Lactate Medium

Fig 2: B- Yeast Extract Medium

Purification of vitamin B12 organisms:

Fig 3: C-Purified strains

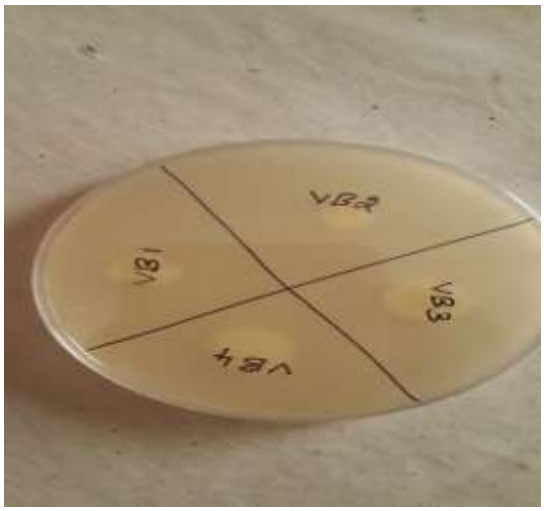

Fig 4: D- Selected strains for optimization

Molecular Identification Based on 16s r-RNA sequencing the strains of VB1, VB2 were identified as P. freudenreichii, P. shermanii. From phylogenetic analysis, the closely related strains were compared with identified strains shown in the figure 6&7. Molecular level of identification carried out by using Cbi gene and results showed that base pair size of ~400bp shown in the figure 5. In previous research work author as conducted the experiment of vitamin B_{12} using Cob gene with base pair size of ~478bp (Piao, Y., et al., 2004).

International Journal of Advances in Engineering and Management (IJAEM)
Volume 3, Issue 7 July 2021, pp: 2863-2873 www.ijaem.net ISSN: 2395-5252

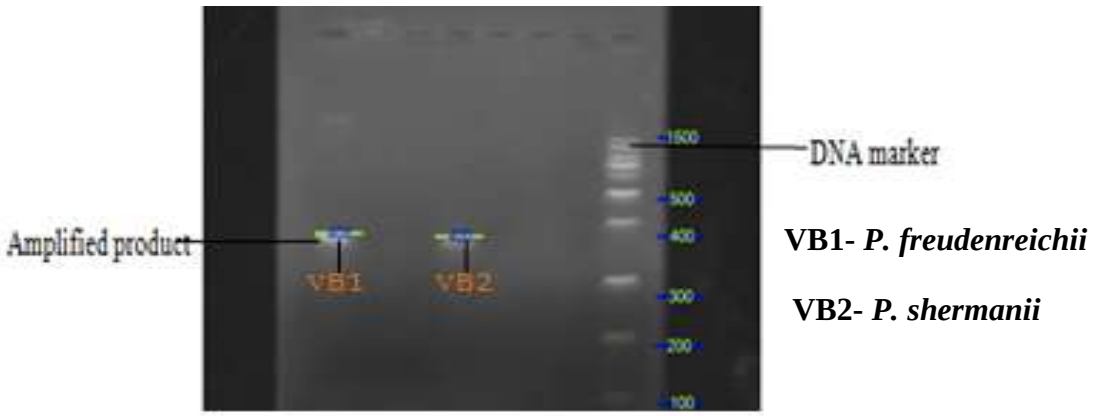

Fig 5: Amplification of Cbi gene

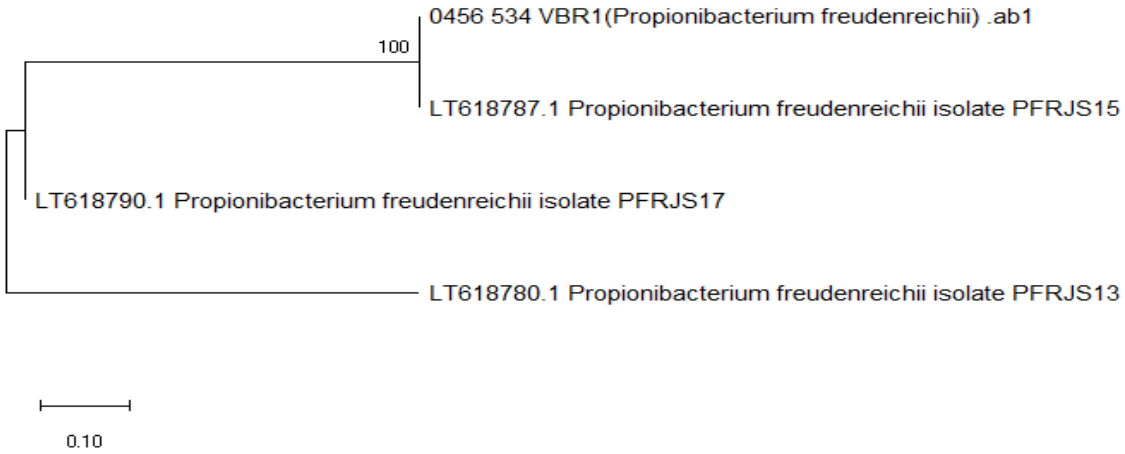

Fig 6: Phylogenetic tree for *P. freudenreichii*

International Journal of Advances in Engineering and Management (IJAEM)
Volume 3, Issue 7 July 2021, pp: 2863-2873 www.ijaem.net ISSN: 2395-5252

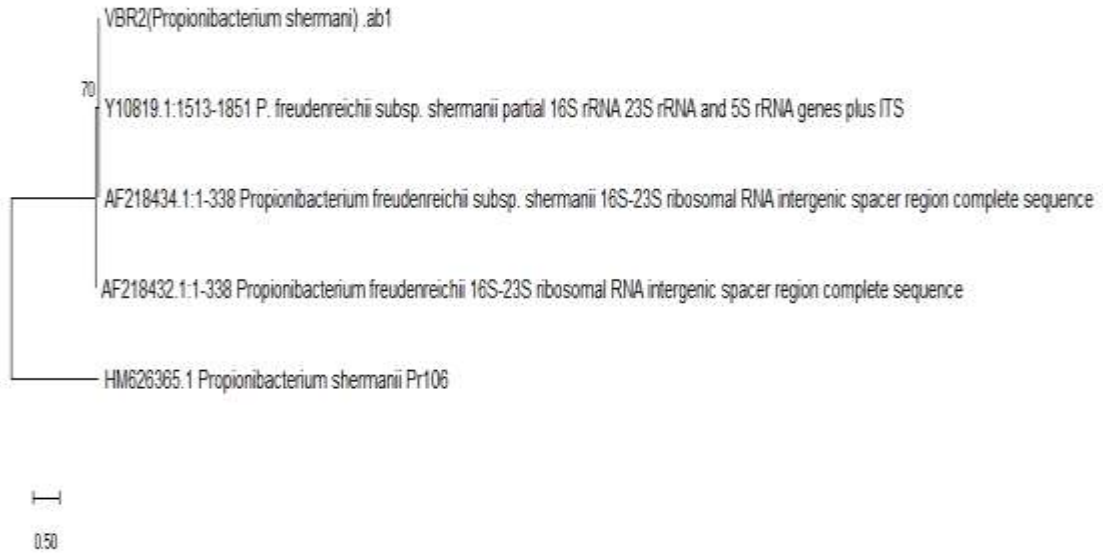

Fig 6: Phylogenetic tree for *P. shermanii*

Optimization studies using RSM methodology

To estimate the vitamin B_{12} production on culture medium of P. freudenreichii, P. shermanii were engaged using Placket Burmann design. In this design elements of 7 were tested. In this case, amount of vitamin B_{12} production in experiment on 8 runs showed highest percentage achieved in this design was 34.57 mg/L. To perform the experiment, ANOVA variance of statistical tools to find the relationship between significant variables and response. Based on this response only 4 tested variables influences significantly discovered from Plackett–Burman design from p-value represented in Table 1.

From table 2 showed that each parameters of variables in regression analysis, if the variable is positive, it influence the vitamin B_{12} production at higher level indicates (H). At the same time if it is negative, then the amount of concentration of vitamin B_{12} is low (L). In this next stage of research was examined based on significant variables mentioned above.

Central composite design

In the First level of Placket Burmann design, CCD was established based on screening variables that influences vitamin B_{12} production. In the first factor, to determine the optimal concentration of $NaH_2PO_4.2H_2O$ and calcium pantothenate having highest affect on vitamin B_{12} production was found.

International Journal of Advances in Engineering and Management (IJAEM)
Volume 3, Issue 7 July 2021, pp: 2863-2873 www.ijaem.net ISSN: 2395-5252

Fig 7: Optimization of Vitamin B_{12} producing organisms:

In this experiment, second factor considered as casein hydrolysate and $Fe_2SO_4.7H_2O$ concentration were used for optimization and maintained in the tested culture medium at the levels corresponding to the calculated value of response in maximum of CCD and the rest of medium components (non-significant factors) were still maintained at the lower (L) level concentration of the levels used in the Plackett–Burman design. The design matrix, experimental responses, dry cell weight amount are given in Table 2. Some of the runs produced substantially improved results for the vitamin B_{12} production over those achieved in the Plackett–Burman design or in the first phase of the central composite design. The amount of vitamin B_{12} obtained 31.67 mg/L. and it achieved better results obtained when compared with the previous experiments as shown in Table 3. The two significant factors correlated based on experimental responses executed by using statistical analysis of variance (ANOVA) according to regression equation (1):

$$[\text{Vitamin } B_{12}] = 2.97+0.24A+0.21B-0.26AB-0.21A^2-0.29B^2$$

Effect	-1.5775	1.5775	5.4125
SE	0.365963	0.365963	0.365963
t-value	0.6831	-0.6831	-0.798
P-value	1.000	0.516	0.451
Confidence level	0.990	0.994	0.995

D=Dummy constituents/variable. Effect = significant for confidence level > 0.95. H−high; L−low

Table 2: Design of experiments and significance of effects of constituents in Plackett-Burman Screening for Propionibacterium freudenreichii

K3PO4 (g/L)	$NaH_2PO_4 2H_2O$ (g/L)	Tryptone (g/L)	Casein Hydrolysate (g/L)	Ca panthothenate (mg/L)	$FeSO_4.7H_2O$ (mg/L)	Biotin (mg/L)	Yield $\mu g/ml^{-1}$
H	L	H	L	H	L	H	31.67
L	H	H	L	L	H	H	24.26

International Journal of Advances in Engineering and Management (IJAEM)
Volume 3, Issue 7 July 2021, pp: 2863-2873 www.ijaem.net ISSN: 2395-5252

L	H	H	L	H	L	H	19.46
H	L	L	H	L	H	L	22.53
L	H	L	H	L	H	L	18.27
H	L	L	H	H	L	L	14.38
L	H	H	L	H	L	H	11.51
L	H	H	L	L	H	H	10.35

Table 3: Central Composite Design (Coded and Uncoded test variables) with observed and predicted yield (Each row corresponds to a single experiment) for Propionibacterium freudenreichii

Run	Ca panthothenate		Casein Hydrolysate (g/L)		$NaH_2PO_42H_2O$ (g/L)		$FeSO_4.7H_2O$ (mg/L)			
	Coded	Uncoded	Coded	Uncoded	Coded	Uncoded	Coded	Uncoded	Observed Yield $\mu g/ml^{-1}$	Predicted Yield $\mu g/ml^{-1}$
1	-1	3.5	-1	4.5	1	6.5	1	3.5	32.68	34.57
2	-1	3.5	1	7.5	1	6.5	-1	3.5	26.25	28.52
3	-1	3.5	1	7.5	-1	3.5	1	4.5	18.56	24.45
4	0	5	0	6.5	0	5	0	6.5	23.54	23.63
5	0	5	0	6.5	0	5	1	4.5	17.34	20.42
6	1	6.5	-1	4.5	0	5	0	7.5	13.26	15.61
7	1	6.5	1	7.5	-1	6.5	-1	5	10.41	17.43
8	1	6.5	-1	4.5	-1	6.5	1	4.5	9.54	13.72

Table 4: Result of the second order response surface model (same for coded and Uncoded test variables) fitting in the form of analysis of variable (ANOVA) for Propionibacterium freudenreichii

	DF	SS	MS	F-test	P-value
Regression	5	13.50000	2.700000	1.801	0.000
Lack-to-fit	3	8.71875	1.743750	4.50	0.500
Total	7	22.2187			

$R^2 = 0.7936$, R = 0.2817, Adj. $R^2 = 0.740$

DF=Degree of freedom, SS=Sum of squares, MS =Mean square

The maximum response surface obtained from three dimensional plot used for evaluation and it indicates introduced model showed significant (p=0.000) and insignificant lack-of-fit was stated (p = 0.500). Shown in Table (4). The regression analysis of the data showed an R^2 value of (0.7936) and an adjusted R^2value of (0.740) was obtained from analysis of variance. The experiment model validity verified from vitamin B_{12} production in culture medium based on the predicted value obtained at confidence interval 95% was 2.85-3.55 mg/L as shown in figure 8. Several studies showed that pantothenic acid is essential for Propionibacterium species (Kośmider, A. et al., 2012). In the present study, the optimal concentration of this compound increased vitamin B_{12} production that was not accompanied by higher cellular biomass. This suggests that Ca pantothenate influences not only primary bacteria metabolism but also processes involved in vitamin B_{12} formation. The validity of the model was verified experimentally, vitamin B_{12} concentration of 34.57 mg/L was attained in culture medium and factors corresponding to the maximum value of response were evaluated. Although, no reports of vitamin B_{12} production using this substrate were published previously. Many of the research reports exist concerning microbial propionic acid production on media containing crude glycerol (Himmi et al., 2000). However, this factor affects the overall substrate utilization of 93% increase in vitamin B_{12} production in optimization of fermentation medium led to be completed when

compared to values attained before initial screening. In the previous research article (Chiliveri et al., 2010) a 43% increase in vitamin B_{12} production by medium optimization with the use of statistical methods is reported.

IV. CONCLUSIONS

The statistical experimental design of two step process of response surface methodology enabled to increase the Vitamin B_{12} production by 93% when compared with highest value attained in initial screening of the culture samples. The concentrations of casein hydrolysate, $Fe_2SO_4.7H_2O$, Ca pantothenate and NaH2PO4. $2H_2O$ influences the production of Vitamin B_{12} synthesis from P. freudenreichii sp. and P. shermanii.

REFERENCES:

[1]. Park, Y.W., Marnet, P.G., Yart, L. and Haenlein, G.F. (2013) Mammary Secretion and Lactation, In: Park, Y.W. and Haenlein, G.F., Eds., Milk and Dairy Products in Human Nutrition: Production, Composition and Health, Wiley, Blackwell, 31-45. http://dx.doi.org/10.1002/9781118534168.ch2

[2]. Schaafsma, G. (2008) Lactose and Lactose Derivates as Bioactive Ingredients in Human Nutrition. International Dairy Journal, 18, 458-465. http://dx.doi.org/10.1016/j.idairyj.2007.11.013

[3]. Ramani, W.-B. and Barbara, B. (2013) Milk and Dairy Product Composition. In: Muehlhoff, E., Bennett, A. and McMahon, D., Eds., Milk and Dairy Products in Human Nutrition, Food and Agriculture Organization of the United Nations, Chapter 3, 42-102. http://www.fao.org/docrep/018/i3396e/i3396e.pdf. Accessed 23-07-2014

[4]. Badui Dergal, S. (2006) Química de los alimentos. Pearson Educación, México, 603-617.

[5]. Papademas, P. and Bintsis, T. (2010) Food Safety Management Systems (FSMS) in the Dairy Industry: A Review. International Journal of Dairy Technology, 63, 489-503. http://dx.doi.org/10.1111/j.1471-0307.2010.00620.x

[6]. Ostojić, S., Pavlović, M., Zivić, M., Filipović, Z., Gorjanović, S., Hranisavljević, S. and Dojcinovic, M. (2005) Processing of Whey from Dairy Industry Waste. Environmental Chemistry Letters, 3, 29-32. C.-S. A. De Jesus et al. 232 http://dx.doi.org/10.1007/s10311-005-0108-9

[7]. Valencia, E. and Ramírez, M.L. (2009) La industria de la leche y la contaminación del agua. Elementos: Ciencia y Cultura, 73, 27-31.

[8]. Smithers, G.W. (2008) Whey and Whey Proteins—From "Gutter-to-Gold". International Dairy Journal, 18, 695-704. http://dx.doi.org/10.1016/j.idairyj.2008.03.008

[9]. Morales, J., Choi, J.S. and Kim, D.S. (2006) Production Rate of Propionic Acid in Fermentation of Cheese Whey with Enzyme Inhibitors. Environmental Progress, 25, 228-234. http://dx.doi.org/10.1002/ep.10153

[10]. Parra Huertas, R.A. (2009) Whey: Importance in the Food Industry. Revista Facultad Nacional de Agronomia Medellin, 62, 4967-4982.

[11]. Tovar, J.X., Téllez, J.A., Mercado, F.Y., Abreu, C.A., Muro, U.C., Gómez, A.C.A. and Arana, C.A. (2013) Suero lácteo ¿Solo un residuo o una oportunidad? Ciencia y Desarrollo, 39, 64-69.

[12]. Poopathi, S. and Abidha, S. (2012) The Use of Clarified Butter Sediment Waste from Dairy Industries for the Production of Mosquitocidal Bacteria. International Journal of Dairy Technology, 65, 152-157. http://dx.doi.org/10.1111/j.1471-0307.2011.00745.x

[13]. Kothari, R., Pathak, V.V., Kumar, V. and Singh, D.P. (2012) Experimental Study for Growth Potential of Unicellular Alga Chlorella pyrenoidosa on Dairy Waste Water: An Integrated Approach for Treatment and Biofuel Production. Bioresource Technology, 116, 466-470. http://dx.doi.org/10.1016/j.biortech.2012.03.121

[14]. Neal, C. and Jarvie, H.P. (2005) Agriculture, Community, River Eutrophication and the Water Framework Directive. Hydrological Process, 19, 1895-1901. http://dx.doi.org/10.1002/hyp.5903

[15]. Anderson, T.M., Bodie, E.A., Goodman, N. and Schwartz, R.D. (1986) Inhibitory Effect of Autoclaving Whey-Based Medium on Propionic Acid Production by Propionibacterium shermanii. Applied and Environmental Microbiology, 51, 427-428.

[16]. Woskow, S.A. and Glatz, B.A. (1991) Propionic Acid Production by a Propionic Acid-Tolerant Strain of Propionibacterium

acidipropionici in Batch and Semicontinuous Fermentationt. Applied and Environmental Microbiology, 57, 2821-2828.

[17]. Sánchez Toro, Ó.J., Ortiz Buriticá, M.C. and Betancourt Garcés, A.L. (2004) Citric Acid Production from Whey by Fermentation Using Aspergillus spp. Revista Colombiana de Biotecnologia, 6, 43-54.

[18]. Wan, C., Li, Y., Shahbazi, A. and Xiu, S. (2008) Succinic Acid Production from Cheese Whey Using Actinobacillus succinogenes 130Z. Applied Biochemistry and Biotechnology, 145, 111-119. http://dx.doi.org/10.1007/s12010-007-8031-0

[19]. Spalla, C., A. Grein, L. Garofano, G. Ferni, 1989. Microbial production of vitamin B12. In: Vandamme, E.J. (Ed.), Biotechnology of Vitamins, Pigments and Growth Factors. Elsevier, London, pp: 257-284

[20]. Martins, J.H., Barg, H., Warren, M.J., Jahn, D., 2002. Microbial production of vitamin B12. Appl. Microbiol. Biotechnol. 58, 275–285.

[21]. Allen, L. H. (2010). Bioavailability of vitamin B12. Int J Vitam Nutr Res, 80(4-5), 330-335.

[22]. Rucker, B. R., Suttie, J. W., McCormick, B. D., & Machilin, L. J. (2001). Handbook of vitamin. New York: 322 Marcel Dekker, Inc.

[23]. Rossi, F., Torriani, S., & Dellaglio, F. (1999). Genus-and species-specific PCR-based detection of dairy propionibacteria in environmental samples by using primers targeted to the genes encoding 16S rRNA. Appl. Environ. Microbiol., 65(9), 4241-4244.

[24]. Kośmider, A., Białas, W., Kubiak, P., Drożdżyńska, A., & Czaczyk, K. (2012). Vitamin B12 production from crude glycerol by Propionibacterium freudenreichii ssp. shermanii: optimization of medium composition through statistical experimental designs. Bioresource technology, 105, 128-133

[25]. Himmi, E. H., Bories, A., Boussaid, A., & Hassani, L. (2000). Propionic acid fermentation of glycerol and glucose by Propionibacterium acidipropionici and Propionibacterium freudenreichii ssp. shermanii. Applied Microbiology and Biotechnology, 53(4), 435-440.

[26]. Chiliveri, S.R., Yeruva, T., Panda, S.H., Linga, V.R., 2010. Optimization of fermentation parameters for vitamin B12 production using Propionibacterium freudenreichii sub sp. shermanii OLP-5 by Taguchi Method. J. Pure Appl. Microbiol. 4 (2), 647–658.

[27]. Piao, Y., Yamashita, M., Kawaraichi, N., Asegawa, R., Ono, H., & Murooka, Y. (2004). Production of vitamin B12 in genetically engineered Propionibacterium freudenreichii. Journal of bioscience and bioengineering, 98(3), 167-173.