Optimization of Vitamin B_{12} Synthesis Using *Propionibacterium* sp. Utilizing Dairy Wastes

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

Abstract:

The medicinal and food industries have long used vitamin B12, a vital nutrient. Numerous bacterial strains, including Propionibacterium freudenreichii, Propionibacterium shermani, and Pseudomonas denitrificans, have been effectively exploited in the industrial production of vitamin B12 because chemical synthesis is too expensive, labor-intensive, and time-consuming. The major goal of this work was to identify microbial strains that produce vitamin B12 and to synthesize vitamin B12 from industrial wastewater, particularly dairy waste water. Propionibacterium species were isolated, identified, and characterized using molecular techniques, particularly polymerase chain reaction (PCR). The purpose of this study was to investigate how to increase vitamin B12 yield from Propionibacterium species utilizing RSM approach. Recent developments in biotechnology have produced techniques that limit modification, deliver results faster, and are less expensive.

Keywords: vitamin B $_{12}$, microbial strains, dairy wastewater, *Propionibacterium* species, PCR, RSM methodology.

Introduction:

Milk has generally been regarded as the most complete food produced by nature and a significant source of the vital elements required for growth and development. Due to its great nutritional content, it is also strongly advised in the human diet [1]. Water, proteins, lipids, carbs, vitamins, and minerals make up the majority of its make-up. The main carbohydrate present in milk is lactose. According to some reports, lactose can increase the absorption of certain minerals, such as calcium and magnesium.[2] Furthermore, caseins and whey proteins, which are biologically active chemicals, have been found to be present in milk in addition to nutritive ones. 80% of the protein in milk is made up of caseins, and the remaining 20% is made up of whey proteins like immunoglobulins, α--lactalbumin, and β--lactoglobulin.[3]

Milk can be used to create a wide variety of dairy products, including whole, skim, lactose-free, and fat-free milk, as well as whole or skimmed milk powder, thanks to its high nutritional value. Condensed and evaporated milk, which can be pasteurized or ultra pasteurized, are additional products.[4][5] The fermented dairy products cheese, yogurt, kefir, and bulgaro are also made from milk. The primary products we receive from milk include butter, milk whey, sweet/sour cream, casein acid type, lacticorrenin, caseinates, lactose, enzymatically modified milk

components, cream, ice cream, and other dairy beverages. Due to pollution and environmental harm, the production of dairy products is a major source. This issue is brought on by waste production from animal producers and byproducts from dairy products [6]–[7].

Whey is a liquid by-product of cheese making. Equivalent to 85% to 90% of original milk yield and retains 55% of nutrients. Whey contains proteins, lipids, soluble vitamins, minerals and carbohydrates. 75% of total whey solids is lactose. Lactose is one of the most environmentally hazardous by-products as it has a biochemical oxygen demand (BOD) of less than 35,000 ppm and a chemical oxygen demand (COD) of less than 60,000 ppm [8]. Disposal of this industrial wastewater into the soil without pretreatment can alter the physical and chemical composition of the soil, reducing crop yields and oxygen availability in water [9][10].]. It has been estimated that 40,000 liters of unprocessed whey will produce as much pollution as a population of 250,000 produces daily [11]. Another by-product from the dairy industry is the clarified butter sediment waste resulting from the production of clarified butter or ghee butter. This butter waste is mainly composed of fat in the form of fatty acids and a small amount of protein [12]. Dumping milk whey into river or lake water can cause serious pollution problems due to high nitrogen and phosphorus levels and high BOD (40 - 48,000 mg/L) and COD (89 - 95,000 mg/L). [13].

However, in this analysis, it was shown that the manufacturing process of many dairy products generates large amounts of by-products.[14][15] Dairy by-products can be used to obtain valuable chemical compounds useful in healthcare, pharmaceuticals, food, plastics and fuels. These additives can act as acidifiers, stabilizers, flavor enhancers or preservatives. Some examples of additives are organic acids (citric, lactic, succinic and propionic acids) [16] - [18]

Vitamin B 12 also known as cyanocobalamin belongs to the cobalamin family, is synthesized by prokaryotes and inhibits the development of pernicious anemia in animals. Since the production of vitamin B12 by chemical synthesis is too costly, several bacterial strains such as Propionibacterium freudenreichi, Propionibacterium shermani and Pseudomonas denitrificans have been successfully used in the commercial production of vitamin B12 in industry. [19] While Pseudomonas reject tool has been successfully and exclusively used in industries because of its rapid growth and high productivity. Propionibacterium species have been shown to have a high potential for vitamin B 12 accumulation and are considered GRAS (generally recognized as safe). In general, high yields of vitamin B12 have been achieved by further treating potentially mutagenic organisms such as UV rays or chemical agents and by selecting strains with practical advantages such as stability. genetics, yield, reasonable growth rate and resistance to high concentrations of toxic substances. in environment. [20]

Vitamin B12, which is one of the water-soluble vitamins and naturally occurring organometallic compounds of cobalt, contains substances involved in a wide range of biochemical processes such as DNA synthesis, fatty acid regulation, amino acid metabolism as well as energy production. Vitamin B12 deficiency causes mitotic disorders, neuropathy, nervous system disease, and pernicious anemia. [21] To prevent such deadly illnesses, you should take a daily 2.4 mcg vitamin B12 supplement. [22]. The aim of this study was to isolate and identify vitamin

B12-producing microorganisms from dairy waste. To screen and characterize the organism's ability to produce vitamin B12 by HPLC method.

Materials and Methods:

Sample Collection and isolation of strains:

The samples were obtained from a variety of industrial dairy waste sites in Chennai, Tamil Nadu, India. For experimentation, the samples were kept in sterile vials at 4°C. Saline (0.9%) was used to homogenize and serially dilute the materials, and three different mediums-Yeast Extract Medium (YEM), MRS Agar, and Sodium Lactate Medium were used for plating procedures. 48 hours were spent incubating the plates at 37°C. The strains were kept in 50% glycerol stock that was kept at -20°C, and working cultures were made using sodium lactate medium stabs that were kept at 4°C.

Screening of vitamin B12 producing organism:

Screening by plating method

Identifying the organism in process of developing vitamin B12 is aided by screening the organisms that produce the vitamin. The colonies that displayed a zone of clearance on starch agar plates were kept on MRS agar plates for additional tests. Strains were cultured in MRS broth at 25°C from high dilution in the MRS agar plates. By streaking on an appropriate agar medium, purity was examined.

Optimization of Vitamin B_{12} using RSM methodology:

Inoculum preparation:

1 ml of the previously preserved culture was diluted into nine milliliters of fresh glucose medium, and the culture was then incubated at 30°C for 48 hours. 200 mL of preculture received this culture in addition glucose-containing medium was incubated at 30C for 48 hours. For the purpose of fermenting vitamin B12, 200 mL of various culture medium were inoculated with an aliquot (10% v/v) of crude glycerol. The culture was kept at 30 °C, and a 25% ammonia solution was used each day to bring the pH down to 6.8. The vitamin B12 precursor, DMB, was introduced after 96 hours, and after 168 hours, the vitamin B12 content of cultures was assessed. Every fermentation experiment was carried out twice.

Experimental design:

P. freudenreichii and P. shermanii were used to maximize the production of vitamin B12 using a two-step optimization technique. The first phase involved using the Plackett-Burman design to determine which variables significantly affect the production of vitamin B12. Seven variables (A–G) were chosen as factors: K3PO4, NaH2PO4.2H2O, casein hydrolysate (acid), tryptone, biotin, calcium pantothenate, and FeSO4.7H2O. Two dummy factors were used to assess the experiment's standard errors. For each factor, low (-1) and high (+1) levels were assigned (Table 1). After 168 hours of fermentation, average vitamin B12 concentration values were employed as the response in this design.

The second phase involved optimizing the amounts of the significant variables chosen by the Plackett-Burman design using the response surface methodology. In order to enable statistical investigation of potential interactions between components in a smaller number of experiments than would be required for factor design, a five level (-a, -1, 0, +1, +a) two-factor Central composite design was used twice. Tables 3 and 4 for the first and second phases of the experiment, respectively, demonstrate the experimental designs and results.

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:

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

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.

Statistical analysis:

All statistical evaluations were performed using STATISTICA 6.0. The significance level for the statistical tests was set at 0.05.

Results and Discussion:

Isolation and screening of Vitamin B_{12} producing bacteria

Out of the 55 isolated strains used in the study, 4 strains displayed the most clear zone on the screening plate when compared to the standard strain. The zone index was compared to the already established effectiveness of vitamin B12 in dairy products, Lactobacillus plantarum (MTCC 1325). Dairy products are not the only source for isolating these strains of P. freudenreichii and P. shermanii. The existence of the aforementioned organisms can also come from other sources, such as human skin, the digestive tract, ruined citrus fruits, etc. Previous research has indicated that Lactobacillus sp. produced more vitamin B12 than other bacteria (Rossi, F., et al., 1999).

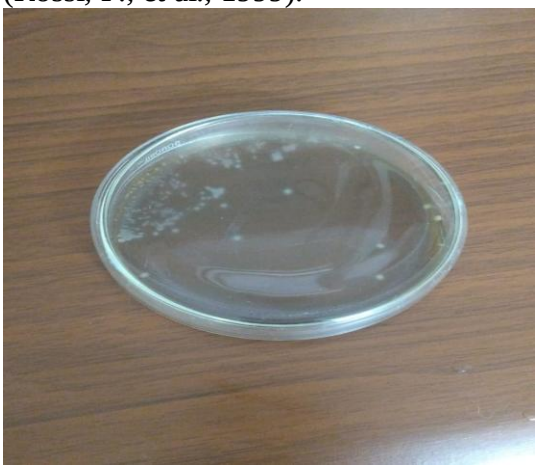

Fig 1: A- Sodium Lactate Medium

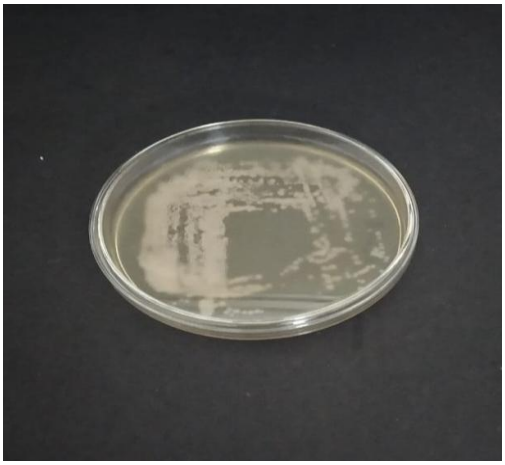

Fig 2: B- Yeast Extract Medium

Purification of vitamin B12 organisms:

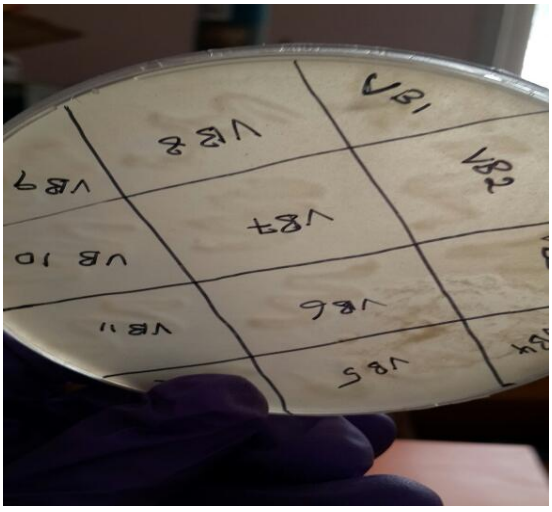

Fig 3: C-Purified strains

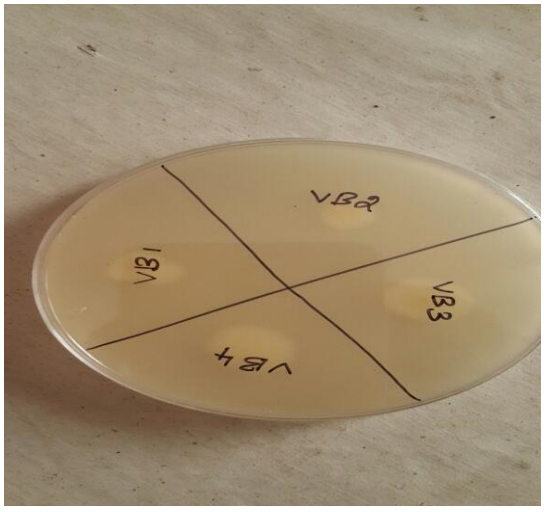

Fig 4: D- Selected strains for optimization

Optimization studies using RSM methodology.

Using the Placket Burmann design, P. shermanii were added to the growth medium of P. freudenreichii to quantify the production of vitamin B12. Seven design elements were tested in this project. The amount of vitamin B12 produced in this experiment across eight runs revealed that 34.57 mg/L was the greatest percentage attained with this technique. ANOVA variance is a statistical tool used to conduct the experiment and determine the association between significant variables and response. Based on this answer, only 4 of the investigated variables substantially influenced the Plackett-Burman design, as shown by the p-value in Table 1.

From Table 2: demonstrated that each parameter of a variable in a regression analysis influences the production of vitamin B12 at a higher level if the variable is positive (H). In addition, if it is negative, there is a low concentration of vitamin B12 (L). Using the important factors described above, the research's next phase was analyzed.

Central composite design

It was created during first level of the Placket Burmann design based on screening variables that affect the production of vitamin B12. The ideal concentration of calcium pantothenate and NaH2PO4.2H2O were shown to have the greatest effects on the formation of vitamin B12 in first factor.

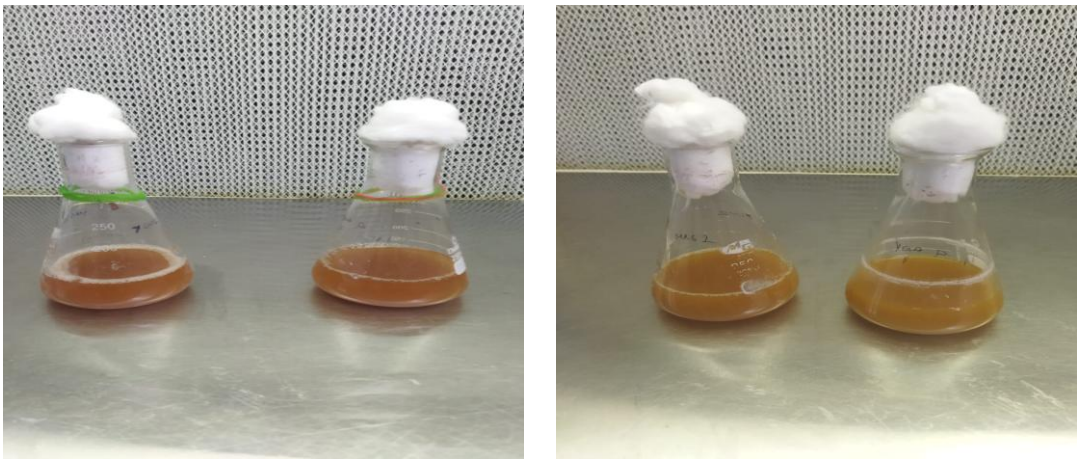

Fig 7: Optimization of Vitamin B12 producing organisms:

Under this experiment, casein hydrolysate and Fe2SO4.7H2O concentration were the second factor taken into consideration. They were used for optimization and kept in the tested culture medium at levels that matched the calculated value of response in the maximum of CCD, while the other medium components (non-significant factors) were kept at the lower (L) level concentration of the levels used in the Plackett-Burman design. Table 2 provides the design matrix, experimental results, and dry cell weight quantity. Some of the runs significantly outperformed the Plackett-Burman design or the first stage of the central composite design in terms of the generation of vitamin B12, according to the data.

When compared to earlier studies, the amount of vitamin B12 obtained, 31.67 mg/L, produced better results, as indicated in Table 3. Based on experimental results analyzed statistically using analysis of variance (ANOVA) in accordance with the regression equation, the two significant factors were correlated.

$$[\text{Vitamin B12}] = 2.97+0.24A+0.21B-0.26AB-0.21A^2-0.29B^2$$

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)	$NaH_2PO_42H_2O$ (g/L)	Tryptone (g/L)	Casein Hydrolysate (g/L)	Ca panthothenate (mg/L)	$FeSO_4.7H_2O$ (mg/L)	Biotin (mg/L)	Yield µg/ml^{-1}
H	L	H	L	H	L	H	31.67
L	H	H	L	L	H	H	24.26
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 µg/ml^{-1}	Predicted Yield µg/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 improved response surface from the three-dimensional plot was used to analyze the model, and it revealed both a substantial (p=0.000) and an insignificant (p=0.500) lack of fit. depicted in Table (4). The data's regression analysis revealed an R2 value of (0.7936), while analysis of variance yielded an adjusted R2 value of (0.740). Figure 8 illustrates how the experiment model's validity was confirmed by vitamin B12 production in culture media based on the anticipated value at a 95% confidence level, which was 2.85-3.55 mg/L. According to a number of studies (Komider, A. et al., 2012), pantothenic acid is necessary for Propionibacterium species. The recommended dosage of this chemical in the current investigation boosted vitamin B12 synthesis without increasing cellular biomass. This shows that Ca pantothenate affects mechanisms involved in the synthesis of vitamin B12 in addition to primary bacterial metabolism. Experimental validation of the model's validity involved achieving a 34.57 mg/L vitamin B12 concentration in culture media and analyzing the variables that would produce the highest possible response. Despite this, there haven't ever been any instances of this substrate being used to produce vitamin B12. Numerous studies have been conducted on the microbial generation of propionic acid on medium containing crude glycerol (Himmi et al., 2000). Furthermore, as compared to the values achieved prior to initial screening, this component has an impact on the overall substrate utilization of the 93% increase in vitamin B12 production that was completed as a result of the modification of the fermentation medium. An increase in vitamin B12 production of 43% was reported in the earlier research paper (Chiliveri et al., 2010) by medium optimization with the use of statistical methods.

Conclusions

The statistical experimental design of two step process of response surface methodology enabled to increase the Vitamin B12 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 B12 synthesis from *P. freudenreichii sp.* and *P. shermanii*.

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