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Media optimization studies and production of adenosylcobalamin (Vitamin B12) by environment friendly organism *Rhizobium* spp

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ABSTRACT

Production of Vitamin B12 from microbial sources has many advantages over conventional chemical synthesis. In the present investigation, an attempt was made to isolate and characterize the environment friendly symbiotic *Rhizobium* species from its natural host, *Sesbania sesban* (L) root nodules, as source of adenosylcobalamin producer. A total of 75 isolates of *Rhizobium* were obtained and characterized by morphological, biochemical, and molecular methods. All the isolates obtained, produced the compound of interest in the range of 0.5–7 ppm. Two isolates, namely, AMB and PMT4 showed higher production of Adenosylcobalamin than the others. These isolates, on optimization showed increased production (28±0.26 ppm and 19±0.26 ppm). Beet Molasses, Cobalt Nitrate, and 5,6 DMB were found to be essential components for adenosylcobalamin production. Further, although betaine and Choline Chloride were revealed to affect the cell growth, they could elicit Adenosylcobalamin production. Thus, *Rhizobium* species has dual advantage as Vitamin B12 producer and as nitrogen fixing environment friendly organism. Further studies are warranted for genetic improvement to enhance Vitamin B12 production without affecting its nitrogen fixing ability.

1. INTRODUCTION

There is an increasing consciousness about nutrition and health all over the globe. However, the developing world is still burdened with the problem of under and malnutrition. This directly relates to vitamin deficiencies [1]. Vitamin B12 is highly essential and one of the most fascinating molecule in the world of nutrition. It is crucial in the production pathways of fatty acids and thus the bioenergetics. It was initially discovered as a treatment of pernicious anemia[2]. Prolonged deficiency of Vitamin B12 leads to irreversible neurological damage. Clams, eggs, oysters, fishes, meat, and milk are known sources of Vitamin B12. However, under specific conditions, intestinal microbes produce Vitamin B12, anaerobically [3,4].

Hydroxycobalamin (OH-Cbl), 1,5–deoxyadenosylcobalamin (Ado-cbl), and methylcobalamin (Me-Cbl) are the natural forms of Vitamin B12 which are produced by microbes. Cyanocobalamin (CN-Cbl) is not the natural form of Vitamin B12 but is commercially synthesized because of its stable structure. Physiologically, Vitamin B12 is essential to maintain myelin sheath of the nerve cells. In addition, it is an essential nutrient for fat and carbohydrate metabolism, and synthesis of DNA in the bone marrow during the formation of red blood cells (RBCs). The deficiency of Vitamin B12 affects growth and development of RBCs leading to megaloblastic anemia [5]. Ado-Cbl and Me-Cbl act as cofactors for the enzymes methyl malonyl coenzyme A (CoA) mutase and methionine synthase [2]. The production of Vitamin B12 from microbial sources involves, approximately, 30 enzymatic steps either through anaerobic pathway as observed in *Lactobacillus reuteri, Propionibacterium shermanii, Salmonella typhimurium,* and *Bacillus megaterium* or through aerobic pathway as apparent in *Pseudomonas denitrificans* [6]. Chemical synthesis of Vitamin B12, in contrast, is a 60 step extensive process [7]. The production of Vitamin B12 from microbial sources is considered as an alternative method because of its simple process. The production of Vitamin B12 has been commercially achieved using bacterial strains such as *Pseudomonas, Nocardia and Propionibacterium* [8,9] and a higher productivity has been reported from Cobalt-resistant strain of *Propionibacteria* [4]. Selection of natural Vitamin B12 producers is an endorsed strategy as it does not have any legislative hurdles [10,11]. Various metabolic engineering strategies have been reported for enhanced production of Vitamin B12 in *P. freudenreichii* [12]. A genetically modified *P. Freudenreichii* strain harboring a plasmid comprising *hem*A, from *Rhodobacter sphaeroides*, and homolog of *hem*B and *cob*A, showed 2.2-fold increase in Vitamin B12 [9,13]. These studies have shown that multigene expression systems increase the Vitamin B12 production in *Propionibacteria.* However, other strategies of enhanced supply of precursors such as aminolevulinic acid and allied intermediates were found to be beneficial [14].

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The genetically engineered strain of *P. denitrificans* gave around 100–300 mg/l of productivity [8]. Commercial productions mostly rely on the strains which show rapid growth and high productivity and therefore adoption of genetically modified microbes is advocated.

The main focus of this study was to isolate the root nodulating, nitrogen fixing bacterial strains having potential to produce Vitamin B12 and optimization of media for its high recovery.

2. MATERIALS AND METHODS

Sesbania sesban was collected from eight industrial areas around Mumbai city [Table 1]. A total of 120 test isolates were obtained from the root nodules. All isolates were morphologically and biochemically characterized, and 75 isolates were confirmed using 16s RNA sequencing [15]. The confirmed stains were preserved in a glycerol stock at −80ºC. These 75 isolates were screened for the production of adenosylcobalamin using Submerged Fermentation Technique. The culture was inoculated into 250 ml Erlenmeyer flasks containing 30 ml of media [Table 2] and was incubated at 30°C on rotary shaker at 200 rpm for 48 h. Then, 10% media of this culture were inoculated in 30 ml of seed medium [Table 3] and incubated at 30°C with 200 rpm for 25 ± 1 h. Then, 10% (v/v) seed culture was transferred to 60 ml of production medium in 500 ml of Erlenmeyer flasks [Table 4].

After screening, eight (one from each industrial area) out of these 75 isolates were selected as high producers of adenosylcobalamin. Two of these highest adenosylcobalamin producing strains, namely, AMB and PMT4 were selected for further studies.

Table 1: Collection site for test sample (*Sesbania sesban*) – A total of eight industrial areas in the Mumbai Metropolitan region

Isolates	Industrial areas	Locations
PMT	Maharashtra Industrial Development Corpration	Panvel
TB	TTC Industrial Area , Pawne	Thane Belapur Road
AN	Maharashtra Industrial Development Corpration	Anand Nagar, Thane
PG	Maharashtra Industrial Development Corpration	Patalganga
AMB	Maharashtra Industrial Development Corpration	Ambernath
BD	Maharashtra Industrial Development Corpration	Badlapur
ZS	Jindal steel plant	Khalapur
MT	Maharashtra Industrial Development Corpration	Taloja

Table 2: Composition of inoculum media

Constituent	Concentration(g/l)
Amonium sulfate	0.2
Diammonium hydrogen phosphate	2.35
Magnous sulfate	0.2
Beet Molasses	120
Magnesium sulfate	2.5
Sucrose	40
Zinc sulfate	0.2

2.1. Media Optimization for Adenosylcobalamin Production

Medium optimization was carried out by the traditional method of taking one variable at a time and keeping other variables fixed, i.e., varying one factor while keeping all others constant. Using this approach, the important media components were identified, and they were further used to optimize the fermentation media. The effect of varying concentrations of Betaine, Cobalt Nitrate, Beet Molasses, Choline Chloride, and 5,6 DMB on cell growth and adenosylcobalamin biosynthesis, was tested by designing set of experiments using Taguchi's method [Table 5]. The flasks were incubated at 200 rpm for 7 days at 30°C. 10% (v/v) of 50 % Sucrose feeding was done in a production flask from log 48 to 120 h at 24 h interval.

2.2. HPLC Analysis

The samples were analyzed using HPLC as per Singh *et al*., from 120 h onward up to 168 h, respectively [16].

2.3. LC MS Analysis

The samples were analyzed using LC MS method, executive Plus – Orbitrap MS, column details – Hypersil gold 3 micron 100 × 2.1 mm, solvent used: Solvent A – 0.1% food acid in milliq water, solvent B – 100% acetonitrile, run time – 30.000 [min], syringe type – Hamilton, flow rate – 3.000 µL/min, inner diameter – 2.303 mm, volume – 250 µL, polarity – Positive, In-source CID – 0.0 eV, microscans 1,

Table 3: Composition of seed media

Constituent	Concentration (g/l)
Beet molasses	50
Magnous sulfate	0.25
Zinc sulfate	0.01
Sodium molybdate	0.02
Magnesium sulfate	0.05
Glycerol	3.5
Nutrient broth	4.5

Table 4: Composition of production media

Constituent	Concentration (g/l)
Beet molasses	10, 60, 120, 180
Calcium carbonate	2
Glycerol	1.5
Sucrose	8
Choline chloride	2.5
Magnesium sulfate	1.5
Diammonium hydrogen phosphate	2
Ammonium sulfate	1.8
Cobalt nitrate	0.1, 0.2, 0.3, 0.4
Ferrous sulfate	0.02
Magnous sulfate	0.02
Monosodium glutamate	3
Zinc sulfate	0.02
Potassium dihydrogen phosphate	0.25
5,6 Dimethyl benzemidiazole	0.02, 0.05, 0.1, 0.15
Betaine monohydrate	0, 1, 2, 5

Table 5: Composition of different sets of Production medium designed using Taguch's method

Set of experiments	Medium constituent (g/l)				
	Betaine monohydrate	Cobalt nitrate	5,6 DMB	Choline chloride	Beet molasses
1	0	0.1	0.02	2.5	10
2	0	0.1	0.05	5.5	60
3	0	0.2	0.1	6.5	120
4	0	0.3	0.15	7.5	180
5	1	0.4	0.05	6.5	180
6	1	0.2	0.02	7.5	120
7	1	0.3	0.15	2.5	60
8	1	0.4	0.1	5.5	10
9	2	0.1	0.1	7.5	60
10	2	0.2	0.15	6.5	10
11	2	0.3	0.02	5.5	180
12	2	0.4	0.05	2.5	120
13	5	0.1	0.15	5.5	120
14	5	0.2	0.1	2.5	180
15	5	0.3	0.05	7.5	10
16	5	0.4	0.02	6.5	60

resolution – 70,000, AGC target – 3e6, Maximum IT – 200 ms, number of scan ranges – 1, scan range – 133.4 to 1800m/z.

2.4. Determination of Dry Cell Weight (DCW)

The liquid culture was centrifuged at 5000 rpm for 10 min after fermentation, followed by three washes with distilled water and pellet was obtained. The biomass was then dried to a persistent weight at 100°C.

2.5. Statistical Analysis

The analysis of the data was done with the help of SPSS 24.0 software (SPSS Inc., Chicago, IL, USA). Primary and secondary variables under study were analyzed and statistical such as like percentages; standard deviation and mean were calculated. Logistic regression was applied considering amount of adenosylcobalamin produced by the isolates as the dependent variable and medium components as an independent variable. $P < 0.05$ was statistically significant.

3. RESULTS AND DISCUSSION

3.1. Screening of Isolates and Media Optimization for Adenosylcobalamin Production

A total of 75 isolates were screened for adenosylcobalamin production [Figure 1]. Out of these, eight significant producers were selected based on their performance and were further taken for characterization and optimization studies [Figure 2]. Finally, two highest producing strains were used for further studies.

For media optimization important components such as Betaine, Cobalt Nitrate, 5,6 DMB, Choline Chloride, and Beet molasses were evaluated at various concentrations [Table 6]. It was observed that betaine was a necessary compound for adenosylcobalamin production, although it had a negative impact on cell growth. A maximum DCW of 22 (± 0.01) g/l only was obtained in presence of betaine with the highest adenosylcobalamin titer (5g/L). With the increased concentration of Choline Chloride in fermentation medium, the DCW decreased gradually, confirming that Choline also has an undesirable effect on cell growth. The low yield after addition of Choline Chloride may be due to change in pH during fermentation. This perhaps had a negative effect on activities of enzymes involved in biosynthesis. For the formation of Methionine, Choline Chloride, and betaine are essential and this amino acid is then converted to S-adenosylmethionine by the action of methionine adenosyltransferase enzyme. Betaine, although, has undesirable effect on cell growth, its feeding during fermentation was found effective to enhance production of adenosylcobalamin. The higher synthesis of Vitamin B12 precursors such as methionine, glutamate, and glycine was reported only after addition of betaine to the production media [9,17]. Choline and betaine increased the formation of adenosylcobalamin in *Agrobacterium* species by, as much as five to six folds [8,18]. As reduction of production cost is an essential aspect, a low cost carbon source, namely, Beet Molasses was screened. The addition of precursors such as DMB, Cobalt ions, or compatible solutes such as Choline and betaine for adenosylcobalamin was found to be beneficial. Under optimal fermentation conditions approximately 28.57 ± 0.26 mg/l of adenosylcobalamin were accumulated in the fermentation medium during 7 days run [Table 6] while, *Rhizobium cobalaminogenum* was reported as the most active producers of Cyanocobalamin (16.5 mg/l) [2,14]. Margaret *et al*. screened 70 strains representing six species of *Rhizobium*, namely, *R. trifoli, Rhizobium meliloti, R. japonicum, R. leguminosarum, R. phaseoli, and R. lupine* for Vitamin B12 production in which, *R. meliloti* showed the highest production (1000 µg/ml) of Vitamin B12 under the experimental conditions. Addition of 1 mg/L of Cobalt Chloride in media for *R. meliloti* and *Bradyrhizobium japonicum* was found essential for maximum production of Vitamin B12 [13,19].

The adenosylcobalamin produced in the media was evaluated using HPLC and MS. The UV-vis spectra data retention time (11 min) obtained by HPLC analysis showed the peak matching to adenosylcobalamin [Figures 3 and 4]. The LC MS analysis of the isolated compound produced by *Rhizobium* isolate was carried out. The mass of the ionized peak confirmed the presence of adenosylcobalamin in isolated compound produced by *Rhizobium* isolate. The mass of adenosylcobalamin is 1579.58m/z and its spectra show that it is doubly charged. Mass of 790 m/z was observed instead of their singly charged mass of 1581 m/z. The mass spectrometry data [Figures 5 and 6] showed that the spectra of the compound produced by *Rhizobium* isolate matched the values of Vitamin B12 analog, adenosylcobalamin.

Vitamin B12 is a highly essential and indispensable component for normal functioning of human body. Different producer-strains synthesis pathways for production of Vitamin B12 are under consideration by research institutions. New Technologies and strategies are being developed to increase the potential of the microbes for its production.

In the recent years, a lot of interest on this subject has been shown by the researchers in various parts of the world. In one such metagenomic analysis study, it was observed that less than 10% of soil archaea and bacteria (Predominantly *Nitrospirae*, *Proteobacteria*, *Thaumarchaeota, Actinobacteria*, and *Firmicutes*) could initiate the coding process of the genetic makeup required for *de novo* production of the enzyme cofactor, which is required for manufacturing adenosylcobalamin. In the same study, the enrichment of DMB and corresponding DMB synthesis genes, relative to corrin ring synthesis genes, suggests an important role for cobalamin remodelers in terrestrial habitats [20].

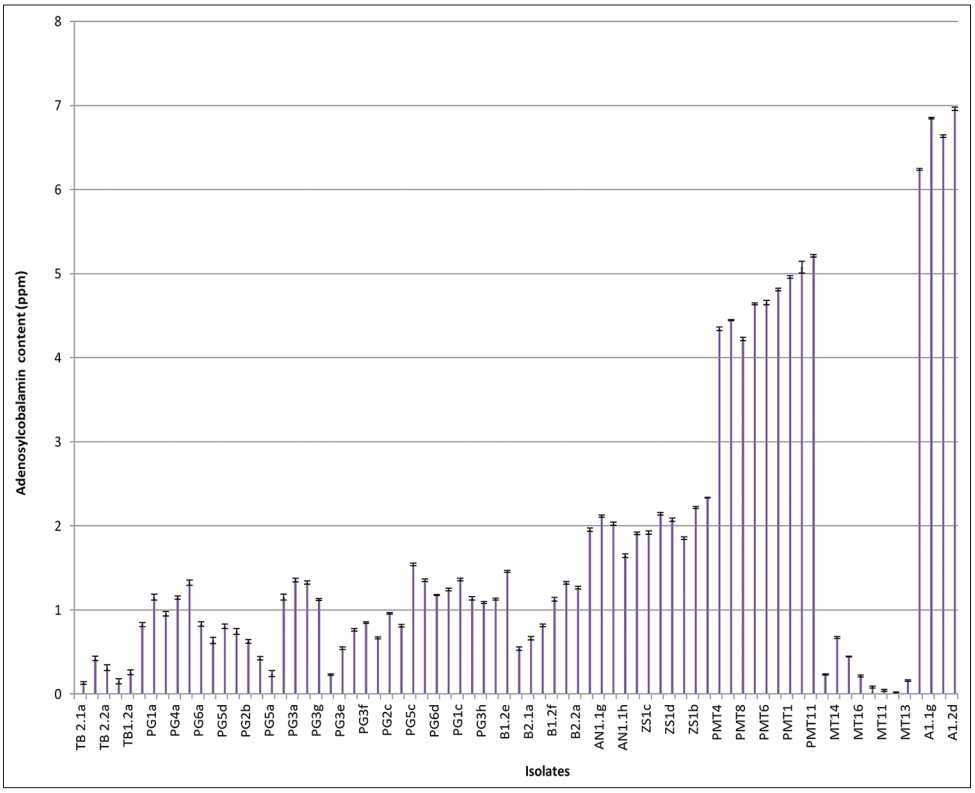

Figure 1: Adenosylcobalamin production efficiency of different isolates.

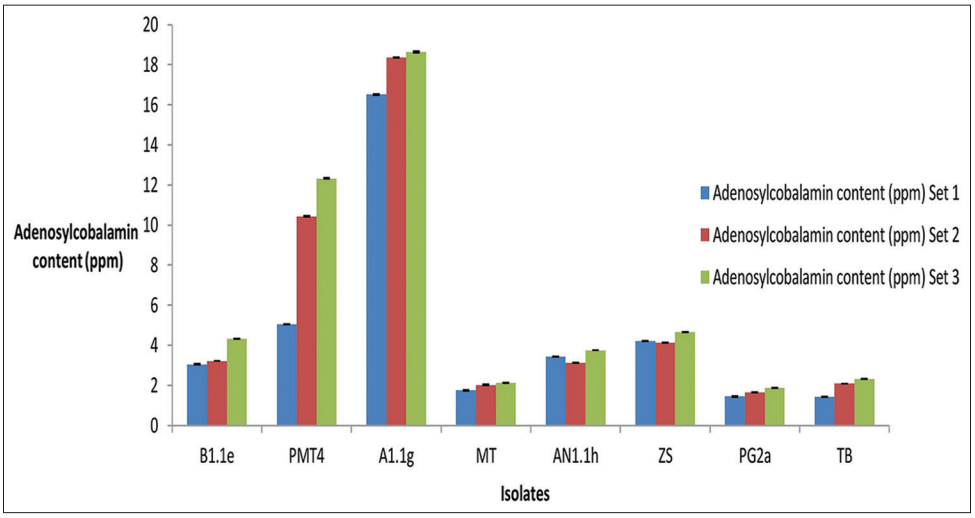

Figure 2: Media optimization for high producers of adenosylcobalamin.

In another study, the corrinoid compounds production in *Lactobacillus* (*L*.) strains (such as *L. reuteri* CRL 1098 and *L. coryniformis* CRL 1001) was increased by adding 5,6-dimethylbenzimidazole and Co^{2+}. Whereas, when L-threonine was added, it only increased the production of corrinoid compounds by CRL 1001 strain [21]. As stated above, the biosynthesis of Vitamin B_{12} is limited to only few bacteria and archaea and depends as such on microbial fermentation. Current innovations in metabolic engineering and synthetic biology are being involved to efficiently construct many microbial chemical factories [22].

In one more study, genes responsible for biosynthesis of adenosylcobinamide phosphate from *Rhodobacter capsulatus* were studied *in vitro* and/or *in vivo*. The analysis suggests that the biosynthetic steps from co(II)byrinic acid a,c-diamide to adocobalamin are same in both the anaerobic and aerobic pathways. The yield of Vitamin B_{12} from a genetically engineered, recombinant *E. coli* strain could be increased by more than ~250-fold to 307.00 $\mu g\, g^{-1}$ DCW by metabolic engineering and optimizing the favorable conditions required for fermentation [23].

3.2. Statistical Analysis

Selection of media components plays a key role in adenosylcobalamin production. Sixteen sets of experiments were performed using different

Table 6: Media optimization for high producers of adenosylcobalamin (AMB and PMT4) and DCW (at 168 h) under four various concentrations of betaine, DMB, beet molasses, choline chloride, and cobalt nitrate

Set of experiments	Amount of adenosylcobalamin produced by AMB isolate	DCW g/l of AMB	Amount of adenosylcobalamin produced by PMT4 isolate	DCW g/l of PMT4
Set 1	2.20±0.11	22±0.01	1.16±0.03	20.26±0.19
Set 2	5.07±0.60	21.49±0.15	3.15±0.03	19.54±0.10
Set 3	5.21±0.37	20.50±0.16	3.66±0.21	18.36±0.14
Set 4	8.37±0.25	18.28±0.18	6.25±0.17	15.38±0.33
Set 5	15.30±0.40	15.56±0.11	12.29±0.19	13.36±0.40
Set 6	10.37±0.26	14.45±0.24	8.59±0.18	12.52±0.41
Set 7	9.2±0.29	15.33±0.19	8.93±0.06	13.29±0.29
Set 8	8.07±0.44	18.57±0.22	7.06±0.05	15.39±0.31
Set 9	18.37±0.26	15.48±0.11	14.44±0.21	13.64±0.21
Set 10	10.36±0.15	14.19±0.17	9.40±0.24	12.42±0.44
Set 11	15.51±0.22	13.82±0.15	12.5±0.19	11.5±0.17
Set 12	19.27±0.42	14.45±0.35	16.47±0.21	12.40±0.48
Set 13	28.62±0.26	10.43±0.42	19.29±0.26	8.61±0.33
Set 14	20.1±0.25	10.43±0.39	17.72±0.11	8.03±0.04
Set 15	12.37±0.26	12.46±0.29	10.45±0.21	10.5±0.42
Set 16	15.48±0.17	12.69±0.29	13.62±0.16	10.51±0.42

The results were means ± SD (standard deviation) of triplicate determinations

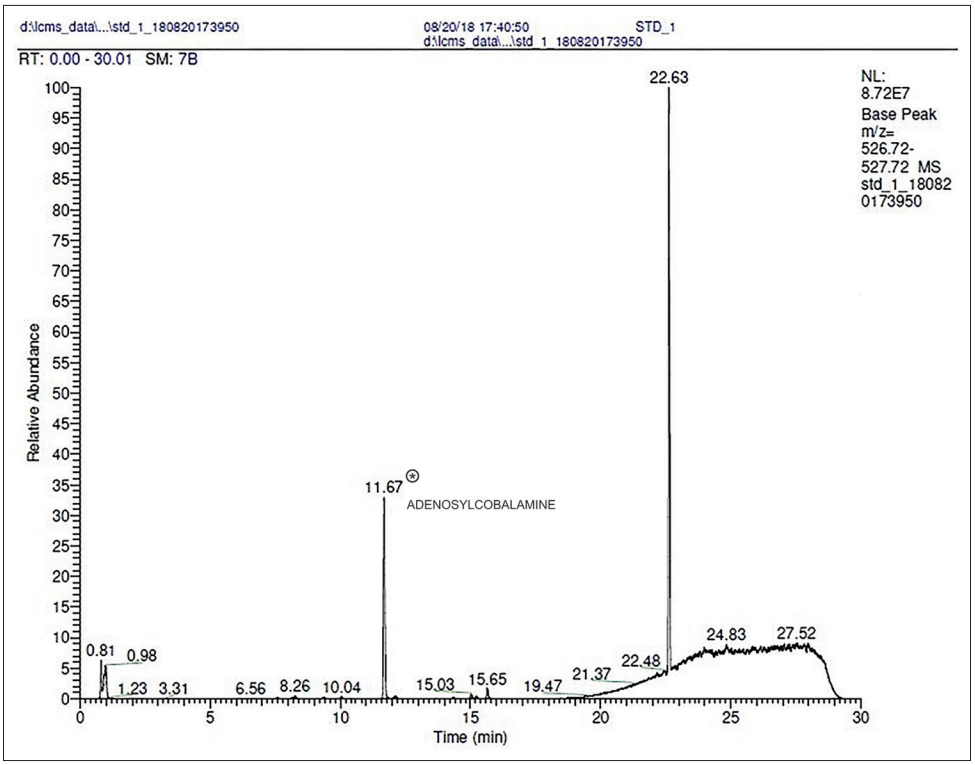

Figure 3: HPLC spectrum of the adenosylcobalamin standard.

combinations of variables – Beet Molasses, 5,6 DMB, Choline Chloride, and Cobalt Nitrate as per Taguchi's method. Independent T-test was performed and *P*-value represents that the model was significant [refer in supplementary file Table S1]. Correlation analysis was performed and the relationship between the significant variables and the response was determined. It was observed that the Pearson Correlation was positive and the variable under study had beneficial impact on the adenosylcobalamin production. Negative Pearson Correlation for the variable was also observed which had its beneficial effect at the lower concentrations. The above-mentioned relationship between the variables and the response for adenosylcobalamin production was considered in the next stage for regression analysis.

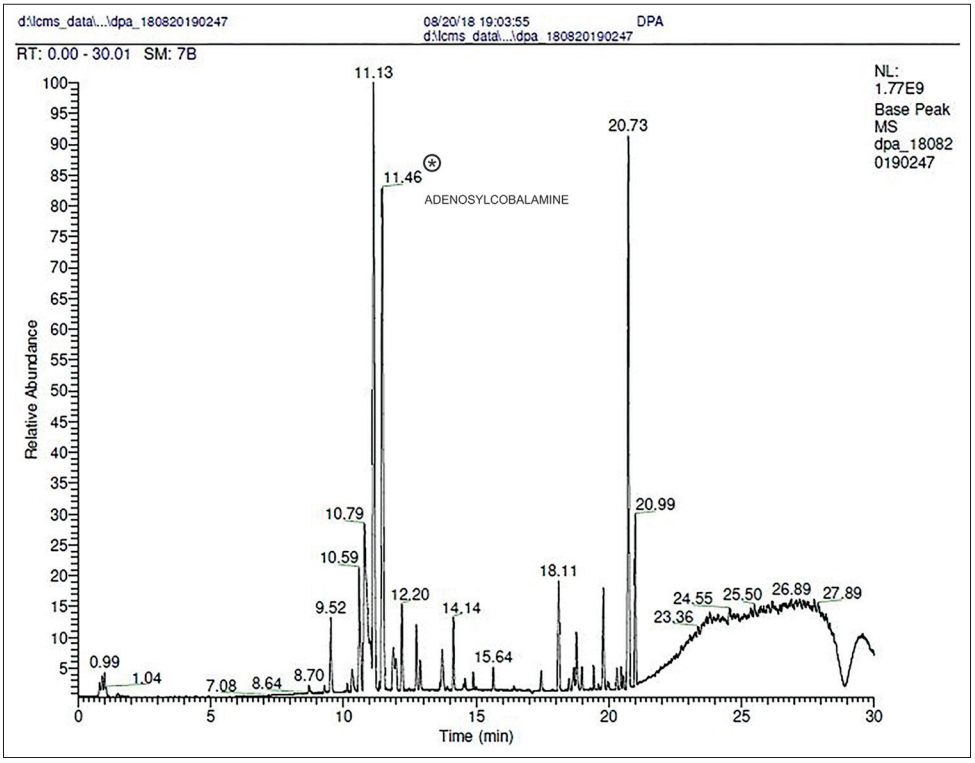

Figure 4: HPLC spectrum of the extract from *Rhizobium* isolates AMB.

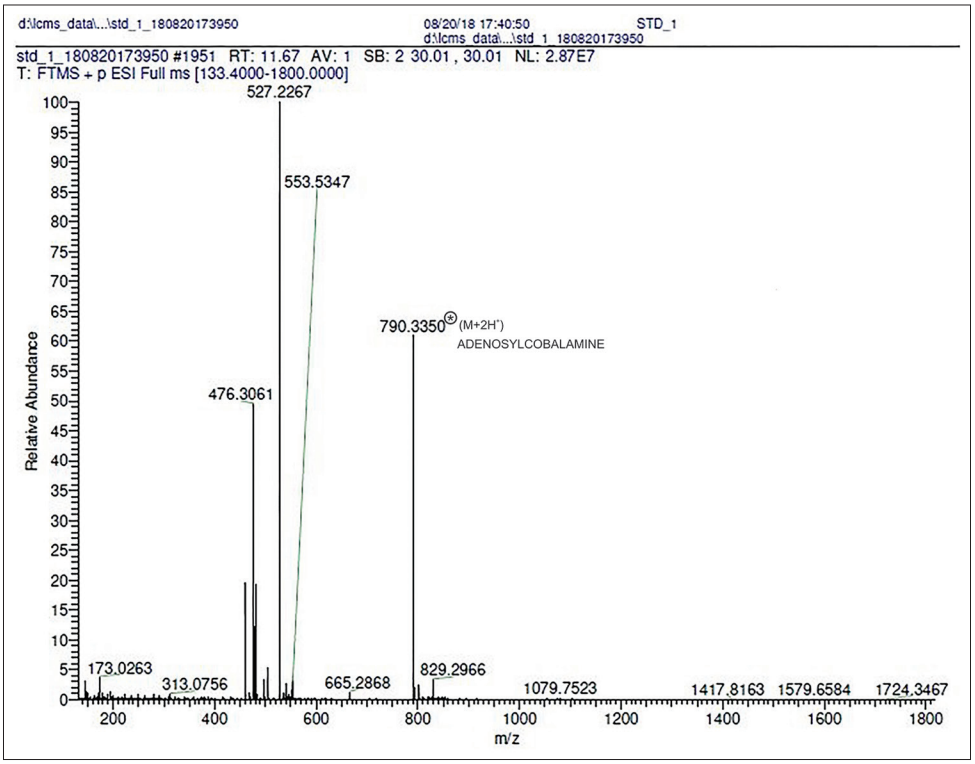

Figure 5: Mass spectrometry spectrum of the standard adenosylcobalamin.

Multiple regression analysis was used to analyze the data for adenosylcobalamin production [refer in supplementary file Table S2 - S5]. The regression model's goodness of fit was checked by multiple correlation coefficients (R^2). The model proved to have accuracy, precision and reliability as the R^2 value is close to 1. The *P*-value of the model revealed that the model was significant.

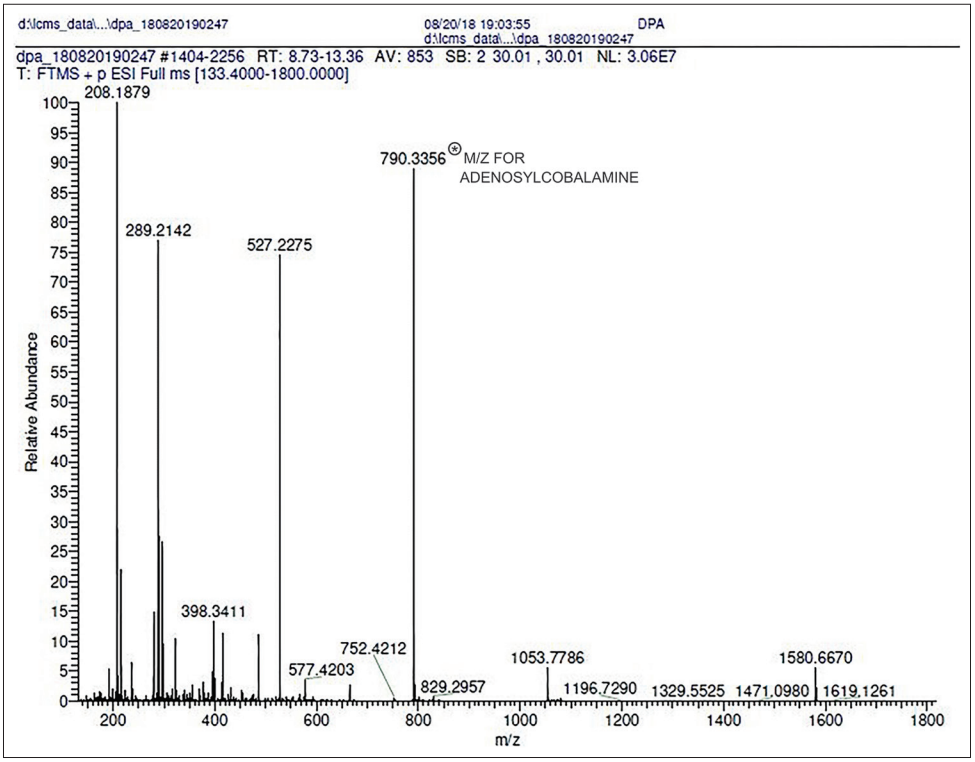

Figure 6: Mass spectrometry spectrum of the extract from *Rhizobium* isolate AMB.

4. CONCLUSION

A total of 75 *Rhizobium* species were screened for the production of adenosylcobalamin using submerged fermentation technique. All the isolates were capable of producing in the range of 2–28 ppm. In comparison to others, isolates AMB and PMT showed high yield (28 ± 0.26 ppm and 19±0.26 ppm). In this study, we reported higher yield of adenosylcobalamin from *Rhizobium* isolate than that of earlier research work. Betaine and Choline Chloride were found to affect the cell growth; however, they could stimulate the adenosylcobalamin production. It was observed that the Beet Molasses, Cobalt Nitrate, and 5,6 DMB are essential components in production media. Media optimization is necessary for each fermentation process. Thus, *Rhizobium* species has dual advantage as Vitamin B12 producer and as nitrogen fixing environment friendly organism. Further studies will be warranted for genetic improvement to enhance Vitamin B12 production without affecting its nitrogen fixing ability.

5. AUTHORS' CONTRIBUTIONS STATEMENT

Neha Nohwar have made substantive intellectual contributions to the content of this manuscript in the areas of concept and design, data acquisition, data analysis/interpretation, drafting manuscript, critical revision of manuscript, statistical analysis, funding, admin, technical or material support, and final approval.

Rahul V. Khandare have made substantive intellectual contributions to the content of this manuscript in the areas of concept and design, data acquisition, data analysis/interpretation, drafting manuscript, critical revision of manuscript, admin, technical or material support, supervision, and final approval.

Neetin S Desai have made substantive intellectual contributions to the content of this manuscript in the areas of concept and design, data acquisition, data analysis/interpretation, drafting manuscript, critical revision of manuscript, admin, technical or material support, supervision, and final approval.

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7. CONFLICT OF INTEREST

Authors declared that there are no conflicts of interest.

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None.

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SUPPLEMENTARY TABLES

Table S1: Independent *t*-test

Set of experiments	Group	*n*	Mean	Standard deviation	*P* value
Set 1	AMB isolate	3	2.2033	0.11930	0.0001
	PMT4 isolate	3	1.1600	0.03000	
Set 2	AMB isolate	3	5.0767	0.60178	0.005
	PMT4 isolate	3	3.1500	0.03606	
Set 3	AMB isolate	3	5.2167	0.37581	0.003
	PMT4 isolate	3	3.6667	0.21032	
Set 4	AMB isolate	3	8.3733	0.25423	0.0001
	PMT4 isolate	3	6.2500	0.17349	
Set 5	AMB isolate	3	15.3033	0.40079	0.0001
	PMT4 isolate	3	12.2933	0.19140	
Set 6	AMB isolate	3	10.3700	0.26627	0.001
	PMT4 isolate	3	8.5967	0.18877	
Set 7	AMB isolate	3	9.2000	0.29614	0.207
	PMT4 isolate	3	8.9367	0.06658	
Set 8	AMB isolate	3	8.0733	0.44736	0.018
	PMT4 isolate	3	7.0600	0.05292	
Set 9	AMB isolate	3	18.3700	0.26627	0.0001
	PMT4 isolate	3	14.4433	0.21008	
Set 10	AMB isolate	3	10.3633	0.15948	0.005
	PMT4 isolate	3	9.4033	0.24583	
Set 11	AMB isolate	3	15.5100	0.22605	0.0001
	PMT4 isolate	3	12.5000	0.19157	
Set 12	AMB isolate	3	19.2700	0.42532	0.001
	PMT4 isolate	3	16.4700	0.21633	
Set 13	AMB isolate	3	28.6267	0.26764	0.0001
	PMT4 isolate	3	19.2933	0.26083	
Set 14	AMB isolate	3	20.1000	0.25060	0.0001
	PMT4 isolate	3	17.7233	0.11930	
Set 15	AMB isolate	3	12.3733	0.26160	0.001
	PMT4 isolate	3	10.4533	0.21548	
Set 16	AMB isolate	3	15.4800	0.17692	0.0001
	PMT4 isolate	3	13.6233	0.16503	

Table S2: Correlations (PMT4)

AMB isolate	Betaine monohydrate	Cobalt nitrate	5,6 DMB	Choline chloride	Beet molasses
Pearson Correlation	0.736**	0.088	0.157	−0.035	0.394
P value	0.001	0.747	0.561	0.896	0.131
n	16	16	16	16	16

**Correlation is significant at the 0.01 level (2-tailed).

Table S3: Correlations (PMT4)

PMT4 isolate	Betaine monohydrate	Cobalt nitrate	5,6 DMB	Choline chloride	Beet molasses
Pearson correlation	0.767**	0.220	0.124	−0.094	0.375
P value	0.001	0.413	0.646	0.730	0.152
n	16	16	16	16	16

**Correlation is significant at the 0.01 level (2-tailed)

Table S4: Linear regression (For dependent variable: PMT4 isolate)

	Unstandardized coefficients		*t*	*P* value	*R* square change	95% confidence interval for B	
	B	Standard error				Lower bound	Upper bound
Constant	6.102	1.290	4.732	0.0001		3.336	8.868
Betaine Monohydrate	2.106	0.471	4.473	0.001	0.782	1.096	3.116

Table S5: Linear regression **(**For dependent variable: AMB isolate)

	Unstandardized coefficients		*t*	*P* value	*R* square change	95% confidence interval for B	
	B	Standard error				Lower bound	Upper bound
Constant	7.564	1.744	4.336	0.001		3.823	11.305
Betaine Monohydrate	2.590	0.637	4.067	0.001	0.852	1.224	3.956