RESEARCH PAPER | Biotechnology | Volume : 3 | Issue : 7 | July 2013 | ISSN - 2249-555X

Evaluation of Vitamin B12 Synthesis by isolated *Rhizobium* sp. from *Sesbania sesban* found in Mumbai and its suburban areas

KEYWORDS: Vitamin B12, *Sesbania sesban*, *Rhizobium* sp., Fermentation

Nishtha K. Singh	Dr. Umesh Luthra	Dr. Neetin Desai
IPCA Laboratories Ltd. Biotech, Kandivali, Mumbai- 400067	IPCA Laboratories Ltd. Biotech, Kandivali, Mumbai- 400067	Department of Biotechnology and Bioinformatics, Padmashree Dr. D. Y. Patil University, Sector No. 15, C.B.D. Belapur, Navi-Mumbai- 400 614

ABSTRACT *ABSTRACT Vitamin B12 is most chemically complex of all the Vitamins .It was produced in 1926, as an anti-pernicious anemia factor. Its synthesis is restricted to micro-organism which engrossed complicated biosynthetic pathway. In present study Vitamin B12 production was studied with newly isolated Rhizobium species obtained from Sesbania sesban. The six isolates of Rhizobium spp were screened for the production of Vitamin B12. Submerged fermentation was applied for the evaluation of Vitamin B12 Synthesis. All six isolates were capable of producing Vitamin B12, on an average range of approx 2-3 mg/kg. Isolate CHA was able to produce higher yield of 1.35 mg/kg in comparison to control culture (Pseudomonas denitrificans ATCC 19244) 1.05 mg/ kg. Maximum activity of 5.08 mg/kg was attained with CHA culture by media optimization with literature media while in control culture the activity was mg/kg. Production of Vitamin B12 depends on the proper balance of medium constituents and their utilization by isolates during the fermentation process. Addition of Beet molasses in fermentation medium shows positive impact on Vitamin B12 Yield.*

INTRODUCTION

Vitamin B12 is one of the most alluring and fascinating molecule in the world of science, food and medicine. It was discovered as the anti pernicious anemia factor. It is normally involved in the metabolism of every cell in the human body especially fatty acid synthesis and energy production. (4)

Submerged fermentation applied for the production of Vitamin B12 by using isolated *Rhizobium* culture. *Rhizobium* is a genus of Gram negative soil bacteria that fix nitrogen. *Rhizobium* forms an endosymbiotic nitrogen fixing association with roots of legumes. Nodulated legumes have potential for nitrogen fixation, reforestation and to control soil erosion. (Lorite,et. al.,2010;Elsoni and Osman, 2011). Plant diversity significantly enhances rate of microbial process that mediate C and N cycling. (Mukherjee et al.2006) *Rhizobium* with their ability to fix nitrogen in symbiosis with legumes, play central role in the Nitrogen supply of soil ecosystem.Nitrogen fixation is a result of intimate Symbiotic relationship between the root nodule bacteria and the host plants.

The objective of this study was to isolate, characterize the *Rhizobium* species isolated from *Sesbania sesban* from different regions of Mumbai and its suburban areas and evaluation of Vitamin B12 synthesis by the isolated *Rhizobium* cultures. Besides biological nitrogen fixation, some strains of *Rhizobium* are involved in the production of Vitamin B12. Isolation of new cultures for production of industrial product has been the hallmark for fermentation processes. Fermentation economics are driven by the profitability of a marketed product. A key component of this value is based on manufacturing cost per unit of product (Parekh et al., 2000). Natural isolates usually produce commercially important products in very low concentrations and, therefore, every attempt is made to increase the productivity of the chosen organism. Increased yields may be achieved by optimizing the culture medium and growth conditions, but this approach will be limited by maximum ability of the organisms to synthesize the product (Yuzura and Omura, 1981).

The product yield with culture depends upon providing the optimum growth conditions. Therefore, it is necessary to avail such nutrient medium to microbes, which helps them in increase the productivity of requisite metabolite. The nutritional requirements also get changed slightly as per the requirement of metabolite needs to be synthesized. Hence, they are modified according to the new requirements to ensure maximum product yield.

Thus, the further assessment of Rhizobial genetic diversity is contributing both to the worldwide knowledge of biodiversity of soil microbes and the usefulness of Rhizobial cultures in the industrial field for the metabolites production like Vitamin B12.

MATERIALS AND METHODS

1. Procurement of culture-

Recommended control culture of Pseudomonas denitrificans ATCC 19244 Were procured from ATCC U.S.A.

2. Sample collection

Sesbania sesban plants were collected from different sites of Mumbai and its suburban areas in the spring season in year 2010. (Table no. 1).

Table 1. Different sites of sample collection -*Sesbania sesban* plant

Culture ID	Locations	Collection sites
CHA	Charkop goun Kandivali	Near charkop goun
VIR	Virar	Agashi
PAL	Palghar	Agricultural dept. Palghar
GHO	N. B. Mehta College, Nursery	Near Railway station
BEL	Belapur	Near River
TUR	Turbhe	Near Railway station

3. Isolation of *Rhizobium* species from Root nodules of *Sesbania sesban*

To isolate Rhizobia from the collected root nodules sample, pink and healthy root nodules of *Sesbania sesban* plant were selected.Isolation of *Rhizobium* sp. was done by the method described by (P.Somasegaran et al.,1994). Nodules were

Volume : 3 | Issue : 7 | July 2013 | ISSN - 2249-555X

surface sterilized by immersing in Collected samples were washed several times with sterile distilled water. After washing 70% ethanol followed by 3% sodium hypochlorite solution for 3-4 minutes. Then surface sterilized nodules were immediately washed with 5-6 times with sterile distilled water to remove the traces of Sodium hypochlorite solution.

Surface sterilized nodules were crushed with a sterile glass rod in a sterile test tube containing 100µl. Loopful of nodule containing culture suspension was streaked on Yeast Extract Mannitol Agar(YEMA) plates containing 0.0025% congo red.(Vincent ,1970)and plates were incubated at 28 ± 1 °c temperature in dark condition .Single isolated colonies were selected and streaked on CREYMA medium for purity. (JORDAN 1984).Isolated pure Rhizobial cultures were preserved in -80°C with 15-20% glycerol until further use. (Elbanna et al.2009;EI-Akhal et al.,2009).

4) Phenotypic Characterization of *Rhizobium* isolates
a) Morphological characteristics
The colony morphology of the isolates was examined on Yeast extract Mannitol agar plate and CREYMA. After incubation of 48-72 hrs at 28°C isolated colonies were characterized on the basis of colony morphology. *Rhizobium* colonies appeared white glistening, gummy, mucous producing, small circular, elevated and transparent. (Aneja.,2003).

Gram stain reaction was carried out to confirm that culture is gram negative and does not contain any gram positive bacteria. Gram's procedure was done as per the method described by Somasegaran and Hoben 1994.

b) Biochemical Characterization
Glucose peptone agar assay (GPA)
GPA assay was performed to determine the capability of micro-organisms to utilize glucose as a sole carbon source for its growth. The Glucose peptone agar (40g/L glucose, 5g/L peptone, 15g/L agar, pH 7.0) medium was inoculated with *Rhizobium* isolates and growth was observed (Singh B. et al, 2008).

Bromothymol Blue Test (BTB)
To differentiate between fast and slow growers of *Rhizobium* species this assay was performed. YMA plates containing

Bromothymol blue agar (10g/lMannitol, 0.5gm/l, Potassium thiophosphate, 0.2gm/l Magnesium sulphate, 0.1gm/l Sodium chloride, 0.5gm/l yeast extract, 3gm/l

Calcium carbonate, 20gm/l agar, 5 % bromothymol blue, and pH 6.8) Initially media was green in color. reaction in the medium, while fast growing bacteria are acidic in nature which changes colour from green to yellow. (Vincent, 1970).

Lactose assay (LA assay)
Lactose assay was performed to determine the capability of the micro-organisms to utilize lactose present in medium ((10gms/l lactose, 5gms/l peptone, 3gms/l beef extract, 20gms/l agar) as the sole carbon source for its growth.

Fluorescence assay
Fluorescence assay was performed (King et al, 1954) to determine the ability of isolates to fluoresce. King's medium (2g/l Peptone, 1.5g/l $MgSO_4$,1.5g/l K_2HPO_4 1.5G/l Glycerol, 15g/L agar, pH7) was prepared and inoculated aseptically with *Rhizobium* cultures. It was incubated at 28°c for 48hrs and observed under U.V. Light source. This test is a confirmatory test for the presence of Pseudomonas bacteria (Singh B.et al, 2008).

Methylene blue and Gentian violet Assay
Methylene blue and Gentian violet assay was performed to check the growth of the isolates .(SinghB. et al.,2008).Dye methylene blue (0.1%) was added in the medium and inoculated with isolated culture. Incubation was given at 28°c for 72hrs and observed the results. Similarly, Gentian violet treatment was done with the concentration of 0.1% (Gao et al, 1994).

Detection of Keto-lactose Production
Keto-lactose Production assay was specifically done for detection of *Agrobacterium* species. Test is based on the ability of *Agrobacterium*, common *Rhizobium* contaminant to produce Ketolactose enzyme which converts Lactose to 3-ketolactose .It is detected by the Benedicts reagent. *Rhizobium* culture were streaked on Lactose agar plates (10gms/l lactose, 5gms/l peptone, 3gms/l beef extract, 20gms/l agar) after incubation of 3-4 days at 28°C, plates were flooded with Benedict's reagent and kept at ambient temperature for 1-2 hrs.

If *Agrobacterium* species were present, then *Agrobacterium* growth was surrounded with yellowish zone of Copper di oxide. Where as yellow zone was absent on *rhizobium* growth. (Stowers and Elkans, 1980).

Characterization of culture on the basis of nutrient utilization
The screened isolates were inoculated into biochemical media of the BIOLOG MicroPlate™ to test the ability of these *Rhizobium* strains to utilize different 92carbon, nitrogen, phosphorus, sulfur, nutrient supplements, peptide, nitrogen sources, osmolytes, pH and chemical sensitivity.

Biolog microplates were used for the characterization of microbial cells. A microbial cell can be characterized for nearly 1000 cellular phenotypes which are Preconfigured into 10 micro plates with 96 well formats. Each well represents an independent test centre. Cells were cultured and added to the wells (independent test centre) in different plates. Cells metabolize a substrate present in a well, respire, and release energy, which reduces colorless tetrazolium redox dye. The reduced dye which is purple in color can be detected visually.

Molecular Characterization
DNA Isolation
Isolation and purification of DNA was carried out according to the method described in (Sambrook and Rusell, 2001). The total genomic DNA was extracted from *Rhizobium* species and purified with the help of Agarose Gel Electrophoresis.

Identification of the isolated bacteria by 16s r-DNA gene sequencing
The most powerful tool to identify the unknown bacteria is to sequence the gene (DNA) coding for 16S-rdna, which is present in the chromosome of the bacteria. The prokaryotic specific primers used for 16S r-DNA gene amplification were as follows- FD1 (5' AGA GTT TGA TCC TGG CTC AG 3') and RP2 (5' ACG GCT ACC TTG TTA CGA CTT 3').These forward and reverse primers were used to amplify 16s r-DNA genes from the bacterial colony under standard PCR conditions

Bacterial Identification by nif H gene Amplification
Nirogenase enzyme plays an essential role in the reduction of N_2 to NH_3. It is composed of Fe (dinitrogenase) and Mo-Fe protein (dinitrogenase reductase) which is encoded by Nif gene present in *Rhizobium* species (Raymond et al., 2004).

For the gene amplification, nifHF (5'TAC GGN AAR GGS GGN ATC GGC AA 3') and nifHR (5' AGC ATG TCY TCS AGY TCN TEC CA 3') were used to amplify nif genes from the bacterial colony under standard PCR conditions such as and initial denaturation 95°C for 3 min., followed 30 cycles of denaturation at 94°C at 45 sec, annealing 62°Cat 45min., elongation at 72°C for 1 min with final extension at 72°Cfor 7 min (Sara Nour et al,2001).which amplifies 601- bp fragement (Mullis.1990).

The amplified product has been subjected to sequencing with closely related sequences retrieved from EMBL by using

Volume : 3 | Issue : 7 | July 2013 | ISSN - 2249-555X

CLUSTALW (Felsenstein,1993). Phylogenetic tree was constructed by using Neighbor joining method.

Molecular Characterisation
DNA isolation
DNA isolation was carried out on 0.8% Agarose gel. The bands were obtained visualized under UV-transilluminator .In this case the isolated DNA was compared with the bacterial DNA. All the 8 samples showed the presence of DNA.

The almost complete 16S r-DNA gene sequences (1500bp) were aligned. Analysis of the 16s rDNA on the 8 isolates was performed using NCBI BLAST (National Centre for Biotechnology Information) with

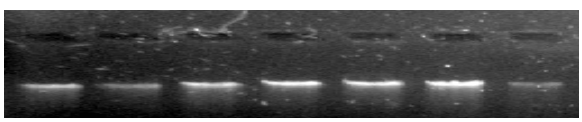


Figure 4. DNA isolation by AGE

Following primers -
(1 6 S - F , 5 ' A G A G T T T G A T C C T G G C T C A G 3'16SR,5'ACGGCTACCTTGTTACGACTT 3'). The complete sequences were aligned to the homologous sequences available for *Rhizobium* strains.The BLAST (NCBI) search using the sequences showed 99% homology of isolates to other GenBank as Rhizobia with 16s r-DNA gene sequences.

The 16s r-DNA genes are directly linked to the phylogenic of micro-organism (Schwieger and Tebbe, 1998).

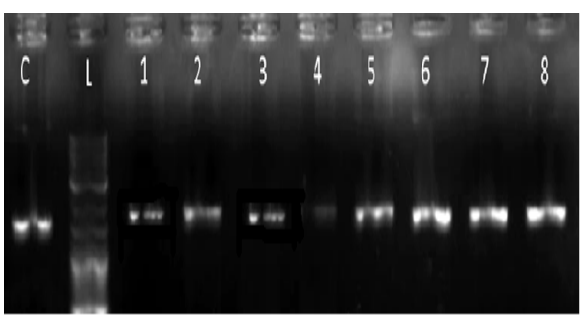


Figure 5. Nif gene amplification

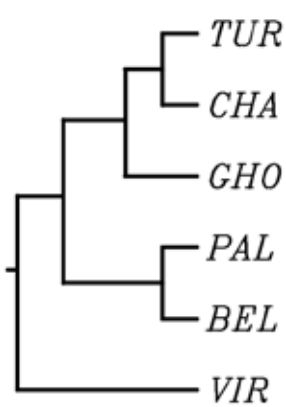


Figure 5 Phylogenetic Tree view of *Rhizobium* isolates by neighbor joining method

Inoculum Preparation
The culture suspension was prepared in normal saline for each culture by using 48 hrs old slant culture. The cells were grown in 250 ml flask containing 30 ml of medium and incubated in orbital shaking incubator at 200 rpm and 30°C for 24±4 h. For inoculum preparation, the culture was grown in NB medium (Ammonium sulphate 0.2 Di Ammonium hydrogen Phosphate 2.35, Magnous sulphate 0.2, Beet Molasses 120, Magnesium sulphate 2.5, Sucrose 40, Zinc sulphate 0.2,g/l) at 30°C and 220 rpm for 24 h. 1 % of lab inoculum at the age of 32 ± 4h was transferred into the different seed media .

S1 (Ammonium Sulphate 0.5g/l, Diammonium Hydrogen Phosphate 1.5, Magnesium Sulphate 0.5, Zinc Sulphate 0.1). pH adjust 7.2 to 7.4 with 5N NaoH. Seperately autoclaved glucose as the carbon source was added into the medium as the final concentration of 18.0g/l just before inoculation.

S2 (Beet Molasses20, Magnous Sulphate 0.005, Magnesium Sulphate 1.1, Zinc Sulphate 0.02)

S3 (Ammonium Sulphate 0.25, Corn Steep liquor 7.5, Ferrous Sulphate 0.02,Glucose 5.0, Magnesium Sulphate 0.45, Zinc Sulphate 0.02)

S4 (Magnous sulphate 0.15, Magnesium sulphate 3.0, Sodium molybdate 0.005, Sucrose 25, Zinc sulphate 0.05)

S5 (Ammonium sulphate 0.65, Beet Molasses 45,Cobalt Chloride 0.01, Di-Methyl Benzemedazole (5,6DMB) 0.002,Magnous sulphate 0.1,Magnesium sulphate 1.3, Sucrose 50,Zinc sulphate 0.1).

S6 (Beet Molasses 50, Magnous sulphat 0.25, Zinc sulphate 0.01, Sodium molybdate 0.02, Magnesium sulphate 0.05 ,Glycerol 3.5 Nutrient Broth 4.5 ,ph 7.4) and flasks were incubated at 30oC at 180 rpm up to 24 ± 4 h or till growth appears.

The production medium:
PM1 (Glucose 40.0,Betaine 2.5, Potassium chloride 0.8,sodium diHydrogen Phosphate1.5, Magnesium Sulphate 1.4, Diammonium Hydrogen Phosphate 10.0, Ammonium Sulphate 9.0,TES 80.0 ml

PM 2 (Glucose 40.0,Betaine 2.5, Potassium chloride 0.8,sodium diHydrogen Phosphate1.5, Magnesium Sulphate 0.8, Diammonium Hydrogen Phosphate 6.0, Ammonium Sulphate 5.0,TES 80.0 ml .g/l)

PM3 (Glucose 70.0,Betaine 2.5, Potassium chloride 0.8,sodium diHydrogen Phosphate2.0, Magnesium Sulphate 1.4, Diammonium Hydrogen Phosphate 10.0, Ammonium Sulphate 9.0,TES 120.0 ml .g/l)

PM4 (Glucose 40.0,Betaine 5.5, Potassium chloride 1.0,Sodium diHydrogen Phosphate2.0, Magnesium Sulphate 1.4, Diammonium Hydrogen Phosphate 10.0, Ammonium Sulphate 9.0,TES 80.0 ml .g/l)

PM 5 (Glucose 40.0,Betaine 5.5, Potassium chloride 1.0,Sodium diHydrogen Phosphate1.5, Magnesium Sulphate 1.4, Diammonium Hydrogen Phosphate 6.0, Ammonium Sulphate 9.0,TES 120.0 ml .g/l)

PM6 (Glucose 40.0,Betaine 5.5, Potassium chloride 0.2,Sodium diHydrogen Phosphate2.0, Magnesium Sulphate 0.8, Diammonium Hydrogen Phosphate 10.0, Ammonium Sulphate5.0.0,TES 120.0 ml .g/l)

PM 7 (Glucose 40.0,Betaine 5.5, Potassium chloride 0.2,Sodium di Hydrogen Phosphate1.5, Magnesium Sulphate 0.8, Diammonium Hydrogen Phosphate 6.0, Ammonium Sulphate 9.0.0,TES 120.0 ml .g/l)

PM 8 (Glucose 70.0,Betaine 2.5, Potassium chloride 1.0,Sodium di Hydrogen Phosphate1.5, Magnesium Sulphate 0.8, Diammonium Hydrogen Phosphate 10.0, Ammonium Sulphate 5.0.0,TES 120.0 ml .g/l)

Volume : 3 | Issue : 7 | July 2013 | ISSN - 2249-555X

PM 9 (Glucose 70.0,Betaine 2.5, Potassium chloride 1.0,Sodium di Hydrogen Phosphate2.0, Magnesium Sulphate 0.8, Diammonium Hydrogen Phosphate 6.0, Ammonium Sulphate 9.0.0,TES 80.0 ml .g/l)

PM 10 (Glucose 70.0,Betaine 5.5, Potassium chloride 1.0,Sodium di Hydrogen Phosphate 1.5, Magnesium Sulphate 1.4, Diammonium Hydrogen Phosphate 10.0, Ammonium Sulphate 5.0.0,TES 80.0 ml .g/l)

PM 11 (Beet Molasses 20, Sucrose 8.0, Choline chloride 2.5, Magnesium sulphate 1.5 Di Ammonium hydrogen Phosphate 2.0, Ammonium sulphate 1.8, Betain monohydrate 1.0 ,Cobalt nitrate 0.1,Ferrous sulphate 0.02, Magnous sulphate 0.02, Monosodium glutamate 3.0, Zinc sulphate 0.02 Di-Methyl Benzemedazole (5,6DMB) 0.005, Calcium Carbonate 2.0, Potassium Dihydrogen Phosphate 0.25,Glycerol 1.5 g/l)

Sucrose 50 % (ph as such) 10% (v/v) feeding was done in production flask from log 48 to 120 h at 24 h interval to maintain the carbon source.Samples were analyzed by HPLC at different hours 144, 168,192, 216 and 240 h respectively.pH, PMV and Microscopy needs to be checked at an alternate of 24 hours to understand the morphological features of isolated culture.

Table 3: In process parameter of Lab inoculum (NB-Merck dehydrated media)

Lab Inoculum	Age (log h)	pH	PMV (%)
NB	20	7.79	01
	24	7.85	02
	28	7.93	02

Table 4: Analysis of process parameter in Seed medium

Seed Inoculum	Age (log hrs)	pH	PMV (%)
S1	20	7.23	-
	24	7.81	01
	28	8.12	01
S2	20	6.95	-
	24	7.29	01
	28	7.65	02
S3	20	6.85	01
	24	6.91	02
	28	7.13	02
S4	20	6.96	01
	24	7.19	01
	28	7.28	02
S5	20	7.12	01
	24	7.51	02
	28	7.65	03
S6	20	7.45	02
	24	7.86	04
	28	8.25	04

Table 5: Maximum Yield of Vitamin B12 of all isolated cultures in different Production media

	Activity (mg/Kg) at 192hrs										
Culture ID	**PM1**	**PM2**	**PM3**	**PM4**	**PM5**	**PM6**	**PM7**	**PM8**	**PM9**	**PM10**	**PM11**
CHA	0.031	0.048	0.059	0.121	0.256	0.231	0.294	0.325	0.456	0.831	1.354
VIR	0.001	0.051	0.098	0.12	0.129	0.158	0.171	0.185	0.223	0.453	0.485
PAL	-	-	-	-	-	-	-	-	-	-	-
GHO	0.01	0.059	0.101	0.151	0.18	0.195	0.201	0.208	0.216	0.225	0.234
TUR	0.015	0.068	0.085	0.127	0.149	0.166	0.175	0.181	0.189	0.192	0.285
BEL	0.041	0.318	0.31	0.325	0.361	0.376	0.385	0.404	0.411	0.435	0.483
(P.denitrificans ATCC 19244)	0.029	0.038	0.05	0.111	0.216	0.401	0.051	0.259	0.416	0.801	1.051

Table 6: Activity of all isolated culture at different hrs in control media (mg/kg)

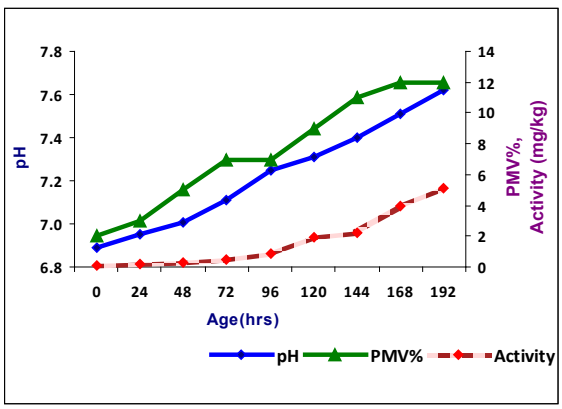
Sample Details	144	168	192	216
CHA	2.258	3.954	5.088	4.151
VIR	0.018	1.429	1.682	0.985
PAL	-	-	-	-
GHO	0.125	0.859	1.296	0.885
TUR	0.095	0.983	1.354	0.961
BEL	0.097	1.212	1.456	0.896
(P.denitrificans ATCC 19244)	1.956	3.125	2.059	3.598

Table7: Analysis of process parameter in Optimized Production medium Containing Beet Molasses 120 gm/ltr

Age (log h)	pH	PMV (%)	Activity (g/l)
0	6.89	02	0.100
24	6.95	03	0.190
48	7.01	05	0.268
72	7.11	07	0.505
96	7.25	07	0.890
120	7.31	09	1.929
144	7.40	11	2.258
168	7.51	12	3.954
192	7.62	12	5.088

Volume : 3 | Issue : 7 | July 2013 | ISSN - 2249-555X

Graph 1: Graphical representation of vitamin B12 yield (pH ,PMV verses Activity/Yield)



Quantification of Vitamin B12 production by HPLC analysis

Quantification of Vitamin B12 production was done by HPLC analysis.After the removal of cell mass methanol extract of culture broth was analyzed by HPLC .Hypersil BDS C18, 250X4.6mm, 5ul column was used, 0.1% Trifluoroacetic acid in Acetonitrile was used as mobile phase at the flow rate of 1.0ml/min.Detection wavelength was 260nm and the total sample run time was 30mins. The column temperature was maintained at 30°C.

Result and Discussion

The key enabling finding of this study was the identification of bacterial culture for the Vitamin B12 production. In this study we also attempted significantly design more efficient fermentation media for the higher yield of Vitamin B12 production. Six cultures of *Rhizobium* were isolated from *Sesbania Sesban* found in Mumbai and its suburban areas. Their morphological, biochemical and molecular characterization was done to confirm them as *Rhizobium* species. We had experiment with many different seed and production media in order to check the production of Vitamin B12.

Among all six isolated cultures,CHA isolate was found to synthesized maximum amount of vitamin B12 i.e. 5.08 mg/kg whereas control (Pseudomonas denitrificans ATCC 19244) strain shows 2.059mg/kg The isolate produced higher yield was further subjected for Vitamin B12 production with the final media containing 10-12 % of Beet Molasses in production media. Concentration of Beet Molasses shows major impact on Vitamin B12 production and hence Vitamin B12 yield can be improved by optimizing its concentration in production media .However outcomes of this study shows that Beet molasses concentration plays a important role in Vitamin B12 production.

The culture was quantitatively tested for Vitamin B12 production on the basis of HPLC assays. The isolated culture was grown in minimal medium and then incubated at 30°C and 220 rpm for specified incubation period. The results of HPLC assays with different isolated cultures shows that the CHA isolates produced 1.35 mg/kg of Vitamin B12 titer on minimal medium produced only 1.05 mg/kg of Vitamin B12. Therefore, this strain was selected for further studies on Vitamin B12 production. Medium optimization is necessary for each fermentation process. It is necessary to optimize each and every component of fermentation media by varying the constituents concentration in the medium, in order to achieve the higher product yield. Purpose of medium optimization is to support the efficient growth of culture as well to maximize the product yield. Medium should comprise of a suitable carbon and nitrogen source. providing carbon- and nitrogen containing compounds to the microorganism in such a way that the microorganism may use/convert these compounds for growth/development/ reproduction and for the production of secondary metabolites, representing compounds of metabolism that are not essential for normal growth, development or reproduction of said microorganism (EP 1 860 194 A1). Previous research has confirmed that cobalt ion and dimethylbenzimidazole (DMBI) are the precursors of vitamin B12 biosynthesis.

Conclusion

In conclusion we have successfully identified new *Rhizobium* isolate capable of producing Vitamin B12. Biostastistical experiment needs to be performed to understand the hidden complexity of ingredients role in production of Vitamin B12.Morework will be carried out to understand the isolates activities with respect to other fields like Bioremediation as culture is capable to perform high oxidation and reduction reaction associated with the enzyme production. Inspite of nitrogen fixation and Vitamin B12 production it additionally brings new opportunities as an industrial product.

REFERENCE

Aneja K. R. (2003). Experiments in Microbiology Plant pathology and Biotechnology. 4th edition, New age International Publishers, New Delhi,India | Aurag, J. and Sasson, A. 1992. Tolerance of Rhizobium leguminosarum by Phaseoli to acidity and drought. World J. Microbiol. Biotechnol., 8: 532-535. | Baoling, H., Q.L. Cheng W, Bo and L. F.Qin, (2007). A Rhizobia strain isolated from root nodules of Gymnosperm Podocarpus macrophyllus. Sci. chin. Ser. C-Life Sci., 50: | De Vries G. E., Kinjne J., Ineke A. M., Schaal V., (1980). Pea lectins and the recognition of Rhizobium leguminosarum, Research group of biological nitrogen fixation, Botanical laboratory, Leiden , the Netherlands, DOI: 10.1016/0304-4211(80)900022-X. | El Sheikh EAE and Wood M., (1989). Response with of Chickpea and specific ion effects of salts. Soil Biol. Biochemistry 21: 889-985. | Elsoni E. and Osman A., (2011). Effects of biofertilization on field, physical characteristics and chemical composition of pigeon pea (cajanus cajan L.). Pakistan journal of nutrition 10(10): pp1-6.978-981. | Elbanna K., Medhat and Eldin H. (2009). Genotypic and phenotypic characterization of Rhizobia that nodulate snap bean (Phaseolus vulgaris L.) in Egyptian soils, Systematic and Applied Microbiology, Vol. 32, issue 7, pp 522-530. | El-akhal R, Ana Rincon, El Mourabit N., Pueyo J. and Barrijal S. (2009). Phenotypic and Genotypic characterizations of Rhizobia isolated from root nodules of peanut (Arachis hypogaea L.) grown in Moroccan soils; Journal of Basic Microbiology, vol. 49; issue5; pp:415-425. | Gao et al, 1994, Characterization of Rhizobium strain isolated from the roots of Trigonella foenumgraecum, African Journal of Biotechnology Vol.7(20) pp.3671-3676, 20 October 2008. | King et al, 1954, Isolation, characterization and effect of fluorescent Pseudomonads on micropropagated sugarcane, Can.J.Microbial. 55: 1007-1011 (2009), NRC Research Press. | P.Somasegaran et al.,1994, Methods in legumes Rhizobium technology,University of Hawaii NifTAL' Project and MRCEN', Department of Agronomy and Soil Science. | Raymond et al., 2004, Molecular evolution of the nif gene cluster carrying nifl1 and nifl2 genes in the Gram-positive phototrophic bacterium Heliobacterium chlorum, The International Center for Biotechnology, Osaka University, 2-1 Yamada-oka, Suita-shi, Osaka 565-0871, Japan | Sambrook and Rusell, 2001, Phenolic DNA Purification --- | Background and Protocol, acc. to Sambrook and Russell, Molecular Cloning, Third Edition, Cold Spring Harbour Laboratory Press, Cold | Spring Harbour, New York, 2001 | Sara Nour et al,2001, Epidemiology, variable genetic organization and regulation of the EDIN-B toxin in Staphylococcus aureus from bacteraemic patients, Microbiology (2010), 156, 860–872, DOI 10.1099/mic.0.030304-0 | Schwieger and Tebbe, 1998, A survey of the methods for the characterization of microbial consortia and communities, Can. J. Microbiol. Vol. 51, 2005, doi: 10.1139/W05-003 | Stowers and Elkans, 1980, A systems level analysis of the effects of light quality on the metabolism of a cyanobacterium, Plant Physiology Preview. Published on September 16, 2009, as DOI:10.1104/pp.109.144824