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Synthesis of vitamin B_{12} by actinomycetes isolated from forest soils and its occurrence in wild animals feces

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ABSTRACT

A total of 16 actinomycetes were isolated from vegetative soil samples of Nallamala forest and screened for their efficiency for the production of vitamin B_{12}. Among the 16 actinomycetes, *Streptomyces* DVB1 (1.328µg/ml) and *Streptomyces* DVB11 (1.217µg/ml) yielded higher titers of vitamin B_{12}. In addition to this, various animals' fecal samples were screened and found highest content of vitamin B_{12} in tiger feces with 1.057µg/mg of fecal matter.

KEYWORDS

Actinomycetes;
Vitamin B_{12};
Wild animals;
Nallamala forest;
Fermentation.

INTRODUCTION

Actinomycetes form a large and important segment of the microflora of most natural environments. Soils, freshwater, lake and river bottoms, manures and compost contain an abundance of these organisms. They are of universal occurrence in nature, living and multiplying in both cold and tropical zones, and have been reported to occur even under the most extreme conditions of the desert. The temperate zones are, however, generally most favorable for their development. Actinomycetes have provided important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive substances. These searches have been remarkably successful and approximately two-thirds of naturally occurring antibiotics, including many of medical importance, have been isolated from actinomycetes[2,8]. Cyanocobalamin is a vitamin commonly known as vitamin B_{12}. In a broad sense it refers to a group of cobalt-containing compounds known as cobalamins-cyanocobalamin. Cyanocobalamin, which is the principal B_{12} form used for foods and in nutritional supplements[12] Vitamin B_{12} is a red crystalline cobalt complex synthesized by microorganisms[5]. Vitamin B_{12} is present in small amounts in almost every animal tissues, it originates from microorganisms. Depending on the nature of their nutritional habits and digestive physiology, animals obtain the vitamin from their own intestinal flora or from other animals through their meat diet[11].

In this present study we collected various soil samples and wild animals fecal samples from Nallamala forest area. Soil samples were collected for the isolation of actinomycetes and producing vitamin B_{12} from them. Wild animals fecal samples were collected for the determination of vitamin B_{12}.

MATERIALS AND METHODS

Soil samples

Soil samples were collected by sterile method from various locations visited throughout this scientific expe-

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TABLE 1 : Total number of soils collected and their physical and chemical parameters

S. No	Soil particulars	Depth (cm)	pH	Electron conductivity (dS/m)	Organic carbon (%)	Available nutrients (kg/ha)		
						N	P_2O_5	K_2O
1	Teak plant soil	0-15	7.9	0.009	1.50	125	41.0	352
2	Bombax cieba plant soil	0-15	7.0	0.075	1.14	138	31.0	494
3	Cluster fig plant soil	0-15	8.1	0.247	3.00	125	15.3	2022
4	Marking nut plant soil	0-15	8.1	0.302	2.40	188	107.7	1313
5	Indian mulberry plant soil	0-15	8.3	0.171	3.18	138	71.8	261
6	Laurel plant soil	0-15	8.2	0.163	2.82	138	71.8	529

TABLE 2 : Vitamin B_{12} producing actinomycetes isolated from various soil samples

Sl.No	Source	Collection date	Isolate	Yield (µg/ml)	No. of isolates source wise
1	Marking nut plant soil	09/09/07	DVB1	1.328	
2	Marking nut plant soil	09/09/07	DVB5	0.171	
3	Marking nut plant soil	09/09/07	DVBMB	0.146	5
4	Marking nut plant soil	09/09/07	DVBSR	0.131	
5	Marking nut plant soil	09/09/07	DVB8	0.534	
6	Indian mulberry plant soil	08/09/07	DVBS	0.106	
7	Indian mulberry plant soil	08/09/07	DVBC	0.170	3
8	Indian mulberry plant soil	08/09/07	DVBN	0.423	
9	Teak plant soil	11/09/07	DVB4	0.512	
10	Teak plant soil	11/09/07	DVB10	0120	4
11	Teak plant soil	11/09/07	DVBVR	0.046	
12	Teak plant soil	11/09/07	DVBM	0.192	
13	Cluster fig plant soil	12/09/07	DVB11	1.217	2
14	Cluster fig plant soil	12/09/07	DVB12	0.258	
15	Laurel plant soil	13/09/07	DVB13	0.480	1
16	Bombox ceiba plant soil	09/09/07	DVB14	0.382	1
	Number of isolates				16

dition to Nallamala forest area (Figure 1). Soil samples were air-dried under room temperature for about 5-30 days before isolation.

Wild animal fecal samples

All the wild animal fecal samples were collected in the mid summer of 2008. Fresh fecal samples were collected in a new polythene bags and brought to the laboratory and transferred into Petridishes. Fresh tiger and panther fecal samples were mixed with bones, remaining animals fecal samples were mixed with leaves, seeds and stones. After bringing samples to the laboratory only feces were separated and remaining dust was discarded. The samples were air dried at 30°C for ten days. After drying the samples were immediately assayed for the presence of vitamin B_{12}.

Isolation of actinomycetes

1g of soil samples was suspended in 10ml of sterile distilled water and was 10 fold diluted.0.1ml of diluted was spread on Starch Casein Agar medium with pH.7.5[7]. The plates were incubated at 38ºC for 1week.Actinomycete colonies on the isolation plates were examined and picked based on morphological features and colours of pigmentation including diffusible pigments. Selected colonies were picked using sterile needles, transferred to Bennett's agar plates and incubated at 35 ± 3°C for two weeks to observe further morphological characteristics and purity[10]. All actinomycetes isolates were indexed and pure cultures grown on Bennett's agar were used to prepare spore suspensions. Stock cultures were maintaining in a refrigerator.

Figure 1 : Soil samples and wild animals fecal samples collected area (Nallamala forest)

Vitamin B_{12} fermentation

Fermentations were conducted in 100ml of Bennett's media in 500ml Erlenmeyer flasks kept in a reciprocal shaker equipped with automatic temperature control, and air sparger, traveling speed at 200rpm through a circular path. Fermentation media was adjusted to 7.8 and sterilized at 121°C for 15min.After Inoculation the flasks were incubated at 38°C for 4days. Inocula were prepared from sporulated stock cultures on Bennett's agar. The inoculum medium was the same as the final fermentation medium. A loopful of spores was transferred to 100ml of fermentation broth.

Determination of vitamin B_{12} in fermented media

Quantitative determination of vitamin B_{12} was made by following the procedure of Gardner and Champagne[3], using the assay organism, *Lactobacillus delbrueckii* subsp *lactis* ATCC 7830. 5ml of each fermented sample was taken in a test tube and added to the tubes containing 5ml of vitamin B_{12} assay medium (Hi-media). 0.05% Thiomalic acid and 0.5µg KCN were added to each tube as a reducing agents. The tubes were plugged which were then shaken and autoclaved at 15lbs pressure at 121C for 15min. After cooling the tubes were inoculated with 100µl of the *Lactobacillus* culture using sterile gals syringe, and incubated at 37°C for 24hrs. Growth of the test microorganism was then measured by spectrophotometer at 540 nm. The entire procedure was carried out in dim light to minimize light destruction of vitamin B_{12}.

Determination of vitamin B_{12} in fecal samples

Ten grams of fecal sample pulverized and from that 1g portion was taken for the determination of vitamin B_{12}.1g of sample homogenized with 9.0ml extraction buffer (0.1 M acetate buffer, pH 5.5 containing 0.1%KCN) and autoclaved for 15 min. The samples were cooled and centrifuged at 7,000 rpm for 10 min. 5ml of the sample and 5ml of the Vitamin B_{12} assay media (Himedia) makeup into 10ml and autoclaved at 121°C for 15min[4]. Vitamin B12 in feces was determined by the microbiological assays method with *Lactobacillus delbrueckii* subsp. *lactis* ATCC 7830 as the test organism. Test tubes were then inoculated with 100µl of the *Lactobacillus* culture and incubated at 37°C for 24 h. Growth of the test microorganism was then measured by spectrophotometer at 540nm[3].

RESULTS AND DISCUSSION

All soil samples were slightly alkaline in reaction except *Bombax cieba* which is in neutral in reaction. Organic carbon content is very high and available ni-

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TABLE 3 : Characterization of vitamin B_{12} producing actinomycetes organisms

Isolates	Gelatin hydrolysis	H_2S production	Nitrate reduction	Casein hydrolysis
DVB1	+	-	+	+
DVB5	+	-	-	+
DVBMB	+	-	-	+
DVBSR	+	-	+	+
DVB8	+	+	-	-
DVBS	+	-	+	-
DVBC	+	-	+	-
DVBN	-	-	-	-
DVB4	+	-	-	+
DVB10	-	-	-	-
DVBVR	+	-	-	+
DVBM	+	+	-	-
DVB11	+	+	+	-
DVB12	+	+	-	+
DVB13	-	-	-	-
DVB14	+	-	+	+

trogen is low. Available phosphorus is medium to high and potassium is very high in all the samples except Indian mulberry plant soil (TABLE 1). Sixteen actinomycetes were isolated from six plant soils, and all the isolates were positive for vitamin B_{12}. As per the Saunders[1,9] all actinomycetes organisms can produce vitamin B_{12} Among all the isolates DVB1 and DVB11 isolates are the potential for the production of vitamin B_{12} (TABLE 2). These two organisms are the *Streptomyces* organisms. DVB1 was isolated from marking nut plant soil and DVB11 isolated from Cluster fig plant soil. Marking nut plant soil and cluster fig plant soils were highly litter containing soils. Their organic carbon content and nitrogen, phosphorous and potassium contents were also very high. Five actinomycetes organisms were isolated from marking nut plant soil. Among five organisms DVB1 and DVB5 are the two potential isolates for the production of vitamin B_{12} and from teak plant soil DVB4 was isolated and its production was also high. Four isolates were isolated from teak plant soil. And three actinomycetes from Indian mulberry plant soil, two isolates from cluster fig plant soil. DVB11 which was isolated from cluster fig soil has given more amount of vitamin B_{12} (TABLE 2). All the actinomycetes organisms isolated for the production of vitamin B_{12} were characterized. Among all the sixteen isolates DVB1 and DVB11 are showing more potential than other isolates (TABLE 3).

TABLE 4 : Wild animal dung samples analysis

S.No	Wild animal	Sample collection date	Dung pH	vitamin B_{12} presence (µg/mg)
1	Tiger	24/04/08	5.5	1.057
2	Bear	25/04/08	5	0.038
3	Panther	16/05/08	6	0.705
4	Stag	24/04/08	6.4	0.010
5	Rabbit	25/04/08	5	0.241
6	Porcupine	26/04/08	6.2	-0.086
7	Race dog	29/04/08	6	0.069
8	Fox	03/05/08	5.4	0.435

We have collected all the three types of herbivorous animal, carnivorous animals and omnivorous animals. From all the eight wild animals' fecal samples tiger fecal sample is containing 1.057µg/ml amount of vitamin B_{12} (TABLE 4). This is the highest amount of all the remaining animals. Tiger is an obligate carnivorous animal. The large urinary excretion of vitamin B_{12} by rabbits and herbivorous consuming a diet practically devoid of the vitamin indicates bacterial synthesis in the gut. It has previously been reported by Kulwich et al.[6] that rabbits excrete between 50 and 100 Mg of vitamin B_{12} daily in feces and that the soft or night feces (which are eaten) contain two to three times as much of the vitamin as hard feces. Presumably, vitamin B_{12} is synthesized in the gastrointestinal tract by microorganisms. In the experiments reported here, the animals were permitted to consume the night feces, thus obtaining considerable quantities of vitamin B_{12}. Even during starvation, small amounts of feces, presumably night feces, are produced and eaten immediately and are never observed in the metabolism cages unless the animals are collared. Actually the vitamin B_{12} is bound to enzymes in food and must be released by the action of gastric enzymes and acid prior to being bound by intrinsic factor, a protein synthesized by gastric parietal cells, which is taken up in the distal ileum[1]. PA is due to the autoimmune loss of secretion of intrinsic factor, which causes a severe, previously fatal, vitamin B_{12} loss and excretion.

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