International Journal of ChemTech Research
CODEN(USA): IJCRGG ISSN : 0974-4290
Vol.4, No.3, pp 976-982, July-Sept 2012

Microbial Production of Vitamin B12 and Antimicrobial Activity of Glucose Utilizing Marine Derived Streptomyces Species

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Abstract: One of the most alluring and fascinating molecules in the world of science, food and medicine is vitamin B_{12} (cobalamin), which was originally discovered as the anti pernicious anemia factor. In current study, fifteen marine derived *Streptomyces* species were isolated from the marine sediment and identified by the morphology biochemical analysis. The batch fermentation process was used for the production of vitamin B_{12} at optimal conditions with specific fermentation medium. Among fifteen *Streptomyces* isolates, seven produced vitamin B_{12} and quantified its concentration range from 1.9µg to 45.3µg per ml. *Streptomyces rochei* produced maximum amount of Vitamin B_{12} (45.3 µg per ml). Among the seven vitamin B_{12} produced *Streptomyces* species four used for the antibacterial activity and exhibited broad antagonistic spectrum against all tested selective human pathogenic bacteria. These activity measured by zone of inhibition (mm), by agar well diffusion method. Altogether, the results indicated that the natural marine environment is also good sources for isolation of novel varieties of antagonistic *Streptomyces* species and to produce Vitamin B_{12}.
Key words: *Streptomyces* species, Vitamin B_{12}, Antibacterial activity and Marine sediment.

INTRODUCTION

Marine microbial technology has opened a wide area for finding novel organisms for trapping their potentiality. The microorganisms growing in marine environments are metabolically and physiologically diverse from terrestrial organisms (1). The novel marine actinomycetes are valuable source of new bioactive compounds (2, 3). Vitamins are regarded as organic compounds required in the diet in small amounts to perform specific biological functions for normal maintenance, optimum growth and health of the organisms. Vitamin B_{12} (cobalamin, anti-pernicious anemia factor) is a water soluble vitamin with a key role in the normal functioning of the brain and nervous system and for the formation of blood. It is normally involved in the metabolism of every cell of the human body, especially fatty acid synthesis and energy production. It is the largest and most structurally complicated vitamin and can be produced industrially only through bacterial fermentation-synthesis (4). *Streptomyces* is the largest genus of Actinobacteria , over 500 species of *Streptomyces* bacteria have been described. *Streptomycetes* are characterised by a complex secondary metabolism (5) and they produce over two-thirds of the clinically useful antibiotics of natural origin (e.g., neomycin, chloramphenicol). Streptomycetes are infrequent pathogens, though infections in human such as

mycetoma can be caused by *S. somaliensis* and *S. sudanensis* and in plants can be caused *by S. caviscabies* and *S. scabies* (6). It is a unique vitamin, synthesized by only microorganisms and not by animals and plants. Vitamin B_{12} has been isolated from fermentation broths of numerous microorganisms, including antibiotic-producing actinomycete (7). Industrial production of Vitamin B_{12} is through fermentation of selected microorganisms **(**8). Determination of vitamin B_{12} production was carried out using spora, *Mycobacterium*, *Nocardia*, *Propionibacterium*, *Proteus*, *Pseudomonas*, *Rhizobium*, *Salmonella*, *Serratia*, *Streptomyces*, *Streptococcus* and *Xanthomonas*. Vitamin B_{12} is provided as a supplement in many processed foods and is also available in vitamin pill form, including multi-vitamins. Vitamin B_{12} can be supplemented in healthy subjects also by liquid, transdermal patch, nasal spray, or injection and is available singly or in combination with other supplements. It is a common ingredient in energy drinks and energy shots, usually at several times the minimum recommended daily allowance of B_{12}. *Streptomycetes* are the most well known genus of *Actinomycete* family which always has been notified because of their ability to produce and secrete a large variety of industrial, medical, biotechnological and agricultural secondary metabolites (9). Based on several studies among bacteria, the actinomycetes are noteworthy as antibiotic producers, making three quarters of all known products; the *Streptomyces* are especially prolific (10, 11,12). The marine derived antibiotics are more efficient at fighting microbial infections than the terrestrial bacteria have not developed any resistance against them (13). The present study was aimed to isolate *Streptomyces* species from marine sediment and assess their vitamin B_{12} productivity and antibacterial activity against selected human pathogenic bacteria.

MATERIALS AND METHODS

SAMPLE COLLECTION

The marine sediment sample was collected from muttam sea, Nagercoil, kanyakumari District, Tamil nadu, India at 10 m length and 5 m depth in sterilized glass bottle. The collected marine sediment sample was stored in ice box and then transported to the laboratory within 3 hours.

PREPARATION OF MARINE SEDIMENT SUSPENSION AND SERIAL DILUTIONS

20 grams of marine sediment was transferred into 250 ml conical flask containing 100 ml of sterilized physiological saline and this sample was serially diluted up to 10^{-4} dilution. Each test tube of diluted samples were vortexed vigorously for 15 minutes.

PREPARATION OF BENNETT MEDIA

Bennett Media composition

S.NO	INGREDIENTS	QUANTITY
1	Beef extract	1g
2	Yeast extract	1g
3	Casein digest	2gms
4	Agar	17gms
5	Glucose solution (25% w/v)	20ml
6	Maltose solution (25% w/v)	20ml
7	Nystatin solution (0.25%w/v)	20ml
8	Sea water	470ml
9	Double distilled water	470ml

After adding the ingredients, the pH was adjusted to 7.4 before autoclaved.

ISOLATION AND IDENTIFICATION OF *STREPTOMYCES* SPECIES

The serially diluted samples were spread evenly on the media and kept it for incubation at 37°C for 96 hours**.** After incubation, *Streptomyces* species were identified by morphological and biochemical methods. Morphological methods consist of macroscopic and microscopic methods. The microscopic characterization was done by cover slip culture method (14).The mycelium structure, color and arrangement of conidiospore and arthrospore on the mycelium was observed through the oil immersion (1000X). The observed structure was compared with Bergey's manual of Determinative Bacteriology, Ninth edition (2000) (15) and the organisms were identified. Various biochemical tests performed for the identification of the potent isolates are as follows: Casein hydrolysis, Starch hydrolysis, Tween 20 hydrolysis, Urea hydrolysis, Esculin hydrolysis, Acid production from sugar, NaCl resistance, Temperature tolerance.

INOCULUM PREPARATION

The suspension was prepared for each strain and 24 hours old slant culture was used. The inoculum was prepared by transferring 5ml of suspension into

100ml conical flask containing 45ml of sterile inoculum medium. 50% of sea water and equal volume of double distilled water was used to prepare the inoculum. Then it was kept in electric shaker with 100 rpm for 24 hours**.** The medium described by Tanaka et al., 1974(16) was modified and contained NH_4PO_4, 0.2%; KH2PO4, 0.2%; CaCl2.2H2O, 0.001%; FeSO4.7H2O, 0.003%; MnSO4.n H2O; 0.002g; CoSO4.7H2O, 0.005%; and 0.5 g glucose per 50ml.

FERMENTATION PROCESS

Fermentation studies were carried out in the conical flasks. Fifty milliliters of 24 hours aged inoculum at the ratio of 10% (v/v) was added to 200 ml of production medium in 250 ml of conical flask. The glucose was used as a carbon source. The pH of culture was adjusted to 7.4 and the fermentation was carried out at 30^0C for 5 days. The growth of bacterium and vitamin B_{12} production was measured. The fermentation medium used contained (g/l) NH_4PO_4, 2g; KH_2PO_4, 2g; $CaCl_2.2H_2O$, 0.01g; $FeSO_4.7H_2O$, 0.005g; $MnSO_4.n\ H_2O$, 0.005g; $CoSO_4.7H_2O$, 1.0mg; $CoSO_4.7H_2O$, 1.0mg. The glucose was used as a carbon source.

BIOMASS ESTIMATION

2ml of culture sample was centrifuged at 8500 rpm at 4^0C for 15 minutes then the cells were collected and washed with sterilized distilled water. The pellet was then desiccated in an electric oven at 105^0C until constant weight obtained. The biomass was measured for each strain at 12 hours interval.

EXTRACTION OF VITAMIN B_{12}

The extraction of vitamin B_{12} was done by harvesting the cells from fermentation broth and centrifuged at 10,000 rpm at -4^0C for 10 minutes. The pellets were washed with 0.2M potassium phosphate buffer (pH 5.5) and suspended in the same buffer containing 0.1% of KCN.

The suspension was autoclaved for 15 minutes at 121^0C. The supernatant containing extracted vitamin B_{12} was filtered through a cellular acetate membrane filter with 0.2µm and stored at -4^0C.

ESTIMATION OF VITAMIN B_{12}

The vitamin B_{12} was estimated by UV-VIS Spectrophotometer (Double beam, I-1029) with optimum absorbance of vitamin B_{12} (360nm) with 0.2M Potassium Phosphate Buffer as reference. Production of vitamin B_{12} was estimated for each isolated *Streptomyces* species. The concentration of vitamin B_{12} was quantified with the help of standard graph and the formula.

(Test O.D / Standard O.D) × Concentration of standard solution.

PREPARATION OF CELL FREE MICROBIAL EXTRACT

The culture medium was prepared, sterilized and inoculated with fresh culture of the four selected *Streptomyces* strain. The cultured flasks were incubated at 37 ºC for 24 hours. After incubation time the cultures were centrifuged at 12000 rpm and their supernatant was used for further experiments.

ANTIBACTERIAL ACTIVITY

Muller Hinton Agar was prepared according to the manufacturer's instructions. The medium was sterilized by autoclaving at 121°C for 15 minutes at 15 psi pressure and was used to determine the antibacterial activity of Streptomyces species. Sterile molten cool (45°C) agar was poured aseptically into sterile Petri plates (15 ml each) and the plates were allowed to solidify at room temperature in sterile condition. After solidification and drying, the plates were seeded with appropriate micro organisms by streaking evenly on to the surface of the medium with a sterile cotton swab and wells (8 mm diameter) were cut out from the agar plates using a sterile stainless steel bore and filled with 0.1ml of the each supernatant solution of *Streotomyces* species in respective wells. Tetracycline used as a control. Then the plates were incubated at 37°C for 24 hrs in the next day the zones of inhibition were measured with a measuring scale. This experiment was carried out in triplicate for their confirmation. The results were read by the presence or absence of zone of inhibition.

RESULTS AND DISCUSSION

Glucose utilizing 15 *streptomyces* species were isolated from marine sediment. The isolates were identified as *S.filementosus*(S1), *S.ruber*(S2), *S.niveus*(S3), *S.lusitanus*(S4), *S.aureofaciens*(S5), *S.gougeroti*(S6), *S.albus(S7), S.eurocidicus(S8), S.nitrosporeus(S9), S.erythreus(S10), S.gougeroti (S11), S.rochei*(S12), *S.candidus*(S13), *S.fulvissimus* (S14) and *S.olivaceus*(S15) based on the morphology of growth, colour appearance and biochemical analysis.

GROWTH OF *STREPTOMYCES* SPECIES

The growth and biomass was measured for each strain at 12 hours regular interval, were reported in Table 1. A particular carbon source, should be effectively utilized by the Streptomyces for their

growth (17). In the present study, glucose is utilized as the carbon source. Among the 15 species isolated, five showed maximum growth from 2.19 to 2.24 g dry weight / litre. The growth and biomass of S1, S5, S9, S11 and S15 was high at 96, 84, 96, 108 and 96 hour respectively represented in graph 2.

VITAMIN B_{12} CONCENTRATIONS

The *Streptomyces* is one of the few microorganisms producing significant amount of vitamin B_{12} reported by Hall *et al*, 1950 (18). Seven *Streptomyces* species from the fifteen isolates produced vitamin B_{12} among which S12 produced remarkably high amount (45.3 µg/ml) of vitamin B_{12}. Other *Streptomyces* species produced vitamin B_{12} in the range of 1.9 to 6.4µg/ml. bykhorsky et al., 1998 listed 12 species of microorganisms that are most active producers of vitamin B_{12}. In which *Streptomyces* olivaceus produced 6.0 µg/ml of vitamin B_{12} with glucose as carbon source. In our investigation, *Streptomyces rochei* produced 45.3µg/ml, which is 7.5 times higher than that represented in Table 2.

Table 1: Growth of *Streptomyces* species

Time(hrs)	Growth(g dry weight / litre)														
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	0.10± 0.005	0.10± 0.005	0..06± 0	0.04± 0	0.08± 0.005	0.10±0. 005	0.10± 0.005	0.11± 0.05	0.06± 0.001	0.10±0 .005	0.12±0 .001	0.06±0 .015	0.12±0 .005	0.10±0 .005	0.11± 0.005
24	0.29± 0.005	0.21± 0	0.18± 0.002	0.31± 0.01	0.26± 0.015	0.31±0. 01	0.30± 0.005	0.36± 0.015	0.25± 0.01	0.25±0 .03	0.30±0 .005	0.25±0 .01	0.33±0 .015	0.33±0 .015	0.32± 0.01
36	0.64± 0.01	0.51±0. 015	0.32± 0.005	0.82± 0.02	0.43± 0.005	0.6± 0	0.6± 0	1.05± 0.05	0.40± 0.005	0.51±0 .01	0.52±0 .015	0.52±0 .025	0.65±0 .01	0.61±0 .01	0.43±- .025
48	1.55± 0.05	0.91±0. 01	0.56± 0.01	1.36± 0.01	1.45± 0.03	0.76±0. 01	0.75± 0.03	1.35± 0.05	1.08± 0.035	1.1± 0.1	1.06±0 .04	0.95±0 .03	1.07±0 .03	1.05±0 .03	1.55± 0.04
60	2.2± 0.2	1.22±0. 01	1.05± 0.05	1.43± 0.01	2.05± 0.05	1.32±0. 025	1.31± 0.01	1.35± 0.03	1.7± 0.2	1.45±0 .035	1.42±0 .02	1.43±0 .03	1.45±0 .035	1.43±0 .03	2.24± 0.035
72	2.3± 0.1	1.41±0. 01	1.31± 0.01	1.44± 0.03	2.19± 0.01	1.45±0. 03	1.44± 0.04	1.25± 0.025	1.87± 0.075	1.83±0 .02	1.76±0 .015	1.72±0 .02	1.53±0 .025	1.52±0 .02	2.26± 0.03
84	2.22± 0.01	1.54±0. 04	1.65± 0.03	1.37± 0.02	2.20± 0.015	1.48±0	1.48± 0	1.15± 0.15	2.14± 0.04	1.87±0 .025	1.91±0 .005	1.84±0 .04	1.67±0 .025	1.66±0 .015	2.21± 0.01
96	2.3± 0.1	1.63±0. 02	1.74± 0.04	1.21± 0.01	2.16± 0.015	1.31±0. 01	1.32± 0.025	0.84± 0.02	2.19± 0.07	1.84±0 .0845	1.92±0 .02	1.87±0 .025	1.73±0 .02	1.65±0 .035	2.56± 0.335
108	2.05± 0.05	1.66±0. 01	1.82± 0.02	1.05± 0.05	2.07± 0.02	0.82±0. 025	0.79± 0.005	0.75± 0.03	2.03± 0.025	1.72±0 .03	1.94±0 .02	1.85±0 .03	1.71±0 .01	1.67±0 .01	2.12± 0.025
120	2.05± 0.05	1.63±0. 03	1.83± 0.025	0.08± 0	1.77± 0.02	0.30±0. 005	0.27± 0.025	0.59± 0.005	1.76± 0.04	1.62±0 .025	1.84±0 .035	1.83±0 .025	1.66±0 .02	1.64±0 .025	1.76± 0.04
132	1.77± 0.02	1.65±0	1.73± 0.03	0.67 ±0.01	1.57± 0.02	0.22±0. 02	0.2± 0	0.53± 0.02	1.45± 0.045	1.1± 0.1	1.81±0 .005	1.67±0 .025	1.44±0 .02	1.46±0 .025	1.62± 0.02
144	1.21± 0.015	1.52±0. 02	1.62± 0.02	0.54± 0.02	1.05± 0.05	0.16±0. 01	0.16± 0.02	0.41± 0.01	1.15± 0.03	0.83±0 .015	1.52±0 .025	1.52±0 .025	1.05±0 .05	0.94±0 .0035	1.23± 0.03
156	1.05± 0.05	1.22±0. 02	1.22± 0.02	0.47± 0.02	0.82± 0.025	0.09±0. 005	0.09± 0	0.30± 0.005	0.93± 0.025	0.53±0 .03	1.05±0 .05	1.22±0 .025	0.68±0 .035	0.67±0 .025	0.83± 0.025

Graph 2. Maximum biomass in 15 isolated *Streptomyces* strains

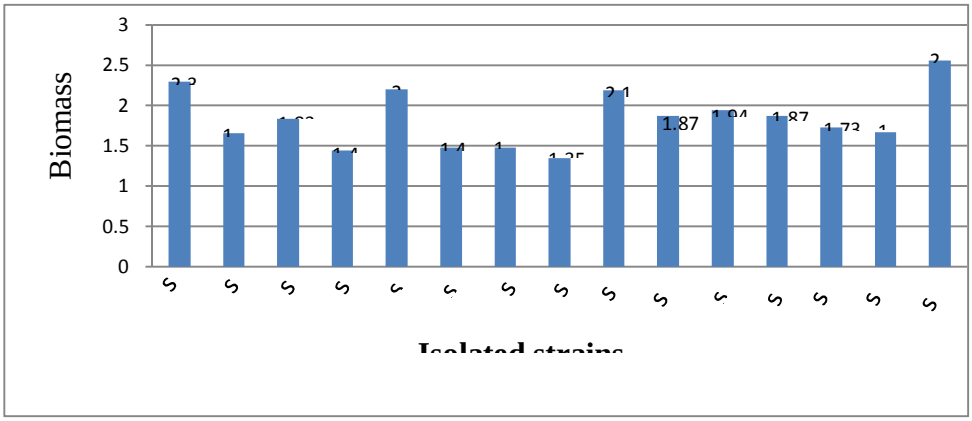
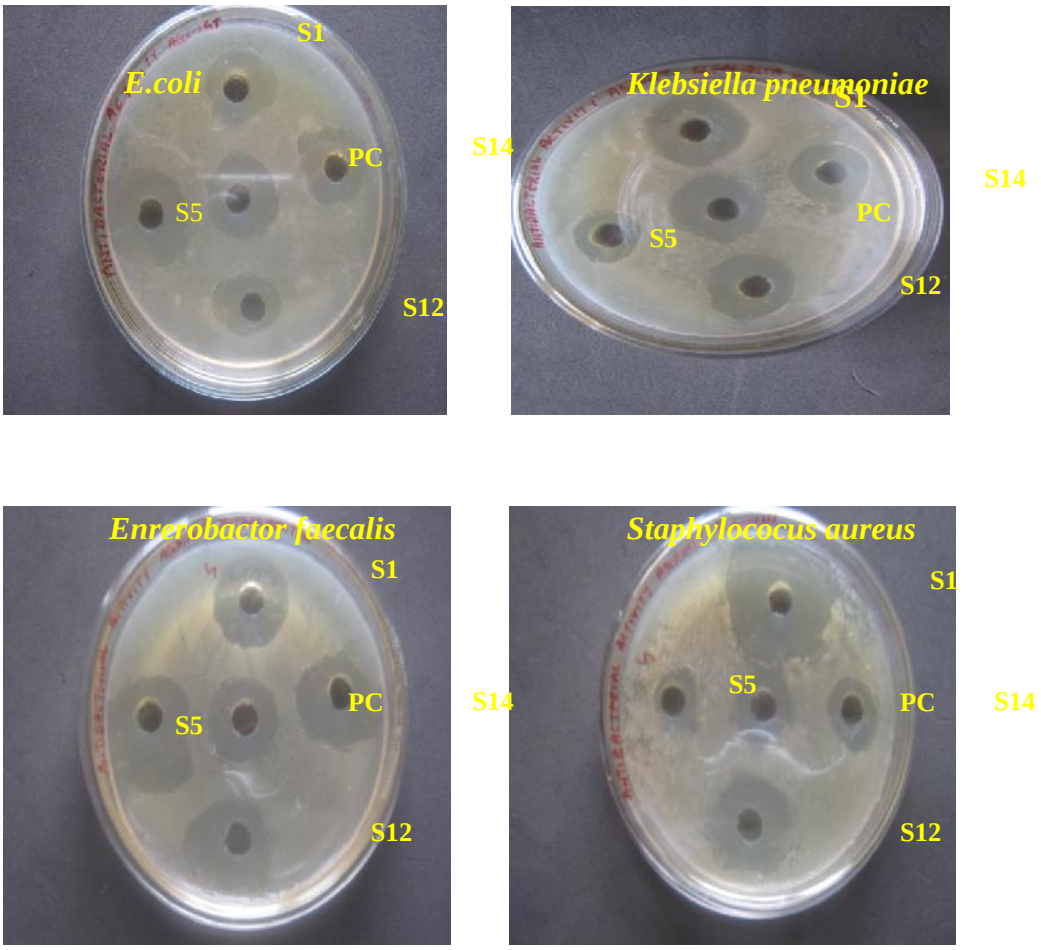


Table. 2. Vitamin B_{12} Concentrations of isolated *Streptomyces* species

Strain name	Absorbance at 360nm	Concentration of Vitamin B_{12} (µg/ml)
S1	0.17	5.8
S5	0.1	3.5
S10	0.06	1.9
S11	0.1	3.3
S12	1.48	45.3
S14	0.18	6.4
S15	0.13	5

ANTIBACTERIAL ACTIVITY OF *STREPTOMYCES* SPECIES AGAINST PATHOGENIC BACTERIA

The antibacterial activity of *S.filementosus*, *S.fulvissimus*, *S.rochei*, *S. aureofaciens*, were investigated against some selected Gram negative *(E.coli ,Klebsiella pneumoniae, Entero bactorfaecalis*) and Gram positive (*Staphylococcus aureus*) human pathogenic bacteria by agar well diffusion method figure1.



S1- *S.filementosus* ; S14- *S.fulvissimus* ; S12- *S.rochei* ; S5- *S.aureofaciens* ; PC-Positive Control.

In the positive control wells zone of inhibition were observed. From the result it is observed that the strain S1 shows highest activity against the entire test organism, the zone of inhibition was measured as (18, 20, 20 and 24 mm) against *E.coli, Klebsiella pneumoniae, Enterobactor faecalis* and *Staphylococcus aureus*. The strain S5 shows maximum activity (23, 22 mm) against *Escherichia coli* and *Enterobactor faecalis.* Strain S12 and S14 showed the moderate activity against the entire test organisms. Oskey, 2009 (19) reported that the certain *Streptomyces* strains showed strong antibacterial activity against gram positive (*S.aureus*) and gram negative bacteria (*E.coli*). Different types of novel *Streptomyces* strains identified by Sunanda *et al.,* (2009) (20), were reported that, has been showed various degrees of antibacterial activity against gram-positive as well as gram negative bacteria.

CONCLUSION

Microorganisms of the genus *Streptomyces* produce a wide spectrum of bioactive compounds with application in pharmaceutical and food industries, in biotechnology and laboratory practice. The present study reveals that the marine derived *Streptomyces* species produced maximum amount of Vitamin B_{12} (1.9-45.3 µg/ml) and shows the highest antibacterial activity (10-24mm in diameter) selected human pathogenic bacteria.

	Zone of inhibition in Diameter (mm)			
Strains	S1	S5	S12	S14
Escherichia coli	18	23	14	10
Klebsiella pneumoniae	20	11	18	14
Enterobactor faecalis	20	22	19	12
Staphylococcus aureus	24	10	20	15

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