PRODUCTION OF VITAMIN B_{12} USING ENRICHED OIL CAKES BY *STREPTOMYCES SPP* ISOLATED FROM SOIL SAMPLES

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ABSTRACT

Vitamin B_{12}, also called Cobalamin, is a water-soluble vitamin involved in metabolism. It is essential in the formation of Red Blood Cells and its deficiency causes Pernicious Anemia. It is an essential vitamin widely used in Medical and Food Industries. Vitamin B_{12} is synthesised by microorganisms. There are four forms of vitamin B_{12}. Of which three are naturally occurring forms which are Methylcobalamin (MeCbl), Adenosylcobalamin (AdCbl), Hydroxycobalamin (OHCbl) and Cyanocobalamin (CNCbl) is a synthetic form. In the recent days, cold-pressed oils have gained prevalence. The waste that is generated by this process is the oil cake which is used as an animal feed. This oil cake is a rich source of protein. In the present study, two Streptomyces spp were isolated from Sewage soil and Sugarcane soil samples and produced methylcobalamin using alternate substrates such as Soybean powder and supplemented Oil cake medium by the fermentation process and the residues at the end of the process can be used as vitamin B_{12} enriched animal feed. The concentration of vitamin B_{12} was found to be 57.5 µg/ml in the filtrates as well as in the residual substrates. RP-HPLC also confirmed the presence of methylcobalamin in the filtrates. The production of certain enzymes along with this process also results in the enrichment of the animal feed. Some industrially important enzymes such as amylase and protease are found to be produced by these isolates. Enzymes such as amylases and proteases in animal feed help in easy starch digestibility and breaking down storage proteins, respectively thereby allowing the animals to extract more energy from the feed which can be efficiently converted into meat and egg production.

KEYWORDS: *Vitamin B_{12}, Cobalamin, Enzymes, Supplemented oil cake medium and Animal feed.*

INTRODUCTION

The synthesis of vitamin B_{12} is restricted to microorganisms exclusively by the fermentation process **(Survase *et al.*, 2006)**. Several bacteria and archaea such as *Pseudomonas denitrificans, Propionibacterium freudenreichii, Propionibacterium shermanii, Bacillus megaterium, Streptomyces olivaceus, Streptomyces griseus* are found to synthesise vitamin B_{12}. However, *Pseudomonas denitrificans* and *Propionibacterium freudenreichii* are involved in the large scale industrial production. There are only few reports of vitamin B_{12} production in plants and fungi **(Martens *et.al.*, 2002)**.

There are two biosynthetic pathways for the production of vitamin B_{12}. They are:

- the aerobic pathway or oxygen-dependent pathway found in *P.denitrificans*
- the anaerobic pathway or oxygen-independent pathway in *P.shermanii*, *Bacillus megaterium*

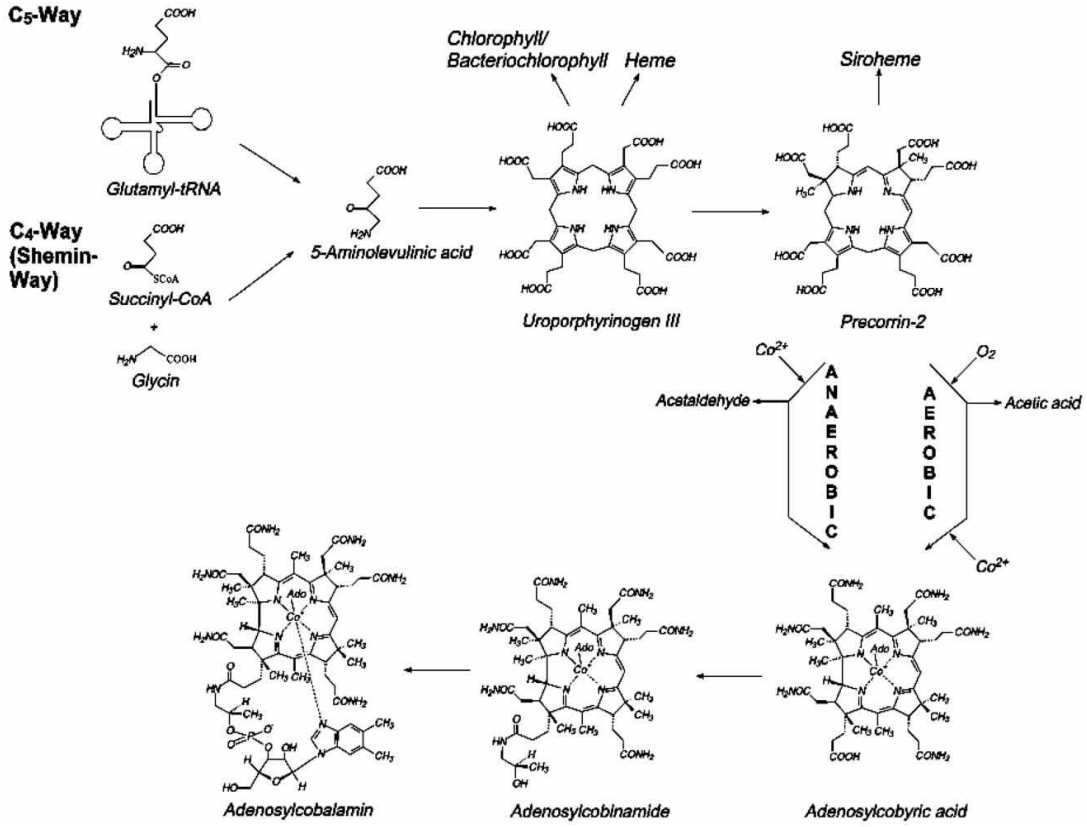


Figure 1: Synthesis of Vitamin B_{12} (Survase *et al.*, 2006)

Streptomyces belonging to the Actinomycetes group of organisms are found to be involved in the production of vitamin B_{12} and are considered to produce a high yield **(A.P. Saunders *et al.*, 1952 & Hall *et al.*, 1953)**. Actinomycetes are widely found in terrestrial and aquatic ecosystems although soil is considered to be the main source. They are filamentous Gram positive aerobic bacteria which have high guanine + cytosine in their DNA. They produce two types of branching mycelium, aerial and substrate mycelium. These closely resemble fungi in their morphology. They produce spores and have a distinct earthy odour which is a result of the production of a volatile metabolite, geosmin **(Mukesh Sharma *et al.*, 2014)**. *Streptomyces* accounts for about 90% of the total Actinomycetes population. They are involved in the production of a wide range of secondary metabolites and almost 70% of the naturally derived antibiotics are derived from soil actinomycetes. *Streptomyces* has the remarkable ability to produce bioactive secondary metabolites such as antibacterials, antifungals, antivirals, antitumoral, anti-hypertensives and mainly antibiotics and immunosuppressives **(Al-Saadi *et al.*, 2013; Islam *et al.*, 2014; Narendra Kumar *et al.*, 2010)**. Some of the antibiotics produced include Streptomycin by *S. griseus*, Neomycin by *S. fradiae*, Kanamycin by *S. kanamyceticus*, Tetracycline by *S. rimosus*, etc. Some of the antifungals include Nystatin produced by *S. noursei*, Amphotericin B produced by *S. Nodosus* and Natamycin by *S. natalensis*. They are also involved in the production of enzymes such as amylase, protease, lipase, cellulase, chitinase, urease, etc **(Mukesh Sharma *et al.*, 2014 & Salma Mukhtar *et al.*, 2017)**. They are also a source of agroactive compounds as they are involved in the production of insecticides and herbicides. Examples are: Kasugamycin produced by *Streptomyces kasugaensis* is used for the control of rice blast and bacterial *Pseudomonas* diseases in several crops. Polyoxin B and D produced by *Streptomyces cacaoivar* is used against fungal pathogens in fruits, vegetables and in the control of rice sheath blight **(Mukesh Sharma *et al.*, 2014)**.

All of the four forms of B_{12} are found to be reduced to the core cobalamin molecule inside the cytosol and is then converted to the two active forms of B_{12}—Methylcobalamin and Adenosylcobalamin—irrespective of the form of B_{12} ingested. Thus, it is considered as a better option to produce any one of the active forms directly. Since, cyanocobalamin – a synthetic form of vitamin B_{12}, although considered to be stable, contains the cyanide group which when consumed in the long run can have toxic effects **(Paul & Brady 2017)**.

Studies have been conducted on the production of vitamin B_{12}. Some of which include: production of vitamin B_{12} by *Klebsiella pneumoniae* and *Citrobacter freundii* using agricultural wastes such as wheat bran, wheat straw, rice bran, etc as substrates and production of vitamin B_{12} by *Streptomyces spp* using different protein sources such as distiller's soluble, soybean meal. It was found that the amount of vitamin B_{12} produced varied with the different protein sources **(El-Sheekh *et al.*, 2013 & Hall *et al.*, 1953, Gandhi *et.al.*,1964, Muniappan *et.al.*, 2012, Selvakumar *et.al.*, 2012, Atta MH 2007, Chandani. D. Patel. 2014, Karima *et.al.*, 2015, Poovarasan *et.al.*, 2015, Rózycki H and Strzelczyk E. 1986, Jeffrey LSH. 2008)**.

Based on this, the present study has attempted to produce vitamin B_{12} from alternate substrates such as Soybean powder and supplemented Oil cake medium using *Streptomyces spp* isolated from soil sources.

MATERIALS AND METHODOLOGY

COLLECTION OF SAMPLE

Sewage soil sample was collected from Anna Nagar, Chennai. Sugarcane soil sample was collected from Uthukottai, Tamil Nadu. The soil samples were collected in a sterile polythene bag and stored in -20°C freezer until the isolation process.

ISOLATION OF ACTINOMYCETES

The sewage soil and sugarcane soil samples were dried on a hot plate at 70ºC for 3 minutes. One gram of the soil samples were sprinkled onto starch casein agar. The plates were kept for incubation at room temperature for 10 days. One gram of the soil samples were suspended in 9 ml of sterile distilled water and then the dilution was carried out up to 10^{-6} dilutions. Aliquots (0.1 ml) of 10^{-4}, 10^{-5}, 10^{-6} were spread on the starch casein agar. The plates were incubated at room temperature for 10 days. After incubation, actinomycetes colonies which were morphologically distinct were picked from the starch casein agar plates and further purified by repeated streak plate method on starch casein agar with rifampicin (30 µg/ml) and amphotericin B (5 mg/ml) to minimise bacterial and fungal growth.

IDENTIFICATION OF ACTINOMYCETES

Morphological identification was carried out by Simple Staining and Lactophenol cotton blue staining technique. Biochemical identification was confirmed by conducting Indole Test, Methyl Red Test, Voges-Proskauer Test, Catalase test, Oxidase test, Nitrate reduction test, Triple sugar iron test and Lactose utilization test. The sewage and sugarcane soil isolates were given for the 16S rDNA sequencing for identification of the organism.

VITAMIN B_{12} PRODUCTION USING ALTERNATE SUBSTRATES

Vitamin B_{12} production was carried out using the basal medium (Glucose, Calcium carbonate, $CoCl_2.6H_2O$) with two alternate substrates – 5% Soybean powder (SB) and 10% Oilcake (OC). This production medium was sterilized and rifampicin (30 µg/ml) and amphotericin B (5 mg/ml) were added to minimise the bacterial and fungal growth. The two isolates were inoculated in the medium separately. The conical flasks were kept for incubation at room temperature for 10 days on shaker. At the end of 6 days incubation, the pH of the medium was checked and cooling centrifugation (4ºC) at 7200 rpm for 25 minutes was carried out for the media with sugarcane soil isolate (SB 6, OC 6). At the end of 10 days incubation, the pH of the medium was checked and cooling centrifugation (4ºC) at 7200 rpm for 25 minutes was carried out for the sewage soil isolate (SB 10, OC 10). The supernatant was separated from the pellet and filtered using Whatman filter paper and was analysed.

DRIED SUBSTRATES

The pellets (spent substrate) were dried in a hot air oven at 60ºC for 2 hours (SB, OC). The dried substrates were soaked overnight in sterile distilled water. The suspension was centrifuged at 6000 rpm for 25 minutes. The supernatant was separated from the pellet and was analysed.

ESTIMATION UV-VISIBLE SPECTROPHOTOMETER

Methycobal 500 mcg/ml (Wockhardt Limited, India) was used as standard. Standard OD values were taken for 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml concentrations of standard methylcobalamin at 353 nm and standard graph was plotted. The OD values of the filtrates (SB 6, OC 6, SB 10, OC 10, SB, OC) were taken at 353nm and the concentrations calculated using the standard graph.

RP-HPLC

The filtrates (OC 6, SB 10, OC 10) were given for RP-HPLC for the confirmation of methylcobalamin. Methycobal (Wockhardt Limited, India) was given as standard.

Antibacterial Activity Assay and Antifungal Activity Assay were conducted. Tests for other products from fermentation were also conducted such as Estimation of Organic Acids - Citric Acid Estimation, Oxalic Acid Estimation. Protein Estimation by Lowry's method and Qualitative estimation of Enzymes were carried out by Protease assay, Amylase assay and Lipase Assay.

RESULTS AND DISCUSSION

COLLECTION OF SAMPLE

The Soil Samples collected comprised of Sewage Soil and Sugarcane Soil **(Figures 2 & 3)**

Figure 2: Sewage soil (SW)

Figure 3: Sugarcane soil (SC)

ISOLATION OF ACTINOMYCETES

SW soil isolate was isolated from the sewage soil sample by sprinkling method. Morphologically distinct actinomycetes colonies were picked and pure culture was obtained by streaking on starch casein agar medium with Rifampicin and Amphotericin B **(Figures 4 & 5).**

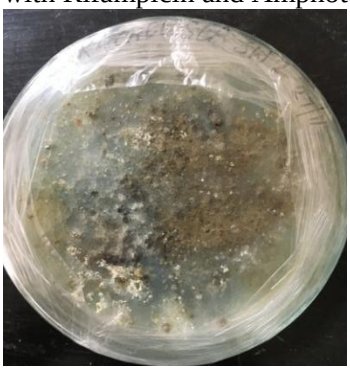


Figure 4: Isolation from Sewage soil (SW)

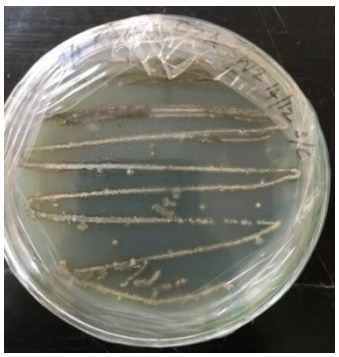


Figure 5: Pure culture of SW soil isolate

SC soil isolate was isolated from the sugarcane soil sample by serial dilution method. Morphologically distinct actinomycetes colonies were picked and pure culture was obtained by streaking on starch casein agar medium with Rifampicin and Amphotericin B **(Figures 6 & 7)**

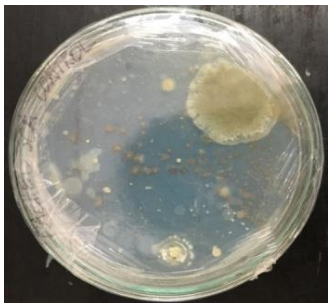


Figure 6: Isolation from Sugarcane soil (SC)

Figure 7: Pure culture of SC soil isolate

IDENTIFICATION OF ACTINOMYCETES

MORPHOLOGICAL IDENTIFICATION

Simple staining

SW soil isolate was stained using crystal violet and actinomycete-like morphology was observed under the microscope **(Figure 8).**

Figure 8: Microscopic image of the SW isolate

Lactophenol cotton blue Staining

SW and SC soil isolates were stained using Lactophenol cotton blue and actinomycete-like morphology was observed under the microscope **(Figures 9 & 10).**

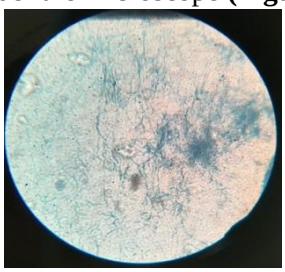


Figure 9: Microscopic image of SW isolate

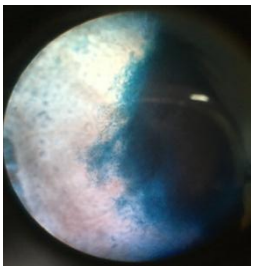


Figure 10: Microscopic image of SC isolate

BIOCHEMICAL IDENTIFICATION

The Biochemical results are tabulated in **Table 1**.

BIOCHEMICAL TEST	SW ISOLATE	SC ISOLATE
INDOLE	NEGATIVE	NEGATIVE
METHYL RED	POSITIVE	POSITIVE
VOGES-PROSKAUER	POSITIVE	POSITIVE
CATALASE	POSITIVE	POSITIVE
OXIDASE	POSITIVE	POSITIVE
NITRATE REDUCTION	POSITIVE	POSITIVE
TSI	POSITIVE	POSITIVE
LACTOSE UTILIZATION	POSITIVE	NEGATIVE

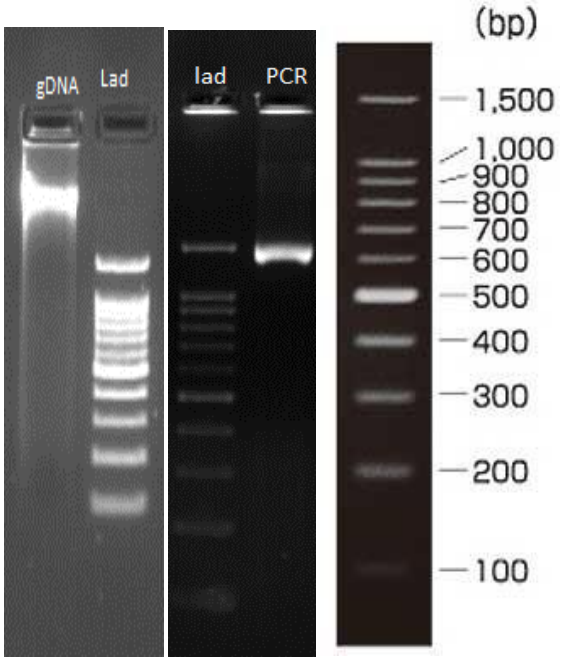
Table 1: Biochemical tests

16S rDNA SEQUENCING

The sewage soil isolate and the sugarcane soil isolate were given for the 16S rDNA sequencing commercially, for taxonomic identification of the species. The following results were obtained **(Figures 11 - 14).**

SW SOIL ISOLATE

gDNA **16S PCR amplicon**



Ladder specification

Figure 11: 16S rDNA sequencing of SW Soil Isolate

Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI genbank database.

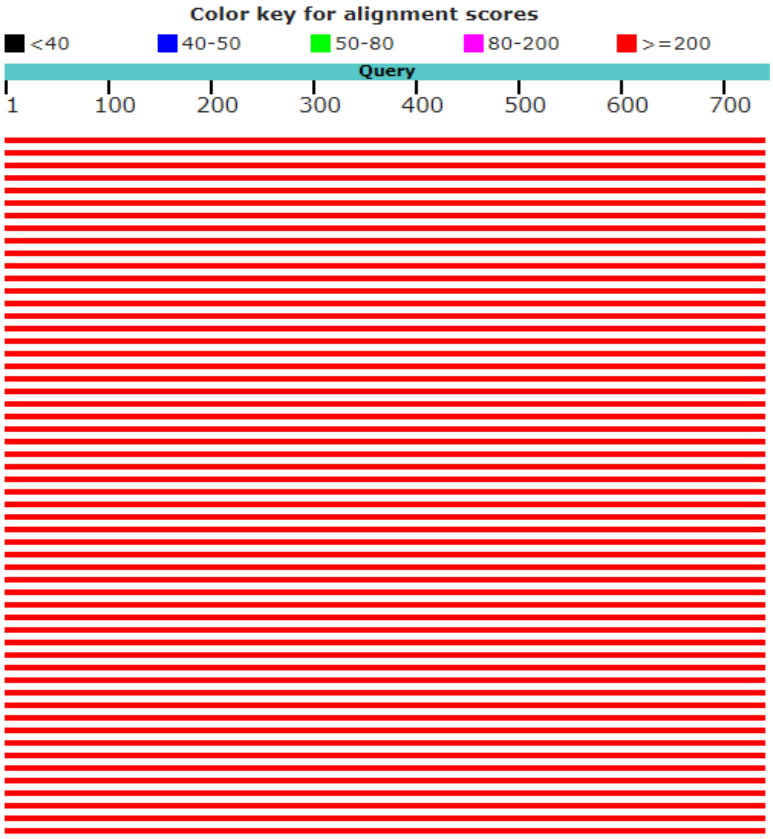


Figure 12: BLAST result of SW Soil isolate

The **SW soil isolate** showed high similarity with ***Streptomyces asenjonii*** based on nucleotide homology and phylogenetic analysis.

SC SOIL ISOLATE

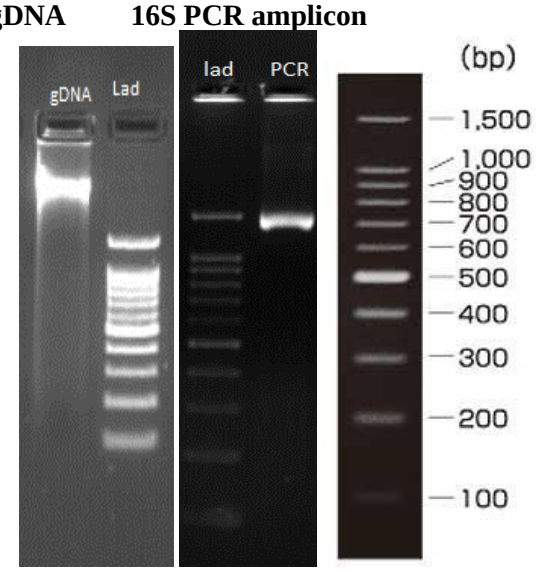


Figure 13: 16S rDNA sequencing of SC Soil Isolate

Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI genbank database.

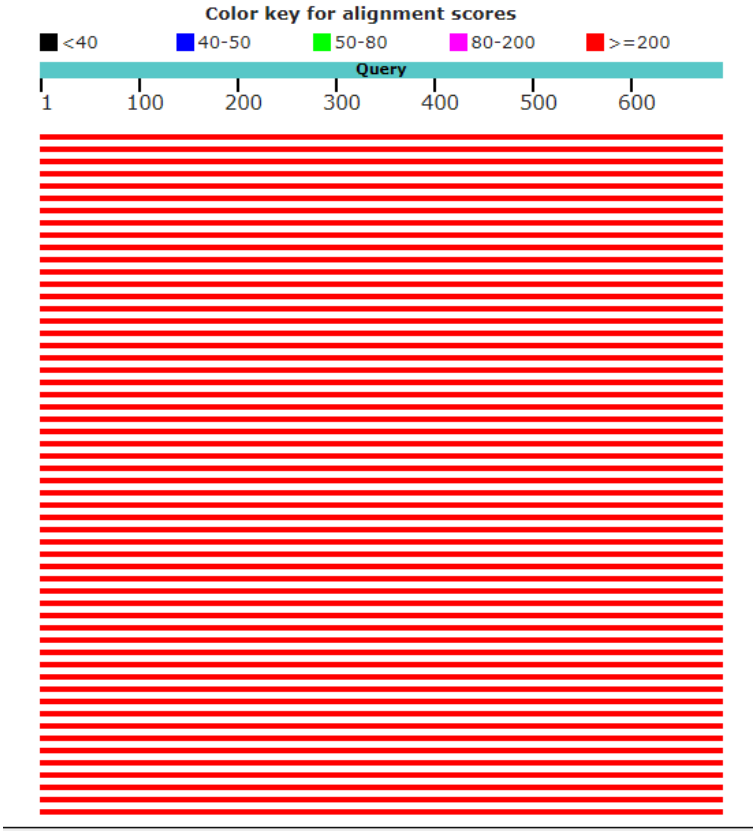


Figure 14: BLAST result of SC Soil isolate

The **SC soil isolate** showed high similarity with ***Streptomyces djakartensis*** based on nucleotide homology and phylogenetic analysis.

VITAMIN B_{12} PRODUCTION USING ALTERNATE SUBSTRATES

SUBSTRATES USED

A study was done by Gandhi & Freitas (1963) in which soybean meal, sesame and coconut oilcakes were used for the production of vitamin B_{12} like substances. Another study was conducted by Hall *et al.*, (1953) in which different proteinaceous substrates were used. In the present study, alternate substrates such as Soybean powder and Oilcake – Groundnut & Sesame were used for the production of vitamin B_{12} keeping soybean powder as the standard **(Figures 15 & 16).**

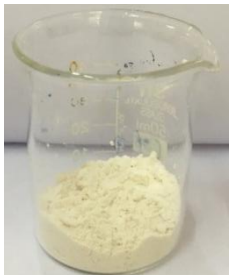


Figure 15: Substrate - Soybean powder

Figure 16: Substrate – Oilcake

FERMENTATION

Fermentation was done using both the SW and SC isolates using both the substrates **(Figures 17 & 18).**

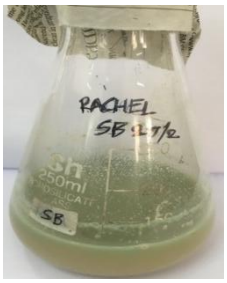


Figure 17: Fermentation medium with Soybean powder (SB)

Figure 18: Fermentation medium with Oilcake (OC)

For fermentation using the SC isolate, at the end of 6 days incubation, the pH of the medium was found to be: SB 6 – pH 8 and OC 6 – pH 5.
For fermentation using the SW isolate, at the end of 10 days incubation, the pH of the medium was found to be: SB 10 – pH 5 and OC 10 – pH 5. A study conducted by Hall *et al.*, (1953) revealed that the production of vitamin B_{12} was obtained throughout the pH range of 5 to 9.
Cooling centrifugation was carried out for the four media to separate the supernatant from the pellet **(Figure 19).**

Figure 19: After centrifugation – SB & OC

The supernatants were filtered using Whatman filter paper, followed by membrane filtration and were further analysed **(Figure 20).**

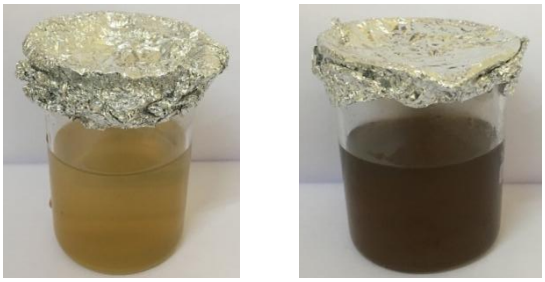


Figure 20: Filtered supernatant – SB; OC

DRIED SUBSTRATES
The pellets (spent substrate) were dried in a hot air oven at 60ºC **(Figure 21).**

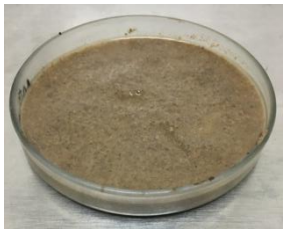


Figure 21: Pellet for drying

The dried substrates were weighed to find out the quantity of the substrate used during fermentation. The dried substrates were further tested for the presence of residual vitamin B_{12} so that it can be used as a vitamin B_{12} enriched animal feed **(Figures 22 & 23, Table 2).**

Figure 22: Dried Substrate – SB

Figure 23: Dried Substrate – OC

SUBSTRATE	GRAMS ADDED FOR FERMENTATION	GRAMS AFTER FERMENTATION
SOYBEAN	5g	1.57g
OILCAKE	10g	7.47g

Table 2: Dry weight of the substrates

The dried substrates were soaked overnight in sterile distilled water and after cooling centrifugation was carried out, the supernatants were filtered using Whatman filter paper, followed by membrane filtration and were further analysed **(Figures 24 & 25).**

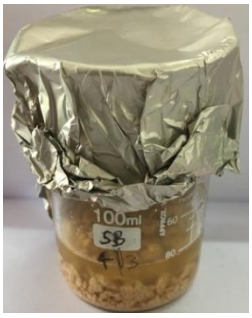


Figure 24: Dried Substrates soaked overnight – SB; OC

Figure 25: After Centrifugation

ESTIMATION OF VITAMIN B_{12}
UV-VISIBLE SPECTROPHOTOMETER

Methycobal 500 mcg/ml (Wockhardt Limited, India) was used as the standard. Standard OD values were taken for 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml and 50 µg/ml concentrations of standard methylcobalamin at 353 nm and standard graph was plotted. In a study conducted by Ganesan *et al.*,(2012) on the estimation of methylcobalamin using spectrophotometer, maximum absorption was recorded at 353 nm and thus this nm was adopted **(Figure 26 and Table 3)**

Figure 26: Different concentrations of the Standard Methylcobalamin

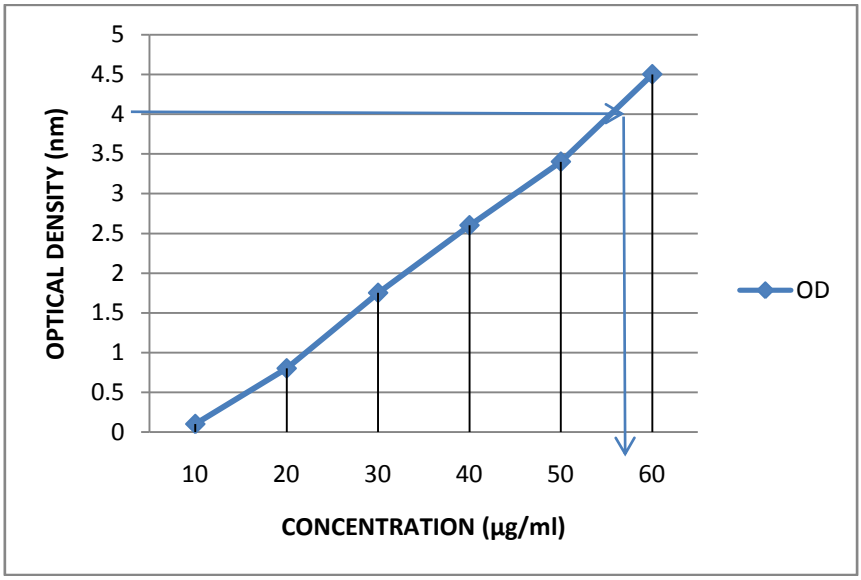
CONCENTRATION (µg/ml)	OD (nm)
10	0.1
20	0.8
30	1.75
40	2.6
50	3.4

Table 3: OD values for the Standard at 353 nm

The OD values of the filtrates (SB 6, OC 6, SB 10, OC 10, SB, OC) were taken at 353 nm and the concentrations calculated using the standard graph **(Table 4 and Graph 1)**

SAMPLE	OD (nm)
SB 6	4.0
OC 6	4.0
SB 10	4.0
OC 10	4.0
SB	4.0
OC	4.0

Table 4: OD values for the samples at 353 nm



Graph 1: Standard graph showing sample concentration

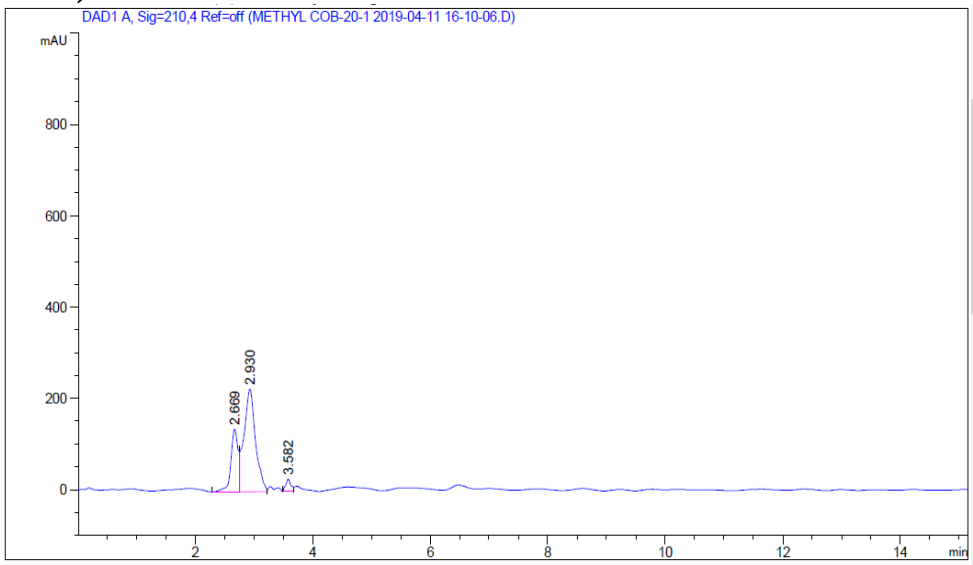
From the standard graph, the concentration of vitamin B_{12} in each of the samples was found to be 57.5 µg/ml. In one study conducted by Hall *et al.,* (1953) on vitamin B_{12} production by *Streptomyces olivaceus* using different proteinaceous substrates, the yield was reported to be 3 µg/ml. Another study on vitamin B_{12} production by actinomycetes in a soybean meal medium fortified with cobalt reported the yield to be 1 µg/ml (A. P. Saunders *et al.,* 1952). A study conducted by Gandhi & Freitas (1963) on the production of vitamin

B_{12}-like substances by marine microorganisms using coconut oilcake reported the yield to be 2 µg/ml. A study on vitamin B_{12} production by marine *Streptomyces spp* reported the yield to be 45.3 µg /ml (Selvakumar *et al.*, 2012). A study conducted by H M Atta 2007 on vitamin B_{12} production by *Streptomyces fulvissimus* using soybean meal medium fortified with cobalt reported the yield to be 64.57 µg/ml.

RP-HPLC

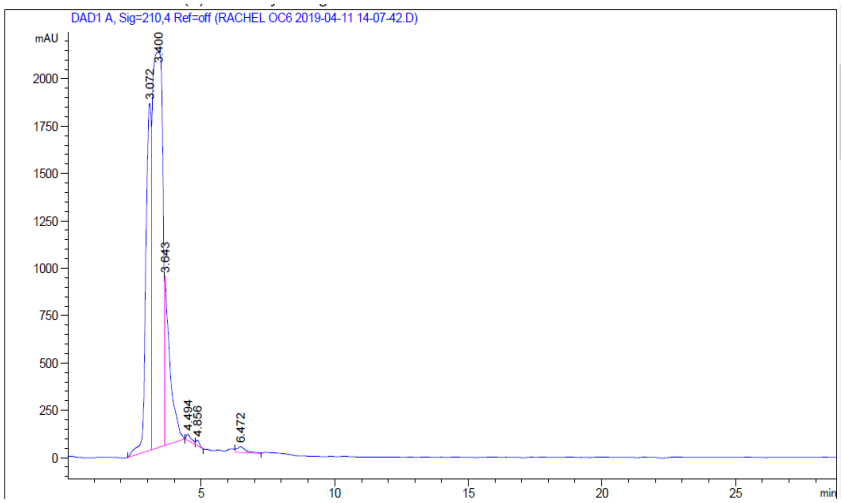
The filtrates (OC 6, SB 10, OC 10) were given for RP-HPLC for the confirmation of methylcobalamin. C18 column (250X4.6mm, 5micron particle size) was used. Methanol : water (80:20) was used as mobile phase with detection at 210 nm (Chandani. D. Patel *et al.*, 2014).

Methycobal (Wockhardt Limited, India) was given as standard. The filtrates OC 6, SB 10, OC 10 were taken as test **(Graph 2 - 5) Standard**



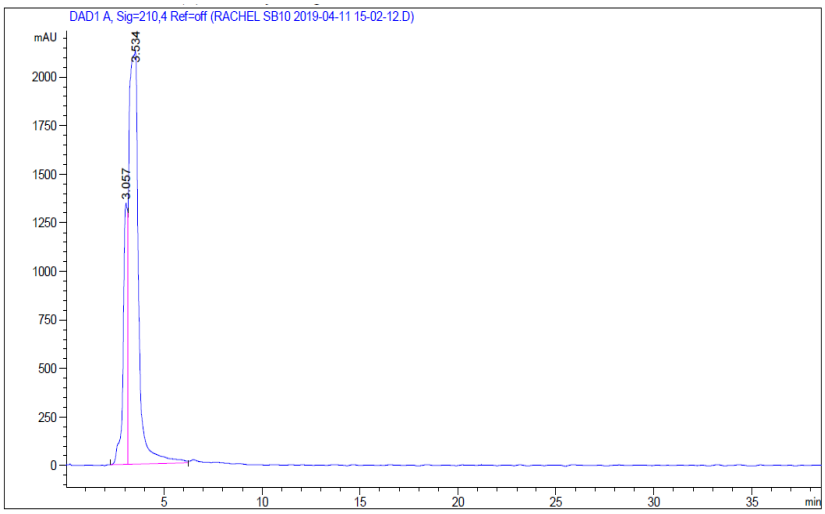
Graph 2: Chromatogram showing peaks for Standard

OC 6



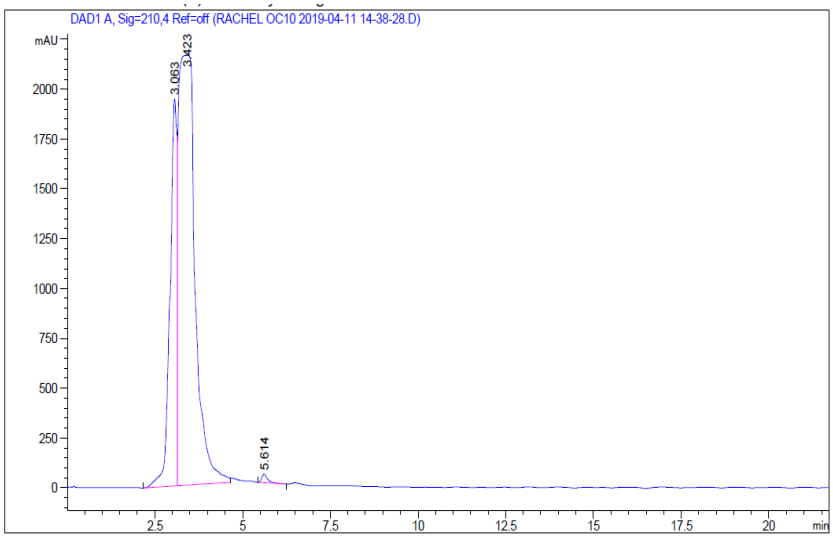
Graph 3: Chromatogram showing peaks for OC 6

SB 10



Graph 4: Chromatogram showing peaks for SB 10

OC 10



Graph 5: Chromatogram showing peaks for OC 10

The retention time (in min) of:
Standard was found to be 2.669, 2.930, 3.582
OC 6 was found to be 3.072, 3.400, 3.643, 4.494, 4.856, 6.472
SB 10 was found to be 3.057, 3.534
OC 10 was found to be 3.063, 3.423, 5.614

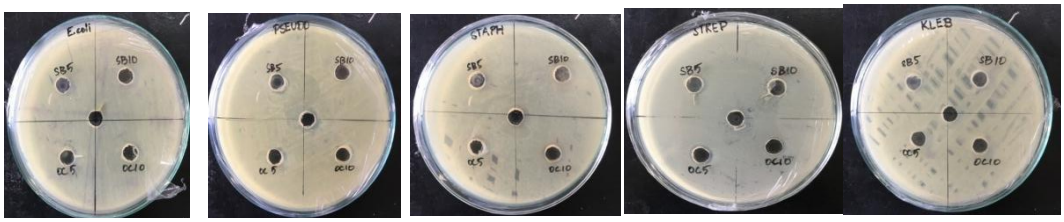
The retention time of the standard and the test filtrates (OC 6, SB 10, OC 10) are found to be in the same range. This confirms that Methylcobalamin is present in the filtrates and that the isolated organisms are capable of producing methylcobalamin.

ANTIBACTERIAL ACTIVITY ASSAY

Antibacterial activity of all the filtrates (SB 6, OC 6, SB 10, OC 10) against *E.coli, Pseudomonas aeruginosa, Staphylococcus spp., Streptococcus spp., Klebsiella spp* was checked. There were no zones of inhibition formed indicating that both the isolates do not have antibacterial activity. A study conducted by Selvakumar *et al.*,(2012) revealed that the marine *Streptomyces spp.* showed antibacterial activity against *E.coli, Klebsiella pneumoniae, Enterobacter faecalis* and *Staphylococcus aureus* **(Figures 27 and 28).**

(a) *E.coli* (b) *Pseudomonas aeruginosa* (c)*Staphylococcus spp* (d) *Streptococcus spp* (e)*Klebsiella spp*

Figure 27 (a) to (e): Antibacterial activity – SB 6, OC 6 – No zones of inhibition

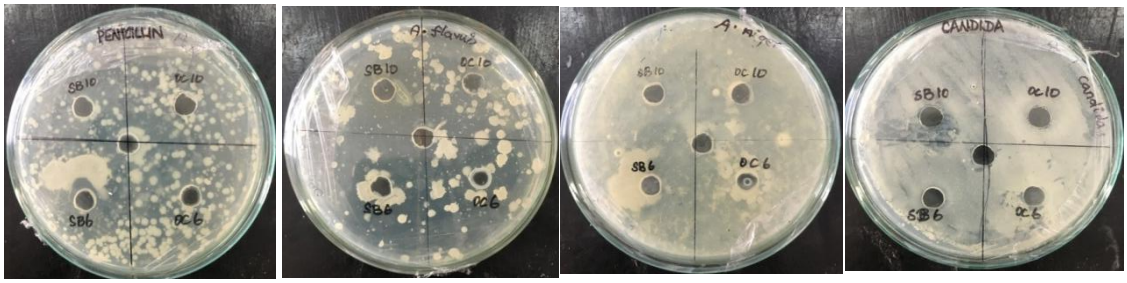


(a) *E.coli* (b) *Pseudomonas aeruginosa* (c)*Staphylococcus spp* (d) *Streptococcus spp* (e)*Klebsiella spp*

Figure 28 (a) to (e): Antibacterial activity – SB 10, OC 10 – No zones of inhibition

ANTIFUNGAL ACTIVITY ASSAY

Antifungal activity of all the filtrates (SB 6, OC 6, SB 10, OC 10) against *Penicillium spp.*, *Aspergillus flavus*, *Aspergillus niger*, *Candida spp.* was checked. There were no zones of inhibition formed indicating that both the isolates do not have antifungal activity. A study conducted by Bensouici Karima *et al.*, (2015) revealed that *Streptomyces spp* has antifungal activity against *Tramates spp a*nd *Memnoniella spp* **(Figure 29).**



(a) *Penicillium spp.* (b)*Aspergillus flavus* (c) *Aspergillus niger* (d)*Candida spp*

Figure 29 (a) to (d): Antifungal activity – SB 6, OC 6, SB 10, OC 10 – No zones of inhibition

TESTS FOR OTHER PRODUCTS FROM FERMENTATION
ESTIMATION OF ORGANIC ACIDS

CITRIC ACID ESTIMATION

Citric acid estimation was carried out for the filtrates (SB 6, OC 6, SB 10, OC 10) by the titrimetric method against sodium hydroxide solution with phenolphthalein indicator. The titration was stopped when the contents of the flask turned red/pink colour which is the end point. The concentration of citric acid was found to be the highest for the OC 6 filtrate – 0.2 g/L **(Table 5).**

SAMPLE	CONCENTRATION OF CITRIC ACID (g/L)
SB 6	0.004
OC 6	0.2
SB 10	0.008
OC 10	0.016

Table 5: Concentration of Citric acid

OXALIC ACID ESTIMATION

Oxalic acid estimation was carried out for the filtrates (SB 6, OC 6, SB 10, OC 10) by the titrimetric method against $KMnO_4$. The contents of the flask changed from colourless to pink colour which is the end point **(Table 6).**

SAMPLE	CONCENTRATION OF OXALIC ACID (g/L)
SB 6	0.0002
OC 6	0.0004
SB 10	0.0002
OC 10	0.0004

Table 6: Concentration of Oxalic acid

A study conducted by Poovarasan *et al.,* (2015) revealed that the actinomycetes isolated from the Arbuscular mycorrhiza fungal spores were capable of producing organic acids. Another study revealed that *Streptomyces spp* isolated from soil, rhizosphere and mycorrhizosphere of pine were capable of producing organic acids (Rozycki & Strzelczyk 1986).

PROTEIN ESTIMATION BY LOWRY'S METHOD

This was carried out for OC 6, SB 10, OC 10 filtrates. BSA was used as standard and OD values were taken at 660 nm and standard graph was plotted. The sample OD values were also taken at 660 nm and calculations were done using the standard graph. Protein concentration in OC 6, SB 10 and OC 10 was found to be 600 mg/ml, 375 mg/ml and 600 mg/ml, respectively. Protein estimation by Lowry's method was carried out to further test for the presence of enzymes **(Figure 30 and Graph 6).**

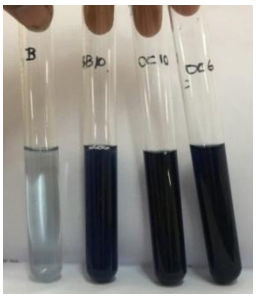
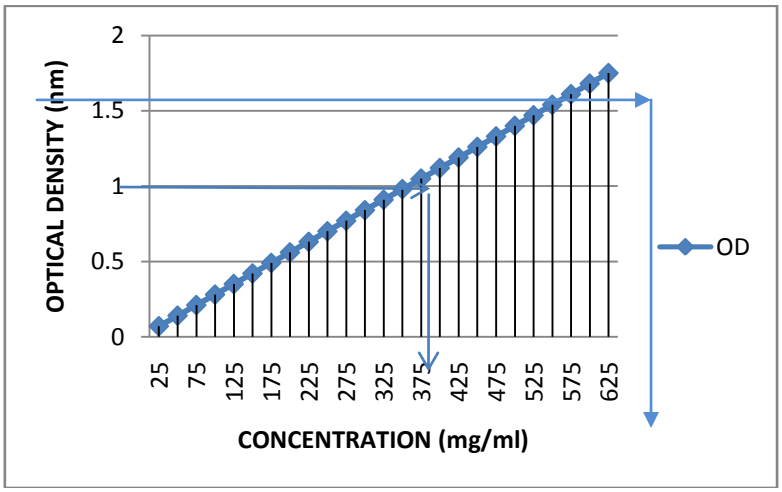


Figure 30: Protein estimation by Lowry's method



Graph 6: Standard graph showing sample protein concentration

QUALITATIVE ESTIMATION OF ENZYMES PROTEASE ASSAY

Protease assay was carried out for the filtrates (SB 6, OC 6, SB 10, OC 10) using the skim-milk agar. After incubation, zones of clearance were observed around the wells indicating that both the isolates were capable of producing the enzyme protease **(Figure 31).**

Figure 31: Protease assay plate showing zone of clearance

AMYLASE ASSAY

Amylase assay was carried out for the filtrates (SB 6, OC 6, SB 10, OC 10) using the starch agar. After incubation, iodine crystals were sprinkled on the plate. There was appearance of yellow colour around the wells indicating that starch has been degraded by amylase. Thus, it was found that both the isolates were capable of producing the enzyme amylase **(Figure 32).**

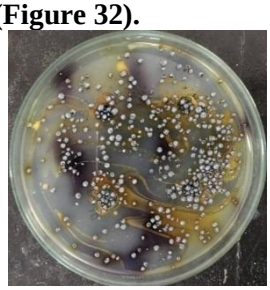


Figure 32: Amylase assay plate showing yellow colour around the wells

LIPASE ASSAY

Lipase assay was carried out for the filtrates (SB 6, OC 6, SB 10, OC 10) using the tributyrin agar with coconut oil. After incubation, there was no zone of clearance observed around the wells indicating that both the isolates were not capable of producing the enzyme lipase **(Figure 33).**

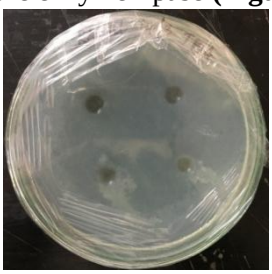


Figure 33: Lipase assay plate showing no zone of clearance

A study conducted by Jeffrey, L. S. H. (2008) revealed that actinomycetes isolated from soil were capable of producing enzymes such as cellulase, lipase and protease. Actinomycetes are capable of producing several enzymes such as amylase, protease, catalase, lipase, chitinase, etc (Mukesh Sharma *et al.*, 2014). Salma Mukhtar *et al.*, 2017 revealed that actinomycetes are a source of industrially important enzymes.

CONCLUSION

In the present study, production of vitamin B_{12} was carried out by *Streptomyces asenjonii* (SW isolate) and *Streptomyces djakartensis* (SC isolate) using alternate substrates such as Soybean powder and Oilcake (Groundnut & Sesame) supplemented media. The concentration of vitamin B_{12} was found to be 57.5 µg/ml in the filtrates as well as the residual substrates. This shows that the residual substrates are enriched with vitamin B_{12} and thus they can be utilised as vitamin B_{12} enriched animal feed. RP-HPLC results of the filtrates confirmed the presence of vitamin B_{12}. Antimicrobial activity is commonly associated with this group of organisms but in this study, it was found that both the isolates lack antimicrobial activity. These isolates were found to be capable of producing organic acids such as citric acid and oxalic acid. Some industrially important enzymes such as amylase and protease are also found to be produced by these isolates. Since vitamin B_{12} is not synthesised by the body, microorganisms are the main source for its production. By

using alternate cost effective substrates, vitamin B_{12} can be produced efficiently. Thus, this process can be developed into a large scale process by which vitamin B_{12} and vitamin B_{12} enriched animal feed can be produced in large amounts.

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