BIOFORTIFICATION OF SEAWEED WITH VITAMIN B12 USING BACTERIAL FERMENTATION

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By

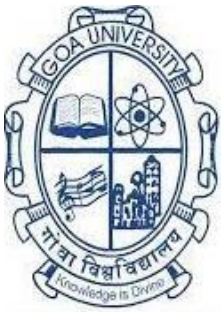
Sanjay S

21P050020

Under the Supervision of

Dr. Meghanath Prabhu

School of Biological Sciences and Biotechnology



GOA UNIVERSITY

April 2023

DECLARATION

I hereby declare that the work incorporated in her dissertation entitled **"BIOFORTIFICATION OF SEAWEED WITH VITAMIN B12 USING BACTERIAL FERMENTATION "**. Submitted in partial fulfilment of the degree of Master of Science in Biotechnology in original and carried out by me at the School of Biological Sciences and Biotechnology, Goa University.

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Mr. Sanjay S

21P050020

Marine Biotechnology

School of biological sciences and Biotechnology

SBSB: Biotechnology Taleigao Plateau, Goa 403206 GOA UNIVERSITY

Seal of the School

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CERTIFICATE

This is to certify that the dissertation entitled "**BIOFORTIFICATION OF SEAWEED WITH VITAMIN B12 USING BACTERIAL FERMENTATION**" submitted by Mr. Sanjay S is an authentic piece of work carried out in partial fulfilment of the requirement for the degree of Master of Science in Biotechnology, Goa University in the year 2022-2023, is according to the results obtained by him under my guidance and supervision.

Date: 24/04/2023

Dr. Meghanath Prabhu
School of Biological Sciences and Biotechnology,
Goa University.

sanita kerkar 24/4/23

Dr. Savita Kerkar
Marine Biotechnology
School of Biological Sciences and Biotechnology.

Dean of School of Biological Sciences & Biotechnology
Goa University, Goa-403206
Office Ph. 8669609246

Date:

Place: Goa University

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ABSTRACT:

This research investigates the biofortification of seaweed with Vitamin B12 by co-fermentation of *Lacticaseibacillus rhamnosus* and *Lactococcus lactis* for use as a probiotic supplement. The work included the collection of seaweed samples and the isolation of *Lacticaseibacillus rhamnosus* and *Lactococcus lactis* from cheese samples and their proliferation for the fermentation process. The basic idea is to connect the de Novo type Vitamin B12 synthesis and transformation of Vitamin B2 to DMB and ultimately to Vitamin B12 and the enzyme BluB is used as a connecting factor. The fermented samples were quantified for Vitamin B12 using HPLC with a C18 column at 361nm having acetonitrile and water as mobile phase solvents. The quantification of Vitamin B12 produced in the fermented seaweed was 19.6 mcg/mL, the highest concentration at the optimum time period identified to be 72hrs. Whereas the amount of Vitamin B12 in control was 4mcg/mL. The research investigations conclude the potential application of seaweed as a substrate for probiotic fermentation in the fortified production of Vitamin B12.

LIST OF ABBREVIATIONS:

mcg - Microgram

mL – Millilitre

µg – Microgram

MRS Agar - De Man, Rogosa and Sharpe Agar

Pvt. Ltd – Private Limited

℃ - Degree Celsius

OD – Optical Density

LAF – Laminar Air Flow

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1. INTRODUCTION:

Vitamin B12, also known as cobalamin, is a water-soluble vitamin that plays an essential role in many bodily functions. It is a necessary nutrient that cannot be produced by the human body and therefore must be obtained through diet or supplements (Allen, 2012). Vitamin B12 is found naturally in various animal-based foods, including meat, fish, eggs, and dairy-based products including cured, cheese, etc., and is quite absent in plant-based food sources (Stabler, 2020).

Vitamin deficiencies, including a lack of vitamin B12, have recently been reported to affect people of various ages including pregnant and lactating women worldwide. Strict vegetarians are even more at lay on the line for the need for vitamin B12. Approximately 62% of vegetarians experience vitamin B12 deficiencies, compared to 25 to 86%, 21 to 41%, and 11 to 90% of children, adolescents, and the elderly (Hunt et al., 2014).

Adults recommended daily intake (RDI) is 2.4 mcg of vitamin B12. Pregnant or nursing women may need higher doses of vitamin B12, with an RDI of 2.6–2.8 mcg daily. The RDIs for infants and young children range from 0.4 mcg to 1.8 mcg per day. Vitamin B12 is also essential for maintaining optimal cognitive function and preventing cognitive decline. Vitamin B12 and folate work together to produce healthy red blood cells. Megaloblastic anemia, caused by vitamin B12 deficiency, causes enlarged red blood cells and results in fatigue and reduced oxygen transport (Allen,1998).

Seaweeds are a diverse group of multicellular marine macroalgae found in various marine environments across the globe. They are classified into three briny groups based on their pigment composition - red, green, and brown algae. Seaweeds are a vital component of marine ecosystems, which supports a wide range of marine plants and animals, and play a crucial role in the global carbon cycle (Hurd et al., 2014).

Seaweed can grow in a broad range of environmental conditions, from shallow waters that are warm with excess nutrients to those that are cold with fewer nutrients. They are the substantive sources of food, medicine, and industrial products and are widely used in various industries, including biotechnology, agriculture, and cosmetics (Holdt & Kraan, 2011).

Seaweeds have been used for centuries for their nutritional and curative benefits, such as boosting the immune system, lowering blood pressure, and promoting healthy skin.

They are also rich in vitamins and minerals and are a promising source of bioactive substances with the potential to be leveraged in the creation of novel medications, cosmetics, and solid food additives (Shannon & Abu-Ghannam, 2019).

Seaweeds are a crucial asset for many industries and aid marine ecosystems, and their study and preservation are important fields of research. The increasing demand for natural and safe food products has resulted in an increased interest in the use of functional foods that supply added health benefits. Vitamin B12, an essential micronutrient, is produced only by certain bacteria and is not available from plant-based sources. Therefore, the need for sustainable and efficient methods to produce Vitamin B12 is of high importance (VELUCHAMY & PALANISWAMY, 2020).

Seaweeds are naturally known to contain small amounts of Vitamin B12 (concentration here) and are a promising substrate for the production of Vitamin B12 by microbial fermentation. *Lactobacillus* and *Propionibacterium* species are lactic acid bacteria that are unremarkably used in the fermentation of dairy and other functional food products (Capozzi et al., 2012).

These bacteria can synthesize Vitamin B12 for their biochemical needs and are known to metabolize a range of substrates including those derived from seaweeds. Commercial uses of these bacteria include the production and selling of vitamin B12 supplements. A de novo pathway involving a series of enzymatic reactions using amino acids such as glutamate as the initial source is used by bacteria, including *Lactobacillus sp*., to synthesize vitamin B12. Conversely, *Propionibacterium* species synthesize vitamin B12 by converting vitamin B2 to DMB (5, 6-dimethyl benzimidazole), which is then converted to vitamin B12(Javanainen & Linko, 1993).

Both of the aforementioned types of bacteria are used in the strategy for the enhanced synthesis of vitamin B12, with BluB enzyme (5, 6-dimethyl benzimidazole synthase) serving as a connecting link between both pathways. *Lactobacillus* sp. lacks the gene factor encoding the BluB enzyme, which is necessary to transition riboflavin (vitamin B2) to cyanocobalamin (vitamin B12), but *Propionibacterium* sp. type bacteria do (Balabanova et al., 2021).

In addition to the synthesis of vitamin B12, other biomolecules such as vitamin B2 are also produced. Therefore, in summation to the de novo pathway for synthesizing vitamin B12, bacteria with genes for the BluB enzyme also carry out the BluB enzyme's transition of vitamin B2 to vitamin B12 (Mohedano et al., 2019).

Vitamin B12 synthesis is anticipated to occur as a result of this fortification. Studies on this topic have shown that the co-fermentation of the antecedently stated bacterial types can make more vitamin B12 than either type of fermentation alone (Xie et al., 2019).

Ulva lactuca, green seaweed, was selected for the role because it is edible, promptly available, and has high protein content. *Ulva lactuca* has been found to hold a high proportion of essential amino acids, including lysine, leucine, and valine.

It also contains non-essential amino acids, such as aspartic acid, glutamic acid, glutamate, and proline. The amino group acid content of *Ulva lactuca* may mostly depend on the season, location of the seaweed, and environmental conditions. Nevertheless, it is considered to be one of the most nutritionally beneficial seaweeds due to its high protein and amino acid content (Machado et al., 2020).

The overarching aim of this research study is to look into the co-fermentation of seaweed using *Lactobacillus* and *Propionibacterium* species for the enriched synthesis of Vitamin B12.

And the objectives towards reaching the goal are as follows:

1. Collection and morphological characterization of green seaweed *Ulva* sp.

2. Isolation, and identification of *Lactobacillus* and *Propionibacterium* species.

3. Fermentation of *Ulva* sp. for the production of Vitamin B12 and its Quantification.

2. Literature Review:

Throughout the world, seaweeds, which are multicellular algae, can be found in a range of marine habitats. Red seaweed, brown seaweed, and green seaweeds are the three groups into which seaweeds fall according to the makeup of their pigments (Guiry,2012).

1. Red algae (Rhodophyta): This is the group of seaweeds with the greatest variety and abundance. They can be found in colder waters as well as the warm waters closest to the equator. Phycobiliproteins, chlorophyll a, and carotenoids are a few of the pigments that give red algae their distinctive red color. Due to their high nutritional value and abundance in vitamins, minerals, and antioxidants, they are also well-known for this. Red algae are frequently used to make cosmetics, medications, and food products (Yanshin et al., 2021).

2. Green algae (Chlorophyta): This group of seaweed is typically found in shallow waters close to the shore. Green algae are thought to be the ancestors of higher plants. They are very similar to land plants, including chlorophyll a and b. They also contain pigments like carotenoids and phycobilins. Green algae, like other seaweeds, are a valuable source of nutrition due to their substantial vitamin, mineral, and antioxidant content. Additionally, they are employed in the production of a wide range of goods, such as cosmetics, dietary supplements, and food additives (Biris-Dorhoi et al., 2020).

3. Brown algae (Phaeophyta): Of all seaweed subclasses, this one is the most intricate. They can grow up to 60 meters in length and are found worldwide in cold and temperate waters. Fucoxanthin, chlorophylls a and c, and carotenoids are just a few of the pigments found in brown algae that help to give them their distinctive brown hue. They are renowned for their distinctive substances, which include alginates, which are used as thickeners, and fucoidans, which are produced to make a variety of pharmaceuticals (Li et al., 2021).

Seaweeds can be divided into three groups according to their habitat in addition to the pigments they contain (Tittley, 1991):

1. Rocky shore seaweeds: These are predominantly red and brown seaweeds that are found in rocky intertidal areas.
2. Soft sediment seaweeds: These are predominantly green seaweeds found in mud or sand sediment in shallow water areas.
3. Open ocean seaweeds: These are predominantly brown and green seaweeds that can be found in the ocean's depths.

Among the many nutritional and practical advantages of seaweeds are their sources of dietary fiber, protein, vitamins, minerals, and phytochemicals. The compounds and their concentrations vary greatly between seaweed species.

Among the most prevalent bioactive substances discovered in seaweeds are:

1. Carbohydrates: Seaweeds are abundant in carbohydrates, which are important for preserving general health. Sulfated galactans (agar and carrageenan), cellulose, laminarin, fucoidan, and alginate are the main sources of carbohydrates in seaweeds. 20% to 80% of the dry weight of seaweed can be made up of carbohydrates (Goni et al., 2020).
2. Proteins: Red and green seaweeds are the richest sources of essential amino acids, which are the building blocks of proteins, in seaweeds. About 5–47% of the dry weight of different seaweed species is made up of proteins (Černá, 2011).
3. Vitamins: Seaweeds are a good source of several vitamins, including B12, C, A, E, and K. Seaweeds contain a variety of vitamins, with some species being higher in a particular vitamin than others (Glassman, 2023).

4. Minerals: Minerals like iodine, potassium, calcium, magnesium, and iron are prevalent in seaweeds. Seaweed's mineral content varies greatly depending on its species, harvesting area, and season (Circuncisão et al., 2018).
5. Polyphenols: Phlorotannin and flavonoids are two examples of polyphenolic substances found in seaweeds. The antioxidant and anti-inflammatory properties of seaweeds are influenced by these compounds. From 0.1% to 10% of their dry weight, polyphenols can be found in seaweeds (Cotas et al., 2020).
6. Fatty acids: Omega-3 and omega-6 fatty acids, such as EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), are abundant in seaweeds. Species, seasons, and places of harvest all have an impact on how much fatty acids are found in seaweeds (Ginneken et al., 2011).
7. Additional bioactive substances: Terpenoids, alkaloids, and polysaccharide-protein complexes are among the additional bioactive substances found in seaweeds, which support their nutritive and practical qualities (Khalid et al., 2018).

Ulva lactuca, more commonly referred to as sea lettuce, is prevalent green seaweed that is found worldwide in coastal areas. Rich in lipids, proteins, and carbohydrates, *Ulva lactuca* is a good source of these macronutrients.

Ulva lactuca has an average of 323.34 ± 14.90 mg/g of carbohydrates, 247.74 ± 0.08 mg/g of proteins, and 21.22 ± 0.02 mg/g of lipids per gram of dry biomass. Other micronutrients, such as vitamins and minerals, are also abundant in *Ulva lactuca*. *Ulva lactuca* had 24.3 ± 0.6 μg/g of vitamin C, 193 ± 1 μg/g of vitamin E, and 6.5 ± 0.2 μg/g of vitamin B12 per gram of dry biomass. *Ulva lactuca* contains 3803 ± 20 μg/g of iron, 947 ± 7 μg/g of calcium, and 506 5 μg/g of magnesium per 1 gram of dry biomass.

Per 1 gram of dry biomass, *Ulva lactuca* contained 38.3 1.8 mg/g of total carotenoids, 185.4 2.4 mg/g of total chlorophylls, and 2.4 ± 0.1 mg/g of total phlorotannin (Yaich et al., 2011).

Various forms of the intricate vitamin B12 exist, and each one has a unique chemical makeup and biological activity. The most prevalent kinds are methylcobalamin, cyanocobalamin, hydroxocobalamin, and adenosylcobalamin. These vitamin B12 forms each function a specific purpose with specific importance in the human body.

1. Cyanocobalamin: The most widely used form of vitamin B12 in dietary supplements and foods that have been fortified is cyanocobalamin. It is a synthetic form of vitamin B12 that is created through the fermentation process. Because cyanocobalamin is stable and has a long shelf life, it is the perfect form for use in supplements and fortification. Cyanocobalamin is transformed into methylcobalamin and adenosylcobalamin in the body (O'Leary & Samman, 2010).

2. Hydroxycobalamin: A naturally occurring form of vitamin B12 called hydroxocobalamin is created by bacteria in the soil. A common treatment for vitamin B12 deficiency is hydroxocobalamin, a precursor to other forms of vitamin B12. It is additionally employed as a cyanide poisoning antidote (Minigh, 2017).

3. Methylcobalamin: Methylcobalamin is a biologically active form of vitamin B12 necessary for the body's numerous biochemical processes, such as the production of DNA and red blood cells. Additionally frequently found in dietary supplements, methylcobalamin is thought to be more bioavailable than other forms of vitamin B12 (Zhang et al., 2013).

4. Adenosylcobalamin: Another biologically active form of vitamin B12 is adenosylcobalamin, which is crucial for the body's metabolism of fatty acids and amino acids among other metabolic processes. Numerous animal-based foods, especially organ meats, as well as some plant-based foods, contain it. Since adenosylcobalamin is less stable than other forms of vitamin B12, it is frequently combined with them in dietary supplements and foods that have been fortified (Marsh & Meléndez, 2012).

The human body uses vitamin B12 in various forms, each of which has its own purposes and significance. All forms of vitamin B12 can be transformed by the body into biologically active forms, but some forms may be more bioavailable and offer more health advantages than others.

For instance, methylcobalamin has been linked to cardiovascular and neuroprotective advantages, whereas adenosylcobalamin may be more useful in the management of specific anemia types.

While humans are unable to synthesize Vitamin B12, numerous bacteria, including *Lactobacillus*, are able to do so via a convoluted series of enzymatic processes (Lawrence & Roth, 1995).

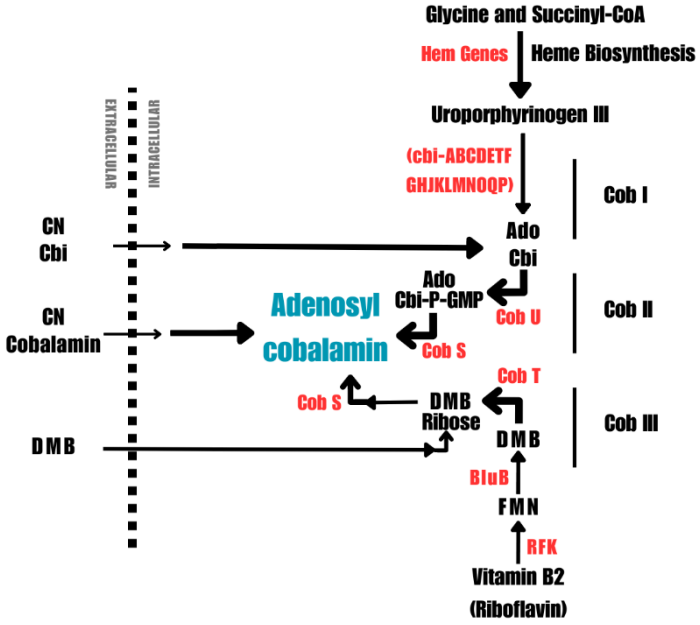

Figure.1 – Schematic representation of all the reactions involved in the synthesis of Vitamin B12 via Co-fermentation.

3. Materials and Methods:

3.1: Collection and morphological characterization of green seaweed *Ulva* sp.

Depending on the type of seaweed, the location, and the intended use, seaweed can be collected from the ocean environment using a variety of techniques. The Goan shores of Vagator Beach were used to collect the *Ulva lactuca*. The green seaweed was manually harvested using the common hand harvesting technique by wading through shallow water and scooping it out of the rock surfaces to which it was attached. The seaweed was identified based on morphological characteristics. After collection, freeze storage is a popular technique for maintaining the quality and nutritional value of green seaweed. If it is not stored in an optimum way, seaweed, which is a highly perishable product, can quickly lose its quality and nutritional value. The activity of enzymes and other biological processes that lead to spoilage are slowed down by freeze storage (Harrysson et al., 2021).

In order to get rid of any sand, dirt, or impurities, the collected seaweed was thoroughly cleaned and rinsed with filtered seawater. To avoid moisture loss and freezer burn, it was then drained and packaged in airtight plastic bags. Before packaging, it's crucial to make sure the seaweed is completely dry because any moisture left behind could lead to the formation of ice crystals, which could harm the seaweed when frozen. When the seaweed was packaged; it was put in a freezer and kept there at a constant -20°C.

3.2: Isolation, and identification of *Lactobacillus* and *Propionibacterium* species.

3.2.1: Isolation of *Lactobacillus* species bacteria:

Numerous dairy products, including yogurt, cheese, and kefir drinks, contain *Lactobacillus* species (Widyastuti et al., 2021). Cheddar cheese was thereby used in *Lactobacillus* separate isolation. The MRS agar is a specific media for *Lactobacillus* species. MRS media is an exclusive culture medium made to encourage *Lactobacilli's* prolific development for laboratory research.

For the purpose of this work, the company SRL's selective media *Lactobacillus* MRS Agar with catalog number "79562" was used.

The steps of the workflow are as follows:

- Sterile MRS agar plates were prepared.
- A sample of 1 gram of cheddar cheese was taken and placed in a sterile test tube. It was diluted 10^{-1} by adding 9 mL of water. It was then homogenized to create a smooth paste.
- In order to promote individual growth on petri dish media, the homogenized cheese sample was streaked with the streaks being distinct and well-spaced apart.
- The plates were then taped shut and wrapped in parafilm. To prevent any contamination, all of these processes were carried out within the LAF.
- The inoculated MRS agar media plates were incubated for 48-72 hours at 37 °C.
- The plates were examined for the development of bacterial colonies following the incubation period.
- The bacterial colonies were picked out based on the morphology and re-streaked on fresh plates with MRS agar media for purification.

3.2.2: Isolation of *Propionibacterium* species bacteria:

The *Propionibacterium* species were cultured and isolated using the same procedure as described in section "3.2.1" above, with the exception of the culture medium used. The presence of *Propionibacterium* freudenreichii, a probiotic grade *Propionibacterium* species is proposed to be available in products that are milk-based like cheese.

Therefore, the same cheddar cheese was used as the sample for the isolation. A selective medium that promotes *Propionibacterium* species' growth while inhibiting that of other bacterial species is yeast extract lactate agar with cadmium chloride.

The preparation of this media involves the incorporation of cadmium chloride into yeast extract lactate agar followed by autoclaving, inoculation, incubation, and sub-culturing procedures.

Further evaluation was done on the pure isolates that were obtained to determine the *Propionibacterium* species.

3.2.3: Identification of the isolated bacterial cultures:

3.2.3.1: Gram Staining:

The most prevalent staining method in microbiology is gram staining, which is employed to initially characterize and identify bacterial species based on the makeup of their cell walls

First, a bacterial smear was prepared and air-dried, and the slide was heat-fixed to create a bacterial smear of the pure isolate. The slide was then submerged in crystal violet solution and left to sit for one minute. For a further minute, Gram's iodine solution was flooded onto the slide after it had been cleaned with distilled water to permanently adhere the crystal violet stain to the bacterial cell walls.

The slide was then briefly flooded with safranin for a minute as a counterstain, washed with distilled water, and briefly passed under a stream of 95% ethanol to complete the decolorization process.

After these staining procedures, the slide was examined under a 100x objective lens with oil immersion, and the findings were interpreted (Becerra et al., 2016).

3.2.3.2: Molecular identification using 16s ribosomal RNA gene sequencing:

A reliable method for identifying and classifying bacteria according to their genetic makeup is 16s ribosomal RNA gene sequencing. The technique is based on the sequencing of a conserved region of the 16s ribosomal RNA gene of bacteria, which experiences minute changes over time and enables the determination of taxonomic relationships among various bacterial strains. In order to produce accurate data, the protocol calls for DNA extraction, PCR amplification of the 16s ribosomal RNA gene, and DNA sequencing of the amplicons. More accurate bacterial identification can be achieved by comparing the generated sequence with reference databases and using bioinformatics tools to find the closest-matching bacterial sequence (Johnson et al., 2019).

Freshly streaked pure isolate sample was packed and sent to HiMedia Laboratories Pvt. Ltd. of Thane, Maharashtra, India for the Hi-Gx360 Service of 16s ribosomal RNA sequencing. The sequence obtained was subjected to BLAST in the NCBI database for identification and phylogenetic tree based on the NJ (Neighbor-joining) algorithm.

3.3: Fermentation of *Ulva* sp. for the production of Vitamin B12 and its Quantification:

3.3.1: Proliferation of Cultures in Broth:

As directed by the manufacturer, the required quantity of MRS media broth powder and Yeast Extract Lactate broth powder was added to distilled water.

In order to see and dissolve the broth, the solution was kept in an autoclave for a temperature of 121 ℃ and a pressure of 15psi for 15 minutes. After it had cooled down, a loopful of bacterial culture was then transferred to the media broth using a sterile inoculation loop in the LAF. The flask was gently shaken to thoroughly combine the inoculum with the broth. The flask was incubated at an ideal temperature range of 35 to 37 ℃.

This was carried out for both isolated cultures. Assuming a linear relationship between OD 600 and bacterial cell density, an OD 600 value of 1 typically corresponds to a bacterial cell density in a bacterial broth culture of about $1*10^8$ cells/mL. In order for both of the chosen bacterial cultures, which were inoculated in broth, to reach the $1*10^8$ cell count, they were incubated for a period of time of approximately 16 to 20 hours. The incubation ceased once the broths' respective OD at 600 nm values crossed the OD - 600 value of 1 (Beal et al., 2020).

3.3.2: Seaweed Processing:

During this step, seaweed is processed into a homogenized sample for fermentation. The seaweed and water were formulated in a 1:4 ratio following 5g of seaweed taken and homogenized with 20 mL of water. Here, the homogenized mixture serves as the sample of the substrate, and 5 mL of the sample was transferred to four sterile 20 mL flasks. For the "Test" and the "Control," there are two flasks each. Given that vitamin B12 is photosensitive and thus susceptible to deterioration, the flasks were covered with aluminum foil.

3.3.3: Fermentation of the seaweed:

The "Test" flasks containing seaweed samples as substrate were inoculated with both of the isolated bacterial cultures from the broth. In the ratio of 5%, 250 µL of each bacterial culture from the broth was transferred to the two 'Test' flasks for the co-fermentation process.

Five hundred µL of sterile distilled water was added to the ‘Control’ flasks. The flasks were placed in a shaker incubator set to 37 degrees Celsius and 100 rpm. Under the aforementioned conditions, incubation took place continuously for 5 days.

3.3.4: Quantification of Vitamin B12 in the fermented seaweed:

3.3.4.1: Collection and Storage:

Five hundred µL of the fermented seaweed sample from each flask was collected once every 24 hours for five days in 1 mL vials covered with aluminum foil, and it was then stored at -20 degrees Celsius for the quantification of vitamin B12.

3.3.4.2: Extraction Vit B12 from the fermented seaweed sample:

Hundred µL of fermented *Ulva* sp. samples from days 1, 3, and 5 of both the "Test" and "Control" flasks were pipetted out. The samples were then transferred to 2 mL centrifuge tubes (wrapped in aluminum foil) to which 100 µL of 0.05 mol/L acetate buffer with a pH of 4.8 was added. Next, 20 mg of potassium ferro cyanide was added, and the total volume was then brought to 1 mL by using distilled water.

After that, the samples were kept in a 98 °C boiling water bath for 30 minutes followed by 10 minutes of 10000g centrifugation, and the supernatant was collected for further analysis (Watanabe, 1999).

3.3.4.3: HPLC Analysis for the Quantification of Total Cyanocobalamin.

The HPLC analysis using the C18 column proved to be a reliable and accurate method for the analysis of cyanocobalamin in vitamin B12 samples. The combination of the C18 column and gradient system produced the high-resolution separation of the analytes with minimal interference from other compounds. The method's excellent detection and quantification limits allowed for an accurate and precise analysis of cyanocobalamin in the sample.

Using a C18 column at 361 nm and a gradient of acetonitrile and water as the mobile phase solvents, the samples were analyzed. samples (20 µL) were injected at a flow rate of 1 mL/min. The instrumentation utilized was a "SHIMADZU LCMS-2020" with a 150 mm x 4.6 mm C18 column and its own software for data integration. The integrated data was then analyzed for the quantification of Cyanocobalamin (Watanabe, 1999).

4. RESULTS AND DISCUSSION:

4.1: Identification of the Isolated Bacteria:

4.1.1: Isolate 1:

The first isolate which was cultivated on the *Lactobacillus* MRS agar and expected to be of any *Lactobacillus* species underwent a preliminary analysis involving gram staining followed by 16S rRNA sequencing. The results were interpreted.

4.1.1.1: Gram Staining:

The gram staining technique was carried out and the result was found to be Gram-positive Bacteria. Therefore, the isolate was selected for further procedures.

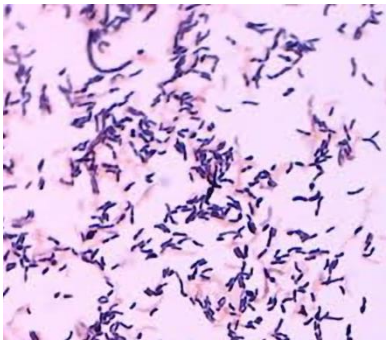

Figure.2 – The bacteria retained crystal violet and therefore appearing purple in colour confirming that it is Gram-Positive. And the rod shape of the bacteria supports it possibly could be of *Lactobacillus* species.

4.1.1.2: 16s rRNA Sequencing:

The freshly streaked isolates were sent for 16s rRNA sequencing as mentioned in the section "3.2.2.2". The sequence was obtained for the sequencing process involved with primer "27R" and the BLAST results showed 95.39% similarity with '*Lacticaseibacillus rhamnosus*'. And the culture was identified as similar to that of the bacterial species *Lactobacillus rhamnosus*.

The Neighbor-Joining based phylogenetic tree was constructed for result interpretation. The phylogenetic tree constructed depicted the relativeness of the isolate with that of others and based on the results of the phylogenetic tree analysis for 16S rRNA gene sequence that has 95.3% similarity to the bacterial species Lacticaseibacillus rhamnosus, the sequence is likely to belong to the same genus as *L. rhamnosus*, which was previously classified as *Lactobacillus* (Zhang,2000).

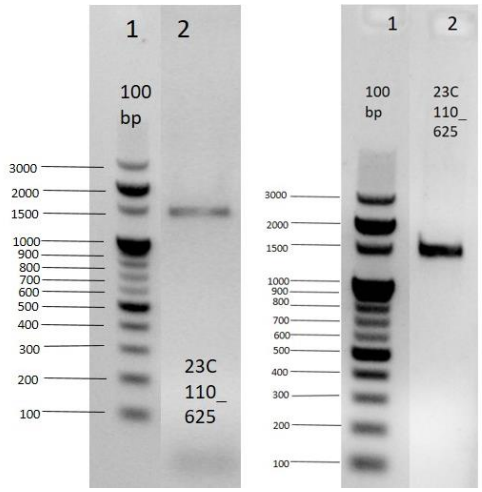

Figure.3 – Resulted Electrophoresis Gel-Image of PCR product of the Isolate 1 confirming the isolation of 16S rRNA gene of 1550 base pairs with that of reference

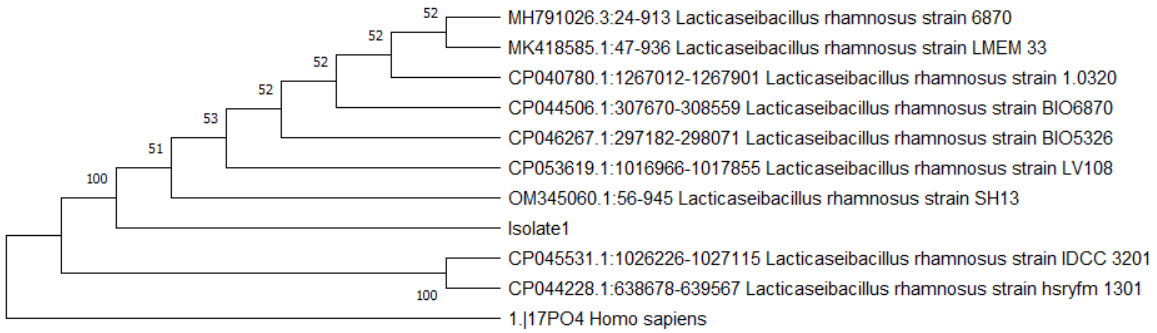

Figure.4 – The constructed phylogenetic tree using Neighbour Joining method. With the incidence of 95.3% similarity, it can be grouped under the species of *Lacticaseibacillus rhamnosus*.

4.1.2: Isolate 2:

The first isolate which was cultivated on the Yeast Extract Lactate agar and expected to be of any *Propionibacterium* species underwent a preliminary analysis involving gram staining followed by 16S rRNA sequencing. The results were interpreted.

4.1.2.1: Gram Staining:

The gram staining technique was carried out and the result was found to be Gram-positive Bacteria. Therefore, the isolate was selected for further procedures.

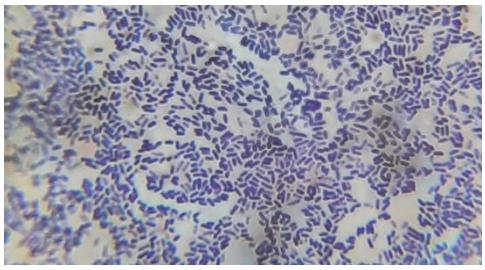

Figure.5 – The bacteria retained crystal violet and therefore appearing purple in colour confirming that it is Gram-Positive. And the morphology appears short- rods.

4.1.2.2: 16s rRNA Sequencing:

The freshly streaked isolates were sent for 16s rRNA sequencing as mentioned in the section "3.2.3.2". The sequence was obtained for the sequencing process involved with primer "907R" and the BLAST results showed 100% similarity with the species *'Lactococcus lactis'*. And therefore, probably predicted in high confidence to be the bacterial species *Lactococcus lactis*.

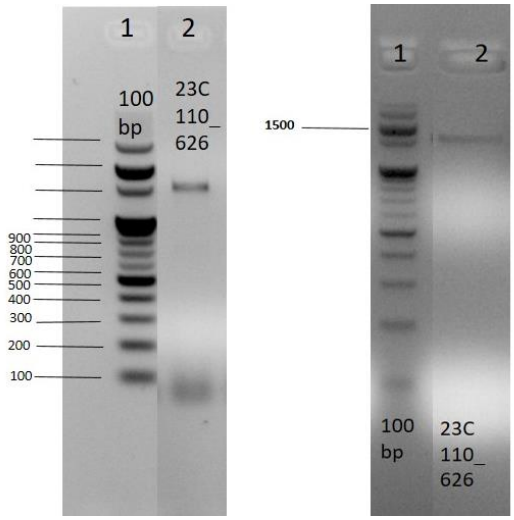

Figure.6 – Resulted PCR Gel-Image of the Isolate 1 confirming the isolation of 16S rRNA gene of 1550 base pairs with that of reference (Clarridge, 2004).

The Neighbor-Joining based phylogenetic tree was constructed for result interpretation. The result of phylogenetic tree analysis for a 16S rRNA gene sequence having 100% similarity with the bacterial species *Lactococcus lactis* indicates that the sequence belongs to the same species as *Lactococcus lactis*.

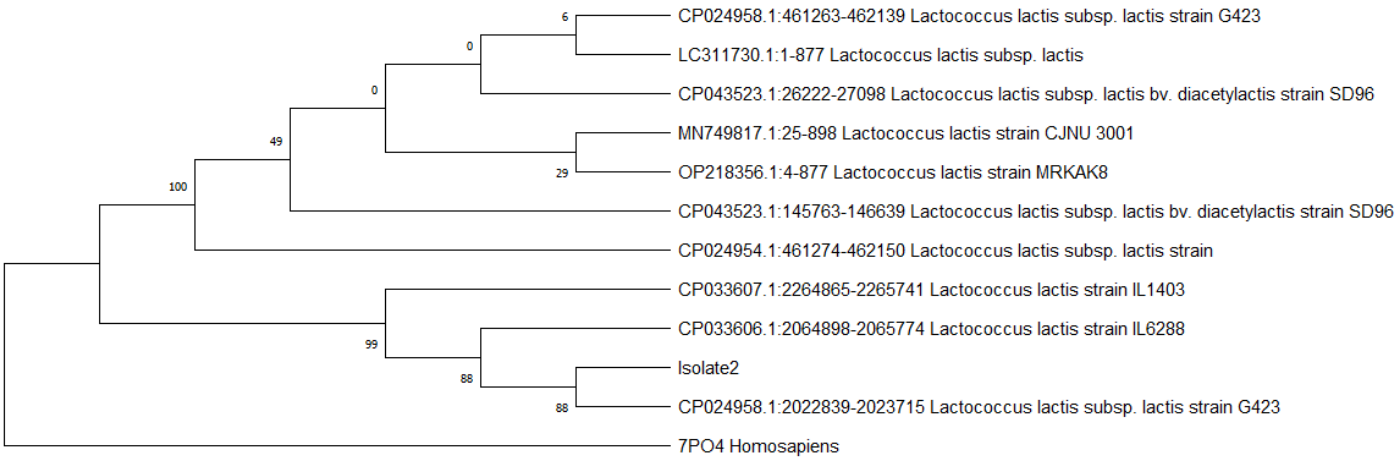

Figure.7 – The constructed phylogenetic tree using the Neighbour Joining method. With the incidence of 100% similarity, it can be grouped under the species of *Lactococcus lactis*.

Despite the fact that we isolated the *Propionibacterium* species using specialized conditions with high metal concentrations, the isolate has been identified as *Lactococcus lactis*. As a result, research demonstrates that *Lactococcus lactis* may thrive in heavy metal media, however many other bacteria cannot tolerate and grow.

The reason for choosing Propionibacterium species was the ability to possess the 'BluB' gene, which is essential for the synthesis of Vitamin B12 by converting FMN to DMB. Therefore, a database search was conducted to see if the 'BluB' gene has been reported in *Lactococcus lactis*, and the findings were positive. Multiple entries in UniProt for the presence of the 'BluB' gene in *'Lactococcus lactis*' is reported and one such entry for confirmation is with an ID - A0A0H1RRD1.

4.2: HPLC Analysis for the Quantification of Cyanocobalamin:

The other forms of cobalamin eventually degrade because they are unstable during extraction and analysis. Therefore, the other forms of cobalamin are converted into the stable cyanocobalamin form, which makes it simple to measure total cobalamin, with the addition of a cyanide compound, Potassium Ferro Cyanide.

The method of preparation was carried out using one of the standards and the cyanocobalamin's peak was seen and noted around the retention time of 6.6 mins. Then all the standards and samples were extracted and injected likewise by the same method as explained in section "3.3.4".

For all the standards and samples (Test and Control) the peaks were observed in the range of 6.4 to 6.7 mins. The peak areas of the respective were marked down and used for further analysis.

The calibration curve was plotted using Microsoft Excel with the Standard Concentration vs Peak Area. And the equation of the straight was calculated to be $Y = 1441.7X + 7607.8$ with a Coefficient of determination, $R^2 = 0.971$.

The concentration of all the individual samples was calculated using this equation substituting 'Y' with the value of the sample's peak area obtained. The mean was taken for the calculated concentration of each 'Control' and 'Test' respectively at Day 0, 3, and 5.

S1 to S6 represent the standards starting from 0.0001 mcg to 10 mcg. The 'C.1 and C.2' represent the Control in duplicates. In the same way the 'T.1 and T.2' denote the test in duplicates.

The determined concentration for all the samples is given in Table.1, Table.2, and Table 3.

Standard Number	Concentration (mcg/mL)	Peak Area
S1	0.0001	7402
S2	0.001	7115
S3	0.01	8056
S4	0.1	6479
S5	1	10746
S6	10	21867

Table.1 – Standard Concentration and its respective peak area.

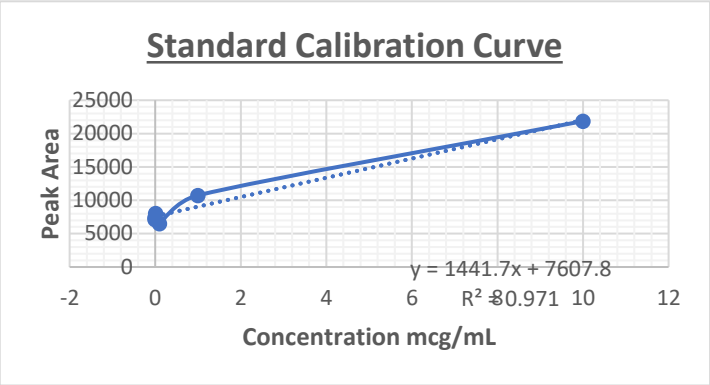

Figure.8 – Standard Calibration Curve of Vitamin B12. Concentration Vs Peak Area.

CONTROL	PEAK AREA	CONCENTRATION
C.1.0	7957	0.242214053
C.1.3	17300	6.722757855
C.1.5	17698	6.998820837
C.2.0	11034	2.376499965
C.2.3	9687	1.442186308
C.2.5	9346	1.205659985

DAYS	CONCENTRATION
C.0	1.309357009
C.3	4.082472082
C.5	4.102240411

Table.2 – 1. Peak area and the calculated concentration for Control. 2. And the mean calculated concentration over 3 days.

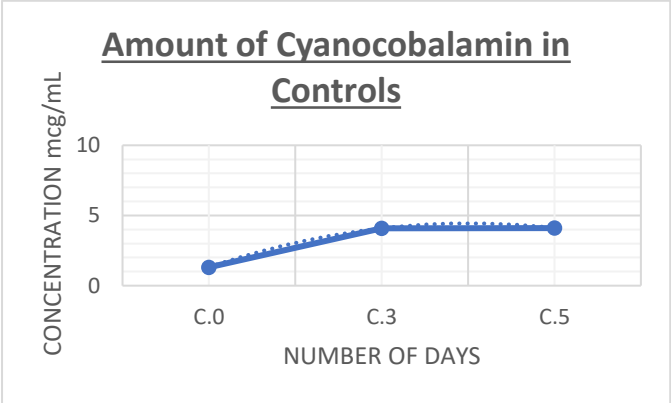

Figure.9 – Plot of Concentration over Days for CONTROL. Number of Days vs Concentration.

CONTROL	PEAK AREA	CONCENTRATION
T.1.0	11133	2.445168898
T.1.3	31091	16.28854824
T.1.5	27481	13.78455989
T.2.0	17557	6.90101963
T.2.3	40843	23.05278491
T.2.5	25267	12.24887286

DAYS	CONCENTRATION
T.0	4.673094264
T.3	19.67066657
T.5	13.01671638

Table.3 – 1. Peak area and the calculated concentration for Test. 2. And the mean calculated concentration over 3 days.

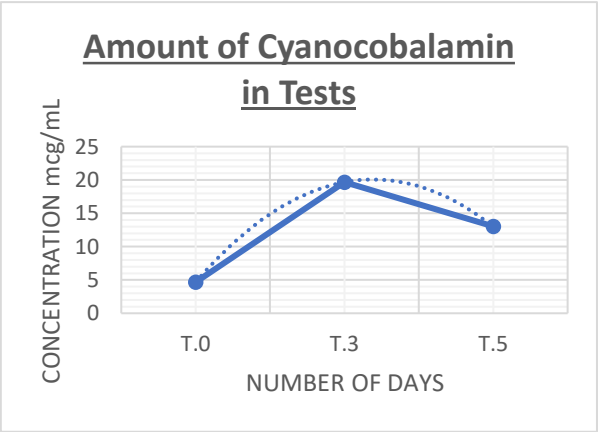

Figure.10 – Plot of Concentration over Days for TEST. Number of Days vs Concentration.

The amount of cyanocobalamin on the 0th day, 3rd day, and 5th day of the 'Control' was determined to be 1.3, 4.08, and 4.1 mcg/mL. The amount of cyanocobalamin in the 'Control' did not have much deviations in the value over a period of time. The amount of cyanocobalamin determined is in the range of 4 mcg/mL. This confirms that seaweed has not been contaminated post-sterilization or during fermentation. This also confirms the fact that seaweed does not have the capability to produce Vitamin B12 on its own.

The amount of cyanocobalamin on the 0th day, 3rd day, and 5th day of the 'Test' was determined to be 4.6, 19.6, and 13.01 mcg/mL of the fermented seaweed sample. This depicts the fortified production of Vitamin B12 by the probiotic bacteria used on the seaweed as substrate.

The concentration of cyanocobalamin increases from the 0th day to the 3rd day and gradually decreased on the 5th day maybe due to the fact that the bacteria utilize the produced Vitamin B12 for its proliferation. Therefore, it can be concluded that the optimum time for the maximum concentration of Vitamin B12 (19.6 mcg/mL), is around 72 hours which is during 3rd day. A similar experiment has been carried out using coconut milk as substrate fermented by *Lactobacillus casei* L4 and the highest concentration of Vitamin B12 produced was 1.47 mcg/mL. This shows the reliability of the experiment conducted (Giri et al., 2018).

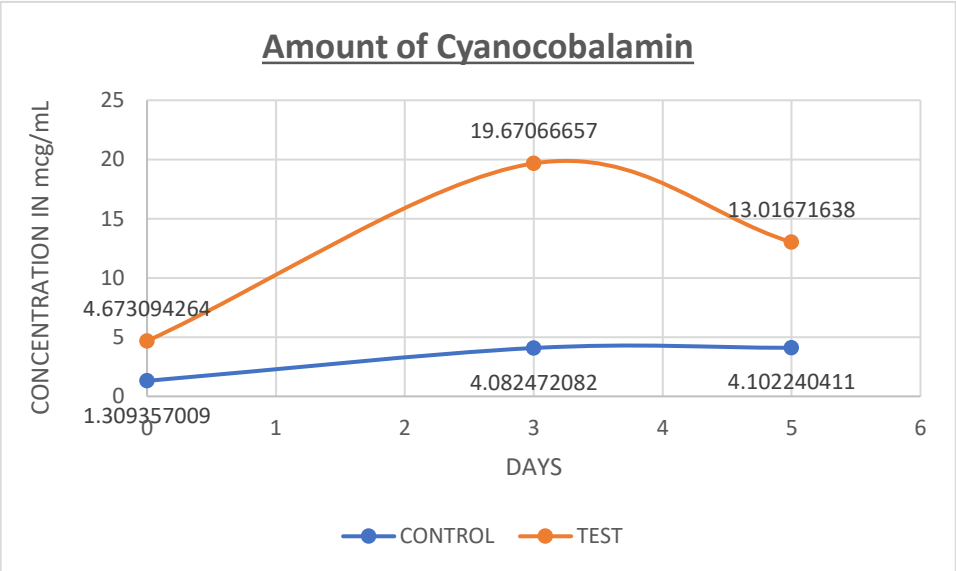

Figure.11 – Graphical representation depicting the comparative values of the amount of Vitamin B12 produced on the 0th, 3rd, and 5th day.

5. CONCLUSION:

The co-fermentation of seaweed as a substrate for the production of Vitamin B12 using *Lacticaseibacillus rhamnosus* and *Lactococcus lactis* proved to be successful. The concentration of Vitamin B12 determined was 19.6 micrograms per mL of the fermented seaweed at the optimum period i.e., 72 hours indicating that this method can be employed as a feasible and cost-effective way for the production of Vitamin B12 as a probiotic supplement. The use of seaweed as a substrate for fermentation presents a promising alternative to conventional methods that commonly use animal-derived products. The results of this study are particularly significant in light of current concerns about the availability and sustainability of Vitamin B12 sources. As this vitamin is essential for human health, particularly for those following a vegan or vegetarian diet, developing new and sustainable methods for its production is crucial. This fermentation product can be used as a probiotic supplement with a fortified amount of Vitamin B12 which can meet the quantity of daily requirement.

Overall, this research provides important insights into the potential use of co-fermentation with seaweed as a means of producing Vitamin B12. Further studies can help to optimize the process and determine its commercial feasibility, paving the way for a more sustainable and accessible source of this crucial vitamin.

Future Prospects:

The same approach can be devised and investigated on microalgae like spirulina which is much richer in protein content and therefore can be used as the substrate of purpose. This research was intended to make a commercial product as a fusion of probiotics and products of nutrient supplements into one. Further research can be done in the processing of the product in order to make the product better for consumable grade with added flavors.

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Appendix:

A] Media Composition:

1. *Lactobacillus* MRS agar – SRL:

Ingredients	gm/L
Agar	12.00
Peptone	10.00
Yeast extract	5.00
Dextrose	20.00
Meat extract	8.00
Triammonium citrate	2.00
Sodium acetate	5.00
Magnesium sulfate heptahydrate	0.20
Manganese sulfate tetrahydrate	0.05
Dipotassium phosphate	2.00
Sorbitan monooleate (Tween 80)	1.00

2. Yeast Extract Lactate Agar:

Ingredients	gm/L
Enzymatic Digest of Casein	10.0
Yeast Extract	10.0
Potassium Phosphate, Monobasic	2.5
Sodium Lactate	10.0
Magnesium Sulphate	0.005
Cadmium Chloride	1.9
Agar	15.0

Final pH 7.0 ± 0.2 at 25°C

B] List of Genes involved in the Vitamin B12 synthesis and their enzymatic name:

1. Genes involved in conversion of Amino acid/ Succinyl-CoA to Uroporphyrinogen III:

1. HemA	Delta-aminolevulinic acid synthase (ALA synthase)
2. HemB	Delta-aminolevulinic acid dehydratase (ALA dehydratase)
3. HemC	Dorphobilinogen synthase (PBG synthase)
4. HemD	Uroporphyrinogen III synthase (UROS)

2. Genes involved in the De Novo Biosynthesis of Vitamin B12:

1. cbiA	Cobyrinic acid a,c-diamide synthase
2. cbiB	Cobalt-precorrin-2 methyltransferase
3. cbiD	Precorrin-2 dehydrogenase
4. cbiE	Cobalt-precorrin-3B synthase
5. cbiF	Cobalt-precorrin-3A synthase
6. cbiG	Sirohydrochlorin ferrochelatase
7. cbiH	Sirohydrochlorin cobaltochelatase
8. cbiI	Sirohydrochlorin cobaltochelatase
9. cbiJ	Cobalt reductase
10. cbiK	Cobalt-precorrin-5B synthase
11. cbiL	Cobalt-precorrin-4 methyltransferase
12. cbiM	Precorrin-6A synthase
13. cbiN	Cobalt-precorrin-6X reductase
14. cbiP	Cobalt-precorrin-6B synthase
15. cbiQ	Cobalt-precorrin-6A synthase
16. cbiR	Cobalt-precorrin-7A synthase
17. cbiS	Cobalt-precorrin-7B synthase
18. cbiT	Cobalt-precorrin-8X reductase
19. cbiU	Cobalt-precorrin-8B C12-methyltransferase
20. cobA	Adenosylcobinamide-GDP ribazoletransferase
21. cobB	Cobinamide kinase
22. cobC	Nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase
23. cobD	Adenosylcobalamin synthase
24. cobE	Cobalamin synthase
25. cobF	Cobamide-phosphate synthase
26. cobG	Cob(I)amide adenosyltransferase
27. cobH	B12-binding protein and CobF coenzyme
28. cobI	B12-dependent ribonucleotide reductase
29. cobM	B12-dependent methionine synthase
30. cobS	Adenosylcobalamin synthase

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Submitter email	mprabhu@unigoa.ac.in
Similarity	0%
Analysis address	mprabhu.unigoa@analysis.urkund.com

Sources included in the report