STUDIES ON VITAMIN B12 PRODUCTION BY *LACTOBACILLUS* SPECIES ISOLATED FROM MILK PRODUCTS

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A thesis submitted to Faculty of Basic Sciences in partial fulfillment of the requirements for the degree of

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IN

MICROBIOLOGY

Division of Microbiology, Faculty of Basic Sciences

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CERTIFICATE-I

This is to certify that the thesis entitled **"Studies on Vitamin B12 production by *Lactobacillus* species isolated from milk products"** submitted in partial fulfillment of the requirements for the degree of **Master of Science in Basic Sciences (Microbiology)**, to the Faculty of Basic Sciences Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, is original work and has similarities with published work not more than minor similarities as per UGC norms of 2018 adopted by the university. Further the level of minor similarities has been declared after checking the manuscript with Urkund software provided by the University.

This work has been carried out by **Ms. Shruti Sharma**, under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma. It is further certified that help and assistance received during the course of thesis investigation have been duly acknowledged.

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We, the members of Advisory Committee of **Ms. Shruti Sharma**, Registration No. **J-19-MBS-42**, a candidate for the degree of **Master of Science in Basic Sciences (Microbiology),** have gone through the manuscript of the thesis entitled "**Studies on Vitamin B12 production by *Lactobacillus* species isolated from milk products"** and recommend that it may be submitted bythe student in partial fulfillment of the requirements forthe degree.

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ABSTRACT

Title of Thesis	**: Studies on Vitamin B12 production by *Lactobacillus* species isolated from milk products**
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Major Subject	**: Microbiology**
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An investigation entitled "Studies on production of Vitamin B12 by *Lactobacillus* species isolated from milk products" was conducted in the Division of Microbiology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, to study the capability of mutants generated out of UV and EMS mutagens of native *Lactobacillus* isolates, for Vitamin B12 production potential. In this study 8 isolates of *Lactobacillus* were isolated from curd and kaladi. Screening and Quantification of isolates was done on Vitamin B12 assay medium. The Vitamin B12 production by Lb-7 was 0.39 mg/g DCW. Lb-7, the fastest growing strain was selected for strain improvement, that was done by UV and EMS mutagens. Based on performance during screening, UV mutant was taken up for fermentation optimisation studies. The Vitamin B12 production by UV mutant was 0.63mg/g DCW. Three fermentation parameters i.e temperature, pH and inoculum load were optimized for mutant to enhance Vitamin B12 production. The Vitamin B12 production at 25°C was 2.05 mg/g DCW ,5.5 pH was 1.55mg/g DCW and 10^8 inoculum load was 1.53mg/gDCW. Best results were recorded at 25°C, 5.5 pH and 10^8 cfu/ml of microbial load. An increase in temperature, pH and inoculum load caused decrease in Vitamin B12 production due to death of cells.

Key words: Vitamin B12, *Lactobacillus* sp., UV and EMS Mutagenesis, Fermentation

Signature of Major Advisor

Signature of the Student

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LIST OF ABBREVIATIONS

pH	Power of Hydrogen	***et.al***	et alia=and other
°C	Degree Celsius	**Fig**.	Figure
Dw	Dry weight	**Temp**.	Temperature
Mg	Milligram	**G**	Gram
µg	Microgram	**CN-cbl**	Cyanocobalamin
OD	Optical density	**CFU/ml**	Colony Forming Unit
S.No.	Serial number	**UV**	Ultraviolet
Lb	*Lactobacillus*	**EMS**	Ethyl methane sulphonate
%	Percent	**Nm**	Nanometer
m	Meter	**Cbl**	Cobalamin
Max	Maximum	**Me-Cbl**	Methylcobalamin
Min	Minimum	**mcg**	microgram
Cm	Centimeter	**Vit. B12**	Vitamin B12
No.	Number	**µM**	Micrometer
NB	Nutrient Broth	**MRS**	De Man, Rogosa and Sharpe agar
LB	Luria Bertani Broth	**DMB**	Dimethyl benzimidazole
OH-cbl	Hydroxocobalamin	**Me-cbl**	Methylcobalamin
PBS	Phosphate Buffered Saline	**Ado-Cbl**	Deoxyadenosylcobalamin

CHAPTER -1

INTRODUCTION

Vitamin B12 is a crucial water-soluble biological compound that plays a vital role in cell metabolism, red blood cell formation, nerve function and the production of DNA. It is one of the most attractive and interesting molecules in the worlds of science and medicine therefore widely used in medical and food industries. It helps in the formation and regeneration of red blood cells thus preventing a type of anaemia mainly called megaloblastic anaemia that makes people weak and tired and is also necessary as a dietary supplement for animals and human beings (Jajodia *et al*., 2017). The human body produces millions of red blood cells every minute. These cells cannot multiply properly without vitamin B12.Therefore in consolidation with Vitamin B6 and folate (Vitamin B9) it controls high levels of homocysteine in the blood. Elevated homocysteine might increase your risk of diseases of the heart and blood vessels (cardiovascular disease). It plays an important role in cell maturation by maintaining healthy nerve cells and formation of Red Blood Cells. The human body can store Vitamin B-12 for up to 4 years. Any excess or unwanted Vitamin B12 is excreted in the urine. Thus, it is an important additive in animal foods also. It is synthesized by prokaryotes and inhibits the development of pernicious anaemia in animals and generally used to describe a cobalt corrinoid family. It is also known as cyano-cobalamin, which refers to the cobalamin family of compounds, which are composed of a corrinoid ring and an upper and lower ligand (Piwowarek *et al.*, 2018). Vitamin B12 or cyanocobalamin is a member of the corrinoids that contain a corrin ring hydroxocobalamin, methylcobalamin, and 5′-deoxyadenosylcobalamin are chemically more versatile than cyanocobalamin. It is also explored in free vitamin form ($C_{63}H_{90}N_{14}O_{14}$ PCO) (Jajodia *et al*., 2017). Its deficiency is associated with dermatia and low cognitive function and mostly obtained from the milk, cheese, yoghurt, dairy products, rice beverages, fish, meat, fortified soy products. Some breakfast cereals, nutritional yeasts and other food products are fortified with Vitamin B12. It exists in animal tissue at a very low concentration (e g. 1 ppm in the liver). It is mainly used as a food supplement and is extremely important in the treatment of pernicious anaemia. In addition, it plays an important role in the normal functioning of the nervous system and for the formation of blood. Deficiency of vitamin B12 is correlated with hematological and neurological disorders, causing peripheral neuritis, anaemia, coronary disease, stroke, hyper homocysteine anaemia and myocardial infarction (Pawlak, 2015). Strict vegetarians with low

intakes of animal-source foods and elderly populations with certain gastric dysfunctions are at higher risk of developing B12 deficiency. B12 deficiency also occurs commonly in countries, such as India, due to lacto-vegetarianism and a scarcity of meat (Green, 2009; Pawlak, 2015; Watanabe *et al.*,2013). B12-fortified foods and B12-containing dietary supplements have been considered to be good alternatives to prevent this deficiency in recent years (Watanabe *et al.,* 2013). It is extensively used as a dietary supplement, as medicine for treating hematologic and neurological disorders, and as important feed additives (growth enhancer) for fowls and domestic animals. However, most of the B12 for fortification are chemically synthesized, which is costly and may cause unacceptable side effects. In comparison, use of vitamin-producing microorganisms for in situ fortification is achievable and inexpensive alternative, and it is less likely to cause side effects from elevated concentrations of vitamins. Vitamin B12, a water-soluble vitamin, is a necessary molecule for human nutrition and its dietary reference intake (D.R.I.) of 2.4/day in adults is a general selected value (Rizzo *et al.*, 2016). Its deficiency is a general problem worldwide, leading to many clinical conditions. Plants, animals and fungi cannot synthesize this vitamin; it is produced by microorganisms (Linares *et al.,* 2017). Conceptually, the definition of vitamin B12 generally describes a type of cobalt corrinoid, belonging to the cobalamin (Cbl) group. On the other hand, vitamin B12 is the form of the vitamin acquired during the process of industrial production but this form does not exist in nature (Rucker *et al.,* 2001). Naturally, this compound is found as deoxyadenosylcobalamin (coenzyme B12), methylcobalamin or pseudo cobalamin, among other forms. Concerning the structure, the cobalamin molecule present 3 parts main: (i) the central corrinoid ring with the four ligands of a cobalt ion, (ii) a superior (or beta) ligand that is attached to adenosyl o methyl group, and (iii) the lower ligand (or alfa), usually dimethyl benzimidazole (DMB). It has been reported that in some anaerobic bacteria, the adenine and other ligands can replace DMB giving as a result pseudo cobalamin (pseudo-B12) and other active cofactors (Martens *et al.,* 2002). A diet adequate in vitamin B12 is essential to prevent severe pathologies (megaloblastic anaemia, pancytopenia, peripheral neuropathy, increased risk of myocardial infarction and stroke, others) some of which are irreversible (Derin *et al.*, 2016). Membrane proteins that are tangled in the uptake of vitamin B12 are also yet to be identified (Rempel *et al.,* 2018). In view of the instability of Me Cbl and Ado Cbl to the light, they are easily transfigured to OH Cbl at room temperature in aqueous solution (Martens *et al.,* 2002). For this reason, almost all profitable vitamin B12 products (powder, tablets, capsules or granules) having longer shelf life are produced as the air-stable cobalamin form CN Cbl via a reaction with cyanide through industrial manufacture,

which further is transformed by animal and human organisms into the coenzymes MeCbl and AdoCbl (Biedendieck *et al.,* 2010). Therefore, the term vitamin B12 is usually used to refer to CNCbl. It is considered as a crucial nutrient for humans and animals, which plays a lead coenzyme role in numerous mitochondrial and cytosolic pathways (tricarboxylic acid cycle, One-carbon metabolism including methionine and folate cycles), methylation-mediated Regulation (metabolites, DNA, RNA, and proteins), and regulation of sex steroids due to the host–microbe metabolic interactions, therefore it is an important for gut microbiota themselves (Froese *et al*., 2019). Vitamin B12 is the name used for all corrinoids revealing the qualitative biological activity of cyanocobalamin. Cobalamin is unique in the fact that its de novo synthesis appears to be confined only to some bacteria and archaea. It forms a nutritional requirement for animals and protists though they do not amalgamate, while plant and fungi putatively neither require nor synthesise it (Martens *et al*., 2002). It is linked to Compounds of the cobalt corrinoid group "cobalamins", whose structure includes: cobalt-containing cyclic tetra pyrrolidine in the core (corrinoid ring); the common lower ligand 5,6-dimethylbenzimidazole (DMB) in the α-position; and one of four upper ligands in the β-position (cyano, hydroxyl, methyl or 50-desoxyadenosyl radical) forming methyl- Cobalamin (MeCbl), 50-desoxyadenosilcobalamin (AdoCbl), hydroxocobalamin (OHCbl)and cyanocobalamin (CNCbl), respectively (Acevedo-Rocha *et al.,* 2019). The natural forms of vitamin B12, MeCbl and AdoCbl, are synthesized only by prokaryotes (via aerobic/anaerobic and/or salvage pathways), which are required as a principal cofactor for two enzymes: cytosolic methion-ine synthase (formation of methionine) and mitochondrial methylmalonyl-CoA mutase (formation of succinyl-CoA) in the human and animal metabolism (Froese *et al.,* 2019). In bacteria, the list of B12-dependent enzymes is commendated by glycerol dehydratase and ethanolamine ammonia lyase for anaerobic fermentation of glycerol, propanediols and ethanolamine; aminomutases for conversion of amino acids; ribonucleoside diphosphate reductases for DNA synthesis, and still growing (Danchin *et al*., 2017; Shelton *et al.*,2019). Membrane proteins that are tangled in the uptake of vitamin B12 are also yet to be identified (Rempel *et al.,* 2018).

Chemical synthesis of Vitamin B12 requires more than 70 steps. The industrial production by chemical method and subsequent purification steps made its industrial production too difficult, expensive, unsafe to the operators and the process is not environment friendly .So its biosynthesis is confined to few bacteria and archaea , and such its production relies on microbial fermentation, therefore, it has been produced on an industrial scale using

the batch or fed batch process by microbial fermentation(Fang *et al.,* 2017).Various microorganisms including those of the genera *Propionibacterium shermanii* , *Pseudomonas denitrificans*, *Streptomyces griseus* , *Lactobacillus*, *Proteus*, *Rhizobium* , *Saccharomyces cerevisiae* , *Propionibacterium freudenereichii* , *Streptococcus* , *Klebsiella* and *Methanobacterium* are producers of Vitamin B12 on an industrial scale. Among the above microorganisms in general *Propionibacteriumsp*. are preferred for vitamin B12 as these species produces neither endotoxins nor exotoxins and produce highest yield of 20 mg /lit vitamin B 12(Fang *etal.,* 2017). Large scale industrial production of Vitamin B12 in via microbial fermentation, predominantly utilising *Pseudomonas denitrificans, Propionibacterium shermanii or Sinorhizobium meliloti,* however these strains have several shortcomings such as long fermentation cycles, complex and high cost media requirements and a lack of suitable genetic systems for strain engineering. To date, most of the research on these producers has focused on traditional strategies, such as random mutagenesis and fermentation process optimization. (Fang *et al.,* 2017). Propionibacterium revealed that these bacteria are capable of biosynthesising valuable metabolites such as bacteriocins, propionic acid, Vitamin B12 and trehalose. This suggests that these are important group of microorganisms that are industrially important in the future (Piwowarek *et al.,* 2018). B12 is synthesized by certain bacteria and archaeon, but not by plants or animals (Watanbe *et al.,* 2003). *Propionibacterium freudenreichii* is a generally recognized as safe (GRAS) bacterium having the ability to make active vitamin B12 in different plant-based matrices (Chamlagain *et al.,* 2018; Signorini *et al.,* 2018; Wolkers-Rooijackers *et al.,* 2018). Meanwhile, the experimentally established and bioinformatically predicted vitamin B12 requirements for growth (auxotrophy) have been shown for human gut bacteria, namely: *Ruminococcus bromi, Clostridium spiroforme, Shigella sonnei, Shigella flexneri ,Serratia marcescens, Serratia fonticola , Escherichia fergusonii, Shigella dysenteriae Lactobacillus delbrueckii, Escherichia coli, Lactobacillus sakei, Bacteroides thetaiotaomicron, Bacteroides ovatus, Bacteroides caccae* (Rodionov *et al.,* 2019). By metabolic reconstruction, the molecular genetics and capability of B12 biosynthesis were anticipated in 60–80% and no more than 40% (Fusobacteria, Actinobacteria, Proteobacteria, Bacteroidetes, Firmicutes) of the human gut microbial genomes, respectively (Radionov *et al.,* 2019, Magnúsdóttir *et al.,* 2015). It also has been reported that certain strains belonging to Lactic acid bacteria group are also capable of synthesising waters soluble vitamin such as those included in the B group mainly as Vitamin B 12. Since Lactic acid bacteria (LAB) are Gram-positive rods or cocci. They have low (G+C) content in their chromosomes. Classifically, LAB comprises a relatively

large subgenus of the Bacilli, one of the three classes within the Firmicutes phylum. They all have the common feature of producing lactic acid as the major end product of carbohydrate metabolism. More precisely, the genera that make up LAB belong to the order of the Lactobacillales, and include *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus,* and *Weisella*. LAB occupy several ecological positions and some of the species of LAB are natives of the gastro-intestinal tract (GI-tract) of humans and other animals but by large they are found in decomposing plant material (Vaughan *et al.,* 2002). Historically, humans have subjugated LAB for thousands of years by integrating them in our food fermentation process (Bolotin *et al.,* 2004). This long history of safe use contributed greatly to their major role in the food industry, being responsible for the manufacturing of several fermented foods and beverages. Their industrial, economic and nutritional value is clearly indicated by the wide variety of applications. For example: LAB are the main components of dairy starter cultures by transforming milk into products like cheese or yoghurt and expanding its life span by lowering the pH due to lactic acid production. Lactic Acid Bacteria are Gram (+), non-sporulating, acid-tolerant, catalase negative and facultative anaerobic organisms. Except for a few species, LAB members are considered as non- pathogenic organisms with a renowned generally recognized as safe status. Many species of *Lactobacillus* are used for the manufacture and conservation of fermented feed and foods from raw agricultural materials in which they are either present as pollutants or purposely added as starter cultures. Some Due to their safety and beneficial effects on health, many strains of LAB can be considered as probiotics which can be considered as 'live microorganisms' that when taken in adequate amounts, confer a health benefit to the host' (WHO/FAO 2002). In counting to their intrinsic properties, certain strains of LAB have the capability of releasing and/or increasing specific beneficial compounds in foods. Despite these benefits, it has been reported that certain strains of Lactic Acid Bacteria have the ability to synthesize B group vitamins (folates, thiamine, riboflavin and cobalamin) (LeBlanc *et al.,* 2011). Glycerol has been regarded as a best and sufficiently available carbon source to synthesize many essential products, including vitamins. Many members of *Lactobacillus* sp. reduce glycerol and possess the mechanism for the synthesis of vitamin B12 in a single chromosomal gene cluster. *Lactobacillus* species hide the genes responsible for glycerol utilization and Vitamin B12 production (Santos *et al.,* 2011). It is a Complex compound that is naturally produced by specific Lactic acid Bacteria. Thus, the intake of foods, particularly dairy foods, with Vitamin B12 appears to be one of the best foods for providing a good source of vitamin B12. Hypercholesterolemia is one of the

major causes of cardiovascular disease. Supple-mentation with probiotic bacteria subsidizing improved lipid metabolism is one of the best measures to fight with hypercholesterolemia (Ly *et al.,* 2019]. Cholesterol metabolism is controlled by the activity of *Lactobacillus* species on microflora and the general action of human gut microbiota (Khare *et al.,* 2020).

Thus, the use of vitamin-producer bacteria appears as a suitable alternative to produce innovative foods with added nutritional value (Hugenholtz *et al.,* 2002). The production of Vitamin B12 is strain specific and is confined to certain *Lactobacillus* genus. Hence in the light of above facts the present study was designed to investigate the production of vitamin B12 by *Lactobacillus* sp. with the following objectives:

1. Isolation of *Lactobacillus* sp. producing vitamin B12 from milk and fermented products.
2. Strain improvement by random mutagenesis
3. Shake flasks standardization of Vitamin B12 production by potent mutants

CHAPTER- 2

REVIEW OF LITERATURE

2.1 Structure and Functions of Vitamin B12

Sobczyńska-Malefora *et al.* (2021) revealed that Vitamin B12 is the largest and most structurally complex non-polymeric biomolecules described in nature, which has a molecular weight of 1355 Daltons for its regularly used cyano- form. The generic term cobalamin refers to a set of structures named corrinoids, consisting of one central cobalt (Co) atom coordinated with 4 equatorial nitrogen atoms joined with pyrrole residues. The 5^{th} coordination site (α) in B12, below the planar structure, is inhabited by a 5′,6′-dimethyl benzimidazole residue connected to a α-ribosyl-3-phosphate. The 6^{th} coordination site (β), above the corrin ring, is tenanted by a methyl- (methylcobalamin [Me-Cbl]), 5′-deoxyadenosyl (5′-deoxyadenosylcobalamin [Ado-Cbl]), aquo- (hydroxocobalamin [OH-Cbl]), or a cyano-group (cyanocobalamin [CN-Cbl]). The metabolic utility of B12 in humans is conferred by an upper ligand consisting of either a methyl or adenosyl moiety. Cyanocobalamin undergoes enzymatic conversions during intracellular processing in human cells and it is eventually converted to the two cofactors, Me-Cbl and Ado-Cbl (Gherasim *et al.*, 2013). Me-Cbl and Ado-Cbl consequently undergo catalytic oxidations and reductions and sometimes generate OH-Cbl, which returns to the catalysis via reactivation cycle (Kim *et al*., 2009). Vitamin B12 also called cobalamin consists of a number of forms including hydroxy, methyl, cyano, deoxy adenosyl cobalamin. The cyano form which is used in supplements is found in defined amounts as food (J.M. ,1997).

Bridwell- Rabb and Drennan, (2017) explained the ability of cobalamin to coordinate different upper axial ligands give rise to a heterogeneity of reactivity. Generally, adenosylcobalamin is related with radical-based rearrangements, and methylcobalamin with methyl cation transfers. Recently, however, a new role for adenosyl cobalamin has been explored as a light sensor, and a methylcobalamin-dependent enzyme has been acknowledged, suggested to transfer a methyl anion. Moreover, recent studies have provided a treasure of new information about a third class of cobalamin-dependent enzymes that do not mainly use an upper ligand

Allen *et al.* (2008) explained that Vitamin B12 is a water-soluble vitamin found in considerable quantities only in animal foods. If the consumption of animal foods is very low

or absent, its sparse presence in plant foods makes its introduction essential, either through fortified foods or supplements. Its deficiency is common among vegetarians because of the result of a very low intake.

Froese *et al.* (2019) indicated that Vitamin B12 (cobalamin, Cbl) is a nutrient crucial for human health. Due to its complex structure and dual cofactor forms, Cbl undergoes a convoluted series of absorptive and processing steps before serving as cofactor for the enzymes methylmalonyl CoA mutase and methionine synthase. Methylmalonyl CoA mutase is mandatory for the catabolism of certain (branchedchain) amino acids into an anaplerotic substrate in the mitochondrion, and dysfunction of the enzyme itself or in production of its cofactor adenosyl Cbl result in an inability to successfully undergo protein catabolism with associated mitochondrial energy disruption. Methionine synthase catalyzes the methyl Cbl dependent (re)methylation of homocysteine to methionine within the methionine cycle; a reaction required to produce this essential amino acid and generate S adenosylmethionine, the most essential cellular methyl donor. Splitting of methionine synthase has wide-ranging involvement for all methylation dependent reactions, including epigenetic modification, but also for the intracellular folate pathway, since methionine synthase uses 5 methyltetrahydrofolate as a one carbon donor. Folate bound one-carbon units are also compulsory for deoxythymidine monophosphate and de novo purine synthesis; therefore, the flow of single carbon units to each of these pathways must be regulated based on cellular needs. This review provides an overview on Cbl metabolism with a brief description of absorption and intracellular metabolic pathways. It also provides an explanation of folate mediated one carbon metabolism and its intersection with Cbl at the methionine cycle.

Rizzo *et al.* (2020) revealed that Cobalamin (Cbl), also known as vitamin B12, is a crucial water-soluble molecule. As far as it is known at present, its function is restricted to cofactorial activity for only two enzymes. However, they are important for anabolic and catabolic processes of the body with more stress for strong cell replication events like haematopoiesis and tissue expansions. Moreover, Cbl reveals a central function in neuronal health such as the maintenance of the myelin sheath. Even if a balanced diet is able to provide a sufficient amount of Cbl, some diseases can still lead to a vitamin deficiency status.

Smith *et al.* (2018) explained that biosynthesis of B12, involving up to 30 different enzyme-mediated steps, only occurs in Bacteria. Thus, most eukaryotes need an external source of B12, and yet the vitamin develops to have only two functions in eukaryotes: as a cofactor for the enzymes and methyl malonyl Coa mutase and methionine synthase. These two

functions are necessary for normal health in humans, and in particular, the formation of methionine is important for providing methyl groups for over one hundred methylation processes. Interference with the methionine synthase reaction not only depletes the body of methyl groups but also leads to the assemblage of homocysteine, a risk factor for many diseases. The syndrome pernicious anaemia, characterised by lack of intrinsic factor, leads to a severe, sometimes fatal form of B12 deficiency. However, there is no intense cut-off for B12 deficiency; rather, there is a ceaseless inverse relationship between serum B12 and a variety of undesirable outcomes, including Neural tube defects, dementia and stroke. The brain is particularly endangered; in children, inadequate B12 stunts brain and intellectual development. Substandard B12 status (serum B12 < 300 pmol/L) is very common, occurring in 30-60% of the population, more specific in pregnant women and in less-developed countries. Thus, many tens of millions of people in the world may suffer harm from having a poor B12 status. Public health steps are importantly needed to correct this deficiency.

2.2 Bacteria capable of producing Vitamin B12.

Several literatures are available on the isolation of microorganisms efficient in vitamin B12 production. Jajodia *et al.* (2017), reported that the different strains of Lactic Acid Bacteria were isolated from curd samples and additionally screening and qualitative assay of isolates were done by the ability of the isolates to grow in Vitamin B12 assay medium. The best Vitamin B12 microorganisms were chosen and the recognition of the microorganisms were carried out. The best potent Vitamin B12 producer was sequenced and characterized.

Piwowarek *et al.* (2018) reported that Bacteria from the *Propionibacterium* genus are capable of integrating numerous valuable compounds and also capable of producing vitamin B12. Trehalose and bacteriocins from the vitamin B group. These bacteria are also efficient in synthesising organic acids such as Propionic and acetic acids. Till date numerous studies have been conducted regarding the use of the bacteria from *Propionibacterium* genus which disclosed that these are capable of biosynthesized vitamin B12. Propionic acid etc. They laid stress on that cobalamin (Vitamin B12) synthesised via both the two mechanisms anaerobic and aerobic conditions to effectively produce vitamin B12.

Piao *et al.* (2004) reported that the chemical synthesis of vitamin B12 requires more than 70 steps, the production of vitamin B12 has been achieved by microorganism fermentation with additional brief chemical adjustments. In a try to increase the productivity

of vitamin B12, we attempted to express 10 genes belonging to the hem, cob and cbi gene families that intricate the synthesis of vitamin B12 in *Propionibacterium freudenreichii*, which is a studied producer of vitamin B12. In a recombinant *P. freudenreichii* clone that concealed the expression vector containing a cob A, cbi LF, or cbi EGH, we obtained an increase in vitamin B12 production of 1.7, 1.9-, and 1.5-fold higher, respectively, than that in the microorganism without any cloned genes in the expression vector pPK705. The cob U and cob S genes caused a minute increase in the production of vitamin B12. Moreover, we achieved multigene expression in *P. freudenreichii*. In a recombinant *P. freudenreichii* clone that sheltered an exogenous gene, hem A, from *Rhodobacter sphaeroides* and endogenous hem B and cob A genes, we profitably achieved the production of about 1.7 mg/l vitamin B12, 2.2fold more than the *P. freudenreichii* suppressing pPK705.

Selvakumar *et al.* (2012) said that the natural environment is the best source for isolates of a typical variety of antagonistic *Streptomyces* sp. and to produce Vitamin B12 and also reported that by batch fermentation process the isolated *Streptomyces sp.* can produce vitamin B12 at specific optimal conditions with a specific fermentation medium. The vitamin B12 produced by *Streptomyces sp.* ranges from (1.9ug to 45.3ug per ml) while the *Streptomyces rochei* isolate produced maximum amount of Vitamin B12 i.e. 45.3 ug per ml).

Taranto *et al.* (2003) found that Lactic Acid Bacteria isolated from sourdough are capable to produce cobalamin the sugar glycerol cofermentation in vitamin b12. Fundamental genetic studies of cobalamin biosynthesis genes from *L. reuteri* allowed the recognition of cob genes which code the Cbi A, Cbi J, Cbi K enzymes involved in the cobalamin pathway. The cobalamin produced by *L. reuteri* isolated its cyanide form by HPLC analysis to produce Vitamin b12. Among many microorganisms *Propionibacterium sp.* are preferred mostly for the production of vitamin B12 particularly *Propionibacterium freudenereichii* is mostly chosen as this species neither produce endotoxins nor exotoxins and produce higher yield of vitamin b12. But *Propionibacterium sp.* produce Vitamin B12 intracellularly and excrete mainly propionic acid and acetic acid extracellularly. This is the major problem in vitamin B12 production using Propionibacterium as the cell growth initials due to get together of inhibitory metabolites such as propionic acid and acetic acid produced extracellularly. To overcome the situation physical mutagenesis (UV radiation) develops a desirable resistant character in *P. freudenreichii* organised in the leading content product of vitamin b12. (Ali *et al.*,2011). *Propionibacterium freudenreichii* is a dairy-associated bacterium, primarily used in

the production of Swiss type cheeses and also for industrial-scale production of vitamin B12 (Martens *et al.*, 2002).

Kaneko *et al.* (1994) reported the discovery of its probiotic and bifidogenic properties lead to a broadening number of studies focusing on the species. As a result, the first genome sequence of the *P. freudenreichii* CIRM-BIA1^T was published (Falentin *et al.*, 2010) and then followed by further studies providing deeper understanding into the modification mechanisms and long-term survival of this species (Thierry et al., 2011; Aburjaile *et al.*, 2016). Vitamin B12 contributes to many substantial metabolic cycles in the living organisms. Since human beings cannot produce such co-factor by their metabolism, they have to get this vitamin from foods and supplements.

2.3 Vitamin B12 production by *Lactobacillus* species.

De Angelis *et al.* (2014) investigated that *Lactobacillus rossiae* is an obligately hetero-fermentative lactic acid bacterium, which can be isolated from a broad range of environments including sourdoughs, vegetables, fermented meat and flour, as well as the gastrointestinal tract of both humans and animals. In order to resolve distinctive genomic features of this particular species and explored the ancestral positioning within the genus *Lactobacillus*, comparative genomics and genetic approaches, followed by functional analyses were performed on *L. Rossiae* DSM 15814^T, showing how this type strain not only occupies an independent hereditary branch, but also carry genomic features emphasising its biotechnological potential. This strain in fact represents one of a small number of bacteria known to encode a complete de novo biosynthetic pathway of vitamin B12 in addition to other B vitamins such as folate and riboflavin. In addition, it possesses the capacity to utilize a substantial set of carbon sources, a characteristic that may contribute to environmental adjustment perhaps enabling the strain's ability to populate different niches.

Burgess *et al. (*2009) reported that Lactic acid bacterial strains isolated from fermented foods have been found to be cobalamin producers which offers the advantage of exploiting their 'generally regarded as safe' (GRAS) status (Santos *et al.*, 2007). A restriction to this however is that the vitamin is not Secreted and therefore to increase bioavailability autolytic mutants Would be needed (Martens *et al.*, 2002; Taranto *et al.*,2003). *Lb. reuteri*, unlike other bacteria, has been shown to encode all the genes crucial for the synthesis of vitamin B12 in one continuous stretch of its chromosome. This provides a major advantage for consideration of Metabolic engineering strategies for transferring the B12 production

capability to other bacteria (Dos Santos, 2008). Major studies on this organism have indicated that modification of the growth Medium such as supplementation with glycerol or omission of single Amino acids such as cysteine can significantly enhance production of the vitamin providing further avenues for investigation for industrial Production (Dos Santos, 2008).

Bhushan *et al.* (2017) demonstrated suitable selection of *Lactobacillus* strain (probiotic/starter/functional) on the basis of its techno-functional characteristics is needed before developing a novel fermented functional food. We contrasted vitamin B12 (B12, cobalamin) producing *Lactobacillus plantarum* isolates, BHM10 and BCF20, for functional (vitamin overproduction, genomic insight to B12 structural genes, and probiotic attributes) and technological [milks (skim and soy) fermentation and B12 bio-fortification] characteristics. Incorporation of B12 precursors (5-aminolevulinate and dimethyl benzimidazole) to cobalamin-free fermentation medium increased vitamin production in BHM10, BCF20, and DSM20016 (a positive standard) by 3.4-, 4.4-, and 3.86-folds, respectively.

Hati *et al.* (2019) reported that Lactic acid bacteria produce necessary vitamins like folate, riboflavin, SCFA and cobalamin which have health impacts (anti-obesity, anti-diabetics, anti-microbial, and other chronic diseases prevention) to the host. These vitamins are crucial for cellular and metabolic growth of the living system. In the study, five potent *Lactobacillus* isolates viz., KGL2 (*Lactobacillus fermentum*), KGL3A (*Lactobacillus plantarum*), KGL4 (*Lactobacillus fermentum*), RNS4 (*Lactobacillus rhamnosus*), and WTS4 (*Lactobacillus fermentum*) were considered for vitamins (B2, B12, and B9) and SCFA productions (lactate, butyrate, and acetate). However, KGL3A had shown highest B2 production (0.7 μg/ml) while KGL2 exhibited maximum B12 production (0.05 μg/ml) after 36 h. Moreover, WTS4 allotted highest folate production (0.09 μg/ml) after 24 h. In addition, RNS4 reported the maximum short-chain fatty acid production (0.77 g/l acetic acid, 0.26 g/l lactic acid, and 0.008 g/l butyric acid respectively). Dominant *Lactobacillus* isolates from traditional fermented foods of Garo Hills, Meghalaya, India (North East Part of India) showed maximum production of B2, B9, and B12 as well as short-chain fatty acids and could be used for their implementation as health beneficial functional fermented dairy products.

Taranto *et al.* (2003) found that *Lactobacillus reuteri* CRL1098, a lactic acid bacterium isolated from sourdough, is able to manufacture cobalamin. The sugar-glycerol cofermentation in vitamin B12-free medium showed that this strain was able to lower glycerol through a well-known cobalamin-dependent reaction with the formation of 1,3-

propanediol as a final product. The cell extract of *L. reuteri* rectified the coenzyme B12 requirement of *Lactobacillus delbrueckii subsp*. Lactis ATCC 7830 and allowed the growth of *Salmonella enterica serovar Typhimurium* (metE cbiB) and *Escherichia coli* (metE) in the smallest medium. Prefatory genetic studies of cobalamin biosynthesis genes from *L. reuteri* allowed the identification of cob genes which encode the CobA, CbiJ, and CbiK enzymes involved in the cobalamin pathway. The cobamide manufactured by *L. reuteri*, isolated in its cyanide form by using reverse-phase high-pressure liquid chromatography, exhibited a UV-visible spectrum identical to that of standard cyanocobalamin (vitamin B12).

Li *et al.* (2017) investigated extracellular vitamin B12-producing *Lactobacillus* strains and their characteristics in tolerance to environmental stresses, gastric acid and bile salts. Two isolates, *Lactobacillus plantarum* LZ95 and CY2, exhibit high extracellular B12 production, 98 ± 15 μg/L and 60 ± 9 μg/L respectively. Extracellular B12 from LZ95 were identified as methylcobalamin and adenosylcobalamin using a combination of reverse-phase HPLC and solid phase extraction while that from CY2 was adenosylcobalamin. Both strains grew under environmental stresses, and LZ95 exhibited better tolerance to low temperature and high ethanol concentration. LZ95 also showed good viability when exposed to gastric acid (pH 2.0 and 3.0) and bile salts (0.3%) as well as good adhesion to Caco-2 cells. The viability of CY2 was significantly reduced under low pH and exposure to bile salt. Together, extracellular B12 producer LZ95 with good probiotic properties might be a candidate for in situ B12 reinforcement in the food industry.

2.4 Screening of lactic Acid Bacteria.

Masuda *et al*. (2012) investigated the extracellular production of vitamin B12, folate and thiamine in cultures of Lactic acid bacteria (LAB) isolated from nukazuke, a traditional Japanese pickle, and the relationships between the vitamin production and such properties of LAB as tolerance to salts, ethanol, etc. Among the 180 isolates of LAB, two strains of *Lactobacillus* (Lb.) sakei and a strain of *Lb*. *Plantarum* extracellularly produced high levels of folate (about 100 μg/L). A strain of *Lb. Coryniformis* and one of *Lb*. *Plantarum* produced about 2 μg/L of vitamin B12, though the level was not high. No isolates produced a high level of thiamine. The type cultures of LBA (53 strains) did not show any higher production of these vitamins. Some isolates showed resilience to high concentrations of salts and alcohol, and low initial pH. No significant relationships between folate or vitamin B12 productions and these properties of LAB were apparent.

Hugenschmidt *et al.* (2010) described that Lactic acid bacteria (LAB) and Propionic acid bacteria (PAB) are known for the production of several Important nutraceuticals. They screened and 100 PAB and 151 LAB of different origins (fermented foods and Feeds) for and intracellular vitamin B12 and extracellular folate production in supplemented whey permeate using a standardized microbiological assay (folate) and HPLC (vitamin B12). Five LAB strains belonging to the species *Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus brevis* and *Lactobacillus fermentum* revealed high extracellular folate productions, with a maximum yield of 397±0 ng ml-1 for *L. plantarum* SM39. The highest vitamin B12 production was measured for *Propionibacterium freudenreichii* DF15 with 2.5 mg mL. Screening a large biodiversity of PAB and LAB led to an illustrative image of the distribution of folate and vitamin B12 production by these genera and enabled the identification of high.

Mohammed *et al.* (2014_a) demonstrated that there has been considerable debate on the ability of *Lactobacillus reuteri* to produce the active form of vitamin B12. In this study, they indicated the ability of two wild type strains of *Lactobacillus reuteri* to produce α-(5, 6-dimethylbenzimidazole)-cobamidcyanide or cyanocobalamin which are the active form of vitamin B12. This was attainable by providing the required compounds of vitamin B12, δ-aminolevulinic acid (ALA) and 5, 6-dimethylbenzimidazole (DMB) under the worthy conditions for bacteria to induce and complete the formation of vitamin B12 with DMB as the lower (Coα) ligand. It was amazing to find a microorganism possessing probiotic properties along with the capability to produce an active vitamin B12. High performance liquid chromatography (HPLC) coupled with mass spectrometry were used to identify vitamin B12 forms and confirm our results.

2.4. Strain improvement by mutagenesis

Abdelsalam, (2018) reported the production of vitamin B12 and folate under solid state fermentation was explored by using different mutants of *Klebsiella pneumonia.* The results showed that the isolate no (EM33TE) produced the maximum B12 and folate yields (77.45 and 88.22 ug/ml) respectively. The addition of the fermentation medium by different carbon sources (fructose, glucose, sucrose, mannose, maltose, lactose, starch and dextrin) indicated that the best carbon source was glucose and the maximum B12 yield (356.9ug/ml) was obtained by the successive supplementation of glucose (10g) to the fermentation medium. PCR studies revealed clear differences at bands number and size between the original strain and its mutants using RAPD analysis by three different random primers, these

differences in RAPD profiles proved the evidence of genetic variations of mutants and*K.pneumonia* genome after EMS-mutagenesis.

Ali *et al*. (2011) explored that vitamin B12 production was carried out with parental and mutant strains of *Propionibacterium freudenreichii.* Physical mutagenesis was carried out by three different doses of U.V. Irradiation- 200, 300 and 400erg/mm2 and five different irradiation time periods were selected- 30sec, 60sec, 90sec, 120 sec and 150sec. Acquired 15 mutant strains were used to produce vitaminB12 by Aerobic and Anaerobic fermentation techniques at 30°C, 32°C and 34°C. 200 erg/mm2. Short time exposure and U.V. dose haven't developed any desirable character in organisms but mutants which were naked to 400 erg/mm2 for 60 seconds developed desirable (resistant to propionic acid) character. High yield of vitmainB12 was produced at 30°C by *Propionibacterium freudenreichii.*

2.5 Fermentation and Optimization of Vitamin B12.

Hedayati *et al.* (2020) reported that Dimethylbenzimidazole (DMBI) renowned the active form of vitamin B12 from pseudo-vitamin B12. De Novo total biosynthesis of vitamin B12 in the bacteria should include DMBI biosynthesis through riboflavin pathway. *Propionibacterium freudenreichii* can produce vitamin B12 through anaerobic biosynthesis pathway. As vitamin B12 production by *P. freudenreichii* is the growth-associated phenomena, the effect of different carbon sources (rice bran oil, argon oil), nutrients (DMBI) and amino acids (L-Serin, L-Tryptophan, L-cysteine, L-Methionine) on the growth of *Propionibacterium freudenreichii* PTCC1674 (pfre) were explored.

Chamlagain *et al*. (2018) explored the effect of co-fermentation on vitamin B12 content and microbiological composition of wheat bran. *Propionibacterium freudenreichii* DSM 20271 was used as the producer of vitamin while *Lactobacillus brevis* ATCC 14869 was chosen to assure the microbial safety of the bran dough. Fermentation trials were managed in bioreactors to monitor and adjust the pH of the ferments. Vitamin B12 level reached 357 ± 8 ng/g dry weight (dw) after one day of pH-controlled fermentation with *P. freudenreichii* monoculture and remained stable thereafter. In co-fermentation with *L. brevis*, slightly less vitamin B12 (255 ± 31 ng/g dw) was produced in one day and an effective obstruction of the growth of total Enterobacteriaceae and *Bacillus cereus* was obtained. On day 3, vitamin B12 content in pH-controlled co-fermentation increased to 332 ± 44 ng/g dw. On the other hand, without a pH control, co-fermentation resulted in a stronger inhibition of

Enterobacteriaceae and *B. cereus* but a lower level of vitamin B12 (183 ± 5 ng/g dw on day 3).

Xu et *al*. (2018) investigated that the vitamin B12-dependent riboswitch is a critical factor that regulates gene transcription to mediate the growth of and vitamin B12 synthesis by *Propionibacterium freudenreichii*. They explained the effect of various wavelengths of light on the growth rate and vitamin B12 synthesis was studied. Red, green, and blue light-emitting diodes (LEDs) were selected, and a dark condition was used as the control. The microorganism growth rate was measured using a spectrophotometer and plate counting, while the vitamin B12 content was discovered using an HPLC-based method. The optical density at 600 nm (OD600) values indicated that *P. freudenreichii* grew better under the continuous and discontinuous blue light conditions. Moreover, under the blue light condition, *P. freudenreichii* contributed to have a higher growth rate (0.332 $h^{-}1$) and vitamin B12 synthesis (ca. 10 μg/mL) in tofu wastewater than in dark conditions. HPLC analysis also showed that more methylcobalamin was produced under the blue light conditions than in the other conditions. The cbiB gene transcription results showed that blue light promoted the synthesis of this vitamin B12 synthesis enzyme. Furthermore, the results of inhibiting the expression of green fluorescent protein indicated that blue light removed the inhibition by the vitamin B12-dependent riboswitch. This method can be used to reduce fermentation time and produce more vitamin B12 in tofu wastewater.

Xia *et al.* (2015) described the aerobic *Pseudomonas denitrificans* as widely used for industrial and commercial vitamin B12 fermentation, due to its higher productivity compared to the anaerobic vitamin B12-producing microorganisms. This paper aimed to develop a cost-effective fermentation medium for industrial vitamin B12 production by *P. denitrificans* in a 120,000-l fermenter. It was found that maltose syrup (a low-cost syrup from corn starch by means of enzymatic or acid hydrolysis) and corn steep liquor (CSL, a by-product of starch industry) were greatly applicable to vitamin B12 production by *P. denitrificans*. Under the optimal fermentation medium performed by response surface methodology, 198.27 ± 4.60 mg/l of vitamin B12 yield was obtained in 120,000-l fermenter, which was close to the fermentation with the refined sucrose (198.80 mg/l) and was obviously higher than that obtained under beet molasses utilization (181.75 mg/l). Therefore, maltose syrups and CSL were the well organised and cost-effective substrates for industrial vitamin B12 fermentation by *P. denitrificans*.

Deptulla *et al. (*2015) *P. freudenreichii* is a food grade bacterium that has attention as a producer of considerable amounts of cobalamin, a cobamide with activity of vitamin B12. Production of active form of vitamin is essential for attempts to naturally fortify foods with B12 by microbial fermentation. Active vitamin B12 is distinguished from the pseudovitamin by the presence of 5,6-dimethylbenzimidazole (DMBI) as the lower ligand. Genomic data indicate that *P. freudenreichii* possesses a fusion gene, bluB/cobT2, coding for a predicted phosphoribosyltransferase /nitroreductase, which is presumably involved in production of vitamin B12. Understanding the mechanisms influencing the synthesis of different vitamin forms is useful for rational strain selection and essential for engineering of strains with improved B12 production properties. Here, we investigated the activity of heterologously expressed and purified fusion enzymes BluB/CobT2. Our results show that BluB/CoBT2 is responsible for the biosynthesis of the DMBI base and its activation into α-ribazole phosphate, preparing it for attachment as the lower ligand of cobalamin. The fusion enzyme was found to be well organised in metabolite channeling and the enzymes' inability to react with adenine, a lower ligand present in the pseudovitamin, disclosed a mechanism favoring the production of the active form of the vitamin. In vivo experiments also revealed a clear preference for incorporating DMBI over adenine into cobamide under both microaerobic and anaerobic conditions. The described BluB/CobT2 is responsible for the production and activation of DMBI. Fusing those two activities results in high pressure towards production of the true vitamin B12 by effectively activating DMBI formed within the same enzymatic complex. This showed that BluB/CobT2 is the crucial enzyme in the B12 biosynthetic pathway of *P. freudenreichii*. The GRAS organism status and the preference for synthesizing active vitamin form make *P. freudenreichii* a distinctive candidate for the in-situ production of vitamin B12 within food products.

Jalillian *et al.* (2019) explained that *Chlorella Vulgaris* is enriched in vitamin B12 and cobalt ion which is placed in the center of the vitamin molecule. This study focused to investigate how different concentrations of cobalt chloride salt affected the vitamin B12 production by utilizing CO_2 gas, to assess *C. Vulgaris* biomass. Therefore, Bold's basal medium used as the medium and 0.5, 1.5, 2 and 2.5 μM cobalt chloride salt was added to *C. Vulgaris* culture. Under four cobalt chloride salt treatments, the best growth rate was acquired at the 2 μM of cobalt chloride salt (0.186 + 0.07 g /Ld). CO2 gas was supplied by a 5% CO2 gas cylinder and fermented milk as a novel biological CO2 gas generator (CO2,10%). Use of fermented milk is a practical approach for elimination of waste gas emission and converting

CO2 into biomass. The results revealed that, in the presence of 5% CO2 gas, C. Vulgaris vitamin B12 content at 2 and 2.5 μM cobalt chloride, were 166.23 ± 1.78 and 173.32 ± 4.23 μg /100 g of dry biomass (7 and 12% higher than control), respectively. However, under controlled conditions (ambient air and 2 μM cobalt chloride) vitamin B12 content was 154.9 ± 1.14 μg / 100 g of dry biomass.

Mohammed *et al. (*2014$_b$) reported that Vitamin B12 is an interesting molecule which acts as a cofactor in the metabolism of many organisms, especially affecting DNA synthesis and regulation, fatty acid synthesis and energy production. The synthesis of vitamin B12 is limited to a few archaea and bacteria. Therefore, industrial microbial fermentation is used to meet annual demands worldwide of vitamin B12 and worked as a substituted method to the chemical synthesis which requires at least 60 steps that is extravagant. *Bacillus megaterium* is one of vitamin B12 producers and perfect host for many biotechnology applications and being one of the best tools for the industrial production of several enzymes. Therefore, a two-step optimization strategy was established to produce high yield of vitamin B12 by *B. Megaterium* through the supplying of the production requirements and the suitable conditions for the biosynthesis of vitamin B12. They achieved the optimum conditions for the fermentation process of *B. Megaterium* to produce high yield of vitamin B12 in a practical way based on statistical design and analysis which allowed vitamin B12 production to increase up to 759-fold (204.46 μg/l) as compared with control without parameters (0.26 μg/L). High performance liquid chromatography combined with variable wavelength detector and mass spectrometry has been used to identify vitamin B12 forms and confirm the results. We developed the fermentation process of B. Megaterium to increase the production of vitamin B12 by providing the required supplements for the synthesis of vitamin B12 (CoCl2, δ-aminolevulinic acid (ALA) and 5,6-dimethylbenzimidazole (DMB)) and dividing the fermentation process into three stages. In addition, the optimum incubation times of the three fermentation stages were investigated and performed with a shortening number of experimental and evaluated multiple parameters and their interactions by using statistical experimental design and analysis. All of these plans has proven successful in increasing the production of vitamin B12 up to 204.46 μg/l and revealed that *B. Megaterium* could be a good candidate for the industrial production of vitamin B12.

Biedendieck *et al.* (2010) revealed that Cobalamin (vitamin B12) production in *Bacillus megaterium* has served as a model system for the systematic estimation of single and multiple directed molecular and genetic optimization strategies. Plasmid and genome-based

overexpression of genes involved in vitamin B12 biosynthesis, including cbiX, sirA, modified hemA, the operons hemAXCDBL and cbiXJCDETLFGAcysGAcbiYbtuR, and the regulatory gene fnr, significantly increased cobalamin production. To reduce alteration along the heme branch of the tetrapyrrole pathway, an antisense RNA strategy involving silencing of the hemZ gene encoding coproporphyrinogen III oxidase was successfully employed. Feedback inhibition of the initial enzyme of the tetrapyrrole biosynthesis, HemA, by heme was overcome by stabilized enzyme overproduction. Similarly, the removal of the B12 riboswitch upstream of the cbiXJCDETLFGAcysGAcbiYbtuR operon and the recombinant production of three different vitamin B12 binding proteins (glutamate mutase GlmS, ribonucleotide triphosphate reductase RtpR and methionine synthase MetH) partly abolished B12 dependent feedback inhibition. All these arrangements increased cobalamin production in *B. Megaterium*. Finally, combinations of these plans enhanced the overall intracellular vitamin B12 concentrations but also reduced the volumetric cellular amounts by placing the organism under metabolic stress.

Tanaka *et al.* (2017) Ribosome engineering has been widely utilized for strain improvement, especially for the activation of bacterial secondary metabolism. This study estimated ribosome engineering technology to regulate primary metabolism, taking vitamin B12 production as a representative example. The introduction into *Propionibacterium shermanii* of mutations giving resistance to rifampicin, gentamicin, and erythromycin, respectively, increased per cell production (μg/L/OD600) of vitamin B12 5.2-fold, although net production (μg/L) was unchanged, as the cell mass of the mutants was reduced. Real-time qPCR analysis indicated that the genes involved in vitamin B12 fermentation by *P. Shermanii* were activated at the transcriptional level in the drug-resistant mutants, providing a mechanism for the higher yields of vitamin B12 by the mutants. These results signified the efficacy of ribosome engineering for the production of not only secondary metabolites but of industrially important primary metabolites.

Chamlagain *et al.* (2018) reported the in-situ production of active vitamin B12 was explored in aqueous cereal-based matrices with three strains of food-grade *Propionibacterium freudenreichii*. Matrices prepared from malted barley flour (33% w/v; BM), barley flour (6%; BF), and wheat aleurone (15%; AM) were fermented. The effect of cobalt and the lower ligand 5,6-dimethylbenzimidazole (DMBI) or its natural precursors (riboflavin and nicotinamide) on active B12 production was calculated. Active B12 production was confirmed by UHPLC–UV–MS analysis. A B12 content of 12–37 $\mu g \cdot kg^{-1}$

was produced in BM; this content increased 10-fold with cobalt and reached 940–1,480 μg·kg⁻1 with both cobalt and DMBI. With riboflavin and nicotinamide, B12 production in cobalt-supplemented BM increased to 712 μg·kg⁻1. Approximately, 10 μg·kg⁻1 was achieved in BF and AM and was increased to 80 μg·kg−1 in BF and 260 μg·kg⁻1 in AM with cobalt and DMBI. The UHPLC and microbiological assay (MBA) results agreed when both cobalt and DMBI or riboflavin and nicotinamide were added. However, MBA gave ca. 20%–40% higher results in BM and AM supplemented with cobalt, designating the presence of human inactive analogues, such as pseudovitamin B12. This study denoted that cereal products can be naturally adjoining with active B12 to a nutritionally relevant level by fermenting with *P. freudenreichii*.

Signorini *et al*. (2018) reported the fermentation represents a commercial and economic approach for food stabilisation and nutritional improvement. Tempeh is an example of soybean solid-state fermentation. In this work, they investigated the possibility of producing a tempeh analogue holding high amounts of vitamin B12 using seeds of three different species of the legume lupin, namely *Lupinus albus*, *L. Angustifolius* and *L. Mutabilis*, with *Rhizopus oligosporus* and *Propionibacterium freudenreichii* co fermentation. Synergic effects of *Rhizopus* and *Propionibacterium* in increasing vitamin B12 up to 1230 ng/g dw was observed. These findings specified that this co fermentation can improve lupin nutritional quality and safety to provide a tempeh analogue with added value for vegetarian communities and low-income populations. The level of potentially toxic lupin alkaloids was also observed during the tempeh preparation.

Wang *et al.* (2020) reported the Vitamin B12 and propionic acid that were simultaneously produced by *Propionibacterium freudenreichii* are both advantageous chemicals widely used in food preservatives, medicine, and nutrition. While the carbon source and propionic acid accumulation reflected fermentation efficiency. In this study, using corn stalk as a carbon source and fed-batch fermentation process in an expanded bed adsorption bioreactor was studied for structured and economic biosynthesis of acid vitamin B12 and propionic. With liquid hot water pretreated corn stalk hydrolysates as carbon source, 28.65 mg L⁻1 of vitamin B12 and 17.05 g L⁻1 of propionic acid were attained at 168 h in batch fermentation. In order to optimize the fermentation outcomes, fed-batch fermentation was performed with hydrolyzed corn stalk in expanded bed adsorption bioreactor (EBAB), giving 47.6 mg L⁻1 vitamin B12 and 91.4 g L⁻1 of propionic acid at 258 h, which correspond to product yields of 0.37 mg g−1 and 0.75 g g−1, respectively. The present study provided a

favourable strategy for economically justifiable production of vitamin B12 and propionic acid by *P. Freudenreichii* fermentation using biomass cornstalk as carbon source and expanded bed adsorption bioreactor.

Kustyawati *et al.* (2020) reported that most studies have found that vitamin B12 in tempeh is produced by contaminating bacteria specifically, *Klebsiella sp*. And *Citrobacter freundii*, during fungal fermentation. This study is to calculate the effect of starter culture on the vitamin B12 and isoflavone aglicone content of soybean fermentation for tempeh production. In this study, soybeans were washed, soaked in water overnight, dehulled and sterilized by boiling at 100 °C for 30 min. Three starter cultures (103 CFU $g^{-}1$) namely *Rhizopus oligosporus*, *Klebsiella sp*. and *Saccharomyces cerevisiae* were then inoculated as follows, soybeans + *R. oligosporus* + *S. cerevisiae* (SRSc), soybeans + *R. oligosporus* + Klebsiella sp. (SRK), soybeans + *R. oligosporus* + *S. cerevisiae* + *Klebsiella sp*. (SRScK), and Soy + *R. oligosporus* (SR) and soybeans + *Klebsiella sp* (SK). Inoculated soybeans were then incubated at 30 ± 2 for 40 hours (tempeh-style). The growth of *Klebsiella sp*., *S. cerevisiae*, *R. oligosporus* and the production of vitamin B12 as well as isoflavone aglicones were observed. The results showed the highest vitamin B12 3.15 mg 100 $g^{-}1$ was found in tempeh SRSc, followed by 2.88 mg 100 $g^{-}1$ and 1.64 mg 100 $g^{-}1$ in tempeh SR and SRScK, respectively. In addition, vitamin B12 in tempeh SRK was the lowest (0.81 mg 100 $g^{-}1$). All the starter cultures were able to hydrolyze daidzin and genistin, but the amount of daidzein and genistein was tripled and doubled, respectively when *Klebsiella sp* was inoculated to the soybean fermentation. The study indicated that *S. cerevisiae*. contributes to the production of vitamin B12, while *Klebsiella sp.* contributes to production of daidzein and genistein in soybean fermentation for tempeh production.

Pereira *et al.* (2019) demonstrated that a network of components from different metabolic pathways is the building scaffold of an indispensable compound in the human organism—vitamin B12. The biosynthesis of this compound is constricted to a limited number of representatives of bacteria and archaea, while vitamin B12 dependent enzymes are spread through several domains of life. Different attempts have been performed to increase vitamin B12 levels in dietary products, particularly in vegetarian and vegan dietary regimes. The integration of vitamin B12 in microalgae through symbiosis with microorganisms generally recognized as safe, for example the probiotic *Lactobacillus reuteri*, can even expand the nutritional value of the micro algal biomass. This study reviews the microbial production of vitamin B12 based on genetic analyses and chemical studies. Recent genetic

approaches are focused, particularly potential metabolic engineering targets to increase vitamin B12 production. The bio incorporation of vitamin B12 in microalgae as an attempt to provide a superfood is also reviewed.

Wang *et al*. (2012) A new type of in situ product removal (ISPR) technique of expanded bed adsorption (EBA) bioreactor was studied to simultaneously produce and intracellular vitamin B12 and extracellular Propionic acid by *Propionibacterium freudenreichii* CICC 10019. Resin screening experiments showed that the ZGA330 resin has the best biocompatibility and highest adsorption for propionic acid. Through the EBA bioreactor, propionic acid could be recovered efficiently by semi-continuous recirculation of the unfiltered broth, which abolished the feedback inhibition of propionic acid. Fed-batch fermentation was carried out using the EBA system, resulting in a propionic acid concentration of 52.5 g L^{-1} and vitamin B12 concentration of 43.04 mg L^{-1} at 160 h, which correspond to product yields of 0.66 g g^{-1} and 0.54 mg g^{-1}, respectively. The present study suggests that the EBA bioreactor can be utilized for the simple and economical production of propionic acid and vitamin B12 in a single fermentation process.

Li *et al.* (2017) investigated extracellular vitamin B12-producing *Lactobacillus* strains and their characteristics in tolerance to environmental stresses, gastric acid and bile salts. Two isolates, *Lactobacillus plantarum* LZ95 and CY2, exhibit high extracellular B12 production, 98 ± 15 μg/L and 60 ± 9 μg/L respectively. Extracellular B12 from LZ95 were identified as methylcobalamin and adenosylcobalamin using a combination of reverse-phase HPLC and solid phase extraction while that from CY2 was adenosylcobalamin. Both strains grew under environmental stresses, and LZ95 exhibited better tolerance to low temperature and high ethanol concentration. LZ95 also showed good viability when exposed to gastric acid (pH 2.0 and 3.0) and bile salts (0.3%) as well as good adhesion to Caco-2 cells. The viability of CY2 was significantly reduced under low pH and exposure to bile salt. Together, extracellular B12 producer LZ95 with good probiotic properties might be a candidate for in situ B12 reinforcement in the food industry.

Turlo *et al.* (2008) reported the preparations derived from *Lentinula edodes* (*Berk.*) *Pegl*. are widely used as dietary supplements, they contain compounds with immune system enhancing and chemopreventive properties. These preparations contain a blend of minerals and vitamins, including vitamins A, D, B1, B2, C, and niacin, but only scant vitamin B12. Our goal was to improve the growth conditions of submerged mycelial cultures of *L. edodes* in order to obtain a new dietary supplement enriched in Vitamin B12.They designed a

biotechnological process in which cobalamin precursors, cobalt chloride and the methyl donors' betaine, methionine, betaine and choline, were added to the culture medium. The vitamin B12 content in mycelial extracts was determined by RP-HPLC, while total Co2+ content was determined by HPIC. At the optimal Co2+ concentration (40 μg/mL), the vitamin B12 level in *L. edodes* mycelium reached 95 μg/g of dry weight. All methyl donors had a positive effect on cobalamin biosynthesis, at least doubling its concentration in mycelia as compared to mycelia grown in non-enriched medium. The amount of cobalamin in submerged cultivated mycelia of *L. edodes* was 10,000-fold higher than that recorded for fruiting bodies. Our results suggest that these optimized culture conditions could be applied to obtain a new cobalamin-enriched dietary supplement derived from *L. edodes*.

Hajfarajollah *et al.* (2015) explained that experimental designs were used to develop a medium based on waste frying sunflower oil (WFO) and other nutrient sources for vitamin B12 (VB12) production by *Propionibacterium freudenreichii* subsp. *freudenreichii* PTCC 1674. The production of propionic acid and acetic acid were also evaluated using the same microorganism. The amount of WFO in the media was initially optimized. The amount of 4 % w/v of oil found to be an appropriate amount for production of VB12. A Plackett Burman design was then employed to identify nutrients that have remarkable effect on the production of VB12 in the WFO media. Dimethylbenzimidazole (DMB), cobalt chloride, ferrous sulfate, and calcium chloride where the most important compounds. The level optimization of nutrients as the significant factors was finally performed using response surface methodology based on a central composite design. The model predicted that a medium containing 35.56 mg/L DMB, 14.69 mg/L CoCl2.6H2O, 5.82 mg/L FeSO4.7H2O, and 11.41 mg/L CaCl2.2H2O gives the maximum VB12 production of 2.60 mg/L. The optimized medium provides a final concentration of vitamin 170 % higher than that by the original medium. This study offers valuable insights on a cost-effective carbon source for industrial production of food-grade VB12.

Wang *et al.* (2010) demonstrated the effects of different oxygen transfer rates (OTR) on the cell growth and vitamin B12 biosynthesis of *Pseudomonas denitrificans* were first investigated under dissolved oxygen limiting conditions. The results demonstrated that high OTR accelerated cell growth and initial vitamin B12 biosynthesis rate, while lower OTR was critical for higher productivity in the late fermentation process. The oxygen uptake rates (OUR) correlated well with OTR. Based on the metabolic halfway analysis, a step-wise OUR control strategy was proposed. The strategy was successfully implemented in scale-up to an

industrial fermenter (120,000 l). A stable maximum vitamin B12 production of 208 ± 2.5 mg/l was achieved, which was increased by 17.3% compared with the control. Furthermore, the glucose consumption coefficient to vitamin B12 was 34.4% lower than that of the control. A structured and economical fermentation process based on OUR criterion was established for fermentation of industrial vitamin B12 by *P. denitrificans*.

Bao *et al.* (2019) revealed that in order to solve problems of fluctuant vitamin B12 contents in furu, the effect of L. Reuteri inoculation on vitamin B12 content of furu in a large-scale experiment was studied in addition to evaluating the microbiomes of furus. Results showed that vitamin B12 content in furu inoculated with *L. reuteri* was gradually increased up to 141.7 ng/g (wet weight), which is higher than the control group (36.0 ng/g). After principal component analysis, samples for bacterial composition in L. R. Groups were gathered together, but those for the composition of fungi were dispersed. *L. reuteri* inoculation obviously increased the relative profusion of *Firmicutes* and *Actinobacteria*, but reduced the relative amount of Proteobacteria. Based on spearman analysis, *Enterococcus*, *Lactobacillus*, *Streptococcus*, and *Corynebacteria* were reported to be positively related with vitamin B12 contents and supported each other, vice versa Rhodotorula and Penicillium. Regarding the results of PICRUSt, vitamin B12 related genes pathways were enhanced after *L. reuteri* was applied. In this study, the novel start culture of *L. reuteri* was successfully introduced to a traditional soybean fermented food to improve the content of vitamin B12 in furu.

Li *et al.* (2008) explained that previous research has committed cobalt ion and dimethylbenzimidazole (DMBI) which are the precursors of vitamin B12 biosynthesis, and porphobilinogen synthase (PBG synthase) is a zinc-requiring enzyme. In this paper, the effects of Co2+, Zn2+ and DMBI on vitamin B12 production by *Pseudomonas denitrificans* in shake flasks were studied. Present experimental results revealed that the addition of the above mentioned three components to the fermentation medium could remarkably restore the biosynthesis of vitamin B12. The concentrations of zinc sulphate, cobaltous chloride and DMBI in the fermentation medium were further optimized with nutrients WA orthogonal central composite design and statistical analysis by Data Processing System (DPS) software. As a result, vitamin B12 production was increased from 69.36 ± 0.66 to 78.23 ± 0.92 μg/ml.

Shi *et al.* (2018) reported the Soybean products are favoured because of its taste, digestibility, and health benefits. However, soybean lacks vitamin, mainly the low water-soluble vitamin B12. This study revealed the effects of fermentation conditions on the

synthesis of vitamin B12, production of metabolites, and growth of *Lactobacillus reuteri* and *Propionibacterium shermanii* in fermented soy-milk. A Lotka Volterra model was successfully worked to describe the competition relationship between the two microorganisms under various fermentation conditions. A quadratic function between the ratio of interchange coefficients and vitamin B12 content was found. Higher vitamin B12 in soy-milk can be manufactured when the ratio of interaction coefficients is applicable to one. Contrasted with other fermented soybean products, fermented soy-milk contains more acetate, ethanol, and propionic acid. This study successfully demonstrated a mathematical model to increase soy-milk vitamin B12 production.

Massoud *et al.* (2020) explored the fermented dairy products produced by various microorganism's activity provide valuable nutrients for humans. Fermentation affects the physicochemical and organoleptic characteristics of foods as well as human health. In the present review, they describe the production of vitamin B12 in a fermented dairy product by *Propionibacterium* sp. The effect of the process variables on vitamin B12 production in fermented dairy products e.g., temperature, pH, different nitrogen and carbon sources as well as the type and size of inoculum, the fermentation time and fermentation strategy, etc. are discussed. Finally, fermentation strategy, inoculum preparation of *Propionibacterium freudenreichii subsp*. *Shermanii* methods of determining biomass and Vitamin B12 concentration are reviewed and their outcomes on vitamin B12 production are mentioned. Propionibacteria are so popular due to their unique characteristics such as being suitable, safe and economical. Among all, the highest efficiency was observed by *P. freudenreichii*. To achieve this purpose, some proper situations need to be considered. The best carbon source for this bacterium to produce biomass is lactate, the suitable pH for growth of the *Propionibacterium* species is in the range of 6 to 7 and the fed-batch is most preferable for vitamin B12 production.

Khosravi-Darani *et al.* (2019) discussed the production of fermented functional foods containing micronutrients is required for their health beneficial properties. Impact of 11 process variables on vitamin B12 production in a dairy beverage containing propionic acid was explored. *Propionibacterium freudenreichii ssp*. *Shermanii* was applied in a 3-l fermentor in a fed-batch fermentation system. The most appropriate conditions for vitamin B12 production were attained by 5% v/v inoculum size containing *Propionibacterium freudenreichii* (without *Lactobacillus acidophilus*) and continuous feeding of lactose with the rate of 0.04 l/h at 36 C in a medium containing 25 g/l molasses, 10 g/l corn steep liquor, at

pH= 6.5, after 96 h fermentation. Maximum vitamin concentration (30 mg/l) and productivity (7.5 mg/l. Day) were obtained in trial 9. Organoleptic properties of the fermented beverage were also acceptable for panelists and no remarkable difference was observed between samples and control during 6 days of refrigerated storage.

CHAPTER-3

MATERIALS AND METHODS:

The present research work was carried out in the Laboratory at Division of Microbiology, Faculty of Basic Sciences, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu. Materials and methods used for conducting experiments are elucidated as under:

3.1 Sample Collection

The potent *Lactobacillus* strain capable of producing Vitamin B12 were isolated from curd and kaladi samples collected from local markets of District Jammu and Udhampur respectively. Samples were collected in sterilized plastic bags and brought to the laboratory and kept at 4°C in the refrigerator for further studies.

Lyophilised *Lactobacillus* culture (L-1408), procured from IMTECH-Chandigarh was taken up as check during the studies. Culture was revived on MRS agar media and stored at 4ºC.

3.2 Sterilization:

All glassware and media were sterilised for experiment purposes. Glassware sterilization was done in hot air oven at a temperature of 180ºC for 20 minutes. All media were sterilised at 121 ºC for 15 minutes in autoclave.

3.3 Isolation from Curd sample

Isolation of cultures was done using serial dilution method under aseptic conditions. Serial dilution method involves the process of taking a sample and diluting it through a series of standard volumes of sterile diluent, which can either be distilled water or 0.9% saline. Then a small measured volume of each dilution is used to make a series of pour or spread plates. This technique is much known for the isolation and culturing of bacteria. In the serial dilution method, bacterial suspensions are taken and diluted serially in the successive test tubes. This technique is much known for the isolation and culturing of bacteria. Following the serial dilution, add 1 ml of the sample to the neighbouring culture tube sequentially in a series 10^{-1}, 10^{-2}, 10^{-3} and so on. The curd sample was taken in a 10 ml culture tube and the stock solution was prepared by diluting it with 9 ml distilled water. The 1ml of the sample was transferred to the neighbouring *culture tube* in a series of 10^{-1},10^{-2},10^{-3},10^{-4},10^{-5},10^{-6},10^{-7} ,10^{-8}

and 10^{-9} under aseptic conditions. Dilutions 10^{-5}, 10^{-7} and 10^{-9} were selected and spreading was done on pre-poured MRS agar Petri plates. They were incubated at 37°C from 24 to 48 h in the incubator to obtain *Lactobacillus* cultures.

3.4 Isolation from Kaladi sample

Kaladi was mashed and diluted with water in a sterilized flask. The Kaladi sample was taken in a 10 ml culture tube and the stock solution was prepared by diluting it with 9ml distilled water. The 1ml of the sample was transferred to the neighbouring culture tube in a series of 10^{-1},10^{-2},10^{-3},10^{-4},10^{-5},10^{-6},10^{-7} ,10^{-8} and 10^{-9} under the aseptic conditions.

From these 9 dilutions 10^{-5},10^{-8},10^{-9} were selected then spreaded on MRS Agar Medium by spread plate technique. They were further incubated at 37°C from 24 to 48 h. Pure cultures were transefered to MRS agar slants and stored at 4°C for further use.

3.5 Purification of the isolates

5 curd samples and 3 Kaladi samples were isolated and incubated at 37°C for 24 to 48 h. Streak plate technique was further used for the purification of *Lactobacillus*. After purification, plates were observed for isolated colonies. For preserving isolated Colonies, Nutrient Agar stabs were prepared and stored at 4°C.

3.6 Identification of the isolates

Bacterial isolates were identified on the basis of morphology and Gram's staining technique.

3.6.1. Morphological identification

With the help of a sterilized inoculation loop individual bacterial colonies were streaked on the plates and incubated at 28±2ºC for 24 hours and colony characteristics were observed visually viz, shape, colour and mucosity.

3.6.2. Microscopic Identification by Gram's Staining Technique

The isolated cultures were examined using Gram's staining technique and was observed under microscope for Gram's reaction and cell shape. In this method bacterial smears were prepared from 24 hrs colonies, grown on MRS agar on grease free glass slides. Smears were heat fixed and flooded with crystal violet for 30 seconds, then washed with water. Gram's iodine was applied on smear for 60 seconds and then washed with ethyl

alcohol. Finally, safranin was applied on the smear for 60 seconds and then washed with distilled water. After air drying it was observed under a microscope. Finally, it was observed 10x, and then a drop of immersion oil was placed on the smear and observed under 100x magnification of a binocular microscope in order to record the microscopic characteristics (Bathlomew, 1962).

3.7. Screening and Quantification of Isolates for the Ability to Produce Vitamin B12: Preparation of bacterial inoculum: Different individual microorganisms were inoculated. In 100 ml of Nutrient Broth and Luria Bertani (LB) broth and incubated at 37°C for 10 days (Martens *et al.,* 2002).

3.7.1 Assay Fermentation for Vitamin B12 Production

The isolated bacteria were allowed to grow in vitamin B12 assay broth and MRS broth (Hi Media) of 100 mL by inoculating the Colony in the fermentation media for 5 to 7 days and after few days of growth the precursor cobalt chloride was added and the cells was harvested by centrifugation at 7000 rpm after 72 h of growth. (Chowdhury *et al.,* 2012). The ability of the microorganisms to produce vitamin B12 was tested by growing the organisms in vitamin B12 assay medium plates. The plates were incubated at 37°C for 24 to 48 h and check for growth (Jajodia *et al*., 2017).

3.8. **Vitamin B12 Quantification by Spectrophotometer**

UV Visible Spectrophotometer.

3.8.1. Preparation of standard solution

Standard curve of different concentrations was made by dissolving 1 tablet of Me-Cbl containing 1500Mcg working standard in 10mL distilled water by boiling at 60°C for 5 min. Different aliquots were prepared and absorbance was measured from 300 nm to 750 nm. (Jajodia *et al*., 2017).

3.9. Extraction of Vitamin B12

3.9.1. Extraction of vitamin B12 (From supernatant):10 ml of fermented broth each was taken in microfuge tube and centrifuged at 7,000RPM; 10 min at 4°C.After centrifugation, cell free supernatant was collected into new microfuge tube. The O.D. of supernatant was measured spectrophotometrically.

3.9.2. Extraction of Vitamin B12 (From pellet): 1 mL of fermented broth was taken in microfuge tube and centrifuged at 7,000RPM; 10 min at 4°C. After centrifugation, the supernatant was discarded and to the pellet PBS buffer was added and mixed by vortexing. Three times the pellet was washed with a PBS buffer, mixed by vortexing and centrifuged. The bacterial cell pellet was resuspended into 1 mL PBS, followed by ultrasonic cell disruption for five times at 1 min interval under ice. The mixture was cleared by centrifugation (7,000RPM; 10 min) and the supernatant of lysed cells were taken for Spectrophotometric analysis (Bhushan *et al*., 2016).

3.11. Strain improvement by random mutagenesis

3.11.1. UV Mutagenesis:

Culture was grown overnight in 50 ml of nutrient broth (NB) medium in 500 ml flask,5 ml of the suspension was placed in sterile Petri dish and shall be exposed to UV rays (235 nm) at a distance of 10 cm. At regular intervals, the samples were taken out and different dilutions were plated on the NA plate to determine viable count. (Ali *et al.,* 2011).

3.11.2. EMS Mutagenesis:

10 ml suspension of exponential phase growth culture was centrifuged to get a pellet.Then it was washed with saline and resuspended in 10 ml of phosphate buffer (pH 7.0). Then suspension was treated with 80 μM of EMS with constant shaking. At different time intervals samples was withdrawn and to it 5% sodium thiosulphate was added to stop the mutagenesis. Next the cells were washed and plated on a NA plate to determine the viable count (Abdelsalam, 2018).

After doing mutagenesis again Screening and quantification on Vitamin B12 was done using visible spectrophotometer

Screening for High Vitamin B12 producing Potents

Cell suspension was spreaded on selective medium (i.e Vitamin B12 assay medium) and the medium must be supplemented with glycerol (CM Burgess et al., 2009). It was also shown that *Lactobacillus reuteri* CRL1098 was able to Metabolize glycerol in a B12-free medium; this being the first hint that a LAB might be able to produce cobalamin (LeBlanc *et al*., 2000). The plates were incubated for 24 h at 37°C. After that the fast-growing colonies were taken for fermentation studies.

3.12. Shake Flasks Standardisation of Vitamin B 12 Production: Fermentation was carried out using shake flasks. Classical one -factor-at -a-time method was used for studying. In the present study three parameters of fermentation were optimised and used for study and these were Inoculum load, pH and temperature. For all the factors i.e low, middle and high was evaluated for each parameter in terms of product recovery after 48 hrs. All the experiments were performed in triplicates and blank was maintained simultaneously as check (Qiang *et al*., 2013).

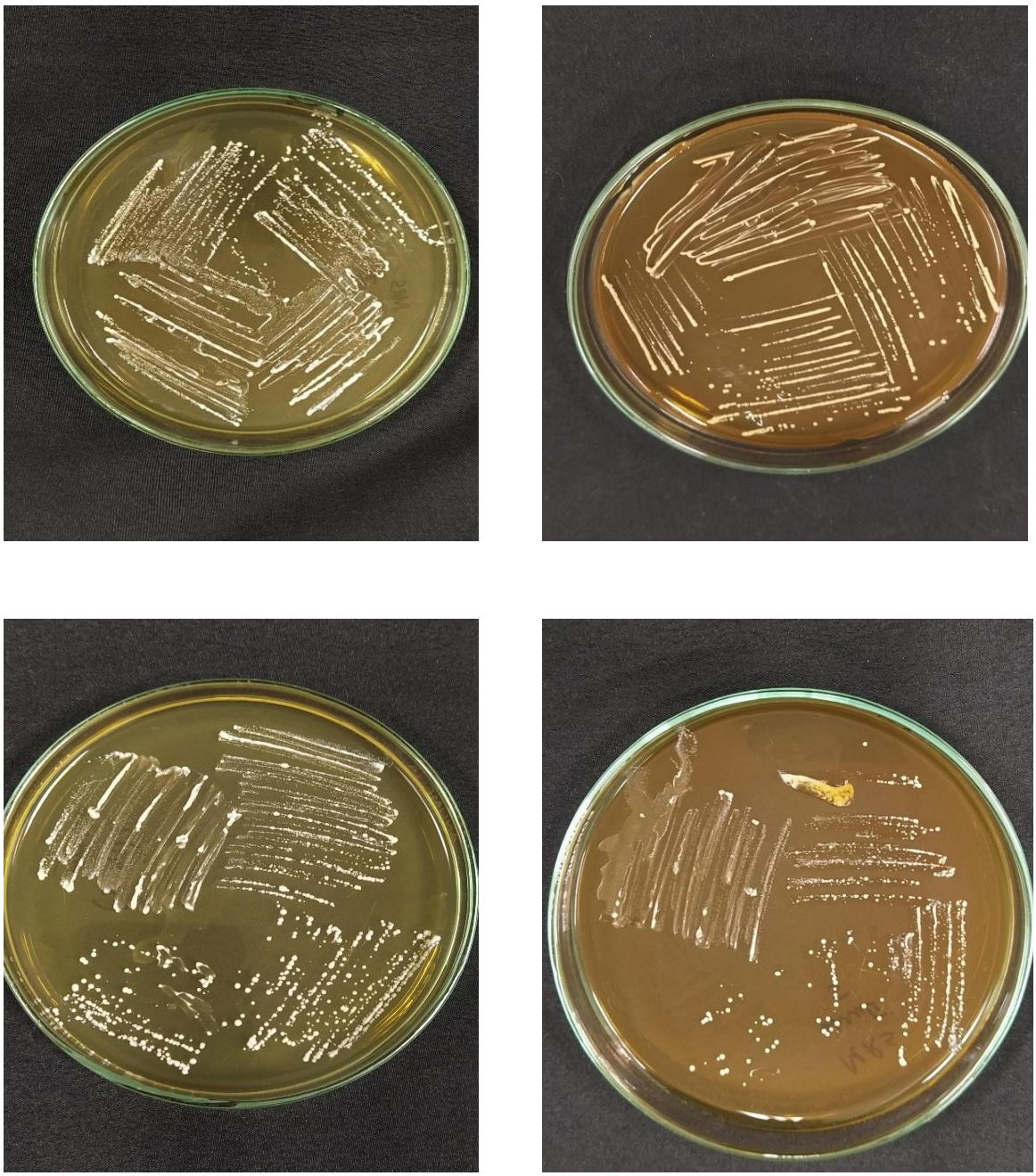


Plate 1: Purification of colonies by streaking on MRS Agar

Plate 2: Vitamin B12 Screening media for assaying the fermentation of *Lactobacillus*

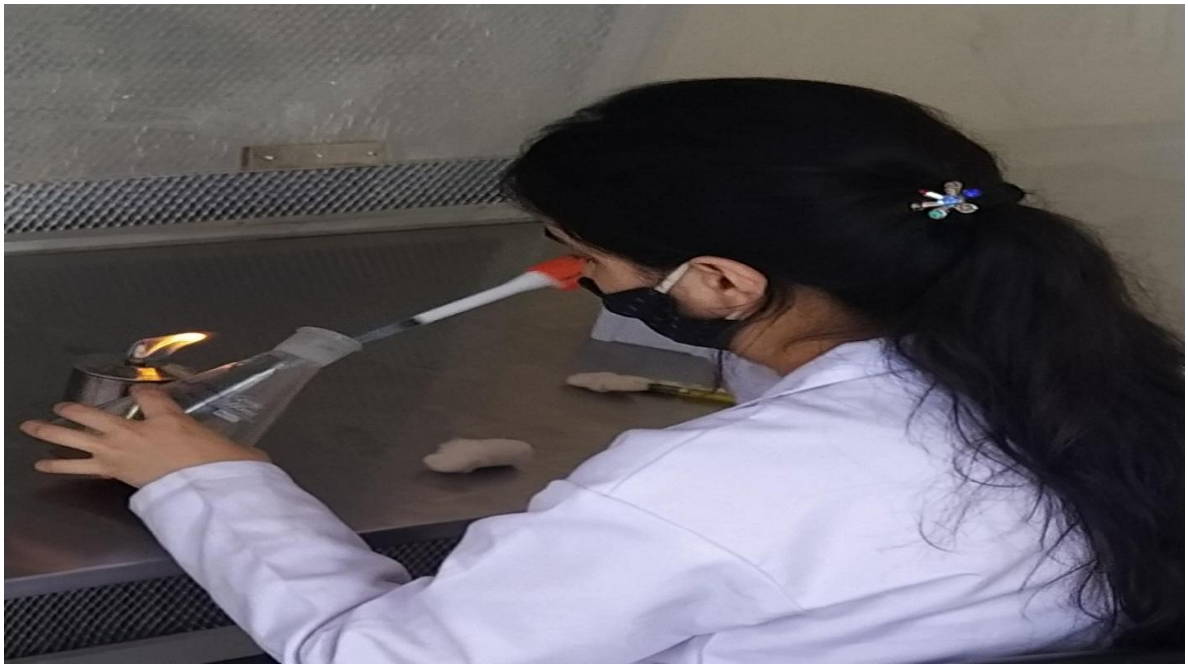


Plate3: Inoculation of the *Lactobacillus* culture for the fermentation studies

Optimization at different pH

Optimization at different temperatures

Optimization at different Inoculum load

Plate 4: Optimization of fermentation parameters for *Lactobacillus* culture

CHAPTER-4

RESULTS

In the present study, lactic acid bacteria were isolated from curd and Kaladi samples. The potential isolates were identified on the basis of morphology and microscopic studies. Further the isolates were screened on Vitamin B12 assay medium and potential isolates i.e Lb – 1,2 ,6 and 7 showed positive results and the best isolated i.e Lb-7 was taken for further mutation studies. After doing mutagenesis the mutated strain was taken for an optimization process.

4.1 Isolation of Desirable Probiotic Bacteria Producing Vitamin B12

Isolation of Lactic Acid Bacteria was carried out from curd samples and Kaladi samples named as Lb - 1,2,3,4,5,6,7 and 8 in which moderate to high growth was shown in different samples. Spread plate technique and Streak plate technique was further used in order to isolate the desirable colonies. The best isolated colonies were further used for the screening process. (Table-1) & Plate-5 ,6 and 7

Table -1: Isolates o*f Lactobacillus* collected from curd and kaladi sample

S. No.	Isolates	Sample collection
1	Lb -1	Curd sample
2	Lb -2	Curd sample
3	Lb -3	Curd sample
4	Lb -4	Kaladi sample
5	Lb -5	Kaladi sample
6	Lb -6	Kaladi sample
7	Lb -7	Curd sample
8	Lb -8	Curd sample

4.2. Identification of Potential Bacteria

Gram's stain reaction is an important tool developed by Dr. Hans Christian Gram, a Danish Physician, in 1884 for the classification remains an important and useful technique till today. The bacteria identified under the microscope was Gram+ve and long rods shaped.

The identification of Bacteria are based on colony morphology Gram's staining and biochemical tests. Morphological characteristics of single cell colony were used for preliminary identification of Bacterial genus. In the present study the isolates Lb -1,4,6 and 8 showed Creamish white colonies, Lb -2,3 and 5 showed off white colonies and Lb -7 showed dark Creamish white colonies and all the isolates were Gram +ve and long rod shaped. (Table-2), Plate-8 and 9

Table -2: Morphological and Microscopic Characteristics of *Lactobacillus* Isolates

Isolates	Morphology	Microscopic
Lb -1	**Creamish white**	**Gram +ve, long rods**
Lb -2	**Off white**	**Gram +ve, long rods**
Lb -3	**Off white**	**Gram +ve, long rods**
Lb -4	**Creamish white**	**Gram +ve, long rods**
Lb -5	**Off white**	**Gram +ve, long rods**
Lb -6	**Creamish white**	**Gram +ve, long rods**
Lb -7	**Dark Creamish white**	**Gram +ve, long rods**
Lb -8	**Creamish white**	**Gram +ve, long rods**

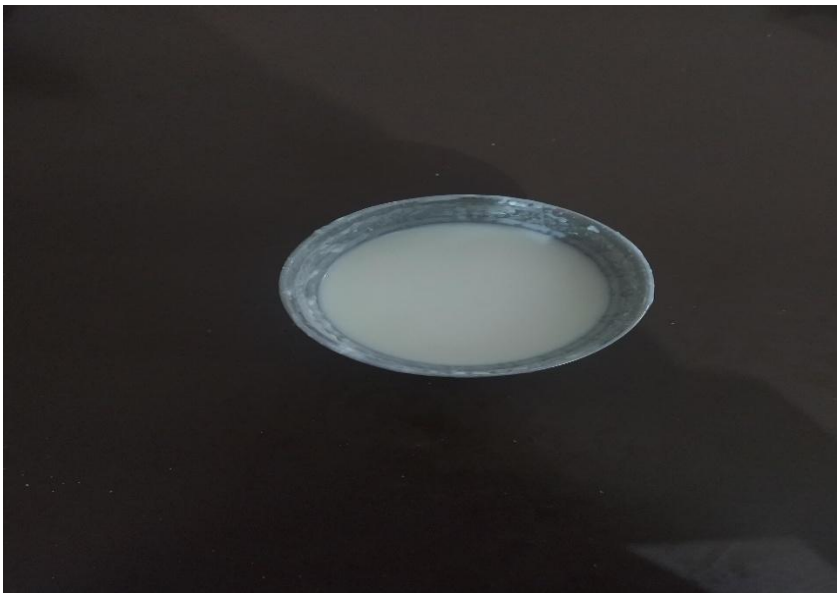
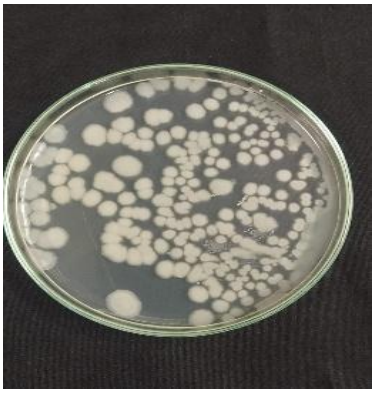
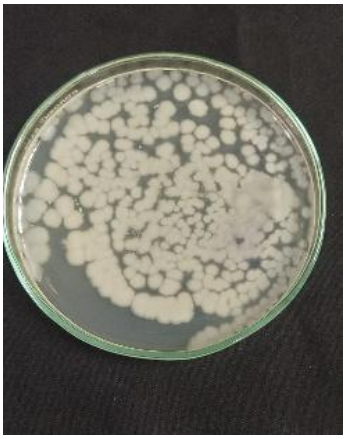


Plate 5: Homemade curd sample

Plate 6: Kaladi sample



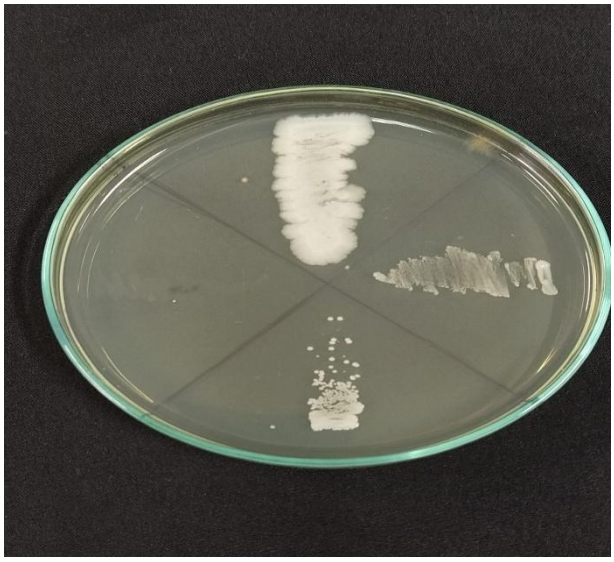
Lb -1 **Lb -2** **Lb -3**



Lb -4. **Lb -5.** **Lb -6**

Lb -7. **Lb-8**

Plate 7: Isolates of *Lactobacillus* from curd and Kaladi sample

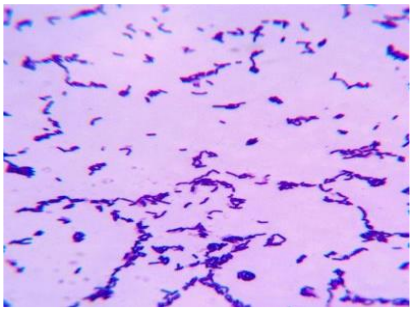


Lb -1, Lb -2 and Lb - 3

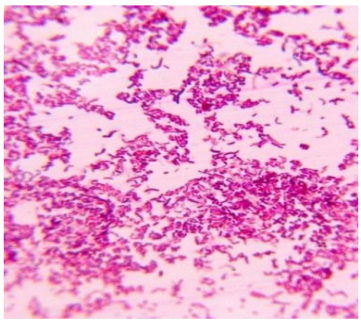
Lb-4, Lb -5 and Lb -6

Lb -7 and Lb -8

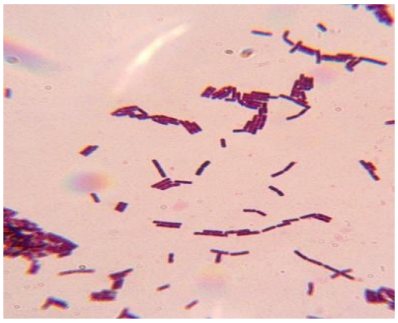
Plate 8: Morphological Characteristics of Isolates - Lb-1,2,3,4,5,6,7 and Lb -8.



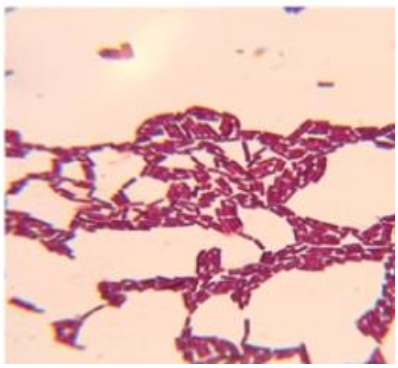
Lb -1.



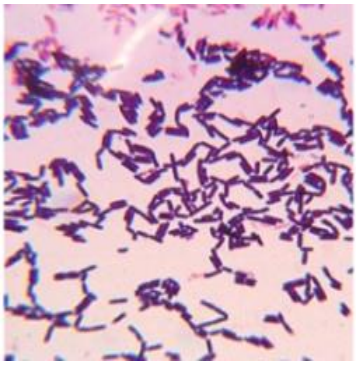
Lb -2.



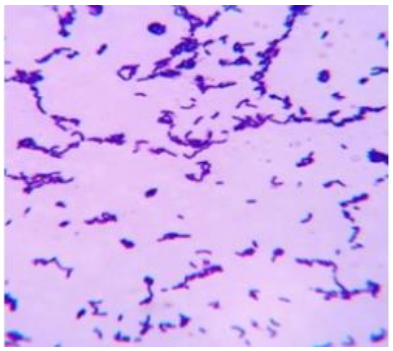
Lb-3



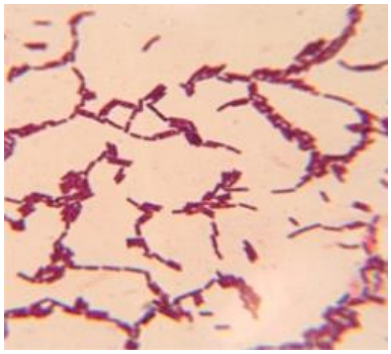
Lb -4



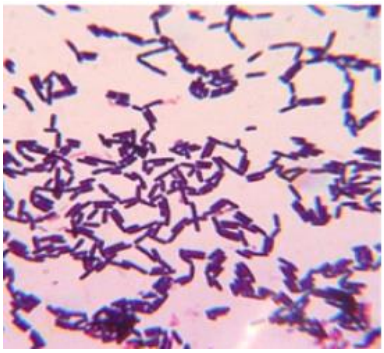
Lb-5



Lb -6



Lb -7



Lb -8

Plate 9: Microscopic Characteristics of *Lactobacillus* Isolates -Lb-1,2,3,4,5,6,7 and 8

4.3 Screening of the *Lactobacillus* bacteria on the Vitamin B12 Assay medium

The– **Lactic Acid Bacteria - Lb -1, Plate 2 – Lb – 2, Plate 3 – Lb -6, Plate 4 – Lb -7** showed positive result on Vitamin B12 assay medium while the organisms in other plates showed no rowth on Vitamin B12 assay medium. (Plate -10)

Hence the Lb -1, Lb- 2, Lb -6 and Lb-7 showed positive result on Vitamin B12 assay medium having the ability to produce Vitamin B12. From all the above samples i.e the Lb-1,2,6 and 7, the fast-growing colonies of the Lb -7 were taken for mutagenesis.

4.4 Strain improvement by random Mutagenesis

4.4.1 UV Mutagenesis

Lb -7 isolate was treated with UV radiations at 235nm at different time intervals (1min, 5min and 10min) from a distance of 10 cm. It was observed that at short duration of UV radiations, there was no effect on isolate. When the isolate Lb -7 was UV radiated for longer duration of 15min and 20 min, the cell count reduced. From the above observations least, cell count was recorded with UV exposure for 15min. (Plate-11)

4.4.2 EMS Mutagenesis

Lb-7 was treated with 80µM of EMS at different time intervals of 5m,10m and 20m. It was observed that with short durations of EMS, there was no effect on isolate. When the isolate Lb-7 was EMS treated for longer duration of 30 min and 35min, the cell count reduced. The least cell count was recorded at 30 min. (Plate-12)

4.5 Quantification of Vitamin B12 using visible Spectrophotometer

After doing mutagenesis, Screening and Quantification of Vitamin B12 was done again, Spectrophotometrically at 361 nm wavelength.

The colonies of UV mutated plates showed more dense and fast-growing colonies on screening Vitamin B12 Assay Media than the EMS mutated plates. (Plate-13). Hence the UV mutated plate was chosen further for the optimization.

Table 3: Quantification of Isolates and mutants for the production of Vitamin B12

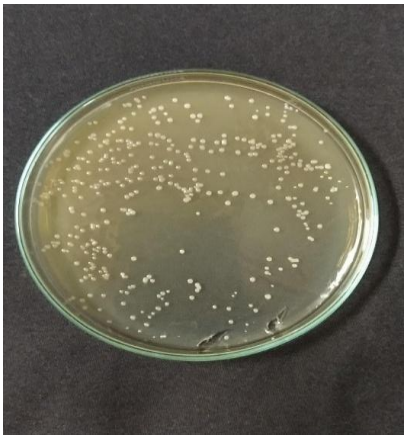
S.No.	Isolate	Vitamin B12 content mg/g DCW
1.	Lb -7	0.39±0.14mg/g DCW
2.	Lb – 7 Mutant	0.63 ± 0.11mg/g DCW
3.	L- 1408	0.84± 0.15mg/g DCW
4.	L- 1408 Mutant	1.05± 0.29mg/g DCW

***All the results were taken in mean of their triplicates**

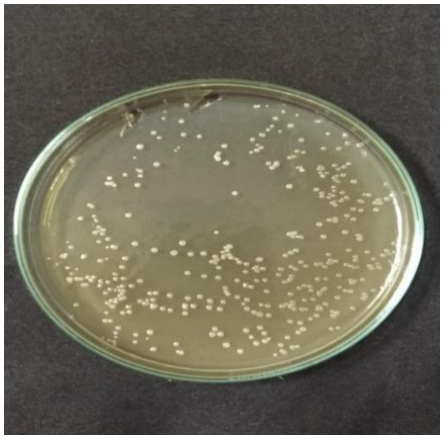
In our Study the isolate of Lb -7 showed production of Vitamin B12 (0.39 mg/g dcw) and L-1408 produced 0.84 mg/g dcw. On other hand Lb -7 mutant showed Vitamin B12 production of 0.63 mg/g dcw and L-1408 mutant produced 1.05 mg/g dcw of Vit B12 (Table-3). From the above result it was observed that the UV mutant strains of Lb-7 and L-1408 showed increased in the Vitamin B12 production than normal isolates. It was estimated that after mutagenesis was done the rate of vitamin B12 production in Lb -7 mutant was 0.24 mg/g dcw higher than normal strain and that of L-1408 mutant by 0.81 mg/g dcw over normal strain. This showed that strain improvement of both the isolates i.e Lb-7 and L-1408 both the mutants developed the resistance against the lactic acid production normally which that leads to vitamin B12 production increase. So, after mutation the rate of production of Lactic acid decreases therefore the vitamin B12 production increases. Before mutation the isolates produced lactic acid together with Vitamin B12 which reduces the production and the vitamin B12 produces less effectively. Hence after UV mutation was done the more production of Vitamin B12 was observed because the resultant strain developed the resistance against the lactic acid production. Lactic acid production caused reduction in production of Vitamin B12 in bacteria (Ali *et al*., 2011).

Lb – 1

Lb -2

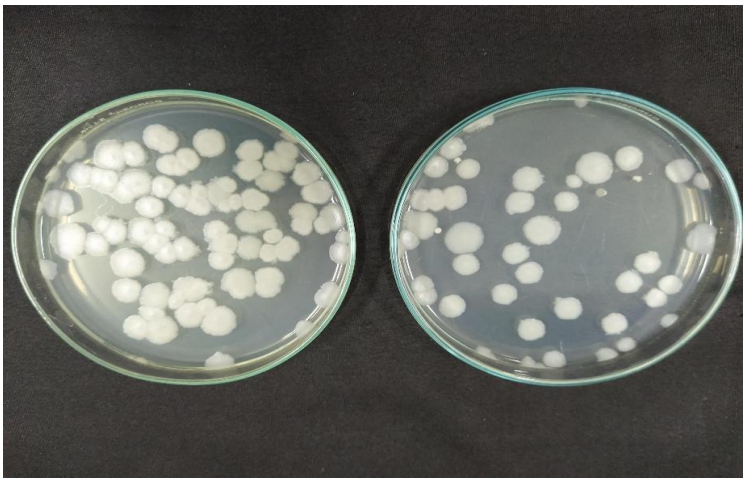


Lb-6

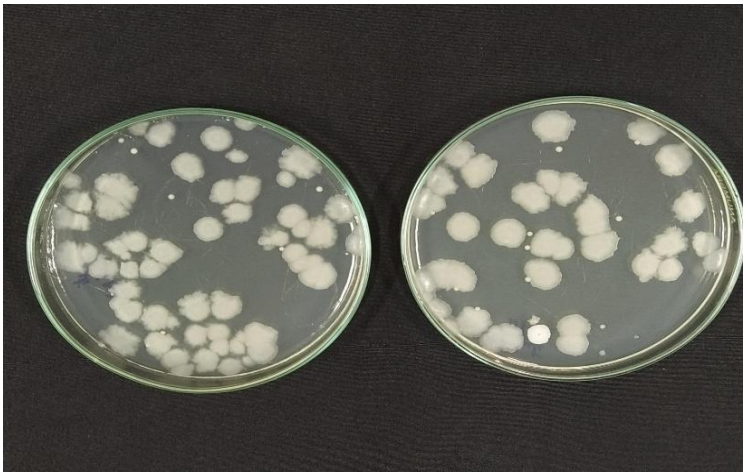


Lb -7

Plate 10: Screening of *Lactobacillus* isolates on Vitamin B12 Assay medium



Lb -7 A: Control. B: Treated



Lb -1408 A: Control B; Treated

Plate 11: UV mutagenesis

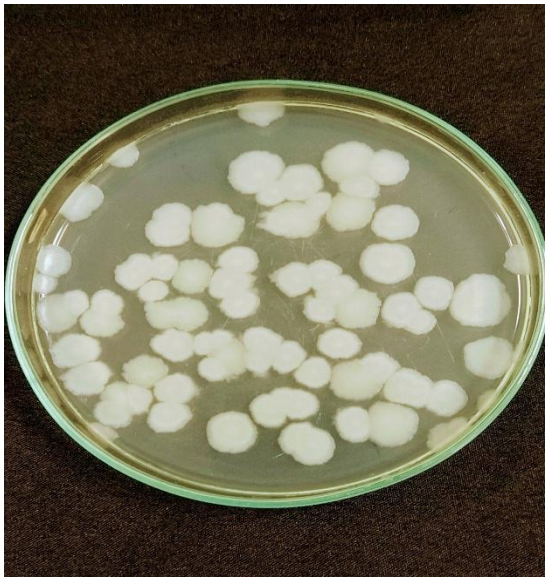
Lb-7: A. Untreated

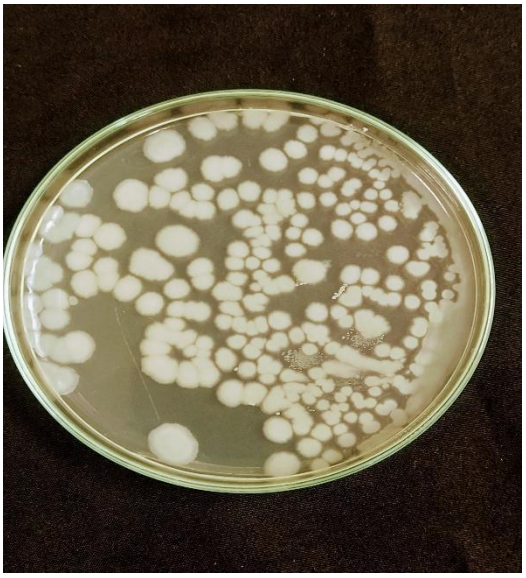
Lb-7: B Treated

Lb -1408: A Control

Lb – 1408: B Treated

Plate 12: EMS Mutagenesis





EMS Mutate of Lb -7 **UV Mutate of Lb- 7**

Plate: 13: Screening of mutants on vitamin b12 assay medium

4.6 Fermentation process Optimization: Fermentation studies were done by the shake flask method. Fermentation parameters i.e temperature. Inoculum load and pH were optimized using the classical one factor at one time method. Three factors were analysed for each parameter. Total Vitamin B12 content was determined and the absorbance was recorded at 361 nm.

In case of Lb -7 the conditions for fermentation were temperature – 15°C, 25° C ,35°C, pH – 4.5,5.5,6.5 and inoculum load - 10^4 cfu/ml ,10^6cfu/ml and 10^8cfu/ml.

During studies performed with Lb-7 mutant, maximum increase in Vitamin B12 content was shown in 25° C of temperature and the total Vitamin B12 content showed 2.05 mg/g dcw. However, from 15°-25°C the vitamin B12 content improved gradually from (1.27 to 2.05 mg/g dcw) but decreased significantly at 1.5 mg/g dcw at 35°C. The decreased in Vitamin B12 production could be due to death of cells due to further rise in temperature. Hence the production gets decreased.

In case of pH maximum increase in Vitamin B12 content was at 5.5 pH and the total Vitamin B12 content showed 1.55 mg/g dcw. However, from 4.5 pH to 5.5 pH the vitamin B12 content improved gradually from (0.79 mg/g dcw to 1.55 mg/g dcw) but decreased significantly to 1.05 mg/g dcw at 6.5 pH. Also, in case of Inoculum load the maximum increased in Vitamin B12 content showed at 10^8cfu/ml and the total Vitamin B12 content increased was 1.53 mg/g dcw. The vitamin B12 content showed improvement in growth gradually. The vitamin B12 growth was 0.96 mg/g dcw at 10^4cfu/ml, 1.05 mg/g dcw at 10^6 cfu/ml and 1.53 mg/g dcw at 10^8 cfu/ml.

The growth content of Vitamin B12 was maximum at 25°C which was 2.05 mg/g dcw.

Table 4: Effect of temperature on vitamin b12 content after fermentation by Lb- 7 mutant

S.No.	Temperature	Vitamin B12 content mg/g DCW
1	15°C	1.27±0.35mg/g DCW
2	25°C	2.05±0.68mg/g DCW
3	35°C	1.52 ±0.35mg/g DCW

Table 5: Effect of pH on Vitamin B12 content after fermentation by Lb-7 mutant

S.No.	pH	Vitamin B12 content mg/g DCW
1	4.5	0.79±0.17mg/gDCW
2	5.5	1.55±0.53mg/gDCW
3	6.5	1.05±0.20mg/gDCW

The growth content of Vitamin B12 was maximum at 6.5 pH which was 1.05 mg/g dcw.

Table 6: Effect of Inoculum load on Vitamin B12 content after fermentation by Lb-7 mutant

S.NO.	Inoculum load	Vitamin B12 Content mg/g DCW
1	10^4	0.96±0.20 mg/g DCW
2	10^6	1.09±mg/g DCW
3	10^8	1.53±0.11mg/g DCW

The growth content of Vitamin B12 was maximum at 10^8 Inoculum load which was 1.53 mg/g dcw

Table 7: Effect of temperature, pH and Inoculum load on Vitamin B12 content after fermentation by Lb-7 mutant

S.No.	OPTIMIZED CONDITIONS		Vitamin B12 content (mg/g DCW)
1	Temperature	15°C	1.27
		25°C	2.05
		35°C	1.52
2	pH	4.5	0.79
		5.5	1.55
		6.6	1.05
3	Inoculum load	10^4 CFU/ml	0.96
		10^6 CFU/ml	1.09
		10^8 CFU/ml	1.53

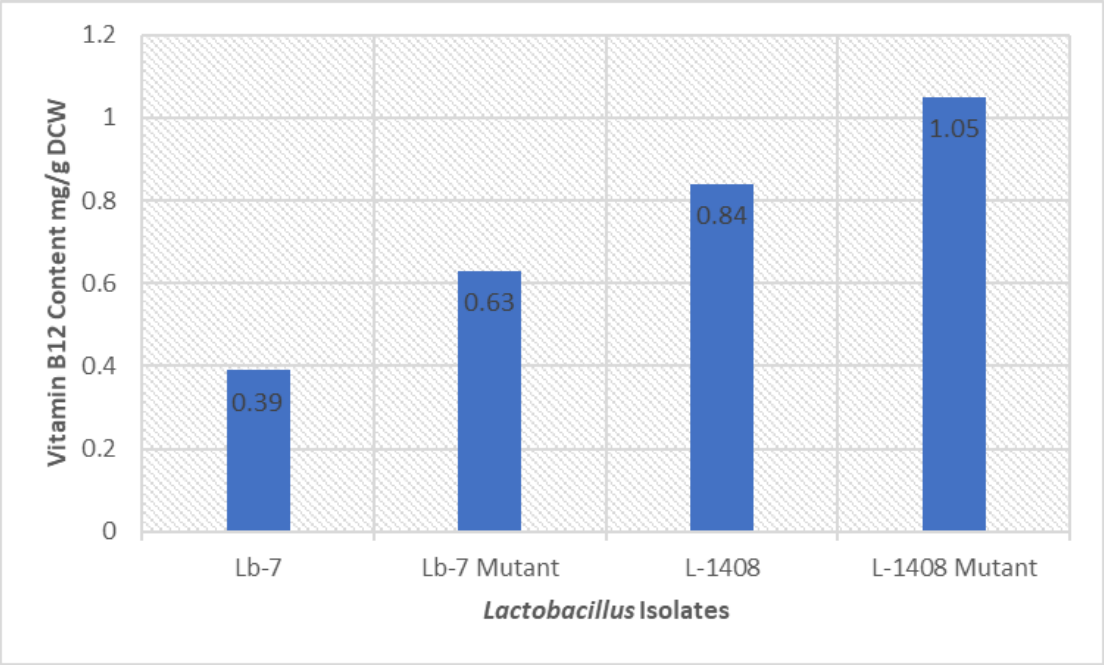


Fig.1: Quantification of Isolates and mutants for the production of Vitamin B12

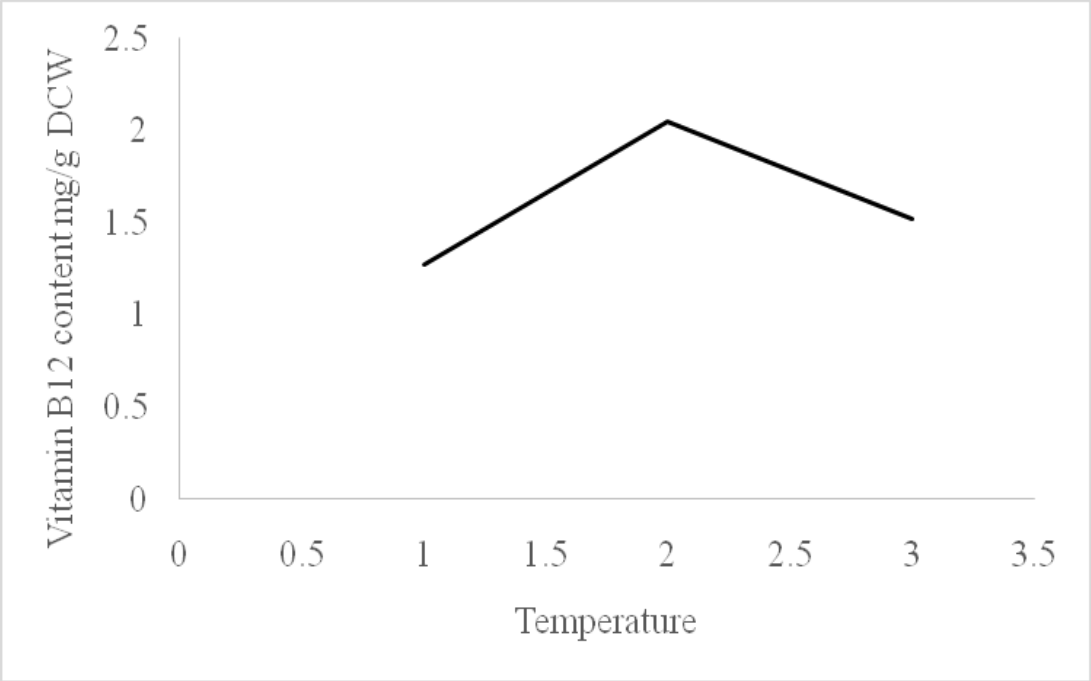


Fig: 2. Effect of temperature on Vitamin B12 content after fermentation by Lb-7 mutant

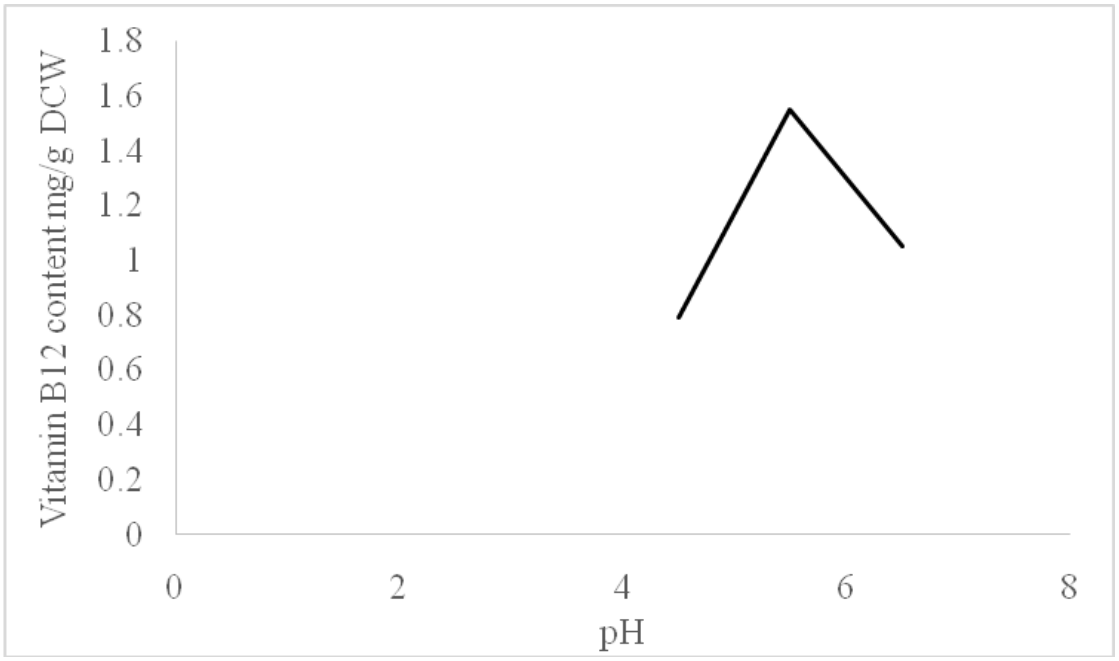


Fig 3: Effect of pH on Vitamin B12 content after fermentation of Lb-7 mutant

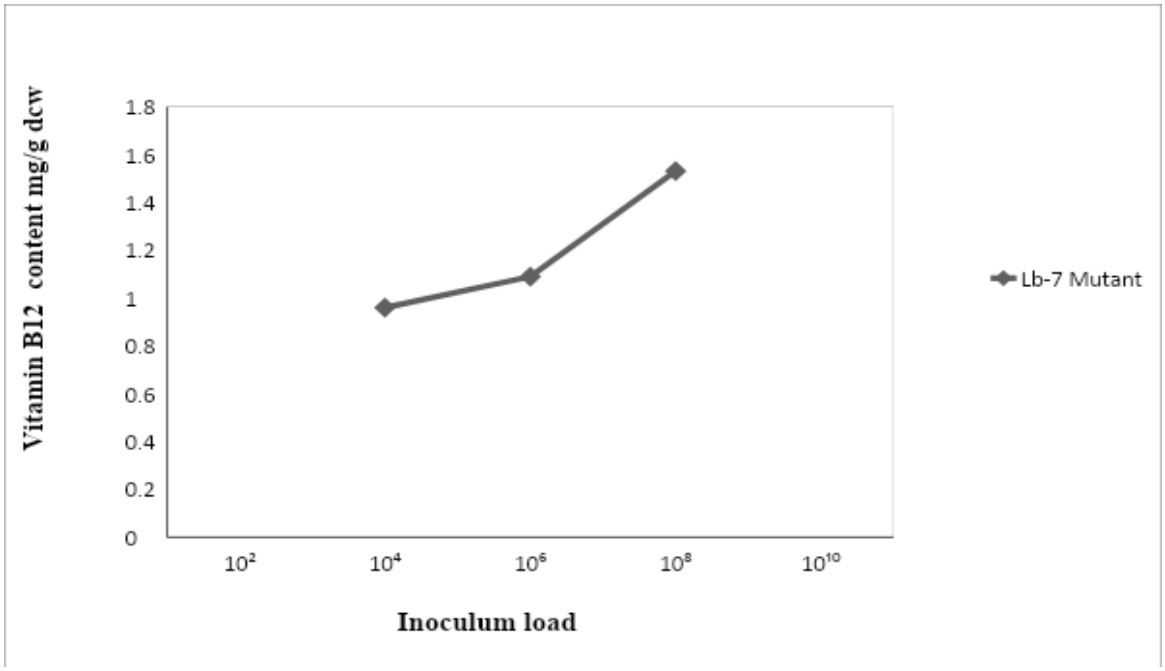


Fig. 4: Effect of Inoculum load on Vitamin B12 content after fermentation by Lb-7 mutant

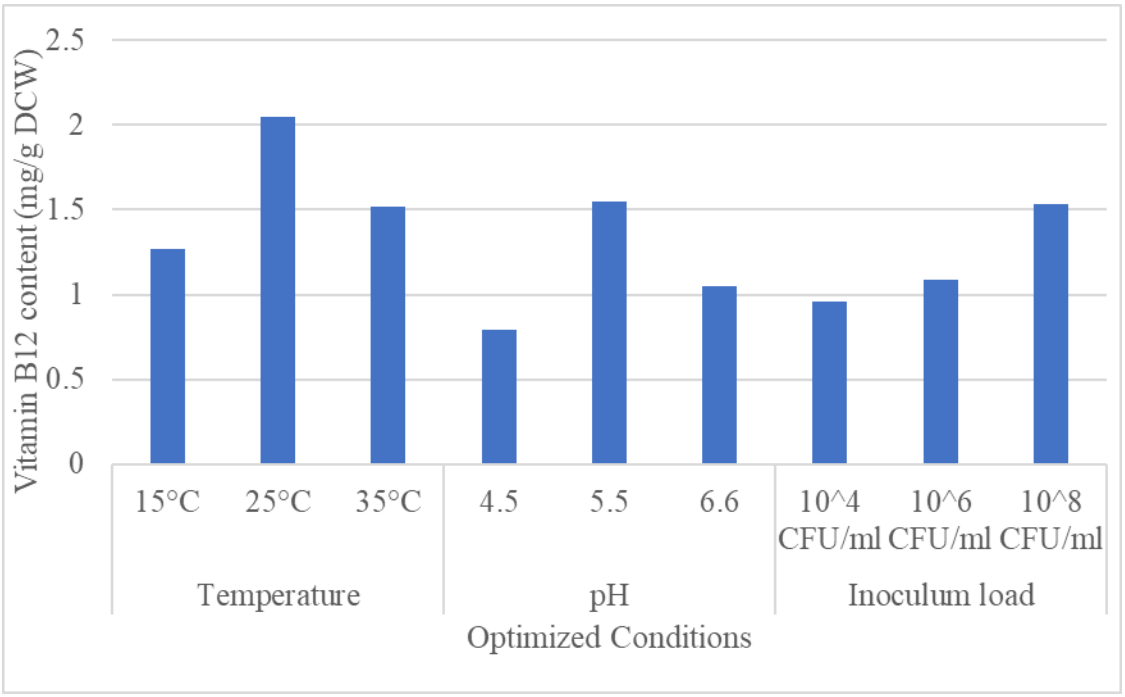


Fig 5: Effect of temperature, pH and Inoculum load on Vitamin B12 content after fermentation by Lb-7 mutant

CHAPTER -5

DISCUSSION

Lactobacillus consists of genetically and physiologically diverse group of Gram positive, rod shaped, non-spore forming bacteria capable of producing Vitamin B12(Kumar and Kumar ,2014). The source of *Lactobacillus* is mainly from dairy and milk products. Isolation of *Lactobacillus* was done from home-made curd and Kaladi samples collected from local markets of district Jammu and Udhampur respectively. Eight different isolates were obtained from different homemade curd samples and kaladi samples. All the isolates were studied for their microscopic and morphological characteristics and Gram's staining of bacterial isolates were done for identification. All the bacteria were purple stained and were Gram positive and long rods shaped.

Screening of the isolates were done for the ability to produce Vitamin B12 on Vitamin B12 assay medium. Lb – 1, Lb -2, Lb-6 and Lb-7 showed growth on Vitamin B12 assay medium. Isolates which showed positive growth on Vitamin B12 assay medium were further streaked on B12 assay medium plates to check the ability of isolates to produce Vitamin B12. LB- 7 showed maximum and fastest growing colonies on B12 medium, hence it was chosen for production B12 medium. Quantification of Vitamin B12 produced by Lb-7 was done by UV – Visible Spectrophotometer. The Vitamin B12 production by Lb-7 was 0.39 mg/g DCW. Then the Strain improvement of Lb-7 was done by mutagenesis. Random mutagenesis has been done to generate mutant strains which can positively produce high yield of product. UV radiations of wavelength 235nm were given to the isolate Lb -7 at different time intervals of 1min, 5min and 10min from a distance of 10 cm. It was observed that at short duration of UV radiations, there was no effect on isolate. When the isolate Lb -7 was UV radiated for longer duration of 15min and 20 min, the cell count reduced. From the above observations least, cell count was recorded at UV exposure for 15min (Plate 11). Moreover, the genetic effect of EMS-mutagenesis on nucleotide sequence by random amplified polymorphic DNA (RAPD) analysis is also considered (Abdelsalam,2018). Mutagenesis of industrial microbial strains is widely used for the improvement of the microbial productivity and Vitamin B12 production (Li *et al*., 2008). In EMS Mutagenesis, Lb-7 was treated with 80µM of EMS at different time intervals of 5m,10m and 20m. It was observed that short duration of EMS, there was no effect on isolate. When the isolate Lb-7 was EMS treated for longer duration of 30 min and 35min, the cell count reduced. The least

cell count was recorded at 30 min (Plate 12). Hence, on the basis of less densed colonies it was observed that the Lb-7 gets mutated. After mutation again, Screening of UV treated Lb-7 sample and EMS treated Lb-7 sample was done by growing both the samples on Vitamin B12 assay medium for growth and the plates were incubated for 24 to 72 hrs. The UV mutated (Lb-7) sample showed fast and densed growth than the EMS mutated (Lb-7) sample. Hence UV mutated Lb-7 sample was further chosen for fermentation studies. The isolate Lb -7 showed Vitamin B12 production at 0.39 mg/g dcw and Lb -1408 showed production of Vitamin B12 at 0.84 mg/g dcw whereas the Lb -7 mutant showed Vit. B12 production at 0.63 mg/g dcw and L-1408 mutant showed Vit. B12 production at 1.05 mg/g dcw (Table -3). From the above table we observed that before doing mutation of the isolates the production of Vitamin B12 was less as compared to Mutants. So, the present study revealed that when mutation was done the productivity of vitamin B12 gets increased. The more production of Vitamin B12 was observed because the resultant strain developed the resistance against the lactic acid production. Lactic acid production caused reduction in production of Vitamin B12 in bacteria (Ali *et al*., 2011).

In our study we took three parameters for fermentation of Vitamin B12 i.e temperature, pH and Inoculum load. Temperature is sensitive parameter for Lb-7 mutant as it showed variation in the production of Vitamin B12 at different temperatures. However, the maximum production of Vitamin B12 was observed at 25°C. Actually, the Vitamin B12 improved gradually from 15°C to 25°C from 1.27 mg/g dcw to 2.05 mg/g dcw but decreased significantly at 35°C to 1.52 mg/g dcw (Table-4). Masuda *et al*., 2012 also reported maximum production of Vitamin B12 at 25°C. At low temperature the Lb-7 mutant initiates the production of Vitamin B12 and showed the maximum production at optimum temperature i.e 25°C. Further increase in temperature than the optimum temperature causes the denaturation of cells and also at higher temperature cells comes under the stress and therefore the rate of production of Vitamin B12 at 35°C was observed low.

We also observed the rise in Vitamin B12 production of Lb-7 mutant from 0.79 mg/g dcw to 1.55 mg/g dcw from 4.5 pH to 5.5 pH but decreased significantly at 6.5 pH to the maximum Vitamin B12 production of Lb-7 mutant was 1.55 mg/g dcw at 5.5 pH to 1.05 mg/g dcw (Table 5). Masuda *et al*., 2012 reported that the Vitamin B12 production was maximum at 5.5 pH. Though the range of Vitamin B12 production by *Lactobacillus* is between (5-7) pH.

In our study it was reported that inoculum load also causes the effect in Vitamin B12 production by Lb-*7* mutant. The three parameters i.e 10^4cfu/ml. 10^6cfu/ml and 10^8cfu/ml was taken as inoculum load. The maximum production of Vitamin B12 was reported in 10^8cfu/ml which was 1.53mg/g dcw. The increasing trend of Vitamin B12 production was observed from 0.96 mg/g dcw to 1.53 mg/g dcw at inoculum load of 10^4cfu/ml to 10^8cfu/ml. The reason for this increase in production from 10^4cfu/ml to 10^8cfu/ml could be due to the presence of more cells for fermentation and further increase the inoculum load had also followed the same trend. It was observed that the Vitamin B12 production increases on increasing the inoculum load. More inoculum load added to the fermentation medium caused the more Vitamin B12 production (Table-6).

CHAPTER-6

SUMMARY AND CONCLUSION

The present study entitled "Studies on Vitamin B12 production by *Lactobacillus species* isolated from milk products" was conducted at Department of Microbiology, SKUAST Jammu with a view to enhance the production of Vitamin B12 by *Lactobacillus* species, doing UV and EMS Mutation of *Lactobacillus* strain and also to optimize the fermentation parameters i.e temperature, pH and inoculum load for Vitamin B12 production. The Vitamin B12 is the most fascinating molecule that plays an essential role in red blood cell formation, cell metabolism, nerve function and the production of DNA.

Lactic Acid Bacteria have the ability to synthesize B group vitamins (folates, thiamine, riboflavin and cobalamin). Thus, the enrichment of foods, particularly dairy foods, with Vitamin B12 appears to be one of the best approached to providing a good source of vitamin B12. In our study we focussed on the isolation of *Lactobacillus* from milk products (home-made curd and kaladi sample) which have the ability to produce Vitamin B12. We have isolated eight samples from home-made curd and kaladi sample. Characterisation of isolates were done both morphologically and microscopically. Screening of the isolates were done on Vitamin B12 Assay Medium. Among 8 isolates, Lb-1,2,3 and Lb-7 showed positive result on Vitamin B12 Assay medium. Quantification of the Lb-7 isolate was done spectrophotometrically. Among the isolates the best isolate i.e Lb-7 was taken further for mutagenic studies. The Lb -7 strain was treated with UV and EMS and it was confirmed further by growing both the UV and EMS treated mutant on screening media that UV mutated strain has shown best potential for taking up for fermentation studies because the more fast and densed colonies were obtained on Screening media plate.

Fermentation studies were carried out taking three parameters i.e temperature, pH and inoculum load. Fermentation procedures were carried out by using flasks standardization and filterates were used to determine the Vitamin B12 production. This gave an idea about the ideal conditions for the fermentation of *Lactobacillus* and this can be used further in enhancing the production of Vit. B12 from the sample. Further many studies have reported the same trends as we observed during our experimental work. The vitamin B12 production by Lb-7 strain was maximum at 25°C, pH 5.5 and inoculum load of 10^8cfu/ml. Also, it was

seen that increasing temperature, pH and inoculum load showed positive effect on Vitamin B12 production however prolonged fermentation would result in the decrease in the vitamin B12 production because of the competition among cells for the usage of fermentation media and hence declined the production of Vit. B12.

To sum up the following conclusions were drawn from the present investigation:

- *Lactobacillus* bacteria is an heterofermentative lactic acid bacterium which can be isolated from milk products i.e curd and Kaladi and has the ability to produce Vitamin B12.
- Total 8 isolates were obtained from curd and kaladi samples.5 isolates were obtained from curd and three were obtained from kaladi samples.
- The isolates were identified on the basis of colony morphology and microscopic identification. Further Gram's staining showed that all isolates are Gram positive and long rods.
- Out of 8 isolates 4 isolates named as (Lb -1 ,2 ,6 and 7) showed positive growth on Vitamin B12 screening media.
- Further Quantification of isolates were done by UV visible spectrophotometer.
- The best strain i.e Lb -7 on the basis of colony characteristics was further taken for the treatment of both UV and EMS mutagenesis.
- The UV mutated strain was taken for fermentation studies on the basis of observance on Screening media (i.e Vitamin B12 assay medium). The UV mutated strain showed fast growing colonies on media as compared to EMS mutated strain.
- Quantitative analysis revealed the enhancement of production after fermentation and it was observed that the optimum temperature for the production of Vitamin B12 by Lb-7 mutant was 25°C, at 5.5 pH and 10^6inoculum load.

It has been reported that metabolic engineering and synthetic biology strategies as well as other traditional strategies that either have been or could be applied to vitamin B12 production. These strategies have been extensively applied in microbial strain engineering to improve the production of the vitamin B_{12} metabolism in microbes, the utilization of these strategies should promote an improved microbial vitamin B_{12} production. Mutant strain i.e Lb-7 developed during this study can further be explored for industrial application. Upscaling of Vitamin B12 for this mutant can be done to replace strains already in use.

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APPENDIX -1

Nutrient Broth (**NB**)

Peptone	:5.00g
Sodium Chloride	:5.00g
Beef extract	:1.50g
Yeast extract	:1.50g
Distilled water	:1000ml
pH	:7.4

Luria Bertani Broth (LB)

Casein enzymic hydrolysate	:10.000g
Yeast extract	:5.000g
Sodium Chloride	:10.000g
pH	:7.5

MRS Agar

Proteose peptone	:10.000g
Beef extract	:0.000g
Yeast extract	:5.000g
Dextrose	:20.000g
Polysorbate 80	:1.000g
Ammonium citrate	:2.000g
Sodium acetate	:5.000g
Magnesium sulphate	:0.100g
Manganese sulphate	:0.050g
Dipotassium phosphate	:2.000g
Agar	:12000g
pH	:6

Vitamin B12 Assay medium

Acicase	:10.000g
Dextrose	:40.000g
Asparagine	:0.200g
Sodium acetate	:20.000g
Ascorbic acid	:4.000g
L-Cystine	:0.400g
L-Tryptophan	:0.200g
Adenine sulphate	:0.020g
Uracil	:0.020g
Xanthine	:0.020g
Riboflavin (Vitamin B12)	:0.001g
Thiamine Hydrochloride	:0.001g
Biotin	:0.004g
Niacin	:0.004g
p-Amino benzoic acid (PABA)	:0.0008g
Calcium pantothenate	:0.0002g
Pyridoxine hydrochloride	:1.000g
Pyridoxal hydrochloride	:1.000g
Pyridoxamine dihydrochloride	:0.400g
Folic acid	:0.020g
Potassium dihydrogen phosphate	:0.020g
Dipotassium hydrogen phosphate	:0.020g
Magnesium Sulphate	:0.020g
Polysorbate 80	:2.000g
Guanine hydrochloride	:0.020
pH	:5.50-6.00

CERTIFICATE-IV

Certified that all the necessary corrections as suggested by the external examiner and the advisory committee have been duly incorporated in the thesis entitled **"Studies on Vitamin B12 production by *Lactobacillus* species isolated from milk products"** submitted by **Ms.Shruti Sharma**, Registration No.**J-19-MBS-42.**

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