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Vitamin B12 Production by Lactobacillus Species Isolated from Milk Products

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ABSTRACT

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/g DCW. 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.

Keywords- Vitamin B12, *Lactobacillus* sp., UV and EMS Mutagenesis, Fermentation.

I. 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 attracting 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 as megaloblastic anaemia that make 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, 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

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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 synthetized, 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).

II. MATERIALS AND METHODS

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

2.1 Sample Collection

The potent Lactobacillus microbial strain capable of producing Vitamin B12 were isolated by serial dilution method from different homemade Curd samples and kaladi samples made of different milk obtained from cow and buffalo and were stored at 4°C.

2.2 Sterilization:

Sterilization of glass wares were done using ethyl alcohol.

2.3 Isolation from Curd sample

Curd is the best source for *lactobacillus* sp**.** Curd is taken in sterilized flask. Under the aseptic conditions curd was serially diluted from 10^{-1} to 10^{-9} from these 9 dilutions $10^{-}5$, 10^{-7},10^{-9} were selected then futher spreaded by spread plate was done on MRS Agar Medium. They were further incubated at 37°C from 24 to 48 h which is the optimum temperature for *Lactobacillus*.

2.4 Isolation from Kaladi sample

Kaladi was firstly crushed and then diluted with water in sterilized flask. Under the aseptic conditions Kaladi was serially diluted from $10^{-1}$to 10^{-9} 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 which is the optimum source for *Lactobacillus*.

2.5 Purification of the isolates

5 curd samples and 3 kaladi samples was isolated and incubated at 37°C for 24 to 48 h (optimum temperature for *lactobacillus*) (Bhushan *et al*., 2017). Streak plate technique was further used for the purification of *lactobacillus*. After purification, plates was observed for isolated colonies. For preserving isolated Colonies, Nutrient Agar stabs was prepared and stored at 4°C (lajodia *et al*., 2017).

2.6 Identification of the isolates

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

2.6.1. Morphological identification

With the help of 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.

2.6.2. Microscopic Identification by Gram's Staining Technique

The isolated bacteria were examined using Gram staining technique and was observed under microscope for their gram 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 microscope. Finally it was observed 10x, and then a drop of immersion oil was placed on smear and observed under 100x magnification of binocular microscope in order to record the microscopic characteristics (Bathlomew,1962).

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2.7. 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 (Hi Media) and incubated at 37°C for 10 days (Martens *et al.*, 2002).

2.7.1 Assay Fermentation for Vitamin B12 Production:

The isolated bacteria wasallowed for growth in vitamin B12 assay broth (Hi Media) 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 (Hi Media) 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 (Hi Media) plates. The plates were incubated at 37°C for 24 to 48 h and check for growth (Jajodia *et al*., 2017).

2.8. Vitamin B12 Quantification by Spectrophotometer

UV Visible Spectrophotometer:

2.8.1. Preparation of standard solution

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

2.9. Extraction of Vitamin B12

2.9.1. 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 (Eppendorf Bio Spectrometer).

2.9.2. 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 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)

2.10 HPLC Analysis*:*

Then the HPLC analysis was done by Shimadzu UFLC system with UV -detector at 361 nm in which Mobile phase – ACN: distilled water (40:60), stationary phase – Mark RP18E column was used and flow rate was set 0.5 ml/ min at 25°C temperature. The run time was 12 minutes and the length of column was 250 ml.

2.11. Strain improvement by random mutagenesis

2.11.1. 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 was plated on NA plate to determine viable count. (Ali *et al.*, 2011).

2.11.2. 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 NA plate to determine the viable count (Abdelsalam *et al.*, 2018).

After doing mutagenesis again Screening and quantification o Vitamin B12 was done using visible spectrophotometer Screening for High Vitamin B12 producing Potents Cell suspensions 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 Lact. 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 fastgrowing colonies was taken for fermentation studies.

2.12. 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 ferrmentation was 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 triplets and blanks was maintained simultaneously for comparison (Qiang et al., 2013).

III. RESULTS

In the present study, Lactic Acid Bacteria was isolated from curd and Kaladi samples for the production of Vitamin B12. The potential isolates were identified on the morphological and microscopic basis. 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 optimization process.

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3.1 Isolation of Desirable Probiotic Bacteria Producing Vitamin B12

Isolation of Lactic Acid Bacteria was carried out from different homemade curd samples and Kaladi samples named as Lb - 1,2,3,4,5,6,7 and L8 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 screening process.

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

3.2 Identification of Potential Bacterias

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. On the basis of Gram stains bacteria are classified as Gram positive and Gram negative.

The identification of Bacterias 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 in Table 1- 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.

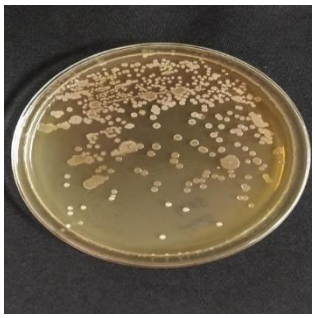
Table 2: Morphological and Microscopic Characteristics of *Lactobacillus* Isolates

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

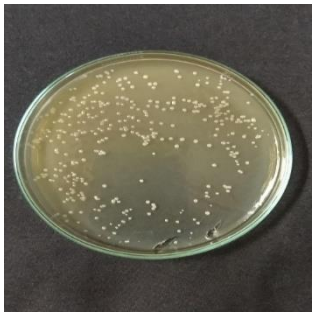
3.3 Screening of the Lactobacillus bacteria on the Vitamin B12 Assay medium

The Lactic Acid Bacteria in **Plate– 1 - 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 growth on Vitamin B12 Assay medium.

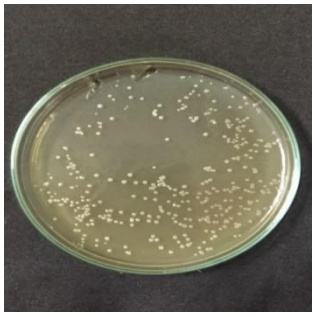
Lb – 1



Lb -2



Lb-6



Lb -7

Screening of Isolates of *Lactobacillus*

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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 grown on **Plate -4 of *Lactobacillus* -7** was taken for mutagenesis.

3.4 Strain improvement by random Mutagenesis

The **Lactobacillus -7** was both **UV and EMS mutated.** In **UV mutagenesis** in plate-7. Culture was grown overnight in 50 ml of NB medium in 500 ml flask. 5 ml of this suspension was placed in sterile Petri dish and was exposed to UV rays. At regular intervals , the sample was taken out and different dilutions was placed on NA to determine the viable count. (M.N. Ali , 2011).

It was observed that mutated strain showed more densed colonies as compared to control of Lb -7 . UV radiation was given to the isolate of Lb -7 at different time intervals of 30s, 60s, 90s ,120s and 150s. The results indicate that short duration of UV radiation didnot develop any resistant characters in mutant strain towards the production of Vitamin B12 . Also when the Lb -7 was UV radiated with higher dose for longer duration i.e 90s, 120s and 150s caused decrease in the Vitamin B12 production and when the Lb-7 was UV radiated for short duration i.e 30s , 60 s caused increase in the production of Vitamin B12. So at higher doses of UV with short duration developed the resistance in mutant for the production of vitamin B12. Hence the rate of production of Vitamin B12 increased in mutant strain.(M.N. Ali et al., 2011)

In 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 was washed and plated on NA plate to determine the viable count(Abdelsalam *et al.*, 2018).

In **EMS mutagenesis** it was observed the eight fold increase in the production of Vitamin B12 in both i.e Lb- 7 and Lb -1408 as compared to untreated sample. However the production in Lb-1408 mutant was more than that of Lb-7 mutant. Though the viability of cells decreases but the production of Vitamin B12 gets increased after when EMS Mutation was done. Also from Various studies it was reported Mutations are induced randomly in a microbial DNA by the implementation of chemical and physical mutagenic agents. Among the most popular mutagens used for the bacterial strain improvement is EMS which typically produces a variety of point mutations in the bacterial DNA. Through these point mutations, the different mutants are produced at high level in comparison of the natural mutations. Mutagenesis of industrial microbial strains is widely used for the improvement of the microbial productivity and Vitamin B12 production (Li *etal.*, 2008).

Table 3: Quantification of Isolates 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

In our Study in table -3 the isolate of Lb -7 showed production of Vitamin B12 at 0.39 mg/g dcw and L-1408 showed Vitamin B12 production at 0.84 mg/g dcw where as Lb -7 mutant showed Vitamin B12 production at 0.63 mg/g dcw and L-1408 mutant showed production at 1.05 mg/g dcw . 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 the normal isolates . It was estimated that after when mutagenesis was done the rate of vitamin B12 production was increased of Lb -7 mutant by 0.24 mg/g dcw and L-1408 mutant by 0.81 mg/g dcw . This showed that by doing the 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 also gets produced along with vitamin B12 . So after mutation the rate of production of Lactic acid decreases therefore the vitamin B12 production increases. Before mutation the isolates produces lactic acid together with Vitamin B12 which reduces the production and the vitamin B12 produces less effectively. Hence after when UV mutation was done the more production of Vitamin B12 was observed because the resultant strain gets developed the resistance against the lactic acid production. Also it was reported in study M.N Ali etal ., 2011 that lactic acid production caused reduction in production of Vitamin B12 in bacteria.

3.5 Fermentation process Optimization:

Fermentation studies were done by shake flask method. Fermentation parameters i.e temperature, Inoculum load and pH were optimized using 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 *Lactobacillus* -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 *Lactobacillus* bacteria , maximum increased in Vitamin B12 content was showed in 25° C of temperature and the total

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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 get decreased.

In case of pH maximum increased 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 10^6cfu/ml and 1.53 mg/g dcw at 10^8 cfu/ml.

Table 4: Effect of Temperature on Vitamin B12 Content After Fermentation by Lactobacillus Species Total Vitamin B12 content mg/g DCW

Temperature	Lb -7 Mutant
15°C	1.27±0.35mg/g DCW
25°C	2.05±0.68mg/g DCW
35°C	1.52 ±0.35mg/g DCW

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

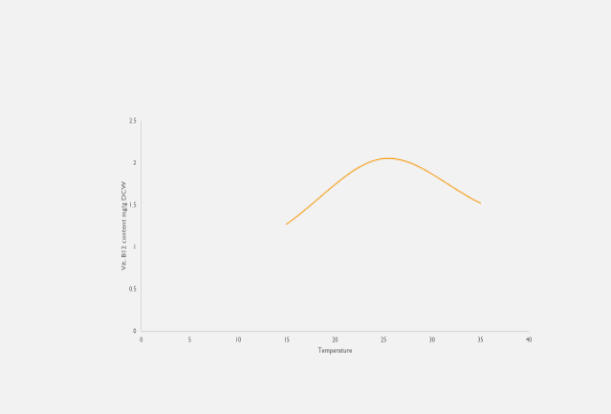

Figure 1: Effect of temperature on Vitamin B12 content after fermentation by *Lactobacillus* species.

Table 5: Effect of pH on Vitamin B12 content after Fermentation by *Lactobacillus* species Total Vitamin B12 content mg/g DCW

pH	Lb -7 Mutant
4.5	0.79±0.17mg/gDCW
5.5	1.55±0.53mg/gDCW
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.

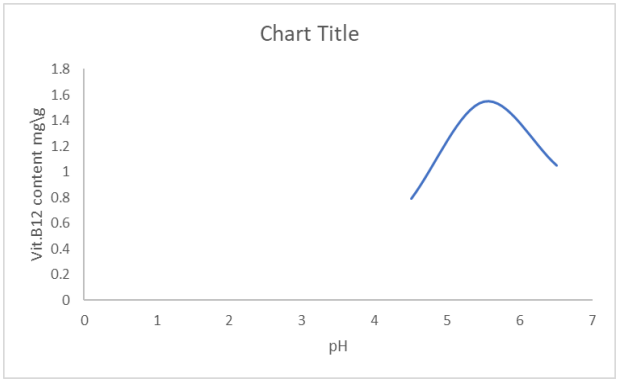

Figure 2: Effect of Vitamin B12 content after fermentation by *Lactobacillus* species.

Table 6: Effect of Inoculum load on Vitamin B12 content After Fermentation by *Lactobacillus* species

Inoculum load	Lb -7 Mutant
10^4	0.96±0.20 mg/g DCW
10^6	1.09±mg/g DCW
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.

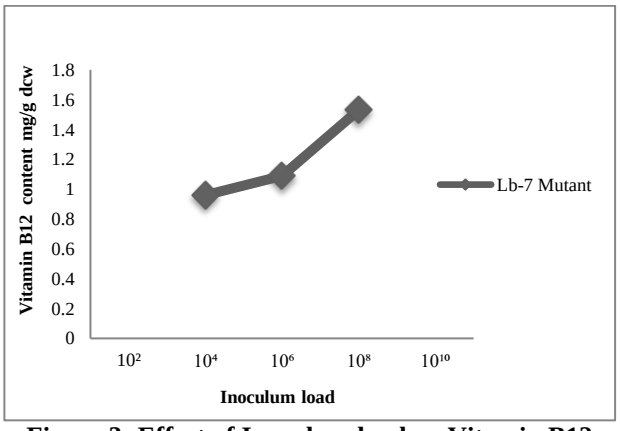

Figure 3: Effect of Inoculum load on Vitamin B12 content after Fermentation by *Lactobacillus* specie

IV. DISCUSSION

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 staining of bacterial isolates were done for distinguishing between Gram positive and Gramnegative bacteria. All the Bacterias were purple stained and were Gram positive and long rods shaped.

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Screening and Quantification of the isolates was done for the ability to produce Vitamin B12 by inoculating the isolates on Vitamin b12 assay medium. Further then the organisms 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 – 1, Lb -2, Lb-6 and Lb-7 showed positive result on Vitamin B12 assay medium having the ability to produce Vitamin B12. Further Quantification of Vitamin B12 was done by UV – Visible Spectrophotometer for extraction of Vitamin B12 from both supernatant and pellet by harvesting the cells by centrifugation and ultrasonic cell disruption. The qualitative analysis for Vitamin B12 was done with eight isolates from supernatant of fermentation media.

Then the HPLC analysis was done for qualitative confirmation of Vitamin B12. From all the above samples i.e the Lb -1,2 6 and Lb -7, the fastgrowing colonies (i.e Lb-7) was chosen further for mutation. Then the Strain improvement of Lb-7 was done by mutagenesis. The Lb -7 and Lb -1408 both were treated with UV mutation and after mutation was done both the samples were compared with untreated samples. Hence it was reported that the treated samples showed more growth than the untreated sample which explained that the strain gets improved after doing mutation.Similarly in case of EMS mutagenesis, the Lb-7 and Lb-1408 was treated with Ethyl methane sulfonate (EMS). After Mutation both the samples were compared with untreated samples and EMS treated samples showed more growth compared to untreated samples. After Mutation again Screening of UV treated Lb-7 sample and EMS treated Lb-7 sample was done by growing on Vitamin B12 assay medium and the medium was supplemented 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 where as 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 in 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 after when mutation was done the productivity of vitamin B12 gets increased. Ali etal., 2018 find out the increase in Vitamin B12 production after doing UV Mutagenesis.

In our study we took three parameters for fermentation of Vitamin B12 i.e temperature, pH and Inoculum load Temperature is sensitive parameter for *Lactobacillus* 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. M Masuda etal., also reported maximum production of Vitamin B12 at 25°C. At low temperature the *Lactobacillus* mutant- 7 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. Ide M 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 *Lactobacillus* species. The three parameters i.e 10^4 cfu/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 10^8 cfu/ml could be due to the presence of more cells for fermentation and further increase the inoculum load had also followed the same trend. From table -6 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.

V. 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 check the capability of *Lactobacillus* bacteria of producing Vitamin B12 and enhance the Production by doing UV and EMS Mutation of Lactobacillus Strain and also to optimize the fermentation parameters i.e temperature, pH and inoculum load. 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

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isolation of *Lactobacillus* from dairy products which have the ability to produce Vitamin B12.The qualitative screening and substantiation for the vitamin B12 production were done on the isolates obtained from kaladi and curd samples. 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 10^6cfu/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.

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