Journal Homepage: -www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI:10.21474/IJAR01/3350
DOI URL: http://dx.doi.org/10.21474/IJAR01/3350
ISSN NO. 2320-5407

RESEARCH ARTICLE

BIOSYNTHESIS OF POLY-β-HYDROXYBUTYRATE AND DISTRIBUTION OF *phbC* GENE IN LACTOBACILLUS PLANTARUM.

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Manuscript Info

Manuscript History

Received: 28 December 2016
Final Accepted: 20 January 2017
Published: February 2017

Key words:-
poly-β-hydroxybutyrate, *Lactobacillus*, *phbC* gene, bioplastics.

Abstract

Accumulation of synthetic plastics in the environment has become a worldwide problem. Polyhydroxy alkanoates (PHA) are biodegradable plastics, an alternative to petroleum-based synthetic plastics. In this investigation, the best known PHA, poly-β-hydroxybutyrate (PHB) accumulating strains of *Lactobacillus plantarum* were isolated from various food and environmental samples using MRS agar medium. Among the 31 isolates, 22 strains were identified as *Lactobacilli* by comparing the biochemical profile with *Lactobacillus plantarum* MTCC6160. The *L. plantarum* strains were confirmed with 16S rRNA identification method and 11 among the 22 *Lactobacillus* strains were revealed the characteristic band at 735 bp. The partially amplified product was sequenced and the comparison of the sequence in NCBI BLAST obtained 94% similarity with the strain, *L. plantarum* IMAU70089. The isolated strains of *L. plantarum*was subjected to PHB production using nitrogen limited minimal medium (NLMM).The inclusion bodies were stained with Sudan Black B and the organism was also plated on Nile blue A medium, revealed the presence of a lipid material, which was confirmed as PHB by the analysis of molecular fragments by FTIR spectroscopy in comparison with reference material. In addition, the existence of *phbC* gene (578 bp) was identified, which encodes the enzyme, PHB synthase for accumulating PHB. This investigation confirmed that 1 mg of the cell dry weight constitutes the maximum of 40.4 µg of PHB. This study revealed that *L. plantarum*has been recognized as a good candidate for PHB.

Introduction:-

Synthetic plastics produced from the petrochemical source are non-degradable and cause waste disposal problems leading to environmental pollution (Muller et al., 2001). Polyhydroxyalkonoates and their co-polymericderivativeshave emerged as very attractive substitutes for synthetic plastics due to their complete

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biodegradability (Kumar and Prabakaran, 2006) and similar physical properties to synthetic plastics (Chee et al., 2010).

Polyhydroxyalkanoates comprise a large class of polyesters that is synthesized by many bacteria as an intracellular carbon and energy compound (Lee and Chang, 1994). PHBs are synthesized when the cells surrounding contains unbalanced growth conditions such as limited concentrations of oxygen, nitrogen, phosphorous, sulfur or trace elements like magnesium, calcium, ferrous and high concentrations of carbon.

PHB is a linear polyester of D (-) β-hydroxybutyrate and the best-known polymers of polyhydroxyalkanoates. Bacteria produce PHAs with average molecular mass up to $4.0x10^6$ Da with a polydispersity of around 2.0. Many organisms accumulate polyoxoesters of short carbon chain length hydroxyalkonic acids comprising of three to five carbon atoms called polyhydroxybutyrates.

More than 250 different microorganisms are reported to accumulate PHB. Only several of these such as *Alcaligeneseutrophus*(Kim et al., 1994), *Alcaligenes latus* (Yamane et al., 1996), *Azotobactervinelandii*(Page and Knosp 1989), methylotrophs (Kim et al., 1996), *Pseudomonas oleovorans*(Brandl et al., 1988) and recombinant *Escherichia coli* (Lee and Chang, 1994) are suitable for the production of PHAs to a high concentration with high productivity.

The major focus of many investigators is to make their production economically competitive with polypropylenes. Many soil and environmental microorganisms were estimated for the PHB production by many researchers, very less study were reported in *Lactobacillus*. Hence, this study is an attempt to reveal the percentage production of PHB in *L. plantarum* isolated from the food and environmental sources. These are capable of supplying rich protein and also they have the ability to accumulate some amount of PHB. Biosynthetic processes of producing polymers are often financially uncompetitive. Thus, research has focused on modifying microorganisms to improve product yields and increase substrate ranges. Many controlling factors need to be considered in an effort to modify metabolic fluxes responsible for bioplastic production.

The *phb*C gene is responsible for the production of PHB synthase in an organism apart from *phbA* and *phbB* which are responsible for the production of β-ketothiolase and acetoacetyl-CoA reductase respectively (Sharma et al., 2007). Identification of these genes is the confirmation of PHB production in the respective strain.

Materials and Methods:-

Sample collection:-

The food and environmental samples such as raw milk (n=6), pasteurized milk (n=6), butter (n=3), cheese (n=1), curd (n=6), buttermilk (n=3), grey water (n=4) and soil (n=2) were collected from various locations in Erode, South India in sterile screw cap tubes and processed within 3 h from the time of collection.

Isolation and presumptive identification:-

The collected samples were serially diluted (tenfold dilution) and 0.1 mL of eachdilution was plated on sterile de Man Rogosa Sharpe (MRS) medium by spread plate technique (Emmanuel et al., 2005). The plates were maintained under an aerobic condition at 30°C for 24-48 h. The purified cultures were preserved and maintained at -4°C in MRS agar slants.

The presumptive Identification of *Lactobacilli* was carried out based on the microscopic, cultural, and biochemical identification methods. *L. plantarum* MTCC 6160 (Microbial type culture collection, IMTECH, India) was used as areference strain in this study.

Isolates were identified microscopically on the basis of Gram staining, endospore staining, motility test, and phenotypically on the basis of Kovac's oxidase and catalase test, followed by a series of biochemical tests such as indole production, methyl red test, Voges-Proskauer test, utilization of citrate, reduction of nitrate to nitrite and H_2S production, urease and carbohydrate fermentation.

Extraction of genomic DNA:-

Aliquots of 1 mL of 24 h cultures grown in MRS broth were centrifuged at 6000 rpm for 15 min at 4°C in Eppendorf tubes. The supernatant was discarded and the pellet was suspended in 467 µL of TE buffer (10 mMTris

base and 0.5 mM EDTA), 30 µL of 10% sodium dodecyl sulfate, 3 µL of proteinase k and incubated at 37°C for 1 h. An equal volume (500 µL) of phenol–chloroform (24:1) was added to the content and mixed gently. The tubes were centrifuged at 10000 rpm for 10 min at 4°C. The upper aqueous phase was transferred to a fresh Eppendorf tube. The DNA from the aqueous phase was precipitated by the addition of one-tenth the volume of 3 M sodium acetate and 330 µL the volume of ice-cold isopropanol. The precipitated DNA was pelleted by centrifugation at 3000 rpm for 3 min at 4°C and the DNA was washed with 70% ethanol and the pellet was air dried.

PCR amplification of 16S rRNAgene:-

Unique primers were designed for the amplification of the genes coding for the 16S rRNA of *Lactobacillus plantarum*. Primers were designed from the conserved regions and searched for their uniqueness and specificity to the respective 16S rRNA gene from different *Lactobacillusplantarum* by basic local alignment search tool (BLAST). A 750 bp fragment from the *Lactobacillus plantarum* encoding 16SrRNA gene was identified by using a primer set. The amplification was performed with forward primer, 5'-TTCATCCGAATAACCC-3' and reverse primer,5'-CGACAGTAACGGAGGT-3'.

The PCR reaction was performed in 25 µL reaction volume containing 2µL of template DNA (20 ng μL^{-1}), 1 µL (1 µM)each of forwarding primer and reverse primer, 12.5 µL of PCR master mix(Fermentas, USA) contains 0.05 U/µLTaqDNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 M of each dNTP (dATP, dCTP, dGTP and dTTP)and 8.5 µL of nuclease-free water. The reaction conditions were as follows: initial denaturation at 94°C for 5 min, 30 cycles of amplification, each consisting of denaturation at 94°C for 1 min, annealing at 57.9°C for 1 min, elongation at 72°C for 1 min followed by a final extension at 72°C for 10 min.

The PCR products (10 µL) were electrophoresed in 1.5% agarose gel for 2 h at 50V in theTris-acetate-EDTA buffer. The size of the amplification products was determined by comparison with 100 bp DNA ladder (Helini biomolecules, Chennai).Amplified products under optimum conditions with expected size were purified using purification kit (Qiagen, India) and sequenced. The generated nucleotide sequences were subjected to further analysis.

Screening of PHB producers:-

A pure culture of Lactobacilli grown in 250 m flasks containing 50 mlof nitrogen limited minimal medium (NLMM) comprised of the following (per liter); 3.8 g Na_2HPO_4, 2.65 g $KHPO_4$, 2 g NH_4Cl, 0.2 g $MgSO_4$, 2 g fructose and 1 ml trace minerals. Trace element solution contains (per liter): 5 g EDTA, 2.2 g $ZnSO_4$, 5.4 g $CaCl_2$, 5.6 g $MgCl_2$, 0.05 g boric acid, 4.79 g $FeSO_4$, 22.4 g $(NH_4)_6MoO_{24}$, 1.6 g $CoCl_2$, and 1.57 g $CuSO_4$. The pH of the media was maintained at 7.0. The culture flask was kept in ashaker at 250 rpm at 37°C for 48 h. The PHB producers were screened microscopically for the presence of metachromatic granules by Sudan black B staining method (Burdon, 1946) and also by colony morphology by inoculating the isolated strainon nutrient agar medium supplemented with 1% Nile blue A (Ostle et al., 1982).

Extraction of PHB:-

The PHB was extracted from the 24-72h old cultures of *L. plantarum*. The bacterial cultures were harvested from 20 mL by centrifugation at 6000 rpm for 15 min. The cell pellet was dried at 40 ± 1°C for 24 h and the dry cell weight was estimated. The cell pellet was suspended in 5 mL of sodium hypochlorite, 5 mL of chloroform and incubated at 30°C for 1 h. Then the incubated content was centrifuged at 1500 rpm for 15 min at room temperature. Three phases were obtained, the lower PHB containing chloroform phase was recovered without disturbing the other phases. Chloroform was evaporated and the PHB crystals on the walls of the tubes were collected and stored.

Qualitative estimation of PHB:-

The extracted PHB was estimated qualitatively by TLC. The extracted PHB was dissolved in chloroform and 50 µL samples was loaded on the TLC plate and chromatogram was developed with a solvent system consisting of benzene and ethyl acetate (1:1). The resolved spot was visualized by exposing the dried plate to iodine vapor for 10 min. The R_f value of the spot with greenish brown color was measured(Kumar and Prabakaran, 2006).

PHB was qualitatively estimated using FTIR spectrophotometer. The PHB from five different strains were selected randomly for analysis. (Kumar and Prabakaran, 2006). The pure form of PHB (1 mg) was thoroughly mixed with 15 mg KBr (Spectroscopic grade) and the treated pellet was dried at 100°C for 4 hrs. The FTIR spectrum of the compound was analyzed in the range of 400-4000 cm^{-1} ina Perkin-Elmer (USA) model 1720 FTIR spectrometer.

Quantitative estimation of PHB:-

The production of PHB by *L. plantarum* was estimated as crotonic acid. The extracted PHB crystals were dissolved in 5 mL of chloroform and converted to crotonic acid by the addition of 5 mL of concentrated sulfuric acid and boiled at 100°C for 10min. Then the tubes were cooled to 25°C and the absorbance was measured at 235 nm using a UV-spectrophotometer and a standard graph were plotted with pure crotonic acid in the dilutions of 1-10 µg mL.

PCR amplification of *phbC*gene:-

The production of PHB in *L. plantarum*was confirmed by PCR amplification of *phbC* gene which encodes PHBsynthase using the primers F1- 5' CGCAATCCCGTTGATAAG 3' and R1- 5' CGCTTTTCAGGATCAATGTC 3'(Labmate Asia, India).The amplification was performedin 20 µL reaction mixtures, containing 10 µL of PCR master mix (Fermentas, USA) contains 0.05 U/µLTaq DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 M of each dNTP (dATP, dCTP, dGTP and dTTP), 5 µL of nuclease-free water, 1.5 µL (1 µM) each of forward and reverse primer and 2 µL of template DNA (20 ng μL^{-1}). The DNA amplification was performed using a thermal cycler (MJ Research, Model PTC 100 Watertown, Mass., USA). The amplification conditions were initial denaturation at 94°C for 5 min, 30 cycles of amplification, each consisting of denaturation at 94°C for 1 min, primer annealing at 52.3°C for 1 min, and elongation at 72°C for 1 min followed by a final elongation of 72°C for 5 min. Following amplification, the PCR products were subjected to horizontal agarose gel electrophoresis through 1.5% agarose gel supplemented with ethidium bromide solution (0.5 µg/mL). The size of the amplification products was determined by comparison with 100 bp DNA ladder (Helini biomolecules, Chennai).

Results:-

Isolation and identification:-

The distribution of Lactic acid bacteria was enumerated in each sample (n=31). The percentage incidence of *Lactobacilli* was analyzed and tabulated (Table 1). The microscopic identification confirmed the presence of Gram-positive rod, non-spore forming, and non-motile bacteria. On MRS agar plates *Lactobacilli*showed pure white, regular, and small (2 to 3 mm diameter) colonies. The *L. Plantarum*strainswere identified by biochemical profiling by comparing with the MTCC6160.Among the 31 isolates 22 isolates were identified as *Lactobacilli* by biochemical profiling and 11 among the 22 *Lactobacilli* were confirmed to be *L. plantarum*by 16S rRNA identification method. The PCR amplification of 16S rRNA revealed the characteristic band at 735 bp for all the strains. The partially amplified product of one among the 11 isolates was sequenced and designated as *L. plantarum* H1 by comparing phylogenetically with the other *Lactobacillus* strains using public databases (Fig. 1). The comparison of the sequence in NCBI BLAST obtained 94% similarity with the sequence of the strain *L. plantarum* strain IMAU70089 16S ribosomal RNA gene, and their phylogenetic analysis positioned the strain nearer to *L. plantarum*(6Q131205).

The forward sequence of the 16S rRNA gene of *L. plantarum:-*

>read seq. input (1), 409 bases, 29A496B6 checksum.

CAAATGACCACGTTTTTTCCCCATCCTGGCGGACGGGTGAGTAACGCGTAGGAATCTATCCGTGGGTG
GGGGATAACTCTGGGAAACTGGAGCTAATACCGCATGATACCTGAGGGTCAAAGGCGCAAGTCGCCT
GCGGAGGAGCCTGCGTTTGATTAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGATGATCAATAGCT
GGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATATTGGACAATGGGGGCAACCCTGATCCCCAATGCCCGTGTGTGAATAAGTTTTCCTATTGT
ACAGTTTTCCGACATGATACAGGCCGTAATCTACACATATCTGCATCGAGAGGCCACTTCAGACCAGT
ACG

The Reverse sequence of the 16srRNA gene of *L. plantarum:-*

>read seq. input (1), 223 bases, 784537BC checksum.

ACACGTCGAGTGAATACTGCCGGGGATTTTACATCTGACTGTACAACCGCCTACACGCCCTTTACGCCC
AGTCGTTCCGAGAAACGCTAGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTT
CTTCTGCGGGTACCGTCATCATCGTCCCCGCCGAAAGTGCTTTACATCCGAAAACCTTCTTCAAACGGC
TTGTGGAAAAGTGAGCC

Screening for PHB producers:-

The PHB accumulated in the cells was microscopically observed as blue stained metachromatic granules against the pink background of the cells and the colonies were observed as blue color in the Nutrient agar with 1% Nile blue A solution. Among the 11, 8 isolates were reported to be PHB producers using presumptive screening methods (Table

2). About 72.7 % of PHB producers among the strains of *L. plantarum* was reported based on Sudan Black B staining and Nile blue A method indicates a maximum probability of PHB in these strains.

Qualitative estimation of PHB:-

A single spot on TLC of extracted PHB granules indicates the purity of the molecule and the obtained R_f values of 0.81 is higher than the normal. The R_f value of the PHB molecule increases with its rate of polymerization and the decrease in therate of proponalysis(Panda et al., 2008). In this study, the sample is not processed by propanolysis, the value higher.

PHB samples of five strains were randomly selected for FTIR analysis. Comparison of the FTIR results with standard PHB revealed the similarity that exists in the functional group of the molecule. The IR analysis showed the presence of CH_2-CH_3-C=O methyl ester groups confirmed that the extract was PHB. The C-O bond shows thestrong and broadband at 1047-1089 cm^{-1}. The bands found at 1442-1488 cm^{-1} correspond to the asymmetrical C–H bending vibration in CH_3 group, while the series of bands found between1000–1200 cm^{-1} correspond to the stretching of the C–O bond of the ester group. The values of C=O were observed between 1626.05-1637.62 $cm^{-1.}$ The values of C=O are slightly lower than the standard value 1712 and this may be due to polymerization of the molecules (Kadouri et al., 2002). The absorption band at and around 3450 cm^{-1} corresponds to the terminal OH group (Fig. 2) (Table 3).

Extraction and Quantitative estimation of PHB:-

The overall dry weight of the cell was found to be 0.5-1.0 mg. The PHB accumulation of different strains of *L. plantarum*was estimated against the standard crotonic acid curve and the values were tabulated (Table 4). The PHB accumulated was found to increase with nutrient stress and varied considerably with the strains. After 24 h of nutrient stress, the concentration of PHB increased in all the strains. The strain produced PHB from a range between 0.2-8.8 µg/mL. Highest accumulation of 8.8 µg/mL was found in *L. plantarum* H and *L. plantarum* 12 and the lowest and negotiable concentration of 0.2 µg/mL was produced in *L. plantarum* 05.

Confirmation of PHB producers by PCR Method:-

The isoalates which accumulating the PHB in higher amount (LP01, LP02, LP03, LPH1 and LP12) were analyzed for the presence of PHB synthase encoding gene, *phbC*by PCR amplification using unique primers.The conserved sequence of *phb C* gene available in all the strains. The bands in the region of 585bp revealed the presence of *phbC*gene (Kadouri et al., 2002) and the ability of the strains to produce PHB. The amplified DNA products also revealed the minor diversification among the strains.

Table 1:-Distribution of lactic acid bacteria and percentage incidence of *Lactobacilli*

S. No.	Samples	No. of Samples collected	Lactic acid bacteriaIncidence (X 10^4cfu mL^{-1})		Number of *Lactobacilli* Isolated	%
			Maximum	Minimum		
1.	Curd	6	53	19	6	100
2.	Raw milk	6	17	12	4	67
3.	Pasteurized milk	6	9	1	2	33
4.	Butter	3	67	22	2	33
5.	Butter milk	3	19	5	3	100
6.	Cheese	1	12	0	1	100
7.	Grey water	4	7	2	3	75
8.	Soil	2	8	6	1	50

Table 2:-PHB screening of *Lactobacillusplantarum*

Strain No.	Sudan black B	Nile blue A
LP01	+	+
LP02	+	+
LP03	+	+
LP05	+	+
LP07	+	+
LP10	-	-

LP12	+	+
LP21	+	+
LP25	-	-
LP27	-	-
LP31	+	+
L. plantarum MTCC6160	+	+

+ PHB producers; - Non-PHB producers

Table 3:-IR spectrum of PHB

Samples	Peak region	Comments
LP 01	3384.22-3439.34	Intramolecular hydrogen bonding
	2924.18-3056.31	CH_2 stretch
	1626.05-1637.62	C=O
	1258.59	O-H tertiary alcohol
	1061.85	C-O stretching
LP 03	2925.15-3549.14	Intramolecular hydrogen bonding
	2925.14-3549.14	CH_2 stretch
	1636.65-1619.25	C=O
	1255.70	O-H tertiary alcohol
	1063.78	C-O stretching
LP 05	3240.52-3266.56	Intramolecular hydrogen bonding
	2924.18-3087.17	CH_2 stretch
	1636.65	C=O
	1255.70	O-H tertiary alcohol
	1063.78	C-O stretching
LP 12	3388.08-3482.59	Intramolecular hydrogen bonding
	2923.22-2956.97	CH_2 stretch
	1636.65	C=O
	1065.51	O-H tertiary alcohol
	1066.71	C-O stretching
LP31	2856.67-3562.64	Intramolecular hydrogen bonding
	2923.22-2956.97	CH_2 stretch
	1637.62-1618.37	C=O
	1256.67	O-H tertiary alcohol
	1062.81	C-O stretching

Table 5:-Quantitative estimation of PHB of *L. plantarum* in NLMM broth

Bacteria	Crotonic acid concentration (µg/mL) under stress		
	24 h	48 h	72 h
L.plantarum 01	3.0	4.3	4.4
L.plantarum 02	4.7	5.6	7.4
L.plantarum 03	1.5	2.3	4.3
L.plantarum 05	0.2	1.2	3.1
L.plantarum H1	5.7	8.8	8.8
L.plantarum 12	3.9	5.4	8.8
L.plantarum 21	0.7	2.4	3.9
L.plantarum 31	2.7	5.7	5.9

Fig. 1:-Phylogenetic tree of *Lactobacilli.*

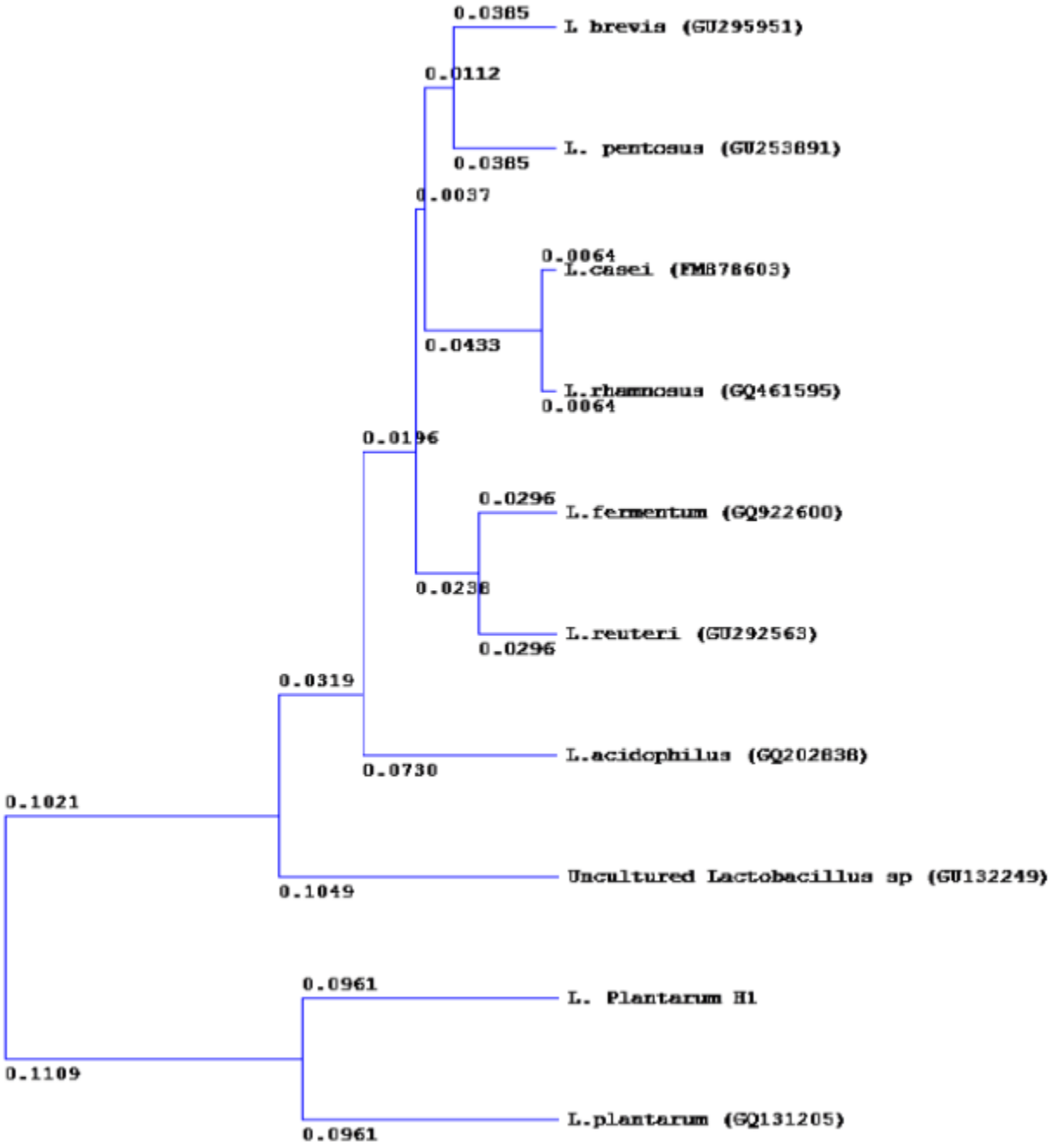


Fig. 2:-FTIR spectrum of PHB (*L.plantarum*01)

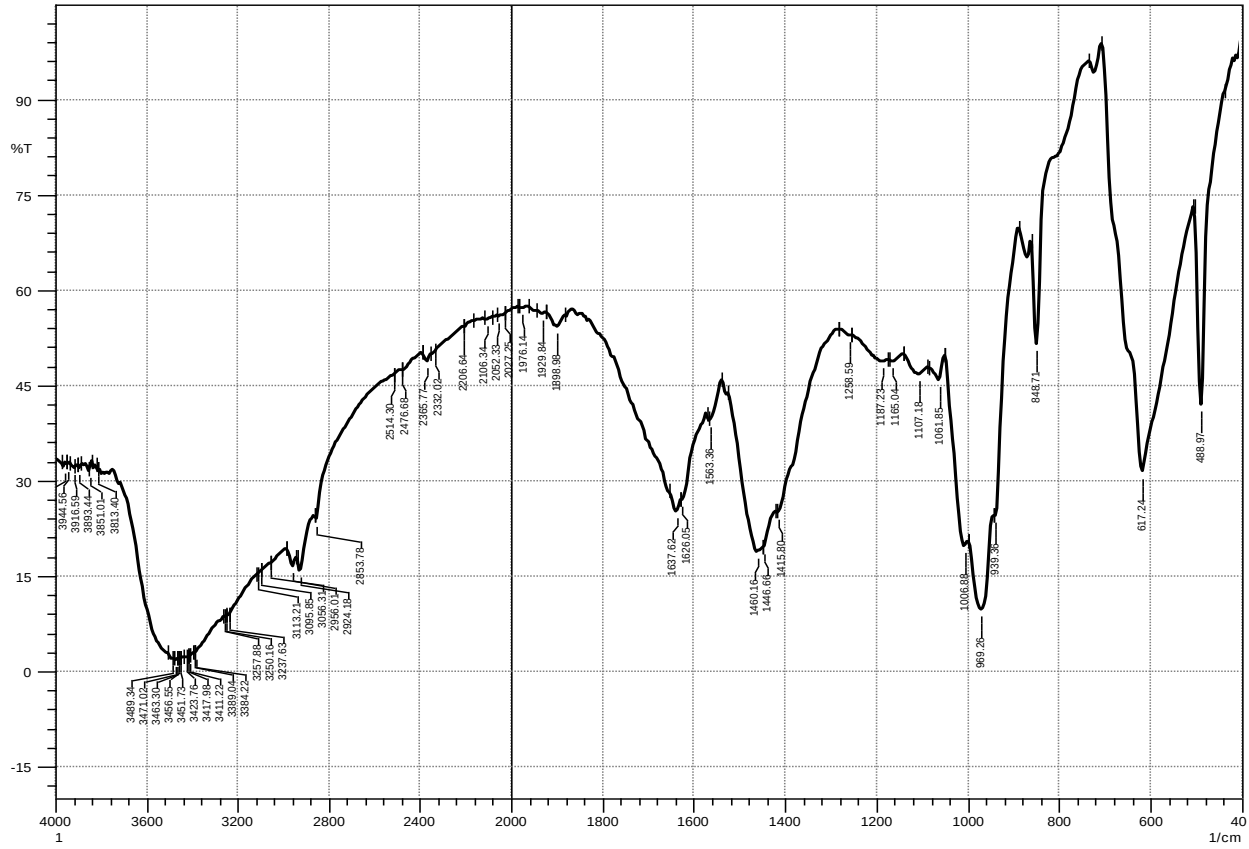
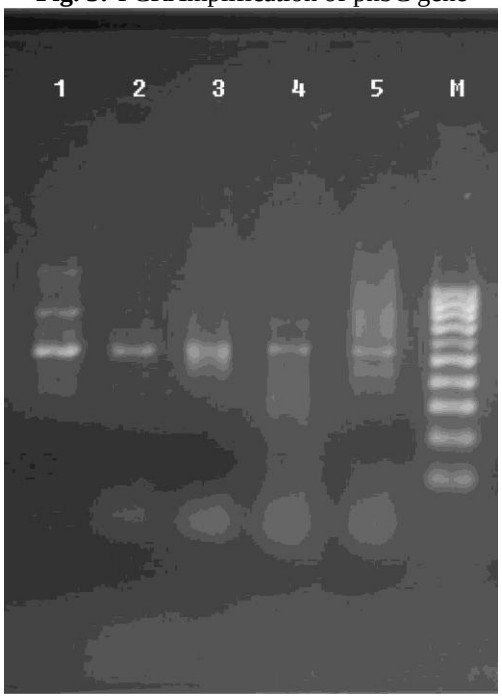


Fig. 3:-PCRAmplification of phbC gene



From Lane 1 - LP01, LP02, LP03, LPH1, LP12, Marker

Conclusion:-

The wide difference between the rates of PHB production in same species indicates that there are several factors that influence the PHB production among the strains. The accumulation of PHB in *Lactobacillus* was not the highest among all the organisms but the *L. plantarum* produced a considerable amount of PHB and to be considered as a part in the choice of organisms for PHB production. The results conclude that nutrient stress conditions can be considered as an optimization parameter for PHB production in high yield. This investigation confirmed that 1mg of the cell dry weight constitutes the maximum of 40.4 µg of PHB.

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