PRODUCTION OF POLYHYDROXYBUTYRATE USING BACTERIAL STRAINS

Thesis submitted in partial fulfillment of the requirements for the degree of

Bachelor of technology

In

Biotechnology and Medical Engineering

By

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111BM0541

UNDER THE SUPERVISION OF

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Certificate

This to certify that the work in the thesis entitled "**PRODUCTION OF POLYHYDROXYBUTYRATE USING BACTERIAL STRAINS OF Lactobacillus acidophilus"** by **Laxmi Badaik (111bm0541)** is a record of an original work carried out with my supervision and guidance in partial fulfillment of the requirements for the degree of Bachelor in Technology in Biotechnology and Medical Engineering. Neither this thesis nor any part of it has been submitted for any degree elsewhere.

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CONTENTS

ABSTRACT:

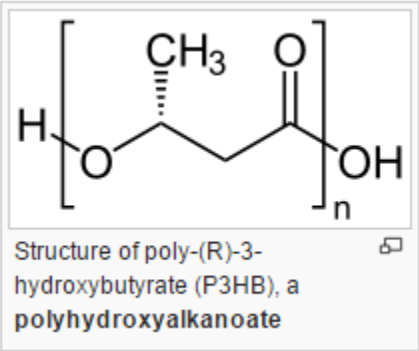
Polyhydroxybutyrate is a polyhydroxyalkanoate (PHA), a polymer having a place with the polyesters classes. That areinterest as biodegradable and bio-derived plastics. The poly-3-hydroxybutyrate (P3HB) sort of PHB ispresumably the most widely recognized sort of polyhydroxyalkanoate, yet different polymers of this class are created by a mixture of life forms: these incorporate poly-4-hydroxybutyrate (P4HB), polyhydroxyvalerate (PHV), polyhydroxyhexanoate (PHH), polyhydroxyoctanoate (PHO) and their copolymers.Synbiotic sachets were obtained from general medical store and Soil was gathered from garden and screened for PHB delivering microscopic organisms. A few unidentified bacterial colonies were confined utilizing serial dilation method. Every bacterial colony was kept up in slants and liquid cultures. A lab strain of Lactobacillus was refined and kinetic studies were performed. Lactobacillus acidophilus produced PHB.

Keyword:

Polyhydroxybutyrate, hydroxybutyrate, Lactobacillus, kinetic studies.

INTRODUCTION

Polyhydroxybutyrate is a polyhydroxualkanoate, which is of a great deal of enthusiasm as it has the attributes of being a bioplastic. It is biodegradable. Remembering the ecological issues of the era, polyhydroxybutyrate creation can be of extraordinary use in conveying these issues to a hindered rate. Polyhydroxybutyrate, not at all like different classes of bioplastics is water insoluble which others are touchy to dampness. It is impervious to bright beams too. It has the qualities of good oxygen porousness. It can disintegrate in chlorinated hydrocarbons. It has a softening purpose of 175 degree Celsius. Above all it is non-harmful and biocompatible which makes it suitable to biomedical applications. Its rigidity is 40 MPa which is near to that of polypropylene. It can be biodegraded an aerobically.



Structure of poly-(R)-3-hydroxybutyrate (P3HB), a **polyhydroxyalkanoate**

PHB is naturaly produced by a few microorganisms for the most part in light of anxiety conditions like supplement constraint. The polymer is principally a result of carbon digestion (from glucose or starch) and is utilized by microorganisms as a type of vitality stockpiling atom to be metabolized when other basic vitality sources are not accessible.

Name of the bacterial strains that is of lactobacillus species:-

- Bacillus mycoids
- Bacillus thiringiensis
- Bacillus subtitis
- Bacillus megatorium
- Lactobacillus plantarum
- Lactobacillus brevis
- Lactobacillus casei
- Lactobacillus bifidus
- Lactobacillus fermentum
- Lactobacillus bulgaricus
- Bacillus sp.

The bacteria in the sachet were:-

- ***Lactobacillus acidophilus***
- ***Lactobacillus rhamnosus***
- ***Biffidobacterum longum***
- ***Biffido bacterium bifidum***
- ***Sachharomyces bouladill***
- ***Frucio oligo sachharides***

These bacteria have been reported to produce high amount of bioplastics (PHB) and are also probiotic .therefore, these are safe organism.

Lactobacillus acidophilus: **-**L.actobacillus acidophilus is a types of gram positive microscopic organisms in the class Lactobacillus. L. acidophilus is a homofermentative, microaerophilic animal types, aging sugars into lactic corrosive, and develops promptly at rather low philus happens characteristically in the human and creature gastrointestinal tract and mouth. A few strains of L. acidophilus may be considered to have probiotic characteristics. These strains are financially utilized as a part of numerous dairy items, infrequently together with Streptococcus thermophilus and Lactobacillus delbrueckii subsp. bulgaricus in the generation of acidophilus-sort yogurt.

Lactobacillus acidophilus

Methods to find out the yield of PHB:-

Gas chromatography

High performance liquid chromatography

Gas chromatography:-

This is a technique is very useful for separating and analyzing compounds which vaporize because of decomposition. It has its application in the field of testing purity of a substance as well as separating various components in a mixture. It helps identify required compound too which is the property that can be used to find out the yield of PHB. It can also assist in making a pure compound from a mixture of substances.

Its mechanism is that it has a carrier gas as its mobile phase which can be helium or nitrogen (inert). Stationary phase consists of a layer of liquid or polymer kept on a solid support which is inert. This setup can be kept within a column. Retention time gives information about each compound to be separated.

HIGH PERFORMANCE LIQUID CHROMATOGRAPH:-

It has same uses as gas chromatography but it has an additional advantage of quantifying each component too. It consists of a pump and a pressurized liquid sample is passed through a column consisting of solid adsorbent. That contains the sample to be separated. The components of the mixture have different reaction rates with the solid adsorbent. Thus different components with ease. It has high pressure at which it operates because of which it is different from gas chromatography.

MATERIALS AND METHODS:-

Isolation of microbial structure:-

Isolation of *E.coli* was done from garden soil using serial dilution method. Each bacterial colony was isolated and maintained in slant culture tubes containing LB agar medium. Liquid culture was prepared by inoculating a loop full of bacterial cells in 100 ml nutrient broth medium and incubating the culture overnight at 37 °C in an incubator shaker set at 200 rpm. The inoculum culture age was 14-16 h..

Batch cultivation of *E.coli* cells in a Stirred tank bioreactor:-

The composition of growth culture medium of *E.coli* has been shown in Table 2. The 5 L fermentor (BIOSTAT B+ Twin Bioreactor, Sartorius-Germany) was inoculated with 110 ml culture from 14 -16 h old primary culture of *E.coli* strain PC-I. Total medium volume in the 5 l fermentor was 2.1 L. The bioreactor was maintained at a constant incubation temperature of 37 °C. The batch cultivation was continued for incubation time of 12h. The Stirred tank reactor impeller was maintained at constant rotational speed of 220 rpm. The pH of the culture was maintained at 6.8 using bio controller.

S.No.	Component	Concentration (g/l)
1.	Yeast extract	1.0
2.	Citric acid	1.7
3.	KH2PO4	6.8
4.	Na2HPO4	8.9
5.	Glucose	3.0
6.	MgSO4	0.2

Measurement of cell optical density:-

The optical density measurement on spectrophotometer is based on light scattering. In spectrophotometerwith Beer Lambert's law, the broth sample should be suitably diluted so that its OD_{600nm} is between 0.1-0.4. The OD of undiluted sample can be obtained by multiplying diluted sample can be obtained by multiplying diluted sample OD with the dilution factor.

Measurement of cell concentration:-

For the measurement of Cell concentration 50ml empty falcon tube was weighed. 1ml broth sample was added to it and centrifuged for 5 minutes at 10,000 rpm. The supernatant was decanted and the residue was dried in tube at 40^0C under vacuum for 24h. Thereafter the tube was placed in a desiccators for cooling. After 30 minutes, the tube was weighed again. Then weight of empty tube was subtracted from it. This gives us cell dry weight in (g/l) of the sample broth.

Measurement of glucose concentration:-

Measurement of glucose concentration was done using di-nitrosalicylic acid (DNS) reagent. Preparation of standard curve for glucose was done in the range of sugar estimation by the DNS method that is 0.1-4.0 mg/ml sugar in sample. 3ml DNS reagent was added to all the tubes, and the tubes were kept in

boiling water bath for exactly 5 minutes. Thereafter, all the tubes were cooled under running tap water. 20 ml distilled water was added to all the tubes and mixed thoroughly. Absorbance at 540 nm wavelength was found on a spectrophotometer using sixth tube as a blank. A plot of OD vs Sugar concentration (mg/ml) was prepared.

Measurement of glucose:

For measurement of glucose in the sample, we centrifuged the sample and diluted the supernatant. DNS treatment was performed and the OD was read against a blank. The sugar standard curve was used for calculation of glucose concentration in the sample of fermentation broth.

Estimation of polyhydroxybutyrate:

1ml freshly prepared phosphate buffer saline was added to the stored cell pellet of *E.coli.* After mixing well the cell pellet was centrifuged for 10 minutes at 15000 rpm at room temperature. PBS was slowly removed from the falcon tube to achieve the target of washing the cell pellet. Then the cell pellet was air dried for 20 minutes. After that 1ml concentrated H2SO4 was added and boiled at 100 degree Celsius for 20 minutes. After cooling the sample to room temperature dilution was prepared from 10microlitre/ml to 100microlitre/ml. Absorbance at 235nm was taken for all the dilutions using distilled water as blank.

Standard curve for polyhydroxybutyrate:-

200mg PHB was dissolved in 1 ml of concentrated H2SO4 and boiled for 10 minutes. In this event, PHB is converted into crotonic acid and cool it. Concentration of the solution is 200mg/ml

(crotonic acid).solution was used as stock solution. Now prepared a dilution from 10mg/ml distilled water used as a diluent.took absorbance at 235nm, distilled water is used as a blank. Stocks solution is stored at 4c for further use.

Result and description:

Isolation of microbial structure:-

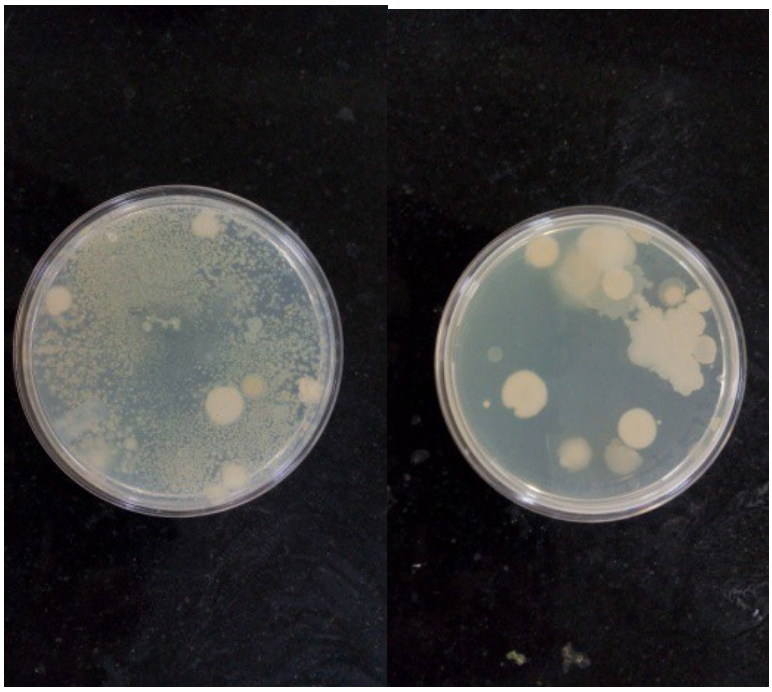


Fig1

Cultivation of *E.coli* cells in a Stirred tank

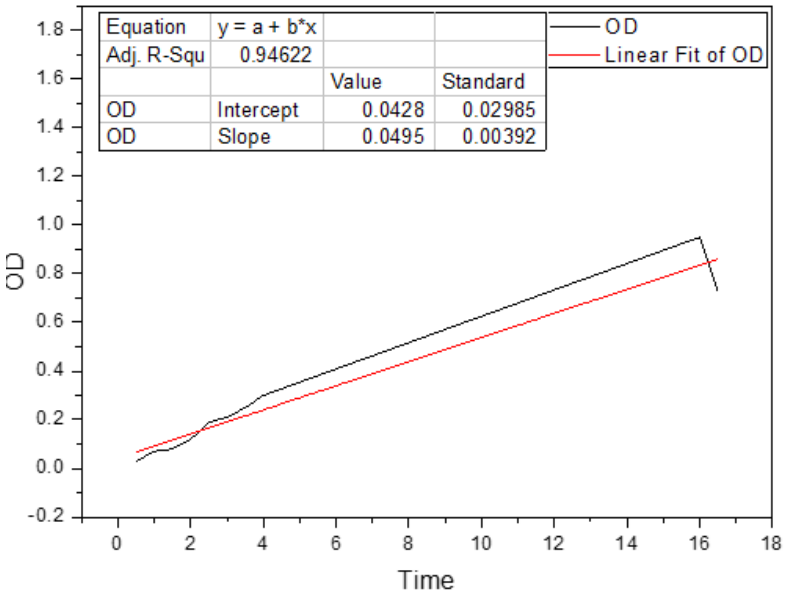
Bioreactor:-

Stirred tank reactor is a preferred mode of cultivation which provides adequate oxygen supply to growing aerobic bacterial cultures. *E. coli* cells were cultivated in a 5-L stirred tank reactor for 17 hours till stationary phase. Biomass concentration and substrate concentration were estimated at an interval of 30 minutes. The specific growth rate was found out to be 0.065 which is the slope of graph of ln(DW) vs time.

The average growth yield coefficient was found out to be 0.0725 which can be calculated from the formula:

$Y_{X/S} = (X_F - X_0)/ (S_F - S_0)$

Where X_F& S_F represent cell dry weight and substrate concentration at the end of fermentation respectively, and X_0 and S_0 represent dry cell weight and substrate concentration at the beginning of the fermentation process.



Time	od
0.5	0.03
1	0.07
1.5	0.08
2	0.12
2.5	0.19
3	0.21
3.5	0.25
4	0.3
16	0.95
16.5	0.728
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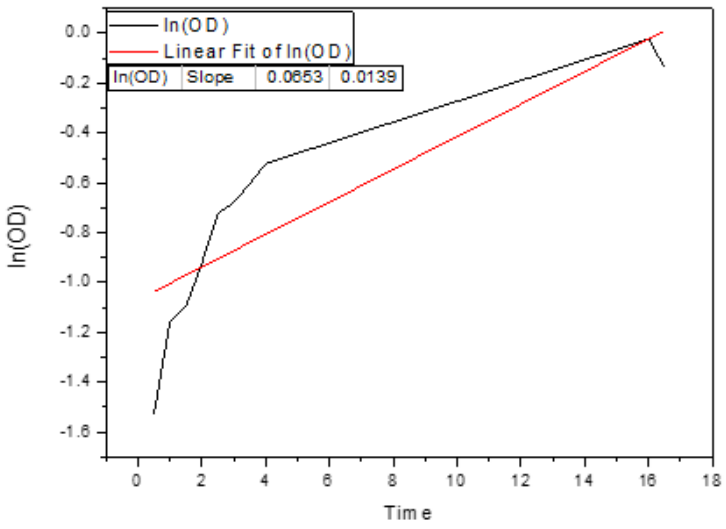
Fig2:- OD vs TIME



0.5	-1.52287874528034
1	-1.15490195998574
1.5	-1.09691001300806
2	-0.920818753952375
2.5	-0.721246399047171
3	-0.677780705266081
3.5	-0.602059991327962
4	-0.522878745280338
16	-0.0222763947111523
16.5	-0.137868620686963
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Fig-3 :-ln(OD) vs Time

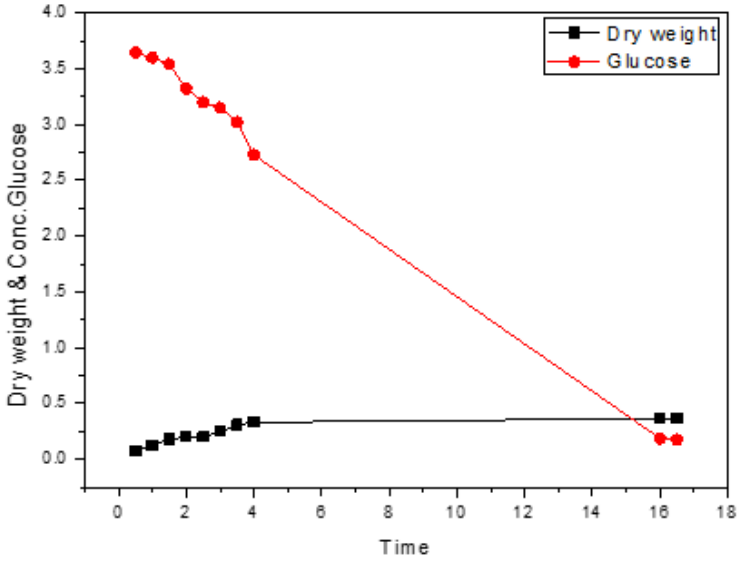


Fig-4:-Graph of dry weight and concentration of glucose vs time

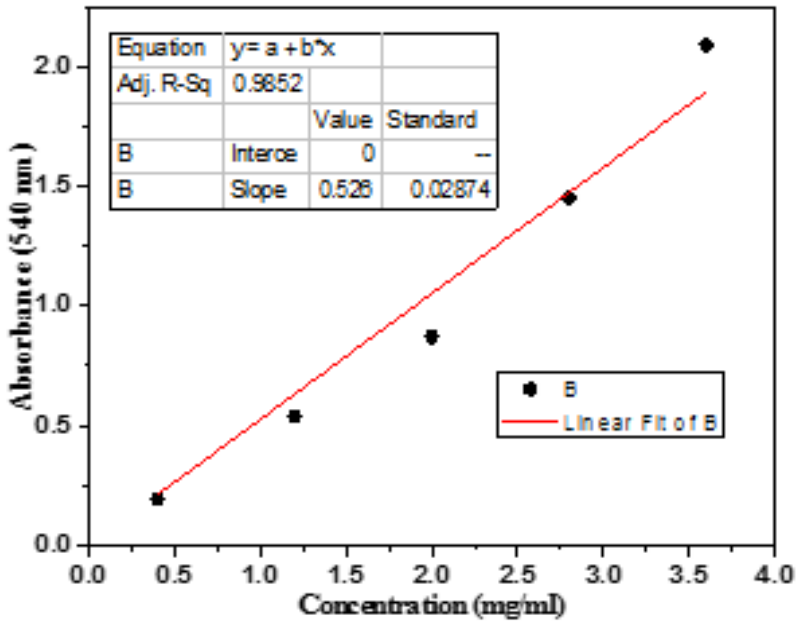
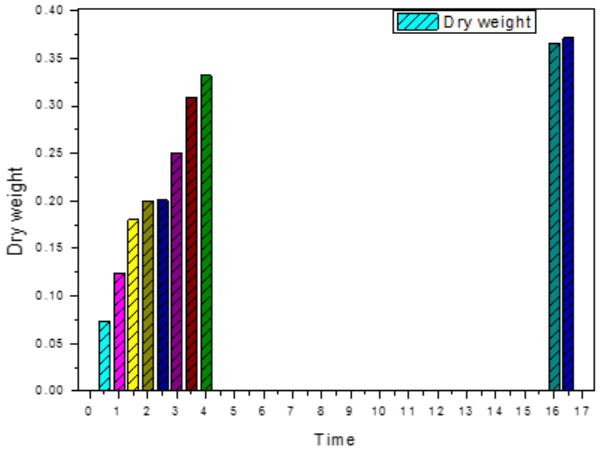


Fig-5: Standard sugar curve



Time	dry weight
0.5	0.074
1	0.124
1.5	0.18
2	0.2
2.5	0.201
3	0.251
3.5	0.309
4	0.332
16	0.366
16.5	0.372
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Fig6: Dry cell weight vs. time

Standard Curve for polyhydroxybutyrate:

The absorbance readings table at 235nm for different concentrations of Standard sample of PHB is as follows:-

Concentration in ug/ml	Absorbance at 235nm			Ave.
blank	0	0	0	0
10	0.028	0.028	0.029	0.028333
20	0.058	0.058	0.058	0.058
30	0.098	0.098	0.098	0.098
40	0.135	0.136	0.135	0.135333
50	0.171	0.172	0.172	0.171667
60	0.222	0.222	0.222	0.222
70	0.265	0.265	0.265	0.265
80	0.318	0.319	0.319	0.318667
90	0.349	0.349	0.348	0.348667
100	0.401	0.403	0.403	0.402333
110	0.423	0.423	0.424	0.423333
120	0.437	0.437	0.438	0.437333
130	0.451	0.451	0.452	0.451333
140	0.478	0.476	0.477	0.477
150	0.535	0.536	0.535	0.53533

				3
160	0.564	0.564	0.565	0.564333
170	0.59	0.589	0.59	0.589667
180	0.632	0.632	0.632	0.632
190	0.664	0.664	0.663	0.663667
200	0.696	0.695	0.697	0.696

Estimation of polyhydroxybutyrate:

Absorbance readings for different dilutions of E.coli at 235nm:-

DILUTION(microliter/litre)	ABSORBANCE
20	0.095, 0.094, 0.095

40	0.205, 0.205,0.205
60	0.315, 0.314,0.314
80	0.360,0.360,0361
100	0.428,0.428,0.428

Conclusion

In this thesis, PHB was produced by serial dilution method. We estimated PHB and plotted standard curve of PHB. The dry cell weight was taken 0.247gm and estimation of PHB was 11 mg.The yield of PHB was 5.725mg/DW.

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