PRODUCTION OF POLYHYDROXYBUTYRATE USING BACTERIAL STRAINS OF *Escherichia coli* AND *Bacillus subtilis:* A COMPARISON

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENT FOR THE DEGREE OF

Bachelor of Technology
In
Biomedical Engineering
By

IPSITA PANDA
111BM0536

Under the Supervision of

Dr. Nivedita Patra
Department of Biotechnology and Medical Engineering

National Institute of Technology Rourkela
Rourkela, Odisha, 769 008, India

Department of Biotechnology and Medical Engineering

National Institute of Technology Rourkela

Rourkela - 769008, Odisha, India.

Certificate

This is to certify that the thesis entitled "**PRODUCTION OF POLYHYDROXYBUTYRATE USING BACTERIAL STRAINS OF *E.coli* and *B.subtilis*: A COMPARISON**" by **Ipsita Panda (111BM0536),** in partial fulfillment of the requirements for the award of the degree of Bachelor of Technology in Biomedical Engineering during session 2011-2015 in the Department of Biotechnology and Medical Engineering, National Institute of Technology Rourkela, is an authentic work carried out by her under my supervision and guidance. To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University/Institute for the award of any degree or diploma.

Place: Rourkela **Dr. Nivedita Patra**
Date 3rd May, 2015 **Assistant Professor**

Acknowledgement

I take this golden opportunity to thank my guide Dr. Nivedita Patra, Department of Biotechnology and Medical Engineering, NIT Rourkela for her kindest cooperation and guidance and having the largest contribution in the successful fulfillment of this project.

I am extremely grateful to Dr. Krishna Pramanik, HOD, Department of Biotechnology and Medical Engineering for providing me with all the important facilities of the department which are a reason of the completion of this project.

I also would like to thank Dr. Kunal Pal and Dr. Indranil Banerjee for their supporting nature and kindly allowing me to access their labs for my work. I also want to express my gratitude to Mr. Gautham Harinarayana (Mtech Scholar), Mr. Jay Prakash Yadav (Ph.D Scholar) and Mr. Senthil (Ph.D Scholar) for their timely help whenever it was needed. Without their motivation this project would have not come to successful completion.

Finally I would like to thank my friends Miss Laxmi Badaik, Mr. Utkarsh Srivastava, Miss Meghasmita and Miss Deepshikha for always being with during problems and encouraging me to work even in difficult situations.

Not to mention I would like to thank my family for their mental support which also plays an important role in the completion of this project.

PLACE: Rourkela
DATE: 3RD May, 2015

Ipsita Panda
111BM0536

CONTENTS

LIST OF TABLES:

LIST OF FIGURES:

ABSTRACT

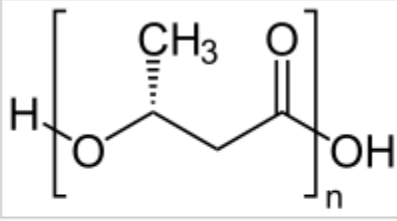
Polyhydroxybutyrate (PHB), a polyhydroxyalkanoate (PHA), is of interest as bio-derived plastics. PHB is obtained from renewables and is compostable and biodegradable. An extensive literature review indicated that a number of microorganisms produce PHB for the purpose of energy generation. The source of these micro-organisms was investigated. Garden soil was collected and screened for PHB producing bacteria. Several unidentified bacterial colonies were isolated using serial dilution method. Each bacterial colony was maintained in slants and liquid cultures. A lab strain of *E.coli* was cultured in a 5-L stirred tank bioreactor and kinetic studies were performed till it reached stationary phase at 17 hours. The maximum biomass obtained was 3.7 g/l. Similarly sample of *B.subtilis* was taken and bacterial colonies were isolated. Slant streaks and liquid cultures were maintained. Same procedure was carried out for *B.subtilis.* The maximum biomass obtained in case of *B.subtilis* was. The polyhydroxybutyrate produced in both cases were analyzed and compared. *B.subtilis* produced more amount of polyhydroxybutyrate as compared to *E.coli.*

KEYWORDS: Polyhydroxybutyrate, *E.coli, B.subtilis*

CHAPTER 1

INTRODUCTION

Polyhydroxybutyrate is a polyhydroxualkanoate, which is of a lot of interest as it possesses the characteristics of being a bioplastic. It is biodegradable. Keeping in mind the environmental problems of the generation, polyhydroxybutyrate production can be of great use in bringing these problems to a retarded rate. Polyhydroxybutyrate, unlike other categories of bioplastics is water insoluble which others are sensitive to moisture. It is resistant to ultraviolet rays also. It has the characteristics of good oxygen permeability. It can dissolve in chlorinated hydrocarbons.It has a melting point of 175 ° C. Most importantly it is non-toxic and biocompatible which makes it suitable to biomedical applications. Its tensile strength is 40 MPa which is close to that of polypropylene. It can be biodegraded anaerobically.



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

Figure 1 Polyhydroxybutyrate

PHB is naturally produced by several microorganisms mainly in response to stress conditions like nutrient limitation. The polymer is primarily a product of carbon assimilation (from glucose or starch) and is employed by microorganisms as a form of energy storage molecule to be metabolized when other common energy sources are not available. The various bacterial strains which are reported to produce PHB are reported in Table 1.

Table 1: Strains of bacteria that produce large amount of PHB

Sl.No.	Name of bacterial strain	Source	Yield of PHB (g/l)	Reference
1.	*Bacillus mycoides*	Garden soil	1.8	[1]
2.	*Cupriavidus taiwanensis*	Purchased from BCRC	7	[2]
3.	*Bacillus thuringiensis*	Chlorine contaminated soil	0.72	[3]
4.	*Sacharococcus thermophilus*	Marine soil	0.60	[4]
5.	*Veillonella sp.*		0.05	
6.	*Curtobacterium sp.*		0.43	
7.	*Sarcina sp.*		0.34	
8.	*Syntrophococcus sp.*		0.53	
9.	*Lactobacillus acidophilus*	Whey protein	3.24	[5]
10.	*Bacillus subtilis*	Garden soil	6.24	
11.	*Rhotobacter spaeroides*	Marine natural resource	8.02	[6]
12.	*Bacillus megaterium*	Soil	0.93	[7]
13.	*Ralstonia eutropha*	Soil	3	[8]
14.	*Enterobacteriaceae bacterium*	Organic waste contaminated soil	7.2	[9]
15.	*Pseudomonas stutzeri*	Microbial sources	0.1-3	[10]
16.	*Lactobacillus plantarum*	Dairy and food products	0.66	[11]
17.	*Lactobacillus brevis*		0.86	
18.	*Lactobacillus casei*		1.90	
19.	*Lactobacillus bifidius*		0.03	
20.	*Lactobacillus fermentrum*		0.21	
21.	*Lactobacillus bulgaricus*		0.86	
22.	*Bacillus sp.*	Not available for public download	1.9	[12]
23.	*Hypomicrobium zavarzini*	-	0.64	[13]
24.	*Pseudomonas aeruginosa*	Stock culture of Obafami University, Nigeria	0.93	[14]

Thus many other bacterial strains including above all strains are reported to produce polyhydroxybutyrate. *E.coli* and *Bacillus subtilis* were taken into consideration for this study.

E.coli

It is a gram negative bacteria with the scientific name of *Escherichia coli* which was discovered by a pediatrician Theodor Escherich from Germany. It is a rod shaped bacterium. Mostly it can be found in moist soil and lower intestine of some animals which are warm blooded. They have some pathogenic effects like causing food poisoning. But they have beneficial effects as well which include production of vitamin K. It has been the most widespread studied gram negative bacterium. They display a wide range of genetic and phenotypic diversity.

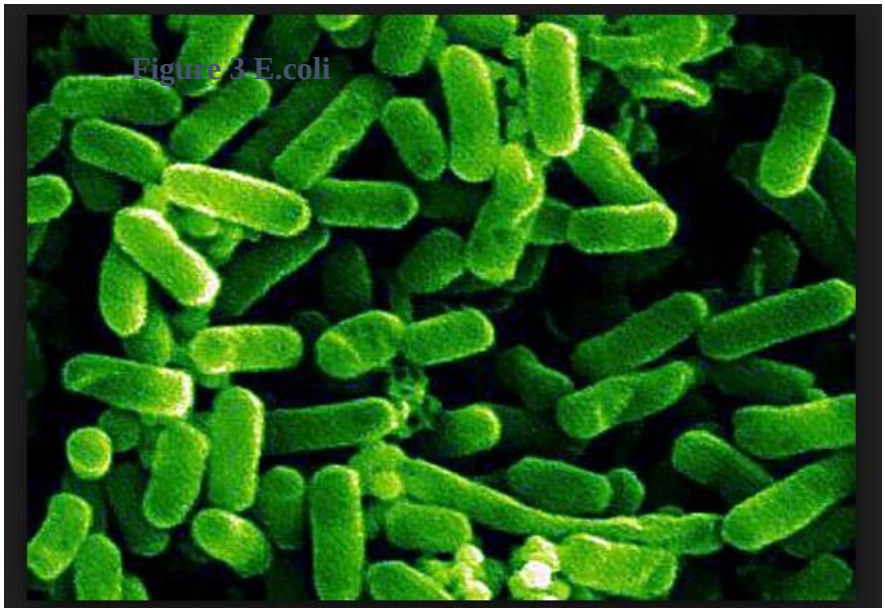


Figure 2 *E.coli*

Bacillus subtilis

It is a widespread studied gram positive bacteria which is the representative for studying properties of gram positive bacteria. Christian Gottfried Ehrenberg named it *Vibrio subtilis* which was later renamed as *Bacillus subtilis* by Ferdinand Cohn in 1872.It is also found in moist soil and intestine of some animals. In matter of shape it is also rod shaped like *E.coli*. It

can form protective endospores which provide it protection against tough conditions of environment. It is the best bacteria to study cell differentiation. It produces a lot of enzymes too. It has a lot of flagella. These bacteria reproduce through binary fission. They are well known for having been a cure for urinary tract diseases. It is ideal for space-testing as its endospores can survive in space with ease for nearly 6 years if they are coated with a layer of dust. A very important property of this bacteria is that it can convert many explosives into harmless substances. Strains of this bacteria is known to produce good yield of polyhydroxybutyrate because of which it is selected by us for this study. It can indicate when the gas sterilization procedure comes to an end.

Figure 4 Bacillus subtilis

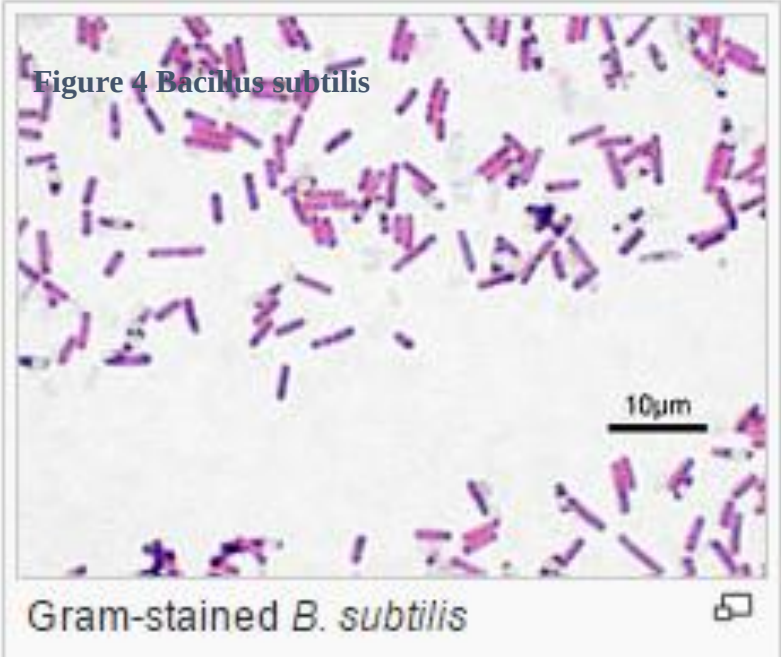


Figure 3 *B.subtilis*

Techniques for Finding out the Yield of PHB:

GAS CHROMATOGRAPHY

This is a technique is very useful for separating and analyzing compounds which vapourize 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 CHROMATOGRAPHY

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.

CHAPTER 2

LITERATURE REVIEW

This chapter is an explanation to why production of polyhydroxybutyrate is important. Polyhydroxybutyrate is a very important polymer as it is a bioplastic which can be produced as a result of fermentation reaction of bacteria when there are deficiency of nutrients as the process goes on. Keeping in account the need some biodegradable material which can take the load of environment, polyhydroxybutyrate seems like a very good option. Reviewing the research in this field the fact becomes clear that research in this field is not more than 30 decades old. So many more areas remain unveiled in this field. Studies in this field have revealed that many bacterial strains like that of *Bacillus myocoides, Cupriavidus taiwanensis Bacillus thuringiensis, Sacharococcus thermophiles, Veillonella sp., Curtobacterium sp, Sarcina sp., Syntrophococcus sp., Lactobacillus acidophilus, Bacillus subtilis, Bacillus megaterium, Ralstonia eutropha, Enterobacteriaceae bacterium, Pseudomonas stutzeri, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus casei, Lactobacillus bifidius, Lactobacillus fermentrum, Lactobacillus bulgaricus, Bacillus sp., Hypomicrobium zavarzini, Pseudomonas aeruginosa* produce a good yield of polyhydroxubutyrate. It has been found out through studies that there are many advantages of this method of usage of microbes for the production of this polymer rather than using other conventional processes. Genetic modification of bacteria can even lead to enhancement of the production process more. Amid the previous decades, metabolic engineering has been utilized broadly to enhance the cellular processes in microorganisms which are used for industrial purpose with the motivation behind enhancing the yield of it, expand the scope of substrate usage, decrease of by-product development and presentation of novel pathways prompting new items. Metabolic engineering includes the investigation of metabolic pathways utilizing metabolic flux for the examination to recognize targets for control and coordinated hereditary adjustment with a specific end goal to accomplish the required phenotype. With the presentation of recombinant DNA innovation, particularly to adjust the focused on gene(s) or metabolic pathways, strain change has steadily moved from traditional rearing or irregular mutagenesis to a more level headed methodology. At present, through the propel in frameworks science and the developing of manufactured science, metabolic building has continuously advanced and gets to be more effective in outlining cell producing factories. Techniques like batch fermentation, fed-batch fermentation and continuous fermentation are known to produce fermentation products from which polyhydroxybutyrate can be estimated. There are various methods which were found out by reviewing the research work done earlier that methods like gas chromatography, high pressure

liquid chromatography spectrophotometric analysis can be useful to quantify the amount of polyhydroxybutyrate produced by the bacteria. Even methods like Fourier transform infrared spectroscopy can also be used for the estimation purpose by statistically studying the similarities between sample content and spectrum obtained.

According to a study done by Pablo I. Nikel, Alejandra de Almeida, Evelia C. Melillo, Miguel A. Galvagno, and M. Julia Pettinari , Appl Environ Microbiol. 2006 Jun; 72(6): 3949–3954 it shows that by usage of a recombinant strain of *E.coli* (K24K) as bacterial strain and whey protein and corn steep liquor as the main source of carbon and nitrogen, a yield of 72.9% of dry cell weight of the polymer was produced. This shows that recombinant strains produce a good yield of polyhydroxybutyrate. Another study by F Wang and S Y Lee , Appl Environ Microbiol. 1997 Dec; 63(12): 4765–4769, reveals that Recombinant Escherichia coli XL1-Blue can produce a very high yield of Polyhydroxybutyrate which amounts to 3.4g/l/h. This gives a possibility of industrial production of Polyhydroxybutyrate. *E.coli* is a very widespreadly available bacterium which can be extracted and isolated from garden soil. Techniques like ethyl methylene blue agar can be used for screening *E.coli* as it gives shiny green colour in presence of E.coli.

Coming to *B.subtilis* it even has a higher yield of polyhydroxybutyrate according to studies. With respect to a study done by Gulab Singh, Anish Kumari, Arpana Mittal, Anita Yadav, and Neeraj K. Aggarwal, BioMed Research International,Volume 2013 (2013), Article ID 952641, 10 pages the yield of polyhydroxybutyrate using the strain of B.subtilis NG220, and keeping carbon source as waste water from sugar industry was found to be 5.29g/l. This value is greater than that of E.coli too. Also it was reviewed according to Sathiyanarayanan G, Saibaba G, Seghal Kiran G, Selvin J., Int J Biol Macromol. 2013 Aug;59:170-7. doi: 10.1016/j.ijbiomac.2013.04.040. Epub 2013 Apr 18., isolation of B.subtilis was done from sponge Callyspongia diffusa and identification was carried out by the process of 16S r RNA analysis. Different cheap raw materials like tamarind powder, jaggery, pulp waste were used to increase polyhydroxybutyrate composition which went as high as 19.08g/l after an incubation period of 40hrs. The screening of *B.subtilis* were done using sudan black staining method in most of the cases. Thus later on starting from gas chromatography, high pressure liquid chromatography, UV spectrophotometer and FTIR spectrophotometer techniques amount of the polymer can be detected.

These data encouraged us to carry forward with the two selected bacterial strains of *E.coli* and *B.subtilis* as they are available easily and can facilitate industrial production of the polymer if the production conditions, growth medium components, carbon and nitrogen source are properly optimized using proper techniques.

CHAPTER 3

MATERIALS AND METHODS

3.1 For *E.coli* strain

3.1.1. Isolation and maintenance of microbial cultures

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 culture inoculum age was 14-16h.

3.1.2 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 biocontroller.

Table 2. Components of growth medium for E.coli

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

3.1.3 Estimation of optical density, cell concentration and glucose concentration

The optical density measurement on spectrophotometer is based on light scattering. In accordance with 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 OD with the dilution factor.

For the measurement of Cell concentration 50ml empty falcon tube was weighed. 1ml broth sample was added to it and centrifuged at 10,000 rpm for 5 minutes. 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 desiccator 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 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.

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.

3.1.4 Standard Curve for Polyhydroxybutyrate

200mg of PHB was dissolved in 1ml of concentrated H2SO4 and then heated for 10 minutes to convert PHB into crotonic acid. This produced a concentration of 200mg/ml of crotonic acid which was used as a stock. The sample was cooled before use. Working standard solutions were prepared by diluting the sample to different concentrations ranging from 10 microgram/ml to 200 microgram/ml. Readings for absorbance were taken for different concentrations of solution at 235nm using distilled water as blank. Then a standard curve for PHB was plotted utilizing the absorbance data.

3.1.5 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.

3.2 For *B.subtilis* strain

3.2.1. Isolation of *B.subtilis* and Storing for Use

The sample of *B.subtilis* was taken and serially diluted. Then the bacteria was plated using LB agar as well as stored as slant streaks. Also broth culture was made by taking a loop full of bacteria in by using nutrient broth and incubating it at 37° C and 200rpm for 24 hrs. These were stored for future use.

3.2.2. Batch Cultivation of *B.subtilis* using Bioreactor

The composition of growth culture medium of B.subtilis has been shown in Table 3. The 5 L fermentor (BIOSTAT B+ Twin Bioreactor, Sartorius-Germany) was inoculated with 110 ml culture from 14 -16 h old primary culture of B.subtilis strain. Total medium volume in the 5 l fermentor was 2.1 L. A constant temperature of 37 °C was maintained in the bioreactor. The batch cultivation was continued for incubation time of 17h. The Stirred tank reactor impeller was maintained at constant rotational speed of 220 rpm. pH of the culture was maintained at 7.4 using biocontroller (acids and bases).

Table 3. Components of nutrient broth

Sl. No.	Components	Concentration (g/l)
1.	Peptic digest of animal tissues	5
2.	Sodium chloride	5
3.	Beef extract	1.50
4.	Yeast extract	1.50

Figure 4. The setup of Bioreactor for fermentation of *B.subtillis*

3.2.3 Measurement of Cell Concentration

In the end of the fermentation the end products were filled in 50ml falcon tubes and centrifuged for 10minutes. At once not more than 40ml was taken. Four such tubes were used repeatedly to

centrifuge the entire end product. Every time the supernatant was discarded and cells were stored. After the completion of the centrifugation process, all the contents in the falcon tube were air dried for 20minutes. Then an empty tube was weighed and the four tubes with cell content were weighed. Dry cell weight was calculated by subtracting mass of empty tubes from filled tubes.

3.2.4 Measurement of Sugar Concentration

Sugar concentration was estimated in the same way as described in case of *E.coli* by using DNS reagent and following the same protocol.

3.2.5 Standard Curve of Polyhydroxybutyrate

The same method was followed to plot standard curve of PHB as described in case of *E.coli*. In fact the same absorbance readings were used for *B.subtilis* too.

3.2.6 Estimation of Polyhydroxybutyrate

1ml freshly prepared phosphate buffer saline was added to the stored cell pellet of *B.subtilis*. 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° Celsius for 20 minutes. After cooling the sample to room temperature dilution was prepared from 10µl/ml to 100µl/ml. Absorbance at 235nm was taken for all the dilutions using distilled water as blank.

CHAPTER 4

RESULTS

AND DISCUSSIONS

4.1. *E. Coli*

4.1.1 Isolation and maintenance of microbial cultures

Figure 5 Plate 1 of maintained *E.coli*

Figure 6 Plate 2 of maintained *E.coli*

PHB is produced by microbes (such as *Ralstonia eutrophus, Bacillus subtilis* or *Bacillus megaterium*) apparently in response to conditions of physiological stress, mainly conditions in which nutrients are limited. The polymer is primarily a product of carbon assimilation (from glucose or starch) and is employed by microorganisms as a form of energy storage molecule to be metabolized when other common energy sources are not available. A close observation of the source of origin of these microorganisms (Table 1) suggests that soil is one of the rich sources of these microbes. Using serial dilution method four different bacterial colonies were isolated and maintained in slant cultures for further analysis and experimentation. Out of these four colonies two were used for further work.

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

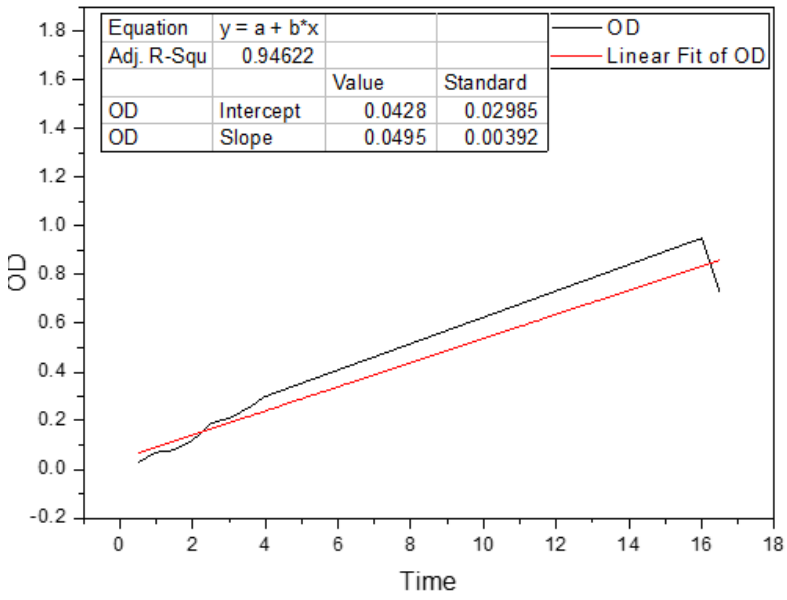
Stirred tank reactor is a preferred mode of cultivation which provides adequate oxygen supply to growing aerobic bacterial cultures. *E. coli* cells were cultivated in a 5-L stirred tank reactor for 17 hours till stationary phase. Biomass concentration and substrate concentration were estimated at an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

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

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

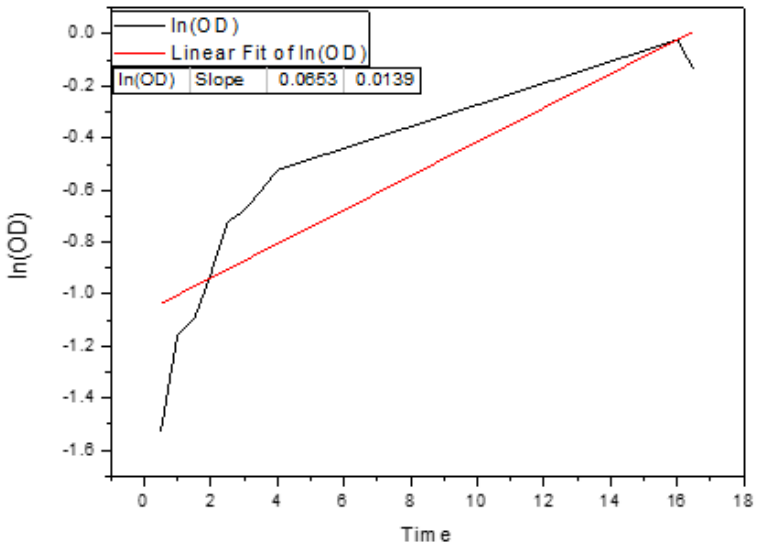
Where X_F & S_F represent cell dry weight and substrate concentration at the end of fermentation respectively, and X_0 and S_0 represent dry cell weight and substrate concentration at the beginning of the fermentation process.

4.1.3. Estimation of optical density, cell concentration and glucose concentration



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
--	--

Figure 7 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
--	

Figure 8 ln (OD) vs Time

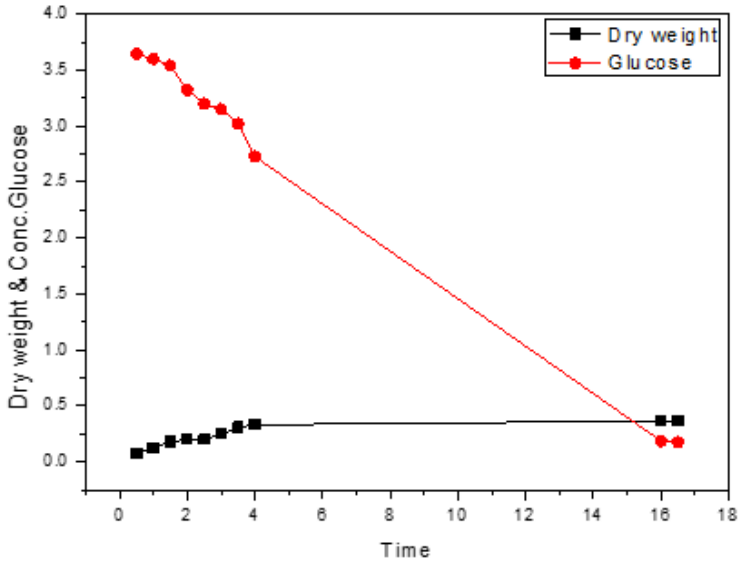


Figure 9 Graph of dry weight and concentration of glucose vs time

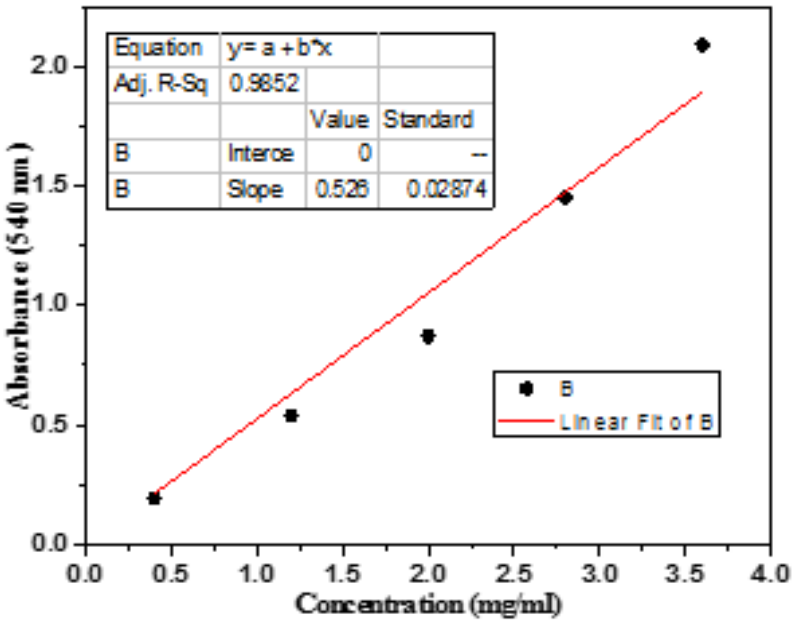
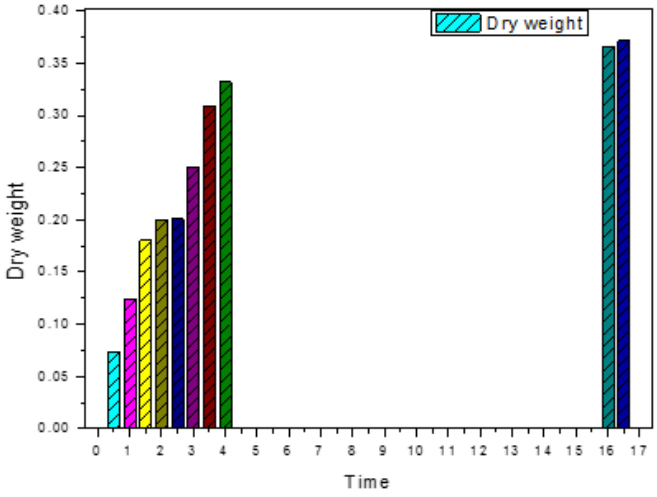


Figure 10 Standard sugar curve



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
--	

Figure 11 Dry cell weight vs Time

4.1.4. Standard Curve for Polyhydroxybutyrate

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

Table 4. Absorbance readings for different concentrations of PHB

Sl. No.	Concentration (µg/ml)	Absorbance
1.	10	0.028, 0.028, 0.029
2.	20	0.058, 0.058, 0.058
3.	30	0.098, 0.098, 0.098
4.	40	0.135, 0.136, 0.135
5.	50	0.171, 0.172, 0.172
6.	60	0.222, 0.222, 0.222
7.	70	0.265, 0.265, 0.265
8.	80	0.318, 0.319, 0.319
9.	90	0.349, 0.349, 0.348
10.	100	0.401, 0.403, 0.401
11.	110	0.423, 0.423, 0.423
12.	120	0.437, 0.437, 0.437
13.	130	0.455, 0.455, 0.455
14.	140	0.482, 0.482, 0.482
15.	150	0.507, 0.507, 0.507
16.	160	0.527, 0.526, 0.527
17.	170	0.535, 0.536, 0.535
18.	180	0.564, 0.564, 0.565
19.	190	0.590, 0.589, 0.590
20.	200	0.632, 0.632, 0.632

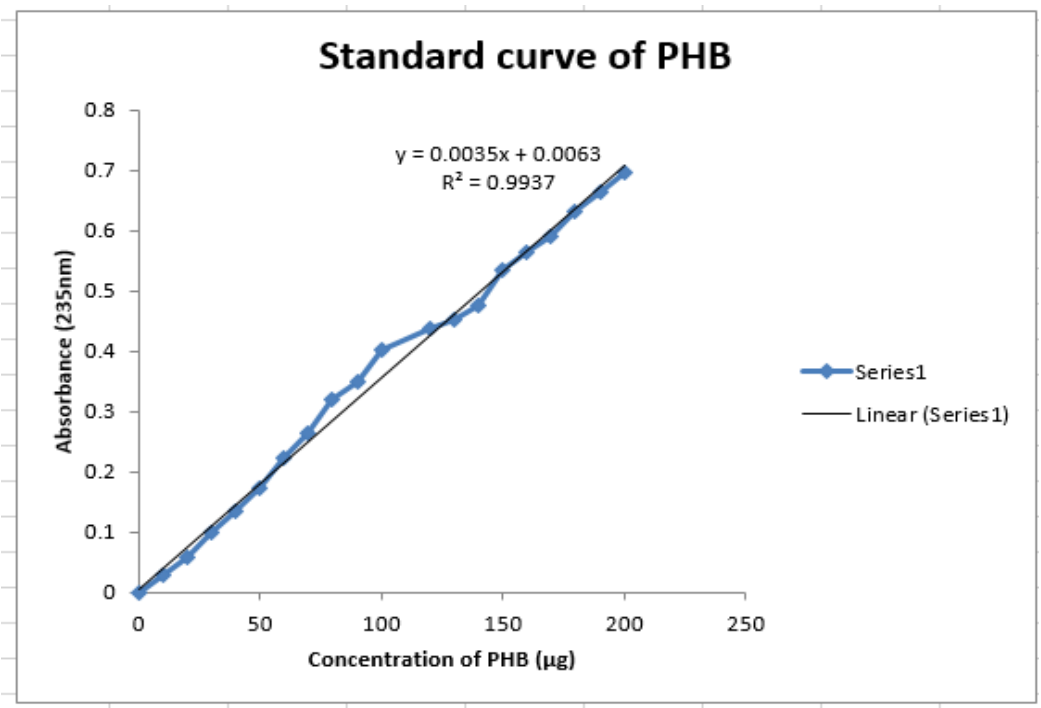


Figure 12 Standard curve for PHB

4.1.5. Estimation of Polyhydroxybutyrate

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

Table 5. Absorbance readings for different dilutions of the sample of E.coli dry cell

SL. NO.	DILUTIONS(µl/l)	ABSORBANCE
1.	20	0.036, 0.036, 0.036
2.	40	0.085,0.085,0.085
3.	60	0.119,0.119,0.118
4.	80	0.152, 0.151, 0.152
5.	100	0.181,0.181, 0.181
6.	120	0.243,0.243, 0.242
7.	140	0.272, 0.272,0.271
8.	160	0.319,0.319, 0.318

Dry cell weight taken was 0.181gm.

Estimated PHB in 0.181gm *E.coli* was 0.5mg.
Yield of PHB was 2.76 mg/ gDW of *E.coli*

4.2. *B.subtilis*

4.2.1. Isolation of *B.subtilis* and Storing for Use

Plates of bacteria had developed colonies of *B.subtilis* which were round in an undulated manner. Slant streaks had also developed colonies. There was greenish turbidity in the liquid culture.

4.2.2. Batch Cultivation of *B.subtilis* using bioreactor

Thus fermentation process of *B.subtilis* was completed till stationary phase was reached. There was a lot of bubble formation as all nutrients had depleted in the bioreactor.

4.2.3. Measurement of Cell Concentration

Measuring the dry cell weight of all the end products present it was found out the total dry cell weight of *B.subtilis* after air drying was 11.022g.

4.2.4. Measurement of Sugar Concentration

It was found out that all the glucose had ended up in the end product by using DNS method. This was because all the glucose was utilized by the bacteria for their metabolic purpose.

4.2.5. Standard Curve for Polyhydroxybutyrate

The same standard curve was used for *B.subtilis* also which was used for *E.coli* as same standard sample of PHB was considered to get the products.

4.6.2. Estimation of Polyhydroxybutyrate

Absorbance readings for different dilutions of *B.subtilis* at 235nm

Table 6. Absorbance readings for different dilutions of *B.subtilis* dry cell weight

Sl. No.	Dilutions(µl/l)	Absorbance
1.	20	0.111, 0.112, 0.110
2.	40	0.209, 0.209, 0.210
3.	60	0.307, 0.306, 0.306
4.	80	0.368, 0.368, 0.366
5.	100	0.465, 0.464, 0.464
6.	120	0.557, 0.556, 0.555
7.	140	0.630, 0.631, 0.629
8.	160	0.723, 0.726, 0.726

Dry cell weight of *B.subtilis* taken was 0.417g.

PHB estimated in 0.417g *B.subtilis* was 1.43mg.

Yield of PHB was 3.42mg/gDW of *B.subtilis*.

CONCLUSION

E.coli is the model organism for the study of gram negative microbes. So is *B.subtilis* for gram positive bacteria. The strains of both were used for the production of bioplastic polyhydroxybutyrate. It was found out that under same conditions *E.coli* produced less dry cell weight than *B.subtilis* as a whole. It was also found out that the yield of polyhydroxybutyrate in *B.subtilis* was higher than *E.coli*. According to calculations the yield of PHB for *E.coli* was 2.76mg/g DW and for *B.subtilis* was 3.42mg/g DW. Thus production of this polymer at an industrial scale can be very beneficial for our environment. Not only these two strains but strains of many bacteria can be useful for industrial production of this polymer. Using recombinant DNA technology can also be very helpful in increasing yield of PHB. Optimized production conditions like nutrient composition, addition of carbon and nitrogen source also plays a dominant role in the enhancement of yield. With future explorations of the microbial production technology of this polymer, the environmental problems cause due to non-biodegradable products can surely come to an end.

References

[1] A. Narayanan, K.V. Ramana. Polyhydroxybutyrate production in *Bacillus mycoides* DFC1 using response surface optimization for physico-chemical process parameters. *3 Biotech.* 2012, **2**(4):287-296.

[2] Y-H Wei, W-C Chen, C-K Huang, et al. Screening and Evaluation of Polyhydroxybutyrate-Producing Strains from Indigenous Isolate *Cupriavidus taiwanensis* Strains. *International Journal of Molecular Sciences.* 2011, **12**(1):252-265.

[3] T. Charen, P. Vaishali, M. Kaushalya, K. Amutha , V. Ponnusami and D. Gowdhaman. Isolation and identification of Polyhydroxybutyrate producing bacterial strain (Bacillus thuringiensis GVP) from chlorine contaminated soil. *International Journal of ChemTech Research*. 2014, **6** (5): 3197-3202.

[4] R. Kalaivani and V. Sukumaran. Isolation and identification of new strains to enhance the production of biopolymers from marine sample in Karankura, Tamil Nadu. *European Journal of Experimental Biology*. 2013, **3**(3):56-64.

[5] Alyaa Hamieh, Zakia Olama and Hanafi Holail. Microbial production of polyhydroxybutyrate, a biodegradable plastic using agro-industrial waste products. *Global Advanced Research Journal of Microbiology*. 2013, **2**(3) 054-064.

[6] Kanokphorn Sangkharak and Poonsuk Prasertsan. Nutrient optimization for production of polyhydroxybutyrate from halotolerant photosynthetic bacteria cultivated under aerobic-dark condition. *Electronic Journal of Biotechnology*. 2008, **11** (3): 1-12.

[7] T. Prasanna, P. Ajay Babu, P. Dhanavara Lakshmi, R. Chakrapani and CSV Ramachandra Rao. Production of Poly (3-hydroxybutyrates) by Bacillus species isolated form Soil. *Journal of Pharma Research & Reviews.* 2011, **1**: 15-18.

[8] R. Preethi, P. Sasikala, J. Aravind. Microbial production of polyhydroxyalkanoate (PHA) utilizing fruit waste as a substrate. *Research in Biotechnology*. 2012, **3**(1): 61-69.

[9] Nighat Naheed, Nazia Jamil, Shahida Hasnain and Ghulam Abbas. Biosynthesis of polyhydroxybutyrate in Enterobacter sp. SEL2 and Enterobacteriaceae bacterium sp. PFW1 using sugar cane molasses as media. *African Journal of Biotechnology*. 2012, **11**(16): 3321-3332.

[10] Zhong Zheng . Ming Li . Xiao-Jing Xue . Hong-Lei Tian . Zhi Li. Guo-Qiang Chen. Mutation on N-terminus of polyhydroxybutyrate synthase of Ralstonia eutropha enhanced PHB accumulation. *Appl Microbiol Biotechnol* 2006, **72**: 896–905.

[11] Z.N. Yuksekdag, Y. Beyatli, B. Aslim. Determination of Poly-ß-Hydroxybutyrate(PHB) production by some mesophilic and thermophilic lactic acid bacteria. *TUBITAK, Tr. J. Biology*. 2003, **27:** 37-42.

[12] M. Thirumala and S. Vishnuvardhan Reddy, Production of Polyhydroxybutyrate Using Cane Molasses as a Sole Carbon Substrate by Bacillus sp. 112A. *The IUP Journal of Biotechnology*. 2012, **6**(1): 25-33.

[13] S. Zhao, C. Fan, X. Hu, J. Chen, H. Feng. The microbial production of polyhydroxybutyrate from methanol. *Applied biochemistry and biotechnology*. 1993, **40**(1):191-199.

[14] T.V. Ojumu, J. Yu, and B.O. Solomon. Production of Polyhydroxyalkanoates, a bacterial biodegradable polymer. *African Journal of Biotechnology*. 2004, **3** (1). 18-24.

[15] Kiran Nehra1, Ashish Jaglan, Anjum Shaheen, Jyoti Yadav, Priyanka Lathwal and Manpreet. Production of Poly-β-Hydroxybutyrate (PHB) by bacteria isolated from rhizospheric soils. *International Journal of Microbial Resource Technology*.2015, 2(3): 38-48.

[16] Chakraborty, P.; Gibbons, W.; Muthukumarappanl K. Conversion of volatile fatty acids into polyhydroxyalkanoate by *Ralstonia eutropha*. *J. Appl. Microbiol.* **2009**, *106*, 1996–2005.

[17] 2. Yu, P.H.; Chua, H.; Huang, A.L.; Ho, K.P. Conversion of industrial food wastes by *Alcaligenes latus* into polyhydroxyalkanoates. *Appl. Biochem. Biotechnol.* **1999**, *77–79*, 445–454.

[18] 3. Zhang, H.F.; Ma, L.; Wang, Z.H.; Chen, G.Q. Biosynthesis and characterization of 3-hydroxyalkanoate terpolyesters with adjustable properties by *Aeromonas hydrophila*. *Biotechnol. Bioeng.* **2009**, *104*, 582–589.

[19] 4. Ren, Q.; de Roo, G.; Witholt, B.; Zinn, M.; Thöny-Meyer, L. Influence of growth stage on activities of polyhydroxyalkanoate (PHA) polymerase and PHA depolymerase in *Pseudomonas putida* U. *BMC Microbiol.* **2010**, *10*, 254.

[20] Chakraborty, P.; Gibbons, W.; Muthukumarappanl K. Conversion of volatile fatty acids into polyhydroxyalkanoate by *Ralstonia eutropha*. *J. Appl. Microbiol.* **2009**, *106*, 1996–2005.

[21] 2. Yu, P.H.; Chua, H.; Huang, A.L.; Ho, K.P. Conversion of industrial food wastes by *Alcaligenes latus* into polyhydroxyalkanoates. *Appl. Biochem. Biotechnol.* **1999**, *77–79*, 445–454.

[22] 3. Zhang, H.F.; Ma, L.; Wang, Z.H.; Chen, G.Q. Biosynthesis and characterization of 3-hydroxyalkanoate terpolyesters with adjustable properties by *Aeromonas hydrophila*. *Biotechnol. Bioeng.* **2009**, *104*, 582–589.

[23] 4. Ren, Q.; de Roo, G.; Witholt, B.; Zinn, M.; Thöny-Meyer, L. Influence of growth stage on activities of polyhydroxyalkanoate (PHA) polymerase and PHA depolymerase in *Pseudomonas putida* U. *BMC Microbiol.* **2010**, *10*, 254.

[24] Khanna S, Srivastava A (2005). Recent Advances in Microbial Polyhydroxyalkanoates. Process Biochem., 40: 607-619.

[25]Pablo I. Nikel, Alejandra de Almeida, Evelia C. Melillo, Miguel A. Galvagno, and M. Julia Pettinari , Appl Environ Microbiol. 2006 Jun; 72(6): 3949–3954

[26] F Wang and S Y Lee , Appl Environ Microbiol. 1997 Dec; 63(12): 4765–4769

[27] Gulab Singh, Anish Kumari, Arpana Mittal, Anita Yadav, and Neeraj K. Aggarwal, BioMed Research International,Volume 2013 (2013), Article ID 952641, 10 pages

[28] http://en.wikipedia.org/wiki/Polyhydroxybutyrate

[29] http://www.geneticliteracyproject.org/wp-content/uploads/2014/09/e.coli-ed01.jpg

[30] http://en.wikipedia.org/wiki/Escherichia_coli

[31] http://en.wikipedia.org/wiki/Bacillus_subtilis

[32]https://www.google.co.in/search?q=bacillus+subtilis&espv=2&biw=1280&bih=699&source=lnms&tbm=isch&sa=X&ei=O35OVfHNKMfguQTo0oDwDg&sqi=2&ved=0CAYQ_AUoAQ#imgrc=RkjtosN-L9NGRM%253A%3BaZk-cTzyWHoyYM%3Bhttp%253A%252F%252Fupload.wikimedia.org%252Fwikipedia%252Fcommons%252Fb%252Fb9%252FBacillus_subtilis_Gram.jpg%3Bhttp%253A%252F%252Fen.wikipedia.org%252Fwiki%252FBacillus%3B500%3B375

[33] http://en.wikipedia.org/wiki/Gas_chromatography

[34]http://en.wikipedia.org/wiki/High-performance_liquid_chromatography