PROCESS OPTIMIZATION FOR POLYHYDROXY BUTYRATE (PHB) PRODUCTION FROM RENEWABLE CARBON SOURCE

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Submitted

in partial fulfillment of the

Degree of

Bachelor of Technology

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS,

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY, WAKNAGHAT

MAY 2009

CERTIFICATE

This is to certify that the work entitled, "Production and process parameter optimization of PHB " submitted by Isha Sodhi & Sonali Garg in fulfillment for the award of degree of Bachelors of Technology in Biotechnology of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Ms.Runni Mukherjee
25/05/09

ACKNOWLEDGEMENT

We hereby acknowledge with deep gratitude for the co-operation and help given to us by all the members of this organization (Jaypee University of Information Technology) in completing the final year project.

With proud privilege and profound sense of gratitude, we acknowledge our indebtedness to our guide Ms. Runni Mukherjee, for her valuable guidance, suggestions, constant encouragement and cooperation.

We express our heartfelt thanks to our Head of Department Dr.R.S Chauhan for providing us with the opportunities of doing this final year project.We extend our gratitude to Mr. Baleshwar (Lab. Attendant), and all other staff members of Deptt. of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Distt. Solan, H.P. for their numerous helps.

Last, but not the least, we express our indebtedness to our parents, brothers and sister whose support, love and affection has been a source of encouragement, which always motivates us to move ahead in life.

Place: JUIT, Waknaghat

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Chapter 1

ABSTRACT

1.1 Introduction

Polyhydroxyalkanoates, or PHAs, are homo- or heteropolyesters synthesized and intracellularly stored by numerous prokaryotes. They can be produced in large quantities from renewable resources by means of well known fermentation processes and the imposition of particular culture conditions, and a number of physical or chemical methods are known to extract them from the producing biomass. Production processes such as batch, semi-batch and continuous fermentation are all known to work. PHAs have properties similar to those of some polyolefins. This combined with the fact that they are fully and rapidly biodegraded under the appropriate conditions has generated a high interest in them as substitutes to petroleum-based polymers in many applications (Fuller and Lenz, 1990).

The majority of PHAs are aliphatic polyesters of carbon, oxygen and hydrogen. Their general formula is shown in Fig. 1.1. (Braunegg *et al.*, 1998). The composition of the side chain or atom R and value of *x* determine together the identity of a monomer unit. For poly(3-hydroxyalkanoates) (or poly(b-hydroxyalkanoates)), the most common PHAs, x=1. Pure P(3HB) is composed of monomers with a methyl group as side chain, and the P(3HV) units of P(3HB-*co*-3HV)s contain an ethyl group on carbon number 3. A large number of PHAs other than P(3HB) and P(3HB-*co*-3HV)s are now known.

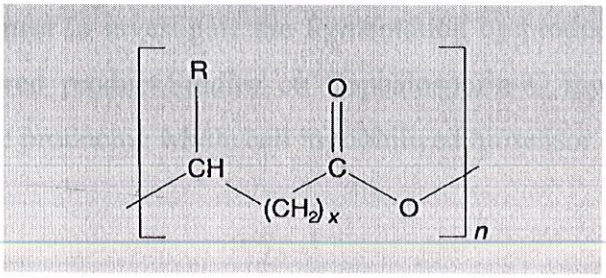


Fig. 1.1 .a: General formula for PHAs

Due to the stereo-specificity of biosynthetic enzymes, the chiral centers have the (R) stereochemical configuration, so that they are isotactic and optically active.

PHB is a carbon storage polymer widely distributed among prokaryotes including *Azotobacter sp.*. PHB and other PHAs have been considered commercially important because of their possible use as biodegradable thermoplastics. Poly-β hydroxyl-butyrate, (PHB) is a biodegradablethermoplastic which can be extracted from a widerange of bacteria. PHB belongs to the class of bacterial polyesters collectively called polyhydroxyalkanoates, (PHAs). PHAs have properties similar to polypropylene and are important due to their complete biodegradability, with recognised potential applications in reducing disposable waste problems and in certain medical applications (Gostomski, and Bungay, 1996). These biodegradable thermoplastics have many applications. They can be used as packaging material and can be utilized as drug delivery systems, since these polymers are immunologically inert. Biodegradable polymers would help to reduce solid waste disposal problems associated with most plastics. The following fifteen years saw intense research on the subject and featured important developments in knowledge about the polymer's widespread occurrence in micro-organisms including the bacterial genera *Bacillus*, *Pseudomonas* (Doudoroff and Stanier, 1959; Delafield *et al.*, 1965), *Azotobacter* , *Hydrogenomonas* , *Chromatium* (Schlegel, 1962), a cyanobacterium , and many others (reviewed in Dawes and Senior, 1973). Future scope is more stable protein engineered β-lactamase. Further stability studies in field of biosensors, Highly discriminative minuscule Transducer.Studies on biosensors fabricated using purified immobilized β-lactamase from *C. freundii* and *P. aeruginosa*.Smart signaling processing system. Studies on enhancing sensitivity of the biosensorTo investigate the fermentation by-products, which can interfere in monitoring of, desired product.Studies on Cephalosporin–C monitoring in continuous mode using β-lactamase producing whole cell immobilized biosensor.

Chapter 2

2.1 OBJECTIVE

- Selection of strain for PHB production
- Selection of a renewable and low cost carbon source
- Production of PHB using the selected strain
- Extraction and Quantification of PHB
- Optimization of process parameters
  - ✓ Carbon source
  - ✓ Ph
  - ✓ Temperature
  - ✓ Time

CHAPTER 3

LITERATURE REVIEW

3.1 Introduction

3.1.1 Pathway of PHA synthesis

P(3HB) is produced from acetyl-CoA by the sequential action of three enzymes, 3-ketothiolase, acetoacetyl-CoA reductase and PHA synthase (Pathway I) (Oeding and Schlegel, 1973). 3-Ketothiolase reversibly combines two acetyl-CoAs into acetoacetyl-CoA and is competitively inhibited by high concentrations of CoASH, which is released when acetyl-CoA enters the TCA cycle (Doi *et al.*, 1988b). NADPH-dependent acetoacetyl- CoA reductase reduces its substrate to R-3-hydroxybutyryl-CoA, and this is incorporated by PHA synthase into the polymer chain. 3HB units can also be synthesized form butyric acid directly via acetoacetyl-CoA without its prior degradation to acetyl-CoA (Pathway II) (Doi *et al.*, 1988b). In this sequence of reactions featuring β-oxidation of the substrate, both NADH-linked and NADPH-linked acetoacetyl-CoA reductases affect the epimerization of S-3-hydroxybutyryl-CoA to the R isomer, and 3-ketothiolase is not involved.

The key enzyme of PHA biosynthesis is PHA synthase.*a* and many other microorganisms synthesize poly(3HB) from acetyl-CoA via R(−)-3-hydroxybutyryl-CoA, employing a three-step pathway with β-ketothiolase, an NADPH-dependent acetoacetyl-CoA reductase and PHA synthase (Fig 2.3). Acetyl-CoA is a central intermediate in the metabolism of any organism and is therefore formed not only from carbohydrates and fatty acids but from any carbon source. Carbon sources that are degraded via acetoacetyl-CoA or R(−)-3-hydroxybutyryl-CoA will shortcut the pathways for PHAs.

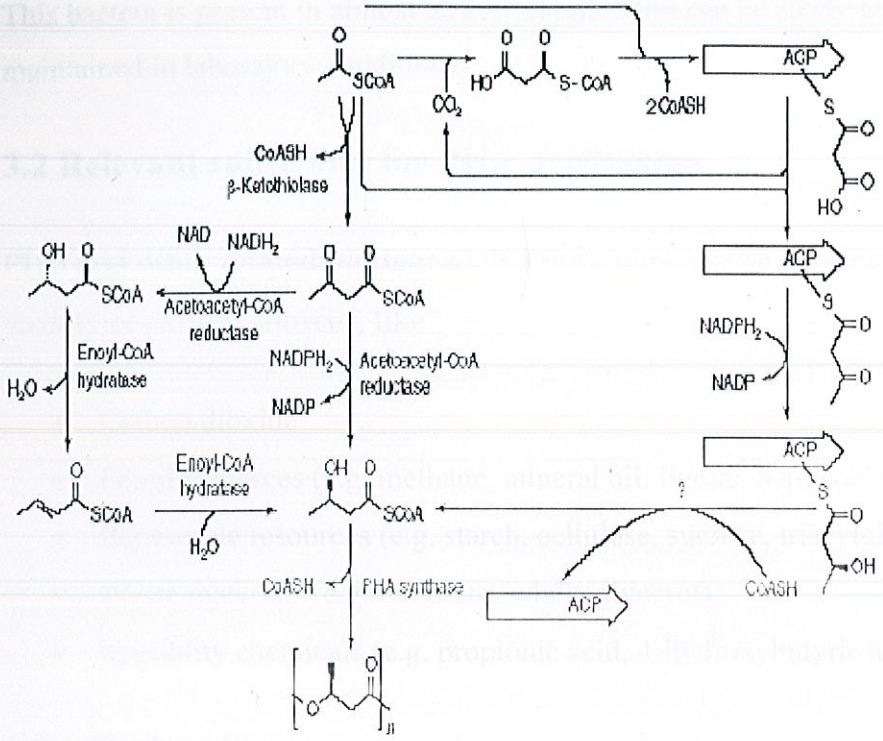


Fig 3.1.1.a Pathway of biosynthesis of PHB

3.1.2 Bacterial description

Alcaligenes is a genus of Gram-negative, aerobic, rod-shaped bacteria.

Alcaligenes species have been used for the industrial production of non-standard amino acids; *A. eutrophus* also produces the biopolymer polyhydroxybutyrate .

Fig 3.1.1.a: Intercellular PHB accumulation in *Alcaligenes latus* cell

This bactera is present in almost all soil samples and can be easily grown and maintained in laboratory conditions.

3.2 Relevant substrates for PHA production

PHB and other related biodegradable polymers can be produced from a variety of carbon sources, like

- Carbon dioxide
- Fossil resources (e.g. methane, mineral oil, lignite, hard coal)
- Renewable resources (e.g. starch, cellulose, sucrose, triacylglycerols)
- Waste materials (e.g. molasses, whey, glycerol)
- Speciality chemicals (e.g. propionic acid, 4-hydroxybutyric acid)

3.2.1 Carbon Source

- Kitchen waste as carbon source will cut down the production cost of PHB
- Easily available
- No costly pretreatment is required like molasses or hard coal
- Contains starch in sufficient amount
- Provides an alternative for waste treatment

3.4 Properties and features of PHB

(Steinbüchel and Füchtenbusch, 1998):

- Thermoplastic (melting point is around 177°C)
- Biodegradable
- Can be produced from renewable resources
- Nontoxic
- Biocompatible
- High degree of polymerization
- Insoluble in water
- Highly crystalline, if extracted from its natural environment
- Optically active
- Isotactic (i.e. stereochemical regularity in its repeating units)

3.5 Applications of Biopolymers

- Manufacture of bottles, films and fibers for biodegradable packaging materials and as mulch films for agriculture (Hocking and Marchessault, 1994).
- Latex of PHAs may be applied to paper or cardboard to form a water resistant layer and to produce a completely biodegradable compound (Lauzier *et al.*, 1993).
- PHAs as osteosynthetic materials, bone plates, surgical sutures and other materials in medicine has also been considered.
- PHAs and PLA can also be applied as a matrix in retardant materials for the slow release of drugs, hormones, herbicides, insecticides, flavors and fragrances in medicine, pharmacy, agriculture and the food industry.
- Sources for the synthesis of enantiomerically pure chemicals and as raw materials for the production of paints (Muller and Seebach, 1993).

Chapter 4

MATERIALS AND METHODS

4.1MATERIALS

4.1.1Microorganism

The following microbial culture was used:

Alcaligenes latus	**MTCC– 2239**

Strains were procured from Institute of Microbial Technology (IMTECH), Chandigarh.

4.1.2 Medium composition for maintenance of cultures

Medium for *Alcaligenes latus*

Constituents	g/l
Yeast extract	2.0
Peptone	5.0
Soil Extract	50 ml
Distilled Water	950 ml

pH : 6.8 – 7.2

4.1.3 Instruments used

- Digital pH Meter
- UV- Visible Spectrophotometer
- Incubator
- General purpose Balance
- Centrifuge
- Vertical autoclave
- Laminar Flow Cabinet
- Orbital Shaker
- Magnetic Stirrer & Hot Plate

4.1.3Media composition for production of polymer by *A.latus*

Constituents	
$NA_2HPO_4.12H_2O$	9.0 g/l
$MgSO_4.7H_2O$	0.2 g/l
KH_2PO_4	1.5 g/l
$CaCl_2.2H_2O$	0.01 g /l
Trace solution	1 ml
Carbon source (Starch)	To be optimized
Trace solution	
H_3BO_4	0.3 g/l
$CoCl_3.6H_2O$	0.2 g/l
$ZnSO_4.7H_2O$	0.3 g/l
$MnCl_2.7H_2O$	0.03 g/l
$NH_4MoO_4.2H_2O$	0.03 g/l
$CuSO_4$	0.01 g/l
$FeSO_4.7H_2O$	20.0 g/l
$NiSO_4$	0.01 g/l

A control media was produced with all above nutrients along with sucrose as the carbon source instead of potato peels.

4.1.4 Conditions for production of polymer using sucrose:

- ✓ pH – 6.7 – 7.0
- ✓ Temperature - 30°C
- ✓ Shaker speed – 150 rpm
- ✓ Time – 72 hours

4.2. Methodology-

4.2.1 Standard curve

A standard curve (OD vs. concentration) was plotted using pure PHB. This curve helps in determining the amount of PHB produced.

4.2.1.1 Preparation of standard curve

Pure PHB stock solution was prepared of concentration 10 µg/ml. From this stock various dilutions were made in order to plot the standard curve.

4.2.1.2 PHB stock dilutions

Stock (ml)	Chloroform (ml)
0.1	0.9
0.2	0.8
0.3	0.7
0.4	0.6
0.5	0.5
0.6	0.4
0.7	0.3
0.8	0.2

4.2.1.3 Calculation of standard curve

In all the test tubes that were diluted 0.1 ml chloroform was added and they were dried it at 40°C. Then 5ml conc. H_2SO_4 was added and kept at 100°C for 20 min. OD values were taken at 235 nm with sulfuric acid as blank.

4.2.2 Optimization of amount of potato peels

Potato peels were pretreated by first chopping them into small pieces and then boiling them in 1L of double distilled water and then they were homogenized. Growth was observed at varying concentrations of potato peels ranging from 30g/l to 100 g/l by monitoring the OD at different time intervals. The production media was used along with varying concentrations of potato peels as carbon source.

4.2.3 Carbon source

In Experiment (flask 1 & flask 2)

Potato peels 100g/L

(homogenized in chloroform)

In Control (flask 3)

Sucrose 20 g/L

4.3 PHB Extraction Procedure

- Take cultures and centrifuge them at 6000 rpm for 30 min.
- Dry pellets at 100°C for 24 hr.
- Resuspend in sterile water and homogenize
- Destroy cells walls by ultrasonication (5 min)
- Add 2 ml 2 N HCL in 2 ml cell suspension and dry it at 100°C for 2 hrs
- Centrifuge at 6000 rpm for 20 min
- Add 5 ml chloroform and leave overnight at 28°C, 150 rpm
- Centrifuge at 6000 rpm, 20 min
- Add 0.1 ml chloroform and dry it at 40°C
- Add 5ml conc. H2SO4 and keep at 100°C for 20 min
- Take OD at 235 nm with sulfuric acid as blank.

4.4 Optimization procedure

4.4.1 pH optimization

Experiment was conducted by taking seven bacterial cultures and varying the pH values from 6.6 to 7.2. Amount of PHB was obtained for each pH value after 72 hours. Temperature was maintained at 30°C. Media used was same as mentioned earlier. The output in the form of optical density was given as input in minitab software to generate a probability graph that will give optimized pH.

4.4.2 Temperature optimization

Experiment was conducted by taking seven bacterial cultures and varying the temperature conditions ranging between [illegible] Media was same as mentioned earlier. The output in the form of optical density was given as input in minitab software to generate a probability graph that will give optimized temperature.

4.4.3 Time optimization

Experiment was conducted by taking seven bacterial cultures and varying the number of hours of growth, keeping other parameters constant as above .Optical density was measured after every 24 hours. Media was same as mentioned earlier. The output in the form of optical density was given as input in minitab software to generate a graph that will give optimized time for growth.

Chapter 5

5.1 RESULTS AND DISCUSSION

5.1.1 Optimized Amount of Carbon Source

Amount of potato peels	*Observation*
30 g per litre of media	No significant growth
50 g per litre of media	No significant growth
75 g per litre of media	No significant growth
100 g per litre of media	Maximum growth

Thus potato peels were added to the production media in concentration of 100g/L

5.1.2 Standard Curve Plotting

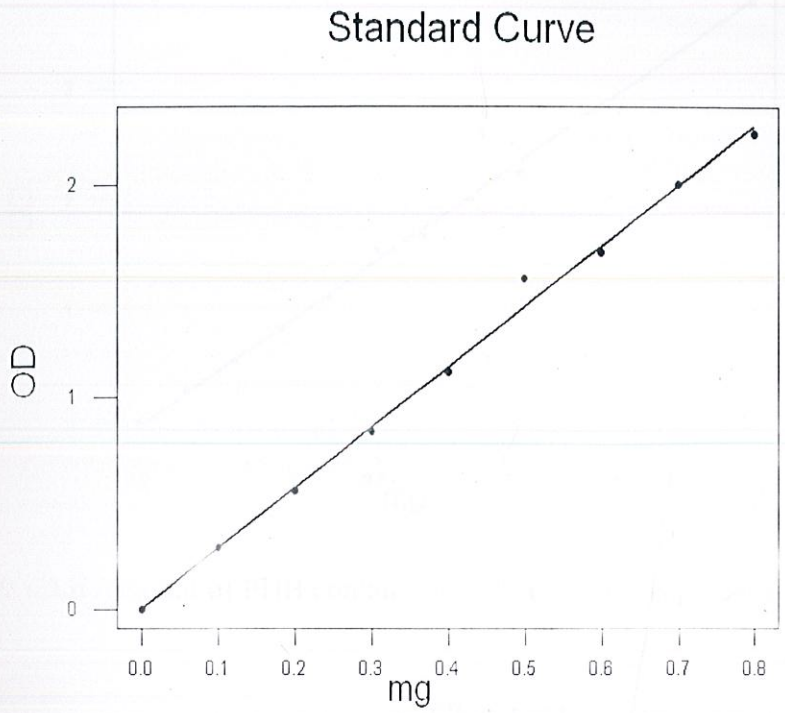


Fig 5.1.2.a Standard curve for pure PHB

5.1.3 Production Results

- ODs were taken at 235 nm and the values were plotted on the standard curve by extrapolating it in order to get the amount of PHB produced.
- 1.4 mg PHB was produced from 250 ml media inoculated with *Alcaligenes latus* **(Flask 1,Fig 5.1.3.b)**
- 1.29 mg PHB was produced from another set of same media. **(Flask 2, Fig 5.1.3.c)**
- 1.15 mg PHB was produced in control containing carbon source as sucrose. **(Flask 3, Fig 5.1.3.d)**

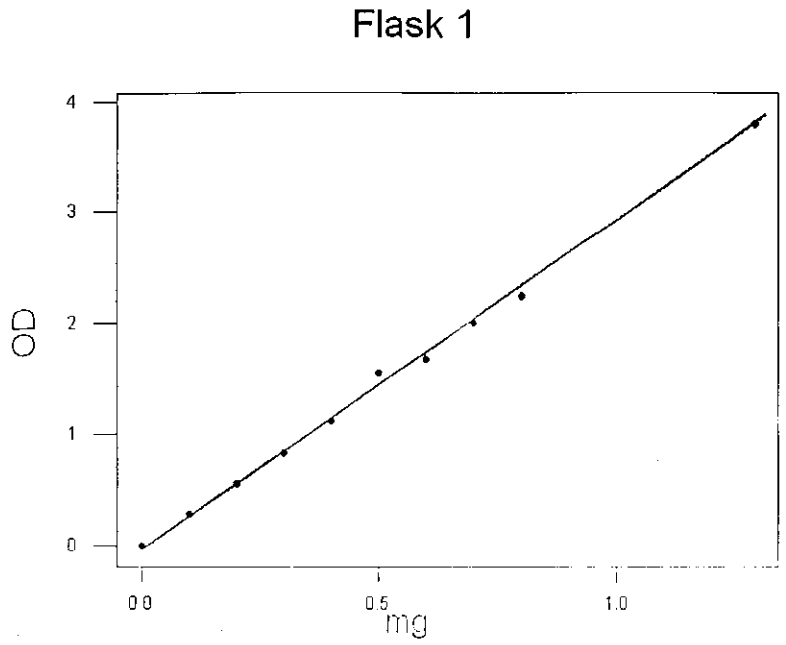


Fig 5.1.3.b Amount of PHB containing carbon source as potato peels in flask 1

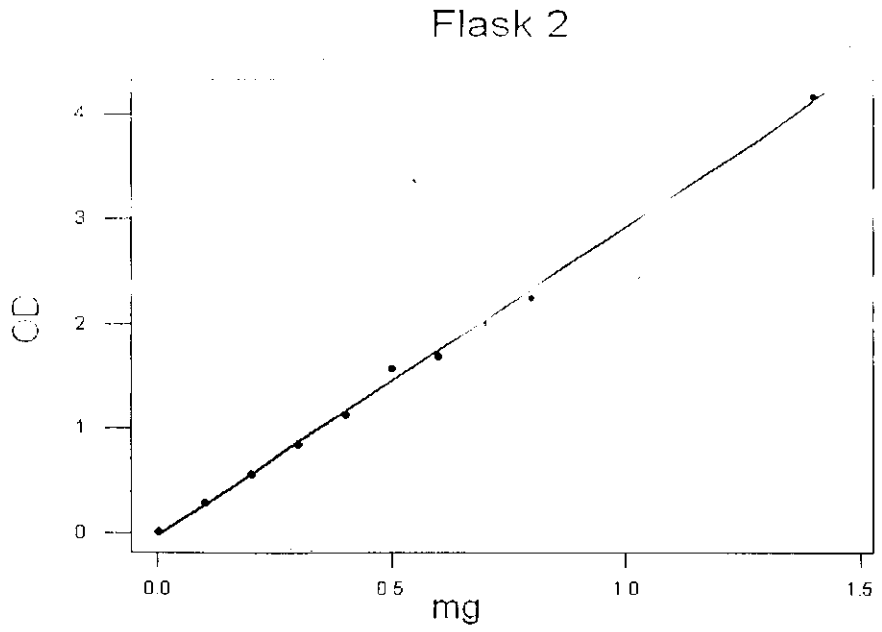


Fig 5.1.3.c Amount of PHB containing carbon source as potato peels in flask 2

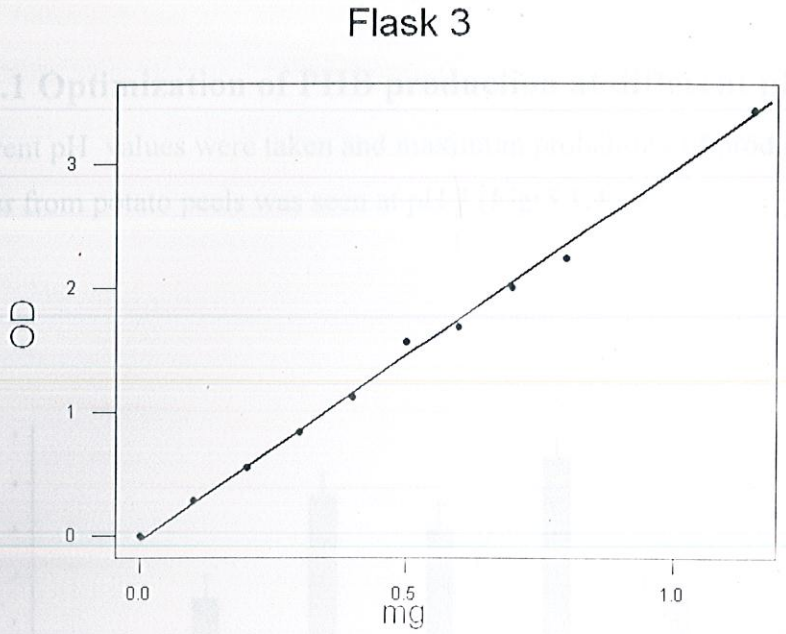
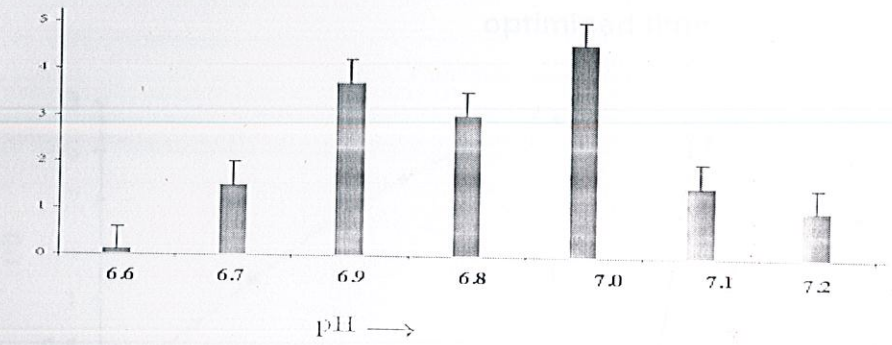


Fig 5.1.3.d Amount of PHB containing carbon source as sucrose in flask 3

5.1.4 Optimisation results

5.1.4.1 Optimization of PHB production at different pH

Different pH values were taken and maximum probability of production of PHB by *A.latus* from potato peels was seen at pH 7 **(Fig:5.1.4.a)**



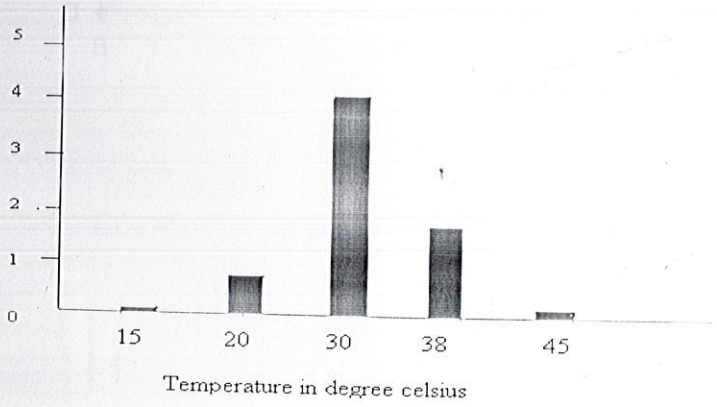


Fig 5.1.4.a: pH optimization plot **and 5.1.4.b:** Temperature optimization plot

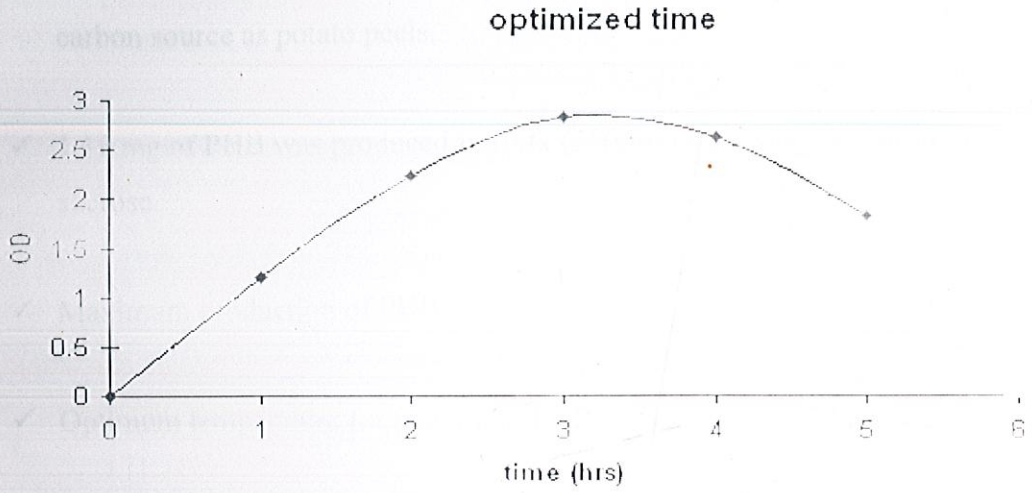
5.1.4.2 Optimization of PHB production at different temperatures

Different temperature ranges were taken and maximum probability was seen at 30°C. **(Fig 5.1.4.b)**

5.1.4.3 Optimization of PHB production at different time periods

It was observed that the amount of PHB showed a gradual increase till 3rd day of incubation and then decreased after 72 hours. **(Fig 5.1.4.c).** Thus 72 hours of incubation is the optimum time for production of PHB from potato peels using *A.latus*.

Fig 5.1.4.c Incubation time optimization



Chapter 6

6.1 CONCLUSION

- ✓ It observed that PHB can be successfully produced from potato peels as carbon source at temperature 30°C, pH 7, within 72 hours
- ✓ 1.4 and 1.29 mg of PHB in 250ml of media were produced in flasks having carbon source as potato peels.
- ✓ 1.15 mg of PHB was produced in flask (250ml) containing carbon source as sucrose.
- ✓ Maximum production of PHB was seen at ph 7
- ✓ Optimum temperature for maximum PHB production was 30 degree Celsius
- ✓ Maximum growth was seen after 72 hours and it declined after that.
- ✓ It was observed that production using potato peels as carbon source was more as compared to sucrose as carbon source.

Chapter 6

6.1 CONCLUSION

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Chapter 7

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