ESTIMATION OF KINETIC PARAMETERS FOR PRODUCTION OF PHB

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Bachelor of Technology In

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CERTIFICATE

This is to certify that the thesis entitled, "**ESTIMATION OF KINETIC PARAMETERS FOR PRODUCTION OF PHB"** submitted by Ms **Prayashree Bahalia** in partial fulfillment of the requirement for the award of **Bachelor of Technology** Degree in **Biotechnology Engineering** at the National Institute of Technology, Rourkela (Deemed University) 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.

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ABSTRACT

High use of plastics lead to environmental pollution as this undergoes a very slow process of degradation. To eradicate this problem there is a demand of bioplastics. Polyhydroxyalkanoates (PHAs) are bioplastics with most common form as Polyhydroxybutyrates. In this study, Bacillus subtilis is used to produce PHB using LB broth media. Glucose is used as carbon source and Ammonium sulphate is used as nitrogen source. The cells were cultured in optimal conditions with 37°C temperature, 200rpm in shaking flask incubator. With the estimation of PHB, the biomass formed and glucose utilized were also analyzed. With the references of the plots drawn with the data obtained, different kinetic parameters like μ, K_s, K_i, K_1, K_2 were determined.

Keywords: Polyhydroxybutyrate, Kinetic parameters, *Bacillus subtilis*

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Plastic is highly used by humans because of its properties like low cost, durability, etc,. But it shows very slow degradation; because of this its accumulation in environment adversely affects waterways and oceans, lands, wildlife, marine life and even humans. In humans, plastic can lead to disruption of thyroid hormone axis and hormone levels.

To avoid such adverse conditions, there is a need of production of bioplastics. Bioplastics are generally synthesized from renewable biomass sources like molasses, vegetable oils and fats, and different agricultural byproducts. These are biodegradable in nature and can break down in aerobic or anaerobic conditions, depending on their properties. Bioplastics can be of different types based on their manufacturing- starch based; cellulose based, aliphatic polyesters, genetically modified plastics, etc.

Polyhydroxybutrate also termed as PHB is the main focus of this study. PHB is the most common type of polyhydroxyalkanoates (PHA). Other derivatives of this polymer are produced by a variety of microorganisms: these include poly-4-hydroxybutyrate (P4HB), polyhydroxyvalerate (PHV), polyhydroxyhexanoate (PHH), polyhydroxyoctanoate (PHO) and their copolymers. The properties of PHB are the reason of interest for its production. This bioplastic shows optical activity, piezoelectricity and also high degree of crystallinity. Its degree of brittleness depends on the degree of its crystallinity, glass temperature and microstructure. The time for which it is stored in room temperature is directly proportional to its brittleness.

This is not soluble in water and also shows resistance to hydrolysis. This property makes it different from other biodegradable plastics. Other bioplastics are soluble in water and even shows sensitivity to moisture. PHB is non-toxic and shows good compatibility with blood and tissues and thus, can be used for medical purposes- surgical implants, seam threads, wound and blood vessel healing. This is also used in food industry for the purpose of packaging.

LITERATURE REVIEW

In 2001 Azza E. Swellam produced PHB by *Bacillus megaterium* strain using sugarcane molasses and corn steep liquoir as carbon and nitrogen source. In this the yield was found to be 46.2% per mg cell dry weight with 2% molasses [3]. Nazime Mercan in 2002 produced PHB by different *Rhizobium* species, among which *Rhizobium spp* 2426 showed highest yield of 74.03% dry cell weight after 48 hours [1]. Songsri Kulpreecha in 2008 [9] by a new isolate of *Bacillus megaterium* BA-019 did fed batch cultivation to produce PHB. In this yield found was 42% of cell dry weight. In 2002, L.H. Mahishi [5] synthesized PHB by recombinant *E.Coli* harbouring *Streptomyces aureofaciens* (PHB biosynthesis genes). The medium was provided with glycerol, glucose, yeast extract, palm oil, peptone, ethanol and corn steep liquior. In this the PHB yield was found to be 60% of dry cell weight after 48 hours. S P Valappil in 2007 [8] produced PHB by *Bacillus cereus* SPV and found an yield of 38% of its dry cell weight in shake flask cultures using glucose as main carbon source. Chih-Ching Chein in 2007 isolated *Vibrio spp*. M11 from marine environment [2]. He used media of acetate, glycerol, succinate, glucose and sucrose for production of PHB and found a yield of 41% of dry cell weight. Municipal waste water activated sludge with different carbon sources was used by Qiuyan Yuan in 2015 to produce PHB [6]. Phosphorous accumulating organisms were used in this and yield found was 15%. In 2012, Fereshteh Rahanama used *Methylocystis hirusta* from natural gas in bubble column and vertical loop for production of PHB and the yield found was 51.6% of dry cell weight.

PHB production was tried by *Methylobacterium organophilum* isolated from methanotriphic consortium in a two phase partition bioreactor [4]. In this the *Methylobacterium organophilum*, grown on methane was found to accumulate PHB of 57% dry cell weight. In 2012, Javier M. Naranjo, evaluated PHB production from glycerol with *Bacillus megaterium*. At industrial conditions the production of PHB was simulated using glycerol, then valorization of glycerol was assessed. In this the PHB accumulation was found to be 60% of its dry cell weight [7].

Comamonas species were isolated from dairy effluents by Thunoli Poyyanvalappil Prabish in 2015[10]. It was then given a suitable media for PHB production. The media consisted of MgSO4.7H2O, (NH4)2SO4, Na2HPO4, KH2PO4, glucose, yeast extract. The yield seen in this was 52.3% of its dry cell weight.

Optimization of production of PHB with tailor made molecular properties in *Azohydromonas lata* bacteria was done. This optimization process enhanced the intracellular accumulation of PHB. The PHB accumulation was found up to 95% of its dry cell weight in grams [16]. In a study done by Dan Tan, in 2011, it was seen that *Halomonas* TD01, a halophilic bacterium, showed PHB accumulation of 80% CDW when glucose salt was used in fed batch process for 56 hours. And when this cell was cultured on same media in an open unsterile continuous process for 14 days the PHB accumulation was found to be 40% of CDW [14].

Polyhydroxybutyrate was also tried to produce from bio waste. In 2011, Sanjay K.S. Patel analyzed PHB production using 23 different bacterial strains in different combinations belonging to *Bacillus spp*., *Enetrobacter aerogenes, Bordetella arium, Proteus mirabilis* and also marine bacterium. It was found that *Bacillus cereus* EG044 accumulated PHB of 62.5gm/Kg TS fed [18]. In a study done by A. Gacia in 2014, alteration of PHB regulating mutant was done in *Azobacter Vinelandii*. This was then cultured in fed batch fermentation on PY-sucrose media. To this PHB yield of 30% of CDW was observed [12].Under two stage fermentation *Cupriavidus taiwanensis* strains were cultiured on media containing gluconic acid and NH4Cl. This showed a very high yield of PHB, i.e. the PHB accumulation was found to be 72% of dry cell weight [15].

A strain *Bacillus subtilis* G1S1 was isolated from soil and was used in optimization of production of polyhydroxybutyrate in 2014 by K.R.Shah. The media used as carbon source was glucose. This showed a yield of 55% of dry cell weight [11].

From the above literature review it can be seen that many studies and experiments has been done for the production and optimization of PHB using different strains of microorganism and alternating media compositions.

CHAPTER 2

MATERIALS AND METHODS

Microorganisms and culture maintenance:

In this study *Bacillus subtilis* strain was used for the production of PHB. This strain was ordered from HiMedia, india and maintained in glycerol stock. It was taken out and grown on nutrient culture whenever required before processing it for further tests. For primary culture it is once grown in broth culture, again 1ml of broth culture was inoculated in another broth culture.

2-Step Culture:

Cells before use were cultured first in primary media and then in secondary media. The composition of both the media are same, but in primary media cell takes time to grow, that is the growth is not as fast as in secondary media, because the cells in this first take time to get adapted to new environment. Then culture from this is used to inoculate in secondary media, in this a fast growth of cells are seen and cells in this stage can be used for different kinetic studies.

Medium and culture conditions:

In this LB broth was used for the cultivation of bacillus subtilis. This media contains Casein enzymic hydrolysate, yeast extract, sodium chloride. This media is used as it provides complete nutrient to the strain and acts as a great source for carbon. Liquid culture was prepared by inoculating a loop full of bacterial cells in 100 ml LB broth medium and incubating the culture overnight at 37 °C in an incubator shaker set at 200 rpm. For preparing media for PHB production 5g of LB media was added to 250ml of distilled water. To it 2.5gm of glucose was added as carbon source and 0.25g of ammonium sulphate as nitrogen source. The media was sterilized in autoclave at 15lb pressure, 121°C temperature and for 15minutes.

From the liquid culture 2ml of sample was inoculated into 200ml of media prepared for PHB production. Other 50ml of media was kept in same temperature to be used as control for further analysis. These were kept in shaking incubator at 200rpm at 37°C for 24 hours.

Sample collection:

At the time of inoculation 3ml of sample was collected in 3 eppendorf tube- 1ml in each. Further samples were collected in every two hour intervals for 12hours. This experiment was repeated for 72 hours, collecting samples at an interval of 12 hours. These samples were stored in 4°C in freezer.

Stock solution preparation:

PHB stock of 200mg/ml was prepared. For this 200mg PHB powder was dissolved in 1ml H_2SO_4. After that stock was put in water bath at 100°C for 20minutes. In that incubation period, PHB converted into crotonic acid. Then the test tube was left in room temperature. After cooling, the solution was serially diluted from 20µg/ml to200µg/ml and absorbance was taken at 235nm and the standard curve was plot.

DNS reagent preparation:

Solution A was prepared with 40g of Sodium potassium tartarate (Rochelle salt) in 75 ml of H_2O. In solution B, 1.5g of DNS was added to 30ml of NaOH. To prepare 50 ml of 2M NaOH 4g of NaOH was added to 50 ml of H_2O. Solution A and B were mixed and the volume was made up to 150ml.with distil water. This was stored at room temperature.

Reducing glucose test:

Sugars are assayed by using their reducing properties. One such compound 3,5-dinitrosalicylic acid which in alkaline solution, gets reduced to 3-amino,5-nitro salicylic acid. The degree of color intensity formed due to the presence of reduced compound in the reaction mixture can be directly correlated to the amount of reducing sugar present in that. For plotting DNS standard curve at first glucose stock solution was prepared.. 5ml of stock solution with 10g/l

concentration was prepared by adding 50mg of glucose to 5ml of water. Dilutions of this stock solution from 0.2 to 2 were prepared by adding 20µl of stock solution to 980µl of water, 40µl of stock in 960µl of water and so on. To each test tube 1ml of distilled water is added followed by 3ml of DNS to each test tube. Test tubes were then placed in hot water bath in boiling water for exactly 5minutes. After taking out the test tubes were cooled under running tap water. Then 20ml of distill water was added to each test tube. OD of these samples was measured at 540nm.

Cell dry weight estimation:

Before collecting the samples, weight of each eppendorf tube was taken. After collecting the sample, one set of the sample was put to centrifuge at 8000rpm for 15 minutes. The supernatant was removed to another tube for DNS test. The pellet was washed with distilled water by adding 1ml of water to each tube and centrifuging it again for 15minutes at 8000rpm. The water was carefully removed by micropipette without disturbing the pellets. The tubes were left for air drying for 2-3 days. After it gets completely dried up, weight of each tube was again measured and was compared with the initial weight.

Reducing glucose test of samples:

The supernatant collected after centrifugation is used to study reducing sugar or the glucose utilized by the cells. 100µl from each eppendorf were taken into different test tubes using pipette. 900µl of distil water is added to it and mixed. To each test tube again 1ml of distil water was added followed by 3ml of DNS reagent. Then these test tubes were kept in hot water bath in boiling water for 5 minutes. Then the test tubes were kept under running tap water for cooling. At last 20ml of distil water was added to each of it and mixed properly. OD of these samples was measured at 540nm.

PHB estimation:

Other set of samples collected was centrifuged at 8000 rpm for 10minutes. After removing the supernatant, the pellet was washed with acetone and methanol for 10 minutes each. During this process the lipids, proteins and other components other than PHB is washed away. This is now kept for air drying for 2-3 days. After it gets completely dried up, 1ml of H_2SO_4 was added to each tube and this was kept in hot water bath for 10minutes at 100°C. During this process PHB was converted into crotonic acid. The crotonic acid is then diluted in distil water. 100µl was taken from each test tube and mixed with 900µl water in different test tubes. These samples were then used for OD measurement in spectrophotometer. OD was measured at 235nm.

Analytical methods:

PHB estimation was done by measuring OD at 235nm and was then by correlating the absorbance values with the standard curve of PHB plot. Similarly glucose estimation was done by measuring the OD of processed sample at 590nm and then by putting the absorbance value found, in the equation of standard curve of DNS plot.

CHAPTER 3

RESULTS AND DISCUSSION

RESULT:

The absorbance of PHB at different concentration was taken by UV spectrophotometer and the data obtained was used to plot PHB standard curve that would be further used for estimation of kinetic parameters. The standard curve plotted is shown in figure 1 and the absorbance obtained for plotting of the graph is written under table 1.

Table 1:

Absorbance for standard curve:

PHB Concentration (ug/ml	Absorbance(235nm)
0	0
20	0.089333333
40	0.164833333
60	0.264666667
80	0.3465
100	0.4195
120	0.487
140	0.560666667
160	0.623666667
180	0.699666667
200	0.747666667

Figure 1:

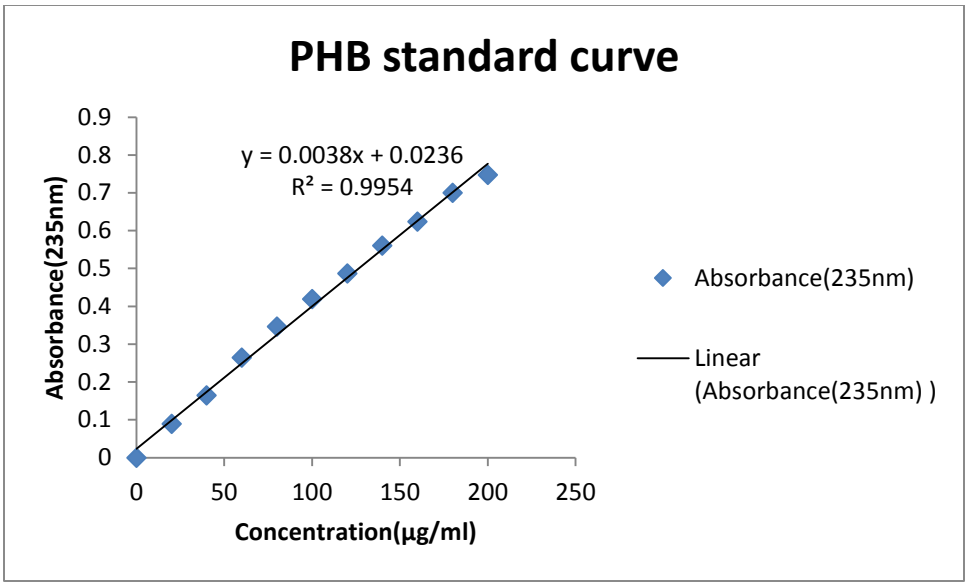


Table 2:

The standard curve for DNS is required to estimate the reducing glucose. The absorbance of glucose stock solution with DNS reagent was measured under visible spectrophotometer and the observation is shown in table 2.

Absorbance for DNS standard curve:

CONC(mg/ml)	ABSORBANCE(540nm)
0	0
0.2	0.053
0.4	0.097
0.6	0.152
0.8	0.19
1	0.250333333
1.2	0.323
1.4	0.388
1.6	0.444
1.8	0.492
2	0.542

Figure 2:

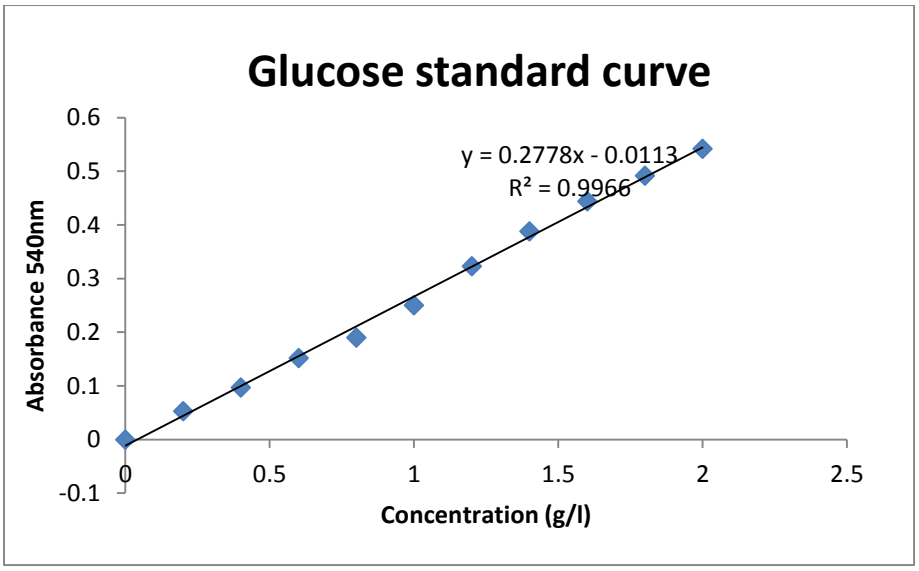


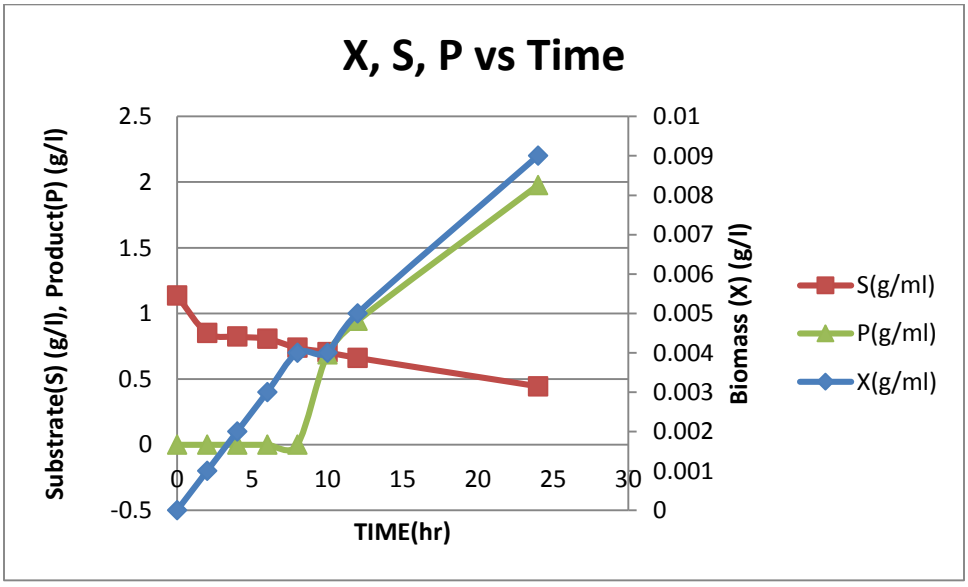
Figure 2 shows glucose standard curve, the equation of this plot is further used for analyzing the reducing glucose during PHB production.

The samples were collected in set of 3 at the intervals of 2hours were processed for 12hours and the samples were used for measuring biomass(X), Substrate(s) i.e. glucose used up by cells and PHB i.e. product (P) with respect to time. The culture used in this experiment was a solid culture taken up from the agar plates. The value of X, S, P with respect to time is tabulated under table 3.

Table 3:

TIME	X(g/ml)	S(g/ml)	P(g/ml)
0	0	1.135981	0
2	0.001	0.851986	0
4	0.002	0.823105	0
6	0.003	0.806258	0
8	0.004	0.737665	0
10	0.004	0.703971	0.688333
12	0.005	0.66065	0.943333
24	0.009	0.444043	1.976667

Figure 3:



Here it was seen that, with increasing time the biomass(X) and the product (P) increases and the glucose present in the substrate decreases as it is continuously utilized by the cells. Particularly in this case, the product formation is not much in first 8 hours. As the culture used in this was in solid form because of which it takes time for its adaptation and multiplication.

Table 4 shows the values of X, S, P with respect to time, each taken at interval of 12 hours for 36 hours.

Table 4:

TIME	x	s	P
0hr	0	0.008303	0.018333
12hr	0.5	0.005884	1.033333
24hr	0.8	0.003863	1.376667
36hr	1.1	0	1.413333

Figure 4 shows the plot of X, S, P with respect to time.

Figure 4:

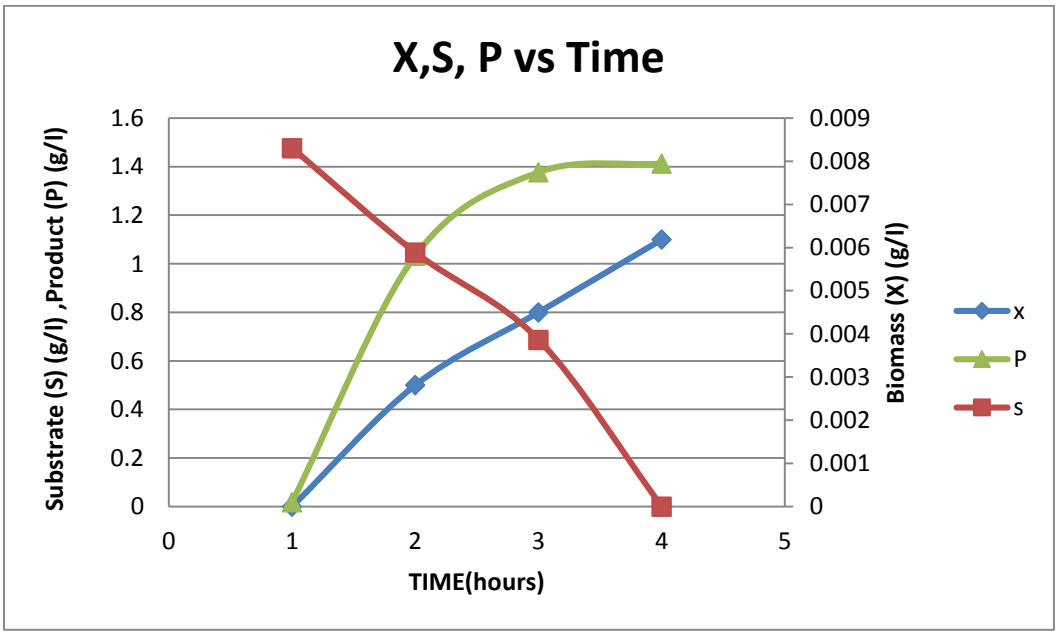


Table 5 shows the values of X and P with of samples collected each at the interval of 12 hours. The sample collection was done till 72 hours.

Table 5:

TIME(hr)	X(g/l)	P(g/l)
0	0	0
12	0.006	1.151667
24	0.017	1.391667
36	0.002	1.535
48	0.026	1.861667
60	0.038	1.938333
72	0.052	1.95

Figure 5:

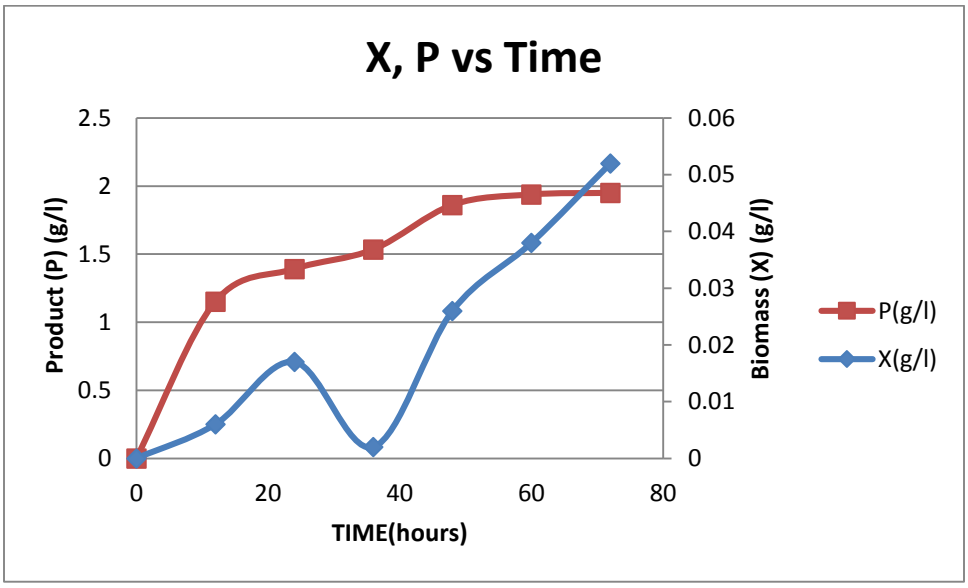


Figure 5 shows the plot of X, P with respect to time (at 12 hour interval) up to 72 hours.

1ml of culture from broth culture was added to media with glucose. Table 6 shows the value of X, S, P at the interval of 2hours up to 12 hours.

Table 6:

Time	X(g/l)	S(g/l)	P(g/l)
0	0	0.920578	0.00000
2	0.005	0.844765	0.01923
4	0.006	0.812274	0.24861
6	0.008	0.711191	0.36791
8	0.009	0.6787	0.54949
10	0.01	0.610108	0.63765
12	0.011	0.534296	0.84072
24	0.016	0.231047	1.27493

Figure 6:

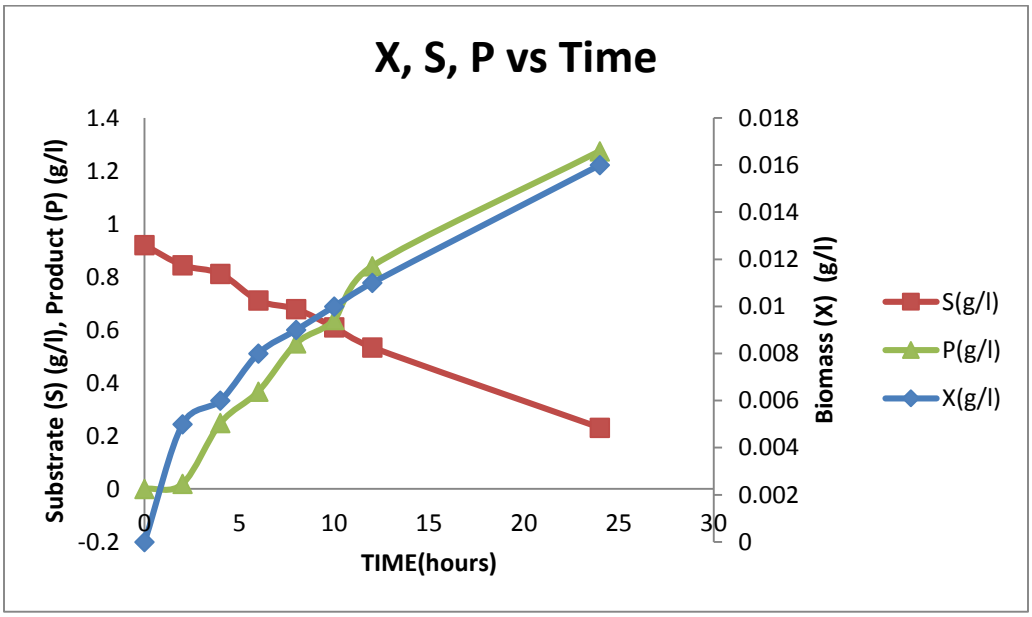


Figure 6 shows the plot of X, S, P in table 6 with respect to time.

Equations used for estimation of kinetic parameters:

$(1/x)*(dx/dt) = \mu = \mu_m (S/(Ks + S))(Ks/(Ki + S))$

$(1/x)*(ds/dt) = q_s = (1/y)\ \mu + m$

$(1/x)*(dp/dt) = q_p = K_1 (\mu) + K_2$

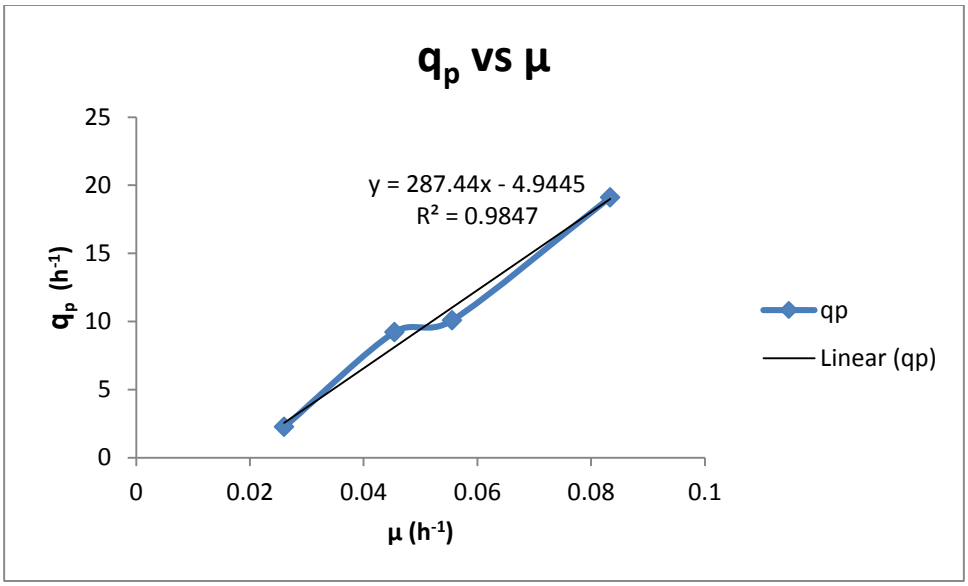
q_p is the specific rate of product formation calculated by multiplying (1/x) and dp/dt.

Figure 7 shows plot between q_p and μ. The value of K_1 and K_2 can be derived from this equation where K_1 is growth associated and K_2 is non-growth associated.

Table 7:

$\mu(h^{-1})$	$q_p\ (h^{-1})$
0.083333	19.11549708
0.055556	10.0877193
0.045455	9.23046252
0.026042	2.261513158

Figure 7:



y= 287.4x-4.944

K_1=287.4

K_2= -4.944≈ 0

As the value of K_2 is much less as compared to the value of K_1 it can be taken as 0.

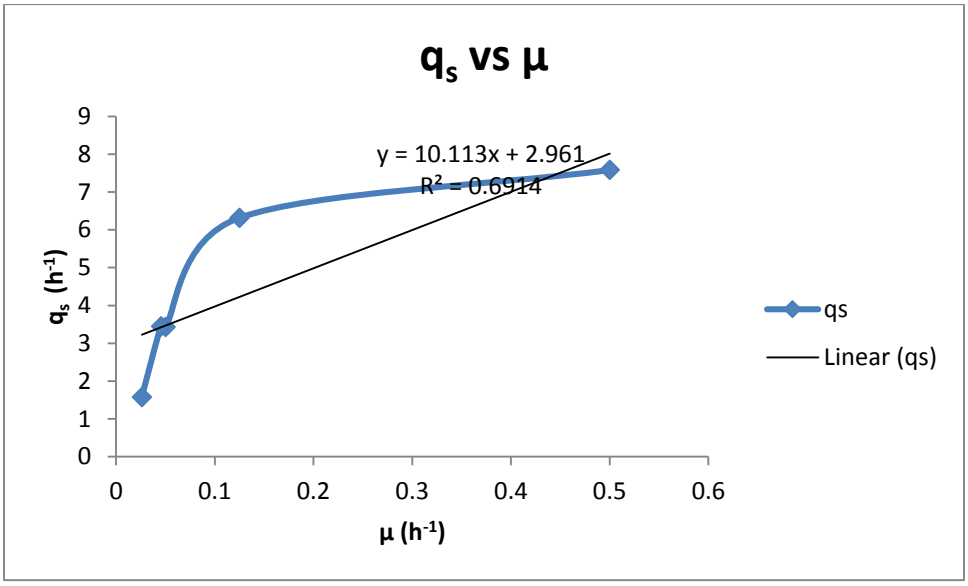
The specific rate of substrate consumption is q_s. It can be calculated by multiplying (1/X) with ds/dt. The plot q_s vs μ is shown in figure 8 and is used to get the value of Y (yield) i.e. the biomass formed from per unit of substrate. The slope of figure 8 gives 1/Y while its intercept gives the value of m.

Graph of q_s vs μ:

Table 8:

μ (h^{-1})	q_s (h^{-1})
0.5	7.581227
0.125	6.31769
0.05	3.429603
0.045455	3.446012
0.026042	1.579422

Figure 8:



y= 10.11x + 2.961

(1/y) = 10.11

m = 2.961

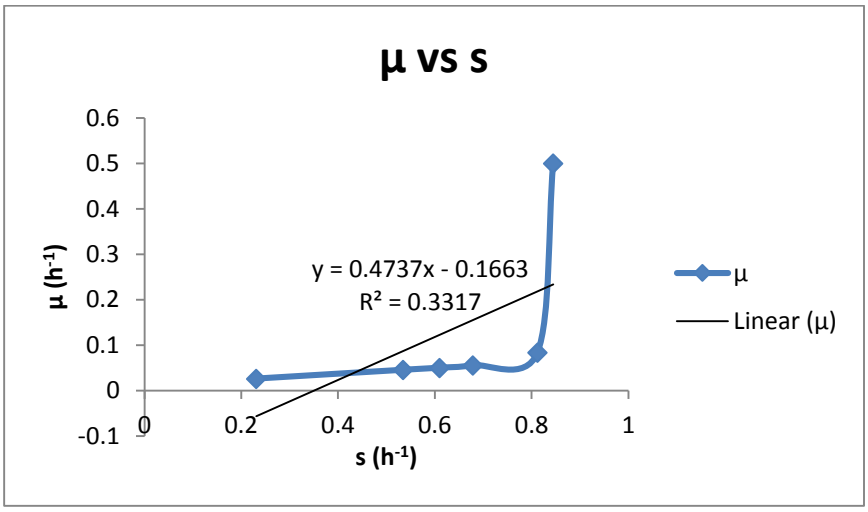
Graph of μ vs S:

Ks can be calculated from the plot μ vs S. Ks is the concentration of substrate at which the specific growth rate is half of the maximum specific growth rate.

Table 9:

S (g/l)	μ (h^{-1})
0.844765	0.5
0.812274	0.083333
0.6787	0.055556
0.610108	0.05
0.534296	0.045455
0.231047	0.026042

Figure 9:



μm = o.5

μm/2 = 0.25

y= 0.473x-0.166

Here x= K_s

K_s= (0.25+0.166)/0.473

K_s= 0.87949

K_i= 180

Table 10:

Kinetic parameters	value	Unit
μ_m	0.5	h^{-1}
K_1	287.4	g/g
K_2	0	g/g/h
K_i	180	
m	2.961	g/g/h
1/Y	10.11	g/g
K_s	0.879	g/l

DISCUSSION:

In this study the kinetic parameters like maximum specific growth rate (μ_m), growth associated constant (K_1), non growth associated constant (K_2), maintenance coefficient (m), growth inhibition constant (K_i), and yield (Y). These values were estimated from different other parameters like specific rate of product formation (q_p), specific rate of substrate consumption (q_s), specific growth rate (μ), and substrate concentration.

From figure 7 we observed that the specific rate of product formation i.e q_p increases with increasing specific growth rate (μ). The slope of this plot gives the value of growth associated constant and non growth associated constant. This equation of this plot helps to know if the relation is growth associated or non growth associated. Growth associated suggests that the product formation takes place along with the cell growth while non-growth associated states that the product formation takes place in stationary phase only. In figure 7 the K_2 value is approximately zero, this indicates that in this case, the product formation is growth associated that is the PHB production takes place at the time of cell growth only.

A non linear relationship was seen between specific rate of substrate consumption and specific growth rate was seen in figure 8. The specific rate of substrate consumption for maintenance of cells that is the maintenance coefficient (m) is estimated from this plot. Its value was found to be 2.961. The biomass obtained per unit of substrate (Y) is calculated from its slope and was found that for every 1 gm of substrate the biomass formed is0.099g.

The growth inhibition constant K_i and K_s is obtained from plot in figure 9. K_s is the substrate concentration when the specific growth rate is equal to half of the maximum specific growth rate. Thus the K_s was found to be 0.879 g/l. K_i is calculated to know the growth inhibition that is caused by the inhibitory components that might be present in the substrate. K_i was determined as 180. As the K_i value is greater than 1. It indicated non-competitive growth.

CHAPTER 4

CONCLUSION

CONCLUSION:

In this study production of PHB was done by *Bacillus subtilis*. At first, standard curves for PHB and glucose were plot. *Bacillus subtilis* were cultured on LB media with glucose as carbon source and ammonium sulphate as nitrogen source. Samples were collected and used for estimation of X, S, P. Using the value of X, S, P further values of q_s, q_p and μ were calculated. The growth parameters-maximum specific growth rate (μ_m), growth associated constant (K_1), non-growth associated constant (K_2), maintenance coefficient (m), Yield (1/Y), inhibition constant (K_i) and K_s were found to be 0.5(1/h), 287.4 g/g, 0, 2.961 g/g/h, 10.11 g/g, 180, 0.789 g/l.

The PHB was produced and its kinetic parameters were obtained. Polyhydrxybutrate is a biodegradable plastic and can be helpful in eliminating the plastic pollution. Also its properties like piezoelectricity, brittleness, non-solubility in water, etc make it an option of replacement of plastics in pharmacology (in microcapsules), in hospitals (as surgical implants), in industries (for packaging, making up of lamina ted foils, bottles, fishnets, fibres). So this can easily be used as a substitute for plastics without compromising with its characteristics.

CHAPTER 5

REFERENCES

1. Nazime Mercan, Production of Poly-b-Hydroxybutyrate (PHB) by Some Rhizobium Bacteria, (2002), 215-219
2. Chih-Ching Chien , Chang-Chieh Chen, Meng-Hui Choi, Shieh-Shiuh Kung, Yu-Hong Wei, Production of poly-_-hydroxybutyrate (PHB) by *Vibrio* spp. isolated from marine environment,(2007), 132, 259-263.
3. Mona K. Gouda, Azza E. Swellam, Sanaa H. Omar**,** Production of PHB by a *Bacillus megaterium* strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources, (2001), 201-207
4. C. Zúˇniga, M. Morales, S. Le Borgne, S. Revah , Production of polyhydroxybutyrate (PHB) by Methylobacterium organophilum isolated from a methanotrophic consortium in a two-phase partition bioreactor, (2011), 876-882
5. L. H. Mahishi, G. Tripathi, S. K. Rawal, Poly(3-hydroxybutyrate) (PHB) synthesis by recombinant *Escherichia coli* harbouring *Streptomyces aureofaciens* PHB biosynthesis genes: Effect of various carbon and nitrogen sources,(2003), 158, 19-27
6. Qiuyan Yuan, Richard Sparling and Jan Oleszkiewicz, Polyhydroxybutyrate Production from Municipal Wastewater Activated Sludge with Different Carbon Sources,(2015), 8, 53-58.
7. Javier M. Naranjo, John A. Posada, Juan C. Higuita, Carlos A. Cardona, Valorization of glycerol through the production of biopolymers: The PHB case using Bacillus megaterium, (2013) , 133, 38-44.
8. S.P. Valappil, S.K. Misra, A.R. Boccaccini, T. Keshavarz, C. Bucke, I. Roy, Large-scale production and efficient recovery of PHB with desirable material properties, from the newly characterized *Bacillus cereus* SPV, (2007), 132, 251-258
9. Songsri Kulpreecha, Atipol Boonruangthavorn,Boonyarit Meksiriporn,and Nuttha Thongchul, Inexpensive fed-batch cultivation for high poly(3-hydroxybutyrate) production by a new isolate of *Bacillus megaterium*, (2009), 107, 240-245.
10. Thunoli Payyanvalappil Prabisha, Raveendran Sindhua, Parameswaran Binoda, Vandana Sankar, Kozhiparambil Gopalan Raghu, Ashok Pandeya, Production and characterization of PHB from a novel isolate Comamonas sp. from a dairy effluent sample and its application in cell culture,(2015), 101, 150-159.

11. K.R.Shah, Optimization and production of Polyhydroxybutarate(PHB) by *Bacillus subtilis* G1S1from soil, (2014),3(5), 377-387
12. A. García, D. Segura, G. Espín, E. Galindo, T. Castillo a, C. Pe˜na, High production of poly--hydroxybutyrate (PHB) by an Azotobacter vinelandii mutant altered in PHB regulation using a fed-batch fermentation process, (2014),82, 117-123.
13. Franziska Hempel, Andrew S Bozarth, Nicole Lindenkamp, Andreas Klingl, Stefan Zauner, Uwe Linne, Alexander Steinbüchel and Uwe G Maier, Microalgae as bioreactors for bioplastic production,(2011),2-6.
14. Dan Tan, Yuan-Sheng Xue , Gulsimay Aibaidula , Guo-Qiang Chen, Unsterile and continuous production of polyhydroxybutyrate by Halomonas TD01 (2011), 102, 8130-8136
15. Yu-Hong Wei, Wei-Chuan Chen, Chin-Kuei Huang, Ho-Shing Wu, Yi-Ming Sun, Chi-Wei Lo and Om-Murugan Janarthanan, Screening and Evaluation of Polyhydroxybutyrate Producing Strains from Indigenous Isolate *Cupriavidus taiwanensis* Strains, (2011), 12, 252-265
16. Giannis Penloglou, Christos Chatzidoukas, Costas Kiparissides, Microbial production of polyhydroxybutyrate with tailor-made properties: An integrated modelling approach and experimental validation, (2012),30, 329-337.
17. Elsayed B. Belal, Production of Poly-β-Hydroxybutyric Acid (PHB) by *Rhizobium elti* and *Pseudomonas stutzeri,* (2013), 5(6), 273-284.
18. Sanjay K.S. Patel, Mamtesh Singh, Prasun Kumar, Hemant J. Purohit ,Vipin C. Kalia, Exploitation of defined bacterial cultures for production of hydrogen and polyhydroxybutyrate from pea-shells (2012), 36, 218-225.