Optimization of the Yield of Thermoplastic PHB Using Statistical Approach: Validation by Pilot Scale Fermentation Process.

By

ANOOP GOEL - 031556
ARCHANA SHARMA - 031546

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CERTIFICATE

This is to certify that the work entitled, "**Optimization of the yield of thermoplastic PHB using statistical approach: Validation by pilot scale fermentation process**" submitted by Mr. Anoop Goel and Miss Archana Sharma in partial fulfillement for the award of degree of Bachelor of Technology in Biotechnology and Bioinformatics 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.

Dr. Sudhir Syal
(Project Coordinator)
Senior Lecturer
Dept. of Bioinformatics and Biotechnology
Jaypee University of Information Technology
Waknaghat, Solan, Himachal Pradesh, India

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Hope our as well as their efforts will bite the fruit of success.

Thanking You!

(The Authors)

Anoop Goel

Archana Sharma

Anoop Goel

Archana

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Place : JUIT, Waknaghat

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LIST OF SYMBOLS AND ABBREVIATIONS

3HB	d-(±)-3-hydroxybutyryl-CoA	**Oxy Perm**	Oxygen Permeability ($cm^3m^{-3}atm^{-1}d^{-1}$)
A	Acetic acid	**P**	Propanoic acid
C/N	Ratio of Carbon to Nitrogen source in the growth media	**P-(3HB-co-3HV)**	Poly-(3-hydroxybutyrate-co-3-hydroxyvalerate)
C	Carbon source	**PHA**	Polyhydroxyalcanoate
Conc	Concentration ($g \cdot lt^{-1}$)	**PHB/ Poly-(3HB)**	Poly-β-hydroxybutyric acid
Cry	Crystallinity (%)		
ρ	Density (g/cm^3)	**PHV/Poly-(3HV)**	Poly-3-hydroxyvalerate
DCW	Dry Cell Weight (g)		
F	Fructose	**S**	Sucrose
FM	Flexural Modulus (GPa)	**Sod**	Sodium acetate
F_t	Fermentation time (hrs.)	**Sor**	Sorbose
G	Glucose	**Temp**	Temperature (°C)
Inc	Seed inoculum (%)	**T_{GT}**	Glass Transition Temperature (°C)
L	Lactic acid		
M Wt	Molecular weight	**T_M**	Melting point (°C)
N	Nitrogen source	**U**	Urea
NC	Ammonium chloride	**X**	Xylose
NN	Ammonium nitrate		

ABSTRACT

Poly-β-hydroxybutyric acid (PHB) is a natural, biodegradable polymer, which is accumulated as an energy reserve material by a large number of bacteria when, nutrients such as nitrogen or phosphorous sources are available in limiting concentrations in the presence of excess carbon source. The production process of PHB heavily depends on five crucial parameters – pH of the growth media, the amount of seed inoculum taken, the fermentation time, operational temperature and the ratio of carbon to nitrogen content in the media provided. From the literature review, a data set was generated providing the information of the PHB yielded from different organisms under various combinations of the aforementioned parameters. The organisms were distributed among five categories on the basis of their PHB yielding capacities. Using the statistically derived linear regression model for each category, generated upon the data set using the software 'MINITAB Ver.13.1', an equation was formulated which is used to predict the yield of PHB under the various growth conditions. This model was used to optimize the yield of PHB in a pilot scale production process. Using Sucrose and Ammonium sulphate as Carbon and Nitrogen source respectively for A. latus, the predicted yield was 1.618 g/l in 108 hours at pH 7.2 and 33°C temperature with 3% inoculum under C/N ratio of 16.0. Similarly using Glucose and Ammonium sulphate as Carbon and Nitrogen source respectively for R. eutropha, a yield of 1.3041 g/l was predicted at (pH=6.8, Ft=72 hrs, Temp=27°C, Inc=2.5%, C/N=15.5).

1
INTRODUCTION

Plastics, due to their versatile qualities of strength, lightness, durability and low production cost, have now become the essential ingredients to enhance the comfort and quality of our life. Plastic materials have become an integral part of contemporary life because of their many desirable properties including durability and resistance to degradation. The current global plastic industry turn over is about $1 trillion/year and represent 3.7% of the world GDP. These non degradable plastics accumulate in the environment at a rate of millions of ton per year causing several problems. Recently, issues concerning the global environment and solid waste management have created much interest in the development of biodegradable plastics (Anderson, A.J. Dawes, 1990).

Poly (3-hydroxybutyrate) (Poly-(3HB)) was first isolated and characterized in 1926 by Lemoigne at the Pasteur Institute in Paris (Lemoigne, 1926). PHAs are natural biodegradable thermoplastics. One of such polymers is poly-β-hydroxybutyrate (PHB), accumulated by many bacteria as carbon and energy reserve material (Anderson, A.J. Dawes, 1990). The production of PHA from renewable resources, offers additional ecological advantages of the polymer as compared to thermoplastics and elastomers produced from fossil carbon sources (Byrom,1992) (Table 1). The suitability of a bacterium for the PHA production depends on many different factors such as stability and safety of the organism, growth and accumulation rates, achievable cell densities and PHA contents, extractability of PHA, molecular weights of accumulated PHA, range of utilizable carbon sources, costs of the carbon source and other components of the medium, and occurrence of by-products (Hrabak, 1992). As thermoplastic polymer PHA can be processed like other synthetic thermoplastics however some polymer properties thermal stability, brittleness and relatively high cost - are reasons limiting its processing and use.

Whereas P-(3HB) is somewhat brittle and thus limited in its commercial scope, a closely related copolymer, poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) (P-(3HB-co-

3HV)), is more flexible because of reduced crystallinity and is suitable for many commercial applications (Fig.1).

Table 1
Comparison of PHB with traditional plastic

Parameter	Polypropylene (pp)	PHB
T_m (°C)	171-181	171-182
T_{GT} (°C)	~15	5-10
Cry (%)	65-70	65-80
ρ (g/cm^3)	0.905-0.95	1.23-1.25
M Wt (* 10^{-5})	2.2-7	1-8
M Wt Distribution	5-12	2.2-3
FM (GPa)	1.7	3.524
Tensile strength (MPa)	39	40
Extension to Break (%)	400	6-8
UV Resistance	Poor	Good
Solvent resistance	Good	Poor
Oxy. Perm ($cm^3m^{-3}atm^{-1}d^{-1}$)	1700	45
Biodegradability	------	Good
Other	Due to low density floats in aquatic system	Due to more density goes to sediment in aquatic system.

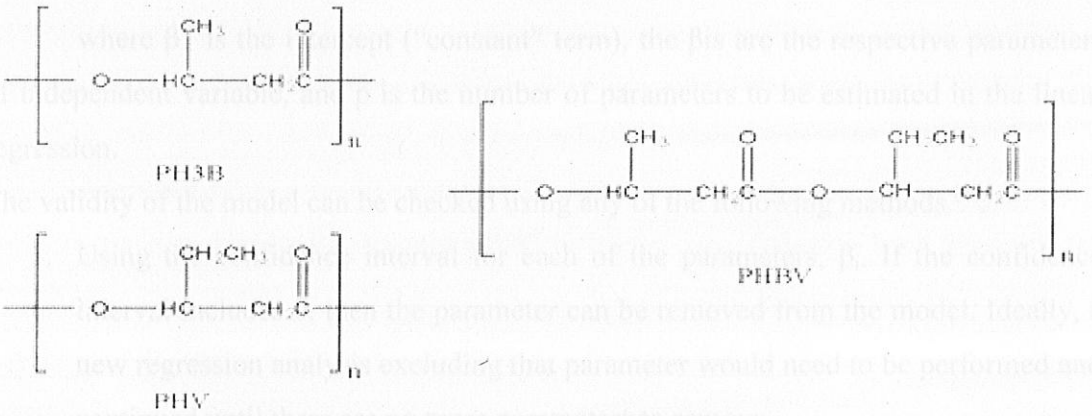

Fig.1 Structures of PHB, PHV and P-(3HB-co-3HV) polymers

In the past a different bacterium, Ralstonia eutropha, has been the focus of attention as a producer of PHB, but that microorganism requires an expensive two stage cultivation (Hrabak,1992) a non growth associated Serial Process. Unlike R. eutropha, A. latus is a growth-associated producer of PHB; hence, single-stage fermentation is sufficient (Ejiofor, 1996) referred to as Parallel Process (Fig. 2). Furthermore, A. latus grows

readily on sucrose which is less expensive than the glucose-based media typically used for R. eutropha.

For generating a linear regression model, we used MINITAB (Ver.13.1), a computer program designed to perform statistical functions. In statistics, linear regression

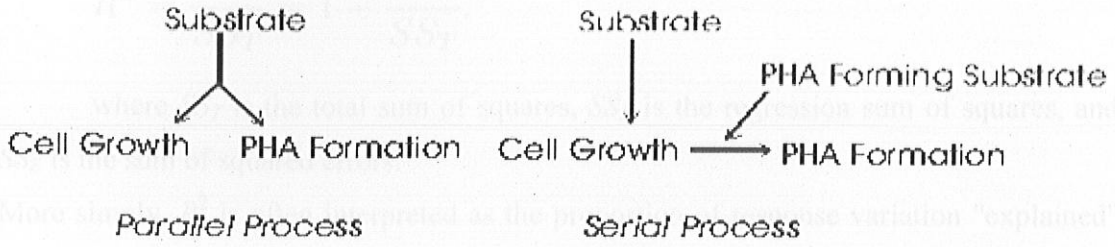

Fig.2 Showing the two pathways for PHB production using different organisms.

is a regression method that models the relationship between a dependent variable Y, independent variables Xp, and a random term ε. The model can be written as [1]

$$Y = \beta_1 + \beta_2 X_2 + \cdots + \beta_p X_p + \varepsilon$$

where β1 is the intercept ("constant" term), the βis are the respective parameters of independent variable, and p is the number of parameters to be estimated in the linear regression.

The validity of the model can be checked using any of the following methods [1]:

1. Using the confidence interval for each of the parameters, β_i. If the confidence interval includes 0, then the parameter can be removed from the model. Ideally, a new regression analysis excluding that parameter would need to be performed and continued until there are no more parameters to remove.
2. Calculate Pearson's co-efficient of regression (R^2). The closer the value is to 1, the better the regression is. This co-efficient gives what fraction of the observed behavior can be explained by the given variables.
3. Examining the observational and prediction confidence intervals. The smaller they are the better.
4. Computing the F-statistics.

In statistics, the **coefficient of determination** R^2 is the proportion of variability in a data set that is accounted for by a statistical model. In this definition, the term "variability" stands for variance or, equivalently, sum of squares.

$$R^2 = \frac{SS_R}{SS_T} = 1 - \frac{SS_E}{SS_T}.$$

where SS_T is the total sum of squares, SS_R is the regression sum of squares, and SS_E is the sum of squared errors.

More simply, R^2 is often interpreted as the proportion of response variation "explained" by the regressors in the model. Thus, $R^2 = 1$ indicates that the fitted model explains all variability in y, while $R^2 = 0$ indicates no 'linear' relationship between the response variable and regressors. An interior value such as $R^2 = 0.7$ may be interpreted as follows: "Approximately seventy percent of the variation in the response variable can be explained by the explanatory variable. The remaining thirty percent can be explained by unknown, lurking variables or inherent variability."

A caution that applies to R^2, as to other statistical descriptions of correlation and association is that "correlation does not imply causation." In other words, while correlations may provide valuable clues regarding causal relationships among variables, a high correlation between two variables does not represent adequate evidence that changing one variable has resulted, or may result, from changes of other variables.

Adjusted R^2 is a modification of R^2 that adjusts for the number of explanatory terms in a model. Unlike R^2, the adjusted R^2 increases only if the new term improves the model more than would be expected by chance. The adjusted R^2 can be negative, and will always be less than or equal to R^2. The adjusted R^2 is defined as [1]

$$1 - (1 - R^2)\frac{n-1}{n-p-1}$$

where p is the total number of regressors in the linear model, and n is sample size. As for any microbial production process, the performance of A. latus culture and Ralstonia eutropha is susceptible to many influences, including temperature, pH, carbon-to-nitrogen ratio in the feed etc (Schlegel,1992). In the present study, the fermentation

process was set up as reported in the literature for the microbial strains R.eutropha and A.latus (Grothe, 2000). The yield of the PHB was correlated with the regression model in order to validate the equation.

1.1
INDUSTRIAL HISTORY OF PHB

- ICI, the UK Chemical group, started producing PHB in 1975-76. They started marketing BIOPOL in 1982. They launched BIOPOL® resin in 1990. At present, BIOPOL costs about £10/kg, 20 times more than conventional plastic.
- Australian raw sugar industry is planning to produce plastic (PHB) from sugarcane, based on the technology held by Procter & Gamble Co.
- BIOPOL has been used in Germany since 1990 to make the bottles of Wella's Sanara Shampoo.
- In Japan, BIOPOL was introduced in 1991 as a container for Ishizawa Kenkyujo's Earthic Alga shampoos and conditioners. Kai used it for disposable razors.

Table 2 Key players in the industry

No	COMPANY	AREAS OF INTEREST
1	Berlin Packaging Corp. (USA)	Marketing.
2	Bioscience Ltd. (Finland)	Medial Applications.
3	Bio Ventures Alberta Inc. (Canada)	Production in Recombinant E. coli.
4	Metabolics Inc. (USA)	Production in transgenic plants.
5	Monsanto (USA)	Production in transgenic plants.
6	Polyfirm Inc (Canada)	Production from cheap substrates.
7	ZENECA Bio-products (UK)(former ICI,UK)	Production using R. eutropha.
8	ZENECA seeds (UK)	Production in transgenic plants.
9	Petrochemia Danubia (PCD)	Production using A.latus.

Some of the patents owned by Monsanto Inc. (USA) are [2]:

- US 6,709,848 - Methods for Isolating Polyhydroxyalkanoates from Plants
- US 6,623,749 - Polyhydroxyalkanoates for In Vivo Applications
- US 6,689,589 - Biological Systems for Manufacture of Polyhydroxyalkanoate Polymers Containing 4-hydroxyacids
- US 6,610,764 - Polyhydroxyalkanoate Compositions Having Controlled Degradation Rates
- US 6,429,285 - Polymer Compositions Providing Low Residue Levels and Methods of Use Thereof (previously Metalization of Phosphor
- US 6,586,658 - Modification of Fatty Acid Metabolism in Plants
- US 6,605,262 - Methods and Apparatus for Production of Amorphous Polymer Suspensions
- US 6,576,450 - Polyhydroxyalkanoate Production from Polyols
- US 6,593,116 - Transgenic Microbial Polyhydroxyalkanoate Producers

- US 6,828,376 - Aqueous Coating Composition Giving Coatings having an Improved Early Hardness and Tack Resistance
- US 6,548,569 - Medical Devices and Applications of Polyhydroxyalkanoate Polymers
- US 6,838,493 - Medical Devices and Applications of Polyhydroxyalkanoate Polymers
- US 6,867,247 - Medical Devices and Applications of Polyhydroxyalkanoate Polymers
- US 6,780,911 - Low Molecular Weight Polyhydroxyalkanoate Molding Compositions
- US 6,753,384 - Polyurethanes Obtained from Polyhydroxyalkanoates and Isocyanates

The world's first biodegradable credit card was made from Biopol (Fig.3).

The Greenpeace Visa card was launched in May1997 and Monsanto claimed it decomposed in compost within 12 weeks.

Fig.3 Greenpeace Visa card made from BIOPOL

1.2
PHB BIOSYNTHETIC PATHWAY

The pathway involves the condensation of two molecules of Acetyl-CoA by ß-ketothiolase to form Acetoacetyl-CoA which is subsequently reduced by Acetoacetyl-CoA reductase to form d-(±)-3-hydroxybutyryl-CoA. This monomeric 3HB is then polymerized to form Poly-(3HB) by PHB synthase. The three enzymes that catalyze these reactions are encoded by genes organized as an operon in this organism designate PhbA, PhbB, and PhbC for the ketothiolase, reductase, and synthase, respectively (Slater et al., 1988) (Fig.4).

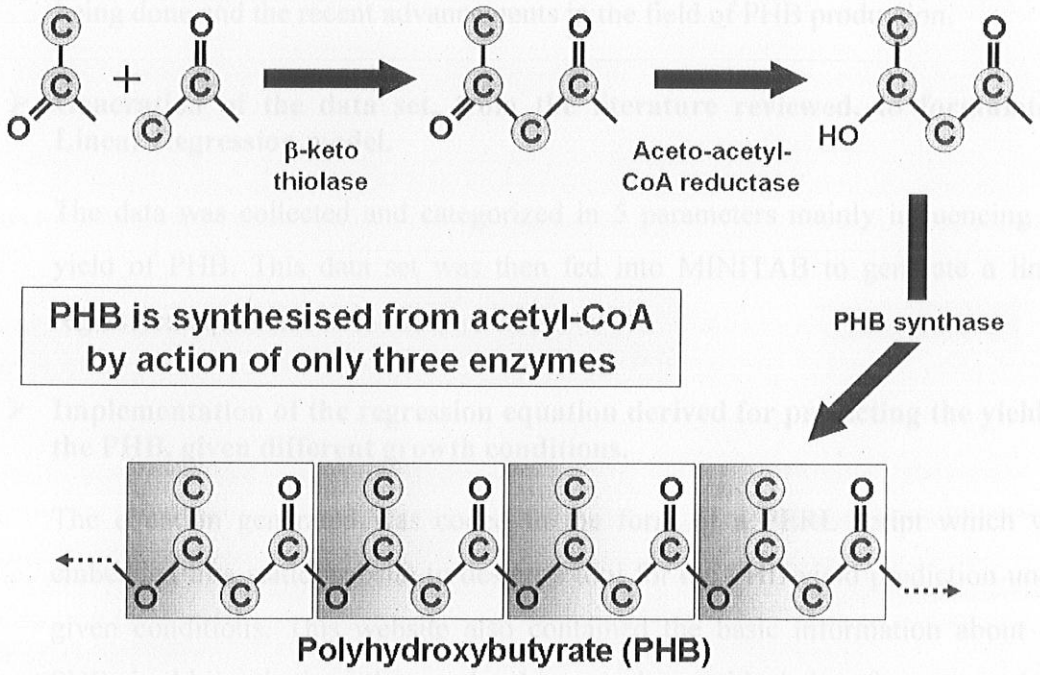

Fig. 4 The 3-step process for the biosynthesis of PHB from microbial strains.

The percentage of PHB in bacterial cells is normally low, i.e. 1-30%, but under controlled fermentation conditions of Carbon excess and Nitrogen limitation, overproduction of polymer can be encouraged to produce yields of up to 80% of the dry cell weight (Dawes and Senior, 1973; Ward et al., 1977).

2
PLAN OF ACTION

- **Review the available literature on PHB production.**

  In this step we studied the literature available in various journals like Science Direct, Indian Journal of Biotechnology, Process biochemistry, World Journal of Microbiology and Biotechnology, Journal of Bacteriology, Applied Journal of Microbiology, Turkish J. of Biotechnology, Letters in Applied Microbiology, Pakistan J. of Biological Sciences, Applied and Environmental Microbiology, Applied Microbiology and Biotechnology etc. to know about the various works being done and the recent advancements in the field of PHB production.

- **Generation of the data set, from the literature reviewed, to formulate a Linear Regression model.**

  The data was collected and categorized in 5 parameters mainly influencing the yield of PHB. This data set was then fed into MINITAB to generate a linear regression equation.

- **Implementation of the regression equation derived for predicting the yield of the PHB, given different growth conditions.**

  The equation generated was coded in the form of a PERL script which was embedded in a static website to design a tool for the PHB yield prediction under given conditions. This website also contained the basic information about the PHB, its biosynthetic pathway, the data set along with their references and the statistical algorithm used.

- **Validation of the prediction by setting up a pilot scale fermentation processes.**

  For validation purpose we chose model microbial strains viz. Alcaligenes latus and Ralstonia eutropha, the key producers of PHB in the industry. These strains were cultivated to produce PHB under the controlled conditions (as mentioned in the literature) to cross validate the prediction form the optimization tool.

2.1 LITERATURE REVIEW

A summary of milestones and important works done in the field of PHB production (Table 3) is as follows:

Table 3 Literature Review on PHB

WORK DONE	YEAR	AUTHOR
PHB was first isolated and characterized at the Pasteur Institute in Paris. (Lemoigne,1926)	1926	Lemoigne
Small quantities of PHB were produced for commercial evaluation.(Asrar,2000)	1958	W.R.Grace
Study of occurrence of PHB in Azotobacteriaceae. (Stockdale, Ribbons, Dawes, 1968)	1968	H. Stockdale, D. W. Ribbons, AND E. A. Dawes
Study of kinetics and effect of carbon and nitrogen source feeding on production of PHB by fed-batch culture.(Suzuki,Yamane,Shimizu,1986)	1986	Takahiro Suzuki, Tsuneo Yamane, and Shoichi Shimizu
Study of substrate inhibition kinetics for microbial growth and synthesis of PHB. (Mulchandani,Luong,Groom,1989)	1989	A. Mulchandani, J. H. T. Luong, and C. Groom
Study of batch culture of mucoid strain of *Azotobacter vinelandii.* (Brivonese,Sutherland,1989)	1989	Anne C. Brivonese and Ian W. Sutherland
Study of effect of culture conditions on the PHB production (in *Haloferax mediterranei*) (Lillo,Valera,1990)	1990	Jose Garcia Lillo And Francisco Rodriguez-Valera
Study of methanol utilizing bacterial strains (e.g. *Methylobacterium extorquens)* (Bourque,Ouellette,Andre,Groleau,1992)	1992	D. Bourque, B. Ouellette, G. Andre, and D. Groleau
Study of production of PHB in *Acinetobacter* sp. (Rees, Vasiliadis, May, Bayly,1993)	1993	Gavin N. Rees, George Vasiliadis, John W. May, Ronald C. Bayly
Study of biosynthesis of PHB in mutant of *Sphaerotilus natans.* (Takeda,Matsuoka,Ban,Ohasi,1995)	1995	M. Takeda, H. Matsuoka, H. Ban, Y. Ohashi, M. Hikuma, J-i. Koizumi

Attempts were made to make the process cost effective by using potato processing waste for the production of PHB (Rusendi,Sheppard,1995)	1995	D. Rusendi & John D. Sheppard
Production of PHB by fed-batch culture of recombinant species esp. E.coli was studied.(Wang,Lee,1997)	1997	F Wang and SY Lee
Study of cheaper media like molasses, plant oils, organic nitrogen substrates, formates and other organic acids for the production of PHB (Leu,Li,Ridgway,Gu,1998;Yellore,Thakur,Desai,1998; Fukui,Do,Bormann,1998)	1998	Fang Liu, Wenqing Li, Darin Ridgway, and Tingyue Gu, T. Fukui, Y. Do,E. J. Bormann , M. Leiûner, B. Beer, V.S. Yellore, N.B. Thakur and A.J. Desai
Use of mathematical models and fed-batch strategies for the production of PHB (Raje,Srivastava,1998)	1998	Poonam Raje & Ashok K.Srivastava
Production of PHB from industrial effluents. (Nonato,2001; Fuchtenbusch,2001; Ahn, Park, Lee, 2001)	2001	R. V. Nonato, P. E.Mantelatto, C. E. V. Rossell, B. Fuchtenbusch, D. Wullbrandt, A. Steinbuchel, Woo Suk Ahn, Si Jae Park & Sang Yup Lee
Production of PHB by eukaryotic organisms (Safak,Mercan, Beyatli,2002)	2002	Sibel Safak, Nazime Mercan, Yavuz Beyatli
The influence of nutritional and environmental conditions on the accumulation of PHB in *Bacillus mycoides* , *Rhizobium, Streptomyces* species. (Borah,2002;Mercan,Aslim,2002;Ugur,Beyatli,2002)	2002	B. Borah, P.S. Thakur and J.N. Nigam, Nazime Mercan, Belma Aslim, Z. Nur Y.Ksekdau, Yavuz Beyatli, Aysel Ugur, Nurettin Sahin
Production of PHB in Transgenic Alfalfa (Saruul,Somers,2002)	2002	Purev Saruul, Friedrich Srienc, David A. Somers, and Deborah A. Samac
Simulation and optimization of PHB production in fed-batch culture.(Shahhosseini,2004)	2004	Shahrokh Shahhosseini
Statistical media optimization studies for growth and PHB production by *Ralstonia eutropha* (Khanna,Srivastava,2005)	2005	Shilpi Khanna, Ashok K. Srivastava
Study of continuous stirred tank reactors for the production of PHB (Yu,Lin,Too,2005)	2005	S.T. Yu , C.C. Lin, J.R. Too

Statistical optimization of a culture medium for biomass and PHB production (Nikel,Pettinari,Galvangno,2005)	2005	Pablo I. Nikel M. Julia Pettinari Beatriz S. Méndez Miguel A. Galvagno
Neural network designs for PHB production optimization under simulated industrial conditions (Patnaik,2005)	2005	P.R.Patnaik
Production of PHB using solid-state fermentation (Oliveria,Castilho,Frerie,2006)	2007	Fabiane C. Oliveira, Marcos L. Dias , Leda R. Castilho, Denise M.G. Freire
Process optimization for PHB production in a nitrogen fixing cyanobacterium using response surface methodology (Sharma,Mallick,2007)	2007	Laxuman Sharma, Akhilesh Kumar Singh, Bhabatarini Panda, Nirupama Mallick
Synthesis of PHAs from waster under various C:N ratios (Wang, Hua,Ren,2007)	2007	Y.J. Wang, F.L. Hua, Y.F. Tsang, S.Y. Chan, S.N. Sin, H. Chua, P. H.F. Yu , N.Q. Ren
Utilization of various industrial wastes for the production of PHB (Arun,Ravindran,Balaji,2007)	2007	A. Arun, Rm. Murrugappan, A. D. David Ravindran, V. Veeramanikandan and Shanmuga Balaji
Dispersion optimization to enhance PHB production in fed-batch cultures (Patnaik,2007)	2007	Pratap R. Patnaik

2.2
FORMULATION OF THE LINEAR REGRESSION MODEL

The data set (Table 3) was used to formulate the Linear Regression Model using the statistical software MINITAB (Ver. 13.1).

Table 4 Data set for Linear Regression Model

Organism	pH value	Inoculum (%)	Fermentation Time (hrs.)	Temperature (^{0}C)	C/N ratio	Yield (g/l)
Bacillus mycioides (Borah,2002)	7.0	0.5	24	30	20	2.50
Azotobacter insigne (Stockdale, Ribbons, Dawes, 1968)	7.7	1	36	27	4.8	1.46
Azotobacter insigne (Mercan,Aslim,2002)	7.7	1	36	27	2.28	1.05
Streptomyces anulatus (Ugur,Beyatli,2002)	6.8	1	72	30	4.3	5.7
Haloferax mediterranei (Lillo,Valera,1990)	7.2	2	60	38	8.0	6.0
Methylobacterium sp. ZP24 (Yellore,Thakur,Desai,1998)	7.0	1	40	29	12.7	1.1
Rhizobium sp. 2426 (Mercan,Aslim,Beyatli,2002)	7.0	4	48	30	3.41	0.285
Rhizobium sp. 2426 (Mercan,Aslim,Beyatli,2002)	7.0	4	24	30	3.41	0.159
Rhizobium sp. 2426 (Mercan,Aslim,Beyatli,2002)	7.0	4	72	30	3.41	0.139
Rhizobium sp. 2426 (Mercan,Aslim,Beyatli,2002)	7.0	4	96	30	3.41	0.101
Rhizobium sp. 2426 (Mercan,Aslim,Beyatli,2002)	7.0	4	120	30	3.41	0.227
Protomonas extorquens (Suzuki,Yamane,Shimizu,1986)	7.0	1	170	30	1.1	1.49
Comamonas testosteroni (Thakor, Patel, Trivedi, 2003)	7.2	2	24	30	67.8	0.35
Halomonas boliviensi LC1 (Quillaguerman,2006)	7.5	1	24	35	6.428	7.7
Azotobacter vinelandii (Page,1998)	6.8	4	24	29	36.5	6.5
Azotobacter vinelandii (Page,1998)	6.8	4	24	29	36.5	6.5

Azotobacter vinelandii (Page,1998)	6.8	4	24	29	36.5	6.5
Ralstonia eutropha (Khanna,Srivastava,2005)**cF**	7.2	1	60	30	18.8	1.4
Ralstonia eutropha (Khanna,Srivastava,2005)**cL**	7.2	1	60	30	18.8	0.089
Ralstonia eutropha (Khanna,Srivastava,2005)**cS**	7.2	1	60	30	19.85	0.042
Ralstonia eutropha (Khanna,Srivastava,2005)**cA**	7.2	1	60	30	18.8	0.031
Ralstonia eutropha (Khanna,Srivastava,2005)**cX**	7.2	1	60	30	18.8	0.023
Ralstonia eutropha (Khanna,Srivastava,2005)**cSod**	7.2	1	60	30	13.8	0.001
Ralstonia eutropha (Khanna,Srivastava,2005)' **cP**	7.2	1	60	30	22.9	0.001
Ralstonia eutropha (Khanna,Srivastava,2005)**nU**	7.2	1	60	30	4.99	3.84
Ralstonia eutropha (Khanna,Srivastava,2005) **nNC**	7.2	1	60	30	15.2	30.45
Ralstonia eutropha (Khanna,Srivastava,2005)**nNN**	7.2	1	60	30	0.69	11.5
Ralstonia eutropha (Khanna,Srivastava,2005)**cSor**	7.2	1	60	30	18.8	0.007
Ralstonia eutropha (Khanna,Srivastava,2005)**cG**	7.2	1	60	30	18.8	0.003
Azotobacter vinelandii (Page,1998)	6.8	4	24	29	36.5	6.5
Azotobacter vinelandii (Page,1998)	6.8	4	24	29	26.86	7.5
Azotobacter vinelandii (Page,1998)	6.8	4	24	29	14.97	7.0
Bacillus brevis M2 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.005
Bacillus brevis M4 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.059
Bacillus brevis M6 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.025
Bacillus sphaericus M3 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.040

Bacillus sphaericus (Aslim,Beyatli,2002)[1]	6.8	2	48	30	15.5	0.690
Bacillus cerus M5 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.095
Bacillus cerus M10 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.059
Bacillus cerus M15 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.097
Bacillus cogulans M8 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.046
Bacillus cogulans M25 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.032
Bacillus cogulans M35 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.064
Bacillus megaterium M14 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.087
Bacillus megaterium M21 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.048
Bacillus megaterium M22 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.052
Bacillus megaterium M26 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.036
Bacillus megaterium M28 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.066
Bacillus circulans M16 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.033
Bacillus subtilis M17 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.036
Bacillus subtilis M24 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.021
Bacillus subtilis M29 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.038
Bacillus subtilis M33 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.084
Bacillus subtilis ATCC 6633 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.042
Bacillus licheniformis M19 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.042
Bacillus licheniformis M20 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.042

Bacillus licheniformis M27 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.042
Bacillus licheniformis M30 (Aslim,Beyatli,2002)	6.8	2	48	30	15.5	0.042
Alcaligenes latus (Grothe,2000)	7.2	2.5	108	33	10.87	1.8

The model generated using the aforementioned data set is as follows:

The regression equation is

Yield = - 1.0 + 2.61 pH + 1.13 Inoculum (%) - 0.0582 Fermentation Time (hrs.) - 0.615 Temperature + 0.163 C/N ratio

Predictor	Coef	SE Coef	T	P
Constant	-1.03	13.74	-0.08	0.940
pH	2.6149	0.8579	3.05	0.004
Inoculum	1.1325	0.1443	7.85	0.000
Fermentation Time	-0.05823	0.01444	-4.03	0.000
Temperature	-0.6154	0.3063	-2.01	0.050
C/N ratio	0.16288	0.01952	8.35	0.000

S = 0.8364 R-Sq = 86.0% R-Sq (adj) = 84.6%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	5	222.584	44.517	63.63	0.000
Residual Error	52	36.378	0.700		
Total	57	258.962			

2.3
IMPLEMENTATION OF THE REGRESSION EQUATION

The equation generated from the Linear Regression Model formulated using the MINTAB statistical software was used to code a program in PERL script which was then embedded into a static web page. The website so formed includes the basic information and introduction about the PHB and its biosynthetic pathway. It also consists of the dataset along with their respective references to the journals (Fig.5). The PERL script was used to design a tool which takes an input of five parameters, i.e. the pH of the growth media, the Seed Inoculum, Fermentation time, Temperature of the media and the C/N value, to predict the yield of the PHB with a variation of approximately ±10% in the results (Fig.6).

The regression model was then implemented to predict the yield of the PHB from the microbial strains A. latus and R. eutropha, corresponding to the existing literature, under the specified growth conditions (Fig. 7, 8) respectively.

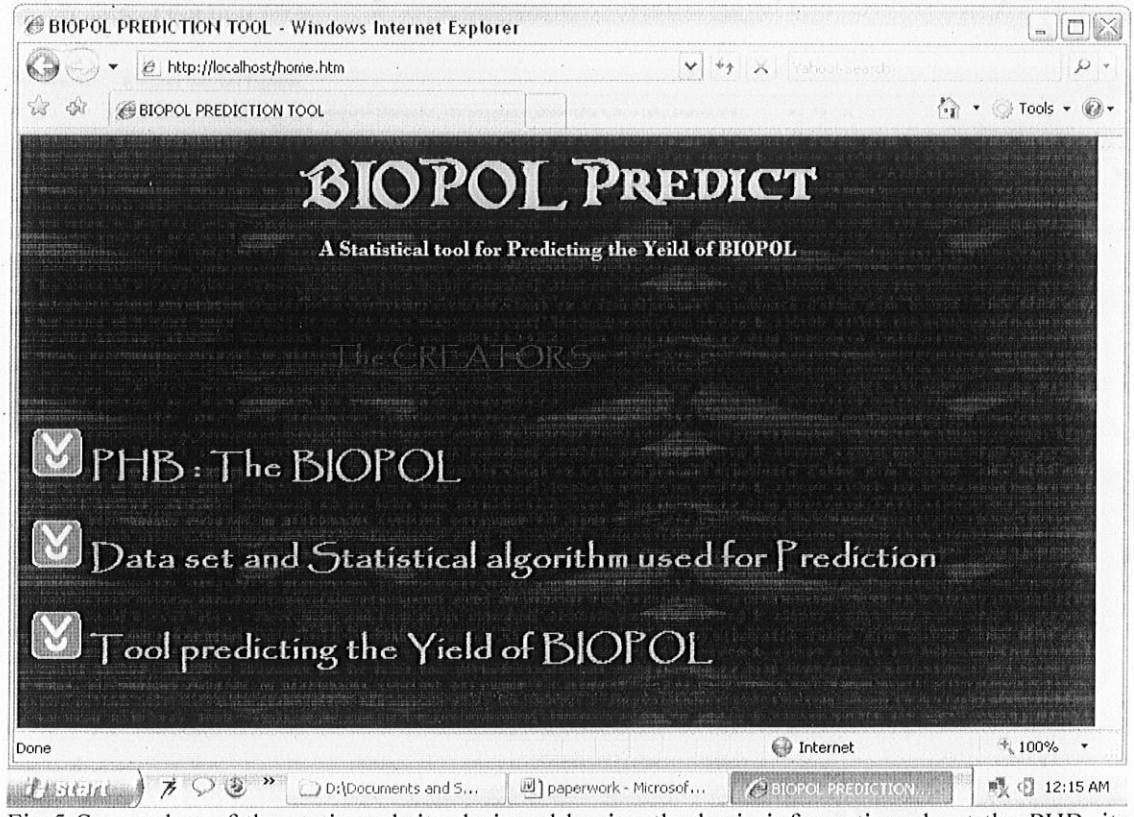

Fig.5 Screenshot of the static website designed having the basic information about the PHB, its biosynthetic pathway and Data set along with the algorithm implemented.

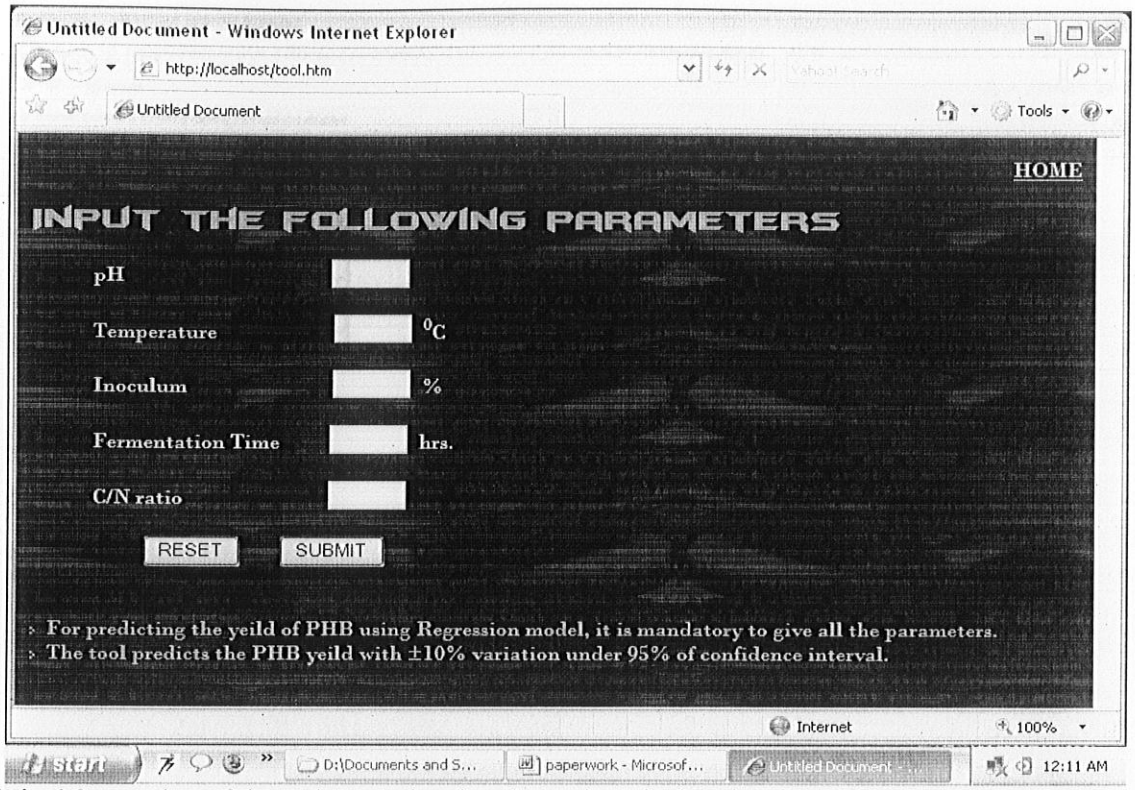

Fig.6 Screenshot of the web page showing the tool predicting the yield of PHB having the PERL script embedded in it.

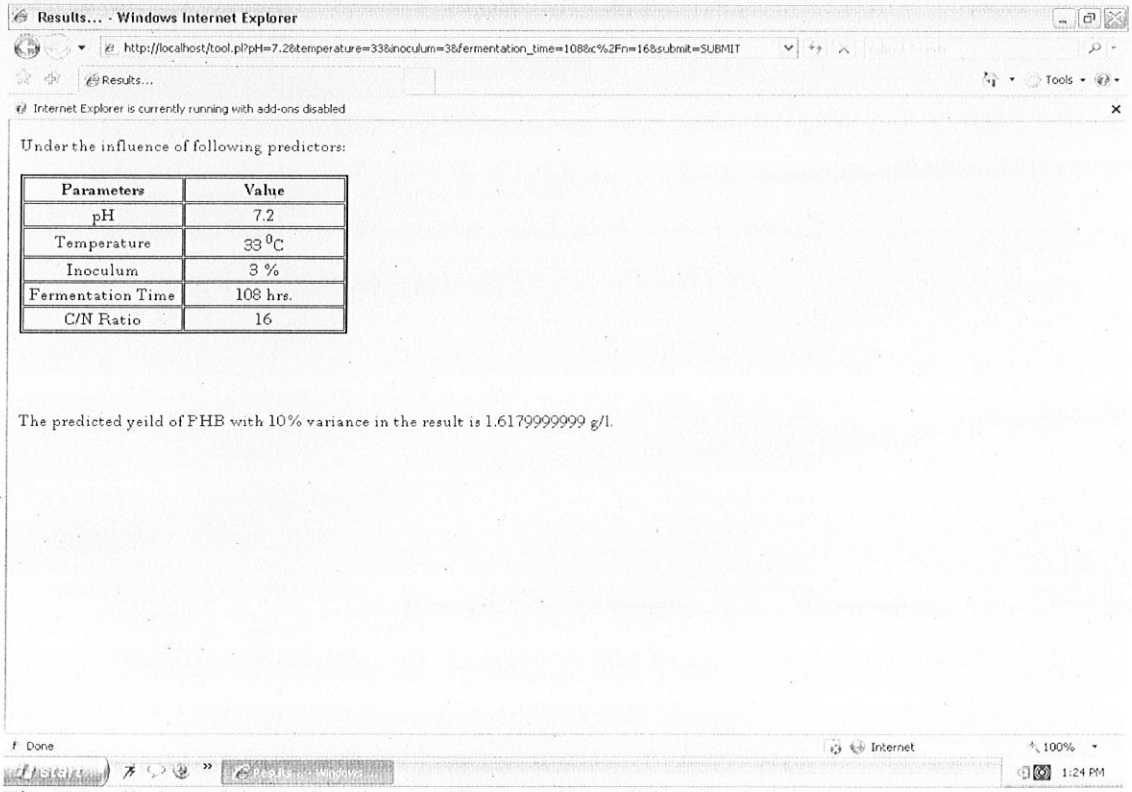

Fig. 7 Prediction of the tool under specific growth condition for A. latus

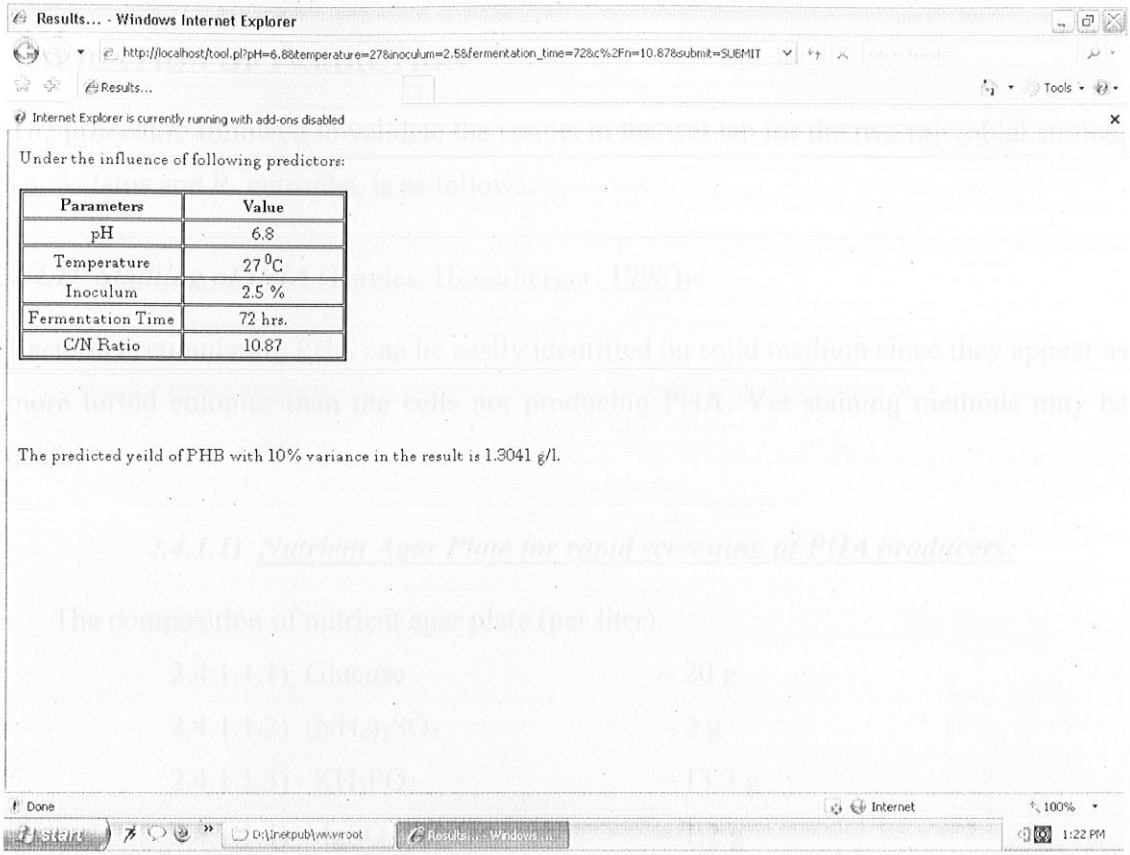

Fig. 8 Prediction of the tool under specific growth condition for R. europha

2.4
VALIDATION OF PREDICTION

The procedure followed to validate the results in the wet lab for the two microbial strains, i.e. A. latus and R. eutropha, is as follows:

2.4.1) Staining of PHA (Davies, Hershberger, 1995):-

Bacteria accumulating PHA can be easily identified on solid medium since they appear as more turbid colonies than the cells not producing PHA. Yet staining methods may be useful.

2.4.1.1) Nutrient Agar Plate for rapid screening of PHA producers:

The composition of nutrient agar plate (per liter):

2.4.1.1.1) Glucose – 20 g

2.4.1.1.2) $(NH_4)_2SO_4$ – 2 g

2.4.1.1.3) KH_2PO_4 – 13.3 g

2.4.1.1.4) $MgSO_4.7H_2O$ – 1.2 g

2.4.1.1.5) Citric acid – 1.7 g

2.4.1.1.6) Trace element solution – 10 ml

(per lt: $FeSO_4.7H_2O$ – 10 g, $ZnSO_4.7H_2O$ - 2.25 g, $CuSO_4.5H_2O$ – 5 g, $CaCl_2.2H_2O$ – 2 g, $Na_2B_4O_2.10H2O$ - 0.23 g, $(NH_4)_6Mo_2O_{24}$ - 0.1 g, 35%HCl – 10 ml)

2.4.1.1.7) Bacto agar – 15 g

Glucose can be replaced by other carbon source as per the requirement of the organism producing PHA, e.g. Glucose for R. eutropha and Sucrose for A. latus.

Staining with Sudan black B:

- Prepared a heat fixed film of the specimen grown under PHA accumulating conditions on a slide and immerse it in a filtered solution of 0.3%(w/v) Sudan black B (in ethylene glycol). Stain it for 5 to 15 min.
- Drained and air dried the slide.

- Immersed and withdrew the slide several times in Xylene, and blot dry with absorbent paper.
- Added the counter-stain for 5 to 10 s. with 0.5 %(w/v) aqueous Safranin.
- Rinsed the slide with tap water and blot dry.
- Examined the slide under oil immersion. PHA inclusions appear as blue black droplets, while cytoplasmic parts of the organism appear pink.

2.4.2.) ***Production of PHA by Fermentation Process*** (Grothe, 2000):-

2.4.2.1) Prepared a minimal media called Medium 1018 and a nutrient media named as Media 1 having following composition:

COMPONENT	**Medium 1018 (per liter)**	**Medium 1**
Sucrose/Glucose	20g	20g
$(NH_4)_2SO_4$	1g	2g
KH_2PO_4	4.4g	1.5g
Na_2HPO_4	4.8g	3.6g
$MgSO_4.7H_20$	0.5g	0.2g
Trace elements solution:	1ml/l	0.5ml/l
Composition (per liter)		
Ammonium Fe (3) citrate	50g	60g
$CaCl_2.2H_2O$	5g	10g
H_3BO_3	-	0.3g
$CoCl_2.6H_2O$	-	0.2g
$ZnSO_4.6H_2O$	-	0.1g
$MnCl_2.4H_2O$	-	0.03g
$Na_2MoO_4.2H_2O$	-	0.03g
$NiSO_4.7H_2O$	-	0.02g
$CuSO_4.5H_2O$	-	0.01g

2.4.2.2) Inoculated the shake flask containing 50ml of medium 1018 with a loop full of culture from agar plates to prepare the seed. Let the culture grow at 33°C at 200 rpm for 3-4days.

2.4.2.3) Transferred 5ml of this seed to a 2l shake flask containing medium 1 with 0.5 ml/l of trace element solution at pH 7.2 (for A. latus) or 6.8 (for R. eutropha). Then these flasks were kept for 4 days at 33°C, 200 rpm for the growth of the culture.

Due to unavailability of $NiSO_4 \cdot 7H_2O$, we have modified the media using nickel Chloride without referring to literature.

2.4.2.4) After 4 days transferred the entire content to inoculate the 4.5l fermentation media (medium 1) in the bioreactor.

Conditions: 300-800 rpm

dO2 – Above 15%

Aeration rate – 1vvm

pH – 6.5 (maintained by adding aqueous ammonia 20% w/v)

Fermentation time – 35-40 hrs.

*2.4.3) **Extraction and Purification of the Polymers*** (Davies, Hershberger, 1995):-

After the cells were collected, they were lyophilized. The dried cells were extracted for 6 hrs in chloroform to achieve the destruction of the cells and solubilization of polymer. Purification of the polymers was done by precipitation of the polymers in alcohol. The precipitated polymers were collected by filtration and dried at room temperature. The basic procedure followed is given below:

- Collect bacterial cells by centrifugation and measure the dry cell weight.
- Remove lipids by adding methanol (40 times the volume of the collected cells) and incubate at 95°C for 1 hr.
- Recover cells by vacuum filtration. To remove methanol completely, incubate cells in a dry oven for several minutes.
- Add chloroform (50 times the dry cell weight) and incubate at 95°C for 10 min.
- Cool to room temperature, and mix solution overnight with stirring.
- Filter the solution to remove cell debris.
- Precipitate P-(3HB), wash with acetone, and dry.

When cyclic carbonates are used as solvents, Poly-(3HB) can be precipitated by simply cooling the solution. However, cyclic carbonates are relatively expensive and have a high boiling point, which makes the reuse of these solvents infeasible. Because PHAs consisting of different monomer units have different properties, the specific pairs of extraction solvent and precipitation non-solvent should be used for efficient recovery. It makes the extraction method economically unattractive, even after recycling of the

solvents. Therefore, the extraction method is useful only for small scale analytical purposes. For large scale purposes, hypochlorite digestion method can be used.

2.4.4) Crotonic Acid Assay (Law John H, Slepecky Ralph A., 1961):-

The method is a simple and specific one that can be used to measure PHB in any form. The principle of this method lies in two observations –

- First that PHB can be converted quantitatively to crotonic acid by heating in conc. H_2SO_4 and
- Second that the UV absorption maximum of crotonic acid is shifted to 235 mμ when conc. H_2SO_4 is the solvent.

For the spectrophotometric assay of the polymer a sample containing 5-50μg polymers in chloroform is transferred to a clean test tube. The chloroform is then evaporated and 10 ml of conc. H_2SO_4 was added, the tube is capped with a glass marble and heated for 10 min at 100 ^{0}C in water bath. The solution is cooled and after thorough mixing the sample is transferred to a silica cuvette and the absorbance at 235 mμ is measured against a sulfuric acid blank. The amount of crotonic acid is calculated from the molar extinction coefficient (1.55 X 10 ^{4}).

The formula used for calculating the conc. of the PHB after the spectrophotometric analysis of Crotonic acid formed by the reaction of Poly-(3HB) with the boiling conc. H_2SO_4 is as follows:

O.D./Absorbance (at 235 n.m.) = Molar Extinction coefficient * Concentration * Path length of the cuvette (1cm)

$$\text{Concentration of PHB} = \frac{\text{O.D./Absorbance (at 235 n.m.)}}{\text{Molar Extinction coefficient}}$$

$$\text{Concentration of PHB} = \frac{\text{O.D./Absorbance (at 235 n.m.)}}{1.55 \times 10^4}$$

3
RESULTS AND DISCUSSION

3.1
For Alcaligenes latus

Growth conditions:

pH = 7.2
F_t = 108 hrs.
Temp = 33 °C
C/N = 16.0
Inc = 3 %

DCW of the pellet	: 8.207 g
Absorbance at 235 nm	: 3.151
Conc. of PHB	: 1.813 g/l
PHB in biomass	: 22.09 %
Reported yield in literature	: 2.048 g/l
Predicted yield	: 1.6179g/l

3.2
For Ralstonia eutropha

Growth conditions:

pH = 6.8
F_t = 72 hrs.
Temp = 27 °C
C/N = 10.87
Inc = 2.5

DCW of the pellet	: 6.337 g
Absorbance at 235 nm	: 1.829
Conc. of PHB	: 1.169 g/l
PHB in biomass	: 18.44 %
Reported yield in literature	: 1.40 g/l
Predicted yield	: 1.3041g/l

From the above statistics, it is clear that there is a variation in the yield predicted by the tool and that observed in the pilot scale fermentation process of the microbial strains. This variation can be explained on the basis of -

- the R-square value of the regression equation which implies that the prediction can be up to 86% accurate because five independent variables are used to predict one dependent variable based upon their coefficient values derived from the 68 equations i.e. the coefficients are the average values.
- the outliers that do not fit into the regression line result in the diversion of the regression equation from its mean path.
- each species has a different genetic construct resulting in varied response to the stress condition producing different concentrations of PHB. The response can be approximated but cannot be determined accurately on basis of statistics which is completely based on probability.

4
CONCLUSION

- The parameters pH, temperature, inoculum, C/N ratio and fermentation time play an important role in determining the yield of the PHB though there are several other parameters like agitation rate, concentration of trace elements, concentration of substrates, dissolved oxygen content etc. also affect the PHB production.

- The linear regression model of MINITAB can help in approximating the yield for the pilot scale process only whereas for industrial purpose, we must implement some logistic approach.

- Since the PHB content in the biomass ranges up to 25-30% only we may conclude that fed-batch process is superior to the batch process for producing PHB as it is produced under the stress conditions with limited Nitrogen and excess Carbon supply i.e. a high C/N ratio is maintained throughout the process easily.

5
FUTURE ASPECTS

- The tool can be better optimized to predict the yield more accurately by considering some additional parameters affecting the PHB production as agitation rate, dissolved oxygen content, concentration of trace elements, solubility of substrates etc.

- The prediction of the tool can be made more precise by comparing the strains for which yield is to be predicted at genetic levels. For example, by using BLAST analysis.

- Since the data set and the microbial strains producing the PHB are very diverse in nature therefore some logistic approach can also be applied to find a better algorithm to generate a non linear regression model for more accurately predicting the yield. Implementation of neural networks can also be equally effective provided the data set is sufficiently large.

- Microbial strains other than bacteria like algae e.g. Nostoc, Synechocystis etc. have been reported to produce high amount of PHB in lesser C/N ratio quite contrary to the bacterial species. A separate data set can be devised for algal producers also.

- Keeping scale up constraints in view, the statistical models can also be devised to optimize the large scale fermentation processes.

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