Response Surface Methodology for Optimization of Polyhydroxybutyrate Production from Agribyproducts

Mukesh Jangra[1], Virendra K Sikka[2] and Akanksha Jain[3]

[1]Ph.D. Student, (MBBB) Department of Molecular Biology, Biotechnology & Bioinformatics College of Basic Sciences & Humanities CCSHAU, Hisar, Haryana-125004
[2]Department of Molecular Biology, Biotechnology & Bioinformatics College of Basic Sciences & Humanities CCSHAU, Hisar, Haryana-125004
[3]Ph.D. Student (FST), Centre of Food Science and Technology, CCSHAU, Hisar, Haryana-125004
E-mail: [1]jangra.mukesh02@gmail.com, [2]vksikka@hau.ernet.in, [3]akankshajain972@gmail.com

Abstract—*Stastical media optimization using surface response methodology was employed to optimize the growth towards Polyhydroxybutyrate (PHB) production. Media were developed using nutrients from agribyproducts to expedite PHB production and high growth potential. Strain B3 on fructose as carbon sources and mustard cake and yeast extract as nitrogen source, exhibited a maximum biomass of 0.031g/l with PHB recovery of 0.45g/l. A significant increase in the PHB production using renewable carbon and nitrogen sources in a time period of just 3 days was an important outcome of the experiments.*

Keywords*: Response surface methodology, Polyhydroxybutyrate, Agribyproducts, Optimization.*

1. INTRODUCTION

PHA are the polyster of hydroxyacids which is naturally synthesized by bacteria as carbon reserve under stress conditions. It is beneficial for bacteria to store surplus nutrients within their cells, especially as their general physiological health is not affected. Its accumulation in cytoplasmic inclusion has occurred due to unbalanced growth conditions usually characterized by an excess supply or the stress conditions. PHB is the first and most studied PHA to be discovered. In their metabolism, three enzymes play important role. Bacteria produce acetyl-coenzyme-A (acetyl-CoA), which is converted into PHB by three biosynthetic enzymes [11]. In the first step, 3-ketothiolase (*PhaA*) form acetoacetyl-CoA by combining two molecules of acetyl-CoA which is reduced to 3-hydroxybutyryl-CoA by CoA reductase (*PhaB*) Finally, PHB synthase (*PhaC)* polymerizes 3-hydroxybutyryl-CoA to PHB, coenzyme-A being liberated. Only (R)-isomers are accepted as substrates for the polymerizing enzyme [12, 14]. These are gaining importance as biopolymers to the petroleum based plastic due to increasing environmental pollution and its ecofriendly nature. This polymer is easily degraded in the soil and sewage, and is at present used in making polyethylene or polypropylene films [1].The PHA content and its composition are affected mainly by the strain of the microorganism, the type of substrate used and its concentration and other growth conditions [16]. The high cost of PHB can be curtailed by strain development, improving fermentation, separation process and using low cost carbon sources like agribyproducts. An optimal medium might enhance and maintain PHB recovery and help to maximize the cell growth in terms of optical density and PHB production. The parameters like carbon source and nitrogen source of medium affects metabolism of these bacteria and accumulation of PHB. Response surface methodology is a stastical tool, helps to study the interactive effect of all the parameters on the cell growth and PHB recovery with improved product yield. In the present study, bacterial isolate B3 was grown under conditions favoring maximum PHB production. Attempts were made to use economical strategies to reduce the production costs of PHB as well as its expeditious production from agribyproducts and its applications in various fields. The aim of this study is to optimize PHB production and to study the interaction of various factors which help to enhance the PHB production through stastical media optimization for its possible applications in various scale up studies.

2. MATERIALS AND METHODS

2.1 Bacterial strains, media, growth conditions and screening

The PHB producing bacteria B3 was used in this study were isolated from garden soil at Hisar, India. One mg of different soil samples were dissolved in 1 ml of sterile distilled water and serial dilutions were made 5-6 times. 100 microliter of samples from these dilutions were taken and spread on plates containing TY media. Bacterial colonies appeared after 24 hrs.

A single colony was taken from the plate and streaked on a fresh TY plate for isolation of pure colony. The process of streaking is repeated several times for obtaining the single colony. The isolated pure cultures were stored at 4ºC in plates containing GSY media. For screening the PHB producing bacterial isolates Nile Blue A staining method was used. Nile Blue A stain was added to the autoclaved medium at the final concentration of 25 µg/ml were screen under UV light after 3 or 4 days of incubation. The PHB producing stain gave an orange/yellowish color florescence. Extent of florescence indicated the intracellular quantity of PHB.

2.2 Characterization of selected bacterial strain

Morphological and biochemical tests were performed for characterization of selected PHB producing bacterial strain B3 for its tentative generic identification. Various biochemical tests namely Triple Sugar Iron test (TSI), Nitrate reduction test, Urea hydrolysis test, Carbohydrate fermentation test, Citrate utilization test, Methyl red test, Gelatin hydrolysis test, Catalase test, Indole test and Hydrogen Sulfide production test etc. and morphological test like spore staining, gram staining, color, shape and morphology.

2.3 Analytical Procedures

Determination of the amount of PHB was performed by chemical method. Cell mass is collected by centrifuging bacterial culture broth at 8000 rpm. The cell mass collected from two liter broth was treated with 10 ml sodium hypochlorite (4%) by vortexing it and samples were boiled for five to ten minutes in boiling water bath. After cooling add 20 ml of chloroform to the boiled samples. Keep it at 65ºC. Chloroform evaporates and dissolved PHB recovered. 50 µl of dissolved PHB added to 2 ml of sulphuric acid and then incubated for one day at 65ºC. After that the spectrophotometer reading is taken at 235 nm.

2.4 Comparison of PHB production in different media by selected strains

It was found that test isolate B3 was producing maximum PHB by weight so these were used for further media optimization studies towards scaled up production of PHB. Twenty four different media were investigated to determine the suitable composition for the maximum PHB accumulation by B3 strain. One liter of media inoculated with equal starter culture for media optimization studies and incubated for 72 hrs. Based on the results of these experiments, for B3 strain carbon source fructose when used in combination with nitrogen source mustard cake and yeast extract gave the maximum PHB production.

2.5 Experimental design and optimization using Response Surface Methodology

The optimization of process parameters in growth associated PHB production by B3 strain was studied using central composite design (CCD) of RSM (Stat Ease, Inc Design Expert software, trial version 9.0.3, Minneapolis, USA).The CCD for three independent variables fructose (A), Mustard cake (B) and yeast extract (C) each at five levels including 6 replicates at the center point, 6 axial points and 8 factor points leading to a total no. of 20 experiments was in work for optimization. Each variable was calculated at two different levels (-1, +1) and center point (0) which is midpoint of each factor range (table 1). The initial concentration was varied to 2 to 4 g/l, the concentration of mustard cake and the yeast extract was varied from 0.5 to 1 to observe the effect of culture conditions on PHB production. Concentration range for the variables was determined on the basis of literature reports for PHB production. Responses were measured in terms of O.D. and PHB recovery. This data was used to fit in the design of the experiments for response surface optimization.

Table 1: Experimental design matrix for B3 in terms of actual, coded factors for the responses- O.D. and PHB recovery

Factor	Name	Units	Type	Min.	Max.	Coded	Values
A	Fructose (C-source)	g/l	Numeric	1.318	4.681	-1.00=2	1.00=4
B	Mustard cake (N-source)	g/l	Numeric	0.159	1.840	-1.00=0.5	1.00=1.5
C	Yeast Extract (N-source)	g/l	Numeric	0.159	1.840	-1.00=0.5	1.00=1.5

The experiments were done in Erlenmeyer flasks containing 250 ml media incubated for 72 hrs for B3. The experimental results were fitted with a second-order polynomial function:

$Y = b_0 + b_1x_1 + b_2x_2 + b^{11}x^2_1\ b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3$

where, Y is the predicted response, b_0 the intercept, b1$b_1, b_2, b_3$the linear coefficient, $b_{11}, b_{22}, b_{33}$the squared coefficient and $b_{12}, b_{13}, b_{23}$the interaction coefficient.

2.6 FTIR spectrum analysis

Fourier-transform infrared spectroscopy (FTIR) has been demonstrated to be a powerful tool for screening various types of PHAs. It is used to detect functional groups in an organic compound. FTIR spectra of PHBs that were produced using different carbon and nitrogen sources were recorded in KBr (GJUS&T, Hisar).

3. RESULTS AND DISCUSSION

3.1 Screening of bacteria accumulating PHB

Microbial strains were isolated from different sample that effectively accumulated PHB. By Nile Blue A staining method, about 300 hundreds of colonies were screened for

their ability to produce PHB in minimal media. Based on the cultural, morphological features, growth rate, the intensity of fluorescence under UV-light, kinetic studies, expeditious and maximum PHB producers were screened. Then twenty six different media with a combination of organic and inorganic nitrogen and carbon source with agribyproducts were used for maximum PHB production. Based on the dry weight and Crotonic acid estimation of the extracted PHB, an effective producer of PHB was chosen. From a comparison of their cell growth in terms of optical density and PHB recovery (data not shown), B3 with a highest PHB content for given cell dry weight was selected for further study.

3.2 Identification and characterization of strain B3 for PHB production

An isolate B3 was identified using a series of morphological characteristics and biochemical tests. Microbiological properties were studied and investigated according to the methods described in ‘‘Bergey’s manual of systematic bacteriology” [8] and the bacterial isolate selected in this study was identified as a member of the genus *Pseudomonas*.

3.3 Production and characterization of PHAs

By inoculating a 2% seed culture of B3 into the minimal media, the growth curve was determined and the result showed that the organism reached the log and the stationary phases at 56^{th} and 69^{th} hour, respectively. Subsequently, the organism was inoculated into a production medium containing agribyproducts like mustard cake, cotton cake as nitrogen sources and fructose as carbon source. Interestingly, PHB production gets increased as compared to the reference media. The accumulated PHB was extracted and subjected to FT-IR analysis. The spectroscopic analysis revealed 4 major peaks at 2920, 1722, 1461, 1250 cm^{-1}, whereas the remaining peaks are closely lying between 3430 cm^{-1} and 649 cm^{-1}. The predominant peak at 2921 and 1461 which represents the methane groups, followed by a peak at 1722 corresponds to C=O stretch of an ester group present in highly ordered crystalline structure whereas the peak at 1276 correspond to CH group. The presence of these marked peaks demonstrated the presence of PHB in strain B3 [4, 6]. PHB is the most common member of the PHAs family and it belongs to the medium-chain-length PHAs (mcl- PHAs) [12].

3.4 Response surface methodology

3.4.1 Effects of process variables on bacterial growth and PHB recovery of B3 strain: Table 2 is showing the experimental results of PHB production by a complete three factor- two-level factorial experiment design with six replications of the central point and six axial points. The quadratic model obtained from regression analysis for bacterial growth in terms of optical density (O.D) in terms of coded level of the variables was developed as follows:

Optical density of B3 strain (nm) (Y_1) = +0.65+0.012*A +0.052*B -0.073*C -0.033*AB +0.015*AC –0.012*BC +0.014*A^2 - 0.010*B^2 -0.031*C^2

PHB recovery of B3 strain (g/l) (Y_2)= +0.024 +8.504E-003*A +3.728E-003*B +3.545E-003*C+2.750E- 003*AB +2.250-003*AC +7.500E-004*BC +7.501E-004*A^2 - 8.409E-004*B^2 -3.105E-004*C^2

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors were coded as +1 and the low levels of the factors were coded as -1. The coded equation was useful for identifying the relative impact of the factors by comparing the factor coefficients.

Table 2: Response Surface Methodology yield of PHB by strain B3

Run	A:fructose (C- source)	B:mustard cake (N-source)	C:yeast extract (N- source)	O.D.	PHB Recovery
	g/l	g/l	g/l	nm	mg/l
1	4	1.5	1.5	0.925	45
2	4	0.5	0.5	0.599	19
3	3	1	1	0.625	21
4	3	1	1	0.648	23
5	3	1	1	0.656	23
6	4.6	1	1	0.897	41
7	1.3	1	1	0.427	16
8	3	1	1	0.696	29
9	3	1	1	0.689	28
10	3	0.1	1	0.487	13
11	3	1	0.1	0.407	20
12	3	1	1	0.623	21
13	3	1	1.84	0.659	25
14	2	0.5	0.5	0.495	15
15	4	0.5	1.5	0.774	34
16	2	0.5	1.5	0.559	17
17	2	1.5	0.5	0.468	12
18	4	1.5	0.5	0.752	31
19	2	1.5	1.5	0.629	21
20	3	1.84	1	0.699	29

The significance of coefficient of fitted quadratic model was evaluated by using F-value and P- value. The analysis of variance (ANOVA) for optical density of quadratic model Eq. (1) of B3 strain is given in (table 3 & 4). The Model F-value 18.94 implied the model was significant. There was only a 0.01 % chance that an F-value this large could occur due to noise for B3 strain. Significant model terms were indicated by the values of "Prob > F" which is less than 0.0500. A, B, C, C^2 were significant model terms. Non significant terms were indicated by the values greater than 0.1000. Model reduction may improve the model, if there were many insignificant model terms (not counting those required to support

hierarchy), The "Lack of Fit F-value" was 3.27. It implied the Lack of Fit was not significant relative to the pure error. Non-significant lack of fit was good - we wanted the model to fit. The fit of model was expressed by the coefficient of determination R^2, which was found to be 0.94. The analysis of variance for B3 (Tables 3, 4) indicated that A, B, CB as significant terms ($p<0.05$) for cell optical density and PHB recovery. In fig.1 (7-12) were shown the surface plots for the interactive factors fructose, mustard cake and yeast extract.

The maximum predicted optical density (0.897) increased with increase of fructose and mustard cake up to 4.6 g/l and 1 g/l (Fig. 1, 7-9). PHB recovery (Fig. 1, 9-12) also increased with increase of fructose, mustard cake and yeast extract up to 4g/l, 1.5g/l and 1.5g/l respectively. The graphs showing the interaction between carbon source fructose, nitrogen sources mustard cake and yeast extract. So, the effect of interaction of various nutrients on the PHB production (z axis) was studied by plotting three dimensional response surface curves.

Table 3: Analysis of variance (ANOVA) for B3 strain for Optical Density

	F	p-value	
Model	18.94	< 0.0001	significant
A-fructose (C- source)	102.03	< 0.0001	
B-mustard cake (N-source)	17.69	0.001	
C-yeast extract (N- source)	35.52	0.000	
AB	4.16	0.068	
AC	0.92	0.359	
BC	0.55	0.475	
A^2	1.43	0.259	
B^2	0.72	0.414	
C^2	6.92	0.025	
Lack of Fit	3.27	0.110	not significant

Table 4: Analysis of variance (ANOVA) for B3 strain for PHB recovery

	F-value	**p-value**	
Model	**12.57**	**0.0002**	**significant**
A-fructose (C-source)	75.71	< 0.0001	
B-mustard cake (N-source)	14.55	0.0034	
C-yeast extract (N-source)	13.15	0.0046	
AB	4.64	0.0567	
AC	3.10	0.1085	
BC	0.34	0.5700	
A^2	0.62	0.4487	
B^2	0.78	0.3975	
C^2	0.11	0.7508	
Lack of Fit	1.14	0.4430	not significant

These curves were plotted against any two independent variables while keeping the other independent variable at their ‘‘0” levels. Therefore two response surfaces were obtained by considering all three possible combinations. From the response surface 3D plots (Fig. 1) it is understandable that all nutrients has considerable effect on PHB production with the optimized medium the production of PHB obtained was 0.45 g/L.

3.5 FTIR analysis conformed presence of PHB

The FTIR spectroscopic analysis of B3 strain was done Fourier Transform Infra-Red (FTIR) spectrum of the PHB sample revealed 4 major peaks at 2920, 1722, 1461, 1250 cm-1, whereas the remaining peaks are closely lying between 3430 cm-1 and 649 cm-1. The predominant peak at 2921 and 1461 which represents the methane groups, followed by a peak at 1722 corresponds to C=O stretch of an ester group present in highly ordered crystalline structure whereas the peak at 1276 correspond to CH group. The presence of these marked peaks demonstrated the presence of PHB in strain B3 (fig. 2). The FTIR results of pseudomonas B3 strain was in agreement with the earlier works [10].

4. DISCUSSION

A new efficient Polyhydroxybutyrate (PHB) producer, that utilized Mustard cake as nitrogen sources for production was isolated from garden soil, and medium conditions were optimized for high PHB production. Response surface methodology is a promising method to optimize medium concentration and the interactions of other variables involved in the production. The validity of the model was proved by fitting the values of the variables into a model equation [3]. Additionally, the benefit of RSM is the study of interactions between the coded variables which is very difficult to be studied in conventional one-factor-at-a-time method. Although PHB production was enormous in earlier studies, the raw materials used in those studies are expensive. For the production of PHB (PHAs), the cost of the carbon and nitrogen source is supposed to be low and yield should be the maximum.

According to [9], many carbon sources derived from wastes like whey, cane molasses and sugar beet molasses were used for production of PHB and for mineral source mixture of different salts had been used. However, mustard cake, one of the least expensive, renewable and easily available resources was supplied as a nitrogen source. In this study we demonstrate the production of PHB on industrial and large scale towards low cost bioplastics as a solution to environmental problems due to petrochemical plastics has created a renewed interest in biologically derived polymers. To apply PHAs as product plastics, the utilized raw materials should be inexpensive [7] and it has to be easily available. The media and PHB production process developed on the novel pseudomonad has enabled to achieve these goals to help the environment of the globe at large.

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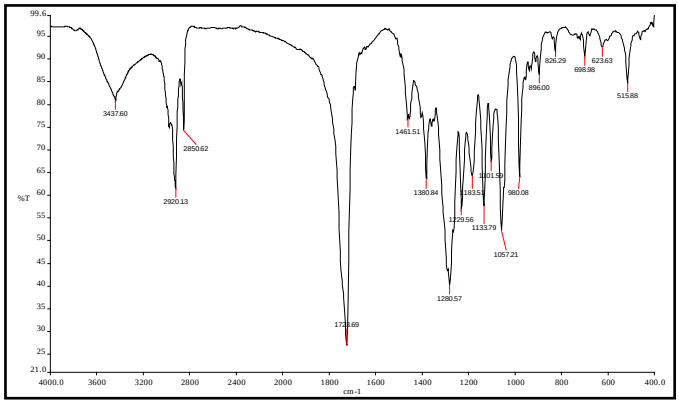
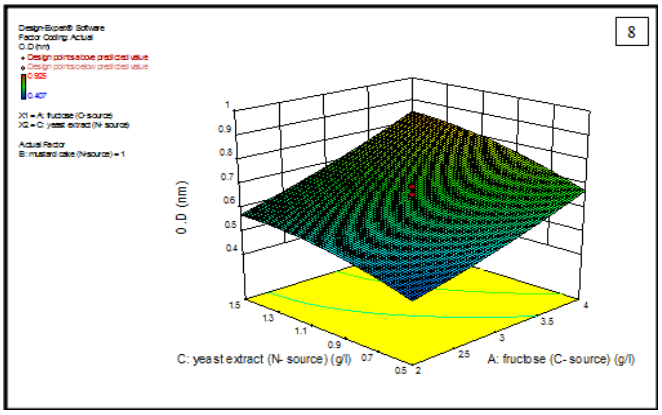
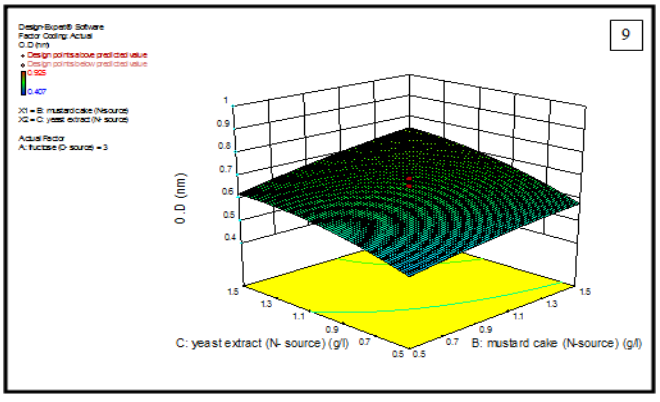
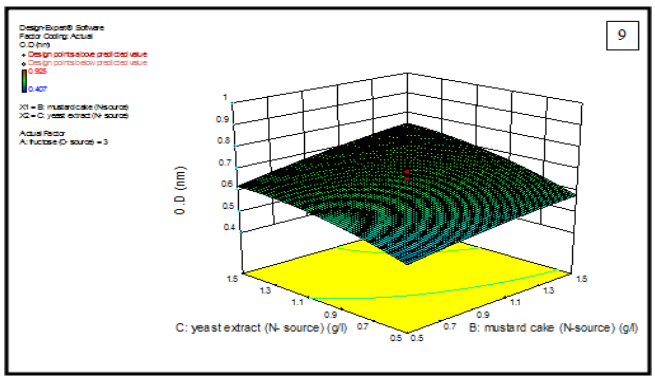
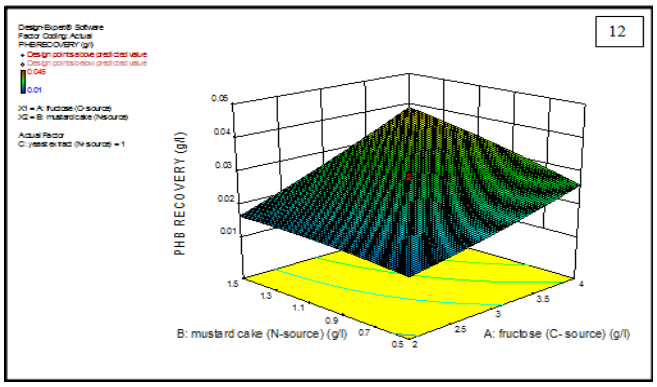


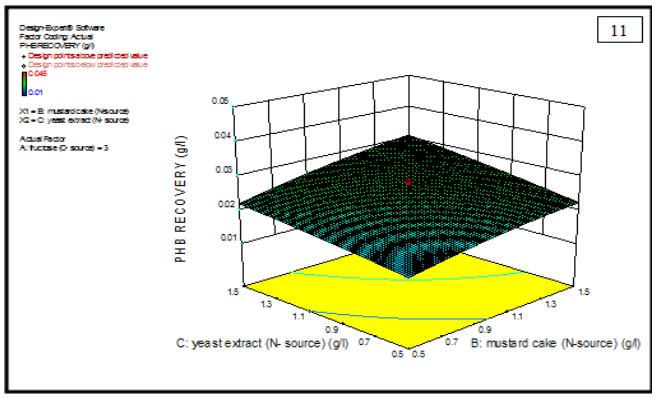
Fig. 2: FTIR spectrum of PHB produced by strain B3











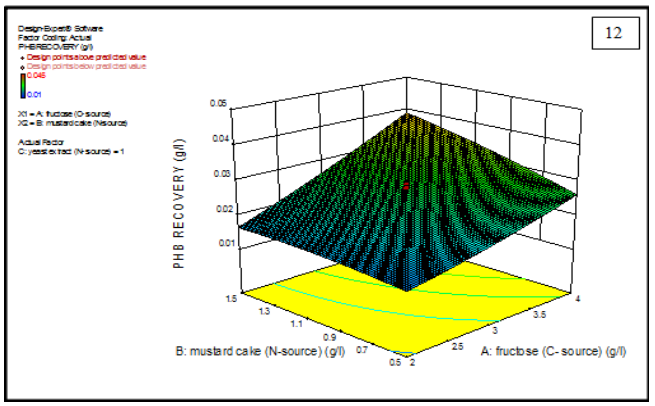


Fig. 1: 3-D plots showing the interactive effect of mustard cake, yeast extract and fructose for the responses Optical Density and PHB Recovery in strain B3 (7-12)

REFERENCES

[1] Byrom D. (1987). Polymer synthesis by microorganisms: technology and economics. Trends Biotechnol.5:246-250.

[2] Choi GG, Kim HW, Rhee YH (2004). Enzymatic and non-enzymatic degradation of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) copolyesters produced by Alcaligenes sp. MT-16. J. Microbiol. 42 :346–352.

[3] Deepak V, Kalishwaralal K, Ramkumarpandian S, Venkatesh BS, Senthilkumar SR, Sangiliyandi G (2008). Optimization of media composition for Nattokinase production by Bacillus subtilis using response surface methodology. Bioresour. Technol. 99:8170–8174.

[4] Hong K, Sun S, Tian W, Chen GQ, Huang W (1999). A rapid method for detecting bacterial polyhydroxyalkanoates in intact cells by Fourier transform infrared spectroscopy. Appl. Microbiol. Biotechnol. 51:523–526.

[5] Kansiz M, Jacobe HB, Mcnaughton D (2000). Quantitative determination of the biodegradable polymer poly (b-hydroxybutyrate) in a recombinant Escherichia coli strain by use of mid-infrared spectroscopy and multivariative statistics. Appl. Microbiol. Biotechnol. 44:3415–3420.

[6] Khanna S, Srivastava AK (2005). Statistical media optimization studies for growth and PHB production by Ralstonia eutropha. Process Biochem. 40:2173–2182.

[7] Kreig NR, Holt JG (1984). Bergey's Manual of Systematic Bacteriology. Williams & Wilkins, Baltimore.

[8] Lee SY, Middelberg, APJ, Lee YK (1997). Poly (3-hydroxybutyrate) production from whey using recombinant Escherichia coli. Biotechnol. Lett. 19:1033–1035.

[9] Muthazhagan K, Thangaraj M (2014). Production and ftir analysis of bio-polymer by *bacillus sp* isolated from vellar estuary sediment. IJSIT. 3:625-638.

[10] Pouton CW, Akhtar S (1996). Biosynthetic polyhydroxyalkanoates and their potential in drug delivery. Adv Drug Deliv Rev. 18:133–162.

[11] Steinbuchel A, Schlegel HG, 1991. Physiology and molecular genetics of poly (bhydroxyalkanoic acid) synthesis in Alcaligenes eutrophus. Mol. Microbiol. 5:535–542.

[12] Sujatha K, Mahalakshmi A, Shenbagarathai (2005). Analysis of PCR products for PHB production in indigenous pseudomonas isolates LDC-5 and LDC-25. Int. J. Biotechnol. 4:323-335.

[13] Tsuge T, Saito Y, Kikkawa Y, Hiraishi T, Doi Y. (2004). Biosynthesis and compositional regulation of poly [(3-hydroxybutyrate)-*co*-(3- hydroxyhexanoate)] in recombinant Ralstonia eutropha expressing mutated polyhydroxyalkanoate synthase genes. Macromol Biosci. 4: 238 – 242.