Optimization of Bacterial Poly – β – Hydroxy Butyrate (PHB) Production from Different Industrial Waste using Central Composite Design

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ABSTRACT
Poly (ß-hydroxybutyrate) (PHB) belongs to a family of microbial energy/carbon storage compounds collectively known as poly hydroxyalkanoates. About 150 different PHAs have been identified as constituents of bacterial polyesters. PHB is natural, biodegradable polyester which is accumulated in the form of intracellular granules by a variety of heterotrophic and autotrophic aerobic bacteria, photosynthetic anaerobic bacteria, gliding bacteria, Actinomycetes sp, cyanobacteria and recently an anaerobic fatty acid oxidizing gram negative bacterium. Until now there are only few reports on marine PHAs producing microorganisms. With this background an optimization of media components for the effective PHB production using different industrial waste has been attempted for this study.

Keywords
Poly-β-hydroxy butyrate, PHB, *Alcaligenes eutrophus*, CCD, RSM, Industrial waste, bacterial polyesters.

1. INTRODUCTION

Poly (ß-hydroxybutyrate) (PHB) belongs to a family of microbial energy/carbon storage compounds collectively known as poly hydroxyalkanoates. About 150 different PHAs have been identified as constituents of bacterial polyesters [1]. PHB is a natural, biodegradable polyester which is accumulated in the form of intracellular granules by a variety of heterotrophic and autotrophic aerobic bacteria, photosynthetic anaerobic bacteria, gliding bacteria, Actinomycetes sp, cyanobacteria and recently an anaerobic fatty acid oxidizing gram negative bacterium [2]. Until now there are only few reports on marine PHAs producing microorganisms[3,4].

Current worldwide dependence on fossil fuels for plastic manufacture (approximately 270 million metric tones of fossil fuels), the scarcity of space for disposal and growing environmental concerns for non-biodegradable synthetic plastics have fuelled research towards development of ecofriendly biopolymer materials[5,6].considerable emphasis have been laid on the development of five different types of biopolymers which include fibre-reinforced composites, starch based materials, plant produced polymers, microbially produced polymers and biologically based resins, coatings and adhesives[7]. Of these maximum attention has been laid on the development of microbially produced polymer such as Polyhydroxyalkanoates (PHAs).

Despite these decisive benefits, history of research into PHAs has had a slow and chequered history. The composition of PHAs was first described by Lemoigne [8,9] as an unknown material in the form a homopolyseter of 3-hydroxybutyric acids, called PHB synthesized by a soil bacterium - Bacillus. During the following 30 years, interest in this unknown material was negligible. The first report on function of PHB was explained in 1958 by Macrae and Wilkinson[10]. They reported the rapid biodegradability of PHB produced by *Bacillus megaterium* and *Bacillus cereus* itself. From here on, the interest in PHB increased dramatically. In the following years, research on PHB and other forms of PHAs included investigations with other microorganisms and potential use of these biopolymers was realized [11,12,13,14,15].

Moreover processing PHB into articles of commerce does not require new investments in technology; existing equipment developed for originally processing polyethylene and polypropylene can be used. However a major draw back to the commercialization of PHB is its higher cost compared with petrochemical-based plastic material[16]. Low cost production of PHB requires improved fermentation and cultivation strategies, inexpensive media [17] and easier downstream processing [18,19,20]. Thus using cheap substrates and low cost nutrients at an optimized concentration can feature an improvement in the productivity of PHB at an economical cost.

2. MATERIALS AND METHODS

2.1. Organism

Alcaligenes eutrophus MTCC1285 was obtained from the Microbial type culture collection, Chandigarh, India. The PHB producing capability of the organism was confirmed by Sudan black staining method[21].

2.2. Central Composite Design and Response Surface Methodology

The levels of the significant parameters and interaction effects between four industrial waste and the bacterial strain viz., *Alcaligenes eutrophus* was used for the production of PHB were analyzed and optimized by using a central composite design in response surface methodology. The experimental design was carried out by using "Stat-Ease Design-Expert" software (version 8.1, Stat-Ease Corporation, USA). The four independent factors were investigated at five different levels

(−2, −1, 0, +1, +2). The response Y (yield of PHB) was analyzed by using a second order polynomial equation in four independent variables and the data were fitted into the equation by multiple regression procedure.

The model equation for analysis is given below Eq.

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X^2_i + \Sigma\beta_{ij} X_i X_j$$

where Y is the predicted response, X_i, X_j represent the independent variables which influence the response variable Y, and $ß_0$, $ß_i$, $ß_{ii}$, and $ß_{ij}$ represent the offset term, the ith linear coefficient, the ith quadratic coefficient and the interaction coefficient, respectively. "Design-Expert"8.1 was used for regression and graphical analyses of the data obtained. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The student's t-test permitted the checking of the statistical significance of the regression coefficient, and the Fischer's test determined the second-order model equation. The quality of the fit of the polynomial model equation was given by the coefficient of determination (R2). The optimum concentration of the variables were calculated from the data obtained by using the response surface regression procedure of the SAS statistical package (Version 8.1, SAS institute inc. NC. USA).

2.3. PHB production and extraction

Four different industrial waste substrates such as (seasame, molasses, sago and paper waste) were collected from industries and were used for the PHB production in different percentage (10, 20, 30, 40 and 50%). The PHB production by *A. eutrophus* on different industrial wastes [22] under aerobic conditions was studied. *A. eutrophus* was grown at 37°C for 72 h. PHB produced were extracted as described in the method of Ramsay et al.[23, 24].

2.3. Estimation and qualitative analysis of PHB

The amount of PHB in the extracted samples was determined spectrophotomertrically at 235 nm [25,26]. The pure form PHB was collected [27] and qualitatively analyzed by infrared method [28] and by NMR method [29].

3. RESULTS AND DISCUSSION

3.1. Central Composite Design and Response Surface Methodology

Central Composite Design is powerful method for screening significant factor in the presence study, 30 runs were carried out to investigate the production optimization of PHB using four different factors viz., seasame oil waste, sago waste, molasses waste and paper waste. The PHB production varied from 1.7 grams/litre to 3.6 grams/litre in industrial waste used for the study. Response surface methodology help in evaluation of relationship between the dependent (PHB yielded) variable and independent variables and predicted values of the PHB production are shown. The accuracy of the model can be seen by the different between observed and predicted value. The co-efficient and the analysis of variance are presented in table. Fitness of the model was expected by the value of the determination co-efficient in the present PHB comes out to be 2.8 for PHB production high value of adjust co-efficient determination this adjusted 3.6 indicate high significance of model (Fig. 1 and Fig. 6).

3.5. FTIR spectrum

Figure 7 shows the FTIR spectrum of the extracted polymer isolated in the study. The FT-IR spectrums obtained were compared with the spectrum of commercially available PHB. The large absorption peak at 3395.07 cm^{-1} – 3452.34 cm^{-1} was OH stretching and C-H was between 2924.25 cm^{-1} – 2994.59 cm^{-1}. The absorption band at 1723.45 cm^{-1}- 1728.87 cm^{-1} attributed to the stretching vibration of the carboxyl bond (C=O). The band at 2321.87 cm^{-1}- 2359.02 cm^{-1} was assigned to the C≡C stretching of alkynes. Absorption peaks between 1537.95 cm^{-1} and 1655.59 cm^{-1} indicates the presence of nitro compounds. The bands between 1547.59 cm^{-1} and 1597.11 cm^{-1} arise from N-H vibration of amines. Intense bands centered at 1078.01 cm^{-1} – 1283.39 cm^{-1} were assigned to C-N vibrations of amine group. The obtained IR absorption peaks correlated with the literature value and with the spectrum of pure PHB. From the above details it is concluded that the compound should be PHB.

3.6. ^{1}H NMR spectral analysis

The obtained spectrum for the *Alcaligenes eutrophus* PHB showed the following results (Figure 8).

The NMR spectra identified the polymer as an isocratic homopolymer. The spectrum revealed the presence of three group of signals characteristic of PHB homopolymer. The doublet at 1.25 ppm was attributed to the methyl group coupled to one proton; the doublet of the quadruplet around 2.5 ppm to the methylene group adjacent to an asymmetric carbon atom bearing a single proton and the singlet at 5.6 ppm to the methyne group. Chloroform-d gave a chemical shift signal at 7.26 ppm.

4. CONCLUSION

RSM was used to estimate and optimize the PHB production. All the independent variables, quadratic of all the independent variables had highly significant effects on the response values ($p < 0.03$). The optimal media composition for PHB production was obtained through a central composite design in response surface methodology as 1.7 to 3.6. Under these conditions, the experimental yield of PHB was 3.6 gms in the factor with preliminary media optimization experiments with the use of industrial wastes (Table 1).

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Figure 1. – PHB Model Graph of *Alcaligenes eutrophus* in Sago and seasame oil waste

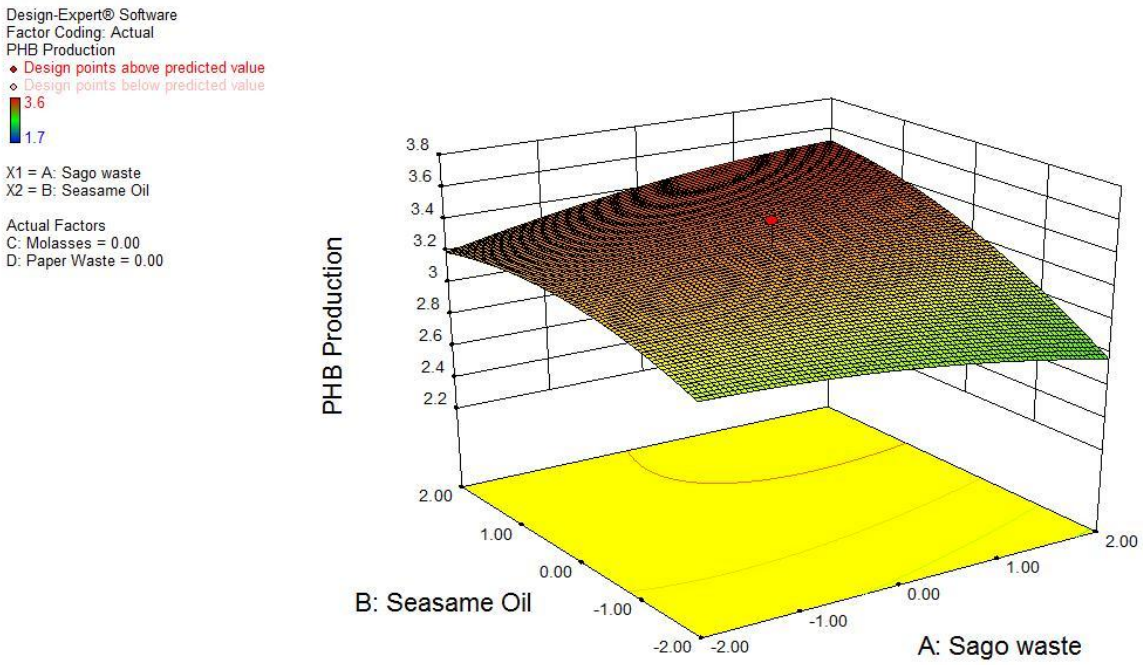

Figure 2. – PHB Model Graph of *Alcaligenes eutrophus* in Sago and molasses waste

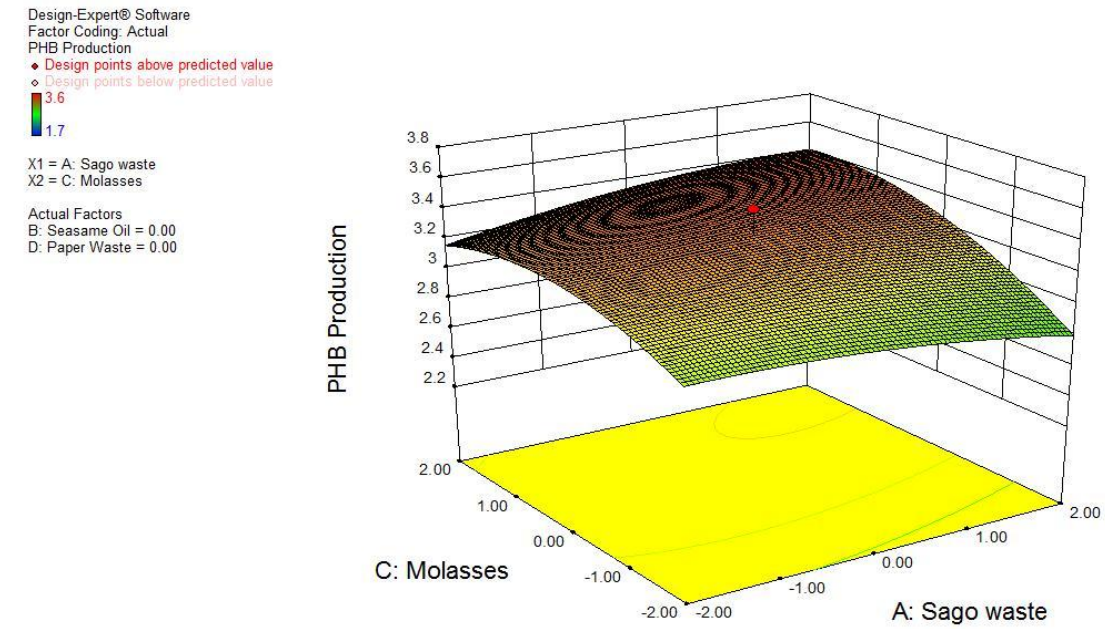

Figure 3. PHB Model Graph of *Alcaligenes eutrophus* in Sago and paper waste

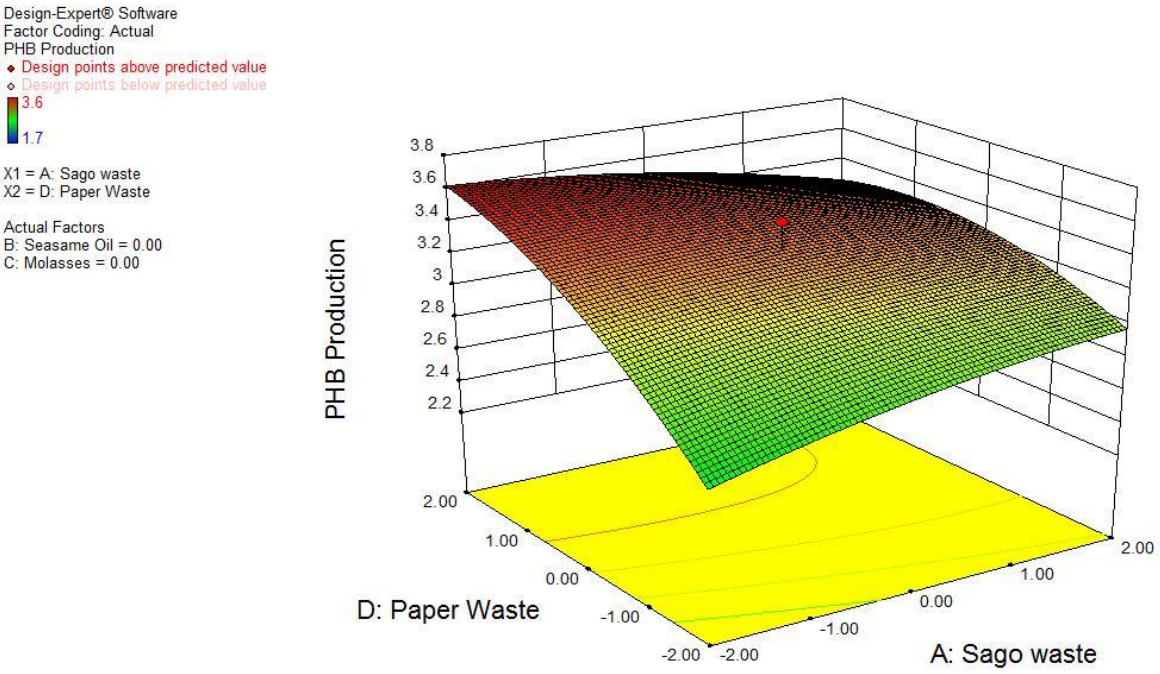

Figure 4. PHB Model Graph of *Alcaligenes eutrophus* in Seasame oil and molasses waste

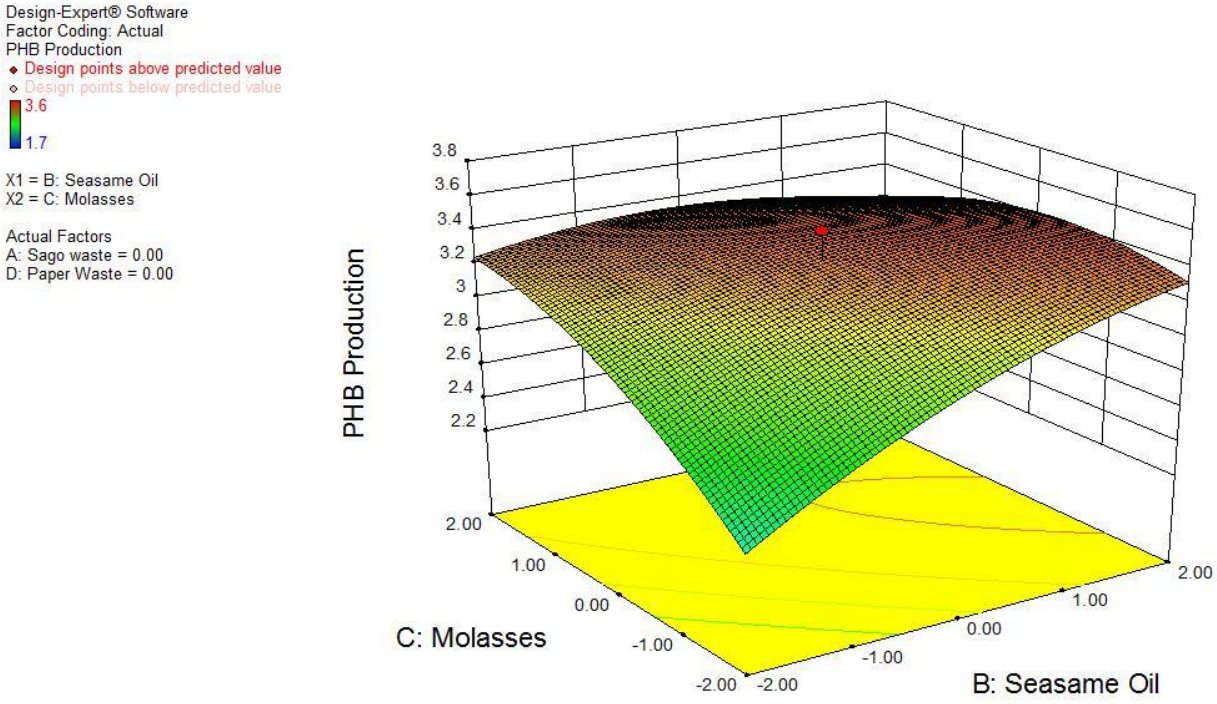

Figure 5. PHB Model Graph of *Alcaligenes eutrophus* in Seasame oil and paper waste

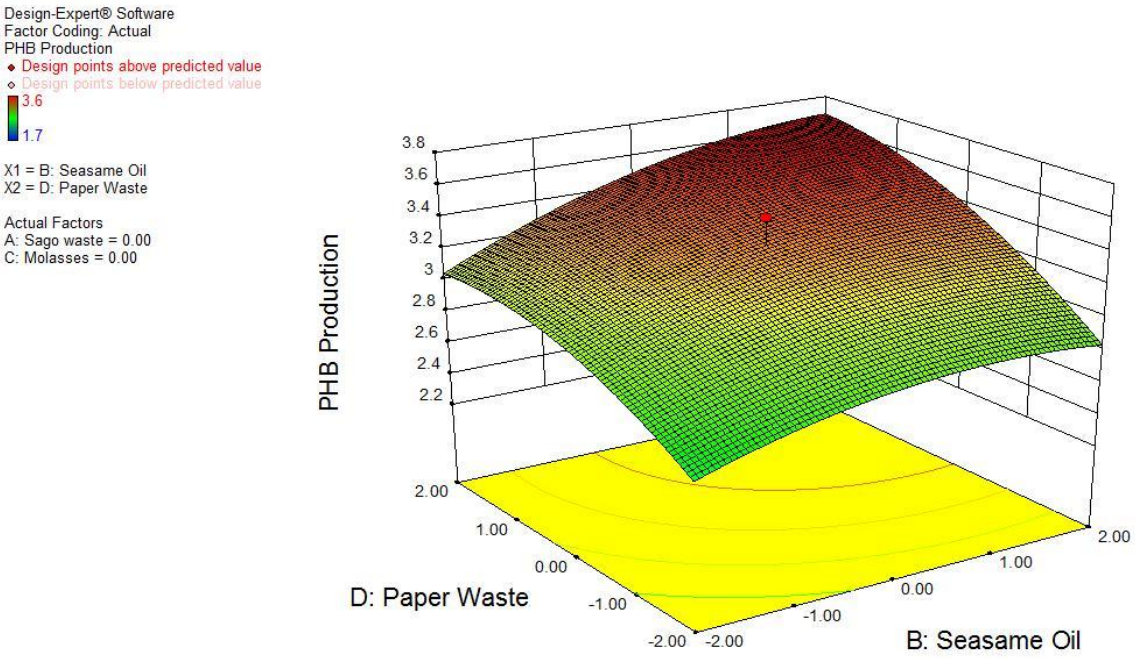

Figure 6. PHB Model Graph of *Alcaligenes eutrophus* in Molasses and paper waste

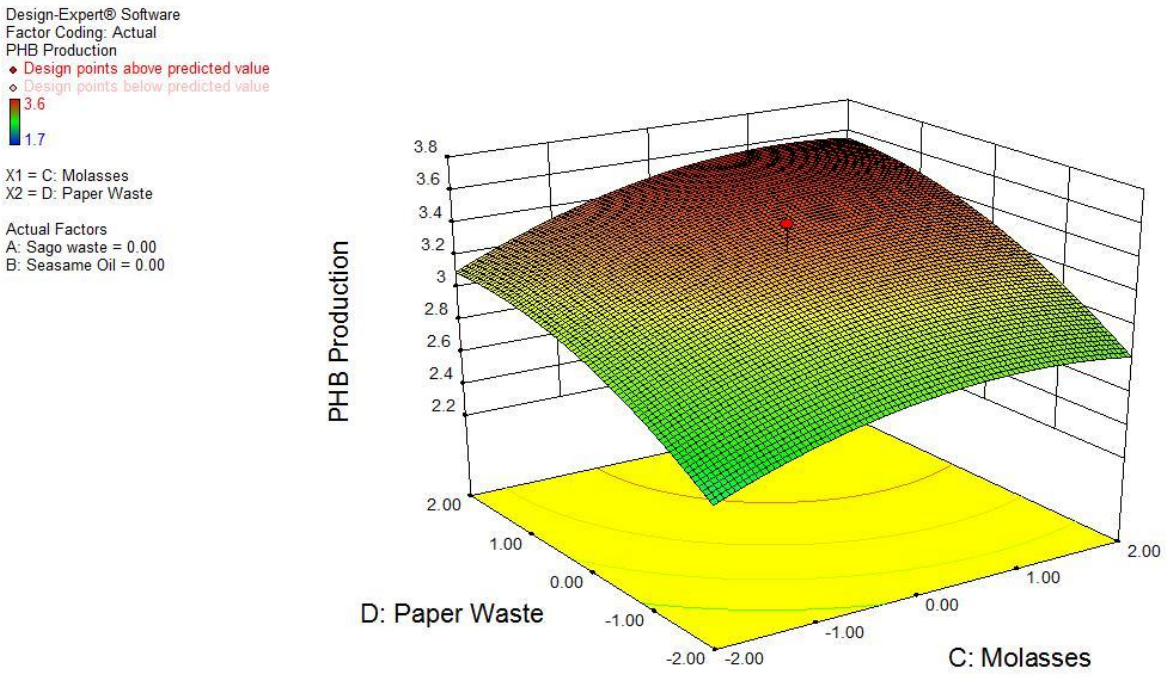

Figure 7. FTIR Spectrum of PHB produced by *Alcaligenes eutrophus*

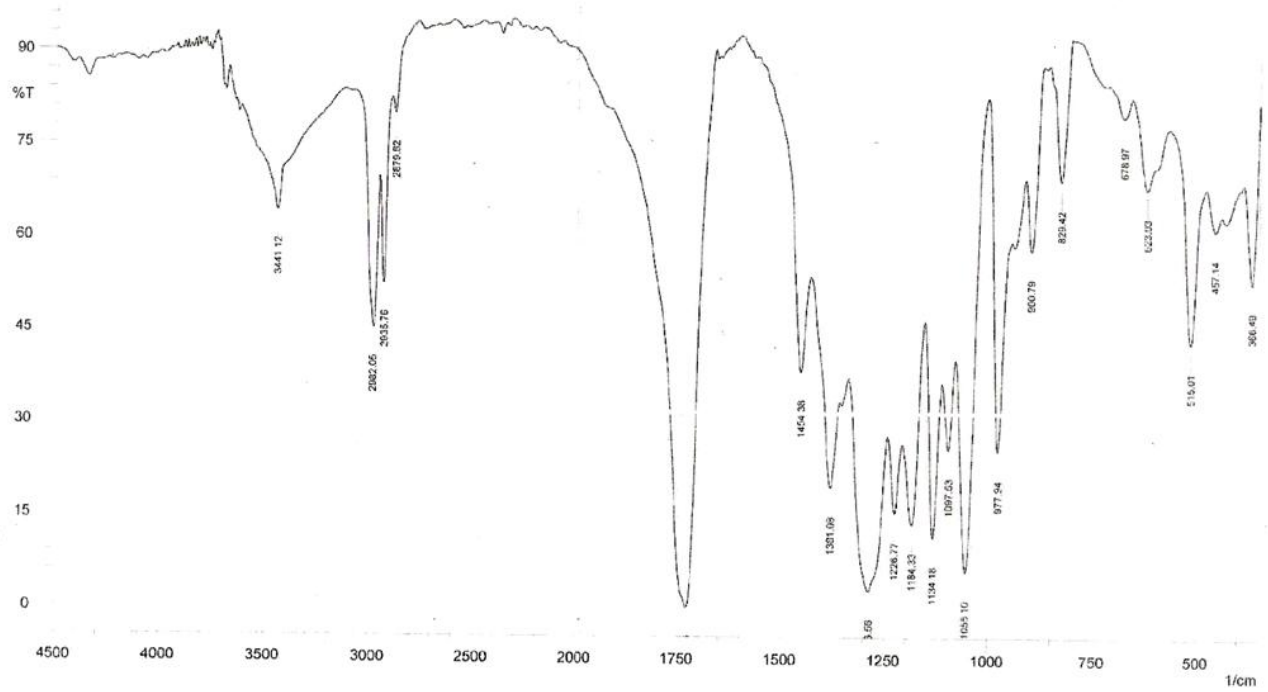

Figure 8. ^{1}H NMR Spectrum of PHB produced by *Alcaligenes eutrophus*

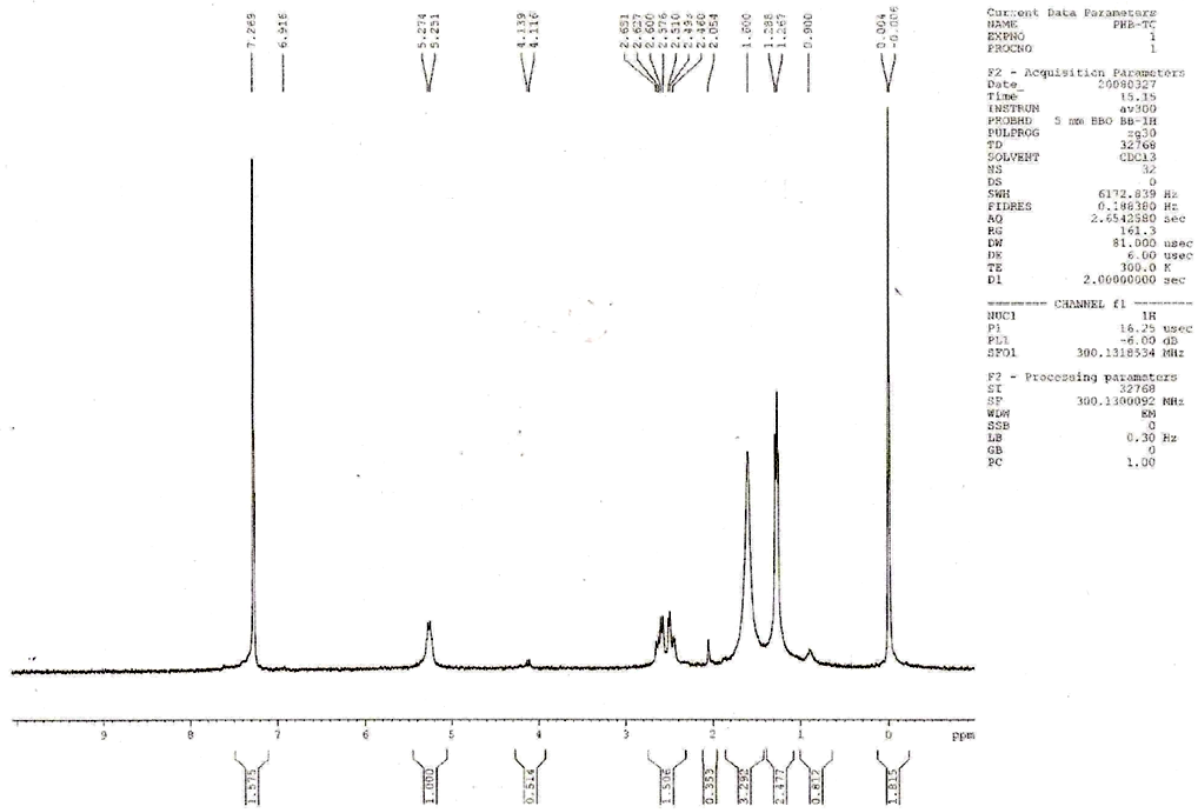

Table 1. Response surface methodology *yield of PHB by Alcaligenes eutrophus*

Run	Factor 1 A:Sago waste Grams (g)	Factor 2 B:Seasame Oil Grams (g)	Factor 3 C:Molasses Grams (g)	Factor 4 D:Paper Waste Grams (g)	Response 1 PHB production g/litre
1	0.00	-4.00	0.00	0.00	2.5
2	-4.00	0.00	0.00	0.00	3.5
3	-2.00	-2.00	2.00	2.00	3.1
4	4.00	0.00	0.00	0.00	3.1
5	2.00	2.00	-2.00	2.00	3.5
6	-2.00	2.00	2.00	2.00	3.5
7	-2.00	2.00	-2.00	2.00	3.5
8	0.00	0.00	-4.00	0.00	2.5
9	0.00	0.00	0.00	-4.00	2.5
10	2.00	-2.00	2.00	2.00	3.6
11	2.00	2.00	-2.00	-2.00	2.9
12	0.00	0.00	0.00	0.00	3.5
13	0.00	0.00	0.00	0.00	3.5
14	0.00	0.00	0.00	0.00	3.5
15	-2.00	-2.00	2.00	-2.00	2.45
16	-2.00	-2.00	-2.00	2.00	2.75
17	0.00	0.00	0.00	0.00	3.1
18	2.00	2.00	2.00	-2.00	3
19	0.00	0.00	0.00	0.00	3.1
20	2.00	-2.00	2.00	-2.00	2.6
21	0.00	0.00	0.00	4.00	3.3
22	2.00	2.00	2.00	2.00	3.1
23	0.00	4.00	0.00	0.00	3.5
24	2.00	-2.00	-2.00	-2.00	1.7
25	2.00	-2.00	-2.00	-2.00	1.7
26	0.00	0.00	0.00	0.00	3.1
27	-2.00	2.00	2.00	-2.00	1.8
28	-2.00	-2.00	-2.00	-2.00	1.96
29	-2.00	2.00	-2.00	-2.00	1.98
30	0.00	0.00	4.00	0.00	3.2