International Journal of Computer Applications (0975 – 8887)
Volume 68– No.25, April 2013

# Optimization of Poly β-Hydroxy Butyrate Production by *Alcaligenes Latus* MTCC2311 using Central Composite Design

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## ABSTRACT
Considering the industrial interest of poly-b-hydroxy butyrate (PHB) and its high production cost, work has been undertaken for the production of PHB by *Alcaligenes latus* (2311). Different industrial wastes (sesame, molasses, sago and paper waste) were used as a cheap substrate to minimize the production of cost and nitrogen limited minimal agar synthetic medium was also used for comparison. Accumulation of PHB granules in the organism was analyzed by sudan black method. The PHB production in various industrial waste based medium and nitrogen limited minimal agar synthetic medium was studied by crotonic acid method. The pure form of PHB was collected and qualitatively analyzed by infrared and nuclear magnetic resonance methods. Highest PHB production was found in nitrogen limited minimal agar synthetic medium. Among the various industrial wastes based media, highest yield was obtained with sesame oil waste as carbon source

## Keywords
Poly-β-hydroxy butyrate, PHB, *Alcaligenes latus* MTCC 2311, CCD, bacterial polyesters.

## 1. INTRODUCTION

For more than seventy years petroleum based plastics have been used in a variety of industrial and day to day applications owing to their versatility and durability [1, 2]. However, they bear negative attributes including recalcitrance to biodegradation [3] toxicity after incineration and massive waste accumulation into the landfills and the marine environment. In response to the problems associated with the synthetic polymers, the public tendency, the scientific interest and the governments determination has worked in unison over the past two decades to support the development of a new class of plastics. The challenge for the new polymers is to retain the physicochemical characteristics of the traditional plastics but benefit from biocompatibility and biodegradability [4,5,6].

Polyhydroxyalkanoates (PHA) are polyesters synthesized by a variety of bacterial strains as intra cellular carbon and energy storage compounds grown usually under stress conditions [7]. As biodegradable and biocompatible materials, PHA have drawn a lot of industrial attention for the potential applications in many fields [8, 9, 10, 11]. Poly (3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-co-4HB)] was first found from Ralstonia eutropha cultivated with 4-hydroxybutyric or 4-chloro butyric acid as carbon sources by Doi et al. [12]. The incorporation of 4HB units into P (3HB) improves the material application potentials, and the copolymers show a wide range of physical properties ranging from highly crystalline plastic to elastic rubber, depending on the polymer composition[13,14]. P (3HB-co-4HB) is hydrolyzed by both PHA depolymerases and lipases, and its degradation rate is relatively rapid compared with other PHA. Thus, P(3HB-co-4HB) with various 4HB compositions are promising materials that have favorable biodegradability and mechanical properties. P (3HB-co-4HB) has been produced in a few bacteria, including *R. eutropha, Alcaligenes latus* [15,16,17,18], *Comamonas acidovorans* [19,20], *Comamonas testosteronii* [21] and *Hydrogenophaga pseudoflava* [22]. Generally, carbon sources structurally related to 4HB are required to generate 4HB-containing PHA, such as 4-hydroxy butyric acid, g-butyrolactone and1,4-butanediol [23,24,25,26]. However, these carbon sources are much more expensive than glucose or other 3HB-generating carbon sources are for example, 4-hydroxy butyric acid is considered to be the most effective precursor for forming 4HB monomer, but it is difficult to obtain since 4-hydroxy butyric acid is a controlled substance in countries like USA and China. The high cost of raw material for the co-polymer production has become an obstacle for the wide production and application of P(3HB-co-4HB).

Biological waste water treatment technology has been extensively applied to treat both municipal sewage and industrial waste water. However, the active microorganisms may be exposed to alternating feast and famine conditions due to the fluctuations in the hydraulic loading and nutrient concentrations of the waste water treatment plants [27]. It has been reported that, after heavy rainfall or on weekends, a municipal waste water treatment plant (WWTP) can temporarily receive low concentrations of sewage and a high hydraulic loading, which could lead to the active microorganisms suffering famine periods for hours [28]. As for the industrial waste water (e.g. the dairy and food processing industries, paper mills and abattoirs), the WWTPs may be more prone to suffering large fluctuations in the waste water composition and flow, and even complete interruptions of waste water flows due to the nature of the industrial activities[29,30]. Therefore, the biomass may be in famine periods for hours, and even for days or weeks. Such famine periods can significantly affect the amount and activity of active microorganisms and performance of a waste water treatment system [31, 32, 33, 34]. Therefore, the ability of the biomass to survive under such conditions needs to be investigated in order to achieve reliable biological waste water treatment [35].

The British Chemical giant, Imperial Chemical Industries (ICI) began investigating in 1976; the possibility of producing PHB from carbohydrate feed stocks. The use of renewable substrates added to the biological advantages of producing PHB, but the project slowed down, mainly because of high cost of production and insufficient demand [36,37]. The process has been, however, revived many years later and now *Alcaligenes eutrophus* and *Alcaligenes latus* are currently utilized and commercially produced under the trade name Biopol by Monsanto, which acquired the business from Zeneca bioproducts, an offshoot of ICI [38].

# 2. MATERIALS AND METHODS

## 2.1. Organism

*Alcaligenes latus* MTCC2311 was obtained from the Microbial type culture collection, Chandigarh, India. The PHB producing capability of the organism was confirmed by Sudan black staining method [39].

## 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.

$$Y = \beta_0 + \Sigma\beta_{i}X_i + \Sigma \beta_{ii}X^2_{i} + \Sigma \beta_{ij}X_iX_j$$

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. latus* on different industrial wastes [40] under aerobic conditions was studied. *A. latus was* grown at 37°C for 72 h. PHB produced were extracted as described in the method of Ramsay et al.[41,42].

## 2.3. Estimation and qualitative analysis of PHB

The amount of PHB in the extracted samples was determined spectrophotomertrically at 235 nm [43,44]. The pure form PHB was collected [45] and qualitatively analyzed by infrared method [46] and by NMR method [47].

# 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 1.96 grams/litre to 3.21 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 1.95 for PHB production high value of adjust co-efficient determination this adjusted 3.21 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 latus* 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 1.96 to 3.21. Under these conditions, the experimental yield of PHB was 3.21 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 latus* MTCC 2311 in Sago and seasame oil waste**

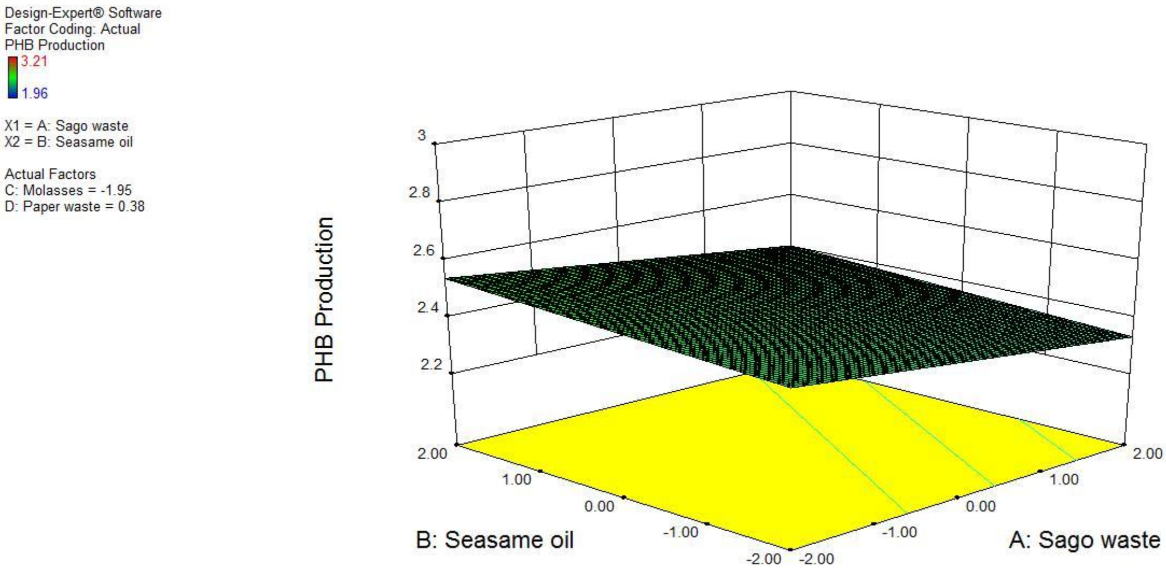


**Figure 2. – PHB Model Graph of *Alcaligenes latus* MTCC 2311 in Sago and molasses waste**

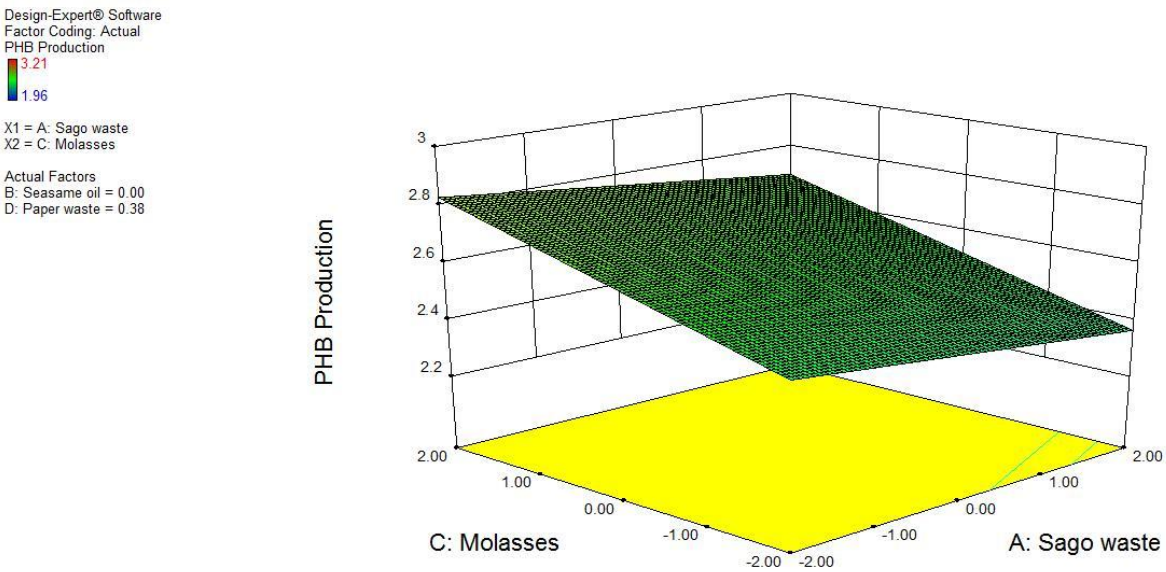

**Figure 3. PHB Model Graph of *Alcaligenes latus* MTCC 2311 in Sago and paper waste**

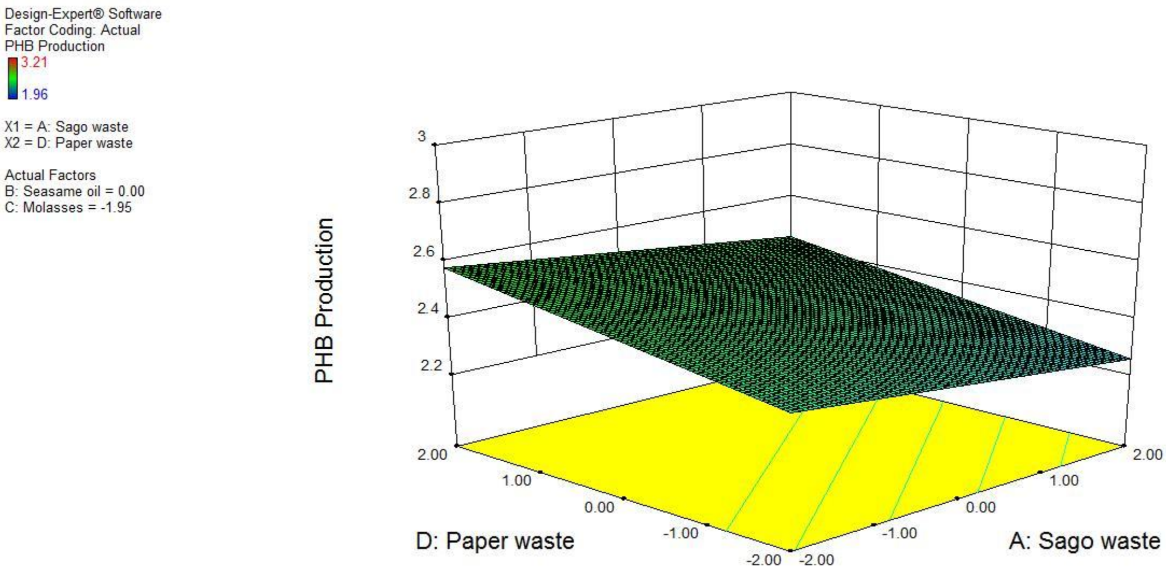


**Figure 4. PHB Model Graph of *Alcaligenes latus* MTCC 2311 in Seasame oil and molasses waste**

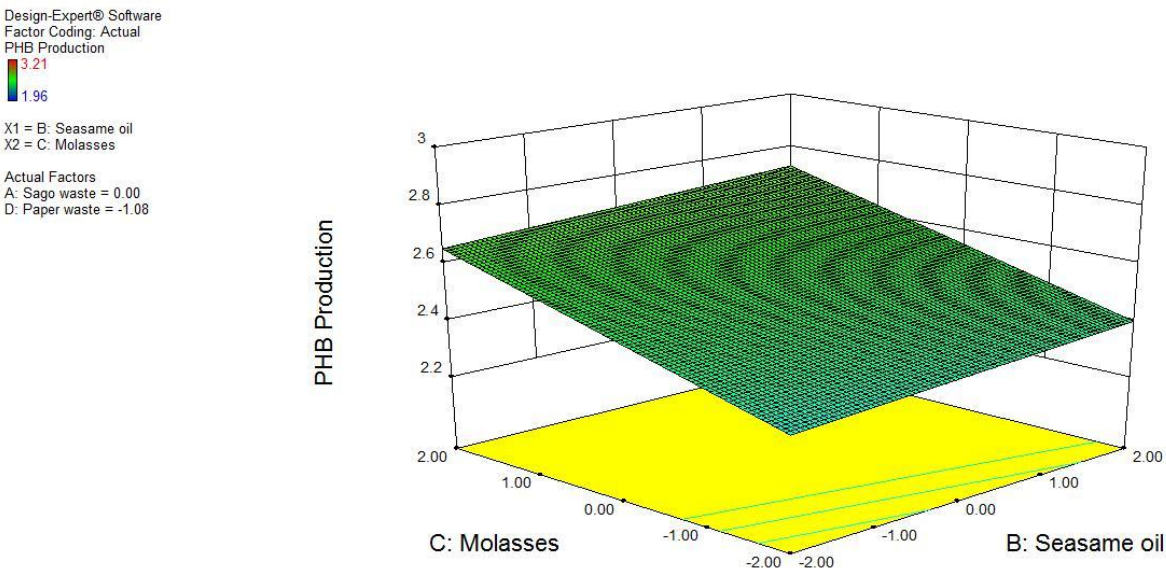

**Figure 5. PHB Model Graph of *Alcaligenes latus* MTCC 2311 in Seasame oil and paper waste**

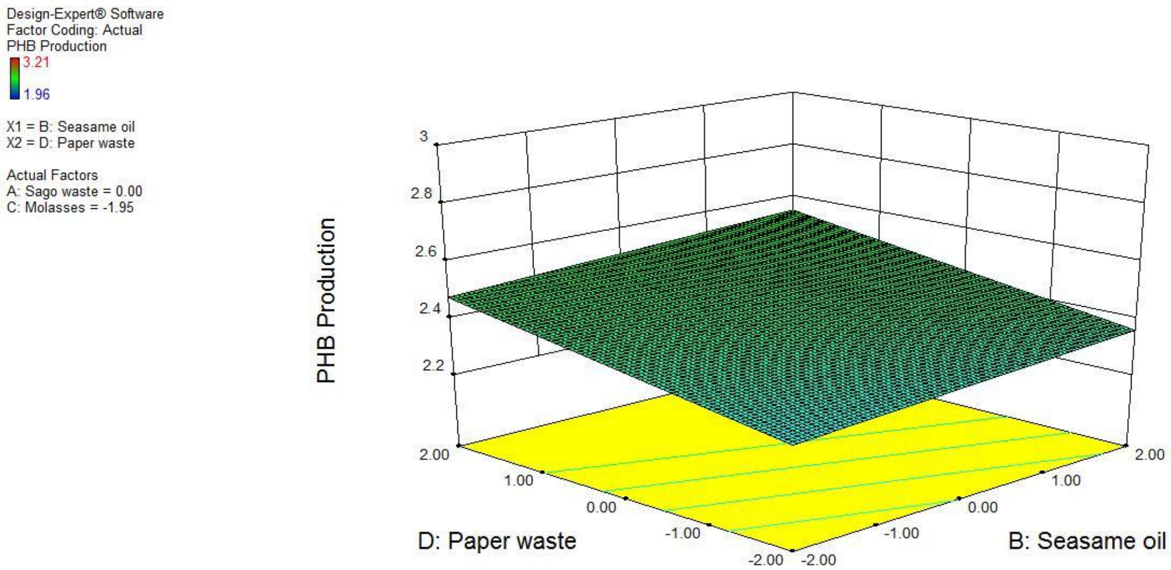


**Figure 6. PHB Model Graph of *Alcaligenes latus* MTCC 2311 in Molasses and paper waste**

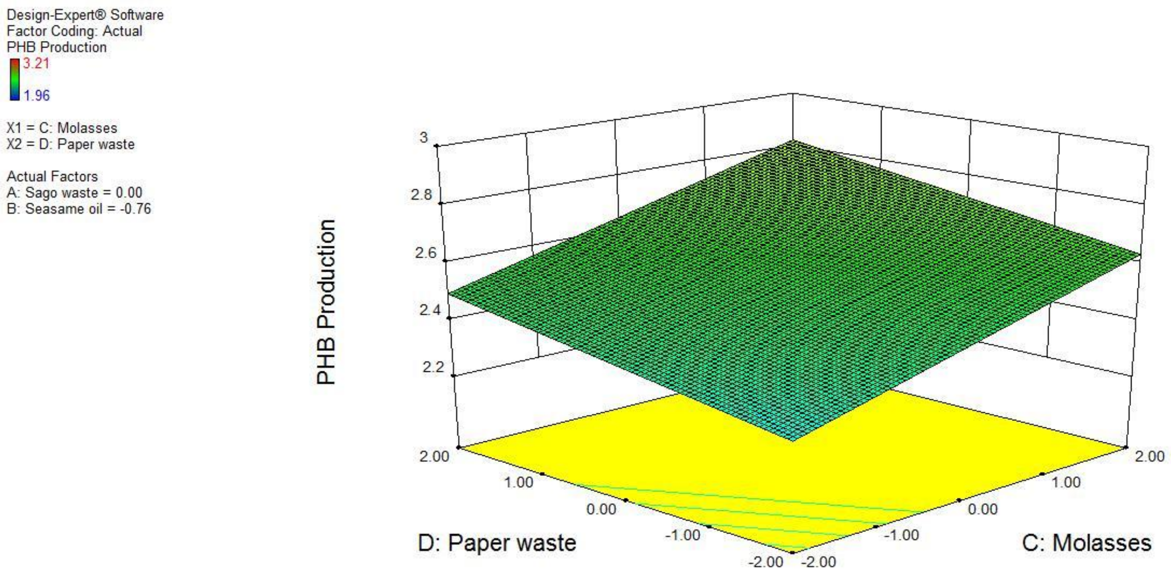

**Figure 7. FTIR Spectrum of PHB produced by *Alcaligenes latus* MTCC 2311**

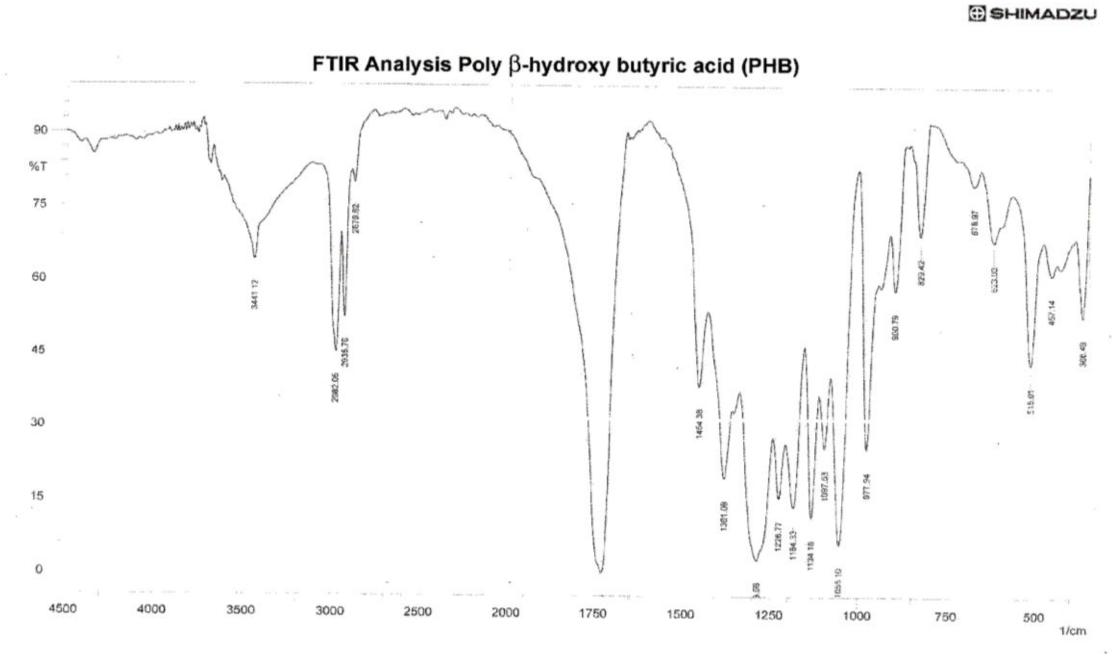


**Figure 8. $^{1}$H NMR Spectrum of PHB produced by *Alcaligenes latus* MTCC 2311**

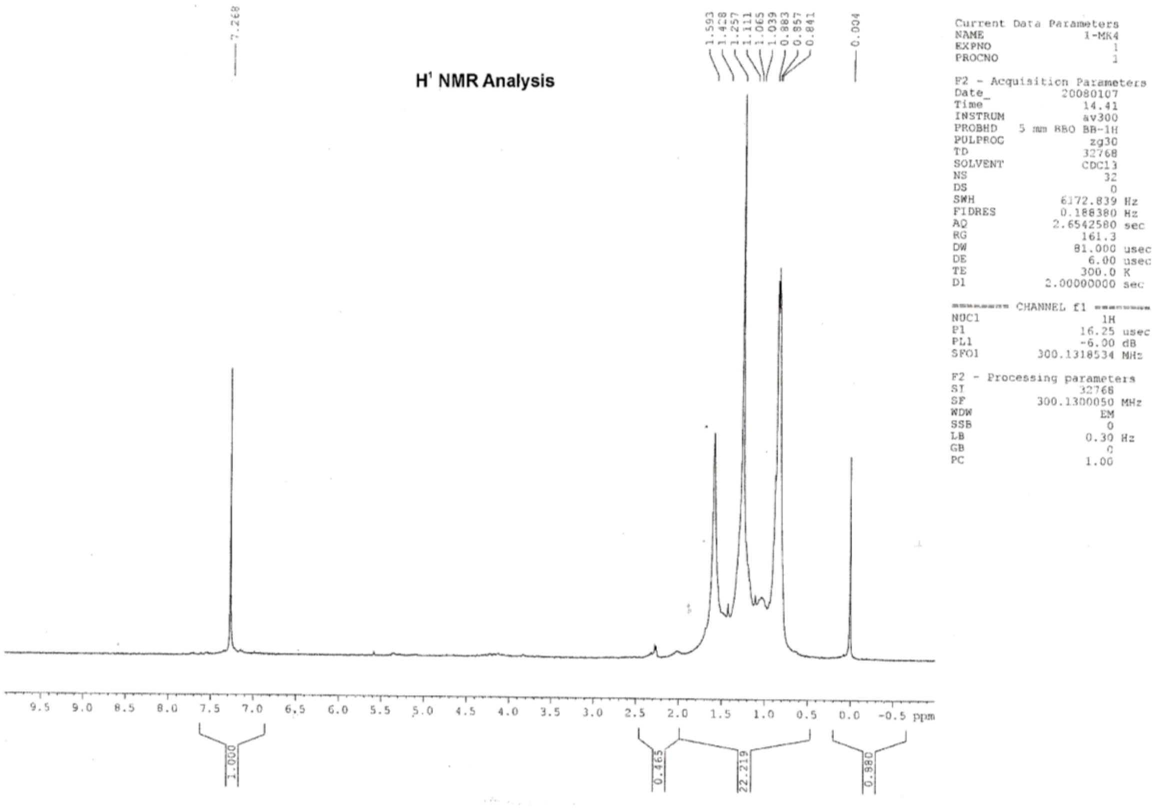

**Table 1. Response surface methodology *yield of PHB by Alcaligenes latus* MTCC 2311**

| Run | Trial | Sago waste | Seasame oil | Molasses | Paper waste | PHB production (g/litre) |
|---|---|---|---|---|---|---|
| 14 | 1 | 2 | -2 | 2 | 2 | 2.17 |
| 12 | 2 | 2 | 2 | -2 | 2 | 2.17 |
| 23 | 3 | 0 | 0 | 0 | -4 | 2.25 |
| 18 | 4 | 4 | 0 | 0 | 0 | 2.25 |
| 7 | 5 | -2 | 2 | 2 | -2 | 2.17 |
| 1 | 6 | -2 | -2 | -2 | -2 | 2.17 |
| 4 | 7 | 2 | 2 | -2 | -2 | 2.17 |
| 6 | 8 | 2 | -2 | 2 | -2 | 3.04 |
| 21 | 9 | 0 | 0 | -4 | 0 | 2.25 |
| 16 | 10 | 2 | 2 | 2 | 2 | 3.21 |
| 25 | 11 | 0 | 0 | 0 | 0 | 2.25 |
| 11 | 12 | -2 | 2 | -2 | 2 | 2.32 |
| 13 | 13 | -2 | -2 | 2 | 2 | 2.52 |
| 26 | 14 | 0 | 0 | 0 | 0 | 2.76 |
| 20 | 15 | 0 | 4 | 0 | 0 | 3.21 |
| 10 | 16 | 2 | -2 | -2 | 2 | 3.04 |
| 15 | 17 | -2 | 2 | 2 | 2 | 3.1 |
| 3 | 18 | -2 | 2 | -2 | -2 | 3 |
| 19 | 19 | 0 | -4 | 0 | 0 | 3 |
| 5 | 20 | -2 | -2 | 2 | -2 | 3 |
| 24 | 21 | 0 | 0 | 0 | 4 | 3 |
| 30 | 22 | 0 | 0 | 0 | 0 | 3 |
| 2 | 23 | 2 | -2 | -2 | -2 | 1.96 |
| 29 | 24 | 0 | 0 | 0 | 0 | 2.28 |
| 17 | 25 | -4 | 0 | 0 | 0 | 2.91 |
| 8 | 26 | 2 | 2 | 2 | -2 | 2.4 |
| 22 | 27 | 0 | 0 | 4 | 0 | 2.92 |
| 9 | 28 | -2 | -2 | -2 | 2 | 2.17 |
| 28 | 29 | 0 | 0 | 0 | 0 | 2.25 |
| 27 | 30 | 0 | 0 | 0 | 0 | 2.43 |