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PII: S1369-703X(12)00352-X
DOI: doi:10.1016/j.bej.2012.12.015
Reference: BEJ 5629

To appear in: *Biochemical Engineering Journal*

Received date: 23-9-2012
Revised date: 6-12-2012
Accepted date: 19-12-2012

Please cite this article as: R. Sindhu, N. Silviya, P. Binod, A. Pandey, Pentose-rich hydrolysate from acid pretreated rice straw as a carbon source for the production of poly-3-hydroxybutyrate, *Biochemical Engineering Journal* (2010), doi:10.1016/j.bej.2012.12.015

Pentose-rich hydrolysate from acid pretreated rice straw as a carbon source for the production of poly-3-hydroxybutyrate

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ABSTRACT

The aim of this work was to evaluate three different bacterial strains for their ability to accumulate poly-3-hydroxybutyrate (PHB) using pentose sugar rich hydrolysate generated from acid pretreated rice straw as the sole carbon source. Out of these, *Bacillus firmus* NII 0830 showed maximum PHB production. Acid pretreated black liquor contained sugars and sugar degradation products such as formic acid, acetic acid, furfural and hydroxymethyl furfural. The bacterium grew in the hydrolysate medum without any detoxification and it could accumulate 1.9 g/l biomass with 1.697 g/l PHB and the PHB content in the cell was 89%. This was the highest value ever reported from *Bacillus* species. The optimum condition for the fermentation media were an inoculum concentration of 6.5%, 90 h of incubation and 0.75% of xylose concentration. The characterization of extracted polymer was carried out by FTIR, ^{1}H and ^{13}C NMR which showed characteristics similar to that of the standard PHB from Sigma.

Key words: Fermentation, microbial growth, biosynthesis, submerged culture, poly-3-hydroxybutyrate, *Bacillus firmus*

1. Introduction

Research on biodegradable polymers has been increasing rapidly during the past few years due to rise in petroleum prices as well as environmental concerns related to plastic pollution. Poly-3-hydroxybutyrate (PHB) can be used as an alternative for plastics as its structural properties are similar to polypropylene, with the advantage of its biodegradability, biocompatibility and can be produced from renewable carbon sources. Depending upon the application, the biopolymer requires different material properties. These properties can be triggered by fine tuning of the composition of PHB during the biosynthesis. One of the major obstacles in the partial

replacement of petrochemical derived plastics by the biopolymer is the cost difference between the PHB and petrochemical derived plastics [1]. The cost of carbon substrate used in fermentation accounts for more than 50% of the production [2]. Therefore the production of PHB utilizing the cheaply available agro-residues and waste stream from various industries may contribute significantly to lower manufacturing costs. From an ecological point of view, they are renewable and from an economic point-of view, many of the waste co products being studied are derived from surplus or low-cost processing streams.

Several reports are available for the production of PHB using carbon source from waste materials. Waste by-products such as glycerol as well as low quality fatty acid esters from biodiesel industry, residues form bioethanol factories can be utilized for the production of biopolymer. Several reports are published on PHB production utilizing biodiesel industry generated crude glycerol [3, 4], residual waste cooking oil and other lipid wastes [5], waste tallow and waste plant oils [6], olive oil mill effluent [7], olive mill waste water ie. Alpechin [8] and whey from the dairy industry [9]. However, few reports are available on utilizing hemicellulosic hydrolysate from forest biomass [10, 11]. Yu and Stahl [12] reported biopolyester synthesis using bagasse hydrolysate by *Ralstonia eutropha*. The initial studies showed an inhibition for PHB synthesis which can be overcome by increasing inoculum concentration, diluted hydrolyzate or utilizing a tolerant strain. To the best of our knowledge, there is no report available on utilization of hemicellulose rich acid hydrolysate from rice straw for PHB production.

Mild acid pretreatment of lignocellulosic biomass removes hemicelluloses in the aqueous solution along with other organic and inorganic reaction byproducts constitute the black liquor.

The utilization of black liquor for the production of PHB makes the process economic and environmentally possible.

One of the major limiting factors for the industrial production of biopolymer is the expense associated with the carbon source and this accounts for about 50% of the overall production cost [2]. Therefore the simplest approach is to select a cheapest as well as readily available carbon source. The hydrolysate from lignocellulosic biomass, which is abundantly available as a renewable source, seems to be promising.

The objective of the present study was to utilize the hemicelluloses rich, mild acid pretreated hydrolysate from rice straw (black liquor) for PHB production using *Bacillus firmus* NII 0830. Media engineering for optimization of process parameters for enhanced production and characterization of the extracted polymer obtained from fermentation were also evaluated.

2. Materials and Methods

2.1. Microorganisms and growth conditions

Bacillus firmus NII 0830, *Bacillus sphaericus* NII 0838 and *Paracoccus denitrificans* were maintained on nutrient agar slants. Inoculums were prepared in LB media. The microbial growth conditions were 24 hours of incubation at 30°C in a rotary shaker at 250 rpm. The seed cultures (1% v/v) were transferred to 100 ml of fermentation media as and when required.

2.2. Acid pretreatment of rice straw

Rice straw received from Hyderabad, Andhra Pradesh, India was used in this study. The raw materials were air dried and milled to a size less than 1 mm. The dried materials were stored at

room temperature until further use. The acid pretreatment of rice straw was carried out with 2% (w/w) H_2SO_4, biomass loading of 15 % w/w and incubation time of 60 minutes. Pretreatment was carried out in a laboratory autoclave at 121˚C, 15 lb pressure. The pretreated sample was made into slurry by the addition of 800 ml of water. After pretreatment the solids and liquids were separated by squeezing through cheese cloth and was neutralized by adding 1N NaOH. The filtrate (black liquor) was used as such in the fermentation media as carbon source.

2.3. Compositional analysis of black liquor

Black liquor was analyzed for sugars and inhibitors using Bio-Rad Aminex HP-87H HPLC column using RI detector [13].The composition of BL is shown in Table -1.

2.4. Primary screening of organisms for PHB production

Primary screening of various microorganisms for PHB production were carried out in submerged culture condition using black liquor (BL) containing 0.5% xylose supplemented with 0.4% $(NH_4)_2SO_4$ and 0.08% KH_2PO_4 salts. *Bacillus firmus* NII 0830, *Bacillus sphaericus* NII 0838 and *Paracoccus denitrificans* were inoculated (1% v/v) in BL media and incubated at 30˚C for 72 hours and total PHB production were analyzed. Highest PHB accumulating strain was selected for further studies.

2.5. Estimation of pentose sugar

Estimation of pentose sugar concentration was carried out by Orcinol method [14].

2.6. PHB assay

Assay of PHB was carried out by the method of Law and Slepecky [15]. After fermentation, the media was centrifuged and the pellet was lyophilized and digested with 30% sodium hypochlorite solution at 37°C for 30 minutes. The sample was centrifuged at 8,000 rpm for 30 minutes and washed with distilled water, acetone, methanol and chloroform. The supernatant was collected and vaporized. It was further treated with concentrated H_2SO_4 and incubated at 100 °C for 10 minutes. Absorbance was taken at 235 nm using a UV-Visible spectrophotometer (Shimadzu UV-1601, Japan) with crotonic acid as standard.

2.7. Optimization of process parameters for PHB production

A Plackett - Burman [16] experimental design was employed for the identification of significant parameters that influence PHB production under submerged culture condition. The variables chosen include inoculum age, inoculum concentration, agitation, temperature, incubation time and xylose concentration. The experiment consists of 20 runs. The results were analyzed by software Minitab 15.

A Box- Behnken design [17] was employed to determine the effects of independent variables on the response and factor interactions with different combinations of variables. Three independent variables – inoculum concentration, incubation time and xylose concentration were studied at three levels -1, 0 and +1 which corresponds to low, medium and high values respectively. The variable input factors were inoculum concentration (5 - 8% v/v), incubation time (60 -120 hours) and xylose concentration (0.5 -1%) and the PHB and biomass yield were the output factors. The results were analyzed by software Minitab 15 (Minitab Inc, USA). Both the linear and quadratic effects and possible interactions of the three variables were calculated.

The statistical as well as numerical analyses of the model were evaluated by analysis of variance (ANOVA) which included Fishers F test, probability P, correlation coefficient R and R^2 which explains the quality of the model.

2.8. Characterization of PHB

2.8.1. FTIR

FTIR analysis was carried out to detect different functional groups in PHB. FTIR spectrum was recorded between 4000 and 400 cm^{-1} using a Shimadzu (Japan) spectrometer with detector at 4 cm^{-1} resolution and 25 scan per sample. Extracted PHB (2mg) and standard PHB (2mg) from Sigma were dissolved in 500µl of chloroform and layered on a NaCl crystal. After evaporation of chloroform, the PHB polymer film was subjected to FTIR [3].

2.8.2. Proton and carbon NMR

The ^{1}H- NMR spectra was recorded at 30 ˚C with a Bruker Advance II 500 MHz spectrometer equipped with ^{1}H and ^{13}C dual probe to study the structural elucidation. Proton spectra were recorded at 300.13 MHz with a spectral width of 2,840 Hz over 16 K data points. A 66˚pulse angle was used. The ^{13}C- NMR spectrum was also recorded at 30 ˚C with a Bruker Advance II 500 MHz spectrometer equipped with ^{1}H and ^{13}C dual probe to study the structural elucidation. 20 mg of the standard and extracted PHB was dissolved in $CDCl_3$ and subjected to analysis.

3. Results and Discussion

3.1. Screening of PHB producing microorganisms

PHB production was carried out by batch fermentation using *Bacillus firmus* NII 0830, *Bacillus sphaericus* NII 0838 and *Paracoccus denitrificans*. BL was used to provide desired

concentrations of xylose ie. (0.5%) in the fermentation medium. Among the three isolates, *Bacillus firmus* NII 0830 proved to be the best candidate for PHB production from BL as sole carbon source and thus it was selected for further study. The results indicate that *Bacillus firmus* NII 0830 showed a total PHB productivity of 0.16 g/l from a total biomass of 0.35 g/l where as *Bacillus sphaericus* NII 0836 and *Paracoccus denitrificans* showed a total PHB productivity of 0.08 g/l and 0.04 g/l from a total biomass of 0.225 g/l and 0.221 g/l respectively. Deepthi et al. [4] observed PHB production from *Bacillus firmus* NII 0830 utilizing biodiesel industry generated crude glycerol as sole carbon source without any pretreatment. This indicates the potential of *Bacillus firmus* NII 0830 to grow in presence of salts, methanol and fatty acid esters. Similarly in the present study *Bacillus firmus* NII 0830 produced PHB utilizing pentose stream generated from acid pretreatment of rice straw without any detoxification indicating the potential of this strain to grow and produce PHB under stress conditions. The utilization of inexpensive substrates for the production makes the process economically sustainable.

3.2. Optimization of process parameters for PHB production

Optimization of process parameters influencing the production of PHB and biomass were carried out using statistical design. Effect estimate on PHB yield based on Plackett- Burman design indicate inoculums concentration, incubation time and xylose concentrations is having a positive control and inoculums age, incubation temperature had a negative impact on PHB production (data not shown).

The objective of the experimental design was to optimize process parameters for maximum PHB yield. Since step wise optimization by one parameter at a time cannot examine all the possible combinations of independent variables, statistical experimental design tools for optimization are

important. Experimental design and experimental PHB and biomass yields are presented in Table 2. Polynomial equation for the model used was as below: PHB (g/l) = $1.458 + 0.092X_1 + 0.353X_2 + 0.068X_3 – 0.146\ X_1^2 – 0.321\ X_2^2 – 0.0978X_3^2$

$– 0.060\ X_1X_2 + 0.128\ X_1X_3 – 0.013\ X_2X_3$ Eq (1)

Where X_1, X_2 and X_3 are inoculum concentration, incubation time and xylose concentration respectively.

Biomass (g/l) = $1.896 + 0.016X_1 + 0.475X_2 - 0.013X3 – 0.009\ X_1^2 – 0.252\ X_2^2 + 0.035X_3^2$

$– 0.065\ X_1X_2 - 0.012\ X_1X_3 – 0.035\ X_2X_3$ Eq (2)

Where X_1, X_2 and X_3 are inoculum concentration, incubation time and xylose concentration respectively.

Response surface curves were plotted to find out the interaction of variables and to determine the optimum level of each variable for maximum response. Surface plot showing the interaction between a pair of factors on PHB as well as biomass yield are given in Figs 1a – c.

The effect of incubation time and inoculum concentration on PHB yield when the other factor is kept at the center point is shown in Fig 1a. At low levels of inoculum concentration and low levels of incubation time the PHB yield was low. Maximum PHB yield (1.5g/l) was observed with middle level of inoculum concentration ie. in the range of 6.0 - 7.5% and high level of incubation time (100 -120 hrs). Jiang et al. [18] reported an optimum incubation time of 4 days for PHB production by *Pseudomonas fluorescens* A2a5. PHB concentration of 22g/l was obtained when batch cultivation was conducted at 25˚C. Biosynthesis of PHB takes place either in the logarithmic phase or in the stationary phase. The long duration of incubation time for PHB

accumulation indicate that the PHB synthesis might be occurring during stationary phase of bacterial growth and it requires limited nutrients. An identical observation was reported by Pal et al. [19] for PHB production by *Bacillus thuringensis* IAM 12077.

The interaction of xylose concentration and incubation time on PHB yield is presented in Fig 1b. At low levels of incubation time the PHB yield was low. This surface plot explains that high level of incubation time (100-120 hrs) and low to high level of xylose concentration (0.5 -1%) gives maximum PHB (1.45g/l). An identical observation was earlier reported for *Pseudomonas pseudoflava* by Bertrand et al. [10] utilizing xylose and arabinose in a concentration of 1.0 – 1.2 g/l. Utilization of xylose as primary carbon source were earlier reported by Ramsay et al. [20] and Young et al. [21] in *Burkholderia* species. Carbon source serve three important functions – a source for biomass synthesis, an energy source for biosynthesis and cell maintenance and for PHB depolymerization [22]. There have been few reports for the production of PHB from a single carbon source. Composition of the carbon substrate as well as utilization of suitable strain of bacterium controls the production of co-polymers of PHB [23]. Under nutrient (phosphorous and nitrogen) limited conditions *Bacillus firmus* NII 0830 uses excess carbon sources for biosynthesis of PHB. An identical observation was earlier reported by Yu and Stahl [12] for PHA production by *Ralstonia eutropha*.

Fig 1c indicates PHB yield as a function of xylose concentration and inoculum concentration. High level of inoculum concentration (7.0 – 8.0% v/v) and high level of xylose concentration (0.8 -1%) gave maximum PHB yield (1.4g/l). PHA biosynthesis from the hemicelluloses fraction of poplar wood was reported by Bertrand et al. [10] with *Pseudomonas pseudoflava* ATCC 33668 where the production rate was low and this may be due to higher substrate utilization for maintenance of energy supply by the organisms when they are cultivated on pentoses instead of

hexoses. A contrary observation was reported by Keenan et al. [11] for PHA synthesis by *B. cepacia* grown in presence of 2.2% (w/v) xylose and different concentrations of levulinic acid. Growth as well as PHB accumulation was enhanced by increasing concentrations of levulinic acid to 0.52% (w/v). Ramsay et al. [20] demonstrated improvement in PHB accumulation using detoxified hemicellulosic hydrolysate using *P. cepacia* ATCC 17759 which can grow and produce PHB from xylose up to 60% of the cell dry mass under nitrogen limiting conditions. Yu and Stahl [12] reported PHA synthesis by *Ralstonia eutropha* using bagasse hydrolyzate. At low inoculum concentration (1.2 g/l), no PHA was formed and accumulated by the cells in the hydrolyzate, even though acetate is available for direct biosynthesis of PHB.

The ANOVA of response for PHB is shown in Table - 3. The R^2 value explains the variability in the PHB yield associated to the experimental factors to the extent of 74.22%.

Though there were few reports on PHB production utilizing pentoses, in most reports either pure xylose or detoxified samples were used [11]. BL used in the present study contained inhibitors like furfural (0.11 g/l), hydroxymethylfurfural (0.02 g/l), formic acid (0.03 g/l) and acetic acid (4.87 g/l). The findings of the present study reveal the potential of *Bacillus firmus* NII 0830 to produce PHB using BL as carbon source without any detoxification as well as minimal nutrients. This provides a breakthrough for the cost–effective production of biopolymer.

The most favorable condition found in the considered range of PHB production were inoculums concentration (6.5% v/v), incubation time (90 hrs) and xylose concentration (0.75%) with 89% PHB accumulation within the bacterial biomass. This is the highest value ever reported form *Bacillus* species. Sangkharak and Prasertsan [24] reported PHB content of 95.4% of the biomass from a mutant strain of *Rhodobacter sphaeroides*.

For the validation of the model, three confirmation experiments were performed within the range of levels defined previously. Correlation analysis was performed on the actual responses and predicted values for each solution and the correlation coefficient was found to be 0.943, hence the empirical models developed were reasonably accurate. The details are shown in Table 4.

3.3. *Characterization of PHB produced from Bacillus firmus NII 0830*

3.3.1. FTIR

FTIR spectrum of the extracted polymer from *Bacillus firmus* NII 0830 show peaks at 1724 cm^{-1} and 1279 cm^{-1} corresponding to specific rotations around carbon atoms (Fig 2). The peak at 1724 cm^{-1} corresponds to C=O stretch of the ester group present in the molecular chain of highly ordered crystalline structure [25]. There was a strong adsorption band at 1279 cm-1 which is characteristic for ester bonding. Other adsorption bands at 1379, 1454, 2928, 1724 and 3750 cm-1 for - CH_3, -CH_2, -CH, C=O, and O-H groups respectively [26].

3.3.2. NMR analysis

The ^{1}H NMR spectra obtained from PHB samples produced from BL, is compared with the commercial PHB (Sigma-Aldrich, USA). Both spectra were found to match perfectly with each other. The peaks observed in the spectra coincide, corresponding to the different types of carbon atoms in the PHB structure (Fig 3). The ^{13}C NMR spectrum was also recorded at room temperature with a Bruker Advance II 500 MHz spectrometer. The ^{13}C NMR spectrum obtained from PHB samples produced from BL is compared with commercial PHB (Sigma-Aldrich, USA). The spectrum shows a doublet at 1.29 ppm which is attributed to the methyl group coupled to one proton. The doublet of quadruplet at 2.57 ppm is attributed to the methylene

group adjacent to an asymmetric carbon atom bearing a single atom. The multiplet at 5.27 ppm is characteristic of methylene group. Two other signals are observed, a broad one at 1.56 ppm which is due to water and another one at 7.25ppm attributed to the solvent used i.e. chloroform (Fig 3).

4. Conclusions

The present study shows that hemicellulose rich hydrolysate from mild acid pretreated rice straw can be utilized in fermentation media for the synthesis of PHB using *Bacillus firmus* NII 0830. The results indicate that the optimum conditions for PHB production were inoculum concentration of 6.5% v/v, incubation time of 90 hrs and xylose concentration of 0.75%. Under these optimized conditions *Bacillus firmus* NII 0830 produced the highest amount of PHB (89% of biomass). This is the highest value ever reported from *Bacillus* species. Fermentative production of PHB using BL as sole carbon source revealed that acid pretreated hydrolysate without any detoxification can be used for PHB production by *Bacillus firmus* NII 0830. To the best of our knowledge this is the first report on utilization of pentose rich hydrolysate from mild acid pretreated rice straw as a sole carbon source for the production of biopolymer, PHB. However further research need to be carried out to develop a suitable and efficient downstream processes as well as to reduce the energy consumption and carbon dioxide emissions.

Acknowledgements

The authors are grateful to Department of Biotechnology, Government of India and Council of Scientific and Industrial Research (CSIR), New Delhi for financial support.

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Table 1 Composition of black liquor

Component	Concentration (g/L)
Glucose	1.01
Xylose	22.56
Arabinose	0.48
Formic Acid	0.03
Acetic Acid	4.87
Furfural	0.11
Hydroxymethyl Furfural	0.02

Table 2 PHB and biomass yields from individual runs of the RSM design

Run				Total	
	Inoculum conc	Incubation time	Xylose conc	Biomass(g/l)	Total PHB (g/l)
1	6.5	60	1	1.2	0.672
2	6.5	90	0.75	1.86	0.997
3	6.5	120	0.5	2.23	1.43
4	8	120	0.75	2.03	1.25
5	5	90	1	1.9	1.03
6	8	90	0.5	1.97	1.14
7	6.5	120	1	2.11	1.39
8	8	60	0.75	1.24	0.701
9	5	120	0.75	2.16	1.4
10	6.5	60	0.5	1.18	0.66
11	5	90	0.5	1.88	0.998
12	5	60	0.75	1.11	0.61
13	8	90	1	1.94	1.687
14	6.5	90	0.75	1.93	1.68
15	6.5	90	0.75	1.9	1.697

Box- Behnken experimental design at three levels for inoculum concentration (5, 6.5 and 8 % v/v), incubation time (60, 90 and 120 hrs) and xylose concentration (0.5, 0.75 and 1%)

Table 3 Analysis of Variance for PHB

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	1.63943	1.63943	0.18216	1.98	0.234
Linear	3	1.10539	1.10539	0.36846	4.01	0.085
Square	3	0.45254	0.45254	0.15085	1.64	0.293
Interaction	3	0.08150	0.08150	0.02717	0.30	0.828
Residual Error	5	0.45978	0.45978	0.09196		
Lack-of-Fit	3	0.14085	0.14085	0.04695	0.29	0.830
Pure Error	2	0.31893	0.31893	0.15946		
Total	14	2.09921				

S = 0.303242 PRESS = 2.97121

R-Sq = 78.10% R-Sq (pred) = 0.00% R-Sq (adj) = 38.67%

Table 4 Model predicted value for PHB yield at optimum condition

Inoculum concentration	Incubation time (hrs)	Xylose conc	PHB yield (g/l)	
			Predicted	Experimental
6.5	90	0.75	1.23	1.68
6.5	120	1	1.29	1.39
5	60	0.75	0.55	0.61

HIGHLIGHTS

- First report on biopolymer production from mild acid pretreated rice straw.
- *B. firmus* can grow in hydrolysate media without detoxification.
- Maximum PHB production was 1.697 g/l from 1.9 g/l biomass.
- Highest value (89% of biomass) reported from *Bacillus* species.

Figure captions

Fig. 1 Surface plots of PHB vs. (a) inoculum concentration and incubation time (b) xylose concentration and incubation time (c) xylose concentration and inoculum concentration

Fig. 2 FTIR spectrum of PHB extracted from *Bacillus firmus* NII 0830

Fig. 3 ^{1}H NMR spectrum of (a) Standard PHB (Sigma – Aldrich) and (b) PHB extracted from *Bacillus firmus* NII 0830; Inset: ^{13}C NMR spectrum of (a) Standard PHB (Sigma – Aldrich) and (b) PHB extracted from *Bacillus firmus* NII 0830

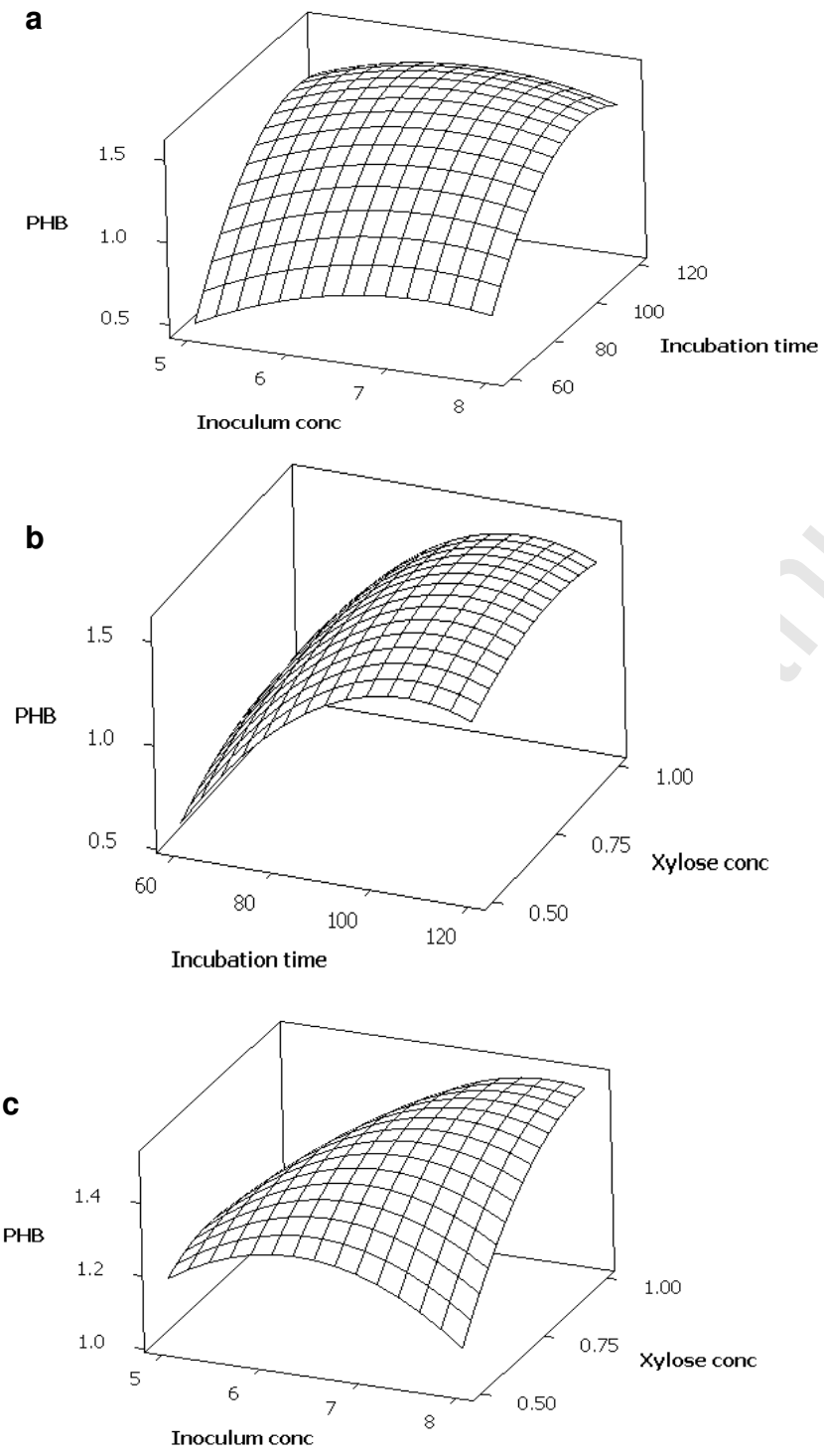

Fig. 1

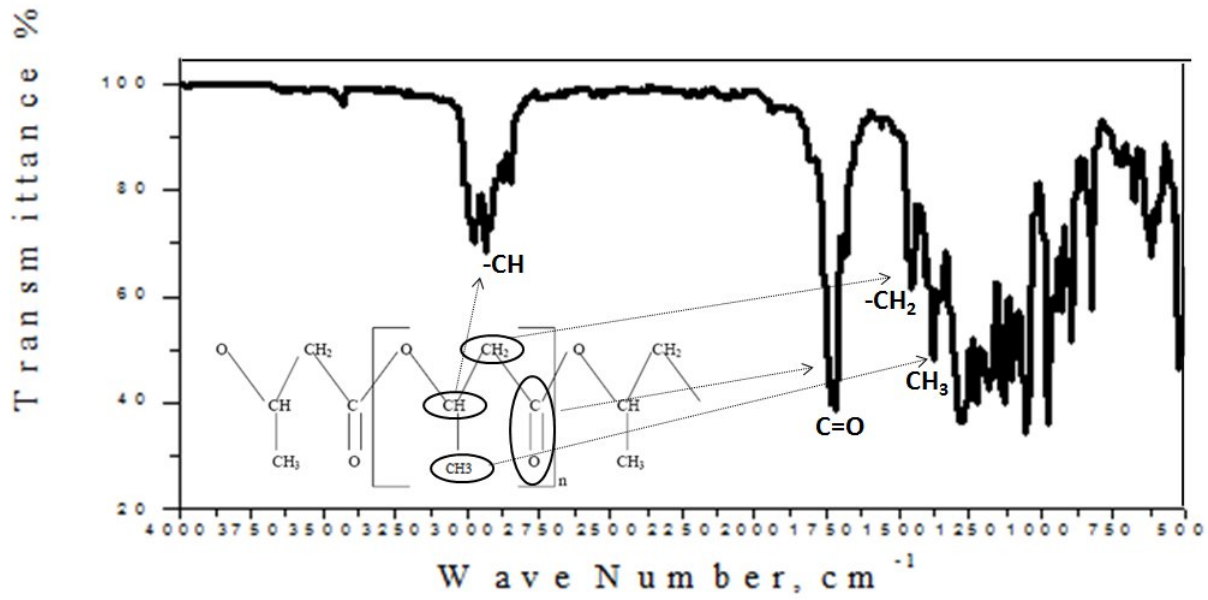

Fig. 2

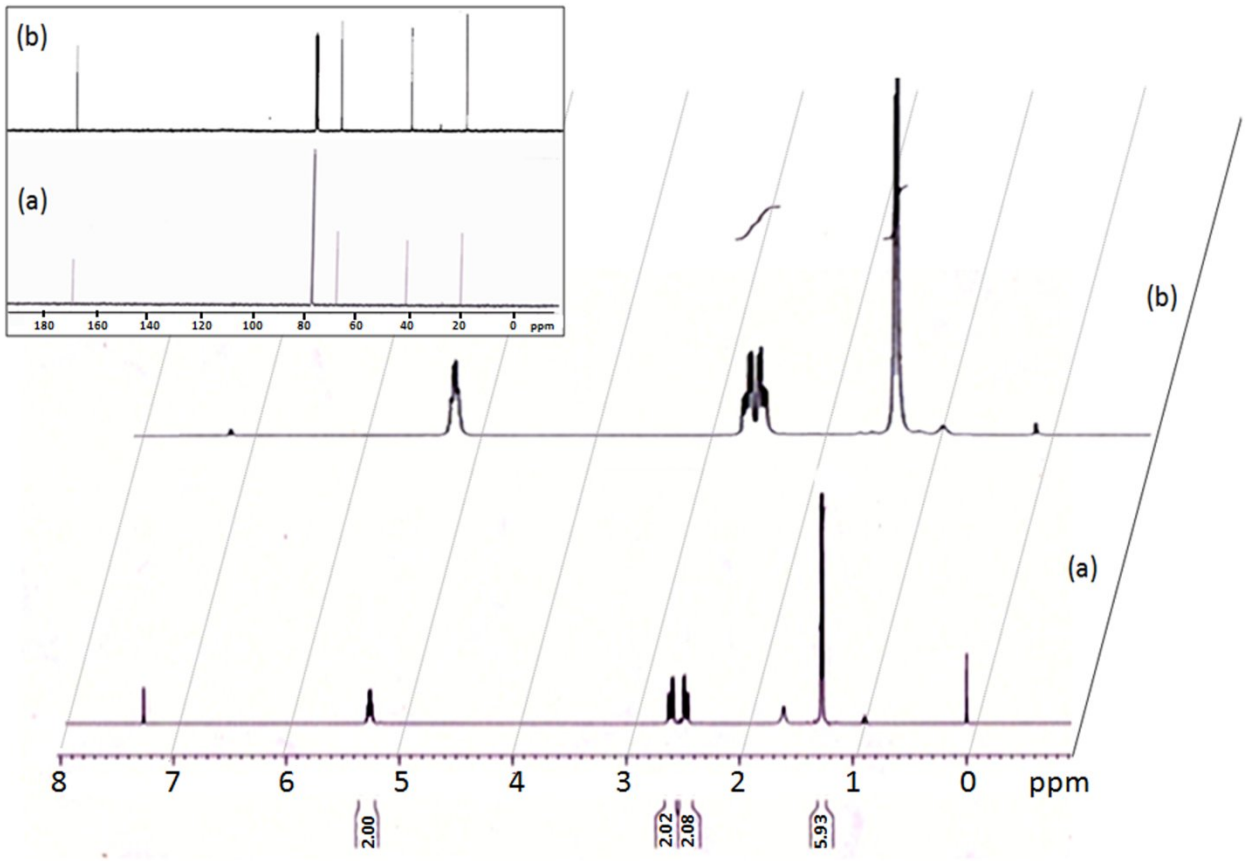

Fig. 3