Poly-3-hydroxybutyrate (PHB) production from alkylphenols, mono and poly-aromatic hydrocarbons using Bacillus sp. CYR1: A new strategy for wealth from waste

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Abstract

In the present study five different types of alkylphenols, each of the two different types of mono and poly-aromatic hydrocarbons were selected for degradation, and conversion into poly-3-hydroxybutyrate (PHB) using the *Bacillus* sp. CYR1. Strain CYR1 showed growth with various toxic organic compounds. Degradation pattern of all the organic compounds at 100 mg/l concentration with or without addition of tween-80 were analyzed using high pressure liquid chromatography (HPLC). Strain CYR1 showed good removal of compounds in the presence of tween-80 within 3 days, but it took 6 days without addition of tween-80. Strain CYR1 showed highest PHB production with phenol (51±5%), naphthalene (42±4%), 4-chlorophenol (32±3%) and 4-nonylphenol (29±3%). The functional groups, structure, and thermal properties of the produced PHB were analyzed. These results denoted that the strain *Bacillus* sp. CYR1 can be used for conversion of different toxic compounds persistent in wastewaters into useable biological polyesters.

Keywords: Poly-3-hydroxybutyrate (PHB); *Bacillus* sp. CYR1; alkylphenols; aromatic compounds; Transmission Electron Microscopy (TEM).

1. Introduction

Alkylphenols are toxic compounds and are considered as endocrine disrupters that can cause various harmful effects, including reproductive effects by imitating the typical female sex hormone, estrogen in aquatic life and in humans (Ogata et al., 2013; Toyama et al., 2011). Therefore, the industrial effluents containing alkylphenols should be properly treated to remove them. In the similar way, substantial amount of phenol and phenolic compounds are discharged through effluents from a variety of industries including leather processing, textiles, pharmaceutical, and oil plants (Haddadi & Shavandi, 2013). Phenol pollution is of great alarm since this chemical is toxic, mutagenic, and carcinogenic. Because of its several toxic effects, removal of phenol from industrial wastewaters before their release is considered to be obligatory (Lu et al., 2012). Polycyclic aromatic hydrocarbons (PAHs) are classified as priority pollutants by the United States Environmental Protection Agency and are ubiquitous in environment. They pose a significant threat to human health due to their mutagenic and carcinogenic properties (Xia et al., 2015). Wastewaters with these organic compounds can be treated mainly by physicochemical processes, but these methods are costly and in-appropriate for large wastewaters volumes (Lobo et al., 2013). Biological degradation has been utilized as alternative, since it has low associated costs and is more effective in degradation of organic compounds (Park et al., 2013).

On the other side, conventionally large fraction of synthetic plastic waste is buried in the soil due to its non-biodegradable property. Bioplastics in the form of polyhydroxyalkanoates (PHA) are an alternative to synthetic plastics and use of these bioplastics could lower the contribution of synthetic plastics to municipal landfills. Poly-3-hydroxybutyrate (PHB) is a type of PHA produced by many species of bacteria that serves as a storage form of carbon and energy (Koller & Braunegg, 2015; Venkateswar Reddy and Venkata Mohan, 2012; Keshavarz and Roy, 2010; Venkata Mohan et al, 2010). PHA are synthesized and stored intracellularly by a wide variety of bacteria such as, *Bacillus* sp., *Pseudomonas* sp., *Serratia* sp., *Azobacterium* and many recombinant strains through the fermentation of sugars, lipids, and volatile fatty acids (Amulya et al., 2014; Venkateswar Reddy et al., 2012, 2014, 2015; Keshavarz and Roy, 2010).

To overcome the above mentioned two problems (*i.e.,* toxic compounds degradation, and plastic disposal) the search for new bacterial strains that are capable of growth on unconventional/toxic substrates and that are more efficient and cost effective PHB producers is an active area of present research. Although the isolation of PHB producing bacteria from environments contaminated with different pollutants has been studied, only a few reports describe the microbial production of PHB using pollutants.

Mono-aromatic hydrocarbons such as styrene and phenyl acetic acid have been evaluated as feedstock for the production of PHA by *Pseudomonas putida* CA-3 (Ward et al., 2005). Nikodinovic et al. (2008) reported degradation and conversion of BTEX (benzene, toluene, ethylbenzene, and xylene) compounds in to PHA using single and defined mixed cultures. Objective of the present study is conversion of toxic compounds into useful bioplastics *i.e.*, PHB using *Bacillus* sp. CYR1. In this study, we examined the capacity of *Bacillus* sp. CYR1 to degrade different toxic compounds. Furthermore the effect of surfactant (Tween-80) addition on degradation of different toxic compounds was evaluated. Degradation of toxic compounds was analyzed using high pressure liquid chromatography (HPLC). The amount of PHB produced was calculated based on bacterial cell dry mass (CDM) using gravimetric method. The PHB granules accumulated inside the bacteria were observed using transmission electron microscopy (TEM). The functional groups, primary and higher-order structures, thermal properties of the produced PHB were confirmed using fourier-transform infrared spectroscopy (FT-IR), ^{1}H and ^{13}C nuclear magnetic resonance (NMR), gel permeation chromatography (GPC), X-ray diffraction (XRD), thermogravimetric analysis (TGA), and differential scanning calorimetry (DSC) respectively.

2. Materials and methods

2.1. Chemicals

We selected three different types of toxic organic compounds, *i.e.*, alkylphenols, mono and poly-aromatic hydrocarbons for degradation studies using the bacteria *Bacillus* sp. CYR1 (DDBJ accession number, LC049103). In alkylphenols we selected five different types of compounds *i.e.*, 4-n-butylphenol (4-BP), 4-sec-butylphenol (4-s-BP), 4-tert-butylphenol (4-t-BP), 4-tert-octylphenol (4-t-OP), and 4-n-nonylphenol (4-NP). In mono-aromatic hydrocarbons phenol (phe) and 4-chlorophenol (4-CP) were selected. In poly-aromatic hydrocarbons we selected two rings and three rings compounds such as naphthalene (naph), and phenanthrene (phen) respectively. All chemicals used were of analytical grade and were purchased from Tokyo Chemical Industry (Tokyo, Japan).

2.2 Culture media

For the growth of *Bacillus* sp. CYR1 nutrient broth, and mineral salt medium (MSM) were used as the media. The MSM contained 5.0 g CH_3COONa, 0.1 g $(NH_4)_2SO_4$, 0.1 g K_2HPO_4, 0.2 g NaH_2PO_4, 0.2 g $MgSO_4$ $7H_2O$, 0.05 g NaCl, 0.05 g $CaCl_2$, 8.3 mg $FeCl_3.6H_2O$, 1.4 mg $MnCl_2.4H_2O$, 1.17 mg Na_2MoO_4 $2H_2O$, and 1 mg $ZnCl_2$ per one liter of deionized water. The pH of the medium was adjusted to 7 and autoclaved before adding to the flasks.

2.3 Toxic compounds degradation and PHB production

Bacillus sp. CYR1 was cultivated in MSM at 30^{o}C by supplementing with different

types of toxic organic compounds, *i.e.*, alkyl phenols (4-BP, 4-s-BP, 4-t-BP, 4-t-OP, and 4-NP), mono-aromatic compounds (phenol, 4-chlorophenol) and poly-aromatic compounds (naphthalene, phenanthrene) at 100 mg/l final concentration. Substrate stock solution (10 g/l) was prepared using methanol. For preparation of naphthalene and phenanthrene stock solution *n*-hexane was used. A loop of *Bacillus* sp. CYR1 strain was initially inoculated into 50 ml of nutrient broth in 500 ml flasks, and kept in shaking incubator under dark condition at 30^{o}C for overnight at 180 rpm. For initial experiments 4 ml (4%v/v) of the overnight grown culture was inoculated into different shake flasks containing 100 ml of MSM with different toxic compounds at 100 mg/l concentration. Further studies were conducted by adding 0.02% Tween-80 as co-substrate and as surfactant in MSM without changing any conditions. The experiments were conducted for 3 days with Tween-80 addition, and for 6 days without Tween-80 addition. Samples were collected at different time intervals, and growth was monitored spectrometrically by measuring the absorbance at 600 nm using UV-spectrometer (UV-1800, Shimadzu, Japan). The degradation patterns of different toxic organic compounds were analyzed on HPLC as described in section 2.4.1. Cultures grown with Tween-80 addition were collected and the PHB was extracted and analyzed at 72 h as described in section 2.4.2. Results reported are the averages of three replicates for all experiments.

2.4 Analysis

2.4.1 Analysis of toxic compounds degradation by HPLC

The degradation patterns of different toxic organic compounds were analyzed on HPLC (Shimadzu) with an SPD-10AV UV/Vis detector at 277 nm and Shim-pack VP-ODS column (4.5×150 mm diameter, particle size 5 μm; Shimadzu, Kyoto, Japan). Samples collected for HPLC analysis were acidified with phosphoric acid (10 %, wv^{-1}) to stop the biological reaction, extracted with an equal volume of 1:1 (v v^{-1}) ethyl acetate, shaken for 3 minutes, and centrifuged at 8,000×g for 10 min. The organic layer was filtered and then analyzed directly by HPLC. Filtered and degassed mobile phase mixture composed of acetonitrile: water (4:1) was used as mobile phase at a flow rate of 1.0 ml/min. The column was maintained at a temperature of 40°C in a thermostat chamber. Conditions maintained for HPLC analysis were described in Table S1 (see supplementary information Table S1). Degradation concentrations were calculated from the area of the curve obtained for 1 mM of the standards. The detection limit was 0.03 mg/l. Recovery of samples was 99.5% in percent. All results were presented as average and standard deviation of the data from three independent experiments.

2.4.2 Extraction and estimation of PHB

Extraction and estimation of PHB was performed following the procedure reported (Law and Slepecky, 1960; Venkata Mohan & Venkateswar Reddy, 2013). The biomass pellet was separated from the substrate by centrifugation (6000×g for 10 min at 10^{o}C)

and the resulting pellet was washed with acetone and ethanol separately to remove unwanted materials. The pellet was suspended in 4% sodium hypochlorite and incubated at room temperature for 3 h. The resulting mixture was centrifuged (6000×g for 10 min at 10^{o}C) and the supernatant was discarded. The pellet with lysed cells after washing simultaneously with acetone and ethanol was dissolved in hot chloroform and was passed through filter paper (0.45 µm pore size) to separate the polymer from cell debris. PHB was separated from the chloroform by filtration, and concentrated using Rotary evaporator (Eyela N-1000) followed by drying to a constant mass. The mass of PHB was calculated gravimetrically. Final PHB production was quantified as cell contents (wt %) and as a concentration (g/l). Chloroform filtrate obtained was precipitated with cold ethanol and used for analysis of functional groups, structure, and thermal properties. Spectrometric analysis was conducted according to the method described by Law and Slepecky, (1960). The extracted PHB was dissolved in 10 ml of sulfuric acid (36 N), and the resultant solution was heated at 100^{o}C on a water bath for 10 min. Addition of sulfuric acid converts the PHB to crotonic acid. The solution was cooled and the absorbance was measured at 235 nm for determining the PHB concentration against a sulfuric acid blank. Standard curve was prepared using pure PHB (Sigma-Aldrich).

2.4.2.1 Electron microscopy

For transmission electron microscopy (TEM) observation, cultured cells (50 µl) were collected in to a 1.5 ml tube, embedded the cells in iPGell (GenoStaff, Japan) according to the manufacturer's instructions. The cells were fixed at 4ºC in 6% glutaraldehyde (TAAB, UK) in 0.1% phosphate buffer (Wako, Japan) at pH 7.2 for 2 days, then rinsed three times for 5 min each at room temperature in the same buffer. After three additional rinses at room temperature, samples were fixed at room temperature in 1% osmium tetroxide (Nisshin EM, Japan) in the same buffer. After three rinses of 5 min each in the same buffer, and in distilled water at the room temperature, dehydration was conducted using ethanol (Wako, Japan) with different concentrations of 40%, 50%, 70% at 4ºC, 90%, 99.5%, and three times of 100% at room temperature for 10 min each. Following three rinses in 100% acetone (Wako, Japan), samples were infiltrated with Epon 812 resin (TAAB, UK) according to the following schedules: 1 hour in 3:1 acetone: resin; 1 hour in 1:1; 1 hour in 1:3, then 1 hour, overnight, and 5 hours in pure resin. Samples were embedded in resin with accelerator and polymerized at 60ºC for 3 days. 70 nm sections were cut using an UC6 ultra microtome (Leica), post-stained with 2% uranyl acetate followed by Reynold`s lead citrate. The sections were imaged in an H-7650 (Hitachi, Japan) operated at 80 kV.

2.4.2.2 Fourier Transform Infrared (FT-IR) spectroscopy

FT-IR spectrum were measured by Attenuated Total Reflection (ATR) method using FT/IR-4100 (Jasco, Japan). Neat PHB samples were pressed on KRS-5 crystal for analysis.

2.4.2.3 Nuclear Magnetic Resonance (NMR) spectroscopy

^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on a JNM-ECA500 NMR spectrometer (JEOL, Japan) at 20°C. Samples of produced PHB and standard PHB (Aldrich) were prepared by dissolving in deuterated chloroform ($CDCl_3$), and then were filtrated with cotton. The signals of tetramethylsilane (TMS) and $CDCl_3$ were used as the standards for chemical shift of ^{1}H and ^{13}C spectra, respectively.

2.4.2.4 Molecular mass determination

Number and weight average molecular mass (M_n and M_w) of standard and sample PHBs were measured using GPC 900-1 (JASCO, Japan) equipped with two Shodex K-806L columns and an RI detector. Chloroform was used as an eluent at 40ºC and polystyrene standards (M_n = 1,680-3,065,000) were employed for calibration.

2.4.2.5 Thermal analysis

Thermogravimetric analysis (TG/DTA7300, Hitachi, Japan) was used to determine the decomposition temperature (T_d) of PHB. PHB powder was added into an aluminum pan and subjected to a heating rate of 20°C/min from 40°C to 600ºC. Differential scanning calorimetry (DSC, DSC-60, SHIMADZU, Japan) was used to characterize the melting temperature (T_m). The temperature range for DSC varied from 40ºC to 240ºC at a

heating rate of 10ºC/min.

2.4.2.6 X-ray diffraction (XRD) analysis

X-ray diffraction spectra of PHB were recorded on RIGAKU RINT2200 Ultima employing nickel-filtered Cu Kα radiation (λ = 1.5406 E; 40 kV, 40 mA) in the 2θ range of 2-40º at 25ºC, using a scan speed of 0.6º/min. The XRD spectra were used to estimate the crystallization degrees of polymers.

3. Results and discussion

In the present study we have used *Bacillus* sp. CYR1 for degradation of different toxic compounds and subsequent conversion of their metabolites into PHB. This is an exceptional bacteria for studying the biosynthesis of PHB under the conditions of stress induced by the toxic compounds.

3.1 Growth curve

3.1.1 Growth curve with alkylphenols

Bacillus sp. CYR1 was cultivated in MSM at 30ºC by supplementing with five different types of alkylphenols (4-BP, 4-s-BP, 4-t-BP, 4-t-OP, and 4-NP), at 100 mg/l final concentration. Among all the compounds, bacteria showed higher growth with 4-t-BP followed by 4-s-BP, 4-NP, and 4-t-OP. Bacteria grown with 4-BP not showed any significant growth, the OD values showed decrement from 0^{th} day to 6^{th} day. *Bacillus* sp. CYR1 not showed significant growth with alkylphenols, so further studies were carried out by adding 0.02% Tween-80 without changing the conditions. *Bacillus* sp.

CYR1 with 0.02% Tween-80 addition showed rapid growth with all alkylphenols. Bacteria showed short lag phase and then it entered in to log phase (Figure S1). Among all the alkylphenols, bacteria grown with 4-s-BP showed highest growth followed by 4-BP, 4-t-BP, 4-NP and 4-t-OP. Bacteria grown with 4-s-BP showed highest growth, at 3rd day it was 2.8 times higher with Tween-80 addition than without Tween-80 addition. The growth of *Bacillus* sp. CYR1 was increased by 3.13 times higher with 4-BP, and 1.4 times with 4-t-BP respectively by the addition of Tween-80 (Figure S1). It clearly indicates the positive effect of Tween-80 on growth of bacteria.

3.1.2 Growth curve with mono and poly-aromatic compounds

Two different types of mono-aromatic compounds *i.e.,* phenol, and 4-chlorophenol at 100 mg/l final concentration were used for *Bacillus* sp. CYR1 growth. Among the two compounds, bacteria showed higher growth with phenol. Bacteria grown with 4-chlorophenol did not showed any significant growth without Tween-80 addition. After addition of Tween-80, bacteria incubated with phenol showed rapid growth (Figure S1). Bacteria at 3rd day showed highest growth, it was increased by 1.18 times after Tween-80 addition. Interestingly bacteria grown with 4-chlorophenol also followed similar type of trend, the growth was enhanced by 2.80 times after addition of Tween-80.

Two different types of poly-aromatic compounds *i.e.,* naphthalene, and phenanthrene were used for the growth of *Bacillus* sp. CYR1. Bacteria with both the compounds

showed similar growth pattern, the OD values showed little increment from 0^{th} day to 6^{th} day. It indicates that *Bacillus* sp. CYR1 cannot degrade and utilize naphthalene and phenanthrene. After addition of Tween-80, bacteria incubated with naphthalene and phenanthrene showed rapid growth (Figure S1). Many authors reported Tween-80 addition will improve the bacterial growth. Brar et al. (2005) reported addition of Tween-80 to non-hydrolyzed sludge resulted in increase in cell and spore count of *Bacillus thuringiensis* by 1.67 and 4 times respectively, maximum specific growth rate (μmax) increased from 0.19 to 0.24 h^{-1}. They indicated that Tween-80 improved *B. thuringiensis* growth in non-hydrolyzed sludge which will have tremendous impact on its use as raw material for fermentation and finally bio-pesticide formulations. Budde et al. (2011) conducted experiments to know the effect of Tween-80 at 0.2% concentration on the growth of *Ralstonia eutropha.* They reported that Tween-80 did not inhibit the growth of *R. eutropha*, the bacteria were able to use this surfactant as a sole carbon source. They reported that Tween-80 cultures showed considerably higher optical densities than the controls (without Tween-80), and Tween-80 can serve as an effective carbon source for *R. eutropha*. Tween molecules contain a fatty acid group. They hypothesized that enzyme esterase may cleave the fatty acid from Tween, allowing it to be used as a carbon source by the bacteria.

3.2 Degradation of toxic organic compounds

3.2.1 Alkylphenols degradation

HPLC results denoted that there is a marked difference on the degradation of different alkylphenols by *Bacillus* sp. CYR1. Among all the compounds, bacteria showed highest degradation of 4-NP (51±4%), followed by 4-t-BP (47±2%), 4-s-BP (41±3%), and 4-t-OP (8±2%) at 6^{th} day of operation (Figure 1). Bacteria were not able to degrade 4-BP (0% degradation) up to 6^{th} day, and it was well supported by growth study results.

Figure 1

Tween-80 addition showed improvement in degradation of alkylphenols by *Bacillus* sp. CYR1 except 4-t-OP, and 4-t-BP (Figure 2). Among all the alkylphenols, bacteria showed highest degradation of 4-BP (92±6%). Interestingly, the degradation efficiency of *Bacillus* sp. CYR1 was 92 times increased by addition of Tween-80. After the 4-BP, bacteria showed highest degradation of 4-s-BP (66±5%), the degradation was increased by 1.6 times after Tween-80 addition. Bacteria showed 54±4% removal of 4-NP, the degradation was increased by 1.05 times after Tween-80 addition. Contrary to this, addition of Tween-80 also did not showed any significant influence on the degradation of 4-t-OP and 4-t-BP. The degradation efficiency of *Bacillus* sp. CYR1 was coordinated with other bacteria according to the existing literature reports. Ogata et al. (2013) reported 100% degradation of 4-BP, 4-s-BP using the bacteria *Sphingobium fuliginis* within 3 days at 75 mg/l concentration. They reported 50% degradation of 4-NP and 4-t-

OP at the concentration of 110 and 103 mg/l using the same bacteria. Toyama et al. (2011) reported 100% degradation of 4-t-OP using the bacteria *Stenotrophomonas* strain IT-1 within 5 days at 1000 mg/l concentration.

Figure 2

3.2.2 Mono-aromatic compounds degradation

Among the two mono-aromatic compounds, *Bacillus* sp. CYR1 showed highest removal of phenol (93±5%), and it did not showed degradation (0%) of 4-chlorophenol (Figure 2). *Bacillus* sp. CYR1 without Tween-80 did not showed significant degradation with 4-chlorophenol, so further studies were carried out by adding 0.02% Tween-80. The degradation of 4-chlorophenol was 76 folds increased after addition of Tween-80. Strain CYR1 with Tween-80 addition showed rapid degradation of phenol *i.e.,* within 3 days it showed 91±5% removal (Figure 2). Haddadi & Shavandi, (2013) reported 100% degradation of phenol using the bacteria *Halomonas. sp* within 7 days at 1100 mg/l concentration.

3.2.3 Poly-aromatic compounds degradation

Among the two poly-aromatic compounds, bacteria showed 26±4% removal of naphthalene. Bacteria did not showed degradation (0%) of phenanthrene, and it was supported by growth study results, the OD values showed decrement from 0^{th} day to 6^{th} day. *Bacillus* sp. CYR1 showed good degradation of naphthalene and phenanthrene after addition of Tween-80, the degradation was increased by 1.42 times and 39 times

respectively (Figure 2). Growth study and degradation results clearly indicated that the strain *Bacillus* sp. CYR1 has marginal degradation capacity of naphthalene and phenanthrene. Tween-80 addition showed significant influence on the growth and degradation of naphthalene and phenanthrene by *Bacillus* sp. CYR1.

Many authors reported about efficiency of bacteria belongs to the *Bacillus* group for degradation of various toxic compounds. Toledo et al. (2006) reported that strains *B. pumilus*, and *B. subtilis* were able to grow and utilize PAH compounds such as naphthalene, phenanthrene, fluoranthene and pyrene. Kuang et al. (2013) reported 62% degradation of phenol at 200 mg/l concentration using *B. fusiformis* within 7 h. Fayidh et al. (2015) reported 43% and 94% degradation of phenol at 200 mg/l concentration using *Bacillus* sp. and *B. cereus* respectively. Devi et al. (2011) reported biodegradation of a crude oil using anaerobic mixed culture and involvement of *Bacillus* group of organisms in degradation process.

3.3 PHB production

PHB was accumulated by *Bacillus* sp. CYR1 when various compounds were supplied to the culture. PHB production and CDM were evaluated at 72 h of time interval for all the compounds. Biomass levels (CDM) varied (0.19 to 1.01 g/l) according to type of substrate (Table 1), and PHB was also accumulated to different levels ranging from 0%

to 51% CDM (Table 1). The best accumulation of PHB was achieved when phenol (51±5%) was used in MSM. Strain CYR1 accumulated the good level of PHB when supplied with other compounds like naphthalene (42±4%), 4-CP (32±3%), 4-NP (29±3%), and 4-BP (24±3%) (Table 1). On the other side strain CYR1 unable to accumulate PHB when supplied with compounds like 4-t-BP and 4-t-OP.

PHB accumulation ability of *Bacillus* sp. CYR1 is carbon source dependent. Although all toxic substrates supported biomass growth to the comparable levels, but some substrates were poorer substrates for the PHB accumulation (Table 1). This may be due to the different bioavailability of these substrates to the medium and different efficiency in utilization of these substrates by the *Bacillus* sp. CYR1. Hoffmann et al. (2000) reported many bacteria can consume a large number of aromatic compounds, but they cannot always accumulate PHB.

Table 1

The possibility to convert mono-aromatic compounds (BTEX, styrene) into PHA has been previously reported using *Pseudomonas putida* strains (Ward et al., 2005; Nikodinovic et al., 2008). Berezina et al. (2015) reported that benzoic acid was found to be a crucial compound for the bioremediation by *Cupriavidus necator,* and this strain transforms benzoic acid to the PHB showed that this bioremediation process is more suitable for the purification of waste streams. Koller & Braunegg, (2015) reported PHB

and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) production by *Cupriavidus necator* strain DSM 545 using animal-based crude glycerol phase (CGP) and the saturated biodiesel share (SFAE).

3.4 Identification of PHB produced by *Bacillus* sp. CYR1

3.4.1 Transmission Electron Microscopy

The ability of the strain to accumulate PHB using phenol (see supplementary information Figure S2) and naphthalene (Figure S3) was investigated under TEM. PHB production was assessed by growing the strains in 500 ml conical flasks containing 100 ml nitrogen and phosphorous limited (100 mg/l) MSM medium with phenol or naphthalene. Bacterial cultures were incubated on a rotary shaker for 3 days at 30°C and 180 rpm. After the incubation period cells were collected and the accumulated PHB was analyzed as described in section 2.4.2.1. A series of thin sections of *Bacillus* sp. CYR1 containing PHB inclusions are shown in Figure S2 &S3. Under electron microscope, cells occurred singly and gradually swollen during polymer accumulation.

3.4.2 Functional groups identification

FT-IR has been demonstrated to be a powerful tool for studying microorganisms and their cell components in intact form. It has been reported that PHB is observable in FT-IR spectra in intact bacteria (Hong et al., 1999). FT-IR is sensitive to local molecular environment; consequently it has been widely applied to reveal the conformational changes of macromolecules during melting and crystallization (Xu et al., 2002). In

addition, FT-IR has been applied to the qualitative studies of PHA (Xu et al., 2002). The FT-IR spectra were analyzed in order to confirm the practically identical structure of commercial PHB with the PHB produced by *Bacillus* sp. CYR1 (see supplementary information Figure S4 A & B). The PHB extracted from *Bacillus* sp. CYR1 had the same C-H and carbonyl bands as standard PHB. The band appeared at 1456 cm^{-1} corresponded to the blending of C-H bond in CH_2 groups including CH_3 groups at 1378 cm^{-1}. The band at 1721 cm^{-1} corresponded to the stretching of the C=O bond, whereas a series of intense bands located at 1000-1300 cm^{-1} corresponded to the stretching of the C-O bond of the ester group. All bands in the sample were identical to that of standard PHB. The methylene C-H stretching vibration near 2925 cm^{-1} also observed. The presence of absorption bands at 1721 cm^{-1} and 1277 cm^{-1} in extracted PHB sample were characteristic of C=O and C-O stretching groups and were identical to PHB.

3.4.3 Structure determination with ^{1}H and ^{13}C NMR

The ^{1}H NMR spectrum of $CDCl_3$ soluble part of PHB extracted from *Bacillus* sp. CYR1 grown with phenol as substrate was measured at 20°C to deduce its chemical structure and primary sequence of polymer chain. Based on their peak positions, splitting patterns and integral ratio of these signals, each peak can be assigned to the protons on methane (5.25 ppm), methylene (2.59-2.48 ppm) and methyl (1.58 ppm) groups as shown in Figure S5A. Figure S5B (see supplementary information Figure S5) shows the ^{13}C

NMR spectrum of $CDCl_3$ soluble part of PHB extracted from *Bacillus* sp. CYR1 grown with phenol as substrate measured at 20°C. These four peaks were assignable to the carbonyl (169.13 ppm), methine (67.59 ppm), methylene (40.76 ppm), and methyl (19.75 ppm) carbon resonances of PHB. Additionally, these spectra were almost the same as PHB standard measured at the same conditions (see supplementary information Figure S6). Therefore, we concluded that PHB with highly stereoregular sequence and single chemical structure as repeating unit, and relatively high molecular mass PHB produced by *Bacillus* sp. CYR1.

3.4.4 Gel permeation chromatography (GPC)

To confirm the PHB produced from *Bacillus* sp. CYR1 was high molecular mass, we measured both number average molecular mass (M_n) and weight average molecular mass (M_w) of thin PHB solvent-cast films using by GPC. M_W and polydispersity index (PDI, M_w/M_n) of PHB produced by *Bacillus* sp. CYR1 is 709 kDa and 2.25, respectively. The PDI of the sample PHB was nearly half to the standard PHB (purchased from Aldrich (PDI: 4.28)), irrespective of the M_w was almost the same value, *i.e.*, 709 and 725 kDa, respectively. These results indicate that *Bacillus* sp. CYR1 can produce PHB with relatively lower dispersity.

3.4.5 Thermal properties evaluation with TGA, DSC, and XRD

Thermogravimetric analysis (TGA) was used to evaluate the thermal stability of

polymers, *i.e.*, decomposition temperature, especially focus on the temperature of 5% weight loss (T_{d5}). Around 7 milligrams of PHB powder was added into an aluminum pan and subjected to a heating rate of 20°C/min from ambient to 600°C (Figure 3). The weight loss of standard PHB started at around 230°C and its T_{d5} was at 274°C, completely decomposed at 310°C. However the starting temperature for weight loss of the PHB grown by *Bacillus* sp. CYR1 was significantly low compared with that of standard PHB, *i.e.*, at around 100°C. In addition, the PHB was completely not decomposed at 600°C, indicating that the PHB extracted from *Bacillus* sp. CYR1 composed of some inorganic material which may came from bacterial dry mass.

Figure 3

Differential scanning calorimetry (DSC) was used to characterize the melting temperature (T_m) of the PHB extracted from *Bacillus* sp. CYR1, and it was compared with standard PHB. The temperature range for DSC varied from 30°C to 250°C at a heating rate of 10°C/min (Figure 4). From the endothermic peaks in each DSC traces, it denoted that PHB extracted from *Bacillus* sp. CYR1 contains the T_m of 171°C, it was matched with standard PHB (T_m, 178°C). From XRD patterns it can be seen that the characteristic peak positions at 2θ of 13, 17, 20, 21, 22, 25 and 27 found in extracted polymer were confirming the presence of PHB (see supporting information Figure S7). However, the peak intensities of extracted PHB were extremely weak due to its low

crystallinity which may influenced by the lower temperature for initial degradation. Nevertheless, based on the results shown above, we concluded that the produced polymer is PHB.

Figure 4

4. Conclusion

Aromatic compounds are widely distributed in nature and cause serious environmental problems if they are not degraded properly. In the present study, the bacteria *Bacillus* sp. CYR1 showed good removal of toxic substrates within three days in the presence of Tween-80. Moreover strain CYR1 successfully converted the different compounds into PHB. Among different compounds CYR1 showed highest PHB production with phenol, naphthalene, and 4-chlorphenol. The ability to degrade a wide range of aromatic substrates and convert their metabolites into PHB will make the *Bacillus* sp. CYR1 as robust bacterium and a potent candidate for the chemo biotechnological conversion of waste to valuable bioplastics.

Acknowledgements

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Table 1: Cell dry mass (CDM) and PHB production from *Bacillus* sp. CYR1. Bacteria were incubated at 100 mg/l concentration of different aromatic compounds and 0.02% Tween-80 in MSM media at 30ºC. For CDM measurement cells were harvested by centrifugation, washed twice with phosphate-buffer and dried for 24 h at 80ºC until a stable weight was reached. PHB was extracted from the CDM using sodium hypochlorite-chloroform extraction method.

Substrate		CDM (g/l)	PHB productivity (mg/g CDM)	PHB production (% CDM)
Alkylphenols	4-BP[1]	0.82	240	24
	4-s-BP[2]	0.74	160	16
	4-t-BP[3]	0.25	ND	ND
	4-t-OP[4]	0.19	ND	ND
	4-NP[5]	0.81	290	29
Poly-aromatic hydrocarbons	Nap[6]	0.91	420	42
	Phen[7]	0.62	180	18
Mono-aromatic hydrocarbons	Phe[8]	1.01	510	51
	4-CP[9]	0.85	320	32

4-BP[1]: 4-butylphenol; 4-s-BP[2]: 4-secondary butylphenol; 4-t-BP[3]: 4-tertiary butylphenol; 4-t-OP[3]: 4-tertiary octylphenol; 4-NP[5]: 4-Nonylphenol; Naph[6]: Naphthalene; Phen[7]: Phenanthrene; Phe[8]: Phenol; 4-CP[9]: 4-chlorophenol

ND: Not detected

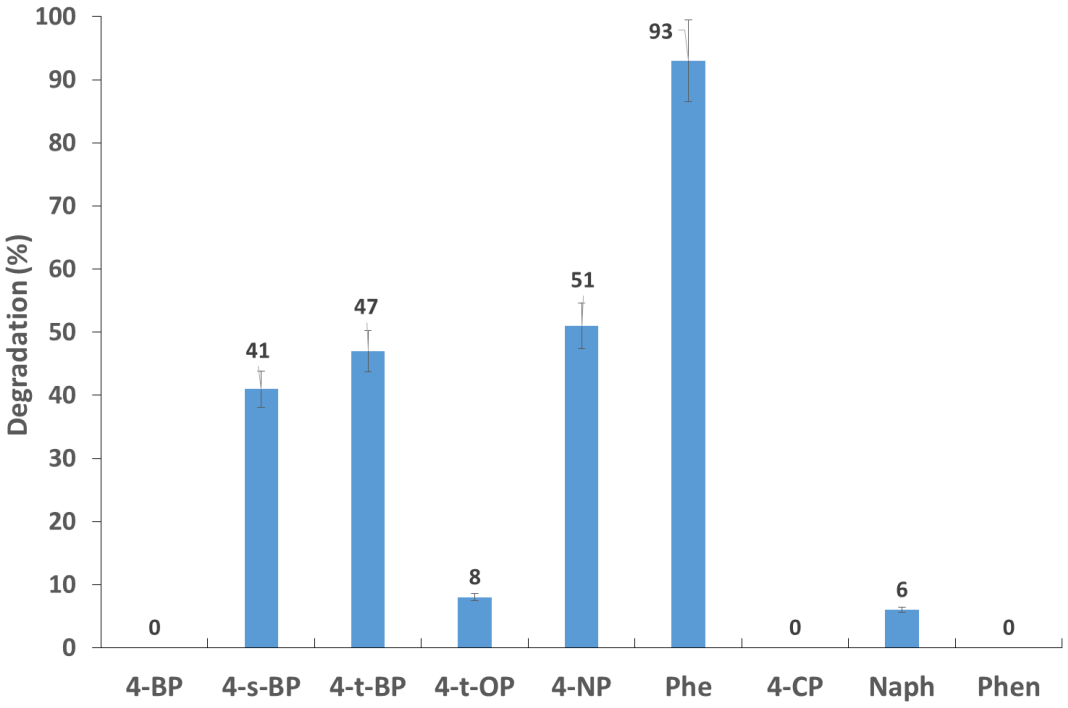
Figure Captions

Figure 1: Alkylphenols, mono-aromatic, and poly-aromatic compounds degradation using *Bacillus sp.* CYR1 without addition of Tween-80 in MSM media.

Figure 2: Alkylphenols, mono-aromatic, and poly-aromatic compounds degradation using *Bacillus* sp. CYR1. Bacteria were incubated at 100 mg/l concentration of different toxic compounds and 0.02% Tween-80 in MSM media at 30^{o}C. Samples were collected at different time intervals and residual concentration of toxic compounds in the medium were analyzed by HPLC.

Figure 3: Thermo gravimetric analysis (TGA) analysis of PHB extracted from *Bacillus* sp. CYR1 cultivated on 100 mg/l phenol in MSM medium.

Figure 4: Differential scanning calorimetry (DSC) of PHB extracted from *Bacillus* sp. CYR1 cultivated on 100 mg/l phenol in MSM medium.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100
90
80
70
60
50
40
30
20
10
0
0
41
47
8
51
93
0
6
0
4-BP
4-s-BP
4-t-BP
4-t-OP
4-NP
Phe
4-CP
Naph
Phen

Fig. 1

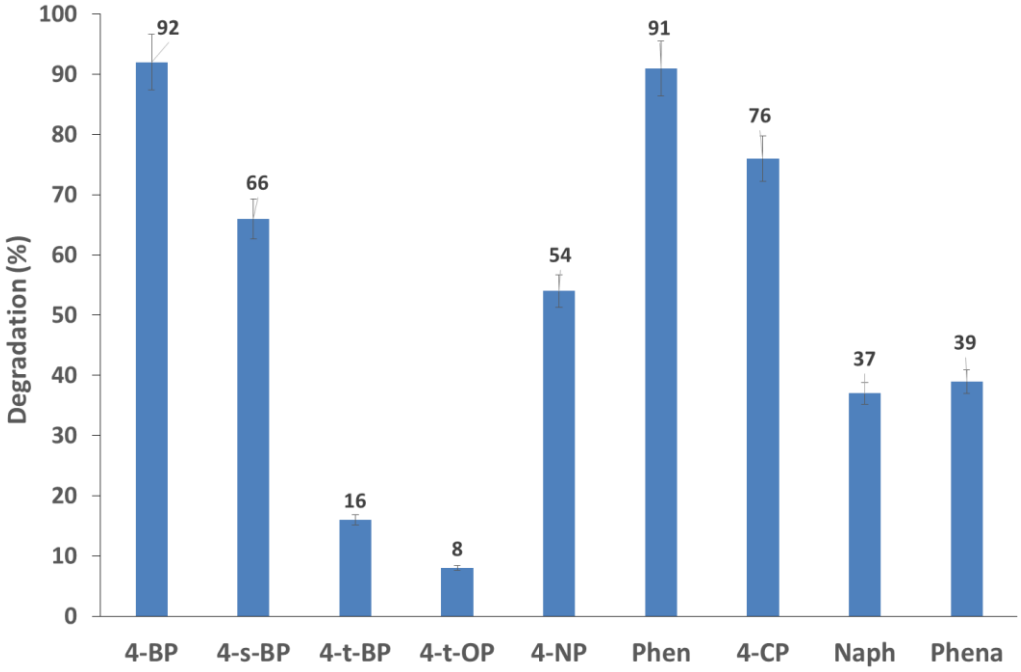
Degradation (%)
100
90
80
70
60
50
40
30
20
10
0
92
66
16
8
54
91
76
37
39
4-BP
4-s-BP
4-t-BP
4-t-OP
4-NP
Phen
4-CP
Naph
Phena

Fig. 2

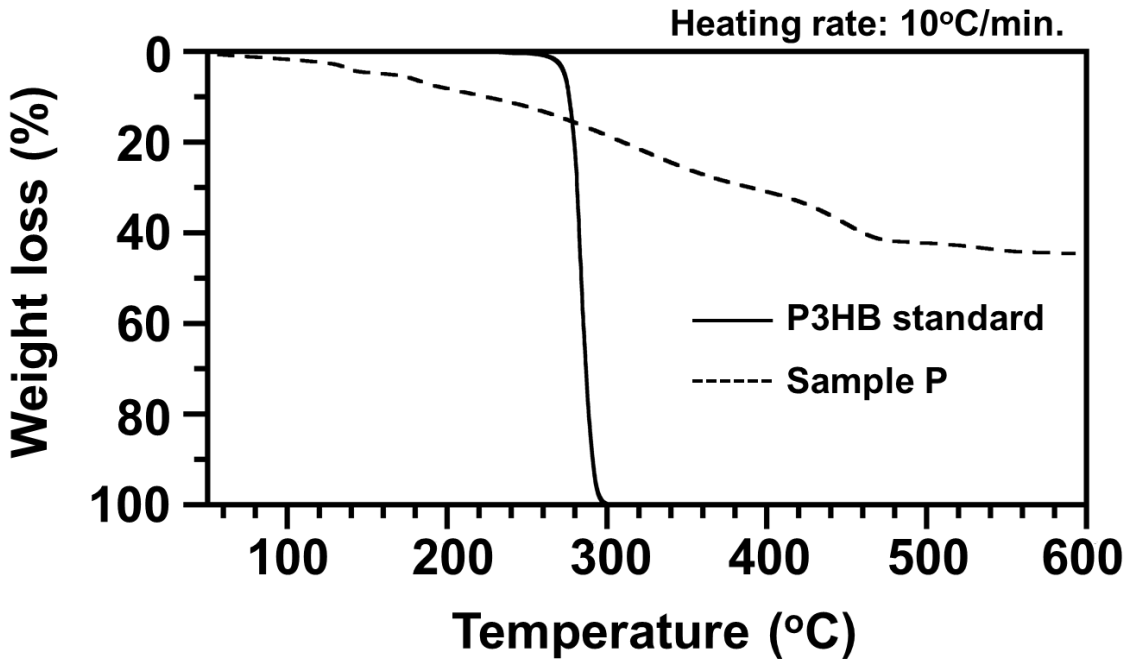

Figure 3

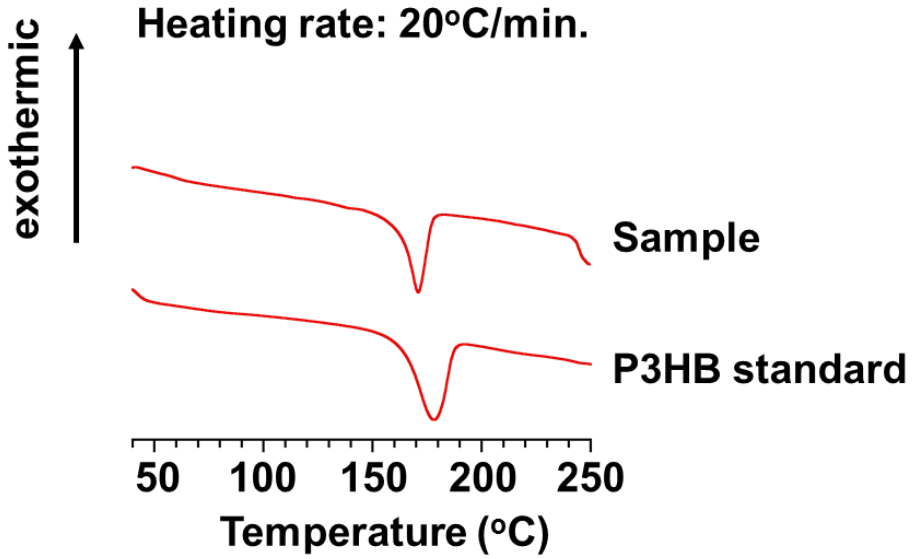

Figure 4

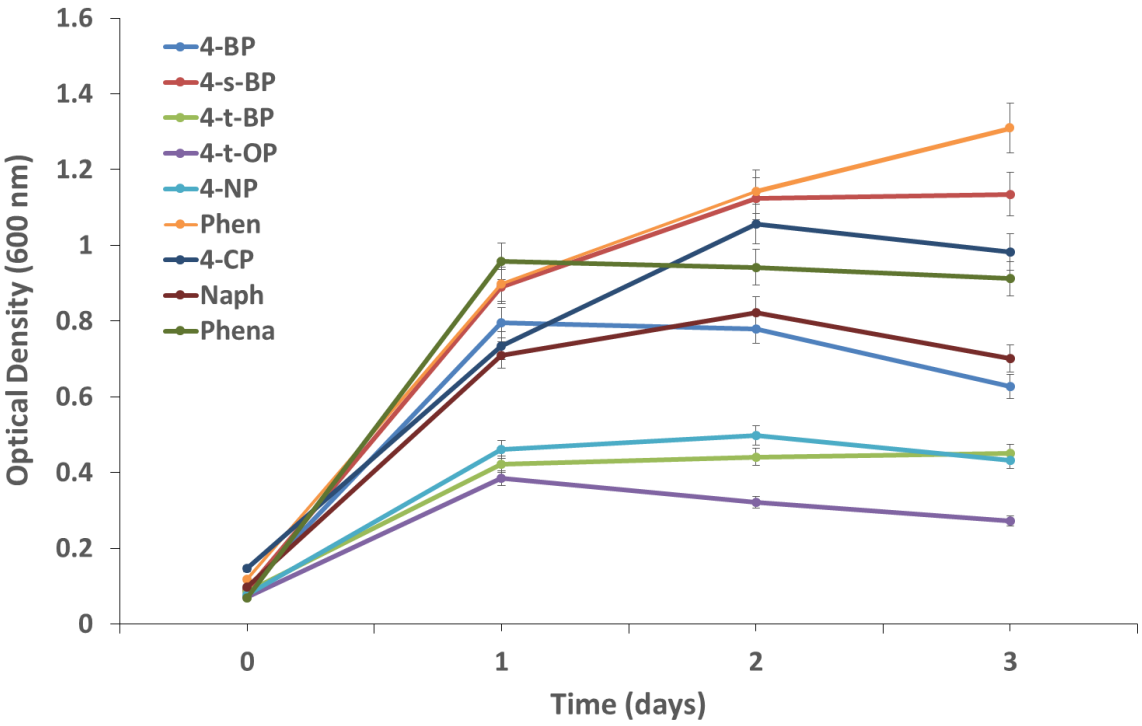

Figure S1: Growth curve of *Bacillus* sp. CYR1with alkylphenols, mono-aromatic, and poly-aromatic compounds. Bacteria at 4% inoculum were incubated at 100 mg/l concentration of different toxic compounds and 0.02% Tween-80 in MSM media at 30°C. The optical density (OD at 600 nm) of each sample was measured at different time intervals.

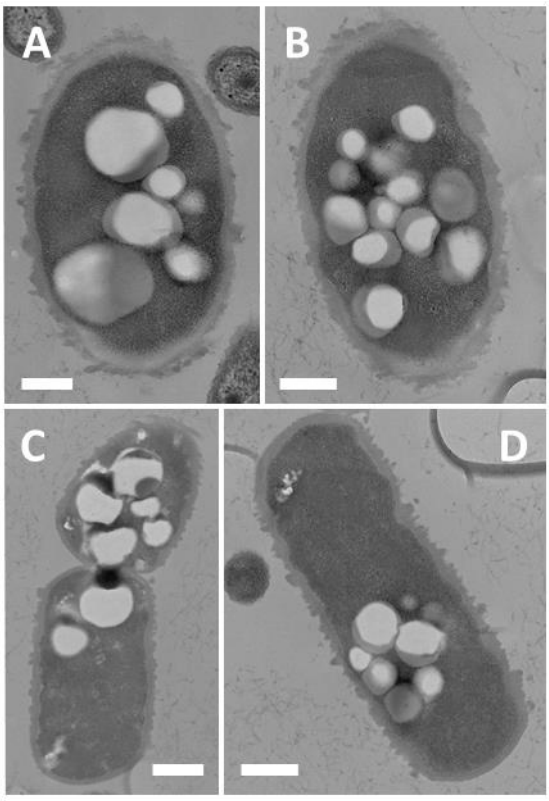

Figure S2: Transmission electron micrograph images of thin sections of *Bacillus* sp. CYR1. Bacteria were grown in MSB medium with phenol as carbon source. Images were taken at 72 h time intervals. Bars: A, B, 0.25 µm; C, D, 0.4 µm

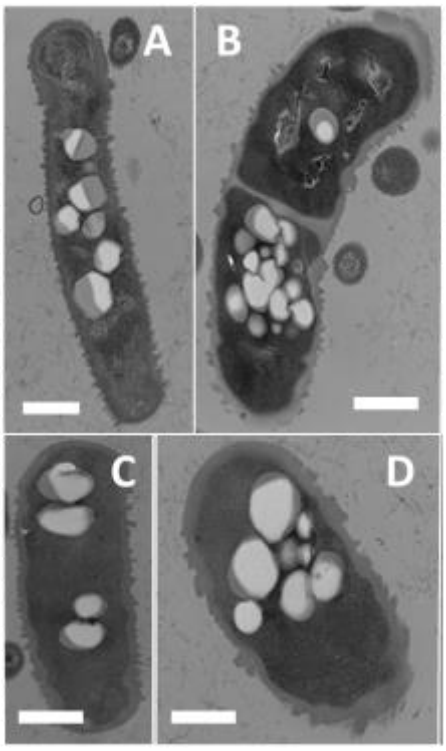

Figure S3: Transmission electron micrograph images of thin sections of *Bacillus* sp. CYR1. Bacteria were grown in MSB medium with naphthalene as carbon source. Images were taken at 72 h time intervals. Bars: A, B, C 0.5µm; D 0.3µm.

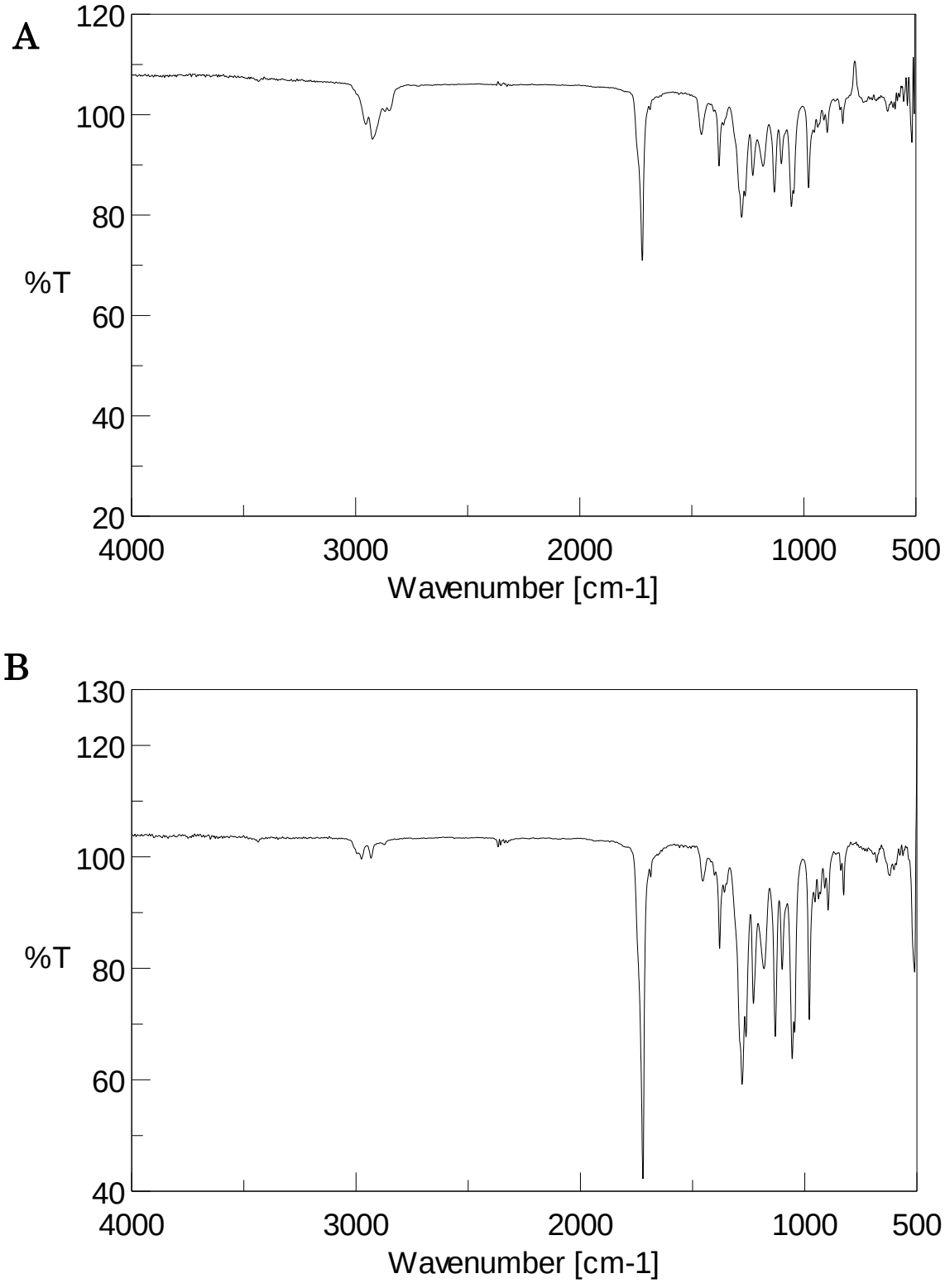

Figure S4: Fourier-transform infrared (FT-IR) spectrum of (A) pure PHB extracted from *Bacillus* sp. CYR1; and (B) standard PHB

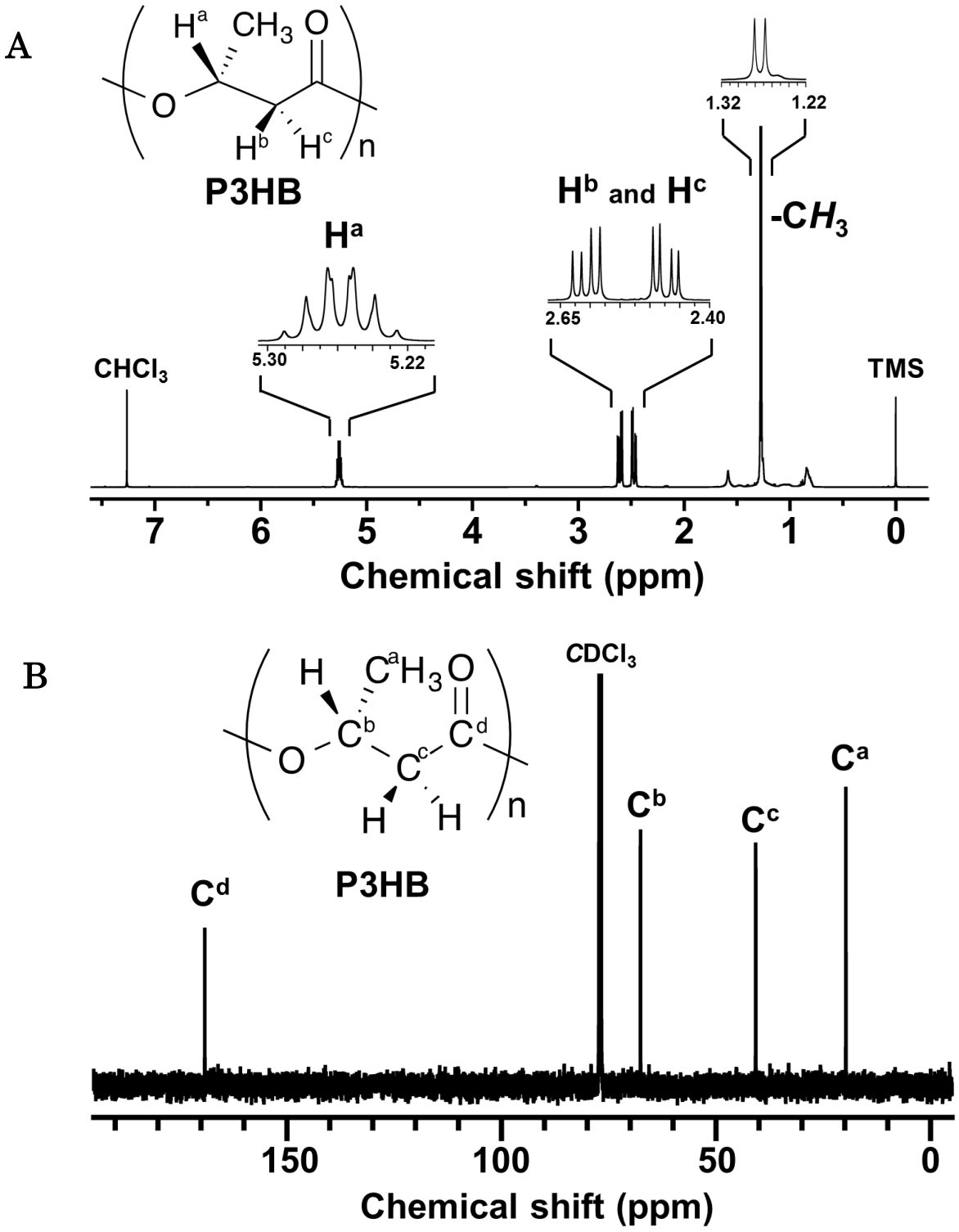

Figure S5: (A) ^{1}H and (B) ^{13}C NMR spectra of PHB extracted from *Bacillus* sp. CYR1 cultivated on 100 mg/l phenol in MSM medium.

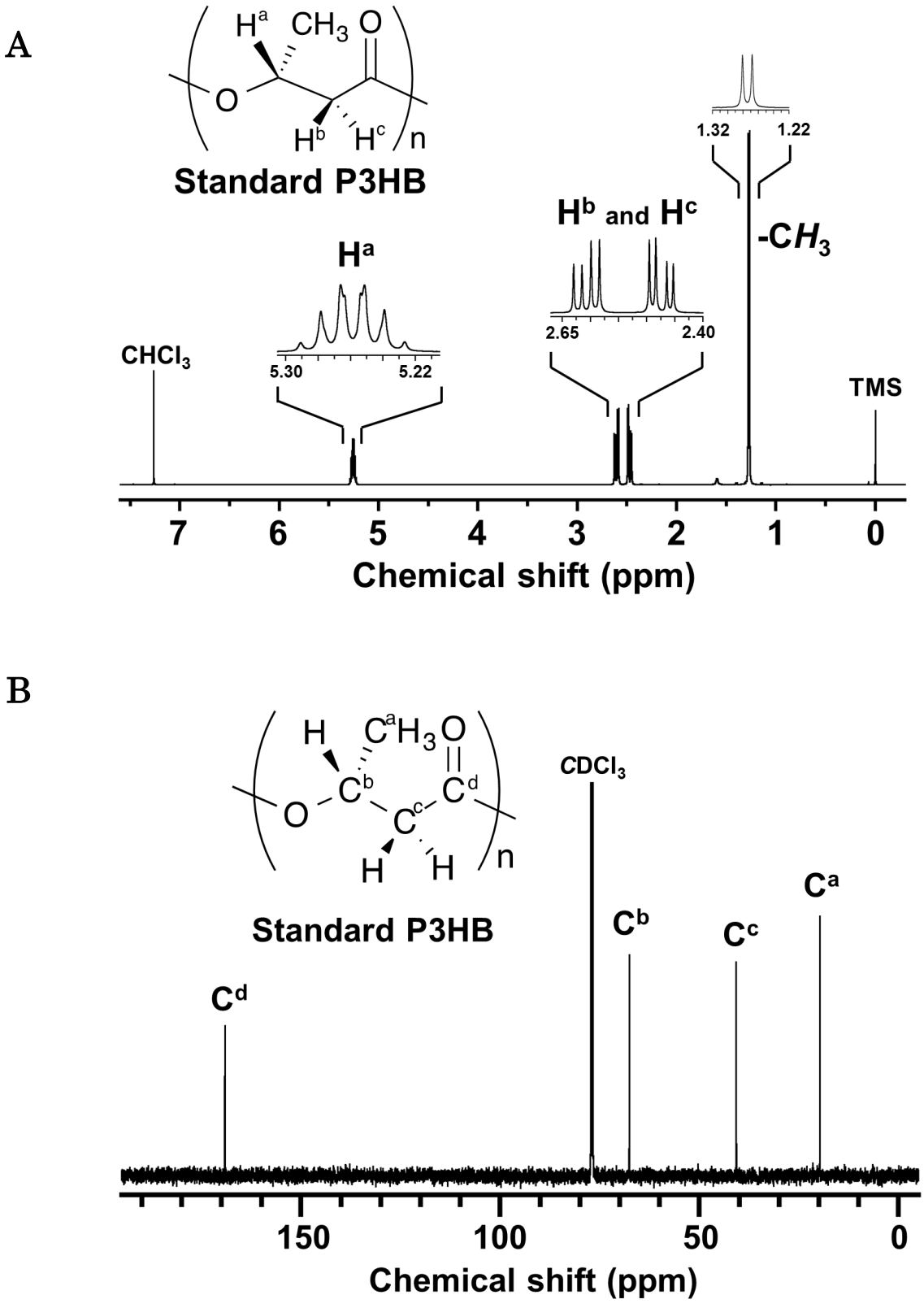

Figure S6: (A) ^{1}H and (B) ^{13}C NMR spectra of standard PHB.

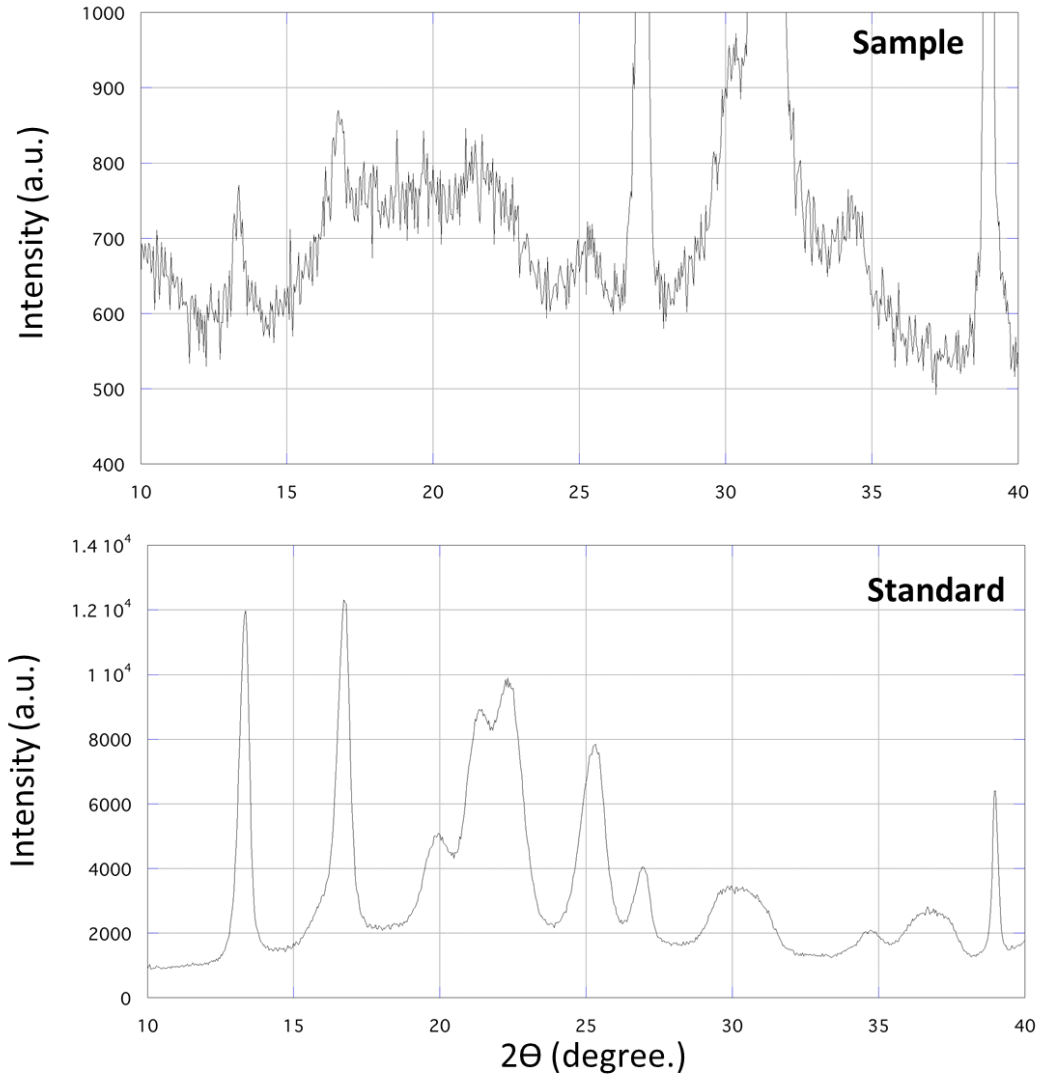

Figure S7: X-ray diffraction (XRD) patterns of pure PHB extracted from *Bacillus* sp. CYR1 and standard PHB.

Table S1: Conditions maintained for analysis of various toxic compounds using HPLC

S. NO	Compound name	HPLC conditions		
		Detector (nm)	Mobile phase (% ratio)	Retention time (min)
1	4-BP[1]	277	Acetonitrile: water (8:2)	3
2	4-s-BP[2]	277	Acetonitrile: water (8:2)	3
3	4-t-BP[3]	277	Acetonitrile: water (8:2)	3
4	4-t-OP[4]	277	Acetonitrile: water (8:2)	5.3
5	4-NP[5]	277	Acetonitrile: water (8:2)	7.2
6	Phe[6]	277	Acetonitrile: water (8:2)	3
7	4-CP[7]	280	Acetonitrile: 1% Ethylacetate (1:1)	3
8	Naph[8]	254	Acetonitrile: water (8:2)	2.1
9	Phen[9]	254	Acetonitrile: water (8:2)	5.5

4-BP[1]: 4-butylphenol; 4-s-BP[2]: 4-secondary butylphenol; 4-t-BP[3]: 4-tertiary butylphenol; 4-t-OP[3]: 4-tertiary octylphenol; 4-NP[5]: 4-Nonylphenol; Phe[6]: Phenol; 4-CP[7]: 4-chlorophenol; Naph[8]: Naphthalene; Phen[9]: Phenanthrene.