Degradation and conversion of toxic compounds into useful bioplastics by Cupriavidus sp. CY-1: relative expression of the PhaC gene under phenol and nitrogen stress

Degradation and conversion of toxic compounds into useful bioplastics by Cupriavidus sp. CY-1: relative expression of the PhaC gene under phenol and nitrogen stress

Degradation and conversion of toxic compounds into useful bioplastics by *Cupriavidus* sp. CY-1: Relative expression of PhaC gene under phenol and nitrogen stress

M. Venkateswar Reddy[1], Yuka Yajima[2], Yasuteru Mawatari[3], Tamotsu Hoshino[4,5], Young-Cheol Chang[1]*

[1]Department of Applied Sciences, College of Environmental Technology, Muroran Institute of Technology, 27-1 Mizumoto, Muroran, 050-8585, Japan,
[2]Graduate School of Medicine, Kyoto University, Yoshidakonoe-cho, Sakyo-ku, Kyoto-shi, Kyoto, 606-8501, Japan,
[3]Research Center for Environmentally Friendly Materials Engineering, Muroran Institute of Technology, 27-1 Mizumoto-cho, Muroran, Hokkaido 050-8585, Japan,
[4]Biomass Refinery Research Center, National Institute of Advanced Industrial Sciences and Technology (AIST), 3-11-32 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-0046, Japan,
[5]Bioproduction Research Institute, National Institute of Advanced Industrial Sciences and Technology (AIST), 2-17-2-1Tsukisamu-Higashi, Toyohira-ku, Sapporo 062-8517, Japan
E-mail: *ychang@mmm.muroran-it.ac.jp*

Abstract

In this study different types of toxic compounds *i.e.*, alkylphenols, mono and poly-aromatic hydrocarbons were converted into polyhydroxybutyrate (PHB) using the isolated bacteria *Cupriavidus* sp. CY-1. Influence of Tween-80 on toxic compounds degradation ability of CY-1 were analyzed using high pressure liquid chromatography. Among all the compounds, CY-1 showed highest removal of naphthalene (100±6%), phenol (96±7%), and lowest removal of alkylphenols without Tween-80 addition. However, Tween-80 addition enhanced the degradation capacity of CY-1, and showed highest removal of 4-tertiary-butylphebol (74±5%), followed by phenol (69±5%), 4-chlorophenol (59±3%), 4-tertiary-octylphenol (53±5%), and naphthalene (48±5%). Further experiments were carried out for conversion of toxic compounds into PHB. CY-1 grown with phenol (48±6%) and naphthalene (42±4%) showed highest PHB production. The functional groups, structure and thermal properties of the produced PHB were analyzed. In addition the expression of PhaC gene was quantified at transcriptional level through real time quantitative PCR. The results showed up-regulation of PhaC gene in the presence of phenol, up and down-regulations in the presence of nitrogen. The maximum PhaC transcript expression was 5.37 folds at 100 mg/l nitrogen concentration.

Keywords: *Cupriavidus* sp. CY-1; Real-time PCR; Naphthalene; Bioplastics; PHA synthase; NMR spectroscopy.

1. Introduction

Increasing concern for the environment has recently highlighted two major problems to be resolved *i.e.*, pollution and scarcity of resources[1]. Pollution is a serious issue as a cause of many health related problems. Among all the pollutants, aromatic compounds gaining particular concern, because their chemical structure makes them hazardous and resistant to bioremediation[1]. Aromatic compounds like phenol and phenolic compounds are discharged through effluents from a variety of industries including leather processing, textiles, pharmaceutical, and oil plants[2]. Phenol pollution is of great alarm since this chemical is toxic, mutagenic, and carcinogenic to microorganisms, animals and human beings. Because of its several toxic effects, removal of phenol from industrial wastewaters before their release is considered to be obligatory. In similar way alkylphenols are toxic and are considered as endocrine disrupters. Polycyclic aromatic hydrocarbons (PAH) are also classified as priority pollutants by US Environmental Protection Agency and are ubiquitous in the environment. They pose a significant threat to human health due to their mutagenic and carcinogenic properties[3]. Wastewaters with high concentration of these organic compounds can be treated mainly by physicochemical processes, but these methods are costly and in-appropriate for large wastewater volume[4]. Bioremediation have been shown to be practical, economical as it leads to complete mineralization of organic compounds.

On the other hand side 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. Polyhydroxybutyrate (PHB) is a type of PHA produced by many species of bacteria that serves as a storage form of carbon and energy that is metabolically equivalent to fat in animals[5-7]. PHB are synthesized and stored by a wide variety of bacteria such as, *Bacillus* sp. and *Pseudomonas* sp. through the fermentation of various substrates[8-10]. Among the pure cultures the bacteria *Cupriavidus necator* has been widely studied because of its potential in producing significant amounts of PHB from organic carbon substrates such as waste glycerol[11], and in-organic carbon source like carbon dioxide[12]. The amount of PHB accumulation from *C. necator* can be widely varied from 40-90% of their cell dry weight depending on the type and concentration of substrate[13-15].

One promising route for sustainable development is the combination of bioremediation with production of bioplastics. Some pioneering work in this field has already been reported[1, 16-19], but there is no information about the type of genes involved in this process, and the relative expression of specific genes at different stress conditions. Therefore,

objective of the present study is to evaluate degradation capacity of *Cupriavidus* sp. CY-1 for different toxic organic compounds, and conversion of the metabolites in to useful bioplastics like PHB. In addition, the gene (PhaC) encoding for the PHA synthase was identified, and the expression levels of PhaC gene under phenol and nitrogen stress was evaluated by Real-Time PCR.

2. Materials and Methodology

2.1 Isolation and identification of *Cupriavidus* sp. CY-1

Soil collected from the forest located in Muroran city, Hokkaido, Japan were used as inoculum source for isolation of *Cupriavidus* sp. CY-1. Bacteria were isolated using enrichment culture method. The purity of isolated culture was confirmed by an inverted microscope (Diaphot TMD300, Nikon, Tokyo, Japan) equipped for simultaneous recording of cell length. Total genomic DNA was extracted from the isolated colonies using Takara, Nucleospin kit. The 16S rRNA gene fragment was amplified by polymerase chain reaction (PCR) with a pair of universal primers: 27 F (5'-GAGTTTGATCMTGGCTCAG-3') and 1392 R (5'-ACGGGCGGTGTGTRC-3'), under standard conditions. DNA sequencing and phylogenetic analysis was performed according to the previously reported method[20]. The physiological characteristics of the isolates were also determined using commercially available identification systems (API 20 E and API 20 NE; bioMérieux, Japan). The 16S rRNA gene sequence of the isolate

(strain CY-1) determined in this study was deposited in the DDBJ under accession no: AB604176.

2.2 Culture media and chemicals

For the growth of *Cupriavidus* sp. CY-1 nutrient broth, and mineral salt medium (MSM) was used as the media. MSM was prepared according to the composition reported[21]. The medium pH was adjusted to 7 and autoclaved before adding to the flasks. Three different types of toxic organic compounds, *i.e.,* alkylphenols, mono and poly-aromatic hydrocarbons were selected for degradation studies. In alkylphenols we selected five different types of compounds *i.e.,* 4-butylphenol (4-BP), 4-secondary-butylphenol (4-s-BP), 4-tertiary-butylphenol (4-t-BP), 4-tertiary-octylphenol (4-t-OP), and 4-nonylphenol (4-NP). In mono-aromatic hydrocarbons phenol and 4-chlorophenol were selected. In poly-aromatic hydrocarbons naphthalene, and phenanthrene were selected. All chemicals used were of analytical grade and were purchased from Tokyo Chemical Industry (Tokyo, Japan).

2.3 Growth curve and degradation studies

Cupriavidus sp. CY-1 was cultivated in MSM at 30^{o}C by supplementing with above mentioned toxic organic compounds at 100 mg/l final concentration. Substrate stock solution was prepared as previously described[21]. For growth curve and degradation

studies, a loop of CY-1 strain was inoculated into 50 ml of nutrient broth in 100 ml flasks, and kept in shaking incubator under dark condition at 30^{o}C for overnight at 180 rpm. For initial experiments 2 ml (2%v/v) of the overnight grown culture was inoculated into different shake flasks containing 100 ml of MSM with different toxic compounds. Further studies were conducted by adding 0.02% Tween-80 as co-substrate and as surfactant without changing the conditions. The experiments were conducted for 6 days. 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). Samples collected for high pressure liquid chromatography (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.

2.4 PHB production

The ability of the strain CY-1 to accumulate PHB was investigated using different toxic compounds along with Tween-80 in MSM. Stress conditions were created by using lower nitrogen and phosphorous concentrations (100 mg/l) which induce PHB accumulation by CY-1. Bacterial cultures were incubated on a rotary shaker for 72 h, after the incubation period cells were collected by centrifugation, washed with acetone and ethanol. The

polymer was extracted and analyzed as described in 2.6.3 section. Experimental results reported here are the averages of three replicates for all fermentation experiments.

2.5 Real time PCR

2.5.1 Isolation of total RNA

Cupriavidus sp. CY-1 was grown in different flasks with MSM by adding phenol (100 mg//l), and nitrogen at different concentrations (0 to 500 mg/l) to isolate RNA. Control experiments were carried out without adding phenol and nitrogen. To induce the PhaC gene, the cells were incubated at 30°C for 72 hours and were harvested by centrifugation at 5,000 rpm for 10 minutes at 4^{o}C. Total RNA was extracted with the Trizol reagent (Invitrogen, USA) according to the manufacturer's recommended protocol. The RNA concentration was quantified using a UV-spectrometer (UV-1800, Shimadzu, Japan).

2.5.2 Complementary DNA (cDNA) synthesis and real time PCR

Amplification of complementary DNA (cDNA) was carried out using Prime Script II 1st strand cDNA Synthesis Kit (Takara, Japan). Synthesis of cDNA was done by amplification at 42^{o}C for 1 hour, followed by enzyme (RTase) inactivation at 95^{o}C for 5 minutes. The samples were stored at 4^{o}C until further analysis. Real-time PCR amplification was carried out using the Applied Biosystems StepOne^{TM} and StepOnePlus^{TM} Real-Time PCR systems with 20 µl reaction mixture containing 10 µl of 2×SYBR premix Ex *Tag* (Takara, Japan), 0.5 µM of primers, 0.04 µg of DNA template,

and nuclease free water. The amplification program consisted of one cycle of 95°C for 10 min and then 30 cycles of 95°C for 30 sec, 60°C for 40 sec, and 72°C for 60 sec. A melting curve analysis was done after amplification to distinguish the target PCR product from the non-target PCR product. The melting curves were obtained by slow heating at temperatures from 65 to 95°C at a rate of 0.2°C/s, with continuous fluorescence collection. For real-time PCR, the primers for PhaC and housekeeping genes were selected and designed by the software Primer3 (http://primer3.ut.ee/). The target gene PhaC was amplified using forward (5'-ATCGAGTCGGGCGGCGAAT-3') and reverse (5'-AGTACTCGTTCTCGAAGACCA -3') primers. All gene expressions were normalized using 16S rRNA as housekeeping gene. Housekeeping gene was amplified using primers 16S-F (5'-AGACTCCTACGGGAGGCA-3') and 16S-R (5'-TACCGTCATCGACCCCGG-3'). The specificity of the primers was confirmed by the presence of a single peak of the melting curve. Each target mRNA level was evaluated from the real-time threshold cycle and compared with the amount of 16S as an internal control. Three independent experiments were performed, and each sample was tested in triplicate.

2.6 Analysis

2.6.1 High pressure liquid chromatography

The degradation patterns of different toxic organic compounds were analyzed on HPLC

(Shimadzu) with an SPD-10AV UV/Vis detector and Shim-pack VP-ODS column (4.5×150 mm diameter, particle size 5 μm; Shimadzu, Kyoto, Japan). Conditions maintained for HPLC analysis were described in Table S1. The column was maintained at a temperature of 40°C in a thermostat chamber. 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.

2.6.2 Transmission electron microscopy (TEM)

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 4^{o}C 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 4^{o}C, 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 60^{o}C 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.

2.6.3 PHB extraction, FT-IR and NMR spectroscopy

Extraction and estimation of PHB was performed following the procedure reported[21-22]. Spectrometric analysis was conducted according to the method described[22]. In briefly, 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). 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. ^{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 (Sigma-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.

2.6.4 Gel permeation chromatography (GPC), TGA and DSC

Number and weight average molecular weights (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. 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 10°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 -50ºC to 250ºC at a heating rate of 10ºC/min.

3. Results and discussion

3.1 Taxonomy of isolated bacteria CY-1

The isolated strain CY-1 is aerobic, Gram-negative, and rod-shaped bacterium. Results of

physiological and biochemical tests denoted that the strain was able to produce urease and cytochrome oxidase but not indole or β-glucosidase. The carbon sources glucose, arabinose, mannose, and mannitol did not support growth. The strains displayed a negative esculin and gelatin hydrolysis. Based on these characteristics as well as carbon utilization test, we considered CY-1 belongs to the genus Cupriavidus. On the other hand, the 16S rRNA gene sequence and phylogenetic analysis also showed that strain CY-1 was closely related to *Cupriavidus oxalaticus* AB680453 (99% sequence identity). Furthermore, the 16S rDNA sequence data revealed that strain CY-1 was very similar to *Ralstonia* sp. among the known PHB producing bacteria.

In the present study we have used *Cupriavidus* sp. CY-1 for degradation of different toxic compounds and subsequent conversion of their metabolites into PHB. Even though these bacteria were able to tolerate and can grow with different toxic compounds, little information is available about the PHB production using this bacteria with feeding of toxic compounds. This is an exceptional candidate for studying the biosynthesis of PHB under the conditions of stress induced by the toxic compounds. So, we selected these bacteria for degradation of toxic organic compounds and subsequent conversion of their metabolites into useful bioplastics.

3.2 Growth curve

3.2.1 Alkylphenols

CY-1 was cultivated in MSM 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 concentration. Strain CY-1 could not showed significant growth with alkylphenols (Figure S1), so further studies were carried out by adding 0.02% Tween-80 in MSM without changing the conditions. CY-1 with Tween-80 addition showed good growth with all the alkylphenols (Figure 1A). Among all the alkylphenols, CY-1 grown with 4-t-BP and 4-t-OP showed highest growth followed by 4-NP, 4-s-BP, and 4-BP. After addition of Tween-80, the growth of CY-1 was increased by 5.8 times with 4-t-BP, 6.6 times with 4-t-OP, 5.1 times with 4-NP, 4.9 times with 4-s-BP, and 5.3 times with 4-BP respectively at 6^{th} day (Figure 1A). It clearly indicates the positive effect of Tween-80 on growth of bacteria.

3.2.2 Phenol and 4-chlorophenol

Among the two compounds, CY-1 showed higher growth with phenol, and lower growth with 4-chlorophenol. The growth of CY-1 was 2.1 times higher with phenol, when growth was compared with 4-chlorophenol. CY-1 with phenol showed higher growth than acetate (Figure S2). Strain CY-1 could not showed significant growth with 4-chlorophenol, so further studies were carried out by adding 0.02% Tween-80 without changing the conditions. After addition of Tween-80, bacteria incubated with phenol showed rapid

growth, it showed short lag phase and within 1 day it entered in to log phase (Figure 1A). Bacteria at 6th day showed highest growth (OD at 600 nm is 0.92), and growth was increased by 3.5 times after Tween-80 addition. Interestingly, bacteria grow with 4-chlorophenol also followed similar trend. Bacteria showed 5.5 times higher growth after addition of Tween-80. Growth study results with Tween-80 clearly indicating that the strain CY-1 has good growth capacity with phenol, and 4-chlorophenol.

3.2.3 Naphthalene and phenanthrene

Two different types of poly-aromatic compounds i.e., naphthalene, and phenanthrene were used for CY-1 growth. Bacteria showed almost similar growth with both naphthalene and phenanthrene (data not shown). Further studies were carried out by adding 0.02% Tween-80. After addition of Tween-80, CY-1 incubated with naphthalene showed rapid growth, at 6th day CY-1 showed highest growth (OD at 600 nm is 0.78), it was increased by 3.6 times after Tween-80 addition (Figure 1A). Bacteria grown with phenanthrene also showed highest growth at 6th day (OD at 600 nm is 0.712), it was 3.6 times increased after Tween-80 addition. 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[23]. Budde et al. (2011) conducted experiments to know the effect of Tween-80 at 0.2% concentration on the growth of *Ralstonia eutropha*[24]. They reported that Tween-80 did not inhibit growth of *R. eutropha*, but 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.

3.3 Degradation of toxic compounds using *Cupriavidus* sp. CY-1

Existing literature shows *Cupriavidus* group of bacteria involved in the degradation of various toxic compounds. According to Perez-Pantoja et al. the strain *C. necator* JMP134 can grow and utilize 60 different aromatic compounds like., benzoate, 4-hydroxybenzoate, ferulate, 3-hydroxyphenylpropionate, phenol, benzene, toluene, 3-nitrophenol and hydroquinone as the sole carbon and energy source[25]. Shi et al. used *C. basilensis* B-8 as biocatalyst and studied degradation of different aromatic compounds such as phenol,

ferulate, cinnamate, benzoate, 3, 4- dihydroxybenzoate, 4-hydroxycinnamate, vanillate, 4-methylcatechol, 2-methylphenol, salicylate, 3-hydroxybenzoate, ethyl salicylate, and 3-hydroxbenzylalcohol[26]. Based on this information, in the present study we evaluated the degradation efficiency of CY-1 using alkylphenols, mono and poly-aromatic compounds.

3.3.1 Alkylphenols

Among all the alkylphenols, strain CY-1 showed 29±4% degradation of 4-s-BP. CY-1 was unable to degrade other alkylphenols such as 4-BP, 4-t-BP, 4-t-OP and 4-NP up to 6th day. It indicates that strain CY-1 was unable to degrade alkylphenols due to the toxic nature of the compounds, so further studies were carried out by adding Tween-80 without changing the alkylphenols concentration. Tween-80 addition showed improvement in degradation of two alkylphenols such as 4-t-BP, and 4-t-OP, but not in degradation of 4-BP, 4-s-BP, and 4-NP (Figure 1B). Among all the alkylphenols, CY-1 incubated with 4-t-BP showed highest degradation (74±5%), followed by 4-t-OP (53±6%). Contrary to this, addition of Tween-80 also not showed any significant influence on the degradation capacity of CY-1 towards 4-BP and 4-NP. Interestingly, Tween-80 addition decreased the degradation capacity of CY-1 towards 4-s-BP, further research was needed to explore the mechanism.

3.3.2 Phenol and 4-chlorophenol

Among the two compounds, strain CY-1 showed highest removal (96±5%) with phenol

and lowest removal (15±3%) with 4-chlorophenol without Tween-80 addition. Tween-80 addition showed contrary results with degradation of phenol and 4-chlorophenol. Interestingly, Tween-80 addition showed negative influence on the degradation of phenol, even though growth was enhanced. CY-1 with Tween-80 addition showed 1.4 times lower removal than without Tween-80 addition, this may be due to the utilization of Tween-80 and phenol simultaneously as carbon source for growth of bacteria instead of only phenol. There are many reports on the degradation of phenol using *C. necator*. Tepe and Dursun reported the biodegradation of phenol from aqueous solution by immobilized *Ralstonia eutropha* (formerly named as *C. necator*) in a batch stirred and packed bed reactor[27]. Maximum phenol removal efficiency was determined as 68% in the batch studies. Addition of Tween-80 showed significant influence on 4-chlorophenol degradation. The degradation was 3.9 times increased after addition of Tween-80 (Figure 1B). These results clearly indicating that the strain CY-1 has good degradation capacity of phenol and 4-chlorophenol, it can be used for remediation of phenol contaminated wastewaters.

3.3.3 Naphthalene and phenanthrene

Degradation studies denoted that there is a marked difference on the degradation of two compounds. Among the two compounds, CY-1 showed highest removal (100±5%) with naphthalene (Figure 1B). Strain CY-1 did not showed degradation (0%) of phenanthrene.

Previous reports also showing naphthalene degradation ability of *C. necator*. Jones et al. reported *nag* genes of *Ralstonia* sp. strain U2 code for the enzymes for the catabolism of naphthalene, which converts naphthalene to fumarate and pyruvate via salicylate and gentisate and they are organized in a continuous sequence of adjacent genes[28]. But according to our knowledge there are no reports on degradation of phenanthrene using *C. necator*. CY-1 without Tween-80 unable to degrade phenanthrene, so further studies were carried out by adding Tween-80. CY-1 showed 15±3% degradation of phenanthrene after addition of Tween-80 (Figure 1B).

Figure 1

3.4 PHB production

PHB was accumulated by CY-1 when various toxic organic compounds were supplied in MSM. Biomass levels and PHB was accumulated to different levels. CY-1 showed highest dry cell weight with phenol (0.41±0.1 g/l), followed by naphthalene (0.4±0.08 g/l), 4-chlorophenol (0.38±0.05 g/l), 4-t-BP (0.34±0.05 g/l), and 4-t-OP (0.32±0.05 g/l) (Figure 2A). CY-1 showed negligible amount of dry cell weight with the compounds like phenanthrene, 4-BP, 4-s-BP and 4-NP. The best accumulation of PHB was achieved when phenol (48±4% DCW) was used as carbon sources (Figure 2B). CY-1 accumulated the PHB with other compounds like naphthalene (42±3% DCW), 4-t-BP (23±3% DCW), 4-CP (13±4% DCW) and 4-t-OP (11±3% DCW). CY-1 was not accumulated PHB with

compounds like 4-BP, 4-s-BP, 4-NP, and phenanthrene. According to Hoffmann et al. many bacteria can consume a large number of aromatic compounds, but they cannot always accumulate PHB[29]. This is a possible reflection on the metabolic routes employed in the breakdown of these substrates and also the substrate range and efficiency of the PHB polymerase[30].

Although the isolation of PHB producing bacteria from environments contaminated with different toxic compounds has been studied, only a few reports describe the microbial production of PHB using pollutants. The possibility to convert mono-aromatic compounds (BTEX, styrene) into PHA has been previously reported by *P. putida* strains [17,18]. Narancic et al. reported PHA production by *Pseudomonas* sp. TN301 from a wide range of poly aromatic and mono aromatic hydrocarbons[16]. Thakor and his co-workers reported *Comamonas testosteroni* was found to produce 85% of the dry cell mass as PHB during its growth on Bushnell Haas mineral salts medium containing 0.5% w/v naphthalene as a carbon source[19]. Very recently, Berezina et al. reported benzoic acid was found to be a crucial compound for the bioremediation by *C. necator* and this strain transforms benzoic acid into PHB[1]. Dahman and Ugwu, reported 65% PHB production with *C. necator* using heat straws as carbon substrates[31]. Wang et al. used lignin monomers as substrate for production of PHB with *C. necator*[32]. Mozumder et al. and

Garcia-Gonzalez et al. reported 62% and 61% PHB production with *C. necator* DSM 545 using waste glycerol and CO_2 as substrates respectively[11,12]. Many authors reported PHA production with different wastewaters like sugar cane molasses[33], biohydrogen reactor effluent[34], food waste[35] and dark fermentation effluents[36] using various types of biocatalysts.

Figure 2

3.5 PhaC gene expression analysis using Real-Time PCR

Expression pattern of the PhaC gene in CY-1 under phenol and nitrogen stress was analyzed by Real-Time PCR. After evaluation of the appropriate Real-Time PCR conditions, RNA expression levels of reference gene and a target gene were measured. The relative expression of PhaC gene is shown in Figure 3. The PhaC expression was up-regulated with phenol, it was induced by 1.13 folds. The PhaC expression was up and down-regulated with nitrogen concentration. The expression levels of PhaC was induced by 5.3 folds at 100 mg/l, 0.8 folds at 250 mg/l, and 1.9 folds at 500 mg/l nitrogen concentration (Figure 3). The PHB production values were very well corroborated with PhaC gene expression where 100 mg/l nitrogen concentration showed highest PHB production and gene expression levels. This indicates that this gene is working as functional gene for the production of PHB.

Figure 3

3.6 Biopolymer characterization

3.6.1 Transmission electron microscopy

The ability of the strain CY-1 to accumulate PHB was investigated under TEM using phenol (Figure 4A) and naphthalene (Figure 4B-E) in MSM. PHB production was assessed by growing the strains in 500 ml conical flasks containing 100 ml nitrogen and phosphorous limited MSM medium. Bacterial cultures were incubated on a rotary shaker for 72 h at 30°C and 180 rpm. After the incubation period of 72 h, cells were collected and the accumulated PHB was analyzed as described in section 2.6.2. A series of thin sections of CY-1 containing PHB inclusions are shown in Figure 4. Under electron microscope, cells occurred singly and gradually swollen during polymer accumulation. CY-1 grown with phenol showed more number of PHB granules, than naphthalene.

Figure 4

3.6.2 Functional groups identification by FT-IR

The FT-IR spectra were analyzed in order to confirm the practically identical structure of standard PHB with the PHB produced by CY-1 (Figure 5). The PHB extracted from CY-1 (Figure 5A) had the same C-H and carbonyl bands as standard PHB (Figure 5B). The band appeared at 1457 cm^{-1} corresponded to the blending of C-H bond in CH_2 groups including CH_3 groups at 1376 cm^{-1}. The band at 1723 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 2922 cm^{-1} also observed. The presence of absorption bands at 1723 cm^{-1} and 1278 cm^{-1} in extracted PHB sample were characteristic of C=O and C-O stretching groups and were identical to PHB.

Figure 5

3.6.3 Structure determination

The ^{1}H NMR spectrum of $CDCl_3$ soluble part of PHB extracted from CY-1 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 methine (5.26 ppm), methylene (2.65-2.45 ppm) and methyl (1.28 ppm) groups as shown in Figure 6A. The chemical shifts of sample peaks were almost identical to standard PHB which measured at the same conditions. Standard PHB showed methine (5.26 ppm), methylene (2.65-2.40 ppm) and methyl (1.28 ppm) groups as shown in Figure 6B.

Figure 6

Figure 7A shows the ^{13}C NMR spectrum of $CDCl_3$ soluble part of PHB extracted from CY-1 grown with phenol in MSM measured at 20°C. The four peaks symbolized in Figure 7A were assignable to the carbonyl (169.15 ppm), methine (67.61 ppm), methylene

(40.78 ppm), and methyl (19.77 ppm) carbon resonances of PHB. Additionally, the chemical shifts of these peaks were almost identical to standard PHB which measured at the same conditions (Figure 7B). Therefore, we concluded that extracted polymer is undoubtedly PHB with highly stereoregular sequence and single chemical structure as repeating unit. The other small peaks were observed in both ^{1}H and ^{13}C NMR spectra of sample PHB. Based on the region of these peaks, these impurities can be assigned to hydrocarbons although the origin is still unknown.

Figure 7

3.6.4 Gel permeation chromatography (GPC)

To confirm the PHB produced from CY-1 was exact high molecular weight material, we measured both number average molecular weights (M_n) and weight average molecular weights (M_w) of the extracted PHB produced by CY-1 using by GPC. The GPC chromatogram was composed of two peaks, indicating the extracted PHB consisted of two kinds of polymers having a different chain length. M_W and polydispersity index (PDI, M_w/M_n) of high molecular weight part in the PHB is 269 kDa and 2.09, respectively. The *Mw* of standard PHB is 725 kDa, and PDI of the sample PHB was nearly half to the standard PHB (purchased from Sigma Aldrich (PDI: 4.28)). In the case of low molecular weight part, M_w and PDI are 9 kDa and 1.99, respectively. These results indicate that CY-

1 produced PHB with relatively lower dispersity.

3.6.5 TGA and DSC

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 5 milligrams of PHB powders were added into an aluminum pan and subjected to a heating rate of 20°C/min from ambient to 600°C. The weight loss of standard PHB started at around 230°C, its T_{d5} was at 274°C, and was completely decomposed at 310°C (Figure 8). However the starting temperature for weight loss of the PHB extracted from CY-1 was significantly low compared with that of standard PHB, *i.e.*, at around 100°C (Figure 8). In addition, the PHB was completely not decomposed at 600°C, indicating that the PHB extracted from CY-1 composed of some inorganic material which may came from bacterial dry mass.

Figure 8

Differential scanning calorimetry (DSC) was used to characterize the melting temperature (T_m) of the PHB extracted from CY-1, and it was compared with standard PHB. The temperature range for DSC varied from -50°C to 250°C at a heating rate of 10°C/min (Figure 9). From the endothermic peaks in each DSC traces, it denoted that PHB extracted from CY-1 contains the T_m of 160°C (Figure 9A), and it was matched with standard PHB

(T_m, 178°C) (Figure 9B).

Figure 9

4. Conclusions

PHB is gaining importance as a promising biodegradable plastics, and other side pollution caused by aromatic compounds creating many problems to environment and human health. To fulfill these two objectives, bacteria *Cupriavidus* sp. CY-1 was isolated and identified with the ability to degrade the toxic compounds and simultaneous accumulation of bioplastics. Results from this study showed that CY-1 utilized different toxic compounds as sole carbon and energy source, grew well and converted in to useful bioplastics. The results of relative expression studies clearly indicate that the gene PhaC could be involved in PHB production and up-regulated during phenol and nitrogen stress conditions.

Acknowledgements

This work was partially supported by a Grant-in-Aid for Scientific Research (No. 26340067) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

The authors have declared no conflict of interest.

References

1. N. Berezina, B. Yada and R. Lefebvre, *New Biotechnol.*, 2015, **32,** 47-53.

2. M. Haddadi and M. Shavandi, *Int. Biodet. Biodeg.*, 2013, **85,** 29-34.
3. X. Xia, N. Xia, Y. Lai, J. Dong, P. Zhao, B. Zhu, Z. Li, W. Ye, Y. Yuan and J. Huang, *Chemosphere.,* 2015, **128,** 236–244.
4. A.M.D Elahwany and M.E.M. Mabrouk, *Acta Biologica Hungarica.,* 2013, **64**, 106-117.
5. M. Guo, D.C. Stuckey and R. J. Murphy, *Green Chem.*, 2013, **15,** 706–717.
6. Y. Parulekar and A.K. Mohanty, *Green Chem.*, 2006, **8,** 206-213.
7. A.J. Anderson and E.A. Dawes. *Microbiol. Rev.*, 1990, **54**, 450-472.
8. K. Amulya, M. Venkateswar Reddy and S. Venkata Mohan, *Bioresour. Technol.*, 2014, **158,** 336-342.
9. M. Venkateswar Reddy, K. Amulya, M.V. Rohit, P.N. Sarma and S. Venkata Mohan, *Int. J. Hydrogen Energy.,* 2014, **39,** 7616-7626.
10. M. Venkateswar Reddy, G.N. Nikhil, S. Venkata Mohan, Y.V. Swamy and P.N. Sarma, *Bioresour. Technol.*, 2012, **123,** 471-479.
11. M.S.I. Mozumder, H. De Wever, E.I.P. Volcke and L. Garcia-Gonzalez, *Proc. Biochem.*, 2014, **49**, 365-373.
12. L. Garcia-Gonzalez, M.S.I. Mozumder, M. Dubreuil, E.I.P Volcke, H. De Wever, *Cat. Today.*, 2014, DOI: 10.1016/j.cattod.2014.05.025.
13. C. Morais, F. Freitas, M.V. Cruz, A. Paiva, M. Dionísio and M.A.M. Reis, *Int. J. Biol. Macromol.*, 2014, **71,** 68-73.
14. P. Passanha, S.R. Esteves, G. Kedia, R.M. Dinsdale and A.J. Guwy, *Bioresour. Technol.*, 2103, **147,** 34-352.
15. R.A.J. Verlinden, D.J. Hill, M.A. Kenward1, C.D. Williams, Z. Piotrowska-Seget and I.K. Radecka, *AMB Express.*, 2011, **1,** 1-8.
16. T. Narancic, S.T. Kenny, L. Djokic, B. Vasiljevic, K.E. O'Connor and J.N. Runic, *J. Appl. Microbiol.*, 2012, **113,** 508-520.
17. J. Nikodinovic, S.T. Kenny, R.P. Babu, T. Woods, W.J. Blau and K.E. O'Connor, *Appl. Microbiol. Biotechnol.*, 2008, **80,** 665-673.
18. P.G. Ward, G. de Roo, K.E. O'Connor, *Appl. Environ. Microbiol.*, 2005, **71**, 2046-2052.
19. N.S. Thakor, M.A. Patel, U.B. Trivedi and K.C. Patel, *World. J. Microbiol. Biotechnol.*, 2003, **19,** 185-189.
20. Y.C. Chang, K. Ikeutsu, T. Toyama, D.B. Choi and S. Kikuchi, *J. Ind. Microbiol. Biotechnol.*, 2011, **38,** 1667-1677.
21. M. Venkateswar Reddy, Y. Mawatari, Y. Yajima, C. Seki, T. Hoshino and Y.C. Chang, *Bioresour. Tecnol.*, 2015, **192,** 711–717.

22. J.H. Law and A.S. Slepecky, *J. Bacteriol.*, 1960, **82,** 33-36.
23. S.K. Brar, M. Verma, S. Barnabe, R.D. Tyagi, J.R. Valero and R. Surampalli, *Proc. Biochem.*, 2005, **40,** 2695–2705.
24. C.F. Budde, S.L. Riedel, F. Hübner, R. Risch, M.K. Popović, C.K. Rha and A.J. Sinskey, *Appl. Microbiol. Biotechnol.,* 2011, **89,** 1611-1619.
25. D. Perez-Pantoja, R.D.L. Iglesia, D.H. Pieper and B. Gonzalez, *FEMS. Microbiol. Rev.*, 2008, **32,** 736-794.
26. Y. Shi, L. Chai, C. Tang, Z. Yang, H. Zhang, R. Chen, Y. Chen and Y. Zheng, *Biotechnol. Biofuels*, 2013, **6**, 1-14.
27. O. Tepe, A.Y. Dursun, *J. Haz. Mat.*, 2008, **151,** 9-16.
28. R.M. Jones, B. Britt-Compton and P.A. Williams, *J. Bacteriol.,* 2005, **185,** 5847-5853.
29. N. Hoffmann, A. Steinbuchel and B.H.A. Rehm, *Appl. Microbiol. Biotechnol.*, 2000, **54,** 665-670.
30. B. Kessler and B. Witholt, *J. Biotechnol.,* 2001, **86,** 97-104.
31. Y. Dahman and C.U. Ugwu, *Bioprocess. Biosyst. Eng.*, 2014, **37,** 1561-1568.
32. W. Wang, S. Yang, G.B. Hunsinger, P.T. Pienkos and D.K. Johnson, *Front. Microbiol.*, 2014, **5,** 1-10.
33. M.G.E. Albuquerque, C.A.V. Torres and M.A.M. Reis, *Water. Res.*, 2010, **44,** 3419–3433.
34. S. Venkata Mohan, M. Venkateswar Reddy, G.V. Subhash and P.N. Sarma, *Bioresour. Technol*., 2010, **23,** 9382–9386.
35. M. Venkateswar Reddy and S. Venkata Mohan, *Bioresour. Technol.,* 2012, **103**, 313–321.
36. M. Venkateswar Reddy, K. Amulya and S. Venkata Mohan, *Eng. Life Sci.*, 2015, DOI:10.1002/elsc.201500016.

Figure Captions

Figure 1: (A) Growth curve of *Cupriavidus* sp. CY-1 with various toxic compounds. (B) Degradation of various toxic compounds using *Cupriavidus* sp. CY-1. Bacteria were incubated at 100 mg/l concentration of different toxic compounds and 0.02% Tween-80 in MSM media at 30^{o}C. Residual concentration of compounds at different time intervals in the medium were analyzed by HPLC.

Figure 2: (A) Dry cell weight; (B) and PHB production from *Cupriavidus* sp. CY-1. Bacteria were incubated with different aromatic compounds and 0.02% Tween-80 in MSM media at 30^{o}C. For dry cell weight (DCW) measurement cells were harvested by centrifugation, washed twice with phosphate-buffer and dried for 24 h at 80^{o}C until a stable weight was reached. PHB was extracted from the DCW using sodium hypochlorite-chloroform extraction method.

Figure 3: Real-time PCR result of PhaC gene expression induced by phenol in *Cupriavidus* sp. CY-1 during PHB production at varying (0-500 mg/l) initial nitrogen concentrations.

Figure 4: Transmission electron micrograph images of thin sections of *Cupriavidus* sp. CY-1. Bacteria were grown in MSB medium with (A) phenol; and (B-E) naphthalene; as carbon source. Bars: A, E - 0.5µm. B, C - 0.2µm. D - 0.25µm.

Figure 5: Fourier-transform infrared (FT-IR) spectrum of (A) PHB extracted from *Cupriavidus* sp. CY-1; and (B) standard PHB.

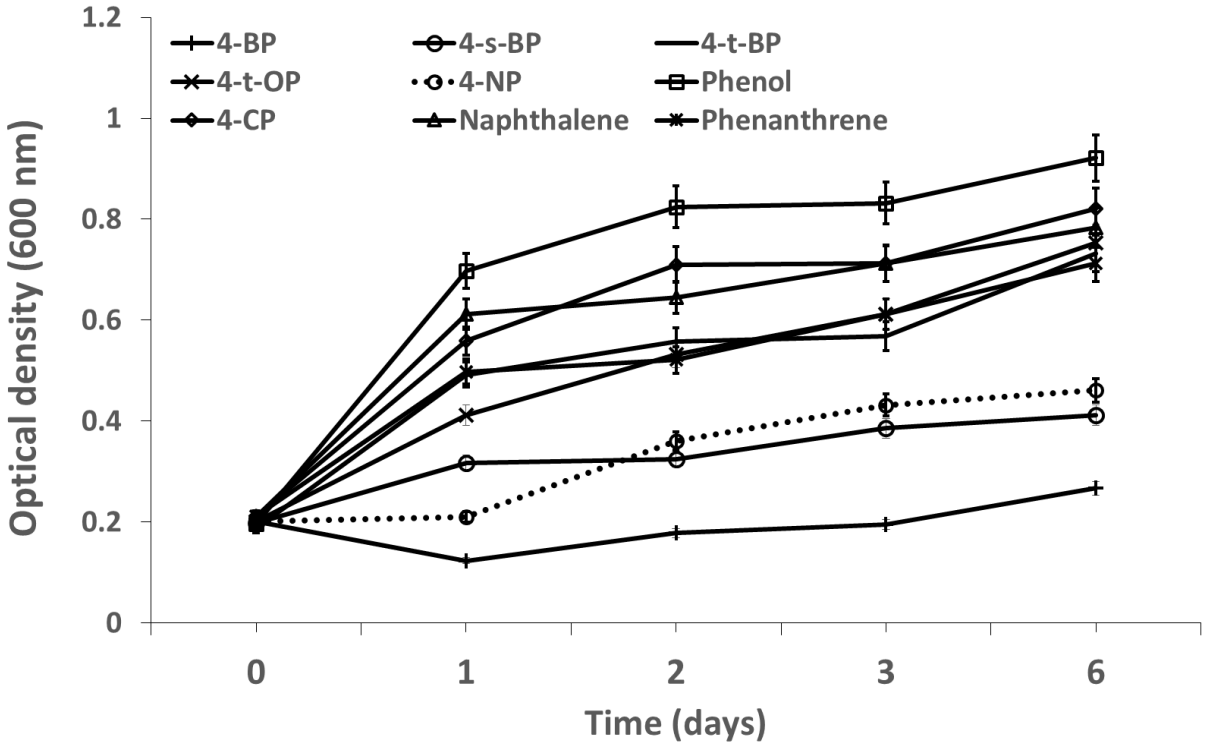
Figure 6: ^{1}H NMR spectra of (A) PHB extracted from *Cupriavidus* sp. CY-1; and (B) standard PHB.

Figure 7: ^{13}C NMR spectra of (A) PHB extracted from *Cupriavidus* sp. CY-1; and (B) standard PHB.

Figure 8: Thermogravimetric analysis (TGA) of PHB extracted from *Cupriavidus* sp. CY-1 and standard PHB.

Figure 9: Differential Scanning Calorimetry (DSC) of PHB extracted from *Cupriavidus* sp. CY-1 and standard PHB.

A


4-BP
4-s-BP
4-t-BP
4-t-OP
4-NP
Phenol
4-CP
Naphthalene
Phenanthrene
Optical density (600 nm)
Time (days)

B

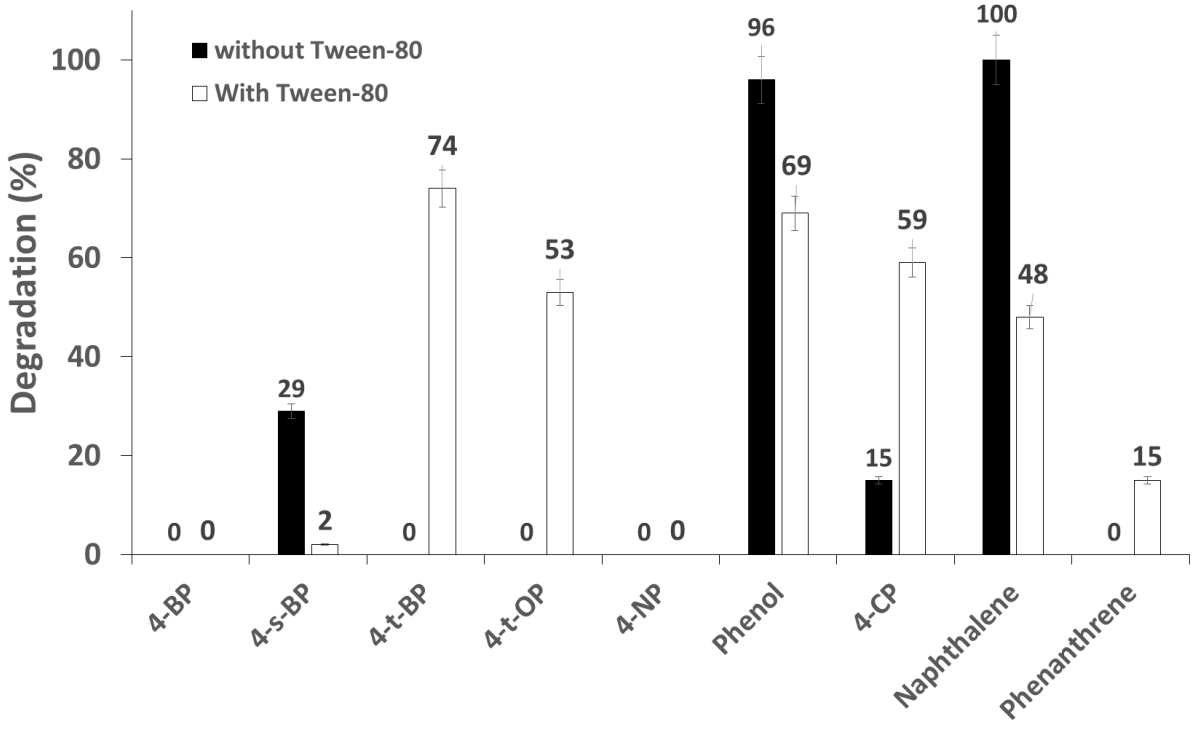
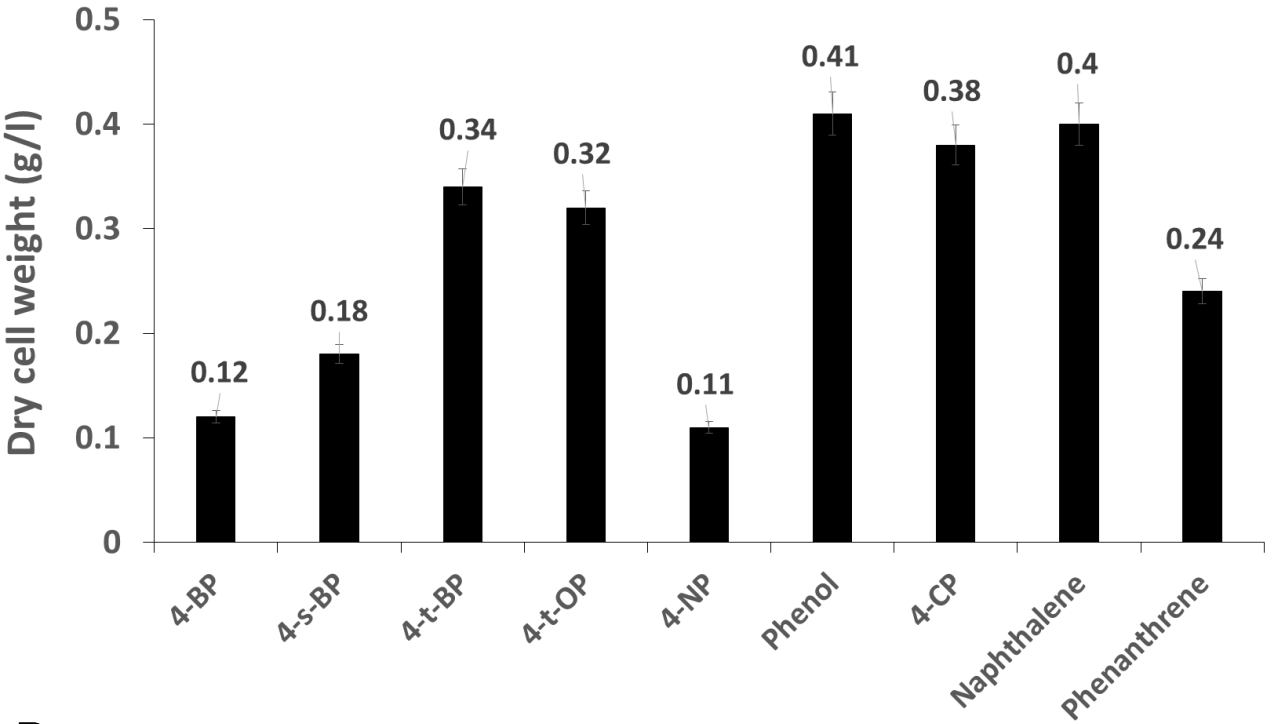

without Tween-80
With Tween-80
Degradation (%)
4-BP
4-s-BP
4-t-BP
4-t-OP
4-NP
Phenol
4-CP
Naphthalene
Phenanthrene

Figure 1

A


Dry cell weight (g/l)
0.5
0.4
0.3
0.2
0.1
0
0.12
0.18
0.34
0.32
0.11
0.41
0.38
0.4
0.24
4-BP
4-s-BP
4-t-BP
4-t-OP
4-NP
Phenol
4-CP
Naphthalene
Phenanthrene

B

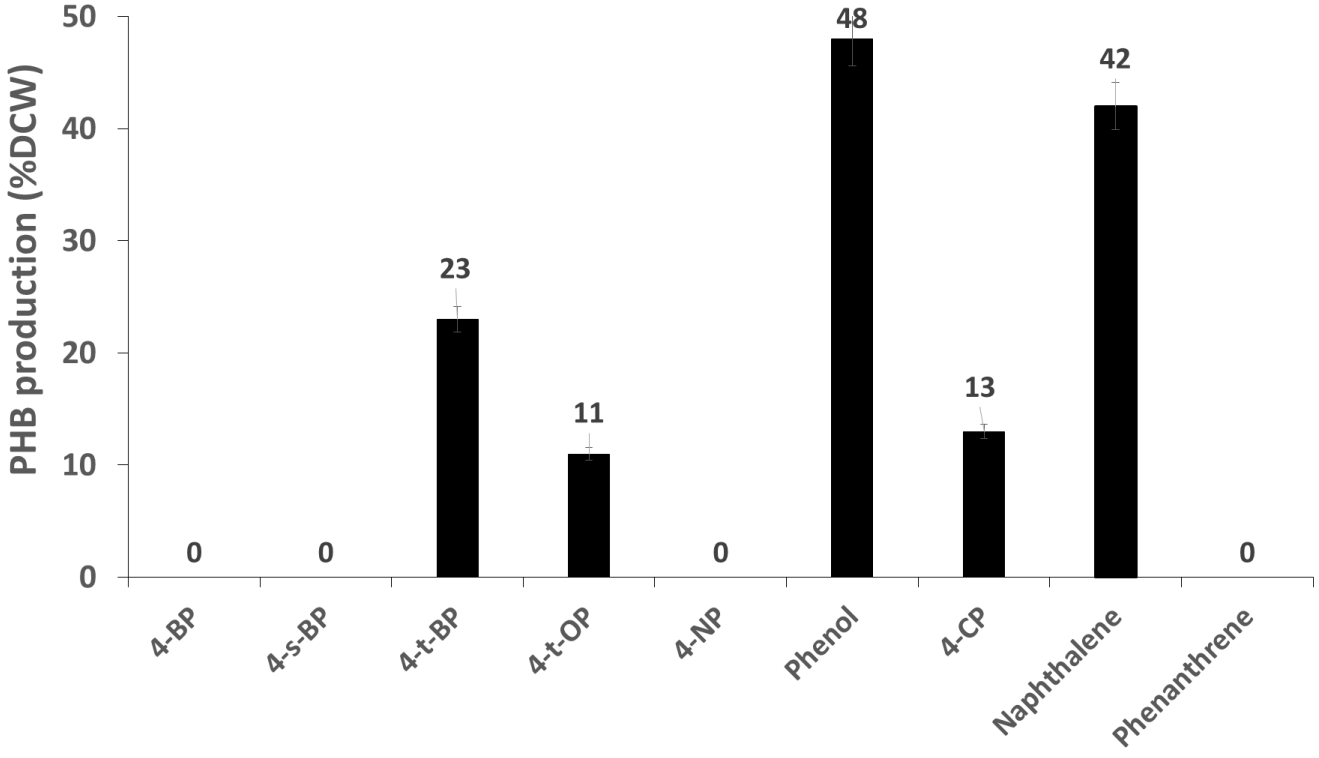

PHB production (%DCW)
50
40
30
20
10
0
0
0
23
11
0
48
13
42
0
4-BP
4-s-BP
4-t-BP
4-t-OP
4-NP
Phenol
4-CP
Naphthalene
Phenanthrene

Figure 2

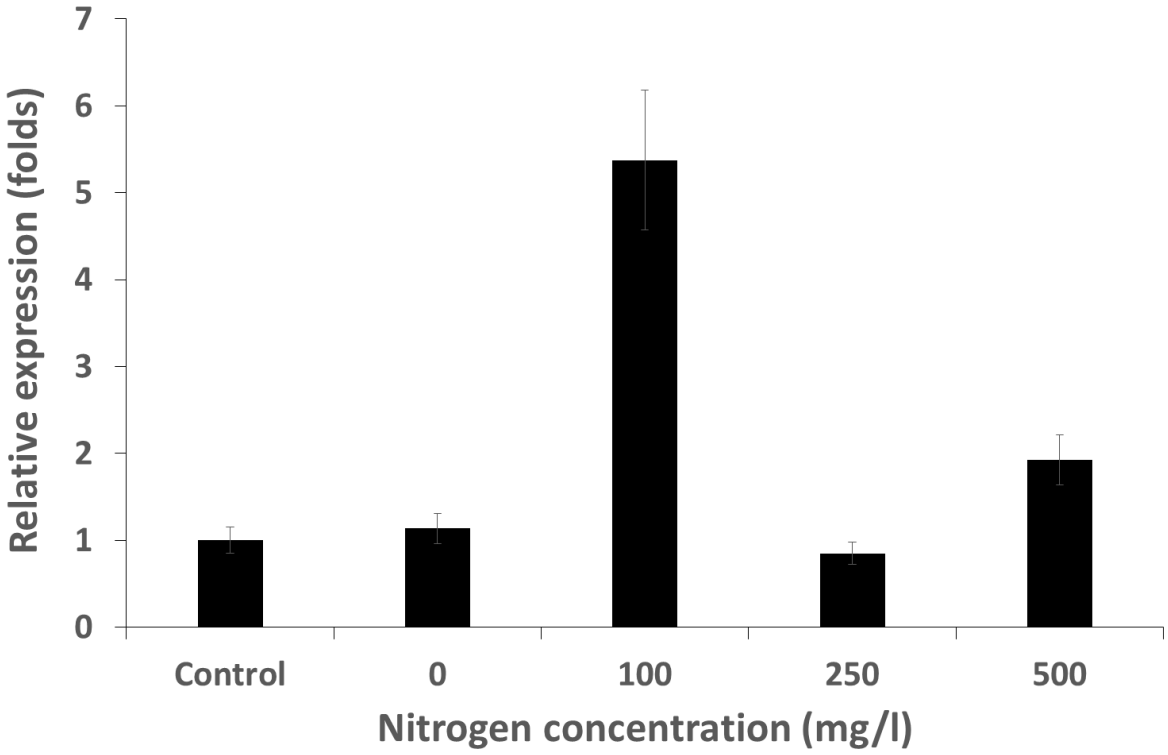

Relative expression (folds)
7
6
5
4
3
2
1
0
Control
0
100
250
500
Nitrogen concentration (mg/l)

Figure 3

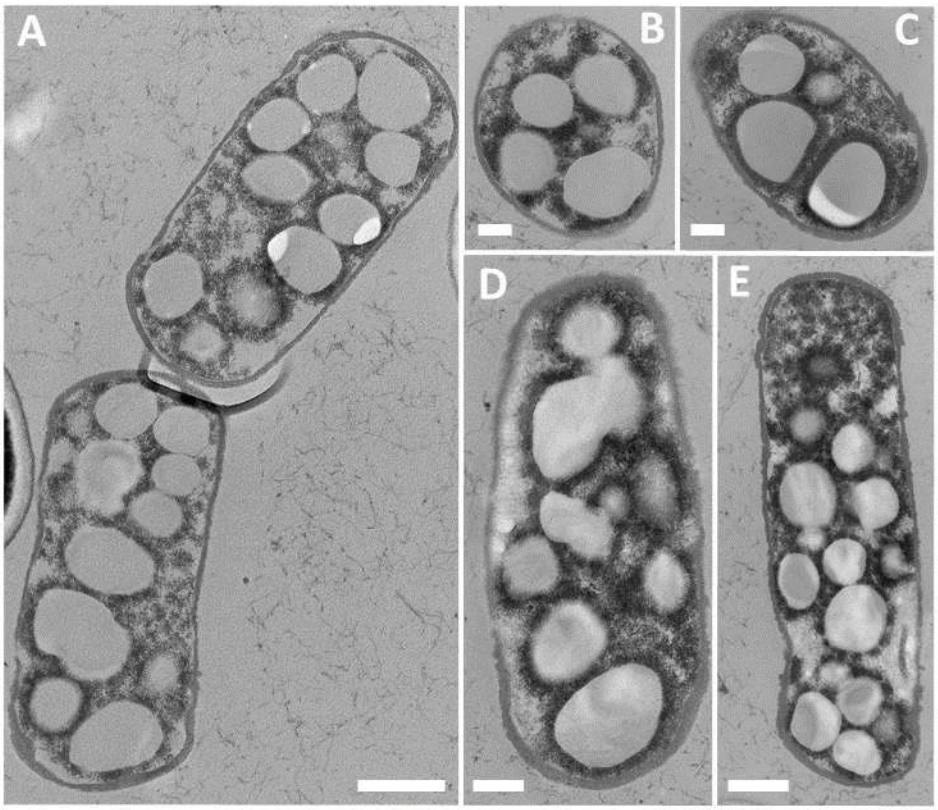


Figure 4

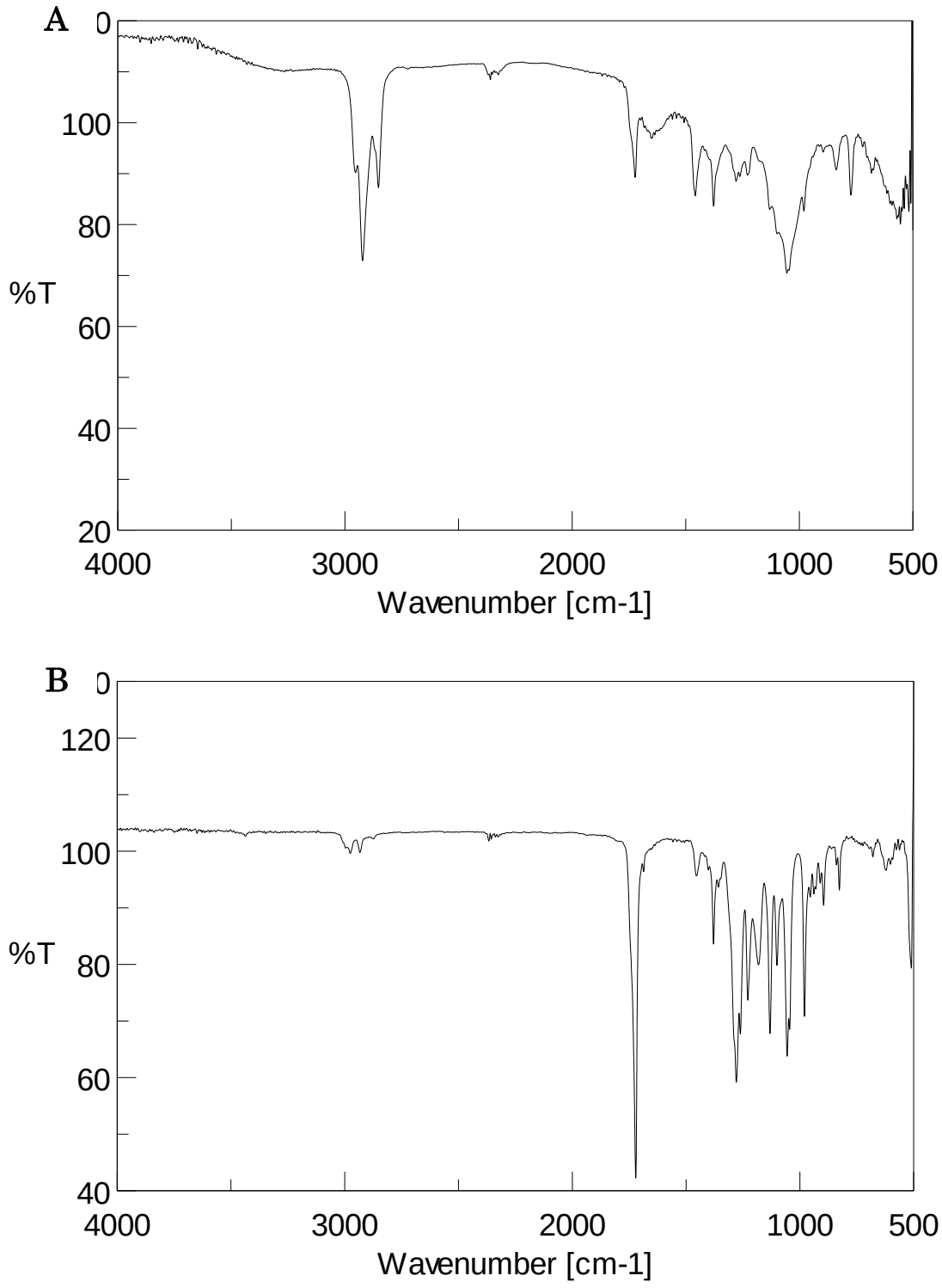

A
0
100
80
%T
60
40
20
4000
3000
2000
1000
500
Wavenumber [cm-1]
B
0
120
100
%T
80
60
40
4000
3000
2000
1000
500
Wavenumber [cm-1]

Figure 5

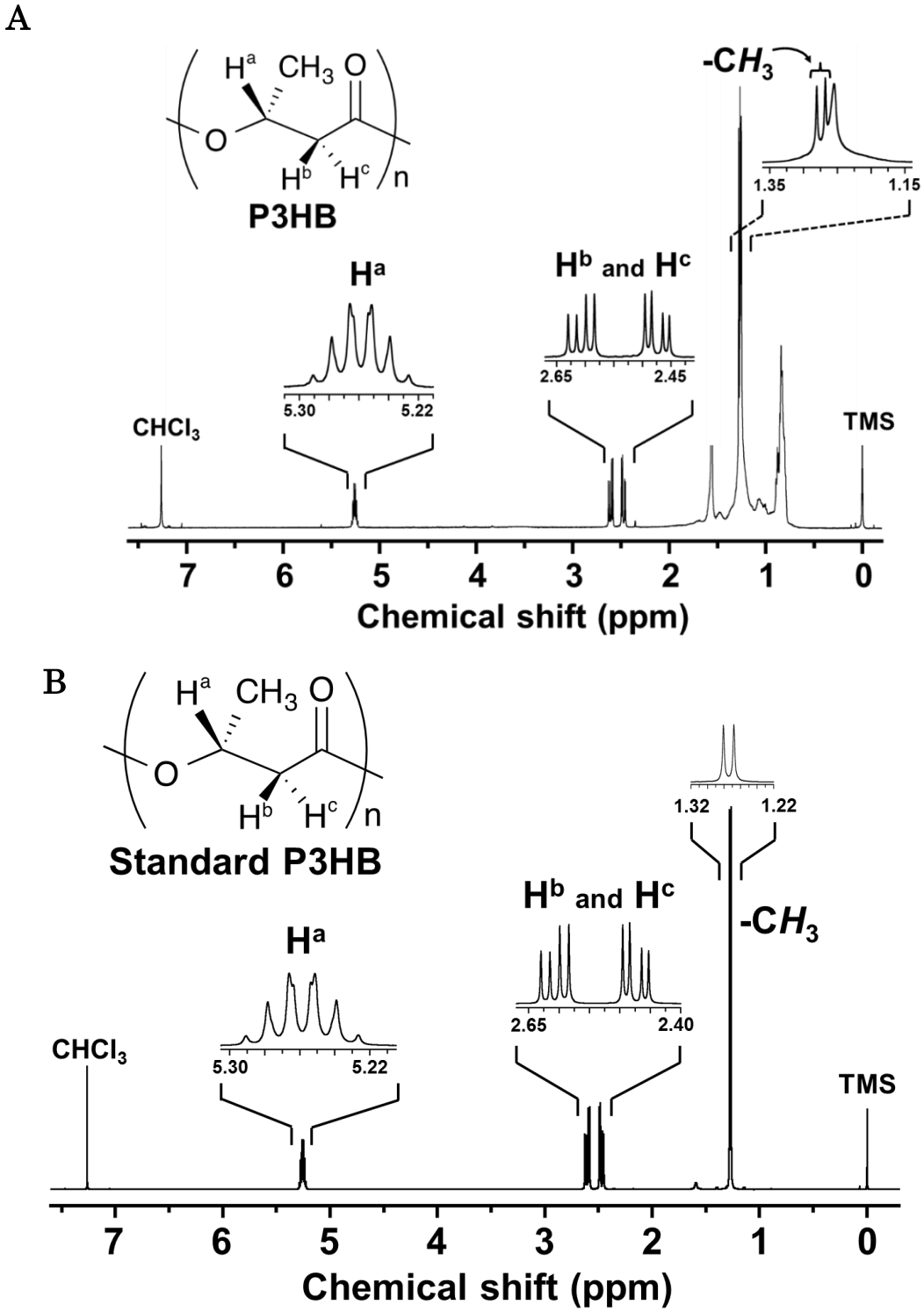

A
P3HB
-CH3
1.35
1.15
Ha
Hb and Hc
5.30
5.22
2.65
2.45
CHCl3
TMS
7
6
5
4
3
2
1
0
Chemical shift (ppm)
B
Standard P3HB
1.32
1.22
Hb and Hc
-CH3
Ha
2.65
2.40
CHCl3
5.30
5.22
TMS
7
6
5
4
3
2
1
0
Chemical shift (ppm)

Figure 6

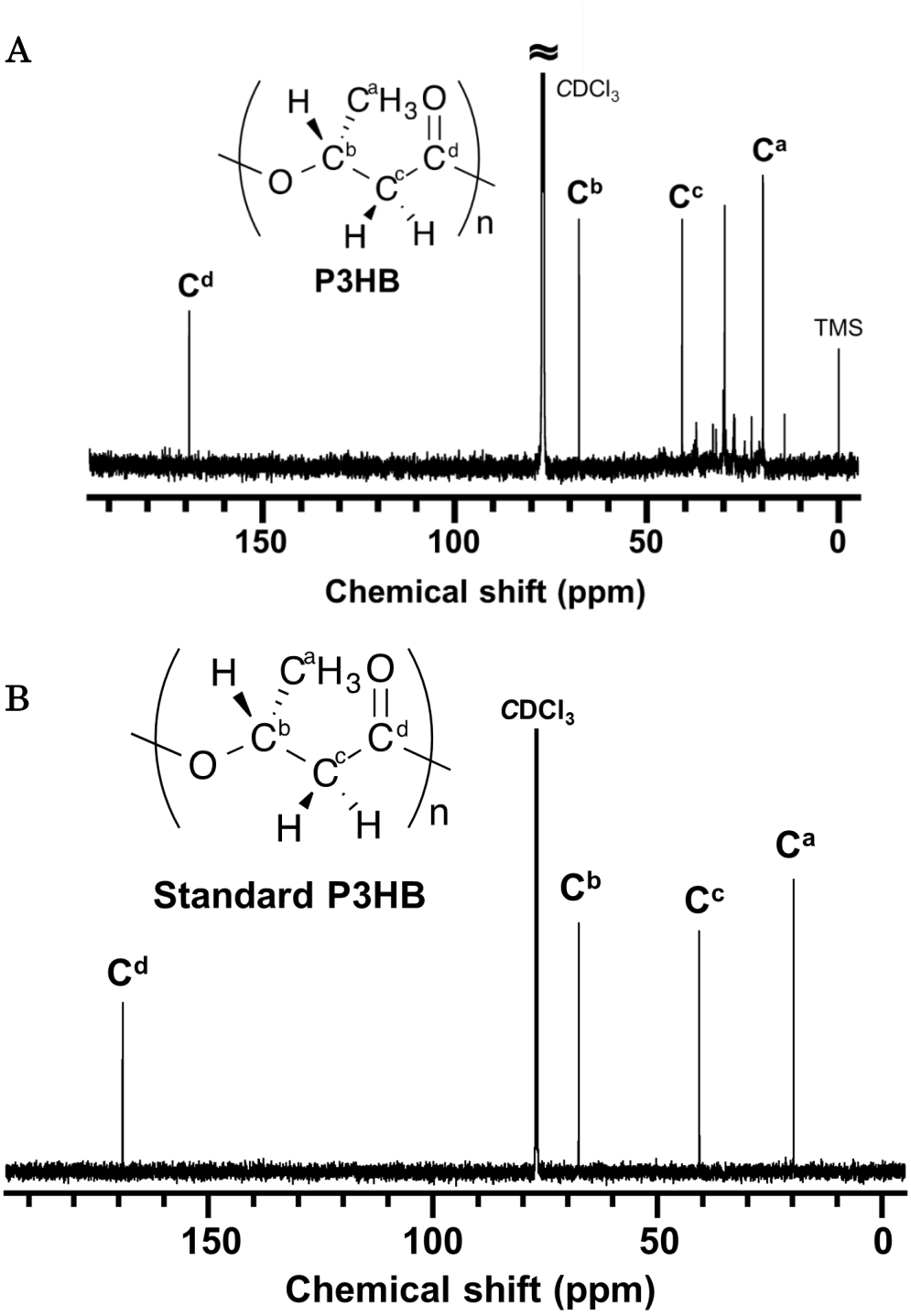

A
≈
CDCl3
Ca
Cb
Cc
Cd
TMS
P3HB
150
100
50
0
Chemical shift (ppm)
B
CDCl3
Standard P3HB
Chemical shift (ppm)

Figure 7

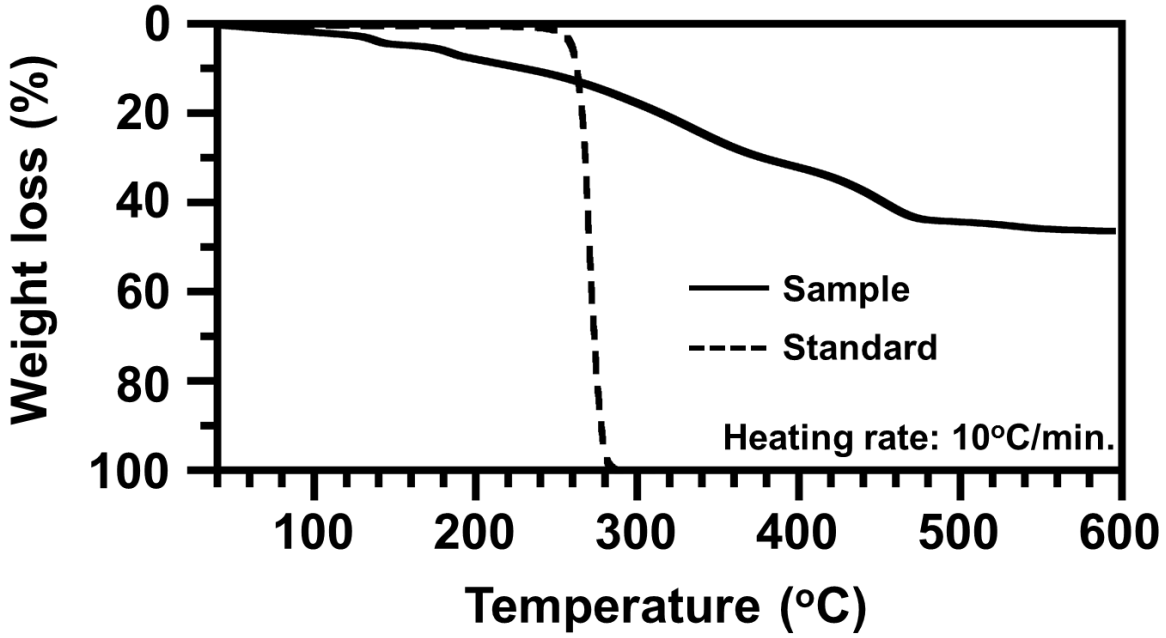

Weight loss (%)
0
20
40
60
80
100
100
200
300
400
500
600
Temperature (°C)
Sample
Standard
Heating rate: 10°C/min.

Figure 8

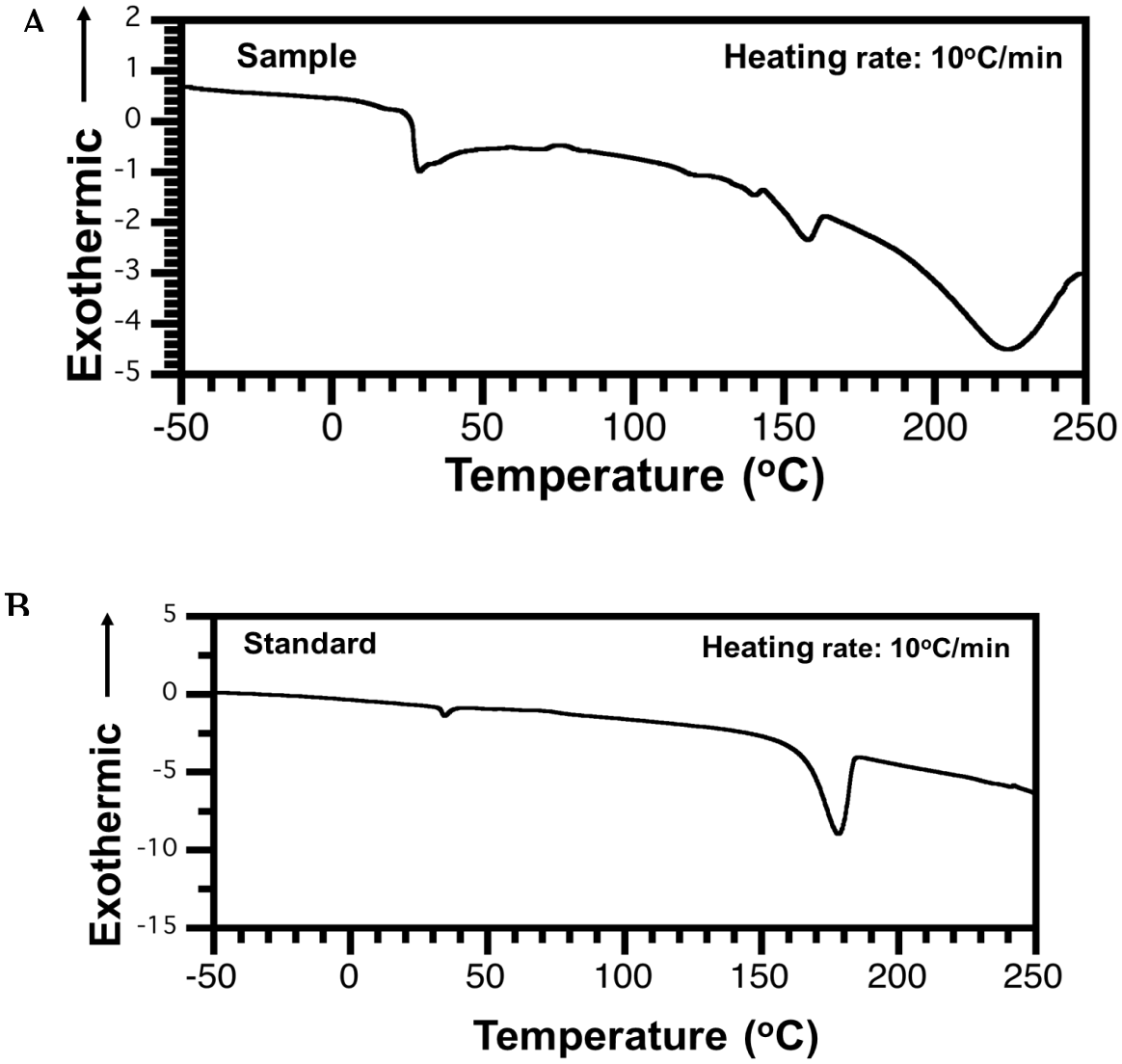

A
Sample
Heating rate: 10°C/min
Exothermic
2
1
0
-1
-2
-3
-4
-5
-50
0
50
100
150
200
250
Temperature (°C)
B
Standard
Heating rate: 10°C/min
Exothermic
5
0
-5
-10
-15
-50
0
50
100
150
200
250
Temperature (°C)

Figure 9

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	Phenol	277	Acetonitrile: water (8:2)	3
7	4-chlorophenol	280	Acetonitrile: 1% Ethylacetate (1:1)	3
8	Naphthalene	254	Acetonitrile: water (8:2)	2.1
9	Phenanthrene	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.

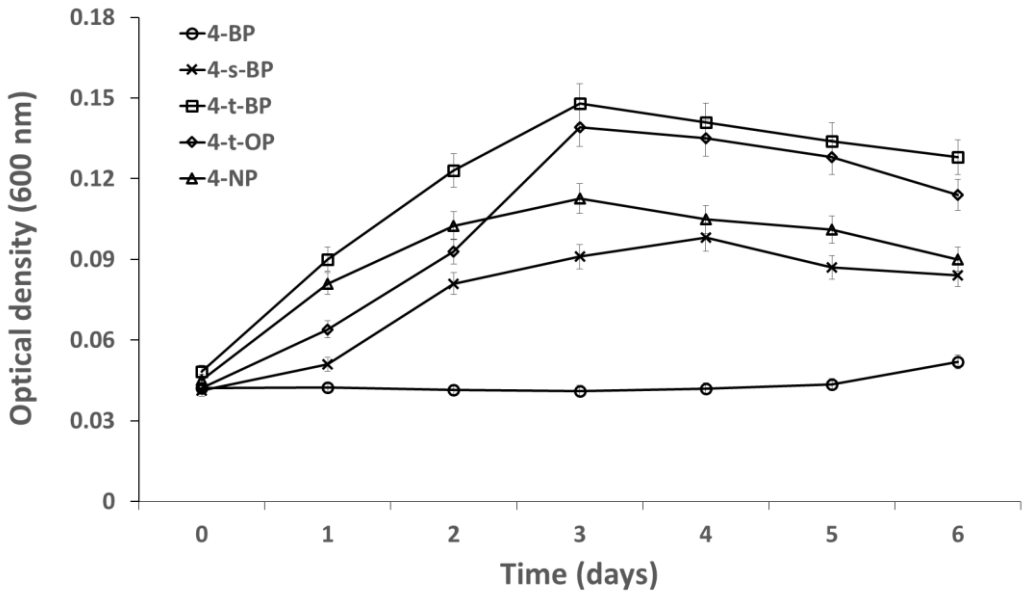


Figure S1: Growth curve of *Cupriavidus* sp. CY-1 with various alkylphenols. Bacteria were incubated at 100 mg/l concentration of different toxic compounds in MSM media without Tween-80 at 30ºC. The optical density (OD at 600 nm) of each sample was measured at different time intervals.

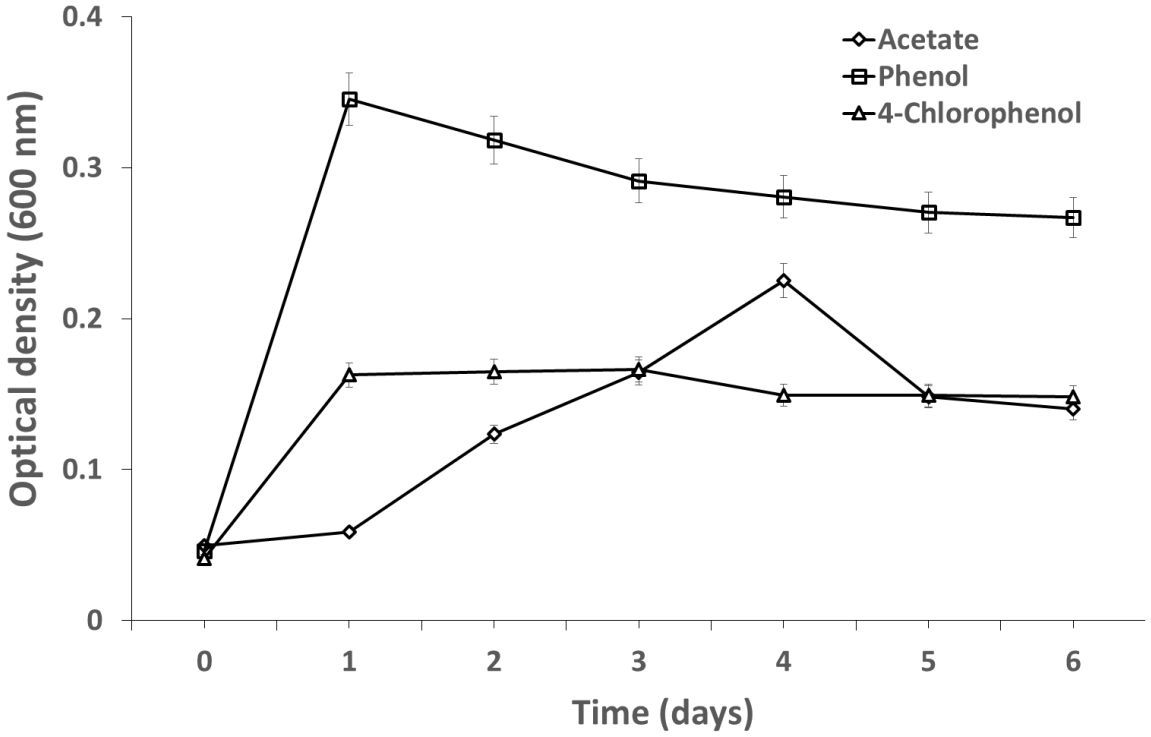


Figure S2: Growth curve of *Cupriavidus* sp. CY-1 with phenol, 4-chlorophenol and sodium acetate. Bacteria were incubated at 100 mg/l concentration of substrate in MSM media without Tween-80 at 30ºC. The optical density (OD at 600 nm) of each sample was measured at different time intervals.