Journal of Applied Biological Sciences 3(2): 79-84, 2009
ISSN: 1307-1130, www.nobel.gen.tr

Biodegradation of Bisphenol a by *Chlorella vulgaris* and *Aeromonas Hydrophilia*

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Received: April 25, 2009
Accepted: June 07, 2009

ABSTRACT

Bisphenol A (BPA) is an important raw material which has been used in plastic industry and released into environment by industrial applications. Endocrine disruptor chemicals (EDS) disturb endocrine functions in animals. BPA is a well known to be estrogenic-like EDS. In this study, biodegradation of BPA by *Aeromonas hydrophilia* and *Chlorella vulgaris* investigated. Biodegradation test result showed that BPA easily biodegraded by *A. hydrophilia* at 60 and 120 mgL^{-1} concentrations within 6 day and *C. vulgaris* at 20 mgL^{-1} concentrations within 7 day. No inhibitory effect on cell growth seen for *A. hydrophilia* but cell growth inhibition determined for *C. vulgaris* above 20 mgL^{-1} concentrations of BPA. The first-order kinetic model used to describe the biodegradation kinetic and it was fitted well with test results. 1-(3-methylbuthyl)-2, 3, 4, 6-tetramethylbenzene and 4-(1-hydroxy-2-methylprop-1-enyl) phenol were found to be biodegradation intermediate products. The estrogenic activities of BPA decrease after biodegradation and it could not be detect after 6 and 7 days.

Key Words: Aeromonas hydrophila, Bisphenol A, Biodegradation, Chlorella vulgaris, Endocrine Disrupter

INTRODUCTION

Environmental pollutants such as alkylphenolic compounds, polychlorinated biphenyls, dioxins, pesticides, textile dyes and heavy metals have been release into the environment. Most of these chemicals and/or their degradation products are toxic, mutagenic, carcinogen and endocrine disrupting (EDC) for aquatic and terrestrial life. Bisphenol A (BPA) is widely used monomer to produce of polycarbonate, epoxy resins, phenol resins, polycarbonates, polyacrylates, polyesters, powder paints and in thermal paper. It is also be used in dental sealing and as an antioxidant in plastics [1]. The contamination sources of BPA in aquatic environment are deriving from plastic waste such as polycarbonate bottles [2].

BPA is a well-known, estrogenic-like, environmental endocrine disruptor chemical (EDS). Mutagenesis and acute toxicity of BPA indicated between 1–10 μgmL^{-1} for freshwater and marine species [3]. BPA can harm to aquatic organisms and also cause brain damage and stimulates mammary gland development [4]. According to the U.S. Centres for Disease Control, 95% of Americans have detectable levels of BPA in their bodies [5]. Recent studies show that BPA related with breast cancer [6]. BPA exposure is linked to an error in cell division called aneuploidy, which causes 10-20% of all birth defects in people. In studies with mice, BPA causes aneuploidy even at extremely low doses [7].

Wenzel et al. reported that BPA concentrations on the surface waters in some E.U. countries, such as Austria,

Belgium, Switzerland, Germany and Netherlands reach up to 0.8 μgL^{-1} [8]. BPA is an important environmental chemical, for this reason removal of BPA from aqueous solution and fate of BPA in the aquatic environment is very important for human health. In this study, algal (*Chlorella vulgaris*) and bacterial (*Aeoromonas hydrophylia*) biodegradation of BPA and remove of estrogenic activity of BPA were determined.

MATERIALS and METHODS

Chemicals and Biodegradation Experiments

Analytic grade Bisphenol A (>99%) was obtained from Sigma–Aldrich Inc. BPA degrader bacteria were isolated from oil spilled soil by using enrichment culture [9]. API 20 NE (Biomerieux) was used to identification of BPA degrader bacteria and selected BPA degrader bacteria was identified as *Aeromonas hydrophylia*. Fresh water green algae *Chlorella vulgaris* obtained from algae culture laboratory faculty of Fisheries, Adana Turkey.

Algal and bacterial biodegradation experiments were conducted screw top flasks. PAS media was used to bacterial biodegradation study. Composition of PAS media are K_2HPO_4, 4.35gL^{-1}; KH_2P04, 1.7 gL^{-1}; NH_4Cl, 2.1 gL^{-1}; $MgSO_4$, 0.2 gL^{-1}; $MnSO_4$, 0.05 gL^{-1}; $FeSO\text{-}7H_20$, 0.01 gL^{-1}; and $CaCl_2\text{-}\ 2H_20$, 0.03 gL^{-1} [10]. Jaworsky media was used to algal BPA biodegradation study. Jaworsky media was prepared as 1 mL each of stock solution dilute to 1000 mL with distilled water. Stock solutions of $Ca(NO_3)_2{\cdot}4H_2O$ (4.0 g), KH_2PO_4 (2.48 g), $MgSO_4{\cdot}7H_2O$ (10.0 g), EDTAFeNa (0.45 g), $EDTANa_2$ (0.45 g), H_3BO_3 (0.496 g), $MnCl_2{\cdot}4H_2O$ (0.278 g), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.20 g), Cyanocobalamin (0.008 g), Thiamine-HCl (0.008 g), Biotin (0.008 g), $NaNO_3$ (16.0 g), $Na_2HPO_4{\cdot}12H_2O$ (7.2 g) were prepared in in 200 mL^{-1} distilled water. The pH of the culture media adjusted to 7.0±0.1 before sterilizations with dilute H_2SO_4 or NaOH solutions. Algal and bacterial growth was determined at OD_{660} and OD_{600}, respectively. Bacterial BPA biodegradation experiments were run at 60 and 120 mgL^{-1} initial BPA concentrations during 168 h at 25 °C. Algal biodegradation experiments were run 20 mgL^{-1} initial BPA concentrations during 216 h at 25 °C. Algal culture irradiate with cool day light (Philips) at 14 h light/10 h dark cycle. Variations of pH during biodegradation experiments were determined per day. Biomass free BPA contain Jaworsky and PAS media used to sterile control experiment to determine physico-chemical affects on BPA degradation. All experiments were run at least in duplicate and results were the means of the duplicate experimental results.

Analysis of Bisphenol A

Bisphenol A and intermediate products of biodegradation were determined by using GC and GC-MS (Thermo Trace GC and GC-MS).

BPA concentration was determined by GC after liquid-liquid extraction by dichloromethane (DCM). DCM liquid-liquid extracts were silylated by adding 50 µl of the Derivatisation mixture (N-Methyl-N-(trimethylsilyl)-trifluoroacetamide /trimethylsilylimidazole /dithioerytrol, (1000+2+2; v/v/w) at 60 °C for 1 h. The GC conditions were column ZB 35; 30 m x 0.25 mm ID; 0.25 µm film, (zorbax), helium as carrier gas flow rate 1.0 ml/min, injector 250 °C, detection 290 °C, and the oven temperature was initially held at 40 °C for 5 min, and then increased at a rate of 20 °C/min to 180 °C and held for 1 min, again increased at a rate of 2 to 250 °C and held for 10 min, again increased at a rate of 2 °C/min, and then finally held for 10 min. GC-MS results were obtained in the EI mode (70-eV).

Endocrine Disrupter Effects of BPA: Yeast Assay

The dichloromethane (Liquid-liquid) extracts were used to determine estrogenic activity by using yeast assay. Yeast assay was performed according to the method of Routledge and Sumpter [11]. Yeast estrogen screen (YES) assay is a widely used recombinant gene assay. Stock solutions of bisphenol A and degradation products were serially diluted in Dimethyl sulfoxide (DMSO). 5 µL samples were transferred to a 96-well optically flat bottom microtitre plate. Recombinant yeast stock was added to 45 mL growth medium and incubated overnight at 28 °C in until turbid. Assay medium was prepared by adding of the substrate Chlorophenol Red-β-D-Galactospyranoside (CPRG) to fresh growth medium. This medium was seeded with the overnight culture of yeast cells. 200 µL of the seeded assay medium was transferred to each well by using a multi-channel pipette. The plates were sealed with sterile sealing film and incubated at 32 °C in a naturally ventilated heating cabinet. After 3-day incubation of plates, the color development of the medium was measured by the absorbance at 540 nm for CPRG and 620 nm for turbidity.

RESULT and DISCUSSION

Biodegradation of Bisphenol A

A sterile control experiment was made in order to determine of BPA decomposition by physico-chemical reactions such as hydrolysis and oxidation. GC and GC-MS analysis showed that abiotic physico-chemical degradation was not occur. Initial concentrations of BPA were not change during biodegradation time. Similar results are reported by various researchers [12-13].

Inhibitory effects of BPA on bacterial growth was determined using 0.5-120 mgL^{-1} BPA contain PAS agar [10]. No inhibitory effect of BPA was seen at studied concentrations [10, 14]. Oil spill soil isolates bacteria *A. hydrophila* was well growth at 120 mgL^{-1} BPA concentrations and bacterial biodegradation experiments were studied at two different 60 and 120 mgL^{-1} initial BPA concentrations.

Effect of BPA concentrations on algal growth was determined in screw top flasks 5-80 mgL^{-1} BPA contain Jaworsky medium. Cell growth of *C. vulgaris* was limited above 20 mgL^{-1} BPA concentrations. For these reason 20

mgL^{-1} BPA concentrations was chosen for algal biodegradation experiment. Nakajima et al., (2007) indicate that, no inhibitory effect was seen up to 5 mgL^{-1} BPA concentrations for algal species but 10 mgL^{-1} BPA concentration limited growth of *P. Subcapitata*.

Biodegradation is quite an effective technique to remove of pollutants from aqueous solution. Bacterial biodegradation results were given in Figure 1. BPA concentrations were decrease with increasing of optical densities of culture. Optical densities of culture rapidly increase first 72 h. In parallel to increasing of biomass concentrations removal of BPA concentrations was screened. High amount of BPA was removed from aqueous solution between 48-72 h. Decreasing of BPA concentrations for 60 mgL^{-1} initial BPA concentrations was determined as 63 and 79% for 48 and 72 h, respectively. Decreasing of BPA concentrations for 120 mgL^{-1} initial BPA concentration was determined as 37 and 69% for 48 and 72 h, respectively. Kang and Kondo [15] report that, biodegradation of spiked BPA in the river water samples shows BPA is rapidly degraded under aerobic conditions and *Pseudomonas* sp. and a *Pseudomonas putida* strains are very effective microorganisms for biodegradation of BPA (about 90% within 5 days) in the river water.

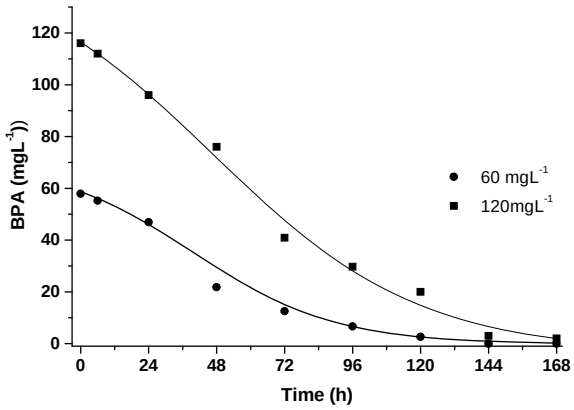

Figure 1. Bacterial biodegradation of BPA

Algal BPA degradation was studied at 216 h at 20 mgL^{-1} BPA concentrations. Algal BPA degradation results were given in Figure 2. Optical densities of algal culture are increase to first 120 h and optical densities of culture no more change after 120 h. High amount of BPA (>50%) degrade after 96 h and the concentration of BPA decrease below detection limit after 144 h. To determine whether reduction or accumulation of BPA by algal cultres radiolabeled BPA was used by Nakajima et al., [13]. They indicate reduction of BPA concentrations to be degree of reduction in the level of BPA in the culture was higher than the degree of accumulation in the cells.

Solution pH is very important parameter for cellular growth and dissolving of chemicals. pH variations of biodegradation media were determined during biodegradation study. pH of the microbial media was decrease from 7 to 6.5 ± 2 during bacterial biodegradation experiments.

Decreasing of the pH is not effect bacterial growth. During algal biodegradation study growth pH of the biodegradation media was increase from 7 to 9. This is result from dissolving of CO_2 as bicarbonate ion.

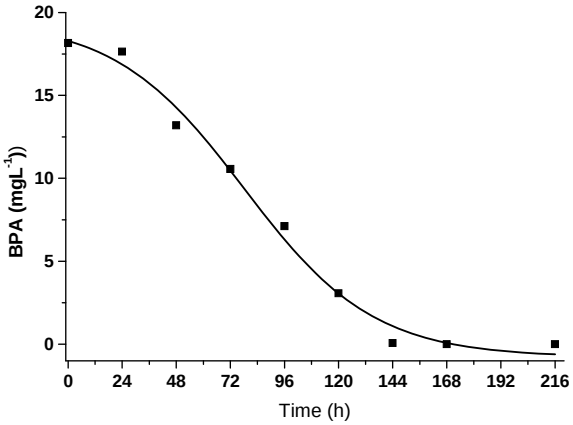

Figure 2. Algal biodegradation of BPA

Kinetic Modelling

First order kinetic model was used to determine biodegradation kinetic. The first-order kinetic equation is,

$$\ln C = -Kt + A \quad (1)$$

Where C is initial concentration of BPA, K is first-order kinetic constant, t time and A is constant. The biodegradation half-life of first-order reaction of the BPA can be expressed as:

$$t_{1/2} = \ln 2/K \quad (2)$$

Plots of linear form of the first order kinetic model were given in Figure 3. The results are indicating that the first-order model give a better fit. The biodegradation kinetics of BPA can be described by a first-order reaction model under the experimental conditions. The algal BPA degradation kinetic constant K, calculated BPA concentrations C and correlation coefficient r were determined as 0.0122, 21.5 mgL^{-1} and 0.973, respectively. Bacterial biodegradation kinetic results (60 mgL^{-1} BPA concentration) were determined as 70.1 mgL^{-1}, 0.257 and 0.980 for constant K, calculated BPA concentrations C and correlation coefficient r, respectively. Bacterial biodegradation kinetic results (120 mgL^{-1} BPA concentration) were determined as 121.5 mgL^{-1}, 0.0151 and 0.978 for constant K, calculated BPA concentrations C and correlation coefficient r, respectively.

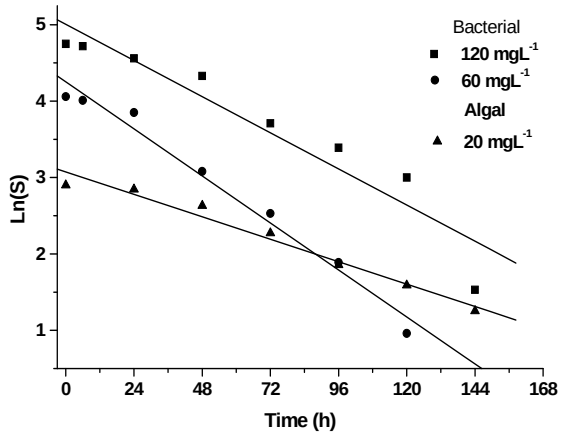

Figure 3. First-order kinetic model of bacterial and algal BPA biodegradation

Algal and bacterial biodegradation half lives of BPA shows BPA can not persisted in aquatic environment. Half-lives of the bacterial BPA biodegradation were determined as 27 h and 46 h for 60 and 120 mgl^{-1} concentrations, respectively. Half-life of algal BPA biodegradation was determined 57 h. Kang and Kondo [15] was indicate that half-lives of BPA degradation at 25 and 35°C were 96 and 72 h, respectively.

Endocrine Disrupter Activity and Biodegradation Products of BPA

The removal of estrogenic activities of BPA is very important. Endocrine disrupter chemicals have adverse effects on aquatic organisms and human health. In-vitro YES test is very effective and easily application for determine estrogenic activity of any chemical. In this test, human oestrogen receptor (hER) is expressed in yeast [11]. Comparison of estrogenic activity of BPA and 17β-estradiol was given in Figure 4. According to Figure 4 estrogenic activity of BPA 100 times lower than 17 β-estradiol. Removal of estrogenic activity of BPA was determined during algal and bacterial biodegradation experiments. Estrogenic activity results were given in Figure 5-6.

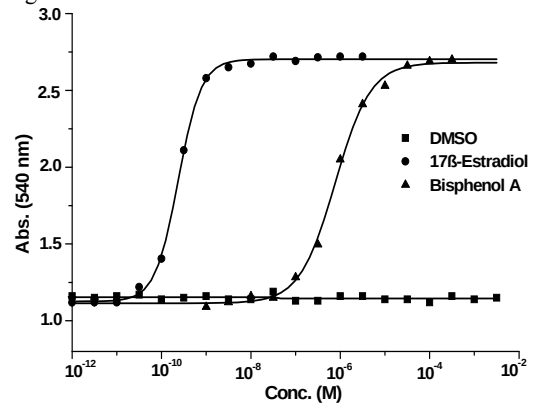

Figure 4. Comparison of estrogenic activity of BPA and 17β-estradiol

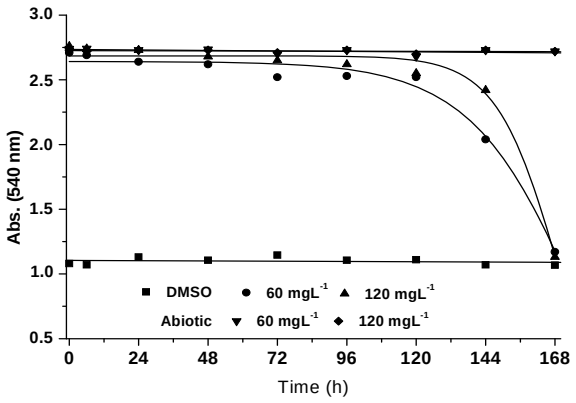

Figure 5. Estrogenic activity results of bacterial biodegradation study

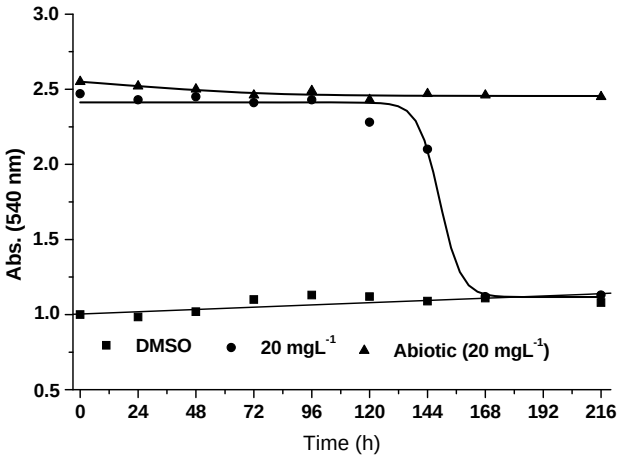

Figure 6. Estrogenic activity results of algal biodegradation study

Estrogenic activity of BPA decreases with microbial treatment of *A. hydrophila* and *C. vulgaris*. Estrogenic activity decreases after 72 and 120 h at 60 and 120 mgL^{-1} BPA concentrations with bacterial biodegradation (Figure 5), respectively. Similar result obtained from algal biodegradation studies and estrogenic activity decreases after 120 h. No estrogenic activity was detect after 168 h treatment in both Algal and bacterial biodegradation experiments (Figure 6). Estrogenic activity of the control samples (abiotic) not changed during biodegradation experiments. The estrogenic activity of BPA is greatly decrease by algal and bacterial biotransformation. Similar result indicated by previous publication of Nakajami et al. [13].

Degradation products formed during the biodegradation of BPA were analyzed by GC/MS. Three main compounds detected in the solution were presented in Figure 7.

Determined bacterial biodegradation product of BPA was 1-(3-methylbuthyl)-2,3,4,6-tetramethylbenzene. Bacterial biodegradation products of BPA were indicated by various researchers as 4-hydroxy benzoic acid, 4-hydroxy acetophenon [10], 4-hydroxybenzaldehyde [16].

Algal degradation product of BPA was determined as 4-(1-hydroxy-2-methylprop-1-enyl) phenol. Hirooka et al. [17] reported that *Chlorella fusca* transformed BPA to monohydroxyl BPA.

Primary degradation products of BPA was determined to be 4-hydroxybenzoic acid and 4-hydroxyacetophenone by various researchers [14-16]. Bisphenol A easily degrades by microorganisms and biodegradation products have not estrogenic activity [10,16, 18].

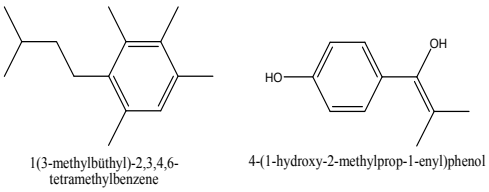

Figure 7. Bacterial and algal biodegradation intermediate products

CONCLUSIONS

Bisphenol A (BPA) is well known environmental chemical which is used variety industrial applications. Algal (*Chlorella vulgaris*) and bacterial (*Aeromonas hydrophilia*) biodegradation of bisphenol A were studied in batch biodegradation experiments. First-order kinetic model was fit well to the algal and bacterial biodegradation of BPA. The primary degradation products were 1(3-methylbuthyl)-2,3,4,6-tetramethylbenzene and 4-(1-hydroxy-2-methylprop-1-enyl)phenol. In this study, algal and bacterial biodegradation results shows that BPA concentrations was decrease to below detection limits after 168 h. this indicate that BPA can easily be eliminated in the aquatic environment. *Aeromonas hydrophila* and *Chlorella vulgaris* are effective organisms for removal of estrogenic activity of BPA.

ACKNOWLEDGEMENTS

This study (PhD thesis) was supported by the Cukurova University research found (FBE 2004-D14).

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