

## Evaluation of Cyto-, Geno- and Ecotoxicity of Bio-oil from the Fast Pyrolysis of Radiata Pine

Sun-Young Park, Joo-Sik Kim, Young-Kwon Park and Jinhee Choi\*

Faculty of Environmental Engineering, College of Urban Science, University of Seoul,  
Dongdaemun-gu, Seoul 130-743, Korea

### Radiata pine의 fast pyrolysis 공정에서 얻어진 bio-oil의 세포독성, 유전독성 및 생태독성 평가

박선영, 김주식, 박영권, 최진희\*

서울시립대학교 환경공학부 환경독성학연구실

#### 요 약

국내에서 목질계 바이오매스는 유망한 재생가능한 자원이다. Fast pyrolysis을 통한 radiata pine 톱밥의 bio-oil의 전환은 벤치스케일의 유동층 반응기를 이용하였다. 이 실험에서 얻어진 bio-oil은 주로 산, 페놀, 알킬페놀 등을 포함하고 있었고, 세포생존율실험, comet assay, 물벼룩 급성유영저해실험을 이용하여 각각 세포독성, 유전독성 및 생태독성을 평가하였다. Bio-oil의 액상부분은 타르 부분보다 세포독성과 유전독성이 더 높게 나타났고, 반면 타르부분은 액상부분에 비해 생태독성이 높게 나타났다. 본 연구에서 얻어진 결과를 통해 pyrolysis 생성물에 대한 다양한 독성영향을 확인할 수 있었으나, 보다 다양한 독성 지표의 적용이 필요할 것으로 보인다.

**Key words** : bio-oils, radiata pine, fast pyrolysis, toxicity

#### INTRODUCTION

The need for resource conservation has promoted the replacement of fossil fuels whose reserves can not meet future demands. Biomass is recognized as a promising alternative fuel, which is renewable and environmentally friendly. New technologies exist for converting biomass into valuable products, of which thermochemical processes are the most common route

for converting biomass into energy. Pyrolysis, one of these processes, is a promising tool for providing bio-oil that can be used as an alternative fuel oil or chemical feedstock. Fast pyrolysis, in particular, can provide a higher heating rate and short residence time of gas-phase products for obtaining bio-oil in high yields. Over the last two decades, comprehensive research on fast pyrolysis has focused on understanding the complexity of the mechanism in relation to the use of biomass, obtaining the optimal reaction conditions for bio-oil production, and the quantitative and qualitative analyses of its composition (Evans and Miline, 1987; Radlein *et al.*, 1991; Demirbas, 2000).

\* To whom correspondence should be addressed.  
Tel: +82-2-2210-5643, Fax: +82-2-2244-2245  
E-mail: jinchoi@uos.ac.kr

Despite the intensive attention paid toward pyrolysis products as promising renewable energy sources, little information is available on the potential toxic effects of these chemicals on living organisms. The potential deleterious effects of pyrolysis product on human and ecological health need to be identified, prior to their widespread use as renewable energy. However, the high complexity of pyrolysis products limits the direct analysis and identification of their components using a single technique; thus, we used a combined chemical analysis method exploiting GC-MS and short-term bioassays using cyto-, geno-, and ecotoxicity tests. Short-term bioassays, which are considered complementary to conventional chemical analysis, constitute valuable tools for the evaluation of the toxic effects of diverse types of pollutants on living organisms. This method is especially appropriate for quickly screening any hazardous effect of a large number of chemicals.

The aims of this work were: 1) to determine the components of the bio-oil produced from the fast pyrolysis of radiata pine using a GC-MS method, 2) to evaluate the cyto- and genotoxicity of the bio-oil using the MTT test and comet assay on mammalian cells, L5178Y and 3) to evaluate the ecotoxicity of the bio-oil on an environmental monitoring species, the freshwater crustacean, *Daphnia magna*.

## MATERIALS AND METHODS

### 1. Radiata pine

A sample of radiata pine was obtained from a sawmill, the characteristics of which are shown in Table 1. The size of the feed material was around 1~2 mm. The sawdust contained no sulfur and was almost free of nitrogen; the ash content was about 0.2 wt %. Among the alkali and alkali earth metals, the contents of calcium and potassium were both relatively high.

### 2. Bench scale pyrolysis of plant and bio-oil

For the pyrolysis experiments, a bench scale plant

**Table 1.** Analysis data of radiata pine

Composition (wt %)	
Cellulose	44.8
Hemicellulose	34.1
Lignin	27.5
Proximate analysis (wt %)	
Water	7.61
Organics	92.20
Ash	0.19
Elemental analysis (wt %)	
C	44.8
H	5.9
N	0.1
O	46.2
Alkali and alkali earth metal concentration (ppm)	
Na	46.8
Mg	165.3
Ca	491.4
K	891.7

with a 3 kg/h capacity fluidized bed was used. The plant was equipped with a char removal system, a series of quencher and a gas-regulating system. Sand, with a mean particle size of about 0.4 mm, was used as the fluidized bed material, and nitrogen gas was served as the fluidizing medium, with a reaction temperature 550°C. Fig. 1 shows a schematic diagram of the plant. The bio-oil produced after pyrolysis was separated into two phases in a collecting vessel. The upper bio-oil phase was clear and fluid; the other was composed mainly of tar, with a small amount deposited in the vessel.

### 3. GC-MS analysis

For identification and quantification of the bio-oil components, a GC-MS system was employed. In the GC-MS analysis, a sample of the bio-oil was initially well mixed, and then diluted with 500 mL of acetone to dissolve the high molecular weight tar. After dilution, 0.2 µL of the bio-oil sample was injected with a microsyringe on to an HP-5973 column (50 m × 0.32 mm × 0.52 µm) employing a total ion current MS detector.

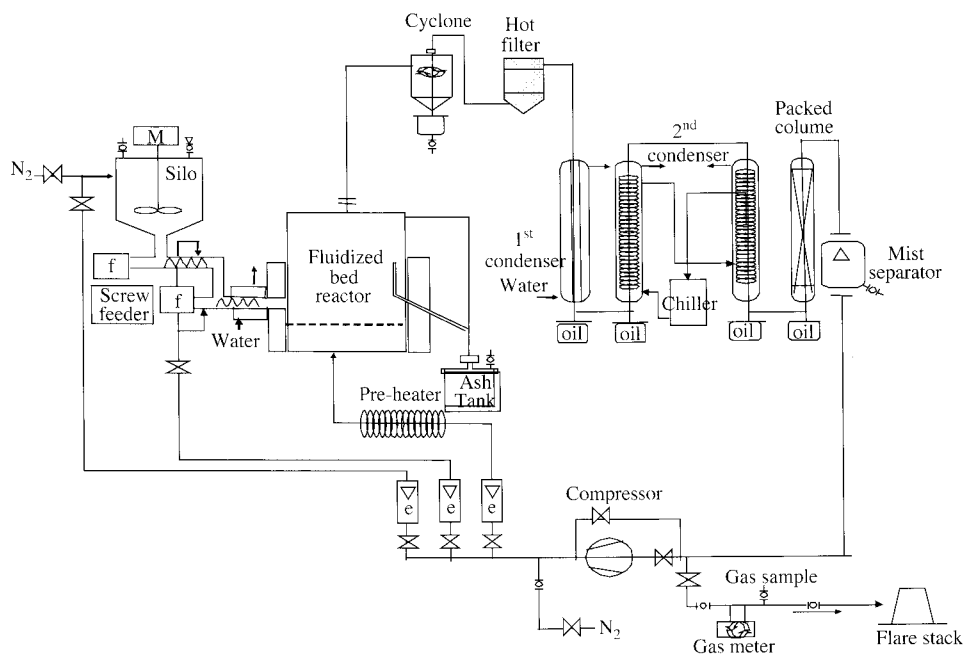


Fig. 1. Schematic diagram of bench-scale pyrolysis plant.

#### 4. Cell culture and cell treatment

The mouse lymphoma L5178Y cells were maintained in RPMI1640 (GIBCO BRL Life Technologies), supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotics, at 37°C in a 5% CO<sub>2</sub> atmosphere. The bio-oils from the upper (liquid fraction; 200, 100, 50, 25, 12.5 mg/L and lower phases (tar fraction; 500, 200, 100, 25, 12.5, 6.25 mg/L) and lower phases (tar fraction) were used to treat the L5178Y cells. Treated and control cells were incubated for 2 hours, and then harvested for the analysis.

#### 5. Trypan blue exclusion assay

For the determination of cell viability, about 10<sup>6</sup> cells were treated for 2 hours with the bio-oil. After the staining of 0.4% trypan blue (GIBCO BRL life technologies, MD, USA), the total number of cells and the number of unstained cells were counted of four of the major sections of a hemocytometer. The average number of cells per section was calculated. Cell viability was treated with the solvent.

#### 6. Comet assay

About 1 × 10<sup>6</sup> cells/well were seeded in six well plates and incubated for 24 h prior to treatment with the bio-oils. After treatment, the cells were washed with PBS, the cell suspension immediately mixed with 100 μL of 1% low-melting-point (LMP) agarose for use in the comet assay. An alkaline comet assay was performed, as described by Singh *et al.* (1988). Briefly, 100 μL of 1% LMP agarose was spread onto a normal agarose pre-coated microscope slide and incubated at 4°C for 5 min to allow solidification. The cells were lysed in high salt and detergent (10 mM Tris, 100 mM EDTA, 2.5 NaCl, 10% DMSO, 1% Triton × 100, 1% N-lauroylsarcosinate, pH 10), and subsequently exposed to alkali condition (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 min at 4°C to allow the DNA to unwind and alkali-labile sites to be expressed. For electrophoresis, an electric current of 300 mA (25 V) was applied for 20 min, after which the slides were neutralized and dehydrated in 70% ethanol. The slides were stored in a dry place prior to image analysis. Before their analyses, the slides were

stained with 50- $\mu$ L ethidium bromide (5  $\mu$ g/mL), then analyzed using a fluorescence microscope (Nikon, Kanagawa, Japan) equipped with an excitation filter of BP 546/12 nm and a barrier filter of 590 nm at 400  $\times$  magnification. Approximately, 25 cells per slide (3 slides per treatment) were examined. DNA damage was expressed as the olive tail moment using an image analysis computerized method (Komet 5.5, Kinetic Imaging Limited, Nottingham, UK).

### 7. *Daphnia* culture

Using an original strain provided by the Korea Institute of Toxicology (Daejeon, Korea), *D. magna* larvae were obtained from adults reared in our laboratory. *D. magna* were individually placed in glass beakers, containing aerated M4 culture media, for 2 days. Cultured daphnids were fed every 2 days on the green alga, *Chlorella* sp., at concentrations of  $1 \times 10^6 \sim 10^9$  cells/mL. The *D. magna* culture was maintained at  $20 \pm 1^\circ\text{C}$ , with a 16 h light and 8 h dark cycle photoperiod regime.

### 8. *Daphnia* immobility test

The experiment was conducted at a constant temperature of  $20 \pm 1^\circ\text{C}$  under 16 h light and 8 h dark conditions. Neonates less than 24 h old were used to assess acute toxicity. Three replicates were prepared for each concentration. Acute toxicity tests were performed in accordance with the standard protocol for *D. magna* acute tests (OECD, 1984). Ten neonates aged less than 24 h were individually transferred into 100 mL glass beakers filled with 50 mL of test solution and incubated at  $20 \pm 1^\circ\text{C}$  for 24 h. The 24 h EC50 value was determined for swimming inhibition of daphnids using the probit method recommended by the OECD (1984).

### 9. Data analysis

The data passed the normality test and the equal variance test. Statistical differences between the control and the treated larvae were examined using varia-

tion analysis with Dunnett's multiple comparison test. A parametric Pearson test was conducted to study correlations among the parameters. All statistic tests were performed using SPSS<sup>®</sup> 12.0 KO (SPSS Incorporated, Chicago, IL, USA).

## RESULTS AND DISCUSSION

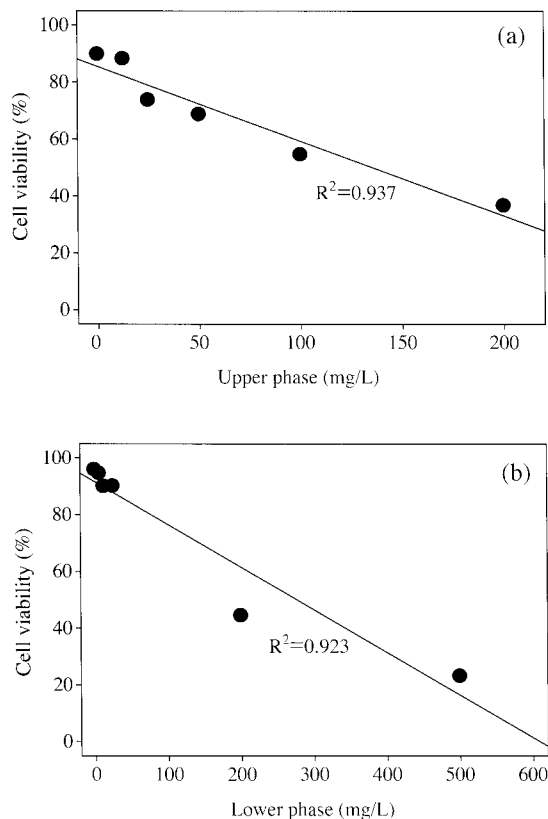
### 1. Characteristics of bio-oil

The bio-oil had a high water content, about 40 wt%, and about a 0.1 wt% solid content. The alkali and alkali earth metal contents of the bio-oil were less than 1 mg/L, with the exception of Na, most of which seemed to result from the glass ware used. Bio-oil

**Table 2.** Major composition of the bio-oil from radiata pine

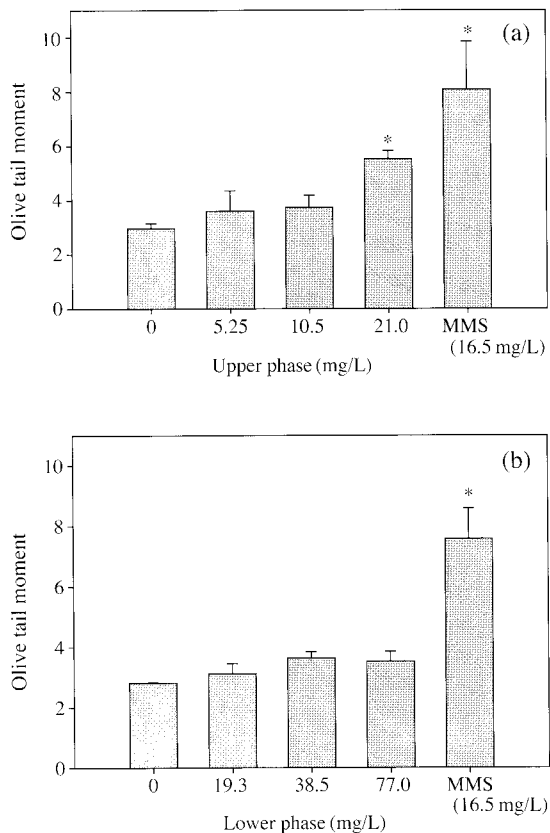
Components	Area (%)	
	Upper phase	Lower phase
Propanal	0.42	0.89
2-Butenal	0.97	0.15
Acetic acid	3.46	n.d.
Propanoic acid	0.87	n.d.
1,4-dimethylpyrazole	3.54	n.d.
2-cyclopenten-1-one	5.42	n.d.
2-Furancarboxaldehyde	0.23	1.87
2-Furanone	n.d.	0.69
2-hydroxy-2-cyclopenten-1-one	1.04	0.78
2-ethylfuran	0.09	n.d.
Phenol	4.62	0.58
Methyl-2-cyclopenten-1-one	2.77	0.70
Methyl-2-Furancarboxaldehyde	2.11	0.72
2-hydroxy-3-methyl-2-cyclopenten-1-one	4.64	0.85
Methylphenol	5.62	1.62
2-methoxyphenol	0.88	3.07
2-methoxy-4-methoxyphenol	0.64	4.35
Ethyl-4-methoxyphenol	0.54	2.10
1,2-benzenediol	2.1	1.47
2-methoxy-4-(2-propenyl)-phenol	0.25	3.10
2-methoxy-4-propylphenol	0.29	2.82
2-methoxy-4-(1-propenyl)-phenol	0.35	9.13
Dimethylnaphtalene	2.82	n.d.
Methylbenzenediol	0.94	1.41
Fluorene	n.d.	0.2

n.d.: non detected



**Fig. 2.** Cell viability measured after treated with of upper phase (a) and lower phase (b) bio-oil (wood) for 2 hr in L5178Y cells (solvent: DMSO).

contains hundreds of components with widely varying polarities and volatilities; therefore, accurate quantitative and qualitative analyses of bio-oil, using only a GC-MS system, currently seem almost impossible. However, the results shown in Table 2 give the general composition of the bio-oil produced from fast pyrolysis. In Table 2, only the area percentage of each components obtained from the total ion current of MS detector is indicated due to the difficulty in obtaining the response factor of every component. The components with a small area percentage have been omitted. The main components found in the upper phase (liquid fraction) were acetic acids, dimethylpyrazole, cyclopenten-1-one, phenol, hydroxy-3-methyl-2-cyclopenten-1-one and methylphenol. In contrast, the lower phase (tar fraction) contained large



**Fig. 3.** Olive tail moment value measured in cells pretreated with the upper phase (a) and the lower phase (b) of bio-oil (wood) for 2 h in L5178Y cells (solvent: DMSO). Olive tail moment value: mean  $\pm$  standard deviation. \*Statistically significant difference and duncan's multiple range test ( $p < 0.05$ ). ONE WAY ANOVA). MMS (Methylmethanesulfonate) was used as the positive control.

amounts of various phenols, especially methoxy-4-(1-propenyl)-phenol. The results indicated that the degradation products from the lignin fraction in the wood are mainly deposited as tar in the lower phase.

## 2. Cytotoxicity and genotoxicity

The potential human health effects of the wood pyrolysis products were preliminary screened by cell viability (Fig. 2) and genotoxicity tests (Fig. 3) using the mammalian cell line, L5178Y. EC50s values of the cell viability for the liquid and tar fractions were

135 and 279 mg/L, respectively (Fig. 2). The cytotoxic effects of bio-oil may indicate early cellular changes, with possible biological consequences, which should be considered in the preliminary evaluation of the risk of populations exposed to these chemicals *in vivo*. Despite the constraints in the extrapolation of *in vitro* to *in vivo* data in humans, the cytotoxicity of the studied compounds requires special attention in view of the major damage they cause to cell function, which result in the inability of cells to proliferate. These disturbances frequently appear long before genotoxic effects manifest, or even in the absence of the latter. Thus, the cytotoxic effect may be considered an earlier indication of cellular damage, with possible biological consequences; therefore, this should be taken into account in the preliminary evaluation of the risk to populations exposed *in vivo*, as has already been suggested.

DNA damage, particularly DNA strand breaks, was measured using a single cell gel electrophoresis (comet) assay to evaluate whether the pyrolysis products induced any genetic toxicity (Fig. 3). Genotoxic parameters are currently the most valuable biomarkers for environmental risk assessment, and many reports linking the DNA damage to subsequent molecular, cellular and tissue level alteration have been published (Ohe *et al.*, 2004). DNA strand breaks are potential pre-mutagenic lesions, which are sensitive markers of genotoxic damage. Among the available genotoxicity indicator tests, the comet assay has recently attracted much attention. The comet assay, also called the single-cell gel electrophoresis (SCGE) assay, primarily measures DNA strand breakage in single cells. The comet assay has been shown to respond quickly and accurately, and its findings are easily measured. Thus, since the protocol was first published by Singh *et al.* (1988), it has been increasingly used in different fields of study: clinical applications, human monitoring, radiation biology and genetic toxicology (Cotelle and Ferard, 1999; Tice *et al.*, 2000; Brendler-Schwaab *et al.*, 2005; Møller, 2006). Most related studies conducted so far have demonstrated the genotoxic effects of wood smoke associated with

**Table 3.** Estimation of effective concentrations of bio-oil in *D. magna*

		24 h EC (mg/L)	95% Confidence interval
Upper phase	EC10	38.51	21.12 < EC50 < 46.71
	EC50	55.50	45.19 < EC50 < 68.96
	EC90	80.01	67.02 < EC50 < 134.35
Lower phase	EC10	14.12	6.816 < EC50 < 18.51
	EC50	23.17	17.34 < EC50 < 30.88
	EC90	38.02	29.04 < EC50 < 78.27

emissions from combustion, gasification, pyrolysis systems or slow pyrolysis products (Mezerette and Girard 1991; Pimental *et al.*, 2000; Glatt *et al.*, 2005), but none have evaluated the chemical compounds found in the products from the fast pyrolysis of wood. Fig. 3 shows that the liquid fraction of bio-oil (upper phase) seems to have a genotoxic property, since the Olive tail moment obtained from the comet assay increased significantly for the cell exposed to the highest concentration, whereas, no genotoxic effect was observed for the tar fraction (lower phase) at any of the concentrations studied. Taking into account the overall cytotoxicity and genotoxicity data, exposure of L5178Y cells to bio-oil is sufficient for the expression of noticeable genotoxic and cytotoxic effects and the tar seems to be less toxic than the liquid fraction, which might be due to the main components of the upper phase of the bio-oil, these being acetic acid, cyclopenten-1-one, phenol and methylphenol (Table 2). However, caution should be taken if using the present results to estimate the risk to populations exposed to low doses of these compounds, as the appropriate assessment of the genotoxic potential of an agent requires the use of different assays that will permit the evaluation of different genetic events in different cell types.

### 3. Ecotoxicity

The deleterious effects on ecological health were evaluated using a simple ecotoxicity test of the total pyrolysis product utilizing the water flea, *D. magna*. The small-sized crustacean *Daphnia* plays a pivotal

role in aquatic food webs and has been widely used as a model species for water quality biomonitoring (Dutka *et al.*, 1983; Giesy, *et al.*, 1988; Atienzar *et al.*, 2001). The use of *D. magna* as an ecotoxicity model species may provide an insight into the potential toxic effect of bio-oil in water, since this species holds an important position in aquatic ecosystems. The acute toxicity of the bio-oil on *D. magna* was studied using EC50 derived from a probits analysis (Table 3). The 24 h EC50s of the pyrolysis product on *D. magna* were 55 and 23 mg/L for the liquid and tar fractions, respectively. As far as the ecotoxic-properties are concerned, the tar fraction of the pyrolysis products seems to be more toxic than the liquid fraction, but the opposite was the case with the cyto- and genotoxic results, as shown in Fig. 2 and 3. These phenomena suggest that *Daphnia* might react more sensitively to methoxy substituted phenols, which were abundantly presented in the lower phase of the bio-oil, than to the other compounds found in the upper phase (Table 1). Given the importance of pyrolysis products as promising renewable energy sources, studies of the potential toxic effects of these chemicals on living organisms can provide valuable information.

## CONCLUSION

In this study, the components of the bio-oil from the fast pyrolysis of radiata pine were determined and their cyto-, geno- and ecotoxic properties were investigated. The main components in the upper phase of the bio-oil were acetic acid, cyclopenten-1-one, phenol and methylphenols. In contrast to the upper phase, the lower phase of the bio-oil contained a large amount of methoxy substituted phenols. The upper phase seemed to be more cyto- and genotoxic than the lower phase, whereas, the lower phase seemed to be more ecotoxic than the upper phase of the pyrolysis products. The results obtained from this study could constitute an important contribution to knowledge of the toxicology of pyrolysis products, inclu-

ding aquatic toxicity, for which little data are available.

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