

Degradation of Humic Acid in Soil Aqueous Extract Using the Fenton Reaction and a Microbiological Technique

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ABSTRACT

In this study, the degradation of humic acid (HA) extracted from soil in aqueous solution was carried out using the Fenton reaction and a microbiological technique. The Fenton reaction produced hydroxyl radicals ($\bullet\text{OH}$) from the catalytic decomposition of hydrogen peroxide (H_2O_2) by the soluble ferrous ion (Fe^{2+}) for use in the destruction of organic pollutants. At pH 3.0, the ratios of HA and H_2O_2 of 1:560 and the H_2O_2 and Fe^{2+} ratios of 5:1 showed the maximum percentage of HA removal. Thus, the optimal ratio of $\text{HA}:\text{H}_2\text{O}_2:\text{Fe}^{2+}$ for HA removal appeared to be 1:560:112. However, the highest HA removal per gram of soil was 30% after 3 h when using a ratio of humic acid to reagents (H_2O_2 and Fe^{2+}) of 1:11200:2240. The comparative evaluation of two microorganisms *Pseudomonas fluorescens* and *Lactococcus lactis* for the degradation of HA indicated the highest removal of about 28% when using *Lactococcus lactis* as a single culture for soil samples within 20 d.

Keywords: humic acid, fenton reaction, *Pseudomonas fluorescens*, *Lactococcus lactis*

INTRODUCTION

Humic substances (HS) are components in the environment and can be isolated readily from nearly all types of soil, water (ground and surface water) and sediment. They are formed by the decay of plants, animals and microbial cells. Humic substances are composed of three basic components, namely, humins, humic acids (HAs), and fulvic acids (FAs) which are defined based on their solubility. HAs are comprised of high molecular weight organic substances soluble in water at a high pH value, whereas FAs are comprised of moderate molecular weight organic substances soluble in water at any pH value (Trump *et al.*, 2006).

Humic substances are known to be complex compounds composed of large numbers of different bioorganic molecules that can interact with organic and inorganic substances in the environment. The presence of humic substances may result in the formation of trihalomethanes (THMs) during the chlorination process to make drinking water. THMs have been identified as a human carcinogenic compound by the US Environmental Protection Agency (US EPA). The US EPA suggested the maximum contamination level of THMs for drinking water should be 80 $\mu\text{g/L}$ (Kim *et al.*, 2002).

Several processes have been reported for the degradation of HA and FA in soil using fungi (Rezacova *et al.*, 2006) and cultivation

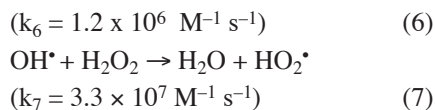
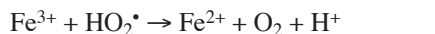
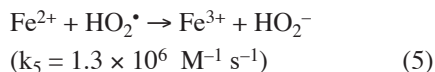
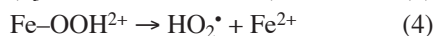
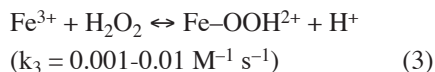
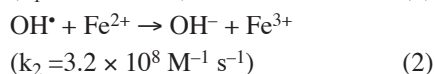
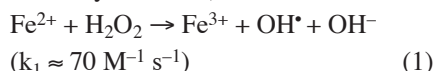
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(Bongiovanni and Lobartini, 2006). One of the most advanced oxidation processes is the Fenton technique that has been the subject of considerable interest for the remediation of contaminated soils. The Fenton reaction involves the formation of hydroxyl radicals ($\bullet\text{OH}$) from the catalytic decomposition of hydrogen peroxide (H_2O_2) by a soluble ferrous ion (Fe^{2+}). The hydroxyl radical ($\bullet\text{OH}$) is a strong oxidizing agent for the destruction of organic pollutants (Villa and Nogueira, 2006). The Fenton technique is a promising chemical oxidation reaction due to its high efficiency and low cost (Sun and Yan, 2007).

Fenton's reagent is a mixture of H_2O_2 and ferrous ions which generates hydroxyl radicals according to the reactions in Equations 1-7 (Neyens and Baeyens, 2003):



Moreover, microorganisms have a basic role in the biogeochemical cycles of the elements and in the formation of soil structure (Bastida *et al.*, 2007). Microorganisms are the main agents for biodegradation of substances in the environment. Current evidence suggests that in aquatic and terrestrial environments, microorganisms are the chief agents for the biodegradation of substances of environmental concern. Thus, the microbiological techniques appear to be the most promising for application with a variety of organic contaminants. The use of microbiological techniques for the removal of

pollutants typically is less expensive than the physical and chemical methods (Balba *et al.*, 1998) and is also environmentally friendly.

Soil organic matter is a complex mixture which influences a number of soil properties and nutrient cycling. The degradation of the humic substances is considered to be related to the soil fertility and ecological processes (Mishra and Srivastava, 1986; Mackowiak *et al.*, 2001; Katsumata *et al.*, 2008; Badis *et al.*, 2009). However, little information is available with regard to the characteristics of soil HAs and their degradation by microorganisms. Therefore, this study aimed to evaluate and compare the application of both chemical and microbiological methods for the removal of humic acid in the soil and its aqueous extract.

MATERIALS AND METHODS

Standard humic acid (HA)

Technical grade HA (in solid form) was purchased from Sigma-Aldrich Co. A standard HA solution of 100 mg/L concentration was prepared by mixing 25 mg of solid HA with 10 mL of 0.5M NaOH solution and 240 mL of distilled water. Subsequently, the prepared standard HA solution was diluted to 5, 10, 15, 20, 25 and 30 mg/L concentrations, using distilled water.

Analysis of humic acid (HA)

The prepared HA samples of different concentrations were analyzed by measuring the absorbance with an UV/VIS spectrophotometer (Perkin Elmer Lambda 20) at a wavelength of 272 nm (Traina *et al.*, 1989; Haderlein *et al.*, 2001; Young *et al.*, 2004).

Preparation of the HA samples

Soil samples were obtained from the surface horizon along the Mahasawat canal in Nakhonpathom province, 1-1.5 m away from the river banks. Soil samples were air-dried, passed

through a 1.70-mm sieve and stored in closed containers in a dry place under dark conditions at room temperature in order to prevent photo-oxidation of soil organic matter and any changes in the major soil characteristics prior to use.

Extraction of humic acid (HA) from soil samples

Humic acid was extracted from the soil samples by a modified Essington method. The 50g-soil samples were placed in conical flasks and washed with 50 mL of 0.1M HCl. The HA in the soil samples was extracted by 100 mL of 0.5M NaOH on an orbital shaker at 120 rpm for 2 h. Subsequently, the extracted solutions were filtered and adjusted approximately to pH 1 by concentrated 6M HCl. As a result of the pH adjustment, a solid precipitate was produced representing the crude HA fraction, while the crude fulvic (FA) fraction remained in the filtrate.

The crude HA fraction was first dissolved in 0.5M NaOH and then re-precipitated by acidification with 6M HCl. Additionally, the filtrate containing the crude FA was combined with the earlier fraction. The solid precipitate was re-dissolved in 0.5M NaOH to obtain the humic acid (HA) solution.

Treatment of HA by Fenton solution

Part 1: Humic acid solution extracted from soil samples

This part of the study was carried out to determine the optimum conditions for the Fenton technique.

Effect of pH values

The pH of extracted HA solutions was adjusted to three different levels (pH 2.5, 3 and 4) by adding 0.5M H_2SO_4 . Each extracted HA solution was treated with 0.097M H_2O_2 and 0.02M FeSO_4 at a ratio 1:140:14.

Effect of HA:H₂O₂ ratio

The HA:H₂O₂ ratio was varied at 1:140, 1:280, 1:420, 1:560 and 1:700, respectively. The

pH was adjusted to 3 for all ratios. The H_2O_2 : Fe^{2+} ratio was maintained constant at 10:1.

Effect of H₂O₂:Fe²⁺ ratio

The H_2O_2 : Fe^{2+} ratio was varied to 5:1, 10:1 and 20:1, respectively. The ratio HA:H₂O₂ was maintained constant at 1:560.

Part 2: Soil samples

Soil samples were treated with the optimum conditions of the Fenton technique determined from part 1.

Treatment of HA by microbiological technique

The performance of *Pseudomonas fluorescens* and *Lactococcus lactis* was evaluated for the degradation of HA from soil and a synthetic HA solution. Nutrient agar (NA) and MRS medium (MRS) were used for the growth of *P. fluorescens* and *L. lactis*, respectively. The viable cell concentration of *P. fluorescens* for growth conditions with nutrient agar, 30°C and 24 h was 3.1×10^6 cell/mL. Likewise the viable cell concentration of *L. lactis* for growth conditions with MRS medium, 37°C and 72 h was 4.2×10^6 cell/mL. The efficiency of the microbiological treatment process was investigated in terms of the single pure culture and mixed culture of two microorganisms at the ratio of 1:1. The batch experiments were carried out simultaneously in an autoclave (121°C for 15 min). The control experiments were set up by preparing a series of flasks with the same composition but excluding the bacteria. The soil samples were taken at regular time intervals for the extraction and analysis of the HA.

RESULTS AND DISCUSSION

Fenton technique

Effect of pH on the degradation of extracted HA solution

The effect of pH on the degradation of HA in extracted solution was investigated for three pH values (pH 2.5, 3 and 4). The ratio of

HA:H₂O₂:Fe²⁺ was kept at 1:140:14 for all tests. These results are shown in Figure 1. The percent of extracted HA solution removal increased with an increase in the reaction time for all pH values. The maximum percent removal from the extracted HA solution was at pH 3. Neyens and Baeyens, (2003) also stated that the highest efficiency of the Fenton reaction was at pH 3 and that Fe(OH)₃ may be formed at pH 4 and would not react with H₂O₂. This may have resulted in decreased oxidation efficiency of the Fenton's reagent. On the other hand, at the lower pH and the higher hydrogen ion (H⁺) concentration, the formation of Fe-OOH²⁺ would be slowed down with a possible decrease in the production rates of Fe²⁺

and hydroxyl radicals (•OH).

Effect of H₂O₂ ratio on the degradation of extracted HA solution

The extracted HA samples were treated with different ratios of HA and H₂O₂. The H₂O₂:Fe²⁺ ratio was maintained constant at 10:1 and the pH was adjusted to 3. The efficiency of extracted HA solution removal increased with an increase in the reaction time. The amounts of H₂O₂ per HA content were 140, 280, 420, 560 and 700. It was found that the ratio of 1:560 resulted in the maximum percent removal of HA in extracted solution (Figure 2). This result may have been due to the self-decomposition of H₂O₂ to oxygen and

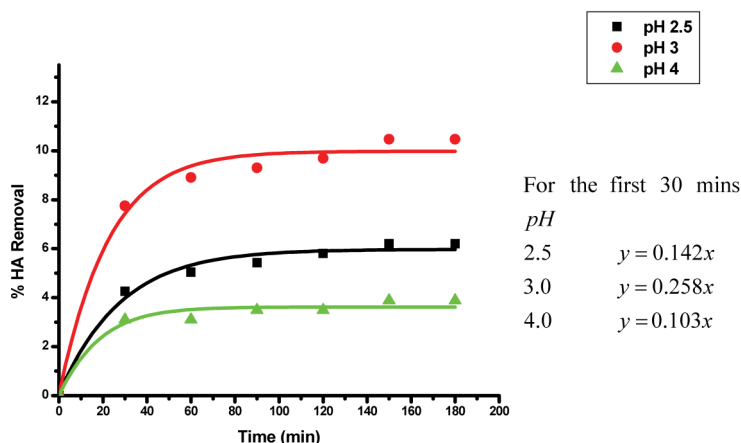


Figure 1 Effect of pH on the degradation of extracted HA solution.

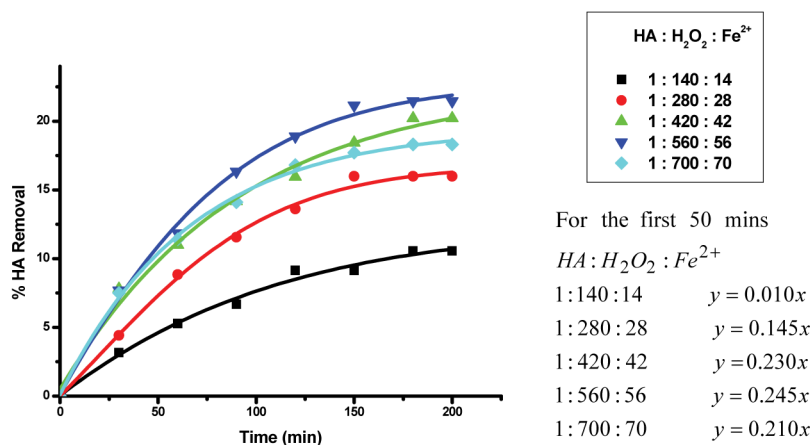


Figure 2 Effect of H₂O₂ ratio on the degradation of extracted HA solution.

water, as well as the recombination of the $\bullet\text{OH}$ radical at the rate of $3.3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ (Katsumata *et al.*, 2007). The other ratios of $\text{HA}:\text{H}_2\text{O}_2$ of 1:140, 1:280, and 1:420 showed lower percentage removal of extracted HA solution compared to the ratio of 1:560. This could have been due to the amount of H_2O_2 , which was not enough for the degradation of extracted HA.

Effect of H_2O_2 and Fe^{2+} ratio on the degradation of extracted HA solution

This was investigated using three different ratios of H_2O_2 and Fe^{2+} (5:1, 10:1 and 20:1) with a constant ratio of HA and H_2O_2 at 1:560. The results presented in Figure 3 show clearly that the percent HA removal increased with an increase in the reaction time and remained constant roughly up to 180 min for all ratios. The 5:1 ratio of $\text{H}_2\text{O}_2:\text{Fe}^{2+}$ was the most effective for extracted HA degradation. This may be explained by the reaction between H_2O_2 and Fe^{2+} , which generated hydroxyl radicals ($\bullet\text{OH}$). Therefore, the addition of Fe^{2+} may not result in more $\bullet\text{OH}$ radicals. Subsequently, the extracted HA solution would be degraded. Neyens and Baeyens, (2003) considered that at a high ratio of H_2O_2 and Fe^{2+} , the hydroxyl radicals ($\bullet\text{OH}$) could react with the greater amount of H_2O_2 to produce HO_2^\bullet radicals. Subsequently, the HO_2^\bullet radicals could participate

in the radical chain reactions and cause the reduction of ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) ions, resulting in the higher consumption of H_2O_2 .

Effect of optimum conditions in the Fenton technique on the degradation of HA from soil

It was observed that the two ratios of HA to H_2O_2 (1:560) and H_2O_2 to Fe^{2+} (5:1) resulted in the maximum percent removal in the extracted HA solution at pH 3. Thus, the optimal ratio of $\text{HA}:\text{H}_2\text{O}_2:\text{Fe}^{2+}$ appeared to be 1:560:112 for about 8.2% HA removal from the soil. In order to find a higher efficiency of Fenton's reagents for the degradation of HA from soil, the ratio of $\text{HA}:\text{H}_2\text{O}_2:\text{Fe}^{2+}$ was varied to 1:2,800:560 and 1:5,600:1120 and 1:11,200:2240 and 1:16,800:3360 to match 5-, 10-, 20- and 30-fold increases in the optimum Fenton's reagent ratio (560:112), respectively. The results showed that the percent HA removal was about 30% corresponding to the $\text{HA}:\text{H}_2\text{O}_2:\text{Fe}^{2+}$ ratio of 1:11,200:2240.

Effect of concentration of H_2O_2 and Fe^{2+} on the optimal ratio of Fenton's reagent for HA removal from extracted solution and soil

The concentrations of the $\text{HA}:\text{H}_2\text{O}_2:\text{Fe}^{2+}$ ratio at 1:560:112 were 0.000714, 0.4 and 0.08

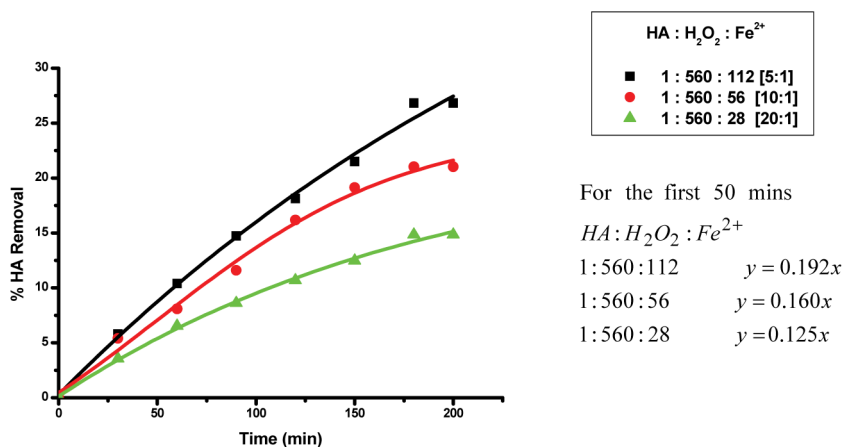


Figure 3 Effect of H_2O_2 and Fe^{2+} ratio on the degradation of extracted HA solution.

mM, respectively. Correspondingly, the concentrations the $\text{HA:H}_2\text{O}_2\text{:Fe}^{2+}$ ratio at 1:11200:2240 were 25 μg , 0.8 and 0.16 mM/g soil, respectively.

Microbiological technique

HA removal efficiency of *Pseudomonas fluorescens*

The soil inoculated with *P. fluorescens* was sampled for analysis at selected time intervals. The HA removal increased with an increase in the incubation time until 20 d for the autoclaved and non-autoclaved soil samples. The percent HA removal did not show a significant increase after 20 d of incubation. The highest HA removal was observed to be 14.9% for the autoclaved soil and 13.8% for the non-autoclaved soil, as shown in Figure 4. These results appear to be in good

agreement with those reported by Hertkorn *et al.* (2002) who demonstrated that a pure culture of *P. fluorescens* degraded groundwater HA (11%) in the aerobic liquid nutrient broth. However, the humic materials are slowly degraded by microbial populations in aerobic environments (Trump *et al.*, 2006). Moreover, Filip and Tesarova (2004) demonstrated that HA from permanent meadow soil was degraded in the range of 12 to 26% by soil microorganisms after 12 months incubation, as a result of the nutrient conditions of the individual cultures and the source of the HA.

HA removal efficiency of *Lactococcus lactis*

Following the inoculation of soil samples with *L. lactis*, the samples were analyzed at selected time intervals. The results in Figure 5

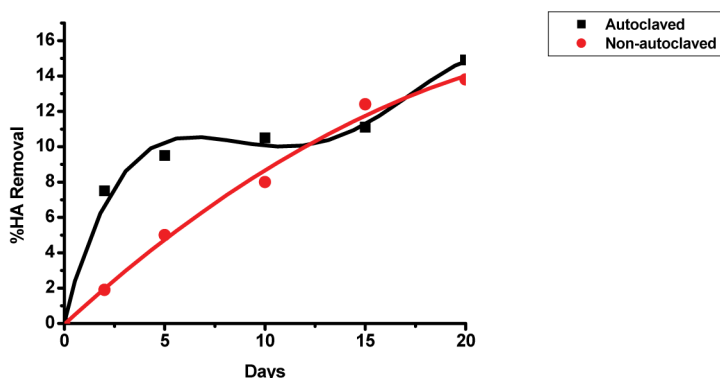


Figure 4 Percent HA removal with time by *Pseudomonas fluorescens*.

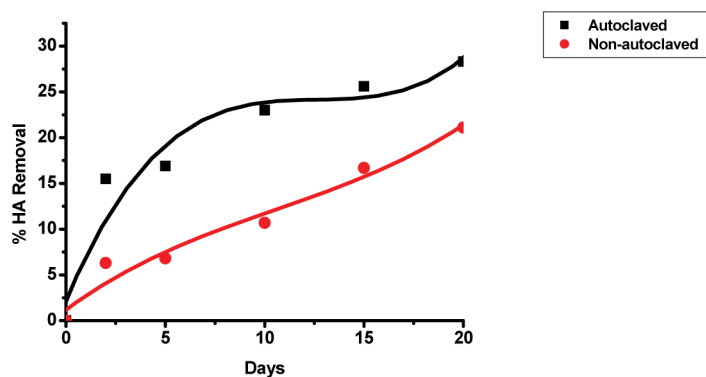


Figure 5 Percent HA removal with time by *Lactococcus lactis*.

show that the HA removal was maximized after 20 d incubation for both batch experiments (28.3% for the autoclaved and 21.1% for the non-autoclaved soil samples). Benz *et al.* (1998) proposed that in the presence of humic acids (HA), *L. lactis* could shift the fermentation patterns towards more oxidized products. In addition, there was good agreement with the results of Liang *et al.* (2009), who reported that the detected bacteria showed high oxidation-reduction potential as a degrader of aquatic humic substance in landfill leachate without molecular oxygen. However, the HA removal did not increase after 20 d incubation.

Removal efficiency of *Pseudomonas fluorescens* and *Lactococcus lactis*

Soil samples were inoculated with *P. fluorescens* and *L. lactis* as a mixed culture in equal amounts (a ratio of 1:1). The mixed culture could degrade the HA both for autoclaved and non-autoclaved soil as shown in Figure 6. The HA removal for the autoclaved and non-autoclaved soil was 18.5% and 13.4%, respectively. Hertkorn *et al.* (2002) reported that the concentration of humic substances (HS) decreased up to 27% in broth cultures inoculated with the groundwater microflora (mixed culture).

Comparison of HA removal by different microorganisms

The results on maximum HA removal by

each microorganism type investigated in this study achieved after 20 d incubation are summarized in Table 1. *L. Lactis* (pure culture) appeared to have the highest efficiency for HA removal within 20 d incubation for autoclaved and non-autoclaved soil samples. These results may be explained by the fact that *L. lactis* is a fermenting bacterium and probably has a high ability to use humic substances as an electron acceptor. Benz *et al.* (1998) also reported that humic acid could be reduced by fermenting bacteria; they demonstrated that there was a significant shift toward more oxidized products in fermentation by *L. lactis* when humic acids were available as electron acceptors. Trump *et al.* (2006) implied the rate of bacterial degradation of different varieties of organic compounds depending on the nature of the carbon compounds, environmental conditions and bacterial physiology, such as bacterial growth phase. The different bacterial communities have different abilities to utilize high- versus low-molecular-weight natural organic matter components (Young, 2005). Additionally, it was stated that the removal of humic substances may be inhibited in the absence of dissolved oxygen (Trump *et al.*, 2006).

Comparison between the Fenton reaction and the microbiological treatment technique

The maximum HA removal by the Fenton technique was higher (30.4%) than that of

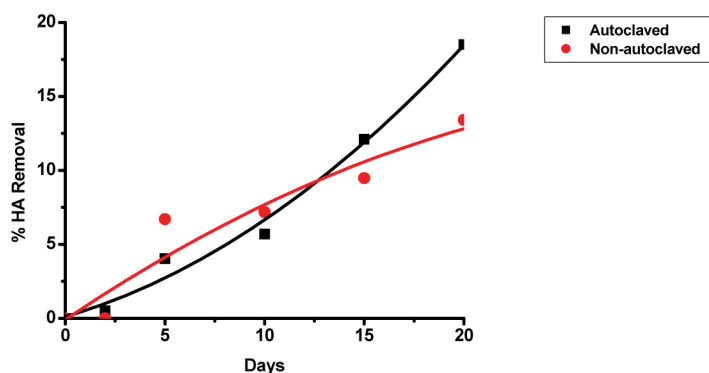


Figure 6 Percent HA removal with time by *Pseudomonas fluorescens* and *Lactococcus lactis*.

Table 1 HA removal efficiency of different microorganisms.

Reference	Microorganism type	Time	Condition	HA Removal (%)
The present study	<i>P. fluorescens</i>	20 d	Autoclaved soil	14.9
			Non-autoclaved soil	13.8
	<i>L. lactis</i>	20 d	Autoclaved soil	28.3
			Non-autoclaved soil	21.1
	Mixed culture (<i>P. fluorescens</i> and <i>L. lactis</i>)	20 d	Autoclaved soil	18.5
			Non-autoclaved soil	13.4
Filip and Tesarova, 2004	Soil microorganism	12 month	Meadow I soil (full-strength nutrient broth)	12.3
			Meadow I soil (deficient in carbon)	26.3
			Meadow I soil (deficient in nitrogen)	17.3
			Groundwater HA	11
Hertkorn <i>et al.</i> , 2002	<i>P. fluorescens</i>	3 w	Groundwater HA	11
Rezacova <i>et al.</i> , 2006	<i>Clonostachys rosea</i>	1 month	Soil	19.2
	<i>Paecilomyces lilacinus</i>			16

the microbiological degradation technique (21.1%).

CONCLUSION

Application of the Fenton technique was investigated for the degradation of HA extracted from the soil. Results showed a decrease in the absorbance with a variation in the pH and reagent ratios (H_2O_2 and Fe^{2+}). The highest treatment efficiency was obtained at about pH 3.0. The optimal ratio between HA and H_2O_2 appeared to be 1:560 and between H_2O_2 and Fe^{2+} , it was 5:1. The maximum decrease in concentration of HA in the extracted solution was found after 3h under the optimal conditions with the HA: H_2O_2 : Fe^{2+} ratio being 1:560:112. The optimal ratios of HA, H_2O_2 and Fe^{2+} were determined to be 0.000714, 0.4 and 0.08 mM, respectively.

Subsequently, soil samples that had been treated with the Fenton technique, under the optimum conditions that had been determined earlier, showed HA degradation of about 8.2%. However, the maximum ratio of humic acid to H_2O_2 and Fe^{2+} for degradation of HA in soil was found to be 1:11200:2240, with a reaction time of 3 h. Under these conditions, the HA was determined to be 25 $\mu\text{g/g}$ soil, along with concentrations of H_2O_2 and Fe^{2+} of 0.8 and 0.16 mM/g soil, respectively. Thus, under these conditions, the Fenton technique could be useful for HA degradation of soil.

The results of the microbiological treatment technique showed that *Lactococcus lactis* had good potential for the degradation of HA in soil. These results also indicated that the microbial degradation of HA depended on the species of microorganism.

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