

Characterization of humic acid extracted from solid fermentation of rice straw using *Trichoderma viride* and *Phanerochaete chrysosporium*

(Pencirian asid humik yang diekstrak daripada fermentasi pepejal jerami padi menggunakan *Trichoderma viride* dan *Phanerochaete chrysosporium*)

M.S. Umi Kalsom* and D. Noor Shita*

Key words: humic acids, *Trichoderma viride*, *Phanerochaete chrysosporium*, fourier transform infrared and nuclear magnetic resonance, rice straw

Abstract

The basidiomycetes, *Phanerochaete chrysosporium* and ascomycetes, *Trichoderma viride* decompose rice straw in solid state fermentation. Degradation of rice straw by these fungi resulted in the formation of humic acid (HA). The HA extracted from two fermentations using *P. chrysosporium* (HAP) and *T. viride* (HAT) were characterized using i) elemental analysis ii) fourier transform infrared (FTIR) and iii) solid state cross polarization magic angle spinning carbon-13 nuclear magnetic resonance (CP/MAS ¹³C NMR) spectroscopy. From elemental analysis it was shown that, HAP has a lower C/N ratio (13.9) as compared to HAT (16.2). The O/C ratio was higher in HAP (0.6) than in HAT (0.5). Result of CP/MAS ¹³C NMR analysis showed that 27.2% of the total component of HAT was made up of aliphatic alkyl groups, 24.7% N-alkyl, 33.4% O-alkyl, 6.1% aromatic, 1.1% phenolic and 7.5% carboxylic. On the other hand, 24.6% of the total component of HAP was made up of aliphatic alkyl groups, 17.8% N-alkyl, 33.9% O-alkyl, 5.2% aromatic, 1.4% phenolic and 17.1% carboxylic. The FTIR spectra of both HAT and HAP contained all the major characteristic absorption peaks of humic acid which indicate the presence of the major structural elements of HA namely phenolic- OH, CH₂, CH₃, and aromatic ring. Results from both elemental and spectroscopic analysis suggest that HAP has a higher degree of humification as compared to HAT.

Introduction

Humic acids have played a vital role in soil fertility and environmental quality. The roles played by these materials can greatly benefit plant growth. These include their contributions in plant growth enhancement, increasing fertilizer efficiency, or reducing soil compaction. The benefits of humic acid in agricultural soils are well established

(MacCarthy 2001), especially in soils with low organic matter (Chen and Aviad 1990). They are an integral part of all ecosystems and play an important role in global cycling of nutrients and carbon (MacCarthy 2001).

Humic acids are extremely versatile. They provide a concentrated and economical form of organic matter that can replace humus depletion caused by conventional

*Strategic Resources Research Centre, MARDI Headquarters, Serdang, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia

Authors' full names: Umi Kalsom Md. Shah and Noor Shita Desa

E-mail: umishah@mardi.my

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fertilization methods (Burdick 1965). The addition of humic acids to soils, including calcareous soils, can stimulate growth beyond the effects of mineral nutrients alone (Chen et al. 1999). Depending on the form of fertilizer applied, nitrogen may become a structural component of humic acid as a stable organic material, preventing it from leaching through the soil (Haworth 1971; Kelly and Stevenson 1996). In their natural state, humic acids contain 1–5% nitrogen.

The ability of basidiomycete

Phanerochaete chrysosporium to degrade and metabolize polymeric lignin is well known (Gold and Alic 1993; Reddy and D'Souza 1994) and the cellulase enzyme system from ascomycete *Trichoderma viride* has been well studied (Selby and Maitland 1967). As such, they are promising candidates for the decomposition process of plant wastes such as rice straw for humic acid production. The chemical composition of rice straw consists of 33.8% cellulose, 20.9% hemicellulose and 5.2% lignin (Xing 1995). In this work, two experiments on solid state fermentation of rice straw were conducted, using *P. chrysosporium* and *T. viride*, respectively as inoculum. The HA from both fermentation were extracted and characterized.

Materials and methods

Microorganism

Trichoderma viride was isolated from a compost. *Phanerochaete chrysosporium* were obtained from University of Manchester Institute of Science and Technology culture collection. The fungi were sub-cultured every month on potato dextrose agar (PDA) and stored at 4 °C.

Solid fermentation

One hundred g of rice straw (1–2 cm size) was mixed with 0.1% $(\text{NH}_4)_2\text{SO}_4$, 0.5% yeast extract, 0.2% K_2HPO_4 and 65% (v/w) distilled water in 2-litre Erlenmeyer flask and sterilized by autoclaving at 121 °C, 15 p.s.i. for 30 min. After cooling at room

temperature, the substrate mixture was then inoculated with 10^8 spores of *T. viride*.

A similar set of experiment was conducted using *P. chrysosporium*. Three replicates of culture flasks were incubated stationary at 30 °C. Samples were harvested after 5 weeks of incubation.

Extraction of humic acid

HA was extracted following the method described by Olk et al. (1996). Briefly, 45 g of the fermentation substrate was added with 450 ml 0.2 M KOH under an atmosphere of N_2 . The mixture was then shaken at room temperature and left to stand for 18 h. The extract was separated by centrifugation at 10,000 rpm for 20 min. The supernatant was collected and the pH adjusted with 6 M HCl to pH 1–2 to precipitate out humic acid. The precipitate was allowed to stand for 12 h, centrifuged and the HA recovered by dissolving in a small volume of 0.1 N KOH. The HA was then purified with a mixture of hydrochloric and hydrofluoric acid (0.1 M HCl/0.3 M HF) and finally dialysed using dialysis Visking tubing against distilled water for more than 24 h. The HA gel was air dried before grinding to pass a 0.25 mm sieve.

Elemental analysis

The solid HA was analysed for its elemental composition. The percentage of H was analysed using an auto element-analyser (CHNS-932 Model Lecco, ST Joseph, MI, USA). Other elements such as C, N, O, and S were analysed using Various Pressure SEM, Model EELS attached to LEO 912AB energy filter transmission EM 120 kv integrated with EDS.

Fourier transform infrared (FTIR) analysis

FTIR analysis was performed on a Perkin Elmer FTIR spectrophotometer. KBr pellets were made by accurately weighing 2 mg of dried HA and 300 mg of dried KBr and pressing the mixture under vacuum at 10 t for 10 min. Measurement was done at

wavelength range of 4,000–400 cm^{-1} . Data collection and processing was performed by GRAMS/386 version 3.02 software.

Solid state cross polarization magic angle spinning carbon-13 nuclear magnetic resonance spectroscopy (CP/MAS ^{13}C NMR)

Solid-state ^{13}C CP/MAS NMR spectrum was recorded at room temperature on a Bruker Avance 400 MHz NMR operating at a static magnetic field of 9.4 T. Magic angle spinning was performed at 5.0 kHz, making use of 4 mm ceramic Si_3N_4 rotor. The ^{13}C CP/MAS experiment was performed using a 3.8 microsecond 90° pulse with a delay time of 5 s, a contact time of 1 ms and spinning rate of 5 kHz and 2000 transients. Chemical shifts were measured with respect to tetramethylsilane. The Bruker Win NMR software was used to measure peak areas of the following chemical shift regions: 0–40 ppm (alkyl), 40–60 (N-alkyl), 60–90 ppm (O-alkyl), 90–140 ppm (aromatic), 140–160 ppm (phenolic), and 160–190 ppm and (carboxyl).

Results and discussion

Elemental analysis using SEM

The percentage amount of elements present in the solid HA is shown in Table 1. The elemental analysis revealed that the solid HA derived from solid fermentation of rice straw using *T. viride* was primarily composed of carbon (48.5%), oxygen (37.0%), hydrogen (6.3%), nitrogen (3.0%), sulphur (1.1%) and silica (4.1%). The solid HA derived from solid fermentation of rice straw using *P. chrysosporium* was primarily composed of carbon (50.0%), oxygen (39.5%), hydrogen

(6.1%), nitrogen (3.6%), sulphur (0.3%) and silica (0.5%). The elemental composition of the HA obtained in this study was almost similar to HA extracted from refuse compost as reported by Chien et al. (2003) which consisted of carbon (55.2%), oxygen (32.4%), nitrogen (6.5%), hydrogen (4.8%) and sulfur (1.0%). The C/N ratio of HA from *P. chrysosporium* was lower (13.9) as compared to C/N ratio of HA derived from *T. viride* (16.2). The O/C ratio which indicates level of oxidation was higher in HA derived from *P. chrysosporium* (0.6) as compared to O/C of HA derived from *T. viride* (0.5).

FTIR spectra

The IR spectra of HA extracted from solid fermentation of rice straw using *T. viride* and *P. chrysosporium* are shown in Figure 1. Interpretation of the spectra was based on Pavia et al. (1996). Both spectra showed similar trends. Major characteristic bands recorded included: a broad O-H band obtained around $3,436\text{ cm}^{-1}$ for intermolecular hydrogen bonding (H-bonded OH groups) attributed to phenolic groups. Methylene hydrogens ($-\text{CH}_2-$) gave rise to two aliphatic C-H stretching bands; a sharp peak at $2,929\text{ cm}^{-1}$ for asymmetric stretching and a shoulder at $2,852\text{ cm}^{-1}$ for symmetric stretching. A pronounced peak at $1,653\text{ cm}^{-1}$ (HA from *T. viride*) and $1,638\text{ cm}^{-1}$ (HA from *P. chrysosporium*) could be attributed to aromatic C=C, C=O and/or C=O of conjugated ketones or to C=N amide stretching. The peak intensity was higher in HA obtained from *T. viride* than that from *P. chrysosporium*. The weak band at the vicinity $1,236\text{ cm}^{-1}$ obtained in HA from

Table 1. The elemental composition of humic acid extracted from solid fermentation of rice straw using *Trichoderma viride* and *Phanerochaete chrysosporium* measured by various pressure SEM integrated with EDS detector

	C	N	O	H	S	Si	O/C (Atomic ratio)	C/N
	%							
<i>T. viride</i>	48.5	3.0	37.0	6.3	1.1	4.1	0.55	16.2
<i>P. chrysosporium</i>	50.0	3.6	39.5	6.1	0.3	0.5	0.6	13.9

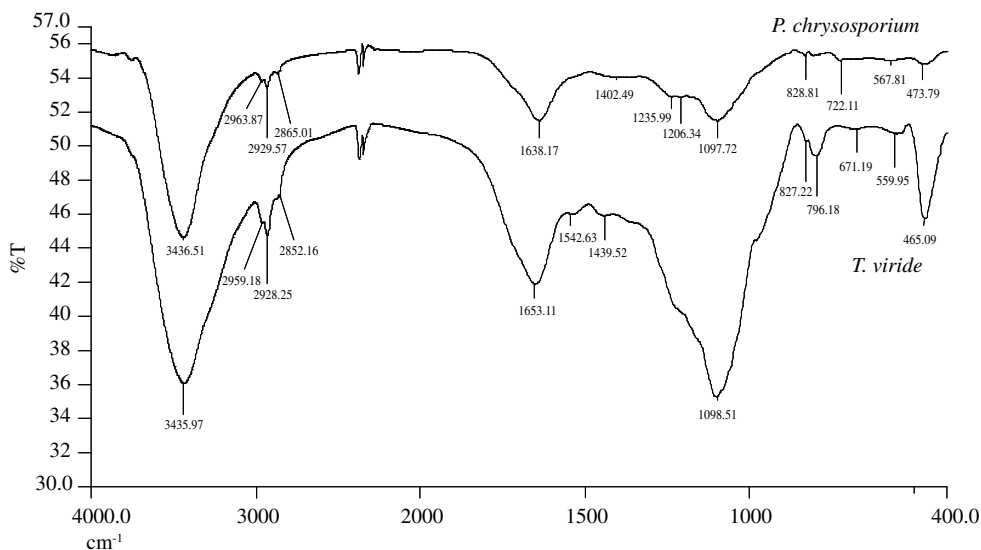


Figure 1. Fourier transform infrared spectra of humic acid extracted from solid fermentation of rice straw using *Phanerochaete chrysosporium* (top) and *Trichoderma viride* (bottom)

P. chrysosporium was due to deformation vibration of C=O of the carbonyl group mainly the carboxyl (COOH). Peaks at 1,098 cm^{-1} could be attributed to C-O stretching of alcohol, sulfoxides, carbohydrates or polysaccharides-like substances, or Si-O of silicates. The peak intensity was higher in HA obtained from *T. viride* than that from *P. chrysosporium*. The FTIR spectra of the extracted HA contained all the major characteristic absorption peaks of humic acid.

Solid state cross polarization magic angle spinning carbon-13 nuclear magnetic resonance spectroscopy (CP/MAS ^{13}C NMR)

NMR spectroscopy has become a major tool in investigations of organic matter and humification processes in soils and composts (Wilson 1989). To get a structural components of the humic samples, solid state cross polarization magic angle spinning (CP/MAS) carbon-13 NMR spectroscopy ^{13}C NMR was used for estimating the concentrations of the main functional groups.

The CP/MAS ^{13}C NMR spectra of HA extracted from rice straw using *T. viride* and *P. chrysosporium* is presented in Figure 2. Spectra were acquired at the end of the first month of solid fermentation. The CP/MAS ^{13}C NMR spectra of HA extracted from *T. viride* exhibited major peaks at 19 and 29 ppm attributed to alkyl group which consist of paraffinic carbons from lipid and biopolymers, 42, 52 and 57 ppm attributed for N-alkyl which consist of methoxyl group mainly from lignin and amino groups, 71 and 90 ppm attributed to O-alkyl which consist of carbohydrate carbons, 101 and 138 ppm attributed for aromatic groups which consist of carbon-substituted aromatic carbons from lignin and non hydrolysable tannins, 149 and 155 ppm attributed for phenolic groups which consist of oxygen substituted aromatic carbons from lignin and non hydrolysable tannins, and 170 ppm attributed to carboxyl and aliphatic amide carbons from degraded lignin and fatty acids. The CP/MAS ^{13}C NMR spectra of HA extracted from *P. chrysosporium* exhibited major peaks at 16, 21, 26 and 29 ppm (alkyl), 43, 52 and 58 ppm (N-alkyl),

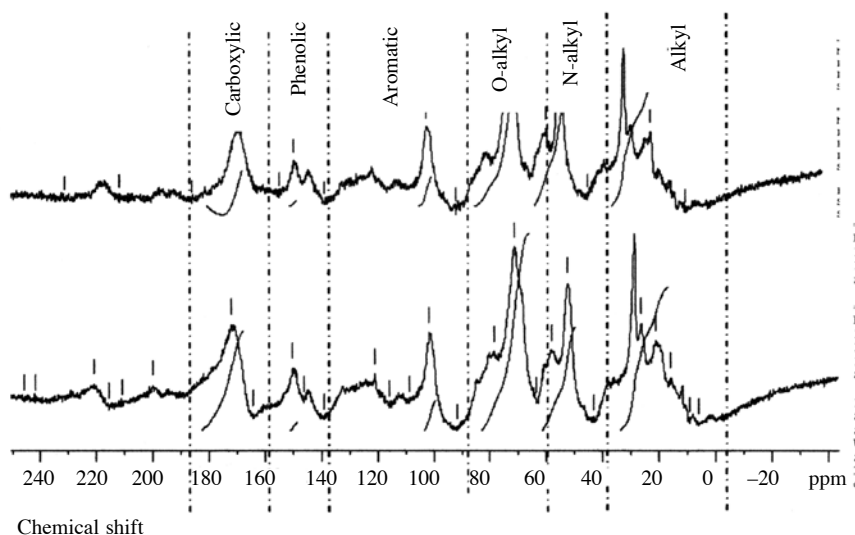


Figure 2. The CP/MAS ^{13}C NMR spectrum of HA extracted from rice straw using *Phanerochaete chrysosporium* (top) and *Trichoderma viride* (bottom)

Table 2. Distribution percentage of the characteristic carbons in CP/MAS ^{13}C NMR of humic acid extracted from fermented rice straw by *Trichoderma viride* and *Phanerochaete chrysosporium*

Humic acid	Carboxylic	Phenolic	Aromatic	O-alkyl	N-alkyl	Alkyl
<i>T. viride</i>	7.5	1.1	6.1	33.4	24.7	27.2
<i>P. chrysosporium</i>	17.1	1.4	5.2	33.9	17.8	24.6

71 and 79 ppm (O-alkyl), 102 and 121 ppm (aromatic C), 146 and 150 ppm (phenolic) and 172 ppm (carboxyl).

Generally, the most prominent signals are in the range between 0 and 90 ppm. Peaks in this area correspond to C-N and C-O groups as found in amines (e.g. amino acids), aliphatic alcohol groups (like sugars and other carbohydrates) and aliphatic ethers (methoxy and ethoxy groups), typical degradation products of plant litter in the early stage of humification. Aromatic and unsaturated carbon (90–160 ppm) and very highly oxidized carbon such as carboxyl group (160–190 ppm) can be detected only in very small quantities.

The resonance integrals of six common spectral regions are shown in Table 2. For HA extracted from *T. viride*, 27.2% of the total component was made up of aliphatic alkyl groups and 6.1% of the

total component was made up of aromatic groups. The other components were N-alkyl (24.7%), O-alkyl (33.4%), phenolic (1.1%) and carboxylic (7.5%). For HA extracted from *P. chrysosporium*, 24.6% of the total component was made up of aliphatic alkyl groups and 5.2% of the total component was made up of aromatic groups. The other components were N-alkyl (17.8%), O-alkyl (33.9%), phenolic (1.4%) and carboxylic (17.1%).

The substantial amount of alkyl-C in the HA extracted from rice straw was very similar to those of HA extracted from refuse compost (Chien et al. 2003). The HA extracted from fermented rice straw exhibited a higher level of C-substituted aromatic C and a lower content of phenolic C. The 150 ppm peak could be assigned to C-3 and C-5 of syringyl units (Chefetz et al. 2002). The relationship between the

chemical nature of the polymethylenic domains in humic acid and the degree of humification is not yet well understood and should be further investigated.

Conclusion

The study demonstrated that HA extracted from solid fermentation of rice straw using *P. chrysosporium* has a higher degree of humification as compared to HA extracted from fermentation by *T. viride*, as shown in the elemental and spectroscopic analysis. From elemental analysis it was evidence that, HA from *P. chrysosporium* has a lower C/N ratio (13.9) as compared to C/N ratio of HA derived from *T. viride* (16.2). Furthermore, the O/C ratio was higher in HA derived from *P. chrysosporium* (0.6) as compared to O/C of HA derived from *T. viride* (0.5). The lower C/N ratio and higher O/C ratio indicated higher degree of humification.

From CP/MAS ¹³C NMR analysis, the higher proportion of carboxylic groups in HA from *P. chrysosporium* (17.1%) indicated higher degree of humification. The FTIR spectra of the extracted HA contained all the major characteristic absorption peaks of humic acid which indicated the presence of the major structural elements of HA namely phenolic -OH, CH₂, CH₃ and aromatic ring.

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Abstrak

Basidiomiset *Phanerochaete chrysosporium* dan askomiset *Trichoderma viride* didapati berupaya untuk mengurai jerami padi dalam keadaan fermentasi pepejal. Penguraian jerami padi oleh kulat ini akan menghasilkan asid humik (HA). Dalam kajian ini HA yang diekstrak daripada dua fermentasi menggunakan *P. chrysosporium* (HAP) dan *T. viride* (HAT) telah dicirikan menggunakan kaedah (i) analisis elemen (ii) spektroskopi penular gelombang infra merah fourier (FTIR) dan (iii) kekutuban campuran magik sudut berpusing karbon 13-gema magnetik nuklear (CP/MAS ¹³C NMR) secara pepejal. Daripada analisis elemen, HAP mempunyai nisbah C/N lebih rendah (13.9) berbanding dengan HAT (16.2). Nisbah O/C HAP (0.6) lebih tinggi daripada nisbah O/C HAT (0.5). Daripada analisis CP/MAS ¹³C NMR, sebanyak 27.2% daripada jumlah komponen HAT terdiri daripada kumpulan alifatik alkyl, 24.7% N-alkyl, 33.4% O-alkyl, 6.1% aromatik, 1.1% fenolik dan 7.5% karboksilik. Di samping itu, 24.6% daripada jumlah komponen HAP terdiri daripada kumpulan alkyl, 17.8% N-alkyl, 33.9% O-alkyl, 5.2% aromatik, 1.4% fenolik dan 17.1% karboksilik. Spektra FTIR bagi kedua-dua HAT dan HAP mengandungi semua puncak penyerapan penting bagi asid humik yang menandakan kehadiran struktur elemen HA khususnya fenolik –OH, CH₂, CH₃ dan lingkaran aromatik. Hasil keputusan daripada kedua-dua analisis elemen dan spektrokopi mencadangkan bahawa HAP mempunyai darjah humifikasi lebih tinggi berbanding dengan HAT.