



Preliminary study on treatment of waste organic matter from livestock by bacteria-mineral technology

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Abstract

The present study dealt with relationships between the degradation and humification process that the organic matter underwent during bacteria-mineral technology. An inverse correlation was found between the protein, lipid, and some of the humification indices considered, suggesting that the humification theory is actually humic substances produced from simple-structured natural organic substrates. Weight-average molecular weight (M_w), number-average molecular weight (M_n), and the ratio M_w/M_n of dissolved organic matters at different stages of the process were measured by gel permeation chromatography. The results showed that M_n and M_w increased with reaction time from 352 to 17,191, and from 78,707 to 104,564, respectively. The ratio of M_n/M_w decreased from 223.3 to 6.1. This reflected the growth of the polymerization degree of dissolvable organic matters in the process; furthermore, it indicated the formation of complex molecules (humic substances) from more simple molecules. Bacteria-mineral water (BMW) (the effluent of the process) treatments can exert hormone-like activity for enhanced seed germination of wheat and rice and greatly improved chlorophyll synthesis in wheat and rice leaves. Major polyamines (plant regulators) putrescine, spermidine, and spermine, were found in BMW by a high performance liquid chromatography (HPLC) method, which may explain the hormone-like activity of BMW.

Key words: livestock liquid wastes treatment; bacteria-mineral technology; recycles; humification; biogenic amines; seed germination

Introduction

Bacteria-mineral technology (BMT) was initially introduced in Japan based on an organic wastewater treatment technology (Nagasaki, 2001). Bacteria-mineral water (BMW) is produced in several bioreactors containing water, humus soil, and natural rocks. During the bio-reaction, bacteria utilize manure, releasing unknown factors, such as, hormone-like agents, antimicrobial agents, antioxidants, and immunity-promoting agents. Natural minerals not only accommodate bacteria in the reactors, but also supply essential inorganic saline nutrients for them and stimulate microbial activity (Nagasaki, 2001). Finally, the clean and safe BMW is produced. Thus, BMW could be used for various agricultural purposes.

Bacteria-mineral water has been proved effective and safe through actual application in agriculture fields. However, no research has been conducted to investigate the BMT process and examine the effect of BMW on planting agriculture. There were only two research articles about the application of BMW: one stated that supplementation of BMW that originated from bio-reacted swine urine into the culture medium increased the activities of quinine reductase (QR) and glutathione S-transferase (GST) of

Scenedesmus spp., a green algae that has great potential as bioresources for fishes, alternative and health foods, and pharmaceutical products (Shon *et al.*, 2004); the other study examined the BMW produced from bio-reacted swine manure that would stimulate rumen fermentation increasing microbial growth and cellulose degradation (Kim *et al.*, 2005). If BMW is applied on crop seed treatment, it would be an affordable and effective way to optimize seed germination, plant establishment, early growth, and yield potential.

According to Claus *et al.* (1999), aquatic humic substances (HS) may arise from microbial production from decomposing autochthonous biomass. The study also demonstrated the contributions of microorganisms to the release of HS from simple-structured natural organic substrate of animal urine such as carbohydrate, protein, and lipid.

Many studies have demonstrated that humic substances have exhibited hormone-like activity on plant growth (Chen *et al.*, 2000; Nardi *et al.*, 2000; Nardi *et al.*, 2002; Chen *et al.*, 2004). Through the standard procedures (Thurman and Malcolm, 1981) for isolating HS that International Humic Substances Society (IHSS) recommended, HS can be separated from the BMW, and accounted for more than 79% of total organic carbon of BMW. Therefore,

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the presence of HS in BMW may play a key role. Soluble HS, which are natural biologically active compounds, are of great importance for plant growth processes and seed germination (Cacco and Dellagnola, 1984; Malik and Azam, 1985; Young and Chen, 1997; Muscolo *et al.*, 1999; Loffredo *et al.*, 2005; Pouneva, 2005). It was shown that HS could exert a direct influence on plant growth (Chen *et al.*, 2004), photosynthesis (Nardi *et al.*, 2002), and activity of some enzymes (Yang *et al.*, 2004). Therefore, BMT process is an aerobic biological decomposition of organic substrates, with putrescible materials converted to a stabilized end-product by the active contribution of aquatic microorganisms (Claus *et al.*, 1999). Through the BMT process, organic matter undergoes partial mineralization and, to a varying degree, transformation into more refractory materials such as HS. Thus, BMW can be used directly in agriculture as a “nature” growth-promoting substance.

Biogenic amines (plant regulators) function similar to the recognized plant hormones (Galston and Sawhney, 1990; Young and Chen, 1997) and may occur as biodegraded products of organic materials, such as proteins and amino acids or other nitrogen-containing compounds (Lloret *et al.*, 2002). Polyamines may also be bound to the structure of humic substances. Young and Chen (1997) found polyamines existed in humic acid, which were found to be regulators of physiological processes of lettuce seedlings. In this study, the major polyamines, putrescine, spermidine, and spermine, were found in BMW with a high performance liquid chromatography (HPLC) (Smith and Davies, 1985).

Growth in the livestock and dairy industries has made it necessary to pay close attention to increasingly large quantities of animal urine wastewater, which cause nasty and offensive odors resulting in environmental pollution. On the other hand, in agricultural practices, pesticides seed treatments pose certain risks. There is a growing concern to the chemical inputs in agriculture. Consequently, the use of biological control with introduced microorganisms and/or their metabolites can provide more environmentally sound and economically feasible alternatives for eradication of seed-borne pathogens. Protection of seeds and seedlings has been increased in response to demands for using more “nature” substances. Therefore, the aim of the present study was to investigate recycling methods such as BMT for treating urine and other liquid wastes more efficiently to convert them to a material that can be reused as a safe and environmental-friendly seed-treatment material.

1 Materials and methods

1.1 Experiments

The bacteria-mineral process was prepared through six bio-reaction steps, each of the treating steps were aerated by means of aeration pipes. In the first bioreactor containing pelletized soil humus and crushed igneous rocks, 0.10 kg fowl dung (0.2%, W/V) and water (50 L) were added, then the bioreaction was carried out for 5 d with aeration.

After the first step, liquid in the first bioreactor flowed into the second bioreactor containing natural rocks, while fresh 0.10 kg fowl dung and water were added into the first bioreactor. The reaction was carried out for another 5 d. In the other bioreactors containing natural rocks, a similar reaction to the second bio-reaction was carried out for 5 d in each step. After 30 d (six bioreaction steps), the resultant aqueous solution contained high concentrations of activated soil microorganisms and their metabolic products, which can be used as an agricultural material.

1.2 Analysis and measurements

Carbohydrate was measured using phenol-sulfuric method with glucose as standard (Herbert *et al.*, 1971). Soluble protein was determined using Lowry-Folin method with BSA (bovine serum albumin) as standard (Lowry *et al.*, 1951). Lipid was then measured gravimetrically after the solvent was evaporated at 80°C (Clesceri *et al.*, 1998).

Aquatic humic acid (HA) and fulvic acid (FA) were isolated according to standard procedures (Thurman and Malcolm, 1981) for isolation of HS that IHSS recommended. A Shimadzu TOC analyzer (Shimadzu Corporation, Japan) was used for organic carbon analysis.

Gel permeation chromatography (GPC) was applied for recognizing the humic acid and fulvic acid in BMW. The GPC system consisted of a column (TSK gel G4000PWXL, Shimadzu Corporation, Japan), a peristaltic pump, and a refractive index detector. The flow rate of the eluents was 1.0 ml/min. Samples were filtered through 0.45 µm GF/F glass fiber filters (Millipore, USA) before analysis.

A Varian HPLC (Varian Inc., USA), equipped with a fluorescence detector, was used for separation of biogenic amines (plant hormones) from the effluent of the process. An Agilent ODS-C18 (250 mm × 4.6 mm, 5 µm particle diameter, from Thermo Electron Corporation, USA) was used. The polyamines were eluted from the column 60%–95% acetonitrile in water gradient for 20 min, and detected with a fluorescence spectrophotometer (excitation at 365 nm, emission at 510 nm) (Smith and Davies, 1985). Dansyl choride as fluorescent derivation agent was used. The optimal reaction conditions were determined as follows: concentration of dansyl chloride 3 mg/L; temperature 60°C; 15 min; pH 9.78.

The seeds of the cereals were surface sterilized with 55°C warm water for 5 min. Next, seeds were soaked in an eight-level treatment using concentrations of 100%, 10%, 1%, 0.5%, 0.25%, 0.125%, 0.025%, and 0 (control) BMW. The cereals were prepared by diluting BMW with distilled water, rice (*Oryza sativa* L.) for 96 h and wheat (*Triticum aestivum*) for 12 h. After imbibition, the seeds were placed in Petri plates of 12 cm diameter containing sterile filter sheets, moistened with deionized water. Fifty seeds for each treatment were uniformly distributed on the filter article and a factorial design with three replications was used. The plates were incubated at 25±1°C in a seed germinator with 12 h light period. The procedure based on the rules for seed testing (ISTA, 2006) was used. The number of germinated seeds was counted daily until

the end of the germination process, then calculated as a percentage of seeds sown. Germination percentage was calculated as the total number of seedlings that emerged versus the total number sown. The criterion to establish germination was the emergence of a primary root and a coleoptile the same length as the seed.

Chlorophyll *a*, *b*, and total chlorophyll content levels in seedling leaf tissue were measured using the method of Baker and Hipkins (1986). Fifty milligrams of leaf tissue (at the end of the germination) was added to 3.0 ml of 100% methanol and incubated in the dark for 2 h. Next, each sample was homogenized and centrifuged at 13,000 r/min for 10 min. Absorbance of the samples at 650 and 665 nm was measured with UV spectrophotometer. Absolute methanol (100%) was used as blank. The calculation of chl-*a* ($\mu\text{g/ml}$), chl-*b* ($\mu\text{g/ml}$), and total chl ($\mu\text{g/ml}$) content was as follows:

$$\text{Chl-}a = 16.5 \times A_{665} - 8.3 \times A_{650} \quad (1)$$

$$\text{Chl-}b = 33.8 \times A_{660} - 12.5 \times A_{665} \quad (2)$$

$$\text{Total chl} = 22.8 \times A_{650} + 4.0 \times A_{665} \quad (3)$$

The chlorophyll levels were subsequently converted to micrograms of chlorophylls per gram of fresh weight (fw tissue by the equation ($\mu\text{g chlorophyll/ml methanol}$) \times 3 ml methanol/(g fw tissue) (Horii *et al.*, 2007). The experiment was conducted in triplicate. Calculation concerning data of the treatments and evaluation of the significance of differences between the mean values of the treatments were subjected to the analysis of variance (Snedecor and Cochran, 1980), whereas the control of statistical significance of differences between treatment means was performed by Duncan's test (Dawson and Trap, 2001). *P* value at *P* < 0.05 was considered significant.

2 Results and discussion

2.1 Organic matter degradation

The organic matter of the six reactors degraded because of the activity of the microorganisms that had a large quantity of easily degradable substances available such as carbohydrate, protein, and lipid. Fig.1a shows the water-soluble carbohydrates' evolution during the process. The method for measuring water-soluble carbohydrates mainly quantified hexoses and pentoses, this fraction of the water-solution organic carbon is the most labile and sensitive in

reflecting the biochemical transformations (Brink *et al.*, 1960). In the process, the concentration of carbohydrates fell sharply in the first 5 d by more than 74% of the initial values. Although there was very little variation during the rest of the reaction process, carbohydrate content linearly decreased by increasing reaction time after the first 5 d. The concentration of protein strongly decreased during the process but stabilized in the last 5 d (Fig.1b). Lipid strongly degraded with reaction time, 22.09 mg/L in influent and 7.89 mg/L in effluent, and followed a linearly trend (Fig.1c).

2.2 Humification process

An individual study of the HS, HA, and FA is useful for following the evolution of organic matter during reaction process. However, in studying the humification process, it may be more accurate to consider the ratios between these parameters and TOC (total oxidizable carbon) or between themselves. Humification ratio (HR = HS/TOC \times 100), humification index (HI = HA/TOC \times 100), and HA/FA ratio, were calculated according to Jimenez and Garcia (1992).

Table 1 shows these indices and the humification degree. The increase noted in the humification ratio suggested that the proportion of HS increases in the process, whereas the rise in the humification index indicates an increase in the structural complexity of the molecules studied, which therefore showed more accentuated humic characteristics. The increase of HA/TOC and FA/TOC ratios reflected the formation of complex molecules (HA, FA) from more simple molecules and a diminution in the nonhumic components of the total organic matters, which are the most easily degraded by microorganisms. The HA and FA increase from an initial 39.09 and 17.1 mg/L to final values of 40.02 and 20.65 mg/L, behaved similarly. The increase of HS shows the reaction is a humification process.

2.3 Correlation between the humification indices and carbohydrate, protein, and lipid

The possible role of the water-soluble carbohydrate, protein, and lipid as precursors in the humification process were studied by correlating their amounts with the humification indices during the reaction. An inverse correlation was found between the protein, lipid, and some of the humification indices considered, suggesting that the humification theory is actually a HS produced from

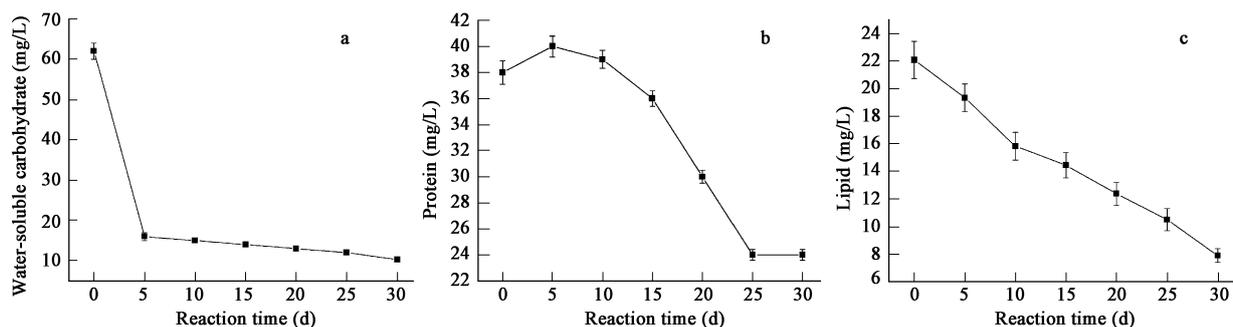


Fig. 1 Degradation of water-soluble carbohydrates (a), protein (b), and lipid (c) in different reaction stages.

Table 1 Humification indices in the process

Reaction time (d)	TOC (mg/L)	HS (mg/L)	HA (mg/L)	FA (mg/L)	HS/TOC (%)	HA/TOC (%)	FA/TOC (%)	HA/HS (%)	FA/HS (%)	HA/FA
0	112.34	56.19	39.09	17.10	50.02	34.80	15.22	69.57	30.43	2.29
5	110.41	58.30	39.76	18.54	52.80	36.01	16.79	68.20	31.80	2.14
10	108.23	59.14	40.57	18.57	54.64	37.48	17.16	68.60	31.40	2.18
15	105.20	56.19	39.09	18.80	53.41	37.16	17.87	69.57	33.46	2.08
20	91.51	59.54	40.69	18.85	65.06	44.47	20.60	68.34	31.66	2.16
25	80.84	61.29	41.54	19.75	75.82	51.39	24.43	67.78	32.22	2.10
30	76.79	60.67	40.02	20.65	79.01	52.12	26.89	65.96	34.04	1.94

HS: humic substance; HA: humic acid; FA: fulvic acid.

simple-structured natural organic substrates of plant, animal, and microbial origin such as starch and peptone (Claus *et al.*, 1999). The most significant correlations were found between protein, lipid and HS/TOC, HA/TOC, and FA/TOC (Table 2), suggesting that the most significant correlation with the humification indices occurred where HA and FA fractions were involved. The water-soluble carbohydrate would have been preferentially degraded by the microbial flora into carbon and energy sources, thus diminishing their role as precursors in the humification process.

However, no type of correlation was observed between the water-soluble carbohydrate content and that of the earlier-mentioned humification indices (Table 2). Because soluble carbohydrates are the main carbon and energy sources of the microorganisms, these types of molecule participate more in the degradation processes and their role as precursors in the synthesis of humic substances is thus masked.

2.4 Molecular mass distributions of dissolved organic matter in the process

Separation by means of gel permeation chromatography is based on differential permeation of various molecular sizes into a porous matrix. As the sample traverses the column, the small compounds permeate the matrix pores to a greater degree than the larger components, and retained longer. Elution order therefore depends on molecular size, with the largest materials eluting first, and the smallest last. In the case of an individual compound, molecular weight can be uniquely defined by a single value, whereas molecule weight of natural random macromolecular compounds such as HS is always a distribution. Weight-average molecular weight (M_w) and number-average molecular weight (M_n) are the most common parameters employed to characterize polymeric substances. The ratio M_w/M_n is a measure of the breadth of the molecular mass distribution (Janos, 2003).

Under the same conditions, all water samples measurements were performed triplicate, and the average values

are shown in Table 3.

Table 3 Molecular mass distributions of dissolved organic matters in different stages of the process

Reaction time (d)	M_w	M_n	M_w/M_n
0	78,707	352	223.3
5	89,451	1,605	55.7
10	92,784	3,073	30.2
15	116,402	6,822	17.1
20	125,545	14,658	8.6
25	136,761	16,140	8.5
30	104,564	17,191	6.1

M_w : weight-average molecular weight; M_n : number-average molecular weight.

The decrease of the ratio M_w/M_n indicated the shortening of the breadth of the molecular mass distribution. The ratio M_w/M_n fell sharply during the first 5 d by more than 75% of the initial values, suggesting the main organic components was strongly degraded in the first 5 d. There was very little variation during the final 10 d of the reaction process. The increase of M_n and M_w reflected the growth of the polymerization degree of dissolvable organic matters in the process; furthermore, it indicated the formation of complex molecules from more simple molecules.

2.5 Polyamines content at different stages of the process

As HS originate from chemical and biological degradation of plant, animal residue, and from metabolic activities of microorganisms, they might be expected to show hormonal character. The main identifiable compounds in hydrolyates of humic acid and fulvic acid are amino acids and amino sugars. Other nitrogenous biochemicals are also present, but specialized techniques are required for their separation and identification.

Large amounts of polyamines are known to be present in seeds of a number of gymnosperms and angiosperms, the polyamine contents during development and germination change significantly (Sen *et al.*, 1981). Polyamines also can be produced by some rhizobia (Wheeler *et al.*, 1994). In addition, they occur in free form or bound to phenolic

Table 2 Correlation between the humification indices and carbohydrate, protein, and lipid

		HS/TOC	HA/TOC	FA/TOC	HA/HS	FA/HS
Carbohydrate	Pearson correlation	NS	NS	NS	NS	NS
Protein	Pearson correlation	-0.972**	-0.981**	-0.958**	NS	NS
Lipid	Pearson correlation	-0.904**	-0.906**	-0.930**	NS	NS

** Correlation is significant at the 0.01 level (2-tailed). NS: not significant.

acids and other low MW compounds or macromolecules (Galston and Sawhney, 1990). Polyamines may also be bound to the structure of humic acid. Humic acid can exert stimulating effect similar to auxins, gibberellin, and cytokinin-like activity. However, there is no direct chemical evidence that can prove that these hormone-like activities of humic acid are the presence of PF plant regulators. The present study represents the first investigation on qualitative determination and quantitative measurement of putrescine, spermidine, and spermine from the process effluent, which are the regulators of physiological processes of crop seeds germination.

Techniques such as HPLC were employed in a search for plant hormones, but failed to provide evidence of the presence of IAA (3-indoleacetic acid) or GA₃ (gibberellin). Biogenic amines (plant regulators) function is similar to the recognized plant hormones (Galston and Sawhney, 1990; Young and Chen, 1997) and may occur as biodegradation products of organic material such as proteins and amino acids or other nitrogen-containing compounds (Lloret *et al.*, 2002). In this study, the major polyamines, putrescine, spermidine, and spermine are found in BMW with HPLC method. The individual content of polyamines in water samples are shown in Table 4.

Table 4 Polyamines content at different stages of the process

Reaction time (d)	Putrescine (µg/L)	Spermidine (µg/L)	Spermine (µg/L)
0	4.029	0.001	0.002
5	19.710	0.028	0.004
10	21.927	0.032	0.029
15	27.619	0.031	0.031
20	29.477	0.056	0.033
25	30.196	0.058	0.039
30	63.751	0.059	0.044

2.6 Effect of BMW on seed germination and chlorophyll content in the leaves of wheat and rice

All BMW treatments enhanced germination energy and final germination percentage. BMW (0.25%) treatment would be the most suitable concentration level to stimulate germination of wheat seeds (Fig.2a), and in the concentration level from 0.25% to 0.125% BMW were suitable to stimulate germination of rice seeds (Fig.2b). Germination energy being close to final germination percentage represented that seeds sprouted quickly and seedlings emerged uniformly. It is shown in Fig.2 that BMW treatments could accelerate germination; especially for rice (*Oryza sativa* L.) seeds, germination energy was equal to final germination percentage of all BMW treatments except for control and 0.25% BMW treatment.

Chlorophyll content in the leaves of wheat and rice differed in accordance with the treatments (Tables 5 and 6). The measured chlorophyll levels in wheat leaves were 0.93, 1.21, 1.27, and 1.25 mg/g (fw) for the control, 0.125%, 0.25%, and 0.5% BMW, respectively, and in rice leaves were 0.61, 0.83, 0.76, and 0.73 mg/g (fw) for the control, 0.125%, 0.25%, 0.5% BMW, respectively. The

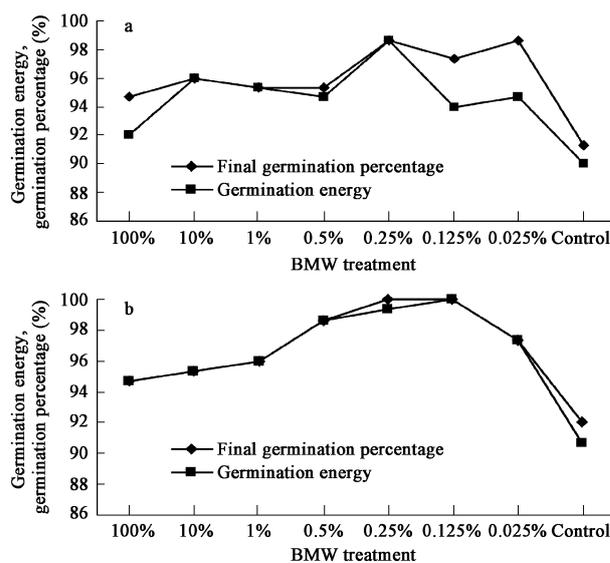


Fig. 2 Effects of BMW treatments on germination energy and final germination percentage. (a) *Triticum aestivum*; (b) *Oryza sativa* L.

Table 5 Effect of BMW on chlorophyll content in leaves of wheat (mg/g fw)

BMW treatment	Chl-a	Chl-b	Chl-a/Chl-b	Total Chl
0.5%	1.03 b	0.22 b	4.64 a	1.25 b
0.25%	1.05 b	0.22 b	4.86 b	1.27 b
0.125%	1.00 b	0.21 b	4.78 b	1.21 b
0 (control)	0.76 a	0.17 a	4.56 a	0.93 a

Different letters a and b in a column indicate significant differences ($P = 0.05$).

Table 6 Effect of BMW on chlorophyll content in leaves of rice (mg/g fw)

BMW treatment	Chl-a	Chl-b	Chl-a/Chl-b	Total Chl
0.5%	0.53 a	0.19 a	2.79 a	0.73 a
0.25%	0.56 b	0.20 a	2.84 a	0.76 b
0.125%	0.62 b	0.22 b	2.85 a	0.83 b
0 (control)	0.44 a	0.16 a	2.72 a	0.61 a

Different letters a and b in a column indicate significant differences ($P = 0.05$).

presence of BMW greatly improved chlorophyll synthesis in wheat and rice leaves.

3 Conclusions

Carbohydrate, protein, and lipid play an important role in soluble organic matter degradation. As a consequence of microbial activity, their concentrations are in continuous decrease as a result of degradation, and their contribution on formation of more complex polymers. Both protein and lipid were inversely correlated with some of the humification indices throughout the reaction process, which strongly suggests they acted as precursors in the humification process and implies, therefore, a decrease in concentration with the increase in humic substances in the process. No mathematical correlations were observed between the soluble carbohydrate fraction and principal humification indices, probably because such carbohydrates are the principal carbon sources of the microbial flora,

which are mainly responsible for the degradation that takes place. The participation of soluble carbohydrates in the humification process is therefore masked.

The increase of M_n and M_w reflected the growth of the degree of polymerization of dissolvable organic matters in the process; furthermore, it indicated the formation of complex molecules from more simple molecules. The decrease of the ratio M_w/M_n suggested the shortening of the breadth of the molecular mass distribution in the process.

Bacteria-mineral water treatments can enhance seed germination of wheat and rice and greatly improved chlorophyll synthesis in their leaves. The major polyamines (plant regulators), putrescine, spermidine, and spermine that are found in BMW with HPLC method may explain the hormone-like activity of BMW.

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