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COMPARISON OF MICROBIAL HUMIC ACID PRODUCED BY *Aspergillus niger* X_1 AND SOIL HUMIC ACID

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ABSTRACT

A comparison is drawn between fungal humic acid produced by *Aspergillus niger* X_1 and soil humic acid regarding elementary and functional groups analysis. Elementary and functional groups analysis show that unhydrolyzed fungal humic acid contained more H and N per unit weight, more alcoholic OH but lower CO_2H and phenolic OH groups than unhydrolyzed soil humic acids. The C and N content of 6(N) HCl hydrolyzed humic acid are higher but the O content lower than the soil humic acids. Acid hydrolysis also increase the content of phenolic groups in fungal humic acid, this is accompanied by a decrease in alcoholic OH groups. There exist a number of similarities in analytical characteristics, especially surface functional groups.

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KEYWORDS

Humic acid, functional groups, alcoholic oH, phenolic OH

INTRODUCTION

Humic acids appear to be polymers of phenolic and other aromatic units complexed with amino acid compounds, polysaccharides and possibly other organic constituents of biological origin[1-4]. It may constitute 50 to 80 percent of the soil humus [2,5].

The phenols that participate in the synthesis of humic acids in soil come from three sources : (i) phenolic materials such as flabonoids leached from the plant debris[1,6,7]. (ii) phenolic units formed during the decomposition of lignin[1,2,8,9]. (iii) phenolic substances synthesized by soil microorganisms which may have been utilizing carbohydrates [2,10,11,12]. The phenolic substances may be transformed by enzymatic hydroxylation, demethylation and oxidation of mythylgroups to form numerous mono-, di- and trihydroxy phenols and benzoic acid[2,8].

The most acceptable theory at present is that humic acid is a polycondenste of C_6, C_6-C_1, C_6-C_3 and other phenolic units which are released during microbial degradation of plant phenolic polymers[13,14,15]. Polymerization presumably takes place either by oxidation on more probably under the influence of microbial-phenol oxidase and other enzymes[2,13,15,16].

The possible occurrence in humic materials of complex chemical structures of microbial origin has been stressed by Haider, K. & Martin, J. P. (1967) and Bondietti, E. et al. (1971)[17,18]. These workers have reported that cultures of Epicoccum nigrum, Stachybotrys atra, Stachybotrys chartarum, Aspergillus sydowi and Hendersonula toruloidea when provided with relatively simple organic C and N sources, were able to produce dark-coloured polymeric substances which resembled humic materials in resistance to decomposition in the soil, elementary analysis, exchange capacity, total acidity, molecular weight distribution, release in amino acids on hydrolysis with 6(N) HCl or proteolytic enzymes and phenols, toluenes and phenolic acids released after Na-amalgam reduction of 6(N) HCL-hydrolysed meterials. Both enzymatic and non-enzymatic oxidative and coupling mechanisms were assumed to be active in the formation of the fungal polymers, which were thought to be arisen from interactions of phenols synthesized by the fungi wit peptide on amino acids.

Haider, K. & Martin, J. P. (1967 and Bondietti, E. et al. (1971) used two approaches to demonstrate similarities between fungal humic acids and natural humic acids : (i) they measured oxygen containing functional groups which provided information on the chemical properties of the polymeric surface and (ii) they degraded the two types of materials with Na-amalgam in order to find out what the chemical make up of the 'cores' or 'inside' of the different polymers was[17,18].

A review of the literature shows that oxidative and thermal degradation of humic substances have so far provided more useful information on the chemical structure of humic materials than have reductive degradations. Zetsche, F. & Reinhart, H. (1939) pointed out that it is difficult to reduce humic acids, they have a natural tendency to be oxidized[19]. Zetsche, F. & Reinhart, H. (1939) reduced a number of synthetic and natural humic acids with Na-amalgam in aqueous solutions at about 100^{o}C[19]. The solution underwent a seriese of colour changes which ranged from dark brown, through green, reddish orange to light yellow. The reduction products were found to be extremely unstable and were readily reozidixed by air-oxygen. It was therefore, necessary to completely exclude oxygen. The reduced humic materials could be stabilized by methylation, followed immediately by reduction with Zi-dust in glacial acetic acid

and additional methylation with diazomethane.

Burges, A. et al. (1963) reported recoveries upto 30% of initial soil umic acids of enter-soluble substances after reduction cleavage with Na-amalgam. They described the ether extracts as rich mixtures of phenolic acids and aldehydes including C_6-C_1 and C_6-C_3 units[10].

From what has been stated above, it can be concluded that Na-amalgam reduction is an experimentally difficult procedure that a relatively little is known about the reactions involved in it. Thus, it was set out to produce a fungal humic acid according to the medium composition : glucose, 1%; asparagines, 0.2%; L(+) lysine, 0.75 mg/ml (0.75%); K_2HPO_4, 0.05%; $MgSO_4.7H_2O$, 0.05%; $CaCl_2.2H_2O$, 5 μg/ml and thiamine, 7 μg/ml. The pH was adjusted to 7.4. The fungal humic acid was characterized by a number of analytical method and by permanganate oxidation of methylated materials, one of the most degradative procedures presumably available for the characterization of humic materials. Methylation prior to oxidation protects phenolic OH groups against attack by electrophilic $KMnO_4$ and so permits the isolation of phenolic in addition of alphatic and benzene-carboxylic acids. This procedure has been widely used for the characterization of a large number of humic acids extracted from soils of widely different geographical origins and pedological histories. Present procedure entailed oxidation of the methylated materials with 4% aqueous $KMnO_4$ solution, extraction of the oxidation products into ethyl acetate, remethylation, separation by preparative gas chromatography into relatively pure compounds which were identified by mass spectrometry and microinfrared spectophotometry. The analytical data for fungal humic acids were then compared with those obtained by identical methods on soil humic acids. It was hoped that the results so obtained by identical methods on soil humic acids. It was hoped that the results so obtained would shed additional light on the contribution of microbes to the synthesis of naturally occurring humic acids, a subject that has been of concern to soil scientists for many years.

MATERIALS AND METHODS

Microorganism : In previous experiments a mutant strain A. niger X_1 was obtained by mutagenic treatment with ethylene imine and X-ray irradiation which gave improved production of humic acid[20]. The mutant was stable in the medium having composition : Malt extract, 0.5%; yeast extract, 0.5%; agar, 3%, pH-4.0 and maintained at 4°C. The composition of the medium for humic acid production was same as given before.

Inoculum preparation : cultural condition, isolation and purification procedure of humic acids were same as Ghosh, N. & Banik, A. K. (1988)[21]. The yield of humic acid from 10 litres of culture solution was 14.0 gms.

Determination of Carbon and hydrogen : Carbon and hydrogen were determined by dry combustion, N- by the automated Dumas method, S by oxygen flask combustion and 0 was calculated by difference. The OCH_3 content was measured by the Zeisel method. Moisture was determined by heating separate samples at 105°C for 24 hours and ash by heating at 700°C for 4 hours. Total acidity and carboxyl groups were measured methods described by Schinitzer, M. & Gupta, U.S. (1965), carboxyl groups by oximation and total hydroxyls by acetylation[22,23,24]. Phenolic and alcoholic hydroxyls were considered to equal to the difference between total acidity and carboxyls and between total carboxyls and phenolic hydroxyls respectively. Quinone groups were measured amperometrically[25]. The procedure used for oxidation, separation and identification of oxidation products were identical to that described by Khan, U.S. & Schnitzer, M. (1972)[26].

RESULTS & DISCUSSION

Elementary Analysis : Table – 1shows that the carbon content of untreated fungal humic acid synthesized by A. niger X_1 was somewhat below the range of that for soil humic acids. The soil humic acids were

extracted from samples taken from Canning soils and North Bengal soils of West Bengal. The H content of the untreated fungal humic acid was higher than that for soil humic acids. N-content of the fungal humic acid was slightly higher while the C-content was somewhat lower than the corresponding ranges for the soil humic acids. The OCH_3 content of the fungal humic acid fell within the range of that for soil humic acids.

In order to lower the N-content and to remove carbohydrates, phenols, phenolic acids, metals and other absorbed materials, each humic preparation was hydrolyzed with 6(N) HCl. Following 6(N) HCl hydrolysis, the carbon content of fungal humic acid increased significantly than soil humic acids. Hydrolysis with 6(N) HCl solution reduced the N-content of te fungal humic acid by 80%. Lossess of N on 6(N) HCl hydrolysis were approximately 70% for the soil humic acids. Both fungal and soil humic acids lost S-content on acid hydrolysis. Methoxyl groups in fungal humic acid were less resistant to acid hydrolysis than were those occurring in soil humic acids.

Table - 1

Elementary analysis of untreated and 6(N) HCl hydrolyzed fungal humic acid and of soil humic acids (% dry, ash free)

Element or groups	A. niger X_1 humic acid		Soil humic acid	
	a	b	a	b
C	52.5	63.0	54.0	58.0
H	5.8	4.1	4.5	3.6
N	9.0	1.2	3.2	1.5
S	1.5	0.7	1.0	0.6
O	31.0	30.4	33.1	35.3
OCH_3	1.2	0.5	1.5	1.0
Ash	1.0	0.0	1.8	0.6
Weight loss on 6(N) HCl hydrolysis (%)		47.0		48.1

a = before 6(N) HCl hydrolysis

b = after 6(N) HCl hydrolysis

Functional Groups : Table – 2 shows that the total acidity and CO_2H groups of untreated fungal humic acid (Table 2) were lower but total OH groups, specially alcoholic OH groups were higher than those of soil humic acids. Phenolic OH and total C = O groups fell approximately within the range for naturally occurring humic acids. Following acid hydrolysis, total acidity, to a lesser extent CO_2H groups increased. Total acidities were well within the range for soil humic acids whereas CO_2H groups were still below the range. Acid hydrolysis appear to have little effect on total OH groups. C = O group increased on acid hydrolysis.

Elementary and functional groups analysis show that unhydrolyzed fungal humic acid contained more H and N per unit weight, more alcoholic OH but lower CO_2H and phenolic OH groups than did unhydrolyzed soil humic acids. The C and N content of 6(N) HCl hydrolyzed humic acid were higher but the O content lower than the soil humic acids. Acid hydrolysis also increased the content of phenolic groups in fungal humic acid, this was accompanied by a decrease in alcoholic OH groups. The data in Tables 1 and 2 show a number of similarities in analytical characteristics, especially surface functional groups.

Considering the relatively high phenolic OH content of the hydrolyzed fungal humic acid (Table 2), the yields of phenolic isolates were low. This suggests that eigher most phenolic OH groups in the fungal preparations occur in complex structures that do not yield simple phenolics on permanganate oxidation or that the phenols and phenolic acids formed are destroyed during oxidation.

Table - 2

Major oxygen-containing functional groups in untreated and 6(N) HCl hydrolyzed fungal humic acid and in soil humic acids (μg/g, dry ash free)

Type of humic acid	Total acidity		CO_2H		Total OH		Phenolic OH		Alcoholic OH		Total C = O	
	a	b	a	b	a	b	a	b	a	b	a	b
A. niger X_1 humic acid	4.5	7.8	1.5	2.0	9.6	9.6	3.0	5.8	6.5	3.8	2.5	5.1
Soil humic acid	8.8	8.0	8.0	4.7	4.5	6.1	4.0	3.1	3.0	4.0	2.7	4.7

	Quinone		Ketonic C = O	
	a	b	a	b
A. niger X_1 humic acid		2.7		2.5
Soil humic acid	1.5		1.5	

a = before 6(N) HCl hydrolysis

b = after 6(N) HCl hydrolysis

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