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CHARACTERIZATION OF HUMIC ACID SYNTHESIZED FROM WHEAT BRAN THROUGH MICROBIAL STRAINS ASSOCIATED WITH SHILAJIT

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ABSTRACT
Humic acid (HA) is a dark brown or black colored complex organic molecule with high molecular weight soluble in alkaline solution and insoluble in acidic condition. The specific properties of humic acid products enable their application in industry, agriculture, environmental remediation and biomedicine. In the present study different bacterial strains isolated from *Shilajit* oozing out from rocks were explored for humic acid production by cultivating them on wheat bran with 80% moisture content for 25 days. Humic acid, thus synthesized (as indicated by blackening of wheat bran) was then extracted with 0.1M NaOH, 0.1M $Na_4P_2O_7$ and 6M HCl in a series of steps. The extracted humic acid fraction was lyophilized and the powdered sample was subjected to FTIR analysis. Elemental analysis was also performed to predict the quality of the synthesized humic acid. Furthermore, to understand the pathway of humic acid production from wheat bran, polyphenols were also estimated using UV-Visible spectrophotometer.

KEYWORDS: Bacterial isolates, FTIR, Humic acid, Polyphenols, *Shilajit*.

INTRODUCTION

Humic acids are complex aggregates of brown to dark colored amorphous high molecular weight substances, containing carboxylic, phenolic, amino and quinone with aromatic nuclei of low degree of condensation. The presence of aromatic nuclei with mobile p-type electrons and various functional groups attribute to their ability of ionic exchange, complex formation and oxidation-reduction reactions. It is an organic acid component of humus which act as mineral chelators and are resulted from decomposition of humin component of soil humus by soil bacteria (Senesi *et al*., 1991). Different salts of humic substances, such as calcium humate, and Ammonium humate are used to increase soil fertility as well as growth-stimulating effect (Buckau *et al*., 2000, Lotosh, 1991).

Antibacterial (Skliar *et al*., 1998) and antiviral (Klocking *et al*., 2002) properties of humic substances represent new possibilities for their medical application. The main reason for the increasing attention devoted to humic acids can be explained by their antiviral, profibrinolytic, anti-inflammatory and estrogenic activities (Yamada *et al*., 1998). Currently a variety of veterinary and human medicines employ commercial humic substances. Several studies of the medicinal properties of humic materials have been reported (Mund-Hoym 1981, Brzozowski *et al*., 1994). It has been found that humic acids significantly accelerate the healing process in case of gastric and duodenal ulcers in experimental models (Brzozowski *et al*., 1994). Preincubation of cell cultures with ammonium humate have been reported to avoid infection by the herpes virus (Thiel *et al*., 1981). Humic materials in aquatic systems and water sediments have been observed to be closely connected with efficacy of hydrotherapy and balneotherapy (Gadzhieva *et al*., 1991; Hampl *et al*., 1994).

Apart from this, they show numerous benefits in crop production and have electrically charged sites on their surfaces which function to attract and inactivate pesticides and other toxic substances. For this reason the Environmental Protection Agency recommends the use of humates for cleanup of toxic waste sites (Pettit, 2004). However, their structure varies depending upon their origin; major elements in their composition *viz.* C, H, O, N, and S are always present regardless of their origin and country or continent (Kurkova *et al*., 2004). Although formation process of humic substances has been studied hard and for a long time their formation is still the subject of long-standing and continued research. In the present study, humic acid synthesis from wheat bran has been reported for the first time with the help of different bacterial isolates from *Shilajit*. Wheat bran is one of the richest sources of fibre, and non-starch polysaccharide (NSP) out of which 70%are arabinoxylan, 24% cellulose and 6% beta-glucan (Maes and Delcour, 2002). According to Pomeranz (1988), the wheat caryopsis, by weight, is composed of an outer branny husk (14-16% of the grain), the germ or embryo (2-3%), and the central endosperm (mainly starch 81-84%). Wheat bran fractions also contain almost all of the B-group vitamins as well as phenolic acids, carotenoids and tocopherols that can perfectly support the growth of microflora (Shewry *et al*., 2010). Therefore, submerged fermentation of wheat bran was explored for production of humic acid through different microbial strains isolated from crude *Shilajit* reported earlier (Mishra *et al*., 2015).

MATERIALS AND METHODS

Samples origin

Shilajit samples were collected in zip lock polythene bags from different sources i.e. *Shilajit* (exudates) deposited over leaves, exuding out from mountain and deposited on rocks. After serial dilution, the samples were spread onto Luria Bertani (LB) Agar plates (HiMedia) followed by incubation at 30±2ºC for 24-48 hours. Colonies were selected initially according to their morphology and isolation of well-defined single unit of colonies resulted in the isolation of five different microbial strains as pure cultures.

Cultivation of microbial strains

All the samples were preliminarily identified using morphological and biochemical examination and were further identified using 16S rRNA and ITS gene sequencing. Pure cultures were initially cultivated on LB broth for 24 hours and then 1 ml of each sample was inoculated to 250 ml Erlenmeyer flask containing 5g autoclaved wheat bran with 80% moisture content. The flasks were then incubated at 30±2ºC with continuous shaking at 150 rpm for different days. Initial pH at the time of inoculation was also recorded.

Total Polyphenols’ content

The polyphenols’ content was determined by the Folin-Ciocalteu method according to the method described by the International Organization for Standardization (ISO, 2005). The culture filtrate was harvested at regular intervals of 2, 4, 6, 8, and 10 days, which were further analyzed for polyphenols’ content. From each harvested filtrate, 250 µl was diluted with sterilized distilled water to 1 ml. Then 5 ml of 10-fold-diluted Folin-Ciocalteu reagent was added, followed by the addition of 4 ml of 7.5% sodium carbonate after 3 min. Mixtures were incubated in water bath at 40 °C for 30 min. The absorbance was then measured at 734 nm. The total polyphenols content was calculated and expressed as gallic acid equivalents (GAE: g/100 g dry mass) using a gallic acid (5-25 mg/l) standard curve.

Humic substances extraction

To maximize the extraction of humic acid, some modifications were made to the method described by Schnitzer (1982). After 25 days cultivation of different bacterial cultures over the wheat bran, the culture extracts were harvested by adding 30 ml sterilize double distilled water to the flask followed by 1 hr shaking to ensure proper mixing of the cultures broth. The mixture was filtered through the muslin cloth and then centrifuged at 8000 rpm for 10 min. The filtrate was then used for humic acid extraction as follows:

Weighed NaOH and $Na_4P_2O_7$ were added one after another into the harvested solution in such a way that final concentration of each additive in harvested solution resulted into 0.1 M. The mixture was then kept on a shaker at 150 rpm for 18 hrs at 25°C followed by the centrifugation of the suspension at 10,000 rpm for 25 min. The supernatant was carefully decanted into a clean glass beaker and retained for separation into humic acid (HA) and fulvic acid (FA) fractions; whereas, the pellet was washed with deionized water and freeze dried for humin analysis. Further on, the supernatant was subjected to pH adjustment with 6 M HCl till the pH of 1.5 was obtained. The solution was then centrifuged to isolate humic acid from the mixture. Yellowish colored supernatant was fulvic acid fraction, while the humic acid fraction precipitated down. The wheat bran with 80% moisture content kept for 25 days without any microbial strain was used as control for comparison with fermentation through different microbial strains.

Humic acid purification

25 ml of 0.1 M NaOH was added to the precipitated residue to redissolve it and then the resulting solution was lyophilized. Dried samples were stored and further used for FTIR analysis.

UV-Visible spectroscopy

UV-Visible spectra in the region between 200-800 nm were obtained using Labtronics Double Beam UV-VIS Spectrophotometerwith pure water as a reference. Samples were diluted to maintain the maximal response and all measurements were made in triplicates in quartz cuvettes.

Fourier-transform infrared spectroscopy

The lyophilized samples were then subjected to FTIR analysis and prepared by weighing 0.8 mg of lyophilized samples and 80 mg of spectroscopy grade KBr. FTIR spectra of humic fraction samples were collected with a Nicolet 380 spectrophotometer in the 400 to 4000 cm^{-1} range with a 4 cm^{-1} resolution and 32 scans per sample.

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Elemental analysis
Different elements were determined with the aid of a CHNS analyzer (Thermo Finnigan) by packing the humic acid powder in the tin boats after careful weighing. Obtained values were expressed as dry weight of powder in mass %.

RESULTS AND DISCUSSION
Different researchers have proposed three theories to elucidate the mechanism of humic acid formation, *viz.* Sugar-amine Condensation Theory, the Lignin Theory and the Polyphenols Theory. Most investigators suppose that humic substances have originated from lignin degradation (Oglesby *et al*., 1967). Lignin biodegradation often results in polyphenols formation which probably plays a key role in the humic acid synthesis process. Polyphenols can be considered as humic acid precursors and regarded as main agents in the formation of humic substances from some plants that do not contain much lignin. They themselves possess enough reactive sites to permit further transformations, as in some condensation reactions. In the present study, amount of polyphenols from the different microbial isolates in the wheat bran fermentation were estimated on the subsequent days. As proposed by Stevenson (1982), cellulose and other compounds, on partial degradation by microbial species, synthesize polyphenols which contribute to humic acid synthesis on combining with different amino compounds. So, it can be hypothesized that the humic acid production, in the present study, is attributed by the series of biodegradation of components of wheat bran, carried out by the microbial isolates in accordance to the theories of humic acid production put forth by Waksman (1932) and Stevenson (1982).

Humic acid production
Change of color of wheat bran from creamish yellow to black was the first indication of humic and fulvic acids formation. Moreover, liquefaction of wheat bran and decrease in pH from 6.8 (day 1) to around 4 (upto day 10) was also observed in comparison to the control which is indicative of degradation in the wheat bran and also acid production from fermentation up to day 10. However, increase in pH to 9, 10, 11 and 12 with the respective strains (upto day 25) was the indication of production of humic acid as it needs alkaline pH to aggregate. According to Pettit (2004), in alkaline soils, a large percentage of organic matter is present in the form of humic acids whereas in acidic soils fulvic acid is predominantly available.

Total Polyphenols'content
The estimated amount of polyphenols present in the extract was compared among all the strains as well as with the control. It was observed that maximum polyphenols were produced on 4thday with all the strains. Among them *Bacillus subtilis* is the most promising strain for humic acid production and showed maximum amount of polyphenols (966 μg/ml) followed by *B. cereus* then *Enterococcus lactis* and *Fereydounia sp.* isolate S5Act, while the *B. clausii*, showed no polyphenols production as compared to the control (Figure 1). Previously, similar method has been adopted for estimation of polyphenols in the pomegranate peels (Wissam *et al*., 2012).

UV-Visible spectroscopy
The absorbance of humic acid fractions in UV-VIS spectral range was recorded. *E*4/*E*6 ratio (OD at 465nm/665nm) were below 4 in all the samples, out of which two samples, *B. subtilis* and *B. cereus* showed values ranging from 3 to 4 that correspond to the highest humus quality in humic acid (Table 1). Our results are consistent with previous reported data which reports that *E*4/*E*6 ratio between 3 to 4 indicates good quality, equal to 4 indicate moderate quality and above 6, indicate a worse HA quality, as well as the presence of more aliphatic and fewer aromatic compounds (Trompowsky *et al*., 2005; Agarwal *et al*., 2010).
In *Fereydounia sp.* isolate S5Act, *E*4/*E*6 ratio was less than 3 which indicated low quality humic acid, while in case of *B. clausii*, no humic acid or humus substances were formed (Table 1).

FTIR analysis
The absorption spectra were obtained for the fraction of the synthesized humic acids from wheat bran using five microbial strains (Figure 2). The FTIR spectrum of the humic product was characterized by a number of sharp peaks and was interpreted with reference to already reported data. From the spectra, it is evident that all the bacterial isolates were capable of humic acid production out of which *B. subtilis* was found to be the most promising strain and was showing all the characteristic peaks of humic acid. The broad intense band, at 3400 cm^{-1} due to the stretching vibration of bonded hydroxyl groups, was present in all the samples (Solomon *et al*., 2007; Meccozi *et al*., 2009).

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The sample *B. subtilis* exhibited broad peak in absorption range 2960 cm^{-1}which was attributed to C-H strong stretch. Two peaks were observed in a region around 2924 and 2852 cm^{-1} which might be attributed to asymmetrical and symmetrical stretching of methylene ($-CH_2-$) groups and is a characteristic of aliphatic and non-strained cyclic hydrocarbons present in humic acid (Silverstein *et al*., 1991; Goivanela *et al*., 2004). Shoulder peak was observed in a region of 2179 cm^{-1} corresponding to asymmetrical alkyne variable stretch and a peak around 1639 cm^{-1}, corresponding to alkene (C═C) variable stretch or unsubstituted amides-NH bending in quinones was also observed (Coates, 2000).These peaks are in agreement with previous reported HA peaks from *Shilajit* and sediments (Agarwal *et al*., 2010; Goivanela *et al*., 2004) (Table 2). Agarwal *et al*. (2010) also reported intense peak in the absorption range 1564 corresponds to stretching of aromatic ring as HA peak from *Shilajit* and is consistent with our data. Sharp peak at 1116 cm^{-1} corresponding to C-O strong stretch and a peak near 1023cm^{-1}attributed to O-Si-O linkage reported in HA from Brazilian soils (Dick *et al*., 2003).

Peak at 932 cm^{-1} is due to N-O stretch and diffused peaks in the range 866 and 881 cm^{-1} are indicative of 1, 3-Disubstitution at Meta position in aromatic compounds (Coates, 2000).Weak bands observed in a region of 694 cm^{-1}, 623 $cm^{-1}$and medium band in 780$cm^{-1}$is indicative of strong alkyl halide stretch.

In case of *B. cereus*, most of the peaks were similar to that of *B. subtilis* like 3433, 2931, 2493, 2169 , 1446, 1119, 1028 and 933 cm^{-1} except a sharp peak at 994 cm^{-1} attributed to polysaccharide chains (Figure 2). Earlier, Pospisilova and Fasurova, (2009) have reported similar peak in HA from lignite (Table 2). Unlike the other isolates, *B. clausii* did not exhibit the consistent peaks, corresponding to humic acid, according to previously reported data, which has been discussed above, except for 1647, 2181, 1026 and 1119 cm^{-1}.

The presence of these four peaks is insufficient to confirm humic acid production by this strain. Absorption spectra of *Enterococcus lactis* and *Fereydounia sp.* isolate S5Act are similar to *B. subtilis* in range of 3433, 2924-2918, 2853-2851, 2171-2180, 1646-1658, 1445-1449, 1118-1123,1022-1030 and 919-933 cm^{-1} and these peaks are characteristic humic acid peaks, as already discussed (Figure 2). Apart from this, *Enterococcus lactis* exhibit two unique peaks in 1564 cm^{-1} and 695 cm^{-1} which are attributed to vibration of aromatic ring due to carboxylates and alkyl halide stretch i.e. C-Cl. In *Fereydounia sp.* isolate S5Act, peaks at 2957, 2494, and 701, were observed which correspond to methyl C-H asymmetric/symmetric stretch and *cis* C-H out-of-plane bend (Coates, 2000).However a peak, unique to all other strains, in the range of 1774-1775 was found in both *Enterococcus lactis* and *Fereydounia sp.* isolate S5Act which correspond to carbonyl C═O stretch of anhydride or ester fatty acids (Mecozzi *et al*., 2009). Similar peaks were observed in humic acid from *Shilajit* and soil (Agarwal *et al*., 2010; Ahamadou *et al*., 2013).

Table 1. Comparison of E4/E6 ratio of HA produced from different microbial strains.

Bacterial Strain	OD 465nm (E4)	OD 665nm (E6)	E4/E6 ratio
Bacillus subtilis	0.138	0.455	3.033
B. cereus	0.118	0.039	3.026
B. clausii	0.013	0.021	0.619
Enterococcus lactis	0.084	0.022	3.818
***Fereydounia sp.* isolate S5Act**	0.336	0.194	1.732

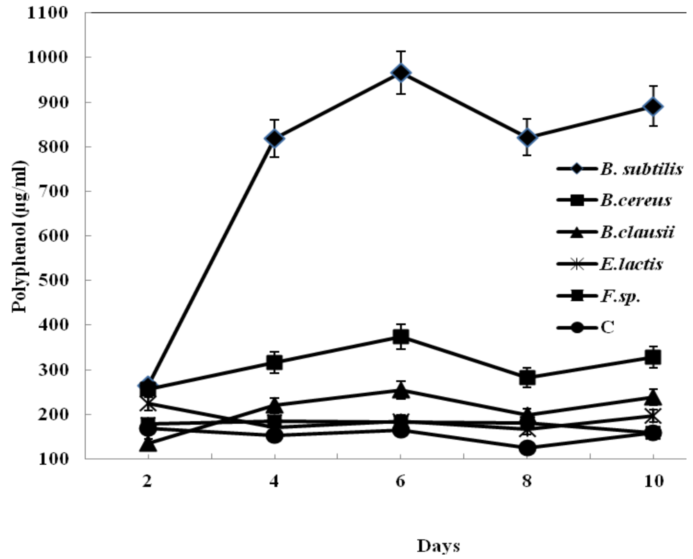

Figure 1. Day profiling of Polyphenols' production during wheat bran fermentation through different microbial strains as compared to control.

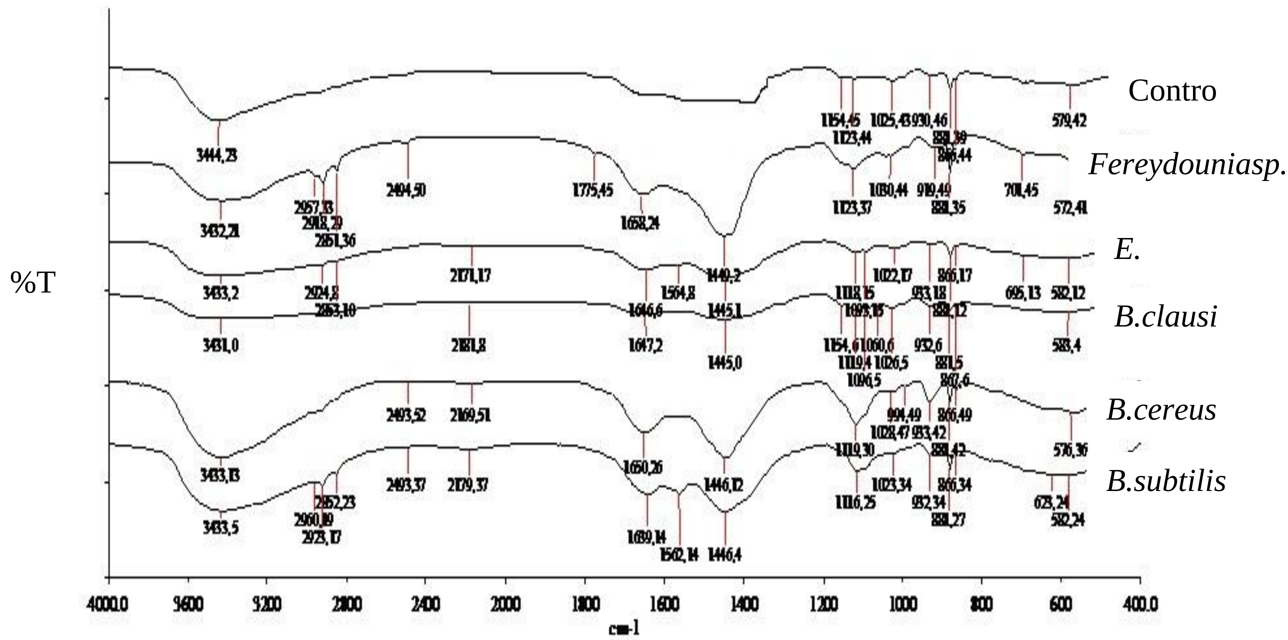

Figure 2. FTIR spectra of humic acid from different microbial strains as compared to control.

Table 2. Major FTIR peaks obtained and suggested assignments for humic acid from different microbial strains

Microbial strain showing respective peak	Absorption Range(cm^{-1})	Assignment	Comparison	Reference
***Bacillus subtilis, B. cereus, B. clausii, Enterococcus lactis, Fereydounia sp.* isolate S5Act**	3433-3431	stretching vibration of bonded hydroxyl groups of phenols	Previous reported HA peaks from coal and compost .	Solomon et al., (2007); Meccozi et al., (2009);
***B. subtilis,Fereydounia sp.* isolate S5Act**	2960	C-H strong stretch		Silverstein et al., (1991)
***B. subtilis, B. cereus, Enterococcus lactis, Fereydounia sp.* isolate S5Act**	2931-2917	asymmetrical stretching of methylene ($-CH_2-$) groups	Characteristic of aliphatic and non strained cyclic hydrocarbons present in humic acid.	Silverstein et al., (1991); Goivanela et al., (2004)
***B. subtilis, B. cereus Enterococcus lactis, Fereydounia sp.* isolate S5Act**	2852-2851	symmetrical stretching of methylene ($-CH_2-$) groups		
***B. subtilis , B. cereus , B. clausii , Enterococcus lactis, Fereydounia sp.* isolate S5Act**	2181-2171	asymmetrical alkyne variable stretch.		Silverstein et al., (1991)
***Enterococcus lactis, Fereydounia sp.* isolateS5Act**	1775-1774	carbonyl C=O stretch of anhydride or ester fatty acids.	Reported HA peaks from *Shilajit* and soil	Agarwal et al., (2010); Ahamadou et al., (2013)
***B. subtilis , B. cereus , B. clausii , Enterococcus lactis, Fereydounia sp.* isolate S5Act**	1639-1658	alkene (C=C) variable stretch or unsubstituted amides-NH bending.alsorefered as carboxylic peak.	Previously reported HA peaks from *Shilajit*	Agarwal et al., (2010)
B. subtilis , Enterococcus lactis	1562-1565	stretching of aromatic ring		
***B. subtilis , B. cereus , B. clausii , Enterococcus lactis, Fereydounia sp.* isolate S5Act**	1446-1449	CH and NH (amide II) bending motions, molecule skeleton vibrations and CO-bond vibration		Antil et al., (2005)
B. subtilis , B. cereus , B. clausii , Enterococcus lactis	1116-1119	Si-OR strong stretch		Silverstein et al., (1991);
***B. subtilis , B. cereus , B. clausii , Enterococcus lactis, Fereydounia sp.* isolate S5Act**	1023-1030	O-Si-O linkage peak or C-O stretch	Reported in HA from Brazilian soils.	Dick et al., (2003)
B. cereus	994	polysaccharide chains	HA from lignite	Pospisilova and Fasurova, (2009)
***B. subtilis , B. cereus , B. clausii , Enterococcus lactis, Fereydounia sp.* isolate S5Act**	881	1, 3-Disubstitution at Meta position in aromatic compounds		Coates, (2000)
B. subtilis , B. cereus , B. clausii , Enterococcus lactis	866			
B. subtilis , B. cereus , B. clausii , Enterococcus lactis,	780	alkyl halide stretch		Ding et al.,(2002)
***Fereydounia sp.* isolate S5Act**	701	*Cis*C-H out-of-plane bend		Coates, (2000)
B. subtilis , Enterococcus lactis	694	strong alkyl halide stretch		Filip and Demnerova, (2007); Coates, (2000)
B. subtilis	623			

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Table 3. Elemental analysis of humic acid produced from different microbial strains and comparison with crude and pure *Shilajit*.

Atomic ratios	*Bacillus subtilis*	*Bacillus cereus*	*Enterococcus lactis*	*Fereydounia sp.* isolate S5Act	Crude Natural *Shilajit*	Rock *Shilajit* (*Dabur*)*
% C	28.704	21.614	20.097	26.491	23.788	36.46
% N	2.427	2.982	1.889	3.614	1.891	3.03
% H	3.375	2.448	1.867	3.180	2.277	ND
% O	65.494	72.843	76.147	66.715	72.044	ND
H/C ratio	0.118	0.113	0.093	0.120	0.095	ND
N/C ratio	0.085	0.138	0.093	0.136	0.079	0.083
C/N ratio	11.827	7.248	10.639	7.330	12.580	12

* adapted from Agarwal *et al*., 2010.

Elemental analysis

All the samples had high carbon and oxygen content due to organic mass, but low nitrogen and nil sulphur percentage (Table 3). Terrestrial humic substance samples, due to the higher plant inputs are, in general, rich in lignin moieties presenting, consequently, low H/C and N/C ratios (Stuermer *et al*., 1978). However, C/N ratio of sample *B. subtilis* and crude natural *Shilajit* is comparable to the rock *Shilajit* extract, which is processed for making edible *Shilajit* by Dabur India Limited (Agarwal *et al*., 2010).

The low value of the C/N ratio in organic treatments could probably be attributed to the high levels of products arising from protein decomposition and hence may indicate a greater level of humification. According to Baddi *et al*. (2004), high values of nitrogen and sulphur are indicative of high contents of non-humified biomolecules (polysaccharides and polypeptides) in the material due to incomplete hydrolysis of proteinaceous constituents. Previously reported data on HA from sediments reveals that high N/C (>0.06) and H/C ratio (>1.35) is indicative that HA is formed by receiving, constant algal inputs while lower ratio indicates higher plants input (Goivanela *et al*., 2004). In the present data, N/C ratio comes out to be very high, while H/C ratio is quite lower (Table 3) which is indicative of an entirely different source of humic acid and is consistent with our study, as we have used wheat bran as a source. According to Stevenson (1994), higher C/H, C/O, and C/N atomic ratios are associated with higher degrees of humification due to decreased acid, carbohydrate, and amino acid/protein content. So, the humic acid produced by these microbial strains can be regarded as highly humified and degraded product.

CONCLUSION

Humic acid production from wheat bran using microbial strains was investigated for the first time and the study demonstrated that *B. subtilis* was most promising among all the strains. It was evident from atomic ratios as well as E4/E6 ratios that the fermentation on wheat bran with known isolates resulted in good quality humified product as compared to HA from commercially available sources. Maximum amount of polyphenols were also observed in *B. subtilis*, exhibiting all the characteristics peaks for HA. Henceforth, the NMR studies of the HA produced and its yield from wheat bran through *B. subtilis* using bioreactors is underway.

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