Assessment of microbial roles in the bioconversion of paper mill sludge through vermicomposting

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Abstract

Purpose Main thrust of the present study is to determine the role of microbes in changing the proportion and turnover of nutrients such as carbon and nitrogen during vermicomposting of paper mill sludge through the assessment of β -glucosidase and Leucine arylamidase activities.

Methods The change in the ratio of Total Organic Carbon (TOC) and Total Kjeldahl Nitrogen (TKN) during sludge composting using paper mill sludge, cow dung, straw in the ratio of 5:4:1 have been determined alongside observing β -glucosidase and Leucine arylamidase activities in different phases (0th, 30th and 60th days) of vermicomposting. The present study also assessed the bacterial assemblages in order to predict their role as major producers of these enzymes as estimated by Vitek 2 system.

Results A declining trend of C/N ratio was observed which exhibited significant negative and positive correlations with the activity of β -glucosidase and Leucine arylamidase respectively. Twenty-four strains were isolated under two genus *Bacillus* spp. and *Lysinibacillus* spp. which revealed differential sensitivity towards major carbon and nitrogen turnover mediating enzymes.

Conclusion Change in the activities of carbon and nitrogen turnover enzymes due to microbial growth and proliferation are the mediator for the change in C/N ratio.

Keywords Cowdung · Bacterial isolates · C/N ratio · Turnover enzymes · Sludge composting

Introduction

India shares about 2.6% of global paper production catering to the need of socio-economic development of the country. However, in most of the developing countries, use and recycle of paper mill wastes like discarded papers; wood pulps etc. have been contributing a lot of adverse effects on the environment due to improper management of wastes. Such industries used to release very toxic polychlorinated dibenzodioxins, dibenzofurans, and suspended solids [1]. Wastes of paper industries are generally classified as primary sludge or primary effluent or non-activated sludge produced in a raw form prior to treatment and secondary sludge or secondary effluent or activated sludge obtained after several treatments (charging, settling, slanting, drying etc.) of primary sludge. In such

context, alongside abatement and controlling of sludge mediated pollution by existing mechanical or physical technologies, developing of biological methods especially by way of microbial amendments has appeared to act not only as an acceptable tool for bio-conversion but also to ensure sustainability in the eco-management of solid organic wastes [2–4].

Standardizing and up-gradation of existing vermitechology process involve an intricate interaction between earthworms and microorganisms which greatly modify its physical and biochemical properties of the compost [5]. This eco-friendly and user-friendly biotechnological tool is now being used not only for bioconversion of wastes but also to ensure sustainable environmental management. Therefore, they are used as a sustainable tool for decomposition of different wastes [6–8].

Generally, earthworms feed on that organic stuff supplemented with necessary ingredients and in oblivion consume a large number of bacterial populations along with it. These microbes under ideal microenvironment of gut tend to become activated and increase the population of plant growth-promoting rhizobacteria (PGPR) [9]. This specific group of bacteria has been found to stimulate plant growth through

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solubilization of nutrients through the breakdown of complex organic polymers of carbohydrates and polypeptides [10]. This suggests the presence of carbohydrate (carbon) and peptide (nitrogen) degrading enzymes within vermicompost [11]. In such context, the present study has attempted to pinpointedly focus on the declining trend of C/N ratio over a period of 60 days of vermicomposting and also correlated the change with the activity of two selected carbon and nitrogen turnover enzymes including β -glucosidase and arylamidase enzymes respectively in random as their representatives. Since both earthworms and microbes tend to interact in the vermicomposting process, the present study has also isolated and enumerated microbes in order to understand their contribution towards production of these enzymes along with their phylogeny.

Materials and methods

Collection of sludge

Sludge samples were collected from UNIGLOBAL paper mill at Jhargram, India (22° 27' 0" N, 86° 59' 0" E) in plastic zip bags under aseptic conditions and marked as primary and activated sludge respectively. Sludge samples after being properly processed, were sundried and used for vermicomposting for the present study.

Experimental design and chemical analysis

Two kilograms of each sundried sample was grounded properly and mixed with cow dung with straw in the ratio of waste: cow dung: straw as 5:4:1. In the present study, sludge samples were not directly used due to its toxic effect and heavy metal content [12]. Therefore, the samples were supplemented with dried cow dung as a bulking agent and were placed in separate ceramic tubs (24 in. × 18 in. × 18 in.) along with thirty potential breeders of *Eisenia foetida* for 60 days in three replicates [13, 14]. The ratio used here was optimized in the laboratory with several trials (considering least mortality rate) under proper environmental conditions. The temperature during the entire process of composting was maintained at 25 °C with a moisture content of around 60% and pH (7.4–7.6) through the regular sprinkling of water [2].

Chemical analyses of two nutrients (Carbon and Nitrogen) were considered during the vermicomposting. The Total Organic Carbon (TOC) and Nitrogen (TKN) were measured using Walkley-Black and Kjeldahl methods respectively [15]. C/N ratios were calculated for both types of sludges using the estimated results of C and N at 0th, 30th and 60th days of vermicomposting. C/N ratio on the 0th day of vermicomposting was treated as control.

Growth of bacteria from two samples of vermicompost

1.0 g of the sample from each vermicompost prepared from both types of sludge (primary and secondary) were taken at different times during the process and subjected to serial dilution up to 10^{-6} in normal saline solution. 200 μ l of the sample was added in Nutrient Broth (HIMEDIA) and kept at incubation for 48 h at 37 °C. 10 μ l of samples were taken and further spread over minimal agar plate made up of Nutrient Agar (HIMEDIA) and kept at incubation for 24 h at 37 °C for proper isolation of colonies.

Analysis of enzyme activity

β -Glucosidase (EC 3.2.1.21) activity: 0.2 g of vermicompost obtained from the bioconversion of primary sludge was incubated at 37 °C with 2 mL of maleate buffer at pH 6.5 along with 2 mL of 0.05 M 4-nitrophenyl- β -D-glucopyranoside (PNG) as the substrate for 2 h. To stop the reaction, samples were kept at 2 °C for 15 mins, and the amount of p-nitrophenol (PNP) formed in this given reaction was determined at 398 nm (SHIMADZU UV - 1601). Amount of PNP formed is directly proportional to the β -glucosidase activity of the sample. This procedure was also followed for the vermicompost of secondary sludge [16].

Leucine arylamidase (EC 3.4.11.2) activity: 1 g of vermicompost from respective composting material was taken in an Erlenmeyer flask and was treated with 3 mL of 0.1 M, tris-hydroxymethyl aminomethane (THAM) buffer (pH 8.0) and 1 mL of 8.0 mM L-leucine b-naphthylamide hydrochloride. Contents were mixed properly and then placed on a shaker cum incubator at 37 °C for 1 h. The reaction was then allowed to stop by adding few drops of ethanol (95%). The suspension formed was centrifuged at 17,000 g for 5 min and the resultant supernatant was then treated with 1 mL of ethanol, 2 mL of acidified ethanol and 2 mL of the p-dimethyl-amino cinnamaldehyde reagent. The solution was mixed well by using vortex mixer and the intensity of the red azo compound was determined at 540 nm (SHIMADZU UV - 1601) [17]. All the experiments were conducted in three replicates at 0th, 15th, 30th and 60th days of vermicomposting process from two different waste materials. Experiments on 15th day were done only to emphasize the rate of bioconversion during an early phase of vermicomposting [11, 18].

Biochemical characterization and enzyme sensitivity

Microbial contribution towards the enzymes was ascertained through Vitek 2 system (bioMérieux, India), an automated system which measures the growth potential of bacteria calorimetrically. By using an applicator stick, colonies from pure slant culture were allowed to suspend in 3.0 ml of sterile saline

Table 1 C/N ratio of vermicompost prepared from primary and secondary sludge at 0th, 30th, 60th days respectively

Type of sludge's	Total Organic Carbon (gm/kg)			Total Organic Nitrogen (gm/kg)			Ratios (C/N)		
	0	30	60	0	30	60	0	30	60
Primary sludge (PS)	750.97 ± 1.17	407.08 ± 4.28	282.69 ± 2.67	5.40 ± 0.50	11.29 ± 1.05	21.18 ± 0.57	138.92 ± 12.77	36.06 ± 3.73	13.35 ± 0.48
Secondary sludge (SS)	402.31 ± 1.84	242.47 ± 2.87	152.78 ± 1.47	4.68 ± 0.30	15.65 ± 0.30	23.10 ± 0.22	86.21 ± 5.75	15.49 ± 0.33	6.61 ± 0.09

Values are taken as mean ± SD; $n = 3$

(0.50% NaCl, pH adjusted to 6.5) and turbidity was adjusted according to McFarland Turbidity Range 1.80 to 2.20. BCL identification cards were inoculated with microbial suspension followed by card sealing, incubation at 36 °C and the readings are taken accordingly. Since BCL cards were impregnated with several isozymes of arylamidases, glucosidases, galactosidases and mannosidases, positive responses from Vitek 2 data reflect these microbes as a potential contributor towards these set of specific carbon and nitrogen turnover enzymes.

16srRNA gene sequencing and phylogeny construction

16srRNA sequencing technique was used here for identification of different bacterial samples isolated during the process of vermicomposting. Three heat shock cycles (20 mins at −85 °C and 15 mins at 95 °C) were used for genomic DNA isolation. Amplification of DNA samples through PCR (Polymerase chain reaction) was carried out using two universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-AGAGCCCGATCMTGGCTCAG-3'). Unincorporated PCR primers, dNTPs from PCR products were removed by Montage PCR Clean-Up Kit (Millipore). Sequencing was performed using ABI PRISM Big Dye TM Terminator Cycle Sequencing Kit with Ampli Taq DNA polymerase (FS enzyme) (Applied Biosystems). Single-pass sequencing was performed using 27F/1492R primers and fluorescently label fragments were purified and subjected to

electrophoresis in an ABI 370X1 sequencer (Applied Biosystems) [19].

16srRNA gene sequence was BLAST using NCBI BLAST search tool. MUSCLE 3.7 programs used for multiple alignments of sequence and were cured using program G blocks 0.91b. Finally, PhyML3.0Airt was used for phylogeny analysis using HKY85 as substitution model and program Tree Dyn 198.3 was used for tree rendering [20].

Statistical analysis

Tukey test (One-way ANOVA) and co-relational analysis were performed using SPSS version 25. These tests were performed to find out any significant relation between the change in C/N ratio and enzyme activity.

Data availability Currently the data is unavailable because it is a part of our ongoing work.

Results and discussion

Interpretation of C/N ratio

C/N ratio was calculated at regular intervals during the entire period of vermicomposting. This ratio represents the rate of decomposition and stability of organic compounds during its bioconversion through vermicomposting [2]. Initially, at the 0th day of vermicompost, C/N ratio was found to be 138.92

Fig. 1 **a** Represents the C/N (carbon / nitrogen) ratio change of vermicompost prepared from primary sludge. **b** Represents the C/N (carbon/nitrogen) ratio change of vermicompost prepared from secondary sludge. Error bars represent the standard deviation where $n = 3$. * indicates the significance of test using Tukey test at $p = 0.05$

Fig. 2 **a** Represents the change in β -glucosidase activity in vermicompost prepared from primary sludge. **b** Represents the change in β -glucosidase activity in vermicompost prepared from secondary sludge. Error bars denote standard deviation at $n=3$. * shows significance of the data at $p=0.05$

(PS) and 86.21 (SS) but it significantly decreased over the period of time and subsequently attained a value of 13.35 (PS) and 6.61 (SS) at 60th day of vermicomposting (Table 1 and Fig. 1). A significant decrease of C/N ratio was also reported previously by several authors [21–23].

Identification of bacteria

Microbial studies including screening and identification of bacteria were done only to enumerate the role of microbes as a producer of certain enzymes. RSC and RG comprise of strains isolated from the vermicompost of primary and secondary sludges. These were found to comprise of total of twenty-four strains of bacteria which were subjected to 16srRNA sequencing. It revealed that RSC1, RSC5, RG3, RG8 were *Lysinibacillus sphaericus*; RSC2, RSC6, RG4, RG9 were *Lysinibacillus xylaniliticus*; RSC3, RSC7, RG5, RG10 were *Lysinibacillus macroides*; RSC4, RSC8, RG1, RG7 were *Bacillus cereus*; RSC10, RSC11, RG6, RG11 were *Bacillus thuriangiensis*; RSC9, RSC12, RG2, RG12 were *Lysinibacillus fusiformis*. However, application of medium and other chemicals are supposed to disturb the actual distribution of bacteria over these samples. Relevant bioinformatics tools were

applied for deducing phylogenetic affiliation of isolated bacterial strains (Fig. 4). Earlier studies have advocated the occurrences of many bacterial species such as *Bacillus*, *Clostridium*, *Lactobacillus*, *Acetobacter*, and *Thermoactinomyces* in the vermicomposting process [24–27].

Analysis of enzyme activity

β -glucosidase, an enzyme required for the breakdown of the polymer of glucose mainly cellulose and other forms of it over the entire period of vermicomposting have tended to focus on the carbon turnover during vermicomposting. In comparison with the 0th day of vermicomposting, β -glucosidase activity was found to be always high during vermicomposting of both the sludges and at 15th day, a significant maximum rise in the enzyme activity was noticed which thereafter decreased gradually. Increase in enzyme activity at the beginning was due to huge substrate availability which started decreasing with time upon enzyme-substrate reaction [11] (Fig. 2). Correlation analysis depicts an overall negative correlation with the C/N ratio and β -glucosidase activity during vermicomposting as prepared from primary sludge ($P < 0.05$, $r = -0.687$) and secondary sludge ($P < 0.05$, $r = -0.845$) respectively. Negative

Fig. 3 **a** Represents the change in Leucine arylamidase activity in vermicompost prepared from primary sludge. **b** Represents the change in leucine-arylamidase activity in vermicompost prepared from secondary sludge. Error bars represent standard deviation at $n=3$. * shows significance of the data at $p=0.05$

Fig. 4 Top to down order: *Lysinibacillus sphaericus*, *Lysinibacillus macroides*, *Bacillus cereus*, *Bacillus thuringiensis*, *Lysinibacillus xylanilyticus*, *Lysinibacillus fusiformis*

correlation ascertains the fact that there is a rapid breakdown of organic carbon polymer specifically ligno-cellulose with the help of these enzymes during the process.

Arylamidases are the exo-amino peptidases which hydrolyze the N-terminal amino acid from a nitrogen polymer. This study focuses on its activity by taking leucine arylamidase as a representative of this group of enzymes. Enzyme activity was found to be maximum at 15th day and then decreases gradually for both types of sludges. However, it was always high as compared with the 0th day of vermicomposting. This decrease in enzyme activity after 15th day depicts a decrease in turnover rate upon substrate utilization. The sharp decrease at the end of vermicomposting was previously noticed for urease and protease activity which suggest depolymerisation and degradation of organic nitrogen by earthworms and

microorganisms. [11]. (Figure 3) Correlation study indicates an overall significant positive relation between the C/N ratio and enzyme activity during vermicomposting as prepared from primary sludge ($P < 0.05$, $r = 0.745$) and secondary sludge ($P < 0.05$, $r = 0.658$) respectively. Positive correlation reflects a continuous breakdown of complex nitrogen to its simple products during vermicomposting (Fig. 4).

Screening of carbon and nitrogen turnover enzyme sensitive bacteria

The decrease in C/N ratio intensifies a rapid bio-degradation of total organic carbon and mineralization of organic nitrogen which are presumed to remain in the form of simple amino acids to the compost. Such observations have necessitated for

Table 2 Differential sensitivity of bacterial isolates (RSC 1–12) towards several isozymes of Arylamidase, Mannosidase, Glucosidase and Galactosidase using Vitek 2 isolated from vermicompost of primary sludge

Well	Test	RSC1	RSC2	RSC3	RSC4	RSC5	RSC6	RSC7	RSC8	RSC9	RSC10	RSC11	RSC12
3	L-Lysine-Arylamidase	–	–	–	–	–	–	–	–	–	–	–	–
4	L-Aspartate Arylamidase	–	–	–	–	–	–	–	–	–	+	+	–
5	Leucine Arylamidase	+	+	+	+	+	+	+	+	+	+	+	+
7	Phenylalanine Arylamidase	+	+	+	+	+	+	+	+	+	+	+	+
8	L-Proline Arylamidase	+	+	+	+	+	+	+	+	+	–	–	+
9	Beta-Galactosidase	+	+	+	+	+	+	+	+	+	+	+	+
10	L-Pyrrolydonyl-Arylamidase	–	–	–	–	–	–	–	–	–	–	–	–
11	Alpha Galactosidase	+	+	+	+	+	+	+	+	+	+	+	+
12	Alanine Arylamidase	–	–	–	–	–	–	–	–	–	+	+	–
13	Tyrosine Arylamidase	+	+	+	+	+	+	+	+	+	+	+	+
15	Ala-Phe-Pro Arylamidase	–	–	–	+	–	–	–	+	–	+	+	–
27	Alpha-Mannosidase	–	–	–	–	–	–	–	–	–	–	+	–
30	Glycine Arylamidase	–	–	–	–	–	–	–	–	–	–	–	–
41	Beta- Glucosidase	+	+	+	+	+	+	+	+	+	+	+	+
43	Beta-Mannosidase	–	–	–	–	–	–	–	–	–	–	–	–
46	Alpha-Glucosidase	+	+	+	+	+	+	+	+	+	+	+	+

+ and - denotes presence and absence of sensitivity towards these enzymes respectively

finding out any microbial contribution regarding the change in C/N ratio. Vitek 2 system characterization of bacteria obtained from the vermicompost of primary and secondary sludges have revealed sensitivity towards all isozymes for

galactosidase and β -glucosidase activity in all isolates of *Lysinibacillus* spp. and *Bacillus* spp. (Tables 2 and 3). These reflect an extracellular digestion of waste materials and their polymers of carbohydrates [28]. Most of the isolates have

Table 3 Differential sensitivity of bacterial isolates (RG 1–12) towards several isozymes of Arylamidase, Mannosidase, Glucosidase and Galactosidase using Vitek 2 isolated from the vermicompost of secondary sludge

Well	Test	RG1	RG2	RG3	RG4	RG5	RG6	RG7	RG8	RG9	RG10	RG11	RG12
3	L-Lysine-Arylamidase	–	–	–	–	–	–	–	–	–	–	–	–
4	L-Aspartate Arylamidase	–	–	–	–	–	+	–	–	–	–	+	–
5	Leucine Arylamidase	+	+	+	+	+	+	+	+	+	+	+	+
7	Phenylalanine Arylamidase	+	+	+	+	+	+	+	+	+	+	+	+
8	L-Proline Arylamidase	+	+	+	+	+	–	+	+	+	+	–	+
9	Beta-Galactosidase	+	+	+	+	+	+	+	+	+	+	+	+
10	L-Pyrrolydonyl-Arylamidase	–	–	–	–	–	–	–	–	–	–	–	–
11	Alpha Galactosidase	+	+	+	+	+	+	+	+	+	+	+	+
12	Alanine Arylamidase	–	–	–	–	–	+	–	–	–	–	+	–
13	Tyrosine Arylamidase	+	+	+	+	+	+	+	+	+	+	+	+
15	Ala-Phe-Pro Arylamidase	+	–	–	–	–	+	+	–	–	–	+	–
27	Alpha-Mannosidase	–	–	–	–	–	+	–	–	–	–	–	–
30	Glycine Arylamidase	–	–	–	–	–	–	–	–	–	–	–	–
41	Beta- Glucosidase	+	+	+	+	+	+	+	+	+	+	+	+
43	Beta-Mannosidase	–	–	–	–	–	–	–	–	–	–	–	–
46	Alpha-Glucosidase	+	+	+	+	+	+	+	+	+	+	+	+

+ and - denotes presence and absence of sensitivity towards these enzymes respectively

been found to show negative response towards isozymes of mannosidases except *Bacillus thuringiensis*. Earlier findings show that earthworm facilitates the digestion of carbohydrates or polysaccharides which result in the decrease of carbon content [29]. Several authors had also evaluated the potential of various microorganisms like fungi and bacteria regarding degradation of cellulosic substrates into its monomers [30].

An increase in total nitrogen content was supposed to be due to an elemental conversion of nitrogen as free amino acids into the compost. Results obtained through Vitek2 system have confirmed a differential sensitivity towards several isozymes of arylamidase and at the same time, pin-pointedly focussed these bacterial isolates as a potential contributor of enzyme synthesis (Tables 2 and 3). The earlier studies with regard to the change of nitrogen content in the vermicomposting process have highlighted the significance of extracellular and intracellular enzymes of bacteria with respect to complex protein assimilation [31–33]. Based on the results of the present study, it can be inferred that cumulative effect of microbial enzymes alongside protein assimilation are instrumental for the change in C/N ratio [34].

Conclusion

Changes in the activity of microbial enzymes arylamidase and β -glucosidase have appeared to be the significant triggering factors for determining the change of C/N ratio in the process of vermicomposting. This pioneering study has also documented a number of gram-positive isolates such as *Lysinibacillus sphaericus*, *Lysinibacillus xylaniliticus*, *Lysinibacillus macroides*, *Bacillus cereus*, *Bacillus thuringiensis* and *Lysinibacillus fusiformis* from different phases in vermicomposting of paper mill sludges. Potentiality of these bacterial isolates have been found to rely upon differential sensitivity in terms of several isozymes of arylamidase, glucosidase, galactosidase and mannosidase suggesting a functional roles of microbes in the decomposition of organic matters as reflected in the decrease of C/N ratio of the respective vermicompost. The present study is expected to open a new vista on the role of microbes with different enzymatic performances in microbial ecology towards waste management however, in depth study on enzymatic kinetics along with their functional role towards waste management are supposed to generate more definite conclusion in future science.

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Author's contributions All the authors participate fully in performing this work. Most of the work like isolation and identification of bacteria, enzyme activity analysis and Vitek compact2 sensitivity test was done by RKG. Vermiculture bed maintenance, the collection of sample and writing of manuscript were done together by both the authors.

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Compliance with ethical standards

Conflict of interest Authors have no conflict of interest.

Ethics approval and consent to participate Not applicable for this study.

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