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# Changes in earthworm gut associated enzymes and microbial diversity on the treatment of fermented tannery waste using epigeic earthworm *Eudrilus eugeniae*

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ABSTRACT

Tanneries produce enormous quantities of solid waste including animal fleshing (ANFL) which is a major solid waste generated during pre-tanning operations of leather processing and needs to be disposed of in a safe and environmentally sound way. The aim of this study was to evaluate how the combination of earthworms and microorganisms could enhance the biodegradation of fermented tannery waste mixed with cow dung and leaf litter over a period of 25 days. In our previous study we focused on enzyme activity in fermented waste degradation while this current study revealed the significant role of microbial diversity and population in the *Eudrilus eugeniae* gut and in vermicompost manure. The maximum microbial population in both studies was recorded on day 21 of the vermicomposting process. Results in this study showed that substantial changes were observed with solid state ferment (SSF) > submerged state ferment (SmF) > control mixtures ($p < 0.05$). The same trend was identified in earthworm gut enzyme activity. The phytohormones (indole 3-acetic acid [IAA], gibberellic acid [$GA_3$], kinetin) were detected in all treatment vermicompost products. The germination index showed that the vermicomposts from all treatments had no phytotoxic effect on carrot seed (*Daucus carota*). The overall results confirmed that the microorganisms role were dominant in the vermicomposting process and that it is possible to produce rich manure from fermented tannery waste mixtures.



## 1. Introduction

Earthworms are believed to have a relationship with soil microorganisms ranging from commensalism to species-specific mutualism (Sampedro and Whalen, 2007). Parle (1963) first reported the presence of microbes in the earthworm gut and several researchers followed and attempted to study earthworm gut microbes using direct culture methods (Karsten and Drake, 1995). Garg et al. (2006a) suggested that vermicomposting is a waste management technique that promotes the production of organic fertilizers from organic wastes. Earthworms are the crucial drivers of the vermicompost process. They cause fragmentation of the ingested material through muscular action increasing the surface area for microbial activities (Edwards, 1988 and Lazcano et al., 2008). In the earthworm gut a variety of intestinal microorganisms which produce digestive enzymes such as amylase, proteases, lipases, and cellulases enhance the biodegradation of organic matter (Aira et al., 2006). In addition, earthworms combine with microbes to secrete growth promoting plant hormones such as gibberellins, auxins and cytokinins, which help to mineralise the nutrients and make them bioavailable in the final product (Sinha et al., 2011). Bhat and Limaye (2012) suggested that plant hormones such as auxin and gibberellins and enzymes found in vermicompost stimulate plant growth and discourage plant pathogens. Blair et al. (1997) reported that the waste material consumed by an earthworm is excreted as 85% vermicompost and the other 5–10% is distributed for growth and metabolic activities. Several researchers have demonstrated the management of wastes through vermicomposting using earthworms in areas such as: industrial sludge (Yadav and Garg, 2009), herbal pharmaceutical industry waste (Singh and Suthar, 2012), food industry waste (Garg et al., 2012), biosolids (Contreras-Ramos et al., 2005), sludge of

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beverage industry (Singh et al., 2010), tannery sludge (Vig et al., 2011), paper mill sludge (Kaur et al., 2010), human feces waste (Yadav et al., 2011).

Animal fleshing (ANFL) is an untanned tannery solid waste which contains a rich source of nutrients (Ravindran and Sekaran, 2010). During the vermicomposting process, major constituents such as cellulose and protein compounds present in the waste mixtures are hydrolysed by specific enzymes. The correlation of the enzyme activities with the changes of microbial type and number are helpful to ascertain the maturity of the vermicompost product (Tiquia, 2005). In our previous study (Ravindran et al., 2014), we discussed the nutrient and enzymatic changes of hydrolysed tannery waste during the vermicomposting process. The main objectives of the current study were to monitor the changes of earthworm gut enzymes and microbes and determine the value-added products such as indole 3-acetic acid (IAA), gibberellic acid ($GA_3$) and kinetin generated during the vermicomposting of fermented tannery waste.

## 2. Materials and methods

### 2.1. Earthworms and waste materials

Earthworms (*Eudrilus eugeniae*) were randomly picked from a stock culture maintained in the vermicomposting unit, Environmental Technology Division, Central Leather Research Institute, Adyar, Chennai, India. The animal fleshing (ANFL) was collected from a local commercial tannery and fermented through anaerobic submerged (SmF) and solid state fermentation processes (SSF) using the bacterium *Selenomonas ruminantium*. These fermentative processes were described in previous studies (Ravindran et al., 2011). The main chemical parameters at the end of the fermentation process of SmF ANFL were: pH: $6.8 \pm 0.5$; total organic carbon (TOC): $269 \pm 21$ mg/L$^{-1}$; total nitrogen (TN): $203 \pm 14$ mg/L$^{-1}$; and of SSF ANFL were: pH: $6.9 \pm 0.5$; total organic carbon (TOC): $275 \pm 22$ mg/L$^{-1}$; total nitrogen (TN): $214 \pm 15$ mg/L$^{-1}$. Cow dung (CD) was procured from a local cowshed and the chemical parameters were: pH: $7.9 \pm 0.3$; total organic carbon (TOC): $431 \pm 23$ g/kg$^{-1}$; total nitrogen (TN): $6.4 \pm 0.4$ g/kg$^{-1}$and total C:N ratio: $67.3 \pm 5.9$. Partially decomposed leaf litter was collected from the garden area and the main chemical parameters were: pH: $7.6 \pm 0.4$; total organic carbon (TOC): $510 \pm 43$ g/kg$^{-1}$; total nitrogen (TN): $9.2 \pm 0.6$ g/kg$^{-1}$and total C:N ratio: $55.4 \pm 4.5$.

### 2.2. Experimental design

Three different waste mixture compositions were prepared (cow dung plus leaf litter and with or without hydrolysed ANFL). Vermicomposting processes were conducted in circular plastic containers with a working capacity of 1 kg were used for experiment. Each experiment unit consist of one kg of cow dung and leaf litter (1:1 ratio), and 1 L of hydrolysed ANFL extract (SmF or SSF) was added in respective treatments except in control, more details could be found in Ravindran et al. (2014) for more detail. Prior to the experiment, the initial mixtures were turned manually for 3 days in order to eliminate toxic volatile substances to the favor of earthworms. 50 non-clitellated earthworms were introduced into the three different treatment containers (control, SSF, SmF). The moisture content of the mixtures was maintained at 70–75% (w/v) throughout the experiment and vermicontainers were kept and maintained in darkness at room temperature (25–30 °C). A sub-sample (5 g) of each treatment (control, SmF, SSF) was collected from the vermicomposting reactor at 0, 7, 14, 21 and 25 days for microbiological analysis i.e., total bacteria, fungi, actinomycetes, proteolytic bacteria and cellulolytic bacteria. The initial and final vermicompost products were identified as well as the pH and C:N ratio. The microbial diversity and enzyme activities were determined in the gut of earthworms from control, SmF and SSF treatments on 0, 7, 14, 21 and 25 days of the vermicomposting process. The final product of the different treatment was analysed for plant growth hormones (indole acetic acid, gibberellic acid and kinetin).

### 2.3. Physico-chemical and plant growth hormones analysis

The pH of the sample in distilled water (1:10 w/v) was determined using a Systronics pH meter (Model μ Systronocis 369) fitted with a glass electrode. Total kjeldhal nitrogen (TKN) was estimated following the method of Bremner (1996). Total organic carbon (TOC) was determined by the Walkley and Black (1934) rapid titration method. The C:N ratio was calculated from the measured values of C and N. For the determination of plant hormones, the vermicompost samples (5 gm) were homogenized using 100 mL methanol:chloroform:ammonium 2N (12:5:3 v/v/v). The homogenised extracts were transferred into a conical flask 22.4 mL of double distilled water was added. After this the mixed homogenized extracts were centrifuged at 6000 rpm for 15 min at 27 °C. The extraction and quantitative determination of phytohormones (indole 3-acetic acid [IAA], gibberellic acid [$GA_3$], kinetin) were carried out according to the method of Unyayar et al. (1996).

### 2.4. Microbiological analysis

The total number of bacteria, fungi, actinomycetes, proteolytic bacteria and cellulolytic bacteria were estimated using the serial dilution and standard pour plate method (Dubey and Maheshwari, 2005). The number of colony forming units (CFU) was expressed as CFU g$^{-1}$. One gram of each treatment sub-sample was suspended in 10 mL of sterile water. The serial dilutions were made up to $10^{-4}$ and an aliquot of 1 mL was poured onto plates in agar media such as nutrient agar for bacteria, potato dextrose agar for fungi, actinomycetes agar for actinomycetes, skim milk agar for proteolytic bacteria and cellulose agar for cellulolytic bacteria. Fecal coliforms were determined by inoculation of tube media using the most probable number (MPN) method (APHA, 2005). All samples were assayed by dilution with at least three replicates of each suspension.

For microbial counts of earthworms gut contents, earthworms were collected on 0, 7, 14, 21 and 25 days of vermicompost process. The peripheral surfaces of earthworms were disinfected with sodium hypochlorite (25 ppm) for 10 min before dissection. The gut contents of each segment were squeezed into sterile test tubes using a sterile scalpel, forceps, and sterile knife under aseptic condition. The collected gut content (1 g) was diluted in saline solution (NaCl, 0.9% (w/v)) and microbial diversities were determined (Prakash and Karmegam, 2010). The serial dilutions were made up to $10^{-4}$ dilution and an aliquot of 0.1 mL was spread onto plates of the agar media. The respective media and procedures used for vermicompost product was used for gut microbial content i.e., media for bacteria, fungi, actinomycetes, proteolytic bacteria, cellulolytic agar and fecal coliforms.

### 2.5. Enzymatic analysis

The earthworms were collected on 0, 7, 14, 21 and 25 days of vermicompost process from control, SmF and SSF treatments. The gut content was cleared by feeding the worms with wet blotting paper for 10–12 h. The gut (4–5.5 cm, ranging from about 18–185 segments) of the earthworm *E. eugeniae* were selected for enzymatic analysis (Parthasarathi and Ranganathan, 2000). The

enzyme extract was obtained after homogenizing the gut tissues (20 mg) free from gut contents with respective enzyme buffers. The homogenate was centrifuged at 2000 rpm for 15 min to remove the debris. The supernatant was used as an enzymatic extract for quantifying the dehydrogenase, protease and cellulase activities. Dehydrogenase enzyme activity was measured by following the method of Klein et al. (1971). Three percent of 2,3,5-triphenyl tetrazolium chloride (TTC) was used as a substrate. Triphenyl formazan (TPF) produced in the reduction of TTC was measured with a spectrophotometer at 485 nm. The cellulase activity was estimated by the method of Miller (1959) using carboxy methyl cellulose as the substrate. The reaction mixture was filtered and filtrate was assayed by 3,5-dinitrosalicylic acid (DNS) reagent to determinate the amount of reducing sugar formed due to cellulolytic activity, and this was determined using a spectrophotometer at 540 nm. Protease activity was quantified by method of Nannipieri et al. (1980) to determine the amount of amino acids released after incubation of each treatment of earthworm gut content. This method is based on the determination of trichloroacetic acid-soluble tyrosine derivatives with the Folin reagent.

### 2.6. Determination of amino acid in earthworm biomass

Amino acids in earthworm biomass was determined following the method of Ding et al. (2002). In the end of vermicomposting process, 10 mature earthworms of *E. eugenia* were collected from each treatment container i.e., control, SmF and SSF. Earthworms were washed, weighed, and dried for 3 h to obtain constant weight. The samples were parched again after grinding. 10 mg of dried earthworms were added into a hydrolysis tube in 10 mL HCl (6 M). The tubes were vacuumized and then sealed under alcohol spurt flame. The contents were hydrolysed at 110 °C for 24 h, transferred to a centrifuge tube and then centrifuged at 10,000 rpm for 15 min. The supernatant was collected and used to determine the total amino acid composition using a $C_{18}$ column in an Agilent model 1100HPLC analyzer following the method of Ramakrishnan et al. (1996).

### 2.7. Phytoxicity test

Seed germination studies were used to assess the toxicity and maturity of vermicomposts obtained with each treatment. The methods followed were as per Araujo and Monteiro (2005). Vermiextracts were prepared from final vermicomposts with distilled water in the ratio of 1:10 (w/v) (Araujo and Monteiro, 2005). Two pieces of Whatman® filter paper were placed inside a 15 × 100 mm sterilized petridish and wetted with the vermicompost extracts. Fifteen seeds of carrot (*Daucus carota*) were placed on top of the filter paper and incubated for 5 days in a dark condition at 20 °C.

### 2.8. Statistical analysis

Data reported here are the means of three replicates ($n = 3$). All obtained results were subjected to a two-way analysis of variance (ANOVA) using PROC GLM (SAS, 1989) to test significant differences between treatments at different time along with Tukey's studentized range (HSD) test and Duncan's multiple range test. Data of microbial counts were transformed by log $(x+1)$ to normalize the data previously to ANOVA analysis. The probability levels used for statistical significance were $p < 0.05$ for all tests. A PROC MIXED was applied to all data in order to test statistical significance between time and treatments, which is appropriated when repeated measurements are taken on the same experimental unit, and these repeated measurements are correlated or exhibit variability that changes (SAS, 1989).

## 3. Results and discussion

### 3.1. Changes in physico-chemical of the feed mixtures

The earthworms had modified the physico-chemical properties of the waste materials by the end of the vermicomposting process (25 days). The final products of vermicompost were odor free, much darker and homogeneous than the initial mixtures. The pH was reduced in all the treatments and the highest reduction was recorded in the SSF treatment with pH 6.56 and the lowest was pH 6.84 in the control treatment (Table 1). The vermicompost from fermented waste mixtures had more decomposed material than the control treatment. Ndegwa et al. (2000) and Garg et al. (2006b) indicated that the decrease of pH can be attributed to mineralization of organic material and also to the production of organic acids and $CO_2$ by microbial metabolism. Elvira et al. (1998) reported that the joint action of earthworms and microbial decomposition lead a low pH of the substrate through the vermicomposting process. The C:N ratio significantly decreased at the end of the process and reduced to below 20 in all vermicomposts, indicating the stabilisation of vermicompost products. A significant reduction in C:N ratio was observed in SSF (10.3) followed by SmF (11.6), compared to the control (17.32). Significant differences were observed in all the feed applications ($p < 0.05$) (Table 1). According to Atiyeh et al. (2000), an advanced degree of stabilization of organic matter in organic waste might be obtained with C/N ratio less than 20. The result obtained in the present study indicated that the mineralization and stabilization of organic wastes occurred during the vermicomposting process. Suthar and Singh (2008) reported that a decrease of C:N ratio could be due to an increase in N content and simultaneously a decrease in TOC contents of waste mixtures. There could be a loss of carbon as carbon dioxide possibly due to respiratory activities of earthworms and microbes, as the same time as there is an addition of nitrogen to the substrate material by earthworm excretions such as mucus, enzymes and nitrogenous compounds.

### 3.2. Quantification of hormones in the vermicompost

In the present investigation, three different plant growth promoters (IAA, GA3 and kinetin) were quantified in the vermicomposts from all treatments as shown in Table 2. Several researchers have reported that plant growth hormones are available in composted manure. Tomati et al. (1988) demonstrated that a high concentration of plant hormones such as auxins, gibberellins and cytokinins were available in earthworm-

**Table 1**
Changes in pH and C:N ratio content on initial and final vermicompost from different treatments.

| Treatments | pH | | C:N ratio | |
|---|---|---|---|---|
| | Initial | Final | Initial | Final |
| Control | 7.09(0.12)a* | 6.82(0.27)a* | 57.7(4)a* | 17.37(1.2)a* |
| SmF# | 7.11(0.2)a* | 6.60(0.15)b* | 55.6(3.8)b* | 11.60(.82)b* |
| SSF# | 7.27(.012)b* | 6.56(0.11)b* | 53.4(3.73)c* | 10.30(.72)b* |

Values between by parenthesis are standard errors of estimates ($n = 3$).
# See text. Means within a column followed by the different lowercase letter indicate significant difference between treatments at same time. Means with in row of each parameter.
* Significant differences between time (initial and final) in the same treatment ($p < 0.05$).

**Table 2**
Distribution of plant growth regulators (mg/kg) in vermicomposts derived fromdifferent feed mixtures.

| Treatments | IAA[#] | GA3[#] | Kinetin[#] |
|---|---|---|---|
| | (mg/kg) | | |
| Control | 5.6 (0.4)b[*] | 2.2(.16)c[*] | 0.9(.06)c[*] |
| SmF[#] | 7.32(0.53)a[*] | 3.1(.27)b[*] | 1.7(0.11)b[*] |
| SSF[#] | 7.37(0.6)a[*] | 5.4(.38)a[*] | 2.8(.19)a[*] |

Values between by parenthesis are standard errors of estimates ($n = 3$).
[#] See text. Means within a column followed by the different lowercase letter indicate significant difference between treatments at same time.
[*] Significant differences between plant growth regulators in the same treatment ($p < 0.05$).

processed sewage sludge. In our study, three plant hormones were recorded with the highest values in SSF vermicompost as IAA (7.37 mg $kg^{-1}$), kinetin (4.9 mg $kg^{-1}$) and gibberellic acid (2.7 mg $kg^{-1}$). Perumal et al. (2006) reported that cow pat pit manure contained the three plant growth hormones IAA (28.6 mg $kg^{-1}$), kinetin (7.6 mg $kg^{-1}$) and gibberellic acid (23.6 mg $kg^{-1}$) whereas IAA (8.2 mg $kg^{-1}$), kinetin (5.7 mg $kg^{-1}$) are available in vermicompost manure. Miezah et al. (2008) reported that a range of 42.0–248.8 mg $kg^{-1}$; 33.1–198.3 mg $kg^{-1}$ and 10.1–200.2 mg $kg^{-1}$ of auxins, cytokinins and gibberellins, respectively, were present in compost manure. These plant hormones must be being produced by microorganisms in the vermicompost manure. Arshad and Frankenberger (1993) and Tomati et al. (1988) suggested that

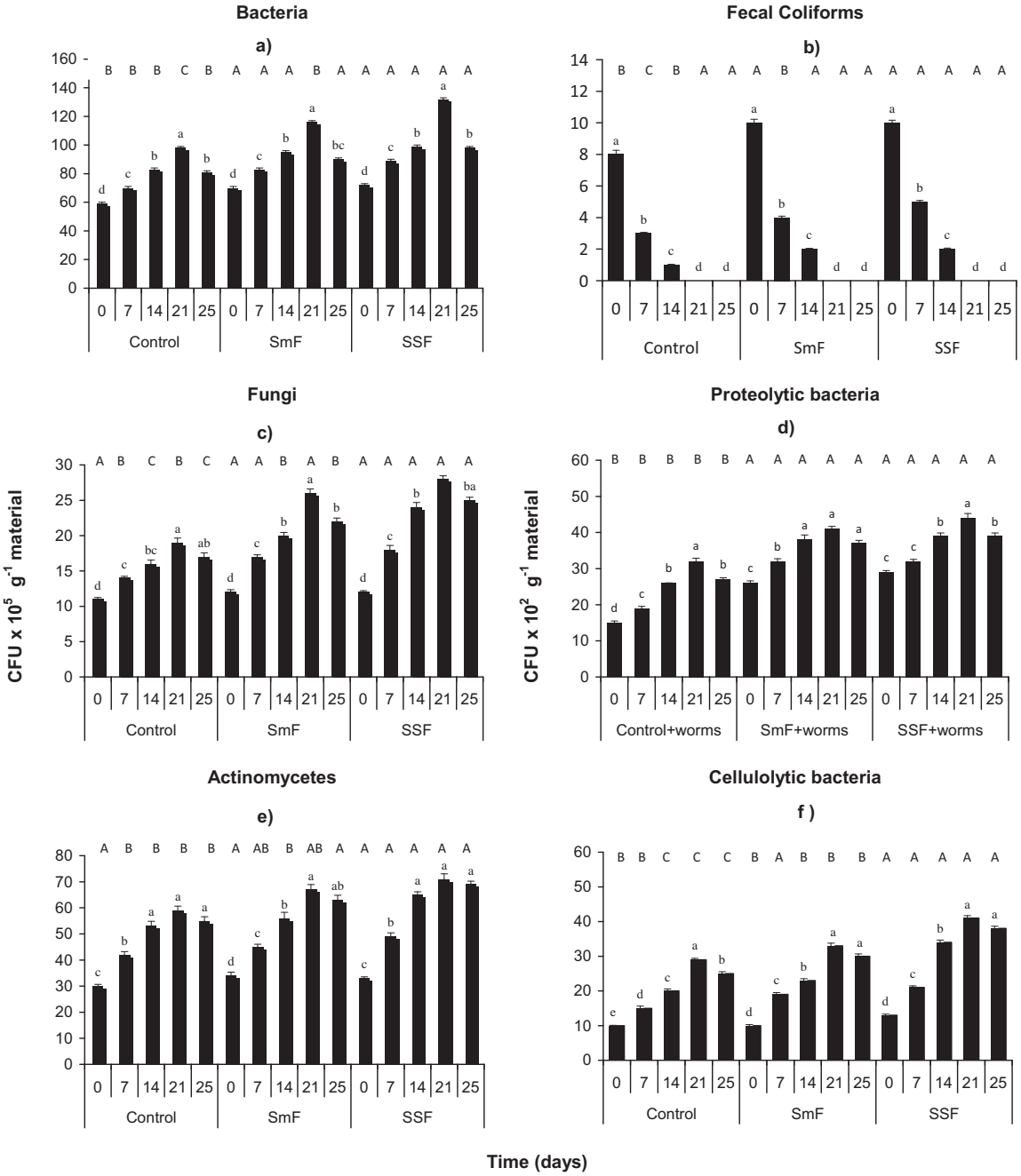


**Fig. 1.** Changes in microbial count from vermicompost in different treatments (control, SSF and SmF) during 25 days. (a) Bacteria; (b) fecal coliforms; (c) fungi; (d) proteolytic bacteria; (e) actinomycetes and (f) cellulolytic bacteria. Different lowercase letter indicate significant difference between time at same treatment. Different capital letters indicate significant differences between treatments at same time ($p < 0.05$).

different type of microorganisms (bacteria, fungi, yeasts, actinomycetes) are capable of producing plant hormones. Siripin (2000) has concluded that many plant hormones can be produced by $N_2$ fixing bacteria such as *Pseudomonas, Azotobacter* and *Bacillus*. Perumal et al. (2006) also reported that high loaded microorganisms from manure can produced a maximum quantity of phytohormones. The SSF treatment product had a greater quantity of plant growth hormones compared to the other treatments which may be due to the availability of more available nitrogen fixing bacteria. Krishnamoorthy and Vajrabhiah (1986) showed that earthworms could significantly promote the production of the phytohormones, auxins and cytokinins from organic wastes. The vermicompost obtained in this study had a significant amount of phytohormones, possibly derived from microorganisms or the earthworms themselves.

### 3.3. Microbial populations in vermicompost

Microbial populations were determined during the vermicompost (VC) process and the results were correlated with the microbial concentration and maturity of vermicompost (Fig. 1a–f). The highest microbial count was observed on day 21 of the VC process, with a bacterial count of $132 \times 10^5$ CFU/g; fungi $28 \times 10^5$ CFU/g and actinomycetes $71 \times 10^5$ CFU/g in SSF treatment, while in SmF treatment the microbial diversity for bacteria was $116 \times 10^5$ CFU $g^{-1}$, fungi $26 \times 10^5$ CFU $g^{-1}$; and actinomycetes $67 \times 10^5$ CFU $g^{-1}$. The control treatment had microbial diversities in bacteria of $98 \times 10^5$ CFU $g^{-1}$, fungi $19 \times 10^5$ CFU $g^{-1}$ and actinomycetes $59 \times 10^5$ CFU $g^{-1}$. This increase in the microbial population may be due to the conductive environment provided by the earthworm digestive tract and the nutrient rich organic wastes, which provide

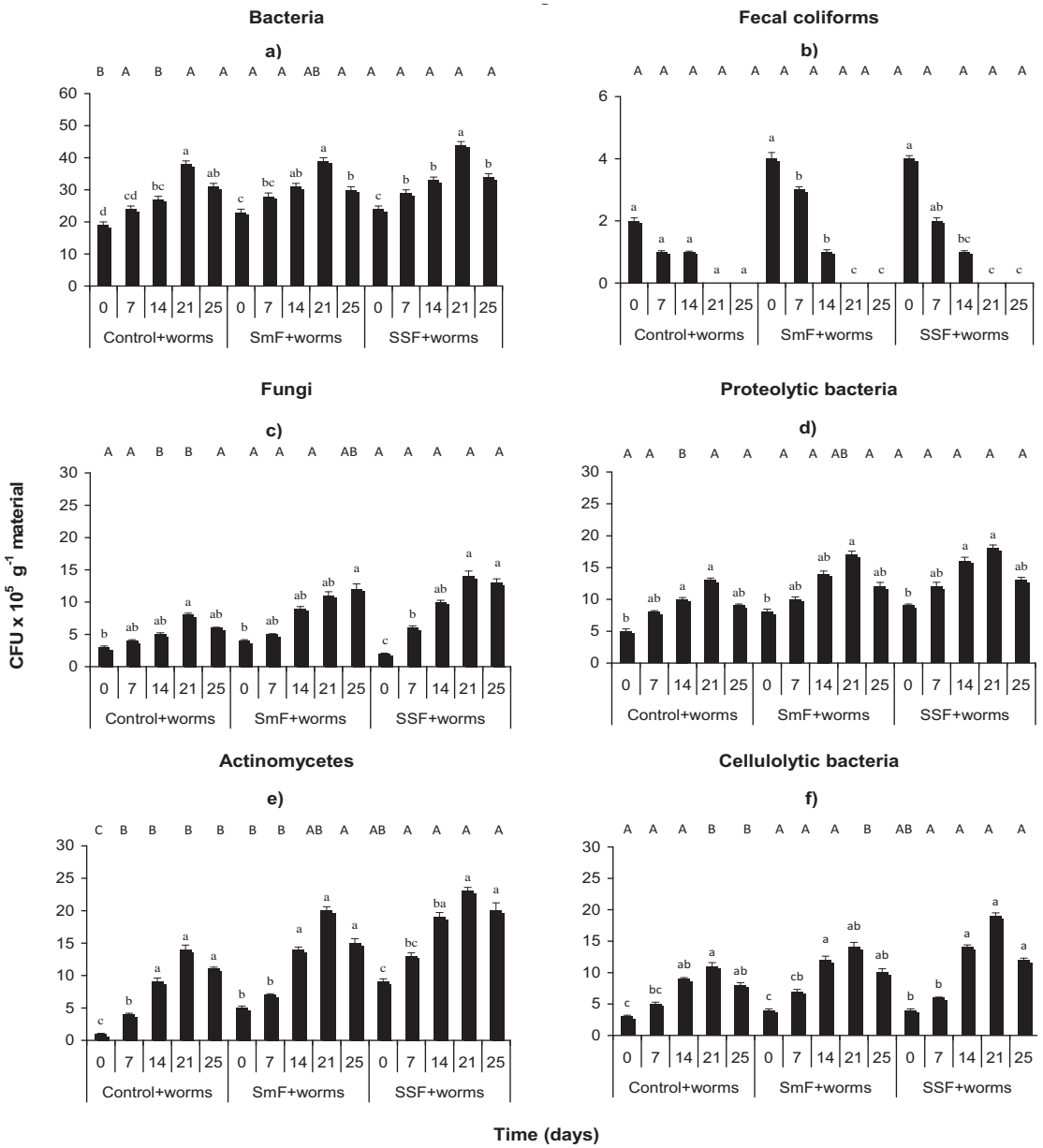


**Fig. 2.** Changes in microbial count from earthworm gut in different treatments (control, SSF and SmF) during 25 days process. (a) Bacteria; (b) fecal coliforms; (c) fungi; (d) proteolytic bacteria; (e) actinomycetes and (f) cellulolytic bacteria. Different lowercase letter indicate significant difference between time at same treatment. Different capital letters indicate significant differences between treatments at same time ($p < 0.05$).

energy and nutrient for the growth of microorganisms such as was reported by Tiwari et al. (1989). Parthasarathi and Ranganathan (2000) also reported the increase in microbial population in the vermicompost using the earthworm of *E. eugeniae*. It was observed that after 21 days of VC process, the microbial population decreased which may be attributed to the nutrient limitation condition of the feed mixtures. The proteolytic bacteria count was significantly higher in SSF treatment ($44 \times 10^2$ CFU $g^{-1}$) than that of all other treatments at 21 days and was followed by SmF and control ($p < 0.05$). However, no significant differences were found between SSF and SmF. Proteolytic bacterial species are commonly related to casein-hydrolyzing protease activity. These results were similar to that reported by Devi et al. (2009). They reported that protease activity was gradually decreased after 28 days owing to depletion in the protein content in the feed mixture during the vermicompost process of 49 days. In this study, a significant increase was observed in the cellulolytic bacteria until 21 days of vermicomposting process ($p < 0.05$). The maximum cellulolytic bacteria count were identified in SSF followed by SmF and control. These results revealed that the microbial activities also correlated with cellulase enzyme activities and cellulose contents in feed mixtures (Ravindran et al., 2014).

The presence of fecal coliforms in the initial treatment mixtures was probably inherited from the cow dung. The average numbers of fecal coliform were found to be from $8 \times 10^5$ to $10 \times 10^5$ CFU/g in all treatments in the initial stage of the vermicompost process. The fecal coliforms were drastically reduced by day 21 of the vermicompost process in all mixtures. Khwairakpam and Bhargava (2007) suggested that the coliforms were eliminated when they entered the food chain of the earthworm in the vermicomposting process.

### 3.4. Microbial activities in earthworm gut

The maximum microbial count (bacteria, fungi, actinomycetes, cellulolytic and proteolytic bacteria) in earthworm gut of different treatment mixtures was reached at 21 days (Fig. 2a–f) and was similar to the maximum found in the vermicomposts. The variations in the microbial populations in the earthworm gut may be due to their nutritional needs and digestion ability (Kadalli et al., 2000). According to Senapati and Dash (1984), the increase in bacterial populations might be due to the environmental conditions prevailing and nutritional status in the earthworm gut. The microbial population significantly ($p < 0.05$) increased in the SmF and SSF applied earthworm gut compared to earthworms from control. The results indicate that the hydrolysed ANFL may act as a favorable feed mixture to earthworm *E. eugeniae*. In particular, the SSF treated earthworm gut showed the maximum microbial count of bacteria $44 \times 10^4$ CFU $g^{-1}$; fungi $14 \times 10^4$ CFU $g^{-1}$; actinomycetes $23 \times 10^4$ CFU $g^{-1}$; cellulolytic bacteria, $19 \times 10^2$ CFU $g^{-1}$ and proteolytic bacteria $18 \times 10^2$ CFU $g^{-1}$, while fecal coliforms were absent after 21 days of treatment. Eastman et al. (2001) reported that the fecal coliforms were reduced after 90 days of vermicomposting. Also, in this study the microbial population decreased by day 25 of the VC process which may be attributed to the limitations of nutrients in the feed mixtures; however, the count was higher on day 25 in the earthworm gut than that of the initial feed mixtures.

### 3.5. Enzymatic analysis in earthworm gut

Digestive enzymes were secreted by earthworm *E. eugeniae* and/or in association with gut microflora, and they might be involved in cleaving the macromolecules in the feed mixtures. The enzymes (cellulase, protease, dehydrogenase) were analysed in the earthworm gut (Fig. 3a–f) during the VC process in the different

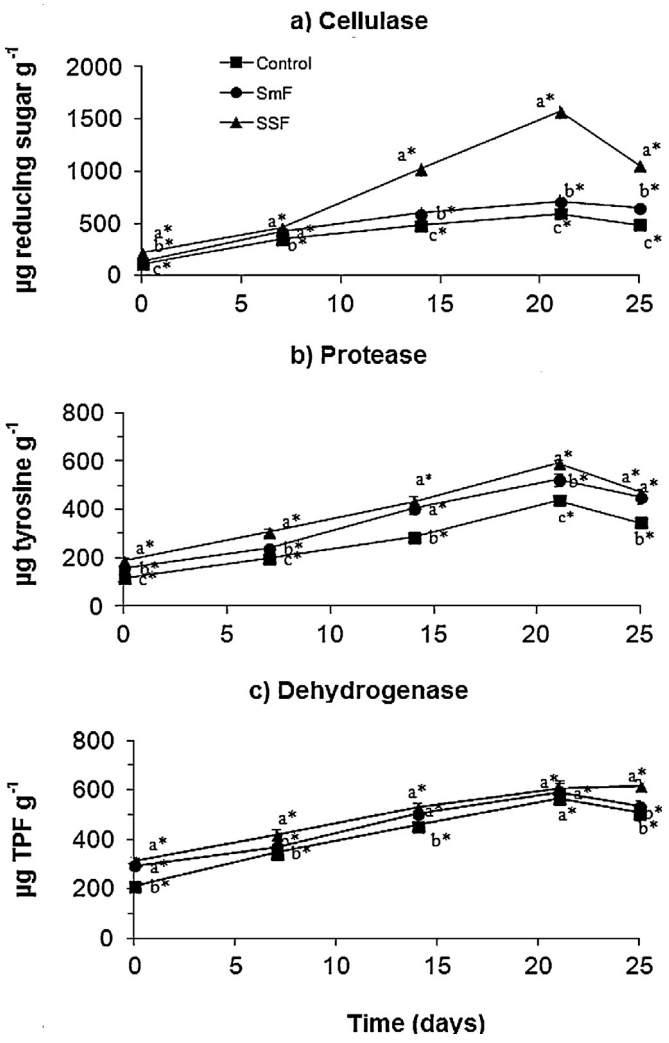


**Fig. 3.** Changes of enzyme activities in earthworm gut from different treatments (control, SSF and SmF) during 25 days process. (a) Cellulase; (b) protease; and (c) dehydrogenase. Small letters show significant differences between treatments at same time, and asterisk shows significant differences between days in the same treatment ($p < 0.05$).

treatments. The enzyme activities in the initial treatment mixtures were detected in the range cellulases, 112–223 (µg $g^{-1}$ reducing sugar); protease, 116–187 (µg tyrosine $g^{-1}$) and dehydrogenase 210–313 (µg TPF $g^{-1}$) (Fig. 3a–f). The maximum enzyme activities were recorded in earthworm gut of SSF for cellulases (1569 µg $g^{-1}$ reducing sugar); protease (589 µg tyrosine $g^{-1}$) and dehydrogenase, (603 µg TPF $g^{-1}$). The hydrolytic enzymes such as protease and cellulase were responsible for the hydrolysis of proteins and cellulose respectively in feed while dehydrogenase activity accounts for the energy metabolism of microbes in the gut of the earthworms. Domínguez (2004) indicated that during the vermicomposting process earthworms and microorganisms secrete certain extracellular enzymes required for the decomposition of organic solid waste within the worm gut. Benítez et al. (2002) suggested that the increase in hydrolytic enzymes and overall microbial populations during the VC process indicated the biodegradation of the substrates and thereby the disappearance of the initial phytotoxicity of the substrate. In this study, the enzyme activity declined at the end of 25 days of the vermicomposting process. Ganesh Kumar et al. (2008) asserted that microorganisms reduced the enzyme synthesis due to disappearance of available substrates.

**Table 3**
Aminoacid content in the earthworm biomass from different treatment at end of vermicomposting process.

| Amino acids | Earthworms in different treatments (g/100 g of crude protein) | | |
|---|---|---|---|
| | Control | SmF[#] | SSF[#] |
| Aspartic acid | 6.38 (0.44)bC | 8.25 (0.41)aC | 8.25 (0.33)aCD |
| Glutamic acid | 8.23 (0.41)bB | 10.3 (0.51)aB | 9.71(0.29)aB |
| Serine | 4.62 (0.18)bE | 5.04 (0.33)abE | 5.46 (0.33)aE |
| Histidine | 21.7 (0.08)bA | 27.9 (0.84)aA | 28.5 (1.14)aA |
| Alanine | 3.20 (0.16)bF | 4.09 (0.16)aF | 3.56 (0.11)bGH |
| Arginine | 8.01 (0.40)bC | 8.71 (0.35)aC | 9.05 (0.27)aBC |
| Tyrosine | 3.26 (0.19)bH | 2.17 (0.06)cH | 4.34 (0.34)aFG |
| Valine | 2.10 (0.08)aGH | 2.57 (0.10)aGH | 2.57 (0.08)aH |
| Methionine | 2.98 (0.12)aI | 0.60 (0.03)bI | 0.29 (0.01)bI |
| Phenylalanine | 2.95 (0.09)aG | 2.97 (0.12)aG | 2.97 (0.11)aH |
| Isoleucine | 2.09 (0.10)bG | 2.88 (0.11)aG | 2.88 (1.17)aH |
| Leucine | 6.03 (0.18)bD | 7.08 (0.21)aD | 7.34 (0.22)aD |
| Lysine | 4.09 (0.20)bE | 5.26 (0.16)aE | 4.97(0.19)aEF |
| Total amount | 75.7 (2.27)b | 87.9 (2.64)a | 89.9 (2.69)a |

Values between by parenthesis are standard errors of estimates ($n = 3$).
[#] See text. Means within a row followed by the different lowercase letter indicate significant difference between treatments at same time. Means with in column, uppercase letter shows significant differences between aminoacids in the same treatment ($p < 0.05$).

### 3.6. Amino acid content of Eudrilus eugeniae

*Eudrilus eugeniae*

After 25 days of the vermicomposting process, the amino acid content was monitored in the earthworm body of *E. eugeniae* from all treatment mixtures by acid hydrolysis, and their composition was listed (see Table 3). The total amounts of amino acids were recorded from the different feed mixtures earthworm body of SSF (89.99 g/100 g of crude protein), SmF (87.887 g/100 g of crude protein) and control (75.749 g/100 g of crude protein). Overall, SSF and SmF mixtures had a higher content of different amino acids compared to the control mixture, with the exception of methionine where control had a higher value. In addition, only alanine, methionine and tyrosine were significantly different between SSF and SmF mixtures, while no significant differences were found for other amino acids ($p < 0.05$) (Table 3). This result suggests that hydrolysis of ANFL feed mixtures played a major role in increasing the aminoacid content in earthworm guts compared to control (Table 3). Dedeke et al. (2010) reported the total amount of amino acids in *E. eugeniae* earthworm body as being 77.87 g/100 g of crude protein. Accordingly, the hydrolyzed ANFL feed mixtures in those two treatments will have increased the earthworm protein levels in those treatments. This result reflects the major role of protein content in initial feed mixtures. Xiang et al. (2006) reported that the amino acid content of the earthworm increased with addition of a nitrogen source (i.e., rice straw plus chemical fertilizer) in paddy field experiments. Accordingly, our results suggest that including organic waste such as hydrolyzed ANFL with high levels of protein into feed mixtures could increase the crude protein content in earthworms in terms of free amino acids.

**Table 4**
Effect of different treatment vermicompost extracts on carrot by seed germination bioassay ($n = 3$).

| Treatment | Carrot (%) | | |
|---|---|---|---|
| | RSG[#] | RRE[#] | GI[#] |
| Control | 94 (6.5)aA | 55(4)cB | 51(4)cB |
| SmF[#] | 95(6.7)aA | 92 (6)bA | 87(5)bB |
| SSF[#] | 99 (7)aB | 132(9)aA | 130(9)aA |

Values between by parenthesis are standard errors of estimates ($n = 3$).
[#] See text. Means within a column followed by the different lowercase letter indicate significant difference between treatments at same time. Means with in row, uppercase letter shows significant differences between bioassay (RSG, RRE, GI) in the same treatment ($p < 0.05$).

### 3.7. Effect of vermicompost extracts on seed germination

The vermicompost extracts from the final products of all three mixtures had no harmful effect on carrot seed germination (Table 4). Better results were shown in relative seed germination, relative root elongation and germination index in the treatments as follows: SSF > SmF > control. The germination of carrot seed was recorded as being highest in the SSF treatment (99%) while control had the lowest (94%). The relative seed germination treatment results of SSF, SmF and control were significantly different ($p < 0.05$). According to relative root elongation, the highest significant value was recorded in SSF treatment vermiextract (132%) followed by SmF (92%) and control (55%). The germination indexes of all treatment vermiextracts was recorded as being above 50%. In particular the fermented vermiextracts (SSF and SmF) showed good effects and recorded above 80%. The highest germination index value was recorded in SSF (130%) and the lowest was control (51%). Alvarez and Grigera (2005) suggested that a germination index > 50% indicates that the worm leachate is mature. It also suggests the absence of pathogens, i.e., coliforms (*Escherichia coli*), *Salmonella* sp. and *Shigella* sp. The overall results revealed the vermiproduct from all mixtures had no phytotoxic effect and these are confirmed through the germination index analysis.

## 4. Conclusions

The present study proved that vermicomposting is an effective and eco-friendly technology for fermented tannery waste to be converted into rich manure with the help of earthworms and their associated microbes. The microbial population and its activity was increased to significant levels in vermicompost product derived from fermented waste mixtures compared to the control mixture. This study concludes that the earthworm *E. eugeniae* can utilize tannery fermented waste mixtures through their gut and can digest it with enzyme activity to produce a nutrient rich manure.

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