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Effect of Cow Urine on the Biochemical and Microbial Properties of Cow Dung Derived Biogas Slurry

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ABSTRACT

Biogas slurry (BGS) is an anaerobic digested organic material that can be used as an organic fertiliser. As cow urine (CU) is rich in plant nutrients, it may be used as diluting agent in biogas production to enhance the fertiliser quality of BGS. To explore the potency of CU on the fertiliser quality of BGS, four experimental trials were constructed by mixing cow dung (CD) and CU in varying proportion designated as T_0 (50% CD + 50% Water) as control, T_1 (50% CD + 50% CU), T_2 (40% CD + 60% CU), T_3 (30% CD + 70% CU) for biogas production. The quality of BGS was evaluated by studying its biochemical and microbial properties. The enzymatic activities revealed that all the CU amended samples showed better activities than control and were increased with the increase in CU. Compared to the control, the increase in urease, protease and phosphatase activities were 11.6% to 64.6%, 4.6% to 29.6% and 22.1% to 50.0%, respectively while cellulase activities were decreased from 25.9% to 3.1%. Most of the bacterial populations also increased in CU amended samples; total bacteria (TB) 20% to 60%, phosphate solubilizing bacteria (PSB) 33% to 67% and nitrogen fixing bacteria (NFB) 0% to 33%. Phytohormone, indole acetic acid (IAA) content and glycemic index (GI) were also increased with increase in CU (IAA- 23.5% to 59.5% and GI- 6.2% to 100.5%). With respect to all parameters analysed, CU amended samples can be considered superior to the control one except for their cellulase activities. Thus, utilization of cow urine improves the quality of BGS as organic fertiliser. This finding will help in reducing environmental pollution by utilizing hazardous cow urine as well as improving fertiliser quality of biogas slurry for agronomic use.

Keywords: Bacterial population; biogas slurry; cow dung; cow urine; enzyme activity

INTRODUCTION

Fertiliser applied to soil for enriching plant nutrients, can be derived from natural sources as organic fertilisers or synthetic sources as inorganic fertilisers (FAO, 2009). Inorganic fertilisers dramatically improved production of food but it has created so many grievous issues such as soil and air pollution, nutrient imbalance, increased dependency on fossil fuel, decline of nutritional quality of food and soil fertility, destruction of beneficial soil organisms, and impairment of biological resistance in crops by making them more susceptible to pests and diseases. Organic fertilisers are thought to be the answer for the 'food safety and farm security' in future by adding nutrients of biological origin, increasing of the yield of agricultural crops without harming environment (Bulluck Iii *et al.*, 2002; Bulluck Iii & Ristaino, 2002; Arancon *et al.*, 2003; Bondarenko *et al.*, 2018). Organic fertilisers are significant components of integrated nutrients which play a vital role in promoting productivity and sustainability of soil. It is also a cost-effective input for the farmers, while being ecofriendly to the atmosphere (Rokhzadi *et al.*, 2008; Bhunia *et al.*, 2021). Their use improves the physical, chemical and biological characteristics of the soil even though they are lower in nutrients compared to inorganic fertilisers (Wu *et al.*, 2005). Biogas slurry (BGS) is an anaerobic digested organic material released as byproduct from the biogas plant after the production of combustible biogas that contribute to the mitigation of climate change by replacing fossil fuels (Islam & Momin, 2004). It can be used as an organic fertiliser as it is a rich source of both plant nutrients and organic matter (Ishikawa *et al.*, 2006). Its use as a soil amendment could offer a win-win opportunity to improve crop production and soil physical properties. In addition, it prevents adverse

environmental impacts of chemical fertilisers and pesticides use; reduce cultivation cost and waste disposal.

In the last few decades, the Government of Bangladesh has promoted biogas production at individual and community levels. The organic materials generally used as raw materials for biogas plant are cow dung, poultry litter and other easily decomposable materials such as kitchen refuses, farm wastes, and crop residues after dilution with water (Islam & Momin, 2004). Livestock dung such as cattle manure is the most commonly available substrate for biogas production (Mdlambuzi *et al.*, 2021) precisely in rural areas. It mainly consists of lignin, cellulose and hemicelluloses and 24 different minerals like nitrogen, potassium, along with trace amount of sulphur, iron, magnesium, copper, cobalt and manganese (Garg & Mudgal, 2007; Randhawa & Kullar, 2011). It harbors a wealthy microbial diversity, containing different species of bacteria, protozoa and yeast (Behera & Ray, 2021). Like other potential agrobiotech waste for fertiliser production, cow dung (CD) is accessible than any other agro-materials and the appearance is superior to other substrates considering cost and availability of the substrate material (Pandey *et al.*, 2000). Thus, CD diluted with water is the main raw materials for biogas production in Bangladesh. Cow urine (CU) is rich in nitrogen, sulphur, ammonia, copper, phosphorus, sodium, potassium, manganese, carbolic acid, iron, uric acid, urea, silicon, chlorine, magnesium, calcium, lactose, enzymes, creatinine, aurum hydroxide (Jain et al., 2010). Therefore, addition of CU with CD as a diluting agent instead of water could not only enrich the nutritive value of BGS but also helps in managing pollution. In addition, extract of BGS amended with CU may be used as pest repellent as fermented cattle urine is rich in chloride, sulphate and nitrite (Miah *et al.*, 2017). Considering the above facts, we previously reported the impact of urine on biogas production and the cumulative gas volume of CU amended sample was found to be 28% higher than control (Fardous *et al.*, 2020). In the present study, we explore the potency of CU on fertiliser quality of CD derived BGS by evaluating their important biochemical and microbial properties.

MATERIALS AND METHODS

Materials

All chemicals used in enzymatic analysis were of analytical grade. Redistilled and deionized water were used for solution preparation. Working solutions were prepared by sequential dilution of each standard solution with ultrapure water. The substrate CD and CU were collected from a local farm located at Meherchandi, Motihar, Rajshahi, Bangladesh. Biogas slurry collected from local biogas plant was used as seeding material.

Methods

Experimental design for BGS production

To evaluate the effects of urine on the biochemical and microbial properties of BGS, four experimental trials were constructed by mixing CD and CU in varying proportion designated as T_0 (50% CD + 50% distilled water, DW) as control, T_1 (50% CD + 50% CU), T_2 (40% CD + 60% CU) and T_3 (30% CD + 70% CU). Each of the mixtures was taken in 5 L flask for digestion. Seeding material, biogas slurry was added into the raw materials for its rapid fermentation. These mixtures were digested under anaerobic condition around 37 ℃ for 40 days and were terminated after stopping gas production. Before collection, each BGS sample was made homogeneous by shaking for 5 minutes and kept at 8 ℃ for analysis. Quality of the BGS produced was evaluated by biochemical parameters e.g., enzyme activity (urease, protease, phosphatase and cellulase), microbial parameters (total bacteria (TB), phosphate solubilizing bacteria (PSB) and nitrogen fixing bacteria (NFB), phytohormone, indole acetic acid (IAA) and phytotoxicity as Germination index (GI) were analysed by standard methods.

Enzymatic activities

Urease activity: BGS sample (0.5 g) was added to 0.25 ml of toluene and kept for 15 minutes. Four ml of 0.1 M phosphate buffer (pH 7) and 0.5 ml of 64 mg/ml (6.4%) urea was added to BGS sample and incubated at 37 ℃ for 1 hour. After incubation, 10 ml of 2 M KCl containing

100 µg/ml Ag_2SO_4 was added and the mixture was kept at 4 ℃ for 10 minutes to stop the enzymatic reaction. Suspensions were centrifuged for 10 minutes at 4000 rpm and the NH_4^+ ion formed by ureases in the supernatant was determined by phenol hypochlorite method (Fawcett & Scott, 1960). Controls were prepared by adding urea and 0.5 g of sterilized BGS sample after incubation.

Protease activity: Four ml of 1.0% egg albumin (BDH, UK) was added to 0.5 g of BGS sample and was mixed. After incubation at 51 ℃ for 60 minutes, the reaction was terminated by the addition of 4.0 ml of 10% trichloroacetic acid. After centrifugation at 4000 rpm for 10 minutes, 2.5 ml of supernatant was pipetted out. The amino acids formed were determined by the Folin-colorimetric method (Nannipieri *et al.*, 1980). Controls were prepared by adding egg albumin and 0.5 g of sterilized BGS sample after incubation.

Phosphatase activity: BGS sample (0.5 g) was added to 0.250 ml of toluene and was kept for 15 minutes. Four ml of 0.1 M maleate buffer (pH 6.5) and 1.0 ml of 0.115 M *p*-nitrophenyl phosphate (*p*-NPP) solution was added. The flask was swirled for few seconds and then incubated at 37 ℃ for 1 hour. After incubation, 1.0 ml of 0.5 M calcium chloride solution and 4.0 ml of 0.5 g sodium hydroxide solution were added to the mixture to stop the reaction. Suspensions were centrifuged for 10 minutes at 4,000 rpm. The released *p*-nitrophenol (*p*-NP) was determined by the method of Tabatai and Bremner (1969). Controls were made in the same manners, but the substrate and 0.5 g of sterilized BGS sample were added after incubation.

Cellulase activity: Four ml of carboxymethyl cellulose (CMC, BDH, UAE) dissolved in 0.1 M sodium citrate buffer (pH 5.0) was added to 0.5 g of BGS sample and incubated at 55 ℃ for 60 minutes. Then the reaction mixture was centrifuged at 4000 rpm for 10 minutes and passed through Whatmann No.1 filter paper. The filtrate was assayed by dinitrosalicylic acid method for amount of reducing sugar formed due to cellulolytic activity (Miller, 1959). Controls were prepared by adding CMC and 0.5 g of sterilized BGS sample after incubation.

Bacterial populations

The total number of fungi, actinomycetes and bacteria present in BGS sample of each trial was estimated by counting colony forming unit (CFU) through "Serial dilution plate technique" (Allen, 1958). One g of the freshly collected BGS sample was mixed with 9.0 ml of sterilized NaCl solution (0.85%) in sterilized test tube and serial dilutions were carried out up to suitable ranges of diluted solution. Then 0.1 ml of the diluted solution was spread into a sterile petri dish containing suitable agar media by spread plate technique and was incubated at 37 ℃ for 24 hours for TB, 28 ℃ for 5 days for PSB and 37 ℃ for 7 days for NFB. Nutrient agar medium for TB, Pikovskaya medium (Pikovskaya, 1948) for PSB, mannitol ash by agar medium (Subba-Rao *et al.*, 1995) for NFB were used.

Phytohormone, IAA

IAA in BGS was measured by a colorimetric assay technique using Salkwoski reagent based on the method of Gordon & Weber (1951) after extraction from BGS sample (5.0 g) using in a mixture (50 ml) of DW and acetone (1:1) as extracting solvent. Then one part of the supernatant and two part of the Salkowski's reagent was added. The absorbance was determined at 530 nm in a spectrophotometer. A standard curve was prepared from serial dilutions of IAA stock solution.

Phytotoxicity as GI

Phytotoxicity as GI was determined according to the method described by Zucconi (1981). Ten g of each BGS sample was added to 100 ml of DW and the solution was agitated for 30 minutes. The solution was centrifuged at 3000 rpm for 10 min and then filtered using Whatman 41 (20 – 25 µm). Ten ml of aliquot or extract was added to a petri dish with a Whatman no.1 ashless filter paper and 20 radish seeds were placed in each dish. The control was prepared by placing Whatman no.1 ashless filter in petri dish containing 10 ml of DW. The plates were incubated at 25 - 28 °C in the dark for 6 days. Seed germination and root length in each plate were measured on the 6th day. The results are expressed as GI [GI = (% G × % L)/100] combining relative germination (% G) and

relative root elongation (% L), compared to a DW control.

Statistical analysis

All the experimental analyses were carried out in triplicate and the mean values with standard deviation are presented. One-way ANOVA was used to analyse the differences between treatments. The statistical difference between the control and treatments was measured using a paired-sample t-test. Data processing, linear regression and other statistical analyses were conducted using Microsoft Excel 2019.

RESULTS AND DISCUSSION

Enzyme Activities

The quantification of enzyme activity during composting can reflect the dynamics of the composting process in terms of the decomposition of organic matter and nitrogen transformations, and may provide information about the quality and maturity of the composted product (Sen & Mahapatra, 2009). In this study, the main enzymatic activities of BGS in different trials, the activities of some enzymes such as urease, protease, phosphatase and cellulase were first analyse to evaluate the impacts of urine on its compost quality as their substrate, cellulose, nitrogen, phosphate are the main ingredients of CD and CU (Misra *et al.*, 2003).

Urease regulates the soil-N transformation and is involved in the hydrolysis of urea into ammonia and CO_2. So, the enzyme assay is important in understanding mineralization process of urea nitrogen (Luo *et al*., 2020). In different trials of BGS sample, the urease activity was in the range of 1034.3 ± 33.2 to 3702.2 ± 84.5 µg NH_4^+-N. g^{-1} hr^{-1} (Table 1). The urease activities of all the samples were much higher than other composted organic fertilisers (Devi *et al.*, 2009). This may be because of the substrate availability in sample as fermented CD and CU are rich in its substrate, urea (Miah *et al.*, 2017). From this finding, it is also clear that CU has positive impact on the urease activity of BGS as all the samples amended with urine were found to be 11.6% to 64.6% higher (t-test: $P < 0.05$, for all trials) than that of control (Figure 1(a)). Jia *et al.* (2011) showed that the urease activity had significant positive correlation with activities of microorganism, soil organic matter, total nitrogen and available nitrogen content.

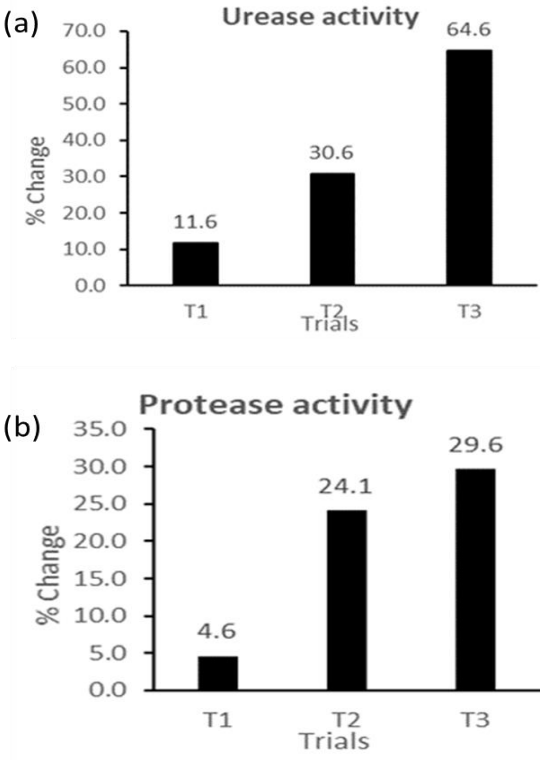

Figure 1. Percentage change in (a) urease and (b) protease activities over control in different trials

Table 1. Activities of various enzymes in different trials of BGS (mean ± SD, n = 3)

Enzyme activities	Trials			
	T_0 (50% CD+50% DW)	T_1 (50% CD+50% CU)	T_2 (40% CD+60% CU)	T_3 (30% CD+70% CU)
Urease[a]	1034.3 ± 33.2	1154.1 ± 66.8	1350.2 ± 64.2	1702.2 ± 84.5
Protease[b]	1.08 ± 0.04	1.13 ± 0.06	1.34 ± 0.03	1.40 ± 0.06
Phosphatase[c]	6.81 ± 0.56	8.32 ± 0.20	9.33 ± 0.03	10.20 ± 1.48
Cellulase[d]	2029 ± 68	1967 ± 57	1651± 54	1507 ± 51

a: µg NH_4^+-N. g^{-1} hr^{-1}, b: µmol amino acid g^{-1} hr^{-1}, c: µmol p-NP g^{-1} hr^{-1}, d: µg glucose g^{-1} hr^{-1}

Plants are capable of using a wide range of N forms as N sources, such as NO^{3-} and NH^{4+} as inorganic forms while proteins, peptides, amino acids as organic forms. Protease is another hydrolytic enzyme involved in hydrolyzing proteins and peptides to its simpler amino acids form that are the sources of both C and N for plants. Along with urease activity assay, it is also widely used in compost quality evaluation (Luo *et al*., 2020). In this study, in the case of protease activity, the range was 1.08 ± 0.04 to 1.40 ± 0.06 µmol amino acid g^{-1} hr^{-1} and their order was $T_3>T_2>T_1>T_0$ (Table 1, Figure 1(b)). The activity was enhanced up to 29.6% over control (t-test: P < 0.05, for all trials).

The phosphatase activity has been widely used for evaluating fertiliser quality and maturity assessment because of its importance in organic P mineralization, releasing orthophosphates that are readily assimilated by plants and soil microorganisms (Luo *et al*., 2020). The phosphatase activity was in the range of 6.81 ± 0.56 to 10.20 ± 1.48 µmol p-NP g^{-1} hr^{-1} (Table 1) and also showed the same pattern of changes from 22.1% to 50.0% (t-test: P < 0.05, for all trials) reflecting positive correlation with its substrate availability (Figure 2) as CU is rich in P (Kilande *et al*., 2015).

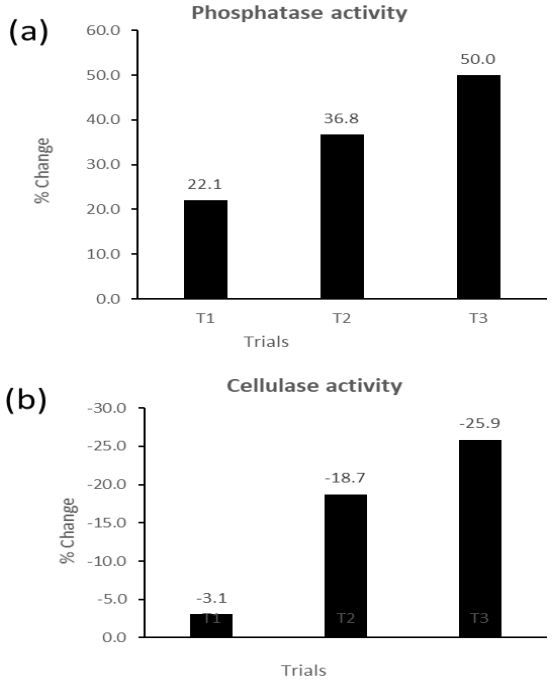

Figure 2. Percentage change in (a) phosphatase and (b) cellulase activities over control in different trials

Cellulase is a class of carbohydrate splitting enzyme hydrolysing cellulose to glucose and play a crucial role in carbon recycling during composting process. In contrast with other enzyme activities, interestingly cellulase showed the opposite pattern of change in activity and their order was $T_0>T_1>T_2>T_3$ (Table 1). This data clearly implies that CU has negative correlation with BGS quality as the activity of all CU amended samples (1507 ± 51 to 1967 ± 57 µg glucose g^{-1} hr^{-1}) were found to be lower than the control, T_0 (2029 ± 68 µg glucose g^{-1} hr^{-1}) and the activities of all amended samples were decreased from 25.9% to 3.1% (t-test: P < 0.05, for all trials) with the increased in CU percentage (Figure 2). This may be because of the substrate unavailability with the increase in CU due to absence of cellulose in CU (Miah *et al*., 2017). The cellulase activity of all the samples were comparable with other compost (Devi *et al*., 2009).

Bacterial Populations

Soil microorganisms play a crucial role in the biogeochemical cycling of carbon, nitrogen and phosphorus through the production of organic matters degrading enzymes (Luo *et al*., 2020). Thus, in addition to enzyme activity, microbial population study is also an important marker to evaluate the potency of organic fertiliser. In different trials of BGS, the number of TB was in the range of 5.0 ± 0.3 to 8.1 ± 0.5 ($\times 10^{11}$ CFU g^{-1}) (Table 2). Urine has positive impact on the bacterial population of BGS as all the samples amended with urine (T_1 to T_3) were found to be higher counts (t-test: P < 0.05, for all trials) than control, T_0. This may be because of the presence of bacteria in higher quantity in urine (Rawat *et al*., 2019). Among urine amended samples, T_1 showed the highest population (60% increased over control). However, the population was decreased with increasing CU due to lower bacterial populations in fermented CU compared to CD (Rawat *et al*., 2019).

Study of PSB and NFB showed that there was significant effect of CU on the BGS quality and followed the same pattern of change like TB. Both the number of PBS and NFB in T_1 trial (50% CD+50% CU) were increased by 67% and 33%, respectively (t-test: P < 0.05, for both trials) over control, T_0 (50% CD+50% DW) and then decreased with the increase in CU over CD (Figure 3) due to the same reason as mentioned above in the case of TB.

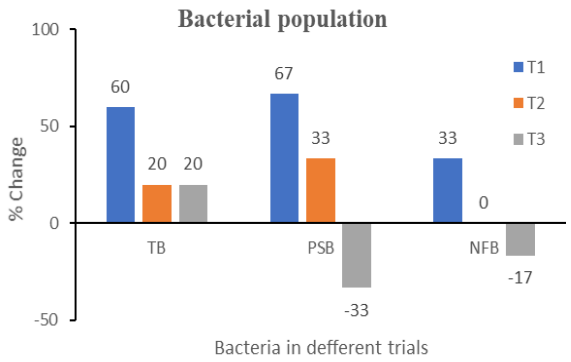

Figure 3. Percentage change in bacterial population over control in different trials

Phytohormone, IAA and its impacts as GI

IAA is an auxin of L-tryptophan metabolism produced by several microorganisms including Plant Growth-Promoting Rhizobacteria (PGPR) that colonise the rhizosphere and plant roots, and enhance plant growth by increasing nutrients uptake through the production of longer roots with increased number of root hairs and root laterals (Wu *et al.*, 2016). In different trials of BGS sample, the amount of IAA was in the range of 0.796 ± 0.002 to 1.270 ± 0.007 mg/g (Table 2). Urine has positive impact on the IAA producing microbial populations of BGS as all the samples amended with urine, (T_1 to T_3) were found to show higher IAA from 0.803 ± 0.004 to 1.270 ± 0.007 mg g^{-1} (t-test: $P < 0.05$, for all trials) than the control, T_0 (0.796 ± 0.002 mg g^{-1}) due to the presence of IAA in CU (Mubarik & Maslahah, 2019). The results further revealed that among all samples amended with urine (T_1 to T_3), the amount of IAA was increased to 23.5%, 53.6% and 59.5% (t-test: $P < 0.05$, for all trials) over the control, T_0 (Figure 4) with the increase in CU.

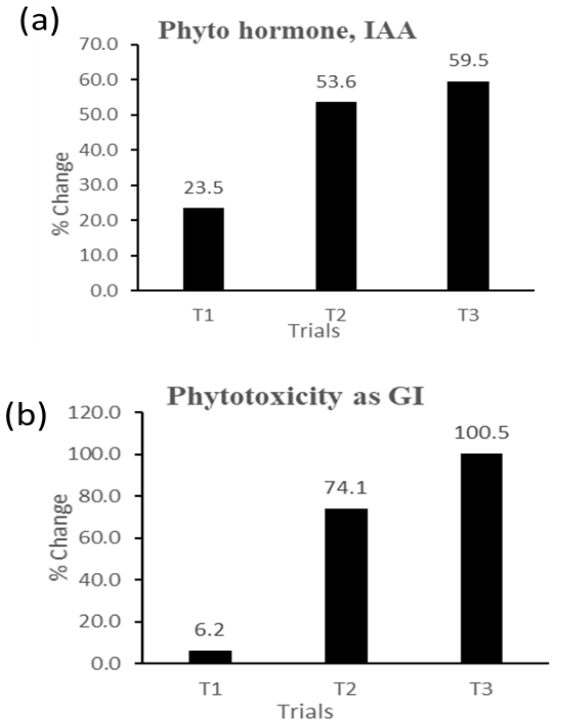

Figure 4. Percentage changes in (a) IAA and (b) GI over control in different trials

The values of GI in different trials were ranging from 193 ± 3 to 287 ± 6 and also increased from 6.2% to 100.5% (t-test: $P < 0.05$, for all trials) with the increased in CU concentration (Figure 4) as CU is known to have beneficial effect on germination, growth and yield components (Chawla, 1986; Joseph & Nair, 1989) due to presence of IAA. Study of the GI, implied that the values of all the trials of BGS exceeded 100% (Table 2) demanding being a phytostimulant. Therefore, CU can be used as diluting agents as it improves the fertiliser quality of BGS by increasing IAA as well as GI.

Table 2. Bacterial populations as CFU g^{-1} in different trials of BGS (mean ± SD, n = 3)

Trials	Total bacteria ($\times 10^{11}$ CFU g^{-1})	Phosphate solubilizing bacteria ($\times 10^{7}$ CFU g^{-1})	Nitrogen fixing bacteria ($\times 10^{4}$ CFU g^{-1})	IAA/mg g^{-1}	Glycemic index (%)
T_0 (50% CD+50% DW)	5.0 ± 0.3	3.2 ± 0.1	6.1 ± 0.7	0.796 ± 0.002	193 ± 3
T_1 (50% CD+50% CU)	8.1 ± 0.5	5.3 ± 0.3	8.0 ± 0.9	0.803 ± 0.004	205 ± 5
T_2 (40% CD+60% CU)	6.2 ± 0.2	4.1 ± 0.2	6.4 ± 0.6	1.223 ± 0.005	336 ± 6
T_3 (30% CD+70% CU)	6.0 ± 0.2	2.5 ± 0.1	5.3 ± 0.5	1.270 ± 0.007	387 ± 6

CONCLUSION

From the biochemical and microbial analysis, it was found that CU amended BGS (T_1-T_3) were superior to the control, T_0 and all the parameters such as enzyme activity (urease, protease and phosphatase), microbial parameters (TB, PSB and NFB), phytohormone, IAA, and phytotoxicity as GI were increased with the increase in CU. So, it can be concluded that CU can be used to improve the quality of BGS. This process will help in reducing environmental pollution by utilising hazardous CU as well as improving BGS quality for agronomic use.

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