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Full Length Research Paper

Effectiveness of inoculation with isolated *Anoxybacillus* sp. MGA110 on municipal solid waste composting process

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***Anoxybacillus* sp. MGA110, a thermophilic cellulolytic bacterium, was isolated from municipal solid waste (MSW) composting and characterized by 16S rRNA sequencing. A pure culture of the isolated strain was applied to municipal solid waste composting as inoculum. The results of inoculation showed that the biodegradation of organic matter was quicker than the control as indicated by more reduction in C/N ratio. The inoculation increased duration of thermophilic phase, cellulase activity and number of thermophilic bacteria during the composting process. Hence, it can be concluded that inoculation with the isolated strain can accelerate composting process and promote biodegradation of organic matter as this reduces C/N ratio faster and increases cellulase activity more.**

Key words: *Anoxybacillus* sp., cellulolytic, compost, inoculation and thermophilic.

INTRODUCTION

Municipal solid waste composting is an aerobic decomposition of organic materials by a variety of microorganisms under controlled conditions to convert into humus-like product which are used in agriculture as fertilizer (Sarkar et al., 2010).

Since degradation of cellulosic compounds like fruits, vegetables, kitchen refuses and industrial food processing wastes in the MSW composting process is difficult and takes considerable period of time (Nair and Okamitus, 2010), the main concern for MSW composting is the shortening of composting period.

Many efforts have been made to accelerate composting period. Microbial inoculation is one of these attempts. Microbial inoculation can increase the microbial population, improve microbiological quality, generate various desired enzymes and thus enhance the degradation of organic materials (Ohtaki et al., 2000). Inoculation efficiency is usually affected by competition with indigenous microorganisms (Xi et al., 2005) and variation of temperature during composting process (Neklyudov et al., 2006). Therefore, selection of suitable microorganisms is an important factor on effectiveness of inoculation.

In compost, many cellulose-degrading bacteria are predominant and play a key role in the process of biodegradation in thermophilic stage (Karita et al., 2003;

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Abbreviations: **MSW,** Municipal solid waste; **CMC,** carboxy methyl cellulose; **CMCase,** carboxy methyl cellulase; **FPase,** filter paper Cellulase; **C/N ratio,** water soluble organic carbon/ total organic nitrogen ratio.

Sarkar et al., 2010; Strom, 1985). They were proved to have the ability to adapt to the changing environment. Thus, their isolation and inoculation may intensify the biodegradation of recalcitrant cellulosic compounds and accelerate the process of composting.

In this study, we isolated a cellulolytic thermophilic bacterium, *Anoxybacillus* sp.MGA110, from compost piles and subsequently re-applied it in the beginning of thermophilic stage of MSW composting process in order to assay the effects of inoculation with an indigenous cellulolytic bacterium on composting parameters such as temperature, C/N ratio, population of thermophilic bacteria, and cellulase activity.

MATERIALS AND METHODS

Isolation of bacterium

To isolate thermophilic cellulolytic bacteria, sampling was done at a depth of about 50 cm from MSW piles surface on the 14th day of composting process when temperature reached to 65 to 70°C. 10 g of samples were mixed with 100 ml of saline solution. 5 ml of suspension was inoculated into 100 ml of medium prepared for isolation of cellulolytic bacteria and incubated at 55°C in incubator shaker (120 rpm) for 6 days. This medium contained (l^{-1}): 0.1 g nitrilotriacetic acid, 1-ml $FeCl_3$ solution (0.03%), 0.05 g $CaCl_2.2H_2O$, 0.1 g $MgSO_4.7H_2O$, 0.01 g NaCl, 0.01 g KCl, 0.3 g NH_4Cl, 1.8 g of 85% H_3PO_4, 0.005 g methionine, 0.05 g yeast extract, 1 ml of Nitsch's trace element solution and 0.5 g L^{-1} carboxy methyl cellulose (CMC) as sole source of carbon. Then, an aliquot of broth was spread on agar medium containing CMC as sole source of carbon, incubated at 55°C for 3 days (Rastogi et al., 2009). The cellulolytic bacteria were recognized by clear zone formation around the colony after staining with Congo red (Liang et al., 2010).

Carboxy methyl cellulase (CMCase) and filter paper cellulase (FPase) activities

In order to assay cellulases activities, 0.5 ml of cell-free supernatant of the culture was added to 0.5 ml of 0.05 g CMC solubilized in 200 mM Tris-HCl buffer pH 5.0 and 0.05 g Whatman No.1 filter paper strip in 1 ml of 100 mM phosphate buffer pH 5.0 and incubated at 50°C for 30 min. In order to stop the reaction, 3 ml of 3, 5-dinitrosalicylic acid (DNS) solution were added to mixture and boiled for 5 min. The released glucose was measured by optical density at 540 nm. One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 µmol of glucose per minute (Ghose, 1987). The activity of enzyme was assayed for temperature between 50°C and 80°C.

Molecular characterization of the bacterium and analysis of 16S rRNA gene sequence

For molecular characterization of the bacterium, 16S rRNA sequence analysis was performed. DNA was extracted using the method described by Weisburg et al. (1991) and amplified with universal bacterial primers fD1 (5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3') and rD1 (5'- CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC -3'). PCR products were cloned in pBleuescript SK plasmid and nucleotide sequence of cloned gene was determined in an automated 3730 analyzer (Applied Biosystem) (Rastogi et al., 2009). This sequence data has been submitted to the GenBank databases under accession number HQ696615.

Bacterial inoculation and analysis

In order to assay the effect of inoculation on composting process, a pure culture of the bacterium in the medium described above was applied as the inoculum. A stack of MSW composting with a weight of about 500 kg was inoculated with 10 ml liquid inoculants per kg of MSW on the 5th day of composting process while temperature was 50°C. The concentration of microbial inoculants was 2×10^7 CFU ml^{-1}. A stack without inoculum was marked as control. The trials were conducted in triplicates. The initial C/N ratio of the stacks was 25 and the moisture was measured about 54%. The aeration was done once a week during composting process. Changes in temperature in the center of MSW during the composting were monitored daily. The organic carbon content of the compost was analyzed by combustion method (Nelson and Sommers, 1982) and total nitrogen by Kjeldahl method (Bremner and Mulvaney, 1982). To determine cellulase (CMCase) activity of the compost, 5 g samples were extracted with 50 ml deionized water under rotary shaking (200 rpm) for 1 h. Then, the homogenate was centrifuged at 10.000 rpm for 10 min at 4°C and the filtrate was analyzed for activity of CMCase (Ghose, 1987). One unit (U) of CMCase activity was defined as the amount of enzyme that released 1 µmol of glucose per minute. The bacterial biomass was assessed by counting the colony forming units plated on nutrient agar supplied with cycloheximide (100 mg L^{-1}) after incubation at 50°C. All analyses were carried out in triplicates. In order to determine the effect of inoculation on C/N ratio, paired samples T test were used for the content of C/N ratio during composting process. Paired samples T test were completed using SPSS software.

RESULTS AND DISCUSSION

Isolation, CMCase and FPase activities and molecular identification of cellulolytic thermophilic bacterial strain

Among the screened cellulolytic isolates from thermophilic stage of the MSW composting, a strain with highest cellulase activity was selected for inoculum preparation. The temperature range for its growth was between 45 and 75°C and the optimum temperature for growth was at 55°C. 16S rRNA sequence analysis showed that there was a great similarity (99%) between the isolated strain and representative strains in GenBank of *Anoxybacillus rupiensis* strain DSM (AJ879076) and *A. rupiensis* strain R-32636 (AM988775). To our knowledge there is no previous report on the production of cellulases enzymes by *A. rupiensis.* The activity of cellulases enzymes against filter paper and CMC was detected as 0.146 U ml^{-1} (FPase) and 0.011 U ml^{-1} (CMCase) at the end of exponential growth phase. Both enzymes demonstrated good activity between 50 and 80°C with maximum activity at 70°C. The results are in conformity with the findings of Liang et al. (2010) about *Anoxybacillus* sp. The ability to grow in high temperature and the activity of CMCase as well as FPase on variations of temperature were the advantages of this novel cellulolytic strain for

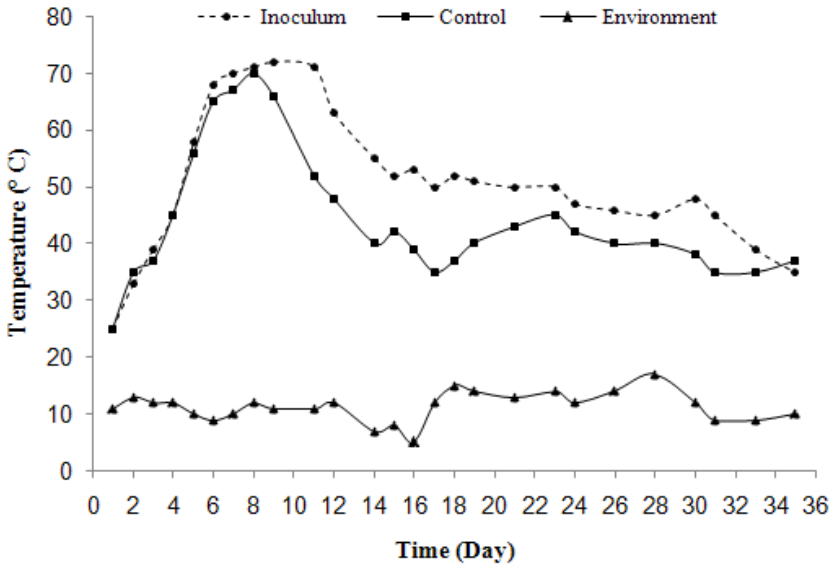

Figure 1. Effect of inoculation on temperature during composting process.

selection.

Effect of inoculation on the temperature of composting process

Effects of inoculation on the temperature of composting process are illustrated in Figure 1. In the control, the temperature increased to a peak of about 70°C on day 8 and dropped thereafter to 52°C on day 11. In inoculated stacks, maximum temperature was about 71°C on day 6 and this temperature remained for almost 6 days. According to Xi et al. (2005), the temperature in inoculated stack with a complex cellulolytic microbial inoculum could reach a maximum of 61°C and remain for 1 day while selection of a single indigenous cellulolytic thermophilic bacterium such as *Anoxybacillus* MGA110 could result in a higher and more durable temperature during composting process after inoculation. Vargas-Garcia et al. (2006) observed, in heaps made of olive-oil mill waste which were inoculated by lignocellulolytic microorganisms, a higher temperature for a longer period of time during composting process. According to Jouraiphy et al. (2005), more heat output was possibly the result of biological activity in compost. Therefore, it can be concluded that in this study, the high temperature after inoculation was due to the improvement of biological activity in compost.

Effect of inoculation on C/N ratio during composting process

Data regarding the C/N ratio of compost with and without inoculation are shown in Figure 2. The C/N ratio of the stacks was 25 at the beginning of the composting process and reached 23 after 5 days. After inoculation on day 5 some differences in the control and treatment were detected. The C/N ratio of the stacks inoculated with *Anoxybacillus* sp. MGA110 decreased to 18.5 on day 13, while in the control it reduced to 20. Afterward, the C/N ratio decreased slowly and reached 16.63 and 19 in the inoculated stacks and control on day 35, respectively. Nair and Okamitus (2010) reported that after 4 weeks of household organic waste composting, final C/N ratio reached about 27 in the inoculated stacks with EM inoculum (a complex of effective microorganisms). Sarkar et al. (2010) also obtained a remarkable decrease in C/N ratio during the decomposition of vegetable waste composting inoculated by amylolytic and cellulolytic thermophilic bacteria. The C/N ratio below 20 is an indicative of acceptable compost maturity that during efficient composting is obtained due to degradation of organic matter (Raut et al., 2008). In our results, considering the decrease in the C/N ratio from 25 to less than 20 both in inoculated stacks and in the control, lesser final C/N ratio with inoculant is an indicator of compost with better quality. Furthermore, according to Hirai et al. (1983), C/N ratio is not an absolute indicator of assessing compost maturity, the comparative decrease in C/N ratio implies that inoculation with the isolated strain could promote the rapid degradation of organic materials in composting process.

Effect of inoculation on CMCase activity during composting process

Cellulose decomposition limits the rapid production of compost more than any other substrates (Raut et al.,

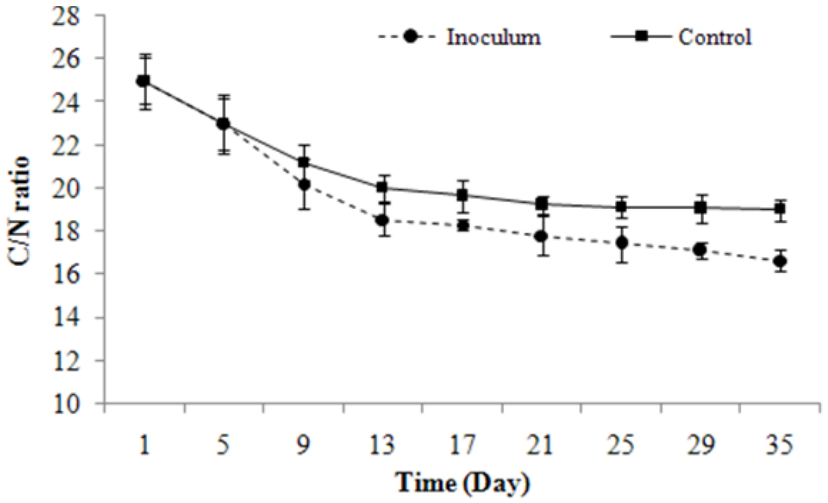

Figure 2. Effect of inoculation on C/N ratio during composting process (Vertical bars show standard deviation, n=3).

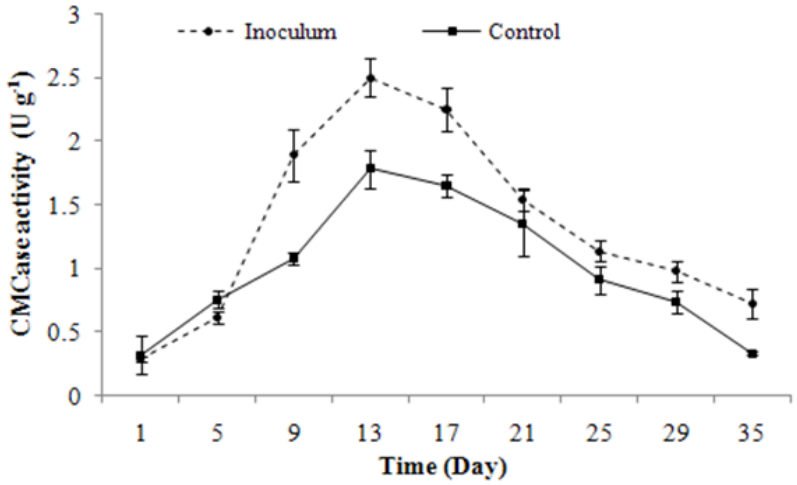

Figure 3. Changes in the CMCase activity during composting process.

2008). Hence, CMCase is one of the important enzymes involved in the acceleration of composting process. CMCase activity changes during composting process are given in Figure 3. The activity of CMCase both in inoculated stack and in the control increased until day 13, and then declined. The values of CMCase activity in inoculated stack were higher than those in the control which could be due to the growth of the inoculated strain and reduction of C/N ratio. Ming et al. (2008) observed a significant increase of Cellulase activity in MSW composting stacks inoculated with a microbial inoculum, which was originated from MSW leachate. According to Raut et al. (2008) reduction of C/N ratio increases the growth of microbial biomass and stimulates secretion of CMCase. In the studies of Zeng et al. (2010), it was reported that after inoculation with *Phanerochaete chrysosporium* (a lignocellulolytic fungus) during first fermentation phase of composting process, CMCase activity in the inoculated stacks was lower than in the control which is attributed to secretion of inhibitor by inoculated fungus.

Effect of inoculation on thermophilic bacterial biomass

Figure 4 shows the effect of inoculation on population of cultivable thermophilic bacteria during MSW composting process. It was found that the proliferation rate of thermophilic bacteria in the inoculated stacks was much

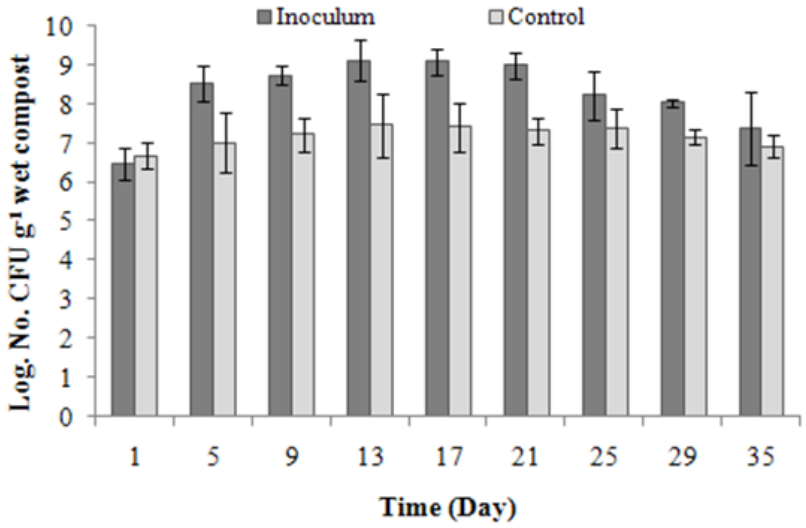

Figure 4. Changes in the thermophilic bacterial biomass during composting process.

larger than that in the control. The population of thermophilic bacteria in the inoculated stacks increased until 17 days of composting followed by a steady decline. In the control stack the population of thermophilic bacteria showed a slight increase until 13 days of composting followed by marginal decrease. Increase in population of thermophilic bacteria coincided with thermophilic stage of composting process. This result is not surprising given the fact that the strain MGA110 of *Anoxybacillus* sp. grows at an optimum and a maximum temperature of 55 and 75°C, respectively. Following the inoculation on day 5 composting process, the population of thermophilic bacteria and subsequently the temperature increased. It could be speculated that the addition of thermophilic cellulolytic inoculum enhanced the biological activity. This enhancement led to more decrease in C/N ratio and more increase in cellulase activity in inoculated stacks (Zeng et al., 2010).

Conclusion

The efficiency of inoculation with *Anoxybacillus* sp. MGA110 was confirmed by the decrease in C/N ratio, increase in temperature, duration of thermophilic phase, CMCase activity and number of thermophilic bacteria during the composting process. So far, researches have shown that complex cellulolytic microbial inoculum could promote the biodegradation of organic matter and accelerate the composting process (Raut et al., 2008; Sarkar et al., 2010; Wei et al., 2007; Xi et al., 2005), whereas in this study it was revealed that a single indigenous cellulolytic bacterium could be effective in acceleration of composting process as well. This research will hopefully make a basis for further research on effectiveness of indigenous bacteria of compost to accelerate the composting process and intensify the biodegradation process.

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