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Research Article

Isolation, Characterization and Rapid Composting of Cellulolytic Nitrogen-Fixing Bacteria for Biofertilizer Preparation

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- DNS method
- Composting
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- Biofertilizer

Abstract

In this research, twenty isolated strains of cellulolytic nitrogen-fixing bacteria were isolated from different soil sources. Cellulase activity of each selected strain was detected by screening of clear zone formation and by using DNS (Dinitrosalicylic colorimetric) method. The clear zone formation were not significant among these strains but amongst them, for cellulose substrate isolated strain K-7 was considered as the most potent isolate in term of their high cellulase productivity in the concentration of 0.452 mg/0.5 ml and in carboxymethyl cellulose substrate, M-1 strain showed maximum yield for cellulase production in the concentration of 0.493 mg/0.5ml. Nitrogen fixation activity of those selected strains was measured by the color changes in G-NFMM (Glucose-Nitrogen Free Mineral Medium) supplemented with BTB (Bromothymol Blue) and ammonium test kit. Composting was studied by using six strains on straw waste. Finished composting was observed for a month and nitrogen rich compost was obtained. The finished compost was used as carrier system and six selected bacterial strains for nitrogen fixation were used as formulated biofertilizer and applied in rice field. They improve rice plant growth, yield and nitrogen content in soil when compared with other rice plant used in chemical fertilizer. Our research can fulfill the aspect of enhancing the usage of environmentally friendly biofertilizer without reducing farmers' profit by maintaining the good yield.

INTRODUCTION

Waste disposable cellulosic materials (e.g., newsprints, disposable diapers, shopping bags, fast food containers) contribute significantly to the total volume of waste in domestic landfills [1]. There is intense interest in the biomass conversion of waste cellulosic products into alternative fuels. The availability of a commercially feasible means of converting cellulosic materials into a usable energy source would reduce the volume of solid waste deposited in landfills and reduce our dependence on foreign fossil fuels [2]. The alternative process is the use of cellulase enzyme that can degrade cellulose and it is produced by a number of microorganisms which include fungi (e.g. *Trichoderma reseei*, *Humicola insolens*, etc.) and bacteria (e.g. *Clostridium thermocellum*, *Cellulomonas* spp, *Thermonospora* spp, *Bacterioides* spp, *Microbispora bispora*, etc.) [3]. In nature, fungi produced more cellulase than bacteria and they are effective catalysts [4]. However, bacteria can grow easily than fungi and its cellulase more tolerates to very high temperature and also have a valuable characteristic that are generally faster and more cost effective at elevated temperature for chemical reactions. Another important concern is that bacteria have the potential that can easily be genetically engineered than fungi [5]. Cellulose is a consortium of free enzymes comprised of endoglucanase, exoglucanases and β-glucosidases. Among them, endocanases can hydrolyze internal β-1,4-glycosidic bonds in cellulose. The enzyme system of some microorganisms for cellulose is not the same. Some microorganisms can produce large amount of enzyme in culture media and others can grow on media containing cellulose but they can excrete little or no enzymes into the media [6].

Organic production systems represent an economically significant niche in Myanmar agriculture. These systems require intensive management of essential soil nutrients to be a sustainable system that maintains soil fertility status [7]. This study utilized cellulolytic nitrogen-fixing microorganisms to produce inoculants to enrich the N-content of straw residues. The more efficient organisms will be developed into inoculants where the cellulose degrading microorganism's break down the resistant components of the straw residues into more readily used organic substrates so that the nitrogen-fixing bacteria can convert atmospheric nitrogen into organic nitrogen. This biological inoculants may then be used by organic producers on farm sites to help manage and recycle organic residues and to create an N-enriched fertilizer. The degradation of the cellulose is interfered by a nitrogen limitation and it could be overcome by decomposing microorganisms that have dual

functions of cellulolytic and nitrogen fixation activity. Thus, our research mainly focused on selecting the potential strains in the biodegradation of natural polymers and environmental pollutants such as agricultural and organic municipal solid waste. The role of nitrogen-fixing bacteria on cellulose degradation may be useful in developing technologies to convert solid waste into nitrogen rich compost.

MATERIALS AND METHODS

Collection of samples

The samples for isolation of microorganisms were collected from various paddy fields under cultivation, cultivated conditions, and natural compost where the natural process of cellulose degradation is taking place, soil under rice straw from Kyaukse District, and around Mandalay Technological University, Mandalay Division, and Myanmar. The samples were then brought to the laboratory for microbiological study.

Isolation of cellulolytic nitrogen fixing bacteria

Cellulolytic nitrogen-fixing bacterial strains were isolated by using G-NFMM supplemented with the sole carbon sources are Avicel Cellulose and Sodium Carboxymethyl cellulose (CMC). One gram of soil samples was inoculated to 100 ml of sterile nitrogen free cellulose medium and nitrogen free CMC medium in each 250 ml conical flasks and then was incubated at 30°C in a shaker for 6 days. After the incubation period, 0.1 mL of the broth of each tube containing suspension of soil and culture media were inoculated in on solid nitrogen free cellulose medium and CMC medium and incubated for a week. After that, all different bacterial colonies that appeared on the plates of the two selective media were selected and subjected to the purification process.

Screening on cellulase producing activity

The screening for qualitative analysis of cellulolytic activity of isolated strains was performed by using Congo red dye. The pure cultures were inoculated in the centre with almost equal amounts and incubated at 37°C for one week to allow for the secretion of cellulose. After incubation, the agar plates were flooded with 0.1% (w/v) Congo red solution, and after 15 minutes the Congo red solution was discarded, and the plates were washed with 1 M NaCl solution allowed to stand for 15-20 minutes. The formation of clear zone was observed around the colony because of the indication of cellulose degradation. The ratio of the clear zone diameter to colony diameter was measured in order to select for the highest cellulases activity producer. The largest ratio was indicated to contain the highest activity [8].

Quantitative determination of cellulolytic activity of bacterial isolates

The cellulase enzyme production activity was determined according to the Dinitrosalicyclic colorimetric method recommended by Miller [9]. Firstly, 3 ml of DNS reagent was mixed with 3 ml of glucose sample in a test tube. The mixture was boiled at 90ºC for 5-15 minutes to develop the red-brown color. Then 1 ml of a 40% potassium sodium tartrate (Rochelle salt) solution was added to stabilize the color. After boiling, transfer was carried out immediately to a cold water bath; the absorbance was recorded with a spectrophotometer at 575 nm.

Detection of nitrogen-fixing activity

The isolated strains were screened for nitrogen fixing-activity by using Glucose Nitrogen Free Mineral Agar Medium as well as Broth Medium and Ammonia Test Kit (VISCOLOR Alpha Ammonium reagent, MACHEREY-NAGEL GmbH & Co. KG, Germany). The pure cultures were inoculated into GNFMM containing BTB (bromothymol blue solution) and without BTB and incubated in a shaker for a week and changing the color of medium from green to blue was recorded [10]. For the determination of nitrogen fixing activity from NFGMM broth medium without BTB, broth culture was centrifuged at 8000 rpm for 10 minutes and the pellet was discarded. Two drops of ammonium test kit Reagent I was mixed with 1 ml of supernatant. And one fifth of Reagent II was added and incubated for five minutes. After that, one drop of Reagent III was poured and incubated again for 5 minutes. Finally, color development was noted by comparing the color chart from the test kit.

Identification and characterization of selected bacterial strains

Morphological characteristics of bacterial isolates were examined by Gram's staining according to the method by Salle [11]. Biochemical tests, including triple sugar iron agar test, Methyl Red (MR) test, utilization of citrate, nitrate reduction test, gelatin agar test, and starch hydrolysis test were studied [12-14].

Composting experiment

In this research work, raw materials such as rice straw, water hyacinth and cow dung were used for composting process. The rice straw and water hyacinth were chopped into small particles to enable fast and efficient process. Nine kg of straw, 3kg of cow dung and 3 kg of hyacinth were used as raw materials for composting. Firstly, straw and hyacinth were chopped into small pieces. Then each raw material was placed layer by layer by the volume of 3:1:1 as a heap. The straw layer was covered by cow dung layer. Above the two layers, hyacinth was then added. The procedure was repeated with another 3 layers. Moisture content was maintained at 60% throughout the active composting period and 30ml of bacterial broth per 1 kg of raw material was added. The mixtures were turned at 7 days intervals to maintain porosity. The heap was covered with plastic sheet. When the heat was evolved in the heap, it was turned over to circulate air underneath the composting heap and then covered with plastic sheet. The compost heap was sited away from, and downwind of, the house and placed in a shady shuttered place to give protection from sun and wind. The volume was fallen to nearly half of the original heap and the color was changed to dark brown- black. The heap was reached to a mature stage after four weeks and used for direct incorporation into the soil preparation before sowing. The chemical composition of matured compost was analyzed in the Soil of Analysis Department, Ministry of Agriculture and Irrigation, Myanmar. 2.8. Field Trial for Rice

Field experiments of the isolated bacterial strains were conducted at the agriculture land near Technological University (Kyaukse), Mandalay Division. The total area was 0.50 acre. The experiment period was from July 12nd 2008 to November 24th 2008, altogether 150 days; the average temperature was 34ºC.

Each single colony of six bacterial isolates (the best nitrogen fixing activity) was inoculated in 10 ml G-NFMM in test tubes and incubated at 37ºC for three days on a shaker. The starter inoculum of 10 ml of broth culture was inoculated into one liter of G-NFMM broth. One liter broth culture solution was separately inoculated with four 500 ml flasks and incubated on a shaker until optimum growth. For P-solubilizing, *Saccharomyces cerevisiae* was inoculated together with the best nitrogen fixing strains. For biofertilizer preparation for field trial, the culture broth of the best six bacterial strains incubated for one week and then it was inoculated into the carrier system, the compost. This prepared biofertilizer was applied to the field at 10 days interval. Rice plant height, length of panicle, grains per panicle, weight of thousand grains and yield per acre were also recorded.

RESULTS AND DISCUSSION

Isolation and purification of cellulolytic nitrogen fixing bacteria

Twenty strains of cellulolytic nitrogen fixing bacteria were isolated from different soil samples. They were all well grown on C-NFMM, and CMC-NFMM media. But, cellulase producing activity was different among them. All bacterial isolates can utilize different carbon sources, glucose, cellulose and carboxymethyl cellulose. According to the previous studies, the results are harmony, which the cellulolytic microorganisms have been isolated from diverse environments such as, soil [15,16] organic waste [17,18]. They were cultured at 0.7% Cellulose, CMC and Glucose at 37ºC. By visual detection, their growth rates were higher on G-NFMM medium than C-NFMM and CMC-NFMM media. They grew well on G-NFMM medium after overnight incubation. But, their growth rates were good at 0.2% Cellulose or CMC than 0.7% Cellulose or CMC. So, 0.2% of Cellulose or CMC was used for detection of cellulolytic activity of isolated strains.

Detection of cellulase production activity

For qualitative assay of cellulase producing activity of the isolated strains, Congo red was used. All isolated strains of cellulolytic nitrogen-fixing bacteria showed the growth on CMC agar and gave positive results in hydrolysis by Congo red. When the sole carbon source was CMC (cellulose), the clear zone is stronger than in media containing cellulose. The clear zone diameter of all isolated strains on 0.2 % CMC or Cellulose Nitrogen Free Mineral medium when detected by flooding 0.1 % Congo-red solution were shown in Figure 1 and Figure 4. The diameter of clear zone on CMC Nitrogen Free Medium is higher than that of Cellulose Nitrogen Free Mineral medium. Many researchers have reported agar media containing cellulose or CMC for the screening of cellulases producing bacteria through the formation of zone of hydrolysis [19]. However, the diameter of the clearing zone may not be accurately reflecting the true cellulases activity [20]. Therefore, all the bacterial colonies having the large clear zones were screened again by colorimetric method to determine the most potent isolates for cellulase and CMCase production. So, six selected strains were selected and tested for cellulolytic activity by DNS method and they gave various amount of reducing sugar concentrations (Figure 2). 0.2% cellulose and 0.2% CMC were used as substrates for this method. After one week incubation, most of six strains utilized

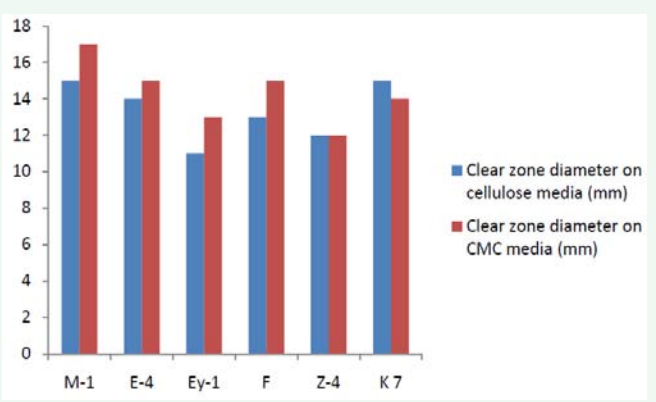

Figure 1 The clear zone diameter of six selected strains on two different media.

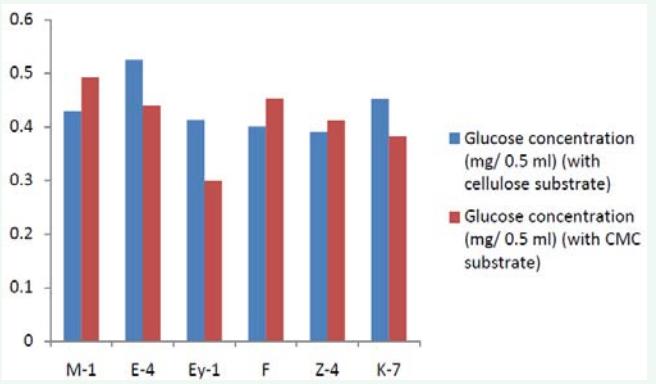

Figure 2 Glucose concentrations of six selected strains using cellulose and CMC as substrates by DNS method.

CMC more than cellulose by the observation of turbidity of broth culture solution. The best strain K-7 for cellulolytic activity gave glucose concentration at 0.452 mg/0.5 ml utilizing cellulose as substrate and the best strain M-1 for CMCase activity gave reducing sugar concentration at 0.493 mg/0.5 ml utilizing CMC as substrate. Reducing sugar concentration was not significantly different among six selected strains but they utilized CMC more than cellulose according to these data. These results are very similar with previous studies, which also recorded a CMCase activity greater than cellulose [21,22].

Detection of nitrogen fixing activity and strain selection

Leschine et al. [23], found the first anaerobic bacteria known to use cellulose as an energy source for nitrogen fixation. The isolated strains in this research work can also perform dual activities; nitrogen fixing and cellulolytic activities. For screening of nitrogen fixation activity, all bacterial strains were incubated in G-NFMM solid and broth medium containing BTB and incubated for one week. After one week incubation, the best isolates for nitrogen fixation produced significant amounts of ammonia into the media by changing the color of the medium from green to blue as the pH of the medium was increased. All isolated strains gave nitrogen fixing activity when activity was tested by ammonium test kit. M-1, E-4 and Ey-1 showed higher amount of ammonia

than other three strains in detection with ammonium test kit and the color intensity greater than 3 mg/l were estimated by the dilution of sample color solution (Figure 3) (Figure 5).

Identification of selected strains

The selected isolates were identified depending on their morphological and biochemical properties based on Bergey's Manual of systematic Bacteriology 2nd ed. (vol. 3: The Firmicutes) according to [24]. The colonial morphology, microscopic morphology, and biochemical tests of the best selected six strains were described in Table 1. The preliminary characteristics suggested that the isolated strains were *Azotobacter* species. Azotobacter is a broad spectrum biofertilizer and can be used as inoculants for most agricultural crops. Inoculation of soil with *Azotobacter* species lead to increase in crop yields due to the increase in the concentration not only of nitrogen, but also of other substances, such as vitamins, gibberellins, naphthalene and acetic acid, which improve plant growth. *Azotobacter* also synthesizes growth-promoting substances, produces group B vitamins such as nicotinic acid and pantothenic acid, biotin and heteroauxins, gibberellins and cytokinin-like substances, and improves the seed germination in several crops.

Composting experiment

This study was conducted to assess the combinations of 6 strains of cellulolytic nitrogen-fixing bacteria for their ability to degrade straw waste. Saha et al. [25], found that rice straw has many characteristics for production of feedstock of several valuable products through microbial biodegradation processes. The chemical composition of straw mainly includes cellulose (32–47%), hemicellulose (19–27%) and lignin (5–24%). And Binod [26], said that the common manner adopted by farmers to eliminate these materials is opening ignition in fields that promote the air pollution and also have the impact on public health. Chamhuri [27], suggested that efficient utilization of rice straw *via* environmentally and friendly waste management approach including zero waste and zero burning has become quite important candidates. This point of view focused on the minimal waste, re-use and recycling of farm products, composting, and utilization as energy source, or re-use for landscaping and other many useful impacts. The finished compost was observed after one month which contained C/N ratio 14:1 (Figure 6). To prepare organic biofertilizer, the best nitrogen fixing strains were added to compost which was used as a carrier system. The constituents after composting was measured and described in Table 2. Treatment II showed higher in Total N% and Total P_2O5% while Treatment I was better in Total K_2O % and Total S%. C:N ratios of these two treatments were not different. [28-30], have been reported that in this process, microorganisms degrade organic matter that can produce ammonia (NH_4), carbon

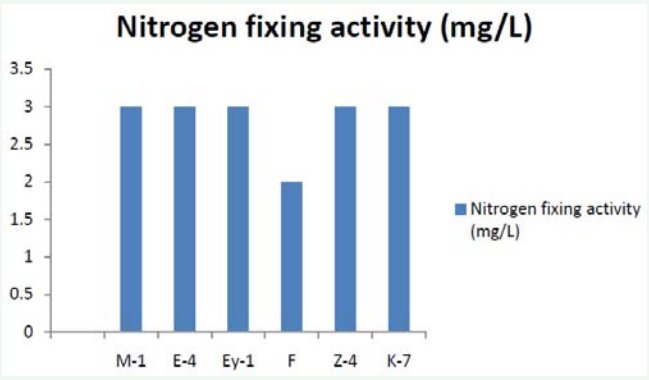

Figure 3 Quantities of Nitrogen fixed by the six selected strains as detected by ammonium test kit.

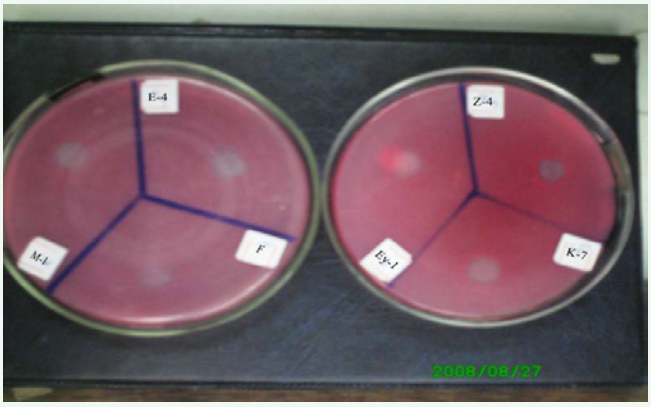

Figure 4 Screening of Cellulase activities of isolated strains on 0.2 % CMC-NFMM.

Figure 5 Detection of nitrogen fixation activity of isolated strains by test kit.

Figure 6 Initial (a) and final (b) composting material.

Table 1: Colonial morphology and biochemical characteristics of six selected bacterial strain.

No.	Strains	Colony Morphology and Gram's staining reaction	Triple Sugar Iron (TSI)	Methyl Red	Citrate Utilization	Nitrate Reduction	Motility	Gelatin Liquification	Starch hydrolysis
1	M-1	Pale yellow, Mucoid, ~1 mm, Gram negative	+	-	+	+	+	+	+
2	E-4	Pale white, Mucoid, ~ 0.5 mm, Gram negative	+	-	+	+	+	+	+
3	F	White, Mucoid, ~1 mm, Gram negative	+	-	+	+	+	+	+
4	K-7	Pale yellow, No Mucoid, ~ 0.5 mm, Gram negative	+	+	+	+	+	+	+
5	Z-4	White, Mucoid, ~1 mm, Gram negative	+	-	+	+	+	+	+
6	Ey-1	Pale white, No Mucoid, ~1-1.2 mm, Gram negative	-	-	+	+	+	+	+

Table 2: Nutrient content of the compost made using six selected strains.

Sample	Treatment I	Treatment II
Moisture %	43.43	14.37
Total N%	0.323	1.097
Total P_2O_5%	0.5884	0.7153
Total K_2O %	1.47	1.38
Total Ca %	2.766	4.04
Total S%	7.192	4.669
Total organic Matter %	20.91	27.34
Total organic carbon%	12.127	15.857
C:N	14.735	14.459
Total Mg%	1.258	0.272

Treatment I = Rice straw, fresh water weed and cowdung; Treatment II = Rice straw, water hyacinth and cowdung

Table 3: Comparison on the effect of Different Fertilizers on Rice Plant.

No.	Parameters	Treatment I	Treatment II
1.	Dosage of biofertilizer (kg)	110	-
2.	Dosage of chemical fertilizer (kg)	20	50
3.	No. of grain per panicle	147	115
4.	No. of filled grain per panicle	128	95
5.	Weight per panicle (g)	1.786	1.857
6	1000 grains weight (g)	23.0	20
7.	Length of panicle (cm)	23	29
8.	No. of unfilled grains per panicle	19	20
9.	Number of Tillers	14	10
10.	Plant height (cm)	145	150
11.	Yield (basket per acre)	101	90
12.	Yield (kg per ha)	5212	4644
13.	Located area	Kyaukse	Kyaukse
14.	Field area (acre)	1	1
15.	Duration	July to Nov	July to Nov
16.	Rice species	Ayarmin	Ayarmin

Treatment I = Formulated biofertilizer + Chemical fertilizer; Treatment II = Chemical fertilizer (Armo + Urea)

dioxide (CO_2), heat, water and humus and they are stable organic end products. Composting has many advantages that can enhance soil fertility, increase agricultural yield, improve soil biodiversity, reduce ecological risks and ameliorate the environment. Before inoculating bacteria, content in the compost was studied and the amount of bacteria $1x10^5$ CFU/g was observed. Treatment II was better in Total N% because water hyacinth contains more nitrogen content than fresh water weed. Roper and Ladha [31], also used straw to detect the biological N_2 fixation by heterotrophic and phototrophic bacteria. Normally, N_2-fixing bacteria that can utilize cellobiose carried out cellulolysis and diazotropy and it can produce glucose from straw by cellulolytic microorganisms [8]. Roper and Ladha, Chen Li et al. [32], found that the C:N ratio gradually decreases from around 30:1 to 10-15:1 for the finished product when composting proceeds. This is due to the fact that each time microorganisms consumed organic compounds, two thirds of Carbon is given off as Carbon dioxide. This is due to the fact that each time microorganisms consumed organic compounds, two thirds of Carbon is given off as Carbon dioxide. The remaining one third is absorbed along with N into microbial cells and then later release for further use once those cells die. Compost is considered finished if the C:N ratio has the value reaching to 20:1. In my research work, C: N ratio was in the range 14-15: 1.

Effects of six selected strains on rice plants (Ayarmin)

The effectiveness of organic biofertilizer was observed by applying the selected strains on rice cultivation. From the data mentioned in Table 2, usage of chemical fertilizer could reduce up to 50% when supplement with formulated biofertilizer with the yield nearly the same. Therefore, it was shown that using biofertilizer together with lower amount of chemical fertilizer improved rice plant growth; yield and nitrogen content in soil when compared with other rice plant where chemical fertilizer only was used. And also, nitrogen-fixing bacteria on cellulose degradation are very useful for advanced technology that converts solid waste into nitrogen rich compost. Although the agriculture industry plays a vital role in the developing countries,

such as feeding the population and economic exports, it can cause the degradation of the environment by using chemicals to improve yields. The use of biofertilizers and biopesticides in place of chemicals is likely to reduce the impact on soil, air and water [33]. Raja [34], also described nowadays, it becomes developing technology that organic farming using biofertilizers and biopesticies and it is a growing demand for safe and healthy food and long term sustainability and also concerns on environmental pollution associated with indiscriminate use of agrochemicals. Therefore, this research can enhance the understanding of the effects of cellulolytic nitrogen fixing bacteria for degradation of solid waste into nitrogen rich compost which can be used as biofertilizer.

CONCLUSION

Among 20 isolated strains, M-1, E-4, Z-4, F, K-7 and Ey-1 were the best strains for cellulolytic activity. When cellulose was used as substrate, glucose concentrations of six strains were lower than the concentration of glucose when using CMC as a substrate. These six bacterial strains had dual activities for cellulose degradation and nitrogen fixation because they grew well on Nitrogen Free Mineral Medium (CMC-NFMM). In this context decomposition of organic material or waste by suitable microorganisms with concern to produce high value compost and incorporation in alternation of chemical farming practices. Composting of organic waste material and utilization in farming practices is an alarming issue for future prespectives of research which could mitigate the pollutants indirectly and benefit the agro-industry directly by adopting environment friendly approaches for agricultural sustainability. It was shown that using biofertilizer together with lower amount of chemical fertilizer improve rice plant growth, no. of panicles, yield and nitrogen content in soil when compared to other treatment using chemical fertilizer only. Biofertilizers are environmentally friendly and leading to sustainable agriculture by maintaining soil fertility.

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