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Full Length Research Paper

Bioconversion of agricultural cellulose waste to valuable product by cellulolytic nitrogen-fixing bacterial isolates

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Six strains of cellulolytic nitrogen-fixing bacteria were isolated from various agricultural soil sources of Kyaukse District, Mandalay Division, Myanmar by using nitrogen free Cellulose and Carboxymethyl Cellulose (CMC) medium. From the 16S rDNA sequencing results, three strains showed high similarity to *Lysobacter enzymogenes* and three strains showed high similarity to *Azotobacter chroococcum*. These six isolates excreted significant amounts of ammonium (30.03 – 100.32 ppm). Cellulolytic activity of isolated strains was detected by DNS colorimetric method using CMC and cellulose as substracts and significant amount of glucose was observed. Phosphate solubilizing, IAA (Indole Acetic Acid) production activity and antifungal activities of isolated strains were also detected. The phosphate solubilizing amount of these isolates was detected form 11 to 26 ppm. Among six strains, four bacterial isolates can also produce IAA. These strains gave positive results to Indole test. Antifungal activity of isolated strains was tested with *Fusarium, Rhizotonia* and *Pythirium*. M-2 and M-3 isolates can inhibit the growth of *Fusarium* and *Rhizotonia*. M-1 isolate can inhibit the growth of *Fusarium,* Ey-1 can inhibit *Rhizotonia* and E-4 can inhibit *Pythirium*. Composting was studied by using 6 strains on straw waste and complete composting was detected for a month. Formulated Biofertilizer based on compost by adding 7 strains including *Sachromycese cerevisiae* was applied on rice cultivation and good yield was obtained.

Key words: Cellulolytic nitrogen-fixing bacteria, *Lysobacter enzymogenes*, *Azotobacter chroococcum* composting, biofertilizer, rice cultivation.

INTRODUCTION

Cellulose is the most abundant of all naturally occurring organic compounds, which accumulates every year in large quantities in the form of agricultural, industrial, forest and residential wastes. Cellulosis materials, as agricultural and forest residues are available in huge quantity, scattered over wide areas in many agro-based countries including Myanmar. Crop residues are generated in large quantities and constitute an abundant but underutilized source of renewable biomass in agriculture. Half the quantity of agro-residues thus produced finds use as roofing material, animal feed (Wanapat, 1984), fuel and packing material, while the other half is disposed of by burning in the field. Burning agro-residues in the field is considered a cheap and easy means of disposal of excess residues (Levine, 1996). This practice appends to air pollution, increases soil erosion and decreases the efficacy of soil-applied herbicides like isoproturon (Andreae and Crutzen, 1997). Moreover, it also causes respiratory problems and increases the fog incidences even in distant cities. Direct incorporation of agro-residues like rice straw in field solves the problem of air pollution but it is not feasible due to the short time gap between harvesting of rice and sowing of wheat. Besides, it involves additional cost of labour, irrigation and extra tillage. Moreover, observations of long term experiments indicate that though

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incorporation of agro-residues in soil improves soil health significantly, it decreases the subsequent crop yields due to production of microbial phytotoxins and allelochemicals and immobilization of the available nitrogen. Incorporation of agro-residues like paddy straw increases the CH_4 emission from field especially in irrigated soils, which in turn adds to the malice of global warming (Andreae and Merlet, 2001).

Composting is an attractive prospective in a policy of waste recycling to produce humus-like compounds to be used for improvement both soil and plant growth. Composting is an aerobic process in which microorganisms convert a mixed organic substrate into carbon dioxide, water, minerals and stabilized organic matter under controlled condition, particularly of moisture and aeration are required to yield temperatures conductive to the microorganisms involved in the composting process (Chen and Inbar, 1993). This process has many advantages including sanitation, mass and bulk reduction and decrease of C/N ratio. The stabilized compost produced should benefit the plant growth and be suitable for agricultural applications (Campbell et al, 1995). Rice straw is rich in carbon and poor in nitrogen, which limits the composting process. The ratio of carbon to nitrogen (C:N) is a common index used for assessing feedstocks and the maturity of any given compost. Nitrogen becomes more concentrated as carbon in organic materials is broken down and liberated as carbon dioxide. C: N ratios in finished compost range from 12:1 - 20:1, but are ideally between 14:1 - 18:1 (Jimenez and Garcia, 1992).

The application of compost in soil improves soil physical, biological and chemical properties, and also restores soil organic matter and carbon pools. Tremendous information is available on use of compost in organic farming. Several experiments were conducted throughout the world to monitor the effect of compost application on vegetable fruits, ornamental crops, legumes and cereal crops. Most of the researchers reported significant benefit in terms of increased crop yield and better mineral nutrition under integrated nutrient management practices (Khater et al., 1997). Composts can be further enriched with minerals (rock phosphate and mica) and microbial inoculants (N fixers, PSB and K solubilizing microbes) to enhance the overall quality of these organo-mineral fertilizers. The application of such bioaugmented nutrient enriched compost in soil leads to a significant increase in the soil fertility status (in terms of microbial biomass, N and available P) enhancing the overall chemical and biological activity of soil (Stamatiadis et al., 1999). The application of mineral enriched compost indirectly satisfied the N and P needs of plants in nutrient deficient soils. Apart from being a source of macro and micro nutrients for plants, compost is also believed to suppress soil-borne diseases in plants (Jusoh et al., 2013). The exact mechanisms of disease suppression is not clear but it may involve antibiotic production by beneficial microorganisms present in compost, activation of disease-resistant genes in plants by microorganisms (induced systemic resistance), improved plant nutrition and vigour leading to enhanced disease resistance, presence of toxic or stimulatory volatile compounds in compost. Composting transforms organic "waste" products into a nutrient-rich soil amendment capable of improving depleted or disturbed soil environments (He et al., 2000).

The decomposition of the cellulose is inhibited by a nitrogen limitation and that this inhibition could be overcome by decomposer organisms which combined both cellulolytic and nitrogen-fixing functions. The main aim of this study was to isolate the cellulolytic nitrogen-fixing bacteria for conversion of agricultural cellulose waste to valuable product. This biological inoculant may then be used by organic producers on farm sites to help manage and recycle organic residues and to create an N-enriched biofertilizer.

MATERIALS AND METHODS

General isolation methodology for cellulolytic nitrogen-fixing bacteria

To isolate the cellulolytic nitrogen-fixing bacteria, soil samples were collected from various paddy fields under cultivation and cultivated condition and natural compost from Kyaukse district, Mandalay Division in Myanmar. Strains of both cellulolytic nitrogen-fixing characteristics were selected by using a nitrogen-deficient medium where in the sole carbon sources are Avicel Cellulose and Sodium Carboxymethy Cellulose.

One gram of soil samples was added to 100ml sterile nitrogen free cellulose medium and nitrogen free CMC medium in each 250 ml conical flasks. The flasks were shaken once and incubated on water bath shaker at room temperature for a week. Then the broth culture was plated on solid nitrogen free cellulose medium and CMC medium. The plates were labeled and incubated for a week. After incubation, they were analyzed and colonies with different morphologies were subcultured onto new plates. The isolated colonies were characterized for their morphological and biochemical characters and were also detected for nitrogen fixation and enzyme production activities.

16S rDNA Identification

DNA extraction was performed using the Miniprep DNA Extraction and Purification Kit (TaKaRa, Tokyo, Japan). Bacterial 16S rDNA was amplified by using universal primers and 35 PCR cycles. Each cycle consisted of denaturation for 1 min at 94°C, annealing for 30 s at 60°C, and extension for 4 min at 72°C. DNA purification

was done using DNA Extraction Kit (Roche Diagnostics GmbH, Mannheim, Germany). Nucleotide sequences were analyzed using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and BLAST on the NCBI BLASTN.

Detection of nitrogen-fixing activity

The visual detection of nitrogen-fixing activity was observed by using Nitrogen Free Glucose Mineral Agar Medium (NFGMM) as well as Broth Medium and Ammonia Test Kit. Individual purified colony growing on Cellulose nitrogen free medium was taken and inoculated into NFGMM containing BTB (bromothymol blue solution) and without BTB.. After one week incubation, changing the color of the BTB containing medium was recorded. To detect nitrogen-fixing activity from the broth culture without BTB, the reagents of ammonia test kit were added and the appeared color was noted by comparing with the color chart from the test kit. The quantitative analysis was done by comparing the value of absorbent of known concentration of ammonium sulphate solution at 625 nm.

Detection of cellulase producing activity

A modification of the Saleh-Rastin et al. (1991) plate assay was used to detect the crude enzyme activity. The purified colony of each isolates was plated on 0.2% Avicel Cellulose and Sodium Carboxymethyl Cellulose media and incubated at room temperature for a week. Cellulose activity and CMCase activity were recognized by zones of clearing after flooded with 0.1% Congo-red solution for 1 h and washed with 1M NaCl. A clear zone was evident around the strain while the hydrolyzed portions remain colorless. Then those bacteria that were of good clearance beyond the area of growth were selected for compost study as potential secretors. Cellulose was degraded to glucose by cellulase enzyme, therefore cellulase activity was quantitatively analysed by DNS method. 1 to 2 ml sample solutions was mixed with 3 ml DNS reagent and placed on boiling water bath for 5 min. Then cooled to room temperature and the absorbent read at 540 nm. The absorbance values were translated into glucose equivalent using a standard curve obtained by plotting glucose concentration against absorbance.

Detection of anti-fungal activity

Fusarium, Rhizotonia and *Pythium* were used as target soil borne pathogenic fungi. One loopful of target fungus was spread on the Potato Dextrose Agar Medium. Then tested strains were placed onto the fungus medium and incubated at room temperature for a week. After incubation the inhibition zones appeared around the tested strains were recorded.

Detection of phosphate solubilizing activity

Detection and estimation of the phosphate solubilizing ability of microorganisms have been possible using plate screening methods. Phosphate solubilizers produced clearing zones around the microbial colonies on the medium containing insoluble mineral phosphate such as tricalcium phosphate. Also the bromothymol blue method was used for detection of pH dropping activity that was caused by the releasing of organic acids and showing yellow halos around the tested colonies. The quantitative bioassay was carried out using Erlenmeyer flasks (100 ml) containing 10ml of NBRIP broth medium inoculated with the bacteria at around 10^8 - 10^9 CFU/ml. Autoclaved uninoculated NBRIP medium served as control. The flasks were incubated for 3 days at 37°C on a shaker at 180 rpm. The cultures were harvested by centrifugation for 15 min at 8,000 rpm. Supernatant was decanted and autoclaved at 121°C for 20 min. Autoclaved samples were then filtered through a 4.5 *µm* membrane. Available phosphorus content in the culture supernatant as well as control (supernatant obtained from no bacteria inoculation) was estimated using the vanadomolybdate colorimetric method by measuring the absorbance at a wavelength of 420 nm.

Detection of plant growth hormone (IAA) producing activity

Purified single colony was added into the NFGM liquid medium with 0.5mg/ml tryptophan and incubated at room temperature for a week. After incubation culture was centrifuged at 10000rpm for 5minutes. One ml of supernatant was taken and mixed with one drop of orthophosphoric acid and 2 ml of Solkoski's reagent (150ml of 35% puchlnic acid and 1ml of 0.5%$FeCl_3$). Development of pink color was indicated that IAA production and recorded.

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. 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. Then each raw material was placed layer by layer by the volume of 3:1:1 as a heap in the size of 6'x4'x3'. Moisture content was maintained at 60% throughout the active composting period. The mixtures

Figure 1. Screening of nitrogen-fixing activity by pouring by test-kit reagent.

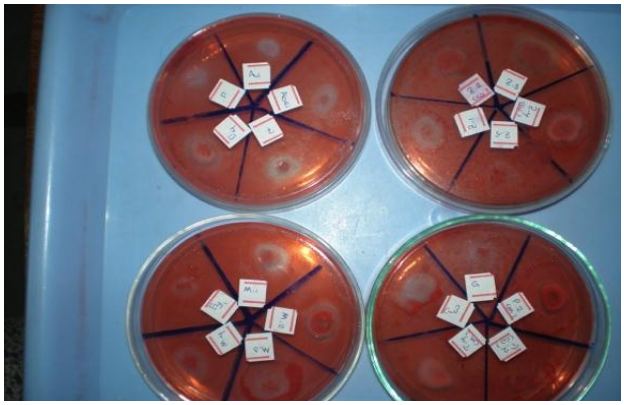

Figure 2. Screening of cellulase activity congo-red solution.

were turned at 7 days intervals to maintain porosity and complete composting were achieved at one month.

Organic biofetilizer preparation based on compost and field trial

To prepare organic biofertilizer, compost was used as a carrier system. 6 strains of nitrogen-fixing cellulolytic bacteria and 1strain of *S. cerevisiae* which can provide all the essential factors and nutrients for the growth of the 6 strains were added into this carrier. The effectiveness of formulated organic biofertilizer was studied on rice (variety; Manawthukha) cultivation on the period of March to July.

RESULTS

Cellulolytic nitrogen-fixing bacteria were isolated from different soil sources and their colonial morphology, microscopic morphology and some biochemical characteristics were studied. Among six bacteria isolates, the nucleotide sequences of E-4, Ey-1 and M-2 showed high similarity (99%) to *Lysobacter enzymogenes* and M-1, M-3 and M-4 showed high similarity (99%) to *Azotobacter chroococcum.* The screening tests of nitrogen fixing activity and cellulose producing activity are shown in Figures 1 and 2. After screening 6 strains were selected based on their visual detection results. The phosphate solubilizing activity and plant growth hormone (IAA) producing activity were also detected and are presented in Figures 3 and 4. In addition antifungal activity of selected bacteria was observed and the recorded photo is mentioned in Figure 5. Some biochemical characteristics of these strains are recorded in Table 1. The activities of isolated bacteria for composting and biofertilizer preparation are presented in Table 2. The amount of nitrogen fixation and phosphate solubilizing amount of selected bacteria is mentioned in

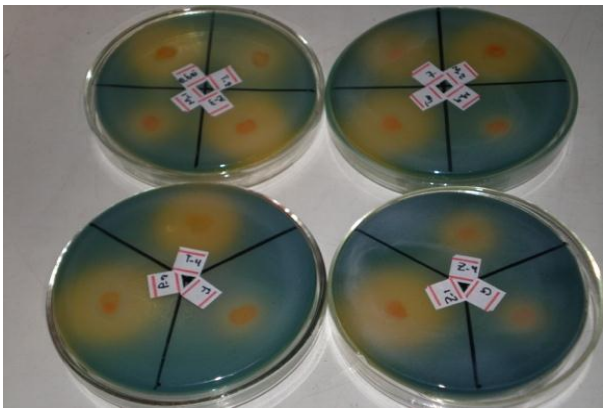

Figure 3. Screening of phosphate solubilizing activity of isolated strains.

Figure 4. Screening of IAA activity.

Figure 5. Screening of antifungal activity of Isolated strains against *Rhizotonia*

Table 3. The cellulase producing activity of the selected strains was detected as reducing sugar concentration by DNS method and the results are mentioned in Table 4. Composting was studied by using 6 selected strains and is mentioned in Figure 6. The comparative study of the effectiveness of formulated biofertilizer and others are

Table 1. Some biochemical characteristics of isolated bacteria.

No.	TSI	Catalase	Citrate	Gelatin	MR	Nitrate	Motility	Starch
M-1	Y/Y(+)	+	+	+	-	-	+	+
M-2	Y/R(+)	+	+	+	-	-	+	+
M-4	Y/R(++)	+	+	+	-	-	+	+
Ey-1	Y/Y(-)	+	+	+	-	-	+	+
E-4	Y/R(++)	+	+	+	-	-	+	+
M-3	Y/R(++)	+	+	+	-	-	+	+

Table 2. The activities of selected bacteria for biofertilizer preparation.

No.	Selected Bacteria	N-2 Fixing	P-Solubilizing	Cellulose Degradation	Antifungal Activity			IAA Production
					Fusarium	*Rhizotonia*	*Pythium*	
1.	M-1	+	-	+	+	-	-	+
2.	M-2	+	+	+	+	+	-	+
3.	M-4	+	+	+	-	-	-	-
4.	Ey-1	+	+	+	-	+	-	+
5.	E-4	+	+	+	+	-	+	+
6.	M-3	+	+	+	+	+	-	-

Table 3. Nitrogen fixing amount and phosphate solubilizing amount of six selected strains.

No	Strains	Nitrogen fixing amount (ppm) at 625 nm	Phosphate solubilizing amount (ppm)
1	M-1	100.32	11.7
2	M-2	46.64	16.1
3	M-3	27.46	20
4	M-4	89.03	16.2
5	E-4	30.03	27
6	Ey-1	32.22	11

Table. 4. Determination of reducing sugar by six selected strains using cellulose and CMC as substrate.

No	Strains	Glucose concentration (mg/ 0.5 ml)(using cellulose substrate)	Glucose concentration (mg/ 0.5 ml)(using CMC substrate)
1	M-1	0.429	0.493
2	M-2	0.590	0.456
3	M-3	0.402	0.461
4	M-4	0.498	0.439
5	E-4	0.525	0.439
6	Ey-1	0.413	0.300

Figure 6. Composting by using 6 strains on rice straw.

Table 5. Comparative study of rice cultivation by using various fertilizers.

No.	Parameter	Field trial-1	Field trial-2	Shwe Myae	Chemical
1.	Dosage of biofertilizer (Kg/ha)	268.8	268.8	268.8	-
2.	Dosage of chemical fertilizer (Kg/ha)	80.64	56	80.64	168
3.	No. of grains per panicle	151	143	119	188
4.	No. of filled grains per panicle	137	139	112	171
5.	% of fill grains per panicle	90.73%	97.20%	94.09%	91.08%
6.	Weight per panicle (gm)	1.786	1.857	1.420	2.258
7.	1000 grains weight (gm)	20.52	20.51	19.89	20.17
8.	Length of panicle (cm)	23.91	22.25	21.04	23.36
9.	No. of unfilled grains per panicle	14	4	7	17
10.	Tiller with panicles	14	14	13	16
11.	Plant height (cm)	95.5	93.7	90.8	97
12.	Yield (Kg/ha)	2510.45	2532.28	2095.68	2554.11

presented in Table 5.

Discussions

Nitrogen fixing bacteria were obtained from different soil sources. Among the different carbon sources, Cellulose, Sodium Carboxylmethyl Cellulose and Glucose, they utilized glucose more than the other two carbon sources. 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, but their growth rates at 0.2% cellulose or CMC were not as good as 0.7% Glucose. So, glucose was used as a carbon source for detection of nitrogen fixation of isolated strains.

20 bacterial isolates were screened visually for nitrogen fixation by ammonium test kit in G-NFMM broth medium. All isolates gave nitrogen fixing activity by the development of green color of the medium. But, color intensities were different among the isolated strains when compared with the color chart on ammonium test kit. So, it was noted that their nitrogen fixing activities were different. And, 20 bacterial isolates were also cultured on G-NFMM solid and broth medium containing BTB and incubated for one week for screening of nitrogen fixing activity and strain selection. 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 rose. In this way, the best strains for nitrogen fixation were selected based on these observations.

From this selection, six bacterial isolates were selected and calculated their accurate ammonium concentrations by constructing a glucose standard curve at 600 nm, assayed with the same reagent. Six selected strains produced significant amounts of ammonium and the ammonium concentrations were from 30 to 100 ppm.

For cellulase production activity in nitrogen free media, all 20 bacterial isolates grew well on 0.2% C-NFMM 0r 0.2% CMC-NFMM media but enzyme production activity couldn't be measured. They provided clear zones on medium when activity was detected by pouring 0.1% Congo-red solution, but clear zone diameters were not different among isolates. So, six selected strains were tested for cellulolytic activity by dinitrosalicyclic colorimetric method (DNS). 0.2% cellulose and 0.2% CMC were used as substrates for this method. After one week incubation, most of six strains utilized cellulose more than CMC by the observation of turbidity of broth culture solution. The best strain (M-2) for cellulolytic activity gave glucose concentration at 0.590 mg/0.5ml utilizing cellulose as substrate and the best strain (M-3) for CMCase activity gave reducing sugar concentration at 0.461 mg/0.5 ml utilizing CMC as substrate. Reducing sugar concentration was not significantly different among six selected strains but they utilized cellulose more than CMC according to these data.

Biochemical characterizations of six selected strains were mostly similar among them. Some results were different, eg, TSI, and Indole. Microscopic morphology was slightly different with one another strain.

Besides nitrogen fixation and cellulolytic activities, six bacterial strains also gave P-solubilizing activities by producing acid phosphatase. When activity was recorded by measuring the pH of the medium (NBRIP media) and the yellowish zone diameter on NBRIP solid media after three days incubation. The pH of the medium fell from 8.9 to 2.6 by E-4. Other culture broth solution fell in the range of 2.9 to 5.1. Bacterial strains were still survive at this pH level of the medium when survival was tested by motility. So, it was noted that six bacterial strains had P-solubilizing activity. On the other hand, P-solubilizing activity was tested by ion-exchange method and recorded absorbance at 830 nm.

Some of six strains had antifungal activity against *Fusarium*, *Rhizotonia* and *Pythium*. *Fusarium* was sensitive to M-1, M-2 and M-3 strains. *Rhizotonia* was sensitive to M-2, M-3 and Ey-1, and *Pythium* was

sensitive to E-4. Although they had antifungal activities, they couldn't provide inhibition zones largely. So, although it was said that they had antifungal activities, they didn't totally suppress the growth of these fungus on Czapek Dox Agar medium.

By visual detection, four out of six bacterial strains gave IAA production activity. Quantitative was not measured. But, activities were coincided with Indole test results. They also gave positive results to Indole test. But qualitative might be different among six strains by visual detection of color intensities.

Six selected wild type strains were combined in G-NFMM broth media and labeled as Shweziwa for effective application on rice plants (Manawthukha). They grew well together on G-NFMM media and improved actual rice plant growth and yields when rice plant growth and yields were compared with another rice plants used with Shwemyay biofertilizer and used with only chemical fertilizer. Rice plant height used with Shweziwa biofertilizer was 93.7 cm but height of other rice plants used with Shwemyay biofertilizer and only chemical fertilizer was 91.5 cm and 95.0 cm. Height of rice plant used with Shweziwa biofertilizer was higher than height used with Shwemyay biofertilizer and lower than height used with only chemical fertilizer. Length of panicle was not significant among them. But, grains per panicle were different. But there was not a significant difference of yield among them. Average yield per acre for rice plant used with Shweziwa biofertilizer was 5336 lb per acre and the other treatments were 4324 lb per acre and 5290 lb per acre. By measuring, rice plant height, yield, length of panicle, grains per panicle and costs of fertilizers, Shweziwa biofertilizer was the best among them.

Conclusions

Among six strains which have good nitrogen fixing activity and cellulolytic activity, M-1 has highest nitrogen fixing activity and it can also produce plant growth hormone (IAA) and can inhibit the growth of soil borne pathogenic fungus, *Fusarium*. The main aim of this research was to degrade agricultural cellulosic waste by using cellulolytic nitrogen-fixing bacteria to obtain nitrogen rich compost. Composting was studied on rice-straw by using six effective strains mixture and complete composing which contained CN ratio 14:1 was observed after one month inoculation. To prepare organic biofertilizer, 7 strains were added to compost as a carrier system. Before inoculating of 7 strains, bacteria containing in compost was studied and it consisted $1x10^5$ cfu/g was observed. The effectiveness of organic biofertilizer was observed by applying on rice cultivation. From the data mentions in Table 5, usage of chemical fertilizer could reduced up to 50% when supplemented with new formulated biofertilizer with the yield nearly the same.

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