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CHARACTERIZATION AND DETERMINATION OF LOCAL *TRICHODERMA SP* ISOLATES AS BIOFERTILIZER

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ABSTRACT

Application of chemical fertilizer for increased yields has largely affecting human health, normal flora, soil and environment. Such a scenario welcomes the use of bio- fertilizers as an alternative and as such, current research is focused on the development of such an alternative using *Trichoderma* sp. In this study, various *Trichoderma* sp were checked for their ability to compost municipal solid waste in vitro and in vivo. The results were highly promising and this could, in future, replace the chemical fertilizers.

KEYWORDS: *Trichoderma*, Biofertilizer, Composting, Municipal Solid Waste Management (MSWM).

INTRODUCTION

Municipal Solid Waste (MSW) is one of the major problems worldwide. Improper collection, recycling or treatment and uncontrolled disposal of waste in dumps lead to severe hazards, such as health risks and environmental pollution (Eawag, 2008; Collivignarelli *et al*., 2004).Municipal solid waste management continues to be a major challenge for local governments in both urban and rural areas (Wang *et al*., 2011).

Trichoderma sp are cosmopolitan fungi, frequently present in all types of soil, decaying vegetables and most of the plant particles. The major problem related to municipal solid waste is that effect air, land and also water. To survive these problems non hazardous, environmentally friendly and stabile techniques only possible with biological agents based treating. The present study focuses on isolation, identification, and characterization of local *Trichoderma* sp isolates and their premises in composting. Physico-chemical characters of sample were analyzed, and checked the correlation between before and after the biodegradation process. The biodegradative ability of *Trichoderma* isolates was also evaluated on kitchen solid waste in laboratory conditions **(**Ilias *et al*.,2005).

Compost is the product resulting from the controlled biological decomposition of organic material that has been sanitized through the generation of heat and stabilized to the point that it is beneficial to plant growth. Compost bears little physical resemblance to the raw material from which it originated. Compost is an organic matter resource that has the unique ability to improve the chemical, physical, and biological characteristics of soils or growing media. Aerobic composting of organic material is a humification process of unstable organic matter (C/N ratio >25) to become stable, characterized by the release of heat and gas from composted substrates. Composting duration varies from 2 to 7 weeks, depending on the type of decomposers and composting techniques used. Level of maturity and stability in compost determine compost quality shown by the various changes in the physical, chemical and biological compost substrate (Sant *et al*.,2010).

MATERIALS AND METHODS

Collection of sample

Samples were collected from different locations of north Kerala. The collected samples were transferred aseptically to the laboratory.

Isolation of microorganism

The serially diluted sample where plate out on *Trichodema* specific medium. Incubate at 25±1 C for 72 hours. The 1000ml media includes Magnesium sulphate (0.2g), Dipotassium hydrogenphosphate (0.9g), Potassium chloride (0.15g), Ammonium nitrate (1g), Glucose (3g), Chloramphenicol (0.25g), p-dimethylaminobenediazo sodium sulfonate (0.2g), Agar (20g).

Identification of *Trichoderma*

The isolated fungi were inoculated on to culture plate containing PDA medium, and incubate at 25±1^{o}C for up to experimental days. The morphology of fungi was observed based on colour intensity during incubation period.

Lacto Phenol Cotton Blue Staining
About mount was prepared by suspending cultures in a few drops of LPCB solution, the preparations were examined under high power and low power magnification with a microscope.

Fungal inoculum preparation
The identified *Trichoderma sp* was cultured on potato dextrose agar plates. The fungi were purified separately by transferring the tip of the mycelia into PDA slants and maintained as stock cultures for further studies.

Growth parameters
Effect of pH and Temperature
Effect of pH and temperature on the growth of *Trichoderma* was studied using Potato Dextrose Broth at different pH levels, by adding 0.1N NaOH or 0.1N HCl. *Trichoderma* was measured for biomass production during intervals of 4th, 7th, 10th, and 14th days at pH 5, 6, 7, and 7.5. Flasks were sterilized and each flask was inoculated with each isolate using 5 mm diameter mycelia disc in sterile conditions. Inoculated flasks were incubated at various temperature 20°C, 25°C, 30°C, 35°C and 40°C for ten days. Dry mycelial weight was measured after the incubation period by subtracting the initial weight of the conical flask and weight of the conical flask along with the mycelial mat.

Starch hydrolysis
The isolated *Trichoderma* were inoculated on sterile starch agar plates and incubated at 25°C for 72 hours. After incubation, iodine solution was added in to the culture plates. Clear halos surrounding the colonies indicated positive result.

Cellulase test
The activity of cellulase was tested by using CMC agar media (carboxymethyl cellulose). The isolates were spotted on the surface of agar plates and then incubated at 25^0C for 72 hours. After incubation the fungal growth was inundated with Lugol's iodine solution [iodine + potassium iodide]. A clear zone around the colonies indicates positive reaction.

Phosphate Solubilization test
All fungal isolates were screened for inorganic phosphate solubilization. A loop full of fresh fungal cultures was spotted on to Pikovaskaya's medium amended with inorganic phosphates and plates were incubated at 27^0C for 2-4 days. A clear halo around the fungal colony indicated solubilization of mineral phosphate.

Flask Assay
Substrate preparation for composting
A flask assay experiment was carried out to study the efficiency of biodegradation of the selected isolates using kitchen waste as substrate. Initially the substrates were cut into small pieces, weighed and mixed thoroughly with coir pith to maintain moisture content. Then, 400g of the mixed substrate was taken in a 1000ml conical flask, and was sterilized for the removal of surface contaminants. After cooling it was inoculated with 10ml ($1^{x}10^8$ cfu/ml) of the isolates and an uninoculated flask was used as control. These flasks were kept at room temperature for 20 days. The height reduction of the substrate during degradation was measured regularly.

Physico-chemical characterization of substrates
Determination of pH and moisture
A 25g of sample was mixed with 50ml distilled water and was shaken on a rotary shaker (ELICO Heavy rotary shaker) for 2 hours. It was filtered through Whattmann's No. 1 filter paper. The pH of the filtrate was determined using calibrated pH meter. To estimate the moisture, 5gm of the samples were weighed in a pre weighed clean, dry petri dish. It was heated for about 5 hours at 65 ± 1^oC to constant weight, cooled in desiccator and weighed. The moisture content was estimated as percentage loss in weight by using the formula;

$$\text{Moisture percentage by weight} = \frac{100(B-C)}{B-A}$$

A = Weight of petri dish. B = Weight of the petri dish + material before drying. C = Weight of petri dish plus material after drying.

Estimation of Electrical Conductivity (EC) and Total Dissolved Solids (TDS)
20 g of sample was added to 100 ml of distilled water to give a ratio of 1:5. It was stirred for about an hour at regular interval. The conductivity meter was calibrated using 0.01 M potassium chloride solution and the conductivity and TDS of the sample were measuring using EC-TDS analyser (ELICO CM 183EC-TDS Analyser).

Estimation of organic carbon:- A 10g of the sample was weighed and dried in oven at 105^0C for 6 hours in a pre weighed crucible and ignited the material in a muffle furnace at 650-700^0C for 6-8 hours. It was cooled at room temperature and kept in desicator for 12 hours. The content was weighed and total organic carbon was calculated using the following formula.

$$\text{Total organic matter (\%)} = \frac{Initial\ wt - Final\ wt}{wt\ of\ sample\ taken} \times 100$$

$$\text{Total organic carbon (\%)} = \frac{Total\ organic\ matter}{1.724}$$

Estimation of total nitrogen
Double acid - extract preparation
A 1g of dried sample was transferred into 100ml conical flask and about 10-15ml of diacid mixture (H_2SO_4 & $HClO_4$; 5: 2) was also added. The mouth of the flask was covered with a funnel. The content of the flasks were digested over a sand bath till a clear solution was obtained. It was filtered through a Whattmann's No. 41 filter paper to 250ml flask and washed with hot distilled water. After cooling it was made up to 250ml.

Determination of nitrogen

A 10 ml of the di-acid extract was pipetted out into a distillation flask and 10 ml of 40 % sodium hydroxide was also added and connected the distillation unit. 20 ml of 2 % boric acid was pipetted out into a clean conical flask and added 2 drops of double indicator (bromo cresol green and methyl red) and was kept at the delivery end of the distillation set. The completion of distillation was tested with a moistured red litmus paper. Absence of blue color indicated that complete ammonia has been distilled. The delivery tube was washed with distilled water and collected the washing in ice tumbler. Titrate the content with 0.01N sulphuric acid. End point was the color change from blue to reddish green. Percentage of Nitrogen on moisture was calculated by using the formula:

$$\%\text{of Nitrogen on moisture free basis} = \frac{\%\text{of Nitrogen on moisture free basis}}{10 \times \text{Weight of sample } (100-M)} \times 100$$

RESULT AND DISCUSSION

Isolation and screening of *Trichoderma*

For this study 5 isolates were obtained from rhizospheric soil sample and then they were cultured on potato dextrose agar. The isolates were purified and identified on the basis of their morphological characteristics. The characterization of these microorganisms was also studied by visual observation of colonies, microscopic observations.

Plant growth promotional tests

Assay for phosphate solubilization

All isolates showed clear halo zone of clear phosphate solybilization. Isolate 2 showed highest clear halo zone in Pikovskaya medium.

Phosphate solubulization index: Phosphate solubilization activities were screened by measuring the clear zone surrounding the developed fungal colony via calculation of phosphate solubilization index. The isolate 2 shows high phosphate solubilization index value (21.1mm) (Table-1).

Phosphate solubilization index = A/B

A = Total diameter (colony + halo zone) B = colony diameter only.

Table 1: Phosphate solubulization index

Isolates	Total Diameter(mm)	Colony Diameter(mm)	PI(mm)
1	16.0	11.0	14.5
2	19.0	09.0	21.1
3	15.0	10.0	15.0
4	17.0	12.0	14.1
5	13.0	08.0	16.2

Cellulase production test

Cellulase production activities were screened by measuring the clear zone surrounding the developed bacterial colony via calculation of cellulase production index. Isolate1and 3 shows high cellulase production index value (20.0mm) (Table-2).

cellulase production index = A/B

A = Total diameter (colony + halo zone) B = colony diameter only.

Table 2: Cellulase production index (CI)

Isolates	Total Diameter(mm)	Colony Diameter(mm)	CI(mm)
1	20.0	10.0	20.0
2	15.0	10.0	15.0
3	18.0	09.0	20.0
4	16.0	10.0	16.0
5	10.0	07.0	14.2

Flask Assay – Lab scale biodegradation

The ability to hastening the process of biodegradation was analyzed by flask assay method using kitchen waste as substrate. Initial and final physico-chemical characterization of substrate and the presence of pathogenic microbes in the substrate were determined.

Physico-chemical characterization of substrates used for biodegradation

During the initial stages of decomposition, organic acids are formed. The acidic conditions are favorable for growth of microbes and breakdown of cellulose. As composting proceeds, the organic acids become neutralized, and mature compost generally has a pH between 6.5-7.5. Compost organisms need water to live. Initially, composting material with moisture content between 20-30% and 100% will be aerobic. In the aerobic composting, high moisture content must be avoided. C:N ratio of 8:1 after harvest was obtained in compost applied with combined species of *Trichoderma* which is comparable to that of control. However, the compost combined with *Trichoderma* reached its maturity within 20 days as compared with the compost without activator that reached its maturity within 25

days. Meanwhile, a higher C:N ratio level of 11:1 result when *Trichoderma* sp.2 was added to the compost which is within the acceptable level for matured compost of less than 12:1, indicating a good degree of maturity.

Table 3: Initial reading of substrate

Characters	Initial value	Final value					
		Isolate 1	Isolate 2	Isolate 3	Isolate4	Isolate5	Control
pH	4.92	5.5	6.8	6.1	5.8	6.5	5.4
Moisture (%)	84	22	20	22	23	22	24
Electrical conductivity (ms)	1.306	3.2	3.4	3.19	3.22	3.18	3.35
TDS (ppm)	1.795	1.687	1.453	1.81	1.67	1.354	1.58
Organic carbon (%)	11.1	8.86	8.99	9.28	10.23	8.99	10.75
Total nitrogen (%)	3.0	2.03	0.91	0.85	2.8	1.9	2.6

The activity of isolates on biodegradation

The height of compost was measured periodically within an interval of 20 days. After incubation period, it was found that all the isolates were able to degrade the kitchen wastes by the production of more than one enzymes and ability to degrade crystalline cellulose (Table-4). The **isolate 2** and **isolate 4** showed a potential decrease in the height of substrate when compared with the activity of other microbes. The height of substrate inoculated with **isolate 4** was found to be decreased from 5.4cm to 2.1cm on 19th day of incubation. The height of substrate inoculated with **isolate 2** was decreased from 4.5cm to 2.9cm. From the results obtained the isolate 4 was found to be efficient to convert cellulose into reducing sugars.

Table 4- Biodegradation activity of isolates

Days	Height of substrate in cm				
	Isolate 1	Isolate 2	Isolate 3	Isolate4	Control
1st	4.7	4.5	4.8	5.4	5.6
3rd	4.6	4.0	4.1	5.0	5.5
5th	4.0	3.6	3.9	4.0	4.5
7th	3.7	3.4	3.7	3.5	4.1
9th	3.6	3.1	3.5	3.0	4.0
11th	3.5	3.0	3.5	2.6	4.0
13th	3.4	3.0	3.4	2.3	3.9
15th	3.3	3.0	3.4	2.2	3.9
17th	3.2	2.9	3.3	2.1	3.8
19th	3.2	2.9	3.2	2.1	3.8

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