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Microbial Characterization of Sulphate Oxidizing Bacteria Isolated from Cattle Manure Compost

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ABSTRACT

The current study includes the collection, isolation and characterization of sulphate oxidizing bacteria from the cattle manure compost dumpings from Namakkal district. Thereafter, three different sulphate oxidizing bacterial isolates such as *Bacillus* sp., *Klebsiella* sp., *Pseudomonas sp.* were isolated. The sulphate oxidation by these bacteria was optimized and it estimated quantitatively. The present study was aimed in studying the application of sulphate oxidizing bacteria as biofertilizers. Also, if the cattle manure compost studied are used as biofertilizers they would improve and enhance the soil vitality and fertility. They would be a best alternative for chemical fertilizers.

Keywords: Cattle Manure, Sulphate Oxidizers, Biofertilizer, Biomanuring

I. INTRODUCTION

Composting is one of the most successful methods for treating organic waste such as animal manure. Livestock manure accounts for a large part of the total waste generated and can cause environmental problems (e.g., air, water, and soil pollution) but, using composting treatment can reduce these problems and apply to agricultural soil as nitrogen fertilizer (Khan et al., 2009). Cattle manure compost (CMC) (mixture of dung and urine in the ratio of 3:1) is the daily dumped waste plant matters with undigested fecal residue of cow‘s. It is a good natural fertilizer because it contains a variety of essential nutrients for plant growth (generally, It contains crude fiber, crude protein, cellulose, hemicellulose and minerals such as N, K, S, traces of P, Fe, Co, Mg, P, Cl, Mn, etc. (Swain et al., 2009).

Cowdung is a mixture of dung and urine, generally in the ratio of 3:1. It contains crude fibre, crude protein, cellulose, hemicellulose and 24 types of minerals such as N, K, S, traces of P, Fe, Co, Mg, P, Cl, Mn, etc. (Nopparat*et al.*, 2007). It is normally used as an organic fertilizer for enhancing soil fertility, as a source of fuel, for dressing seeds, plastering cut ends of vegetatively propagated sugarcane, dressing plant wounds, sprinkling diluted suspension of cowdung on plant surface, etc. from ancient times. Besides, although the potential of cowdung in enhancing soil fertility is known to Indian sub-continental farmers for centuries (Nopparat*et al*2007), little is known whether cowdung microorganisms mediate nutrients cycling such as sulphur (S) oxidation and phosphorus (P) solubilization in soil.

Sulphur is one of the basic building blocks in microorganisms, plants and animals and hence it is considered to bevital for life. In plants, it is considered as the fourth essential nutrient next to N, P and K. However, its wide spread deficiency in soils and consequent losses on crop productivity have been reported during last three decades due to the continuous use of sulphur free fertilizers. The sulfur bacteria comprise a heterogeneous group of organisms which share the ability to oxidize reduced or partially oxidized inorganic sulfur compounds. *Thiobacilli*play an important role in sulphur oxidation in soil which improves soil fertility. It results in the formation of sulphate, which can be used by the plants, while the acidity produced by oxidation helps to solubilize plant nutrients and improves alkali soils (Hitsuda*et al.*, 2005). Thus, the current study was designed to

demonstrate the beneficial activities of cow dung microflora and to study the ability of microorganisms to oxidize sulphates, solubilize phosphate and reduce iron.

II. METHODS AND MATERIAL

1. Collection of Cattle Manure Compost

Cattle manure compost samples were collected from cattle farm dumping in different areas of Namakkal district during December 2014. Generally, the cow dung was dumped nearby the farms approximately 1 to 2 months of period. During that time, the cow dung gets transformed into compost (ready to apply in the crop fields); Samples were withdrawn from one-month-old compost, the 50cm depth of the pits and they were immediately transferred into sterilized polyethylene bags and were brought to the laboratory for processing. They were stored at room temperature until use.

2. Microbiological Analysis of Cattle Manure Compost

The microbial flora present in the cattle manure composts were enumerated by serial dilution and pour plating method. For serial dilution 1 gram of the compost was added in 10ml of the sterile distilled water and serially diluted upto 10^{-6} dilutions. Then, the dilutions were pour plated and were incubated at 37˚C for 24 hours. After incubation, the colonies were counted and expressed as colony forming units (cfu) and the results were tabulated.

3. Identification of bacterial isolates

The identification tests were carried out by standard laboratory procedures. The preliminary identification of the bacterial isolates was performed using Gram's staining, motility test, Catalase test and oxidase test. It was followed by biochemical tests such as IMViC tests, Nitrate test,TSI test, Urease test and by colony characterization.

4. Isolation of Sulphate Oxidizing bacteria (SOB) from Cattle Manure Compost (CMC)

The compost was composed of cattle feces, urine, and sawdust. For isolation of sulphur oxidizing bacteria, thiosulphate medium was employed by enriching with 5% of sodium thiosulphate. The thiosulphate medium contained 5.0 g $Na_2S_2O_3$, 0.1 g K_2HPO_4, 0.2 g $NaHCO_3$, 0.1 g NH_4Cl and 5.0 g of glucose in 1000 ml distilled water, with pH 8.0. Bromocresol purple was used as an indicator. The plates were inoculated with CMC and were incubated for 24 hours at 37°C. Later, the plates were examined for colour change due to sulphate oxidation which indicated the presence of SOB in the sample.

5. pH reduction test for SOB

The acquired isolates were inoculated in the thiosulphate broth with initial pH adjusted to 8.0. After 40 hours of incubation, the final pH of the growth media was measured. The isolates were screened based on their efficacy to reduce the pH from 8.0 to 4.5. The isolates capable of reducing the pH were selected and used for further studies.

6. Quantitative estimation of Sulphate oxidation

In order to evaluate the utililsation of sulphur by the isolates, turbidimetry method was followed. A quantity of 5ml of 24 hours old culture was taken and 0.15% $CaCl_2$ (25ml) was added and incubated at room temperature in a rotary shaker for 30 minutes. After incubation, the medium was filtered through Whatman filter paper and the sulphate content in the clear supernatant was estimated.

About 5ml of the extract was taken in a 25ml volumetric flask and sulphate in the supernatant solution was precipitated with barium chloride (BaCl2) salt as barium sulphate, which imparted turbidity. The light transmission through the turbid solution was measured at a wavelength of 440nm in a UV–Vis Spectrophotometer. A reagent blank was run consisting of the clear supernatant but without the addition of $BaCl_2$.

7. Phospahtesolubilization ability of SOB

One loopful of the 24 hours culture was inoculated onto the solid bromophenol containing Pikovaskaya'smedium(g/l): glucose, 10.00; $Ca(PO_4)_2$, 5.00; $(NH_3)_2SO_4$, 0.50; NaCl, 0.20; $MgSO_4$. $7H_2O$, 0.10; KCl, 0.20; yeastextract, 0.50; $MnSO_4$, 0.005; $FeSO_4$. $7H_2O$,0.005; bromophenol, 25ml; agar, 15.00; and pH adjusted to 7.0–7.2 and incubated for 96 hours at room temperature. After the incubation period,

yellow-coloured halo zones were formed around the individual bacterial colony in response to the pH drop produced by the release of organic acids by microorganisms, which were responsible for P-solubilization.

III. RESULTS AND DISCUSSION

Cow dung is traditionally used as organic fertilizer in Indian sub-continental farming for centuries. The addition of cow dung increases the mineral status of soil, enhances resistance of plant against pests and diseases; stimulate plant growth and other beneficial activities such as sulphur oxidation and phosphorous solubilization. Sulphur in plants, is considered as the fourth essential nutrient next to N, P and K. However, it's wide spread deficiency in soils and consequent losses on crop productivity have been reported during last three decades.Chemically processed fertilizers are costly and make it unaffordable for a large proportion of farmers involved in food production. Keeping this in mind, the present study was aimed in isolating and identifying the sulphate solubilizing bacteria from the cattle waste compost.

The study was conducted in Namakkal district and uniform collection procedure was followed. The cattle manure compost were collected and the total bacterial population were enumerated by serial dilution and plating methods. About 5 samplings were done and the results were shown in figure.1.

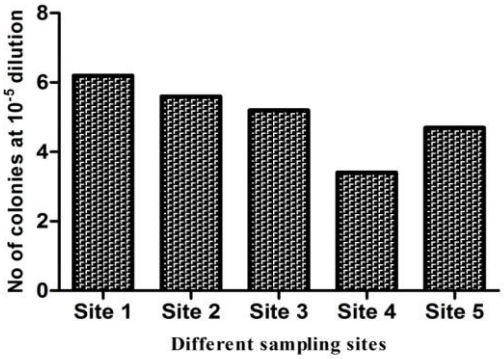

Figure1. Enumeration of bacterial population from Cattle manure compost

The above figure indicates the total bacterial populations isolated from the CMC. The bacterial populations were almost similar in about three sites and were found to decrease in site 4 alone. This may be due to the aging of the cowdung compost and similar results were noted bySwain *et al.,* 2009 in their studies conducted at Orissa.

Preliminary Identification of the isolates:

The bacterial isolates were considered for the preliminary tests such as gram's staining, motility, catalase and oxidase. The results of the bacterial isolates were tabulated in table-1.

Table-1: Preliminary identification tests

Isolate No	Gram's Staining	Motility	Catalase	Oxidase	Suspected Organism
Isolate 1	+ve	Motile	+ve	+ve	*Bacillus sp*
Isolate 2	-ve	Non Motile	+ve	-ve	*Klebsiella sp*
Isolate 3	-ve	Motile	+ve	+ve	*Pseudomonas sp*

The identified suspected organisms were identified by various biochemical tests for further confirmation.

Biochemical identification of the isolates:

Various biochemical characteristics of the isolated strains were shown in table.2. Based on the biochemical tests, the suspected isolates were identified according to the Bergey's Manual of Systemic Bacteriology.

Table-2: Biochemical identification tests

Name of the test	**Isolate - 1**	**Isolate -2**	**Isolate -3**
Indole test	-ve	-ve	-ve
Methyl red test	+ve	-ve	-ve
VogesProskauer test	-ve	+ve	-ve
Citrate utilization test	+ve	+ve	+ve
Nitrate reduction test	-ve	+ve	-ve
TSI agar	No reaction	A/Ak,G+	AK/AK
Organism	***Bacillus sp.***	***Klebsiella sp.***	***Pseudomonas sp.***

Morphological Characteristics of the Isolates

Selective medium were prepared, sterilized and inoculated with the respective organisms such as *Bacillus sp,Klebsiella sp,* and *Pseudomonas sp.* The inoculated plates were incubated at 37˚C for 24 hours. After incubation, the plates were examined for morphological characteristics. The results were tabulated in Table-3.

Table 3: Morphological Characteristics

S.no	Bacterial isolates	Selective medium	Morphological observation
1	*Bacillus sp.*	Blood agar	α hemolytic colonies
2	*Klesiella sp.*	Mac Conkey agar	Pink coloured, lactose fermenting colonies
3	*Pseudomonas sp.*	King's B medium	Colourless colonies , fluorescing under UV illumination

Isolation of Sulphate oxidizing bacteria (SOB) from Cattle Manure compost:

The identified isolates were detected for their ability to oxidize sulphates in the medium. They were inoculated in Thiosulphate medium supplemented with bromocresol purple indicator and incubated at 37˚C for 24 hours. After incubation, sulphate oxidizers were identified based on the yellow colour zone around the colonies indicating acidic pH. The indicator bromocresol purple turned yellow due to the oxidation of thiosulphate into sulphuric acid. Similar such identification was done by PeriyasamyRameshkumar*et al.,* 2014, in their studies done from cattle manure compost.

pH reduction tests for SOB

The three isolates were screened based on the pH reduction by inoculating them in the thiosulphate broth supplemented with bromocresol purple with an initial pH of 8.0. From the initial pH 8.0, the purple colour turned orange followed by yellow colourbuilded on the pH reduction to 2.5.The results were tabulated in table-4.

Table 4:pH reduction tests

S.No	Time (hrs)	pH	Colour of medium
1	0	8.0	Purple
2	20	4.9	Orange
3	30	3.7	Yellow
4	40	2.5	Pale yellow

In a study conducted at the TNAU, Coimbatore, Vidhyasri*et al.,* 2011, reported that a total of ninesulphur oxidizing bacterial isolates were obtained from different sources and they were screened by pH reduction tests. The isolates capable of changing colour due to sulphuric acid production were identified and used for further studies.

Quantitative estimation of sulphate oxidation of the isolates:

The quantitative estimation of sulphate was calculated using $BaCl_2$ precipitation. All the three isolates showed sulphate oxidization ability. 5ml of the 24 hours culture supernatant was mixed with $BaCl_2$ and if the sulphate is found in the supernatant, then it reacts with the $BaCl_2$ and forms $BaSO_4$. The amount of Barium sulphate formed was measured using turbidometry method by reading at 440 nm using UV-Vis spectrophotometer. The results are shown in figure.2.

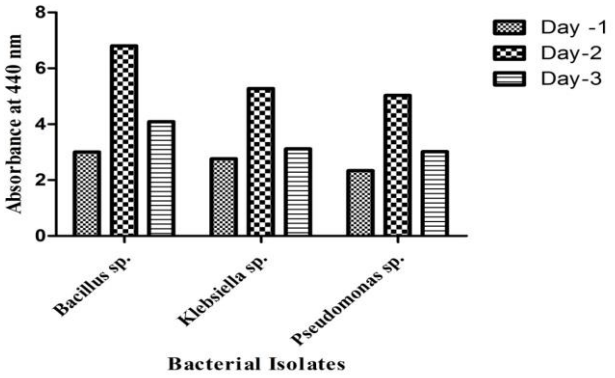

Figure.2: Estimation of sulphate oxidation using $BaCl_2$

From the above table it was identified that among the three isolates studied, all the isolates had the maxiumum absorbance on the second day and later the OD value started to reduce. Also, out of the three bacterial isolates studied, *Bacillus sp.* showed the maximum results and was found to be an active sulphate oxidizer. Similarly, Greene *et al.,* 2003 reported that sulphate production from thiosulphate over 20 days of aerobic incubation at 15˚C. Also, RajagopalVidhyalakshmi *et al.,* 2006, in their work reported that the sulphate oxidizers enhanced the sulphur availability of the soil and hence can be incorporated in the alkali soils. In another work done by Priyanka *et al.,* 2014 and Claudia *et al* 2008, isolated sulphate oxidizing bacteria and noted them as farmer friendly bioinioculants for enhancing the sulphur nutrition of the soil.

Phosphate solubilization ability of SOB:

The sulphate oxidizing bacteria were also tested for their ability to solubilizing phosphates using Pikovskaya's medium supplemented with bromophenol blue. The SOB were inoculated and incubated for 24 hours at room temperature. After incubation, the plates were examined for yellow coloured halo zones.All the three isolates were capable of phosphate oxidation.

IV. CONCLUSION

Thus, as sulphur is propably important plant nutrition and the deficiency of sulphur in the soil is a global problem. This problem could be overcome by application of cheap and reduced forms of sulphur such as elemental sulphur and thiosulphates along with these kind of sulphate oxidizing microorganisms as biofertilizers. As cowdung manure are used as fertilizers, the addition of such SOB capable phosphate solubilizing and iron reducing ability would be useful in enhancing soil vitality and fertility.

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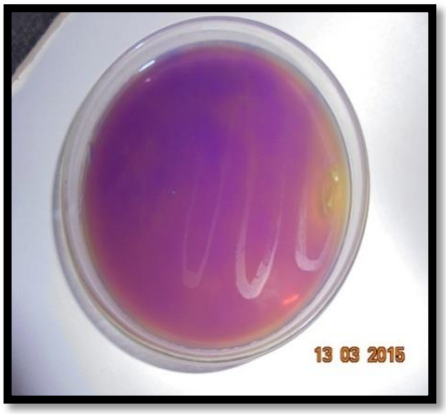
PLATE: 1

Thiosulphate Agar with Bromo Cresol Purple

Bacillus sp.

Klebsiella sp.

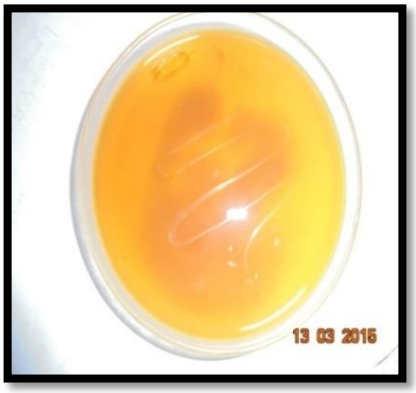
Pseudomonas sp.



Thiosulphate Agar with Bromo Phenol Blue

Bacillus sp.

Klebsiella sp.



Pseudomonas sp.