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### **RESEARCH ARTICLE**

#### **DEVELOPEMNT OF MICROBIAL DIAGNOSTIC KIT FOR QUALITATIVE DETECTION OF MICROBIAL POPULATION IN BIOMANURES**

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#### **Abstract**

To fulfil the demand of quality biomanures and composts containing vibrant beneficial microbes, the qualitative check for the presence of effective microflora in biomanures is very much significant commercially. For checking the microbial contents of the compost products on site easily, qualitatively and cost-effectively, a portable microbial diagnostic kit for qualitative detection of microbial populations in biomanures has been scientifically conceptualized, designed, tested, developed and commercialized for the use by rural industrialists and entrepreneurs especially producing and selling composts and biomanures etc. The kit can give appropriately precise results within 24hours, by which an entrepreneur can decide about the microbial quality of the compost. The details regarding this invention are discussed herewith.

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#### **Introduction:-**

Composts are produced and used by farmers for maintaining soil fertility and agricultural productivity in sustainable manner. Due to world-wide upsurge of interest in organically grown food, composts have gained broad acceptability for its use and therefore, it is finding a financial value in the local, regional markets due to its inherent importance in enriching soil organic carbon and amending soil with a beneficial micro-biota. The economic value to these products of composting has resulted into its trade and there exists a perfect producer-consumer relationship. This necessitated it to have well-documented and studied technologies of production, sampling, quality control and assurance, storage etc. All the methods in relation to above topics are there except easy methods for determination of microbial properties.

The laboratory procedures for the detection of different microbial populations in the biomanure are lengthy, time, space and labour consuming. It also needs sophisticated instruments and costly media. Therefore, it is necessary to have certain means of detection of atleast quality of biomanure from microbial point of view (Phirke *et al.*, 2004; Phirke, 2012). These efforts have been initiated for the development of microbial population testing kit.

The preliminary investigations in this regard, have revealed that microbes bear the capacity to reduce certain dyes through respiration. These dyes act as final hydrogen acceptor. There are several organic dyes available in market, which were tested. Among it, Bromocresol green, Malachite's green, safranine, methyl red, methylene blue were there for testing (Collins *et al.*, 1980). The readymade formulations were available in alcohol. This alcohol hindered

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the microbial activity and therefore, aqueous dilute solutions were used. The aqueous solution of methylene blue was found effective.

### **Materials and methods:-**

#### **Composite compost sampling:-**

To get good results it is very essential to collect uniform samples. Compost heap should be mixed thoroughly and 5-6 samples from each heap, should be collected in such a way that the total heap gets proper chance to be sampled (Tandon, 1993; Phirke, 2002; Phirke, 2012). The samples should be properly packed in polythene bag and labelled. Each sample should be dried (shade dry) grounded and sieved before test. The samples should be stored in dry place to avoid absorption for moisture. If compost contains moisture it continues to decompose over a period of time due to microbial activity and degrades the quality of compost. Therefore, it is essential to test the samples immediately after collection and store it in a dry condition. Minimum 10-20 g of compost is required for the tests. For determination of microbial count, always fresh compost sample, having minimum 10-12 % moisture should be used.

#### **Media Preparation:-**

Nutrient broth [with composition Beef extract 3g, Peptone 5g, Methylene blue (0.01 % w/v) 100ml, Distilled water 800 ml, pH 7.5 adjusted using 0.1N NaOH/ 0.1N HCl] amended with methylene blue was distributed in each vial in the quantity of 9ml, sealed and autoclaved at 15lb pressure, 121 °C for 15 m (Salle, 1954).

Potato dextrose medium [with composition: Boiled aqueous peeled off potato mash 1Kg was squeezed through clean cheese cloth to collect 200g of extract, 100 g dextrose added, pH adjusted to 5.5 and volume was finalized to 800ml. To it, 100 ml methylene blue (0.01 % w/v) solution was added] amended with methylene blue was distributed in each vial in the quantity of 9ml, sealed and autoclaved at 15lb pressure, 121 °C for 15 m.

Jensen's medium [with composition Sucrose 20g, K<sub>2</sub>HPO<sub>4</sub> 1g, MgSO<sub>4</sub> 0.05g, NaCl 0.5g, FeSO<sub>4</sub> 0.001g, Na<sub>2</sub>MoO<sub>4</sub> 0.005g, Ca<sub>3</sub>(CO<sub>3</sub>)<sub>2</sub> 2g, Distilled water 800ml, Methylene blue (0.01 % w/v) 100ml, pH 7.5] amended with methylene blue was distributed in each vial in the quantity of 9ml, sealed and autoclaved at 15lb pressure, 121 °C for 15 m (Phirke, 2002).

Compost decoction has to be obtained by suspending approximately 1g fresh compost composite sample in 10ml distilled water, vigorously shaking it to suspend all the microbes in water. And it was allowed to stand for a while to allow settling of coarse particles at the bottom, while microbes appeared in Brownian motion in the suspension. The control was also kept without inoculation of any microbial inoculum.

Graduated syringes of 2ml capacity are provided with the kit for sucking exactly 1ml of compost decoction with the help of needle. Using this needle, the sealed vial has to be pierced and exactly 1ml compost decoction has to be introduced into the vial. Along with this volume of compost decoction, all the microorganisms present are introduced in the medium, which provides nutrients for their growth. As per the selectivity of microbial medium, the respective microbial populations are selected, utilize the nutrients from the medium and get dominated to increase their number. This results into higher rate of metabolism, altering the colour through reduction of the dye.

Therefore, the sterilized vials (volume 30ml) containing 9ml liquid nutrient medium, amended with aqueous solution of non-toxic, easily reducible organic dye in appropriate quantity to impart the colour to medium has to be inoculated with 1 ml quantity of compost aqueous decoction.

The inoculated vials were kept for observation at room temperature. The colour was observed for twelve to twenty four hours and the time of discoloration of medium was noted every after half hour's interval. The microbes grow in the medium, respire and reduce the dye by changing its colour to original colour of the medium. This depends on the quantity of microbes present in the biomanure. More the microbes; earlier the colour will fade. Lower the microbes are present in compost; late the colour will fade. From the time required, it could be observed that what level of microbial group was present in the compost.

This process can be repeated to test 50 samples from one kit. Similar sterilized vials can be replaced to test more samples.

### Results and Discussion:-

The time required for fading of colour of the dye in the inoculated medium with the corresponding level of compost-microbe-inoculum was optimized and the whole system was made in the ready-made form. Such sterile vials were designed and made available through the kit on the site where compost operators could just prepare the compost decoction and introduce it in the vial through syringe and wait for the colour change.

From the time required, it can be observed that the tested compost contains very high population, high level or moderate level or low-level population. From the time chart, it would be able to detect the levels of microbes present in a particular sample.

By using the selective media for the growth of desired organism, the presence and level of microbial population in the compost could be checked. Such as for the presence of bacterial population, fungal population, *Azotobacter* and *Rhizobium* population can be anticipated in given sample through kit to prove good for testing on site qualitatively. This microbial kit can also be added to the Biomanure diagnostic kit.

#### Observations of the nutrient broth amended with methylene blue:

The diluted compost decoction was inoculated at 10 % v/ v level in the nutrient broth tubes with methylene blue and incubated at room temperature for 24 hours. The  $10^{-1}$  level showed speedily colour change within six hours, while rest took atleast 8 to 24 hours to get decolorize. This again depended upon the level of dilution. As the dilution level increased, overall time required for reduction of methylene blue also increased. But all the tubes were decolorized completely in 24 hours. The pH of the medium was kept 7.2 to selectively allow the growth of bacteria maximally. After 24 hours (Table 1.), one can observe the turbidity in the test tube. From these results, one can develop the qualitative broad ranges for determining the bacterial loads of the composts.

**Table 1:-** Observations of vials filled with the nutrient broth amended with methylene blue.

Time (h) taken to get decolorized	Approximate bacterial population (cfug <sup>-1</sup> )	Bacterial load of compost
6 – 8 hours	$> 10^9$	Very high
8 – 12 hours	$10^7 - 10^9$	High
12 – 16 hours	$10^5 - 10^7$	Moderate
16 – 20 hours	$10^3 - 10^5$	Low
20 – 24 hours	$10^1 - 10^3$	Very low

#### Observations of the potato dextrose broth added with methylene blue:

The diluted compost decoction was inoculated at 10 % v/ v level in the PDB tubes with methylene blue and incubated at room temperature for 24 hours. The  $10^{-1}$  level showed speedily colour change within six hours, while rest took atleast 8 to 24 hours to get decolorize. This again depended upon the level of dilution. As the dilution level increase, overall time required for reduction of methylene blue also increased. But all the tubes were decolorized completely in 24 hours. The pH of the medium was kept 5.5 to selectively allow the growth of fungi only. After 24 hours (Table 2.), one can observe the surface mycelial mat in the test tube. From these results, one can develop the qualitative broad ranges for determining the fungal load of the composts.

**Table 2:-** Observations of vials filled with potato dextrose broth added with methylene blue.

Time (h) taken to get decolorized	Approximate fungal population (cfug <sup>-1</sup> )	Fungal load of compost
6 – 8 hours	$> 10^5$	Very high
8 – 12 hours	$10^4 - 10^5$	High
12 – 16 hours	$10^3 - 10^4$	Moderate
16 – 20 hours	$10^2 - 10^3$	Low
20 – 24 hours	$10^2 - 10^1$	Very low

#### Observations of the Jensen's broth amended with methylene blue:-

The diluted compost decoction was inoculated at 10 % v/ v level in the nutrient broth tubes with methylene blue and incubated at room temperature for 24 hours. The  $10^{-1}$  level showed speedily colour change within six hours, while rest took atleast 8 to 24 hours to get decolorize. This again depended upon the level of dilution. As the dilution level increased, overall time required for reduction of methylene blue also increased. But all the tubes were decolorized

completely in 24 hours. The pH of this nitrogen free medium was kept 7.2 to selectively allow the growth of *Azotobacter* only. After 24 hours (Table 3.), one can observe the turbidity in the test tube. From these results, one can develop the qualitative broad ranges for determining the bacterial loads of the composts.

**Table 3:-** Observations of vials filled with Jensen's broth amended with methylene blue.

Time (h) taken to get decolorized	Approximate <i>Azotobacter</i> population (cfug <sup>-1</sup> )	<i>Azotobacter</i> load of compost
6 – 8 hours	$> 10^7$	Very high
8 – 12 hours	$10^6 - 10^7$	High
12 – 16 hours	$10^5 - 10^6$	Moderate
16 – 20 hours	$10^4 - 10^5$	Low
20 – 24 hours	$10^3 - 10^4$	Very low

The determination of corresponding approximation in each microbial population with the time (h) taken to get decolourised and have turbidity in each vial have been established between the colony forming units per gm of compost samples (cfug<sup>-1</sup>) using standard spread and pour plate technique inoculation in respective solid culture media (Collins *et al.*, 1980; Phirke, 2002).

### Conclusions:-

This kit (Photo 1.) would be a portable and easy for carrying anywhere in the field. This is anticipated to serve beneficial to rural entrepreneurs with a limited to nil facilities of compost testing. This kit would be a cost-effective and qualitatively precise. The kit is under trial and needs to be testified at sites by real end users and their suggestions in this feedback would be able to upgrade its applicability.

**Photo 1:-** Microbial diagnostic kit for qualitative detection of microbial populations in a biomanures.



### Recommendations:-

One drawback in using methylene blue in the system was found that the reduction of methylene blue was found reversible. If the tubes are shaken vigorously, the reduced and hyaline methylene blue turns blue again imparting its colour to the medium. Accidental shaking may hinder the observations, if such oxidation occurred. Hence, it is found necessary to search an alternative to this dye. The resazurin will be checked instead of methylene blue.

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