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FORMULATION AND DEVELOPMENT OF BIOFUNGICIDE

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ABSTRACT: *Biofungicide are biologically pest (fungi) control agent and one of the solutions for sustaining agricultural output and environmental quality. In order to implement these environmental friendly biofungicide on disease causing fungi of crop plant effectively, it can be important to play attention to way of formulation and application. Biofungicide have many advantages over chemical fungicide like it is biodegradable, cheaper and harmful residues are not detected. In present study, two pathogenic fungi Fusarium oxysporum and Alternaria species were isolated from carnation(Dianthus caryophyllus) plant and anthurium leaves respectively. Bacillus bacterial strain was used to ascertain affectivity against these fungi Fusarium oxysporum and Alternaria species for antifungal activity. Both fungi growth were inhibited by this bacterial strain at different incubation period. Maximum inhibition percentage was found in Alternaria sp.than Fusarium oxysporum. Also it was made to evaluate the population dynamics of spawn based bacterial strain in different organic substrates so as to determine the shelf life of substrate based bioformulation of bacteria. There was a gradual decline in population in Bacillus bacteria in different carriers. After 90 days of storage maximum population of bacteria was observed in soil. Compost ranked 2nd talcum powder ranked 3rd but in case ash there was no growth after one month.*

KEYWORDS: Biofungicide,Bioformulation, Antifungal activity, Carriers, *Fusarium oxysporum* and *Alternaria*.sp.

INTRODUCTION

Biofuncide means fungicides of biological origin.It may be microorganism such as bacteria,fungi and animal or plant based product like secondary metabolite.Indian economy is dependent upon agriculture and agriculture has major problems of fungal diseases.Fungi can cause serious damage in agriculture, resulting in critical losses of yield, quality and profit. . Fortunately, agriculture has made tremendous progress during the last century, and part of this progress has been the development of modern means of plant disease control. In particular, the introduction of chemical disease control agents has contributed to a substantial increase in crop production, to a smoothing of annual undulations in crop yields, and, ultimately, to today's high level of food security. Agrochemicals control diseases of crops. But the constant and regular use of chemicals has resulted in detrimental effects to the environment and human health (Mendgen, K and Schiewe, *et.al*.,1992.) 0.1% of agrochemicals used crop protection reach the target pest leaving the remaining 99.5% to enter the environment to cause hazards to non-target organisms including

human. Broad spectrum activity of agrochemicals has increase the risk of residue toxicity, prices and pathogen resistances (DekaBoruah, HP *et al* , 2002).

This has urged agriculturists to look for available alternatives. Use of naturally–occurring beneficial microorganism may be safe and alternative approach to compensate multiple effects of fertilizers, pesticides. In nature microorganism grow in various associations ranging from antibiosis, commensalism, parasitism, symbiosis. This association can be exploited in biological control of plant pathogen. Microbes that are either antagonistic to a pathogen itself are used for biological control.Some microorganisms have biocontrol potential. A few bacteria belong to genus *Bacillus* and *Pseudomonas* have been to be found as bio-control agents primarily due to their ability to produce antimicrobial metabolites (Li WJ *et al*,2007).Biocontrol bacteria suppressed the different fungal growth by the producing secondary metabolites like antibiotic, cell wall degrading enzyme and hydrogen cyanide (Bakker, *et al*,1987). The present study involed isolation of potential microbe and evaluated antifungal activity against two major pathogens *Fusarium oxysporum* and *Alternaria sp*.are a major pathogenic fungi cause's wilt disease, blight disease in many agricultural and horticultural crops. So we used in vitro testing against two major disease causing pathogen.Currently, there are two formulated fungal isolates active against powdery mildews which are available commercially. AQ10 Biofungicide, developed by Ecogen, Inc, USA, contains conidia of an Ampelomyces isolate formulated as water-dispersible granules (Paulitz, *et al*, 2001). Biological management of soil borne pathogens has been reported successfully reported in many crops (BenuzzI, *et al* ,2000).AQ 10 is the first biofungicide based on the spores of the hyperparasitic fungus *Ampelomycesquisqualis* registered in Italy and Europe for control of powdery mildew (Uncinulanecator) on grapes. AQ 10 is formulated as Water Dispersible Granules and can be sprayed by conventional spray equipment.(Mathivanan, *et. al*.2000) A strain of *Trichoderma viride* with high antagonistic potential against *Rhizoctoniasolani* and *Sclerotiumrolfsii* [*Corticiumrolfsii*] in dual culture was isolated from soil (kong,et al 1999) . The inhibitory action of *B. subtilis* strain B-903 on plant pathogenic fungi is reported. The antibiotic substance in B-903 culture filtrate was inhibitory to >10 species of pathogenic fungi, such as *Fusarium oxysporum* and *Physalosporapiricola* (Chiou ,*et al*,2001).Of the 700 microorganisms isolated from lily plants and screened by dual and concomitant cultures, 10 isolates (B99, B111, B128, B131, B171, B190, B196, B203, B501 and BS) had antagonistic effects against *Botrytis elliptica* on three lily cultivars (Acapulco, Casa Blanca and Marco Polo) in greenhouse trials in 1998. There are a few 'fungicides' (more strictly termed disease control agents) in agricultural use that do not affect the viability, growth or reproduction of the target pathogen directly. Tricyclazole and pyroquilon, used to control rice blast disease, specifically affect the penetration of the pathogen (Magnaporthe grisea) into the host plant through inhibition of reductase steps in melanin biosynthesis needed for the normal function of appressoria. So far, no resistance problems have arisen with these melanin biosynthesis inhibitor-reductase (MBI-R) fungicides, but there is no obvious reason why not; resistance to carpropamid (melanin biosynthesis inhibitor–dehydratase or MBI-D fungicide), occurred soon after its introduction into Japan (Kaku *et al*., 2003). Probenazole, which also is used against rice blast, acts primarily on the plant, and is known to induce a set of defence reactions known as systemic acquired resistance (SAR). Advantages of biofungicide have biodegradable in nature, no effect on non-target species, cheaper than agrochemicals, less toxic and less time to develop. Present study was also made to evaluate the population dynamics of

spawn based bacterial strain in different in different organic substrates so as to determine the shelf life of substrate based bioformulation of bacteria.

Our objective is to find out suitable organism for formulation of biofungicide and find out suitable carrier for formulation of biofungicide.

MATERIALS AND METHODS

Isolation of potential bacteria and maintenance

The spoiled spawn sample was obtained from the mushroom spawn and compost production centres R.A.U Pusa.Spoiled mushroom sample was serially diluted up to 10^{-4} dilution factor and spread plated on full strength nutrient agar .It was incubated at 28-30^{0}c for 48 hrs.At the end of incubation period distinct colony morphology was recorded on incubation plate. The isolated colonies were purified by streaking on nutrient agar.

Isolation of pathogenic fungi

The diseased leaves spot were cut into small pieces. It was surface sterilized with 0.1% $HgCl_2$ solution for 1 minute.It was washed in three change of sterilized water to remove the disinfectant. Then it was transferred into PDA slant with the help of sterilized inoculating needle incubated for a week at room temperature (28-30^{0}c).After 4 to 5days of mycelia growth was observed, hyphal tip was transferred to PDA slant to obtain pure culture. The regular transfer of hyphal tips and examination gave the pure culture of *Alternaria sp*. and *Fusarium oxysporum respectively*.

Dual culture method for screening of antagonism

The interaction between pathogenic or non-pathogenic fungi and bacteria were done by dual culture method (Dennis and Webster, 1971) on PDA media. The bacteria were screened for in vitro antagonism against two fungal pathogen of carnation plant and anthurium leaves. In this technique a 4 mm mycelia disc of the fungus taken advancing zone of growing hyphe was inoculated at the centre of a 9cm diameter petriplates containing PDA medium. The bacterial isolate was streaked at a distance of 2-3 cm either in semi-circular or in a circular pattern.The culture plates were incubated at 28±2^{0}C and zone of inhibition was checked after 24 hrs, 48 hrs, 72 hrs and 96 hrs.

Lactophenol cotton blue staining Technique

The lactophenol cotton blue (LPCB) wet mount preparation is most widely used method of staining and observing fungi and is simple to prepare. The preparation has three components: phenol, which will kill any live organisms; lactic acid which preserves fungal structures, and cotton blue which stains the chitin in the fungal cell walls.

Gram Staining Technique

This test used a differential stain called gram stain. Gram staining is method used for differentiating between gram positive and gram negative bacteria.

Screening of suitable carrier for formulation

The bacteria were formulated with the soil, telcom powder, ash and spent compost. All the four carriers were autoclaved (121^0C, 15psi) for one hour twice. In the above carriers 100ml/kg bacterial broth was added separately. Bacterial broth was prepared as, samples were serially diluted in Sterile distilled waterupto10^{-4}dilution factor and each dilution were used for spread on nutrient agar. After incubation at 96 hrs at 30^0C, 100 colonies were picked from each nutrient agar plates. 100 colonies were transferred to nutrient broth and transferred separately to each carrier. Colony forming unit (CFU) of the bacteria was counted serve as the survival of biocides in different substrate. The bags were stored at room temperature after incubation for mass multiplication. One gram sample was drawn at 7days, 30days, 60days and 90days and colony forming unit was estimated by serial dilution technique by Waksman, 1922.

$$CFU\ of\ Sample = \frac{Average\ colonies\ X\ Dilution\ Factor}{Sample\ size}$$

Percentage inhibition of test pathogen

When pathogen achieved full growth, percentage inhibition of the test pathogens was calculated by the following formula.

$$Percentage\ inhibition\ of\ the\ radial\ growth = \frac{\{Growth\ in\ control\ (mm) - Growth\ in\ treatment\ (mm)\}\ X\ 100}{Growth\ in\ control\ (mm)}$$

RESULTS AND DISCUSSION

In the present study the potential bacteria was isolated on NA Media and identified as gram negative rod shaped bacteria(Bacillus). The detailed morphological, biochemical characterization,screening and evaluation of antifungal activity, formulation is described below.

Table.3.1 Morphological characteristics of bacterial isolate.

SI.NO.	Parameters	Characteristics
1	Colony characteristics	Hard crust , opaque, off white, irregular margin initially but after two days it became pigmented.
2	Cell features	Gram –ve short rods, scattered arrangement of cells.

Table3.2 3.2.1 Morphological Characteristics of Fungal Isolates

SI.NO.	Parameters	Characteristics
1	Colony Characteristics	Cottony growth, dark brown to black
2	Cell features	Transverse and longitudinal septa

Table.3.2.2

SI.NO.	Parameters	Characteristics
1	Colony characteristics	Cottony growth,initally white after some days appeared as grayish.
2	Cell features	Sickled shaped macroconidia, septed 40-50 X 4.5 micron in size.

On the basis of above morphological characteristics fungus was identified as *Fusarium oxysporium* and *Alternaria species.*

3.3Biochemical properties of bacterial isolate

SI NO	BIOCHEMICAL TEST	RESULTS	OBSERVATIONS
1	Oxidase	Positive	Purple to Black
2	Catalase	Negative	$Noo_{2\ bubble}$
3	Citrate	Positive	Blue colour
4	MR test	Positive	Red colour
5	VP test	Negative	No pink colour
6	TSI agar	Positive	Yellow colour
7	Nitrate reduction	Negative	No red layer
8	Amylase	Negative	No clear zone
9	Casein	Negative	No clear zone
10	Gelatin	Negative	No clear zone
11	Indole	Negative	No red layer

Table.3.4 .1 Screening and evaluation of antifungal activity

organism	24 hr (RD)	48(RD)	72(RD)
Control *fusarium*	12.2mm	20mm	30mm
Control *Bacillus* bacteria	15mm	30.2	70.4 mm
Fusarium ×bacillus	5mm	12 mm	20 mm
Inhibition percentage	60	40	29.3

Table.3.4.1.1

organism	24 hr(RD)	48(RD)	72(RD)
Control *Alternaria sp*	10.5mm	25mm	40mm
Control *Bacillus*	15mm	30.2mm	70.4mm
Alternaria ×Bacillus	2mm	2mm	2mm
Inhibition percentage	90	92	95

On the basis 0f inhibition % it proved that rod shaped bacteria (bacillus)Inhibited more in case of *Alternaria sp.* than *FusariumOxysporoum.*

Percentage inhibition = $\left[\frac{\text{Growth in control (mm) - growth in treatment (mm)} \times 100}{\text{Growth in control (mm)}}\right]$

*Bacillus- rod shaped

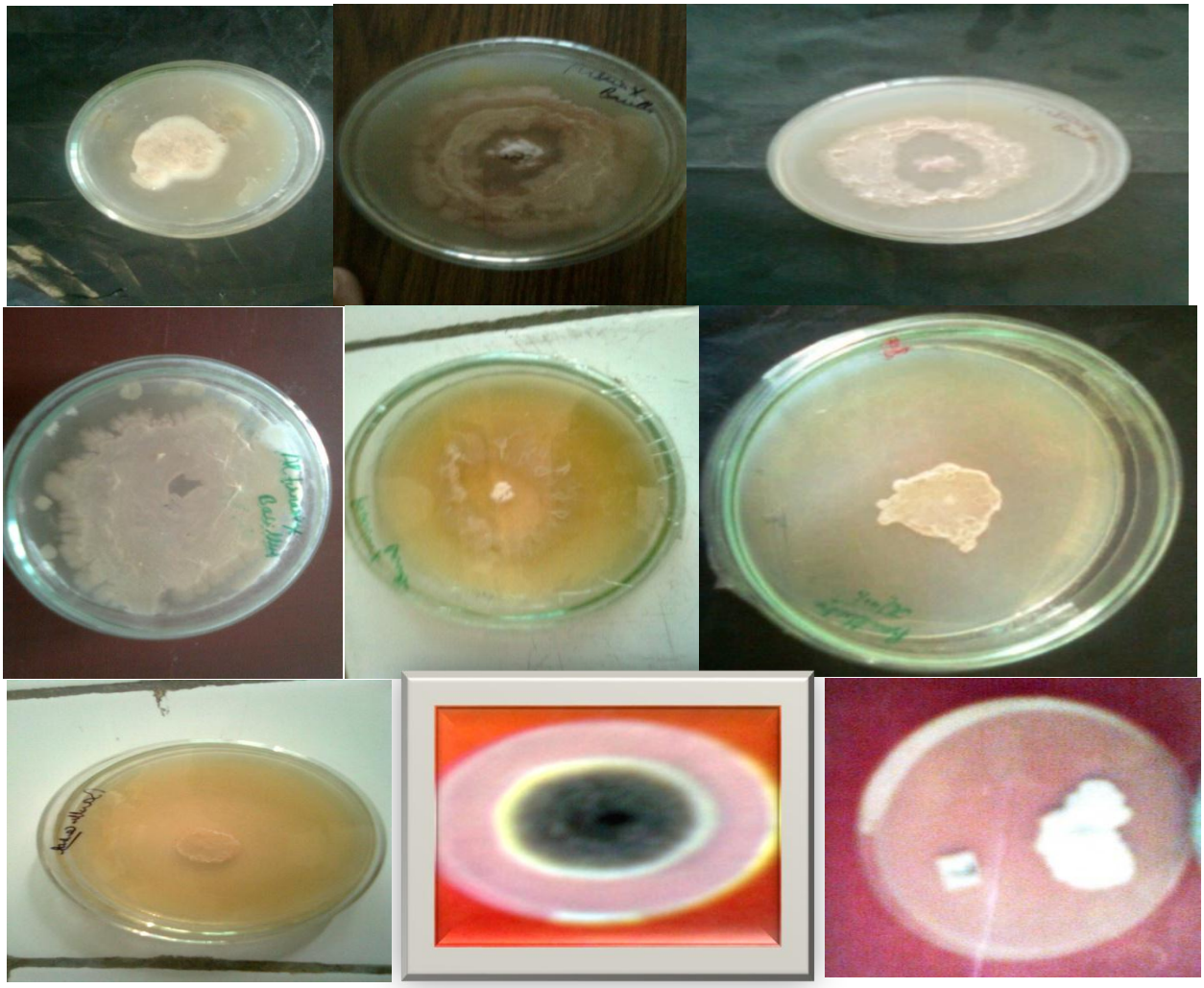

Fig 1. Showing *Fusarium* × *Bacillus* , *Alternari*× *Bacillus and fusarium* ,*Alternaria,Bacillus* control at different incubation period

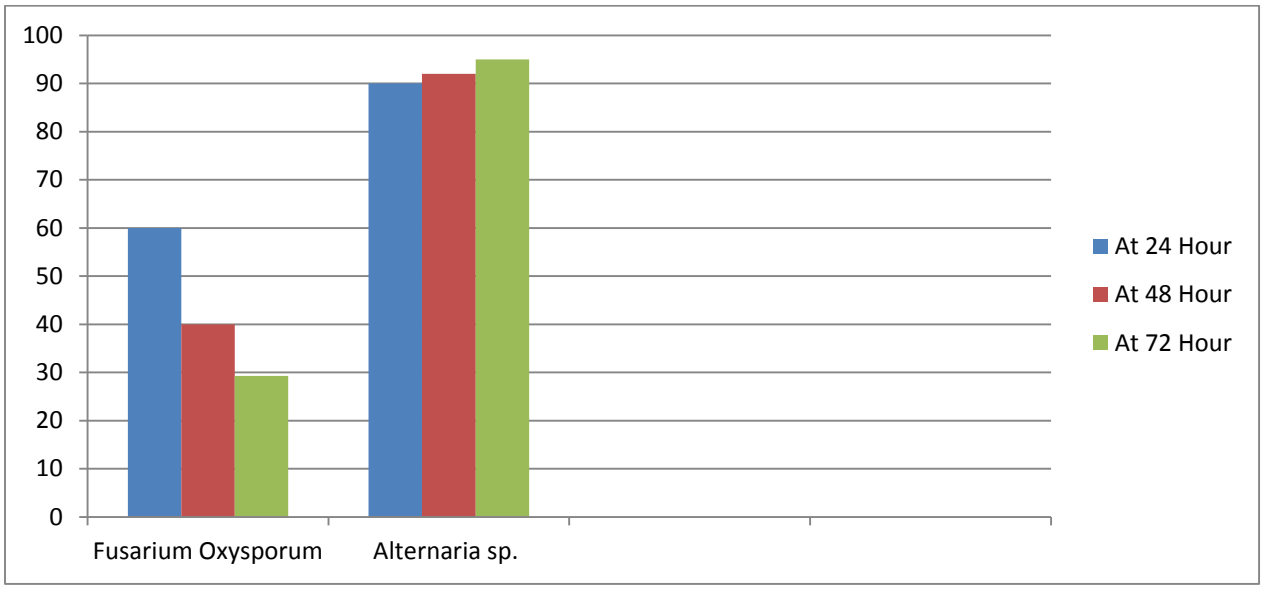

Fig.2. **Inhibition % of fungi at different incubation period**

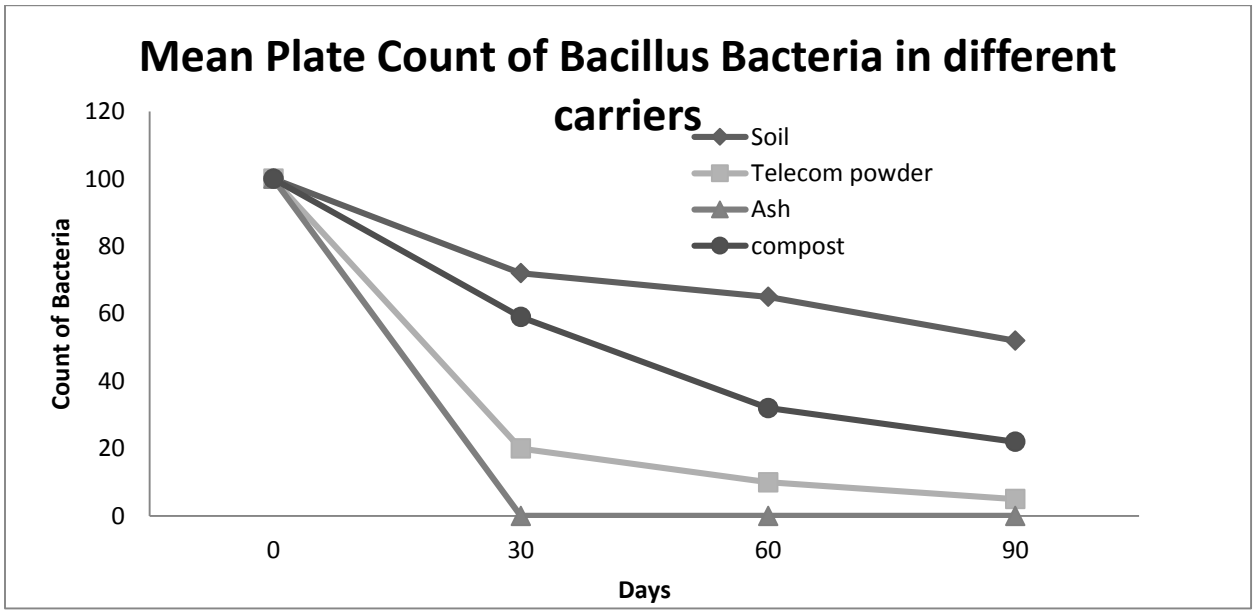

Fig 3.showing mean plate count of bacteria in different carriers.

it is apparent from graph ,the population of antagonist in formulation of three substrate decreased up to 90 days of storage gradually. Initial mean count of bacteria in soil, compost,telcom powder, ash 100. After 90 days of storage population was 52 in soil, 22 in compost, $5x10^{-5}$ in telcom powder , o in ash respectively.

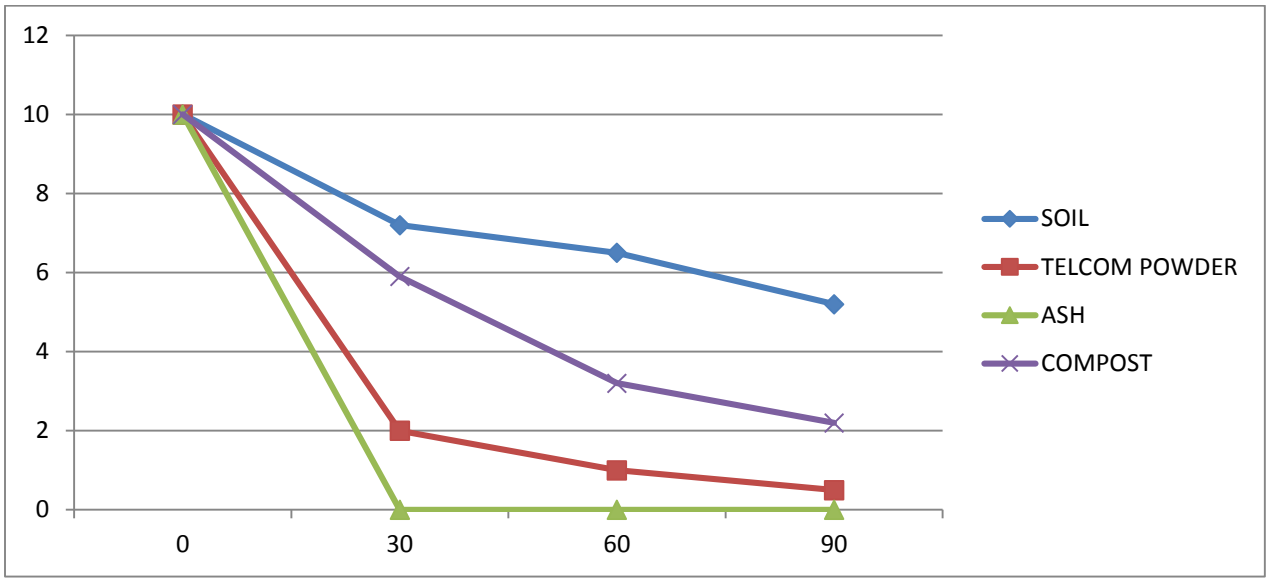

Fig.3 c.f.u value of bacreia in different carriers

On the basis of the above graph it concluded that there was a gradual decline population of bacillus bacteria in different carrier. After 90 days of storage maximum population of bacteria was observed in soil. Compost ranked second, Telcom powder ranked third but incase of ash there was no growth at month.

The results of analysis of morphological characteristics suggest that bacterial isolate colonies were whitish opaques,hard irregular margin after some days it became pigmented ,small rod shaped, scattered arrangement of cell. For fungal species identification, slide culture technique was followed(1950) and isolate were identified on the basis of morphological characteristics as *Fusarium* and *Alternaria sp*. Bacterial isolates were oxidase positive, citrate positive, MR positive, utilizing all types of sugar . Nallathanbi and thakore(2002) reported that P. Fluorescens CIAH 196.Inhibited 70 percentage growth of *A. alternata* which causes fruit rot in ber. it was found that *Bacillus* bacteria inhibited *Alternaria* and *Fusarium oxysporum* respecteviely.Inhibitory effect of bacterial isolate is due to antibiotic, sidophere and other such as cyanide (loper and byer 1991) . Inhibitory action of *B. Subtilis* has been attributed to its ability to produce antibiotic like bulliformin(*Vasudva et al* 1958). The population of antagonist(c.f.u) in formulation of three substrate decreased up to 90 days of storage gradually.initial population was $10x10^5$ gm/ml(c.f.u) in soil, compost,telcom powder, ash, respectively. After 90 days of storage population was $5.2x10^{-5}$ gm /ml in soil, $2.2x10^{-5}$ gm/ml in compost, $0.5x10^{-5}$gm/ml in telcom powder , o gm/ml in ash respectively. Multiplication Of bacteria was maximum in soil based formulation, followed by compost, telcom powder, ash.Soil has the rich source of nutrients.So maximum population was observed in soil. Compost has also the rich source of nutrient. Soil born microrganism multiplies in soil (literature).

CONCLUSION

Screening of antifungal activity proved that *Bacillus* bacteria (gram negative rod shape bacteria) inhibited *Fusarium oxysporum* and *Alternaria species* respectively. Maximum inhibition % was found in *Alternaria* than *Fusarium oxysporum*.Mass multiplication for bacteria was found maximum in soil based carrier. On the basis of c.f.u. value it was found that soil was the best carrier after 90 days of storage at room temperature. Compost ranked second position in terms of c.f.u value for multiplication of bacteria. Ash had least number of bacteria.

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