EVALUATION OF DIFFERENT CARRIERS AND PHYSICAL FACTORS FOR THE GROWTH OF *Pseudomonas fluorescens*

THESIS

Submitted to
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola
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MASTER OF SCIENCE

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(PLANT PATHOLOGY)

BY

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DECLARATION OF STUDENT

I hereby declare that, the experimental work and its interpretation of the thesis entitled **"EVALUATION OF DIFFERENT CARRIERS AND PHYSICAL FACTORS FOR THE GROWTH OF *Pseudomonas fluorescens*"** or part there of has neither been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis / publication of any University or scientific organization. The source of materials used and all assistance received during the course of investigation have been duly acknowledged.

Place : Akola.

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Date : 31 / 05/2013

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CERTIFICATE

This is to certify that, the thesis entitled **"EVALUATION OF DIFFERENT CARRIERS AND PHYSICAL FACTORS FOR THE GROWTH OF *Pseudomonas fluorescens* "** submitted in partial fulfilment of the requirement for the degree of "**Master of Science in Agriculture (Plant Pathology)**" of Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola is a record of bonafide research work carried out by **Chaithanya B.H** under my guidance and supervision.

The subject of thesis has been approved by the Student's Advisory Committee.

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Date : 31/05/2013

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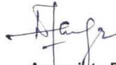

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chaithanya

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D) ABBREVIATIONS

%	–	Per cent
/	–	Per
^{0}C	–	Degree celcius
C.D.	–	Critical difference
Cfu	–	Colony forming unit
DAI	–	Days after incubation
e.g.	–	Exampli gratia (For example)
et. al.	–	Et alia (and others)
etc.	–	Et cetra
EC	_	Eelectrical conductivity
Fig.	–	Figure
g	–	Gram
h	–	Hours
HCl	–	Hydrochloric acid
HCN	–	Hydrocynic acid
i.e.	–	That is
J.	–	Journal
KB	–	King's B
Kg	_	Kiligram
M	–	Molar
ml	–	milliliter

mm	–	millimeter
mM	–	Mill molar
Ppm	_	Parts per million
pH	_	- log H^+
Pf	_	*Pseudomonas fluorescens*
SE (m) $\pm$	–	Standard error of mean
Viz	_	Videlicet (Namely)
μ	–	Micron
μg	_	Microgram
μl	–	micro liter
Viz;	_	Videlicet (namely)

(E) **Thesis Abstract**

a)	**Title of the thesis**	:	**"EVALUATION OF DIFFERENT CARRIERS AND PHYSICAL FACTORS FOR THE GROWTH OF *Pseudomonas fluorescens* ."**
b)	**Full name of student**	:	**Chaithanya B. H**
c)	**Name and address of Major Advisor**	:	**Dr. R.M. Gade** Professor, Department of Plant Pathology Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola - 444 104 (M.S.)
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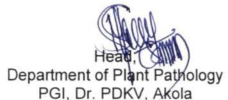

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ABSTRACT

Pseudomonas fluorescens is potential growth promoters and antagonist against soil borne pathogens. It dominate in the

rhizosphere and possess several properties that have made them as biocontrol agent of choice. The mechanisms by which these bacteria affect the plants involve the production of diverse metabolites including siderophore, hydrocynic acid (HCN), phytohormones and the other associated activities which include phosphate solubilization, iron competition in soil and root colonization resulting in plant growth promotion.

Research findings have established that Fluorescent pseudomonads as an acceptable green approach for the management of plant diseases beyond doubt. But they cannot be used as cell suspension under field conditions as it is done in green house or in research plots with a limited area. Hence, the cell suspensions of these Fluorescent pseudomonads should be immobilized in certain carriers and prepared as formulations for easy application, storage, commercialization and field use. Also the carrier should be such that it should support considerable bacterial population for a longer period of time and cost effective.

P. fluorescens isolate was collected from Department of Plant Pathology Dr. PDKV., Akola. and was conformed on the basis of morphological and biochemical tests. The bacterial cells were Gram negative and rod shaped. It showed positive for Starch hydrolysis, gelatin liquefication, catalase, KOH, citrate utilization, siderophore production and IAA production tests.

Physico-chemical properties of carriers were analysed and maximum water holding capacity, low bulkdensity values recorded with talc (189%, 0.3 g/cm^3) followed by spent mushroom substrate(93%, 0.35 g/cm^3).

In viability test it was observed that talc based carrier formulations supports better viable count of 18.33×10^8 cfu/g than other carrier materials at 180days of storage. However spent mushroom

substrate under test and has mean population value of 12.33×10^8 cfu/g at the end of 180 days.

The survival of bacterium in all carrier materials was found better at 0^0c temperature than the other temperature ranges.There was significant performance of carriers under different temperature ranges. 91×10^8 cfu/g viable count was observed in talc carrier at 25^0c after 15 days of storage.

Highest mean viable count was observed in all carrier material at 16hrs light+ 8hrs dark than any other light regimes.

CHAPTER I

INTRODUCTION

1.1 Background Information

The genus *Pseudomonas* was first named by migula in 1894. *Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas chlororaphis, Pseudomonas corrugate* and *Pseudomonas straita* were identified as important organisms with the ability of plant growth promotion and effective disease management (Nakkeran *et al.,* 2005; Nakkeran *et al.*, 2006; Chen *et al.*, 2000 and Mugilan *et al.*, 2011). Several strains of *Pseudomonas* were demonstrated to protect plants against many fungal, bacterial and viral diseases (Chen *et al.*, 2000; Ramamoorthy *et al.*, 2001; Saravanakumar *et al.*, 2009).

The word *Pseudomonas* means false unit being derived from Greek word pseudo-false, monas- single unit. The name *fluorescens* refers to the microbes secretion of a soluble fluorescent pigment called pyoverdin (formely called fluorescein),which is a type of siderophore. (Cox and Adams,1985).It belongs to the kingdom Bacteria, Phylum Proteobacteria, Class Gamma proteobacteria, Order Pseudomonadales and family Pseudomonadaceae.

P.fluorescens is an obligate aerobe, but certain strains are capable of using nitrate instead of oxygen as a final electron acceptor during cellular respiration. *P.fluorescens* has multiple flagella at one end, gram negative, straight or curved rod shaped with size 1.50-1.49 to 0.51-0.03. It has an ability to grow at 4°C and hydrolyse gelatin. Optimum temperature requirement for growth is 25-30°C.It produces circular, tabular colonies with greenish center on gelatin media.

The biocontrol mechanism of *P. fluorescens* is mainly antibiosis that able produce a suit of antibiotics including compounds

such as 2,4 diacetylphioroglucinol (DAPG), phenazine, pyrrolnitrine, pyoleuteorin and biosurfactant antibiotics (Angayarkanni *et al.,* 2005). *P.fluorescens* has been successfully used for biological control of several plant pathogens because it has twin adavantage of faster multiplication and higher rhizosphere competence (Ramamoorthy *et al.,* 2002). Its applicability as bioagent has drawn wide attention because of production of secondary metabolites such as siderophore, antibiotics, volatile compounds, HCN, enzymes and phytohormones (Nagarajkumar *et al.,* 2004).

Several reports have shown the potential of *Pseudomonas* species as biocontrol agents for controlling plant and fruit diseases (Whipps *et al.,* 2001; Okubara *et al.,* 2004; Jayaraj *et al.,* 2005; Trivedi *et al.*, 2008).

It is important that the biogent needs to be incorporated in a suitable carrier medium for its storage in a viable state and for field application at a later stage. Materials selected as carrier media are sometimes inert materials such as the talc powder, vermiculite, perlite, sand etc or agricultural wastes or by products.

Carrier is medium matrix on which the inoculated micro-organism grows at reasonably higher population for an initial period and thereafter decline. The nature of carrier often determines the subsequent performance of the inoculant. The criteria for a good carrier material are no toxicity to the introduced microorganism, good absorption capacity, suitable pH, fine particle size for better adherence to seed, good water holding capacity and availability at cheaper cost, addition to these the another important characteristics of good carrier should to support the survival of introduced organism at high temperature conditions during crop growing season.

1.2 Importance of study

As agricultural practices become more sustainable, there is an increasing need for ecologically sound methods of disease control. Biological control which exploits the natural antagonistic activity

of certain root colonizing bacteria against fungal pathogens, is one such approach. Biological control agents often perform inadequately under field conditions, however and this has impeded acceptance of the technology as an alternative to chemical pesticides.

The talc based powder formulations of *P. fluorescens* was effective against chickpea wilt, pigeon pea wilt, rice blast and rice sheath blight (Vidhyasekaran & Muthamilan, 1999).

In India *Pseudomonas fluorescens* is the important bacterial bioagent exploited commercially and being used by farmers as seed treatment, soil application and foliar spray in many crops. Development of powder based inorganic carriers (talc, vermiculite, perlite, ground rock phosphate, diatomaceous earth and calcium sulphate) and organic carrier based (charcoal, peat) formulations of *Pseudamonas fluorescens* have been reported by several workers (Fravel *et al.,* 1998; Sharma *et al.,* 2005; Wiyano *et al.,* 2008).The feasibility of endophytic bacteria *i.e Pseudomonas fluorescens* in greenhouses or the field is determined largely by its formulation, shelflife and delivery techniques (Stephens and Rask., 2000; Albareda *et al., 2008;* Khavzi *et al.,* 2007).

Formulation of biological control agent for commercial use generally involves the mixing of viable biological control agent cells with carrier based materials in liquid or dry form that not only stabilize and enhance the growth of biological control agent but also convenient for storage (Ilibazilah *et al.,* 2011).In addition to these the another important characteristic of good carrier should support the survival of introduced organism in higher temperature conditions for considerable time during crop growing season.

Survival of microorganisms is a changeable feature, depending most of all on the environmental factors like moisture and temperature. In Vidarbha region of Maharashtra, particularly in Akola, Amravati and Nagpur districts temperature always goes above 40° C in

summer and that's why in the present carrier based inoculants, *Pseudomonas fluorescens* population always goes on decreasing due to loosing moisture from them. This condition is more probable when the inoculants are prepared in advance of the season and not stored in proper conditions.

1.3 Objectives of the study

Considering the importance of 'Evaluation of different carriers and physical factors for the growth of *Pseudomonas fluorescens*' the present investigation was taken with the following objectives.

1. To study viability of *Pseudomonas fluorescens* in different carriers.
2. To study physical (Temperature and Light) requirements for the growth of *Pseudomonas fluorescens.*

1.4 Scope and limitation

Chemical fertilizers were used intensively around the world to increase crop yield. However, they started displaying their harmful effects to the environment. Therefore, the biofertilizers were introduced as a supplement and alternative fertilizers for reducing usage of the chemical fertilizers and preserving the environment in the long run.

The *rhizobial* biofertilizer technologies are more developed and advanced, and their markets are larger than any other types of the biofertilizers today. Because of their huge markets, many biofertilizer companies invest a large amount in Research and Development (R&D) of rhizobial biofertilizer technology. Research and developments of Fluorescent pseudomonads is being limited when compared to *rhizobium* inoculants.

Biofertilizers manufactured in India presently carrier based, in general, it suffers from short shelf life, poor quality, high contamination and unpredictable field performance. However research

conducted on the inoculants production and formulation technologies is limited. A break through is needed in the inoculants technology to improve shelf life and field efficacy of carrier based formulations in order to make them commercially viable and acceptable to farmers.

At present, there are many scientific challenges in the field of biocontrol (Pseudomonads). Although biocontrol strains of Fluorescent pseudomonad have contributed greatly to the understanding of the mechanisms that are involved in disease suppression, these strains have disadvantage from application point of view, they generally lose viability when stored for a period of several weeks.

The seemingly inherent variable performance of most biocontrol strains between field location and cropping season has hampered commercial development and relatively few biocontrol agents are registered for use in agriculture (Cook, 1996). Thus, the market for biocontrol PGPR is relatively small at present, but has the potential to grow as they provide an environmental friendly means to control pathogens.

Formulation is a challenging and often success limiting step in the successful commercialization of microbial inoculants. Although formulation research is progressing slowly. Several developments including liquid and granular formulations have contributed to the ease of use at the farm combined with the economic benefits of increased crop yield levels.

Improvement in formulation is key to the development of enhanced high end inoculants as the identification of new isolates with specific beneficial activities is often not difficult.

Commercial manufacturing and distribution of bioagents is now a days increasing to a great extent but the market coverage is very little as compared to the well established chemical pesticides.

1.4 Hypothesis

P. fluorescens possess a variety of promising properties which make them better biocontrol agent. Although the present study is not an initiative but helps in better understanding of *P. fluorescens* survival in different carriers.

Carrier based inoculants produced in India are generally lignite coal or charcoal based. The major disadvantages associated with these carriers are short shelf life, poor quality, high contamination and unpredictable field performance.

Different formulations of *P. fluorescens* have been developed. Talc and diatomite were good carrier materials for developing formulations of *P. fluorescens* because of their surface and pore structure (Li Hui *et al.*, 2009).

Now days, agro wastes are being tried as carrier materials for bio control agents, either individually or in combination with the conventional carriers(Vyas *et al.*, 2001; Thara & Nassema, 2002 and Bharathi *et al.*, 2003).

Spent mushroom substrate (SMS) has good physical properties, it includes the water holding capacity, soil pH, soil porosity, salt content i.e electrical conductivity. Addition of SMS will add great amount of macro nutrients but it is in little quantity (Kim *et al.*, 2011).The biological properties of SMS enhance its marketability as a soil conditioner(Brady *et al.*, 2004).Spent compost is believed to be a source of humus formation and humus is provided to the plants with micronutrients improve the soil aeration, soil water holding capacity and contributes the maintenance of soil structure (Kediri and Mustapa, 2010).

By considering all good properties of SMS, it has been tested in the present study for survival of *P. fluorescens* along with other carriers like talc, lignite, charcoal, farm yard manure and flyash.

CHAPTER II

REVIEW OF LITERATURE

Studies regarding the "Evaluation of different carriers and physical factors for the growth of *Pseudomonas fluorescens*" were taken up study. The viability of *Pseudomonas fluorescens* in different carriers like talc, lignite, charcoal, fly ash, farm yard manure and spent mushroom substrate was detected. The effect of physical factors *i.e* temperature and light on the viability of *Pseudomonas fluorescens* in different carrier based formulations was evaluated .The isolate of *P. fluorescens* was characterized on the basis of morphological and biochemical characterization.

Attempt therefore, were made to put forth recently published work reviewed critically under the following major heads

2.1 Morphological and biochemical characters of *Pseudomonas fluorescens*

2.2 Growth promoting ability of *Pseudomonas fluorescens*

2.3 Viability of *Pseudomonas fluorescens* in different carriers

2.4 Physical (temperature and light) requirements for the growth *Pseudomonas fluorescens.*

2.1 Morphological, Physiological and biochemical characters of *Pseudomonas fluorescens*

Yeole and Dube (2000) isolated the 73 Fluorescent *Pseudomonads* from rhizoplane of four crops (Chilli, cotton, groundnut and soybean) and diagnostic tests were followed for identification of Fluorescent *Pseudomonas* on the basis of morphological, physiological and biochemical characters which involves staining reaction, levan production, oxidase test, arginine dihydrolase, tobacco hypersensitivity and production of siderophore.

Hagedoron (2001) identified characteristics of PGPR which are strictly aerobic straight or curved rods, without resting spore, Gram negative, chemoorganotrophic and catalase positive. Colonies

are usually white to cream to yellow pigmented while many species produce extra cellular fluorescent pigment.

Shinde (2003) reported that among ten *P. fluorescens* isolates, seven produced H_2S gas, eight were able to hydrolyse starch, nine liquefied the gelatin and nine isolates gave positive test for auxin production (IAA).

Bhosale (2005) studied biochemical test of two *Pseudomonas fluorescens* isolates. Both the isolates were Gram negative and rod shaped, positive for starch hydrolysis and gelatin liquefaction.

Ipper *et al.* (2005) characterized that the Korean strain Gpf01 is closely related with *Pseudomonas fluorescens* strains on the basis of physiological and biochemical tests. They observed that Gpf01 isolates were positive for levan production, oxidase and arginine dihydrolase activity, and utilizations of 2- ketogluconate, mannitol and D- arabinose. However, negative for gelatin liquefaction and growth at higher temperatures.

Shivani *et al.* (2005) characterized ten *fluorescent Pseudomonas* isolates on the basis of biochemical characteristics. All the ten fluorescent *Pseudomonas* isolates were gram negative and showed positive test for casein hydrolysis, gelatin liquefaction and oxides test. Starch hydrolysis and H_2S production test were negative for all the isolates.

Gyung *et al.* (2006) reported that *P. fluorescens* isolates, showed negative reaction for hydrolysis of starch and gelatin liquefaction.

Mahesh (2007) worked on physiological and biochemical test of ten *P. fluorescens* isolates and stated that all isolates were gram negative and rod shaped. All *P. fluorescens* isolates showed variable reaction in case of starch hydrolysis, gelatin liquefaction and H_2S production.

Tiwari and Thrimurthy (2007) studied the morphological and biochemical characteristics of seven isolates of *Pseudomonas fluorescens* and found that all the isolates showed positive reaction to

gelatin liquefaction, catalase test, oxidase test, ammonification, starch hydrolysis, alkali reaction in litmus milk and strictly aerobic.

Gate (2009) collected soil samples from rhizosphere of different field crops and ten *P. fluorescens* were isolated and observed that all isolates were Gram negative and rod shaped and had an ability to produce H_2S gas, liquefy gelatin. Six were positive for starch hydrolysis and five isolates were positive for casein hydrolysis.

Siddiqui and Shakeel (2009) selected twenty one isolates of rhizobacteria belonging to *Pseudomonas* from pigeonpea area in Aligarh district of Uttar Pradesh. For identification of *Pseudomonas* some characterization tests was taken and found that six were positive for gelatin liquefaction, growth at $4^{\circ}C$ and catalase test while negative for starch hydrolysis and growth at $40^{\circ}C$. These characteristic were confirmed with Bergey's manual of determinative Bacteriology and identified as *P. fluorescens*.

Jayaprakashvel *et al.* (2010) isolated Fluorescent pseudomonads (FPs) and reported that all the isolates were gram negative, produced fluorescence on KBA also positive for catalase, oxidase, and gelatin liquefaction tests.

Sherris and Shoesmith (2010) tested cultures of 351 miscellaneous Gram-negative rods including 54 pseudomonads for their capacity to break down arginine rapidly by Mosller's qualitative technique, by a new qualitative method, and by a quantitative technique. All but *5* of the pseudomonads gave positive results by both qualitative techniques.

Armarkar (2011) studied twenty six isolates of *P.fluorescens* and reported that all were rod shaped and gram negative,ninteen isolates were Found positive for gelatin liquefication and thirteen isolates were able to hydrolyse starch.

Koche (2011) reported that among thirty isolates of *P.fluorescens* all were positive for oxidase, catalase, urease test, alkali reaction in litmus milk and ninteen isolate were positive for gelatin liquefication and only six isolates were able to hydrolyse starch.

Karuna (2012) studied that All isolates of *P.fluorescens* were gram negative and rod shaped, all isolates were positive for Arginine dihydrolysis, catalase and H_2S gas production and four isolates showed their ability to hydrolyse starch. Two isolates showed positive reaction against citrate utilization and also reported that Thirteen isolates showed positive reaction against KOH test. Thirteen isolates showed positive reaction against urease test. Four isolates showed positive reaction against oxidase test.

2.2 Growth promoting characters of *Pseudomonas fluorescens*

Kloepper *et al.* (1980) undersigned that specific strain of *Pseudomonas fluorescens* and *Pseudomonas putida* group are used as seed inoculants to crop plant to promote growth and increased yield. They produces extra cellular siderophores which efficiently complex environmental iron, making it less available to certain native microflora

Schroth and Hancock (1982) observed that increase in plant growth and yield was associated with the capacity of *Pseudomonas* to produce iron binding compound called siderophore.

Leong (1986) stated that the PGPR especially *Pseudomonas fluorescens* produce siderophore, which may stimulate plant growth either by improving the Fe nutrition to the plant or by inhibiting the establishment of plant pathogen or other harmful microorganism through deprivation of iron.

Anith *et al.* (1999) accessed the ability of wild and mutant strains of *Pseudomonas* spp. in details with respect to the antifungal activity, both strains produced similar levels of siderophore, HCN and fluorescent pigments.

Dave and Dube (2000) examined siderophore production by 6 rhizobacterial fluorescent *Pseudomonads* and characterized for their chemical nature and binding properties and found that isolate P1, P2 and P3 are strong siderophore producer.

Yeole and Dube (2000) obtained twelve (12) rhizobacterial fluorescent *Pseudomonas* isolates from chilli, cotton,

groundnut and soybean rhizosphere. All the isolates produced siderophore under iron deficient condition.

Ramamoorthy and Samiyappan (2001) tested twenty *Pseudomonas* isolates. Seven isolates viz., Pf1. FP 7, PB 2, ATR, PSV, COP and COT were found to increase plant vigour and produced higher amount of IAA under laboratory conditions. While PF1 effectively increased the plant vigour and produced the maximum amount of IAA in the culture medium.

Jayakumar *et al.* (2004) reported that the increase in plant growth of cotton by *Pseudomonas fluorescens* may be due to production of gibberellins, cytokinin and IAA.

Dibypual *et al.* (2005) conducted experiment for root proliferation activity of the *P. fluorescens* and found that bacterial strains increased the root biomass of the plant. Strains were also found to produce plant growth harmones viz; IAA and GA.

Shivani *et al.* (2005) reported that all the isolates of *Pseudomonas fluorescens* obtained from sunflower, Potato, Maize and groundnut produced fluorescent pigment in succinate broth and exhibited HCN and Indole acetic acid (IAA) by all the isolates, besides phosphate solublization. Further they estimated the production of siderophore by chrome azurol (CAS) assay and stated that appearance of an orange coloured zone after 48 h of incubation indicated the strains of fluorescent *Pseudomonas* were able to chelate Fe^{3+} from chrome azurol sugar medium.

Mahesh (2007) studied the biochemical properties of four *P. fluorescens* for HCN produciton, IAA production, siderophore production and phosphate solubilization. All isolates showed negative test for HCN production, 3 isolate showed positive test for IAA production. While four isolates were positive for siderophore production.

Muthukumar and Bhaskaran (2007) evaluated 12 *P. fluorescens* isolates from rhizosphere of rice for their efficacy against *Rhizoctonia solani* causing sheath blight of rice. Isolates 3 and 4 showed the greatest production of siderophore, salicylic acid and HCN.

Muzumdar *et al.* (2007) compared nine fluorescent *Pseudomonas* isolates on King's B from rhizosphere of tea plants with strain *P. fluorescens* MTCC -103 for their biochemical and functional characteristics. It produced IAA like substances, siderophore and soluble P.

Umamaheswari *et al.* (2008) studied the production of HCN by *Pseudomonas fluorescens* quantitatively and observed that there was strong production of HCN in PfCIAH-196 followed by moderate level in PfC6. They also studied plant growth promoting characteristics i.e. production of IAA, cytokinin and gibberellin. Among the four bacterial antagonists tested, production of IAA was more in *B. subtillis* (BsW 1) and in *P. fluorescens* (PfCIAH-196) which might be responsible for the enhanced plant growth .

Gate (2009) assayed 10 isolates of *P. fluorescens* for their ability to HCN, IAA and siderophore production. He found the positive reaction for siderophore production in all isolates, two isolates produce IAA and five isolates are able to solubilize phosphate and all isolates showed negative reaction for HCN production.

Suresh *et al.* (2010) screened Fluorescent pseudomonads for their plant growth promoting activity based on their ability to produce hydrogen cyanide (HCN), siderophores, proteases, indole acetic acid (IAA). All the 10 tested isolates of Fluorescent pseudomonads were positive for the production of IAA, protease, siderophores and HCN.

Ahemad and Khan (2011) studied that *P. putida* strainPS9 exhibited the plant growth promoting traits like phosphate solubulization, production siderophore phytoharmone and exo-polysacchrides in substantial amount.

Armarkar (2011) studied twenty six isolates of *P.fluorescens* and reported that eight isolates were able to produce siderophore and thirteen isolates showed positive test for IAA production.

Koche (2011) assayed thirty isolates of *P. fluorescens* and reported that twenty four isolates were able to produce siderophore and seventeen isolates showed positive test for IAA production.

Karuna (2012) studied plant growth promoting characteristics of *P. fluorescens* and reported that four isolate shows IAA production and seven isolate showed siderophore production.

2.3 Viability of *Pseudomonas fluorescens* in different carriers:-

Jauhri *et al.* (1979) reported higher survival of *Rhizobium* and *Azotobacter* was observed in modified charcoal carrier.

Kloepper and Schroth (1981) demonstrated the potentiality of talc to be used as a carrier for formulating rhizobacteria. The Fluorescent pseudomonads did not decline in talc mixture with 20% xanthum gum after storage for two months at 4^{0}c.*Pseudomonas fluorescens* isolate pf1 survived upto 240 days in storage. The initial population of pf1 in talc based formulation was 37.5×10^{7} cfu/g and declined to 1.3×10^{7} cfu/g after 8 months of storage.

Caesar and Burr (1991) reported better shelf life of *Pseudomonas fluorescens* (P7NF, TL3) in talc based formulations ammended with sucrose (0.72M) upto 240 days.

Heijnen *et al.* (1993) tested various organic carriers *i.e* peat, turf. Talc. Lignite, kaolinite, pyrophyllite, zeolite, montmorillonite, alginite, pressmud, sawdust, and vermiculite etc for the development of PGPR formulations and also reported the surviaval of *P. fluorescens* (2-79RN10, W4F393) in montmorillonite, zeolite and vermiculite with smaller particle size increased the survival rate than in kaolinite,pyrophylite and talc with bigger particle size.

Van Elsas *et al.* (1993) reported that genetically engineered *Pseudomonas fluorescens* encapsulated and released into soil microcosms showed significantly longer survival rates than those of unencapsulated cells after 3 months.

Dandurand *et al.* (1994) reported that clays with a small particle size such as montmorillonite, vermiculite and zeolite support a

higher survival *of Peudomonas fluorescens* compared to clays with a larger particle size such as talc, kaolinite and pyrrophilite.

Vidhyasekaran and Muthamilan (1995) studied the shelf life of *P.fluorescens* (Pf1) in various carriers and recorded shelf life for a period of 8 months with a viable load of bacteria in vermiculite of 1×10^{6} cfu/g ,2.8×10^{6} cfu/g in peat based formulation, 1×10^{6} cfu/g in farm yard manure and 1.3×10^{7} cfu/g in talc. They also recorded a shelf life of Pf1 for four months with a viable load of bacteria as 2.8×10^{6} cfu/g in both kaolinite and lignite.

Muthukumarasamy et al. (1996) suggested that vermicompost was an alternative carrier to lignite in the inoculants preparation of phosphobacteria, *Azospirillum* and *Acetobacter diazotrophicus.*

Narendranath (1996) studied the suitability of different carrier materials for inoculants preparation and reported that incorporation of amendments like soymeal (1%) and molasses (1%) in the carrier enhanced the survival of *Rhizobium* and also suggested that pressmud amended with soymeal, as an alternative carrier to peat in inoculation preparation.

Vidhyasekaran and Muthamilan (1997) developed various powder formulations of two efficient *P. fluorescens* strains i.e talc, peat, vermiculite, lignite and kaolinite and reported that talc formulations were effective even after six months of storage, while peat formulations were effective upto 60 days of storage. The shelf life of *P. fluorescens* was short in vermiculite, lignite and kaolinite formulations.

Bashan *et al.* (1998) reported that alginate beads have more advantages over peat, encapsulating and proctecting the microorganisms (*Pseudomonas fluorescens)* against environment stress and releasing them to the soil gradually when the polymers are degraded by soil microbes and also stated that long term survival of inoculants is a matter of commercial secrecy.

Fravel *et al.* (1998) reported that the high survival rate of Pf B5 in the wet materials infers that it would be possible to develop

these materials as wet carriers in formulations of PfB5. and also stated that peat as a good carrier for rhizobacteria formulations.

Bashan *et al.* (1999) showed that plant growth promoting bacteria (*P.fluorescens)* can survived (10^5 - 10^6cfu/g beads) in dry alginate beads at ambient temperature i.e.10-28°c for 14days.

Widayati and Premono (1999) studied the viability and population stability of *Pseudomonas putida* Lp.112-1 and *P. putida* Lp.312-1 in the various carrier carrier composition, size and granulation, the carriers consist of peat, zeolite, soil dominated by montmorillonitic clay and lime in various compositions were used. The results showed that *P. putida* Lp.112-1 and *P. putida* Lp.312-1 were viable and stable within 7 weeks incubation at room temperature (28-30 degrees C) in peat:soil: zeolite (1:1:1, w:w:w) carrier composition.

Amer and Utkhede (2000) recorded the shelf life of *P.putida* for 45 days in talc based formulations and the bacterial load was 1.0×10^3 cfu/g.

Sivakumar *et al.* (2000) recorded that out of three carriers studied for the survival of *P.fluorescens*, peat and talc maintained the highest population level of 19.5×10^7 and 18.3×10^7 cfu/g respectively after forty days of storage than other carriers.

Van Dyke *et al.* (2000) studied preinoculation of *Pseudomonas fluorescens* in sterile soil carrier for 0, 7 and 14 days. Results shows that establishment and growth using nutrients in sterile soil adapt cells soil environmental conditions, there by increasing survival of bacteria preioculated for 7 or 14 days and even it detected upto 56 days.

Georgrakapoulas *et al.* (2002) claimed that *Pseudomonas fluorescens* B5 survived in a peat carrier at ambient temperature for 2 years with a decrease of only 1 log cfu/g peat.

Kavitha *et al.* (2003) reported that peat carrier was retained the populations of *P. chlororaphis* (PA23) and *B. subtilis* (CBE4) for more than six months.

Bora *et al.* (2004) observed the survival of *P.putida* strain upto six moths in talc based formulations and the bacterial load at the end of six month was 10^8 cfu/g of product.

Gaind and Gaur (2004) evaluate fly ash as a carrier for *diazotrophs* and phosphobacteria, Fly ash and its different combination with soil (1:1) were tested to explore its possibility to use as a potential carrier for micro-organisms, *Azotobacter chroococcum, Azospirillum brasilense* and *Bacillus circulance* showed their maximum viability in fly ash alone where as *Pseudomonas striata* proliferated most in soil: fly ash (1:1) combination.

Nakkeeran *et al.* (2005) studied that the potential plant growth promoting rhizobacteria isolates are formulated using different organic and inorganic carriers either through solid and liquid fermentation technologies and results showed that shelf life of *Pseudomonas fluorescens* in talc based formulations was maintained upto 12 months than in peat, vermiculite, FYM, kaolinite formulations.

Nakkeeran *et al.* (2006) reported that peat, lignite, lignite+flyash and bentonite paste based formulations maintained higher propagule number of *P.fluorescens* upto 2 months of storage at ambient temperature(28°c).

Jayaraman *et al.* (2007) evaluated the shelf life of a highly antagonistic *Pseudomonas fluorescens* (PFT-8) in seven different carrier formulations viz., talc, lignite, peat, lignite+ flyash, wettable powder, bentonite paste and polyethylene glycol (PEG) paste up to six ,months of storage and results showed that Peat, lignite, lignite +flyash and bentonite paste based formulations maintained higher populations than others. However, the population decreased in all formulations with time.

Okereke and Okeh (2007) evaluated the suitability of decomposting rice husk, charcoal and coal as *bradyrhizobia* carrier for inoculant production. Three bradyrhizobial strains (tAL-209,TAL-379 and TAL- 173) were incubated for 2 weeks and observed that decomposed rice husk was found better carrier material.

Arora *et al.* (2008) conducted experiment with five carriers i.e. alginate beads, charcoal, sand, sawdust and sugarcane bagasse..Results showed that sawdust was the best carrier in maintaining *Pseudomonas fluorescens* uptc 180days.

Gade *et al.* (2008) tested shelf life of *Trichoderma* spp. In four carriers viz., talc, lignite, charcoal & flyash.Talc was found as the best to retain maximum number of viable population.

Wiyaono *et al.* (2008) reported that the long term survival of *P.fluorescens* B5 was always higher in wet (60% moisture) than in dry (10% moisture) carrier materials. The survival of Pf B5 estimated after 12 months in the carriers, especially in the types of wood flour and peat was as higher as the initial rate of 9log cfu/g dry materials.

Gandhi and Saravanakumar (2009) reported the shelf life of *Pseudomonas fluorescens* in vermicompost and lignite carriers and results found that vermicompost supports the colony count of 18.80 $\times 10^8$ cfu/g than lignite (0.24 $\times 10^8$ cfu/g) at 240 days of storage.

Li Hui *et al.* (2009) studied diatomite and talc carriers for *Pseudomonas fluorescens* P13 strain and results showed that both diatomite and talc were good carrier materials for formulations.

Liza and Barua (2009) observed population dynamics of *P. fluorescens* and *Trichoderme harzianum* in two different substrates viz., vermicompost and wheat bran carrriers and reported that the population density significantly increased upto 45 days of storage. The substrate comprising of vermicompost recorded highest population after 15, 30 and 45 days of storage.

Mythukumar (2009) studied seven carriers namely flyash, lignite, peatsoil, ricebran, sugarcane baggasse, talc powder and wheat bran to test the shelf life of *Pseudomonas fluorescens* and found that talc based formulations was superior in supporting its survival and recorded a population of 17×10^7 cfu/g at 150 days of storage.

Gandhi and Sivakumar (2010) reported that vermicompost carrier was alternative for both lignite and charcoal for the preparation of carrier based formulations of *Azospirillum lipoferum*, *Bacillus megaterium* and *Pseudomonas fluorescens*.

Mankandan *et al.* (2010) developed liquid formulation of *P. fluorescens* strain (Pf1) in nutrient broth medium with the addition of different chemicals such as trehalose, polyvinylpyrrolidone and glycerol. Among them, glycerol amendment maintained the greater population level of Pf1 upto six months of storage.

Meena *et al.* (2010) reported that talc based and talc +gypsum based formulations supported better survival of *Pseudomonas fluorescens.*

Salaheddin *et al.* (2010) reported that the shelf life of *Pseudomonas fluorescens* isolates (Pf32,Pf93) survived with required cfu(10^8cfu/ml) in the talc based formulations upto 90days of storage.

Rangeswaran *et al.* (2010) studied the effect of adding nutrient additives to talc based formulation on the shelf life of *P. fluoresens* (PDBCAB2) stored under room temperature and reported that highest count of log 1×104.2 cfu/g was obtained with formulations amended with 2 per cent tryptone and 2 per cent glycerol at 240th day, and also the results indicated that 2 per cent peptone or 2 per cent tryptone supplemented with 2 per cent giycerol helped *P. fluorescens* for its better survival.

Chakravarty *et al.* (2011) conducted experiment on viability of *Pseudomonas fluorescens* in various organic formulations like vermicompost, FYM, wheat bran, rice straw and banana leaf and found that vermicompost and FYM supports highest population count of 45×10^8 cfu/g and 42×10^8 cfu/g respectively after 120 days of storage at room temperature.

Ilibazilah *et al.* (2011) reported that the luria broth based and vermiculite based formulation of *P. fluorescens* were found to be most stable by sustaining 86% of viable bacterial cells after 6 months of storage.

Mugilan *et al.* (2011) studied the survival of *P. striata* in different formulations viz., lignite, vermiculite and liquid inoculants were estimated over a period of three months of storage. Liquid inoculants supported the maximum population of 28.3×10^9 cfu per ml.

Devi *et al.* (2012) observed maximum viable cell count of *Pseudomonas fluorescens* in lignite carrier from 0 days to 120 days of storage.

Rajalaxmi *et al.* (2012) recorded the shelflife of *Pseudomonas fluorescens* (PF-4) and *Pseudomonas putida* (RFP-13) in different carrier materials and stored at two different temperature regimes viz., room and refrigerator over a storage period of 10 months. The population of both isolates increased significantly in talc, vermicompost, FYM up to 60 days and up to 30 days in King's B broth. Refrigerator temperature supported better population of both Pf-4 and RFP-13 in the carrier materials. Talc was found to be the best carrier material that maintained better population of the both isolates till the end of storage period.

Sangeetha *et al.* (2012) determined the survival of *Pseudomonas fluorescens* population in four different carriers i.e lignite, vermiculite and pressmud, Results showed that pressmud support the population of 66.82×10^8 cfu per gram than vermiculite (61.23×10^8 cfu/g) and lignite (61.23×10^8 cfu/g) upto 3 months of storage and also reported that vermiculite supports the population of (5.43×10^{8}) than pressmud (3.93×10^8 cfu/g) and lignite(2.33×10^8 cfu/g) upto 6 months of storage.

Sivakumar *et al.* (2012) conducted experiment with four carriers peat, talc, vermiculite, lignite. Results showed that peat based formulations of *P. fluorescens* Pf(5) supported the survival of bacterial strains upto 270 days with a viable population of 4.3×10^7 cfu per gram.

2.4 Physical (temperature and light) requirements for the growth *Pseudomonas fluorescens*.

The shelf life of environmentally sensitive micro organisms continues to be a challenging and success limiting step in development of biocontrol products (Paau.,1998).While Fluorescent pseudomonads have significant potential as biocontrol agents, they are very sensitive to environmental factors, in particular temperature and acidity (Callaghan *et al.*, 2001).

Roughly (1968) reported that effect of storage on growth and survival of organism is influenced by both the purity of the culture and the amount of moisture lost during storage and prepared cultures with sterilized carriers incubated at 26°C, immediately after inoculation promotes initial rapid growth of organisms and has little or no effect on long term survival if moisture content was maintained.

Wodzinski *et al.* (1960) studied the effect of temperature on the growth of *P. fluorescens* and reported that at low temperature(15ºC) the apparent lag phase and generation time of *P.fluorescens* was shortened to a relatively greater extent than at higher temperature(30º C).

Kloepper and Schoth (1981) reported that the populations of Fluorescent pseudomonads did not decrease in the talc mixture with 20% xanthum gum after storage for 2 months at 4ºC.

DeConnick (1988) studied Fluorescent pseudomonads formulations and indicated that vermiculite based dried formulations retained effective levels of population after storage for 6 months at 4ºC.

Wessendorf *et al.* (1989) studied the influence of laboratory growth conditions i.e temperature,pH, carbon and nitrogen sources on survival of *P. fluorescens* and reported that viability of *P. fluorescens* maintained at 4º C was more than maintained at 20º C.

Callaghan *et al.* (2001) worked on *P. fluorescens* (HAO-Rif) and inoculated into soil microcosms at three soil moistures and temperature, survival was determined at regular intervals and reported that *P. fluorescens* was not recovered after 54days at 20ºC .

Sandikar and Awasthi (2009) carried out experiment with formulations of four *Pseudomonas* and three *Bacillus* isolates and were stored at room temperature (30 degrees) and cold conditions (4 degrees) and reported that both bacterial isolates were durable in formulations which were stored at 4 ºC .

Chakravarty *et al.,* (2011) conducted experiment on viability of *Pseudomonas fluorescens* in various organic formulations like vermicompost, FYM, wheat bran, rice straw and banana leaf at two different temperatures and reported that vermicompost based

formulations retains highest viable count of 890×10^{8} cfu/g at 4^{0} c than 45×10^{8} cfu/g at at room temperature.

Ilibazilah *et al.* (2011) reported dry and liquid formulations of endophytic bacteria i.e *Burkholderia sp* (UPMB3) and *Pseudomonas* (UPMP3) stored at 10, 20 and 30°C. Formulations stored at 10 and 20°C provided longer shelf life than those stored at 30 °C based on viability at monthly intervals over a 9 months storage period.

Rajyalaxmi *et al.* (2012) recorded refrigerator temperature (4^{0}C) was found to be better than the room temperature (32±2^{0}C) in all carrier materials i.e talc, vermicompost and farm yard manure in maintaining the viable populations of *P.fluorescens* and *P.putida* upto ten months of storage.

Sadi and masoud (2012) carried out experiment under in vivo conditions .Formulations were stored at 90 consecutive days at two different temperatures, room temp (26°C) and refrigerator temp (4°C). Longest stability of UTPF61 strain of *P.fluorescens* was observed in talc based formulations which were stored at 4°C.

Sangeetha *et al.* (2012) studied influence of storage temperature i.e 25^{0}C, 30^{0}C, 35^{0}C and 40^{0}C for the viability of *P. fluorescens* in different carrier material and results showed that the carrier formulation which was kept under 30^{0}C storage temperature found as effective for the viability of bacteria.

Phiromtan *et al.* (2013) tested *Azotobacter vinelandii* NDD-CK-1 for its survival in carriers i.e peat, peat mixed with corn stubble compost ,peat mixed with golden flamboyant leaf compost and peat mixed with mushroom waste compost at -16^{0}C, 5^{0}C, 25±2^{0}C,30±2^{0}C and 37.5±2.5^{0}C and reported that most suitable temperature for longer term (30 to 90 days) was at 5^{0}C followed by -16^{0}C, 25±2^{0}C, 30±2^{0}C and 37.5±2.5^{0}C.

CHAPTER III

MATERIAL AND METHODS

The present investigation entitled "Evaluation of different carriers and physical factors for the growth of *Pseudomonas fluorescens*" was carried out in the laboratory of Department of Plant Pathology, Post Graduate Institute, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola.

3.1 Collection of Carrier materials:

The carrier materials like Talc,lignite, charcoal,farm yard manure (FYM),flyash and spent mushroom substrate (SMS) were collected from the following sources. (Plate.3).

Carrier material	Source
Talc & lignite	Department of plant pathology
Charcoal & Flyash	Thermal Power Station, PARAS.
FYM	Department of Diary Science, Dr PDKV, Akola.
Spent Mushroom Substrate	Vitthal Mushrooms, Akola.

3. 2 Analysis of carrier materials:

All the carrier materials were dried in shade, powdered sieved through 60 micron sieve. These materials were analyzed for their physical and chemicals characters *viz.,* pH, organic carbon content, water holding capacity, bulk density and electrical conductivity.

3.2.1 Water holding capacity (Emmanuel *et al.,* 2010)

The air dried sample of the carrier material (20g) was placed in a plastic container (with wire mesh at the bottom), in a dish of water. The carrier material was allowed to become saturated, for approximately six hours. The container was removed from the water and covered with

cling film to prevent loss of evaporation. It was then hung on a retort stand overnight to allow drainage. Next carrier sample was carefully removed, put in a pre weighed container (M1) and total weight of the moist carrier and moisture container (M2) was taken. The sample were dried in an oven at 105^{0}C until no further water loss occurred and reweighted to record the oven-dried sample (M3).The water holding capacity was calculated as :

$$WHC(\%) = \frac{(M2 - M3)}{(M3 - M1)} X100$$

3.2.2 Bulk density (Emmanuel., 2010)

Bulk density was determined after drying the core samples in an oven at 105^{0}C for 24hrs and calculated as:

$$\text{Bulk density } (g/cm^3) = \frac{W2 - W1}{V}$$

Where W2 and W1 are weights of moist and oven-dry soils, respectively and V is the volume of the cylindrical core.

3.2.3 pH (Piper 1966)

Twenty gram of each carrier material was weighed and transferred to 100ml beaker. Forty ml of distilled water was added and stirred well with a glass rod and allowed to stand for 30 min with intermittent stirring. pH was determined using a pH stick by immersing it in the beaker containing carrier-water suspension.

3.2.4 Organic carbon content: (Walkley and Black, 1934)

One gram of finely powdered carrier material was taken in a 500ml conical flask, 10 ml of 1NK_2Cr_2O7 and 20 ml of conc. H_2SO_4 were added and swirled and allowed to stand for 30min.This oxidizes carbon in the sample to CO_2 . The unused chromic acid in the oxidation reaction was estimated by back titration against standard ferrous ammonium sulphate. Simultaneously blank was also run. Organic carbon content was estimated and expressed in percent.

3.2.5 Electrical conductivity: (Piper 1966)

Transfer 20 g of carrier material was transfer into 50ml beaker, add 40ml of distilled water and stir the suspension intermittently for an half an hour and keep it 30 min without touch. Then insert the conductivity cell in suspension or solution and note the electrical conductivity of the carrier material

3.3 Eqipments:

Standard laboratory equipments used for different experiments Autoclave, Laminar air flow,Hot air oven, Orbitory rotary shaker, pH meter,Glass electrode, Qubec colony counter, Weighing balance, Single and double distillation unit, Reffigerator,Incubator

3.4 Chemicals & Miscellaneous materials:

Chemicals used during the course of research work were Spirit, conc H_2SO_4, $K_2Cr_20_7$, Ferrous ammonium sulphate,H_20_2,KOH etc.

Miscelllaneous materials were Inoculation needle, slides, cover slip, forceps, micropipette, non absorbant cotton,Filter paper,tray, scissors,thread,sieves and polythene bags.

3.5 Sterilization of glass wares, media and water

Petriplates, test tubes and conical flask of different capacities i.e., 500ml, 250 ml of "Borosoil" make were used. The glasswares were sterilized in hot air oven at 180^0C for one hour. The material *viz.* needles, inoculating needle, forcep, scarpel were sterilized on flame by direct heating.

The media and distilled water were sterilized in autoclave at 1.05 kg/cm^2 for 15 minutes.

3.6 Precaution to eliminate contamination

All inoculation work of microbial culture was carried out in laminar air flow. The laminar air flow was sterilized by glowing ultraviolet lamp ½ hr prior to comencement of work. The working surface and side glasses of laminar airflow were also sterilized with denatured spirit.

3.7 Source of bio agent *Pseudomonas fluorescens*

The bio agent involved in the study i.e *Pseudomonas fluorescens* was collected from Department of Plant Pathology, Post Graduate Institute, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola.

3.8 Maintenance of *Pseudomonas fluorescens*

The bio agent required for the experiment i.e. *Pseudomonas fluorescens* was maintained on selective medium i.e. Kings B medium at room temperature by adopting subsequent sub culturing at periodical, regular intervals. Three days old culture was used for further studies.

3.8.1 Composition of KingsB :

For the growth of *Pseudomonas fluorescens* King's B medium was used (King *et al.*, 1954)

Peptone	-	20g
Glycerol	-	10 ml
K_2HPO_4	-	1.5g
$MgSO_4$ 7 H_2O	-	1.5g
Agar agar	-	20g
Distilled water	-	1000 ml

3.9 Morphological studies

The confirmations of the *Pseudomonas fluorescens* isolate was performed with the following studies (Buchanon and Gibbson,1974). Pure culture of *Pseudomonas fluorescens* isolate was streaked on King's B medium petriplate separately for colony development. The individual colonies were examined for colony colour, and pigmentation.

3.10 Biochemical studies (Aneja, 2003)

Biochemical tests viz., starch hydrolysis, gelatin liquefaction, citrate utilization, KOH test, catalase activity, siderophore production, and Gram's reaction were carried out for biochemical characterization of *P. fluorescens.*

The isolate of *P. fluoresens* were also evaluated for plant growth promoting properties viz., IAA production. etc.

3.10.1 Starch hydrolysis

Starch is a complex carbohydrate (polysaccharide) composed of two constituents – amylose, a straight chain polymer of 200-300 glucose units, and amylopectin, a larger branched polymer with phosphate groups. The positive test indicates by the presence of amalyse enzyme, an exoenzyme that hydrolyses (cleaves) starch, into maltose (disaccharide) and some monosaccharides such as glucose.

Medium : Starch Agar – nutrient agar + 0.2% soluble starch

Test reagent : Gram's iodine

Bacterial culture was inoculated on starch agar plates and incubated for 7 days. After incubation, the plates were flooded with Lugol's iodine solution. Presence of starch hydrolysis indicated by the appearance of clear zone. Reddish zone indicated the starch was partially hydrolysed to dextrin.

3.10.2 Gelatin liquefaction

Gelatin is a protein produced by hydrolysis of collagen, a major component of connective tissue and tendons in humans and other animals. Hydrolysis (liquefaction) of gelatin is brought about by bacterium capable of producing a proteolytic exoenzyme known as gelatinase, which acts to hydrolyze this protein to amino acids.

Medium : Nutrient gelatin = Nutrient broth + 1.5% gelatin

Bacterial culture were inoculated through stab of a nutrient gelatin tube and incubated for 7 days uninoculated tubes serves as control and observed for liquefaction. Deep gelatin inoculated tubes that remain liquified produce gelatinase and show positive test for gelatin hydrolysis and those tubes that remain solid demonstrate negative reaction for gelatin hydrolysis.

3.10.3 Gram's reaction

Identification was made by gram staining and by studying the morphological characters of the isolates.

Procedure :

i) First a smear was prepared of bacterial cells by holding a clean slide by grapping at edges.

ii) A loopful of bacterial suspension was transferred in the center of slide, with the help of wire loop

iii) The drop was smeared over slide and air dried.

iv) Then dried smear was fixed by passing the slide 3-4 times rapidly over the flame.

v) The smear was flooded with crystal violet for 30 seconds, washed in the tap water.

vi) Then the smear was immersed in potassium iodide/ Lugol's iodine solution for 30 second,s washed in tap water then decolorized with 95% alohol and rinsed with water.

vii) Counterstained with saffranin for 10 second, again washed with tap water and air dried.

viii) Drop of cedar wood oil was placed on the slide and examined the smear under oil immersion lense.

3.10.4 Citrate utilization

Citrate test is used to differentiate among enteric bacteria on the basis of their ability to utilize or ferment citrate as the sole carbon source. The utilization of citrate depends on the presence of an enzyme citrase produced by the organism, that breaks down the citrate to oxaloacetic acid and acetic acid. These products are later converted to pyruvic acid and carbon dioxide.

Medium : Simmon's citrate agar medium

The citrate test was performed by inoculating the microorganisms into an organic synthetic medium, Simmon's citrate agar, where sodium citrate was only source of carbon and energy. Bromothymol blue was used as an indicator. When the citric acid was metabolised, the CO_2 generated combines with sodium and water to

form sodium carbonate an alkaline product, which changes the colour of the indicator from green to blue and this constitutes a positive test.

3.10.5 Catalase test

During aerobic respiration in the presence of oxygen, microorganisms produce hydrogen peroxide (H_2O_2) which is lethal to the cell. The enzyme catalase present in some microorganisms breaks down hydrogen peroxide to water and oxygen and helps them im their survival.

Medium : Trypticase soy agar medium

Catalase test was performed by adding H_2O_2 to Trypticase soy agar slant culture. Release of free oxygen gas (O_2) bubbles is a positive catalase test.

3.10.6 IAA production

Tryptophan, an essential amino acid, is oxidised by some bacteria by the enzyme tryptophanase resulting in formation of indole, pyruvic acid and ammonia. The indole test is performed by inoculating a bacterium into tryptone broth, the indole produced during the reaction was detected by adding Kovac's reagent which produces a cherry-red reagent layer.

Medium : 1% tryptone broth : 10 gm of peptone in one litre of distilled water.

Test reagent :Kovac's reagent (P- di-methylamino benzaladehyde 50 g, amyl alcohol 750 ml, HCL 250 ml)

The medium was distributed in test tubes and autoclaved. The bacteria was inoculated and incubated for 48 hrs. After 48 hours of incubation, Kovoc reagent (1 ml) was added in incubated test tube. The tubes were allowed to stand to permit the reagent to come to top.Development of a cherry (deep) red colour in the top layer of the tube was a positive test for indole production. Absence of red colouration was indole negative.

3.10.7 Siderophore production

Evaluation of the isolates with universal chrome – Azurol assay (CAS) helps in detecting the siderophore production by *Pseudomonas fluorescens.* This assay mainly depends on the colour zone i..e orange zone against dark blue background a positive indication for the presence of siderophore. All the ten isolates were screened by CAS method (Schwyn and Neilands.,1987) for their ability to produce siderophores. Six ml of 10 mM strength HDTMA (hexadecyl trimethyl ammonium bromide) solution was poured in to 100 ml volumetric flask and further diluted up to 50 ml with de ionized water. One and half ml of Fe_3 solution (prepared by mixing 1 mM $FeCL_3$,6 H_2O + 10 mM HCL) and 7.5 ml 2 mM aqueous CAS solutions were added into the volumetrix flask which subsequently turned the colour of the solution to dark blue. This entire content was added to 41.0 ml distilled de ionized water so as to make up the final volume to 100 ml. King's B medium (KMB) were added which turned the medium colour to blue. The medium was sterilized and poured in the Petriplates @ 20 ml per plate. After solidification, spot inoculation was done with each bacterial isolates and the plates were incubated at 28 ± 2^0Cfor 24 hour. Four replicated plates were maintained for each isolates. The observations were recorded for the development of orange halo region surrounding the incubated spot.

3.11 Preparation and Sterilization of carrier material:

Pseudomonas fluorescens survival was tested in six different carriers: Talc, spent mushroom substrate,lignite,charcoal,FYM and fly ash.Ten grams of carboxymethyl cellulose was added to 1 kg of the carrier and mixed well.The pH of all carrier materials was adjusted to 7.0 by adding calcium carbonate.The carriers were autoclaved for 30min on each of two consecutive days.

3.12 Preparation of carrier based formulations of *Pseudomonas fluorescens* :

Pseudomonas fluorescens isolate was grown on liquid KingsB broth for 48 h as shake culture incubating in orbitory rotary shaker at 150 rpm at room temperature (25±2°C). Four hundred milliliters of the bacterial suspension containing 9×10^8 colony forming units (cfu) per ml was added to 1 kg of the carrier and mixed well under sterile conditions.The materials were packed in polythene bags seald,and incubated at room temperature (25±2°C).(Plate. 3& Plate. 4)

3.13 Evaluation of *Pseudomonas fluorescens* population in carrier based formulations:

Evaluation of *Pseudomonas fluorescens* in carrierbased formulations was done by serial dilution method

Test tubes with 9 ml distilled water were sterilized in autoclave for preparation of water blank procedure

1) Labeled the dilution blank as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 3 plates marked with marking pencil.
2) Prepared initial dilution by adding 1 g of each carrier based formulations of *Pseudomonas fluorescens* into 9 ml distilled sterilized water and dilution blank labeled 10^{-1}. Thus diluting original sample 10 times (1:10).
3) Vigorously shaken the dilution and from the first dilution transferred 1 ml of suspension to the dilution blank number of 10^{-2} by sterile pipette shaken well for minutes.
4) From 10^{-2} suspension transferred 1 ml of suspension to 10^{-3} dilution blank with a fresh sterile pipette.
5) Repeated the procedure till the original sample had been diluted to 10^{-8} using every time a fresh sterile pipette.
6) From the appropriate dilution (10^{-7} and 10^{-8}) transfered 1 ml of suspension with respective pipette to sterile petriplates. Three petriplates were used for each dilution.

Plate 1: Carrier materials used for shelf life study

(a) Broth of *Pseudomonas fluorescens*

(b) Growth of *Pseudomonas fluorescen*
in rotary shaker

(c) Carriers inoculated with *P.fluorescens*

(d) Stored in incubator

Plate 2: Broth and Carrier based formulation of *Pseudomonas fluorescens*

7) Approximately 20 ml melted Kings B medium added to each petridishes, containing the diluted sample. Mixed the content of each dish by rotating gently to distribute the cell through out the medium.

8) Allowed the plates to solidify and incubate these plates in a inverted position for 2 days at 28±2^{0}C.

9) The bacterial colonies were counted as colony forming units (c.f.u) per ml and expressed as c.f.u per gram substrate.

10) The plate count were carried out in triplicates and final value of cfu was the average of three readings.

3.14 Counting colonies of *P.fluorescens* :

The number colonies were conted on a Qubec colony counter after the incubation peroid of 48 hrs as colony forming units (cfu) per ml and expressed as cfu per gram of carrier material. The plate count was carried out in triplicates and final value of c.f.u will be the average of three readings. (Aneja, 2003)

$$\text{Cfu/g of carrier} = \frac{\text{Number of colonies}}{\text{Amount plated x dilution}}$$

3.15 Effect of Temperature and light for the growth of *Pseudomonas fluorescens*:

Temperature and light requirement determined for all carrier based formulations and c.f.u was determined (as cfu/g of carrier) by keeping carrier based inoculum at different temperature viz.,0°C, 15°C, 25°C and 30°C for 15 days and under different light regimes viz., 12hrs dark+ 12hrs light, 18 hrs light+6hrs dark, 6hrs light+18 hrs dark for 15 days. Population of bacterium was counted after 15 days of storage by dilution plate method.

AKOLA

CHAPTER IV

RESULTS AND DISCUSSION

An investigations were carried out on " Evaluation of different carriers and physical factors for the growth of *Pseudomonas fluorescence* " during 2012-2013. The results and its interpretation of the present studies are given in this chapter.

Inoculum carriers serve as media in bioinoculant production, controlling quality and shelflife of bacterial inoculants by serving as microenvironment for microorganisms. Besides, types of carrier, and storage temperatures are important factors determining shelf life of bioinoculants (Kremer & Peterson, 1983).

One of the main problem in inoculant technology is the survival of microorganisms during storage and several parameters such as the culture medium, physiological state of the microorganisms when harvested (Chen and Alexander, 1973), the process of dehydration rate of drying (Mary *et al.*,1985), the temperature of storage and water activity of the bacterial inoculum (Hahn-Hagerdal, 1986) have an influence on their shelf life. So studies to increase the shelf life of inoculants or finding an alternate formulations for carrier based inoculants are important.

Results of present investigation along with statistical analysis have been presented under the different headings.

4.1. Morphological & Biochemical characterization of *Pseudomonas fluorescens*

The microbial inoculant *P. fluorescens* used in the present study was obtained from the Department of Plant pathology, Post Graduate Institute, Dr PDKV, Akola. The above strain was conformed by performing morphological and biochemical tests. The results are presented in Table 1. Based on the results, *P. fluorescens* was rod shaped, gram-negative and also produced yellow color colonies on Kings B medium. It showed positive for catalase activity,

starch hydrolysis, citrate utilization, gelatin liquefication, siderophore and IAA production tests. (Table 1) (Plate 1& Plate2).

Gate (2009) also reported that all fifteen isolates of *P. fluorescens* were rod, gram negative which produced round to irregular colonies with yellowish, dull yellowish and greenish yellowish, water soluble pigment production. All isolates were found positive for catalase, gelatin liquefication and able to hydrolyse starch (Tiwari and Thrimurthy, 2007).Six isolates were positive for gelatin liquification (Siddiqui and Shakeel, 2009). Two isolates were positive to citrate utilization (Shivani *et al.*, 2005; Gate., 2009; Nisharani, 2010). All thirty isolates were positive for catalase test and nineteen isolates were positive for gelatin liquefication and six were able to hydrolyse starch (Koche, 2011; Armarkar, 2011).Thirteen isolates were positive for gelatin liquefication (Armarkar, 2011).Four isolates were showed their ability to hydrolyse starch, two isolates showed positive reaction against citrate utilization and thirteen isolates showed positive for KOH test (Karuna, 2012).

Gate (2009) also found siderophore production in all ten rhizobacterial *P. fluorescens*. Siderophore are iron chelating compound which bind to available form of iron Fe^{3+} in rhizosphere, thus making it unavailable to phytopathogens. It also makes iron available to plant in soluble form and protecting the plant health (Umamaheswari *et al.*, 2008). The involvement of siderophore in disease suppression, ISR and plant growth promotion has been well established (Kloepper *et al.*, 1980). In our study 7 isolates of *P. fluorescens* were positive for siderophore production. This result are also inline with the findings of Ahemad and Khan (2011) who reported that *P. putida* strain PS9 exhibited the plant growth promoting traits like phosphate solubulization, production of siderophore phytoharmone and exo-polysacchrides in substantial amount.

Out of 15 isolates 4 isolates showed positive test for IAA production. Production of IAA and some other auxins has also been reported in *Pseudomonas cepacia* and *P. fluorescens* isolated from maize, bean and tobacco rhizosphere (Hass *et al.*, 1991). One of the

proposed mechanisms of plant growth promotion by bacteria was production of IAA, cytokinin and gibberallin (Glick, 1991). The effect of IAA produced by *P. fluorescens* on root development of black current cutting was observed by Dubeikousky *et al.* (1993). Shinde (2003) noted IAA production of nine isolates out of ten isolates of *P. fluorescence*. Deokar (2009) observed that IAA production was positive in only one isolates out of four. Increase in IAA might be responsible for enhancement of plant growth. Koche (2011) observed that twenty four isolates were able to produce siderophore and seventeen isolates showed positive test for IAA production. Armarkar, (2011) reported that eight isolates were able to produce siderophore and thirteen isolates showed positive test for IAA production. Karuna (2012) stated hat four isolate showed IAA production and seven isolate showed siderophore production.

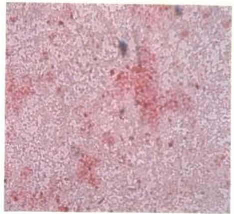
Table 1: Morphological and Biochemical characterization of *Pseudomonas fluorescens*

Sr.No.		Character	*P.fluorescens*
1.	Morphological tests	Shape	Rod
2.		Gram reaction	Negative
3.		Pigmentation	Yellow
4.	Biochemical tests	Starch Hydrolysis	Positive
5.		Citrate utilization	Positive
6.		Catalase Activity	Positive
7.		Gelatin liquefaction	Positive
8.	Growth Promoting characterization	IAA Production	Positive
9.		Siderophore Production	Positive

4.2. Physico-chemical properties of carrier materials

The physico-chemical properties such as pH, organic carbon content, water holding capacity, Bulk density and electrical conductivity were estimated for the carrier materials like Talc, Spent mushroom substrate, Lignite, Charcoal, FYM and Fly ash which were used in this study and the results are presented in Table 2

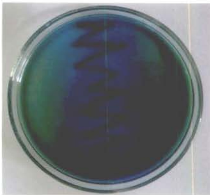
Pseudomonas fluorescens on King's B media



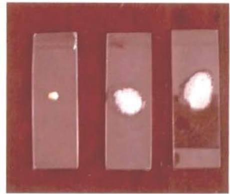
Gram negative reaction

Starch hydrolysis

Geletine liquefaction



Citrate utilization



Catalase test

Plate 3: Biochemical characterization of *Pseudomonas fluorescens*

Siderophore production

IAA production

Plate 4: Growth promoting characters of *Pseudomonas fluorescens*

Table 2. Physico-chemical properties of different carrier materials

S. No	Carrier	pH	Organic carbon content (%)	Water holding capacity (%)	Bulk density (gcm^{-3})	Electrical conductivity (ds/m)
1.	Talc	7.2	-	189	0.30	0.67
2.	Spent mushroom substrate	7.0	0.43	93	0.35	0.28
3.	Lignite	7.0	7.90	65	0.82	0.50
4.	Charcoal	6.9	5.30	52.6	0.75	0.49
5.	Farm yard manure	7.1	18.7	85	0.65	0.30
6	Fly ash	6.9	0.36	63	1.2	0.65

The physico-chemical properties revealed that the pH values of carrier materials were in the range of 6.9-7.3 and maximum organic carbon content was recorded with farm yard manure (18.7%) followed by lignite, charcoal, spent mushroom substrate and fly ash (7.90%, 5.30%, 0.43%, and 0.36%). Maximum water holding capacity was recorded in talc (189%) followed by spent mushroom substrate, farmyard manure, lignite, fly ash and charcoal (93%, 85%, 65%,63% and 52.6%).

Date (1976) suggested that high organic carbon content, high water holding capacity and neutral in reaction were the desirable characters of carriers.

Low bulk density values were recorded in talc (0.3 g/cm^3) followed by spent mushroom substrate, fly ash, FYM, charcoal and lignite (0.35, 0.47,0.65, 0.75 and 0.82 g/cm^3).Low electrical conductivity value was recorded in spent mushroom substrate (0.28 ds/m) followed by FYM, lignite, charcoal, fly ash and charcoal.

The physico-chemical characters of carrier materials have got profound influence on the survival of inoculants. The ideal characteristics of an inoculant carrier include more surface area, rich in

organic matter, high water holding capacity, neutral pH, easy availability and inexpensiveness (Arangarasan *et al.*, 1998). Physico chemical properties of carriers i.e pH of spent mushroom substrate was 6.8 (Polat *et al.*, 2009), organic matter between 40-60% on dry weight basis (Liu *et al.*, 2006) and electrical conductivity of the sample of spent mushroom substrate was 0.28 which was considered as good for seed Germination (Enrique *et al.*, 1988).

Nigussie and Kissi (2011) recorded the bulk density of charcoal was 1.12 and water holding capacity was 52.77%. The bulk density of fly ash was 1-1.5 g/cm3 (Prem Kishore, 2010).These findings are confirmative with present study.

4.3. Survival of *Pseudomonas fluorescens* in different carrier materials at room temperature

The survival of *P. fluorescens* in different carrier materials *viz.*, Talc, Spent Mushroom Substrate, Lignite, Charcoal, Farm Yard Manure and Fly ash were estimated under controlled conditions over a period of six months of storage period at room temperature by serial dilution technique on Kings B plate. The results are presented in Table 3 and Fig. 1.

Initially (at 0 days) all the carriers revealed non significant differences in colony forming units (cfu) and it was ranged between 83 to 86 cfu/g in six different carriers. The population of *P. fluorescens* was increase up to 60 days of storage in all carrier materials on storage and there was slow decline in number of viable propagule after 60 days of storage. Shelf life studies revealed that among six carrier materials Talc, FYM, Lignite supported maximum mean population of *P.fluorescens* till 60 days of storage and recorded 25.65%, 23.38% and 14.75% addition of bacterial propogules compared to initial population.

Among the different carriers tested, the talc powder supported the maximum population of 18.00×10^{8} cfu /g at 180 days of storage. It was significantly superior over all treatments.

Talc was followed by FYM of 15.67×10^{8} cfu /g, Lignite of 14.67×10^{8} cfu /g, SMS of 12.33×10^{8} cfu/g, Charcoal of 11.00×10^{8} cfu /g

and fly ash of 9.00×10^8 cfu /g. (Table.3 and Plate.5)The results clearly indicate that the formulation can be stored for 180 days. Minimum number of cfu was recorded in fly ash at 180 days of storage as compared to other carrier materials. But the population of *P. fluorescens* was well maintained upto 60 days (88.67×10^8 cfu /g). However the population decreased drastically at 90 days of storage. Nakkeeran *et al.* (2006) reported same results that peat, lignite, lignite + fly ash and bentonite paste based formulations maintained higher propagule number of *P. fluorescens* upto 2 months of storage at ambient temperature(28ºc).Fly ash formulation can be used upto 60 days as it will be the cheaper source for *P. fluorescens*.(Fig.1 &Fig.2)

Table :3 Viability of *Pseudomonas fluorescens* in different carriers

Sr. No.	Treatments	Population of *Pseudomonas fluorescens* ($\times 10^8$ cfu/g) of a carrier						
		0 days	30 days	60 days	90 days	120 days	150 days	180 days
1	Talc	86.00	97.33	115.67	86.33	52.00	31.00	18.00
2	SMS	83.33	86.33	97.00	78.00	48.33	25.33	12.33
3	Lignite	84.67	87.67	99.33	80.00	49.33	26.67	14.67
4	Charcoal	84.00	85.67	95.00	64.00	55.67	23.00	11.00
5	FYM	84.33	90.33	108.33	81.67	53.00	28.00	15.67
6	Fly ash	83.00	84.00	88.67	53.67	43.67	21.33	8.00
	'F 'test	NS	Sig	Sig	Sig	Sig	Sig	Sig
	SE(M)±		0.60	0.59	0.62	1.13	1.03	0.45
	CD (p=0.01)		2.59	2.53	2.68	4.90	4.49	1.93

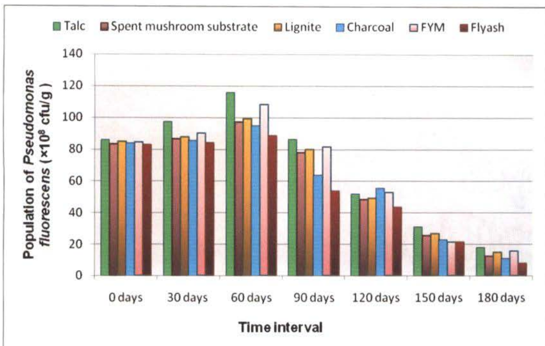


Fig. 1. Viability of *P. fluorecens* in different carrier materials for 180 days at monthly interval.

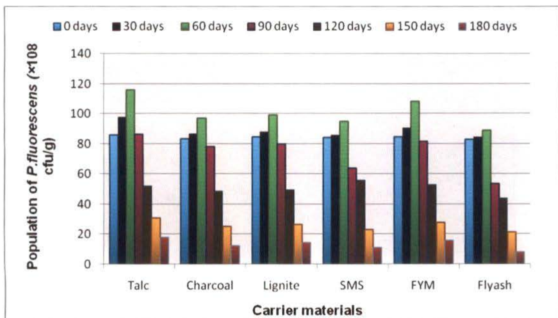
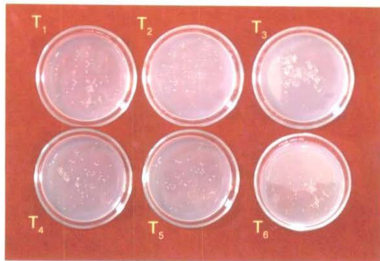


Fig. 2 Population of *P. fluorescence* in different carriers

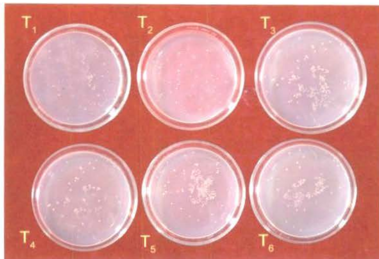
The results indicate that spent mushroom substrate maintained viable population count (78×10^{8} cfu /g) at 90 days of storage. when it compare with lignite and farm yard manure there was little difference in population level was observed (80×10^{8} cfu /g ,81×10^{8} cfu /g) as SMS has good physical factors includes water holding capacity, electrical conductivity (Kim *et al.*,2011) at it is of cheaper cost, we can use SMS as a carrier for *P. fluorescens* . Rebah *et al.* (2007) also used agro by products and agro industrial wastes (plant compost, fliter mud) as a carriers for *Rhizobial* inoculants.

The results of this study are in conformity with the findings of Rajalaxmi *et al.* (2012) who reported talc based formulations of *P. fluorescens* retains mean population of 11×10^{7} cfu/g at 300 days of storage. Caesar and Burr (1991) reported better shelf life of *Pseudomonas fluorescens* (P7NF, TL3) in talc based formulations ammended with sucrose (0.72M) upto 240 days. Sivakumar *et al.* (2000) studied three carriers for the survival of *P. fluorescens* and reported that talc maintained the highest population level of 18.3×10^{7} cfu/g after forty days of storage and Amer and Utkhede (2000) also recorded the shelf life of *P. putida* for 45 days in talc based formulations and the bacterial load was 1.0×10^{3} cfu/g. Bora *et al.*, (2004) observed the survival of *P. putida* strain upto six moths in talc based formulations and the bacterial load at the end of six month was 10^{8} cfu/g of product.

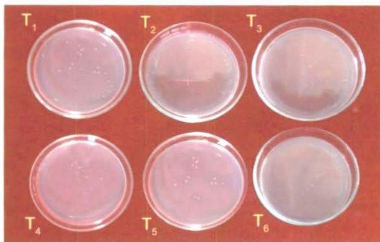
Nakkeeran *et al.* (2005) examined shelf life of *Pseudomonas fluorescens* in talc based formulations and found that it was maintained upto 12 months than in peat, vermiculite, FYM, kaolinite formulations. Mythukumar, (2009) tested the shelflife of *Pseudomonas fluorescens* in seven carriers namely flyash, lignite, peatsoil, ricebran, sugarcane baggasse, talc powder and wheat bran and found that talc based formulations was superior in supporting its survival and recorded a population of 17×10^{7} cfu/g at 150 days of storage.Similar findings were also recorded by Gade *et al.* (2008) in respect of *Trichoderma* spp in talc based formulations.



0 days



90 days



180 days

T_1 = Talc, T_2 = Spent mushroom substrate, T_3= Lignite,
T_4=Charcoal, T_5 = Farm yard manure, T_6= Fly ash

Plate 5: Population of *Pseudomonas fluorescens* in different carrier materials

4.4 Effect of temperature on the growth of *P. fluorescens* in different carrier materials:

Temperature is one of the most important factor that governs the physiology and growth of bacteria. Therefore, the experiment was conducted and growth of bacteria was observed by storing carrier based formulations of *P. fluorescens* at 0^0C, 15^0C,25^0C and 30^0C and cfu count was recorded after 15 days of inoculation and data presented in Table. 4 and Plate.6

4.4.1 Effect of temperature:

Incubation of carrier based formulations of *P. fluorescens* in different temperatures had shown significant effects on its population. Maximum population was observed at 25^0C temperature (85.83×10^8 cfu/g of carrier), while at 30^0C population was poor (70.05×10^8 cfu/g of carrier).Better survival of *P. fluorescens* was observed at 0^0C in all carriers. But 25^0C temperature was found to be favourable for storage of inoculum to induce maximum growth of *P. fluorescens.* (Table 4) and was found significantly superior to all other temperature regimes.

4.4.2 Effect of carriers:

Different carrier inoculants had also significantly affected the population of *P. fluorescens* and showed that significant increase in population of *P. fluorescens* was observed in talc based formulations (86.09×10^8 cfu/g of carrier). Furthermore, inoculants prepared with FYM(A_5),lignite(A_3) and SMS(A_2) also performed well to support the growth of bacteria i.e 81.67×10^8 cfu/g , 79.75×10^8 cfu/g and 78.41×10^8 cfu/g of carrier. Lowest population was recorded in fly ash (75.50 cfu/g)

Table 4. Effect of temperature ranges on the growth of *P. fluorescens* in different carriers.

	Factor-A (carrier)	Population of *P. fluorescens* ($\times 10^8$ cfu/g) of a carriers					Mean of factor A
		At 0 days	After 15 days				
			Factor-B(Temperature)				
			0^0C (B_1)	15^0C (B_2)	25^0C (B_3)	30^0C (B_4)	
A_1	Talc	86.00	87.67	85.00	93.00	78.67	86.09
A_2	SMS	83.33	83.00	77.33	84.00	69.33	78.41
A_3	Lignite	84.67	84.00	79.67	85.33	70.00	79.75
A_4	Charcoal	84.00	83.67	75.33	82.33	65.00	76.58
A_5	FYM	84.33	84.33	81.00	88.33	73.00	81.67
A_6	Flyash	83.00	82.33	73.33	82.00	64.33	75.50
	Mean of factor B		84.17	78.61	85.83	70.05	
				Factor-A	Factor-B	Int (A×B)	
	SE (m)±			0.21	0.24	0.59	
	CD at 1 %			0.80	0.92	2.26	
	'F' test	NS		Sig	Sig	Sig	

4.4.3 Effect of interaction (Carriers × Temperature) :

There were significant differences in survival of *P. fluorescens* among carriers, storage temperatures at initial date to 15 days. The interaction between carriers and storage temperature during 0 to 15 days showed that talc stored at 25^0C gave highest viable count (93×10^8 cfu/g) which was significantly superior to all other treatments. Farm yard manure, lignite and spent mushroom substrate gave 88.33×10^8 cfu/g, 85×10^8 cfu/g and 84×10^8 cfu/g of carrier.(Fig.3)

The factors that affect the longevity of the cells of bioinoculants include temperature, moisture, carrier material, etc. The

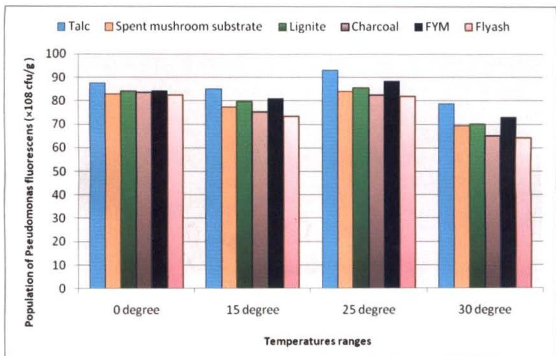


Fig. 3 Effect of temperature ranges on the growth of *P. flourescens* at 15th day of incubation in different carrier materials.

optimum moisture level of 35 to 50 per cent and a temperature of 30°C is required for maximum survival of the cells in the carrier based inoculants for longer period of storage and was found that upto 40°C there was no serious mortality (Baj Pai *et al.*, 1978).

Generally it was experienced that exposure of inoculums at different temperature, gradually declined the bacterial population with increasing temperature (Kandasamy and Prasad, 1971; Baipai *et al.*, 1978; Eckart and Raguse,1980). Wessondorf, (1989) reported lower temperatures (4^0C) showed extended bacterial survival by reducing metabolic activity.

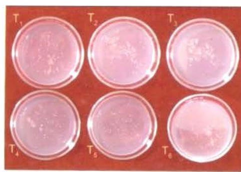
Sandikar and Awasthi. (2009) found that *P. fluorescens* isolate which were stored at 4^0C showed its maximum population. Formulations of *P. fluorescens* stored at 10^0C & 20^0 C provided longer shelf life than those stored at 30^0C (Illibazilah *et al.*, 2011).

Rajyalaxmi (2012) recorded the talc based formulations supports maximum mean population of *P. fluorescens* at 4^0c was 13×10^8 cfu/g than those at 32 ± 2^0c was 11×10^8 cfu/g after 300 days of storage. Sadi and Masoud (2012) reported the same results as above that Longest stability of UTPF61 strain of *P. fluorescens* was observed in talc based formulations which was stored at 4^0c than at 26^0c. The present findings also in line with the findings of Temprano *et al.*, 2002;Sadi and Mousad., 2012; Sangeetha *et al.*, 2012 .It clearly indicates that at o^0C temperature can be congineal for storage but for getting optimum growth 25^0C is preferable.

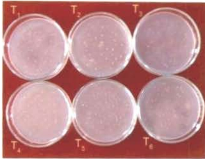
4.5 Effect of light on the growth of *P. fluorescens* in different carrier materials for 15 days:

Light effect and length of day however may affect the survival of inoculum during length of incubation period.

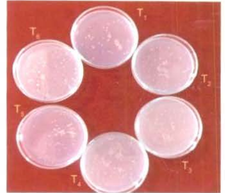
This study indicates the interaction effect between carriers and light for the growth of *P. fluorescens* in different carriers over a period of 15 days of storage and data presented in Table.5 and Plate 6 and also graphically presented in Fig. 4.



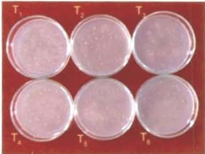
0 days



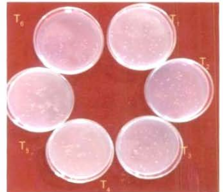
0°C temperature



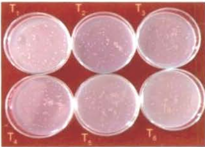
16 hours light + 8 hours dark



15°C temperature

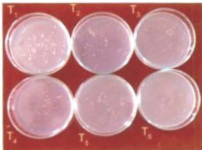


16 hours dark + 8 hours light



25°C temperature

12 hours light + 12 hours dark



30°C temperature

T_1 = Talc, T_2 = Spent mushroom substrate, T_3= Lignite,
T_4=Charcoal, T_5 = Farm yard manure, T_6= Fly ash

Plate 6: Effect of physical factors on the growth of *Pseudomonas fluorescens*

Table 5. Effect of light regimes on the growth of *P. fluorescens* in different carriers

	Factor-A (carrier)	Population of *Pseudomonas fluorescens* ($\times 10^8$ cfu/g) of a carrier				Mean of factor A
		At 0 days	After 15 days Factor –B(light)			
			16hrs light +8 hrs dark (B_1)	8hrs light + 16 hrs dark (B_2)	12hrs light +12 hrs dark (B_3)	
A_1	Talc	86.00	94.67	78.33	90.00	87.67
A_2	SMS	83.33	85.33	72.00	83.33	80.22
A_3	Lignite	84.67	87.33	73.00	84.00	81.44
A_4	Charcoal	84.00	84.67	71.00	82.33	79.33
A_5	FYM	84.33	91.33	75.00	86.33	84.22
A_6	Flyash	83.00	83.33	69.33	78.33	76.99
	Mean of factor B		87.78	73.11	84.03	
			Factor-A	Factor- B	Int (A×B)	
	SE (m)±		0.29	0.34	0.82	
	CD at 1 %		1.13	1.30	3.20	
	'F' test	NS	Sig	Sig	Sig	

4.4.1 Effect of light:

Carrier based formulations of *P. fluorescens* in different light regimes had shown significant effects on its population. Significantly maximum population was observed at 16hrs light +8 hrs dark(B_1) (87.78×10^8 cfu/g of carrier),while at 8hrs light +16 hrs dark(B_2) population was poor (73.11×10^8 cfu/g of carrier). Better survival of *P. fluorescens* was observed at 16hrs light +8 hrs dark(B_1) in all carriers. Thus, 12 hrs light+12 hrs dark was found to be favourable for storage of inoculum to induce optimum growth of *P. fluorescens.*

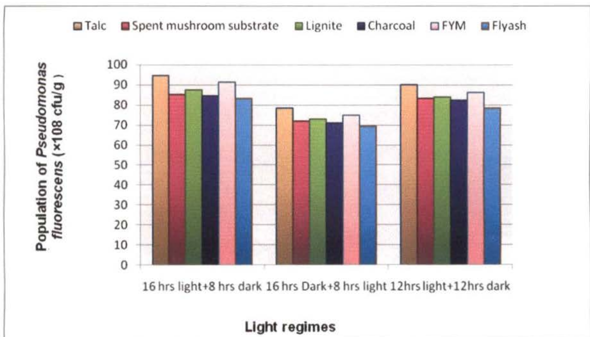


Fig. 4 Effect of light regimes on growth of *P.fluorescens* at 15th day of incubation in different carrier materials

4.4.2 Effect of carriers:

Different carrier inoculants had also significantly affected the population of *P. fluorescens.* Results represented in Table-5 (Mean factor A) showed that maximum population of *P. fluorescens* was observed in talc based formulations (87.67×10^8 cfu/g of carrier). Furthermore, inoculants prepared with FYM (A_5),lignite (A_3) and SMS(A_2) also performed well to support the growth of bacteria i.e 84.22×10^8 cfu/g , 81.44×10^8 cfu/g and 80.22×10^8 cfu/g of carrier. Population of *P. fluorescens* in the range of 79.33×10^8 cfu/g to 76.99 $\times10^8$ cfu/g was recorded incase of remaining carriers.

4.4.3 Effect of interaction (Carriers×Light) :

There were significant differences in survival of *P. fluorescens* among carriers, light regimes at initial date to 15 days and interaction. The interaction between carriers and storage temperature during 0 to 15 days showed that talc stored at 16hrs light+8hrs dark gave highest viable count (94.67×10^8 cfu/g) and was found significantly superior to all other treatments. However farm yard manure, lignite and spent mushroom substrate (91.33×10^8 cfu/g, 87.33×10^8 cfu/g and 85.33×10^8 cfu/g) also showed better survival of *P. fluorescens*. The present study clearly indicated that 16hrs light +8hrs dark will be good for survival of *P. fluorescens.* Konstantinova (1991) stated that there is correlation between sporulation of *Trichoderma spp* and light regimes and also Wardhe (2007) reported that growth of *Trichoderma* was faster at alternate hours of 12hrs light and 12 hrs dark.

CHAPTER V

SUMMARY AND CONCLUSIONS

Pseudomonas fluorescens belong to Plant Growth Promoting Rhizobacteria (PGPR), the important group of bacteria that play a major role in the plant growth promotion, induced systemic resistance, biological control of plant pathogens *etc.*

Pseudomonas fluorescens isolate was collected from Department of Plant Pathology Dr. PDKV Akola then maintanence of these isolate was done. It was selected on the basis of their morphological and biochemical tests.

The isolate was Gram negative and rod shaped and positive for catalase and also showed its ability to hydrolyze starch, which was evident from the zones formed. The isolate also showed positive reaction against citrate utilization which was evident from the blue zone formed and also showed positive reaction against KOH test and it showed positive results for IAA production and siderophore production.

Physico chemical properties like pH, organic matter content, water holding capacity and electrical conductivity of carier materials was determined. The talc has high water holding capacity and low bulk density values followed by Spent mushroom substrate.

Viability study was undertaken to asses the suitability of different carrier materials. The carriers used were talc, spent mushroom substrate, lignite, charcoal, farm yard manure and flyash. The cfu count was taken upto 180 days in three replicates. Generally in all carriers survival of *P.fluorescens* was decreased over a different period of storage. Results showed that talc was found as best carrier among all the carrier material to give maximum viable cfu count at 180 days after storage while flyash was recorded minimum cfu count.

Among four test temperatures 25^0c proved best for maximum growth of *P. fluorescens* in all carriers. Considering interaction between temperature and carriers for the growth of

P.fluorescens after 15 days, significant results were observed. Talc maintained good survival of bacteria followed by FYM, lignite, SMS, charcoal and flyash.

Effect of different light regimes on growth of *P. fluorescens* was examined and observed that light is the limiting factor for its growth. At 16hrs light and 8hrs dark maximum growth was recorded in all carriers and talc

Conclusions

1. *Pseudomonas fluorescens* was rod shaped and gram negative. It showed positive reaction for gelatine liquefaction, starch hydrolysis, citrate utilization, catalase activity and KOH test
2. *P. fluorescens* was positive towards siderophore production and IAA production.
3. PH of all carriers was in range of 6.9 to 7.3.Maximum organic carbon content was found in Farm yard manure. Maximum water holding capacity (189%),low bulk density (0.3 g/cm3) was found in talc .
4. Among all treatments, maximum mean population of *P. fluorescens* (18×10^{8} cfu/g) was observed in talc based formulations followed by FYM (15.67×10^{8} cfu/g) at 180 days of storage. However, spent mushroom substrate also retains viable mean population upto 90 days. As it is very cheap and locally available, it can be used as a carrier for short storage period.
5. Population of *P. fluorescens* was well maintained in flyash upto 60 days . Therefore it can be used as carrier upto 60 days, moreover it will be the cheaper source as a carrier for *P .fluorescens*
6. Among all temperature ranges, 0^{0}c degree temperature better survival and at 25 degrees temperature better growth of *Pseudomonas fluorescens* was observed in all carrier materials
7. Among all light ranges, 16hrs light+ 8hrs dark was better for the growth *Pseudomonas fluorescens* in all carrier materials than at 12hrs light+ 12hrs dark and 16hrs dark+8hrslight.

CHAPTER VI

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APPENDIX- I

1) Media

i) King's B medium content

Peptone	-	20 g
K_2HPO_4	-	1.5 g
$MgSO_4$	-	1.5 g
Glycerol	-	10 ml
Agar	-	15 g
Distilled water	-	1000 ml

ii) Nutrient agar media

Beef extract	-	3 g
Yeast extract	-	2 g
Peptone	-	5 g
Sodium chloride	-	5 g
Agar	-	20 g
Distilled water	-	1000 ml

iii) Simmons citrate agar media

Ammonium dihydrogen phosphate	-	1g
Dipotassium hydrogen phosphate	-	1g
Sodium chloride	-	5 g
Sodium citrate	-	2 g
Magnesium sulphate	-	0.2 g

Bromtymol blue	- 0.08 g
Agar	- 15 g
Distilled water	-1000 ml

v) Starch agar

Starch (soluble)	- 20 g
Peptone	- 5 g
Beef extract	- 3 g
Agar	- 15 g
Distilled water	- 1000 ml