"ISOLATION AND IDENTIFICATION OF ENDOPHYTIC BACTERIA FROM GARLIC

A

PROJECT REPORT

BY

MS. ASHWINI SHIVAJI KHOT

DEPARTMENT OF MICROBIOLOGY

VIVEKANAND COLLEGE, KOLHAPUR
(AUTONOMOUS)

KOLHAPUR – 416003

2021-2022

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ROLL NO :- 5044

"Dissemination of education for Knowledge, Science and Culture"

-Shikshanmaharshi Dr. Bapuji Salunkhe

Shri Swami Vivekanand Shikshan Sanstha's

VIVEKANAND COLLGE, KOLHAPUR (AUTONOMOU)

Department of Microbiology

LABORATORY CERTIFICATE

This is to certify that **Ms. Ashwini Shivaji Khot** studying in B. Sc. III Microbiology at Vivekanand College, Kolhapur. She have sincerely completed Project Work entitled as **"ISOLATION AND IDENTIFICATION OF ENDOPHYTIC BACTERIA FROM GARLIC"** prescribed by Vivekanand College, Kolhapur during academic year 2021 –2022.

Mr. S. D. Gabale
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ACKNOWLEDGEMENTS

I wish to express my deep sense of appreciation to Prof. Mr. S. D. Gabale Department of Microbiology, Vivekanand College, Kolhapur for his valuable support and expert guidance during the course of this study. He has been extremely understanding and cooperative and has always taken great interest in this work.

I wish to express my sincere thanks to Head of the Microbiology Department Dr. G. K. Sontakke and Principal Dr. R. R. Kumbhar, Vivekanand College, Kolhapur for providing the laboratory facilities in the Department to carry out the experimental work.

I express my thanks to my teachers, Ms. V. V. Misal and Ms. S. A. Pise for their valuable suggestions and help during the work. My special thanks to my teacher Mr. S. D. Gabale for giving me the culture for study and his expert guidance throughout.

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Finally I thank my family members who had enclosed upon their blessing and moral and economical support because of which this work has proved satisfactory to me.

Place: Kolhapur

Date:

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Place: Kolhapur

Date: **Ms. Ashwini Shivaji Khot**

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CHAPTER - 1

INTRODUCTION

INTRODUCTION

Garlic (Allium sativum) is a species in the onion genus, Allium. Its close relatives include the onion, shallot, leek, chive, and Chinese onion. Garlic is native to Central Asia and northeastern Iran, and has long been a common seasoning worldwide, with a history of several thousand years of human consumption and use. It was known to ancient Egyptians, and has been used both as a food flavoring and as a traditional medicine [McGee, Harold 2004].

Garlic (Allium sativum L), a member of the family Alliaceae, enjoys the reputation of “antibiotics grown out of the land” [Raghu, Lu, & Sheen, 2012]. Rahman (2007) reported that fructose-containing carbohydrates were the main component of dry garlic, followed by sulfur compounds, proteins, fibers, and free amino acids. Garlic has a wide range of purposes on account of high nutritional value and unique flavor, regarded as one of the daily best healthy food, as demonstrated9by some researchers [Ban et al., 2009; Benkeblia, 2004; Kim, 2016; Raghu et al., 2012].

Symbiotic interaction is the driving force in an ecosystem. Symbiosis ranges from parasitism to mutualism & includes everything in between. The fitness of outcomes for plants differs accordingly. If a plant is highly susceptible to pathogens, it's fitness is likely to be low in pathogen reach environments; if a plant cooperates with mutualism, it is likely to thrive even in adverse environments. Bacteria, which colonize the interface between living plant route & soil, namely the rhizosphere are abundant symbiotic partners of plants. These so called "Rhizobacteria" are said to be plant growth promoting (PGP). These microbes able to colonize plant root internally without negatively affecting the host are called as "Endopytes" [Schulz & Boyle 2005]. Although all of the approximately 300000 plants species have been estimated to harbor one or more

endophytes [Strobel et al, 2004] very few such relationship have been studied in details.

"Endophytes can be defined as those microbes that colonize the internal tissues of healthy plants, showing no obvious external sign of infection or negative effect on their host". There have been a hundred years of history on the research of endophytes, with endophytes found in almost every plant studied [Ryan, Germaine, Franks, Ryan, & Dowling, 2008]. Plant endophytes which coexist with host plants for a long term can produce a series of the same bioactive secondary metabolites as the host10plants, such as antitumor bioactive substances, with great potential for medical, agricultural, and industrial exploitation [Kim et al. 2007].

Endophytic bacteria associated with plants:

Endophytic bacteria have been found associated with nervous plant species, with most being members of common soil bacterial genera such as *pseudomonas, Bacillus, & Azospirillum* [Chanway 1996]. As cited extensively by Kobayashi & Palumbo (2000), endophytic bacteria exits is variety of tissues type within numerous plant species, suggesting ubiquitous existence in most in not all higher plant species. Moreover, endophytic bacteria have been isolated from both monocotyledon & dicotyledon, woody tree species such as oak [Brooks et al 1985] & pear [Whiteside & Spotts 199] to herbaceous crop plants such as sugar beets [Jacob et al 1985] & maize [Fischer et al 1992 ; Mc Inroy & Kloepper 1995]

The role of endophytic bacteria:

Bacterial endophytes can accelerate seeding emergence, promote plant establishment under adverse conditions & enhance plants growth [Chanway &

Bent 1998]. Endophytic bacteria are believed to elicit plant growth promotion in one of two ways; either-

1) Indirectly by acquire nutrients, via nitrogen fixation, phosphate solublization [Wakelin et al 2004] or iron chelating [Costa & Loper 1994], by preventing pathogen infection via antifungal or antibacterial agents, by out-competing pathogens for nutrients by siderophore production or by establishing the plants systemic resistance [Van Loon et al 1998] or

2) Directly by producing phytoharmones such as auxins or cytokinins [Mabhaiyan et al 2006], or by producing the enzymes 1 - aminocyclopropane - 1 carboxylate (ACC) deaminase, which lowers plant ethylene level [Click 1965]. In addition to these plant growth promoting traits, endophytic bacteria must also be Compatible with host plant & able to colonize the tissues of the host plants without being recognized as pathogens [Rosenblueth 2006]. A particular bacterium may affect the plant growth & development using one or more of these mechanisms, & may use different ones at various times during the life cycle of the plant [Long et al 2008].

Genomics of endophytic bacteria:

Endophytic bacterial genome sequences have been published; however, genome sequencing of a number of endophytes including *Enterobacter sp*.638, *Stenotrophomonas maltophilia* R551-3, *Pseudomonas putida* W619, *Serratia proteamaculans* 568 and *Methylobacterium populi* BJ001 is underway at the United States Department of Energy Joint Genome Institute (www.jgi.doe.gov). Recently, the complete genome sequence of the nitrogen-fixing endophyte, *Azoarcus sp.* strain BH72 [Hurek & Reinhold Hurek., 2003, Krause et al., 2006] has been compared with that of the related soil bacterium strain *Azoarcus sp.* strain EbN1 and other plant-associated bacteria. The BH72 genome lacks genes encoding type III and type IV secretion systems, toxins, nodulation factors,

common enzymes that hydrolyses plant cell walls and the N-acyl homoserine lactone-based quorum-sensing system, which is found in many plant-associated bacteria and plant pathogens [Rainey, 1999; Preston et al., 2001; Buttner & Bonas, 2006]. However, Krause et al. (2006) identified other factors encoded by the BH72 genome that may be involved in the host interaction. These include type IV pili, surface polysaccharides, type I and II protein secretion systems, flagella and chemotaxis proteins and a large number of ferric siderophore uptake systems. The BH72 genome provides valuable insights into the biology of bacterial endophytes, and as more endophyte genome sequences become available, this will provide a rational basis to design experiments to investigate the mechanisms involved in successful endophyte colonization.

Plant growth promoting endophytes:

Research has been conducted on the plant growth-promoting abilities of various rhizobacteria. They differ from biocontrol strains in that they do not necessarily inhibit pathogens but increase plant growth through the improved cycling of nutrients and minerals such as nitrogen, phosphate and other nutrients. Endophytes also promote plant growth by a number of similar mechanisms. These include phosphate solubilization activity [Verma et al., 2001; Wakelin et al., 2004], indole acetic acid production [Lee et al., 2004] and the production of a siderophore [Costa & Loper, 1994]. Endophytic organisms can also supply essential vitamins to plants (Pirttila et al., 2004). Moreover, a number of other beneficial effects on plant growth have been attributed to endophytes and include osmotic adjustment, stomatal regulation, modification of root morphology, enhanced uptake of minerals and alteration of nitrogen accumulation and metabolism [Compant et al., 2005]. The recent areas where these plant growth-promoting bacterial endophytes are being used are in the developing areas of forest regeneration and phytoremediation of contaminated soils.

Biocontrol and endophytic:

Endophytic bacteria are able to lessen or prevent the deleterious effects of certain pathogenic organisms. The beneficial effects of bacterial endophytes on their host plant appear to occur through similar mechanisms as describes for rhizosphere-associated bacteria. These mechanisms have been reviewed in great detail by Kloepper et al. (1999) or, more recently, by Gray & Smith (2005) and Compant et al. (2005). Diseases of fungal, bacterial, viral origin and in some instances even damage caused by insects and nematodes can be reduced following prior inoculation with endophytes [Kerry, 2000; Sturz et al., 2000; Ping & Boland, 2004; Berg & Hallmann, 2006]. It is believed that certain endophyte bacteria trigger a phenomenon known as induced systemic resistance (ISR), which is phenotypically similar to systemic-acquired resistance (SAR). SAR develops when plants successfully activate their defense mechanism in response to primary infection by a pathogen, notably when the latter induces a hypersensitive reaction through which it becomes limited in a local necrotic lesion of brown desiccated tissue [Van Loon et al., 1998]. ISR is effective against different types of pathogens but differs from SAR in that the inducing bacterium does not cause visible symptoms on the host plant [Van Loon et al., 1998].Bacterial endophytes and their role in ISR have been reviewed recently by Kloepper & Ryu (2006).13

Route of entry of endophytes and their colonization:

Endophytes can enter in to the plant tissue primarily through the root part area. However arial parts or upper portion (except root) like flowers, leaves, stems and cotyledons also can used for entry of endophytes too [Zinniel et al 2002].

Specifically, the bacteria enter tissue via germinating radicals, secondary roots, stomata or a result of foliar damage. Endopytes inside a plant may either

become localized at the point of entry or spread throughout the plant. These microorganisms can reside within cells, in the intracellular spaces, or in the vascular system. Generally bacterial populations are larger in roots and decrease in the stems and leaves. Natural endophytic concentration can vary between 40 to 250 Cfu per gram [Zinniel et al 2002].

Bacterial endophytes can be isolated from surface disinfected plant tissue or extracted from internal part of tissue.

Interest in the use of the microorganisms for biological control of plant diseases has increased to the growing environmental and health concerns created by the use of pesticides. The importance of environmental plant protection methods has been greatly emphasized in sustainable agriculture. The resent increase in publication on bacterial endophytes reflects an interest in their potential benefits in agriculture [Kobayashi n Palumbo 2000].

Endophytic bacteria help plants by preventing pathogen infection via antifungal or antibacterial compounds produced by them. They greater increased resistance I plants to pathogens and parasites. Some species such as *Curtobacterium luteum* and *Bacillus amyloliquefaciens* were reported to control plant pathogenic bacteria like *Clavibacter michiganensis* and *Erwinia carotovara* [Van Buren et al 1993 and Sturz and Matheson 1996].

Wild relatives of eggplants (*Solanum spp.*) are well known to be resistance to some major soil borne phytopathogens such as bacterial wilt caused by *Ralstonia solanacearum* and *Verticillum* wilt [Kondou at al 2001]. *Solanum aethiopicum* was reported to carry resistance to bacterial wilt, one of the most important diseases of egg plant (*Solanum melongena*) and traits of resistance against bacterial wilt have also been identified in *solanum torvum*, *S. Sisymbrifolium* and *S. aethiopicum* [Collonier et al 2001]. However the

resistance mechanism associated with *Solanum spp*. has not been elucidated and little attention was paid to *Solanum spp*. endophytic bacterial population.

Other studies on endophytic bacteria:

Zinger:

Microbially unexplored medicinal plants can have diverse and potential microbial association. The rhizome of ginger is very remarkable because of its metabolite richness, but the physiological processes in these tissues and the functional role of associated microorganisms remain totally unexplored. Through the current study, the presence of four different endophytic bacterial strains was identified from ginger rhizome. Among the various isolates, ZoB2 which is identified as Pseudomonas sp. was found to have the ability to produce IAA, ACC deaminase and siderophore. By considering these plant growth promoting properties, ZoB5 can expect to have considerable effect on the growth of ginger. [B. Jasim, Aswathy Agnes Joseph, C. Jimtha John, Jyothis Mathew, E. K. Radhakrishnan, 2014]

Moso Bamboo:

We analyzed cultural endophytic bacteria from Moso bamboo (Phyllostachys edulis) using traditional bacterial isolation and culture methods and then studied the colony characteristics and diversity with a 16S rRNA sequence analysis. We isolated 82 endophytic bacteria strains belonging to 47 species in 26 genera from the root, rhizome, stem and leaves of Moso bamboo species from populations on Wuyi Mountain, and in the Jiangle and Changting regions. There were significant differences in the composition of the culturable endophytic bacteria isolated from the different areas and from different tissues. The dominant bacteria strains from the Wuyi Mountain samples were *Arthrobacter*, *Staphylococcus*, *Bacillus* and *Enterobacter*, while the dominant bacteria from the Jiangle samples were *Bacillus*, *Staphylococcus* and

Curtobacterium, and the dominant bacteria in the Changting samples were *Alcaligenes, Pseudomonas, Staphylococcus* and *Bacillus*. Our results demonstrate the abundant diversity of endophytic bacteria in Moso bamboo. [Zong-Sheng Yuan, Fang Liu and Guo-Fang Zhang, 2015.]

Chili:

The antagonistic potentials of endophytic bacteria isolated from chili plants were determined in vitro against four pathogens, viz., *Sclerotium rolfsii, Fusarium oxysporum, Colletotrichum capsici* and *Pythium spp*. The effect of endophytic bacteria towards these fungi revealed that most of the isolates showed antagonistic activity against *Pythium spp.* (37.8%), followed by 35.1% isolates against *F. oxysporum* and *C. capsici*, and 21.6% to *S. rolfsii.* The identification of potential bacterial isolates through Microbial Identification System (Biology) and 16S rRNA sequencing of the isolates revealed the presence of 8 genera. Among them Bacillus species were the dominant antagonists. Characterized by BOX-PCR fingerprints, the 23 antagonistic endophytic bacterial (AEB) isolates represented 19 different cluster types. To explore the antagonistic mechanisms, the agar diffusion method was used to detect cell-wall degrading enzyme activity and siderophore secretion. The isolates BECS7, BECS4 and BECL5 showed clearly the growth promoting activity, reduction of disease incidence and high yield under field conditions. Hence, these isolates are promising plant growth promoting isolates showing multiple attributes that can significantly influence the chilli growth. The results of present study provide a strong basis for further development of this strain as bio-inoculants to attain the desired plant growth promoting activity in chili growing fields.

Common Bean

The common bean is one of the most important legumes in the human diet, but little is known about the endophytic bacteria associated with the leaves of this plant. The objective of this study was to characterize the cultural endophytic bacteria of common bean (*Phaseolus vulgaris*) leaves from three different cultivars (Vermelhinho, Talisma, and Ouro Negro) grown under the same field conditions. The density of endophytic populations varied from 4.5 x 10(2) to 2.8 x 10(3) CFU /gm of fresh weight. Of the 158 total isolates, 36.7% belonged to the *Proteobacteria*, 32.9% to *Firmicutes*, 29.7% to *Actinobacteria*, and 0.6% to *Bacteroidetes*. The three *P. vulgaris* cultivars showed class distribution differences among *Actinobacteria, Alphaproteobacteria* and *Bacilli*. [Oliveira Costa LE, et al. Braz J Microbiol, Oct. 2012]

CHAPTER 2

AIMS AND OBJECTIVE

AIMS & OBJECTIVES:

Endophytes:

Microorganism able to colonize plant root internally without negatively affecting the host are called as "Endopytes" [Schulz & Boyle 2005].

Roles of endophytes:

Bacterial endophytes can accelerate seeding emergence, promote plant establishment under adverse conditions & enhance plants growth [Chanway & Bent 1998]. Endophytic bacteria must also be Compatible with host plant & able to colonize the tissues of the host plants without being recognized as pathogens [Rosenblueth 2006]. A particular bacterium may affect the plant growth & development using one or more of these mechanisms, & may use different ones at various times during the life cycle of the plant [Long et al 2008].

Endophytic bacteria are believed to elicit plant growth promotion indirectly by acquire nutrients, via nitrogen fixation, phosphate solublization [Wakelin et al 2004] or iron chelation [Costa & Loper 1994], by preventing pathogen infection via antifungal or antibacterial agents, and Directly by producing phytoharmones such as auxins or cytokinins [Mabhaiyan et al 2006], or by producing the enzymes 1 - aminocyclopropane - 1 carboxylate (ACC) deaminase, which lowers plant ethylene level [Click 1965]. In addition to these plant growth promoting traits.

Garlic was shows much best antimicrobial activity, but there are some bacteria, fungus, viruses can colonize in their tissue or in their cells to live. It shows resistance against garlic.

Endophytes studied from Potato, Chili, Moso bamboo, Zinger, Rice seed, common bean. However there are very few reports on endophytes from Garlic and their role. Therefore to fulfill this aim following objectives were defined;

1. Collection of sample of garlic.
2. Isolation of endophytic bacteria from garlic.
3. Studies on morphological, cultural, biochemical and physiological characteristics of the isolates.

CHAPTER 3

MATERIAL AND METHODS

METHODS AND MATERIALS:

Collection of sample:

Garlic plant collected during January of 2018, from Banvadi road, behind the Holly family school, Vidyanagar, Karad. Garlic plant was just pull out from the soil surface of the farm, in such a way that the whole garlic bulb was obtained and that plant was taken in to the sterile plastic bag to the laboratory.

Preparation of sample:

Healthy white garlic was chosen, with the outermost epidermis removed. The pre-sterilization procedure was conducted in a sterile Petri plate as follows:

Initial sterilization with 75% alcohol for 10 min.

↓

Soak with 0.3% NaClO for 20 min.

↓

Immersion with 75% alcohol for 20 min.

↓

Rinse with sterile water for 20 min twice.

↓

Dry for 5 min.

↓

25 g of dried garlic sample was homogenized with 225 ml of sterile saline (0.9% NaCl, w/v).

↓

The mixture was stood for 15 min subsequently,

↓

Then 0.1 ml of this mixture was spread inoculated on the surface of plates containing Beef extract peptone agar, and Gauze's medium no. 1.

↓

Then incubated at room temperature for 2 days.

↓

After colony growth, the single colonies were picked up to two corresponding medium. Cultured at room temperature for 48 hours. [Biscola et al. Wei et al. 2013]

Storage and Maintenance of the pure culture:

The isolated organisms were streaked on the Beef extract peptone agar and Gauze's medium no. 1 agar slants. Further the slants were incubated at room temperature for 48 hours. Then the pure culture is then stored in the refrigerator at 4°C and the slant was subcultured periodically and the culture was maintained.

Characterization of endophytic bacteria:

1. **Colony characters**

The colony characterization of the endophytic bacteria on Beef extract peptone agar Beef extract peptone agar & Gauze's medium no. 1 plates were observed and recorded.

2. **Morphological Characterization:**

The fresh suspension of each isolates from the plates were used to study Gram staining by Hucker - Cohn (1923) Gram staining method and motility was observed by hanging drop motility test and sporulation property by Dorner's staining method, [Desai and Desai , 1980].

3. **Biochemical characteristics:**

Test for Biochemical and physiological characteristics of isolates were carried out as described in standard methods.

A. Hugh and Leifson's Test:

Two tubes with Hugh and Leifson's agar medium were stab inoculated with each isolates was inserted. One tube overlayed with sterile paraffin oil up to 2cm above medium. The tubes were incubated at 37°C for 24 hours. After incubation color changes from bluish green to yellow was taken as positive.

B. Carbohydrate Utilization Test:

The one loopful suspension of each isolate was inoculated in Norris (1965) nutrient agar medium containing 1% solution of carbohydrates such as glucose, sucrose, maltose, mannitol, galactose, with bromothymol blue (BTB) as an indicator and inverted Durham's tubes. Tubes were incubated at 37°C for 24 hours and observe the change in the color and acid gas production in the tube.

C. Indole Production Test:

Loopful of suspension of each isolate was inoculated into sterile 1% tryptone water medium and was incubated at 37°C for 48 hours. After incubation first xylene was added and then 1ml Kovacs reagent. Positive test was indicated by development of pink colored ring at top of medium.

D. Methyl Red Test:

Loopful suspensions of each isolate were incubated in to sterile glucose phosphate broth medium and were incubated at 37°C for 24 hours. After incubation 5 drops of the methyl red indicator was added. Positive test was indicated by development of red color in the medium.

E. Voges Proskauer test:

Loopful suspension of each isolate was incubated in to sterile glucose phosphate broth medium and was incubated at 37°C for 24 hours. After incubation 0.6ml of α- naphthol and 0.2ml of 40% of KOH were added. Positive test was indicated by development of red color in the medium.

F. Citrate Utilization Test:

Sterile Koser's broth was prepared. Loopful suspension of each isolate

was inoculated in sterile Koser's citrate broth tube and was incubated at 37°C for 24 hours. After incubation tubes were observed for turbidity in the tubes.

G. Enzymatic Test:

1. Catalase Test:

One ml of 30% of hydrogen peroxide was taken in a small test tube and growth of each isolates was picked up with sterile nichrome wire loop and dipped into 30% hydrogen peroxide containing test tube for observations of evolution of the gas bubbles for the positive test. [Rakesh patel., 2008].

2. Gelatin hydrolysis test:

The nutrient agar medium containing 1% gelatin was prepared. One Loopful suspension of test culture was spot inoculated in gelatin agar plate. The plates were then incubated at 37°C for 24 hours. After incubation plates were flooded with Frazier's reagents [Harrigan 1976] and observed for zone of clearance around the colonies against the opaque background and indicated the gelatin hydrolysis activity.

3. Casein Hydrolysis Test:

A loopful suspension of test culture was spot inoculated on the milk agar plate separately with each isolate and plates were incubated at 37°C for 24 hours. After incubation clear zone was observed around the colony indicated the casein hydrolysis activity [Bradshaw, 1979].

4. Starch Hydrolysis Test:

Starch plate was prepared by using 1% starch in nutrient agar medium. A loopful suspension of test culture was spot inoculated at 37°C temperature for 24 hours. To test the amylase activity of isolates the plates flooded with iodine solution. The clear zone of hydrolysis with purple background indicated the amylase activity [Aneja, 1990].

5. Urease Production Test:

A loopful suspension of each isolate was streak inoculated on sterile Christensen's urea agar slant and incubated at 37°C for hrs. The slant color was change to pink and indicated the urea hydrolysis.

CHAPTER 4

RESULT AND DISCUSSION

RESULT AND DISCUSSION

1. Collection of sample:

Garlic plant was just pull out from the soil surface of the farm, in such way that the whole garlic bulb was obtained and that plant was taken in to the sterile plastic bag to the laboratory. The sample was stored at 4°C in refrigerator till further use.

2. Isolation of endophytic bacteria:

The two isolates were obtained on Beef extract agar and Gauze's medium no. 1. Then the isolates were coded as G-1 and G-2. Isolated organisms were streaked on the Beef extract agar and Gauze's medium no. 1. Slants. After streaked on the nutrient agar slants. Further the slants were incubated at 30°C for 48 hours. Then the pure culture is then stored in the refrigerator at 4°C and the slant was subcultured periodically and the culture was maintained.

Characterization of Endophyte:

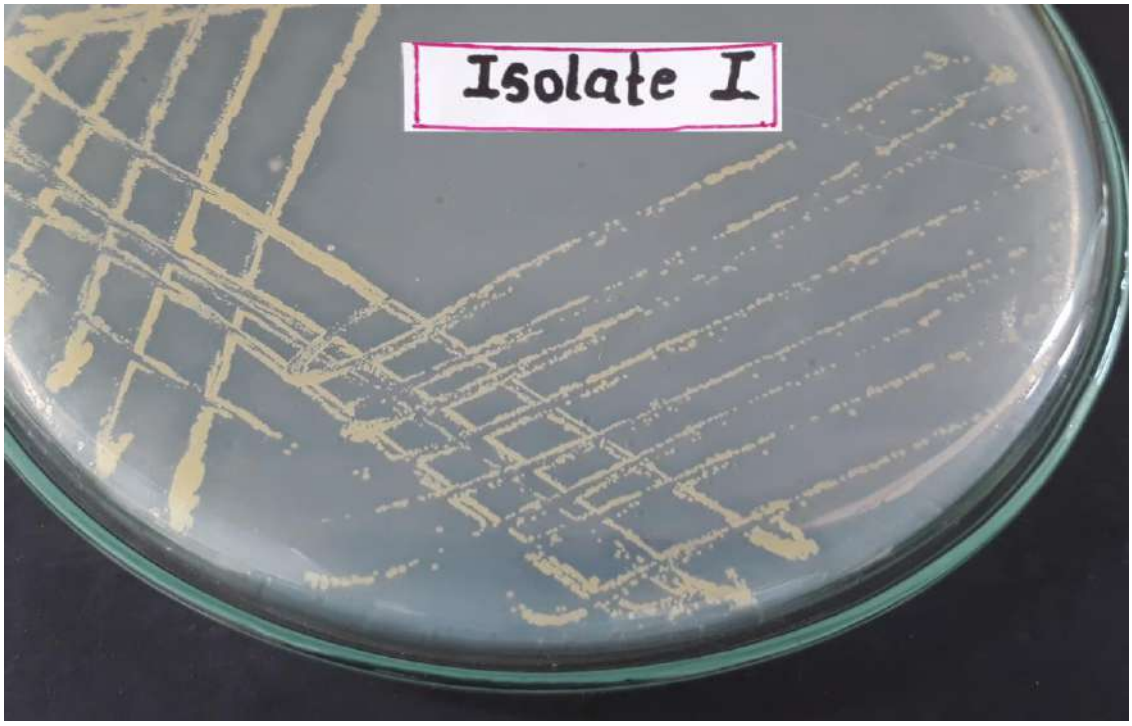
Colony characteristics:

The colony characters of the isolates on BPA medium were presented in Table no. 1.

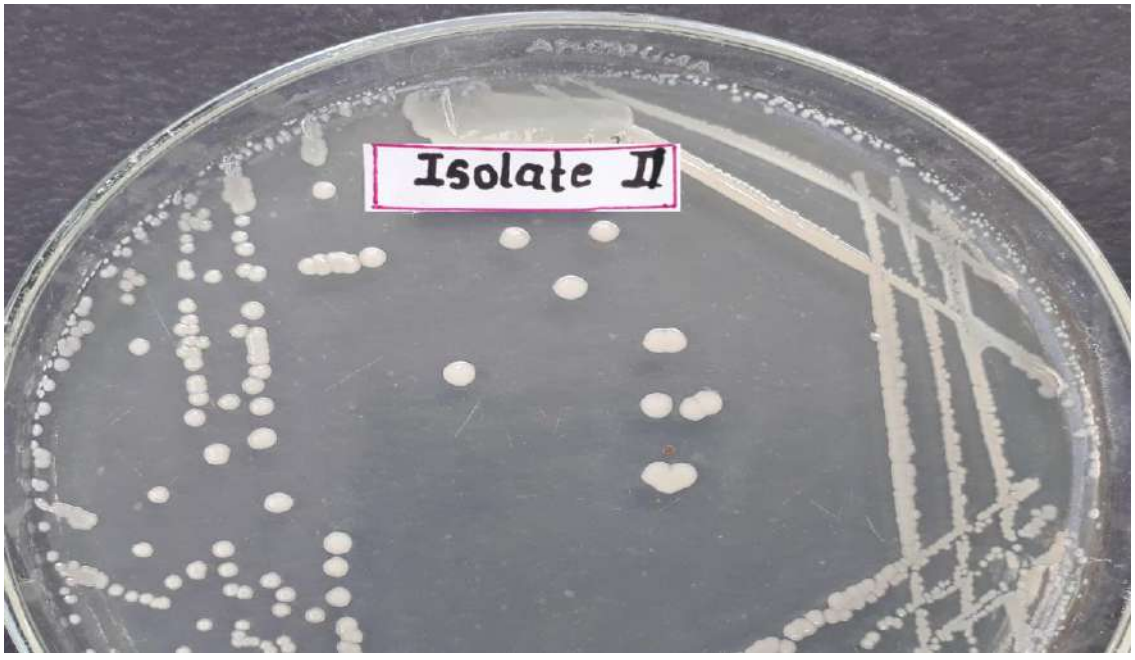
Table no. 1 – Colony characteristics of the endophytic bacteria isolated on sterile Beef Extract Peptone Agar plates.

Isolate Code	Size	Shape	Color	margin	elevation	Opacity	consistency
G1	1mm	Circular	Yellow	Entire	convex	Opaque	moist
G2	2mm	Circular	White	Entire	flat	Translucent	Moist

The shape of colonies of the isolates in the above table was circular. The isolates showed 1mm in size, circular in shape, entire margin with moist consistency. The isolate G-1 has yellow color, convex elevation, and opaque density. While the isolate G-2 showed white color, flat elevation, translucent opacity.



Photograph No. 1: Colonial Morphology of isolate 1 (G-1) on Beef extract peptone agar medium.



Photograph No. 1: Colonial Morphology of isolate 2 (G-2) on Gauze's medium no. 1.

Morphological Characteristics:

On Gram Staining, all the isolates appeared Gram positive, Bacilli and Cocci, non motile and non spore former.

Table no. 2: Gram property, morphology and motility properties of the isolates:

Property	Isolate Code	
	G -1	**G -2**
Gram Staining	Gram positive	Gram positive
Morphology	Cocci	Bacilli
Motility	Non motile	Non motile
Endospore staining	Non spore former	Non spore former

2. Huge and Leifson's Test:

Fermentative and oxidative ability of isolates were studied by using Huge and Leifson's Test, the results are shown in table no.3

Table No 3: Huge and Leifson's Test:

Isolate code	Incubation Condition	
	Aerobic condition	**Anaerobic condition**
G -1	**+**	**-**
G -2	**-**	**+**

(Note: + = positive; - = negative)

2. Carbohydrate Utilization:

All the isolates were studied for their carbohydrate utilization capacity. The results were listed in the table no. 4

Table No. 4: Carbohydrate Utilization:

Sr. No	Sugars	Isolate Code	
		G -1	G -2
1.	Glucose	+	+
2.	Sucrose	-	-
3.	Maltose	-	+
4.	Mannitol	-	-
5.	Galactose	-	-
6.	Lactose	-	-

(Note: - = No acid production, + = Acid production)

From the above table it can be seen that Isolate G-1 and G-2, ferment Glucose sugar with acid production, while the isolate G-1 shows remaining sugars negative result.

Isolate G-2 fermented glucose, maltose, sugars with acid production, while the remaining sugars shows negative results.

Biochemical characters:

All the isolates were studied for biochemical characters the results are listed in the table no. 5.

Table No 5: Biochemical characters of the isolates:

Sr. No	Biochemical Test	Isolate Code	
		G -1	G-2
1.	Indole Production Test	-	-
2.	Methyl Red	-	-
3.	Voges Proskeur	-	+
4.	Citrate utilization	-	+

(Note: + = Positive; - = Negative)

From the above table it can be seen that both isolates G-1 shows all test negative, isolate G-2 shows Indole production test and methyl red test negative while VP and citrate utilization test positive.

1. Study of Enzymatic Properties of isolates:

All the isolates were studied for the enzymatic activities. The results were listed in table no.6

Table No. 6: Enzymatic Characteristics of endophytic Bacteria:

Sr. No	Test	Isolate Code	
		G -1	G -2
1.	Catalase Production	+	+
2.	Oxidase test	+	-
3.	Gelatin Hydrolysis	+	-
4.	Starch Hydrolysis	+	+
5.	Casein Hydrolysis	+	-
6.	Urea Hydrolysis	+	-
7.	Arginine Hydrolysis	-	-

(+ = Positive; - = Negative)

From the above isolates, isolate G-1 shows all test positive except Arginine Hydrolysis, while the isolate G-2 shows Catalase production test and starch hydrolysis test positive and remaining tests negative.

2. Tentative identification of isolates and detection of isolates:

From all morphological, cultural and biochemical character using Bergey's manual two isolates were tentatively identified. Results are cited in the table no 7:

Table No. 7: Tentative identification of the isolates:

Sr. No.	Isolates Code	Tentative Name
1.	G – 1	*Micrococcus spp.*
2.	G – 2	*Bacillus spp.*

From the above table it can be seen that isolate G-1 was resemble with *Micrococcus spp.* and *Bacillus spp*.

Comparison between the isolated endophytes with other endophytic bacteria present in the garlic:

Compared with the positive controls, *Trichodermin hasbroad*- spectrum and strong antifungal activity microorganisms from garlic that can produce bioactive compounds isolate 0248 subsequently identified a *T. brevicompactum* showed strong antifungal activities. This is the first study of the endophytic fungus from garlic. *T. brevicompactum* a new species was first confirmed by morphological, molecular and phylogenetic analyses in 2004, and related research has been very rare. *Trichoderma* species are common soil borne fungi. One of the most significant ecological niches occupied by *Trichoderma* species is the plant rhizosphere [Harman et al., 2004]. The concept of *Trichoderma* as an endophyte has received a less attention. Although *Trichoderma* isolates were isolated from live sapwood below the bark of trunks of wild and cultivated. The *obroma cacao* and other *Theobroma* species [Evans et al., 2003] only a small number of *Trichoderma*.

In this study, the endophytes in garlic characterized and identified based on conventional morphological approaches & molecular biological approaches. 16S rRNA sequencing was used for the first identification of endophytic isolates. Representatives of the different types based on 16S rRNA sequencing were selected for further identification by gyrA and rpoB sequencing and phylogenetic analysis based on these concatenated house-keeping sequences.

Research on endophytes from plants of the family *Allium* is a few. So, an endophytic fungus isolated from garlic has a pronounced inhibitory effect on phytopathogens *Rhizoctonia solani* and *Botrytis cinerea* [Khassanov, 1996].

Endophytic fungi have been a promising source for bioactive compounds, especially their anticancer potential. As great demand arises for new drug leads for cancer, there arises a need to exploit the endophytic fungi associated with

medicinal plants. In 37the present study, we collected *A. schoenoprasum* bulbs from snow mountain regions of the Western Himalayas for the isolation of endophytic fungi. In 1965, Chopra et al. reported an enhancement of seven times in the medicinal properties of the garlic species growing at higher elevations due to a high content of organo-sulphur compounds [Shah NC, 2004]. It is also known to have abundant biological activities including immune-modulatory and anticancer properties. The endo-symbiotic nature of many endophytic fungi is to inherit their bioactive secondary metabolite producing capability from their host. Considering this a major reason, we selected it as a host plant [Brown AG, 1996] and isolated the endophytic fungus *P. pinophilum* (MRCJ-326) growing from its bulbs. Endophytic fungal research is focused on *Penicillium* sp. for its secondary metabolites displaying various pharmacological effects including anticancer activity [Rukachaisirikul V., 2007]. Previously, it has been reported that a multitude of anticancer agents have been produced from *Penicillium* sp., e.g., Brefeldin A, Wortmanin, and Chloctans the present study, we collected *A. schoenoprasum* bulbs from snow mountain regions of the Western Himalayas for the isolation of endophytic fungi. In 1965, Chopra et al. reported an enhancement of seven times in the medicinal properties of the garlic species growing at higher elevations due to a high content of organo-sulphur compounds. It is also known to have abundant biological activities including immuno-modulatory and anticancer properties. The endo-symbiotic nature of many endophytic fungi is to inherit their bioactive secondary metabolite producing capability from their host. Considering this a major reason, we selected it as a host plant and isolated the endophytic fungus *P. pinophilum* (MRCJ-326) growing from its bulbs.

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CHAPTER 5

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION:

- ✓ Two endophytic bacterial species were isolated from garlic bulb.
- ✓ The isolate coded as G-1has yellow color, convex elevation and opaque density. While the isolate coded as G-2 showed white color, flat elevation and translucent opacity. All the isolates G-1 and G-2 appeared Gram positive, Bacilli and cocci, non motile and non spore former and isolate G1 shows HL test positive (Aerobic) while isolate G-2 shows negative (Anaerobic). The isolate G-1 were ferment glucose and isolate G-2 ferment mannitol only.
- ✓ Isolate G-1 shows all the test catalase production, oxidase test, gelatin hydrolysis, starch hydrolysis, casein hydrolysis, urea hydrolysis tests positive, while the isolate G-2 shows Catalase production test and starch hydrolysis test positive.
- ✓ On basis of morphological, biochemical & physiological characteristics of isolate G-1 and G-2 were tentatively identified as *Micrococcus spp.* and *Bacillus spp.* respectively. However further taxonomic confirmation by using 16s rRNA is necessary.

CHAPTER 5

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CHAPTER 6

APPENDIX

APPENDIX

Beef extract peptone agar:

Composition:

Beef extract	:	3.0 g
Peptone	:	5.0 gm
Agar	:	25.0 gm
pH	:	7.2 ± 2
Distilled water	:	1000 ml

[John Weley, 1992]

Gauze's medium No. 1:

Composion:

Soluble starch	:	20.0 gm
KNO_3	:	1.0 gm
K_2HPO_4	:	0.5 gm
$MgSO_4$	:	0.5 gm
NaCl	:	0.5 gm
$FeSO_4$	:	0.01 gm

[Ronald m. Atlas, 2010]

Peptone water

Composition:

Peptone	:	20.0 gm
Sodium chloride	:	5.0 gm
pH	:	7.3

Biochemical Media:

Sugar fermentation medium:

Composition:

Peptone water	:	100 ml
Test sugar	:	1.0 gm
Bromothymol blue	:	0.25 ml

Hugh and Liefson's medium:

Composition:

Peptone	:	2.0 gm
NaCl	:	5.0 gm
K_2HPO_4	:	0.3 gm
Bromothymol blue (1% aqueous solution)	:	3.0 ml
Glucose (10 % solution)	:	100 ml
Agar agar	:	3.0 gm
Distilled water	:	900 ml
pH	:	7.3

[Atlas 1993]

Enzymatic activity:

Starch Agar (Deshmukh A.M.):

Composition:

Starch	:	20.0gm
Peptone	:	5.0 gm
Beef extract	:	3.0 gm
Distilled water	:	1000ml
pH	:	7.0

Gelatin Agar (Deshmukh A.M)

Composition :

Gelatin	:	1.5 gm
Peptone	:	0.4gm
Yeast extract	:	1.0 gm
Agar-Agar	:	1.5 gm
pH	:	7.0

Stains and staining reagents

Grams iodine (Lugols iodine)

Composition:

Iodine	:	1.0 gm
Potassium iodide	:	2.0 gm
Distilled water	:	100 ml

Frazier's reagent

Composition:

Mercuric chloride	:	15.0 gm
HCl	:	20.0 ml
Distilled water	:	100 ml

Oxidase test reagents

Composition:

Dimethyl - P- phenylene Diamine dichloride	:	1.0 gm
Distilled water	:	100 ml

Catalase test reagent

10% hydrogen peroxide solution

Crystal violet solution

Composion:

Solution A:

Crystal violet	:	20 gm
95 % ethanol	:	20 ml

Solution B

Ammonium oxalate	:	0.8 gm
Distilled water	:	80 ml

Basic fuchsin stain

Composition

Basic fuchsin	:	0.5 gm
Distilled water	:	1000 ml