ISOLATION AND IDENTIFICATION OF BACTERIA FROM MUSHROOM SUBSTRATES AND EVALUATION OF THEIR BIOLOGICAL CONTROL POTENTIAL AGAINST THREE SELECTED PATHOGENIC FUNGI

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CERTIFICATE

This is to certify that the thesis entitled "**ISOLATION AND IDENTIFICATION OF BACTERIA FROM MUSHROOM SUBSTRATES AND EVALUATION OF THEIR BIOLOGICAL CONTROL POTENTIAL AGAINST THREE SELECTED PATHOGENIC FUNGI**" submitted to the Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE** in **PLANT PATHOLOGY**, embodies the result of a piece of bona fide research work carried out by **H. M. IFTAKHAR**, **Registration No. 10-04099,** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

I further certify that such help or source of information, as has been availed of during the course of this investigation has duly been acknowledged.

Dated: :25th September,2017.
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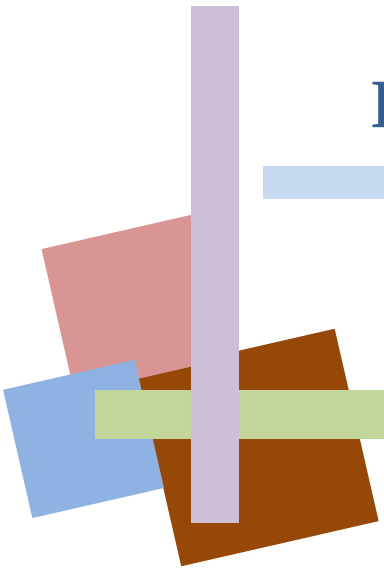

Dedicated To

My Beloved Parents & Respected Research Supervisor

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The Author

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ABSTRACT

A study was conducted at the Molecular Plant Pathology Laboratory, Department of Plant Pathology, Faculty of Agriculture, Sher-e-Bangla Agricultural University, (SAU), Dhaka-1207, during the period of January to June, 2017 to isolate the bacteria from different mushroom substrates and to evaluate their biological activities against selected fungal pathogens (*Fusarium oxysporum, Sclerotium rolfsii, Colletotrichum corchori*). For this purpose three types of mushroom substrate viz. sawdust, rice straw and newspaper were used. Four bacteria were isolated and identified from mushroom substrate;those were *Bacillus subtilis, Bacillus cereus, Paenibacillus polymyxa and Pseudomonas* sp. Among the bacterial isolates, *Pseudomonas* sp. has showed the best significant result against all three tested fungal pathogens, whereas *Bacillus subtilis* has the least significant result against the pathogens. *Pseudomonas* sp. showed highest inhibition against *Sclerotium rolfsii* (72.25%) followed by *Fusarium oxysporum* (68.96%) and *Colletotrichum corchori* (48.15%).

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CHAPTER I
INTRODUCTION

Mushroom belongs to the kingdom of fungi under the sub-division of *Basidomycotina* due to unique fungal characteristics (Song, 2004). Among the edible fungi, mushroom is a large reproductive structure, which is the most popular nutritious, delicious and medicinal vegetable around the world. The term mushroom applies mostly to those fungi that have stem (stalk), cap (pileus), hymenium (lamellae) and pores (gills) on the underside of the cap. The interest of oyster mushroom is increasing day by day due to its taste, nutrient and medicinal properties (Garcha *et al*., 1993). *Pleurotus* species can efficiently degrade agricultural wastes and they grow at a wide range of temperatures (Sánchez *et al*., 2010). *Pleurotus* species require carbon, nitrogen and inorganic compounds as their nutritional sources. The main nutrients are less nitrogen and more carbon so materials containing cellulose, hemicellulose and lignin (i.e., rice and wheat straw, cotton seed hulls, sawdust, waste paper, leaves, and sugarcane residue) can be used as mushroom substrates (Chang, 1989). Presently, in Asia (including Taiwan), the main substrate used for the commercial cultivation of oyster mushroom is saw dust (Rizki *et al*., 2011).

Substrates in mushroom cultivation have the same function as soil in plant production (Kwon and Kim, 2004). Many species of *Pleurotus* are commonly grown on a wide range of lignocellulosic materials (Sanchez, 2004). Different substrates can be recommended per region due to local availability of agricultural wastes (Cohen *et al*., 2002). Most commonly used substrates include sawdust, cotton seed straw, cereal straw, corncob, sugar cane straw and other plant fibres with high cellulose content (Ragunathan *et al*., 1996; Kwon and Kim, 2004). According to Labuschagne *et al*. (2000), wheat straw has been the main substrate used for cultivating *Pleurotus ostreatus*. However, Bughio (2001) successfully planted *Pleurotus ostreatus* on a combination of wheat straw, cotton boll straw, paddy straw, sugarcane and sorghum leaves. Different substrates can, therefore, be

recommended per region depending on local availability of agricultural wastes (Cohen *et al*., 2002).

Various agricultural by-products are being used as substrates for the cultivation of the oyster mushroom. Some of these wastes include banana leaves, peanut hull and corn leaves, mango fruits and seeds, sugarcane leaves, wheat and rice straw (Cangy and Peerally, 1995). The widely used substrate for cultivation of the oyster mushroom in Asia is rice straw (Thomas *et al*., 1998). Combinations of soybean straw and groundnut haulms were inferior to the combinations of wheat straw, leaves and stalks of pigeon pea and cotton stalks. An increase in yield was recorded by the addition of rice bran, gram powder and groundnut oilseed cakes in the substrate (Mane *et al*., 2007). Poppe (1974) cultivated *P. ostreatus* on manure, wood and straw substrates at a temperature of 10-20^{o}C with light intensities of 40-80 Iux. Imbernon *et al.,* (1977) grew several species of *Pleurotus* on tree bark and concluded that such substrates could replace corn cobs and straw for commercial cultivation. Khan and Ali (1981) cultivated oyster mushroom on cotton boll locules in polythene bags and kept in semi-dark thatched cottage with the atmospheric temperature range of 16-20ºC. Tan (1981) studied cotton waste as a substrate for the cultivation of *P. ostreatus* along with other *Pleurotus* species.

In Bangladesh, about 30 million tons of agricultural wastes like paddy straw, wheat straw, saw dust and sugarcane bagasse are being lost by improper utilization (Ahmed, 2001). Oyster mushroom are reported to be easily grown on different lignocellulose wastes such as banana leaves, cereal straw, paper wastes, sawdust, rice, wheat straw and other agro-wastes (Bhuyan, 2008). Rice straw, paper and saw dust are available and cheap to use as a substrate for mushroom production in Bangladesh.

Considering the nutritional, medicinal and economic importance of mushroom it is important to improve the production techniques by using local low cost materials. Remarkable various factors are responsible for lower mushroom production in Bangladesh. Among the different factors, bacterial disease and their antagonistic effect on mushroom is one of the major influential factor, which can initiate from

the mushroom substrate. There are several distinct bacteria can be found in mushroom substrate viz; *Bacillus*, *Enterobacter*, *Sphingomonas*, *Staphylococcu*, *Moraxella* and *Pseudomonus* (Yunjung, 2008). Even proper substrate promotes the development of a number of saprophytic soil microorganisms. Species of some bacteria viz; *Bacillus, Enterobacter,Flavobalistinum, Pseudomonas, Streptomyces, Nocardia, Rhodococcus* and species of some fungi *Penicillium, Trichoderma and Gliocladium* have been reported by several authors (Gbolagade, 2006; Anastasi *et al.*, 2005; Charest *et al*., 2004; Taiwo and Oso, 2004; Ryckeboer *et al*., 2003; Fordyce, 1970).

Antifungal agents produced by some bacteria have shown to be beneficial to control pathogenic fungi (Chang and Kim, 2007). However, they are harmful for the mushroom industry in general. *Pseudomonas tolaasii* is particularly notorious for causing brown blotch disease in the cultivation of edible mushrooms, including *Agaricus bisporus*, *P. ostreatus*, *P. eryngii*, *P. Pleurotus* and *Flammulina velutipes* (Rainey *et al.,* 1991). The causative agent of the disease was identified as a lipopeptide toxin, tolaasin, which has been shown to disrupt the cell membrane via the formation of membrane pores (Rainey *et al*., 1991; Nutkins *et al*., 1991). Unlike *P. tolaasii*, some bacteria belonging to the species *Pseudomonas* and *Bacillus* have been reported to exert promoting effects on the growth of mushrooms, including *P. eryngii* (Kim *et al*., 2007), *P. ostreatus* (Cho *et al.*, 2003), and *A. biporus* (Eger, 1972; Rainey *et al*., 1990). They also showed some antagonistic effects against fungi reported by Santoso *et al*., (2007) and gladiol (Goszczynska *et al.*, 2008). *Bacillus* spp. stains like *B. subtilis*, *B. atrophaeus*, *B. amyloliquefaciens*, *B. cereus*, *B. licheniformis* and *B. pumilis* were used as potential biocontrol agents against different *Fusarium* sp. (Marten *et al*., 2000; Siddiqui, 2005). Among bacteria commonly used as plant biological control, *Bacillus* spp. strains are highly promising (Choudhary and Johri, 2009; Hobley *et al.*, 2013). In particular, *Bacillus subtilis* and *Bacillus amyloliquefaciens* strains are considered safe for the environment, with excellent colonization capacity and versatility to protect plants from phytopathogenic fungi (Zhao *et al.*, 2013).

Therefore, the analysis of the bacterial community in association with the cultivated mushrooms substratum becomes crucial for the mushroom cultivation industry.

Keeping these facts in mind the study was undertaken to achieve the following objectives:

1. To isolate, identify and characterize the bacteria from three different mushroom substrates.
2. To evaluate the biological activities of isolated bacteria against selected pathogenic fungi (*Fusarium oxysporum, Sclerotium rolfsii, Colletotrichum corchori*).

CHAPTER II
REVIEW OF LITERATURE

2.1. Present status of mushroom in Bangladesh

Mushroom cultivation in Bangladesh began in 1979 with assistance from Japanese organization JOCDV. In early 1980s commercial mushroom cultivation was initiated by Bangladesh Agricultural Research Council and Mushroom Culture Centre at Savar. Apart from Savar, mushroom is being cultivated in Dinajpur, Jessore, Barisal, Chittagong, Sylhet, Comilla, Khulna, Mymensingh, Bandarban, Rangamati, Chapainawabganj and Rangpur (Asia Pulse News, 2008).

Currently 13 species of mushroom are cultivated in Bangladesh of which oyster mushroom is produced commercially to a large extent. Mushroom farming is in fact a very easy job. There is an opportunity to make good profit by investing a little amount of capital and labour. One can earn Tk 4-5 thousand a month by investing only Tk 10- 15 thousand (Barnett, 1980).

During the last few years about 2700 people are trained for mushroom cultivation by the Mushroom Culture Centre of Savar. Recently government has taken a project of Tk. 7 crore in Rangamati to increase and popularize the cultivation of edible mushrooms (Kabir, 1999).

2.2. Bacteria associated with mushroom substrate

Adedeji and Aduramigba (2016), presented the results of bacteriological tests for the differentiation of *Pseudomonas fuscovaginae*, *Pseudomonas syringae*, *Pseudomonas avenae* and *Pseudomonas glumae* are commonly isolated from mushroom substrate prepared by rice straw.

Yunjung (2008) stated that there are several distinct bacteria can be found in mushroom substrate such are *Bacillus*, *Enterobacter*, *Sphingomonas*, *Staphylococcu*, *Moraxella* and *Pseudomonus.*

Jandaik *et al.* (1993) reported that during 1989-93, *Pseudomonas agarici*, was observed at various mushroom substrate which cause yellow blotch disease in Himachal Pradesh, India.

Pattnaik *et al.* (1998) reported that *Bacillus* sp., *E. coli, Pseudomonas* spp., *Alealigenus* sp., *Klebsiella* sp., *Staphylococcus* sp., *Streptococcus* sp. and *Acenetobacter* sp. were identified from mushroom substrate.

2.3. Biological efficacy of bacteria against different fungi

Among bacteria commonly used as plant biological control, *Bacillus* spp. strains are highly promising (Choudhary and Johri, 2009; Hobley *et al*., 2013; Ongena and Jacques, 2008). In particular, *Bacillus subtilis* and *Bacillus amyloliquefaciens* strains are considered safe for the environment, with excellent colonization capacity and versatility to protect plants from phytopathogenic fungi (Zhao *et al*., 2013).

Bacillus spp. stains like *B. subtilis*, *B. atrophaeus*, *B. amyloliquefaciens*, *B. cereus*, *B. licheniformis* and *B. pumilis* were used as potential biocontrol agents against different *Fusarium* sp. (Marten *et al.,* 2000; Siddiqui, 2005).

Bacillus subtilisis is an antagonistic bacterial biological agent which control many air borne, seed borne and soil borne diseases of rice, wheat, sugarcane, jute, groundnut, cotton, rubber, soybean, tobacco, and vegetables etc (Kim *et al*., 2007).

Bacillus strains are well-known antibiotic producers, which have advantage over other biocontrol microorganisms due to their inherent property to form endospores and resistance to extreme conditions. The antagonistic effects of *Bacillus* strains have been shown by *in vitro* antibiosis (Chang *et al.,* 1981) and *in situ* disruption of spikelet infection leading to reduced disease severities, and identifying the lipopeptides (Crane *et al*., 2013) regarding antimicrobial mechanism study,

production of antifungal compounds is thought to be the main mode of action by the antagonistic bacteria.

The *Bacillus* spp. has shown strong antagonistic activity against *Fusarium oxysporum* in *in vitro* antagonistic activity tests, and it was used to effectively control muskmelon *Fusarium* wilt under greenhouse and field conditions (Zhao *et al*., 2011, 2013).

B. subtilis showed antagonistic effect against *Rhizoctonia solani*, the causal agent of damping-off of tomato seedlings by producing a small peptide(s) with a long fatty acid moiety, the so-called lipopeptide antibiotics (Asaka and Shoda, 1996).

B. subtilis and *B. polymyxa* showed distinct antagonism against *B. cinerea*, *Pythium aphanidermatum*, *P. mamillatum* and *P.ultimum* (Walker *et al*., 1998; Jiang *et al.,* 2001).

Singh *et al*. (1976) reported that *Bacillus* spp. has broad range antifungal activity showed 71% and 78% mycelial growth inhibition of *F. oxysporum* and *C. gloeosporioides* respectively.

Adebayo *et al*. (2013) reported that soil inoculation with *Bacillus cereus, Bacillus subtilis* and *Trichoderma* species, prevent seed infection caused by *Fusarium* species and also in this way, the antagonist activity of the root increases.

Several species of *Bacillus* are known to produce toxins that are inhibitory to the growth and activities of fungal and nematode pathogens of plants, where in most thoroughly studied species are *B. subtilis* (Schaad *et al.,* 2002).

Bacillus species have been extensively used against many soil-borne plant pathogens. *Bacillus substilis, Bacillus cereus, Bacillus polymyxa, Bacillus megaterium* and *Bacillus pumillus* have been identified as biocontrol agents to reduce disease caused by a variety of soil-borne plant pathogens, including *Rhizoctonia* (Yu *et al*., 2002) and *Fusarium* (Schisler *et al*., 2002).

Bacterial species like *Bacillus, Pseudomonas* have been proved in controlling the fungal diseases. Several bacteria thrive on abundant nutrients in the rhizosphere and some of these possess antagonistic action, which safeguard plants from pathogens and stimulate growth (Gray *et al.,* 2005).

Bacillus strains showed significant inhibition activity against *Botrytis cinerea* (Walker *et al.,* 1998), *Puccinia pelargonii zonalis* germination (Rytter *et al*., 1989) and *Fusarium oxysporum* (Lang *et al*., 2002).

Bacillus subtilis was discovered to produce antifungal compound which was antagonistic effect to the growth of *S. rolfsi* (Nalisha *et al.,* 2006).

Abdallah *et al.* (2016) reported *P. polymyxa* strains were shown to cause antibiosis and produce polymyxins, colistin and hydrolytic enzymes, which play important roles in the biocontrol of plant pathogens .

Srivastava (2009) studied the antifungal activity of different strains of *Pseudomonas flurescens* against some plant pathogens such as *Alternaria cajani, curvularia lunata, Fusarium* sp., *Bipolaris* sp. and *Helminthosporium* sp. in *in vitro* with diffrent concentrations (1000-2000, 3000, 4000 and 5000 ug/ml) of *Pseudomonas fluorescens* and concluded that all the strains of *Pseudomonas fluorescens* presented a most significant value against *Alternarja cajani* and *Curvularia lunata*.

Jayaswal *et al*. (1993) studied two strains of *Pseudomonas capacia*, RJ3 and ATCC 52796, have been identified as potential antagonists of fungal plant pathogens. They compared the antagonistic activity of these two strains against various fungal pathogens. Although both strains displayed high levels of antagonism, ATCC 52796 was slightly more antagonistic than RJ3.

Pseudomonas fluorescens is one of rhizospheric microorganisms that could be used as biological control agent (Singh *et al*., 2003).

P. fluorescens P60 could inhibit formation of new microsclerotia of *Verticillium dahliae* and supress the wilt disease caused by *V dahliae* on *Arabidopsis thaliana* and eggplant (Soesanto, 2000; Soesanto and Termorshuizen, 2001).

The bacteria suppress sclerotial germination of *Sclerotium rolfsii in vitro* as high as 92% with the most effective soaking time of 10 minute; for *in planta* test, the bacteria could supress stem rot disease intensity as 92% and decrease late sclerotial population density in soil as 86.3% (Soesanto *et al*. 2003).

The *Pseudomonas* spp. could also decrease some plant pathogens such as *Fusarium* wilt on shallot (Palleroni, 2007).

Kim *et al*. (2012) published a report that stated the insertion of Tn7 based ChiA gene into *P. fluorescens*, the resultant construct exhibited improvement in antagonistic activity against *Rhizoctonia solani*.

A report by Ajit *et al*. (2006) provided evidence for enhancement of plant growth by chitinases of *fluorescent Pseudomonas* which was responsible for preventing the proliferation of *Fusarium oxysporum* causing carnation wilt.

Hoffmaster *et al*. (1992) reported the chitinolytic activity of *B. cereus* strain which significantly reduced the severity of the leafspot caused by *Cercospora arachidicola.*

Fermor *et al*. (2005) reported a cloning and purification of the ChiCW gene of *Bacillus cereus* 28-9 strain. The purified ChiCW protein demonstrated inhibition of conidial germination of *Botrytis elliptica*, a major fungal pathogen of lily leaf blight.

Chen *et al.* (1996) stated that *P. fluorescens* is a root colonist which has been shown to reduce the incidence of *Fusarium* wilt cotton.

Some rhizobacteria have been used to control *S. rolfsii* such as *Pseudomonas* sp., *Burkholderia cepacia, Bacillus subtili* stated by Ali *et al.* (2014).

Fluorescent pseudomonads have been reported as promising biological control agents against *S. rolfsii* in betelvine (Singh, 2003) and bean (Fuente *et al.,* 2004).

Bacillus subtilis secrete antifungal substance which is highly antagonistic against *S. rolfsii* (Nalisa *et al.,* 2006).

Abd-Allah (2005) revealed that *Bacillus subtilis* control *Sclerotium rolfsii* by 92% under greenhouse condition in peanut.

Pastor *et al.* (2010) observed that *Pseudomonas* sp. were more potent antagonistic activity against *Sclerotium rolfsii* in the rhizospheric soil of groundnut.

Several species of *Pseudomonas* produce antifungal antibiotics such as 2, 4 diacetyl phloroglucinol, oligomycin, phenazine, pyolyteorin, pyrrolnitrin and pyocyanin which inhibit fungal activity (Gupta *et al*., 2001).

CHAPTER III
MATERIALS AND METHODS

3.1. Experimental site

The experiment was conducted in the Molecular Plant Pathology Laboratory, Department of Plant Pathology, Faculty of Agriculture, Sher-e-Bangla Agricultural University, (SAU), Sher-e-Bangla Nagar, Dhaka-1207, Bangladesh .

3.2. Experimental period

The experiments was conducted during the period of January to June, 2017.

3.3. Preparation of mushroom substrate for mushroom production

3.3.1. Selection of substrate for spawn production

Consideration of literature and checking the local availability resulted in a general consensus that the following three types substrates rice straw, newspaper and sawdust are highly appropriate for the proposed study. Major consideration involved were availability of the materials, cost and ease of obtaining it. Sawdust, rice straw and newspaper were used to make three different types of spawn packets.

3.3.2. Preparation of saw dust

Saw dust was collected from the nearest saw mill. After sun drying by adding distilled water the moisture was increased. Then polypropylene bags (25×18 cm) were filled with 500 g prepared substrate and packed tightly. A 3 to 5 cm hole was made with pointed steel at the centre for space to put the inoculums. The packets were plugged with cotton and bind with rubber band to prevent the displacement of colored paper. The packets were sterilized in the autoclave for 15 minutes at 120°C with 1.5 kg/cm^2 atmospheric pressure were kept 24 hours for cooling. One tea spoonful of mycelia containing mother culture material was placed aseptically in through the hole of each packet separately and each treatment was replicated 4 times. The packets were then marked treatment wise and kept on the self in an

incubation room at 25±1°C under 80% to 85% relative humidity and were allowed to complete the whitish mycelial growth (Parvez, 2008).

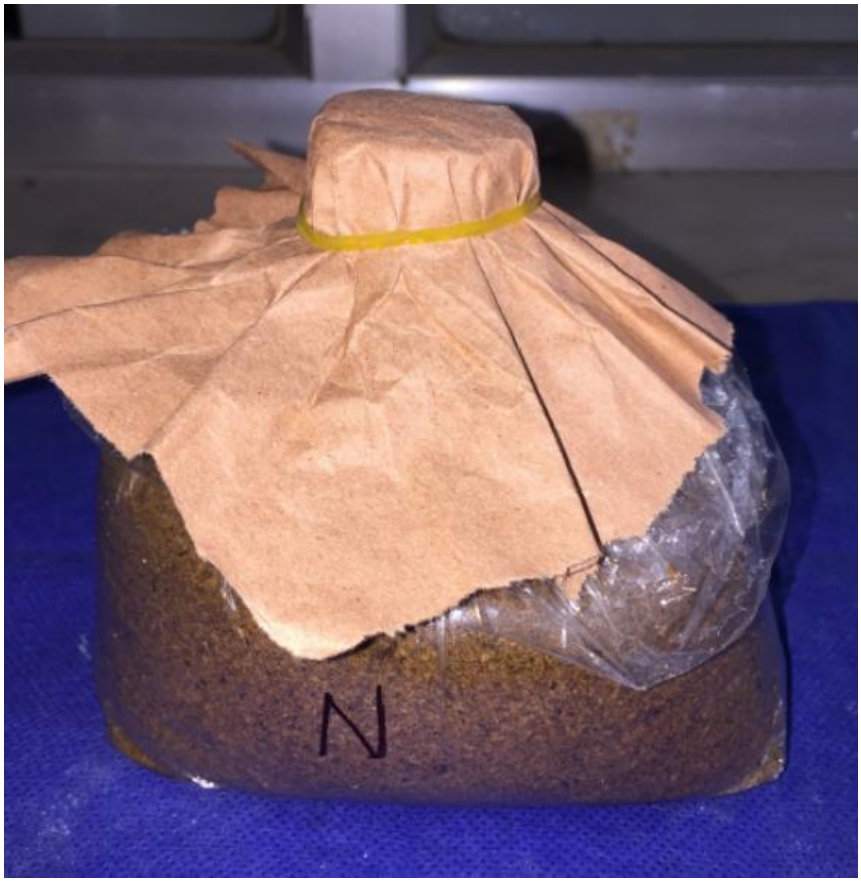

Figure 1. Sawdust packet

3.3.3. Preparation of rice straw

After sun drying, rice straw was fragmented into small pieces (less than 3 cm particle size) with a sickle. The materials were mixed thoroughly with mixture machine and the moisture was increased by adding distilled water until it reached around 65% moisture content. Then polypropylene bags (25×18 cm) were filled with 500 g prepared substrate and packed tightly. A hole of 3 to 5 cm was made with pointed steel at the centre for space to put the inoculums. The packets were plugged with cotton and bind with rubber band to prevent the displacement of colored paper. The packets were sterilized in the autoclave for 15 minutes at 120°C with 1.5 kg/cm^2 atmospheric pressure and kept 24 hours for cooling. One tea spoonful of mother culture materials containing mycelia was placed aseptically in the hole of each packet separately and each treatment was replicated 4 times. The

packets were then marked treatment wise and kept on the self in an incubation room at 25±1°C under 80% to 85% relative humidity and were allowed to complete the whitish mycelial growth (Parvez, 2008).

Figure 2. Rice straw packet

3.3.4. Preparation of newspaper

Newspaper was cut into small pieces. Then polypropylene bags (25×18 cm) were filled with 500 gm newspaper and packed tightly. A hole of 3 to 5 cm was made with pointed steel at the centre for space to put the inoculums. The packets were plugged with cotton and bind with rubber band to prevent the displacement of colored paper. The packets were sterilized in the autoclave for 15 minutes at 120°C with 1.5 kg/cm^2 atmospheric pressure and kept 24 hours for cooling. One tea spoonful of mother culture materials containing mycelia was placed aseptically through the hole of each packet separately and each treatment was replicated 4 times. The packets were then marked treatment wise with a marker pen and were kept on the self in an incubation room at 25±1°C under 80% to 85% relative

humidity and were allowed to complete the whitish mycelial growth (Poppe, 1995).

Figure 3. Newspaper packet

3.4. Inoculation of spawn

The spawn packets with complete mycelium were transferred to the culture house and the colored paper, rubber bands, cotton plug and plastic neck of the spawn packets were removed and the mouths of the polypropylene bags were wrapped and tied with rubber bands. The plastic bags were opened by “D” shaped cut on the shoulder side and removed the sheet. The opened surface of substrate was scraped slightly with a blade for removing the thin whitish mycelial layer. The packets were placed separately side by side on the rack in the culture house. The relative humidity (RH%) was maintained by watering four times daily. The average temperature (22-27^{0}C) and relative humidity (70-85%) were measured.

3.5. Selection of spawn packet

The packets were randomly selected from each (rice straw, saw dust, waste paper) substrate that were previously inoculated with oyster mushroom.

3.6. Isolation of bacteria on NA media

3.6.1. Preparation of Nutrient Agar (NA) media

Nutrient agar media was prepared according to the method followed by Schaad (1988). At first 15 g bacto agar was taken in an Erlenmeyer flask containing 1000 ml distilled water. Then 5 g peptone, 3 g beef extract and 5 g NaCl were added to it. The nutrient agar was shaken thoroughly for few minutes for mixing properly. The mixture was then autoclaved at 121^0C under 15 PSI pressure for 15 minutes.

3.6.2. Isolation of bacteria

Randomly selected packets were diluted to a composite sample (from each 3 poly bags). On this dilatation process 1 g of substratum was taken into a test tube containing 9 ml of distilled water and shaken thoroughly resulting 10^{-1} dilution. Similarly, final dilution was made up to 10^{-3}. Then 0.1 ml of each dilution was spread over NA plate at three replications as described by Goszczynska and Serfontein (1998). The solution was spread with the help of alcohol flame sterilized glass-rod. The inoculated NA plates were kept in an incubation chamber at 30^0C. The plates were observed after 24 hrs and 48 hrs. In order to get pure colony, single unmerged colony grown over NA plate was restreaked on another plate with the help of a sterile loop.

3.6.3. Growth of bacteria on nutrient agar (NA) media

Freshly prepared Nutrient agar (NA) medium was poured into a sterile petri dish and cooled. Pure colony of bacterium was streak inoculated on the plate with the help of a sterile transfer loop. It was incubated at 30^0C for at least 24 hrs in incubation chamber and observed the colony characters.

3.7. Preservation of bacteria

A slant culture of purified bacteria was done on NA slant in small screw-cap test tubes in order to preserve the bacteria for future use and kept it in refrigerator at 4^0C.

3.8. Identification of the bacteria

Bacteria were identified on the basis of morphological, biochemical and cultural features as per standard microbiological procedures and grew the bacteria over selective media.

3.8.1. Morphological characters

Morphological characteristics of the pathogen such as cell shape, Gram's reaction and pigmentation were studied as per the standard procedures described by Schaad (1992); Gerhardt (1981). It includes the cultural characteristics.

3.8.2. Biochemical characters

Biochemical tests such as oxidase test, gelatine liquefaction test, starch hydrolysis test, catalase test and cetrimide agar base test were studied as per the methods described by Schaad (1992) and Salle (1961).

3.8.2.1. KOH solubility test

A single drop of 3% KOH (aqueous) was placed on a glass slide. One loop full of a single colony (18-24 hrs old) was taken from the NA plate using a cooled, sterile loop and it was mixed with KOH solution until an even suspension was obtained. The loop was raised a few centimeters from the glass slide and repeated strokes to have strands of viscid materials as described by Suslow *et al*. (1982).

3.8.2.2. Gram's staining

A single drop of sterile water was placed on a clean microscope slide. Small amount of a young colony (18-24 hrs old) was taken with a cold, sterile loop from the nutrient agar medium and the bacteria were smeared on to the slide very thinly.

The thinly spread bacterial film was air dried. Underside of the glass slide was heated by passing it four times through the flame of a sprit lamp for fixing the bacteria on it. After that the slide was flooded with crystal violet solution for 1 minute. The slide was rinsed under running tap water for a few seconds and excess water was removed by air. Then it was flooded with lugol's iodine solution for 1 minute. After that it was decolorized with 95% ethanol for 30 seconds and again rinsed with running tap water and air dried. Then it was counterstained with 0.5% safranine for 10 seconds. It was rinsed under running tap water for a few seconds and excess water was removed by air. Then the glass slide was examined at 40x and 100x magnification using oil immersion. The Gram negative cells appeared red in color and Gram positive cells appeared violet in color (Pastor *et al.*,2010).

3.8.2.3. Oxidase test

One ml 1% aqueous (w/v) solution of NNN'Ntetramethyl-p-phenylene-diamine-dihydrochloride solution was spread on the middle of filter paper and the paper placed on a petri dish. Then some colony part of the bacteria was picked with a sterile tooth pick and smeared onto the moistened filter paper and observed up to 60 seconds whether it changed color to dark purple or not.

3.8.2.4. Gelatin liquefaction test

A tube containing 12% (w/v) gelatin was stub inoculated with one loop-full bacterial culture with the help of a sterile transfer loop. It was incubated at 30^0C for 24 hrs. By the formation of liquid culture after keeping it at 5^0C in refrigerator for 15 minutes, gelatin liquefied microorganism was determined. The development of yellow halo around the growth indicates utilization of gelatin (Stolpe and Godkeri, 1981).

3.8.2.5. Starch hydrolysis test

Nutrient agar plate containing 0.2% soluble starch was spot inoculated with pure colony of bacterium. It was then incubated at 30^0C for at least 48 hours in incubation chamber. Then the plates were flooded with lugol's iodine solution and observed whether a clear zone appeared around the colony or not. The positive

result of starch hydrolysis test was mentioned by clear zone surrounding the bacterial colony. The zone showed that starch in the media could be hydrolyzed because of an ezymatic reaction, *i.e*., amylase, secreated by the antagonist (Karkalas, 1985).

3.8.2.6. Catalase test

Some drops of freshly prepared 3% H_2O_2 (Hydrogen peroxide) was added with 48 hours old pure culture of bacterium grown on NA plate and observed whether it produced bubbles within a few seconds or not.

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \text{ (gas bubbles)}$$

3.9. *Bacillus cereus* agar base test

Suspended 20.5 gm agar base powder mixed in 475 ml distilled water. Heated it to boiled to dissolved the medium completely. Sterilized it by autoclaving at 15 lbs pressure (121ºC) for 15 minutes. Cooled to 45-50ºC and aseptically added rehydrated contents of 1 vial of Polymyxin B selective supplement and 25 ml of sterile egg yolk emulsion. Then it was mixed well and poured into sterile petri plates. Inoculated with pure colony of bacterium. It was then incubated at 30^0C for at least 48 hours in incubation chamber. Different species of *Bacillus* spp. were separated on cultural response on *Bacillus Cereus* Agar Base at 30°C after 18-48 hrs incubation described by Harmon (1992). These are as follows :

Table.1. Reaction of *Bacillus* spp. on *Bacillus cereus* agar base test

Microorganism	Expected Results	
	Growth	Reaction
Bacillus subtilis	Fair to good	Cream to light yellow colonies
Bacillus cereus	Fair to good	Blue colonies (halo)
Paenibacillus polymyxa	Fair to good	Yellow colonies

3.10. Cetrimide agar test

On this test at first in a Erlenmeyer flask 46.5 gm cetrimide agar was taken in 1000 ml water. Then 10 ml glycerin was added in it. The mixture was boiled to mix the elements properly. After that, it was autoclaved at 121^0C under 15 PSI pressure for 20 minutes. The pure colony that grew over NA medium was transferred on cetrimide agar medium by streak plate method. After inoculation the plates were kept in an incubation chamber at 30^0C. Virulent colonies of *Pseudomonous* were selected on the basis of growth of bacteria on cetrimide agar medium.

3.11. Preparation of Potato Dextrose Agar (PDA) media for antagonistic test

PDA was prepared as described by Islam (2009). 200g peeled and sliced potato was boiled in 500 ml water in a bowl for about half an hour. Then the extract of the potato was filtered through was cheese cloth. The other two ingredients viz. 20g dextrose and 20g agar were added in the extract and the volume was made up to 1L mark. Then the prepared standard PDA was poured in 1000ml conical flask and sterilized (121˚C, 15 psi for 15 min.) in an autoclave.

3.12. Dual culture method for evaluation of antagonistic effect of isolated bacteria against three selected fungi

Bacterial isolates were screened for their ability to suppress the mycelial growth of fungal *in vitro* dual culture assays on potato dextrose agar media (Lahlali *et al.*, 2007). Each combination of pathogen and antagonist was replicated three times and plates were randomly placed in the dark chamber and incubated at 25°C for 7 days. The radial mycelial growth of fungus towards the antagonistic bacteria (T) and that on a control plate (C) were measured and the mycelial growth inhibition was calculated according to the formula:

$$\% \text{ inhibition of growth} = \frac{C-T}{C} \times 100$$

Each fungal mycelia plug was cut by cork borer and placed on the center of PDA plate. Each bacterium was grown on nutrient agar slant for 24 hrs before inoculated by streaking two horizontal lines on PDA at the position of 20 mm

from the rim of petri dish upper and lower of inoculated fungi. And for control each fungal mycelia plug was cut by cork borer placed on the center of PDA plate without any streak of bacterium. The growth and reduction in mycelial growth of the pathogenic fungus was calculated according to (Fokemma, 1973). Radial mycelial growth of fungi was recorded. Inhibition percent of growth was calculated using the following formula: Growth reduction (%) = (Growth in control - Growth in treatment / Growth in control) x 100.

3.13. Statistical analysis of data

Data collected during experiment period were tabulated and analyzed following Duncan's Multiple Range Test (DMRT) (Gomez, K.A. and Gomez, 1984).

Chapter IV
RESULTS

4.1. Isolation and identification of different bacteria

Several cultural, physiological and biochemical tests were performed and some selective and semi-selective media were used to identify and differentiate the bacteria.

4.2. Isolation and purification of different bacteria on NA media

Isolation was done to determine the cultural characteristics of bacteria as an identifying and classifying bacteria into taxonomic groups. When grown on a variety of media, bacteria exhibit differences in the microscopic appearance of their growth. These differences called cultural characteristics which were used as the basis for separating bacteria into taxonomic group (Table 2).

To identify the bacteria existed on the mushroom substrate, dilution plate method was used to isolate. Colonies of bacterium on nutrient agar medium were found after 48 hours of incubation at 30^0C (Figure 4). Colonies were purified by restreaking the isolated colony on nutrient agar plate (Figure 5).

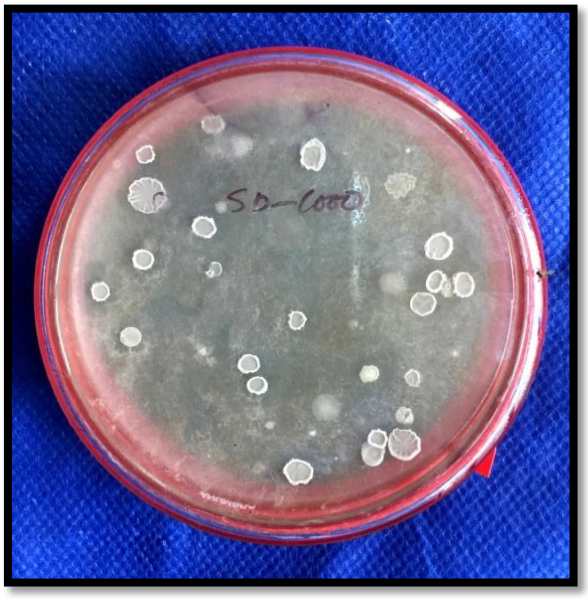

Figure 4. Isolation of bacteria by spread plate method

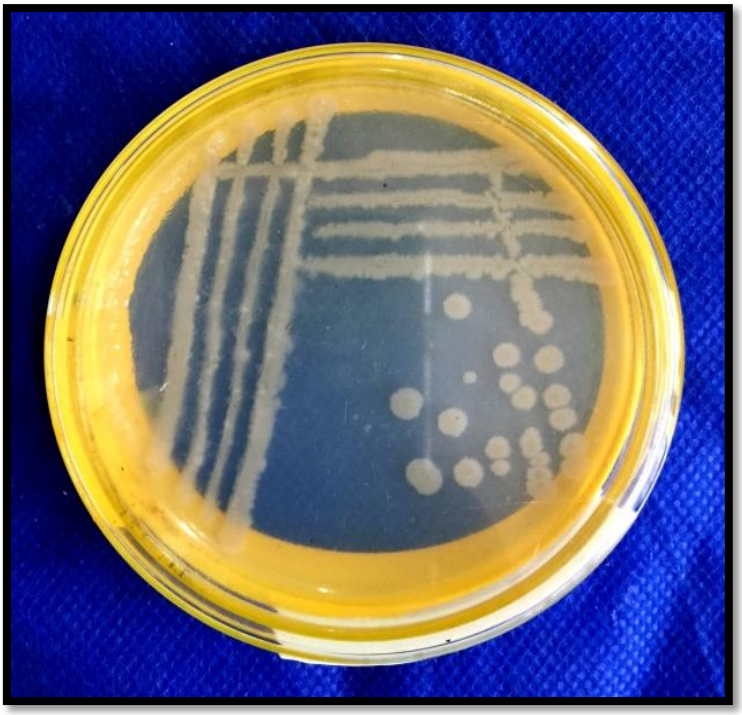

Figure 5. Isolation of bacteria by streaking plate method

4.3. Preservation of bacteria

Purified bacteria on NA slant separated and were kept in refrigerator at 4^0C in test tubes. It was served as a stock culture for further studies (Figure 6).

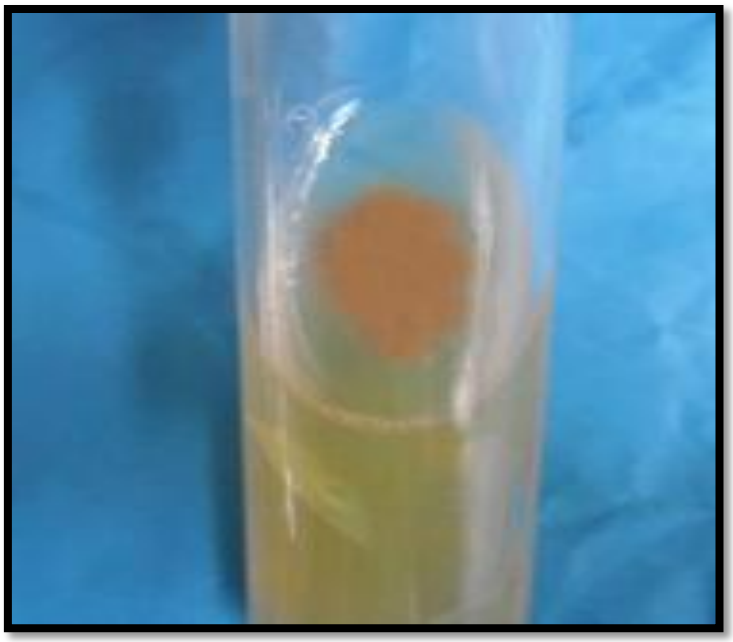

Figure 6. Slant culture of pathogenic bacteria

4.4. Identification of the Bacteria

The bacteria was identified by the morphological, biochemical and cultural characteristics as per standard microbiological procedures. The identified bacteria were *Bacillus subtilis*, *Bacillus cereus*, *Paenibacillus polymyxa* (Figure 7) and *Pseudomonas* sp. (Figure 8).

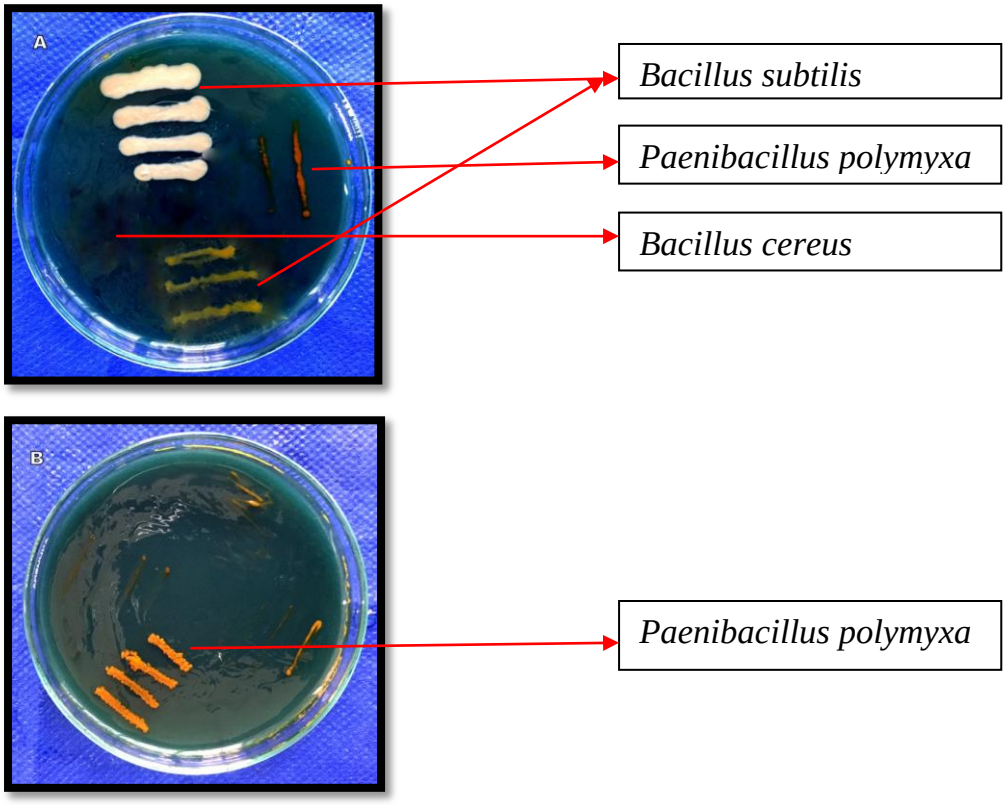

Figure 7. A and B showing isolation of Bacillus spp. on *Bacillus cereus* agar base test

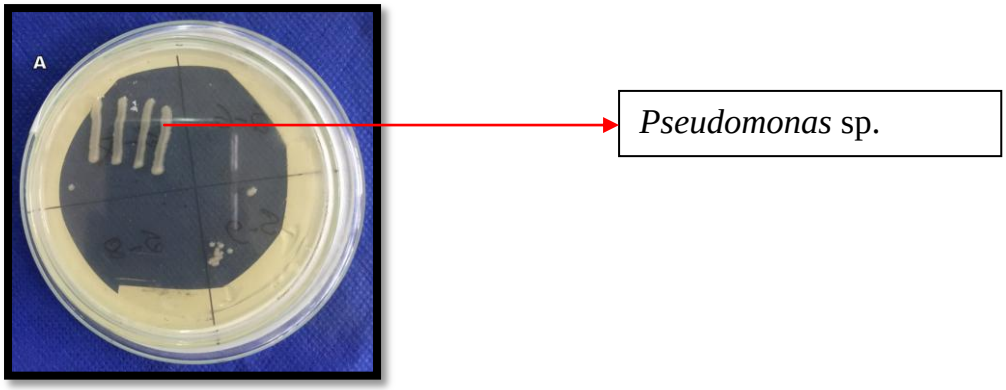

Figure 8. Growth of *Pseudomonas* sp. on cetrimide agar base medium

4.4.1. Morphological characters

A Motic binocular compound microscope was used for morphological study. For microscopic examination, the antagonistic bacteria were prepared according to the method of Logan and Berkeley (1984).

4.4.2. Cultural characterization and physiological tests

Four different kind of bacterial isolates were found from all three types of substrates. Under light microscope their cultural characteristics on NA plates have been very cleared. Cultural characteristics of different bacteria were presented in Table 2.

Table 2. Cultural Characterization of different bacteria on NA plates

Bacterial isolates	**Size**	**Pigment**	**Form**	**Margin**	**Elevation**	**Texture**
Bacillus subtilis	Large	White, dull	Irregular	Undulate	Umbonate	Dry
Bacillus cereus	Medium	Opaque	Irregular	Undulate to curled	Flat to raised	Smooth
Paenibacillus polymyxa	Small	Pale	Circular to irregular	Undulate	Raised	Matt
Pseudomonas sp.	Medium	Light yellow to transpar-ent	Circular	Smooth to wavy	convex	Shiny , smooth

4.4.3. Biochemical characters

The results of biochemical tests for four different kind of isolates (*Bacillus subtilis, Bacillus cereus, Paenibacillus polymyxa* and *Pseudomonas* spp.) were presented in figure 9-10 and in the table 3.

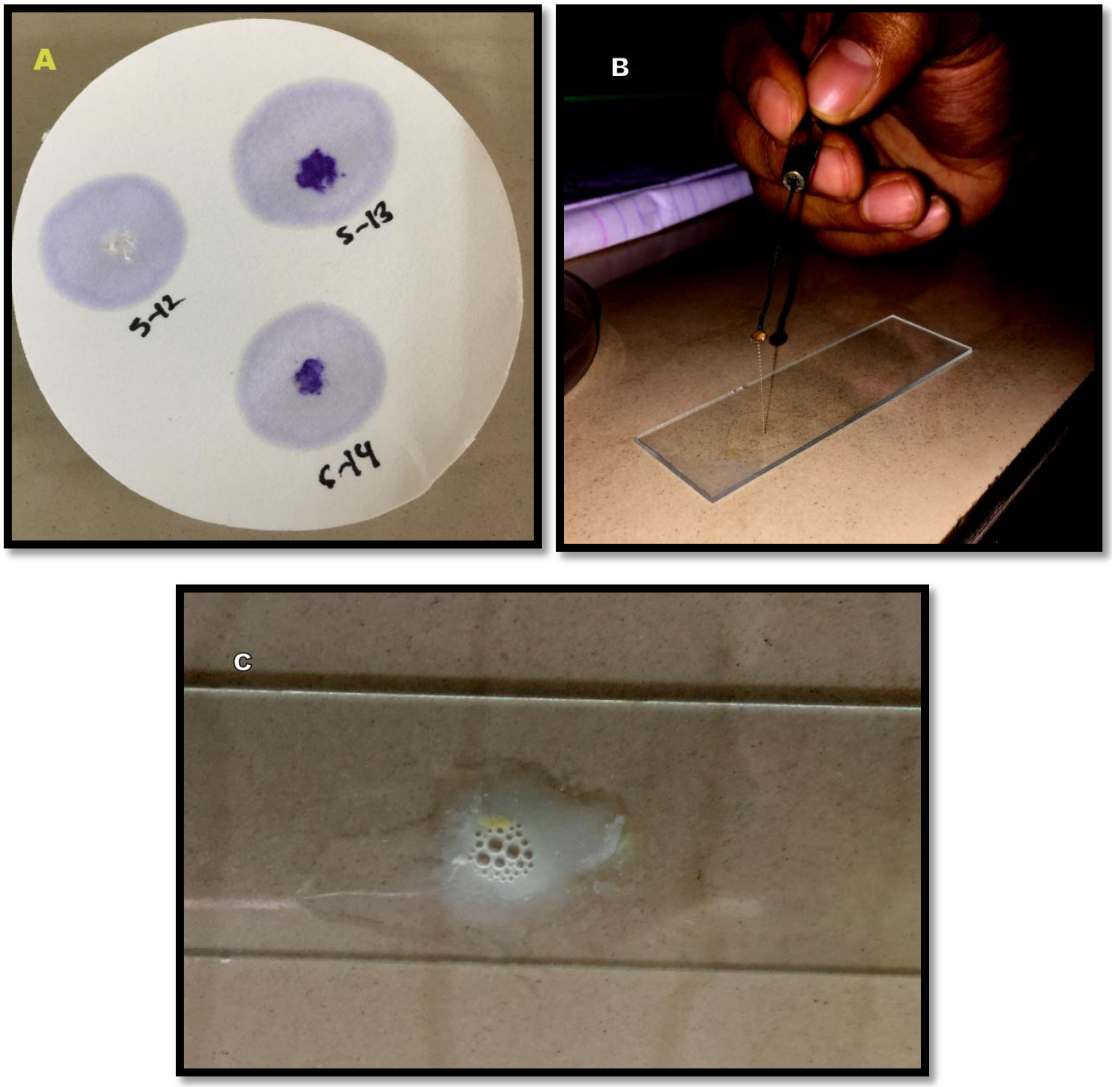

Figure 9. Biochemical tests- A. Oxidase test. B. KOH test. C. Catalase test.

Figure 10. Gelatin liquefaction test

Table 3. Biochemical Tests for identification of different bacteria

Bacterial isolates	**Gram Staining**	**KOH solubility test**	**Oxidase Test**	**Gelatin liquefaction Test**	**Starch Hydrolysis Test**
Bacillus subtilis	+	-	+	+	+
Bacillus cereus	+	-	+	+	+
Paenibacillus polymyxa	+	-	+	+	+
Pseudomonas sp.	-	+	+	+	-

4.5. Efficacy of isolated bacteria as a bioagent against three selected pathogenic fungi

Table 4. Efficacy of the bacterial isolates in inhibition of mycelial growth of *Sclerotium rolfsii* .

Bacterial isolates	Radial mycelial growth (mm) at 7 DAI*	% Inhibition of mycelial growth at 7 DAI*
Bacillus subtilis	66.29	20.14
Bacillus cereus	49.81	40.18
Paenibacillus polymyxa	42.46	48.85
Pseudomonas sp.	23.24	72.25
Control	83.00	-

*In column, DAI = Days after inoculation

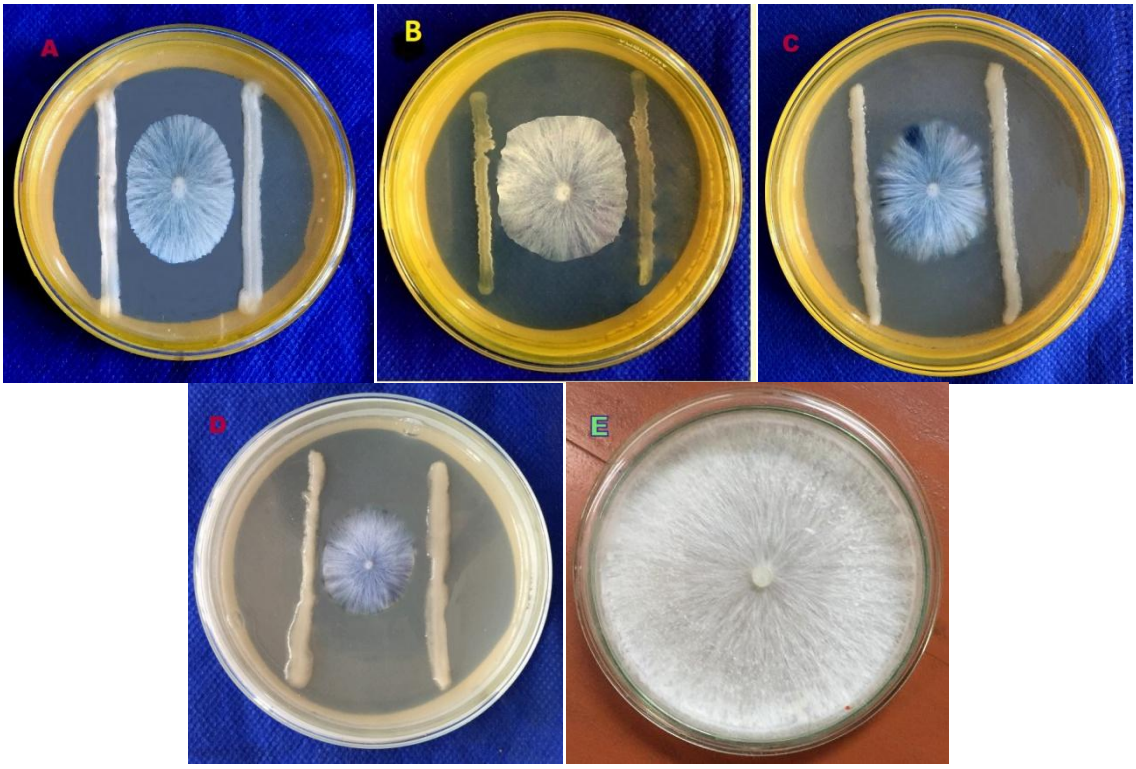

Figure 11. Radial mycelial growth of *S. rolfsii* against (a) *Bacillus subtilis* (b) *Bacillus cereus* (c) *Paenibacillus polymyxa* (d) *Pseudomonas* sp. and (e) Control after 7 DAI (Only pathogen)

Table 5. Biological efficacy of the isolates in inhibition of mycelial growth of *Fusarium oxysporum* .

Bacterial isolates	**Radial mycelial growth (mm) at 7 DAI***	**% Inhibition of mycelial growth at 7 DAI***
Bacillus subtilis	15.93	63.12
Bacillus cereus	18.92	56.20
Paenibacillus polymyxa	23.22	46.24
Pseudomonas sp.	13.40	68.96
Control	43.2	-

*In column, DAI = Days after inoculation

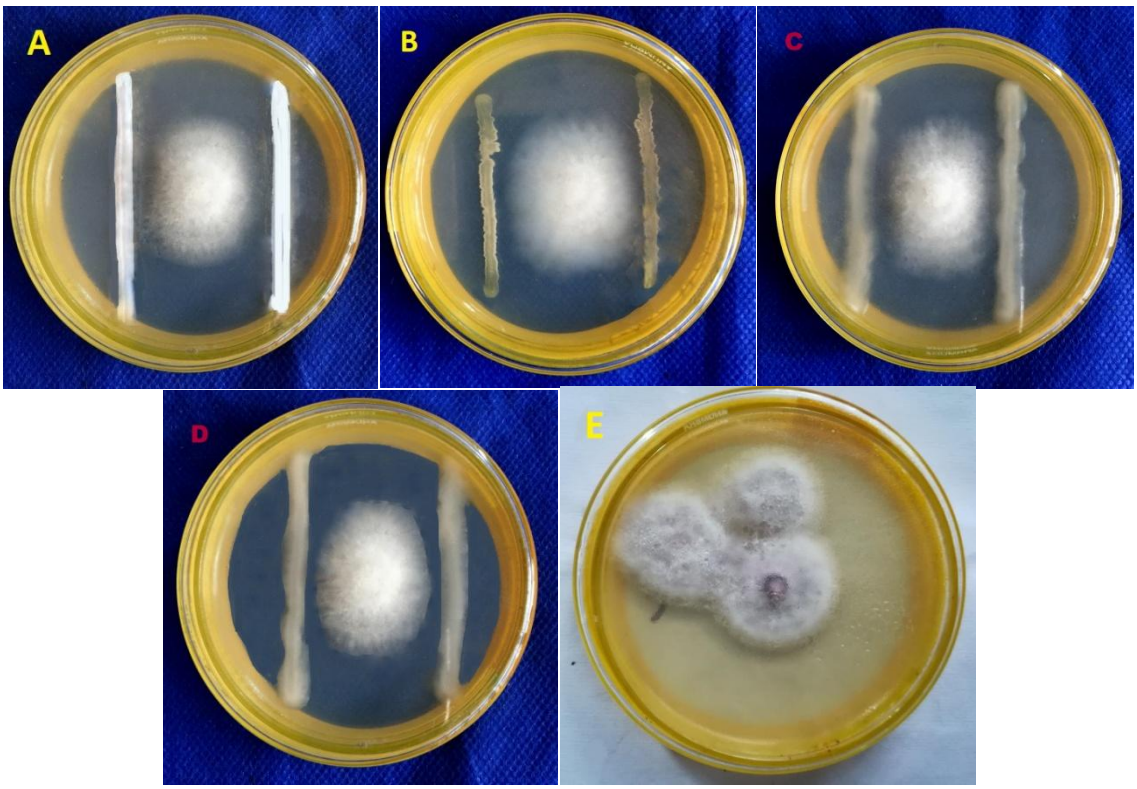

Figure 12. Radial mycelial growth of *Fusarium oxysporum* against (a) *Bacillus subtilis* (b) *Bacillus cereus* (c) *Paenibacillus polymyxa* (d) *Pseudomonas* sp. and (e) Control after 7 DAI (Only pathogen)

Table 6. Biological efficacy of the isolates in inhibition of mycelial growth of *Colletotrichum corchori* .

Bacterial isolates	**Radial mycelial growth (mm) at 7 DAI***	**% Inhibition of mycelial growth at 7 DAI***
Bacillus subtilis	42.95	20.15
Bacillus cereus	33.28	38.14
Paenibacillus polymyxa	34.81	35.28
Pseudomonas spp.	27.89	48.15
Control	53.8	-

*In column, DAI = Days after inoculation

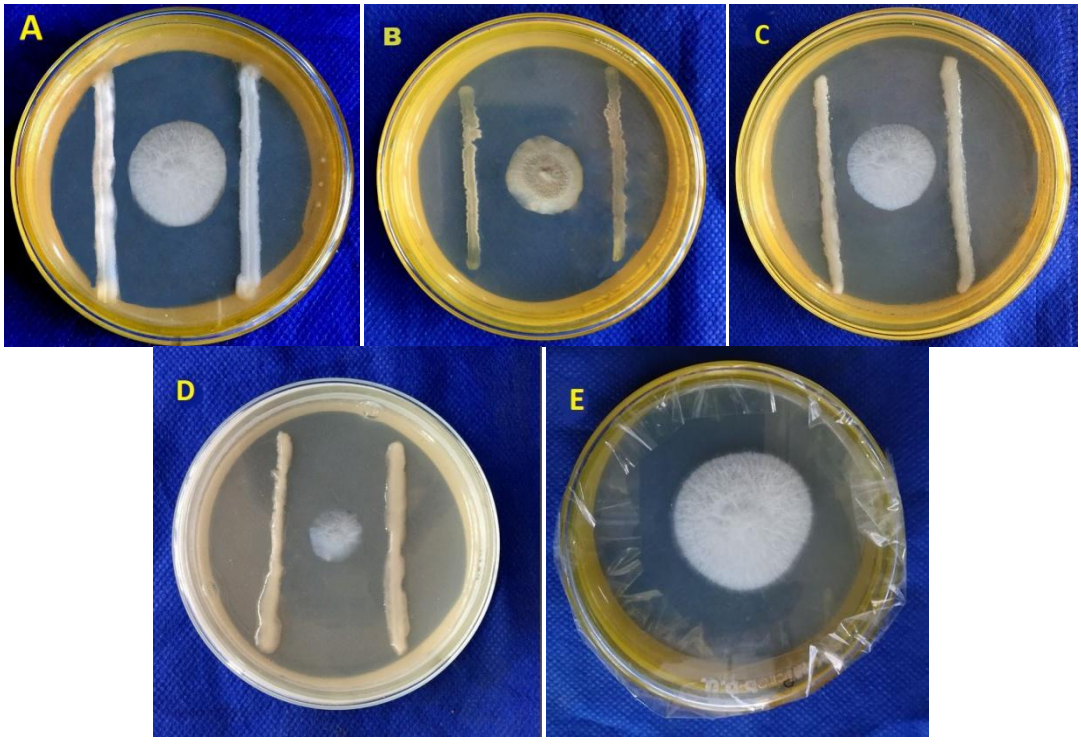

Figure 13. Radial mycelial growth of *Colletotrichum corchori* against (a) *Bacillus subtilis* (b) *Bacillus cereus* (c) *Paenibacillus polymyxa* (d) *Pseudomonas* sp. (e) Control after 7 DAI (Only pathogen)

4.6. Antagonistic effect of isolated bacteria against three selected pathogenic fungi

Biological efficacy of bacteria isolated from mushroom substrate against three pathogenic fungi were studied and found significant variations in terms of percent inhibition of radial mycelial growth of pathogenic fungi (Table 7). In case of *Fusarium oxysporum* the highest inhibition observed against *Pseudomonas* spp. (68.96 %) and the lowest against *Paenibacillus polymyxa* (46.24 %). In case of *Sclerotium rolfsii* the highest inhibition observed against *Pseudomonas* spp. (72.25 %) and the lowest against *Bacillus subtilis* (20.14 %). In case of *Colletotrichum corchori* the highest inhibition observed against *Pseudomonas* spp. (48.15 %) and the lowest against *Bacillus subtilis* (20.15 %). Results are presented in figure 11-13. In this study it has been observed that among the bacterial antagonists used against pathogenic fungi the most effective was *Pseudomonas* sp.

Table 7. Biological efficacy of bacteria isolated from mushroom substrate against three pathogenic fungi.

Bacterial isolates	**% Inhibition of mycelial growth**		
	Fusarium oxysporum	*Sclerotium rolfsii*	*Colletotrichum corchori*
Bacillus subtilis	63.12 b	20.14 d	20.15 c
Bacillus cereus	56.20 c	40.18 c	38.14 b
Paenibacillus polymyxa	46.24 d	48.85 b	35.28 b
Pseudomonas sp.	68.96 a	72.25 a	48.15 a
LSD (0.50)	2.71	2.19	3.27

Chapter V
DISCUSSION

The present study was conducted to isolate bacteria from mushroom substrates and their characterize and evaluation of biological activities against three selected pathogenic fungi (*Fusarium oxysporum, Sclerotium rolfsii, Colletotrichum corchori).*

In the present study four different types of bacteria were isolated and identified from mushroom substrate viz; *Bacillus subtilis, Bacillus cereus, Paenibacillus polymyxa* and *Pseudomonas* sp. Isolated four bacteria were evaluated against the selected pathogenic fungi viz. *Fusarium oxysporum*, *Sclerotium rolfsii* and *Colletotrichum corchori* (Siddiqui, 2005), *Pseudomonas* sp. showed more potent antagonistic activity against *Sclerotium rolfsii* in the rhizospheric soil of groundnut (Pastor *et al.,* 2010). For isolation of bacteria NA medium was used following dilution plate method. After isolation, different bacterial genera were separated by growing them on selective and semi selective media. Cultural characteristics of bacteria were also recorded as they were isolated on NA medium. Mainly three genus of bacteria *Bacillus, Paenibacillus* and *Pseudomonous* sp. were found on this study. In the present study *Bacillus* were differentiated from *Pseudomonous* using two selective media, *Bacillus cereus* agar base and cetrimide agar medium following the protocol described by Kumar *et al.* (2007).

The bacteria was identified as a *Bacillus* spp. through the good growth on *Bacillus cereus* agar base media. The colonies of *Bacillus subtilis* was found to be white umbonate elevation and dry in texture, same as by Harmon (1992), the colonies of *Bacillus cereus* was found opaque, flat to raised elevation and smooth in texture and *Paenibacillus polymyxa* are pale, raised and matt in texture same as by Orhan *et al.* (2005). On the other hand the colonies of *Pseudomonas* spp. was found to be light yellow to transparent in color, convex elevation and shiny smooth in texture same as by Beji *et al.* (1987). Under the compound microscope at 100x all of them

are rod shaped and variations found on margin and it was confirmed according to the description of Chen *et al*., (2009). In KOH solubility test for *Bacillus* spp. a mucoid thread was not formed when lifted with the loop that supports the result of Gram's staining test i.e., the bacteria was Gram positive and a mucoid thread formed for *Pseudomonas* sp. which is Gram's negative. Olivieri *et al*. (2004) and Mahmoud *et al*. (2008) in their separate works reported the similar result. Smear culture with a drop of hydrogen peroxide (H_2O_2) produced bubbles indicating positive result on catalase tests for all four bacteria. Similar biochemical test results were found by Ashmawy *et al*. (2015). *Bacillus* spp. produced a clear zone around the growth which is a positive reaction and indicates that the starch has been removed in the area around the bacterial inoculum . Bird *et al.* (1954) reported the similar result. *Pseudomonas* sp. does not produced any clear zone around the growth which is a negative reaction and indicates that the starch has not been removed in the area around the bacterial inoculum. Georgia *et al.* (1931) reported the similar result. On Gelatin liquefaction test both *Bacillus* spp. and *Pseudomonas* sp. gives the positive result same as the result reported by Johnson (2012).

In case of biological activity the highest inhibition was measured against *Fusarium oxysporum.* Among the tested bacteria the best result in terms of inhibition was found in case of *Pseudomonas* sp. Ajilogba *et al.* (2013) showed that *Bacillus* sp. and *Pseudomonas* sp. had antagonistic activity against *Fusarium oxysporum.* The tested *Pseudomonas* sp. caused 68.96% growth inhibition of *F. oxysporum* and the growth inhibition against *F. oxysporum* was very high, which is supported by Srivastava (2012), who reported that *Pseudomonas* sp. grew over the colonies of *Fusarium oxysporum*. The tested *Bacillus subtilis*, *Bacillus cereus* and *Paenibacillus polymyxa* also caused growth inhibiton of *F. oxysporum* and it was 63.12%, 56.20% and 46.24% respectively. Which is nearly similar as Borriss, (2011).

In-vitro screening, *Pseudomonas* sp. showed best performance by reducing the growth of *Sclerotium rolfsii. Pseudomonas* sp. caused 72.25% growth inhibition of *Sclerotium rolfsii,* which is supported by Nutkins *et al*. (2007). Respectively *Paenibacillus polymyxa* (48.85%), *Bacillus cereus* (40.18%) and *Bacillus subtilis* (20.14%) caused growth inhibiton of *Sclerotium rolfsii,* which is closely similar as Nair *et al*. (2002).

Pseudomonas sp. showed best performance by reducing the growth of *Colletotrichum corchori* in *in-vitro.* The inhibition is 48.15%, which was the highest. *Bacillus cereus, Paenibacillus polymyxa* and *Bacillus subtilis* showed inhition 38.14%, 35.28% and 20.15% respectively. The present findings agreed with the findings of kim *et al.* (2007).

Under this study, all four isolated bacteria showed statistically different level of inhibition on all of the selected pathogenic fungi except *Paenibacillus polymyxa.* Here *Pseudomonas* sp. showed significantly the best antagonistic activity against all the selected pathogenic fungi. Other bacteria had also potential effect against all tested pathogenic fungi.

This result clearly indicates that the *Pseudomonas* sp. have the strong antagonistic effect and could be used as an eco-friendly management against *Fusarium. oxysporum, Sclerotium rolfsii* and *Colletotrichum corchori.*

Chapter VI
Summary and Conclusion

The experiment was conducted in the Molecular Plant Pathology Laboratory of the department of Plant Pathology, Sher-e-Bangla Agricultural University (SAU), Sher-e-Bangla Nagar, Dhaka, during the period of January, 2017 to June, 2017 to isolate the bacteria from mushroom substrate and to evaluate the biological activities of isolated bacteria against selected plant pathogenic fungi (*Fusarium oxysporum, Sclerotium rolfsii, Colletotrichum corchori).* For this purpose three types of mushroom substrate were prepared and four bacterial isolates were found from that substrates. *Bacillus subtilis, Bacillus cereus* and *Paenibacillus polymyxa* were identified by selective medium *Bacillus cereus* agar base media and *Pseudomonas* sp. was identified through cetrimate agar base test.

Among the four isolates all *Bacillus* spp. showed Gram positive reaction in Gram staining test and only the *Pseudomonas* sp. showed Gram negative reaction. Except *Pseudomonas* sp. all other *Bacillus* spp. showed negative reaction in KOH solubility test and made clear zone in starch hydrolysis test. In catalase test, all the four bacteria showed the positive result in reaction with 3% H_2O_2. All of the four bacteria showed positive result in Gelatin liquefaction test. These above result revealed that, they are *Bacillus subtilis, Bacillus cereus, Paenibacillus polymyxa and Pseudomonas* sp.

To evaluate the antagonistic effect of four isolated bacteria against three selected plant pathogenic fungi (*Fusarium oxysporum, Sclerotium rolfsii, Colletotrichum corchori)* a dual culture method was conducted. Among the bacterial isolates, *Pseudomonas* spp. had the most capability to inhibit the growth of *F. oxysporum* which was 68.96%. *Bacillus subtilis* also inhibited the growth of *F. oxysporum* after than *Pseudomonas* sp. and *Paenibacillus polymyxa* had less capability to inhibit the growth of *F. oxysporum* which was 46.24%. Against *Sclerotium rolfsii, Pseudomonas* sp. had the most desirable inhibition of growth of mycelia which

was around 73%, where *Bacillus subtilis* has the less capability to inhibihit the growth, it was only 20.14%. Against the other fungi *Colletotrichum corchori*, *Pseudomonas* sp. had the highest inhibition of growth of mycelia which was 48.15% and *Bacillus subtilis* had the less capability to inhibihit the growth, its was 20.15%. *Bacillus cereus* and *Paenibacillus polymyxa* had a moderate inhibition of growth 38.14% and 35.28% respectively.

On a comparative study among the four isolated bacteria *Pseudomonas* sp. showed the best significant antagonistic activity against all the selected plant pathogenic fungi. After *Pseudomonas* sp. *Bacillus subtilis* had the most significant antagonistic activity. And the other two bacteria showed less significant antagonistic activity than *Pseudomonas* sp. and *Bacillus subtilis.*

The present study was based on the presence of bacteria on mushroom substrate and their antagonistic effect against *Fusarium oxysporum*, *Sclerotium rolfsii* and *Colletotrichum corchori*. These four bacteria had potential antagonistic effect and can be used as bio-control agent. Further works to be conducted again to find out an effective method of mass production of these bacteria.

Chapter VII
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APPENDICES

Preparation of culture media

The composition of the media used in this thesis work are given below: Unless otherwise mentioned all media were autoclaved at 121^{o}C for 20 minutes at 15 lb pressure.

Nutrient Agar (NA)

Beef extract	3.0 g
Peptone	5.0 g
Bacto agar	15.0 g
Distilled water	1000 ml

KOH solubility reagent

3% aqueous solution of KOH was prepared from the KOH granules.

Catalase reagent

3% aqueous solution of H_2O_2 was prepared from the H_2O_2 absolute solution.

Oxidase reagent

1% aqueous solution of NNN'N-tetramethyl-p-phenylene-diaminedihydrochloride was prepared from the absolute solution.

Gram's staining reagents

Gram's Crystal violet (Hucker's modification)

Solution A : Crystal violet (90% dye content)	2.0 g
Ethyl alcohol	20.0 ml
Solution B : Ammonium oxalate	0.8 g
Distilled water	80.0 ml

Solution A and B in equal volume to prepare crystal violate solution.

Gram's Iodine (Gram's modification of Lugol's solution)

Iodine	1.0 g
Potassium iodide (KI)	2.0 g
Distilled water	300.0 ml

Add iodine after KI is dissolved in water to prepare Gram's Iodine solution.

Gram's alcohol (decolorizing agent)

Ethyl alcohol (95%)	98 ml
Acetone	2 ml

Safranin (counter stain)

Safranin (2.5% solution in 95% ethanol) 10 ml

Distilled water	100 ml

Cetrimide agar

Pancreatic digest of gelatin	20 gm
Magnesium chloride	1.4 gm
Potassium sulfate	10 gm
Agar	13.6 gm
Cetyl trimethyl ammonium Bromide	0.3 gm
Glycerin	10 ml
Water	1000ml
pH after sterilization	7.2

***Bacillus cereus* agar base**

Peptone	1 gm
Manitole	10 gm
Sodium chloride	2 gm
Magnesium sulphate	0.10 gm
Disodium phosphate	2.50 gm
Monopotassium phosphate	0.25 gm
Sodium pyruvate	10.00 gm
Bromo thymol blue	0.12 gm
Agar	15gm