MOLECULAR CHARACTERISATION OF BACTERIAL ISOLATES FROM ACID SOILS OF ODISHA AND THEIR BIOTIC POTENTIAL

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HOD, Microbiology

CERTIFICATE

This is to certify that the thesis entitled **"Molecular characterisation of bacterial isolates from acid soils of Odisha and their biotic potential "** submitted here in partial fulfilment of the requirements for the award of the degree of **Doctor of Philosophy in Microbiology** of the **Orissa University of Agriculture and Technology**, Bhubaneswar, Odisha, India is a faithful record of bonafied research work carried out by Mr. Suraja Kumar Nayak under my guidance and supervision and that no part of this thesis has been submitted for any other degree or diploma. The assistance and help received during the course of investigation has been duly acknowledged.

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CERTIFICATE-II

This is to certify that thesis entitled "**Molecular characterisation of bacterial isolates from acid soils of Odisha and their biotic potential"** submitted by the student bearing Registration No. 01 MICRO/10/Ph.D. to the Orissa University of Agriculture and Technology, Bhubaneswar, for partial fulfilment of the requirements for the award of the **Doctor of Philosophy** in Microbiology, has been approved by the advisory committee after an oral examination on the same.

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DECLARATION

I do here by declare that the thesis entitled **"Molecular characterisation of bacterial isolates from acid soils of Odisha and their biotic potential"** is a record of original research work conducted by me and that no part of the thesis has been presented before for award of any other degree or diploma of any university.

Suraja Kumar Nayak

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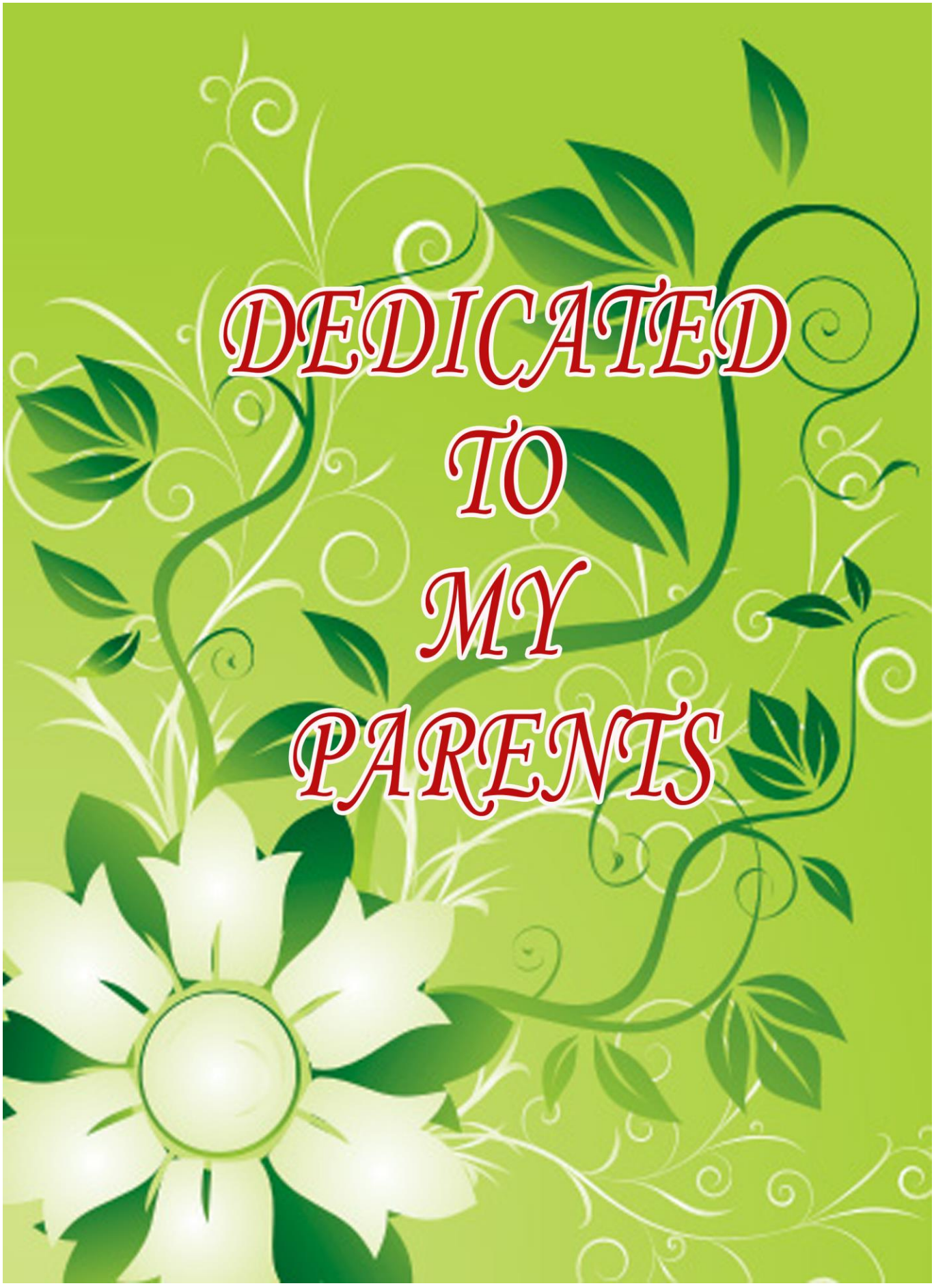
%	Percentage
°C	Degree Centigrade
µg	Microgram
µl	Microlitre
AM	ante meridiem
bp	Base pair
C.D.	Critical Difference
CFU	Colony forming Unit
Cm	Centimeter
Da	Dalton
KDa	Kilo Dalton
EPS	Extracellular polymeric substance
F	Forward primer
Fig.	Figure
FTIR	Fourier Transform infrared spectroscopy
g	gravitation (used for rotation)
gm	Gram(s)
GRAS	Generally recognized as safe
Ha	Hectares
Hrs	Hours
kb	Kilobase
kg	Kilograms

l	Litre
M ha	Million hectors
m	Meters
m/z	Mass-to-charge ratio
mg	Milligram(s)
MHz	megahertz
min	Minute
ml	Milliliter
mm	Milimiter
mM	Milimolar
MS	Mass Spectroscopy
MT	Metric tons
MTCC	Microbial Type Culture Collection
N	Normality
NB	Nutrient Broth.
ng	Nanogram
nm	Nanometer
NMR	Nuclear Magnetic Resonance
No.	Number
PBS	Phosphate Buffer Solution
PM	Post meridiem
pmole	picomole
ppb	Parts per billion
ppm	Parts per million
R	Reverse primer

rpm	Revolutions per minute
s	Second
S	Svedberg unit
UV	Ultra violet
V	Volt
v/v	Volume by volume
VIS	Visible
w/v	Weight by volume
λmax	absorption maxima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CHAPTER -1

INTRODUCTION

CHAPTER 1

INTRODUCTION

Man has been exploring the valuable gift of nature viz. air, water and soil for social benefits since time immemorial. Microbe that inhihabit in air, water and soil are also no exception to that. During ancient days microbes were being used for various purposes, without being realised of its origin and existence. A vedic epic depicts existence of such organisms, obviously not seen at that time but realised through the microbial activities and designated it as natural activities or natural phenomena. Vedic microbiology cites many such episodes. But the real breakthrough in microbial study was brought by Sir Antony van Leeuwenhoek, who first discovered microbes initiating a new era on microbial study. Since then millions of microbes have been identified and their potential established though it accounts to 10% of the total microbial diversity of the world, leaving a scope to study. Microbes with established potential have been of immense use to human civilisation but still air, water & soil need to be studied and explored for potential microbial sources of human benefits.

1.1 Soil as natural resource in study

The soil is one of the most important natural resources among the national treasure of any country. It is one of the basic source to produce food, fodder, fuel and fibre - the necessities of the human being. Soil is not only the source of minerals and water to the living organisms but also harbours innumerable microbes which are essential for maintenance of soil fertility vis-à-vis crop productivity. The agriculture and the other allied activities and in turn the prosperity and economic growth of both developing and developed countries depend on the soil resources and thereby is the inherent soil potential. Feeding, predation, degradation of soil macromolecular

substrates and absorption of nutrients have drawn attention about biogenic chemical processes in soil. These interactions involve release of secondary metabolites, which are not strictly needed for the survival and reproduction of the producer. Secondary metabolites are structurally highly diverse and each of them is produced only by a small number of species. They exert various biological effects, often at very low concentrations, and can be regarded as carriers of chemical communication among soil inhabitants. Soil with a complex environment, is also a major reservoir of microbial genetic diversity (Robe *et al.,* 2003). The complexity of microbial diversity results from multiple interacting parameters, which include pH, water content, soil structure, climatic variations and biotic activity. Soil environment is not an exception, but biotic interactions dominating soil biology differ from those in other systems because of the dominating role of sessile organisms and the lack of autotrophy in soil (chemolithoautotrophs being an interesting but not significant exception).

Soil bacteria show antifungal properties because of the production of different enzymes which may be a part of their lytic system that enables bacteria for living on hyphae as actual growth substrate (De Boer *et al.*, 1998). Due to production of antibiotics they have been used as biocontrol agents against pathogenic fungi (Yilmaz *et al.*, 2005; Gebreel *et al.*, 2008). It is, however, arguably still true: comparisons of the information presented on sources of new drugs from 1981 to 2007 indicate that almost half of the drugs approved since 1994 are based on natural products.

Soil is the most essential resource for sustained quality of human life and related activities, therefore, soil resource and agro-ecology based agricultural development should be the strategy for exploiting renewable resources. Soil is regarded as very heterogeneous with respect to conditions for microbial growth and for the distribution of microorganisms and matrix substances. This heterogeneity results in a wide variety of microbial niches and a high diversity of soil microorganisms. The microbial diversity in soils exceeds that of other environments

and is far greater than that of eukaryotic organisms; one gram of soil can contain up to 10 billion microorganisms of possibly thousands of different species.

The diversity of soil microorganisms has been exploited for many years based on the cultivation and isolation of microbial species. Most natural products of economic value such as antibiotics or other pharmaceuticals are derived from cultured soil microorganisms (Daniel, 2004). The complexity of microbial diversity results from multiple interacting parameters which include pH, water content, soil texture and structure, climatic variations and biotic activity. Soil texture and structure are mainly determined by sand, silt, clay and organic matter content through the organization of micro and macro aggregates. Microorganisms are heterogeneously distributed inside microaggregates and in macroporosities outside microaggregates (Hattori, 1988; Ranjard *et al.*, 1998).

1.2 Survey of acid soil

Acid soil is a matter of great concern not only in India but also at global basis as more than 800 M ha (million hectares)of acid soils are present. Australia has more than 7 M ha of acid soils whereas, in India it is 49 M ha, out of which 26 M ha of land soil pH is less than 5.6 and other 23 M ha of land have soil pH range 5.6 to 6.5 (Sharma and Singh, 2002). Acid soils are heavily weathered and characteristic of leaching (Alloway, 2008). About 70% of land in Odisha (previously Orissa) is acidic in nature.

The state of Odisha lies in the tropical belt in the eastern region of India at 17°31'- 22°27' latitudes and 81°27'- 87°30' longitudes. The adjoining states to Odisha are West Bengal in the north-east, Jharkhand (erstwhile Bihar) in the north, Andhra Pradesh on the south-west, Chhattisgarh (erstwhile Madhya Pradesh) on the north-west and on the east it is having a long coast line of 480 KMs with the Bay of Bengal.

It is the tenth largest state in India. The climate is characterized by high temperature and medium rainfall. Out of 1482 mm annual average rainfall, 85% is received during July through October. Mean annual temperature for the state is 26.2°C. Mean summer temperature from April to June is 30.3^{o}C and mean winter temperature from December to February is 21.3^{o}C. Odisha covering 15.57 M ha geographical area, of which 8.67 M ha is acidic. Out of 6.1 M ha cultivated area, about 70% area is acidic. Odisha has wide variations in climate, geology, land forms and vegetation, which give rise to large variations in soils. Based on stratigraphy, tectonic history, relief feature and erosion process, the state represents four broad and well-defined physical regions viz. Northern plateau, Central tableland, Eastern Ghats and Coastal plains. The geological sequences responsible for the present topography are the Archean to recent through Pleistocene. The Archeans dominate the rock system with other system like Proterozoic, Cretaceous and Carboniferous. Integrating the effects of land form, topography, climate, soil and crop adaptability the state has been divided into 10 Agro Climatic Zones. The National Bureau of Soil Survey and Land use Planning (NBSS & LUP) in collaboration with the Soil Conservation (Survey) Department have categorized the soils of Orissa into 3 Agro-Ecological Regions(AER), and 6 Agro-Ecological Sub-regions(AESR) (Sehgal *et al.*, 1993). This is based on the variability in rainfall, potential evapo-transpiration and actual evapo-transpiration. Laterite soils (Hapustalfs, Plinthustalfs, Orchaqualfs) constitute 0.70 M ha and are mildly to strongly acidic. Apart from these acid soils, there are Black soils, Deltaic alluvial soils, mixed red and black soils, which are mildly acidic to slightly alkaline.

Acid soils occupy about 30% of cultivated land in India, whereas 70% of the cultivated land in Odisha is acidic. Acid soils have poor base saturation; which generally varied from 16% to 17% in the pH range of 5.0 to 6.0. The active species of naturally occurring ions bound to the clay are H^{+} and Al^{3+}. The KCl extractable Fe^{2+}

has minor role in soil acidity compared to Al^{3+}. Humic acid, hymetamalonic acid, fulvic acid and humus contribute to acidity in various ways.

The most common of the problems in acid soils in respect of chemical properties are low pH, low CEC, nutrient imbalance, low level of base saturation percentage, high Fe^{2+}, Al^{3+}, Mn^{2+} saturation percentage, high P fixing capacity and clay fraction constituting of rather surface inactive minerals. All these problems could be managed by liming, which improves base status, inactivates Fe^{2+}, Al^{3+}, and Mn^{2+} in soil solution and reduces P fixation markedly (Panda and Kosy, 1982). Management of acid soils should aim at realization of production potential either by addition of amendment or manipulation of agricultural practices. Soil acidity in the uplands, which is caused mostly by leaching losses of bases and high percolation of water, create problems of crust formation particularly in light textured red soils.

Soil pH not only affects chemical, physical properties and crop productivity but also affects or influences soil microbial diversity. Soil pH is probably at least as important as soil C and N concentration in influencing the microbial biomass, which has acclimatized in such area, can have some novel properties. Acidophilic bacteria being nitrogen reducer or sulphur reducer may have some antimycotic activity. The central role that soil microorganisms play in the nutrient cycling and energy flow in soil has brought into consideration microbial parameters such as microbial biomass as an indicator of soil functioning (Beelen and Doelman, 1997). Soil pH largely influences the activity of soil microorganisms. Microbial and biochemical properties in addition to nutrient turnover in the soil are significantly reduced under an acidic pH condition because of the combined effect of H^+ and Al^{3+} (Baath *et al.*, 1980; Walse *et al.*, 1998; Blagodatskaya and Anderson, 1999).

1.3 Soil microflora and their potential

Prokaryotes are the most ubiquitous organisms on earth, represented in all habitats, including soil, sediment, marine and terrestrial subsurface, animals & plant tissues and play a key role in the biogeochemical cycles of the biosphere and represent an enormous reservoir of novel valuable molecules for health or industry. The vast majority of enzymes and antimicrobial products have been isolated from microorganisms cultivated on artificial media in the laboratory.

Most secondary metabolites produced by soil microbes appear to be secreted an observation which corroborates their role if controlling biotic interactions. Concerning soil microorganisms, the antibiotics paradigm has dominated experimental approaches to the ecological role of secondary metabolites so far, followed by pathogenic interactions between microorganisms. Many bacterial species are known to produce antimicrobial agents. These could be bacteriocins, inhibitory peptides or proteins (Jack *et al.*, 1995); cationic peptides, glycopeptides, ß-lactams, quinolones, streptogramins, glycylcidenes, ketolides, and oxazolidinones (Bush, 1997). With the rapid appearance of antibiotic resistant pathogens, the search is continuously on for the development of newer drugs. A large hitherto unexplored reservoir of microbial flora is being tapped as a source of various natural products. During most of the geological past of the Earth, microorganisms have primary roles in shaping the environmental conditions that exist today. They are the major drivers of the biogeochemistry, the nutrient cycles and the degradation of natural and anthropogenic waste products of the planet (Rodriguez-Valera, 2004).

Soil is considered one of the most suitable environment for microbial growth (Cavalcanti *et al.*, 2006). Production of antimicrobial compounds seems to be a general phenomenon for most bacteria. Admirable arrays of microbial defense systems are produced, including broad-spectrum classical antibiotics, metabolic by-

products such as organic acids and lytic agents like lysozyme. In addition, several types of protein exotoxins and bacteriocins, which are biologically active peptide moieties with bactericidal mode of action, are of microbial origin (Riley and Wertz, 2002; Rodríguez *et al.*, 2000). Biosurfactants are compounds of microbial origin that exhibit pronounced surface and emulsifying activities. Although most biosurfactants are considered to be secondary metabolites, some may play essential roles for the survival of biosurfactants producing microorganisms through facilitating nutrient transport or microbe–host interactions or by acting as biocide agents. Biosurfactants are suitable alternatives to synthetic medicines and antimicrobial agents and may be used as safe and effective therapeutic agents (Rodrigues *et al.*, 2006). Members of the *Bacillus* sp. are generally found in soil and most of these bacteria have proteolytic activity with the ability to disintegrate proteins (Aslim *et al.*, 2002). While many antibiotics are known to exist, efforts to discover new antibiotics still continue. Thus numerous species of *Streptomyces*, *Bacillus* and *Penicillium* have been studied due to their inherent properties of antibiotics production (Brock and Madigan, 1991) with relevant to agriculture and medical realm (Yoshiko *et al.*, 1998; Sharga *et al.*, 2004). Among the most promising candidates for bacterial biocontrol agents are the several species of the genus *Bacillus*, ubiquitously occurring safe microorganisms with proven excellent colonization aptitudes (Shoda, 2000) with an outstanding ability to sporulate, which assures their prevalence in the environment and guarantees future suitable formulation strategies (Schallmey *et al.*, 2004). In addition, they produce antibiotics of soluble protein structure and which are found to be cheaper and more effective and are preferable for commercial production, with the target to produce antibiotics such as polymyxin and bacitracin from *Bacillus* sp. (Debavov, 1982). Bacteria from various environmental habitats produce antimicrobial substances which are active against other bacteria & fungi (Risoen *et al.*, 2004). Rhizobacteria are present in the soil in an average of about 108 cells per gram (Stein, 2005). The

potential of *B. subtilis* to produce antibiotics has been recognized for 50 years. Peptide antibiotics represent the predominant class. However, systematic studies that survey the complete spectrum of antibiotic activities by different *B. subtilis* strains are rare (Pinchuk *et al.*, 2002).

Several *Bacillus* strains have been considered to be natural factories of biologically active compounds such as lipopeptides and the significance of their involvement in plant microbial disease control have been demonstrated (Asaka and Shoda, 1996; Emmert and Handelsman, 1999). Lipopeptides, oligopeptides synthesized in a non-ribosomal manner by large multienzyme complexes are the most frequent antibiotic compounds produced by Bacilli, exhibiting a wide antimicrobial spectrum and exceptional surfactant activities (Magnet-Dana *et al.*, 1992; Vanittanakom *et al.*, 1986; Vater *et al.*, 2002; Vollembroich *et al.*, 1997). These amphiphilic compounds share a common cyclic structure consisting of a β-amino or β-hydroxy fatty acid integrated into a peptide moiety. The main differences rely on the amino acid sequence and fatty acid branching, criteria that allow their classification in three families. The iturin family, represented by iturin A, mycosubtilin and bacillomycin, are heptapeptides with a β-amino fatty acid which exhibit strong antifungal activity (Duitman *et al.*, 1999; Moyne *et al.*, 2004; Thimon *et al.*, 1995; Tsuge *et al.*, 2001)

Bacteriocins are ribosomaly synthesized antimicrobial peptides that are usually inhibitory to strains closely related to the producing bacteria. These antimicrobial compounds are thought to provide the producer strain with the selective advantage over other strains. Bacteriocins produced by gram-positive bacteria are often membrane permeabilizing cationic peptides with fewer than 60 amino acid residues (Jack *et. al.*, 1995). *Bacillus* sp. includes a variety of species with a history of safe use in industry. Commercial products that are currently obtained from *Bacillus* sp. include enzymes, antibiotics, amino acids, and insecticides. The potential of *Bacillus* sp. to

produce antibiotics has been recognized for more than 50 years, and peptides antibiotics represent the predominant class. Many bacteriocins or bacteriocin-like substances (BLS) in the genus *Bacillus*, such as thuricin, cerein 7, cerein 8A, subtilosin A, and surfactin have been reported (Jack *et. al.*, 1995; Bizani and Brandelli, 2002; Stein, 2005).

The negative influence exerted by the type and concentration of carbon source on the fermentative production of secondary metabolite, has been well documented for several antibiotics (Hu *et al.*, 1984).Nitrogen source have long been known to suppress the biosynthesis of a variety of chemically unrelated antibiotics and secondary metabolites (Brana and Demain, 1988). Biosynthesis of several antibiotics was controlled by phosphate concentration (Martin, 1977). Production of gentamycin by *Micromonospora purpurea* was inhibited by higher phosphate concentration (11.5mM), while biomass production was unaffected (Obregon *et al.*, 1994) and similarly production of antibiotic tylosin by *Streptomyces* sp. was inhibited by high phosphate concentration (30 mM), while biomass was unaffected (Madry and Pape, 1982).

1.4 Molecular identification of bacteria

Morphological and physiological characteristics have traditionally provided a wealth of *Bacillus* systematic information for establishing *Bacillus* classification systems (Claus and Berkely, 1986; Wisotzkey *et al.*, 1992). The development of molecular techniques such as DNA sequencing in bacterial taxonomy have permitted objective determination of inter and intra relatedness amongst species. Genomic analyses of microbial communities in the environment have underscored the tremendous genetic diversity, potentially available to evolving members of these communities. Bacteria are excellent models for novel gene synthesis due to their high level of genomic plasticity. In addition, the recent

development in sequencing bacterial genomes and their bioinformatic analysis support the relative importance of LGT (Lateral Gene Transfer) and genetic rearrangement in microbial evolution (Lawrence and Ochman, 1998). DNA fragments have also been shown to be exchanged with the intervention of genetic elements and could influence the evolution of microbial genomes via mosaic structures (Burrus and Waldor, 2004; Tauch *et al.*, 2000). Nevertheless, while most studies speculate about the importance of LGT based on the analyses of genomic sequences and some studies measure the potential frequency of gene transfer between microorganisms, observations of real-time bacterial adaptation through DNA shuffling are limited. This limitation stems in part from the difficulty to characterize the contemporary environmental pressure necessary for selecting successful novel genes. Genetic analysis provides a successful paradigm for forward gene discovery based on phenotypic properties of organisms (Shuman and Silhavy, 2003).

Comparisons of rRNA sequences, pioneered by Woese and his colleagues, defined the main lineages in the evolution of microorganisms (Woese, 1987). An advantage of rRNA sequence comparisons is the generation of an increasingly expanding data base against which newly determined sequences may be compared (De Rijk, 1992; Maidak *et al.*, 2001). Divergence of the primary lines of bacterial descent occurred early in biotic history so highly conserved molecular chronometers are best suited to the task of reconstructing bacterial phylogeny (Woese, 1987). Ribosomal RNAs are integral elements of the protein synthesizing apparatus, the basic components of which are present in all primary kingdoms and are among the most highly conserved cellular molecules. Yet, rRNAs also contain sufficient sequence variability so that relationships between closely related groups can be determined. The abundance of rRNAs in actively growing cells made them readily obtained in the purified form needed for the earliest methodologies which determined their sequences directly. Over the last two decades, bacterial nucleic acid sequence data (genomic and

ribosomal) was obtained and included in numerous databases. At first, rRNAs were compared by oligonucleotide cataloguing (Woese, 1987). This entailed the digestion of purified rRNA with a ribonuclease to generate fragments that were then electrophorectically separated and individually sequenced. Comparison of rRNA oligonucleotide catalogs and contiguous sequences determined from cloned genes, led to identification of highly conserved nucleotide tracts in dispersed regions of the 16S rRNA. The conserved tracts, serving as priming sites, made it possible to rapidly determine nearly complete sequences using rRNA as the template for reverse transcriptase in dideoxynucleotide terminated sequencing reactions (Lane *et al.*, 1985; Lane, 1991). Highly conserved nucleotide tracts have also now been identified in the 23S rRNA (Lane, 1991).

The Molecular Evolutionary Genetics Analysis (MEGA) software was developed with the goal of providing a biologist centric, integrated suite of tools for statistical analyses of DNA & protein sequence data from an evolutionary standpoint. Over the years, it has grown to include tools for sequence alignment, phylogenetic tree reconstruction and visualization, testing an array of evolutionary hypotheses, estimating sequence divergences, web based acquisition of sequence data, and expert systems to generate natural language descriptions of the analysis methods and data chosen by the user (Kumar *et al.*, 1994, 2008; Kumar and Dudley, 2007).

1.5 Dermatophytes

Infectious diseases especially of fungal origin, are major health hazard all over the world and in some cases they cause premature deaths. Reports available indicate nearly 50,000 people die per day due to dermatophytic infections (Mulligen *et al.*, 1993) which has increased remarkably during the last decade (Terrell, 1999; Meis and Verweji, 2001). The remedial use of commercially available antifungal drugs also induces various side effects (Chotomongkol and Sukeepaisarncharoen, 1997) thereby

demanding a distinct need for the discovery of new, safer and more effective antifungal agents (Frontling and Rathway, 1987).

Dermatophytes are pathogenic fungi specialized in the infection of skin, hair and nails that utilize keratinous substrates as the source of carbon, nitrogen and sulphur sources. They belong to three anamorphic (asexual or imperfect) genera, *Epidermophyton*, *Microsporum* and *Trichophyton*, and have long been classified as anthropophilic, zoophilic and geophilic species on the basis of their primary habitat associations. Thus, anthropophilic dermatophytes are associated with humans and rarely infect other animals, zoophilic dermatophytes usually infect animals or are associated with animals, but occasionally infect humans (Nilce *et al.*, 2008). The disease generally known as tinea or ringworm may be a consequence of the inflammatory reaction of the host to the enzymes secreted by the fungus during its invasive process (Ellis *et al.*, 2000).

Once invade, the dermatophytes scavenge nutrients for their growth, a process based on the induction of structural proteins, permeases and enzymes of the cell wall, in addition to the secretion of a variety of proteins and hydrolytic enzymes such as nucleases, lipases, nonspecific proteases and keratinases, among others, which occur in response to a short supply of essential nutrients in the host (Giddey *et al.*, 2007; Giddey *et al.*, 2007). The successful initiation of infection is a process closely related to the capability of the infecting dermatophyte to overcome the host resistance mechanisms. Different kinds of mycoses, especially invasive, have become an important public health problem as their incidence has increased dramatically in the last decades in relation to AIDS (Acquired Immunodeficiency Syndrome), hematological malignancies, transplant recipients and other immunosuppressed individuals (Quindós, 2002; Pontón *et al.*, 2000; Walsh *et al.*, 2004). Fungal infections remain a major direct cause of death in patients who are treated for a malignant

disease and emerging resistance is also a serious problem (Giusiano *et al.*, 2004, 2005). These immunocompromised patients are mainly infected by *Candida* sp., *Aspergillus* sp., *Cryptococcus* sp. and other opportunistic fungi. *Candida albicans* is very often associated with serious invasive fungal infections, but other *Candida* sp. and yeast-like organisms (*Trichosporon*, *Blastoschizomyces* and *Malassezia*) have emerged as etiological agents of severe mycoses.

In the beginning of the last century, the major causes of human death were infectious diseases, but their incidence started to decrease with the improvement of basic sanitation conditions vis-a-vis discovery and widespread use of vaccines & antimicrobial agents. Although fungi do not cause outbreaks or pandemics, the incidence of severe systemic fungal infections has increased significantly, mainly because of the explosive growth in the number of patients with compromised immune system. Opportunistic fungal infections are common among patients who have AIDS or who had medical procedures that suppress the immune system, such as organ transplantation and chemotherapy. The indiscriminate use (over use and under use) of antibiotics also contributes to the worsening of this picture, leading to the installation of fungal infections. Hence, fungal infections may become an important cause of human death or at least a significant cause of reduced quality of human living standards. On this basis, it is necessary to have antifungals available for the efficient control of fungal infections.

Antibiotic applications for treatment have several side effects. The remarkable increase in antibiotics resistant bacteria species (Kina, 2003; Motta *et al.*, 2004) lead to search for new sources of antibiotics through the isolation and identification of new types of microorganisms such as bacteria, fungi and actinomycetes (Alexander, 1982). The antibiotics produced by bacteria have been gaining importance by many investigators. Bacterial species producing antibiotics have been used as biocontrol

agents against pathogenic fungi (Yilmaz *et al.*, 2005; Gebreel *et al.*, 2008). Soil is considered one of the most suitable environments for microbial growth (Cavalcanti *et al.*, 2006) and source of microbes with antimicrobial novality. The genus *Streptomyces* which is antibiotics producer has been isolated from the soil of Yemen (Ahmed, 2003). Also, of the one hundred bacterial isolates from six different soil samples collected from Egypt, 20 of them could antagonize some selected plants and human pathogenic fungi such as *Aspergillus* sp., *Fusarium oxysp*orum, *Penicillium digitatum* and *Alternaria solani* (Gebreel *et al.*, 2008). Twenty bacterial strains isolated from soil stressed ecological niches of Eastern Uttar Pradesh, India showed strong antimicrobial activities (Singh *et al.*, 2009).

Many bacteria of different taxonomic branches and residing in various habitats produce antimicrobial substances that are active against other bacteria. Both Gram -ve and Gram +ve bacteria produce bacteriocins, which constitute a heterologous subgroup of ribosomally synthesized antimicrobial peptides. In general, these are cationic peptides that display hydrophobic or amphiphilic properties and the bacterial membrane in most cases is the target for their activity (Risøen, 2004).

Huck *et al.*, 1991 and Marahiel *et al.*, 1993 reported that *Bacillus* sp. generally produced polypeptide type bacteriocins that generally affect Gram +ve bacteria and Yeast cells. It was also reported that since most *Bacillus* sp. populate the same ecosystems as *Streptomyces* and other antibiotic producers, they might have acquired resistance to antibiotics produced under natural conditions. Studies conducted till revels resistance in many bacteria against antibiotics, bacteriocin production fertility and many specific biochemical functions were controlled by plasmid DNAs. Currently, antimicrobials are perceived as an essential adjunct to both human and animal health systems worldwide. A range of antimicrobial products have been used to both treat and prevent infectious diseases of animals for over a half-century (Gustafson and Bowen, 1997). It is now widely acknowledged that for most bacterial

species, the use of antimicrobials favors the selection of resistant strains (Prescott, 1999). Indeed, some authors consider increasing resistance to be the inevitable outcome of the use of antimicrobials in both animal agriculture and human health alike (Levy, 2002). For over 30 years, antimicrobial resistance has been a serious issue of concern to animal health pharmaceutical regulators in the United States (Gustafson, 1993).

Although, many antifungal drugs have been developed during the last two decades, most are confined to a relatively few chemical classes. In addition, the occurrence of resistance in clinical isolates leads to failure in the treatment of mycosis. Thus, the effective control of dermatophytes necessitates development of a new generation of potent broad-spectrum antifungals with selective action against new targets in the fungal cells. Antifungal agents have been the focus of pharmaceutical industries, since life-threatening and irritating superficial mycoses are increasing with time at global basis. Current strategies to identify new antimicrobial drugs include the screening for natural or synthetic products that inhibit fungal growth and the designing of new molecules capable of interfering with the target fungus, affecting its viability. This strategy is also used in the searching for new molecular and biochemical targets (Jiang *et al.*, 2002).

The topic antifungal medications used by the end of the nineteenth century in the treatment of superficial mycoses consisted of some inorganic salts such as potassium permanganate, lead arsenate, mercuric chloride and potassium iodide in various cream or ointment bases. Acriflavin, gentian violet and the acids benzoic, acetylsalicylic, undecanoic, undecylenic, among others, were introduced in medical practice early in the last century as the first organic antifungal medications of topical use. It is noteworthy that the search for new antifungals was influenced by the discovery of penicillin and its clinical use during the 1940s, a time in which the idea of synthetic chemotherapy was being introduced. Systemic antifungal agents to treat

mycoses were rare until the advent of modern chemotherapy. Although number of antifungal drugs was small, fungal infections were easily treated before the 1980s because they were often limited to superficial mycoses, athlete's foot thrush caused by *C. albicans*, cryptococcosis, ringworms (keratomycoses) and a few cases of deep-seated mycoses (Pena-Muralla *et al.*, 2002)

Antibiotics are low molecular-weight (non-protein) molecules produced as secondary metabolites, mainly by microorganisms that live in the soil. A natural assumption is that soil microbes produce antibiotics in their natural habitat and use them to gain advantage over their competitors; that is, antibiotics are presumed to be involved in naturally occurring amensal relationship in the soil. Regardless of the toxicity of some antibiotics produced by bacteria from *Bacillus* sp. to the cells of mammals (e.g. polymyxines, bacitracin, etc.), they were and continued to be in the focus of attention of scientists. The amount of antibiotics produced by the group *Bacilli* approaches 167. From that more, than 66 derived from *B. subtilis* and about 23 originated from *B. brevis* (Katz and Demain, 1977). As generally recognized, these antibiotics are mainly polypeptides. Most of the peptide antibiotics produced by *Bacillus* are active against gram positive bacteria (Ming and Epperson, 2002). However, compounds such as polymyxin, colistin, and circulin exhibit activity almost exclusively against Gram -ve forms, whereas bacillomycin, mycobacillin and fungistatin are effective agents against molds and yeasts (Katz and Demain, 1977). Peptide antibiotics fall into two broad classes whose evolutionary biology is very different. The first is a large and heterogeneous category of peptides that are synthesized on very large, modular enzyme complexes (Peptide synthetases) by bacteria and fungi (Stachelhaus *et al.*, 1996; Konz and Marahiel, 1999; Eppelman *et al.*, 2001). Since these and other synthetases complexes possess enzymatic activities toward the syntheses of secondary metabolite, these are potential target for drug discovery in the production of potential bio-active peptide and polyketide as well as

their hybrids (Stachelhaus *et al.*, 1999; Maraheil, 1997). Examples of peptide antibiotics include some well known or commonly used drugs, such as bacitracin, polymyxin, amphomycin, actinomycin, gramicidin, vancomycin, penicillin, and cephalosporin (D'Aversa *et al.*, 1997). Peptide antibiotics form an unique group of "bio-active molecules" (Hancock and Chapple, 1999).

Among the several categories of biosurfactants, Lipopeptides are particularly interesting because of their high surface activities and antibiotic potential. Lipopeptides can act as antibiotics, antiviral and antitumour agents, immunomodulators or specific toxins and enzyme inhibitors. Lipopeptide profile and bacterial hydrophobicity vary greatly with the strains, iturin A being the only lipopeptide type produced by several *Bacillus* sp. strains (Ahimou *et al.*, 2001). Surfactin was found to be more efficient than iturin A in modifying the *B. subtilis* surface hydrophobic character. This aspect appears essential, in association with the antifungal properties of lipopeptides involved, in the biological control of plant diseases. A biosurfactant, arthrofactin, produced by *Arthrobacter* sp., was found to be seven times more effective than surfactin (Morikawa *et al.*, 1993). Lipopeptide surfactin was also reported to have an antitumor activity against Ehrlich's ascite carcinoma cells (Kameda *et al.*, 1974) and an antifungal activity as well as various pharmacological applications such as inhibiting fibrin clot formation and haemolysis (Bernheimer and Avigad, 1970) and formation of membrane ion channels.

The clinical resistance of microbes has been defined as the persistence or progression of an infection despite appropriate antimicrobial therapy. *In vivo*, resistance is also correlated with antifungal misuse because patients often fail to finish the full course of treatment. Thus, the indiscriminate use or dosage of drugs contributes to the failure in eliminating the disease agent completely, encouraging growth of the most resistant strains, which may lead to hard to treat fungal infections. The *in vitro* resistance of an isolate can be classified as either intrinsic or acquired.

Intrinsic resistance allows all normal members of a species to tolerate a particular drug. In this case, a specific characteristic responsible for resistance is inherent to the species and has arisen through the process of evolution. Acquired resistance is a term used when a resistant strain emerges from a population that was previously drug-sensitive

Most of the reported yeast and bacteria antagonists were naturally occurring. Microbial biocontrol agents of postharvest diseases have been criticized mainly for not providing as consistent or broad-spectrum control as synthetic fungicides. The "first generation" of biological controls agents for postharvest spoilage relied on the use of single antagonists. Perhaps it is unrealistic for us to expect disease control comparable to synthetic fungicides by the use of single antagonists. The mechanism(s) by which microbial antagonists exert their influence on the pathogens has not yet been fully understood. It is important to understand the mode of action of the microbial antagonists because; it will help in developing some additional means and procedures for better results from the known antagonists. It will also help in selecting more effective and desirable antagonists or strains of antagonists (Wilson and Wisniewski, 1989; Wisniewski and Wilson, 1992). Several modes of action have been suggested to explain the biocontrol activity of microbial antagonist. Still, competition for nutrient and space between the pathogen & the antagonist is considered as the major modes of action by which microbial agents control pathogens causing diseases (Filonow, 1998; Ippolito *et al.*, 2000; Jijakli *et al.*, 2001). In addition, production of antibiotics (antibiosis), direct parasitism and possibly induced resistance are other modes of action of the microbial antagonists by which they suppress the activity of postharvest pathogens on fruits and vegetables (Janisiewicz *et al.*, 2002; Barkai-Golan, 2001; El-Ghaouth *et al.*, 2004). *Bacillus* sp. are often considered microbial factories for the production of a vast array of biologically active molecules potentially inhibitory for phytopathogens growth, such as kanosamine or zwittermycin A from *B. cereus*. Their

spore forming ability also makes these bacteria some of the best candidates for developing efficient biopesticide products from a technological point of view. *Bacillus* spores have a high level of resistance to the dryness necessary for formulation into stable products. *B. subtilis* strains are a rich source of antimicrobial peptides with a high potential for biological control applications. *Bacillus* lipopeptides are synthesized non-ribosomaly via large multi-enzymes (non-ribosomal peptide synthetases, NRPSs) (Kowall *et al.*, 1998; Stein, 2005, Finking and Marahiel, 2004). These biosynthetic systems lead to a remarkable heterogeneity among the lipopeptides products generated by *Bacillus* with regards to the type and sequence of amino acid residues, the nature of the peptide cyclization and the nature, length and branching of the fatty acid chain (Ongena and Jacques, 2008).

B. subtilis has been used for genetic and biochemical studies for several decades and is regarded as paradigm of Gram +ve endospore forming bacteria (Moszer *et al.*, 2002). Several hundred wild-type *B. subtilis* strains have been collected, with the potential to produce more than two dozen antibiotics with an amazing variety of structures. All of the genes specifying antibiotic biosynthesis combined amount to 350 kb; however, as no strain possesses them all, an average of about 4% to 5% of a *B. subtilis* genome is devoted to antibiotic production. Peptide antibiotics, also named lipopeptides, represent the predominant class resistant to hydrolysis by peptidases and proteases (Katz & Demain, 1977). Lipopeptides are classified into three families depending on their amino acid sequence: iturins, fengycins and surfactins (Perez García, *et al.*, 2011).

1.6 Spectroscopic analysis of purified active component

Iturin A, produced by the strains of *B. subtilis*, is a potent antifungal lipopeptide taken from the soil in Ituri (Zaire) and its structure was elucidated with many properties, of which antimicrobial activity was the first reported (Besson *et al.*, 1976;

Ahimou *et al.*, 2001). Mechanism of action of Iturin A is related to the disruption of the plasma membrane by the formation of small vesicles and the aggregation of intra membranous particles in yeast cells. Iturin A has been proposed as an effective antifungal agent for profound mycosis (Tanaka *et al.*, 1996). Other members of the iturin group, including bacillomycin D and bacillomycin Lc, were also found to have antimicrobial activity against *A. flavus*, but the different lipid chain length apparently affected the activity of the lipopeptide against other fungi (Moyne *et al.*, 2001). Thus, the members of the iturin-like biosurfactant group have the potential to be used as alternative potent antifungal agents.

Ongena and Jacques, (2008) reported isolation from other strains of *B. subtilis* of five other lipopeptides such as iturin AL, mycosubtilin, bacillomycin L, D, F and L_C (or bacillopeptin). All have a common pattern of chemical constitution, led to the adoption of the generic name of "iturins" for this group of lipopeptides. The iturin groups of compounds are cyclic lipoheptapeptides which contain ß-amino fatty acid in its side chain. Lipopeptides belonging to the iturin family are potent antifungal agents. Iturin A and C, bacillomycin D, F, L and L_C and mycosubtilin were described as the seven main variants within the iturin family. They are heptapeptides linked to ß-amino fatty acid chain with a length of 14 to 17 carbons. The biological activity of iturins is different to surfactins: they display a strong *in vitro* antifungal action against a wide variety of yeast and fungi but only limited antibacterial and no antiviral activities (Moyne *et al*., 2001; Phae *et al*., 1990). This fungitoxicity of iturins almost certainly relies on their membrane permeabilization properties (Deleu *et al*., 2003). After extraction, the purification procedures of lipopeptides included chromatography methods (TLC and Column). Each step of purification will be monitored by bioassays. The bioassays could be bioautographic methods, dual culture plate. Methanol, the best solvent for extraction of antifungal compound (Kumar *et al.*, 2009)

Identification of the relative percentage of the lipid and protein portions is carried out using simple colorimetric assays, such as Bradford assay for protein determination and spectroscopic methods (FTIR). The molecular mass determination of the compounds of interest may be facilitated by MALDI-TOF MS (mass spectrometry using assisted laser desorption ionization time of flight mass spectrometry). Rf values of the spots of the standards antibiotics fengycin (0.09), iturin A (0.3), and surfactin (0.7) (Romero *et al.*, 2007 and Arrebola *et al.*, 2010). To determine which lipopeptides were directly involved in fungal inhibition, the bioautographies were performed using the pathogens as revealing microorganism.

It is reported that most of peptide antibiotics exhibit absorbance maxima at 210-230 and 270-280 nm (Motta and Brandelli, 2002; Kurusu and Ohba, 1987). A peptide antibiotic cerein, obtained from *Bacillus cereus*, shows UV absorbance peak at 250 and 273 nm.

The infrared spectrum of the antibiotic was measured as a KBr (potassium bromide) pellet. Characteristic absorption valleys at 1,540; 1,650, and 3,300cm^{-1} indicate that the antibiotic contains peptide bonds. A lactone ring is suggested by the absorption at 1,740 cm^{-1} and valleys that result from C-H stretching (2,950; 2,850; 1,460 and 1,400 cm^{-1}) indicate the presence of an aliphatic chain (Bechard *et al.*, 1998). Romero *et al.* (2007) performed active extracts from *B. subtilis* strains. The Fourier transform-infrared spectrum (FT-IR) analysis showed bands in the range of 1,630 to 1,680 cm^{-1}, resulting from the stretching mode of the CO-N bond (amide I band), and at 1,570 to 1,515 cm^{-1}, resulting from the deformation mode of the N-H bond combined with C-N stretching mode (amide II band), both indicating the presence of a peptide component and also bands at 2,855 to 2,960 cm^{-1}, resulting from typical CH stretching vibration in the alkyl chain. Also was observed at 1,730 cm^{-1}due to the lactone carbonyl absorption typical for surfactin and fengycin families of

lipopeptides. FTIR spectra of antifungal compound had a broad band centering around 3,421.5 cm^{-1} indicated an amino and hydroxyl group of amino acids (Kumar *et al.*, 2009). Analysis of the spectrum also shows typical absorption bands (1,670.5 and 1,539.8 cm^{-1}) corresponding to N-H stretching of proteins and peptides bonds (Maquelin *et al.*, 2002). Additional absorption valleys 1,418.4 and 1,488.6 cm^{-1}indicating (C-H) aliphatic side chain may be related with predominance of hydrophobic amino acids such as Val, Leu and Ile or its contains fatty acids in their structure (Bizani *et al.*, 2005).

The lipopeptide molecules are detected, in their protonated form or as Na^+ or K^+ adducts, by MALDI-TOF mass spectrometry in the m/z range of 1,400-1,550 Da (Deleu *et al.*, 2003). The molecules were separated according to their mass and were detected by the ion detector set in reflector mode (Maneerat and Phetrong, 2007; Leenders *et al.*, 1999). Although methods like ion exchange chromatography (Mukherjee *et al.*, 2006), thin layer chromatography (Desai and Banat, 1997), gel permeation chromatography (Mukherjee *et al.*, 2009) and ultrafiltration (Sen and Swaminathan, 2005; Lin *et al.*, 1998) have been used for the purification of lipopeptide biosurfactants, these techniques have a serious limitation as they don't separate individual isoforms present in the crude lipopeptide mixture.

1.7 Scope of the present Work

The antibiotic research from the discovery of Fleming to till date has been a fascinating, exciting, continuously changing and developing adventure. Antibiotics are low molecular weight (non-protein) molecules produced as secondary metabolites, mainly by microorganisms that live in the soil. The secondary metabolites isolated from microbes and exhibits either antimicrobial (antibacterial, antifungal, antiprotozoal), antitumor and/or antiviral activities, used to be called as antibiotics. The large hitherto unexplored reservoir of microbial flora is being tapped as a source

of various natural products. Several bacterial species produce antibiotics, mainly polypeptides or active metabolites from Bacillus sp. that are active against gram positive pathogens. The need for new, safe and effective antidermatophytic antibiotics is a major challenge to the Pharmaceutical industry today, especially with the increased opportunistic infections in immunocompromised host and lack of non toxic antifungal antibiotics (Suzuki *et al.*, 1991). The history of new drug discovery processes shows that novel skeletons have, in majority of cases, come from natural sources (Gupte *et al.*, 2002). This involves the screening of microorganisms using a variety of models. In the last few years, great attention has been paid to the bioactive compounds due to their ability to promote benefits for human health, such as the reduction in the incidence of some degenerative diseases antioxidant, anti-mutagenic, anti-allergenic, anti-inflammatory, and antimicrobial effects (Balasundram *et al.*, 2006; Ham *et al.*, 2009; Parvathy *et al.*, 2009). Due to the increasing serious problems of chemotherapy (multi-resistant strains, reappearing mycobacteria, HIV, etc.), new challenges in the therapy of physiological diseases and in the agriculture, the renovation of the classical screening methods, allowed by the new technologies, were highly required. The natural products may exhibit various effects or are without any discovered interaction with other living organisms. In other words, they may show some kind of biological activity. The activity may be highly specific, exhibiting usually in low concentration (representing the usual bioactivities), or may be very unspecific (toxic) action.

The most important, inherent characteristics of the bioactive microbial metabolites are their microbial origin, that is to say their specific microbial producers; their interaction with the environment, namely their various biological activities and last but not least their unique chemical structures.

Keeping the above facts in mind the present piece of work deals with the microbial diversity of lateritic soil (acid soil) of Odisha, as well as the inherent

potential of the bacterial isolates against the fungal pathogens. To find out the active compound (if any) showing antifungal activity along with the phylogenetic relatedness of the isolates using bioinformatics tool. It is pertinent to mention here that microbes in the lateritic soil of Odisha have acclimatized in the acid pH of the soil for millions of years there by undergoing certain biochemical and (or) molecular change, which might have developed some potential ability that ability that need to be explored. The present work is aimed at the exploration of antimycotic activity of microbes (bacteria) from such lateritic soil of Odisha.

1.8 Plan of Work

(i). Survey of soil sampling in acid soil region of Odisha and determination of sampling sites.
(ii). Collection of soil samples from the sampling sites at Dhenkanal, Cuttack, Jajpur and Nayagarh districts of Odisha adopting standard sampling technique.
(iii). Analysis of physico-chemical parameter like soil pH.
(iv). Isolation of bacteria from soil following standard microbiological procedures.
(v). Morphological, physiological and biochemical identification of isolates.
(vi). Collection and maintenance of human dermatophytes and phytopathogens.
(vii). Screening against the pathogens through bioassay test.
(viii). Identification of potent bacterial isolates following different biochemical tests.
(ix). Standardisation of the growth medium for the mass production of active metabolites from the most potent bacterium.
(x). Bioassay test of the metabolites against the pathogens.
(xi). Partial purification of the active component from the active metabolites through Silica gel Column and bioassay.
(xii). Purification of metabolite through Thin layer Chromatography and bioassay.
(xiii). Spectroscopic analysis of purified active component through

NMR (both C^{13} and H^{1}), UV-VIS, FTIR, Mass Spectroscopy, CHN Analysis

(xiv). Interpretation of Spectral Data.

(xv). Molecular characterisation and Phylogenetic relatedness of bacterial isolates.

(xvi). Future prospective of the work

CHAPTER -2

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITRETURE

2.1 Soil as resource in study

Of the basic natural resources soil, water, air, flora and fauna constitute the basic natural resources and the national treasure of any country. Soil is the most important natural resource because it is the medium which directly or indirectly supports the growth of almost all land life forms. Thus soil health, an assessment of the ability of soil to (i) produce, sustain plant, animal and microbial productivity and diversity, (ii) maintain or enhance water and air quality, and (iii) support human health and habitation, is important for sustenance of life.

The unconsolidated mineral or organic matter on the surface of the Earth that has been subjected to and shows effects of genetic and environmental factors of: climate (including water and temperature effects) and macro and microorganisms, conditioned by relief, acting on parent material over a period of time. A product soil differs from the material from which it is derived in many physical, chemical, biological and morphological properties & characteristics (Soil Science Glossary, Soil Science Society of America). Soil is a very complex habitat dominated by the soil solid phase. In contrast to water systems, soil is relatively recalcitrant to mixing, but soluble components of the solid soil matrix may dissolve in soil water and reprecipitate at other sites. The soil microorganisms are localized in close association with soil particles, such as complexes of clay–organic matter (Foster, 1988; Robe *et al.*, 2003). The complexity of microbial diversity results from multiple interacting parameters, which include pH, water content, soil structure, climatic variations and biotic activity. Soil can be regarded as very heterogeneous with respect to conditions

for microbial growth and for the distribution of microorganisms and matrix substances. This heterogeneity results in a wide variety of microbial niches and a high diversity of soil microorganisms (Daniel, 2004).

Acidity, associated soil infertility and mineral toxicities are major constraints to agricultural production in several parts of the world (Rao *et al.*, 1993). Acid soils are distributed over extensive areas of the humid tropics and subtropics (Van Wambeke, 1976), where they represent an important but fragile resource covering 850 mha in tropical America, 450 mha in tropical Africa, and 210 M ha in tropical Asia (Rao *et al.*, 1993). India is gifted with heterogeneous landforms and variety of climatic conditions such as the lofty mountains, the raverine deltas, high altitude forests, peninsular plateaus, variety of geological formations endowed with temperature varying from arctic cold to equatorial hot and rainfall from extreme aridity with a few cms i.e. less than 10 cms (Jaisalmer, in the state of Rajasthan) to per humid with world's maximum rainfall up to 1187.2 cms (Mawsynram, in the state of Meghalaya). Depending upon the soil, bioclimatic type and physiographic situations, the country has been grouped into 20 agro-eco regions (AER) and 60 agro-eco subregions (AESR) (Gajbhiye and Mandal, 1999).

Microorganisms in soil are essential for functioning of biogeochemical cycles, plant growth and to maintain environmental equality because of involvement in mineralizing organic molecules, regenerating nutrients to avail to plants, destroying toxic chemicals and releasing products to destroy contemporaries. Thus, microbes play a critical role in modulating global biogeochemical cycles and influence all lives on Earth (Garbeva *et al.*, 2004). In soil ecosystems, microorganisms are pivotal also in suppressing soil-borne plant diseases, promoting plant growth and promoting changes in vegetation (Garbeva *et al.*, 2004).

Substantial denitrification potential exists on acid soils, although potential denitrification-N loss is generally less than observed in comparable soils of neutral pH (Parkin *et al.*, 1985). Acid soil infertility can be caused by toxic levels of hydrogen (H^+), aluminum (Al_3^+) and manganese (Mn_2^+) and deficiencies of calcium, magnesium, phosphorus and molybdenum (Foy *et al.*, 1978; Foy, 1984). These infertility factors have been shown to affect symbiotic nitrogen fixation through their effects on any stage of the legume - rhizobium symbiosis (Alva *et al.*, 1987, 1988). In acidic soil (pH<5), there are a large amount of Aluminium ion(Al^{3+}), Al-F complexes such asAlF_2^+ , AlF^{2+}, AlF_4^-, AlF_3^0 are the main species of Al and F in soil solution of acidic soils(McLean, 1976; Wenzel and Blum, 1992; Xie *et. al.*, 2000). The analysis of microbial populations in natural habitats such as soil is one of the cornerstones of current research on the functioning of natural ecosystems (Elsas and Boersma, 2011). The diversity of soil microorganisms has been exploited for many years based on the cultivation and isolation of microbial species (Daniel, 2004). Microorganisms are key players in important ecological processes such as soil structure formation, decomposition of organic matter and xenobiotics and recycling of essential elements (e.g., carbon, nitrogen, phosphorous, and sulfur) & nutrients. An understanding of microbial dynamics and their interactions with biotic and abiotic factors is indispensable in bioremediation techniques, energy generation processes and in biotechnological industries such as pharmaceuticals, food, chemical & mining.

2.1.1 Soil microflora and their potential

Most natural products of economic value such as antibiotics or other pharmaceuticals are derived from cultured soil microorganisms, but the rate of discovery of novel natural products derived from isolated microorganisms has significantly decreased during the past couple of years (Strohl, 2000).

Fungi produce several mycotoxins which can cause economic losses and may affect human health. Mycotoxins are toxic secondary metabolites produced under appropriate environmental conditions by filamentous fungi, mainly by species of *Aspergillus*, *Penicillium* and *Fusarium* (Bennett and Klich, 2003). Soil bacteria are well known natural sources of remedies, used in the treatment of innumerable diseases since antiquity. Ethanolic extract from *Bacillus subtilis* from acid soil of Dhenkanal have potential antifungal activity against human dermatophytes (Nayak *et al.*, 2012).

Amongst several microbes including bacteria and fungi, like species of *Pseudomonas*, *Bacillus* have been investigated because their properties to produce antifungal metabolites and protect plants from fungal infection (Radheshyam *et al.*, 1990; Moita *et al.*, 2005; Siddiqui *et al.*, 2005; Nourozian *et al.*, 2006). The materials based on microorganisms have following properties: high specificity against target plant pathogens; easy degradability and paves way for mass multiplication which could be utilized for proper subject to its economical return. There is a need for new solutions to plant disease problems that provide effective control while minimizing negative consequences for human health and the environment. Biocontrol seems to be a reliable alternative to chemical fungicides, which have raised serious concerns of food contamination and environmental pollution. Biocontrol is eco-friendly, safe and may provide long term protection to the crop. Some saprotrophic bacteria (*Actinobacillus* sp., *Clostridium* sp., *Streptococcus* sp.) can serve as excellent biocontrol agents against plant pathogens and human pathogens (Fernando *et al.*, 2005).

2.2 Survey of acid soil

Vast tract of acid soil not only in Odisha or India but also across the Globe is a problem from agricultural point of view. At global basis more than 800 M ha of soils are acidic in nature. Australia has more than 7 M ha of acid soils whereas acid soils in India covers about 49 M ha of area (Sharma and Singh, 2002), out of which 26 M ha

of land having soil pH less than 5.4 and rest 23 M ha of land having soil pH range of 5.6 to 6.5 (Das, 1996). The State of Odisha covering geographical area of 15.57 M ha, lies in the tropical belt in the eastern regions of India between 17°47'-22°33' N latitude and 81°.31'-87°30' E longitudes. The climate is characterized by high temperature and medium rainfall. The average annual rainfall of the State is 1500mm and the mean annual temperature is 26.2°C. The mean summer and winter temperatures are 30.3°C and 21.3°C respectively. Out of 15.57 mha, 12.45 mha of lands are acidic in nature covering the districts of Cuttack, Kendrapada, Jajpur, Jagatsinghpur, Dhenkanal, Nayagarh, Nawarangpur, Mayurbhanj (Policy paper, 48), which constitute around 80% Acid soil region(ASR). Laterite and lateritic (high clay Indian soil) soil which covers approx area 0.70 mha (Panda, 2009) in between 20.11-20.85 (latitude) and 85.01-85.54 (longitude), are mild acidic in nature having pH in between 4.0 to 5.5. The acidity of soil is due to the following reasons:

1. Region with high rainfall, the surface soils get are leached out leaving the Fe and Al present in it. These substances are highly toxic and its oxides and hydroxides reacts with water to release hydrogen (H^+) ions and the soil becomes acidic.

2. It may be due to the parent material like granite which contributes to the soil acidity.

3. The use of fertilizers like ammonium sulphate $(NH_4)_2SO_4$ and ammonium nitrate $(NH_4)NO_3$ increases the soil acidity.

4. Humus during decomposition by the microbes sometimes contain carboxylic (-COOH), phenolic (-OH) which attracts and dissociates H^+ ions.

5. Soil containing high concentrations of CO_2, are low in pH. Root activity and metabolism helps increase in CO_2 concentration and soil acidity.

6. Under favorable condition oxides of Iron and Aluminium undergoes stepwise hydrolysis and releases hydrogen (H^+) and develop soil acidity.

2.3 Soil microbes and their potential

Microorganisms represent the largest and most diverse biotic group in soil, with an estimated one million to one billion microbes per gram (gm) of agricultural top soil (Tugel and Lewandowski, 1999). Microorganisms from all three kingdoms (Monera, Protista and fungi) have been described to thrive under extreme conditions. Acidophilic organisms can grow optimally at pH values between l.0-4.0 (Extreme acidophilic), 4.0-5.9 (Moderate acidophilic) and 5.9-6.9 (Mild acidophilic). The acid soil with pH range 4.8 to 6.2 favors growth and sustenance of variety of microbes especially bacteria having adaptability to acidic environment (Nayak *et al.*, 2012). Certain fertilizer applications, such as the injection of anhydrous ammonia, can temporarily harm some soil organisms at the injection site (Tugel and Lewandowski, 1999). In their natural habitats, prokaryotes exist in mixed populations. It is extremely difficult to study morphological, physiological and molecular properties of populations of prokaryotes when they are growing and interacting with other populations. This is why microbiologists study prokaryotes in pure cultures. Dilution and spread plate technique is the method often used when mixed populations of prokaryotes exist in a natural sample (i.e. soil, pond water, fecal material, etc.) and the researcher would like to obtain isolated colonies and enumerate the number of cultivable prokaryotic cells in the sample. Endospore forming soil bacilli have been shown to form a large percentage of the bacterial flora developing on dilution plates made from many soils, especially forest soils, but they are rare in low temperature soil (Baker and Smith, 1972).

Natural products have been the source of most of the active ingredients of medicines. This is widely accepted to be true when applied to drug discovery in ‘olden times’ before the advent of high throughput screening (HTS) and the post

genomic era when more than 80% of drug substances were natural products or inspired by a natural compound (Sneader *et al.*, 1996). Biological control, using microorganisms to suppress plant disease, offers a powerful alternative to the use of synthetic chemicals. The rich diversity of the microbial world provides a seemingly endless resource for this purpose. Increasing the abundance of a particular strain in the vicinity of a plant can suppress disease without producing lasting effects on the rest of the microbial community or other organisms in the ecosystem. Fungal infections have been gaining prime importance because of the morbidity of hospitalized patients (Beck-Sague and Jarvis, 1993). Fungi are a large group with about 250,000 species, of which more than 300 species have been reported to be potentially pathogenic to humans (Guarro *et al.*, 1997). *Aspergillus* sp. is common contaminants of agriculture, producing ochratoxin and aflatoxin cause human colonial cancer. Citrinin is isolated from several species of *Penicillium* and *Aspergillus* and fumonisin is produced notably by *Fusarium* sp. (Moss, 1996; Etzel, 2002). Sabouraud Dextrose agar medium was used for fungal growth (Georg *et al.*, 1954).

2.4 The Dermatophytes

Skin diseases, especially ringworm, are an important problem in India. Most antifungal creams used in India and other developing countries are imported & very expensive. Therefore, the development of an antifungal agent from local raw material indigenously is contemporary to the necessary requirement of the country. Members of the genus *Bacillus* are generally found in soil and most of these bacteria have the ability to disintegrate protein (proteolytic activity). The high proportion of antimicrobial compound producing strains may be associated with ecological role, playing a defensive action to strains into an established microbial community (Strahl *et.al*, 2002).

Pathogenic fungi present a threat not only to immunocompromised patients with immune systems weakened by AIDS, aggressive cancer chemotherapy or drugs

aimed at foiling rejection of transplanted organs but also to others particularly when microbes are resistant to antifungal agents (McGinnis and Rinaldi, 1991; Odds, 1993).

The dermatophytes are a group of fungi that have the capacity to invade keratinized tissue (skin, hair, and nails) of humans and other animals to produce an infection, dermatophytosis, commonly referred to as ringworm. They utilize keratinous substrates as the Carbon, Nitrogen and Sulphur sources**.** They belong to three anamorphic (asexual or imperfect) genera, *Epidermophyton, Microsporum* and *Trichophyton* and have long been classified as anthropophilic, zoophilic and geophilic species on the basis of their primary habitat associations. Thus, anthropophilic dermatophytes are associated with humans and rarely infect other animals, zoophilic dermatophytes usually infect animals or are associated with animals but occasionally infect humans and geophilic dermatophytes are primarily associated with keratinous materials such as hair, feathers, hooves and horns but as a part of their decomposition process (Nilce *et al.*, 2008). Infection is generally cutaneous and restricted to the nonliving cornified layers because of their inability to penetrate the deeper tissues or organs of immunocompetent hosts (Dei Cas and Vernes, 1986; King *et al.*, 1975). Dermatophytes derepress unspecific proteolytic enzymes and keratinases with optimum activity at acidic pH during the initial stages of infection probably because human skin has an acidic pH. The successful initiation of infection is a process closely related to the capability of the infecting dermatophyte to overcome the host resistance mechanisms. Approximately 90% of human fungal infections are caused by *Aspergillus*, *Candida*, *Cladosporium*, *Epidermophyton*, *Microsporum* and *Trichophyton* sp. (Dasgupta, 1998; Pathak, 1998). Of major concern are the cases of systemic mycoses caused by *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Fusarium* sp., *Histoplasma capsulatum* and *Pneumocystis carinii*, which have been increasing over the years. Dasgupta (1998) reported an increase over 400% in mycotic infection during the past two decades. The frequency of *Candidiasis* has

increased ten-fold and as such *C. albicans* has become the fourth most common culture isolate. Dermatophyte colonization is characteristically limited to the dead keratinized tissue of the *Stratum corneum* and results in either a mild or intense inflammatory reaction. Although, the cornified layers of the skin lack a specific immune system to recognize this infection and get rid itself, nevertheless, both humoral and cell-mediated reactions and (non)specific host defense mechanisms respond and eventually eliminate the fungus, preventing invasion into the deeper viable tissue (Weitzman and Summerbell, 1995). The dermatophytes must scavenge nutrients for growth, a process based on the induction of structural proteins, permeases and enzymes of the cell wall, in addition to the secretion of a variety of proteins and hydrolytic enzymes such as nucleases, lipases, nonspecific proteases and keratinases etc., which occur in response to a short supply of essential nutrients in the host (Giddey *et al.*, 2007).

The real founder of dermatomycology was David Gruby on the basis of his discoveries during 1841 to 1844 as evident from his communications to the French Academy of Science and his publications during this period. Independently and perhaps unaware of the work of Remak and Schönlein, he described the causative agent of favus, both clinically & in microscopic details of the crusts and established the contagious nature of dermal disease.

C. neoformans, an encapsulated yeast, is the second most common cause of opportunistic fungal infection in patients with AIDS and also can cause disease in normal hosts (Subramanian & Mathai, 2005). The clinical manifestations of this infection can range from an asymptomatic colonization of the respiratory tract to widespread dissemination, depending on the host immune response. When dissemination occurs, the central nervous system is commonly involved. Risk factors include: advanced HIV stage, use of corticosteroids, lymphomas, solid organ transplants and immunosuppressive diseases or drugs (Krick, 1981). In *Trycophyton*

spp macroconidia present, have smooth, usually thin walls with one to 12 septa, are borne singly or in cluster, and may be elongate & pencil shaped, clavate, fusiform, or cylindrical. They range in size from 8 to 86 by 4 to 14mm. Microconidia, usually more abundant than macroconidia which may be globose, pyriform or clavate or sessile or stalked and are borne singly along the sides of the hyphae or in grape like clusters.

2.5 Antagonism of bacteria and fungi

The antagonism between microbial strains can be expressed in a number of ways; the most common are production of metabolites, competition and direct parasitism, but other mechanisms are involved, for example induced resistance sometimes associated with reduction of pathogen enzyme activity. The dual solid cultures have indicated the existence of an antagonism between several microbial strains. Different sensitivities of the fungi to the various bacteria may indicate the production of different metabolites or antifungal products. The performed experiments based on agar diffusion technique showed that some fungi are resistant to biocontrol agent, while others are sensitive to inhibition. The inhibitory effects of different strains on the growth of various phytopathogens were tested in Petri dishes containing the PSA medium, following the published procedure (Gong *et al.*, 2006). Inhibition of fungal growth was evaluated as the percentage reduction in the growth of the mycelia to that of control.

Bacteria of different taxonomic branches and residing in various habitats produce antimicrobial substances that are active against other bacteria and fungi. Both Gram -ve and Gram +ve bacteria produce bacteriocins, which constitute a heterologous subgroup of ribosomaly synthesized antimicrobial peptides. In general, these substances are cationic peptides that display hydrophobic or amphiphilic properties and the bacterial membrane in most cases is the target for their activity (Risøen *et al.*, 2004).

Soil bacteria showed antifungal properties because of the production of chitinase which may be a part of a lytic system that enables bacteria for living on hyphae as actual growth substrate (De Boer *et al.*, 1998). Due to production of antibiotics they have been used as biocontrol agents against pathogenic fungi (Yilmaz *et al.*, 2005; Gebreel *et al.*, 2008). It is, however, arguably still true: comparisons of the information presented on sources of new drugs from 1981 to 2007 indicate that almost half of the drugs approved since 1994 are based on natural products. Currently, antimicrobials are worldwide perceived as an essential adjunct to both human and animal health systems. A range of antimicrobial products have been used to both treat and prevent infectious diseases of animals for over a half-century (Gustafson and Bowen, 1997). It is now widely acknowledged that for most bacterial species the use of antimicrobials favors the selection of resistant strains (Prescott, 1999). Indeed, some authors consider increasing resistance to be the inevitable outcome of the use of antimicrobials in animal, agriculture and human health alike (Levy, 2002). There is widespread agreement that antimicrobial resistant bacteria is increasingly ubiquitous and that its costs are immense and growing. There is a continuing need for more effective and safer fungicides, especially those with novel modes of action, which have resistance breaking properties, and natural products have a key role to play in the search for such compounds (Liu *et al.*, 2007).

2.6 Soil *Bacillus* species

The concern over toxicity and the development of resistance to some fungicides makes it necessary to find safe and effective fungicides. Microbial metabolites have attracted attention as potential alternatives to synthetic pesticides (Saxena and Pandey, 2001; Joshi *et al.*, 2008; Zhao *et al.*, 2010). Additionally, the exploration of the bioactivity of natural products continues to provide novel chemical scaffolds for further drug inventions (Dayan *et al.*, 2009). *Bacillus* sp. can produce more than 24

antifungal substances with a wide variety of structures, such as bioactive peptides and volatiles (Stein, 2005; Joshi *et al.*, 2008).

Sporulating Gram-positive microorganisms, such as *Bacillus* and *Streptomyce*s, produces heat and desiccation resistant spores, this can be formulated readily into stable products. There are substantial base of industrial experience with *Bacillus* and *Streptomyces* sp. which have been used for insect biocontrol, industrial enzyme production and antibiotic production for many decades. This experience can be applied to the use of members of these same genera for biocontrol to overcome current obstacles to fermentation, formulation, and storage. Some of these organisms have been the subject of intense study at the genetic and biochemical level, providing a basis for study of them as biological control agents.

Bacillus, established by Chon in 1872, has undergone considerable taxonomic changes over the years. In the 2ndedition of the Taxonomic Outline of Bergey's Manual of Systematic Bacteriology (Ludwig *et al.*, 2009) phylogenetic classification schemes, accomplished mainly by the analysis of 16S rDNA sequence similarities, included in the family of *Bacillaceae*, the genus *Bacillus* made up by 94 species. *Bacillus* sp. are an important source of fine biochemicals, antibiotics and insecticides. Moreover, the ability of *B. subtilis* and close relatives to secrete grams per litre of proteins directly into the growth medium and their well proven safety have also made them prime candidates for the production of heterologous proteins. In fact, about two-thirds of the enzyme market (proteases, amylases, rennet substitutes, endonucleases, glucose-dehydrogenase and pullulanase) for industrial applications are produced by fermentation from species of *Bacillus*. *B. subtilis* has been used for the production of nucleotides, sold as food flavour enhancers, amino acids (such as tryptophan, histidine and phenylalanine) and vitamins such as biotin, folic acid and riboflavin (Queener and Lively, 1989). Although, δ-endotoxins from *B. thuringensis* are the most known and

used proteinaceous metabolites derived from *Bacillus*, recently a large variety of antimicrobial peptides have been discovered in these bacteria. Some of these peptides can play a role in competence and in the de-repression of various stationary-phase genes involved in sporulation.

2.6.1: (Active) Metabolites from *Bacillus* sp.

Representatives of the *Bacillus* spp and biologically active metabolites produced by them in addition to their practical application in various branches of the economy have been widely studied in the scientific literature (Egorov, 1986). *Bacillus* genus is made up of Gram +ve aerobic or facultative endospore forming rod shaped bacteria that includes both mesophiles and extremophiles. These microorganisms are metabolically chemoorganotrophs being dependent on organic compounds as sources of carbon and energy. In addition, their ability to form highly resistant endospores is the key for their successful colonization of a wide variety of environments. Due to their wide ubiquity in nature and genetic and metabolic diversity leading the production of several antibiotics and enzymes, these bacteria have become increasingly important for different biotechnological applications ranging from the production of fermented foods to engineered industrial enzymes used in food and detergent industries (Priest *et al.*, 1993). Secondary metabolites are structurally highly diverse and each of them is produced only by a small number of species. They exert various biological effects, often at very low concentrations and can be regarded as carriers of chemical communication among soil inhabitants. These are not strictly needed for the survival and reproduction of their producers (Karlovsky, 2008). Most secondary metabolites produced by soil microbes appear to be secreted an observation which corroborates their role if controlling biotic interactions. They possess unusual chemical linkages, such as lactam rings, cyclic peptides made of normal and modified amino acids, unsaturated bonds of polyacetylenes & polyenes and large macrolide

rings. They include mycotoxins, antibiotics, pigments, and pheromones. An important characteristic of secondary metabolism is that it is usually suppressed by high specific growth rates of the producing cultures. In addition to growth rate control, individual biosynthetic pathways are often affected by regulatory mechanisms such as induction, nutrient repression, synthetase decay, and end-product regulation (Demain, 1986). Primary metabolites are chemical components of living organisms that are vital for their normal functioning, while secondary metabolites are compounds which are dispensable. A distinguishing feature of secondary metabolites is that their production is limited to a group of species or genera and is rarely conserved over a wide taxonomical range, while primary metabolism is conserved among phyla and across kingdoms. Some authors also use terms like "extrolites, i.e. an outwardly producers directed chemically differentiated product of a living organism", "special metabolites" (Gottlieb, 1990), "idiolites" (Demain, 1986), "ecological metabolites" instead of secondary metabolites.

Extraction and production of bioactive compounds by fermentation is also an interesting alternative that merits attention since it is able to provide high quality and high activity extracts while precluding any toxicity associated to the organic solvents. In this process, bioactive compounds are obtained as secondary metabolites produced by microorganisms after the microbial growth is completed (Nigam, 2009). Studies on liquid culture shows that the production of these compounds starts when growth is limited by the exhaustion of one key nutrient: Carbon(C), Nitrogen (N) or Phosphate (P) source (Barrios-González *et al.*, 2005).

The 16S rRNA gene sequence has been widely used as a molecular method to estimate phylogenetic relationships among bacteria. More recently its use has also been proposed for the identification of unknown bacteria. The 16S rRNA gene is highly conserved among bacterial species, but it has some variable zones that

can be used for identification purposes. These zones can be amplified by specific primers and the sequence can be introduced in available online data bases. The identification is based on the similarity with other sequences within the data base. Although it is very useful and simple method for the identification of genus and species of bacteria, it does not allow differentiation of subspecies (Sato *et al.*, 2001; du Plessis *et al.*, 2004; Moreno-Arribas *et al.*, 2008). Acid nucleic hybridization techniques are important for the detection and identification of microorganisms allowing higher resolution than those based on the 16S rRNA gene sequence. From a taxonomic point of view the 16S-23S rRNA region from the bacteria genome has been proposed as a useful tool for species identification (Rodas *et al.*, 2003, 2005). The common principle of all the techniques based on "nucleic primers" is to reveal the presence of a DNA or RNA fragments complementary to those of the primer by hybridization. Therefore, the choice of the primer sequence is an outstanding decision, since it will determine the taxonomic level of the study. The main objective of any microbial classification system is to identify the species level, which is the basic unit of the taxonomic grouping. Nevertheless, from an industrial point of view, the discrimination or classification of different strains or genotypes of a same species are of increasing interest. This is mainly due to the differences in transformation products (metabolites) and technological properties the strains of the same species could exhibit (Ángeles Pozo-Bayón *et al.*, 2009).

Biologist-friendly software tools are crucial in this age of burgeoning sequence databases. These tools not only make it possible to use computational and statistical methods but also allow scientists to select methods and algorithms best suited to understand the function, evolution and adaptation of genes and species. MEGA (Molecular Evolutionary Genetics Analysis) is an integrated tool for conducting sequence alignment, inferring phylogenetic trees, mining web-based databases,

estimating rates of molecular evolution, inferring ancestral sequences, and testing evolutionary hypotheses. MEGA is used in a large number of laboratories for reconstructing the evolutionary histories of species and inferring the extent and nature of selective forces shaping the evolution of genes and species. Most of the currently used methods of phylogenetic inference from molecular data are based on some form of optimization principle (Swofford *et al.*, 1996). Under this principle, the preferred tree is determined by assigning an optimality score to all possible topologies (or all potentially correct topologies) according to a certain procedure and choosing the topology that shows the highest or lowest optimal score. For ex-ample, in the case of maximum-parsimony (MP) methods (Eck and Dayhoff, 1966; Fitch, 1971), the minimum number of evolutionary changes that explains the entire sequence evolution (tree length [TL]) is computed for each topology, and the topology showing the smallest TL value is chosen as the preferred tree (MP tree). In this paper, we used five different algorithms for constructing MP trees that are available in the computer program PAUP* (Swofford, 1998). The simplest algorithm used was stepwise addition with the closest option (SA). This algorithm is a rough search of MP trees, and it often fails to find the true optimal (MP) tree.

2.7 Antifungal antibiotics

Among the different types of drug prevailing in the market, antifungal antibiotics are very small but significant group of drugs and have an important role in the control of mycotic diseases. The history of new drug discovery processes shows that novel skeletons have, in the majority of cases, come from natural sources (Bevan *et al.*, 1995). This involves the screening of microorganisms and plant extracts, using a variety of models (Shadomy, 1987). Now a days emphasis is being given on the exploration of unusual and previously ignored ecosystems, using a variety of selected novel targets (Cragg *et al.*, 1997; Von Dohren and Grafe, 1997; Hegde *et al.*, 2001;

Phoebe *et al.*, 2001). The discovery of antifungal antibiotics dates back when Oxford *et al.* (1939) reported the isolation of the first antifungal antibiotic, griseofulvin or "curling factor". Griseofulvin inhibits the growth of various species of *Epidermophyton*, *Macrospore* and *Trichophyton*. In 1950, fungicidin (later renamed nystatin) was discovered by Hazen and Brown. Antifungal agents now constitute 15–16% of the total activity in the infective area (Dasgupta, 1998) and the world market for the antifungals is reported to be expanding at a rate of 20% per annum (Khan and Gyanchandani, 1998). One reason for the slow progress in development of antifungals compared to antibacterials is that, like mammalian cells, fungi are eukaryotes for which the agents that inhibit protein, RNA or DNA bio-synthesis in fungi have greater potential for toxicity to the host as well (Georgopapadakou and Walsh, 1994). Antifungal agents have a wide application in human medicine, agriculture and veterinary medicine (Misato and Yamaguchi, 1977; Vandamme, 1984). Five major classes of systemic antifungal compounds are currently in clinical use; the polyene antibiotics, the azole derivatives, allylamines and thiocarbamates, the morpholines and the nucleoside analogs (Georgopapadakou and Walsh, 1994). Among biopreservatives, more than 500 antimicrobial compounds have been described so far. *Bacillus* genus has been reported to produce more than 45 antimicrobial molecules; some of these compounds are of clinical value, others are assayed *in vitro* to control food microbes and the remaining ones control plant diseases (Stein, 2005; Urdaci and Pinchuk, 2004). Notwithstanding the failures, the exponential increase in the total number of discovered compounds in the last decades has surprisingly become constant. In 1940 only 10~20, in 1950 300~400, in 1960 approximately 800~1000 and in 1970 already 2500 antibiotics are known. From that time the total number of known bioactive microbial metabolites has doubled in every ten years. In 1980 about 5000, in 1990, 10000 and in 2000 already almost 20000 antibiotic compounds are known. By

the end of 2002 over 22000 bioactive secondary metabolites (including antibiotics) were published in the scientific and patent literature.

Presently efforts are being made to inhibit specific enzymes in the metabolic pathway of the fungus. For example, inhibition of glucan and/or chitin synthase, which leads to a lack of cell wall formation. Inhibition of squalene epoxidase and/or squalene synthetase is another target that has been chosen (Tanimoto *et al.*, 1996; Wills *et al.*, 2000). Antifungal therapy often involves the use of azole compounds among which fluconazole is the major drug commercially. As these agents are generally fungistatic and used for lifetime therapy of AIDS patients, resistance has emerged as a significant problem, particularly for *Candidiasis* in over 10% of late stage AIDS patients (Baily *et al.*, 1994). Virulence is defined as the relative infectiousness of a microorganism causing disease combined with the capability to overcome the host natural immune defenses. The most important virulence factors for dermatophytes described so far are the enzymes released during *Stratum corneum* and nail invasion, including keratinases, proteinases, lipases, mucinolytic enzymes, elastases and DNases (Brasch *et al.*, 1994).

Although many antifungal drugs have been developed during the last two decades, they are confined to a relatively few chemical classes. In addition, the occurrence of resistance in clinical isolates leads to failure in the treatment of mycosis. Thus, the effective control of dermatophytes will necessarily involve the development of a new generation of potent broad spectrum antifungals with selective action against new targets in the fungal cells, without irreversible side effects in the host. The metabolic responses that govern homeostatic pH and extracellular pH sensing represent an interesting area to be studied in dermatophytes, since these mechanisms are possibly involved in the installation, development and survival of dermatophytes in humans. The topic antifungal medications used by the end of the nineteenth century in the treatment of superficial mycoses consisted of some inorganic

salts such as potassium permanganate, lead arsenate, mercuric chloride and potassium iodide in various cream or ointment bases. Acriflavin, gentian violet and the acids benzoic, acetylsalicylic, undecanoic, undecylenic, among others, were introduced in medical practice early in the last century as the first organic antifungal medications of topic use. It is noteworthy that the search for new antifungals was influenced by the discovery of penicillin and its clinical use during the 1940s, a time in which the idea of synthetic chemotherapy was being introduced. Systemic antifungal agents to treat mycoses were rare until the advent of modern chemotherapy. Although, the number of antifungal drugs was small, fungal infections were easily treated before the 1980s because they were often limited to superficial mycoses, athlete's foot, thrush etc. caused by *C. albicans*, *Cryptococcosis*, ringworms (keratomycoses) and a few cases of deep-seated mycoses (Pena-Muralla *et. al.*, 2002).

Biofilms are differentiated masses of microbes that adhere to surfaces and are surrounded by a matrix of extracellular polymers, increasing resistance to standard antimicrobials. It is well known that *C. albicans* biofilm are highly resistant to most of the antifungals (Peltroche *et. al.*, 2006). The concept of biofilm for dermatophyte was introduced by Burkhart *et al.* (2002) to explain dermatophytomas, a form of onychomycosis refractory to standard antifungal therapies. Dermatophytomas are characterized by a dense white mass of fungus tenaciously adherent to the surrounding nail plate, which may require surgical removal (Burkhart *et. al.,* 2002).

2.8 Molecular study of antifungal bioactive compounds

Progress in methods of isolation and structure elucidation has led to an increase in the number of scientific publications pertaining to pharmacological examination of individual compound of plant as well as of microbial origin. Validation and selection of primary screening assays are pivotal to ensuring the sound selection of extracts or compounds with relevant pharmacological action and worth following up. Use of

ethnopharmacological knowledge is one attractive way to reduce empiricism and enhance the probability of success in new drug finding efforts (Patwardhan *et al.*, 2005). One of the most important challenges before using (or selling) a bioactive molecule is to purify it from the originating medium.

Peptide antibiotics, also named lipopeptides, represent the predominant class. They exhibit highly rigid, hydrophobic and/or cyclic structures with unusual constituents like D-amino acids and are generally resistant to hydrolysis by peptidases and proteases (Katz & Demain, 1977). Furthermore, cysteine residues are either oxidized to disulphides and/or are modified to characteristic intramolecular C–S (thioether) linkages, and consequently the peptide antibiotics are insensitive to oxidation (Stein, 2005). Lipopeptides are classified into three families depending on their amino acid sequence; iturins, fengycins and surfactins (Perez García *et al.*, 2011). The surfactins (Family: Iturin) are powerful biosurfactants which show antibacterial activity but no marked fungitoxicity (with some exceptions) (Ongena & Jacques, 2008). Iturin A, the first compound discovered of the iturin group and its best known member, was isolated from a strain of *Bacillus subtilis* strain taken from the soil in Ituri (Zaire) and its structure was elucidated. The subsequent isolation from other strains of *Bacillus subtilis* of five other lipopeptides such as iturin AL, mycosubtilin, bacillomycin L, D, F and LC (or bacillopeptin) was reported (Ongena & Jacques, 2008). These are potent antifungal agents which can be used as biopesticides for plant protection and against human dermatophytes. The iturin groups of compounds are cyclic lipoheptapeptides which contain a ß-amino fatty acid in its side chain.

Iturins display strong antifungal action against a wide variety of yeasts and fungi but only limited antibacterial activity. Fengycins also show a strong fungitoxic activity, specifically against filamentous fungi (Ongena & Jacques, 2008). The ability of various *Bacillus* strains to control soil borne, foliar and postharvest fungal diseases

has been attributed mostly to iturins and fengycins (Ongena & Jacques, 2008; Romero *et al.*, 2007; Arrebola *et al.*, 2010). The surfactin family encompasses structural variants but all members are heptapeptides interlinked with a ß-hydroxy fatty acid to form a cyclic lactone ring structure (Peypoux *et al.*, 1999). Because of their amphiphilic nature, surfactins can also readily associate and tightly anchor into lipid layers and can thus interfere with biological membrane integrity. Iturin A and C, Bacillomycin D, F, L and LC and Mycosubtilin were described as the seven main variants within the iturin family. They are heptapeptides linked to a ß-amino fatty acid chain with a length of 14 to 17 carbons. The biological activity is different to surfactins; they display a strong *in vitro* antifungal action against a wide variety of yeast and fungi but only limited antibacterial and no antiviral activities (Moyne *et al.*, 2001; Phae *et al.*, 1990). This fungitoxicity of iturins almost certainly relies on their membrane permeabilization properties (Deleu *et al.*, 1999). However, the underlying mechanisms based on osmotic perturbation owing to the formation of ion conducting pores and not membrane disruption or solubilization as caused by surfactins (Aranda *et al.*, 2005).

2.8.1. Purification and spectroscopic study of active compound

After extraction, the purification procedures of lipopeptides included chromatography methods (TLC, Ion Exchange Chromatography and RP-HPLC). Each step of purification will be monitored by bioassays. The bioassays could be bioautographic methods, dual culture plate, etc. Different solvents are used for extraction of lipopeptides from cell free supernatant. The solvents used are n-hexane, ethyl acetate, petroleum ether, chloroform and methanol to determine the best solvent for extraction of antifungal compound (Kumar *et al.*, 2009). The lipopeptide produced by cultures of *Bacillus mojavensis* strain ROB-2 was used to compare the efficiencies of two purification methods. One of the methods involve acid precipitation using 1 N HCl to adjust the pH of the cell-free culture fluid to 2 (Mc Keen *et al.*, 1986; Yakimov

et al., 1995) followed by TLC. The second method use ammonium sulfate precipitation (40%) followed by acetone extraction and TLC (Youssef *et al.*, 2005).

Thin layer chromatography is a simple method, which can be used to detect the presence of lipopeptides while preparative TLC can be used to purify small quantities (Symmank *et al.*, 2002). TLC requires only a few nano grams (ng) of sample for a successful analysis and can be accomplished in a matter of minutes. Like all chromatographic methods, TLC takes advantage of the different affinity of the analyte with the mobile and stationary phases to achieve separation of complex mixtures of organic molecules. Silica gel (SiO_2), the most commonly used stationary phase. The methanolic fractions are analyzed using TLC (Razafindralambo *et al.*, 1993) with direct view developed using distilled water spraying. A white spot formed with the same Rf value when the plate was sprayed with water, indicating that the compound is lipophilic. Cell free supernatant of *Bacillus subtilis*UMAF6614, UMAF6619, UMAF6639 and UMAF8561 and *Bacillus amyloliquefaciens*PPCB004 were evaluated by TLC and the spots with Rf values similar to the standards fengycin (0.09), iturin A (0.3), and surfactin (0.7) (Romero *et al.*, 2007 and Arrebola *et al.*, 2010). To determine which lipopeptides were directly involved in fungal inhibition, the bioautographies were performed using the pathogens as revealing microorganism. Iturin A inhibits almost all fungi. Maldonado *et al*., 2009 run silica gel plates 60 F254 (Merck, 2 mm) and are carried out with a chloroform–methanol–acetic acid (40:4:1) mixture (Batrakov *et al.*, 2003). Plates are developed under UV light at 254 and 365 nm and only one spot of Rf 0.67 was detected (Kumar *et al.*, 2009). The inhibitory activity of the spot was confirmed after TLC by bioautographic assay. Besides the TLC plates were developed with ninhydrin and no spot was observed.

2.8. 2. UV-VIS Spectroscopy

Gueldner *et al.,* 1988 assayed the crude material dissolved in 50:50 methanol-water, and the solution was chromatographed on a column of C-18 reversed-phase absorbent (Waters Prep Pak 500). Elution with a stepwise gradient of methanol and water (from water up to 80% methanol-water) eluted most of the lipopeptides. Published by Isogai *et al.,* 1982 for *B. subtilis* metabolites and collected from an analytical C-18 column were also active in the bioassay test *Monilinia fructicola*, a causative agent of brown rot disease in stone fruits. The absorbance spectrum for the lipopeptide is measured in acetonitrile: 1% acetic acid (68:32) between 200 and 600 nm. The antibiotic shows absorbance maxima at 235, 278, and 285 nm and there was no appreciable absorbance above 300 nm (Bechard *et al.,* 1998). Kumar *et al*., 2009 dissolved 1 mg extract in 10 ml of methanol and the spectra were recorded at 190-600 nm range. UV spectral data of antibiotic exhibited strong absorption maxima (λ max) at 254, 255 and 277 nm and there was no appreciable absorbance above 300 nm, which was corresponding to characteristic absorption of peptide bond. It is reported that most of peptide antibiotics exhibit absorbance maxima at 210-230 and 270-280 nm (Motta & Brandelli, 2002; Kurusu & Ohba, 1987). A peptide antibiotic cerein, obtained from *B. cereus*, shows UV absorbance peak at 250 and 273 nm.

2.8.3. Infrared (IR) spectroscopy

The infrared spectrum of the antibiotic was measured as a potassium bromide (KBr) pellet. Characteristic absorption valleys at 1540, 1650 and 3300 cm^{-1} indicate that the antibiotic contains peptide bonds. A lactone ring is suggested by the absorption at 1740 $cm^{-1}$and valleys that result from C-H stretching (2950, 2850, 1460 and 1400 cm^{-1}) indicate the presence of an aliphatic chain (Bechard *et al.,* 1998). Romero *et al.,* 2007 performed Fourier transform-infrared spectrum (FT-IR) analysis of active extracts from *Bacillus subtilis* strains which showed bands in the range of

1,630 cm^{-1} to 1,680 cm^{-1}, resulting from the stretching mode of the CO-N bond (amide I band), and at 1,570 cm^{-1} to 1,515 cm^{-1}, resulting from the deformation mode of the N-H bond combined with C-N stretching mode (amide II band), both indicating the presence of a peptide component and also bands at 2,855 cm^{-1} to 2,960 cm^{-1}, resulting from typical CH stretching vibration in the alkyl chain. Also was observed at 1,730 cm^{-1}due to the lactone carbonyl absorption typical for surfactin and fengycin families of lipopeptides. The one at 1,650 cm^{-1} assigned to the vibrational amida I mode which shows the peptide link; another at 1,710 cm^{-1} to 1,740 cm^{-1}characteristic of carbonyl groups in ester or ketone groups and another at 2,850 cm^{-1} to 2,950 cm^{-1} bands corresponding to saturated CH links assigned to long chain fatty acids. The IR absorption pattern for fractions from *Bacillus circulans* revealed the presence of peptide and carboxyl groups that indicated their lipopeptide nature (Desai & Bannat, 1997; Thaniyavaran *et al.*, 2003; Das & Mukherjee, 2005; Pueyo, 2009). FTIR spectra of antifungal compound had a broad band centering around 3,421.5 cm^{-1}indicated an amino and hydroxyl group of amino acids (Kumar *et al.*, 2009). Analysis of the spectrum also shows typical absorption bands (1,670.5 and 1,539.8 cm^{-1}) corresponding to N-H stretching of proteins and peptides bonds (Maquelin *et al.*, 2002). Additional absorption valleys 1418.4 cm^{-1} and 1488.6 cm^{-1} indicating (C-H) aliphatic side chain may be related with predominance of hydrophobic amino acids such as Val, Leu and Ile or its contains fatty acids in their structure (Bizani *et al.*, 2005).

2.8.4. Mass spectrometry

The lipopeptide molecules are detected, in their protonated form or as Na^+ or K^+ adducts, by matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF-MS) in the m/z range of 1,400–1,550 Dalton (Da). Several reports are available that highlight the analysis and purification of lipopeptide

biosurfactants (Desai & Banat, 1997; Maneerat & Phetrong, 2007; Mukherjee *et al.*, 2009; Sen & Swaminathan, 2005). Although, methods like ion exchange chromatography (Mukherjee *et al.*, 2006), thin layer chromatography (Desai & Banat, 1997), gel permeation chromatography (Mukherjee *et al.*, 2009) and ultrafiltration (Sen & Swaminathan, 2005; Lin *et al.*, 1998) have been used for the purification of lipopeptide biosurfactants, these techniques have a serious limitation as they don't separate individual isoforms present in the crude lipopeptide mixture. Mass spectra were recorded by (MALDI-TOF-MS).

Sivapathasekaran *et al.*, 2009 analyzed the HPLC purified isoforms by matrix-assisted laser desorption/ionization time-of-flight analysis (MALDI-TOF) for molecular mass determination. A great deal of research has been carried out the properly methods for fully characterization of cyclic lipopeptides and their structures. In this regard, reverse-phase high-performance liquid chromatography (RP-HPLC) could be extensively used because it is efficient in separation and purification of isoforms (Lin *et al.*, 1998; Thaniyavaran *et al.*, 2003). Mass spectrometry methods developed to rapidly characterize the lipopeptides nature. An efficient high-resolution HPLC method is a prerequisite for the purification up to the individual isoform level, required for subsequent commercialization of a particular lipopeptide isoform as a potential therapeutic agent. The products obtained with these methods shows a greater level of purity and profound biological activity (Sivapathasekaran *et al.*, 2009). The lipopeptides and moreover the particular isoforms could be exploited as a powerful tools for the selection of useful strains in the context of biocontrol.

It is evident from the review of literature that soil not only encompasses a great microbial diversity but also harbor microbes that have been exploited in past for human welfare and still leaves a futuristic scope for elucidation of unidentified compounds of microbial origin to be of use of mankind.

CHAPTER - 3

SOIL SAMPLING, ISOLATION AND MAINTAINACE OF CULTURES

CHAPTER 3

SOIL SAMPLING, ISOLATION AND MAINTENANCE OF CULTURES

3.1 Introduction:-

Odisha is a state with different physiographic and agro-climatic zones. Soils of Odisha are mainly developed by the relief, climate and parent material of varying composition of sandstone, quartzite, granite gneiss and khondalities either *in situ* or over transported material. The red & laterite and lateritic group of soils generally constitute about two-third of the total areas in the state. The lateritic mass is characterized by compact to vesicular sometimes honey-combed structure, composed essentially of a mixture of hydrated oxides of Fe and Al with small amount of Mn and Ti oxides and SiO_4 (quartz) as a necessary diluent. These soils are highly permeable and are poor in NPK and Ca. The texture of these soils varies from sandy loam to sandy clay loam with depth. The available water holding capacity of these soils is low. Soils are highly permeable and susceptible to droughts during frequent dry spell in the rainy season emergence of seedling is obstructed due to a shallow crusting developed after beating action of a rain because of cementation of colloidal Fe oxides. They are generally acidic in nature, being leached and heavily weathered (Alloway, 2008) and affecting crop productivity.

Soil microbiologists and microbial ecologists differentiated soil microorganisms as ‘beneficial’ or ‘harmful’ depending how they affect soil quality, crop growth and yield. Beneficial microorganisms are those that fix atmospheric N, decompose organic wastes and residues, detoxify pesticides, suppress plant diseases and soil-borne pathogens, enhance nutrient cycling and produce bioactive compounds

such as vitamins, hormones and enzymes that stimulate plant growth. A natural assumption is that soil microbes produce antibiotics in their natural habitat and use them to gain advantage over their competitors; that is, antibiotics are presumed to be involved in naturally occurring amensal relationship in the soil (Jamil *et al.*, 2007) which are low molecular weight (non-protein) molecules produced as secondary metabolites, mainly by microorganisms that live in the soil.

Because of current public concerns about the side effects of agrochemicals, there is an increasing interest in improving the understanding of microbial interaction activities among rhizospheric microbes and how these can be efficiently used for the benefit of agriculture, environment and human civilization (Barea *et al.*, 2004; Lucy *et al.*, 2004). In soil ecosystems, beneficial microbial interactions are responsible in the regulation of key environmental phenomena, such as the mineralization of complex organic matters into simpler available N and the regulation of plant growth and productivity (Barea *et al.*, 2004).

A wide range of microbial community participates in decomposition, mineralization, and nutrient availability (microbe-mediated unusable P-availability), and therefore influence the efficiency of nutrient cycles. The populations of bacteria and actinomycetes in acid soils have been reported to be lower than those in neutral soils. Microbial diversity in acid soils needs to be explored and utilized.

3.2 Material and Methods:-

3.2.1 Study sites & collection of sample

Lateritic soil region of Odisha including four different districts of Dhenkanal, Cuttack, Jajpur and Nayagarh were selected as experimental soil sampling sites. Samples were collected during the Month of December to February in the morning hours (6AM to 8AM). Top 0-15 cm of soil was collected aseptically, covered with

plastic bags and were transported to the laboratory in a thermal box. Collected soil samples were air dried in the Laboratory of Department of Microbiology, OUAT, Bhubaneswar for 48hrs, powdered and processed for physico chemical analysis.

3.2.2 Physico chemical parameter analysis

The physico chemical parameter like soil pH and temperature were measured. 10gms soil sample was mixed with double distilled water (soil: water = 1:2, w/v) for the pH measurement with the help of pH meter. Standard isolation method was followed taking specimens randomly, without any prior knowledge of the microbial composition of the source under investigation (Donadio *et al.*, 2002).

3.2.3 Isolation of bacteria

Most of the media used for the isolation, enumeration and identification of bacteria were prepared as per the manufacturer's instructions by using de-ionized water. Some of the pseudo selective media were prepared in the laboratory using synthetic chemicals and basal media. The samples were them processed in the laboratory at an earliest isolate the bacteria, study their morphology and other characteristics features required for their identification. Enumerations of heterotrophic bacteria were conducted through serial dilution method and plate technique. 1gm of soil was added to 9ml of de-ionized water and serially diluted up to 10^{10}. Aliquots of 0.1ml from each dilution was spread on Nutrient Agar (NA) media (Himedia, India) plates in triplicate with pH 5.4, 5.0, 4.9 and 5.6 for Dhenkanal, Cuttack, Jajpur and Nayagarh samples respectively. The plates were incubated at 37°C for 24hrs. Colonies were compared before proceeding for pure culture (prepared to rule out any possible contamination). The CFUs of different morphology were then selected and subcultured once or twice on fresh NA plates & incubated at 37ºC to obtain pure culture.

3.2.4. Identification of bacteria

Identification of bacterial isolates carried out by their colony characteristics on basal media plate and Gram's staining. Gram staining followed by microscopic observation of all the bacterial isolates were done for Gram's variability, morphology and arrangement of bacteria.

Colony Morphology and Gram's staining

Shape, size, color, margin, surface, elevation and transparency of the bacterial colonies were observed with the 24hrs incubated cultures on NA plates with acidic pH. To study Gram's variability of the isolates, diluted suspensions of the bacteria were smeared on clean glass slides, air dried, heat fixed by passing over a flame for 2 to 3 times. The slides, were flooded with crystal violet solution for 1min, washed with water and flooded with Gram's iodine for 1min. The slide were washed with water and decolorized with 95% ethyl alcohol dropped from a dropping bottle until no violet colour was visible from drain off solution. The slides were washed with water and counter stained with saffranin stain for about 30s and washed with water. The slides were air dried and examined under a microscope using 100x objectives using a daylight filter. Cells were then identified by the colour observed purple for Gram +ve and pink or red for Gram -ve cells.

3.2.5 Fungal pathogens (phytopathogens and dermatophytes) isolation

A total of seven fungal cultures were used in this study some of them were brought from IMTECH (Institute of Microbial Technology), Chandigarh. Others were collected from the microbiology laboratory. All were successfully revived prior to experimental use.

The dermatophytes viz. *Candida albicans* MTCC 854, *C. tropicalis*, *Epidermophyton floccosum*, *Trycophyton rubrum* and *T. mentagrophytes* were grown

and maintained on Sabouraud medium(Himedia, Mumbai) (Venkateswarlu *et al.*, 1997) containing agar (2% w/v, Himedia) in Petri dishes for 24-48hrs at room temperature. The phytopathogens (*Aspergillus fumigatus, Penicillium notatum* and *Fusarium* sp.) were grown in different medium. *A. fumigatus* and *P. notatum* were grown aerobically for 48hr at 25°C in PDA (potato dextrose agar) (Fateixa *et al.*, 2009). *Fusarium* sp. was grown on RBCA (Rose Bengal Chloramphenicol agar) (Himedia, India).

3.3 Results:-

A survey was conducted on four sampling sites in lateritic soil regions of Odisha cover (districts of Dhenkanal, Cuttack, Jajpur and Nayagarh) a minimum 20.11 to a maximum 20.85 latitude and a minimum 85.01 to a maximum 85.54 longitude. The Soil was found to be acidic in nature ranging pH in a minimum 4.8 to a maximum 6.2. The area ranges to a lowest of 19m to highest of 90m altitude (Table-1, Fig-1 to Fig-5).

Table-1: Geographical distribution of different acid soil sampling region of Odisha

Sampling site	Geographical Indications			pH	Samples collected in g
	Latitude	Longitude	Altitude MSL in m		
Mahishapat, Dhenkanal	20.67	85.54	76	5.3-6.2	150
Barang, Cuttack	20.27	85.52	23.5	5.0-5.1	150
Chandikhol, Jajpur	20.85	86.33	19	4.8-5.5	150
Ranpur, Nayagarh	20.11	85.01	90	5.3-5.9	150

MSL- Mean Sea Level

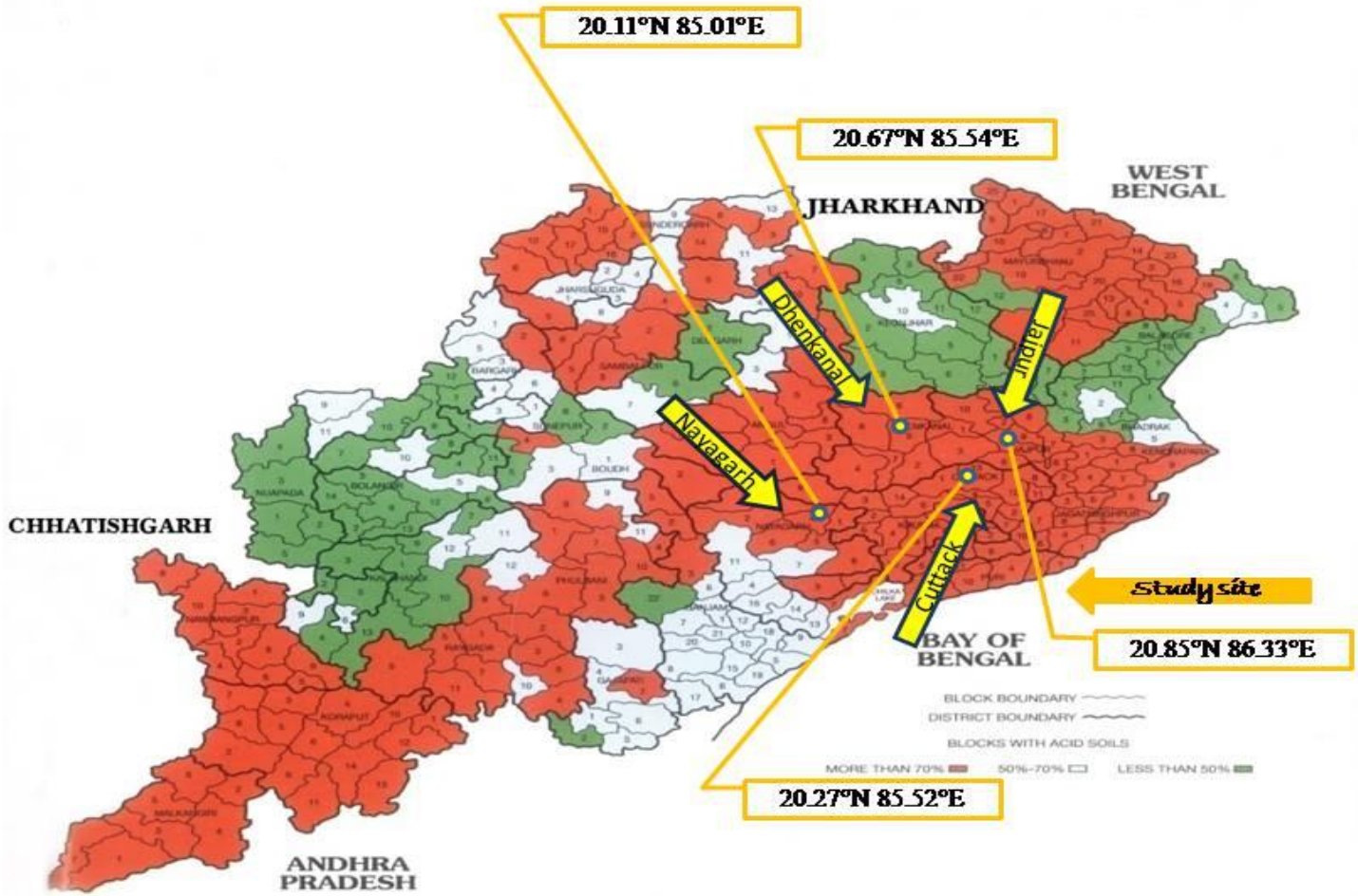


Fig. 1. Map of Odisha indicating the study site (soil sampling sites)

Fig. 2. Soil sampling site at Mahishapat, Dhenkanal district

Fig. 3. Soil sampling site at Barang, Cuttack district

Fig. 4. Soil sampling site at Chandikhol, Jajpur district

Fig. 5. Soil sampling site at Ranpur, Nayagarh district

A total of 10 bacterial isolates were found coded as DOD-1 to DOD-10(coded as DOD: 'D' denotes Dhenkanal and Odisha abbreviated as "OD") each for 10 independent colonies. Colony characteristics ranged from circular small size to circular large size and colour ranged from white to off white and fade yellow colour. Colonies were with erose/entire or lobate margins. Except two all were lustreless and these colonies were either convex or flat but all opaque. The rod shaped bacterial colonies were tested for Gram's variability and found positive (Table-2, Fig-6).

Table-2: Morphological characteristic of bacterial isolates from acid soil of Dhenkanal district

Sl. No.	Colony Characteristic	Gram's Variable
DOD-1	Circular, Medium size, Off white colour, Erose margin, Lustreless surface, Convex colonies, Opaque	Positive, Rods
DOD-2	Circular, Medium size, Fade yellow colour, Entire margin, Lustreless surface, Flat colonies, Opaque	Positive, Rods
DOD-3	Circular, Medium size, Off white colour, Entire margin, Lustreless surface, Flat colonies, Opaque	Positive, Rods
DOD-4	Circular, Medium size, White colour, Erose margin, Lustreless surface, Flat colonies, Opaque	Positive, Rods
DOD-5	Circular, Large size, White colour, Lobate margin, Lustreless surface, Convex colonies, Opaque	Positive, Rods
DOD-6	Circular, Medium size, Fade yellow colour, Entire margin, Glossy surface, Flat colonies, Opaque	Positive, Rods
DOD-7	Circular, Large size, Cream colour, Entire margin, Lustreless surface, Flat colonies, Opaque	Positive, Rods
DOD-8	Circular, Small size, White colour, Entire margin, Lustreless surface, Flat colonies, Opaque	Positive, Rods

DOD-9	Circular, Large size, Cream colour, Entire margin, Glossy surface, Flat colonies, Opaque	Positive, Rods
DOD-10	Circular, Large size, Off white colour, Entire margin Lustreless surface, Flat colonies, Opaque	Positive, Rods

"D" denotes Dhenkanal District and Odisha abbreviated as "OD"

Five different bacterial colonies were isolated from acid soil of Cuttack district starting from COD-1 to COD-5(coded as COD: "C" denotes Cuttack and Odisha abbreviated as "OD"). The colony characteristics ranged from circular small size to medium size with colour ranged from white to off white to fade yellow to yellow. All were with entire margin. Colonies were with either glossy or lustreless surface, low

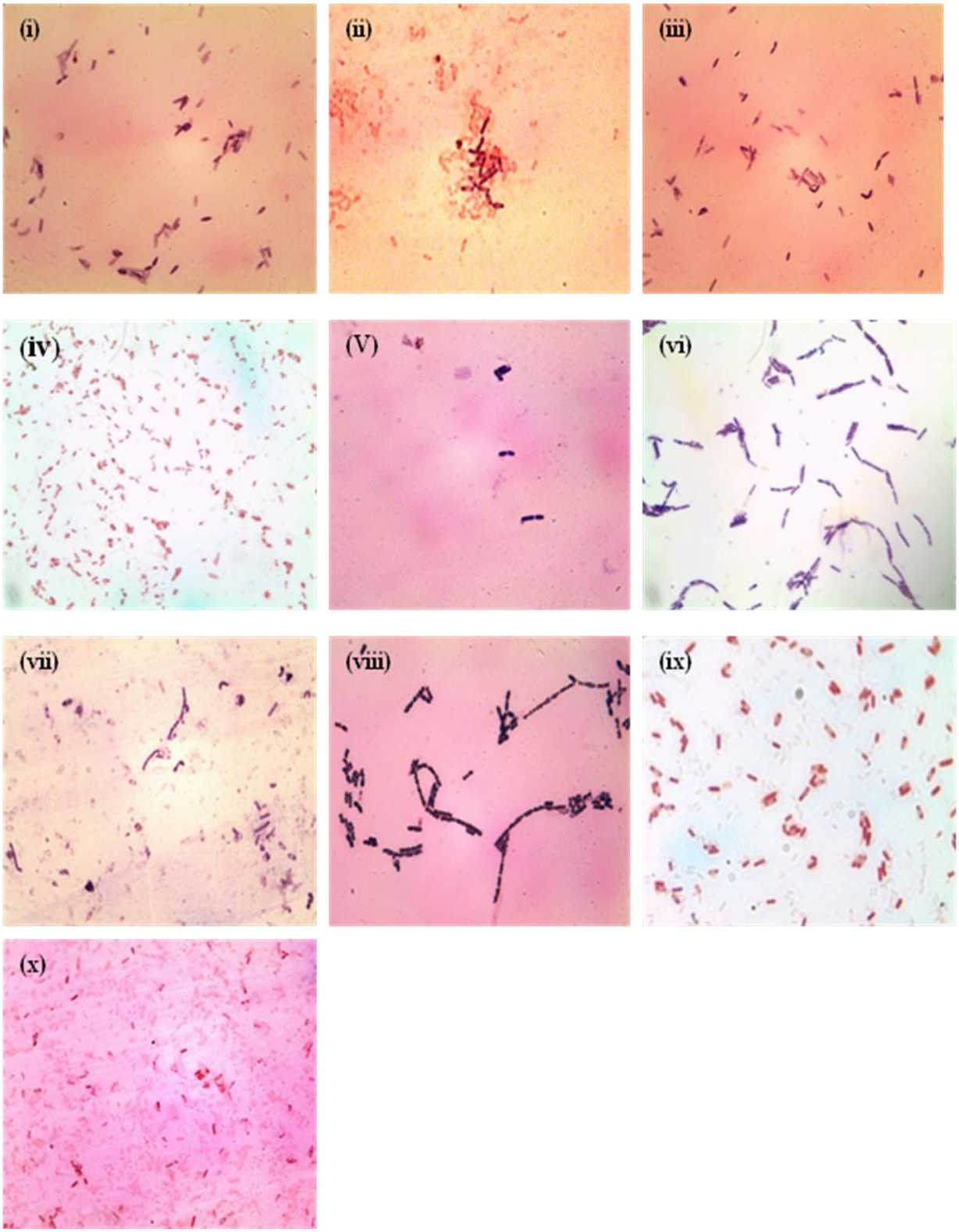


Fig. 6. Plates showing staining micrograph of bacterial isolates from acid soil of Dhenkanal, Odisha (DOD- i TO x)

convex to convex elevation with opaque except one semitransparent. After Gram's reaction three positive with two cocci and one rods along with two negative cocci were found (Table-3; Fig-7).

Table-3: Morphological characteristic of bacterial isolates from acid soil of Cuttack district

Sl. No.	Colony Characteristic	Gram's Variable
COD-1	Circular, Medium size, Off white colour, Entire margin, Glossy surface, low convex elevation, Opaque	Negative, Cocci
COD-2	Circular, Medium size, Fade yellow colour, Entire margin, Smooth surface, low convex, Semi transparent	Negative, Cocci
COD-3	Circular, Small size, Yellowish white colour, Entire margin, Smooth, low convex elevation, Opaque	Positive, Rods
COD-4	Circular, Small size, White colour, Entire margin, Glossy surface, Convex elevation, Opaque	Positive, Cocci
COD-5	Circular, Medium size, Yellow colour, Entire margin, Glossy surface, Convex elevation, Opaque	Positive, Cocci

"C" denotes Cuttack and Odisha abbreviated as "OD"

Total of 10nos. of bacterial colonies were isolated from acid soil of Jajpur district starting from JOD-1 to JOD-10 (coded as JOD: "J" denotes Jajpur and Odisha abbreviated as "OD"). The colony characteristics ranged from circular small size to medium size with colour ranged between off white, white, Cream, Silvery greenish, Fade orange and Golden orange. The margins of the colonies were either undulate or entire. All are with glossy surface with convex to low convex to flat colonies. Out of ten colonies three were semitransparent, rest opaque. Gram's reaction showed that all are rods with five negative and rest positive to the reaction (Table-4; Fig-8).

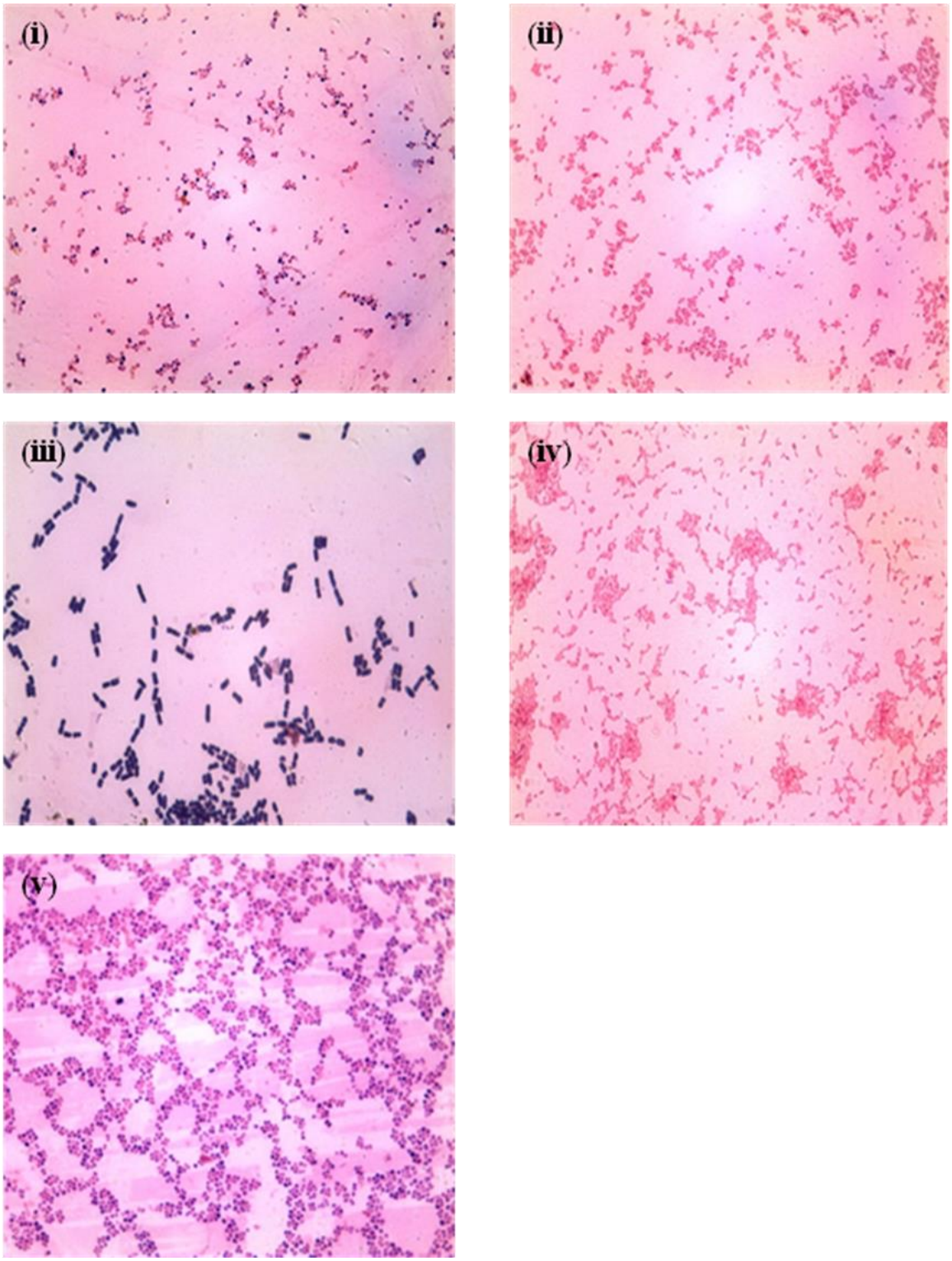


Fig. 7. Plates showing staining micrograph of bacterial isolates from acid soil of Cuttack, Odisha (COD- i TO v)

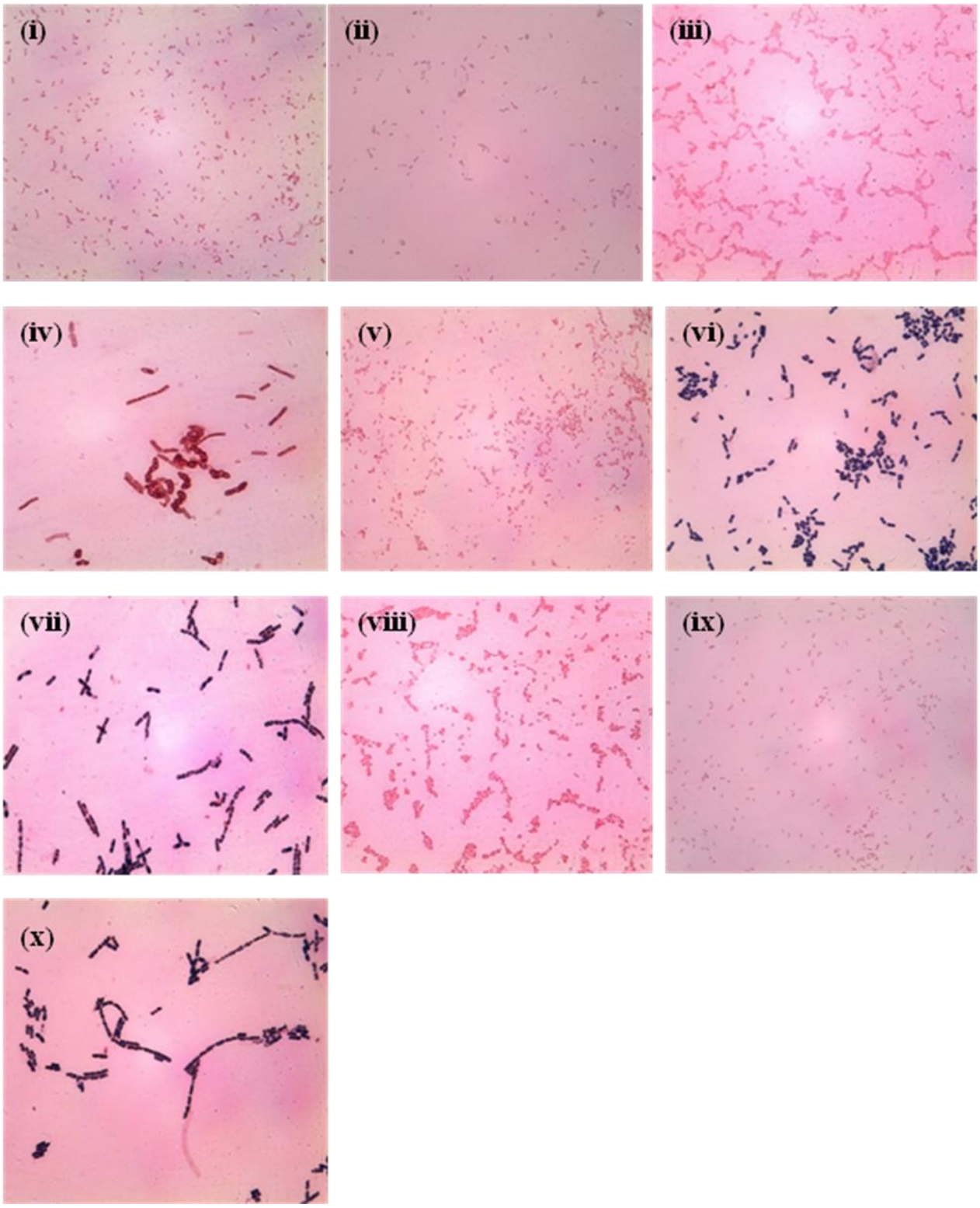


Fig. 8. Plates showing staining micrograph of bacterial isolates from acid soil of Jajpur, Odisha (JOD- i TO x)

Table-4: Morphological characteristic of bacterial isolates from acid soil of Jajpur district

Sl. No.	Colony Characteristic	Gram's Variable
JOD-1	Circular, Medium size, Silvery greenish colour, Entire margin, Glossy surface, convex elevation, Opaque	Negative, Rods
JOD-2	Circular, Small size, Fade yellow colour, Entire margin, Glossy surface, convex, Semi transparent	Negative, Rods
JOD-3	Circular, Medium size, Off white colour, Entire margin, Glossy surface, convex elevation, Semi transparent	Negative, Rods
JOD-4	Circular, Medium size, Cream colour, Undulate margin, Glossy surface, convex elevation, Opaque	Positive, Rods
JOD-5	Circular, Medium size, Off white, Irregular/Erose margin, Glossy surface, Flat, Semi transparent	Negative, Rods
JOD-6	Circular, Medium size, Off white colour, Undulate margin, Glossy surface, Flat colonies, Opaque	Positive, Rods
JOD-7	Circular, Medium size, Off white colour, Undulate margin, Glossy surface, low convex colonies, Opaque	Positive, Rods
JOD-8	Circular, Medium size, Golden greenish colour, Entire margin, Glossy surface, Flat colonies, Opaque	Negative, Rods
JOD-9	Circular, Medium size, Cream colour, Entire margin, Glossy surface, convex elevation, Opaque	Positive, Rods
JOD-10	Circular, Medium size, Fade Orange colour, Undulate margin, Glossy surface, convex elevation, Opaque	Positive, Rods

"J" denotes Jajpur and Odisha abbreviated as "OD"

A total of 23nos of bacterial colonies were isolated from acid soil of Nayagarh district starting from NOD-1 to NOD-23 ("N" denotes Nayagarh District and Odisha abbreviated as "OD"). The colony characteristics ranged from circular small size to circular large size and irregular spreading configuration with colour ranged between

off white, white, foggy white, fade yellow and media yellow. Colonies were with either entire or dented margin. Some were glossy surface and some were with lustreless surface, 4 nos of colonies were convex leaving rest flat colonies with one traumatized surface. All colonies were opaque with one centre traumatized. Gram's staining results in to two negative and rest positive. Out of 23 bacteria 1 was cocci and rests were rods in shape (Table-5; Fig. 9).

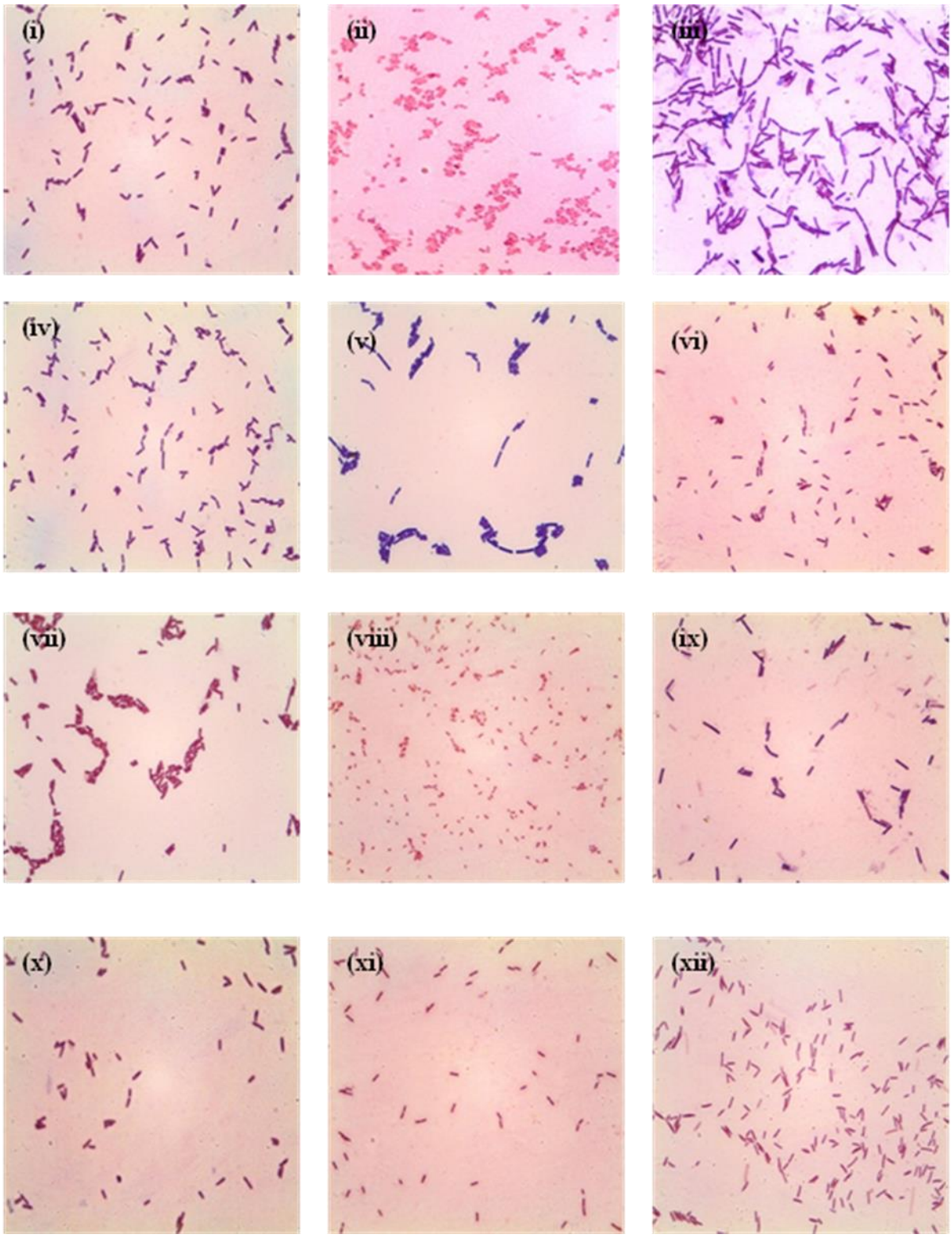


Fig. 9. Plates showing staining micrograph of bacterial isolates from acid soil of Nayagarh, Odisha (NOD- i TO xii)

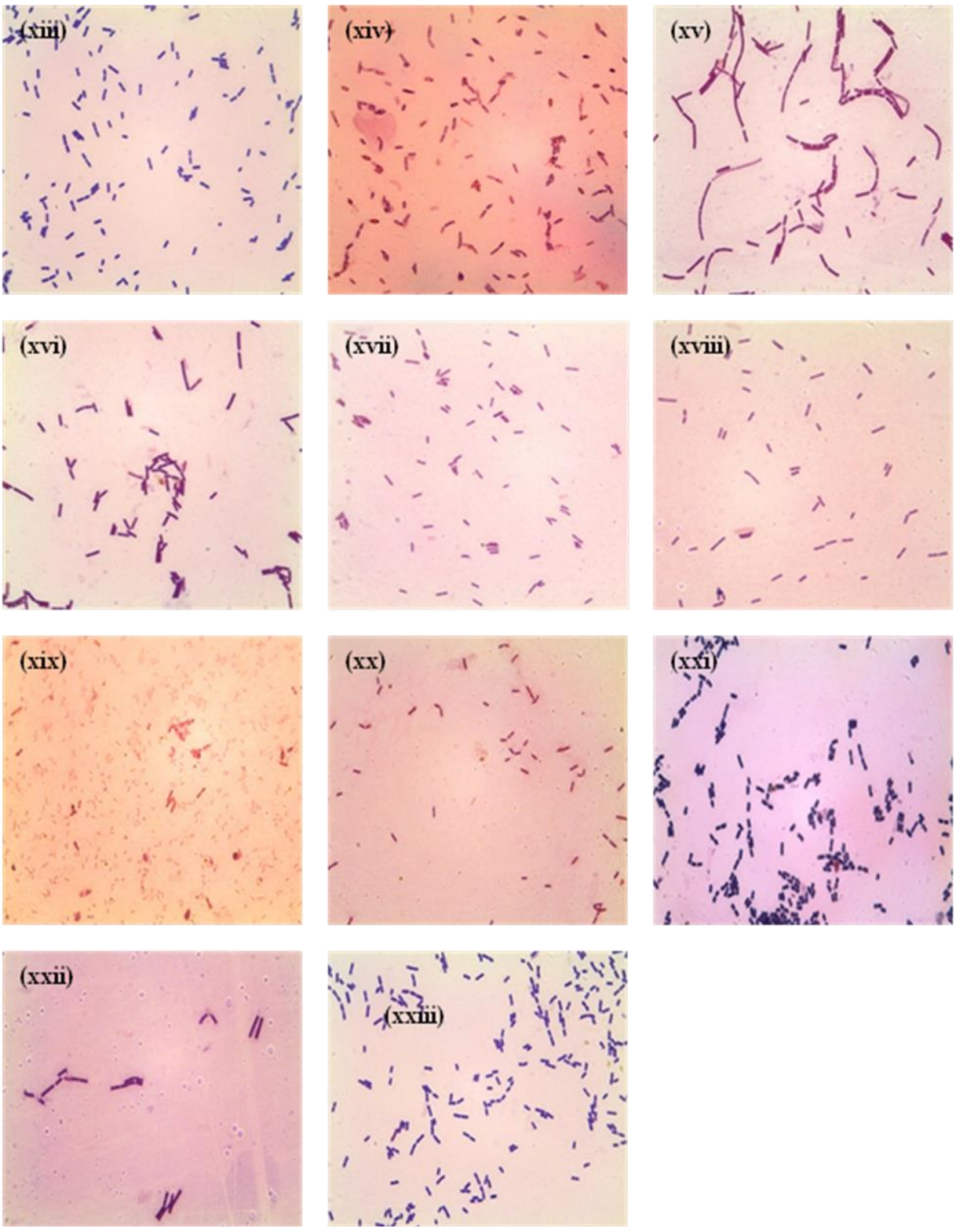


Fig. 9. Plates showing staining micrograph of bacterial isolates from acid soil of Nayagarh, Odisha (NOD- xii to xxiii)

Table-5: Morphological characteristic of bacterial isolates from Acid soil of Nayagarh District

Sl. No.	Colony Characteristic	Gram's Variable
NOD-1	Off white Colour, Large size, Entire margin, Circular shape, Flat appearance, Opaque	Positive, Rods
NOD-2	Fade Yellow Colour, Large size, Entire margin, Circular shape, Flat appearance, Opaque	Negative, Cocci
NOD-3	Off white Colour, Large size, Dented margin, Circular shape, Flat appearance with traumatized surface, Opaque	Positive, Rods
NOD-4	Fade Yellow Colour, Small size, Entire margin, Circular shape, Glossy surface, Flat appearance, Opaque	Positive, Rods
NOD-5	Foggy white Colour, Large size, Entire margin, Circular shape, Convex colonies, Opaque	Positive, Rods
NOD-6	Media Yellow Colour, Medium size, Entire margin, Circular shape, Flat appearance, Opaque	Positive, Rods
NOD-7	Cream Colour, Small size, Entire margin, Circular shape, Flat appearance, Opaque	Positive, Rods
NOD-8	Fade Yellow Colour, Large size, Entire margin, Circular shape, Convex colonies, Opaque	Negative, Rods
NOD-9	Off white Colour, Large size, Dented margin, Circular shape, Flat appearance, Opaque, Centre traumatized	Positive, Rods
NOD-10	Fade Yellow Colour, Medium size, Entire margin, Circular shape, Flat appearance, Opaque	Positive, Rods
NOD-11	White Colour, Small size, Entire margin, Circular shape, Convex colonies, Glossy appearance, Opaque	Positive, Rods
NOD-12	Fade Yellow Colour, Small size, Dented margin, Circular shape, Convex colonies, Opaque	Positive, Rods

NOD-13	Fade Yellow Colour, Medium size, Entire margin, Circular shape, Flat appearance, Opaque	Positive, Rods
NOD-14	White Colour, Large size, Entire margin, Circular shape, Flat appearance, Opaque	Positive, Rods
NOD-15	Fade Yellow Colour, Large size, Entire margin, Circular shape, Flat appearance, Opaque	Positive, Rods
NOD-16	White Colour, Large size, Entire margin, Circular shape, Flat appearance, Opaque	Positive, Rods
NOD-17	Fade Yellow Colour, Large size, Entire margin, Circular shape, Flat appearance, Opaque	Positive, Rods
NOD-18	Fade Yellow Colour, Large size, Entire margin, Circular shape, Flat appearance, Opaque	Positive, Rods
NOD-19	Off white Colour, Irregular and spreading configuration, Lobate margin, Flat, Opaque	Positive, Rods
NOD-20	Fade Yellow Colour, Large size, Entire margin, Circular shape, Flat appearance, Opaque	Positive, Rods
NOD-21	Fade Yellow Colour, Large size, Entire margin, Circular shape, Flat appearance, Opaque	Positive, Rods
NOD-22	Fade Yellow Colour, Large size, Entire margin, Circular shape, Flat appearance, Opaque	Positive, Rods
NOD-23	White Colour, Large size, Entire margin, Circular shape, Flat appearance, Opaque	Positive, Rods

"N" denotes Nayagarh District and Odisha abbreviated as "OD"

3.4 Discussion:-

Microbial diversity of extreme environment is of significant importance to soil microbiologist as they play an important role in acclimatized to that environment. Soil is considered one of the most suitable environments for microbial growth

(Cavalcanti *et al.*, 2006). Soil microorganisms contribute a wide range of essential services to the sustainability of all ecosystems, by acting as the primary driving agents of nutrient cycling, regulating the dynamics of soil organic matter, soil carbon sequestration and green house gas emission, modifying soil physical structure and water regimes, enhancing the efficiency of nutrient acquisition by the vegetation and enhancing plant health. These services are not only essential to the functioning of natural ecosystems but constitute an important resource for the sustainable management of agricultural and environmental ecosystems. Soil microbial communities mediate both the synthesis and decomposition of soil organic matter and therefore, influence cation exchange capacity, the soil N, S, P reserve, soil acidity and toxicity and soil water holding capacity. Acidophiles acclimatize themselves in natural environment where the soil is lateritic moderate to highly acidic. The acid soil with pH range 4.8 to 6.2 favours growth and sustenance of variety of microbes especially bacteria having adaptability to acidic environment (Nayak and Mishra, 2012). Soil pH also appears to lead to selection of different bacterial communities (Nicol *et al.*, 2008; Lehtovirta *et al.*, 2009) suggesting adaptation of particular phylogenetic group in acidic soils (de Boer and Kowalchuk, 2001).

In the present investigation, a survey was conducted on bacterial flora of acid soil region having a minimum 20.11 to a maximum 20.85 latitude and a minimum 85.01 to a maximum 85.54 longitude of Odisha covering Dhenkanal, Cuttack, Jajpur and Nayagarh districts.

Changes in soil structural properties brought about by natural or manmade activities might change the interactions between microorganisms and their substrates in the soil matrix (Hojati and Farshid, 2009). By using morphological characteristics and simple staining reaction Eikelboom (1975) distinguished 27

bacteria in activated sludge samples. This identification key has some general limitations. Morphology and staining reaction of microbial cell vary in broad range depending upon environmental conditions. A total of 48nos. bacteria were isolated from 4 districts of laterite soil regions of Odisha. 10nos. of bacteria from Dhenkanal district indicating all positive to gram's reaction and rods in shape. Out of 5nos. bacterial colonies from the soil of Cuttack district two were negative cocci under microscope while other three were positive to Gram's reaction leaving one rod and rest cocci. All bacteria isolated from Jajpur district were rods in shape, leaving equally positive and negative to gram's staining among 10 colonies. Out of 23nos of isolated bacterial colonies from acid soil of Nayagarh district, Odisha 1 colony showed cocci in shape leaving rest 22nos rods under microscope at 100X magnification. A total of 2nos colonies were negative to Gram's reaction and all others were positive.

CHAPTER - 4

SCREENING AND IDENTIFICATION OF BACTERIA

CHAPTER 4

SCREENING AND IDENTIFICATION OF BACTERIA

4.1 Introduction

Interactions among organisms are central to understanding any ecosystem, perhaps with the exception of a short period when a newly created niche is colonized by its first inhabitants (Karlovsky, 2008). Aerobic *Bacillus* bacteria are widely distributed in nature (Ismailov, 1996). They participate in various biological processes and are very resistant to various physicochemical actions of microorganisms (Smirnov *et al.*, 1982; Safiyazov and Mannanov, 1997). They can also adapt to varied environmental changes (Shukri and Sattarova, 1989; Shukri *et al.*, 1992; Mannanov and Safiyazov, 1996). A natural assumption is that soil microbes produce antibiotics in their natural habitat and use them to gain advantage over their competitors; that is, antibiotics are presumed to be involved in naturally occurring amensal relationship in the soil (Jamil *et al.*, 2007).

Fungal diseases became recognized as being of clinical importance in the second half of the last century, mainly due to advances in medical technologies. Many species of *Bacillus* including *B. cereus*, *B. subtilis* and *B. mycoides* are known to suppress several fungal pathogens growth such as *Rhizoctonia* sp., *Fusarium* sp., *Pythium* sp. and *Phytophthora* sp. The bacterial antagonists assume their antagonistic effects mainly by the production of antifungal antibiotics (Katz and Demain, 1977; Korzybski *et al.*, 1978), which seem to play a major role in the biological control of plant pathogens (McKeen *et al.*, 1986; Phae *et al.*, 1990) and post-harvest spoilage fungi.

The search for new substances of pharmaceutical or agricultural importance can be conducted through screening large collections of diverse chemical entities (generally defined as a library), employing assays designed to detect modulators of pharmacologically or agriculturally relevant targets (Donadio *et al.*, 2002). The discovery and characterization of antimicrobial compounds produced by organisms isolated from extreme environments are of interest and potentially important to industry. Owing to the unique nature of the compounds produced by these organisms, they might provide new or more efficient means for the inhibition of selected microorganisms.

The simplest direct test and the one most widely used for the preliminary screening of large numbers of strains is the "spot-on-lawn" antagonism, based on the method devised by Gratia, (1946) in which the test and indicator cultures are grown simultaneously and the demonstration of antagonism is dependent upon the release of a diffusible inhibitor early in the growth of the test culture. The density of the indicator lawn is an important determinant of the sensitivity of the method (Kuttner, 1966). The lawn is generally seeded before inoculation of the test strains (Tagg *et al.*, 1973). This method is well suited to the examination of single colonies and has widespread application in genetic studies.

The genus *Bacillus* is characterized by Gram +ve, aerobic or facultative anaerobic, rod shaped bacteria that form spores, and contains more than 60 species that have quite different phenotypes (Claus & Berkeley, 1986). Most of the tests conducted for identification of bacteria have been based on physiological and nutritional tests (Claus & Berkeley, 1986). Methods such as DNA (including PCR fingerprinting) and RNA analysis are useful for identification and classification of bacteria. *Bacillus* species have been identified and characterized, where the 16S rRNA gene of several *Bacillus* sp. was sequenced. The study revealed the presence of five

highly different lines within the genus; based on sequence homologies, *B. amyloliquefaciens*, *B. subtilis* and *B. pumilus* belong to the same group (group 1).

Several reviews have provided the good characteristic traits desired in microbial antagonist in the disease controlling process (Droby *et al.*, 2009; Sharma *et al.*, 2009). Wilson and Wisniewski (1988) recommended a guideline to select an ideal antagonist, which are as follows:

1. Must be stable

2. Should be effective at low concentrations

3. Must not be demanding in terms of required nutrients

4. Must be able to survive under adverse environmental conditions

5. Should be effective against a wide spectrum of commodities and pathogens under different conditions

6. Should be amenable to production on inexpensive growth media

7. Should be amendable to formulations with a long shelf life

8. Should be easy to dispense without being hazardous to human health

10. Must be environmentally friendly

One of the modern approaches is screening of bacteria from relatively unknown areas. In this regard acid soil (lateritic soil) of Odisha carries significant importance as no/little microbiological work has been done earlier. The present study aims at screening of soil bacterial isolates against some potent phytopathogens and dermatophytes.

4.2 Materials and methods

4.2.1 Pathogens

Virulent strain of phytopathogens *Aspergillus fumigatus*, *Penicillium notatum*, *Fusarium* sp. maintained in the Department of Microbiology, OUAT was used for antimicrobial assay. The dermatophytes as *Trycophyton mentagrophytes*, *T. rubrum*, *Epidermophyton floccosum* and *Candida tropicalis*, were obtained from Microbiology division, Center for Biotechnology. Virulent strain of *Candida albicans* MTCC No.854 were obtained from IMTECH, Chandigarh. *A. niger* and *P. notatum* were maintained on PDA while *Fusarium* sp. was maintained on RBCA (Rose Bengal Chloramphenicol Agar). *T. mentagrophytes*, *T. rubrum*, *E. floccosum*, *C. tropicalis* and *C albicans* were maintained on SDA and periodically revived and stored at 4°C.

Bacterial inocula preparation

Bacterial crude cultures used for bioassay were prepared in NB with appropriate acidic (low pH) condition. Cultures were incubated at 37°C for 24hrs on a shaker incubator at 125 rpm. For testing antimicrobial activity, SDA (Sabouraud dextrose agar) medium was used.

4.2.2 *In vitro* antifungal assays

Antifungal activity of the bacterial isolates was determined by agar diffusion technique. Antifungal assays were performed on 9cm Petri plates (Merck, Germany) containing 25ml of SDA medium. A well was made by sterile Cork Borer (diameter, 6mm) in the centre of the Petri plate. 100-µl aliquot of 24hr old crude bacterial broth culture was pipetted into the well. Plates were incubated at 30°C for 24-48hr. The results were reported by measuring diameter of the inhibition zone. Three plates with equal measurement were used for each sample and the experiment was repeated thrice. Effectivity of the isolates was assessed by growth inhibition of pathogenic fungi.

Similar antifungal assay was conducted with medicines like Clotrimaoxazole, Ketconazole and Itraconazole generally recommended by physicians for the disease *Candidiasis*.

4.2.3 Biochemical Characterization

After the microscopic examination the Gram negative and Gram Positive processed separately for identification, they were subjected to biochemical identification by standard biochemical identification tests (Collins and Lyne, 2005); some enzymatic tests and test for sugar utilization as per the requirement of the bacterial identification software ABIS (Advanced Bacterial Identification Software) online (Costin and Ionut, 2007) and Bergey's manual of determinative bacteriology (1994). Gram +ve and -ve (rods & cocci) bacteria were identified from the Gram's reaction, morphology and colony characteristics on basal media up to generic level. For all biochemical tests 24hr old cultures were used. One control was kept on each case.

NaCl tolerance

Growth of the organisms on NA medium supplemented with 1 to 11% NaCl was carried out. Highly diluted suspensions of the organisms of the organisms were spotted on the plates, incubated at 37°C for 72hr and growth was recorded.

Oxidase test

To detect presence of the enzyme cytochrome 'C' oxidase in the bacterial isolates which catalyses transport of electrons between bacteria and redox dye e.g. N-tetramethyl para phenyldiamine dihydrochloride or dimethyl-p-phenylene diamine dihydrochloride or dimethyl-p-phynylene diamine or methylene blue was studied. Development of purple or blue colour within 10-30s of rubbing bacterial culture on

oxidase discs was counted as positive while no/delaying change in colour was considered as negative.

Catalase test

Presence of the enzyme catalase which breakdowns (catalyze) hydrogen peroxide in to water and oxygen was done. Bacterial cultures were flooded with hydrogen peroxide. Effervescence of from the plate was indicated as positive.

Urease test

Presence of the enzyme urease which splits urea into ammonia and CO_2 was detected by inoculating bacterial cultures in to tubes containing Christensen's urea agar medium. Change of colour from yellow to purplish pink coloration of the medium indicated a positive reaction while no change of colour was regarded as negative.

Indole test

The test is used to check ability of the organisms to produce indole from tryptophan by the action of enzyme tryptophanase. The test was performed by inoculating the bacterial culture in to tubes containing tryptone broth incubated at 30°C for 24hrs. After inoculation Kovac's or salkowski's reagent was added (1:1 v/v). Appearance of a pink colored ring at the interface of the two solutions indicates indole production which leads to positive result. Absence of pink ring considered negative result.

Methyl Red test

Methyl red test detects production of acid from glucose by fermenting glucose. Production of acid in the medium lowers the pH ≤4.2 and detected by the presence pH indicator (Methyl Red). Bacterial isolates were inoculated into tubes containing Methyl Red-Voges Proskauer (MR-VP) broth and incubated at 30°C for 48hr. 0.04%

alcoholic methyl red indicator was added to the incubated tube. Positive reaction was indicated by change in colour of the medium yellow to bright red. No colour change indicated as negative result.

Voges-Proskauer (acetoin production) test

Ability of many bacteria to ferment carbohydrates (glucose) with production of acetyl methyl carbinol ($CH_3.CO.CHOH.CH_3$) or its reduction product (2,3-butylene glycol) in to neutral products and CO_2 instead of organic acid is assessed. Bacteria were inoculated in to tubes containing MR-VP broth and incubated at 30°C for 24 to 48hr. After incubation a mixed solution of α-napthol and KOH (potassium hydroxide) were added to 2.5 to 5ml of the culture. Development of crimson red colour of the medium indicated positive result while copper colour indicated as negative.

Nitrate Reduction test

The ability of the microorganisms to reduce nitrate (NO_3^-) to nitrite (NO_2^-) using enzyme nitrate reductase is detected through the test. The ability of the isolates to denitrify nitrate to nitrite ultimately producing ammonia and molecular nitrogen was also tested simultaneously. Bacteria were inoculated into nitrate broth and incubation at 30°C for 96hr. After incubation sulphanillic acid (in 30% acetic acid) and α- napthylamine (in 30% acetic acid) mixture (1:1, v/v) was added to the broth culture. Appearance of deep pink colour indicated positive result for nitrate reduction. In case of no change in colour, a pinch of powdered Zinc was added to the broth. Development of red colour in this step indicated a negative result while no change of colour in this step, indicated a positive complete showing that denitrification took place and ammonia or molecular nitrogen was formed.

Manitol motility test

The capacity of the isolate to utilize manitol, reduce nitrate indicating the presence of the nitrate reductase &their motility was studied using this medium. The fermentation of manitol was indicated by acid production which changes the colour of the butt from red to yellow. Presence of air bubbles was taken as positive indication of nitrate

utilization. Diffused growth along the stubbed line was considered positive test while only on the stabbed line indicated a negative test for motility.

Citrate utilization test

The test aims at ability of the bacteria to grow in a medium containing citrate as a sole carbon and energy source. Citrate utilization is monitored by appearance of growth and increase of pH from 6.8 which is indicated by the change in colour of Bromothymol blue indicator of the medium. This test was performed by inoculating the bacterial cultures in Simmon's citrate medium as slant culture and incubated at 30°C for 96hr. Growth on the slant accompanied by change of colour from green to royal blue indicated positive result. No change of colour indicated negative result.

Carbohydrate metabolism (Acid-gas production):

Ability of microorganism to ferment carbohydrate and related compounds (monosaccharides, disaccharides, polysaccharide, polyhydric alcohols, glycosides and organic acids) such as arabinose, lactose, rhamnose, maltose, salicin, Trehalose, mannose, sucrose, cellobiose, fructose, dextrose, D glucose, glycerol, inositol, xylose, raffinose, glycogen, galactose etc. resultant acid and gas production is assessed by this test. Yellow coloration of the medium (inside test tube) was considered positive while negative test was indicated by no colour change of the medium. The bacteria were inoculated in tubes containing OF medium and incubated at 30°C for 72hr.

Extracellular Enzymatic Activity

The activities of various extra cellular enzymes produced by the isolates were studied by the following tests:

Starch hydrolysis test

Capacity of the organism to hydrolyze starch into dextrin, glucose, maltose, etc. by the amylase enzymes were detected by spot inoculating the bacterial cultures on

NA plates containing 1% (w/v) soluble starch and 1.5% (w/v)agar power. After incubation for 24hr at 30°C, the plates were exposed to iodine cubes for few minutes; positive amylolytic activity caused by production of amylase was observed from a clear zone formed around the CFU as unhydrolysed starch on exposure to iodine vapors turns blue black. The diameter of the clear zone was measured using antibiotic inhibition zone scale and the ratio calculated from diameter from the clear zone and the diameter of the bacteria growth gave the activity level.

Lipase test

Tween NA plates were prepared using N/5 Nutrient broth, 1%(v/v) tween 20 & 1.5% agar. All the isolates were spot inoculated on plates and incubated at 37°C for 24hrs. All the plates were then stored at 4°C for 2-3hr to precipitate the lipid in presence of $CaCl_2$. A clear zone around a CFU indicated positive lipolytic activity of the isolate caused by production of lipase. Ratio of the diameter of the clear zone and the bacterial growth indicated the activity index.

Protein hydrolysis test

Proteolytic activity of bacteria was determined by the following tests on different media plates containing the protein sources.

Gelatin liquefaction test

Gelatin liquefaction was detected by spot inoculating the bacteria on N/5 Nutrient broth tubes containing 1% (w/v) gelatin followed by the incubation at 30°C for 24-48hr. A clear liquification of gelatin in the test tube indicated positive gelatinolytic activity of the isolate caused by production of gelatinase.

Casein hydrolyzing test

The skim milk agar plates were prepared on which all the isolates were spot inoculated and incubated at 30°C for 24-48hr. A clear zone around CFU on an opaque

background indicated hydrolysis of the milk protein, Casein by the isolates due to production caseinase. The ratio of clear zone and growth of bacteria gave enzymatic activity of the organism.

Pectin hydrolysis test

The ability of the isolates to hydrolyze pectin to pectic acid was assessed by the test. Pectin NA plates were prepared using N/5 Nutrient broth, 1% (w/v) pectin and 1.5% (w/v) agar power. All isolates were spot inoculated on the plates and incubated at 30°C for 24-48hr. All the plates were then exposed to 0.1% Congo red solution for 1min and decanted off. Then the plates surface was immersed in 1N HCl and 1N NaOH for 5mins respectively and decanted off. A clear zone around each CFU on red background indicated positive pectinolytic activity of the isolate caused by the production of pectinase.

Chitin hydrolysis test

The ability of microorganisms to hydrolysis chitin is determined by the test. Chitin plates were prepared using N/5 Nutrient broth, 1% (w/v) chitin and 1.5% agar. All isolates were spot inoculated and incubated at 30°C for 24-48hr. All the plates were then exposed to 0.1% Congo red solutions for 10 minutes and decanted off. Then the plate surface was immersed in 1N HCl and 1N NaOH for 5mins respectively and decanted off. A clear zone around a CFU on a blue background indicated positive chitinolytic activity of the isolate caused by the production of chitinase. The ratio of clear zone to the growth of the bacteria gave the activity levels of the organisms.

Esculin Hydrolysis

Esculin in the medium is hydrolyzed to esculetin and other compounds. The esculetin (released from esculin by ß-glucosidase) reacts with ferric chloride in the medium to form a black-brown colour. Bile Esculin agar medium was prepared and

bacterial isolates were stabbed in the medium and then zigzag streaked on the surface of the slant, then incubated at 37°C for 24-48hr. Reaction is considered positive if the slant turned blue-black and negative if no change from the original colour occurred. Bile-esculin is a selective and differential medium used in the identification of catalase-negative bacteria

Antibiotic sensitivity test

The selected bacterial isolates were screened for antibiotic resistance following Kirby- Bauer (1966) disc diffusion method. The isolates were exposed to different antibiotics viz. Polymyxin-B, Chloramphenicol, Co-Trimaoxazole, Gentamycin, Nalidixic Acid, Ciprofloxacin and Streptomycin to observe the effect of these antibiotics on physiology of the bacterial isolates of the acid soil area. The bacterial isolates were revived in Nutrient broth and 24hrs fresh culture was used. Muller Hinton Hi veg Agar (No. 2) plates were prepared. A sterile cotton swab was dipped in to the bacterial suspension and used evenly to inoculate the entire surface of Muller Hinton Agar. After the agar surface was dried for about 5minutes, the antibiotic disc was placed on it by a pre sterilized forceps. The plates were incubated at 37°C for 24hrs. After the incubation period, diameters of the zones of inhibition were measured to nearest mm. Test results were interpreted using a table that relates zone diameter to degree of microbial resistance.

4.2.4 Molecular identification of bacteria

By comparing 16s rRNA partial gene sequence to a database of known sequences, molecular identification of the unknown bacteria is determined. Sequencing of 16S rRNA of the isolate was out sourced and sequence data was BLAST in NCBI database, dendrogram was constructed using MEGA version 5. The 16S rRNA genes of many bacterial species have already been sequenced, the sequence

of the 16S rRNA gene was submitted to NCBI, USA for its novelity as a new bacterial sequence.

4.2.4.1. Bacterial Genomic DNA Isolation

The bacteria registering highest inhibition zone against *C. albicans* was cultured overnight in NB medium at 150rpm & 30°C and 1.0 ml of broth samples were withdrawn aseptically and centrifuged at 5000g for 10 min and the respective pellets were processed for genomic DNA extraction using XcelGen bacterial genomic DNA isolation kit, XG 2411-01, following the manufacturer's protocol.

4.2.4.2. Quantitation and Quality Assessment of DNA

The DNA stock samples was quantified using Nanodrop spectrophotometer at 260 and 280 nm using the convention that one absorbance unit at 260 nm wavelength equals 50 µg DNA per ml. The Ultra violet (UV) absorbance was checked at 260 and 280 nm for determination of DNA concentration and purity. Purity of DNA was judged on the basis of optical density ratio at 260:280 nm. The DNA having ratio between 1.8 to 2.0 was considered to be of good purity. Concentration of DNA was estimated using the formula.

Concentration of DNA (mg/ml) = OD 260 x 50 x Dilution factor

Quality and purity of DNA were checked by agarose gel electrophoresis. Agarose (Biogene, USA) 0.8% (w/v) in 0.5X TAE (pH 8.0) buffer (Sambrook and Russel, 2001) was used for submarine gel electrophoresis. Ethidium bromide (1%) was added @ 10µl /100ml. The wells were charged with 5µl of DNA preparations mixed with 1µl gel loading dye. Electrophoresis was carried out at 80V for 30 min at

room temperature. DNA was visualized under UV using UV transilluminator. The DNA was used further for PCR.

4.2.4.3. Polymerase Chain Reaction

16S rDNA fragments of bacterial DNA samples of NOD-19 were amplified by using universal 16S rRNA gene primers 8F as forward primer & 1492R as reverse primer. Details of 16S Universal Primer Sequence given below

8F: 5' AGA GTT TGA TCC TGG CTC AG 3'

1492R: 5' ACG GCT ACC TTG TTA CGA CTT 3'

PCR was carried out in a final reaction volume of 25 µl in 200 µl capacity thin wall PCR tube in Eppendorf Thermal Cycler. Composition of reaction mixture for PCR is given in Table 6. PCR tubes containing the mixture were tapped gently and spin briefly at 10,000 rpm. The PCR tubes with all the components were transferred to thermal cycler. The PCR protocol designed for 30 cycles for the primers used is given in Table 7.

Table-6: Composition of reaction mixture for PCR

Components	**Quantity**	**Final**
DNase-RNase free water	7.50µl	---
2X PCR master mix (MBI Fermentas)	12.50 µl	1X
Forward Primer (10pmole/µl)	1.00 µl	10 pmole
Reverse Primer (10pmole/µl)	1.00 µl	10 pmole
Diluted DNA(30ng/µl)	3.0 µl	---
GRAND TOTAL	25.00 µl	---

Table-7: Steps and conditions of thermal cycling for PCR

Steps	Temperature(°C)	Time	Cycles
Initial Denaturation	95	2 min	1
Final Denaturation	94	30 sec	30
Annealing	52	30 sec	
Extention	72	90 ec	
Final Extention	72	10 min	1

4.2.4.4. Visualization and Purification of PCR Product

To confirm the targeted PCR amplification, 5 µl of PCR product from each tube was mixed with 1 µl of 6X gel loading dye and electrophoresed on 1.2 % agarose gel containing ethidium bromide (1 per cent solution @10 µl/100 ml) at constant 5V/cm for 30 min in 0.5 X TAE buffer. The amplified product was visualized as a single compact band of 1500bp size under UV light and documented by gel documentation system (Biorad). These 16S rDNA fragments were further purified using Min Elute Gel Extraction kit (QIAGEN) following the manufacturer's protocol

4.2.4.5. Sequencing of Purified DNA

The concentration of the purified DNA was determined and was subjected to automated DNA sequencing on ABI 3730xl Genetic Analyzer (Applied Biosystems, USA). Sequencing was carried out using BigDye Terminator v3.1 Cycle sequencing kit following manufacturer's instructions. Cycle sequencing was performed following the instructions supplied along with BigDye Terminator v3.1 Cycle Sequencing Kit. The reaction was carried out in a final reaction volume of 20µl using 200µl capacity

thin wall PCR tube. The cycling protocol (Table 8) was designed for 25 cycles with the thermal ramp rate of 1^{o}C per second.

Table-8: Cycling protocol for sequencing reaction

Step*	Temperature(^{o}C)	Time
Denaturation (1)	96	10 sec
Annealing (2)	52	5 sec
Extension (3)	60	4 min

* Repeat step 1 to 3 for 25 cycles

After cycling, the extension products were purified and mixed well in 10 µl of Hi-Di formamide. The contents were mixed on shaker for 30mins at 300g. Eluted PCR products were placed in a sample plate and covered with the septa. Sample plate was heated at 95^{o}C for 5 min, snap chilled and loaded into auto sampler of the instrument. Electrophoresis and data analysis was carried out on the ABI 3730xl Genetic Analyzer using appropriate Module, Basecaller, Dyeset/Primer and Matrix files.

4.2.4.6. Sequence analysis

The resulting sequence was searched against the National Center for Biotechnology Information (NCBI) nonredundant (nr) nucleotide database by using the Basic Local Alignment Search Tool (BLAST). All sequence similarity searches were performed by using BLASTN at NCBI website. The Bioedit Sequence Alignment Editor (An Abbott company) was used to generate the contig sequence from sequenced PCR products. The software package MEGA version 5 was used to

sequence alignment and Cluster analysis for phylogenetic and molecular evolutionary analysis (Tamura *et al.*, 2011).

4.2.5. Statistical analysis

Balanced Analysis of Variance of were conducted on the efficacy trials using CropStat for windows version 7.2.2007.3 (IRRI, Philippines). Statistical significance was judged at the $P<0.05$ level and critical difference (C.D) procedure was used to compare means.

4.3 Results

4.3.1 *In vitro* antifungal assay

On evaluation of antifungal activity of the bacterial isolates by agar diffusion technique, it was found that amongst the total isolates, two from DOD, three from JOD and three from NOD were able to inhibit the dermatophyte, *Trycophyton rubrum*. The bacterial isolates sl. no. 37 (NOD-19) produced maximum of inhibition up to 16mm and found significantly superior to all. The NOD-19 was able to produce an

Table-9: Inhibitory zone of the bacterial isolates from varying sampling sites with specific agro eco regions (sub-regions) against *Trycophyton rubrum*

Soil sampling (Sl. No.)		Bacteria Isolated	Inhibition Zone(mm)
Sampling site -1 'a'	1.	DOD-1	14
	2.	DOD-2	14
	3.	DOD-3	-
	4.	DOD-4	-
	5.	DOD-5	-
	6.	DOD-6	-
	7.	DOD-7	-
	8.	DOD-8	-

	9.	DOD-9	-
	10	DOD-10	-
Sampling site -2 'b'	11.	COD-1	<10
	12.	COD-2	12
	13.	COD-3	-
	14.	COD-4	-
	15.	COD-5	-
Sampling site -3 'c'	16.	JOD-1	-
	17.	JOD-2	-
	18.	JOD-3	<10
	19.	JOD-4	-
	20.	JOD-5	<10
	21.	JOD-6	-
	22.	JOD-7	<10
	23.	JOD-8	-
	24.	JOD-9	-
	25.	JOD-10	-
	26.	NOD-1	14
	27.	NOD-2	-
	28.	NOD-3	-
	29.	NOD-5	-
	30.	NOD-6	-
	31.	NOD-7	-
	32.	NOD-8	-
	33.	NOD-9	-

Sampling site -4 'd'	34.	NOD-10	-
	35.	NOD-11	-
	36.	NOD-12	-
	37.	NOD-13	-
	38.	NOD-14	12
	39.	NOD-15	-
	40.	NOD-16	-
	41.	NOD-17	-
	42.	NOD-18	-
	43.	NOD-19	16
	44.	NOD-20	-
	45	NOD-21	-
	46.	NOD-22	-
	47.	NOD-23	-

'a'- sub humid to humid eastern & south eastern upland; 'b'- Eastern Ghats hot moist sub-humid eco-sub-region 'c'- Eastern plateau (chhotanagpur) and Eastern Zone; 'd'- Eastern Ghats, hot moist sub humid eco sub region; Unidentified bacteria are numbered where alphabet represents their respective places of sample collected; "D"-Dhenkanal, "N"- Nayagarh, "J"-Jajpur, "C"-Cuttack, "OD"-Odisha. C.D.at $p<0.05=0.15$ for interaction between bacterial isolates.

inhibition zone up to 16mm *in vitro* plate assay as shown in Fig. 10. On comparison, the zone of inhibition by NOD-19 was more and superior as compared to others. The zone of inhibition of other bacteria didn't produce significant difference between control and treatments (in triplicate) and found at par with control 89.9±0.6 (C.D. at $p<0.05=0.15$).

Inhibitory strength of the bacteria were tested against *T. mentagrophytes* and it is evident that the bacteria NOD-19 was the most potent against this pathogen also out of 8 bacteria exhibiting zone of inhibition (Table-10). Cells showed that NOD-19

inhibited the fungal growth significantly higher in comparison with other isolates and untreated control (C.D. at $p<0.05=0.97$) (Fig. 11).

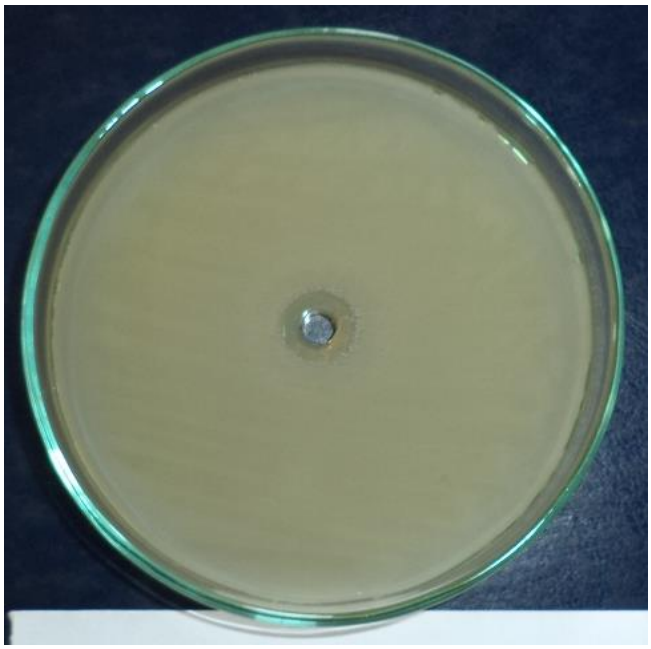


Fig. 10: Zone of inhibition by the bacterium NOD-19 against *T. rubrum*

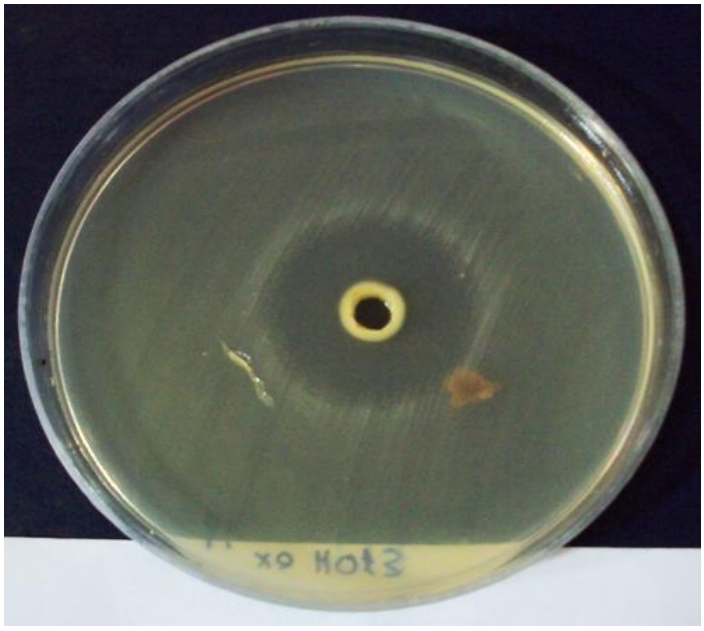


Fig. 11: Zone of inhibition by the bacterium NOD-19 against *T. mentagrophytes*

Table-10: Inhibitory zone of the bacterial isolates from varying sampling sites with specific agro eco regions (sub-regions) against *T. mentagrophytes*

Soil sampling (Sl. No.)		Bacteria Isolated	Inhibition Zone(mm)
Sampling site -1 'a'	1.	DOD-1	10
	2.	DOD-2	12
	3.	DOD-3	-
	4.	DOD-4	-
	5.	DOD-5	-
	6.	DOD-6	-
	7.	DOD-7	-
	8.	DOD-8	-
	9.	DOD-9	-
	10	DOD-10	-
Sampling site -2 'b'	11	COD-1	-
	12	COD-2	-
	13.	COD-3	-
	14.	COD-4	-
	15.	COD-5	-
Sampling site -3 'c'	16.	JOD-1	-
	17.	JOD-2	-
	18.	JOD-3	10
	19.	JOD-4	-
	20.	JOD-5	-
	21.	JOD-6	10
	22.	JOD-7	-
	23.	JOD-8	-
	24.	JOD-9	-
	25.	JOD-10	12
Sampling site -4 'd'	26.	NOD-1	-
	27.	NOD-2	14
	28.	NOD-3	-
	29.	NOD-5	-
	30.	NOD-6	-
	31.	NOD-7	-
	32.	NOD-8	-
	33.	NOD-9	-
	34.	NOD-10	-
	35.	NOD-11	-
	36.	NOD-12	-
	37.	NOD-13	-
	38.	NOD-14	12
	39.	NOD-15	-

	40.	NOD-16	-
	41.	NOD-17	-
	42.	NOD-18	-
	43.	NOD-19	16
	44.	NOD-20	-
	45.	NOD-21	-
	46.	NOD-22	-
	47.	NOD-23	-

'a'- sub humid to humid eastern & south eastern upland; 'b'- Eastern Ghats hot moist sub-humid eco-sub-region; 'c'- Eastern plateau (chhotanagpur) and Eastern Zone; 'd'- Eastern Ghats, hot moist sub humid eco sub region; Unidentified bacteria are numbered where alphabet represents their respective places of sample collected; "D"-Dhenkanal, "N"- Nayagarh, "J"-Jajpur, "C"- Cuttack, "OD"-Odisha. C.D.at $p<0.05=0.97$ for interaction between bacterial isolates.

Epidermophyton floccosum is a dermatophyte able to cause several dermal diseases in human being as well as in animals. Out of 47 isolates 9 bacteria were able to inhibit the growth of the pathogen *in vitro*. NOD-19 and NOD-14 were able to inhibit the pathogen maximally up to 18mm. (Table-11). The bacteria isolated from JOD and COD were failed to prove their potentiality against *E. floccosum* while from DOD showed inhibition maximum up to 14mm. NOD-19 inhibited the fungal growth significantly higher in comparison with other isolates and untreated control (C.D. at $p<0.05=0.70$).

Table-11: Inhibition zone of the bacterial isolates from varying sampling sites with specific agro eco regions (sub-regions) against *Epidermophyton floccosum*

Soil sampling (Sl. No.)		**Bacteria Isolated**	**Inhibition Zone(mm)**
Sampling site -1 'a'	1.	DOD-1	14
	2.	DOD-2	13
	3.	DOD-3	-
	4.	DOD-4	-
	5.	DOD-5	-
	6.	DOD-6	-
	7.	DOD-7	-
	8.	DOD-8	-
	9.	DOD-9	-
	10.	DOD-10	-
	11.	COD-1	-
	12.	COD-2	14

Sampling site -2 'b'	13.	COD-3	<10
	14..	COD-4	-
	15.	COD-5	-
Sampling site -3 'c'	16.	JOD-1	-
	17.	JOD-2	<10
	18.	JOD-3	-
	19.	JOD-4	<10
	20.	JOD-5	-
	21.	JOD-6	-
	22.	JOD-7	-
	23..	JOD-8	-
	24.	JOD-9	-
	25.	JOD-10	-
Sampling site -4 'd'	26.	NOD-1	-
	27.	NOD-2	14
	28.	NOD-3	-
	29.	NOD-5	-
	30.	NOD-6	-
	31.	NOD-7	-
	32.	NOD-8	-
	33.	NOD-9	-
	34.	NOD-10	-
	35.	NOD-11	-
	36.	NOD-12	-
	37.	NOD-13	-
	38.	NOD-14	18
	39.	NOD-15	-
	40.	NOD-16	-
	41.	NOD-17	-
	42.	NOD-18	-
	43.	NOD-19	18
	44.	NOD-20	-
	45.	NOD-21	-
	46.	NOD-22	-
	47.	NOD-23	-

'a'- sub humid to humid eastern & south eastern upland; 'b'- Eastern Ghats hot moist sub-humid eco-sub-region; 'c'- Eastern plateau (chhotanagpur) and Eastern Zone; 'd'- Eastern Ghats, hot moist sub humid eco sub region; Unidentified bacteria are numbered where alphabet represents their respective places of sample collected; "D"-Dhenkanal, "N"- Nayagarh, "J"-Jajpur, "C"- Cuttack, "OD"-Odisha. C.D. at $p<0.05=0.70$ for interaction between bacterial isolates.

The bacteria isolated from acid soils of Odisha were screened against one of the most potent dermatophytes, *Candida albicans*. Through dual culture method the

bacteria and the test pathogens were cultured simultaneously. Two bacteria from DOD (Fig. 12), three from JOD (Fig. 14) and three from NOD (Fig. 15) exhibited the inhibition zone. Out of 5 different bacterial colonies commencing from COD-1 to COD-5 failed to inhibit the dermatophyte *in vitro* (Fig. 13). NOD-19 bacteria significantly inhibited growth of *C. albicans* MTCC 854. While with other bacteria no significant difference was found between control and treatments (in triplicate) being at par with control 89.9±0.6 (C.D. at $p<0.05=1.10$).

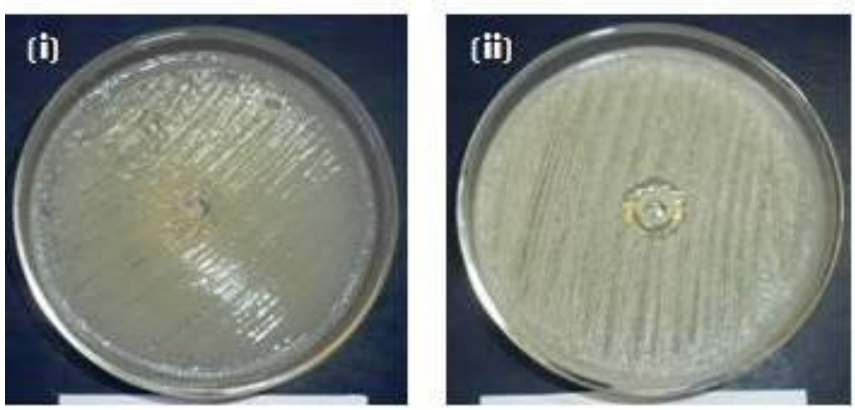


Fig. 12: Primary screening of bacteria from Dhenkanal district, Odisha against *C. albicans*

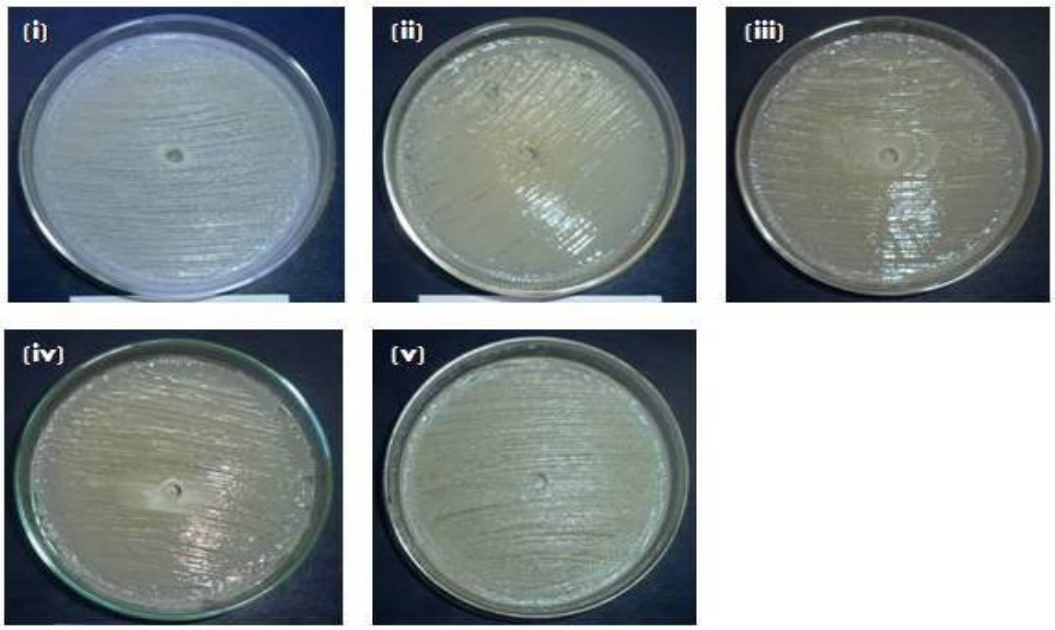


Fig. 13: Primary screening of bacteria from Cuttack district, Odisha against *C. albicans*

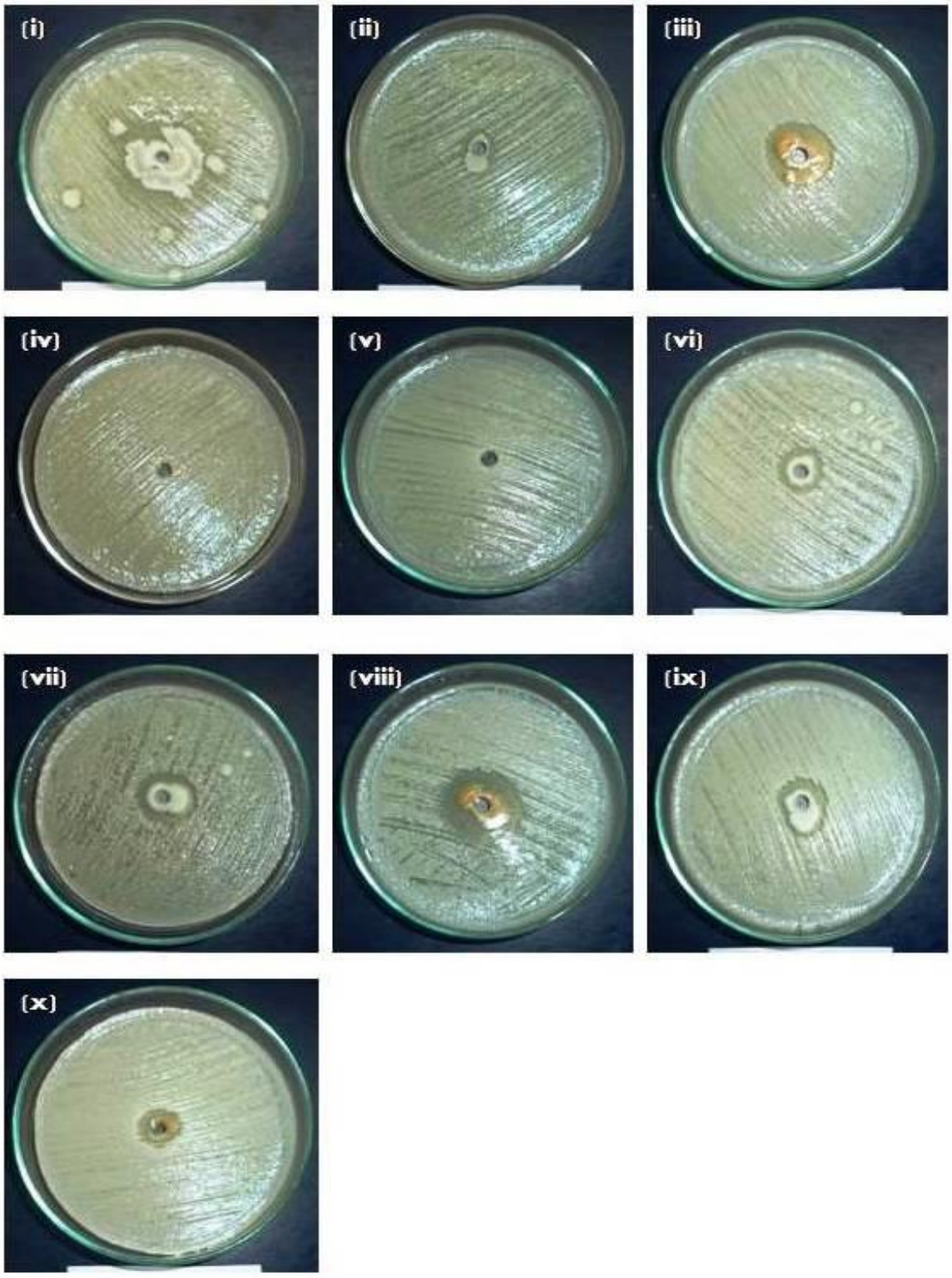


Fig. 14: Primary screening of bacteria from Jajpur district, Odisha against *C. albicans*

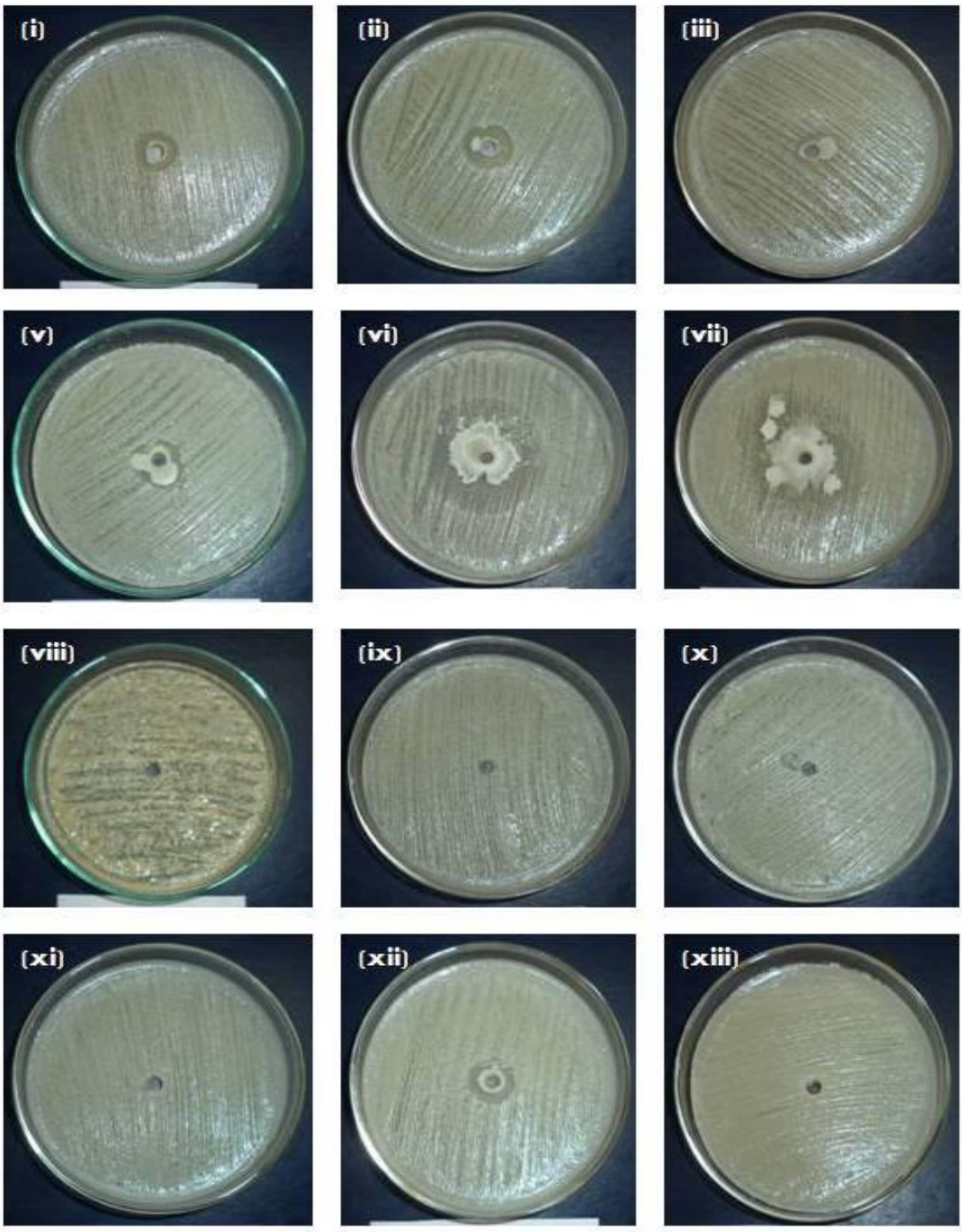


Fig. 15: Primary screening of bacteria from Nayagarh district, Odisha against *C. albicans*

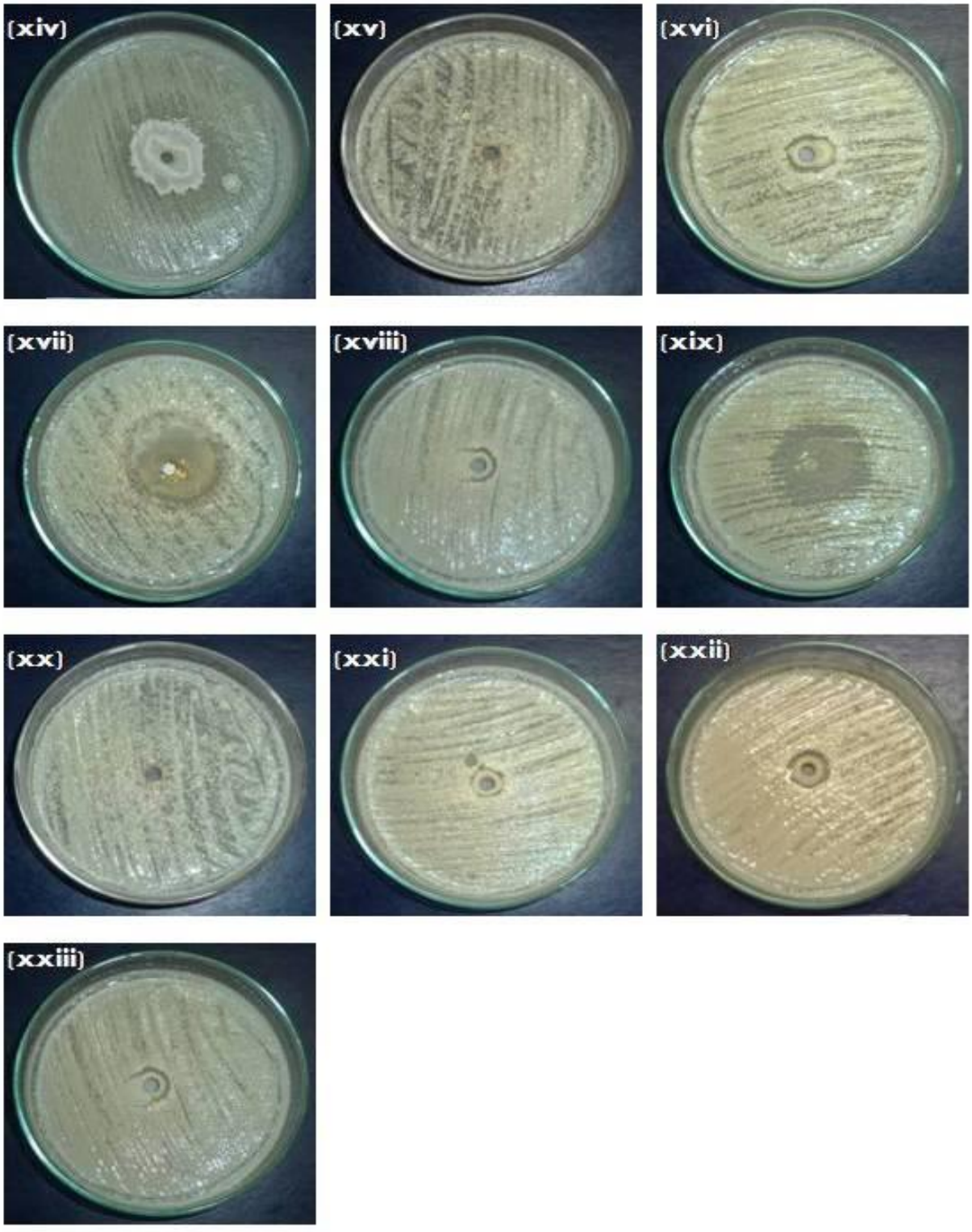


Fig. 15: Primary screening of bacteria from Nayagarh district, Odisha against *C. albicans*.

Table-12: Zone of inhibition by the bacterial isolates from varying sampling sites with specific agro eco regions (sub-regions) against *Candida albicans*

Soil sampling (Sl. No.)		Bacteria Isolated	Inhibition Zone(mm)
Sampling site -1 'a'	1.	DOD-1	12
	2.	DOD-2	14
	3.	DOD-3	-
	4.	DOD-4	-
	5.	DOD-5	-
	6.	DOD-6	-
	7.	DOD-7	-
	8.	DOD-8	-
	9.	DOD-9	-
	10.	DOD-10	-
Sampling site -2 'b'	11.	COD-1	-
	12.	COD-2	-
	13.	COD-3	-
	14.	COD-4	-
	15.	COD-5	-
Sampling site -3 'c'	16.	JOD-1	-
	17.	JOD-2	-
	18.	JOD-3	12
	19.	JOD-4	-
	21.	JOD-5	-
	21.	JOD-6	10
	22.	JOD-7	-
	23.	JOD-8	14
	24.	JOD-9	-
	25.	JOD-10	-
Sampling site -4 'd'	26.	NOD-1	-
	27.	NOD-2	14
	28.	NOD-3	-
	29.	NOD-5	-
	30.	NOD-6	-
	31.	NOD-7	-
	32.	NOD-8	-
	33.	NOD-9	-
	34.	NOD-10	-
	35.	NOD-11	-
	36.	NOD-12	-
	37.	NOD-13	-
	38.	NOD-14	12

	39.	NOD-15	-
	40.	NOD-16	-
	41.	NOD-17	-
	42.	NOD-18	-
	43.	NOD-19	25
	44.	NOD-20	-
	45.	NOD-21	-
	46.	NOD-22	-
	47.	NOD-23	-

'a'- sub humid to humid eastern & south eastern upland; 'b'- Eastern Ghats hot moist sub-humid eco-sub-region; 'c'- Eastern plateau (chhotanagpur) and Eastern Zone; 'd'- Eastern Ghats, hot moist sub humid eco sub region; Unidentified bacteria are numbered where alphabet represents their respective places of sample collected; "D"-Dhenkanal, "N"- Nayagarh, "J"-Jajpur, "C"-Cuttack, "OD"-Odisha. C.D.at $p<0.05=1.10$ for interaction between bacterial isolates.

The bacteria isolated from acid soils were screened against one of the most potent dermatophytes, *C. tropicalis*. Through dual culture method the bacteria and the test pathogens were culture simultaneously. One bacterium from DOD and three from NOD were exhibit the inhibition zone. Out of 4 the NOD-19 bacterium showed the least inhibition zone while other 3 were showed the equal zone of inhibition (Table-13). NOD-8 and NOD-14 showed significantly equal range of inhibition zone. C.D.at $p<0.05=0.97$ for interaction between bacterial isolates.

Table-13: Inhibitory zone by the bacterial isolates from varying sampling sites with specific agro eco regions (sub-regions) against *C. tropicalis*

Soil sampling (Sl. No.)		Bacteria Isolated	Inhibition Zone(mm)
Sampling site -1 'a'	1.	DOD-1	-
	2.	DOD-2	14
	3.	DOD-3	-
	4.	DOD-4	-
	5.	DOD-5	-
	6.	DOD-6	-
	7.	DOD-7	-
	8.	DOD-8	-
	9.	DOD-9	-
	10.	DOD-10	-
Sampling site -2 'b'	11.	COD-1	-
	12.	COD-2	-
	13.	COD-3	-
	14.	COD-4	-

	15	COD-5	-
Sampling site -3 'c'	16	JOD-1	-
	17.	JOD-2	-
	18.	JOD-3	-
	19.	JOD-4	-
	20.	JOD-5	-
	21.	JOD-6	-
	22.	JOD-7	-
	23.	JOD-8	-
	24.	JOD-9	-
	25.	JOD-10	-
Sampling site -4 'd'	26.	NOD-1	-
	27.	NOD-2	-
	28.	NOD-3	-
	29.	NOD-5	-
	30.	NOD-6	-
	31.	NOD-7	-
	32.	NOD-8	14
	33.	NOD-9	-
	34.	NOD-10	-
	35.	NOD-11	-
	36.	NOD-12	-
	37.	NOD-13	-
	38.	NOD-14	14
	39.	NOD-15	-
	40.	NOD-16	-
	41.	NOD-17	-
	42.	NOD-18	-
	43.	NOD-19	12
	44.	NOD-20	-
	45.	NOD-21	-
	46.	NOD-22	-
	47.	NOD-23	-

'a'- sub humid to humid eastern & south eastern upland; 'b'- Eastern Ghats hot moist sub-humid eco-sub-region; 'c'- Eastern plateau (chhotanagpur) and Eastern Zone; 'd'- Eastern Ghats, hot moist sub humid eco sub region; Unidentified bacteria are numbered where alphabet represents their respective places of sample collected; "D"-Dhenkanal, "N"- Nayagarh, "J"-Jajpur, "C"-Cuttack, "OD"-Odisha. C.D.at $p<0.05=0.97$ for interaction between bacterial isolates.

To inhibit the growth of *Aspergillus fumigatus in vitro* all bacterial were screened. Most of the bacteria failed to produce any inhibition zone against the pathogen. Bacteria isolated from Cuttack and Jajpur were proved impotent to produce any kind of inhibition zone. Out of 47 bacteria 5 bacteria were having capability to

inhibit the pathogen and NOD-19 was the most significant inhibitor amongst other isolates as compared to the control (C.D. at $p<0.05=0.91$) (Table-14, Fig.16 & 17).

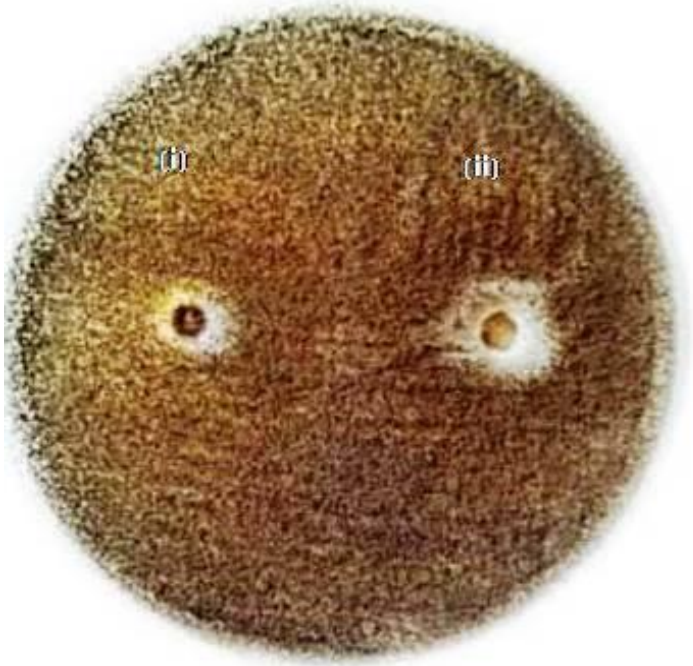


Fig. 16: Screening of antifungal bacterial isolates (i) & (ii) shows NOD-19 & NOD-14 against *Aspergillus fumigatus*

Fig. 17: Screening of antifungal bacterial isolates (i) & (ii) shows DOD-2 & NOD-8 against *Aspergillus fumigatus*

Table-14: Inhibitory zone by the bacterial isolates from varying sampling sites with specific agro eco regions (sub-regions) against *Aspergillus fumigatus*

Soil sampling (Sl. No.)		Bacteria Isolated	Inhibition Zone(mm)
Sampling site -1 'a'	1.	DOD-1	-
	2.	DOD-2	<10
	3.	DOD-3	-
	4.	DOD-4	12
	5	DOD-5	-
	6.	DOD-6	-
	7.	DOD-7	-
	8.	DOD-8	-
	9.	DOD-9	-
	10.	DOD-10	-
Sampling site -2 'b'	11.	COD-1	-
	12.	COD-2	-
	13.	COD-3	-
	14.	COD-4	-
	15.	COD-5	-
Sampling site -3 'c'	16.	JOD-1	-
	17.	JOD-2	-
	18.	JOD-3	-
	19.	JOD-4	-
	20.	JOD-5	-
	21.	JOD-6	-
	22.	JOD-7	-
	23.	JOD-8	-
	24.	JOD-9	-
	25.	JOD-10	-
Sampling site -4 'd'	26.	NOD-1	-
	27.	NOD-2	-
	28.	NOD-3	-
	29.	NOD-5	-
	30.	NOD-6	-
	31.	NOD-7	-
	32.	NOD-8	12
	33.	NOD-9	-
	34.	NOD-10	-

	35.	NOD-11	-
	36.	NOD-12	-
	37.	NOD-13	-
	38.	NOD-14	14
	39.	NOD-15	-
	40.	NOD-16	-
	41.	NOD-17	-
	42.	NOD-18	-
	43.	NOD-19	17
	44.	NOD-20	-
	45.	NOD-20	-
	46.	NOD-21	-
	47.	NOD-22	-

'a'- sub humid to humid eastern & south eastern upland; 'b'- Eastern Ghats hot moist sub-humid eco-sub-region; 'c'- Eastern plateau (chhotanagpur) and Eastern Zone; 'd'- Eastern Ghats, hot moist sub humid eco sub region; Unidentified bacteria are numbered where alphabet represents their respective places of sample collected; "D"-Dhenkanal, "N"- Nayagarh, "J"-Jajpur, "C"-Cuttack, "OD"-Odisha. C.D.at $p<0.05=0.91$ for interaction between bacterial isolates.

Table-15: Inhibitory zone by the bacterial isolates from varying sampling sites with specific agro eco regions (sub-regions) against *Penicillium notatum*

Soil sampling (Sl. No.)		**Bacteria Isolated**	**Inhibition Zone(mm)**
Sampling site -1 'a'	1.	DOD-1	12
	2.	DOD-2	12
	3.	DOD-3	-
	4.	DOD-4	-
	5.	DOD-5	-
	6..	DOD-6	-
	7.	DOD-7	-
	8.	DOD-8	-
	9.	DOD-9	-
	10.	DOD-10	-
Sampling site -2 'b'	11.	COD-1	-
	12.	COD-2	-
	13.	COD-3	-
	14.	COD-4	-
	15.	COD-5	-
	16.	JOD-1	-

Sampling site -3 'c'	17.	JOD-2	-
	18.	JOD-3	-
	19.	JOD-4	-
	20.	JOD-5	-
	21.	JOD-6	-
	22.	JOD-7	-
	23.	JOD-8	-
	24.	JOD-9	-
	25.	JOD-10	-
Sampling site -4 'd'	26.	NOD-1	-
	27.	NOD-2	-
	28.	NOD-3	-
	29.	NOD-5	-
	30.	NOD-6	-
	31.	NOD-7	-
	32.	NOD-8	14
	33.	NOD-9	-
	34.	NOD-10	-
	35.	NOD-11	-
	36.	NOD-12	-
	37.	NOD-13	-
	38.	NOD-14	14
	39.	NOD-15	-
	40.	NOD-16	-
	41.	NOD-17	-
	42.	NOD-18	-
	43.	NOD-19	18
	44.	NOD-20	-
	45.	NOD-21	-
	46.	NOD-22	-
	47.	NOD-23	-

'a'- sub humid to humid eastern & south eastern upland; 'b'- Eastern Ghats hot moist sub-humid eco-sub-region; 'c'- Eastern plateau (chhotanagpur) and Eastern Zone; 'd'- Eastern Ghats, hot moist sub humid eco sub region; Unidentified bacteria are numbered where alphabet represents their respective places of sample collected; "D"-Dhenkanal, "N"- Nayagarh, "J"-Jajpur, "C"-Cuttack, "OD"-Odisha. C.D.at $p<0.05=0.81$ for interaction between bacterial isolates.

The bacteria isolated from Nayagarh district were potent against the pathogens *Penicillium notatum*. From plate assay it is confirmed that the bacteria from Jajpur and Cuttack district were unable to inhibit the growth of the fungus. Out of the total 47 bacteria from the acid soil region, 5 isolates registered zone of inhibition against *P. notatum* as 2 from Dhenkanal and 3 from Nayagarh. Maximum inhibition was reported in NOD-19 followed by NOD-14 and NOD-8 (Fig. 18 & Fig. 19). Other organisms didn't produce any insignificant difference between the control and the treatment and found at par with control 89.9±0.6 (C.D. at $p<0.05=0.62$) (Table-15).

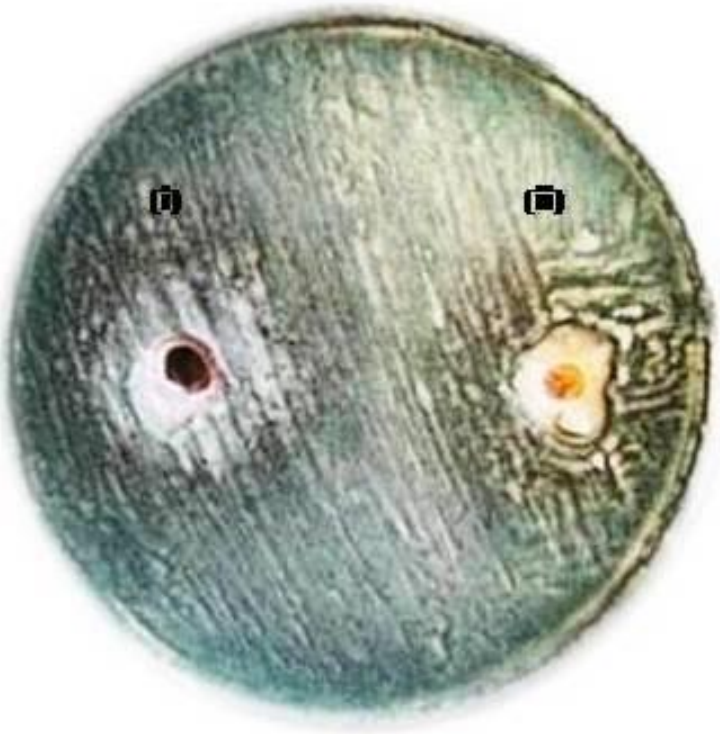


Fig. 18: Screening of antifungal bacterial isolates (i) & (ii) shows NOD-14 & NOD-8 against *Penicillium notatum*

Fig. 19: Screening of antifungal bacterial isolates (i) & (ii) shows NOD-19 & DOD-1 against *Penicillium notatum*

Table-16: Inhibitory zone by the bacterial isolates from varying sampling sites with specific agro eco regions (sub-regions) against *Fusarium* sp.

Soil sampling (Sl. No.)		Bacteria Isolated	Inhibition Zone(mm)
Sampling site -1 'a'	1.	DOD-1	-
	2.	DOD-2	-
	3.	DOD-3	-
	4.	DOD-4	12
	5.	DOD-5	-
	6.	DOD-6	-
	7.	DOD-7	-
	8.	DOD-8	-
	9.	DOD-9	-
	10.	DOD-10	-
Sampling site -2 'b'	11.	COD-1	-
	12.	COD-2	-
	13.	COD-3	-
	14.	COD-4	-

	15.	COD-5	-
Sampling site -3 'c'	16.	JOD-1	-
	17.	JOD-2	-
	18.	JOD-3	-
	19.	JOD-4	-
	20.	JOD-5	-
	21.	JOD-6	-
	22.	JOD-7	-
	23.	JOD-8	-
	24.	JOD-9	-
	25.	JOD-10	-
Sampling site -4 'd'	26.	NOD-1	-
	27.	NOD-2	-
	28.	NOD-3	-
	29.	NOD-5	-
	30.	NOD-6	-
	31.	NOD-7	-
	32.	NOD-8	16
	33.	NOD-9	-
	34.	NOD-10	-
	35.	NOD-11	-
	36.	NOD-12	-
	37.	NOD-13	-
	38.	NOD-14	16
	39.	NOD-15	-
	40.	NOD-16	-
	41.	NOD-17	-
	42.	NOD-18	-
	43.	NOD-19	14
	44.	NOD-20	-
	45.	NOD-21	-
	46.	NOD-22	-
	47.	NOD-23	-

'a'- sub humid to humid eastern & south eastern upland; 'b'- Eastern Ghats hot moist sub-humid eco-sub-region; 'c'- Eastern plateau (chhotanagpur) and Eastern Zone; 'd'- Eastern Ghats, hot moist sub humid eco sub region; Unidentified bacteria are numbered where alphabet represents their respective places of sample collected; "D"-Dhenkanal, "N"- Nayagarh, "J"-Jajpur, "C"-Cuttack, "OD"-Odisha. C.D.at $p<0.05=1.003$ for interaction between bacterial isolates.

The inhibitory strength of the bacteria from the lateritic soil region was tested against *Fusarium* sp., the producers of fumonisins. Only 4 bacteria out of a total of 47 isolates were able to exhibit antifungal activity in plate assay by agar diffusion method. NOD-14 produces 16mm zone of inhibition followed by NOD-19 (Fig. 21). The bacteria isolated from Cuttack and Jajpur district did not show any kind of growth inhibition. Only a single bacterium (DOD-4) from Dhenkanal district produce inhibition zone. There was no significant difference between the control and the treatment as regards inhibition by other bacteria 89.9±0.6 (C.D. at $p<0.05=1.003$) (Table-16).

During assay of the antifungal disc as in the medicines prescribed by physicians, it has found that ketoconazole showed 13mm of zone of inhibition while Clotrimaoxazole and Itraconazole produce no inhibition zone (Fig. 20).

4.3.2 Biochemical Identification of bacterial isolates

The main objective of any microbial classification system is to identify at species level, which is the basic unit of the taxonomic grouping. The bacteria which were active against human dermatophytes as well as phytopathogens were biochemically characterized. Bergey's manual of systemic bacteriology 2nd edition was followed for the identification. There are two bacteria from Dhenkanal district which were active against almost all pathogens and these were biochemically characterized. DOD-1 showed positive to VP test (Fig. 22) and negative to indole test (Fig. 23) among other IMVIC tests. It had also showed positive response to the catalase and nitrate enzyme activity leaving all other enzyme negative. The sugar utilized by the bacterium as negative.

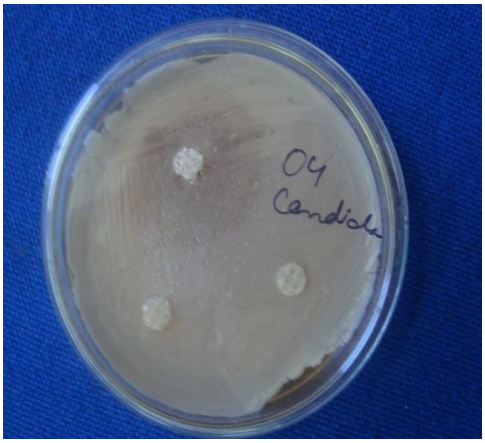


Fig. 20: Antifungal disc against *C. albicans*

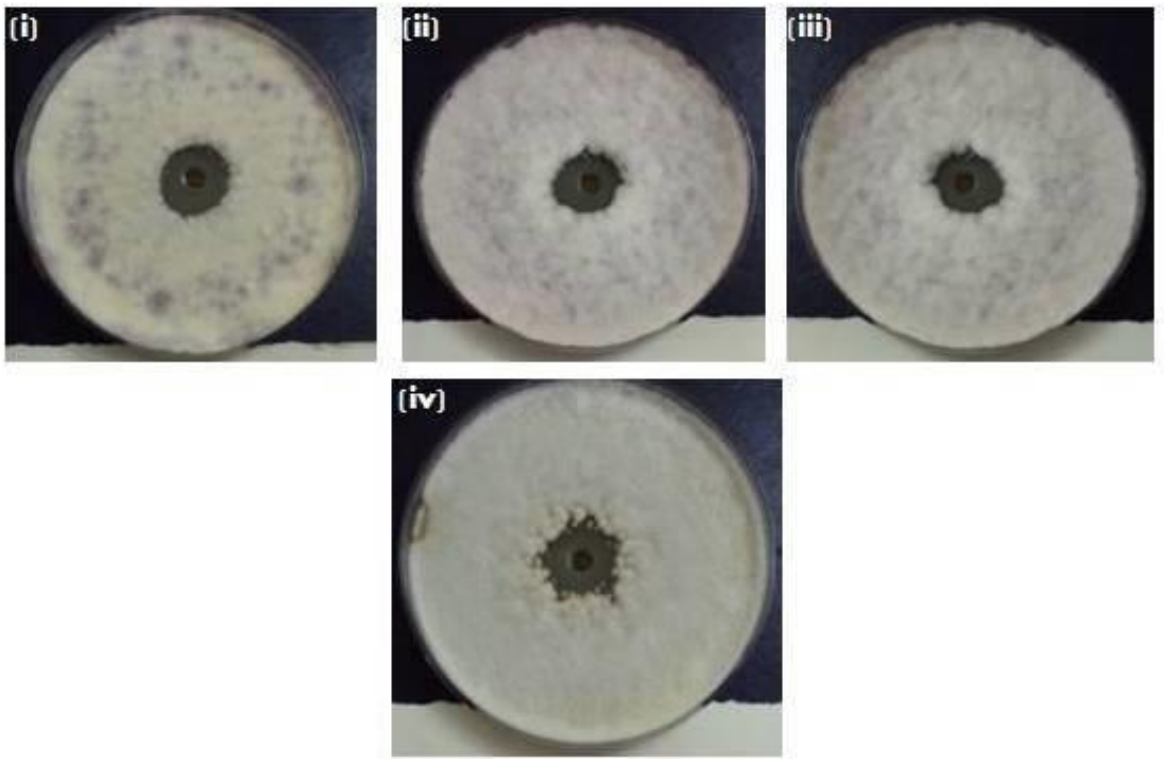


Fig. 21: Primary screening of antifungal bacterial isolates from acid soil of Odisha against *Fusarium* sp. (i) shows DOD-4 while (ii),(iii) &(iv) shows NOD-8, NOD-14 & NOD-19 respectively

Fig. 22: Voges-Proskauer (acetoin production) test of DOD-1 and DOD-2

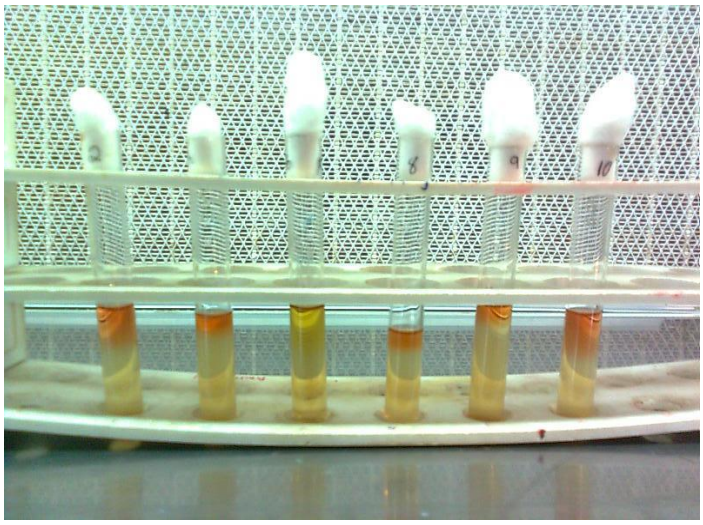


Fig. 23: Indole test of DOD-1 and DOD-2 bacteria

Fig.24: Citrate utilization test of NOD-8 and NOD-19 bacteria

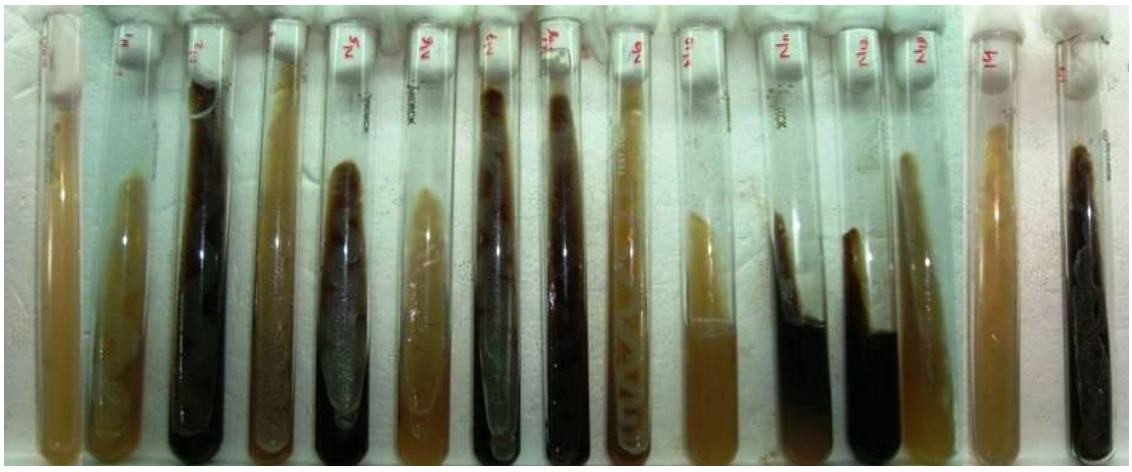


Fig.25: Esculin hydrolysis test of NOD-8 and NOD-19 bacteria

Likewise the bacterium coded as DOD-2 showed negative response to IMVIC tests. Negative results in sugar tests followed by in enzyme activity. Salt tolerance (NaCl) test was positive in both the bacterial case (Table-17). After manipulating the biochemical result in the Bergey's of manual of systemic bacteriology 2nd edition the bacterium coded as DOD-1 was found to be *Bacillus subtilis* and DOD-2 was *Bacillus brevis*.

The bacteria isolated from Cuttack and Jajpur failed to prove their activity against the phytopathogens and dermatophytes. The isolates from acid soil of Nayagarh were biochemically characterized. Out of 23 bacteria only two were active against almost all pathogens. Amongst these two, NOD-19 was most potent. NOD-19 and NOD-14 showed negative Citrate utilization (Fig. 24) and others of IMVIC test except Methyl red. In the enzyme test (both extracellular and intracellular) NOD-14 was positive to Oxidase and Starch hydrolysis while NOD-19 was positive to all (Urease, Oxidase, Catalase, Esculin (Fig. 25) and Starch degradation) tests. Extracellular enzyme activity as Pectin hydrolysis **(**Fig. 26) and Casein hydrolysis (Fig. 27) were positive in case of NOD-19 while NOD-14 gives negative result. The

Table-17: Biochemical characteristics of the most potent bacterial isolates from Dhenkanal district active against *Candida albicans*

Sl. No.	IMVIC Tests				Enzyme Activity (Extracellular and Intracellular)							Salt Tolerance (NaCl)	Sugar Utilization				Identification as per Bergey's Manual
	MR	VP	IP	CU	SD	GL	PH	Oxi	Cat	Ure	Nit		Glu	Suc	Fru	Gal	
DOD-1	-	+	-	+	+	+	+	+	-	+	-	+	-	-	-	-	*Bacillus subtilis*
DOD-2	-	+	-	-	+	-	-	-	-	-	-	+	-	-	-	-	*Bacillus azotoformans*

"D" denotes Dhenkanal District and Odisha abbreviated as "OD": MR- Methyl Red, VP- Voges-Proskauer, IP- Indole Production, SD- Starch Degradation, CU- Citrate Utilization, GL- Gelatin Liquefaction PU- OXi- Oxidase, Cat-Catalase, Ure- Urease, Nit- Nitrate. Glu- Glucose, Suc- Sucrose, Fru- Fructose, Gal- Galactose.

Table- 18: Biochemical characteristics of the most potent bacterial isolates from Nayagarh District against *Candida albicans*

Sl. No.	IMVIC Tests				Enzyme Activity (Extracellular and Intracellular)							Mot	Sugar Utilization															Antibiogram Profile						
	MR	VP	IP	CU	SD	EH	Ure	Oxi	Cat	PH	CH		A	L	R	M	S	T	Mo	Su	C	F	D	G	Gy	I	X	Rh	PB	C	CT	G	NA	CIP	ST
NOD-14	+	-	-	-	+	-	-	+	-	-	-	+	-	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	R	R	R	S	S	S	R
NOD-19	+	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	+	-	+	+	-	-	-	-	-	R	R	S	R	R	S	R

"N" denotes Nayagarh District and Odisha abbreviated as "OD" '+'symbolizes Utilizes Sugar; '-' symbolizes unable to utilize sugar; MR- Methyl Red, VP- Voges-Proskauer, IP- Indole Production, CU- Citrate Utilization, SD- Starch Degradation, EH- Esculin Hydrolysis, Ure- Urease, Oxi- Oxidase, Cat-Catalase, PH- Pectin Hydrolysis, CU- Casein Hydrolysis Mot- Motility Test; A-Arabinose; L-Lactose; R-Rhamnose; M-Maltose; S-Salicin; T-Trehalose; Mo-Mannose; Su-Sucrose; C-Cellobiose; F-Fructose; D-Dextrose; G- D Glucose; Gy- Glycerol; I- Inositol; X- Xylose; Rh- Raffinose. S-Sensitive; R-Resistance; PB-Polymyxin-B; C-Chloramphenicol; CT-Co-Trimaoxazole; G-Gentamycin; NA-Nalidixic Acid; CIP- Ciprofloxacin; ST-Streptomycin

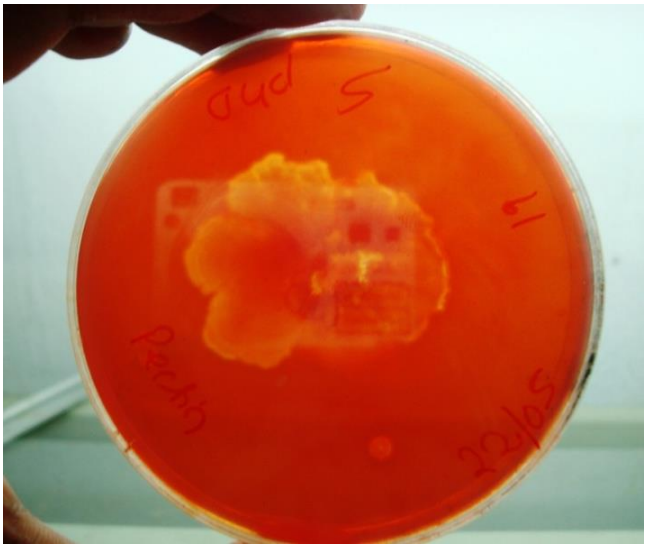


Fig. 26: Pectin hydrolysis of NOD-19 bacteria

Fig. 27: Casein hydrolysis of NOD-19 bacteria

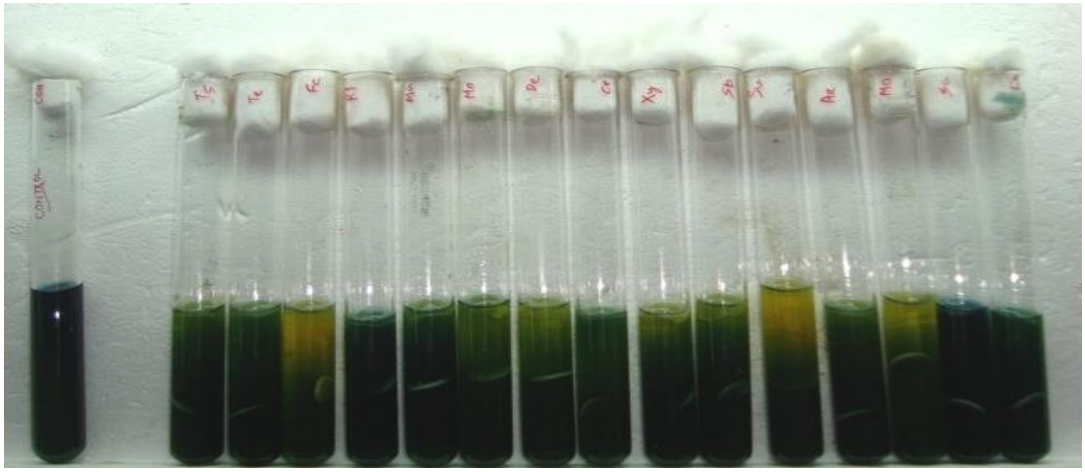


Fig. 28: Sugar Utilization of NOD-8 and NOD-19 bacteria

sugar utilization test by NOD-14 was negative in all most all sugar except lactose, sucrose and fructose. NOD-19 showed positive to sugar utilization test in case of Mannose, Sucrose, Fructose and Dextrose (Fig. 28). NOD-14 showed resistance against Polymyxin-B (PB), Chloramphenicol (C), Co-Trimaoxazole (CT) and Streptomycin (ST) antibiotics. NOD-19 was sensitive to Co-Trimaoxazole (CT) and Ciprofloxacin (CIP) antibiotics. Following Advanced bacterial identification software (ABIS) online it is found that these two bacteria NOD-14 and NOD-19 were *Bacillus* sp. (Table18).

4.3.3 Molecular identification of bacteria

The genomic DNA of the final isolate NOD-19 was extracted and found to be of good purity as (OD260/OD280) ~1.91. The quality and purity of these DNA samples were further confirmed with agarose gel electrophoresis resulting in the single band of high molecular weight DNA as it was observed under UV illumination. The 16S rRNA genes of the isolate NOD-19 was amplified by PCR with 10pmole of both 8F and 1492R primer set separately using Eppendorf Thermal Cycler and the resulting PCR amplicons were visualized as a single compact band of expected 1500bp DNA using 1.2% agarose gel electrophoresis as shown in the Figure 29.

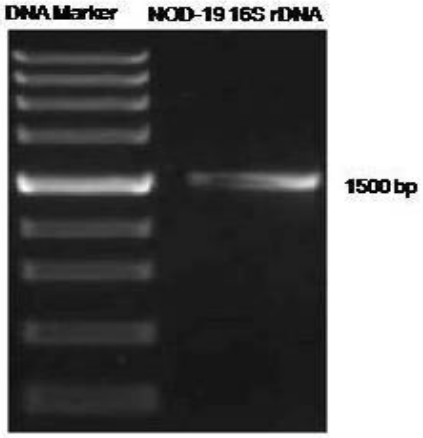


Fig. 29: Visualization of amplified16S rDNA fragments of 1500bp of the NOD-19 microbial isolate

These PCR amplicons were purified and subjected to automated DNA sequencing. The resultant forward and reverse sequences of 16S rRNA genes were aligned with CAP3 aligner software separately for isolate NOD-19, and constructed the corresponding consensus sequences. The longest (Forward/Reverse) sequence can be taken as consensus sequence as instructed in our case as shown in the Fig. 30.

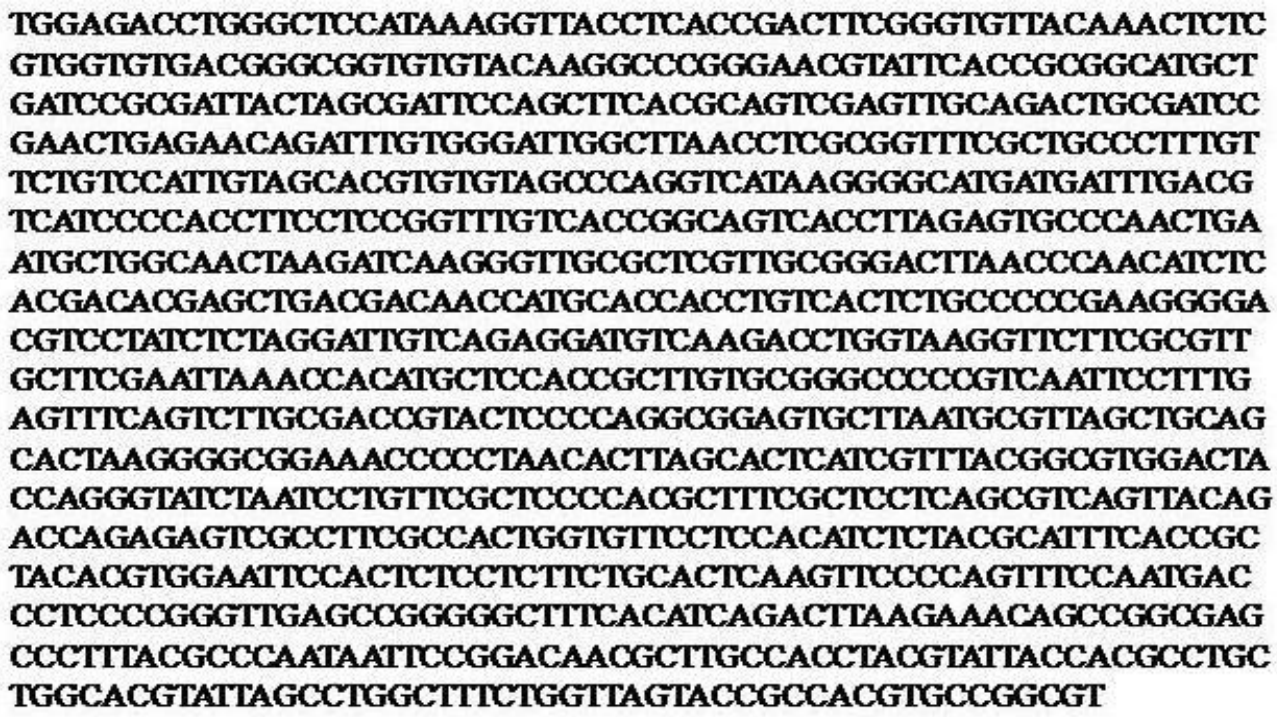


Fig. 30. Reverse sequences of 16S rDNA fragments of the isolate, NOD-19

The consensus sequences were analyzed with BLASTN search tool using nr database of NCBI GenBank for the identification of bacterial isolate, NOD-19. The homologous 16S rRNA gene sequences of the selected strains with respect to isolate NOD-19 was obtained from the microbial nucleotide databases through NCBI facility. These selected homology sequences of 16S rRNA genes along with the respective NOD-19 16S rRNA gene sequences were aligned using alignment tool viz. multiple

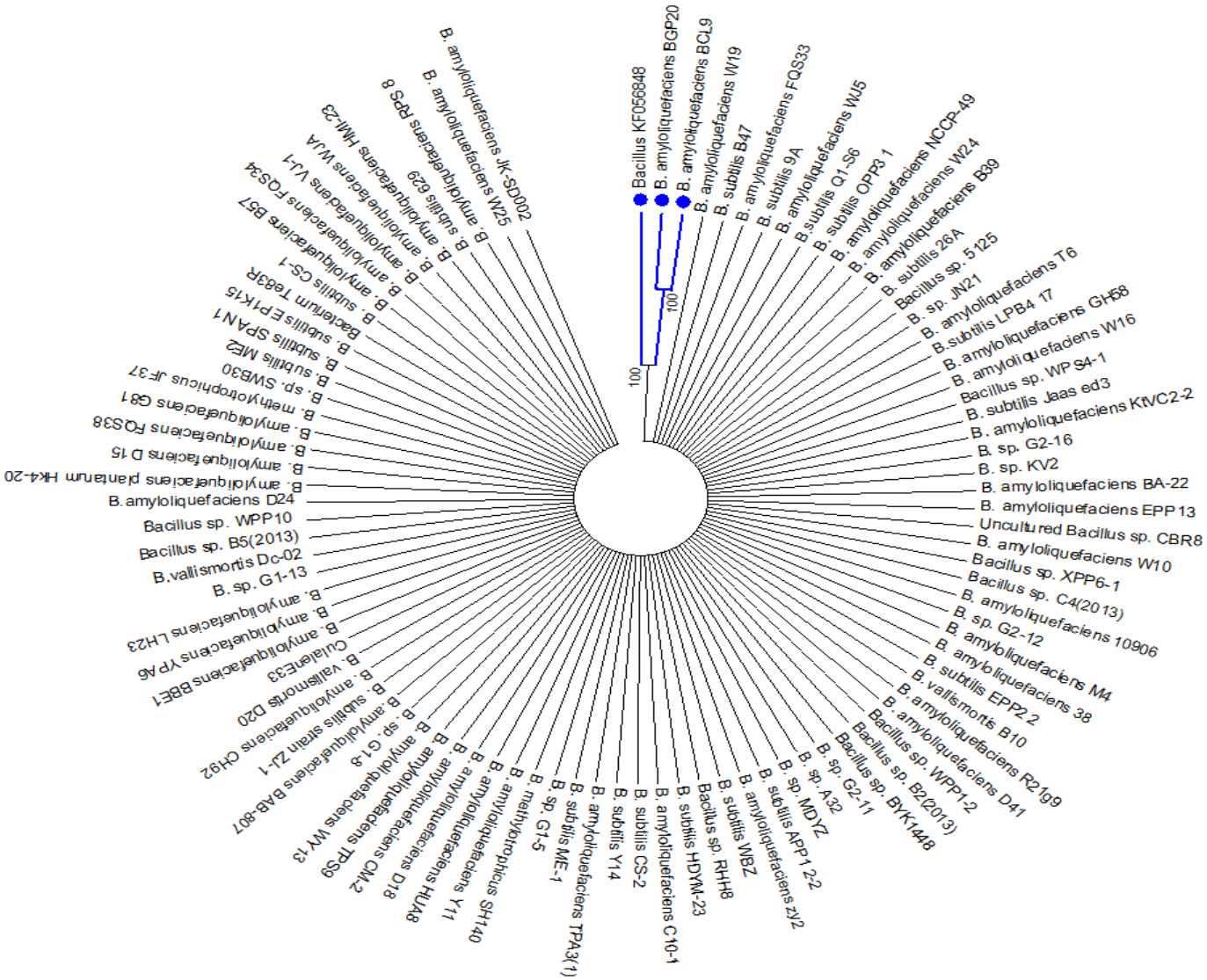


Fig. 31: Phylogenetic tree of the isolate NOD-19 with the homologous known bacterial strains developed using MEGA 5.0 Software

sequence Alignment by muscle and the results obtained were processed for generation of phylogenetic tree through MEGA version 5 software (Fig. 31). The results thus revealed that the bacterium belongs to genus Bacillus of the family Bacillaceae. The 16S rRNA genome sequence of the *Bacillus* sp. submitted to Genbank with an accession number KF056848.

4.4 Discussion

Although India has a vast tract of acid soil the microbial diversity of acid soil and their biotech potential has not been sincerely attempted so far. NAAS (National Academy of Agricultural Sciences, New Delhi) in its policy paper 48 has recommended study and utilization of microbial diversity of acid in 2010 which has been expanded even for biotech potential. Nevertheless the present finding seems to of its kind in the country as very few or no references are available on antimicrobial potential of bacteria isolated from acid soil. Even at global basis reports available are scanty. Some of the information available on the topic are discussed below.

Antagonistic interactions between different bacteria probably date back to Pasteur and Joubert. Application of this screening procedure gives useful preliminary information for the identification of possible potent organisms. These methods suffer from the limitation of not specifically demonstrating bactericidal activity. The antifungal activities of bacteria were evaluated by agar diffusion method as it is simple and cost effective (Scorzoni *et al.*, 2007). The present study emphasizes on screening of bacterial isolates from Acid soil region comprising of four districts namely Dhenkanal, Cuttack, Jajpur and Nayagarh of Odisha and biochemical and molecular characterization of most potent isolates against some plant and dermatophytes causing infections in immunocompromised patients.

Antifungal activity of bacterial isolates from acid soil by dual culture method on SDA plates showed that NOD-19 was the most potent isolates from the region. Out

of 47 bacterial isolates bacterium NOD-19 was most potent isolate from acid soil bacteria against *Trycophyton rubrum* giving a inhibition of 16mm (Fig. 10) followed by DOD-1, DOD-2 & NOD-1, 14mm each. *T. mentagrophytes* main causal agent of ringworm in rabbits was screened by the acid soil bacterial isolates. Among the bacterial population isolated, the bacterium NOD-19 at serial no. 43 (Table-10, Fig. 11) has produced maximum and very strong zone of inhibition, 16mm with respect to a dermal pathogen *T. mentagrophytes* where as other bacterial isolates from the region produce an inhibition zone between 10 to 14mm. The zone of inhibition of other bacteria didn't produce significant difference between control and treatment and found at par with control 89.9±0.6 (C.D. at $p<0.05=0.97$). Nayak and Mishra (2012) while studying antimycotic activity of *Bacillus* sp. isolated from acid soil of Nayagarh, Odisha reported 16mm zone of inhibition against the pathogen *T. mentagrophytes*. The organism in agar well diffusion method showed significant inhibition against the dermatophyte.

The biocontrol potential of NOD-19 was again proved as maximum growth inhibition up to 18mm. against *E. floccosum* followed by DOD-1, COD-2 & NOD-2 (all are having inhibited up to 14mm), while statistical analysis revealed C.D. at $p<0.05=0.70$. *C. albicans* is one of the most potent dermatophyte found in tropical countries. Among the bacterial population isolated, the bacterium at serial no.43 (Table 12) has produced maximum and very strong zone of inhibition, 25 mm against *C. albicans* MTCC 854 (Fig. 12, 13, 14, 15) where as other bacterial isolates from the region produce an inhibition zone between 12 to 14mm. However, other didn't produce the growth inhibition in plate assay. The *Bacillus* sp. producing maximum inhibition zone thus holds the promise for controlling development and growth of *C. albicans* and hence could be utilized in pharmacology as a novel source for management of the disease caused by *C. albicans*. The zone of inhibition of other bacteria didn't produce significant difference between control and treatment and

found at par with control 89.9±0.6 (C.D. at p<0.05=1.10). When screened against *C. tropicalis* the acid soil bacteria were failed to produce inhibition of growth except a few. Out of 47 bacteria 4 were having inhibition in the range of 12mm to 14mm. Interaction between treatments and respective control results C.D. at p<0.05=4.056.

The bacteria isolated from Jajpur and Cuttack districts were unable to inhibit the phytopathogen *A. fumigatus*, P. notatum and Fusarium sp. a serious rice pathogen. Sandikar and Awasthi (2010) reported a no. of soil bacteria isolated from rhizospheric region were able to inhibit the *Fusarium* sp. in dual culture method up to 60.00% which do not corroborates with the present finding that out of five bacteria 3 bacteria from Nayagarh and 2 from Dhenkanal districts were inhibiting the fungus in the range of 12 to 18mm. NOD-19 produces the maximum of 18mm inhibition zone against *P. notatum* followed by 17mm against *A. fumigatus*. The maximum zone of inhibition with 16mm was registered by NOD-8 while DOD-4 comes with the lowest inhibition of 12mm against *Fusarium* sp. However, the biocontrol potential of *B. subtilis*, have been reported as effective against a broad spectrum of plant diseases caused by soil borne (Asaka and Shoda, 1996) and foliar fungal pathogens (Romero *et al.*, 2007)

Amongst the 48 isolates obtained in primary screening, four potent antifungal isolates were gone through biochemical characterizations. Two isolates form Dhenkanal district (DOD-1 and DOD-2) and two isolates from Nayagarh districts were inhibiting almost all pathogens. Biochemical characters of two Dhenkanal isolates revealed that they belongs to *Bacillus* sp. were identified as *Bacillus subtilis* and *Bacillus azotoformans* according to Bergey's manual of determinative bacteriology (1994). The biochemical characteristics of Nayagarh isolates showed that NOD-19 as *Bacillus amyloliquefaciens* (probability 82%) and NOD-14 as *Bacillus farraginis* (probability 80%) by ABIS ONLINE software (Thangaraj *et al.*, 2013).

Antifungal activity of *Bacillus* sp. isolates against *T. rubrum*, *T. mentagrophytes*, *E. floccosum*, *C. albicans* MTCC 854, *C. tropicalis*, *A. fumigatus*, *P.*

notatum and *Fusarium* sp. was related to its ability to produce antifungal compounds similar as previously was reported by other *Bacillus* sp. The growth of *Penicillium* was inhibited by >50% by 14 strains of *Bacillus* sp. *in vitro* as reported by Arras and D'hallewin, 1994. *B. subtilis* strain GA1 has potential to control disease of apple caused by fungus (Toure *et al.*, 2004).

Most of the *Bacillus* are nonpathogenic, with high secretion capacity and are competent in producing various biological substances such as secretory proteins, enzymes, biofilm, biosurfactants and antibiotics. Screening of different antibiotics from natural sources is increasingly essential for the pharmaceutical industry as pathogenic bacteria develops resistance against commonly used therapeutic agents. Amongst these 4 isolates from acid soil of Odisha, NOD-19 was showing the best antifungal properties which leads to its molecular characterization viz. 16s rRNA sequencing. This is the first report from the acid soil region of Odisha.

The genomic DNA of the *Bacillus* sp. was extracted and found to be of good purity as (OD260/OD280) ~1.91, resulting the single band (1500bp) of high molecular weight DNA as observed under UV illumination. The 16S rRNA genes of was amplified by PCR with both 8F and 1492R primer set separately and electrophoresed using 1.2% agarose gel & results single compact band (Mandepudi, 2013) (Fig. 29). These PCR amplicons were purified and subjected to automated DNA sequencing. These sequences analysis was done with BLASTN, NCBI GenBank for the identification of bacterial isolates. These selected homology sequences of 16S rRNA genes aligned using multiple sequence alignment by Alignment by muscle and generated the dendrogram of through MEGA version 5 software as shown in fig. 31. The homologous organisms for isolate NOD-19 as shown in the dendrogram were of maximum similarity (99%-100%) to the genus *Bacillus* having the similar characters to its nearest neighbors *Bacillus amyloliquefaciens* BCL9 (JQ734537) & *Bacillus amyloliquefaciens* BGP20 (JQ734535) that are isolated from soil samples and were showed for the excellent antifungal activity against fungal pathogens. 16S rRNA sequencing of potent antifungal *Bacillus* isolate identified as *Bacillus* sp. KF056848. However the final results of the most probable nearest neighborhood strain may be considered as the *B. amyloliquefaciens* BGP20, which is also evident from the phylogenetic tree (Fig. 31).

CHAPTER -5

MASS PRODUCTION, EXTRACTION AND PURIFICATION OF THE METABOLITE

CHAPTER 5

MASS PRODUCTION, EXTRACTION AND PURIFICATION OF THE METABOLITE

5.1 Introduction

Bioactive compounds are extra nutritional constituents that naturally occur in small quantities in microbes, plant and food products (Kris-Etherton *et al.*, 2002). Most common bioactive compounds include secondary metabolites such as antibiotics, mycotoxins, alkaloids, food grade pigments, plant growth factors, and phenolic compounds (Hölker *et al.*, 2004; Kris-Etherton *et al.*, 2002; Nigam, 2009). Secondary metabolites are also biosurfactants; some may play essential roles for survival of the biosurfactants producing microorganisms through facilitating nutrient transport or microbe–host interactions or by acting as biocide agents. Work on biosurfactants applications has been focused to their diversity, environmental friendly nature, suitability for large-scale production and selectivity. Many of the potential applications of biosurfactants depends their production (economically); however, much effort in process optimization and at the engineering & biological levels have been carried out.

It is believed and also true that novel antibiotics and other bioactive secondary metabolites can still be discovered from microbial sources. The probability of finding novel bioactive compounds depends on a series of critical factors. On one hand, there are number of strains screened and their degree of diversity; on the other hand, their uniqueness and their potential to produce secondary metabolites. These last two criteria are extremely important and must be considered as intensively screened microbes are less likely to yield novel metabolites than unexploited groups.

Peptide antibiotics form an unique group of "bioactive molecules" (Hancock and Chapple, 1999). Regardless of the toxicity of some antibiotics produced by bacteria from *Bacillus* genus to the cells of mammals (e.g. polymyxines, bacitracin etc.), they were and continued to be in the focus of attention of researchers and scientists. Antibiotics and similar natural products, being secondary metabolites produced by all most all living organisms. They are produced by *Bacillus* sp. and *Pseudomonas* sp. as prokaryotic organisms.

The present study was aimed to check the ability of *Bacillus* sp. isolates for the production of antibiotics and to optimize different physical and chemical parameters with special reference to this bacterium *Bacillus* sp. isolated from specific environment (NOD-19, Table-5) for antibiotic production and to identify, extract and concentrate the so produced antibiotic. Antibiotics are mainly polypeptides; compounds such as bacillomycin, mycobacillin, fungistatin etc. are effective agents against molds and yeasts (Katz and Demain, 1977) isolated from *Bacillus* sp. Peptide antibiotics fall into two broad classes first is a large and heterogeneous category of peptides that are synthesized on very large, modular enzyme complexes (Peptide synthetases) by bacteria and fungi (Stachelhaus *et al.*, 1996; Konz and Marahiel, 1999; Eppelman *et al.*, 2001) and second category is quite different which comprises linear peptides consisting almost entirely of conventional amino acid residues that are produced by all major kind of organisms (including microbes). These are translated using ribosomes in the usual fashion of protein synthesis, and therefore called as RAMPs, for ribosomally synthesized Anti Microbial Peptides (Hancock and Chapple, 1999) to distinguish them from the non-RAMPs of the first category.

One of the most important challenges before using (or marketing) a bioactive molecule is to purify it from the originating medium. This step generally constitute a bottleneck in the development of a process. Usually, bioactive compounds are recovered

from natural sources by solid–liquid extraction employing organic solvents in heat-reflux systems (Wang and Weller, 2006). However, other techniques have been recently proposed to obtain these compounds including the use of supercritical fluids, high pressure processes, microwave-assisted extraction and ultrasound-assisted extraction (Cortazar *et al.*, 2005; Markom *et al.*, 2007; Wang and Weller, 2006). Extraction/production of bioactive compounds by fermentation is also an interesting alternative that merits attention, since it is able to provide high quality and high activity extracts while precluding any toxicity associated to the organic solvents. In this process, bioactive compounds are obtained as secondary metabolites produced by microorganisms after the microbial growth is completed (Nigam, 2009). Studies on liquid culture show that the production of these compounds starts when growth is limited by the exhaustion of one key nutrient: carbon, nitrogen or phosphate source (Barrios-González *et al.*, 2005). Mostly the production yields of secondary metabolites can be improved with a suitable choice of substrate or mixture of substrates with appropriate nutrients (Nigam, 2009). As a whole, the support material must present characteristic favorable for the microorganism development and be of low cost.

Finally, the selection of the most appropriate downstream process for the obtained product is also crucial when submerged fermentation processes are performed. The product obtained by submerged fermentation may be recovered from the submerged fermented mass by extraction with solvents (aqueous or other solvents mixtures). The type of solvent and its concentration, as well as the ratio of solvent to the liquid and pH are important variables that influence in the product extraction. The cost of purification depends on the quality of the obtained extract. For example, the presence and concentration of inert compounds in the extract increase the cost of purification and therefore the cost of recovery is increased. Particularly those secondary metabolites which are used in bulk in the pharmaceutical and health industry and whose purity is governed by stringent regulations need to go through specific purification strategy (Nigam, 2009).

5.2 Materials and Methods

5.2.1. Bacterial growth medium

The bacteria was identified as Bacillus sp. and characterized as described elsewhere (chapter-4). The *Bacillus* sp. was successfully revived on NA medium (Himedia, Mumbai) having pH 5.5±0.1. The revival of cultures was performed aerobically.

To prepare the *Bacillus* sp. of the invention for use as an antifungal agent, the microorganism is grown to stationary phase in a defined medium that favors the production of the antifungal substance(s).

5.2.2. Production of antifungal compounds

For the isolation and enrichment of the antifungal compounds slightly modification in the procedure of Neyra *et al.* (1996) was followed. The *Bacillus* sp. was grown on NA slants (acidic pH) 24hrs prior to transferring in the minimal salt medium (MSM) for antibiotic production (Table-19). The pH of the medium was adjusted to 5.9±0.1. The cultures were grown in batch cultures in 500ml conical flask (Merck, India) containing 200ml. of the medium. After inoculation with 1ml of the washing from NA slant, the flasks were incubated at 30°C in a gyratory shaker (Satyam, India), at a speed of 150 rev min^{-1} for 3days. The incubator is preferably affected under the condition of aerial stirring.

5.2.3. Acid precipitation and collection of metabolites

After 3 days of incubation the cells were removed by centrifugation at 14,500 g for 25mins at 4°C. A fractioned precipitation with conc. HCl was performed with culture supernatant containing the metabolites by adjusting the pH to 2.5. The HCl was slowly added to the medium at room temperature with constant stirring. Slow stirring was

continued for an additional 1-2hrs at low temperature. The precipitate/pellet was collected by centrifugation at 14,500 g for 15mins at 4°C.

The pellets containing the active metabolites were then suspended and extracted with absolute ethanol (Hong Yang Chemical Corporation, China, 1:4 wt/v) with polarity 6.8.

Table- 19: Composition of Minimal salt medium for the mass multiplication of the *Bacillus* sp. to collect metabolites

Chemicals Used	Amount (g)*	Utilization
Bacto Dextrose	20	Main constituent of cellular material
L-Glutamic acid	5	Constituent of amino acids, nucleic acids nucleotides, and coenzymes
Magnesium Sulfate ($MgSO_4.7H_2O$)	1.02	Inorganic cellular cation, cofactor for certain enzymatic reactions
Di-Potassium Hydrogen Phosphate (K_2HPO_4)	1.0	Main cellular inorganic cation and cofactor for certain enzymes
Potassium Chloride (KCl)	0.5	
Trace Elements**		
Manganese Sulfate (MnSO4)	0.5	As metal ions, the trace elements usually act as cofactors for essential enzymatic reactions in the cell
Copper sulfate pentahydrate ($CuSO_4.5H_2O$)	0.16	
Ferrous sulfate heptahydrate ($FeSO_4.7H_2O$)	0.015	

*Amount in gram per 1000ml. of water **Amount in gram per 100ml. of dH_2O water

The crude ethanolic extract (CEE) was later evaporated to syrup form. The syrup was stored in vials at 4°C until further use.

5.2.4 Antimicrobial spectra of CEE

The antifungal activity of the CEE was performed on SDA plates. It was determined by agar well diffusion method in which a well of 6mm diameter was made at the center of previously *C. albicans* MTCC 845 seeded plate. A 100µl of CEE was loaded

in the plate and incubated at 30°C for 24-48hrs along with untreated control. The growth inhibition was measured as zone diameter with the help of Zone diameter measuring scale (Himedia, India).

Having conformity of bioactivity in the ethanol extract of metabolites, 45ml of such biologically active metabolites was collected from *Bacillus* sp. and utilized for isolation of active principle through partial purification by column chromatography followed by pure active ingredient through single spot isolation on TLC.

5.2.5 Antifungal compound Purification

Partial purification of the CEE was carried out by Column Chromatography and purification of active ingredient through Thin Layer Chromatography (TLC)

5.2.5.1 Column Chromatography

The column was loaded with CEE precaution to keep the same column saturated for extraction of the active principle through bioactive metabolites through various solvents (Hexane, Benzene, Chloroform, Methanol and Acetone) following polarity gradient based solvent extraction system (Table-20)

Table-20: Polarity of Organic solvents used for extraction

Solvents	Polarity
Hexane	1.8
Benzene	2.3
Chloroform	4.8
Methanol	5.1
Acetone	5.1
Ethanol	6.8

Polarity parameter collected from www.macro.Isu.edu

Loading of column: - Clean Silica Gel (60-120 mesh, Merck, Germany) was loaded to the 2/3 of the clean vensil column with 95cm in length (from sintered glass bottom). The column was loaded with silica gel saturated in hexane solvent and allowed to settle over night with care taken that there is no air bubble in the loaded column. The air bubble if any in the column was taught to remove through the solvent in a clean beaker on the packed Silica Gel saturated with hexane even in the minutes of the air bubble. This way to regulate the constant flow of solvent.

Loading of biologically active metabolites:- The bioactive metabolite extraction i.e. CEE as stated above was loaded to the column slowly on the top of the Silica Gel saturated with hexane. The care to be taken while loading of metabolites on to the top of the silica gel saturated with hexane in such way that the top layer was not disturbed in any way.

Fractionation of metabolites and isolation of active principle:- Polarity gradient based solvent extraction system was followed for the isolation of active principle through compact column. The rate of flow of solvent was taught to regulated from 18-22 drops per minute and up to 25ml (elute) per container. The selection of metabolites is purely based on bioassay test.

Pull of active elutes:- The elute showing maximum activity through bioassay test were pulled together.

5.2.5.2 Thin layer Chromatography

To purify the isolated compound collected through intermediate partial separation Thin layer Chromatography (TLC) was adopted.

Preparation of TLC plates:- Known amount of Silica Gel G (without binder) was taken along with distilled water to make the slurry with the help of glass rod in a beaker. Precaution was taken that the slurry was not so liquid or hard paste. It must be a intermediate stage in between them. Some amount of slurry was poured on the clean

glass plate (8'×8'). Care should be taken that the distribution of the slurry should be uniform and presence of no air bubble was there. The plates were dried under fan followed by activation in oven.

Activation of TLC plates:- After air dried the silica gel G coated TLC the plates were oven dried at 50°C. To remove the moisture the plates were oven dried for 20mins.

Standardization of developer solvent:- The principle behind the developer solvent selection is movement and separation of spots in solvent. Slides were coated with Silica gel G for the TLC plates. The trough (Glass chamber) was saturated by the developer solvent. Elute showed excellent antimycotic activity was spotted on the slide (One spot per slide). The slide was putted in the developer solvent saturated beaker and dipped up to 0.5 cm in solvent and covered. The plate was taken out after movement of solvents to a distance of 5cm and placed in iodine chamber for colour development.

Spotting and identification of active compound(s):- The activated plates were picked up and spotted with active column elute. The plates were putted into the saturated developer chamber. After some movement in the plates these plates were brought to outside and allowed to evaporation of developer solvent. The plates were then putted inside the iodine chamber for colour development. The Rf value was calculated using the formula;

$$Rf = \frac{\text{Distance travelled by spot (midpoint of the spot)}}{\text{Total distance travelled by the solvent}}$$

Side by side other plates were also kept outside for the evaporation of developer solvent and scrapped as the separated spot in the iodine reacted plate. The spot was

scrapped and tested for its activity against test pathogens. After conformation of pure compound on the spot the mass collection was done.

5.3 Results

During optimization of various parameters it was observed that after 72hrs of incubation at 30°C the zone of inhibition started decline and after 96hrs the growth ceased completely thereafter (Fig. 32(A)). Although, significantly good amount of metabolite was also found at 3-4 days of prolonged incubation, after that antibiotic production started declining. The data exhibited (Table-19) an important role in chemical constituents such as Carbon and Nitrogen sources of the fermentation medium for antibiotic production. Maximum antibiotic production was obtained when dextrose was used as carbon source. However, significant antibacterial activity was also achieved when maltose, sorbitol, lactose and glucose were employed as carbon sources. For further experiments dextrose was used as the carbon source.

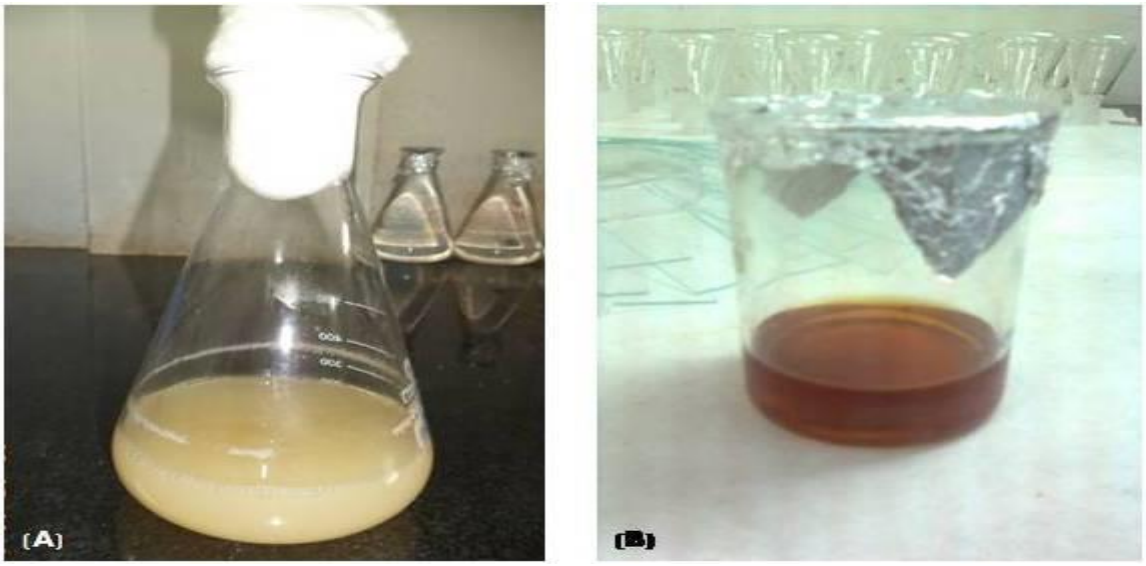


Fig. 32. (A)Antibiotic production in Minimal Salt Medium. (B)Crude Ethanolic extract

Antimicrobial properties of the Crude Ethanolic Extract (CEE)

CEE was collected (Fig. 32(B)) and the antimicrobial spectrum of the CEE was observed (Table-21). *C. albicans*, a potential dermatophyte that causes several dermal infections was found sensitive to CEE (Fig. 33) along with another dermatophyte *T. mentagrophytes* (Fig. 34), but the extract was least effective against *Fusarium* sp.

(Fig. 35) The extract was observed to be fungicidal not fungistatic *in vitro*. The zone of inhibition was increased as the CEE get in to the syrup form. The effectiveness against *C. albicans* MTCC 854 was the most as compared to the pathogens despite of day interval.

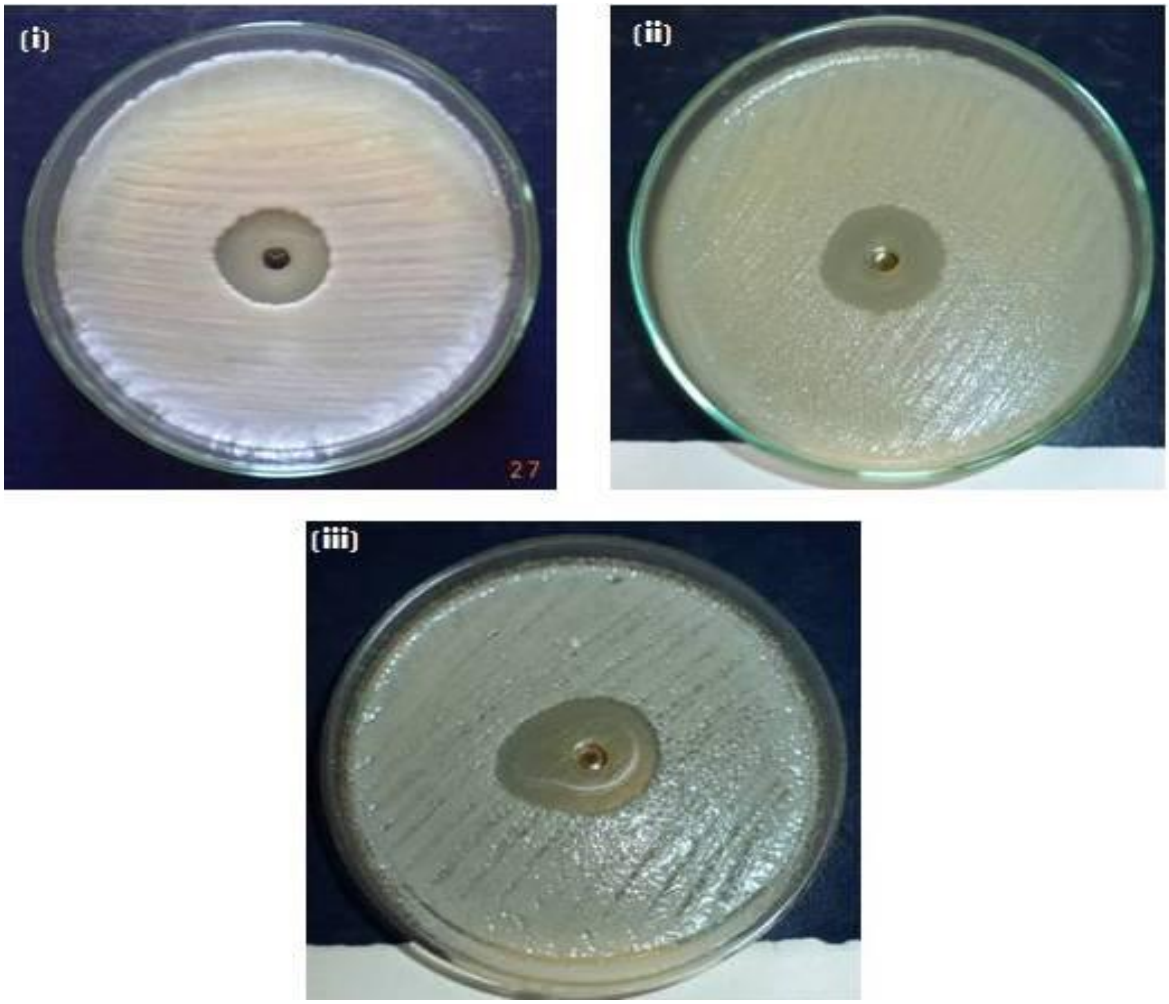


Fig. 33. Ethanolic extract of metabolites against *C. albicans*. Plates (i) to (iii) shows inhibition at interval from 1 to 3 days respectively

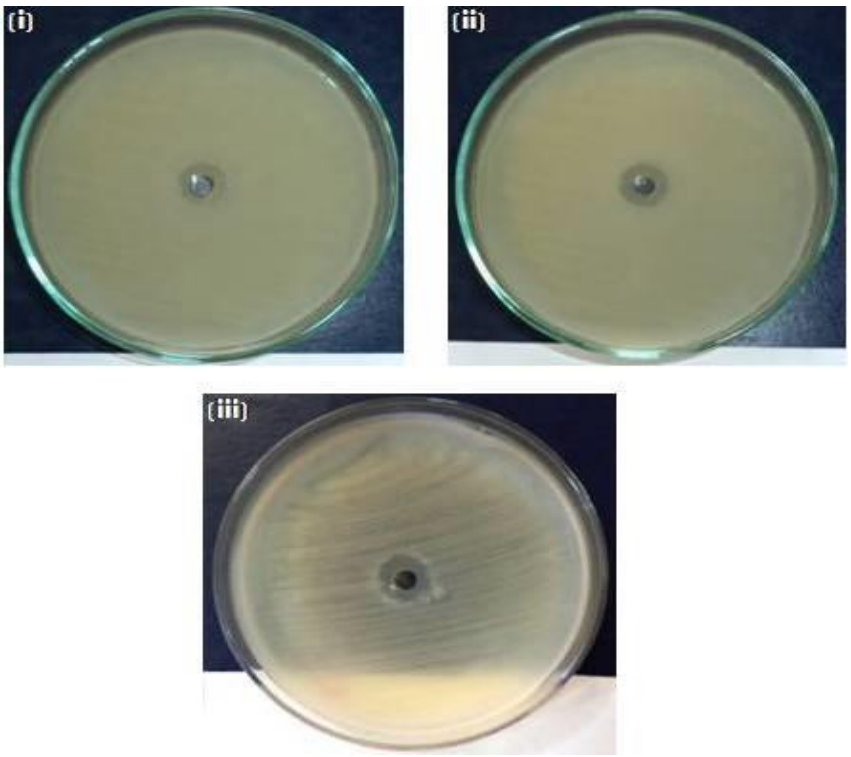


Fig. 34. Ethanolic extract of metabolites against *T. mentagrophytes*. Plates (i) to (iii) shows inhibition at interval from 1 to 3 days respectively

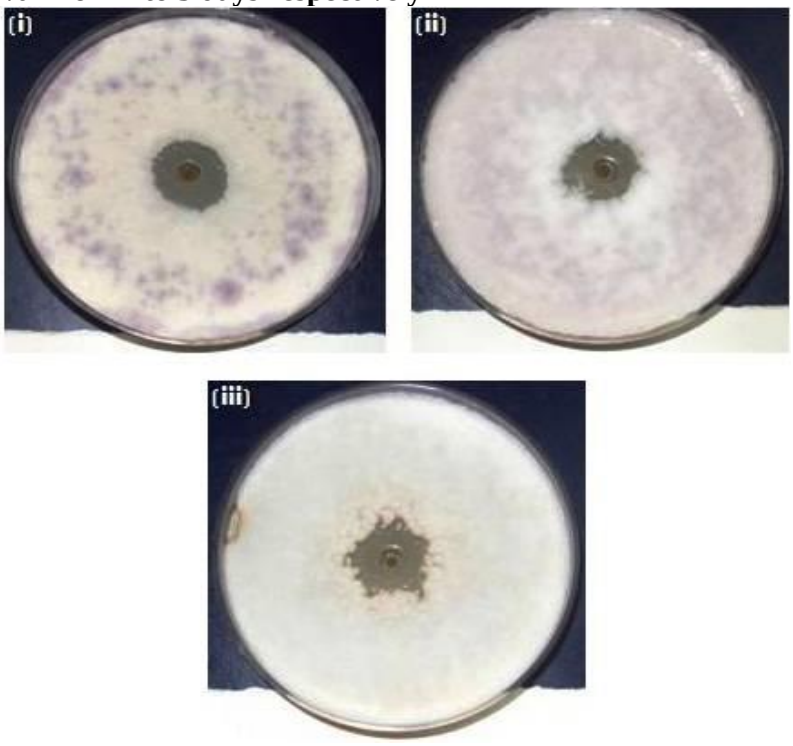


Fig. 35. Ethanolic extract of metabolites against *Fusarium* sp. Plates (i) to (iii) shows inhibition at interval from 1 to 3 days respectively

Table-21: Antimicrobial activity of the Crude Ethanolic Extract from *Bacillus* sp. isolate against fungal pathogens

Test organisms		Inhibition Zone(mm)
Candida albicans MTCC 854	D_1	22
	D_2	25
	D_3	27
Trycophyton mentagrophytes	D_1	14
	D_2	16
	D_3	16
Fusarium sp.	D_1	14
	D_2	14
	D_3	14

D_1: Day-1, D_2: Day-2, D_3: Day-3

Polarity gradient based solvent extraction system was followed for extraction of active compound from bacterial metabolites. Organic solvents with different polarity were illustrated (Table 20). Hexane is having least polar (1.8) while ethanol shares highest polarity (6.8). The Data was collected from www.macro.Isu.edu.

Silica gel column chromatography was used for partial purification of the active compound (Fig. 36). After loading the silica gel column with the ethanol extract subsequently polarity gradient solvent system was used for further fractionation (Fig. 37, Fig. 38). Column elutes (Hexane) were bioassayed. The Hexane elutes were bioassayed against *C. albicans* and proved ineffective against the pathogens (Fig. 39). Commencing from H_1 to H_{30} were fail to produce any inhibition zone (Table-22).

Table-22: Bioassay of *Bacillus* sp. metabolites against *C. albicans* in hexane fractions from Silica Gel column Chromatography

Hexane (100%)	Inhibition Zone(mm)
H_1	-
H_2	-
H_3	-
H_4	-
H_5	-
H_6	-
H_7	-
H_8	-
H_9	-
H_{10}	-
H_{11}	-
H_{12}	-
H_{13}	-
H_{14}	-
H_{15}	-
H_{16}	-
H_{17}	-
H_{18}	-
H_{19}	-
H_{20}	-
H_{21}	-
H_{22}	-
H_{23}	-
H_{24}	-
H_{25}	-
H_{26}	-
H_{27}	-
H_{28}	-
H_{29}	-
H_{30}	-
CONTROL	-

H- Hexane fractions and numbered serially

Fig. 36. Unloaded Silica gel column ready for loading of bacterial metabolite for partial purification

Fig. 37. Metabolite loaded for partial purification

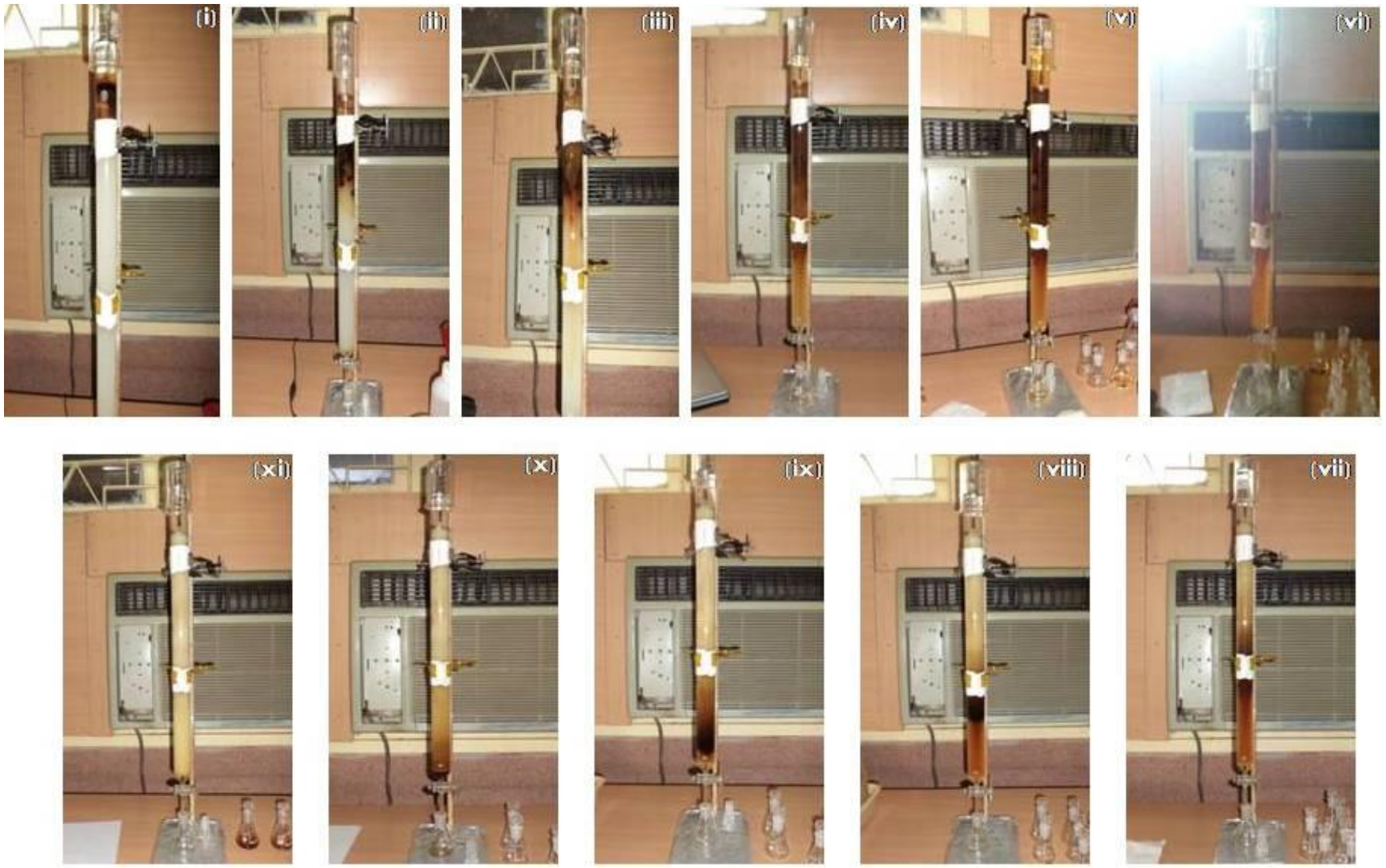


Fig. 38. Stage wise fractionation and collection of elutes at regulated time interval

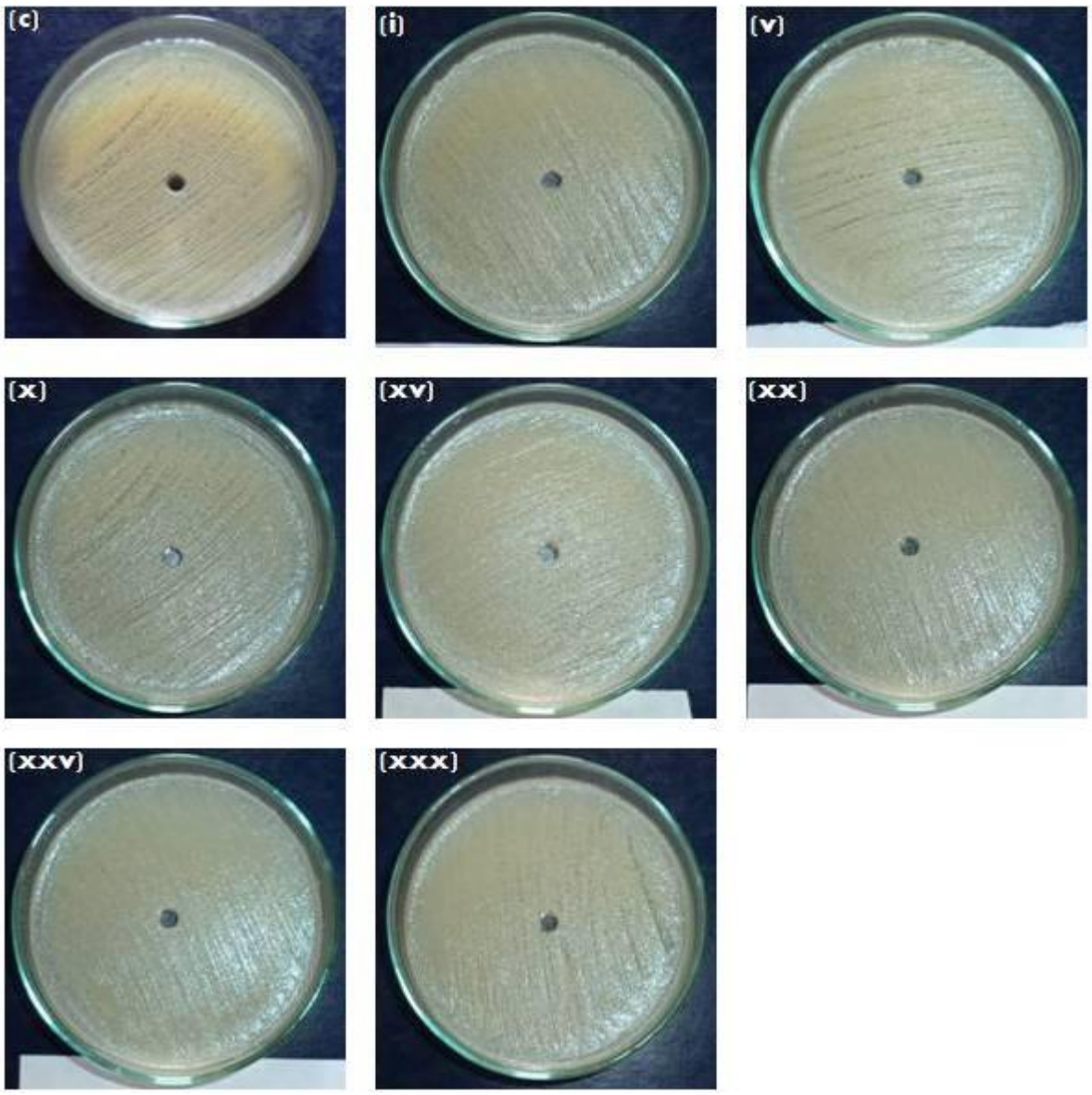


Fig. 39. Bioassay test of hexane fractions against *C. albicans*. (C)-Control, (i) to (xxx) showing no inhibition zone in elutes from hexane fractions.

The Crude ethanolic extract of the metabolites were loaded in the column for the partial purification of active compound. After the Hexane, Benzene was loaded in to the silica gel column. The benzene elutes were collected, bioassayed and gradually numbered B_1 to B_{20}. Fraction 1 to 20 failed to produce any kind of inhibition *in vitro* (Table-23; Fig. 40).

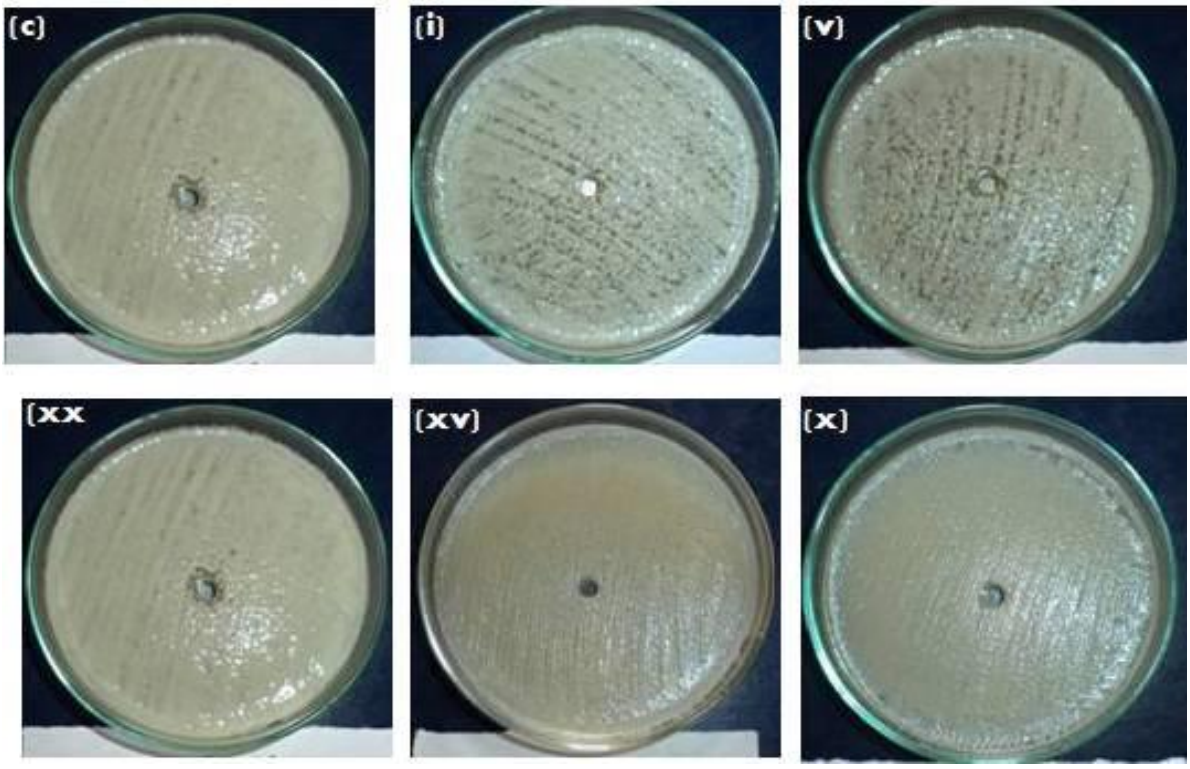


Fig. 40. Bioassay test of benzene fractions against *C. albicans*. (C)-Control, (i) to (xx) showing no inhibition zone in elutes from benzene fractions

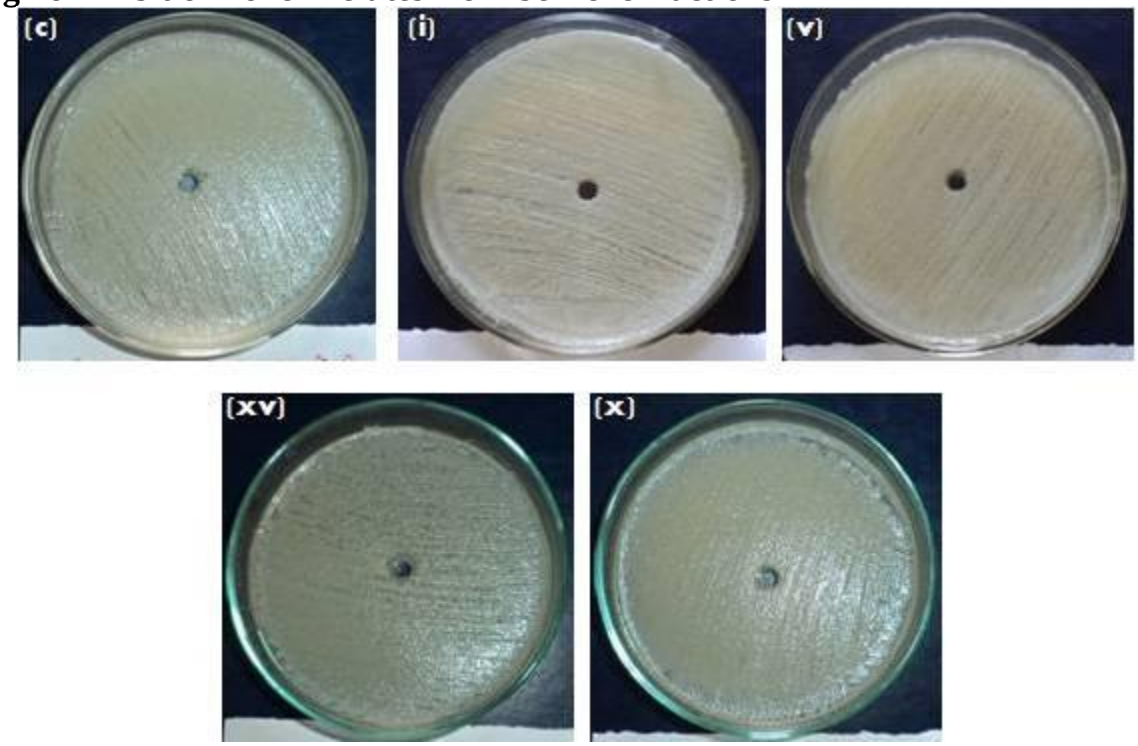


Fig. 41. Bioassay test of chloroform fractions against *C. albicans*. (C)-Control, (i) to (xv) showing no inhibition zone in elutes from chloroform fractions

Table-23: Bioassay of *Bacillus* sp. metabolites against *C. albicans* in Benzene fractions from Silica Gel column Chromatography

Benzene (100%)	Inhibition Zone(mm)
B_1	-
B_2	-
B_3	-
B_4	-
B_5	-
B_6	-
B_7	-
B_8	-
B_9	-
B_{10}	-
B_{11}	-
B_{12}	-
B_{13}	-
B_{14}	-
B_{15}	-
B_{16}	-
B_{17}	-
B_{18}	-
B_{19}	-
B_{20}	-

B- Benzene fractions and numbered serially

Following polarity gradient solvent system for partial purification of metabolites chloroform was poured in to the column next to benzene and elutes were collected. Starting from C_1 to C_{15} (Chloroform was symbolizes as C and numbered gradually) each elute was bioassayed (Table-24). There is no inhibition of growth observed in any of such elutes (Fig. 41).

Table-24: Bioassay of *Bacillus* sp. metabolites against *C. albicans* in Chloroform fractions from Silica Gel column Chromatography

Chloroform (100%)	Inhibition Zone(mm)
C_1	-

C_2	-
C_3	-
C_4	-
C_5	-
C_6	-
C_7	-
C_8	-
C_9	-
C_{10}	-
C_{11}	-
C_{12}	-
C_{13}	-
C_{14}	-
C_{15}	-

C- Chloroform fractions and numbered serially

The pre *Bacillus* sp. ethanolic extract loaded silica gel column was poured with the solvent which was having higher polarity than chloroform. The methanol fractions were gradually numbered and bioassayed against *C. albicans*. Starting from M_1 to M_{16} (Methanol was symbolized as M and numbered gradually) each fraction was able to inhibit the pathogen. While M_{10} was able to inhibit the highest inhibition zone up to 27mm followed by M_{11}, 26mm (Fig. 42). The inhibition zone gradually decreases and disappears after M_{16} (Table-25).

Table-25: Bioassay of *Bacillus* sp. metabolites against *C. albicans* in methanolic fractions from Silica Gel column Chromatography

Methanol (100%)	**Inhibition Zone(mm)**
M_1	15
M_2	13
M_3	11
M_4	12
M_5	15
M_6	10
M_7	10
M_8	10
M_9	11
M_{10}	27
M_{11}	26

M_{12}	19
M_{13}	18
M_{14}	19
M_{15}	15
M_{16}	12
M_{17}	--
(CONTROL)	--

M- Methanol fractions and numbered serially

In polarity table acetone comes next to methanol. Acetone was poured to the preloaded column and elute was collected. Commencing from A_1 to A_{17} each such elute was bioassayed and proves its inability to inhibit the growth of the pathogen, *C. albicans* (Table-26; Fig. 43).

Table-26: Bioassay of *Bacillus* sp. metabolites against *C. albicans* in Acetone fractions from Silica Gel column Chromatography

Acetone (100%)	Inhibition Zone(mm)
A_1	-
A_2	-
A_3	-
A_4	-
A_5	-
A_6	-
A_7	
A_8	-
A_9	-
A_{10}	-
A_{11}	-
A_{12}	-
A_{13}	-
A_{14}	-
A_{15}	-
A_{16}	-
A_{17}	-
CONTROL	-

A- Acetone fractions and numbered serially

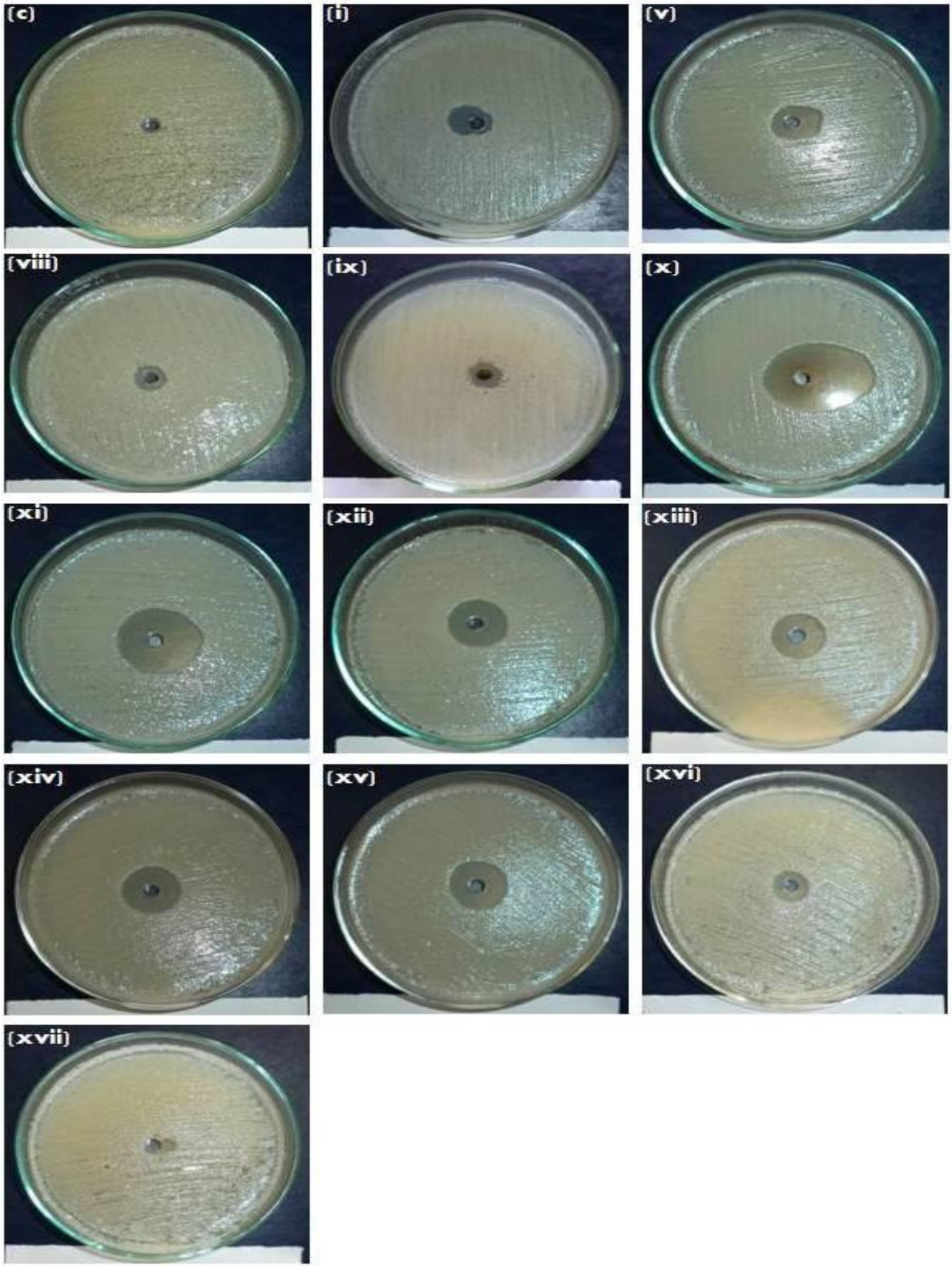


Fig. 42. Bioassay test of Methanol fractions against *C. albicans*. (C)-Control, (i) to (xvii) showing inhibition zone in elutes from methanol fractions

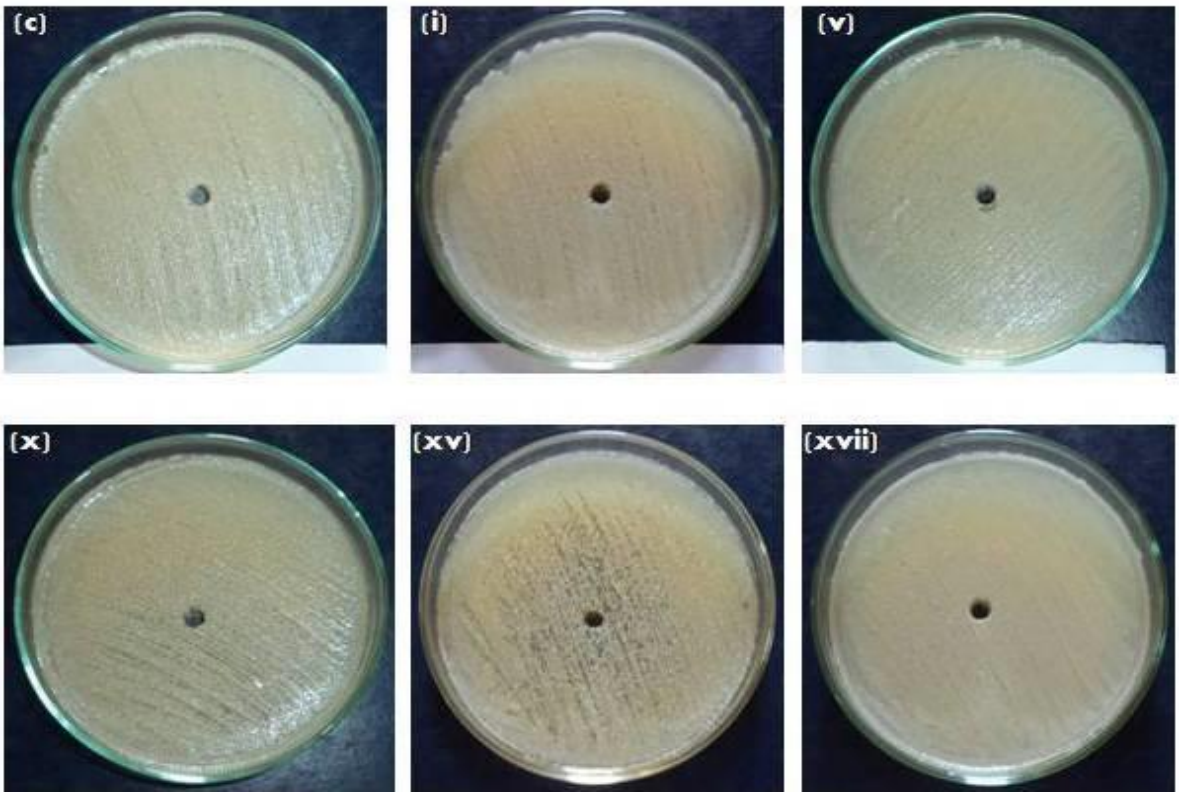


Fig. 43. Bioassay test of Acetone fractions against *C. albicans*. (C)-Control, (i) to (xvii) showing no inhibition zone in elutes from acetone fractions

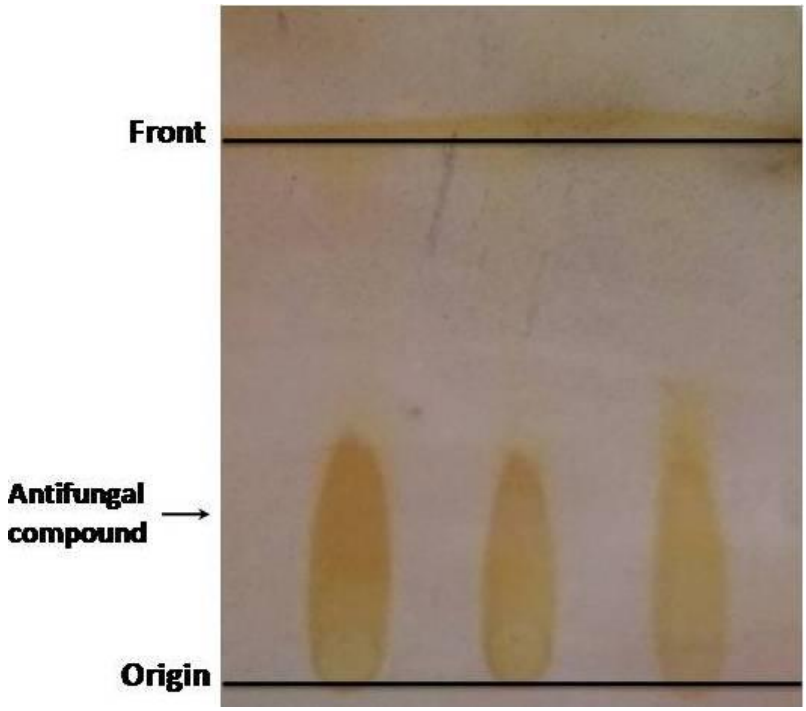


Fig. 44. Isolation of antifungal substances. TLC bioautography of antifungal substances

Table-27: Antimycotic activity of *Bacillus* sp. metabolites extracted in polarity gradient based solvent systems against *C. albicans*, a potential dermatophyte.

Sl. No.	Treatments	Polarity	Growth (mm)
1.	T_1	0.1	89.9
2.	T_2	2.7	89.9
3.	T_3	4.1	89.9
4.	T_4	5.1	64.9
5.	T_5	5.1	77.9
6.	T_6	Respective Solvents in each case	89.9

C.D. at $p<0.05=0.43$ for interaction between individual treatments of solvents; T_1: Hexane, T_2: Benzene, T_3: Chloroform, T_4: Methanol, T_5: Acetone, T_6: Control; 89.9 Represents Complete growth.

The *Bacillus* sp. posses a number of toxins such as thuricin, subtilosin A, and surfactin (Bizani and Brandelli, 2002; Stein, 2005). Keeping this in view, metabolites of the *Bacillus* sp. was fractionated through polarity gradient solvent system viz. Hexane, Benzene, Chloroform, Methanol & Acetone and bioassayed. Through fractionation and bioassay test it is established that the solvent with polarity range 0.1 to 5.1 (www.marco.Iso.edu) though were able to extract the compound causing inhibitory effect in the range 64-89.9±0.6, the best solvent was found to be Methanol followed by Acetone. On comparison, the zone of inhibition by the methanol extract was superior as compared to acetone extract. The zone of inhibition of other solvent extract didn't produce significant difference between control and treatment and found at par with control 89.9±0.6 (C.D. at $p<0.05=0.43$) (Table-27).

The methanol fractions showing maximum inhibition zone up to 27mm viz. M_{10}, M_{11} fractions were pulled together. The Standardization of developer solvent was carried out using TLC coated slides. Solvents with different polarity were tested as developer solvent both in pure form (100%) and in proportions with other solvents. The movement of spots was indicated when the slides were taken from the beaker containing organic solvent as developer solvent and put inside the iodine chamber as

because of development of colour due to reaction of iodine with the compound. Starting from hexane to chloroform along with ethyl acetate and dichloromethane were failed to move the compound from the spot. Methanol and Acetone both move from the slide but a trail was indicated in case of acetone showing Rf value 0.32 while methanol shows no trail with Rf value 0.23. When methanol was proportioned with hexane commenting from 5:5, 6:4, 7:3, 8:2, 9:1, v/v trail was also indicated with different Rf values (Table-28). When ethanol was taken as developer solvent a clear spot was indicated without any trail with Rf value 0.26 (Fig. 41). As because of the clear spot and no trail indication ethanol was selected as developer solvent.

Table-28: Standardization of developer solvent and separation of active compound on TLC coated slide

Solvent	Movement of active compound	Rf Value
Hexane	-	-
Benzene	-	-
Chloroform	-	-
Methanol	+	0.23
Acetone	+	0.32 (A trail was indicated)
Ethyl acetate	-	-
Ethanol	+	0.26
Dichloromethane	-	-
M:H (5:5)	+	0.23 (A trail was indicated)
M:H (6:4)	+	0.21(A trail was indicated)
M:H (7:3)	+	0.25(A trail was indicated)
M:H (8:2)	+	0.20(A trail was indicated)
M:H (9:1)	+	0.17(A trail was indicated)

‘+’-Movement indicated; ‘-’No movement

The mass collection of the compound has been carried out from the 8'×8' of TLC plate by scrapping the spot area as same as in the iodine reacted plate.

5.4 Discussion

Amongst several microrganism inhabitiing a range of soil characterised by diversed textured and structural value and organic matter the soil form Nayagarh district, Odisha was thought to be investigated for its microbial presence specially the th most commonly occuring Bacilli. As this evident that the later produces metabolites which are studied and utilized by human being to their advantage. The production of metabolite is a natural process by bacteria in thr struggle for survival as the principle of survival of fittest that eliminates the colonisation of other microbial entities (Talaro and Talaro, 1996; Jensen and Wright, 1997). There has been established evidence that genera *Bacillus*, *Streptomyces* and *Pseudomonas* the soil bacteria have produce antibiotics of agricultural and medical importance (Yoshiko *et al.*, 1998; Sharga *et al.*, 2004). In continuation of same series of research *B. subtilis* has been found to produce antibiotics and has been acknowledged since past 50 years. Amongst which peptide antibiotics represents the predominant class. However, a systematic study with respect to specificity of antibiotics by variety of *B. subtilis* strains are only rare (Pinchuk *et al.*, 2002).

In an effort to explore the antibiotic properties of *Bacillus* sp. isolated from acid soil of Nayagarh, Odisha has not been reported except of course where T. mentagrophytes that was found to inhibit as repoted by Nayak and Mishra, 2012. As it is evident that *Bacillus* genus has been the one that investigated, studied and examined for its production of antibiotics owing to the facts of its numerosity of natural isolates. Although, the *B. brevis* and *B. amyloliquefaciens* have been found to produce certain antibiotics but the less number of as compared to *B. subtilis*. These secondary metabolites which are extracellular in its origin are basically extracellular polymeric substances (EPS) in nature and composed of polysaccharides, proteins and/or other

biopolymers at different rations (Volk and Furkert, 2006; Mishra and Jha, 2009; Trabelsi *et al.*, 2009).

Study was also made to determine a specificity of elements, essential minerals and metal ions to determine optimal growth then biological activities as it has been recorded that most antibiotics are independently produce various biological activities without the need of metal ions such as Mn^{+2}, Co^{+2} or Zn^{+2}. However, in order to maintain proper structure and function of these antibiotics a coordinated metal ion in these antibiotics have been indicated to play an important role especially for maintenance of antibiotic activities. Such antibiotics are referred as metaloantibiotics (Epperson and Ming, 2002; Ming, 2003). Interestingly, Haavik (1974) reported the reversal of inhibitory effect of EDTA by addition of excess of Mn^{+2}, Co^{+2} or Zn^{+2} to the culture. Here a care has also to be taken from this angel while optimizing the growth and production of metabolites specific in the present study.

Different parameters for the optimum production of antibiotics from *Bacillus* sp. were optimized in the present study. Incubation time optimization gave 72hrs as optimum time for antibiotic production. As antibiotics are secondary metabolites so these are formed usually when organism has passed rapid growth phase. The same thing was discussed by (Demain, 1986). Demain concluded that the synthesis of peptide antibiotics is initiated after the organism has passed the rapid growth phase. Although antibiotic formation usually follows logarithmic growth (presumably due to some type of repression of antibiotic synthetases in the growth phase), this is not universally observed. It is clear that antibiotics are sometimes produced during growth and that both genetic and nutritional modifications can shift the time of antibiotic synthesis in relation to the growth phase. Hanlon and Hodges (1981), concluded that bacteria and proteases may under certain conditions, arise throughout the phase of

growth which is truly exponential rather than the phase of active but non exponential growth.

In the instant case of studies optimization was made by employing carbon sources that supported antibiotics formation maximum and them dextrose was used as carbon source Although good activity was also obtained with maltose, glucose, sorbitol and lactose sugar source of carbon. Qadeer *et al.*, (1988) has reported similarly the production of antibiotics bacitracin in starch-glucose-soybean meal medium where glucose has been found an excellent carbon source for bacterial growth but also is reported interferences synthesis of secondary metabolites coupled with decrease in pH in certain cases. Interestingly bacitracin production in B. subtilis has been found to be pH dependent where the inhibitory effect of glucose was due to acidification as a result of the accumulation of organic acids (Montserrat *et al.*, 2000).

Jamil *et al.* (2007) reported that L-glutamic acid was most condusive for the antibiotics formation. Whereas Egorov *et al.*, (1986) concluded that glutamate substitution with tryptone resulted in a dramatic deceleration of the bacterial growth and biomass accumulation where the process of the antibiotic biosynthesis ceases.

The conventional approach to isolate EPS involves a number of steps, including concentration under reduced pressure, elimination of impurities, and precipitation by chemical solvent (Li *et al.*, 2011). Such methods include, but are not limited to various chromatographic procedures, such as Column chromatography, Thin Layer Chromatography (TLC) using standard preparative protocol.

Silica gel Column Chromatography and Thin layer Chromatography was followed for the purification of the antifungal active compound from the CEE of *Bacillus* sp. The broad spectrum of antifungal against *C. albicans* renders the antifungal active compound producing strain of *Bacillus* sp. particularly useful as

biological control agents to reduce the dermatophytic infection where as prolonged clinical trials needs to be made for some conclusive statement. In silica gel of 60-120mesh compacted column, loaded with CEE and polarity gradient based solvent extraction system was deployed for partial purification, the compound was methanol soluble that gave strong antifungal inhibition zone against the test pathogen *C. albicans* MTCC 854 and found least effective against agronomically important phytopathogenic fungi *Fusarium* sp. Kumar *et al.*, (2009) also opined that solvent methanol was the best solvent for extraction of antifungal compound which is in curiosity with the present finding. Thin layer chromatography method was adopted to detect the presence of lipopeptides while preparative TLC was suggested to be used to purify small quantities (Symmank *et al.*, 2002). Standardization for selection of developer solvent was based on the principle of movement of compound in the solvent and cessation of movement in solvent. Ethanol was the only solvent which gave single spot movement without any trail. The other solvents (listed in table 25) were not giving any clear movement or no movement. The Silica Gel G (without binder) was used so as to avoid the interference of binder in active compound movement. The methanolic fractions were analyzed using TLC (Razafindralambo *et al.*, 1993) with direct view developed using reaction in iodine chamber. A brown spot formed with the Rf value 0.26 when the plates were reacted with iodine, indicating that the compound is same in all plates because of movement on TLC plate. Since the Rf value of the methanolic fraction of *Bacillus* sp. were similar to the standards iturin A (0.3) hence the method was developed for isolation of active principle was similar to Arrebola *et al.*, (2010) and inhibitory activity was confirmed by developing agar diffusion method. Mass collection was made by scrapping and collecting the mixing spot into methanol solvent and compound recovered after evaporation of methanol in dust proof chamber at room temperature. The compound was collected approx. 800mg and stored in glass vials for further spectral analysis.

It is evident from the above finding that the bacterium from acid soil of Nayagarh, Odisha has the potential to produce bioactive substance/compound that is highly effective against dermatophyte *Candida albicans*. Higher zone of inhibition was shown in the elute as compared to control set with medicine recommended by physicians for the disease is axiomatic that the bioactive compound produced by the bacterium is more potential against the dermatophytes than the medicines available.

CHAPTER - 6

MOLECULAR ANALYSIS OF THE ACTIVE COMPOUND

CHAPTER 6

MOLECULAR ANALYSIS OF THE ACTIVE COMPOUND

6.1 Introduction

Many microbes are potential to produce a wide range of amphipathic compounds, with both hydrophilic and hydrophobic moieties present, which allow them to exhibit surface activities at interfaces and are generally called biosurfactants or bioemulsifiers. These surface-active compounds (SAC) are mainly classified according to their mode of action, molecular weight and general physico-chemical properties. The former group includes low-molecular-weight compounds, such as lipopeptides, glycolipids, proteins, while the latter includes high-molecular-weight polymers of polysaccharides, lipopolysaccharides proteins or lipoproteins (Smyth *et al.*, 2010a, 2010c). During the past two decades research on biosurfactants have gained momentum as a potential replacement for synthetic surfactants and are expected to have several industrial and environmental applications (Banat *et al.*, 2000). As such these compounds have several applications and traditionally are used in pharmaceutical, and cosmetics industries (Nitschke *et al.*, 2005). Chemical surfactants are widely used in industry but the increasing environmental issues and restrictive laws led to the development of biodegradable and naturally produced surfactants, such as microbial surfactants. Biosurfactants appears as a reliable alternative due to their lower toxicity, higher biodegradability and effectiveness at extreme temperature, salinity & pH conditions (Van Hamme *et al.*, 2006). Several groups of biosurfactants have been reported in recent pasts, but the most important group includes glycolipids and lipopeptide (Nitschke *et al.*, 2005).

Unlike chemically synthesized surfactants, which are classified according to the nature of their polar group, biosurfactants are categorized mainly by their chemical composition and their microbial origin. In general, their structure includes a hydrophilic moiety consisting of amino acids or peptides anions or cations; mono-, di- or polysaccharides; and a hydrophobic moiety consisting of unsaturated, saturated or fatty acids. Accordingly, the major classes of biosurfactants include glycolipids, lipopeptides and lipoproteins, phospholipids and fatty acids, polymeric surfactants, and particulate surfactants. Although there are a number of reports on the synthesis of biosurfactants by hydrocarbon-degrading microorganisms, some biosurfactants have been reported to be produced on water-soluble compounds such as glucose, sucrose, glycerol, or ethanol. Bacteriocins are proteins produced by prokaryotes that are bactericidal and/or bacteristatic against organisms related to the producer strain (Jack *et al.*, 1995). Depending on their structure, mode of action and chemical properties, four distinct classes are recognized (Klaenhammer, 1993).

Molecular structure is determined by observing and analyzing how electromagnetic radiation interacts with matter by spectrometer (instrument) measures radiation absorbed at various energies. There are numerous kinds of spectrometers, using different ranges of radiation energy as visible radiation, infrared radiation and with microwave radiation. IR (Infrared) spectroscopy can provide information about the nature of the bonds in a substance because infrared absorptions of a substance reveal a great deal about the bonding between the atoms in its molecules. It can reveal the presence/absence of certain types of bonds. However, it can seldom reveal by itself the location of the bonds within the molecule.

MS (Mass spectrometry) with ESI (electrospray ionization) is a powerful analytical technique that has been commonly used for identification and analysis of several bio molecules such as lipids and carbohydrates. Using this approach it is

possible to obtain the information about the structural details of the analyzed molecules. This technique has advantage that it requires very small amount of sample, even when present in mixture without derivatisation. MS spectra allowed the identification of the molecular weight of the compound.

NMR (Nuclear magnetic resonance) spectroscopy is extremely useful in determining the arrangement of atoms in a molecule. This form of spectroscopy relies on properties of the nuclei in a molecule. NMR spectroscopy is a very powerful tool for determining the structure of molecules. Because many nuclei have spin, the arrangements of these nuclei in molecules can be investigated by NMR spectroscopy. Combined with infrared spectroscopy, NMR spectroscopy can reveal both the arrangements atoms in molecules and how they are bonded together.

Elemental analysis determines the molecular formula (Dhanasekaran *et al.*, 2008). UV absorption spectroscopy can be used for the quantitative determination of compounds that absorb UV radiation based on Beer's law.

6.2 Materials and methods

After single spot isolation from TLC plates the active compound were subjected for mass collection.

Mass collection and spectral analysis.

The pure active compound were mass collected by evaporating the solvent (soluble) in mg. Spectral analysis of active compound of the *Bacillus* sp. isolate was out sourced. The compound was sent for the spectral analysis to SAIF, Panjab University, Chandigarh, Punjab and STIC, CUSAT, Cochin, Kerala.

FT-Infra Red spectrum

The FT-Infra Red (FTIR) spectrum of active compound was analyzed. The pure compound of *Bacillus* sp. KF056848 was subjected to IR spectral analysis. IR spectrum was recorded on a Perkin Elmer - Spectrum RX-IFTIR instrument.

Mass Spectrometry

Analysis of the active compound from *Bacillus* sp. was carried out by MS using ESI ionization obtained in a Q-TOF (Waters, Milford, USA). ESI conditions in the electrospray Q-TOF mass spectrometer were as follows: electrospray voltage was 3kV in positive mode.

NMR spectra

For proper structural elucidation, it is important to have a good NMR spectroscopic data. In this study, isolated and purified compound was dissolved in $CDCl_3$ for ^1H NMR spectrum. Nuclear Magnetic Resonance (NMR): Avance II-400 MHz Bruker (Ultrashield ^{TM}) model of SAIF Punjab University, Chandigarh was used for this purpose.

UV spectrum

The ultra violet spectral measurement of the pure active compound from *Bacillus* sp. was made 200-400nm by using Hitachi (330) instrument (Japan); with ethanol was used as the solvent.

Elemental (C-H-N) Analysis

C-H-N analysis of the active compound from *Bacillus* sp. has been carried out in Elementar Vario EL III instrument. The temperature was varying in between 950-1200°C based on sample. From the values (%) of CHN, O was calculated.

6.3 Results

6.3.1 Infrared Spectral Analysis (FTIR)

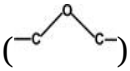
The FTIR spectrum of the antibiotic was measured as a KBr pellet. The FTIR spectrum of the purified active compound (antibiotic) is shown in Fig. 45. Characteristic absorption valleys at 1,632cm^{-1} and 3,306cm^{-1} indicate that the active compound contains peptide bonds. There is a peak near 1700cm^{-1} which may be due to the presence of keto () group. This keto () group may be in lactone ring or may be an amide group. Valleys at 2921cm^{-1} may be due to C-H stretching vibration of alkane/alkyl moiety. Presence of ether () stretch may be due to 1079cm^{-1}. One groove is also indicated at 1400cm^{-1} which may be due to alkenes or free carboxylate ion (Table-29).

Table-29: Infrared Spectral Analysis data

Spectroscopic Analysis	Peaks(cm^{-1})/Wave number	Characterisation
FT-IR Spectroscopy	3306	(Broad peak) It may be due to stretching vibration of -OH or -NH molecule. It denotes that OH/NH molecules are in H-bonded condition.
	2921	This is due to C-H stretching vibration
	1632	Due to Amide –C=O vibration. It may be secondary amide.
	1700	May be due to presence of lactone ring.
	1079	It may be ether(C-O-C) stretching vibration
	1400	It may be due to alkenes or free carboxylate ion

6.3.2 Mass Spectrometry

The Q-ToF MS Spectra, of the metabolite obtained from the isolate *Bacillus* sp. are shown in Fig. 46. The most abundant ions observed in the extract at 685.28 m/z,

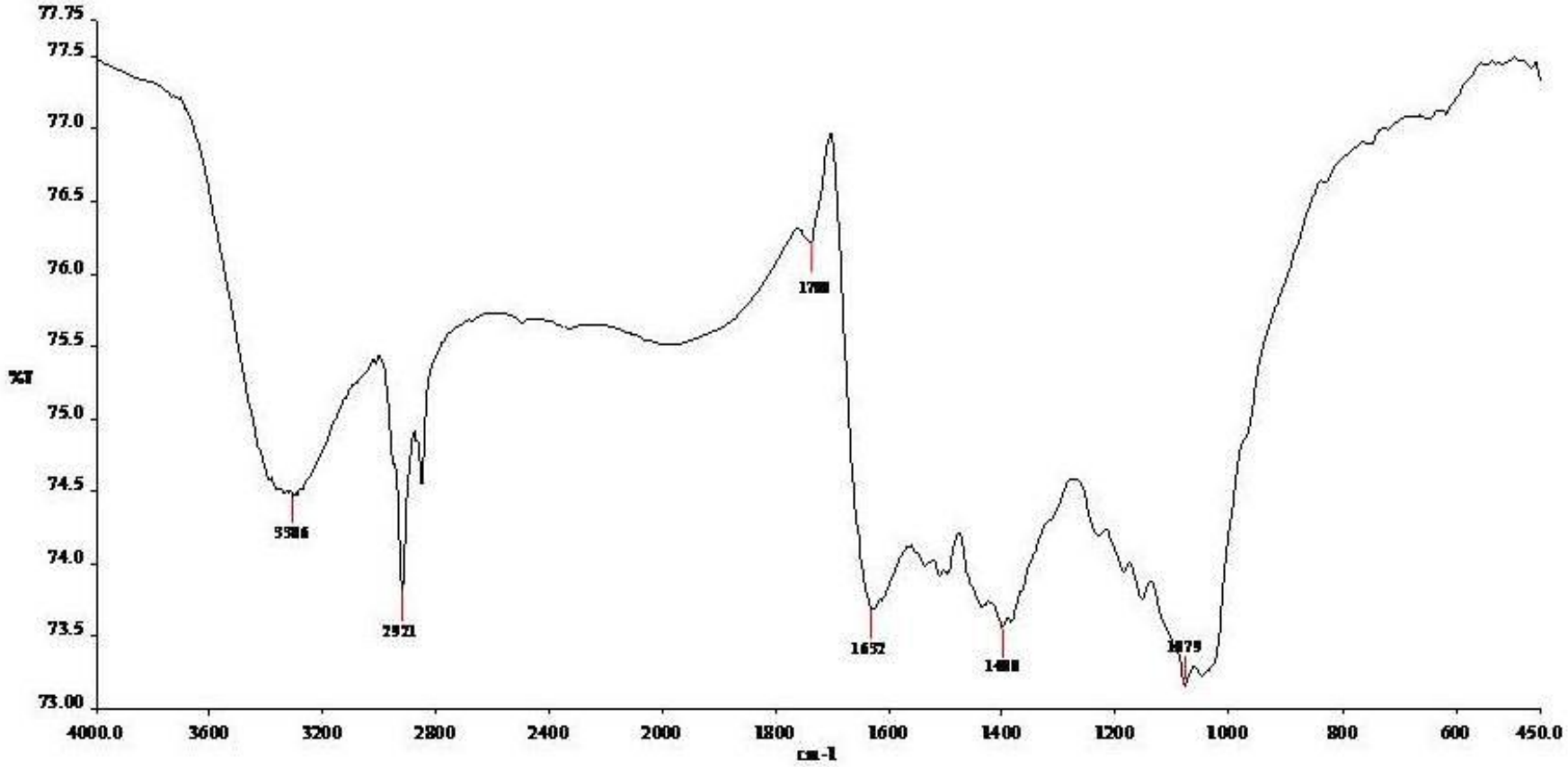


Fig. 45. IR spectrum of purified surface active compound produced by *Bacillus* sp.

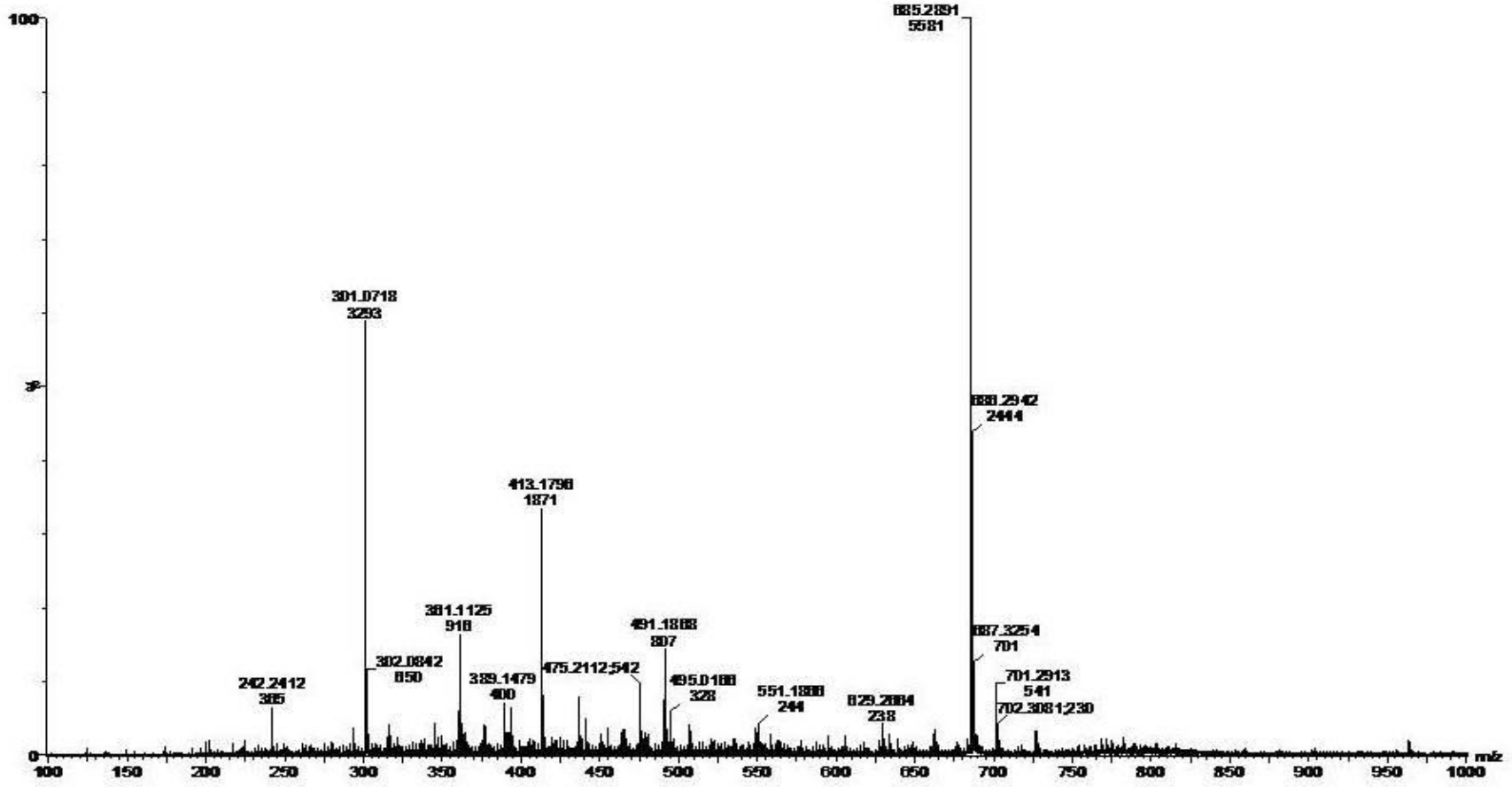


Fig. 46. MS Spectra obtained using Q ToF mass spectrometer of active compound of *Bacillus* sp. Y-axis: Relative abundance considering the highest abundant ion as 100%; X-axis: m/z for each ion.

followed by 686.29 m/z, 687.32 m/z and 701.29 m/z. Some low abundance ions were also observed at 702.30 m/z followed by 629.26 m/z, 663.21 m/z, 507.19 m/z and 551.18 m/z. Although, ToF MS is a qualitative not quantitative method. A product ion at m/z 301 formed due to loss of residue (112 Da). Other product ions are observed at m/z 242 due to loss of one residue (57 Da) and at m/z 413 due to loss of two residue ions (138 Da) with subsequently formation of ions at m/z 301 and m/z551 respectively. From mass spectra we can predict that presence of a alkyl group with benzene ring.

6.3.3 NMR Spectroscopic analysis

The ^{1}H NMR spectrum of the active purified compound displayed in Fig. 47(a) & 47(b). The different signals obtained were at δ 7.2624 (Aromatic H, may be four H). Signals of δ 2.17 (1H, s) and δ 2.03 (1H, s) may be due to presence amide (-NH) bond. Signals at δ 1.67 (1H, s), δ 1.56 (27H, s), δ 1.33-1.20 (7H, s) and δ 0.88-0.86 (2H, m) may be due to the presence of alkyl moieties, as these are maximum down fielded (Table-30).

Table-30: NMR Analysis data

Spectroscopic Analysis	Peaks	Characterisation
NMR spectroscopy	7.2624	Aromatic H, may be 4H
	2.17	1H, s
	2.03	1H, s may be due to presence amide (-NH) bond
	1.67	(1H, s),
	1.56	(27H, s)
	1.33-1.20	(7H, s)
	0.88-0.86	(2H, m) may be due to the presence of alkyl moieties

s: singlet; m: multiplet (denotes complex pattern for a single proton)

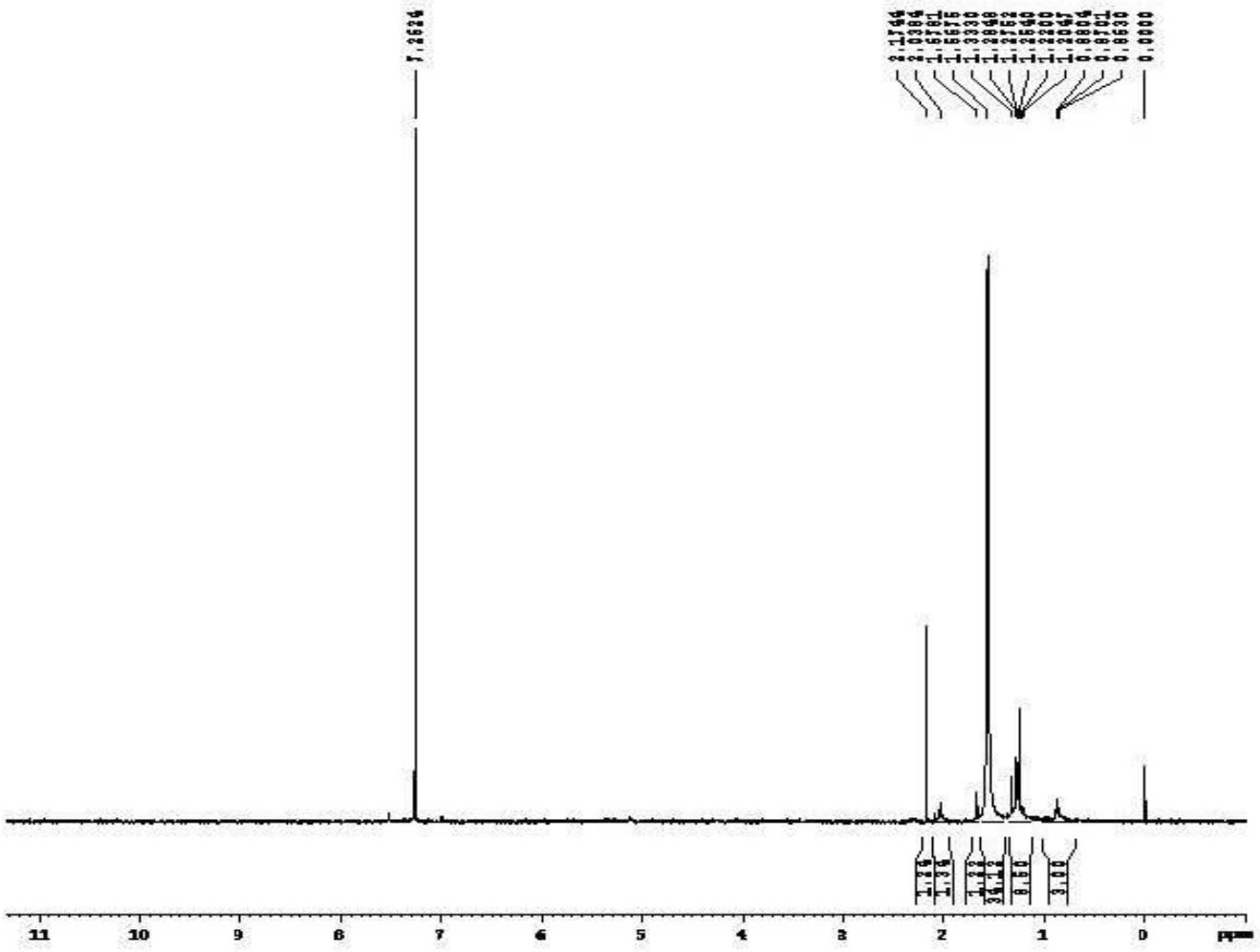


Fig. 47(a). ^{1}H-NMR spectrum for active purified compound

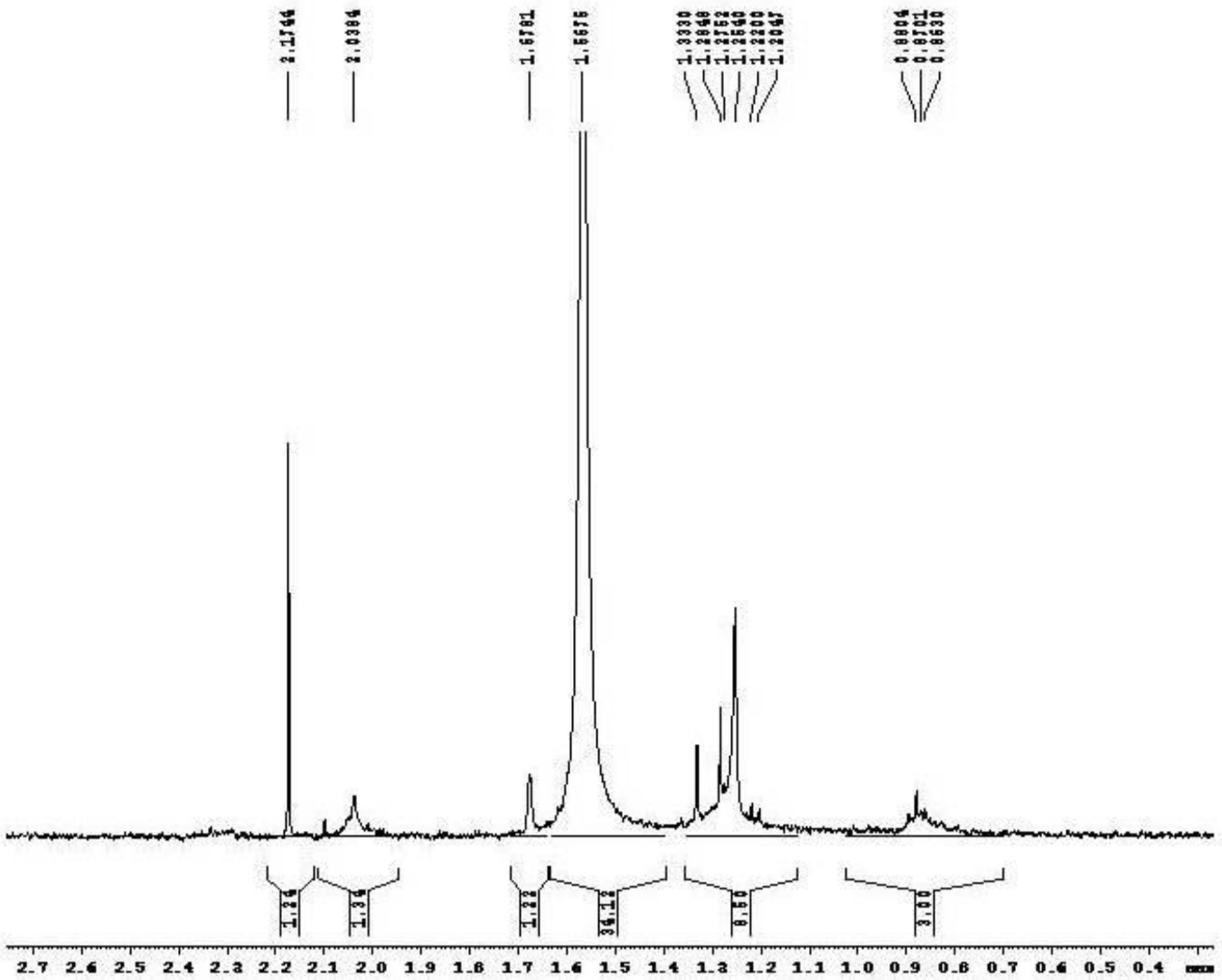


Fig. 47 (b). ^{1}H-NMR spectrum for active purified compound

6.3.4 UV spectrum

The active compound isolated from *Bacillus* sp. was subjected to UV spectral analysis which showed the absorbance peak at 230nm in ethanol (Fig. 48).

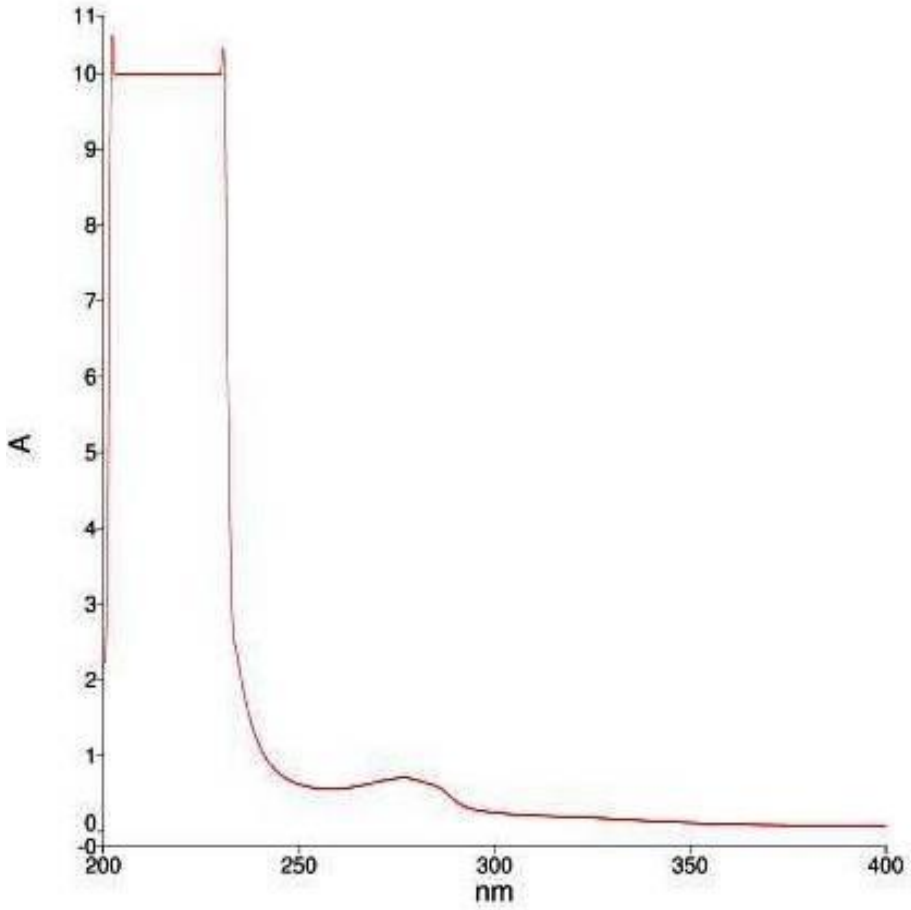


Fig. 48. Ultraviolet spectrum of the peptide antibiotic in ethanol

6.3.5. Elemental (CHN O) Analysis

Elemental analysis of *Bacillus* sp. active compound speaks about presence of nitrogen but at vicinity. The percentage of Hydrogen (H) is 11.19 so as Oxygen (O) and Carbon (C) as 3.72% and 84.72% respectively (Table-31).

Table-31: Elemental analysis data

Sample	N%	C%	H%	O%
1	0.37	84.72	11.19	3.72

6.4 Discussion

A broad peak was observed at 3306cm^{-1} which may be due to vibration of -OH or -NH molecule. It denotes that OH/NH molecules are in H-bonded condition. There is also a peak at 1632cm^{-1} which indicates the probable presence of Amide vibrations. Characteristic absorption valleys at 1,540; 1,650, and 3,300 cm^{-1} indicate that the antibiotic contains peptide bonds (María and Maldonado, 2012). This was also confirmed by Maldonado *et al.* (2009). There is a peak at 1400 cm^{-1} which indicates alkenes or free carboxylate ion. Das & Mukherjee (2005) optioned that the transmittance at 1,390cm^{-1} range may be due to the aliphatic chain of C–H group. The absorption at 1,024cm^{-1} indicated a C–O stretch which is similar to the experimental peak obtained at 1079 cm^{-1}. A lactone ring is suggested by the absorption at 1,740 cm-1 (Bechard *et al.*, 1998) as there is a peak at 1700cm^{-1}.

An MS spectrum of the active compound showed product ions at m/z 301 formed due to loss of residue (112 Da), m/z 242 due to loss of one residue (57 Da) and at m/z 413 due to loss of two residue ions (138 Da) identified the compound may be having the one benzene ring –Glycine(G)-Isolucine/Leucine(I/L)-Histidine(H)- Histidine(H). These fragmentation pathways allow identifying the molecular weight and tentative structure of the compound and their position. It may be cyclic in structure with one alkyl moiety.

^{1}H NMR spectrum was used to predict the structure of the active principle. The protons of the aromatic hydrogen appeared as a singlet at δ 7.2624. It is obvious that active principle has an aromatic ring. The two protons at δ 2.17 (1H) and δ 2.03 (1H) may be due to –NH protons. As predicted from the mass spectroscopy the active compound has peptide bonds. These two signals may be due to peptide NH of the molecules. Most of the protons appear downfield, this prove that the molecule has long chain alkyl moieties. Down fielded protons are also at different chemical shifts, denoting different adjoining environment of the hydrogen molecules. It is being predicted from this proton NMR that the active

compound consists of aromatic as well as long chain aliphatic groups with the presence of secondary amine in the form of peptides.

UV spectral analysis was carried out in ethanol for *Bacillus* sp. revealed the absorption maxima at 230nm which may represents the peptidic nature of the compound. The absorbance spectrum for the lipopeptide is measured in solvent between 200 and 600 nm. The antibiotic shows absorbance maxima at 235, 278, and 285 nm and there is no appreciable absorbance above 300 nm as determined by Bechard *et al.*, 1998.

Emperical formula based on elemental analysis of C, H, O and N as well as ^{1}H NMR data suggested that

C (30-50) H(48-75) N(1-3)

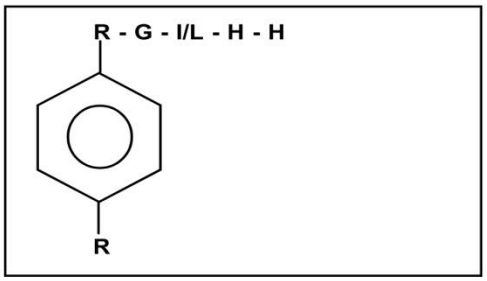


Fig. 49 Tentative structure of active compound

For further conformation the laboratory synthesis as well as comparison with the standard is required.

CHAPTER - 7

SUMMARY & CONCLUSION

CHAPTER 7

SUMMARY & CONCLUSION

7.1 Summary

Acclimatizing to extreme environment including adverse conditions like soil acidity is an adaptation by microorganisms as they originated and evolved in an uncongenial environment. This adaptation leads to production of some metabolites which help microbes to sustain vis-à-vis the society in benefied at a large. A vast tract of land not only in India but also around the globe is acidic in nature. The acid soils are sedimentary in nature belonging to lateritic (high clay Indian soil), ferruginous red and other red soil groups. The state of Odisha (20.11 to 20.85 latitude and 85.01 to 85.54 longitude) lies in the tropical belt in the eastern region of India contains 15.57 M ha geographical area, of which 8.67 M ha is acidic. Acid soils occupy about 30% of cultivated land in India, whereas 70% of the cultivated land in Odisha is acidic comprises the districts of Cuttack, Dhenkanal, Jajpur, and Nayagarh which having the soil pH in a range of 4.8 to 6.2. Acidophilic microbes sustaining in these areas are exposed to high acidity over years and adapt to the adverse environmental condition. The soil samples were collected and isolated. Out of a total of 48nos. of bacterial isolates on Gram's staining it was found that of 10nos. of isolates from Dhenkanal district were positive to gram's reaction and rods in shape, 5nos. of isolates from Cuttack district from which 2nos. were negative cocci and 3 were positive to gram's reaction leaving one rod and rest cocci. Out of 10nos. isolates from Jajpur 5 were positive and equally negative to gram's staining and of the 23nos of isolates from Nayagarh district of Odisha 1 colony showed cocci in shape while rest 22nos rods, a total of 2nos colonies were negative to gram's reaction leaving all others were positive.

All the bacterial isolates were screening of their antifungal potential by agar well diffusion method *in vitro* against some phytopathogens and dermatophytes. While two bacterial isolates from Dhenkanal and two from Nayagarh were able to inhibit almost all pathogens with various inhibitory zones. The bacteria isolated from Cuttack and Jajpur were failed to prove their potentiality against the pathogens. Maximum inhibition of *Candida albicans* MTCC 854 was produced by NOD-19. whereas the inhibition of growth by the isolates against phytopathogens viz. *Penicillium notatum*, *Aspergillus fumigatus* and *Fusarium* sp. were not good as compared to the dermatophytes *Trycophyton mentagrophytes*, *T. rubrum*, *Epidermophyton floccosum*, *Candida albicans* and *C. tropicalis*. The Four potent bacteria were biochemically characterized. DOD-1 and DOD-2 were identified as *Bacillus subtilis* and *Bacillus azotoformans* according to Bergey's manual of determinative bacteriology respectively while the Nayagarh isolates were identified as NOD-19 as *Bacillus amyloliquefaciens* (probability 82%) and NOD-14 as *Bacillus farraginis* (probability 80%) by ABIS Online software. Due to excellent antimycotic activity of the NOD-19 (*Bacillus* sp.) it was molecularly characterized and found as a bacterium with different 16S sequence.. One accession no. was assigned to it by NCBI, USA as KF056848. The Bacillus sp. showed maximum similarity with *B. amyloliquefaciens* BCL9 (JQ734537) and *B. amyloliquefaciens* BGP20 (JQ734535) isolated from soil samples and showed excellent antifungal activity against fungal pathogens.

The *Bacillus* sp. were mass cultivated in MSM medium for production of bioactive metabolite. The growth parameters were standardized like sugar composition of the medium, incubation period, pH for the production of metabolite etc. By the process of acid precipitation the metabolites were collected from the fermenting medium. The Crude ethanolic extract (CEE) was collected from the cell free metabolite and subjected to bioassay test to check the presence of antimycotic activity. The CEE was bioassayed in day interval. Chromatographic method was followed for purification and isolation of active compound from the active

metabolite. Loading of metabolites on silica gel column. Collection of elutes following polarity gradient based solvent extraction system and bioassay. Standardization of developer solvent was carried out and found that the ethanol (absolute) was the best. Purification of compound was done from the active elute by Thin Layer Chromatography. Single spot isolation of the active compound was done from the TLC plates having ethanol as developer solvent with Rf value 0.26. Mass collection of active compound was done by scrapping the spot as another plate was spotted and iodine reacted. The scrapped spot was again solubilized with ethanol and the supernatant was collected and allowed to evaporate. The active compound were weighed and stored in weighing bottle for molecular (spectral) analysis.

Spectral analysis of active compound of the *Bacillus* sp. isolate was out sourced. The compound was sent for the spectral analysis like FT-IR, Mass Spectra, NMR and UV-VIS to SAIF(Sophisticated Analysis and Instrumentation Facility), Panjab University, Chandigarh, Punjab and Elemental analysis to STIC(Sophisticated Test and Instrumentation Centre), CUSAT, Cochin, Kerala. The FT-IR peaks revealed that the compound may contain stretching vibration of -OH (3306 cm^{-1}), -C-H stretch vibration (2921 cm^{-1}), Amide -C=O vibration (1632 cm^{-1}), ether (C-O-C) stretching (1079 cm^{-1}), alkanes or free carboxylate ion (1400 cm^{-1}). The UV-VIS spectra shows strong absorption in the UV region (200-230nm) can be attributed to compound having peptidic in nature with antifungal properties. The MS analysis revealed that the antifungal compound isolated and purified from soil *Bacillus* sp. was having molecular weight of 702.30 Da. The compound may consist of one benzene ring along with one alkyl moiety. When the alkyl moiety was fragmented it may appears with the product ions of one Glycine, one residue of Isoleucine/Leucine and two residues of Histidine. The presence of aromatic ring was conformed from the ^{1}H NMR spectrum as a singlet at δ 7.2624. As the active compound has the peptide bonds which was predicted from the mass spectroscopy. There are two protons at δ 2.17 (1H) and δ 2.03 (1H) may be due to –NH protons.

These two signals may be due to peptide NH of the molecules. It is being predicted from ^{1}H NMR that the active compound consists of aromatic as well as long chain aliphatic groups with the presence of secondary amine in the form of peptides. Emperical formula based on elemental analysis as well as ^{1}H NMR data suggested that the compound may be cyclic peptide

C (30-50) H (48-75) N (1-3)

7.2 Conclusion

It is evident from the experimental observations that the acid soil of Odisha with special reference to the study sites contain bacterial diversity with novel properties. Bacteria isolated from one of the study site is having potential to inhibit growth of the human dermatophytes *Candida albicans* in vitro. This paves the path for selecting the bacteria for isolation, mass multiplication and production of bioactive metabolite. The chromatographic method leads to purification and isolation of active component from the metabolite which was exhibiting its solubility in organic solvents with higher polarity showing antimycotic activity against the predominant fungal pathogen, *C. albicans*. The spectral analysis data of the active compound leads some sings of cyclic peptide which are now the predominant class antibiotics. The exact structure of the compound requires laboratory synthesis of the compound and comparison with the standard. However at laboratory scale the compound is exhibiting excellent antimycotic activity in comparison to some locally available medicines recommended by physicians, it may be a source of antifungal agent after the development of formulation along with clinical trials which may helps the society in combating against dermatophytic infections. The work can explore biotic potential of acidophiles from the natural acid soil habitats which has not seen studied in depth and systematically.

Future line of work

1. Structural elucidation of the compound is need to be undertaken as the compound is need to be undertaken as the propose structure is a tentative one.

2. Clinical trial of the purified isolates from *Bacillus* sp. metabolites based active ingredient.

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APPENDICES

APPENDIX-I

	Reagents	Composition
1.	Methyl red indicator	Methyl red 0.1gm dissolved in 300ml ethanol and added to 200ml distilled water.
2.	α – napthol solution	5% α-napthol in absolute ethanol mixed with 1ml 40% KOH.
3.	Kovac's indole reagent	By dissolving 10gm of p-dimethyl aminobenzaldehyde in 150ml of isoamyl alcohol and then slowly adding 50ml of concentrated HCl.
4.	Sulphanilic acid	Sulphanilic acid 08gm Dissolve 8gm of sulfanilic acid in 1l 5 N acetic acid
5.	α –napthylamine solution	Dissolve 5gm of α-naphthylamine in 1l 5N acetic acid
6.	Hydrogen Peroxide (3%)	50% H_2O_2 (w/v) 0.6ml added to 9.4ml of distilled water

APPENDIX-II

1. Urea Agar Base

Ingredients	**gm/l**
Peptone	01.00
NaCl	05.00
K_2HPO_4	02.00
Glucose (sterilized separately)	01.00
Urea	20.00
Phenol red (filter sterilized)	0.012
Final pH (at 25°C) 6.8 ± 0.2	

2. Simmon's Citrate agar

Ingredients	**gm/l**
NaCl	05.00
$MgSO_4.7H_2O$	00.20
$NH_4H_2PO_4$	01.00
KH_2PO_4	01.00
Sodium Citrate	05.00
Bromothymol Blue	00.08
Agar	15.00
Final pH (at 25°C) 6.8 ± 0.2	

3. Potato Dextrose agar

Ingredients	**gm/l**
Potatoes infusion	200.00
From Dextrose	20.00
Final pH (at 25°C) 6.1 ± 0.2	

4. MR-VP Medium

Ingredients	gm/l
Peptone	05.00
Dextrose	05.00
K_2HPO_4	05.00
Final pH (at 25°C) 6.9 ± 0.2	

5. Nitrate broth

Ingredients	gm/l
Peptic digest of animal tissue	05.00
NaCl	30.00
Meat extract	03.00
KNO_3	01.00
Final pH (at 25°C) 7.0 ± 0.2	

6. Manitol Motility agar

Ingredients	gm/l
Peptic digest of animal tissue	20.00
Manitol	02.00
KNO_3	01.00
Phenol Red	00.04
Agar	02.00
Final pH (at 25°C) 7.6 ± 0.2	

7. Starch-NA Medium

Ingredients	gm/l
NB	40.25
Corn starch	10.00
Agar powder	02.00

Final pH (at 25°C) 7.6 ± 0.2

8. Skimmed Milk Agar

Ingredients	gm/l
Casein Enzyme hydrolysate	05.00
Yeast extract	02.50
Dextrose	01.00
Skim milk powder	28.00
Agar powder	02.00

Final pH (25°C) 7.0 ± 0.2

9. Bile-esculin medium

Ingredients	gm/l
Peptic digest of animal tissue	05.00
Beef extract	03.00
Oxgall	40.00
Ferric citrate	00.50
Agar	15.00

Final pH (at 25°C) 6.6 ± 0.2

12. Sabouraud Dextrose Agar

Ingredients	gm/l
Dextrose	40.00
Mycological, peptone	10.00
Agar	15.00

Final pH (at 25°C) 5.6 ± 0.2

8. Rose Bengal Chloramphenicol Agar

Ingredients	gm/l
Mycological peptone	05.00

Dextrose	10.00
KH_2PO_4	01.00
$MgSO_4$	00.50
Rose Bengal	00.05
Chloramphenicol	00.10
Agar	15.50

Final pH (at 25°C) 7.2 ± 0.2

13. OF Basal Medium

Ingredients	**gm/l**
Casein enzymic hydrolysate	02.00
NaCl	05.00
K_2HPO_4	00.30
Bromo thymol blue	00.08
Agar	02.00

Final pH (at 25°C) 6.8 ± 0.2

14. Nutrient Gelatin

Ingredients	**gm/l**
Peptic digest of animal tissue	05.00
Beef extract	03.00
Gelatin	120.00

Final pH (at 25°C) 6.8 ± 0.2

15. Mueller Hinton HiVeg Agar No. 2

Ingredients	**gm/l**
HiVeg hydrolysate	17.50
HiVeg infusion	02.00
Starch, soluble	01.50
Agar	17.00

Final pH (at 25°C) 7.3±0.2