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MOLECULAR DIVERSITY AND BIOTECHNOLOGICAL POTENTIAL OF HALO-TOLERANT AND HALOALKALIPHILIC ACTINOMYCETES FROM SALINE HABITATS ALONG THE COASTAL GUJARAT

A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

MICROBIOLOGY

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I take pleasure in forwarding the thesis entitled "MOLECULAR DIVERSITY AND BIOTECHNOLOGICAL POTENTIAL OF HALO-TOLERANT AND HALOALKALIPHILIC ACTINOMYCETES FROM SALINE HABITATS ALONG THE COASTAL GUJARAT" of Ms**. Sangeeta Gohel** for the acceptance of the degree of Doctor of Philosophy in **Microbiology**. Thesis presented here embodies records of original results and investigations carried out by her.

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Sangeeta

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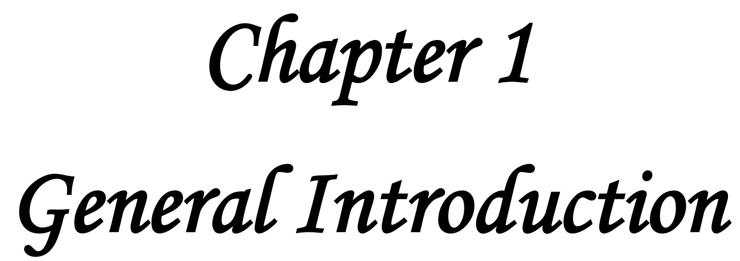

Chapter 1
General Introduction

The analyses of soil communities are usually performed through culture-independent DNA based methods. Among them, Terminal Restriction Fragment Length Polymorphism (T-RFLP) is an extensively explored tool used to analyze the genetic diversity of amplified 16S rRNA genes of a microbial community (Mengoni *et al.,* 2007, 2004; Berg *et al.,* 2005; Liu *et al.,* 1997; Dunbar *et al.,* 2001; Marsh, 1999). However, cultivation-independent approaches cannot provide insights into the phenotypes of the bacteria within the community, which are directly linked to community function and fitness with respect to environmental stressful conditions. In particular, salt resistance of bacteria, is an important parameter for assessing functionality of soil ecosystems in arid regions (Trabelsi *et al.,* 2009). Bacteria dwelling under extreme conditions might offer important research tool for investigating the relationships and interactions between environmental factors and microbial evolution at metabolic and gene level (Gould and Corry, 1980). Moreover, a recent metagenomic analysis found that the major environmental determinant of microbial community composition is salinity rather than extremes of temperature, pH, or other physical and chemical factors (Lozupone and Knight, 2007). Finally, bacteria from saline environments may be considered models for biological salt tolerance (Lanyi, 1979). These organisms have evolved in saline environments and are able to overcome the deleterious effects of salts up to saturating concentrations.

Although large number of new reports on bacteria and archea from extreme habitats are available, the extremophlic actinomycetes have been largely overlooked for their occurrence, phylogeny, diversity and biotechnological potential. It is, therefore, important to pay more attention toward extreme actinomycetes as a possible way to explore their diversity, distribution and new secondary metabolites. Actinomycetes are gram positive bacteria, with a high G+C ratio in their DNA (>55 mol %), which are phylogenetically related from the evidence of 16S ribosomal cataloguing and DNA:rRNA pairing studies (Goodfellow and Williams, 1983). The name “Actinomycetes” was derived from Greek “atkis” (a ray) and “mykes” (fungus), and has features of both bacteria and fungi. However, they are now generally considered to be more closely related to bacteria. The chemical composition of their cell wall is

similar to that of gram-positive bacteria but because of their well-developed morphological (hyphae) and cultural characteristics, actinomycetes have been considered as a group, well separated from other common bacteria. The studies on extremophilic actinomycetes started, when Gochnauer and coworkers (1975) isolated *Actinopolyspora halophila*, an extremely halophilic actinomycete. However, there was a long gap since then in pursuing the research on such organisms. It's only during the last several years that some reports on the commercial significance of novel metabolites obtained from these organisms have appeared in the literature (Xu *et al.,* 2006a; Imada, 2005; Li *et al.,* 2005c; Manam *et al.,* 2005).

Despite the advantages of soil actinomycetes in comparison with other bacteria, mycelial bacteria traditionally were not considered very resistant to extreme factors of the soil environment. A study of the ecological features of representatives from the order Actinomycetales showed that their ecological niches are extremely diverse. Specialists have no doubt about the probability of being existence of acidophilic and alkaliphilic, psychrophilic, thermophilic, halophilic, and haloalkaliphilic actinomycetes (Ventosa *et al.,* 1998; Jiang and Xu, 1993; Goodfellow and Williams, 1983). Study of soil actinomycetes having unusual salt, temperature, and pH requirements makes it possible not only to expand our knowledge about the diversity of the microbial world, but to reveal new organisms that can be used in the search for producers of biologically active substances (Zvyagintsev *et al.,* 2009). Halophilic actinomycetes were known for a long time only as contaminants of nonmycelial bacteria. However, halophilic forms *Actinopolyspora halophila* (Gochnauer *et al.,* 1975), *Nocardiopsis halophila, N. halotolerans, N. kunsanensis, N. tropica, N. trehalosi, N. dassonvillei* subsp. *albirubida, Streptimonospora salina* (Al-Tai and Ruan, 2001, 1994; Al-Zarban *et al.,* 2002a; Cui *et al.,* 2001; Chun *et al.,* 2000; Evtushenko *et al.,* 2000), *Prauserella halophila* and *P. alba* (Li *et al.,* 2003b) were described among the actinomycetes, which can grow in media containing 10–20% or even 15–25% NaCl. Actinomycetes isolated from saline soils and grown under moderate NaCl concentrations are among the small number of heterotrophic bacteria that synthesize compatible osmoregulatory substances glycine-betaine

(*Actinopolyspora halophila*) hydroxy-derivatives of beta-glutamate (*Nocardiopsis halophila*) (Ventosa *et al.,* 1998), the accumulation of which in a cell provides osmotic adaptation for this cell. The osmotic stress induces numerous cytoplasmatic effects, each of which might act as a signal for adaptive gene expression. It is probable that the main factors enhancing the gene expression are changes in the DNA topology and an increased level of the synthesis of the sigma factor responsible for the gene expression in dependence on the growth phase. It cannot be excluded that there are genes regulating the biosynthesis or transfer of specific osmosis-regulating substances (especially, potassium ion and betaine, trehalose, glycerin, saccharose, L-proline, D-mannitol, Dglucitol, L-taurine, ectoine, and small peptides). The high concentration of these substances ensures sufficient intracellular turgur pressure and protects enzymes from denaturation. Thus, the normal development of the main physiological capacity of proteins of halophilic organisms for water retention is 2 to 4 time higher than the analogous capacity of proteins of organisms adapted to media with lower osmotic pressure. Halophilic organisms are also characterized by the presence of specific enzymes and by a surface structure more adapted to the high osmotic pressure in comparison with osmosis-tolerant organisms.

It is indisputable that new drugs, notably antibiotics, are urgently needed to halt and reverse the relentless spread of antibiotic resistant pathogens which cause life threatening infections and risk undermining the viability of healthcare systems (Talbot *et al.,* 2006). Filamentous bacteria belonging to the order *Actinomycetales*, especially *Micromomospora* and *Streptomyces* strains, have a unique and proven capacity to produce novel antibiotics (Bentley *et al.,* 2002; Omura *et al.,* 2001; Watve *et al.,* 2001), hence the continued interest in screening such organisms for new bioactive metabolites is going on worldwide (Hong *et al.,* 2009; Berdy 2005; Lazzarini *et al.,* 2000). However, it is becoming increasingly difficult to discover commercially significant secondary metabolites from well known actinomycetes as this practice leads to the wasteful rediscovery of known bioactive compounds. Thus, it is critical that new groups of microbes from unexplored habitats be pursued as sources of novel antibiotics and other small-molecule therapeutic agents (Bull *et al.,*

2000) and thereby the demand emphasizing the need to isolate, characterize and screen representatives of undiscovered actinomycete taxa.

The most powerful approaches to taxonomy are through the study of nucleic acids. Because these are either direct gene products or the genes themselves and comparisons of nucleic acids yield considerable information about true relatedness. The genetic diversity of soil microorganisms is an indicator of the genetic resource, which is the basis of all actual and potential functions. Molecular tools have a great potential to identify microorganisms from unexplored habitats. One of these molecular tools is the PCR amplification of variable regions of the genes encoding 16S rRNA (16S rDNA) by use of species specific primers homologous to conserved regions of the gene. Subsequent electrophoretic separation of the PCR products in a polyacrylamide matrix over a denaturing gradient is a technique introduced in microbial ecology by Muyzer *et al* (1993). In contrast to patterns from other fingerprinting methods, TGGE and DGGE patterns offer the possibility to analyze bands of interest in depth by sequencing or probing (Muyzer *et al.,* 1998, 1993). In present study, we applied a direct approach, to analyze the fragments generated from actinomycete specific PCR on DGGE.

The recent discovery of novel primary and secondary metabolites from taxonomically unique populations of extremophilic actinomycetes suggested that these organisms add new dimensions to the microbial natural product research (Jensen *et al.,* 2005). Continued efforts to characterize halotolerant alkaliphilic actinomycete diversity and how adaptations to the extreme environment affect metabolite production will create a better understanding of the potential utility of these organisms as a source of useful products for biotechnology. To date, the findings and research on salt-tolerant and alkaliphilic actinomycetes is largely based on phylogeny and only limited attempts have been made to explore their enzymatic potential and other biotechnological implications. A great number of microbes belonging to archaea, bacteria, fungi and yeast are well known to produce alkaline proteases (Cannio *et al.,* 2010; Haddar *et al.,* 2009). However actinomycetes with salt tolerant and alkaliphilic nature are less

explored for the production of extracellular alkaline protease (Thumar and Singh, 2007). They hold significance in the field of enzymology, pharmaceuticals, degradation of biomolecules, food technology and microbial enhanced oil recovery (Starch *et al.,* 2005; Kampfer *et al.,* 2002; Abd-Allah, 2001; Horikoshi, 1999; Eltem and Ucar, 1998; Basaglia *et al.,* 1992; Richmond, 1990; Zajic and Spence, 1986). The enzymatic spectrum of such organisms, though not systematically explored, appears to be quite promising based on the preliminary indications. We explored haloalkaliphilic proteases from actinomycetes as proteases occupy a pivotal position with respect to their significance from physiological and commercial view points. However, there are many parameters involved in the selection of a good alkaline protease, such as activity and stability at washing pH, high salt, temperature, compatibility with detergent components like surfactants and oxidizing agents. The demand prompts us to explore new alkaline proteases with enhanced properties of actinomycetes isolated from coastal region of Gujarat, India. Therefore, capturing enzymatic potential of actinomycetes from saline and alkaline environments of coastal Gujarat would be of prime importance from ecological and biotechnological stand point.

In the light of above consideration recombinant DNA technology along with many other molecular biological techniques are used to improve and evolve enzymes leading to new opportunities for biocatalysts. Protein engineering and directed evolution provide approaches to improve enzyme stability and modify specificity in ways that may not exist in the natural world. Genes from the extremophiles often cloned and over expressed in domestic host systems to obtain large quantities of enzymes (Yan *et al.,* 2009; Ni *et al.,* 2009; Carolina *et al.,* 2008). However, cloning of extremophilic enzymes from salt tolerant alkaliphilic actnomycetes has focused considerable attention. Therefore, as an extension of our research, we planned to clone and express alkaline protease genes from salt tolerant alkaliphilic actinomycetes which could be potential source as a recombinant enzyme suitable for various industrial applications and may highlight on the regulation of gene expression in these organisms.

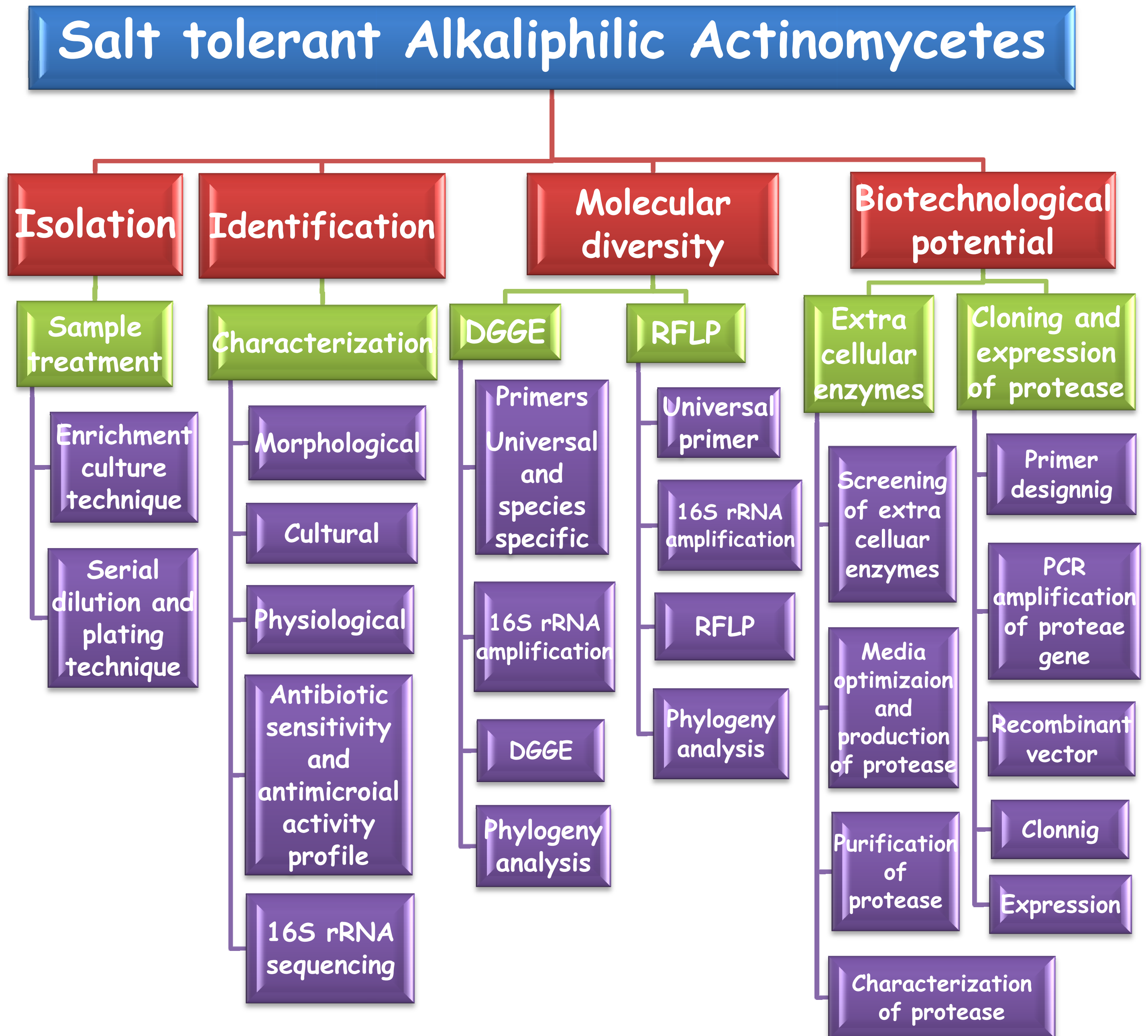
The halotolerant alkaliphilic actinomycetes are explored in limited senses. Therefore, we explored saline habitats of coastal Gujarat as a possible way to isolate taxonomically diverse groups of actinomycetes. Further, to study their unique enzymatic properties using thermodynamic parameters of purified proteases would further strengthen the data as thermodynamic properties of purified proteases are rarely reported in literature especially in actinomycetes. Besides, studies on antimicrobial potential of extreme actinomycetes as well as exploring the diversity of the isolates using different molecular techniques would lead to understand the unique prospects for molecular identification of acinomycetes at the genus level. Overall the present work highlighted occurrence, phylogeny and biotechnological potential of extreme actinomycetes from the saline habitats.

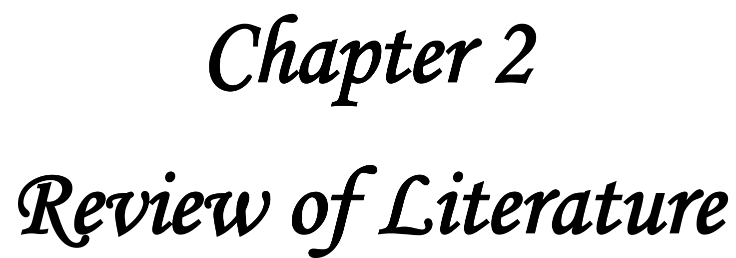
Objectives

- Isolation of salt tolerant alkaliphilic actinomycete from saline habitats along the coastal Gujarat
- To study morphological, cultural and biochemical properties of the isolated actinomycetes towards judging the diversity
- 16S rRNA gene sequencing and polygenetic analysis based on 16S rRNA gene sequence.
- Diversity based on antibiotic sensitivity profile and antimicrobial activity profile of actinomycetes
- PCR amplification of variable regions of the genes encoding 16S rRNA using universal and species specific primers homologous to conserved regions of the genes.
- Further analysis of the diversity among the isolated organisms by using denaturing gradient gel electrophoresis (DGGE) as well as restriction fragment length polymorphism (RFLP) of the amplified 16S rRNA gene.

- Detection and screening of extra cellular enzymes (e.g. protease, amylase, cellulase, lipase etc) among the salt tolerant alkaliphilic actinomycetes obtained from saline environment.
- Optimizing media components and characterization of crude and partially purified alkaline proteases
- Studies on the catabolite and nitrogen repression of proteases
- Purification and characterization of the potent alkaline proteases and their comparative studies with respect to enzyme stability, thermodynamics, kinetic behavior and protein denaturation
- Cloning and expression of alkaline protease genes into mesophilic host

Entire spectrum of work




Chapter 2
Review of Literature

2.1 Extremophiles

A major thrust that has been driven extensive and intensive research efforts on extremophiles during last few decades is the potential biotechnological applications associated with microbes and their products. The ever increasing uses of the extremozymes in the industries have converted them into bioindustries. This is mainly due to the discovery of novel enzymes from extremophilic microorganisms. Due to the unusual and unique properties, these extremozymes are expected to fill the gap between biological and chemical processes. Recent developments clearly show that extremophiles could be good source of novel catalysts of industrial interest. However, the ability to withstand the rigorous environments is not sufficient for commercial success. In additions, number of other factors must also be considered and investigated.

2.2 Extremophiles and extreme environment

Extremophiles have been categorized in to various groups on the basis of their survival under different extremities. They grow optimally in some of earth's most hostile environments of temperature (-2ºC to 15ºC; **Psychrophiles**; and 60ºC to 115ºC; **Thermophiles**), salinity (2-5M NaCl; **Halophiles**), pH (<4 **Acidophiles** and >9; **Alkaliphiles**), anaerobicity (**Methanogens**), and/or pressure (**Barophiles**). Ultra extreme environments are generally stable and allow growth of only extremophiles while an extreme environment are periodic and sometimes supports the growth of mesophiles as well. The groups of organisms capable of growing at moderate salt concentration under alkaline conditions are known as halo-tolerant alkaliphilic organisms. They can be isolated from the carbonate rich springs and alkaline soils, Soda lakes, sea water and man made saline and hyper alkaline environments.

2.3 Saline and alkaline soils

Saline soils appear to yield mostly halotolerant rather than halophilic microorganisms, arguably reflecting adaptation to periodic changes of dilution (Quesada *et al.*, 1990; Quesada *et al.*, 1985). However, isolation of novel halophilic

Actinopolyspora and *Nocardiopsis* species from salty soils in Death Valley (Calif.), Alicante, and Iraq (Yorkovsky and Silver 1997; Al-Tai and Ruan 1994) suggests that a wealth of interesting unknown halophilic microorganisms may be present in such saline soils. Similarly, many actinobacterial species have been isolated from soil collected from Xinjiang, China including *Nocardiopsis xinjiangensis* (Li *et al*., 2003a); *Prauserella halophila* and *Prauserella alba* (Li *et al.,* 2003b) and *Streptomonospora alba* (Li *et al*., 2003c). Similarly, *Saccharomonospora halophila,* able to grow at 20% salt, was isolated from Marsh soil of Kuwait (Al-Zarban *et al*., 2002).

2.4 Actinomycetes - the diverse group of bacteria

Gram-positive bacteria with a high mole % G + C composition (55%) are grouped as the actinomycetes. These bacteria typically exhibit filamentous growth and many also produce spores. The filaments they produce are much narrower than fungal hyphae and, like all bacteria, are composed of prokaryotic (non-nucleated) cells. Some bacteria of this group, such as some species of *Nocardia*, exhibit only a limited degree of filamentous growth followed by fragmentation. Actinomycetes exhibit extensive cellular differentiation than most other bacteria. Growth of actinomycetes is very different than the progress of cell enlargement and binary fission that occurs in unicellular bacteria. Vegetative hyphae grow into long, often branched masses of tangled filaments without undergoing cell division, finally producing long cells with multiple copies of the genome. Separation of cytoplasm and bacterial chromosome occurs sporadically and without apparent pattern. Reproduction occurs by several mechanisms of fragmentation of hyphae to form haploid spherical cells in the non spore-forming genera such has *Nocardia* or by differentiation of the fragments to spores in other genera. The genomes of actinomycetes, which may be circular or linear in diverse species, are large. In addition, there are various plasmids, which exist in a wide range of sizes and copy numbers. Most commonly, actinomycetes have GC-rich plasmids with 10 - 40 Kb size and copy numbers less than 30. Actinomycetes are physiologically diverse bacteria, as evident by their production of numerous extra

cellular enzymes and by the thousands of metabolic products they synthesize and excrete. Actinomycetes are the major antibiotic producers in the pharmaceutical industry (Manam *et al*., 2005). While the production of antibiotics is of great benefit in medicine, some actinomycetes are pathogens and some cause allergic reactions (Kageyama *et al*., 2004a; Kageyama *et al*., 2004b; Conville *et al*., 2004; Palmer *et al*., 2003). In nature, biodegradation by actinomycetes plays an extremely useful role in waste removal and is an integral part of the recycling of materials. Most actinomycetes live in aerobic soils, where they biodegrade organic substrates and decompose lignocellulose from plant residues. They secret extra cellular enzymes such as cellulases, xylanases, amylases, proteases and ligninases (Starch and Bull, 2005; Moreira *et al.,* 2001) that degrade the complex macromolecule substrates commonly found in soils.

2.5 Extremophilic actinomycetes

Waksman (1923) gave a descriptive account on the existence of actinomycetes in Bergey's Manual of Determinative Bacteriology and contributed significantly to the nomenclature as well as classification of actinomycetes. Till date, thousands of new species have been isolated in different genera of normal mesophilic actinomycetes, while in comparison, extremophilic actinomycetes are largely overlooked for their occurrence, phylogeny, diversity and biotechnological potential. The studies on extremophilic actinomycetes started, when Gochnauer and coworkers (1975) isolated *Actinopolyspora halophila*, gen. et. sp. nov., an extremely halophilic actinomycete. However, there was a long gap since then in pursuing the research on such organisms and it's only during the last 5-6 years, that some reports on the commercial significance of novel metabolites obtained from these organisms (Xu *et al*., 2006a; Imada, 2005; Li *et al*., 2005c; Manam *et al*., 2005) have appeared in the literature.

2.6 Taxonomy and diversity of halophilic and halo-tolerant actinomycetes

One of the most fascinating and attractive aspects of the microbial world is its extraordinary diversity. The diversity of an ecosystem is dependent on the physical

characteristics of the environment, the diversity of species present, and the interactions among the species and the environment. Environmental disturbance on a variety of temporal and spatial scales can affect the species richness and, consequently, the diversity of an ecosystem. Currently microbial diversity is in ferment because of the use of new molecular techniques in classifying microorganisms. Even these new advances have generated much excitement and are drastically changing microbial taxonomy, more traditional approaches still have value.

The diversity of Halophilic and halotolerant microorganisms is expressed both at the phylogenetic and at the physiological level; Halophiles are found in all the 3 domains of life: Archaea, Bacteria, and Eucarya. Most of the energy generation modes known in non-halophiles are also used by halophilic counterparts. Extreme halophiles are not the only representative of the group Archaea. Instead, they are also found among the *Cyanobacteria* (Oren, 2000), the Flavobacterium – Cytophaga branch, the spirochetes, and the actinomycetes. However, archaea dominate among the halophilic populations. According to the clone libraries, the archaeal assemblage was dominated by microorganisms related to the cosmopolitan square archaeon "Haloquadra walsbyi," although a substantial number of the sequences in the libraries (31% of the gene archaeal clones) were related to Halobacterium sp., which is not normally found in clone libraries from solar salterns. Several extremely halophilic coccoid archaeal strains of genus *Halococcus* were isolated from pieces of dry rock salt obtained 3 days after blasting operations in an Austrian salt mine (Stan-Lotter *et al*., 2000).

The halophilic actinomycetes, like all the actinobacteria, are difficult to isolate from the saline and marine environment. However, their ecological role in the marine ecosystem is largely neglected and various assumptions meant there was little incentive to isolate strains for search and discovery of new drugs. However, as a recent trend, the saline habitats have become a prime resource in search and discovery for novel natural products and biological diversity, and marine actinomycetes turn out to be important contributors. Similarly, striking advances have also been made in

marine microbial ecology using molecular techniques and metagenomics, and certainly actinomycetes emerged as an often significant, sometimes even more dominant (Ward and Bora, 2006). Both approaches - cultivation methods and molecular techniques are leading to new insights into marine actinobacterial biodiversity and biogeography. Different views on actinobacterial diversity emerged from these approaches, however, are still to be analyzed further since their biogeography are still not clear.

2.7 Adaptations to grow at high salt concentration

Osmosis, the physical process by which water moves from a region of high concentration to a region of low concentration, is one of the first fundamental processes encountered in Biology classes. Water is fundamental for life, but it is also important to balance its content critical to being alive. Organisms manage their water content differently depending on the environment they live in, as terrestrial organisms have evolved a mean to prevent dehydration, organisms living in fresh water have developed strategies to counteract the osmotic pressure that forces water inside their cells, while marine organisms fight against the reverse tendency. Sea water is about 3% NaCl solution and halophilic organisms can live in salt solutions even more concentrated than that. The optimal growing conditions for some, called extreme/hyper halophiles, are nearly saturated salt solutions (about 35% salts).

Most halophiles are highly adapted both externally and internally to combat the osmotic pressure of salty solutions and to live in this extreme environment. An interesting group of extreme halophiles actually use an ion pump to actively maintain a high concentration of potassium ions (K+) in their cytoplasm. Halophiles live in approximately 4M NaCl containing much lower amounts of K^+, Mg^{2+} and accumulate K^+ inside and keep the Na^+ outside (Oren *et al.,* 2002). The H^+ gradient is used for either the excretion of Na^+ or ATP formation (Roberts, 2004; Lanyi, 1993). The enzymes mediating internal processes in halophilic organisms have high salt tolerance (Muller and Oren, 2003) and may be completely inactivated in its absence.

2.8 Cell wall of halophiles

Halophilic organisms also adapt externally by modifying the composition of their cell wall as compared to those adapted to more conventional environments. For example, the proteins that are found in the cell walls of *Halobacteria* tend to be negatively charged and stabilized by attracting the abundant positive sodium ions (Na^+) of the salty water. In fact, an extremely high concentration of sodium ions is required for the integrity of the cell wall. In lower salt concentrations, there are insufficient sodium ions and the cell walls of these highly adapted organisms, therefore, become structurally unstable and hence they are susceptible to break down.

2.9 Lipids of halophiles

The cytoplasmic membranes of halophiles contain unusual lipids, made up from C5 isoprenoid units (as for the side chains of ubiquinone) rather than C2 units as in normal fatty acids. The major components are the diphytanyl ether analogs of phosphatidyl glycerol (4%) phosphatidyl glycerol phosphate (65%), phosphatidyl glycerol sulfate (4%), and a 3-sugar glycolipid sulfate (25%). Neutral lipids make up around 10% of the lipids and include squalene and its derivatives (C30), menaquinone-8, carotenoids (C40), bacterioruberins (C50 analogs of carotenoids) and diphytanyl-glycerol - all isoprenoid derivatives. The isoprenoid lipid chains of 40-50 carbon atoms can stretch across the whole membrane. Bacterioruberin has hydroxyl groups both inside and outside of the membrane. Some halophiles do contain a vestigial fatty acid synthetase which is strongly inhibited by high NaCl concentrations. In contrast the mevalonate pathway for isoprenoid synthesis is dependent on high salt concentrations (4M).

2.10 Compatible solutes

Some halophiles tend to maintain a highly concentrated internal environment by accumulating compatible solutes (sugars, alcohols, glycine, betaine, ectoines, glycerol and amino acids) in their cytoplasm. They are polar, highly soluble molecules and uncharged or zwitterionic at physiological pH. They are strong water structure

formers and probably excluded from the hydration shell of proteins. Compatible solutes display a general stabilizing effect by preventing the unfolding and denaturation of proteins caused by heating, freezing, and drying (Madern and Zakai, 2004; Nyyssola and Leisola, 2001). Haloalkaliphilic archaea *Natronobacterium* species accumulates a novel osmolyte, 2-sulfotrehalose for osmotic balance (Desmarais *et al.,* 1997). Most halophilic bacteria such as *H. elongata* and obligate halophilic archaeon *Methanohalophilus portucalensis* accumulate glycine betaine from the enviornment, and found as the sole or main osmotic solute (Lai *et al.,* 1999). However, certain halophilic and moderate halophiles can use glycine betaine as a carbon and energy source along with an osmotic stabilizer (Madern *et al.,* 2004). The extremely halophilic actinomycete *Actinopolyspora halophila* is a rare example of a heterotrophic eubacterium producing betaine from simple carbon sources (Nyyssola and Leisola, 2001). *Actinopolyspora halophila* synthesizes remarkably high intracellular concentrations of betaine. The highest betaine concentration, determined at 24% (w/v) NaCl, is around 33% of the cellular dry weight. *Actinopolyspora halophila* synthesizes trehalose as a compatible solute, accounting for up to 9.7% of the cellular dry weight. The betaine concentration increases with increasing NaCl concentration, whereas the trehalose concentration attains highest level at the lowest NaCl concentration (15% w/v). *Actinopolyspora halophila* is capable of accumulating betaine from the medium, while at the same time betaine was also excreted back into the medium by the cells. Along with the de novo synthesis of betaine, *Actinopolyspora halophila* can take up choline from the medium and oxidize it to betaine.

2.11 Diversity of alkaliphilic actinomycetes

The term “alkaliphile” is used for microorganisms that grow optimally or very well at pH above 9 but cannot grow or grow only slowly at the near-neutral pH value of 6.5. Alkaliphiles include prokaryotes, eukaryotes, and archaea. Many different taxa are represented among the alkaliphiles, and some of these have been proposed as new taxa (Horikoshi, 1999). Alkaliphiles can be isolated from normal environments such

as garden soil, although the number of alkaliphiles is higher in samples from alkaline environments. The cell surface may play a key role in keeping the intracellular pH value in the range between 7 and 8.5, allowing alkaliphiles to thrive in alkaline environments, although adaptation mechanisms have not yet been clarified. It is believed that the actinomycete complex of alkaline soils is dominated by many novel genera of actinomycetes including S*treptomyces*, which showed maximal radial rates of colony growth at pH 9-10. The research on actinomycetes surviving under extreme environments is still limited. However, alkaliphilic actinomycetes have gained considerable attention in recent years. There is tremendous diversity and novelty among the alkaliphilic actinomycetes present in alkaline habitats including soda lake and desert soils (Hozzein *et al*., 2004; Al-Zarban *et al*., 2002a, b).

2.11.1 Molecular diversity

DNA is a molecule responsible for preserving genetic information across species and across time. It consists of a meaningful arrangement of chemicals called nucleotides that are symbolized by “A”, “T”, “C” and “G.” These arrangements tell us a story of each organism or individual, in that the code they produce, represent a detailed instruction book for that organism or individual. Any change introduced in this sequence is called a mutation, and whilst mutations occur randomly, many endure as an organism “acclimatizes” to a new environment. Hence, observing and understanding these mutations would undoubtedly improve our understanding of residual and transient organisms in a variety of environments when observation is difficult. For instance, this would be useful for the monitoring of microbial populations, where culture dependant methods fall short.

2.11.2 16S rRNA PCR amplification

One of these molecular tools is the PCR amplification of variable regions of the genes encoding 16S rRNA (16S rDNA) by use of primers homologous to conserved regions of the gene. Initially, the analysis of the diversity of natural microbial populations relied on direct extraction, purification, and sequencing of 5S rRNA molecules from

environmental samples. Although these studies yielded interesting insights, the information content of the 120 nucleotides long 5S rRNA is relatively small, and its paucity of independently varying nucleotide positions limits its usefulness to less complex ecosystems. An average bacterial 16S rRNA molecule has a length of approximately 1500 nucleotides and thus contains considerably more information for reliable analyses than the 5S rRNA molecule. Consequently, the use of the larger rRNA molecules for studies in microbial ecology was suggested. In addition, the development of robust DNA cloning techniques and the polymerase chain reaction (PCR) have facilitated higher resolution analyses of more complex communities using 16S rRNA sequence analysis.

The 16S rRNA gene has been widely used for phylogenetic and diversity studies for several reasons. It consists of conserved and variable regions, which allows the development of primers and probes with variable levels of specificity. The conserved regions carry information about phylogenies at the higher taxonomic levels, since they have evolved slowly and are highly similar among the different taxa, whereas the variable regions have undergone more mutations during evolution, and are more useful for classification at the interspecies level. The rRNA genes are essential, and therefore present in all organisms. Mehling *et al* (1995) used 16S rDNA sequencing to determine those regions suitable for detection of *Streptomycetes*, and proposed a genus-specific probe and primers targeting the 16S rRNA gene. Selective restriction of the *Streptomyces* 16S rRNA genes via the suicide polymerase endonuclease restriction PCR method was employed to remove the amended DNA (Stefan, 2005). PCR-based detection methods are culture-independent and potentially more sensitive than culturing, and thus, can provide better tools for exposure assessment (Zhou *et al.*, 2000). PCR based methods for detection of some indoor microbes, though not for *Streptomycetes*, has been reported (Cruz-Perez *et al.*, 2001b; Zhou *et al.*, 2000).

The sequence analysis of the genes coding for the ribosomal subunits (16S, 23S, and 5S rRNA), in particular the 16S rRNA gene has become an important tool in bacterial identification, since it provides information about the phylogenetic placement of

species (Brenner *et al*., 2001). The DNA sequences of the ribosomal genes are highly conserved, but the genes also contain variable regions, which sometimes can be useful for species discrimination (Rossello-Mora and Amann, 2001). However, the 16S rDNA sequence information alone is not sufficient for species identification (Rosselló-Mora and Amann, 2001). Also repetitive intergenic DNA sequences (rep-PCR) and PCR-RFLP of the 65-kDa heat shock protein gene have been used for the classification and identification of pathogenic and other clinically important *Streptomyces* species (Rintala, 2002).

It has been reported that up to 32% of PCR products may be chimeric (Wang and Yang 1995). This is a problem especially with the highly conserved ribosomal genes. PCR based methods for the detection and identification of microbes are widely used in clinical microbiology and food hygiene. Applications in environmental microbiology, especially in the soil environment are also increasing. A few methods have been published for applications in indoor environments, aiming at the detection of major fungal species, such as *Stachybotrys chartarum* and *Aspergillus fumigatus* (Zhou *et al*., 2000).

Antony-Babu *et al* (2008) reported that Twenty-four isolates representing the colour and rep-PCR groups grew well from pH 5 to 11, and optimally at pH 9, as did phylogenetically close members of the *Streptomyces griseus* 16S rRNA gene clade. One hundred and twelve representative alkaliphilic *streptomycetes* formed a heterogeneous but distinct clade in the *Streptomyces* 16S rRNA gene tree. An analysis of complete 16S rRNA gene sequences demonstrated that the two strains analyzed in detail are most closely related to actinobacteria in the *Thermomonosporaceae* and the *Micromonosporaceae* (Valdes *et al*., 2005).

Analysis of 16S rDNA of A novel alkaliphilic actinomycetes strain TOA-producing PrpSc-degrading keratinase indicated that the strain belonged to the genus *Nocardiopsis*, but genetically differed from the other *Nocardiopsis* species (Mitsuiki *et al.,* 2007). The nucleotide sequence of the 16S rRNA gene (1.5 kb) of the most

potent strain evidenced a 99% similarity with *Streptomyces* spp. and *S. aureofaciens* 16S rRNA genes, and the isolated strain was ultimately identified as *Streptomyces* sp. MAR01 (Moustafa *et al*., 2006). The analysis of the nucleotide sequence of the 16S rRNA gene (1480 bp) of the isolate indicated that this strain is identical to *Streptomyces violaceusniger* (accession number EF063682) and then designated *S. violaceusniger* strain HAL64 (Moustafa *et al.,* 2007).

Thirteen isolates of *Nocardia asteroides* from both soils and aquatic samples (lake and moat sediments, as well as scum from activated sludge), together with a type strain and two known clinical isolates of this species, were characterized by repetitive extragenic palindromic - PCR fingerprinting with the BOX-A1R primer. Analysis of the 16S rRNA sequence of strain SK4-6 showed a high similarity, 99%, with S. qinlingensis (Laidi *et al*., 2008). The 16S rRNA gene of this aerobic, gram-positive, mycelium- and spore-forming microorganism was amplified, and molecular phylogenetic analysis of the DNA sequence showed less than 93% similarity with its closest relative, indicating differentiation at the genus level (Saha *et al*., 2005). Analysis of the 16S rDNA sequence indicated that strain P1B belongs to the genus *Desulfovibrio*, with *Desulfovibrio halophilus* as its closest relative. Based on physiological properties strain P1B could not be assigned to this species. Therefore, a new species, *Desulfovibrio oxyclinae*, was proposed (Daniel *et al*., 1997).

2.11.3 Denaturing gradient gel electrophoresis

The search for sequence variations in genomic DNA becomes increasingly important in the study of inherited disease genes as well as of genes that play a role in the development of cancer. A wide variety of different methods to detect DNA sequence variations has been developed during the past few years. One of these methods, denaturing gradient gel electrophoresis (DGGE), has been shown to be very sensitive and the method of choice in studying mutations in large genes. The detection of mutations by DGGE is based on the sequence-dependent electrophoretic mobility of double stranded DNA fragments in a polyacrylamide gel that contains a linear

denaturing gradient. A key requirement of DGGE is that the DNA fragment of interest is composed of at least two melting domains (blocks of sequence with a discrete melting temperature, or Tm. DGGE involves electrophoresis of double-stranded DNA fragments through a polyacrylamide gel containing a linear gradient of DNA-denaturing agents (e.g., a combination of formamide and urea) at a fixed temperature (usually 60°c). Initially, the migration rate of the fragment depends on its molecular weight. However, at a specific point in the gel, the combination of denaturant concentration and temperature equals the Tm of the lowest melting domain, resulting in a partially single-stranded fragment. The mobility of these branched fragments in the polyacrylamide gel is abruptly retarded. The fact that the Tm for a given domain is determined by its sequence and base composition means that two DNA fragments that differ by a single base change (and thus in Tm) in the lowest melting domain will be separated from each other at the end of the run. In DGGE (Myers *et al.,* 1987; Fischer and Lerman, 1979) as well as in TGGE (Riesner *et al*., 1989; Rosenbaum and Riesner, 1987), DNA fragments of the same length but with different sequences can be separated. Separation is based on the decreased electrophoretic mobility of the single stranded DNA (Muyzer *et al*., 1998).

Different hypervariable (V) regions of the archaeal 16S rRNA gene (rrs) were compared systematically to establish a preferred V region(s) for use in Archaea-specific PCR-denaturing gradient gel electrophoresis (DGGE) (Yu*, et al*., 2008). Using this approach, members or close relatives of the genera *Halomonas*, *Clostridium*, and *Frankia* were identified (Rolleke *et al*., 1996). DGGE analysis of the amplified fragments distinguished *Colletotrichum circinans from Colletotrichum coccodes* isolates. This result provides molecular evidence that supports the current treatment of *C. circinans* as a species distinct from *C. coccodes* (Fagbola *et al*., 2004).

PCR amplification of 16S ribosomal DNA fragments from the co-culture, analyzed by DGGE, resulted in two distinct 16S ribosomal DNA bands, indicating two different bacterial components. Sequencing showed that the bands were derived from a *Desulfovibrio* strain and an *Arcobacter* strain (Teske *et al*., 1996). DGGE profiles

displayed no differences between them. In contrast, the comparison of band patterns of endocytic bacteria and free-living marine bacteria were different, indicating the development of a specific bacterial population within *N. scintillans* (Seibold *et al*., 2001). The 16S rDNA fragments of uniform length obtained from the different bacterial species were separated according to their sequence differences by DGGE. By sequencing excised and re-amplified individual DNA bands, the phylogenetic affiliations of the corresponding bacteria were characterized (Rolleke *et al*., 1996).

Some of the major disadvantages or limitations of conventional culture-dependent analysis, in terms of speed, detection level, taxonomic resolution and reproducibility can be overcome through culture-independent approaches. Sequence-dependent electrophoresis (SDE) techniques like Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Eletrophoresis (TGGE) are molecular tools that have been used to study microbial popu-lations in aquatic and terrestrial environments, food fermentations, gastro-intestinal tracts etc. The generated population fingerprints provide information on the microbial diversity and can also be used in assessing stability of microbial populations.

Denaturing gradient gel electrophoresis has been shown to detect differences in the melting behavior of small DNA fragments (200-700bp) that differ by as little as a single base substitution. When a DNA fragment is subjected to an increasingly denaturing physical environment, it partially melts. As the denaturing conditions become more extreme, the partially melted fragment completely dissociates into single strands. Rather than partially melting in a continuous zipper-like manner, most fragments melt in a step-wise process. Discrete portions or domains of the fragment suddenly become single-stranded within a very narrow range of denaturing conditions. The rate of mobility of DNA fragments in acrylamide gels changes as a consequence of the physical shape of the fragment. Partially melted fragments migrate much more slowly during electrophoresis through the polyacrylamide matrix than completely double-stranded fragments. When a double-stranded fragment is electrophoreses into a gradient of increasingly denaturing conditions, it partially melts

and undergoes a sharp reduction in mobility because it changes shape. In practice, the denaturants used are heat (a constant temperature of 60°C) and a fixed ratio of formamide (ranging from 0-40%) and urea (ranging from 0-7 M). The position in the gradient where a domain of a DNA fragment melts and thus nearly stops migrating is dependent on the nucleotide sequence in the melted region. Sequence differences in otherwise identical fragments often cause them to partially melt at different positions in the gradient and therefore 'stop' at different positions in the gel. By comparing the melting behavior of the polymorphic DNA fragments side-by side on denaturing gradient gels, it is possible to detect fragments that have mutations in the first melting domain.

Many fragments can be analyzed simultaneously on a single denaturing gel in which the direction of electrophoresis is perpendicular to that of the denaturing gradient. When a large number of different fragments is electrophoresed, the fragments can be identified by their molecular weight in the low denaturant side of the gel. By following the S-shaped curves, the characteristic denaturant concentration at which the first domain melts can be determined. When two nearly identical sets of fragments are mixed together and electrophoresed into a 'perpendicular' denaturing gradient gel, the melted domains that have sequence differences between each other will melt at slightly different positions and produce double bands.

Sequence differences are often easily detected in DNA fragments when nearly identical digests are electrophoresed in the same direction as that of the denaturing gradient. These 'parallel' gels permit the simultaneous comparison of as many sets of fragments as there are lanes on the gel, unlike the perpendicular gels. The procedures below refer almost entirely to parallel denaturing gradient gels.

Gradient Gel Electrophoresis (DGGE) separates mixtures of amplified 16SrRNA gene segments, which are all the same size, based on nucleotide sequence. *Denaturing* - breaking apart the two strands of the DNA molecule. *Gradient gel* with an increasing concentration of a chemical (denaturant) which breaks apart the DNA molecule.

Electrophoresis - application of an electric current across a gel. In response to the current, double - stranded DNA migrates (moves down) the gel. Denaturing the DNA molecule forms Y- and T- shaped structures greatly slowing migration. DNA contains four nucleotide bases which bond across the two strands of the molecule - "G" form three hydrogen bonds with "C"; "A" forms 2 hydrogen bonds with "T". Thus, DNA segments with more GC base pairs (high GC content) form stronger bonds between the DNA strands than those with less GC base pairs. Consequently, high GC content DNA segments require a greater concentration of the denaturing chemical before the DNA strands break apart.

The mixture of amplified DNA segments is loaded at the top of the gel. DNA migrates from the top (low denaturant concentration) toward the bottom of the gel (high denaturant concentration). DNA segments with low GC content denature near the top of gel and stop migrating. DNA segments with higher GC content denature further down the gel. DNA with the identical sequences migrates the same distance forming a "band". Individual bands are excised for sequencing and results are compared to a database of 16S rRNA genes to identify the dominant organisms.

2.11.4 Applications of PCR-DGGE

PCR-DGGE is classified as part of the new discipline of molecular microbial ecology (Muyzer *et al.,* 1998). Microbial ecology aims at studying interactions among microorganisms and between microorganisms and their environment. This involves long-term study, which includes various and numerous environmental sample analysis (Muyzer *et al.,* 1998). However, conventional cloning, hybridization and culture methods as mentioned above are not always practical for such investigations. Moreover, these techniques do not provide any information on the dynamics of the microbial populations in complex ecosystems and potential effects of environmental changes on such populations (Sheffield *et al.,* 1989; Muyzer *et al.,* 1993, 1998). Most importantly, these methods require an extended knowledge of the microorganisms to

develop adapted probes that target particular individuals among diverse populations (Muyzer *et al.,* 1998).

PCR-DGGE has the advantage of not requiring previous knowledge on microbial populations. It is a fingerprinting approach that can generate a pattern of genetic diversity in complex microbial ecosystems such as gastrointestinal tracts, soils, sediments, deep seas, rivers, hot springs and biofilms (Muyzer *et al.,* 1998, 1993). A major advantage of this method is its potential to visually profile and monitor changes occurring in various microbial communities that are undergoing different treatments or modifications. It is a rapid and efficient separation technique of same length DNA sequences (amplified by PCR), which may vary as little as a single base pair modification (Muyzer *et al.,* 1998, 1993; Sheffield *et al.,* 1989).

Furthermore, PCR-DGGE is a flexible method that allows a unique combination of different approaches for a more accurate identification of, for example, functional genes present in particular bacterial populations or specific bacterial species by using hybridization or species-specific probes (Walter *et al.,* 2000; Muyzer *et al.,* 1998, 1993). This methodology can be utilized in diverse subject areas such as clinical and environmental microbiology and food safety.

2.11.4.1 PCR-DGGE in clinical microbiology

Examples of DGGE applications in clinical microbiology abound. For instance, DGGE allowed the identification of over 65 *Mycoplasma* species of human and veterinary origins in less than 24 hours (Laura *et al.,* 2005). *Mycoplasmas* are fastidious organisms that require many weeks to culture and other serological tests to be identified. They cause various diseases associated with pneumonia, arthritis, conjunctivitis, infertility and abortion (Laura *et al.,* 2005). This application of PCR-DGGE could potentially allow considerable savings of time, life and treatment costs. This method was also able to demonstrate that animals, such as the Nile crocodile, baboon, red panda, wolf and Taiwan beauty snake, could also be infected by

Helicobacter species, bacteria suspected to be responsible for stomach ulcers (Abu *et al.,* 2003).

2.11.4.2 PCR-DGGE in environmental microbiology and food safety

The recent outbreak of *E. coli* on spinach in California is a painful recall that even vegetarians are not safe from the damages caused by a degraded health condition of food producing animals. The need for rapid and accurate methods for screening of total microbial populations in complex ecosystems is more evident than ever. PCR-DGGE has proved to be a powerful tool in assessing total gut microbial populations and was also used to detect previously unknown bacteria species in the GI tract of animals (McAuliffe *et al.,* 2005; Abu *et al.,* 2003; Gong *et al.,* 2002; Walter *et al.,* 2000). Understanding the relationship between the host and the disease-causing organisms will certainly assist us in defining efficient pathogens control measures. This is of paramount importance in food safety and food processing where quality control and assurance programs necessitate proficient methods to discontinue the transmission cycle of life-threatening microbes.

DGGE is undeniably a valuable approach in screening complex ecosystems on a large scale and in analyzing various environmental samples in a reduced amount of time. Using this technique, diagnosis of emerging infections could become easier and faster and identification of uncultivable pathogens can also now be facilitated. Although there are limitations, DGGE is an interesting and unique approach that bridges many molecular biology tools together, and its limitations are primarily attributable to the fact that it is still a relatively new technique.

Although the Denaturing Gradient Gel Electrophoresis (DGGE) concept was originally developed for detecting single base changes and DNA polymorphisms, it has now become one of the most important molecular tools in the field of microbial ecology. At present, there is considerable interest in the use of culture-independent methods for the characterization and monitoring of total microbial populations in

different niches. Alone or in combination with conventional culturing techniques, molecular techniques such as DGGE most certainly contribute to a more complete and focused description and exploration of microbial ecosystems.

2.11.4.3 Intestinal microbiota

The human colon is one of the most complex natural ecosystems usually containing 10^{10} to 10^{12} bacteria per g content. In a recent study performed at the Laboratory of Microbiology of Ghent University (LM-UG), the temporal stability of both the predominant population and a number of specific subpopulations of the fecal microbiota of four healthy volunteers was monitored over a 3 month period (Walter *et al.,* 2000). For this purpose, a combination of different universal (V_3 and V_6-V_8) and group-tspecific (targeting the *Bacteroides fragilis* subgroup, the genus *Bifidobacterium* and the *Lactobacillus*-*Leuconostoc*-*Pediococcus*-*Weissella* group) 16S rRNA gene primers was used. DGGE profiles generated with universal primers showed subject-specific clustering with numerical analysis which clearly indicates that the predominant fecal microbiota is host-specific and relatively stable over a prolonged time. In contrast, the specific primers yielded profiles with varying degrees of temporal stability. The autochthonous groups (e.g. the bifidobacteria and the *Bacteroides fragilis* subgroup) did not undergo major population shifts during the study period whereas other subpopulations (e.g. *Lactobacillus* and related genera) tend to show temporal variations even within a two-week period and resulted in complete loss of subject-grouping.

2.11.4.4 Bacteria in food products/foods

Various Gram-positive bacteria including lactic acid bacteria (LAB) are associated with various natural and fermented foods. In many foods products, LAB play a key role in the production process or contribute to their health-promoting or probiotic properties.

At LM-UG, a series of probiotic products (i.e. dairy products, dried food supplements, etc.) were subjected to microbial analysis in which DGGE was used as an identification method based on DGGE band position comparison of reference strains in a computational database (Creighton *et al.,* 1999). This approch was developed further into a nested PCR-DGGE method which allowed taxonomic characterization of bifidobacterial communities at species level in combination with the monitoring of its temporal changes (Muyzer *et al.,* 1993). The nested PCR-DGGE method was also used for qualitative analysis of 58 commercial probiotic products claiming to contain bifidobacteria (Muyzer *et al.,* 1998). In this regard, DGGE is used in a study in which the micro-organisms in Flemish artisanal cheeses are monitored in order to gain more insights in population dynamics during the production process and ripping.

Since the introduction in microbial ecology in the early nineties (Muyzer *et al.,* 1993), DGGE has been successfully used to analyze microbial communities from extremely diverse environments. The success of the DGGE technology lays in the fact that it allows visualization of the total biodiversity including the unculturable/uncultured bacteria. The generated genomic fingerprints permit to study species diversity and microbial community dynamics. Through its relative simplicity and reproducibility, DGGE will definitely conquer its place in every microbial ecologist's laboratory and will certainly broaden our current views on the overall microbial biodiversity.

2.11.4.5 DGGE- finding polymorphisms with PCR amplified DNA

Although we have yet to try it in our lab, the DGGE should provide an excellent method of finding polymorphisms when DNA samples amplified from different individuals are run on parallel denaturing gradient gels. The mobility shifts of these amplified fragments are visible with ethidium staining, eliminating the need for autoradiography. The polymorphisms detected in such a screen could be pursued with genotypic data collection in the CEPH pedigrees. Richard Myers advocates the PCR amplification of larger fragments, e. g. 2-3kb in size, followed by restriction digestion of the amplified products with two different frequent cutting enzymes such as HaeIII and Sau3AI, then running the samples on two different denaturing gradient gels (0-

50% denaturant and 40-80% denaturant) with different running times. The gels are then examined under UV illumination after staining with ethidium bromide. Myers estimates as many as 75% of the base pair changes in a 2-3 kb fragment could be detected in this screen.

Advantages

- Very sensitive to variations in DNA sequence
- Allows simultaneous analysis of multiple samples
- Useful method for monitoring shifts in community structure over time;
- Community profiles can be analyzed with cluster analysis
- The use of universal primers allows the analyses of microbial communities without any prior knowledge of the species
- The fragments separated by DGGE can be excised, cloned and sequenced for identification
- It is possible to identify constituents that represent only 1 % of the total community
- DGGE can be applied to phylogenetic and functional genes

Disadvantages

- DGGE analysis is rather time consuming
- DGGE analysis suffers from the same drawbacks as all PCR-based community analysis techniques, including biases from DNA extraction and amplification
- The variation in 16S rRNA gene copy number in different microbes makes this technique only "semi-quantitative"
- Microheterogeneity in rRNA encoding genes present in some species may result in multiple bands for a single species and subsequently to an overestimation of community diversity
- Heteroduplexes can cause biases to the observed diversity
- No method for automated analyses currently available
- Works well only with short fragments (<600 bp), thus limiting phylogenetic characterization

- Gels of complex communities may look smeared due to the large number of bands;
- Band position does not provide reproducible taxonomic information;
- Results difficult to reproduce between gels and laboratories.

For bioremediation assessment, DGGE profiles and sequence analysis are commonly used for evaluating the similarities/differences in the microbial community composition (dominant bacterial or fungal groups). DGGE highlights differences between samples and changes or “shifts” in microbial community composition over time or following a treatment. For example, DGGE can be used to determine the differences in the dominant bacterial groups in contaminated versus non-contaminated groundwater monitoring wells to evaluate which groups are enriched in impacted zones. Likewise, DGGE can be utilized to determine which bacterial groups are stimulated following a corrective action such as addition of a growth substrate or nutrient.

2.11.5 Restriction fragment length polymorphism (RFLP)

Amplicons from all aerobic actinomycete isolates lacked *Bst*EII recognition sites, thereby distinguishing them from those of mycobacteria that contain one or more such sites. Of 29 restriction endonucleases, *Msp*I plus *Hin*fI produced RFLP patterns that differentiated 16 of the 20 taxa. A single RFLP pattern was observed for 15 of 20 taxa that included 65% of phenotypically clustered isolates. Multiple patterns were seen with *Gordona bronchialis*, *Nocardia asteroides* complex type VI, *Nocardia otitidiscaviarum*, *Nocardia transvalensis*, and *Streptomyces* spp. *Streptomyces* RFLP patterns were the most heterogeneous (five patterns among 19 isolates), but exhibited a unique *Hin*fI fragment of >320 bp (Steingrube, *et al*., 1997). Pedro *et al* (2001) reported that a number of molecular fingerprinting techniques are available that can be used to detect microbial groups at or below the species level (Bull *et al*., 2000). Of these, the technique of choice in our laboratory has been polymerase chain reaction–restriction fragment length polymorphism–single-strand conformational polymorphism (Clapp *et al*., 2001a, b; Starch *et al*., 2001; Clapp 1999).

The RFLP fingerprinting of selected strains by HhaI-digestion of the 16S rRNA genes resulted in 11 different patterns. The HhaI-RFLP analysis gave good resolution for the identification of the actinobacteria isolates at the genus level (Zhang *et al*., 2006). Frederic *et al* (1999) reported all *Nocardia* strains exhibited one *Mln*I recognition site but no *Sac*I restriction site. The soil that supported a higher number of indigenous organisms resulted in wheat roots with higher actinobacterial diversity and levels of colonization within the plant tissue. Sequencing of 16S rRNA clones, obtained using the same actinobacterium-based PCR primers that were used in the T-RFLP analysis, confirmed the presence of the actinobacterial diversity and identified a number of *Mycobacterium* and *Streptomyces* species. An amplified rRNA restriction analysis confirmed that these actinomycetes are distinct from *Frankia*, a finding substantiated by a 16S rRNA gene phylogenetic analysis of two of the Mexican isolates (Valdes *et al*., 2005).

2.12 Enzymatic potential of halophilic and alkaliphilic actinomycetes

During the last decade, there has been a dramatic increase in the need for bioactive compounds with novel activities. Enzymes, after antibiotics are the most important biologically derived product having immense potential in catalytic reactions of commercial interest. By remaining active when other enzymes would fail, enzymes from extremophiles – referred as “extremozymes” can potentially eliminate the need for those added steps, thereby increasing efficiency and reducing costs. During the last few years, some extracellular enzymes from halophilic and alkaliphilic bacteria and actinomycetes have been studied. However, from the literature, it is evident that the exploration of the enzymatic potential of these microbes is just the beginning and till date only few enzymes are investigated in depth.

2.12.1 Alkaline proteases

Alkaliphiles have made a great impact in industrial applications. As stated earlier in chapter 1, proteases are among the commercially most viable enzymes and exploration of further novel microbial sources of this enzyme has enthused scientific

community during the last several years. Proteases are among the most important class of industrial enzymes, which constitute >65% of the total industrial applications such as food additives, leather (dehairing agents), enhanced oil recovery, hyper saline waste treatment and in detergent formulation (Ito *et al*., 1998; Gupta and Roy, 2002). Studies concerned with production and purification of alkaline protease were carried out from *Streptomyces clavuligerus* (Moreira *et al*., 2001).

2.12.2 Alkaline amylases

Starch is a major storage form of carbohydrates in nearly all green plants. Various organisms have developed different strategies for starch breakdown, but all involve an initial attack by glycosidase which converts the dextrins of oligosaccharides to glucose and amylases are among the most effective enzymes known. The majority of amylases from mesophilic and extremophilic organisms depend on calcium ions for their activity and stability, which leads to increase in process costs. Alkaliphilic *Bacillus halodurans* A-59 (ATCC 21591), Ammar and colleagues (2002) explained new action pattern of a maltose-forming alpha-amylase from alkaliphilic *Streptomyces* sp. and its possible application in bakery.

2.12.3 Alkaline cellulases

Cellulose, the most abundant component of plant biomass, is found in nature almost exclusively in plant cell walls, although it is produced by some animals (e.g., tunicates) and a few bacteria. Commercially available cellulases display optimum activity over a pH range from 4 to 6. No enzyme with an alkaline optimum pH for activity (pH 10 or higher) was reported before the rediscoveryof alkaliphiles. Alkaliphilic *Bacillus* sp. strains N4 and 1139 secreted alkaline CMCases that were active over a broad pH range (Fukumori *et al.,* 1985; Horikoshi *et al.,* 1984). Cellulase production has also been reported in an alkaliphilic *Streptomyces* strain S36-2. Van and coworkers (2001) described the cloning and expression of an endocellulase gene from a novel alkaliphilic *Streptomycete* isolated from an East African soda lake.

2.12.4 Microbial Proteases

Proteases represent the class of enzymes which occupy a pivotal position with respect to their physiological roles as well as their commercial applications. Since they are physiologically necessary for living organisms, proteases occur ubiquitously in a wide diversity of sources such as plants, animals, and microorganisms. The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases. Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation.

2.12.5 Classification of Proteases

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases) (IUBMB, 1992). Depending on the site of action, proteases are mainly subdivided in to two major groups, i.e., exopeptidases (cleave the peptide bond proximal to the amino or carboxy termini of the substrate) and endopeptidases (cleave the peptide bonds distant from the termini of the substrate). Based on the functional group present at the active site, proteases are further classified into four prominent groups, i.e., serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Hartley, 1960).

2.12.5.1 Metalloproteases

Metalloproteases are the most diverse of the catalytic types of proteases (Barett, 1995). They are characterized by the requirement of a divalent metal ion for their activity. They include enzymes from a variety of origins such as collagenases from higher organisms, hemorrhagic toxins from snake venoms, and thermolysin from bacteria (Shannon *et al.,* 1989). Based on the specificity of their action, metalloproteases can be divided into four groups, (i) neutral, (ii) alkaline, (iii) *Myxobacter* I, and (iv) *Myxobacter* II. All of them are inhibited by chelating agents such as EDTA but not by sulfhydryl agents or diisopropylfluorophosphate (DFP).

2.12.5.2 Aspartic proteases

Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Most aspartic proteases show maximal activity at pH; 3 to 4 and have isoelectric points in the range of pH 3 - 4.5. Their molecular masses are in the range of 30 - 45 kDa. The aspartic proteases are inhibited by pepstatin (Fitzgerald *et al.,* 1990).

2.12.5.3 Cysteine/ Thiol proteases

The activity of all cysteine proteases depends on a catalytic site consisting of cysteine and histidine. Generally, cysteine proteases are active only in the presence of reducing agents such cysteine. Based on their side chain specificity, they are broadly divided into four groups: (i) papain-like, (ii) trypsinlike with preference for cleavage at the arginine residue, iii) specific to glutamic acid, and (iv) others. Papain is the best-known cysteine protease. Cysteine proteases have neutral pH optima, although a few of them, e.g., lysosomal proteases, are maximally active at acidic pH. They are susceptible to sulfhydryl agents such as p-chloromercuribenzoate (pCMB) but are unaffected by DFP and metal-chelating agents.

2.12.5.4 Serine proteases

Serine proteases are characterized by the presence of a serine group in their active site. They are numerous and widespread among viruses, bacteria, and eukaryotes, suggesting that they are vital to these organisms. Serine proteases are subdivided in to four class; chymotrypsin (SA), subtilisin (SB), carboxypeptidase C (SC), and *Escherichia* D-Ala.D-Ala peptidase A (SE). Serine proteases are recognized by their irreversible inhibition by 3, 4 - dichloroisocoumarin (3,4-DCI), L-3-carboxytrans 2,3-epoxypropyl-leucylamido (4-guanidine) butane (E.64), DFP, phenylmethylsulfonyl fluoride (PMSF) and tosyl-L-lysine chloromethyl ketone (TLCK). Their molecular masses range between 18-35 kDa, exceptions are the serine protease from *Blakeslea trispora* (Govind *et al.,* 1981) and *Natronococcus occultus* (Studdert *et al.,* 2001), having molecular mass of 126 kDa and 130 KDa respectively.

2.12.5.5 Serine alkaline proteases

Alkaline proteases are defined as those proteases, which are active in a neutral to alkaline pH, with optimum at pH 7.11, although higher pH optima (10-12.5) from *Bacillus* sp. YaB have also been reported (Shimogaki *et al*., 1991). The alkaline serine proteases are the most important group of enzymes exploited commercially. It is produced by several bacteria, molds, yeasts, and fungi. They are inhibited by DFP or a potato protease inhibitor but not by tosyl-L-phenylalanine chloromethyl ketone (TPCK) or TLCK. They hydrolyze a peptide bond, which has tyrosine, phenylalanine, or leucine at the carboxyl side of the splitting bond. Their molecular masses are in the range of 15 - 30 kDa.

2.12.5.5.1 Subtilisin

Subtilisin-like serine proteases are generally bacterial in origin; however, there are reports from other sources as well (Siezen and Leunissen, 1997). The microbial proteases are generally secreted extracellularly for the purpose of scavenging nutrients (Graycar, 1999) and are specific for aromatic or hydrophobic residues such as tyrosine, phenylalanine and leucine. They are highly sensitive towards PMSF. There are two major classes of Subtilisin as highlighted below.

2.12.5.5.2 Subtilisin Carlsberg

Gutenlberg and ottesen (1952) discovered enzyme capable of converting ovalbumin to plakalbumin. This enzyme was known as subtilisin carlsberg. It was the first microbial protease isolated in crystalline form. The source of this enzyme was *Bacillus pumilis* and *Bacillus licheniformnis.* Subtilisin Carlsberg is widely used in detergents. Enzyme has a wide pH range 5 -11 for stability. They are most active at pH 10 with molecular weight between 15- 39 KDa (Gupta *et al.,* 2002). The Carlsberg enzyme has broader substrate specificity and does not depend on Ca^{2+} for its stability.

2.12.5.5.3 Subtilisin Novo

This alkaline serine protease was first purified and crystallized by Hagihara (1958). It is usually present as a side activity in commercial preparation of *Bacillus* amylases. However, an amylase preparation free from proteases is now available. *Bacillus amyloliquefaciens* is the best source for this enzyme (Rao *et al.,* 1998). It is also produced by *Flavobacterium* and the enzyme was stable at pH 5-11 up to 60ºC and was dependent on Ca^{2+} for stability (Morita *et al.,* 1998).

2.12.6 Production of alkaline protease from halo-tolerant alkaliphilic actinomycetes

Extracellular enzymes can be produced easily and effectively. However, halotolerant alkaliphilic actinomycetes and their extremozymes such as alkaline proteases have been the subject of extensive investigation and valuable information for scientists during the last few years. The culture conditions that promote protease production are usually significantly different from the culture conditions promoting cell growth (Moon and Parulekar, 1991), so it is essential to optimize the physical and nutritional conditions for the industrial production of alkaline proteases. According to Kumar and Takagi (1999), to develop an economically feasible technology, research efforts are mainly focused on: (i) improvement in the yields of alkaline proteases; and (ii) optimization of the fermentation medium and production conditions. Production and optimization of the enzyme from certain alkaliphilic *Bacillus* spp. and a few actinomycetes have been investigated (Mehta *et al*., 2006; Kanekar *et al*., 2002; Keila *et al*., 2001).

2.12.6.1 Salt and pH requirement

Halo-tolerant and alkaliphilic microorganisms exhibit strong dependence on the extracellular pH and salt for the cell growth and enzyme production. Two extreme halophilic actinomycetes *Prauserella halophila* sp. nov. and *Prauserella alba* sp. nov. grew optimally at 20 % NaCl, w/v (Li *et al.,* 2003b). The pH of the medium also plays an important role in growth and enzyme production. It is necessary to maintain

the pH of the medium above 8 throughout the fermentation period to increase protease yields (Li *et al*., 2005c; Tsuchiya *et al.,* 1992). *Nocardiopsis alkaliphila*, an extremely alkaliphilic actinomycete has been reported to grow best at pH 10 (Hozzein *et al*., 2002). pH 9 was optimum for the production of protease from Nocardiopsis sp. TOA-1(Mitsuiki *et al*., 2002) and pH 9.5- 10 was more suitable for an alkaliphilic actinomycete (Mehta *et al.,* 2006).

2.12.7 Physiological functions of proteases

Proteases execute a large variety of complex physiological functions. Their importance in conducting the essential metabolic and regulatory functions is evident from their occurrence in all forms of living organisms. Proteases play a critical role in many physiological processes. In general, extracellular proteases catalyze the hydrolysis of large proteins to smaller molecules for subsequent absorption by the cell whereas intracellular proteases play a critical role in the regulation of metabolism. In contrast to the multitude of the roles contemplated for proteases, our knowledge about the mechanisms by which they perform these functions is very limited. Extensive research is being carried out to unravel the metabolic pathways in which proteases play an integral role; this research will continue to contribute significantly to our present state of information.

2.12.8 Purification of the alkaline protease

As described above, proteases are among the most commercially exploited enzymes. A number of alkaline proteases have been purified and characterized. However, to reach the current industrial demand for the enzymes with specific features, further sources need to be explored. Generally, crude preparations of alkaline proteases are widely used in industries such as detergent; however, purification of the proteases is important for the better understanding of the structural and functional relationship. Besides, many applications would require enzyme in homogeneity.

2.12.8.1 Protein precipitation

The first step in the conventional purification is the precipitation of the enzyme. The culture supernatant containing enzyme is concentrated by means of ultrafilteration and precipitated with different salts such as ammonium sulfate. However, ammonium sulfate is not a good choice of salt for the precipitation of alkaline protease and as an alternative, sodium sulfate has been promoted, but due to poor solubility at low temperature, it has limited use. Precipitation by solvent extraction using acetone and ethanol is also widely used. In addition, other methods, such as use of PEG - 35,000 (Larcher *et al.,* 1996), temperature-sensitive hydrogel (Han and Damodaran, 1998) and lyophilization (Manonmani and Joseph, 1993) are also used for concentration of alkaline proteases. In order to purify the enzymes many chromatographic techniques have been used in different combinations.

2.12.8.2 Ion Exchange Chromatography

Alkaline proteases are generally positively charged and thus could not bind to anion exchangers (Kumar *et al.,* 2004; Fujiwara *et al.,* 1993). However, cation exchangers can be a rational choice and the bound molecules are eluted from the column by an increasing salt or using pH gradient (Tsuchiya *et al.,* 1992). The matrices for ion exchange contain ionizable functional groups such as diethyl amino ethyl (DEAE) and carboxy methyl (CM). The adsorbed protein molecule is eluted by a gradient change in the pH or ionic strength of the eluting buffer or solution.

2.12.8.3 Affinity Chromatography

Reports on the purification of alkaline proteases by different affinity chromatographic methods showed that an affinity adsorbent hydroxyapatite was used to separate the neutral protease as well as purify the alkaline protease from a *Bacillus* sp. (Kobayashi *et al.,* 1996). *Streptomyces griseus* metalloprotease II (SGMP II) was purified by ammonium sulphate precipitation (Chaphalkar *et al*., 1998) followed by affinity chromatography on carbobenzoxy-L-alaninyl-triethylenetetraminyl-sepharose (Tsuchiya *et al.,* 1992). Other affinity matrices used were Sephadex-4-

phenylbutylamine, casein agarose, or N-benzoyloxycarbonyl phenylalanine immobilized on agarose adsorbents (Larcher *et al.,* 1996). However, the cost of enzyme supports and the labile nature of some affinity legends limit the use of this technique at large scale.

2.12.8.4 Affinity precipitation

Affinity precipitation is a function of a soluble macromolecule (ligand polymer and macroligand) that has two functions: (1) it contains an affinity ligand (polyvalent macromolecule), and (2) it can be precipitated in many ways, i.e., by change in pH, temperature or ionic strength. After elution of proteins the polymer can be recycled. An alkaline protease from *Streptomyces pactum* DSM 40530, used as a washing powder additive, was earlier purified by affinity precipitation (Bockle *et al.,* 1995).

2.12.8.5 Gel filtration

In addition to the above chromatographic techniques, gel filtration is used for rapid separation of macromolecules based on size. Recently, many new agarose based and more rigid and cross-linked gels, such as Sephacryl, Superose, Superdex and Toyopearl are also being used for purification purposes. They are generally used either in the early-to-middle stage of purification (Chakrabarti *et al.,* 2000) or in the final stages of purification. Major disadvantages of this method are the lower capacity for loading proteins and that the desired protein gets too diluted.

2.12.8.6 Hydrophobic interaction chromatography

This approach exploits the variability of external hydrophobic amino acid residues on different proteins. These hydrophobic interactions are strengthened by high salt concentrations and higher temperatures, and are weakened by the presence of detergents or miscible organic solvents. Hydrophobic interactions are much more variable in behavior than ion exchangers and, thus, resolution is generally poor than ion exchange. The most commonly used hydrophobic adsorbents are octyl- (C8-) and phenyl substituted matrices.

2.12.8.7 Aqueous two-phase systems

Apart from the conventional methods of enzyme purification, some recent approaches have also been employed. Among them Aqueous biphasic system has focused considerable attention. This system results from the incompatibility between aqueous solution of two polymers (i.e. PEG and dextran) or between one polymer and an appropriate salt (i.e. K_2HPO_4). Aqueous biphasic system also has been employed for In situ production of enzymes (Sinha, 1993). Thus, this approach has been taken as one of the purification step and the results, particularly for alkaline proteases are quite encouraging. This technique has been applied for purification of alkaline proteases using mixtures of polyethylene glycol (PEG) and dextran or PEG and salts such as H_3PO_4, MgSO4 (Hotha *et al.,* 1997; Sinha, 1993).

2.12.9 Characterization of alkaline Proteases

2.12.9.1 Influence of pH and Temperature

Alkaline proteases are generally active in the range of pH 8-12. An extracellular alkaline serine protease from *Natronococcus occultus* (Studdert *et al.,* 2001) was active and stable in a rather wide range of pH between pH 3-12. In general, most of the alkaline proteases used in detergent industries are thermostable having optimum temperature between 50 -70ºC (Adinarayana *et al.,* 2003). Currently, a new trend in detergent industries have come up that required alkaline proteases active at low temperature, for example, cold active serine alkaline protease from *Pseudomonas* sp. which was active at 10-40ºC with optimum at 30ºC (Zeng *et al.,* 2003).

2.12.9.2 Influence of salt on protease activity and stability

Generally, proteases from extreme halophiles and haloalkaliphiles require higher salt concentrations (up to 4M) for their activity and stability, optimum being at 1-2M (Gimenez *et al.,* 2000) and lost their activity in the absence of salt. In comparison, the enzymes from halo-tolerantorganisms may not require salt for their activity; however, its presence may even cause the adverse effects. More recently, an alkaline protease from a halophilic actinomycete *Streptomonospora salina* was reported to be active up

to 4M NaCl (Cui *et al.,* 2004). Novel protease from haloalkaliphilic *Natronococcus occultus* was active in the presence of 1-2 M NaCl or KCl and lost its activity below 0.5M NaCl (Studdert *et al.,* 2001).

2.12.9.3 Thermostability

Alkaline proteases are generally thermally stable in the range of 37 - 70ºC (Bayoudh *et al.,* 2000). Alkaline serine protease from *Bacillus subtilis* was highly thermostable and retained 100% activity at 60ºC for 350 min (Adinarayana *et al.,* 2003). Alkaline protease from haloalkaliphilic archaea *Natronococcus occultus* was optimally active at 60ºC in presence of 2M NaCl (Studdert *et al.,* 2001). The temperature stability of the enzyme can also be enhanced by addition of some stabilizers such as polyethylene glycol (PEG), polyhydric alcohol, starch (Gupta *et al.,* 2002) and glycerol (Bayoudh *et al.,* 2000).

2.12.9.4 Cation requirement

Alkaline proteases require divalent cations like Ca^{2+}, Mg^{2+} and Mn^{2+} or a combination of these cations, for maximum activity. Activity of an alkaline serine protease from *Bacillus subtilis* increased in the presence of Ca^{2+}, Mg^{2+} and Mn^{2+}(Adinarayana *et al.,* 2003). However, as per few reports, Ca^{2+} inhibited the activity of alkaline protease (Bayoudh *et al.,* 2000) and in one case, Ca^{2+} did not exhibit any effect on activity at higher temperature (70ºC) (Gessesse *et al.,* 2003). Above citations clearly indicate the extreme nature of alkaline proteases and their stability at various physical extremes. Thus it is quite interesting to know how these proteins maintain their active confirmation at the extreme conditions. Some mechanisms of enzyme stability at extremes of temperature are described below.

2.12.9.5 Effect of surfactants and detergents

Some alkaline proteases are reported to remain stable with sodium dodecyl sulphate (Surface active agent) and sodium linear alkyl benzene sulphonate (Muderrizade *et al.,* 2002; Horikoshi, 1999) and with laundry detergents (Adinarayana *et al.,* 2003;

Singh *et al.,* 1999). Alkaline protease from the moderate halophile, *Halomonas* sp Es 10 was stable with Triton X- 100 and activity was slightly increased with SDS, Tween 20, and tween 80 (Kim *et al*., 1992).

2.12.9.6 Effect of inhibitors

Inhibition of enzyme was first studied by Aunstrup (1980). An alkaline protease from alkaliphilic Thermoactinomycetes sp.HS682 was inhibited by DFP and PMSF, but not by EDTA (Tsuchiya *et al*., 1997). An alkaline protease from the moderate halophile, *Halomonas* sp Es 10 was inhibited with EDTA but not with cysteine and PMSF (Kim *et al*., 1992). Alkaline protease from haloalkaliphilic archaeon *Natronococcus occultus* was also inhibited with PMSF and chymostatin but not with EDTA (Studdert *et al.,* 2001). Similarly, a thermostable alkaline protease from *Bacillus lichniformis* MIR29 was inhibited by PMSF but remained unaffected with EDTA, SDS and urea (Ferrero *et al*., 1996).

2.12.9.7 Thermophilic adaptation of proteins

Certain microorganisms are capable of growing at high temperatures at which proteins are commonly denatured. It has been known that many enzymes obtained from such thermophilic bacteria are unusually thermostable, while possessing other properties identical with enzymes found in mesophilic bacteria (Mandrich *et al.,* 2004). The sequencing, structure, and mutagenesis information accumulated in the last 20 years confirm that hydrophobicity (Vieille and Zeikus, 2001); hydrogen bond, ion pairs and hydrophobic interactions (Scandurra *et al.,* 1998) are the main force in protein stability at higher temperature. Another mechanism is the decrease in the uncharged polar residues and increase in charged polar residues in the polypeptide chain of the thermophilic protein. In addition, the hydrophobic effect was also considered to be a major driving force of protein folding and thus it may be responsible for the protein stability. Moreover, many thermophilic and hyperthermophilic proteins show a statistically increased number of salt bridges and salt bridge networks. As the three dimensional structures of many thermophilic and

mesophilic proteins are known, mutational analysis and comparison of protein structure from two groups may provide some batter explanation for the adaptation mechanisms of thermophilic proteins (Cambillau and Claverie, 2000).

2.12.9.8 Protein folding

Enzymes are very sensitive to certain environmental conditions. Denaturation of enzyme occurs naturally or the enzyme can be subjected to denaturation by various chemical denaturants. To have biologically active protein, it must fold into proper secondary and tertiary structures. These structures are held together by chemical interactions between the side chains of the amino acids, including; hydrogen bonds, hydrophobic interactions, and, at times, covalent bonds. Regardless of its function, a protein must be properly folded to carry out its biological role. Now days genes from extremophiles are being cloned in mesophilic bacteria to generate the protein in large amount. Fast and highlevel expression of heterologous proteins in bacterial hosts often results in the accumulation of almost pure aggregates, inclusion bodies of the target protein. Hence, recently, renaturation of the over expressed but wrongly folded proteins have gained considerable attention. Protein can also be denatured under *in vitro* conditions by exposing it to 6-8 M urea or 6M Guanidine HCl. The denaturants not only prevent the aggregation of the protein but also arrest protein folding leading to non-functionality. Despite their use, the mode of action of these denaturants is still not well understood.

These denaturants apparently disrupt hydrogen bonds, which hold the protein in its unique structure. However, there are evidences suggesting that urea and guanidine hydrochloride may also disrupt hydrophobic interactions by promoting the solubility of hydrophobic residues in aqueous solutions. An alkaline protease from haloalkaliphilic *Bacillus* sp., was sensitive to urea denaturation and denatured within 30min (Patel *et al.,* 2006). However, this finding was in contrast with some of our own studies with other strains of haloalkaliphilic bacteria and halo-tolerant alkaliphilic actinomycetes, where the extracellular proteases were highly resistant to

urea denaturation (Dodia *et al.,* 2008). Denaturation profile of the alkaline proteases from our own group, clearly suggested the highly resistant nature of these enzymes against urea.

Recovery of the denatured protein can be achieved by various methods. *In vivo* solubilization includes co expression of molecular chaperons, low level of induction, low growth temperature and error prone PCR. *In- vitro* renaturation could be achieved by gentle removal of denaturant by dialysis (Maeda *et al.,* 1995), a resin bound dialysis, rapid dilution method and folding in immobilized state by FPLC (Singh *et al*., 2002). The renaturation of urea-denatured alkaline protease from haloalkaliphilic *Bacillus* sp., *in-vitro* conditions was significantly enhanced at lower protein concentrations (Patel *et al.,* 2006). In contrast, the renaturation of another alkaline protease from haloalkaliphilic *Bacillus* sp., was not achieved by conventional dialysis and even at lower protein concentrations (Dodia *et al.,* 2008).

2.12.10 Applications of alkaline protease

Proteases are used extensively in the pharmaceutical industry for preparation of medicines such as ointments for debridement of wounds. Proteases that are used in the food and detergent industries are prepared in bulk quantities and used as crude preparations; whereas those that are used in medicine are produced in small amounts but require extensive purification before their usage.

2.12.10.1 Detergent Industries

Alkaline proteases are one of the standard ingredients of all kinds of detergents ranging from those used for household laundering to reagents used for cleaning contact lenses or dentures. Fungal alkaline proteases are also advantageous due to the ease of downstream processing to prepare a microbe-free enzyme. The evaluation of detergent proteases is mainly dependent upon parameters such as the pH and ionic strength of the detergent solution, the washing temperature and pH, mechanical handling, level of soiling and the type of textile (Gupta *et al.,* 2002). Many reports

have been published on the compatibility of alkaline proteases with detergent (Dodia et al., 2008; Patel *et al.,* 2006; Adinarayana *et al.,* 2003). Recently, Stoner and his coworkers (2004) have studied the denaturation and autolysis of protease in heavy-duty liquid detergent (HDLD) and the factors affecting the stability of protease.

2.12.10.2 Leather industry

Leather processing involves several steps such as soaking, dehairing, bating, and tanning. The major building blocks of skin and hair are proteinaceous. The use of enzymes as alternatives to chemicals has proved successful in improving leather quality and in reducing environmental pollution. Scientists have demonstrated the successful use of alkaline proteases in leather tanning from *Bacillus amyloliquefaciens* (George *et al.,* 1995), *Bacillus subtilis* (Varela *et al.,* 1997) and *Bacillus subtilis* K2 (Hameed *et al*., 1996). An alkaline protease with dehairing function has been purified and characterized from *Streptomyces clavuligerus* (Keila *et al*., 2001). However, the selection of the enzyme depends on its specificity for matrix proteins such as elastin and keratin, and the amount of enzyme needed depends on the type of leather (soft or hard) to be produced.

2.12.10.3 Food Industries

The use of proteases in the food industry dates back to antiquity. They have been routinely used for various purposes such as cheese making, baking, preparation of soya hydrolysates and meat tenderization. Alkaline proteases have broad substrate specificity and can hydrolyze proteins from varied sources to produce hydrolysates of peptides with high nutritional value. Recently, Cavaicanti *et al* (2004) demonstrated a new milk clotting enzyme produced by *Nocardiopsis* sp. The commercial alkaline protease *Alcalase*, was used in the production of a less bitter hydrolysate (Adler-Nissen, 1986) and a debittered enzymatic whey protein hydrolysate which play an important role in blood pressure regulation, in infant food formulations and therapeutic dietary products (Neklyudov *et al.,* 2000).

2.12.10.4 Pharmaceutical industry

The wide diversity and specificity of proteases are used to get advantage in developing effective therapeutic agents. Alkaline proteases are also used for developing products of medical importance. Chaphalkar and Deys (1998) exploited the elastolytic activity of *Streptomyces diastaticus* for the preparation of elastoterase, which was applied for the treatment of burns, purulent wounds, carbuncles, furuncles and deep abscesses. Kim *et al* (1996) reported the use of alkaline protease from *Bacillus* sp. strain CK 11-4 as a thrombolytic agent having fibrinolytic activity. Clostridial collagenase or subtilisin is used in combination with broad-spectrum antibiotics in the treatment of burns and wounds. Alkaline protease from *Conidiobolus coronatus* was found to be able to replace trypsin in animal cell cultures (Chiplonkar *et al*., 1985).

2.12.10.5 Waste treatment

Alkaline proteases provide a potential application for the management of waste from various food processing industries and household activities. Proteases solubilize proteinaceous waste and thus help lower the biological oxygen demand of aquatic systems. Kampfer and Busse (2002) described *Nocardiopsis compostus* sp. nov. which produced an alkaline protease active in composting of solid waste. Recently, a novel alkaline protease has been studied from the alkaliphilic bacteria grown on the chicken feather that can be effectively used in the hydrolysis of feather waste (Gessesse *et al.,* 2003). Tannery effluents are highly polluting and contain chromium and high COD and BOD. Alkaliphilic actinomycetes, NCIM 5080 and NCIM 5142, have been shown to tolerate and accumulate chromium (More *et al*., 2001). During their growth, they also produced alkaline protease in the presence of chromium ions and reduce the COD by 70-80%. These properties of the actinomycetes make them suitable for treatment of tannery effluents which are alkaline and contain chromium and proteinaceous matter.

2.12.10.6 Silk degumming

One of the least explored areas for the use of proteases is the silk industry and only a few patents have been filed describing the use of proteases for the degumming of silk (Kanehisa, 2000). The traditional methods for the removal of sericin from the inner core of fibroin by starch (Kanehisa, 2000) are expensive and therefore, an alternative method could be the use of enzyme preparations, such as protease, for degumming the silk prior to dyeing.

2.13 Molecular approaches

Now a day's recombinant DNA Technology in conjunction with the directed evolution tools is extensively being used to improve and evolve enzymes for various unconventional applications. Molecular imprinting through conditional lyophilization is coming up to match molecular approaches in protein engineering. Knowledge of full nucleotide sequences of the enzyme genes has facilitated the deduction of the primary structure of the encoded enzymes and, in many cases, identification of various functional regions. These sequences also serve as the basis for phylogenetic analysis of proteins and assist in predicting the secondary structure of proteins, thereby helping in the study of structure and functional relationships of the enzyme. The nucleotide sequences of subtilisin and other serine alkaline proteases have been extensively reviewed by Gupta and coworkers in 2002. Tsuchiya and coworkers (1997) demonstrated the cloning and sequencing of an intracellular alkaline protease gene from alkaliphilic Thermoactinomyces sp. HS682. Page and coworkers (2003) chose *Streptomyces griseus* trypsin (SGT) as a model scaffold for the development of serine proteases with enhanced substrate specificity. Recombinant SGT has been produced in a *Bacillus subtilis* expression system in a soluble active form. Halolysin R4, a serine proteinase from the halophilic archaeon *Haloferax mediterranei* was cloned and studied for expression and structural properties (Kamekura *et al.,* 1996). Fernandez *et al* (2003) demonstrated the posttranslational processing of the xylanase Xys1L from *Streptomyces halstedii* JM8 by secreted alkaline serine protease. Recently, a novel calciumindependent serine protease from an alkaliphilic bacterium,

Nesterenkonia sp. AL20, has been purified and crystallized by X- ray analysis using sodium formate as the main precipitant (Bakhtiar *et al.,* 2002). Due to the advancement in molecular tools and increasing realization on the potential of the extremophiles, much of the work in this area is being done at molecular level to expand the horizon of genomics and proteomics in these organisms. Regulation of gene expression of various enzymes from extremophiles would pave the way for further molecular evolution to achieve unexplored and non-existed features of biocatalysts.

2.13.1 Cloning and expression of enzymes

Among the enzymes from extremophilic organisms, relatively limited awareness exists about enzymes from haloalkaliphilic bacteria. Extremozymes offer new opportunities for biocatalysis and biotransformations as a result of their extreme stability (Niehaus *et al.,* 1999). From recent work, major approaches to extending the range of applications of extremozymes have emerged. Both the discovery of new extremophilic species and the determination of genome sequences provide a route to new enzymes, with the possibility that these will lead to novel applications. The enzymes from extremophilic organisms are relatively less explored in the aspect of novel enzymology. All that is known /explored about the extremophilic enzymes is its character to work at relative high/elevated temperatures. The well known examples are the *Taq polymerases* from various thermophilic organisms. But one of the most important characters to be explored is their ability to act under alkaline conditions at industrial levels and here come the Haloalkaliphilic organisms and their enzymes which can be coined as *Halo-alkaline Enzymes.* In the past few decades, biocatalysts have been successfully exploited for the synthesis of complex drug intermediates, specialty chemicals and even commodity chemicals in the pharmaceutical, chemical and food industries. Recent advances in recombinant DNA technologies, high-throughput technologies, genomics and proteomics have fuelled the development of new catalysts and biocatalytic processes. In particular, gene cloning & directed evolution have emerged as powerful tools for biocatalyst engineering in order to

develop enzymes with novel properties, even without requiring knowledge of the enzyme structure and catalytic mechanisms.

The approach of directed evolution has been reviewed several times by a number of researchers. Also very important is the cloning of these important genes which in turn code for extremophilic enzymes (Yan *et al.,* 2009). Then gradually the cloned gene is over expressed in stable host or suitable mesophilic host which produces our desired protein in bulk quantities. Recent work suggests that the diversity of organisms in extreme environments is far greater than was initially suspected. The majority of extremophiles have not yet been isolated in pure culture, however, and thus it is difficult to determine the stability characteristics of these enzymes.

2.13.2 Expression Systems

Expression can be induced from a host strain without a source of T7 RNA polymerase by infection with Bacteriophage CE6. CE6 is a lambda recombinant that carries the cloned polymerase gene under control of the phage pL and pI promoters, the cI857 thermolabile repressor, and the Sam7 lysis mutations. When CE6 infects an appropriate host, the newly made T7 RNA polymerase transcribes target DNA so actively that normal phage development cannot proceed. Although this method is less convenient than induction of DE3 lysogens, it can be used if target gene products are too toxic to be maintained any other way. No T7 RNA polymerase is present in the cell before infection, so it should be possible to express any target DNA that can be cloned under control of a T7 promoter.

2.13.3 Induction of λDE3 Lysogens with IPTG:

After a target plasmid is established in a λDE3 lysogen, expression of the target DNA is induced by the addition of IPTG to a growing culture. For pET constructions carrying the “plain” T7 promoter, a final concentration of 0.4 mM IPTG is recommended for full induction, while 1 mM IPTG is recommended for full induction with vectors having the T7lac promoter. IPTG induction results in uniform,

concentration-dependent entry into all cells in the population. A range of IPTG concentrations from 25 μM to 4 mM should be tested, and the induced cultures examined for target protein activity and solubility to establish the optimal IPTG concentration.

2.13.4 Recombinant Protein Purification

The methods chosen for protein purification depend on many variables, including the properties of the protein of interest, its location and form within the cell, the vector, host strain background, and the intended application for the expressed protein. Culture conditions can also have a dramatic effect on solubility and localization of a given target protein. Many approaches can be used to purify target proteins expressed with the pET System. One advantage of the system is that in many cases the target protein accumulates to such high levels that it constitutes a high percentage of the total cell protein. Therefore, it is relatively straightforward to isolate the protein in two or three chromatographic steps by conventional methods (ion exchange, gel filtration, etc.).

A variety of affinity purification methods are available that take advantage of the various peptide fusion tags available with pET vectors. In many cases, the use of an affinity method enables the purification of the target protein to near homogeneity in one step. Purification may include cleavage of part or all of the fusion tag with enterokinase, factor Xa, thrombin, or HRV 3C proteases. Before purification or activity measurements of an expressed target protein, preliminary analysis of expression levels, cellular localization, and solubility of the target protein should be performed. The target protein may be found in any or all of the following fractions: soluble or insoluble cytoplasmic fractions, periplasm, or medium. Depending on the intended application, preferential localization to inclusion bodies, medium, or the periplasmic space can be advantageous for rapid purification by relatively simple procedures.

2.13.5 Solubilization and Refolding Proteins

A variety of methods have been published describing refolding of insoluble proteins (Willis *et al.,* 2005; Machida *et al.,*2004; Singh *et al.,* 2004; Vincentelli *et al.,* 2004). Most protocols describe the isolation of insoluble inclusion bodies by centrifugation followed by solubilization under denaturing conditions. The protein is then dialyzed or diluted into a non-denaturing buffer where refolding occurs. Because every protein possesses unique folding properties, the optimal refolding protocol for any given protein must be empirically determined (Dodia *et al.,* 2008). Optimal refolding conditions can be rapidly determined on a small scale by a matrix approach, in which variables, such as protein concentration, reducing agent, redox treatment, divalent cations, etc., are tested. Once the optimal concentrations are found, they can be applied to a larger scale solubilization and refolding of the target protein.

The Protein Refolding require CAPS buffer at alkaline pH in combination with N-lauroylsarcosine to achieve solubility of the inclusion bodies, followed by dialysis in the presence of DTT to promote refolding. Depending on the target protein, expression conditions, and intended application, proteins solubilized from washed inclusion bodies may be >90% homogeneous and may not require further purification. Purification under fully denaturing conditions (before refolding) is possible using His•Tag fusion proteins and His•Bind immobilized metal.

2.13.6 Purifying target proteins

Fusion proteins solubilized from inclusion bodies using 6 M urea can be purified under partially denaturing conditions by dilution to 2 M urea or 1 M ureaprior to chromatography on the approriate resin. Refolded fusion proteins can be affinity purified under native conditions using His•Tag, S•Tag, Strep•Tag II, and other appropriate affinity tags (e.g., GST•Tag, and T7•Tag).

2.13.7 Examples of successful cloning approaches

With reference to halophilic proteins particularly; maintenance of stability and activity in high salt is major challenge (Ueda *et al*., 2008; Wang *et al*., 2008). Most typical halophilic enzymes from extremely halophilic archaea and bacteria require high concentrations of salt for their activity and stability and are inactivated in Escherichia coli unless refolded in the presence of salts under in-vitro conditions. Recombinant DNA Technology in conjunction with many other molecular techniques is being used to improve and evolve enzymes leading to new opportunities for biocatalysis (Caralina, 2008; Battestein, 2007). Therefore, cloning of the potential genes coding for different enzymes would be an attractive approve to begin with.

Several examples are available in literature where successful cloning and expression has been analyzed; the gene encoding a ferredoxin of nucleoside diphosphate kinase from a moderately halophilic eubacterium was cloned and protein was over expressed in *E.coli*. Sequence analysis of the cloned gene revealed an open reading frame of 387 nucleotides encoding 129 amino acids. The deduced amino acid sequence of *Ha.Japonica* Fd showed 84 to 98% identity with the corresponding sequences of other extremely halophilic archaea (Matsuo *et al.,* 2001).

The extracellular-amylase-encoding amyH gene isolated from a moderate halophile, is understood to be the first extracellular-amylase with significant biotechnological potential. Besides, *H. meridiana* and *H. elongata* were also able to secrete the thermostable α-amylase from *Bacillus licheniformis*, indicating that members of the genus *Halomonas* could be good candidates for the production of heterologous extracellular enzymes.

Some alkaline protease-encoding bacterial genes have been cloned and expressed in new hosts, the two major organisms for cloning and over-expression being *E. coli* and *B. subtilis.* The gene of a highly thermostable alkaline protease from an alkaliphilic bacillus was cloned by PCR and nucleotide sequence was determined. Similarly, around 1242 base pair DNA fragment from *Bacillus halodurans* isolated from

alkaline sediments coding for a potential protease was cloned and sequenced (Zang *et al.,* 2008a and b). As deduced from amino acid sequence, it was an active monomer of 46.5 kDa (Calik *et al*., 2003). This recombinant F1 protease was efficiently secreted into the culture medium using *E. coli* harboring two vectors with its lac promoter–operator system. A new strain of *B. pumilis*, c172-14 (pBX96) was engineered by introducing the pBX 96 plasmid (carrying the α-amylase *amy* gene) into the host strain of alkalophilic *B. pumilis* c172 through transformation. The level of alkaline protease production was improved to 43% of new strain compared to the parent strain (Feng *et al.,* 2001). Another alkaline protease gene, *apr*, from *Bacillus licheniformis* 2709 was cloned into a *Bacillus* shuttle expression vector, pHL and expressed in *Bacillus subtilis* WB600. The expression of alkaline protease increased by 65% in the engineered strain BW-016 relative to the original strain (Tang *et al.,* 2004). The cloning, sequencing, and specific amplification of a protease gene on the chromosome of an alkaliphilic bacillus was carried out with increase in gene copy number by an improved gene amplification technique.

Further, the gene encoding ferric uptake regulator protein (fur gene) of *Vibrio* (2088bp fragment), encoding a protein of 147 amino acids, and homologous with fur, was identified, cloned and sequenced (Colquhouna, 2002). Earlier, to study gene expression in halophilic archaea, a reporter system was analyzed by β–glycosidase enzyme. The developments related to cloning and expression of the genes from halophilic organisms in heterologous hosts will certainly boost the number of enzyme-driven transformations in chemical, food, pharmaceutical and other industrial applications (Singh *et al.,* 2009).

The gene encoding the protease Nep from haloalkaliphilic archaeon Natrialba magadii was cloned and sequenced. The nep gene was expressed in Escherichia coli and Haloferax volcanii resulting in production of active Nep protease. The nep-encoded polypeptide had a molecular mass of 56.4 kDa, a pI of 3.77 and included a 121-amino acid propeptide not present in the mature Nep. The primary sequence of Nep was

closely related to serine proteases of the subtilisin family from archaea and bacteria (50–85% similarity).

The gene encoding a ferredoxin of nucleoside diphosphate kinase from a moderately halophilic eubacterium was cloned and protein was over expressed in *E.coli.* Sequence analysis of the cloned gene revealed an open reading frame of 387 nucleotides encoding 129 amino acids. The deduced amino acid sequence of Ha.Japonica Fd showed 84 to 98% identity with the corresponding sequences of other extremely halophilic archaea (Matsuo *et al*., 2001).

The member of halo tolerant alkaliphilic actinomycetes are currently in culture is limited. The challenge today is to isolate, purify and cultivate microorganisms that have been so far remained "uncultivated". The challenge will be to taxonomically classify them using both classical and molecular methods and exploit their genetic potential to yield novel bioactive molecules. So, it is of great value to discover new organisms from unexplored regions by developing suitable enrichment techniques and to study their unique biotechnological potential and molecular diversity.

Chapter 3

Isolation and Identification of Salt tolerant Alkaliphilic Actinomycetes along the Coastal Gujarat (India)

3.1 INTRODUCTION

Actinomycetes are widely distributed in natural and manmade environments. They are well known as a rich source of antibiotics and bioactive molecules, and are of considerable importance in industry. Filamentous soil bacteria belonging to the genus *Streptomyces* is continue to be a rich source of novel bioactive natural and commercially significant compounds representing some 70-80% of the all isolated compounds (Berdy *et al.,* 2006; El-Naggar *et al.,* 2006; Dietera *et al.,* 2003). These compounds were discovered by applying recent advances in understanding the genetics of secondary metabolism in actinomycetes and new screening technologies (Gullo *et al.,* 2006). *Streptomyces* are remarkable, and merit special consideration with regard to the morphological and metabolic differentiation phenomena they manifest during later stages of development. *Streptomyces* species generally synthesize a sizeable number of diverse natural secondary metabolites, the best known of which are antibiotics currently used worldwide as pharmaceutical and agrochemical products (Thumar *et al.,* 2010; Ben-Fguira *et al.,* 2005; Pamboukian *et al.,* 2004).

Saline soils appear to yield mostly halo tolerant rather than halophilic microorganisms, arguably reflecting adaptation to periodic changes of dilution (Quesada *et al*., 1990; Quesada *et al*., 1985). However, isolation of novel halophilic *Actinopolyspora* and *Nocardiopsis* species from saline soils in Death Valley (Calif.), Alicante, and Iraq (Yorkovsky and Silver 1997; Al-Tai and Ruan 1994) suggested that a wealth of interesting unknown halophilic microorganisms may be present in such saline soils. Similarly, many actinobacterial species have been isolated from soil collected from Xinjiang, China including *Nocardiopsis xinjiangensis* (Li *et al*., 2003a); *Prauserella halophila* and *Prauserella alba* (Li *et al.,* 2003b) and *Streptomonospora alba* (Li *et al*., 2003c). Adding together, *Saccharomonospora halophila,* able to grow at 20% salt, was isolated from Marsh soil of Kuwait (Al-Zarban *et al*., 2002). Many strains of moderately halophilic actinomycetes, belonging to genera; *Nocardiopsis, Kocuria and Saccharomonospora*, were isolated from these lakes (Li *et al*., 2003d; Chun *et al*., 2000). Therefore we tried to capture the diversity

profile of haloalkaliphilic actinomycetes by microbiological and molecular biology approaches.

While most of the attention related to diversity, phylogeny and bioactive molecules has focused on neutrophilic actinomycetes, only limited work is evident on these organisms from extreme environment. Although alkaliphilic bacteria are studied extensively, the similar account on alkaliphilic actinomycetes is quite rare. Recent findings from culture-dependent and culture-independent methods have demonstrated that there is tremendous diversity and novelty among the halo tolerant and alkaliphilic actinomycetes present in saline and alkaline environments. Members of the genus *Nocardiopsis* have been reported to predominate in saline or alkaline soils (Tang *et al.,* 2003). It is, therefore, necessary to focus attention to extreme actinomycetes, as a possible way to discover novel taxa and consequently, new secondary metabolites and biocatalysts. Besides, their diversity and phylogeny would be explored with the emphasis on their ecological significance.

In the present study, occurrence and diversity of novel halophilic/halotolerant and alkaliphilic actinomycetes from saline habitats along the coastal Gujarat have been investigated. The population heterogeneity in terms of extreme habitats, the morphological features, growth patterns, cultural and physiological characteristics and enzyme secretion were included as crucial parameters to judge the diversity, distribution and strength of actinomycetes from saline habitats along the coastal Gujarat (India).

3.2 MATERIALS AND METHODS

3.2.1 Sample collection

For the isolation of salt tolerant and alkaliphilic actinomycetes a series of 22 soil samples were collected from the diverse areas of coastal Gujarat, including 8 sites were Kurunga, Okha-madhi (A), Okha madhi (B), Okha, Mithapur, Tata disposal site and Somnath. The samples were collected in clean plastic bags; the pH and temperature of all the samples were measured manually at the time of the sample collection. The physical characteristics of the samples were also recorded. The collected samples were stored at 4 to 8°C immediately upon retrieval of the samples.

3.2.2 Isolation of salt tolerant alkaliphilic actinomycetes

Each sample was processed by mixing air dry soil with $CaCO_3$ in 1:1 ratio and leaved it at 28°C for 10 days. Heating of air dry soil sample for 2h at 60 to 65°C was done for the processing of samples prior to isolation, to eliminate bacterial growth. For the isolation, enrichment culture technique and standard serial dilution and plating techniques with dilutions at 1:100, 1:1000, and 1:10,000 were used in this study, for the purpose of isolating and quantifying actinomycetes. The processed soil samples were plated on actinomycetes isolation agar and oat meal agar (ISP-3) with (0-20% w/v NaCl and pH 9) for 3-4 weeks. In enrichment process processed soil was first inoculated to 25 mL of sterile actinomycetes broth (Hi Media Ltd) containing 0-20% w/v NaCl. The final pH of the medium was adjusted to 9 by adding separately sterilized 20% Na_2CO_3. The inoculated broth was incubated at 28°C under shaking conditions for 4 to 5 days and then enriched culture was plated with dilutions at 1:100, 1:1000, and 1:10,000 on actinomycetes agar plates and oat meal agar (ISP-3) with (0-20% w/v NaCl and pH 9). After incubation at 28°C for 3-4 week, a typical chalky white colony was picked and sub cultured until purification. These strains were maintained on ISP-2, ISP-3 and some on ISP-4 (5% w/v NaCl, pH 9) slant at 4°C and as 20% (w/v) glycerol suspensions at -20°C.

3.2.3 Identification of actinomycetes

Actinomycetes were identification based on their morphological, cultural and physiological characteristics. For that, the organisms were characterized on the basis of gram's reaction, light microscopy scanning electron microscopy, in situ characterization of isolates as well as cultural and physiological characterization.

3.2.3.1 Morphological characterization

Morphological methods consist of macroscopic and microscopic methods. The detailed cell morphology and growth behavior of the isolates, at different age of their growth, were studied by light and scanning electron microscopic (SEM) examinations. Light microscopy of different isolates was carried out using zeiss microscope attached with computer. Scanning electron microscopy was performed from the cultures of different age. The colony was directly suspended in a drop of sterile D/W and observed under the scanning electron microscope. The microscopic characterization was also done by cover slip slide culture method by inoculating a loopful of spore onto thin film of 3% ISP medium-2 which was earlier being taken on sterile glass slide surface and then it was covered by sterile cover slip to facilitate direct observation under microscope. The cultures were incubated at 28°C and examined periodically for the formation of aerial mycelium, spore-bearing hyphae and spore chains by using direct microscopic examination of the culture surface.

3.2.3.2 Cultural characterization

Cultural characteristics were determined after 4 weeks at 28°C by using the International Streptomyces Project (ISP) methods (Shirling & Gottlieb, 1966). All purified isolates were transferred onto yeast extract-malt extract agar (ISP-2)/streptomycetes agar, oatmeal agar (ISP-3), inorganic salt starch agar (ISP-4), glycerol-asparagine agar (ISP-5), actinomycetes isolation agar (AIA), starch casein agar (SCA) and starch agar (SA) and incubated for 14 days at 28°C. The different media were supplemented with 5% NaCl (w/v) at pH 9.

3.2.3.3 Physiological characterization

Physiological features were observed on media commonly used for characterization of *Streptomyces* species (Shirling & Gottlieb, 1966) prepared with 5% NaCl. The media and procedures used for physiological features and carbon source utilization of the isolates were those described by Shirling & Gottlieb (1966) and Locci (1989). Various biochemical tests such as ammonia production, nitrate reduction, methyl red, catalase, oxidase were performed in presence of 5% w/v NaCl. To determine hydrolytic properties of actinomycetes, gelatine hydrolysis, starch hydrolysis, casein hydrolysis and lipid hydrolysis was done by adding gelatine, starch, casein and tributyrine for respective tests. In addition to substrate, each plate also contained 0.5% peptone, 0.5% yeast extract, 10% NaCl and pH 9. Plates were then incubated at 37°C for 4 days to observe the results. Similarly, fermentation of sugars such as glucose, arabinose, mannitol, xylose, raffinose, rhamnose, sucrose, galactose and fructose was studied.

3.2.3.4 16S rRNA identification

For 16S rRNA gene sequencing, genomic DNA was isolated from the pure cultures of OM-6, OK-5 and OK-7 strains and subjected to high-fidelity 16S rRNA amplification using consensus universal primers designed. The PCR product was bi-directionally sequenced using the forward, reverse and an internal primer. Sequence data was aligned and analyzed for closest homologous for the actinomycetes using Mega 3.1 by neighbor joining method.

3.2.4 Salt and pH profile

To check salt and pH profile, all isolates were grown on gelatin agar containing (g/liter); gelatin, 30; peptone, 10; yeast extract, 10; NaCl, 0-200 and agar, 30. The pH of the medium was adjusted to 9 by adding separately autoclaved Na_2CO_3 (20% w/v). Similarly for pH profiling salt concentration of agar medium was remained 5% while pH of the medium was adjusted to 7-11.

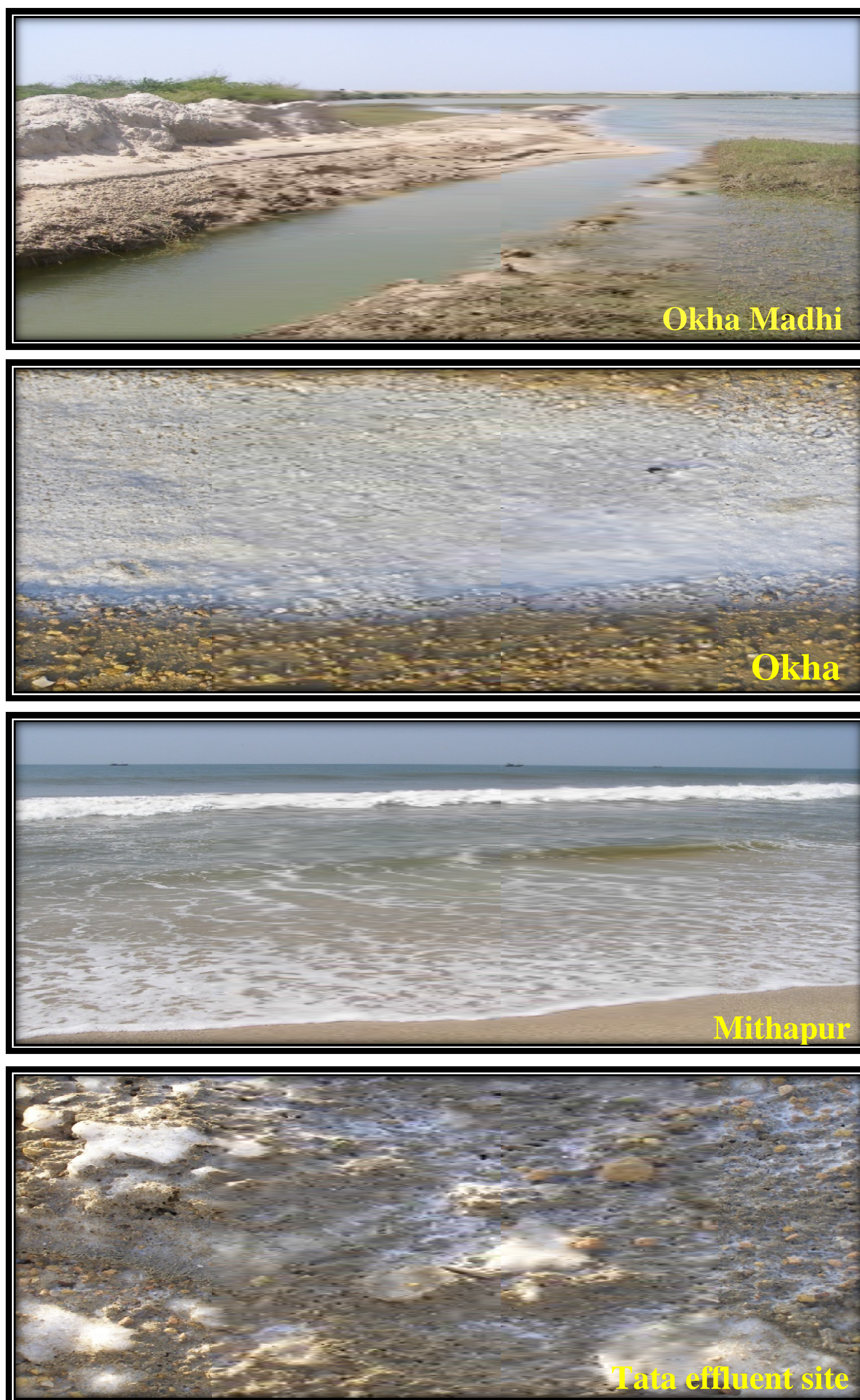
3.3 RESULTS

3.3.1 Sample collection

A series of 22 soil samples collected from the diverse areas of the coastal region of Gujarat, including 8 sites were Kurunga, Okha-madhi(A), Okha madhi(B), Okha, Mithapur, Tata disposal site and Somnath (Picture 3.1). The pH of the samples varied from 8-10 and the temperature was around 20-30°C at the time of collection. Total twenty two soil samples were differentiated mainly on the basis of color of soil and texture of soil samples that whether it is in stone/crystallized form or it is smooth, whether it is particulate or it is powdery. The detailed description of the samples is given in Table 3.1.

3.3.2 Isolation of salt tolerant alkaliphilic actinomycetes

A series of 22 soil samples collected from the diverse areas of the coastal region of Gujarat, including 8 sites were Kurunga, Okha-madhi(A), Okha madhi(B), Okha, Mithapur, Tata disposal site and Somnath. Among 40 actinomycetes isolates, 12 were from Okha-Madhi (OM-1 to 12), 10 from Okha (OK-1 to 10), 8 from Mithapur (Mit-1 to 8) and 10 isolates from Tata disposal site (Tata-1 to 10) were isolated having tolerance to different conditions of pH and salinity. For each isolate, type of soil sample, dilution of soil sample and concentration of salt at which isolates gained is mentioned in Table 3.2. Though majority of isolates were observed at 0% NaCl (w/v), they can tolerate up to 10-15% NaCl (w/v) indicating halo-tolerant rather than halophilic nature of the isolates. Forty isolates putatively assigned to the actinomycetes on the basis of their tough, leathery colony, branched vegetative mycelia, and when present aerial mycelia, spore formation, and checked for purity by microscopic examination of gram-stained smears.



Picture 3.1: Photographs represent salt-enriched soil collected from Okha Madhi, Okha, Mithapur and Tata effluent sites Gujarat, India

No.	Sample collection Site	No. of samples	Description
1.	Kurunga	1	Lotus site, low salt concentration
2.	Okha-madhi	2.1	Flowing water, high salt concentration
		2.2	Stagnant water, high salt concentration
		2.3*	Hard soil, dull white
		2.4*	Salty soil, black after first layer
		2.5*	Powdery soft soil, dark sandy color
3.	Okha	3.1	High salt conc., flowing water
		3.2	Static water
		3.3*	Sandy soil, brownish
		3.4	Flowing water
		3.5*	Big crystalline sand
		3.6^{Δ}	Stone with pigmented growth
4.	Mithapur	4.1*	black colored hard crystalline soil
		4.2*	Brownish powdery soil
		4.3	Water having high salt conc.
5.	Tata-disposal site	5.1	High salt effluent
		5.2*	Light brown soft soil(smooth)
		5.3*	soft and light brownish greenish soil
		5.4*	Blackish particulate dry soil,
		5.5*	Smooth black soil
6.	Okha-madhi	6.1	Water with pinkish color tinch
		6.2*	Crystalline soil collected from red ring
		6.3*	Soil from the layer after red ring
		6.4^{Δ}	Grayish porous hard soil with salt layer
		6.5*	Sticky and smooth mud soil
		6.6*	Reddish brown particulate soil
		6.7	Flowing water
		6.8	Water from non- pinkish area
		6.9^{Δ}	salty hard and porous grayish black stone
		6.10*	Creamish, sticky soil
		6.11*	Grayish mud soil
		6.12	salt crystals
		6.13^{Δ}	Hard salt crystals like stone
7.	Madhavpur	7.1	From petrol pump on porbandar highway
8.	Somnath	8.1	Somnath sea
		8.2*	Brownish black soil

∗ - soil samples
Δ - stone samples
No sign indicates water samples

Table 3.1: Description of sites and samples

Isolates	Soil Samples	Dilution	NaCl (%)	Isolates	Soil Samples	Dilution	NaCl (%)
	Okha Madhi				**Mithapur**		
OM-1	2.4*	C-10^{-4}	0	Mit-1	4.1*	T-10^{-2}	0
OM-2	2.5*	T-10^{-4}	0	Mit-2	4.1*	T-10^{-2}	0
OM-3	2.5*	C-10^{-4}	0	Mit-3	4.2*	C	0
OM-4	2.4*	T-10^{-4}	5	Mit-4	4.2*	T-10^{-2}	0
OM-5	2.5*	T-10^{-4}	5	Mit-5	4.2*	T-10^{-2}	0
OM-6	2.5*	T-10^{-4}	5	Mit-6	4.2*	T-10^{-2}	0
OM-7	2.3*	T-10^{-4}	10	Mit-7	4.2*	C-10^{-2}	5
OM-8	2.5*	C	10	Mit-8	4.1*	T	0
OM-9	2.4*	C-10^{-4}	10	Mit-1	4.1*	T-10^{-2}	0
OM-10	2.3*	T-10^{-4}	5	Mit-2	4.1*	T-10^{-2}	0
OM-11	2.4*	T-10^{-4}	5		**Tata effluent**		
OM-12	2.3*	T-10^{-4}	5	Tata-1	5.3*	T	0
	Okha			Tata-2	5.3*	T	0
OK-1	3.3*	T-10^{-2}	0	Tata-3	5.5*	T	0
OK-2	3.3*	T-10^{-2}	0	Tata-4	5.5*	T	0
OK-3	3.3*	T-10^{-2}	0	Tata-5	5.5*	T-10^{-2}	0
OK-4	3.6▲	T-10^{-4}	0	Tata-6	5.5*	T-10^{-2}	0
OK-5	3.3*	T	5	Tata-7	5.5*	T-10^{-2}	0
OK-6	3.5*	C-10^{-2}	5	Tata-8	5.4*	T-10^{-2}	0
OK-7	3.5*	C-10^{-2}	5	Tata-9			5
OK-8	3.5*	T-10^{-4}	5	Tata-10	5.4*	T-10^{-2}	5
OK-9	3.6▲	T	10	Tata-11	5.2*	T	5
OK-10	3.6▲	T-10^{-4}	10	Tata-12	5.3*	T	5
				Tata-13	5.3*	T	5
				Tata-14	5.3*	T	5
				Tata-15	5.3*	T	5

C-10^{-4}**:** Control (soil sample) without $CaCO_3$ treatment (10,000times dilution)

T-10^{-4}**:** Test (soil sample) with $CaCO_3$ treatment (10,000times dilution)

C **:** Control without $CaCO_3$ (no dilution)

T **:** Test with $CaCO_3$ (no dilution)

Table 3.2: Isolation conditions including soil characteristics, dilution of soil sample and salt concentration at the time of isolation for each isolates

3.3.3 Identification of actinomycetes

All isolated were identified as actinomycetes based on their morphological, cultural and physiological characteristics as described below.

3.3.3.1 Morphological characterization

3.3.3.1.1 Light microscopy

The morphological characterization of the organisms was based on colony and cell morphology, gram's reaction and morphogenesis. Colony characteristics were recorded after growing the organisms on starch agar (5% w/v, NaCl; pH 9) until sporulation (Table 3.3). The cellular morphology and growth behavior of few isolates was further studied by light microscopy. The light microscopic examination of isolates revealed their gram positive character having long filamentous structure as well fragmentation of hyphae (Picture 3.2).

3.3.3.1.2 Scanning electron microscopy

Morphological characteristics of some selected isolates were observed by scanning electron microscopy. OM-4, OK-5, Tata 5 and Tata 13 were observed by scanning electron microscopy after 15 days of growth on ISP-2 medium agar containing 5% NaCl (w/v) and pH 9. The filamentous mycelia structure as well as long chain of smooth ovel shaped spores of isolates can be observed in Picture 3.3. Interestingly, there was the deposition of the salt crystals on the spore chain of Tata 5 and Tata 13 as well as on the vegetative mycelia of OM-4 with scanning electron microscopy.

3.3.3.1.3 *In-Situ* observation of colony morphology

In-situ observation of the colony development reflected diversity in terms of the structure and growth behavior of the isolates. OM-1, OM-6 and OM-8 started sporulation in starch agar medium (5%, NaCl; pH 9) after 10 days of incubation (Picture 3.4). The spores were oval to elongated having smooth surface and arranged in chain followed by fragmentation in short chains of spores. Interestingly, OM-1 and OM-6 displayed curved shaped mycelia, a typical characteristic of genus

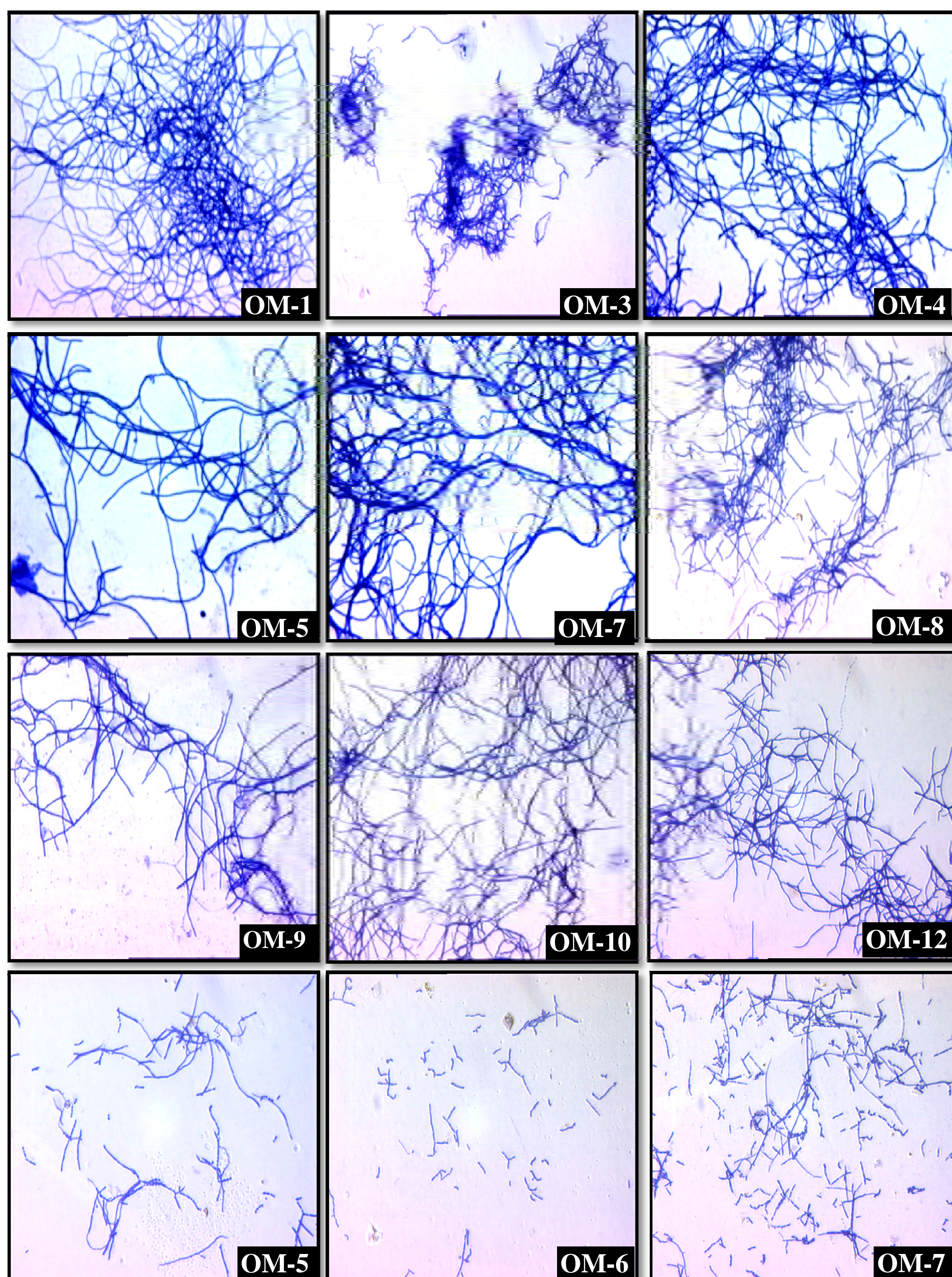
Streptomyces. On the other hand, OM-8 displayed relatively long and strait mycelia. After 4 days of inoculation fragmentation of mycelia was observed in both isolates and after 10 days oval shaped spores were observed producing brown colored pigmentation in OM-1, while light blue colored road shaped spores in OM-8 arranged in the long chain of spores.

3.3.3.2 Cultural characterization

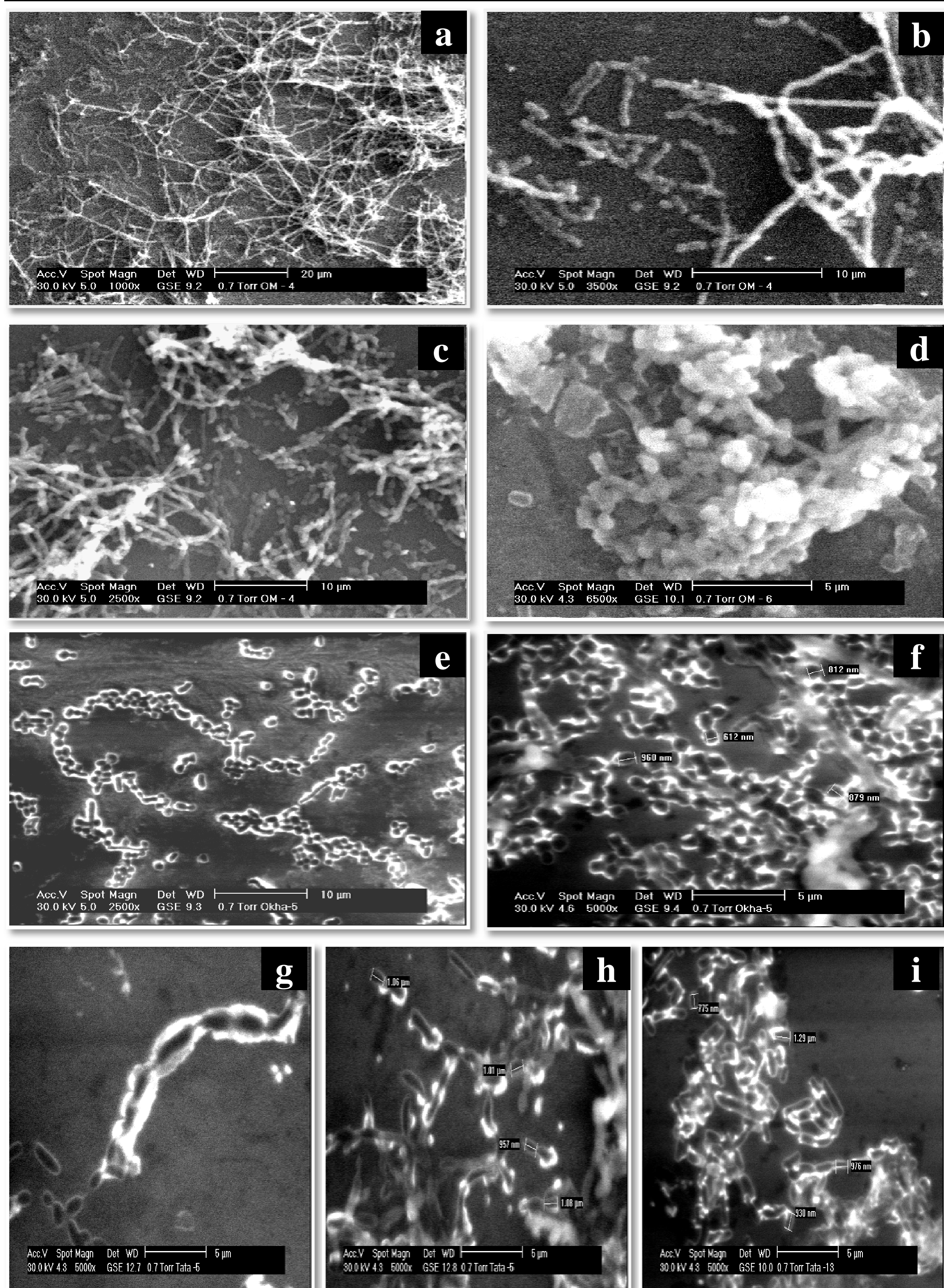
Cultural characteristics of most active actinomycetes were identified according to the protocol suggested by the International Streptomyces Project (Shirling & Gottlieb., 1966). Total 40 isolates were transferred on to yeast extract-malt extract agar (ISP-2)/streptomycetes agar, oatmeal agar (ISP-3), inorganic salt starch agar (ISP-4), glycerol-asparagine agar (ISP-5), actinomycetes isolation agar (AIA), starch casein agar (SCA) and starch agar (SA) and incubated for 14 days at 28°C. Plates were examined by eye for the state of growth, aerial spore mass color, substrate mycelia color and pigment production (Picture 3.5, 3.6, 3.7). The growth behavior of actinomycetes was examined on different cultural media specific for actinomycetes and *Streptomyces*. Almost all strains of actinomycetes were able to grow well on most of the tested organic and synthetic media. Typically, some of the colonies were elevated, spreading and were covered with gray to light pink and light blue colored aerial mycelia and spores. The spores were in short to long chains, having evidenced smooth surfaces and were morphologically spherical to cylindrical. Most of the pigmented colonies developed on ISP-2 and ISP-3 media followed by starch casein agar and starch agar (Table 3.4, 3.5, 3.6, 3.7).

Isolates	Size (mm)	Shape	Margin	Texture	Elevation	Consistency	Opacity	Earthy aroma
OM-1	4	Round	Entire	Rough	Raised	Powdery	Opaque	+
OM-2	2	Round	Entire	Smooth	Slightly raised	Rigid	Opaque	-
OM-3	2	Round	Entire	Smooth	Slightly raised	Rigid	Opaque	-
OM-4	8	Round	Irregular	Smooth	Raised	Rigid	Opaque	-
OM-5	3	Round	Entire	Rough	Raised	Powdery	Opaque	-
OM-6	4	Round	Entire	Rough	Slightly raised	Powdery	Opaque	-
OM-7	4	Round	Entire	Rough	Raised	Rigid	Opaque	-
OM-8	4	Round	Entire	Smooth	Raised	Rigid	Opaque	-
OM-9	4	Round	Entire	Rough	Raised	Powdery	Opaque	-
OM-10	3	Round	Entire	Rough	Slightly raised	Powdery	Opaque	+
OM-11	5	Round	Entire	Rough	Raised	Powdery	Opaque	-
OM-12	4	Round	Irregular	Smooth	Slightly raised	Powdery	Opaque	-
OK-1	3	Round	Entire	Smooth	Slightly raised	Powdery	Opaque	-
OK-2	3	Round	Entire	Smooth	Slightly raised	Powdery	Opaque	-
OK-3	5	Round	Entire	Smooth	Slightly raised	Smooth	Opaque	-
OK-4	7	Round	Irregular	Smooth	Raised	Powdery	Opaque	+
OK-5	8	Irregular	Irregular	Rough	Raised	Powdery	Opaque	-
OK-6	6	Round	Irregular	Rough	Raised	Powdery	Opaque	-
OK-7	8	Round	Irregular	Rough	Raised	Powdery	Opaque	+
OK-8	6	Round	Entire	Rough	Raised	Powdery	Opaque	-
OK-9	8	Round	Entire	Rough	Slightly raised	Powdery	Opaque	-
OK-10	8	Round	Entire	Rough	Raised	Powdery	Opaque	-
Mit-1	2	Irregular	Irregular	Rough	Slightly raised	Rigid	Opaque	+
Mit-2	6	Round	Entire	Smooth	Slightly raised	Powdery	Opaque	+
Mit-3	6	Irregular	Irregular	Rough	Slightly raised	Rigid	Opaque	-
Mit-4	8	Round	Entire	Smooth	Slightly raised	Rigid	Opaque	+
Mit-5	2	Round	Entire	Rough	Slightly raised	Powdery	Opaque	+
Mit-6	6	Round	Entire	Rough	Slightly raised	Powdery	Opaque	+
Mit-7	5	Round	Entire	Smooth	Raised	Powdery	Opaque	-
Mit-8	2	Round	Entire	Smooth	Slightly raised	Powdery	Opaque	+
Tata-1	2	Round	Irregular	Rough	Slightly raised	Rigid	Opaque	-
Tata-2	2	Round	Irregular	Rough	Slightly raised	Rigid	Opaque	+
Tata-3	3	Irregular	Irregular	Rough	Slightly raised	Powdery	Opaque	-
Tata-4	4	Irregular	Entire	Rough	Slightly raised	Rigid	Opaque	+
Tata-5	8	Round	Irregular	Smooth	Slightly raised	Powdery	Opaque	+
Tata-6	2	Round	Entire	Smooth	Raised	Powdery	Opaque	+
Tata-7	7	Irregular	Irregular	Rough	Raised	Powdery	Opaque	-
Tata-8	4	Round	Entire	Rough	Raised	Powdery	Opaque	-
Tata-9	2	Round	Entire	Rough	Raised	Powdery	Opaque	-
Tata-10	4	Round	Irregular	Rough	Raised	Powdery	Opaque	-
Tata-11	2	Round	Entire	Rough	Slightly raised	Powdery	Opaque	-
Tata-12	2	Round	Entire	Rough	Raised	Powdery	Opaque	-
Tata-13	3	Round	Entire	Rough	Slightly raised	Powdery	Opaque	-
Tata-14	2	Round	Irregular	Rough	Slightly raised	Powdery	Opaque	-

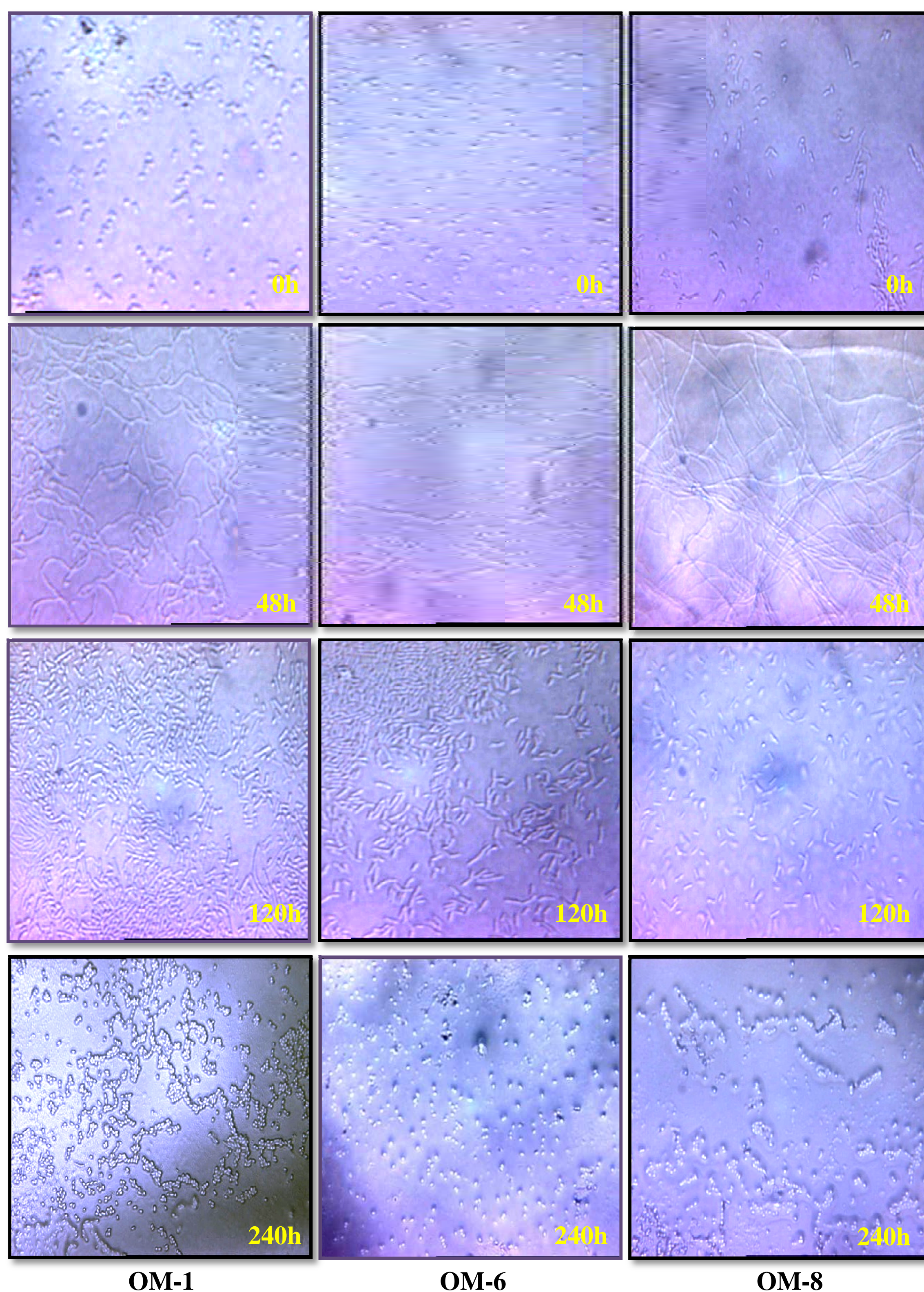
Table 3.3: Colony characteristics of all isolates



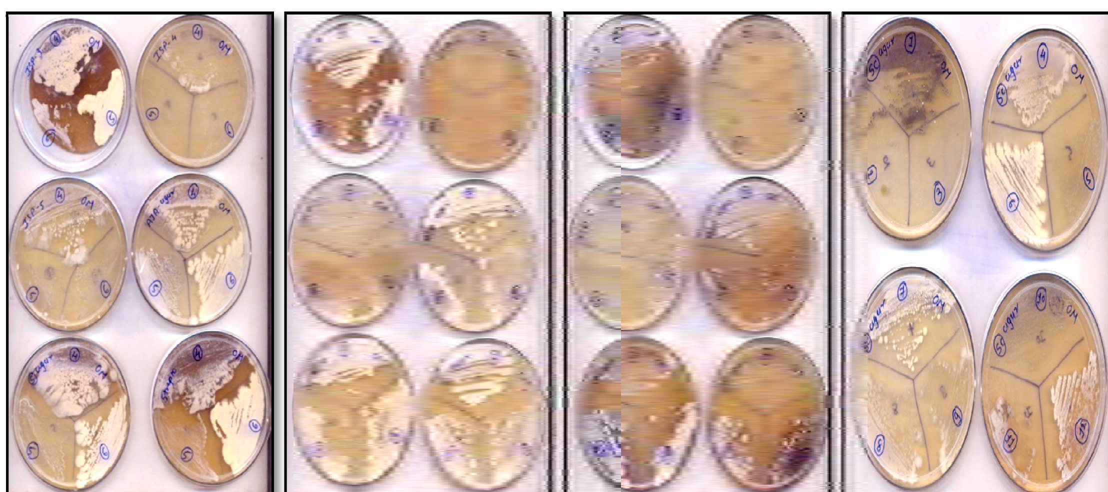
Picture 3.2: Light microscopic examination (1000x) of isolates form Okha Madhi after gram's staining



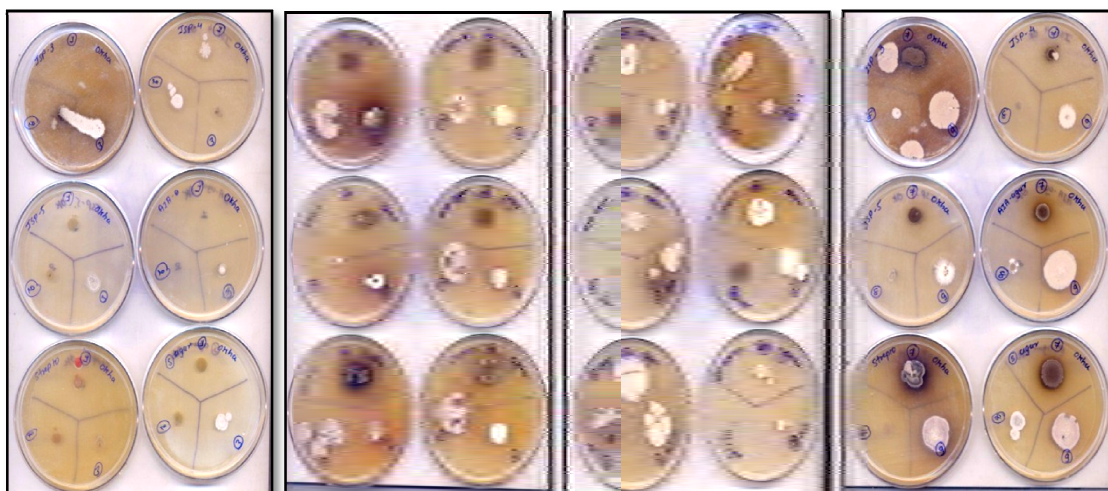
Picture 3.3: SEM analysis of actinomycetes from different sites demonstrating a) vegetative mycelia of OM-4; b, c, d) vegetative mycelia with spore chain; e, f, g, h, i) spore chain and fragmented spores



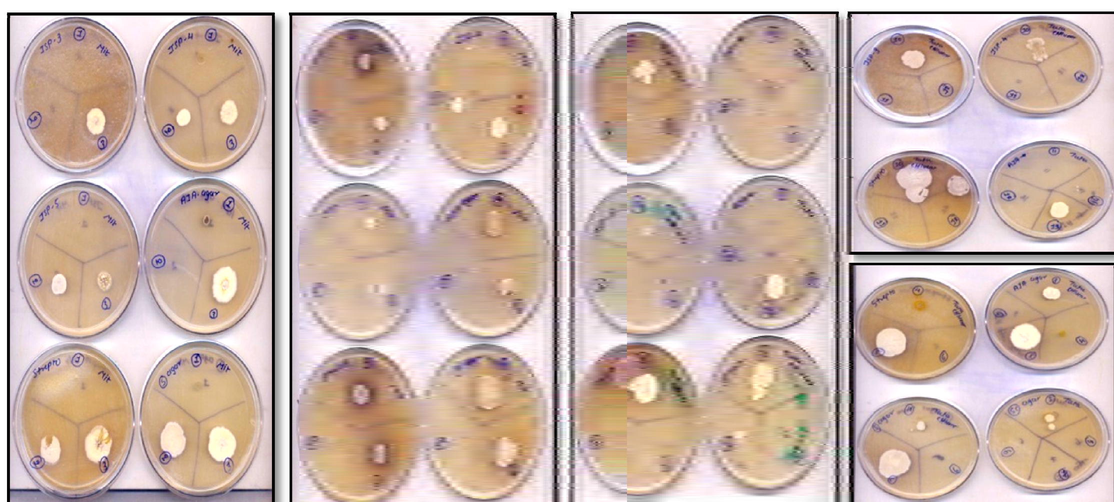
Picture 3.4: In situ study of OM-1, OM-6 and OM-8 isolates under oil immersion lense (1000X). Growth after 48h showing typical, curved and strait mycelia; growth after 120h, fragmentation of mycelia; growth after 240 days, mature spores



Picture 3.5: Cultural characterization of actinomycetes from Okha Madhi



Picture 3.6: Cultural characterization of actinomycetes from Okha



Picture 3.7: Cultural characterization of actinomycetes from Mithapur and Tata effluent site

Isolates	Yeast extract –malt extract agar (ISP 2)				Oatmeal agar (ISP 3)			
	Growth	**Aerial mycelia**	**Reverse side**	**Pigmen-tation**	**Growth**	**Aerial mycelia**	**Reverse side**	**Pigmen-tation**
Okha Madhi (OM)								
OM-1	Good	Dark gray	Dark brown	Dark Brown	Good	Dark gray	Brown	Dark brown
OM-2	-	-	-	-	Moderate	Pinkish red	Light red	-
OM-3	-	-	-	-	Good	Gray	Greenish yellow	-
OM-4	Good	Pinkish white	Brown	-	Good	Creamish white	Brown	-
OM-5	Poor	Light bluish white	Light creamish	-	Moderate	Bluish grayish white	Creamish yellow	-
OM-6	Good	Yellowish Creamish white	Creamish yellow	-	Good	Yellowish Creamish white	Light brownish	-
OM-7	Good	Yellowish bluish white	Creamish yellow	-	Good	Yellowish Creamish white	Creamish brown	-
OM-8	Good	yellowish	Light creamish	-	Moderate	Bluish white	Light creamish	-
OM-9	Good	Yellowish Creamish white	Creamish yellow	-	Good	Yellowish Bluish white	brownish yellow	-
OM-10	Moderate	Light bluish white	Creamish yellow	-	Good	Grayish bluish white	Light creamish yellow	-
OM-11	Good	Grayish white	Light greenish		Good	grayish white	Greenish brown	
OM-12	Good	Grayish white	Light greenish		Good	Gray	Light greenish	Greenish brown
Okha (OK)								
OK-1	Poor	Creamish	Creamish	-	-	-	-	-
OK-2	Poor	Creamish	Creamish	-	Good	Grayish white	Brownish	-
OK-3	Poor	Light creamish	Creamish	-	Good	Light bluish	Creamish	-
OK-4	Good	Grayish white	Greenish brown	-	Good	Dark Grayish	greenish brown	brown
OK-5	Good	White	Creamish brown	-	Good	pinkish white	Pinkish cream	-
OK-6	Good	Creamish white	Creamish red	-	Good	White	yellowish brown	-
OK-7	Good	Light grayish white	Dark greenish brown	Dark brown	Good	Dark gray	Dark greenish black	Dark brown
OK-8	Poor	Creamish	Creamish white	-	Good	bluish white	Creamish	-
OK-9	Good	Pinkish Grayish white	Light creamish brown	-	Good	Light pinkish white	Light creamish brown	-
OK-10	Good	Grayish white	Light grayish white		Moderate	Yellowish Creamish white	Dark creamish	-
Mithapur (Mit)								
Mit-1	-	-	-	-				
Mit-2	Good	White	Light brownish yellow					
Mit-3	Good	grayish white	Light brownish yellow		Good	Yellowish Creamish white	Dark brownish yellow	
Mit-4	Good	Light grayish white	Dark greenish brown	Light brown	Good	Light grayish white	Dark green	Dark green
Mit-5	-	-	-	-	-	-	-	-
Mit-6	Good	Light grayish white	Dark greenish brown	Light brown	Good	White	Light Grayish creamish	-
Mit-7	Good	White	Dark brown	-	Good	Grayish white	greenish brown	-
Mit-8	Moderate	Gray	Brown	-	-			
Tata effluent (Tata)								
Tata-4	Poor	Yellow	Creamish yellow	Creamish yellow	-	-	-	-
Tata-10	Good	Grayish white	Brownish	-	-	-	-	-
Tata-13	Good	white	Brown	-	-	-	-	-

Table 3.4: Comparison of growth and cultural characteristics of actinomycetes isolates on yeast extract – malt extract agar (ISP2) and oatmeal agar (ISP3)

Isolates	Inorganic salts starch agar (ISP 4)				Glycerol asparagine agar (ISP 5)			
	Growth	Aerial mycelia	Reverse side	Pigmen-tation	Growth	Aerial mycelia	Reverse side	Pigmen-tation
Okha Madhi (OM)								
OM-1	Good	Grayish white	Dark greenish	greenish brown	Good	Light gray	Light greenish yellow	-
OM-2	-	-	-	-	-	-	-	-
OM-3	-	-	-	-	-	-	-	-
OM-4	Poor	White	Creamish	-	Good	White	Creamish yellow	-
OM-5	Poor	Creamish	Light creamish	-	Poor	Light bluish	Light creamish	-
OM-6	Poor	White	Light creamish	-	Poor	White	Light creamish	-
OM-7	Poor	Creamish white	Creamish	-	Moderate	Grayish White	Creamish	-
OM-8	Poor	Light bluish white	Light creamish yellow	-	Poor	Grayish white	Light creamish	-
OM-9	Poor	Creamish	Creamish	-	Poor	Grayish white	Creamish	-
OM-10	Poor	Creamish	Creamish	-	Moderate	Light bluish white	creamish	-
OM-11	Poor	Creamish	Creamish	-	-	-	-	-
OM-12	Poor	Creamish	creamish	-	Moderate	White	Creamish	-
Okha (OK)								
OK-1	Good	Light bluish white	Creamish	-	Moderate	Cream	Creamish	-
OK-2	Good	Grayish white	Creamish	-	Poor	Creamish	Light creamish	-
OK-3	Poor	Creamish	Light creamish	-	Moderate	Light blueish white	Light creamish	-
OK-4	Good	Grayish white	Dark greenish	Greenish yellow	Good	Grayish white	Greenish	Greenish yellow
OK-5	Good	Light pinkish white	Pinkish creamish brown	-	Moderate	Creamish white	Creamish	-
OK-6	Good	Yellowish creamish white	Creamish	-	Good	Creamish White	Dark brown	Light brown
OK-7	Moderate	Dark gray	Dark Green	-	Good	Dark gray	Dark green	Light green
OK-8	Poor	Light creamish	Light creamish	-	Poor	Creamish white	Light creamish white	-
OK-9	Good	White	Creamish white	-	Good	White	Creamish	-
OK-10	Good	Yellowish Creamish white	Creamish	-	Good	Creamish white	Creamish brown	Yellow
Mithapur (Mit)								
Mit-1	Poor	Light creamish	Light creamish		-	-	-	-
Mit-2	Good	Creamish White	Light creamish	-	-	Light grayish white	Creamish brown	-
Mit-3	Good	Creamish, Yellowish white	Light brownish yellow		Good	Creamish white	Dark brownish yellow	-
Mit-4	Poor	Creamish	Light creamish	-	Moderate	Light bluish white	Light creamish brown	-
Mit-5	Good	White	Creamish white	-	-	-	-	-
Mit-6	Good	Creamish white	Creamish	-	Moderate	White	Creamish white	-
Mit-7	Good	Light bluish white	Creamish	-	Good	Light bluish white	Light creamish	-
MIT-8	Good	Dark gray	Dark greenish	Light greenish	Moderate	Dark gray	light greenish	-
Tata effluent (Tata)								
Tata-10	Moderate	White	Creamish white	-	-	-	-	-
Tata-13	-	-	-	-	Moderate	White	Brown	-

Table 3.5: Comparison of growth and cultural characteristics of actinomycetes isolates on inorganic salts starch agar (ISP4) and glycerol asparagine agar (ISP5)

Isolates	Starch casein agar				Actinomycetes isolation agar			
	Growth	Aerial mycelia	Reverse side	Pigmen-tation	Growth	Aerial mycelia	Reverse side	Pigmen-tation
Okha Madhi (OM)								
OM-1	Good	Dark grayish	Greenish yellow	greenish yellow	-	-	-	-
OM-2	-	-	-	-	-	-	-	-
OM-3	-	-	-	-	-	-	-	-
OM-4	Moderate	Grayish white	Creamish	-	Good	Creamish white	creamish yellow	-
OM-5	Poor	Creamish yellow	Light creamish	-	Moderate	Light bluish white	Light creamish yellow	-
OM-6	Good	yellowish white	Creamish yellow	-	Good	Creamish white	Creamish yellow	-
OM-7	Good	Creamish white	Light Creamish yellow	-	Good	Yellowish Creamish white	Creamish yellow	-
OM-8	Poor	grayish white	Creamish	-	Moderate	bluish white	creamish yellow	-
OM-9	Poor	grayish white	yellowish	-	Good	bluish white	Creamish yellow	-
OM-10	-	-	-	-	Good	bluish white	Creamish yellow	-
OM-11	Poor	Grayish white	Creamish yellow	-	Good	Bluish, creamish white	Creamish yellow	-
OM-12	Moderate	Creamish	Creamish	-	Good	Bluish white	Creamish yellow	-
Okha (OK)								
OK-1	-	-	-	-	-	-	-	-
OK-2	-	-	-	-	-	-	-	-
OK-3	Poor	Creamish	Creamish	-	Moderate	white	Creamish	-
OK-4	Moderate	Grayish white	Green	Greenish yellow	Good	Grayish white	Green	Greenish yellow
OK-5	Good	White	Cream	-	Good	Pinkish white	Creamish brown	-
OK-6	Good	Creamish white	Dark brownish black	Light brown	Good	Creamish white	Reddish brown	-
OK-7	Poor	Creamish white	Creamish white	-	Good	Grayish white	Dark brownish black	Brownish Yellow
OK-8	Poor	Creamish	Creamish	-	Poor	Creamish white	Creamish	-
OK-9	Good	Creamish white	Creamish	-	Good	Light pinkish white	Light Creamish brown	-
OK-10	Good	White	creamish brown		Moderate	Creamish white	Creamish	
Mithapur (Mit)								
Mit-1	-				Moderate	Dark gray	Gray	-
Mit-2	Good	White	Light creamish	-	-			
Mit-3	Moderate	Creamish yellowish white	Creamish		Good	Grayish Yellowish creamish white	Creamish yellow	-
Mit-4	Good	White	Creamish white	-	Good	Grayish white	Dark brown	Light yellow
Mit-5	-	-	-	-	-	-	-	-
Mit-6	Poor	Light Creamish	Light Creamish	-	Good	Grayish white	Dark brown	Light yellow
Mit-7	Good	White	Light brownish Creamish	-	Good	Dark gray	Dark brown	Light brown
MIT-8	poor	Light grayish	Creamish	-	Poor	White	Creamish	-
Tata effluent (Tata)								
Tata-2	-	-	-	-	Good	Dark pink	Dark pink	-
Tata-3	-	-	-	-	Good	White	Creamish white	-
Tata-5	Good	Yellowish Creamish white	Brownish yellow	-	Good	Yellowish Creamish white	Dark brown	-
Tata-6	-	-	-	-	Good	Grayish white	Creamish gray	-
Tata-7	-	-	-	-	Moderate	Yellow	Creamish yellow	-
Tata-8	-	-	-	-	Good	Dark creamish	Creamish	-
Tata-10	-	-	-	-	-	-	-	-
Tata-13	-	-	-	-	Good	Yellowish white	Dark brown	Light yellow

Table 3.6: Comparison of growth and cultural characteristics of actinomycetes isolates on starch casein agar and actinomycetes isolation agar

Isolates	Starch agar							
	Growth	Aerial mycelia	Reverse side	Pigmentation	Growth	Aerial mycelia	Reverse side	Pigmentation
Okha Madhi (OM)					**Okha (OK)**			
1	Good	Light grayish white	Creamish yellow	-	Poor	Cream	Cream	-
2	-	-	-	-	Poor	Cream	Cream	-
3	-	-	-	-	Good	White	Cream	-
4	Good	Light pinkish bluish	Light brown	-	Good	Grayish white	Green	Light brown
5	Poor	White	Creamish yellow	-	Good	Light pinkish white	Pinkish creamish brown	-
6	Good	Creamish grayish white	Creamish yellow	-	Good	White	Creamish red	Light yellow
7	Good	Yellowish creamish white	Dark creamish yellow	-	Good	Dark gray	Dark brown	Brownish yellow
8	Good	Light grayish creamish white	Light creamish yellow	-	Good	Light bluish white	Light creamish	-
9	Moderate	Light grayish creamish white	Light creamish yellow	-	Good	Light pinkish white	Creamish white	-
10	Moderate	Light bluish white	Light creamish	-	Good	Bluish creamish white	Creamish	-
11	Good	Grayish white	Creamish brown					
12	Moderate	creamish Grayish white	Creamish brown	Light brown				
Mithapur (Mit)					**Tata effluent (Tata)**			
1	-	-	-	-				
2	Good	Good	White	Creamish	-	-	-	-
3	Good	Yellowish white	Light creamish yellow	-	-	-	-	-
4	Good	Grayish white	Creamish	Light yellow	Good	White	Creamish	-
5	-	-	-	-	Good	Grayish white	Dark brown	-
6	Good	Grayish white	Brown	Light brown	-	-	-	-
7	Good	Yellowish creamish white	Creamish white	-				
8	Moderate	Dark gray	Dark brown	-	-	-	-	-
10				Creamish	Good	Yellowish white	Brownish	-
13					Good	Yellowish white	Dark brown	Light yellow
14					Good	Yellowish white	Brownish	-

Table 3.7: Comparison of growth and cultural characteristics of actinomycetes isolates on starch agar

2.3.3.3 Physiological characterization

For further differentiation and characterization, biochemical and metabolic activities of all the isolates were studied. However, extent of the result varied among the isolates. Figure 3.1A and 3.1B represents primary graphs of biochemical test

performed as well as sugar utilization respectively from the isolates of Okha Madhi and Okha sites. Besides, the maximum positive reaction obtained with ammonia production, triple sugar iron (TSI) fermentation, catalase and oxidase (100%) followed by nitrate reduction (91%), H_2S production (18%) and methyl red (9%) while none of the isolates under study produced indol or utilized urea (Figure 3.2A). Among the twelve cultures tested for hydrolysis, 100% of them displayed strong proteolytic activity on gelatin agar while 91% isolates displayed hydrolytic activity with casein, starch and cellulose indicating that ability to hydrolyze different substrates is consistent trait among the strains. However, only 18% isolates displayed moderate to weak ability to hydrolyze lipid. The extent of sugar utilization was highly varied among the isolates of Okha-Madhi site. Maximum isolates were able to ferment fructose extensively (92%) followed by glucose (83%), xylose (50%), sucrose (42%), galactose and mannitol (33%), lactose (25%), maltose (17%), arabinose and raffinose (8%). While studying biochemical properties of isolates from Okha site, the maximum positive reaction obtained with ammonia production, nitrate reduction, catalase and oxidase (100%) followed by methyl red (90%), triple sugar iron (TSI) fermentation (80%), H_2S production (40%) while none of the isolates under study produced indol or utilized urea. Among the ten cultures tested for hydrolysis, 100% of them displayed strong hydrolytic property against gelatin, starch and cellulose while 80% isolates displayed hydrolytic activity with casein indicating that ability to hydrolyze different substrates is consistent trait among the strains. However, only 30% isolates displayed moderate to weak ability to hydrolyze lipid. The extent of sugar utilization was highly varied among the isolates of Okha site. Maximum isolates were able to ferment glucose extensively (70%) followed by maltose (50%), galactose, mannitol, fructose (40%), sucrose lactose and xylose (20%), raffinose (10%) while none of the isolates were not able to utilize arabinose.

On the whole, profile of biochemical characterization and sugar fermentation of all actinomycetes is presented as secondary graph in Figure 3.2A and 3.2B.

Figure 3.1: A) Biochemical characterization B) Sugar utilization of salt tolerant alkaliphilic actinomycetes from Okha Madhi and Okha site (1 → +, 2 → ++, 3 → +++)

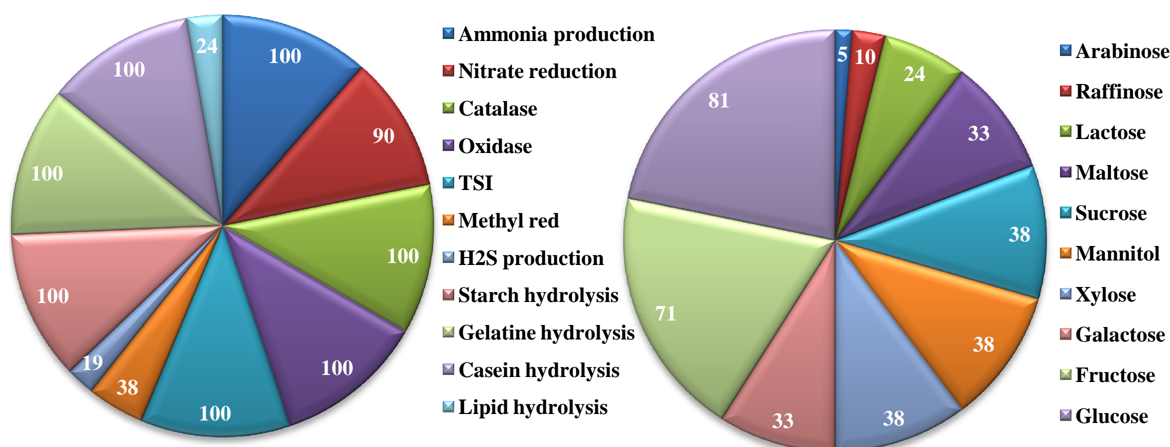


Figure 3.2: Secondary graphs presenting profile of A) Biochemical characterization B) Sugar utilization of all actinomycetes isolates

2.3.3.4 16S rRNA identification

Actinomycete isolates OM-6, OK-5 and OK-7 were identified by 16S rRNA gene sequencing. Based on nucleotides homology and phylogenetic analysis, OM-6 was related to *Brachystreptospora xinjiangensis* (GenBank Accession Number: AF251709). The nearest homolog genus-species was *Nocardiopsis kunsanensis* (Accession No. AB368716). The GenBank accession number for OM-6 isolate was EU710555.1. Based on nucleotides homology and phylogenetic analysis, OK-5 was detected as *Nocardiopsis alba* (GenBank Accession Number: GU985439). The nearest homolog was *Prauseria sp.* (Accession No. AB188209). The GenBank accession number for OK-5 was HM560975. Furthermore, OK-7 was detected as *Streptomyces* sp. (GenBank Accession Number: EF527834) while nearest homolog was found to be *Streptomyces* sp. (Accession No. EU368779). The GenBank accession number for OK-7 isolate was HM560976. Information about other close homologs for the microbe can be found from alignment view the phylogenetic tree (Table 3.8, 3.9; Figure 3.3).

Aligned Sequence Data of OM-6: (1474 bp)

GAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAGCGGTAAGGCCCTTCGGGGTACACGAGCGGCGAACGGGTG
AGTAACACGTGAGCAACCTGCCCCTGACTCCGGGATAAGCGGTGGAAACGCCGTCTAATACCGGATACGACCCCC
CGGCTCATGCCGGAGGGTGGAAAGTTTCTCGGTTGGGGATGGGCTCGCGGCCTATCAGCTTGTTGGTGGGGTAAA
GGCCTACCAAGGCGATTACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGA
CTCCTGCGGGAGGCAGCAGTGGGGAAGCTTGCGCAATGGGCGAAAGCCTGACGCAGCGACGCCGCGTGGGGGAT
GACGGCCTTCGGGTTGTAAACTCCTTTTACCACTCACGCAGGCCTCCAGTTCTCTGGGGGTTGACGGTAAGTGGGG
AATAAGGACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTCCGAGCGTTGTCCGGAATTATTGGGC
GTAAAGAGCTCGTAGGCGGCGTGTCACGTCTGCTGTGAAAGACCGGGGCTCAACCTCGGTTTTGCAGTGGATACG
GGCATGCTAGAGGTAGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGCCCAC
CGGTGGCGAAGGCGGGTCTCTGGGCCTTACCTGACGCTGAGGAGCGAAAGCATGGGTAGCGAACAGGATTAGATA
CCCTGGTAGTCCATGCCGTAAACGATGGGCGCTAGGTGTGGGGACTTTCCACGGTTTCCGCGCCGTAGCTAACGCA
TTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGC
GGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGCAGAGAT
GTGGGGTCATTTAGTTGGTGGGTGACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
CCCGTAACGAGCGCAACCCTTGTTCCATGTTGCCAGCACGTAGTGGTGGGGACTCATGGGAGACTGCCGGGGTCA
ACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCAAACATGCTACAATGGCC
GGTACAGAGGGCGTGCGATGCCGTAAGGTGGAGCGAATCCCTAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCA
ACTCGACCCCATGAAGGTGGAGTCGCTAGTAATCGCGGATCAGCAACGCCGCGGTGAATACGTTCCCGGGCCTTG
TACACACCGCCCGTCACGTCATGAAAGTCGGCAACACCCGAAACTTGCGGCCCAACCCCTTGTGGGAGGGAGTGA
GTGAAGGTGGGGCTGGCGATTGGGACGAAGTCGTAACAAGGTAGCCGTA

Aligned Sequence Data of OK-5: (1506bp)

AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAGCGGTAAGGCCCTTCGG
GGTACACGAGCGGCGAACGGGTGAGTAACACGTGAGCAACCTGCCCCTGACTCTGGGATAAGCGGTGGAAACGC
CGTCTAATACCGGATACGACCTTCCGCCTCATGGTGGAGGGTGGAAAGTTTTTCGGTCAGGGATGGCTCGCGGC
CTATCAGCTTGTTGGTGGGGTAACGGCCTACCAAGGCGATTACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACA
CTGGGACTGAGACACGGCCCAGACTCCTGCGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGCGAAAGCCTGA
CGCAGCGACGCCGCGTGGGGGATGACGGCCTTCGGGTTGTAAACCTCTTTACCACCAACGCAGGCTCCGGGTTCT
CTCGGGGTTGACGGTAGGTGGGGAATAAGGACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTCCG
AGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCGTGTCGCGTCTGCTGTGAAAGACCGGGGCTTA
ACTCCGGTTCTGCAGTGGATACGGGCATGCTAGAGGTAGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAA
TGCGCAGATATCAGAGGAACACCGGTGGCGAACGCGGGTCTCTGGGCCTTACCTGACGCTGAGAGCGAAAGCATG
GGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAACGTTGGGCGCTAGGTGTGGGGACTTTCCACGGT
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CAAACATGCTACAATGGCCGGTACAATGGGCGTGCGATACCGTAAGGTGGAGCGAATCCCTTAAAGCCGGTCTCA
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AATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGCAACACCCGAAACTTGCGGCCTAACC
CTTCGGGGAGGGAGTGAGTGAAGGTGGGGCTGGCGATTGGGACGAAGTCGTAACAAGGTAGCCGTACCGGAAGG
TGCG

Aligned Sequence Data of OK-7: (1405bp)

GCTTACACATGCAAGTCGAACGATGAACCGGTTTCGGCCGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGC
AATCTGCCCTGCACTCTGGGATAAGCCCGGGAAACTGGGTCTAATACCGGATACGACACTCCAAGGCATCTTGGG
GTGTGGAAAGTTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGC
GACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGT
TGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAT
CATCCGCGGTAATACGGAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGC
GTCGATTGTGAAAGCCCGGGGCTTAACCCTGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGCAGGGGAGAC
TGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCCG
ATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGT
GGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTAC
GGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGC
AACGCGAAGAACCTTACCAAGGCTTGACATACATCGGAAACGGCCAGAGATGGTCGCCCCCTTGTGGTCGGTGTA
CAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCC
TGTGTTGCCAGCGGAGCCTTCGGGCTGCCGGGGACTCACGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGG
ACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATG
CCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGG
AGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCA
TGAAAGTCGGTAACACCCGAAGCCGGTGGCCTAACCCCTTGTGGGAGGGAGCTGT

A

Alignment View	ID	Alignment results	Sequence description
	OM-6	0.87	Studied sample
	AF251709	0.87	*Brachystreptospora xinjiangensis*
	AB368716	0.87	*Nocardiopsis kunsanensis*
	AF195412	0.84	*Nocardiopsis kunsanensis* HA-9T
	EF392847	0.96	*Nocardiopsis lucentensis* isolate CNR-712
	AY373031	0.87	*Nocardiopsis salina*
	AJ539401	0.94	*Nocardiopsis aegyptica* strain DSM 44442
	AJ290448	0.94	*Nocardiopsis halototerans* strain DSM 44410
	X97888	1.00	*Nocardiopsis lucentensis*
	X97886	1.00	*Nocardiopsis dassonvillei*
	AB184655	1.00	*Streptomyces flavidofuscus* st. NRRL B-16366

B

Alignment View	ID	Alignment results	Sequence description
	OK5	1.00	Studied sample
	GU985439	0.99	*Nocardiopsis alba* strain JKNS-02
	AB188209	0.98	*Prauseria sp.* TUT1202
	EU430537	0.98	*Nocardiopsis exhalans* strain VTT E-063001
	EU430534	0.98	*Nocardiopsis dassonvillei* strain VTT E-062968
	EU430536	0.97	*Nocardiopsis dassonvillei subsp. albirubida* strain VTT E-062983
	NR_025517	0.98	*Nocardiopsis metallicus* strain R2A
	EU882851	0.97	*Actinomycetales bacterium* XJ-1
	NR_025589	0.98	*Nocardiopsis aegyptia* strain SNG49
	AB368711	0.98	*Nocardiopsis synnemataformans*
	NR_026342	0.98	*Nocardiopsis lucentensis* strain DSM 44048

C

Alignment View	ID	Alignment results	Sequence description
	OK7	1.00	Studied sample
	EF527834	1.00	*Streptomyces* sp. 160317
	EU368779	0.99	*Streptomyces* sp. A452 Ydz-DS
	AB184641	0.99	*Streptomyces carpaticus* strain: NBRC 15390
	AY822606	0.98	*Streptomyces cheonanensis* strain VC-A46
	FJ406109	0.98	*Streptomyces niger* strain AS 4.1244
	EF012099	0.97	*Streptomyces xiamenensis* strain MCCC 1A01550
	AY331685	0.96	*Streptomyces sannurensis*
	AB184865	0.96	*Streptomyces morookaensis* strain: NBRC 13461
	EU570376	0.96	*Streptomyces fragilis* strain 173823
	EU841632	0.95	*Streptomyces bingchengensis* strain HBUM174849

Table 3.8: Alignment view of A) OM-6, B) OK-5 and C) OK-7 using combination of NCBI GenBank and RDP database

A

	1	2	3	4	5	6	7	8	9	10	11
AF195412	---	0.973	0.995	0.969	0.975	0.974	0.983	0.973	0.971	0.978	0.983
AJ539401	0.027	---	0.978	0.982	0.990	0.989	0.974	0.988	0.986	0.980	0.974
AB368716	0.005	0.022	---	0.974	0.980	0.979	0.988	0.978	0.976	0.983	0.988
AJ290448	0.031	0.018	0.026	---	0.983	0.983	0.970	0.988	0.991	0.979	0.970
EF392847	0.025	0.010	0.020	0.017	---	0.996	0.975	0.986	0.986	0.980	0.975
X97888	0.026	0.011	0.021	0.017	0.004	---	0.974	0.987	0.986	0.979	0.974
AF251709	0.017	0.026	0.012	0.030	0.025	0.026	---	0.975	0.971	0.981	1
X97886	0.027	0.012	0.022	0.012	0.014	0.013	0.025	---	0.995	0.981	0.975
AB184655	0.029	0.015	0.024	0.009	0.015	0.014	0.029	0.005	---	0.979	0.971
AY373031	0.022	0.020	0.017	0.021	0.020	0.021	0.019	0.019	0.021	---	0.981
OM-6	0.017	0.026	0.012	0.030	0.025	0.026	0.000	0.025	0.029	0.019	---

B

	1	2	3	4	5	6	7	8	9	10	11
EU430534	0.0000	0.0006	0.0069	0.0069	0.0131	0.0138	0.0138	0.0215	0.0230	0.0138	0.0124
EU882851	0.0006	0.0000	0.0062	0.0062	0.0138	0.0145	0.0145	0.0208	0.0222	0.0131	0.0124
EU430536	0.0069	0.0062	0.0000	0.0034	0.0152	0.0159	0.0159	0.0208	0.0222	0.0152	0.0124
AB368711	0.0069	0.0062	0.0034	0.0000	0.0145	0.0152	0.0152	0.0215	0.0215	0.0152	0.0145
AB188209	0.0131	0.0138	0.0152	0.0145	0.0000	0.0006	0.0006	0.0096	0.0131	0.0131	0.0117
OK5	0.0138	0.0145	0.0159	0.0152	0.0006	0.0000	0.0000	0.0103	0.0138	0.0138	0.0124
GU985439	0.0138	0.0145	0.0159	0.0152	0.0006	0.0000	0.0000	0.0103	0.0138	0.0138	0.0124
EU430537	0.0215	0.0208	0.0208	0.0215	0.0096	0.0103	0.0103	0.0000	0.0048	0.0145	0.0152
NR_025517	0.0230	0.0222	0.0222	0.0215	0.0131	0.0138	0.0138	0.0048	0.0000	0.0145	0.0180
NR_025589	0.0138	0.0131	0.0152	0.0152	0.0131	0.0138	0.0138	0.0145	0.0145	0.0000	0.0117
NR_026342	0.0124	0.0124	0.0124	0.0145	0.0117	0.0124	0.0124	0.0152	0.0180	0.0117	0.0000

C

	1	2	3	4	5	6	7	8	9	10	11
EF012099	0.0000	0.0220	0.0251	0.0251	0.0228	0.0321	0.0282	0.0353	0.0392	0.0360	0.0385
AB184641	0.0220	0.0000	0.0045	0.0045	0.0007	0.0114	0.0070	0.0329	0.0321	0.0313	0.0305
OK7	0.0251	0.0045	0.0000	0.0000	0.0037	0.0143	0.0113	0.0330	0.0353	0.0345	0.0353
EF527834	0.0251	0.0045	0.0000	0.0000	0.0037	0.0143	0.0113	0.0330	0.0353	0.0345	0.0353
EU368779	0.0228	0.0007	0.0037	0.0037	0.0000	0.0113	0.0075	0.0322	0.0329	0.0321	0.0314
AY822606	0.0321	0.0114	0.0143	0.0143	0.0113	0.0000	0.0037	0.0354	0.0377	0.0353	0.0345
FJ406109	0.0282	0.0070	0.0113	0.0113	0.0075	0.0037	0.0000	0.0314	0.0337	0.0314	0.0306
AY331685	0.0353	0.0329	0.0330	0.0330	0.0322	0.0354	0.0314	0.0000	0.0322	0.0275	0.0244
EU570376	0.0392	0.0321	0.0353	0.0353	0.0329	0.0377	0.0337	0.0322	0.0000	0.0252	0.0260
gAB184865	0.0360	0.0313	0.0345	0.0345	0.0321	0.0353	0.0314	0.0275	0.0252	0.0000	0.0143
EU841632	0.0385	0.0305	0.0353	0.0353	0.0314	0.0345	0.0306	0.0244	0.0260	0.0143	0.0000

Table 3.9: Distance Matrix based on Nucleotide Sequence Homology (Using Kimura-2 Parameter): Table indicates nucleotide similarity (above diagonal) and distance (below diagonal) identities between the studied sample A) OM-6, B) OK-5 and C) OK-7 with ten other closest homologs microbe

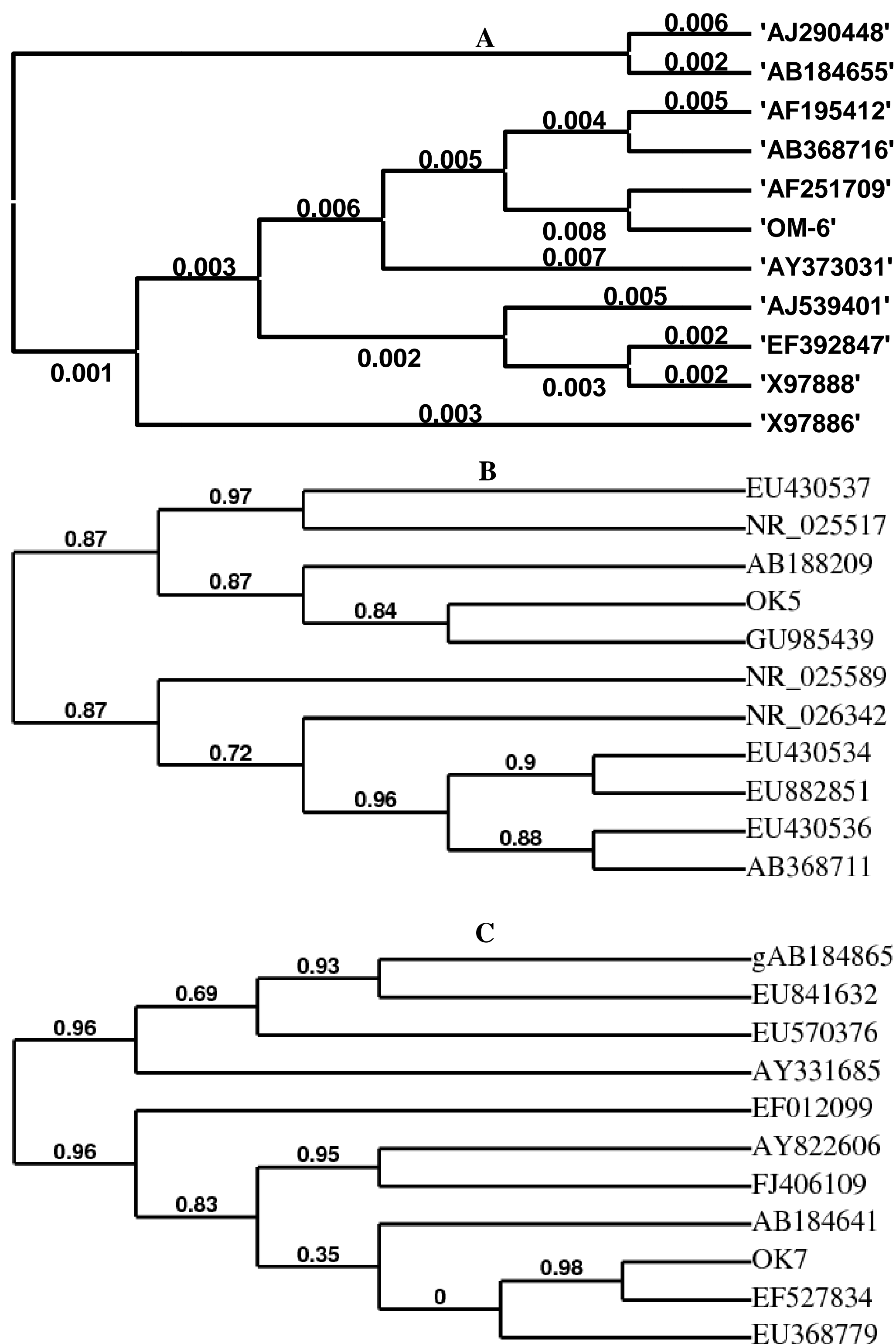


Figure 3.3: Phylogenetic tree of A) OM-6, B) OK-5 and C) OK-7 made by using neighbor joining method

2.3.5 Salt and pH profile

Among 40 isolates 1 isolate was able grow up to 20% NaCl, 5 isolates were able grow up to 15% NaCl, 1 isolate was able grow at 5-15% NaCl, 1 isolate was able grow at 5-10% NaCl 17 isolates were able grow up to 10% NaCl, 9 isolates were able grow up to 5% NaCl, and remaining 6 isolates were able to grow only at 0% NaCl at pH 9 on gelatin agar medium. Among total isolates 15 isolates were able to grow at 8-11 pH, 14 were able to grow at 7-11 pH, 1 isolate was able to grow at 8-10 pH While remaining isolates were able to grow at 7-10 pH at 5% (w/v) NaCl on gelatin agar medium indicating the possibility of new strain of alkaliphilic actinomycetes (Figure 3.4).

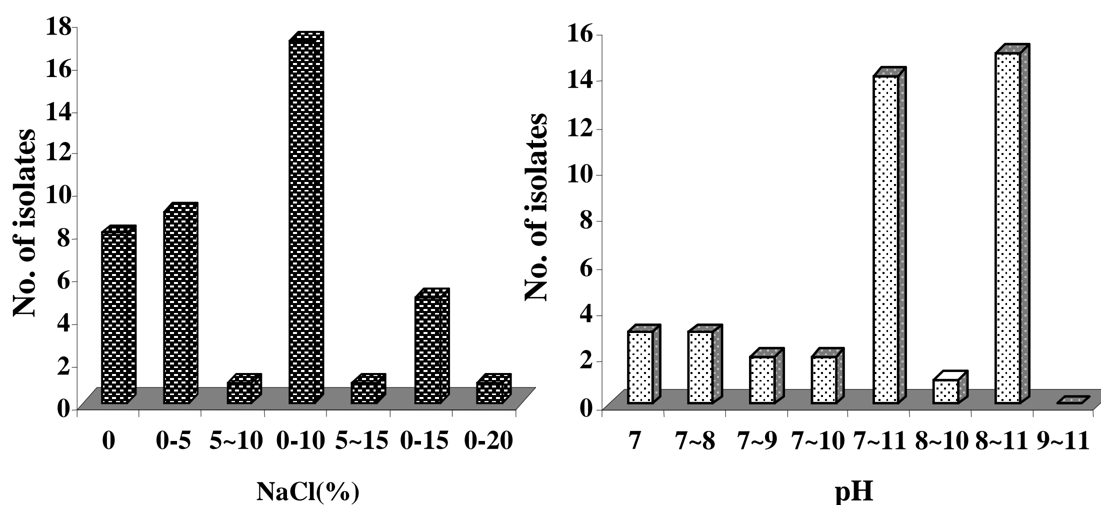


Figure 3.4: Salt (▩) and pH (░) profile of total isolates at 0-20% NaCl and pH 7-11

2.4 DISCUSSION

However, salt tolerant alkaliphilic actinomycetes have gained considerable attention in recent years, the research on actinomycetes surviving under extreme environments is still limited. There is tremendous diversity and novelty among the alkaliphilic actinomycetes present in saline and alkaline habitats including soda lake and desert soils (Li *et al.,* 2006, 2005b; Hozzein *et al.,* 2004; Al-Zarban *et al.,* 2002a, b; Miyashita *et al.,* 1984). Therefore, we explored saline and alkaline habitats of coastal Gujarat including eight sites comprising diverse saline soil samples with different texture and color to discover new taxa of actinomycetes and consequently new secondary metabolites. The soil habitat is inherently in homogeneous containing wide range of salinities. Many actinobacterial species have been isolated from soil collected from Xinjiang, China including *Nocardiopsis xinjiangensis* (Li *et al.,* 2003a); *Prauserella halophila* and *Prauserella alba* (Li *et al.,* 2003b) and *Streptomonospora alba* (Li *et al.,* 2003c). Similarly, *Saccharomonospora halophila,* able to grow at 20% salt, was isolated from Marsh soil of Kuwait (Al-Zarban *et al.,* 2002b).

One of the most fascinating and attractive aspects of the microbial world is its extraordinary diversity. The diversity of an ecosystem is dependent on the physical characteristics of the environment, the diversity of species present and the interactions among the species and the environment. Environmental disturbance on a variety of temporal and spatial scales can affect the species richness and, consequently, the diversity of an ecosystem. Therefore, in order to explore the microbial diversity of saline habitats, we employed various dilution techniques and enrichment culture conditions. It is clear that questions concerning the true numbers of actinomycetes and the best methods for their quantification remain unanswered. Based on the problems reported in this study and elsewhere in the literature we caution the use of serial dilution and plating for the quantification of actinomycetes since the number of isolates increases and the contamination of other bacteria and mainly of fungi decreases with increasing dilution up to 100 times. However, the actinomycetes counts were not a quantitative function of dilution as would be expected when using these techniques. A similar observation was reported by Weyland (1981) for

actinomycetes isolation from deep sea sediments. By this method 1000 time more colonies were formed than by control without $CaCO_3$ as $CaCO_3$ stops the growth of vegetative cells so that only spore forming organisms would stay alive. On the basis of our study, we revealed ISP-2 as a superior media to the other media tested for initial isolation of actinomycetes.

During research expedition, strains representing all observed variations in pigmentation, colony size, shape, texture and level of sporulation were selected and cultured along the saline habitats of coastal Gujarat for further study. Enrichment cultures frequently produced visible aggregates of filamentous bacteria that upon microscopic examination were recognized actinomycetes by the presence of branching filaments. These actinomycetes blooms could be harvested and upon transfer to solid media yielded actinomycetes of different morphological and biochemical characters. However, actinomycetes were observed only while using original cultivation techniques (heat shock or drying methods), supporting the use of enrichment culture for the recovery of new actinomycetes taxa. Further, morphological, cultural, physiological and molecular characterization was conducted for identification and classification of actinomycetes isolates.

The isolates in the present study were aerobic and gram positive with filamentous structures and well developed aerial and substrate mycelia when grown on YEME (5% w/v NaCl and pH 9.0). All the isolates were conformed to the group actinomycetes and clearly distinguished from each other on the basis of morphological characterization. *In-situ* colony development studies revealed that they produced abundant aerial mycelium with white, gray and yellow color while substrate mycelium was light yellow with soluble pigments of various colors. Results are in accordance with *Streptomycetes* sp. MARO1 and *Streptomyces violaceusniger* strain HAL64 with white, yellow to grey mycelia (El-Naggar 2007; El-Naggar *et al.,* 2006). Further, dark grey to pale yellow colored aerial mycelium and grey, dark grey or black colored substrate mycelium of actinomycetes has also been described (Saleh *et al.,* 2007). However, beside grey and white colored mycelia, actinomycetes with

reddish to pinkish mycelia were also observed with some of our strains (Thangapandian *et al.,* 2007).

While studying cultural characteristics on different media, most of the isolates exhibited good growth on ISP-2 media, ISP-3 media, starch agar media and starch casein agar media. At the same time ISP-4, ISP-5 and actinomycetes isolation agar supported comparatively good growth in few isolates. They showed good sporulation with compact, chalk like dry colonies of different colors from white to grey with light yellow to dark brown substrate mycelia and brown colored pigmentation in a few (Thangapandian *et al.,* 2007; Li *et al.,* 2006). All the isolates started sporulation at different incubation stages. Some reports in the literature tinted these types of results (Saleh *et al.,* 2007; Mistuiki *et al.,* 2007; Kokare *et al.,* 2004; Matthew *et al.,* 2002; Al - zarban *et al.,* 2002).

Cultural and physiological characters of our isolates are in correspondence with Thangapandian *et al* (2007) and Li *et al* (2006) describing catalase positive and indol production strictly negative by majority of actinomycetes and *Nesterenkonia* sp. studied. In addition nitrate reduction was found positive in *Actinomyces* and *Streptomyces* isolates by (Thangapandian *et al.,* 2007 and Dastager *et al.,* 2006). However, Li *et al* (2006) found oxidase and nitrate reduction negative in many of *Nesternkonia* sp. that is in contrast with our findings. Furthermore, Li *et al* (2006) and Yoon *et al* (2006) described hydrogen sulphide negative in most of *Nocardiopsis* and *Nesterenkonia* sp. studied whereas Li *et al* (2005a) described urease negative *Streptomyces sodiiphilus* sp. nov., that is in accordance with our results. While studying hydrolytic properties of actinomycetes, observation of our results found similar with *Streptomyces* isolates displaying hydrolysis of gelatin, starch and casein (Dastager *et al.,* 2006). Recently, Bull *et al* (2000) reported marine actinomycetes capable of secreting extra cellular hydrolytic enzymes in the range of 0-10% NaCl (Li *et al.,* 2006c). Pattern of sucrose utilization was in accordance with Yoon *et al* (2006) describing utilization of sucrose by most of the *Nesterenkonia* sp., and Dastager *et al* (2006) relating utilization of mannitol by all *streptomyces* isolates as well as with Li

et al (2004b) suggesting nov. *Nesternkonia Xinjiangensis* sp. that utilized sucrose and galactose as carbon source while in contrast with Thangapandian *et al* (2007) describing sucrose and mannitol negative in most of the actinomycetes isolates. Moreover, literature described some of the actinomycetes and *Streptomyces* species giving arabinose positive (Thangapandian *et al.,* 2007; Dastager *et al.,* 2006) likewise Yoon *et al* (2006) observed maltose positive *Nesternkonia* sp. Consequently, utilization of disaccharides, as compared to simple carbon sources, suggested the adaptation of actinomycetes towards different metabolic pathways for their energy generation. Therefore, depending on morphological and physiological characterization, most of actinomycetes isolates appeared to be *Streptomyces, Nocardiopsis and Nesterenkonia* species. However, molecular characterization of isolates would provide evidence to the results obtained by morphological, cultural and physiological characterization of actinomycetes.

Three actinomycete cultures, OM-6, OK-5 and OK-7 were identified as *Brachystreptospora xinjiangensis*, *Nocardiopsis alba* and *Streptomyces* sp. respectively based on 16S rRNA identification. However, based on the results of morphological, cultural and biochemical properties described above, we further differentiate cluster of actinomycetes comprising three genus *Streptomyces, Nocardiopsis* and *Nesterenkonia.* Further, our finding was also supported while microbiological methods were combined with molecular methods like DGGE and RFLP described in Chapter 3. That can be very useful to differentiate microbial communities.

The isolation and characterization of halophilic and halo tolerant actinomycetes appears to be fairly recent (Kim *et al.,* 2005; Starch *et al.,* 2005; Montalavo *et al.,* 2005; Zhang *et al.,* 2005; Kim *et al.,* 2004; Chen *et al.,* 2004; Li *et al.,* 2004b). Majority of our isolates could grow in the range of 0-10% and 0-15% salt while pH in the range of 7-11 and 8-11 with scarce growth at pH 7 hence they have been referred as halo tolerant and alkaliphilic. The salt tolerance of the isolates in the present study was comparable to *Nocardiopsis halotolerans* sp. nov., a halo-tolerant actinomycetes

which grew at salt concentration of 0-10% w/v NaCl (Al-Zarban *et al.,* 2002), while less in comparison to *Streptimonospora salina* gen nov., sp. Nov. and *Streptomonospora alba* sp. nov., the halophilic actinomycetes (Li *et al.,* 2003c; Cui *et al.,* 2004). pH requirement of our isolates are in agreement with *Nocardiopsis alkaliphila* in which optimum growth occurred at pH 10 (Hozzein *et al.,* 2004). Recently, a novel alkaliphilic actinomycetes *Streptomyces sodiphilus* sp. nov., was reported to grow with an optimum pH of 9-10 (Li *et al.,* 2005b). Quite recently, Li and coworkers (2006a) reported 5 novel species of the genus Nocardiopsis from hyper saline soil in China. Similarly Li *et al* (2006c) isolated a coccoid, non-motile novel actinomycete, Kocuria aegyptia, from a saline, desert-soil sample from Egypt having tremendous salt-tolerance. The fairly better salt and pH tolerance of these actinomycetes, along with their capacity to secrete commercially valuable primary and secondary metabolites, can be considered as attractive features of these organisms.

The salt-tolerant and alkaliphilic actinomycetes are much less explored for their occurrence, growth characteristics of enzymes secretion. It is, therefore, important to pay attention to extremophilic actinomycetes from newer and unexplored habitats for their significance in ecological sustenance and search for novel secondary metabolites. Numerical assessment of aerial mycelium color appears as strong phenotypic trait in *Streptomycetes* classification and thus could be a powerful tool in finding the mutual relationship. The pigmentation of *Streptomyces* is distinct enough to allow ready delineation in most *Streptomyces* cultures when combined with other fundamental features, such as color of the surface aerial mycelium after sporulation, sporophore morphology and spore surface. Overall, present chapter has demonstrated wide occurrence of salt-tolerant and alkaliphilic actinomycetes and that they can be diversified based on their growth patterns as well as morphological, cultural and physiological features.

Chapter 4

Antibiotic Sensitivity and Antimicrobial Activity Profile of Actinomycetes

4.1 INTRODUCTION

Actinomycetes, the group of gram positive filamentous bacteria, are excellent of biotechnological products such as antibiotics, industrial enzymes and other bioactive compounds (Thumar *et al.,* 2010; Lam, 2006; Sacramento, 2004; Ndonde and Semu 2000). Although, actinomycetes exist as resource for novel bioactive compounds in various aquatic and terrestrial habitats (Sibanda *et al.,* 2010; Glen *et al.,* 2008; Parungawo *et al.,* 2007; Pathome *et al.,* 2006; Zhang *et al.,* 2005; Williams *et al.,* 2005), only few reports on diversity and activity of actinomycetes from saline and alkaline habitats are available (Thumar and Singh 2009; Thumar and Singh 2007a, 2007b; Mehta *et al.,* 2006; Tsujibo *et al.,* 2003; Mitsuiki *et al.,* 2002). Vasavada *et al.,* (2006) reported secretion of potent antibiotic by salt tolerant alkaliphilic *Streptomyces sannnanensis* strain RJT-1. Suthindhiran and Kannabiran, (2009) reported halophilic *Saccharopolyspora salina* VITSDK4 producing extracellular compound that inhibited the growth of tumor cells as well as microbial cells. Recently, Thumar *et al* (2010) reported halotolerant alkaliphilic *Streptomyces aburaviensis* strain Kut-8 producing antibiotic. Therefore, exploration of extreme actinomycetes with novel and unique antimicrobial properties is fairly recent and active area of research. In this context, we explored antimicrobial potential of actinomycetes as a function of salt and pH.

In addition to the differentiation reflected by species and strains, production of secondary metabolites by microorganisms differs qualitatively and quantitatively depending on their nutritional and cultural conditions (Lam *et al.,* 1989). Approaches to discover new antibiotics are generally based on screening of naturally occurring microorganisms, being able to produce a wide range of molecules with broad spectrum of activities; such as, antibacterial, antifungal, antitumour, antiparasitic and antiviral (Atta and Ahmad, 2009; Naeimpoor and Mavituna, 2000). However, isolation of novel secondary metabolites for therapeutic applications poses great challenge. Therefore, improvement in the growth by manipulating the nutritional and physical parameters plays a vital role.

In recent years, increasing prevalence of infectious diseases resistant to chemotherapy has caused an urgent need to discover and develop new antibiotics. However, development of new antibiotics against antibiotic-resistant pathogenic strains requires extensive survey of unexplored habitats for novel antibiotic-producing actinomycetes strains. In the present study, our main objective was to survey saline and alkalline habitats of coastal Gujarat, India for isolating promising actinomycete strains.

4.2 MATERIAL AND METHODS

4.2.1 Isolation and identification of salt tolerant alkaliphilic actinomycetes

Total twelve halo-tolerant and alkaliphilic actinomycetes from Okha-Madhi and ten from Okha were isolated from the region of coastal Gujarat. Actinomycete colonies were recognized by morphological, cultural and physiological characterization as described in chapter 3. We further differentiate our isolates based on antimicrobial characteristics as described below.

4.2.2 Antibiotics sensitivity test

Standard antibiotic discs, Azithromycin, Amikacin, Gentamicin, Ciprofloxacin, Cephadroxil, Cefuroxime, Roxithromycin, Ampicillin/Cloxocilin, Cephotaxime, Cefaperazone, Clarithromycin, Sparfloxacin of different units were placed on the plates containing ISP 1 media swabbed with young culture of actinomycetes. The plates were then incubated for 4-5 days followed by the measurement of zones of inhibition.

4.2.3 Antimicrobial activity profile of actinomycetes

4.2.3.1 Test organisms for antimicrobial characterization

Antimicrobial activity of the selected isolates was tested against four gram-positive bacteria i.e., *Bacillus cereus; Bacillus megaterium, Bacillus subtilis* (MTCC 121) and *Staphylococcus aureus* (MTCC 96), three gram-negative bacteria i.e., *Escherichia coli* (MTCC 739), *Enterobacter aerogenes* and *Salmonella typhimurium (*MTCC 98), a Yeast, *Saccharomycetes cerevise* and a Candida, *Candida albicans* (MTCC 227).

4.2.3.2 Effect of different media on antimicrobial activity

Antimicrobial activities of the actinomycete isolates from Okha Madhi and OKha were detected using ISP-2 media, ISP-3 media, nutrient agar, starch agar, starch casein agar and tyrosine agar (5% w/v NaCl, pH 9). Using inoculation loop, each actinomycete isolate was spotted on the plate with medium and incubated for 4 days

till the beginning of sporulation. Thereafter, the molten nutrient agar with each activated test culture was poured on already grown actinomycetes and incubated for 24 h at 37°C. The zone of inhibition was measured for each test organism.

4.2.3.3 Salt and pH profile for antimicrobial activity production

Effect of salt on antibiotic production was studied by inoculating spore suspension of each isolate on starch agar medium supplemented with different salt concentrations (0–10% w/v NaCl, pH 9). After 4 days, each test organism in molten agar was poured into the plates and the zone of inhibition was measured after 24 h incubation at 37°C. Similarly, the pH profiling of all isolates for antibiotic production was studied in the range of pH 6–11on starch agar with 5%, w/v NaCl.

4.3 RESULTS

After isolation and identification, we further studied our isolates for the production of antimicrobial activity as described below.

4.3.1 Antibiotic sensitivity test

Antibiotic sensitivity profile of total isolates from Okha Madhi and Okha site was generated by using dodeca disc on starch agar plate. After 5 days of incubation, zone diameter (mm) of each isolate against each antibiotic was measured as shown in (Table 4.1, 4.2). Observation reveled that among total isolates majority of isolates were sensitive toward Azithromycin (86%), followed by Clarithromycin, Roxithromycin (81%), Ciprofloxacin (76%), Sparfloxacin (57%), Gentamicin (52%), Amikacin (19%), Cefaperazone, Cephotaxime (14%), Cefuroxime, Cephadroxil (10%). The values in the brackets indicated percentage of the isolates. The isolates, however, exhibited great resistance against Cloxocilin, as no zone of inhibition was visible in presence of Cloxocilin (Figure 4.1; Picture 4.1 and 4.2).

Antibiotic	Mcg	OM-1	OM-3	OM-4	OM-5	OM-6	OM-7	OM-9	OM-10	OM-11	OM-12
Azithromycin (At)	15	14	15	18	18	18	R	16	16	19	30
Amikacin (AK)	30	25	10	R	R	R	R	R	R	R	15
Gentamicin (G)	10	10	10	R	R	12	18	9	R	R	13
Ciprofloxacin (Cf)	5	30	39	R	16	38	31	40	20	32	R
Cephadroxil (Cq)	30	R	R	R	R	R	R	R	R	R	R
Cefuroxime (Cu)	30	R	R	R	R	R	R	R	R	R	R
Roxithromycin (Ro)	30	11	11	15	15	16	R	12	18	12	18
Cephotaxime (Ce)	30	R	R	R	R	9	R	R	R	R	R
Cefaperazone (Cs)	75	R	R	R	R	R	9	9	R	R	0
Clarithromycin (Cw)	15	11	14	14	19	14	R	15	17	10	0
Sparfloxacin (Sc)	5	25	35	R	9	28	26	30	R	35	0
Ampicillin/ Cloxocilin (Ax)	10	R	R	R	R	R	R	R	R	R	R

Table 4.1: Antibiotic sensitivity of actinomycetes from Okha Madhi

Antibiotics	Mcg	OK-1	OK-2	OK-3	OK-4	OK-5	OK-6	OK-7	OK-8	OK-9	OK-10
Azithromycin (At)	15	R	12	20	17	18	14	15	20	22	35
Amikacin (AK)	30	R	R	22	R	R	R	R	R	R	R
Gentamicin (G)	10	20	8	20	R	R	15	12	R	R	R
Ciprofloxacin (Cf)	5	R	31	28	47	17	37	47	8	R	10
Cephadroxil (Cq)	30	R	11	12	R	R	R	R	R	R	R
Cefuroxime (Cu)	30	R	R	9	R	R	R	14	R	R	R
Roxithromycin (Ro)	30	R	10	15	15	R	10	12	13	9	27
Cephotaxime (Ce)	30	R	10	15	R	R	R	R	R	R	R
Cefaperazone (Cs)	75	9	R	R	R	R	R	R	R	R	R
Clarithromycin (Cw)	15	R	12	13	12	18	12	19	17	12	32
Sparfloxacin (Sc)	5	R	40	25	0	22	34	R	12	R	R
Ampicillin/ Cloxocilin (Ax)	10	R	R	R	R	R	R	R	R	R	R

Table 4.2: Antibiotic sensitivity of actinomycetes from Okha

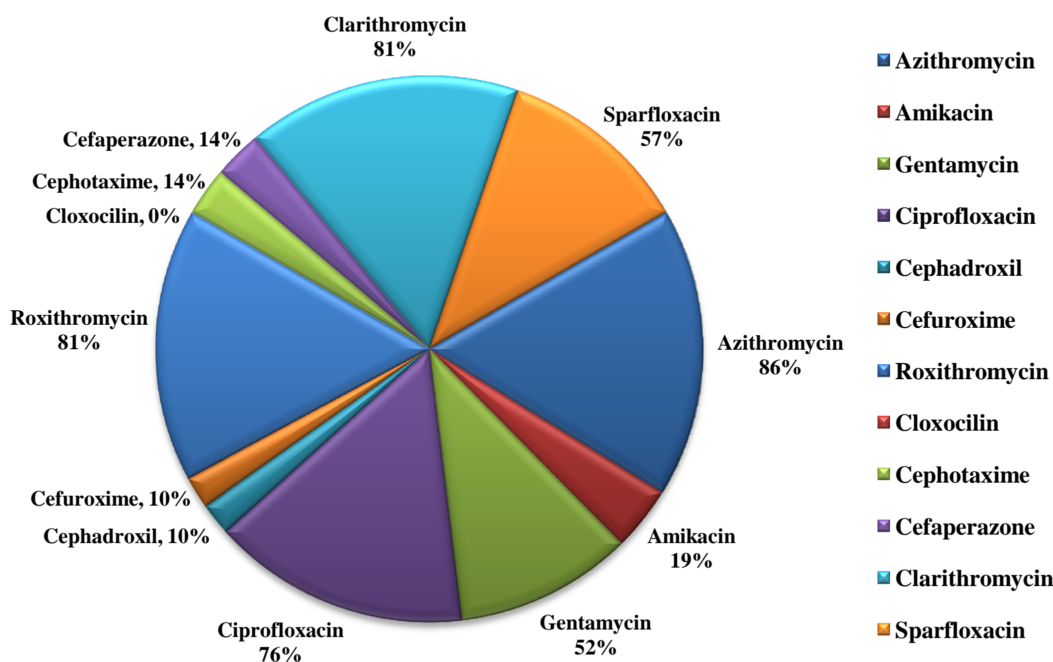
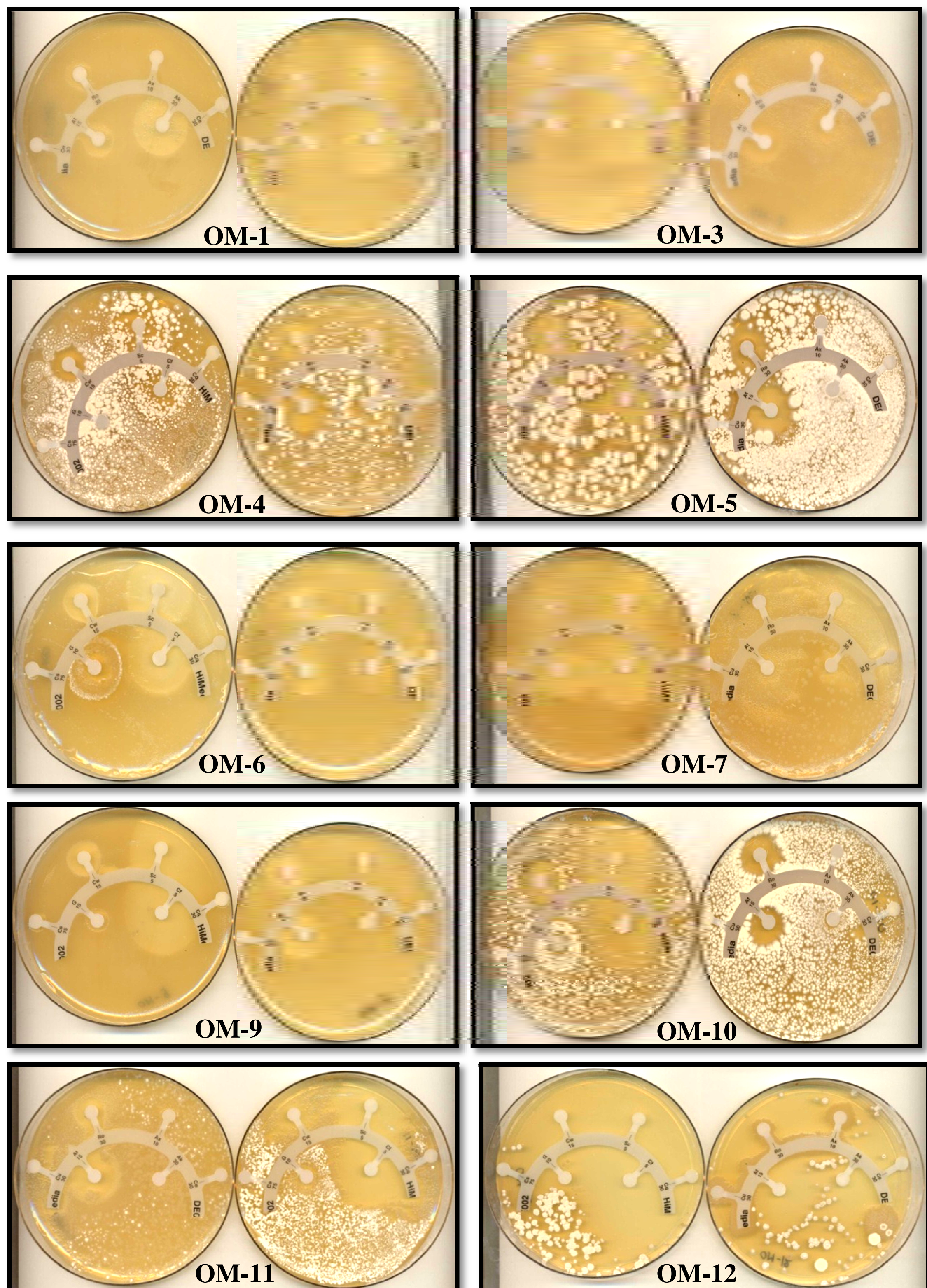
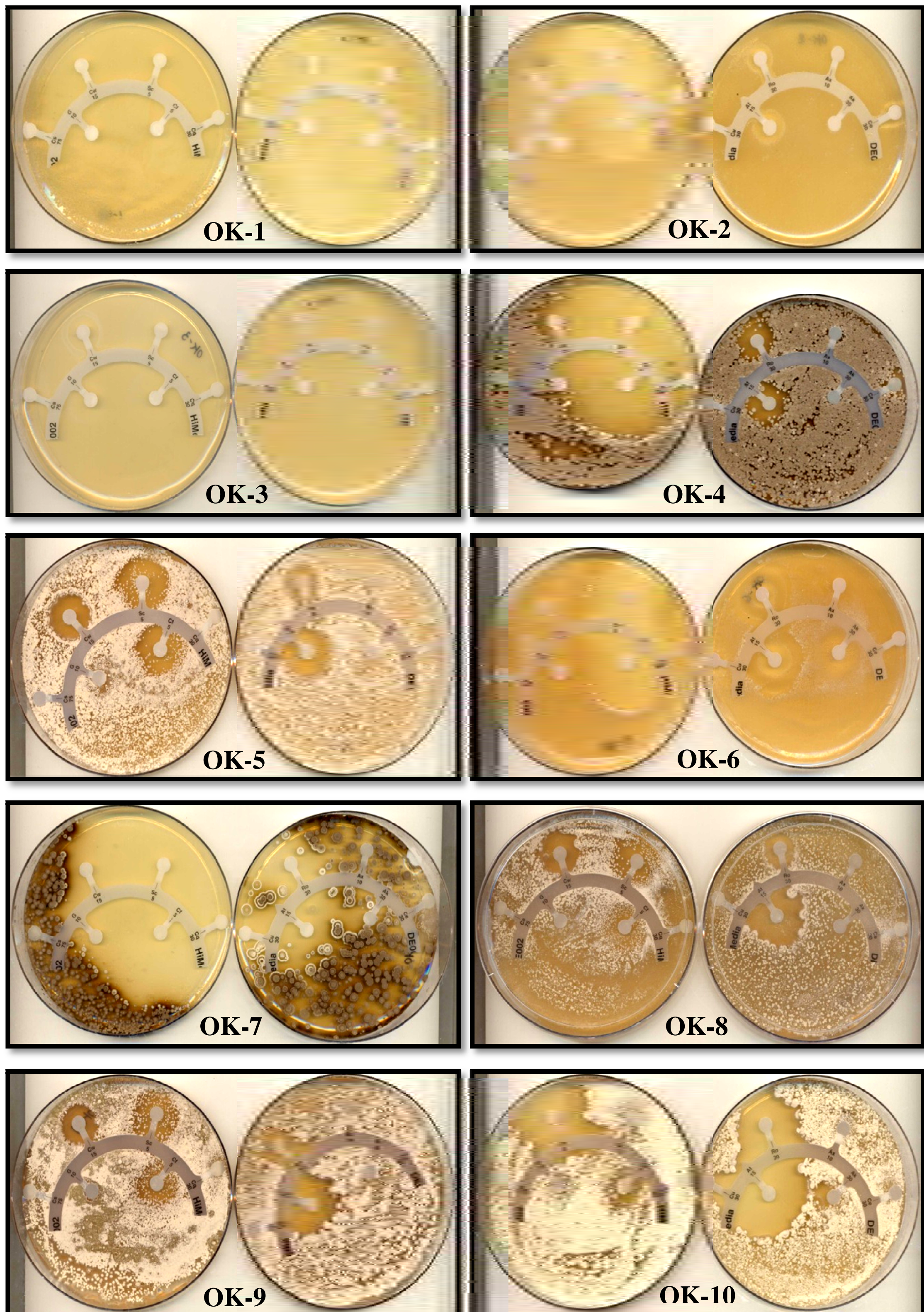


Figure 4.1: Antibiotic sensitivity profile of salt tolerant alkaliphilic actinomycetes from Okha Madhi and okha



Picture 4.1: Detection of zone of inhibition among the selected actinomycetes isolates from Okha Madhi against gram positive antibiotics



Picture 4.2: Detection of zone of inhibition among the selected actinomycetes isolates from Okha against gram positive antibiotics

4.3.2 Effect of different media on antimicrobial activity

The antimicrobial activity of all actinomycetes was screened on a range of media against various gram-positive, gram-negative bacteria as well as yeast and candida. Among the isolates majority exhibited antimicrobial potential against gram positive bacteria while few i.e., OM-1, OM-3, OM-4, OM-5, OM-10, OK-8 also displayed antimicrobial activity against gram negative bacteria *Salmonella typhimurium*. Some of the isolates i.e., OK-1 and OK-2 produced antimicrobial activity even against yeast *Saccharomycetes cerevise*. Interestingly, many isolates i.e., OM-1, OM-5, OM-10, OK-1, OK-2, OK-5, OK-6, OK-8, OK-9 produced antimicrobial activity against *Candida albicans*. However growth was evident on most of the media used, as described in chapter 3, maximum isolates from both Okha Madhi and Okha displayed antimicrobial activities on starch casein agar media, starch agar media and ISP-2 followed by tyrosine agar, N-agar and ISP-3 agar media (Figure 4.2, 4.3).

4.3.3 Effect of NaCl on growth and antibiotic production

The isolates from Okha Madhi and Okha were studied for their antimicrobial activities in the presence of 0-10% NaCl and pH 7-11. Although study was carried out with all test organisms described in the materials and method part, the antimicrobial activity was observed only against gram positive bacteria and candida. While no antimicrobial activity was evident against gram negative test organisms or yeast. Among the Okha Madhi isolates, only OM-10 and form Okha site isolates; OK-5, OK-9 and OK-10 exhibited antimicrobial activities against *Bacillus cereus* at 5-10% NaCl, while no antimicrobial activity was observed in presence of 0% NaCl. On the contrary, majority of the Okha Madhi isolates secreted antibiotic against *Bacillus megaterium* and *Bacillus subtilis* at 5-10% NaCl. Interestingly, some of the isolates from Okha also produced antibiotics against *Candida albicans*. In addition, it grew but did not secrete antibiotic at the salt concentrations above 10% (Figure 4.4).

4.3.4 Effect of pH on growth and antibiotic production

Majority of the isolates displayed antibiotic action in the wide range of pH 7–11, optimum at pH 9. On the other hand, poor growth was evident at pH below 7. Further, majority of isolates exhibited antimicrobial activity against *S. auresus* and *B. cereus* (gram positive test organisms) as well as against *E.Coli* followed by *E. aerogenes* and *S. typhimurium* (gram negative test organisms). Many actinomycetes isolates exhibited antimicrobial activity against *C. albicans*, while none produced antibiotics against *S. cerevise* (Figure 4.5).

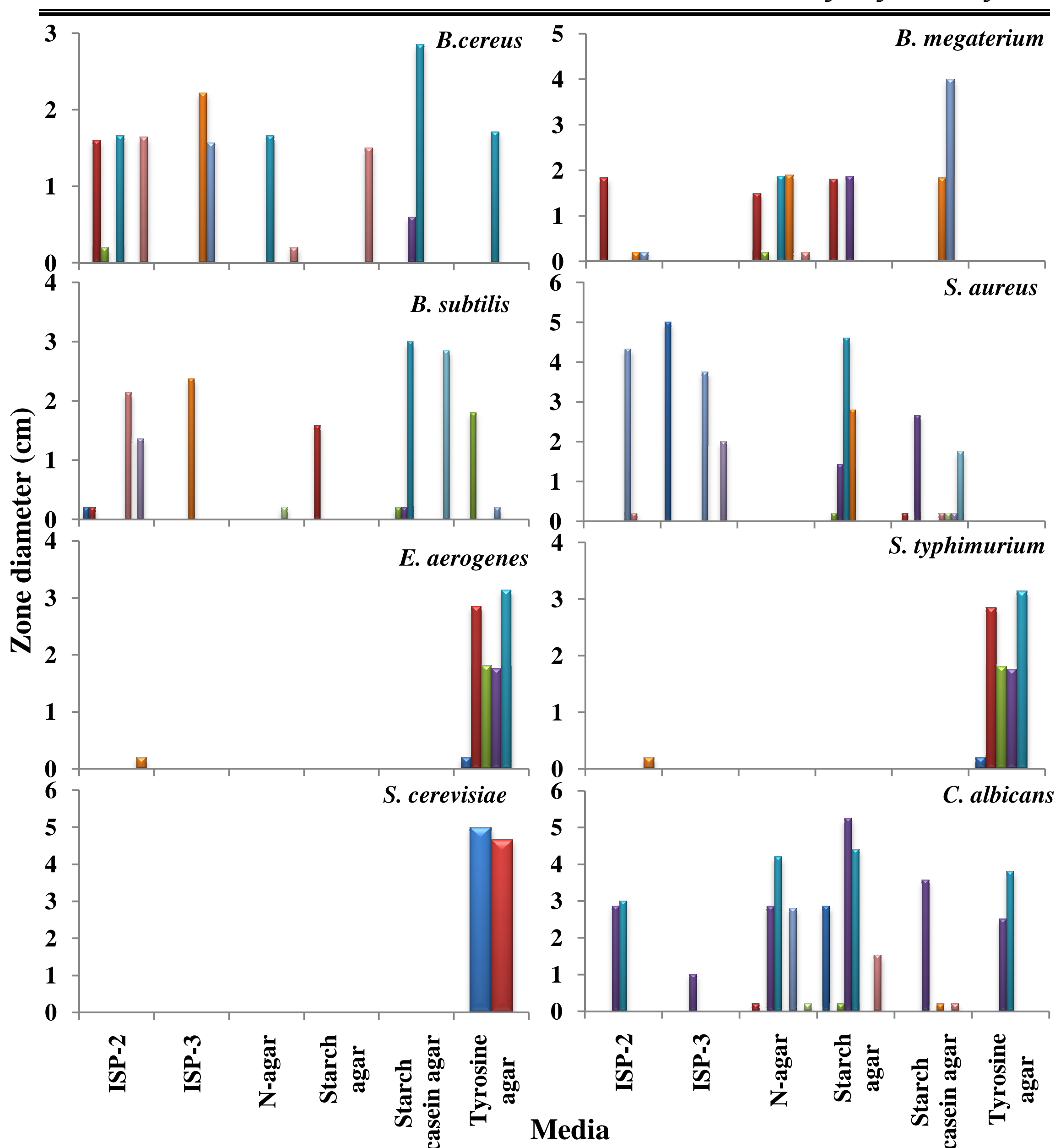


Figure 4.2: Effect of media on the antimicrobial activities among the isolates of Okha Madhi and Okha

B. cereus: OM-1 OM-3 OM-7 OM-8 OM-10 OK-3 OK-5 OK-10
B. megaterium: OM-1 OM-3 OM-6 OM-7 OM-8 OM-10 OM-11 OM-12
B. subtilis:
OM-1 OM-3 OM-5 OM-6 OM-10 OM-11 OK-1 OK-3 OK-5 OK-9 OK-10
S. aureus:
OM-1 OM-3 OM-7 OM-10 OM-11 OK-1 OK-3 OK-4 OK-8 OK-9 OK-10
E. aerogenes: OK-5 OK-8 OK-9;
MTCC: OM-1 OM-3 OM-4 OM-5 OM-10 OK-8, *S. cerevisiae*: OK-1 OK-2
C. albicans: OM-1 OM-5 OM-10 OK-1 OK-2 OK-5 OK-6 OK-8 OK-9

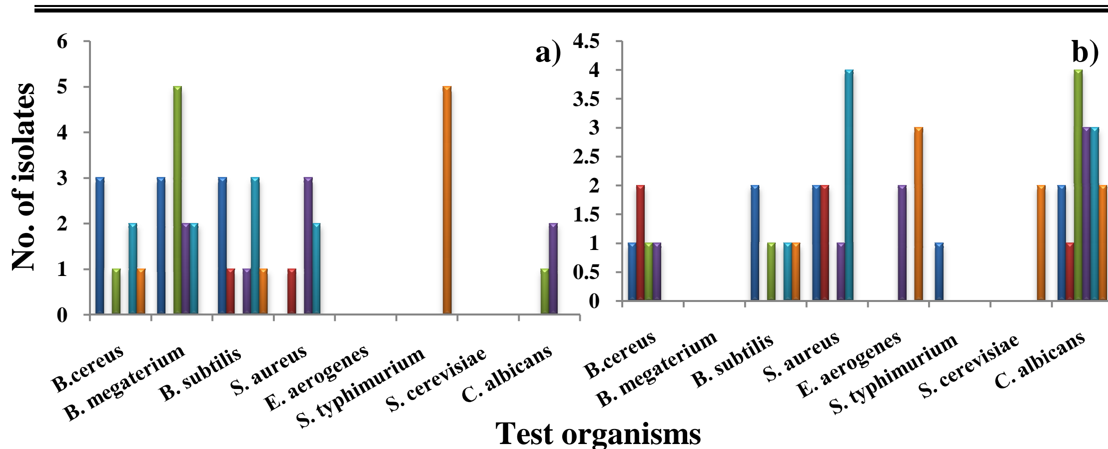


Figure 4.3: Antibiotics production of isolates from a) Okha Madhi and b) Okha using various media ISP-2 ISP-3 N-agar Starch agar Starch casein agar Tyrosine agar

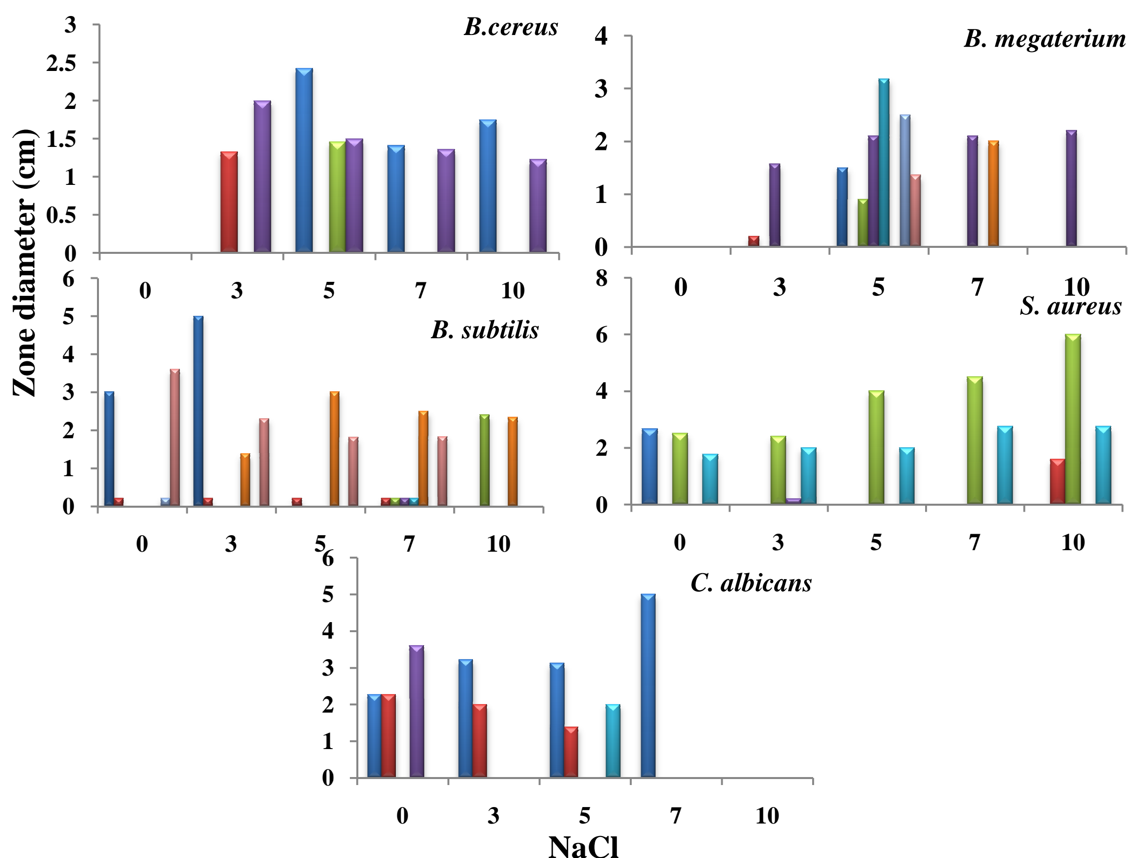


Figure 4.4: Effect of salt on the antimicrobial activities of the isolates from Okha Madhi and Okha

B. cereus: OM-10 OK-5 OK-9 OK-10

B. megaterium: OM-6 OM-7 OM-8 OM-10 OM-11 OM-12 OK-3 OK-9

B. subtilis: OM-1 OM-5 OM-6 OM-7 OM-8 OM-10 OM-11 OK-10

S. aureus: OM-1 OM-6 OM-10 OM-12 OK-10

C. albicans: OK-1 OK-2 OK-5 OK-6 OK-9

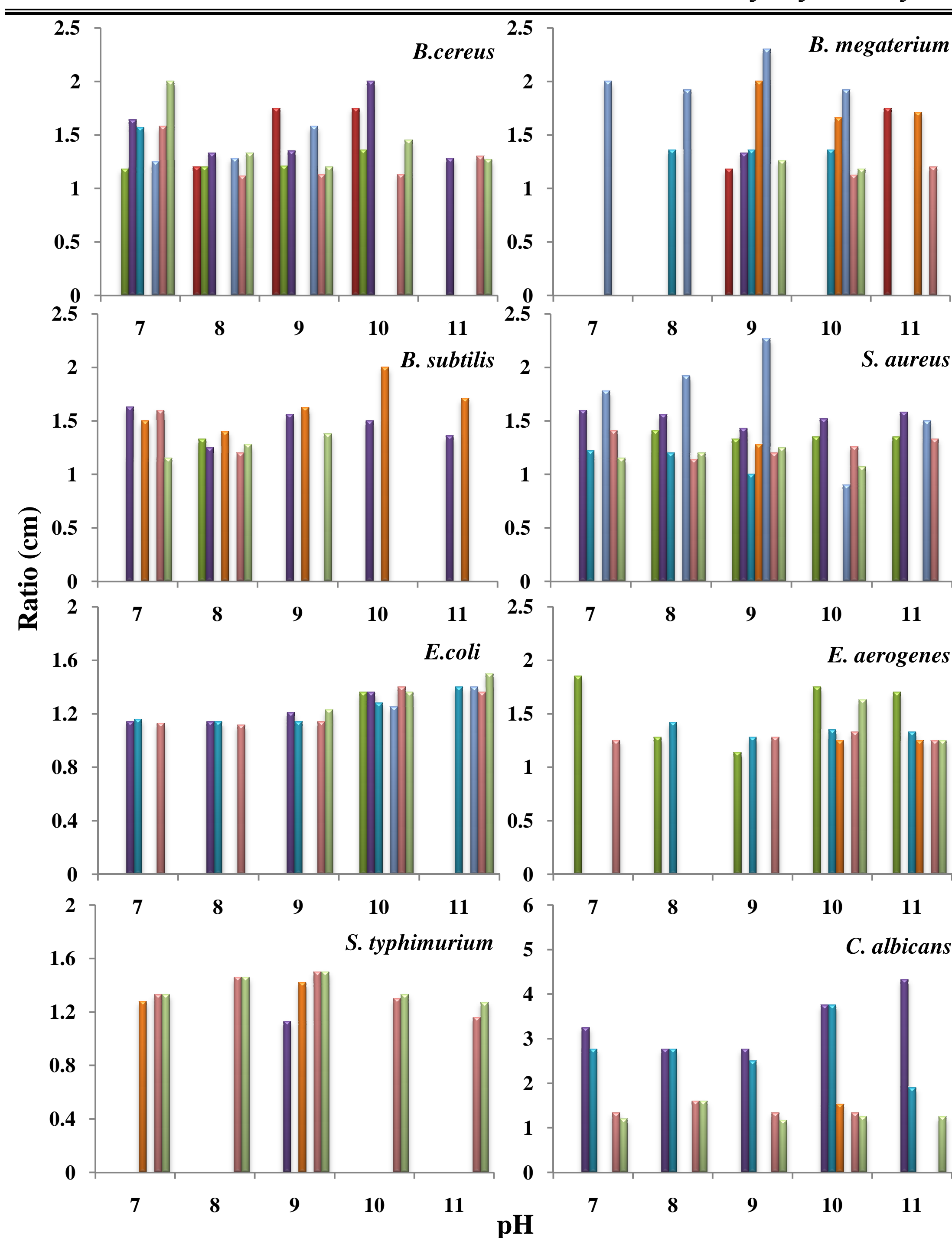


Figure 4.5: Effect of pH on the antimicrobial activities of the isolates of Okha Madhi and Okha

B. cereus, B.megaterium, B. subtilis, S. aureus:

OM-1 OM-3 OM-4 OM-5 OM-10 OM-11 OK-5 OK-8 OK-9

E.coli, E. aerogenes, S. typhimurium:

OM-1 OM-3 OM-4 OM-5 OM-10 OM-11 OK-5 OK-8 OK-9

C. albicans:

OM-1 OM-6 OM-10 OK-1 OK-2 OK-5 OK-6 OK-8 OK-9

4.3.4 Salt and pH profile of actinomycetes

On the whole salt profile (0-10% Nacl) and pH profile (7-11 pH) of actinomycetes among Okha Madhi and Okha site are presented in (Figure 4.6, 4.7). Antibiotic production on various media among the selected isolates of Okha Madhi and Okha site is shown in picture 4.3.

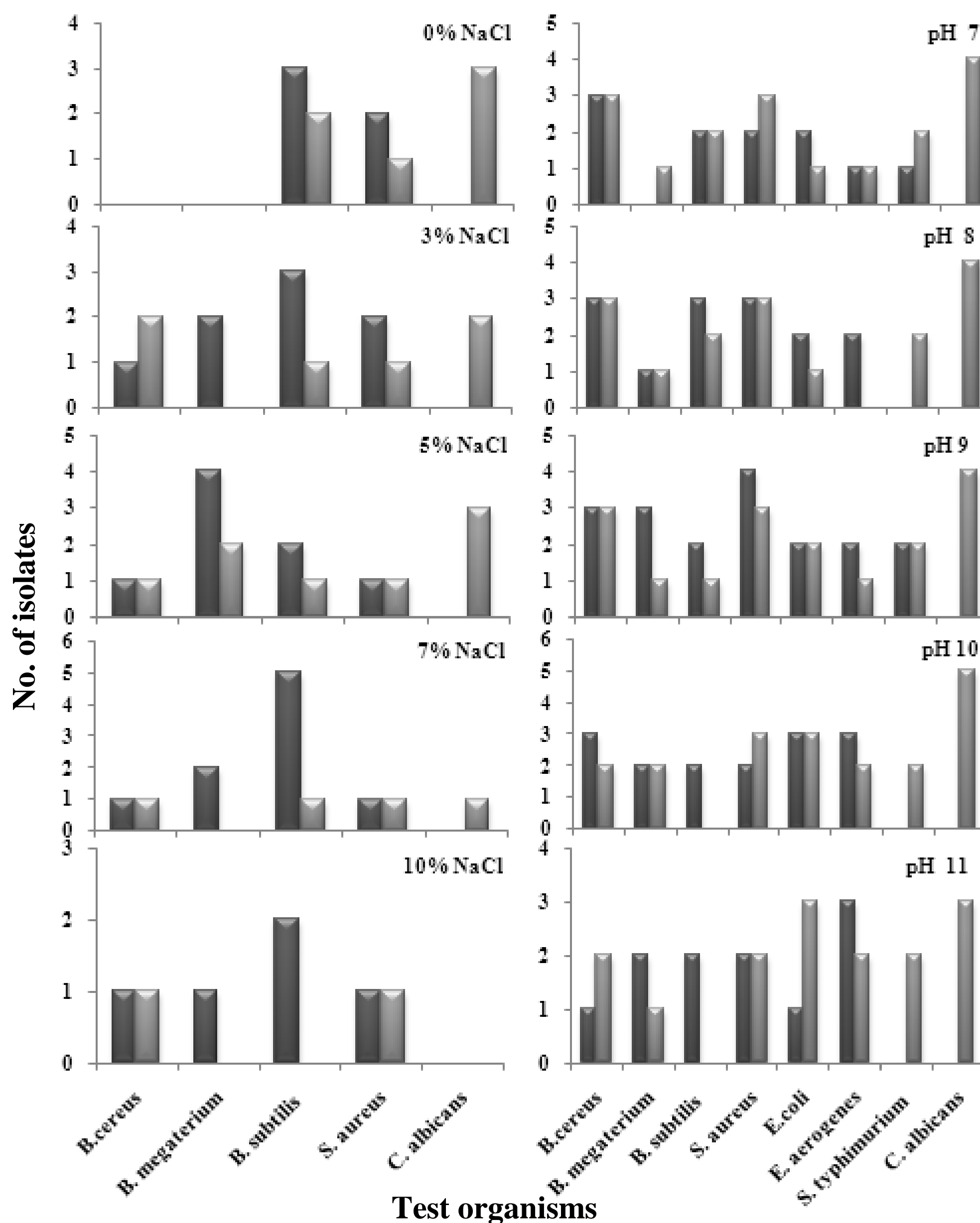


Figure 4.6: Salt profile and pH profile of actinomyces from Okha Madhi (■) and Okha (■)

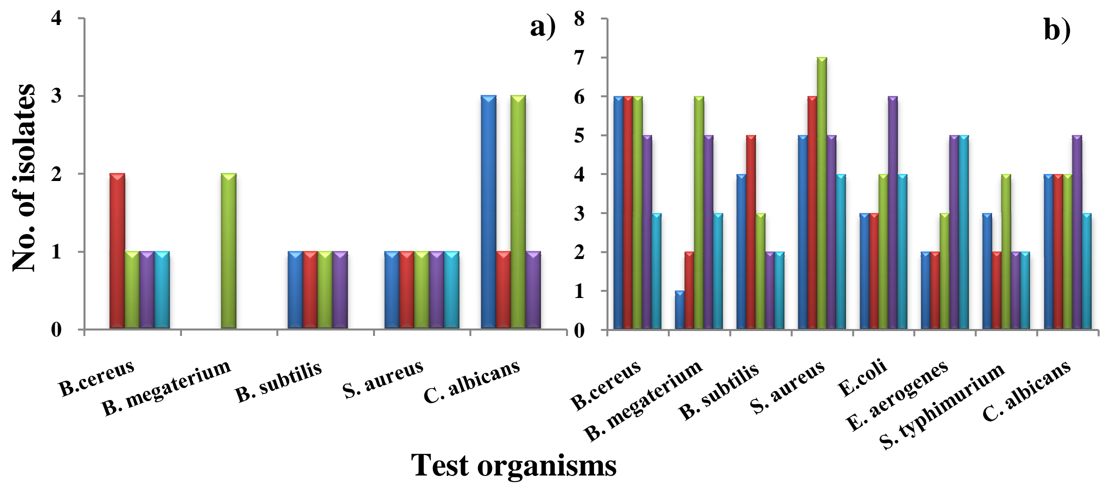
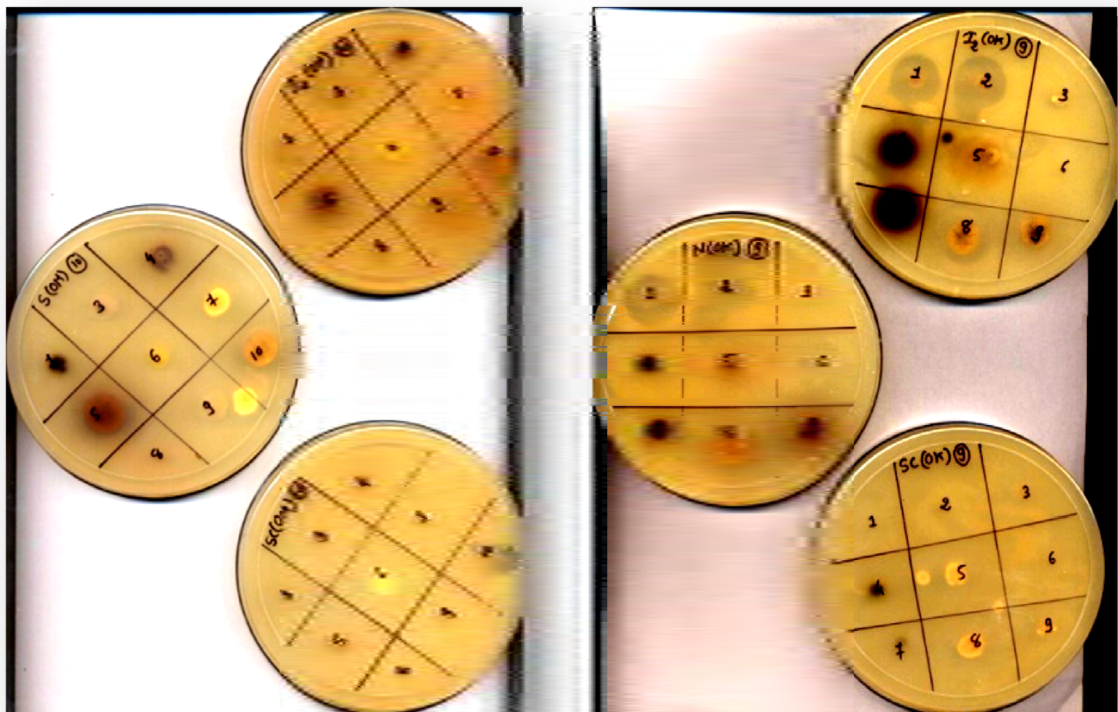


Figure 4.7: a) Salt profiling 0 3 5 7 10and b) pH profile 7 8 9 10 11 of total isolates



Picture 4.3: Antibiotic production on various media from the selected isolates of Okha Madhi and Okha

4.4 DISCUSSION

During the recent years, pathogenic microorganisms have acquired resistance against antimicrobial agents and hence search for new, safe and more effective antimicrobial agents has intensified. Though, there are many reports on mesophilic actinomycetes (Muiru *et al.,* 2008; Pandey *et al.,* 2008; Cowen, 2008), there are only few reports on bioactive compounds from salt tolerant alkaliphilic actinomycetes (Hong *et al.,* 2009; Fiedler *et al.,* 2005; Uyeda 2004). Selection of potential antagonistic actinomycetes depends upon the huge diversity of the strains. To explore the diversity of promising strains, the present investigation focused on the isolation of actinomycetes from various sites of less explored saline and alkaline habitats of coastal Gujarat, India. Consequently, salt tolerant alkaliphilic actinomycetes displaying spectrum of antimicrobial potential were isolated and characterized.

Actinomycetes had good growth on yeast extract malt extract agar medium with good aerial and substrate mycelium formation. Based on the antibiotic susceptibility pattern, majority of actinomycetes were found to be resistant against Cloxocilin. Pearson and Carol (2008) reported that antibiotics, such as penicillin and erythromycin, used as a one-time miracle to cure, are now less effective due to bacteria having acquired resistance. As an evolutionary process, bacteria have developed antibiotic resistance mechanisms against most of the natural antimicrobial agents (Cowen, 2008).

Media composition plays a vital role in the efficiency and economics of the biological processes. It influences not only growth and metabolism of the culture, but also increases product titer and consequently process economy. Maximum isolates displayed antimicrobial activity on starch casein agar media, starch agar media and ISP-2, followed by tyrosine agar, N-agar and ISP-3 agar media. However, while growth was evident on most of the media used, the antimicrobial activity depended on the medium used for growth (Takahashi *et al.,* 1994; Bernan *et al.,* 1994). Kokare *et al* (2004) reported that AH1 species grown on tyrosine agar showed good

antibacterial activity against *S. aureus* as compared to when grown on maltose yeast extract agar. Among the isolates, majority exhibited antimicrobial potential against gram positive bacteria while the isolates also displayed antimicrobial activity against gram negative bacteria, yeast and even against candida. Atta and Ahmad (2009) and Naeimpoor and Mavituna (2000) reported actinomycetes with broad spectrum of antibacterial, antifungal, antitumural, antiparasitic and antiviral activities.

Majority of the isolates were halo tolerant and some were able to grow up to 20% w/v NaCl, the optimum being in the range of 5–10%. Some were also capable to grow in the absence of salt indicating their halo tolerant nature. The results are quite comparable with Guan *et al* (2009) and Syed *et al* (2008). However, the salt dependence of the isolates was less compared to *Prauserella salina* sp. nov., a truly halophilic actinomycete (Li *et al* 2009b). Majority of the isolates from Okha madhi secreted antibiotics against *Bacillus subtilis* and *Bacillus megaterium* at 5-10% NaCl. However, Thumar *et al* (2010) and Vasavada *et al* (2006) reported *Bacillus subtilis* as a most sensitive against the antimicrobial agents for the salt-tolerant alkaliphilic actinomycetes. Further, as reported by Radhakrishnan *et al* (2010), a great part of antibiotic compounds exhibited exclusive activity against gram-positive bacteria and only 1.5% of the compounds had antimicrobial activities against gram-negative bacteria (Berdy, 2005). While observing the trends of salt profile, it was evident that majority of isolates produced antimicrobial activity against gram positive test organisms however; no antibiotic production against gram negative test organisms or yeast was observed amongst the isolates of Okha Madhi or Okha site. Some of the isolates from Okha also produced antibiotics against *Candida albicans*. Sundaram *et al* (2010) reported 31 strains out of 63 isolates of actinomycetes having sensitivity against *Candida albicans* and hence it was confirmed that actinomycetes from saline and alkaline habitats would have novel antimicrobial compounds than those from normal microflora. As per a report, while a haloalkaliphilic actinomycete grew but did not secrete antibiotics at the salt concentrations above 10% (Thumar *et al.,* 2010). Among the isolates in the present study, only OM-10 from Okha Madhi and from Okha; OK-5, OK-9 and OK-10 exhibited antimicrobial activity against *Bacillus*

cereus at 5-10% NaCl, while no antimicrobial activity was observed in the absence of NaCl. This signifies the role of biologically competitive environment with unique conditions of pH, temperature, pressure, oxygen, light, nutrients and salinity in antibiotic production. Earlier studies (Suthindhiran and Kannabiran, 2009, Sarkar *et al.,* 2008, Li *et al.,* 2005, Manam *et al.,* 2005 and Kokare *et al.,* 2004) also revealed that halophilic actinomycetes from saline (marine) habitats are rich in bioactive antibiotics.

The isolates in the present study grew optimally at pH 9 with slow growth at neutral pH. Further, majority of the isolates exhibited antimicrobial activity against gram positive and gram negative test organisms at the wider range of pH 7–11. Recently, *Nesterenkonia alba* sp. nov., an alkaliphilic actinobacterium was reported to grow with an optimum pH of 9–10 (Luo *et al.,* 2009). A novel alkaliphilic *Streptomyces* strain has been reported to secrete pyrocoll, an antimicrobial compound, under alkaline conditions (Dietera *et al.,* 2003). Our results are also comparable with some *Streptomyces* species recorded to secrete antibiotics against bacteria, fungi and yeasts at higher salinity and alkaline pH (Basilio *et al.,* 2003). It is also observed in pH profiling that both, gram positive and negative target bacteria were inhibited by the strains of actinomycetes. Interestingly, many of the present isolates also produced compounds against candida at wider range of alkaline pH (pH 7-11), besides affecting the growth of bacteria and yeast. The trends indicated broad spectrum antimicrobial potential of the actinomycetes. Recently, Dhanasekaran *et al* (2009) reported a *Streptomyces* sp. secreting a broad spectrum antibiotic against some pathogenic bacteria and fungi. *Candida* sp. has emerged as the third most usually present isolates in hemocultures in developed countries. Continuous use of antifungal drugs may also produce resistant strains of *C. albicans* and many infections due to *Candida* species are refractory to antifungal therapy (Sangamwar *et al.,* 2008; Cowen *et al.,* 2002). Despite the long list of currently available antibiotics, there are only limited reports on anticandidal activities (Susithra *et al.,* 2009; Khalesi *et al.,* 2006; Moosa *et al.,* 2004). Consequently; new, safe and more effective sources of antimycotic agents are quite in need, particularly in view of the opportunistic capabilities of candida and

yeast in patients suffering from terminal diseases. Hence, our attempts on the search of broad spectrum antibiotics against bacteria, yeast and candida exploring extreme actinomycetes would be an important step.

Chapter 5

Assessment of Molecular Diversity among Actinomycetes using DGGE and RFLP

5.1 INTRODUCTION

Actinomycetes, phylogenetically defined as a number of taxa within the high G+C subdivision of the gram-positive phylum, are involved in important processes with a wide range of habitats. Till date, thousands of new species have been isolated in different genera of normal mesophilic actinomycetes, while in comparison, extremophilic actinomycetes are largely overlooked for their occurrence, phylogeny, diversity and biotechnological potential. The studies on extemophilic actinomycetes started, when Gochnauer and coworkers (1975) isolated *Actinopolyspora halophila* an extremely halophilic actinomycete. However there was a long gap since then in pursuing their research on such organisms and it's only during the last 5-6 years that some reports on the commercial significance of novel metabolites obtained from these organisms have appeared in the literature (Thumar and Singh 2009, 2007; Mehta *et al.,* 2006). According to Bergey's Manual (Garrity & Holt, 2001), the phylum Actinobacteria comprises 39 families and 130 genera, making it one of the largest groups within the Bacteria. However, except for their distinct clustering in 16S rRNA trees, no other reliable biochemical or molecular characteristics are presently known which can clearly distinguish species belonging to the phylum Actinobacteria from other bacteria. Therefore, our recent work has focused on identifying novel molecular characteristics that are useful for biochemical, taxonomic and phylogenetic purposes to differentiate actinomycetes.

In addition, due to pharmaceutical, industrial and environmental importance of actinomycetes, current understanding of the taxonomy and evolutionary relationships of the extremophilic actinomycetes is of a great importance. Therefore we attempted differentiation of salt tolerant alkaliphilic actinomycetes species like *Streptomyces*, *Nocardiopsis* and *Nesterenkonia* based on the branching patterns of 16S rRNA trees as well as morphological, cultural, physiological and molecular identification. Among actinomycetes species presented, *Streptomyces* and *Nocardiopsis* are well known whereas phylogenetic and chemotaxonomic re-analysis of the genus *Micrococcus* resulted in the proposal of the *Nesterenkonia* gen. nov. and the reclassification of *Micrococcus halobius* (Onishi and Kamekura 1972) as *Nesterenkonia halobia* (Stackebrandt *et al.,* 1995). All *Nesterenkonia* species identified in present study are gram positive, strictly aerobic and moderately halophilic or halotolerant. The remarkable adaptability to saline environment makes the *Nesterenkonia* an important

group in the microbial community of saline habitat. Furthermore, PCR, using specific probes as selective amplification primers offer an alternative approach for the rapid identification of large numbers of strains (Yoon *et al.,* 1996; Mehling *et al.,* 1995). At the same time application and usefulness of the genus-specific primers based on 16S rRNA genes has already been reported for PCR identification of genus *Streptomycetes* (Mehling *et al.,* 1995), *Nocardiopsis* (Salazar *et al.,* 2002), *Streptomonospora* (Zhi *et al.,* 2006) and *Nesterenkonia* (Zhi *et al.,* 2008). Following electrophoretic separation of the PCR products, denaturing gradient gel electrophoresis is a technique recently introduced in microbial ecology by Muyzer *et al* (1993). The denaturing gradient can be achieved either chemically with urea and formamide in denaturing gradient gel electrophoresis (DGGE) (Myers *et al.,* 1985) or physically by temperature in temperature gradient gel electrophoresis (TGGE) (Riesner *et al.,* 1989). Both techniques are reported to be interchangeable, giving comparable fingerprints of microbial communities (Heuer and Smalla 1997). In a DGGE gel the number, precise position, and intensity of the bands in a gel track give an estimate of the number and relative abundance of numerically dominant ribotypes in the sample. However, distinct band pattern generated by pure cultures of actinomycetes isolates in our study may indicate heterogeneity of 16S rRNA genes in the genome and this would be first report citing application of species specific primers as well as fingerprinting technique like DGGE in diversifying actinomycetes species from saline and alkaline habitat.

In addition to DGGE, we applied PCR amplification and restriction fragment length polymorphism (RFLP) as an identification method, in which amplified 16S rRNA gene was digested by selected restriction endonuclease *HhaI*. The restriction patterns of the unknown isolates were easily compared to the established patterns on the data bases. This method allowed actinomycete isolates to be identified to the genus level in less than a week, following DNA isolation from a pure culture. Traditional methods for differentiation of species and taxa of aerobic actinomycetes are laborious and time-consuming and frequently require specialized testing that is beyond the capabilities of clinical labor. Therefore, a rapid method like DGGE and RFLP would be extremely useful to identify actinomycetes to the genus level.

5.2 MATERIALS AND METHODS

5.2.1 Actinomycetes and culture conditions

Twelve halo-tolerant and alkaliphilic actinomycetes were isolated from Okha-Madhi site, the region of coastal Gujarat. For the isolation of actinomycetes, all soil samples were processed in the field as soon as possible after collection by using desiccation and heat shock as selective cultivation methods (Mincer *et al.,* 2002).These methods were designed to reduce the numbers of gram negative bacteria and to enrich for slow growing spore forming actinomycetes. Detailed cultural conditions are described in Chapter 3 (Section 3.2.2). Actinomycetes colonies were recognized by the presence of branching, vegetative filaments and the formation of tough, leathery colonies that adhered to the agar surface. Hence, only mycelium forming bacteria belonging to the order *Actinomycetales* were included in the present study.

5.2.2 DNA extraction

For the DNA isolation, isolates were grown on YEME media (Yeast Extract-Malt Extract broth) with 5% salt at pH 9.0 for 3-4 days at 28-30°C. The preparation of total genomic DNA was conducted in accordance with the methods described by Sambrook *et al* (1989) with the little modifications. For that, the culture aliquots were subjected to centrifugation for 15 minutes at 10000 rpm to obtain cell pallets, which were then suspended in STE buffer. The samples were again centrifuged at 10000 rpm. Subsequently, the pellets were re-suspended in GET buffer with SDS (20%) and lysozyme solution (10mg/ml in Tris-Cl pH 8) and incubated for 2 hours. After performing P: C: I (phenol:Chloroform:isoamyl:propanol) and C:I extraction steps, the DNA was pooled by adding 3M potassium chloride and chilled ethanol. The DNA was then suspended in appropriate volume of distilled water and preserved at -20°C. The quality of the DNA was checked by spectrophotometry and agarose gel electrophoresis.

5.2.3 Primers

The sequence of the 16S rRNA genes from a large number of isolates were isolated using two different universal primer sets U1 and U2, two *Streptomyces* specific primers sets, Strep B/E, Strep B/F and one *Nesterenkonia* specific primer set N F/R. Quality and amount of DNA were suitable for successful PCR amplification. Primer pair U1F 5'-AGAGTTTGATCCTGGCTCAG-3' and U1R 5' AAGGAGGTGATCCAGCCGCA-3' (*Edwards et al.,* 1989) and primer pair U2F 5' CCAGCAGCCGCGGTAATACG-3' and U2R 5' ATCGGCTACCTTGTTACGACTTC (Lu *et al.,* 2000) were used for universal amplification of actinomycetes 16S rRNA as a control, to ensure that DNA extracts contained actinomycetes DNA and the quality and amount of DNA were suitable for successful PCR amplification. *Streptomyces* specific PCR primer Strep B 5'-ACAAGCCCTGGAAACGGGGT-3' (forward), StrepE 5'-CACCAGGAATTCCGATCT-3' (reverse) and StrepF 5'-ACGTGTGCAGCCCAAGACA-3' (reverse) (Rintala *et al.,* 2001; Suutari *et al.,* 2001) in two primer sets were used for the amplification of 16S rRNA gene from DNA isolated. The primer pairs StrepB/StrepE and StrepB/StrepF amplified 520 bp and 1070 bp fragments, with nucleotides position 139-657 and 139-1212 respectively (Pernodet *et al.,* 1989). *Nesterenkonia* specific Primer pair N F/R 5'-CGCATAGGGTGCTGGTGGAAAG-3' (forward) 5'-GAGGTCGGGTTGCAGACTTCG-3' (reverse) was used for selective amplification of the 16S rRNA gene, corresponding to the region 194–215 and 1308 - 1328 respectively with 1120bp fragment of 16S rRNA gene (Zhi *et al.,* 2008). The primers used in this study were synthesized by Sigma Aldrich USA.

5.2.4 PCR amplification

The gradient PCR method was developed to specifically amplify 16S rRNA sequences from twelve pure cultures of actinomycetes using universal primers as well as *Streptomyces* specific and *Nesterenkonia* specific primer sets. Optimization of PCR amplification was done using Eppendorf Master Cycler Gradient. PCR amplification

conditions used for all actinomycetes isolates, with five different sets of primers are listed in (Table 5.1). Gel electrophoresis of PCR amplified product was carried out using 0.8% agarose gel with 5µg/ml Et-Br and 6x gel loading buffer. After loading samples, gel was run with 1x TAE buffer at 55 volt for 90 minutes followed by the observation of DNA bands under Gel doc system (Biorad USA).

Primer	Denaturation	Annealing	Extension	No. of Cycles	Amplification (Expected bp)
U1 F/R	94°C-10min	94°C-30s 56°C-30s 72°C-1min	72°C-10min	30	1500bp
U2 F/R	94°C-5min	94°C-1min 55°C-1min 72°C-1min	72°C-10min	30	1000bp
Strep B/E	94°C-8min	95°C-1min 54°C-40s 72°C-2min	72°C-10min	30	520bp
Strep B/F	95°C-8min	95°C-1min 58°C-40s 72°C-2min	72°C-10min	30	1170bp
N F/R	94°C-4min	94°C-30s 56°C-30s 72°C-1min	72°C-10min	30	1120bp

Table 5.1: PCR amplification conditions and expected base pair amplification of five different primers used

5.2.5 Denaturing gradient gel electrophoresis (DGGE)

For DGGE analysis, 16S rRNA regions of actinomycetes sp. were directly amplified with two universal and three different species specific primer pairs. The DNA concentration of samples was determined and 400 nanogram of DNA were loaded onto 8% (w/v) polyacrylamide gel in TAE buffer (40 mM Tris, 20 mM acetate, 1 mM EDTA, pH 8.0). The 8% polyacrylamide gel (40% acrylamide-N, N'-methylenebisacrylamide stock, 37:1) was made with denaturing gradients ranging 20-

50% (100% denaturant contains 7M urea and 40% formamide). DGGE was performed at 200V. DNA bands were visualized by staining with ethidium bromide.

5.2.6 RFLP analysis

For restriction pattern analysis, each amplified 16S rRNA gene was digested using the four cutter restriction enzyme *HhaI* (Genei, Bangalore) for 2h. After electrophoresis on 2% agarose gel at 50V for 3 h, band patterns were captured and analyzed using a Gel doc system (Bio Rad, USA).

5.3 RESULTS

5.3.1 Isolation and 16S rRNA identification

Morphological, cultural and physiological characteristics of all actinomycetes have been studied in Chapter 3 (Section 3.3.3). However, molecular characterization of isolates would provide evidence to the results obtained by morphological, cultural and physiological characterization of actinomycetes. Therefore, we attempted identification of actinomycetes using molecular tools and techniques specifically from the isolates of Okha Madhi and Okha sites.

5.3.2 DNA isolation

To isolate DNA in its pure form, actinomycetes were needed to proceed for molecular biology techniques. Standardization of protocol was required as it differs from bacteria due to its cell wall composition and filamentous structures. DNA isolation of all isolates were done by using modified method and DNA, obtained good quality and purity which was judged on the basis of spectrophotometric results as well as agarose gel quantification. The result of spectrophotometric analysis showed that DNA samples of nearly all the isolates from Okha Madhi and Okha sites had good purity and ratio (A_{260}/A_{280}) in between 1.4 - 1.8.

5.3.3 PCR amplification

After DNA isolation, the next step was to amplify 16S rRNA gene. For that we used two universal primers (U1, U2) and three species specific primers (Strep B/E, Strep B/F, NF/R). The detailed results of amplification from the isolates of Okha Madhi and Okha sites are described below.

5.3.3.1 16S rRNA amplification of isolates from Okha Madhi with universal and species specific primer sets

Among total twelve isolates from Okha Madhi site, 16S rRNA gene of 7 isolates (58%) were able to amplify with U1 primer giving 1500bp amplification while 9 isolates (75%) were amplified with U2 primer giving 1000bp amplification. However,

OM-1, OM-2, OM-7, OM-10 and OM-12 were not amplified with U1 primer. Similarly, OM-2, OM-5 and OM-10 were not amplified with U2 primer. OM-2 and OM-10 were not amplified with any of the primer U1 or U2. Annealing temperature (Ta) had huge impact on amplification pattern as with change of temperature; size and concentration of amplified product differed. Three different gradient of annealing temperature 53.1°C, 56.3°C, 59.3°C were used in view of the melting temperature of U1 primer (Tm) (Figure 5.1a, 5.2a) while 52.7°C, 55.9°C, 59.2°C were used for U2 primer (Figure. 5.1b, 5.2b).

Steptomyces specific primer sets StrepB/E, StrepB/F were used for the amplification of 16S rRNA gene fragments obtained from DNA isolated as of pure culture of actinomycetes. We got 520 bp and 10870 bp sized amplified product with StrepB/E, StrepB/F primer sets. Gradient of annealing temperature used was 50.7°C, 53.9°C, 56.7°C while amplifying with StrepB/E primer and 54.1°C, 58.1°C, 60.0°C while amplifying with StrepB/F primer set (Figure 5.1c, 5.3a). Among total twelve isolates seven isolates i.e., OM-3, OM-4, OM-5, OM-8, OM-9, OM-10, OM-11 were amplified with StrepB/E while only OM-2 isolate was amplified with StrepB/F primer set (Figure 5.1d, 5.3a). However, concentration of amplified product was too low in OM-8, OM-9, OM-10 and OM-11 that band was not clearly visualized on DGGE. With *Nesterenkonia* specific primer set N-F/R, 16S rRNA gene of OM-8, OM-9, OM-11 and OM-12 was amplified having 1120 bp product at annealing temperatures; 53.3°C, 56.4°C and 60.0°C. However, concentration of amplified product was very poor in OM-8 and OM-12 (Figure 5.1e, 5.3b).

5.3.3.2 16S rRNA amplification of isolates from Okha with universal and species specific primer sets

Among total twelve isolates, 16S rRNA gene of 5 isolates (50%) from Okha site were able to amplify with U1 primer giving 1500bp amplification while 7 isolates (70%) were amplified with U2 primer giving 1000bp amplification. However, OK-1, OK-4, OK-5, and OK-7 were not amplified with U1 primer. Similarly, OK-1 and OK-5 were

not amplified with U2 primer. OK-1 and OK-5 were not amplified with any of the primer U1 or U2. Annealing temperature (Ta) had huge impact on amplification pattern as with change of temperature; size and concentration of amplified product differed. Three different gradient of annealing temperature 53.1°C, 56.3°C, 59.3°C were used in view of the melting temperature of U1 primer (Figure 5.1a, 5.4a) while 52.7°C, 55.9°C, 59.2°C were used for U2 primer (Figure 5.1b, 5.4b).

Steptomyces specific primer sets StrepB/E, StrepB/F were used for the amplification of 16S rRNA gene fragments. We got 520 bp and 1170 bp sized amplified product with StrepB/E, StrepB/F primer sets. Gradient of annealing temperature used was 50.7°C, 53.9°C, 56.7°C while amplifying with StrepB/E primer and 54.1°C, 58.1°C, 60.0°C while amplifying with StrepB/F primer set (Figure 5.1c, 5.4a).. Among total ten isolates five isolates i.e., OK-1, OK-2, OK-3, OK-4, OK-8 were amplified with StrepB/E while OK-1, OK-2, OK-3, OK-4, OK-8, OK-9 was amplified with StrepB/F primer set (Figure 5.5b). However, concentration of amplified product of Strep B/E Strep B/F was too low in OK-2, OK-4, OK-1 and OK-9 that band was not clearly visualized on DGGE. With *Nesterenkonia* specific primer set N-F/R, 16S rRNA gene of OK-1 and OK-8 were amplified having 1120 bp product at annealing temperatures; 53.3°C, 56.4°C and 60.0°C. However, concentration of amplified product was very poor in OK-8 (Figure 5.1e, 5.5c).

5.3.3.3 16S rRNA amplification profile of isolates from Okha Madhi and Okha

While generating amplification profile of 16S rRNA gene using all five primer sets we found maximum amplification with U2 primer (75%) followed by U1 (58%), StrepB/E (50%), StrepB/F (42%) and NF/R (33%) among the isolates of Okha Madhi site (Figure 5.6A). While among the isolates of Okha, we found maximum amplification with U2 primer (80%) followed by StrepB/F (60%), StrepB/E (50%), U1 (50%), and NF/R (20%) (Figure 5.6B).

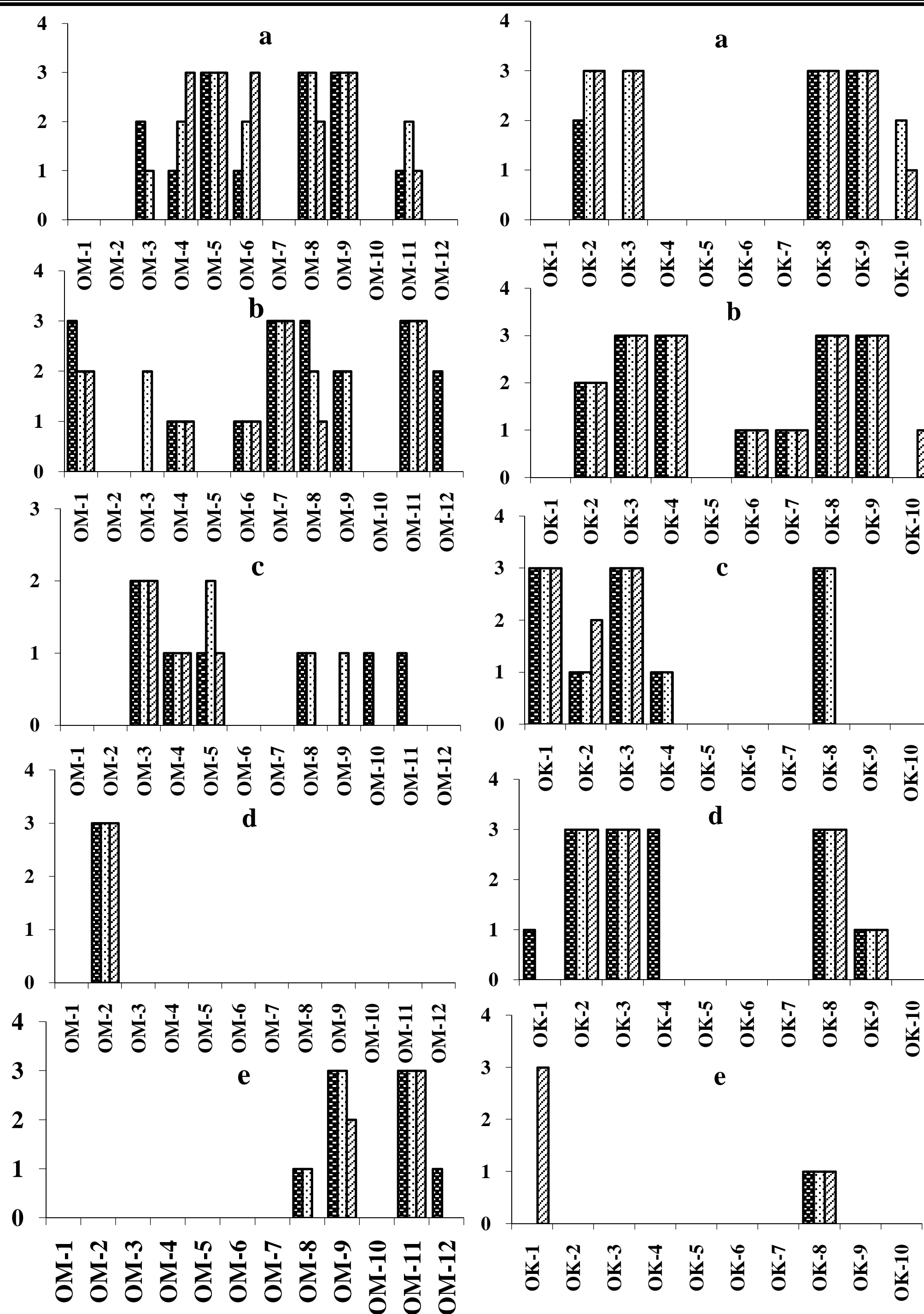


Figure 5.1: 16S rRNA amplification profile of isolates from Okha-Madhi and Okha site using five different primer sets and gradient of annealing temperatures for each primer. a) U1: 53.1°C (▩), 56.3°C (▤), 59.3°C (▨); b) U2: 532.7°C (▩), 55.9°C (▤), 59.2°C (▨); c) StrepB/StrepE: 50.7°C (▩), 53.9°C (▤), 56.7°C (▨); d) StrepB/StrepF: 54.1°C (▩), 58.1°C (▤), 60.0°C (▨); e) N-F/R; 53.3°C (▩), 56.4°C (▤), 60.0°C (▨). (0 - no amplification, 1- slight amplification, 2 - medium amplification and 3 - intence amplification)

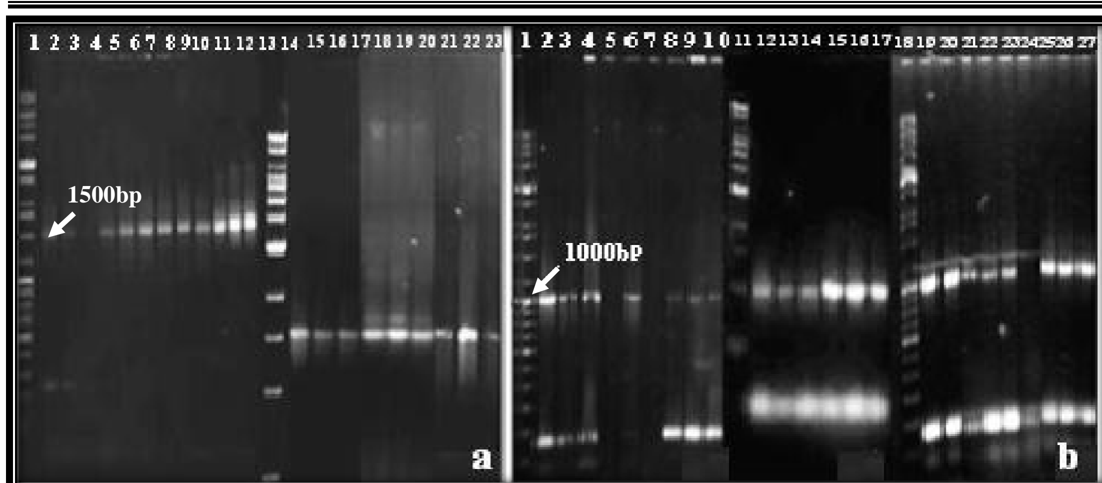


Figure 5.2: 0.8% agarose gel show PCR products of Okha Madhi isolates amplified with (a) U1 primer at 52.3°C 55.3°C 59.4°C: lane-1 Medium range DNA ruler lane-2,3,4 OM-3, lane-5,6,7 OM-4 lane-8,9,10 OM-5 lane-11,12,13 OM-6 lane-14 Super Mix DNA ladder, lane-15,16,17 OM-8 lane-18,19,20 OM-9 lane-21,22,23 OM-11 (b) U2 primer at 52.7°C, 55.9°C, 59.2°C: lane-1 High range DNA ruler lane-2,3,4 OM-1 lane-5,6,7 OM-3 lane-8,9,10 OM-4 lane-11 Super Mix DNA ladder, lane-12,13,14 OM-6 lane-15,16,17 OM-7 lane-18 High range DNA ruler lane-19,20,21 OM-8 lane-22,23,24 OM-9 lane-25,26,27 OM-11

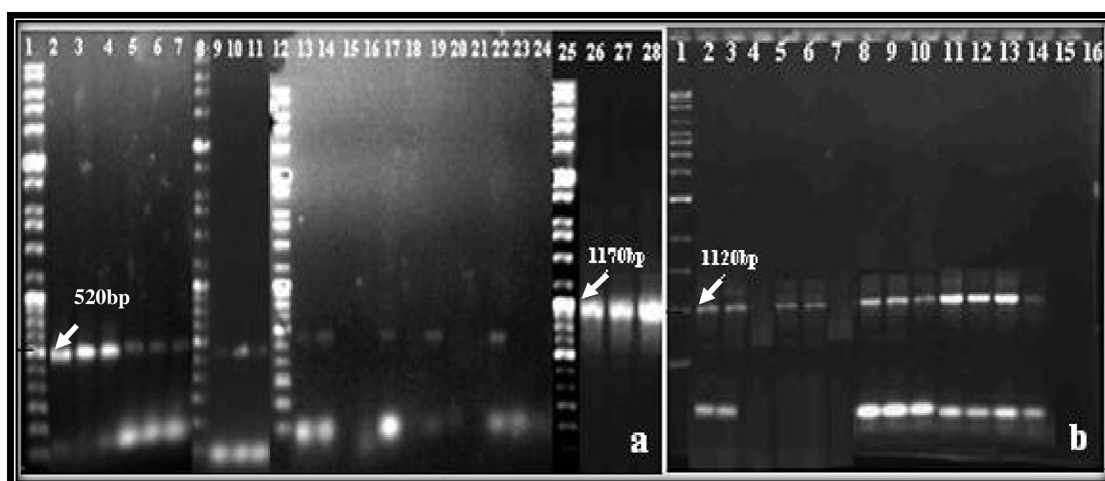


Figure 5.3: 0.8% agarose gel show PCR products of Okha Madhi isolates amplified with (a) StrepB/E (Lane 2-24) at 50.7°C, 53.9°C, 56.7°C and StrepB/F (Lane 26-28) at 54.1°C, 58.1°C, 60.0°C lane-1 High range marker (10 kb), lane-2,3,4 OM-3 lane-5,6,7 OM-4 lane-8 High range marker, lane-9,10,11 OM-5 lane-12 High range marker, lane-13,14,15 OM-8 lane-16,17,18 OM-9 lane-19,20,21 OM-10 lane-22,23,24 OM-11 lane-25: High range marker, lane-26,27,28 OM-2 (b) N F/R primer at 53.3°C, 56.4°C, 60.0°C lane-1 high range marker (10 kb), lane-2,3,4 OM-6 lane-5,6,7 OM-8 lane-8,9,10 OM-9 lane-11,12,13 OM-11 lane-14,15,16 OM-12

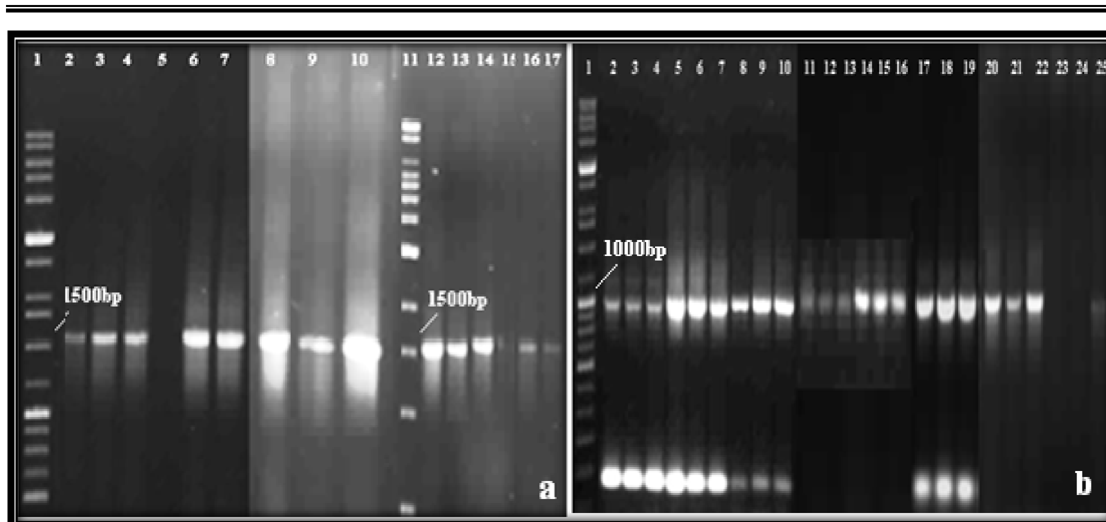


Figure 5.4: 0.8% agarose gel show PCR products of Okha isolates amplified with (a) U1 primer at 52.3°C 55.3°C 59.4°C: lane-1 Medium range DNA ruler lane-2,3,4 OK-2, lane-5,6,7 OK-3 lane-8,9,10 OK-8 lane-11 Super Mix DNA ladder, lane-12,13,14 lane-15,16,17 (b) U2 primer at 52.7°C, 55.9°C, 59.2°C: lane-1 High range DNA ruler 2,3,4 OK- 2 lane-5,6,7 OK-3 lane-8,9,10 OK-4 lane-11,12,13 OK-6 lane-14,15,16 OK-7 lane-17,18,19 OK-8 lane-20,21,22 OK-9 lane-23,24,25 OK-10

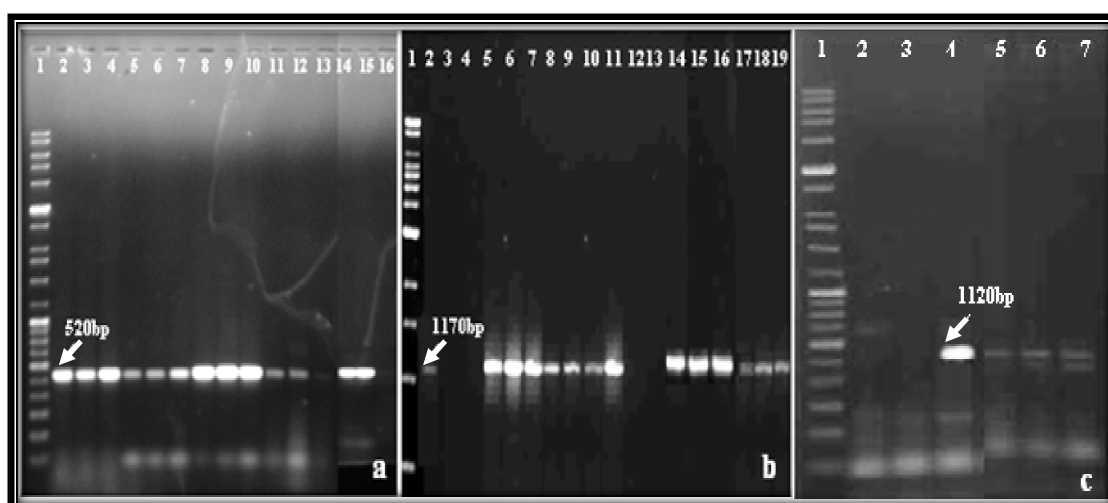


Figure 5.5: 0.8% agarose show PCR products of Okha isolates amplified with (a) StrepB/E at 50.7°C, 53.9°C, 56.7°C: lane-1 high range marker (10 kb), lane-2,3,4 OK-1 lane-5,6,7 OK-2 lane-8,9,10 OK-3 lane-11,12,13 OK-4 lane-14,15,16 OK-8 (b) StrepB/F at 54.1°C, 58.1°C, 60.0°C: **l**ane-1 Super mix DNA ladder, lane-2,3,4 OK-1 lane-5,6,7 OK-2 lane-8,9,10 OK-3 lane-11,12,13 OK-4 lane-14,15,16 OK-8 lane-17,18,19 OK-9 (c) N-F/R primer at 53.3°C, 56.4°C, 60.0°C: lane-1 high range marker (10 kb), lane-2,3,4 OK-1 lane-5,6,7 OK-8

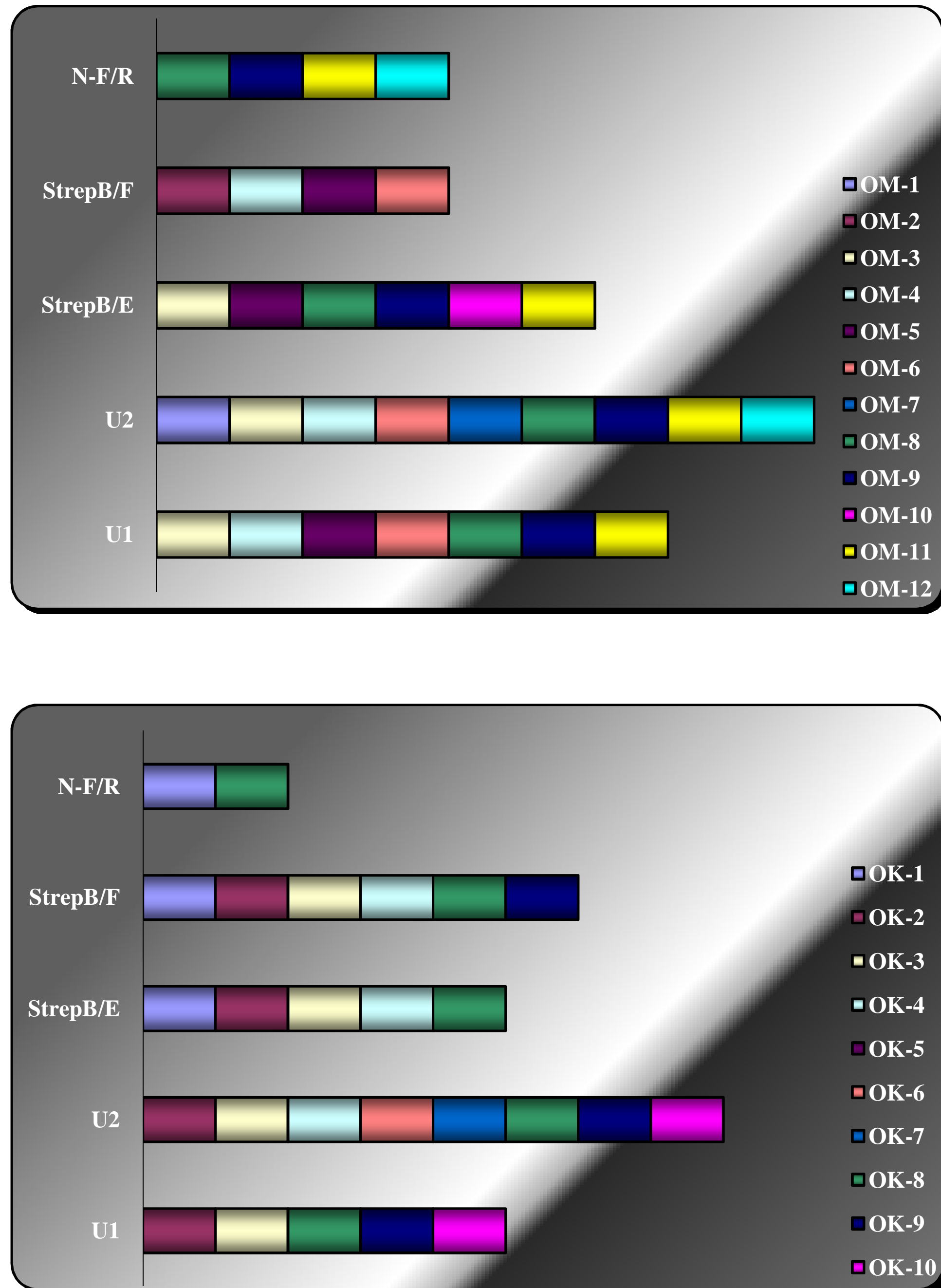


Figure 5.6: 16S rRNA amplification profile of isolates from A) Okha Madhi and B) Okha site

5.3.4 Denaturing gradient gel electrophoresis (DGGE) with universal (U1, U2) and species specific (Strep B/E, Strep B/F, NF/R) primer sets

5.3.4.1 Analysis of DGGE pattern among the isolates of Okha Madhi

The sensitivity of DGGE to resolve DNA fragments with different sequences was performed and comparison of DGGE profile was made among as well as between U1 and U2 primer sets. While observing DGGE pattern of U1 primer (Figure 5.7a), actinomycetes were divided into two major groups one group was having similar band pattern with one dominating band in case of OM-4, OM-5, OM-6 and OM-7 while another group was having distinct band pattern in each isolate with one major band at the same position as first group. Similarly while studying DGGE profile of U2 primer set (Figure 5.7b), we found same four band pattern in OM-1, OM-6, OM-7 and OM-12 with three dominating bands put into one group while OM-3, OM-4, OM-8 and OM-11 were put into second group as having three bands with two dominating bands and OM-9 and OM-11 were put into third group. However two dominating bands were observed in each isolate suggesting that each isolate is of same order actinomycetes and variation among the groups would be there at species level. Therefore the results obtained by DGGE band pattern of U1 primer are in compliance with the results obtained by DGGE band pattern of U2 primer set. Interestingly we found a single DGGE band of the isolates amplified 520bp with streptB/E primer at the same electrophoresis distance suggesting strains of a common *Streptomyces* species. However distinct band was observed of OM-2 isolate amplified with SteptB/F primer as size of amplified product was 1070 bp suggesting a different species of *Strepotmyces* (Figure 5.7c). Similarly, while studying DGGE profile of isolates amplified with *Nesternkonia* specific primer set, we found similar band pattern of all isolates studied that proves that isolates are of common genus *Nesternkonia* (Figure 5.7d). However, identification at the species level would generate some more incites in detection of polygenetic status of *Nesterenkonia* in a clade of actinomycetes. Phylogenetic status of actinomycetes based on microbiological and molecular techniques was predicted as shown in Table 5.2.

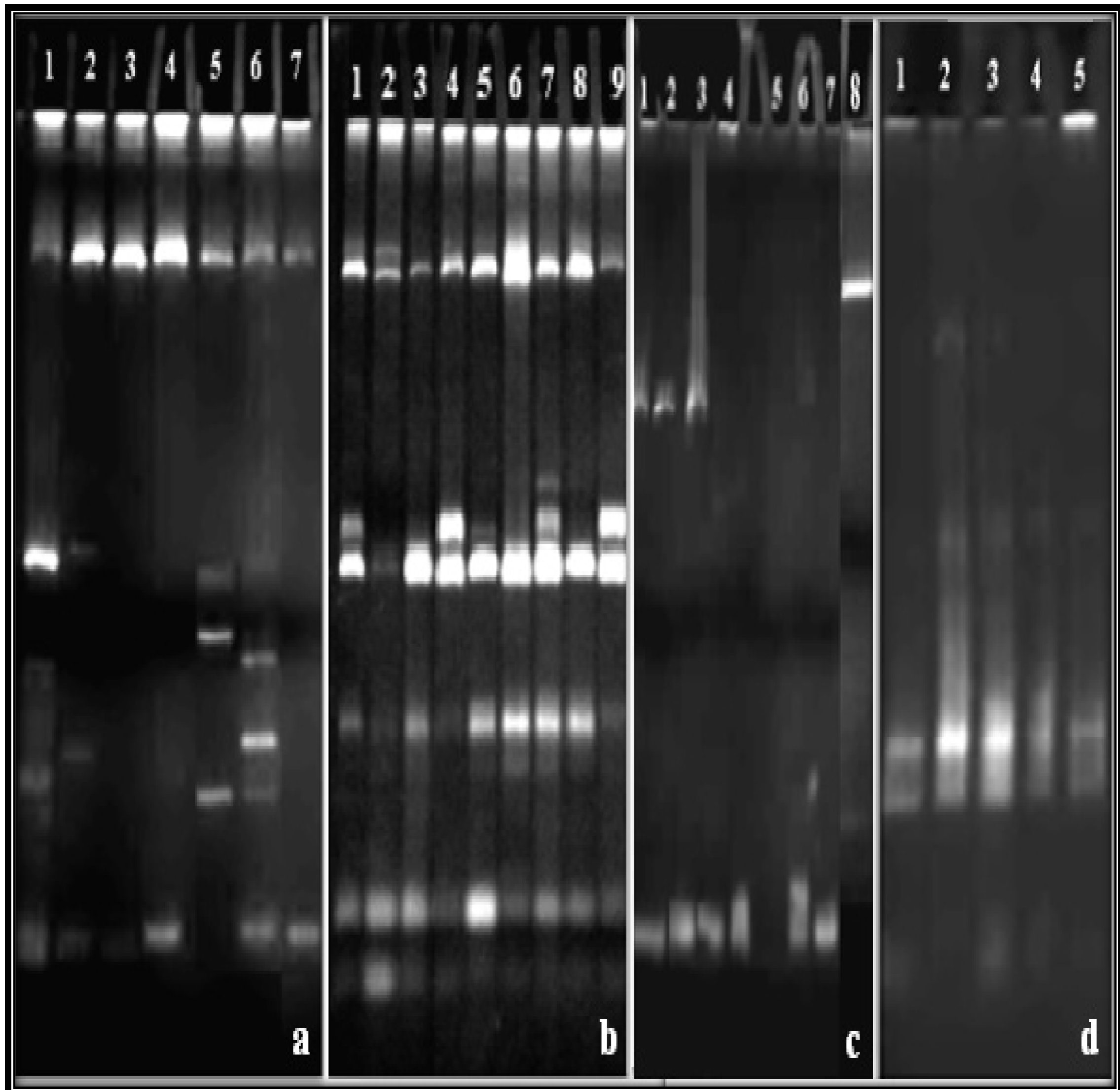


Figure 5.7: DGGE pattern of 16S rRNA amplified products with (a) Universal primer U1; lane-1: OM-3, lane-2: OM-4, lane-3: OM-5, lane-4: OM-6, lane-5: OM-8, lane-6: OM-9, lane-7: OM-11 (b) Universal primer U2; lane-1: OM-1, lane-2: OM-3, lane-3: OM-4, lane-4: OM-6, lane-5: OM-7, lane-6: OM-8, lane-7: OM-9 lane-8: OM-11 lane-9: OM-12 (c) *Streptomyces* specific primer (StrepB/StrepE); lane-1: OM-3, lane-2: OM-4, lane-3: OM-5, lane-4: OM-8 lane-5: OM-9, lane-6: OM-10, lane-7: OM-11 and *Streptomyces* specific primer (StrepB/StrepF); lane 8: OK-2 (d) *Nesterenkonia* specific primer (NF/R); lane-1: OM-6, lane-2: OM-8, lane-3: OM-9, lane-4: OM-11, lane-5: OM-12

5.3.4.2 Analysis of DGGE pattern among the isolates of Okha site

The sensitivity of DGGE to resolve DNA fragments with different sequences was performed and comparison of DGGE profile was made among as well as between U1 and U2 primer sets. While observing DGGE pattern of U1 and U2 primer (Figure 5.8a, 5.8b), we found distinct band pattern. However two dominating bands were observed in majority of isolates suggesting that each isolate is of same order actinomycetes and variation among the groups would be there at species level. Interestingly, we found a single DGGE band of the isolates amplified 520bp with StrepB/E primer at the same electrophoresis distance suggesting strains of a common *Streptomyces* species. However distinct band was observed of OK-1, OK-2, OK-3, OK-4, OK-8 and OK-9, isolate amplified with StrepB/F primer as size of amplified product was 1170 bp suggesting a different species of *Strepotmyces* (Figure 5.8c). Similarly, while studying DGGE profile of isolates amplified with *Nesternkonia* specific primer set, we found similar band pattern of all isolates studied that proves that isolates are of common genus *Nesternkonia* (Figure 5.8d). However, intensity of band was too low. So identification at the species level would generate some more incites in detection of polygenetic status of *Nesterenkonia* in a clade of actinomycetes. Phylogenetic status of actinomycetes based on microbiological and molecular techniques was predicted as shown in Table 5.3.

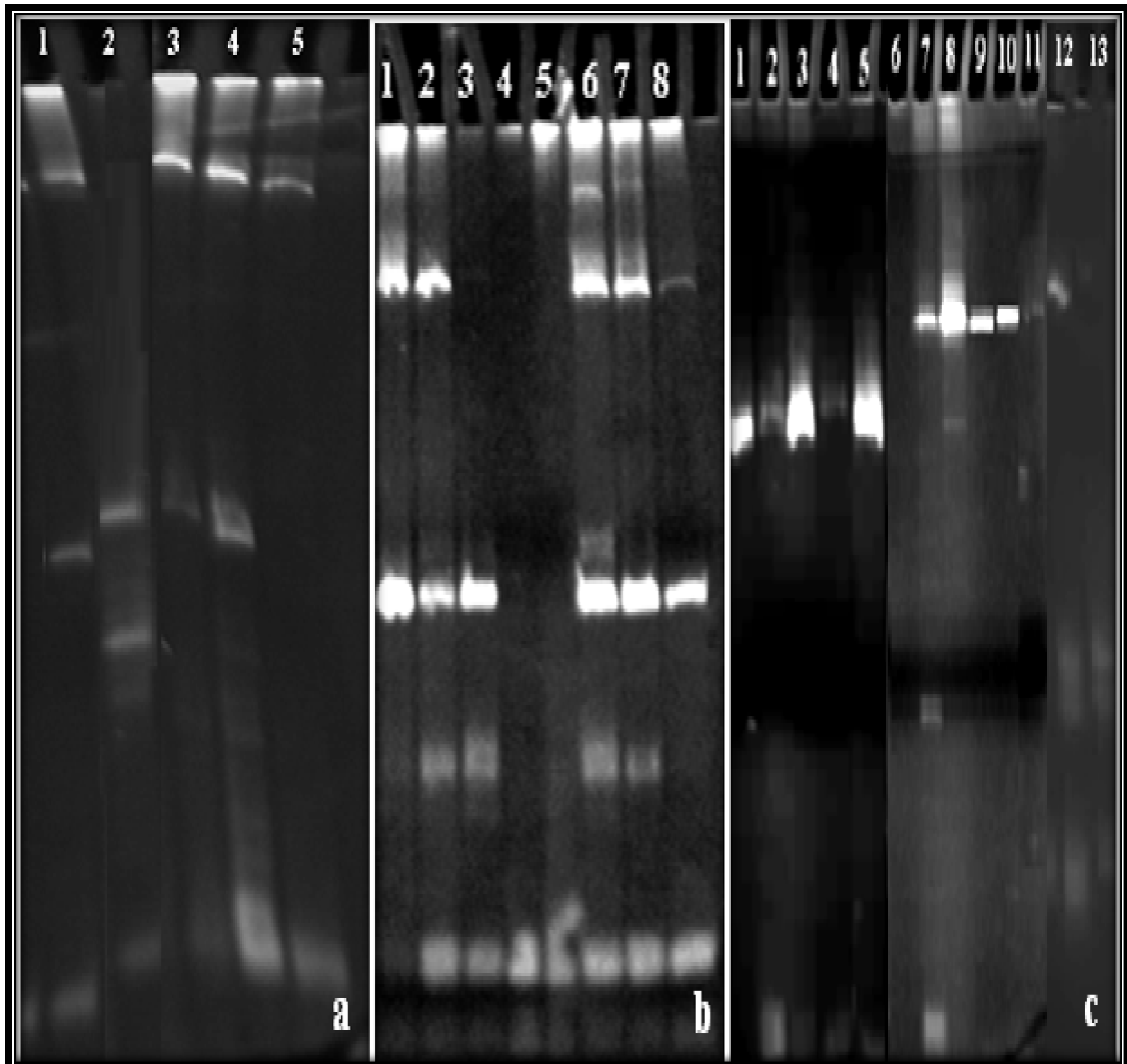


Figure 5.8: DGGE pattern of 16S rRNA amplified products with (a) Universal primer U1; lane-1 OK-2, lane-2 OK-3, lane-3 OK-8, lane-4 OK-9, lane-5 OK-10 (b) Universal primer (U2); lane-1 OK-2, lane-2 OK-3, lane-3 OK-4, lane-4 OK-6, lane-5 OK-7, lane-6 OK-8 lane-7 OK-9, lane-8 OK-10 (c) *Streptomyces* specific primer (StrepB/StrepE); lane-1 OK-1, lane-2 OK-2, lane-3 OK-3, lane-4 OK-4, lane-5 OK-8 (d) *Streptomyces* specific primer (StrepB/StrepF); lane 6 OK-1 lane 7 OK-2, lane 8 OK-3, lane 9 OK-4, lane 10 OK-8, lane 11 OK-9 (e) *Nesterenkonia* specific primer; lane-12 OK-1, lane-13 OK-8

Group	Test organisms	Color	Closest database match
I	OM-1	Dark gray	***Nocardiopsis lucentensis***
	OM-6	Yellowish white	***Nocardiopsis kunsanensis***
	OM-7	Yellowish white	***Nocardiopsis halototerans***
	OM-12	Gray	***Nocardiopsis salina***
II	OM-2	Pinkish red	***Streptomyces griseus***
	OM-3	Gray	***Streptomyces anulatus***
	OM-4	Light Yellow	***Streptomyces coelicolor***
	OM-5	Gray	***Streptomyces griseus***
	OM-8	Bluish white	***Streptomyces griseus***
	OM-10	Grayish bluish white	***Streptomyces griseus***
III	OM-9	Yellowish Bluish white	***Nesterenkonia Sp.***
	OM-11	Light grayish white	***Nesterenkonia Sp.***

Table 5.2: Phylogenetic status of actinomycetes based on microbiological and molecular techniques from Okha Mdhi

Group	Test organisms	color	Closest database match
I	OK-5	Light pinkish	***Nocardiopsis alba***
	OK-6	Dark gray	***Nocardiopsis kunsanensis***
	OK-9	Light pinkish	***Nocardiopsis halototerans***
	OK-10	Creamish white	***Nocardiopsis salina***
II	OK-1	Creamish	***Streptomyces griseus***
	OK-2	Light Gray	***Streptomyces anulatus***
	OK-3	Bluish white	***Streptomyces coelicolor***
	OK-4	Dark Gray	***Streptomyces griseus***
	OK-7	Dark gray	***Streptomyces* sp.**
III	OK-8	Light bluish	***Nesterenkonia Sp.***

Table 5.3: Phylogenetic status of actinomycetes based on microbiological and molecular techniques from Okha

5.3.5 RFLP analysis of isolates from Okha Madhi and Okha

Result of 16S rRNA amplification showed amplification of more than 50% actinomycetes isolates. However, no amplification was observed in OM-2, OM-7, OM-10, OM-12, OK-1, OK-3, OK-5 and OK-6. The PCR product of each 16S rRNA genes from actinomycetes isolates was about 1.5kb long. After purification, the amplicons were digested with the four cutter endonuclesse *HhaI* and restriction pattern was analyzed on agarose gel electrophoresis. For different strains of actinomycetes analyzed, 3 different RFLP patterns were differentiated (Figure 5.9) Further, RFLP band pattern was correlated with the results of denaturing gradient gel electrophoresis profile and based on that we found out that each typical RFLP pattern represented a group of isolated actinobacteria, as shown in Table 5.4. Strains yielding RFLP patterns 1 belonged to genus *Nocariopsis*; strains yielding patterns 2 belonged to *Streptomyces*. The strain yielding pattern 3 belonged to *Nesterenkonia*. Therefore, using the *HhaI* based 16S rRNA - RFLP fingerprinting analysis, the actinmycetes isolates could be identified to the genus level.

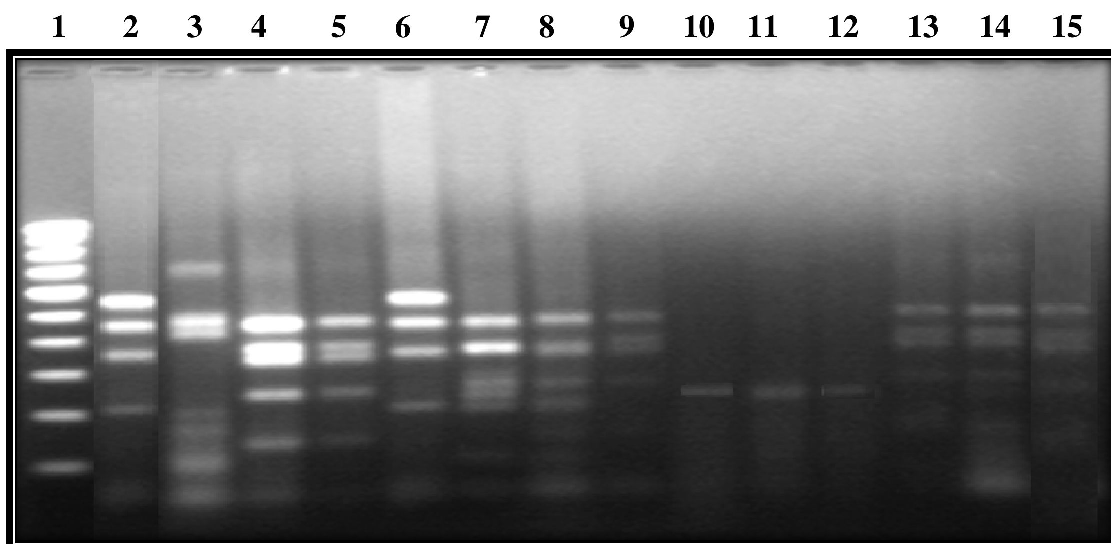


Figure 5.9: Restriction fragment length polymorphism (RFLP) patterns of the 16S rRNA gene PCR products of actinomycetes digested with the restriction enzyme *HhaI*. Lane 1: 100bp DNA Ladder, Lane 2: OM-1, Lane 3: OM-3, Lane 4: OM-4, Lane 5: OM-5, Lane 6: OM-6, Lane 7: OM-8, Lane 8: OM-9, Lane 9: OM-11, Lane 10: OK-2, Lane 11: OK-4, Lane 12: OK-7, Lane 13: OK-8, Lane 14: OK-9, Lane 15: OK-10

Group	Test organisms	Closest database match
I	OM-1	***Nocardiopsis lucentensis***
	OM-6	***Nocardiopsis kunsanensis***
	OK-8	***Nocardiopsis sp.***
	OK-9	***Nocardiopsis halototerans***
	OK-10	***Nocardiopsis salina***
II	OM-3	***Streptomyces anulatus***
	OM-4	***Streptomyces coelicolor***
	OM-5	***Streptomyces griseus***
	OK-2	***Streptomyces anulatus***
	OK-4	***Streptomyces griseus***
	OK-7	***Streptomyces* sp.**
III	OM-8	***Nesterenkonia Sp.***
	OM-9	***Nesterenkonia Sp.***
	OM-11	***Nesterenkonia Sp.***

Table 5.4: Phylogenetic status of actinomycetes based on microbiological and molecular techniques from Okha Madhi and Okha

5.4 DISCUSSION

Molecular biological approaches are used to characterize bacterial population in the field of microbial ecology and which include 16S rRNA gene cloning and genomic fingerprinting techniques. Genomic fingerprinting techniques are valuable tools for the characterization of complex microbial communities in the environment. We employed molecular methods like 16S rRNA gene amplification using universal and species specific primers in combination with DGGE as a fingerprinting technique supported by cultural and physiological characters of salt tolerant alkaliphilic actinomycetes.

PCR which allows the specific and sensitive amplification of a preselected DNA region has been intensively applied to the species identification of numerous organisms (Crotchefelt *et al.,* 1997; Garnier *et al.,* 1997; Ramzan 1997; Koide and Saito 1995; White *et al.,* 1992). However, its use require the detailed examination of the molecular genetics of organisms, especially the identification of the 16S rRNA sequences that are specific for the organism tested. Moreover, species specific primer profiles make it easy to identify organisms at genus and species level. Therefore, we generated 16S rRNA gene amplification profile of total isolates using five different primer sets. Two sets were universal primers specific for eubacteria and three primer sets were group specific; two *Streptomycetes* specific and one *Nesterenkonia* specific.

The results of 16S rRNA amplification profile using universal primers suggest that the PCR based assay described in this study is a reliable tool for detection of actinomycetes. This is particularly important since recent work indicates considerable diversity and phylogenetic relatedness within the salt tolerant alkalliphilic actinomycetes. Literature supported the results as phylogenetic characterization of culturable actinomycetes and taxonomic characterization of *Streptomyces* strain CH54-4 was completed using universal primer U1 (Nithyanand *et al.,* 2010; Srivibool *et al.,* 2010). Identification of novel alkaliphilic actinomycetes *Nocardiopsis valliformis* sp. as well as other alkaliphilic actinomycetes was completed by

amplifying 16S rRNA gene using U1 primer at 52°C annealing temperature (Yang *et al.,* 2008; Mitsuikli *et al.,* 2007). Li *et al* (2006) found 16S rRNA gene sequences of five novel *Nocardiopsis* isolates ranged between 438bp and 1490bp using universal primer. Similarly Magarvey *et al* (2004) also found nearly complete 16S rRNA gene sequences (averaging 1,445 nucleotides) at 55°C annealing temperature from novel marine derived actinomycetes while almost complete 16S rRNA gene sequence (1494bp) of *Nocardia* and *Streptomyces* were found using universal primer by (Roth *et al.,* 2003; Rashidian *et al.,* 2003). In addition, three xylanase producing strains of actinomycetes were amplified using U2 primer at 55°C annealing temperature (Ninawe *et al.,* 2006). Similarly, Yoon *et al* (2006) used universal primers for 16S rRNA gene sequence of nov. *Nesternkonia Jeotgali* sp. Consequently, amplification of the isolates by 16S rRNA universal primers (U1 and U2), specific to eubacteria established the fact that the selected isolates belong to this group.

In addition to universal primers, we diversify our isolates using species specific primers and literature supported our results as members of the *Streptomyces violaceusniger* was analyzed using taxon specific primer (Sahin *et al.,* 2010; Goodfellow *et al.* 2007). El-Naggar *et al* (2006), amplified 16S rRNA gene of *Streptomyces* sp. strain MAR01 using Strep B/F primer. However annealing temperature was 53°C that was lower than ours. Similarly, 16S rRNA gene of *Strepomyces vilaceusniger* strain HAL64 and *Streptomyces* sp. was amplified using StrepB/F primer by (El-Naggar 2007; Park *et al.,* 2006). Furthermore, Rintala *et al* (2002) used both *Streptomyces* specific primer pairs to find diversity of Streptomycetes based on 16S rDNA sequences. Zhi *et al* (2008) found similar results while studying genus-specific PCR for molecular identification of novel isolates of the genus *Nesterenkonia.* Another novel species, *Nesterenkonia jeotgali*, has been described validly by Yoon *et al* (2006). However, to date, few *Streptomyces* isolates have been tested using these assays, so further testing should be done to confirm their wider applicability. Therefore, we applied DGGE as a fingerprinting tool to project phylogenetic relationship among the isolates of Okha Madhi site.

Now pure cultures should theoretically produce a single band in DGGE analysis. However all the groups formed a specific side product pattern type that was same for all the strains of a specific group. The extra bans did not disappear by dilution or with DNA purified by alcohol extraction, and could be due to 16S rRNA heterogeneity. Heterogeneity among rRNA genes within a single organism has been investigated with denaturing gel electrophoresis (Nubel *et al.,* 1996) and DNA sequencing of the cloned gene (Wang *et al.,* 1997; Liefting *et al.,* 1996; Fox *et al.,* 1992). Earlier it was believed that mutations are introduced by misincorporation and misrepair by DNA polymerase during DNA replication is the cause of heterogeneity. However driving force for the origin of heterogeneity in the group is not mutation during DNA replication (Ueda *et al.,* 1999). It is found that conjugative plasmids are generally found in *Streptomyces* and the plasmids can mobilize the host chromosome at high frequency (Hopwood and Kieser, 1993). One possible explanation for the mechanism that generates the heterogeneity in the large group is horizontal gene transfer mediated by conjugative plasmids. This consideration is not limited to the genus *Streptomyces*. In other microorganisms, gene flux mediated by conjugative plasmids is thought to have an important role in increasing genetic diversity (Syvanen, 1994; Mabile and Chicurel 1992), suggesting that it is a mechanism common to many microorganisms. From the viewpoint of phylogenetic analysis, it is difficult to consider that one or two base heterogeneity within the α-region affects the topology of the phylogenetic relationship reconstructed with 16S rRNA sequences in small groups (Ueda *et al.,* 1999). In spite of the fact that almost identical 16S rRNA sequences have been reported in phynotypically divergent bacteria (Fox *et al.,* 1992), sequence analysis of the 16S rRNA gene is of great importance for modern bacterial taxonomy.

The 16S rRNA amplification profile using species specific primer and DGGE analysis suggests that majority of the isolates can be clustered in one group while analyzing phylogeny. However, the results indicate the presence of several different types of *Strepotomyces* and some of the *Nesterenkonia* strains. We found literature describing role of species specific primers in phylogeny study as cited above however we don’t find literature describing application of DGGE as a finger printing tool to evaluate

phylogenetic relatedness among the isolates of same genus or species especially in *Streptomyces* and *Nesterenkonia.* Moreover, while PCR amplification profile and DGGE fingerprinting profile was compared with morphological and physiological properties of actinomycetes isolates and we found majority of isolates from the species *Streptomyces* followed by *Nocardiopsis* and *Nesterenkonia* respectively. So by combining the 16S rRNA amplification profile, DGGE fingerprinting profile and cluster analysis, together with morphological, cultural and physiological characterization clearly differentiate cluster of actinomycetes comprising three genus *Streptomyces, Nocardiopsis* and *Nesterenkonia.* From our finding it is shown that the combination of molecular and microbiological methods can be very useful to differentiate microbial communities.

When there are too many isolates and the sequencing costs of all strains are high, RFLP analysis can effectively reduce the number of isolates needing to be sequenced (Zhang *et al.,* 2006). RFLP analysis of the 16S rRNA gene is a rapid and inexpensive method that can be applied to study diversity of microbial communities. Actinbacterial strains could be identified at the genus level using restriction endonucleases without sequencing (Magdy *et al.,* 2010; Cook and Meyers 2003). In our earlier studies, diversity of actinomyctes was find out using molecular approach, the predominant actinomycetes groups identified were *Streptomycetes, Nocardiopsis and Nesterenkonia* on the basis of 16S rRNA amplification using five different sets of primer (universal and species specific) followed by denaturing gradient gel electrophoresis (DGGE). Further, our current study supported the results of DGGE analysis. In the current study, we only used one restriction endonuclease *HhaI* that specifically recognize and cut the sequence “GCGC”. The resulting different RFLP patterns obtained allowed us to differentiate the strains into distinct groups of actinobacteria. When the *HhaI* RFLP fingerprints were compared with the corresponding DGGE band pattern the isolated actinomycetes could be identified at the genus level and for two genera *Streptomyces* and *Nocardiopsis* at the subgenus level. Literature supports our findings as Ninawe *et al* (2006) identified three xylanase producing actinomycetes using PCR – RFLP method. Zhang *et al* (2006) used 16S

rRNA amplification - RFLP analysis for culturable actinobacteria from the marine sponge *Hymeniacidon perleve*. Further, Cook and Meyers (2003) used PCR-restriction pattern analysis for rapid identification of actinomycetes to the genus level. Lu *et al* (2000) used PCR with universal primers and restriction endonuclesase digestions for detection and identification of common bacterial pathogens in cerebrospinal fluid. Consequently, this rapid and convenient method can be very useful in the large scale identification of actinomycetes isolates.

The objective of the present work was to develop molecular methods for assessing molecular diversity among salt tolerant alkaliphilic actinomycetes. In this chapter we described optimization of PCR reaction system, especially annealing temperature, can relieve the interference of pseudopositive results and raising the annealing temperature can avoid non-specific amplification effectively. In addition to tentative assignment of novel isolates to specific genus, genus-specific PCR combined with DGGE technique distinguish different species belonging to the same genus and promote the development of microbial ecology from a methodological perspective. Moreover, due to its rapid and easy performance, DGGE is useful for identification of extended number of isolates and is also powerful fingerprinting method for revealing sequence heterogeneities in the 16S rRNA gene.

Chapter 6

Extracellular Enzymes from Salt tolerant Alkaliphilic Actinomycetes and Factors Influencing their Production

6.1 INTRODUCTION

Actinomycetes have attracted attention due to their various natural products and specific mechanism of adaptation to extreme environments. While actinomycetes from normal habitats have focused considerable attention during the last decades, exploration of such organisms from extreme habitats is a relatively new horizon (Jiang *et al.,* 2006; Li *et al.,* 2005a; Zhang *et al.,* 2004; Kim *et al.,* 2003; Kampfer *et al.,* 2002; Kim *et al.,* 2001; Heyd *et al.,* 2000). Continued efforts to characterize halo-tolerant alkaliphilic actinomycetes diversity and their adaptations to the extreme environment will create a better understanding of the potential applications of these organisms in biotechnology. To date, the findings on salt-tolerant and alkaliphilic actinomycetes are largely based on phylogeny and only limited attempts have been made to explore their enzymatic potential. They hold significance in the field of enzymology, pharmaceuticals, degradation of biomolecules, food technology and microbial enhanced oil recovery (Starch *et al.,* 2005; Kampfer *et al.,* 2002; Abd-Allah, 2001).

The occurrence and optimization of many industrially important antibiotics have been reported from different actinomycetes. However, the studies relating to the parameters affecting enzyme production among the salt tolerant alkaliphilic actinomycetes are rather scarce. Optimization of the process parameters is important to improve the productivity and yield. Each of the medium components increase the enzyme production up to a threshold level, however, a concentration above it may exert repression. Hence, in the process aimed to produce bulk product, the most important parameters have been shown to be the cultural and nutritional conditions.

Generally protease synthesis and secretion are induced by peptides or other proteinaceous substrates (Litchfield and Prescott, 1970). Enhanced production of alkaline protease from *Bacillus* sp. P-2 was observed in glucose 1% (w/v) along with yeast extract and peptone at 0.25% w/v (Kaur *et al.,* 2001). However, it may also be subjected to feed back inhibition by some of the amino acids, which are end products

of proteolytic degradation (Moon and Parulekar, 1991). Enzyme repression is a mode of regulation through which the synthesis of an enzyme is prevented by repressor molecules. In many cases, the end product of a synthesis chain acts as a feed-back co-repressor in association with an intracellular apo-repressor protein, so that this complex is able to block the function of an operator. As a result, the concerned structural genes are prevented from being transcribed into mRNA. On the other hand it was also found that amino acids were inducer of protease synthesis in species of *Micrococcus* (McDonald, 1966). Pastor and coworkers (2001) studied the effect of amaranth seedmeal, a pseudocereal which contains a good amino acid distribution and a high level content of several vitamins, on the alkaline protease levels in *Bacillus subtilis* 3411. They concluded that an increase in amino acid concentration provided by amaranth exerted an inhibitory effect on the enzyme production.

Studies presented in this report deal with the parameters affecting growth and enzyme production. Besides their biotechnological applications, the enzymes from extremophiles may also provide unique models for biochemical and molecular studies to understand the biochemistry, genetics and physiology of organisms under extreme conditions. In addition, on the basic question that how the organisms regulate the synthesis of such enzymes in relation to changing physicochemical environment, present study describes the repression of alkaline protease production by various cultural factors and their threshold limits. Further, the ability of these organisms to utilize crude sources and pure amino acids as the sole nutrient sources would be important for large scale enzyme production. Thus, studies on process optimization for the enzyme production from actinomycetes seem to be a accompanying to traditionally used protease producer.

6.2 MATERIAL AND METHODS

6.2.1 Microorganisms

Forty salt tolerant alkaliphilic actinomycetes were isolated from the coastal region of Gujarat, India. They were putatively assigned to the actinomycetes on the basis of their morphological, cultural, physiological, antimicrobial and molecular characteristics as described in Chapter 3, 4 and 5. Therefore, our next area of thrust was to check the potential of actinomycetes to produce extracellular enzymes. For that, we screened our isolates for the production of extracellular alkaline protease, amylase and cellulase as described below.

6.2.2 Screening of extracellular enzymes

6.2.2.1 Detection of alkaline protease

Detection of alkaline protease secretion was carried out on gelatin agar medium containing (g/L); gelatin, 30; peptone, 10; NaCl, 50 and agar, 30. The pH of the medium was adjusted to 9 by adding separately autoclaved Na_2CO_3 (20% w/v). As actinomycetes produce hard chalky white colonies, suspension was directly prepared by scraping a slant. Spore suspension prepared in sterile D/W using vortex homogenizer was inoculated in the form of regular spot on gelatin agar followed by the incubation for 4 days (until sporulation) at 30ºC. Detection of alkaline protease was carried out by flooding the plates with Frazier's reagent (g/L: $HgCl_2$, 150g; concentrated HCl, 200mL). The clear zone surrounding the colony indicated the secretion of extracellular protease. The colony diameter and zone of clearance was measured to assess the relative enzyme secretion as a function of colony size. The ratio of clearance zone to the colony diameter was calculated. The data in triplicate were subjected to statistical analysis and standard deviation was calculated.

6.2.2.2 Detection of alkaline amylase

Detection of extracellular amylase was carried out on starch agar. The spore suspensions were inoculated on starch agar plates (g/L: starch, 2; yeast extract, 3;

peptone, 5; NaCl, 50, pH 9; agar, 30) as describe above. The plates were incubated for 4 days at 30ºC and after sufficient growth, colony diameter was measured. Gram's iodine (g/300mL; iodine, 1; potassium iodide, 2) was then poured onto the plates to detect the clear zone surrounding the colony against blue background. Ratio of clearance zone to the colony diameter was calculated and data was analyzed as indicated above.

6.2.2.3 Detection of cellulase

Cellulase secretion was detected on Dubo's cellulose agar medium. The spore suspension was prepared as above and inoculated in the form of regular spot on Dubo's agar medium containing, (g/L): cellulose, 10; $NaNO_3$, 10; K_2HPO_4, 1; $FeSO_4{\cdot}7H_2O$, 0.01; KCl, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5; agar, 30g; NaCl, 100 and pH 9. Plates were incubated at 30ºC until sporulation and detection of alkaline cellulase was carried out by adding gram's iodine. Ratio of clearance zone to the colony diameter was calculated to assess the enzyme activity as indicated above.

6.2.3 Effect of NaCl on growth and enzyme secretion

To study the effect of NaCl on the secretion of various extracellular enzymes; gelatin, starch and Dubo's agar plates were prepared for protease, amylase and cellulase, respectively, at NaCl concentrations, 0-20% w/v. The pH of each medium was adjusted to 9 by adding 20% Na_2CO_3 as mentioned earlier. After the incubation at 30ºC for 4 days at time intervals of 24h, the plates were flooded with appropriate reagents and enzyme activity was detected as described above.

6.2.4 Effect of pH on growth and enzyme secretion

Influence of pH on the secretion of protease, amylase and cellulase was studied on gelatin, starch and dubo's agar medium, respectively at pH 7-11 and NaCl, 5% w/v. After the incubation at 30ºC for 4 days at time interval of 24h, the plates were flooded with appropriate reagents to detect the enzyme activity as highlighted above.

6.2.5 Protease production in liquid culture

6.2.5.1 Inoculum preparation

The inoculum was prepared by adding a loopful of pure culture into 250mL sterile gelatin broth followed by the incubation at 30°C on a rotary shaker for 48h. A 10% inoculum from the culture (O.D. 1.0 at A_{540nm}) was added to the gelatin broth containing (g/L); gelatin 10-30; yeast extract 5; peptone 5; NaCl (w/v), 0-200 and pH 9. After incubation for 48h at 30°C under shaking conditions (120 rpm), the growth was measured at 540nm and the cultures were harvested by centrifugation at 5000 rpm for 10min at 4°C. The cell free extract was used as crude preparation of the enzyme.

6.2.5.2 Enzyme assay

Alkaline protease was estimated by the Anson Hagihara method (Hagihara, 1958). The enzyme (0.5 mL) was added to 3.0 mL casein (0.6% w/v in 20mM borate buffer, pH 10) and the reaction mixture was incubated at 60°C for 10min before the addition of 3.2 mL of TCA mixture (0.11 M trichloroacetic acid, 0.22 M sodium acetate, 0.33 M acetic acid). The terminated reaction mixture was incubated for 30min at room temperature and the precipitates were removed by filtration through whatman no. 1 filter paper. The absorbance of the filtrate was measured at 280nm, where one unit of the alkaline protease was defined as amount of enzyme liberating 1µg of tyrosine per minute under the conditions of assay. Enzyme units were measured using tyrosine (0-100 µg) as standard.

6.2.6 Optimization of media for growth and protease production

Inoculums of 9 cultures were prepared in 5% gelatin broth and 10% of each was further inoculated into sterile gelatin broth supplemented with range of NaCl concentrations and pH. The growth and enzyme production was detected at regular time intervals of 24h for 12 to 15 days.

6.2.6.1 Effect of NaCl on growth and protease production

The effect of salt on growth and protease production was studied by varying the NaCl concentrations (0-20%, w/v) at a constant pH 9 in gelatin broth. The growth and enzyme activity were quantified for 15 days at time intervals of 24h at 30°C temperature under shaking condition (120 rpm).

6.2.6.2 Effect of pH on growth and protease production

The influence of pH on growth and protease production was investigated in gelatin broth by varying pH (7-11) at 5% (w/v) NaCl concentration at 30°C under shaking at 120rev/min. Growth and enzyme activity was quantified for 15 days at definite time interval of 24h.

6.2.6.3 Effect of gelatin on growth and protease production

The effect of substrate concentration on growth and protease activity was investigated by varying gelatin concentrations (0-3% w/v) at optimum NaCl and pH under shaking condition (120 rpm) at 30°C for 15 days at time interval of 24h.

6.2.7 Repressive effect of various medium components on OM-6 and OK-5 protease

6.2.7.1 Repression with peptone

Peptone, one of the key ingredients of production medium, was studied for its effect on protease production. Peptone (0-2%, w/v) was added in gelatin broth and medium was inoculated with actively growing culture followed by the incubation at 30°C under shaking conditions (100 rpm). The enzyme activity was detected after 110 h.

6.2.7.2 Repression by yeast extract and casamino acid

Repression exerted by yeast extract was studied by changing the concentrations of yeast extract in gelatin broth in the range of 0-2%, w/v. The medium contained 5% NaCl and pH was adjusted to 9. The actively growing culture (5%) was inoculated in

the medium followed by incubation under shaking conditions (100 rpm) for 110 h at 30ºC.

6.2.7.3 Repression by inorganic phosphate

The effect of K_2HPO_4 on growth and enzyme secretion was studied in gelatin broth (5% w/v, NaCl; pH 9) supplemented with different concentrations of K_2HPO_4 (0-2%, w/v). After incubation for 110 h, the culture aliquots were withdrawn aseptically and the growth and enzyme activity were measured.

6.2.7.4 Catabolite repression by glucose

Glucose is known to exert catabolite repression on enzyme production. So, an experiment was designed to study the effect of glucose on protease production. Glucose was included at varied (0-2%, w/v) concentrations in gelatin broth (5% w/v NaCl, pH 9). Growth and protease production were measured after 110 h of incubation at 30ºC under shaking conditions.

6.2.7.5 Repression of enzyme production by various amino acids

The repressive effect of amino acids at the concentrations of 0-2% w/v was studied in minimal medium (5% w/v NaCl, pH 9) devoid of any other nitrogen source and having 0.5% w/v glucose as the sole source of carbon. The amino acids were selected from each five classes of amino acids. Methionine, alanine, and leucine were selected from class 1, phenylalanine and tyrosine from class 2, aspartic acid from class 3, histidine and arginine from class 4 and asparagine from class 5. Each of these amino acids was included in the medium independently at varied concentrations to study its repressive effect. After 48 h, the culture was centrifuged and resuspended in sterile D/W followed by homogenization with vortex mixer. This inoculums, at the concentration of 5%, was inoculated in each medium and incubated at 30°C under shaking conditions for 110 h. The growth and protease activity were detected as described earlier in this chapter.

6.2.8 Effect of the increasing number of amino acids on protease production

In order to investigate the influence of increasing number of amino acids on protease production, OM-6 and OK-5 were grown in minimal medium (5%, w/v, NaCl, pH 9) containing following combinations of amino acids as the sole source of nitrogen.

1. Phenyl alanine
2. Phenyl alanine+ leucine
3. Phenyl alanine + leucine + methionine
4. Phenyl alanine + leucine + methionine + tyrosine
5. Phenyl alanine + leucine + methionine + tyrosine + aspartic acid
6. Phenyl alanine + leucine + methionine + tyrosine + aspartic acid + arginine

Inoculum was added at the concentration of 5% and after 110 h, the growth and protease was quantified.

6.2.9 Characterization of crude alkaline protease from actinomycetes

6.2.9.1 Temperature profile

The optimum temperature for protease activity was determined by incubating the reaction mixture at 37-100ºC temperatures. Protease activity was measured by the method described previously and residual activity was then calculated by considering protease activity at 37°C as 100%.

6.2.9.2 Effect of pH on protease activity and stability

The effect of pH on protease activity was determined by preparing the substrate in various buffers (20mM) of different pH. The reaction cocktails were incubated at 60ºC. The buffers used were: Citrate phosphate (pH 7); Tris-HCl (pH 8); Glycine-NaOH (pH 9); Borate (pH 10-11) and KCl - NaOH (pH 12). After incubating the enzyme for 30, 60 and 120 min in respective buffers, the residual activities were measured as per assay procedure and residual activity was then calculated by considering protease activity at pH 7 as 100%.

All media and reagents used were of Himedia pvt limited, Rankem and Merck Life sciences.

6.3 RESULTS

6.3.1 Microorganisms

In the present chapter, we focused on the exploration of enzymatic potential of actinomycetes. For that, forty actinomycetes isolates were judged for enzymatic potential by plate assay method followed by investigation of protease secretion in liquid culture media and repression studies.

6.3.2 Growth and extracellular enzyme production

Forty salt tolerant alkaliphilic actinomycetes discussed in the present study were screened and found to secrete extracellular proteases, amylases and cellulases (Picture 6.1). This implies that the concerned habitats are rich in proteinaceous and carbohydrate substances. The isolates displayed diversity with respect to growth patterns and production of extracellular enzymes at different salt and pH.

6.3.2.1 Growth and protease production at different NaCl concentrations

Salt tolerance and its optimum requirement for growth and alkaline protease production were studied in gelatin agar media at varying NaCl concentrations. Although, nearly 45, 30, 15 and 5% of the isolates required 0, 0-5, 5 and 5-10% NaCl, respectively for optimum growth, on the whole 80% of the isolates could tolerate high NaCl concentrations. For enzyme production, 5% NaCl was optimum for 25% of the isolates, while 8% of the isolates required NaCl at 10% and approximately 13% produced enzyme optimally at 0-5% NaCl (Figure 6.1, 6.2; Figure 6.13A, 6.13A B). Majority of the isolates could grow and produced enzymes with 0-10% salt while some tolerated up to 15 and 20% NaCl.

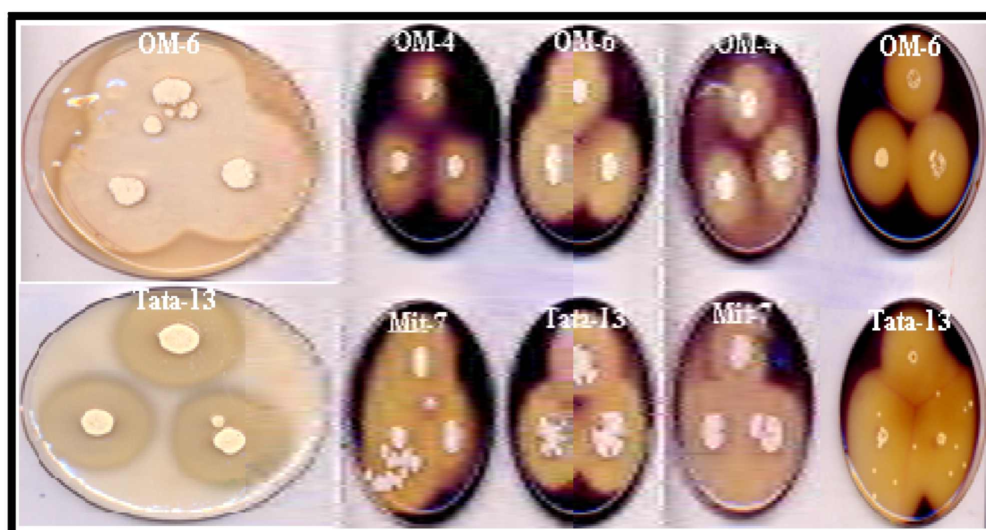
6.3.2.2 Secretion of extracellular amylase at different NaCl concentration

Amylase production was studied on starch agar media at 0-20% NaCl concentrations. It appeared that 50, 23 and 5% of the isolates grew optimally at 0, 0-5 and 5% NaCl, respectively. Only 5% grew optimally at 10% NaCl. However, approximately 60% of the isolates tolerated higher NaCl concentrations for growth. For enzyme secretion, 0-

10, 0-5 and 0-15% NaCl was optimum for 38, 33 and 15% of the isolates respectively. Peculiarly, approximately 90% of the isolates secreted amylase with tolerance to higher NaCl concentrations (Figure 6.3, 6.4; Figure 6.13C and 6.13AD). The results are quite interesting as it reflected diverse salt requirements for different enzymes.

6.3.2.3 Secretion of extracellular cellulase

Detection of cellulase production was studied on Dubo's cellullose agar media at different concentrations of NaCl. It was apparent that 45, 25, 10 and 15% of the isolates grew optimally at 0%, 0-5%, 5% and 5-10% NaCl, respectively; while only 6% of the isolates grew optimally at 10%, 0-20% and 10-20% NaCl. Overall, approximately 80% of the isolates tolerated high NaCl concentrations for their growth. Cellulase was optimally secreted at 5 and 10% NaCl by 35 and 33% of the isolates, respectively, while 10 % of the isolates produced the enzyme in the range of 5-10 and 0-10% NaCl. Only 10% of the isolates had optimum salt requirement at 0-10%, 0-15% and 0-20% for the cellulase secretion. However, approximately 80% of the isolates secreted cellulases with tolerance at higher NaCl concentrations (Figure 6.5, 6.6; Figure 6.13E, 6.13F).



Picture 6.1: Secretion of extracellular alkaline proteases, amylases, and cellulases among selected actinomycetes isolates on gelatin agar, starch agar and dubo's cellulose agar plate

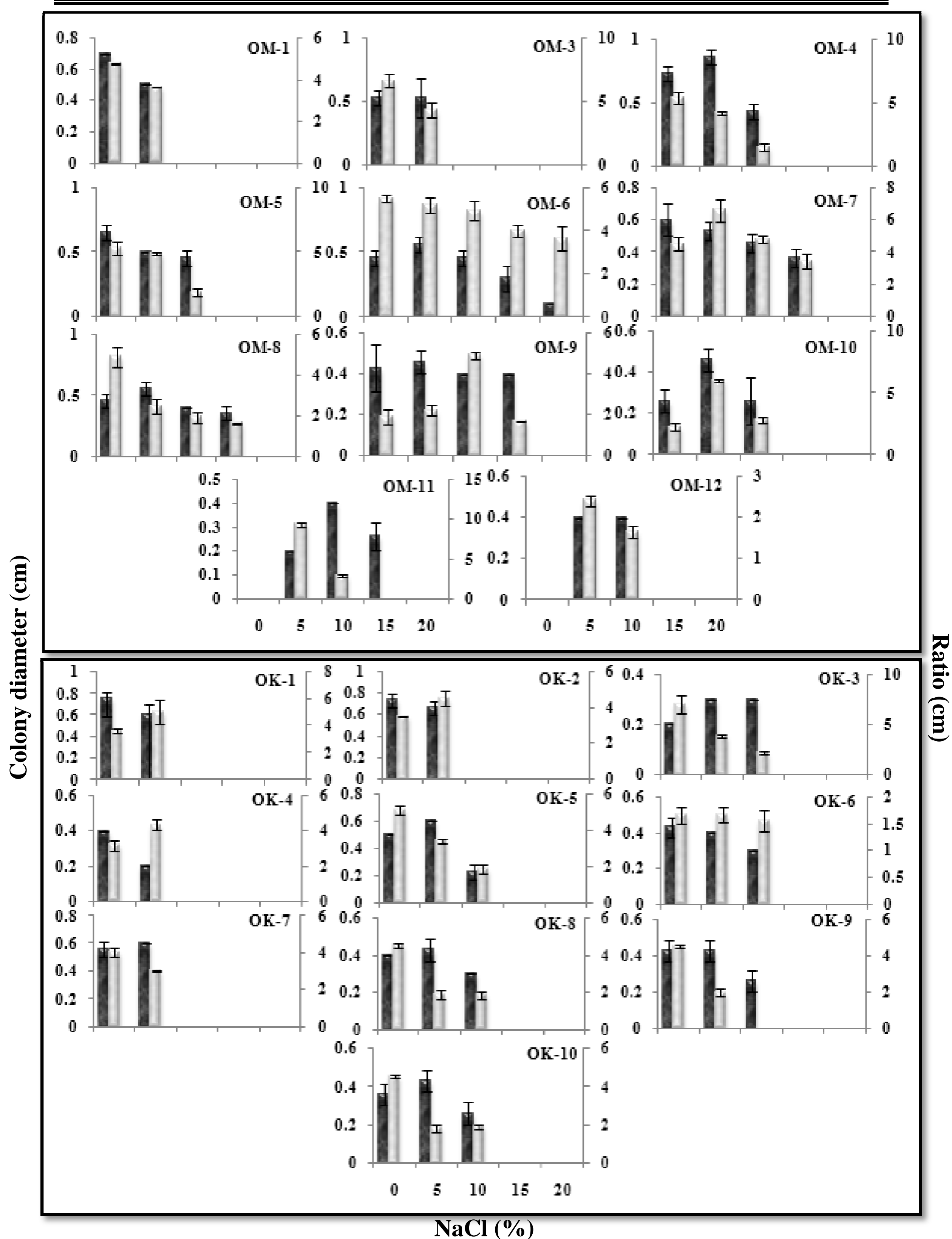


Figure 6.1: Effect of salt on growth (■) and protease secretion (■) among the actinomycetes isolates from Okha Madhi (OM) and Okha (OK) site

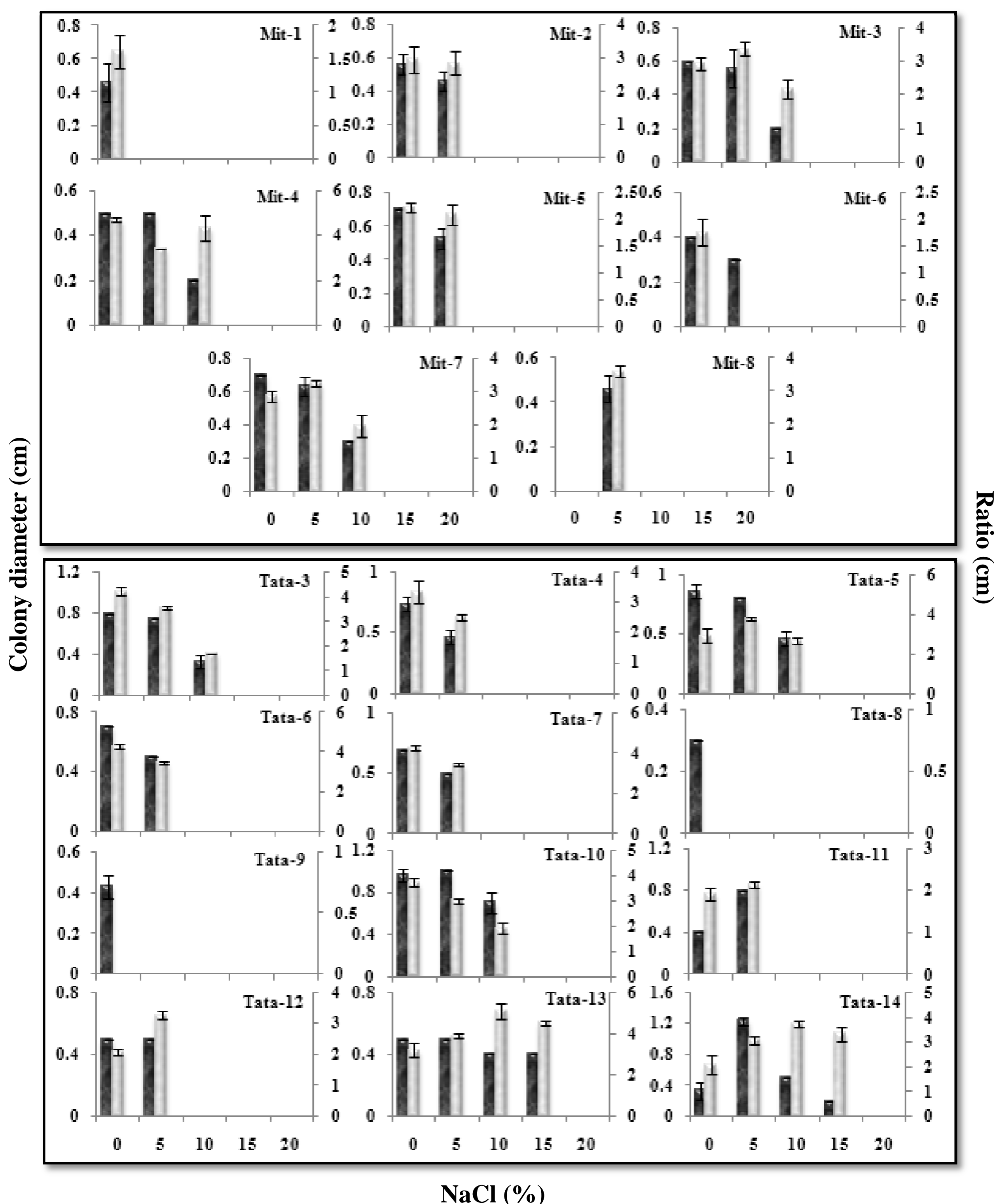


Figure 6.2: Effect of salt on growth (■) and protease secretion (■) among the actinomycetes isolates from Mithapur (Mit) and Tata effluent (Tata) site

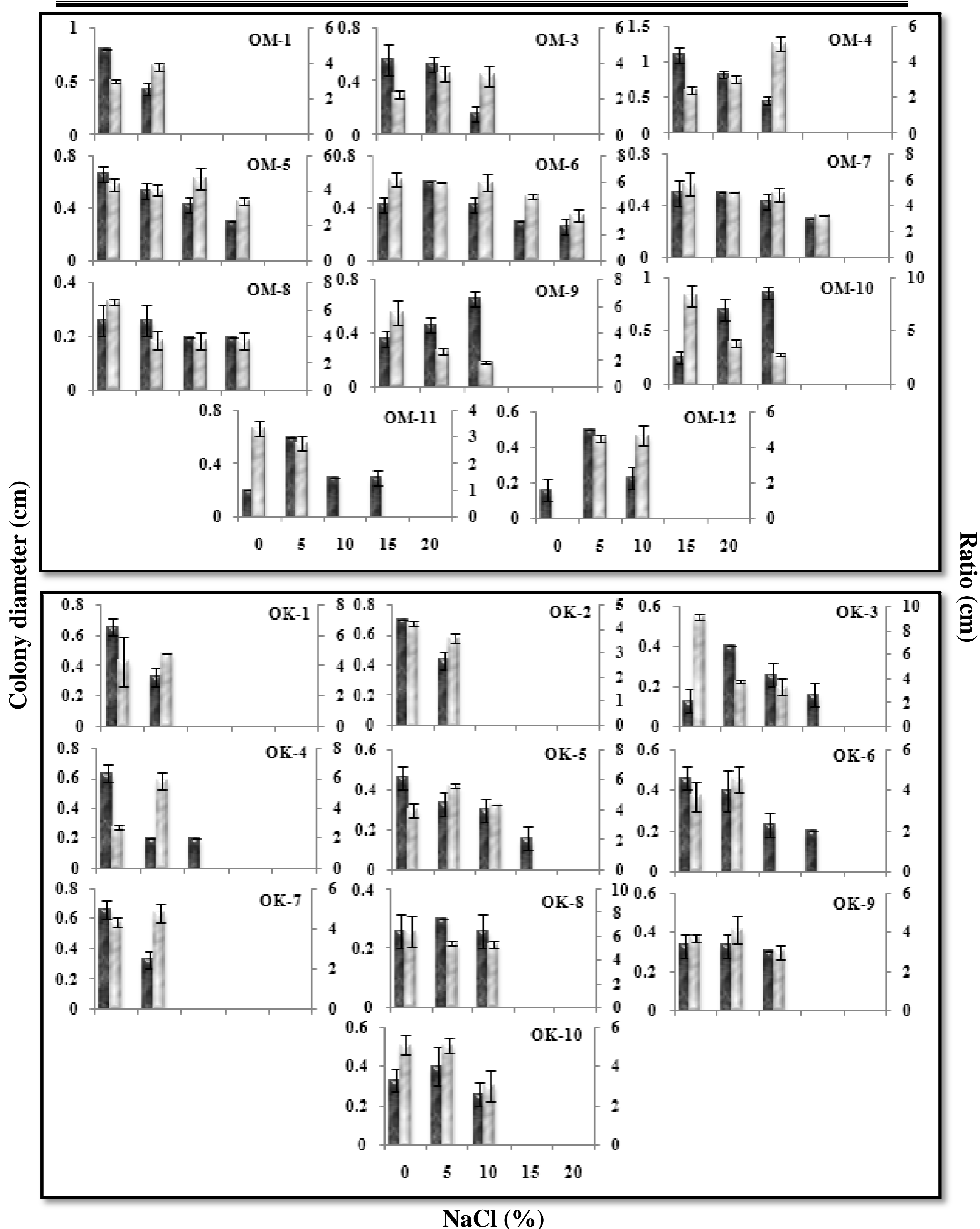


Figure 6.3: Effect of salt on growth (■) and amylase secretion (■) among the actinomycetes isolates from Okha Madhi (OM) and Okha (OK) site

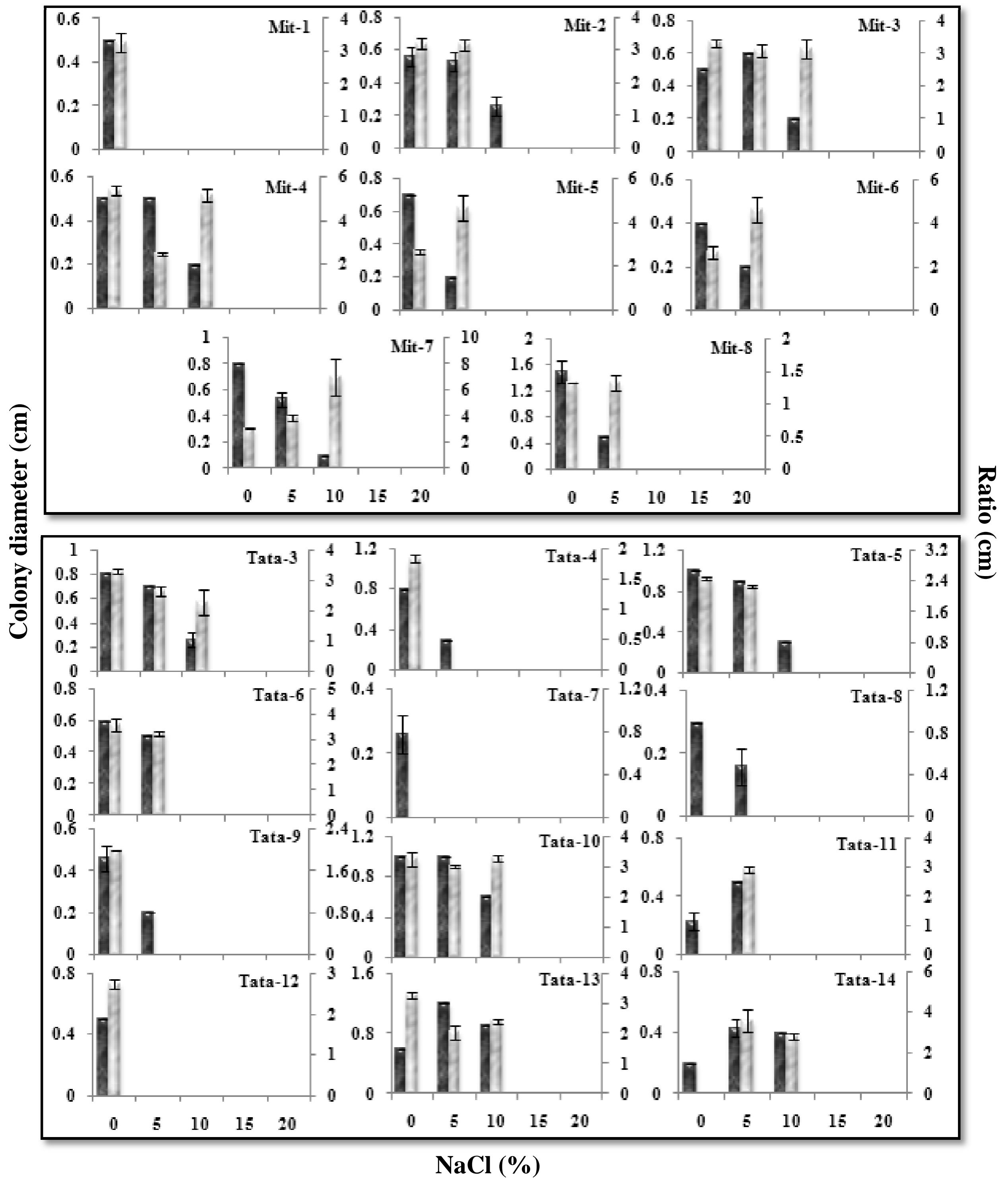


Figure 6.4: Effect of salt on growth (■) and amylase secretion (■) among the isolates of Mithapur (Mit) and Tata effluent (Tata) site

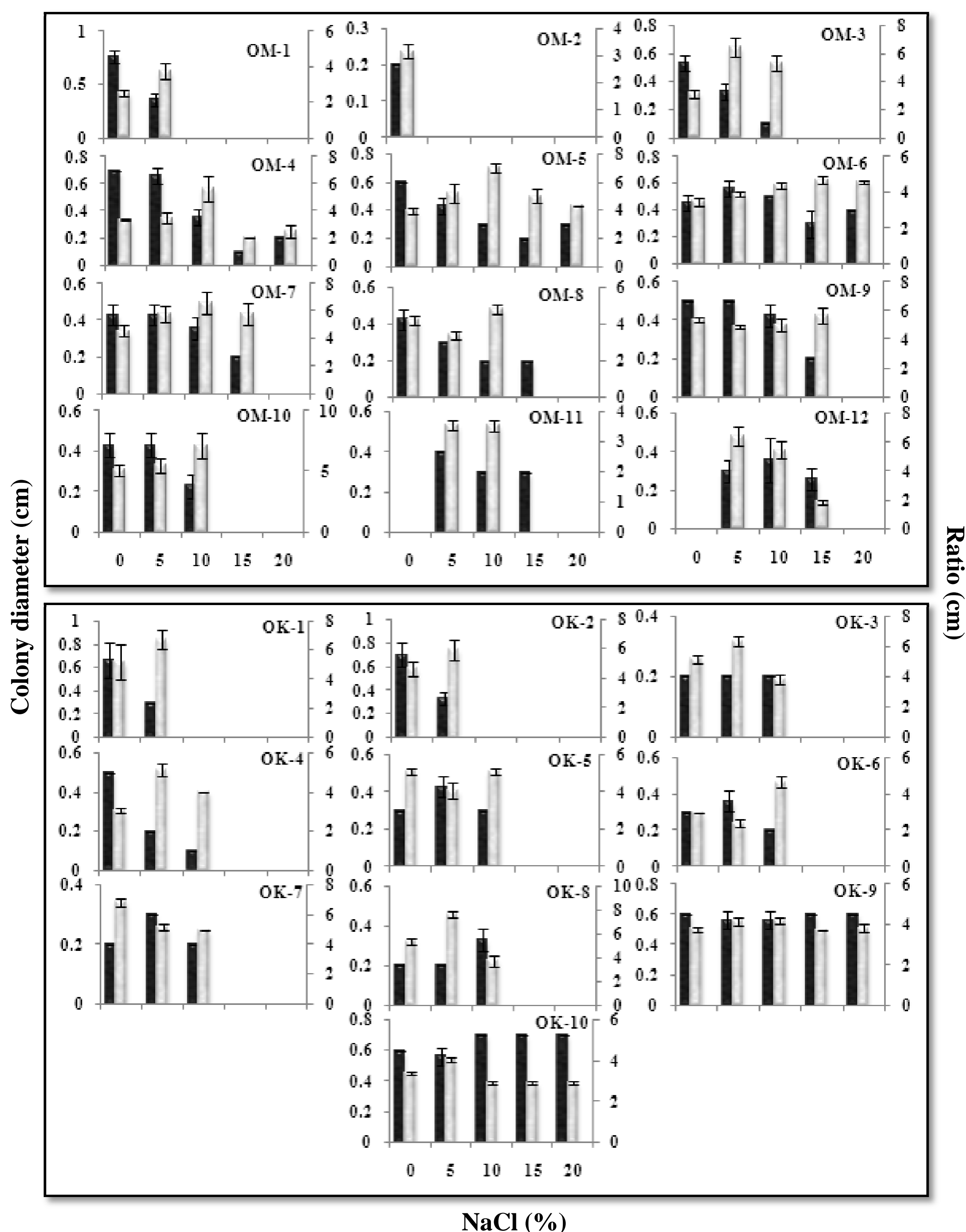


Figure 6.5: Effect of salt on growth (■) and cellulase secretion (■) among the actinomycetes isolates from Okha Madhi (OM) and Okha (OK) site

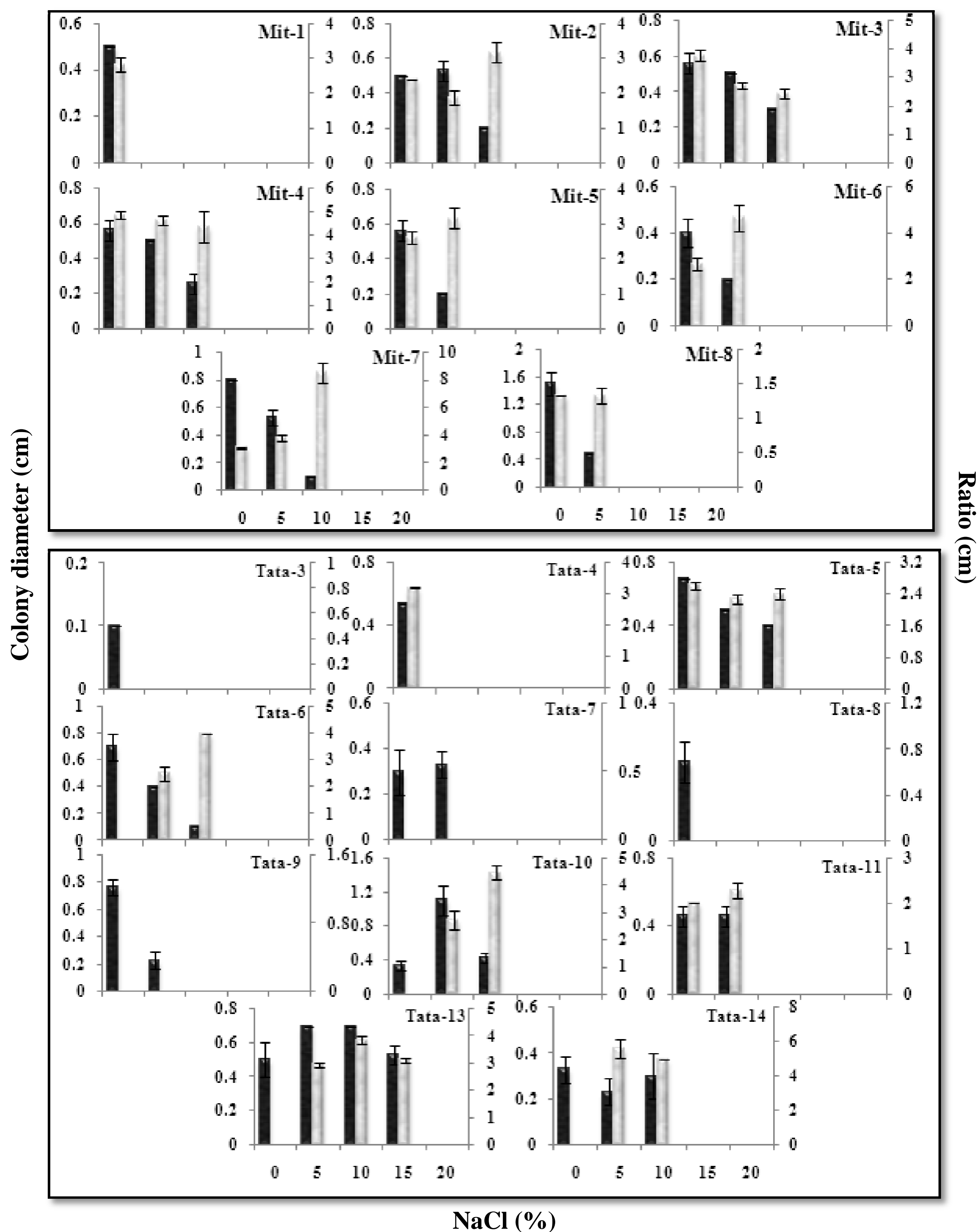


Figure 6.6: Effect of salt on growth (■) and cellulase secretion (■) among the actinomycetes isolates from Mithapur (Mit) and Tata effluent (Tata) site

6.3.2.4 Secretion of extracellular alkaline proteases at alkaline pH

Ability of isolates to tolerate wide range of pH for growth and protease production was studied in gelatin agar media. It appeared that among the total isolates; 20, 30, 8 and 10% of the isolates grew optimally at pH 7, 8, 9 and 11, respectively, while remaining isolates had optimum pH between 8-11. At the same time, 35 and 38% of the isolates tolerated broad range of pH from pH 7-11 and 8-11, respectively. For enzyme secretion, optimum pH at 8-9, 9 and 11 were detected for 33, 15 and 10% of the isolates, respectively, while the remaining 15% of the isolates displayed optimum pH at 10, 8-11 and 9-11. Approximately, 35 and 40% of the isolates secreted proteases at broader ranges of pH, 7-11 and 8-11, respectively, signifying enormous tolerance to alkaline pH for protease production (Figure 6.7, 6.8 6.14A, 6.14B).

6.3.2.5 Secretion of extracellular amylases and cellulases alkaline pH

Growth and amylase production at different pH was studied on starch agar media. It appeared that 25, 20, 12, 13% of the isolates grew optimally at pH 7, 7-8, 8 and 7-11, respectively and rest of the isolates were able to grow at different ranges; pH 10-11 for 8%, 9-11 and 8-10 for 10%, 8-9,9,11,8-11 for the remaining 12% of the isolates. Overall, 70% of the isolates grew at broader range of pH, 7-11 while 8% could grow at 8-11 and 15% had pH 8, 7-9 and 7-10. Though optimum enzyme secretion was detected at pH 7 for 45% of the isolates, 60% produced amylase at the broader range of 7-11 (Figure 6.9, 6.10, 6.14C, 6.14D). Growth and cellulase production at different range of pH was studied on Dubo's cellulose agar media. It was apparent that 22, 12, 12 and 20% of the isolates grew optimally at pH 7, 7-8, 9 and 10-11, respectively, the rest of the isolates were able to grow at different ranges from 9-11 and 8 for 15%, pH 8-10, 10, 7-11 and 8-11 for 20% of the isolates. Among the rest of the isolates 67 and 10% could grow at broad pH range 7-11 and 8-11, respectively. For enzyme secretion, though optimum pH was 7 for 35% of the isolates; pH 7-8 for 20%, 8-10 for 20%, 8 for 15% and pH 9 and 10 for 20% of the isolates were evident. Further, 60, 15 and 10% of the isolates produced amylases at the broader pH; 7-11, 8-11 and 7-9, respectively (Figure 6.11, 6.12, 6.14E, 6.14F).

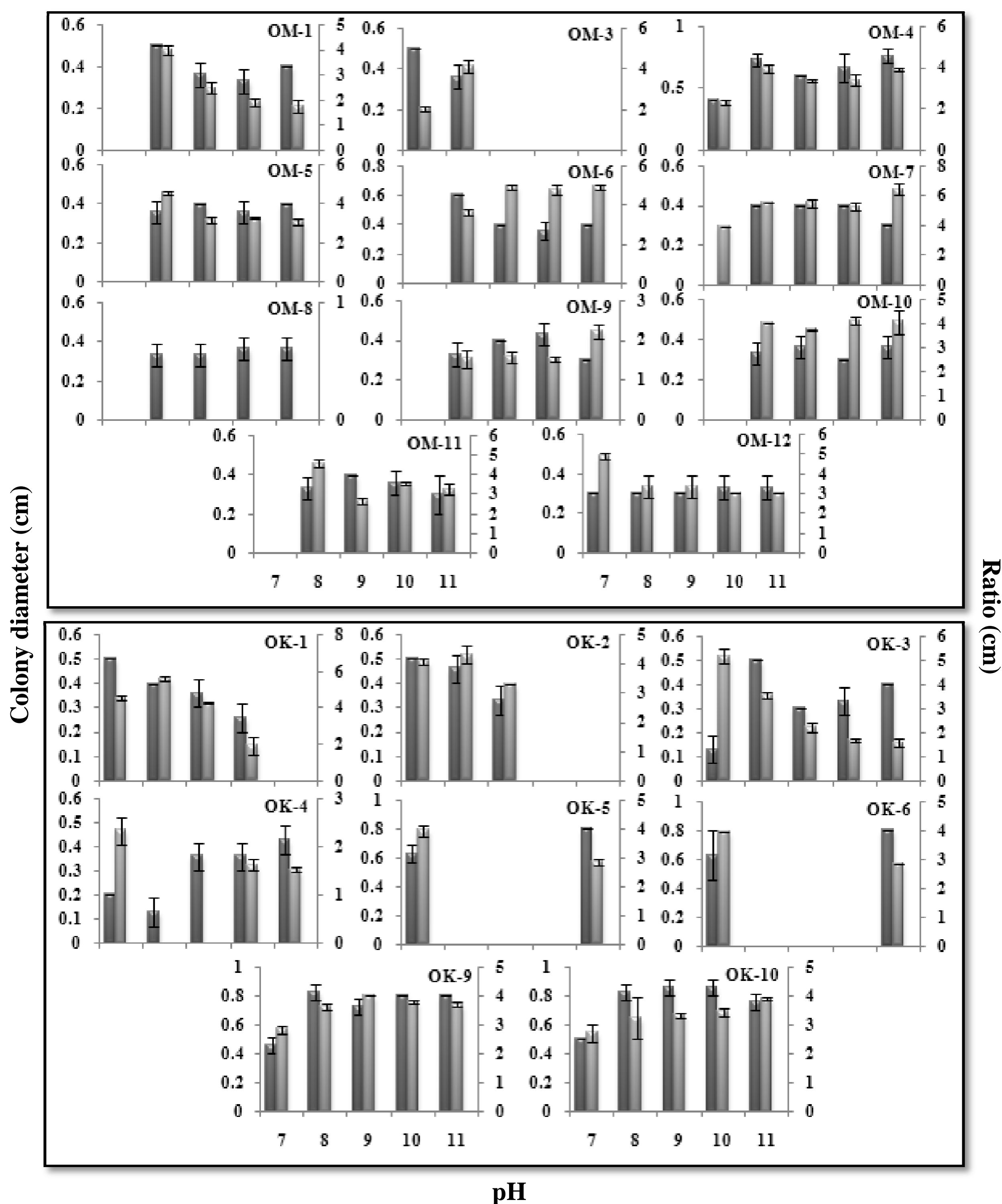


Figure 6.7: Effect of pH on growth (■) and protease secretion (■) among the actinomycetes isolates from Okha Madhi (OM) and Okha (OK) site

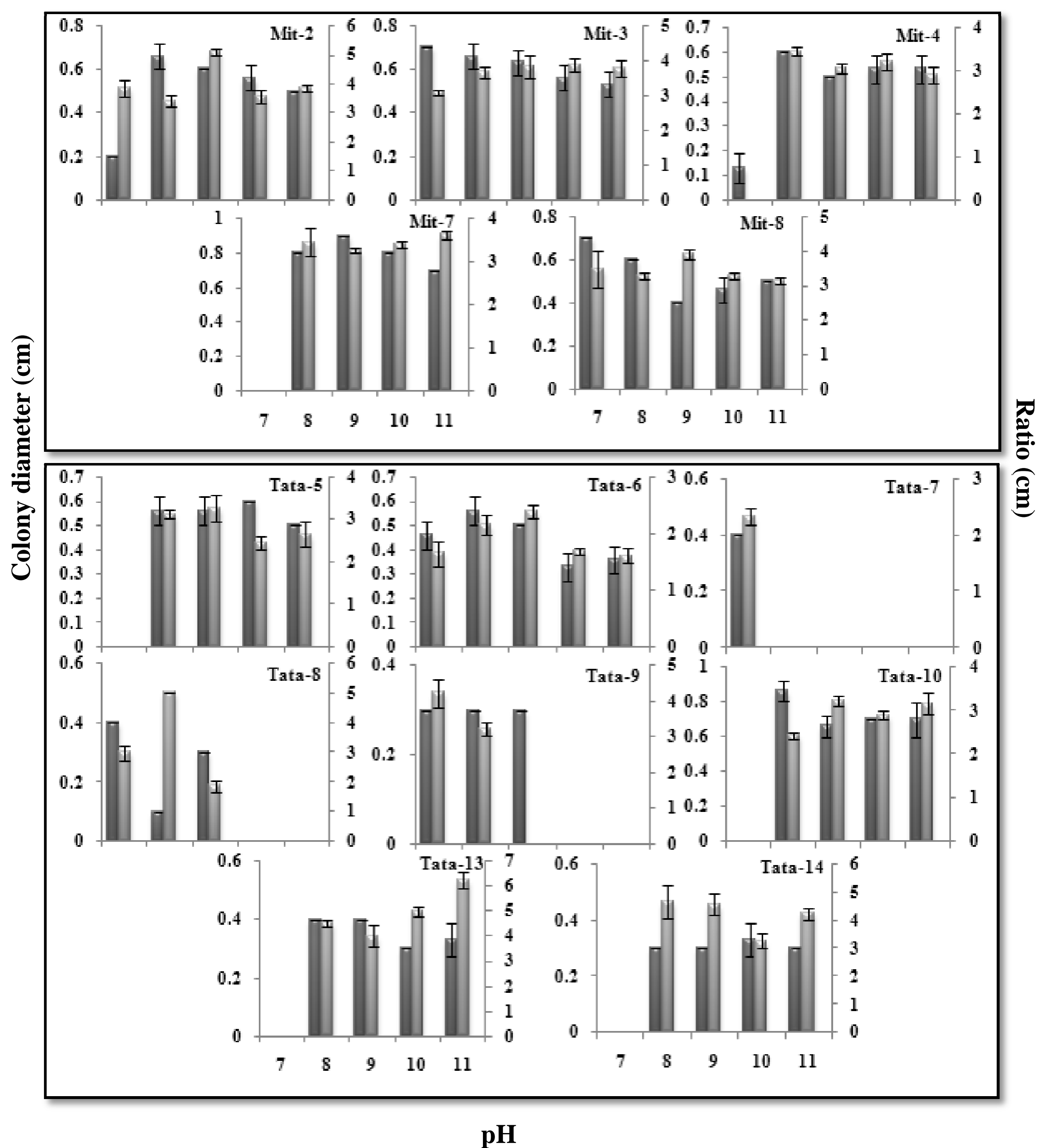


Figure 6.8: Effect of pH on growth (■) and Protease secretion (■) among the actinomycetes isolates from Mithapur (Mit) and Tata effluent (Tata) site

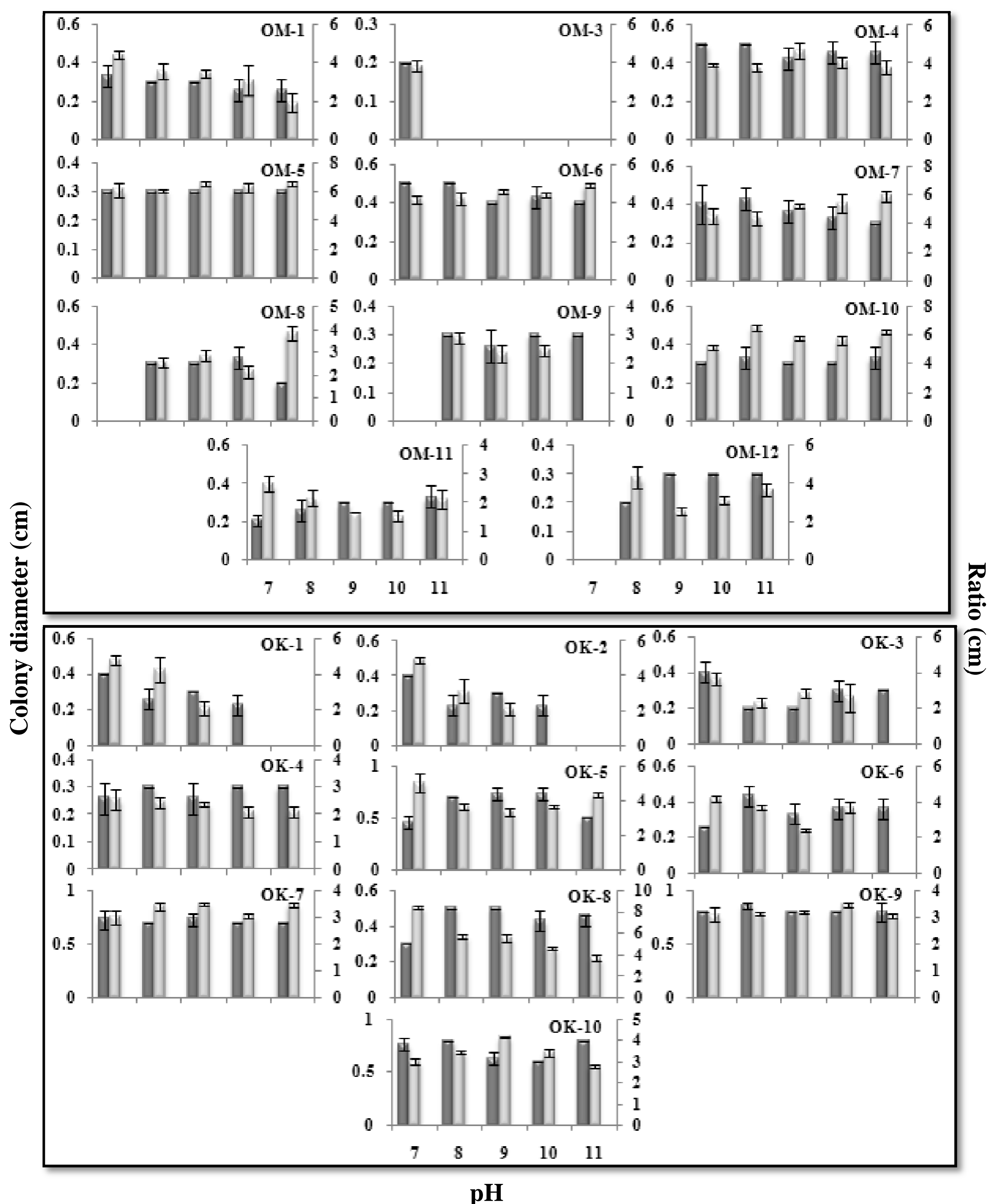


Figure 6.9: Effect of pH on growth (■) and amylase secretion (■) among the actinomycetes isolates from Mithapur (Mit) and Tata effluent (Tata) site

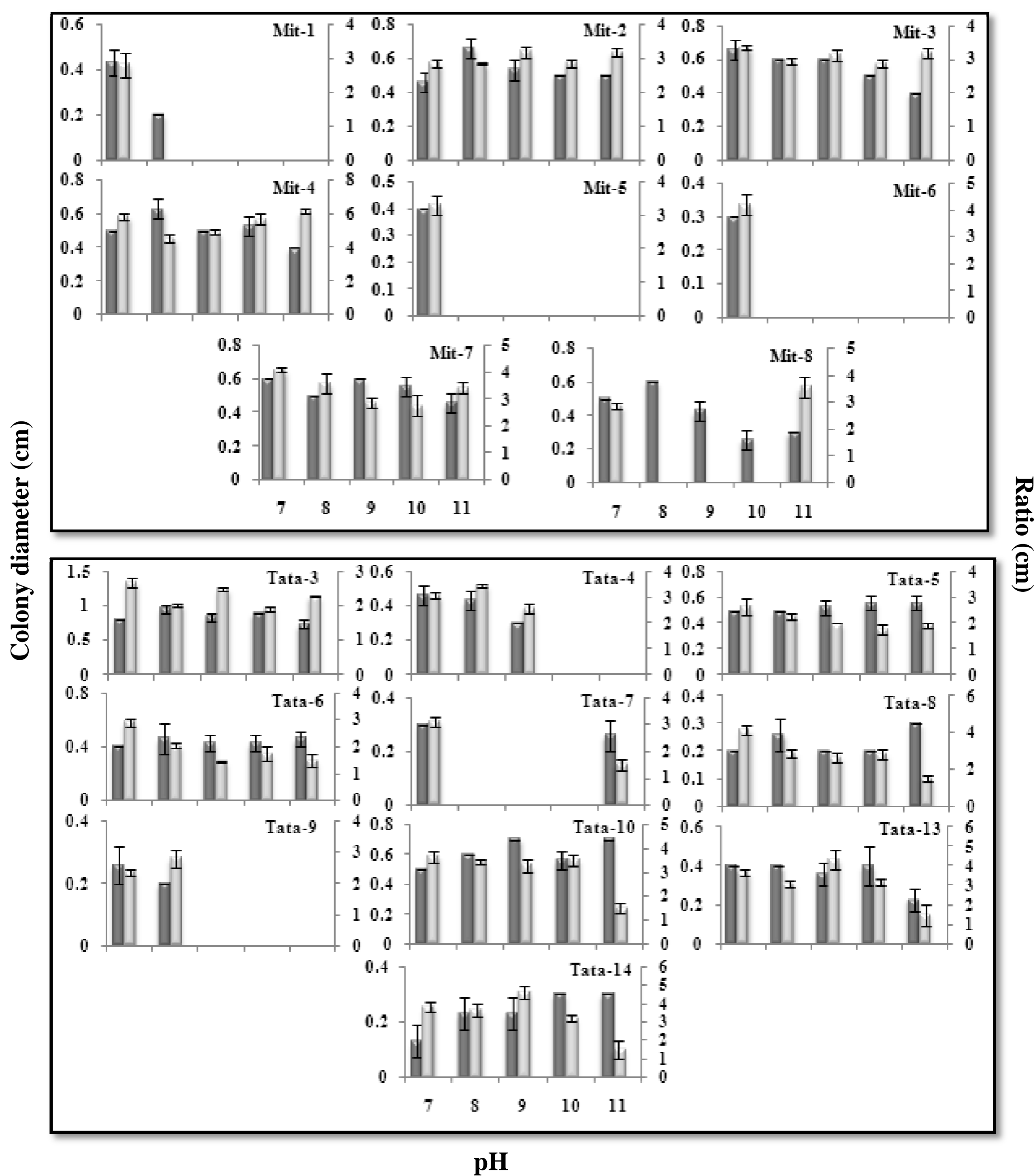


Figure 6.10: Effect of pH on growth (■) and amylase secretion (■) among the actinomycetes isolates from Mithapur (Mit) and Tata effluent (Tata) site

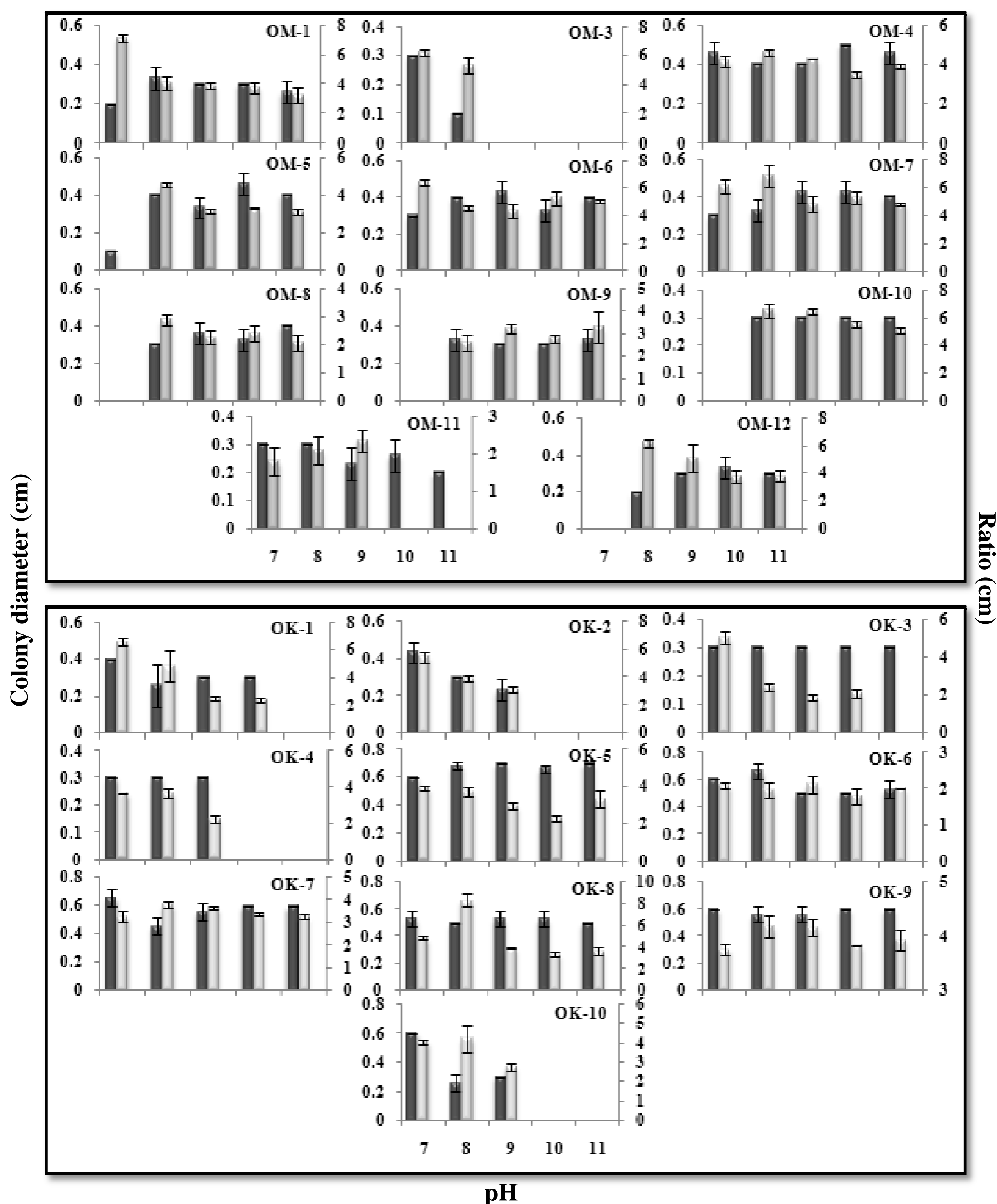


Figure 6.11: Effect of pH on growth (■) and cellulase secretion (■) among the actinomycetes isolates from Mithapur (Mit) and Tata effluent (Tata) site

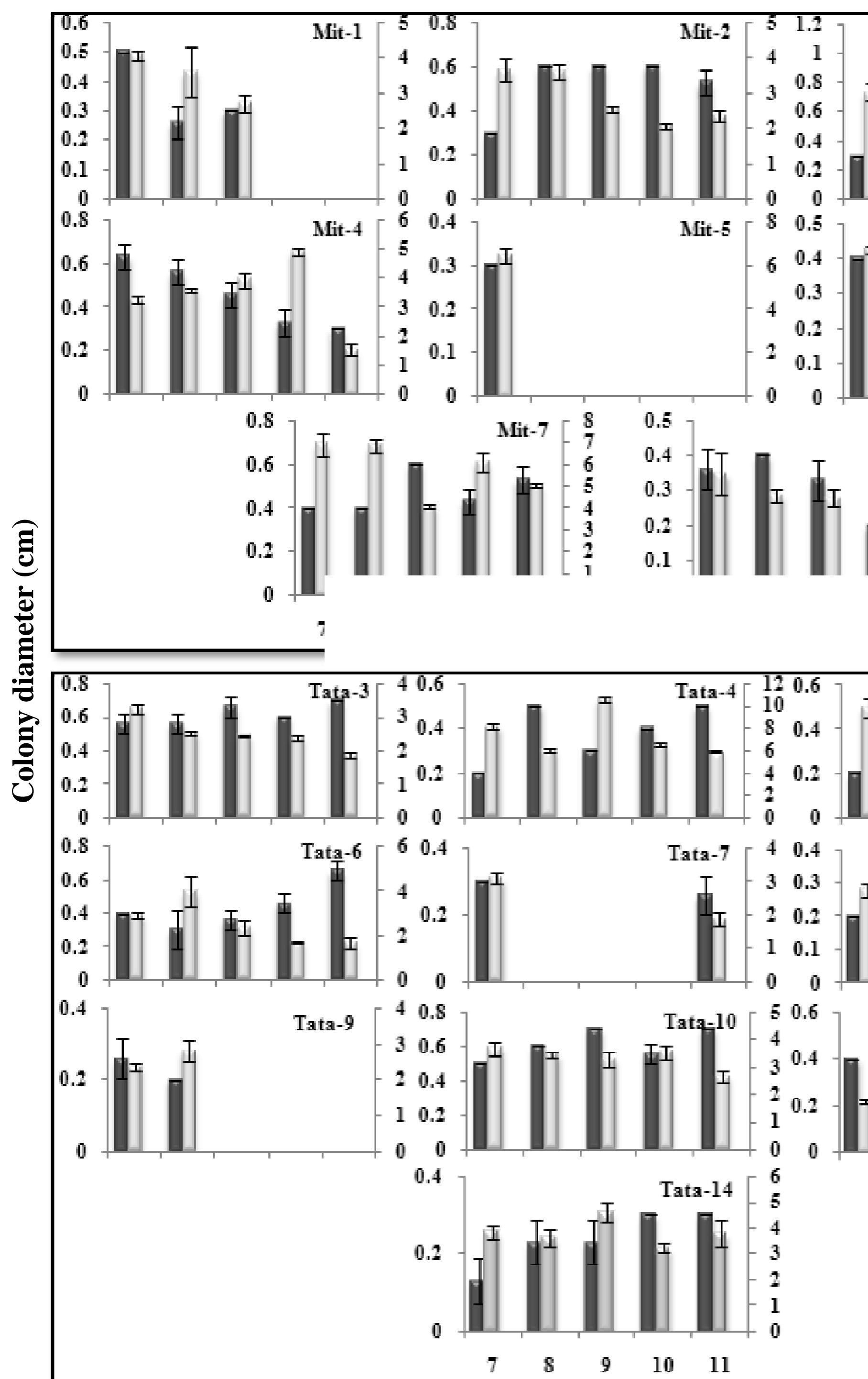


Figure 6.12: Effect of pH on growth (■) and cellulase secreti
actinomycetes isolates from Mithapur (Mit) and Tata effluent (Tata

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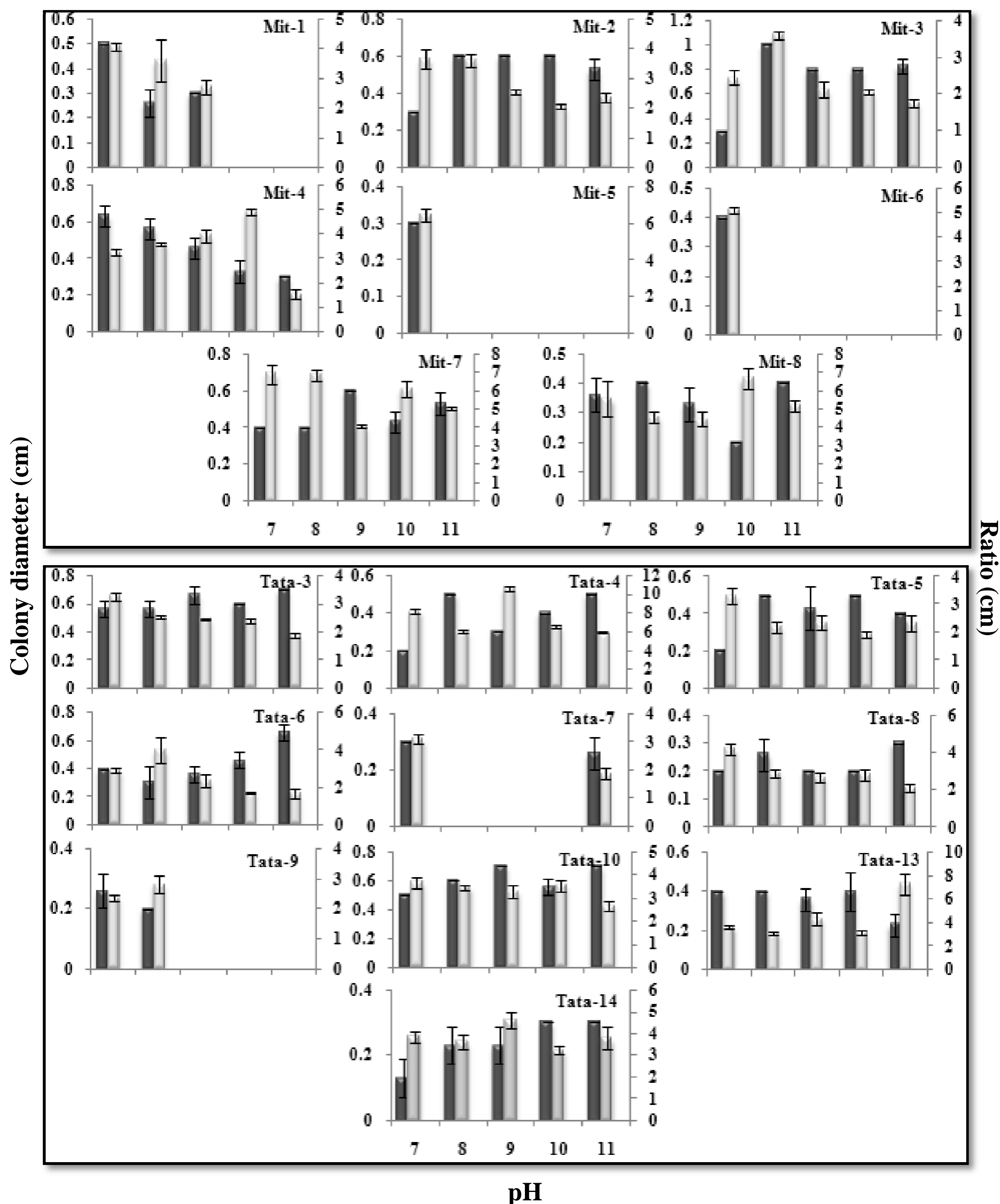


Figure 6.12: Effect of pH on growth (■) and cellulase secretion (■) among the actinomycetes isolates from Mithapur (Mit) and Tata effluent (Tata) site

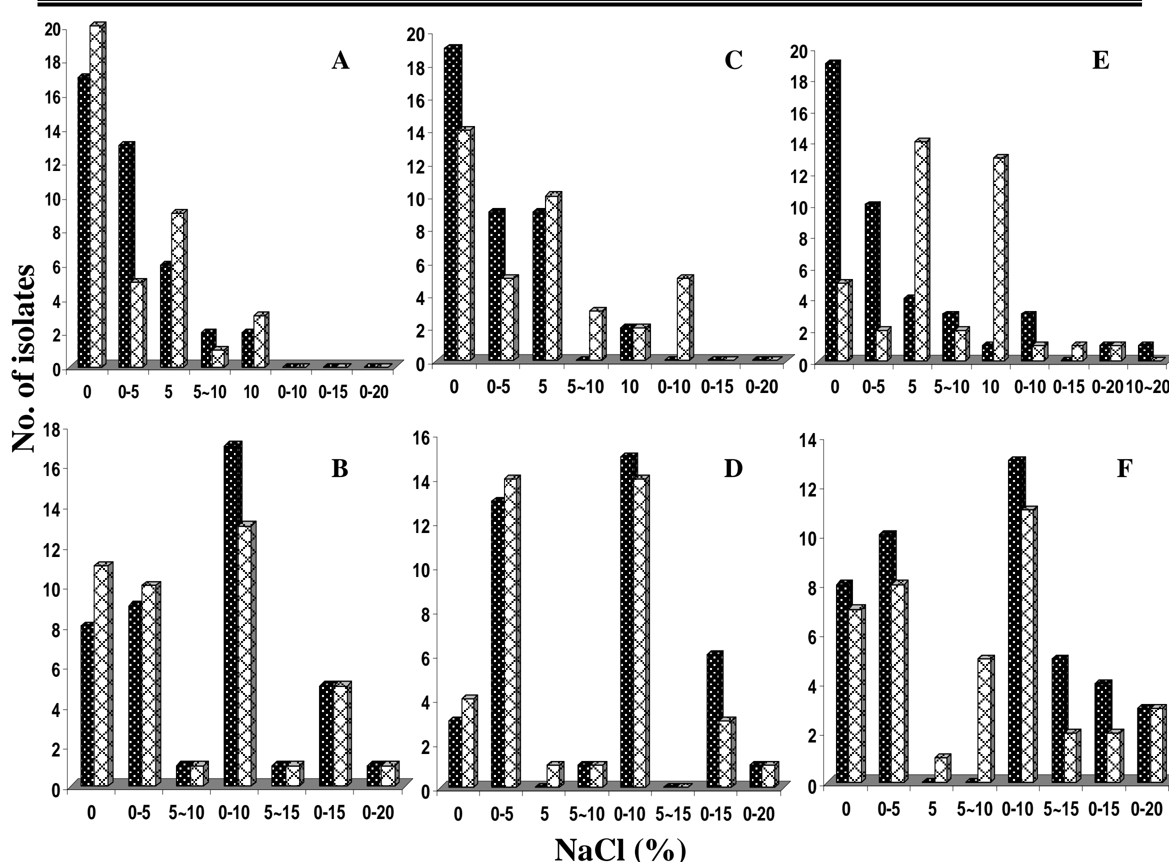


Figure 6.13: Growth (▓) and extra cellular enzyme secretion (×) with reference to NaCl optima and NaCl tolerance of total forty actinomycetes isolates: **A)** Optimum NaCl required for growth and protease secretion, **B)** NaCl tolerance of isolates for growth and protease secretion, **C)** Optimum NaCl required for growth and amylase secretion, **D**) NaCl tolerance of isolates for growth and amylase secretion, **E)** Optimum NaCl require for growth and cellulase secretion, **F)** NaCl tolerance of isolates for growth and cellulase secretion

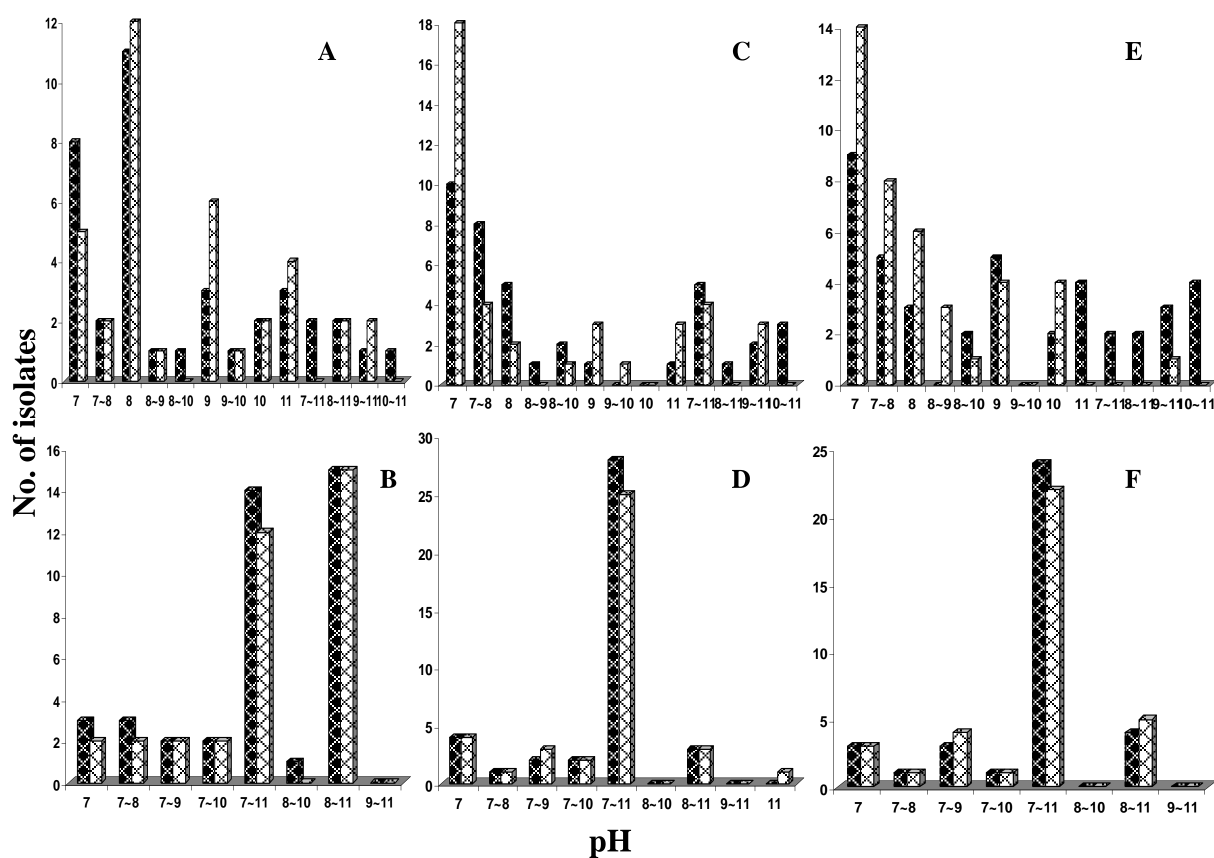


Figure 6.14: Growth (⊠) and extra cellular enzyme (×) secretion of pH optima and pH tolerance in forty different actinomycetes isolates: **A)** pH optima of growth and protease secretion, **B)** pH tolerance of growth and protease secretion, **C)** pH optima of growth and amylase secretion, **D)** pH tolerance of growth and amylase secretion, **E)** pH optima of growth and cellulase secretion, **F)** pH tolerance of growth and cellulase secretion

6.3.3 Growth kinetics and protease production

While alkaline proteases from bacteria are extensively characterized, similar attention has not been paid to actinomycetes. However, the ability to produce a variety of enzymes at significantly higher level appears to be an attractive phenomenon in these prokaryotes. On the basis of the results obtained from the comparative studies of enzyme secretion of the isolates, 9 isolates secreting highest protease were selected for further studies. The growth kinetics along with the alkaline protease production of

all nine isolates was followed up to 15 days of incubation. It revealed that the growth of isolates entered the exponential phase during 48 to 72h of incubation under shake flask conditions. The variation in growth phases was depended on salt concentrations. The production of protease was optimum during stationary phase of growth.

6.3.3.1 Effect of salt on growth and protease production

Optimum NaCl for enhancement in protease activity was 15% for OM-11(835%, 371 U/mL), 10% for OM-4(175%, 311 U/mL), OK-1(195 U/mL), OK-7(267 U/mL), OK-5(245%, 486 U/mL) and 5% for OM-6(317%, 400U/mL), Mit-7(149%, 198U/mL) and Tata-13(131%, 356 U/mL). Only Tata-5 did not require any salt for optimum protease production (Figure 6.15, 6.16). Therefore, with the increasing salt from 0 to 15%, the activity increased, while at 20% salt, it decreased for all isolates. Secondary graph presenting salt profile of all nine actinomycetes isolates is shown in Figure 6.17.

6.3.3.2 Effect of pH on growth and protease production

Among the 9 isolates, the optimum pH for enhancement in the protease activity and protease production were; 11 for OM-4(540%, 258 U/mL) and OK-7(120%, 230 U/mL), 10 for OM-11(390%, 79 U/mL), OK-1(470%, 191 U/mL) and Tata-5(280%, 283 U/mL), 9 for OM-6(490%, 347 U/mL) and OK-5, (600%, 437 U/mL) and pH 8 for Mit-7(150%, 299 U/mL) and Tata-13(150%, 593 U/mL) (Figure 6.18, 6.19). The trends indicated that with the increasing pH from 7 to 11, the residual activities also increased up to pH 11 in case of OM-4 and OK-7, pH 10 for OM-11, OK-1 and Tata-5, pH 9 for OM-6 and OK-5 and pH 8 for Mit-7 and Tata-13. Although the growth was comparable at pH 8-10, optimum protease production was at alkaline range indicating alkaliphilic nature of the majority proteases from the 09 isolates of actinomycetes. At all pH values, the optimum protease secretion was during the late exponential or early stationery phase. Secondary graph presenting pH profile of all nine actinomycetes isolates is shown in Figure 6.20.

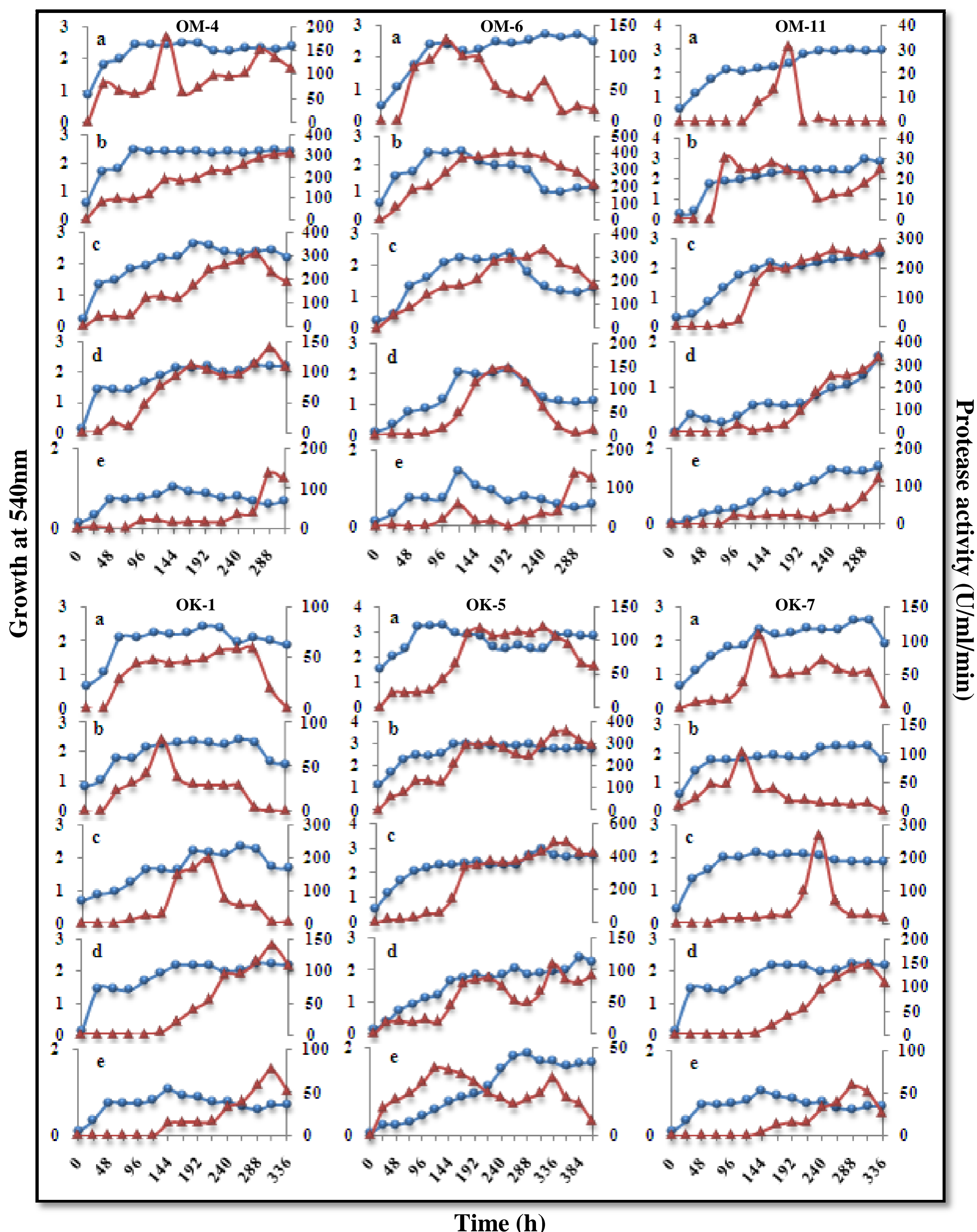


Figure 6.15: Growth (●) and protease production (▲) among the salt tolerant alkaliphilic actinomycetes strains OM-4, OM-6, OM-11, OK-1, OK-5 and OK-7 at 0% (a), 5% (b), 10% (c), 15% (d) and 20% (e) NaCl (w/v)

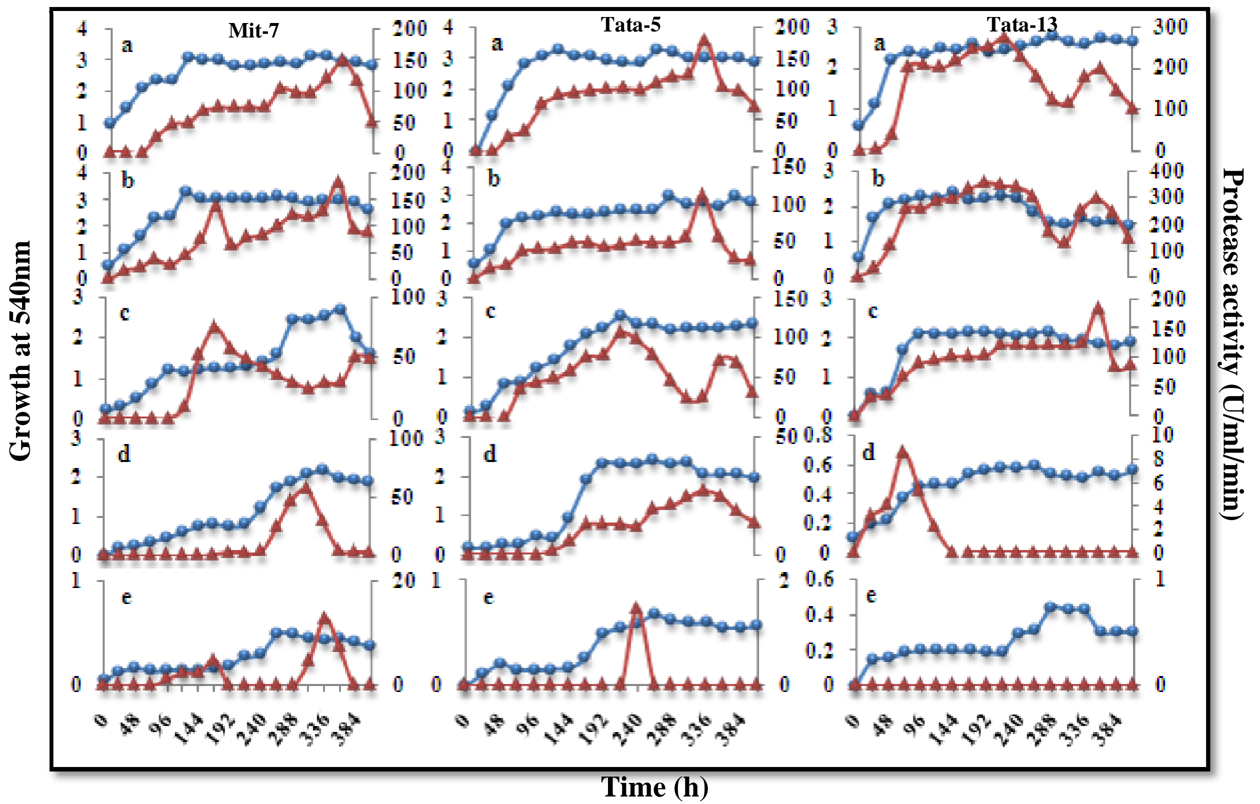


Figure 6.16: Growth (●) and protease production (▲) among the salt tolerant alkaliphilic actinomycetes strains Mit-7, Tata-5 and Tata-13 at 0% (a), 5% (b), 10% (c), 15% (d) and 20% (e) NaCl (w/v).

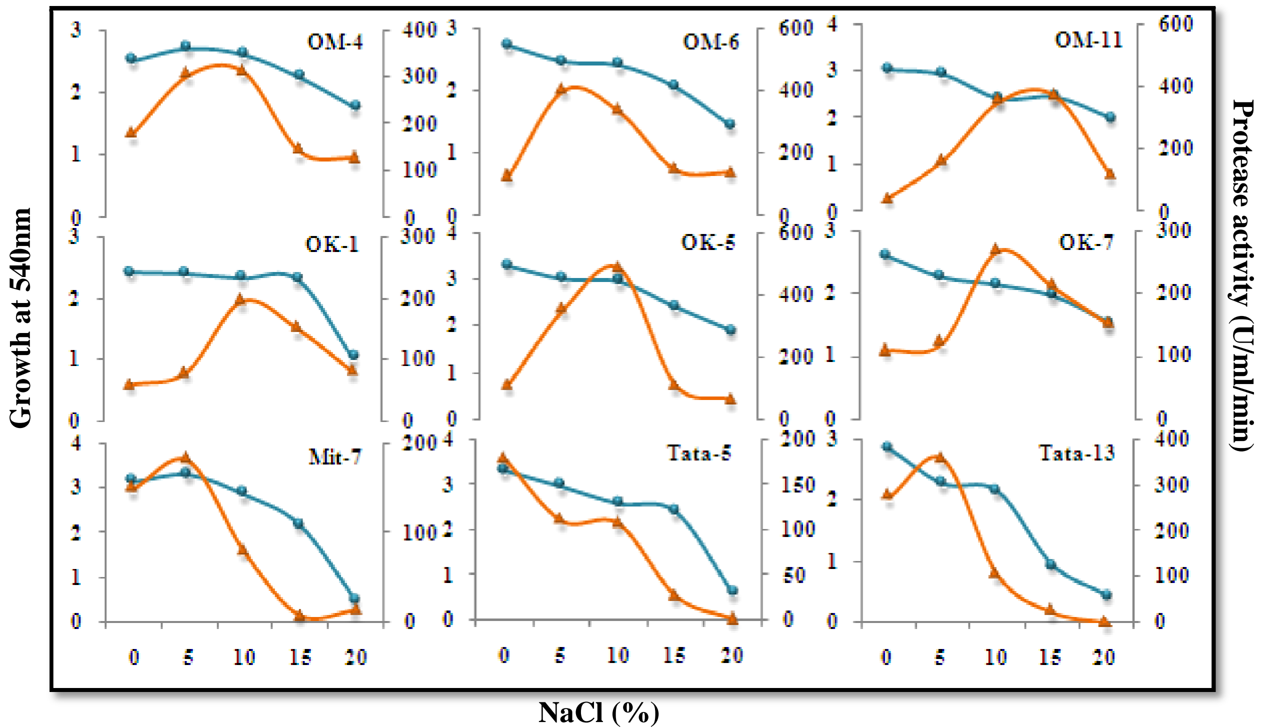


Figure 6.17: Secondary graphs showing optimum growth (●) and optimum protease production (▲) among nine actinomycetes isolates at 0-20% NaCl (w/v)

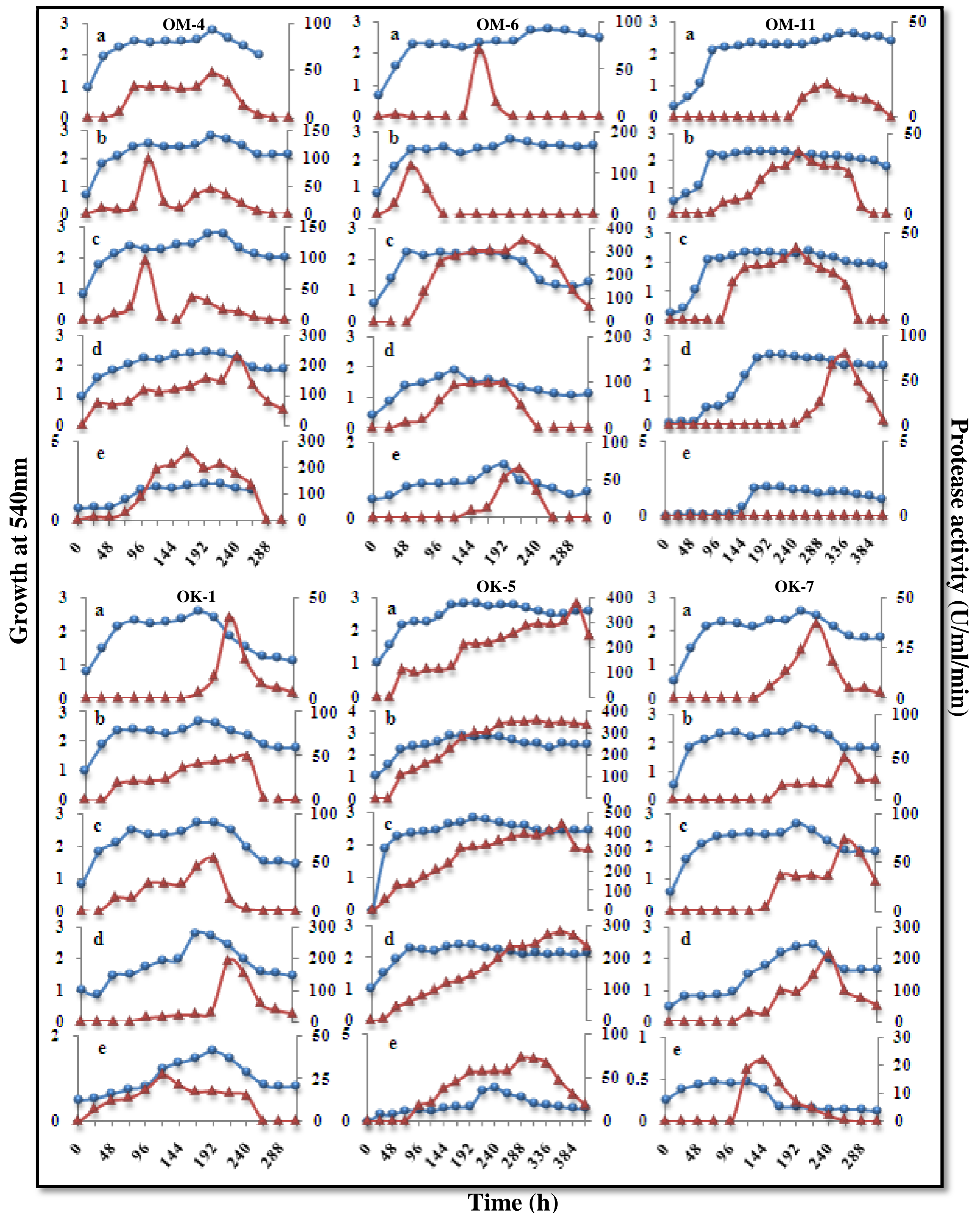


Figure 6.18: Growth (●) and protease production (▲) among the salt tolerant alkaliphilic actinomycetes strains OM-4, OM-6, OM-11, OK-1, OK-5 and OK-7 at pH 7 (a), 8 (b), 9 (c), 10 (d) and 11(e)

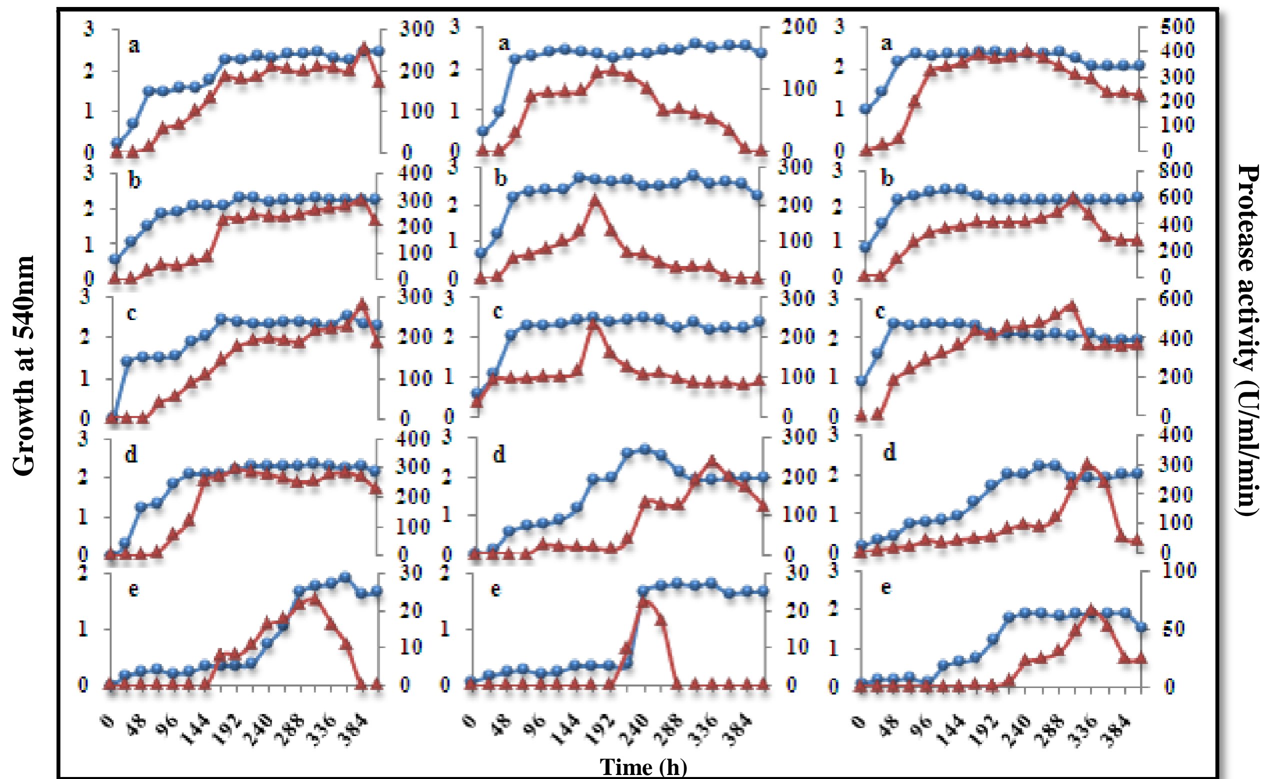


Figure 6.19: Growth (●) and protease production (▲) among the actinomycetes strains Mit-7, Tata-5 and Tata-13 at pH 7 (a), 8 (b), 9 (c), 10 (d) and 11(e)

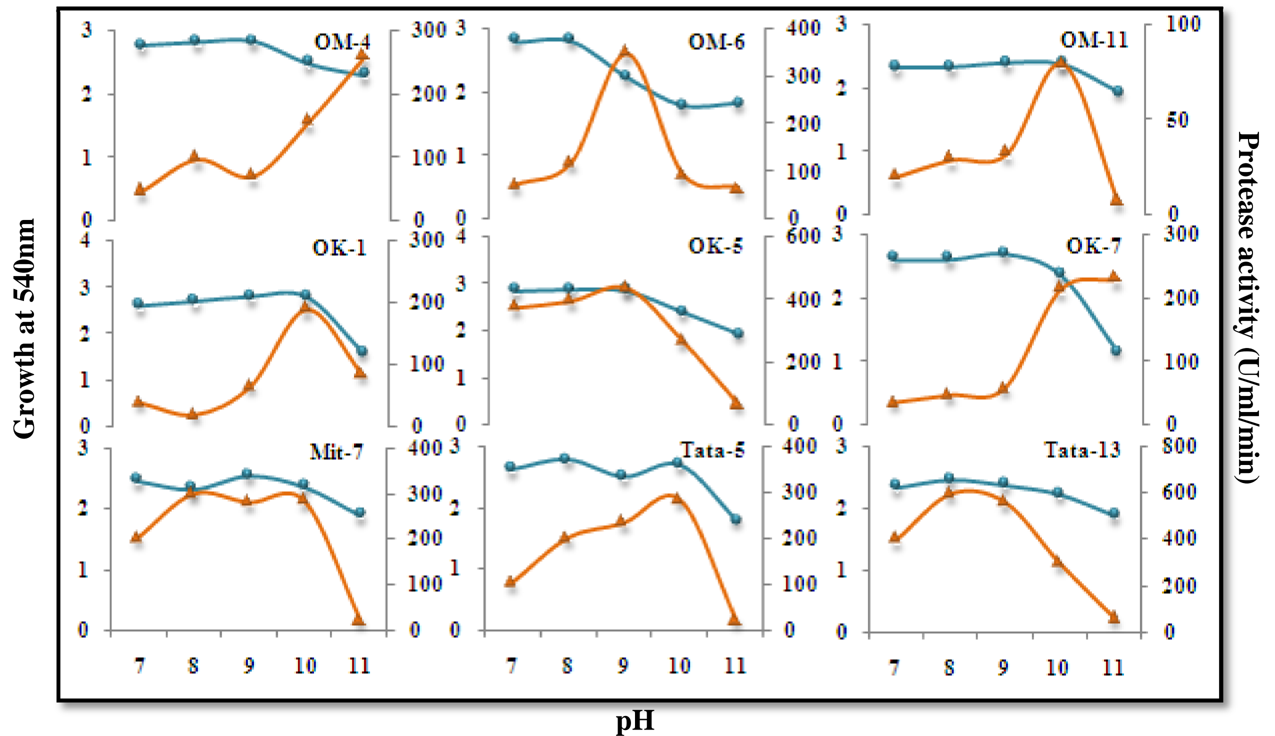


Figure 6.20: Secondary graphs showing optimum growth (●) and optimum protease production (▲) among nine actinomycetes isolates at pH 7-11

6.3.3.3 Effect of gelatin on growth and protease production

Gelatin significantly affected growth and protease production. The growth of the organisms increased with increasing concentrations of gelatin in the range of 0.5 to 3% (w/v), in gelatin broth at the optimum NaCl and pH as described above. It was apparent that while growth increased with increasing gelatin concentrations, its impact on the enzyme production and growth patterns greatly varied among the isolates. In OM-4, the enzyme activity decreased with increasing gelatin concentrations, the optimum (394 U/mL) being at 0.5% gelatin (w/v). Gelatin, however, did not affect the growth pattern. On the other hand, OM-6 grew and produced protease (456 U/mL, 264h) optimally at 3% gelatin. With the increasing gelatin, while protease increased, the time for enzyme production was prolonged. In OM-11, the optimum activity (181 U/mL) was at 3% w/v gelatin with the decreased incubation time. OK-5 displayed nearly similar optimum protease activity at 0.5% and 3% gelatin while incubation time differed; at 0.5%, the optimum protease activity (554 U/mL) was observed at 192h, while at 3%, it was at 168h of incubation. Tata-13 had optimum protease production (462 U/mL) at 1% gelatin at 120h of growth (Figure 6.21). Secondary graphs showing optimum growth and optimum protease production among five actinomycetes isolates at 0-3% Gelatin (w/v) is shown in Figure 6.21. The increase in activity was 100% (0.5% w/v gelatin), 107% (3% w/v gelatin), 100% (0.5% and 3% w/v gelatin), 118% (1% w/v gelatin), for OM-4, OM-6, OM-11, OK-5, and Tata-13, respectively (Figure 6.21).

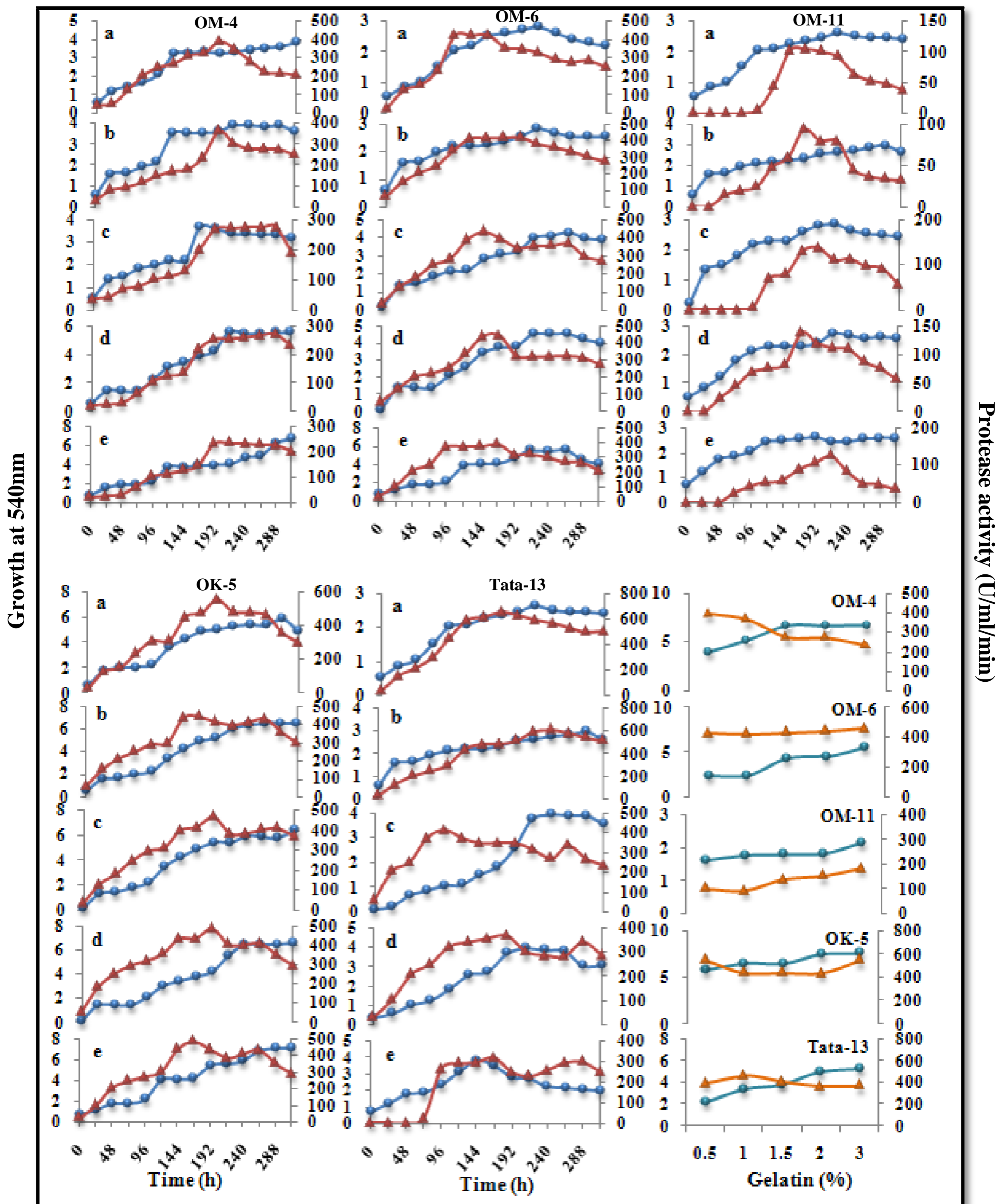


Figure 6.21: Growth (●) and protease production (▲) among the salt tolerant alkaliphilic actinomycetes strains OM-4, OM-6, OM-11, OK-5 and Tata-13 at 0.5% (a), 1% (b), 1.5% (c), 2% (d) and 3% (e) gelatin (w/v) as well as secondary graphs showing optimum growth (●) and optimum protease production (▲) among five actinomycetes isolates at 0-3% gelatin (w/v)

6.3.4 Effect of various medium components on protease production

Six actinomycetes isolates OM-4, OM-6, OM-11, OK-5, OK-7 and Tata-13 were selected for further repression studies on the basis of potential of respective isolates for protease production. The repression of alkaline protease production was studied with varied concentrations of medium ingredients for their repressive effects on protease production.

6.3.4.1 Peptone

As revealed in Figure 6.22, peptone 0-2% (w/v) showed positive correlation with growth in OM-4 while other isolates showed no effect or slightly negative effect of increasing concentration of peptone on growth. The enzyme production slightly increased (40 to 50 U/ml) with the addition of 0.5% (w/v) peptone in case of OM-4 and OK-7 as well as OM-6 showed optimum enzyme production at 1% (w/v) however, further increased up to 2%, w/v caused enzyme repression. While protease production decreased with increasing peptone in case of OM-11, OK-5 and Tata-13.

6.3.4.2 Yeast extract

Yeast extract in the range of 0-2% w/v, exerted positive effect on enzyme production in OM-11 and OK-5. However, the enzyme production was suppressed from 80 to 60 U/ml at 2% w/v yeast extract in OM-4, OM-6, OK-7 and Tata-13 (Figure 6.22). On the other hand, increasing concentrations of yeast extract had slightly adverse or no effect on growth.

6.3.4.3 Inorganic phosphate

To study the effect of inorganic phosphate on enzyme production, K_2HPO_4 was supplemented in gelatin broth. The enzyme production gradually decreased with increasing concentrations of K_2HPO_4 from 0 to 2%, w/v (Figure 6.22) in OM-4, OK-5, OK-7 and Tata-13 however positivity correlation on enzyme activity was found up to 1% w/v yeast extract in OM-6 and OM-11. The growth, on the other hand,

decreased with increasing phosphate in each isolate except OM-11 and Tata-13 where growth was increased up to 1.5% phosphate in Tata-13 while not affected in OM-11.

6.3.4.4 Catabolite repression by glucose

Glucose at higher concentrations inhibited the enzyme production in OM-6, K-5, OK-7 and Tata-13. While OM-4 and OM-11 showed positive correlation of protease production with increasing glucose from 0-2% w/v. the growth increased with increasing concentrations of glucose, in OM-6, OM-11and OK-5 while no effect of increasing concentration of glucose was found on growth in OM-4, OK-7 and Tata-13 (Figure 6.22). In the presence of 2% w/v glucose, the enzyme production was reduced by a factor of two. In addition, organism formed bead shaped cell mass in its presence. The size of these beads decreased with increasing glucose concentrations.

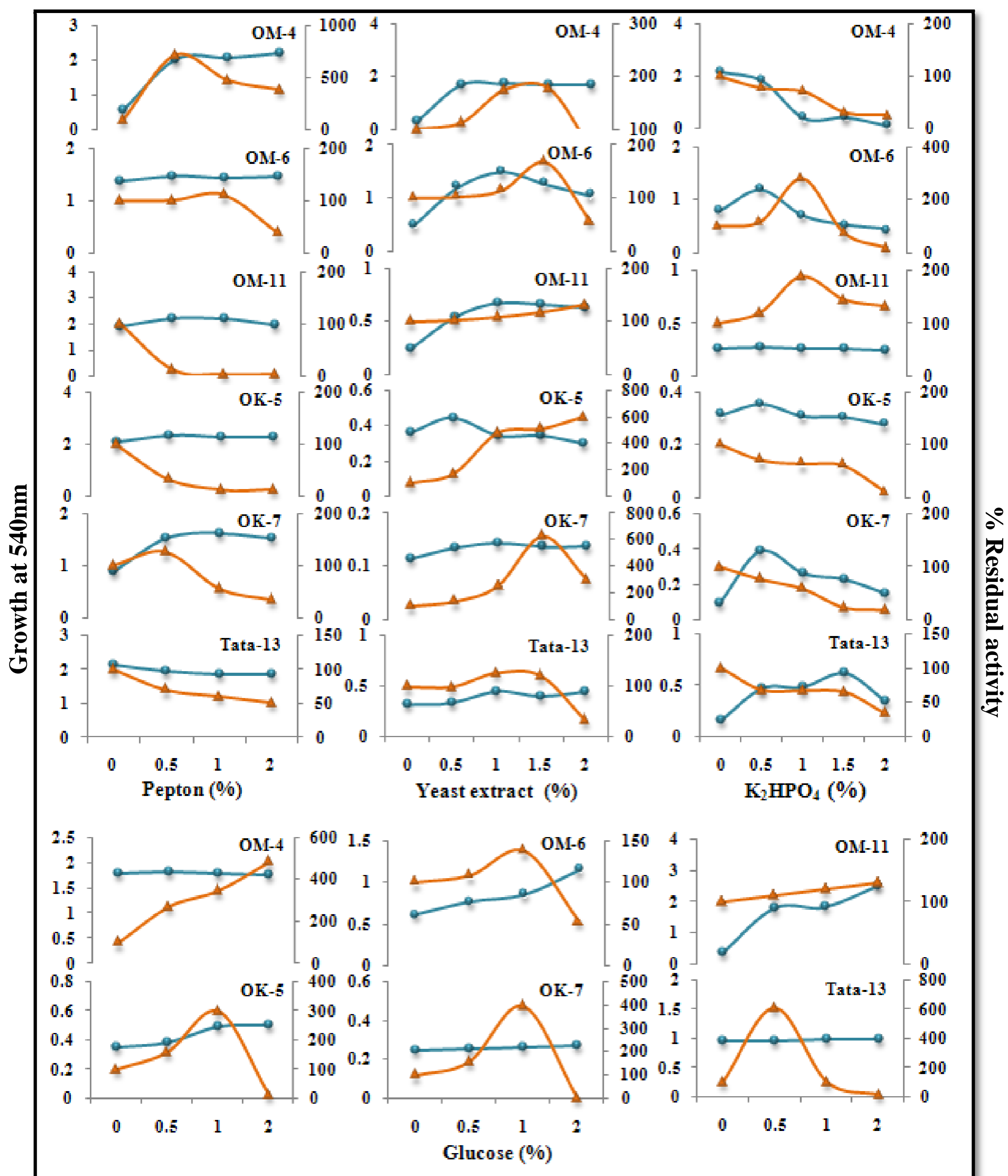


Figure 6.22: Effect of media components (peptone, yeast extract, K_2HPO_4 and glucose) on growth (●) and protease production (▲) among six actinomycetes isolates at 0-2% (w/v) concentration after 5 days of incubation

6.3.5 Repression of protease production by various amino acids

As described in chapter 3, the amino acids as compared to other nitrogen sources had different effects on the enzyme production. This study was further extended to investigate their repressive effect by using various concentrations (0-2% w/v) of methionine, alanine, leucine, phenylalanine, tyrosine, tryptophane, arginine, asparagine, histidine and glutamic acid.

6.3.5.1 Repression by aromatic amino acids

Aromatic amino acids comprise the class 2 of the amino acids. Among them, tyrosine and phenyl alanine were used to study their effect on protease production. Phenylalanine repressed the growth above 1% w/v in both OM-6 and OK-5 however; the enzyme production increased significantly (329.994 U/ml) at 1% followed by a significant decrease (175.203 U/ml) at 2% w/v phenylalanine in OK-5. In comparison, the growth increased with increasing tyrosine concentrations and protease production was optimum at 1% w/v; above which, the enzyme production decreased in both OM-6 and OK-5 (Figure 6.23).

6.3.5.2 Repression by methionine, alanine and leucine

Methionine, alanine and leucine belong to class 1 of amino acids i.e. amino acids with nonpolar, aliphatic R groups. Methionine induced growth in both OM-6 (58.80) and OK-5(150.66) up to 2% w/v while growth was optimum at 1% concentration that gradually decreased with increased methionine concentration up to 2%. While both growth and protease production was favored significantly by alanine up to 2% w/v in OM-6 (54.18 U/ml) and OK-5 (188.81 U/ml). Leucine had not significant effect on growth or protease production in both OM-6 and OK-5 (Figure 6.23).

6.3.5.3 Repression by aspartic acid

Aspartic acid belongs to the class 3 of amino acids having polar uncharged R group. The amino acid significantly supported the growth as well as protease production up

to 1% w/v in OM-6 (40.82). However, in OK-5 protease production decreased drastically from 155.287 U/ml to 35.478 U/ml as concentration of aspartic acid increases from 1% to 2% w/v (Figure 6.23).

6.3.5.4 Effect of arginine

Arginine has charged R groups and it belongs to class 4. It stimulated the growth of OK-5 up to 1% while growth remained stable in case of OM-6 up to 2% w/v arginine. However rather than repression, arginine significantly stimulated the enzyme production up to 2% w/v in both OM-6 (90.88 U/ml) and OK-5 (193.9 U/ml) (Figure 6.23).

6.3.5.5 Repression by Asparagine

Asparagine belongs to class five of amino acids with charged R groups. While the growth increased with increasing asparagine concentrations, protease production slightly decreased from 123.93 U/ml to 70.47 U/ml at 2% w/v asparagines in OK-5 however in OM-6 both growth and enzyme production decreased with increased concentration of asparagines (Figure 6.23).

6.3.6 Effect of the increasing number of amino acids on growth and protease production

The number of amino acids was increased in minimal medium from 0-6. The combinations were selected on the basis of the results described above. Increase in different amino acids in the growth medium resulted in a marked increase the growth. This was most strikingly observed when 5 different amino acids were present, however with six amino acids growth slightly decreased. Protease production increased from 23 U/ml in the absence amino acids to 200 U/ml with 6 amino acids (Figure 6.24). Interestingly, the increase in the number of amino acids also induced the secretion of brownish black pigments in the medium, an observation more evident with 5 and 6 amino acids in the medium.

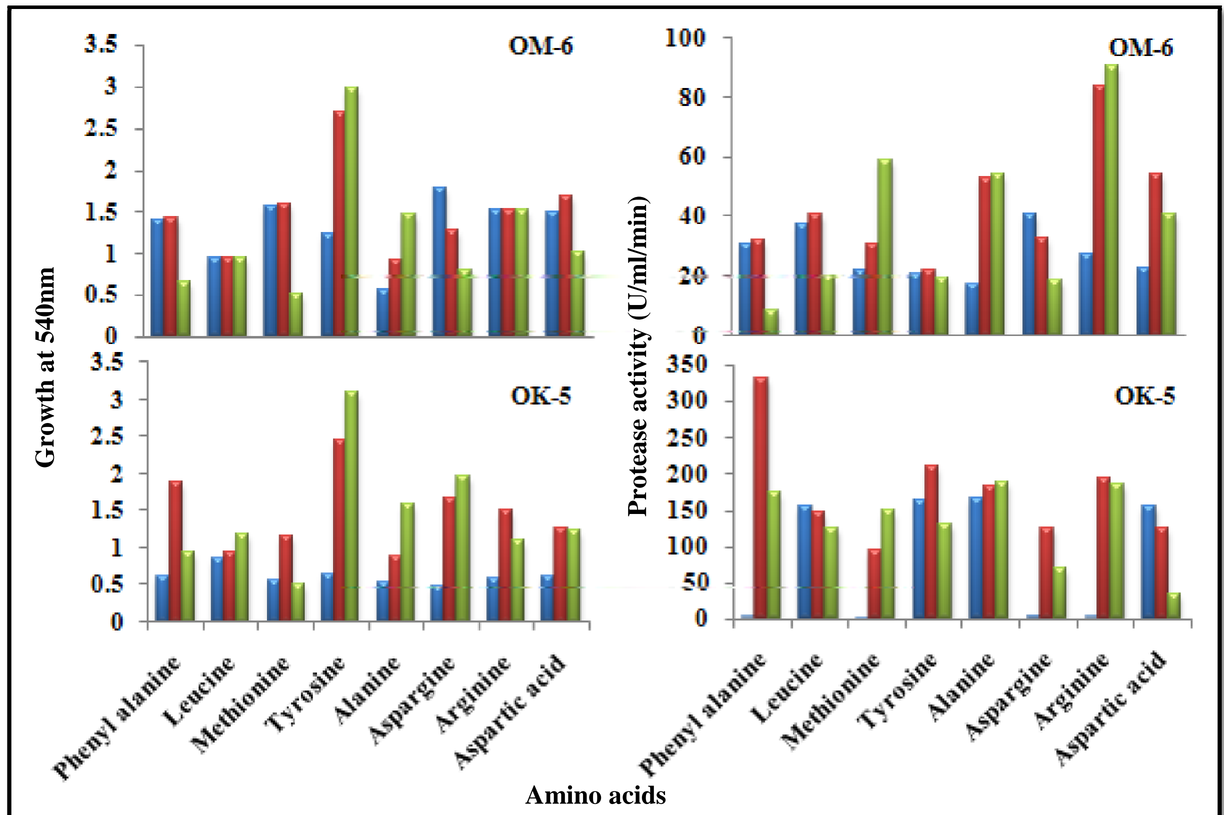


Figure 6.23: Growth kinetics and protease production among OM-6 and OK-5 isolates at 0% (■), 1% (■) and 2% (■) concentration of each amino acid

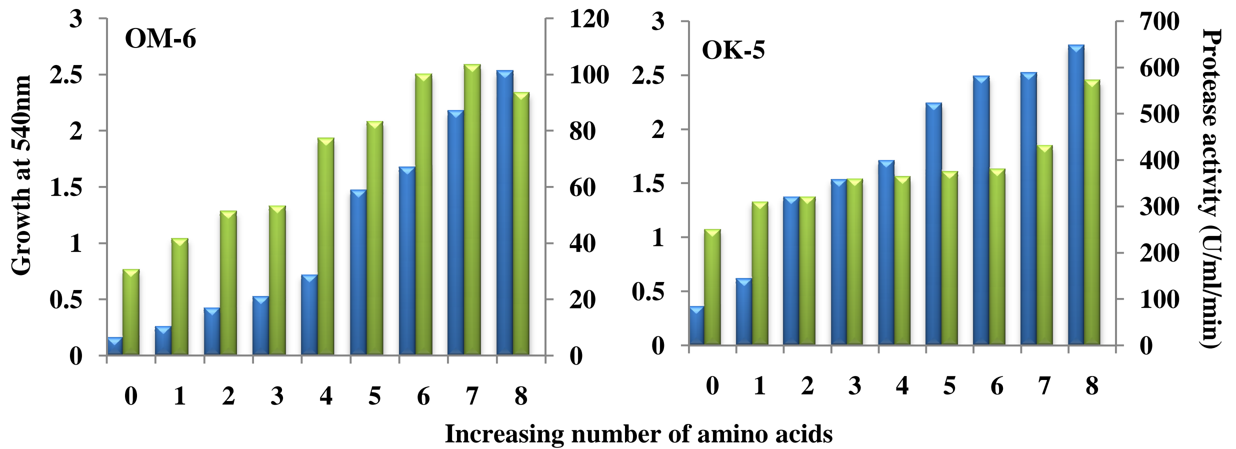


Figure 6.24: Growth (■) and protease production (■) among OM-6 and OK-5 isolates at increasing number of amino acids at 1% concentration of each

6.3.7 Characterization of crude alkaline proteases from actinomycetes

6.3.7.1 Comparative analysis of actinomycetes displaying optimum protease activity

After optimizing medium components for total nine protease producers, we generated comparative profile of all nine isolates for protease production that is displayed in Figure 6.24A. We found that among total isolates OM-6 and OK-5 were best protease producers as it gives very high enzyme activity around 400 U/mL and 500 U/mL respectively. While more than 50% isolates among nine best protease producers produced protease activity above 300 U/mL.

6.3.7.2 Temperature profile

Eight best protease producers were studied for the effect of temperature on protease activity of crude enzyme at 37-90ºC temperature. Interestingly, the crude protease had maximum activity in the range of 60ºC-80ºC temperature and only limited enzyme activity was displayed at temperatures below 50ºC displaying thermophilic nature of enzymes (Figure 6.24B).

6.3.7.3 Effect of pH on protease activity and stability

The effect of pH on protease activity was determined by preparing the substrate in various buffers (20mM) of different pH. The reaction cocktails were incubated at 60ºC. The buffers used were: Citrate phosphate (pH 7); Tris-HCl (pH 8); Glycine-NaOH (pH 9); NaOH-Borax (pH 10-11) and KCl- NaOH (pH 12). After incubating % increase in activities were measured as per assay procedure. As revealed from Figure 6.24C protease is optimally active at alkaline range of pH among all isolates confirm alkaliphilic nature of enzymes.

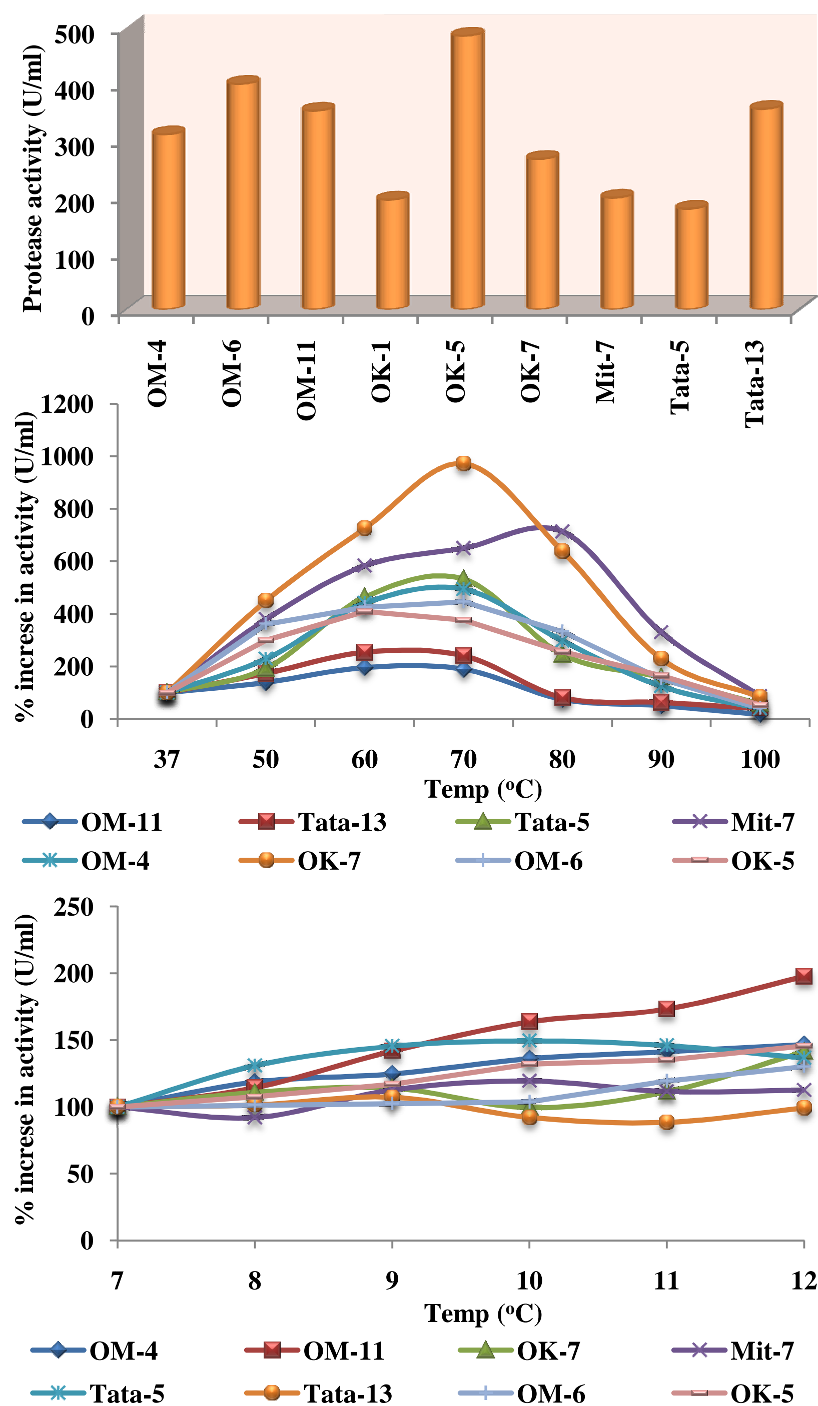


Figure 6.25: A) comparative analysis of actinomycetes displaying optimum protease activity of each isolate B) Temperature optima and C) pH optima of crude alkaline protease in a range of 37-100°C temperature and 7-12 pH, respectively

6.4 DISCUSSION

Most of the halo tolerant alkaliphilic actinomycetes known so far have been isolated from desert soil, soda lakes and other natural habitats are rarely attended in this context. As reflected from the literature, China and Korea are actively involved in the exploration of extremophilic actinomycetes (Li *et al.,* 2006a, 2006b, 2006c, 2006d; Li *et al.,* 2005a, 2005b; Chun *et al.,* 2000; Al-Zarban *et al.,* 2002). Since Gujarat has a very long sea coast and some other saline habitats along with it, it was quite logical to presume that halophilic and haloalkaliphilic actinomycetes might be present in these habitats. In the light of this realization, we focused our investigations on the exploration of enzymatic potential of these unexplored microbes.

In the present study, 40 salt-tolerant and alkaliphilic actinomycetes were studied with reference to extra cellular alkaline protease, amylase and cellulase secretion and the potential of the isolates for protease secretion was explored mainly by studying nine best protease producing actinomycetes in liquid culture media. Based on the comparative analysis on the production of alkaline proteases from the 9 isolates, they were subjected to further detailed studies on optimization and characterization.

Studies on alkaliphilic actinomycetes have led to the discovery of many types of enzymes that exhibit interesting properties (Tsujibo *et al.,* 2003; Fernandez *et al.,* 2003; Tang *et al.,* 2003; Moreira *et al.,* 2001). A number of extra – and intracellular enzymes from moderately halophilic and alkaliphilc actinomycetes have earlier been isolated and characterized which might have potential applications in food, chemical, pharmaceutical, leather, tanning, paper pulp and waste-treatment industries (Starch *et al.,* 2005; Kampfer *et al.,*2002; Petinate *et al.,* 1999; Sampath *et al.,* 1997; Kang *et al.,* 1995). However, amylases, cellulases, proteases, pullulanases, xylanases and lipolytic enzymes produced by halotolerant alkaliphilic actinomycetes have not been investigated in detail.

The halo tolerant alkaliphilic actinomycetes discussed in the present study were screened for the production of extra cellular proteases, amylases cellulases and lipase. They all secreted protease, amylase and cellulase while lipase was less common

among them. This implies that the concerned habitats are rich in proteinaceous and carbohydrate substances occupying the nutritional dynamics where lipids are scares. The isolates displayed varying degree of diversity with respect to growth patterns and potential for extra cellular enzymes at different salt and pH. Majority of our isolates could grow in the range of 0-10% salt while some isolates tolerate 0-15% and even 0-20% NaCl and secrete extra cellular enzymes and hence they have been referred as halo tolerant as they did not satisfy the parameters of the halophilic group in terms of their optimum NaCl requirement. The salt tolerance of the isolates in the present study was comparable to *Nocardiopsis halotolerans* sp. nov., a halo-tolerant actinomycete which grew at salt concentrations of 0-10% w/v NaCl (Al-Zarban *et al.,* 2002), while it was quite less in comparison to *Streptimonospora salina* gen. nov., sp. nov., and *Streptomonospora alba* sp. nov., the truly halophilic actinomycetes (Li *et al.,* 2003c; Cui *et al.,* 2004). The isolates which tolerated higher salt (up to 10% to 15% w/v) were potent enzyme producers in comparison to those grown at lower NaCl concentrations. Recently, Bull *et al* (2005) reported marine actinomycetes capable of secreting extra cellular hydrolytic enzymes in the range of 0-10% NaCl. Most of our isolates produced substrate and aerial mycelia on the growth medium and grow in the range of 8-11 pH, These results are in agreement with *Nocardiopsis alkaliphila* in which optimum growth occurred at pH 10 (Hozzein *et al.,* 2004). Recently, a novel alkaliphilic actinomycetes *Streptomyces sodiiphilus* sp. nov., was reported to grow with an optimum pH of 9-10 (Li *et al.,* 2005b). The pattern of growth with respect to salt and pH requirement of some of our isolates was quite comparable with a novel alkaliphilic and moderately halophilic actinomycete *Streptomyces sodiiphilus* (Li *et al.,* 2005c) which grew in the presence 0-10 % (w/v) NaCl at pH 9-11 while the salt requirement for protease production was quite comparable in some isolates, when compared to haloalkaliphilic bacteria, *Salinicoccus alkaliphiles* sp. which grew in the presence of 0-25 % (w/v) NaCl at pH 6.5-11.5 (Zhang *et al.,* 2002).

Some of our isolates secreted amylase and cellulase optimally at 0-10% and 0-20% NaCl respectively which is contrary in comparison to protease. Protease, amylase and cellulase secretion occurred in the broader range of pH; 7-11 and 8-11. The results as

a whole are quite interesting, as it reflects diverse salt requirement with respect to different enzymes. The salt requirement for some of the isolates for amylase secretion was quite comparable to haloalkaliphilic *Natronococcus* sp. strain Ah-36 and *Streptomonospora alba* sp. nov., which produced an extracellular maltotriose - forming amylase optimally at 10% NaCl (Li *et al.,* 2003c; Kobayashi *et al.,* 1992). Similarly, an alkaliphilic Streptomyces strain KSM-9 has been reported to produce an alkaline cellulase at pH 9 (Park *et al.,* 1993; Damude *et al.,* 1993).

Because of increasing emphasis on the alkaline proteases, several microbes have been looked for their ability to secrete these enzymes (Nascimento *et al.*, 2004; Jones *et al.,* 1998; Chaphalkar and Day, 1998; Paliwal *et al.,* 1994; Nomoto *et al*, 1984; Horikoshi *et al*, 1971). While alkaline proteases from bacteria are extensively characterized, similar attention has not been paid to actinomycetes. However, the ability to produce a variety of enzymes may be an attractive phenomenon in these prokaryotes. Recently, alkaline protease from *Nocardiopsis* sp. NCIM 5124 (Dixit and Pant, 2000) was studied with reference to purification and characterization. Similarly, alkaline protease was purified and crystallized from *Nesterenkoni* sp. (Bakhtiar *et al.,* 2003). Several species from the Streptomyces genus are among the most important industrial microorganisms because of their capacity to produce numerous bioactive molecules, particularly antibiotics and enzymes.

Maximum enzyme production in the present case was achieved during the late exponential and early stationary phases that correspond to the secondary metabolism. These results are in agreement with Bascaran *et al* (1990) and Moreira *et al* (2001), who showed that synthesis of protease by *Streptomyces clavuligerus* starts in the early stationary phase of growth. Salt had pronounced effect on growth and enzyme production. Though all nine isolates could grow up to 20% NaCl, growth in the absence of NaCl indicates the halo-tolerant nature of them. The salt tolerance of one of the isolates was quite similar to *Nocardiopsis kunsanensis* sp. nov., a moderately halophilic actinomycete (Chun *et al.,* 2000). However, Kocuria marina sp. nov., a novel actinomycete, tolerated up to 15 % NaCl, although its presence was not

essential for growth (Kim *et al.,* 2004). However, enzymes (protease, amylase and cellulase) were produced optimally in the range of 5-15% NaCl with optimum protease activity at 15% (OM-11) and 10% (OM-4, OK-1, OK-5 and OK-7). Though growth was evident in the absent of salt, Protease production reduced extensively at 5% (w/v) NaCl. The results clearly indicated the halophilic nature of the protease. Similar results have also been reflected by the haloalkaophilic archaeon, *Natronococcus occultus* in which protease secretion was optimum at 1-2M NaCl (Studdert *et al.,* 2001) and *Bacillus pseudofirmus* with optimum protease production at 10% NaCl (w/v) (Patel *et al.,* 2006). However, in the case of the archaebacterium *Halobacterium mediterranei,* a much higher salt requirement (25%, w/v) for serine protease secretion was reported (Stepanov *et al.,* 1992). Our isolates grew and produce protease over a wider range of pH (7-11) with slower growth at neutral pH. The Optimum pH range for growth and protease production is common among alkaliphilic and haloalkaliphilic organisms (Johnvesly & Naik 2001; Kaur *et al.,* 2001; Studdert *et al.,* 2001; Dodia *et al.,* 2008) but similar response has been quite less observed in alkaliphilic actinomycetes. Nocardiopsis sp. TOA-1 where alkaline protease was produced optimally at pH 9-10 (Mitsuiki *et al.,* 2002). Recently, a novel alkaliphilic actinomycete, *Streptomyces sodiiphilus* sp. Nov., was reported to grow with an optimum pH of 9-10 with scant growth at pH 7 (Li *et al.,* 2005). Similarly, *Nocardiopsis alkaliphila* also grew optimally at pH 9.5-10 (Hozzein *et al.,* 2004).

Generally, complex nitrogen sources are used for protease production. Gelatin stimulated enzyme production in OM-4 and Tata-13 up to 1% concentration beyond which the enzyme activity decreased gradually indicating a threshold level of gelatin. While OM-11 displayed gradual increase in activity with increasing gelatin up to 3%. However, no significant effect of increasing concentration of gelatin was found on enzyme activity in case of OM-6. Enhancement of protease production by gelatin was also reported in a newly isolated haloalkaliphilic Bacillus sp. (Patel *et al.,* 2005). Growth increased with increasing levels of gelatin while growth behavior of the organism in the present study changed along with increasing gelatin concentration.

Optimizing cultural conditions is an approach to study the influence of various cultural parameters on cell growth and synthesis of primary and secondary metabolites. Either excess or a deficiency may cause repression of protease synthesis and therefore, it is essential to study the repressive effect of various cultural parameters on protease production. While these studies have been more common among the rod shaped bacteria, similar investigations are quite limited in actinomycetes (Thumar *et al.,* 2009; Vinogrodova *et al.,* 2003; George *et al.,* 1995; Chakraborty and Srinivasan, 1993).

Complex organic nitrogen sources such as yeast extract and peptone being rich in amino acids and short peptides displayed enzyme repression at higher concentrations. This trend has earlier been shown in *Bacillus firmus* (Moon and Parulekar, 1991). In case of 50% of our isolates, protease secretion slightly enhanced with 0.5-1% (w/v) peptone. However, the enzyme secretion was repressed significantly at higher concentrations of peptone in each isolate. Similarly, Adinarayana and Ellaiah (2002) reported an enhancement of protease production by peptone in a newly isolated alkaliphilic *Bacillus subtilis* PE-11. Our studies revealed that nitrogen sources stimulated protease synthesis up to certain threshold levels beyond which enzyme production was repressed.

As phosphate plays a vital role in carbohydrate metabolism, cellular respiration, and control of adenosine triphosphate (ATP) levels. Excess inorganic phosphate, up to certain levels, has been reported to stimulate glucose utilization, cell growth and production of enzymes in certain organisms (Martin *et al.,* 1977, Yoon *et al.,* 1989). In majority of isolates studied, protease production was repressed gradually with increasing phosphate concentrations. This effect is quite comparable to the results for alkaline protease production by a newly isolated *Bacillus* sp. (Patel *et al.,* 2005), while OM-6 and OM-11 displayed optimum activity at 1% K_2HPO_4 that is in accordance with the findings for *Bacillus firmus* in which increased supply of nitrogen and phosphorus stimulated protease synthesis up to certain threshold levels (Moon and Parulekar, 1991).

Generally, easily metabolizable nutritional sources like glucose induce metabolite production up to a significant level however higher concentrations of glucose may cause catabolite repression of enzyme. In our study, while the growth was quite stable at high glucose concentrations, enzyme production got repressed completely in its presence. The results resembled to earlier reports on the strong repression of protease production by glucose in alkaliphilic Bacillus spp. (Johnvesly *et al.,* 2001; Ferrero *et al.,* 1996; Daatselaar *et al.,* 1974). Repression of protease production by glucose was also reported in *Aeromonas hydrophilla* (O'Reilly and Day, 1983). Catabolite repression of protease synthesis was under a control mechanism in *Arthrobacter* sp. (Hofsten *et al.,* 1965), a phenomenon also reflected by an alkaline protease from alkaliphilic *Bacillus* sp. (Kaur *et al.,* 2001). Similarly, many earlier studies have also reported a reduction in protease production due to catabolite repression by glucose in bacteria (Kaur *et al.,* 2001; Mao *et al.,* 1992; Frankena *et al.,* 1986; O'Reilly and Day, 1983).

The details of the mechanism by which control of protease production is achieved in many prokaryotic systems are not yet known. While protease synthesis and secretion are induced by peptides or other proteinaceous substrates; the amino acids, depending on their nature and levels, may induce or repress protease synthesis and secretion. Amino acids are the end products of proteolytic degradation of complex proteins and the synthesis of protease may be subjected to feed back inhibition by some of the amino acids thus generated. Production of the alkaline protease has earlier been shown under repression by rapidly metabolizable amino acids (Chu *et al.,* 1992; Litchfield and Prescott, 1970).

In our study, the amino acids of class 1 supported the growth of Mit-1. However, protease production was better with methionine even though the growth was adversely affected. Protease activity not affected much with leucine compared to that with methionine. However, alanine increased the protease production which is in contrast with (Thumar, 2007) reported that alanine is likely to be a product of the

action of alkaline protease. Isoleucine and threonine inhibited the production of extracellular proteinase by *Bacillus* sp. (Chaloupka and Kreckova, 1966).

Among the aromatic amino acids (class 2), both phenylalanine and tyrosine induced the growth while protease production was optimum at 1% w/v of both aminocids. Similar results were also reported in *Bacillus megaterium* (Chaloupka and Kreckova, 1962). Interestingly, aspartic acid (class 3 amino acids), at higher concentrations exerted an inhibitory effect on the synthesis of extracellular protease. Arginine (class 4) showed varied effect on growth and protease production. Arginine induced protease production at higher concentrations. Stimulation of protease production at higher concentrations of amino acids has been reported in *Micrococcus* sp. (McDonald and Chambers, 1966) and *Bacillus* sp. (Fayyaz and Chaloupka, 1970). Similarly, O'Reilly (1983) described the induction of protease by various amino acids in *Aeromonas hydrophilla*.

Asparagine (class 5) displayed reduced growth and protease production at higher concentration of asparagine. These results correspond with protease production from *Streptomyces clavuligerus* (Keila *et al.,* 2001). The decrease in extracellular protease synthesis by growth in the presence of asparagine provides another example in which an individual amino acid regulates the production of extracellular enzymes. Thus, it appears that there is a critical limit of stimulation of enzyme synthesis by amino acids.

The additive effect of various amino acids on the protease production was also studied. Protease production increased, when the number of different amino acids in the growth medium increased. This stimulation might be because of cumulative effect of amino acids on the induction of proteases. Possibly small peptides or a combination of different amino acids are better inducers than single amino acids. Daatselaar and Harder (1974) observed the similar results in protease producing marine bacterium in which the protease production increased with the increase in the number of different amino acids in growth medium. Maximum enzyme production was obtained with five different amino acids namely, L-phenylalanine, L-glutamic

acid, L-leucine, Lserine and L-aspartic acid. Interestingly, the production of a soluble brownish pigment was also apparent with the increasing number of amino acids. The pigmentation was quite intense in the presence of asparagine and arginine which reflects that these amino acids might be involved in the synthesis or induction of pigment.

While most of the information about alkaline protease form alkaliphilic actinomycetes is focused on purification and characterization, it would be interesting to generate a bulk of data regarding the production and repression. Further, repression of protease production as affected by the nitrogen and carbon sources in our organism generated valuable information on the enzyme synthesis and production optimization in actinomycetes, where such data are scares.

The temperature optima of crude proteases were higher (60-80ºC). The possible reason is that certain native proteins or peptides present in the crude preparation might be exerting protection against thermal denaturation. Besides, the residual salt concentration in the crude preparation would also have provided certain degree of protection against thermal denaturation of the enzyme. Interestingly, the optimum temperature for almost all proteases was nearly 2-fold higher than those reported from haloalkaliphilic bacteria recently reported from our laboratory (Patel *et al.,* 2006; Dodia, 2005).

Alkaline proteases were active in the optimum pH range of 9-12 indicating the alkaline nature of the enzymes. The findings are in accordance with several recent reports showing pH optima of 10-11 (Joo and Chang, 2005; Nascimento and Martins, 2004; Banik and Prakash, 2004; Adinarayan *et al.,* 2003; Huang *et al.,* 2003; Mane and Bapat, 2001). While a serine protease from the keratin degrading *Streptomyces pactum* DSM 40530 was optimally active in a pH range of 7-10 (Bockle *et al.,* 1995), a thermostable alkaline protease from Thermoactinomycetes sp HS682 was active at pH 11 (Tsuchiya *et al.,* 1997).

The NaCl requirement of alkaline protease for the maintenance of optimum catalysis at higher temperature is well recorded in the literature (Patel *et al.,* 2006; Joshi, 2006; Thumar, 2006; Dodia, 2005). However, alkaline proteases of OM-6 and OK-5 did not require higher salt for the growth. The results indicated the salt tolerant rather than salt requiring nature of the enzyme as the salt requirement for other halophilic proteases is quite obvious (Studdert *et al.,* 2001; Kuniyo *et al.,* 1997; Kamekura *et al.,* 1990; lzotova *et al.*, 1983). More recently, an alkaline protease from a halophilic actinomycetes *Streptomonosporea salina* was reported to be active up to 4M NaCl (Cui *et al*., 2004)

The results discussed above evaluate the current status of research on the biology and biotechnology of salt-tolerant and alkaliphilic actinomycetes. The results are quite interesting in the view of the diversity, novelty and distribution of these actinomycetes. The fairly better salt and pH tolerance of these actinomycetes, along with their capacity to secrete commercially valuable primary and secondary metabolites, can be considered as attractive features of these organisms. Interestingly, the characterization of proteases from the different organisms indicated that although synthesized under similar conditions of growth, the enzymes may display greater variation in their properties. However, the structure elucidation of these enzymes may provide some important clues responsible for varied chemical sensitivity.

Chapter 7

Purification and Characterization of Alkaline Proteases from Actinomycetes

7.1 INTRODUCTION

Proteases are established in enzyme catalysis and have been the subject of numerous texts and reviews. The majorities of halophilic proteases, however, have been studied from halophilic archaea and bacteria (Purohit and Singh, 2011; Cannio *et al.,* 2010; Dodia *et al.,* 2008; Patel *et al.,* 2005, 2006), while representation from haloalkaliphilic actinomycetes in this context is quite restricted (Singh *et al.,* 2010; Thumar and Singh, 2009, 2007; Mehta *et al.,* 2006; Li *et al.,* 2006; Li *et al.,* 2005). Despite their advantages, the application of halophilic proteases is further constrained because of their limited stability under extremes of temperature, pH and ionic strength. The available array is still not sufficient to meet the ever increasing demand for suitable proteases. Halophilic proteases have several applications in food, leather, detergent and antifouling coating industries as well as peptide synthesis in organic media. Haloarchaeal proteases catalyze the reaction at 4 to 5 M NaCl, losing activity rapidly when exposed to low salt concentrations (Lanyi, 1974). This property of enzyme severely restricts the choice of purification methods, making the majority of the conventional procedures unsuitable. The methods used for purification of haloarchaeal proteases include concentrating the enzyme by ethanol precipitation or ultra filtration followed by affinity chromatography and gel filtration. However, many steps make the method more cumbersome and hence, one step purification by hydrophobic interaction chromatography could be a method of choice (Kikani and Singh, 2011; Dodia *et al.,* 2008; Gupta *et al.,* 2005).

Most of the halophilic proteases belong to serine protease family, dependent on high salt concentrations for structural stability and display optimum activity at high salt, neutral to basic pH and temperatures 37-50°C. However, literatures describing halophilic proteases with alkaliphilic and thermophilic properties are quite limited, especially from haloalkaliphilic actinomycetes (Thumar and Singh, 2007). The activity and stability of enzymes are important parameters which codetermine the economic feasibility of applying protease in industrial processes. High stability is generally considered an economic advantage because of reduced enzyme turnover

(Vieille and Zeikus, 2001). A number of strategies are applied to increase the stability of protease enzymes such as mutation, chemical modification at active site, introduction of disulfide bridges, the optimization of helices and helix caps, immobilization, entropic stabilization, changing pH condition and using various salts. Studies on the thermodynamic stability of enzymes have provided insight into the factors that determine enzyme stability (Vieille and Zeikus, 2001). However, thermodynamic properties of purified proteases, particularly from actinomycetes, are not described. Thus, the present investigation addresses upon the thermodynamic approaches (deactivation kinetics, ΔH^*, ΔS^*, E and ΔG^*) to understand the behavior of these enzymes at different temperatures and salts (Gohel and Naseby, 2007).

Halophilic eubacteria accumulate organic compatible solutes such as sucrose, mannitol, trehalose, glycerol, betaine, proline, glutamate, etc. to maintain the protein structure. Vidyasagar *et al* reported the activity of extracellular protease from *Halogeometricum brorinquense* strain TSS101 in presence of compatible solutes; sucrose, mannitol, glycerol and betaine in absence of NaCl. However, no further information is available on the stability of this enzyme in the presence of such solutes. Kinetic studies on the behavior of haloalkaliphilic proteases from actinomycetes in presence of NaCl and compatible solutes would provide insight into their unique properties. The present investigation was undertaken to study the characteristics and thermodynamics of an extracellular alkaline serine protease produced by a salt tolerant alkaliphlic actinomycetes *Brachystreptospora xinjiangensis* OM-6 and *Nocardiopsis alba* OK-5.

7.2 MATERIAL AND METHOD

7.2.1 Purification of OM-6 and OK-5 alkaline protease

7.2.1.1 Strains

On the basis of potential for extracellular enzyme secretion, OM-6 and OK 5 were selected for further purification and characterization studies. The isolates were grown in gelatin broth as described in chapter 3 (Section 3.3.1). The mycelia were separated by centrifugation and the culture filtrates were used as crude enzyme preparation. Alkaline protease activity was measured by modified Anson-Hagihara's method (Hagihara, 1958) as described in chapter 6 (Section 6.2.5.2).

7.2.1.2 Partial purification of OM-6 and OK-5 alkaline protease

The crude Mit-1 protease was fractioned by ammonium sulphate between 30 - 70% saturation. The crude enzyme was precipitated by gradual addition of ammonium sulphate up to 30% (w/v) saturation. The proteins were allowed to precipitate with constant stirring for overnight at 4ºC and the precipitates were separated by centrifugation at 10,000 rpm at 4ºC for 10min. The supernatant was subjected to further precipitation of the residual proteins by addition of ammonium sulphate up to 70% (w/v) saturation. The precipitates were collected by centrifugation (10000 at 4ºC) and resuspended in the minimum volume of 20mM borate buffer (pH 10). This preparation was used as a partially purified protease.

7.2.3 Purification of the OM-6 and OK-5 alkaline protease

Purification was achieved by two-step and a single step purification method using hydrophobic interaction chromatography on a phenyl sepharose 6 fast flow. The affinity column (1 cm × 6.5 cm) was equilibrated with 0.1 M sodium phosphate buffer (pH 8.0) containing 1 M ammonium sulfate. The crude protease preparation [20 ml crude containing 1 M $(NH_4)_2SO_4$] was loaded onto column in case of single step purification, while partially purified protease [1 ml of 70% $(NH_4)_2SO_4$ saturated enzyme sample] was loaded onto the column in two step purification. The bound

enzyme was eluted by 0.1 M sodium phosphate buffer, pH 8.0 containing a decreasing gradient of ammonium sulfate; 1000-100mM. Fractions at a flow rate of 0.8 ml min^{-1} were collected by BIO-RAD fraction collector (BIO-RAD, California, USA) and analyzed for protease activity. The final enzyme preparations from single and two-step purification methods displayed a single band on sodium dodecyl sulfate polyacrylamide gel electorphoresis (SDS-PAGE) (Laemmli, 1970).

7.2.2 Characterization of purified proteases from OM-6 and OK-5

7.2.2.1 Effect of NaCl and Na-glutamate on temperature optima, protease activity and stability

The effect of salt on alkaline protease activity and temperature optima was assessed. The reaction mixtures (pH 10) were separately supplemented with 0-4M NaCl and 30% Na-glutamate and incubated for 10mins at 37-90°C. The thermal stability of the purified enzyme was studied by incubating the enzyme with 20mM borate buffer (pH 10) containing 0-4M NaCl and 30% Na-glutamate at 37-80°C for 5h. The residual activities were expressed as % of the initial activity.

7.2.2.2 Estimation of deactivation rate constant

The deactivation rate of purified alkaline protease was calculated by first order expression:

$$dE/dt = - KdE \qquad (1)$$

So that,

$$Ln\,[E_t/E_0] = - Kdt \qquad (2)$$

The Kd (deactivation rate constant or first order rate constant) values were calculated from a plot of $Ln[E_t/E_0]$ Vs. t at a particular temperature and apparent half lives were estimated using equation (3).

$$t_{½} = \ln 2/K_d \qquad (3)$$

7.2.2.3 Estimation of thermodynamic parameters for protease deactivation

In order to obtain enthalpy and entropies of protease deactivation, absolute rates of reaction theory were used (Eyring and Stearn, 1939) where the rate of any reaction at a given temperature depends only on the concentration of an energy rich activated complex. Thermodynamic data were calculated by rearranging the Eyring absolute rate equation (Cottaz *et al.,* 2000).

The Eyring absolute rate equation is

$Kd = (KbT/h). e (\Delta S^*/R).e(-\Delta H^*/RT)$ (4)

Where,

h (Plank constant) = 6.63×10^{-34} Js,

R (Gas constant) = 8.314 J/K mol,

Kb (Boltzman constant [R/N]) = 1.38×10^{-23} J/K

N (Avogadros no.) = 6.02 x 10-23 mol^{-1}

ΔH^* (Change in enthalpy)

ΔS^* (change in entropy)

To calculate ΔH^* and ΔS^* the Eyring absolute rate equation is rearranged to give

$Ln [Kd/T] = - (\Delta H^*/R) (1/T) + (Ln(Kb/h) + \Delta S^*/ R)$ (5)

ΔH^* and ΔS^* values were calculated from the slope and intercept of a Ln [Kd/T] vs. 1/T plot respectively.

So that,

$\Delta H^* = - (slope) R$ (6)

$\Delta S^* = R [intercept - Ln(Kb/h)]$ (7)

Free energy change (G) for inactivation of protease was calculated by using the following relationship.

$\Delta G^* = - RT [Ln(Kd*T/ Kb*h)]$ (8)

Energy of deactivation was estimated using the Arrhenius equation

$Kd = A e(-E/RT)$ (9)

So that

$Ln[Kd] = -E/RT + LnA$ (10)

Energy involved in this process was calculated from the slope of a linear plot of Ln[Kd] Vs. 1/T (Gohel and Naseby, 2007). Thermal stability of protease in the presence of different salts and pH was determined by incubating the enzyme in the presence of each salt in sealed tubes at 37, 50, 60, 70 and 80°C. Enzyme activity was measured before and after incubation at respective temperature to determine residual activity under each condition. All experiments were conducted in triplicate, results shown are mean values.

7.2.2.4 Substrate specificity and determination of *Km* and *Vmax*

Protease activity with various protein substrates including BSA, casein, egg albumin and gelatin (0.6% w/v) was assayed at 60°C under standard assay condition. The specific protease activity towards casein was taken as a control. *Km* and *Vmax* values of the pure enzyme were determined by measuring the activity with various concentrations of casein substrate (0.025-1 g/100ml). Kinetic constants were calculated from Lineweaver-Burke plot.

7.2.2.5 Effect of pH on protease activity and stability

The effect of pH on alkaline protease activity was determined by assaying the enzyme activity at different pH values from 6-13 using buffers: Sodium phosphate (pH 6-7), Tris-HCl (pH 8-9), Glycine-NaOH (pH 9-11), Borat (pH 10-12) and KCl-NaOH (pH 12-13) at 20mM concentrations. Similarly, for enzyme stability, the enzyme was inoculated with respective buffers at 40°C and activity was measured after 1h, 6h and 24h of incubation. The residual activities were measured by considering the initial activity as 100%.

7.2.2.6 Effect of denaturing agents on protease activity and stability

The effect of chemical denaturants; urea and guanidine hydrochloride on the stability of purified protease was studied at 0-8M. The enzyme was incubated with urea at different temperatures for 5h followed by the measurement of the residual activities to find out loss in enzyme activity.

7.2.2.7 Effect of osmolytes, inhibitors, metal ions, oxidizing agents, reducing agents and surfactants

The reaction mixture was incubated with various osmolytes (5-30%): NaCl, KCl, glycerol, mannitol, trehalose, sucrose and Na-glutamate; metal ions (5mM); Ca^{2+}, Cd^{2+} , Co^{2+}, Cu^{2+}, Fe^{2+}, Sr^{2+}, Ni^{2+}, Ba^{2+}, Hg^{2+}; different inhibitors (1mM, 5mM and 10mM): phenyl methyl sulfonyl fluoride (PMSF), dihiortitol (DTT), ethylene diamine tetraacetic acid (EDTA), thiourea(TU), and (PCMB); oxidizing and reducing agents (0-50mM): H_2O_2 and β-mecaptoethanol; surfactants (0.5%): sodimum dodecyl sulfate (SDS), cetyl trimethyl ammonium bromide (CTAB), tween 80 and triton X-100; The effect was assessed by considering the control (without effectors) as 100%.

7.3 RESULTS

7.3.1 Strains

After completion of media optimization of nine best protease producers, we further selected two best protease producers *Brachystreptospora xinjiangensis* strain OM-6 and *Nocardiopsis alba* strain OK-5 for purification and characterization studies.

7.3.2 Purification of OM-6 and OK-5 alkaline protease

The results of purification of extracellular alkaline protease from strains are summarized in Table 1. The active protease was isolated from the culture filtrate by salt precipitation and hydrophobic interaction chromatography using phenyl sepharose 6FF. In OM-6, the two-step purification method yielded 35 fold purification with a yield of 47% and specific activity of 22938 U/mg protein. On the other hand, single-step purification method resulted in 26 fold purification with 22.30% yield and specific activity at 18590 U/mg protein. In OK-5, two-step purification method yielded 27.34 fold purification of protease with a yield of 34.96% and the specific activity of purified enzyme was 19014.15 U/mg protein. While single-step purification method resulted in 13.03 fold purification with a final yield of 12.95% and specific activity 18589.87 U/mg protein of purified enzyme.

Isolates	Purification steps	Total activity (U)	Total protein (mg)	Specific activity	Purification fold	Yield (%)
OM-6	**Crude**	307314	470.53	653.12	1	100
	70% NH_4SO_4	247802.4	24.64	10056.91	15.39	80.63
	Two Step Purification (Phenyl Sepharose 6FF)	144510.75	6.3	22938.21	35.12	47.02
	One Step Purification (Phenyl Sepharose 6FF)	**1524**	**0.082**	**18589.87**	**26.18**	**22.32**
OK-5	**Crude**	317070	456	695.32	1	100
	70% NH_4SO_4	217589.28	37.90	5741.14	8.25	68.62
	Two Step Purification (Phenyl Sepharose 6FF)	110852.55	5.83	19014.15	27.34	34.96
	One Step Purification (Phenyl Sepharose 6FF)	**1024.67**	**0.099**	**10532.02**	**13.03**	**12.95**

Table 7.1: Purification of OM-6 and OK-5 alkaline proteases

7.3.3 SDS PAGE analysis

Protein purification was successfully achieved to the homogeneity as evident by a single band on SDS PAGE with a molecular mass of 25 KDa in alkaline protease from OM-6 while in OK-5, the results of SDS PAGE analysis revealed a single band with a molecular mass of 20 KDa. The apparent molecular mass of the protease was estimated by using Rf values of the reference proteins (Figure 7.1).

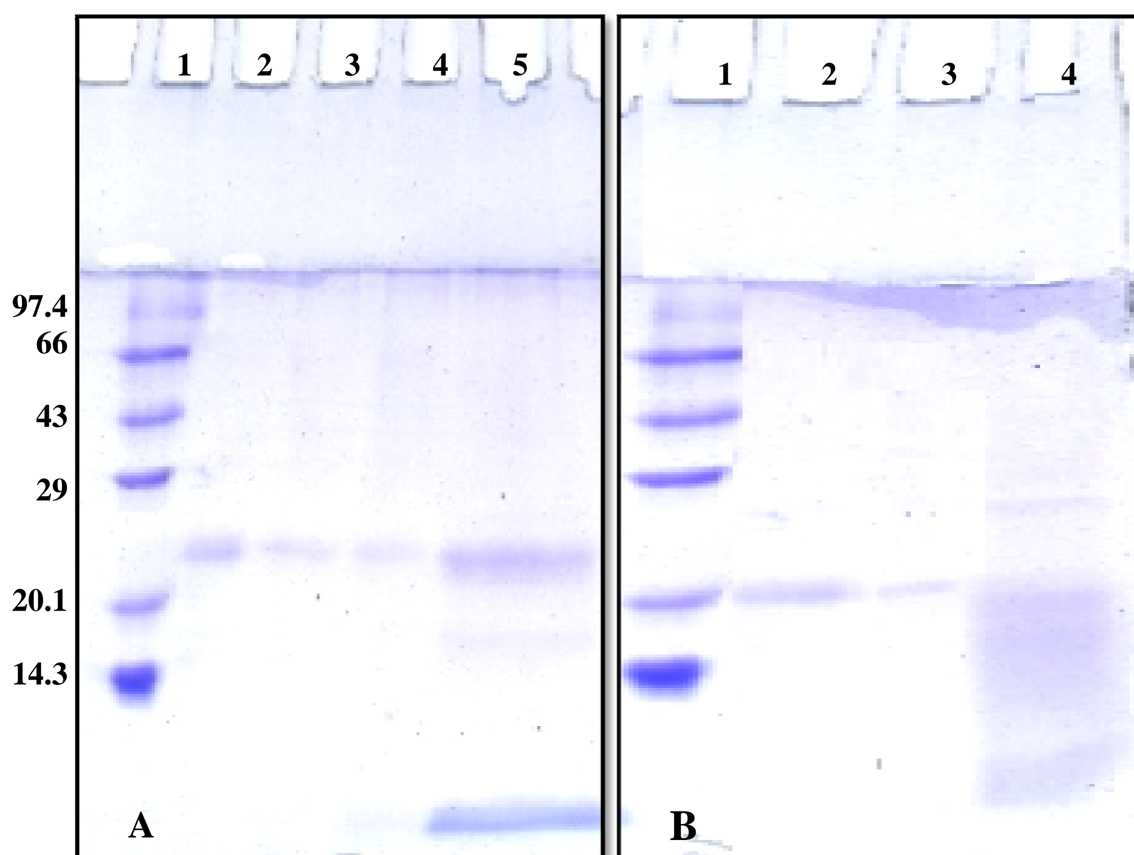


Figure 7.1: SDS-PAGE pattern of purified protease using 12% cross linked polyacrylamide gel stained with coomassie brilliant blue. A) SDS – PAGE of OM-6 alkaline protease Lane 1: protein marker; Lane 2 and 3: electophoretic separation of purified protease by two steps purification method Lane 4: purified protease by one step purification method Lane 5: partially purified protease. B) SDS – PAGE of OK-5 alkaline protease Lane 1: protein marker; Lane 2 and 3: electophoretic separation of purified protease by two steps purification method Lane 4: purified protease by one step purification method Lane 5: partially purified protease

7.3.4 Characterization of crude and partially purified proteases from OM-6 and OK-5

7.3.4.1 Effect of NaCl on temperature optima and stability of OM-6 protease

The effect of NaCl (0-3M) on the temperature profile of the crude and partially purified protease was carried out at various temperatures in the range of 37-90ºC. The temperature optimum for crude and partially purified protease of OM-6 isolate was 60ºC in the absence of salt and it remained unaffected by the salt up to 1M. However, with 3M NaCl, the temperature optima shifted to 70ºC coupled with substantial increase in enzyme activity (Figure 7.2). The crude enzyme remained stable up to 4M NaCl for 120min even at 80°C by substantial loss in activity at higher salt concentrations. However, partially purified enzyme lost almost 50% of the original activity at 4M NaCl and 80°C temperature (Figure 7.3).

7.3.4.2 Effect of NaCl on temperature optima and stability of OK-5 protease

In OK-5, it is apparent from the Figure 7.4 that salt did not have any significant effect on the temperature optima at lower concentrations however at higher concentration optimum temperature of enzyme shifts from 70°C to 80°C reveling halophilic nature of the enzyme. It means that salt and temperature exhibited cumulative effect on the activity of both of the enzymes. Crude enzyme retained stability up to 6M NaCl (w/v) even at 80°C temperature. However, in partially purified protease the loss was more substantial at higher salt concentrations and temperature as enzyme lost more than 50% of the original activity after 10min of incubation at 80°C temperature (Figure 7.5).

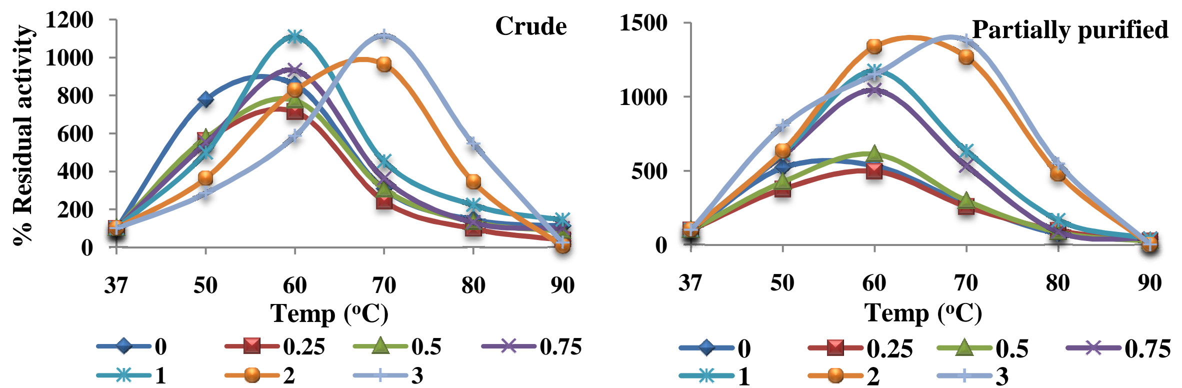


Figure 7.2: Effect of NaCl on temperature optima of crude and partially purified alkaline proteases from actinomycetes strain OK-6

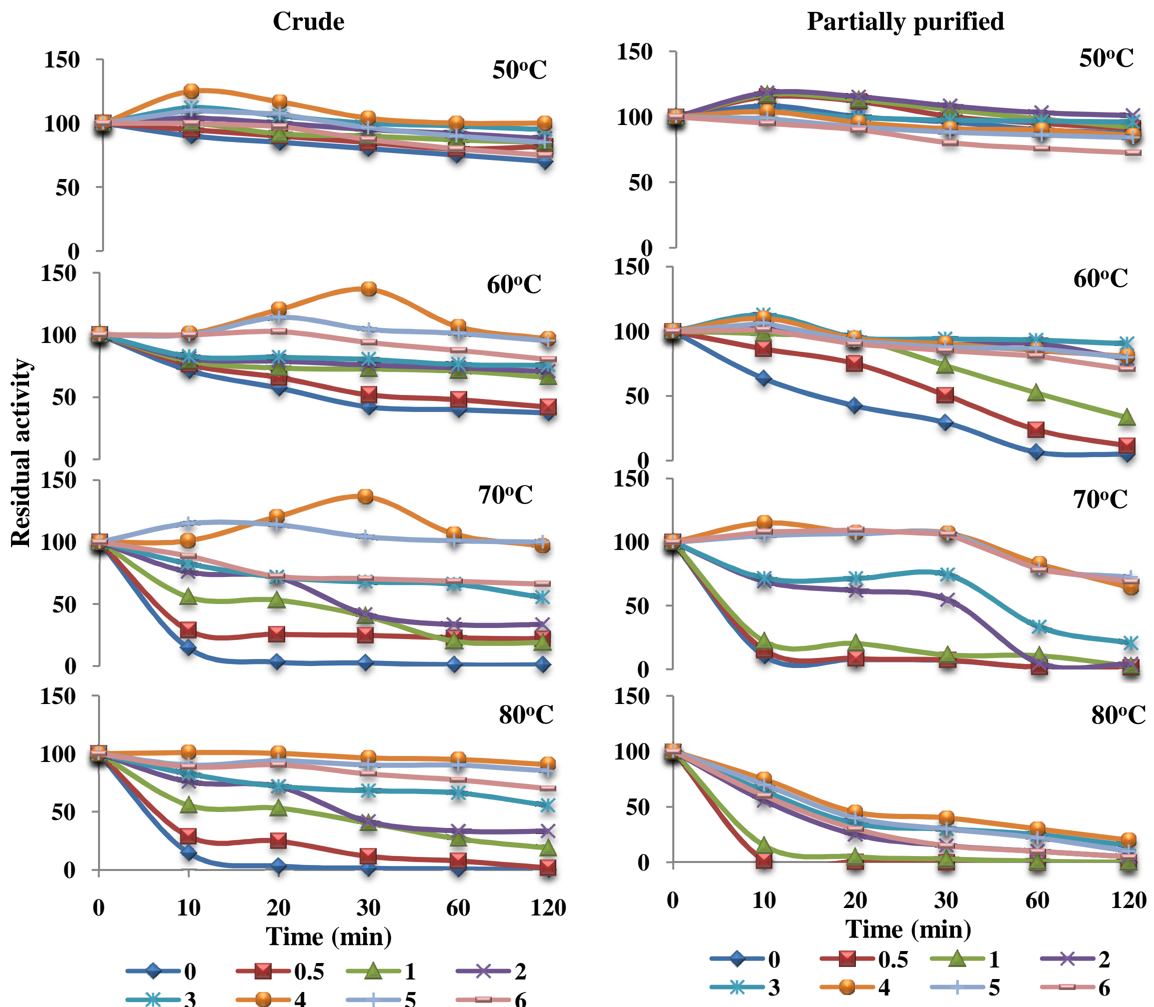


Figure 7.3: Stability of crude and partially purified alkaline proteases of OM-6 in presence of 0-6M NaCl up to 2h

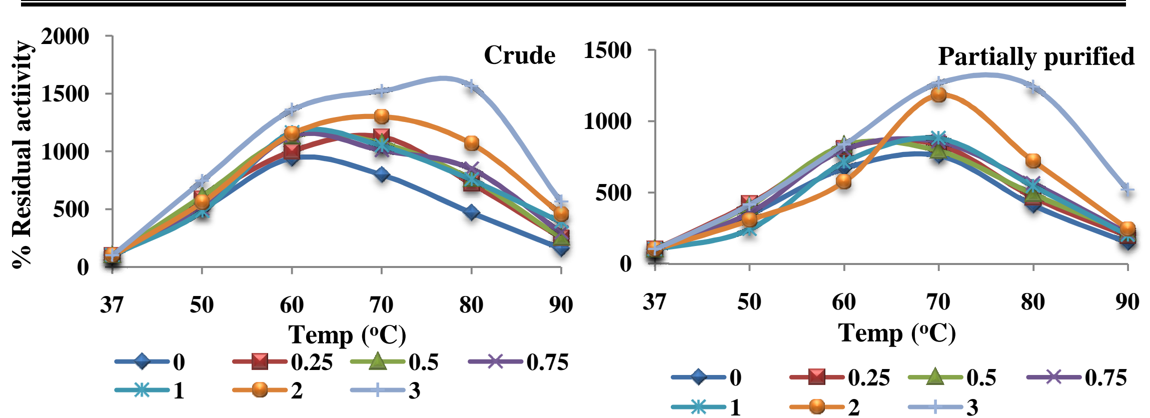


Figure 7.4: Effect of NaCl on temperature optima of crude and partially purified alkaline proteases from actinomycetes strain OK-5

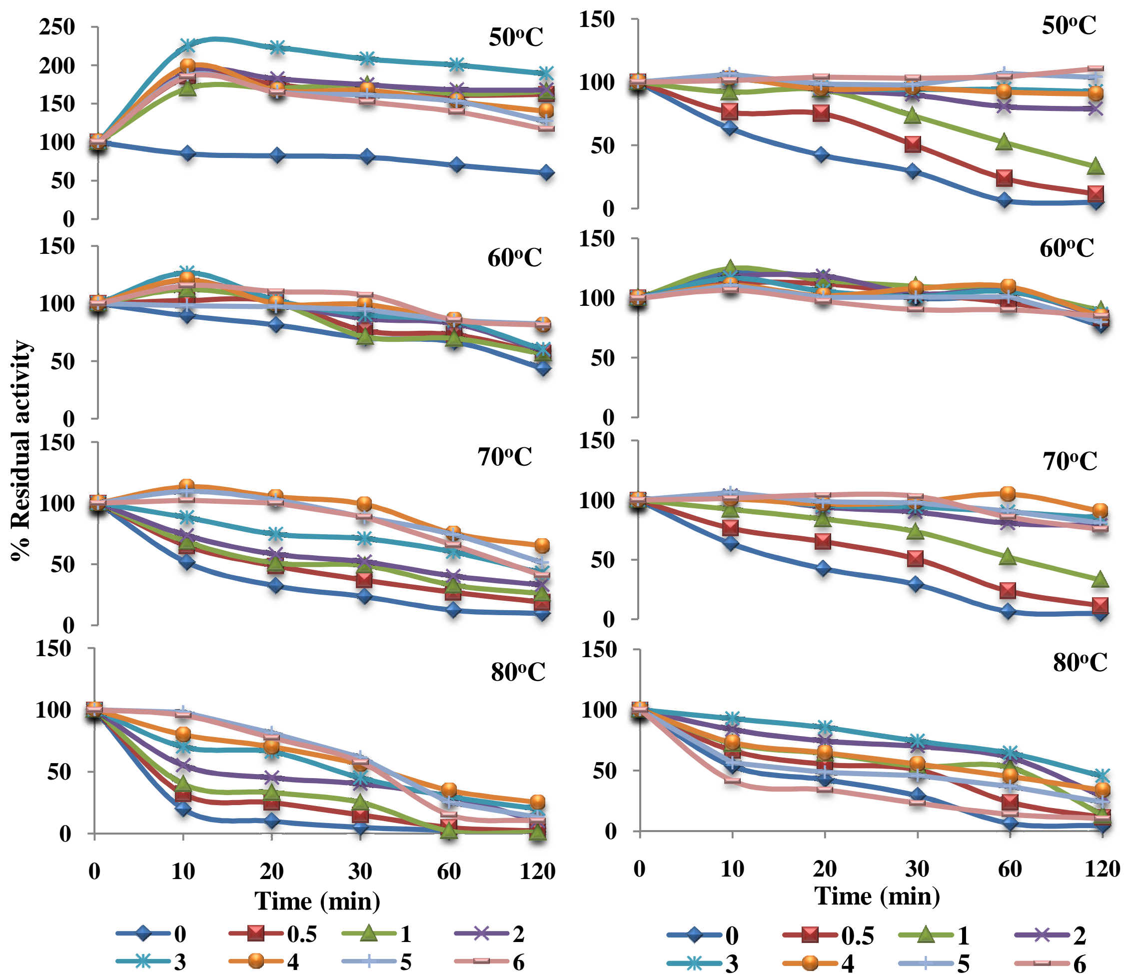


Figure 7.5: Stability of crude and partially purified alkaline proteases of OK-5 in presence of 0-6M NaCl up to 2h

7.3.5 Characterization of purified protease from OM-6 and OK-5

7.3.5.1 Characterization of OM-6 and OK-5 protease in presence of NaCl and Na-glutamate

7.3.5.1.1 Temperature optima and protease activity

The effect of temperature on caseinolytic activity of the purified enzyme from both OM-6 and OK-5 was assessed in presence of 0-2M NaCl and 30% Na-glutamate at pH 10. Interestingly, temperature optima shifted from 60°C to 70°C in presence of 4M NaCl and 30% Na-glutamate and % maximum activity increased more than two fold. Similarly, in *Nocardiopsis alba* strain OK-5 temperature optima shifted from 70°C to 80°C in presence of 4M NaCl and 30% Na-glutamate and % maximum activity increased more than two fold. These trends revealed halophilic and thermophilic nature of the extracellular protease from both the strains. An interesting feature that emerged was the NaCl and Na-glutamate dependences of the enzyme for its optimal activity at higher temperatures in both OM-6 and OK-5 isolates (Figure 7.6).

7.3.5.1.2 Thermal stability and deactivation rate constants for protease

Deactivation rates and half-lives of purified protease from both OM-6 and OK-5 isolates were calculated at 37 - 80°C in presence of 0M, 2M and 4M NaCl and 30% Na-glutamate (Figure 7.7). The deactivation rate constant (K_d) increased and half-life ($t_{1/2}$) decreased with increasing temperature. Highest stability of unified enzyme was observed in Na-glutamate followed by 4M NaCl, 2M NaCl and 0M NaCl in both OM-6 and OK-5. While studying purified protease from OM-6, the enzyme was least stable in the absence of NaCl. This was also apparent from the K_d and $t_{1/2}$ values of protease at 60°C in presence of 30% Na-glutamate (K_d =1.14×10^{-3}; $t_{1/2}$ = 608.02), 4M NaCl (K_d =1.68×10^{-3}; $t_{1/2}$ = 412.58), 2M NaCl (K_d =10.92×10^{-3}; $t_{1/2}$ = 63.47) and 0M NaCl (K_d =27.51×10^{-3}; $t_{1/2}$ = 25.19). The enzyme was stable even at 80°C in presence of 30% Na-glutamate with K_d = 8.66 and $t_{1/2}$ = 80.04 min. In OK-5, highest stability of the enzyme was observed in Na-glutamate followed by 4M NaCl, 2M NaCl and 0M NaCl. This was apparent from the K_d and $t_{1/2}$ values of protease at 60°C in

presence of 30% Na-glutamate (K_d =0.77×10^{-3}; $t_{1/2}$ = 900.19), 4M NaCl (K_d =1.69×10^{-3}; $t_{1/2}$ = 433), 2M NaCl (K_d =2.37×10^{-3}; $t_{1/2}$ = 292.46) and 0M NaCl (K_d =19.15×10^{-3}; $t_{1/2}$ = 36.19). The enzyme was stable even at 80°C in presence of 30% Na-glutamate with K_d = 4.11 and $t_{1/2}$ = 168.64 min (Table 7.2).

7.3.5.1.3 Thermodynamic parameters for protease deactivation

Thermodynamic properties of enzyme are necessary to understand its behavior under different physiological conditions (Table 7.3). In OM-6, the activation energies (E) for protease deactivation calculated in presence of Na-glutamate, 4 M NaCl, 2 M NaCl and 0 M NaCl were 37.14 KJ/mole, 44.01 KJ/mole, 99.57 KJ/mole and 113.92 KJ/mole respectively. While Change in enthalpy (ΔH*) and entropy (ΔS*) for deactivation of protease in presence of 30% Na-glutamate, 4 M NaCl, 2 M NaCl and 0 M NaCl were 34.47 KJ/mole, -196.37 J/mole; 41.34 KJ/mole, -175.17 J/mole; 96.91 KJ/mole, 6.48 J/mole; 111.26 KJ/mole, 57.03 J/mole respectively. The change in free energy (ΔG*) for protease deactivation at 60°C temperature in presence of 30% Na-glutamate, 4 M NaCl, 2 M NaCl and 0 M NaCl was 101.62 KJ/mole, 99.54 KJ/mole, 94.36 KJ/mole, 91.80 KJ/mole respectively. Similarly, in OK-5, the activation energies (E) for protease deactivation calculated in presence of Na-glutamate, 4 M NaCl, 2 M NaCl and 0 M NaCl were 31.97 KJ/mole, 47.66 KJ/mole, 65.34 KJ/mole and 74.08 KJ/mole respectively. While Change in enthalpy (ΔH*) and entropy (ΔS*) for deactivation of protease in presence of 30% Na-glutamate, 4 M NaCl, 2 M NaCl and 0 M NaCl were 29.23 KJ/mole, -211.83 J/mole; 44.91 KJ/mole, -160.12 J/mole; 62.59 KJ/mole, -152.72 J/mole; 71.33 KJ/mole, -69.99 J/mole respectively. The change in free energy (ΔG*) for protease deactivation at 60°C temperature in presence of 30% Na-glutamate, 4 M NaCl, 2 M NaCl and 0 M NaCl was 101.70 KJ/mole, 99.68 KJ/mole, 98.59 KJ/mole, 92.81 KJ/mole respectively (Table 7.4).

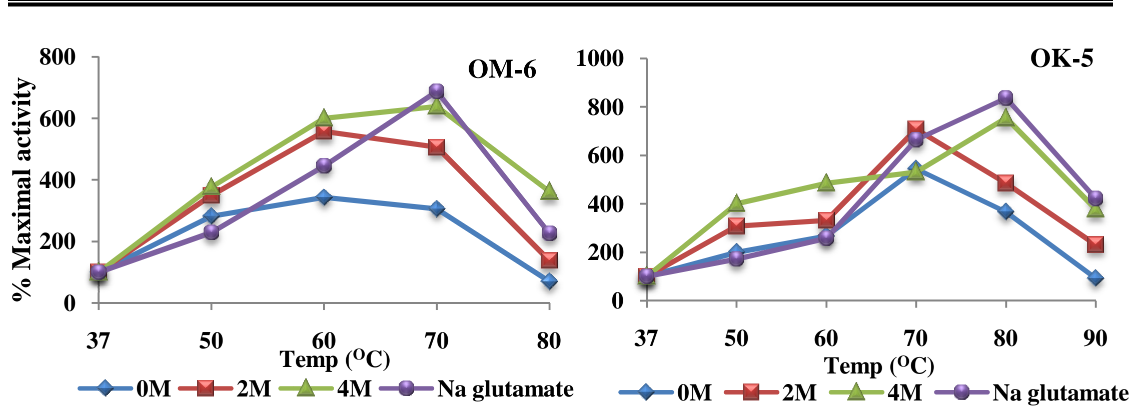


Figure 7.6: Effect of 0 -4M NaCl and 30% Na-glutamate on temperature optima and enzyme activity of purified protease from OM-6 and OK-5

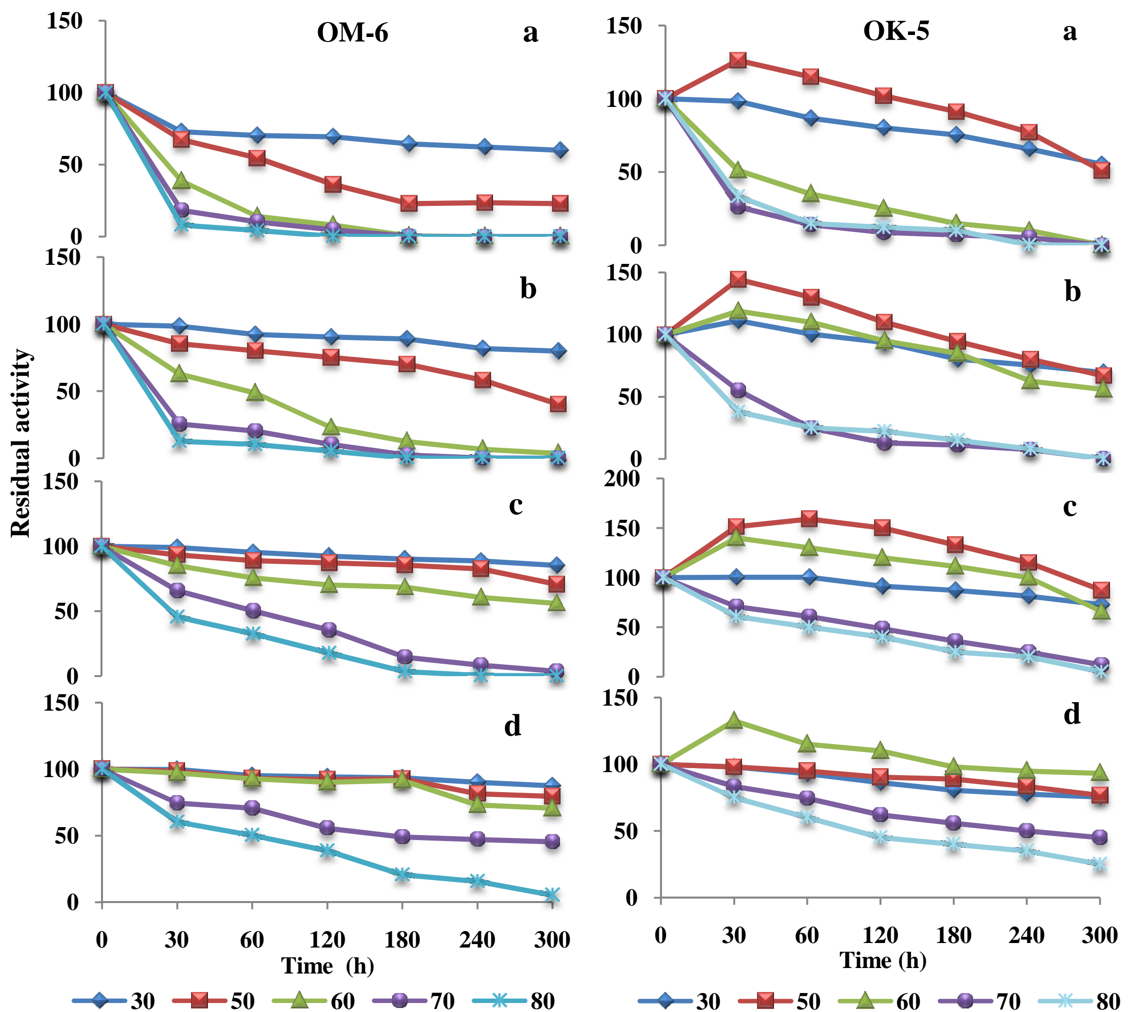


Figure 7.7: Thermal stability of OM-6 and OK-5 purified proteases in presence of (a) 0 M NaCl, (b) 2 M NaCl, (c) 4 M NaCl and (d) 30% Na-glutamate at 37°C - 80°C temperature

Isolates	T (°C)	0 M NaCl		2 M NaCl		4 M NaCl		30% Na-glutamate	
		K_d (×10^{-3})	$t_{1/2}$ (min)	K_d (×10^{-3})	$t_{1/2}$ (min)	K_d (×10^{-3})	$t_{1/2}$ (min)	K_d (×10^{-3})	$t_{1/2}$ (min)
OM-6	37	1.24	558.98	0.73	949.51	0.51	1359.11	0.42	1650.35
	50	4.94	140.31	2.52	274.62	0.91	761.70	0.73	949.51
	60	27.51	25.19	10.92	63.47	1.68	412.58	1.14	608.02
	70	35.86	19.32	27.29	25.39	10.75	64.47	2.41	287.61
	80	41.09	16.86	33.30	20.81	28.50	28.50	8.66	80.04
OK-5	37	1.55	447.19	1.47	471.52	1.08	641.80	0.99	700.14
	50	2.30	301.36	2.01	344.84	1.20	577.62	0.826	839.16
	60	19.15	36.19	2.37	292.46	1.69	433.21	0.77	900.19
	70	22.4	30.94	18.38	37.71	6.23	111.25	2.50	277.25
	80	37.2	18.63	24.31	28.51	8.29	83.61	4.11	168.64

Table 7.2: Deactivation rate constant (K_d) and Half life ($t_{1/2}$) of OM-6 and OK-5 purified proteases in presence of 0 M NaCl, 2 M NaCl, 4 M NaCl and 30% Na-glutamate at the range of 37°C-80°C temperature

Isolates	OM-6			OK-5		
Treatment	ΔH* (KJ/mole)	ΔS* (J/mole)	E (KJ/mole)	ΔH* (KJ/mole)	ΔS* (J/mole)	E (KJ/mole)
0 M NaCl	111.26	57.03	113.92	71.33	-69.99	74.08
2 M NaCl	96.91	6.48	99.57	62.59	-152.72	65.34
4 M NaCl	41.34	-175.17	44.01	44.91	-160.12	47.66
30% Na-glutamate	34.47	-196.37	37.14	29.23	-211.83	31.97

Table 7.3: Values of ΔH*, ΔS* and activation energy for OM-6 and OK-5 proteases deactivation in presence of 0 M NaCl, 2 M NaCl, 4 M NaCl and 30% Na-glutamate at the range of 37°C-60°C temperature

Strains	OM-6				OK-5			
T (^oC)	ΔG* (KJ/mole) for deactivation of protease				ΔG* (KJ/mole) for deactivation of protease			
	0 M NaCl	2 M NaCl	4 M NaCl	30% Na-glutamate	0 M NaCl	2 M NaCl	4 M NaCl	30% Na-glutamate
37	93.27	94.63	95.56	96.06	92.69	92.83	93.62	93.85
50	93.58	94.38	98.12	98.71	95.63	95.99	97.38	98.38
60	91.80	94.36	99.54	101.62	92.81	98.59	99.68	101.70
70	93.89	94.67	97.32	101.59	95.23	95.79	98.88	101.48
80	96.31	96.93	99.85	100.88	96.60	97.85	101.01	103.07

Table 7.4: Values of ΔG* for deactivation of OM-6 and OK-5 proteases in presence of 0 M NaCl, 2 M NaCl, 4 M NaCl and 30% Na-glutamate at the range of 37°C-80°C temperature

7.3.5.2 Characterization of OM-6 and OK-5 purified protease in presence of Ca^{2+}

7.3.5.2.1 Temperature optima and protease activity

The effect of temperature on caseinolytic activity of the purified enzyme was determined in presence of 0M - 3M NaCl and 0 - 100 mM Ca^{2+} at pH 10. An interesting feature that emerged was the NaCl and Ca^{2+} dependences of the enzyme for its optimal activity at higher temperatures in both OM-6 and OK-5. The temperature optima of purified protease from OM-6 was 60°C at the entire concentration of NaCl and Ca^{2+} studied with optimum % increase in activity up to 555% and 455% at 3M NaCl and 100mM Ca^{2+} respectively while retained up to 100% and 55% of original activity even at 90°C up to 3M NaCl and 100mM Ca^{2+} respectively. In OK-5, the temperature optima of purified protease was 70°C at the entire concentration of NaCl and Ca^{2+} studied with optimum % increase in activity up to 650% and 450% at 3M NaCl and 100mM Ca^{2+} respectively while retained up to 500% and 217% of original activity even at 90°C up to 3M NaCl and 100mM Ca^{2+} respectively (Figure 7.8).

7.3.5.2.2 Thermal stability and deactivation rate constants for protease

Deactivation rates and half-lives of purified protease from both OM-6 and OK-5 isolates were calculated at 37°C to 80°C in presence of 0-200mM Ca^{+2} (Figure 7.9). The deactivation rate constant (K_d) increased and half-life ($t_{1/2}$) decreased with increasing temperature (Table 7.5). In OM-6, highest stability of enzyme was observed in 100mM Ca^{2+} at 50°C. This was apparent from the K_d and $t_{1/2}$ values of protease at 50°C in presence of 100mM Ca^{2+} (K_d =1.09×10^{-3}; $t_{1/2}$ = 635.91) and 0mM Ca^{2+} (K_d =7.60×10^{-3}; $t_{1/2}$ = 91.20). The enzyme was stable even at 70°C in presence of 100mM $Ca^{2+}$with K_d = 20×10^{-3}and $t_{1/2}$ = 34 min. Similarly, in OK-5, K_d and $t_{1/2}$ values of purified protease at 50°C in presence of 25mM Ca^{2+} was 0.53×10^{-3} and 1307.82min respectively while in absence Ca^{2+} K_d = 2.17×10^{-3} and $t_{1/2}$ = 319.42. The enzyme was stable even at 80°C in 100mM $Ca^{2+}$with K_d = 22×10^{-3}and $t_{1/2}$ = 31.50 min.

7.3.5.2.3 Thermodynamic parameters for protease deactivation

The activation energy (E) for protease deactivation calculated in presence of 200mM Ca^{2+} was 29.35 KJ/mole in OM-6. While Change in enthalpy (ΔH*) and entropy (ΔS*) for deactivation of protease in presence of 200mM Ca^{2+} were 26.68 KJ/mole and -186.22 J/mole respectively. The change in free energy (ΔG*) for protease deactivation at 60°C temperature in 200mM Ca^{2+} was 95.10 KJ/mole (Table 7.6). Similarly, in OK-5, the activation energy (E) for protease deactivation calculated in presence of 200mM Ca^{2+} was 38.15 KJ/mole. While Change in enthalpy (ΔH*) and entropy (ΔS*) for deactivation of protease in presence of 200mM Ca^{2+} were 35.49 KJ/mole and -183.48 J/mole respectively. The change in free energy (ΔG*) for protease deactivation at 60°C temperature in presence of 200mM Ca^{2+} was 95.88 KJ/mole (Table 7.7). Low values of ΔH* and ΔS* revealed a higher thermal stability of protease in presence 200mM Ca^{2+}. Moreover, negative values of ΔS* designate ordered transition state of protease.

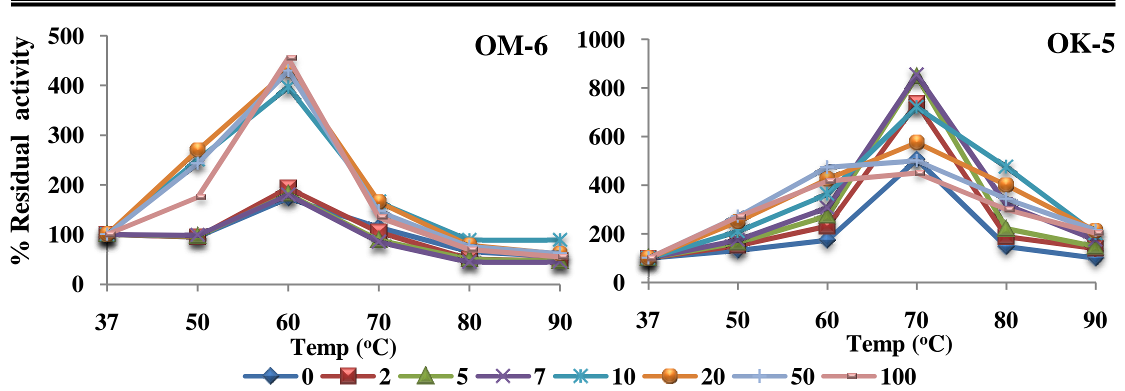


Figure 7.8: Effect of Ca^{2+} on temperature optima and enzyme activity of purified proteases from both OM-6 and OK-5 isolates at 0 - 100mM concentration

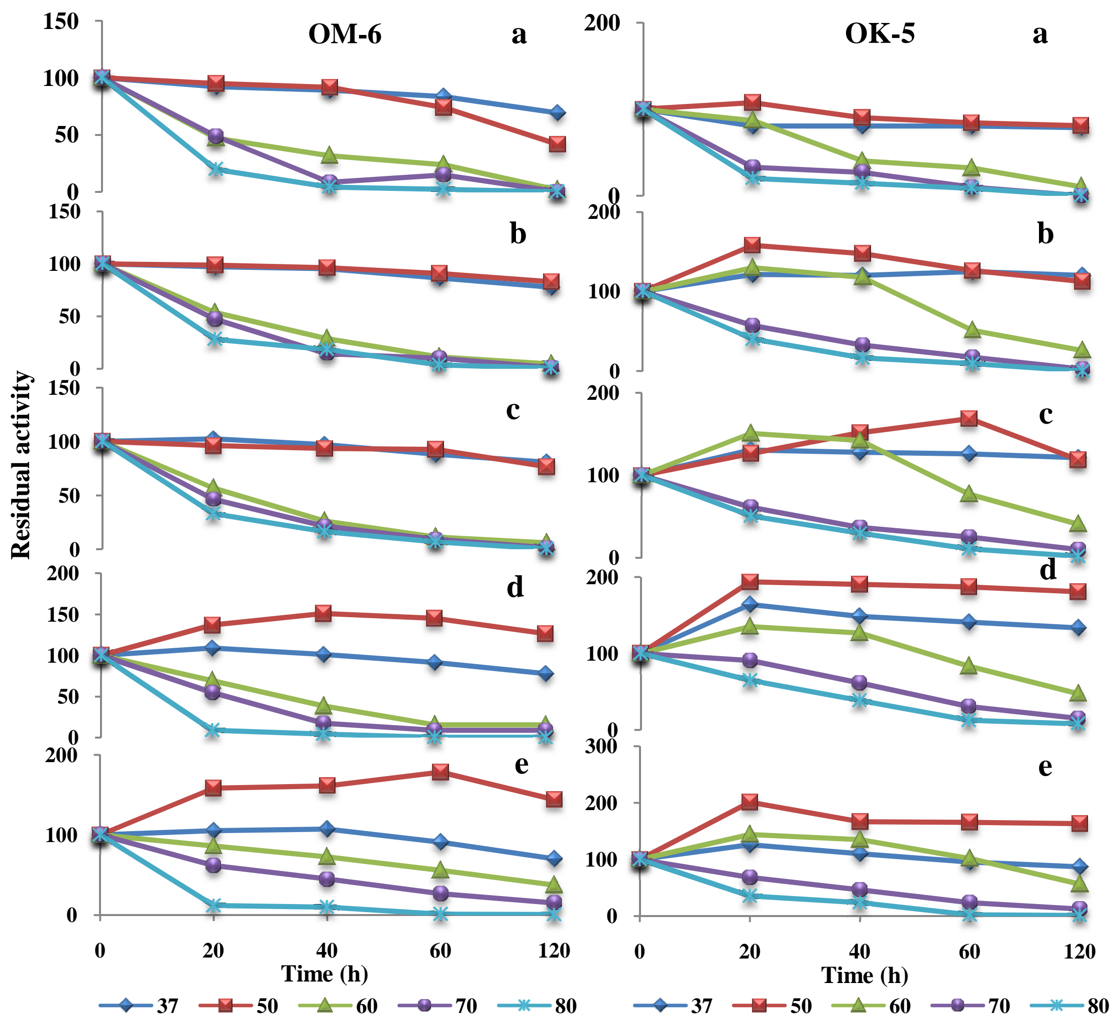


Figure 7.9: Stability of purified protease from both OM-6 and OK-5 isolates in presence of Ca^{2+} at 37°C - 80°C temperature at (a) 0Mm, (b) 25mM, (c) 50Mm, (d)100Mm and (e) 200Mm Ca^{2+}

Isolates	T (°C)	0 Mm Ca^{++}		25 Mm Ca^{++}		50 Mm Ca^{++}		100 Mm Ca^{++}		200 Mm Ca^{++}	
		K_d ($\times10^{-3}$)	$t_{1/2}$ (min)	K_d ($\times10^{-3}$)	$t_{1/2}$ (min)	K_d ($\times10^{-3}$)	$t_{1/2}$ (min)	K_d ($\times10^{-3}$)	$t_{1/2}$ (min)	K_d ($\times10^{-3}$)	$t_{1/2}$ (min)
OM-6	37	3.00	231.04	1.66	417.55	2.00	346.57	2.56	270.76	3.38	205.07
	50	7.60	91.20	2.20	315.06	2.18	317.95	1.09	635.91	1.95	355.46
	60	33.20	20.87	26.31	26.34	23.70	29.24	16.41	42.23	8.35	83.01
	70	49.00	14.14	37.03	18.71	35.00	19.80	20.45	33.89	20.40	33.97
	80	68.90	10.06	41.92	16.53	43.50	15.93	48.05	14.42	48.05	14.44
OK-5	37	1.40	495.10	1.00	693.14	0.85	815.46	0.93	745.31	2.11	328.50
	50	2.17	319.42	0.53	1307.82	1.03	673.61	3.12	222.16	2.08	333.24
	60	19.50	35.54	13.40	51.72	9.60	72.20	7.81	88.75	6.30	110.02
	70	57.00	12.16	31.10	22.28	18.90	36.67	17.00	40.77	18.00	38.50
	80	74.50	9.30	57.30	12.09	32.90	21.06	22.00	31.50	39.90	17.37

Table 7.5: Deactivation rate constant (K_d) and Half life ($t_{1/2}$) of purified protease in presence of 0 mM, 25 mM, 50 mM, 100 mM, and 200 mM Ca^{++} at the range of 37°C-80°C temperature

Isolates	OM-6			OK-5		
Ca^{++} (Mm)	ΔH* (KJ/mole)	ΔS* (J/mole)	E (KJ/mole)	ΔH* (KJ/mole)	ΔS* (J/mole)	E (KJ/mole)
0	85.11	-20.11	87.78	91.14	-8.39	93.81
25	95.02	4.99	99.57	85.41	-32.08	88.08
50	84.06	-175.17	86.73	82.41	-39.73	85.48
100	58.76	-109.74	61.43	76.61	-56.19	79.28
200	26.68	-186.22	29.35	35.49	-183.48	38.15

Table 7.6: Values of ΔH*, ΔS* and activation energy for protease deactivation in presence of 0 mM, 25 mM, 50 mM, 100 mM, and 200 mM Ca^{++}at the range of 37°C-60°C temperature

Strains	OM-6				OK-5			
T (^{o}C)	ΔG* (KJ/mole) for deactivation of protease				ΔG* (KJ/mole) for deactivation of protease			
	0 Mm Ca^{++}	25 Mm Ca^{++}	50 Mm Ca^{++}	100 Mm Ca^{++}	0 Mm Ca^{++}	25 Mm Ca^{++}	50 Mm Ca^{++}	100 Mm Ca^{++}
37	90.99	92.51	92.03	91.40	92.95	93.82	94.24	94.01
50	92.42	95.75	95.77	97.63	95.78	99.57	97.79	94.81
60	91.28	91.93	92.22	93.23	92.76	93.79	94.72	95.29
70	93.00	93.80	93.96	95.49	92.57	94.29	95.71	96.02
80	94.79	96.25	96.14	95.85	94.56	95.33	96.96	98.14

Table 7.7: Values of ΔG* for deactivation of protease in presence of 0 mM, 25 mM, 50 mM, 100 mM, and 200 mM Ca^{++} at the range of 37°C-80°C temperature

7.3.5.3 pH optima and pH stability of OM-6 and OK-5 protease

7.3.5.3.1 pH optima and pH stability of partially purified protease

pH optima and stability of OM-6 and OK-5 partially purified enzyme was studied in the range of pH 5-13. The reduction in activity was more pronounced at the pH below 9. The enzyme was less stable under acidic pH when compared to that at alkaline pH (Figure 7.10). At pH 6 and 7, around 50% of the maximal activity was lost after 2 h of incubation, while at pH 11; the enzyme retained more than 90% of its original activity after 48 h in both OM-6 and OK-5.

7.3.5.3.2 pH optima and pH stability of purified protease

The effect of pH on caseinolytic activity of the purified enzyme was determined in the range of 6-13 pH using different buffer systems. However, glycine-NaOH buffer system exhibited good protease activity; highest activity was found with Borex-NaOH buffer system (pH range 10-11). Likewise maximum stability of enzyme was found at the same range of pH reflecting alkaliphilic nature of the extracellular protease from the strains. Both of alkaline proteases were stable at broad range of pH from 6-11 even after 24 h reflecting alkaliphilic nature of the enzyme. The results clearly indicated the higher stability of pure enzyme when compared with partially purified enzyme in a broader range of pH (Figure 7.11).

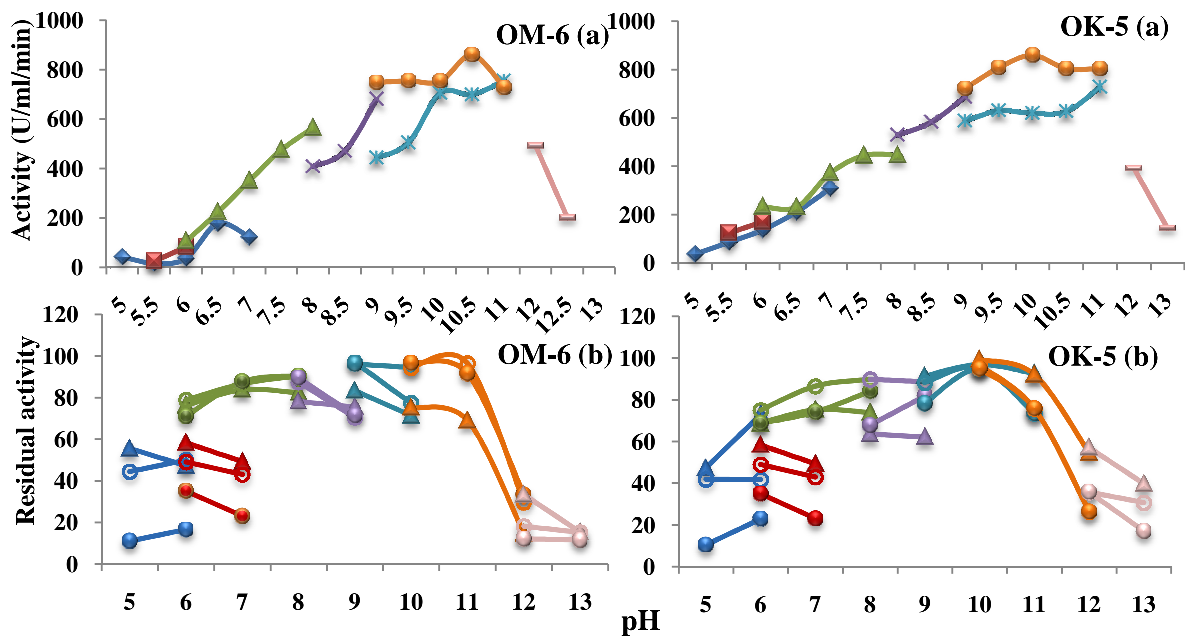


Figure 7.10: (a) pH optima and (b) pH stability of partially purified alkaline protease from OM-6 and OK-5 after 2 h (triangle), 24 h (open circle) and 48 h (close circle) of incubation. The buffers used were Citrate phosphate buffer (pH 5-6), Succinate buffer (pH 6-7), Sodium phosphate (pH 6-8), Tris-HCl (pH 8-9), Glycine-NaOH (pH 9-11), Borex-NaOH (pH 10-12) and KCl-NaOH (pH 12-13)

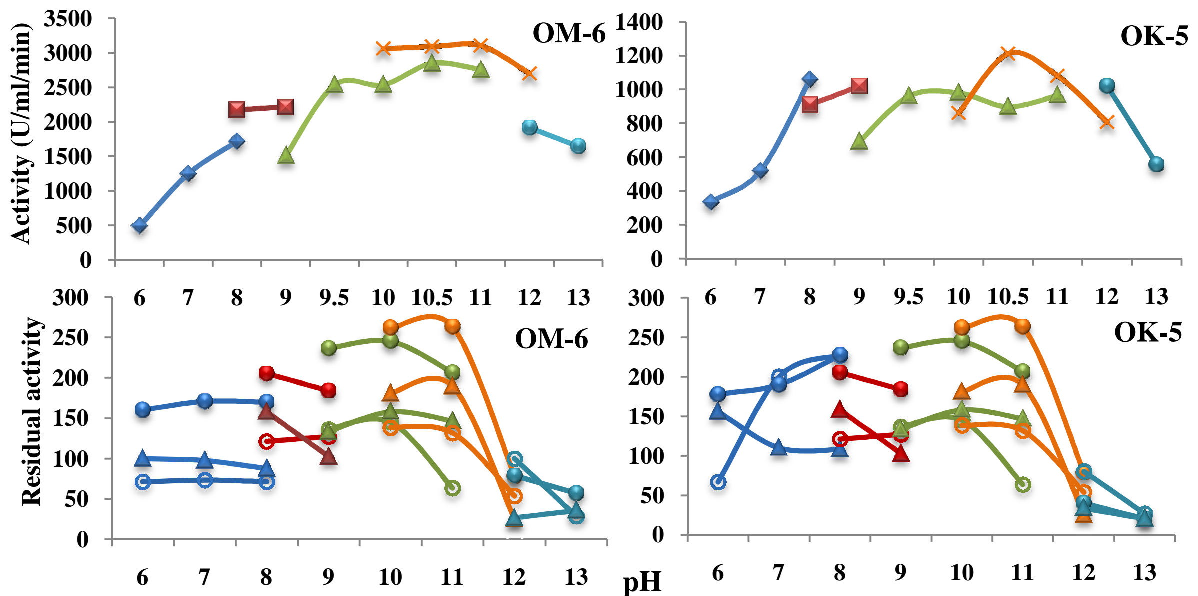


Figure 7.11: (a) pH optima and (b) pH stability of purified alkaline protease from OM-6 and OK-5after 1 h (open circle), 6 h (close circle) and 24 h (triangle) of incubation. The buffers used were Sodium phosphate (pH 6-8), Tris-HCl (pH 8-9), Glycine-NaOH (pH 9-11), Borate (pH 10-12) and KCl-NaOH (pH 12-13)

7.3.5.4 Effect of denaturing agents on activity and stability of purified proteases

Purified proteases from OM-6 and OK-5 were subjected to denaturation at 2M, 4M and 8M urea and guanidine hydrochloride (GH) (Figure 7.12). Purified enzyme was resistant to 8M urea and GH and retained almost 50% of original activity even after 1h of incubation at 60°C while at 2M and 4M concentration both enzymes retained more than 90% of initial activity in presence of urea.

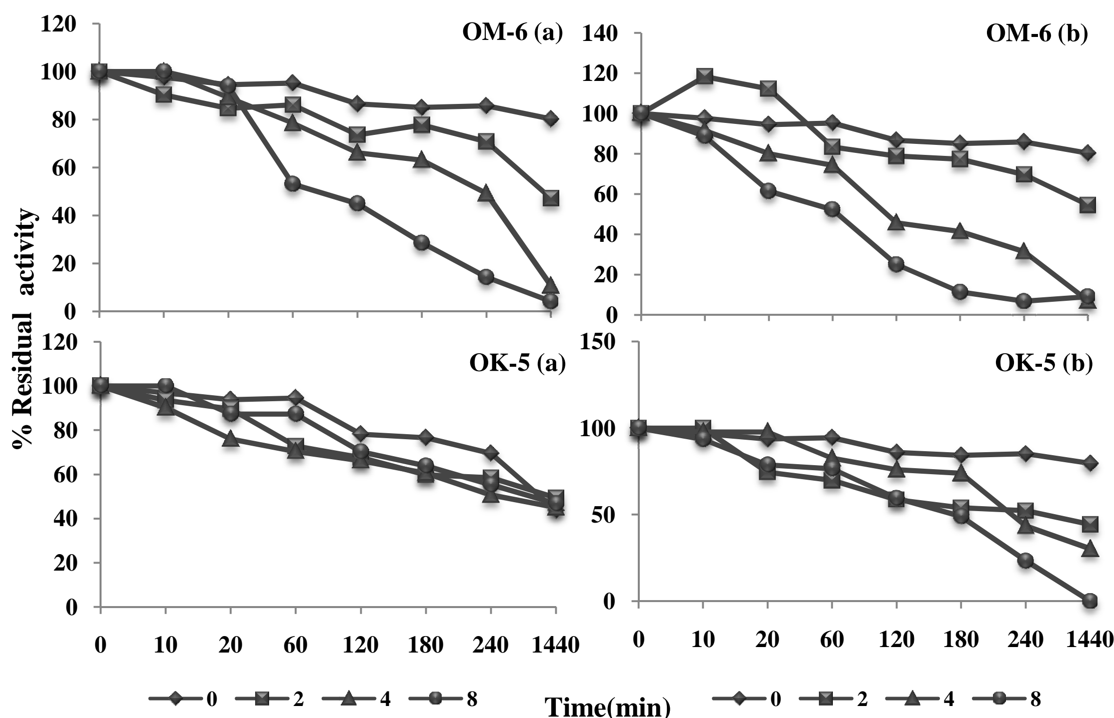


Figure 7.12: Denaturation of alkaline protease in presence of Urea (a) and Guanidine hydrochloride (b) at 0 M - 8 M concentration up to 5 h of incubation in OM-6 and OK-5

7.3.5.5 Hydrolysis of protein substrate

When assayed with native protein as substrates, protease from OM-6 and OK-5 gave 100% hydrolytic activity against casein whereas OM-6 displayed poor to moderate hydrolysis of BSA (68%), gelatin (62%), and egg albumin (30%) and OK-5 displayed BSA (66.66%), gelatin (58.09%), and egg albumin (15.23%).

7.3.5.6 Determination of K_m and V_{max}

The kinetic parameters (K_m and V_{max}) of both purified protease from OM-6 and OK-5 isolates, for the hydrolysis of casein at 60°C and pH 10 were determined by double reciprocal Lineweaver Burk plot. Values for K_m and V_{max} for OM-6 alkaline protease were 0.50 mg/ml and 3634.12 U/min respectively while alkaline protease from OK-5 displayed 0.18mg/ml and 2414.22 U/min values for both K_m and V_{max} respectively. The estimated K_m values indicated the affinity of enzyme towards the substrate. V_{max} is an indication of the catalytic activity of an enzyme which is usually desired to be as high as possible (Figure 7.13).

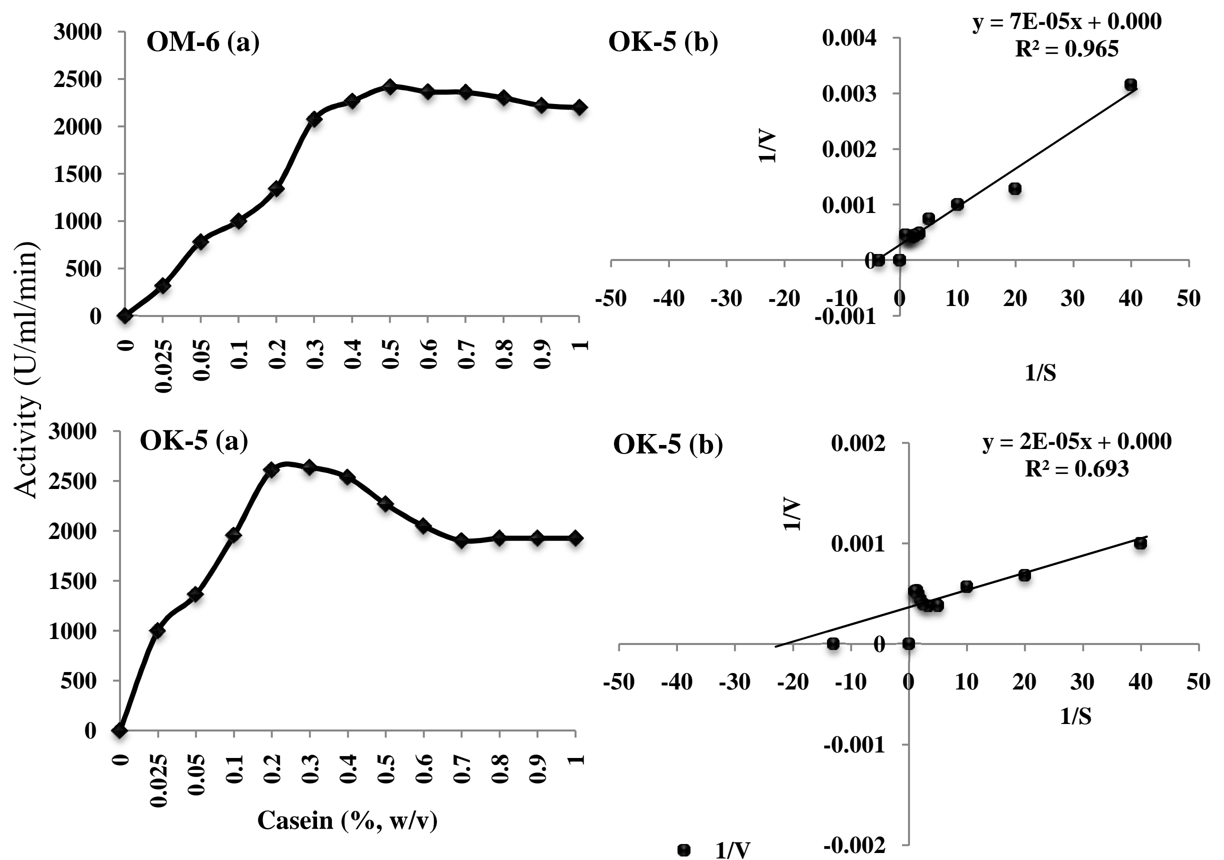


Figure 7.13: **(a)** Effect of substrate concentration on protease activity of OM-6 and OK-5 isolates and **(b)** the reciprocal Lineweaver – Burk plot of OM-6 and OK-5 protease

7.3.5.7 Effect of cations on stability of purified alkaline serine protease

Cations generally protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzymes at high temperatures. The effects of cations on the stability of purified protease from OM-6 are summarized in Figure 7.14. Stimulatory effects were observed for almost all cations used at 100mM concentration up to 3 h of incubation. Stimulatory effects and nearly no effects on the enzyme activity were found for Mg^{2+} and Ca^{2+} even after 24 h of incubation with 100mM concentration at 40°C. While studying effect of cations on the stability of purified protease from OK-5, stimulatory effects were observed for Mn^{+} and Ca^{2+} at 500mM concentration up to 24h of incubation at 40°C. However in presence of Zn^{+} stimulatory effect was seen at 0h while increasing Zn^{+} concentration up to 1000mM (Figure 7.15). However both of the enzymes were not stable in presence of Hg^{2+} at higher concentration suggesting the presence of reactive –SH groups in the active confirmation.

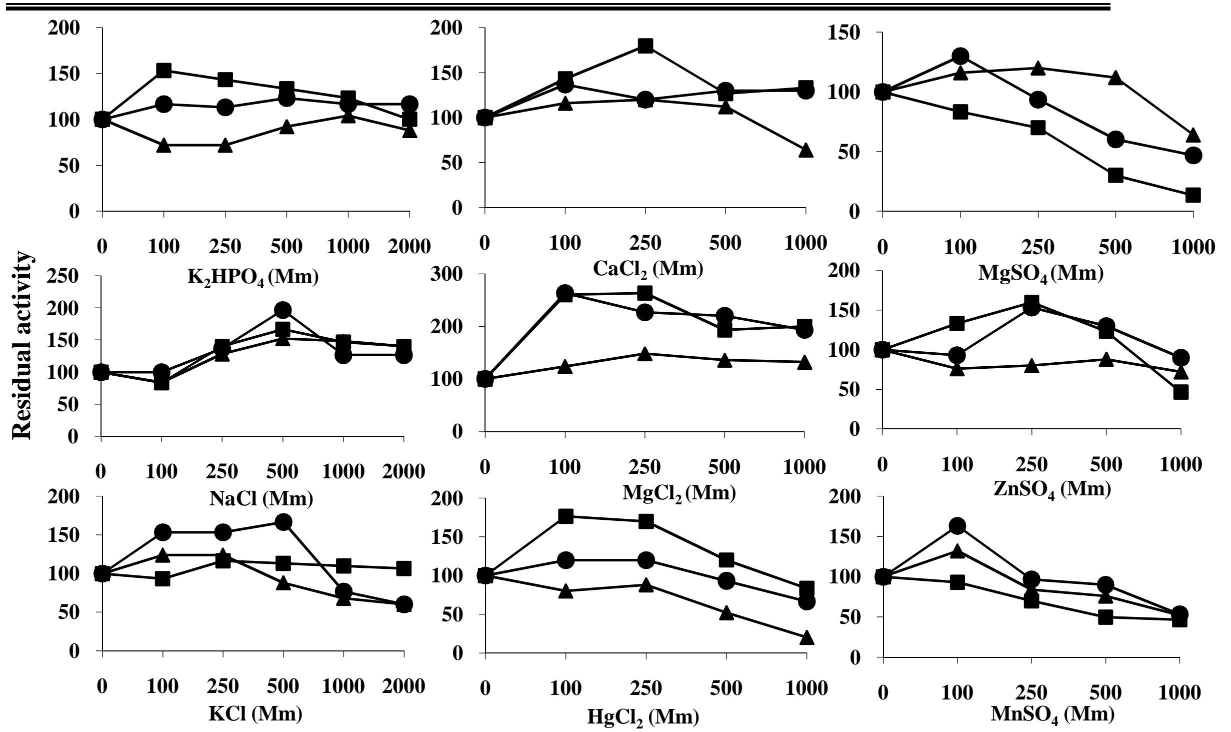


Figure 7.14: Stability of OM-6 purified protease in presence of cations and metal ions after 0 h (♦), 3 h (■) and 24 h (▲) of incubation.

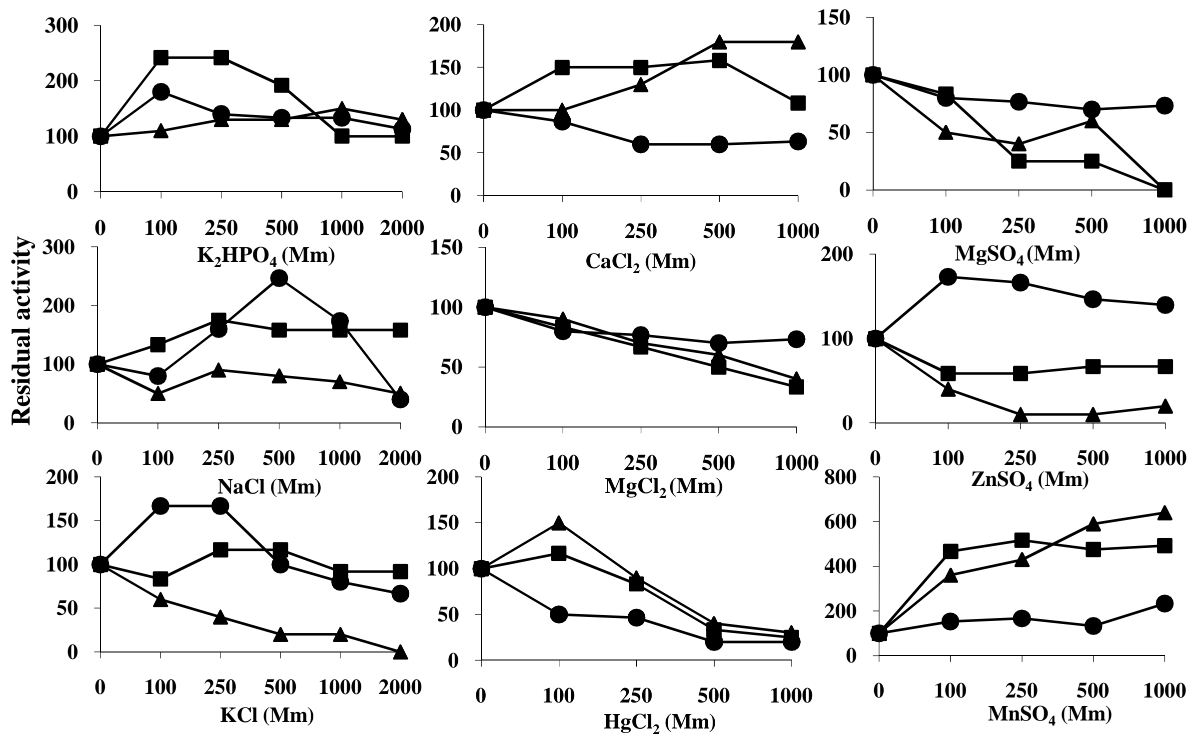


Figure 7.15: Stability of OM-6 purified protease in presence of cations and metal ions after 0 h (♦), 3 h (■) and 24 h (▲) of incubation

7.3.5.8 Effect of osmolytes, inhibitors, metal ions, oxidizing, reducing agents and surfactants on protease activity

The effect of different concentrations of various osmolytes: NaCl, KCl, glycerol, mannitol, trehalsoe, sucrose and Na-glutamate on the activity of purified enzyme in the absence of NaCl was examined (Figure 7.16a). In OM-6, maximum activity in glycerol was observed at 5%, while, the activity in presence of NaCl and KCl was comparable with control up to 30%. Among the osmolytes, Na-glutamate (30%) enhanced the activity by 84% whereas; sucrose (30%) maintained the activity without any enhancement. In OK-5, Na-glutamate (30%) enhanced the activity by 132.43% whereas; sucrose (20%), Trehalose (20%) and Glycerol (5%) enhanced the activity by 5%, 77% and 20%, respectively. The protease was completely inhibited by 10mM PMSF in OM-6 while activity reduced to 25% in OK-5 at the same concentration of PMSF, a serine protease inhibitor and there was no significant effect of EDTA and DTT in both enzymes, indicating that the enzymes were serine proteases (Figure 7.16b). While studying the effect of metal ions on protease activity of OM-6 and OK-5, the metal ions did not have any significant effect on enzyme activity except Cd^{2+}, Cu^{2+} and Hg^{2+}(Figure 7.16c). The effect of H_2O_2 and β-mercaptoethanol was studied on purified protease of OM-6 and OK-5 at 0-50mM concentrations. Interestingly, % maximal activity of OM-6 increased to 218.18% and 186.36% in presence of 10mM H_2O_2 and 5mM β-mercaptoethanol respectively. Similarly % maximal activity of protease from OK-5 increased to 232.43% and 175.67% even in presence of 50mM H_2O_2 and 10mM β-mercaptoethanol respectively (Figure 7.16d). The OM-6 protease also had high % maximal activity in different surfactants at 0.2% concentration; SDS (122.94%), CTAB (109.32), tween 80 (101.47) and triton X-100(137.05). Similarly, OK-5 also had high % maximal activity in different surfactants; SDS (155%), CTAB (134%), tween 80 (136 %) and triton X-100 (180 %) at 0.2% concentration (Figure 7.16e).

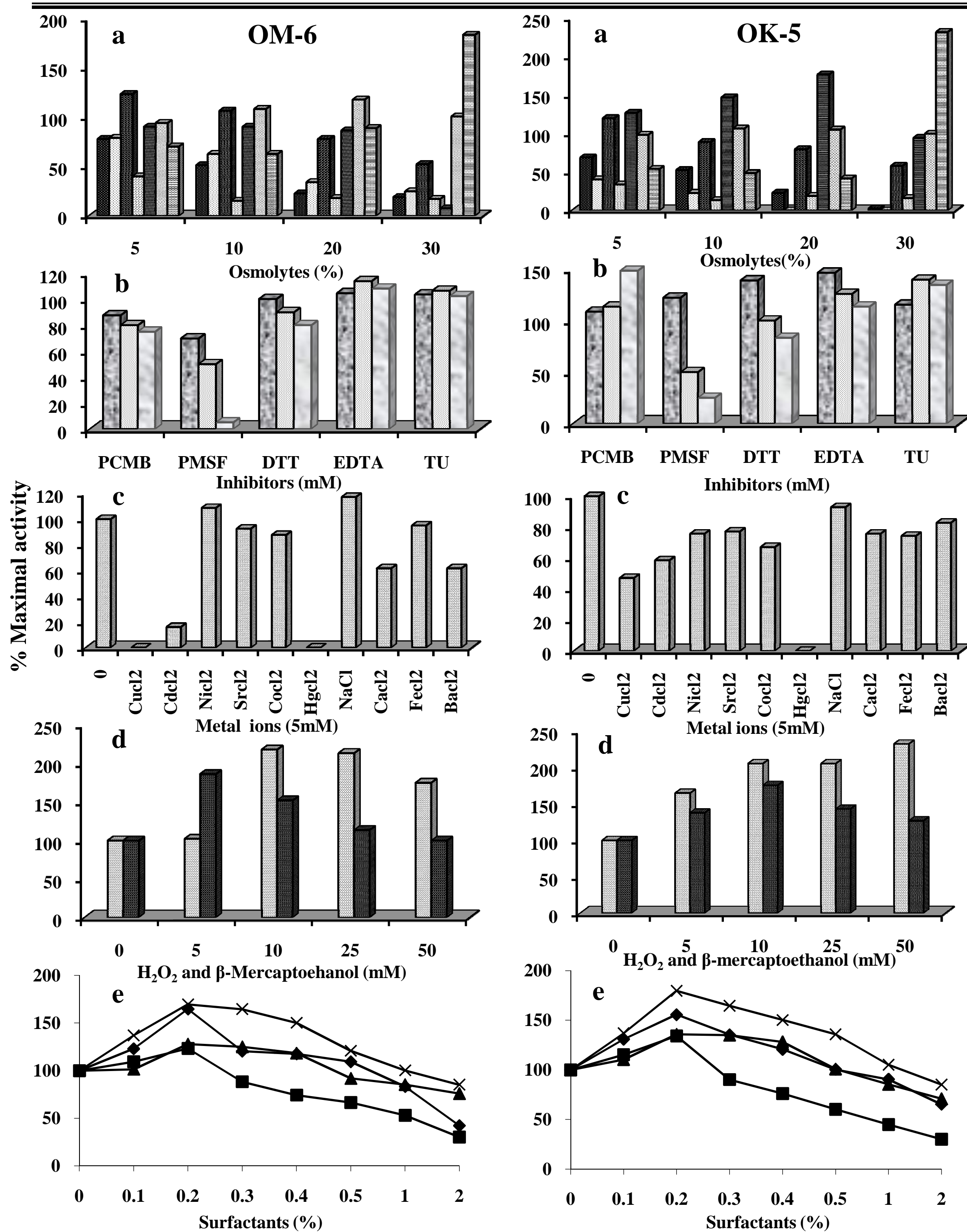


Figure 7.16: Effect of (a) Osmolytes: NaCl (▪), KCl (▫), Glycine (▪), Mannitol (▫), Trehalose (▪), Sucrose (▫) and Na-glutamate (▤); (b) Inhibiotors: at 1mM (▪), 5mM (▫), and 10mM (▫) concentration; (c) Metal ions: at 5mM (▫) concentration; (d): Oxidizing and reducing agents H_2O_2 (▫), β-Mercaptoehanol (▪) from 0-50 mM concentration; (e) Surfactants: SDS (♦), CTAB (■), Tween 80 (▲), Triton X-100 (✕) up to 2% concentration

7.3.5.9 Stability of purified alkaline serine protease in presence of surfactants, oxidizing agent, reducing agent and commercial detergents

Both OM-6 and OK-5 proteases displayed significant stability toward various surfactants. Enzymes were incubated at 40°C for 92 h with 0.5% and 1% surfactants (Figure 7.17a, 7.17b), protease from OM-6 exhibited enhanced residual activities up to 103%, 70%, 144% and 119% with SDS, CTAB, Tween 80 and Triton X-100 respectively up to 2 h of incubation while OK-5 protease exhibited residual activities up to 47%, 60%, 80% and 72% with SDS, CTAB, Tween 80 and Triton X-100 respectively up to 2 h of incubation. Both proteases were even active after 92 h of incubation in presence of 1% Tween 80, and Triton X-100. The enzyme retained stability and catalyzed the reaction with various commercial detergents. OM-6 alkaline protease conserved more than 60% of original activity with each detergent (1%) after 2 h (Figure 7.18a and 7.18b). Even after 24 h of incubation at 40°C enzyme was stable in presence of nirma and surf at both 1% and 10% concentration signifying the role in detergent industry. Similarly in case of OK-5, even after 24 h of incubation at 40°C, enzyme was stable in presence of aerial, tide and wheel at both 1% and 10% concentration. Effect of H_2O_2 and β-mercaptoethanol was studied on purified proteases of both OM-6 and OK-5 isolates at 0-50mM concentration and up to 24 h of incubation at 40°C. Interestingly, up to 2 h of incubation, residual activity of OM-6 protease increased by 450% and 559% in 50mM H_2O_2 and 10mM β-mercaptoethanol respectively. Moreover, even after 24 h, protease retained almost 100% of its original activity with both H_2O_2 and β-mercaptoethanol at highest concentration. Residual activity of OK-5 protease increased by 212.30% and 187.50% in presence of 25mM H_2O_2 and 50mM β-mercaptoethanol respectively and even after 24 h, protease retained almost 70% and 117% of its original activity at 50mM H_2O_2 and β-mercaptoethanol respectively (Figure 7.19).

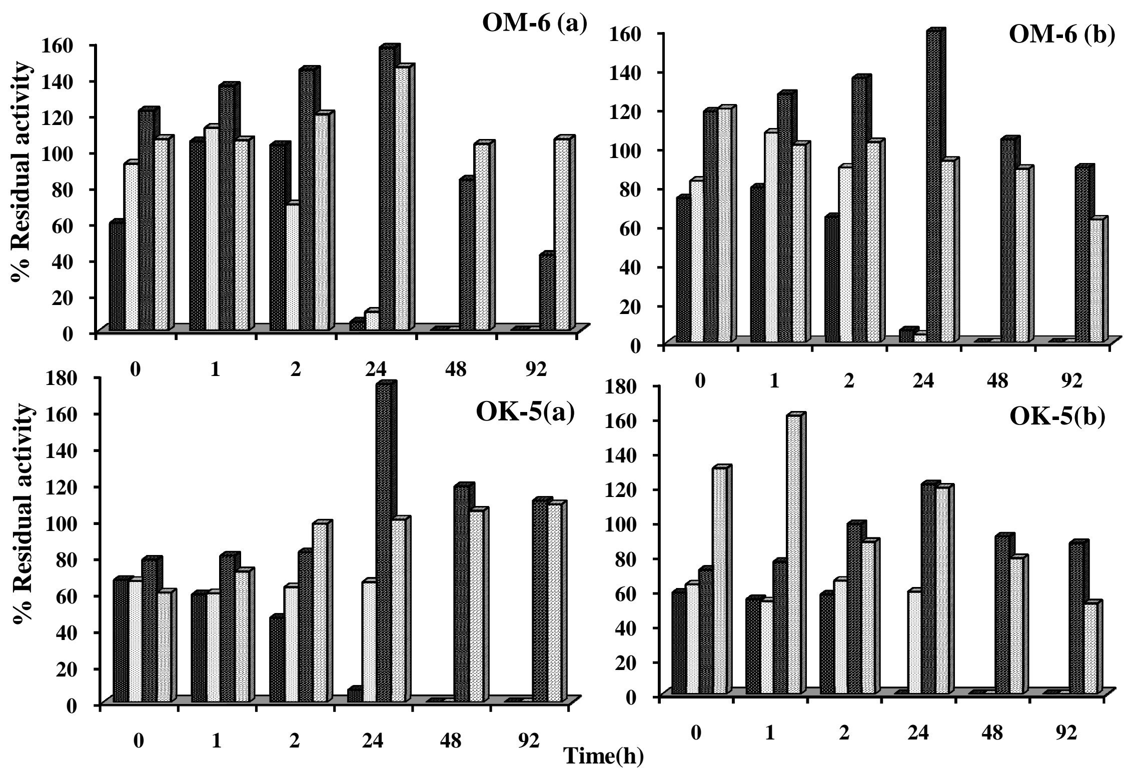


Figure 7.17: Stability of purified protease in presence of SDS (■), CTAB (□), Tween 80 (■) and Triton X-100 (□) up to 92 h of incubation at (a) 0.5% and (b) 1% concentration

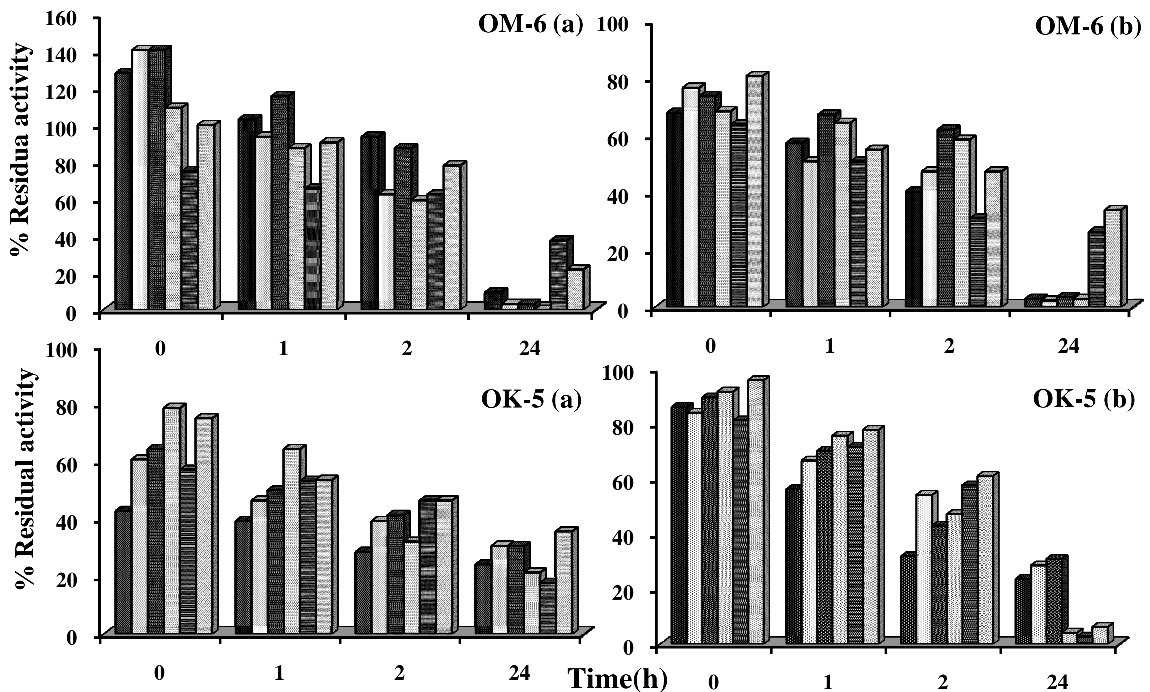


Figure 7.18: Stability of purified protease in presence of Aerial (■), Tide (□), Wheel 80 (■), Rin 80 (□), Surf 80 (■) and Nirma 80 (□) up to 24 h of incubation at (a) 1% and (b) 10% concentration

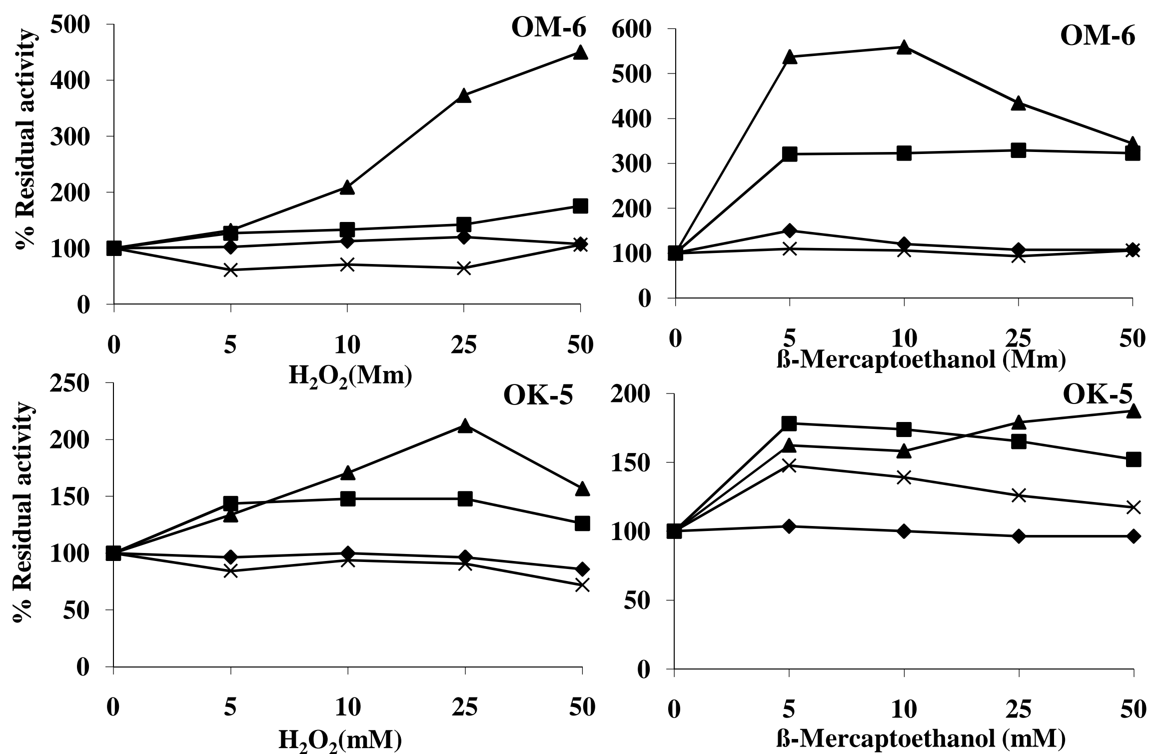


Figure 7.19: Stability of purified protease in presence of (a) H_2O_2 and (b) β-Mercaptoethanol after 0 h (♦), 1 h (■), 2 h (▲) and 24 h (×) of incubation

7.4 DISCUSSION

Alkaline Proteases are one of the largest groups of enzymes used commercially. Purification of extremozymes holds importance to understand cellular metabolism and regulatory pathways. As discussed in the previous chapters, the occurrence and properties of alkaline proteases from actinomyces is rarely reported in the literature. Each enzyme requires specific strategy for purification; it has become more important to develop novel strategies for the purification of individual extremozymes. Similarly, the characterization of the enzymes is important towards understanding how biochemical reactions proceed under extreme conditions and the stability of biomolecules is maintained. Therefore, the studies presented in this chapter are important and aimed at the purification and characterization of alkaline protease from halo-tolerant alkaliphilic actinomycetes with special reference to its unique stability against denaturants and other chemicals.

To pursue the above theme, we purified and characterized alkaline proteases from two isolates; OM-6, and OK-5. The OM-6 and OK-5 proteases were purified to homogeneity by hydrophobic interaction chromatography followed by characterization of purified preparations. The two-step purification method yielded 35 fold and 27.34 fold purification in OM-6 and OK-5, while single-step purification method resulted in 13.03 fold purification. The results are quite encouraging in comparison to an alkaline protease from *Thermoactinomycetes* in which only limited purification was achieved by DEAE SeparoseCL-6B and Toyopearl 650 column chromatography (Gupta *et al.,* 2005). SDS PAGE analysis revealed a single band with a molecular mass of 25 KDa (Fig 2). The value was quite lower than other halophilic alkaline proteases where the molecular weight ranged from 40-130 KDa (Dodia *et al.,* 2008; Thumar and Singh, 2007; Miyaji *et al.,* 2006; Lama *et al.,* 2005) However, there are some reports in the literature on lower molecular weight corresponding to our values (Haddar *et al.,* 2009).

The temperature optima of crude proteases were higher (60-80ºC). The possible reason is that certain native proteins or peptides present in the crude preparation might be exerting protection against thermal denaturation. Besides, the residual salt concentration in the crude preparation would also have provided certain degree of protection against thermal denaturation of the enzyme. Interestingly, the optimum temperature for almost all proteases was nearly 2-fold higher than those reported from haloalkaliphilic bacteria recently reported from our laboratory (Dodia *et al.,* 2008; Patel *et al.,* 2006). Besides the temperature profile for the activity, the enzyme also had greater stability at higher temperatures, a feature quite desirable for biotechnological applications. Han and Damodaran (1998) reported an alkaline protease from *Bacillus pumilus* that was stable only for 20 min at 55ºC.

The temperature optimum of purified protease from OM-6 and OK-5 isolates was 70°C and 80°C respectively at 4M NaCl which was higher than the *Bacillus subtilis* NCIM 2713 (Huang *et al.,* 2003) and *Streptomyces clavuligerus* (Thumar and Singh, 2007). The NaCl requirement of alkaline protease for the maintenance of optimum catalysis at higher temperature is well recorded in the literature (Dodia, 2005; Patel *et al.,* 2006). This trend has earlier been observed for a serine protease from haloalkaliphilic archaea, *Natronococcus occultus* where the enzyme was optimally active at 60°C in the presence of 2M NaCl (Studdert *et al.,* 1997). More recently, an alkaline protease from a halophilic actinomycetes *Streptomonosporea salina* was reported to be active up to 4M NaCl (Cui *et al.,* 2004).

The results of thermal stability revealed the role of Na-glutamate in stabilizing the enzyme at higher temperatures and were consistent with significant activity at higher temperatures. Further, lowering the NaCl concentration from 4 to 2M decreased the stability of enzyme at all temperatures tested suggesting that a low water activity resulting from high salt concentration is required for conformational stability of the enzyme. The protease from both *Brachystreptospora xinjiangensis* strain OM-6 and *Nocardiopsis alba* strain OK-5 showed highest stability in presence of 30% Na-glutamate followed by 4 M NaCl. Enzymes had minimum stability in presence of 0 M

and 2 M NaCl as evidenced from the apparent high deactivation rate constants (K_d) and low $t_{1/2}$ values. The results indicated that the effect of salts on stability of enzymes is related not only to the salt/solute concentration but also to the type of salt/solute. The higher stability of enzymes at 30% Na-glutamate and 4 M NaCl may be because of a decrease in unfavorable electrostatic repulsion. In addition, halophilic enzymes have high negative surface charge with hydrated carboxyl groups which are shielded by high salt concentration which avoids unfolding and maintains the solubility of these proteins (Alolkar and Desai, 2009; Joo and Kim, 2005).

The thermal denaturation of enzymes is accompanied by the disruption of non-covalent linkages, including hydrophobic interactions, with concomitant increase in the enthalpy of activation (Daniel, 1996; Ohnishi *et al.,* 1996). The opening of the enzyme structure is accompanied by an increase in the disorder, randomness or entropy of activation (Vieille and Zeikus, 1996). Low values of ΔH* and ΔS* in Na-glutamate followed by 4 M NaCl revealed a higher thermal stability of protease in presence of Na-glutamate. Moreover, negative values of ΔS* indicated ordered transition state of protease in Na-glutamate followed by NaCl. Similar trends have been observed for protease from *Halobacterium* sp. SP (1) (Alolkar and Desai, 2009); Chitinase from *Pantoea dispersa* (Gohel and Naseby, 2007); ascorbate oxidase of *Cucubita maxima* (Porto *et al.,* 2006) and amylase of *Bacillus lichiniformis* (Declerck *et al.,* 2003). The results indicated that high salt/solute concentrations resulted in a stable conformation of the protease leading to decreased entropy of unfolding which favored the thermodynamic stability. Furthermore, increase in ΔG* revealed that the thermal stabilization of protease was due to the higher free energy (functional energy), as enzyme could resist against unfolding of its transition state (Gohel and Naseby, 2007). Javed *et al* (2009) reported thermal stabilization of endoglucanase from *Aspergillus oryzae* cmc-1 due to high free energy. The lowest ΔG* value for the heat labile enzyme corresponds to the largest ΔH* and ΔS*; while on the other hand, the high ΔG* corresponded to low ΔH* and ΔS* for heat stable enzyme (D'Amico *et al.,* 2003). Since proteases from actinomycetes have not been characterized

thermodynamically, the present report assumes significance. The higher thermal stability of the purified protease from salt tolerant actinomycetes was adjudged on the thermodynamic parameters, which highlighted the novelty of the enzyme.

The effect of temperature on caseinolytic activity of the purified enzyme was determined in presence of 0 - 100 mM Ca^{2+} at pH 10. An interesting feature that emerged was the NaCl and Ca^{2+} dependences of the enzyme for its optimal activity at higher temperatures. The temperature optima of purified protease from OM-6 and OK-5 isolates were 60°C and 70°C respectively at the entire concentration of Ca^{2+} studied with optimum % increase in activity. OK-5 retained up to 55% and 217% of original activity even at 90°C up to 100mM Ca^{2+} which was very high as compared to Bacillus sp. B001 (Deng *et al.,* 2010), alkaline proteases from *Bacillus subtilis* NCIM 2713 (Huang *et al.,* 2003) and *Streptomyces clavuligerus* (Thumar and singh, 2007).

Deactivation rates and half-lives of purified protease were studied at 37°C to 80°C in presence of 0-200mM Ca^{+2}. The deactivation rate constant (K_d) increased with increasing temperature whilst the half-life ($t_{1/2}$) decreased with increasing temperature. The results revealed the role of Ca^{2+} in stabilizing the enzyme at higher temperatures and were consistent with significant activity at higher temperatures. Similar effects of Ca^{2+} on proteolytic activity and thermal stability have previously been reported (Patel *et al.,* 2006).

Till date, thermodynamic parameters are not being calculated for protease in presence of Ca^{2+} ions furthermore this would be the first report sighting importance of Ca^{2+} in stabilizing enzyme structure and function thermodynamically at higher temperature, especially with actinomycetes. However, similar results have been observed for the stability of protease in presence of salt from *Halobacterium* sp. SP(1) (Alolkar and Desai, 2009); Chitinase of *Pantoea dispersa* (Gohel and Naseby, 2007); ascorbate oxidase of *Cucubita maxima* (Porto *et al.,* 2006), amylase of *Bacillus lichiniformis* (Declerck *et al.,* 2003). The results thus, indicated that Ca^{2+} provide a stable conformation to the protease leading to decreased entropy of

unfolding which favored the thermodynamic stability. Furthermore, increase in ΔG* revealed that the thermal stabilization of protease was due to the higher free energy (functional energy) which enabled the enzyme to resist against unfolding of its transition state (Alolkar and Desai 2009). Javed *et al* (2009) had reported thermal stabilization of endoglucanase from *Aspergillus oryzae* cmc-1 due to high free energy. Low values of ΔH* and ΔS* revealed a higher thermal stability of protease in presence 200mM Ca^{2+}. Moreover, negative values of ΔS* designate ordered transition state of protease.

The effect of pH on caseinolytic activity of the purified enzyme was determined in the range of 6-13 pH using different buffer systems. Protease exhibited highest activity in the pH range 10-11 with Borate buffer system. The pH response was in accordance with some other reported proteases (Dodia *et al.,* 2008; Thumar and Singh, 2007; Setyorini *et al.,* 2006; Joo and Chang 2005). The OM-6 and OK-5 alkaline proteases were stable at broad range of pH from 6-12, for 6 h. Significant stability was found in the range of 10-11 pH even after prolong incubation reflecting alkaliphilic nature of the enzyme. The findings are in accordance with several recent reports showing pH optima of 10-11 (Joo and Chang, 2005; Nascimento and Martins, 2004; Banik and Prakash, 2004; Adinarayan *et al.,* 2003; Huang *et al.,* 2003; Mane and Bapat, 2001). Serine protease from the keratin degrading *Streptomyces pactum* DSM 40530 was optimally active in a pH range 7-10 (Bockle *et al.,* 1995), a thermostable alkaline protease from Thermoactinomycetes sp HS682 was active at pH 11 (Tsuchiya *et al.,* 1997).

Cations generally protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzymes at high temperatures. Stimulatory effects were observed for almost all cation used at 100mM concentration up to 3 h of incubation. In case of OM-6 protease, stimulatory effects and nearly no effects on the enzyme activity were found for Mg^{2+} and Ca^{2+} even after 24 h of incubation with 100mM concentration at 40°C. The stimulatory effects of Ca^{2+} and Mg^{2+} have been reported previously (Haddar *et al.,* 2009; Beg and Gupta, 2003). In

OK-5 alkaline protease, stimulatory effects were observed for Mn^{+} and Ca^{2+} at 500mM concentration up to 24h of incubation at 40°C. However in presence of Zn^{+} stimulatory effect was seen on OK-5 protease at 0 h while increasing Zn^{+} concentration up to 1000mM. There are also known serine and cysteine proteases which contain an additional Zn^{+} binding site. However, these do not require Zn^{+} ions for hydrolytic activity, but rather for the stabilization of the active conformation. The stimulatory effects of Ca^{2+} have been reported previously (Haddar *et al.,* 2009; Beg and Gupta, 2003). However none of the enzyme was stable in presence of higher Hg^{2+} at higher concentration suggesting the presence of reactive –SH groups in the active confirmation. The findings gained further ground on the basis of a recent report on two novel haotolerant extrcelular proteases form Bacillus subtilis strain FP-133 which were activated in the presence of Ca^{2+} and inhibited by Hg^{2+} (Joo and Chang 2005).

Purified protease was exposed to urea and guanidine hydrochloride (GH) at 2-8M concentrations. The resistance of OM-6 protease towards urea denaturation was in contrast with one of the previous studies from our own laboratory, where an alkaline protease from a haloalkaliphilic *Bacillus* sp. was highly sensitive to urea denaturation (Patel *et al.,* 2006). The phenomenon of extreme resistance against chemical denaturation appears to be quite rare among the alkaline proteases reported from extremophilic actinomycetes (Setyorini *et al.,* 2006). Urea and guanidine hydrochloride denaturation curves are generally used to obtain an estimate of the conformational stability of proteins by measuring the differences in conformational stabilities between the native (folded) and the denatured (unfolded) states. With respect to the interaction of urea with polar solutes, solvation enhancement is attributed to the more favorable hydrogen bond formation between the peptide amide units and urea than with water (Tobi *et al.,* 2003) because urea itself is a soluble amide. It has also been suggested that urea induces an ‘‘outside-in’’ denaturation process of electrostatic character by adhering on the surface of charged residues, leading to a repulsion between residues. The result of the repulsion is an opening to water into the protein interior that will provoke the unfolding. This explains the need of high urea concentration to achieve denaturation (Wallqvist *et al.,* 1998; Mountain

and Thirumalai, 2003). Herrera *et al.,* (2005) suggested that urea acts indirectly in the denaturation process, decreasing water mobility around the peptide, increasing the ability to form peptide water hydrogen bonds with longer lifetimes, and also directly by giving urea molecules access to the polar groups of the peptide, thus providing a better peptide solvation than pure water.

The enzyme has varying levels of activity at different concentrations of osmolytes. The activity in Na-glutamate increased with increasing concentrations indicating that osmotic pressure or reduced water activity was important in maintaining enzyme. The enzyme was completely inhibited by PMSF, a serine protease inhibitor and there was no significant effect of EDTA and DTT, indicating that the enzyme was a serine protease. Our results correspond with the reported alkaline serine proteases from halophilic and haloalkaliphilic *Bacillus* sp. as well as alkaliphilic *Streptomyces clvuligens* strain Mit-1 (Thumar and Singh, 2007; Patel *et al.,* 2006; Gupta *et al.,* 2005). The metal ions did not have any significant effect on enzyme activity except Cd^{2+}, Cu^{2+} and Hg^{2+} (Fig. 7C). The findings are in agreement with a recent report on two novel halotolerant extrcelular proteases form *Bacillus subtilis* strain FP-133 (Joo and Chang, 2005). The findings are in agreement with a recent report on two novel halotolerant extrcelular proteases form *Bacillus subtilis* strain FP-133 (Javed *et al.,* 2009). The % activity of OM-6 and OK-5 protease significantly increased in the presence of 10mM H_2O_2 and 5mM β-mercaptoethanol. The results indicated that H-bond and disulfide bonds are not directly involved with protein activity or stability (Thumar and Singh, 2007). Joo and chang (2005) reported the existence of oxidant stable alkaline protease in a halo-tolerant *Bacillus clausii* l-52. OM-6 and OK-5 proteases also had high % maximal activity in different surfactants. Haddar *et al* 2009 reported a surfactant stable alkaline serine-protease from a newly isolated *Bacillus mojavensis* A21. High activity in oxidizing agent, reducing agent and different surfactants highlights towards the enzymatic activity and stability under multitude of extremity and hence possible potential applications in varied industries.

This finding is in contrast with (Akolkar and Desai, 2010) where enzyme activity was reduced in presence of both anionic (SDS) and cationic (CTAB) detergent whilst consistent with those reported for alkaline proteases from *B. clausii*, *B. mojavensis*, *Halogeometricum brorinquense* strain TSS101 and *Bacillus sp*. (Vidyasagar *et al.,* 2006; Joo *et al.,* 2003; Gupta *et al.,* 2002). Haddar *et al* (2009b) reported surfactant stable alkaline serine-protease from a newly isolated *Bacillus mojavensis* A21. Joo and chang (2005) reported the existence of oxidant stable alkaline protease in a halo-tolerant Bacillus clausii I-52. Although ionic surfactants and oxidizing agents have been reported to destabilize most alkaline proteases (Beg and Gupta, 2003), the stability of studied protease towards surfactants and oxidant would allow use of this enzyme in detergents.

The present report focused on single step purification of a novel alkaline protease from salt tolerant alkaliphilic actinomycetes, *Brachystreptospora xinjiangensis* strain OM-6 and *Nocardiopsis alba* strain OK-5. Since the halophilic proteases are difficult to purify, development of a simple purification procedure in the present study would be quite useful for purification of other extremozymes. In addition, the present research was an attempt to understand the biochemical, thermodynamic and kinetic properties of extracellular protease. Moreover, such a detailed characterization of protease from actinomycetes have not been conducted till date and to the best of our knowledge this is the first report on the analysis of the thermodynamic and kinetic parameters of protease from salt tolerant alkaliphilic actinomycetes. The findings would add to the understanding of the behavior of extremozymes. Besides, the enzyme had high activity and significant stability at higher salt, temperature, pH and a range of metal ions. The enzyme displayed extreme resistance against urea denaturation, oxidizing and reducing agents and surfactants, a finding which is rather unique and restricted to only few proteins. The results, therefore, would open a new horizon for biotechnological applications of enzymes from less attended haloalkaliphilic actinomycetes.

Chapter 8

Cloning and Expression of Alkaline Proteases from Actinomycetes

8.1 INTRODUCTION

Proteases from extremophiles as mentioned earlier are the most important enzyme from commercial point of view as well as for understanding fundamental cellular features. Proteases are one of the largest selling enzymes accounting about 60% of the total enzyme market worldwide (Dodia *et al.,* 2008a and b; Joshi *et al.,* 2008; Gupta *et al.,* 2005). Alkaline proteases are generated by a wide range of organisms, including bacteria, actinomycetes, molds, yeasts, and mammalian tissues (Boominadhan *et al.,* 2009; Ramesh 2009; Reddy *et al.,* 2008; Thumar *et al.,* 2007; Calik *et al.,* 2003). However, the ability to withstand the rigorous environments is not sufficient for commercial success. In additions, number of other factors must also be considered and investigated. Therefore, Recombinant DNA Technology in conjunction with the protein engineering tools is being extensively used to improve and evolve enzymes suitable for various unconventional applications.

Page and coworkers (2003) chose *Streptomyces griseus* trypsin (SGT) as a model scaffold for the development of serine proteases with enhanced substrate specificity. Recombinant SGT has been produced in a *Bacillus subtilis* expression system in a soluble active form. A gene encoding an alkaline proteinase (subtilisin), was cloned and sequenced from alkaliphilic *Bacillus lentus* NCIB 10309 into *Bacillus subtilis* DN497 (Jorgensen *et al.,* 2000). Similarly, the genes encoding chitinases were cloned and sequenced from alkaliphilic actinomycete, *Nocardiopsis prasina* OPC-131 into *Escherichia coli* (Tsujibo *et al*., 2003). A gene encoding a serine alkaline protease was cloned from alkaliphilic *Nocardiopsis* sp. strain F96 into *Escherichia coli.* Halolysin R4, a serine proteinase from the halophilic archaeon *Haloferax mediterranei* was cloned and studied for expression and structural properties (Kamekura *et al.,* 1996). Cloning and sequencing of an alkaline protease gene from *Bacillus lentus* and amplification of the gene on the *Bacillus lentus* chromosome has also been reported (Jorgensen *et al.,* 2000). Fernandez *et al* (2003) demonstrated the posttranslational processing of the xylanase Xys1L from *Streptomyces halstedii* JM8 by secreted alkaline serine protease. Recently, a novel calcium independent serine

protease from an alkaliphilic bacterium, *Nesterenkonia* sp. AL20, has been purified and crystallized by X- Ray analysis using sodium formate as the main precipitant (Bakhtiar *et al.,* 2002).

Due to the advancement in molecular tools and increasing realization on the potential of the extremophiles, much of the work in this area is being done at molecular level to expand the horizon of genomics and proteomics in these organisms. Regulation of gene expression of various enzymes from extremophiles would pave the way for further molecular evolution to achieve unexplored and non-existed features of biocatalysts. Genes from the extremophiles often cloned and over expressed in domestic host systems to obtain large quantities of enzymes (Yan *et al.,* 2009; Ni *et al*., 2009; Corolina *et al.,* 2008). More recently alkaline protease genes from haloalkaliphilic bacteria and metagenomically derived from saline habitat of Coastal Gujarat were cloned, sequenced and over expressed in functional state into *E. coli* (Purohit and Singh, 2009). The native and recombinant proteases were analyzed for structure and function relationship. However, since only few genes from salt tolerant alkaliphilic actinomycetes have been cloned and analyzed for their expression in heterologus hosts, cloning and expression of alkaline protease from these organisms would be a valuable addition to the knowledge of recombinant biocatalysis from extrmoplies. Further, it would add to the molecular insights into the physicochemical properties of alkaline proteases for its functioning under multitude of extremities.

8.2 MATERIALS AND METHODS

8.2.1 Microorganisms

Alkaline proteases were purified from two halotolerant and alkaliphilic actinomycete strains OM-6 and OK-5 as described in Chapter 7. Purified protease from both of the strains produced high protease activity and stability. Therefore, our next area of thrust was to clone and over express alkaline protease gene from salt tolerant alkaliphiic actinomycetes strains OM-6 and OK-5 into mesophilic host.

8.2.2 Protease assay

Alkaline protease activity was measured by modified Anson-Hagihara's method (Hagihara, 1958) as described in Chapter 6 (section 6.2.5.2).

8.2.3 Genomic DNA extraction

For the DNA isolation, OM-6 and OK-5 were grown on YEME media (Yeast Extract-Malt Extract broth) with 5% salt at pH 9.0 for 48h at 30°C. The extraction of genomic DNA was carried out in accordance with the methods described by Sambrook *et al* (1989) with modifications as described in Chapter 5 (Section 5.2.2).

8.2.4 Primer designing and PCR amplification

According to the previously known whole sequence of the halophilic alkaline protease gene, six pair of primers was designed for cloning the alkaline protease gene in present study. Among the 6 primer pairs, 4 primers (SPS-1F/R, SPS-3F/R, SPS-4F/R, SPS-5F/R) were designed using known sequences of alkaline protease genes from *Bacillus halodurans*, *Bacillus cerus*, *Oceanobacillus iheyensis* and haloalkaliphilic *Bacillus* sp. Two primer pairs (SPS-6F/R, SPS-7F/R) were designed on the basis of conserved sequences of Haliphilic *Bacillus* sp., using multiple sequencing tools followed by block generation with degenerate primer designing bioinformatics tool CODEHOP. Nucleotide sequence of each primer pair is shown in Table 8.1, where the EcoR1 recognition sequence is underlined in case of first four

primer pairs while Nde1 recognition sequence is underlined in case of last two primer pairs. For amplification of alkaline protease gene, the gradient PCR method was developed to specifically amplify protease sequences from OM-6 and OK-5 using Eppendorf Master Cycler Gradient with all six primer pairs. PCR mixture consisted of 100 ng of DNA as the template and 25 pmole of each Forward and oligonucleotides primer (Sigma Aldrich, life sciences), 25 µl of 2X Red Mix Plus (Merk, Life sciences). The PCR program consisted: 1 cycle of initial denaturation at 95°C followed by 30 cycle of at 94^{0}C for 30 secs, Gradient of annealing at 60^{0}Cwith gradient of 8^{0}C for 45 sec, 72^{0}C for 1.5 mins and final extension step of 72^{0}C for 5 min. The amplified products were visualized on 0.8% agarose gel as further purified as discussed below and stored at -20°C till further use.

Primer designation	Sequence
SPS-1F	5’-gga tcc ttg aaa aac aaa atc att-3’
SPS-1R	5’-gtc gac tta aga agc ttt att taa c-3’
SPS-3F	5’-gga tcc ttg aaa aca aaa tca ttg-3’
SPS-3R	5’-gtc gac tta aga agc ttt att taa c-3’
SPS-4F	5’-gga tcc cta ctt gat gta ga-3’
SPS-4R	5’-gtc gac atg cat atc gga aaa c-3’
SPS-5F	5’-gga tcc gcc gcc gag gac gac-3’
SPS-5R	5’-gtc gac atg gga tat tat gac-3’
SPS-6F	5’-gga tcc gcc gcc gag gac gac-3’
SPS-6R	5’-gtc gac gga cca gac cgt cg-3’
SPS-7F	5’-cat atg ccg ccg agg agg ac-3’
SPS-7R	5’-gtc gac ggc ctt cgt gtg g-3’

Table 8.1: Nucleotide sequence of each primer pair

8.2.5 Construction of recombinant clones

8.2.5.1 Plasmid isolation and restriction digestion

Plasmid DNA was isolated by SDS Mini preparation method. *E.coli* Top_{10}, harboring pET $21a^{+}$ plasmid was inoculated in LB broth containing 50µg/ml of ampicillin at $37^{O}C$. 3ml of overnight grown bacterial culture was taken for the extraction of plasmid. The culture was centrifuged at 10,000 rpm for 1min and the pellets were suspended in 1 ml of phosphate buffer saline. The above step was repeated and pellets were re-suspended in 150 µl of solution I. To the above solution, 300 µl of solutions II was added, vortexed and kept on ice for 5mins. To the above mixture, 200 µl of solutions III was added, vortexed and kept on ice for 5 mins. The cell suspension was centrifuged at 10,000 rpm for 15 min. An equal amount of chloroform: isoamyl alcohol (24:1) was added to the supernatant and centrifuged as described earlier. Half volume of pre-cooled isopropyl alcohol was added to the supernatant and incubated at room temperature for 20mins. Above mixture was further centrifuged at 10,000 rpm for 15 min. The supernatant was discarded, pellets were air dried and dissolved in minimum volume of TE buffer. For restriction digestion, 50ng pET $21a^{+}$ vector/1µg insert DNA, 2.5 µl 10X restriction enzyme buffer, 1-2 U each restriction enzyme and x µl Nuclease-free water to volume was assemble in a micro centrifuge tube to make total volume 25 µl and Incubated at $37^{0}C$ for overnight. Followed by RE digestion, 5 µl digested product together with DNA Markers was analyzed on agarose gel to evaluate the extent of digestion. Ethanol purification of amplicon and RE digested product was done after each reaction.

8.2.5.2 Preparation of competent cells

Bacterial cells of *E.coli* $DH5_{\alpha}$ and *E.coli* BL_{21} (DE3) were inoculated into LB broth and incubated at $37^{o}C$ at 120 rpm for 3-4 hours. The bacterial cells were transferred to sterile (50ml) tubes at A_{660} 0.3-0.4 and the cultures were brought to $0^{o}C$ by storing the tubes on ice for 10mins. The cells were recovered by centrifugation at 5000 rpm for 10mins at $4^{o}C$. The tubes were kept in an inverted position for 1min to allow the last traces of media to drain out. The pallets were suspended by swirling or gentle

vortexing in 30ml of ice-cold $MgCl_2$-$CaCl_2$ solution (80mM $MgCl_2$, 20mM $CaCl_2$). The cells were recovered by centrifugation at 5000 rpm for 10mins at 4^0C. The pellets were then resuspended by gentle vortexing in 2ml of ice-cold 0.1M $CaCl_2$ solution for each 50 ml of original culture.

8.2.5.3 Transformation

After competence cells preparation, cells were used directly for transformation and/or stored at -20°C. For transformation, 200µl of competence cells were transferred into sterile, chilled eppendorf tube using a pre chilled tip. Plasmid DNA or ligated vector were added to each tube and mixed gently by swirling. The tubes were incubated on ice for 30min. Heat shock treatment was given for 90 seconds in preheated 42^0C water bath without shaking the tubes. Then the tubes were rapidly transferred on ice bath for 1-2min. To this 800µl of LB media was added and the culture was incubated for 1hour at 37°C. Different volumes: 100, 50 and 25 µl of transformed cells were spreaded on LB agar plates containing ampicillin (30µg/ml) and incubated overnight at 37^0C. Clones containing the insert were screened and identified by colony PCR.

8.2.6 Expression analysis

8.2.6.1 Effect of temperature and IPTG induction on growth and expression of alkaline protease

Positive recombinant clones were further screened for enzyme secretion at different IPTG concentrations and temperature by incubating the recombinant clone on gelatin agar plate (pH-7), with varying IPTG concentration (0.1-3mM) and temperature (27°C and 37°C).

8.2.6.2 Soluble and insoluble fractionation

Positive recombinant clones were inoculated in LB broth containing ampicillin (30µg/ml). At regular interval of 2h, 2 ml of cultures were withdrawn and cells were centrifuged at 5000 rpm for 5 min at 4°C and growth was measured at 660_{nm}. The cell

pellet was mixed with 1 ml of potassium phosphate buffer (pH-8) and subjected to sonication at 30Hz for 30 seconds in 6 cycles. Samples were kept under chilled conditions for 30 seconds between each cycles and the resulted supernatant was treated as soluble fraction. The pellet was treated with 8M urea for 30 min at 30°C, followed by centrifugation at 5000 rpm for 5mins at 10°C to obtain supernatant, which was treated as insoluble fraction. Further, insoluble fractions were dialyzed against phosphate buffer (pH-8), to re-nature the active enzyme present for further analysis.

8.2.6.3 SDS polyacryalamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli using 12% cross-linked polyacrylamide gel. To visualize protease expression, both soluble and insoluble fractions (20µg) were loaded onto gel and molecular weight was determined to by using reference molecular weight marker (Middle range, Merck Life Sciences). The protein bands were visualized on the gel by Coomassie brilliant blue G-250.

8.3 RESULTS

8.3.1 Microorganisms

Salt tolerant alkaliphilic actinomycetes strain OM-6 and OK-5 were isolated from coastal region of Gujarat (India) was gram positive having filamentous structure. Based on the 16S rRNA gene sequence homology and phylogenetic analysis, OM-6 was identified as *Brachystreptospora xinjiangensis* (GenBank Accession Number: AF251709). Similarly, OK-5 was identified as *Nocardiopsis alba (Gene Bank Accession Number:* GU985439) as described in (Chapter 3; Section 2.3.3.4). The isolates hydrolyzed gelatin, as apparent from the zone of hydrolysis on gelatin agar plate on addition of Frazier's reagent (Chapter 6; Picture 6.1). Similarly, high protease production was detected in OM-6 and OK-5 in gelatin broth with 400U/ml and 500U/ml activity respectively (Chapter 6; Figure 6.15). The zone of clearance as well as significant protease activity confirmed proteolytic properties of the strain and thus OM-6 and OK-5 were selected for further studies.

8.3.2 Cloning of alkaline protease gene

8.3.2.1 Amplification OM-6 and OK-5 alkaline protease gene

Spectrophotometric assessment revealed that retrieved DNA was of high purity and yield. DNA concentration of OM-6 and OK-5 was 230µg/ml and 225µg/ml respectively, while the plasmid vector was 250µg/ml. High DNA purity was indicated from the 260/280 ratio around 1.8 and 1.7 in OM-6 and OK-5 respectively. Extracted DNA was further analyzed on 0.8% agarose gel and the results corresponded with the spectrophotometric analysis. Amplification profile of alkaline protease gene was generated using six pairs of primers designed specifically for extracellular alkaline protease genes from the organisms of haloalkaliphilic origin. The primers were designed by using manual primer designing method for SPS-1, 3, 4 SPS-5 was specific to *Bacillus* sp, while SPS-6 and SPS-7 were designed by using CODEHOP primer designing tool. The genomic DNA was used as a template for PCR amplification. PCR reaction was carried out at three gradients of annealing

temperatures i.e., 56.1, 59.8 and 63.7^{o}C using Gradient Thermocycler (Eppendorf). Among three temperature gradients, 56.1^{o}C was most appropriate as SPS 5 generated intense band of 1.2Kb amplicon from OM-6. The concentrations of amplified product gradually decreased as annealing temperature increased. While SPS 6 generated intense band of 0.5Kb amplicon at 59.8^{o}C annealing temperature from OK-5 (Figure 8.1). However, no amplified product was visualized with SPS-1, 3, 4, 6 and 7 primers. Amplification strategies were designed by using bioinformatics based prediction.

8.3.2.2 Restriction digestion of amplicon and vector

In general, enzymes whose sites are 10bp apart and buffers compatible for the reaction are used, or otherwise double digestion should be performed sequentially. In present study, amplified product of OM-6 alkaline protease gene as well as pET 21a^{+} were double digested with BamH1 and Sal I whereas BamH1 and Nde I were used for the digestion of OK-5 alkaline protease and pET 21a^{+} (Figure 8.2). Following the digestion protocol, samples were gel purifed using cleangene PCR purification kit (Banglo GeneI) in order to remove residual restriction enzyme. Selection of right RE pair was predicted on the basis of the bioinformatics based prediction for cutters and non-cutters sites within the vector and insert. Taking all parameters into consideration, BamHI and Sal I were designed into primer pair combination to ensure cohesive end generation**.** pET 21a^{+} was digested by Bam HI and Sal I as their presence was available in multiple cloning site (MCS), as well as they are among the list of non-cutters in alkaline proteases sequences. The inserts were gel-purified using Cleangene PCR purification kit (Banglo Genei) to remove residual restriction enzyme and prevent self-ligation.

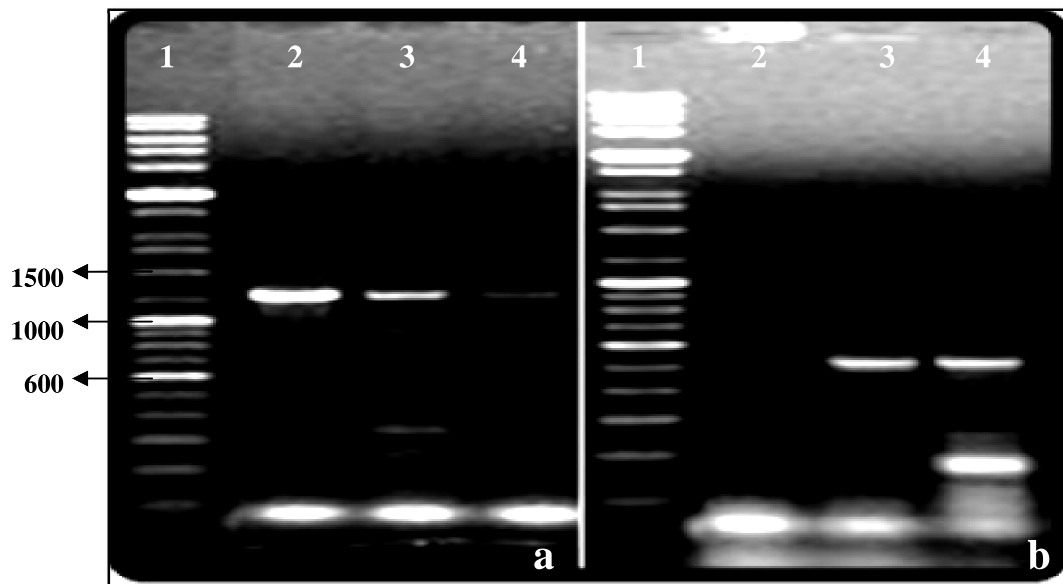


Figure 8.1: Amplification of OM-6 and OK-5 alkaline serine protease with SPS-5 and SPS-6 primer sets respectively. a) Lane 1: medium range DNA ruler, Lane 2: OM-6 (56.1^{o}C), Lane 3: OM-6 (59.8^{o}C), Lane 4: OM-6 (63.7^{o}C). b) Lane 1: medium range DNA ruler, Lane 2: OK-5 (56.1^{o}C), Lane 3: OK-5 (59.8^{o}C), Lane 4: OK-5 (63.7^{o}C)

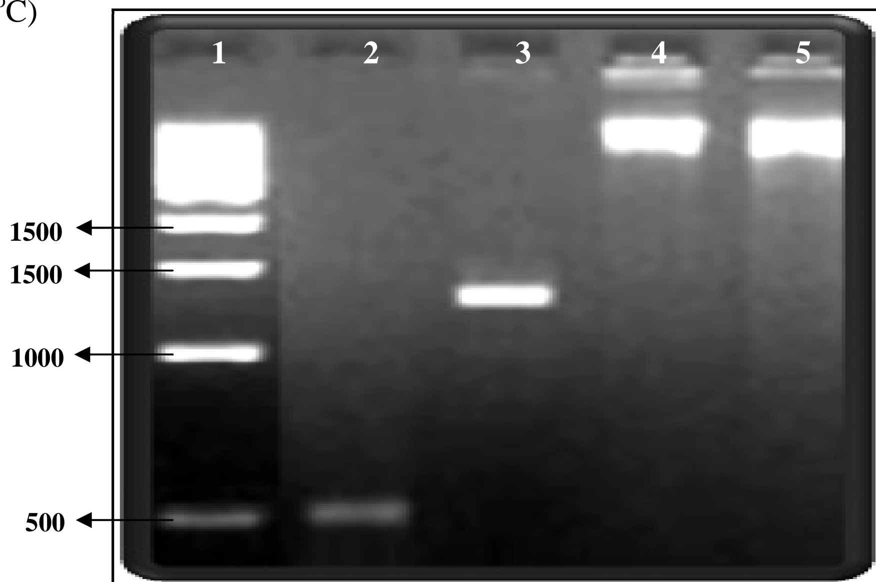


Figure 8.2: Restriction digestion of amplified alkaline serine protease gene. Lane 1: 500bp DNA ladder, Lane 2: Double digestion of amplified alkaline protease gene using Bam HI and Sal I, Lane 3: Double digestion of amplified alkaline protease gene using Bam H1 and Nde I, Lane 4: Double digestion of pET21a+ vector using Bam HI and Sal I, Lane 5: Double digestion of pET21a+ vector using Bam H1 and Nde I

8.3.2.3 Ligation, transformation and induction of alkaline protease

Ligation procedure was carried in 1:3 ratio of Insert:Vector. Ligated plasmids were transformed into *E. coli* DH5α host strain. Selection of non-expression host strain was carried out for their high transformation efficiencies. Positive strains were confirmed on the basis of marker properties of ampicillin resistance of plasmid vector (Figure 8.3, Picture 8.1). Due to the ease of transformation, cloning and high cell density cultivation, *E. coli* is by far the most widely used microorganism for the production of recombinant proteins and enzymes. Hence after confirmation of positive clones, *E. coli* strain BL21 (DE3) was transformed with the expression plasmid pET $21a^+$ which carries strong T7 promoter to over-express alkaline protease gene. Further, positive clones harbouring protease genes were selected on the basis of release of insert and we visualized band size position of insert at aprox 1.2kb and 0.5kb for OM-6 and OK-5 respectively on agarose gel (Figure 8.4). Lateron, all selected positive recombinant clones harbouring alkaline protease gene from haloalkaliphilic actinomycetes, OM-6 and OK-5 were maintained on LB agar plate containing ampicillin (30µg/ml). During each steps of cloning procedure, bioinformatics based tool-CLC- Workbench was used to predict the results as well to standardize the protocols.

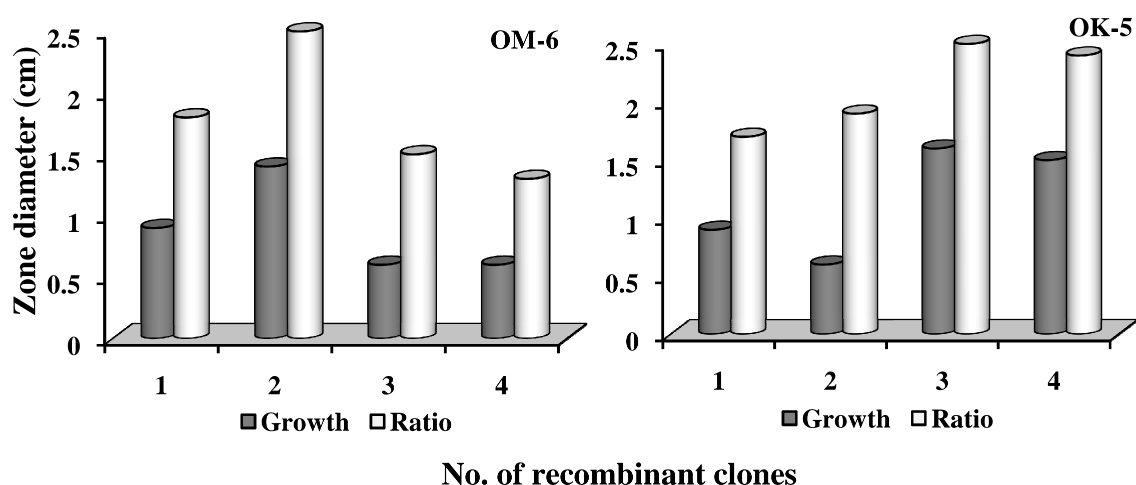
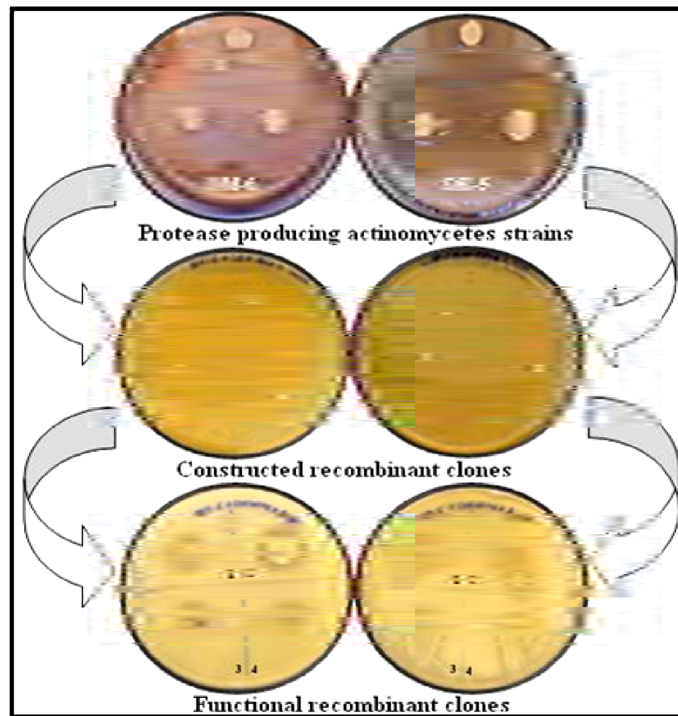


Figure 8.3: Growh and protease production in four selected recombinant clones of OM-6 and OK-5 isolates on gelatin agar plates with amphicilin



Picture 8.1: Construction of alkaline protease producing recombinant clones and confirmation of insert within the pET21a+ vector construct on gelatin agar plate with amphicilin

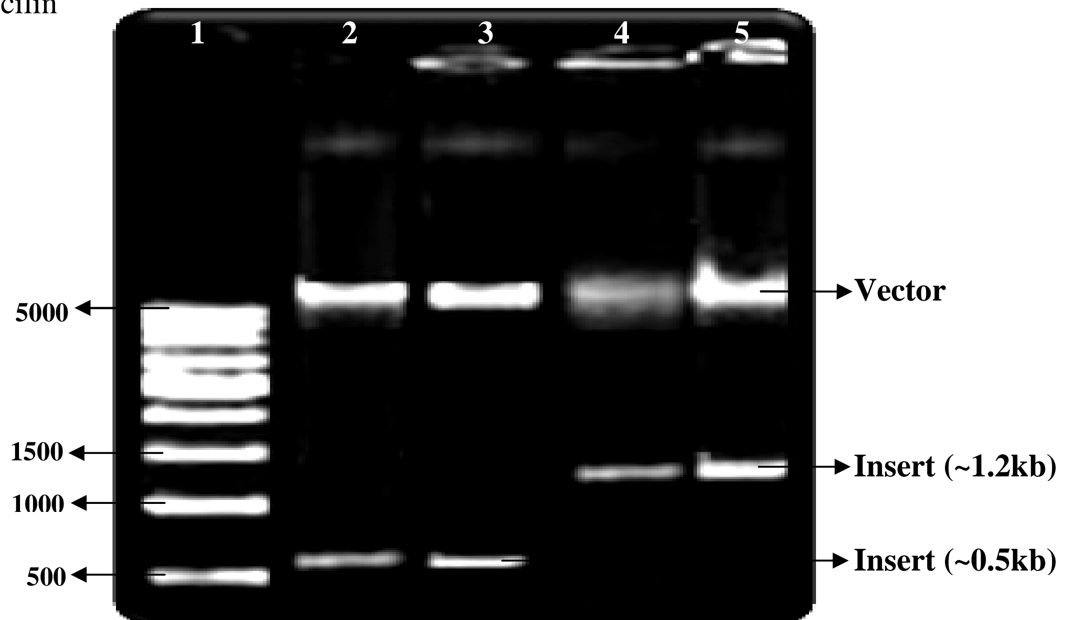


Figure 8.4: Confirmation of inserts within the pET21a+ vector constructs. Lane 1: 500bp DNA ladder, Lane 2 and 3: confirmation of insert within a vector by digesting with Bam H1 and Sal I, Lane 4 and 5: confirmation of insert within a vector by digesting with Bam H1 and Nde I

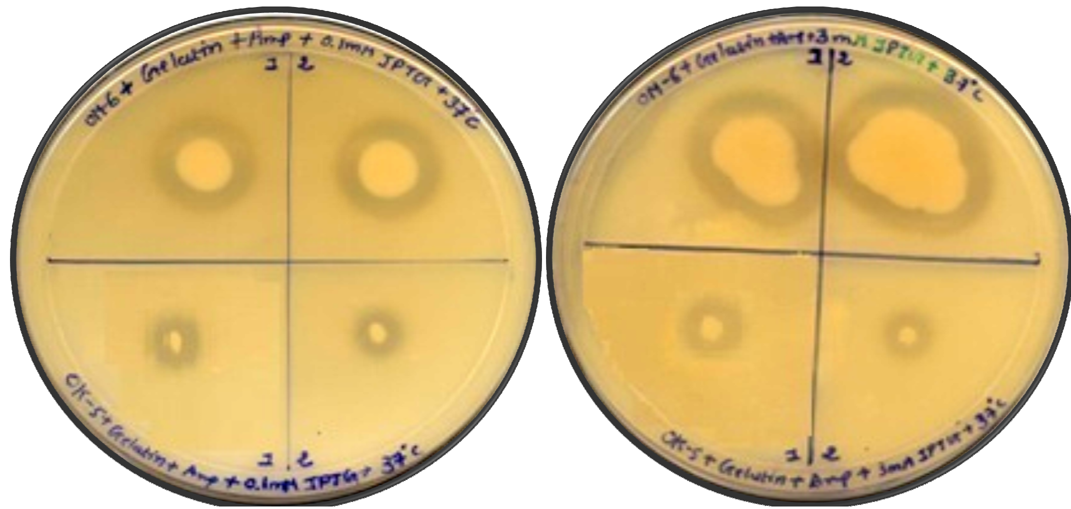
8.3.3 Expression of recombinant protease

8.3.3.1 Effect of IPTG induction

Different concentrations of isopropyl β-d-thiogalactopyranoside (IPTG); 0.1-3.0 mM, were used as inducer to induce the expression of the target protease gene by *E. coli* harboring recombinant plasmids. At 1.0 mM IPTG induction, higher amount of enzyme was produced as compared to 0.1mM in case of OM-6 while in case of OK-5, higher amount of enzyme was produced at 3.0 mM IPTG induction. Optimum enzyme production on gelatin plate was evidently seen at both 27°C and 37°C temperatures with 1mM IPTG. However, IPTG had significant effect on growth of host, as growth increased at higher concentrations of IPTG (Picture 8.2, Figure 8.5).

8.3.3.2 Effect of temperature on growth and induction of protease

To evaluate the effect of temperature on the expression of protease, expression strain was cultivated at 27°C and 37°C in a shaking flask containing LB medium and amphicilin. Protein expression was induced by IPTG at 0.1mM and 1mM concentrations. The expressed protein was fractionated into soluble and insoluble fractions prepared at 0h, 2h, 4h, 6h and 24h of growth and analyzed by measuring protease activity and SDS–PAGE analysis. To determine the amount of soluble and insoluble protease, the cells were harvested and disrupted by sonication in ice water bath. After centrifugation, the pellet represented the insoluble fraction and the supernatant represents the soluble fraction of protease. For insoluble fractions, the urea treated samples were subjected to dialysis to remove 8M urea. The results showed that most of the recombinant protein induced at 25°C or 37°C formed insoluble inclusion bodies. However, growth, as expected, was higher at 37°C as compared to 27°C (Figure 8.6). Synergistic effect of temperature and induction highlighted the significant role of temperature in production of recombinant proteins in soluble fraction while significant expression of protease was evident in presence of 0.1mM IPTG at 25°C after 4h of incubation in insoluble fraction (Figure 8.7). On SDS–PAGE, minor band of OM-6 and OK-5 alkaline protease with respective molecular weight of 25KD and 20KD was apparent (Picture 8.3).



Picture 8.2: a) Effect of 0.1mM IPTG on recombinant clone at 37°C on gelatin agar plate b) Effect of 3mM IPTG on recombinant clone at 37°C on gelatin agar plate

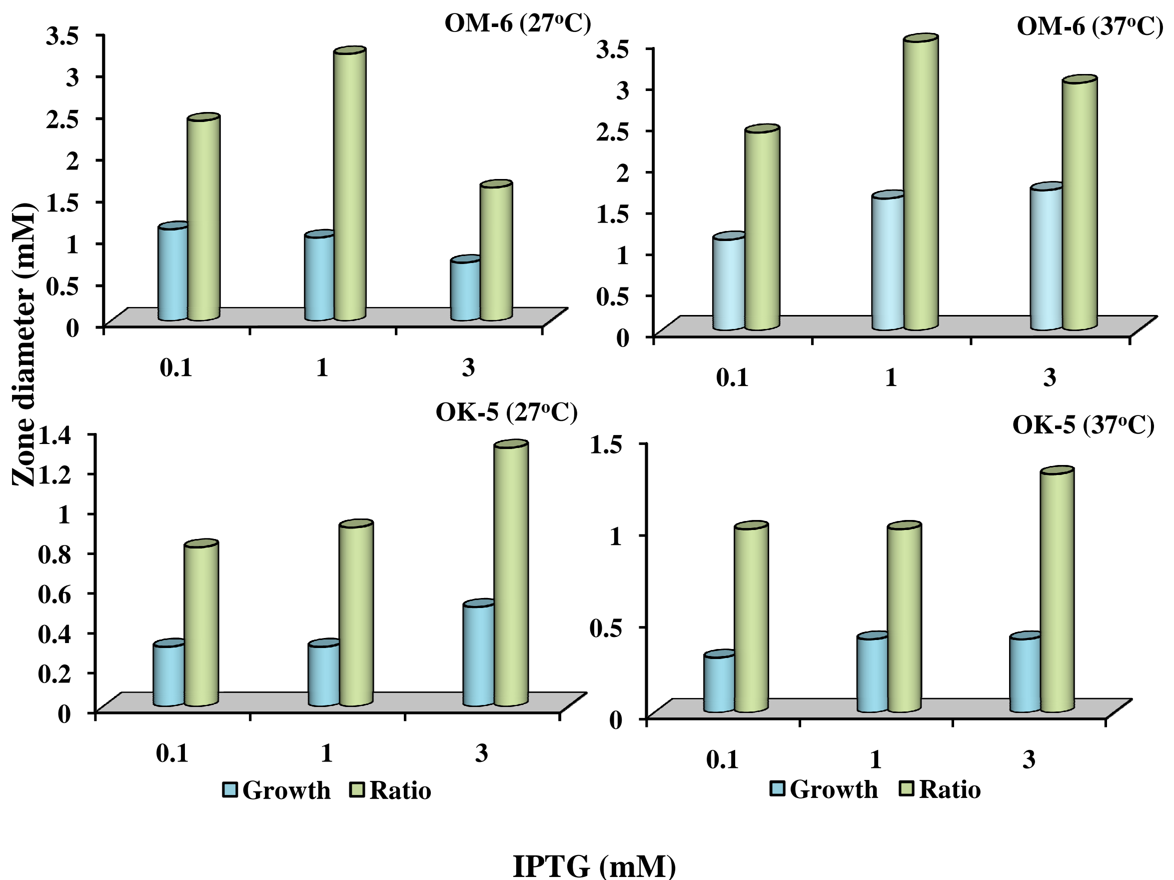


Figure 8.5: Synergistic effect of IPTG and temperature on growth and alkaline protease production

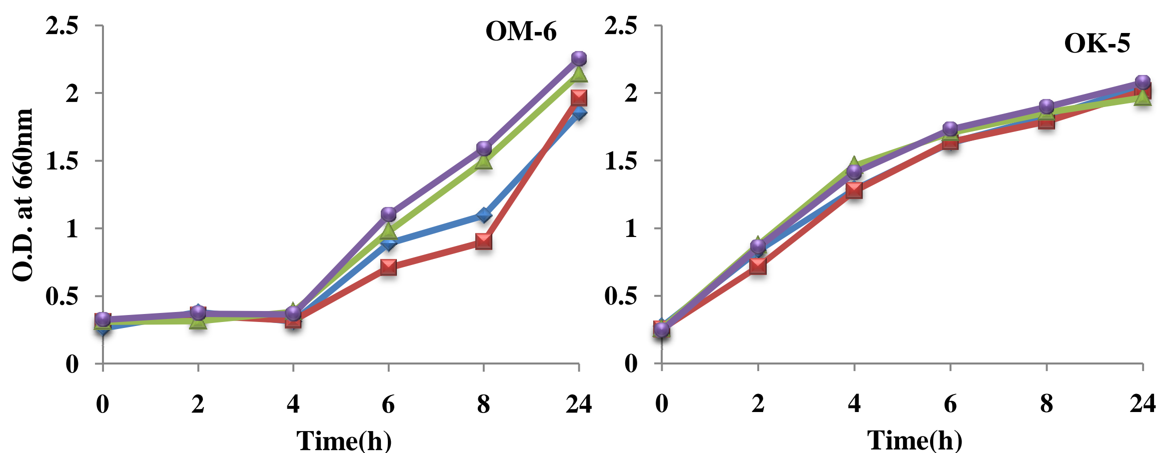


Figure 8.6: Effects of IPTG and temperature on growth of *E. coli* (▲: 37°C, 0.1 mM IPTG; ●: 37°C, 1 mM IPTG; ♦: 25°C, 0.1 mM IPTG; ■: 25°C, 1 mM IPTG)

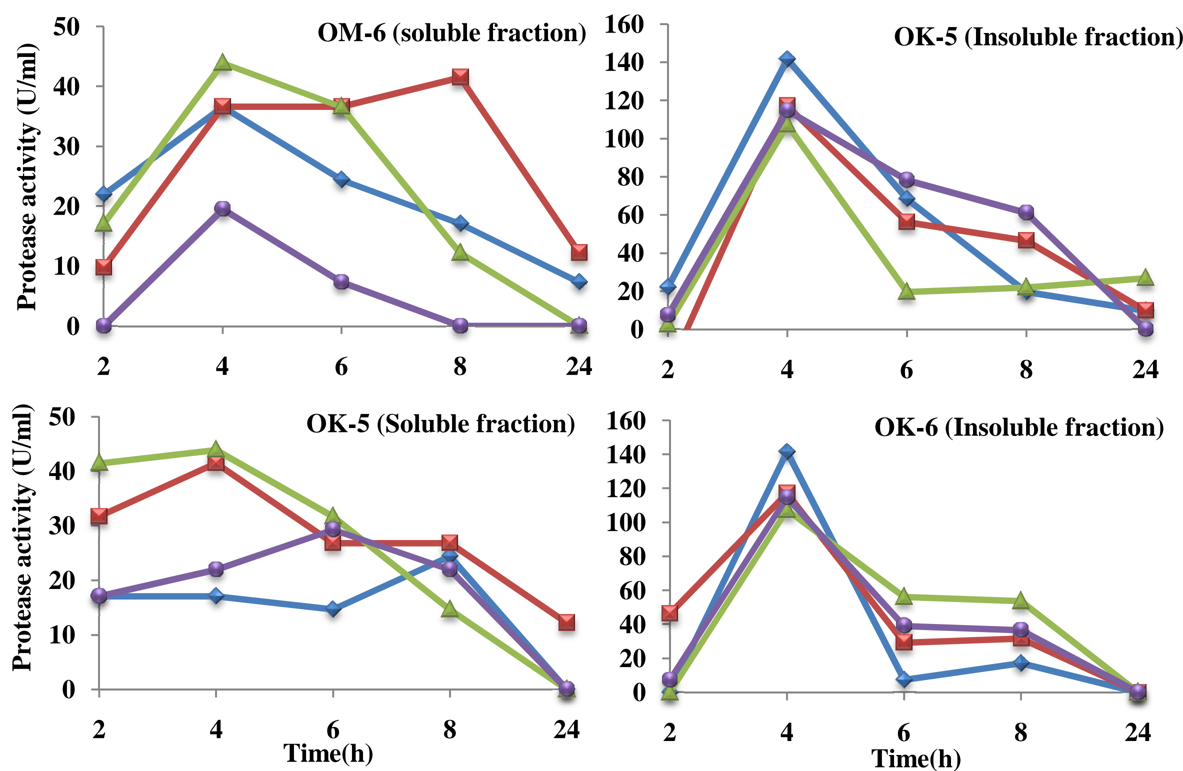
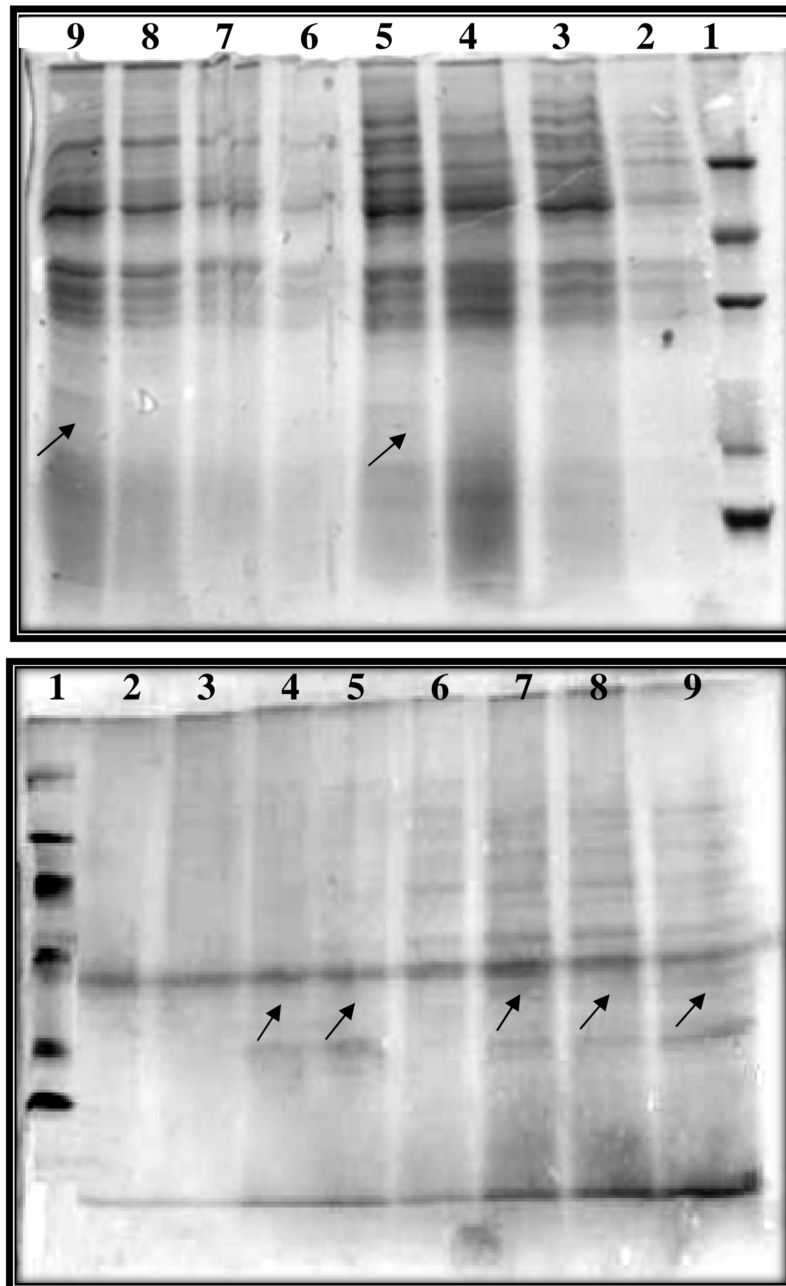


Figure 8.7: Effect of temperature and IPTG induction on the expression alkaline protease gene in *E. coli* (▲: 37°C, 0.1 mM IPTG; ●: 37°C, 1 mM IPTG; ♦: 25°C, 0.1 mM IPTG; ■: 25°C, 1 mM IPTG)



Picture 8.3: SDS-PAGE profile of the expressed protease in soluble fraction (Lane 2-5) and insoluble fraction (Lane 6-9). Lane 1, Marker; Lane 2 BL21 without plasmid; Lane 3 Control (Pre-induction); Lane 4 2 h; Lane 5, 4 h; Lane 6 BL21 without plasmid; Lane 7 Control (Pre-induction); Lane 8 2 h and Lane 9 4 h at 25°C and 0.1mM IPTG

8.4 DISCUSSION

Alkaline proteases constitute a very large and complex group of enzymes; with both nutritional and regulatory role in nature. Various types of alkaline proteases have been characterized and their potential industrial applications have been explored from haloalkaliphilic bacteria (Cannio *et al.,* 2010; Haddar *et al.,* 2009a,b; Dodia *et.al.,* 2008a and b; Joshi *et.al.,* 2008; Nowlan *et al.,* 2006; Patel *et al.,* 2006; Gupta *et al.,* 2005). However, the representation from haloalkaliphilic actinomycetes, as revealed from the literature, is quite limited (Thumar *et al.,* 2009; Thumar and Singh 2007). Further, extremophilic actinomycetes produce enzymes that are functional under extreme conditions and the unique properties of these biochatalysts have potential in several novel applications in industrial processes. Hence, present study focused on alkaline serine protease with halophilic and thermophilic nature as these enzymes form salt tolerant alkaliphilic actinomycetes are rarely explored. Cloning and expression of alkaline proteases from haloalkaliphilic bacteria and especially actinomycetes is further restricted. Therefore, it's of great significance to assess the ecosystem as a whole and to look for novel biocatalysts and metabolites (Yan *et al.,* 2009; Ni *et al.,* 2009; Castro *et al.,* 2008; Zhang *et al.,* 2008). In the present study, after preliminary characterization for secretion of alkaline proteases, gene amplification profile, cloning and expression of enzyme in *E. coli* BL_{21} (DE3), an over-expression host, was attempted from *Brachystreptospora xinjiangensis* strain OM-6 and *Nocardiopsis alba* strain OK-5.

To study cloning and over expression, genomic DNA was isolated from actinomycetes, strains by enzymatic method while plasmid DNA was retrieved by SDS-Miniprep method from *E.coli* BL_{21} (DE3). Retrieved DNA was assessed for its purity and yield by agarose gel electrophoresis and spectrophotometric analysis. High molecular size of genomic DNA was judged with reference to molecular weight marker (High range, Merck Life sciences) while plasmid DNA was confirmed of 5.4kb, which assures isolation of intact pET-21a(+). DNA fragment coding for the desired protein sequence was cloned into the pET-21a (+) vector using BamHI and

Sal I restriction sites as described in material and methods. The plasmid itself provides an N-terminal and a C-terminal His-tag useful for easy purification as well as several potential protease cleavage sites. Due to easy retrieval, in a desired concentration, the above methods used were suitable for further experiments related to cloning.

Further, primers were designed by using bioinformatics based prediction for probable location of primer pair on available protease gene. Primers were designed using CLC Work bench (Daintith, 2004) as well manual interpretation of the datasheets. In order to grab the amplification of probable all the alkaline protease/s particularly of haloalkaliphilic nature, universal degenerate primer pairs were generated by CODEHOP primer designing tool, subjecting motif and blocks result generated by stand alone tool on generating multiple sequence file (Clustal X) of our retrieved FASTA file (Haloalkaliphilic alkaline proteases).

Standardization of annealing temperature was carried out by keeping gradients of temperature calculated on the basis of Tm of primer pair. While the amplification was evident at all set temperatures, the size and concentrations of the products varied with respect to annealing temperature and primer pair used. Higher size (1.2kb) of the amplified product was obtained by using universal designed primer SPS-5 for cloning experiments. Therefore, they were used as amplicon as an assurance of complete amplification of gene. Although, other primer pairs were also capable of generating amplification profile, due to low concentrations and small fragment size, they were not taken into account for further experiments. Selection of *E.coli* $DH5_{\alpha}$ strain was done for transformation prior to its cloning into *E.coli* BL_{21} (DE3) as it is a convenient host for initial cloning of target DNA due to its high transformation efficiency and the high yields of quality DNA that results from recAendA mutations. $DH5_{\alpha}$ contains no source of T7 RNA polymerase; making it ideal for the establishment of recombinant plasmids under non expression conditions.Since we used a plasmid with a T7 lac promoter for expression, a host strain BL21 (DE3) that does not contain a pLysS was used for target gene expression. It was desirable for our

current studies that the host strain was protease deficient, ensuring that the strain will not only facilitate functional product formation but also ensured that there were no chances of false positive results.

Expression of alkaline serine protease was achieved using pET- 21a (+) with a strong promoter T7. Different induction parameters were used to achieve high level of protease expression. This may enhance the feasibility of concomitant transcription and translation which are coupled in prokaryotes (Baneyx, 1999; Ringquist, 1992). In the present study, different induction parameters have been modified to improve the expression levels of soluble proteins: growth temperature was lowered from 37°C to 25°C and incubation time altered from 2h and 4h to 6h. In order to investigate whether the production of the target protein could be increased at higher temperatures, the cultivation was carried out at varied temperatures after induction. Maximal yield of soluble protein was achieved by induction with 1.0 mM IPTG for 4 h at 25°C. The data indicated that solubility of the protein increased with decreasing growth temperatures. Understandably, conditions favoring reduced growth rate could diminish recombinant protein synthesis to a reasonably low speed, facilitating the correct folding into a soluble and biologically active form. This is consistent with the observations in literature (Liu *et al.,* 2010; Balan *et al.,* 2005). However, we observed that Most of the expressed recombinant proteins were insoluble and trapped into inclusion bodies (Luo *et al.,* 2007; Xu *et al.,* 2005; Pittaluga *et al.,* 2005; Kwon *et al.,* 2000; Rajamohan *et al.,* 1999). The inclusion bodies are treated with strong denaturing agents such as urea to proceed for renaturation under in-vitro conditions to refold into its native structure. However, during such refolding process, the protein might re-aggregate or refold incorrectly. Eventually, this could lead to a very low recovery of the active product.

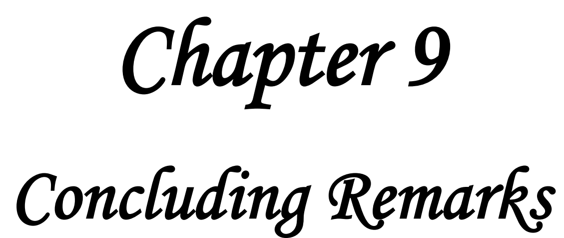
In literature, it’s reported that use of temperature shift strategy combines the advantages of fast growth with reduced expression rates, leading to reduced inclusion body (IB) formation (Gerber *et al.,* 2008; Choudhary *et al.,* 2008; Marx *et al.,* 2007; Jana and Deb 2005; Kwon *et al.,* 2000; Rajamohan *et al.,* 1999). Furthermore,

especially in bacterial cells, aggregation of partially folded intermediates manifests itself in the production of insoluble inclusion bodies, which may be mainly due to unstable folding intermediate of the target protein at higher temperatures and/or during over expression of a gene (Singh *et al*., 2002; Kim *et al.,* 1998). Therefore, when the cells were grown and induced at 25°C, higher activity was evident as compared to that obtained at 37°C, presumably due to rapid accumulation of over-expressed protein at high temperatures. Although, growth at temperatures below its optimum level was low, the fractions of the functional over-expressed protein were enhanced. Temperature appears to be one of the major factors influencing the growth of *E. coli* and expression of proteins. However, the impact was not exerted due to a single factor alone, but combination of various factors interacting together (Strandberg and Enfors, 1991). In addition, it is an obvious phenomenon that protein folding is an optimized process that leads to functional molecules under *in -vivo* conditions, despite various physico-chemical factors. Thus, aggregation of newly synthesized proteins appears to be a process competing with *in- vivo* folding (Machida *et al.,* 2000). When *in-vivo* approaches for protein solubilization fail, folding under *in-vitro* conditions could be alternatively attempted.

Various methods of dialysis have proved successful for renaturation of denatured proteins and towards this end, a modified form of dialysis for the slow removal of denaturant has been particularly attractive (Dodia *et al.,* 2008; Singh *et al*., 2002). Dialysis, rapid dilution and newly devised methods of folding immobilized proteins yielded active enzyme (Dodia *et al.,* 2008, Singh *et al*., 2002; Machida *et al.,* 2000; Kim *et al.,* 1998). In this context, the application of an artificial chaperone appeared as an effective tool in overcoming the folding problem of over-expression of target proteins. The expression of *Bacillus* lipase led to the formation of inclusion bodies in *E. coli* host (Lee *et al*., 2003). However, Rasool *et al* (2005) showed the expression of DBRL-191 lipase in cytoplasm without inclusion body formation. Our results suggested that the formation of inclusion bodies occurred due to high level of expression under the T7 promoter and high hydrophobicity of the protein.

The SDS-PAGE revealed a single band with a molecular mass of 25 KD and 29KD for OM-6 and OK-5 alkaline protease respectively. The value was lower than other halophilic alkaline proteases where the molecular weight ranged from 40-130 kDa (Dodia, *et al.,* 2008; Thumar and singh, 2007) while corresponding with (Haddar *et al.,* 2009a, b).

Successful cloning and expression of alkaline serine protease gene from salt tolerant alkaliphilic actinomycetes *Brachystreptospora xinjiangensiss* **s**train OM-6 and *Nocardiopsis alba* **s**train OK-5 was carried out. Due to the advancement in molecular tools and increasing realization on the potential of the extremophilic actinomycetes, much of the work in this area is being done at molecular level to expand the horizon of genomics and proteomics in these organisms. The results described the simple and inexpensive method of getting recombinant enzymes in active form from lesser explored salt tolerant actinomycetes. The study, therefore, bears significance from biotechnological stand point. Further, cloning and expression of alkaline proteases from actinomycetes would pave the way for further molecular evolution to achieve unexplored features of the biocatalysts.


Chapter 9
Concluding Remarks

Research on extremophilic actinomycetes during the last decade has indicated towards their phylogenetic status and potential biotechnological avenues. In view of the present status of the research in this field, the studies presented in this thesis aimed at the morphological, biochemical and molecular diversity and biotechnological potential of salt-tolerant alkaliphilic actinomycetes from the saline habitats of coastal Gujarat. The work is significant in the light of recent realization that extensive exploration of the newer habitats, particularly the extreme environments, must be carried out for new microbes and potential applications.

In the present study, 40 halo-tolerant alkaliphilic actinomycete strains were isolated from the saline and alkaline habitats of coastal Gujarat using different enrichment and serial dilution conditions of salt and pH. The results revealed that the distribution of these organisms is rather perpetual in these habitats. In addition, the conventional isolation methods in a given medium under varying salt and pH may be sufficient to explore the real population dynamics of extremophilic actinomycetes. The studies on the morphogenesis and growth behavior, salt and pH tolerance, antibiotic susceptibility and production of antimicrobial activity reflected the diversity and heterogeneity among the isolates.

The conventional morphological and biochemical methods may also provide important clues on the diversity of these organisms, as revealed from the study in this thesis. The isolates were identified as *Streptomyces, Nocardiopsis* and *Nesterenkonia* on the basis of their morphological, cultural and biochemical characteristics. Occurrence of catalase and oxidase confirmed the aerobic nature of the organisms, a phenomenon most common in actinomycetes. Sugar fermentation was less common than H_2S and ammonia production, indicating that the concerned habitats might be rich in proteinaceous substances.

The occurrence and optimization of many industrially important antibiotics has been reported from actinomycetes. In addition, the antibiogram profile revealed the extent of diversity among the isolates with respect to antibiotic sensitivity. Extremophilic

microbes have strong potential in commercial applications for the synthesis of antimicrobial agents. In view of the realization that only few extreme actinomycetes are known to secrete antibiotics (Fiedler *et al*., 2005; Saha *et al*., 2005; Manam *et al*., 2005; Maskey *et al*., 2004; Basilio*et al*., 2003; Ellaiah *et al*., 2002), we looked into their possible antimicrobial potential. Some selected isolates secreted a potent broad spectrum antibiotic selective against gram positive organisms, gram negative organisms, yeast and even against candida indicating the antagonistic relationship of the isolates with other organisms in a given ecological niche.

The above accounts present the current status of research on the biology and biotechnology of salt-tolerant and alkaliphilic actinomycetes. The fairly enhanced salt and pH tolerance of these actinomycetes, along with their capacity to secrete commercially valuable primary and secondary metabolites, appeared as attractive features of these organisms. The study on enzymes adapted to extreme conditions may provide indispensable tool in designing the strategies for the rational design of enzymes. Enzymes are so fine-tuned with nature that they often do not coincide with the desired properties and activities useful for biotechnological applications. In view of the significant commercial roles of proteases, it was of significant interest to focus on their characterization from halo-tolerant alkaliphilic actinomycetes which conferred stability under two extremities; salt and pH.

It was of significant interest to purify proteases from rarely explored group of halo tolerant alkaliphilic actinomycetes. In addition, the present research was an attempt to understand the biochemical, thermodynamic and kinetic properties of extracellular proteases. Such a detailed characterization of protease from actinomycetes has not been carried out till date. The conclusions emerged from the biochemical characterization of the purified enzymes in conjunction with the trends supported by various thermodynamic analysis on the enzyme stability would add to our understanding of extremozymes.

The enzymes displayed significant activity and stability at higher salt, temperatures, alkaline pH and a range of metal ions. The enzymes also had extreme resistance against urea denaturation, oxidizing and reducing agents as well as surfactants, the findings which are rather unique and restricted to only few proteins. The results would definitely provide a base for designing the strategies for improvements of extremozymes from actinomycetes. The characterization of proteases from different organisms indicated that although synthesized under similar conditions of growth, the enzymes may display greater variation in their properties. Further, the structural elucidation of these enzymes may provide important clues responsible for varied features.

Optimization of PCR reactions using genus-specific PCR combined with DGGE techniques distinguished species belonging to the same genus. Moreover, due to its rapid and easy performance, DGGE was useful for identification of number of isolates. The technique was also effective as powerful fingerprinting method for revealing sequence heterogeneities in the 16S rRNA genes. One of the foremost objectives of the present work was the development of molecular methods for assessing molecular diversity among salt tolerant alkaliphilic actinomycetes and tentative assignment of novel isolates to specific genus. Further, to confirm the DGGE findings, restriction endonuclease *HhaI* was specifically used to recognize and cut the sequence “GCGC”. The RFLP patterns when compared with the corresponding DGGE band pattern of the same isolates indicated that actionmycetes could be identified at the genus level as *Streptomyces* and *Nocardiopsis*. The PCR amplification profile, DGGE and RFLP fingerprinting patternswere compared with the morphological and physiological properties and it was revealed that majority of the isolates belonged to *Streptomyces* followed by *Nocardiopsis* and *Nesterenkonia*.

While the diversity profile of haloalkaliphilic bacteria by microbiological and molecular biology approaches have been generated and published during the past few years (Thumar *et al.,* 2009; Dodia *et al.,* 2008; Joshi *et al.,* 2008; Dodia *et al.,* 2007; Sinha *et al.,* 2007; Thumar *et al.,* 2007; Patel *et al.,* 2006; Patel *et al.,* 2005), the

similar attention on the salt tolerant alkaliphilic actinomycetes is in infancy. Towards this end, the present work on the molecular approaches to investigate diversity of these actinomycetes along with the cloning and expression of alkaline protease genes will provide insights into the sustainability under multitude of extremity. Recombinant proteins will also satisfy the need of extremozymes for industrial demand. Further, the development of cloning and expression systems can be used for the production of enzymes in large quantity with the possibilities of altering the enzyme for desired traits by protein engineering and random mutagenesis.

In nutshell, we believe that halo-tolerant alkaliphilic actinomycetes described in the present study possess unique adaptive mechanisms such as novel transport mechanisms, osmoregulatory compounds and enzymes. In addition, isolation of these microorganisms will expand our knowledge of the “unseen majority” and will extend our views on microbial diversity beyond the presently described physiochemical boundaries for microbial growth. Further, the unique potential of their enzymes may generate a pool of new applications. The cloning and expression systems can be used for the production of enzymes in large quantity improving biocatalytic properties by protein engineering and random mutagenesis. The trends are quite interesting in view of the diversity, novelty and distribution of these actinomycetes.

Chapter 10

Summary

Isolation and characterization of actinomycetes

- Among the 40 salt tolerant alkaliphilic actinomycetes, isolated from 8 different sites of coastal Gujarat, 12 were from Okha-Madhi, 10 from Okha, 8 from Mithapur and 10 isolates from Tata disposal site. The isolates were able to grow at different conditions of pH and salinity.

- Morphological characteristics of the selected isolates were observed by light microscopy and scanning electron microscopy showing the filamentous mycelia structure and long chain of smooth oval shaped spores. Interestingly, there was the deposition of the salt crystals on the spore chain of Tata 5 and Tata 13 as well as on the vegetative mycelia of OM-4 in scanning electron microscopy.

- *In-situ* observation of the colony development displayed formation of oval shaped spores producing brown colored pigmentation in OM-1; while light blue colored rod shaped spores arranged in long chains were observed in OM-8.

- Cultural characteristics of the actinomycetes revealed that almost all strains of actinomycetes were able to grow well in most of the tested organic and synthetic media. Typically, some of the colonies were elevated, spreading and covered with gray to light pink and light blue colored aerial mycelia and spores. Most of the pigmented colonies developed on ISP-2 and ISP-3 media followed by starch casein agar and starch agar.

- For further differentiation and characterization, biochemical and metabolic activities of all the isolates were studied. While cultures were tested for hydrolysis, majority of them displayed strong hydrolytic activity indicating that ability to hydrolyze different substrates. The extent of sugar utilization highly varied among the isolates of Okha-Madhi site.

- Based on 16S rRNA gene sequence homology and phylogenetic analysis, OM-6 was detected as *Brachystreptospora xinjiangensis* (GenBank Accession Number: AF251709), OK-5 as *Nocardiopsis alba (Gene Bank Accession Number:* GU985439) and OK-7 was detected to be *Streptomyces* sp. (GenBank Accession Number: EF527834).

Antibiotic sensitivity and antimicrobial activity profile

- Among the isolates, majority were sensitive toward Azithromycin, followed by Clarithromycin, Roxithromycin, Ciprofloxacin, Sparfloxacin, Gentamicin, Amikacin, Cefaperazone, Cephotaxime, Cefuroxime, Cephadroxil. However, in general, the isolates exhibited greater resistance against Cloxocilin.

- The antimicrobial activity of the actinomycetes was screened on a range of media as well as in the presence of 0-10% NaCl and pH 7-11 against various Gram-positive and Gram-negative bacteria as well as Yeast and Candida. Among the isolates, majority exhibited antimicrobial potential against gram positive bacteria while few had antimicrobial activity against gram negative bacteria.

- The work in nutshell holds significance as extremophilic actinomycetes have rarely been explored. Further, studies on the enzymes secreted by these actinomycetes may provide important clues to understand their possible ecological role and adaptive strategies. The potential and extraordinary stability of these enzymes would make them economically viable in commercial applications.

Denaturing gradient gel electrophoresis

- We employed molecular methods: 16S rRNA gene amplification using universal and species specific primers in combination with DGGE as a fingerprinting technique to judge the molecular diversity of salt tolerant alkaliphilic actinomycetes.

- The spectrophotometric analysis revealed that the extracted genomic DNA from nearly all the isolates had good purity, reflected by A_{260}/A_{280} ratio being in the range of 1.4 - 1.8.

- Among the 12 isolates, 16S rRNA gene of 7 isolates (58%) from Okha Madhi were amplified with U1 primer yielding1500bp product, 9 isolates (75%) were amplified with U2 primer giving 1000bp amplification product, 7 isolates were amplified with StrepB/E while only OM-2 isolate was amplified with StrepB/F primer set. With *Nesterenkonia* specific primer set N-F/R, 16S rRNA gene of 4 isolates were amplified having 1120 bp product.

- Among the 10 isolates from Okha site, 16S rRNA gene of 5 isolates (50%) were amplified with U1 primer yielding 1500bp product, 7 isolates (70%) amplified with U2 primer with 1000bp product, five isolates were amplified with StrepB/E while 6 isolates were amplified with StrepB/F primer set. With *Nesterenkonia* specific primer N-F/R, 16S rRNA gene of OK-1 and OK-8 were amplified with 1120 bp product.

- PCR amplification and DGGE fingerprinting profile was compared with the morphological and physiological properties of actinomycetes isolates and it was revealed that majority of them belonged to *Streptomyces* followed by *Nocardiopsis* and *Nesterenkonia*.

- Further, different RFLP patterns obtained were compared with the corresponding DGGE band pattern and based on that actinomycetes could be identified at the genus level i.e., *Streptomyces* and *Nocardiopsis*.

Secretion and optimization of extracellular enzymes

- The isolates displayed diversity with respect to growth patterns and production of extracellular enzymes under different conditions of salt and pH. Majority of the isolates could grow and produce enzymes at 0-10% salt while some tolerated up to 15 and 20% NaCl. The isolates grew and produced proteases,

amylases and cellulases over a broader range of pH; 7-11 and 8-11 signifying enormous tolerance to alkaline pH for protease production. Overall, more than 60% of the isolates produced substantial level of extra cellular alkaline proteases, amylases and cellulases at 10% NaCl and pH 9.

- Growth kinetics and alkaline protease production of nine best protease producing isolates were followed up to 15 days at varying concentrations of NaCl and pH. It revealed that proteases were produced in the range of 5-15% NaCl with optimum protease at 15% (OM-11) and 10% (OM-4, OK-1, OK-5 and OK-7).

- Although the growth was comparable at pH 8-10, optimum protease production was at the alkaline pH range indicating alkaliphilic nature of the majority proteases from 9 isolates of actinomycetes. At all pH values, the optimum protease secretion was during the late exponential to early stationery phase.

Repression of alkaline protease

- Further repression studies on the basis of potential of OM-4, OM-6, OM-11, OK-5, OK-7 and Tata-13 for the protease production. Complex organic nitrogen sources such as yeast extract and peptone displayed enzyme repression at higher concentrations in each isolate.

- Further, in majority of the isolates studied, protease production was repressed gradually with increasing phosphate concentrations. While the growth was quite stable at high glucose concentrations, enzyme production was repressed in its presence.

- Amino acids of class 1 category supported the growth. However, protease production was better with methionine even though the growth was adversely affected. Protease production was not significantly affected by leucine compared to that with methionine.

- Among the aromatic amino acids (class 2), both phenylalanine and tyrosine induced the growth, while protease production was optimum at 1% w/v of both aminocids. Asparagine (class 5) displayed reduced growth and protease production at higher concentrations.

- Protease production increased with the increasing number of different amino acids in the growth medium. This stimulation might be due to the cumulative effect of amino acids on the induction of proteases.

Purification and characterization of alkaline proteases

- The active protease was isolated from the culture filtrate by hydrophobic interaction chromatography using Phenyl sepharose 6FF. The two step purification method of purification yielded 35.12 and 27.34 fold purification of alkaline proteases from OM-6 and OK-5, respectively. On the other hand, one step purification method for OM-6 and OK-5 protease exhibited 26.18 and 13.03 fold purification respectively.

- Protein purification was successfully achieved to the homogeneity as evident by a single band on SDS PAGE with molecular mass of 25 KDa and 20 KDa for OM-6 and OK-5 proteases, respectively.

- The optimum temperature of purified protease shifted from 60 to 70°C and 70 to 80°C for OM-6 and OK-5, respectively in 4 M NaCl and 30% Na-glutamate. Significant stability was evident at 60°C-80°C in Na-glutamate.

- Deactivation rate constant (K_d) increased and half life ($t_{1/2}$) decreased with the increasing temperature from 37 to 80°C. The order of stability followed 30% Na-glutamate>4 M NaCl>2 M NaCl> 0 M NaCl.

- The enzyme was stable even at 80°C in 30% Na-glutamate with K_d = 8.66 and $t_{1/2}$ = 80.04 min for OM-6. The values of K_d and $t_{1/2}$ were 4.11 and 168.64 min for OK-5.

- The activation energies (E), enthalpy (ΔH*) and entropy (ΔS*) for protease deactivation calculated in the presence of Na-glutamate were 37.14 KJ/mole, 34.47 KJ/mole and -196.37 J/mole, respectively. While the same parameters calculated in the presence of Na-glutamate for OK-5 were 31.97 KJ/mole, 29.23 KJ/mole and -211.83 J/mole, respectively.

- The changes in free energy (ΔG*) for protease deactivation at 60°C in 30% Na-glutamate were 101.62 KJ/mole and 101.70 KJ/mole for OM-6 and OK-5 respectively.

- The optimum temperature of OM-6 and OK-5 purified proteases were 60°C and 70°C, respectively, with 0-100mM Ca^{2+}. There was significant stability between 50°C-80°C.

- The OM-6 and OK-5 alkaline proteases were stable at 70°C and 80°C in the presence of 100mM Ca^{2+} with $K_d = 20\times10^{-3}$ and $t_{1/2}$ = 34 min for OM-6 and $K_d = 17\times10^{-3}$ and $t_{1/2}$ = 32 min for OK-5.

- The activation energies (E), enthalpy (ΔH*) and entropy (ΔS*) for OM-6 protease deactivation calculated in Ca^{2+} were 29.35 KJ/mole, 26.68 KJ/mole and -186.22 J/mole, respectively. While the same parameters calculated for OK-5 protease in the presence of 100mM Ca^{2+} were 35.49 KJ/mole, 35.49 KJ/mole and -183.48 J/mole, respectively.

- The changes in free energy (ΔG*) for OM-6 and OK-5 protease deactivation at 60°C in 200mM Ca^{2+} were 95.10 KJ/mole and 95.88 KJ/mole, respectively .

- Protease exhibited highest activity and maximum stability at 10-11 pH with Borex-NaOH buffer system. The stability in the range of pH 10-11 even after prolong incubation reflected alkaliphilic nature of the protease.

- In addition, enzyme was highly resistant against chemical denaturation and displayed varied effects towards metal ions, while completely inhibited by 10mM PMSF indicating its serine nature.

- Among the osmolytes examined, Na-glutamate (30%) enhanced the activity by 183.80% and 232% for OM-6 and OK-5, respectively. Interestingly, % maximal activity of serine alkaline protease also increased in H_2O_2, β-mercaptoethanol and different surfactants.

- In addition, enzyme displayed enormous stability towards different cations, oxidizing and reducing agents and various surfactants. These properties make the enzymes ideal choice for application in detergent formulations and enzymatic peptide synthesis.

- K_m and V_{max} for OM-6 alkaline protease were 0.50 mg/ml and 3634.12 U/min respectively while alkaline protease from OK-5 displayed 0.18mg/ml and 2414.22 U/min values for both K_m and V_{max} respectively.

Cloning and expression of proteases

- High quality and yield of genomic DNA of OM-6 and OK-5 and plasmid DNA pET21a$^+$ were obtained. On the basis of assessment and visualization of sample on agarose gel, it was indicative that retrieved DNA was amenable for further molecular biology work.

- Genomic DNA from the both isolates was amplified by six sets of primers. Among different sets of primers, amplification was obtained by SPS-5 and SPS-6. The molecular weights of amplicons were in the range of 0.5-1.0 kb. The specificity of amplification was due to the designed primers based on the haloalkaliphilic protease gene sequences.

- The PCR product of OM-6 was digested with Bam H1 and Sal1 while that of OK-5 with BamH1 and Nde1. Correspondingly, vector was digested with the same REs. The construction of recombinant plasmids was confirmed on the basis of RE digestion followed by the analysis on agarose gel. High transformation efficiency was achieved on LB plates containing ampicillin.

For functional analysis, the transformed clones were analyzed on gelatin agar plate to judge their protease secretion.

- Positive clones secreting substantial level of protease enzyme were used for expression analysis. Effect of induction, temperature and its synergistic effect were analyzed for growth and enzyme secretion.

- Large amount of enzyme was evident in insoluble fraction; however, traces of enzyme were also portioned in soluble fraction. Presence of enzyme in insoluble fraction was due to inclusion bodies formation. Effect of renaturation was analyzed on insoluble fraction and enzyme regained about 5 fold activities as compared to its denatured counterpart.

- Comparative analysis of recombinant enzyme production at optimum conditions with respect to induction and temperature, depicted picture which correlated with the available literature. Based on the SDS-PAGE profile and enzyme activity measurements, soluble and insoluble fractions collected at different hours after induction was analyzed.

- While SDS-PAGE profile displayed the expression of recombinant enzymes, the over-expression of the gene was not evident. Expression of enzyme was attributed to vector harboring properties and no activity was observed before the induction. There was low level of expression at 2h which subsequently increased with time of growth.

Conclusions

Studies on salt tolerant alkalilphilic actinomycetes from saline and alkaline habitats along the coastal region of Gujarat focused on 40 different salt tolerant alkaliphilic actinomycetes. The isolates were diversified on the basis of morphology, growth behavior, cultural characteristics, physiological characters, molecular traits as well as antibiotic production and susceptibility.

PCR amplification profile of 16S rRNA genes and DGGE fingerprinting patterns from actinomycetes proved as useful molecular tools in diversifying the isolates. Comparison of DGGE band patterns with RFLP pattern conformed that majority of isolates belonged to *Streptomyces* followed by *Nocardiopsis* and *Nesterenkonia*.

The isolates were screened for extracellular alkaline proteases, amylases and cellulases in the range of 0-20% NaCl and pH 7-11. More than 60% isolates secreted all the 3 enzymes at 10% NaCl and pH 9.

The growth kinetics and alkaline protease production of nine best protease producing isolates revealed that the isolates tolerating high salt and pH were potent enzyme producers. Protease synthesis was repressed beyond the threshold level of amino acids and other nitrogen sources.

Single step purification and characterization of alkaline proteases from potent protease producing strains; OM-6 and OK-5 exhibited shift in temperature optima from 60°C to 70°C and 60°C to 80ºC, respectively at higher salt concentrations. The enzyme was also stable at higher temperatures for significant time in presence of salt as also revealed from the thermodynamic data, such as half life($t_{1/2}$), deactivation rate constant(K_d), change in enthalpy (ΔH^*), change in entropy (ΔS^*) and free energy change (ΔG^*).

Alkaline protease was highly stable in cations, oxidizing and reducing agents, surfactants, H_2O_2, β-mercaptoethanol and different commercial detergents. Denaturation of proteases indicated its extremely resistant nature against urea. This

property of the enzyme holds significance and further structural elucidation may provide clues on such resistance.

Cloning and expression of alkaline protease genes from OM-6 and OK-5 was carried out followed by the characterization of recombinant enzymes. This would pave the way for further production of recombinant enzymes.

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Zvyagintsev, D. G., Zenova, G. M. and Oborotov, G. V. Moderately Haloalkaliphilic Actinomycetes in Salt-Affected Soils. *Eurasian Soil Science,* 2009, 42(13): 1515-1520.

Appendices

APPENDIX I

I. PAPERS COMMUNICATED/ACCEPTED/PUBLISHED

Manuscripts Submitted

1. **Gohel S.D**. and Singh S.P. Single step purification, characterization and thermodynamic studies on a highly thermo stable alkaline protease from a newly isolated salt-tolerant alkaliphilic actinomycete
2. **Gohel S.D**. and Singh S.P. Purification strategies, characteristics and thermodynamic analysis of a highly thermostable alkaline protease from a salt-tolerant alkaliphilic actinomycete, *Nocardiopsis alba* OK-5

Manuscripts ready to submit

1. **Gohel S.D**. and Singh S.P. Cloning and expression of alkaline proteases among salt tolerant alkaliphilic actinomycetes strains OM-6 and OK-5
2. **Gohel S.D**. and Singh S.P. Characterization of oxidant and denaturant stable purified alkaline protease from salt tolerant alkaliphilic actinomycetes strain OM-6
3. **Gohel S.D**. and Singh S.P. Characterization of oxidant and denaturant stable purified alkaline protease from salt tolerant alkaliphilic actinomycetes strain OK-5
4. **Gohel S.D**. and Singh S.P. Assessment of diversity based on DGGE (Denaturing gradient gel electrophoresis) analysis among the salt tolerant alkaliphilic actinomycetes form Okha Madhi
5. **Gohel S.D**. and Singh S.P. Molecular diversity based on DGGE (Denaturing gradient gel electrophoresis) analysis among the salt tolerant alkaliphilic actinomycetes form Okha

6. **Gohel S.D**. and Singh S.P. Evaluation of diversity among salt tolerant alkaliphilic actinomycetes based on antimicrobial activity profile and RFLP analysis

7. **Gohel S.D**. and Singh S.P. Comparative studies on the production and characteristics of alkaline proteases from the salt-tolerant and alkaliphilic actinomycetes isolated from coastal Gujarat (India)

8. **Gohel S.D**. and Singh S.P. Growth characteristics and enzyme secretion among the salt-tolerant and alkaliphilic actinomycetes isolated from coastal region of Gujarat, India

9. **Gohel S.D.,** Thumar J.T. and Singh S.P. Alkaline exoglucanase from halo-tolerant and alkaliphilic actinomycete, *Streptomyces clavuligerus* Mit-1

10. Trupti T., **Gohel S.D.,** Megha M.K. and Singh S.P. Molecular diversity of protease producing salt tolerant alkaliphilic actinomycetes from the saline habitats of coastal Gujarat

II. BOOK CHAPTERS

PUBLISHED/ACCEPTED/COMMUNICATED

Published

1. Singh, S.P., Thumar J.T., **Gohel S.D.** and Purohit, M.K. Molecular diversity and enzymatic potential of salt-tolerant alkaliphilic actinomycetes. In Current Research Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. (Ed. A. Mendez-Vilas) **Formatex Publications**. pp. 280-286, 2010

Accepted

1. Singh, S.P., Raval, V. H., Purohit, M. K, Thumar J.T., **Gohel, S.D.,** Pandey, S., Rawal C.M. and Akbari V.G. Haloalkaliphilic bacteria and actinobacteria

from the saline habitats: new opportunities for biocatalysis and bioremediation. Microbes in environmental management and biotechnology. **Springer publication** 2010

2. Singh SP, Raval VH, Joshi RH, Dodia MS, Rawal CM, Purohit MK, Pandey S, Thumar JT and **Gohel SD**. Enzymes from haloalkaliphilic bacteria and actinomycetes: Potential candidates for detergent additives. "Detergents" **NOVA Publisher**, New York 2009

III. SEQUENCES SUBMITTED IN NCBI

16S RIBOSOMAL RNA GENE

1. **Gohel S.D.** and Singh S.P. *Brachystreptospora xinjiangensis* strain OM-6 16S ribosomal RNA gene, partial sequence. **GenBank: EU710555.1**
2. **Gohel S.D.** and Singh S.P. *Nocardiopsis alba* strain OK-5 16S ribosomal RNA gene, partial sequence. **GenBank: HM560975**
3. **Gohel S.D.** and Singh S.P. *Streptomyces* sp. OK-7 16S ribosomal RNA gene, partial sequence. **GenBank: HM560976**

APPENDIX II

I PAPER/POSTER PRESENTATIONS

Oral presentations

1. **Gohel S.D.** and Singh S.P. Cloning and expression of alkaline serine protease genes form salt tolerant alkaliphilic actinomycetes isolated from the saline habitats of coastal Gujarat, India - "Biochemisty: Recent advances in molecular research" 2011, Saurashtra University, Rajkot - **Awarded First prize**
2. Sharma A., **Gohel S.D.** and Singh S.P. Assessment of molecular diversity and enzymatic potential of salt tolerant alkaliphilic actinomycetes based on bioinformatics tools - "Biochemisty: Recent advances in molecular research" 2011, Saurashtra University, Rajkot
3. **Gohel S.D.** and Singh S.P. Halo-tolerant and alkaliphilic actinomycetes from the saline habitats of coastal Gujarat: Diversity and biocatalytic potential- Science Excellence, Ahmedabad (India), 2010 - **Awarded second prize**
4. **Gohel S.D.** and Singh S.P. Assessment of molecular diversity and biotechnological potential of salt tolerant alkaliphilic actinomycetes along the habitats of coastal Gujarat - Swarnim Gujarat symposium on trends in biological sciences, Department of Biosciences Saurashtra University, Rajkot 2010
5. **Gohel S.D.** and Singh S.P. Halo-tolerant alkaliphilic actinomycetes from the saline habitats of coastal Gujarat: Diversity based on morphological features, enzyme secretion, antimicrobial activity and molecular parameters - Microbial technology for sustainable environment, Ahmedabad (India), 2009 - **Awarded First prize**
6. Dalsania T.L., **Gohel S.D.,** Purohit, M.K., Singh S.P. Molecular diversity of salt tolerant alkaliphilic actinomycetes from coastal region of Gujarat. Science Excellence at Gujarat University, Ahmedabad (India), 2009

Poster presentation

1. Thakar F., Jadeja, H., **Gohel S. D.,** Singh, S.P., Media optimization and repression analysis of alkaline proteases from salt tolerant alkaliphilic actinomycetes. Swarnim Gujarat symposium on recent trends in life sciences, Department of Biosciences, Saurashtra University, Rajkot 2011
2. Thakar F., **Gohel S. D.,** Singh, S.P. Production of alkaline serine proteases from salt tolerant alkaliphilic actinomycetes strains OM-6, OK-7 and Tata-13 in minimal and complex media. “Biochemistry: A global approach from molecules to cell” Department of Biochemistry Saurashtra University, Rajkot 2011
3. Jadeja, H., **Gohel S. D.,** Singh, S.P. Media Optimization & repression studies of alkaline serine proteases from salt tolerant alkaliphilic actinomycetes strains: OM-4, OK-5 & OM-11. “Biochemistry: A global approach from molecules to cell” Department of Biochemistry Saurashtra University, Rajkot 2011
4. **Gohel S. D.** Enzyme Immobilization. Department of Biosciences, Saurashtra University, Rajkot 2007

II. WORKSHPS/SEMINARS ATTENDED

List of conferences attended

1. Swarnim Gujarat symposium on recent trends in life sciences, Department of Biosciences Saurashtra University, Rajkot 2011
2. “Biochemistry: A goblal approach from molecules to cell” held at Department of Biochemistry Saurashtra University, Rajkot 2010
3. UGC-DSA national conference on Bioresouces: Utilization and Conservation, Department of Biosciences Saurashtra University, Rajkot 2006
4. UGC sponsored national level seminar on “50 years of DNA double helix retrospect ad prospects” Virani Science College, Rajkot 2004
5. 15th state level seminar of micro study circle at Virani Science College, Rajkot 2003

APPENDIX III

I SCHOLARSHIPS/AWARDS

1. **Awarded Meritorious Fellowship (JRF)** by UGC, New Delhi, India from March 2008 - March 2011

2. **Awarded First prize** in National Conference on “Biochemistry: Recent Advances in Molecular Research” Saurashtra University, Rajkot 2011

3. **Awarded second prize** in Science Excellence, Gujarat University, Ahmedabad 2010

4. **Awarded First prize** in Microbial technology for sustainable environment, Gujarat University, Ahmadabad 2009

5. **Ranked 2nd in M.Sc Microbiology**, Saurashtra University, Rajkot 2007

II TRAINING UNDERTAKEN

ORGANIZATION : Indian Institute of Advanced Research (IIAR), Gandhinagar, Gujarat

BRIEF DESCRIPTION : Hands on Training Programme in “Bioinformatics”

DURATION : 3 Days (December 2008)