PROTEINASE PRODUCTION AND HYDROCARBON DEGRADATION BY ACTINOMYCETES

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CONTENTS

DECLARATION

Certified that the work incorporated in the thesis entitled " **Proteinase production and hydrocarbon degradation by actinomycetes**" submitted by **Vaishali .S. Dixit** was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

Dr. Aditi Pant
Research Guide

ACKNOWLEGDEMENT

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LIST OF ABBREVIATIONS

Boc-	t-butyloxycarbonyl
Bz-	Benzoyl
CBZ-	Carbobenzoxy
DAB	Diaminobutyric acid
DAP	Diaminopimelic acid
DEP	Diethyl pyrocarbonate
DFP	di-isopropyl fluorophosphate
DMSO	Dimethylsulphoxide
DTNB	Dithiobisnitrobenzene
EDTA	Ethylene diamine tetraacetic acid
EDAC	1-ethyl-3-(3-dimethylaminopropyl)azonia carbodimide
HNBB	Hydroxy nitro benzyl bromide
IAA	Iodoacetic acid
NAI	N-acetylimidazole
PBS	Phosphate buffered saline
PCMB	p-Chloromercuribenzoate
PMSF	Phenyl methyl sulfonyl fluoride
PNA	paranitoanilide
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TLCK	tosyl lysine chloromethyl ketone
TNBS	Trinitro benzene sulphonate
Tosyl-	4-toluenosulphonyl
TPCK	tosylamido-2-phenylethyl chloromethyl ketone
TRIS	tris-(hydroxymethyl)-aminomethane
WRK	Woodward's Reagent K
Z-	Benzyloxycarbonyl

SUMMARY

Actinomycetes are microorganisms known for their diverse biotechnological potential. They are known to produce a wide variety of antibiotics, extracellular enzymes and enzyme inhibitors and also for their ability to degrade complex biological polymers and hydrocarbons.

Proteases from bacteria and fungi have been extensively studied, whereas those from actinomycetes are not so well-characterized, and also seem interesting owing to their unusual substrate specificities. Even among actinomycetes, proteolytic enzymes from *Streptomyces* species are better characterized as compared to those from the non-streptomycete group of actinomycetes. More studies need to be directed towards proteases from this lesser studied group in order to obtain a clearer understanding about their characteristics and significance. Several actinomycetes are also known to degrade a wide variety of hydrocarbons. Organisms with such capabilities could be useful in the development of biotechnological methods to clean up environmental pollution.

The work presented in this thesis deals with –

1. Isolation of actinomycetes from environmental samples, screening for production of extracellular proteases and for hydrocarbon degradation and identification of the selected isolate.
2. Optimization of culture conditions for production of extracellular protease by the organism and the purification and comparative characterization of two extracellular proteases secreted by the organism.
3. Preliminary studies on hydrocarbon degradation by the isolate.

Chapter 1

General Introduction

The biochemical and ecological diversity of the actinomycetes, proteolytic enzymes from actinomycetes as well as other sources, and hydrocarbon degradation by actinomycetes have been discussed in this chapter. The chapter presents a framework for the studies conducted in this thesis.

Chapter 2

Isolation and screening of actinomycetes for protease production and hydrocarbon degradation and identification of an isolate showing both properties

About forty–five actinomycete isolates were obtained by selective isolation from environmental samples. These were screened independently for the degradation of Bombay High crude oil and for the production of extracellular proteases. Two isolates were found to degrade crude oil and about eleven isolates showed significant amounts of extracellular protease activity. One isolate was found to exhibit both the properties. The genus level identification of this particular isolate indicated that it belonged to the non-streptomycete group of actinomycetes. This particular isolate was selected for further studies. The isolate was assigned to genus *Nocardiopsis* on the basis of the distinct morphology showing long chains of elongated spores formed by the aerial hyphae, a cell wall chemotype IIIC i.e containing meso–diaminopimelic acid without any characteristic sugar, and the absence of mycolic acids. Our isolate was compared with the type culture, *Nocardiopsis dassonvillei* ATCC 23218, with respect to the morphology, cell wall composition, mycolic acid analysis, DNA base composition and biochemical tests and the degradation of specific compounds. The isolate has been deposited with the National Collection of Industrial Microorganisms as *Nocardiopsis* sp. NCIM 5124.

Chapter 3

Production, purification and comparative characterization of proteases from *Nocardiopsis* sp. NCIM 5124

Extracellular protease was maximally produced (about 14 U/ml) in a medium containing 1% starch, 1% casein, 0.1% K_2HPO_4, 0.2% glucose and 1% Na_2CO_3, pH 10.0, at 30°C and 200 rpm, after 4 days of growth. Electrophoretic separation of the crude culture filtrate, and activity staining using casein–agarose showed that there were two protease fractions.

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Protease I was purified to homogeneity by ion exchange chromatography using CM-Sephadex at pH 5.0 and again at pH 9.0 with a yield of about 20%. Protease II was purified by ion exchange using DEAE-cellulose, gel filtration on Sephadex G-50, hydrophobic chromatography using phenyl-Sepharose and colour removal by treatment with hydroxyapatite, with a yield of 43%.

Comparative characterization studies with the two proteases showed that Protease I had a M_r of about 21,000 daltons (SDS-PAGE), pI of 8.3, specific activity of 152 units/mg using casein as substrate and optimum pH and temperature values of 10.0 −11.0 and 65°C respectively. Protease II had a M_r of about 23,000 daltons (SDS-PAGE), pI of 7.0, specific activity of 14 units/mg using casein as substrate and optimum pH and temperature values of 10.0 and 60°C respectively. Both proteases were almost completely inhibited by 1mM PMSF , a specific serine protease inhibitor, indicating that they were both serine proteases. The other class specific protease inhibitors, namely EDTA (metallo proteases), IAA (cysteine proteases) and pepstatin (aspartic proteases) had no effect on either protease I or II. Protease II was found to be inhibited by the chlorides of potassium, barium, calcium, zinc, copper, aluminium, magnessium and iron whereas none of these metal ion salts had any effect on Protease I. Limited substrate specificity studies using synthetic substrates showed that both proteases lacked exopeptidase activities against amino and carboxypeptidase substrates suggesting that both proteases are endopeptidases. Among the endopeptidase substrates tested, tri and tetrapeptide substrates were preferentially hydrolyzed as compared to mono or dipeptide substrates by both proteases, indicating that substrate binding in an extended form is required for efficient hydrolysis. Neither protease showed any esterase activity, as checked with ester substrates. Polyclonal antisera raised in rabbits against protease I did not cross-react with protease II, trypsin, chymotrypsin or subtilisin. Both proteases did not contain free cysteines or disulphides and resembled the subtilisins in this respect.

Chapter 4

Preliminary studies on hydrocarbon degradation by

Nocardiopsis sp. NCIM 5124

Bombay High crude oil was maximally degraded (66%) by *Nocardiopsis* sp. NCIM 5124 when supplied at a concentration of 0.1 to 0.3% w/v with 70 mM NH_4Cl as the inorganic nitrogen source. When organic nitrogen sources were supplied, crude oil was maximally degraded by the organism using yeast extract although the degradation in this case (47%) was less than that with NH_4Cl at similar nitrogen concentrations. Protease activity in media containing organic nitrogen sources was about 0.2 U/ml whereas that with NH_4Cl was as low as 0.02 U/ml. Protease was maximally produced (0.72 U/ml) with coconut press cake, a cheap source of organic nitrogen with 25% degradation of the supplied crude oil. In a different set of experiments when crude oil (0.3%) or C_{14} to C_{18} alkanes (0.5%) were individually supplied instead of starch and glucose in the optimized protease production medium at pH 10.0, an extracellular protease activity of about 0.3 U/ml together with 34% degradation of the crude oil or 60 to 80% degradation of the alkanes was observed.

A composition consisting of coconut press cake, the organism on a carrier and a source of inorganic phosphate is suggested as an alternative biotechnological approach for oil-spill clean up in field trials.

Chapter 5

General Discussion

In this chapter the salient features of the thesis have been discussed with reference to the published literature on actinomycete classification and actinomycete proteases.

CHAPTER 1

GENERAL INTRODUCTION

Actinomycetes are bacteria capable of forming branching filaments at some stage of their development. They are also described as bacteria with a fungal morphology or as filamentous, Gram positive bacteria , although parts of their thallus may be Gram negative. Ferdinand Cohn (1875) first described an actinomycete, when he observed a filamentous organism in a concretion from a human lacrimal duct and named it *Streptothrix foersteri*. C.O. Harz, two years later, gave the name *Actinomyces bovis* to an organism isolated from a case of bovine lumpy jaw. The name actinomyces means ray-fungus in Greek, and actinomycetes are still referred to as Ray-Fungi (Lechevalier and Lechevalier, 1981).

Actinomycetes occur as spores or vegetative forms in a wide variety of habitats such as soil, plant litter, manures and composts, food products, aquatic environments and also in association with plants, animals and man. Thermophilic actinomycetes such as species of *Thermoactinomyces, Thermomonospora, Streptomyces, Pseudonocardia,* and *Streptosporangium* are frequently found in materials where self heating occurs, such as hay, grain, compost, soil, plant litter, food products and water.

The roles actinomycetes play in these environments, as discussed by Goodfellow and Williams (1983) include degradation of complex polymers like chitin, lignin and cellulose, that are found to occur naturally in soil and plant material , biocontrol of pathogenic bacteria and fungi by the production of anti-bacterial and anti-fungal antibiotics, deterioration of stored food products, and pathogenesis in plants, animals and also man. Plant diseases like potato scab and rot of sweet potato are caused by actinomycetes. They are also the causative agents of diseases like tuberculosis, mycetomas, paratuberculosis, actinomycosis, allergic pneumonias and various abscesses in man.

The actinomycetes stand out as a unique group of prokaryotic organisms in two respects: the diversity of their morphology and the diversity of their metabolic products. The prime example of the latter is the plethora of antibiotics produced principally as secondary metabolites late in the growth cycle. These antibiotic molecules, some with

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quite complex structures and with complicated biosynthetic pathways, number in the hundreds (Umezawa, 1967; Ensign, 1978). Actinomycetes are producers of most of the known antibiotics, compounds that are useful not only in human and veterinary medicine but also in agriculture and biochemistry (Waksman and Lechevalier, 1962; Okami, 1973). The actinomycetes are also known to produce a variety of pigments, extracellular enzymes and the terpenoid compounds that give soil its characteristic odour (Ensign, 1978).

A variety of low molecular weight enzyme inhibitors are also produced by actinomycetes. These enzyme inhibitors produced by both actinomycetes and other microorganisms are released extracellularly. They are low molecular weight compounds, and they differ from enzyme inhibitors of plant and animal origin which are macromolecular peptides that often co-exist with the enzymes they inhibit. Enzyme inhibitors isolated from culture filtrates of actinomycetes especially streptomycetes include inhibitors of proteases like trypsin, chymotrypsin, elastase, papain, cathepsin B, glycosidases such as sialidase, β-galactosidase, α and β glucosidases and also several other enzymes like xanthine oxidase, reverse transcriptase, fumarase, glutamate decarboxylase and aspartate amino transferase (Umezawa, 1988).

The actinomycetes are classified into the oxidative and the fermentative subgroups. The morphologically simple fermentative actinomycetes are opportunistic pathogens of animals (Lechevalier and Pine, 1977). The vast majority of actinomycetes are oxidative and aerobic. These are taxonomically characterized on the basis of morphological and chemical criteria (Lechevalier, 1968; Lechevalier and Lechevalier, 1970a; 1970b; Lechevalier *et al*, 1971) using the presence or absence and the type of DAP isomer, whole cell sugar patterns and the presence or absence of mycolic acids for identification. Organisms containing mycolic acids have been further classified depending on the molecular weights of the mycolates (Alshamaony *et al*, 1976; Collins *et al*, 1982).

The biochemical heterogeneity of actinomycetes and their ecological diversity has made them an obvious target for enzymes displaying new activities and/or specificities. Actinomycetes have been found to be a promising source of a wide range

of biotechnologically important enzymes which are of potential interest in areas such as enzyme processes, clinical chemistry and medical therapy, and actinomycete enzymes are sometimes favoured over other sources because they possess greater stability, activity at high temperatures and unusual substrate specificity (Peczynska-Czoch and Mordarski, 1988; Demain, 1988). The genera important in these respects are the *Streptomyces, Nocardia, Rhodococcus, Thermomonospora* and *Thermoactinomyces.*

Several *Streptomyces* species are known to produce glucose isomerase, an important enzyme used for the production of high fructose corn syrups (Demain, 1988). Lipases have been reported from *Rhodococcus erythropolis* (Kurane *et al*, 1984) and some *Streptomyces* strains (Chakrabarti *et al*, 1978). Endonucleases have been isolated from *Streptomyces* and *Nocardia* sps. and about fifteen restriction endonucleases from actinomycetes are now available commercially (Kessler *et al*, 1985). Amylases have been reported from *Streptomyces limosus* (Fairbairn *et al*, 1986), *S. praecox* (Suganuma *et al*, 1980), *S. hygroscopicus* (McKillop *et al*, 1986) and from thermophilic actinomycetes like *Thermomonospora curvata* (Stutzenberger and Carnell, 1977), *T. vulgaris* (Shimizu et al, 1978) and a *Thermoactinomyces* sp. (Obi and Obido, 1984 a,b). Cellulolytic complexes have been reported from *S. antibioticus* (Enger and Sleeper, 1965), *S. flavogriseus* (MacKenzie *et al*, 1984), *S. viridosporus* (Deobald and Crawford, 1987) and *Thermomonospora* species (Stutzenberger, 1972; Crawford, 1975; Waldron and Eveleigh, 1986). Xylanases have been reported from *S. lividans* (Kleupfel *et al*, 1986), other *Streptomyces* species (Nakajima *et al*, 1984) and from *Thermomonospora* sp. (McCarthy *et al*, 1985). Chitinolytic complexes have been isolated from culture filtrates of *S.griseus* (Berger and Reynolds, 1958), *S. antibioticus* (Jeuniaux, 1966) and several other *Streptomyces* species (Price and Storck, 1975; Beyer and Diekman, 1985). β-lactamases have been reported from *S. cacaoi* (Ogawara *et al*, 1981) and *S. cellulosae* (Ogawara and Horikawa, 1979). All these underline the adaptability of the actinomycetes and perhaps explain their almost universal occurrence in the biosphere.

The ability of the actinomycetes to produce a wide variety of proteolytic enzymes (Peczynska-Czoch and Mordarski, 1988) is also another aspect of this adaptability.

Proteases reported from actinomycetes include those from *Streptomyces griseus* (Narahashi, 1970; Awad *et al*, 1972 a), *S. fradiae* (Morihara *et al*, 1967; Nickerson and Durand, 1963; Nadanishi and Yamamoto, 1974; Lagutina and Petrova, 1979), *S. rimosus* (Vitale *et al*, 1986), *S. erythraeus* (Yoshida *et al*, 1971), *Actinomadura* sp.(Rippon, 1968; Norwig, 1971), *Nocardiopsis dassonvillei* (Tsujibo *et al*, 1990a) and also from thermophilic actinomycetes belonging to genera *Thermoactinomyces* (Mordarski *et al*, 1976 ; Kleine, 1982), *Thermomonospora* (Desai and Dhala, 1969) and *Streptomyces* (Mizusawa *et al*, 1969).

A collagenase-like serine protease called Thermitase has been reported from *Thermoactinomyces vulgaris* (Kleine, 1982) , which is capable of degrading native collagen and elastin. It is stable at temperatures of 60-85°C and at pH values ranging from 7.5 to 9.5. Apart from *Clostridium* collagenase, only thermitase has been found to liquify collagen efficiently at pH 8.0. Collagenase like enzymes have also been reported to be produced by *Streptomyces* sps. (Chakraborty and Chandra, 1984) and *Actinomadura* sps (Rippon, 1968; Norwig, 1971). *Streptomyces fradiae* has been reported to produce Keratinase-like enzymes (Nickerson and Durand, 1963; Nadanishi and Yamamoto,1974) . Several other actinomycete proteases have been reported which include aminopeptidase from *S. rimosus* (Vitale *et al*, 1986), carboxypeptidase from *Thermoactinomyces* sp. (Osterman *et al*, 1984), trypsin-like serine protease from *S. erythraeus* (Yoshida *et al*, 1971), elastase-like enzymes from *S. griseus* (Gertler and Trop, 1971) and *S. fradiae* (Lagutina and Petrova, 1979), a highly thermostable protease from *S. rectus var proteolyticus* (Mizusawa *et al*, 1969) and a highly alkalophilic protease from *Streptomyces* sp. (Nakanishi *et al*, 1974).

Proteases are hydrolytic enzymes that catalyze the cleavage of peptide bonds. They are produced both intra and extracellularly by animal and plant cells and microorganisms. Literature on microbial proteases has been extensively reviewed (Aunstrup, 1980; Ward,1983; Kalisz, 1988; Rao *et al*, 1998). Proteases are classified into two major groups on the basis of the nature of attack–the peptidases or exopeptidases and the proteinases or endopeptidases. The exopeptidases cleave peptide

bonds in the terminal region of the peptide chains removing either single aminoacids or dipeptides. Exopeptidases are further subdivided according to whether they act at the carboxy or amino terminal of the peptide chain (carboxy or amino peptidases). The endopeptidases or proteinases cleave internal peptide bonds in the peptide chains and are classified on the basis of the amino acid residues responsible for their catalytic activities, into serine, cysteine, aspartic and metallo proteinases. This classification is based on the reactivity of proteases with active-site directed inhibitors.

Microbial proteinases belonging to these four mechanistic classes have been further classified on the basis of their side-chain specificity towards oxidized insulin β chain (Morihara, 1974) as follows :

I. Serine proteinases:

The serine proteinases are characterized by a reactive serine residue in the active site and are inhibited by DFP and PMSF. They are maximally active in the pH range of 7.0 to 11.0, have molecular masses of about 18-35 kDa and usually have broad substrate specificities. The serine proteinases are divided into four sub-groups according to their side chain specificity towards oxidized insulin β -chain.

a. Trypsin-like proteinases:

These are specific for basic aminoacids on the carboxyl side of the scissile peptide bond.

b. Alkaline proteinases :

These preferentially hydrolyze peptide bonds with aromatic or hydrophobic residues like tyrosine, leucine, phenylalanine on the carboxyl side. The alkaline serine proteinases include–

–the subtilisins, produced by *Bacillus licheniformis* and related bacilli and

–other microbial alkaline serine proteinases like those produced by alkalophilic *Bacillus* sps., by *Aspergillus* sps. like *A. flavus, A. oryzae, A. sojae* and by *Streptomyces* sps. like *S. rimosus* and *S. rectus*.

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c. Myxobacter α–lytic proteinase:

This enzyme is produced by a species of *Sorangium* and exhibits bacteriolytic activity towards a number of soil bacteria. It cleaves peptide bonds involving the carboxyl groups of neutral aliphatic amino acids.

d. Staphylococcal proteinase:

It is produced by a strain of *Staphylococcus aureus* and it cleaves peptide bonds with acidic amino acids at the carboxyl side of the scissile peptide bond.

II. Cysteine proteinases :

Enzymes in this group have cysteine at their active site. They are sensitive to sulphydryl reagents like PCMB, iodoacetic acid, iodoacetamide and are activated by reducing agents such as cysteine and dithiothreitol. Most cysteine proteases are active at pH 5.0 to 8.0. These enzymes are further categorized on the basis of their side-chain specificity as follows–

a. Clostripain : It is derived from the culture filtrates of *Clostridium histolyticum*. It is specific for basic amino acid residues at the carboxyl side of the scissile bond.

b. Streptococcal proteinase :

It is produced by group A *Streptococcus* species as a zymogen, which is converted by autocatalysis into the active enzyme. It has a broad amino acid specificity.

III. Aspartic proteinases:

Proteinases belonging to this class are characterized by a low pH optimum for activity (pH 3.0-4.0) and insensitivity to DFP, PCMB and EDTA, specific inhibitors of the other three groups of proteases. Their catalytic activities involve two aspartic acid residues. They are widely distributed in yeasts and molds but are seldom found in bacteria. Most aspartic proteases have molecular masses in the range of 30-45 kDa and pIs of 3.0–5.0. They are specific for aromatic or bulky amino acids on both sides of the cleavage point. Aspartic proteases are further divided into two groups–

a. Pepsin-like proteinases :

These resemble animal pepsin in their physical and chemical properties. Pepsin-like enzymes are produced by species of *Aspergillus, Penicillium, Rhizopus* and *Neurospora.*

b. Rennin-like proteinases :

These enzymes are capable of clotting milk in a manner similar to animal rennins. They specifically act on the χ-casein fraction of milk and release a soluble peptide, leaving an insoluble fraction which causes milk to clot.

IV. Metallo-proteinases :

These enzymes are inhibited by metal chelating agents like EDTA, but are insensitive to serine proteinase inhibitors and sulphydryl reagents. Most of the bacterial and fungal metallo-proteinases are zinc containing enzymes. The zinc atom is necessary for enzyme activity. Calcium is required to stabilize protein structure. Many of the EDTA inhibited enzymes can be reactivated by zinc, calcium, cobalt. Metallo-proteinases can be divided into five groups–

a. Acid metallo-proteinases :

Enzymes in this group are characterized by low pH optima for activity. Their molecular masses are about 20 kDa. Proteinases produced by *Penicillium roqueforti, P. caseicolum* and *Aspergillus oryzae* are included in this group.

b. Neutral metallo-proteinases :

These enzymes have pH optima around 7.0 and their molecular masses are in the range of 30-40 kDa. They are specific for hydrophobic or bulky amino acid residues. They are widely distributed among *Bacillus* and *Aspergillus species.*

c. Alkaline metallo-proteinases :

These enzymes have pH optima above pH 7.0 for activity and molecular masses of 50-60 kDa. They have very broad substrate specificities and are produced by organisms like *Pseudomonas aeruginosa* and *Serratia marcescens.*

d. Myxobacter proteinase I :

The enzyme has a molecular mass of 14 kDa and optimum pH of 9.0. It is specific for alanine, glycine or serine residues on either side of the cleavage point. This proteinase is capable of lysing cell walls of Gram positive bacteria.

e. Myxobacter proteinase II :

This enzyme has a molecular mass of 17 kDa and pH optimum of 8.5–9.0. It is specific for lysine residues on the amino side of the cleavage point. It does not lyse bacterial cells.

This system for classification of proteases (Morihara, 1974) is based only on side-chain specificity towards oxidized insulin β chain and is applicable only to proteases of microbial origin. More recently proteases have been classified on the basis of their active sites, mechanism of action and three-dimensional structure. In this system, four mechanistic classes of proteases are recognized by the International Union of Biochemistry and within these classes, six families of proteases are recognized (Table 1.1) (Neurath, 1989).

Each family has a characteristic set of functional amino acid residues arranged in a particular configuration to form the active site (Neurath, 1989). A family consists of a group of enzymes that are sequentially homologous and similar in overall 3-dimensional geometry (Kraut, 1977). The serine proteases are further classified into two families–

Serine proteases I comprising of the mammalian serine proteases and serine proteases II, the bacterial serine proteases. Members of the two families differ in the following respects :

1. Complete sequences of the mammalian proteases trypsin and chymotrypsin show no similarities with that of the bacterial serine protease subtilisin BPN' (Kasper and Smith, 1966; Markland and Smith, 1967).

2. Subtilisin contains no disulphide bridges (Markland and Smith, 1967) whereas chymotrypsin contains five (Hartley, 1964) and trypsin contains six (Walsh and Neurath, 1964).

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3. The amino acid sequences adjacent to the 3 amino acids of the charge relay system are different in the two families (Shotton and Hartley, 1970; Smith et al, 1970).

4. Members of these two families also differ with respect to the order of the 3 amino acids of the charge relay system in their primary structures as seen in chymotrypsin and subtilisin, representative enzymes of these 2 families :

Chymotrypsin : His^{57}, Asp^{102}, Ser^{195} (Hartley, 1964 ; Matthews et al, 1967)

Subtilisin BPN': Asp^{32}, His^{64}, Ser^{221} (Smith et al, 1968 ; Wright et al, 1969)

Table 1.1 : Families of proteolytic enzymes (Neurath, 1989)

Family	Representative proteases	Characteristic active site residues *
Serine proteases I	**Chymotrypsin** Trypsin, Elastase, Pancreatic kallikrein	Asp^{102}, Ser^{195}, His^{57}
Serine proteases II	**Subtilisin**	Asp^{32}, Ser^{221}, His^{64}
Cysteine proteases	**Papain** Actinidin, Rat liver cathepsins B and H	Cys^{25}, His^{159}, Asp^{158}
Aspartic proteases	**Penicillopepsin** $Rhizopus$ $chinensis$ and $Endothia$ $parasitica$ acid proteases, Renin	Asp^{33}, Asp^{213}
Metallo proteases I	**Bovine carboxypeptidase A**	Zn, Glu^{270}, Try^{248}
Metallo proteases II	**Thermolysin**	Zn, Glu^{143}, His^{231}

* The number of residues corresponds to the amino acid sequence of the enzymes given in bold letters in column 2.

Between the Chymotrypsin and the Subtilisin families, however, it is very interesting that the reactive serines are in relatively the same positions in the polypeptide

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chain, residue 195 in chymotrypsin and residue 221 in subtilisin (Markland and Smith, 1967). X-ray crystallographic data have demonstrated that the catalytic efficiency of these proteases is due to a charge relay system of single specific aspartyl, histidinyl and seryl residues (Blow *et al*, 1969 ; Wright *et al*, 1969). The three amino acids are widely separated in the primary structures, however in each enzyme tertiary folding brings these amino acids close together to form the catalytic site (Hartley, 1964 ; Matthews *et al*, 1967; Smith *et al*, 1968 ; Wright *et al*, 1969) . Therefore, although these two families differ from each other in amino acid sequence and three-dimensional structure, they share a common active site geometry and catalytic mechanism. It is believed that the two families have descended from unrelated ancestral enzymes and converged upon the same catalytic mechanism (Kraut, 1977 ; Fersht, 1985).

Serine proteases catalyze peptide bond hydrolysis by a two step reaction. The first step involves the formation of a covalent intermediate through an ester bond between the oxygen of serine 195 (chymotrypsin numbering system) and the acyl portion of the substrate, with release of the amino portion of the substrate as the first product or P_1 . In the second step, the nucleophilic attack by a water molecule brings about the deacylation of the covalent intermediate, resulting in peptide bond hydrolysis (Fersht, 1973). A schematic representation of the steps involved in serine protease catalyzed peptide bond hydrolysis is illustrated in figure 1.1.

The best characterized and physiologically most versatile protease family is that of the mammalian serine proteases as exemplified by pancreatic trypsin, chymotrypsin, elastase and kallikrein (Kraut, 1977). Although all these proteases employ the same three amino acid units to hydrolyze peptide bonds, and have similar sequences and 3-dimensional geometry, they differ in their substrate specificity. The differences in their specificity can be attributed to changes in the amino acid residues that line the so-called tosyl pocket, that is, the part of the active site that binds the amino acid side chain which contributes the carboxyl group to the susceptible peptide bond in protein substrates. Such substitutions result from point mutations in the genomic DNA (Kraut, 1977 ; Neurath, 1984).

Figure 1.1

Schematic representation of the steps involved in catalysis by the serine proteases
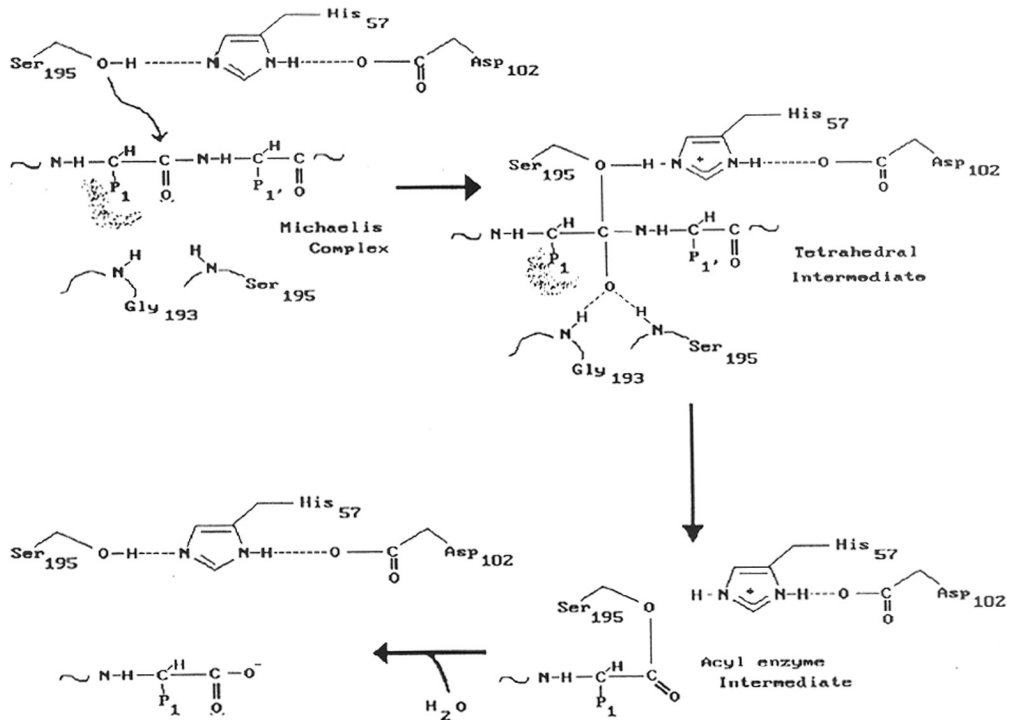
The reaction proceeds through the formation of a tetrahedral intermediate followed by release of the amino portion of the substrate to give an acyl enzyme intermediate. Breakdown of this intermediate occurs by the enzyme-catalyzed attack of water to generate the product. (Reproduced from: Proteolytic enzymes-a practical approach. R.J. Beynon and J.S. Bond ed. IRL Press, Oxford, 1989, p. 58)

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Of the mammalian serine proteases, trypsin is the most selective. It cleaves only the peptide bonds that follow lysine and arginine which are relatively large, carry a positive charge and are hydrophilic. Chymotrypsin is less specific and it hydrolyzes peptide bonds following large hydrophobic amino acids like phenylalanine, tryptophan and tyrosine. Elastase in turn, acts on peptide bonds with small amino acids like glycine, alanine and serine on their carboxyl side. In case of trypsin, the tosyl pocket or the substrate binding pocket has an aspartic acid unit at its back, which carries a negative charge. Thus the positively charged side chains of arginine and lysine of the substrate are held in the pocket by electrostatic forces. In chymotrypsin, the binding pocket is hydrophobic, thereby explaining its specificity for peptide bonds adjacent to amino acids with large, hydrophobic side chains. In elastase, the binding pocket is filled by amino acid side chains and thus, it accepts only the smallest side chains on the substrate molecule. These proteases are believed to have descended from a common ancestor by divergent evolution (Neurath, 1984).

The primary binding site in the substrate binding pocket is however not the only binding site. The enzyme and the substrate need to be secured at several points so that the susceptible bond is oriented in precisely the right configuration at the active site (Stroud, 1974). According to Schecter and Berger (1967), the polypeptide substrate is assumed to bind in an extended form with a length of 3.5 Å per residue and these authors have introduced a nomenclature system to describe the interaction of proteases and their substrates which is used widely in protease literature. According to this system, the binding site for a polypeptide substrate on a protease is envisioned as a series of subsites. Each subsite interacts with one amino acid residue of the substrate. By convention, the amino acid residues on the substrate are called P (for peptide) and the subsites on the protease that interact with the substrate are called S (for subsite). These subsites are in the catalytic site of the protease. As seen in figure 1.1, the amino acid residues on the amino terminal side of the bond to be cleaved (the scissile bond) are numbered P_1, P_2, P_3... counting outwards while the residues on the carboxy-terminal side of the scissile bond are numbered P_1', P_2', P_3'.... The residues can be numbered upto P_6 on each side of the scissile bond. Similarly, the subsites on the enzymes are

numbered as S_3, S_2, S_1, S_1', S_2', S_3' ..... to complement the substrate residues that interact with the enzyme (Fig. 1.2). Thus, the S_1 subsite of trypsin has a marked preference for binding to basic amino acid residues at the P_1 position on the substrate. Differences in substrate specificity can be related to amino acid substitutions in the primary substrate binding site and also to minor differences in the secondary binding sites (Neurath, 1989).

Figure 1.2

The Schecter and Berger nomenclature for binding of a peptide substrate to a protease

The protease is represented as the shaded area. P_3, P_2, P_1, P_1', P_2', P_3' are the side chains of 6 amino acids, and S_3, S_2, S_1, S_1', S_2', S_3' are the corresponding subsites on the enzyme. (Reproduced from: Proteolytic enzymes-a practical approach. R.J. Beynon and J.S. Bond ed. IRL Press, Oxford, 1989, p. 231)

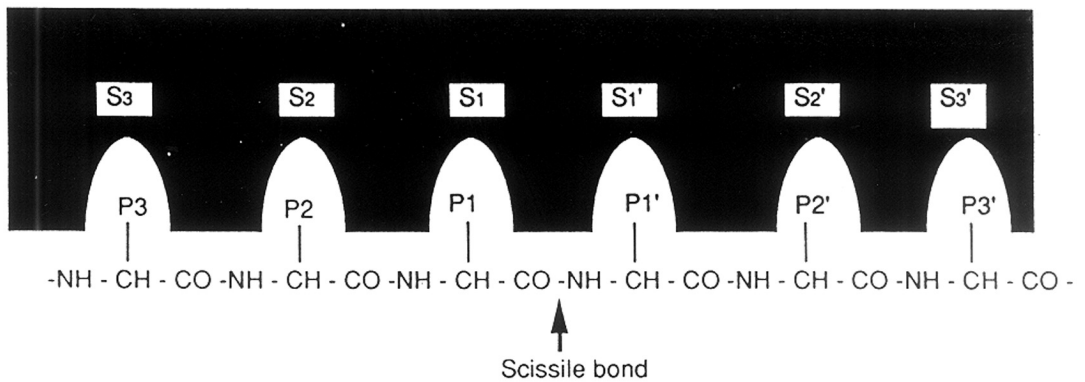Proteases play an important role in metabolic and regulatory processes. Many physiological functions have been attributed to proteolytic enzymes. In higher organisms these range from generalized protein digestion to specific regulated functions such as zymogen activation, blood coagulation, lysis of fibrin clots, release of hormones and pharmacologically active peptides from precursor proteins and the transport of secretory proteins across membranes. In microorganisms extracellular proteases are mainly involved in the hydrolysis of large protein substrates into smaller entities enabling their subsequent absorption by the cell. Besides, microbial proteases are also involved in specific functions like sporulation, spore germination, regulation of other enzymes, protein maturation and secretion.

From an economic point of view, proteases are very important industrial enzymes. They have been successfully used in a wide variety of industries including detergents, leather, food and dairy, breweries and bakery. Proteases have also been found to be medically important. Commercially predominant proteases are usually of bacterial and fungal origin. More recently, proteases from actinomycetes have also gained importance due to their unusual substrate specificities and high temperature and pH stabilities. Pronase has been employed in free and immobilized forms in protein structure studies (Mihalyi, 1978; Church *et al*, 1982) and in the preparation of optically active amino acids (Yamskov *et al*, 1981, 1986). Immobilized Pronase has been used in the pharmaceutical industry to remove protein impurities from preparations of 6-aminopenicillanoic acid (Shaltiel *et al*, 1970). Pronase has also been employed in ophthalmology to liquify hardened material in age-related cataracts before they are surgically removed (Spina and Kent, 1977). Protease from *S. cellulosae* has been used to obtain biologically active peptides (Muro *et al*, 1987). Actinomycete proteases are used in the food industry for protein liquifaction, milk-clotting and as meat tenderizers. Attempts have also been made to introduce actinomycete proteases as fibrinolytic and thrombolytic agents in medical treatment (Peczynska-Czoch and Mordarski, 1988).

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The actinomycetes are therefore, important industrial microorganisms. At the same time the ubiquity of their occurrence in the environment also makes this group ecologically significant. In soil, plant litter, manures and aquatic habitats this group is an important component of the natural population. It is an obvious target group for organisms which may show multiple functionality : for example protease production and oil degradation. In the literature there are few reports of microorganisms which exhibit both functions. *Pseudomonas aeruginosa* is such an organism (Morihara, 1965; Gutcho, 1974). A U.S. Patent by Morihara (1968) has described the production of protease with yeast extract as an organic nitrogen source in a medium containing heavy petroleum oil and propylene glycol. Natural systems, particularly oceanic ecosystems, are highly nitrogen-limited and any organism with the ability to recruit its own nitrogen has an advantage for the development of biotechnological processes in cleaning up oil pollution.

Crude oil is a naturally occurring, complex mixture of hydrocarbons with 4 to 26 or more carbon atoms in the molecules. These include aliphatic or straight chain compounds, branched chain compounds and aromatic compounds with benzene rings. It also contains small quantities of oxygen, sulphur and nitrogen containing compounds and also trace amounts of metallic constituents. The exact composition of crude oil varies from one field to another. The crude oil composition also varies during the life of a single oil field (Gutnick and Rosenberg, 1977; Clark, 1986).

Oil pollution is known to occur in fresh water, marine and soil ecosystems. Sources of oil pollution in the marine environment include tanker operations, other shipping operations, off-shore oil production, coastal oil refineries, industrial and municipal waste, urban and river run-off and natural seeps. The spill of more than 200,000 barrels of crude oil from the oil tanker Exxon Valdez in Prince William Sound in Alaska in 1989 as well as other spills in Texas, Rhode Island at the Delaware bay has refocussed attention on the problem of hydrocarbon contamination in the environment (Leahy and Colwell, 1990).

Oil pollution has varied effects on the natural populations in the ecosystem. In some cases it leads to the enrichment of microbial populations capable of utilizing hydrocarbons while in other cases it results in the suppression of microbial flora. After an oil spill, the low molecular weight components of the oil evaporate, the water soluble components dissolve in water, the insoluble components become emulsified as small droplets and the high molecular weight components, the tars, tend to form tar balls. Water soluble components of crude oil include a large number of compounds that are toxic to a wide range of marine plants and animals. Aromatic components are more toxic than aliphatics and intermediate molecular weight components are more toxic than high molecular weight tars (Clark, 1986). Crude oil is known to contain mutagenic, carcinogenic and growth inhibiting chemicals and even small quantities of certain petroleum fractions destroy microalgae and juvenile forms of several marine organisms. Oil pollution in the oceans and in the coastal waters also presents a serious problem to commercial fisheries, recreational resources and public health (Gutnick and Rosenberg, 1977).

Various approaches have been developed towards oil-spill clean-up. These include chemical treatment with the use of emulsifier dispersants, gelling agents, burning, sinking or physical removal by using floating booms and slick lickers. Besides physical and chemical methods, biodegradation of hydrocarbons by natural populations of microorganisms or by recombinant microorganisms is a viable alternative. Chemical treatments are usually hazardous to the flora and fauna in and around the oil polluted environment whereas physical methods are laborious and expensive, result only in partial cleaning and are of limited use. Therefore, the degradation of hydrocarbon pollutants by microorganisms presents a promising approach towards environmental pollution control.

The hydrocarbon degrading capabilities of microorganisms were recognized by Zobell as early as 1946. According to him, microorganims capable of utilizing hydrocarbons as sole carbon and energy sources were widely distributed in nature and the microbial utilization of hydrocarbons was dependent upon the chemical nature of the

compounds within the petroleum mixture and also on environmental determinants. The hydrocarbon oxidizing cultures isolated from water samples at the bottom of storage tanks containing crude oil or petroleum products, sludge from sedimentation ponds, marine sediments and seawater samples included species of *Pseudomonas, Mycobacterium, Proactinomyces, Actinomyces, Micromonospora, Penicillium, Aspergillus, Bacillus*, yeast-like organisms and molds. However, natural environments usually contain low concentrations of inorganic nutrients and the release of hydrocarbons into such environments results in excessively high carbon to nitrogen or carbon to phosphorus ratios or both, which are unfavourable for microbial growth (Cooney, 1984; Atlas, 1981) and consequently degradation of crude oil in polluted environments is hampered. In general in most bioremediation processes the carbon/nitrogen/phosphorus ratios are adjusted by the addition of nitrogen and phosphorus as their inorganic salts or in the form of oleophilic fertilizers in order to stimulate biodegradation of hydrocarbons in such environments. The process of bioremediation encourages the natural process of biodegradation by utilizing the enzymatic capabilities of indegenous hydrocarbon degraders (Atlas, 1991). This approach is the basic rationale of land-farming for the destruction of oily waste and has been widely used for terrestrial bioremediation (Bossert and Bartha, 1984; Song *et al*, 1990) and for marine bioremediation, especially after the Exxon Valdez oil spill (Prince, 1992).

Use of microorganisms capable of utilizing organic nitrogen sources by producing extracellular proteases is an alternative approach to overcome inorganic nitrogen limitation. An organism capable of both degrading crude oil and producing extracellular proteolytic enzymes may prove useful in the development of biotechnological methods for oil spill clean up. An attempt was therefore made in the present investigation, to isolate actinomycetes from various environmental samples and to screen for their abilities to degrade crude oil and produce extracellular proteases.

Actinomycetes are known for their ability to degrade hydrocarbons (Lacey, 1988), and several actinomycetes have been isolated from oil wells, from sedimentation ponds, water at the bottom of storage tanks, industrial effluents, jet fuel systems and

from hydrocarbon enriched soil. Such actinomycetes include *Corynebacterium* sps., *Nocardioides simplex* (Bushnell and Haas, 1941), *Brevibacterium, Streptomyces* and *Rhodococcus* sps. (Edmonds and Cooney, 1967), *Arthrobacter, Corynebacterium, Mycobacterium* and *Nocardia* sps. (Jones and Edington, 1968). *Corynebacterium, Micromonospora, Nocardia, Mycobacterium* and *Streptomyces* sps. (Purkiss, 1972; Genner and Hill, 1981).

Pathways for the degradation of saturated aliphatic hydrocarbons by actinomycetes have been studied in some detail (Pirnik *et al*, 1974; Gutnick and Rosenberg, 1977). The oxidation of saturated aliphatic hydrocarbons usually occurs first at the C_1 position as in *Brevibacterium erythrogenes*, to form a fatty alcohol, then a fatty aldehyde and then a fatty acid which is degraded by β-Oxidation, but sometimes oxidation at the C_2 position occurs to form a methyl ketone, as in *Mycobacterium smegmatis*. ω-Oxidation can also occur, as in case of *Corynebacterium*, which leads to the formation of dicarboxylic acids (Lacey, 1988).

Keeping in mind that actinomycetes are an obvious target group where multifunctional organisms may be found and that the marine actinomycetes are a relatively poorly studied group of organisms, in the present study, actinomycetes were isolated from various environmental samples and screened for their ability to degrade crude oil and to produce extracellular proteases. An isolate capable of doing both was selected and identified as *Nocardiopsis* sp. on the basis of its morphology, DNA base composition , biochemical tests and degradation of specific compounds. The present *Nocardiopsis* sp. was isolated from an oil-contaminated sea water sample collected from coastal areas near Mumbai. The organism was chosen for study not only for its apparent double role, but also because it was found to belong to the non-streptomycete group of actinomycetes. As discussed earlier, a wide variety of proteases of actinomycete origin have been reported so far. However, the focus has mainly been on *Streptomyces* proteases whereas proteases from the non-streptomycete group of actinomycetes are less well-characterized.

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Proteases have been purified from *Nocardiopsis dassonvillei* OPC-210 (Tsujibo et al, 1990 a) and *N. dassonvillei* ATCC 21944 (Kim *et al*, 1993) and their preliminary characteristics studied. The amino acid compositions and partial sequences of two proteases from *N. dassonvillei* OPC-210 have also been determined (Tsujibo *et al*, 1990b). However, to the best of our knowledge, there are no reports in the literature of a *Nocardiopsis* sp. capable of producing extracellular proteases as well as degrading hydrocarbons.

The major part of the present study deals with the identification of the isolate [chapter2], the optimization of culture conditions for production of extracellular proteases and the purification and characterization of two proteases secreted by the organism [chapter 3]. Chapter 4 presents an alternative biotechnological approach for oil spill clean up based on the ability of the organism to degrade hydrocarbons and produce extracellular protease in media containing hydrocarbons and various organic nitrogen sources. Chapter 5 includes a general discussion to conclude the presentation.

CHAPTER 2

ISOLATION AND SCREENING OF ACTINOMYCETES FOR
PROTEASE PRODUCTION AND HYDROCARBON
DEGRADATION AND IDENTIFICATION OF AN ISOLATE
SHOWING BOTH PROPERTIES

SUMMARY

About forty—five actinomycete isolates were obtained by selective isolation from environmental samples. These were screened independently for the degradation of Bombay High crude oil and for the production of extracellular proteases. Two isolates were found to degrade crude oil and about eleven isolates showed significant amounts of extracellular protease activity. One isolate was found to exhibit both the properties. The genus level identification of this particular isolate indicated that it belonged to the non-streptomycete group of actinomycetes. This particular isolate was selected for further studies. The isolate was assigned to genus *Nocardiopsis* on the basis of the distinct morphology showing long chains of elongated spores formed by the aerial hyphae, a cell wall chemotype IIIC i.e containing meso—diaminopimelic acid without any characteristic sugar, and the absence of mycolic acids. Our isolate was compared with the type culture, *Nocardiopsis dassonvillei* ATCC 23218, with respect to the morphology, cell wall composition, mycolic acid analysis, DNA base composition and biochemical tests and the degradation of specific compounds. The isolate has been deposited with the National Collection of Industrial Microorganisms as *Nocardiopsis* sp. NCIM 5124.

INTRODUCTION

Actinomycetes have been isolated from a wide variety of habitats such as soil, water, plant litter, root nodules and manure. Soil is the most important habitat of actinomycetes their main ecological role being the decomposition of organic matter in the soil (Waksman, 1959). Several reviews have been published on the selective isolation of actinomycetes (Cross, 1982; Wellington and Cross, 1983; Goodfellow and Williams, 1986; Nolan and Cross, 1988). Isolation is usually based on methods which reduce the numbers of bacteria and fungi in enrichment cultures from natural habitats and also involves pretreatment of environmental samples with physical agents such as heat, desiccation, or chemicals like phenol, alkali or sodium propionate. Selective isolation methods make use of growth media containing antibacterial agents, some examples are benzoate for *Micromonospora* sp. (Sandrak, 1977), dihydroxymethylfuratriazine for *Microtetraspora* sp. (Tomita *et al*, 1980), nitrofurazone for *Streptomyces* sp. (Yoshioka, 1952), oxytetracycline for *Streptoverticillium* sp. (Hanka *et al*, 1985), rifampicin for *Actinomadura*, *Saccharomonospora* and *Thermomonospora* sps. (Athalye *et al*, 1981), tunicamycin for *Micromonospora* (Wakisaka *et al*, 1982), tetracyclines for *Nocardia* sp. (Orchard and Goodfellow, 1974) and kanamycin for *Thermomonospora* sp. (McCarthy and Cross, 1981).

The actinomycetes are classified into two large, but rather unequal subgroups– the oxidative forms which are numerous and are generally soil inhabitants and the fermentative types which are primarily found in the natural cavities of man and animals (Lechevalier and Pine, 1977). The fermentative actinomycetes are morphologically simple organisms. Aerial mycelia and spores are usually not formed in this group and the primary mycelium may be either quite well developed or rudimentary. The vast majority of actinomycetes are oxidative and aerobic and have been classified into genera on the basis of morphological and chemical criteria (Lechevalier, 1968; Lechevalier and Lechevalier, 1970a; 1970b; Lechevalier *et al*, 1971) These authors have used the presence or absence of DAP, the type of DAP isomer and the presence of characteristic amino acids and sugars in the cell walls to distinguish 9 distinct cell wall chemotypes in actinomycetes (Table 2.1).

Table 2.1 : Major Constituents of Cell Walls of Actinomycetes

(Lechevalier *et al* , 1971)

Cell Wall type	Major Constituents *
Streptomyces or Type I	L-DAP, Glycine
Micromonospora or Type II	meso-DAP, Glycine, hydroxy DAP may also be present
Actinomadura or Type III	meso-DAP
Nocardia or Type IV	meso-DAP, Arabinose, Galactose
Oerskovia	Lysine, Aspartic acid, Galactose
Actinomyces bovis	Lysine, Aspartic acid
Actinomyces israeli	Lysine, Ornithine
Agromyces	DAB, Glycine
Mycoplana	meso-DAP + numerous amino acids

* All cell wall preparations contain major amounts of alanine, glutamic acid, glucosamine and muramic acid.

DAP– 2,6-Diaminopimelic acid ; DAB– 2,4-Diaminobutyric acid

The cell wall types II,III and IV were further differentiated by whole cell sugar patterns (WCSP) (Lechevalier, 1968; Lechevalier and Lechevalier, 1970a ; 1970b). The WCSP could be of the A,B,C or D type (Table 2.2). Besides these distinguishing compounds, all actinomycete cell wall preparations contain major amounts of alanine, glutamic acid, glucosamine and muramic acid.

Table 2.2 : Cell Wall types and Whole Cell Sugar Patterns of Aerobic

Actinomycetes containing meso-DAP*

(Lechevalier *et al* , 1971)

Cell Wall Type	Distinguishing major constituents of Cell Wall	Whole-Cell Sugar pattern (WCSP) Type	Diagnostic sugars of WCSP
II	Glycine	D	Xylose, Arabinose
III	None	B	Madurose
		C	None
IV	Arabinose, Galactose	A	Arabinose, Galactose

* No differentiation is made between meso-DAP and D-DAP

Madurose= 3-O-methyl-D-galactose

Actinomycetes within the same group are classified into genera on the basis of morphological features for instance the presence or absence of sporangia or the number of spores on the aerial mycelium or on the basis of other chemical criteria like the presence or absence of mycolic acids. Organisms containing mycolic acids have been further classified depending on the molecular weights of the mycolates (Alshamaony *et al*, 1976; Collins *et al*, 1982). There are however some exceptions to this usually employed system for the classification of actinomycetes. There are certain actinomycetes with cell walls containing no DAP. This group includes the genera *Oerskovia* and *Promicromonospora* , while the absence of DAP and presence of xylose and arabinose is characteristic of genus *Actinoplanes*. There are also other actinomycete genera which do not fall into any of the WCSP types designed by Lechevalier (1968) and Lechevalier and Lechevalier (1970a ; 1970b). Organisms containing fucose in their WCSP may belong to the genus *Frankia* or to *Actinoplanes*. Some plant pathogenic corynebacteria also contain fucose . The presence of rhamnose without other diagnostic sugars is assigned to genus *Saccharothrix* (Lechevalier, 1989). The classification of actinomycete genera belonging to cell wall chemotypes I to IV is summarized in figure 2.1.

Fig. 2.1 Generalized scheme for the classification of actinomycetes (cell wall chemotypes I to IV)

ACTINOMYCETES

Type of DAP isomer in cell wall

L-DAP
+Glycine

Cell wall Type I
Streptomyces
Intrasporangium
Nocardioides
Streptoverticillium

meso-DAP

+Xylose
+Arabinose
Actinoplanes

DAP absent

Oerskovia
Promicromonospora

Distinguishing constituents of cell wall

+ Glycine
Cell wall Type II
+Xylose
+Arabinose
(WCSP D)
Micromonospora
Catellatospora
Glycomyces

None
Cell wall Type III

+Arabinose
+galactose
Cell wall Type IV
(WCSP A)

Mycolic acids
present

Mycolic acids
absent
Faenia
Pseudonocardia
Saccharomonospora
Saccharopolyspora

Small
corynomycolates
(22-38 C*)
Corynebacterium

Medium
nocardomycolates
(34-60 C*)
Nocardia
Rhodococcus

Large
mycolates
(60-90 C*)
Mycobacterium

+Madurose
(WCSP B)
Actinomadura
Microbispora
Microtetraspora
Planobispora
Streptosporangium

No sugar
(WCSP C)
Thermoactinomyces
Thermomonospora

| *Nocardiopsis* |

Actinosynnema
Geodermatophilus

* C denotes carbon number

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Besides chemical composition and morphological characteristics, DNA base composition and DNA-DNA as well as DNA-rRNA hybridization have gained significance as diagnostic tools in the recent years. The DNA base composition or the mole% G+C content of the DNA is determined by either thermal denaturation (Marmur and Doty, 1962) or buoyant density (Schildkraut et al, 1962; Mandel and Marmur, 1968). In general, the G+C content of the DNA of actinomycetes is high, the mycobacteria and nocardiae being at the lower end of this high spectrum (60-70 mol %) while the streptomycetes are on the higher side (70-75 mol %). DNA from some thermophilic actinomycetes contains relatively low G+C percentages (44-54 mol %) (Lechevalier et al , 1971). Nucleic acid hybridization studies reveal the degree of relatedness among different genera, among members of the same genus and also within the same species that is to say among different strains of the same species. These methods often reveal poor relationships between biochemically and morphologically similar groups. On the other hand, distantly placed groups may show signiificant degree of homology.

The genus *Nocardiopsis* was first described by J. Meyer in 1976. The name *Nocardiopsis* was coined because of the *Nocardia*-like appearance of the organism. (*Nocardia*: a genus of the order Actinomycetales; opsis: appearance). Meyer has described organisms belonging to genus *Nocardiopsis* as Gram-positive, aerobic, chemo-organotrophic, non-acid fast, with a well developed substrate mycelium, with long and densely branched hyphae which may fragment into coccoid and bacillary elements. The aerial mycelium is also usually well developed and abundant, hyphae are long, moderately branched, straight, flexous or irregularly zig-zagged, completely fragmenting into spores of various lengths. The spores are oval to elongated, spore surface is smooth. Growth temperatures range from 10 to 45°C. The cell wall contains meso-DAP without any diagnostic sugars. Mycolic acids are absent. Menaquinones are predominantly of the MK-10 (H_2, H_4 , H_6) or of the MK-9 (H_4 , H_6) type. Predominant phospholipids are phosphatidylglycerol, phosphatidyline and acylphosphatidylglycerol. The mole % G+C of the DNA is 64-69 (Tm) (Meyer, 1989).

In discussing the history of this genus, Meyer (1976; 1989) has said that the organism that gave its name to the type species of the genus *Nocardiopsis* was originally

isolated from mildewed grain and named as *Streptothrix dassonvillei* by Brocq-Rousseu (1904). This strain was lost. Then, some years later Liegard and Landrieu in 1911, isolated a strain from a case of ocular conjunctivitis which they found was similar to Brocq-Rousseu's *S. dassonvillei* but which they assigned to the genus *Nocardia* . Information on *Nocardia dassonvillei* was scarce until Gordon and Horan (1968) showed that this organism resembled *Streptomyces griseus* in its macroscopic appearance and also several physiological characters. Subsequently, Lechevalier and Lechevalier (1970 a) described the genus *Actinomadura* in which they included *Nocardia madurae* (Vincent) Blanchard (the type species), *N. pelletieri* (Laveran) Pinoy and *N. dassonvillei* (Brocq-Rousseu) Liegard and Landrieu.

The separation of the genus *Nocardiopsis* from the genus *Actinomadura* was based on morphological and biochemical criteria (Meyer, 1976). *Nocardiopsis* has long, scarcely branched hyphae, complete fragmentation of the aerial mycelium into long spore chains and fragmentation of the substrate mycelium into coccoid or bacillary elements, whereas *Actinomadura* has abundantly branched hyphae, spore chains with limited number of spores in distinct parts of the aerial mycelium, and a stable substrate mycelium which shows no fragmentation. The two genera also differ in their cell wall types. *Actinomadura* was characterized by a cell wall type IIIB, i.e meso-DAP with madurose as the diagnostic sugar, whereas the cell wall type of *Nocardiopsis* was IIIC, i.e meso-DAP with no characteristic sugar. On these grounds Meyer (1976) proposed that the genus *Nocardiopsis* should include aerobic actinomycetes with a nocardioform substrate mycelium, an aerial mycelium, and a cell wall containing meso-2,6-DAP but not madurose or other diagnostically important sugars.

Nocardiopsis species have been reported to produce xylanases (Tsujibo *et al*, 1990d; Papeta *et al* , 1995), alkaline α-amylase (El-Meleigy *et al,* 1997) and alkaline serine proteases (Tsujibo *et al*, 1990a ; Kim *et al* , 1993). The amino acid compositions and partial sequences of the proteases and xylanases have been determined (Tsujibo *et al*, 1990b ; Tsujibo *et al* , 1991). Apoptolidin, a specific apoptosis inducer (Hayakawa *et al* , 1998), Kalafungin, an anti-fungal antibiotic which also showed marked cytotoxic activity against

mouse leukamic cells in vitro (Tsujibo *et al*, 1990c), and Portmicin, an antibiotic active against Gram positive bacteria and mycobacteria (Kusakabe *et al*, 1987) have all been isolated from *Nocardiopsis* species.

Degradation of hydrocarbons by actinomycetes has been reviewed extensively by Lacey (1988). Actinomycetes degrading hydrocarbons have been isolated from oil wells, from sedimentation ponds, water at the bottom of storage tanks and from hydrocarbon enriched soil. *Corynebacterium* sps have been isolated from oil-saturated soil and from sedimentation ponds (Bushnell and Haas, 1941). Edmonds and Cooney (1967) have reported *Brevibacterium*, *Streptomyces* and *Rhodococcus* sp isolated from jet fuel systems. Species of *Arthrobacter*, *Corynebacterium*, *Mycobacterium* and *Nocardia* utilizing cyclohexane, n-dodecane, n-hexadecane, n-octane and n-pentadecane were found in soil samples (Jones and Edington, 1968). *Corynebacterium*, *Micromonospora*, *Nocardia*, *Mycobacterium* and *Streptomyces* isolates have also been obtained from rolling mill and other industrial effluents (Purkiss, 1972; Genner and Hill, 1981). *Nocardioides simplex* was found to grow on media containing paraffin wax, light oil, kerosene, toluene and xylene but not on benzene or a mixture of low boiling hexanes (Bushnell and Haas, 1941).

Proteolytic enzymes of actinomycete origin have been well studied, mostly those from *Streptomyces* sp. Proteases from actinomycetes are known for their unusual substrate specificities. The reports include, trypsin-like protease from *Streptomyces erythraeus* (Yoshida *et al*, 1971), keratinase-like protease from *S. fradiae* (Nickerson and Durand, 1963 ; Nadanishi and Yamamoto, 1974), elastase-like enzymes from *S.griseus* (Gertler and Trop, 1971) and *S.fradiae* (Lagutina and Petrova, 1979), collagenase-like enzymes from *Thermoactinomyces vulgaris* (Kleine, 1982), *Streptomyces* sp. (Chakraborty and Chandra, 1984) and *Actinomadura* sp. (Rippon, 1968; Norwig, 1971). The proteolytic complexes from *S.griseus* (Narahashi, 1970; Awad *et al*, 1972 a) and *S.fradiae* (Morihara *et al* , 1967) have been extensively studied and are the best characterized among the actinomycete proteases.

We have isolated an actinomycete from an oil-contaminated sea water sample collected from the coastal areas of Mumbai and have identified it as *Nocardiopsis* sp. The organism apparently plays a dual role: it produces extracellular proteases and it also degrades hydrocarbons. Moreover, this particular isolate belonged to the non-streptomycete group of actinomycetes, from which proteases have been relatively less characterized as compared to those from *Streptomyces* species. Therefore this organism was chosen for further studies.

The present chapter describes the isolation of actinomycetes from different environmental samples, screening for hydrocarbon degradation and extracellular protease production and the identification of the selected isolate on the basis of morphology, chemical composition, DNA base composition, biochemical tests and the degradation of specific compounds.

MATERIALS AND METHODS

MATERIALS

Diaminopimelic acid, lysozyme, pancreatic ribonuclease and ethidium bromide were obtained from Sigma chemicals, USA, glutaraldehyde was obtained from Aldrich chemicals, USA, Casein (Hammerstein), fat free casein, xanthine were purchased from Sisco Research Laboratories, India. Bombay high crude oil was obtained from the Oil and Natural Gas Commission, India. All seawater salts, solvents, and other chemicals used were of analytical grade.

METHODS

Isolation of actinomycetes

Samples were collected from different environments including salt pans, mangroves, seashore sand from Goa and also oil contaminated sea water and soil samples from Mumbai (Bombay). The samples were subjected to the phenol dilution method (Lawrence, 1956) for isolation of actinomycetes modified as follows:

1ml of each sample was mixed with 9 ml of each of 1:100 and 1:150 dilutions of phenol in sterile distilled water. The mixture was vortexed and allowed to stand for about 20 min., after which 0.1 ml from each dilution was spread onto casein starch agar plates (1% starch, 0.03% casein, 0.2% KNO_3, 0.2% NaCl, 0.2% K_2HPO_4, 0.005% $MgSO_4.7H_2O$, 0.002% $CaCO_3$, 0.001% $FeSO_4.7H_2O$, pH 8.0). The medium was prepared in both distilled water and defined sea water. Defined sea water contained per liter of deionized water: 24.53 g NaCl, 1.54 g $CaCl_2$, 0.1 g KBr, 0.003 g NaF, 0.7 g KCl, 0.03 g H_3BO_3, 4.09 g Na_2SO_4, 0.2 g $NaHCO_3$, 0.017 g $SrCl_2.6H_2O$, 11.1 g $MgCl_2.6H_2O$, pH 8.0 adjusted with 1N NaOH. In case of soil samples, 1 g of the sample was suspended in 10 ml of sterile saline, vortexed and then allowed to sediment. 1 ml of the supernatant was subjected to the phenol treatment and then spread onto agar plates as described above. The phenol dilution method resulted in the growth of about 60 colonies. Each of these colonies was observed at

400 X to check for mycelial morphology. Of the 60 colonies, about 45 were actinomycetes which were subcultured and used for further study.

Screening

The actinomycete isolates were screened for their ability to utilize hydrocarbons and to produce extracellular protease. Hydrocarbon degradation was tested by using crude oil from Bombay High oil wells as the sole carbon source in a defined sea water medium containing 0.5% $(NH_4)_2 SO_4$, 0.002% $K_2 HPO_4$, pH 8.0 with 0.5% w/v crude oil. The inoculum of each isolate was developed in sea water nutrient broth at 30°C for 48 h and after pelleting out and washing the cells with sterile sea water, approximately 2.0 g wet weight of each isolate was added to 50 ml of the screening medium and the flasks incubated at 30°C and about 200 rpm for 10 days. The residual crude oil was extracted with dichloromethane and the percentage degradation of crude oil was determined by gravimetry. An uninoculated control determined abiotic losses of the added crude oil.

Protease production was tested in a medium containing 1% starch , 1% fat free casein , 0.3% K_2HPO_4 , 0.05% $MgSO_4$, 1% Na_2CO_3 , pH 10.5 (Nakanishi *et al*, 1974). After 2 weeks incubation at 30°C and about 200 rpm, protease activity in the cell-free supernatant was determined by the method of Kunitz (1947) as described by Laskowski (1955). The method is described in detail in chapter 3. One unit of protease activity is defined as the amount of enzyme which releases one μ mole of tyrosine per min. at 40°C and pH 10.0.

Identification of the isolates testing positive for crude oil degradation and protease production

Determination of whole cell and cell wall composition

The isolates showing positive results were subjected to a rapid genus level identification by the method of Becker *et al* (1964). This method classifies actinomycetes into 2 broad groups depending upon the type of DAP isomer present in their whole cell hydrolysates , i.e the *Streptomyces* group containing L-DAP and the *Nocardia* group

containing meso-DAP. For analysis of the cell wall composition, cell walls were prepared , hydrolysed and subjected to paper chromatography to identify the amino acids and sugars present (Becker *et al*, 1965; Yamaguchi, 1965). Besides the cell wall, whole cell hydrolysates were also used to analyse the sugar pattern.

Morphological studies

Morphological studies were carried out by microscopic examination of slide culture preparations of the isolates. For scanning electron microscopy, coverslip cultures of the isolates were used. The coverslip culture method of Williams and Davies (1967) was slightly modified as follows: small pieces of coverslips were inserted at $45°$ angles into a half-strength nutrient agar medium (nutrient agar: 1% peptone, 0.3% beef extract, 0.5% NaCl, 2.5% agar, pH 8.0). The culture was streaked onto the agar medium, below the coverslip pieces , which were removed after 4 days of growth at $30°C$.

The coverslip culture was fixed overnight with 2% glutaraldehyde at $4°C$ and then gently washed with changes of distilled water to remove the excess glutaraldehyde. The coverslip culture was then dehydrated by successive washing with increasing concentrations of ethanol (10–95% v/v ethanol) and air dried. The specimen was coated with gold using a sputter coater unit (Bio-Rad, UK) and examined with a scanning electron microscope S–120 Cambridge Instruments, UK.

Determination of DNA base composition

For the determination of the DNA base composition (mole% G+C), DNA was isolated from the cultures by the method of Mordarski *et al* (1976) modified as follows : The cultures grown in nutrient broth (1% peptone, 0.3% beef extract, 0.5% NaCl, pH 8.0) at $30°C$ and about 200 rpm were harvested in the exponential phase of growth. The pelleted cells were washed with saline EDTA buffer (0.15 M NaCl, 0.1 M EDTA, pH 8.0) resuspended in buffer A (0.015 M Tris-HCl, pH 8.0, 0.45 M sucrose and 0.008 M EDTA) containing lysozyme (1 mg lysozyme for 0.2 g washed cells) and incubated for 2 h at $37°C$. To this suspension , sodium dodecyl sulphate was added at a final concentration of 2% to

ensure cell lysis and the suspension was heated at 60°C for 30 min. The cell debris was separated by centrifugation at 10,000 rpm for 15 min. and the supernatant containing the DNA was repeatedly treated first with equal volumes of Tris-saturated phenol (pH 8.0) and then with chloroform:isoamyl alcohol (24:1) followed by centrifugation at 10,000 rpm for 10 min at about 4–6°C to precipitate non-DNA material till the interphase appeared clean. DNA was precipitated from the aqueous phase with 0.15 M NaCl and 2 volumes of chilled ethanol, washed with chilled 70 % ethanol, dried and then dissolved in $T_{10} E_1$ buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). The DNA was treated with pancreatic ribonuclease (50 µg/ml) to digest contaminating RNA. DNA was re-precipitated and the quality of the final DNA preparation was checked on 0.8% agarose gels as a single band on staining with ethidium bromide and also by the A_{260}/A_{280} ratios of the preparation. Material thus isolated with ratios of about 1.8 was considered of adequate purity for determination of Tm. DNA samples were extensively dialysed against 0.1X Standard Saline Citrate or SSC (SSC: 0.15 M NaCl, 0.015 M trisodium citrate). The increase in absorbance at 260 nm was recorded using Beckman DU-8B spectrophotometer. The temperature was increased from 25 -102°C, at a rate of 1°C every 10 minutes. A ratio of the A_{260} at temperature t to A_{260} at 25°C was plotted versus the temperature, to determine the Tm value of the DNA samples. The G+C content was then calculated from this value by using the formula of Mandel and Marmur (1968).

Mycolic acid analysis

Mycolic acid analysis was done according to the method of Minnikin *et al* (1975). Mycolic acids extracted similarly from *Nocardia asteroides* ATCC 19247 and *Rhodococcus rhodochrous* ATCC 13808 were used as standards.

Other tests : Biochemical tests were carried out in accordance with Meyer (1989) in the Bergey's Manual of Systematic Bacteriology Vol. IV. The degradation of gelatin, casein, xanthine and tyrosine was checked according to the method of Williams *et al* (1983).

RESULTS AND DISCUSSION

45 actinomycete isolates were obtained by the phenol dilution method (Lawrence, 1956). Of the 45 isolates, 2 were found to degrade crude oil (Table 2.3) and 11 isolates were found to produce extracellular protease in significant amounts (Table 2.4). Isolate No.23 was found to exhibit both properties.

Table 2.3 : Screening for crude oil degraders

Isolate No.	% degradation of 0.5%(w/v) crude oil*
22	60
23	40

* % degradation was determined by gravimetry

Table 2.4 : Screening for protease producers

Isolate No.	Protease activity U/ml
1	21
2	17
3	16
5	21
8	10
11	8
14	24
16	17
19	20
23	6
43	17

The 2 isolates capable of degrading crude oil, namely Nos. 22 and 23 and the best protease producer, i.e isolate No.14 were checked for the type of DAP isomer present in their whole cell hydrolysates. Actinomycetes containing L-DAP are assigned to the *Streptomyces* group, while those with meso-DAP, to the *Nocardia* group (Becker *et al*, 1964). The results are summarized in table 2.5.

Table 2.5 : Type of DAP isomer in whole cell hydrolysates

Isolate No.	DAP isomer	assigned Group
14	L-DAP	*Streptomyces* group
22	L-DAP	*Streptomyces* group
23	meso-DAP	*Nocardia* group

Since isolate No.23 was found to be capable of crude oil degradation and protease production as well and genus level identification indicated that it belonged to the *Nocardia* group or in a broader sense, the non-streptomycete group of actinomycetes, it was chosen for further study.

Among the actinomycetes, streptomycete and non-streptomycete actinomycetes produce proteolytic enzymes, most of the well characterized proteases being from *Streptomyces* species (Awad *et al*,1972a; Narahashi, 1970; Morihara *et al*, 1967 ; Kleine, 1982; Rippon,1968; Norwig, 1971; Tsujibo *et al*, 1990 a; Osterman *et al*, 1984). It is also well recognized that *Streptomyces* sp. and also non-Streptomycete actinomycetes like species of *Arthrobacter, Mycobacterium, Corynebacterium, Brevibacterium, Rhodococcus, Nocardia, Micromonospora* (Bushnell and Haas, 1941; Edmonds and Cooney, 1967; Jones and Edington, 1968; Purkiss, 1972 ; Genner and Hill, 1981) have been reported to degrade crude oil/ hydrocarbons. However, to the best of our knowledge, there is no report in the literature, to date, of an actinomycete that has both these capabilities. Therefore, we chose to study isolate No.23 in greater detail. In the following we discuss the taxonomic characterization of this isolate.

Morphological studies based on slide cultures showed long chains of elongated spores (Figure 2.2A). Scanning electron microscopy also revealed similar morphological features (Figure 2.2B). The whole cell hydrolysate of isolate No.23 showed the presence of meso-DAP and the absence of diagnostic sugars. Cell wall preparations of isolate No.23 hydrolyzed and analysed similarly, also showed the same (Fig. 2.3). These results indicated that the isolate belonged to cell wall chemotype IIIC which includes the genera *Thermoactinomyces*, *Thermomonospora*, *Nocardiopsis*, *Actinosynnema* and *Geodermatophilus*, with distinct morphological features as listed in table 2.6.

Table 2.6 : Distinct morphological features of Genera with Cell Wall of type III C *

Typical key morphological features	Possible generic assignment
Single conidia formed. These are heat resistant bacterial endospores.	*Thermoactinomyces*
Same as above but the spores are not heat resistant.	*Thermomonospora*
Long chains of spores formed by the aerial hyphae.	*Nocardiopsis*
Aerial hyphae often united into synnemata releasing motile spores.	*Actinosynnema*
Multilocular sporangia releasing motile spores.	*Geodermatophilus*

* Modified from Lechevalier (1989).

Therefore, the type IIIC cell wall and the typical morphology showing long chains of spores formed by the aerial hyphae assigned the isolate under study to genus *Nocardiopsis*. Moreover, mycolic acid spots were found to be absent in the whole cell methanolysates of our isolate which is also one of the distinguishing features of the genus *Nocardiopsis*.

Figure 2.2A

Morphology of isolate No. 23 and *Nocardiopsis dassonvillei* ATCC 23218

Slide cultures on half-strength nutrient agar after 4 days of growth at 30°C (1000 X)

Isolate No. 23
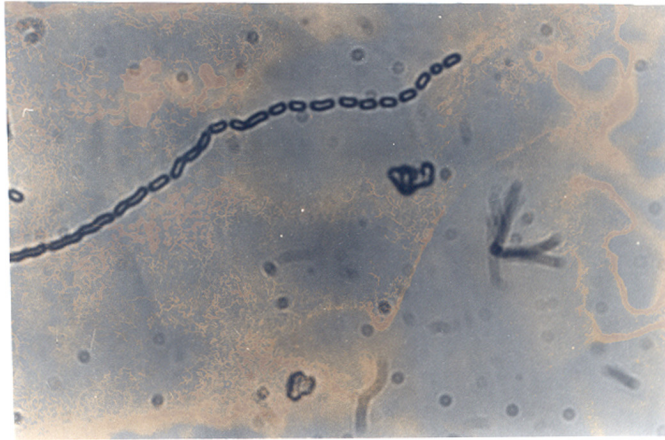
Nocardiopsis dassonvillei ATCC 23218
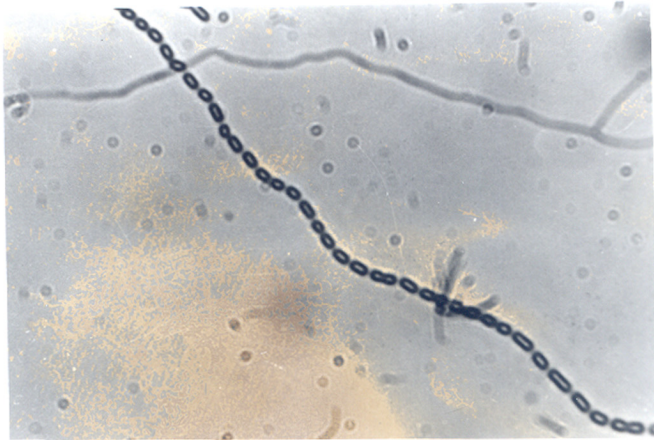
Figure 2.2B

Scanning electron micrographs of Isolate No. 23

The culture was grown on coverslips and treated as described in methods (6,400 X).
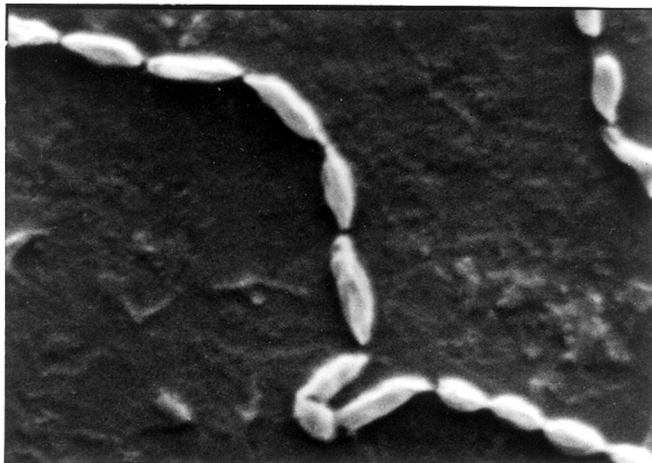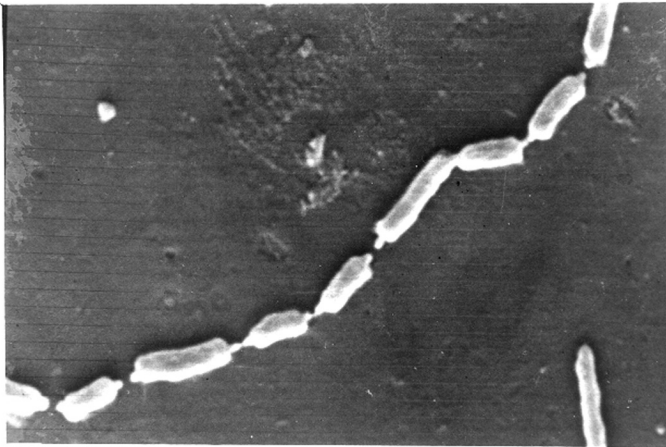
For further confirmation of the generic identification, the type strain of *Nocardiopsis dassonvillei* ATCC 23218 was obtained from the Institute of Microbial Technology, Chandigarh. The two cultures were then compared with respect to their morphology (Fig. 2.2A), cell wall composition, WCSP, morphology and DNA base compositions. Similar results were obtained for both the cultures (Table 2.7).

Table 2.7 : Comparison of *Nocardiopsis* sp. with *N. dassonvillei* ATCC 23218

Character	*Nocardiopsis* sp. (isolate No. 23)	*N. dassonvillei* ATCC 23218
DAP isomer	meso DAP *	meso DAP *
Whole cell sugar pattern	type C	type C
Mycolic acids	absent	absent
Morphological features	long chains of spores observed in slide cultures	long chains of spores observed in slide cultures
mole % G+C	66.1	68.6

* Refer to figure 2.3

The DNA base composition i.e the mole % G+C contents of *N. dassonvillei* ATCC 23218 and the *Nocardiopsis* sp. under study were calculated as 68.6 and 66.1 from Tm values of 82°C and 81°C, respectively (Fig. 2.4). The mole % G+C content of genus *Nocardiopsis* ranges from 64–69 (Tm) (Meyer, 1989).

Figure 2.3

Type of DAP isomer in cell wall preparations of *Nocardiopsis* **sp. and**

N. dassonvillei **ATCC 23218**

1 – *Nocardiopsis* sp. (Isolate No. 23)

2 – *Nocardiopsis dassonvillei* ATCC 23218
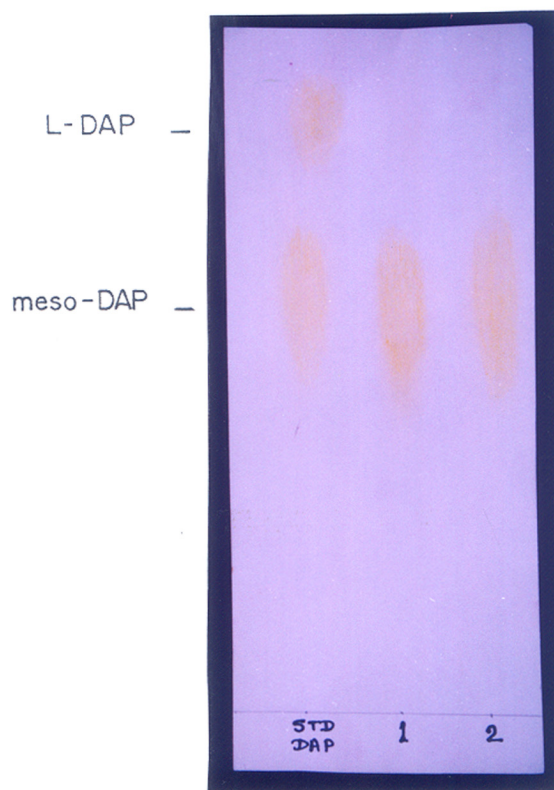
Figure 2.4

Melting patterns of DNA from *Nocardiopsis* sp. and *N. dassonvillei* ATCC 23218
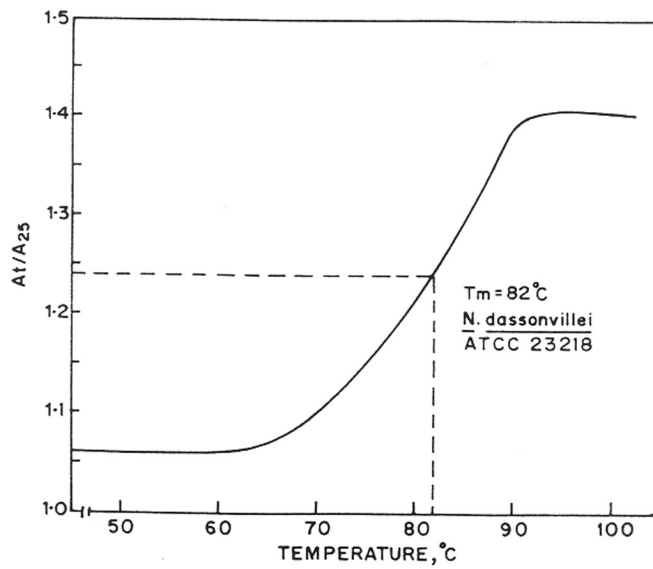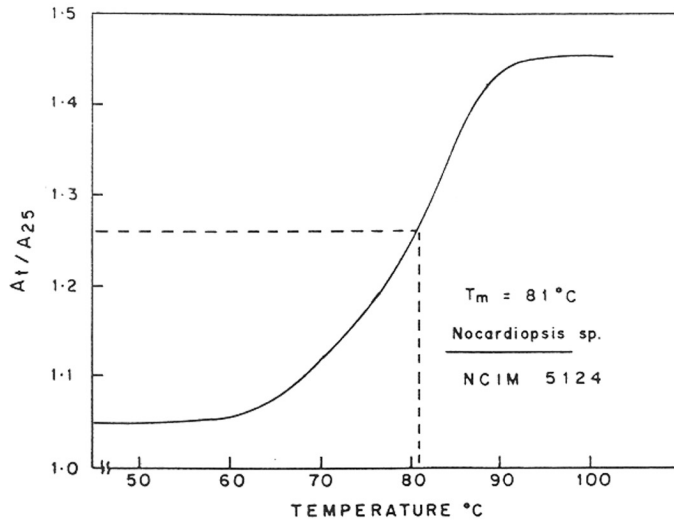
Although several actinomycete genera have overlapping ranges of G+C contents, and DNA base composition by itself is not sufficient to assign an unknown isolate to a particular genus, the calculated values for both the type species and our isolate are in agreement with the reported values for the genus *Nocardiopsis*. Therefore, in view of the morphological similarities between our isolate and *Nocardiopsis dassonvillei* ATCC 23218, the presence of meso-DAP without any diagnostic sugars thus representing a type IIIC cell wall, the absence of mycolic acids and the DNA base composition , our isolate has been characterized as *Nocardiopsis* sp.

Meyer (1989) has stated that the species of *Nocardiopsis* may be distinguished by means of the colour of their mature aerial and substrate mycelia and that additional physiological characteristics may also be helpful. The differences in the colour of the substrate and aerial mycelia of species of genus *Nocardiopsis* are as stated in table 2.8A.

Table 2.8A Differentiating characteristics of the species of genus *Nocardiopsis*
(colour of mycelia)

species	Colour of aerial mycelium	colour of substrate mycelium
N. dassonvillei	W, Cr, Y, Gr	Y, O, Br
N. africana	Bl	O, Br
N. coeruleofusca	Bl	Br
N. flava	W	Y, O
N. longispora	Bl	Y, Br, O
N. mutabilis	W, Y, O	Y, Br
N. syringae	V	Br
N. dassonvillei ATCC 23218	W	Y
Nocardiopsis sp. (isolate No.23)	W	Y

Modified from Meyer (1989)

abbreviations: W-white; Y-yellow; Cr-cream; O-orange; Gr-gray; Bl-blue; Br-brown; V-violet

As seen from table 2.8A, both *N. dassonvillei* ATCC 23218 and our *Nocardiopsis* sp. had a white coloured aerial mycelium and a yellow coloured substrate mycelium. On malt extract glucose yeast extract peptone agar, Starch casein agar and nutrient agar the colours of the mycelia were the same and did not change even after 15 days of incubation at 30°C. Therefore, *N. dassonvillei, N. flava and N. mutabilis* were the 3 possible species to which our isolate could belong, on the basis of the colour of mycelia.

The results of biochemical tests and the degradation of specific compounds by *Nocardiopsis* sp. isolated in our laboratory and *N. dassonvillei* ATCC 23218 are summarized in table 2.8B. *N. flava* does not reduce nitrate to nitrite, and also does not degrade casein, or xanthine, both of which our isolate does, thus ruling out its possible assignment to *N. flava*

Table 2.8B Differentiating characteristics of the species of genus *Nocardiopsis*

(Biochemical tests)

Test	*N.dassonvillei*	*N. flava*	*N.mutabilis*	*N.dassonvillei* ATCC 23218	*Nocardiopsis* sp. (isolate No.23)
Nitrate reduction	d	–	+	+	+
Degradation of:					
Starch	+	ND	+	+	+
Gelatin	+	ND	+	+	+
Casein	+	–	+	+	+
Tyrosine	+	ND	+	+	+
Xanthine	+	–	–	+	+

Modified from Meyer (1989) ; ND= No data ; d= 11-89 % strains are positive

N. mutabilis reduces nitrate and degrades starch, gelatin, casein, tyrosine, but does not degrade xanthine. On the other hand, both *N. dassonvillei* ATCC 23218 and *Nocardiopsis* sp. isolated in our laboratory were found to reduce nitrate, degrade starch, gelatin, casein, tyrosine and also xanthine. Therefore, the most probable species to which the present *Nocardiopsis* could belong was *N. dassonvillei*. However, DNA-DNA hybridization studies have revealed that the genus *Nocardiopsis* is very heterogenous (Meyer, 1989). Poschner *et al* (1985) have reported that *N. coeruleofusca* and *N. longispora* are moderately related to each other (45% homology). *N. africana* and *N. longispora*, are only remotely related (11-13% homology), and interestingly, *N. africana* was found to show a substantial degree of relatedness to certain *Actinomadura* species, for instance, *A. roseola* (64% homology). Furthermore, neither *N. africana, N. longispora* or *N. coeruleofusca* showed any relationship to the type species *N. dassonvillei* (about 8-12% homology). Meyer (1989) has recommended further comparative biochemical and genetic studies of all members of the genus *Nocardiopsis*, which are found to share many morphological and biochemical properties.

Therefore, on the basis of morphological features, cell wall composition, whole cell sugar pattern, mycolic acid analysis and the DNA base composition, the selected isolate was identified as *Nocardiopsis* sp. On account of the limited number of tests recommended for identification of the species of *Nocardiopsis*, and the uncertainity regarding the present state of species level classification for the genus *Nocardiopsis*, the culture has been deposited in the National Collection of Industrial Microorganisms as *Nocardiopsis* sp. NCIM 5124. This isolate was studied in further detail with respect to its proteolytic enzymes and its hydrocarbon degradation capabilities as described in the following chapters.

The type culture *N. dassonvillei* ATCC 23218 was tested for its ability to degrade Bombay High crude oil (0.5% w/v) and its protease production under conditions identical to those used for *Nocardiopsis* sp. NCIM 5124. The type culture does not degrade crude oil. It produces an extracellular protease activity of 0.1U/ml in comparison to 6 U/ml of protease activity produced by *Nocardiopsis* sp.NCIM 5124.

CHAPTER 3

PRODUCTION, PURIFICATION AND CHARACTERIZATION

OF PROTEASES FROM *NOCARDIOPSIS* SP. NCIM 5124

SUMMARY

Extracellular protease was maximally produced (about 14 U/ml) in a medium containing 1% starch, 1% casein, 0.1%K_2HPO_4, 0.2% glucose and 1% Na_2CO_3, pH 10.0, at 30°C and 200 rpm, after 4 days of growth. Electrophoretic separation of the crude culture filtrate, and activity staining using casein–agarose showed that there were two protease fractions. Protease I was purified to homogeneity by ion exchange chromatography using CM-Sephadex at pH 5.0 and again at pH 9.0 with a yield of about 20%. Protease II was purified by ion exchange using DEAE-cellulose, gel filtration on Sephadex G-50, hydrophobic chromatography using phenyl-Sepharose and colour removal by treatment with hydroxyapatite, with a yield of 43%.

Comparative characterization studies with the two proteases showed that Protease I had a M_r of about 21,000 daltons (SDS-PAGE), pI of 8.3, specific activity of 152 units/mg using casein as substrate and optimum pH and temperature values of 10.0–11.0 and 65°C respectively. Protease II had a M_r of about 23,000 daltons (SDS-PAGE), pI of 7.0, specific activity of 14 units/mg using casein as substrate and optimum pH and temperature values of 10.0 and 60°C respectively. Both proteases were almost completely inhibited by 1mM PMSF, a specific serine protease inhibitor, indicating that they were both serine proteases. The other class specific protease inhibitors, namely EDTA (metallo proteases), IAA (cysteine proteases) and pepstatin (aspartic proteases) had no effect on either protease I or II. Protease II was found to be inhibited by the chlorides of potassium, barium, calcium, zinc, copper, aluminium, magnessium and iron whereas none of these metal ion salts had any effect on Protease I. Limited substrate specificity studies using synthetic substrates showed that both proteases lacked exopeptidase activities against amino and carboxypeptidase substrates suggesting that both proteases are endopeptidases. Among the endopeptidase substrates tested, tri and tetrapeptide substrates were preferentially hydrolyzed as compared to mono or dipeptide substrates by both proteases, indicating that substrate binding in an extended form is required for efficient hydrolysis. Polyclonal antisera raised in rabbits against protease I did not cross-react with protease II, trypsin, chymotrypsin or subtilisin. Both proteases did not contain free cysteines or disulphides and resembled the subtilisins in this respect.

INTRODUCTION

A wide variety of proteolytic enzymes have been reported from actinomycetes (Peczynka-Czoch and Mordarski, 1988). Proteolytic complexes have been obtained from culture filtrates of *Streptomyces griseus* (Trop and Birk, 1968 a,b ; Narahashi, 1970), *S. fradiae* (Morihara *et al*, 1967), *S. rimosus* (Pokorny *et al*, 1979) and *S. moderatus* (Chandrasekaran and Dhar, 1987) and these have provided proteases of various substrate specificities. Pronase from *S. griseus* strain K-1 is one of the most studied of such complexes (Peczynka-Czoch and Mordarski, 1988). It is a mixture of several proteases and peptidases (Awad *et al*, 1972a), ten of which have been purified and characterized. Serine and metalloproteases with molecular masses of 15 to 30 kDa dominate the complex. Five of the components were found to be alkaline serine proteases, and have been designated as proteinases A to E (Narahashi and Yoda, 1977). On the basis of substrate specificity these have been classified as chymotrypsin-like (proteases A and C), trypsin-like (protease B), subtilisin-like (protease D) and alkaline serine protease (protease E). The metalloenzymes include carboxypeptidase (Narahashi *et al*, 1980), two zinc endopeptidases and two zinc aminopeptidases (Lofqvist, 1974 ; Vosbeck *et al*, 1975).

Proteases produced by several actinomycetes have been purified and characterized, the majority being from *Streptomyces* species, whereas reports of proteases from the non-streptomycete group are relatively few. Most of the proteases of actinomycete origin are serine proteases of molecular masses ranging from 15 to 50 kDa, with optimum pH values mostly in the alkaline pH range. Some properties of proteases from actinomycetes have been listed in table 3.1.

Table 3.1 : Characteristics of proteases from actinomycetes

Source	Protease class	M$_r$ kDa	pH optimum	Temp. optimum	pI	Reference
Streptomyces rimosus	serine	29	8.4 - 8.8	–	4.5	Renko *et al*, 1989
S. rimosus	metallo-protease	12	8.0	60°C	–	Kïm *et al*, 1989
Streptomyces sp.	serine	50	12.3 or higher	60°C	8.7	Nakanishi *et al*, 1974
Streptomyces griseus var *alkalophilus*	serine	28	11.0	–	8.4	Muro *et al*, 1991
Streptomyces sp.G-157						
Protease I	metal chelator sensitive	36	7.0 - 8.0	45-55°C	–	Sampath *et al*, 1997 ,,
Protease II	Trypsin-like	21	8.4 - 8.6	37°C	–	
S. thermovulgaris						
Protease I	serine	30	–	–	5.8	Yeoman and Edwards, 1997
Protease II	serine	60	–	–	8.4	
S. caespitosus	zinc metallo-protease	15	6.0	50°C	–	Kurisu *et al*, 1997
S. griseoalbus SN-22	serine	26	9.0	45°C	6.4	Murao *et al*, 1994
S. albidoflavus						Kang *et al*, 1995
Protease I	Trypsin-like	32	10.0	40°C	–	
Protease II	metallo-protease	18	8.0	55°C	–	
Streptomyces sp.	serine	33	7.8	35-50°C	6.7	Bono *et al*, 1996
Streptomyces sp.YSA-130	serine	19	11.5	60°C	–	Yum *et al*, 1994
Streptomyces sp. MF-20	serine	28	9.0	75°C	–	Ohishi and Yamagishi, 1996
S. reticuli	metallo-protease	–	7.0 - 7.7	55°C	3.9	Moorman *et al*, 1993

S. corchorusii ST-36						
Protease I	metallo-protease	31	11.0	70°C	–	El-Raheem *et al*, 1995
Protease II	metallo-protease	36	10.0	70°C	–	
S. griseus	serine (acidic amino acid specific)	22	8.8	–	8.4	Yoshida *et al*, 1988
S. moderatus						
Protease I	metal chelator sensitive	21	alkaline pH	–	4.6	Chandrasekaran and Dhar, 1987
Protease II	Trypsin-like	19	optima	–	3.8	
Protease III	alkaline serine	22	,,	–	7.8	
Protease IV	alkaline serine	29	,,	–	8.4	
Protease V	alkaline serine	23	,,	–	9.2	
Nocardiopsis dassonvillei OPC-210						
Protease I	serine	21	10.0-12.0	70°C	6.4	Tsujibo *et al*, 1990a
Protease II	serine (metal chelator sensitive)	36	10.5	60°C	3.8	
N. dassonvillei ATCC 21944	serine	33.5	–	55°C	6.3	Kim *et al*, 1993
Thermoactinomyces sp.	serine	25	11.5-13.0	70°C	>11.0	Tsuchiya *et al*, 1992

Six chymotrypsin-like serine proteases have been identified from *S. griseus*, namely *S.griseus* trypsin (SGT), *S.griseus* proteases A, B, C, D and E i.e. (SGPA), (SGPB), (SGPC), (SGPD) and (SGPE) respectively (Awad *et al*, 1972b; Sidhu *et al*, 1994; Sidhu *et al*, 1995; Yoshida *et al*, 1988). The gene sequences of the enzymes have been determined (Henderson *et al*, 1987; Sidhu *et al*,1993; Kim *et al*, 1991; Sidhu *et al*, 1994; Sidhu *et al*,

1995). The high resolution crystal structures of SGT, SGPA, SGPB and SGPE have also been determined (Read *et al*, 1983; Read and James, 1988; James *et al*, 1980; Nienaber *et al*, 1993). Among these enzymes, SGPD is quite interesting owing to the fact that unlike all previously characterized bacterial chymotrypsin-like serine proteases, SGPD forms a stable α_2 dimer. Otherwise, SGPD has a high degree of sequence homology to SGPA, SGPB, SGPC and SGPE. The amino acid sequence of SGPD in the region of the specificity pocket was also found to be similar to that of SGPA, SGPB and SGPC (Sidhu *et al*, 1995). Some properties of these chymotrypsin-like proteases have been summarized in table 3.2.

Table 3.2 : Some properties of chymotrypsin-like serine proteases from *S. griseus*

- Modified from Sidhu *et al* (1995)

Enzyme	No.of disulphide bonds	Quaternary structure	pI	M_r kDa	Amino acid specificity	Reference
SGPA	2	α	7.1	20	large aliphatic/ aromatic	Henderson *et al*, 1987; James *et al*, 1980
SGPB	2	α	7.01	19	,,	Henderson *et al*, 1987; Read *et al*, 1983
SGPC	3	α	7.59	26	,,	Sidhu *et al*, 1994
SGPD	2	α_2	3.55	36	,,	Sidhu *et al*, 1995
SGPE	2	α	7.86	18	glutamic acid	Sidhu *et al*, 1993; Nienaber *et al*, 1993

The occurrence of chymotrypsin-like enzymes is common in higher eukaryotes although reports of such enzymes from prokaryotic systems are fewer and *S. griseus* is therefore an interesting example. Besides *S. griseus*, several other streptomycete species and few non-streptomycete actinomycetes have been reported to produce proteases having substrate specificities similar to well known microbial and mammalian endopeptidases. They have been classified as having collagenase-like, elastase-like, fibrinolytic, thrombolytic, keratinase-like, trypsin-like or rennin-like activities. Some of these are listed in table 3.3.

Table 3.3 : Actinomycete proteases with unusual substrate specificities

Substrate specificity	Source	Reference
Collagenase-like	*Actinomadura* sp.	Rippon, 1968 ; Norwig, 1971
	Mycobacterium tuberculosis	Takahashi *et al*, 1967
	Streptomyces thermovulgaris	Egorov *et al*, 1976
	Streptomyces griseus	Drake *et al*, 1966
	Streptomyces sp.	Chakraborty and Chandra, 1984
		Chakraborty and Chandra, 1986
		Endo *et al*, 1987
		Demina and Lysenko,1992
Elastase-like	*Streptomyces griseus*	Gertler and Trop, 1971
		Trop and Birk, 1970
	Streptomyces fradiae	Lagutina and Petrova, 1979
	Streptomyces rimosus	Rassulin *et al*, 1974
	Streptomyces griseoalbus SN-22	Murao *et al*, 1994
Keratinase-like	*Streptomyces fradiae*	Nickerson and Durand, 1963
		Nadanishi and Yamamoto, 1974
	Streptomyces sp. A11	Mukhopadhyay and Chandra, 1993

Trypsin-like	*Streptomyces erythraeus*	Yoshida *et al*, 1971
	Streptomyces fradiae	Morihara and Tsuzuki, 1968
	Streptomyces griseus	Trop and Birk, 1968b
		Tashiro *et al*, 1981
Milk-clotting or Rennin-like	*Streptomyces erythraeus*	Sternberg, 1976
	Streptomyces albus	,,
	Streptomyces chimensis	,,
	Streptomyces rimosus	,,
	Streptomyces griseus	Murata *et al*, 1987
	Streptomyces caespitosus	Muro *et al*, 1984
Fibrinolytic	*Streptomyces clavuligerus*	Buckley and Jefferies, 1981
	Streptomyces fulvoviridis	,,
	Streptomyces gedanensis	,,
	Streptomyces griseus	,,
	Streptomyces rimosus	,,
	Streptomyces thermovulgaris	Egorov, 1976; Al-Nuri *et al*, 1984
	Streptomyces sp.	Bono *et al*, 1996
Thrombolytic	*Micromonospora* sp.	Lysenko and Salivonic, 1988
	Nocardia sp.	
	Streptomyces flavus	
	Streptomyces fradiae	
	Streptomyces globisporus	

Among the actinomycetes, most of the reported proteases are from *Streptomyces* species whereas reports of proteolytic enzymes from the non-streptomycete group are relatively few. Very few genera, like for instance *Thermoactinomyces, Nocardia, Nocardiopsis* and *Micromonospora* have been reported in this respect. More studies need to be directed towards proteases from this group of actinomycetes in order to obtain a

clearer understanding of their characteristics and significance. Serine proteases have been reported from *Nocardiopsis dassonvillei* (Tsujibo *et al*, 1990a ; Kim *et al*, 1993). Studies on the amino acid compositions and partial sequences of two serine proteases from *N. dassonvillei* OPC-210 have shown that protease I is a chymotrypsin-like serine protease, whereas protease II was like aqualysin, an alkaline serine protease from *Thermus aquaticus* (Tsujibo *et al*, 1990 b).

The following chapter deals with the optimization of growth conditions for the production of extracellular protease by *Nocardiopsis* sp. NCIM 5124 and the purification and comparative characterization of two proteases secreted by the organism. The proteases have been characterized with respect to their molecular properties and substrate specificities and the data are discussed with reference to the published literature.

MATERIALS AND METHODS

MATERIALS

CM-Sephadex, DEAE-cellulose, Sephadex G-50, phenyl-Sepharose, paranitoanilide substrates, ester substrates, SDS-PAGE and gel-filtration molecular weight markers, bovine serum albumin, ovalbumin, collagen, elastin, fibrin. Freund's adjuvant, all protease inhibitors and chemical modifiers were obtained from Sigma Chemical Company, U.S.A. Hydroxyapatite, Casein (Hammarsten), fat-free casein, SDS and agarose were purchased from Sisco research Laboratories, India. Haemoglobin was obtained from Hi-media chem. India. All other chemicals used were of analytical grade.

Micro organism

Nocardiopsis sp. NCIM 5124 was used for further studies. The culture was maintained on nutrient agar slants (1% peptone, 0.3% beef extract, 0.5% NaCl, 2.5% agar, pH 8.0) and subcultured every 3 months.

METHODS

Enzyme assays

Protease :

Protease activity was determined by the method of Kunitz (1947), as described by Laskowski (1955). The reaction mixture consisted of 1ml of a 1% w/v solution of casein (Hammarsten) and 1 ml of of an appropriately diluted solution of the protease in 50 mM Na-carbonate buffer , pH 10.0. After incubation at 60°C for 20 min., the reaction was stopped with 3 ml of TCA solution (5% w/v TCA, 9% w/v sodium acetate and 9%v/v acetic acid). After 30 min. the residual casein was separated by centrifugation and the absorbance of the TCA soluble peptides liberated from casein was read at 280 nm.

One unit of protease activity is defined as the amount of enzyme which releases 1μ mole of tyrosine per minute at 60°C and pH 10.0. Preliminary studies had shown that 60°C

was the optimum temperature for the crude protease. Protease activity with haemoglobin, bovine serum albumin and ovalbumin as substrates was also determined similarly.

The time course of release of TCA soluble products from casein, haemoglobin, bovine serum albumin and ovalbumin was followed by withdrawing 0.5 ml samples at 10 min. intervals from a reaction mixture consisting of a 5 mg/ml solution of the substrate and 1 μg of the proteases (enzyme to substrate ratio of 1: 5000) in 50 mM Na-carbonate buffer, pH 10.0 incubated at 60°C, over a period of 90 min. The residual substrate was precipitated with 0.75 ml of 10% TCA solution (6% final concentration) and separated by centrifugation. The absorbance of the TCA soluble products was measured at 280 nm.

Assays with insoluble protein substrates

Protease activity against collagen, fibrin, elastin and keratin was determined essentially by the method of Nickerson *et al* (1963). The method was modified as follows :

100 mg of substrate was incubated with 100 μg of proteases I and II in 20 mM Tris-HCl buffer, pH 8.5 (total volume 5 ml) at 37°C for 2 h after which the reaction was stopped by the addition of 5 ml of 0.5 M Na-acetate buffer, pH 5.0. The reaction mixture was filtered through Whatman No. 1 filter paper and the solubilized protein in the clear filtrate was estimated by the method of Lowry *et al* (1951). For each assay the following controls were employed:

1] enzyme in buffer solution; 2] substrate in buffer solution

Activity was calculated from : (substrate+ enzyme+ buffer system) − (controls 1+ 2)

One unit of collagenolytic, fibrinolytic, elastolytic or keratinolytic activity was expressed as mg of protein dissolved per hour per mg of enzyme.

Amidase activity

Amidase activity with various synthetic substrates was determined essentially by the method of Ansari and Stevens (1983). The method was modified as follows :

Different paranitroanilide substrates were incubated at 0.5 mM concentrations with the appropriately diluted proteases in 50 mM Tris-HCl buffer, pH 9.0 in a total volume of

0.76 ml at 60°C for 30 min. in test tubes covered with glass marbles to prevent evaporation. The reaction was stopped with 0.5 ml of 12.6% acetic acid (5% final conc.). The paranitroaniline released was determined by measuring the absorbance at 420 nm. One unit of amidase activity is defined as the amount of enzyme which releases one μ mole of paranitroaniline per min. at 60 °C and pH 9.0.

The Km and Vmax values of the proteases for different paranitroanilide substrates were determined by using different substrate concentrations in the reaction mixtures. The constants were obtained by Lineweaver-Burk double reciprocal plots.

Protein estimation

Protein was determined by the method of Lowry *et al* (1951) with Bovine serum albumin as standard.

Biomass was expressed in terms of dry weight. After growth, the biomass was harvested by centrifugation, washed with distilled water and dried at 40°C, till 3 constant values for dry weight were obtained.

Optimization of conditions for protease production in shake-flask cultures

Culture Conditions

For medium optimization studies, the organism was grown in 250 ml conical flasks with 50 ml medium. The flasks were incubated for 6 days at 30°C on a rotary shaker at about 200 rpm, after which biomass in terms of the dry weight and the protease activity in the cell free supernatant were determined.

The inoculum was developed in 100 ml nutrient broth (1% peptone, 0.3% beef extract, 0.5% NaCl, pH 8.0) for 36 h at 30°C and about 200 rpm. The exponential phase cells were pelleted out, washed with sterile distilled water and resuspended in 100 ml of sterile distilled water. 5 ml of this suspension was inoculated in 50 ml of the culture medium in all experiments (10% v/v inoculum).

Variation of medium constituents

The effect of various carbon sources on protease production was determined in a medium containing 1% fat–free casein, 0.3% K_2HPO_4 , 0.05% $MgSO_4.7H_2O$ and 1% Na_2CO_3 . The carbon sources tried were–

Starch, cellulose, wheat bran, glucose, xylose, fructose, lactose, sucrose, maltose, mollasses at a concentration of 0.5% w/v.

The effect of different nitrogen sources on protease production was determined in a medium containing 1% starch, 0.3% K_2HPO_4 , 0.05% $MgSO_4.7H_2O$ and 1% Na_2CO_3 . Peptone, soyapeptone, yeast extract, beef extract, corn steep liquor, fat free casein and some readily available and cheap sources like ground nut press cake, coconut press cake, cottonseed press cake were tested at a concentration of 1% in the growth medium. Urea, $NaNO_3$, $(NH_4)_2SO_4$, NH_4Cl , NH_4NO_3 and $(NH_4)H_2PO_4$ were used so as to provide 70 mM nitrogen in the medium.

The concentrations of the best carbon and nitrogen sources and of K_2HPO_4 , $MgSO_4.7H_2O$ and Na_2CO_3 were then individually varied step wise to maximize protease production.

Effect of Glucose on protease production

The effect of glucose on growth and protease production was determined by addition of various concentrations of glucose (0.2–1.0%) in the medium containing optimized concentrations of starch, casein, K_2HPO_4, $MgSO_4.7H_2O$ and Na_2CO_3.

Effect of NaCl on protease production

The culture has been isolated from sea water and since NaCl is a major constituent of sea water , its effect on growth and protease production by the culture was checked. Various concentrations of NaCl (0–1.0 M) were added in the optimized medium.

Effect of initial pH of the medium on protease production

The initial pH of the optimized medium was adjusted from pH 4.0 to 10.0 . The medium contains 1% Na_2CO_3 due to which the initial pH is about 10.0. It was adjusted with 1N HCl to the required initial pH values.

Effect of incubation temperature on protease production

The culture was grown in the optimized protease production medium for 6 days at different temperatures (20–50°C) and biomass in terms of dry weight and protease activity in the cell free broth was determined.

Effect of agitation rates on protease production

The culture was grown in the optimized protease production medium for 6 days at 30°C at different agitation rates (0, 50, 100, 150, 200 rpm). The biomass and the protease activity in the cell free supernatant were then determined.

Time course of protease production

The culture was grown in the optimized medium for 8 days at 30°C. Protease production and biomass in terms of dry weight were determined at regular intervals.

Purification of the proteases

The culture was grown in the optimized protease production medium for 6 days at 30°C at about 200 rpm, after which the culture broth was centrifuged at 10,000 rpm for 10 min. to pellet out the cells. The supernatant so obtained was used as the crude protease.

Protease I:

The pH of the crude enzyme preparation was adjusted to 5.0 with 1M acetic acid, in the presence of 1M NaCl. This resulted in the formation of a precipitate, which was removed by centrifugation at 10,000 rpm for 10 min. The presence of 1M NaCl was found to minimize the co-precipitation of the protease. The supernatant was concentrated about

8–10 times by ultra-filtration under reducing atmosphere using a 3,000 cut-off membrane (YM-3 membrane, Amicon).

The concentrate was dialyzed against 20 mM Na-acetate buffer, pH 5.0 and loaded onto a CM-Sephadex column (12.5 cm × 2.5 cm) equilibrated with the same buffer. Most of the activity was found to be retained on the column, while one portion (about 2 to 5 %) of the activity passed unadsorbed through the column. The column was then washed with the above buffer containing 0.2 M NaCl and the adsorbed protein was eluted with a linear gradient of 0.2-1.0 M NaCl in the same buffer.

The active fractions were pooled and dialyzed against 20 mM Na-carbonate buffer, pH 9.0 and re–chromatographed on CM–Sephadex at pH 9.0. The adsorbed protein was eluted with 50 mM NaCl in the same buffer and the active fractions were pooled .

Protease II:

The portion of protease activity that passed unadsorbed through CM-Sephadex at pH 5.0, was applied onto a column of DEAE-cellulose (12 cm × 2.5 cm) equilibrated with 20 mM Na-acetate buffer, pH 5.0. Most of the activity was found to adsorb on the matrix. The column was then washed with the equilibration buffer and the adsorbed protein was eluted with a linear gradient of 0–1.0 M NaCl in the same buffer. The active fractions were pooled and concentrated by ultrafiltration under reducing atmosphere using a 10,000 cut-off membrane (YM -10 membrane, Amicon).

The concentrate was subjected to gel filtration through a column of Sephadex G-50 (145 cm × 2 cm), equilibrated with 20 mM Tris-HCl buffer, pH 8.0 containing 100 mM NaCl. The active fractions were pooled and 12% w/v $(NH_4)_2SO_4$ was added to the enzyme solution at this stage which was then loaded onto a column of phenyl-Sepharose (10 cm × 1.5 cm), equilibrated with 20 mm Tris-HCl buffer, pH 8.0, containing 12% w/v $(NH_4)_2SO_4$. The column was washed first with the equilibration buffer, and then with a linear gradient of 12–0% $(NH_4)_2SO_4$. The bound enzyme was eluted by washing further with 20 mM Tris-HCl buffer, pH 8.0 without $(NH_4)_2SO_4$.

The active fractions were pooled and dialyzed against 10 mM Na-phosphate buffer, pH 7.0 and passed through hydroxyapatite, which removed the yellow colour from the enzyme preparation. The colour was found to adsorb onto the matrix, whereas the protease activity passed unbound through the column. The active fractions were pooled.

The homogeneity of both the proteases was checked by SDS-PAGE (Laemmli, 1970) in 12% w/v acrylamide gels. The protein bands were visualized by silver staining (Blum et al, 1987). The proteases had to be inhibited with 5mM PMSF, before boiling in the SDS sample buffer to prevent autocatalysis.

Characterization of the proteases

The M_r of the proteases were determined by SDS-PAGE and gel filtration chromatography. Determination of M_r by SDS-PAGE was done according to the method of Laemmli (1970). The standard molecular weight markers used were bovine serum albumin-66 kDa, ovalbumin-45 kDa, carbonic anhydrase-29 kDa, soyabean trypsin inhibitor-20.1 kDa and α-lactalbumin-14.2 kDa.

Determination of M_r by gel filtration was done by using a column of Sephadex G-50 (100 cm $\times$ 1 cm) equilibrated with 20 mM Tris-HCl buffer, pH 8.0, containing 0.1M NaCl. Standard molecular weight markers used were carbonic anhydrase- 29 kDa, trypsinogen- 24 kDa , soyabean trypsin inhibitor-20.1 kDa, cytochrome C-12.4 kDa and aprotinin-6.5 kDa .

The isoelectric points of the proteases were determined by the modified straight tube method (Chinnathambi et al, 1995) using ampholines in the range of pH 3.0–10.0.

The optimum pH for enzyme activity was determined by assaying the proteases at pH values ranging from 2.0–12.0 in universal buffers at 60°C.

The optimum temperature for enzyme activity was determined by assaying the proteases at pH 10.0 at different temperatures (10–80°C).

The effect of various protease inhibitors was checked by incubating the proteases with the inhibitor solutions as described in table 3.8 and then determining the residual enzyme activity.

The pH and temperature stabilities were determined by pre–incubating the proteases in universal buffers of various pH values (pH 2.0–12.0) at different temperatures (10–70°C) for 1 hour after which residual enzyme activities were determined at pH 10.0 and 60°C.

The effect of different metal ions was determined by pre-incubating the proteases with the chloride salts of various metal ions for 30 min. and then assaying the enzymes for residual activities.

Chemical modification studies were done by using various amino acid side-chain specific reagents. The proteases were incubated at 2 μM concentrations with different modifiers in the appropriate buffers (Table 3.12) for 30 min. and the residual protease activity was determined. Control tubes without inhibitor were incubated under identical conditions.

Amino acid composition of the proteases was determined by an automated amino acid analyzer (Hewlett Packard Series 1050, with HP fluorescence detector). The samples were hydrolyzed in 200 μl of 6N HCl for 20 h at 110°C and then subjected to analysis. Tryptophan content of the proteases was determined by the method of Spande and Witkop (1967). Total cysteine content was determined according to Cavallini et al (1966).

The proteases were analysed for their carbohydrate content by the phenol-sulphuric acid method for estimation of total carbohydrate (Dubois et al , 1956).

Antibodies were raised against electrophoretically pure protease I in New Zealand white rabbits by subcutaneously injecting 500 μg of protein emulsified with an equal volume of Freund's complete adjuvant. Booster injections were given at fortnightly intervals for 16 weeks. The rabbit was bled when the antibody titre reached 8, as checked by Ouchterlony double diffusion. The IgG fraction of antibodies was separated from the other serum

proteins by precipitation with $(NH_4)_2SO_4$ at 50% concentration and DEAE-cellulose chromatography at pH 7.4 (Dunbar and Schwoebel, 1990).

Ouchterlony double diffusion was carried out in 1.5% agarose gels in phosphate buffered saline (10 mM Na-phosphate buffer, pH 7.0 containing 0.145 M NaCl) at 20°C for 24-48 h, To check cross reactivity against protease II and also other serine proteases, 20 to 100 µg of antigen and 1 mg of IgG was tried. Also, 100 µg of antigen and various dilutions of the antibody (upto 1:16) were tried.

RESULTS AND DISCUSSION

Optimum culture conditions for protease production by *Nocardiopsis sp.* NCIM 5124

1% starch and 1% casein were both used in the medium used for screening for protease production. Therefore both these constituents are important for growth and protease production by this organism. The importance of these substrates was further investigated by replacing each, one at a time, with other substrates and by varying the concentrations in the medium. As seen from table 3.4 and figure 3.1, replacing 1% starch by lower concentrations reduced the activity. Furthermore replacing starch by other sugars or cellulosic substrates also decreased protease activity. Media for production of proteases by *Streptomyces* sp. usually contain starch (Mizusawa *et al*, 1966; Mizusawa *et al*, 1969; Nakanishi *et al*, 1974) whereas glucose has been used for *Thermoactinomyces* sp. (Tsuchiya *et al*, 1992), *Streptomyces cellulosae* (Muro *et al*, 1984) and *Nocardiopsis dassonvillei* OPC 210 (Tsujibo *et al*,1990 a).

Table 3.4 : Effect of Carbon source on protease production

Carbon Source 0.5% w/v *	Protease Activity U/ml
Starch	5.2
Starch (1% w/v)	10.2
Wheat bran	2.7
Molasses	2.0
Cellulose	0.23
Sucrose	1.05
Maltose	0.91
Lactose	1.66
Xylose	0.47
Fructose	0.38
Glucose	0.98

* in a medium containing 1% casein, 0.3% K_2HPO_4, 0.05% $MgSO_4$ and 1% Na_2CO_3

Figure 3.1

Effect of starch concentration on the production of protease by *Nocardiopsis* sp. NCIM 5124

Different concentrations (0–2.5% w/v) of starch were added to a medium containing 1% casein, 0.3% K_2HPO_4 , 0.05% $MgSO_4.7H_2O$ and 1% Na_2CO_3, pH 10.0 and the biomass and protease activity were determined after 6 days of incubation at 30°C and 200 rpm.
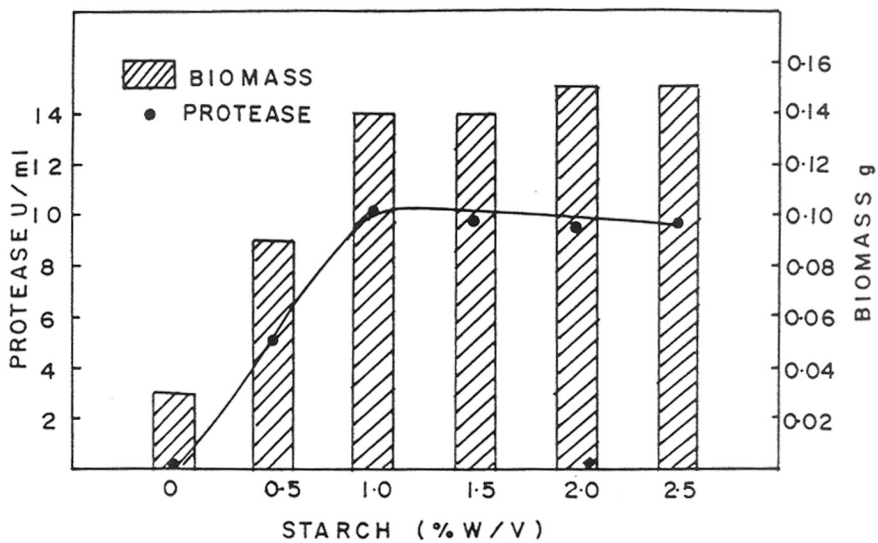
In the presence of 1% starch, casein at 1% w/v concentration enhanced protease production to about 9 U/ml (Table 3.5 and Fig 3.2) from extremely low levels (0.03 to 0.05 U/ml) when inorganic nitrogen sources were used. Tsujibo *et al* (1990 a) have also used casein in the medium used for protease production by *N. dassonvillei* OPC-210 although optimal production conditions have not been reported by them.

Table 3.5: Effect of Nitrogen source on protease production

Nitrogen Source *	Protease activity U/ml
Peptone	0.875
Soya peptone	0.18
Yeast extract	0.19
Beef extract	0.21
Casein	9.02
Corn steep liquor	0.08
Coconut press cake	0.85
Groundnut press cake	2.31
Cottonseed press cake	0.93
Urea	0.02
$NaNO_3$	0.04
$(NH_4)_2SO_4$	0.03
$(NH_4)H_2PO_4$	0.05
NH_4NO_3	0.04
NH_4Cl	0.05

*in a medium containing 1% starch, 0.3% K_2HPO_4, 0.05% $MgSO_4.7H_2O$ and 1% Na_2CO_3

Figure 3.2

Effect of casein concentration on production of protease by *Nocardiopsis* **sp. NCIM 5124**

Different concentrations (0–2.5% w/v) of casein were added to a medium containing 1% starch, 0.3% K_2HPO_4, 0.05% $MgSO_4.7H_2O$ and 1% Na_2CO_3, pH 10.0 and the biomass and protease activity were determined after 6 days of incubation at 30°C and 200 rpm.
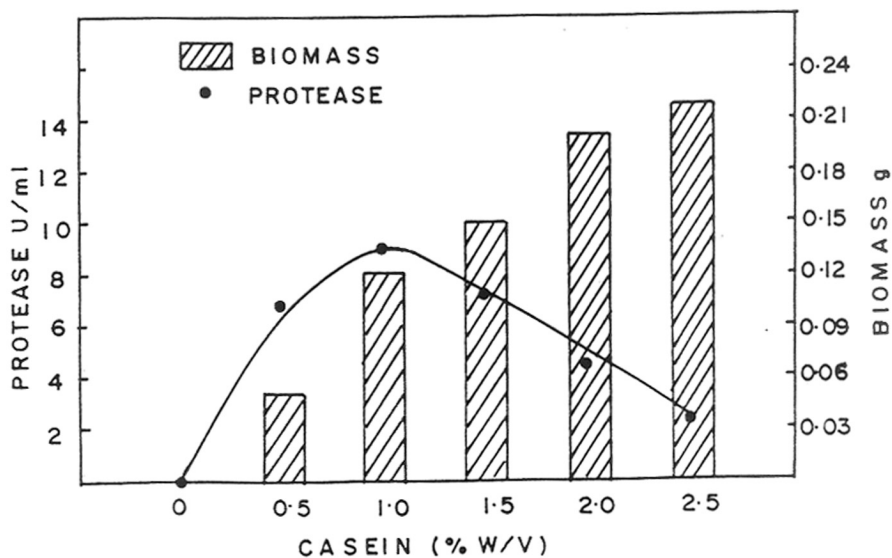
Variation of the concentrations of K_2HPO_4 and Na_2CO_3 (Figures 3.3 and 3.4) further showed that a medium containing 1% starch, 1% casein, 0.1% K_2HPO_4 and 1% Na_2CO_3 was optimum for production of extracellular protease by *Nocardiopsis* sp. NCIM 5124. The medium used for screening for protease production also contained $MgSO_4.7H_2O$. However this salt did not enhance protease production and the same level of protease was secreted at concentrations from 0 to 0.09% $MgSO_4.7H_2O$. Thus, this salt was omitted from the medium in further studies.

There was an increase in both growth (by about 20%) and protease production (by 50%) on addition of 0.2% glucose to the medium (Fig. 3.5). However, higher concentrations of glucose did not affect either. In the present strain, glucose enhances protease production which is different from other bacteria in which the protease production was repressed (Glenn, 1976; Ingram *et al*, 1983; Razak *et al*, 1994).

Nocardiopsis sp. NCIM 5124 grew well upto 0.6 M NaCl, the biomass production being similar to that in a medium without NaCl (Table 3.6). The molarity of NaCl in sea water is 0.4 M and the fact that the organism could grow equally well at this concentration indicated that it is adapted to the sea water environment from where it was isolated. However, above 0.6M NaCl, biomass was found to decrease significantly. A considerable amount of protease (4.7 U/ml) is produced even at 0.6 M NaCl, although protease was maximally produced by the organism in the absence of NaCl in the medium.

Table 3.6 : Effect of NaCl on growth and protease production

NaCl (M) *	Protease U/ml	Biomass (dry wt) g
0	15.0	0.11
0.2	10.6	0.12
0.4	6.5	0.11
0.6	4.7	0.10
0.8	0.25	0.06
1.0	0.05	0.04

* in a medium containing 1% starch, 1% casein, 0.1% K_2HPO_4, 1% Na_2CO_3 ,0.2% glucose, pH 10.0

Figure 3.3

Effect of K$_2$HPO$_4$ concentration on production of protease by *Nocardiopsis* sp. NCIM 5124

Different concentrations (0–0.9% w/v) of K$_2$HPO$_4$ were added to a medium containing 1% starch, 1% casein, 0.05% MgSO$_4$.7H$_2$O and 1% Na$_2$CO$_3$, pH 10.0 and the biomass and protease activity were determined after 6 days of incubation at 30°C and 200 rpm.
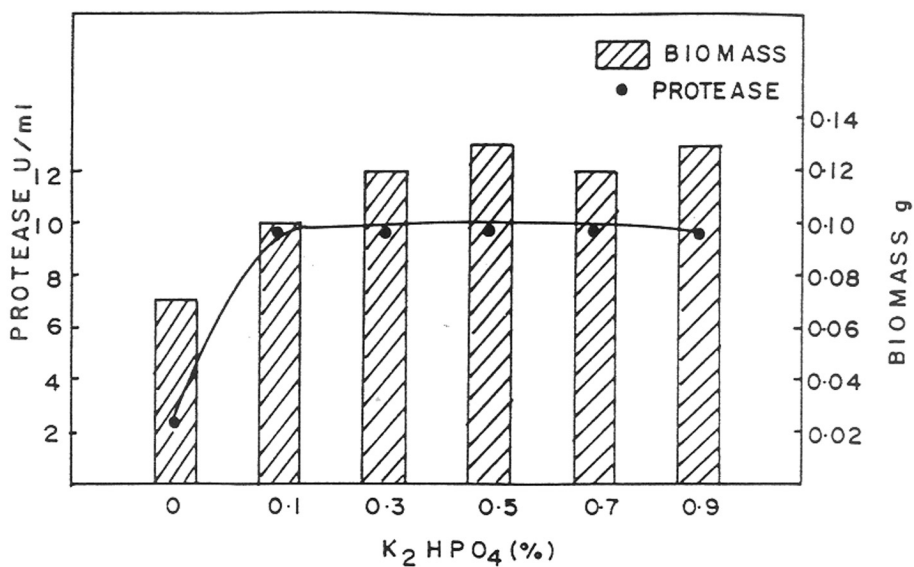
Figure 3.4

Effect of Na₂CO₃ concentration on production of protease by *Nocardiopsis* sp. NCIM 5124

Different concentrations (0.5–2.5% w/v) of Na_2CO_3 were added to a medium containing 1% starch, 1% casein, 0.1% K_2HPO_4 and 0.05% $MgSO_4.7H_2O$, pH 10.0 and the biomass and protease activity were determined after 6 days of incubation at 30°C and 200 rpm.

Figure 3.5

Effect of glucose on production of protease by *Nocardiopsis* sp. NCIM 5124

Different concentrations (0–1.0% w/v) of glucose were added to a medium containing 1% starch, 1% casein, 0.1% K_2HPO_4 and 1% Na_2CO_3, pH 10.0 and the biomass and protease activity were determined after 6 days of incubation at 30°C and 200 rpm.

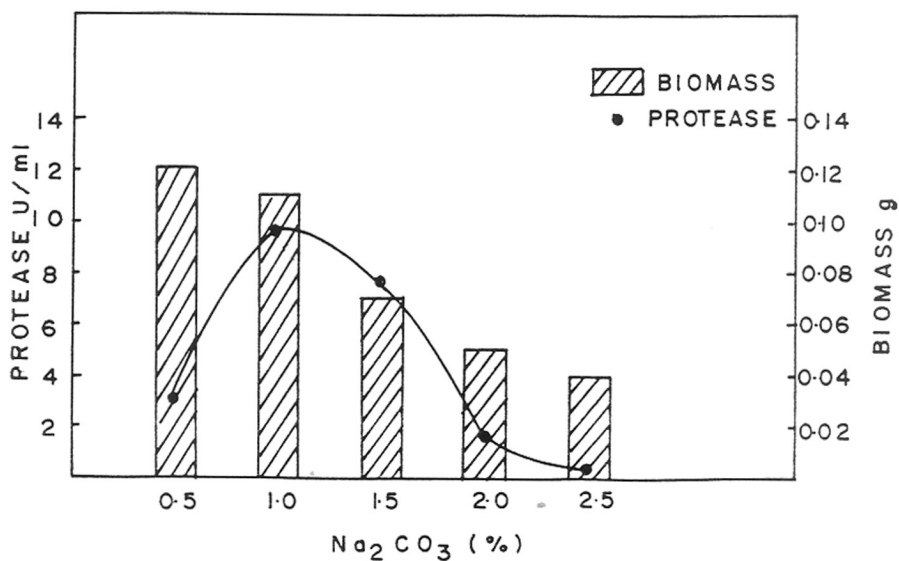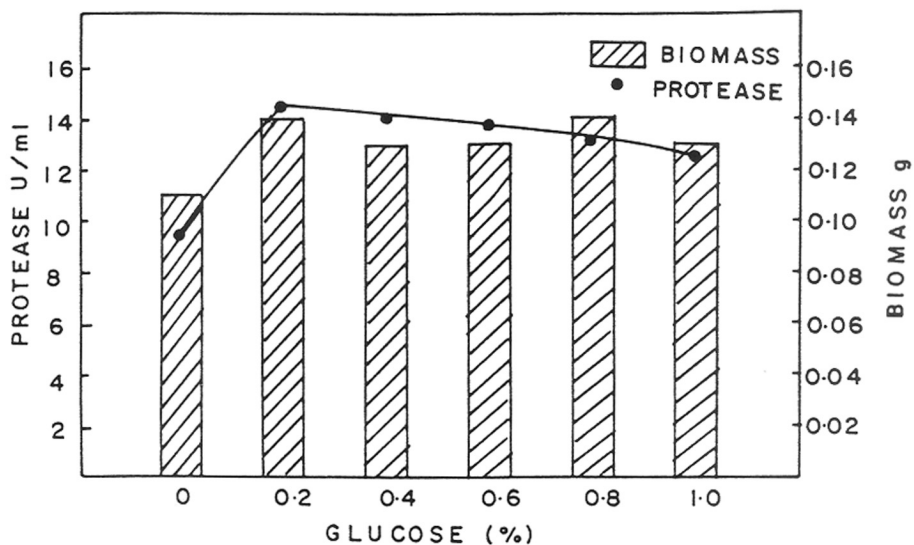**Effect of initial pH of the medium on growth and protease production**

As seen from Figure 3.6, protease was maximally produced (13 U/ml) by *Nocardiopsis* sp. NCIM 5124 when it was cultured at pH 10.0. Less than 5% of the maximum activity was observed when the organism was grown at pH values of 4.0–7.0. Growth in terms of biomass (dry wt) was found to increase with an increase in pH value upto pH 10.0, above which it was found to decrease. Therefore, both growth and protease production by the organism, in the optimized medium, were maximal under alkaline conditions.

Effect of incubation temperature on growth and protease production

The optimum temperature for growth as well as protease production by *Nocardiopsis* sp. NCIM 5124 was found to be 30°C (Fig. 3.7) and this result is consistent with the fact that the organism has been isolated form a tropical marine environment where temperatures range from 28 to 30°C. Most mesophilic microorganisms have been reported to grow and produce proteases at temperatures around 30°C.

Effect of agitation rates on growth and protease production

Almost equal amounts of protease were produced at and above 100 rpm and the growth in terms of biomass was also similar. However, at 50 rpm very low levels of growth and protease production were observed. Neither growth nor protease production was observed under static culture conditions (Fig. 3.8). These results indicate that the organism required substantial aeration for growth and protease production.

Time course of growth and protease production

Extracellular protease was found to be produced by *Nocardiopsis* sp. NCIM 5124 , like most actinomycetes (Peczynska-Czoch and Mordarski, 1988) in the exponential phase of growth (Fig. 3.9). Biomass did not increase after day 4 indicating that the organism had reached the stationary phase. Maximum protease levels were observed a few hours after day 4, and protease production did not increase thereafter.

Figure 3.6

Effect of initial pH on protease production by *Nocardiopsis* sp. NCIM 5124

Initial pH of the medium containing 1% starch, 1% casein, 0.1% K_2HPO_4, 1% Na_2CO_3 and 0.2% glucose was adjusted with 1N HCl at pH values from 4.0 to 11.0. The biomass and protease production were determined after 6 days of incubation at 30°C and 200 rpm.
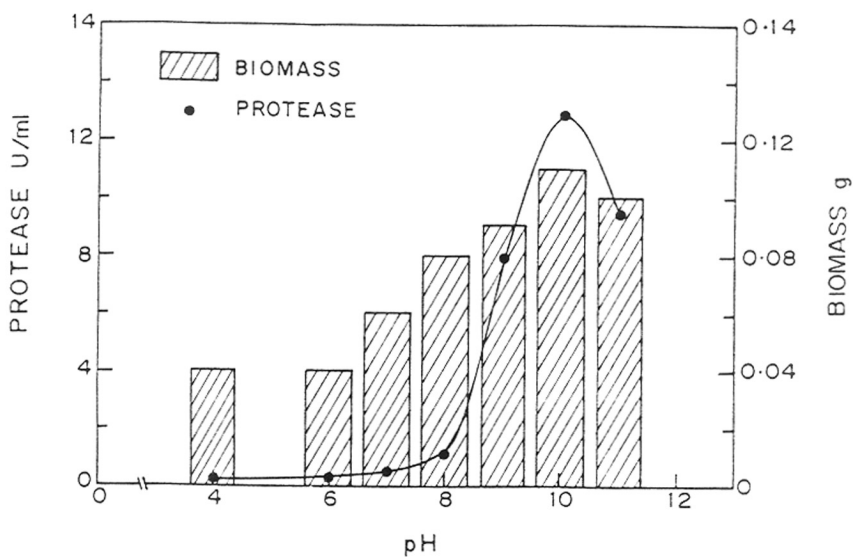
Figure 3.7

Effect of Incubation temperature on protease production by *Nocardiopsis* sp. NCIM
5124

The culture was grown in the optimized medium at different temperatures (20 to 50°C) and
the protease activity and biomass were determined after 6 days of incubation at 200 rpm.
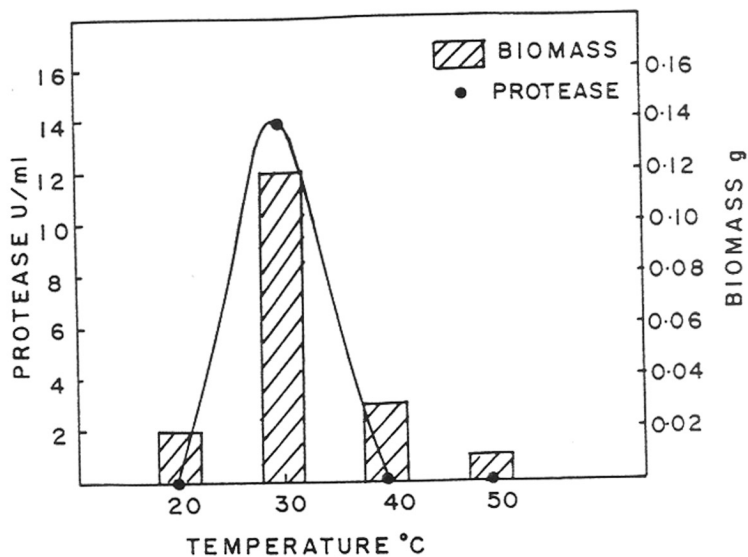
Figure 3.8

Effect of agitation rate on production of protease by *Nocardiopsis* sp. NCIM 5124

The culture was grown in the optimized medium at different agitation rates (0 to 200 rpm) and the protease activity and biomass were determined after 6 days of incubation at 30°C.
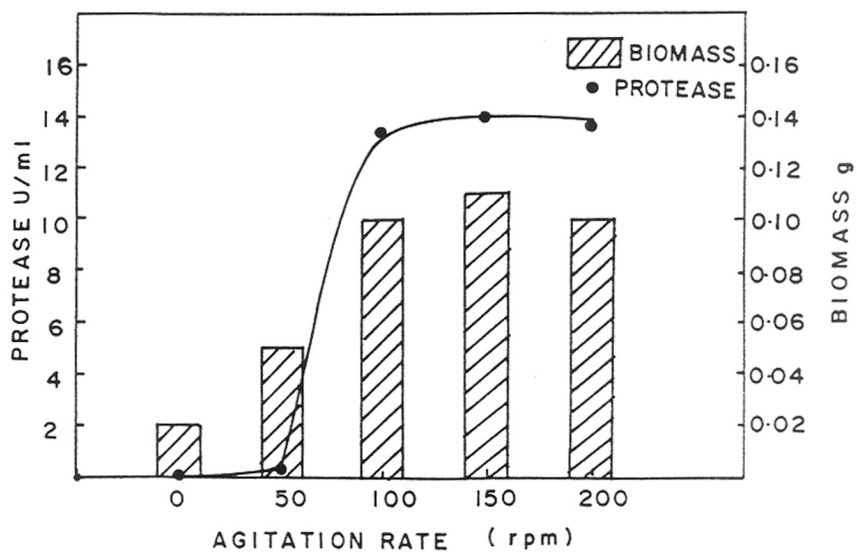
Figure 3.9

Time course of growth and protease production by *Nocardiopsis* sp. NCIM 5124

The organism was grown for 8 days under optimized culture conditions. Protease activity in the culture supernatant and the biomass were determined at regular intervals.
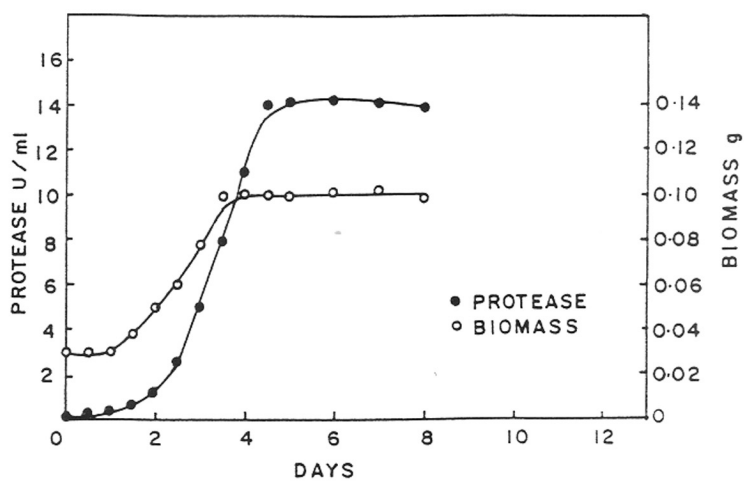
Therefore from these results, the optimum culture conditions for production of extracellular protease by *Nocardiopsis sp.* NCIM 5124 can be summarized as follows :

A medium consisting of 1% Starch, 1% Casein, 0.1% K_2HPO_4 , 1% Na_2CO_3 and 0.2% glucose, pH 10.0 after 4 days of growth at 30°C and 150 to 200 rpm.

Purification of proteases I and II

Electrophoretic separation of the crude filtrate followed by activity staining on casein- agarose showed the presence of two distinct activity bands, indicating the presence of two proteases. The crude culture filtrate contained unutilized casein which on concentration, was found to interfere with the adsorbtion of protease activity on ion-exchange columns. The residual casein was therefore precipitated by adjusting the pH of the culture filtrate to 5.0 and then separated by centrifugation. A large portion of the protease activity was found to co-precipitate, and this could be avoided by the addition of 1M NaCl prior to the precipitation step. Protease I was purified by ion-exchange chromatography using CM-Sephadex at pH 5.0 and then at pH 9.0. Protease I adsorbed onto CM-Sephadex even at pH 9.0 and this property was extremely useful in its purification from the other proteins after the initial chromatography on the same matrix at pH 5.0. Protease II was purified by ion-exchange using DEAE-cellulose, gel filtration on Sephadex G-50, hydrophobic chromatography on phenyl-Sepharose and colour removal on hydroxyapatite. Protease II was found to bind strongly to phenyl-Sepharose at 12% $(NH_4)_2SO_4$ and was not eluted with a gradient of 12–0% $(NH_4)_2SO_4$. It was eluted by washing the matrix further with buffer containing 0% $(NH_4)_2SO_4$. This helped its isolation from other contaminating proteins.

As seen from table 3.7 a and b, protease I and II were purified about 131 and 107 fold and with 20% and 43 % yields respectively. The flow diagram for purification of proteases I and II is illustrated in figure 3.10. The elution profiles of protease I from CM-Sephadex II (pH 9.0) and protease II from phenyl-Sepharose are shown in figures 3.11 and 3.12 respectively. Both proteases were found to be homogenous by SDS-PAGE (Fig. 3.13).

Figure 3.10 : Flow diagram for purification of proteases I and II

```
                              Culture filtrate
                                    |
                            adjusted to pH 5.0
                                    |
                    Centrifugation (10,000 rpm, 10 min.)
                                    |
                    Ultrafiltration (3000 cut– off membrane)
                                    |
                            CM-Sephadex I (pH 5.0)
                                    |
          ┌─────────────────────────┴─────────────────────────┐
  Bound protease fraction                              Unbound protease fraction
          |                                                    |
  elution with 0.2– 1.0 M NaCl                        DEAE-Sephadex (pH 5.0)
                                                      elution with 0 – 1.0 M NaCl
      (linear gradient)                                   (linear gradient)
          |                                                    |
  CM-Sephadex II (pH 9.0)                                  Ultrafiltration
  elution with 50 mM NaCl                          (10,000 cut– off membrane)
          |                                                    |
     ┌─────────┐                                          Sephadex G-50
     │PROTEASE I│                                               |
     └─────────┘                                         phenyl-Sepharose
                                               [ Bound at 12% (NH₄)₂SO₄ ;
                                               washed with 12–0% (NH₄)₂SO₄ ;
                                               eluted with 0% (NH₄)₂SO₄ ]
                                                               |
                                                         Hydroxyapatite
                                                               |
                                                       ┌──────────┐
                                                       │PROTEASE II│
                                                       └──────────┘
```

Bound protease fraction

elution with 0.2– 1.0 M NaCl

(linear gradient)

CM-Sephadex II (pH 9.0)
elution with 50 mM NaCl

PROTEASE I

Unbound protease fraction

DEAE-Sephadex (pH 5.0)

elution with 0 – 1.0 M NaCl

(linear gradient)

Ultrafiltration

(10,000 cut– off membrane)

Sephadex G-50

phenyl-Sepharose

[Bound at 12% $(NH_4)_2SO_4$;

washed with 12–0% $(NH_4)_2SO_4$;

eluted with 0% $(NH_4)_2SO_4$]

Hydroxyapatite

PROTEASE II

Table 3.7 a : Purification of protease I

Purification Step	Volume (ml)	Total Protein (mg)	Total Activity (Units)	Specific Activity (U/mg)	% Yield	Fold Purific- ation
Crude Broth	860	9638	11180	1.16	100	1
Ultraconcentration (YM-10)	145	2129	7535	3.54	67.4	3.05
CM-Sephadex I (pH 5.0)	200	98.3	2571	26.15	23	22.5
CM-Sephadex II (pH 9.0)	60	14.7	2236	152	20	131

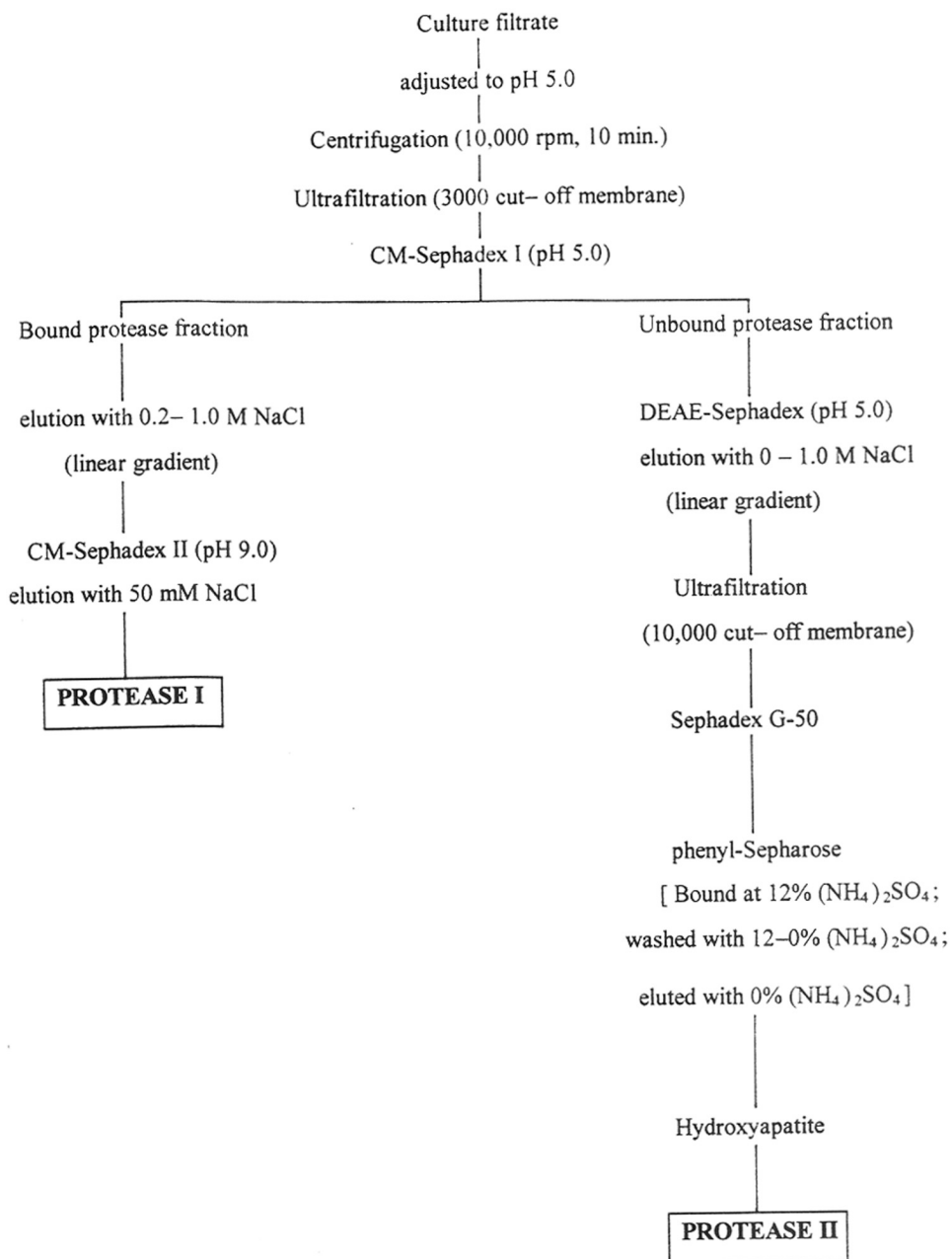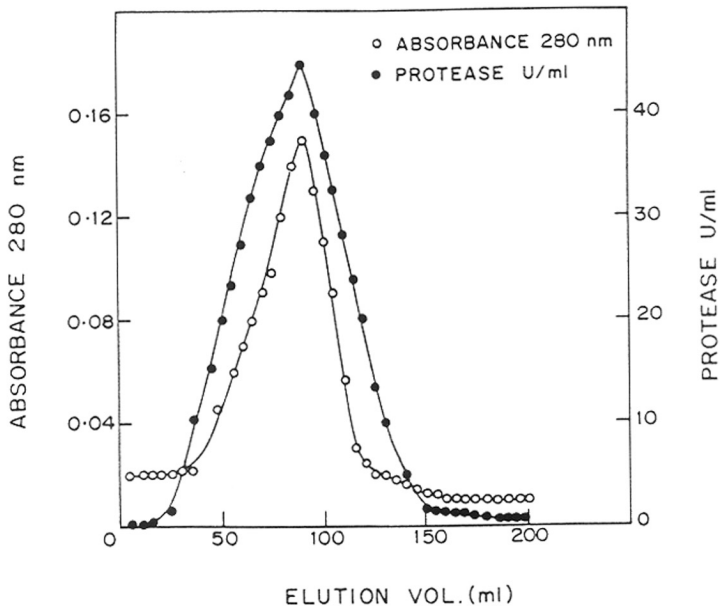Figure 3.11 : Elution profile of protease I from CM-Sephadex II

Table 3.7 b: Purification of protease II

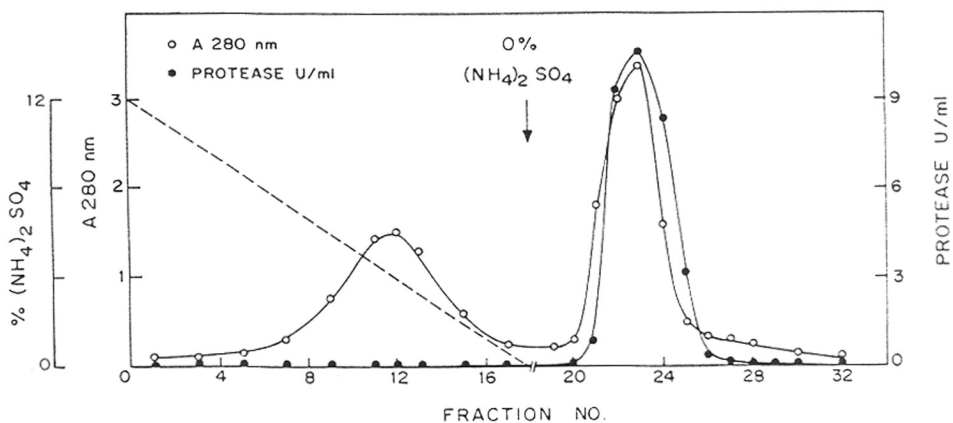| Purification Step	Volume (ml)	Total Protein (mg)	Total Activity (Units)	Specific Activity (U/mg)	% Yield	Fold Purific- ation
Unbound Fraction CM-Sephadex I	146	731	95	0.130	100	1
DEAE Sephadex	50	529	90	0.170	95	1.31
Sephadex G-50	64	166	80	0.482	84.2	3.71
Phenyl Sepharose	14	4.76	42	8.82	44.2	67.84
Hydroxyapatite	27	2.94	41	13.94	43.2	107.2

Figure 3.12 : Elution profile of protease II from phenyl-Sepharose

COMPARATIVE CHARACTERIZATION OF PROTEASES I AND II

Molecular mass (M_r):

The Molecular masses of proteases I and II, as determined by SDS-PAGE were approximately 21,000 and 23,000 daltons (Fig. 3.13). By molecular exclusion chromatography using Sephadex G-50, the apparent molecular mass of protease II was found to be approximately 24,000 (Fig. 3.14), whereas protease I was found to retard on the matrix showing M_r of about 9000. Similar results were obtained in the presence of 100 mM NaCl and also 5% ethylene glycol which are known to rule out ionic and hydrophobic interactions with the matrix. The protease also behaved similarly on Biogel-P30. According to Lane and Rhodes (1990), the greatest source of error in gel-filtration chromatography comes from the requirement that the unknown be similar in shape and density to the protein standards. Since the protein standards used are almost universally compact, globular proteins, this means that fibrous proteins or proteins having fibrous regions can behave anomalously on gel-filtration columns. Protease I was also found to leak out through 10,000 cut-off ultrafiltration membranes and dialysis tubing which further indicated that the anomaly on gel-filtration matrices could be a result of the shape of the molecule. On account of the anomalous behaviour of protease I on gel-filtration matrices, we consider the M_r of protease I and II to be 21,000 and 23,000 daltons respectively, as determined by SDS-PAGE.

Most actinomycete proteases have molecular masses ranging from 15,000-30,000 daltons. Tsujibo *et al* (1990a) reported M_r of 21,000 and 36,000 for proteases from their strain of *Nocardiopsis dassonvillei*. *Streptomyces griseus* proteases have M_r in the range of 15,000-30,000 (Peczynska-Czoch and Mordarski,1988). The trypsin-like protease reported from *S. rimosus* has a M_r of 28,000 (Renko *et al*, 1989), while other trypsin-like proteases from *S. erythraeus* (Yoshida *et al*, 1971) and *S. fradiae* (Morihara and Tsuzuki, 1968) have M_r of about 20,000. Muro *et al* (1984) have reported a protease from *S.cellulosae* having a M_r of about 26,500. An alkaline protease from *Thermoactinomyces* sp. (Tsuchiya *et al*,1992) has a molecular mass of 25,000. Thus, both proteases from *Nocardiopsis* sp. NCIM 5124 follow the same general trend of proteases from actinomycetes, in terms of their M_r.

Fig. 3.1 3

Determination of molecular masses of proteases I and II by SDS-PAGE

Proteases I and II were subjected to SDS-PAGE in the presence of β-ME in 12% w/v acrylamide gels. Lane 1- marker proteins- a) bovine serum albumin (66 kDa), b) ovalbumin (45 kDa), c) carbonic anhydrase (29 kDa), d) soyabean trypsin inhibitor (20.1 kDa), e) α-lactalbumin (14.2 kDa); Lane 2– purified protease I and Lane 3–purified protease II. The gels were stained with silver nitrate.
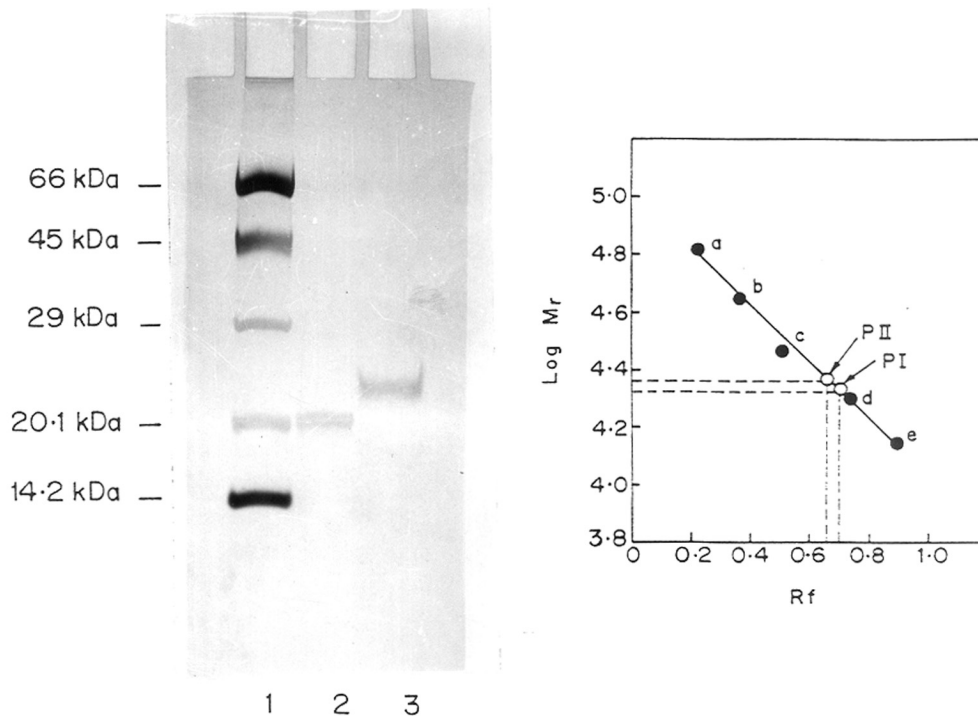
Figure 3.14

Determination of molecular mass of protease II by gel filtration

A Sephadex G-50 column (100 x 1 cm) was equilibrated with 20 mM Tris-HCl buffer, pH 8.0 containing 100 mm NaCl and calibrated with a) bovine serum albumin (66 kDa), b) ovalbumin (45 kDa), c) carbonic anhydrase (29 kDa), d) trypsin inhibitor (20 kDa), e) cytochrome-C (12.4 kDa). V_o - void volume and V_e - elution volume.
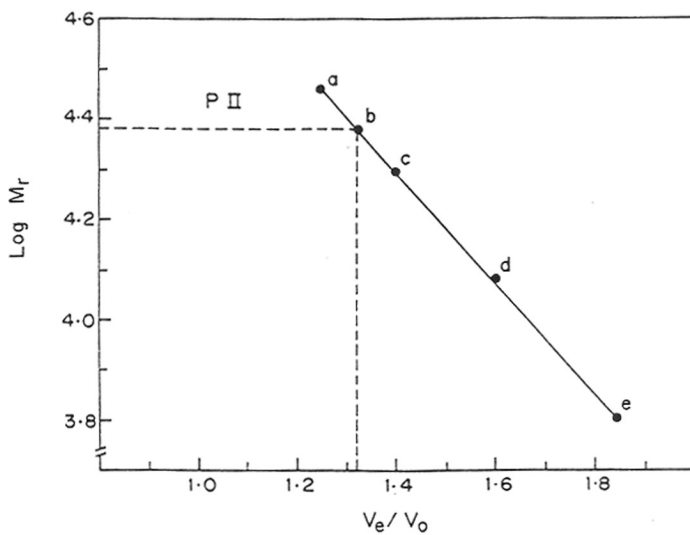
Isoelectric points:

The isoelectric points of protease I and II were determined as 8.3 and 7.0 respectively. Tsujibo *et al* (1990a) have reported isoelectric points of 6.4 and 3.8 for protease I and II from *N. dassonvillei* strain OPC-210. From the literature it is evident that most alkaline proteases of microbial origin have pIs in the range of 6.0-11.0, although there are a few reports of microbial alkaline proteases having acidic pIs like the trypsin-like serine protease from *Streptomyces rimosus* which has a pI of 4.5 (Renko *et al*, 1989) , the alkaline protease from *Candida olea* which has a pI of 5.4 (Nelson and Young, 1987), the alkaline protease D from *Cephalosporium* sp. with a pI of 3.8 (Tsuchiya *et al*, 1987). Protease II from *Nocardiopsis dassonvillei* OPC-210 (Tsujibo *et al*, 1990a) also falls in this class with its acidic pI of 3.8. However, both proteases from our organism have pI values in the range defined for most alkaline proteases.

pH optima :

The optimum pH for enzyme activity with casein as the substrate was pH 10.0-11.0 for protease I and pH 10.0 for protease II (Fig. 3.15). The optimum pH values for the activities of the proteases from *N. dassonvillei* OPC-210 reported earlier by Tsujibo *et al* (1990 a) were pH 10.0 -12.0 for protease I and pH 10.5 for protease II. Nakanishi *et al* (1974) have reported an alkalophilic protease produced by *Streptomyces* sp. which has an optimum pH of around 12.3 with casein as the substrate, while another protease from *S. griseus* had an optimum pH of 11.0 with casein as the substrate (Muro *et al*, 1991). A thermostable alkaline protease from alkalophilic *Thermoactinomyces* sp. had an optimum pH of 11.5 -13.0 (Tsuchiya *et al*, 1992). In fact, most alkaline serine proteases of microbial origin have optimum pH values of around 10.0 (Kalisz, 1988).

Temperature optima :

The optimum temperature for enzyme activity was 65°C for protease I and 60°C for protease II (Fig. 3.16). Tsujibo *et al* (1990 a) have reported optimum temperatures of 70°C for the activity of protease I and 60°C for protease II from their strain of *N. dassonvillei*. Whereas Kim *et al* (1993) have reported an optimum temperature of 55°C for the alkaline

Figure 3.15

pH optima of proteases I and II from *Nocardiopsis* **sp. NCIM 5124**

Proteases I and II were assayed at different pH values (2.0-12.0) in universal buffers at 60°C as described in methods. The relative activities are expressed as percent of protease activities at pH 10.0.
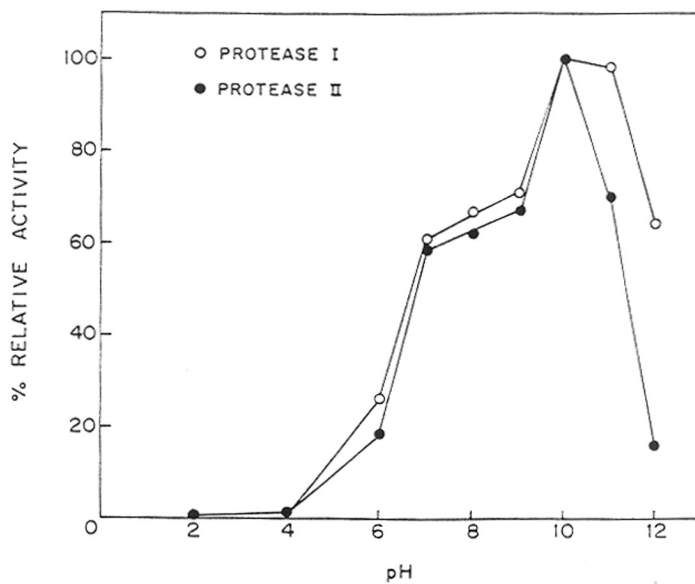
Figure 3.16

Temperature optima of proteases I and II from *Nocardiopsis* sp. NCIM 5124

Proteases I and II were assayed at various temperatures (30-75°C) at pH 10.0 as described in methods. The relative activities are expressed as percent of protease activities at 65°C (protease I) and 60°C (protease II).
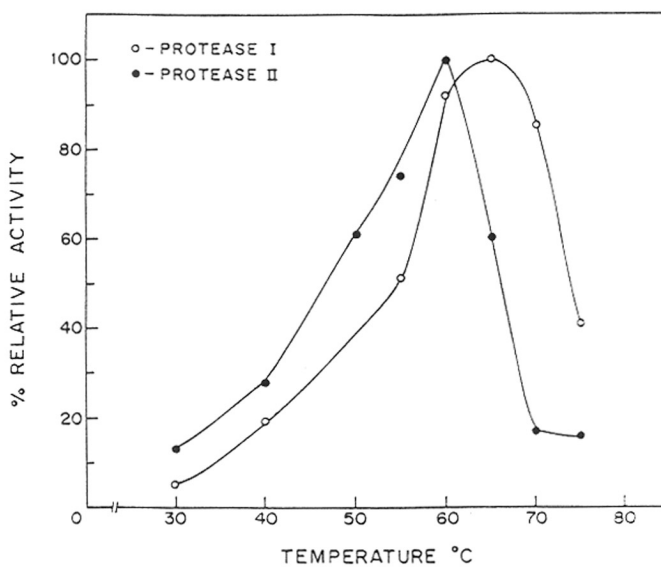
protease from *N. dassonvillei* ATCC 21944. An alkalophilic protease from *Streptomyces* sp. was maximally active at a temperature of 60 ° C (Nakanishi *et al*, 1974), a thermophilic alkaline serine protease produced by *Thermus aquaticus* had an optimum temperature of 80°C (Matsuzawa *et al*, 1988). It appears that both the proteases from the present strain have temperature optima similar to those reported earlier from *N. dassonvillei*.

Effect of various protease inhibitors

As seen from table 3.8, proteases I and II were almost completely inhibited by PMSF but not by the other class specific protease inhibitors namely EDTA, IAA and pepstatin, suggesting that they were both serine proteases. 1 mM PMSF inactivated protease I and II by 97% and 94% respectively. Both proteases reported from *N. dassonvillei* OPC-210 by Tsujibo *et al* (1990a) are also serine proteases whereas protease II from their strain was also found to be almost completely inactivated by 5 mM EDTA besides PMSF and DFP and has been characterized as a chelator sensitive serine protease.

TPCK and TLCK inhibited protease I by 44% and 51% respectively, at 1mM concentration. Protease II, on the other hand, was not affected by either of these.

Protease II was partly inhibited by soyabean trypsin inhibitor (48%) as well as trypsin-chymotrypsin inhibitor (67%) whereas protease I was not significantly inhibited by either of these (only 19% inhibition). Neither protease was significantly inhibited by Aprotinin, a bovine pancreatic trypsin inhibitor.

Therefore, the two proteases from *Nocardiopsis* sp. NCIM 5124 differed with respect to their sensitivity towards TPCK, TLCK, soyabean trypsin inhibitor and trypsin-chymotrypsin inhibitor although their reactivity towards the class-specific protease inhibitors was similar.

Table 3.8 : Effect of various protease inhibitors

Inhibitor	Conc.	Incubation conditions	%Inhibition Protease I	%Inhibition Protease II
PMSF	0.01 mM	50 mM phosphate buffer,	69	74
	0.10 mM	pH 7.0, 20 °C, 15 min.	96.4	90.5
	1.0 mM		96.5	93.8
	10.0 mM		97	94.2
EDTA	1.0 mM	,,	0	0
	3 .0 mM		8	4
	5.0 mM		10	8
IAA	1.0 mM	50 mM phosphate buffer,	0	0
	5.0 mM	pH 7.0, 20°C, 20 min.	0	0
Pepstatin	0.1 mM	50 mM acetate buffer, pH	0	0
	1.0 mM	5.0 20 °C, 60 min	2	6
TPCK	0.1	50 mM acetate buffer,	42	0
	1.0	pH 5.0, 20 °C, 30 min	44	4.5
TLCK	0.1	,,	25	0
	1.0		51	4.5
Soyabean trypsin inhibitor	2.5 mg/ml	,,	19.5	48
Trypsin-chymo -trypsin inhibitor	2.5 mg/ml	,,	19	67
Aprotinin	2.5 mg/ml	50mM phosphate buffer, pH 7.0, 20 °C, 60 min	13	23

Therefore, both proteases from *Nocardiopsis* sp. NCIM 5124 were inhibited by PMSF and had pH optima for activity in the alkaline range, pH 10.0 to 11.0 for protease I and pH 10.0 for protease II. Both the proteases can therefore be classified as **alkaline serine proteases**. Also, proteases I and II have M_r of 21,000 and 23,000 daltons and isoelectric points of 8.3 and 7.0 respectively, which are also in agreement with those reported for most alkaline serine proteases of microbial origin. The preliminary characteristics of proteases I and II have been summarized in table 3.9.

Table 3.9 : Preliminary properties of proteases I and II

Properties	Protease I	Protease II
Protease class	serine (inhibited by PMSF)	serine (inhibited by PMSF)
Optimum pH	10.0-11.0	10.0
Optimum temperature	65°C	60°C
M_r (SDS-PAGE)	21,000	23,000
pI	8.3	7.0

pH and temperature stabilities of the proteases

The proteases were incubated at different pH values and temperatures for 1 hour. The results can be summarized as follows:

(i) As seen from table 3.10, at pH values between 2.0 to 12.0, both the proteases were stable upto 30°C. (ii) At pH values between 2.0 to 10.0, both proteases were stable upto 50°C. (iii) At 60°C protease I was stable in the pH range of 2.0 to 6.0, whereas protease II was stable between pH 4.0 to 8.0 and (iv) At 70°C, both proteases were unstable at all pH values from 2.0 to 12.0

The temperature stability of protease I decreases at alkaline pH values whereas that of protease II decreases at extremes of pH, that is both at pH 2.0 and 12.0. Tsujibo *et al* (1990a) have reported that from their strain of *N.dassonvillei*, protease I was stable in the range of pH 4.0 to 8.0 and protease II was stable in the range of pH 6.0 to 12.0 upto 60 °C.

Table 3.10 : pH and temperature stablities of the proteases

Protease I

pH	% Residual Activity after 1 h incubation at						
	10 °C	20 °C	30 °C	40 °C	50 °C	60 °C	70 °C
2	100	105	104	108	98	93	12
4	102	105	105	103	106	109	23
6	100	103	95	93	92	88	20
8	102	103	96	94	91	30	16
10	101	102	110	112	109	23	8.5
12	103	100	110	62	13	13	13

Protease II

pH	% Residual Activity after 1 h incubation at						
	10 °C	20 °C	30 °C	40 °C	50 °C	60 °C	70 °C
2	100	105	108	111	88	8	8
4	102	105	110	112	113	106	8
6	101	104	112	113	113	115	9
8	101	106	106	110	112	108	9
10	102	101	112	110	89	75	5
12	101	105	98	13	11	5	5

Effect of metal ions on protease activity

As seen from table 3.11, protease I was not inhibited by any of the metal ions tested, in fact Ba^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Fe^{3+} and Al^{3+} were found to slightly stimulate its activity. Protease II, on the other hand, was inhibited 2 0 to 40% by K^+, Ba^{2+}, Mg^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+} ; it was inhibited 50 to 70% by Cu^{2+}, Al^{3+}, Mn^{3+} and completely inhibited by

Fe^{3+}. Neither of these proteases were inhibited by EDTA so metal ions do not appear to play a significant part in the activity of these enzymes.

Tsujibo *et al* (1990a) have reported that protease I from their strain of *N. dassonvillei* was not significantly inhibited by any of the metal ions tested either, and that the activity was slightly stimulated by Mg^{2+}, Mn^{2+} and Zn^{2-}. Protease II from their strain was inhibited by Cu^{2+}, while Ca^{2+} and Co^{2+} slightly stimulated the activity (metal ions were tested at 1 mM concentration). The thermostable alkaline protease from *Thermoactinomyces* sp. (Tsuchiya *et al*, 1992) was almost completely inhibited by Cu^{2-} and Hg^{2-} among the metal ions tested at 10 mM concentration, while the alkaline protease from *S. griseus* var *alkalophilus* was not affected by most of the metal ions tested at 1 mM concentration, although Hg^{2+} inhibited the enzyme activity by about 20% (Muro *et al*, 1991). The activity of a serine protease produced by *Streptomyces* sp. YSA-130 was inhibited by Ag^{2-}, Hg^{2+} and Co^{2+} (Yum *et al*, 1994).

Table 3. 11 : Effect of metal ions on protease activity

Salt used (5 mM)	%Residual activity Protease I	% Residual activity Protease II
None	100	100
KCl	112	69
NaCl	120	87
BaCl₂	118	64
HgCl₂	92	83
MgCl₂	125	76
MnCl₂	120	33
CoCl₂	120	67
CaCl₂	125	69
ZnCl₂	84	79
CuCl₂	95	52
FeCl₃	132	0
AlCl₃	134	49

Effect of chemical modifiers on the proteases

Proteases I and II were modified with various reagents to determine the amino acid side chains necessary for enzyme activity. As seen from table 3.12, proteases I and II were almost completely inhibited by PMSF (97 and 94% respectively), indicating that like all serine proteases, serine was involved in enzyme activity.

DEP inactivated proteases I and II by 70% and 96% respectively, indicating that histidine was also necessary for enzyme activity. Treatment of the DEP-inhibited proteases with 200 mM hydroxylamine at 25°C for 5 h showed about 70% and 85% restoration of the initial activity for proteases I and II respectively, confirming that a histidine residue was being modified by DEP, resulting in the loss of activity in case of both the proteases.

Neither protease was inhibited by TNBS / succinic anhydride, DTNB/ NEM, HNBB or NAI therefore ruling out the role of lysine, cysteine, tryptophan and tyrosine residues respectively, in enzyme activity.

Both proteases I and II were found to be inhibited to some extent (70% and 60% respectively) by phenyl glyoxal and also by 2,3-butanedione (30 and 80%). These reagents are known to modify arginine residues in proteins (Takahashi, 1968; Yankeelov *et al*, 1968). The presence of arginine residues in the active site of various zinc metallo peptidases and their role in substrate and inhibitor binding has been reported (Malfroy and Schwartz, 1982; Jakson and Hersh, 1986; Helene *et al*, 1991). Yum *et al* (1994) have also reported that an alkaline serine protease produced by *Streptomyces* sp YSA-130 was inhibited by phenyl glyoxal and 2,3-butanedione. Since it is well known that most serine proteases have at their active site, a triad of the residues serine, histidine and aspartic acid which are involved in the catalytic mechanism (Stroud, 1974; Fersht, 1985; Zubay, 1993) , this preliminary indication of the involvement of arginine in enzyme activity, in case of proteases from *Nocardiopsis* sp. NCIM 5124 seemed very interesting. However, CD spectra of native and phenyl glyoxal modified proteases I and II showed wide differences, indicating major structural changes in both the proteins , on treatment with phenyl glyoxal (Fig. 3.17). Thus, the loss of protease activity in case of both the proteases I and II , on treatment with phenyl glyoxal

was not a result of specific blocking of essential arginine residues at or near the active site, but due to structural changes in the enzyme molecules.

Therefore, qualitative chemical modification studies suggest that both proteases from *Nocardiopsis* sp. NCIM 5124 require serine and histidine for enzyme activity, like all other serine proteases in which a triad of the residues serine, histidine and aspartic acid is involved in the catalytic mechanism (Stroud, 1974; Fersht, 1985; Zubay, 1993). Blow *et al* (1969) deduced the importance of aspartic acid in the catalytic mechanism from X-ray diffraction measurements and these authors found that the carboxyl group of the aspartic acid residue was buried in a hydrophobic environment and was inaccessible to modifying agents. In the present case also, carboxylate modifying reagents had no effect on both proteases.

**Table 3.12 : Effect of chemical modifiers on the proteases**

Chemical	Conc. mM	Incubation buffer salt, mM, pH	% Inhibition Protease I	% Inhibition Protease II
PMSF	5	phosphate, 50, 7.0	97	94
DEP	5	phosphate, 50, 6.0	70	96
WRK	10	phosphate, 50, 6.0	0	0
EDAC	50	MES/HEPES, 50, 6.0	0	0
Phenyl glyoxal	10	carbonate, 50, 8.0	70	60
2,3-Butanedione	10	borate, 50, 8.0	30	80
HNBB	10	phosphate, 50, 7.0	0	0
DTNB	10	phosphate, 50, 8.0	0	0
NEM	5	phosphate, 50, 6.0	0	0
Succinic anhydride	10	phosphate, 50, 8.0	0	0
TNBS	5	phosphate, 50, 8.0	0	0
NAI	10	phosphate, 50, 7.0	0	0

Figure 3.17

CD spectra of native and phenyl-glyoxal modified proteases I and II

a : protease I ; b: protease II.

_____ native enzyme; ----- modified enzyme.
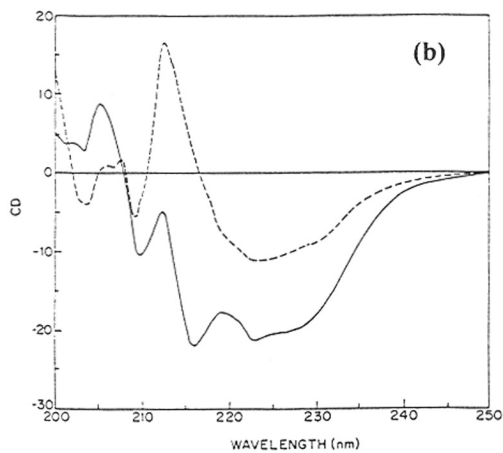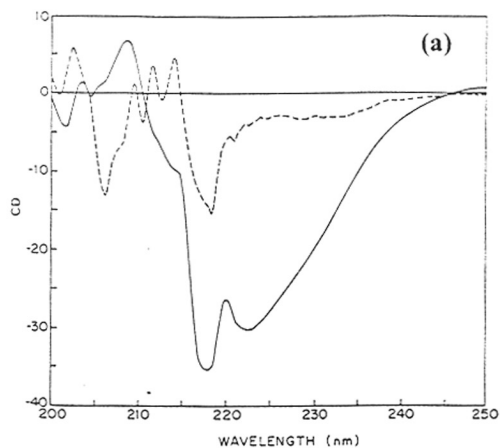
Substrate specificities of proteases I and II

As seen from table 3.13, both proteases I and II had similar substrate preferences against various protein substrates tested, although protease I showed greater specific activities for casein, haemoglobin and bovine serum albumin than protease II. Distinct progressive curves were obtained for the two proteases following a time course of release of trichloroacetic acid soluble products from these protein substrates (Fig. 3.18), which again indicated that proteases I and II differed substantially with respect to the degrees of hydrolysis of these substrates.

Table 3.13 : Protease activities with different protein substrates

Substrate	ProteaseI activity U/mg	ProteaseII activity U/mg
Casein	152	14.2
Haemoglobin	86	10
Bovine serum albumin	22	4.2
Ovalbumin	1.6	2.7

Both proteases from *Nocardiopsis* sp. NCIM 5124 showed collagenolytic and fibrinolytic activity. Protease I also showed elastolytic activity whereas protease II did not. Neither protease had any keratinolytic activity (Table 3.14). In case of both proteases, the specific activity against native collagen was much higher than that observed with fibrin or elastin. Collagenase-like enzymes are characterized by their ability to hydrolyze native collagen. There is usually a preponderance of –Gly–Pro–X– repeats in collagen molecules, and collagenases or collagenase-like enzymes are specific for peptide bonds involving the amino group of glycine (X represents any amino acid, but is either alanine or hydroxy-proline in most repeats). There is no defined specificity for the residue in the carboxyl-donating position of the susceptible peptide bond (Seifter and Harper, 1971).

Fig. 3.18

Time course of formation of trichloroacetic acid soluble peptides from (a) casein, (b) haemoglobin, (c) bovine serum albumin and (d) ovalbumin by proteases I and II.

The proteases were incubated with the substrates at enzyme to substrate ratios of 1: 5000, in 50 mM Na-carbonate buffer, pH 10.0 at 60°C. The absorbance of TCA soluble peptides liberated, was determined at regular intervals as described in methods.

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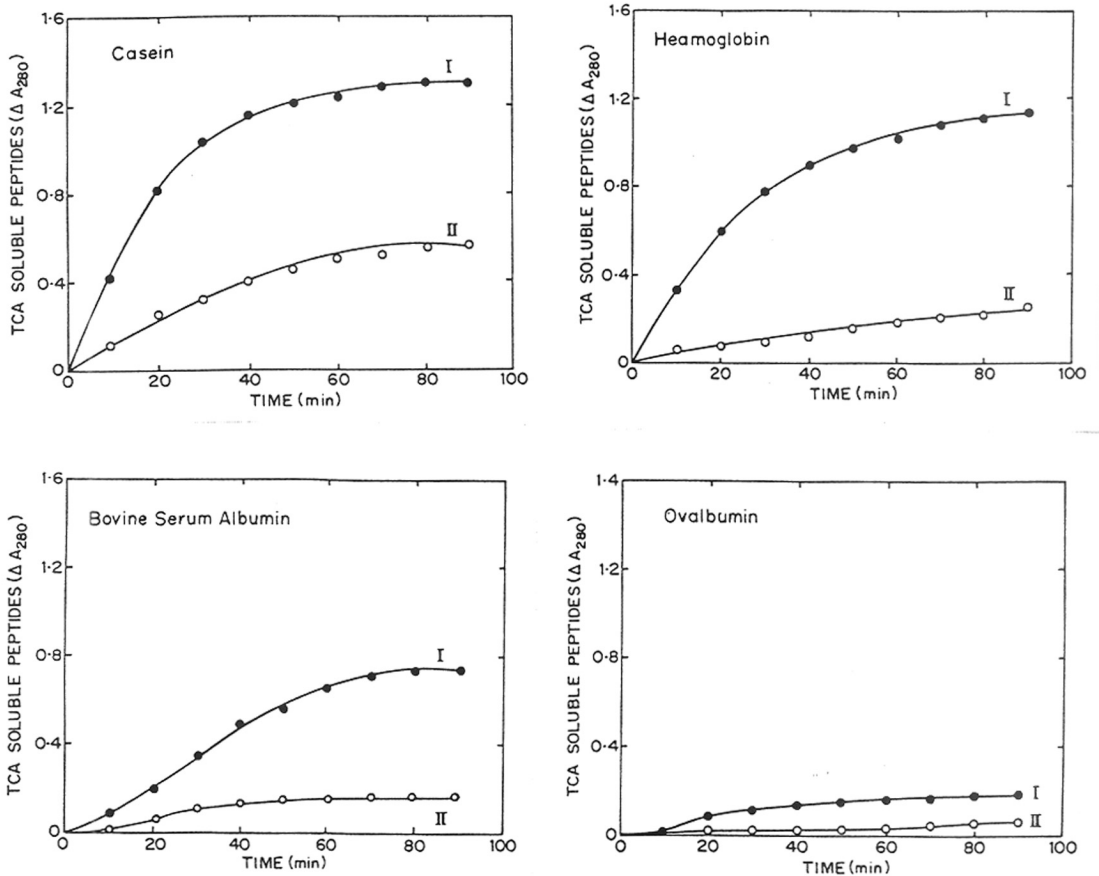
Several actinomycetes have been reported for their ability to produce proteases with unusual substrate specificities. Proteases showing collagenase-like activity have been reported from *Actinomadura* sp. (Rippon, 1968; Norwig, 1971), *Mycobacterium tuberculosis* (Takahashi *et al*, 1967), *Streptomyces thermovulgaris* (Egorov *et al*, 1976), *S. griseus* (Drake *et al*, 1966) and several *Streptomyces* sps. (Chakraborty and Chandra, 1984; Fig. Chakraborty and Chandra, 1986; Endo *et al*, 1987; Demina and Lysenko, 1992). Proteases with fibrinolytic activity have been reported from several *Streptomyces* species (Buckley and Jefferies, 1981; Egorov, 1976; Al-Nuri *et al*, 1984; Bono *et al*, 1996). Proteases with elastase-like activity have been reported from *S. griseus* (Gertler and Trop, 1971; Trop and Birk, 1970), *S. fradiae* (Lagutina and Petrova, 1979), *S. rimosus* (Rassulin *et al*, 1974) and *S. griseoalbus* SN-22 (Murao *et al*, 1994). However, there are no reports of proteases showing such activities from *Nocardiopsis* sp.

Table 3.14 : Assays with insoluble protein substrates

Substrate	Protease I (U/mg)	Protease II (U/mg)
Collagen	15.6	12.2
Fibrin	1.5	0.6
Elastin	1.2	0
Keratin	0	0

Activity towards synthetic substrates

Limited substrate specificity studies using synthetic substrates showed that both proteases I and II from *Nocardiopsis* sp. NCIM 5124 did not have aminopeptidase or carboxypeptidase activities towards any of the substrates tested (XVI to XX) and therefore, both these proteases are characterized as endopeptidases.

Among the endopeptidase substrates tested, proteases I and II preferred tri and tetra peptide substrates as compared to mono or di peptide substrates.

With tripeptide substrates, arginine and lysine were found to be preferred in comparison to leucine, at the P_1 position in both these proteases.

Table 3. 15 :   Activity towards synthetic substrates

Substrate No.	Substrate	Activity (Units/mg) Protease I	Activity (Units/mg) Protease II
	$P_4 - P_3 - P_2 - P_1 - P_1'$		
I	Boc-Leu-Ser-Thr-Arg- pNA	1.32	3.75
II	Tosyl-Gly-Pro-Arg- pNA	1.70	3.53
III	Benzoyl-Phe-Val-Arg- pNA	1.70	4.60
IV	Boc-O-Benzyl-Ser-Gly-Arg- pNA	0.15	0.11
V	Tosyl-Gly-Pro-Lys- pNA	1.60	3.20
VI	CBZ-Ala-Ala-Leu- pNA	0.42	0.35
VII	Benzoyl-Val-Gly-Arg- pNA	0.10	0.13
VIII	Boc-Leu-Gly-Arg- pNA	0.08	0.1
IX	Benzoyl-Pro-Phe-Arg- pNA	0.04	0.06
X	CBZ-Gly-Gly-Leu- pNA	0.02	0.03
XI	Z-Arg-Arg- pNA	0.04	0.05
XII	Z-Phe-Arg- pNA	0.03	0.05
XIII	Benzoyl-Arg- pNA	0.01	0.02
XIV	Benzoyl-Tyr- pNA	0.01	0.03
XV	CBZ-Phe- pNA	0.01	0.02
XVI	Leu- pNA	0	0
XVII	Hippuryl-Phe	0	0
XVIII	Hippuryl-Arg	0	0
XIX	N-CBZ-Glu-Tyr	0	0
XX	N-Benzoyl-Gly-Phe	0	0

Furthermore, even with arginine or lysine at the P_1 position, both proteases preferred threonine, proline or valine as compared to glycine at the P_2 position as in substrates IV, VII and VIII, and the specific activities of the proteases decreased more than 10 fold, in this case. Such an effect was not seen when glycine was present at position P_3 in the peptide substrate (substrates II and V) (Table 3.15).

The specific activities of proteases I and II towards tri and tetrapeptide substrates were about 80 times (protease I) 100 times (protease II) higher than those observed with mono or dipeptide substrates (table 3.15). This indicates, although at a preliminary stage, that substrate binding in an extended form involving atleast 3 subsites, is necessary for hydrolysis in case of both proteases. Kinetic and X-ray crystallographic studies of serine proteases of both bacterial and mammalian origin have shown that the active sites of these enzymes interact with substrates or inhibitors over a rather extended area covering 5,6 or 7 subsites or 18-25 Å and extended active sites are important both for increasing enzyme-substrate affinities and turnover rates (Bauer, 1976; Schecter and Berger, 1967; Abramowitz et al, 1967 ; Atlas et al, 1970; Bauer et al, 1981). Enzymes which are able to form precise S_1–P_1 interactions are little dependent on enzyme-substrate contacts remote from the scissile bond, while the reverse is true for enzymes with less restrictive S_1 subsites. Chymotrypsin and trypsin have S_1 subsites with good to excellent fit for their optimal P_1 residues and thus substrate contacts outside the S_1 subsite are relatively unimportant . In contrast, elastase, S. griseus alkaline proteases A and C and subtilisin BPN, all have S_1 subsites characterized as ill defined, wide, or less restrictive and these enzymes are all found to be highly dependent on substrate contacts outside the S_1 subsite (Bauer, 1978). Both proteases from Nocardiopsis sp. NCIM 5124 therefore, appear to be similar to this latter group of enzymes on the basis of their requirement for extended substrate binding, and are not like trypsin or chymotrypsin in this respect.

Both proteases I and II from Nocardiopsis sp. NCIM 5124 did not show any activity against Benzoyl-Arg-pNA and Benzoyl-Tyr-pNA, which are the substrates cleaved by trypsin-like and chymotrypsin-like proteases. Tsujibo et al (1990 a) have reported that proteases I and II from their strain of N. dassonvillei also did not hydrolyze Benzoyl-Arg-

pNA and thus have claimed that these enzymes were distinguished from the trypsin -like proteases of *Streptomyces* sp. reported by Renko *et al* (1989).

Table 3. 16   Kinetic Constants for preferred pNA Substrates

Protease I :

Substrate No.	Substrate	K_m (mM)	Vmax (U/mg)	k_{cat} min $^{-1}$	k_{cat} / K_m
	$P_4 - P_3 - P_2 - P_1 - P_1'$				
I	Boc-Leu-Ser-Thr-Arg-pNA	6.66	20	420	63
II	Tosyl-Gly-Pro-Arg-pNA	3.33	10	210	63
III	Benzoyl-Phe-Val-Arg-pNA	4	12.5	262.5	65
V	Tosyl-Gly-Pro-Lys-pNA	10	20	420	42

Protease II :

Substrate No.	Substrate	K_m (mM)	Vmax (U/mg)	k_{cat} min $^{-1}$	k_{cat} / K_m
	$P_4 - P_3 - P_2 - P_1 - P_1'$				
I	Boc-Leu-Ser-Thr-Arg-pNA	3.33	25	575	175
II	Tosyl-Gly-Pro-Arg-pNA	0.909	10	230	256
III	Benzoyl-Phe-Val-Arg-pNA	2.86	33.33	766.6	271
V	Tosyl-Gly-Pro-Lys-pNA	2	16.66	383.2	194

The k_{cat} / K_m values determined for proteases I and II with the preferred tri and tetrapeptide substrates I, II, III and V, were found to be almost similar for each protease (Table 3.16).

Although many of the proteases reported from actinomycetes have esterase activities (Renko *et al*, 1989; Tsuchiya *et al*, 1992; Muro *et al*, 1991; Yoshida *et al*, 1971; Narahashi, 1970; Tsujibo *et al*, 1990 a), there are also reports of proteases lacking esterase activity, like the alkalophilic proteinase from *Streptomyces* sp. (Nakanishi *et al*, 1974). In the present case

neither protease from *Nocardiopsis* sp. NCIM 5124 showed any esterase activity against any of the 21 synthetic amino acid ester substrates tested. Both proteases also did not show any non-specific esterase activity against p-nitrophenylacetate.

Amino acid composition of Proteases I and II

Neither protease contained any cysteine residues or disulphides as determined by the method Cavallini (1966). Both proteases therefore resembled the subtilisins in this respect (Ottesen and Svendsen, 1970). Tsujibo *et al* (1990 b) have reported that the amino acid composition of protease I from their strain of *N. dassonvillei* , NDP-I, resembled that of the chymotrypsin-like proteases, while that of NDP-II resembled the subtilisin-like proteases. Higher proline contents of about 10% are observed in both proteases from *Nocardiopsis* sp. NCIM 5124, as compared to about 5% proline content in subtilisin (Delange and Smith, 1968) and also proteases I (NDPI) and II (NDPII) from *N. dassonvillei* OPC-210 reported by Tsujibo et al (1990a) (Table 3.17).

Antigenic relationship of protease I with protease II and with other serine proteases

Polyclonal antisera raised in rabbits against protease I did not cross-react with protease II or with any of the other serine proteases tested including trypsin, chymotrypsin, subtilisin BPN', pronase and proteinase K, as checked by Ouchterlony double diffusion (Fig. 3.19). This indicates that *Nocardiopsis* sp. NCIM 5124 protease I is immunologically unrelated to these proteases and does not share common epitopes with them. Antigen-antibody reactions usually show a high level of specificity between antigens that share some common antigenic determinants or epitopes. Immunological cross-reactivity between proteins, therefore reflects their structural relatedness. The fact that anti-protease I antibody cross-reacts neither with protease II from *Nocardiopsis* sp. NCIM 5124 nor with other well characterized serine proteases is a preliminary indication of the structural unrelatedness of protease I with any of these proteases. However, further evidence based on primary structures and sequence homology between these proteases is required to strengthen these findings.

Table 3.17 Amino acid composition of Proteases I and II

Amino acid Residue	NDP I * mol/mol	NDP II * mol/mol	Subtilisin BPN' • mol/mol	Protease I mol/mol	Protease II mol/mol
As (x)	19	59	28	24	13
Gl (x)	16	22	15	17	20
Ser	20	37	37	24	12
His	4	7	6	2	7
Gly	46	65	33	40	48
Thr	30	18	13	24	9
Ala	19	46	37	17	15
Arg	13	5	2	10	4
Tyr	7	10	10	0	9
Val	18	37	30	18	17
Met	2	1	5	0	8
Cys	6	0	0	0	0
Phe	8	3	3	4	7
Ile	7	11	13	7	7
Leu	5	23	15	6	18
Pro	6	15	14	21	29
Lys	0	4	11	2	10
Try	ND	ND	3	1	3
M_r	21,000	36,000	27,500	21,000	23,000

* NDP I and NDP II stand for *Nocardiopsis dassonvillei* OPC-210 proteases I and II reported by Tsujibo *et al* (1990 b).

• amino acid composition of subtilisin BPN' according to Delange and Smith (1968).

Figure 3.19

Ouchterlony double diffusion to check antigenic cross-reactivity of anti-protease I antibody with protease II and other serine proteases.

Double diffusion was carried out in 1.5% agarose gels in phosphate buffered saline (10 mM Na-phosphate buffer, pH 7.0 containing 0.145 M NaCl) at 20°C for 48 h. 100µg of each antigen and 1 mg of IgG was used.

P1– protease I; P2 – protease II; 1 – subtilisin BPN' ; 2– trypsin; 3 – chymotrypsin; 4 – pronase; 5 – proteinase K.
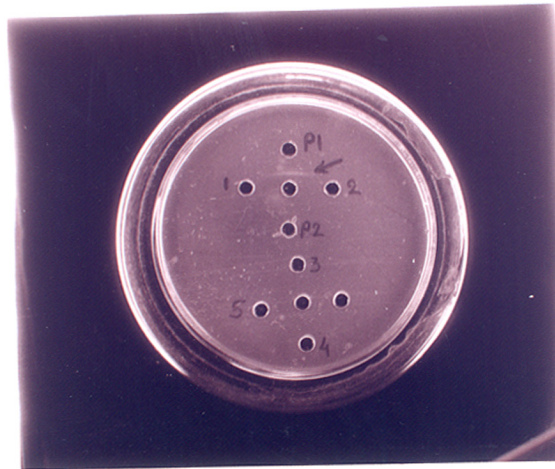
CONCLUSIONS

The culture conditions for production of extracellular protease by *Nocardiopsis* sp. NCIM 5124 have been optimized. Extracellular protease is maximally produced in a medium containing 1% starch, 1% casein, 0.1% K_2HPO_4 , 1% Na_2CO_3 , 0.2% glucose, pH 10.0 after 4 days incubation at $30^{\circ}C$ and 150-200 rpm.

Two protease fractions were purified to homogeneity and characterized with respect to their molecular and biochemical properties. On the basis of their alkaline pH optima and susceptibility to PMSF the two proteases have been classified as alkaline serine proteases. Both proteases were devoid of cysteines, disulphides and carbohydrate and thus resembled the subtilisins in this respect.

Proteases I and II differ from each other with respect to their molecular masses, isoelectric points, pH and temperature optima and stabilities. Protease I was found to be antigenically unrelated to protease II, and also to other serine proteases like subtilisin, trypsin and chymotrypsin.

Substrate specificity studies with synthetic aminopeptidase, carboxypeptidase and endopeptidase substrates suggest that both proteases are endopeptidases, are non-specific in their activites, and show preference for tri and tetrapeptide substrates as compared to mono or dipeptide substrates indicating that substrate binding in an extended form is required for efficient hydrolysis.

CHAPTER 4

PRELIMINARY STUDIES ON HYDROCARBON DEGRADATION
BY *NOCARDIOPSIS* SP. NCIM 5124

SUMMARY

Bombay High crude oil was maximally degraded (66%) by *Nocardiopsis* sp. NCIM 5124 when supplied at a concentration of 0.1 to 0.3% w/v with 70 mM NH_4Cl as the inorganic nitrogen source. When organic nitrogen sources were supplied, crude oil was maximally degraded by the organism using yeast extract although the degradation in this case (47%) was less than that with NH_4Cl at similar nitrogen concentrations. Protease activity in media containing organic nitrogen sources was about 0.2 U/ml whereas that with NH_4Cl was as low as 0.02 U/ml. Protease was maximally produced (0.72 U/ml) with coconut press cake, a cheap source of organic nitrogen with 25% degradation of the supplied crude oil. In a different set of experiments when crude oil (0.3%) or C_{14} to C_{18} alkanes (0.5%) were individually supplied instead of starch and glucose in the optimized protease production medium at pH 10.0, an extracellular protease activity of about 0.3 U/ml together with 34% degradation of the crude oil or 60 to 80% degradation of the alkanes was observed.

A composition consisting of coconut press cake, the organism on a carrier and a source of inorganic phosphate is suggested as an alternative biotechnological approach for oil-spill clean up in field trials.

INTRODUCTION

Marine environments are prone to oil-contamination due to routine shipping operations, off-shore oil production, coastal oil refinery effluents and also accidental oil spills. Since they are chronically polluted environments there is a greater chance of finding useful organisms in these habitats.

The existence of hydrocarbon-degrading microorganisms in natural environments has been recognized since a long time (Zobell, 1946). Considering that growth and therefore degradation of crude oil in such environments is often limited by the availability of nitrogen, use of microorganisms capable of utilizing organic nitrogen sources by producing extracellular proteases is a viable alternative approach for treatment of environmental oil pollution. Hydrocarbon degrading actinomycetes include species of *Corynebacterium, Nocardioides, Brevibacterium, Streptomyces, Rhodococcus, Arthrobacter, Mycobacterium, Nocardia, Micromonospora* and *Streptomyces* (Bushnell and Haas, 1941; Edmonds and Cooney, 1967 ; Jones and Edington, 1968 ; Purkiss, 1972; Genner and Hill, 1981). However, to the best of our knowledge, there are no reports in the literature of an actinomycete capable of degrading hydrocarbons and also producing extracellular proteases.

The actinomycete that we have isolated from a marine oil-polluted environment does both, it degrades crude oil and produces proteases. The present chapter describes preliminary studies on crude oil degradation by *Nocardiopsis* sp. NCIM 5124. Conditions for crude oil degradation by the organism were studied and the ability of the organism to degrade hydrocarbons and produce extracellular proteases was tested in media containing either crude oil or pure alkane compounds as the carbon source with various organic nitrogen sources. The possible use of this organism in oil spill clean up has been discussed.

MATERIALS AND METHODS

MATERIALS

Crude oils were obtained from the Oil and Natural Gas Commission, India. n-alkanes, silica gel G, yeast extract, beef extract, peptone were purchased from S.D fine chemicals. Haemoglobin and tryptone were obtained from Hi-Media chemicals. Casein (Hammarsten), fat-free casein and agarose were purchased from Sisco Research Laboratories, India. All seawater salts, solvents and other chemicals used were of analytical grade.

Microorganism

The culture selected after screening for protease production and crude oil degradation and identified as *Nocardiopsis* sp. NCIM 5124 was used for further studies. The culture was maintained on nutrient agar slants (1% peptone, 0.3% beef extract, 0.5% NaCl, 2.5% agar, pH 8.0) and subcultured every 3 months.

METHODS

Culture conditions

For crude oil degradation studies, the culture was grown in 250 ml conical flasks with 50 ml of the defined sea water (composition given in chapter 2) medium containing 0.5% $(NH_4)_2SO_4$, 0.002% K_2HPO_4, pH 8.0, with crude oil as the sole source of carbon.

The inoculum was developed in sea water nutrient broth (defined sea water containing 1% peptone, 0.3% beef extract, pH 8.0) for 36 h at 30°C and 200 rpm and the exponential phase cells were pelleted out and washed with sterile sea water. Approximately 2.0 g wet weight of cells was added to 50 ml of the medium.

The flasks were incubated at about 200 rpm and 30°C. After 10 days of growth, the residual oil was extracted with dichloromethane. Dichloromethane was evaporated and the crude oil was quantified by gravimetry. The percent degradation of crude oil was

determined with reference to uninoculated controls which determined abiotic losses of crude oil.

Degradation of crude oils from different sources

The degradation of Bombay High, Assam and Gujarat crude oils at 0.5% w/v concentrations was checked in seawater inorganic medium containing 0.5% $(NH_4)_2SO_4$, 0.002% K_2HPO_4, pH 8.0. After 10 days of growth at 30°C and 200 rpm, the residual crude oil was quantified by gravimetry with reference to uninoculated controls. After gravimetric analysis Bombay high crude oil was chosen for further studies.

In further experiments, the culture was grown for 10 days at 30°C and 200 rpm, after which the residual crude oil was extracted as described above and quantified by gas chromatography. For gas chromatographic analysis, the residual crude oil was fractionated by column chromatography using silica gel G by the method of Walker and Colwell (1974). The aliphatic fraction so obtained from the crude oil was concentrated to 2 ml and 1 μ l of the concentrate was loaded on a SE-30 column fitted to a Shimadzu GC RIA gas chromatograph with nitrogen as carrier gas and a Flame Ionization Detector. Temperature programming was from 60-250°C with a 6°C rise per minute. Quantification was done in accordance with the method published by Dibble and Bartha (1976). Percent degradation of the aliphatic fraction of crude oil was determined from the integrated areas of the test samples with reference to those of uninoculated controls, after exclusion of the solvent peaks. The uninoculated controls were incubated under the same conditions as the test samples and checked for the abiotic losses of crude oil.

Effect of crude oil concentration on its degradation

The effect of variation of crude oil concentration on its degradation was checked using different concentrations (0.1-0.9% w/v) of Bombay High crude oil in the medium.

Effect of Nitrogen source on crude oil degradation

The effect of various nitrogen sources on crude oil degradation was studied using $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl, $NaNO_3$ and urea at a concentration of 70 mM

nitrogen in defined sea water containing 0.002% K_2HPO_4, pH 8.0 with 0.3% w/v of Bombay High crude oil.

The effect of organic nitrogen sources on crude oil degradation and protease production by the present *Nocardiopsis* sp. was checked using peptone, beef extract, yeast extract, casein, tryptone and also cheaply available nitrogen sources like coconut press cake at a concentration of 1% in defined sea water containing 0.002% K_2HPO_4, pH 8.0 with 0.3% w/v of Bombay High crude oil. Protease activity in the cell free supernatant and % degradation of the crude oil in terms of the aliphatic fraction (GC) was determined after 10 days incubation at 30°C and 200 rpm.

Production of protease in media containing hydrocarbons

During medium optimization studies for production of extracellular protease by *Nocardiopsis* sp. NCIM 5124, it was found that a medium containing 1% starch, 1% casein, 0.1% K_2HPO_4, 1% Na_2CO_3, 0.2% glucose, pH 10.0 supported maximum protease production. In order to determine protease production in the presence of hydrocarbons, the starch and glucose were replaced by either crude oil (0.3%w/v) or pure alkanes (0.5% w/v) in the above medium. After 10 days incubation at 30°C and 200 rpm, protease activity in the cell free supernatant was assayed and % degradation of alkanes or that of the aliphatic fraction of crude oil was determined by gas chromatography.

Protease assay

Protease activity was measured by the method of Kunitz (1947) as described in chapter 3. One unit of protease activity is defined as the amount of enzyme which releases 1μ mole of tyrosine per minute at 60°C and pH 10.0.

RESULTS AND DISCUSSION

The composition of crude oils from 3 different sources was analyzed. Among these, Bombay High crude oil contained the largest proportion of the aliphatic fraction of hydrocarbons, followed by Assam crude oil and Gujarat crude oil. The aromatic content was found to be the highest in Gujarat crude oil followed by Assam crude and Bombay High crude oils as shown in table 4.1.

Table 4.1 : Composition of Crude oils from different sources

Crude oil	Aliphatic fraction (%)	Aromatic fraction (%)	Ashphaltenes fraction (%)
Bombay High crude	82	17	1
Assam crude	69	22	9
Gujarat crude	55	37	8

Nocardiopsis sp. NCIM 5124 degraded Bombay High crude oil better than Assam crude oil whereas Gujarat crude oil was not degraded at all (Table 4.2). This result is consistent with earlier reports that crude oils with a high saturate and low aromatic, ashphaltenes and sulphur content are degraded better than those with high aromatic and sulphur contents (Walker *et al*, 1976; Jobson *et al*, 1972). Bombay High crude oil was used in all later experiments. Figure 4.1 shows an uninoculated control flask and a flask containing degraded Bombay High crude oil.

Table 4.2 : Degradation of different crude oils

Crude oil 0.5% w/v	%Degradation (Gravimetry)
Bombay High crude	38
Assam crude	12
Gujarat crude	0

Figure 4.1

Flasks showing crude oil degraded by *Nocardiopsis* sp. NCIM 5124 and uninoculated control medium.

The organism was grown in a medium containing defined sea water with 0.5% $(NH_4)_2SO_4$ (70 mM nitrogen) and 0.002% K_2HPO_4, pH 8.0 at $30^\circ C$ and 200 rpm for 10 days.

As seen from table 4.3, Bombay High crude oil was found to be maximally degraded by *Nocardiopsis* sp. NCIM 5124 when supplied at a concentration of 0.1-0.3% w/v. At these concentrations almost 50% of the aliphatic fraction of the supplied oil was degraded. Inhibitory effects of petroleum components are often highly dependent on their solubility and concentration (Leahy and Colwell, 1990; Dibble and Bartha, 1976; Bartha and Atlas, 1977) and in the present study concentrations above 0.3% w/v of Bombay High crude oil resulted in decreased degradation. In all further studies 0.3% w/v crude oil was supplied in the medium.

Table 4.3 : Effect of crude oil concentration on its degradation

% w/v crude oil	% degradation of aliphatic fraction (GC) *
0.1	50 ± 1.7
0.3	51 ± 2.6
0.5	35 ± 2.3
0.7	28 ± 1.9
0.9	15 ± 2.8

* All values are averages $\pm$ standard deviation ; n=2

$(NH_4)_2SO_4$ (70 mM nitrogen) was supplied as the nitrogen source

NH_4Cl was found to be the best inorganic nitrogen source for degradation of Bombay High crude oil (Table 4.4). Generally speaking the nitrogen requirements of most crude oil degraders are satisfied by either ammonium-N or nitrate-N as shown in 1946 by Zobell. The minimum concentration of NH_4Cl for maximum degradation of crude oil was 70 mM. About 67 % of the aliphatic fraction of crude oil was degraded at concentrations between 70 to 110 mM NH_4Cl (Table 4.5). The results of gas chromatographic analysis of the aliphatic fraction of crude oil after growth of *Nocardiopsis* sp. NCIM 5124 with 70 mM NH_4Cl as the nitrogen source are shown in figure 4.2.

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Table 4.4 : Effect of Nitrogen source on crude oil degradation

Nitrogen source (70mM Nitrogen)	% degradation of aliphatic fraction (GC) *
NH_4Cl	66 ± 1.8
NH_4NO_3	60 ± 2.2
$(NH_4)_2SO_4$	51 ± 2.4
$NaNO_3$	37 ± 1.9
Urea	35 ± 2.6

* All values are averages $\pm$ standard deviation; n=2

Table 4.5 : Effect of variation of NH_4Cl on crude oil degradation

NH_4Cl conc. mM	% degradation of aliphatic fraction (GC) *
30	52 ± 1.2
50	58 ± 1.6
70	67 ± 1.7
90	66 ± 1.9
110	67 ± 2.1

* All values are averages $\pm$ standard deviation; n=2

Figure 4.2

Gas chromatographic analysis of the aliphatic fraction of Bombay High crude oil

The organism was cultured in the defined sea water inorganic medium containing 0.3% w/v Bombay High crude oil, 0.002% K_2HPO_4 and 70 mM NH_4Cl as the nitrogen source at 30°C and 200 rpm for 10 days.

INTEGRATED AREA : 25.6×10^4

INTEGRATED AREA : 8.54×10^4
DEGRADATION : 66%
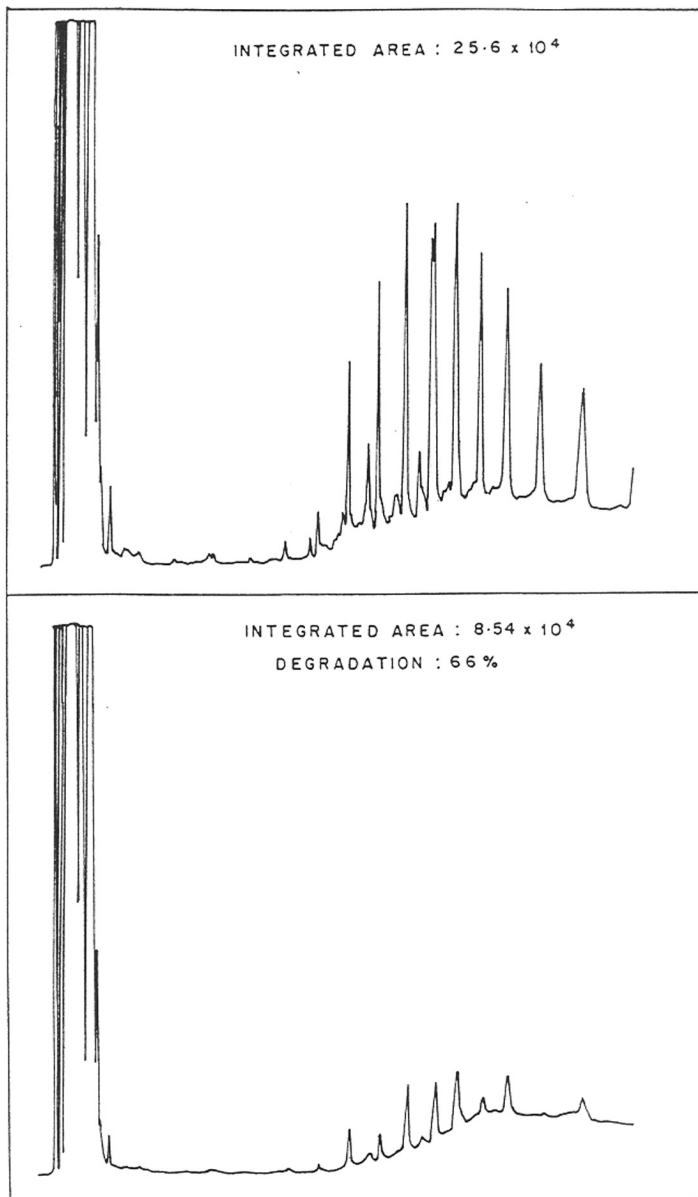
In comparison with inorganic nitrogen, organic nitrogen resulted in decreased values of crude oil or alkane degradation although there was still a substantial decrease in residual hydrocarbon. As seen from table 4.6, crude oil was maximally degraded (47%) when yeast extract was supplied and was least degraded with values as low as 4% when tryptone was provided. The degradation of crude oil with 1% yeast extract (approx. 60 mM nitrogen calculated from the Nitrogen content of yeast extract given in the chemical catalogue) is lower than that achieved with similar concentrations of NH_4Cl nitrogen (Table 4.5) but in terms of costing of these sources is cheap and the best protease production together with substantial crude oil degradation occurs in the presence of coconut presscake which at Rs. 10 per Kg is the cheapest of the available organic nitrogen sources tested. Under these conditions protease production is 0.72 U/ml and oil degradation is 25%. In all other cases, protease activity was about 0.2 U/ml . A suitable composition for use in oil spill clean up would therefore contain coconut press cake , K_2HPO_4 and the organism on a carrier. The applicability of such a composition however, needs to be checked by field studies.

Table 4.6 Degradation of crude oil in the presence of organic nitrogen sources

Nitrogen source (1% w/v)	Protease activity U/ml	% Degradation of aliphatic fraction (GC) *
Casein	0.24	30 ± 1.5
Yeast extract	0.21	47 ± 2.9
Beef extract	0.20	22 ± 2.5
Peptone	0.18	36 ± 2.6
Tryptone	0.21	4 ± 2.3
Coconut press cake	0.72	25 ± 1.3

0.3% Bombay High crude oil was supplied as carbon source in all cases

* All values are averages ± Standard deviation ; n=2

The optimized protease production medium contained starch and glucose (Chapter 3) and in this medium protease production was about 14 U/ml. When these substances were removed from the medium which still contained fat-free casein as the nitrogen source, 0.3 U/ml of protease was still produced. In a set of experiments where starch and glucose were replaced by crude oil (0.3%) or C_{14} to C_{18} alkanes (0.5%), the organism degraded about 34% of the supplied crude oil and 60 to 80% of the alkanes, although the same basal level of 0.3 U/ml of protease was produced. n-Tetradecane was degraded better than n-hexadecane or n-octadecane (Table 4.7). Morihara (1965) reported that the amount of protease produced by a strain of *Pseudomonas aeruginosa* in an alkane containing medium was one-half of that produced in a glucose containing medium. In case of *Nocardiopsis* sp. NCIM 5124, the amount of protease produced in the presence of alkanes is about 3 times less than that in a medium containing glucose (1 U/ml, Chapter 3) and about 50 times less than that under conditions optimal for protease production.

Table 4.7 : Protease production in media containing hydrocarbons

Carbon source*	Protease activity U/ml	% degradation of alkane (GC) *
Bombay High crude	0.28	34 ± 2.5
n-tetradecane	0.26	80 ± 1.8
n-hexadecane	0.30	61 ± 2.3
n-octadecane	0.27	60 ± 2.1

In a medium containing 1% casein, 1% Na_2CO_3, 0.1% K_2HPO_4, pH 10.0

* All values were averages $\pm$ standard deviation ; n=2

It is clear that the organism can both utilize organic nitrogen and degrade crude oil or alkanes in the same medium although the amount of extracellular protease produced with crude oil or alkanes is far less (50 times less) than that observed in the optimized protease production medium. Microorganisms capable of both secreting extracellular proteolytic enzymes and degrading crude oil/hydrocarbons appear to be rare in the environment and

Nocardiopsis sp. NCIM 5124 is such an organism. This particular isolate produces proteases constitutively although the over production of proteases is inducible under appropriate conditions.

CHAPTER 5

GENERAL DISCUSSION

Species of *Nocardiopsis* are distinguished by means of the colour of their mature aerial and substrate mycelium and also additional physiological characteristics. However, as in most cases only one strain has been studied, the value of these criteria in species differentiation is restricted (Meyer, 1989). More recently, Yassin *et al* (1993) have proposed a new species, *Nocardiopsis lucentensis* sp. nov. This organism was found to have a variant of the type C sugar pattern and, glucose and arabinose were detected in the whole-cell extracts. It was found to exhibit only 40 to 50% DNA relatedness to other *Nocardiopsis* species. Al-Tai and Ruan (1994) have proposed another new species of *Nocardiopsis*, *N. halophila* for an organism isolated from a saline soil sample in Iraq. This organism was found to grow in media containing up to 20% (w/v) NaCl and was resistant to lysozyme, unlike the other *Nocardiopsis* sps.

Considerable heterogeneity has been reported within the genus *Nocardiopsis* by Poshner *et al* (1985), using DNA/DNA hybridization studies. These authors found that *N. coeruleofusca* and *N. longispora* were moderately related to each other (45% homology) whereas *N. africana* and *N. longispora* were only remotely related (11-13% homology). Interestingly, *N. africana* was found to show a substantial degree of relatedness to certain *Actinomadura* species especially *A. roseola* (64% homology). Furthermore, neither *N. africana*, *N. longispora* or *N. coeruleofusca* showed any relationship to the type species *N. dassonvillei* (about 8-12% homology). In addition, *N. dassonvillei* itself was shown to be heterogeneous by Fischer *et al* (1983) by studies on four strains of this species by DNA/DNA hybridization. Meyer (1989) has recommended further comparative biochemical and genetic studies of all members of genus *Nocardiopsis*, which share many morphological and biochemical properties. Grund and Kroppenstedt (1989; 1990) have reclassified strains of *N. coeruleofusca*, *N. flava*, *N. longispora*, *N. mutabilis* and *N. syringae* which contain rhamnose and large amounts of galactose in whole cell hydrolysates, in the genus *Saccharothrix*. Tsujibo *et al* (1990a) reported two types of alkaline serine proteases from an actinomycete which they identified as *Nocardiopsis dassonvillei* subsp. *prasina*, however, Grund and Kroppenstedt (1990) have designated *N. dassonvillei* subsp. *prasina* as *N. albus*

subsp. *prasina* comb. nov. from chemotaxonomy and numerical taxonomy studies based on cell wall composition and menaquinone, phospholipid and fatty acid patterns.

Our isolate has been identified as *Nocardiopsis* sp. on the basis of the typical morphology showing long chains of spores formed by aerial hyphae, the presence of meso-DAP without any diagnostic sugars representing the cell wall chemotype IIIC, the absence of mycolic acids, and the DNA base composition and has been compared with the type culture *N. dassonvillei* ATCC 23218 with respect to these criteria. The colour of the mature aerial and substrate mycelium, biochemical tests and degradation of specific compounds indicated that the most probable species to which our isolate belonged was *N. dassonvillei*. However, on account of the uncertainty regarding the classification of members belonging to genus *Nocardiopsis* at the species level and because we have not done DNA/DNA hybridization, the isolate was deposited in the National Collection of Industrial Microorganisms as *Nocardiopsis* sp. NCIM 5124.

The organism produced a maximal protease activity of about 14 U/ml under optimized conditions, and two extracellular proteases, protease I and II, have been purified to homogeneity and characterized. Proteases I and II differed with respect to their M_r which were approx. 21,000 and 23,000 daltons (SDS-PAGE) and their isoelectric points which were 8.3 and 7.0 respectively. Polyclonal antibodies raised in rabbits against protease I do not cross react with protease II suggesting that the two proteases are antigenically unrelated and do not share common epitopes. Protease I was also antigenically unrelated to other serine proteases like subtilisin, trypsin and chymotrypsin. On the basis of inhibition by PMSF and alkaline pH optima, both proteases from *Nocardiopsis* sp. NCIM 5124 are classified as alkaline serine proteases. The isoelectric points and molecular masses of these proteases are also similar to those reported for most alkaline serine proteases of actinomycete origin. Both proteases lacked exopeptidase activity against synthetic aminopeptidase and carboxypeptidase substrates and are thus characterized as endopeptidases. Neither protease from *Nocardiopsis* sp. NCIM 5124 showed any esterase activity. Both proteases lacked trypsin-like or chymotrypsin-like activity as they did not hydrolyze either Benzoyl-arginine-PNA or Benzoyl-tyrosine-PNA respectively, substrates specifically cleaved by trypsin-like and chymotrypsin-like proteases.

Among the actinomycetes, proteases showing collagenase-like activity have been reported from *Actinomadura* sp. (Rippon, 1968; Norwig, 1971), *Mycobacterium tuberculosis* (Takahashi *et al*, 1967), *Streptomyces thermovulgaris* (Egorov *et al*, 1976), *S. griseus* (Drake *et al*, 1966) and several *Streptomyces* sps. (Chakraborty and Chandra, 1984; Chakraborty and Chandra, 1986; Endo *et al*, 1987; Demina and Lysenko, 1992). Proteases having fibrinolytic activity have been reported from *S. clavuligerus*, *S. fulvoviridis*, *S. gedanensis*, *S. griseus*, *S. rimosus* (Buckley and Jefferies, 1981), *S. thermovulgaris* (Egorov, 1976; Al-Nuri *et al*, 1984) and *Streptomyces* sp. (Bono *et al*, 1996). Proteases with elastase-like activity have been reported from *S. griseus* (Gertler and Trop, 1971; Trop and Birk, 1968b), *S. fradiae* (Lagutina and Petrova, 1979), *S. rimosus* (Rassulin *et al*, 1974) and *S. griseoalbus* SN-22 (Murao *et al*, 1994). However, there are no reports of proteases showing such activities from *Nocardiopsis* sp. Both proteases from *Nocardiopsis* sp. NCIM 5124 showed collagenolytic and fibrinolytic activity. Protease I also showed elastolytic activity whereas protease II did not. Neither protease had any keratinolytic activity.

The specific activities of proteases I and II towards synthetic tri and tetrapeptide substrates were about 80 (protease I) to 100 (protease II) times higher than those observed with mono or dipeptide substrates. This result is a preliminary indication that substrate binding in an extended form, involving atleast 3 subsites or atleast 11 Å (Schecter and Berger,1967), is required for efficient hydrolysis by both proteases. The active sites of several proteases of both bacterial and mammalian origin have been shown to interact with substrates or inhibitors over a rather extended area covering 5,6 or 7 subsites or 18 to 25 Å (Bauer *et al*, 1981). The active site of subtilisin BPN' has been reported to extend over atleast 18 Å and can be divided into atleast 5 subsites (Ottesen and Svendsen, 1970). According to Bauer (1981), enzymes which are able to form precise $S_1 - P_1$ interactions are little dependent on enzyme-substrate contacts remote from the scissile bond, while the reverse is true for enzymes with less restrictive S_1 subsites. Chymotrypsin and trypsin have well defined S_1 subsites with good to excellent fit for their optimal P_1 residues and thus substrate contacts outside the S_1 subsite are relatively unimportant. In contrast elastase, *S. griseus* alkaline proteases A and C and subtilisin BPN' all have S_1 subsites characterized as ill defined, wide or less restrictive and these enzymes are all found to be highly dependent on

substrate contacts outside the S_1 subsite (Bauer, 1978). On the basis of their requirement for extended substrate binding, proteases I and II from *Nocardiopsis* sp. NCIM 5124 appear to fall into this latter class of enzymes and therefore differ from trypsin and chymotrypsin.

Both proteases I and II contain no cysteine residues, disulphides, or any associated carbohydrate and therefore are subtilisin-like in this respect. Chymotrypsin-like or trypsin-like proteases on the other hand, are characterized by the presence of cysteines and disulphide bonds. It thus seems that both proteases produced by *Nocardiopsis* sp. NCIM 5124 are closer to the subtilisin family of serine proteases than they are to the chymotrypsin/trypsin family of enzymes.

Nocardiopsis sp. NCIM 5124 is an organism capable of both secreting extracellular proteolytic enzymes and degrading crude oil/hydrocarbons. It can both utilize organic nitrogen and degrade crude oil or alkanes in the same medium although the amount of extracellular protease produced with crude oil or alkanes is far less (50 times) than that observed in the optimized protease production medium. The organism degrades upto 60 to 80% of single alkanes or 34% of crude oil while producing about 0.3 U/ml of protease activity in media containing casein as the organic nitrogen source. The organism can utilize coconut press cake, a cheap source of organic nitrogen, and degrade about 25% of the supplied crude oil. Although the degradation in this case is less as compared to that with NH_4Cl (66%) or with yeast extract (47%) as the nitrogen sources, the fact that the organism is capable of utilizing cheap sources of organic nitrogen by producing proteases makes *Nocardiopsis* sp. NCIM 5124 a good candidate for development of biotechnological methods for oil spill clean up. A composition consisting of coconut press cake, the organism on a carrier and a source of inorganic phosphate is suggested as an alternative biotechnological approach for oil-spill clean up in field trials.

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