# ISOLATION AND CHARACTERIZATION OF *STREPTOMYCES* STRAINS CAPABLE OF DEGRADING LIGNOCELLULOSE FROM *AGROPYRON REPENS*

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**A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY**

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**STRATHCLYDE INSTITUTE OF PHARMACY AND BIOMEDICAL SCIENCES**

**UNIVERSITY OF STRATHCLYDE**

**2010**

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# ACKNOWLEDGEMENTS

I feel moved from the core of my heart to offer my humblest thanks to almighty God who is merciful and compassionate and whose magnanimous blessings have always enabled me to perceive my goals.

I would express my sincere gratitude to my supervisor Dr. Alistair Paterson for his guidance, all-round discussions and giving me the opportunity to work with this fascinating subject. My thanks are also to his encouragement during these years.

I am grateful to Prof Ian Hunter, Dean, for his valuable comments and constructive criticism and encouragement throughout this project.

I would like to take this opportunity to forward gratifying feelings of mine to all of fellows especially Angzzas and Dzeti for their help and advice in the laboratory and with many practical things. My warm thanks are due to the research group in the Microbiology laboratory R521, particularly Dr. Emma Tiely and Vinod for support and advice for samples and information. I will certainly remember all the joys when we were together which surely are life time moments for me.

I am indebted to academic and technical staff in the department for inspiring working atmosphere that made this study possible.

I am also indebted to other staff members in the SIPBS specially Dr. Roth Tate for his help in DNA sequencing.

I would like to avail this opening to pass on my feelings of love and respect to all my family members especially parents, wife Nida and son Nehan as their contributions are multifaceted, words fail to express which.

Rizwan Shahzad

# ABSTRACT

In screening lignocellulose degrading *Streptomyces*, 9 strains were isolated for their ability to achieve degradation of lignocellulosic content of *Agropyron repens*. Isolates from soil samples were subjected to polyphasic systematic characterization to determine taxonomic identity. Strains depicted morphological and biochemical characteristics consistent with an assignment to the genus *Streptomyces*. 16S rRNA gene sequences were determined and compared with those of representative Streptomycetes. A phylogenetic tree from multiple alignment and neighbour-joining algorithms confirmed their membership in the genus *Streptomyces*.

A comparative analysis using the statistical program Minitab further sub-clustered 9 isolates into potential 5 strains. One of the strains 513-5(FJ966269) depicted a low level of similarity to others and its phenotypic characters are different from the reported strains. Thus it could be considered as a possible candidate for a new species.

Production of an extracellular lignocellulose degrading enzyme such as peroxidase was investigated. This was done during the growth phase of *Streptomyces* strains in basal salt-yeast extract medium containing the lignocellulosic content of quack-grass (*Agropyron repens*) and corn (*Zea mays*) and the effects of environmental parameters (carbon source concentration and pH) were recorded. The comparatively high level of enzyme production from cell-free extracellular preparations during the growth phase of strains 513-1(FJ966265), 513-5 (FJ966269) and 513-7 (FJ966271) signify the suitability of these enzymes for possible consideration in industrial applications such as pulp and paper production.

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# 1. Introduction

## 1.1 Preamble

Lignocellulose, the principal structural component of woody and non-woody plants, represents a major source of renewable organic matter and is a complex of three classes of polymer. Cellulose has a structure from repeating glucose (cellobiose) units largely in crystalline fibres. Hemicelluloses are a mixed group of polysaccharides, mainly of pentose monomers. In lignin, monomers are three cinnamyl alcohols and some related acids. Due to structural complexity, both cellulose and lignin are difficult to disrupt. Cellulose is largely crystalline and more difficult to attack than more amorphous hemicelluloses. Lignin is assembled by an oxidative polymerization of free radical species of monomers to yield an irregular three-dimensional polymer with a number of inter-monomeric bond types.

Malherbe and Cloete (2003) concluded lignocelluloses were a substrate of biotechnological value. Large amounts of lignocellulosic waste are generated through forestry, agriculture, paper pulp, timber industries and many agro-industries and accumulate to pose an environmental pollution problem. However biomass considered as waste can potentially be converted into a range of value added products including biofuels, chemicals, energy sources for fermentation, animal feed and human nutrients. Lignocellulose-degrading enzymes also have significant potential industrial applications including chemicals, food, brewing and wine, textiles, pulp and paper (Howard *et al*., 2003).

Apart from an ecological significance in biodegradation actinomycetes are known for a capacity to produce substances of industrial value like antibiotics and enzymes (Okami and Hotta, 1988).

## 1.2. Biodegradation of lignocellulose

Environmental conditions alone are not responsible for most degradation of complex lignocellulose substrates - microbial populations also play key roles in degradation and are well equipped with degradative potential (Waldrop *et al*., 2000). Slow processes of prokaryotic degradation of lignocellulose have been attributed to lack of powerful lignocellulosic degrading enzymes (McCarthy, 1987). Grass lignins are

more susceptible to actinomycete attack than those of wood. Actinomycetes particularly *Streptomyces* strains together with other bacteria hold a significant role in the humification processes associated with soils (Trigo and Ball, 1994).

Lignin is the more recalcitrant of the three lignocellulose components whereas cellulose, due to a crystalline structure, is comparatively resistant to hydrolysis compared to hemicelluloses. Enzymes generally operate under mild reaction conditions acting as specific biocatalysts and are environmental friendly processes. Some useful lignocellulosic bioconversion processes are summarized in Figure 1.1.

## 1.3. Microorganisms and lignocellulolysis

Isolation and/or identification of microorganisms capable of lignocellulolytic activities facilitate understanding of mechanisms by which organisms work as a community to effect breakdown. In this Actinobacteria, especially *Streptomyces,* are key microorganisms (Stackebrandt *et al*., 1997). Streptomycetes are Gram-positive bacteria, of high G-C content, found in a variety of habitats. Actinomycete numbers are high in soil, mostly biodegradative *Streptomyces* which are saprophytes.

Among actinobacteria *Streptomyces* depict greatest morphological differentiation determined by filamentous degree of organization. Although it is quite difficult to summarize all colonial variations, Miyadoh (1997) reviewed different aspects among *Streptomyces*. Colonial forms may be flat or raised and in certain cases surfaces are covered with a leathery layer. Texture varies from soft and pasty to extremely hard. Colouration including white, yellow, red, orange, blue, brown and black. Smooth, wrinkled, rigid or granular surfaces may be observed. Sometimes colonies appear compact, in other cases depict different zones of growth in concentric rings with radial orientation. Species types, age differences and growth conditions influence colony size (Miyadoh, 1997).

Williams *et al*. (1984) discussed the role of *Streptomyces* in recycling of biopolymers such as lignocellulose and also degradation of chemical pollutants including simple and chlorinated hydrocarbons, aliphatic and aromatic hydrocarbons, polycyclic aromatics, nitroaromatics (Warhurst and Fewson, 1994). These organisms also produce industrially important metabolites (Goodfellow *et al*., 1988).

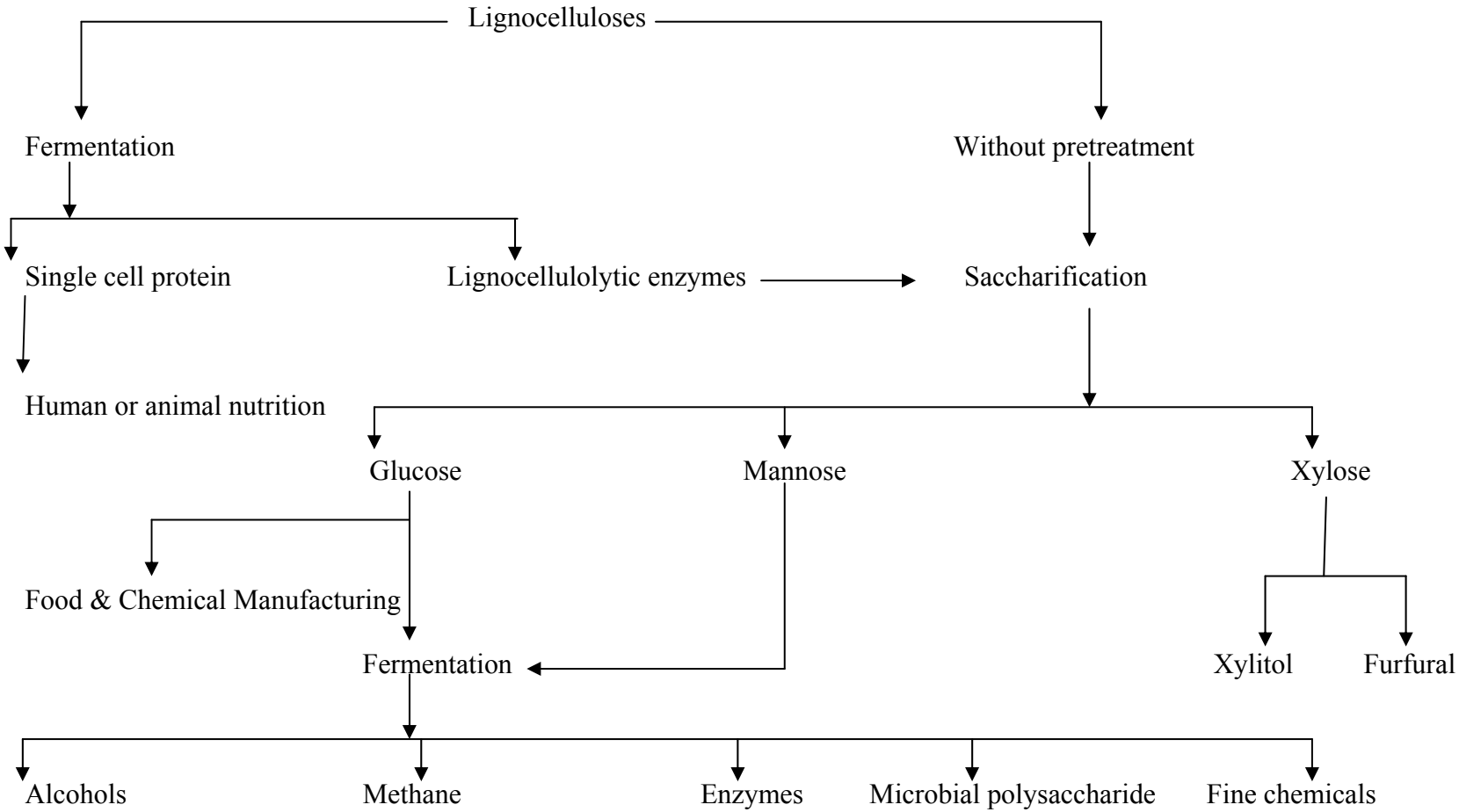



**Figure 1.1: General stages in lignocellulose bioconversion (Modified from Howard *et al*. 2003)**

## 1.4. Identification of isolates

### 1.4.1 Culture –based techniques

Culture based techniques are commonly and conventionally employed for detecting and identifying microorganisms from environments due to simplicity and low cost. Soil environments are heterogenous comprising particles of different sizes surrounding air-filled spaces in which microhabitats are localized by microbial cells (Van Elas and Van Overbeek, 1993). Streptomycete spores reside throughout the soil profile and are present in one of the three living states: free-living conditions between soil particles; on surfaces of soil particles; in pore spaces within soil particles. *Streptomyces* are attached to soil particles by electrostatic interactions.

Different factors are involved in extraction of *Streptomyces* from soil. The free living *Streptomyces* are most easily extracted and those within soil aggregates most difficult to isolate. Because *Streptomyces* filaments are attached to soil particles by polysaccharide adhesions so production of extracellular polysaccharides will adversely affect extraction from such environments (Metcalf *et al*., 1995). Although different methods for the extraction of microorganisms from soil are employed each has two common stages. Firstly soil dispersion can be achieved with an aqueous solution and in certain cases soil particles are separated from microbial cells by centrifugation. Lindahl and Bakken (1995) concluded: 'Maximum dispersion of the soil is vital to release microorganisms resident within the soil particles and aggregates. Dispersion is also required to assist in dissociation of microorganisms from the surfaces of the soil particles'. Actinomycetes present in nature can be cultivated using standard techniques. Different types of non-molecular methods have been developed like microscopic observations, plate culture techniques and selective culture. These are suitable for analyzing streptomycetes from a wide variety of environments such as soil, water and composts. Apart from the differences associated with all the steps, each method helps to highlight the nature and abundance of streptomycetes in a specific environment. The most convenient and convential method for the determination of nature and frequency of microbes in populations is the plate method.

Hunter-Cevera *et al*. (1986) discussed in detail the processes involved in the isolation on different media containing specific nutrients and functionality can be altered by changing physiological parameters such as salinity, pH, temperature and time of incubation. Waksman (1967) concluded that growth factors influence streptomycete abundance. He reported that 'the growth of actinobacteria especially *Streptomyces* may be encouraged by the addition of natural substrates and other biopolymers used as energy sources including: organic acids, sugars, starches, amino acids and to some extent lignin and rubber. The most readily utilizable sources of energy are glucose, mannose and proteins'. The study of Nolan and Cross (1988) highlighted the importance of specific antibiotics and other compounds in isolation media. These authors concluded that addition of antimicrobial substances like cycloheximide and nystatin have effects not only suppressing fungal growth but also give higher counts of actinobacteria. Dietz and Thayer (1980) concluded that air-drying of soils reduces numbers of non-actinobacteria. Cultivation usually involves a small volume of liquid in the form of water, saline and buffer containing microorganisms is usually inoculated in to solid and liquid growth media. Different incubation temperatures can be selected for specific microorganisms. Growth rate of microorganisms is usually dependent on period of incubation - slow growing organisms like *Streptomyces* require more than 96 h, i.e., more than four days to become visible as a distinct colony.

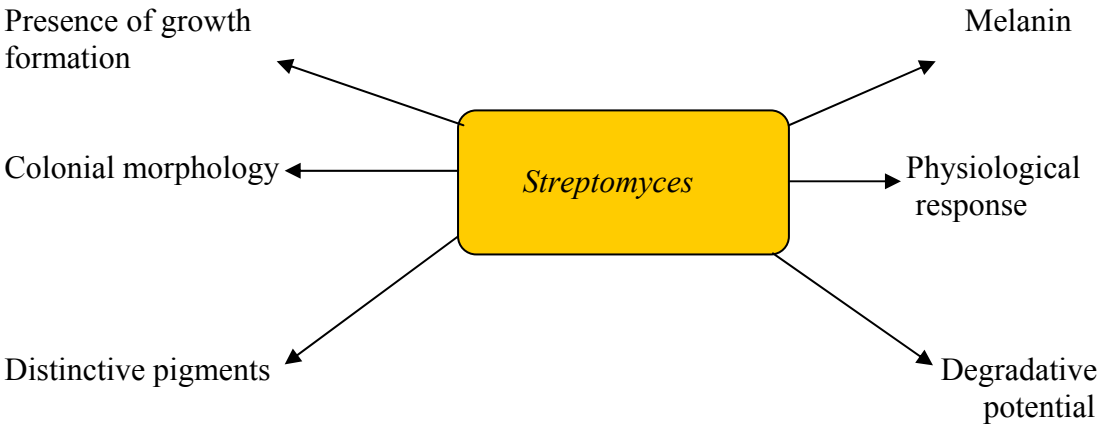



**Figure 1.2: Schematic representation of *Streptomyces* identification.**

An isolation medium can be specifically tailored for nutrient requirements of specific actinomycetes. Rintala (2003) discussed different factors involved in identification and reported that 'Microorganism that are resistant to one or more antibiotics can also be selected and therefore identified by the addition of those antibiotics to the growth medium'. The result of his studies also suggested that distinctive colony morphology enable *Streptomyces* to be identified when grown on solid media (Rintala, 2003).

### 1.4.1.1. Probabilistic identification

In contrast to monothetic classifications stable taxonomies such as those derived by numerical taxonomy could only be achieved when classifications were derived from the analysis of comparatively large numbers of bacteria for many characters. The foundation of numerical taxonomy procedure is based on examining many strains for large numbers of equally weighted characters and final classification is derived from overall similarity (Goodfellow and Dickinson, 1985; Sackin and Jones, 1993).

**1.4.1.2. Computer assisted identification**

The strength of the numerical systematic methodology relies on the ability of computer programs to handle comparatively large amount of data of different strains. The strains to be classified are called operational taxonomic units (OTU). In comparative studies representatives of additional well studied type cultures should be included to provide an internal check on test reproducibility (Sneath and Sokal, 1973). It has always been advisable to include newly isolated strains because previously subcultured organisms may not be good representatives of established taxa (Logan, 1994).

Data must be coded into a format suitable for computation. Usually a binary form is used for results obtained from various tests. Generally unit characters are coded as two character states in which possession of the character is scored as positive (+) or 1 and its absence as minus (-) or 0. A mutually exclusive method of coding is used for multistate characters such as colonial colour and pigmentation. In this procedure OTU having a particular state for a character is usually coded as positive (+) and negative (-) for all the remaining characters. Mutually exclusive characters are

usually kept to a minimum number in numerical systematics (Goodfellow *et al*., 1990).

**1.4.1.3. Computation of resemblance**

Diversity exists in number of ways of calculating similarity and dissimilarity between a pair of OTUs (Austin and Cowell, 1977) but only those most commonly used in bacterial systematics are considered here. The test-statistic, Simple matching coefficient ($S_{SM}$), confers the proportion of characters that two organisms have in common. The symbols w and z are used to account for the number of shared positive and negative matches while x and y accounting for the number of differences between a pair of OTUs:

| | | Result for OTU 2 | |
|---|---|---|---|
| | | + | - |
| Result for OTU 1 | + | w | x |
| | - | y | z |

The $S_{SM}$ coefficient is calculated as:

$$S_{SM} = \frac{W + Z}{W + X + Y + Z}$$

Different workers (Kutzner, 1986; Williams *et al*., 1983; Goodfellow *et al*., 1992; Goodfellow and O'Donnell; Kamfer *et al*., 1991) utilized morphological, biochemical and physiological tests as the basis of streptomycete classification.

Williams *et al*. (1983) devised a probabilistic identification matrix based on characters like spore chain and spore morphology, pigmentation, physiological abilities, antibiosis, and resistance to antibiotics. Kamfer *et al*. (1991) used more characters, revised data analysis and studied more strains and then compared

information with published genetic and chemotaxonomic strategies. By utilizing rapid enzyme tests, Goodfellow *et al*. (1992) supplemented other strategies. However Anderson and Wellington (2001) reported that such approaches were insufficient to discriminate to species level.

According to such classifications, *Streptomyces* can be divided into major, minor and single number clusters. Major clusters comprise six or more type strains and are considered as species group whereas minor clusters contain two to five strains. The degree of variability found among clusters depending upon the approach used (Anderson and Wellington, 2001). *Streptomyces* is the largest of the order Actinomycetales within the class Actinobacteria (Stackerbandt *et al*., 1997). The genera *Kitasatospora* and *Streptoverticillium* have been included in the genus *Streptomyces* (Witt and Stackebrandt, 1990; Wellington *et al*., 1992) although there is confusion about taxonomic position of *Kitasatospora* (Zhang *et al*., 1997).

### 1.4.2. Molecular techniques used in systematics

Identification of a specific gene or nucleic acid sequence is usually employed in molecular based techniques in systematics. DNA is first extracted, achieved by lysing the cell by physical or chemical processes. Cells may be extracted either from cultures and then lysed or lysed in the environmental sample (Leff *et al*., 1995; Kresk and Wellington, 1999).

Polymerase chain reaction (PCR) strategies are usually employed for amplifying sequences of DNA using two typically twenty base primers and a heat stable polymerase. Because newly synthesized DNA strands serve as additional templates for primers, repeated; rounds of primer annealing, strand elongation and dissociation synthesize highly specific amplification of the desired sequence. To confirm the existence of a particular sequence in a DNA sample, PCR can also be used and even a small amount of template DNA in a sample can increase to a level to be studied by other techniques.

Molecular methods based on DNA have been used for identification and differentiation of *Streptomyces* species. Healy and Lambert (1991) concluded that in

certain cases these strategies show good homology with methods based on physiology and morphology but in other cases not.

### 1.4.2.1. Choice of molecule

Woese (1987) pointed out that small and large subunit ribosomal (r) RNA sequence provide most useful chronometer for phylogenetic analysis as they:

- Occur in all organisms
- Depict a high degree of functional constancy which assures relatively good clockwise behaviour
- Show rates of mutation thereby allowing distant phylogenetic comparisons to be made
- Can be sequenced directly

Prokaryotes contain three types of ribosomal RNAs, 5S, 16S and 23S rRNAs. Due to its small size, 5S is not considered to be a suitable molecule for phylogenetic analysis (120 nucleotides) this does not allow statistically significant sampling (Hillis and Dixon, 1991).

Until recently, deductions about the evolution of prokaryotes were almost exclusively based on data derived from 16S rRNA. Reasonably good argument has been made for evolutionary trees based on 16S rRNA sequence data (Ludwig and Schleifer, 1994).

#### 1.4.2.2. Bacterial 16S rRNA genes as a target

The 16S rRNA gene is now mostly used for bacterial systematic purposes. The gene is also designated as 16S rDNA and the terms have been used interchangeably (Bottger, 1989).

Different genetic strategies such DNA-DNA reassociation (Kim *et al*., 1999) and 16s rRNA gene analysis (Kim *et al*., 1996; Takeuchi *et al*., 1996; Hain *et al*., 1997; Kim *et al*., 1999) partly contributed to this classification.

For phylogenetic and diversity studies, the 16S rRNA gene has been extensively used. This gene comprises conserved and variable regions and hence primers and

probes with different levels of specificity could be developed (Woese, 1987). The conserved regions confer phylogenies at the higher taxonomic levels because these have evolved very slowly and are similar among different taxa, whereas due to mutational alterations in evolution, variable regions are useful for delineation at intraspecific level (Woese, 1987).

These rRNA genes are essential and are therefore present in all organisms (Stackebrandt and Gobel, 1994; Rossello-Mora and Amann, 2001). Brenner *et al*. (2001) discussed different aspects of sequence analysis of 16S, 23S and 5S rRNA subunits and concluded that the 16S rRNA gene has become a significant tool in *Streptomyces* identification and extensively utilized for highlighting phylogenetic placement of isolates. The hypervariable region of the 16S rRNA gene was utilized by Kataoka *et al*. (1997) to create an index for identification of *Streptomyces*. However Rossello-Mora and Amann (2001) suggested that 16S rRNA sequence information alone may not be enough for species identification.

Figure 1.3 summarises the processes involved.

## 1.5. Mathematical model of evolutionary history- Phylogenetic trees

Direct determination of evolution in life is difficult so models in the form of phylogenetic trees represent evolutionary pathways. The evolutionary hypothesis representing history of sequences or organisms may be represented as a tree.

A distinction can be made between phenetic and cladistic data. Sneath and Sokal (1973) defined phenetic relationships as similarities based on a set of phenotypic characters whereas cladistic relationships contain information regarding ancestry. Both relationships are well represented as phylogenetic trees.

In a phylogenetic tree history the data used for ordering species are of two types:

- Similarity data or distance data refer to pairs of genes from individuals
- Character data confer information about attributes of genes, individuals or species.

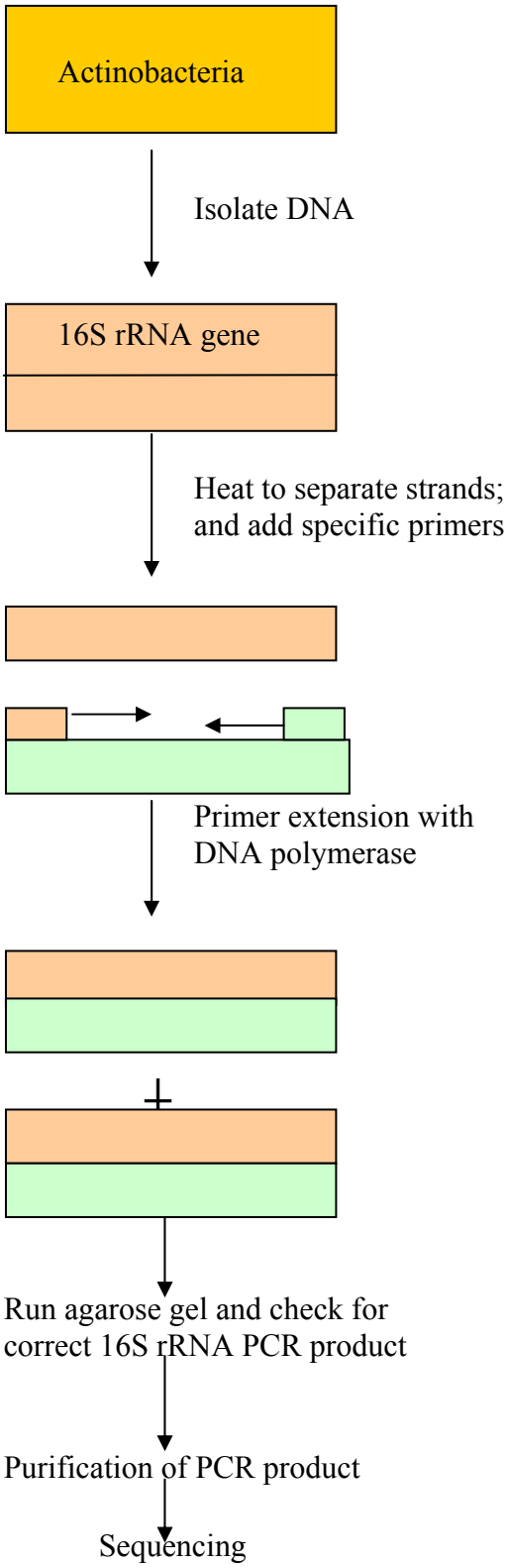



**Figure1.3: Schematic flow of 16S rRNA gene analysis (Modified from Madigan *et al.*, 2003).**

Sets of distances calculated between each pair of taxonomic units are usually employed in distance matrix methods. Distances usually refer to the number of changes between units. The most widely used distance matrix method is that of neighbour-joining (Saitou and Nei, 1987).

Genetic distances between all pairs of operational taxonomic units (OTU) are usually the basis of phylogenetic trees. Clustering is usually formed by using these distances to group OTU's in a phenetic context. The next stage is to align sequences from varied sources in order to obtain maximum homology.

Saito and Nei (1987) devised a method for identification of closest neighbours or pairs of taxonomic units in such a way to minimize the total length of tree. In this mode pairs of species represent neighbours that when joined result in a tree of shortest total length.

### 1.5.1. Statistical evaluation of evolutionary tree

An evolutionary tree should be evaluated to assess the significance of tree topology and the length of branches. The procedures underlying statistical tests of phylogenies have been extensively reviewed (Felsenstein, 1988). Bootstrap analysis is the most extensively used method for evaluating phylogenies. The method is also called 'resampling' as it involves the generation of new data sets by random resampling of positions in the original data set.

Resampling 500-1000 times is a possible compromise between efficacy and accuracy and is usually accepted by most bacterial systematists (Ludwig and Schleifer, 1994).

Chun *et al*. (1997) analyzed phylogenetics of actinomycetes isolated from Korean soil, constructing a phylogenetic tree based on the neighbour-joining method. These authors concluded that 'strains which exhibited possible phenotypic relatedness to strain IMSNU-1 based on probabilistic identification procedure, i.e., *Streptomyces diastaticus* (cluster 19), *S. lincolnensis* (cluster 19), *S. bottropensis* (cluster 19), *S. blnensis* (cluster 19) showed no close phylogenetic relationship to our isolate or among themselves'.

Different steps from sequence to tree inference are summarized in Figure 1.4.

## FROM DNA SEQUENCE TO TAXONOMIC TREES

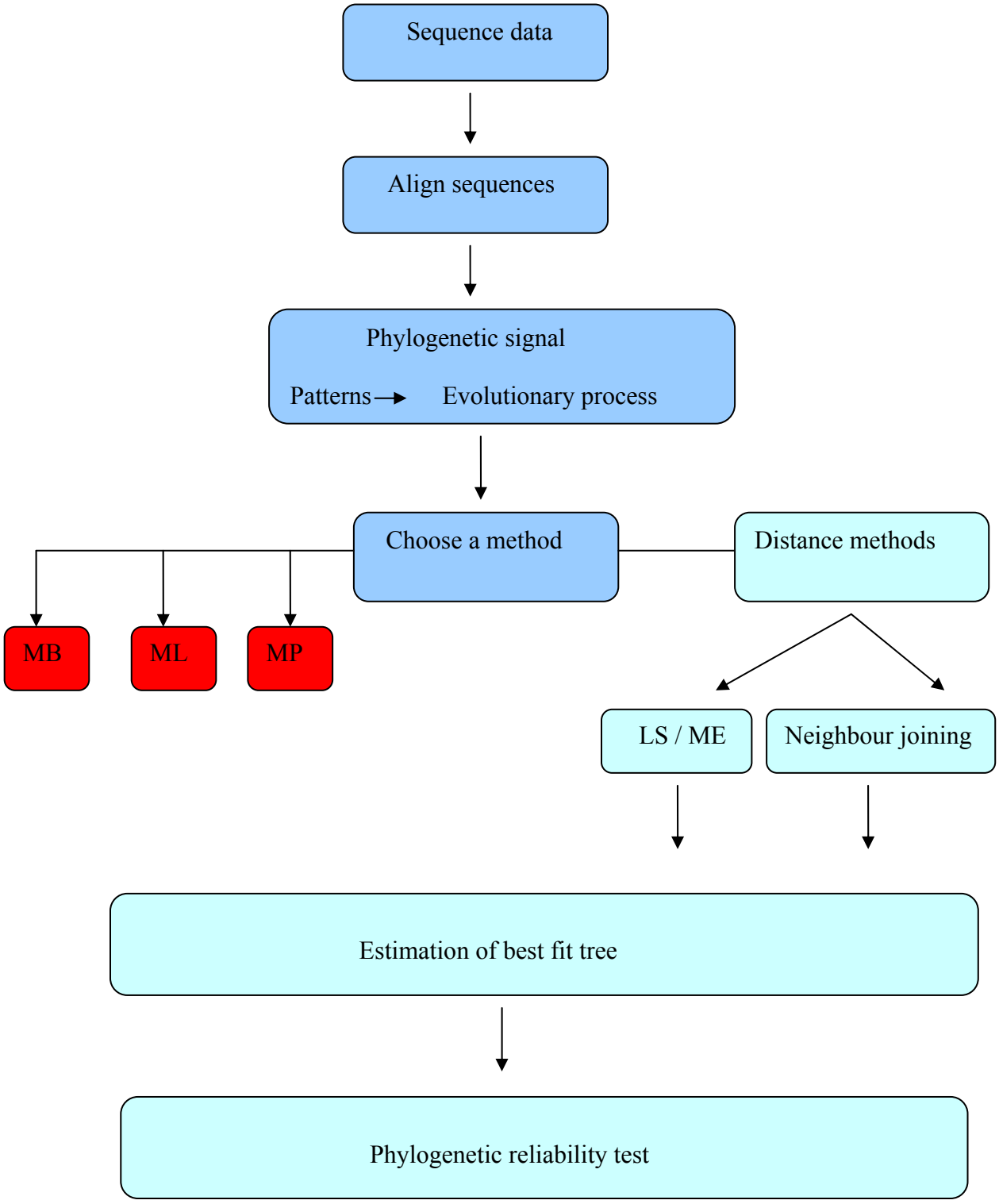



**Figure 1.4: Schematic flow of Tree Inference (Modified from Hillis *et al*., 1993). MB, Max-mini branch; ML, maximum likelihood; MP, Maximum parsimony; LS, Least square; ME, Minimum evolution.**

## 1.6. Filamentous form of actinomycetes with respect to lignocellulose degradation

Colonisation of land by plants provided a new habitat for heterotrophs. The soil habitat is essentially different from sediments in that it contains air-filled voids. The inability of the eubacterial unicellular body form to bridge such voids restricts bacterial colonization of soils, a disadvantage overcome by the mycelial growth form. Hyphal growth of cellulolytic actinomycetes appears an important strategy to access cellulose fibre pores in plant cell-wall material, and to bring secreted cellulases into close contact with substrates. Several groups have suggested this advantageous mode of lignocelluloses decomposition in lignified material (Lynd *et al*., 2002; Daniel and Nilsson, 1998). Filamentous actinomycetes appear thus as likely competitors of cellulolytic fungi.

Waldrop *et al*. (2000) reviewed work on microbial community links to function addressing:

* Can lignocellulose activities be related to changes in community composition?
* In what ways do habitat compositions differ in microbial biomass, community composition and physiological capabilities?.

These authors found that activity of most enzymes, except hydrolytic β-glycosidases, were related to the N content of the soil. A possible reason could be that as plantations age, pH and availability of Ca, Mg, Mn, and P decrease. Conversion of tropical forest to a pineapple plantation decreased soil pH, %C, %N, and the availability of all nutrients except potassium, sulfur, and zinc. Oxidative enzymes such as phenol oxidases and peroxidases are primarly responsible for lignin degradation. Thus enzymes degrading macromolecular substrates may be more closely related to community composition than enzymes that degrade simpler substrates, depicting that enzyme activity fluctuates with the varying contents of soil nutrients. It can be concluded that phenol oxidase, peroxidase and cellobiohydrolase specific activities are more strongly related to the shifts in community composition than β-glycosidase and β-xylosidase. Such trends could be used in a variety of ways to assess issues of environmental quality, such as indices of site fertility, soil quality, pollution effects and nutrient cycling potential.

The concept that production of extracellular enzymes involved in lignocellulose degradation is growth associated in cultures of *Streptomyces* comes from work of Tuncer *et al*. (2004), specifically for *Streptomyces* sp. F2621 grown on xylan. Lignocellulose degrading enzyme activities produced by *Streptomyces* sp. F2621 were higher than those of most bacteria and fungi and generally in accordance with those reported for strains of *Streptomyces albus* (Antonopoulos *et al*. 2001), *S. viridosporus* (Zerbini *et al*., 1999), and *Thermomonospora fusca* (Tuncer *et al*., 1999). This could be attributed to the high thermostability and nearly neutral pH optimum, not usually found in the enzymes of fungi (Tuncer *et al*., 2004).

Only a few lignocellulolytic micro organisms have been studied extensively.

Bacterial genera such as *Cellulomonas*, *Pseudomonas* and the actinomycetes such as *Thermomonospora* and *Microbispora* and bacteria with surface-bound cellulase complexes such as *Clostridium thermocellum* now receive attention as representing a gene pool with possible lignocellulolytic genes that could be used in biotechnology (McCarthy, 1987; Vicuna, 1988; Miller *et al*., 1996).

Apart from some cross activity reported for lignocellulolytic enzymes (Kumar and Deobagkar, 1996), it is conventional to consider lignocellulose-degrading enzymes according to the three components of lignocellulose.

The following tables compiled from the Brenda Enzyme Data Base (http://www.Brenda-enzymes.info) depicting actinomycetes with the highest specific activity under the appropriated conditions.

**Table1.1: Cellulases of Streptomycetes with highest specific activity (μmol.min$^{-1}$mg$^{-1}$)**

| Enzyme (EC Number) | Organism | Substrate | Specific activity (μmol mg$^{-1}$) | Temp ($^0$C) |
|---|---|---|---|---|
| β- Glucosidase (3.2.1.21) | *Streptomyces albaduncus* | Cellulose/Carboxymethyl cellulose | 92.3 | 50 |
| 1,4-β-Glucanase (3.2.1.4) | *S. rochei* | Cellulose + $H_2O$ | 64.8 | 65 |
| 1,6-β-D-glucan glucanohydrolase (3.2.1.4) | *Thermomonospora fusca* | Carboxymethyl cellulose/ Cellotriose/Xylan | 90.2 | 74 |
| 1,4-β-d-Glucosidase (3.2.1.21) | *Streptomyces halsteidii* | 4-β-D- Glucopyranoside | 78.3 | 55 |
| 1,4-β-D-Glucan-4-glucanohydrolase (3.2.1.4) | *Thermobifida fusca* | Glucan/phosphoglucan | 68.0 | 50 |
| 1,4-β-D-Glycosidase (3.2.1.21) | *Streptomyces lividans* | Carboxymethylcellulose + $H_2O$ | 194 | 70 |

Source: Harchand and Singh (1997); Wilson (1988)

**Table1.2: Hemicellulases of Streptomycetes with the highest specific activity (μmol.min$^{-1}$mg$^{-1}$)**

| Enzyme (EC Number) | Organism | Substrate | Specific activity (μmol mg$^{-1}$) | Temp ($^0$C) |
|---|---|---|---|---|
| 1,4-β-Xylosidase (3.2.1.37) | *Streptomyces* sp. | Xylan + $H_2O$ | 12.3 | 55 |
| α-N-Arabinofuranosidase (3.2.1.55) | *Streptomyces diastaticus* | 1,5-L-arabinan + $H_2O$, Oat spelt xylan + $H_2O$ | 105 | 25 |
| Acetyl Xylan esterase (3.11.72) | *Streptomyces lividans* | 2,4,di-o-acetyl beta-D-xylopyranoside + $H_2O$ | 0.175 | 40 |
| Feruloyl esterase (3.1.1.73) | *Streptomyces olivochromogenes* | De- starched wheat bran + $H_2O$ | 1.51 | 30 |
| Pcoumaroyl esterase (3.2.1.73) | *Streptomyces avermitilis* | Destarched wheat bran + $H_2O$/Cinnamic acid | 56.4 | 50 |

Source: Faulds and Williamson (1991); Garcia *et al*. (1998)

**Table1.3: Lignases of Streptomycetes with the highest specific activity ($\mu mol^{-1}mg^{-1}$)**

| Enzyme (EC Number) | Organism | Substrate | Specific activity ($\mu mol\ mg^{-1}$) | Temp ($^0C$) |
|---|---|---|---|---|
| Laccase (1.10.3.2) | *Streptomyces griseus* | 4- Aminoantipyrine +$O_2$ | 4.2 | 40 |
| Monophenol monooxygenase (1.14.18.1) | *Streptomyces castaneoglobisporus* | L- DOPA + $H_2O$ | 1.1 | 35 |
| Monophenol monooxygenase (1.14.18.1) | *Streptomyces* sp. | L- Epicatechin + $O_2$ | 130 | 35 |
| Lignin peroxidase (1.11.1.14) | *Streptomyces viridosporus* | 2,4-Dichlorophenol | 33.8 | 35 |

Source: Endo *et al*. (2003); Kohashi *et al*. (2004)

## 1.7. Lignocellulose solubilisation process:

Actinomycetes live in environments rich in lignocellulose, such as soil, compost, straw and wood chips (Lacey, 1988). Actinomycetes frequently degrade, modify or solubilize the lignin polymer to acid precipitable polymeric lignin (APPL, Adhi *et al*., 1989; Ball *et al*., 1989; Spiker *et al*., 1992). APPL is a high molecular mass water soluble, heterogenous mixture that contains minor amounts of carbohydrates, proteins, organic nitrogen and inorganic materials (Crawford *et al*., 1983). Although lignin mineralization by actinomycetes is not as efficient as by fungi, it is thought more efficient than by unicellular bacteria (Haider and Trojanowski, 1980).

Lignin-carbohydrate complexes (LCCs) are heterogenous structures found in many plant species. Lignin is directly or indirectly bound covalently to carbohydrate and resulting complexes present a barrier to biological degradation (Wallace *et al*., 1991). Lignin- carbohydrate bonds are presumed to exist in high molecular weight lignin fractions that are water insoluble. Softwood LCCs are distinct in that carbohydrate portions consist of galactomannan, arabino-O-4-methylgluronoxylan and arabinogalactan linked to lignin at benzyl positions (Azma *et al*., 1981; Mukoyoshi *et al*., 1981). In contrast, carbohydrate portions of hard wood and grass LCCs are composed exclusively of 4-O-methylglucuronoxylan and arabino-4-O-methylglucuronoxylan respectively (Markwalder and Neukom, 1976; Azma and Koshijima, 1988).

Relatively little attention has been given to enzymes capable of cleaving the covalent linkages between lignin and carbohydrate. Problems associated with studying enzymes that will attack lignocarbohydrate bonds are similar to those involved in studying lignin and hemicellulose degradation. Substances derived from natural polymers are often poorly defined; model compounds may not reflect the structure of native substrates because:

- The bonds are multifarious
- The products can be complex
- Enzymatic activities can be low
- Cofactors and inducers may be required for enzyme activity

Studies on solubilization of lignin from wood labeled with $^{14}C$ phenylalanine have proliferated (Crawford, 1978; Blanchette, 1995). Commonly, biodegradation of the milled wood lignin is followed by trapping the $^{14}CO_2$ respired from active cultures. Between 25% and 40% of $^{14}C$ added to active cultures can be recovered as $^{14}CO_2$. Given that a fully aerobic organism will respire about half of the carbon provided to it while incorporating the other half as cellular material; this represents the metabolism of 50% to 80% of total lignin.

A significant fraction (as much as 30%) of the total lignin can be recovered from cultures as a polymer (Crawford *et al*., 1983). This material precipitates from culture filtrates following acidification to pH 3 to 5. The solubilized acid perceptible polymeric lignin (APPL) shows signs of partial degradation.

Many different enzymatic activities from organisms like *Streptomyces* spp., and *Thermomonospora fusca* have been reported, including endo-glucanase (glucanohydrolase), xylanase (β- xylosidase), esterase (feruloyl esterase) and an extracellular peroxidase (lignin peroxidase) activities (Ramachandra *et al*., 1987). Roles that these enzymes play in lignin solubilization are not clear, but various correlations were made between the appearance of extracellular peroxidase activity and lignin solubilization or mineralization.

A Streptomycete enzyme was reported to cleave a β-aryl ether model in a manner similar to that observed for *Phanerochaete chrysosporium* (Ramachandra *et al*., 1988; Donnelly and Crawford, 1988)).

Schematic modes of action of cellulases and hemicellulases are represented in the Table 1.4, while Table 1.5 summarizes various reactions involving ligninases.

**Table 1.4: Mode of action of cellulases and xylanases in actinomycetes**

| Enzyme | Mode of action |
|---|---|
| Endoglucanase | -G-G-G-G-G-G-G-G-G-G-G-<br>↑ ↑<br>Random cleavage of 1,4-β-linkages (Wood and Bhat, 1988) |
| Cellobiohydrolases | -G-G-G-G-G-G-g-G-G-G-G-<br>↑(Type I) ↑(Type II)<br>Releases cellobiose from non-reducing end(type I) and reducing end(type II) (Barr et al., 1996) |
| B-Glucosidase | -G-G; G-G-G-G; G-G-G-G<br>↑ ↑ ↑ ↑ ↑<br>Releases glucose from cellobiose and hydrolyse cello-oligosaccharides from both reducing and non-reducing ends by releasing one glucose unit at a time. (Bhat and Bhat, 1997) |
| Xylanase | S S<br>X-X-X-X-X-X-X-X-X-X-X-X-X- (Coughlan and Hazlewood, 1993)<br>↑ S ↑ |

**Table1.5: Enzymes involved in the degradation of lignin and their main reactions**

| Enzyme | Cofactor or substrate, mediator | Reaction or main effect |
|---|---|---|
| Lignin peroxidase, LiP | $H_2O_2$, organic acids | Aromatic ring oxidized to cation radical |
| Manganese peroxidase, MnP | $H_2O_2$, Mn, organic acids as chelator | Mn(II) oxidized to Mn(III), chelated Mn(III) Oxidizes phenolic compounds to phenoxyl radicals |
| Laccase | $O_2$; mediators, e.g., hydroxybenzotriazole | Phenols are oxidized to phenoxyl radicals |
| $H_2O_2$ producing enzymes | Organic compounds | $O_2$ reduced to $H_2O_2$ |

Source: Broda *et al*. (1989); Call and Mucke (1997)

## 1.8. Cell-free solubilisation activities:

There is disagreement as to whether enzymic attack is favoured by cell-free and in contact cell systems. McCarthy *et al*. (1986) suggested development of cell-free systems would be a major step towards elucidating actinomycete attack. Mason et al. (1988) studied resolution of different activities from *S. cyaneus* MT813 grown on ball-milled straw and isolated a lignin solubilisation activity.

Tuncer *et al*. (2004) demonstrated an ability of cell- free supernatant fluid to release reducing sugars and aromatic compounds from kraft pulp. Colorimetric analysis of enzyme- treated kraft pulp indicated that overall, a higher hydrolysis of oat spelt xylan (9.3%) occurred after 96 h incubation. Hydroxylation of kraft pulp and ball milled wheat straw were approximately twofold less than for oat spelt xylan under similar conditions.

Leda *et al*. (2003), in a study of effects of aeration on lignin peroxidase production in culture supernatants of *S. viridosporus* T7A, observed media incorporation of 1.0 % corn oil and 400 rpm increased the enzyme concentration 1.8- fold and process productivity 4.8 fold in comparison with the use of 0.3 vvm and 200 rpm. Corn oil, besides its antifoam property, proved not to be a repressor for enzyme production.

Trigo and Ball (1994) proposed cooperativity among actinomycete lignocellulolytic enzymes and observed maximum and minimum APPL levels in culture supernatants exhibiting corresponding levels of all three enzyme activities. Broda (1992) reviewed not only ligninase activity but also xylanase production in thermophilic isolates. Xylanase activity was optimal within the range 60-75 $^{o}$C and pH 5 - 8 and concluded xylan was hydrolysed into a mixture of algometric products indicating primary endoxylanase activity.

Wang *et al*. (1992) used different carbon sources (mono- di- and polysaccharides, aromatic compounds) to elicit expression of cellulase and lignin solubilisation activities as well as xylanase observing both xylan and ball milled straw induced all the three lignocellulosic enzyme activities. Broda (1992) concluded that *S. cyaneus* produced a ‘sensor’ xylanase constitutively that degraded xylan in environments to smaller molecules that act as intracellular inducers.

Lodha *et al*. (1991) evaluated peroxidase activity as non-growth associated secondary metabolite. Peroxidases are primarily intracellular enzymes with important roles in cellular processes (Everse *et al*., 1990), but extracellular peroxidases involved in degradation of complex organic compounds are expected to have improved stability over the intra- cellular, particularly those from thermophiles (Iqbal *et al*., 1994).

## 1.9. Variation among spectrophotometric peroxidase assays

Spectrophotometer peroxidase assays are standardized as far as possible for use. Typically, a final volume of 1.0 ml reaction mixture typically contains 100 mM potassium phosphate buffer (pH 7.0), 50 mM hydrogen peroxide, 200 µl of culture supernatant and 200 µl of peroxidase substrate. Assay reactions are initiated by addition of 200 µl of hydrogen peroxide, changes in absorbance are monitored with a spectrophotometer (at 510nm). One unit of enzyme activity is defined as that producing an increase of absorbance of 1.0 absorbance unit per minute.

The following procedures are normally employed:

### 1.9.1. 2, 4- Dichlorophenol assay:

In this assay procedure, which is mostly employed for peroxidase activity, the reaction mixture (1 ml) contains equal volumes (0.2 ml) of potassium phosphate buffer (100 mM, pH 8), 2,4- DCP (25 mM), 4- aminoantipyrine (16 mM), peroxidase- containing sample and hydrogen peroxide (50 mM). The reaction is normally initiated by addition of $H_2O_2$ and monitored for 1 min at 510 nm. One unit of peroxidase activity is also defined as amount of enzyme required for an increase in absorbance of 1 U $min^{-1}$ (Iqbal *et al*., 1994).

### 1.9.2. Phenol red manganese dependent peroxidase assay:

In this assay a final volume of 5.0 ml of reaction mixture contains 100 mM potassium phosphate buffer (pH 7.0), 0.1 mM $MnSO_4$ , 0.1 mM phenol red, 50 mM $H_2O_2$ and 1.0 ml of concentrated culture supernatant. The reaction again is initiated by addition of $H_2O_2$. At 1 min interval a 1.0 ml sample is removed and then added to 40 ml of 5M NaOH to stop the reaction then the $A_{610}$ is measured (Mercer *et al*. 1996). Mercer *et al*. (1996) reported an extensive study of screening of

actinomycetes for extra cellular peroxidase activity (with prior ultra filtration concentrating of culture supernatants). Concentration of culture supernatants was found necessary as the assay was not sufficiently sensitive to detect low levels of peroxidase activity present in culture supernatants.

A total of 10 conventional peroxidase assays were evaluated with extra cellular enzyme preparations from two *Streptomyces* - strain EC 22 and *S. thermoviolaceous*. Blank assays (absence of hydrogen peroxide) were performed in parallel to demonstrate peroxidase dependency of reaction. With phenol red as substrate, *Streptomyces* strain EC 22 peroxidase activity was significantly higher in the absence of manganese, while vanillylacetone oxidation was stimulated by manganese to a comparable degree. The converse was true for *S. thermoviolaceus*, although the effects were less pronounced. These authors (Mercer *et al*., 1996) also concluded that peroxidase- dependent oxidation of 2,4- DCP assay procedure gave the best combination of optimal activity and reproducibility (Table 1.6).

Also L- DOPA, pyrogallol and guaiacol peroxidase assays resulted in low values of activity for *Streptomyces* EC22 and due to gas bubbles generation these assays could not be applied to *S. thermoviolaceus.* Conclusions were drawn from the study that 2,4- DCP was the more suitable assay probably due to that substrate stablility at extremes of pH and temperature.

**Table 1.6: Extracellular peroxidase activities of *Streptomyces* strain EC22 and *S. thermoviolaceus* enzymes assayed with a range of spectrophotmetric procedures.**

| Peroxidase assay | *Streptomyces* strain EC22 | | *S. thermoviolaceus* | |
|---|---|---|---|---|
| | Mean Peroxidase Activity (U $ml^{-1}$)[a] | SD | Mean Peroxidase activity (U $ml^{-1}$) | SD |
| 2,4- DCP | 0.51 | <0.01 | 0.59 | 0.12 |
| L- DOPA | 0.17 | 0.07 | NA[b] | NA |
| Phenol red + $Mn^{c}$ | <0.01 | <0.01 | 0.10 | 0.01 |
| Phenol red | 0.04 | 0.01 | 0.07 | 0.02 |
| Pyrogallol | 0.27 | 0.01 | NA | NA |
| Vanillylacetone | 0.01 | <0.01 | 0.13 | 0.05 |
| Vanillylacetone + $Mn^{+2}$ | 0.06 | 0.01 | 0.09 | 0.06 |

a For Peroxidase calculation. Data are means of three separate determinations.
b NA, not applicable. The production of gas bubbles in the cuvette prevented accurate measurement of absorbance changes.
c $Mn^{+2}$,with addition of 0.1 mM $MnSO_4$.
Source: Mercer *et al*. (1996).

Ramachandra *et al*. (1987) first reported oxidative enzyme peroxidase activity by *Streptomyces* in solid state fermentation.

Zimmermann (1991) in studies with other *Streptomyces* strains also showed that cell-free extracts could solubilise a variety of lignocelluloses substrates from fractionation of solubilised radiolabelled product from the incubation of culture filtrate from *Streptomyces* with wheat lignocellulose showed 45% of total [$^{14}C$] lignocellulose solubilised in the acid-soluble form, 0.1 % present in the lignocellulose.

## 1.10. Aims of the study

It has been known for years that certain fungi are capable of decomposing lignocellulose. However the roles that non-fungal microbes, specifically actinomycete bacteria, play in lignocellulose decomposition remain to be fully defined. Further, actinomycete lignocellulose degrading systems are at an earlier stage of study.

There has been enhanced interest in recent years in the phylogenetic framework development for actinomycetes, especially *Streptomyces*, as a rapid comparison of isolates to well defined species facilitates decision making as to whether isolates should be included in the screening programmes for industrially interesting enzymes.

Most of the phylogenetic analysis reported previously relates to general strains of *Streptomyces* rather than the complex substrates like lignocellulose decomposing isolates on which this study focused.

Hence this study was conducted to develop a culture screening and PCR- based method for the detection of Streptomycetes capable of degrading lignocellulose. Characterisation of *Streptomyces* isolated from lignocellulosic material like quack-grass (*Agropyron repens* L.) was emphasized.

# Materials and Methods

## 2.1. Sample collection and processing:

Two soil samples were collected from the woodland area of Strathclyde University campus in Glasgow, Scotland. After removing approximately 3 cm soil from the surface, samples were taken to a depth of 10 cm (displacing the soil further at a depth of 10 cm). Soils were air-dried at ambient for 7 days to reduce numbers of non-spore forming bacteria and soil samples were crushed, mixed thoroughly and large debris were removed to get fine soil particles, these samples were used for isolation of *Streptomyces*.

## 2.2. Selective isolation medium:

Soils (1g) sample were suspended in 9 ml saline (0.85% NaCl) dilution blank and vortexed for 1 min. The rationale behind use of saline dilution was that mostly bacteria are unable to grow at neutral pH and *Streptomyces* are well known to be comparatively salt tolerant (Hunter-Cevera *et al*., 1986). The suspension was serially diluted to $10^{-3}$ using the other saline dilution blanks. Aliquots (0.2 ml) were plated on to minimal salt agar medium containing ground mesh grass (*Agropyron repens,* 1.0%) as a carbon source and vitamin free casamino acids (0.6%) as a supplemented nitrogen source. In order to get homogenous suspension ground mesh grass was used. To minimize moulds and eu- bacteria , media were supplemented with filter sterilized rifampicin (1 μg $ml^{-1}$) and to combat more rapidly growing fungi filter sterilized cycloheximide and nystatin (each of 25 μg $ml^{-1}$) were also added to the media. *Streptomyces* are resistant to the antimicrobial compounds so probability of isolation in the chosen medium could be extremely high (Pisano *et al*., 1989).

The minimal salts-casamino acid medium contained (in g $l^{-1}$ ):

Vitamin free casamino acid, 6; $(NH_4)_2SO_4$, 0.1; NaCl, 0.3; $MgSO_4$, 0.1; $KH_2PO_4$ , 0.1; $K_2HPO_4$, 0.1 and 1 ml of trace-elements solution. The trace-elements solution contained (in g $l^{-1}$): $FeSO_4$ , 1; $ZnSO_4$, 0.9; $MnSO_4$, 0.2.

Inoculated plates were incubated at 30 $^0$C for 10 to 14 days. Plated dilutions that gave 20-200 colonies were chosen for further isolation. Different *Streptomyces*

colonies were purified by restreaking on to the same medium. All isolates cleared grass agar to varying degrees within 7 to 14 days. After incubation, typically pigmented, dry, powdery colonies were selected from mixed plate culture and maintained on yeast extract malt extract agar (ISP2) and inorganic salt starch agar (ISP4) plates which were incubated at 30 $^{O}$C for 7 to 10 days. Media plates were examined by eye and aerial spore mass colour, substrate mycelium pigmentation and colour of any diffusible pigments recorded. The compositions of various media used for cultural characteristics are shown in Table 2.1.

**2.3. Strains characterization:**

Characterization of selected isolates was performed using criteria recommended by International Streptomyces Project (Shirling and Gottlieb, 1966). Strains were identified as *Streptomyces* sp. on the basis of the information on actinomycete genera in Bergey's Manual of Systematic Bacteriology (Williams *et al*., 1989; Cross and Lechevalier, 1994). Stock cultures of *Streptomyces* isolates were maintained as a suspension of spores in 20% (v/v) glycerol at -20 $^{0}$C and routinely (3 - 5 week intervals) subcultured on plates or slants containing YEME medium (Table 2.1) at 30 $^{0}$C for 72-96 h.

**2.3.1. Micro-morphological examination:**

The ISP-4 medium was used for culture growth of isolates. Sterilized coverslips were carefully inserted at an angle of about 45 degree in to solidified medium in a Petri dish, until about half of the cover slip was buried in medium. Isolates were inoculated along the line where media meet the upper surface of cover slip. After incubation for 7 - 10 days, the cover slip was carefully removed and placed downwards on the slide and directly examined under the phase contrast microscope (Williams and Cross, 1971). Purified isolates were analysed by comparing their morphological characteristics:

- Spore- bearing hyphae
- Colour of the aerial mycelium
- Colour of the soluble pigments

**Table 2.1. The media compositions used for cultural characteristics**

| Medium (g $l^{-1}$) | 1* | 2* | 3* | 4* | 5* |
|---|---|---|---|---|---|
| Starch | | | 10.0 | | |
| Glycerol | | | | 10.0 | |
| Oatmeal | | 20.0 | | | |
| Yeast extract | 4.0 | | 4.0 | | |
| Malt extract | 10.0 | | 10.0 | | |
| Dextrose | 4.0 | | 4.0 | | |
| L-asparginine | | | | 1.0 | |
| Sodium chloride | | | 1.0 | | |
| $K_2HPO_4$ | | | 1.0 | 1.0 | 5.65 |
| $MgSO_4$ | | | 1.0 | | 1.0 |
| $CaCO_3$ | | | 2.0 | | |
| $(NH_4)_2SO_4$ | | | 2.0 | | 2.64 |
| $KH_2PO_4$** | | | | | 2.38 |
| $FeSO_4$** | | 0.1 | 0.1 | 0.1 | 0.11 |
| $CuSO_4$** | | | | | 0.64 |
| $MnCl_2$** | | | 0.1 | 0.1 | 0.79 |
| $ZnSO_4$** | | 0.1 | 0.1 | 0.1 | 0.15 |

* Medium 1, Yeast extract-malt extract agar (ISP-2) ; Medium 2, Oatmeal agar (ISP-3); Medium 3, Inorganic salt starch agar (ISP-4); Medium 4, Glycerol asparagine agar (ISP-5); Medium 5, Basal mineral salts agar. ** Trace salt solution (Shirling and Gottlieb, 1966)

**Table 2.2. Test attributes used for numeric phenotypic data analysis***

| Unit Characters | Cultural condition | |
|---|---|---|
| | Submerged condition | Agar plate |
| **Morphology and pigmentation:** | | |
| Rectiflexibiles | | + |
| Retinaculam-apertum | | + |
| Aerial mycelial pigments | | + |
| Substrate mycelial pigments | | + |
| Production of diffusible pigments | | + |
| Melanin formation | + | |
| **Degradation of:** | | |
| Casein | | + |
| Xylan | | + |
| Starch | | + |
| **Growth at :** | | |
| 45 $^{0}$C | | + |
| **Growth in the presence of (% w/v, v/v)** | | |
| NaCl (4) | | + |

| | |
|---|---|
| NaCl (7) | + |
| Phenol (0.1) | + |
| **Growth on sole carbon source ( 0.1 %, w/v):** | |
| D-Glucose | + |
| D-Xylose | + |
| D-Fructose | + |
| L-Rhamnose | + |
| Sucrose | + |
| Raffinose | + |
| L-Arabinose | + |
| D-Mannitol | + |
| Dextran | + |
| **Growth on sole nitrogen source ( 0.1 %, w/v):** | |
| L-Asparagine | + |
| L-Histidine | + |
| L-Throenine | + |
| L-Methionine | + |
| L-Phenylalanine | + |
| L-Hydroxyproline | + |
| Potassium nitrate | + |

* PIB programme (Bryant, 2004); + = cultural condition employed

### 2.4. Numerical Taxonomy:

Each strain was examined in duplicate for (29) unit characters. Inocula were prepared from strains grown on ISP-2 for 7 days at 30 $^0$C. It is important to use characters that are genetically stable and not overtly sensitive to experimental uncertainties. Sackin and Jones (1993) recommended at least 25 unit characters but preferably several tests are needed to the effort involved in screening data (Sackin and Jones, 1993).

#### 2.4.1. Degradation tests:

Degradations of starch (1.0%), xylan (0.4%) and casein (1.0%) were determined in modified Bennett's agar after 7 days (Williams *et al*., 1983). Areas of clearance under and around the growth were recorded. Degradation of starch was observed by flooding plates with iodine solutions and scoring zone of clearance.

#### 2.4.2. Physiochemical tests:

The utilization of carbon sources was carried out according to the method of Pridham and Gottlieb (Shirling and Gottlieb, 1966)) with addition of sugars: D-glucose (positive control), L-arabinose, sucrose, D-xylose, D-mannitol, D-fructose, rhamnose, raffinose, dextran and in absence of a carbon source (negative control). A positive result was recorded when growth was greater than of the negative control.

Similarly the capacity of organisms to use 7 compounds as sole nitrogen sources was determined by the method of Williams *et al*. (1983). Growth was scored after 7 days by comparison with positive (basal medium supplemented with L-asparagine (1.0%) and negative (basal medium alone) controls. Growth greater than that of negative control was scored as positive and that to or less than on the negative control as negative.

#### 2.4.3. Tolerance tests:

Isolates were examined for their ability to grow on Bennett's agar supplemented with chemical inhibitors at various concentrations (4% NaCl, 7% NaCl and 0.1% Phenol). Tests were read after 4 and 7 days. Strains were scored as resistant (+) when growth on the test plates was greater or equal to that on positive control plates lacking inhibitors. Visible growth was scored as a positive result (Sahin *et al*., 2002).

### 2.4.4. Melanin formation:

Melanin formation was tested on peptone yeast extract iron agar and in submerged conditions (Arai and Mikami, 1971; Tadashi and Mikami, 1972).

10 ml of suitable liquid media were dispensed in flasks and inoculated with one loop full of spores of *Streptomyces* and subjected to stationary culture at 30 $^0$C for 7 days. Melanin pigment was estimated by taking 2 ml of culture and 1 ml of 0.4% substrate solution (L-dopa). The reaction mixture was incubated at 37 $^0$C for 30 minute for L-tyrosine and 10 min for L-dopa and reddish-brown colouration resulting from dopachrome formation was observed and read spectrophotometrically at 480 nm (Scribners *et al*., 1973).

#### 2.4.4.1. Effect of carbon sources on melanin formation:

The basal medium of the following composition (g ml$^{-1}$) was used with 1% glycerol, starch, glucose, sucrose, lactose or fructose as the sole carbon source.

| | |
|---|---|
| $NaNO_3$ | 2.0 |
| $K_2HPO_4$ | 1.0 |
| $MgSO_4$ | 0.5 |
| KCl | 0.5 |
| $FeSO_4$ | 0.01 |

#### 2.4.4.2. Effect of nitrogen sources on melanin formation:

The effect of nitrogen sources (glutamate, arginine, asparagine, threonine, histidine or ornithine) on the pigment production (melanin) was studied with same basal medium using 1% glycerol as carbon source. All carbon and nitrogen sources were prepared in 10% solution, sterilized with filters and added to basal medium to give final concentration of 1%.

### 2.5. Coding of the data:

Nearly all of the characters existed in one of two mutually exclusive states and were scored positive (+) or negative (-). Qualitative multistate characters such as certain pigmentation and morphological tests were coded as several independent characters and scored plus for character state shown and minus for all alternatives.

#### 2.5.1. Computation:

The binary test data were typed in a +/- format as input to the PIBwin programme (Bryant, 2004) using the simple matching coefficient ($S_{SM}$). Computer assisted identifications are based on Wilcox's implementation of Bayes theorm (Wilcox *et al*., 1973).

Wilcox probability is the likelihood of unknown strains (u) against taxon j divided by the sum of the likelihood of u against all q taxa, i.e.,

**Wilcox probability = $L_{uj}$ / $\square^{q} L_{uj}$** (Wilcox *et al*., 1973)

Where,

Lu = Likelihood of unknown strain

j = individual cluster

q = all clusters

The software is designed to use probabilistic identification matrices that have published in the literature (Langham *et al*., 1989). With the Wilcox probability a score approaching 0.95 indicates a high likelihood of identification; low scores for taxonomic distance (less than 0.3) indicates relatedness (Wilcox *et al*., 1973).

Initially selection of reduced number of characters was carried out by deleting those with no or poor separation values. The software was equipped to screen the distinguishing tests for separation.

Major steps and software tools used in the analysis of numerical phenotypic data are shown in Figure 2.1

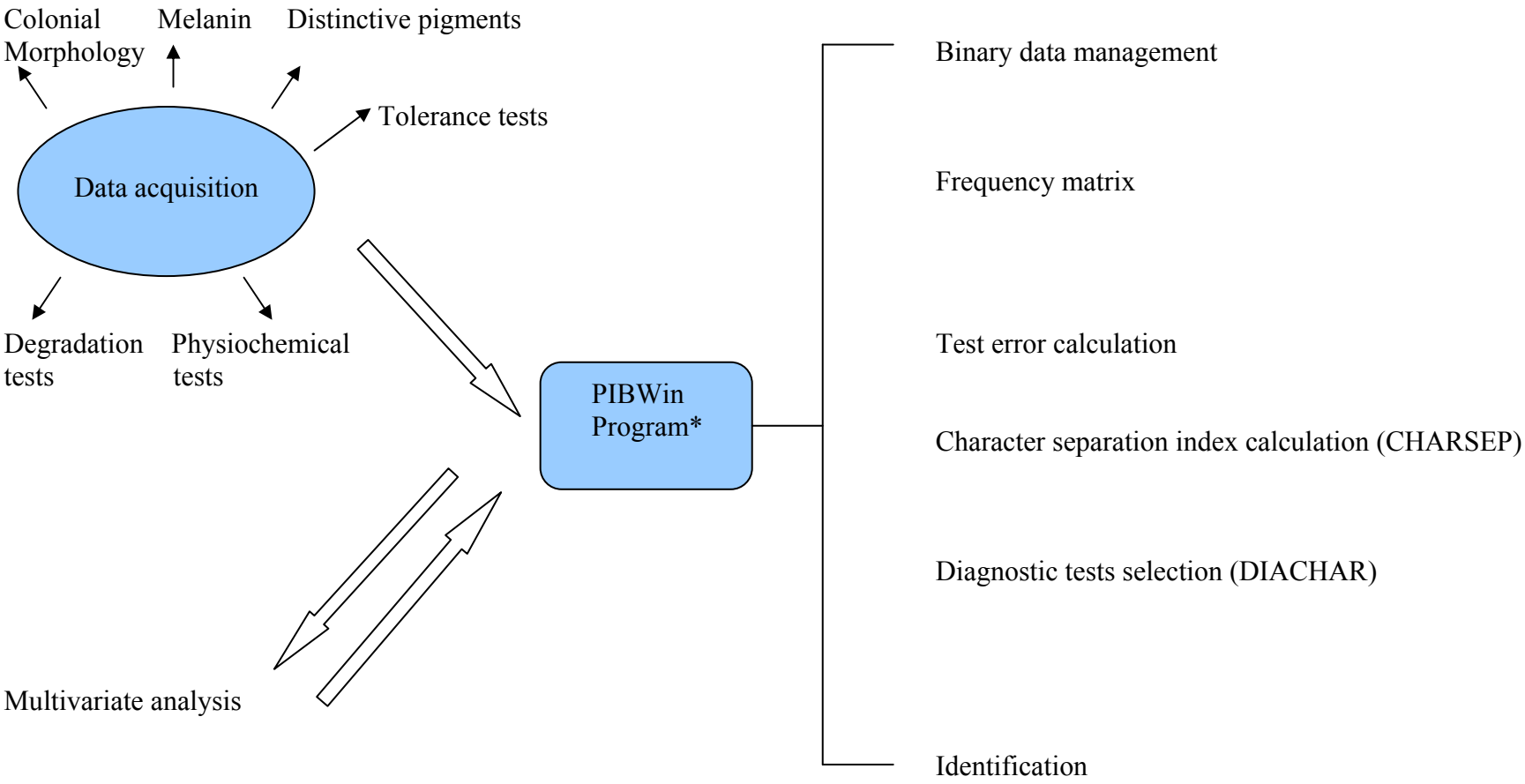



**Fig. 2.1. Major steps and software tools utilized in numerical data analysis (Modified from Chun, 1995). * PIBWin software (Bryant, 2004)**

## 2.6. Lignocellulose substrate:

The following lignocellulosic materials were used for lignin peroxidase assay:

a) Grass (*Agropyron repens* L.)

b) Corn (*Zea mays* L.)

Lignocellulosic materials were chopped and milled in a rotating ball- milled apparatus for 24 h. A fine powder was obtained after sieving ball- milled straw. Ground powder was washed twice with deionised water and once with phosphate buffer (pH 7.0) to remove soluble reducing sugars and soluble residual lignin. The samples were dried overnight at 70.0 $^{O}$C (Tuncer and Ball, 2002).

### 2.6.1. Degradation of lignocellulosic materials by enzymes:

Enzyme production was carried out in submerged conditions.

For estimation of substrate concentration effects on extra cellular lignocellulose degrading enzyme production, pH and incubation time, distilled water suspensions of sporulating growth were used to inoculate 250 ml flasks containing 50 ml of minimal salts- yeast extract nutrient medium (pH 7.5), supplemented with different concentrations of corn straw and grass between 0.2 - 1.0 %. Cultures were incubated for up to 16 days. Cultures were centrifuged at 10,000 *g* for 10 min and culture supernatants were used in enzyme assays (Tuncer *et al*., 2004).

For determination of effect of incubation time on peroxidase production 0.6% each of corn and grass was used in assay. The same concentration (0.6%) was used to determine the effect of pH on peroxidase production.

The culture supernatants from all nine isolates grown on minimal salts- yeast extract media supplemented with corn and grass (concentration of 0.2 – 1.0%) were used for peroxidase production.

### 2.6.2. Harvesting of culture supernatants:

Culture supernatants were harvested by centrifugation at 12,000 *g* for 15 min at 4 $^{O}$C of a 72 h culture of *Streptomyces* spp. grown in the basal mineral salts medium described above. These culture supernatants were used as crude enzyme preparations for peroxidase activities.

## 2.6.3. Enzymes assays:

### 2,4- Dichlorophenol assay:

This assay procedure was employed for peroxidase activity determinations. A final volume of 1ml of the reaction mixture was used for the assay. The reaction mixture contained equal volumes (0.2 ml) of potassium phosphate buffer (100 mM, pH 8), 2,4- DCP (25 mM), 4- aminoantipyrine (16 mM), sample and hydrogen peroxide (50 mM). The reaction was initiated with addition of $H_2O_2$ and monitored for 1 min at 510 nm. One unit of peroxidase activity was defined as enzyme required for an increase in absorbance of 1 U $min^{-1}$ (Iqbal *et al*., 1994).

## 2.7. Molecular methods:

16S rRNA sequencing method based on PCR was used for all nine isolated strains. The quality of 16S rRNA sequence data was assessed by comparing nucleotide sequences in the Gene Bank/ EMBL database.

Phylogenetic lineages were determined based on nucleotide sequences data of all nine isolated strains by using the MEGA software (Tamura *et al*., 2007).

### 2.7.1. DNA extraction

Streptomycetes were grown in 50 ml International *Streptomyces* Project medium 2 (ISP 2) (Shirling and Gottlieb, 1966) with agitation at 30 $^0$C for 5 days. Kirby mix procedure (Kieser *et al*. 2000) was employed with little modification. Cells were harvested by centrifugation (3500 x *g* ,10 min) washed once with TE25S buffer and stored at -20 $^0$C for overnight to get physical disruption of cells. Mycelium was resuspended in 3 ml TE25S buffer, 100 µl of lysozyme solution (2mg $ml^{-1}$) and 31µl of RNase (40µg $ml^{-1}$) was added and incubated at 37 $^0$C for 1 hr. Then 300 µl of SDS solution (10%) was added and incubated at 37 $^0$C for 2 h. Cell debris was removed by centrifugation (3500 x *g*, 20 min) and the supernatant was extracted once with chloroform: isoamylalcohol (24:1 v/v) and once with buffered phenol.

After centrifugation, the aqueous phase was collected in a clean tube and the DNA was precipitated by adding 0.6 volume of isopropanol at ambient. Spooled DNA onto

a sealed Pasteur pipette, washed in 1 ml of ice-cold ethanol (70%), dissolved in TE (100 µl) and stored at -20 $^0$C.

**2.7.2. Polymerase Chain Reaction:**

**2.7.2.1. Primer design**

PCR primers targeting the *Streptomyces* 16S rRNA gene were designed. *Streptomyces* 16S rRNA gene sequences were obtained from public databases (http://www.ncbi.nlm.nih.gov). Primers were designed by selecting sequences homologues among *Streptomyces*. The specificity of the potential primers was determined by comparing sequences against EMBL sequence databases (http://www.ebi.ac.uk/embl) using the FASTA algorithm. Primer candidates were evaluated for predicted PCR performance using an automated search feature of Primer 3® v.0.4.0 package (Rozen and Skaletsky, 2000) that eliminates oligonucleotides with predicted adverse primer performances.

**2.7.2.2. PCR amplification of 16S rRNA gene:**

PCR reactions were carried out in 0.2 ml reaction tubes in a total volume of 50 µl. One positive control (*S. rimosus*) and one tube containing no nucleic acid were included in PCR amplification. *Streptomyces* sp. 16S rRNA gene fragments were amplified from chromosomal DNA isolated from pure cultures of *Streptomyces* isolates (513-1 to 513-9).

The reaction mixture was prepared as follows:

| | |
|---|---|
| Sterilized d. $H_2O$ | up to 50µl |
| DMSO | 5 µl |
| $MgCl_2$ (2mM) | 4 µl |
| Buffer (x 10) | 10 µl |
| dNTP mixture (150 µl of each dNTP) | 8 µl |
| Primer L | 1 µl |
| Primer F | 1 µl |
| Template DNA | 1 µl |
| Taq Polymerase (2.5 U) | 0.25 µl |

The cycling conditions of the PCR programme were as follows:

| Steps | | Temperature | Time |
|---|---|---|---|
| Initial denaturation (1 cycle) | | 94 $^0$C | 5 min |
| Main amplification (29 cycles) | | | |
| | Denaturation | 94 $^0$C | 30 sec |
| | Annealing | 54.8 $^0$C | 50 sec |
| | Extension | 72 $^0$C | 1.30 min |
| Final extension (1 cycle) | | 72 $^0$C | 7 min |
| Cool down | | 4 $^0$C | continuous |

Primers F(5'-TGAGTAACACGTGGGCAATC-3') and primer R(5'-TTCGGGTGTTACCGACTTTC-3') were used to amplify nearly full length (1484bp) 16S rRNA sequences.

Amplified products of each reaction were analysed by electrophoresis for 60 min at 80V in agarose (1.0 %) gels containing 1µg ml$^{-1}$ ethidium bromide to ensure that a fragment of the correct size had been amplified.

**2.7.3. DNA sequencing:**

The PCR products obtained from pure cultures were purified with the QIAquick column extraction kit (Qiagen) and directly sequenced using primers F and R. The sequencing was performed in the Molecular Biology Lab, Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow.

**2.7.4. DNA sequence alignment and Phylogenetics:**

The DNA sequences were compared to other prokaryotic sequences in the Ribosomal database project with the FASTA programme available from the RDP homepage (http://www.rdp.cme.msu.edu). Sequences obtained were aligned with the 16S rRNA sequences of *Streptomyces* type strains obtained from databases by using MEGA software programme (Tamura *et al*., 2007). The evolutionary tree, rooted with an

actinomycete *Nonomuraea* as the outgroup was inferred by using the neighbour joining method (Saitou and Nei, 1987) from the evolutionary distance data corrected by Kimura's two parameter model (Kimura, 1980). The clustal X program (Thompson *et al*., 1994) was used for multiple alignment and phylogenetic analysis. Bootstrap analysis (500 replicates) was putforward to assess validity of obtained tree and to compute standard error of distance estimates by using the program MEGA version 4 (Tamura *et al*., 2007). The entropy plot for estimation of variable regions was generated by using BioEdit software (Hall, 1999).

## 2.8. Statistical analysis:

The statistical program Minitab 15 (Minitab Inc., 2007) was used for comparative analysis (Numeric phenotypic data). In order to establish similarities among the isolated strains and reference strains from Williams *et al*. (1983) a comparison was made. The test statistic was as:

**S (ij) = 100 (1- d (ij) / d (max)**

Where as S (ij) is similarity between two clusters i and j and d (max) is the maximum value in distance matrix D.

To assess statistical significance, results were subjected to analysis of variance (ANOVA) and Tukey post hoc test comparison of the means, $P < 0.05$, using Minitab 15 (Minitab Inc., 2007).

# RESULTS

## 3.1. Selective isolation of lignocellulose degrading *Streptomyces*:

Three soil samples from Toryglen and Govanhill region of Glasgow were used as source of *Streptomyces* isolates. Plating to establish the pattern of growth on surface colonies immensely facilitates identification of organisms and comparisons of isolates. Bacterial and fungal contamination in isolation media is a common problem and several strategies were employed such as pre-treatment of source samples and additions of specific antibiotics to isolation medium. In the present study, serial dilutions were made in saline (0.85%) water and poured on selective media containing rifampicin (1.0µg $ml^{-1}$) and cycloheximide and nystatin each of 25µg $ml^{-1}$ as antibacterial and antifungal agents respectively.

Isolations were made from soil samples plated on to a minimal salt medium containing ball-milled grass as a carbon source (lignocellulosic material). From original plates of isolation different colonies of *Streptomyces* isolates were purified by restreaking on to the same above medium.

Hence it was possible to get Streptomycetes colonies on the isolation plates in an almost exclusively well grown state (Figure 3.1).

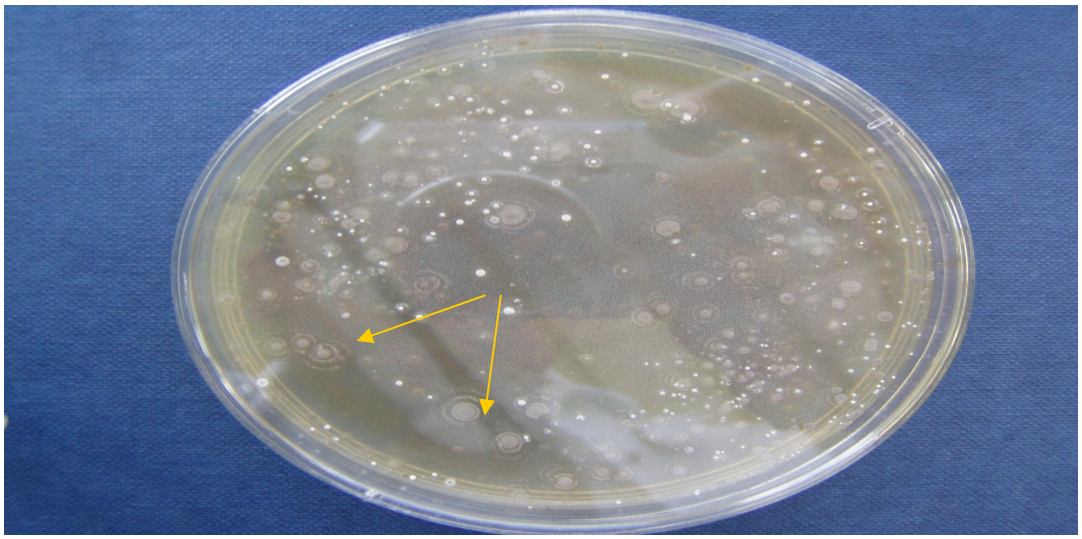
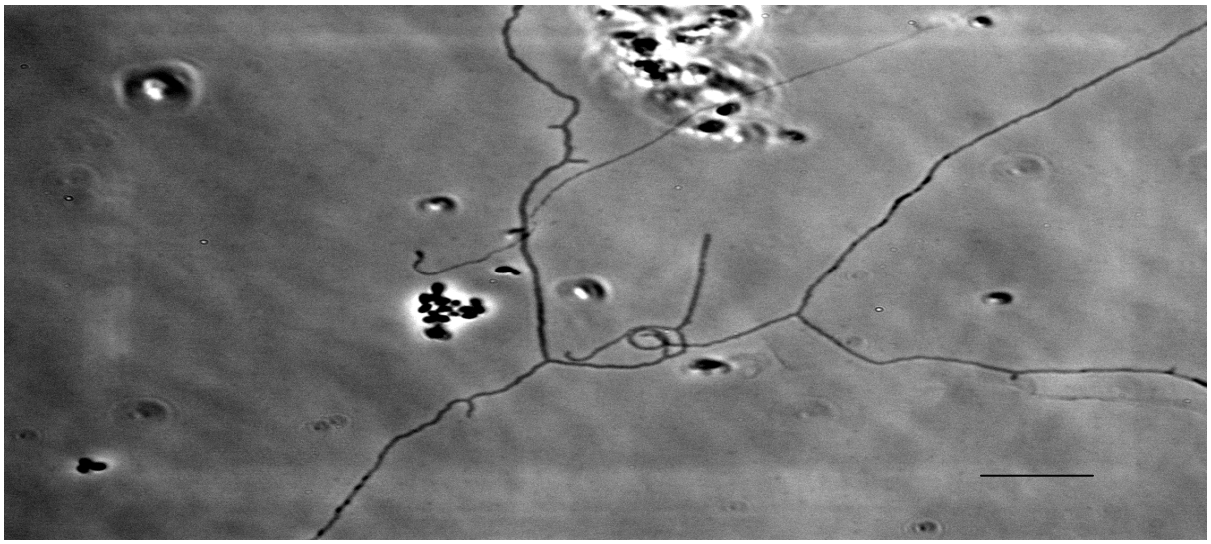


Figure 3.1: Isolation plate after 10 d incubation at 30 $^{0}$C. Arrows clearly indicate the haloes (zone of clearing) formation along the colonies

### 3.2. Micro morphological characteristics:

Fine structures were observed by using phase contrast microscope. Mycelium of all the isolates formed monopodially branched spore- bearing hyphae with the shape of rectiflexibiles (RF) chains but in some cases minor loops were also observed. Spores were found to be round to ellipsoidal in shape. ISP-2 medium was utilised for micromorphological studies. Mostly branched aerial mycelium with short chains of spores were observed. Mostly straight and flexous nature of sporophores (sporogenous hyphae) were observed so the strains may be placed in the 'rectus-flexibilis' group of *Streptomyces* species. But in some cases like in isolate 513-1 loops were also observed so the inclusion of the 'retinaculum-apertum' group could be possible. Although branching nature of mycelia were observed, no septation in the branching pattern was found (Figure 3.2).

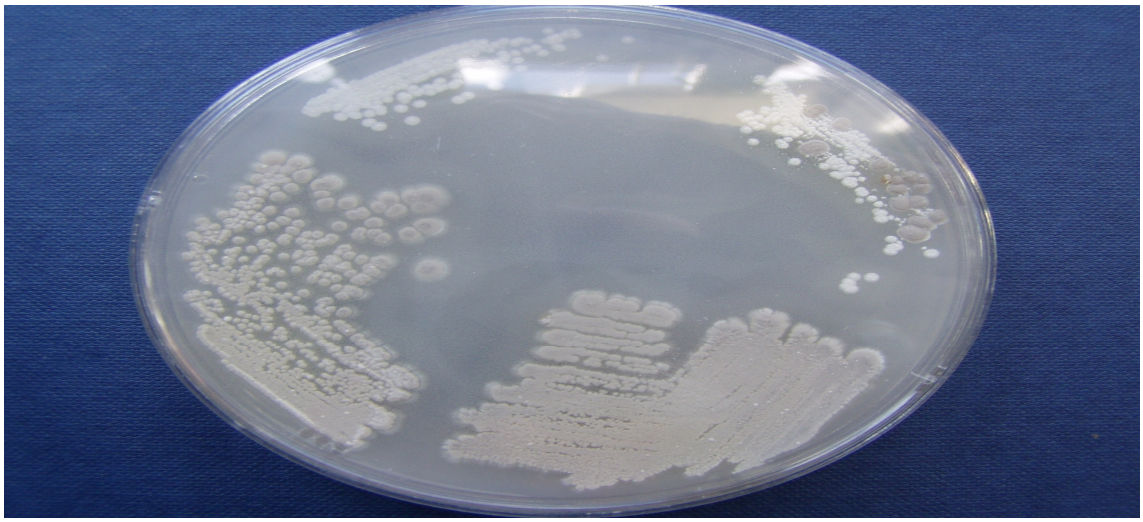


a

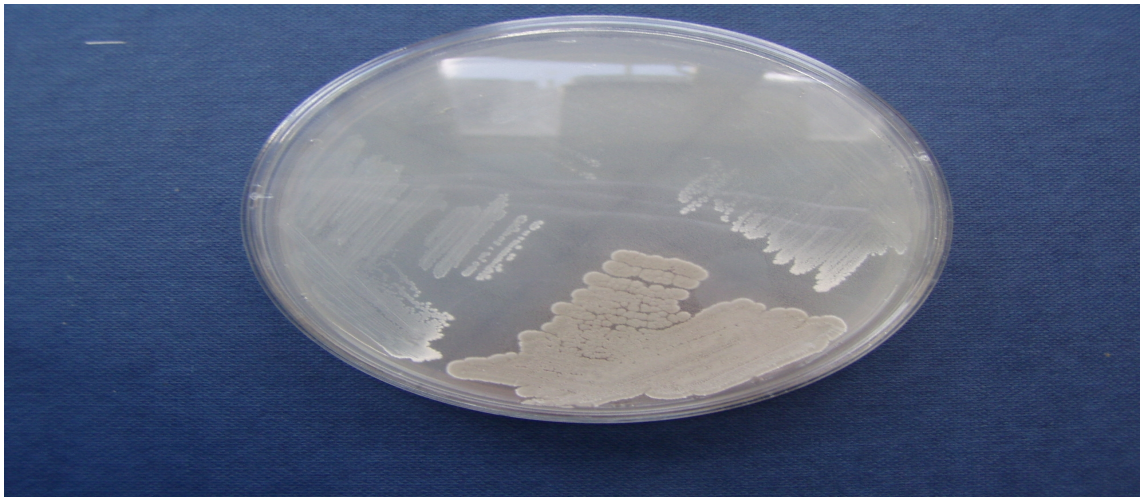
Figure 3.2:Phase contrast micrograph of spore chains of isolate 1 (100 x), bar =10µm

### 3.3. Cultural characteristics:

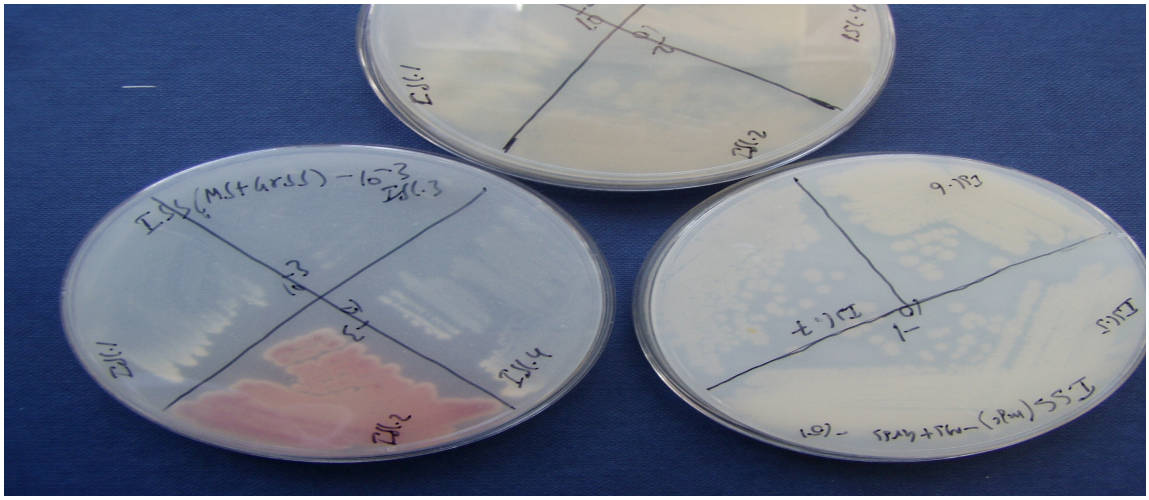
Cultural characteristics of the strains (513-1 to 513-9) were studied with different media recommended by the International Streptomycetes project (Shirling and Gottlieb, 1968) are summarised in Tables (3.1-3.9). All isolates depicted morphology typical of *Streptomyces* on various agar plates and showed polymorphic nature of growth. Different patterns of appearance were observed on various media. Differences of the colonial appearance were observed in profile (raised or flat), shape (ellipsoidal or round), edges (lichenoid or undulating), surface (wrinkled or smooth). For most isolates, colonies were completely covered by aerial mycelium but concentric rings were also observed in some cases. Good growth of the isolates was observed on ISP-2, ISP-3 and ISP-4 while moderate growth was observed on ISP-5 (Figure 3.5). Production of pigment differed with the culture media employed. Also the reverse side of colonies showed different characteristics as compared to aerial mycelia (Tables 3.1-3.9).



a

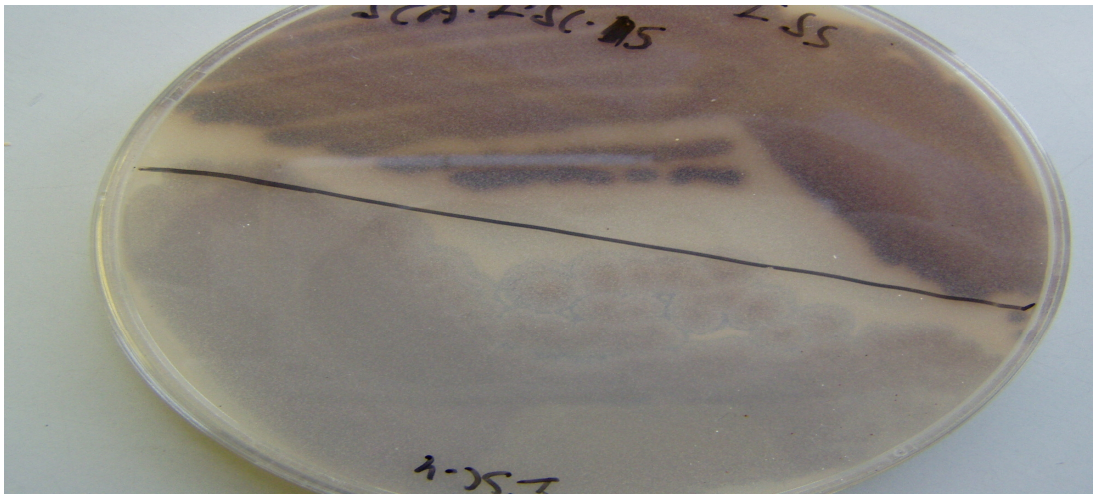


b

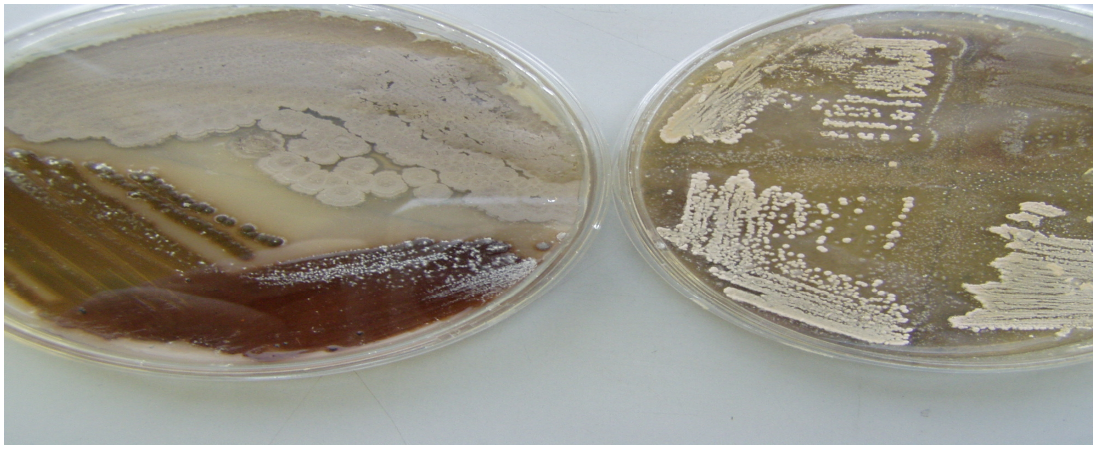


c

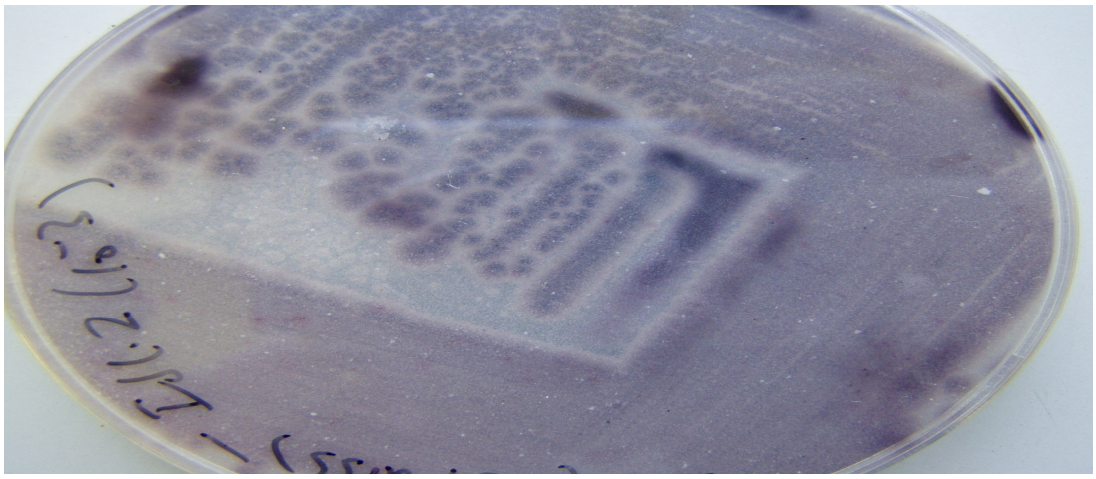
Figure 3.3: Colonial morphology of isolates on ISP4 medium. a, isolate1(aerial mycelium); b, isolate 1(substrate mycelium); c, isolate 2 (top plate), isolate 3(right bottom, aerial mycelium), isolate 3(left bottom, substrate mycelium).



d

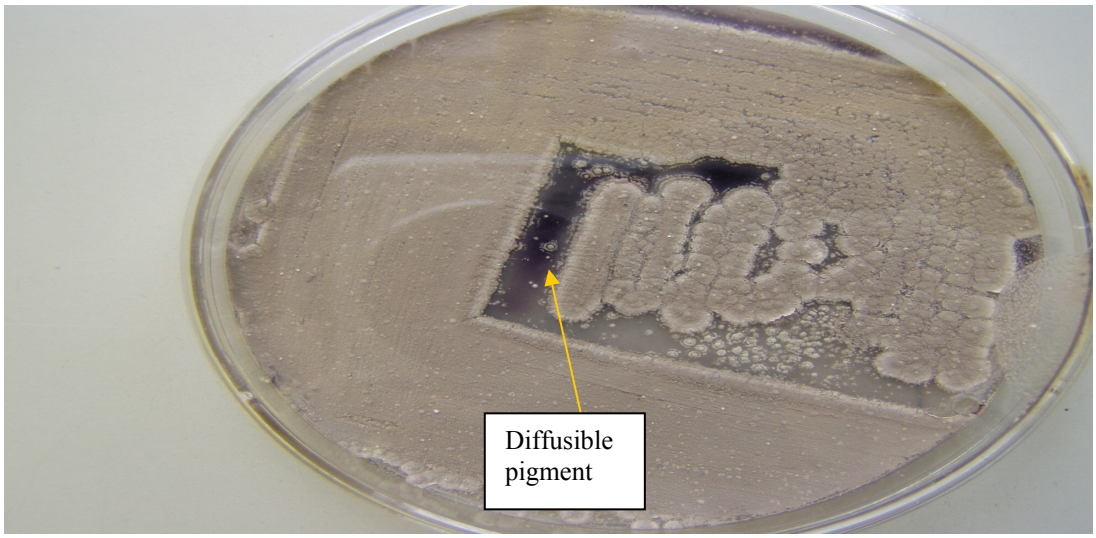


e

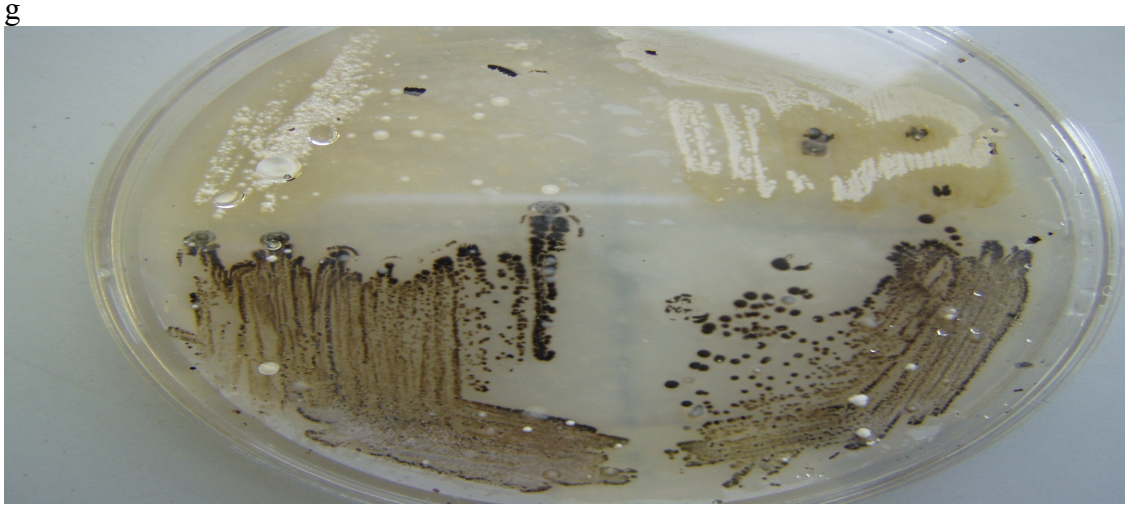


f

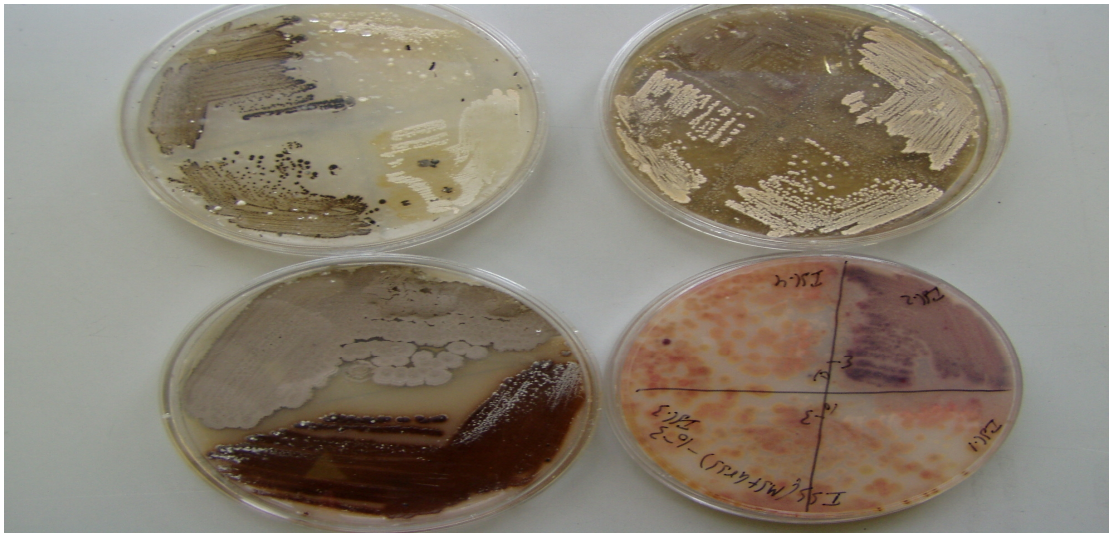
Figure 3.4: Colonial morphology of isolates on ISP4 medium. d, isolate 4; e, isolate 5 (right plate, aerial mycelium; left plate, substrate mycelium); f, isolate 6.




g



h



i

Figure 3.5: Colonial morphology of isolates on ISP4 medium. g, isolate 7; h, isolate 8 and 9 (top of plate isolate 8, bottom of plate isolate 9); i, isolates on minimal medium supplemented with grass.

**Table 3.1. Cultural characteristics of isolate 513-1 on different media**

| Medium | Growth | Spore chain morphology* | Age(days) | Colour of aerial mycelium | Colour of substrate mycelium | Colour of soluble pigments |
|---|---|---|---|---|---|---|
| ISP-2 | Abundant | RA | 7 | White | – | – |
| | | | 14 | White | Grey | – |
| | | | 21 | Whitish grey | Dark grey | – |
| ISP-3 | Abundant | – | 7 | Whitish grey | – | – |
| | | | 14 | Grey | Grey | – |
| | | | 21 | Olive grey | Grey to black | Dark olive |
| ISP-4 | Abundant | – | 7 | White | – | – |
| | | | 14 | Whitish grey | Grey | – |
| | | | 21 | Whitish grey | Leaded grey | – |
| ISP-5 | Moderate | – | 7 | Whitish olive | Grey | – |
| | | | 14 | Whitish olive | Grey | – |
| | | | 21 | Greyish olive | Greyish violet | Olive |

Legend: ISP-2, Yeast extract malt extract; ISP-3, Oatmeal agar; ISP-4, Inorganic salt starch;
ISP-5, Glycerol asparagine agar; *;Only observed on ISP-2;-, absent; Triplicate plates were used for each observation

**Table 3.2. Cultural characteristics of isolate 513-2 on different media**

| Medium | Growth | Spore chain morphology* | Age(days) | Colour of aerial mycelium | Colour of substrate mycelium | Colour of soluble pigments |
|---|---|---|---|---|---|---|
| ISP-2 | Abundant | RA | 7 | White | – | – |
| | | | 14 | White | – | – |
| | | | 21 | Whitish grey | Dark grey | – |
| ISP-3 | Abundant | – | 7 | Whitish grey | – | – |
| | | | 14 | Grey | – | – |
| | | | 21 | Olive grey | – Grey to black | – Olive |
| ISP-4 | Abundant | – | 7 | White | – | – |
| | | | 14 | Whitish grey | Grey | – |
| | | | 21 | Whitish grey | Grey | – |
| ISP-5 | Moderate | – | 7 | Whitish olive | – | – |
| | | | 14 | Whitish olive | Grey | – |
| | | | 21 | Greyish olive | Dark grey | Olive |

Legend: ISP-2, Yeast extract malt extract; ISP-3, Oatmeal agar; ISP-4, Inorganic salt starch;
ISP-5, Glycerol asparagine agar; *;Only observed on ISP-2;-, absent; Triplicate plates were used for each observation

**Table 3.3. Cultural characteristics of isolate 513-3 on different media**

| Medium | Growth | Spore chain morphology* | Age(days) | Colour of aerial mycelium | Colour of substrate mycelium | Colour of soluble pigments |
|---|---|---|---|---|---|---|
| ISP-2 | Abundant | RF | 7 | Light grey | _ | _ |
| | | | 14 | Light grey | _ | _ |
| | | | 21 | Grey | Greyish brown | _ |
| ISP-3 | Abundant | _ | 7 | Grey | _ | _ |
| | | | 14 | Grey | Brown | _ |
| | | | 21 | Greyish brown | Dark brown | _ |
| ISP-4 | Moderate | _ | 7 | White | _ | _ |
| | | | 14 | Whitish grey | _ | _ |
| | | | 21 | Grey | Dark grey | _ |
| ISP-5 | Moderate | _ | 7 | Grey | _ | _ |
| | | | 14 | Olive grey | _ | _ |
| | | | 21 | Olive grey | Olive | _ |

Legend: ISP-2, Yeast extract malt extract; ISP-3, Oatmeal agar; ISP-4, Inorganic salt starch;
ISP-5, Glycerol asparagine agar; *;Only observed on ISP-2;-, absent; Triplicate plates were used for each observation

**Table 3.4. Cultural characteristics of isolate 513-4 on different media**

| Medium | Growth | Spore chain morphology* | Age(days) | Colour of aerial mycelium | Colour of substrate mycelium | Colour of soluble pigments |
|---|---|---|---|---|---|---|
| ISP-2 | Moderate | RF | 7 | White | – | – |
| | | | 14 | Whitish grey | – | – |
| | | | 21 | Whitish grey | Light grey | – |
| ISP-3 | Abundant | – | 7 | Grey | – | – |
| | | | 14 | Dark grey | – | – |
| | | | 21 | Leaded grey | Greyish brown | – |
| ISP-4 | Abundant | – | 7 | White | – | – |
| | | | 14 | White | Grey | – |
| | | | 21 | Whitish grey | Grey | – |
| ISP-5 | Moderate | – | 7 | Grey | – | – |
| | | | 14 | Grey | – | – |
| | | | 21 | Greyish green | Dark grey | – |

Legend: ISP-2, Yeast extract malt extract; ISP-3, Oatmeal agar; ISP-4, Inorganic salt starch;
ISP-5, Glycerol asparagine agar; *;Only observed on ISP-2;-, absent; Triplicate plates were used for each observation

**Table 3.5. Cultural characteristics of isolate 513-5 on different media**

| Medium | Growth | Spore chain morphology* | Age(days) | Colour of aerial mycelium | Colour of substrate mycelium | Colour of soluble pigments |
|---|---|---|---|---|---|---|
| ISP-2 | Abundant | RF | 7 | Light grey | _ | _ |
| | | | 14 | Light grey | Dark grey | _ |
| | | | 21 | Grey | Dark grey | _ |
| ISP-3 | Abundant | _ | 7 | Light brown grey | _ | _ |
| | | | 14 | Light brown grey | Dark Grey | _ |
| | | | 21 | Brown grey | Grey to black | _ |
| ISP-4 | Abundant | _ | 7 | Whitish grey | _ | _ |
| | | | 14 | Whitish grey | Grey | _ |
| | | | 21 | Grey | Dark grey | _ |
| ISP-5 | Moderate | _ | 7 | greyish | Grey | _ |
| | | | 14 | Greyish yellow | _ | _ |
| | | | 21 | Greyish yellow | Greyish olive | _ |

Legend: ISP-2, Yeast extract malt extract; ISP-3, Oatmeal agar; ISP-4, Inorganic salt starch;
ISP-5, Glycerol asparagine agar; *;Only observed on ISP-2;-, absent; Triplicate plates were used for each observation

**Table 3.6. Cultural characteristics of isolate 513-6 on different media**

| Medium | Growth | Spore chain morphology* | Age(days) | Colour of aerial mycelium | Colour of substrate mycelium | Colour of soluble pigments |
|---|---|---|---|---|---|---|
| ISP-2 | Abundant | RF | 7 | Grey | _ | _ |
| | | | 14 | Grey | _ | _ |
| | | | 21 | Grey | Dark grey | _ |
| ISP-3 | Abundant | _ | 7 | Grey | _ | _ |
| | | | 14 | Greyish brown | Brown | _ |
| | | | 21 | Greyish brown | Dark brown | _ |
| ISP-4 | Abundant | _ | 7 | White | _ | _ |
| | | | 14 | Whitish grey | _ | _ |
| | | | 21 | Whitish grey | Dark grey | _ |
| ISP-5 | Moderate | _ | 7 | Grey | _ | _ |
| | | | 14 | Olive grey | _ | _ |
| | | | 21 | Olive grey | Olive | _ |

Legend: ISP-2, Yeast extract malt extract; ISP-3, Oatmeal agar; ISP-4, Inorganic salt starch;
ISP-5, Glycerol asparagine agar; *;Only observed on ISP-2;-, absent; Triplicate plates were used for each observation

**Table 3.7. Cultural characteristics of isolate 513-7 on different media**

| Medium | Growth | Spore chain morphology* | Age(days) | Colour of aerial mycelium | Colour of substrate mycelium | Colour of soluble pigments |
|---|---|---|---|---|---|---|
| ISP-2 | Abundant | RF | 7 | White | – | – |
| | | | 14 | Creamishwhite | Greyish pink | Pink |
| | | | 21 | Creamishwhite | Greyish pink | Pinkish red |
| ISP-3 | Abundant | – | 7 | Whitish grey | – | – |
| | | | 14 | Whitish grey | Greyish red | Pinkish red |
| | | | 21 | Olive grey | Greyish red | Pinkish red |
| ISP-4 | Abundant | – | 7 | White | – | – |
| | | | 14 | Whitish grey | Grey | Creamish pink |
| | | | 21 | Whitish grey | Leaded grey | Creamish pink |
| ISP-5 | Moderate | – | 7 | Whitish olive | Grey | – |
| | | | 14 | Whitish olive | Grey | – |
| | | | 21 | Greyish olive | Greyish violet | Greyish pink |

Legend: ISP-2, Yeast extract malt extract; ISP-3, Oatmeal agar; ISP-4, Inorganic salt starch;
ISP-5, Glycerol asparagine agar; *;Only observed on ISP-2;-, absent; Triplicate plates were used for each observation

**Table 3.8. Cultural characteristics of isolate 513-8 on different media**

| Medium | Growth | Spore chain morphology* | Age(days) | Colour of aerial mycelium | Colour of substrate mycelium | Colour of soluble pigments |
|---|---|---|---|---|---|---|
| ISP-2 | Abundant | RF | 7 | Light grey | _ | _ |
| | | | 14 | Light grey | _ | _ |
| | | | 21 | Grey | Greyish brown | _ |
| ISP-3 | Abundant | _ | 7 | Grey | _ | _ |
| | | | 14 | Grey | Dark brown | _ |
| | | | 21 | Greyish brown | Dark brown | _ |
| ISP-4 | Moderate | _ | 7 | White | _ | _ |
| | | | 14 | Whitish grey | Grey | _ |
| | | | 21 | Whitish grey | Dark grey | _ |
| ISP-5 | Moderate | _ | 7 | Olive grey | _ | _ |
| | | | 14 | Olive grey | _ | _ |
| | | | 21 | Dark grey | Dark olive | _ |

Legend: ISP-2, Yeast extract malt extract; ISP-3, Oatmeal agar; ISP-4, Inorganic salt starch;
ISP-5, Glycerol asparagine agar; *;Only observed on ISP-2;-, absent; Triplicate plates were used for each observation

**Table 3.9. Cultural characteristics of isolate 513-9 on different media**

| Medium | Growth | Spore chain morphology* | Age (days) | Colour of aerial mycelium | Colour of substrate mycelium | Colour of soluble pigments |
|---|---|---|---|---|---|---|
| ISP-2 | Abundant | RA | 7 | White | _ | _ |
| | | | 14 | White | _ | _ |
| | | | 21 | Whitish grey | Dark grey | _ |
| ISP-3 | Abundant | _ | 7 | Grey | _ | _ |
| | | | 14 | Dark grey | _ | _ |
| | | | 21 | Dark grey | Greyish brown | _ |
| ISP-4 | Abundant | _ | 7 | White | _ | _ |
| | | | 14 | Whitish grey | Grey | _ |
| | | | 21 | Whitish grey | Leaded grey | _ |
| ISP-5 | Moderate | _ | 7 | Whitish olive | _ | _ |
| | | | 14 | Greyish olive | _ | _ |
| | | | 21 | Greyish green | Greyish violet | _ |

Legend: ISP-2, Yeast extract malt extract; ISP-3, Oatmeal agar; ISP-4, Inorganic salt starch;
ISP-5, Glycerol asparagine agar; *;Only observed on ISP-2;-, absent; Triplicate plates were used for each observation

### 3.4. Numerical systematics (Biochemical and physiological characteristics):

The phenotypic characteristics of the tested strains are summarized in Table 3.11. All the strains grew at 45 $^{0}$C. All isolates degraded starch, casein and xylan. Some isolates were able to tolerate NaCl levels up to 7 % but isolates 513-3, 513-4, 513-8 and 513-9 were deficient in this regard. Exposure to 0.1% phenol resulted in the growth of isolates 513-1, 513-2, 513-5, 513-6 and 513-7 and inhibition of growth of remaining isolates (Table3.10).

Table 3.11 summarizes the pattern of carbohydrate utilization. A varied pattern of carbohydrate assimilation was observed. All the isolates shared the abundant growth pattern in the presence of glucose, fructose, arabinose and mannitol , whereas isolates 513-4 and 513-9 showed little growth in the presence of dextran, sucrose and rhamnose. Isolates 513-3 and 513-8 showed little growth in the presence of raffinose (Table 3.11).

The utilization of different nitrogen sources also showed a range. Asparagine, histidine and threonine favoured abundant growth for all strains, while poor growth was observed with methionine for isolates 513-4, 513-6 and 513-9; phenylalanine and hydroxyproline for isolates 513-3, 513-4, 513-8 and 513-9; potassium nitrate for isolates 513-4 and 513-9 (Table 3.12).

The **statistical software Minitab 15** (Minitab Inc. 2007) was used in order to establish similarities among the isolated Streptomycetes strains (Figure 3.6).

**Table 3.10. Phenotypic characteristics of isolated strains (513-1 to 513-9)**

| Characteristics | 513-1 | 513-2 | 513-3 | 513-4 | 513-5 | 513-6 | 513-7 | 513-8 | 513-9 |
|---|---|---|---|---|---|---|---|---|---|
| Degradation of: | | | | | | | | | |
| Casein | + | + | + | + | + | + | + | + | + |
| Xylan | + | + | + | + | + | + | + | + | + |
| Starch | + | + | + | + | + | + | + | + | + |
| Physiological tests: | | | | | | | | | |
| Growth at 45 $^{0}$C | + | + | + | + | + | + | + | + | + |
| Growth in 4% NaCl | + | + | + | + | + | + | + | + | + |
| Growth in 7% NaCl | + | + | - | - | + | + | + | - | - |
| Growth in 0.1% Phenol | + | + | - | - | + | + | + | - | - |

Legend: +, Positive or utilization; -, Negative or no utilization. Each isolate was examined in duplicate run of experiment.

**Table 3.11. Carbohydrate utilization by isolated strains (513-1 to 513-9)**

| Carbon source | 513-1 | 513-2 | 513-3 | 513-4 | 513-5 | 513-6 | 513-7 | 513-8 | 513-9 |
|---|---|---|---|---|---|---|---|---|---|
| D-Glucose (Positive control) | + | + | + | + | + | + | + | + | + |
| D-Xylose | + | + | ± | + | + | + | + | ± | + |
| D-Fructose | + | + | + | + | + | + | + | + | + |
| L-Rhamnose | + | + | + | ± | + | ± | + | + | ± |
| Sucrose | + | + | + | ± | + | + | + | + | ± |
| Raffinose | + | + | ± | + | + | + | + | ± | + |
| L-Arabinose | + | + | + | + | + | + | + | + | + |
| D-Mannitol | + | + | + | + | + | + | + | + | + |
| Dextran | + | + | + | ± | + | + | + | + | ± |
| Without Carbon source (Negative control) | - | - | - | - | - | - | - | - | - |

Legend: +, Utilization of tested carbon is equal to or greater than that of positive control; ±, Doubtful or weaker utilization; -, No utilization
Each isolate was examined in duplicate run of experiment.

**Table 3.12. Sole nitrogen source utilization by isolated strains (513-1 to 513-9)**

| Nitrogen source | 513-1 | 513-2 | 513-3 | 513-4 | 513-5 | 513-6 | 513-7 | 513-8 | 513-9 |
|---|---|---|---|---|---|---|---|---|---|
| L-Asparagine (Positive control) | + | + | + | + | + | + | + | + | + |
| L-Histidine | + | + | + | + | + | + | + | + | + |
| L-Throenine | + | + | + | + | + | + | + | + | + |
| L-Methionine | + | + | + | ± | + | ± | + | + | ± |
| L-Phenylalanine | + | + | ± | ± | + | + | + | ± | ± |
| L-Hydroxyproline | + | + | ± | ± | + | + | + | ± | ± |
| Potassium nitrate | + | + | + | ± | + | + | + | + | ± |
| Without nitrogen source (Negative control) | - | - | - | - | - | - | - | - | - |

Legend: +, Utilization of tested nitrogen is equal to or greater than that of positive control; ±, Doubtful or weaker utilization; -, No utilization. Each isolate was examined in duplicate run of experiment.

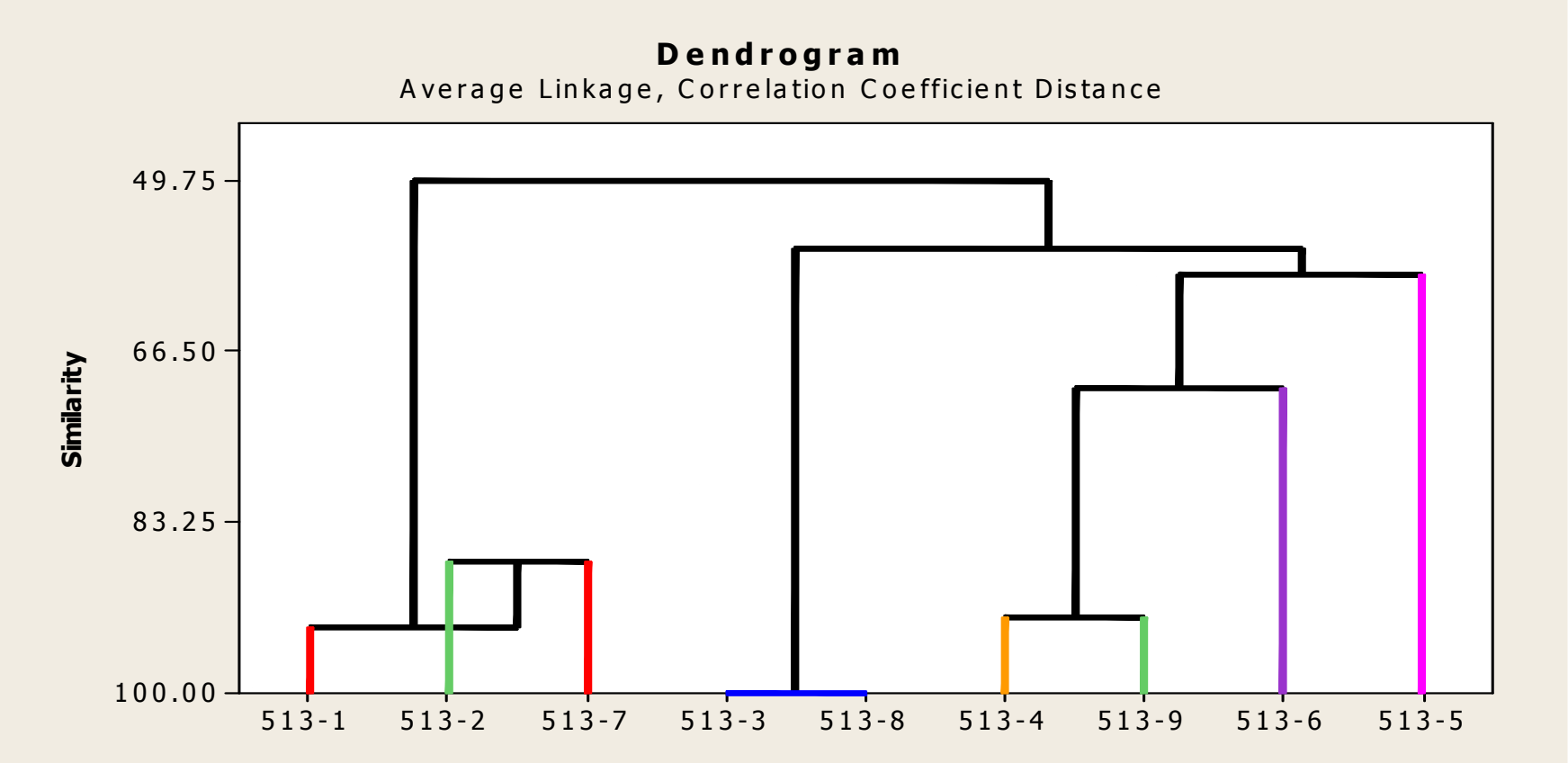



**Figure 3.6. Phenogram based on the multivariate statistical analysis of phenotypic tests listed in Tables 3.10- 3.14. The scale on y-axis shows similarity values in (%). A dendrogram generated by using the software Minitab 15 (Minitab Inc. 2007) with clustering achieved by applying the correlation coefficient distance method with arithmetic average algorithm.**

### 3.5. Melanin formation:

Melanin formation was observed in submerged conditions. Production of a diffusible brown pigment on complex organic media does not necessarily prove that the substrate is melanin (Tadashi and Mikami, 1972). In order to avoid discrepancies regarding melanin formation in agar media, submerged culture conditions were used. For that purpose characteristic chemical test for melanin detection in which reddish-brown colouration resulting from dopachrome formation was observed (Scribners, *et al*., 1973).

Figure 3.7 summarises the variation of pigment (melanin) production with different carbon sources. According to data, no significant differences were observed when glucose and lactose were used as carbon source ($P > 0.05$). Post hoc analysis showed significant differences only with the values of starch.

Starch was found to be the most effective carbon source for melanin formation in strains 513-1, 513-2, 513-3 and 513-8 followed by glycerol and fructose. The influence of nitrogen sources for the production of melanin by different strains is shown in Figure 3.8. In this case different behaviour was observed. Almost all the nitrogen sources showed significant values ($P < 0.05$). Post hoc analysis showed that the best values were found with arginine and glutamate for strains 513-1, 513-2, 513-3 and 513-8.

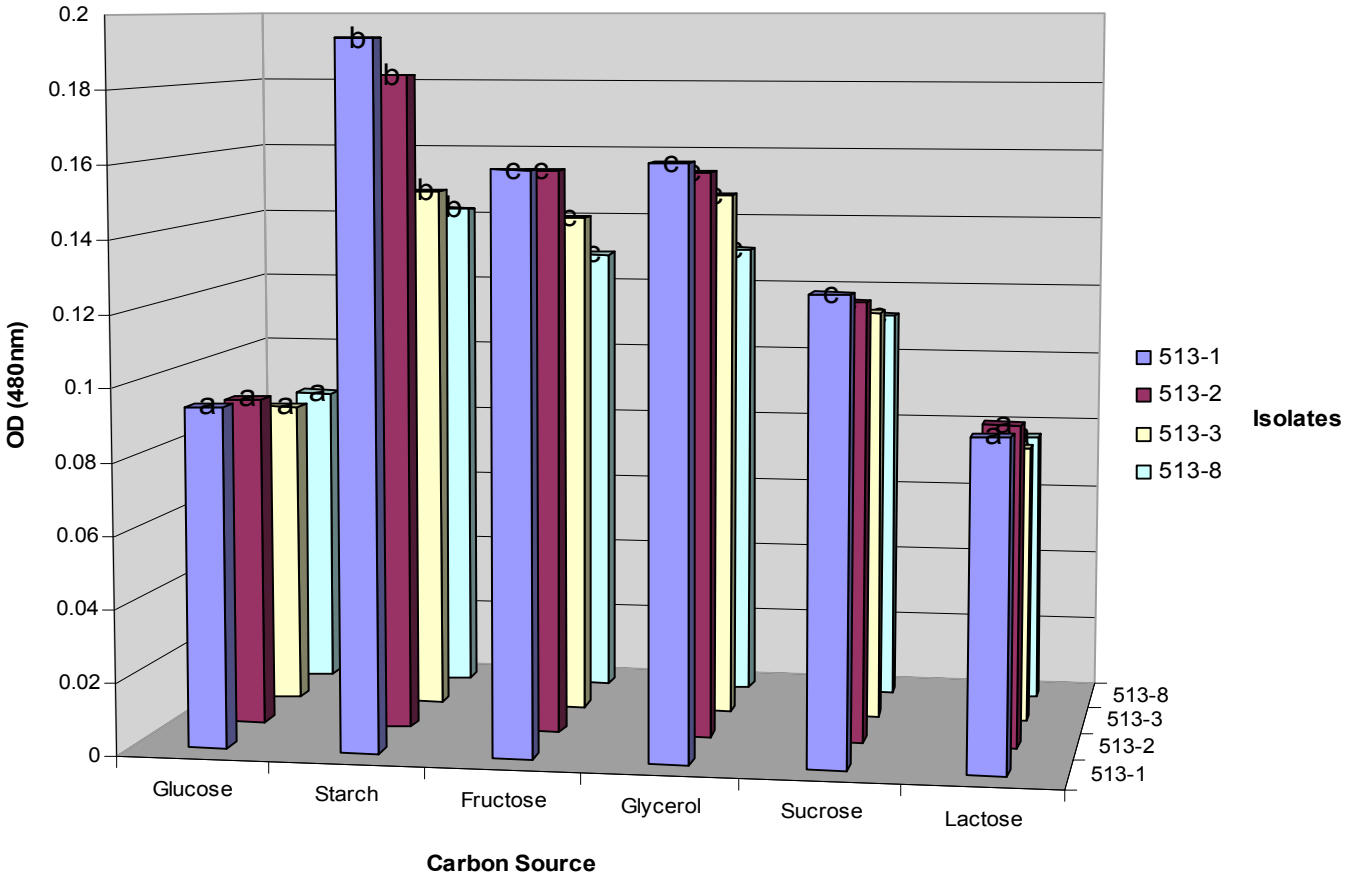



**Figure 3.7: Melanin pigmentation affected by variation in carbon sources. Treatment means of three replicates followed by the same letter are not significant different at α = 0.05**

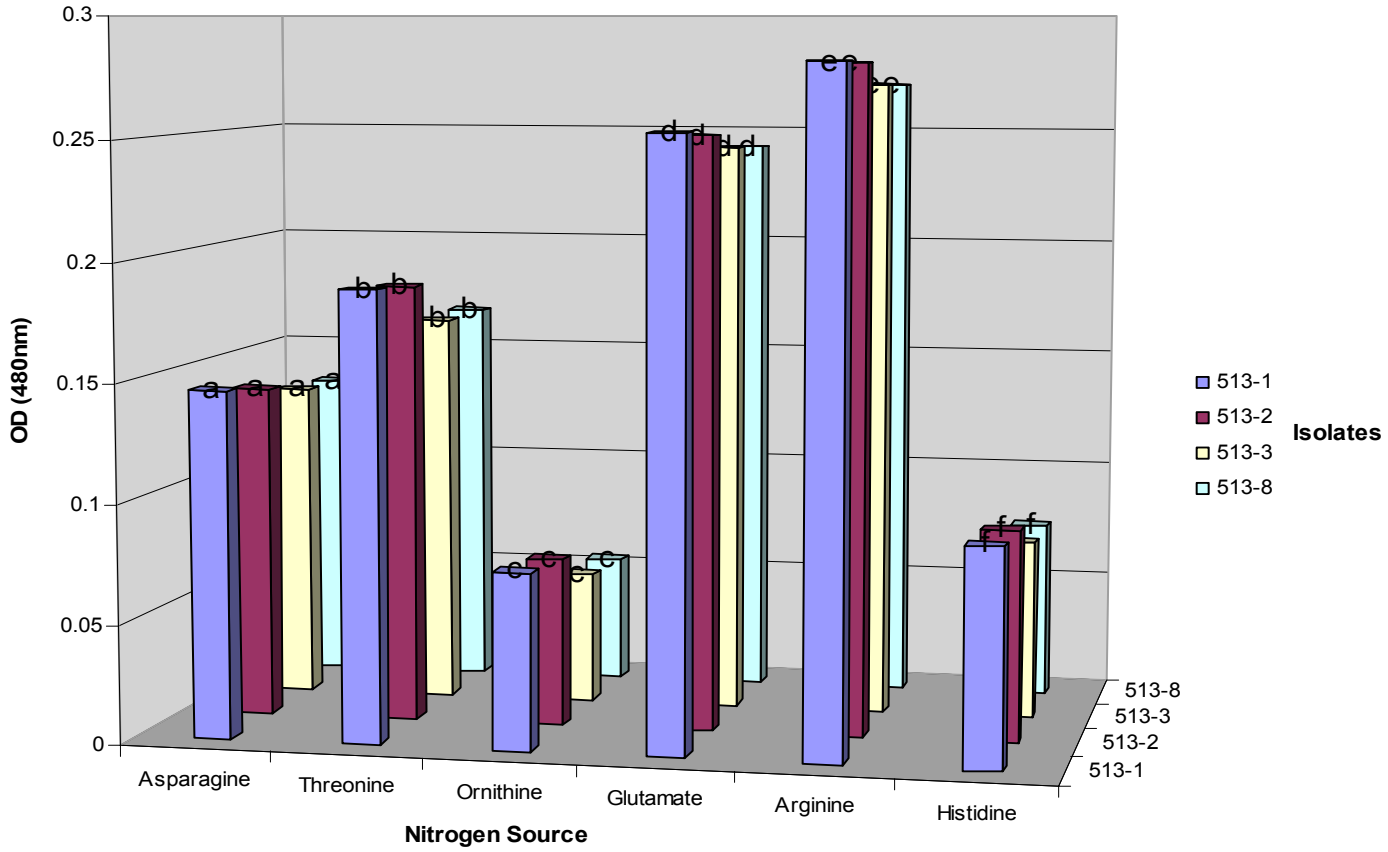



**Figure 3.8: Melanin pigmentation affected by variation in nitrogen sources. Treatment means of three replicates followed by the same letter are not significant different at α = 0.05**

### 3.6. Computer assisted identification:

Figure 3.9 summarizes the results of the probabilistic identification of different strains against the identification matrices of Langham *et al*. (1989) by using the computer software PIBWin. Isolate 513-5 showed its highest Wilcox score (0.89) when it was compared with major cluster of Langham *et al*. (1989). The next highest score was observed for isolate 513-4 (0.51) and isolate 513-9 (0.50) when compared with cluster 38.

Isolates were analysed against identification matrix comprising minor cluster of Langham *et al*. (1989). The highest score was observed for isolate 513-3 (0.9) and isolate 513-8 (0.913) when they were compared with cluster 3. The next highest score was observed for isolate 513-4 (0.88) when it was compared with cluster 3.

In order to establish similarity level among isolated Streptomycetes and strains from clusters of Williams *et al*. (1983), statistical software Minitab 15 (Minitab Inc. 2007) was used. Results are presented in the form of dendrogram (Figure 3.9).

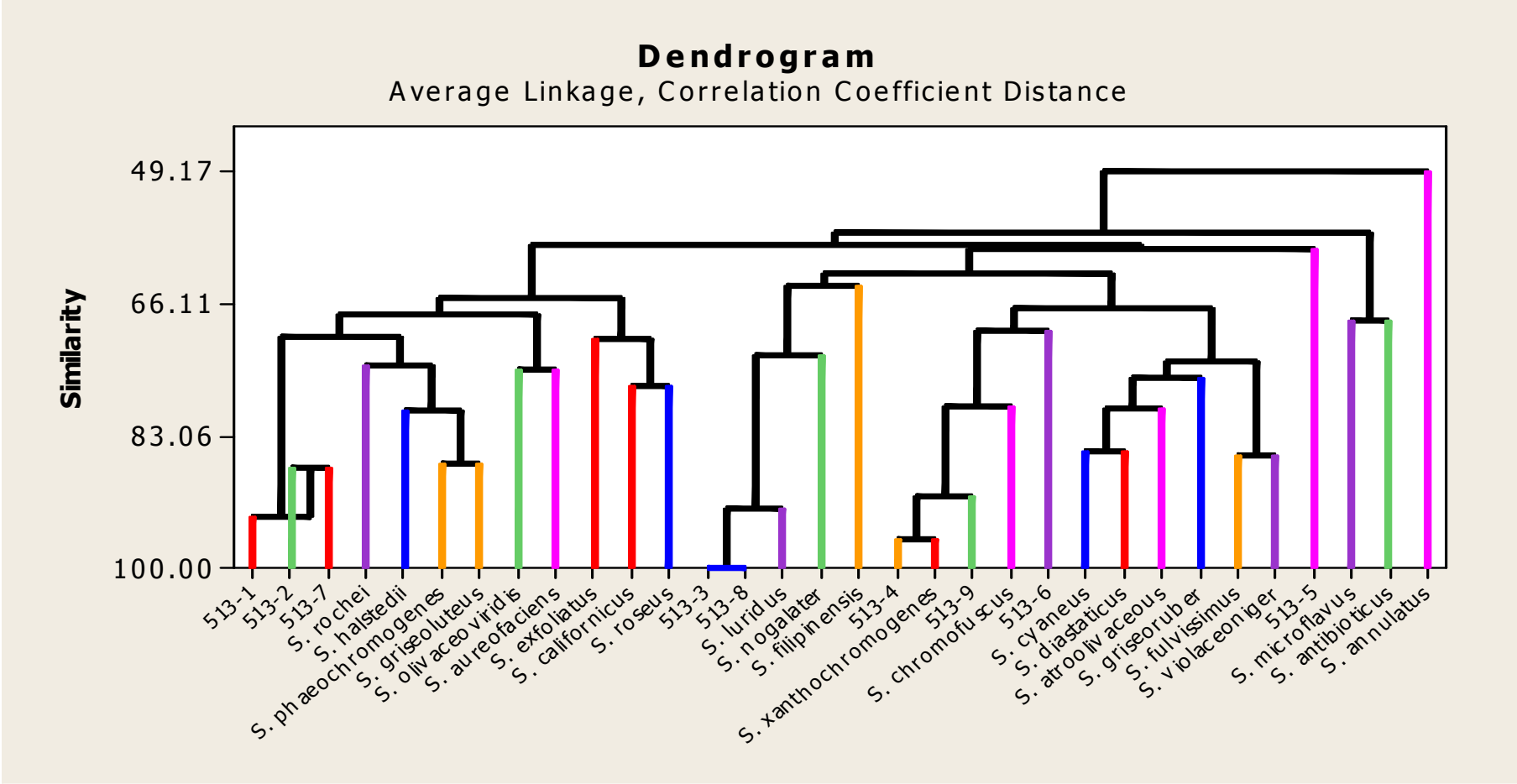



**Figure 3.9: Phenogram showing the relationships among isolated strains and reference strains from Williams *et al*. (1983). A dendrogram generated by using the software Minitab 15 (Minitab Inc. 2007) with clustering achieved by applying the correlation coefficient distance method with arithmetic average algorithm.**

**Table 3.13. Phenotypic properties differentiating strain 513-5 (1) from related type strains of *S. longisporuber* ATCC 27443 (2), *S. rutgersensis* ATCC 3350 (3), *S. antimycoticus* ATCC 27415 (4), *S. melanosporofaciens* ATCC 25473 (5).**

| Characteristics | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Colour of aerial mycelium | Grey | Reddish grey | Yellow-grey | Grey | Grey |
| Colour of substrate mycelium | Dark grey | Not distinctive | Not distinctive | - | - |
| Production of diffusible pigment | - | 33 | 13 | - | - |
| Melanin production | - | - | 14 | - | - |
| Degradation of: | | | | | |
| Casein | + | + | + | + | 33 |
| Xylan | + | - | 4 | - | - |
| Starch | + | 67 | 85 | + | + |
| Growth in the presence of: | | | | | |
| NaCl (4%) | + | 67 | 97 | + | 67 |
| Phenol (0.1%) | + | 44 | 90 | ± | + |
| Sole carbon source: | | | | | |
| D(+) Xylose | + | + | 93 | - | 67 |
| L(+) Rhamnose | + | 22 | 62 | - | 83 |
| Sucrose | + | 34 | 31 | - | 33 |
| D(+) Mannitol | + | + | 94 | + | + |
| Dextran | + | 22 | 59 | - | 17 |

Legend: Data for the strains were obtained from Williams *et al*. (1983)
Numbers indicate the percentage of strains positive for the test

### 3.7. 16s rRNA gene amplification:

Isolates (513-1 to 513-9) were subject to 16S rDNA (16S rRNA gene) sequencing analysis to depict phylogenetic relationships with representatives of *Streptomyces*. Primers F(5'-TGAGTAACACGTGGGCAATC-3') and primer R(5'-TTCGGGTGTTACCGACTTTC-3') were used to amplify DNA isolated from pure cultures of strains (513-1 to 513-9). The primer pair produced fragments of the expected size (1318 bp) successfully in the presence of *Streptomyces* DNA. Hence, the specificity of the primer pair was confirmed experimentally by PCR amplification from DNA isolated from pure cultures of nine isolates. The primer pair successfully amplified the control DNA (*Streptomyces rimosus*) and no amplification was observed in the negative control (Figure 3.10).

To confirm generic identification of strains an almost complete 16S rDNA sequence of strains (1318bp) was determined. A homology search indicated that 16s rDNA sequence had a high level of similarity with those of 24 strains belonging to *Streptomyces*. Hence the isolated strains were assumed to be member of this genus.

Strains 513-1 (FJ966265) and 513-2 (FJ966266) showed relatively high 16s rRNA gene sequence similarity values with the type strains of *Streptomyces sporoclivatus* (97.8%), *S. hygroscopicus* (97.8%) and *S. yatensis* (97.8%). Strains 513-3 (FJ966267) and 513-8 (FJ966272) exhibited levels of similarity (97.2%) with *S. thermocarboxydus*, *S. albogriseolus* and *S. lusitanus*. Strains 513-4 (FJ966268) and 513-9 (FJ966273) were found to be closest to the type strains of *S. paresii*, *S. fulvorubens* and *S. annulatus* (similarity value of 97.0%). Strain 513-5(FJ966269) showed a similarity value of 95.8% with the type strains of *S. enhygrus*. Strain 513-6 (FJ966270) shared the value of 97.5% with *S. aureus*, while strain 7 (FJ966271) exhibited a similarity value of 97.6% with the type strains of *S. humerifus*, *S. rubrogriseus* and *S. caesius*.

16s rDNA sequences of the strains were aligned with those of the type strains from the database using the clustal X programme alignment and subjected to phylogenetic analysis.

The phylogenetic tree was constructed to show the evolutionary relationship of different strains (Figure 3.13).

### 3.7.1. Nucleotide Sequence accession numbers:

The sequences of the strains investigated in this study have been deposited in the GeneBank, under the following accession numbers:

| | | |
|---|---|---|
| FJ966265 | for → | 513-1 |
| FJ966266 | → | 513-2 |
| FJ966267 | → | 513-3 |
| FJ966268 | → | 513-4 |
| FJ966269 | → | 513-5 |
| FJ966270 | → | 513-6 |
| FJ966271 | → | 513-7 |
| FJ966272 | → | 513-8 |
| FJ966273 | → | 513-9 |

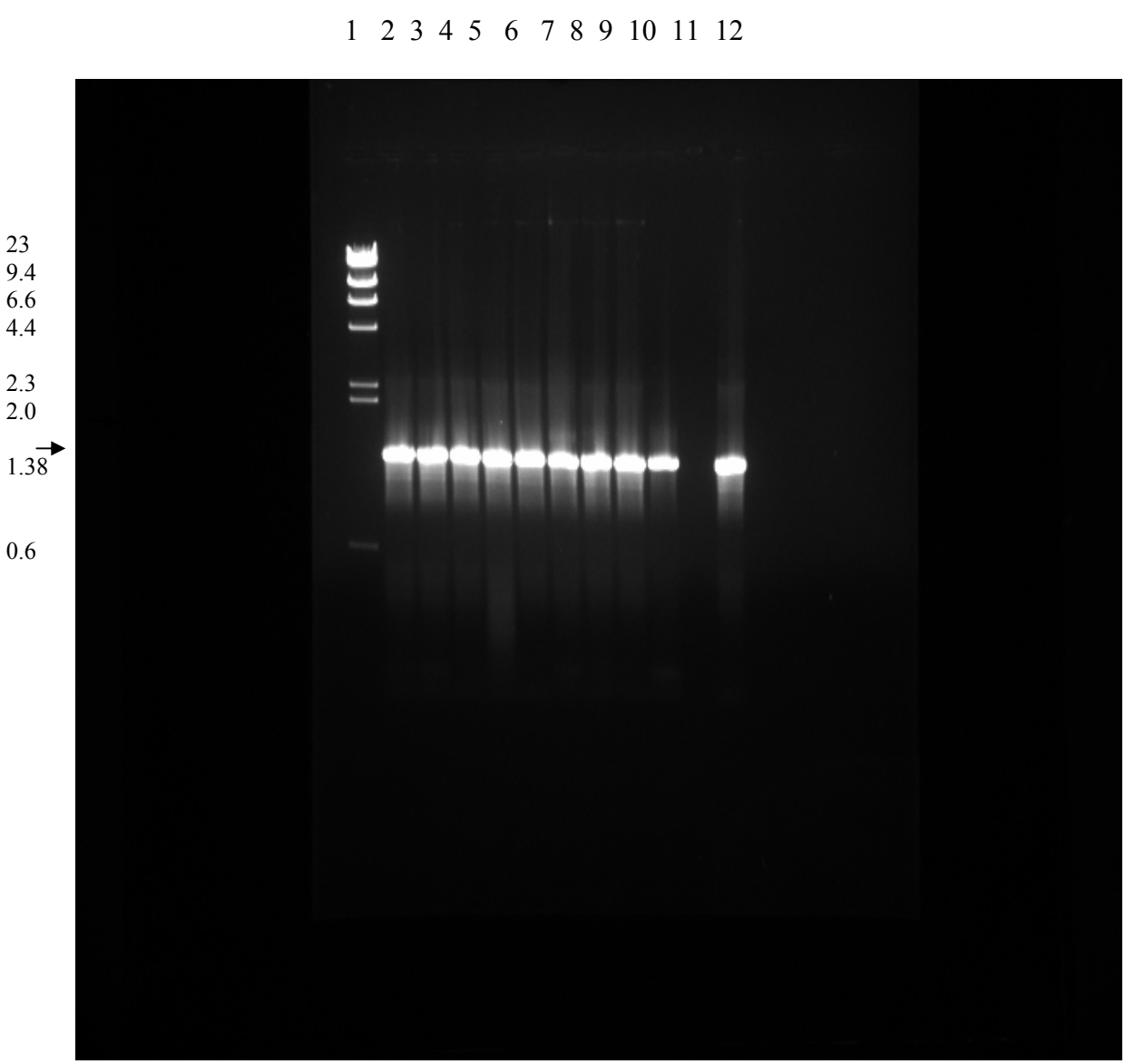



**Figure 3.10: Agarose gel electrophoresis of PCR products obtained by amplication of 16S rDNA gene from pure cultures of isolates 513-1-513-9. Lane 1, λ DNA marker (sizes in kb); Lanes 2-10, isolates 1-9; Lane 11, negative control; Lane 12, positive control (*S. rimosus*).**

**Aligned 16S rDNA sequences of major variable regions of isolated strains and some reference strains. Output, MEGA Version 4 .**

```
513-1                                 ---------- ---------- ---------- ---??????? GG?CTT-GCA CGCTCCCGGG ATTGTGGGCC CGGGCAAGCT  [ 243]
513-2                                 ---------- ---------- ---------- ???????TG? ?.GT..-... .......... .......... ..........  [ 243]
513-3                                 ---------- ---------- ---------- --?????CT? ?.G?.CT.TC GC..TGGCCT -.GTGAC... ..........  [ 243]
513-4                                 ---------- ---------- ---------- ??????????C T.G?GAC..C TC.GG....T C.GTG...TT TTTTGGG.T.  [ 243]
513-5                                 ---------- ---------- ---------- ---??????? C.G?..-... .......... ..C.......  ..........  [ 243]
513-6                                 ---------- ---------- ---------- ---??????? ?.G?.CT.TC GC..TGGCCT -.GTGAC... ..........  [ 243]
513-7                                 ---------- ---------- ---------- ---??T??A? ????.CT..C .T?G.AGCT. -CA.GTC.?? ....A.....  [ 243]
513-8                                 ---------- ---------- ---------- ---??????? ????.CT.TC GC..TGGCCT -.GTGAC... ..........  [ 243]
513-9                                 ---------- ---------- ---------- ????G??TG? ?.G?.A-C.T .TG...T... .CG-G.TA.. ....A.....  [ 243]
S._hygroscopicus(AB184723)            -AAAATGAAC GGTTCCCGGG GATTTAGGCT TGCCAGCTGA C.GT..-... .......... .......... ..........  [ 243]
S._yatensis(AF336800)                 -AAAATGAAC GGTTCCCGGG GATTTAGGCT TGCCAGCTGA C.GT..-... .......... ..C....... ..........  [ 243]
S._sporoclivatus(Eu240413)            ---------C TGATGTCGCG GGATTCGGCT TGCCAGCTG- C.GT..-... .......... .......... ..........  [ 243]
S._sp.(AB373962)                      -AAAATGAAC GGTTCCCGGG GATTTAGGCT TGCCAGCTGA C.GT..-... .......... .......... ..........  [ 243]
S._melanosporofaciens(AB184283)       -AAAATGAAC GGTTCCCGGG GATTTAGGCT TGCCAGCTGA C.GT..-... .......... .......... ..........  [ 243]
S._antimycoticus(AB184185)            -AAAATGAAC GGTTCCCGGG GATTTAGGCT TGCCAGCTGA C.GT..-... .......... ..C....... ..........  [ 243]
S._thermocarboxydus(AJ249627)         -AAAATGAAG CCCTCGGTGG ATT-TAGGCT TGCCAGCTGA C.GT.CT.TC GC..TGGCCT -.GTGAC... ..........  [ 243]
S._albogriseolus(AJ494865)            CAAAATGAAC CACTCTGGGG ATT-TAGGCT TGCCAGCTGA C.GT.CT..C ....TGGCCA -AC.GTC... ..........  [ 243]
S._lusitanus(AB184424)                -AAAATGAAC CACTCTGGGG ATT-TAGGCT TGCCAGCTGA C.GT.CT.TC AT..TGGCCT -.GTGAC... ..........  [ 243]
S._sp.(AF131460)                      -AAGGAAAGG CCCTCGGGTA CTCGCAGACC CTGTAGCGGA CTGT.C-..C GC.C..GC.. G.GTG...TT TTTTGGG.T.  [ 243]
S._sp.(EF216356)                      AAAGGAAAGG CCCTCGG-TA CTCGCAGATC CTGTAGCGGA CTGT..-..C GC.C.AGCT. G.GTG...TT TTTTGGGAT.  [ 243]
S._enhygrus(AB184558)                 -AAAATGAAC GGTTCCCGGG GATTTAGGCT TGCCAGCTGA C.GT..-... .......... ..C....... ..........  [ 243]
S._longispororuber(AB184440)          --AAATGAAC GGTTCCCGGG GATTTAGGCT TGCCAGCTGA C.GT..-... .......... ..C....... ..........  [ 243]
S._aureus(EF371429)                   CAAAATGAAG CCCTCGGTGG ATT-TAGGCT TGCCAGCTGA C.GT.CT.TC GC..TGGCCT -.GTGAC... ..........  [ 243]
S._sp.(EU384278)                      CAAAATGAAG CCCTCGGTGG ATT-TAGGCT TGCCAGCTGA C.GT.CT.TC GC..TGGCCT -.GTGAC... ..........  [ 243]
S._sp.(EF371425)                      --AAATGAAG CCCTCGGTGG ATT-TAGGCT TGCCAGCTGA C.GT.CT.TC GC..TGGCCT -.GTGAC... ..........  [ 243]
S._humiferus(AF503491)                ---------- ---------- -----AGGCT TTCCAGCTGA C.GT.CT..C .T.G.AGCT. -CA.GTC... ....A.....  [ 243]
S._rubrogriseus(AF503501)             -AAAATGAAC CACTCTGGGG ATT-TAGGCT TGCCAGCTGA C.GT.CT..C .T.G.AGCT. -CA.GKC... ..........  [ 243]
S._caesius(AF503495)                  CAAAATGAAC CACTCTGGGG ATT-TAGGCT TTCCAGCTGA C.GT.CT..C .T.G.AGCT. -CA.GTC... ....A.....  [ 243]
S._viridodiastaticus(AY999852)        -AAAATGAAC CACTCTGGGG ATT-TATGCT TGCCAGCTGA C.GT.CT..C ....TGGCCA -AC.GTC... ..........  [ 243]
S._bellus(EU570431)                   --AAATGAAC CACTCTGGGG ATT-TAGGCT TGCCAGCTGA C.GT.CT..C GC..TGGCCA -AGCGTC... ..........  [ 243]
S._althioticus(EU593734)              ------GAAG CCCTCGGTGG ATT-TAGGCT TGCCAGCTGA C.GT.CT.TC GC..TGGCCT -.GTGAC... ..........  [ 243]
S._paresii_type_strain(AJ969177)      -AAAATGAAG CCTTCGGTGG ATT-TAGGCT TTCCAGCTGA C.GT.A-C.T .TG...T... .CG-G.TA.. ....A.....  [ 243]
S._fulvorobeus_(AB184711)             -AAAATGAAG CCTTCGGTGG ATT-TAGGCT TTCCAGCTGA C.GT.A-C.T .TG...T... .CG-G.TA.. ....A.....  [ 243]
S._anulatus(EU570540)                 -TTAATGAAG CCCTCGGTGG ATT-TAGGCT TTCCAGCTGA C.GT.A-C.T .TG....... .CG-G.TA.. ....A.....  [ 243]
S._rutgersensis_(AB184709)            --AAATGAAC GGTTCCCGGG GATTTAGGCT TGCCAGCTGA C.GT..-... .......... ..C....... ..........  [ 243]
S._megasporus_DSM_41476_(type_strain)... -AAACCGGCG CCTCGTGGGG TCGGTAGGCT TGCTACTGGA CCAG.C-..C .T.GGGGCCT -CG.G..... .........G  [ 243]
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513-1                                GGATCTACCA ATGAACGAGA GGCACTTGGA TGGTCTATCA GATTCTAGTC ACTGATACTA AGGACATCGC CCTTGGGCGA  [ 950]
513-2                                .......... .......... .......... .......... .......... .......... .......... ......CGCG  [ 950]
513-3                                AA..TC.... ........A. ...G...?.T ...C...G.. .......... ........GC .......... T..G?.CGCG  [ 950]
513-4                                .AG......G GC.GCTTGAC ....T.G.TC CCA.G.GGGC ..G.A.CAGT CTGTTACT.C G..GTCATC. TGCG.TCGCG  [ 950]
513-5                                .......... .......... .......... ..A....... .......... .......... .......?.. ......CGCG  [ 950]
513-6                                AA..TC.... ........A. ...G.....T ...C...G.. .......... ........GC .......... T..G..CGCG  [ 950]
513-7                                AA..TC.... ........A. .......A.T ...C...G.. .......... ........GC ..A....T.. T..G..CGCG  [ 950]
513-8                                AA..TC.... ........A. ...G.....T ...C...G.. .......... ........GC .......... T..G..C?CG  [ 950]
513-9                                .A....C... ........A. .......A.. ...C.....T A..GT..... ...AT..... .T.......?? T...A.CGCG  [ 950]
S._hygroscopicus(AB184723)           .......... .......... .......... .......... .......... .......... .......... ......CGCG  [ 950]
S._yatensis(AF336800)                .......... .......... .T.....?.. .......... .......... .......... .......... ......CGCG  [ 950]
S._sporoclivatus(Eu240413)           ........T. ..C....... .......... .......... .......... .......... .......... ......CGCG  [ 950]
S._sp.(AB373962)                     .......... .......... .......... .......... .......... .......... .......... ......CGCG  [ 950]
S._melanosporofaciens(AB184283)      .......... .......... .......... .......... .......... .......... .......... ......CGCG  [ 950]
S._antimycoticus(AB184185)           .......... .......... .......... .......... .......... .......... .......... ......CGCG  [ 950]
S._thermocarboxydus(AJ249627)        AA..TC.... ........A. ...G.....T ...C...G.. .......... ........GC .......... T..G..CGCG  [ 950]
S._albogriseolus(AJ494865)           AA..TC.... ........A. ...G...A.T ...C...G.. .......... ........GC .......... T..G..CGCG  [ 950]
S._lusitanus(AB184424)               AA..TC.... ........A. ...G.....T ...C...G.. .......... ........GC .......... T..G..CGCG  [ 950]
S._sp.(AF131460)                     .AG......G GC.GCTTGAC ....T.G.TC CCA.G.GGGC ..G.A.CAGT CTGTTACT.C G...TCATC. TGCG------  [ 950]
S._sp.(EF216356)                     .A.......G .C.GCTTGAC ....T.G.TC CCA.G.GGGC ..G.A.CAGT CTGTTACT.C G..GTCATC. TGCG.TCGCG  [ 950]
S._enhygrus(AB184558)                .......... .......... .......... ...C...... .......... .......... .......... ......CGCG  [ 950]
S._longispororuber(AB184440)         .......... .......... .......... ...C...... .......... .......... .......... ......CGCG  [ 950]
S._aureus(EF371429)                  AA..TC.... ........A. ...G.....T ...C...G.. .......... ........GC .......... T..G..CGCG  [ 950]
S._sp.(EU384278)                     AA..TC.... ........A. ...G.....T ...C...G.. .......... ........GC .......... T..G..CGCG  [ 950]
S._sp.(EF371425)                     AA..TC.... ........A. ...G.....T ...C...G.. .......... ........GC .......... T..G..CGCG  [ 950]
S._humiferus(AF503491)               AA..TC.... ........A. .......A.T ...C...G.. .......... ........GC ..A....T.. T..G..CGCG  [ 950]
S._rubrogriseus(AF503501)            AA..TC.... ........A. .......A.T ...C...G.. .......... ........GC ..A....T.. T..G..CGCG  [ 950]
S._caesius(AF503495)                 AA..TC.... ........A. .......A.T ...C...G.. .......... ........GC ..A....T.. T..G..CGCG  [ 950]
S._viridodiastaticus(AY999852)       AA..TC.... ........A. ...G...A.T ...C...G.. .......... ........GC .......... T..G..CGCG  [ 950]
S._bellus(EU570431)                  AA..TC.... ........A. ...G...A.T ...C...G.. .......... ........GC ..A....T.. T..G..CGCG  [ 950]
S._althioticus(EU593734)             AA..TC.... ........A. ...G.....T ...C...G.. .......... ........GC .......... T..G..CGCG  [ 950]
S._paresii_type_strain(AJ969177)     .A........ ........A. .......A.. ...C.....T A..GT..... ...AT..... .T.......G T...A.CGCG  [ 950]
S._fulvorobeus_(AB184711)            .A........ ........A. .......A.. ...C.....T A..GT..... ...AT..... .T.......G T...A.CGCG  [ 950]
S._anulatus(EU570540)                .A........ ........A. .......A.. ...C.....T A..GT..... ...AT..... .T.......G T...A.CGCG  [ 950]
S._rutgersensis_(AB184709)           .......... .......... .......... ...C...... .......... .......... .......... ......CGCG  [ 950]
S._megasporus_DSM_41476_(type_strain)... ...C.C.T.. C......CA. A.TG.C.... ...C.G.... .G...C.... .........C ...G...... TT.G..CGCG  [ 950]


513-1                                TCAC?GAACA TCCATGGTGC --CG?G?CG? GTAC?-T-TG ?T?GG?GCAT GCT?GGGGA? -A?TC????? CTCGG?????  [1164]
513-2                                C...A..... .......... -C..T.T??- ....??.-.. T.?..?.... ...C...TGA -????C?G?? ?....TC-C?  [1164]
513-3                                ?...A??..G ...?...C.. -C..?.?..? ....?G.T.. TCC..--?C? ??.--.??GC --A?T??G?A AC?C.?CCTC  [1164]
513-4                                .T-.ACG??G CT???.???T -T.TT.G.TG ..GAC?G?.. C.C?CGCAC? ??.A???.GG AGGGATGCGG ?AT.A???--  [1164]
513-5                                ....A..?.- ..TGC?.-.. --..?.?..? ....?-.-.. T??.?G-... ...?...?.? -?????G??A .....C???-  [1164]
513-6                                C...??...G ...?...C.. -C..T.?..T ....?T.GCT G.C???C?G? C..--????C -.?.??GGA? .C??.?C???  [1164]
```

```
513-7                                  ....??..GC AT.?..TGC. -C..?.?..- ....??.?.. ?CC??GCA?C CT.--..?G? --A?T??GA? .???.?A??G  [1164]
513-8                                  ?...?.???T -..?...C.. -C..T.T..T ....???G.. ?CC?.A..?? ?..--.??GC --A?T??G?? .C??.???T?  [1164]
513-9                                  C..TA?..GC AT.?..TGC. --..T.?.?? A...?-?-.. ?.???A.... ..C??....? -.????????  ..??.?A???  [1164]
S._hygroscopicus(AB184723)             C...A..... .......... -C..T.T..T ....AT.G.. T.T..A.... ...C...TGA -.C..CAGAG .....TCACG  [1164]
S._yatensis(AF336800)                  C...A..... .......... -C..T.T..T ....AT.G.. T.T..A.... ...C...TGA -.C..CAGAG .....TCACG  [1164]
S._sporoclivatus(Eu240413)             C...A..... .......... -C..T.T..T ....AT.G.. T.T..A.... ...C...TGA -.C..CAGAG .....TCACG  [1164]
S._sp.(AB373962)                       C...A..... .......... -C..T.T..T ....AT.G.. T.T..A.... ...C...TGA -.C..CAGAG .....TCACG  [1164]
S._melanosporofaciens(AB184283)        C...A....C ..TGCA.G.. -C..T.T..T ....AT.G.. T.T..A.... ...C...TGA -.C..CAGAG .....TCACG  [1164]
S._antimycoticus(AB184185)             C...A....C ..TGCA.G.. -C..T.T..T ....AT.G.. T.T..A.... ...C...TGA -.C..CAGAG .....TCACG  [1164]
S._thermocarboxydus(AJ249627)          C...A....G .......C.. -C..T.T..T ....AT.G.. TCC..A...G ..C-.T.TGC -.C..CGGAG .C...TCACG  [1164]
S._albogriseolus(AJ494865)             C...A....G .......C.. -C..T.T..T ....AT.G.. TCC..A...A ..CC...TGT -.C..CGGAG .C...TCACG  [1164]
S._lusitanus(AB184424)                 C...A....G .......C.. -C..T.T..T ....AT.G.. TCC..A...G ..C-.T.TGC -.C..CGGAG .C...TCACG  [1164]
S._sp.(AF131460)                       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  [1164]
S._sp.(EF216356)                       CTCAC....G .G....-... GCTCGACT.G TG..AT.G.. T.CA.A...A CTCC..A.GT -.C..TGGAG .....TCACG  [1164]
S._enhygrus(AB184558)                  C...A....C ..TGCA.G.. -C..T.T..T ....AT.G.. T.T..A.... ...C...TGA -.C..CAGAG .....TCACG  [1164]
S._longispororuber(AB184440)           C...A....C ..TGCA.G.. -C..T.T..T ....AT.G.. T.T..A.... ...C...TGA -.C..CAGAG .....TCACG  [1164]
S._aureus(EF371429)                    C...A....G .......C.. -C..T.T..T ....AT.G.. TCC..A...G ..C-.T.TGC -.C..CGGAG .C...TCACG  [1164]
S._sp.(EU384278)                       C...A....G .......C.. -C..T.T..T ....AT.G.. TCC..A...G ..C-.T.TGC -.C..CGGAG .C...TCACG  [1164]
S._sp.(EF371425)                       C...A....G .......C.. -C..T.T..T ....AT.G.. TCC..A...G ..C-.T.TGC -.C..CGGAG .C...TCACG  [1164]
S._humiferus(AF503491)                 C...A...GC AT....TGC. -C..T.T..T ....AT.G.. TCC..A...A ..CC...TGT -.C..CGGAG .C...TCACG  [1164]
S._rubrogriseus(AF503501)              C...A...GC AT....TGC. -C..T.T..T ....AT.G.. TCC..A...A ..CC...TGT -.C..CGGAG .C...TCACG  [1164]
S._caesius(AF503495)                   C...A...GC AT....TGC. -C..T.T..T ....AT.G.. TCC..A...A ..CC...TGT -.C..CGGAG .C...TCACG  [1164]
S._viridodiastaticus(AY999852)         C...A....G .......C.. -C..T.T..T ....AT.G.. TCC..A...A ..CC...TGT -.C..CGGAG .C...TCACG  [1164]
S._bellus(EU570431)                    C...A....G .......C.. -C..T.T..T ....AT.G.. TCC..A...G ..C-.T.TGC -.C..CGGAG .C...TCACG  [1164]
S._althioticus(EU593734)               C...A....G .......C.. -C..T.T..T ....AT.G.. TCC..A...G ..C-.T.TGC -.C..CGGAG .C...TCACG  [1164]
S._paresii_type_strain(AJ969177)       C..TA...GC AT....-... CC..T.T..T A...AT.G.. T.T..A.... ..CC...TGA -.C..CAGAG .....TCACG  [1164]
S._fulvorobeus_(AB184711)              C..TA...GC AT....-... CC..T.T..T A...AT.G.. T.T..A.... ..?C...TGA -.C..CAGAG .....TCACG  [1164]
S._anulatus(EU570540)                  C..TA...GC AT....-... CC..T.T..T A...AT.G.. T.T..A.... ..CC...TGA -.C..CAGAG .....TCACG  [1164]
S._rutgersensis_(AB184709)             C...A....C ..TGCA.G.. -C..T.T..T ....AT.G.. T.T..A.... ...C...TGA -.C..CAGAG .....TCACG  [1164]
S._megasporus_DSM_41476_(type_strain)... C...G..C.G C..GC..... TC..G.C..T ....AT.GC. T.C..A...C ..CTC...GT G.C..CGGAG .....TCACG  [1164]
```

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513-1                                  ????------ ---------- ---------- ---------- ---------- ---------- ----- [1498]
513-2                                  A???------ ---------- ---------- ---------- ---------- ---------- ----- [1498]
513-3                                  G?A??GGGA? A-??T?---- ---------- ---------- ---------- ---------- ----- [1498]
513-4                                  ---------- ---------- ---------- ---------- ---------- ---------- ----- [1498]
513-5                                  ---------- ---------- ---------- ---------- ---------- ---------- ----- [1498]
513-6                                  ?GA??AC?A? TCAAGCAT?? G??------- ---------- ---------- ---------- ----- [1498]
513-7                                  ????G???A? T-CA?C???? ?--------- ---------- ---------- ---------- ----- [1498]
513-8                                  G??AGG?C?A ?TCA?C?TA? ????GTG??G CTGGG----- ---------- ---------- ----- [1498]
513-9                                  ??GGA?GTCA ?C?TCTC?TG GCTG????A? G--------- ---------- ---------- ----- [1498]
S._hygroscopicus(AB184723)             AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGA GTGTTACTTT GCCTTTGGCC ATAGT [1498]
S._yatensis(AF336800)                  AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGA GTGTTACTTT GCCTTTGACC ATAGT [1498]
S._sporoclivatus(Eu240413)             AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGA GTGTTACTTT GATGC----- ----- [1498]
```

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S._sp.(AB373962)                      AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGA GTGTTACTTT GCCTTTGGCC ATAGT [1498]
S._melanosporofaciens(AB184283)       AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGA GTGTTACTTT GCCTTTGGCC ATAGT [1498]
S._antimycoticus(AB184185)            AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGA GTGTTACTTT GCCTTTGGCC ATAGT [1498]
S._thermocarboxydus(AJ249627)         AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGT ACGTTACTT- ---------- ----- [1498]
S._albogriseolus(AJ494865)            AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGT ACGTTACTTT GCCCTTGGCT ATAGT [1498]
S._lusitanus(AB184424)                AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGT ACGTTACTTT GCCCTTGGCT ATAGT [1498]
S._sp.(AF131460)                      ---------- ---------- ---------- ---------- ---------- ---------- ----- [1498]
S._sp.(EF216356)                      AAGTGATGAC TCAAGCACTA TTCTTGCACA TGAACGAGTA GTATCTCACC CCAGTCTGCG GTAG- [1498]
S._enhygrus(AB184558)                 AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGA GTGTTACTTT GCCTTTGGCC ATAGT [1498]
S._longispororuber(AB184440)          AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGA GTGTTACTTT GCCTTTGGCC ATG-- [1498]
S._aureus(EF371429)                   AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGT ACGTTACTTT GCCCTTGGCT ATAGT [1498]
S._sp.(EU384278)                      AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGT ACGTTACTTT GCCCTTGGCT ATAGT [1498]
S._sp.(EF371425)                      AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGT ACGTTACTTT GCCCTTGGCT ATA-- [1498]
S._humiferus(AF503491)                AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGT ACATTACTTT GCCCTTGGCT ATAGT [1498]
S._rubrogriseus(AF503501)             AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGT ACATTACTTT GCCCTTGGCT ATAGT [1498]
S._caesius(AF503495)                  AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGT ACATTACTTT GCCCTTGGCT ATAGT [1498]
S._viridodiastaticus(AY999852)        AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGT ACGTTACTTT GCCCTTGGCT ----- [1498]
S._bellus(EU570431)                   AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGT ACGTTACTTT GCCCTTGGCT ATATA [1498]
S._althioticus(EU593734)              AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGT ACGTTACTTT GCCCTTGGCT AT--- [1498]
S._paresii_type_strain(AJ969177)      AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGT GCGCTATTTT GCCCTTGGCT ATAGT [1498]
S._fulvorobeus_(AB184711)             AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGT GCGCTATTTT GCCCTTGGCT ATAGT [1498]
S._anulatus(EU570540)                 AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGT GCGCTATTTT GCCCTTGGCT ATAA- [1498]
S._rutgersensis_(AB184709)            AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGA GTGTTACTTT GCCTTTGGCC ATG-- [1498]
S._megasporus_DSM_41476_(type_strain)... AAGTGACGAC TCAAGCACTA TTCTTGCCCG TGAACATAGG GTGCTACGTT GCCCTCGGTC ACAGT [1498]
```

**Figure 3.11: Sequence alignment of the major variable regions of 16s rDNA encoding for 9 isolates and some reference strains of *Streptomyces*. Only the nucleotides different are shown, whereas identical nucleotides are indicated with dots. Selection of regions was done by using the MEGA software (Tamura *et al*., 2007).**

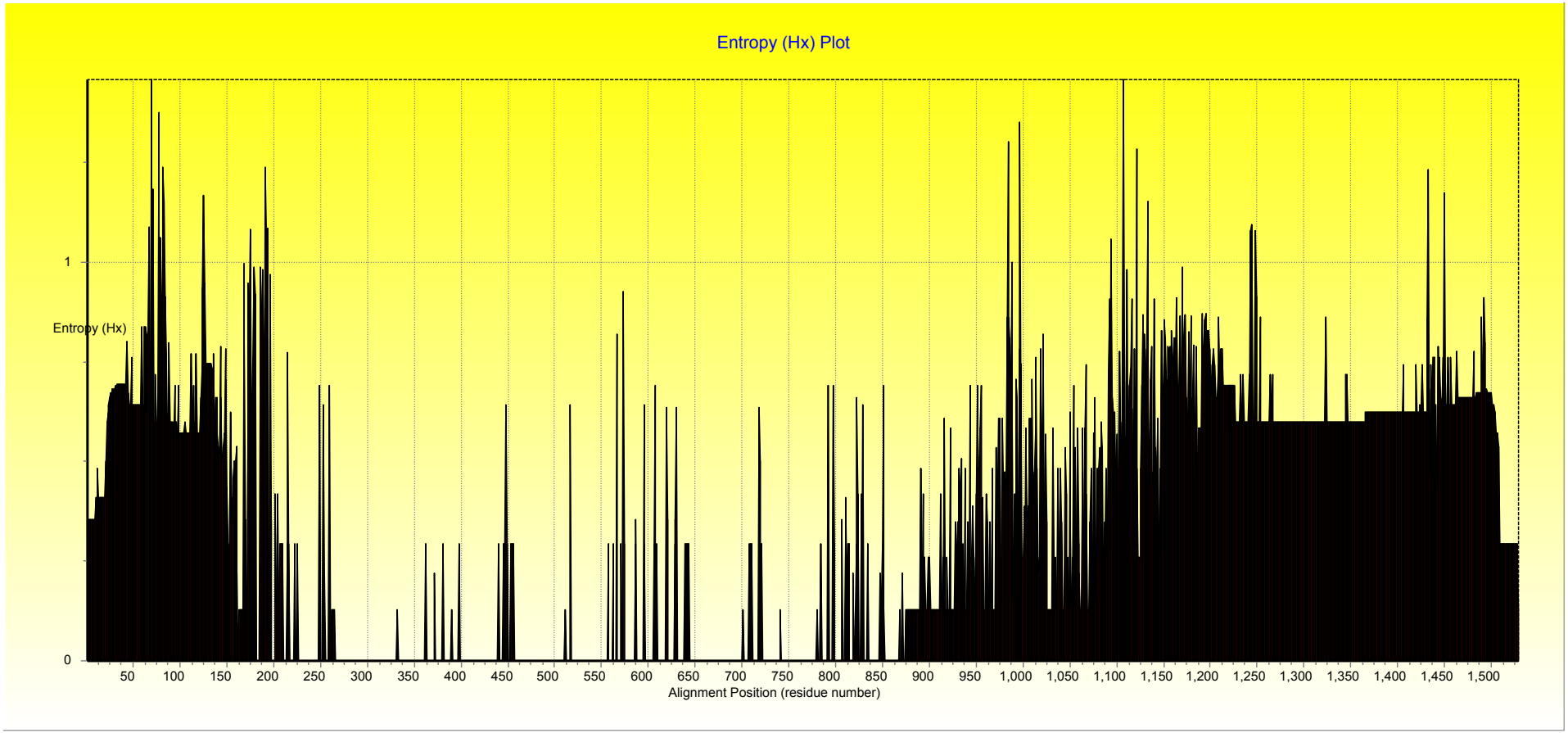


**Figure 3.12: Entropy plot of multiple sequence alignment of 16s rDNA genes of 9 isolated strains and some reference strains of *Streptomyces* depicting accumulation of variable regions. Plot generated by BioEdit Sequence alignment editor (Hall, 1999).**

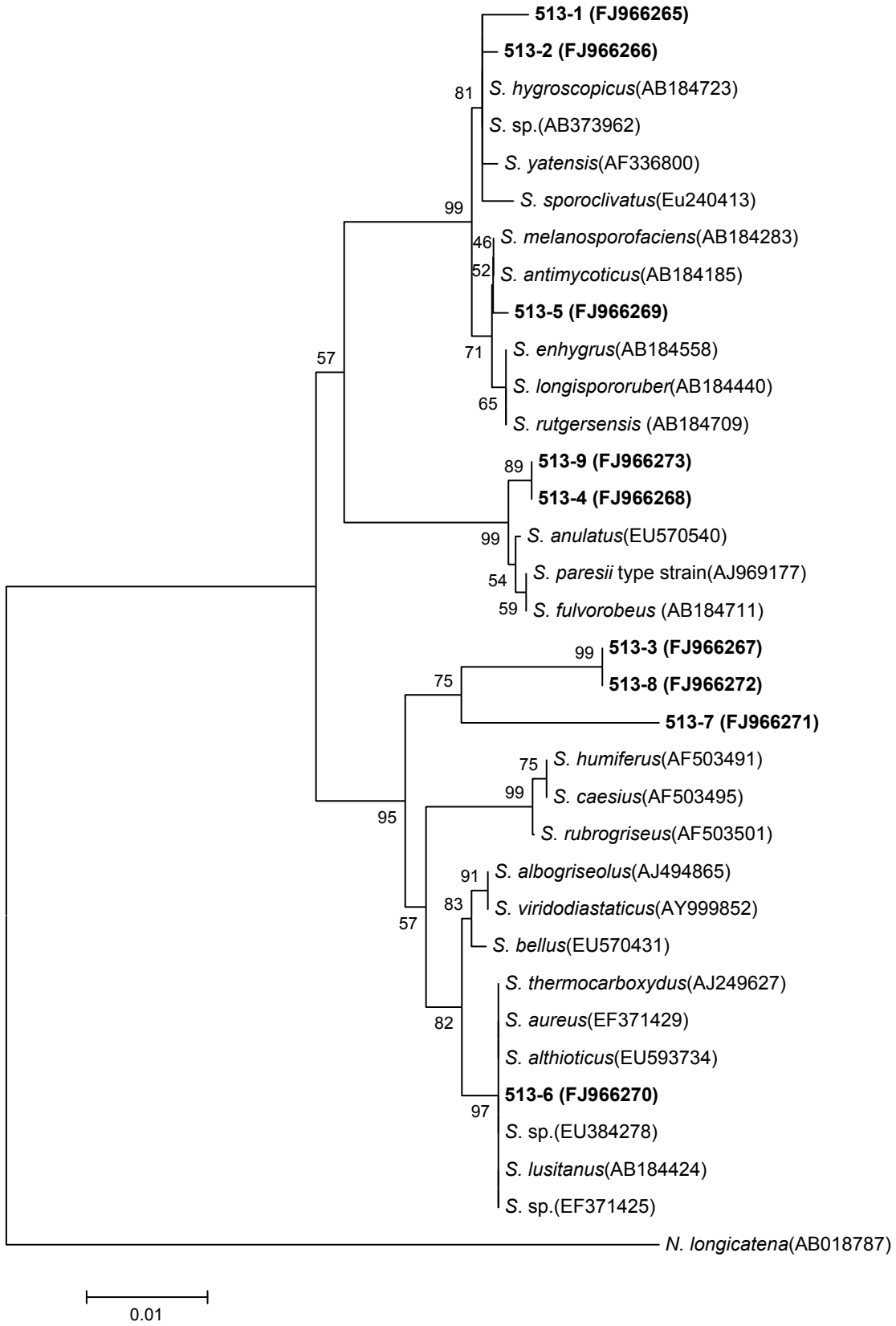


Figure 3.13: Neighbour-joining tree based on 16S rRNA gene sequences showing relationship between strains and the type strains of phylogenetically close *Streptomyces* species. Numbers at the nodes are percentage bootstrap values based on 500 resampled data sets. Bar 0.01 represents substitutions per nucleotide positions.

### 3.8. General pattern of lignocellulose degradation:

Extracellular peroxidase was produced by growing isolates in a basal mineral liquid medium in which the main carbon sources were:

- Quack-grass
- Corn

The first set of experiment was carried out at pH 7.5 with 0.6% substrates (corn and grass as primary carbon source with 0.6% yeast extract as nitrogen source). The liquid medium did not include chemical inducers often used to enhance enzyme activity.

#### 3.8.1. Effect of Incubation time:

Time course profiles of the extracellular peroxidase activities against corn as substrate showed that maximal activities were found during 6 to 8 days of incubation. Extracellular peroxidase activity (0.34 U $ml^{-1}$) increased significantly during the growth phase of culture (6 - 8 days) in the minimal salt medium supplemented with corn. During later stages of growth peroxidase activity decreased. A typical time course profile showing extracellular peroxidase activity in basal salts-yeast extract media containing 0.6% corn is shown in Figure 3.14.

Maximal peroxidase activity during growth phase of culture containing grass was observed during the 8 to 10 days of incubation. Maximal peroxidase activities were observed for strains 513-1 (0.42 U $ml^{-1}$), 513-5 (0.45 U $ml^{-1}$) and 513-7 (0.54 U $ml^{-1}$) during the $10^{th}$ day of incubation.

Time course profile of extracellular peroxidase activity in basal salts- yeast extract media containing 0.6% grass is shown in Figure 3.15.

Grass media showed good response in activity but corn media favoured growth earlier than that observed with grass media.

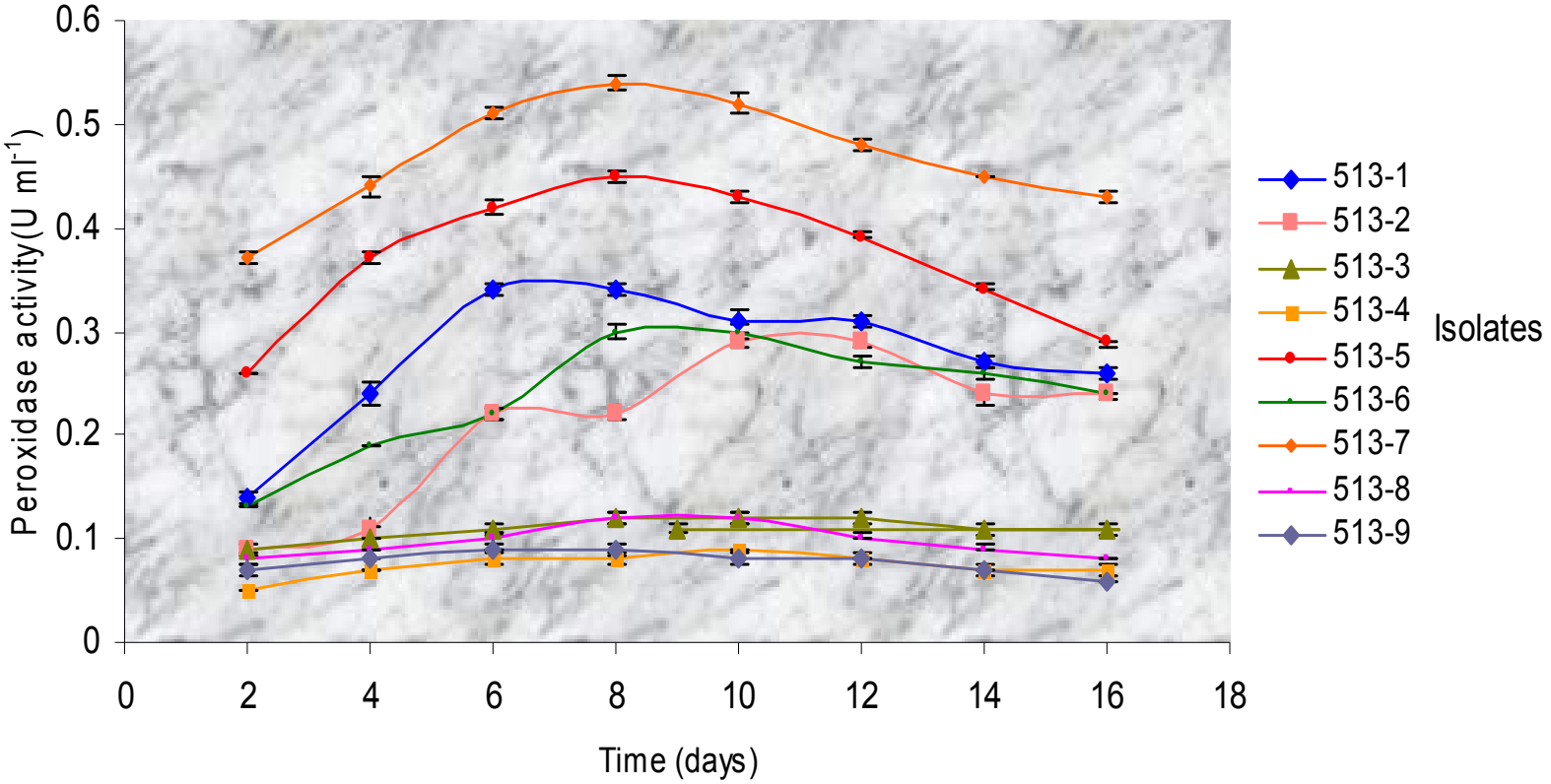


**Figure 3.14: Effects of incubation time on peroxidase production in basal salts-yeast extract medium supplemented with 0.6% corn. Data are presented as mean of three replicates with error bars indicating standard deviations (STDEV).**

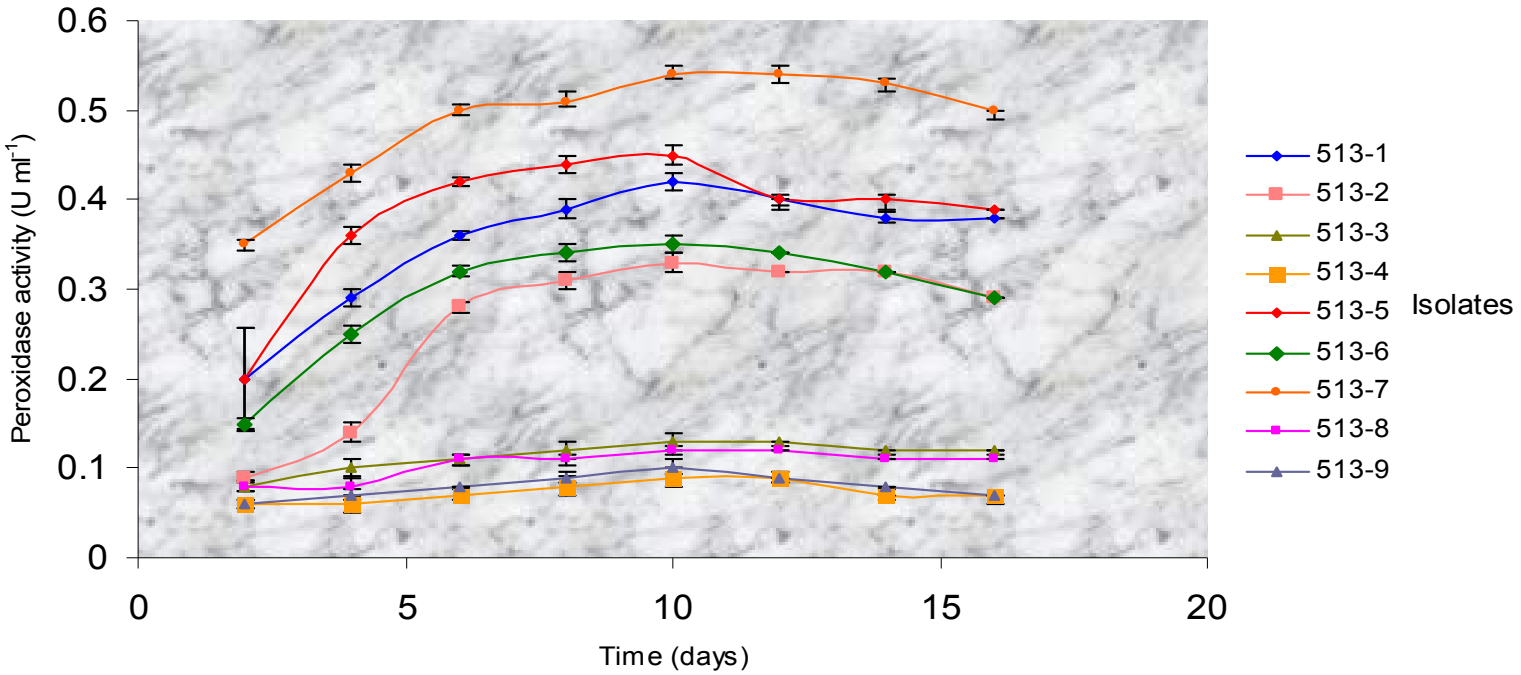


**Figure 3.15: Effects of incubation time on peroxidase production in basal salts-yeast extract medium supplemented with 0.6% grass. Data are presented as mean of three replicates with error bars indicating standard deviations (STDEV).**

### 3.8.2. Effect of substrate concentration:

For estimation of substrate concentration on the production of extracellular peroxidase, concentrations of two components in the media (corn and grass) were investigated. Peroxidase activity was detected optimum at 0.6% corn for all isolates. The activity did not fall drastically on concentration beyond 0.6%, i.e., at 0.8% and 1.0% but was lesser than that observed on the concentration showed high activity (Figure 3.16).

Grass was also a good inducing substrate with the maximum production of peroxidase was detected on minimal salt media supplemented with 1.0% grass.

Different response was observed when the concentration of corn and grass analysed. There was not continuous pattern of activity for corn, attaining high at 0.6 % and then declining after that. Although the decrease in activity was observed beyond the 0.6% but remained at almost constant level at 0.8% and 1.0% substrate concentration (Figure 3.16).

When grass was used as a substrate continuous pattern of peroxidise activity was observed, drastically increasing from 0.6 % and maintaining the same level at 0.8% and 1.0 % substrate concentration (Figure 3.17). This response is in contrary when corn was used as a substrate. Optimum activity was observed when 0.6% concentration of corn was used and beyond that value i.e., at 0.8% and 1.0% peroxidise activity was declined.

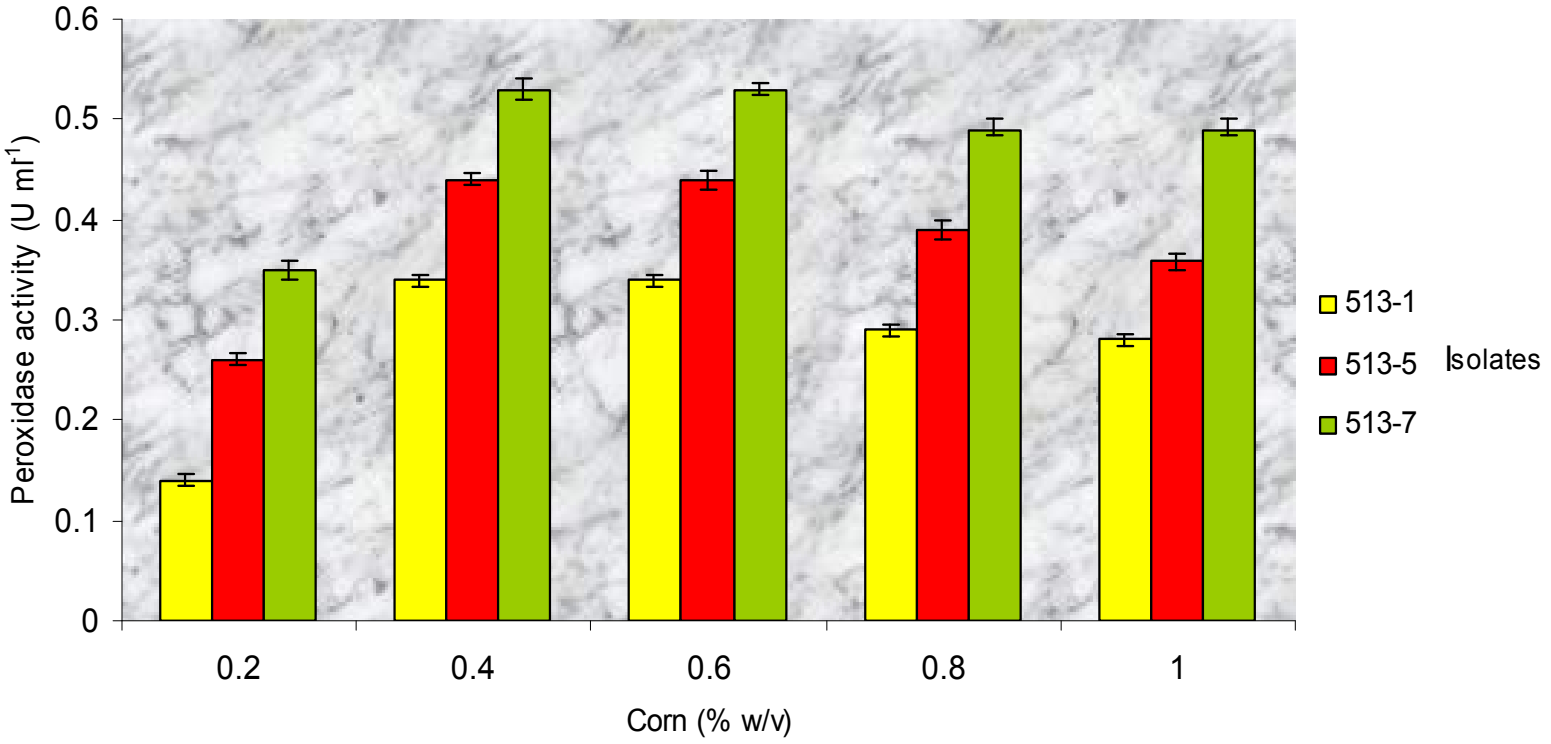


**Figure 3.16: Effects of corn concentration (0.2-1.0% w/v) on the production of extracellular peroxidase in basal salts-yeast extract medium. Data are presented as mean of three replicates with error bars indicating standard deviations (STDEV).**

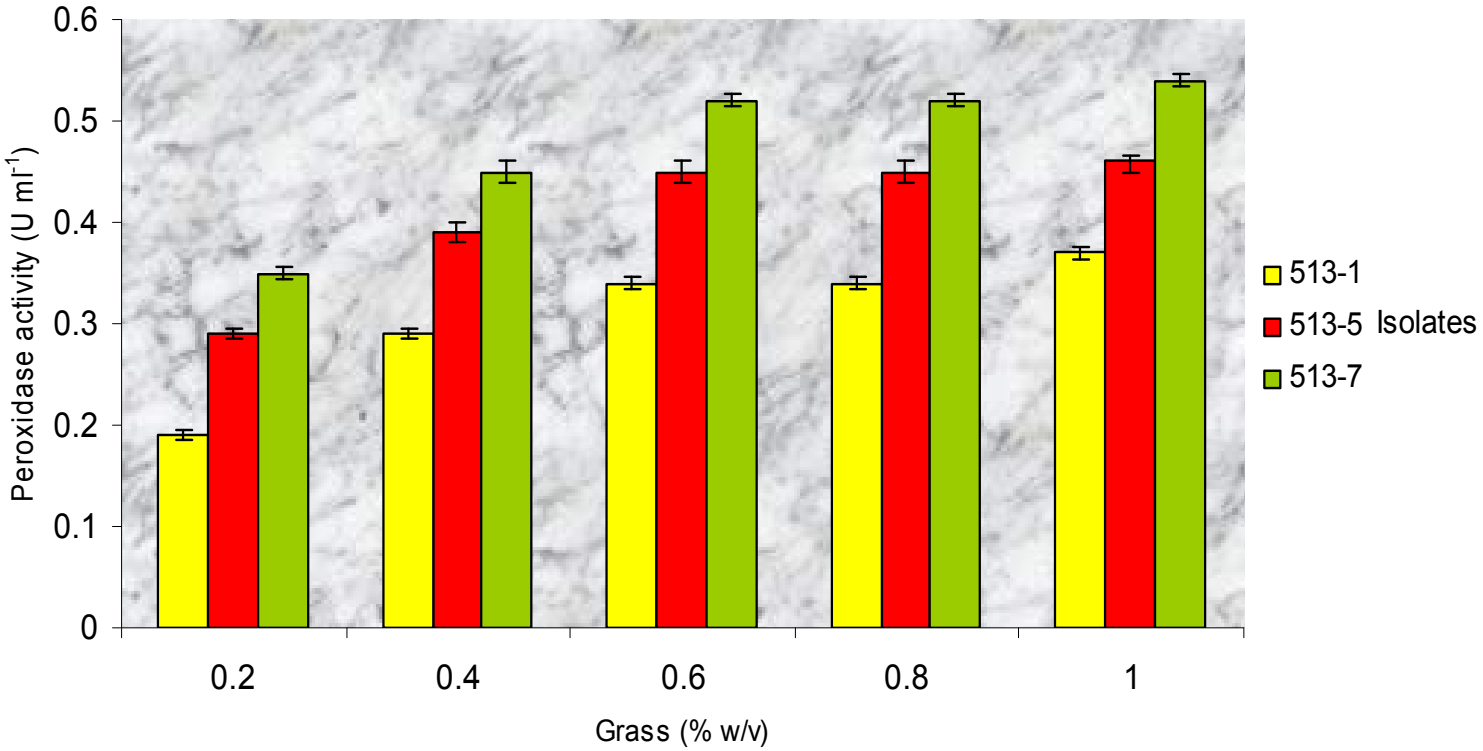


**Figure 3.17: Effects of grass concentration (0.2-1.0% w/v) on the production of extracellular peroxidase in basal salts-yeast extract medium. Data are presented as mean of three replicates with error bars indicating standard deviations (STDEV).**

### 3.8.3. Effect of pH on peroxidase activity:

The pH range (5.5 - 9.5) was analysed to determine the optimum peroxidase activity in the minimal salt-yeast extract medium supplemented with corn. The optimum pH values were found to be 7.5 and 8.0.

The relative production of peroxidase (97.5 % and 89 %) were detected at pH 8.0 and 8.5 respectively for isolate 513-1(FJ966265).While relative peroxidase activity (100% and 97 %) were observed at pH 8.0 and 8.5 respectively for isolate 513-5 (FJ966269). Lower values were observed at pH 5.5 and 6.0. The pH value started to decline at 9.0 and beyond that value. Although the activity values at pH 9.0 and 9.5 were lower but were not statistically different from the values at pH 7.5 (Figure 3.18).

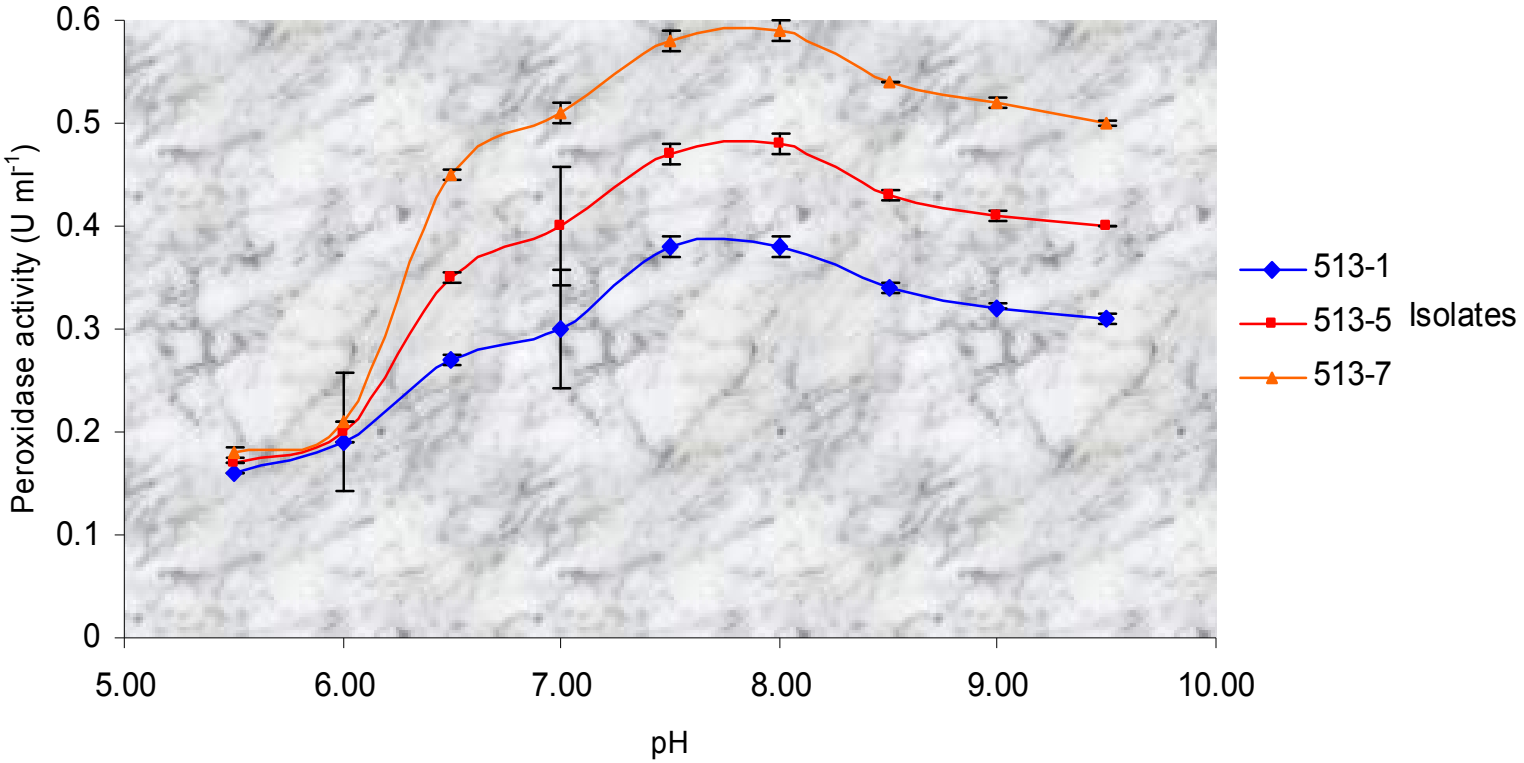


**Figure 3.18: Effects of pH on the activity of peroxidase grown in minimal salt medium supplemented with grass (0.6% w/v). Data are presented as mean of three replicates with error bars indicating standard deviations (STDEV).**

# Discussion

## 4.1. Comparison of PCR and Culture

The results of morphological and biochemical studies clearly placed these isolates in the genus *Streptomyces*. The tested strains were the subject of computer assisted identification using the PIBWin software package (Bryant, 2004) using the phenetic characteristics database generated by Langham *et al*. (1989). The advantage of this software is that it is equipped with statistical packages that calculate the test error and determines the similarity index (SI) values.

It is important to use the Wilcox score algorithm based on a Baysian model (Priest and Williams, 1993; Langham *et al*., 1989). They emphasized the use of these matrices to avoid the assignment of fresh isolates to the incorrect taxon. Bascomb *et al*. (1973) suggested the use of 0.99 threshold value for successful identification but, according to Williams *et al*. (1983), this value is too stringent for the genus *Streptomyces* in which several species are likely to be heterogenous and suggested the threshold value of 0.95.

It was evident from Table 3.13 that no isolate could be assigned to any of the known *Streptomyces* clusters when the results of Wilcox probability analysis were considered. However some isolates such as strain 513- 5 (Wilcox score, 0.89), strain 513- 3 and 513-8 (Wilcox score 0.9 and 0.91 respectively) showed scores very close to the threshold value (Wilcox score 0.95). It is therefore inferred from the study of numerical phenetic identification that the strains showed no apparent relationship with the clusters of Williams *et al*. (1983) with the exception of few strains. Chun *et al*. (1997) also reported that, although the Wilcox score represents the relative relationship of a test strain to the cluster in a given identification matrix, this does not indicate absolute closeness to each cluster.

It was evident from the results of cultural characteristics presented in Table 3.1-3.9 that characteristics varied with different media. These features are known to be typical of *Streptomyces* (Williams *et al*., 1983).

Statistical analysis Minitab (Minitab, 2007) was used to establish uniqueness of isolates based on the morphological and biochemical tests reported in the category

IV of Bergey's Manual of Systematic Bacteriology (Locci, 1989) and cluster analysis of Williams *et al*. (1983).

The analysis depicted that certain isolates were similar, reducing the number from 9 to potentially 5. From the dendrogram it was evident that strains 513-3 and 513-8 showed 100% similarity while strains 513-4 and 513-9 showed 92.5% similarity level. Strain 513-5 showed low level of similarity to other strains (59%).

The same statistical strategy was applied in order to establish similarity to isolates already reported. Each isolate was compared with the corresponding species listed in major and minor cluster of Williams *et al*. (1983). This analysis depicted that 4 isolates had more than 90% similarity with one, two or three reported species. Isolates 513-3 and 513-8 showed a comparatively high similarity (92.3%) to *S. luridus* and isolate 513-4 showed 96.4% with *S. xanthochromogenes*.

The 16s rRNA gene was chosen as the target gene for PCR characterization and sequencing aiming at detection of possible *Streptomyces* presence in the samples. The advantage of rRNA genes as target of PCR detection is that these genes are essential for the cells and are conserved among the prokaryotes (Kim *et al*., 1993). *Streptomyces* spores are readily cultivable from environmental samples and medium selectivity can affect growth (Goodfellow and Simpson, 1987). As evident from the results that cultural characteristics differed from results of phylogenetic analysis with the exceptions of few. Similar results were also reported by other workers (Bentley *et al*., 2002). These authors explained that 16S rRNA genes are located in the conserved part of *Streptomyces* chromosome. While genes coding for morphological, physiological characteristics such as pigmentation and production of extra-cellular enzymes are often located in the chromosome arms which can undergo rearrangements.

Therefore the 16S rRNA gene (16S rDNA) sequence diversity does not reflect the diversity of other properties. Metsa-Ketala *et al*. (2002) compared the phylogenetic trees obtained from 16s rRNA and polyketide synthase gene (involved in biosynthesis of secondary metabolites) sequence of 99 actinomycetes isolated from soil and found no correlation between the two trees.

Strains 513-1 (FJ966265) and 513-2 (FJ966266) showed relatively high 16s rRNA gene sequence similarity values with the type strains of *Streptomyces sporoclivatus*

(97.8%), *S. hygroscopicus* (97.8%) and *S. yatensis* (97.8%). Whereas strains 513-3 (FJ966267) and 513-8 (FJ966272) exhibited levels of similarity (97.2%) with *S. thermocarboxydus*, *S. albogriseolus* and *S. lusitanus*. Strains 513-4(FJ966268) and 513-9 (FJ966273) were found closest to the type strains of *S. paresii*, *S. fulvorubens* and *S. annulatus* (similarity value of 97.0%). Whereas strain 513-5 (FJ966269) showed its similarity value of 95.8% with the type strains of *S. enhygrus*. Strain 513-6 (FJ966270) shared the value of 97.5% with *S. aureus*. While strain 513-7 (FJ966271) exhibited similarity value of 97.6% with the type strains of *S. humerifus*, *S. rubrogriseus* and *S. caesius*.

16s rRNA sequences of the strains were aligned with those of the type strains from the database and subjected to phylogenetic analysis using neighbour-joining method (Saito and Nei, 1987).

When the distribution of variability along the genome was assessed with an entropy plot (Figure 3.10.) it appeared that most variable sites concentrated in 50-200 and 900-1450 nt positions. The variable regions are suitable for the discrimination of *Streptomyces* species. According to Kataoka (1997) sequence analysis of highly variable regions has been applied to *Streptomyces* species identification but has not been able to differentiate to species level.

**4.2. Melanoid pigments detection:**

Production of pigments in *Streptomyces* is sufficiently distinct for delineation in cultures together with other fundamental characteristics like sporophore morphology, colour of the surface mycelium (Dastager *et al*., 2006). It has also been observed that actinomycetes particularly *Streptomyces* synthesize and excrete dark pigments, melanin, which are considered to be a useful criterion for systematic studies (Dastager *et al*., 2006).

Their study also concluded that it could be difficult to determine whether diffusible pigments produced are melanin or just a brown substance particularly when complex organic media are used.

In order to avoid that discrepancy presence of melanin was assayed in sub -merged conditions rather than on agar media in this study. Only 4 out of 9 strains formed melanin and strains found negative for melanin were found previously positive for melanin when grown on solid media. The possible reason for this phenomenon could

be the formation of brown substance on solid media could not be attributed to melanin formation. Our results are in agreement with the findings of Dastager *et al*. (2006). The present study reveals that the method of testing melanin production by L- dopa as a substrate is the good criterion for the identification and classification of *Streptomyces*.

Significant differences were not observed between when glucose and lactose were used as carbon source ($P > 0.05$). Post hoc analysis showed significant differences only with the values of starch. Starch was found to be the most effective carbon source for melanin formation in strains 513-1, 513-2, 513-3 and 513-8 followed by glycerol and fructose. ANOVA showed significant differences when nitrogen sources were used ($P < 0.05$). Post hoc analysis showed that the best values were found with arginine and glutamate for strains 513-1, 513-2, 513-3 and 513-8.

Among the three active lignocellulolytic strains, 513-5 (FJ966269) were subjected to comparative analysis with those of closely related type strains detected in the phylogenetic analysis. The differential morphological and pigmentation features are specially significant for the delineation of members of phylogenetically related Streptomycete species (Manfio *et al*. 2003).

The aerial mass colour of *Streptomyces longispororuber*, *S. antimycoticus* and *S. rutgersensis* were different from those of the strain 513-5 (FJ966269) on 4 different International *Streptomyces* Project media (ISP). In physiological characteristics starch and casein hydrolysis of *S. antimycoticys* are different from those of the strain 513-5 (FJ966269). Differences were also observed in carbon utilisation of dextran and xylose (Table 3.13).

Considering comparative studies of strain 513-5 (FJ966269) with type cultures of the species described above, strain 513-5(FJ966269) was most closely related to S. *antimycoticus*. However there were also differences in cultural and physiological characteristics. Such differences were not enough to establish a new species in the genus *Streptomyces* however it seems appropriate to present this strain as a possible candidate for subspecies.

### 4.3. Strains response to natural substrates:

Under the experimental conditions, the detection of peroxidase activity in non-concentrated supernatant fluid is uncommon because of low level of secretion. However in order to optimise the environmental conditions these studies provide key physiological and nutritional aspects for better understanding of the phenomenon (Trigo and Ball, 1994).

Our results indicate that the production of extracellular enzymes involved in lignocellulose degradation (corn and grass as sole source) was growth associated in culture of different isolates. This is in accordance with previous work for *Streptomyces thermoviolaceus* (Iqbal *et al*., 1994), *Thermomonospora fusca* (Rob *et al*., 1996), *S. viridosporus* (Zerbini *et al*., 1999) all reported that the production of extra cellular lignocellulose degrading enzymes were growth associated in actinobacteria cultures.

Differences in the time of appearance of peroxidase enzymes during growth of *Streptomyces* sp. in corn and grass containing media suggested that initial degradation of corn was rapid, whereas the grass was degraded more slowly

The optimum pH for the production of extracellular peroxidise by our isolates is similar to those reported for *S. badius* (Adhi *et al*., 1989), *T. fusca* (Tuncer *et al*., 1999) and generally in accordance with actinobacteria physiology (McCarthy 1987).

However the optimum pH for peroxidase was found to be in the range of 7.5 to 9.0. This is comparatively higher than the pH range observed for *S. viridosporus* (5.5 to 7.5; Lodha *et al*., 1991), *S. thermoviolaceus* (6.5 to 7.0; Iqbal *et al*., 1994). This suggests that enzyme produced by these strains may be useful for the treatment of alkaline effluents.

From this work optimum conditions for the production of extracellular peroxidase from our isolates can be summarised as:

- Production of extracellular peroxidase increased during growth phase of culture between 6-8 days for corn and 10 for grass. This indicated that production of extracellular peroxidase is growth associated.
- The optimum pH for the production of peroxidase was found to be at pH 7.5-8.5

- The maximum production of peroxidase by strains (513-1, 513-5 and 513-7) occurred when minimal salts-yeast extract media contained corn and grass as sole carbon and energy sources. Under these conditions sufficient enzyme activities were produced to allow detection in non concentrated supernatant fluid.

The capacity of the enzyme to oxidize chlorophenol (2,4-dichlorophenol) from three selected *Streptomyces* isolates suggests the possible use of enzyme in the treatment of effluents from chemically bleached pulp. Most importantly, peroxidase produced by these isolates could be considered as a treatment of alkaline pH effluents where known peroxidases from other species would not be applicable.

Furthermore different efforts have been devoted to the Streptomycete systematics, but we found that still diversity exists in the criteria used for their classification. It would be useful to unify criteria for such purposes even if the diversity of the criteria used is accepted. Despite this, their taxonomy remains somewhat confused and the definition of species is unresolved due to the variety of morphological, cultural, physiological and biochemical characteristics that are observed at both inter- and intraspecific level. It is also worth considering the value of new technologies, while maintaining traditional methodologies that still have value for species applications such as phenotypic identification of species using the probabilistic identification matrix. For a new taxonomic method to be validated a universal standard selection of strains should be used by all groups doing these types of investigations.

# 5. Future perspectives

Developments in modern bacterial taxonomy relied on several fundamental processes such as:

- Generation of quality information
- Development of suitable data handling techniques
- Compilation and management of taxonomic databases

It is believed that the success of numerical phonetic taxonomy partly based on the availability of computers and on the development of a series of computer programs (Sneath and Langham, 1989). It is therefore cardinal that cost effective and suitable software should be developed to handle information in systematic databases.

With respect to the strains studied in this work, further assays are in progress in order to establish the comprehensive classification and identification of these strains. A more exhaustive analysis of the lignocellulolytic system of these strains, employing more concentrated cell extracts and supernatants as well as different cultural conditions and separation techniques will also be performed.

Topics which need further investigation in light of present study are outlined below:

- Upon successful isolation, further analyses would be performed with isolated strains in order to explain specific and comprehensive roles in lignocellulose degradation.
- A next step would be to use species-specific amplification of 16S rRNA genes along with Terminal Restriction Fragment analysis (TRF) and alternative methods like Fluorescent in situ hybridization (FISH) to establish the comprehensive identification of organisms potentially involved in lignocellulose degradation.

In future, global systematic databases together with suitable data handling techniques will prove to be invaluable fo all biologists not only for systematists.

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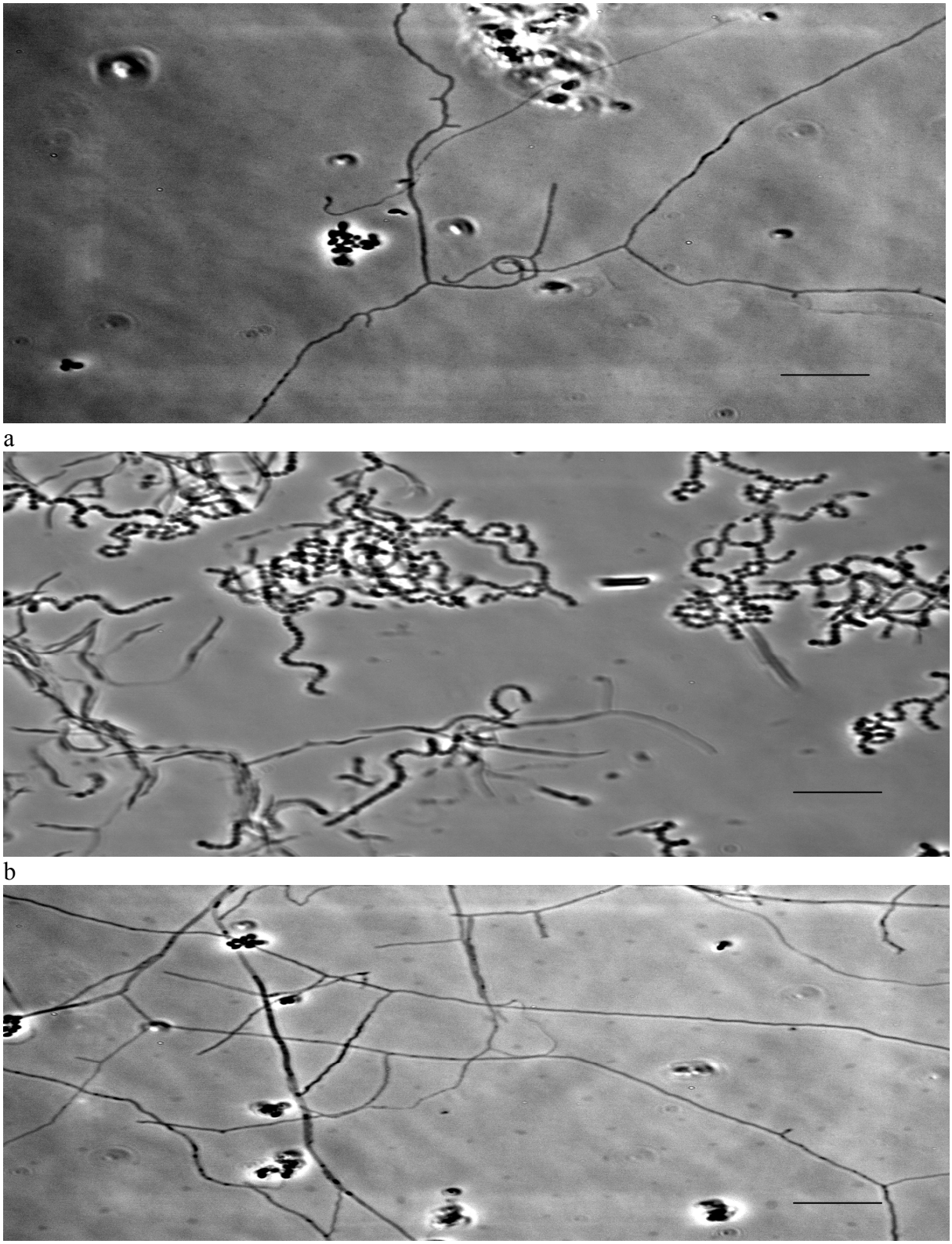
## APPENDIX

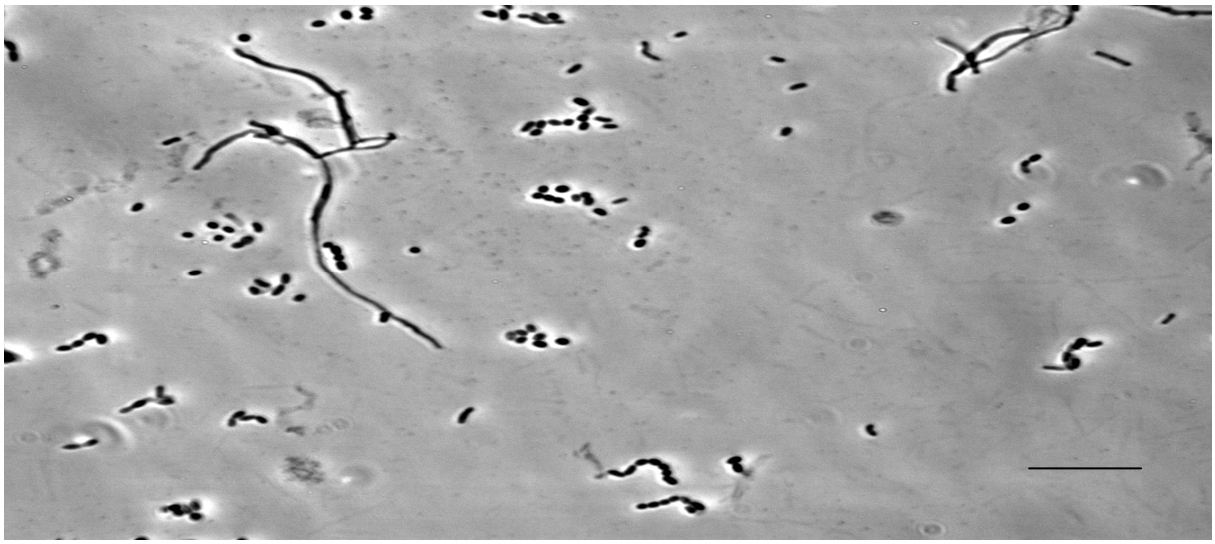


a

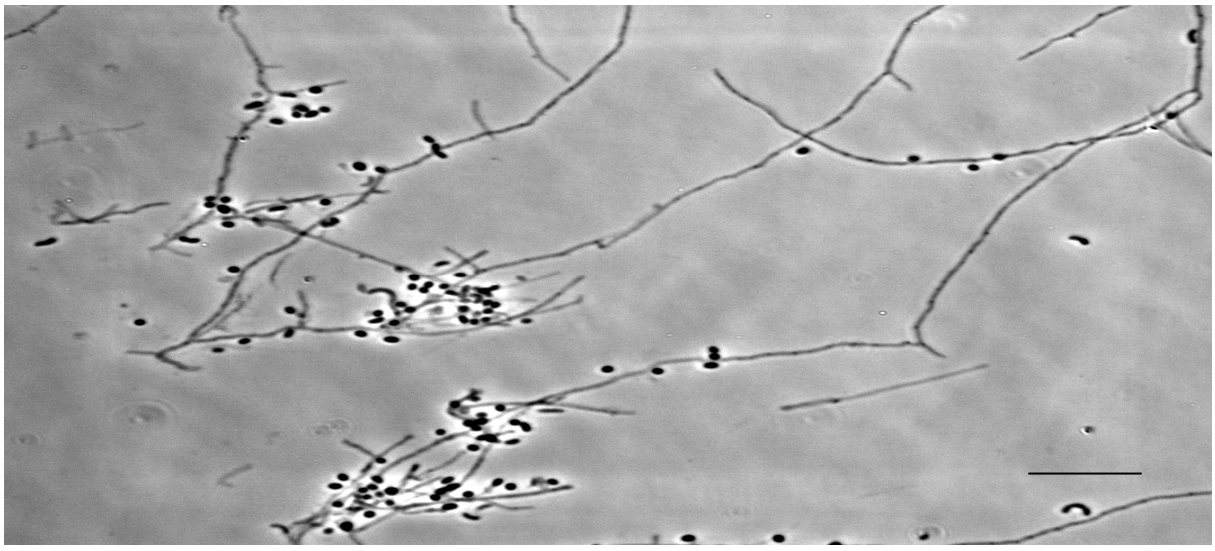
b

c

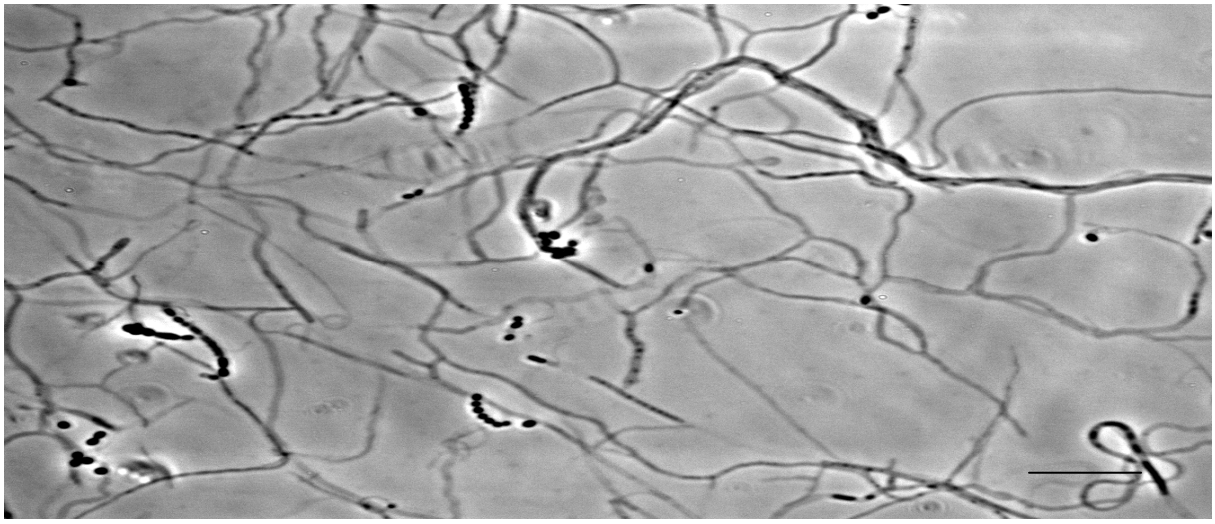
Figure : Phase contrast micrograph of spore chains of isolates. a, isolate 1; b, isolate 2; C, isolate 3 (100 x), bar =10µm



d

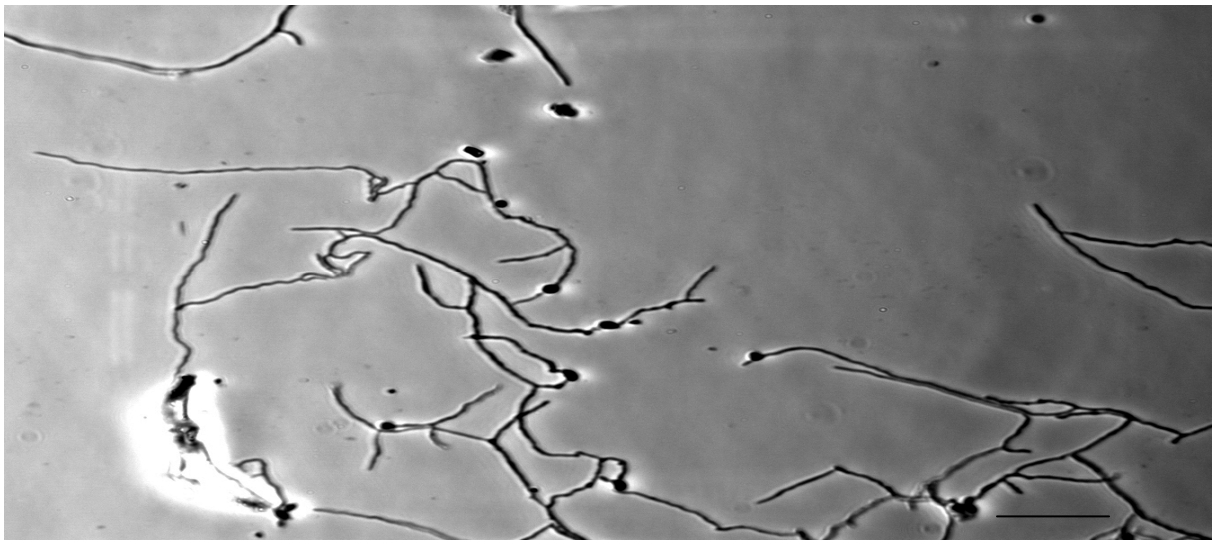


e

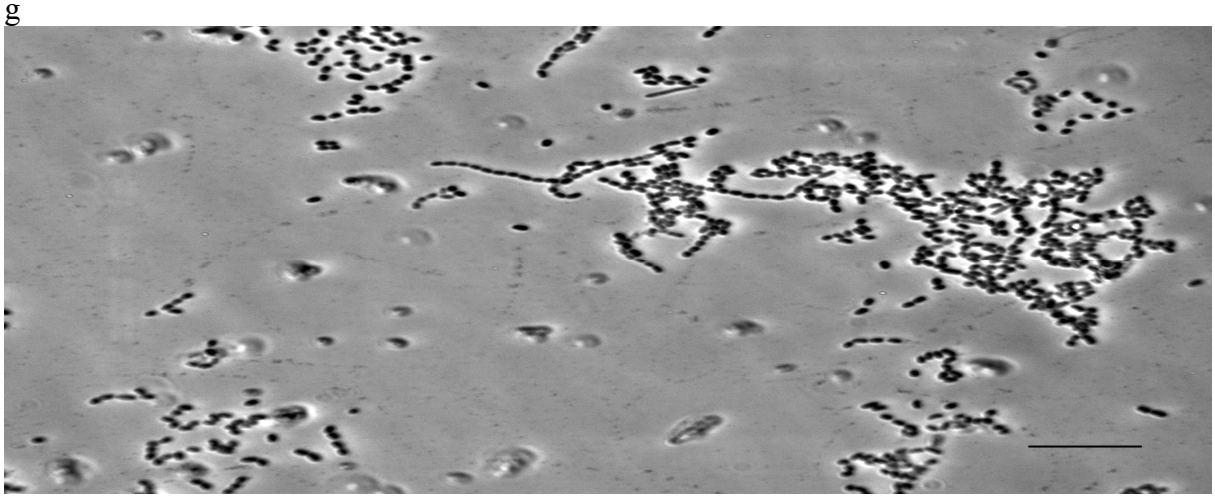


f

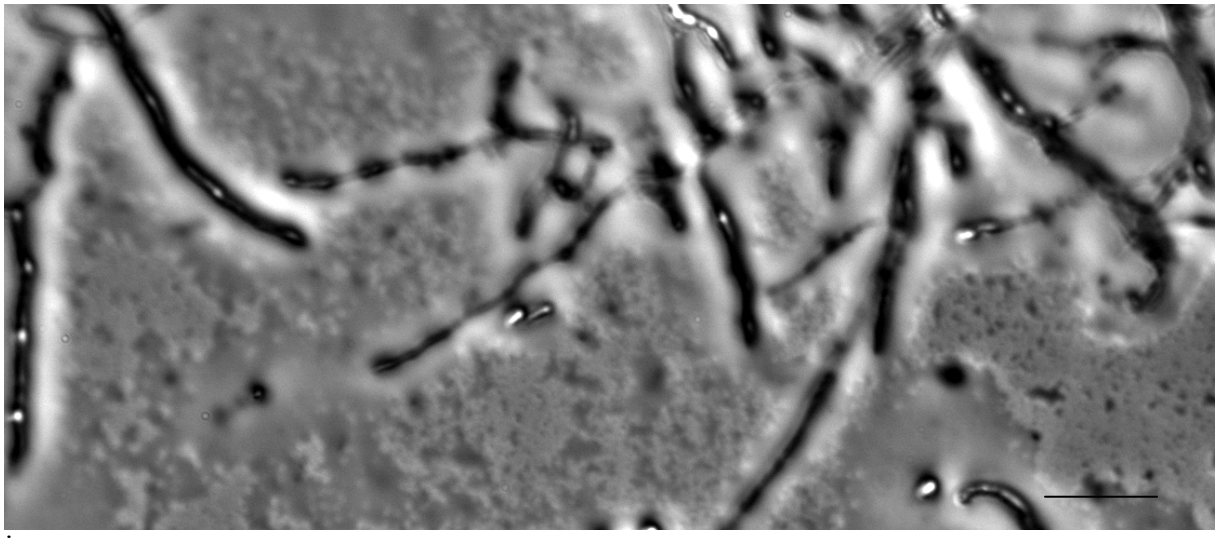
Figure : Phase contrast micrograph of spore chains of isolates. d, isolate 4; e, isolate 5; f, isolate 6 (100 x), bar =10µm



g



h



i

Figure : Phase contrast micrograph of spore chains of isolates. g, isolate 7; h, isolate 8; i, isolate 9 (100 x), bar =10µm

**Culture media**

**1. ISP Medium 2 (Yeast extract- malt extract agar)**

| | |
|---|---|
| Yeast extract | 4.0 g |
| Malt extract | 10.0 g |
| Dextrose | 4.0 g |
| Distilled water | 1.0 l |
| Agar | 20.0 g |
| pH | 7.3 |

The medium components were dissolved in 1 l of cold distilled water and the pH adjusted with 1M NaOH or HCl. The agar was then added and sterilized by autoclaving.

**2. ISP Medium 3 (Oatmeal agar)**

| | |
|---|---|
| Oatmeal | 20.0 g |
| Agar | 18.0 g |
| pH | 7.2 |

Cooked 20.0 g oatmeal in 1 l of distilled water for 20 minutes. Filtered through cheese cloth. Then added 18.0 g agar and made up to 1 l of distilled water. Then added 1 ml of trace salt solution *.

*** Trace salts solution:**

| | |
|---|---|
| $FeSO_4$ | 0.1 g |
| $MnCl_2$ | 0.1 g |
| $ZnSO_4$ | 0.1 g |
| Distilled water | 100.0 ml |

**3. ISP Medium 4 (Inorganic salts-starch agar)**

| | |
|---|---|
| Soluble starch | 10.0 g |
| $K_2HPO_4$ | 1.0 g |
| $MgSO_4.\ 7H_20$ | 1.0 g |
| NaCl | 1.0 g |
| $(NH_4)_2SO_4$ | 2.0 g |
| $CaCO_3$ | 2.0 g |

| | |
|---|---|
| Trace salts solution* | 1.0 ml |
| Distilled water | 1.0 l |
| Agar | 20.0 g |
| pH (Unadjusted) | 7.2 |

*** Trace salts solution:**

| | |
|---|---|
| $FeSO_4$ | 0.1 g |
| $MnCl_2$ | 0.1 g |
| $ZnSO_4$ | 0.1 g |
| Distilled water | 100.0 ml |

**4. ISP Medium 5 (Glycerol-asparagine agar)**

| | |
|---|---|
| Asparagine | 1.0 g |
| Glycerol | 10.0 g |
| $K_2HPO_4$ | 1.0 g |
| Trace salts solution | 1.0 ml |
| Distilled water | 1.0 l |
| Agar | 20.0 g |
| pH | 7.3 |

The medium components were dissolved in 1 l of cold distilled water and the pH adjusted with 1M NaOH or HCl. Agar was then added and sterilized by autoclaving.

**5. Basal mineral salts agar (Pridham and Gottlieb, 1966)**

| | |
|---|---|
| $(NH_4)_2SO_4$ | 2.64 g |
| $KH_2PO_4$ | 2.38 g |
| $K_2HPO_4$ | 5.65 g |
| $MgSO_4.\ 7H_20$ | 1.0 g |
| Trace salts solution (Pridham and Gottlieb)* | 1.0 ml |
| Distilled water | 1.0 l |
| Agar | 15.0 g |
| pH | 7.0 |

*** Trace salts solution:**

| | |
|---|---|
| $CuSO_4.\ 5H_20$ | 0.64 g |
| $FeSO_4$ | 0.11 g |

| | |
|---|---|
| $MnCl_2$ | 0.79 g |
| $ZnSO_4$ | 0.15 g |
| Distilled water | 100.0 ml |

The medium components were dissolved to1 l cold distilled water and the pH adjusted with 1M NaOH or HCl. Agar was then added and sterilized by autoclaving.

**6. Bennett's agar**

| | |
|---|---|
| Yeast extract | 1.0 g |
| Beef extract | 1.0 g |
| N-Z amine | 2.0 g |
| Dextrose | 10.0 g |
| Distilled water | 1 l |
| Agar | 15.0 g |
| pH | 7.3 |

The medium was dissolved in 1 l of distilled water and pH was adjusted by using 1M NaOH or HCl. The agar was then autoclaved. The nitrogen source compounds were sterilised separately by filteration.

**7. ISP Medium 9 (Carbon utilization medium)**

| | |
|---|---|
| $(NH_4)_2SO_4$ | 2.64 g |
| $KH_2PO_4$ | 2.38 g |
| $K_2HPO_4$ | 5.65 g |
| $MgSO_4.\ 7H_20$ | 1.0 g |
| Trace salts solution (Pridham and Gottlieb)* | 1.0 ml |
| Distilled water | 1.0 l |
| Agar | 15.0 g |
| pH | 7.0 |

*** Trace salts solution:**

| | |
|---|---|
| $CuSO_4.\ 5H_20$ | 0.64 g |
| $FeSO_4$ | 0.11 g |
| $MnCl_2$ | 0.79 g |
| $ZnSO_4$ | 0.15 g |

| | |
|---|---|
| Distilled water | 100.0 ml |

The medium was dissolved in 1 l of distilled water and pH was adjusted by using 1M of NaOH or HCl. Aagar was then added and autoclaved. The carbon source compounds were sterilised separately by filtration.

**Table . Numerical phenetic identification of isolates (513-1 to 513-9) based on the probabilistic matrix of Williams *et al*. (1983) as arranged by Langham *et al*. (1989)**

| Cluster (species) | Identification scores* of isolates | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 513-1 | 513-2 | 513-3 | 513-4 | 513-5 | 513-6 | 513-7 | 513-8 | 513-9 |
| **Major clusters (26 clusters)** | | | | | | | | | |
| *S. cyaneus* | 0.465 | 0.145 | 0.333 | 0.012 | - | - | 0.253 | 0.118 | - |
| *S. fulvissimus* | - | - | 0.002 | 0.056 | - | 0.013 | 0.102 | - | 0.008 |
| *S. atroolivaceous* | - | - | - | 0.045 | - | - | - | - | 0.053 |
| *S. rochei* | 0.165 | 0.533 | 0.002 | 0.012 | 0.101 | 0.143 | 0.002 | 0.001 | 0.003 |
| *S. exfoliatus* | - | - | - | - | - | 0.036 | 0.414 | 0.012 | - |
| *S. olivaceoviridis* | 0.137 | 0.244 | 0.017 | 0.013 | - | 0.046 | - | 0.005 | 0.004 |
| *S. halstedii* | 0.079 | 0.006 | - | 0.036 | 0.892 | 0.325 | - | 0.002 | 0.030 |
| *S. phaeochromogenes* | 0.041 | 0.042 | - | - | - | - | 0.093 | - | - |
| *S. chromofuscus* | 0.015 | 0.003 | 0.462 | - | 0.003 | 0.044 | - | 0.753 | - |
| *S. microflavus* | 0.013 | - | 0.001 | 0.004 | - | 0.077 | 0.112 | 0.002 | - |
| *S. diastaticus* | 0.011 | 0.001 | 0.006 | 0.051 | - | 0.113 | 0.008 | 0.002 | 0.010 |
| *S. antibioticus* | 0.006 | 0.016 | 0.149 | - | 0.001 | 0.143 | 0.004 | 0.099 | 0.004 |
| *S. griseoruber* | - | 0.001 | - | - | - | - | 0.002 | - | - |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *S. filipinensis* | - | - | - | - | - | - | 0.004 | - | - |
| *S. annulatus* | - | - | - | 0.515 | - | - | - | - | 0.508 |
| *S. violaceoniger* | - | - | 0.003 | 0.237 | - | 0.042 | - | 0.001 | 0.087 |
| **Minor clusters (28 clusters)** | | | | | | | | | |
| *S. californicus* | 0.523 | 0.69 | - | - | - | - | - | - | - |
| *S. nogalater* | 0.010 | - | - | - | - | - | - | - | - |
| *S. roseus* | - | - | - | 0.006 | - | - | - | - | 0.006 |
| *S. griseoluteus* | - | - | - | - | 0.641 | 0.013 | 0.421 | - | - |
| *S. aureofaciens* | - | - | - | - | - | 0.659 | 0.009 | - | - |
| *S. luridus* | - | - | 0.913 | 0.102 | - | 0.016 | - | 0.913 | - |
| *S. xanthochrom ogenes* | - | - | 0.007 | 0.881 | - | - | - | - | - |

Legend: *, Based on Wilcox score, generated by using the simple matching coefficient algorithm ($S_{SM}$) of Computer program PIBWin (Bryant, 2004).