Polyphasic systematics of marine bacteria and their alpha-glucosidase inhibitor activity

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By

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This is to certify that the work incorporated in this Ph.D. thesis entitled "**Polyphasic systematics of marine bacteria and their alpha-glucosidase inhibitor activity**", submitted by Mr. Rahul Bholeshankar Mawlankar to Academy of Scientific and Innovative Research (AcSIR) in fulfillment of the requirements for the award of the Degree of Doctor of Philosophy, embodies original research work under my/our supervision/guidance. I/We further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table etc., used in the thesis from other sources, have been duly cited and acknowledged.

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Declaration

I hereby declare that the work of the thesis entitled,

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(Rahul B. Mawlankar)

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List of abbreviations

Abbreviation	Full name
AFLP	Amplified fragment length polymorphism
AGI	Alpha-glucosidase inhibitor
AL	Aminolipid
ANI	Average Nucleotide Identity
anti-SMASH	Antibiotics & Secondary Metabolite Analysis Shell
APL	Aminophospholipid
AP-PCR	Arbitrarily primed PCR
BLAST	Basal Local Alignment Search Tool
BOX-PCR	BOX repetitive element
CAG	Cyclitol containing aminoglycoside
CFM-ID	Competitive Fragmentation Modeling For Metabolite Identification
CTAB	Hexadecyltrimethyl ammonium bromide
DDBJ	DNA Data bank of Japan
DDH	DNA-DNA hybridization
DNA	Deoxyribose nucleic acid
DNJ	1-deoxynojirimycin
dNTPs	deoxyribonucleotide triphosphate
DPG	Diphosphatidylglycerol
Dpm	2,6-diaminopimelic acid
EMBL	European Molecular Biology Laboratory
ENA	European Nucleotide Archive
ERIC-PCR	Enterobacterial repetitive intergenic consensus sequences-PCR
FAME	Fatty acid methyl esters
FTIR	Fourier- Transformed Infrared Spectroscopy
G+C	Guanine+Cytosine content
GBDP	Genome Blast distance phylogeny
GC	Gas chromatography
GL	Glycolipid
Glc-HNJ	7-O-β-D-glucopyranosyl-α-homonojirimycin
GLP-1	α-endogenous glycogen like peptide-1
HNJ	1,2-dideoxynojirimycin, α-homonojirimycin
HPLC	High Performance Liquid Chromatography
IC_{50}	Half maximum inhibitory concentration
IR	Infrared
JGI	Joint Genome Institute
MALDI-TOF	Matrix Assisted Laser Desorption/ Ionization Time-of-Flight
MEGA	Molecular evolutionary genetics analysis
MLST	Multilocus sequence typing
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MUMi	Maximal unique matches index
NCBI	National Center for Biotechnology Information
OH-Dpm	2,6-diamino-3-hydroxypimelic acid

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PG	Phosphatidylglycerol
PGL	Phosphoglycolipid
PI	Phosphatidylinositol
PIM	Phosphatidylinositol mannoside
PL	Phospholipid
pNPG	para-nitrophenyl alpha-D-glucopyranoside
RAPD	Randomly amplified polymorphic DNA
RBR	Relative binding ratio
REP-PCR	Repetitive extragenic palindromic-PCR
RFLP	Restriction fragment length polymorphism
RP	Reverse phase
rRNA	Ribosomal RNA
SDS	Sodim dodecyl sulfate
SSC	Saline sodium citrate
SSU rRNA	Small subunit of ribosomal RNA
TE	Tris- Ethylenediaminetetraacetic acid
TLC	Thin layer chromatography
T_m	Melting temperature
T-RFLP	Terminal restriction fragment length polymorphism
ΔT_m	Difference in the melting temperature

Abstract

Microbes are the most diverse group of organisms present on the earth. Microbial diversity is the variety and variability of an organism's and the ecosystem in which they occur. Microbes have been adapted to global environmental changes as and when it happened on the earth. The enormous microbial diversity also gives rise to a largely untapped amount of genetic information, bioactive compounds and biomaterials which could deliver important beneficial traits and applications of societal interest, for example, to improve the medical treatments, fisheries and aquaculture applications, supply of energy for the development of industrial products and processes. It has been estimated that, only one to five per cent of microorganisms have been studied extensively. Identification of a variety of microorganisms is utmost important as it describes the positioning of an organism into the phylogenetic group and various biotechnological applications are dependent on known microorganisms.

Microbial taxonomy is the branch of science that deals with the study of identification, classification and nomenclature of an organism. It classifies and arranges the microorganisms into taxonomical order. With the advancement of molecular, chromatographic and microscopy techniques, knowledge and the number of well characterized microorganisms in taxonomy has been increased in the past two decades. Polyphasic systematics is the recent approach used in the identification of bacteria. It comprises phylogenetic, phenotypic, genotypic and chemotaxonomic methods. This thesis deals with the study of the identification of microorganisms isolated from the sediment sample of Chorao Island, Goa and investigation of these bacteria for their α-glucosidase inhibition activities.

Chapter 1: Introduction

This chapter covers the literature on scope of bacterial systematics, polyphasic approach for the identification of bacteria, potentials of marine bacteria, types of marine sediments, types of alpha-glucosidase inhibitors and alpha-glucosidase inhibitors from marine isolates. This chapter concludes with the objectives and scope of the thesis.

Chapter 2: Polyphasic systematic approach

This chapter focused on the isolation and identification of bacteria from the sediment sample of Chorao Island, Goa. Sixty four unique isolates have been isolated and further characterized with the help of the polyphasic approach. The bacteria were characterized using 16S rRNA gene sequencing, morphological, physiological, biochemical, DNA G+C content, DNA-DNA hybridization, AP-PCR and chemotaxonomical. In this present study, we have been succesfully identified and published ten novel species from the sediment sample using polyphasic taxonomy approach.

Chapter 3: Screening, isolation and identifiaction of alpha-glucosidase inhibitors from marine novel bacteria

Thic chapter focused on screening the marine bacteria for their alpha-glucosidase inhibition activity. Alpha-glucosidase hydrolyzes the breakdown of carbohydrates with α,1-4 linkage into glucose units. Inhibition of these enzymes delays the release of glucose into the blood stream and forms hypoglycemia condition after the meal, thus used in the treatment of diabetes mellitus. In this study, isolated strains were screened for the presence of *sedo*-heptulose 7-phosphate cyclase gene which corelates for α-glucosidase inhibition activity from their crude supernatant and detection of acarbose like molecules and their related products using thin layer chromatography. From overall study, we found that the two potential strains

Arthrobacter enclensis NIO-1008^T and *Deinococcus enclensis* NIO-1023^T showed a highest inhibition towards α-glucosidase with 75.42 ±1.3 and 73.26 ±1.8 % inhibition, respectively compared with standard acarbose (Sigma) which showed 91.11±1.1 % inhibition. Further, these two strains were subjected to purification and characterization of α-glucosidase inhibitors. The purified compound from both the strains was characterized using HPLC, FTIR and tandem mass spectrometry (MS/MS). The purified compound was annotated and identified by a Web tool CFM-ID (Competitive Fragmentation Modeling For Metabolite Identification). From the above analysis, it was confirmed that both the strains produces acarbose like molecule. These compounds showed an inhibition at IC_{50} value of 500 µg/ml and 530 µg/ml for *A. enclensis* NIO-1008^T and *D. enclensis* NIO-1023^T respectively. Further, anti-SMASH analysis of whole genome of *A. enclensis* NIO-1008^T confirms the presence of biosynthetic gene clusters of acarviostatin and acarbose, with 11% and 7% homology respectively.

Chapter 4: Summary and future prospectives

This chapter covers the overall summary and conclusion of polyphasic systematic approach for the identification of bacteria from the sediment sample collected from Chorao island, Goa and the alpha-glucosidase inhibition activity of these isolated strains. These marine isolates could be a source of bioactive molecules. Further, work needs to be anticipated for the identification of enzymes and gene clusters for the inhibitor.

CHAPTER- I:

INTRODUCTION

1.1. Bacterial Systematics

Bacterial Systematics deals with the detailed classification and grouping of bacteria to the species level. It includes classification, identification and nomenclature of bacteria. The classification includes the orderly arrangement of organisms into taxonomic groups on the basis of similarity or relationships. Identification determines the unknown or non-reported organisms belongs to which group of the taxa and nomenclature refers to the giving the name to a defined taxa (Sneath, 1992; Vandamme et.al. 1996).

Since 19th century studies on bacterial taxonomy began with the classification and nomenclature of different bacteria. Earlier work in bacterial classification, as mentioned in the '*Bergey's Manual of Determinative Bacteriology*', was primarily established on morphological, physiological and metabolic characteristics of the bacteria (Bergey et al. 1923). Taxonomy and systematics are often used as synonyms; however, systematics deals with the study of characterizing and arranging of bacteria in an orderly manner (Oren A., 2009). Bacterial taxonomy covers different methods for identification and description of new isolates. With the advancement of molecular techniques have prompted further in depth analysis in taxonomy. These new approaches along with phenotypic data and other determinative chemical markers of bacteria tend to form bacterial classification at a higher hierarchy level. This approach was known as "Polyphasic approach", which was coined by Colwel in 1970 for the successive and simultaneous study of a group of organisms with a different set of procedure to yield a good quality of phenotypic and genotypic data. The polyphasic systematics uses three different analytical methods namely phenotypic analysis, phylogenetic analysis and genotypic analysis. The phenotypic analysis examines the morphological, physiological, biochemical and chemical characteristics of the bacterial cell. Phylogenetic analysis is established on 16S rRNA gene, multi-gene and whole genome sequence study. The genotypic analysis uses methods like DNA-DNA hybridization, genomic

DNA G+C content, multilocus sequence typing (MLST) and DNA profiling with respect related strain and type strain.

The classification of bacteria is based on the system that has been widely accepted by the scientific community and is described in the '*Bergey's Manual of Systematic Bacteriology*' (Brenner et al., 2005). Description of an isolate as a new member of species within same genus requires a specific documentation using polyphasic analysis. The type strain of an isolated species should be deposited into at least two internationally recognized culture collection centres for a taxonomic species description. The step by step procedure for the description of novel species in bacterial taxonomy is summarized in Figure1.

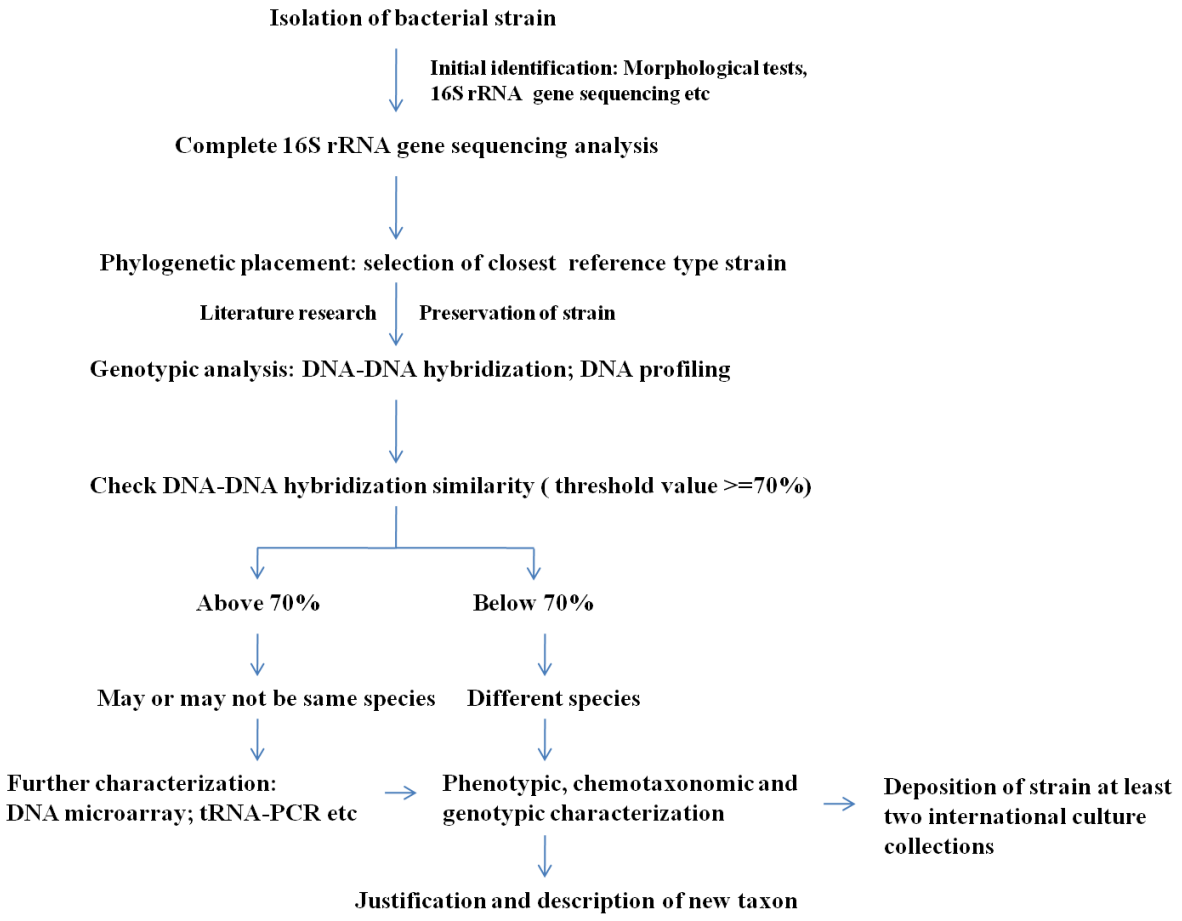


Fig.1. Schematic diagram showing step by step processes for taxonomical characterization of new isolate used in bacterial taxonomy (adapted from Prakash et al., 2007; Rainey, 2011).

1.1.1. Phenotypic analysis

Phenotypes are the observable characteristics of a cell which expressed at the genome level. It describes morphological, physiological and biochemical properties of a cell. Morphological characters include colony (colour, shape, size, margin and surface feature etc.) and cellular properties like cell shape, size, flagella, Gram staining nature and the presence of endospore etc. Physiological test includes growth of an isolate along with its tolerance range at a different temperature, pH, salinity concentration, and in different media used. A biochemical tests contains the utilization of different substrate, oxidation and fermentation of different carbon and nitrogen sources, substrate utilization and enzymatic activity, and susceptibility of antibiotics (Busse et al. 1996). The characteristics of some biochemical tests are given in table 1.

Table.1 Characteristics of some important biochemical test's

Biochemical Test	**Principle**	**Positive reaction**
1. Catalase test	Detects the activity of enzyme catalase, causing breakdown of H_2O_2 to O_2 and H_2O	Bubbles
2. Oxidase test	Detects the activity of cytochrome C oxidase, a component of Electron transport chain of specific organisms	A dark colour develops upon the addition of a specific reagent
3. Gelatinase test	Detects enzymatic breakdown of gelatin to polypeptides	The solid gelatin is converted to liquid
4. Urease	Detects the enzymatic degradation of urea to carbon dioxide and ammonia	Medium becomes alkaline, causing a pH indicator to change colour
5.Casein hydrolysis	Detects the enzymatic degradation of casein to amino acids	Clear zone around the colonies on casein agar plate
6.Starch hydrolysis	Detects the activity of enzyme amylase,causing hydrolysis of starch	A clear zone develops around the colony after flooding the iodine solution
7. Indole test	Detects the enzymatic removal of the amino group from tryptophan	The product, indole reacts with a Kovac's reagent to give a deep red colour

8. Methyl Red test	Detects mixed acids, characteristics end products of a particular fermentation pathway	Medium becomes acidic (pH <4.5); a red colour develops due to pH indicator
9.Voges-Proskauer test	Detects acetoin, an intermediate for 2,3-butanediol production	A red colour develops upon addition of chemicals that detect acetoin
10. Citrate utilization test	Determines whether or not citrate can be used as a sole carbon source	Growth, which is usually accompanied by the colour change of a pH indicator
11.Phenylalanine deaminase test	Detects the enzymatic removal of the amino group from phenylalanine	Product, Phenylpyruvic acid reacts with ferric chloride to give a green colour
12.Lysine decarboxylase test	Detects the enzymatic removal of the carboxyl group from lysine	Medium becomes more alkaline, causing a pH indicator to change colour
13.Malonate utilization test	Determines whether or not disodium malonate can be used as a carbon source	Medium becomes alkaline, causing pH indicator to change colour
14.Sugar Fermentation test	Detects the acidity and gas production	Colour of pH indicator changes if acid is produced
15.Hydrogen Sulphide production	Detects H_2S liberated as a result of the degradation of S-containing amino acids	A black precipitate forms due to the reaction of H_2S with iron salts in the medium

The numbers of tests tested in the phenotypic analysis are common for a particular taxon, while subgrouping determination requires variable traits for the test (Tindall et al. 2010). To classify an isolate as a new species it can be differentiated from a validly known species by array of phenotypic characters (Wayne et al. 1987; Rosselló-Mora and Amann, 2001). Phenotypic characteristics of an isolate to be done simultaneously with the type strain of particular taxon in a given same lab conditions. Because comparison of the previously published data of a type strains may leads false positive or negative results as sometimes the data lacks reproducibility due to the different culturing conditions, methodology and response of strain to a different condition.

Nowadays, commercial available identification kits are commonly referred by research labs to obtain phenotypic data due to their ease of use, for example API (bioMériux), Biolog Microplate (Biolog), Microbact (Oxoid) etc. These systems are available in the forms

of kits:- consist of wells or strips or plates with dehydrated substrate coupled with a required reagents of suspension media. The advantages of such systems are rapid analysis, reduced the time and period of incubation, consumables, reagents, space and storage for the experiment to conduct. The limitations of these systems are interpretation of results may sometimes vary due to confusion at the development of colour gives positive/negative/weak reactions which are interpreted differently by an individual researcher.

Identification and classification of bacteria from the chemical characteristics of the cells are collectively referred as chemotaxonomy. It gives the information about different chemical markers of the bacterial cell from which one can classify bacteria up to genus/species level (Goodfellow and O'Donnell, 1994). The chemotaxonomy includes the analysis of cellular fatty acids, cellular polar lipids, cell wall sugars, peptidoglycan types and types of respiratory quinones (Tindall et al. 2010), Fourier- Transformed Infrared Spectroscopy [FTIR] (Helm et al. 1991; Helm and Naumann, 1995) for the analysis of cellular constituents to form a spectral libraries to analyze and classify the bacteria and Matrix Assisted Laser Desorption/ Ionization Time-of-Flight (MALDI-TOF) mass spectrometry (Conway et.al. 2001) for the study of protein fingerprinting of bacterial cell and classification of bacteria being carried out based on the pattern of protein fingerprinting.

Fatty acids are the carboxylic derivatives of long chain aliphatic group and are mostly present in the cytoplasmic membrane of bacterial cell wall as a constituent of polar lipids and glycolipids (Kates, 1964), and part of the lipid bilayer (Ratledge and Wilkinson, 1988). The chain length of fatty acids, the location of double bonds on unsaturated fatty acids, hydroxylated, 10-methyl fatty acids, branched and unbranched fatty acids provide data for the biosynthetic and taxonomic relationship between bacteria (Suzuki et al., 1983). Detection of cellular fatty acid composition varies with the change in cultivation medium and temperature. Standardization of medium and temperature must follow to quantitate fatty acids

from the cell. Analysis of fatty acids is most commonly done by gas chromatography (GC) in which fatty acid methyl esters are detected using capillary column. The fatty acids is assesed by GC and by comparison of peak retention times of sample with known standards. To identify a large variety of fatty acids from microorganisms, the Sherlock® Microbial Identification System is used.

Peptidoglycan layer of bacteria contains β-1,4 linked unsaturated disaccharides of N-acetylglucosamine and N-acetylmuramic acid chain which are covalently cross-linked to oligopeptides. Gram positive bacteria contains up to 30.0-70.0% of peptidoglycan of total cell wall while Gram negative bacteria contains up to 10.0% of peptidoglycan of the total cell wall. Despite various macromolecules present, peptidoglycan is commonly present in both types of bacteria. Schleifer and Kandler in 1972 proposed the system for the classification of peptidoglycan based on its mode of cross-linkage and distribution of various peptidoglycans within the bacterial kingdom and their taxonomic preference was evaluated. They classify the peptidoglycan into two main types viz., Type A and type B. In type A, cross-linkage is accomplished between position 3 and 4 of two peptide subunit. The cross linkage is extend from ω-amino group of the diamino acid at position 3 to the carboxylic group of D-alanine at position 4 either directly or an interpeptide bridge. In type B, cross-linkage is accomplished between position 2 and 4 of two peptide subunit. The cross linkage is extend from α-carboxylic group of D-glutamic acid at position 2 to carboxyl group of D-alanine at position 4. A tridigital code is used to identify peptidoglycan (e.g. A1α). The classification of peptidoglycan is given in table 2.

Table 2. Classification of peptidoglycan (adapted from Schleifer & Kandier, 1972 and Schleifer and Seidl, 1985)

Position of cross link	Peptide bridge	Amino acid at position 3
Peptidoglycan A: Cross-linkage between two adjacent peptide at position 3 and position 4	1. Direct	α. L-lysine β. L-ornithine γ. Meso-diminopimelic acid:- γa. amidation of α-carboxyl group of D-alanine γb. amidation of carboxyl group of meso-diaminopimelic acid
	2. Polymerised peptide subunit	α. L-lysine
	3. Interpeptides bridge consist of monocarboxylic L-amino acids or glycine or both	α. L-lysine β. L-ornithine γ. L,L-diaminopimelic acid
	4. Interpeptides bridge containing a dicarboxylic amino acid	α. L-lysine β. L-ornithine γ. Meso-diaminopimelic acid δ. L-diaminobutyric acid
	5. Interpeptide brige containing lysine and a dicarboxylic amino acid	α. L-lysine β. L-ornithine
Peptidoglycan B: Cross-linkage between position 2 an 4 of two adjacent peptide subunits	1. Interpeptide bridge containing L-diamino acid	α. L-lysine β. L-homoserine γ. L-glutamic acid δ. L-alanine
	2. Interpeptide bridge containing D-diamino acid	α. L-ornithine β. L-homoserine γ. L-diaminobutyric acid

Polar lipids are the major components of the lipid membrane of bacteria and helps in the permeability and regulation at the membrane. Polar lipids are amphipathic molecules consist of hydrophilic head group in which two hydrophobic aliphatic chains are linked. The polar lipids mainly found in bacteria are phospholipids, glycolipids, aminoglycolipids, aminophospholipids, an amino acid derived lipids, sphingolipids, and sulfur containing lipids. There are vast varieties of polar lipids present in bacteria and are useful for the identification

and classification (Vandamme et al., 1996). For example, the α-β-γ group of proteobacteria generally posses phosphatidylglycerol, phosphatidylethanolamine and diphosphotidylglycerol as a major phospholipids.

Respiratory quinones are mainly found in the cytoplasmic membranes of most of the prokaryotes where they have a role in electron transport, oxidative phosphorylation, and active transport (Collins and Jones, 1981; Collins, 1994). Quinones are divided into two groups, benzoquinones and naphthoquinones. Benzoquinones consists of ubiquinone, plastoquinone, rhodoquinone, caldariellaquinone, sulfolobusquinone and epoxyubiquinone while naphthoquinones consist of menaquinone, dimethylmenaquinone, thymoquinone, chlorobiumquinone, methionaquinone and partially hydrogenated menaquinones. The most common type of quinones are ubiquinone and menaquinone. The variation in length, saturation and hydrogenation in isoprenoid quinones can be used for the characterization of bacteria at different taxonomic level. For example, the α-β-γ group proteobacteria have ubiquinones with variable isoprene chain length (Tindall, 2005).

Cell wall chemotypes such as diagnostic amino acids and sugars were introduced by Lechevalier and Lechevalier (1970a, b; 1980). These are assigned with numbers as wall chemotypes (Table 3). Whole cell hydrolysates gave information on the presence of 2,6-diaminopimelic acid (Dpm) and of 2,6-diamino-3-hydroxypimelic acid (OH-Dpm), on the occurrence of glycolyl residue in the glycan backbone and whole cell sugars. The diagnostic amino acids can be detected on TLC (Staneck and Roberts, 1974) and by gas Chromatography (O'Donnell et al., 1982). The glycolic acid occurs in bacterial cell wall can be detected from the diethyl ether extraction method described by Uchida et al., 1999. Similarly, whole cell sugars can be analyzed by using TLC (Staneck and Roberts, 1974) or by GC separation of the derivatised molecule (Drucker, 1981; Hancock, 1994).

Table 3. Type of cell wall chemotypes (adapted from Kim, 1999).

Cell wall chemotype	Major constituent
I	LL-diaminopimelic acid and glycine
II	meso-diaminopimelic acid and/or hydroxydiaminopimelic acid and glycine
III A	meso-diaminopimelic acid and madurose
III B	meso-diaminopimelic acid
IV A	meso-diaminopimelic acid, arabinose, galactose and mycolic acids
IV B	meso-diaminopimelic acid, arabinose and galactose
V	Lysine and Ornithine
VI	Lysine, aspartic acid and galactose
VII	Diaminobutyric acid, lysine, aspartic acid and glycine
VIII	Ornithine, aspartic acid

Whole cell protein pattern obtained from bacteria has proven to be reliable for comparing and grouping a large number of closely related strains or isolates (Vauterin, 1993; Kersters et al., 1994; Pot et al., 1994). Protein pattern can be compared by matching and clustering algorithms for the classification of bacteria (Freiwald and Sauer, 2009). Currently, two systems are available for species identification, one being MALDI Biotyper (Bruker Daltonics, Germany) and other being VITEK® MS (bioMériux, France). Both systems are useful for the identification of bacteria from clinical as well as environmental samples from their database (Murray, 2010).

Phenotypic analysis, as a whole gives descriptive information of an isolate along with its closest type strain which serves as a piece of basic information coupled with genetic information for the classification of bacteria.

1.1.2. Phylogenetic analysis

Phylogenetics includes sequencing analysis of small subunit of ribosomal RNA (SSU rRNA), phylogenetic tree, multi-gene sequence and whole genome analysis. Classification and identification of bacteria based on sequence analysis of molecular markers were started late

in the 1960s with the pioneering work of Dr. Carl Woese on ribosomal RNA and concept of three domain of life.

1.1.2.1. The 16S rRNA gene sequencing

In 1970s Carl Woese introduced the use of 16S rRNA as a component for the classification of bacteria. Bacteria contains three types of rRNA subunits i.e. 5S, 16S and 23S rRNA. The 5S rRNA subunit is too small for the taxonomic study and 23S rRNA is too large as compared to 16S rRNA. The size and information contains in 16S rRNA, it is widely accepted for the identification of bacteria (Woese and Fox, 1977; Woese et al., 1978; Olsen et al., 1986; Ludwig and Schleifer, 1999; Rosselló-Mora and Amann, 2001).

The use of 16S rRNA gene for phylogenetic analysis has been popularized because of a number of reasons. Firstly, it contains variable region and it is highly conserved over the evolution and is present universally in all bacteria and makes it useful for phylogenetic comparison. Secondly, the lateral gene transfer has occurred very rarely, which makes it as a stable and helps in evolutionary studies among the genera. Thirdly, it is easily isolated from culturable as well as unculturable microorganisms, for identification from natural sources. Fourthly, it is about 1.5 kb size in length throughout the bacterial domain and contains highly conserved and variable regions (Stackebrandt and Woese, 1981). The rare changes in the variable region are for specific group or species in which they occur. Moreover, 16S rRNA gene sequences are readily available in many online databases such as EzTaxon, NCBI GenBank and EMBL etc. which allows comparison and analysis of bacteria.

In bacterial taxonomy, it is suggested that an isolated bacteria will be a new species if its 16S rRNA gene sequencing similarity differs by 3.0% from any known species and for a new genus if it differs by more than 5.0%. The 97.0% cut-off value for species differentiation has been widely accepted for speciation. However, sometimes some distinct species share

more than 97.0% similarity for which DNA-DNA hybridization is mandetory requirement (Fox et.al., 1992; Stackebrandt and Goebel, 1994; Stackebrandt and Ebers, 2006). For the identification and classification of bacteria, 16S rRNA gene sequence is considered as a gold standard tool.

1.1.2.2. Phylogenetic analysis

The 16S rRNA gene sequences provide the data for comparative analysis of bacteria, from which one can determine the phylogenetic relationship between organisms. Phylogenetic analysis is based on the evolutionary relationships of a genetically related group of organism and relation between ancestral sequence and its descendants. The phylogenetic relationship can be visualized by trees which consist of internal and terminal nodes connected by branches. One branch can connect any two adjacent nodes. Nodes are the molecules (taxonomic unit) of the analyzed organisms. The phylogenetic tree has terminal and internal nodes, which represents data of sequence under comparison at terminal nodes and internal nodes represent data in the evolution of these sequences (taxonomic unit). The phylogenetic tree has been constructed in two types i.e. rooted tree and unrooted tree. In a rooted tree, the position of common ancestral is given for a particular taxonomic group from which the evolutionary time can be determined from its path to the nodes. In the unrooted tree, the interrelationship between taxonomic groups is shown on the establishment of the relationship between neighbouring taxa.

Numerous methods have been used for the construction of a phylogenetic tree. The major methods that are widely used are distance based and others are character based (Nei, 1987; Felsenstein, 1978, 1981, 1985, 1988; Saito and Nei, 1987). The distance methods involve two steps in which first sequence similarity data transformed into evolutionary distances and then using information from distance matrices trees are constructed. From the

information in distance matrices construction of tree is carried out by many tree making methods. One method is the neighbour-joining method which is more likely clustering method, which keeps tracks of nodes rather than taxa or clusters of taxa. The tree is constructed on the basis of data provided to the distance matrix in which the partition between nodes is regulated on the basis of their regular discrepancy from other nodes.

The character based method involves maximum parsimony and maximum likelihood method. In maximum parsimony method construction of the tree depends on the sum of changes that occurred to give data sequence in which the alignments are determined. The tree will be constructed from the possible way of one set to another set of alignment data, from which the most parsimonious tree is taken, as it reflects the evolution relation most closely (Stackebrandt and Rainey, 1995). In the maximum likelihood method, the building of the tree is based on a mathematical model (statistical model). It analyzes the sequences on a site to site basis to determine the phylogenetic inference. The model uses nucleotide substitution for tree making to evaluate net likelihood for a given evolutionary model will yield the highest likelihood tree (Felsenstein, 1988; Olsen et al. 1994).

The statistical significance of a phylogenetic tree can be determined by a bootstrapping approach. This approach tells us that how good a dataset fit in our evolutionary model. It resamples the random alignment positions so that some of them included more often as another are not included at all. This method usually repeated with 100 to 1000 times with an alternative data set. The higher the fraction of run, the higher is the significance of individual data set at the branching point. The root of a phylogenetic tree can be determined from a homologous sequence of a different group of related organism, the outgroup. The addition of incomplete or incorrect nucleotide sequences may reduce tree topology, therefore the nucleotide sequence must check carefully. The partial nucleotide sequence should not be used for inferring phylogenetic tree as it contains less information and that the position of

taxa should be assigned from a complete nucleotide sequences. It is therefore evident that the 16S rRNA data must be evaluated carefully for the construction of a phylogenetic tree (Stackebrandt and Ludwig, 1994).

1.1.2.3. Whole genome analysis

Previously, whole genome sequencing of bacteria was considered to be a time-consuming, costly and labour intensive process. But the recent advancement of next-generation sequencing, it is nowadays possible to create whole genome sequence of bacteria within a stipulated period of time with low cost and with less labourious. A large number of whole genome sequences have been deposited into different public databases enabled for the utilisation of genome data for comparison of bacteria. Considering above it has been recommended that, the whole genome sequence data of bacteria should be assigned in novel species description (Whitmann, 2011; Chun and Rainey, 2014; Vandamme and Peeters, 2014).

Whole genome analysis has been correlated with DNA-DNA hybridization data for the species delineation. Few approaches have been used for analysis like Average Nucleotide Identity (ANI) based on similarity type of relatedness index, which is calculated by BLASTn method (Altschul et al. 1990; Goris et al. 2007) or by MuMmer algorithm (Kurtz et al. 2004; Richter and Rosselló-Mora, 2009). Another approach is based on distance type genome relatedness index, in which phylogenetic relationship between two genomes being studied and commonly they named as maximal unique matches index (MUMi) or Genome Blast distance phylogeny (GBDP) [Deloger et al. 2009; Henz et al. 2005].

ANI is the most commonly used techniques among others and is the good tool for the species circumscription (Richter and Rosselló-Mora, 2009; Kim et al. 2014). A threshold value of 95.0% ANI corresponds to the 70.0% value of DDH for species description

(Konstantinidis and Tiedje 2007; Goris et al. 2007). From later studies, it was shown that 95.0-96.0% value of ANI corresponds to species delineation.

1.1.3. Genotypic analysis

This part includes the methodologies which cover the information at genomic level for the circumscription of genus/species of bacteria. It involves the analysis of genomic DNA-DNA hybridization, genomic G+C content, multi-locus sequence typing (MLST) and DNA profiling method.

1.1.3.1. DNA-DNA hybridization (DDH)

The DDH technique is most commonly used for the delineation of species, where 16S rRNA similarity is above 97.0%. In general, DNA gets easily denatured at their melting temperature (T_m) or with a strong alkaline solution, once denatured it gets renatured to form duplex well below T_m temperature or at a neutral solution. This tendency of genomic DNA helps in the analyzation of two genomic DNA of bacteria for the measurement of nucleic acid sequence similarity (Schildkraut et al., 1961; Wayne et al., 1987; Gevers et al., 2005). The *Ad Hoc* Committee on accord of Bacterial taxonomy proposed that a species/strain with approximately 70.0% DDH or more relatedness value with 5 ºC or less divergence value (ΔT_m) should be considered as a species, whereas the DDH value well below 70.0% may be different species or may not be (Wayne et al., 1987; Kreig, 1988; Stackbrandt and Goebel, 1994).

The degree of DNA relatedness of two genomic DNA can be expressed as a relative binding ratio and/or the difference in the melting temperature (ΔT_m). Both parameters are correlated with each other and are used for the species description either together or independently (Johnson, 1989; Rosselló-Mora and Amann, 2001). The relative binding ratio

(RBR) is the measurement of the relative amount of hybrid DNA in comparison with reference DNA, where reference DNA tends to hybridize itself with 100% (Rosselló-Mora and Amann, 2001; Rosselló-Mora, 2006). However, (ΔT_m) is an independent parameter of quantity and quality of DNA used in the hybridization. The denaturation of DNA depends on the ionic strength of the solution, the G+C content of DNA and the temperature. The ionic strength of the solution and G+C content of DNA are constant as compared to a temperature which is variable in the hybridization experiment. The (ΔT_m) can be calculated based on the differences of T_m of homologous DNA and T_m of heterologous DNA (hybrid). The techniques used to measure the stability of hybrid double stranded DNA are categorised into two methods, namely free-solution method and bound DNA methods. These two methods are summarized into table 4.

Table 4. Methods used for DNA-DNA hybridization technique (adapted from Rosselló-Mora 2006).

Method	Measurement	Remarks	Reference
I. free-solution methods			
1. Buoyant density Label: heavy isotopes	RBR	This method was abandoned due to new techniques	Schildkraut et al. 1961
2. Hydroxyapatite Label: radioactive isotopes	RBR; ΔT_m	Multiple hybridization reactions can be performed. It allows for short RBR measurements and long ΔT_m measurement. There is no restriction on temperature.	Brenner et al. 1969b; Lind and Ursing, 1986
3. Hydroxyapatite / microtiter plate Label: Digoxigenin-biotin	RBR	Same advantage as hydroxyapatite method; radiolabeling was not required and double-labelling of DNA required nick translation.	Ziemke et al. 1998
4. Spectrophotometry Label: none	RBR; ΔT_m	No labelling is required; high conc. and an equal amount of DNA are required. Needed special spectrophotometer.	De Ley et al. 1970; Huss et al. 1983
5. Fluorimetric Label: none	ΔT_m	Low amount of DNA is needed; no labelling is required; can perform	Gonzalez and Saiz-Jimenez, 2005

		simultaneous hybridization; the same quantity of DNA is required; can perform in Real time PCR.	
6. Endonuclease Label: radioactive isotopes	RBR	Same advantage as hydroxyapatite method.	Crosa et al. 1973; Popoff and Coynault, 1980
II. Bound DNA methods			
1. Agar embedded Label: radioactive isotopes	RBR	Accuracy of radioactive measurement; difficult to incubate at high temperature, hybridization reaction of labelled DNA to the embedded DNA in agar is difficult.	Bolton and McCarthy 1962; Brenner et al. 1969a
2. Membrane filters Label: radioactive isotopes	RBR; ΔT_m	Multiple reactions of single labelled DNA can be performed; thermal stability calculations are independent of the quality of DNA; DNA can be washed out from the membrane.	Johnson, 1981; Owen and Pitcher, 1985; Tjernberg et al. 1989
3. Membrane filters Label: non-radioactive isotopes	RBR	Same as above but without labelling; requires more incubation; as enzymatic assay, quantification should be done at the time when the reaction is linear.	Jahnke, 1994; Cardinali et al. 2000; Gade et al. 2004
4. Microtiter plate bound DNA Label: photobiotin	RBR	Multiple reactions can be performed; DNA can be washed out; denaturing agents required preventing incubation.	Ezaki et al. 1989; Adnan et al. 1993; Kaznowski, 1995; Christensen et al. 2000
5. Microtiter plate bound DNA Label: digoxigenin	ΔT_m	No need of high conc.of DNA; can be done at microtiter reader; the problem can be derived from amplification reaction; denaturing agents required to prevent incubation.	Mehlen et al. 2004

1.1.3.2. Genomic DNA G+C content

The primary structure of DNA contains four nucleotides bases and it determines genetic information of an organism. Determination of genomic DNA G+C content is a classical method in the bacterial taxonomy and is a part of the standard description of taxa

(Vandamme et al., 1996; Rosselló-Mora and Amann, 2001; Whitmann, 2011). The mol% of G+C is varied from 25.0-80.0% among bacteria and with the value being constant for a given organism. The G+C content of organisms provides additional information for assigning bacterial strains to the taxonomic groups, ranging from low to high G+C content bacteria. It also differentiates bacteria those which have similar morphology but are genetically different (Colwell and Mandel, 1964; Silvestri and Hill, 1965).

Determination of G+C content is done by a chromatographic method in which DNA gets enzymatically hydrolyzed into nucleotides and later analyzed in HPLC as well as estimation from thermal denaturation curves using spectrophotometry (Marmur and Doty, 1962; Mesbah et al., 1989; Tamaoka and Komagata, 1984; Tamaoka, 1994). Generally, well-defined species have not more than 3.0% G+C content similarity and similar, members of species within the genus have not more than 10.0% G+C content similarity (Vandemme et al., 1996; Goodfellow et al., 1997).

1.1.3.3. Multi-locus sequence typing (MLST)

In bacteria, the housekeeping genes are those protein coding genes which are involved in the maintenance of basic cellular functions. These genes are evolved slowly but much faster than the rRNA genes (Schleifer, 2009). The *Ad Hoc* committee suggested the use of multiple housekeeping genes for species delineation whereby at least five genes could be used for species differentiation (Stackebrandt et al., 2002).

MLST was used to characterize the bacteria at the intraspecies level by assessing allelic mismatch of gene sequences (Maiden et al., 1998). Therefore, MLST has been suggested to use for the classification and identification of bacteria at the lower taxonomic group (Stackebrandt et al., 2002; Gevers et al., 2005; Schleifer, 2009; Whitmann, 2011). However,

the selection of different genes and the size of genes being used for the circumscription of species, strain or genus has not been established (Kämpfer and Glaeser, 2012).

1.1.3.4. DNA profiling

DNA profiling methods have been used for the identification and differentiation of bacteria at a species level and strain level. It gave additional information at the intraspecies level and added supportive information to analyse closely related organisms (Vandemme et al., 1996). Initially, DNA based profiling methods includes the use of restriction enzymes to generate fragments from chromosomal DNA. This method, which is known as ribotyping, is based on the fact that rRNA genes are scattered throughout the DNA and are highly conserved and hence produces polymorphic sites for the restriction enzymes. This typing method is known as restriction fragment length polymorphism (RFLP) method. The resulting fragments after the treatment of restriction enzymes are separated and visualized under agarose gel electrophoresis and/or pulsed field gel electrophoresis. The disadvantage of this system is that it produces fragments which are very difficult to compare. In such cases, the fragments generated are separated on the nitrocellulose membrane and hybridized with labelled probes to minimise the number of fragments to detect. The probes may be radiolabeled or fluorescently labelled (Grimont and Grimont, 1986; Grimont et al., 1992).

With the advancement of polymerase chain reaction (PCR) technique, led to the several typing methods for the analysis of amplification of specific nucleic acid sequences. This is the kind of DNA fingerprinting technique. This method includes the use of oligonucleotide primers for the amplification of specific sites or repetitive sites or palindromic sites on chromosomal DNA to generate fragments, which later separated on agarose gel electrophoresis and then analyzed. This type of methods used for the differentiation of two closely relates species/strains. The examples of such typing methods

are arbitrarily primed PCR (AP-PCR), repetitive extragenic palindromic-PCR (REP-PCR), enterobacterial repetitive intergenic consensus sequences-PCR (ERIC-PCR), randomly amplified polymorphic DNA (RAPD) and BOX repetitive element (BOX-PCR) etc. The amplified fragment length polymorphism (AFLP) is a technique of both PCR and restriction enzymes. The restriction is usually done by two to three restriction enzymes to generate fragments which later on combined randomly. Later, to their sticky ends short oligonucleotides (adapters) are ligated to form a template for PCR (Vandemme et al., 1996; Tindall et al., 2010). The above techniques of DNA profiling give additional information for species description at the species/strain level and help in differentiation one from other organisms.

1.2. Marine bacteria and their potentials

The marine environment contains a diverse range of bacteria adapted in low to high temperature, acidic to alkaline water, high pressure, limited substrate availability and symbiotic association with marine organisms. Marine bacteria found in the surface water, offshore to the coastal regime, low surfaces to the deep down region, specialized area of oceans and with the association of plants and animals. Marine bacteria population divided according to mangroves ecosystem, coral reefs, deep sea and extreme environment system (Das et al., 2006).

The number of marine bacteria varies from the different habitat of the marine ecosystem. The most diverse bacteria are found in low surface level to shallow water vents, near shorelines, mangroves and muddy coastal area, where the distribution of water, sediments and organic material are more because of turbulence, wind, runoff water etc. (Reuszer, 1933; Baharum et al., 2010), while the population of bacteria is a low in-depth area of ocean where the distribution of organic material is low.

The distributions of marine bacteria are unique and extreme with respect to salinity (low to high), temperature (low to high), acidity (acidophiles), alkalinity (alkalophiles), pressurized abyssal water (barophiles) and with minerals (low to high). Due to this, it makes the bacteria to adapt in a particular environment and thus producing a diverse range of bioactive compounds. This bioactive compound has drawn special attention in pharmaceutical, food and agricultural industries.

1.3. Marine sediments

Marine sediments are insoluble material or solid material deposited via land, river, wind, ice and volcanic eruption etc. The distribution of sediments near continents, coral reefs and rises etc. gave the information regarding chemical and biological information they possessed (Seibold and Berger, 1982).

Generally, marine sediments are divided into four types namely lithogenous, biogenous, hydrogenous and cosmogenous (Smith, 2011). Lithogenous sediments are derived from lands by the activity of the weathering process on rocks and by means of the volcanic process also. There are two types of lithogenous sediments namely terrigenous and red clay. Terrigenous sediments are those which arise from land by the activity of wind, streams, glaciers and gravity etc. Red clay sediments on the other hands are reddish-brown and are combinations of terrigenous material and volcanic ash.

Biogenous sediments are the most important as they derived from directly or indirectly through biological activity. Majority of these are from shells remains of sea creatures and/or from the skull or leftover parts of organisms from other sources. The distribution of this sediment depends on the precipitating the organism and their dissolution.

Hydrogenous sediments are formed from the oceanic inorganic mineral that precipitates directly or can be formed as a new mineral from chemical reactions between water and sediments that are already present in there. This process is happened because of evaporation and forms precipitate of mineral salts. A hydrothermal deposit also forms sediments as they carry dissolved material to the seafloor. Cosmogenous sediments are from extraterrestrial meteor material that arrives on earth and gets deposited.

Sedimentation formed in mangroves forest areas, which are distributed in the intertidal region are examples of deposition through erosion, freshwater discharge, salinity regime and from the lagoonal or estuarine environments. Mangroves are the salt tolerant wetlands which occur on upper intertidal shorelines of many tropical and subtropical countries (Badarudeen et al., 1996; Woodroffe et al., 2016). Sediments in the mangrove areas can trap nutrients, carbonates from discharge via terrigenous activity. The nutrient recycling of organic matters between marine and terrestrial habitats makes mangroves sediment an important ecosystem as they present in tropical and subtropical areas that typically found in transition between terrestrial, marine and freshwater ecosystem (Chen et al., 2016). Mangrove forests trap energy via photosynthesis and provide it in the form of organic waste. The sediments in the mangrove ecosystem can trap nutrients from terrestrial runoff, decomposed litters from mangrove plants and shrubs, mineralization (dissolved and particulate minerals) and from an estuarine environment. It helps the microorganisms grow and forms a dynamic community. Mangrove-associated microorganisms like bacteria and fungi draw special attention to many researchers. Over the last decades, the number of research publications related to natural products with biopotential activity has been increased. Among 1000 new metabolites which were reported from the last ten years, ~850 derived from fungi and ~120 from bacteria (Blunt et al., 2018). The overall publications describing new/bioactive metabolites from mangrove-derived microorganisms are summarized in figure

2. The number of compounds reported in 2014 was 1378 from 456 papers, in 2015 it's were 1340 from 429 papers, in 2016 it's were 1277 from 432 papers and in 2017 the number of compounds reported were 1490 from 477 papers (Blunt et al., 2016; 2017; 2018; Carrol et al., 2019).

In this study, an attempt was made to isolate and identify the marine organisms from sediment sample using a polyphasic approach to establish their taxonomic position with their possible biological application.

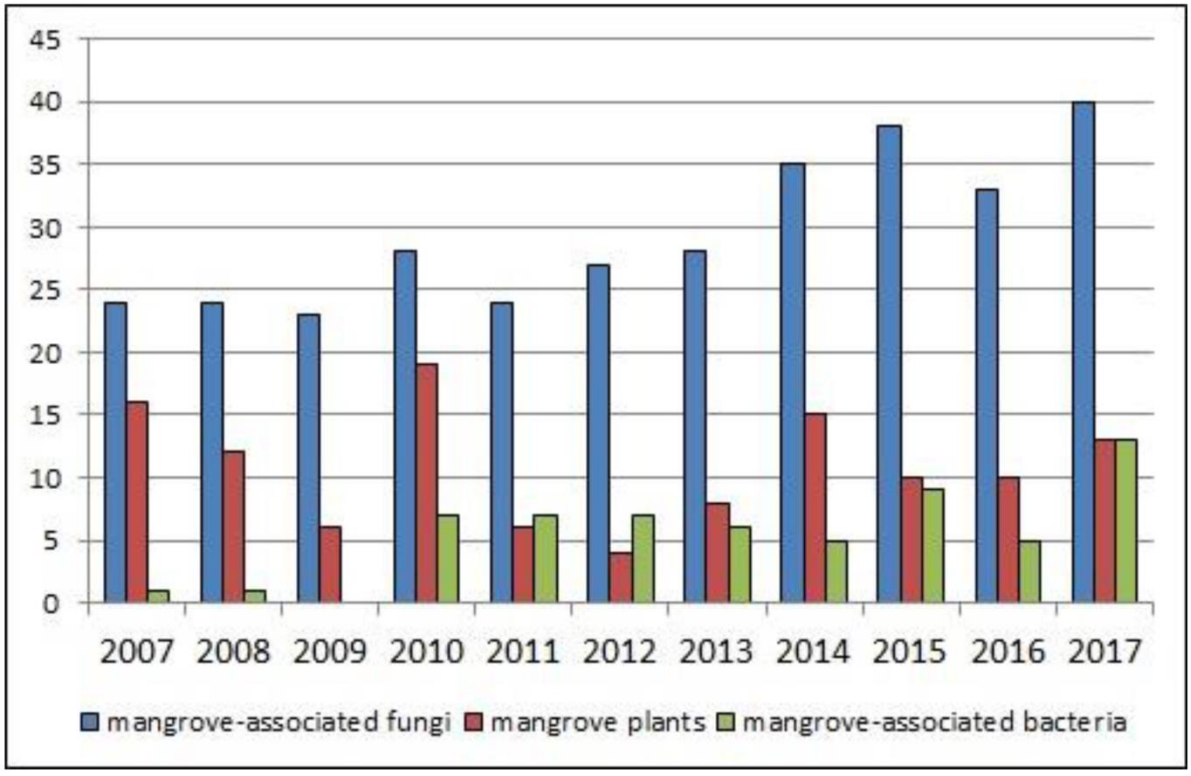


Fig.2. Number of publications describing new metabolites from mangrove associated organisms (source: Ancheeva et al., 2018).

The microorganisms present in the mangrove ecosystem are studied extensively using 16S rRNA gene sequencing, metagenomic aspects, terminal restriction fragment length polymorphism (T-RFLP) along with genetic fingerprinting profiles (Liu et al., 1997; Kent et al., 2003; Brito et al., 2006; Singh et al., 2006; Andreote et al., 2012; Fernandes et al., 2014;

Nogueira et al., 2015; Alzubaidy et al., 2016; Chen et al., 2016; Imchen et al., 2017;; Paingankar and Deobagkar, 2018). The microorganisms of mangrove ecosystem serve as source for many biopotential activities. Microbes from the mangroves sediments have an important role in biogeochemical cycles such as nitrogen, sulfate, carbon, phosphorous (Alongi, 1994; Holguin et al., 2001; Zhou et al., 2017) and biocycling of nutrients (Imchen et al., 2017). Microorganisms from mangrove ecosystem are capable of degrading xenobiotics such as petroleum hydrocarbons and polycyclic aromatic hydrocarbons (Diaz et al., 2000; Yu et al., 2005; Gomes et al., 2008; Santos et al., 2011; Andrade et al., 2012; Muangchinda et al., 2013; Rocha et al., 2013; Cravo-Laureau and Duran, 2014). Some microorganisms found to produce bioactive compound with cytotoxic activity (Arumugam et al., 2010; Li et al., 2011), antibiotic activity (Wang et al., 2010; Li et al., 2012; Hong, 2013), anti-HIV activity (Ding et al., 2010; Ding et al., 2011) and anti-fibrotic activity (Arumugam et al., 2010).

Nitrogen fixing bacteria were found in majority of the mangrove ecosystem in corporation with decayed leaves (Gotto and Taylor 1976; Zuberer and Silver 1978, 1979; van der Valk and Attiwill 1984; Hicks and Silvester 1985; Mann and Steinke 1992), pneumatophores (aerial roots; Zuberer and Silver 1978; Potts 1979; Hicks and Silvester 1985; Toledo et al. 1995; Alfaro-Espinoza and Ullrich, 2015), the rhizosphere soil (Zuberer and Silver 1978; Holguin et al. 1992; Liu et al., 2012; Baskar and Prabakaran, 2015), tree bark (Uchino et al. 1984), cyanobacterial sheets on sediments (Toledo et al. 1995), and the sediments itselves (Zuberer and Silver 1978; Potts 1979; Zhang et al., 2008). The bacteria of genus *Azospirillum*, *Azotobacter*, *Rhizobium*, *Clostridium*, and *Klebsiella* were identified from a mangrove ecosystem as major nitrogen fixers. The correlations of nitrogen fixing bacteria with mangrove trees do not depend on specificity (Sengupta and Chaudhuri 1990, 1991).

Phosphorous is usually available in a precipitate form at a mangrove ecosystem, thus making it unavailable to plants. Phosphate solubilizing bacteria are the only source of soluble phosphorous. Because of the anoxic conditions in lower sediments, the roots of mangrove plants serve oxygenic condition where phosphate solubilization takes place (Holguin et al., 2001). Phosphate solubilizing bacteria were studied and isolated from mangrove ecosystem by many researchers (Vazquez et al., 2000; Dastager and Damare, 2013; Behera et al., 2014; Teymouri et al., 2016; Abhijith et al., 2017; Behera et al., 2017; Tam and Diep, 2017; Prabhu et al., 2018). Sediments in the mangrove ecosystem have upper oxygenic zone and lower anoxygenic zone. Organic matters were usually degraded at the upper zone while mineralization and reduction take place at the lower zone. Sulfate reduction mainly occurs in the lower zone of sediments where as sulfate-reducing bacteria play an important role (Sherman et al., 1998). Sulfate reducing bacteria were reported from various mangrove ecosystems (Zuberer and Silver, 1978; Saxena et al. 1988; Loka Bharathi et al. 1991; Wu et al., 2011; Varon-Lopez et al., 2013; Balk et al., 2016). Moreover, methanogens were also studied from the mangrove ecosystem. Methanogens are archaebacteria which grow in the extreme environment and are one of the major population in mangrove sediments (Shaoo and Dal, 2009; Ahila et al., 2012). They use hydrogen and carbon dioxide as an energy source and convert it into methane, this process generally called methanogenesis (Lekphet et al., 2005). A number of cases of methanogens were reported from mangrove sediments (Ramamurthy et al., 1990; Mohanraju and Natarajan, 1992; Singh et al., 2005; Lyimo et al., 2009; Torres-Alvarado et al., 2013; Zhou et al., 2015).

Apart from the above bioactive potentials, marine bacteria from the mangrove ecosystem also showed various activities. This makes mangrove ecosystem a hotspot for the isolation of bacteria for various applications such as pharmaceutical, bioremediation, food and agriculture etc.

In this study, the mangroves sediments of Chorao Island, Goa were used for the isolation and identification of bacteria using polyphasic systematics approch for their taxonomic grouping and screen these marine bacteria for the alpha-glucosidase inhibition activity.

1.4. Alpha-glucosidase inhibitor

Alpha-glucosidase enzyme catalyzes the cleavage of glycosidic bonds of carbohydrates, specifically depending on the number of a monosaccharide, position and configuration of the hydroxyl group present in the sugar molecule (Park et al., 2008). Alpha-glucosidase [E.C.3.2.1.20] cleaves α-1, 4 linked terminal non-reducing end of a sugar moiety. Alpha-glucosidases produced by animals, plants, microbes and fungi, etc. Alpha-glucosidases are located in the brush border of epithelial cells of the small intestine. They convert the complex carbohydrates into simple sugars. Deficiency of α-glucosidases leads to Pompe disease (Raben et al., 2002). Alpha-glucosidase has drawn special attention to pharmaceutical industries because of the inhibition of α-glucosidases retards the liberation of glucose and decreases the postmeal blood glucose level (Alagesan et al., 2012). This property of inhibitors serves as a therapeutic agent for the treatment of diabetes and obesity.

Diabetes mellitus is a chronic metabolic disorder mediated by either insulin secretion or insulin action. In 2017, India recorded 72.9 million people suffered from diabetes, which is estimated to be reaching 151 million by 2030 (International Diabetes Federation). In type II diabetes mellitus, insulin resistance condition occurs. This happens due to the action of beta cells of the pancreas, which produces an insufficient amount of insulin. The body cells fail to respond to the action of insulin, leading to the elevated level of glucose in the bloodstream thereby causing hyperglycemia. The tissue cells of liver, muscle and fat take the glucose from the bloodstream via action of insulin to produce energy. When these cells fail to respond to circulating insulin, they lose their sensitivity to insulin and blood glucose level rises.

Followings are some examples of α-glucosidase inhibitors used in the treatment of diabetes mellitus and obesity.

1.4.1. Acarbose

Acarbose is an oral α-glucosidase inhibitor isolated from the fermentation broth of *Actinoplanes* sp. SE50 (Truscheit et al., 1981). Acarbose was first launched by Bayer in Switzerland in 1989 for the treatment of diabetes mellitus type 2.

These types of inhibitors are members of pseudo-oligosaccharides. The core element composed of a cyclitol unit and 4-amino-4,6-dideoxy-D-glucopyranose unit. To this core element a varying number of glucose residues linked by α-(1→4) linkage (Truscheit et.al., 1981). The general formula of these types of inhibitors is as follow:

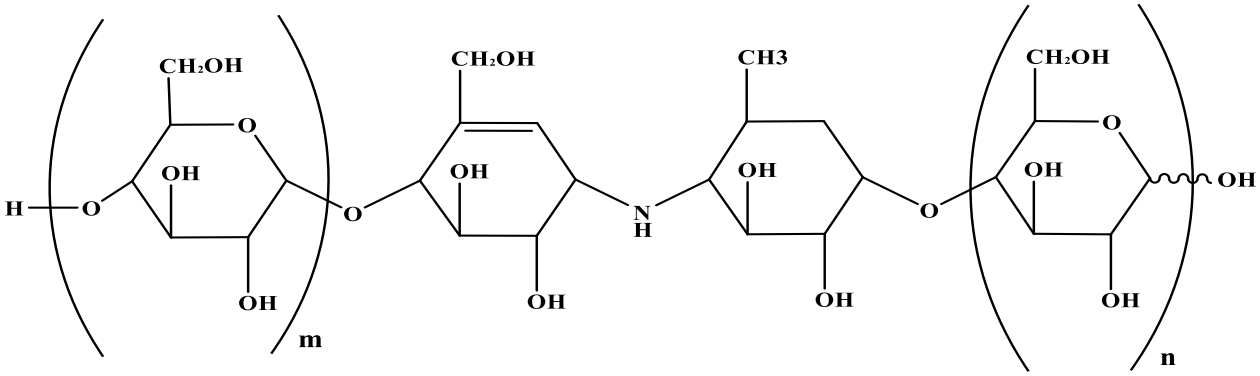


Fig. 3. General formula of pseudo-oligosaccharides α-glucosidase inhibitors isolated from *Actinoplanes* sp. SE50.

The individual member of these series was isolated from the fermentation broth of *Actinoplanes* sp.SE50 either in homogenous form or in isomeric mixtures. These members were tested against inhibitory action on both α-amylase from porcine pancreas and sucrase of the small intestinal mucosa of the pig. The inhibitory pattern was varyied according to the

numbers of glucose residues linked to the core unit (Truscheit et al., 1981). The members of these series had been characterized in details and is summarized in the below table 5.

Table 5. Members of pesudo-oligosaccharides α-glucosidase inhibitors isolated from *Actinoplanes* sp. SE50.

Designation	m	n
Component 2	0	1
Component 3 (acarbose, BAYg (5421)	0	2
Component 4	1	2
Component 5	2	2
Component 6	3	2

The component 3 showed maximum inhibition of α-amylase and sucrase and invested thoroughly with regards to its microbiology, biochemistry, pharmacology and clinical use. This component was given its designation BAYg 5421 and generic naming Acarbose.

The structure of Acarbose consists of an unsaturated cyclitol (valienol or valienamine), a 4-amino-4,6-dideoxyglucose and maltose unit (Fig. 4).The valienol and 4-amino-4,6-dideoxyglucose are linked via an amino bridge mimicking an N-glycosidic bond (Zhang et al., 2002). The molecular formula of acarbose is $C_{25}H_{43}NO_{18}$ and it's molar mass is 645.6048 g/mol.

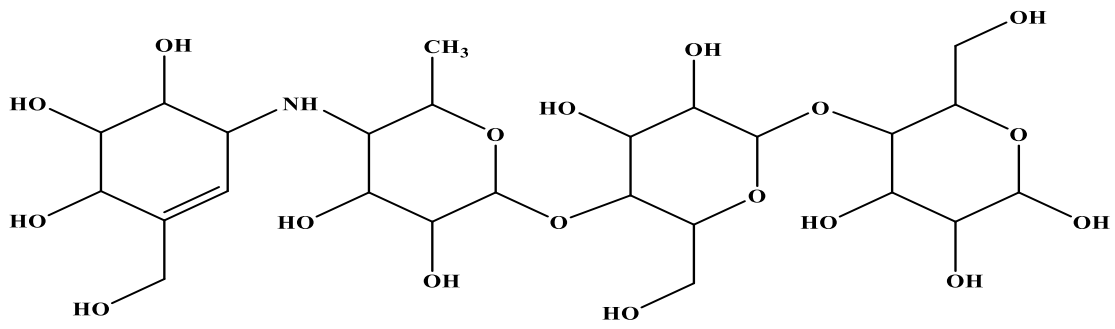


Fig.4. Acarbose

Acarbose belongs to cyclitol containing the aminoglycoside (CAGs) family compound. A recent analysis of gene clusters of CAG and related cyclitol containing metabolites revealed that, their biosynthetic pathways based largely on monofunctional enzymes catalyzing a single step in the reaction. Two distinct pathways are used in the initiation of CAG; one based on myo-inositol (Ca) and other on 1-keto-2-deoxy-cyclitol (Cb) pathway. The precursor for the Cb2 pathway is *sedo*-heptulose-7-phosphate. The biosynthesis of malto-oligosaccharide analogues is based on the formation of a C_7 cyclitol unit (valienol, valieolol and valienamine etc) via the Cb2 pathway and glycosidic attachment of (6-deoxy) hexose unit; they are malto-oligosaccharide analogues up to six (pseudo) saccharide unit linked by glycosidic bonds structurally mimicking to α-1,4-glucosides (Wehmeier and Piepersberg, 2009).

Mahmud et al. (1999) studied the biosynthetic pathway of acarbose by using ^{3}H, ^{2}H and ^{13}C labelled cyclitol. The feeding experiments in acarbose biosynthesis revealed that cyclitols which have the same stereochemistry at C_2 (valienamine, valienone, valiolone, valiolamine and 1-epi-valienol) were not incorporated directly in acarbose synthesis. They demonstrate that 2-epi-5-epi-valiolone was directly incorporated in acarbose.

Acarbose is obtained from the engineered strain of *Actinoplanes* sp. SE50/110 and their acarbose biosynthetic gene cluster was analyzed. It is 32 kb long cluster having 22 genes organized in eight transcriptional (Schwientek et al., 2012). They encode biosynthetic enzymes for the C_7 cyclitol and 6-deoxyhexose components for the formation of acarbose. Another acarbose biosynthetic gene cluster was analyzed from *Streptomyces glaucescenes* GLA.0 named gac-gene cluster. The gac-cluster presented some similarity to the acb-cluster of *Actinoplanes* but some dissimilarity was observed in the C_7N cyclitol synthesis. It is 43 kb long cluster having 25 genes organized from gacO to gacB for acarbose biosynthesis (Rockser and Wehmeier, 2009).

Acarbose binds reversibly to the α-glucosidase enzymes. This effect lasts for 4-6 hrs. It delays the hydrolysis of oligo and di-saccharides by α-glucosidase thus lower the post-prandial blood glucose level. Long term acarbose administrations will increases slowly glucosidase activity in the lower half of the small bowel. Initial therapy often results in the deposition of carbohydrate in the colon, where bacterial fermentation occurs causing flatulence and diarrhoea. Acarbose is poorly absorbed in the blood stream.

1.4.2. Voglibose

Voglibose was first isolated from the culture broth of *Streptomyces hygroscopicus* var. *limonons* in 1981 in Japan. It has been employed in the treatment of diabetes mellitus in 1994 in Japan naming Basen (Dabhi, et al., 2013).

Voglibose reversibly inhibits α-glucosidase enzymes and delays the absorption of glucose from the gastrointestinal tract. Voglibose also facilitates the α-endogenous glycogen like peptide-1 (GLP-1), which increases insulin secretion and sensitivity. It has also an inhibitory activity on glycogen thereby lowers the glucose levels. Voglibose has no inhibitory activity on lactase. The molecular formula of Voglibose (Fig.5) is $C_{10}H_{21}NO_7$ and it's molar mass is 267.28 g/mol.

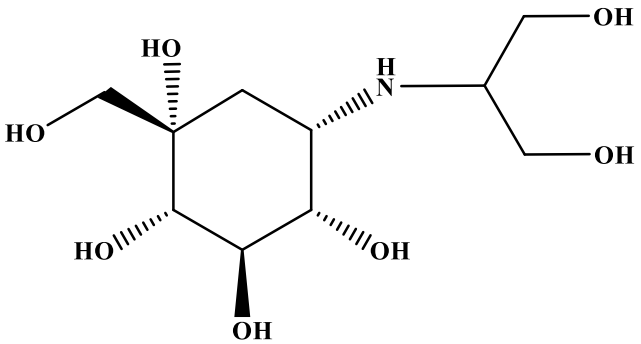


Fig.5. Voglibose

Voglibose used in the treatment of diabetes mellitus and glucose intolerance only when diet, exercise and other drugs do not control the glycemic index. It has been given to patient along with other hypoglycemic agents. In NIDDM patient's voglibose has given in 0.2 mg doses before meals. Maximum dose recommended is 0.6 mg, three times a day. Voglibose is poorly absorbed in the blood and renal excretion has not seen.

Side effects caused by voglibose include flatulence, abdominal pain, diarrhoea and nausea. Voglibose has a very good inhibitory activity on maltose and sucrose than that of acarbose and miglitol. However, it has less inhibitory activity on pancreatic alpha-amylase. Due to its low doses, voglibose is more tolerant as compared to acarbose and miglitol.

1.4.3. Nojirimycin

Nojirimycin was first isolated from *Streptomyces roseochromogenes* R-468 and *Streptomyces lavendulae* SF-425 in 1966 (Truscheit et al., 1981). It was the first member of glucose analogue containing 5-amino sugar isolated from nature and it has antibiotic activity against numerous strains (Niwa et al., 1970; Iida et al., 1987). Nojirimycin (Fig.6) comprises endocyclic nitrogen in place of oxygen pyranosidic atom (Melo et al., 2006). The molecular formula of Nojirimycin is $C_6H_{13}NO_5$ and it's molar mass is 179.172 g/mol.

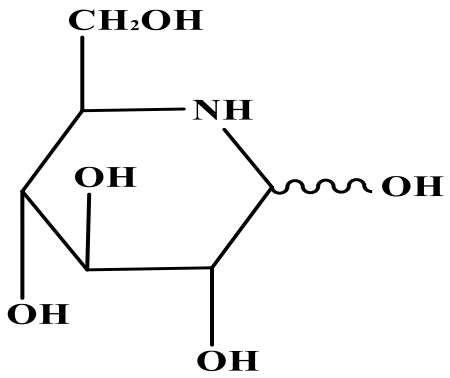


Fig.6. Nojirimycin

It is an iminosugar, which is usually unstable and can be stored as a bisulfite adduct or reduced to 1-deoxynojirimycin by means of hydrogenation or by sodium borohydride. Nojirimycin showed the inhibitory effect on takadiastase and glucoamylase of *Rhizopus niveus* (Niwa et al., 1970). Nojirimycin shows remarkable inhibition on both α and β-glucosidases.

1.4.4. 1-deoxynojirimycin:

1-deoxynojirimycin (DNJ) is a hydrolytic product of nojirimycin. Later on, DNJ was isolated from several *Bacillus* strain, *Streptomyces* strain and from mulberry leaves, which was known as molanoline (Asano, 2009). DNJ was also isolated from silkworm powder and shows higher stability than mulberry leaves DNJ and also has more α-glucosidase inhibitory activity (Yatsunami et al., 2011). The molecular formula of 1-deoxynojirimycin (Fig.7) is $C_6H_{13}NO_4$ and molar mass is 163.173 g/mol.

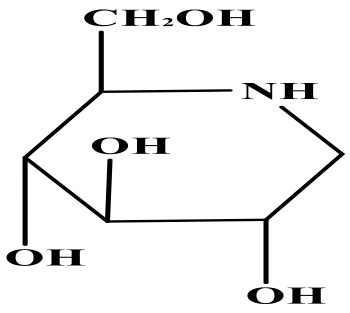


Fig.7. 1-deoxynojirimycin

1-deoxynojirimycin has more potent inhibitory activity than nojirimycin. Later on, DNJ derivatives were prepared in order to develop a potent inhibitor. 1,2-dideoxynojirimycin, α-homonojirimycin(HNJ) and 7-O-β-D-glucopyranosyl-α-homonojirimycin(Glc-HNJ) were tested against the inhibitory activity of glucosidases. 1,2-dideoxynojirimycin showed less activity and HNJ showed identical activity to those of DNJ (Asano, 2009).

DNJ has been reported for the inhibition of both α and β-glucosidases. Li et al. (2013) showed that DNJ modulates hepatic glucose metabolism and gluconeogenesis and it also inhibits the expression of proteins involved in the transepithelial glucose transport system thus prevents glucose absorption using streptozotocin-induced diabetic mice.

1.4.5. Miglitol

Miglitol is derived from the 1-deoxynojirimycin by alkylation to produce an N-hydroxyethyl derivative of DNJ (Fig.8). Miglitol was launched for the treatment of diabetes mellitus in 1998 in Europe. Miglitol is the third AGIs launched in the market after acarbose and voglibose. The molecular formula of miglitol is $C_8H_{17}NO_5$ and it's molar mass is 207.224 g/mol.

Miglitol is the first pseudomonosaccharide drug used in the treatment of diabetes mellitus. Miglitol inhibits α-glucosidases located in the brush border of a small intestine. Miglitol also reduced postprandial insulin level. It is systematically absorbed and excreted through the kidney. The initial dose of 25 mg before the meal is recommended. Side effects include abdominal pain, diarrhoea, skin rash etc. (Scott and Spencer, 2000).

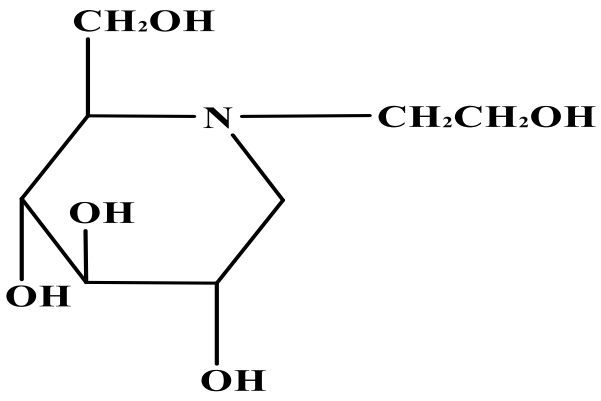


Fig.8. Miglitol

1.5. Alpha-glucosidase inhibitors from marine isolates

Apart from the known α-glucosidase inhibitors present in the market, various studies have been carried from the bacteria, fungus and plants for the isolation of α-glucosidase inhibitors. Here we are summarized some of the inhibition studies of α-glucosidase reported from the marine bacteria.

Suthindhiran et.al. (2009) isolated *Micromonospora* sp.designated as VITSDK3 from marine sediment sample at Marakkanam, India. The ethyl acetate extract of VITSDK3 was subjected to α-glucosidase inhibitory activity and it showed 74.43% inhibition at 100 μg/ml concentration. Ganesan et al. (2011) isolated sediment sample from Parangipettai coast of India. From this sediment sample, they have isolated a total of thirty actinobacteria and tested their activity against yeast and rat alpha-glucosidase. Out of thirty, four strains were found to be active against inhibition of yeast alpha-glucosidase. Among them, PSG22 showed highest inhibition activity (90.0%). Sathiyaseelan and Stella (2012) had isolated marine actinomycetes from Pichavaram mangrove, situated on the southeast coast of India. The strain AP-8 had shown significant inhibitory activity against the α-glucosidase enzyme. Sanjenbam et.al. (2013) isolated two *Streptomyces* strains designated as VITPK9 and VITSTK7 from brine spring, Manipur and marine sediment from Puducherry coast, Tamilnadu, India, respectively. The ethyl acetate extract of both strains subjected to α-glucosidase inhibition activity. Strain VITPK9 showed 57.89% and VITSTK7 showed 64.3% inhibition at 500 μg/ml concentration respectively. Sathish Kumar and Bhaskara Rao (2018) had isolated *Streptomyces coelicoflavus* SRBVIT13 from a soil sample of salterns, collected from a coastal area in Kothapatnam, Ongole, Andhra Pradesh, India. The chloroform extract of SRBVIT13 showed maximum inhibitory activity against mammalian alpha-glucosidase

with an IC_{50} value of 41.24 µg/ml. This extract was used *in vivo* antihyperglycemic study on Streptozotocin (STZ) Induced Diabetic Male Albino Wister Rats.

Apart from marine bacteria, some marine fungi were also showed the inhibitory activity. Ingavat et.al. (2009), isolated *Aspergillus aculeatus* CRI323-04 which was collected from Ton Sai Bay of Krabi Province. The organic extract F-6 was screened for α-glucosidase inhibition activity. The fraction F-6 was identified as Aspergillosol A, which is structurally similar to psammaplin A, tyrosine-derived metabolite. Aspergillosol A inhibited α-glucosidase with an IC_{50} value of 465±2 µg/ml. Chen et al. (2016), isolated the fungus *Talaromyces amestolkiae* from the leaf of mangrove plant *Kandelia obovata* from Zhanjiang Mangrove Nature Reserve in Guangdong Province, China. They have isolated isocoumarins and benzofurans compound from the fungus and tested their inhibitory activity. Isocoumarins and their derivatives were showed good inhibition activity. Wang et al. (2016), isolated the fungus *Aspergillus flavipes* HN4-13 from sediment of Lianyungang, Jiangsu Province of China. They have isolated new compounds from its ethyl acetate extract, namely flavipesolides A-C, aspulvinone Q, monochlorosulochrin, and dihydrogeodin. These compounds showed the non-competitive and mixed type of inhibition against human α-glucosidase enzyme.

Most of the marine bacteria which showed the alpha-glucosidase inhibition activity were from Actinobacteria phylum. Actinobacteria are known to produce many bioactive compounds. In this study, we screened marine bacteria from mangrove marine sediment sample to check their ability for producing any inhibition activity.

The primary aim of this study was to identify and classify the marine bacteria from sediment of Chorao Island, Goa, by using polyphasic systematic approach to decipher the taxonomic positions of the isolates. The second aim of this study was to screen these isolated

marine bacteria for their alpha-glucosidase inhibition activities. To encompass this, two working chapters for polyphasic systematics and screening for alpha-glucosidase inhibition are described.

CHAPTER-II

POLYPHASIC SYSTEMATIC APPROACH

2.1. Overview

This chapter provides a detailed study of marine bacteria collected from sediments from Chorao Island, Goa by using polyphasic systematics approach. The main aim was to examine the taxonomical identification and classification of isolated marine bacteria. Chorao Island is located along the river of Mandovi on the southern side and the river Mapusa on the northern side, both together forms an island in the middle (Fig. 9). It is amongst the 17th largest island of Goa. The total area of this island is 423.75 hectare. The mangrove covers up about 2.5 km^2 of the island (D´Souza et al., 2015). Chorao Island harbours most of the mangroves species found in Goa (Sappal et al., 2014). The land surface of this island extended peripherally because of heavy siltation through the sedimentation of Mapusa river, Norora creek and Mandovi estuary up to the esturian water (D´Souza et al., 2015).

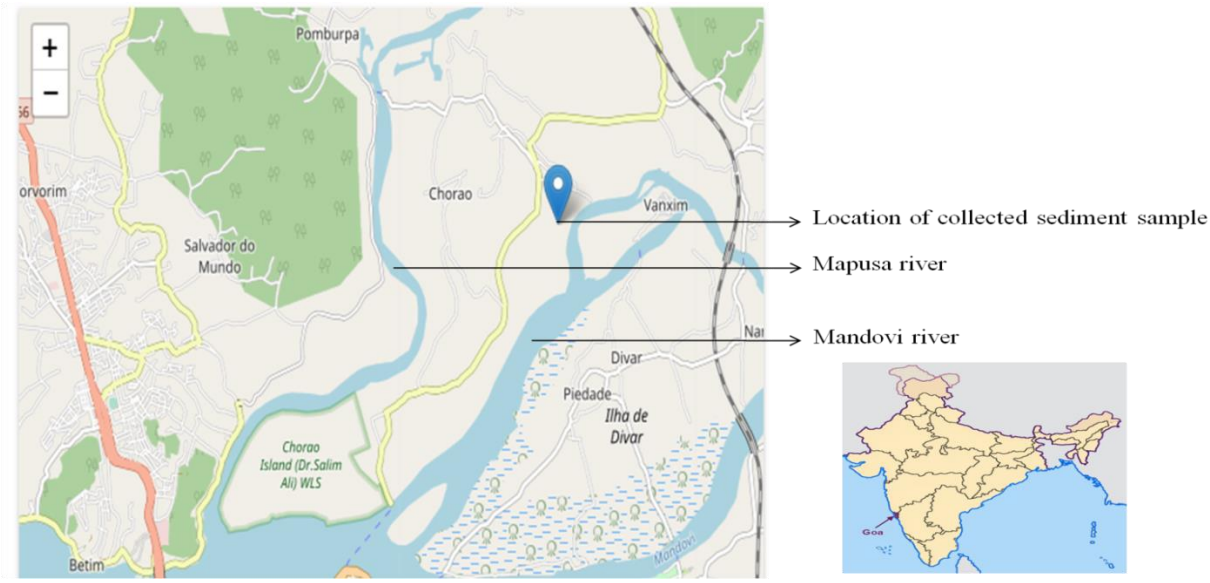


Fig. 9. Map of Chorao island and location of collected sediment sample.

2.2. Isolation of marine sediment sample

The sample of sediments was taken from the intertidal region of mangroves (Fig. 9). The samples were taken in sterile tubes from the 10 cm depth of the intertidal region of mangroves (GPS coordinates 15°32′34″N and 73°55′15″ E). All samples were transported to the laboratory and preserved in 4 ºC until further analysis. The different sediments were dissolved in sterile saline, filtered to remove sediments and other debris. The sterile saline sample was serially diluted and plated on isolation medium viz., marine agar (Difco) and Nutrient agar (Himedia, Mumbai). Due to the nature of sample, marine agar was choosed as a primary media for the isolation of microorganisms.

After primary isolation, the differentiated colonies on marine agar were picked and subcultured on fresh marine agar plates in order to isolate single colonies. The isolated colonies were further purified by repeated subculturing on marine agar plates. The purified colonies obtained were maintained on sterile slants and 20.0% of glycerol suspensions at 4 ºC and -20 ºC respectively.

2.3. Characterization

The isolated bacterial colonies were further subjected to their phylogenetic, phenotypic, genotypic and chemotaxonomic characterization. The methods used in this study were summarized in table 6. Each of these characteristics were describes the behaviour and relationship of bacteria among others with respect to their genus and species levels.

Table 6. Polyphasic methods used in the characterization

Characterization	Methods	Reference
I. Phylogenetic	1. Extraction of genomic DNA 2. 16S RNA gene sequencing 3. Phylogenetic analysis	Li et al., 2007 Ludwig and Schleifer, 1994 Saitou and Nei, 1987; Fitch, 1971; Felsenstein, 1981; Tamura, 2011
II. Phenotypic	1. Colony characteristics: colour, shape and margin etc 2. Cell morphology: size, shape, Gram's reaction and motility etc 3. Growth at different-temperature, pH and saline (NaCl) concentration 4. Biochemical test's	 Gerhardt et.al. 1994; Leifson, 1960 bioMérieux, Marcy l'Etoile, France; Hi-Media, Mumbai
III. Genotypic	1. DNA-DNA hybridization 2. Genomic DNA G+C content 3. DNA profiling (AP-PCR)	De Ley et al. 1970; Huss et al., 1983; Loveland-Curtze et al. 2011 Mesbah et al., 1989; Gonzalez and Saiz-Jimenez 2002 Martinez-Murcia & Rodriguez-Valera, 1994
IV. Chemotaxonomic	1. Cell wall peptidoglycan 2. Cell wall sugars 3. Polar lipids profile 4. Fatty acids methyl esters 5. Menaquinone content	Schleifer and Kandler, 1972; Schleifer ,1985 Staneck and Roberts, 1974 Minnikin et al., 1984 Sasser ,1990; Sherlock version 6.1; MIDI database: TSBA6 Minnikins et al., 1984 ; Kroppenstedt, 1982

2.3.1. Phylogenetic characteristics

2.3.1.1. Extraction of genomic DNA

The genomic DNA of isolates was performed by using a method according to Li et al. 2007 with modified phenol: chloroform extraction method. The resulting DNA products were stored in 1X TE buffer at -20 ºC. The concentration of genomic DNA was determined on

Thermos NANODROP LITE Spectrophotometer and its purity was checked on 0.8% agarose gel electrophoresis.

2.3.1.2. 16S rRNA gene sequencing

The extracted DNA was used as a template for 16S rRNA gene amplification using universal primers by polymerase chain reaction (PCR) [table 7]. The PCR reactions were performed in 50.0 µl which contained approximately 10-50 ng of DNA, 10X PCR buffer, 1.5 U of Taq polymerase, 0.2 µM of each primer, 200µM of each dNTPs and 2.5 mM of $MgCl_2$. The DNA amplification conditions were: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min 30 s, and a final extension step at 72 °C for 7 min. The purity of PCR amplified products were checked by electrophoresis and purified by a method using PEG–NaCl (Hi-Media, Mumbai). The purified PCR products were sequenced on Applied Biosystems 3500xL Genetic Analyzer.

Table 7. Primers used for 16S rRNA gene PCR amplification and sequencing

Primer name	**Sequence (5'-3')**	**Bases**	**Reference**
27F	CCAGAGTTTGATCMTGGCTCAG	22	Lane et al., 1991
530F	GTGCCAGCMGCCGCGG	16	Weisberg et al., 1991
704F	GTAGCGGTGAAATGCGTAGA	20	Tabacchioni et al., 1995
785F	GGATTAGATACCCTGGTA	18	Munusamy et al., 2015
685R	TCTACGCATTTCACCGCTAC	20	Krakova et al., 2012
907R	CCGTCAATTCTTTTGAGTTT	20	Lane et al., 1991
1492R	CGGTTACCTTGTTACGACTT	20	Turner et al., 1999
1525R	AAGGAGGTGWTCCATCC	17	Lane et al., 1991

2.3.1.3. Phylogenetic analysis

The 16S rRNA gene sequences were compared using the BLAST program with available sequences at NCBI GenBank (www.ncbi.nlm.nih.gov/BLAST) and at EzTaxon (www.ezbiocloud.net) using the BLAST program. Multiple sequence alignments with closely related members of the different bacterial genus and their sequence distance were calculated using CLUSTAL_X (Thompson et al., 1997). The phylogenetic tree was constructed by using the Neighbour-Joining method (Saitou and Nei, 1987), Maximum-Parsimony method (Fitch, 1971) and by Maximum-Likelihood method (Felsenstein, 1981) using the software MEGA vs.5.0 (Tamura, 2011). The resulting tree topologies were evaluated by the bootstrap resampling method of Felsenstein (1985).

2.3.2. Phenotypic characterization

The phenotypic characteristics of an organism, which solely depends on observable growth, shape of a colony, growth on a different pH, temperature and saline conditions and these properties can be varied from laboratory to laboratory conditions. These phenotypic characteristics are the expression of genes associated with the genome and it includes the morphological, physiological and biochemical properties of an organism.

Nowaday's phenotypic characterization involves the measuring the cell shape and size by using electron microscopy, observing the presence of motility using conventional motility test and electron microscopy method. The growth of bacteria at various temperature, pH and salt with their tolerance range were tested at the same laboratory conditions along with a type strain of the respective genera. The utilization of different carbon source, nitrogen sources, substrate utilization and hydrolysis patterns of given isolated bacteria were also studied.

We also analyze and compared the phenotypic characterization of isolates with their respective type strains.

2.3.2.1. Morphological characteristics

Gram staining was performed using standard Gram's reaction (Gerhardt et.al. 1994; Beveridge 2001). Cell motility was observed by the presence of turbidity in the semi-solid medium (Leifson, 1960) and with observation under light microscopy. Cell morphology and colony characteristics were observed under light microscope (Leica, Microsystem) and scanning electron microscope (FEI Quanta 200 SEM). Colony characteristics were observed by growing isolates in their respective agar medium for 24-48 hrs. at 30 ºC. For electron microscopy, cultures were grown freshly on Nutrient liquid broth and then the one ml of sample was incubated overnight with 7.0% formaldehyde at 4 ºC. After centrifugation, the cells were washed with 9.0 ml of sterile phosphate buffer saline and then dehydrated in a series of 25, 50, 70 and 100% ethanol solutions. Samples were then dried, mounted on stubs and coated with gold and observed on FEI Quanta 200 scanning electron microscope.

2.3.2.2. Growth on various temperature, pH and salt concentrations

The isolates were initially grown on nutrient agar plates at 30 ºC for 24-48 hrs according to the observable growth. To check the growth parameters at different temperature levels, isolates were grown on a nutrient broth at different temperatures ranging from 25 ºC, 30 ºC, 35 ºC, 40 ºC, 45 ºC and 50 ºC. For the growth at different pH, isolates were grown in nutrient broth set at different pH ranging from pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0, with an addition of appropriate buffer (pH 4.0–5.0: 0.1 M citric acid/0.1 M sodium citrate; pH 6.0–8.0: 0.1 M NaH_2PO_4/0.1M Na_2HPO_4; pH 9.0–10.0: 0.1 M $NaHCO_3$/0.1 M Na_2CO_3). The tubes were incubated at 30 ºC for 24-48 hrs. The salt tolerance test enables the growth and tolerance limit of an organism to a particular range of salt (NaCl, w/v). The isolates were grown at NaCl

concentrations ranging from 0.0% to 15.0 % (at intervals of 1.0%) in nutrient broth at 30 ºC for 24-48 hrs. The growth at a different temperature, pH and salt (NaCl) concentrations were observed visiblly by growth in the form of turbidity and followed by spectrophotometric absorbance at 620 nm. The growth parameters were inferred in good growth to least growth and no growth by '+++', '++', '+' and '-' indications. Growth under anaerobic condition was also determined by growing isolates in the anaerobic chamber.

2.3.2.3. Biochemical test's

Catalase activity of isolates was determined by addition of a drop of 3.0% H_2O_2 in culture broth which gives bubble formation for a positive test. Oxidase test was determined using API oxidase reagent. The utilization of sugars and acid production was performed using API CH50 kit (bioMérieux), with API CHB as an inoculum medium. Enzyme substrate utilization of an isolates were determined using API ZYM test strips (bioMérieux). Hydrolysis of urea was determined on peptone-glucose medium containing 2.0% urea and 0.001% phenol red as an indicator. Hydrolysis of starch was determined on peptone-beef agar containing 0.2% soluble starch by the flooding of the plates with iodine solution. Hydrolysis of gelatine was determined by incubation for one week at nutrient gelatine medium containing 1.2% gelatine. Casein hydrolysis was performed on casein agar by observation of clear zones around the colonies. Indole test, MR-VP test, citrate utilization etc tests were performed by using Hi-Media HiIMViC tets kits, according to manufacturers instructions.

2.3.3. Genotypic characterization

2.3.3.1. DNA-DNA hybridization

DNA-DNA hybridization was used to find out the relatedness between two nearest species of the same genus. This method was done according to De Ley et al. (1970), with an optimized procedure according to Huss et al. (1983) and Loveland-Curtze et al. (2011). The genomic DNA was stored in 0.1X SSC buffer. The genomic DNA was suspended in 2X SSC buffer and 5 µl of each homologous and heterologous DNA (in triplicates) were added into a 96 well PCR plate with a concentration of 0.1 µg/µl. Ten microliter of SYBR green I (diluted in 1: 10000 in 2X SSC buffer) were added to each well. Denaturation and renaturation of DNA was performed in real time PCR system. Initial denaturation was start at 25 ºC for 5s and then increased to 99 ºC for 10 min. After that, the temperature was decreased to 60 or 70 ºC for reassociation for 40 min. Again the temperature was reduced to 25 ºC for 30s. Fluorescence readings were taken at every 10s throught the experiment. The percentage of relative binding ratio (RBR) was calculated by using following formula:

$$D = \frac{4V'_M - (V'_A + V'_B)}{2\sqrt{V'_A V'_B}} \times 100$$

Where v' denoted to renaturation rates expressed as increase in fluorescene per minute. M, A and B denoted to mixture of pairs of DNA and two individual DNAs respectively (Loveland-Curtze et al., 2011).

2.3.3.2. Genomic DNA G+C content

The genomic DNA G+C content is varying from 25.0-75.0% among bacteria and archaea. Several different methods have been reported for G+C content determination (Mesbah et al., 1989; Schildkraut et al., 1962; Owen et al., 1969). Here two methods were used for G+C content analysis. In the HPLC method, genomic DNA was hydrolyzed using P1 nuclease and bovine intestinal mucosa alkaline phosphatase. The nucleosides were separated by reversed phase HPLC method using a C18 column and G+C content was calculated based on the ratio of deoxyguanoside and thymidine (Mesbah et al., 1989). The fluorometric method uses SYBR Green I for the staining of DNA. Thermal denaturation followed by measuring a decrease in fluorescence using a real-time PCR analyzes G+C content (Gonzalez and Saiz-Jimenez 2002).

2.3.3.3. Arbitrary primed (AP) PCR

The AP-PCR fingerprinting was performed using M13F primer (5'-GTAAAACGACGGCCAGT-3') and the PCR program is as follow: two cycles of 94 ºC for 5 min, 40 ºC for 5 min and 72 ºC for 5 min; followed by 40 high-stringency cycles of 94 ºC for 1 min, 60 ºC for 1 min and 72 ºC for 2 min. Amplified DNA products were resolved by electrophoresis on 2.0 %, w/v, agarose gel (Martinez-Murcia & Rodriguez-Valera, 1994).

2.3.4. Chemotaxonomic characterization

2.3.4.1. Cell wall peptidoglycan

Cell wall peptidoglycan was analyzed by a method described by Schleifer and Kandler (1972) and Schleifer (1985). Amino acids from the cell wall were isolated by acid hydrolysis or trypsin treatment and further determined by two dimensional descending or ascending thin layer chromatography (TLC). The hydrolysate was applied as a spot on first system

isopropanol: acetic acid: water (75:10:15, v/v) and for second system α-picoline: 25.0% ammonium hydroxide: water (70:2:28, v/v). The chromatogram was observed by spraying ninhydrin and heated at 100 ºC for 5-10 min.

2.3.4.2. Cell wall sugars

Cell wall sugars were analyzed by following the procedure described by Staneck and Roberts, (1974). Dry cells were hydrolyzed using 1N H_2SO_4 followed by addition of saturated barium hydroxide until pH reaches between 5.2 to 5.5. TLC method was applied for separation and detection of sugars using solvent system n-butanol: water: pyridine: toluene (10:6:6:1, v/v) and spraying system of acid aniline phthalate and heating at 100 ºC for 4 min.

2.3.4.3. Polar lipid profile

Polar lipids were extracted and examined using a procedure described by Minnikin et al., (1984). It was extracted in chloroform: methanol: 0.3% aq. NaCl (1:2:0.8, v/v) for 4 hrs. Polar lipids were recovered into the chloroform phase by adjusting the above mixture to a ratio of 1:1:0.9. It was examined by two-dimensional TLC (silica gel plate 60; Merck). First direction was in chloroform: methanol: water (65:25:4, v/v) and second one in chloroform: methanol: acetic acid: water (80:12:15:4, v/v). Total lipid material and specific functional groups were detected by using Dittmer and Lester reagent (phosphate), ninhydrin (free amino groups), Dragendorff reagent (quaternary nitrogen), and anisaldehyde-sulfuric acid (glycolipids).

2.3.4.4. Fatty acid methyl esters (FAME) analysis

For fatty acid analysis cultures were grown on tryptic soy agar at 30 ºC for 24-48 hrs. Preparation and analysis were performed as described by Sasser (1990) using Microbial Identification System (MIDI) and the Microbial Identification software package (Sherlock

version 6.1; MIDI database: TSBA6). This method contains four steps viz., saponification, methylation, extraction, and washing. Three grams of pure culture biomass or lyophilized powder of previously harvested was used and mixed with 3.0 ml of 3.75 M NaOH in methanol: water (1:1). This mixture was vortexed and kept in a 100 °C water bath for 30 min. Then 6.0 ml of 6M HCl: methanol (1:0.85) was added and then incubated in a water bath for 10 min at 85 °C. Further, 2 to 3 ml of hexane: methyl-tert-butyl ether (1:1) mixture was added to extract the esters into the organic phase. Later on, it was transferred to teflon, sscrew-caped tube and then centrifuged at 4800 rpm for 10 min. The remained organic phase was tensferred to a new tube and further washed by using 3 ml of 0.3M NaOH. The organic phase was subjected to gas chromatography (GC) analysis using capillary column equipped with flame ionization detector. The fatty acids were determined by using the standard library of MIDI.

2.3.4.5. Menaquinone content

It was isolated according to Minnikins et al., 1984 and analyzed by using HPLC method (Kroppenstedt, 1982). About 50.0 mg of dry biomass in teflon lined screw-caped tube was suspended and mixed in biphasic mixture of petroleum ether and methanolic saline (3.0% NaCl in methanol) for 15 min. The upper organic phase containing isoprenoid quinones was collected in glass tube and evaporated under nitrogen atmosphere and stored at -20 ºC. The extracts was resuspended in pet ether and applied on TLC. The TLC plates were developed in hexane:dibutylether (85:15, v/v) and the bands were observed under UV light at 254 nm. The spots were scraped and stored in 1.0 ml of diethyl ether. Thses mixture was then subjected to high performance liquid chromatography (HPLC). The samples were dried and resuspended in n-hexane. The samples were injected in reverse phase RP C-18 column with a mobile phase of acetonitrile: 2-propanol (75:25, v/v) and detected at a wavelenght of 254 nm.

2.4. **Results**

2.4.1. Isolation and identification of marine bacteria

The marine bacteria which were isolated from a marine sediment sample were identified initially by using 16S rRNA gene sequencing method (Table 8.).The focus of this study was mostly on Gram positive bacteria and the selected isolates were identified and classified based on polyphasic approach.

Table 8. The 16S rRNA gene sequence similarity of marine isolates.

Sl.no.	Marine isolate with lab given numbers	Nearest neighbour based on 16S rRNA gene sequencing (NCBI Hit)	16S rRNA sequence similarity with its nearest neighbour
1	R1	*Bacillus mobilis*	99.9%
2	R-2	*Bacillus circulans*	99.80%
3	R-5	*Bacillus flexus*	99.78%
4	R-9	*Bacillus flexus*	99.84%
5	R-10	*Bacillus firmus*	99.68%
6	R-18	*Bacillus cereus*	99.9%
7	R-25	*Bacillus circulans*	99.86%
8	D-5	*Bacillus cereus*	97.04%
9	D-6	*Bacillus halosaccharovorans*	98.30%
10	D-9	*Bacillus flexus*	96.38%
11	D-10	*Bacillus cerues*	99.83%
11	D-12	*Bacillus jeotgali*	99.39%
13	D-17	*Bacillus cereus*	99.92%
14	D-25	*Bacillus cereus*	99.13%
15	DM-1	*Bacillus flexus*	99.37%
16	DM-2	*Bacillus flexus*	99.88%
17	NIO-1001	*Bacillus subtilis*	99.94%
18	NIO-1002	*Microbacterium zeae*	99.22%
19	NIO-1003	*Fictibacillus rigui*	98.2%
20	NIO-1007	*Bacillus stratosphericus*	99.97%
21	NIO-1008	*Arthrobacter equi*	98.5%
22	NIO-1009	*Rhodococcus kroppenstedtii*	99.2%
23	NIO-1011	*Dietzia maris*	99.71%
24	NIO-1013	*Bacillus aerius*	99.93%
25	NIO-1016	*Domibacillus roboginosus*	98.0%

26	NIO-1021	*Kocuria marina*	98.6%
27	NIO-1022	*Bacillus firmus*	99.95%
28	NIO-1023	*Deinococcus ficus*	97.8%
29	NIO-1030	*Bacillus stratosphericus*	98.43%
30	NIO-1051	*Pseudomonas stutzeri*	99.93%
31	NIO-1057	*Glutamicibacter mysorens*	98.42%
32	NIO-1105	*Bacillus niabensis*	99.60%
33	NIO-1109	*Exiguobacterium indicum*	99.5%
34	NIO-1121	*Bacillus nealsonii*	99.98%
35	NIO-1123	*Bacillus aquimaris*	94.5%
36	NIO-1130	*Bacillus niabensis*	98.1%
37	SGD-01	*Bacillus cereus*	99.92%
38	SGD-03	*Bacillus licheniformis*	99.79%
39	SGD-05	*Bacillus mojavensis*	98.33%
40	SGD-06	*Bacillus aerius*	99.79%
41	SGD-07	*Bacillus altitudinus*	99.89%
42	SGD-11	*Bacillus cereus*	99.51%
43	SGD-14	*Bacillus endophyticus*	99.6%
44	V-17	*Bacillus firmus*	98.04%
45	V-19	*Bacillus badius*	99.96%
46	V-25	*Bacillus badius*	99.1%
47	V-68	*Bacillus flexus*	99.87%
48	V-74	*Cohnella ferri*	98.2%
49	V-76	*Bacillus flexus*	99.32%
50	V-82	*Kytococcus sedentarius*	99.84%
51	V-85	*Gordonia terrae*	99.05%
52	V-88	*Bacillus stratosphericus*	91.23%
53	V-93	*Bacillus aerophilus*	99.93%
54	M-37	*Bacillus flexus*	99.46%
55	M-57	*Bacillus flexus*	99.87%
56	19-a	*Paenibacillus pabuli*	99.57%
57	AC-3	*Lysinibacillus macroides*	98.48%
58	AC-6	*Lysinibacillus pakistanensis*	99.97%
59	AC-7	*Lysinibacillus pakistanensis*	96.96%
60	AC-8	*Lysinibacillus cresolivorans*	99.86%
61	AC-9	*Bacillus nealsonii*	99.33%
62	AC-11	*Bacillus aryabhattai*	99.30%
63	AC- 13	*Bacillus velezensis*	98.88%
64	AC-15	*Lysinibacillus xylanilyticus*	98.74%

2.4.2. Description of novel species based on polyphasic approach

In overall we have isolated sixty four unique bacterial colonies isolated and purified based on their apperance and morphology on the isolation medium. Further, all sixty four isolates were subjected for 16S rRNA gene sequencing based identification, based 16S sequencing analysis as mentioned in the earlier table 8. Majority of the strains belongs to *Bacillus* genus (44 strains), *Lysinibacillus* genus (5 strains) and each strain of *Deitzia*, *Microbacterium*, *Fictibacillus*, *Arthrobacter*, *Rhodococcus*, *Domibacillus*, *Kocuria*, *Deinococcus*, *Pseudomonas*, *Glutamicibacter*, *Exiguobacterium*, *Cohnella*, *Kytococcus*, *Gordonia* and *Paenibacillus* genus. Here we are giving description of those isolates which we reported as a novel species.

2.4.2.1. Genus *Bacillus*

Ferdinand Cohn in 1872 described the genus *Bacillus* for his work on boiled hay infusion from which he had isolated a small, motile and aerobic bacterium. He names it *Bacillus subtilis*. *Bacillus* genus consists of rod shaped, Gram positive, aerobic or anaerobic, spore forming bacteria which is widely found in soil and water. It is currently categorised under Phylum *Firmicutes*, Class *Bacilli*, Order *Bacillales* and Family *Bacillaceae*. To date, there are 377 validly species and 8 subspecies in this genus (www.bacterio.net). Most of aerobic, spore forming rod shaped bacteria were initially appointed to a genus *Bacillus* (Claus and Berkeley, 1986). To identify such aerobic, rod shaped, endospore or non-endospore forming bacteria, Logan et al. 2009 proposed minimal standards to characterize new taxan. Therefore, identification and classification of such bacteria required polyphasic taxonomic approach (Claus and Berkeley 1986; Smibert and Krieg 1994).

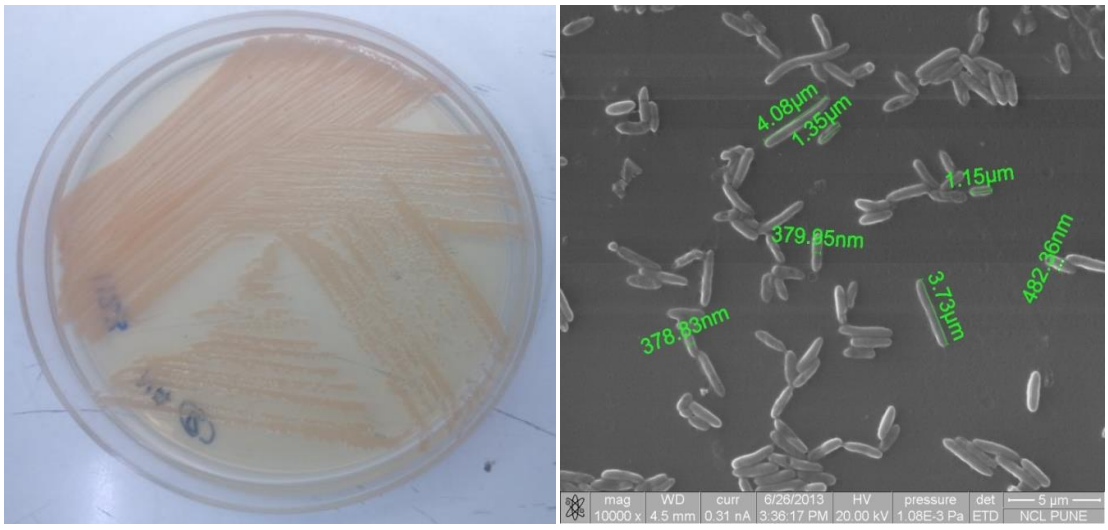
2.4.2.1. a. Strain SGD-1123 *Bacillus* sp.

SGD-1123 were isolated and maintained on marine agar from a marine sediment sample. Colonies of SGD-1123 were observed to be pale orange in colour on nutrient agar and on tryptic soy agar. Smooth, circular to slightly irregular, slightly raised and 2-3mm in diameter colonies were observed (Fig. 10 A). No aerial mycelia and diffusable pigments were found. Cells were observed to be Gram positive and short rods. In Scanning Electron Microscopy cells were found to 0.3-0.4 x 1.3-4.0 μm in size (Fig. 10 B). Cells were observed motile by means of the single sporangium. Central ellipsoidal endospore was observed in swollen sporangia. Strain SGD-1123 was found to grow at 15–42 ºC temperature, pH 5.0–12.0 and with 0–12 % (w/v) NaCl and optimum growth was observed at 30 ºC, pH 7.0–7.5 and 0–5 % (w/v) NaCl concentration. The detailed physiological and biochemical tests are given in the table 9.

The 16S rRNA gene sequence of SGD-1123 (1435 bp) was obtained and deposited in NCBI (GenBank/EMBL/DDBJ accession number KF265350). Strain SGD-1123 showed highest 16S rRNA sequence similarity with *B. aquimaris* JCM 11545^T, *B. vietnamensis* JCM 11124^T, and *B. marisflavi* JCM 11544^T with 94.5, 94.1 and 94.1% and nucleotide differences of 77, 77 and 83 nucleotides, respectively. The phylogenetic tree was constructed from 16S rRNA gene sequence of SGD-1123 and other sequences from related type strains of *Bacillus* species using the neighbour joining algorithm (Fig. 11). Upon comparative analysis of 16S rRNA gene sequence and phylogenetic tree showed that strain SGD-1123 lies in a subclade in a tree with *B. aquimaris* JCM 11545^T and *B. marisflavi* JCM 11544^T supported by the bootstrap value of 70.0%. The affiliation of strain SGD-1123 and its closest neighbours was also supported by the maximum parsimony and maximum-likelihood algorithms with above 70.0 % bootstrap values.

The genomic DNA G+C content of SGD-1123, *B. aquimaris* JCM 11545^T, *B. vietnamensis* JCM 11124^T, and *B. marisflavi* JCM 11544^T were found to be 44.6 mol%, 49.0 mol%, 43.0-44.0 mol% and 38.0 mol%, respectively. The closest strains of SGD-1123 shares less than 97.0% 16S rRNA sequence similarity, therefore DNA-DNA hybridization was not carried out. The AP-PCR amplicon fingerprint (Fig. 12) showed remarkable differences in banding patterns between strain SGD-1123, *B. aquimaris* JCM 11545^T and *B. marisflavi* JCM 11544^T, suggested that the three strains were of different species.

Strain SGD-1123 was found to contain *meso*-diaminopimelic acid as the diagnostic amino acid in the cell wall peptidoglycan. Ribose, glucose and galactose sugars were found in the cell wall. The unsaturated menaquinone with seven isoprene units (MK-7) was identified as a major menaquinone. The polar lipids detected in SGD-1123 (Fig.13) were diphosphotidylglycerol, phosphatidylethanolamine and an unidentified amino lipid. The major fatty acids (>5.0%) detected was iso-$C_{15:0}$ (39.0%), ante-iso-$C_{15:0}$ (30.7%), $C_{16:0}$ (8.0%), iso-$C_{17:0}$ (6.49%) and anteiso-$C_{17:0}$ (5.7%) [Table 10].



A B

Fig.10. A) colony morphology and B) Scanning electron micrograph of strain SGD-1123^T from 3-day old cultures grown on Marine agar.

Table 9. Characteristics that serve to differentiate the novel strain SGD-1123^T from recognized *Bacillus* species

Character	***Bacillus* sp. NIO-1123^T**	***B. aquimaris* JCM 11545^T**	***B. vietnamensis* JCM 11124^T**	***B. marisflavi* JCM 11544^T**
Gram staining	+	+ (or V)	+	V
Flagellum type	Single polar	Single polar	Peritrichous	Peritrichous
Spore position	Central	Sub terminal or central	Central	Central
Colony colour	Pale orange yellow	Pale yellow	Pale yellow	Pale orange-yellow
Growth at 45 °C	–	+	–	–
Optimal pH for growth	7.0–7.5	6.0–8.0	6.5–10.0	6.0–7.0
Growth at pH				
4.5	–	+	–	–
9.0	+	+	+	–
Growth in NaCl at (%, w/v)				
0	+	+	+	W
17	–	–	–	+
Hydrolysis of				
Aesculin	–	+	+	–
Starch	+	–	+	+
Acid production from				
Aesculin	–	+	+	–
Arbutin	+	+	–	–
D-Cellobiose	–	+	–	–
D-Galactose	+	W	–	–
Gentiobiose	+	+	–	–
Glycerol	+	+	+	–
Glycogen	+	–	+	+
5-Ketogluconate	+	–	–	+
D-Mannitol	+	+	+	–
D-Mannose	+	+	–	–
Melibiose	–	+	–	–
Methyl α-D-mannoside	+	+	–	–
D-Raffinose	–	W	–	–
Salicin	+	+	–	–
Starch	+	–	+	+
D-Xylose	–	+	–	–
Major fatty acids	iso-$C_{15:0}$, anteiso-$C_{15:0}$	Anteiso-$C_{15:0}$, iso-$C_{15:0}$	Anteiso-$C_{15:0}$, iso-$C_{15:0}$and anteiso-$C_{17:0}$	iso-$C_{15:0}$, anteiso-$C_{15:0}$
G+C content (mol%)	44.6	49.0	43.0–44.0	38.0
Source of Isolation	Marine sediment	Tidal flat	Fish sauce	Tidal flat

SGD-1123^T (data from this study); Data for *B. aquimaris* from Yoon et al. (2003), *B. vietnamensis* from Noguchi et al. (2004) and *B. marisflavi* from Yoon et al. (2003)

+ positive, - negative, W weakly positive, V variable

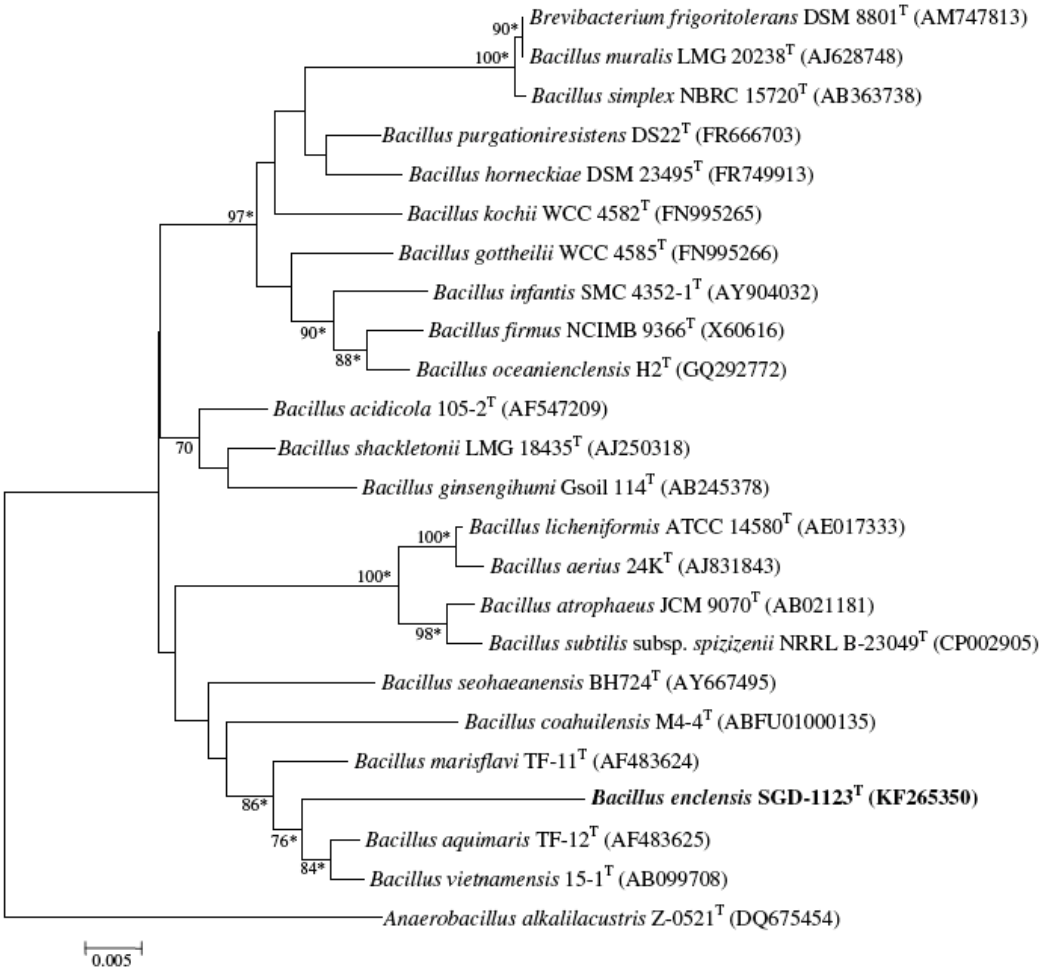


Fig.11. Neighbour-joining tree showing the phylogenetic positions of strains SGD-1123^T and representatives of related taxa based on 16S rRNA sequences. The topology of the entire tree was conserved in all trees using different algorithms. Asterisks indicate the branches found in phylogenetic consensus trees generated with the maximum-parsimony and maximum likelihood methods. Numbers at nodes are levels of bootstrap support from 1,000 resample datasets; values greater than 70.0% are shown at branch-points. Bar 0.005 nucleotide substitutions per position

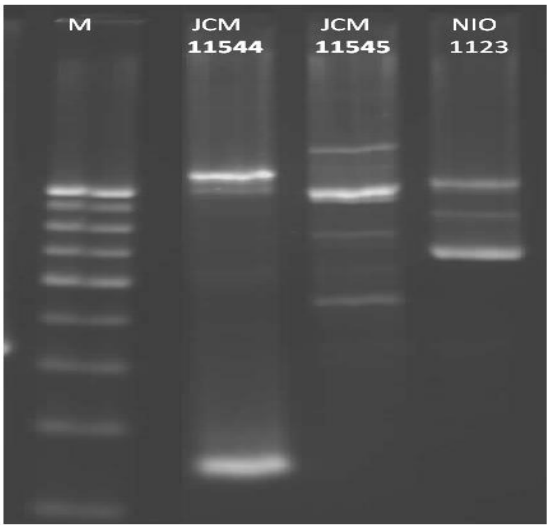


Fig.12. Arbitrary primed PCR (AP-PCR) profile [Lane 1-Marker 100 bp; Lane 2: *Bacillus aquimaris* JCM 11545^{T}; lane 3: *Bacillus marisflavi* JCM 11544^{T}; lane 4: SGD-1123].

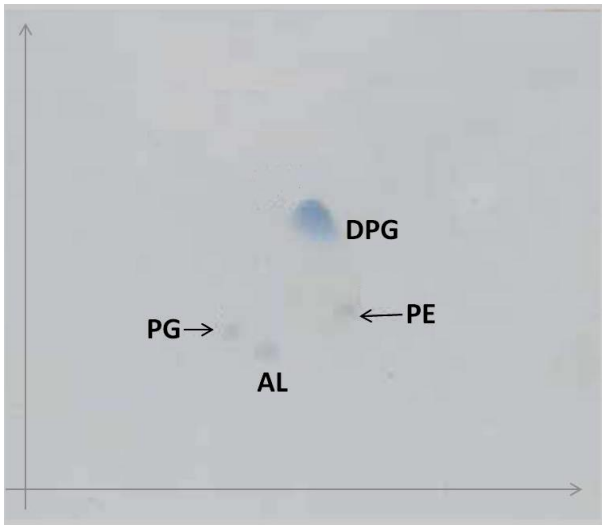


Fig.13. Two-dimensional TLC of the polar lipids of strain SGD-1123^{T}. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; AL, unknown aminolipid.

Table 10. Major fatty acids detected in strain SGD-1123^{T}

Fatty acids	Percentage content (>5.0%)
Straighed chain saturated	
$C_{16:0}$	8.0%
Branched	
Iso-$C_{15:0}$	39.0%
Anteiso-$C_{15:0}$	30.7%
Iso-$C_{17:0}$	6.49%
Anteiso-$C_{17:0}$	5.7%

The phenotypic, genotypic and phylogenetic data distinguish Strain SGD-1123 from other validly named members of genus *Bacillus*. Therefore we proposed SGD-1123^{T} represents a novel species within the genus *Bacillus*, for which *Bacillus enclensis* sp.nov., is proposed. *Bacillus enclensis* (e.ncl.en' sis. N.L. masc. adj. *enclensis* arbitrary name formed from NCL, the acronym for the National Chemical Laboratory, India, where taxonomic studies on this species were performed).

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ORIGINAL PAPER

Bacillus enclensis sp. nov., isolated from sediment sample

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Abstract A novel bacterial strain, designated SGD-1123^T was isolated from Chorao Island, in Goa Province, India. The strain was found to be able to grow at 15–42 °C, pH 5–12 and 0–12 % (w/v) NaCl. The whole cell hydrolysates were found to contain *meso*-diaminopimelic acid, galactose and arabinose. The major fatty acids were identified as iso-$C_{15:0}$ and anteiso-$C_{15:0}$, MK-7 was identified as the predominant menaquinone and the predominant polar lipids were identified as diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and an unidentified aminolipid. The genomic DNA G+C content was determined to be 44.6 mol%. Phylogenetic analysis based on 16S rRNA gene sequences placed the isolate within the genus *Bacillus* and further revealed that strain SGD-1123^T had highest sequence similarity with *Bacillus aquimaris*, and forms a separate clade with its closest relatives i.e. *B. aquimaris*, *Bacillus vietnamensis* and *Bacillus marisflavi,* with which it shares 94.5, 94.1 and 94.1 % similarity respectively. The phylogenetic, chemotaxonomic and phenotypic analyses indicated that strain SGD-1123^T represents a novel species within the genus *Bacillus*, for which the name *Bacillus enclensis* is proposed. The type strain is SGD-1123^T (NCIM 5450^T=CCTCC AB 2011125^T).

Keywords *Bacillus* sp · Marine sediment · Polyphasic

Introduction

Members of the genus *Bacillus* and related genera are ubiquitous in nature. However, *Bacillus* species isolated from marine sediments have attracted less

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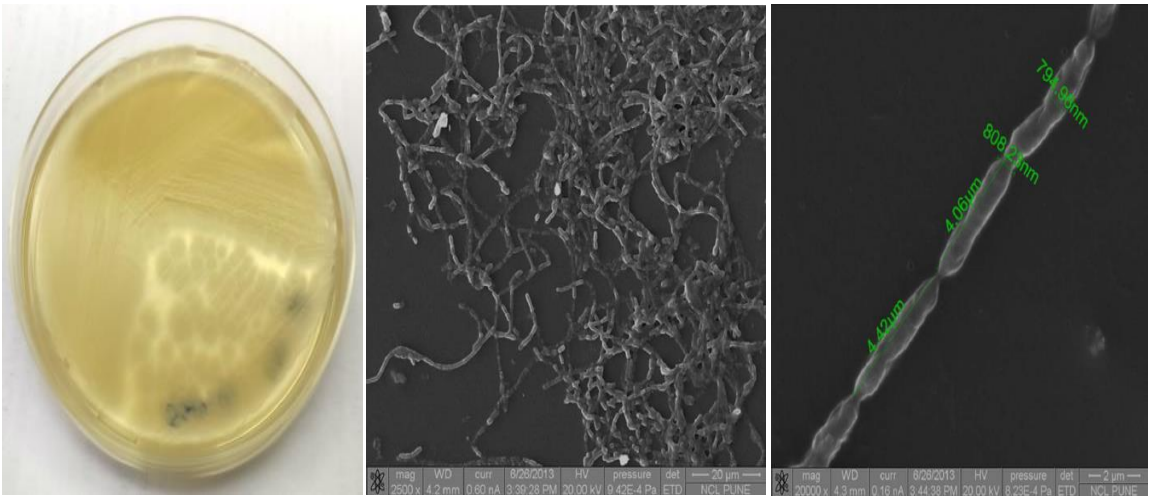
2.4.2.1. b. Strain SGD-14- *Bacillus* sp.

SGD-14 was isolated and maintained on marine agar and nutrient agar. Colonies of SGD-14 was observed as a pale orange colour on nutrient agar and tryptic soy agar, circular, slimy with an entire edge and about 1.5-3 mm in diameter (Fig. 14 A). Cells were observed to be Gram positive rods and non-motile. Terminal ellipsoidal endospore was observed with swollen sporangia. No aerial mycelia and no diffusable pigments were observed. Cells were observed to be 0.7-0.8 x 4.0-4.4 μm in size and were arranged in a long chain of rods which looked like filaments under Scanning Electron Microscope (Fig. 14 B). SGD-14 was found to be grow at temperatures ranging from 20-45 ºC (optimal at 30 ºC) and at pH 7.0-10.0 (optimal at 7.0) with NaCl concentration of 0.0-7.0% (w/v). The detailed biochemical and physiological characteristics of SGD-14 are given in a table 11.

The 16S rRNA gene sequence of SGD-14 (1402 bp) was obtained and subsequently deposited in NCBI (GenBank accession number KF265351). Strain SGD-14 showed highest 16S rRNA sequence similarity with *B. endophyticus* CIP 106778^T with 99.6%. The phylogenetic tree was constructed and it was observed that strain SGD-14 formed a single clade with a type strain *B. endophyticus* CIP 106778^T which was supported by 100% bootstrap values (Fig. 15). The G+C content of strain SGD-14 was determined to be 39.6 mol% whereas strain *B. endophyticus* CIP 106778^T was 35.6 mo%. The DNA-DNA hybridization was carried out in between SGD-14 and *B. endophyticus* CIP 106778^T, which was obtained to be 39.0 ± 3.2%. This value is well below to the threshold cutoff value of 70% for recognition of prokaryotic species (Wayne et al. 1987), thus suggested that strain SGD-14 should be considered as a separate species within *Bacillus* genus. Further, the AP-PCR amplicon fingerprint profiles of SGD-14 and *B. endophyticus* CIP 106778^T showed marked differences in banding pattern (Fig. 16), indicating that SGD-14 was distinguished from its closest neighbour *B. endophyticus* CIP 106778^T.

Strain SGD-14 contains *meso*-diaminopimelic acid in their cell wall peptidoglycan. The cell wall sugars were found to contain ribose, glucose and galactose. The predominant menaquinone was found to be unsaturated menaquinone with seven isoprene units (MK-7). The major polar lipids were identified as diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and aminophospholipid (Fig. 17). The major fatty acids detected (>5%) were anteiso-$C_{15:0}$ (39.3%), iso-$C_{15:0}$ (13.2%), iso-$C_{16:0}$ (10.6%), anteiso-$C_{17:0}$ (10.5%) $C_{16:0}$ (8.9%) and iso-$C_{14:0}$ (8.0%). The fatty acid profile of SGD-14 and *B. endophyticus* CIP 106778^T are shown in the table 12.



A B

Fig.14. A) colony morphology and B) scanning electron micrograph of strains SGD-14^T filamentous structure from 3-day old cultures grown on nutrient agar.

Table 11. Characteristics that serve to differentiate strain SGD-14^{T} from other related *Bacillus* species

Characteristics	***Bacillus* sp SGD-14^{T}**	***B. endophyticus* CIP106778^{T*}**	***B. circulans* ATCC 4513^{T}**	***B. shackletonii* LMG 18435^{T}**	***B.subtilis* subsp *subtilis* CECT 39^{T}**
Spore position	Sub-terminal	Terminal	Terminal	Sub terminal/central	Sub terminal
Spore shape	Ellipsoidal	Ellipsoidal	Oval	swollen	Oval
Oxidase	+	+	−	+	+
Anaerobic growth	−	+	+	−	−
Growth at 50 °C	−	−	−	+	−
Acid from					
L-arabinose	+	+	+	−	+
D-xylose	+	−	+	−	−
Sorbitol	+	+	+	−	+
Methyl α-D-glucoside	−	−	+	−	+
Lactose	−	−	−	+ (weak)	−
Melibiose	+	−	+	−	+
Raffinose	+	−	−	−	+
Trehalose	+	+	+	+(weak)	+
Rhamnose	+	−	−	−	−
Cellobiose	−	+	+	+	+
Glucose	+	+	−	+	+
Mannitol	+	+	+	+(weak)	−
Citrate utilization	+	−	−	−	+
ONPG-hydrolysis	−	−	+	−	+
Aesculin hydrolysis	−	−	+	+	+
G + C mol%	39.6	35.6	43.2	36.8	43.0

Data for *B. circulans* (Venkateswaran et al. 2003; Lim et al. 2007), *B.subtilis* subsp *subtilis* (Ruiz-Garcı´a et al. 2005) and *B. shackletonii* (Logan et al. 2004) taken from the literature. + Positive; - negative

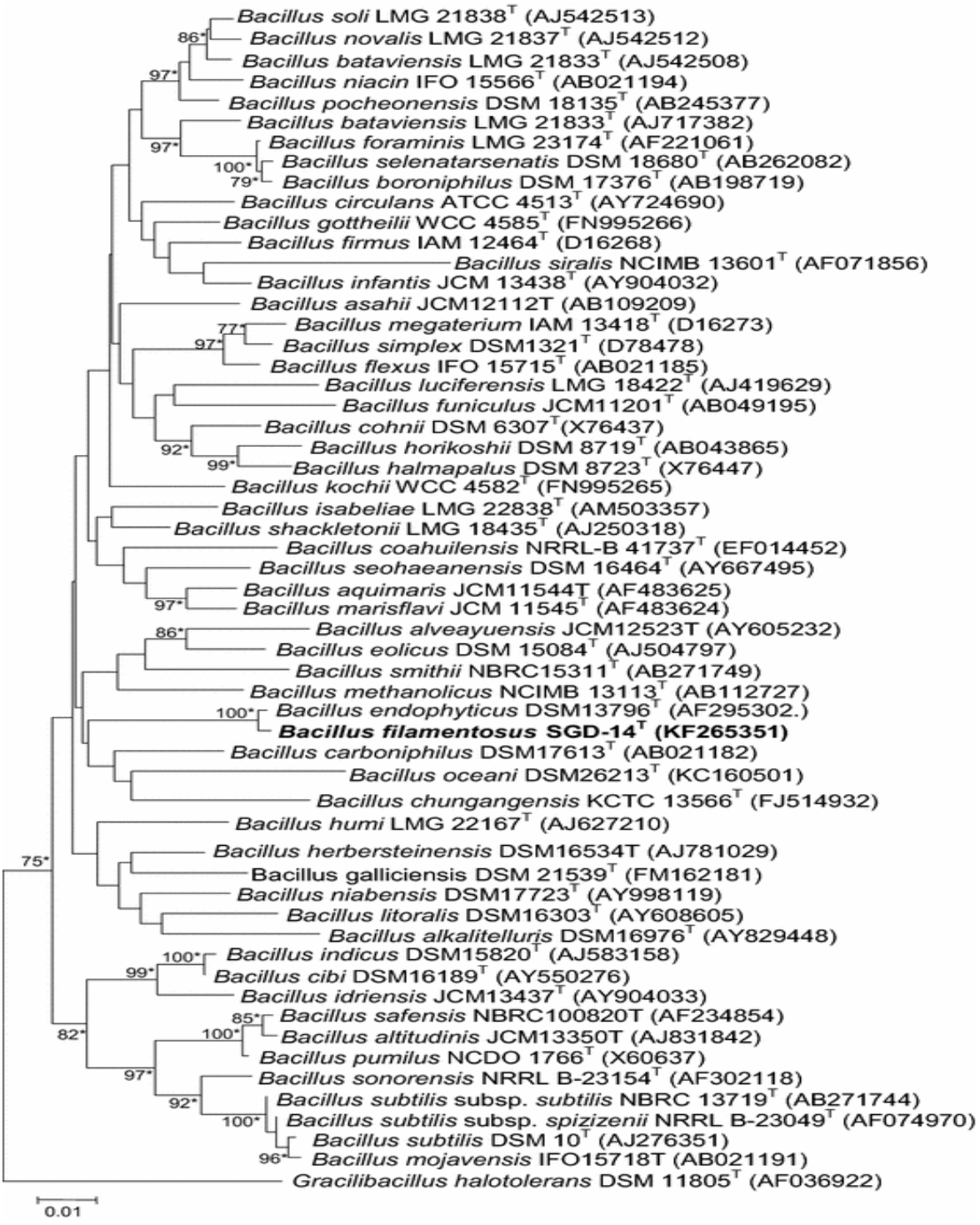


Fig.15. Neighbour-joining tree showing the phylogenetic positions of strains SGD-14^T and representatives of some other related taxa based on 16S rRNA sequences. Bar 0.01 substitutions per nucleotide position. Asterisks indicate that the corresponding nodes (groupings) are also recovered in maximumparsimony and maximumlikelihood trees.

Bootstrap values (expressed as percentages of 1,000 replications) greater than 70.0% are shown at branchpoints.

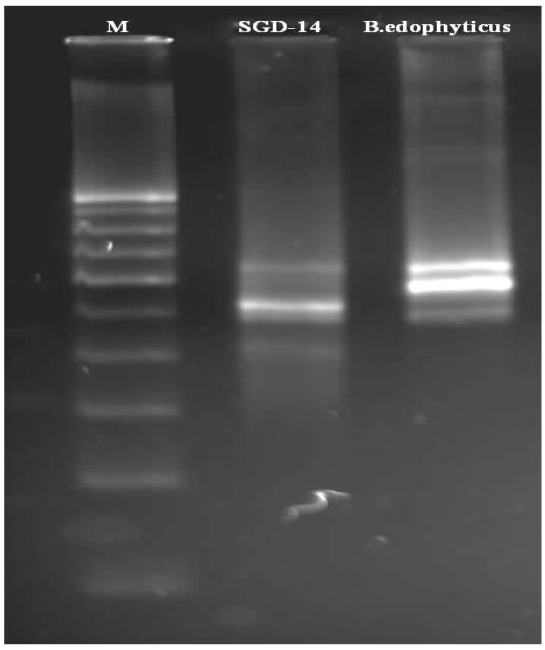


Fig.16. Arbitrary primed PCR (AP-PCR) profile showing different banding pattern of SGD-14^T with its *B. endophyticus* CIP106778^T nearest phylogenetic neighbour. M, Marker 100 bp.

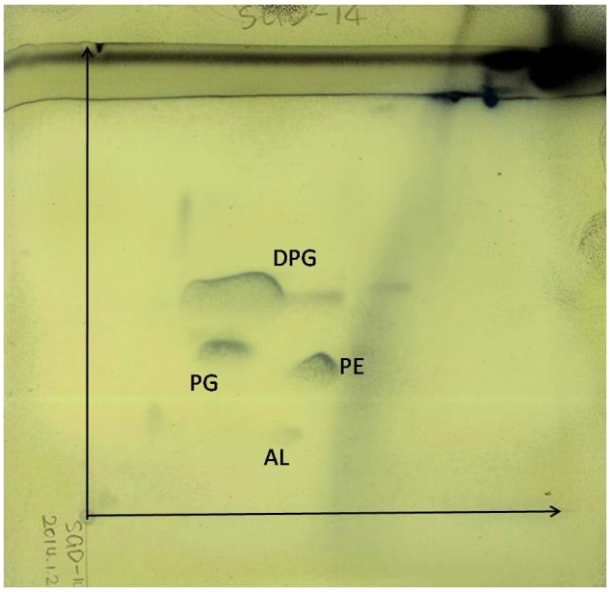


Fig.17. Two-dimensional TLC of the polar lipids of strain SGD-14^T. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; AL, aminophospholipid.

Table 12. Fatty acid composition of SGD-14^{T} and its closest phylogenetic neighbour, *Bacillus endophyticus* CIP106778^{T}.

Fatty acids	***Bacillus* sp SGD-14^{T}**	***B.endophyticus* CIP106778^{T}**
iso-$C_{13:0}$	–	0.4
anteiso-$C_{13:0}$	–	0.8
iso-$C_{14:0}$	8.0	5.5
$C_{14:0}$	2.7	3.9
iso-$C_{15:0}$	13.2	21.2
anteiso-$C_{15:0}$	39.3	34.2
$C_{16:1}\omega7c$	1.6	1.1
iso-$C_{16:0}$	10.6	5.2
$C_{16:1}$ $\omega11c$	2.5	4.9
$C_{16:0}$	8.9	11.9
iso-$C_{17:1}$ $\omega10c$	–	0.4
iso-$C_{17:0}$	1.5	2.0
anteiso-$C_{17:0}$	10.5	6.7
$C_{18:0}$	–	0.3
iso-$C_{19:0}$	–	0.3

Based on phenotypic, chemotaxonomic, genotypic and phylogenetic analysis of strain SGD-14 distinguishes it from its closest neighbour *B. endophyticus* CIP 106778^{T}, suggested that strain SGD-14^{T} considered as a novel member in the *Bacillus* genus for which the name *Bacillus filamentosus* sp. nov., was proposed. *Bacillus filamentosus* (fi.la.men.to'sus. N.L. masc. adj. *filamentosus*, thread-like)

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Bacillus filamentosus sp. nov., isolated from sediment sample

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Abstract A novel Gram-stain positive, endospore-forming bacterium, designated SGD-14^T, was isolated from a marine sediment sample in Goa Province, India. Cells of the isolate were found to be strictly aerobic. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain SGD-14^T showed a similarity of 99.5 % with *Bacillus endophyticus* and similarities to other *Bacillus* type strains were below 96 %. The whole-cell sugar pattern was found to consist of ribose, xylose and glucose. The predominant menaquinone was identified as MK-7 and the major fatty acids as anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$, anteiso-$C_{17:0}$, $C_{16:0}$ and iso-$C_{14:0}$. The strain was found to grow optimally at 30 °C and pH 7.0–7.5. DNA G + C content was determined to be 39.6 mol%. The phospholipid pattern was found to consist of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. DNA–DNA hybridization studies between strain SGD-14^T and *B. endophyticus* CIP106778^T showed that strain SGD-14^T exhibited <70 % similarity with *B. endophyticus* CIP106778^T. Differences in phenotypic and chemotaxonomic characteristics between the novel strain and *B. endophyticus* CIP106778^T further confirmed that this isolate represents novel species. Phylogenetic analysis showed that strain SGD-14^T fits in the same clade with *B. endophyticus* with 100 % bootstrap values. Strain SGD-14^T is therefore considered to represent a novel species of the genus *Bacillus*, for which the name *Bacillus filamentosus* sp. nov. is proposed. The type strain of *Bacillus filamentosus* is SGD-14^T = (=NCIM 5491^T = DSM 27955^T).

Keywords *Bacillus* · Sediment · Filaments · Polyphasic

Introduction

The genus *Bacillus* is composed of rod-shaped, endospore forming bacteria that are members of the phylum *Firmicutes*. The genus *Bacillus* is phylogenetically and phenotypically very heterogeneous (Claus and Berkeley 1986) and in fact over the last two decades nine new genera have been separated from this original taxon (Wisotzkey et al. 1992; Ash et al. 1993; Shida et al. 1996; Heyndrickx et al. 1997; Wainø et al. 1999; Nazina et al. 2001; Yoon et al. 2001). The inability of phenotypic, biochemical and

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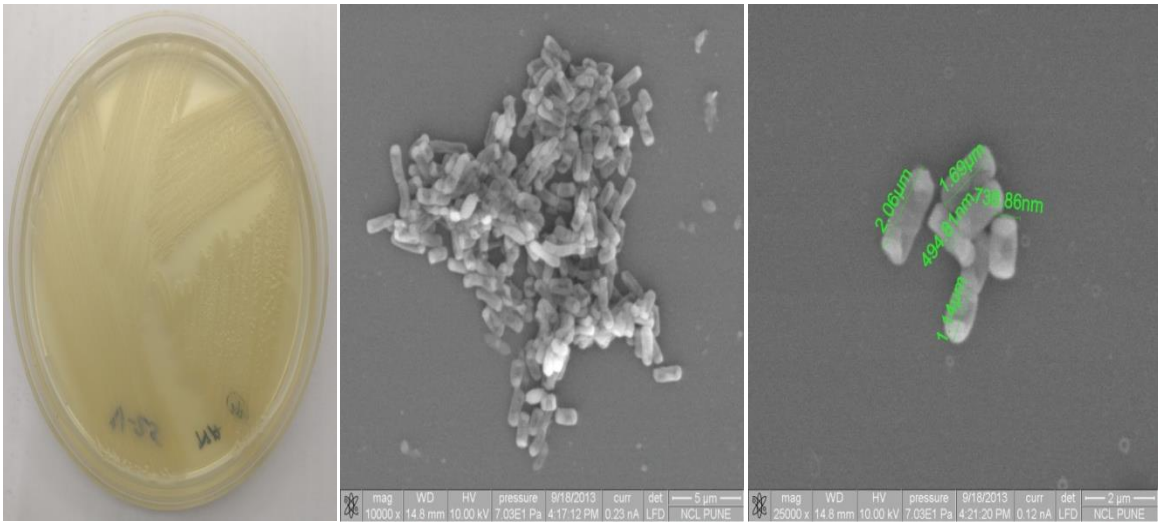
2.4.2.1. c. Strain SGD-V-25- *Bacillus* sp.

Strain SGD-V-25 was isolated from the marine sediment sample of Veraval coast and maintained on marine agar. Colonies of SGD-V-25 were observed to be cream coloured, opaque, circular with entire margin on a nutrient agar medium (Fig. 18 A). Cells were found to be Gram positive rods, aerobic and motile. Under scanning electron microscope cells were appeared in 0.4-0.7 x 1.1-2.0 μm in size (Fig. 18 B). Terminal round endospores were observed. Strain SGD-V-25 was found to be grow at temperatures ranging from 25-50 ºC (optimal at 30 ºC) and pH ranging from 6.0-12.0 (optimal at 7.0) with NaCl concentration of 0.0-5.0% (w/v). The characteristics of SGD-V-25 are given in the table 13.

The 16S rRNA gene sequence of strain SGD-V-25 (1522 bp) was obtained and subsequently deposited in NCBI (Genbank accession no. KF413433). The 16S rRNA sequence was compared and it was found to be matched with the closest similarity with *Bacillus badius* ATCC 14574^T with 99.1%. Initially, by comparing with other strains it was found that the strain SGD-V-25 was assigned to the genus *Bacillus*. Upon construction of the phylogenetic tree (Fig. 19), it was observed that strain SGD-V-25 formed a subclade with *B. badius* ATCC 14574^T which was supported by bootstrap value 100%. Further, the tree was also supported by maximum likelihood and maximum parsimony algorithms. The genomic G+C content of strain SGD-V-25 and *B. badius* ATCC 14574^T was determined to be 37.6 mol% and 43.8%, respectively. The DNA-DNA hybridization value of strain SGD-V-25 and *B. badius* ATCC 14574^T was determined and it was found to be 54.5% 3.0 ±2.0%, suggested that the strain SGD-V-25 shared the highest homology with *B. badius* ATCC 14574^T by 16S rRNA gene sequence and the DNA-DNA hybridization value was well below the threshold value of 70.0% indicates that strain SGD-V-25 should be new member of the genus *Bacillus*.

Strain SGD-V-25 contains *meso*-diaminopimelic acid as a diagnostic diamino acid and A1γ cell wall peptidoglycan. Cell wall sugars were determined as ribose and glucose in

major amount and galactose in a minor amount. The predominant menaquinone was found to be MK-7 in a major amount and MK-6 and MK-8 in a minor amount. The polar lipids were determined (Fig. 20) and it was found to contain diphosphotidylglycerol, phosphatidylglycerol, phosphoglycolipid and two aminophospholipids. The major fatty acids found for strain SGD-V-25 were iso-$C_{15:0}$ (32.1%), anteiso-$C_{15:0}$ (16.0%), anteiso-$C_{17:0}$ (8.9%), iso-$C_{16:0}$ (6.4%), $C_{16:1}\omega11c$ (5.5%), $C_{16:0}$ (4.9%) and $C_{16:1}\omega6c/\omega7c$ (5.6%). The detailed profiling of fatty acids of strain SGD-V25 and *B. badius* ATCC 14574^T is given in the Table 14.



A B

Fig. 18. A) Colony morphology and B) scanning electron mictrograph of strain SGD-V-25^T grown on nutrient agar.

Table 13. Biochemical and physiological characteristics of strain SGD-V-25^T and *B. badius* MTCC 1458^T.

Characteristic	SGD-V-25^T	*B. badius* MTCC 1458^T
Sporangium type	Spherical	Ellipsoidal, swollen
Colony colour	Light yellowish cream	Cream
Optimal pH for growth	7.0–7.5	6.0–8.0
Growth at pH:		
6.0	+	−
12.0	+	−
Maximum NaCl tolerance at 5.0% (w/v)	+	−
Hydrolysis of:		
Aesculin	+	−
Starch	+	−
Urea	+	W
Nitrate reduction	+	−
H_2S production	−	+
Utilized as sole carbon source		
Galactose, lactose, salicin, dulicitol, CM-cellobiose, melezitose, sorbose, methyl α- D-glucoside and methyl α-D-mannoside	+	−
Glucose	−	+
Acid production from:		
L-Arabinose, glucose, inositol, D-mannitol, melibiose, sodium gluconate, sorbitol, sorbose, sucrose	−	+
Glycerol, salicin	+	−
Production of (API ZYM test):		
Alkaline phosphatase, esterase (C4), esterase lipase (C8)	+	−
Leucine arylamidase, valine arylamidase	W	−
Acid phosphatase	−	+
DNA G+C content (mol%)	37.6	43.8
Source of isolation	Marine sediment	Tidal flat

+, Positive; −, negative; W, weakly positive

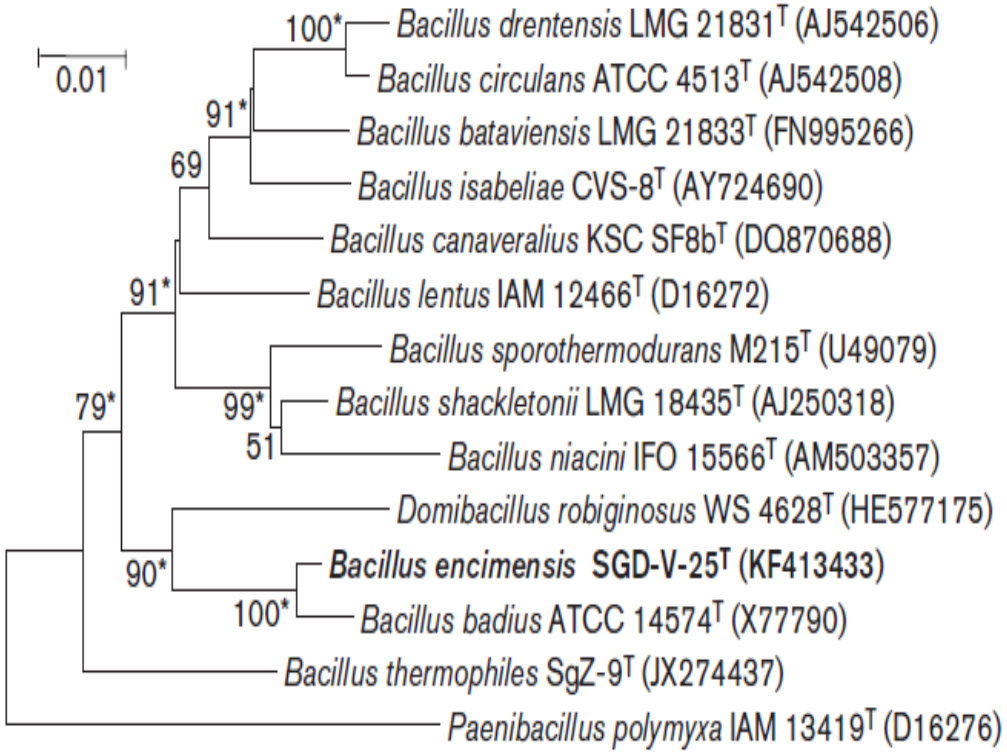


Fig.19. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences, showing the phylogenetic position of strain SGD-V- 25^T and type strains of related species. The sequence of *PaeniBacillus polymyxa* IAM13419^T (D16276) was used as an out-group. Only bootstrap values >50.0% (expressed as percentages of 1000 replications) are shown at branch points. Asterisks indicate that the corresponding nodes (groupings) are also recovered in maximum-parsimony and maximum- likelihood trees. Bar, 0.01 substitutions per nucleotide position.

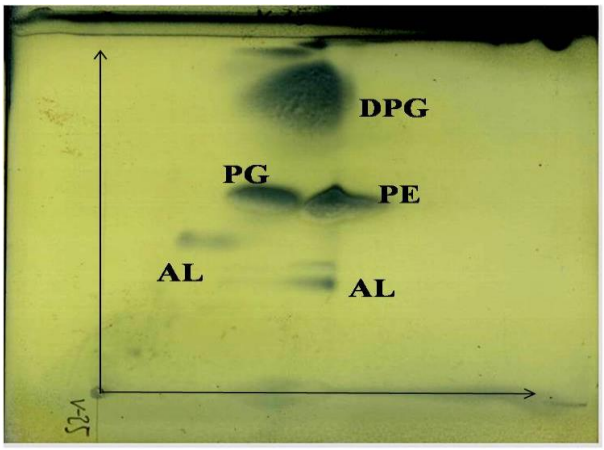


Fig.20. Total polar lipid profile of strain SGD-V-25^T after two dimensional thin layer chromatography staining with molybdatophosphoric acid Abbreviations: DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; AL, unknown phospholipid.

Table 14. Fatty acid composition of SGD-V-25^T compared with *B. badius* MTCC-1458^T

Fatty acid	Profile (%)	
	SGD-V-25^T	*B. badius* MTCC 1458^T
iso-$C_{14:0}$	1.9	2.0
$C_{14:0}$	3.1	2.5
iso-$C_{15:0}$	32.1	42.6
anteiso-$C_{15:0}$	16.0	7.0
$C_{16:1}\omega 7c$ alcohol	3.6	2.6
iso-$C_{16:0}$	6.4	8.6
$C_{16:1}\omega 11c$	5.5	5.9
$C_{16:0}$	4.9	9.0
iso-$C_{17:1}\omega 10c$	3.8	4.3
anteiso-$C_{17:1}$ A	0.9	–
iso-$C_{17:0}$	2.2	4.1
anteiso-$C_{17:0}$	8.9	4.6
$C_{17:0}$	–	0.4
$C_{18:1}\omega 9c$	–	0.2
$C_{18:0}$	–	0.5
Summed feature 3*	5.6	2.8
Summed feature 4*	4.5	2.1

*Summed feature 3 contained $C_{16:1}$ ω7c/ω6c and summed feature 4 contained iso-$C_{17:1}$ I/anteiso-$C_{17:1}$ B.

The phenotypic, chemotypic and 16S rRNA sequencing data and DNA-DNA hybridization suggested that strain SGD-V-25 as a new member of genus *Bacillus*. By comparative study and phylogenetic analysis, it was found that strain SGD-V-25^{T} differs from other validly known *Bacillus* species for which *Bacillus encimensis* sp. nov., the name was proposed. *Bacillus encimensis* (en.cim'en.sis. N.L. masc. adj. *encimensis* arbitrary name formed from NCIM, the acronym for the NCIM Resource Center, CSIR-National Chemical Laboratory, India, where taxonomic studies on this species were performed).

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Bacillus encimensis sp. nov. isolated from marine sediment

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A Gram-stain-positive, rod-shaped, endospore-forming, aerobic bacterium designated SGD-V-25^T was isolated from Veraval sediment sample, India. Strain SGD-V-25^T was capable of growing at 25–50 °C (optimum 37 °C), pH 6–12 (optimum pH 7.0) and with 0–5 % (w/v) NaCl. The taxonomic position of this strain was deduced using a polyphasic approach and the 16S rRNA gene sequence analysis showed that the isolate belongs to the phylum *Firmicutes*, forming the cluster with *Bacillus badius* MTCC 1548^T, with which it shares highest similarity of 99.1 % with 13 nt differences. Other type strains of the genus *Bacillus* showed less than 96 % similarity. The cell wall contained *meso*-diaminopimelic acid as the diagnostic diamino acid. The polar lipid profile of strain SGD-V-25^T showed the presence of diphosphatidylglycerol, phosphatidylglycerol, phsophoglycolipid and two aminophospholipids. The predominant isoprenoid quinone was MK-7. The major cellular fatty acids were iso-$C_{15:0}$, anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{16:0}$, $C_{16:1}\omega11c$ and $C_{16:0}$. The genomic DNA G+C content of strain SGD-V-25^T was 37.6 mol%. On the basis of phenotypic characteristics, phylogenetic analysis and DNA–DNA hybridization, strain SGD-V-25^T could be clearly distinguished from closely related members of the genus *Bacillus*, and the name *Bacillus encimensis* sp. nov., is proposed to accommodate this strain. The type strain is SGD-V-25^T (=NCIM 5513^T=DSM 28241^T).

The genus *Bacillus* in the family *Bacillaceae*, belonging to the phylum *Firmicutes*, includes obligately aerobic or facultatively anaerobic, endospore-forming bacilli and is metabolically and ecologically a diverse group. In the genus *Bacillus*, several moderately halophilic or halotolerant endospore-forming species have been described (de la Haba *et al.*, 2011). Tidal sediments have been utilized as excellent sources for isolating novel and useful micro-organisms (Yi *et al.*, 2003; Yoon *et al.*, 2003a). Some novel genera or species have recently been isolated from tidal sediments in Korea (Yi *et al.*, 2003; Yoon *et al.*, 2003a, b, 2004a, b). These bacteria are frequently isolated from saline and hypersaline environments, such as saline soils and saline aquatic habitats (Arahal & Ventosa, 2002; Márquez *et al.*, 2011; Ventosa, 2006; Ventosa *et al.*, 1998). During the study of the diversity of prokaryotes in coastal sediment, a bacterial strain, designated SGD-V-25^T, was isolated from a sediment sample. The taxonomic position of this strain was determined using a polyphasic approach that included phenotypic properties and phylogenetic analysis based on 16S rRNA gene sequences.

Strain SGD-V-25^T was isolated from a marine sediment sample taken from Veraval coast (GPS position 20° 53′ 51.17 N 70° 22′ 53.00 E). After primary isolation and purification on marine agar 2216 (MA; Difco) at 30 °C for 2 weeks the purified strain was subcultured on the same medium and stored as slants at 4 °C and as 20 % (v/v) glycerol suspensions at −70 °C. Biomass for chemical and molecular studies was obtained by cultivation in shake flasks (about 140 r.p.m.) using trypticase soy broth (Hi-media, Mumbai) medium at 30 °C for 48 h. Gram staining was carried out using the standard Gram reaction, a non-staining method was used to determine the Gram reactions (Buck, 1982) and cell motility was confirmed by the development of turbidity throughout a tube containing semisolid medium (Leifson, 1960). Morphological and physiological characterizations were performed for strain SGD-V-25^T. Cells were grown on MA (pH 7.0) at 30 °C for 48 h. Cell morphology and surface ornamentation was observed by light microscopy and scanning electron microscopy. For scanning electron microscopy examination, 1 ml samples were fixed overnight at

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of SGD-V-25T is KF413433.

Two supplementary figures and one supplementary table are available with the online Supplementary Material.

2.4.2.1. d. NIO-1130- *Bacillus* sp.

Strain NIO-1130 was maintained on marine agar. Colonies of NIO-1130 were observed to be yellow coloured, opaque, circular with entire margin on a marine agar plate (Fig. 21 A). Cells were found to be Gram positive, rods and motile. Ellipsoidal or oval spores were formed by cells terminally with swollen sporangia. Under scanning electron microscope cells were observed to be 0.3-0.7 x 20.-3.0 μm in size (Fig. 21 B). Strain NIO-1130 grows at temperatures ranging from 25-45 ºC (optimal at 28 ºC), at pH ranging from 6.0-12.0 (optimal at 7.0) with NaCl concentration from 0.0-15.0%. The characteristics of strain NIO-1130 is given in the table 15.

The 16S rRNA gene sequence of strain NIO-1130 (1421 bp) was obtained and deposited in NCBI (Genbank accession number HQ858013). Comparative analysis of 16S rRNA sequence was done and it showed similarity with *Bacillus niabensis* 4T16^T with 98.1%. The phylogenetic analysis (Fig. 22) showed that strain NIO-1130 formed a subclade with *Bacillus niabensis* 4T16^T and *Bacillus halosaccharovorans* DSM 25387^T which was supported by a bootstrap value of 80.0%. The genomic G+C content of NIO-1130 was found to be 39.9 mol%. The DNA-DNA relatedness value between NIO-1130 and B. *halosachharovorans* DSM 25387^T and NIO-1130 and B. *niabensis* CIP 109816^T were determined and it was found to be 34.6% (2.0%) and 28.3% (2.2%), respectively.

Strain NIO-1130 contains *meso*-diaminopimelic acid in cell wall diamino acids. Ribose. glucose, galactose and arabinose were detected as cell wall sugars. NIO-1130 contained an unsaturated menaquinone with seven isoprene units (MK-7). Polar lipids of strain NIO-1130 (Fig.23) was observed as diphosphotidylglycerol, phosphatidylethanolamine and phosphatidylglycerol. The fatty acids profiles of NIO-1130 are given in the table 16.

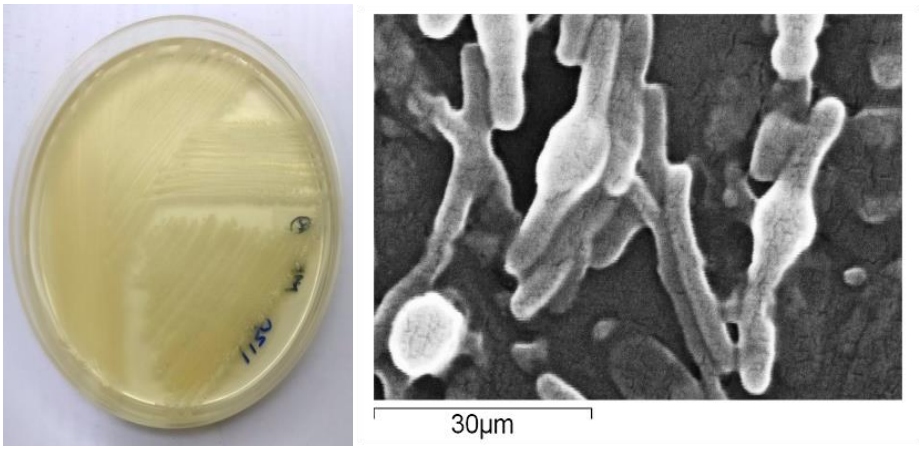

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Fig.21. A) Colony morphology and B) scanning electron micrograph of strain NIO-1130^T from 3 days old cultures grown on nutrient agar.

Table 15. Physiological and biochemical characteristics of NIO-1130^T with respectives strains of *Bacillus*.

Characteristics	***Bacillus*sp NIO-1130^T**	***B. halosaccharovorans* DSM 25387^T**	***B. niabensis* CIP109816^T**	***B. galliciensis* DSM 21539^T**	***B. litoralis* DSM 16303^T**
Spore position	Terminal	Central/subterminal	Terminal	Subterminal	Central/Subterminal/ terminal
Spore shape	Ellipsoidal	Ellipsoidal	Oval	Oval	Ellipsoidal
Oxidase activity	+	+	−	+	+
Anaerobic growth	−	−	+	−	−
pH range	6.0–12.0	6.0–9.0	6.0–8.0	5.5–9.0	5.5–7.5
Optimum pH	7.0–8.0	7.5–8.0	7.0	7.2	7.5
NaCl (%) range	0–15	0.5–25	0–5	0–7	0–10
Temperature (°C) range	25–45	20–45	15–50	10–30	4–45
Acid from					
L-Arabinose	−	−	+	+	+
D-Mannitol	+	+	+	−	−

D-Xylose	−	+	+	−	+
Hydrolysis of					
Casein	+	+	−	−	+
Nitrate reduction	−	−	+	−	−
Urease activity	−	−	+	−	−
DNA G+C content (mol%)	39.9	42.6[a]	37.7–40.9[a]	48.1	35.2
Source of isolation	Marine sediment	Hypersaline lake	Cotton waste compost	Feces of wild seahorses	Tidal flat

[a] Data from Mehrshad et al. (2013) and Kwon et al. (2007). '+' Positive, '−' negative

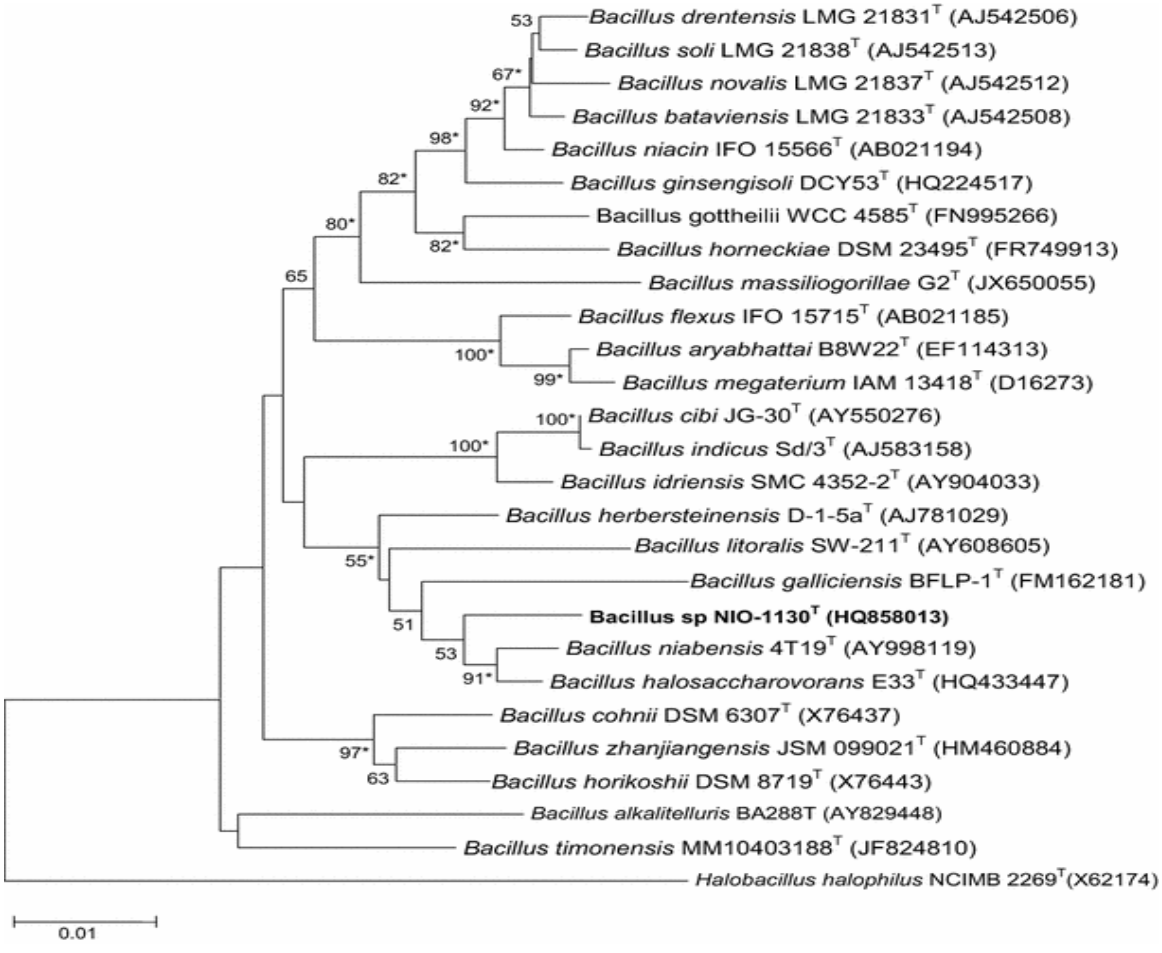

Fig.22. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of the strains NIO-1130^T in relation to selected *Bacillus* species. Bootstrap values (expressed as percentages of 1000 replications) >50.0% are shown at branch points. Asterisks

indicate that the corresponding nodes (groupings) are also recovered in maximum-parsimony and maximum-likelihood trees. Bar 0.01 substitutions per nucleotide position.

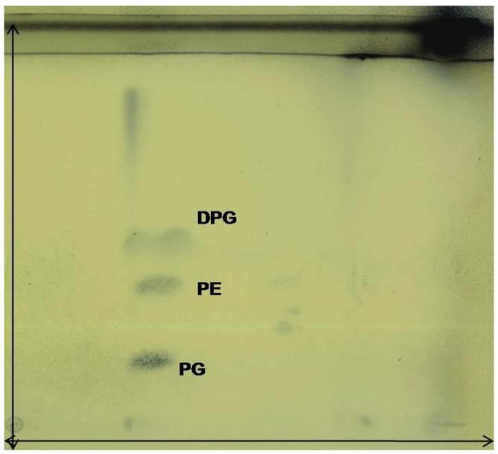

Fig.23. Two-dimensional TLC of the polar lipids of strain NIO-1130^{T}. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine

Table 16. Cellular fatty acid profiles (%) of strain NIO-1130^{T}

Fatty acids	**NIO-1130^{T}**
Straight-chain saturated	
$C_{14:0}$	1.2
$C_{16:0}$	5.4
$C_{18:0}$	0.2
Branched	
iso-$C_{13:0}$	0.3
iso-$C_{14:0}$	1.3
iso-$C_{15:0}$	41.4
iso-$C_{16:0}$	3.6
iso-$C_{17:0}$	12.3
iso-$C_{17:1}$ ω10c	3.7
iso-$C_{19:0}$	0.2
anteiso-$C_{15:0}$	19.7
anteiso-$C_{17:0}$	5.8
Monounsaturated	
$C_{16:1}$ ω 7c	1.0
$C_{16:1}$ ω 11c	2.4
Summed features[a]	
4	0.8

[a] Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 4 contained iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B

The phenotypic, genotypic and phylogenetic analysis of strain NIO-1130T reveals that it forms a new member of genus *Bacillus* for which *Bacillus cellulasensis* sp.nov., was proposed. *Bacillus cellulasensis* (cell.u.la.s.en′sis. N.L. neut. n. cellulase; Gr. adj. pertaining to the cellulase enzyme activity of the strain).

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ORIGINAL PAPER

Bacillus cellulasensis sp. nov., isolated from marine sediment

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Abstract A novel bacterial strain NIO-1130^T was isolated from sediment sample taken from Chorao Island, Goa Province, India, and subjected to a taxonomic investigation. The strain was Gram-positive, aerobic, and motile. Phylogenetic analysis based on 16S rRNA gene sequences placed the isolate within the genus *Bacillus* and strain NIO-1130^T showed highest sequence similarity with *Bacillus halosaccharovorans* DSM 25387^T (98.4 %) and *Bacillus niabensis* CIP 109816^T (98.1 %), whereas other *Bacillus* species showed <97.0 % similarity. Tree based on gyrB gene sequence revealed that strain bacillus group. The major menaquinone was MK-7 and the predominant cellular fatty acids were iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{17:0}$, and anteiso-$C_{17:0}$. The strain showed a DNA G+C content of 39.9 mol%. DNA–DNA hybridization studies revealed that strain NIO-1130^T exhibits 70 % similarity with *Bacillus halosaccharovorans* DSM 25387^T and *Bacillus niabensis* CIP 109816^T. On the basis of physiological, biochemical, chemotaxonomic and phylogenetic analyses, we consider the isolate to represent a novel species of the genus *Bacillus*, for which the name *Bacillus cellulasensis* sp. nov., is proposed. The type strain is NIO-1130^T (=NCIM 5461^T=CCTCC AB 2011126^T).

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Electronic supplementary material The online version of this article (doi:10.1007/s00203-015-1155-7) contains supplementary material, which is available to authorized users.

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Keywords *Bacillus* sp · Polyphasic taxonomy · Cellulase · Marine sediment

Introduction

Members of the genus *Bacillus* are widely distributed in nature and have physiologically diverse characteristics (Claus and Berkeley 1986). For a long time, most aerobic, endospore-forming rods were assigned to the genus *Bacillus* (Claus and Berkeley 1986). The results of genomic analyses proved that the genus *Bacillus* comprised heterogeneous taxa (Priest et al. 1981; Ash et al. 1991; Slepecky and Hemphill 1992; Stackebrandt and Liesack 1993; Nakamura et al. 1999). In particular, 16S rRNA gene sequence analyses have revealed the presence of several phylogenetically distinct lineages within the genus *Bacillus*. Therefore, classification and identification of Gram-positive, endospore-forming rods should be performed by using a polyphasic taxonomic approach that integrates phylogenetic analysis based on 16S rRNA gene sequences, genomic relatedness, and an extensive range of phenotypic characteristics (Smibert and Krieg 1994; Claus and Berkeley 1986). In the course of our study on marine microbial diversity, a Gram stain-positive, endospore-forming bacterial strain, designated strain NIO-1130^T, was isolated from a marine sediment sample and was the subject of a taxonomic investigation.

Materials and methods

Bacterial strains

Sediment sample from 10 cm depth was collected in sterile falcon tubes from the intertidal region of mangroves

2.4.2.2. Genus *Fictibacillus*

Most of the aerobic, rods and Gram positive endospore forming bacteria were initially designate to the genus *Bacillus*. Subsequently, 16S rRNA gene sequencing revealed the several phylogenetically distinct lineages in the *Bacillus* genus. Some of the phylogenetic group has been established as a new genera such as *Alicyclobacillus* (Wisotzkey et al. 1992), *Paenibacillus* (Ash et al. 1993), *Aneurinibacillus* (Shida et al. 1996), *Brevibacillus* (Shida et al. 1996), *Virgibacillus* (Heyndrickx et al. 1998), *Salibacillus* (Wainø et al. 1999), *Ureibacillus* (Fortina et al. 2001), *Marinibacillus* (Yoon et al. 2001), *Alkalibacillus* (Jeon et al. 2005), *Pullulanibacillus* (Hatayama et al. 2006), *Sporolactobacillus* (Hatayama et al. 2006) and *Viridibacillus* (Albert et al. 2007). Recently, Glaeser et al. 2013 further proposed the genus *Fictibacillus* and reclassified *Bacillus nanhaiensis*, *B. barbaricus*, *B. arsenicus*, *B. rigui*, *B. macauensis* and *B. gelatini* as *Fictibacillus nanhaiensis*, *F. barbaricus*, *F. arsenicus*, *F. rigui*, *F. macauensis* and *F. gelatini* comb. nov., respectively. *Fictibacillus* name given to the false rod shaped *bacillus* (false *bacillus*). It is currently categorised under Phylum *Firmicutes*, Class *Bacilli*, Order *Bacillales* and Family *Bacillaceae*. To date, genus *Fictibacillus* contains 11 validly known species (www.bacterio.net).

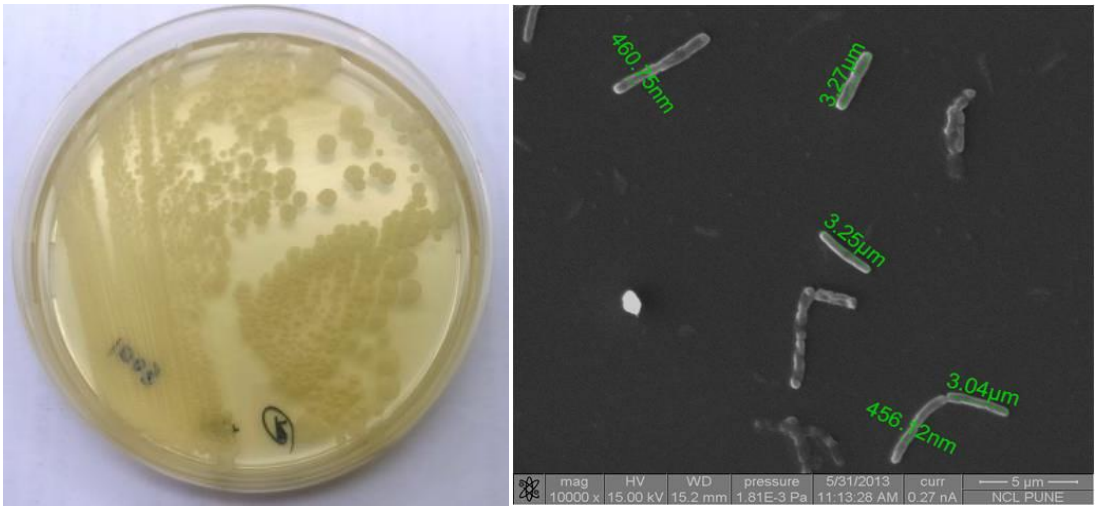
2.4.2.2. a. Strain NIO-1003- *Fictibacillus* sp.

Strain NIO-1003 was isolated from a marine sediment sample from Chorao Island Goa, India. Colonies of NIO-1003 were observed to be cream coloured, circular, opaque, smooth and convex with 0.5-1.0 mm in diameter after two days on incubation on nutrient agar (Fig. 24 A). Cells were observed to be Gram positive, rod shaped, motile with ellipsoidal endospore. Under scanning electron microscope cells were found to be 0.45-0.46 x 3.0-3.2 μm in size (Fig. 24 B). Strain NIO-1003 grows at a temperature ranging from 15-42 ºC (optimal at 30 ºC), at pH ranging from 5.0-12.0 (optimal at 7.0-7.5) with NaCl concentration from 0.0-

12.0%. The detailed physiological and biochemical characteristics of NIO-1003 are given in the table 17.

The 16S rRNA gene sequence of strain NIO-1003 (1419 bp) was obtained and deposited into NCBI (GenBank accession no. JF893461). The 16S rRNA gene sequence of strain NIO-1003 showed similarity with *Fictibacillus rigui* KCTC 13278^T, *Fictibacillus solisalsi* KCTC 13181^T and *Fictibacillus barbaricus* DSM 14730^T with 98.2, 98.0 and 97.2%, respectively. The phylogenetic tree was constructed by using the neighbour joining algorithm along with maximum parsimony and maximum likelihood. The comparative analysis showed that strain NIO-1003 formed a subclade with *F. rigui* KCTC 13278^T in a phylogenetic tree (Fig. 25) which was supported by a bootstrap value of 70.0%. The G+C content of genomic DNA was determined and it was found to be 42.6 mol % for the strain NIO-1003. The DNA-DNA relatedness values were determined and it was found that the value between strain NIO-1003 and *F. rigui* KCTC 13278^T and NIO-1003 and *F. solisalsi* KCTC 13181^T were 53.9 ±1.2 and 54.8 ± 2.1%, respectively. The AP-PCR amplicon of strain NIO-1003, *F. rigui* KCTC 13278^T and *F. solisalsi* KCTC 13181^T showed different fingerprinting banding pattern suggested that the strain NIO-1003 differs from the other strains (Fig. 26).

Strain NIO-1003 contains *meso*-diaminopimelic acid as Diagnostic amino acid in the cell wall peptidoglycan. Cell wall sugars were determined as ribose, glucose and galactose. The unsaturated menaquinone with seven isoprene units (MK-7) was identified as a major menaquinone. The major polar lipids detected in the strain NIO-1003 were diphosphotidyl glycerol, phosphatidylglycerol, phosphatidylethanolamine and an unidentified amino lipid (Fig. 27). The major cellular fatty acids profile of strain NIO-1003 and their closest type strains are given in the table 18.



A B

Fig.24. A) colony morphology and B) scanning electron micrograph of 3 days old culture of strain NIO-1003^T grown on nutrient agar.

Table17. Phenotypic characteristics that differentiate strain NIO-1003^T from its phylogenetic neighbours in the genus *Fictibacillus*.

Characteristics	NIO-1003^T	*F. rigui* KCTC 13278^T	*F.solisalsi* KCTC 13181^T	*F. barbaricus* KACC 12101^T
Motility	+	+	+	−
Anaerobic growth	+	−	+	+
Growth at/in				
15 °C	−	+	+	−
47 °C	−	−	+	−
5.0 % (w/v) NaCl	+	+	+	−
10.0 % (w/v) NaCl	−	−	+	−
Aesculin hydrolysis	+	−	+	−
Malonate utilization	+	+	−	+
Utilization of carbon sources				
Adonitol	W	−	−	−
Fructose	+	−	+	−
Galactose	−	W	−	−
Glucose	+	−	−	−
Inositol	+	−	−	−
Lactose	−	W	−	−
D-Mannitol	−	+	+	−

D-Mannose	+	–	–	–
Maltose	W	+	–	+
Melezitose	+	+	–	–
Melibiose	+	+	–	–
Raffinose	+	+	–	–
Sucrose	+	+	–	–
Xylitol	+	+	–	–
D-xylose	–	+	+	–
Major Fatty acids	ai-$C_{15:0}$, i-$C_{15:0}$, ai-$C_{17:0}$, $C_{16:0}$	ai-$C_{15:0}$, i-$C_{15:0}$, ai-$C_{17:0}$, $C_{16:0}$	ai-$C_{15:0}$, i-$C_{15:0}$, ai-$C_{17:0}$, $C_{16:0}$	ai-$C_{15:0}$, i-$C_{15:0}$; i-$C_{14:0}$ and i-$C_{16:0}$
DNA G+C content (mol %)	42.6	41.9[a]	41.8[a]	42.0[a]
Isolated from	Sediment	Fresh water	Saline soil	Wall painting

All data were generated from present study except that for *F. barbaricus* KACC 12101^T which was taken from Täubel et al. (2003).

[a] Data collected from Täubel et al. (2003), Liu et al.(2009) and Baik et al. (2010)

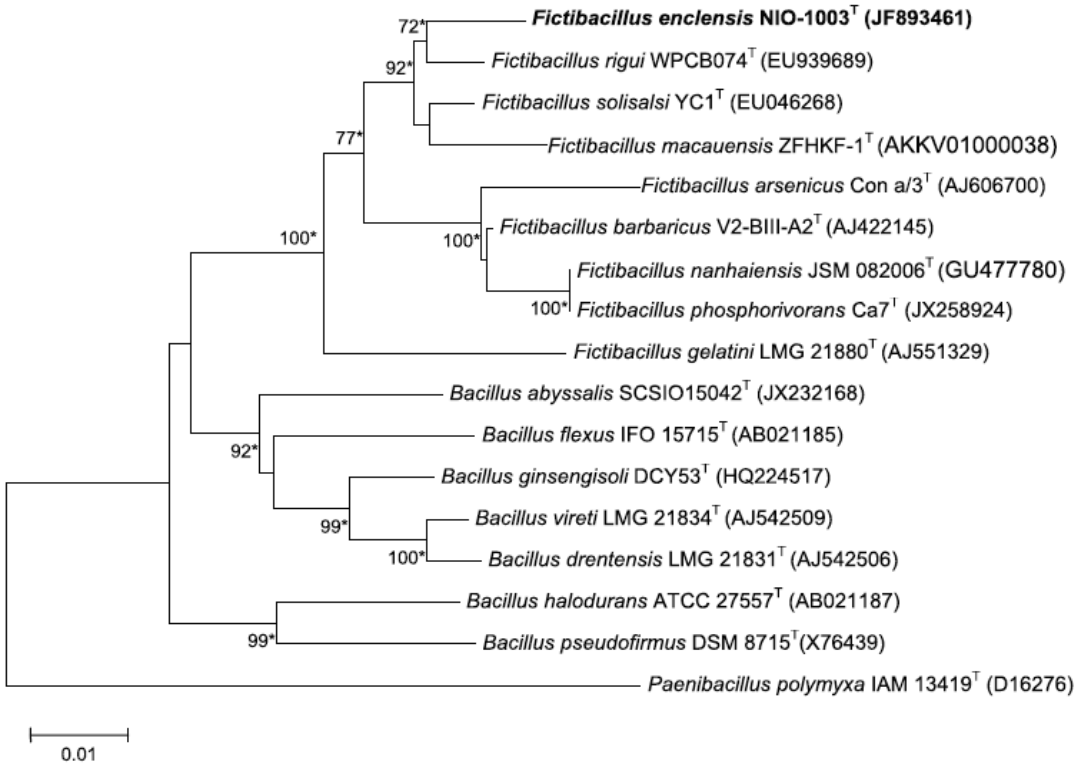


Fig.25. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between strain NIO-1003^T and related members of the genus *Fictibacillus*. Bootstrap values ([>70.0%; 1,000 resamplings) are given at branch points. Astrikes indicate

that the corresponding nodes (groupings) are also recovered in Fitch–Margoliash, maximum parsimony and maximum-likelihood trees. Bar, 0.01 nucleotide substitutions per position.

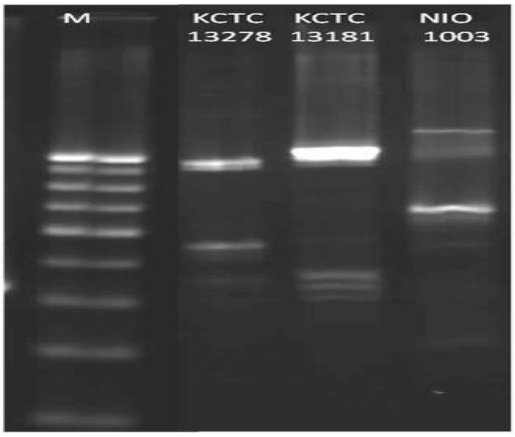


Fig.26. Arbitrary primed PCR (AP-PCR) profile [Lane 1-Marker 100 bp; Lane 2: *F. rigui* KCTC13278^{T}; lane 3: *F. solisalsi* KCTC 13181^{T}; lane 4: Strain NIO-1003^{T}].

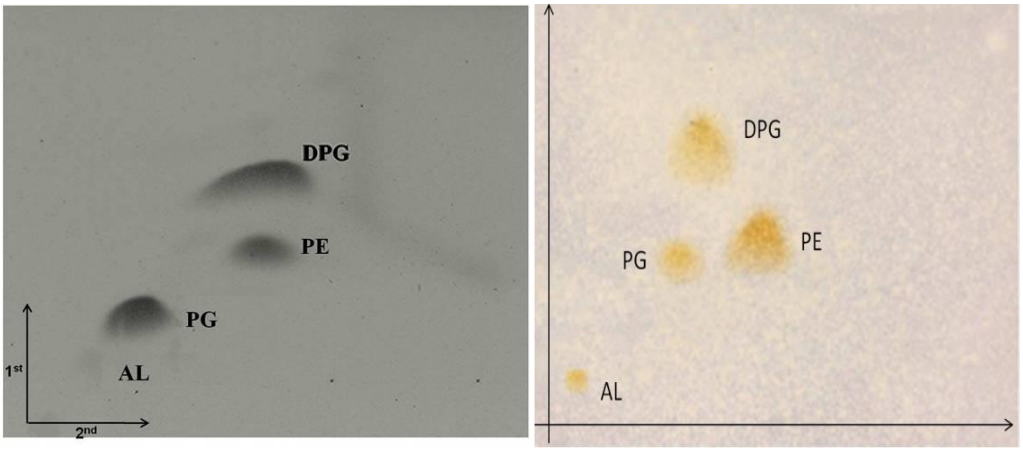


Fig.27. Total polar lipid profile of strain NIO-1003^{T} after two dimensional thin layer chromatography staining with molybdatophosphoric acid and α-naphthol. Abbreviations: DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; AL, unknown aminolipid.

Table18. Cellular fatty acid profiles (%) of strain NIO-1003^T and its phylogenetically related species of the genus *Fictibacillus*.

Fatty acids	1	2	3
Straight-chain saturated			
$C_{12:0}$	–	–	0.6
$C_{14:0}$	1.2	1.0	1.3
$C_{16:0}$	5.0	5.2	7.5
$C_{18:0}$	0.6	1.1	2.1
Branched			
iso-$C_{10:0}$	–	0.4	–
iso-$C_{14:0}$	4.1	1.9	2.7
iso-$C_{15:0}$	13.7	18.6	16.2
iso-$C_{15:1}$ ω 5c	–	0.4	–
iso-$C_{16:0}$	3.3	1.7	3.8
iso-$C_{17:0}$	1.1	1.6	2.7
iso-$C_{17:1}$ ω10c	0.1	0.4	–
anteiso-$C_{15:0}$	60.5	52.0	49.0
anteiso-$C_{17:0}$	7.4	6.7	10.6
anteiso-$C_{17:1}$ω9c	0.1	–	0.1
Monounsaturated			
$C_{16:1}$ω7c	–	0.4	0.2
$C_{16:1}$ω11c	–	1.0	–
Summed features[a]			
3	0.3	0.4	0.3
4	–	1.1	–
5	0.1	–	–
6	–	0.4	–
8	–	0.2	0.2

Strains: 1 NIO-1003^T, 2 *F. rigui* KCTC 13278^T, 3 *F. solisalsi* KCTC 13181^T.

[a] Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained $C_{16:1}$ ω6c/$C_{16:1}$ω7c. Summed feature 4 contained iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B. Summed feature 5 contained ai-$C_{18:0}$/$C_{18:2}$ω6,9c. Summed feature 6 contained $C_{19:1}$ω9c/$C_{19:1}$ω11c. And summed feature 8 contained $C_{18:1}$ω6c/$C_{18:1}$ω7c.

From phenotypic, chemotypic and genotypic data it was found that strain NIO-1003^T as a new member in the genus *Ficitbacillus* for which *Fictibacillus enclensis* sp. nov., was proposed. *Fictibacillus enclensis* (en.clen'sis. N.L. masc. adj. *enclensis* arbitrary name formed from NCL, the acronym for the National Chemical Laboratory, India, where taxonomic studies on this species were performed).

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ORIGINAL PAPER

Fictibacillus enclensis sp. nov., isolated from marine sediment

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Abstract A novel Gram-positive strain, designated NIO-1003^T, was isolated from a marine sediment sample collected from the Chorao Island, Goa Provence, India. Strain NIO-1003^T was found to be strictly aerobic, motile, endospore-forming rods. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain NIO-1003^T belongs to the genus *Fictibacillus* and to be most closely related to *Fictibacillus rigui* KCTC 13278^T, *Fictibacillus solisalsi* KCTC 13181^T and *Fictibacillus barbaricus* DSM 14730^T with 98.2, 98.0 and 97.2 % similarity and 25, 28, 39 nucleotide differences respectively. Strain NIO-1003^T was characterized by having cell-wall peptidoglycan based on meso-diaminopimelic acid and MK-7 as the predominant menaquinone. The polar lipid profile exhibited the major compounds diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. In addition, minor amounts of an aminophospholipid were detected. The major fatty acids were identified as ai-$C_{15:0}$, iso-$C_{15:0}$, ai-$C_{17:0}$ and $C_{16:0}$, supporting the grouping of strain NIO-1003^T into the family *Bacillaceae*. The DNA G+C content of strain NIO-1003^T was determined to be 42.6 mol%. On the basis of phenotypic properties, phylogeny and DNA–DNA hybridisation analysis, strain NIO-1003^T is considered to represent a novel species of the genus *Fictibacillus* for which the name *Fictibacillus enclensis* sp. nov. is proposed. The type strain is NIO-1003^T (= NCIM 5458^T = DSM 25142^T).

Electronic supplementary material The online version of this article (doi:10.1007/s10482-013-0097-9) contains supplementary material, which is available to authorized users.

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2.4.2.3. Genus *Domibacillus*

The family *Bacillaceae* comprises Gram positive, rod shaped, heterogenic and spore forming bacteria while family *Planococcaeae* comprises for Gram positive or Gram variable, motile or non-motile and spore or non-spore forming bacteria. Both families arise from Order *Bacillales* with Class *Bacilli*. Most of the spore forming genera and species were gathered in *Bacillaceae* family while non-spore forming genera and species were gathered in *Planococcaeae* family, but it was not clear whether they are not capable of producing endospore or whether it was not detected due to non-expression of regulon responsible for sporulation. The genus *Domibacillus* was described by Seiler et al., 2013 for a bacterium which was isolated from clean room air of a pharmaceutical manufacturing site in east Germany. *Domibacillus* (Do.mi.ba.ci'lus. N.L. masc. n. domus house; L. masc. n. *bacillus* a rod, and also a bacterial genus name; N.L. masc. n. *Domibacillus* a *bacillus*-like organism isolated from a house). Members of the genus *Domibacillus* are Gram positive, rod shaped, spore forming, oxidative and motile. MK-6 is the major respiratory quinone. It is currently categorised under Phylum *Firmicutes*, Class *Bacilli*, Order *Bacillales* and Family *Bacillaceae*. To date, genus *Domibacillus* contains 8 validly known species (www.bacterio.net).

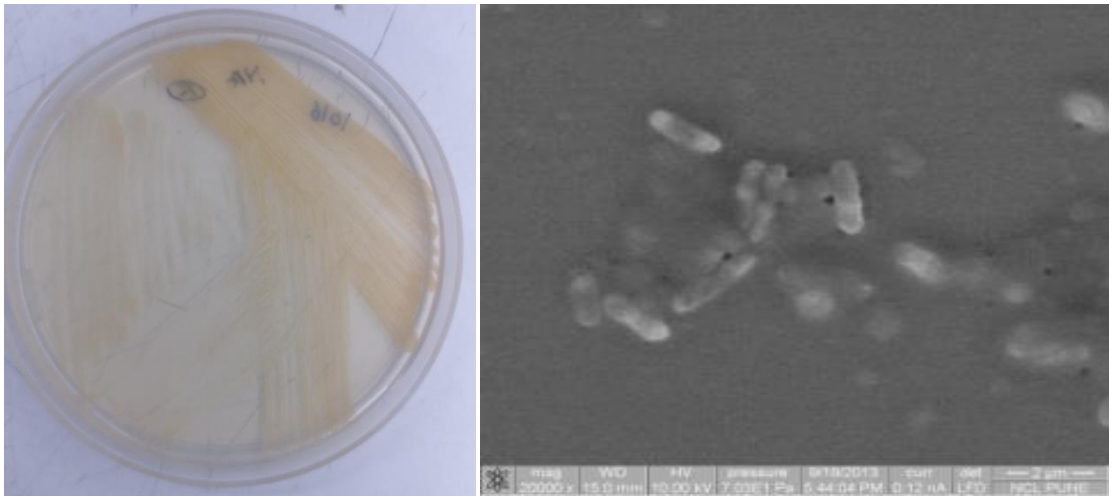
2.4.2.3. a. Strain NIO-1016-*Domibacillus* sp.

Strain NIO-1016 was isolated from a marine sediment sample from Chorao Island Goa, India. It was maintained on marine agar. Colonies of the strain NIO-1016 were observed to be light pink coloured, opaque, circular with an entire margin (Fig. 28 A). The cells of strain NIO-1016 was found to be Gram positive, rod shaped, motile with spherical or ellipsoidal spores arranged at center or subterminal position. The cells were found to be 0.8- 1.15 μm in size (Fig. 28 B). Strain NIO-1016 grows at a temperature ranging from 25-45 ºC (optimal at 30

ºC), at pH ranging from 6.0-12.0 (optimal at 7.0-7.5) with NaCl concentration from 0.0-12.0%. The detailed phenotypic characteristics of strain NIO-1016 are given in the table 19.

The 16S rRNA gene sequence of strain NIO-1016 (1483 bp) was determined and deposited into NCBI (GenBank accession no. JF893466). Comparative 16S rRNA sequence and phylogenetic analysis with other strains revealed that the strain NIO-1016 should be assigned to the genus *Domibacillus*. The phylogenetic tree was constructed by using neighbour-joining method (Fig. 29) and it was found that the strain NIO-1016 formed a subclade with *Domibacillus robiginosus* DSM 25058^T and *Domibacillus indicus* DSM 28032^T, which was supported by the bootstrap value of 99.0 and 96.0%, respectively. The 16S rRNA gene sequence showed similarity with *D. roboginosus* DSM 25058^T and *D. indicus* DSM 28032^T with 98.0 and 97.2% respectively. The genomic G+C content of strain NIO-1016 was found to be 46.9 mol%.

The cell wall of strain NIO-1016 contains *meso*-diaminopimelic acid as diagnostic diamino acid and A1γ type of peptidoglycan. Major cell wall sugars were detected as ribose and glucose while galactose was detected as a minor cell wall sugar. The MK-6 (89.3%) and MK-7 (8.7%) as major and minor menaquinone respectively. The polar lipids of strain NIO-1016 were detected as diphosphotidylglycerol, phosphatidylglycerol and phosphoglycolipid (Fig. 30). The cellular fatty acids profiling of strain NIO-1016 were detected and it's shown in the Table 20.



A B

Fig.28 A) colony morphology and B) scanning electron micrograph of strain NIO-1016^T from 2 days old culture on nutrient agar.

Table 19. Characteristics that differentiate strain NIO-1016^T and other type strains of species of the genus *Domibacillus.*

Characteristic	1	2	3
Spore/sporangium shape	S–E, s	S, S–E, s	S, S–E, s
Spore position	C–S	C, S	T
Pigmentation (on TSBA)	Red pigmentation (after 72 h)	Reddish	Transparent (reddish after 72 h)
Temperature range for growth (°C)	25–45	13–45	10–40
Growth with 12.0% NaCl	+	−	−
Hydrolysis of aesculin	−	+	−
β-Galactosidase (ONPG)	−	+	+
Utilization of gluconate as sole carbon source	−	+	+
Acid production from:			
N-Acetyl-β-glucosamine	+	+	−
L-Arabinose	+	+	−
D-Galactose	+	+	−
Glycerol	+	−	+
Inulin	−	−	+
D-Mannitol	+	+	−
DNA G+C content (mol%)	46.9	44.1	37.4
Major menaquinones	MK-6, MK-7	MK-6	MK-6
Major fatty acids (>5%)	ai-$C_{15:0}$, ai-$C_{17:0}$, i-$C_{15:0}$, $C_{16:0}$, $C_{16:1}\omega11c$, i-$C_{17:0}$	ai-$C_{15:0}$, i-$C_{15:0}$, ai-$C_{17:0}$, i-$C_{17:0}$	$C_{16:0}$, i-$C_{14:0}$, i-$C_{16:0}$, ai-$C_{15:0}$, ai-$C_{15:0}$

Strains: 1, NIO-1016^T; 2, *D. robiginosus* DSM 25058^T (Seiler et al., 2013); 3, *D. indicus* DSM 28032^T (Sharma et al., 2014). +, Positive; −, negative. S, spherical; S–E, spherical to/or ellipsoidal; s, sporangia swollen, C–S, central/subterminal; T, terminal.

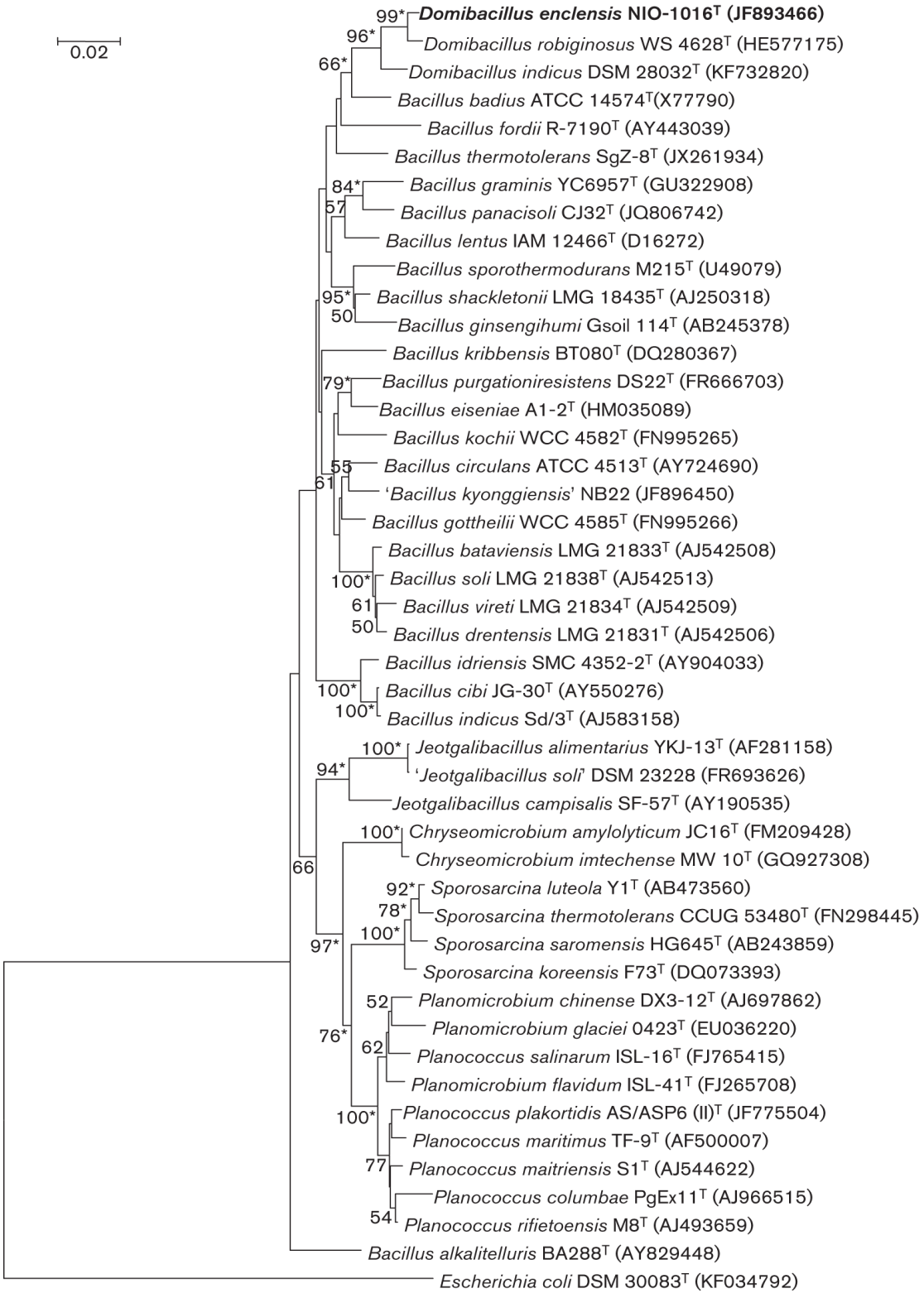


Fig.29. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences, showing the phylogenetic position of strain NIO-1016^T and strains of related species. The sequence of *Escherichia coli* DSM 30083^T (KF034792) was used as an out-group. Only bootstrap values

>50.0%, expressed as percentages of 1000 replications, are shown at branch points. Bar, 0.02 substitutions per nucleotide position.

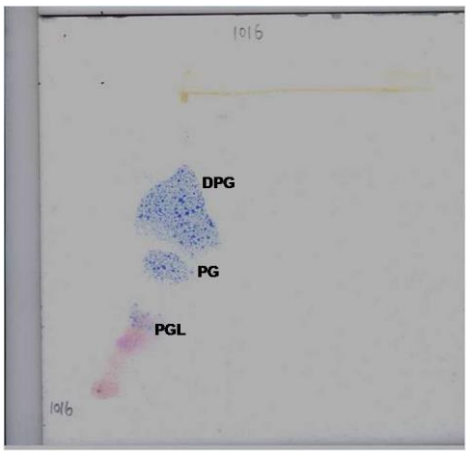


Fig.30. Polar-lipid profile of *Domibacillus enclensis* NIO-1016^T sprayed with molybdenum blue. DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; PGL, unknown phosphoglycolipid.

Table 20. Fatty acid profiling of strain NIO-1016^T

Fatty acids	Percentage content
Straighed chain saturated	
$C_{14:0}$	1.7%
$C_{16:0}$	11.2%
$C_{18:0}$	1.0%
Branched	
Iso-$C_{15:0}$	17.4%
Anteiso-$C_{15:0}$	20.7%
Iso-$C_{17:0}$	5.9%
Anteiso-$C_{17:0}$	19.9%
$C_{16:1}$ ω7c/ω6c	4.9%
$C_{16:1}$ ω11c	5.9%
Iso-$C_{17:1}$ ω10c	2.0%
$C_{18:1}$ ω9c	1.7%

From the above data, it was concluded that strain NIO-1016^T was the newest member of the genus *Domibacillus*, for which *Domibacillus enclensis* sp. nov., was proposed. *Domibacillus enclensi*s (en.clen'sis. N.L. masc. adj. *enclensis* arbitrary name formed from NCL, the acronym for the National Chemical Laboratory, India, where taxonomic studies on this species were performed).

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Domibacillus enclensis sp. nov., isolated from marine sediment, and emended description of the genus *Domibacillus*

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A novel red-pigmented bacterial strain, designated NIO-1016^T, was isolated from a sediment sample from Chorao Island, India and was investigated by a polyphasic taxonomic approach. The strain was Gram-reaction-positive, strictly aerobic, motile and catalase-positive and produced spherical to slightly ellipsoidal endospores with subterminal position on swollen sporangia. The genomic DNA G+C content was 46.9 mol%. The major fatty acids were anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{15:0}$ and $C_{16:0}$. The predominant quinones were MK-6 (89.3 %) and MK-7 (8.7 %). The polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol, and an unidentified phospholipid. *meso*-Diaminopimelic acid (type A1γ) was present in the cell-wall peptidoglycan and the major whole-cell sugars were glucose and ribose. The closest phylogenetic neighbours were identified as *Domibacillus robiginosus* DSM 25058^T (98.0 % similarity) and *Domibacillus indicus* DSM 28032^T (97.2 % similarity), other species of the genus *Bacillus* displayed below 96 % similarity. Phylogenetic, physiological, biochemical and morphological differences between strain NIO-1016^T and its closest neighbours indicate that this strain represents a novel species in the genus *Domibacillus* in the family *Bacillaceae* for which the name *Domibacillus enclensis* sp. nov. is proposed with the type species NIO-1016^T (=DSM 25145^T=NCIM 5462^T=CCTCC AB 2011121^T).

The genus *Bacillus* comprises a phylogenetically and phenotypically heterogeneous group of species. Recently, the systematics of the bacillus group has been greatly modified. A novel genus of the bacillus family, *Domibacillus*, was described by Seiler *et al.* (2013) for a bacterium that forms red-pigmented colonies and was isolated from a pharmaceutical clean room in eastern Germany, based on its morphological, chemotaxonomic and phylogenetic differences from closely related members of the genera *Bacillus*, *Jeotgalibacillus* and *Planococcus*. At the time of writing, two species, *Domibacillus robiginosus* (Seiler *et al.*, 2013) and *Domibacillus indicus* (Sharma *et al.*, 2014) with validly published names are included in the genus *Domibacillus*. Members of the genus *Domibacillus* are Gram-stain-positive, spore-forming, oxidative, motile and strictly aerobic rods. The presence of MK-6 as the dominant quinone is one of the characteristic features of the genus. In the current study we propose that strain NIO-1016^T represents a novel species of the genus *Domibacillus*, and have determined its taxonomic position by using a polyphasic approach.

Strain NIO-1016^T was isolated from a marine sediment sample taken from Chorao Island. After primary isolation and purification on marine agar 2216 (Difco) at 28 °C for 2 weeks, the purified strain was subcultured on the same medium and stored as slants at 4 °C and as 20 % (v/v) glycerol suspensions at −70 °C. Biomass for chemical and molecular studies was obtained by cultivation in shake flasks (shaken at about 140 r.p.m.) using trypticase soy broth (TSB, Hi-Media, Mumbai) medium at 28 °C for 1 week. Gram staining was carried out by using the standard Gram reaction; a non-staining method was used to determine the Gram reactions (Buck, 1982) and cell motility was confirmed by the development of turbidity

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NIO-1016^T is JF893466.

A supplementary figure is available with the online Supplementary Material.

2.4.2.4. Genus *Exiguobacterium*

Genus *Exiguobacterium* was proposed by Collins et al. in 1983 with the characterization of *Exiguobacterium aurantiacum* stain DSM 6208^T from an alkaline potato processing site. The bacterial species within genus *Exiguobacterium* are very diverse and present in cold, hot springs, terrestrial and saline etc. environment. This genus comprises species from psychrotrophic, mesotrophic and thermotrophic conditions (Vishnivetskaya et al., 2009), which showed morphological diversity (ovoids, rods, double rods, chain etc). To date, genus *Exiguobacterium* comprises 17 validly named species (www.bacterio.net). It is categorised under the Phylum *Firmicutes*, Class *Bacilli*, Order *Bacillales* and Family *Bacillacaea.*

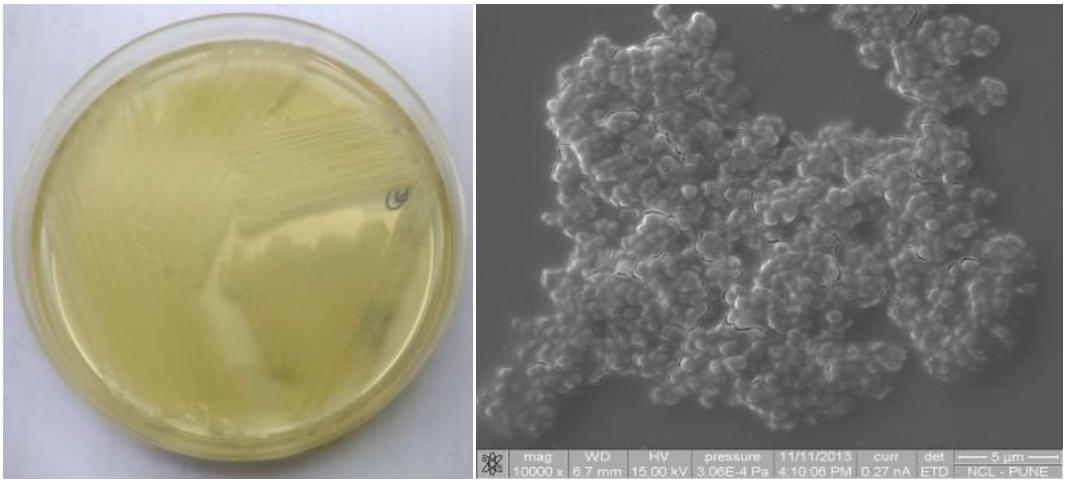
2.4.2.4. a. Strain NIO-1109-*Exiguobacterium* sp.

Strain NIO-1109 was maintained on marine agar and nutrient agar. Colonies of strain NIO-1109 were observed to be light yellowish orange, opaque, circular, convex with an entire margin (Fig. 31 A). Cells were observed to be Gram positive, short rods to coccobacilli, motile and non-spore forming. Under scanning electron microscope cells were observed to be 0.5-0.6 x 0.8-1.3 μm in size (Fig. 31 B). It grows at a temperature ranging from 25-42 ºC (optimal at 30 ºC), at pH ranging from 6.0-12.0 (optimal at 7.0-7.5) with NaCl concentration from 0.0-15.0%. The detailed phenotypic characteristics are given in the table 21.

The 16S rRNA gene sequence of strain NIO-1109 was determined (1415 bp) and deposited into NCBI (GenBank accession no. JF893462). Upon analysis, it was found that strain NIO-1109 showed similarity with *Exiguobacterium indicum* HHS 31^T and *Exiguobacterium acetylicum* NCIMB 9889^T with 99.5 and 99.1% respectively. The phylogenetic tree was constructed by using neighbour-joining method (Fig. 32) which showed that strain NIO-1109 formed a subclade between *E. indcum* HHS 31^T and *E. acetylicum* NCIMB 9889^T supported

by the high bootstrap value of 98.0 and 99.0%. The genomic G+C content was determined which found to be 46.9 mol%. The DNA-DNA relatedness values were determined and it was observed that the homology between strain NIO-1109 and *E. indcum* HHS 31^{T} and between NIO-1109 and *E. acetylicum* NCIMB 9889^{T} were 37.0 ±3.2% and 33.0 ±2.0% respectively which were well below the threshold value of 70.0% for species differentiation.

Strain NIO-1109 contains L-lysine as the diagnostic diamino acid in the cell wall peptidoglycan. Ribose and galactose were observed to be cell wall sugars. MK-7 (91.3%) and MK-8 (8.4%) were detected as a major and minor menaquinone respectively. Polar lipids content were detected as phosphatidylglycerol, diphosphotidylglycerol and phosphatidylethanolamine. The cellular fatty acids profiling are given in the table 22.



A B

Fig. 31 A) colony morphology and scanning electron micrograph of strain NIO-1109 from 2 days old culture on nutrient agar.

Table 21. Phenotypic characteristics that differentiate strain NIO-1109^T from its phylogenetic neighbours in the genus *Exiguobacterium*.

Characteristic	1	2	3
Colony morphology			
Size (mm)	2–4	2–5	2–4
Shape	Round	Irregular	Round
Growth temperature (°C)			
5	−	−	+
37	+	w	−
Optimum growth temperature (°C)	28±2.0	37	30
Gelatinase	−	+	−
Ornithine decarboxylase	−	+	+
Voges–Proskauer reaction	−	+	+
Aesculin hydrolysis	−	+	−
Starch hydrolysis	−	+	−
Lysine decarboxylase	−	−	+
Nitrate reduction	−	−	+
Acid production from:			
D-Glucose, sucrose, D-fructose, trehalose, maltose	+	+	−
Inulin	+	−	−
D-Mannose	−	+	−
Cellobiose	+	+	−
D-Adonitol	−	+	−
Carbon-source utilization			
D-Galactose	+	−	+
L-Rhamnose, melibiose, D-xylose	+	−	+
L-Xylose	−	−	+
D-Mannitol	+	+	−
Raffinose	+	−	+
D-Sorbitol, dulcitol	−	−	+
DNA G+C content (mol%)	46.9	47.0*	48.0*
Quinone(s)	MK-7, MK-8	MK-7*	MK-7, MK-8*
Polar lipids†	PG, DPG, PE	DPG, PS*	PG, DPG, PE*
Source of isolation	Marine sediment	Creamery waste	Glacial water

Strains: 1, NIO-1109^T; 2, *E. acetylicum* DSM 20416^T; 3, *E. indicum* LMG 23471^T. All data were generated from the present study except where indicated. All are motile, Gram-stain-positive and rod-shaped, do not form endospores and are yellowish orange. All are positive for lipase, citrate utilization and cellobiose, but negative for urease, H_2S production and the indole test. +, Positive; w, weakly positive; −, negative.

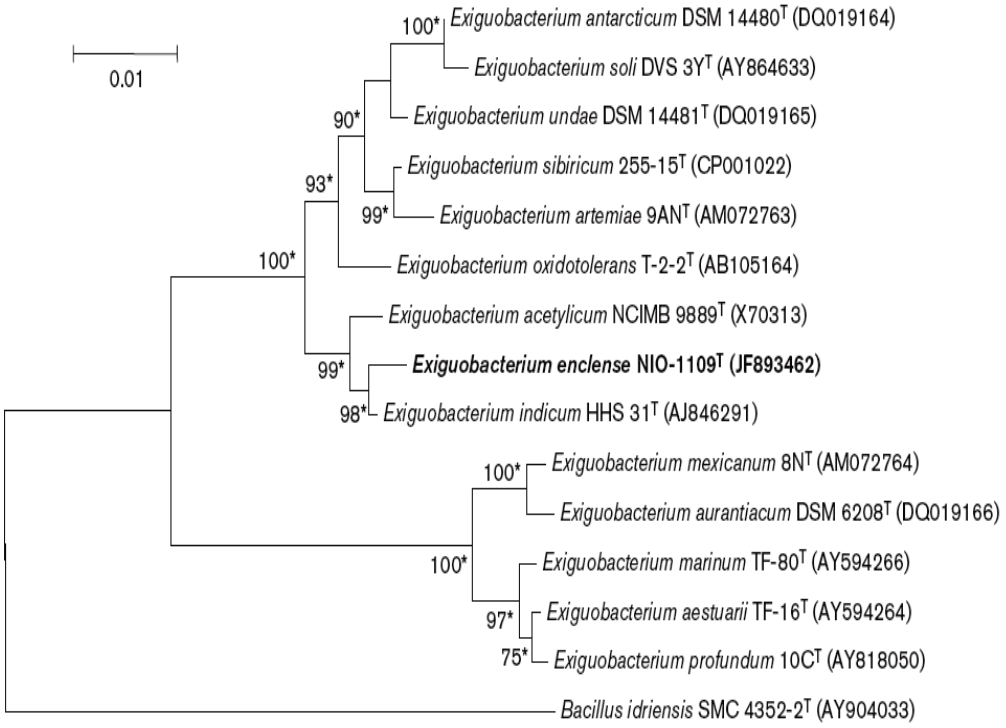


Fig.32 Neighbour-joining phylogenetic tree based on nearly complete (1415 nt) 16S rRNA gene sequences showing the position of NIO-1109^T among members of the genus *Exiguobacterium*. Asterisks indicate that the corresponding nodes (groupings) are also recovered in Fitch–Margoliash, maximum-parsimony and maximum-likelihood trees. Bootstrap values (>70.0%; 1000 resamplings) are given at branch points. *Bacillus idriensis* SMC 4352-2^T was used as an outgroup. Bar, 0.01 nucleotide substitutions per position.

Table 22. Fatty acid profile of strain NIO-1109^T

Fatty acid	Percentage of total
iso-$C_{11:0}$	0.42
iso-$C_{12:0}$	1.49
$C_{12:0}$	0.36
iso-$C_{13:0}$	12.81
anteiso-$C_{13:0}$	7.38
iso-$C_{14:0}$	1.74
$C_{14:0}$	0.93
iso-$C_{15:0}$	13.09
anteiso-$C_{15:0}$	3.28
$C_{16:1}\omega 7c$ alcohol	0.20
iso-$C_{16:1}$ H	0.17

Fatty acid	Percentage of total
iso-$C_{16:0}$	2.21
$C_{16:1}\omega 11c$	0.97
$C_{16:0}$	11.60
anteiso-$C_{17:1}\omega 9c$	0.18
iso-$C_{17:0}$	21.87
anteiso-$C_{17:0}$	2.67
$C_{17:0}$	0.70
iso-$C_{18:0}$	0.76
$C_{18:1}\omega 9c$	0.92
$C_{18:0}$	8.38
iso-$C_{19:0}$	1.06
anteiso-$C_{19:0}$	0.23
$C_{20:0}$	0.26
Summed features*	
1	0.26
3	2.28
4	0.34
8	1.65
9	1.79

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 1 contained iso-$C_{15:1}$H/$C_{13:0}$ 3-OH, summed feature 3 contained $C_{16:1}\omega 7c/\omega 6c$, summed feature 4 contained iso-$C_{17:1}$ I/anteiso B, summed future 8 contained $C_{18:1}\omega 6c/\omega 7c$ and summed feature 9 contained $C_{16:0}$ 10-methyl/iso-$C_{17:1}\omega 9c$.

From the Phenotypic, genotypic and phylogenetic analysis of strain NIO-1109 showed that it lies in the subclade with *E. indcum* HHS 31^T and *E. acetylicum* NCIMB 9889^T and it also shares the DNA-DNA relatedness homology well below the threshold level suggested that stain NIO-1109^T as a new member within the genus *Exiguobacterium* for which *Exiguobacterium enclense* sp.no., were proposed. *Exiguobacterium enclense* (en.clen'se. N.L. neut. adj. *enclense* arbitrary name formed from NCL, the acronym for the National Chemical Laboratory, India, where taxonomic studies on this species were performed).

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Exiguobacterium enclense sp. nov., isolated from sediment

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A Gram-stain-positive bacterium, designated strain NIO-1109^T, was isolated from a marine sediment sample from Chorao Island, Goa, India. Phenotypic and chemotaxonomic characteristics and data from phylogenetic analysis based on 16S rRNA gene sequences indicated that strain NIO-1109^T was related to the genus *Exiguobacterium*. Strain NIO-1109^T exhibited >98.0 % 16S rRNA gene sequence similarity with respect to *Exiguobacterium indicum* HHS 31^T (99.5 %) and *Exiguobacterium acetylicum* NCIMB 9889^T (99.1 %); the type strains of other species showed <98 % similarity. Levels of DNA–DNA relatedness between strain NIO-1109^T and *E. acetylicum* DSM 20416^T and *E. indicum* LMG 23471^T were less than 70 % (33.0±2.0 and 37±3.2 %, respectively). Strain NIO-1109^T also differed from these two closely related species in a number of phenotypic traits. Based on phenotypic, chemotaxonomic and phylogenetic data, strain NIO-1109^T is considered to represent a novel species of the genus *Exiguobacterium*, for which the name *Exiguobacterium enclense* sp. nov. is proposed. The type strain is NIO-1109^T (=NCIM 5457^T=DSM 25128^T=CCTCC AB 2011124^T).

Bacterial species of the genus *Exiguobacterium* are globally diverse organisms, and have been found in a variety of environments, including extreme environments such as microbialites (White *et al.*, 2013), arsenic-rich lakes (Ordoñeza *et al.*, 2013), tidal flats (Kim *et al.*, 2005), freshwater lakes (Raichand *et al.*, 2012), Himalayan ice (Chaturvedi & Shivaji, 2006), Himalayan soil (Singh *et al.*, 2013), hydrothermal vents (Crapart *et al.*, 2007), brine shrimp (López-Cortés *et al.*, 2006) and microbial biofilms (Carneiro *et al.*, 2012). *Exiguobacterium* was proposed by Collins *et al.* (1983) with the description of *Exiguobacterium aurantiacum* as the type species. Farrow *et al.* (1994) transferred *Brevibacterium acetylicum incertae sedis* to the genus *Exiguobacterium*, as *Exiguobacterium acetylicum*. At the time of writing, the genus *Exiguobacterium* comprised 15 recognized species, including the recently proposed *Exiguobacterium aquaticum* (Raichand *et al.*, 2012) and *Exiguobacterium alkaliphilum* (Mohan Kulshreshtha *et al.*, 2013). The genus *Exiguobacterium* comprises psychrotrophic, mesophilic and moderately thermophilic species and strains (Vishnivetskaya *et al.*, 2009), with pronounced morphological diversity (ovoid, rods, double rods and chains) depending on species, strain and environmental conditions (Vishnivetskaya *et al.*, 2007). During an investigation of the bacterial diversity in the marine environment of Chorao Island (15° 32′ 34″ N 73° 55′ 15″ E), Goa State, India, a yellow-pigmented bacterial strain, designated NIO-1109^T, was isolated and subjected to a polyphasic taxonomic investigation.

Strain NIO-1109^T was isolated on marine agar (MA; Himedia) by the standard serial dilution method. MA plates were incubated at 28±2 °C for 3–5 days. Nutrient agar (NA) was used as a growth medium. Isolated colonies were purified by repeated streaking on fresh MA plates, and maintained on NA slants at 4 °C and as glycerol suspensions (20 %, v/v) at −80 °C. Biomass for chemotaxonomic and molecular systematic studies was obtained following growth in trypticase soy broth (Hi-media) under shaking conditions of 150 r.p.m. at 28±2 °C for 72 h. Gram-staining was carried out by using the standard Gram

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Exiguobacterium enclense* NIO-1109^T is JF893462.

Two supplementary figures are available with the online Supplementary Material.

2.4.2.5. Genus *Deinococcus*

The genus *Deinococcus* was described by Brooks and Murray in 1981 for the *Micrococcus radiodurans* and other radiation resistant cocci (Rainey et al. 1997). This genus comprises strictly aerobic, generally stains Gram positive, produces yellow, pink or reddish coloured colonies, have resistance towards radiation, presence of L-ornithine in the cell wall peptidoglycan, and lack teichoic acid ((Brooks and Murray 1981; Kämpfer and Kroppenstedt 1996; Ferreira et al. 1997; Rainey et al. 1997, 2005; Hirsch et al. 2004; Suresh et al. 2004; de Groot et al. 2005; Lai et al. 2006; Shashidhar and Bandekar, 2006; Weon et al. 2007; Im et al. 2008; Kämpfer et al. 2008; Chen et al. 2012; Srinivasan et al. 2012a, b). The 16S rRNA gene sequence analysis showed that *Deinococcus* forms a phylogenetically diverse group in the deeply branching lineage. The genus *Deinococcus* categorised into Phylum *Deinococcus-Thermus*, Class *Deinococci*, Order *Deinococcales*, and Family *Deinococcaceae*. To date, it comprises 76 known validly species (www.bacterio.net).

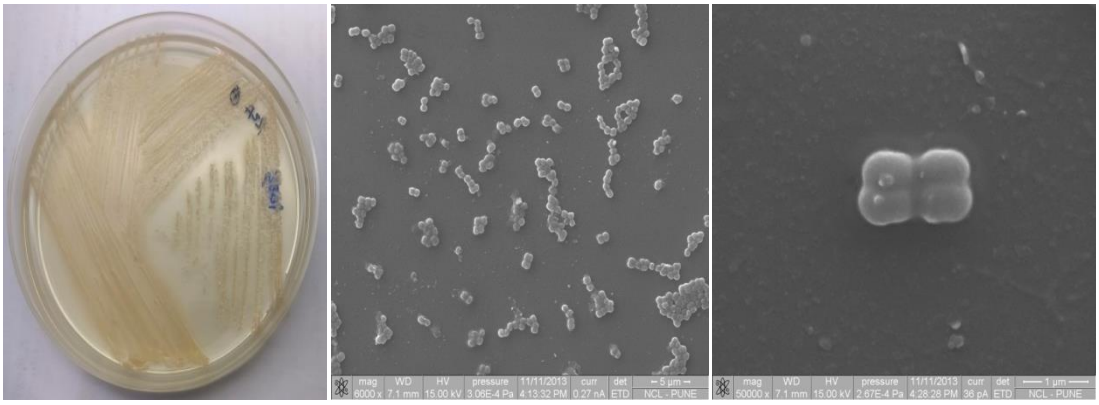
2.4.2.5. a. Strain NIO-1023-*Deinococcus* sp.

Strain NIO-1023 was isolated from the marine sediment sample and maintained on R2A agar. Colonies of NIO-1023 were observed to be pale pink coloured, circular, smooth, convex on two days incubation on R2A agar with 2-3 mm in diameter (Fig. 33 A). Cells were observed to be Gram positive, cocci and non-motile. Under scanning electron microscope cells were observed to be 0.45-0.46 x 3.0-3.2 μm in size (Fig. 33 B). Strain NIO-1023 grow at a temperature ranging from 25-45 ºC (optimal at 30 ºC), at pH 6.0-10.0 (optimal at 7.0-8.0) and with NaCl concentration from 0.0-1.0%. The characteristics of strain NIO-1023 are given in the Table 23.

The 16S rRNA gene sequence of the strain NIO-1023 was obtained (1437 bp) and deposited into NCBI (GenBank accession no. HQ858011). Analysis of 16S rRNA gene

sequence was done and it showed the highest sequence similarity with *Deinococcus ficus* CC-FR2-10*T* with 97.8% match and that other *Deinococcus* species shared less than 95.0% similarity match. Upon construction of the phylogenetic tree (Fig. 34) using the Neighbour-Joining method, it was found that strain NIO-1023 formed a monophyletic clade with *D. ficus* CC-FR2-10^{T} with 100% bootstrap support. The AP-PCR amplicon fingerprinting banding pattern of strain NIO-1023 and *D. ficus* CC-FR2-10^{T} showed marked differences which suggest that both strains were different (Fig. 35). Further analysis of DNA-DNA relatedness between both strains was determined and the values were 23.9 ± 3.0%, which was well below of 70.0% cut-off point for prokaryotic species identification. This suggested that the strain NIO-1023 were different species of genus *Deinococcus*. The genomic DNA G+C content of strain NIO-1023 was determined as 67.2 mol%.

Strain NIO-1023 was found to be containing *meso*-diaminopimelic acid as diagnostic diamino acid in the cell wall peptidoglycan. Ribose, glucose and galactose were found to be whole cell wall sugars. L-ornithine, D-alanine, glycine and D-glutamic acid were found in the cell wall peptidoglycan amino acid in a molar ratio of approximately 1.1:0.8:1.1:1.0. The major menaquinone was identified was MK-8. Polar lipids were detected as two unidentified glycolipids, three unidentified phosphoglycolipids, two unidentified phospholipids, an unidentified aminophospholipids and a brick red pigment spot (Fig. 36). The fatty acids profiling of strain NIO-1023 and its nearest neighbour *D. ficus* CC-FR2-10^{T} are given in the table 24.



A B

Fig.33 A) colony morphology and B) scanning electron micrograph of strain NIO-1023^T from 3-day old cultures grown on R2A agar.

Table 23. Phenotypic properties that separate strain NIO-1023^T from the type strain of the phylogenetically close species *D. Ficus*.

Characteristic	***Deinococcus* sp NIO-1023^T**	***D. ficus* CC-FR2-10^T**
Pigmentation	Pale pink	Pale pink
Nitrate reduction	−	+
Esculin hydrolysis	−	+
Utilization as carbon source for energy		
Glucose	−	+
Lactose	−	+
Xylose	−	+
Maltose	−	+
Galactose	+	−
Raffinose	+	−
Sucrose	+	+ (weak)
Salicin	+	−
Cellobiose	+	−
Acid produced		
Sucrose	+	−
Enzyme activity (API-ZYM system)		
Esterase (C4)	+ (weak)	+
β-Galactosidase	−	+(weak)
α-Glucosidase	−	+
G+C content (mol%)	67.2	71.3
Source of isolation	Marine sediment	Rhizosphere soil

All data were generated from present study. Both strains positive for oxidase, malonate, fructose, trehalose, melibiose, l-arabinose, d-arabinose, mannose, sorbitol, acid phosphatase, and naphthol-AS-BI-phosphohydrolase. Both strains negative lysine utilization, ornithine utilization, urease, phenylalanine deamination, H_2S production, citrate utilization, Voges

Proskauer's test, methyl red, indole production, utilization of xylose, adonitol, and saccharose. Both strains negative for lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-galactosidase, β-glucoronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase, + positive, − negative

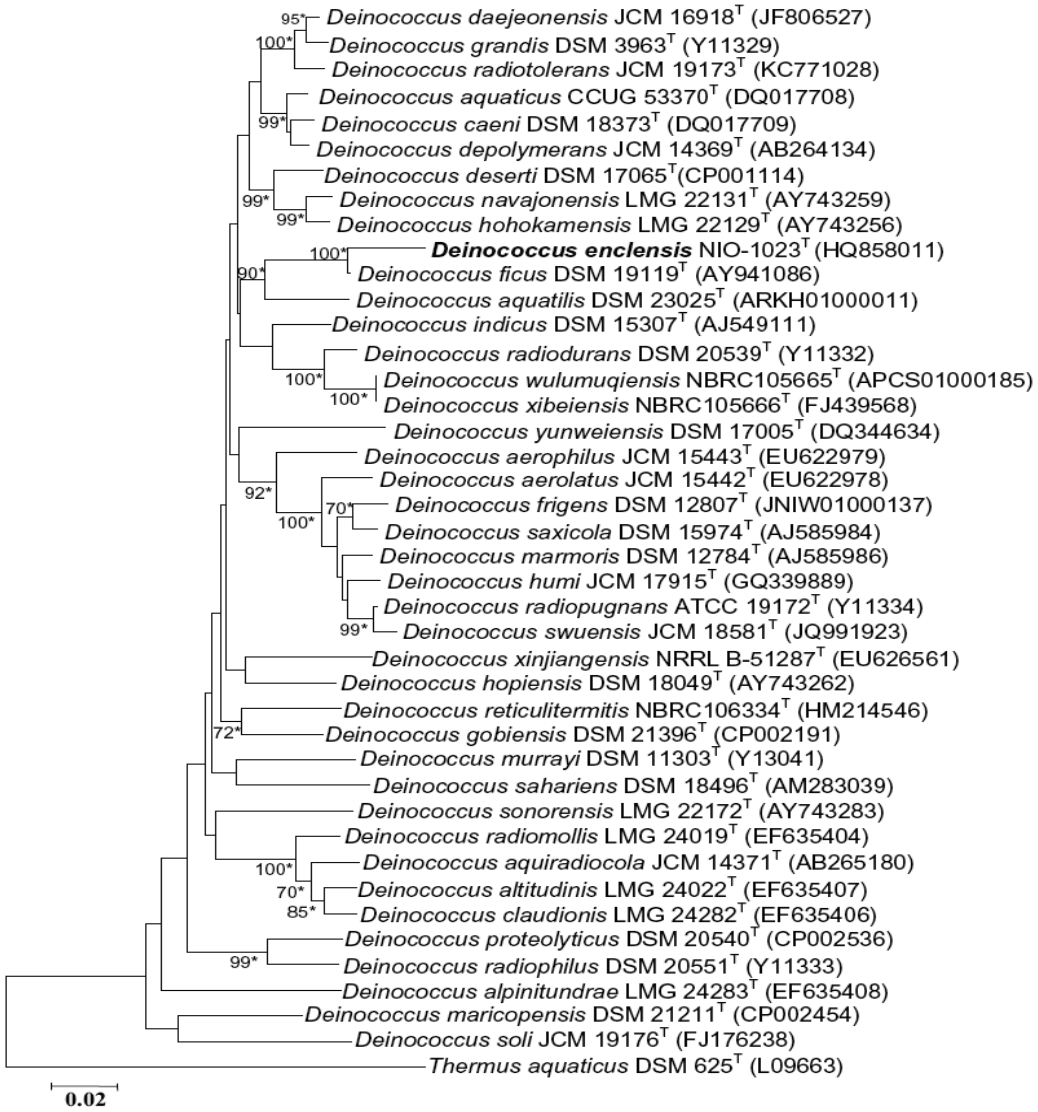


Fig.34 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between isolate NIO-1023^T and representatives of the genus *Deinococcus*. Asterisks indicate phyletic lines that were also recovered using the maximum-parsimony and maximum likelihood algorithms. Numbers at nodes indicate percentages of bootstrap support

based on a neighbour-joining analysis of 1,000 resampled datasets; only values above 70.0% are given. Bar 0.02 substitutions per nucleotide position.

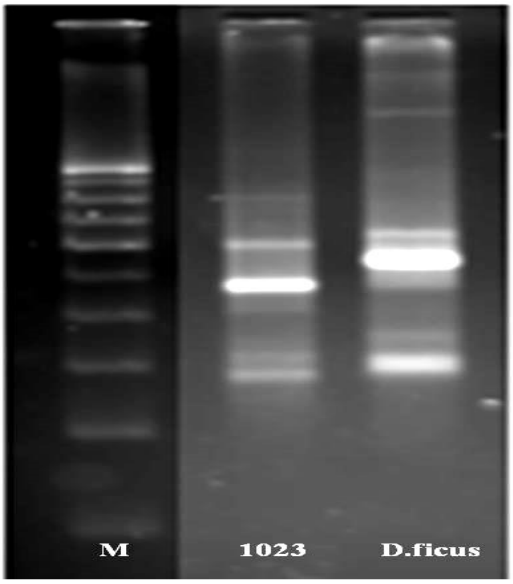


Fig.35 Arbitrary primed PCR (AP-PCR) profile [Lane 1-Marker 100 bp; Lane 2: Strain NIO-1023^{T}; lane 3: *D.ficus* CC-FR2-10^{T}.

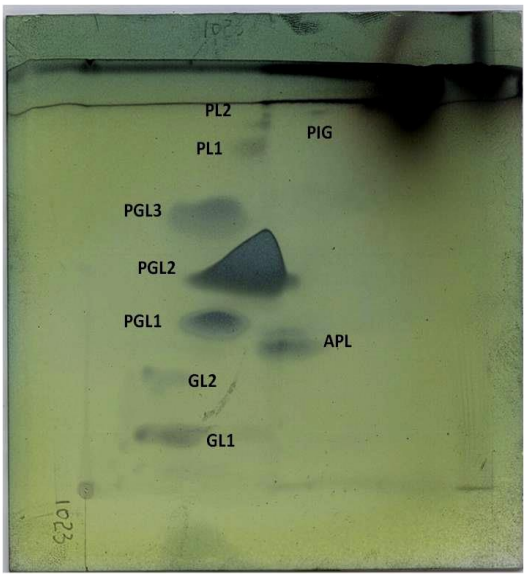


Fig.36 Polar lipid profile of strain NIO-1023^{T}. GL1 and GL2, unidentified glycolipids; PGL1–PGL3, unidentified phosphoglycolipids; PL1–PL2, unidentified phospholipids; APL, unidentified aminophospholipid; PIG brick-red pigments.

Table 24. Fatty acid composition (%) of strain NIO-1023^T and its nearest phylogenetic neighbour *D. Ficus*.

Fatty acid	***D. enclensis* NIO-1023^T**	***D. ficus* CC-FR2-10^T**
iso-$C_{11:0}$	0.4	0.3
$C_{12:0}$	0.2	0.3[b]
iso-$_{13:0}$	0.3	0.3
$C_{14:0}$	0.4	0.4
iso-$C_{15:0}$ F	0.6	0.7
iso-$_{15:0}$	2.2	2.5
$C_{15:1}$ω8c	6.9	4.5
$C_{15:1}$ω6c	12.4	8.2
iso-$C_{16:1}$ H	0.4	0.3[b]
iso-$_{16:0}$	0.5	0.4
iso-$C_{16:1}$ ω9c	3.1	3.9
iso-$C_{16:1}$ ω5c	0.2	0.3
$C_{16:0}$	5.7	8.3
iso-$C_{17:0}$	3.4	4.8
$C_{17:1}$ω8c	26.8	20.6
$C_{17:1}$ω6c	3.0	2.5
$C_{17:0}$	4.2	4.3
$C_{18:1}$ω9c	0.6	1.0
$C_{18:0}$	0.8	0.6[#]
Summed features[a]		
3	15.7	20.8
8	0.5	0.8
9	10.6	13.2

[a] Summed feature 3 consists of $C_{16:1}$w6c/$C_{16:1}$w7c, summed feature 8 consists of $C_{18:1}$w6c/$C_{18:1}$w7c and summed feature 9 consists of $C_{16:0}$ 10-methyl/$C_{17:1}$w9c

[b] Observation was absent and $C_{15:0}$ was present in the original description of *D. ficus* CC-FR2-10^T (Lai et al. 2006).

From the phenotypic, genotypic and phylogenetic analysis of strain NIO-1023^T showed that it differs from its closest neighbour *D. ficus* CC-FR2-10^T, thus the name *Deinococcus enclensis* sp.no., was proposed. *Deinococcus enclensis* (en.clen'sis. N.L. masc. adj. *enclensis* arbitrary name formed from NCL, the acronym for the National Chemical Laboratory, India, where taxonomic studies on this species were performed).

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ORIGINAL PAPER

Deinococcus enclensis sp. nov., isolated from a marine sediment sample

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Abstract A novel pale-pink coloured strain, designated NIO-1023^T, was isolated from a marine sediment sample from Chorao Island, Goa, India. The taxonomic position of strain NIO-1023^T was investigated by using a polyphasic approach. The cells were observed to be Gram-stain positive, coccal shaped and non-spore forming. Phylogenetic analyses using the 16S rRNA gene sequence of the isolate indicated that the organism belongs to the genus *Deinococcus*. The strain NIO-1023^T showed highest 16S rRNA gene sequence similarities with *Deinococcus ficus* (97.8 %), whereas other *Deinococcus* species showed less than 95 % sequence similarity. The DNA–DNA relatedness with respect to *D. ficus* CC-FR2-10^T was 23.9 %. Chemotaxonomic data revealed that strain NIO-1023^T contains only menaquinone MK-8 as the respiratory quinone and a complex polar lipid profile consisting of different unidentified glycolipids and polar lipids, two unknown phospholipids and three unknown phosphoglycolipids. As in other deinococci, one of these phosphoglycolipids was predominant in the profile. The predominant fatty acids were identified as $C_{17:1}$ w8c, $C_{16:1}$ w6c/w7c, $C_{15:1}$ w6c and $C_{17:1}$ w9c. The genomic DNA G + C content of strain NIO-1023^T was determined to be 67.2 mol%. The biochemical and chemotaxonomic properties demonstrate that strain NIO-1023^T represents a novel species, for which the name *Deinococcus enclensis* sp. nov. is proposed. The type strain is NIO-1023^T (=DSM 25127^T = NCIM 5456^T).

Keywords *Deinococcus* · 16S rRNA · Polyphasic · Marine sediments

Electronic supplementary material The online version of this article (doi:10.1007/s10482-014-0311-4) contains supplementary material, which is available to authorized users.

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Introduction

The genus *Deinococcus* was established by Brooks Murray (1981) and at present the genus comprises nearly 50 species with validly published names (http://www.bacterio.net/deinococcus.html). Species of the genus *Deinococcus* are strictly aerobic, have high resistance to ionizing radiation, produce reddish, pink or yellow colonies, generally stain Gram-stain positive, have L-ornithine in the peptidoglycan and lack teichoic acids in the cell wall (Brooks and Murray 1981; Kämpfer and Kroppenstedt 1996; Kämpfer et al. 2008; Ferreira et al. 1997; Rainey et al. 1997, 2005; Hirsch et al. 2004; Suresh et al. 2004; de Groot et al. 2005; Lai et al. 2006; Shashidhar and Bandekar, 2006; Weon et al.

2.4.2.6. Genus *Rhodococcus*

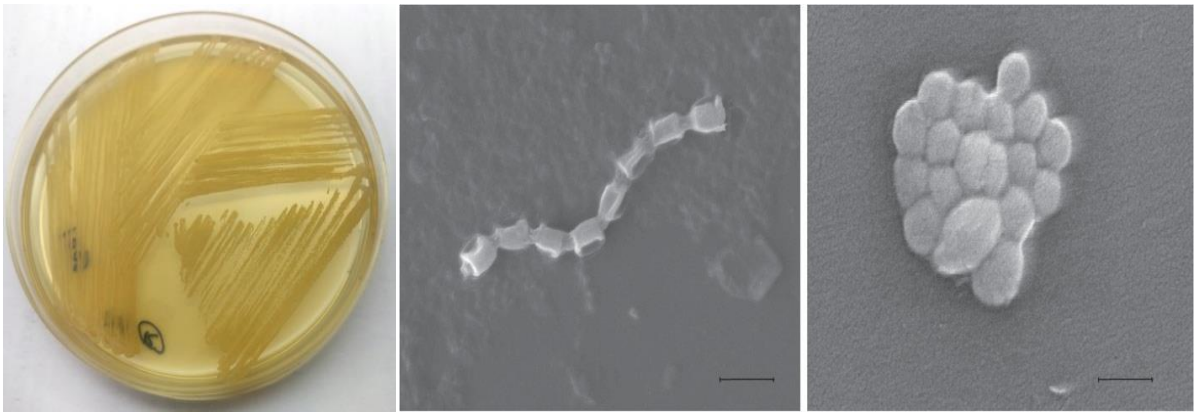
The genus *Rhodococcus* was first used by Zopf in 1891, which was revived and redefined by Goodfellow and Alderson in 1977 to accommodate *rhodochrous* complex which comprised number of strains that did not belong to the genera *Nocardia*, *Corynebacterium* and *Mycobacterium* (Bell et al. 1998). The genus *Rhodococcus* was established on the basis of examination of 177 strains for 92 unit characters and grouped them according to percentage similarity. The genus *Rhodococcu*s was classified by Stackerbrandt et al. in 1997 as a member of suborder *Corynebacterineae* and included in the family of *Nocardiaceae*. The genus *Rhodococcus* has been demonstrated phylogenetically to be grouped in a coherent clade whose members are exclusively contained mycolic-containing genera (Embley and Stackebrandt, 1994). *Rhodococcus* genus was described chemotaxonomically by Goodfellow in 1989 and was updated by Finnerty (1992). The cell wall of the genus *Rhodococcus* contains *meso*-diaminopimelic acid as only diamino acid in the peptidoglycan, which means it has chemotype IV cell wall and the major sugars are arabinose and galactose (Lechevalier & Lechevalier, 1970b). The application of 16S rRNA gene sequencing and phenotypic characteristics facilitate the discovery and classification of novel species within the genus *Rhodococcus* (Rainey et al., 1995; Goodfellow et al., 1998; McMinn et al., 2000; Gürtler et al., 2004). Based on 16S rRNA gene sequencing the phylogenetic tree revealed that the members of the genus *Rhodococcus* can be assigned to four or six subclades (Rainey et al. 1995; Gürtler et al. 2004). The genus *Rhodococcus* is categorised into Phylum *Actinobacteria*, Order *Actinomycetales*, Suborder *Corynebacterineae* and Family *Nocardiaceae*. To date, genus *Rhodococcus* contains 66 validly known species (www.bacterio.net).

2.4.2.6. a. Strain NIO-1009-*Rhodococcus* sp.

Strain NIO-1009 was maintained on marine agar and nutrient agar. Colonies of strain NIO-1009 were observed to be pale orange coloured, circular, opaque, smooth, convex with an entire margin (Fig. 37 A). Cells were observed to be Gram positive, non-motile, non-spore forming. In early-stage cells were observed as thin and filamentous and in the later stage of growth the filaments fragment into the rod to coccoid shaped as reported for the *Rhodococcus* genera. Under scanning electron microscope (Fig. 37 B) cells were observed to be rod shaped (1.22-1.31 x 2.01-2.05 μm) and coccoid shaped (12-.2-2.0 x 1.0-2.0 μm). Strain NIO-1009 grows at a temperature ranging from 20-37 ºC (optimal at 30 ºC), at pH ranging from 6.0-11.0 (optimal at 7.0) with NaCl concentration from 0.0-10.0%. The detailed physiological and biochemical characteristics are given in the table 25.

The 16S rRNA gene sequence of strain NIO-1009 (1374 bp) was obtained and deposited into NCBI (GenBank accession no. HQ858009). Upon comparison, it was found that the strain NIO-1009 showed sequence similarity with *Rhodococcus kroppenstedtii* DSM 44908^T and *Rhodococcus corynebacterioides* DSM 20151^T with 99.2 and 99.1% respectively. The phylogenetic tree was constructed by using Neighbour-Joining method (Fig. 38) which showed that the strain NIO-1009 lies in the subclade between *R. kroppenstedtii* DSM 44908^T and *R. corynebacterioides* DSM 20151^T which supported by a bootstrap value 100%. The affiliation of strain NIO-1009 and its closest neighbour was also supported by Maximum-Likelihood and Maximum-Parsimony method with a bootstrap value of 99.8% and 100% respectively. AP-PCR amplicon fingerprinting of strain NIO-1009 and *R. kroppenstedtii* DSM 44908^T and *R. corynebacterioides* DSM 20151^T showed marked differences in banding pattern (Fig. 39). The DNA-DNA relatedness value between strain NIO-1009 and *R. kroppenstedtii* DSM 44908^T and *R. corynebacterioides* DSM 20151^T were determined as 39.5 ±3.2% and 41.7 ±2.5% respectively. The G+C content of NIO-1009 was 66.9 mol%.

The diagnostic cell wall amino acid of strain NIO-1009 contains *meso*-diaminopimelic acid and galactose and arabinose as cell wall sugars. Mannose, rhamnose, ribose and glucose were also detected as non-diagnostic cell wall sugars. Mycolic acid was present. The polar lipids detected in NIO-1009 were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and one unknown phospholipid (Fig. 40). The fatty acids profile of strain NIO-1009 and their related strains are given in the table 26.



A B

Fig.37 A) colony morphology and B) scanning electron microscopes images showing the rod- to coccoid-shaped cells of NIO-1009^{T} grown in NA medium for (left panel) 24 hrs and (right panel) 72 hrs. Rod- to coccoid-shaped elements are a characteristic property of the members of the genus *Rhodococcus*.

Table 25. Phenotypic characteristics that differentiate strain NIO-1009^T from its phylogenetic neighbours in the genus *Rhodococcus*.

Characteristics	1	2	3
Colony colour	Pale orange	Orange red	Orange red
Cell shape	Rod–coccoid	Rod–coccoid	Rod–coccoid
Nitrate reduction	+	+	−
Hydrolysis of			
Aesculin	+	−	−
Urea	+	−	−
Tolerance to			
8.0% NaCl	+	+	−
10.0% NaCl	+	+ (w)	−
Growth at 42 °C	−	−	+
Acid produced from:			
Raffinose	+	+	−
myo-Inositol	+	+	−
Salicin	−	−	−*
D-Xylose	−	−*	+
Cellobiose	−	−	+
D-Mannose	−	−	−*
Utilization as sole source of carbon			
Sodium lactate	+	+	−
L-Rhamnose	+	+	−
Salicin	+	−	+
DNA G+C content (mol%)	66.9	69.7†	71.4†

*Results differed from results reported by Mayilraj et al. (2006) .†Data from Mayilraj et al. (2006). Taxa: 1, *R. enclensis* NIO-1009^T; 2, *R. kroppenstedtii* DSM 44908^T; 3, *R. corynebacterioides* DSM 20151^T. All data were generated in the present study unless otherwise indicated. All three strains were catalase-positive and negative for tyrosine and citrate utilization. +, Positive; −, negative; w, weakly positive.

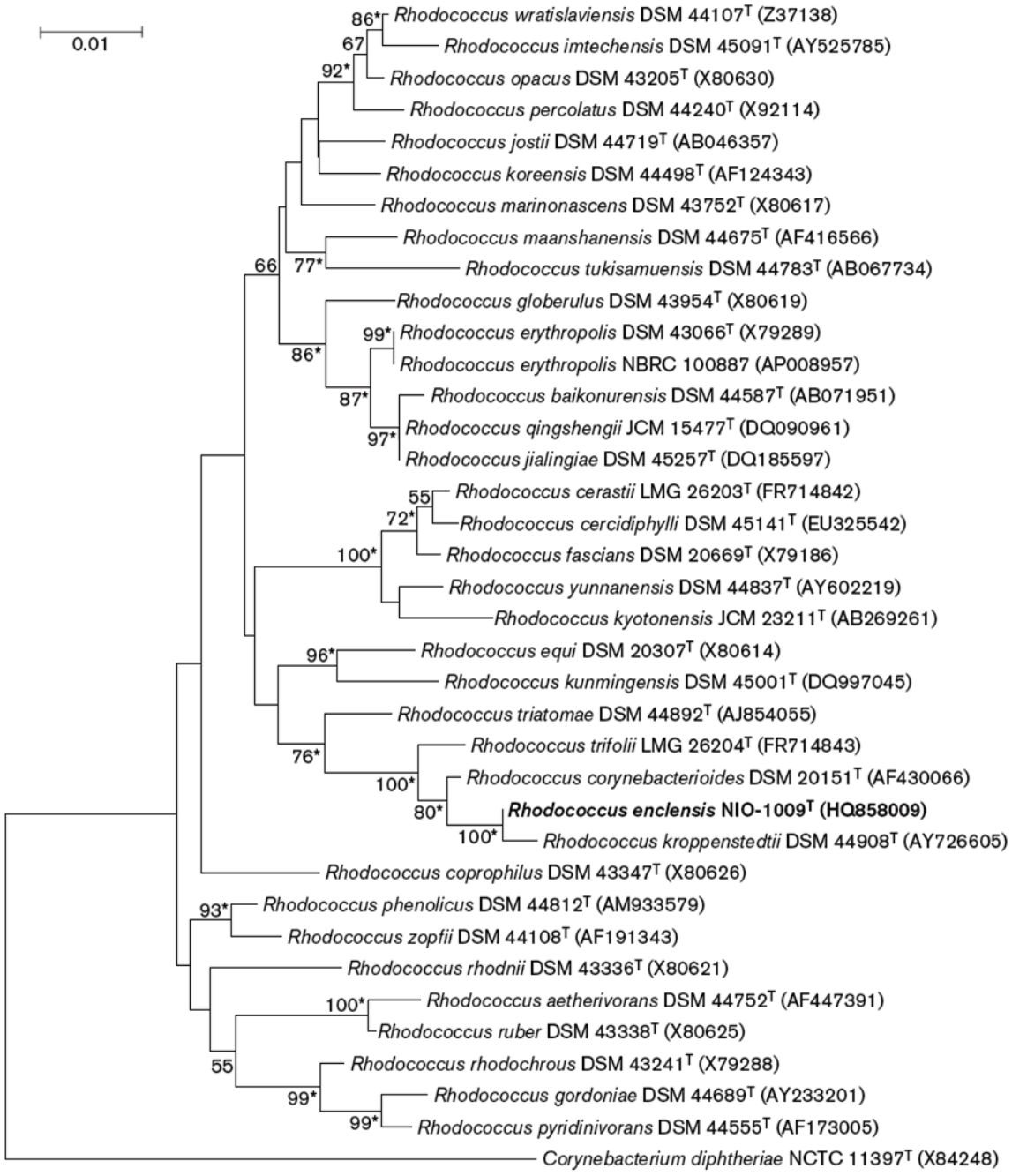


Fig.38 Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between strain NIO-1009^T and related members of the genus *Rhodococcus*. Bootstrap values (>70.0%; 1000 resamplings) are given at branch points. Asterisks indicate that the corresponding nodes (groupings) are also recovered in maximum-parsimony and maximumlikelihood trees. Bar, 0.01 nucleotide substitutions per position.

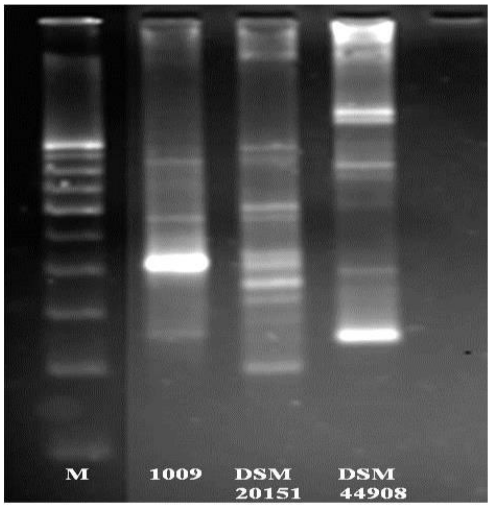


Fig.39 Arbitrary primed PCR (AP-PCR) profile showing the distinct banding pattern of strain NIO-1009^{T} with nearest type strains, which distinguishes the isolates with its closest neighbours.

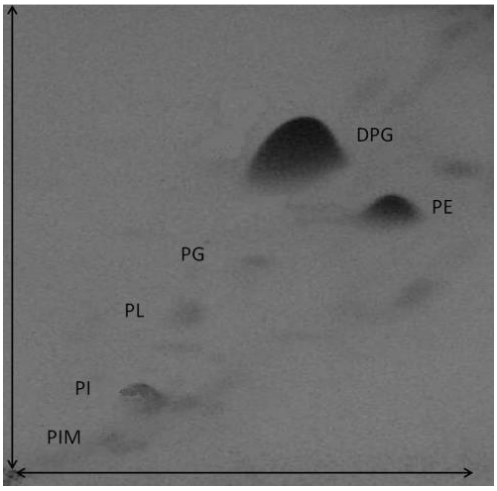


Fig.40 Total polar lipid profile of strain NIO-1009^{T} after two dimensional thin layer chromatography staining with molybdatophosphoric acid Abbreviations: DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol, PIM, phosphatidylinositol mannoside and PL, unknown phospholipid.

Table 26. Fatty acid composition of NIO-1009^T and its closest species *R. kroppenstedtii* DSM 44908^T, *R. corynebacterioides* DSM 20151^T.

Fatty acids	***R.enclensis* NIO-1009^T**	***R. kroppenstedtii* DSM 44908^T**	***R. corynebacterioides* DSM 20151^T**
$C_{12:0}$	0.4	0.1	0.4
$C_{14:0}$ $C_{15:1}$ ω8c $C_{15:1}$ ω5c	4.3 - -	3.3 0.1 0.2	4.6 0.2 0.3
$C_{16:1}$ ω9c $C_{16:0}$	0.4 37.1	0.5 37.9	- 31.2
$C_{17:1}$ ω8c	2.3	5.9	6.4
$C_{17:0}$	0.6	2.8	2.0
$C_{18:1}$ ω9c $C_{18:0}$ $C_{18:0}$ 10-methyl	37.6 0.9 5.2	34.5 2.9 2.6	28.4 1.1 3.8
Summed features *			
3	10.8	7.2	19.2
6 9	- -	1.0 -	0.4 1.0

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained $C_{16:1}$ ω6c /$C_{16:1}$ ω7c. Summed feature 6 contained $C_{19:1}$ ω9c/$C_{19:1}$ω11c, and summed feature 9 contained $C_{16:0}$ 10-methyl/ $C_{17:1}$ iso ω9c.

From Phenotypic, genotypic and phylogenetic analysis of strain NIO-1009 it was found that the strain NIO-1009^T as a member of genus *Rhodococcus* and it differs from its closest neighbours *R. kroppenstedtii* DSM 44908^T and *R. corynebacterioides* DSM 20151^T, so the name *Rhodococcus enclensis* sp.no., was proposed. *Rhodococcus enclensis* (en.clen' sis. N.L. masc. adj. *enclensis* arbitrary name formed from NCL, the acronym for the National Chemical Laboratory, India, where taxonomic studies on this species were performed).

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Rhodococcus enclensis sp. nov., a novel member of the genus *Rhodococcus*

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A novel actinobacterial strain, designated, NIO-1009^T, was isolated from a marine sediment sample collected from Chorao Island, Goa, India. Phylogenetic analysis comparisons based on 16S rRNA gene sequences between strain NIO-1009^T and other members of the genus *Rhodococcus* revealed that strain NIO-1009^T had the closest sequence similarity to *Rhodococcus kroppenstedtii* DSM 44908^T and *Rhodococcus corynebacterioides* DSM 20151^T with 99.2 and 99.1 %, respectively. Furthermore, DNA–DNA hybridization results showed that *R. kroppenstedtii* DSM 44908^T and *R. corynebacterioides* DSM 20151^T were 39.5 (3.0 %) and 41.7 (2.0 %) with strain NIO-1009^T, respectively, which were well below the 70 % limit for any novel species proposal. Phylogenetically strain NIO-1009^T forms a stable clade with and *R. kroppenstedtii* DSM 44908^T and *R. corynebacterioides* DSM 20151^T with 100 % bootstrap values. Strain NIO-1009^T contained *meso*-diaminopimelic acid as the diagnostic diamino acid and galactose and arabinose as the cell wall sugars. The major fatty acids were $C_{16:0}$, $C_{18:1}\omega9c$, $C_{16:1}(\omega6c$ and/or $\omega7c)$ and 10-methyl $C_{18:0}$. The only menaquinone detected was MK-8(H_2), while the major polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannoside and one unknown phospholipid. The G+C content of the genomic DNA was 66.9 mol%. The phenotypic and genotypic data showed that strain NIO-1009^T warrants recognition as a novel species of the genus *Rhodococcus* for which the name *Rhodococcus enclensis* sp. nov., is proposed; the type strain is NIO-1009^T (=NCIM 5452^T=DSM 45688^T).

The genus *Rhodococcus* was classified as a member of the suborder *Corynebacterineae* by Stackebrandt *et al.* (1997) and included in the family *Nocardiaceae* together with the genus *Nocardia*. The genus *Rhodococcus* has been phylogenetically demonstrated to be grouped in a coherent clade whose members are exclusively mycolate-containing genera (Embley & Stackebrandt, 1994). The revived genus *Rhodococcus* was originally proposed on the basis of a study of actinomycetes which examined 92 characters of the test strains and grouped them according to percentage similarity (Goodfellow & Alderson, 1977). Chemotaxonomy defining the genus *Rhodococcus* was described by Goodfellow (1989) and updated by Finnerty (1992). The genus *Rhodococcus* has cell walls of chemotype IV, which means that the only diamino acid in the peptidoglycan is *meso*-diaminopimelic acid and that the major sugars are arabinose and galactose (Lechavalier & Lechavalier, 1970). The application of molecular methods, particularly 16S rRNA gene sequencing, in parallel with improved phenotypic approaches has not only improved the classification of the genus *Rhodococcus* but also has greatly facilitated the discovery of novel species (Rainey *et al.*, 1995; Goodfellow *et al.*, 1998; McMinn *et al.*, 2000; Gürtler *et al.*, 2004). Phylogenetic trees based on the 16S rRNA gene showed that members of the genus

Abbreviation: AP-PCR, arbitrarily primed PCR.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Rhodococcus enclensis* NIO-1009^T is HQ858009.

Two supplementary figures and a supplementary table are available with the online version of this paper.

2.4.2.7. Genus *Microbacterium*

The genus *Microbacterium* was proposed by Orla-Jensen in 1919 for Gram positive, non-spore forming, rod shaped bacteria that was isolated during the study of lactic acid bacteria from dairy products. Members of the genus *Microbacterium* are diverse and isolated from various habitat like soil, sediment, aquatic sample, insect, plants, clinical specimen and food (Behrendt et al., 2001; Kim et al., 2008; Clermont et al., 2009; Yoon et al., 2009; Kim et al., 2010; Zhang et al., 2010; Kaur et al., 2011; Kim et al., 2011; Gao et al. 2013; Ohta et al. 2013; Yu et al. 2013; Zhang et al. 2014; Kook et al. 2014; Lee et al. 2014). The genus *Microbacterium* is categorised into Phylum *Actinobacteria*, Class *Actinobacteria*, Order *Actinomycetales*, Suborder *Micrococcineae* and Family *Microbacteriaceae*. To date, genus *Microbacterium* contains 110 validly known species (www.bacterio.net).

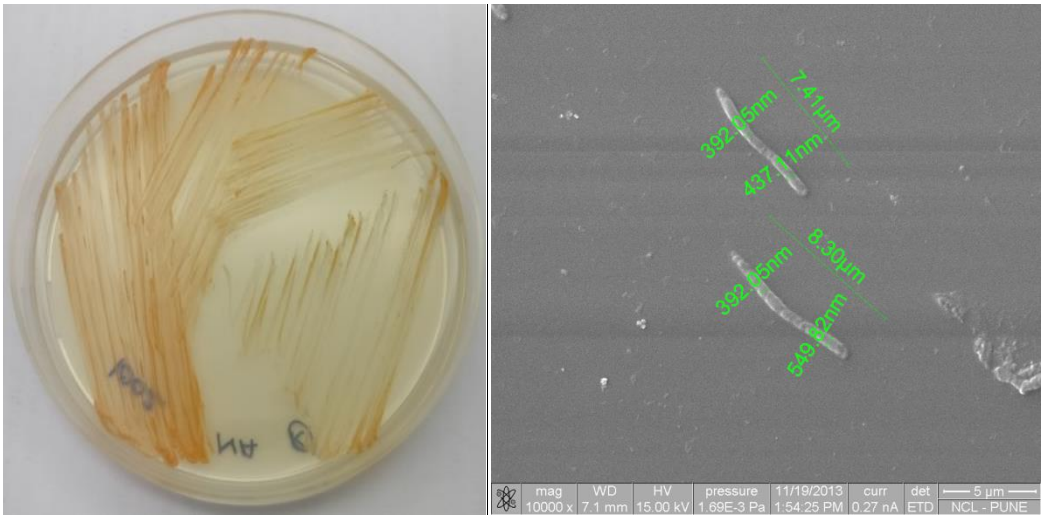
2.4.2.7.a. Strain NIO-1002- *Microbacterium* sp.

Strain NIO-1002 was isolated from marine sediment sample of Chorao island Goa, India and maintained on marine agar. Colonies of strain NIO-1002 were observed to be pale yellow coloured, circular and opaque with an entire margin (Fig. 41). Cells were observed to be Gram positive, short rods and non-motile. Under scanning electron microscope cells were observed to be 0.4-0.6 x 0.5-0.8 μm in size (Fig.). Strain NIO-1002 grows at a temperature ranging from 20-45 ºC (optimal at 30 ºC), at pH from 6.0-12.0 (optimal at 7.0) with NaCl concentration ranging from 0.0-12.0%. The detailed physiological and biochemical characteristics of strain NIO-1002 are given in the table 27.

The 16S rRNA gene sequence of strain NIO-1002 was obtained (1414 bp) and deposited into NCBI (GenBank accession no. JF421612). Comparative analysis of 16S rRNA gene sequence with other strains was matched with *Microbacterium hominis* IFO 15708^T and *Microbacterium testaceum* DSM 20166^T with 98.1 and 98.0% respectively. The phylogenetic

tree was constructed using the Neighbour-Joining method (Fig. 42) which was also supported by maximum-likelihood and maximum-parsimony method with high bootstrap value. The DNA-DNA relatedness values were determined between NIO-1002 and *M. hominis* CIP 105731^{T} and NIO-1002 and *M. testaceum* KCTC 9103^{T} as 39.0 ±2.0% and 41.0 ±2.0% respectively which was well below the cutoff value of 70.0% for prokaryotic species description. Furthermore, AP-PCR amplicon fingerprinting pattern of NIO-1002, *M. hominis* CIP 105731^{T} and *M. testaceum* KCTC 9103^{T} showed marked differences in banding pattern (Fig. 43). The genomic DNA G+C content was determined as 66.5 mol%.

Strain NIO-1002 contains lysine, glutamate plus hydroxyglutamate, glycine and alanine as a cell wall peptidoglycan and ribose, galactose, glucose and a trace amount of xylose as a cell wall sugars. This suggested that the cell wall of strain NIO-1002 has B2β type peptidoglycan (Schleifer & Kandler, 1972). The predominant menaquinone was determined as MK-12 (58.9%), MK-11 (20.9%) as a major menaquinone and MK-13 (13.1%) as a minor menaquinone. The polar lipids detected in strain NIO-1002 were diphosphotidylglycerol, phosphatidylglycerol, two unknown phospholipids and one unknown glycolipids (Fig. 44). The fatty acids profile of NIO-1002 and its closest strains are given in the table 28.



A B

Fig.41. A) colony morphology and B) scanning electron micrograph of strain NIO-1002 grow on nutrient agar.

Table 27. Phenotypic comparison of strain NIO-1002^{T} with its closest phylogenetic neighbours

Characteristic	1	2	3
Colony morphology	Smooth, yellow	Smooth, yellow–white	Cream–yellow
NaCl range (%, w/v) for growth	0–12	0–3	0–5
Motility	−	−	+
Carbon source utilization			
Adonitol	−	−	+
Arabitol	−	−	+
Dulcitol	−	+	+
Erythritol	−	+	+
Glycerol	−	+	+
Cellobiose	−	+	+
Sodium gluconate	−	+	+
Salicin	−	+	+
Sorbitol	−	−	+
Mannitol	−	+	+
Rhamnose	−	+	+
Melezitose	−	+	+
Acid production from:			
Xylose	−	+	+
Raffinose	−	−	+
Trehalose	−	−	+

L-Arabinose	−	−	+
Glycerol	−	+	−
Salicin	−	−	+
Dulcitol	−	−	+
Sorbitol	−	−	+
Adonitol	−	−	+
Arabitol	−	−	+
Rhamnose	−	−	+
Melezitose	−	+	+
Utilization of:			
Malonate	−	+	−
Rhamnose	−	−	+
Sucrose	−	+	+
Salicin	−	+	+
Sorbitol	−	−	+
Adonitol	−	−	+
Arabitol	−	−	+
Erythritol	−	+	+
Xylitol	−	+	+
Hydrolysis of:			
Aesculin	+	−	+
Enzyme activity			
Alkaline phosphate	−	+	−
Valine arylamidase	−	+	−
Naphthol-AS-BI-phosphohydrolase	−	−	+
α-Galactosidase	−	−	+
α-Glucosidase	−	−	+
β-Glucosidase	−	−	+
DNA G+C content (mol%)	66.5	70.9	67.7

Strains: 1, NIO-1002^T; 2, *M. hominis* CIP 105731^T; 3, *M. testaceum* KCTC 9103^T. All strains are positive for H_2S production, esterase (C4) and β-glucuronidase; all are negative for oxidase and nitrate reduction. Acid is produced from maltose, mannitol and cellobiose by all three strains. Xylose, cellobiose, glucose, sodium gluconate, glycerol, dulcitol, mannitol and rhamnose are utilized as sole carbon source by all three strains. +, Positive; −, negative. All tests were performed under similar laboratory conditions except DNA G+C analysis, data for which was taken from Takeuchi & Hatano (1998a, b).

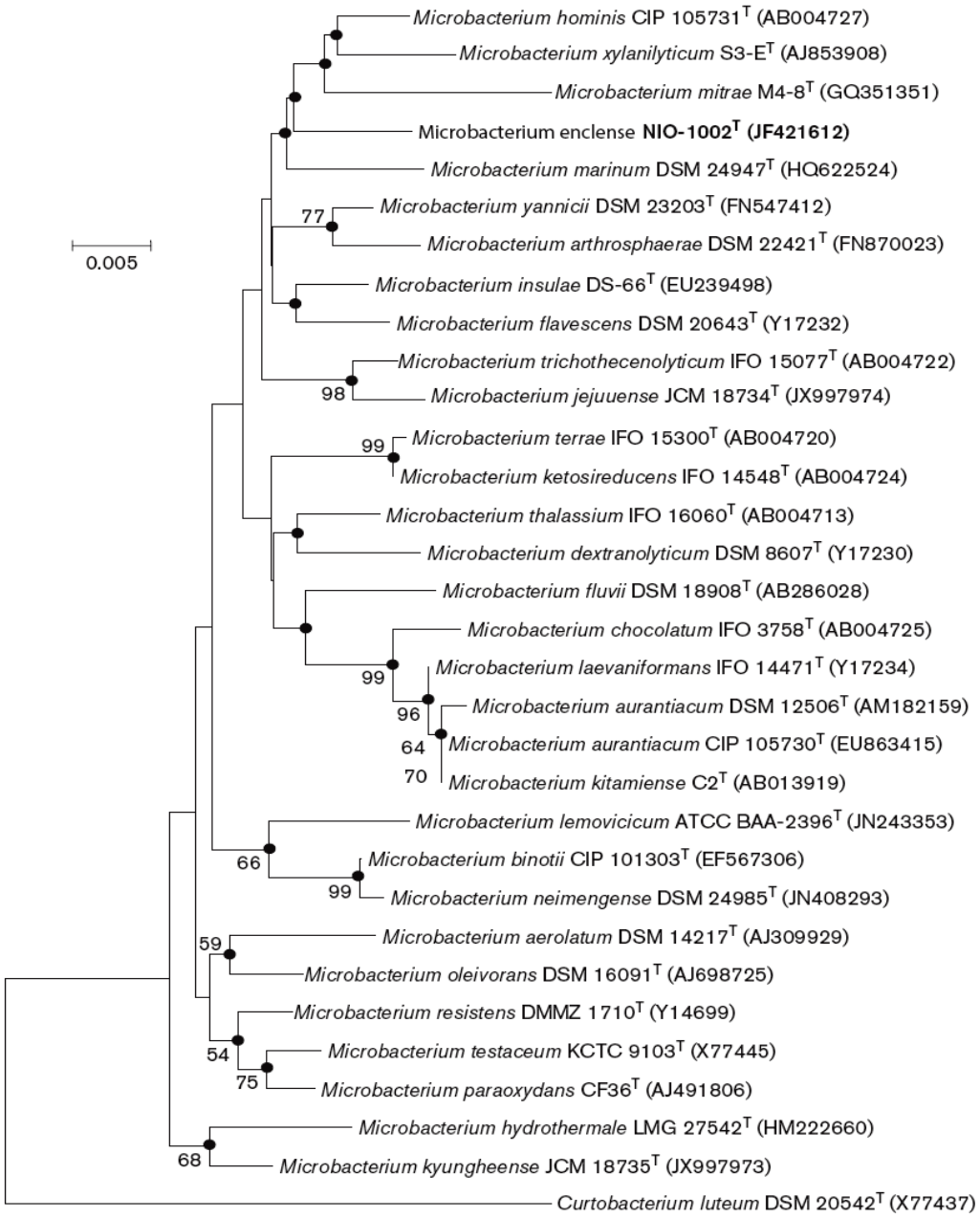


Fig.42. Phylogenetic tree based on 16S rRNA gene sequences. The position of strain NIO-1002^T is shown with respect to other species of the genus *Microbacterium*. Phylogenetic trees were generated using the neighbour-joining and maximumparsimony methods. GenBank accession numbers are shown in parentheses. Filled circles indicate generic branches that were also recovered using the neighbour-joining, minimum-evolution and maximum-parsimony algorithms. Bootstrap values (>50.0%; 1000 resamplings) are given at branch points. Bar, 0.005 % nucleotide substitution.

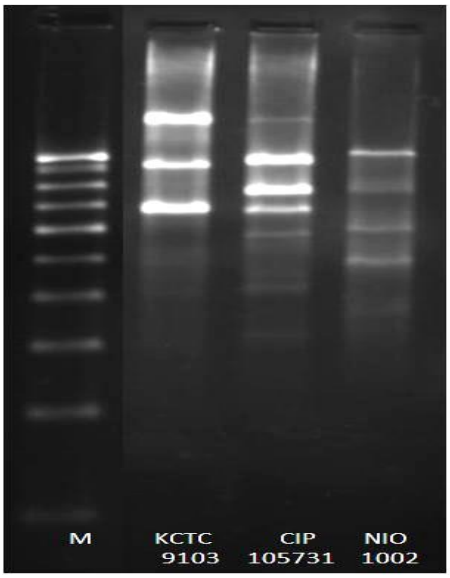


Fig.43. Arbitrary primed PCR (AP-PCR) profile showing the distinct banding pattern of strain NIO-1002^{T} with nearest type strains, which distinguishes the isolates with its closest neighbours. M- Marker 100 bp; KCTC 9103^{T}- *Microbacterium testaceum*; CIP 105731^{T}- *Microbacterium hominis*; NIO 1002^{T} *Microbacterium enclense*.

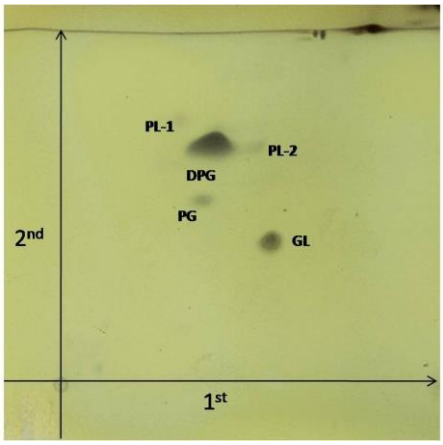


Fig.44. Two-dimensional thin-layer chromatogram of polar lipids of strain NIO-1002^{T} after staining with molybdatophosphoric acid. PG, Phosphatidylglycerol; DPG, diphosphatidylglycerol; PL-1 and PL-2, unknown phospholipid; GL, unknown glycolipid

Table 28. Fatty acid composition of NIO-1002^T and its closest species

Fatty acid	1	2	3
i-$C_{14:0}$	1.28	0.82	1.03
i-$C_{15:0}$	1.97	2.23	1.40
ai-$C_{15:0}$	34.24	35.38	34.69
i-$C_{16:0}$	30.98	25.40	23.43
$C_{16:0}$	4.26	7.80	4.47
$C_{15:0}$ 2-OH	0.62	−	−
ai-$C_{17:1}\omega 9c$	1.08	−	0.5
i-$C_{17:0}$	1.41	1.05	0.93
ai-$C_{17:0}$	23.8	25.2	30.6
$C_{17:0}$	−	0.88	−
i-$C_{18:0}$	−	0.50	−
$C_{18:0}$	−	0.74	−

Strains: 1, NIO-1002^T; 2, *M. hominis* CIP 105731^T; 3, *M. testaceum* KCTC 9103^T. −, Not detected or below 0.5 %. All data are from the present study and represent percentages of total fatty acids.

From the above observation, it was found that the strain NIO-1002 as a new member into the genus *Microbacterium*, for which *Microbacterium enclense* NIO-1002^T sp.nov., were proposed. *Microbacterium enclense* (en.clen'se. N.L. neut. adj. *enclense* arbitrary name formed from NCL, the acronym for the National Chemical Laboratory, India, where taxonomic studies on this species were performed).

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Microbacterium enclense sp. nov., isolated from sediment sample

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A novel bacterium (strain NIO-1002^T) belonging to the genus *Microbacterium* was isolated from a marine sediment sample in Chorao Island, Goa Province, India. Its morphology, physiology, biochemical features and 16S rRNA gene sequence were characterized. Cells of this strain were Gram-stain-positive, non-motile, non-spore-forming rods that formed yellow-pigmented colonies. It grew in 0–12 % (w/v) NaCl and at 25–37 °C, with optimal growth at 30 °C. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain NIO-1002^T is associated with members of the genus *Microbacterium*, with highest sequence similarity with *Microbacterium hominis* CIP 105731^T (98.1 %) and *Microbacterium testaceum* KCTC 9103^T (98.0 %). Within the phylogenetic tree, this novel strain shared a branching point with *M. hominis* CIP 105731^T. The DNA G+C content was 66.5 mol% and DNA–DNA hybridization relatedness between NIO-1002^T, *M. hominis* CIP 105731^T and *M. testaceum* KCTC 9103^T was 39.0±2.0 % and 41.0±2.0 %, respectively. The major fatty acids were ai-$C_{15:0}$, i-$C_{16:0}$ and ai-$C_{17:0}$ and the diamino acid in the cell-wall peptidoglycan of NIO-1002^T was lysine. Data obtained from DNA–DNA hybridization and chemotaxonomic phenotypic analysis support the conclusion that strain NIO-1002^T represents a novel species within the genus *Microbacterium*. The name *Microbacterium enclense* sp. nov. is proposed, with NIO-1002^T (=NCIM 5454^T=DSM 25125^T=CCTCC AB 2011120^T) as the type strain.

The genus *Microbacterium* (type species, *Microbacterium lacticum*) was proposed by Orla-Jensen (1919) for Gram-positive, non-spore-forming, rod-shaped bacteria that were isolated during studies on lactic-acid-producing bacteria. At the time of writing, the genus *Microbacterium*, belonging to the actinobacteria, contains 87 species with validly published names (Euzéby, 1997; Parte, 2014; http://www.bacterio.net/index.html), with the recent addition of *Microbacterium neimengense* (Gao *et al.*, 2013), *Microbacterium saccharophilum* (Ohta *et al.*, 2013), *Microbacterium sediminis* (Yu *et al.*, 2013), *Microbacterium hydrothermale* (Zhang *et al.*, 2014), *Microbacterium jejuense*, *Microbacterium kyungheense* (Kook *et al.*, 2014) and *Microbacterium mangrovi* (Lee *et al.*, 2014). Members of the genus *Microbacterium* are widespread and have been isolated from a broad range of habitats, including soil, sediment, aquatic samples, insect, plants, clinical specimens and food (Behrendt *et al.*, 2001; Kim *et al.*, 2008; Clermont *et al.*, 2009; Yoon *et al.*, 2009; Kim *et al.*, 2010; Zhang *et al.*, 2010; Kaur *et al.*, 2011; Kim *et al.*, 2011). In this study, we describe a novel isolate belonging to the genus *Microbacterium* recovered from a sediment sample collected from Chorao Island, Goa, India.

Strain NIO-1002^T was isolated from a marine sediment sample taken from Chorao Island (GPS coordinates 15° 32′ 34″ N 73° 55′ 15″ E) isolated on marine agar (MA) using the standard serial dilution method. The MA plates were (Hi-Media, Mumbai) incubated at 30 °C for 3–4 days. Isolated colonies were purified by repeated streaking on MA. The purified strain was maintained on MA slants at 4 °C and as glycerol suspensions (20 %, v/v) at −80 °C. Biomass for chemotaxonomy and molecular-systematic studies was obtained following growth in shake flasks (about 200 r.p.m.) of tryptic soy broth at 30 °C for 5 days. Gram staining was carried out using the standard Gram reaction and cell motility was confirmed

Abbreviations: AP-PCR, arbitrarily primed PCR

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Microbacterium enclense* NIO-1002^T is JF421612.

Three supplementary figures are available with the online Supplementary Material.

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2.5. Summary

Chorao island, Goa, India is situated at the confluence of Mandovi river and Mapusa river and it has a well known Salim Ali bird santuary. Chorao is an esturian island that is divided by creeks and backwaters with tidal variations (D'Souza, 2015). The marine sediment sample was taken from the intertidal region of mangroves of this island. It was then washed with sterile saline, filtered and serially diluted to spread on marine agar. After spreading the different isolates were picked and subcultured on a fresh marine agar plate in order to isolate and purify colonies. These bacteria were then initially identified based on the 16S rRNA gene sequencing method. Their phylogenetic positions were determined by using neighbour-joining method. Depending on the 16S rRNA gene pecentage similarity and phylogenetic positions, candidate strains were further characterized using polyphasic approach.

Polyphasic systematics includes phylogenetic, phenotypic, genotypic and chemotaxonomic methods. Phylogenetic characterization was done using 16S rRNA gene sequencing and phylogenetic analysis. These methods assign the position of a strain with its closest type strains Phylogenetic characterization assigns the position of a strain with its closest type strains. Later on, phenotypic characterization likes morphological, physiological and biochemical characteristics were done according to standard methods. Genotypic characterization was done using DNA-DNA hybridization, genomic DNA G+C content and arbitrarily primed-PCR. DNA-DNA hybridization method gaves a value that differentiate one strain to its closest strain on the basis of their genomic reletedness. The AP-PCR generates genomic fingerprinting pattern of a strain along with its closest type strains. The differences in fingerprinting pattern between two strains suggested that they are of different species. Chemotaxonomic characterization includes the study of different chemical markers present in cell from which one can identify bacteria upto genus level. In this study, we characterized

cellular peptidoglycan, cell wall sugars, polar lipids, fatty acids methyl esters and menaquinone content of a strain.

The overall data from the above methods were examined for an isolates and further documented. By using polyphasic systematics, we identified and reported ten novel species from a sediment sample.

CHAPTER-III

SCREENING, ISOLATION AND IDENTIFICATION OF ALPHA-GLUCOSIDASE INHIBITOR FROM MARINE BACTERIA

3.1. Introduction

Alpha-glucosidase is an enzyme that catalyzes the conversion of substrate like starch and different oligosaccharides into monosaccharide residual units. These enzymes cleave the α-1,4 linkage of complex sugars molecules (Melo et al., 2006). Inhibition of α-glucosidase leads to delay in the liberation of glucose from meal thus decreases the post-prandial blood glucose levels. This demonstrated that α-glucosidase inhibitors deliver as a therapeutic agent in the treatment of diabetes and obesity (Alagesan et al., 2012; Wang et al., 2012).

Alpha-glucosidase inhibitors are naturally produced by many organisms. Several α-glucosidase inhibitors have been identified till date, but some of them been used in the treatment of diabetes mellitus. Acarbose is the first discovered α-glucosidase inhibitor consists of an unsaturated cyclitol (valienamine), 4-amino-4,6-dideoxyglucose and a maltose unit linked via an N-glycosidic bond (Mahmud et al., 1999). Acarbose is produced by an industrially developed strain called *Actinoplanes* sp. SE50/110. Voglibose and Miglitol as a alpha-glucosidase inhibitors were obtained from *Streptomyces hygroscopicus* var. *limoneus* and synthetically from 1-deoxynojirimycin, respectively (Kalra, 2014). This inhibitor inhibits the α-glucosidase enzyme in a reversible manner. They have taken with the first bite of meal either alone or in a combination of other therapeutic drugs.

Actinomycetes are known to produce natural bioactive compounds with an application in agricultural, industrial and pharmaceuticals. Screenings of terrestrial actinomycetes have been extensively researched from years in search of bioactive compounds like antibiotics, enzyme inhibitors and antitumour agents etc. (Manivasagan et al., 2014; Lestari et al., 2015). The marine actinomycetes have drawn a special attention in recent years as they produce unique bioactive compounds. These organisms produce secondary metabolites used in the treatment of various diseases. Most of the bioactive compounds

produced by actinomycetes are belongs to class of cyclitol containing aminoglycoside compounds such as neomycin, kanamycin, gabosines, validamycin, pyralomycines and acarbose etc. (Wu et al., 2007). This C_7N aminocyclitol compound shares a unique structure that includes an unsaturated aminocyclitol moiety. It was proposed that the formation of these compounds share a common precursor from which the pathway gets diversed. This precursor is *sedo*-heptulose 7-phosphate, which is derived from the pentose phosphate pathway (Asamizu et al., 2012). The *sedo*-heptulose 7-phosphate is converted to the 2-epi-5-epi valiolone from which C_7N aminocyclitols and their keto analogues derivatives are produced and is converted by *sedo*-heptulose 7-phosphate cyclase (Zhang et al., 2002).

In the present study, we attempted to corelate PCR based functional gene screening method for the detection of *sedo*-heptulose 7-phosphate cyclase gene among marine bacteria and this gene is known to produce C_7N aminocyclitol and their keto analogues such as acarbose and like compounds. Those organisms which showed the presence of *sedo*-heptulose 7-phosphate cyclase gene were further subjected for in detailed study in a fermentation medium to evaluate the α-glucosidase inhibitors potentials. The crude supernatant from the fermentation broth was used for the α-glucosidase inhibition assay. Further confirmation was done using TLC method for the detection of acarbose and their related products from the crude supernatant.

3.2. Screening for the presence of *sedo*-heptulose 7-phosphate cyclase gene:-

The *sedo*-heptulose 7-phosphate cyclase gene is involved in the synthesis of C_7N aminocyclitol and their keto-analogue containing derivatives including a well known inhibitor i.e. acarbose. In this method, degenerated primers were used for the amplification of the desired gene. The primer was designed on the basis of the known sequence of *sedo*-

heptulose 7-phosphate cyclase gene as well as several 3-hydroquinate synthetase genes (Hyun et al., 2005).

The sequence of primers used in this study was VOG-F 5'-(GGSGGSGGSGTSCTSATGGACGTSGCSGG)-3' and VOG-R 5'-(GCCATGTCSACGCASACSGCSGCCTCSCCGAG)-3'. These primers were employed in the amplification of DNA extracted from marine bacteria isolated from marine sediment of Chorao Island, Goa and some of the standard strains of actinomycetes were procured from NCIM resource centre of CSIR-National Chemical Laboratory, Pune. *Actinoplanes* sp. KCTC 9162^{T} was used as a reference strain for screening, as it is known to harbour the same gene and known to produce an acarbose. The DNA of isolates was extracted by the modified cetyl trimethyl ammonium bromide (CTAB) method as described by Wilson, K. (2001). The protocol of DNA isolation by CTAB method is as follow: inoculate a loopful of culture in 564.0 µl of 1X TE buffer. Add 3.0 µl of lysozyme (100 mg/ml) and incubate it at 37 ºC for 2 hrs. Then add 3.0 µl proteinase K of 20.0 mg/ml and 30.0 µl of 10.0% sodium dodecyl sulfate (SDS) and incubate it at 37 ºC for 2 hrs. Add 100 µl of 5M NaCl and mixed thoroughly and incubate at 65 ºC for 2 min. Add 80.0 µl of CTAB/NaCl solution,mix thoroughly and incuabte at 65 ºC for 10 min. Extarct suspension with an equal volume of chloroform: isoamyl alcohol (24:1,v/v) and spin 5 min at 10000 rpm. Remove aqueous upper phase into new tube and extract with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1, v/v). Centrifuge it at 13000 rpm for 5 min. Transfer the supernatant to a fresh tube. Add equal volume of chloroform: isoamyl alcohol (24: 1, v/v) and centrifuge at 10000 rpm for 5 min. Transfer the upper aqueous phase into a new tube. Add $1/10^{th}$ volume of 3M sodium acetate and then add 0.7 volume of chilled isopropanol to precipitate the nucleic acid. Incubate it at -80 ºC for 1 hrs. Remove and kept at room temperature. Centrifuge at 12000 rpm for 30 min. Remov isopropanol carefully to avoid disturbing the pellet. Add 500 µl of 70.0% chilled

ethanol and mix thoroughly. Centrifuge it at 12000 rpm for 30 min. Carefully remove the supernatant and dry teh pellet in vaccum evaporator. Resuspend the pellet in 50 µl of TE buffer and store the DNA at -20 ºC.

The PCR mixture used for the amplification contained 10X PCR buffer, 15mM $MgCl_2$, and 10mM dNTPs, 10 pmol of each primer and 3 U/µl of Taq polymerase. The amplification reaction was conducted using a thermal cycler with fllowing conditions; 5 min initial denaturation at 94 ºC followed by 30 cycles of 20 sec of denaturation at 98 ºC, 1 min of primer annealing at 67 ºC and 1 min of extension at 72 ºC and a final extension of 10 min at 72 ºC. The resultant PCR products were visualized on 1.0% agarose gel (Fig. 45; 46).

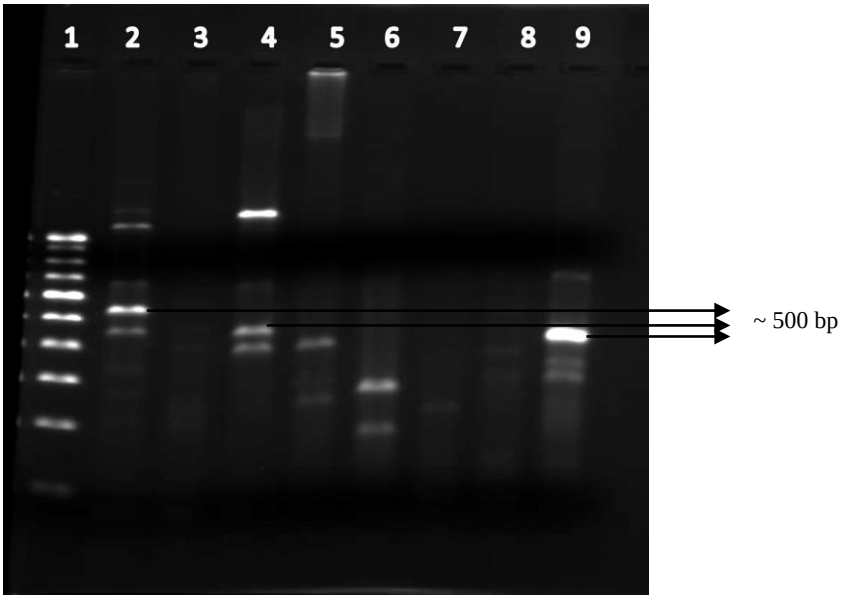


Fig.45. 1% agarose gel electrophoresis of VOG PCR products. The amplicon size was found to be ~500bp. lane 1: 100bp ladder, lane 2: *Arthrobacter enclensis* NIO-1008^T, lane 3: *Glutamicibacter* sp. NIO-1057, lane 4: *Deinococcus enclensis* NIO-1023^T, lane 5: *Streptomyces griseus* NCIM2621, lane 6: *Streptomyces lavendulae* NCIM 2498, lane 7: *Streptomyces lavendulae* NCIM 2499, lane 8: *Actinoplanes missouriensis* NCIM 2838 and lane 9: *Actinoplanes* sp. KCTC 9162^T.

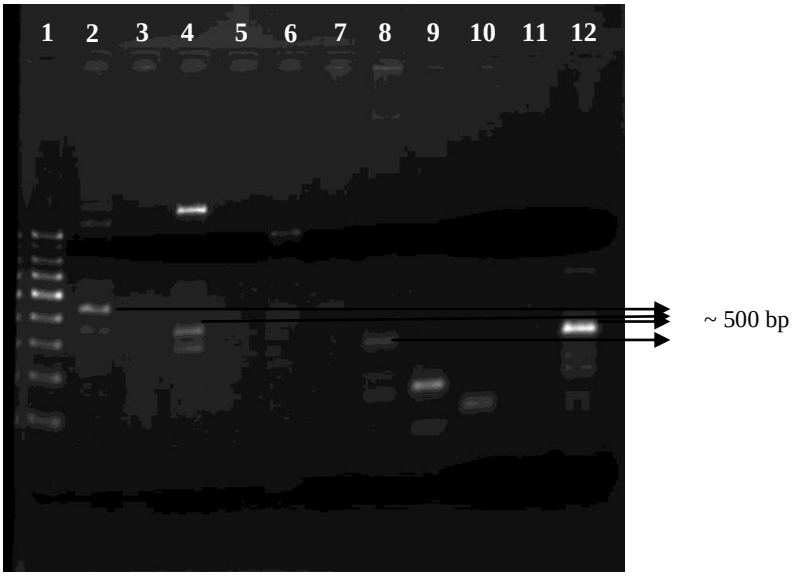


Fig. 46. 1% agarose gel electrophoresis of VOG PCR products. Lane 1: 100 bp ladder; lane 2: *Arthrobacter enclensis* NIO-1008^T; lane 3: *Rhodococcus enclensis* NIO-1009^T; lane 4: *Deinococcus enclensis* NIO-1023^T; lane 5: *Bacillus enclensis* SGD-1123^T; lane 6: *Pseudomonas* sp. NIO-1051; lane 7: *Bacillus cellualsensis* NIO-1130^T; lane 8: *Cohnella* sp. NIO-V-74; lane 9: *Gordonia* sp. NIO-V-85; lane 10: *Streptomyces lavendulae* NCIM 2499; lane 11: *Actinoplanes missouriensis* NCIM 2838 and lane 12: *Actinoplanes* sp. KCTC 9162^T.

The PCR-based screening method was proved to be capable of detecting the presence of the *sedo*-heptulose 7-phosphate cyclase gene involved in the biosynthesis of C_7N aminocyclitol or its keto analogue-containing metabolites. The size of the amplified fragment was found to be ~ 500 bp for *Actinoplanes* sp. KCTC 9162^T. The PCR primers designed by Huyn et al. (2005) for the screening of the C_7N aminocyclitol or its keto analogue-containing metabolites were based on the comparison of the known sequences of *sedo*-heptulose 7-phosphate cyclase (acbC) from the *Actinoplanes* sp. SE50/100, and other several 3-hydroquinate synthetases which were exhibited a higher degree of similarity with the AcbC protein. Therefore, PCR primers for the amplification of the *sedo*-heptulose 7-phosphate cyclase gene were prepared

from amino acid consensus sequences within the known *sedo*-heptulose 7-phosphate cyclase, as well as several 3-hydroquinate synthetases (Mahmud et al. 2001; Chen et al. 2003; Mahmud, 2003).

From the above study, it was observed that *Actinoplanes* sp. KCTC 9162^T harbours the *sedo*-heptulose 7-phosphate cyclase gene as it is known to produce acarbose. The amplified product size was found to be ~500 bp in size, which was also detected in other bacteria as well with an approximate size of 500 bp in length. The following marine isolates showed the presence of amplified product as compared to reference strain *Actinoplanes* sp. KCTC 9162^T, namely *Arthrobacter enclensis* NIO-1008^T, *Glutamicibacter* sp. NIO-1057, *Deinococcus enclensis* NIO-1023^T, *Cohnella* sp. NIO-V-74 and some other actinomycetes of NCIM resource centre like *Streptomyces griseus* NCIM 2621, *Streptomyces lavendulae* NCIM 2498, *Streptomyces lavendulae* NCIM 2499 and *Actinoplanes missouriensis* NCIM 2838. Apart from 500 bp product, some additional amplicon products were also observed in gel electrophoresis indicates that, the primers were used in this study was a degenerate primer and hence some additional products were also produced along with targeted one. Therefore, further analysis for inhibitor was carried by fermentation, HPLC, FTIR and LC-MS methods.

3.3 Fermentation and alpha-glucosidase inhibition

Those bacteria which showed the presence of *sedo*-heptulose 7-phosphate cyclase gene was further cultivated in fermentation medium along with other isolates as well to check whether inhibitor production is there or not. The inoculum medium was composed of (g/L): glucose 15.0g; peptone 7.5.0g; KH_2PO_4 1.0g; NaCl 5.0g and pH 7.0. The cultures were inoculated in inoculum medium for 48 hrs at 28 ºC on a rotary shaker with 150 rpm. Later on, 10.0% of inoculum was inoculated in the fermentation medium. The composition of fermentation medium is: (g/L) glucose-30.0; maltose-15.0; peptone-5.0; monosodium glutamate-3.0;

$K_2HPO_4.3H_2O$-1.0; $MgSO_4.7H_2O$-1.0; $FeSO_4.7H_2O$-0.02 and $NaNO_3$-3.0 and pH were adjusted to 7.2 with 1N NaOH before autoclaving. The fermentation was carried out at 28 ºC for 7 days on a rotary shaker with 150 rpm. After fermentation, the crude supernatant was collected from the fermentation broth by centrifugation at 10000 rpm for 10 min and filtration through whatmann filter paper 1.

The alpha-glucosidase inhibition assay was carried out at a 96 well microtiter plate based spectrometry (Thermo Multiskan EX). A volume of 50µl sample and 100µl of the enzyme (from *Saccharomyces cerevisiae*, Sigma Aldrich) solution (1U/ml) prepared in 0.1M sodium phosphate buffer of pH 6.9, were incubated in microtiter plates for 10 min at 25 ºC. After incubation, 50 µl of para-nitrophenyl alpha-D-glucopyranoside (pNPG) solution prepared in 0.1M sodium phosphate buffer of pH 6.9, was added in each well and further incubated for 5 min at 25 ºC. The absorbance was recorded at 405 nm by using a microtiter plate reader. The absorbance of samples was compared with the absorbance of control, which contains 50 µl of 0.1M phosphate buffer of pH 6.9 in place of sample. The enzyme inhibition was expressed as % inhibition using the following equation.

$$\% \text{ Inhibition} = \frac{[A_{405}\text{Control} - A_{405}\text{Sample}]}{A_{405}\text{Control}} \times 100$$

Inhibition assay of α-glucosidase enzyme were performed in triplicates and results are shown in Table 29. All values are represented as Mean ± Standard Deviation.

Table 29. Strains showing *sedo*-heptulose 7-phosphate cyclase gene product and α-glucosidase inhibition

Sl no.	Strain	*sedo*-heptulose 7-phosphate cyclase gene product	Alpha-glucosidase inhibition (%)
1	*Actinoplanes* sp. KCTC 9162^T	+	64.95
2	*Arthrobacter enlensis* NIO-1008^T	+	75.42
3	*Glutamicibacter* sp. NIO-1057	-	21.25
4	*Deinococcus enclensis* NIO-1023^T	+	73.36
5	*Bacillus* sp. NIO-1027	-	7.38
6	*Bacillus enclensis* SGD-1123^T	-	7.45
7	*Bacillus cellulasensis* NIO-1130^T	-	8.39
8	*Kocuria indica* NIO-1021^T	-	8.54
9	*Rhodococcus enclensis* NIO-1009^T	-	15.36
10	*Cohnella* sp. NIO-V-74	+	24.76
11	*Gordonia* sp. NIO-V-85	+	26.31
12	*Bacillus* sp. NIO-V-19	-	5.42
13	*Bacillus encimensis* NIO-V-25^T	-	6.35
14	*Bacillus* sp. NIO-V93	-	11.94
15	*Bacillus* sp. NIO-V-88	-	5.35
16	*Exiguobacterium enclense* NIO-1109^T	-	9.87
17	*Microbacterium enclense* NIO-1002^T	-	16.35
18	*Fictibacillus enclensis* NIO-1003^T	-	11.21
19	*Bacillus filamentosus* NIO-SGD-14^T	-	8.43
20	*Domibacillus enclensis* NIO-1016^T	-	6.91
21	*Streptomyces lavendulae* NCIM 2498	+	75.8
22	*Streptomyces griseus* NCIM 2621	+	53.20
23	*Streptomyces lavendulae* NCIM 2499	+	29.33
24	*Streptomyces aureofaciens*	-	16.45

	NCIM 2616		
25	*Actinoplanes missouriensis* NCIM 2838	+	65.13
26	*Streptomyces albus* NCIM 2413	-	15.68
27	*Streptomyces aureofaciens* NCIM 2615	-	12.12
28	*Streptomyces olivaceus* NCIM 2503	-	8.65
29	*Streptomyces viridifaciens* NCIM 2506	-	11.23
30	*Streptomyces* sp. NCIM 2631	-	14.76
31	*Streptomyces thermonitrificans* NCIM 2007	-	6.41
32	*Streptomyces fradiae* NCIM 2618	-	9.52
33	*Streptomyces lividans* NCIM 2917	-	5.32
34	*Streptomyces olivaceus*c NCIM 2212	-	12.41
35	*Streptomyces lividans* NCIM 5018	-	9.24
36	*Streptomyces griseus* NCIM 2622	-	17.25
37	*Streptomyces griseus* NCIM 2626	-	18.36
38	*Streptomyces griseus* NCIM 2952	-	16.51
39	*Streptomyces* sp. NCIM 5205	-	11.01
40	*Streptomyces hawiiensis* NCIM 2627	-	12.44
41	*Streptomyces* sp. NCIM 5027	-	8.91
42	*Streptomyces albus* NCIM 2731	-	9.64
43	*Streptomyces* sp. NCIM 2727	-	13.58
44	*Streptomyces aureofaciens* NCIM 2417	-	9.61
45	*Streptomyces diastaticus* NCIM 2115	-	7.41
46	*Streptomyces griseus* NCIM 2020	-	17.58
47	*Streptomyces griseus* NCIM 2952	-	20.21
48	*Streptomyces lividans*	-	8.51

	NCIM 2916		
49	*Strepyomyces lividans* NCIM2964	-	11.21
50	*Streptomyces niveus* NCIM 2502	-	8.69
51	*Streptomyces rimosus* NCIM 2213	-	13.26
52	*Streptomyces hygroscopicus* NCIM 2959	-	26.57
53	*Streptomyces* sp. NCIM 2081	-	8.65
54	*Streptomyces* sp. NCIM 2728	-	9.74

'+', presence of gene *sedo*-heptulose 7-phosphate cyclase; '-', absence of gene *sedo*-heptulose 7-phosphate cyclase

Initial screening of alpha-glucosidase inhibition was carried out for the marine isolates along with *Streptomyces* species from NCIM resource centre, Pune, India. The inhibition study was performed by using crude supernatant from the fermentation broth and expressed as a percent (%) inhibition. From the above results, it was concluded that bacteria isolated from marine sediments have the potentials to inhibit the α-glucosidase enzyme. Two newly identified marine bacteria which showed very good inhibition activity namely *Arthrobacter enclensis* NIO-1008^T (Dastager et al., 2014) and *Deinococcus enclensis* NIO-1023^T, and also showed the presence of *sedo*-heptulose 7-phosphate cyclase gene involved in the synthesis of C_7N aminocyclitol like compounds (e.g. acarbose) in PCR analysis. Standard acarbose was used as a positive control for the assay. The 75.42 ±1.3% and 73.26 ±1.8% inhibition is in respect with two potential strains is in comparison with the standard inhibitor i.e. acarbose (91.11±1.1 %) and also with standard reference strain *Actinoplanes* sp. KCTC 9162^T (64.95±1.5 %) was observed respectively.

Apart from this, *Cohnella* sp. NIO-V-74 and *Gordonia* sp. NIO-V-85 also showed positive for PCR analysis but the inhibition towards α-glucosidase was found to be very less 24.76 ±1.4 % and 26.31 ±1.5 % respectively, which was low as compared to *A. enclensis* NIO-1008^T and *D. enclensis* NIO-1023^T. Some *Streptomyces* sp. was also used for the inhibition assay because they are known to produce large number of bioactive compounds. Some of the *Streptomyces* sp. was found to be positive for PCR reaction and also showed good inhibition activity namely *Streptomyces lavendulae* NCIM 2498, *Streptomyces griseus* NCIM 2621 and *Actinoplanes missouriensis* NCIM 2838 showed 75.8 ± 1.4%, 53.20 ±1.7% and 65.13 ±1.45% inhibitions, respectively as a reference strains. Moreover, *Actinoplanes* sp. KCTC 9162^T which was known to produce acarbose an α-glucosidase inhibitor was used as a standard reference strain. From the above study, it was observed that *A.enclensis* NIO-1008^T and *D.enclensis* NIO-1023^T showed good inhibition activity and also positive for VOG PCR reaction. Further, we analyzed these two strains for C_7N aminocyclitol like compounds using acarbose as a reference with the help of thin layer chromatography (TLC) analysis as mentioned below.

3.4. Thin Layer Chromatography for the detection of acarbose and their related products

A simple TLC technique for the detection of acarbose was developed by Fei Ren et al. (2014). In this method crude supernatant (approximately 5 µl) was spotted on 10 x10 cm Silica gel 60 F_{254} plates. The mobile phase was n-propanol: water (8:2). Acetone containing 10.0% (v/v) phosphoric acid, 2.0% (v/v) aniline and 2.0% (w/v) diphenylamine was used as colour developer. Acarbose on the TLC plate was visualized by the colour developer through a fine spray, followed by heating at 110 ºC for 10 min (Ren et al., 2014). Standard acarbose was used as a positive control (1 mg/ml conc.).

In this study, the crude supernatant after the fermentation was applied on a silica TLC plate for the detection of acarbose and carbohydrate derivatives (Fig. 47). In order to achieve much better separation, the plate was run for two-times with the developing solvent.

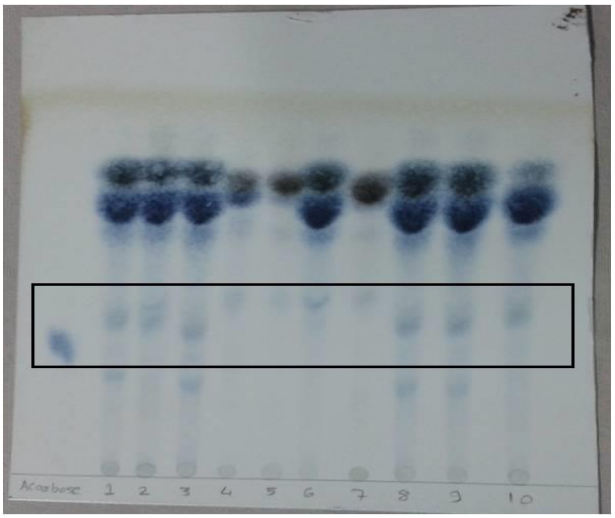


Fig.47. Thin-layer Chromatography of acarbose and Crude supernatant of 1-10: lane 1: *A.enclensis* NIO-1008^{T}, lane 2: *Glutamicibacter* sp. NIO-1057, lane 3: *D.enclensis* NIO-1023^{T}, lane 4: *Cohnella* sp. NIO-V-74, Lane 5: *Gordonia* sp. NIO-V-85, lane 6: *Actinoplanes* sp. KCTC 9162^{T}, lane 7: *Streptomyces griseus* NCIM 2621, lane 8: *Streptomyces lavendulae* NCIM 2498, lane 9: *Streptomyces lavendulae* NCIM 2499 and lane 10: *Actinoplanes missouriensis* NCIM 2838.

From the TLC analysis, it was observed that the bacteria which showed the positive reaction of PCR and good inhibition activity also showed the presence of acarbose like compounds from in its crude supernatant. Further, *A.enclensis* NIO-1008^{T} and *D.enclensis* NIO-1023^{T} were selected for detailed study. The α-glucosidase inhibition and TLC (Fig. 48) were reconfirmed for both the strains.

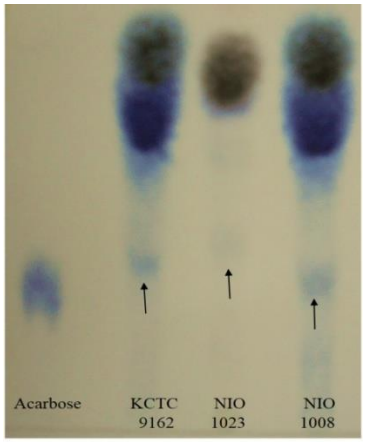


Fig.48. The TLC chromatogram of standard acarbose and crude supernatant of *Actinoplanes* sp. KCTC 9162^{T}, *D. enclensis* NIO- 1023^{T} and *A. enclensis* NIO-1008^{T}.

3.5. Sub-cloning of VOG primer PCR products

The PCR products obtained from the *sedo*-heptulose 7-phosphate cyclase gene by PCR screening method was further used for sub-cloning and sequencing. The PCR products of *A. enclensis* NIO-1008^{T}, *D. enclensis* NIO-1023^{T} and *Actinoplanes* sp. KCTC 9162^{T} were used for the cloning procedure. The PCR products of ~ 500 bp sizes from each organism were extracted and purified using the HiPer Gel extraction kit (HiMedia, Mumbai) and then run on 1.0% agarose gel (Fig. 49). The resulting PCR products were sub-cloned into a pGEM-T vector (Promega) and later transformed into *E. coli* JM109 competent cells according to the method by Mass (1999).

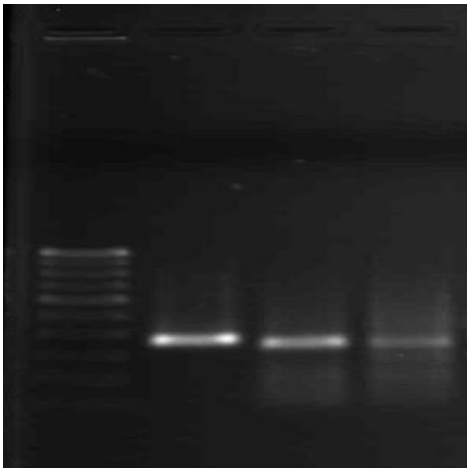


Fig.49. The 1% agarose gel electrophoresis of extracted PCR products of sedo-heptulose 7-phosphate cyclase gene. Lane 1: 100 bp marker; lane 2: *A. enclensis* 1008^{T}; lane 3: *D. enclensis* 1023^{T} and lane 4 *Actinoplanes* sp. KCTC 9162^{T}.

The plasmid DNA which encodes the clones were then isolated and purified with a HiPurA Plasmid purification kit (HiMedia, Mumbai). The resultant products were then sequenced using ABI xL Sequencer. The resultant sequences were then compared with the non-redundant sequence using a BLASTx search [www.blast.ncbi.nlm.nih.gov.com] (Table 30).

Table 30. Sequence similarity of sub-cloned products of *sedo*-heptulose 7-phosphate cyclase PCR method using BLASTx.

Sl.no.	**Similarity hit (BLASTx)**	**% similarity**
A. enclensis **NIO-1008^{T}**	Phospoenol pyruvate synthase	93%
D. enclensis **NIO-1023^{T}**	Aminoglycoside phopshotransferase	100%
Actinoplanes **sp. KCTC 9162^{T}**	2-epi-5epi-valiolone synthase [*sedo*-heptulose 7-phosphate cyclase]	100%

It was observed that the sequences obtained from sub-cloning were compared using BLASTx. *A. enclensis* NIO-1008^T showed the sequence similarity with phosphoenol pyruvate synthase while *D. enclensis* NIO-1023^T showed the sequence similarity with aminoglycoside phosphotransferase. *Actinoplanes* sp. KCTC 9162^T on the other hand showed similarity with *sedo*-heptulose 7-phosphate cyclase as it is known to produce acarbose. This suggests that the primers used for the detection of *sedo*-heptulose 7-phosphate cyclase was degenerate and amplify the other sequences as well. By looking towards sequence similarity of *Actinoplanes* it exactly matches with specific gene sequence. *A. enclensis* NIO-1008^T and *D. enclensis* NIO-1023^T PCR products were found to match with other enzymes apart from *sedo*-heptulose 7-phosphate cyclase. But TLC chromatogram showed that both bacteria were producing acarbose like compound which was not confirmed with the presence of *sedo*-heptulose 7-phosphate cyclase gene. It was observed that two newly identified bacteria from the marine sediment sample showed the inhibition towards alpha-glucosidase enzyme which was not reported previously from any species of *Arthrobacter* and *Deinococcus* genus and were used further for isolation and identification for the alpha-glucosidase inhibitor.

3.6. Microorganisms and fermentation

Arthrobacter enclensis NIO-1008^T and *Deinococcus enclensis* NIO-1023^T were used in this study. Both bacteria were stored at -80 ºC in 20% (v/v) sterile glycerol until further use. Prior to the study, *A.enclensis* NIO-1008^T was activated on the nutrient agar at 30 ºC for about 24 hrs and *D.enclensis* NIO-1023^T was activated on R2A agar plates at 30 ºC for about 24 hrs.

The seed culture was prepared by shaking at 30 ºC for 24 hrs in a medium consisting of (g/L): glucose 15.0; peptone 7.5; $K_2HPO_4.3H_2O$ 1.0 and NaCl 5.0. The seed culture was then transferred to the fermentation medium consisting of (g/L): glucose-30.0; maltose-15.0; peptone-5.0; monosodium glutamate-3.0; $K_2HPO_4.3H_2O$-1.0; $MgSO_4.7H_2O$-

1.0; $FeSO_4.7H_2O$-0.02 and $NaNO_3$-3.0. The fermentation was carried out in shaking condition at 30 ºC for 168 hrs at 150 rpm.

3.7. Purification of alpha-glucosidase inhibitor

The culture broth was centrifuged at 8000 rpm for 10 min. The filtrate was then incubated with *Saccharomyces cerevisiae* beads for 24 hrs at 37 ºC. The *S. cerevisiae* beads were prepared according to Yoon et al. (2003). The *S. cerevisiae* beads were removed by centrifugation at 6000 rpm for 10 min and then mixed with an equal volume of absolute cold ethanol and kept for 30 min to remove any soluble dextran. The mixture was centrifuged again and filtered to remove the precipitated material.

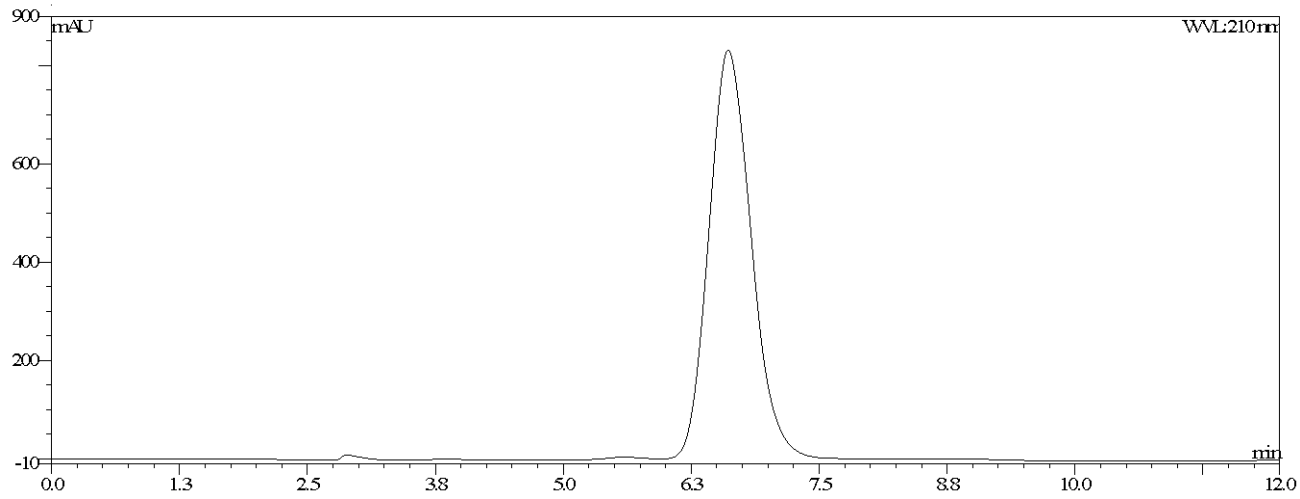
The water fraction was then subjected to the gel permeation chromatography. The fraction was loaded on Biogel P-2 (extra fine) column (1.5 x 90 cm) and eluted with sterile distilled water at a flow rate of 0.1 ml/min, to collecting 10 ml fractions. The fractions which showed the inhibitory activity against α-glucosidase were pooled together and concentrated for further analysis.

3.8. High Performance Liquid Chromatography (HPLC) analyses

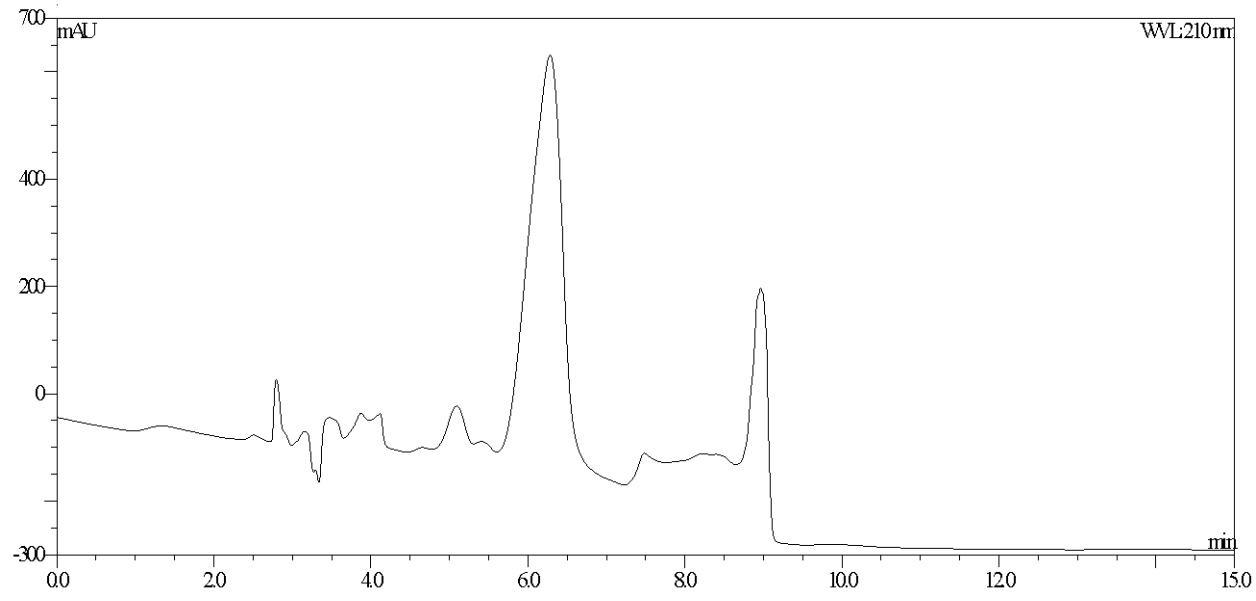
The fractions collected from Biogel P-2 chromatography were further analyzed by HPLC (Fig. 50) along with standard acarbose. The samples were injected into HPLC system (Thermo, UHPLC Ultimate 3000); carbohydrate column (Waters) 3.9 x 300 mm, 10 µl of sample injection volume was employed for HPLC analysis with a flow rate of 1.0 ml/min and a wavelength at 210 nm. The mobile phase used was acetonitrile: phosphate buffer (0.6g of KH_2PO_4 and 0.48 g of Na_2HPO_4) with a ratio of 70:30, v/v.

Fig.50. HPLC chromatogram of (A) acarbose; (B) purified compound of *A.enclensis* NIO-1008^{T} ; (C) purified compound of *D.enclensis* NIO-1023^{T} ; and (D) unfermented broth.

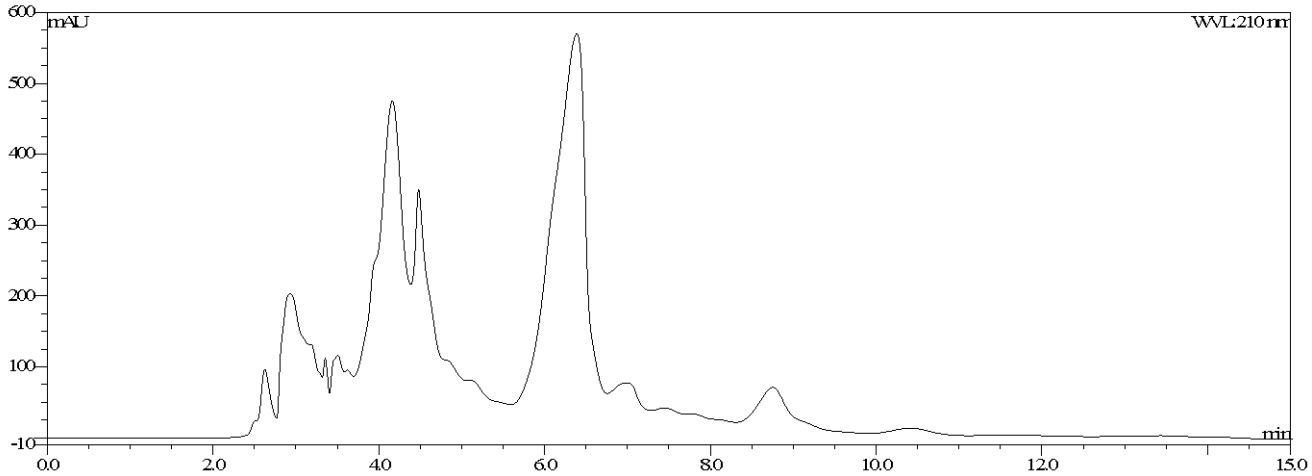
A]



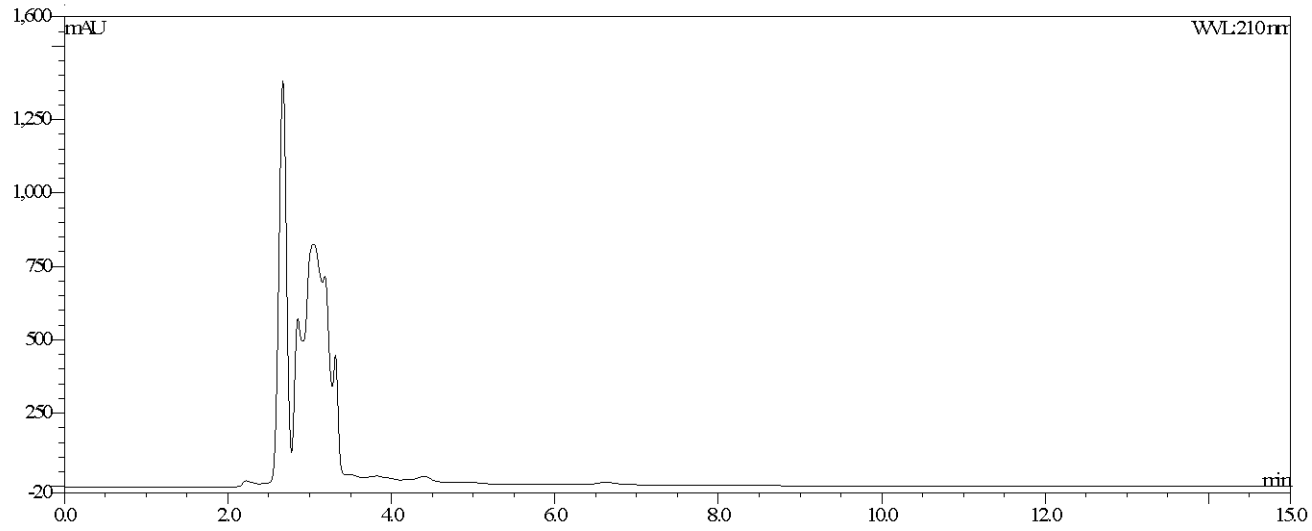
B]



C]



D]



It was observed from the HPLC analysis that the standard acarbose showed a peak at a retention time of 6.61 min. The unfermented broth shows peaks mostly at ranging 2.0- 4.4 min, while purified compounds from both bacteria showed additional peaks at different retention times apart from 2.0- 4.4 min, suggesting that some new peaks appeared which were not observed in unfermented broth. The purified compound of *A.enclensis* NIO-1008^{T},

D.enclensis NIO-1023^{T} showed a peak at retention time 6.28 min and 6.39 min respectively. Later on, further analyses were done for the purified compounds.

3.9. Characterization of the α-glucosidase inhibitor

The isolated compound was identified using FTIR spectrometry, and by tandem mass spectrometry in comparison with standard acarbose. The FTIR was investigated on BRUKER- Tensor37 system with a wavelength ranging from 4000-500 cm^{-1}. The system was controlled by OPUS v.6.5 software. Mass detection (MS) and tandem mass spectrometry (MS/MS) spectra were performed on a Thermo Q-Exactive Orbitrap mass spectrometer, equipped with an ESI source and an ion trap mass analyzer. The mobile phase methanol: water (90:10) with 0.1% formic acid was used at a flow rate of 0.350ml/min. The injection volume of 1.5µl was used for positive mode ionization. The mass range of m/z is from 100 to 1500. For MS/MS analysis the collision energy was set at 35V and system software was X-calibur v.3.0.

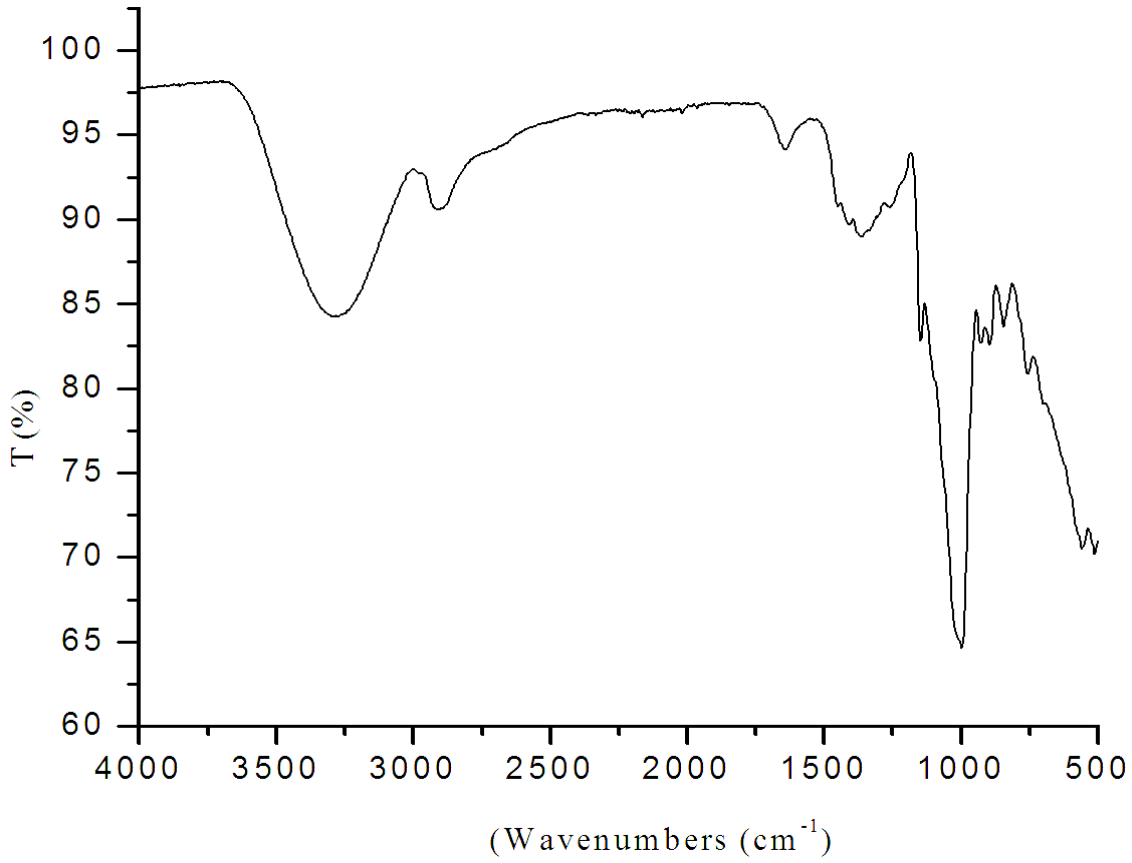
3.9.1. FTIR analyses

The IR spectra of standard acarbose and purified compound of *A.enclensis* NIO-1008^{T} and *D.enclensis* NIO-1023^{T} is shown in (Fig. 51). The IR spectrum of acarbose displayed strong OH stretching (optimal at 3290 cm-1) at wide peak ranging from 3000-3800 cm^{-1}. The C=C weak stretching has appeared at a wide range of 1420-1650 cm^{-1}. The medium C-H bending and C-O stretching was displayed at 1364 and 1034 cm^{-1}, respectively. The IR spectrum of a purified compound of *A. enclensis* NIO-1008^{T} showed a strong OH stretching at 3272 cm^{-1}. The C-H stretching appeared at 2925 cm^{-1}, C=C stretching at 1639 cm^{-1} and C-H bending at 1359 cm^{-1} ranges of a peak. The C-O stretching was displayed at 1012-1144 cm^{-1}. The IR spectrum of the purified compound of *D. enclensis* NIO-1023^{T} showed a strong OH stretching at 3270 cm^{-1}. Weak C-H stretching appeared at 2926 cm^{-1}, C=C stretching at 1558-

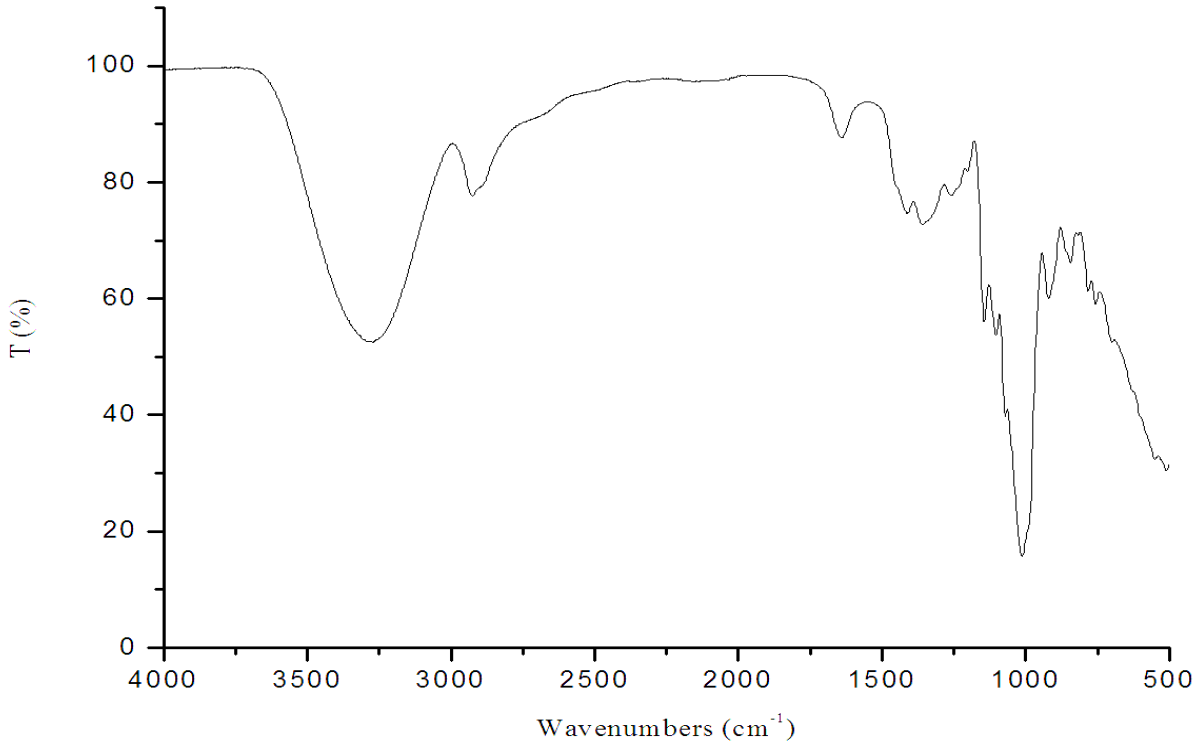
1653 cm^{-1}, C-H bending at 1457 cm^{-1} and C-O stretching at 1013-1016 cm^{-1}. From the above, it was found that most of the groups displayed correspond to the oligosaccharides. Acarbose is a pseudo-oligosaccharide compound, which contains N-H amide linkage, which usually ranges from 3360 to 3310 cm^{-1}, and in case of acarbose, it is occupied by a wide range of OH groups (Saoud et al. 2017). The IR spectra of the purified compound of *A. enclensis* NIO-1008^T and *D. enclensis* NIO-1023^T also showed the presence of an oligosaccharide compound. This suggests that the purified compounds contained an oligosaccharide group which mimics the structure of a substrate for the alpha-glucosidase enzyme and conventionally inhibits the enzyme.

Fig.51. FTIR spectrum of (A) acarbose; (B) purified compound of *A. enclensis* NIO-1008^T; (C) purified compound of *D. enclensis* NIO-1023^T

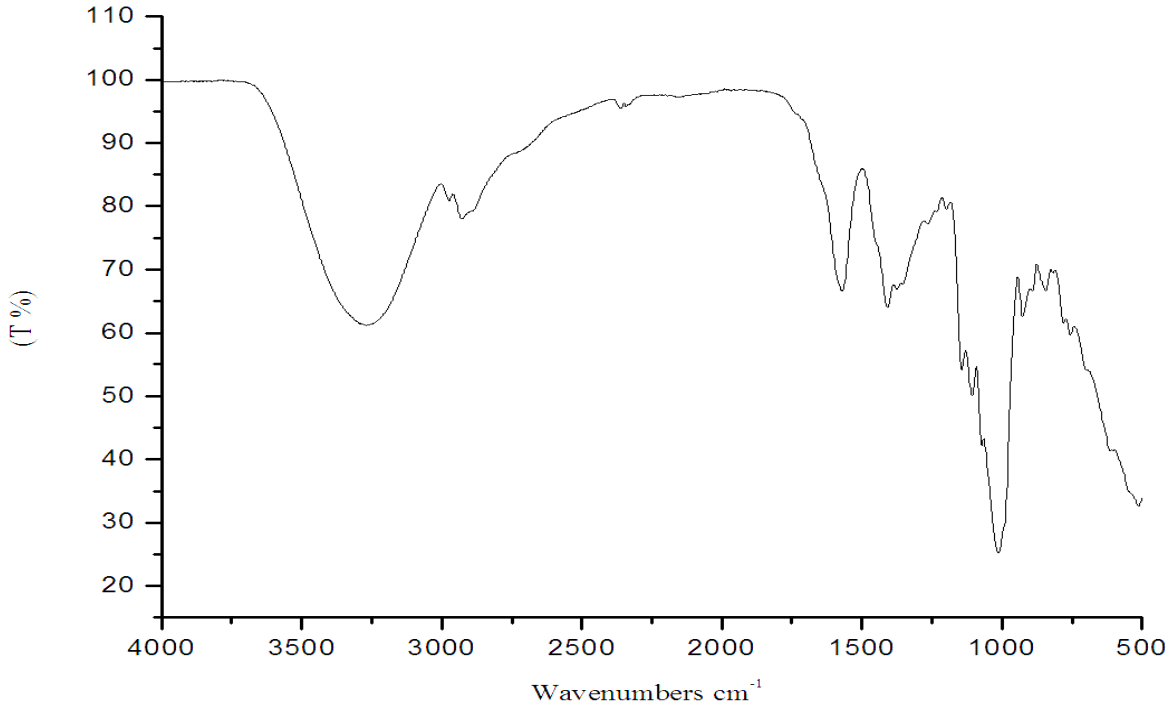
A]



B]


100
80
60
40
20
0
T (%)
4000
3500
3000
2500
2000
1500
1000
500
Wavenumbers (cm-1)

C]

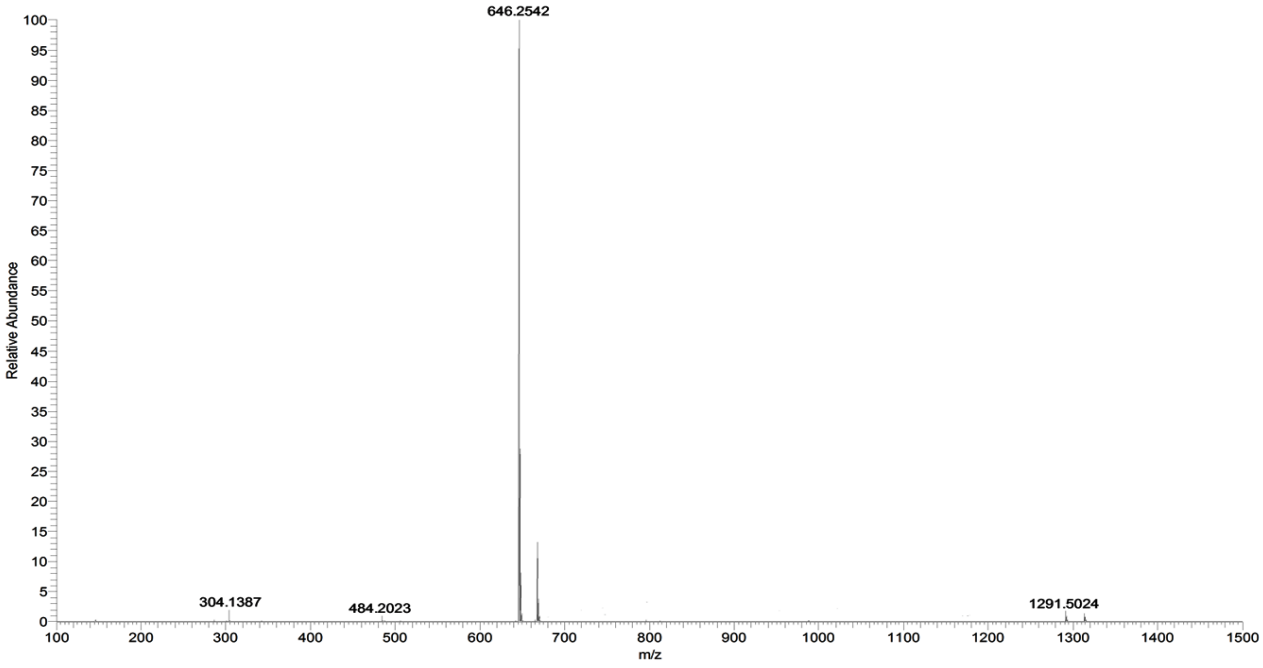

110
100
90
80
70
60
50
40
30
20
(T %)
4000
3500
3000
2500
2000
1500
1000
500
Wavenumbers cm-1

3.9.2. Mass spectrometry (MS) and tandem mass spectrometry (MS/MS) analyses

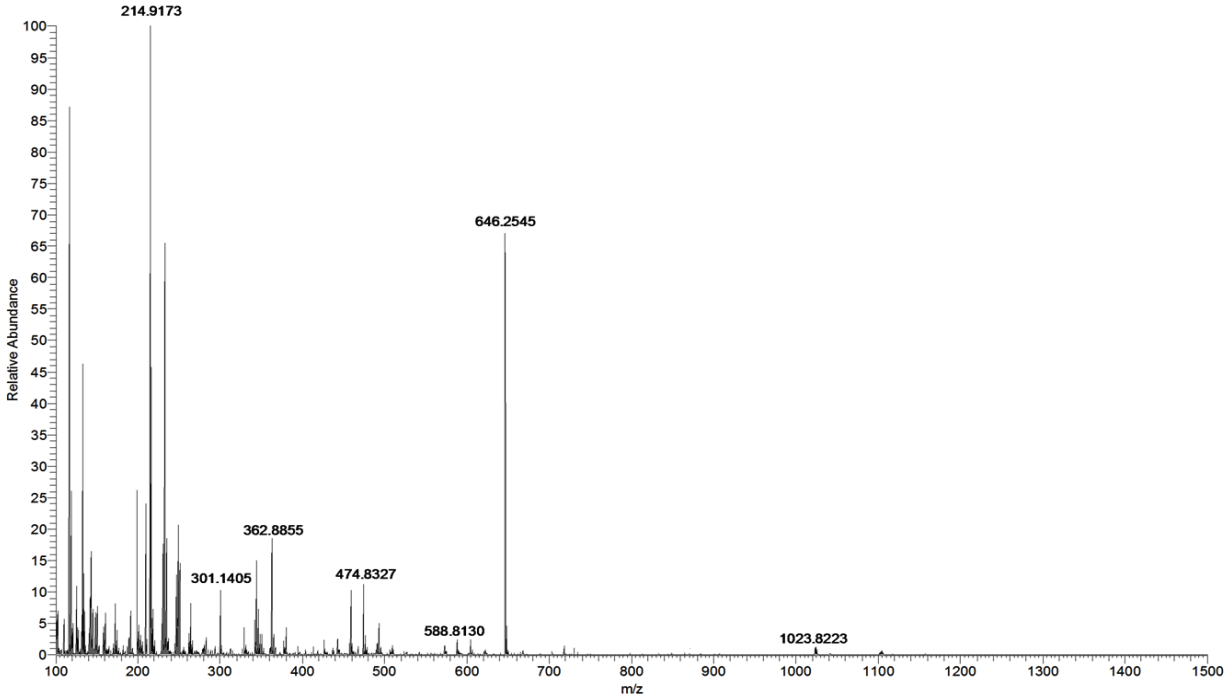
The isolated compound was then identified by positive ion mode electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MS/MS) analysis. The ESI-MS of acarbose was done which showed a peak at m/z 646.2542 $[M+H]^+$ signal is shown in Fig. 52. In full scan of an active compound of *A.enclensis* NIO-1008^T and *D. enclensis* NIO-1023^T showed a peak at m/z 646.2545 $[M+H]^+$ and 646.2545 $[M+H]^+$ signal is shown in Fig. 52. The tandem mass spectrometry (MS/MS) spectrum of the parental compound of standard acarbose and peak of m/z 646.2545 of *A.enclensis* NIO-1008^T and *D. Enclensis* NIO-1023^T was done (Fig. 53). The fragmentation pattern of both the parental compounds was found to be similar. The annotation of peaks in the spectrum and the integration of spectrum were done by using a web tool CFM-ID [Competitive Fragmentation Modeling For Metabolite Identification]. The CFM-ID provides the method for the efficiently identified metabolites in a spectrum for a known chemical structure generated by MS/MS (Allen et al. 2014). The signals at 466 m/z, 304 m/z correspond to the loss of one to two glucose units from the molecule. This indicates the dissociation of the glycosidic bond. The fragment ion 304 m/z, 268 m/z and 286 m/z were matched with the acarviosine unit. The fragment 146 m/z indicates the cleavage at N-H (amide) linkage of the acarviosine moiety. The 146, 128 fragment ions were matched with cyclitol ring of an acarviosine molecule. From the above, it was observed that the fragments produced upon ionization were of dissociation products from a glycosidic bond and from an amide bond. By comparison of the spectrum of acarbose to the active compound of both the bacteria, it was matched with the acarbose, which indicates that the organisms, produces acarbose like compounds.

Fig.52. MS of (A) acarbose; (B) purified compund of *A. enclensis* NIO-1008^{T} and (C) purified compound of *D. enclensis* NIO-1023^{T}.

A]



B]



C]

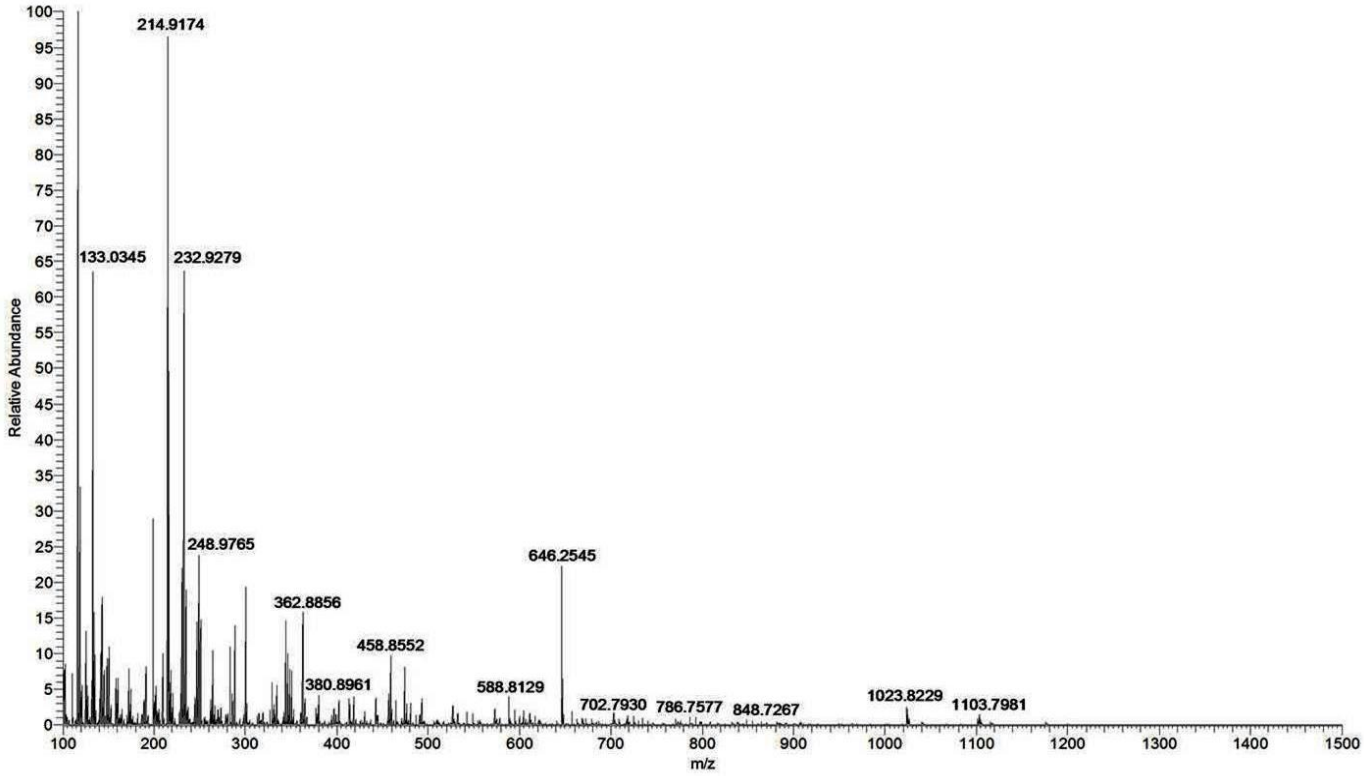
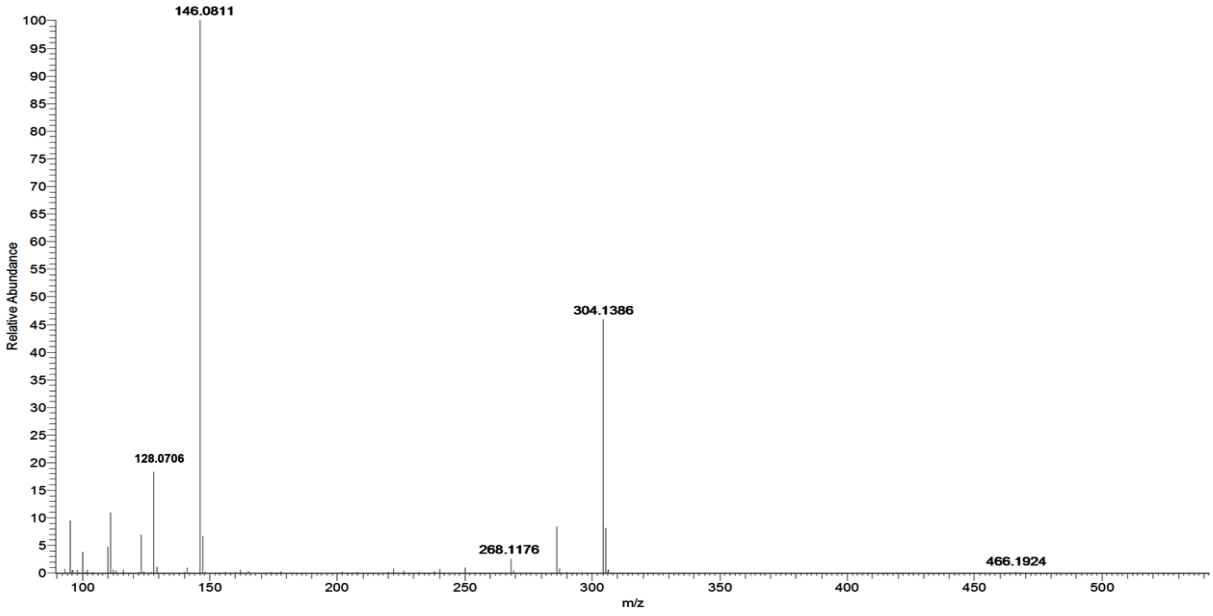
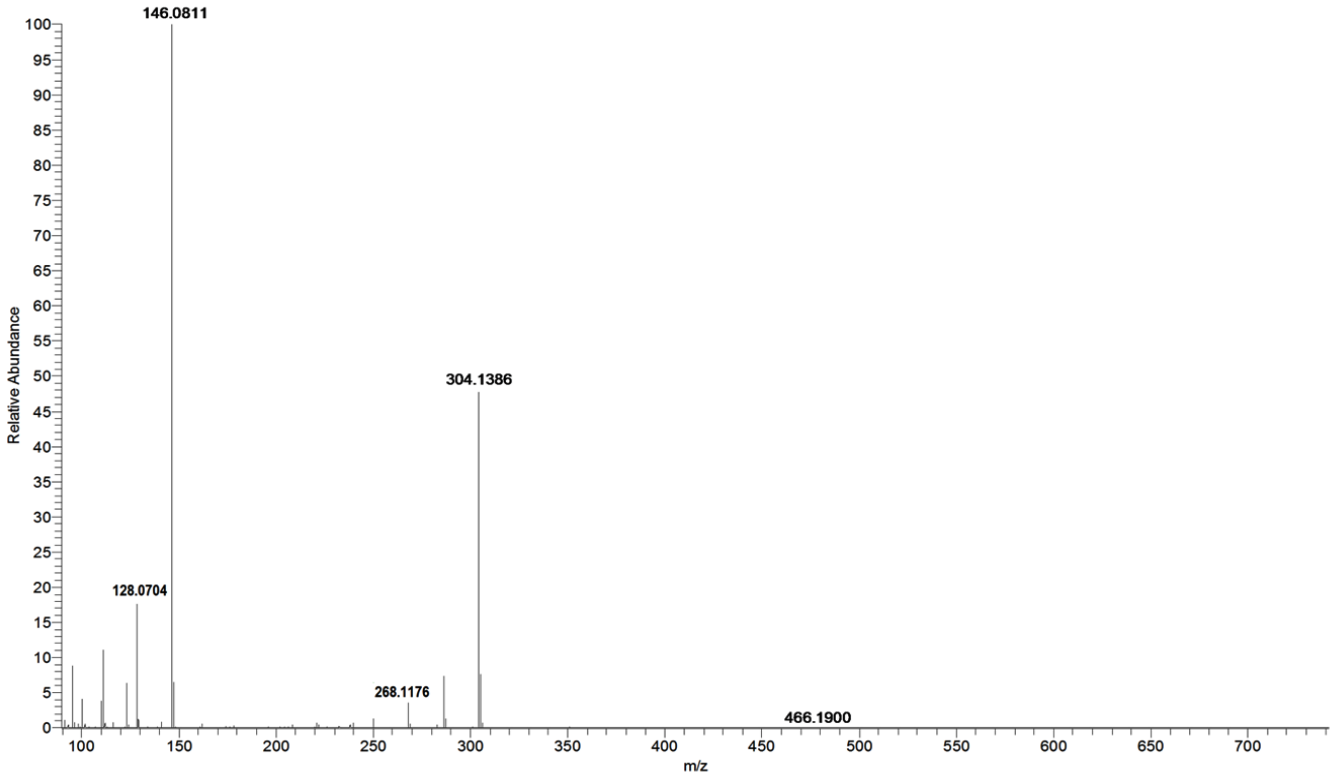


Fig.53. Tandem mass spectrometry (MS/MS) of (A) acarbose; (B) purified compund of *A. enclensis* NIO-1008^{T} and (C) purified compound of *D. enclensis* NIO-1023^{T}.

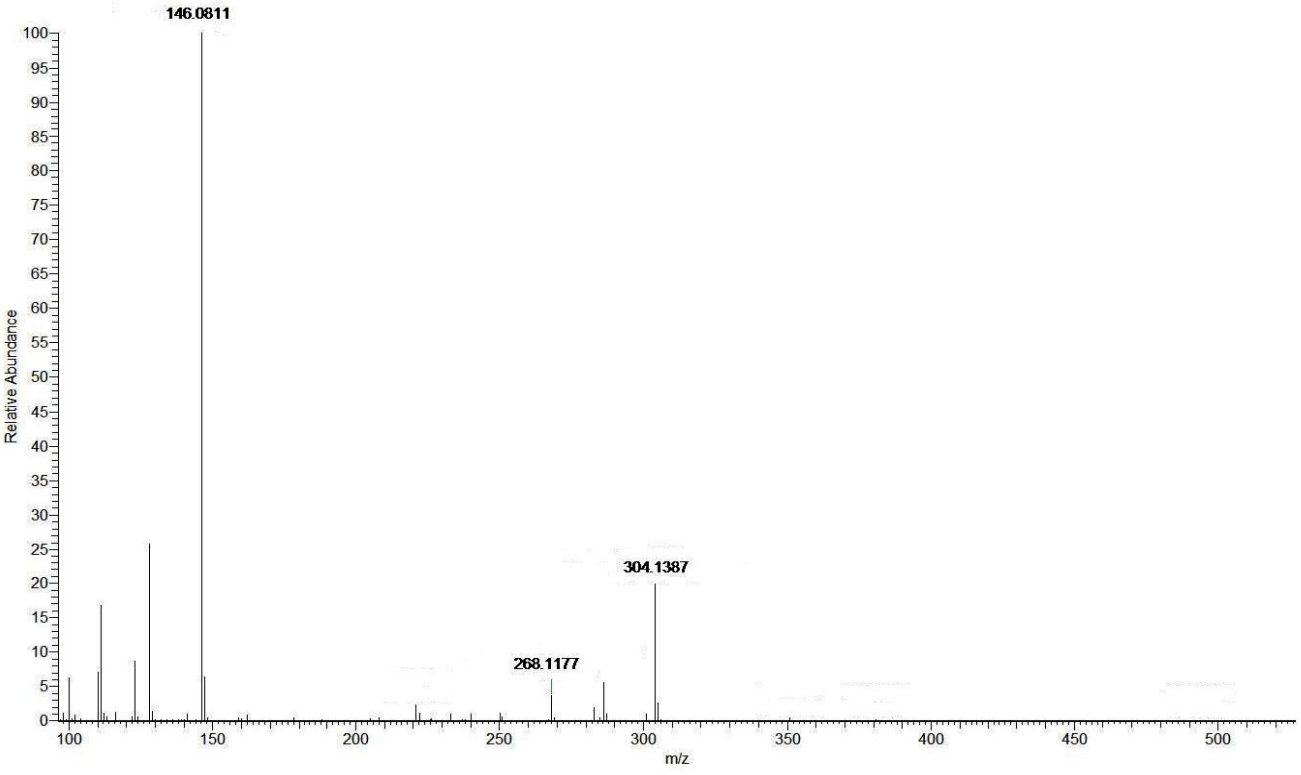
A]



B]


146.0811
304.1386
128.0704
268.1176
466.1900
Relative Abundance
m/z

C]


146.0811
304.1387
268.1177
Relative Abundance
m/z

3.10. Alpha-glucosidase inhibition

We investigated the inhibitory effect of the purified compound from both isolates and standard acarbose on alpha-glucosidase (Table 31). We found that the standard acarbose showed the inhibitory activity with an IC_{50} value of 200µg/ml ± 0.012. While the purified compound from *A.enclensis* NIO-1008^T showed the activity at an IC_{50} value of 500µg/ml ± 0.142 and from *D. enclensis* NIO-1023^T showed the activity at an IC_{50} value of 530 µg/ml ±0.161. The maximum inhibitions were observed 91.11% for acarbose and 75.42% and 73.76% for compound isolated from *A. enclensis* NIO-1008^T and *D. enclensis* NIO-1023^T respectively.

Table 31. Alpha-glucosidase inhibition of acarbose and purified compounds.

Compound	IC_{50} value	Maximum inhibitory activity (%)
Acarbose	200µg/ml ± 0.012 S.D.	91.11
***A. enclensis* NIO-1008^T compound**	500µg/ml ±0.142 S.D.	75.42
***D. enclensis* NIO-1023^T compound**	530 µg/ml ± 0.161 S.D.	73.76

Further study on *A. enclensis* NIO-1008^T was carried out as it showed highest inhibitory activity and the daughter ions of purified compound also matched with the daughter ions of standard acarbose.

3.11. Growth parameters and the inhibitory effect of *A. enclensis* 1008^T

The growth parameter of *A. enclensis* NIO-1008^T and the effect of their crude fermentation broth on α-glucosidase were monitored for 7 days. Growth was measured as an optical density at spectrophotometer with a wavelength of 600 nm and the inhibition was measured as a % inhibition.

The starting inhibitory activity was observed from 24 hrs of fermentation which was then exceeded from 96 hrs to the 168 hrs. The growth pattern of *A. enclensis* NIO-1008^T was measured as an optical density with a wavelength of 600 nm. The maximum growth of an organism was observed at 96 hrs of fermentation on which 50.0% of inhibition was achieved. The decline of growth was observed from 120 hrs of fermentation and the effect of inhibition was seems to be constant to the rest of the days. The correlation between growths of bacteria to the inhibition against an enzyme is shown in (Fig. 54).

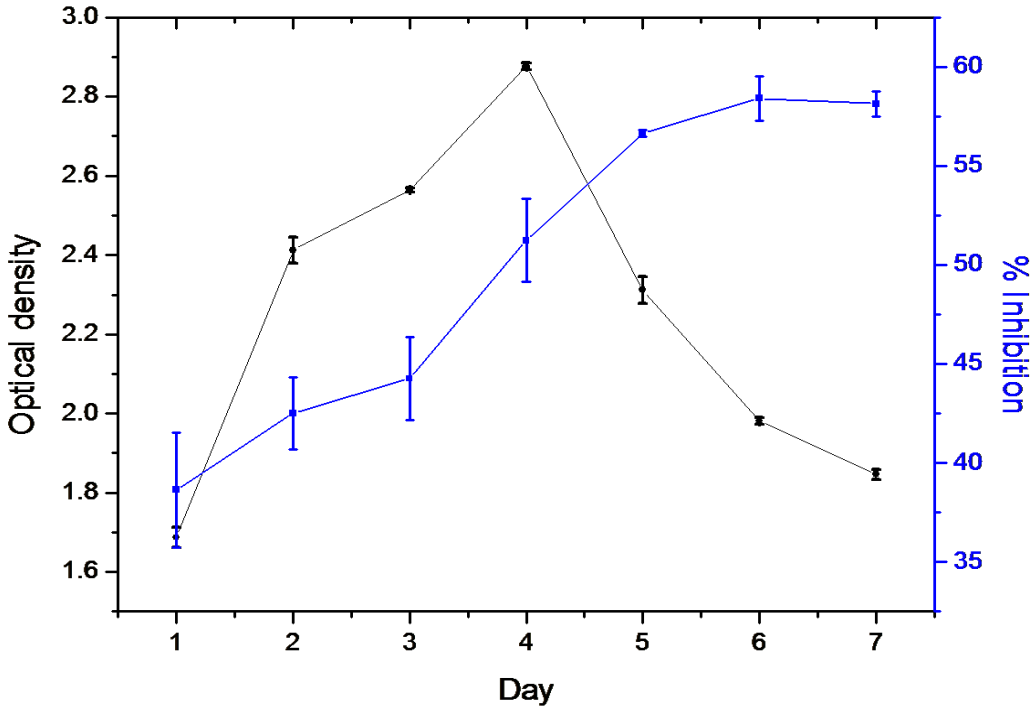


Fig.54. Growth parameter and alpha-glucosidase inhibition activity of *A.enclensis* NIO-1008^T

3.12. anti-SMASH analysis of whole genome of *Arthrobacter enclensis* NIO-1008^T

The genomic DNA of *A. enclensis* NIO-1008^T was isolated from 24 hr old tryptone soy agar plate. The draft genome of *A. enclensis* was generated using Illumina sequencing at DOE Joint Genome Institute (JGI), Walnut Creek, California, USA. By using Illumina HiSeq 2000 platform, shotgun library and sequencing were constructed which generates 8,861,546 reads of totalling 1338.1 Mb data. This draft genome sequence of *A. enclensis* NIO-1008^T was

deposited in DDBJ/ENA/GenBank under the accession number LNQM00000000 (Neurgaonkar et al., 2016).

This draft genome of *A.enclensis* NIO-1008^{T} was analyzed using anti-SMASH (Antibiotics & Secondary Metabolite Analysis Shell) v.4.0. The anti-SMASH helps in the alignment of a query gene cluster with the known gene cluster from a database and to identify biosynthetic gene cluster (Medema et al., 2011; Blin et al., 2017). It showed the gene cluster similarity with Acarviostatin biosynthetic gene cluster (11%) and acarbose biosynthetic gene cluster (7%). In both gene clusters, it matches with acarbose 4-alpha-glucanotransferase AcbQ and alpha-amylase AcbZ genes (Fig. 55). The acarviostatin and acarbose both is C_7N aminooligosaccharide compound and acarviostatin act as alpha-amylase inhibitor and acarbose as alpha-glucosidase and alpha-amylase inhibitor. It suggests that *A. enclensis* NIO-1008^{T} may use another gene cluster apart from known biosynthetic gene cluster for acarviostatin and acarbose for the biosynthesis of acarbose like molecule which was identified previously.

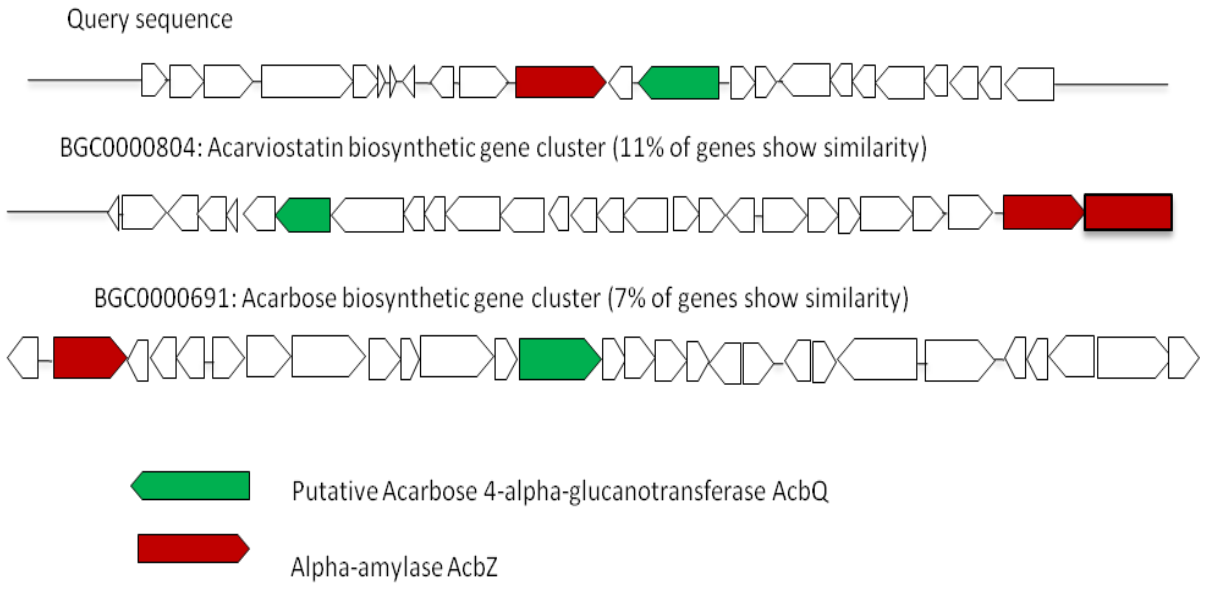


Fig.55. anti-SMASH analysis of draft genome of *A.enclensis* NIO-1008^{T}

3.13. Summary

Alpha-glucosidases are the enzymes which catalyze the α-1,4 linked glycosidic bond of oligosaccharides and convert it into monomer units. They have a primary role in carbohydrate metabolism. Alpha-glucosidase enzymes are used in the pharmaceutical studies to find out the suitable inhibitory compound of it. Inhibition of α-glucosidase leads in the delay of the increase in blood glucose level after a meal. Thus, the inhibitors for α-glucosidase help in the treatment of diabetes mellitus and obesity.

Currently, available drugs in the market are acarbose, voglibose and miglitol for the treatment of diabetes mellitus and obesity as an agent of α-glucosidase inhibitor. Acarbose is produced by an engineered strain *Actinoplanes* sp. SE50/110. Voglibose is derived from *Streptomyces hygroscopicus* var. *limoneus* and miglitol are synthesized from 1-deoxynojirimycin. Many plants and microorganisms are investigated for the inhibitory activity on α-glucosidase and some of them proved to have an inhibitory compound. Many microorganisms have been reported to produce an inhibitor but those which produce sugar mimicking compounds are very efficient. Examples of such case are actinomycetes which produce amino-oligosaccharides that have been proved to show inhibitory activity against α-glucosidase enzymes. These compounds contain an amino group linked to one or more sugar residues or their derivatives. One example of such compounds is C_7N aminocyclitol compounds which include an unsaturated aminocyclitol moiety. Synthesis of such compounds shares a common pathway from which later it gets diverted to form an individual compound in each case. This common precursor is *sedo*-heptulose 7-phosphate which is cyclized to form 2-epi-5-epi-valiolone. From this compound different C_7N aminocyclitol compounds formed in each case. For cyclization of *sedo*-heptulose 7-phosphate cyclase enzyme plays an important role.

In this study, we have screened marine bacteria which were isolated from the sediment sample of Chorao Island, Goa for the isolation and characterization of α-glucosidase inhibitors. The initial screening was based on the PCR method which detects *sedo*-heptulose 7-phosphate cyclase gene. The primers were designed on the basis of the known sequence of *sedo*-heptulose 7-phosphate from *Actinoplanes* sp. SE50/110 and 3-hydroquinate synthetase gene. The *Actinoplanes* sp. KCTC 9162^T was used as a positive control which is known to produce acarbose compound. We found that some marine isolates showed a positive result for PCR method namely *A. enclensis* NIO-1008^T, *D. enclensis* NIO-1023^T, *Cohnella* sp. NIO-V-74 and *Gordonia* sp. NIO-V-85. Further, alpha-glucosidase inhibition activity was carried out for all marine isolates as well as other reference *Streptomyces* sp. collected from NCIM resource centre. We found that after cultivating bacteria in suitable fermentation media, some of them showed inhibitory activity against alpha-glucosidase. *A. enclensis* NIO-1008^T and *D. enclensis* NIO-1023^T showed good inhibitory activity as compared to others. Later on, a thin layer chromatographic technique was employed for the detection of acarbose and their related products from the crude fermentation broth. From overall screening methods, we found that *A. enclensis* NIO-1008^T and *D. enclensis* NIO-1023^T showed positive for PCR based screening, good inhibition activity and acarbose related products in TLC chromatogram.

Further isolation and characterization of α-glucosidase inhibitor was carried out from *A. enclensis* NIO-1008^T and *D. enclensis* NIO-1023^T. The IC_{50} value of α-glucosidase inhibition was found to be 500 μg/ml and 530 μg/ml for a purified compound of *A. enclensis* NIO-1008^T and *D. enclensis* NIO-1023^T respectively as compared to acarbose which showed an IC_{50} value of 200 μg/ml. Characterization was done by using HPLC, FTIR and MS-MS/MS methods. It was found that both the isolates showed to produce an oligosaccharide compound. Upon MS and MS/MS analysis it was found that both bacteria produce acarbose like molecule. This was the first time we found that α-glucosidase inhibitor from the

Arthrobacter enclensis NIO-1008^{T} and *Deinococcus enclensis* NIO-1023^{T}, two newly identified bacteria. From this study we conclude that, marine organisms have potentials to produces pharameceutically important compounds which can be useful for the societal benefits.

CHAPTER-IV

SUMMARY AND FUTURE PROSPECTIVES

4.1. Overall summary

Microbial taxonomy is the branch of science of identification and classification of microorganisms, which involves the morphological, biochemical and phylogenetic study. Most of the biological applications depend on microbial taxonomy. A wide range of methods must be applied to identify an unknown strain of biotechnological important. Bacterial taxonomy provides the new and specific information of the validly described organism for the publicly available databases. It can be used to compare the other members of the genera. Based on the similarity pattern of an organism, and is arranged into groups (Classification) and determines its in defined units (Identification). Nomenclature ensures that, the internationally recognised code of prokaryotes are satisfied and the names are given to particular taxa are validly described.

Bacterial taxonomy was begun in the early 19th century and it was solely depended on morphological, physiological and biochemical aspects of an organism. With the introduction of the use of 16S rRNA gene sequencing method by Dr. Carl Woese, identification and classification of bacteria became easy. With other techniques like pheno-chemotaxonomic (morphological, biochemical, chemotypic etc) and genotypic (DNA-DNA hybridization, G+C content, DNA profiling etc) along with 16S rRNA gene sequencing coupled with phylogenetic analysis, revolutionized the bacterial taxonomy. These combined techniques are known as polyphasic systematics and it's widely accepted by the scientific community for the bacterial taxonomic studies.

In the present study, we used polyphasic systematic approach for the identification of bacteria isolated from the marine sediment samples of Chorao island, Goa. Marine sediments are formed over the seafloor, near seashores, estuaries and coral reefs etc by means of deposition through the action of land, river, wind and volcanic eruption etc. Sediments at

the mangrove ecosystem are rich in organic matter and sulfides. They trap the nutrients in the form of organic matter from dead or decomposing material especially from plants, terrigenous activity and from mineralization. The upper surface layers of mangrove sediments are aerobic in nature whereas the lower layer is an anaerobic. The microorganisms present in the sediments form a dynamic community and they help in the biogeochemical cycles of nitrogen, phosphorous, carbon and sulfate. They also help in the biocycling of nutrients and in degradation of the hydrocarbons. Due to their biopotential activities, mangrove sediment microorganisms draw a special attention to microbiologists. Microorganisms isolated from the sediment of mangroves have been used in the remediation, degradation, medical and agricultural application. In the present study, we isolated different microorganisms from sediment sample of the mangrove ecosystem present at Chorao Island of Goa, India.

The sediments sample was collected in sterile tubes and transported in the laboratory. It was serially diluted and spreads on marine agar in order to isolates bacteria. These marine isolates were subcultured on a fresh marine agar plates and maintained and preserved at refrigerator condition. The initial identification of marine bacteria were done by using 16S rRNA gene sequencing followed by, their phylogenetic analysis to determined the closest neighbour of an isolated strains. Based on the 16S rRNA gene sequence and their phylogenetic positions, candidate strains were selected for further analysis through polyphasic approach. Characterization of these selected bacteria were analysed for phenotypic, genotypic, phylogenetic and chemotaxonomic properties along with their respective closest type strains. The data obtained from each method were analyzed carefully and summarized. These strains were deposited into two internationally recognised culture collections as mandatory deposition for the proposal of novel species description. Documentation and description of these taxons were completed and published in recognised taxonomic journals. With the help of polyphasic systematics, we identified, characterize and

proposed ten novel microbial species isolated from the marine sediment samples. Among ten species, seven belongs to bacillus and three belongs to actinomycetes/actinobacteria mentioned in the below table 32.

Table 32. Proposed novel microbial species from sediment sample of Chorao Island, Goa.

Strain	Proposed novel species	Publications
1. SGD-1123^T	*Bacillus enclensis*	Dastager, S.G., Mawlankar, R., Tang, S.K., Krishnamurthi, S., Ramana,V.V., Shouche, Y.S (2014). Anto van Leuwenhoek, 105(1):199-206.
2. NIO-1003^T	*Fictibacillus enclensis*	Dastager, S.G., Mawlankar, R., Krishnamurthi, S., Tang, S.K., Lee, J.C., Ramana, V.V., Shouche, Y.S. (2014). Anto van Leuwenhoek , 105(3):461–469.
3. NIO-1009^T	*Rhodococcus enclensis*	Dastager, S.G., Mawlankar, R., Tang, S.K., Krishnamurthi, S., Ramana,V.V., Joseph, N., Shouche, Y.S. (2014).IJSEM, 64(8): 2693-2697
4. NIO-1016^T	*Domibacillus enclensis*	Sonalkar, V.,V. Mawlankar, R., Krishnamurthi, S., Tang, S.K., Dastager, S.G. (2014). IJSEM, 64(12): 4098-4102.
5. NIO-1023^T	*Deinococcus enclensis*	Thorat, M.N., Mawlankar, R., Sonalkar, V.V., Raman, V.V., Joseph, N., Shouche, Y.S., Dastager, S.G. (2015). Anto van Leuwenhoek , 107(1):141-148.
6. SGD-14^T	*Bacillus filamentosus*	Sonalkar, V.V., Mawlankar, R., Ramana, V.V., Joseph, N., Shouche, Y.S., Dastager, S.G. (2015). Anto van Leuwenhoek ,107(2):433-441.
7. SGD-V-25^T	*Bacillus encimensis*	Dastager, S.G., Mawlankar, R., Mual, P., Verma, A., Krishnamurthi, S., Joseph, N., Shouche, Y.S. (2015). IJSEM, 65(5):1421-1425.
8. NIO-1109^T	*Exiguobacterium enclense*	Dastager, S.G., Mawlankar, R., Sonalkar, V.V., Thorat, M.N., Mual, P., Verma, A., Krishnamurthi, S., Tang, S.K., Li, W.J. (2015). IJSEM, 65(5):1611-1616.
9. NIO-1002^T	*Microbacterium enclense*	Mawlankar, R., Mual, P., Sonalkar, V.V., Thorat, M.N., Verma, A., Krishnamurthi, S., Dastager, S.G. (2015). IJSEM, 65(7): 2064-2070.
10. NIO-1130^T	*Bacillus cellulasensis*	Mawlankar, R., Thorat, M.N., Krishnamurthi, S., Dastager, S.G. (2016). Arch Microbiol, 198(1): 83-89.

The bacteria which were isolated from marine sediment samples were further subjected for the alpha-glucosidase inhibition screening assay. Alpha-glucosidase is the enzyme of hydrolase family that cleaves the terminal non-reducing α, 1-4 linked glucose residue to release a single glucose molecule. It is located in the brush border of the small intestine. Inhibition of this enzyme delays the breakdown of carbohydrates and leading to the slower release of glucose into the blood stream. This action decreases the blood glucose level after the meal. Because of this property, these inhibitors (alpha-glucosidase) are used in the treatment of diabetes mellitus and obesity.

The alpha-glucosidase inhibitors (AGIs) have been isolated and studied from various organisms. Currently available AGIs in the market as a drug are acarbose, voglibose and miglitol. These drugs mimic the carbohydrate structure, thus inhibits the enzyme reversibly and competitively. Because of their pseudo saccharide structure they are poorly absorbed, digested and excreted from the body. Acarbose is a well studied drug. This drug belongs to the aminooligosaccharide family. This group of the family also include drugs like validamycin, kanamycin, pyralomicin and gabosines etc. Acarbose is a C_7N aminocyclitol compound and synthesis of this kind of compounds are derived from a common C_7 sugar phosphate *sedo*-heptulose 7-phosphate. The compound is cyclized to form 2-epi-5-epi valiolone, an intermediate for C_7N aminocyclitol compounds and catalyzed by an enzyme *sedo*-heptulose 7-phosphate cyclase. Hyun et al. (2005) had designed a primer set for the identification of these cyclase enzymes on the basis of functional PCR assay. This primer was designed on the basis of comparison of the sequence of sedo-heptulose 7-phosphate cyclase (acbC) from *Actinoplanes* sp. SE50/110 and several other 3-hydroquinate synthetases which exhibits high similarity with acbC protein.

In the present study, we used the primer set for the screening and detection of *sedo*-heptulose 7-phosphate cyclase gene from marine bacteria using PCR method. *Actinoplanes*

sp. KCTC 9162^{T} was used as a reference strain as positive during screening study. An approximate 500 bp size of PCR amplicon was reported for *Actinoplanes* sp. KCTC 9162^{T}. In this study, we also observed that, the strain *Actinoplanes* sp. KCTC 9162^{T} showed 500 bp size amplicon product. Along with a positive reference strain, marine bacteria were also showed the positive for the presence of similar 500 bp size amplicon product. These positive strains were further screened along with other strains for the alpha-glucosidase inhibition activity. The strains were cultivated in glucose and maltose contained fermentation media and incubated for 7 days at 30 ºC at 150 rpm. After fermentation, the crude supernatant was used for the inhibition assay. The inhibition study was done in triplicates along with positive control acarbose^{TM} (Sigma). Some *Streptomyces* strains were also used for the screening procedure. The crude supernatants from fermentation broth were spotted on a Silica gel 60 F_{254} plates. This system was employed for the detection of acarbose and their derivatives. Standard acarbose^{TM} was used as a positive control. From the overall screening, it was found that, the strain *Arthrobacter enclensis* NIO-1008^{T} and strain *Deinococcus enclensis* NIO-1023^{T} showed positive for desired PCR amplicon, which confirms the detection of *sedo*-heptulose 7-phosphate cyclase gene with a ~500 bp in size. These strains were also showed good inhibition activity towards alpha-glucosidase enzyme [*Saccharomyces cerevisiae*, Sigma]. The strain *A. enclensis* NIO-1008^{T} and *D. enclensis* NIO-1023^{T} showed 75.42 ±1.3 and 73.26 ±1.8 % inhibition respectively, while *Actinoplanes* sp KCTC 9162^{T} showed 64.95±1.5 % as compared to standard acarbose^{TM} (91.11±1.1 %). On TLC plate strain *A. enclensis* NIO-1008^{T} and *D. enclensis* NIO-1023^{T} showed a spot resembling to acarbose like saccharides. Based on the initial screening methods, two selected strains *A. enclensis* NIO-1008^{T} and *D. enclensis* NIO-1023^{T} were analyzed for further detailed study.

The 500 bp size of PCR product of strain *A. enclensis* NIO-1008^{T} and *D. enclensis* NIO-1023^{T} was extracted and purified from a gel. The purified product was then sub-cloned

into a pGEM-T vector and subsequently transformed into JM109 competent *E.coli* cells. The transformed product was then extracted and purified from the *E.coli* competent cells. The isolated plasmids were sequenced using ABI xL sanger sequencer. The resulted sequences were compared with the non-redundant sequences using the BLASTx program. It was observed that, the strain *Actinoplanes* sp. KCTC 9162^T showed similarity with *sedo*-heptulose 7-phosphate cyclase, while strain *A. enclensis* NIO-1008^T and *D. enclensis* NIO-1023^T showed similarity with phosphoenol pyruvate synthase and aminoglycoside phosphotransferase, respectively. From this analysis, it was concluded that the PCR method used for the screening is capable of detecting the specific gene in *Actinoplanes* sp. KCTC 9162^T showed the presence of the gene and these *Actinoplanes* specific primers. The strain *A. enclensis* NIO-1008^T and *D. enclensis* NIO-1023^T showed similarity with other genes indicating that, they may not have *sedo*-heptulose 7-phosphate cyclase gene but still produces acarbose like molecules, from TLC chromatograms, it was observed that both the bacteria showed the presence of acarbose like saccharides. Further, these bacteria were used for detailed characterization studies of AGIs.

The strain *A. enclensis* NIO-1008^T and *D. enclensis* NIO-1023^T were cultivated in a fermentation medium and incubated at 30 ºC for 7 days at150 rpm. After fermentation, the broth was centrifuged at 8000 rpm for 10 min and the supernatant was collected. The supernatant was incubated with *S. cerevisiae* beads at 37 ºC for 24 hrs. The *S. cerevisiae* beads help in to remove any glucose and maltose moities present in the broth. The *S.cerevisiae* beads were then removed by centrifugation at 6000 rpm for 10 min and the resultant extract was concentrated and subjected to Biogel P-2 gel permeation chromatography and the fractions were collected for 10 ml in each. Those fractions which showed the inhibition activity were further concentrated using rota evaporator. A light yellowish white coloured compounds were obtained from both the strains and it was

characterized with the help of HPLC, FTIR, LC-MS and MS/MS analysis, standard acarbose (sigma) was used as a positive control in each analysis. From HPLC and FTIR analysis it was observed that, the purified compound from both the strains showed an oligosaccharide group. From LC-MS and MS/MS analysis, it was observed that the mass of acarbose was found to be m/z 646.2542 and mass of the purified compound of *A. enclensis* NIO-1008^{T} and *D. enclensis* NIO-1023^{T} was found to be m/z 646.2545 for both the strains.The daughter ions generated from MS/MS analysis of standard acarbose and purified compound of both strains showed a significant matching with each other. This analysis suggests that, the strain *A. enclensis* NIO-1008^{T} and *D. enclensis* NIO-1023^{T} are capable of producing acarbose like molecule, which showed an inhibition towards alpha-glucosidase enzyme. The IC_{50} values for purified compound of both strains were found to be at 500 µg/ml and 530 µg/ml respectively, when compared to standard acarbose, which showed an IC_{50} value of 200 µg/ml. Further, anti-SMASH analysis of the whole genome of *A. enclensis* NIO-1008^{T} showed that it matches 11.0% and 7.0% homology with acarviostatin and acarbose biosynthetic gene clusters respectively. Acarviostatin and acarbose were known as α-amylase and α-glucosidase inhibitor and both fall under C_7N aminocyclitol containing pseudo amino-oligosaccharide family compounds. From the TLC, HPLC, FTIR, LC-MS/MS and whole genome analysis, it is concluded that the strain *A. enclensis* NIO-1008^{T} produces acarbose like saccharide compound and it may be uses different biosynthetic gene clusters for such compound apart from known acarbose and acarviostatin gene clusters.

From the overall study, it is concluded that, the polyphasic systematics is a most useful approach for the taxonomical study of the bacteria. With the help of polyphasic systematic, we reported ten novel species from the marine sediment sample. These isolates showed good inhibitory activity towards the α-glucosidase enzyme. This suggests that marine

environment and microbial isolates of those environments are capable of producing bioactive molecules of pharmaceutical interests.

Overall, thesis dealt with the taxonomic analysis of marine sediment bacteria using a polyphasic approach leading to the proposal of ten novel species belonging to Firmicutes and Actinobacteria. Furthermore, these novel species were screened for a variety of biotechnological application, especially for the isolation of molecules to be used in the treatment of diabetes mellitus II. For the treatment, blood glucose level must be controlled by regulating the action of α-glucosidase, an enzyme responsible for the breakdown of carbohydrates. An inhibitor of this enzyme has significant importance to control the blood glucose level. Various species of bacteria are known to produce inhibitors of α-glucosidase, therefore in the present study; we have screened novel marine isolates for such inhibitor molecules. Furthermore, the potent inhibitor molecule was purified and characterized using various analytical tools.

4.2. Future Prospectives

This work provides valuable information about the importance of polyphasic systematics in bacterial taxonomy. These bacteria could be serving as a source for bioactive molecules.

We used this marine isolates for the screening and characterization of α-glucosidase inhibitor and we found that *A. enclensis* NIO-1008^{T} and *D. enclensis* NIO-1023^{T} showed good inhibitory activity. While these bacteria showed inhibitory activity against yeast α-glucosidase, additional work is anticipated in order to examine the inhibitory effect on mammalian α-glucosidase.To elucidate this, identification of gene clusters and enzymes responsible for such molecules will be necessary which may also allow genetic engineering to be carried out for enhancing the production of such inhibitors.

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APPENDICES

Appendix I

Reagents and buffers:

1. 1X TE buffer (Tris Ethylenediaminetetraacetic acid [EDTA]):-

Tris-HCL	10.0 mM
EDTA	1.0 mM
pH	8.0.

2. 10% sodim dodecyl sulfate (SDS):-

Ingredients	g/100 ml
SDS	10.0

3. 5M NaCl: -

Ingredients	g/100 ml
NaCl	29.2

4. CTAB/NaCl (hexadecyltrimethyl ammonium bromide) solution: -

Ingredients	g/100 ml
NaCl	4.1
CTAB	10.0

5. 3M sodium acetate: -

Ingredients	g/100 ml
Sodium acetate	24.6
pH	5.2

Adjust the pH by slowly adding glacial acetic acid.

6. 10X TBE (Tris-borate EDTA) stock buffer: -

Ingredients	g/1L
Tris	108
Boric acid	55
EDTA	7.5
pH	8.0

7. 1X Phosphate buffered saline (PBS): -

Ingredients	g/1 L
NaCl	8.0
KCl	0.2
NaH_2PO_4	1.44
KH_2PO	0.24
pH	7.4

8. 0.1M citric acid/0.1M sodium citrate buffer: -

   A) 0.1M of citric acid solution: 21.01 g/L

   B) 0.1M of sodium citrate solution: 29.41 g/L

   Mix x ml of A and y ml of B in the proportions indicated and adjust the final volume to 100 ml.

x ml of A	46.5	43.7	40.0	37.0	35.0	33.0	31.5	28.0	25.5	23.0	20.5	18.0	16.0	13.7	11.8	9.5	7.5
y ml of B	3.5	6.3	10.0	13.0	15.0	17.0	18.5	22.0	24.5	27.0	29.5	32.0	34.0	36.3	38.2	41.5	42.8
pH	3.0	3.2	3.4	3.6	3.8	4.0	4.2	4.4	4.6	4.8	5.0	5.2	5.4	5.6	5.8	6.0	6.2

9. 0.1M NaH_2PO_4/ 0.1M Na_2HPO_4: -

   A) 0.2M of NaH_2PO_4 solution: 27.8 g/L

   B) 0.2M of Na_2HPO_4 solution: 53.65 g/L

   Mix x ml of A and y ml of B in the proportions indicated and adjust the final volume to 200 ml.

x ml of A	92.0	87.7	81.5	73.5	62.5	51.0	45.0	39.0	28.0	19.0	13.0	8.5	5.3
y ml of B	8.0	12.3	18.5	26.5	37.5	49.0	55.0	61.0	72.0	81.0	87.0	91.5	94.7
pH	5.8	6.0	6.2	6.4	6.6	6.8	6.9	7.0	7.2	7.4	7.6	7.8	8.0

10. 0.1M $NaHCO_3$/ 0.1M Na_2CO_3 : -

A) 0.2M of Na_2CO_3 solution: 21.2 g/L

B) 0.2M of $NaHCO_3$ solution: 16.8 g/L

Mix x ml of A and y ml of B in the proportions indicated and adjust the final volume to 200 ml.

x ml of A	4.0	9.5	16.0	22.0	27.5	33.0	38.5	42.5
y ml of B	46.0	40.5	34.0	28.0	22.5	17.0	11.5	7.5
pH	9.2	9.4	9.6	9.8	10.0	10.2	10.4	10.6

11. 20X saline sodium citrate (SSC) buffer: -

Ingredients	g/1 L
NaCl	175.0
trisodium citrate	88.0
pH	7.0

12. Acid aniline phthalate reagent: -

Ingredients	g/100 ml of n-butanol saturated with water.
Aniline	0.93
Phthalic acid	1.66

13. Dittmer and Lester reagent: -

a) Solution I:

Ingredients	g/L of 25N sulfuric acid
Molybdenum oxide	40.11

Boil for 3-4 hrs untill molybdenum oxide is completely dissolved and allow to cool ir

b) Solution II:

Ingredients	g/500 ml of solution I
Molybdenum powder	1.78

Boil for 15 min, cool and decant the remaining residues. Add equal volume of I and II to 4.5 volume parts of water.

14. Ninhydrin reagent: -

Ingredients	6 ml of acetone
Ninhydrin	0.2g

15. Dragendorffs reagent: -

Ingredients	g/20 ml of acetic acid
Potassium iodide	0.11
Busmuth subnitrate	0.18

Make the volume upto 100 ml.

16. Anisaldehyde-sulfuric acid solution: -

Ingredients	In 50 ml acetic acid
p-anisaldehyde	0.5 ml
Conc. sulfuric acid	1.0 ml

Appendix II

Media preparations:

1. Nutrient broth: -

Ingredients	g/L
Peptone	10.0
Beef extract	10.0
NaCl	5.0

2. Peptone-glucose medium conaining 2.0% urea: -

Ingredients	g/L
Peptone	1
Glucose	1
NaCl	5
KH2PO4	2
Urea	20
Phenol red	0.01

3. Peptone-beef agar containing starch: -

Ingredients	g/L
Peptone	10.0
Beef extract	5.0
NaCl	5.0
Soluble starch	2.0
Agar	20.0

4. Nutrient gelatine medium: -

Ingredients	g/L
Peptone	5.0
Beef extract	3.0
Gelatine	120.0

5. Casein nutrient agar medium: -

Ingredients	g/L
Peptone	10.0
Beef extract	10.0
NaCl	5.0
Skim milk powder	100.0

6. R2A medium: -

Ingredients	g/L
Casein enzyme hydrolysate	0.5
Yeast extract	0.5
Proteose peptone	0.5
Dextrose	0.5
Soluble starch	0.5
Dipotassium phosphate	0.3
Magnesium sulphate	0.024
Sodium pyruvate	0.3
Agar	15.0

7. Seed medium: -

Ingredients	g/L
Glucose	15.0
Peptone	7.5
Potassium dihydrogen phosphate	1.0
Sodium chloride	5.0

8. Feremntation medium: -

Ingredients	g/L
Glucose	30.0
Maltose	15.0
Peptone	5.0
Monosodium glutamate	3.0
Dipotassium hydrogen phosphate	1.0
Magnesium sulphate	1.0
Ferrous sulphate	0.02
Sodium nitrate	3.0
pH	7.2

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1. **Mawlankar, R.,** Thorat, M.N., Krishnamurthi, S., Dastager, S.G. (2016). *Bacillus cellulasensis* sp. nov., isolated from marine sediment. Archives of Microbiology, 198: 83-89
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