ADVANCES IN BIOTECHNOLOGY

INDEX

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Advances in Biotechnology

Chapter 1

Microbial diversity and syntrophic acetate degradation to methane in a high-temperature petroleum reservoir

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Abstract

The results of our investigation on the microbial community of the high-temperature Dagang oilfield (P.R. China) are summarized. Detailed experimental data are provided on syntrophic acetate degradation by thermophilic associations, on the isolation of pure cultures from these associations, their physiological characteristics, and reconstruction of microbial interactions during acetate degradation to methane. The microbial community of the high-temperature Dagang oilfield was investigated by culture-based, radioisotope, and 16S rRNA gene techniques. Cultivable microorganisms (aerobic oil-oxidizing, anaerobic fermentative, sulfate-reducing, and methanogenic) were found in formation water. Methanogenic enrichments were obtained in media both with H_2+CO_2 and acetate. The process of methane production in formation waters was also registered by radioisotope methods with $Na_2{}^{14}CO_3$ and $^{14}CH_3COONa$. However, pure cultures of thermophilic aceticlastic methanogens were not obtained. The 16S rRNA gene analysis of the formation water and

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The results of our investigation on the microbial community of the high-temperature Dagang oilfield (P.R. China) are summarized. Detailed experimental data are provided on syntrophic acetate degradation by thermophilic associations, on the isolation of pure cultures from these associations, their physiological characteristics, and reconstruction of microbial interactions during acetate degradation to methane. The microbial community of the high-temperature Dagang oilfield was investigated by culture-based, radioisotope, and 16S rRNA gene techniques. Cultivable microorganisms (aerobic oil-oxidizing, anaerobic fermentative, sulfate-reducing, and methanogenic) were found in formation water. Methanogenic enrichments were obtained in media both with H_2+CO_2 and acetate. The process of methane production in formation waters was also registered by radioisotope methods with $Na_2{}^{14}CO_3$ and $^{14}CH_3COONa$. However, pure cultures of thermophilic aceticlastic methanogens were not obtained. The 16S rRNA gene analysis of the formation water and methanogenic enrichments revealed that H_2-utilizing methanogens of the genus *Methanothermobacter* were predominant in the archaeal libraries (97% of archaeal clones). Phylotypes of acetate-utilizing methanogens were detected in the libraries only af-

Keywords: high-temperature petroleum reservoirs, thermophiles, 16S rRNA gene clone library, methanogenesis, syntrophic acetate degradation

1. Introduction

The presence of microorganisms in petroleum reservoirs has been established about 100 years ago. Anaerobic microorganisms reducing sulfate, thiosulfate, Fe(3+), or elemental sulfur, as well as fermentative bacteria, acetogens, and methanogens have been isolated from petroleum reservoirs [1]. The aerobic bacteria isolated from water-flooded oilfields are considered contaminants which arrived from the surface in the course of drilling or with injected water. Abundant data are available concerning the microorganisms of low-temperature (20–45ºC) petroleum reservoirs. The composition of microbial communities in high-temperature reservoirs and methanogenesis under such conditions have been less extensively studied [2-9].

Methanogenesis in oilfields was first reported by Kuznetsov [10]. Belyaev and coworkers isolated the first pure methanogenic cultures from petroleum reservoirs [11-14]. Methanogens oxidizing hydrogen in the course of CO_2 reduction to methane are common in the oilfields with temperatures of 20 to 80°C. Thermophilic H_2-uilizing methanogens isolated from high-temperature petroleum reservoirs of Western Siberia, the North Sea and California belonged to species *Methanothermobacter thermautotrophicus* (previous name *Methanobacterium thermoautotrophicum*), *Methanothermococcus thermolithotrophicus* (previous name *Methanococcus thermolithotrophicus)* and *Methanothermobacter thermoaggregans* (previous name *Methanobacterium thermoaggregans)*. As to organotrophic methanogens, which utilize methanol, acetate, or methylated amines and are often capable of growth on H_2+CO_2, they have been isolated only from oilfields with the temperatures below 50ºC [1,13,14]. However, in the oilfields with the temperatures ranging from 60 to 80°C, methane production from both

bicarbonate and acetate was detected by radioisotope methods [4,6,15-17]. It is an indication of the presence of thermophilic prokaryotes performing acetate degradation to methane in high-temperature oilfields.

Davydova-Charakhch'yan and coauthors [13] were the first to show syntrophic associations to be responsible for methanogenic acetate decomposition in high-temperature oilfields and to isolate the terminal component of this association, a lithotrophic methanogen *M. thermautotrophicus* (formerly *M. thermoalcaliphilum)*. Nilsen and Torsvik [18] observed methane formation in enrichment cultures with acetate at the temperatures of 70, 80, and 92°C, but attempts to isolate acetate-utilizing methanogens were also unsuccessful.

The first molecular studies of microbial diversity of high-temperature oilfields based on 16S rRNA gene analysis were carried out in California [2,3], Western Siberia [4], and China [5,19,20]. Formation water was found to contain 16S rRNA genes of the known thermophilic (*Thermococcus, Thermotoga, Petrotoga, Thermoanaerobacter, Methanothermobacter, Methanococcus*, and *Methanoculleus*) and mesophilic microorganisms, as well as of uncultured archaea and bacteria. In the 16S rRNA gene clone library from a petroleum reservoir in California, the 16S rRNA gene of a novel, probably aceticlastic methanogen of the order *Methanosarcinales* was revealed [2]. Pure cultures of thermophilic aceticlastic methanogens have not yet been isolated from oilfields.

Microbiological, radioisotope, molecular, and biogeochemical techniques have been used to investigate microbial diversity and activity in the oilfields. In most works, these techniques were applied separately; the structure of the microbial community as a whole was therefore not revealed and its geochemical activity was not assessed properly.

The problem of energy recovery from oilfields in the form of methane, based on accelerating natural methanogenic biodegradation is widely discusses in literature. At the high-temperature Dagang oilfield in China, trails of a biotechnology for enhanced oil recovery based on injection of a water-air mixture and of nitrogen and phosphorus mineral salts were carried out [6,7,21,22]. As a result of the trial, 36 thousand tons of additional oil was recovered. Moreover, fundamental studies of the distribution and activity of microorganisms were carried out. When studying the 16S rRNA genes clone library of methanogenic enrichments growing in the medium with acetate, we did not find any phylotypes of aceticlastic methanogens [6,23]. These data did not agree with the numerous results of radioisotope analysis which confirmed formation of $^{14}CH_4$ from $^{14}CH_3COONa$. It was suggested that syntrophic associations were responsible for acetate degradation to methane in the oilfield.

In our previous studies [19,23-25], for the first time pure cultures of both an acetate-oxidizing bacterium (*Thermoanaerobacter ethanolicus*) and an H_2-utilizing methanogen (*Methanothermobacter* sp.) were isolated from thermophilic syntrophic methanogenic enrichments

grown on acetate. For the reconstruction of syntrophic growth on acetate, pure cultures of the methanogen (*M. thermautotrophicus* or *M. wolfeii)* and the acetate-oxidizing bacterium (*T. ethanolicus*) were combined. The binary *Methanothermobacter-Thermoanaerobacter* culture was found to degrade acetate to methane. Methane formation by combined cultures was observed in their subsequent transfers to fresh acetate-containing medium. A range of results of this study was not presented in detail [25].

The aim of our work was to review our data on syntrophic degradation of acetate by thermophilic microorganisms and to present detailed experimental data on the characterization of the microbial community of the high-temperature Dagang oilfield (P.R. China) and on isolation of pure cultures of microorganisms from thermophilic methanogenic enrichments growing on acetate, studying their physiological characteristics and reconstruction of microbial interactions during syntrophic acetate degradation to methane.

2. Materials & Methods

2.1 Reservoir Description and Sample Collection

The Dagang oilfield is situated in the Hebei province (P.R. China). The studied sandstone oil-bearing horizons of the Kongdian bed were located at the depth of 1206–1435 m below sea level; the temperature was 59°C. The natural formation water had a low salinity (5612 mg L^{-1}) and a pH of 7.1–7.6. Accompanying gas contained methane (95–98%), its higher homologues (0.8–1.8%), nitrogen (0.5–3.3%), and carbon dioxide (0.06–0.77%) [5].

Production fluids were collected directly from the production wells into sterilized bottles and immediately prepared for chemical analyses, radioisotope and culture studies. The bottles were filled completely with the oil-water-gas mixture and stored at 4°C prior to analyses. For DNA isolation, water was separated from crude oil by decantation at room temperature. Triton X100 (0.1%) and *n*-hexane were added to the samples. The water phase after hexane extraction was fixed with ethanol (1:1 vol/vol) and kept at 4°C prior to analyses.

2.2 Culture Media and Growth Conditions

A technique for cultivation of strictly anaerobic microorganisms [26] was applied for the enumeration of microorganisms. The numbers of cultivable anaerobic bacteria were estimated by serial decimal dilutions of water samples in test tubes with various enrichment media designed to promote growth of specific functional groups. Anaerobic bacteria were cultivated in test tubes with pure argon as the gas phase; an exception was the medium with H_2/CO_2 for methanogens. Anaerobic organotrophic bacteria with a fermentative type of metabolism were assayed by detection of the H_2 in the highest dilution in the medium with peptone (4 g L^{-1}) and glucose (10 g l^{-1}). Sulfate-reducing bacteria were detected by measuring an increase in sulfide

content in medium B containing sodium lactate (4 g L^{-1}) and reduced with $Na_2S \times 9H_2O$ (0.2 g L^{-1}) [27]. Methanogens were determined by measuring an increase in CH_4 in the media [28] with acetate (2.2 g L^{-1}) or H_2/CO_2 (80:20 % vol/vol), supplemented with $Na_2S \times 9H_2O$ (0.5 g L^{-1}), microelements and yeast extract (1 g L^{-1}). Descriptions of the media were given earlier [4].

Aerobic bacteria were cultivated in Hungate tubes with air as the gas phase. Viable aerobic organotrophs were enumerated in the medium composed of bacto-tryptone (5.0 g L^{-1}), yeast extract (2.5 g L^{-1}), glucose (1.0 g L^{-1}), and distilled water (1 L, pH 7.0). All media were inoculated with samples of formation water using syringes and were incubated at 60° for 30 days. Samples from all the tubes were examined by phase-contrast microscopy.

The content of microbial metabolites (sulfide, CH_4, CO_2, and H_2) in the media and the chemical composition of the formation water were analyzed as described previously [4].

2.3 Isolation and Identification of Pure Cultures

Methanogenic enrichments were obtained by inoculation of mineral medium [28] with various substrates and inhibitors: acetate (40 mM); acetate with ampicillin (2 mg mL^{-1}), acetate with 2-bromo-ethane sulfonate (10 mM); H_2/CO_2; and H_2/CO_2 with ampicillin, methanol (5 mL L^{-1}), propionate (20 mM) or/and benzoate (20 mM).

Pure cultures of methanogens were obtained in a liquid medium [28] supplemented with ampicillin (2 mg mL^{-1}), with H_2/CO_2 mixture as the carbon and energy source. Mineral medium [28] supplemented with sodium pyruvate (40 mM) was used for isolation of anaerobic bacteria from methanogenic enrichments growing on acetate. The isolates were identified by 16S rRNA gene sequencing as described below.

The isolates of H_2-utilizing methanogens (strains KZ3-1 and KZ24a) and members of the genus *Thermoanaerobacter* (strains 1017-7b and 1017-7d) were incubated separately or in binary cultures in the medium [28] with sodium acetate (80 mM) at 60°C. Methane formation was determined by gas chromatography after 14 days of cultivation. The acetate-grown enrichment 24A was used as the positive control.

2.4 Radioisotope Methods

The rates of sulfate reduction and methanogenesis in formation waters were determined by radioisotope methods using labelled $Na_2{}^{35}SO_4$, $^{14}CH_3COONa$, and $Na_2{}^{14}CO_3$ as described previously [4]. Radiotracer analysis was used to determine the rates of methane formation from 2-$^{14}CH_3COONa$ by a binary culture of *Methanothermobacter* sp. KZ3-1 and *Thermoanaerobacter* sp. 1017-7b. The strains were incubated separately (controls) and together in the mineral medium [28] in the presence of 2-$^{14}CH_3COONa$ (70 μg/L) and unlabeled CH_3COONa

(200 mg L^{-1}). The experiment was carried out for 24 h at 60°C. The rate of methane formation was calculated according to the equation: I=r·C/(R·T), where r is the overall count for 60 s, C is the substrate concentration (μg L^{-1}), R is activity of the labeled 2-$^{14}CH_3COONa$ (MBq mL^{-1}), and T is the incubation time (24 h).

2.5 Construction and Analysis of 16S rRNA Gene Clone Libraries

DNA Extraction. Total DNA was extracted from formation water and cultures using DiatomTM DNAprep kit ("BioKom", Russia). Microbial biomass from approximately 2 liters of the water phase of production fluids was collected by centrifugation (Beckman JA10, 8000 g, 1 h). The cell pellet was suspended in MilliQ water and was twice frozen with liquid molecular nitrogen and thawed (at 65°C). The cell lysate was then supplemented with guanidine hydrochloride and incubated at 65°C for 1 h. The sorbent (Diatomid/silica) was added to the cell lysate. After DNA sorption, the supernatant was removed and the adsorbent was washed using a buffer (pH 7.0) and 70% (vol/vol) ethanol. The total DNA preparation was dissolved in MilliQ water and used for 16S rRNA gene amplification.

16S rRNA Gene Amplification and Cloning. 16S rRNA genes were amplified by PCR using primers specific for *Bacteria* (8-27f [5'-AGAGTTTGATCCTGGCTCAG-3'], 519r [5'-G(T/A)ATTACCGCGGC(T/G)GCTG-3'] and 1492r [5'-TACGGYTACCTTGTTACGACTT-3']) [29], and *Archaea* (A109F [5'-ACG/TGCTCAGTAACACGT-3'], A1041r [5'-GGCCATGCACCWCCTCTC-3']) [30]. The final 50-μl reaction mixture contained 5 μl of template DNA, 0.5 μM of each primer, 1× DNA polymerase buffer, 2.5 mM MgCl2, 0.1 mM of each deoxynucleoside triphosphate, and 1 U of Taq polymerase (Perkin-Elmer). Polymerase chain reaction cycles were performed on a Mastercycler (Eppendorf, Germany) as follows: after 5 min of initial denaturation at 94°C, nucleic acids were amplified for 30 cycles (30 s of denaturation at 94°C, 30 s of annealing at 50°C, and 30 s – 1.5 min of elongation at 72°C) followed by a final extension step at 72ºC for 8 min. Archaeal 16S rDNA were amplified at 35 cycles of PCR. PCR products were checked on 0.8% (wt/vol) agarose gel stained with ethidium bromide. Amplicons were cloned with a pGEM-T (pGEM-T Easy Vector Systems, Promega, USA) according to the manufacturer's instructions.

As a result of the PCR analysis, 785 archaeal and 503 bacterial clones were selected.

Sequencing and Phylogenetic Analysis. Inserts of selected clones were amplified by PCR with T7 and SP6 plasmid primers. The same primers were subsequently used to sequence bacterial PCR products. Archaeal PCR products were sequenced with the A109f primer. The sequencing was performed on an ABI 3100 Avant Genetic Analyzer with the BigDye Terminator V3.1 (Applied Biosystems, USA).

Sequence data were aligned using the CLUSTALW v.1.75 package [31], with clones having similarities of 97% or above grouped into operational taxonomic units (OTUs). The

clones were homology-searched using BLAST and the GenBank database of NCBI (http://www.ncbi.nlm.nih.gov). Chimeras were detected using the CHIMERA-CHECK program from the Ribosomal Database Project (http://rdp.cme.msu.edu). The sequences were edited using the BioEdit software package (http://jwbrown.mbio.ncsu.edu/ BioEdit/bioedit.html).

Nucleotide Sequence Accession Numbers. The sequences identified in this study were submitted to the GenBank database under the following accession numbers: DQ657903, and DQ657904 (pure cultures), DQ097666–DQ097668, DQ097671, FJ898357–FJ898360, and FJ898362–FJ898364 (clones).

2.6. Design of Specific Primers

To analyze the predominant phylotypes in the clone libraries from formation water, we designed two specific primers [25]. Primers M400r (GAAAAGCCACCCCGTTAAGA) and Sph196r (CTCGGCGATAAATCTTTGGAC) were developed to detect *Methanothermobacter* sp. and *Sphingomonas* sp., respectively. The primers were designed using the BioEdit and Lasergene v. 5.06 software packages. Melting temperature was analyzed with Oligo v.6 program and used in the PCR procedure. The specificity of the primers was then tested by using DNA extracts from strains and clones with 16S rDNA inserts representing different species. Each specific primer gave positive PCR results for the corresponding target 16S rDNA and negative PCR results for nontarget 16S rDNA.

3. Results

3.1. Physicochemical characteristics and microbial processes in formation water of the Dagang oilfield

The natural formation water of the Kongdian bed of the Dagang oilfield belonged to the sodium hydrocarbonate type; it had low salinity (5.6–6.7 g L^{-1}), was slightly alkaline (pH 7.1–7.6), and contained less than 5 mg of acetate and 433–670 mg of hydrocarbonate per 1 L (Table 1, well 1066-1). Sulfate concentration did not exceed 26 mg L^{-1}. H_2S was not detected.

Since 1975, the North block of the Kongdian bed of the Dagang oilfield was exploited with the water-flooding method for the enhancement of oil recovery. In 2001–2007, MEOR (microbial enhancement of oil recovery) biotechnology was applied at the North block. The biotechnology involved injection of a water-air mixture with dissolved nitrogen and phosphorous mineral salts for the stimulation of the indigenous microorganisms. During the periods of biotechnological treatment, bicarbonate content in formation waster increased from 0.4–0.6 to 0.7–1.8 g L^{-1}, sulfate, from 0 to 12–72 mg L^{-1}, acetate, from 5 to 160.7 mg L^{-1}, formate, from 0 to 67.4 mg L^{-1}, and *iso*-butyrate, from 0 to 98.2 mg L^{-1} [5,6,25]. The chemical characteristics of formation waters in the course of the biotechnological treatment (January, 2002) are given

in Table 1.

In waters of the North block the number of cultivable fermentative bacteria varied from 10 to 10^7 cells mL^{-1}, of sulfate reducers, from 0 to 10^7 cells mL^{-1}, and of methanogens, from 0 to 10^4 cells mL^{-1} (Figure 1a, January, 2002). Aerobic bacteria were present in low numbers in the zone of production wells. The numbers of methanogens obtained on media with H_2+CO_2 were usually 10 times higher than those obtained on media with acetate. Microscopic analysis revealed a dominance of rod-shaped cells growing on both media for methanogens.

The rates of anaerobic processes in formation waters. Thermophilic sulfate reduction and methanogenesis were registered in the formation waters of the Dagang oilfield (Table 1). In January 2002, the rate of sulfate reduction was in the range 0.06–205.8 μg S^{2-} L^{-1} day^{-1}. The rates of methanogenesis from labeled carbonate and 2-^{14}acetate ranged from 0.037 to 6.776 and from 0.002 to 3.16 μg CH_4 L^{-1} day^{-1}, respectively. The maximal values of methanogenesis rates from labeled carbonate and acetate in waters from production wells of the Kongdian bed for the period 06.2001–12.2007 are given in Figure 1b.

3.2. Archaeal 16S rRNA Gene Clone Libraries from Formation Waters

The archaeal clone libraries obtained from the DNA of methanogenic enrichments and formation water collected outside (well 1066-1) and inside the zone of the biotechnological treatment (North Block), contained 785 clones (Table 2, Figure 2).

Our preliminary study revealed predominance of the members of the genus *Methanothermobacter* in combined first generation methanogenic enrichments [5]. Screening of the clones using primers M400r and A109f, as well as direct DNA sequencing of the clones revealed that methanogens of the order *Methanobacteriales* predominated in these communities (97% of all archaeal clones); H_2/CO_2 is the main growth substrate for these organisms. During the trial of the MEOR technology, acetate content in formation water reached 65–160 mg L^{-1} [5,6]. During that period, in the archaeal library from the production waters (785 clones) the phylotypes related to organotrophic methanogens *Methanosaeta thermophila* (3 clones), *Methanomethylovorans thermophila* (2 clones), and uncultured members of the order *Methanomicrobiales* (2 clones) were detected. The results of phylogenetic analysis of archaeal 16S rRNA gene sequences are presented on Figure 2.

3.3. Bacterial 16S rRNA Gene Library from Formation Waters

Bacterial 16S rRNA gene library constructed using the DNA of formation water from the zone of the biotechnological treatment (North Block) contained 453 clones (Figure 3). Preliminary sequencing revealed predominance of bacteria of the genus *Sphingomonas*. Screening of the clones using the *Sphingomonas*-specific primer revealed 333 clones belonging to

this genus. Sequencing of the DNA of the remaining bacterial clones revealed 30 phylogenetic groups belonging to *Proteobacteria*, *Clostridia*, *Thermotogae*, *Dictyoglomi*, *Bacteroidetes*, *Actinobacteria*, and *Nitrospirae*. Representatives of the genera *Sphingomonas*, *Afipia*, *Labrys*, *Phyllobacterium*, *Bradyrhizobium*, *Variovorax*, *Curvibacter*, *Polaromonas*, *Thauera*, *Hydrogenophilus*, *Delftia*, *Leptothrix*, *Achromobacter*, and *Pseudomonas* were revealed in the microbial community. Members of these genera are mesophilic aerobic organotrophs, some bacteria (*Thauera*, *Hydrogenophilus*) are able to grow by nitrate reduction; they probably arrived into the reservoir with cooled injected water. The group of clostridia comprised thermophilic bacteria with fermentative metabolism (*Thermoanaerobacter*, *Thermoterrabacterium*, *Thermovenabulum*), sulfate reducers (*Thermacetogenium*, and *Desulfotomaculum*), and syntrophic bacteria (*Thermovirga*). Thermophilic bacteria with fermentative metabolism belonging to other phylogenetic subdivisions (*Fervidobacterium*, *Thermotoga*, *Dictyoglomus*, *Pedobacter*, and *Dysgonomonas*) were also revealed. Bacteria of the genera *Fervidobacterium* and *Thermotoga* are common in high-temperature oilfields of France, United States, Africa, Japan, and Western Siberia [3,4,32-35]. The phylotype of a sulfate-reducing bacterium *Thermodesulfovibrio* (*Nitrospirae*) was also found in the community. A related phylotype was reported in oilfield formation water [36] and in methanogenic enrichment [5].

3.4. Characterization of the Thermophilic Association Producing Methane from Acetate

Methane formation was observed on the mineral medium [28] with various substrates (acetate, propionate, butyrate, ethanol, or benzoate) inoculated with formation water. Amendment of the medium with ampicillin and/or penicillin resulted in inhibition of growth and methanogenesis from acetate (Figure 4). After treatment with 2-bromo-ethane sulfonate (BES), a specific inhibitor of methanogenesis, methane formation also ceased. These findings demonstrated that both archaeal and bacterial components of the enrichments were necessary for acetate degradation and methane production. Microscopy of methanogenic enrichments growing on acetate revealed rod-shaped cells of different sizes. No cells were found resembling the acetate-utilizing methanogens of the genera *Methanosarcina* or *Methanothrix* (formerly *Methanosaeta*). An enrichment, which exhibited steady methane formation for 3 years, was able to produce $^{14}CH_4$ from $^{14}CH_3COONa$. Addition of unlabeled CH_3COONa (200 mg L^{-1}) resulted in a 4000-fold increased methanogenesis rate (from 0.22 to 880.4 μg CH_4 L^{-1} day^{-1}).

3.5. Isolation of Pure Cultures of Anaerobic Microorganisms from Methanogenic Enrichments

From first-generation methanogenic enrichments grown on media with acetate and H_2+CO_2, two pure cultures of H_2-utilizing methanogens were isolated (strains KZ24a and KZ3-1, respectively). Strain KZ24a was most closely related to the species *Methanothermobacter wolfeii* (DSM 2970, AB104858); another strain, KZ3-1, to *Methanothermobacter*

thermautotrophicus (Delta H, AE000666) (99% similarity of 16S rRNA genes). The 16S rRNA gene of the strain KZ3-1 contained the signature nucleotides (at positions A164, A166, and C188) characteristic of *M. thermautotrophicus* Delta H, and the gene of the strain KZ24a contained the signature nucleotides (C195, A233, C261, T1218, T1274 и T1314) characteristic of *M. wolfeii* [37].

The cells of strains KZ3-1 and KZ24a were nonmotile straight rods with sizes of 0.35–0.5×3–7 μm and 0.35–0.5×2–5 μm, respectively. Both strains grew within the temperature range from 37 to 65°C. The optimum growth temperatures for strains KZ3-1 and KZ24a were 60 and 65°C, respectively. Salinity optimum for both strains was 0–1.0% NaCl; higher NaCl concentrations (1.5–4%) inhibited methane formation. Both strains utilized H_2/CO_2 as the source of carbon and energy; acetate, pyruvate, propionate, methanol, ethanol, and 3-ethylamine were not utilized. Weak growth of strain KZ3-1 occurred on formate. Both strains were resistant to ampicillin and penicillin (2 mg mL^{-1}). Amendment of the medium with BES (2–10 mM) inhibited growth and methanogenesis.

Attempts to isolate thermophilic acetate-utilizing methanogens from enrichment cultures obtained from the Dagang oilfield were unsuccessful.

For isolation of the bacterial component of the thermophilic methanogenic association, which was responsible for acetate oxidation, we used sodium pyruvate as a substrate. Since our previous analysis of the 16S rRNA gene clone libraries obtained from first-generation methanogen cultures [5] revealed a phylotype distantly related to *Thermacetogenium phaeum*, syntrophic acetate degradation was accepted as a working hypothesis. This bacterium is capable of syntrophic acetate degradation to methane in association with H_2-utilizing methanogens. Hattori and co-authors [38] used sodium pyruvate as a substrate for the isolation of *T. phaeum* from a sewage treatment methanogenic reactor. We also used sodium pyruvate, planning to isolate *T. phaeum*.

On media with pyruvate, ten strains of anaerobic rod-shaped motile spore-forming bacteria were isolated from the acetate-utilizing methanogenic association obtained from production well 1017-7. All strains were phylogenetically related to *Thermoanaerobacter ethanolicus* (99.6% similarity of 16S rRNA genes). Two strains, 1017-7b and 1017-7d, were chosen for detailed study. Both strains grew at the temperatures from 40 to 70°C, with an optimum at ~60°C. No growth occurred at 35 and 75°C. Both strains grew at pH range from 5.8 to 8.5 and NaCl concentration in the medium from 0 to 2% with an optimum at 0.5%; higher NaCl concentrations (4–8%) inhibited growth. The strains fermented glucose, fructose, mannose, galactose, ribose, lactose, sucrose, maltose, cellobiose, starch, and pyruvate; growth did not occur on media with acetate, lactate, methanol, ethanol, glycerol, arabinose, raffinose, rhamnose, trehalose, and mannitol. Yeast extract (0.5 g L^{-1}) was required as a growth factor. When

grown with glucose, the strains reduced thiosulfate, sulfite, and sulfur to sulfide; these traits were not previously known for this species. Sulfate, nitrate, and nitrite were not reduced.

3.6. Reconstruction of Syntrophic Acetate Degradation to Methane by the Binary Culture

For the reconstruction of syntrophic growth on acetate (80 мМ), pure cultures of the methanogen *Methanothermobacter* (strains KZ3-1 and KZ24a) and *Thermoanaerobacter* (strains 1017-7b and 1017-7d) were combined. Methane was produced by all four *Thermoanaerobacter – Methanothermobacter* binary cultures (strains 1017-7b + KZ3-1, 1017-7d + KZ3-1, 1017-7b + KZ24a, and 1017-7d + KZ24a), while no individual strain was capable of methanogenesis under these conditions. The experiment with radiolabeled $^{14}CH_3COONa$ confirmed that methane was produced from acetate only by binary cultures (Figure 5a). In this experiment, the methanogenic association 24Ac, which grew steadily on acetate, was used as the positive control. The sulfate-reducing bacterium *Desulfotomaculum kuznersovii* 17^T, which is known to be capable of sulfate-reducing growth on acetate, is often detected in the course of molecular investigation of the microorganisms of high-temperature oilfields. While we expected replacement of sulfate by a biological electron acceptor to result in acetate degradation, the binary culture of *D. kuznetsovii* 17^T and *Methanothermobacter wolfeii* KZ24a was, however, unable to produce methane. Conversion of acetate to methane at 60°C by the *M. thermautotrophicus – T. ethanolicus* co-culture is shown on Figure 5b.

The rates of methane formation from labeled 2-$^{14}CH_3COONa$ by the *M. thermautotrophicus* KZ3-1 – *T. ethanolicus* 1017-7b binary culture and by the 24A enrichment determined in the Zeikus mineral medium supplemented with unlabeled CH_3COONa (200 mg L^{-1}) were similar: 143.75 and 148.34 μg CH_4 L^{-1} day^{-1}, respectively. No formation of methane from 2-$^{14}CH_3COONa$ occurred in pure cultures of strains KZ3-1 and 1017-7b.

3.7. 16S rRNA Clone Gene Libraries from the Methanogenic Association 24A grown on acetate

Two 16S rDNA clone libraries were constructed to examine the phylogenetic diversity of the acetate-degrading methanogenic association 24A. All 96 archaeal clones belonged to one phylotype, *Methanothermobacter thermautotrophicus*. Bacterial clones (46) formed 4 closely related phylotypes belonging to "*Thermoanaerobacter tengcongensis*" MB4 (41 of 46 clones), "*Thermoanaerobacter subterraneus*" (T) SEBR 7858 LA61 (2 clones), *Thermoanaerobacter keratinophilus* 2KXI (2 clones), and *Thermotoga subterranea* SL1 (1 clone). Bacteria "*T. tengcongensis*" and "*T. subterraneus*" are in fact subspecies of the species classified as *Caldanaerobacter subterraneus* [39]. Thus, both H_2-utilizing methanogens of the genus *Methanothermobacter* and anaerobic organotrophic bacteria of the *Thermoanaerobacter – Caldanaerobacter* group are probably responsible for methane formation from acetate in the thermophilic methanogenic association.

4. Discussion

Investigation of formation water from the Dagang high-temperature oilfield by microbiological, radioisotope, and molecular biological methods revealed a diverse, geochemically active microbial community. This community included aerobic organotrophs, as well as anaerobic fermentative bacteria and archaea, sulfate-reducing and syntrophic bacteria, and methanogenic archaea.

In almost sulfate-free waters of the Dagang oilfield, methanogenesis is the main terminal process. Inoculation of formation water into the media with acetate or H_2+CO_2 resulted in methane production. Radioisotope methods also revealed methanogenesis in formation water supplemented with each of the labeled substrates ($^{14}CH_3COONa$ and $Na_2{}^{14}CO_3$). The rates of methanogenesis before the biotechnological treatment were comparable to the values reported for the Mykhpayskoe, Talinskoe, and Samotlor high-temperature oilfields (Western Siberia) and for the Liaohe oilfield (P.R. China), which are also operated with water-flooding [4,5,40]. Injection of the water-air mixture into the oilfield resulted in a significant increase of the rates of methanogenesis from labeled carbonate and from 2-^{14}C-acetate.

These data disagreed with the results of molecular investigation of methanogenic enrichments obtained from the Dagang oilfield [5]. On media with either H_2/CO_2 or acetate, the archaeal component of the enrichments belonged to *Methanothermobacter* sp. (*M. thermautotrophicus*), which consumes hydrogen and carbon dioxide as basic substrates. The genes of acetate-utilizing archaea were not revealed. Phylotypes of *Thermococcus* spp. were also found among archaeal 16S rRNA genes. Bacterial 16S rRNA genes were related to those of the orders *Thermoanaerobacteriales* (*Thermoanaerobacter*, *Thermovenabulum*, *Thermacetogenium*, and *Coprothermobacter* spp.), *Thermotogales*, *Nitrospirales* (*Thermodesulfovibrio* sp.), and *Planctomycetales*. The library was found to contain the 16S rRNA gene with low similarity (95%) to the 16S rRNA gene of *Thermacetogenium phaeum*, a bacterium capable of oxidizing acetate in the course of syntrophic growth with H_2-utilizing methanogens.

Special attention was paid to acetate-utilizing methanogenic associations. Two mechanisms of acetate degradation to methane are theoretically possible. At high acetate concentrations, thermophilic methanogens *Methanosarcina thermophila* and *Methanothrix* (*Methanosaeta*) *thermophila* can carry out direct methane formation by detachment of the methyl group [41,42]. These aceticlastic methanogens, however, have not yet been isolated from high-temperature oilfields.

The mechanism of methane formation from acetate by mesophilic syntrophic associations of an acetate-oxidizing organism and a H_2-utilizing methanogen was originally proposed in 1936 by Barker and Van Niel [see for review 41]. “Oxidation” of acetate to CO_2 and H_2 occurs at the first stage; CO_2 is then reduced to methane with hydrogen. The process of syn-

trophic thermophilic acetate degradation resulting in methane formation was first observed in the course of operating a laboratory reactor for decomposition of lignocellulose-containing solid waste [43,44]. At low acetate concentrations in the system, syntrophic methane production occurs, rather than aceticlastic methanogenesis [42]. Moreover, at high temperature syntrophic acetate oxidation is thermodynamically more preferable [41]. Thermophilic syntrophic methanogenic degradation of propionate, butyrate, and long-chain fatty acids was described. It was shown that the microbes involved in syntrophy have evolved molecular mechanisms to establish specific partnerships and interspecies communication, resulting in efficient metabolic cooperation [45].

Our analysis of archaeal 16S rRNA genes (785 clones) obtained from formation water and methanogenic enrichments of the Dagang oilfield demonstrated that H_2-utilizing methanogens of the order *Methanobacteriales* (*Methanothermobacter* sp., 97% of the total number of clones) predominated in the microbial community. No phylotypes of acetate-utilizing methanogens were revealed in the primary methanogenic enrichments and in the original formation waters. Five phylotypes of methanogens of the order *Methanosarcinales* were found only under considerably increased acetate concentrations in formation water.

The sequences related to the A1m_OTU3 [DQ097668] phylotype of a methanogen from the Kongdian oilfield [5] were subsequently revealed in the course of molecular investigation of gas-associated formation water of a gas-producing well in a natural high-temperature gas reservoir in Japan, as well as in the microbial community of formation water from the high-temperature Shengli oilfield and in enrichment culture derived from this source and producing methane from oil [7,9,46]. Thermophilic hydrogenotrophic methanogens isolated from these environments were assigned to two closely related new species, *Methanothermobacter crinale* and *Methanothermobacter tenebrarum* [7,47]. When analyzing the GenBank data, Cheng and co-authors [7] showed that the phylotypes related to those of A1m_OTU3 and *M. crinale* (GU357468) were also present in the 16S rRNA libraries obtained from various thermophilic anaerobic reactors treating glucose and mixtures of acetate, propionate, and sucrose; propionate and/or acetate; acetate and butyrate, and swine manure; sewage sludge; and a hot-rot compost suspension. The phylotype related to those of *M. crinale* and A1m_OTU3 predominated also in the clone library of the thermophilic crude-oil-degrading methanogenic consortium isolated from the production water of oil reservoirs by Gieg and co-authors [48]; they also suggested that this methanogen could be involved in syntrophic decomposition of crude oil. Syntrophic acetate oxidation as the main methanogenic pathway in a high-temperature Yabase oilfield (Japan) was also shown [49].

In our studies pure cultures of H_2-utilizing methanogens (*M. thermautotrophicus* and *M. wolfeii*) and acetate-oxidizing bacteria (*T. ethanolicus*) were isolated from thermophilic syntrophic methanogenic enrichments. None of the pure cultures was capable of growth on ac-

etate. The binary *Methanothermobacter – Thermoanaerobacter* culture was found to degrade acetate to methane, and its composition remained stable during subsequent transfers to fresh acetate-containing medium. The mesophilic association of *Clostridium ultunense* and an H_2-utilizing methanogen is known to lose its capacity for syntrophic growth after transfers [50]. The thermophilic syntrophic association of a methanogen and *T. phaeum* also retained its ability to form methane from acetate after a series of transfers [38].

Molecular investigation of the new thermophilic methanogenic association 24A, which was maintained on acetate for a long time, revealed that, apart from *Thermoanaerobacter* species (*T. ethanolicus*), *Caldanaerobacter* species (*C. subterraneus*) may also act as an acetate-oxidizing component. *C. subterraneus* uses carbohydrates as fermented substrates, with acetate, L-alanine, H_2, and CO_2 as the major end products [39]. These bacteria are capable of thiosulfate reduction to sulfide. In the presence of thiosulfate, the glucose metabolic profile of *C. subterraneus* changed: acetate production increased and L-alanine was not produced anymore [39]. Ability to degrade acetate was not included in the description of *C. subterraneus* and *T. ethanolicus*. Taking into account similar physiological characteristics of these species and the presence of *C. subterraneus* 16S rRNA gene in the library of associations degrading acetate to methane, we can propose that the bacterium *C. subterraneus* is capable of syntrophic growth on acetate in the presence of hydrogenotrophic methanogens.

Over 30 phylogenetic groups of thermophilic and mesophilic bacteria were revealed in formation water. The representative phylotypes belonged to the classes of *Alpha*-, *Beta*-, *Gamma*-, and *Deltaproteobacteria*, *Clostridia*, *Thermotogae*, *Dictyoglomi*, *Bacteroidetes*, *Actinobacteria*, and *Nitrospirae*. The phylotypes of a range of fermentative and syntrophic bacteria closely related to *Thermovirga lienii* (fam. *Syntrophomonadaceae)* and *T. phaeum* were detected. Representatives of these two metabolic groups could be involved in syntrophic acetate degradation to methane.

Anaerobic organotrophic bacteria, including the members of the *Thermoanaerobacter – Caldanaerobacter* group, are common in the oil-bearing horizons. These bacteria were usually isolated on media with peptone, starch, or glucose; since these substrates do not occur in formation waters, the ecological function of these fermentative bacteria remained unclear.

In our studies, it was shown for the first time that bacteria of the *Thermoanaerobacter – Caldanaerobacter* group in the presence of hydrogenothrophic methanogens are capable of syntrophic acetate oxidation to H_2 and CO_2, which is then reduced to methane. The aceticlastic pathway of methanogenesis is probably not pronounced at high temperature so that direct decomposition of acetate to methane contributes but insignificantly to methanogenesis. In high-temperature oilfields with low concentrations of acetate and sulfate in formation water, other acetate-oxidizing prokaryotes, such as *Coprothermobacter* [51] or sulfate-reducing bacteria

[52], may form acetate-degrading methanogenic associations with hydrogenotrophic methanogens; the syntrophic mechanism of the terminal stage of biodegradation of the oil organic matter is in this case preserved.

Our work suggests important biogeochemical conclusions. Since methane formation in the presence of acetate may be carried out either by aceticlastic methanogens or by syntrophic associations, quantitative assessment of the rates of these processes requires experiments with labeled acetate and no antibiotic, as well as with a penicillin group antibiotic suppressing the growth of bacteria involved in syntrophic acetate oxidation. The difference between these values will indicate the rate of aceticlastic methanogenesis.

Thus, our investigation made it possible to specify the composition of the microbial trophic chain, to estimate the geochemical activity of microorganisms, and to obtain new information concerning the terminal stages of oil biodegradation in high-temperature oilfields.

5. Tables

Table 1: Chemical characteristics and rates of microbial processes in formation waters of the Dagang oilfield

Well No.	Total salinity, mg L^{-1}	K^++Na^+, mg L^{-1}	Mg^{2+}, mg L^{-1}	Ca^{2+}, mg L^{-1}	Cl^-, mg L^{-1}	SO_4^{2-}, mg L^{-1}	HCO_3^-, mg L^{-1}	Acetate, mg L^{-1}	Methanogenesis rate, μg of CH_4 L^{-1} day^{-1}		Sulfate reduction rate, μg of S^{2-} L^{-1} day^{-1}	δ $^{13}C/CH_4$, ‰
									From $Na_2{}^{14}CO_3$	From 2-^{14}C-acetate		
63	6797	2458	46	54	3695	41	503	30.7	0.037	0.290	205.804	-42.0
1002-1	6675	2438	31	39	3572	0	595	29.52	0.880	4.370	0.455[a]	-41.9
1008-1	7024	2589	31	36	3618	35	712	97.6	0.700	1.740	9.367	-42.1
1012	6966	2476	32	52	3766	39	610	0.5	0.250	0.002	22.219	-43.3
1012-1	6999	2548	37	49	3783	18	564	4.2	0.060	0.994	1.629	-43.0
1015-1	6425	2349	33	49	3537	0	457	38.2	0.405	0.425	18.950	-41.7
1017-3	6825	2486	24	34	3519	0	762	21.7	0.604	0.943	0.060	-41.7
1017-4	6584	2384	36	54	3537	24	549	0.76	5.116	0.789	11.163	-41.8
1017-5	7138	2609	28	34	3731	35	701	3.6	0.441	0.120	2.776	-42.7
1017-7	7270	2643	27	30	3695	6	869	2.9	6.776	3.160	4.586	-43.5
1050-3	6897	2501	35	54	3811	37	459	29.1	0.040	0.050	87.288	-42.6
1092	6491	2343	36	44	3396	47	625	4.14	0.708	1.96	23.245	-41.6
1066-1[b]	5658	2049	32	73	3069	0	435	1.4	0	0.004	0.010	-42.1

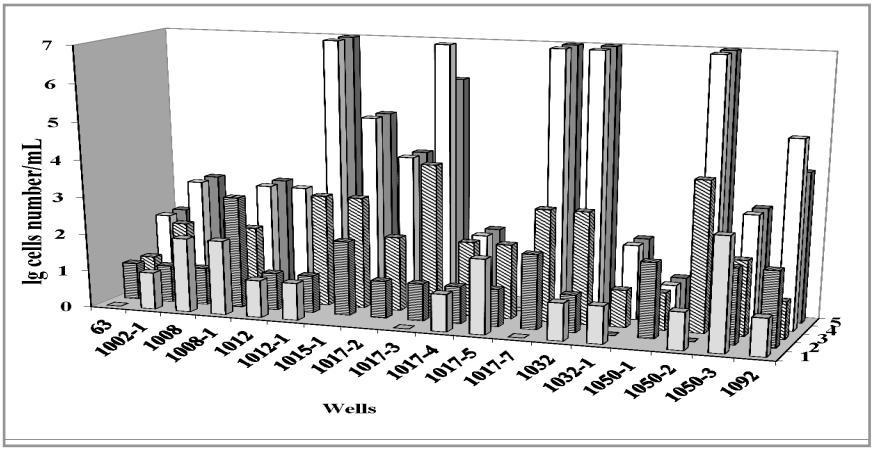
[a] In the absence of sulfate in water the rate of sulfate reduction was calculated using quantity of added label sulfate (20 μg L^{-1}), in the absence of acetate the rate of methanogenesis was calculated using quantity of added label acetate (70 μg L^{-1});
[b] Well located outside of the zone of biotechnological treatment.

Table 2: The total number of sequenced and screened clones of archaeal 16S rRNA genes in the libraries obtained from DNA of methanogenic enrichments and of formation waters from the Dagang oilfield

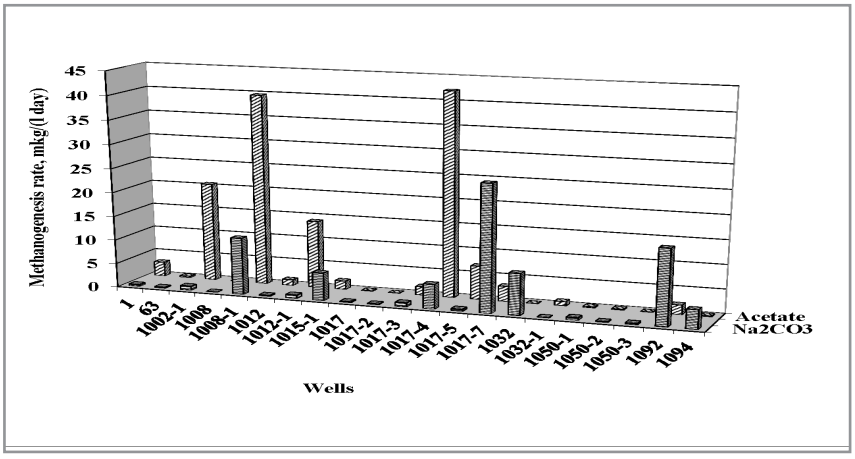
Library of 16S rRNA genes	Number of archaeal 16S rRNA genes in the library	Number of *Methanothermobacter* sp. 16S rRNA genes in the library	References
Combined first-generation methanogenic enrichments	102	101	§
Natural formation water 1066-1	64 + 343*	63 + 343*	#
Formation water from the zone of the pilot trial#	38 + 143*	17 + 143*	#
Methanogenic association 24A grown on acetate	41 + 54*	41 + 54*	#
Total number of clones	785	762	

* Clones revealed positive signal by the PCR with the *Methanothermobacter*-specific primer.
§ The data from the paper [5].
# The data from the paper [25].

6. Figures



(a)



(b)

Figure 1: The number of thermophilic microorganisms in formation water from production wells of the Kongdian bed of the Dagang oilfield in January 2002 (a) and maximal values of methanogenesis rates from labeled $Na_2{}^{14}CO_3$ and 2-$^{14}CH_3COONa$ in the waters from production wells during the period 06.2001–12.2007 (b). Designation on Figure 1a: (1), aerobic organotrophs; (2), methanogens, growing on H_2/CO_2; (3), methanogens, growing on acetate; (4), sulfate-reducers; (5), fermentative bacteria.

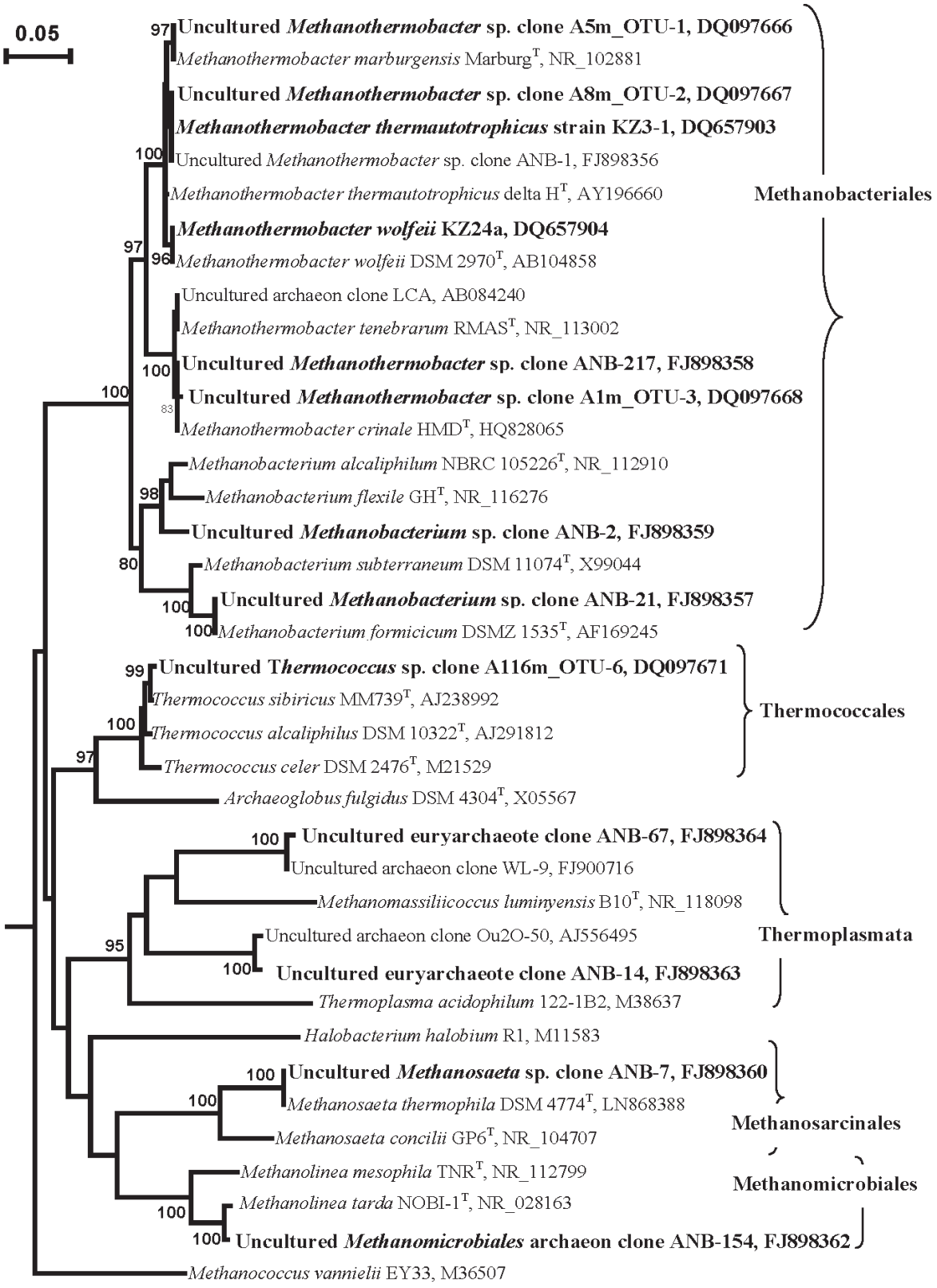


Figure 2: Phylogenetic tree constructed on the basis of analysis of the 16S rRNA gene sequences of pure cultures *Methanothermobacter* KZ24a and KZ3-1 and of representatives of the domain *Archaea* revealed in the clone libraries from methanogenic enrichments (clones designated A1m – A116m) and from formation water (North Block, clones designated ANB). The sequences obtained in our studies are in bold. Scale bar, five nucleotide substitutions for each 100 nucleotide base pairs. Numbers at the nodes indicate the percentage of bootstrap values for the clade in 1000 replications; only values above 80% are shown.

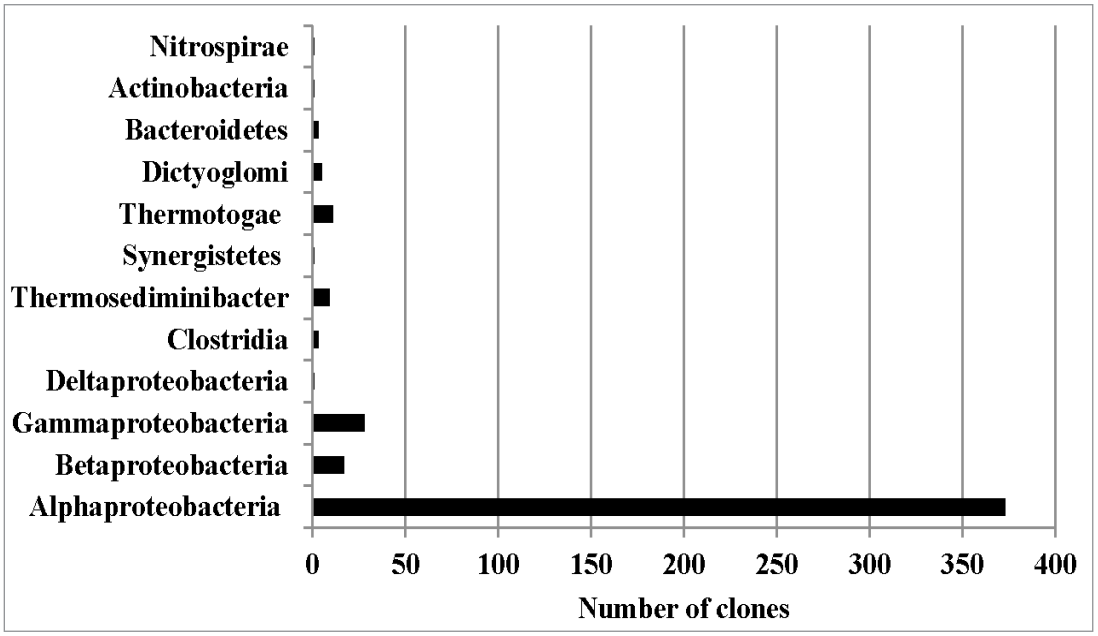


Figure 3: Distribution of 16S rRNA genes of *Bacteria* in clone library of formation water from the zone of the pilot trial of the Dagang oilfield (435 clones total) among high-rank taxa.

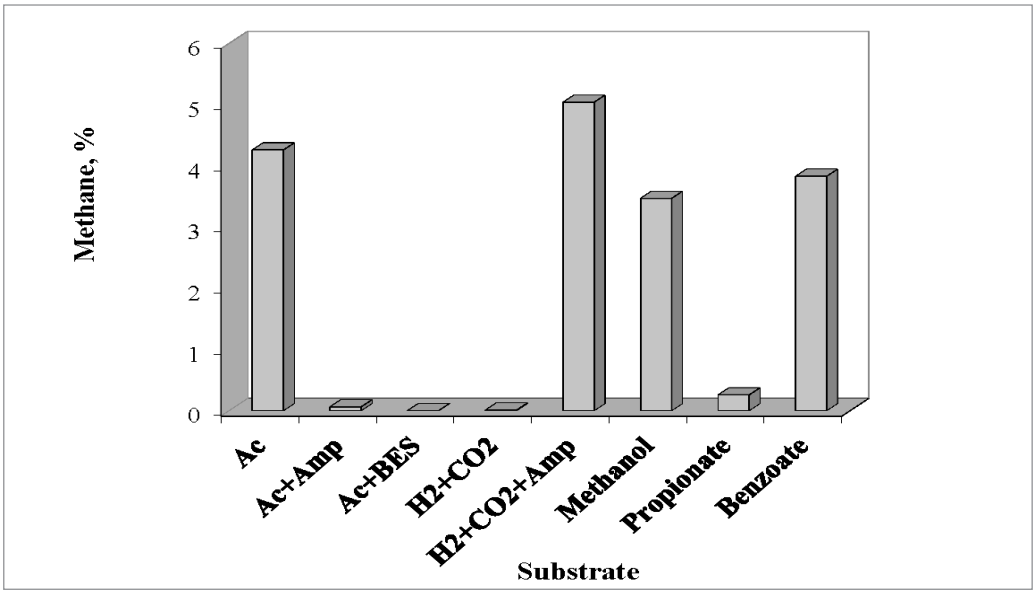
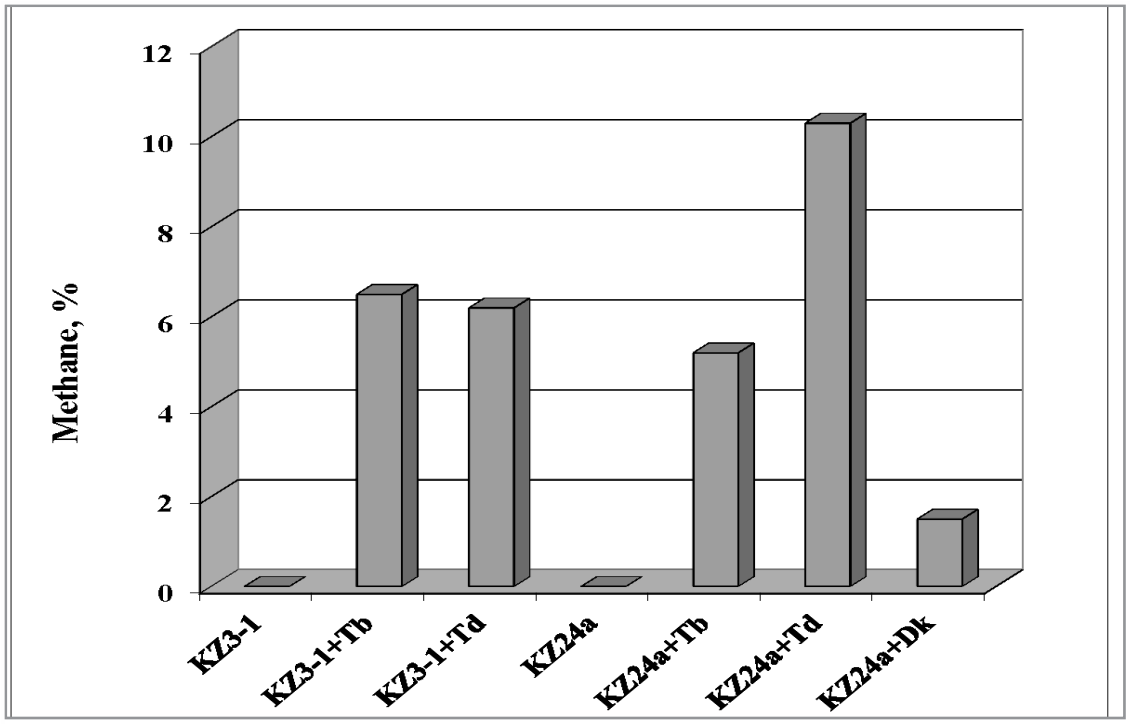
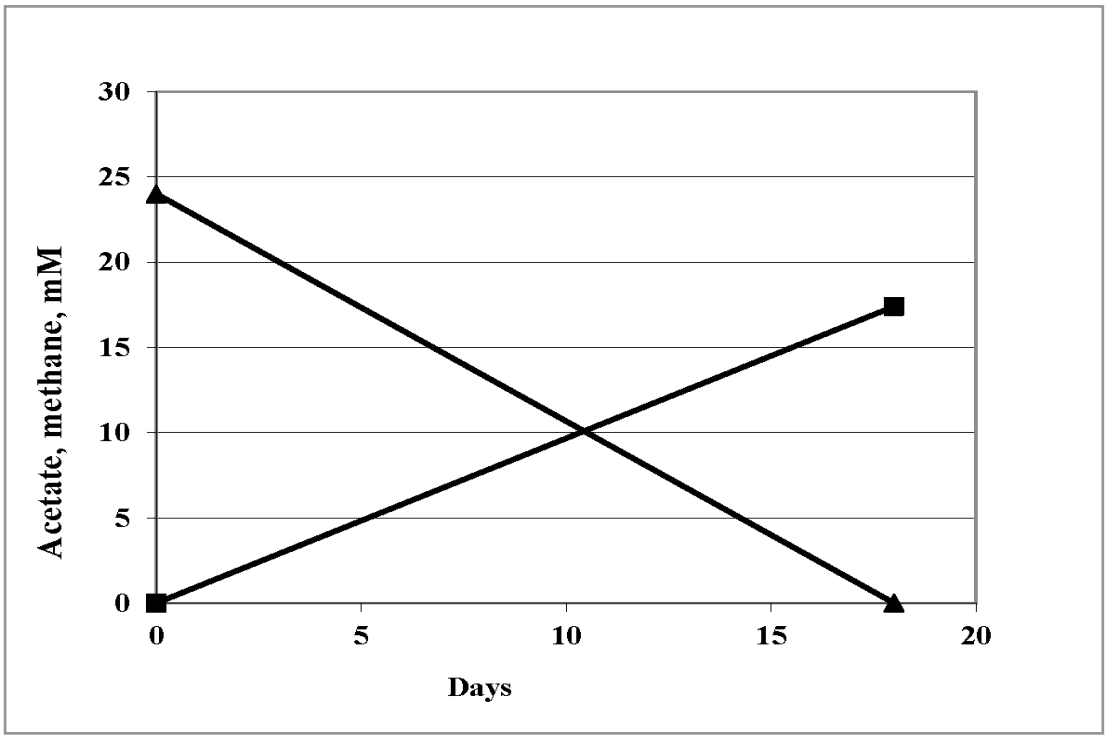


Figure 4: Methane production (%) by enrichments obtained from formation water of the Kongdian bed on media with acetate (Ac), acetate amended with ampicillin (Ac + Amp), acetate amended with 2-bromo-ethane sulfonate (Ac + BES), on H_2+CO_2, H_2+CO_2 with ampicillin (H_2+CO_2 + Amp), methanol, propionate and benzoate.



(a)



(b)

Figure 5: Methane formation in the presence of acetate by pure and binary cultures of the methanogen M. *wolfeii* KZ24a and T. *ethanolicus* strains 1017-7b (Tb) and 1017-7d (Td) and *Desulfotomaculum kuznetsovii* 17^T (Dk) during 18 days of incubation at 60°C (a) and acetate conversion to methane at 60°C the by co-culture of M. *wolfeii* KZ24a – T. *ethanolicus* 1017-7b (b).

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8. References

1. Magot M. Indigenous microbial communities in oil fields. In ‘‘Petroleum microbiology’’ (B. Ollivier and M. Magot, Eds.), ASM, Washington, DC. 2005; 21–34.

2. Orphan VJ, Goffredi SK, Delong EF, Boles JR. Geochemical influence on diversity and microbial processes in high-temperature oil reservoirs. Geomicrobiol J. 2003; 20: 295-311.

3. Orphan VJ, Taylor LT, Hafenbradl D, Delong EF. Culture-dependent and culture-independent characterization of microbial assemblages associated with high-temperature petroleum reservoirs. Appl Environ Microbiol. 2000; 66: 700-711.

4. Bonch-Osmolovskaya EA, Miroshnichenko ML. Lebedinsky AV, Chernyh NA, Nazina TN, Ivoilov VS, Belyaev SS, Boulygina ES, Lysov YuP., Perov AN, Mirzabekov AD, Hippe H, Stackebrandt E, L’Haridon S, Jeanthon C. Radioisotopic, culture-based, and oligonucleotide microchip analyses of thermophilic microbial communities in a continental high-temperature petroleum reservoir. Appl Environ Microbiol. 2003; 69: 6143-6151.

5. Nazina TN, Shestakova NM, Grigor’yan AA, Mikhailova EM, Turova TP, Poltaraus AB, Feng C, Ni F, Belyaev SS. Phylogenetic diversity and activity of anaerobic microorganisms of high-temperature horizons of the Dagang Oilfield (P.R. China). Microbiology. 2006; 75: 55-65.

6. Nazina TN, Grigor’yan AA, Shestakova NM, Babich TL, Pavlova NK, Ivoilov VS, Belyaev SS, Ivanov MV. Feng Q, Ni F, Wang J, She Y, Xiang T, Mei B, Luo Z. MEOR study enhances production in a high-temperature reservoir. World Oil J. 2008, June; 97-101.

7. Cheng L, Dai L, Li X, Zhang H, Lu Y. Isolation and characterization of *Methanothermobacter crinale* sp. nov, a novel hydrogenotrophic methanogen from Shengli oil field. Appl. Environ. Microbiol. 2011; 77: 5212-5219.

8. Jiménez N, Morris BEL, Cai M, Gründger F, Yao J, Richnow HH, Krüger M, Evidence for in situ methanogenic oil degradation in the Dagang oil field. Organic Geochemistry. 2012; 52: 44-54.

9. Mbadinga SM, Li K-P, Zhou L., Wang L-Y, Yang S-Z, Liu J-F, Mu B-Z. Analysis of alkane-dependent methanogenic community derived from production water of a high-temperature petroleum reservoir. Appl Microbiol Biotechnol. 2012. DOI 10.1007/s00253-011-3828-8.

10. Kuznetsov SI. Investigation of the possibility of contemporaneous formation of methane in gas-petroleum formations in the Saratov and Buguruslan regions. Microbiology (in Russian). 1950; 19: 193-202.

11. Belyaev SS, Wolkin R, Kenealy WR, DeNiro MJ, Epstein S, Zeikus JG. Methanogenic bacteria from the Bondyuzhskoe oil field: general characterization and analysis of stable-carbon isotopic fractionation. Appl Environ Microbiol. 1983; 45: 691-697.

12. Obraztsova AY, Shipin OV, Bezrukova LV, Belyaev SS. Properties of the coccoid methylotrophic methanogen *Methanococcoides euhalobius* sp. nov. Microbiology. 1987; 56: 661-665.

13. Davydova-Charakhch’yan IA, Kuznetsova VG, Mityushina LL, Belyaev SS. Methane-forming bacilli from oil fields of Tatarstan and Western Siberia. Microbiology. 1992; 61: 299-305.

14. Davidova IA, Harmsen HJ, Stams AJ, Belyaev SS, Zehnder AJ. Taxonomic description of *Methanococcoides euhalobius* and its transfer to the *Methanohalophilus* genus. Antonie van Leeuwenhoek. 1997; 71: 313-318.

15. Belyaev SS, Borzenkov IA. Microbial transformation of low-molecular-weight carbon compounds in the deep subsurface. In: Biogeochemistry of global change. NY-London: Chapman & Hall. 1993, 825-838.

16. Rozanova EP, Savvichev AS, Miller YM, Ivanov MV. Microbial processes in a West Siberian oil field flooded with waters containing a complex of organic compounds. Microbiology. 1997, 66: 852–859.

17. Nazina TN, Ivanova AE, Borzenkov IA, Belyaev SS, Ivanov MV. Occurrence and geochemical activity of microorganisms in high-temperature water-flooded oil fields of Kazakhstan and Western Siberia. Geomicrobiol J. 1995; 13: 181-192.

18. Nilsen RK, Torsvik *T. Methanococcus thermolithotrophicus* isolated from North Sea oil field reservoir water. Appl Environ Microbiol. 1996; 62: 728-731.

19. Shestakova NM, Grigor’yan AA, Feng Q, Ni F, Belyaev SS, Poltaraus AB, Nazina TN. Characterization of the

methanogenic community from the high-temperature Dagang oil field (P.R. China). In: Kuznetsov AE, Zaikov GE, editors. New Research on the Environment and Biotechnology. New York: Nova Science Publishers Inc. 2006; 159-174.

20. Li H, Yang SZ, Mu BZ, Rong ZF, Zhang J. Molecular phylogenetic diversity of the microbial community associated with a high-temperature petroleum reservoir at an offshore oilfield. FEMS Microbiol Ecol. 2007; 60: 74-84.

21. Nazina TN, Grigor'yan AA, Shestakova NM, Babich TL, Ivoilov VS, Feng Q, Ni F, Wang J, She Y, Xiang T, Luo Z, Belyaev SS, Ivanov MV. Microbiological investigations of high-temperature horizons of the Kongdian petroleum reservoir in connection with field trial of a biotechnology for enhancement of oil recovery. Microbiology. 2007a; 76: 287-296.

22. Nazina TN, Grigor'yan AA, Feng Q, Shestakova NM, Babich TL, Pavlova NK, Ivoilov VS, Ni F, Wang J, She Y, Xiang T, Mei B, Luo Z, Belyaev SS, Ivanov MV. Microbiological and production characteristics of the high-temperature Kongdian petroleum reservoir revealed during field trial of biotechnology for the enhancement of oil recovery. Microbiology. 2007b; 76: 297-309.

23. Shestakova NM, Tourova TP, Feng Q, Ni F, Belyaev SS, Poltaraus AB, Nazina TN. Phylogenetic diversity and activity of microorganisms in the high-temperature petroleum reservoir. In Abstract Book: 7th Int. Symp. for Subsurface Microbiology. Shizuoka, Japan, November 16-21, 2008; 167.

24. Shestakova NM, Nazina TN, Feng Q, Ni F, Tourova TP, Poltaraus AB, Belyaev SS, Ivanov MV. Syntrophic acetate degradation to methane in a high-temperature petroleum reservoir. 2nd International conference on applied microbiology and molecular biology in oil systems. 17-19th June 2009, Aarhus, Denmark. ISMOS^2 Abstract book. 2009; 48

25. Shestakova NM, Ivoilov VS, Tourova TP, Belyaev SS, Poltaraus AB, Nazina TN. Application of Clone Libraries: Syntrophic Acetate Degradation to Methane in a High-Temperature Petroleum Reservoir: Culture-Based and 16S rRNA Genes Characterization. Applied Microbiology and Molecular Biology in Oilfield Systems. Proceedings from the International Symposium on Applied Microbiology and Molecular Biology in Oil Systems (ISMOS-2), 2009. Whitby, C., Lund Skovhus, T. (Eds.). Springer Netherlands. 2011; Chapter 6. 45-53.

26. Hungate RE. A roll tube method for the cultivation of strict anaerobes. Methods in Microbiology. 1969; 3b: 117-132.

27. Postgate JR. The sulfate-reducing bacteria, 2nd ed. Cambridge University Press, Cambridge, United Kingdom. 1984.

28. Zeikus JG, Weimer PJ, Nelson DR, Daniels L. Bacterial methanogenesis: acetate as a methane precursor in pure culture. Arch Microbiol. 1975; 104: 129-134.

29. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol. 1991; 173: 697-703.

30. Kolganova TV, Kuznetsov BB, Turova TP. Selection and testing of oligonucleotide primers for amplification sequencing of archaeal 16S rRNA genes. Microbiology. 2002; 71: 283-285.

31. Thompson J.D., Higgins D.G., Gibson T.J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994; 9: 3251-3270.

32. Jeanthon C, Reysenbach AL, L'Haridon S, Gambacorta A, Pace NR, Glénat P, Prieur D. *Thermotoga subterranea* sp. nov., a new thermophilic bacterium isolated from a continental oil reservoir. Arch Microbiol. 1995; 164: 91-97

33. Fardeau ML, Ollivier B, Patel BKC., Magot M, Thomas P, Rimbault A, Rocchiccioli F, Garcia JL. *Thermotoga hypogea* sp. nov., a xylanolytic, thermophilic bacterium from an oil-producing well. Int J Syst Bacteriol. 1997; 47: 1013-1019.

34. Takahata Y, Nishijima M, Hoaki T, Maruyama T. *Thermotoga petrophila* sp. nov. and T*hermotoga naphthophila* sp. nov., two hyperthermophilic bacteria from the Kubiki oil reservoir in Niigata, Japan. Int J Syst Evol Microbiol. 2001; 51: 1901-1909.

35. Gray ND, Sherry A, Hubert C, Dolfing J, Head IM. Methanogenic degradation of petroleum hydrocarbons in subsurface environments: remediation, heavy oil formation, and energy recovery. Adv Appl Microbiol 2010; 72: 137-161.

36. 36. Li H, Yang SZ, Mu BZ, Rong ZF, Zhang J. Molecular analysis of the bacterial community in a continental high-temperature and water-flooded petroleum reservoir. FEMS Microbiol Lett. 2006; 257: 92-98..

37. Wasserfallen A, Nölling J, Pfister P, Reeve J, Macario E. Phylogenetic analysis of 18 thermophilic *Methanobacterium* isolates supports the proposals to create a new genus, *Methanothermobacter* gen. nov., and to reclassify several isolates in three species, *Methanothermobacter thermoautotrophicus* comb. nov., *Methanothermobacter wolfeii* comb. nov., and *Methanothermobacter marburgensis* sp. nov. Int J Syst Evol Microbiol. 2000; 50: 43-53.

38. Hattori S, Kamagata Y, Hanada S, Shoun H. *Thermacetogenium phaeum* gen. nov., sp. nov., a strictly anaerobic, thermophilic, syntrophic acetate-oxidizing bacterium. Int J Syst Evol Microbiol. 2000; 50: 1601-1609.39. Das S, Ray S, Dey S, Dasgupta S (2001) Optimisation of sucrose, inorganic nitrogen and abscisic acid levels for *Santalum album* L. somatic embryo production in suspension culture. Process Biochemistry 37: 51-56

39. Fardeau ML, Salinas MB, L'Haridon S, Jeanthon C, Verhe F, Cayol JL, Patel B.K.C., Garcia J-L, Ollivier B. Isolation from oil reservoirs of novel thermophilic anaerobes phylogenetically related to *Thermoanaerobacter subterraneus*: reassignment of *T. subterraneus, Thermoanaerobacter yonseiensis*, *Thermoanaerobacter tengcongensis* and *Carboxydibrachium pacificum* to *Caldanaerobacter subterraneus* gen. nov., sp. nov., comb. nov. as four novel subspecies. Int J Syst Evol Microbiol. 2004; 54: 467-474.

40. Nazina TN, Xue Y-F, Wang X-Y, Belyaev SS, Ivanov MV. Microorganisms of the High-Temperature Liaohe Oil Field of China and Their Potential for MEOR. Res Environ Biotechnol. 2000; 3: 109-120.

41. Schink B. Energetics of syntrophic cooperation in methanogenic degradation. Microbial Mol Biol Rev. 1997; 61: 262-280.

42. Schink B, Stams A. Syntrophism among prokaryotes. The Prokaryotes. 2006. On-line version. http://141.150.157.117:8080/prokPUB/index.htm.

43. Zinder S, Koch M. Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. Arch Microbiol. 1984; 138: 263-272.

44. Lee MJ, Zinder SH. Isolation and characterization of a thermophilic bacterium which oxidizes acetate in syntrophic association with a methanogen and which grows acetogenically on H_2-CO_2. Appl Environ Microbiol. 1988; 54: 124-129.

45. Kouzuma A, Kato S, Watanabe K. Microbial interspecies interactions: recent findings in syntrophic consortia. Front. Microbiol., 13 May 2015. https://doi.org/10.3389/fmicb.2015.00477.

46. Mochimaru H, Yoshioka H, Tamaki H, Nakamura K, Kaneko N, Sakata S, Imachi H, Sekiguchi Y, Uchiyama H, Kamagata Y. Microbial diversity and methanogenic potential in a high temperature natural gas field in Japan. Extremophiles. 2007; 11: 453-461.

47. Nakamura K, Takahashi A, Mori C, Tamaki H, Mochimaru H, Nakamura K, Takamizawa K, Kamagata Y. *Methanothermobacter tenebrarum* sp. nov., a hydrogenotrophic thermophilic methanogen isolated from gas-associated formation water of a natural gas field. Int. J. Syst. Evol. Microbiol. 2013; 63: 715-722.

48. Gieg LM, Davidova IA, Duncan KE, Suflita JM. Methanogenesis, sulfate reduction and crude oil biodegradation in hot Alaskan oilfields. Environ Microbiol. 2010; 12: 3074-3086.

49. Mayumi D, Mochimaru H, Yoshioka H, Sakata S, Maeda H, Miyagawa Y, Ikarashi M, Takeuchi M, Kamagata Y. Evidence for syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis in the high-temperature petroleum reservoir of Yabase oil field (Japan). Environ Microbiol. 2011; 13: 1995-2006.

50. Schnürer A, Schink B, Svensson BH. *Clostridium ultunense* sp. nov., a mesophilic bacterium oxidizing acetate in syntrophic association with a hydrogenotrophic methanogenic bacterium. Int J Syst Bacteriol. 1996; 46: 1145-1152.

51. Gagliano MC, Braguglia CM, Petruccioli M, Rossetti S. Ecology and biotechnological potential of the thermophilic fermentative *Coprothermobacter* spp. FEMS Microbiol Ecol. 2015; 91, fiv018. doi: 10.1093/femsec/fiv018.

52. Ozuolmez D, Na H, Lever MA, Kjeldsen KU, Jørgensen BB, Plugge CM. Methanogenic archaea and sulfate reducing bacteria co-cultured on acetate: teamwork or coexistence? Front. Microbiol. 27 May 2015; https://doi.org/10.3389/fmicb.2015.00492.

Advances in Biotechnology

Chapter 2

Limitations in the tissue culture of Indian sandalwood tree (*Santalum album* L.)

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Abstract

The tissue culture of Indian sandalwood (*Santalum album* L.) has been extensively studied, mainly for its rapid propagation technology. *Santalum album* is believed to be the first woody species for which somatic embryogenesis was reported. Despite significant advances, there are still many problems blocking the two basal modes of regeneration, namely, shoot organogenesis and somatic embryogenesis, regardless of other modifications such as protoplast culture or genetic transformation. Without doubt, studies on shoot organogenesis or/and somatic embryogenesis from explants to induce adventitious shoots or somatic embryos of all stages will be continue to be published, but reliable and efficient solutions to the limitations of *S. album* tissue culture are needed. This review highlights these problems, and introduces some physiological and biochemical changes during shoot organogenesis and somatic embryogenesis. Finally, we discuss future research directions for the tissue culture of Indian sandalwood, aiming at a call for more studies that address these problems which hinder the full application of sandalwood tissue culture.

Keywords: *Santalum album*, Indian sandalwood, tissue culture, culture media, callus induction, secondary embryogenesis, maturation of somatic embryos, root formation, plant regeneration

1. Introduction

Indian sandalwood (*Santalum album* L.) belongs to the *Santalum* genus in the Santalaceae, which includes about 18 species [1]. It is believed to be the most valuable species because of a high content of essential oil in heartwood and roots (0.9-8%), which is a higher proportion of α- (41-55%) and β-santalols (16-24%) in essential oil than other sandalwood species [2-6]. So far, Indian sandalwood has been widely planted in India, China and Australia for significant commercial purposes [6]. The trees exhibit great variability and large-scale afforestation provides a feasible way to select superior individual plants [7,8]. *S. album* is a predominantly out-breeding species [9,10], so the traits of offspring are clearly separated. Besides, it takes more than 15 years for Indian sandalwood from planting to harvesting, unlike annual crops which can be selected in time to improve varieties. So, propagating *S. album* trees with desired characters is very important for sustainable development of the sandalwood industry. Although traditional vegetative propagation can be accomplished by grafting, air layering and with root suckers, the production of clones is inefficient, time-consuming and highly dependent on the season [11-13]. The tissue culture of *S. album* is a solution for mass propagation, which is used in limited research on other plant species [6,14,15].

The tissue culture of Indian sandalwood has a history of more than 50 years since 1963 [16], with more than 50 studies published [1,6]. It is believed to be the first woody species for which somatic embryogenesis was reported [1]. Significant advances have been achieved, including a wide range of responsive explants, such as zygotic embryos/endosperms [17-19], hypocotyls [20,21], nodes/internodes [6,22-25], immature leaves [26-29], shoot organogenesis and somatic embryogenesis, cell culture [30], protoplast culture [31-33], and 'synthetic seeds' [34]. Despite all of these, there are still many problems that hinder the full application of tissue culture of Indian sandalwood, such as irregularities in somatic embryos (SEs) [35], low rooting abilities of excised shoots [23,24,36], low survival percentage after regenerated plantlets have been transplanted to the field [20,23,36,37] (Figure 1). So far, the tissue culture of Indian sandalwood has not achieved industrialization. This paper summarizes common problems in the tissue culture of Indian sandalwood, and introduces some physiological and biochemical changes during somatic embryogenesis or shoot organogenesis. Finally, we discuss future research directions of Indian sandalwood tissue culture.

2. Limitations in Indian sandalwood tissue culture

2.1 Callus induction

The main problems during callus induction of Indian sandalwood include the low frequency of callus formation and poor repeatability by different researchers. When Murashige and Skoog (MS) medium was used as the basal medium [38], some researchers used 2,4-dichlorophenoxyacetic acid (2,4-D) alone to induce callus from hypocotyls [35,39] or stem seg

ments [6,20,35], while other researchers found that callus induced from hypocotyls on callus induction medium (CIM) containing 2,4-D alone could not regenerate any callus [21,40] or that the callus induction efficiency of stem segments was extremely low (20% in [14]; <10% in [41]). In another case, callus was efficiently induced in CIM containing α-naphthaleneacetic acid (NAA) or indole-3-acetic acid (IAA) alone [42]. In contrast, callus often died after subculture [22]. In many cases, researchers used CIM supplemented with combinations of auxins and cytokinins, especially 2,4-D and kinetin (KIN), NAA/IAA and 6-benzyladenine (BA) for callus induction [21,22,25,27,36]. However, the combination differed greatly in different experiments, and the effectiveness and validity of callus induction were questionable [14]. There are many factors that affect callus induction, such as the genotype from which explants are derived, explant age, level of endogenous hormones and culture conditions [43]. Genotype is an important factor that greatly affects callus induction in *S. album*. The choice of clone significantly affected callus development [29]. Spontaneous callus formation for A13 may be controlled by genotype, since the addition of proline, arginine or putrescine did not increase the rate of callus induction [14]. Thidiazuron (TDZ) (0.9-4.5 μM) may be the most efficient plant growth regulator, inducing a high percentage of callus from immature leaves or stem segments, even in different groups of researchers [14,28,44]. In five repeated experiments conducted in September and December of 2015, January, March and July of 2016, we found that callus induction percentage from stem segments of a range of genotypes on MS medium with 2.25-4.54 μM TDZ was consistently high, ranging from 82 to 89% (unpublished data). In another experiment, internodes of nine individuals were used to evaluate the effect of genotype on regeneration mode and callus induction: TDZ was effective for callus induction from different genotypes (Table 1). However, it is worth noting that callus induced by TDZ is usually bright yellow, compact and nodular [28] (Fig. 2A) and with a strong ability to differentiate although the ability to disperse callus is poor, thus making it unsuitable for cell suspension cultures. Relying on the efficiency and stability of TDZ-induced callus, the next research objective is to regulate callus growth to obtain suitable callus for cell suspension culture.

2.2 Asynchronized of somatic embryogenesis

S. album somatic embryogenesis is often observed to be asynchronous. This is partly because of the different level of contact between cultures and media (mainly PGRs) leading to the formation of SEs at different times. It is also because of two kinds of secondary embryogenesis, one in which secondary SEs grow directly on primary SEs and the other in which SEs form callus and then form new SEs, i.e., SEs → callus → SEs [18,20,32,36] (Fig. 2B). Secondary SEs have great potential for rapid propagation [18]. Bapat and Rao (1988) [34] induced and isolated secondary SEs from artificial seeds, which then converted into plantlets. Observations from our experiments showed that secondary somatic embryogenesis was usually induced during the first two subcultures (i.e., during the transfer from SE induction

medium (SEIM) to basal MS medium). In this first step, primary SEs seldom induce secondary SEs, even after three subcultures, which may indicate that secondary embryogenesis is due to the residual effect of PGRs. The frequency of somatic embryogenesis was low but the formation of secondary SEs and abnormal SEs decreased on PGR-free SEIM containing cyanobacterial cell extract compared to SEIM containing PGRs [41]. In another experiment, artificial seeds germinated in basal MS medium, producing secondary SEs in the presence of 2.22 μM BA and 2.85 μM IAA [34], indicating that exogenous PGRs may induce repetitive somatic embryogenesis, although other as yet unknown reasons may exist since the occurrence of secondary SEs in woody plants is common [43,45]. SEs that were first separated then transferred to liquid maturation medium containing abscisic acid (ABA) for a month, and then transferred to solid medium for further conversion into secondary SEs [36].

2.3 Somatic embryo irregularities/abnormalities

During the process of Indian sandalwood somatic embryogenesis, abnormalities in SEs are constantly mentioned, including SEs with no roots or roots with an undeveloped shoot axis, asymmetric cotyledons, monocotyledons, or the fusion of cotyledons [35,46] (Fig. 2C). Different types of abnormal SEs can appear in the same medium and in medium supplemented with different PGRs, which may be caused by endogenous factors during callus differentiation [20], an observation also noted with the culture of protoplasts [32]. In SEIM containing 5.36 μM NAA and 2.21-4.42 μM BA, callus initially induced from nodes or shoot tips by NAA can differentiate into globular, heart-shaped or immature torpedo-shaped SEs but not mature SEs, while immature torpedo-shaped SEs germinate precociously, thus forming abnormal plantlets [42]. In that study, MS medium containing 1.44 μM gibberellic acid (GA_3), 2.46 μM indole-3-butyric acid (IBA) and 2.21 μM BA, various stages of SEs, including cotyledonary SEs, were observed, and precocious germination was completely inhibited. The authors of [42] believed that a high concentration of BA not only decreased the number of SEs in each stage, but also increased the amount of abnormal sSEs, which was consistent with a previous report in 2002 [51]. However, a low concentration of GA_3 and IBA inhibited new SE formation, but promoted SE development into a mature stage, and produced high quality SEs [42]. Other studies showed that mature SEs could germinate in medium supplemented with GA_3, which would otherwise produce many abnormal SEs, thus decreasing the conversion percentage from SEs to plantlets [14,27]. In the absence of exogenous ABA, the frequency of SE maturation was very low, while in optimized medium supplemented with 4.95 μM ABA, the efficiency of somatic embryogenesis increased to 57% [39]. The addition of 3 μM ABA, maltose or 2.5% polyethylene glycol (PEG) to maturation medium increased the proportion of normal SEs [36]. In addition, the use of 1/2MS (macro-elements halved) medium instead of full-strength MS decreased the frequency of abnormal SEs, indicating that a low concentration of mineral elements may promote the formation of normal SEs [47].

Most efforts in sandalwood tissue culture have been to modify the culture conditions to solve the problem of abnormal SE formation. However, studies on the internal factors such as the correlation between SE abnormalities and gene/chromosome variation are rare. It is difficult to detect small chromosomes in somatic cells [23], and chromosome aberrations can occur, e.g., in maize [48].

The following conclusions may be inferred from these studies: 1) the formation of abnormal SEs may be caused by their precocious germination, fusion of many secondary SEs or inhibition by a high concentration of mineral elements in or on the SE radical; 2) one solution is to inhibit premature germination, reduce the frequency of secondary SE formation, induce the maturation of SEs, and use a low concentration of mineral elements to promote root development; 3) ABA, GA_3 or TDZ could be used to obtain mature SEs; IBA might inhibit the occurrence of secondary SEs while GA_3 can promote the germination of SEs.

2.4 Difficulty in rooting of regenerated plantlets

Difficulty with rooting is a major problem in the final stage of shoot organogenesis of Indian sandalwood [21,23,24,27,49,50] (Fig. 2D), and is often encountered in the process of somatic embryogenesis as well [51,52] (Fig. 2E).

A study on the effects of basal media (1/2MS, 1/2 Greshoff and Doy (1972) (GD) medium and 1/2 woody plant medium (WPM) [54]) and different concentrations of KIN (0, 1.16, 2.33, 3.49, 4.65 μM) on rooting showed that 1/2MS with 98.40 μM IBA, 5.71 μM IAA and 3.49 μM KIN was the best combination to induce roots from plantlets [55]. Similarly, we found that MS with 24.60 μM IBA, 0.89 μM BA, and 1.07 μM NAA also induced a high frequency of rooting (74.2%) [6]. We proposed that a low concentration of cytokinins may play a significant role in *S. album* root induction. Many excised shoots remain recalcitrant, and take a long time to form roots (Fig. 2D). When excised shoots were treated with 98.4 μM IBA for different periods, then transferred to the PGR-free medium (1/4MS salts with B_5 [56] vitamins and 2% sucrose) for rooting, IBA treatment period had a significant effect on rooting, and shoots treated for 48 h produced the highest rooting rate (41.67%), root number (2.18 ± 0.06), and root length (3.32 ± 0.67 cm) after 8 weeks [24]. Among four kinds of auxins tested, namely, IBA, NAA, 2-naphthoxy acetic (NOA) and IAA, IBA exhibited the best effect on rooting, and shoots treated with 1230 μM IBA for 30 min showed maximum rooting (50%) in soilrite substrate (peat moss, expanded perlite, and vermiculite; 2:1:1) [24]. Their results showed that treatment with a high concentration IBA had a significant effect on rooting, and that a soil-based substrate was superior to agar medium for rooting, consistent with other observations [6]. Our group also successfully induced roots from excised shoots with a high concentrations of IBA (246 μM) [57].

Mature SEs formed good apical and roots faster in White's medium [58] than in MS

medium [22]. Germinated SEs with well-developed shoots and roots could develop into normal plantlets in White's medium with 2.86 μM IAA, indicating significant effects of a low concentration of mineral nutrients [59]. Mature SEs show polarity and develop easily into plantlets with a well-developed radicle and plumule, serving as a method to solve the difficulty of rooting in Indian sandalwood. Another solution to the problem of rooting is *in vitro* micrografting [60], but there is no commercial interest in this method since it requires a seedling to graft a scion.

2.5 Low survival percentage of regenerated plantlets after transplantation into the field

The last step of tissue culture is to transplant regenerated plantlets from sterile culture jars to a field. To date there has been little research on this aspect, mainly because rooting of excised plantlets is difficult, which leads to a shortage of raw materials. One paper [55] studied the effects of different substrates (M1 - volcanic dirt: Purwobinangun, Pakem, Sleman top soils: organic fertilizer = 3:1:1 (v/v/v); M2 - volcanic dirt: Playen, Gunung Kidul top soils: organic fertilizer = 3:1:1; M3 - volcanic dirt: Playen, Gunung Kidul top soils: organic fertilizer = 3:1:1), primary host plants (*Caliandra callotirsus*, *Crotalaria juncea*, and *Capsicum annum*) and their combinations on the survival of transplanted *S. album* plantlets. Results showed that the choice of both substrate and host plant resulted in a slow response to growth and survival of plantlets in a greenhouse. No more than 10% survival of plantlets derived from SEs was shown [23]. The following suggestions were provided to improve this [37]: first, transplanting regenerated plantlets from MS medium to White's medium, then transferring plants from agar medium into sterile substrate, and watering with sucrose-free White's medium to improve root development, and finally placing rooted plants in a mist chamber with high humidity.

3. Biochemical and molecular (DNA/protein) events during Indian sandalwood somatic embryogenesis

Although there are many reports on the culture conditions (especially PGRs) for somatic embryogenesis in *S. album*, the underlying molecular and biochemical events remains unclear. Improving the percentage of SE conversion and the survival percentage of regenerated plants may depend on the elucidation of the metabolic behavior of somatic embryogenesis [61], although related research is still not very profound. The main methods employed have compared the profiles of soluble proteins, amplified DNA fragments, or enzyme (or isozyme) activity in different developmental stages by synchronization or manual separation of cultures. As far as we are aware, only Ca^{2+}-dependent protein kinase (55-60 kD) was identified [62,63] in which Ca^{2+} acted as a secondary messenger in the process of sandalwood somatic embryogenesis while the lack of effective Ca^{2+} (chelated calcium, calcium channel blockers, etc.) significantly reduced the formation of SEs [64]. After 2001, there are no studies that confirm the theories put forward by Anil or Rao.

Fresh callus derived from endosperms in medium containing 2,4-D could partially differentiate into pre-embryogenic masses (PEMs), which developed further into globular SEs in the same medium [17]. In medium containing 2,4-D, however, globular SEs could not develop into bipolar SEs, and bipolar SEs only developed in medium without 2,4-D. In other words, 2,4-D can block somatic embryogenesis in the globular SE stage and bipolar SEs can be obtained in medium without 2,4-D. Based on this, the same authors obtained four stages of cultures, i.e., fresh callus, PEMs, globular SEs, and bipolar SEs. Their experiment showed that during the development process of fresh callus → PEMs → globular SEs → bipolar SEs, the activity of protein kinase gradually increased while the activity of glucosidase and xylanase increased significantly in PEMs → globular SEs and globular SEs → bipolar SEs, indicating that cell wall plasticity was regulated during development [17]. Also, there were differences in protein bands among cultures, indicating possible stage-specific gene expression or post-translational regulation of proteins. The post-translational regulation of proteins was demonstrated in Ca2+-dependent protein kinase [64].

Callus originated from shoot tips and nodes in NAA-fortified media and could differentiate into spherical SEs, heart-shaped SEs and immature torpedo-shaped SEs, but not beyond this stage [42]. Callus could develop into cotyledonary SEs in medium containing 1.44 μM GA_3, 2.46 μM IBA and 2.21 μM BA [42]. Protein analysis of embryogenic tissues by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed that some specific proteins (26, 34 and 56 kD) appeared during the period from heart-shaped SEs to torpedo SEs, which may play an important role in the maturation of SEs.

A tissue culture process, namely, compact callus → friable callus → embryo induction stage → embryo mature stage → mature embryos, based on different media, was established [65]. Protein electrophoretic profiles, detection of isozymes (peroxidase, esterase, malate dehydrogenase and shikimate dehydrogenase) and RAPD analysis revealed metabolic changes and genetic variation during somatic embryogenesis. Soluble protein analysis showed that small molecular weight proteins (< 43 kD) were relatively intense in the third stage, which may be a characteristic of embryogenic potential. Changes in the isozyme patterns of these five stages, i.e., the appearance and disappearance of isozyme bands, but without determining the activity of the enzymes, showed that the genes related to these enzymes were differentially activated as development progressed. RAPD patterns in these five stages also showed numerous changes, indicating that under tissue culture conditions, high variability in gene expression took place during somatic embryogenesis.

4. Conclusions and future perspectives

The tissue culture of *S. album* was recently intensively reviewed [1]. The two known regeneration pathways are blocked to some extent (Fig. 1). In addition, since biochemical

and molecular (DNA/protein) events during Indian sandalwood somatic embryogenesis have seldom been exploited, it is necessary to solve key problems mentioned above to achieve the rapid propagation of *S. album*.

Genotype plays an important role in somatic embryogenesis and callus induction [14,29] (Table 1), and provides new solutions but brings new challenges. For example, in most experiments, explants originating from different genotypes or individuals raised from seed were mixed, and the resulting optimal medium may be not useful for select individual plants with desired characters. However, we can use different individual plants to establish different research systems, such as the use of explants to induce somatic embryogenesis easily, allowing for the study of the factors limiting SE germination and the survival of transplanted plantlets. Callus formed easily from individual lines, e.g. line A13 [14], can be employed to detect factors that influence callus differentiation into normal SEs or for screening callus resistant to salts. Since several parameters of regenerated plants derived from somatic embryogenesis differed considerably, including vigor, leaf length and width, phyllotaxy and chlorophyll content [20], it is importance to carry out research that assesses variability in somatic embryogenesis (Fig. 2E). We can use a single genotype (i.e. individual) to construct RAPD/RFPL/SSR fingerprints of different stages of somatic embryogenesis, including regenerated plants, to elucidate the stages that are prone to genetic changes, which will aid the rapid propagation of individuals with excellent and desired characteristics.

In addition, the selection of superior individuals also depends on the development of molecular markers for assessing desired traits. Can we use SaSSy and SaCYP736A167 genes as probes to evaluate the oil production capacity of Indian sandalwood [66,67]? Is it reliable to use peroxidase (POD) activity in bark as a marker of oil yield [68]? These are problems beyond tissue culture which shall be studied as well.

5. Figures

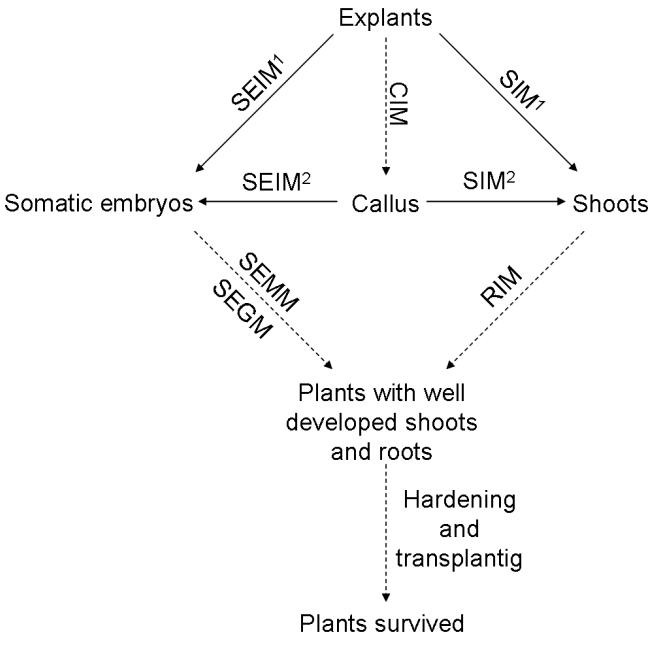

Figure 1: A simplified schematic representing sandalwood tissue culture procedure. SEIM-somatic embryo induction medium; CIM-callus induction medium; SIM-shoot induction medium; SEMM-somatic embryo maturation medium; SEGM-somatic embryo germination medium; RIM-root induction medium. Solid lines indicate easy or effective media while dashed lines indicate ineffective methods or methods with wide variation.

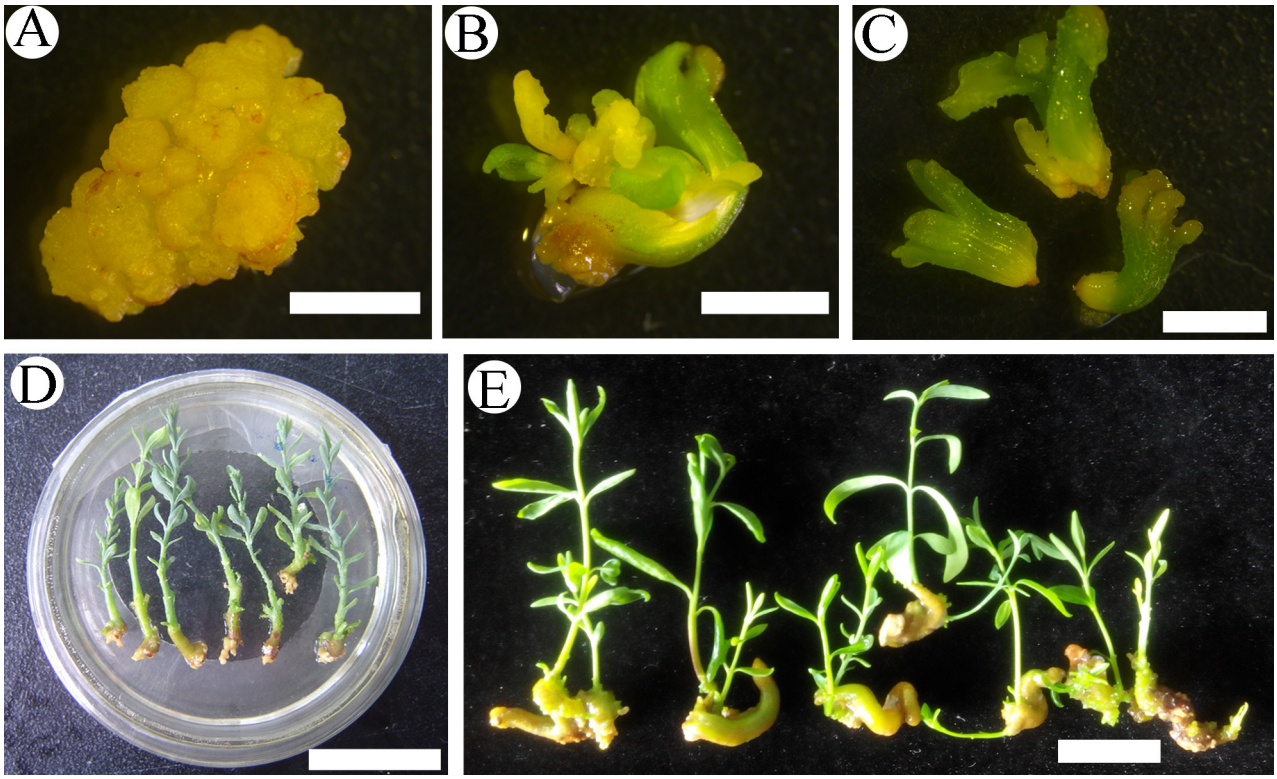

Figure 2: Limitations in tissue culture of Indian sandalwood. (A) Bright yellow, compact and nodular callus was induced from shoot nodes in MS + TDZ (4.54 µM) after one month, bar = 2 mm. (B) Secondary embryos formed directly on the primary somatic embryo, bar = 2 mm. (C) Abnormal embryos with obvious root tip and multi cotyledons, bar = 2 mm. (D) Excised shoots recalcitrant to form roots after 2 months in MS+ IBA (9.84 µM)+ BA (0.89 µM)+ NAA (1.07 µM), bar = 3 cm. (E) Plants raised from somatic embryos with poor roots, the left one showing 3 whorled phyllotaxis different from others, bar = 1 cm.

6. Table

MS media (µM)	Inducing Percentage (%)	Genotype (Sample)								
		1	2	3	4	5	6	7	8	9
BA (2.22)+ NAA (0.27)	Shoots	65	67	62	59	100	100	100	100	100
	Somatic embryos	35	33	38	41	0	0	0	0	0
	Callus	0	0	0	0	0	0	0	0	0
BA (4.44) + NAA (0.54)	Shoots	61	45	58	56	58	100	100	100	100
	Somatic embryos	39	51	44	44	42	34	55	26	45
	Callus	21	19	20	32	11	12	18	12	21
TDZ (4.54) + NAA (0.54)	Shoots	56	35	37	48	100	100	87	91	88
	Somatic embryos	44	65	63	52	89	56	13	9	12
	Callus	87	77	56	67	77	74	78	69	66

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8. References

1. Teixeira da Silva JA, Kher MM, Soner D, Page T, Zhang X, Nataraj M, Ma G (2016) Sandalwood: basic biology, tissue culture, and genetic transformation. Planta 243, 847-887

2. ISO (2002) Oil of sandalwood (*Santalum album* L.) II edition, 3518, 2002 (E), 2002-03-01, Geneva. Switzerland, pp 1–5.

3. Howes MJR, Simmonds MSJ, Kite GC (2004) Evaluation of the quality of sandalwood essential oils by gas chromatography- mass spectrometry. Journal of Chromatography A 1028: 307-312

4. Jain SH, Arya R, Kumar H (2007) Distribution of sandal (*Santalum album* L.), current growth rates, predicted yield of heartwood and oil content and future potential in semi arid and arid regions of Rajasthan, India. Forests, Trees and Liveliloods 17: 261-266

5. Jones CG, Plummer JA (2007) Non-destructive sampling of Indian sandalwood (*Santalum album* L.) for oil content and composition. Journal of Essential Oil Research 19: 157-164

6. Zhang X, Zhao J, Teixeira da Silva JA, Ma G (2016) *In vitro* plant regeneration from nodal segments of the spontaneous F1 hybrid *Santalum yasi* × *S. album* and its parents *S. album* and *S. yasi*. Trees 30: 1983-1994

7. Kulkami HD, Srimathi RA (1982) Variation in folia characteristics in sandal. In Biometric Analysis in Tree Improvement of Forest Biomass (ed. Khosla PK), International Book Distributors, Dehra Dun, pp 63-69

8. Arun Kumar AN, Joshi G, Mohan Ram HY (2012) Sandalwood: history, uses, present status and the future. Current Science 103: 1408-1416

9. Sindhu Veerendra HC, Anantha Padmanabha HS (1996) The breeding system in sandal (*Santalum album* L.). Silvae Genetica 45: 188-190

10. Ma GH, Bunn E, Zhang JF, Wu GJ (2006) Evidence of dichogamy in *Santalum album* L. Journal of Integrative Plant Biology 48: 300-306

11. Srimati RA, Venkateshan KR, Kulkarni HD (1995) Guidelines for selection and establishment of seed stands, seed production areas, plus trees and clonal seed orchards for sandal (*Santalum album* L.). In: Srimati RA, Venkateshan KR, Kulkarni HD (eds) Recent advances in research and management of sandal (*Santalum album* L.) in India. Associated, New Delhi, pp 281–299

12. Li YL (1997) Preliminary studies on grafting of *Santalum album*. Journal of Chinese Medicinal Materials 20: 543-545

13. Chen FL (1999) Cuttage of *Santalum album*. Journal of Chinese Medicinal Materials 22: 109-111

14. Rugkhla A, Jones MGK (1998) Somatic embryogenesis and plantlet formation in *Santalum album* and *S. spicatum*. Journal of Experimental Botany 49: 563-571

15. Baiculacula S (2012) Micropropagation of sandalwood. In: Thomson L, Padolina C, Sami R, Prasad V, Doran J (Eds) Regional workshop on sandalwood resource development research and trade in the pacific and asian region. European Union, Secretariat of the Pacific Community, James Cook University and the Australian Centre for International Agricultural Research, Port Vila, pp 111–116

16. Rangaswamy NS, Rao PS (1963) Experimental studies on *Santalum album* L. establishment of tissue culture of endosperm. Phytomorphology 4:450–454

17. Rao KS, Chrungoo NK, Sinha A (1996) Characterization of somatic embryogenesis in sandalwood (*Santalum album* L.). In Vitro Cellular & Developmental Biology-Plant 32: 123-128

18. Rai VR, McComb J (2002) Direct somatic embryogenesis from mature embryos of sandalwood. Plant Cell Tissue and Organ Culture 69: 65-70

19. Rai VR (2005) Somatic embryogenesis in sandalwood. In: Protocol for Somatic Embryogenesis in Woody Plants. Springer, the Netherlands pp 497-504.

20. Bapat VA, Rao PS (1984) Regulatory factors for in vitro multiplication of sandalwood tree (*Santalum album* Linn.) I. Shoot bud regeneration and somatic embryogenesis in hypocotyl cultures. Plant Science 93: 19-27

21. Crovadore J, Schalk M, Lefort F (2012) Selection and mass production of *Santalum album* L. calli for induction of sesquiterpenes. Biotechnology & Biotechnological Equipment 26: 2870-2874

22. Lakshmi Sita G, Ram NVR, Vaidyanathan CS (1979) Differentiation of embryoids and plantlets from shoot callus of sandalwood. Plant Science Letters 15: 265-270

23. Rao PS, Bapat VA, Mhatre M (1984) Regulatory factors for *in vitro* multiplication of sandalwood tree (*Santalum album* Linn) II. Plant regeneration in nodal and internodal stem explants and occurrence of somaclonal variation in tissue culture raised plants. Proceedings of the Indian National Science Academy 50: 196-202

24. Muthan B, Rathore TS, Rai VR (2006a) Micropropagation of an endangered Indian sandalwood (*Santalum album* L.). Journal of Forest Research 11: 203-209

25. Peeris MKP, Senarath W (2015) *In vitro* propagation of *Santalum album* L. Journal of the National Science Foundation of Sri Lanka 43: 265-272

26. Mujib A (2005) *In vitro* regeneration of sandal (*Santalum album* L.) from leaves. Turkish Journal of Botany 29: 63-67

27. Bele D, Tripathi MK, Tiwari G, Baghel BS, Tiwari S (2012) Microcloning of sandalwood (*Santalum album* Linn.) from cultured leaf discs. Journal of Agricultural Technology 8: 571-583

28. Singh CK, Raj SR, Patil VR, Jaiswal PS, Subhash N (2013) Plant regeneration from leaf explants of mature sandalwood (*Santalum album* L.) trees under *in vitro* conditions. In Vitro Cellular & Developmental Biology-Plant 49: 216-222

29. Herawan T, Na'iem M, Indrioko S, Indrianto A (2014) Somatic embryogenesis of sandalwood (*Santalum album* L.). Indonesian Journal of Biotechnology 19: 168-175

30. Lakshmi Sita G, Shobha J, Vaidyanathan CS (1980) Regeneration of whole plants by embryogenesis from cell suspension cultures of sandalwood. Current Science 49: 196-198

31. Lakshmi Sita G, Sobharani B (1983) Preliminary studies on isolation and culture of protoplasts from sandalwood (*Santalum album*). Experientia Supplementum 45: 4-5

32. Rao PS, Ozias-Akins P (1985) Plant regeneration through somatic embryogenesis in protoplast cultures of sandalwood (*Santalum album* L.). Protoplasma 124: 80-86

33. Bapat VA, Gill R, Rao PS (1985) Regeneration of somatic embryos and plantlets from stem callus protoplasts of sandalwood tree (*Santalum album* L.). Current Science 54: 978-982

34. Bapat VA, Rao PS (1988) Sandalwood plantlets from 'synthetic seeds'. Plant Cell Reports 7: 434-436

35. Ilah A, Abdin MZ, Mujib A (2002) Somatic embryo irregularities in *in vitro* cloning of sandal (*Santalum album* L.). Sandalwood Research Newsletter 15: 2-3

36. Balasundaran M, Muralidharan, EM (2001) Development of spike disease resistant sandal seedlings through Biotechnology involving ELISA technique and tissue culture. DBT project completion report, Kerala Forest Research Institute, Peechi, Trichur, Kerala, 64.

37. Bapat VA, Rao PS (1992) Biotechnological approaches for sandalwood (*Santalum album* L.) micropropagation. Indian Forester 118: 48-54

38. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum 15: 473-497

39. Das S, Ray S, Dey S, Dasgupta S (2001) Optimisation of sucrose, inorganic nitrogen and abscisic acid levels for *Santalum album* L. somatic embryo production in suspension culture. Process Biochemistry 37: 51-56

40. Muralidharan EM (1997) Micropropagation of teak, rosewood and sandal. Kerala Forest Research Institute (KFRI) Research Report 119. KFRI, Peechi,Thrissur, India, pp 1–20

41. Bapat VA, Iyer RK, Rao PS (1996). Effect of cyanobacterial extract on somatic embryogenesis in tissue cultures of sandalwood (*Santalum album*). Journal of Medicinal and Aromatic Plant Sciences 18: 10-14

42. Ilah A, Syed MI, Reyad AM, Mujib A (2016) Gibberellic acid and indole-3-butyric acid regulation of maturation and accumulation of storage proteins (56, 34 and 26 KD) in somatic embryos of *Santalum album* L.. International Journal of Science and Research 5: 2263-2268

43. Isah T (2016) Induction of somatic embryogenesis in woody plants. Acta Physiologiae Plantarum 38: 1-22

44. Singh CK, Raj SR, Jaiswal PS, Jaiswal PS, Patil VR, Punwar BS, Charda JC, Subhash N (2016) Effect of plant growth regulators on *in vitro* plant regeneration of sandalwood (*Santalum album* L.) via organogenesis. Agroforestry Systems 90: 281-288

45. Park YS, Bonga JM, Moon HK (eds) (2016) Vegetative Propagation of Forest Trees. National Institute of Forest Science (NIFoS). Seoul, Korea. pp 56-74

46. Rao PS (1965) *In vitro* induction of embryonal proliferation in *Santalum album* L. Phytomorphology 15: 175-179

47. Rao PS, Ram NVR (1983) Propagation of sandalwood (*Santalum album* Linn) using tissue and organ culture technique. In: Plant Cell Culture in Crop Improvement. Springer US, pp 119-124

48. Edallo S, Zucchinali C, Perenzin M, Salamini F (1981) Chromosomal variation and frequency of spontaneous mutation associated with *in vitro* culture and plant regeneration in maize. Maydica 26: 39-56

49. Rao PS, Bapat VA (1978) Vegetative propagation of sandalwood plants through tissue culture. Canadian Journal of Botany 56: 1153-1156

50. Sarangi BK, Golait A, Thakre R (2000) High frequency *in vitro* shoot regeneration of sandalwood. Journal of Medicinal and Aromatic Plant Sciences 22: 322-329

51. He H (2002) The study on the factors affecting embryogenesis of *Santalum album* L. Primary Journal of Chinese Materia Medica 16: 17-19 (In Chinese)

52. Mo XL, Zeng QQ, Qiu WF, Chen YZ (2008) Study on somatic embryogenesis from sandalwood and plantlet regeneration. Food and Drug 10: 35-37 (In Chinese)

53. Greshoff PM, Doy CH (1972) Developmental and differentiation of haploid *Lycopersicon esculentum* (tomato). Planta 107: 161-170

54. Lloyd G, McCown B (1980) Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. International Plant Propagators' Society Proceedings 30: 421-427

55. Herawan T, Na'iem M (2006) Effect of medium and growth regulator concentration of kinetin on rooting phase (*Santalum album* Linn.). Agrosains 19: 193-199

56. Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Experiment Cell Research 50: 151-158

57. Ma GH, Hu YJ, Xu QS (2008) Tissue culture and rapid propagation of *Santalum album* L. Plant Physiology Communications 44: 296. (In Chinese)

58. White PR (1954) The cultivation of animal and plant cells. The Ronald Press Company, New York

59. Lakshmi Sita G (1986) Forest and nut trees. 8. Sandalwood (*Santalum album* L.). Biotechnology in Agriculture and Forestry, Springer, Germany, pp. 363-374

60. Muthan B, Rathore TS, Rai VR (2006b) Factors influencing *in vivo* and *in vitro* micrografting of sandalwood (*Santalum album* L.): an endangered tree species. Journal of Forest Research 11: 147-151

61. Rao PS, Bapat VA (1995) Somatic embryogenesis in sandalwood (*Santalum album* L.). In: Somatic Embryogenesis in Woody Plants. Springer Netherlands pp 153-170

62. Anil VS, Rao KS (2000) Calcium-mediated signaling during sandalwood somatic embryogenesis. Role for exogenous calcium as second messenger. Plant Physiology 123: 1301-1312

63. Anil VS, Rao KS (2001) Purification and characterization of a Ca^{2+} dependent protein kinase from sandalwood (*Santalum album* L.): evidence for $Ca2^{+}$-induced conformational changes. Phytochemistry 58: 203-212

64. Anil VS, Harmon AC, Rao KS (2000) Spatio-temporal accumulation and activity of calcium-dependent protein kinases during embryogenesis, seed development, and germination in sandalwood. Plant Physiology 122: 1035-1044

65. Suma TB, Balasundaran M (2002) Studies on genetic polymorphism in *Santalum album* L. Cochin University of Science and Technology

66. Jones CG, Moniodis J, Zulak KG, Scaffidi A, Plummer JA, Ghisalberti EL, Barbour EL, Bohlmann (2011) Sandalwood fragrance biosynthesis involves sesquiterpene synthases of both the terpene synthase (TPS)-a and TPS-b subfamilies, including santalene synthases. Journal of Biological Chemistry 286: 17445-17454.

67. Celedon JM, Chiang A, Yuen M, Diaz-Chavez ML, Madilao LL, Finnegan PM, Barbour EL, Bohlmann J (2016) Heartwood-specific transcriptome and metabolite signatures of tropical sandalwood (*Santalum album*) reveal the final step of (*Z*)-santalol fragrance biosynthesis. The Plant Journal 86: 289-299

68. Parthasarathi K, Angadi VG, Shankaranarayana KH, Rajeevalochan AN (1986) Peroxidase isoenzyme activity in living bark tissue as a marker for the oil-bearing capacity in sandal. Current Science 55: 831-834

Advances in Biotechnology

Chapter 3

Microorganisms in environmental biotechnology application

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Abstract

Microorganisms are essential to our very existence. They are ubiquitous, found in common environments such as soil, water, and air as well as exotic locales as diverse as deep sea hydrothermal vents and soda lime lakes. They are abundant in natural environments associated with wide range of activities like fuel and biomass production, mineral, nutrient recycling and energy recovery etc. Concerns of microbial studies have been identified as important area where background information is essential for recovery of natural component for our use. Useful applications of microbes, study of environment balance and their correlation are valuable for living organism. Microbes are used for environmental restoration and biomediation processes which are needed to lead more sustainable lifestyles and use of resources more justifiably. Microbes are everywhere in the biosphere, and their presence invariably affecting the environment. The effects of microorganisms on environment can be beneficial or harmful. Since a good part of this text concerns with a discussion of the beneficial activities of microorganisms as they relate to human welfare. Microorganisms are very diverse and their jobs are highly specific in the environment. Recognizing the numerous invaluable functions of microbes, this book chapter give the information about the environment and role of microbes in various applications

like biomass degradation, fuel production, mineral recovery and nutrient recycling.

Keywords: microbes; biomass; biochemical; environmental application

1. Microbes in Biofuel Production

Our environment provides us natural resources that include, air, water, soil, minerals, along with the climate and solar energy, which form the non-living or abiotic component of nature. The 'biotic' or living parts of nature consist of plants, animals, and microbes (Nejat and Mantri 2017). Plants and animals survive by using abiotic component and microbial help. Microbes play a major role in the balance of environmental biotic and abiotiv components. They break up/decompose dead plant/animal (biomass) and act on the detritus to reform/recycle soil nutrients. Biomass is a very good and abundant renewable source of energy. Trees, crops, garbage, and animal waste all are biomasses but most of the biomass is lignocellulosic biomass of the plants that we use as a source for energy production. The major types of biomass for ethanol production recognized to date are monoculture crops grown on fertile soils (such as sugarcane, corn, soya beans, oilseed rape, switch grass, willow, and hybrid poplar; Farrell et al., 2006), waste biomass (such as straw, corn, and waste wood; Kim and Dale, 2004), and municipal solid waste (such as processed paper and newspaper; Kuhad et al., 2010). Another type of biomass is cellulose of woods, viz. *Eicchornia crassipes, Lantana camara, Prosopis juliflora,Saccharum spontaneum, Typha latifolia, Crofton, Chromolaena odorata*, etc., which are promising and cheaper feedstocks for fuel ethanol production. Plants store the solar energy in terms of photosynthesis a form that can later be converted into fuel with the help of microorganism (Figure 1).

Fuel derived from biological origin is termed as biofuel. Biofuel is a fine alternate of conventional energy sources like Coal, Petrol, and Diesel etc which is actually derived from lignocelluloses component of the plant. The main constituents of lignocellulose are cellulose, hemicellulose and lignin (Reddy and Yang, 2005). Cellulose is the main structural component of plant cell walls. It is a long chain of glucose molecules, linked by glycosidic bonds. Hemicellulose, the second most abundant constituent of lignocellulosic biomass, is a family of polysaccharides, composed of monosaccharide units. After cellulose, hemicellulose is the most potent constituent of biomass to serve as a source of bioethanol production. Lignin is a three dimensional polymer of phenylpropanoid units (Van, 2001). The carbohydrate fractions of the plant cell wall can be converted into fermentable monomeric sugars through acidic and enzymatic (hemicellulase/cellulase) reactions, which have been exploited to produce biofuels like ethanol, butanol, osobutanol via microbial fermentation processes. Thus simple sugars are subsequently converted into fuels by microorganisms (Bacteria, cyanobecteria, yeast).

We need for efficient breakdown of lignocellulosic biomass for biofuel production. A variety of organisms have evolved to take advantage of this source, including the free-living

organisms and symbiotic animal–microbe consortia invariably present in biomass-rich environments. Increasing our knowledge of the biochemical machinery used by these organisms for the breakdown of biomass offers new avenues for the development of biologically based processes that could potentially accomplish biomass conversion at an industrial scale (Edward, 2008). The capture of solar energy through photosynthesis is a process that enables the storage of energy in the form of polymers (cellulose, hemicellulose and lignin). The high energy content and portability of biologically derived fuels, and their significant compatibility with existing petroleum- based transportation infrastructure, helps to explain their attractiveness as a fuel source. Despite the increasing use of biofuels such as biodiesel and sugar- or starch-based ethanol, evidence suggests that transportation fuels based on lignocellulosic biomass represent the most scalable alternative fuel source (Hill et al., 2006). Lignocellulosic biomass in the form of plant materials offers the possibility of a renewable, geographically distributed and relatively greenhouse-gas-favourable source of sugars that can be converted to ethanol and other liquid fuels. Calculations of the productivity of lignocellulosic feedstocks, in part based on their ability to grow on marginal agricultural land, indicates that they can probably have a large impact on transportation needs without significantly compromising the land needed for food crop production (Tilman et al., 2006).

Microbial strategies for degrading lignocellulose are diverse. Our current understanding of the enzymes involved in these processes of biomass degradation is limited to a handful of model organisms such as the fungus *Trichoderma reesei* and the bacterium *Clostridium thermocellum* (DelRio et al., 2007). Research is going on to know about more biomass degraders microbes. The knowledge of biomass degradation pathways is soon to be increased even more by a number of large-scale genomic studies. Thus, enzymes derived from thermophilic and acidophilic organisms known to degrade lignocellulose, hold significant promise for industrial processes (Viikari et al., 2007). Many novel enzymes and enzyme systems that have evolved to make use of cellulosic biomass are present in those microbes which is difficult-to-culture (Hugenholtz et al., 2002). The availability of a wide range of naturally occurring lignocellulose-degrading enzymes increases the chances of successful enzyme optimization for industrial processes. Optimization of the saccharification process is crucial because the cost of cellulases remains a key barrier to economical production of biofuels (Himmel et al., 2007). A more diverse set of candidate enzymes identified through a combination of conventional cultured microbial studies coupled with environmental prospecting methods will improve the likelihood of obtaining enzymes with activities and stability suited to a variety of industrial processes.

Final steps of ethanol production from cellulosic mass will require much of the same infrastructure developed for the production of sugar- and starch-based ethanol, changes will need to be made to exploit the diversity of sugars generated from the breakdown of biomass.

Whereas the conversion of starch-based biomass results primarily in hexoses, and also the pentose sugars D-xylose and L-arabinose. In contrast to the hexose sugars, the pentose sugars cannot be fermented by wild-type *Saccharomyces cerevisiae* (Van Maris et al., 2007). Now a days genetically engineered *Escherichia coli* has been used because it has capacity for the conversion of all hexose and pentose sugars both (Görke B, Stülke, 2008). *E. coli* has recently been engineered to produce isobutanol and other alcohols via a non-fermentative pathway that may be more readily adapted to large-scale production. In the future, genomics studies and pathway engineered microbes will considerably facilitate the process of biodegradation and biofuel production. This perspective has focused on the production of biofuels derived from lignocellulosic biomass with the help of microbial activity. Thus biofuels produced by microorganisms from renewable materials are promising substitutes for traditional fuels derived from fossil sources. The demand for sustainable alternative fuels based on renewable resources is already high today but will dramatically increase in the future. Today biofuel industry primarily produces ethanol from corn starch or sugar cane, and biodiesel is generated from vegetable oils and animal fats. However, these first generation biofuels, especially ethanol produced from starch, are in competition with the food and animal feed industry. In contrast, lignocellulosic biomass like crop wastes, forestry residues and municipal solid waste offers a high potential as feedstock for biofuels because it is the most abundant sustainable raw material worldwide. Fuels produced by microbes should help meeting energy- crisis world over.

2. Microorganism in Metal & Mineral Recovery

Since high grade ore deposits are easily accessible, so these ores become rapidly depleted. It thus becomes necessary to recover mineral resources from low grade ore deposits or extract valuable metals from industrial wastes. However, no appropriate technology is still available for recovery of metals from low- grade deposits. It is encouraging to find some microorganisms that could do it efficiently. This potential of microbes could only be realized recently and efforts are being made to use them for enhanced recovery of mineral resources from natural deposits. The process of extraction of metals from ores or concentrates, using microorganisms is called as bioleaching or microbial mining. In other words Microbial mining is the process of bioleaching recovers metals from ores that are not suitable for direct smelting due to their low metal content. Only ores containing sulfur can be bioleached because the bacteria feed on sulfur. Microbes have been used for recovery of two important natural resources - metals and petroleum. Metals are extracted economically from low grade ore by exploiting metabolic activities of bacteria *Thiobacilli*, particularly *T. ferrooxidans*, *T. thiooxidans, Acidithiobacillus ferrooxidans and Acidithiobacillus thiooxidans* (Pradhan et al., 2010). They are thermoacidophilic autotrophic archaebacteria grow in acidic and hot environments. They thrive at extremely low pH (pH 1–2) and fixes both carbon and nitrogen from the atmosphere. It solubilizes copper and other metals from rocks and plays an important role in nutrient and

metal biogeochemical cycling in environments. It has been demonstrated that these *Thiobacillus spp*. can be used for extraction of copper and uranium from insoluble minerals. This implication of microbial activity in leaching and deposition of mineral ores could develop into a recent field of biotechnology known as biohydrometallurgy.

Under optimal conditions in the laboratory, 97% of the copper in low-grade ores has been recovered by bioleaching. Laboratory experiments could show that recovery of other metals such as Ni, Zn, Co, Sn, Cd, Mb, Pb, Sb, As and Se from their low- grade sulphide-containing ores is also possible through bioleaching. The leaching process can also be used to separate the insoluble lead sulphate ($PbSO_4$) from other metals that occur in the same ore. The recovery of uranium, a nuclear fuel, can also be enhanced by microbial activities, which should help overcoming global energy crisis. Insoluble tetravalent uranium oxide (VO_2) occurs in low-grade ores. VO_2 can be indirectly converted to leachable hexavalent form VO_2SO_4 by *T. ferrooxidans* and release S^{2-} in presence of some growth substrate. S^{2-} is oxidized and to sulphur (S), utilized in the metabolism of *T. ferrooxidans* and completes bioleaching cycale (Figure 2).

These microorganisms actually gain energy by breaking down minerals into their constituent elements. Bioleaching uses bacterial microorganisms to extract precious metals, such as gold, from ore in which it is embedded. The bacteria feeds on nutrients in minerals, thereby separating the metal that leaves the organism's system; then the metal can be collected in a solution. The bacterium uses a chemical reaction called oxidation to turn metal sulphide crystals into sulfates and pure metals. These constituent parts of ore are separated into valuable metal and leftover sulphur and other acidic chemicals. Eventually, enough material builds up in the waste solution to filter and concentrate it into metal. In addition to metal recovery, microorganisms are also used in petroleum recovery called Microbially Enhanced Oil Recovery (MEOR) and *Xanthomonas campestris* is used in this process.

Several species of fungi can be used for bioleaching. Fungi can be grown on many different substrates, such as electronic scrap, catalytic converters, and fly ash from municipal waste incineration. Experiments have shown that two fungal strains (*Aspergillus niger, Penicillium simplicissimum*) were able to mobilize Cu and Sn by 65%, and Al, Ni, Pb, and Zn by more than 95%. *Aspergillus niger* can produce some organic acids such as citric acid. This form of leaching does not rely on microbial oxidation of metal, but rather uses microbial metabolism as source of acids which directly dissolve the metal. Some advantages associated with bioleaching are (i) Bioleaching is generally simpler and therefore cheaper to operate and maintain than traditional processes, since fewer specialists are needed to operate complex chemical plants. (ii) The process is more environmentally friendly than traditional extraction methods. For the company this can translate into profit, since the necessary limiting of sulfur dioxide emissions during smelting is expensive. Less landscape damage occurs, since

the bacteria involved grow naturally, and the mine and surrounding area can be left relatively untouched. As the bacteria breed in the conditions of the mine, they are easily cultivated and recycled. Yet overall, bioleaching creates less air pollution and minimal damage to geological formations, since the bacteria take place there naturally. Microorganisms play a significant role in the recovery of metal and minerals which is extensively used for our need and survival.

3. Environmental Nutrients and Microbes

All living organism require nutrients for growth. Nutrients are the chemical elements consumed by plant and animals in the largest quantities. Nutrients are of two types (i) Organic nutrients: include carbohydrates, fats, proteins and vitamins. (ii) Inorganic chemical compounds such as carbon, hydrogen, oxygen, nitrogen, phosphorus, and sulphur, water, and oxygen may also be considered nutrients (Sizer & Whitney, 2007). Carbon (C), Hydrogen (H), Oxygen (O), nitrogen (N), phosphorus (P), and Sulphur (S) are the elements considered basic for cell growth. An organism needs nutrients to live and grow because they are used in an organism's metabolism which must be taken in from its environment (Elanor & Rolfes, 2005). They are used to build and repair tissues, regulate body processes and converted to and used as energy. Methods for nutrient intake vary, with animals consuming foods that are digested by an internal digestive system, but most plants ingest nutrients directly from the soil through their roots or from the environment. Microbes (most frequently bacteria) play an important role as mineralizers of organic detritus and recyclers of essential nutrients in environment.

Bacteria constitute the foundation of ecosystem, being responsible for degradation and recycling of elements. They are often integrally involved in the chemical alteration of minerals. Minerals, or intermediate products of their decomposition, may be directly or indirectly necessary to their metabolism. The dissolution of sulphide minerals under acidic conditions, the precipitation of minerals under anaerobic conditions, the adsorption of metals by bacteria or algae, and the formation and destruction of organometallic complexes are all examples of indirect micro-organism participation. Where minerals are available as soluble trace elements, serve as specific oxidizing substrates, or are electron donors/acceptors in oxidation-reduction reactions, they may be directly involved in cell metabolic activity. There are three categories of oxidation-reduction reactions for minerals with micro-organisms: (i) Oxidation by autotrophic or mixotrophic organisms. Energy derived from the oxidation reaction is utilized in cell synthesis. (ii) Electron acceptance by minerals (reduction) for heterotrophic and mixotrophic bacteria. Chemical energy is used to create new cell material from an organic substrate. (iii) Electron donation by minerals (oxidation) for bacterial or algal photosynthesis (reaction is fuelled by photon energy).

Nutrient recycling occurs when nutrients are released into the environment. Carnivorous animals feed on herbivorous animals that live on plants. When animals defecate, this

waste material is broken down by worms and insects mostly beetles and ants. These small soil animals break the waste material into smaller bits on which microscopic bacteria and fungi can act. This material is thus broken down further into nutrients that plants can absorb and use for their growth. Similarly the bodies of dead animals and plants are broken down into nutrients so that plants can absorb the nutrients through their roots. Thus nutrients are recycled back from animals to plants and other organism by microbial consumers. If the dead material, or detritus, is not broken down by microbes, those nutrients will never become available to help sustain the life of other organisms. As we know that C, H, O, N, P and S are important for living organism. Recycling of these elements in environment is called as biogeochemical cycle or nutrient cycle. Biogeochemical cycle is a pathway by which a chemical element or molecule moves through both biotic (biosphere) and abiotic (lithosphere, atmosphere, and hydrosphere) compartments of Earth. All are recycled in environment mainly by microbial activity. Microbes like photosynthetic algae and bacteria are the most important agents of CO_2 fixation and recycle carbon in atmosphere. Recycling of H and O is actively involved with the other cycles like the carbon cycle, nitrogen cycle, sulfur cycle and phosphorous cycle as well. Among all biogeochemical elements recycling, most studies on recycling have focused on N, P, S because these elements often limit primary production (Vanni 2002).

Nitrogen is a substance that is essential for all life on the earth. Most nitrogen can be found in air in the gaseous form (78%), but nitrogen can also be found in water and soil in different forms. There, it will be decomposed by bacteria (decomposer) and absorbed by plants and animals. Nitrogen is a part of vital organic compounds because it is chief constituents of amino acids, proteins and DNA. Nitrogen in the gaseous form cannot be absorbed and used as a nutrient by plants and animals; it must first be converted by nitrifying bacteria, so that it can enter food chains as a part of the nitrogen cycle. During the nitrogen fixation process cyanobacteria first convert nitrogen into ammonia and ammonium (ammonium fixation). Plants use ammonia as a nitrogen source. Ammonium fixation is carried out according to the following reaction:

$$N_2 + 3\ H_2 = 2\ NH_3$$

After ammonium fixation, the ammonia and ammonium is converted into nitrite (NO_2^-) by Nitrosomonas bacteria and subsequently Nitrobacter convert nitrite to nitrate (NO_3^-) through the nitrification process. Nitrite and nitrate are the main plant nutrients.

Nitrification is carried out according to the following reactions:

$$2\ NH_3 + 3O_2 = 2\ NO_2 + 2\ H^+ + 2\ H_2O$$

$$2\ NO_2^- + O_2 = 2\ NO_3^-$$

During the assimilation process, plants absorb ammonium and nitrate, after which they are converted into nitrogen-containing organic molecules, such as amino acids and DNA.

Animals cannot absorb nitrates directly. They receive their nutrient supplies by consuming plants. When nitrogen nutrients have served their purpose in plants and animals, specialized decomposing bacteria will start a process called ammonification, to convert them back into ammonia and water-soluble ammonium salts. After the nutrients are converted back into ammonia, anaerobic bacteria will convert them back into nitrogen gas, during a process called denitrification. Finally, nitrogen is released into the atmosphere again (Figure 3). Denitrification is carried out according to the following reaction:

$$NO_3^- + CH_2O + H^+ = ½\ N_2O + CO_2 + 1½\ H_2O$$

Phosphorus is found on earth in water, soil, rock and sediments. Phosphorus is taken by plants and animals in the form of phosphate (PO_4^{3-}) and Monohydrogen phosphare (HPO_4^{2-}) ions. It is a part of DNA, store energy molecules like ATP and ADP, and of fats of cell membranes. Phosphorus is also a building block of bones and teeth of the human and animal body. Phosphorus is usually liquid at normal temperatures and pressures. In the atmosphere phosphorus can mainly be found as very small dust particles. Phosphate salts that are released from rocks through weathering process usually dissolve in soil water and is absorbed by plants. The phosphorus cycle is the slowest one of the matter cycles that is described here. Because the quantities of phosphorus in soil are generally small, it is often the limiting factor for plant growth. That is why humans often apply phosphate fertilizers on farmland. Animals absorb phosphates by eating plants or plant-eating animals. When animals and plants die, phosphates will return to the soils or oceans (environment) again during decay of dead bodies (either plant or animal) by microbial enzymatic activity. Important organisms active in phosphate recycling are bacteria and fungi such as species of *Bacillus, Pseudomonas, Micrococcus, Flavobacterium, Aspergillus, Penicillium, Fusarium*. The enzymes involved in cleaving phosphate from organic phosphorus compounds are collectively known as phosphatases. These enzymes show a broad range of substrate specificity and are grouped into two groups based on their pH optima the alkaline phosphatases and the acid phosphatases. Bacteria play the role of disintegrator in the phosphorus cycle.

Sulphate ion (SO_4^{2-}) is taken up from soil by plants, which incorporate it into protein, and plant protein is consumed by animals that convert plant protein to animal protein. Death of plants and animals allows bacterial decomposition of protein in remains to produce hydrogen sulphide and other products. Members of the genus Thiobacillus are the main orga¬nisms involved in the oxidation of elemental sulphur. The ability to oxidize sulphur is not restricted to only the genus Thiobacillus. Heterotrophic bacteria (*Proteus vulgaris*), actinomycetes and fungi are also reported to oxidize sulphur compounds. For example species of Bacillus,

Pseudomonas, Arthrobacter and Flavobacterium are known to oxidize elemental sulphur or thiosulphate to sulphate. Some bacteria can function in the transition zone between aerobic and anaerobic environments. Hydrogen sulphide may be oxidized to sulphur by such bacteria which deposit elemental sulphur in their cells while using oxygen as the terminal electron acceptor. Hydrogen sulphide may also be oxidized to sulphate photosynthetically by the bacteria, Chromtiacceae and Chlorobiaceae. Sulhur is first converted enzymatically to sulphite which is then oxidized to sulphate.

It is believed that some of the sulphite from the first reaction reacts with sulphur to yield thiosulphate which is then either cleaved to sulphur and sulphite or converted into tetrathionite. The latter is then metabolized to sulphur or sulphite which is then oxidized to sulphate. Under anaerobic conditions, sulphate is first reduced to H_2S by sulphate reducing microorganisms, mostly the bacteria. Many bacteria including species of Bacillus and Pseudomonas are known to reduce sulphur or sulphate to H_2S but among these, *Desulfovibrio desulfuricans* seems to be the most important.

Various studies demonstrate that there are a variety of bacteria (chemo- and phototrophs, auto and heterotrophs) involved in mineralization and in the cycling of C, H, O, N, S, and P. Microbes actively preserve and protect the environment. Microbes make a balance in environment by performing a lot of activity like production, degradation, recovery, recycling (as discussed above).

4. Figures

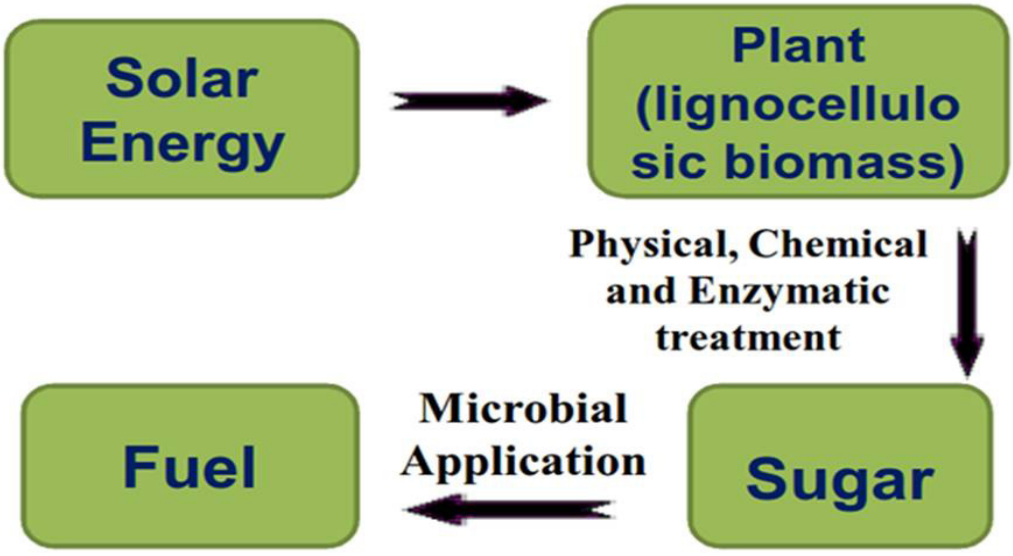

Figure 1: Microbial conversion of solar energy into fuels

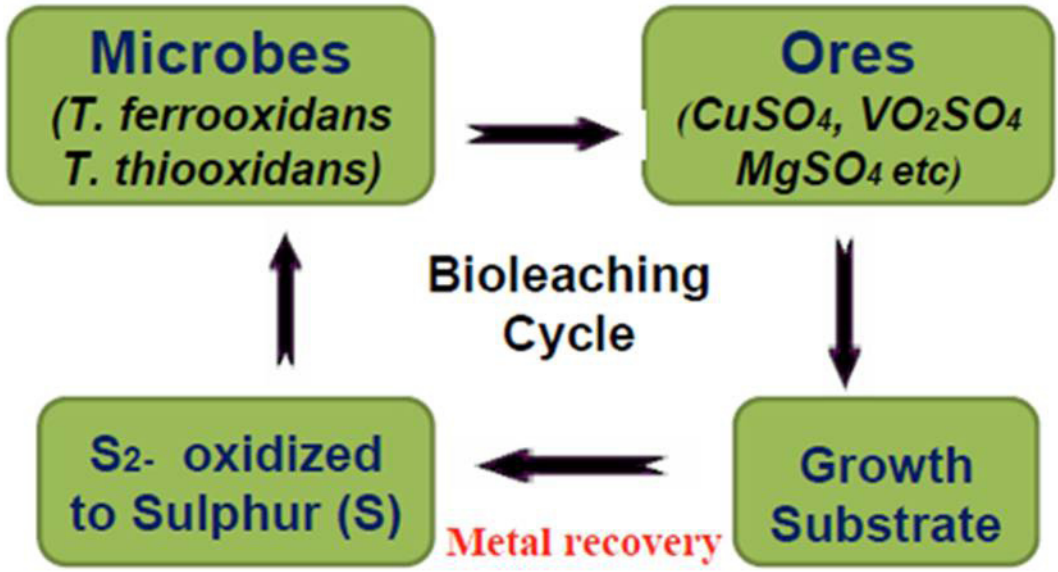

Figure 2: Bioleaching cycle

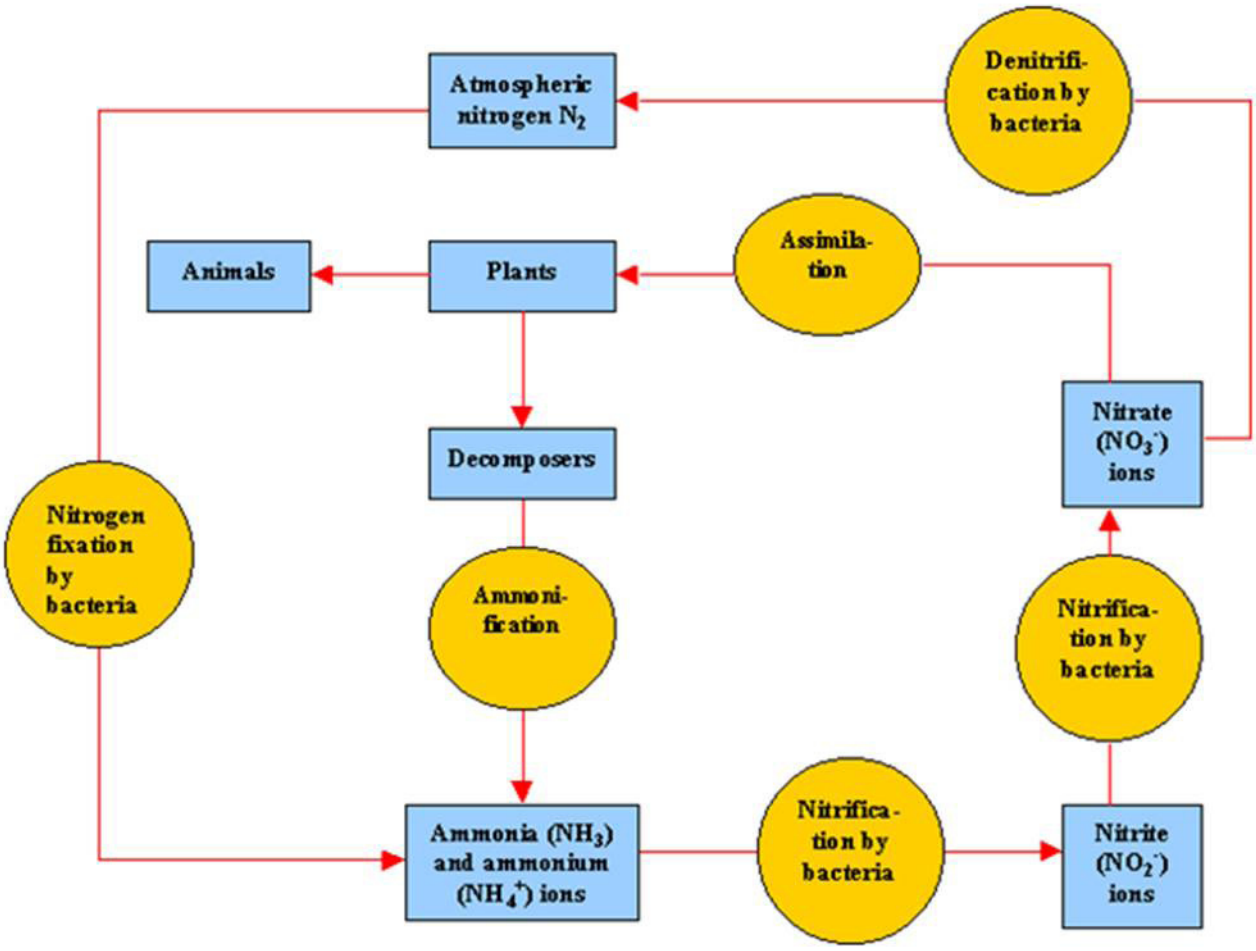

Figure 3: Nirogen cycle

5. References

1. DelRio JC, Marques G, Rencoret J, Martinez, AT & Gutierrez A (2007). Occurrence of naturally acetylated lignin units. J. Agric. Food Chem 55: 5461–5468.

2. Edward M. Rubin (2008). Genomics of cellulosic biofuels. Nature 454: 841-845.

3. Elanor W & Rolfes S (2005). Understanding Nutrition. Thomson-Wadsworth, 10th edition: pp 6.

4. Farrell A, Plevin R, Turner B, Jones A, O'Hare M, Kammen D (2006). Ethanol can contribute to energy and environmental goals. Science 311:506–508.

5. Görke B & Stülke J (2008). Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. Nat Rev Microbiol 6:613–624.

6. Hill J, Nelson E, Tilman D, Polasky S & Tiffany D (2006). Environmental, economic,and energetic costs and benefits of biodiesel and ethanol biofuels. Proc. Natl Acad. Sci. USA 103: 11206–11210.

7. Himmel ME et al., (2007). Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315: 804–807.

8. Hugenholtz, P (2002). Exploring prokaryotic diversity in the genomic era. Genome Biology 3, REVIEWS0003.

9. Kim S, Dale EB (2004). Global potential bioethanol production from wasted crops and crop residues. Biomass Bioenergy 26:361–375.

10. Kuhad RC, Mehta G, Gupta R, Sharma KK (2010). Fed batch enzymatic saccharification of newspaper cellulosics improves the sugar content in the hydrolysates and eventually the ethanol fermentation by Saccharomyces cerevisiae. Biomass Bioenergy 34:1189–1194.

11. Nejat N, Mantri N (2017). Plant Immune System: Crosstalk Between Responses to Biotic and Abiotic Stresses the Missing Link in Understanding Plant Defence. Curr Issues Mol Biol 3;23:1-16.

12. Pradhan D, Mishra D, Kim DJ, Jong GA, Chaudhury GR, Lee SW(2010). Bioleaching kinetics and multivariate analysis of spent petroleum catalyst dissolution using two acidophiles. Journal of Hazardous Materials 175: 267–273.

13. Reddy N and Yang Y (2005). Biofibers from agricultural byproducts for industrial applications. Trends Biotechnology 23: 22–27.

14. Sizer F & Whitney E (2007). Nutrition: concepts and controversies. Cengage Learning pp 26.

15. Tilman D, Hill J & Lehman C (2006). Carbon-negative biofuels from low-input highdiversity grassland biomass. Science 314: 1598–1600.

16. Van Maris AJ (2006). Alcoholic fermentation of carbon sources in biomass hydrolysates by Saccharomyces cerevisiae: current status. Antonie Van Leeuwenhoek 90: 391–418.

17. Van W (2001). Biotechnology and the utilization of biowaste as a resource for bioproduct development. Trends Biotechnol. 19 : 172–177.

18. Vanni M (2002). Nutrient Cycling by Animals in Freshwater Ecosystems. Annu. Rev. Ecol. Syst 33: 341-370.

19. Viikari L, Alapuranen M, Puranen T, Vehmaanpera J & Siika-Aho M (2007). Thermostable enzymes in lignocellulose hydrolysis. Adv. Biochem. Eng. Biotechnol 108: 121–145.

Advances in Biotechnology

Chapter 4

RNAi based strategies for enhancing plant resistance to virus infection

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Abstract

Viruses are the most potent parasitic entities that are detrimental to all animal and plant groups with no exceptions known so far. The viral genome though small is sufficient for sustaining the infection and its propagation inside the host organism. The sessile inhabitants of the plant kingdom have developed their strategies to counter this threat too with variable degree of success. The most important and significant of these strategies involves the RNA silencing mechanism. The viruses have evolved successfully in parasitizing and evading this plant defense strategy, by their ability to encode various "suppressor" molecules, which are able to target different components of the silencing pathway in plants. Besides suppression activity, these proteins also perform functions essential for virus multiplication and pathogenicity. In this chapter we briefly discuss about the plant defense strategies with the help of RNAi mediated processes with special focus on Virus Induced Gene Silencing (VIGS) and the viral suppressors as countermeasures to combat this strategy, while describing the probable mechanisms of suppressor action and the variations that exist in their mode of action. We have also tried to elucidate certain assays that are commonly used to detect and quantify the activity and strength of

these suppressor proteins. Then, we describe the specific applications of the RNAi based strategies used to counter virus attack.

Abbreviations: AGO: Argonaute; amiRNA: Artificial microRNA; CaLCV: Cabbage Leaf Curl Virus; Chs: chalcone synthetase gene; CMV: Cucumber Mosaic Virus; CP: Coat Protein; CP-MR: Coat Protein Mediated Resistance; DCL: DICER-Like; PTGS: Post-Transcriptional Gene Silencing; PVX: Potato Virus X; PVY: Potato Virus Y; ra-siRNA: Repeat associated siRNA; RdRP: RNA dependent RNA polymerase; REP: Replication-associated protein; RISC: RNA Induced Silencing Complex; RSS: RNA Silencing Suppressor; siRNA: Small interfering RNA; SVISS: Satellite-virus-induced silencing system; dsRNA: Double stranded RNA; GUS: β-glucoronidase; Hc-Pro: potyvirus encoded helper component proteinase; miRNA: microRNA; MYMIV: Mungbean Yellow Mosaic India Virus; nat-siRNA: Natural antisense siRNA; Pds: phytoene desaturase; ta-siRNA: Trans acting siRNA; TE: Tobacco Etch Virus; TGMV: Tomato Golden Mosaic Virus; TGS: Transcriptional Gene Silencing; TMoV: Tomato Mottle Virus; TMV: Tobacco Mosaic Virus; ToLCV: Tomato Leaf Curl Virus; TRV: Tobacco Rattle Virus; TYLCV: Tomato Yellow Leaf Curl Sardinia Virus; vi-siRNA: Viral siRNA; VIGS: Virus Induced Gene Silencing

Keywords: RNA Silencing; Viral suppressor; Mechanism; double stranded RNA; VIGS

1. Introduction

Viruses are recognized as the primary cause of nearly half of the infectious diseases in plants. Transmitted by insects such as leafhoppers, treehoppers, whiteflies etc the viruses are capable of infecting almost all types of plants. Infected plants may show a range of symptoms depending on the disease but often there is yellowing of leaf (streaking, vein clearing or mosaic), curling of leaf and other growth distortions like plant stunting, abnormalities in flower or fruit formation etc. The infecting viruses can damage up to 70% to 100% of yield [1] and rough predictions indicate that the total worldwide damage due to plant viruses may be to the tune of US$ 60 billion per year. The accumulation of vast amount of data from across the globe has revealed the devastating potential of the different viruses and have established their identity as notorious plant pathogens.

Plants have developed strategies to counter this threat with variable degree of success. The strategy based on the RNA silencing pathway being the most important and significant. It is a method of sequence-dependent gene regulation involving suppression of transcription, transcript degradation or translation inhibition [2,3]. The phenomena of RNA silencing was first observed during loss in petunia flower pigmentation while over expressing the *chalcone synthetase (chs)* gene [4]. An important observation on RNA silencing was made during experiments with pathogen-derived resistance when it was shown that virus resistance correlated with reduction of viral mRNA in the cytoplasm. Later three independent reports demonstrated that untranslatable viral RNA was sufficient to produce virus resistant plants and the expression of viral proteins was not required [5-7].

These findings launched the search for the “resistance factors” and in the year 1999, it was explicitly proved that plants accumulated small double stranded RNA (dsRNA) molecules whose sequence was identical to the silenced transgene [8]. Similarly sequence-specific

small RNAs were observed in *Potato Virus X* (PVX) infected plants, suggesting a role of these molecules in antiviral defense mechanism. This triggered the discovery of the components and pathways of the silencing machinery [8]. Since then, it has evolved as a gene silencing tool with great potential for virus resistance. The silencing mechanism is involved in adaptive defense response, gene regulation and chromatin maintenance [9-11]. Though the virus can act as both inducers and targets of gene silencing, they have evolved successfully in parasitizing and evading plant defence strategies, by their ability to encode various "suppressor" molecules which are able to repress different components of the silencing pathways. An understanding of both components is necessary for developing effective antiviral strategies for enhancing plant resistance.

2. RNA silencing in plant antiviral defense

RNA silencing or RNA interference is the natural strategy of switching off gene expression during fundamental processes like development, genome maintenance and defense against foreign molecules e.g. viruses. With the rapid advancement in science, a lot of information has emerged regarding the mechanisms and machineries of RNA silencing [12]. This is being exploited as a new tool for developing antiviral products, which have large applications in field of medicine, agriculture and basic biology [13]. In the medical sector, several studies have demonstrated efficient *in-vivo* delivery of siRNAs and therapeutic benefit in mice or bovine models. Presently several companies are engaged in developing RNA silencing based drugs for clinical use [14]. In the agricultural sector studies have been performed on a number of plants to improve nutritional content, increase yields and remove undesirable metabolites [15]. The emphasis is on deciphering gene functions and identifying pathways that can be directed to protect plants from environmental perturbations and pathogen attack. The potential of RNAi has been recently demonstrated in developing effective resistance against many coleopteran and lepidopteran insect-pests of crops [16] and in managing plant-parasitic nematodes [17].

The RNAi mechanism exhibits an array of diversity in different components for its mode of action but the basic mechanism involves the cleavage of a stem-loop like or a perfect dsRNA structure into small RNA molecules of about 21-24 nt length. This inducer dsRNA can be endogenous like annealed overlapping transcript of opposite polarity, triggered by transcription of tandem or inverted repeat sequences or else exogenous, like RNA virus replication intermediates [18-23]. The dsRNAs are diced into RNA duplex of 20-24 nts with the characteristic 2 nt 3' overhangs by DICER, which is a key component of the microprocessor complex [8,19,24,25]. The small RNAs associate with a set of proteins to form RNA Induced Silencing Complex (RISC), which directs the silencing pathway. The small RNAs include the miRNAs (microRNAs) and the siRNAs (small interfering RNAs) with their various sub-types, viz., ta-siRNA (trans acting siRNAs), ra-siRNA (repeat associated siRNAs), vi-siRNA (viral siRNAs), nat-siRNA (natural antisense siRNAs) etc. Several excellent reviews are available

detailing the nature, classification, biogenesis and function of these small RNAs [3,26-29].

The key protein molecules involved in the process of small RNA biogenesis and function are DICER (DCL), RNA DEPENDENT RNA POLYMERASE (RdRP) and ARGONAUTE (AGO). The silencing mainly occurs at two different stages in cells and hence is categorized accordingly. The cytoplasmic RNA silencing also known as Post-Transcriptional Gene Silencing (PTGS), targets mRNA for degradation or translation repression with the help of 21-22 nt species of RNA molecules generated from inducing dsRNA [2]. This involves various small RNA classes (miRNA, siRNA, trans-acting siRNA) and DCLs. Subsequently the diced small RNAs are loaded into the RISC effector complex to guide specific localized silencing [18-22,30]. In several cases, the primary siRNAs prime the RdRP6 mediated synthesis of secondary dsRNA for generation of secondary siRNA or transitive siRNAs. This results in the amplification of siRNAs and the spread of silencing beyond the site of its initiation to bring about systemic silencing [2].

The other silencing pathway operates at the nuclear level and is called Transcriptional Gene Silencing (TGS). This pathway is directed by the 24 nt siRNAs and miRNAs and it involves heterochromatin silencing by cytosine methyation of DNA and post-transcriptional modifications of histone proteins (e.g. H3- methylation at lysine – 9). The siRNA involved in this case are generated by DCL-3 with the help of AGO-4 and RdRP-2 [2,3]. This pathway is considered significant in preventing rearrangement in centromeric and telomeric repeats by suppressing transposons and other invasive DNAs and thus maintaining genetic integrity [2, 3].

2.1 Virus induced gene silencing

The observations that viruses act as inducer as well as target of RNAi machinery lead to the theory that the silencing mechanism is primarily a defense system in plants [31,32]. The invading viral RNAs can precondition this response, even though the natural response is adaptive and requires recognition of ‘foreign’ nucleotides for initiation [33-35]. The small RNAs triggered in response to one mild virus also serve as “molecular memory” to cross-protect the infected plant against virulent infection by another related virus carrying sequences homologous to the first virus [33]. This cross protection phenomenon was reported by the plant virologists as early as 1920, though the mechanisms were worked out much later.

In fact, the term Virus Induced Gene Silencing (VIGS) was first used to describe the phenomenon of recovery from virus infection [36]. Soon after virologists observed that over expression of certain genes using viral vectors led to the degradation of the desired mRNA resulting in genotypes resembling a nearly knockout mutation of the corresponding gene. This was explained to be due to RNA silencing and subsequently the phenomenon of VIGS was exclusively used to describe the ability of recombinant viruses to knock down expression of

endogenous genes [37,38]. It was speculated that majority of plant viruses replicate via a dsRNA intermediate which may serve as the principal inducer of the siRNA/RNase system resulting in VIGS. The secondary structure or convergent transcription of viral RNAs can also serve as a potent trigger of RNA silencing and the reaction is further amplified by host encoded RdRPs [39,40]. Thus RNA silencing stands as a very important as well as efficient tool for plants to maintain their defence strategy, especially against viruses.

VIGS as a tool is gaining immense popularity in the field of functional genomics as it is a simple method when compared to agroinfiltration or biolistic gene guns. The method does not involve stable transformation and the results can be obtained rapidly within a period of 2-3 days only. Furthermore, it is easy to use and provides a high throughput characterization of phenotypes that might be lethal in stable lines. It also has the advantage of being very specific to the target and has been successfully used for rapid silencing of one or more genes in a large number of species. However, it may have limitations on availability of infectious clones, its host range, regions of silencing and size restrictions on the inserts. Furthermore, the virus in the VIGS vector needs to be disarmed to avoid any symptom development due to infection [41].

VIGS is being used as a tool that employs the RNA mediated antiviral defence mechanism to produce gene knockouts. A number of VIGS vectors have also been constructed by cloning the gene to be silenced in a minimal portion of the viral DNA (or cDNA in case of RNA virus) that could efficiently replicate and cause infection [38]. This vector is then introduced mechanically into the system and whenever a dsRNA structure or a secondary RNA structure is formed during the course of infection, silencing process is efficiently initiated against the infecting virus. In the process the host silencing response also silences the target-gene(s) at the post-transcriptional levels, in a sequence-specific manner [42]. The first VIGS vector was developed using *Tobacco Mosaic Virus* (TMV) which has an RNA genome. Transcripts of recombinant TMV carrying a sequence encoding *phytoene desaturase* (*pds*) were produced *in vitro* and inoculated to *Nicotiana benthamiana* plants leading to successful silencing of *pds*. The leaves of these plants exhibited characteristic white patches due to photo-bleaching as PDS enzyme is envolved in biosynthesis of carotenoids that protect the chlorophyll from photo-bleaching [43].

Tobacco Rattle Virus (TRV) based VIGS vectors are the most widely used. These are usually cloned between the T-DNA borders and introduced in plants by agroinfiltration [44-48]. TRV-based VIGS vectors have been used to silence genes in a number of Solanaceous plant species [47,49,50]. PVX based VIGS vector have also been used, however they have more limited host range as only three families of plants are susceptible to this virus [46]. The geminiviruses like bipartite *Cabbage Leaf Curl Geminivirus* (CaLCV), *Mungbean Yellow Mosaic India Virus* (MYMIV), *Tomato Golden Mosaic Virus* (TGMV), etc have emerged as

very promising DNA virus based VIGS vectors, as they can be delivered by direct plasmid DNA infection. The *Tomato Leaf Curl Virus* circular replicon based VIGS vector was shown to silence the *pds* and *pcna*genes of tomato in a long-lasting manner [51]. A list of commonly used VIGS vectors is provided in Table 1.

The Satellite-virus-induced silencing system, SVISS, was also demonstrated as an efficient gene silencing in plants.It employs the dual-component of a Satellite-virus-based vector and a helper virus. The first SVISS was based on a Satellite virus which uses the TMV strain U2 as a helper [53]. In other studies, modified satellite DNA were used for silencing genes along with *Tomato Yellow Leaf Curl China Virus* in *N. Benthamiana* [65], *African Cassava Mosaic Virus* in cassava [67], *Pea Early Browning Virus* in pea [56] and *Bean Pod Mottle Virus* in soybean [58]. This method has the advantage of being easily clonable (small genome size), highly stable and showing attenuated symptoms of virus infection.

3. Suppressor of RNAi

Viruses have evolved a defense measure for evading the RNA silencing mechanisms. They encode for protein molecules known as 'suppressors' [68,69] which interfere at different stages of RNA silencing pathway thus helping in efficient infection and replication of virus in the host cell and spreading the infection systemically. These suppressors molecules are usually ordinary viral proteins e.g., coat protein, movement protein or proteases that carry the suppressor activity as their secondary function. It has been suggested that the suppressor activity is casually coupled with transcription factor activity [70]. As a result there is extensive assortment in the RNA Silencing Suppressors (RSS) documented from the distinct viruses. A number of suppressors discovered so far in various systems have been listed in Table 2.

3.1. History of RNA silencing suppression

The vital role played by specific virus encoded proteins in augmenting virulence provided the first indication on the presence of RSS. In the classical study it was shown that PVX by itself, causes mild symptoms but the symptoms show a vigorous enhancement during co-infection with the *Potato Virus Y* (PVY) and *Tobacco Etch Virus* (TEV) [31]. Subsequently several other reports showed that co-infection with combination of viruses caused increased symptom severity compared to each of the viruses alone. This phenomenon was denoted as synergism [71] and it is now understood that the enhanced synergism is mainly due to weakening of the host defense by RSS targeting the silencing pathway at multiple points [31,72].

In 1998 several independent reports showed the involvement of a *potyvirus* encoded helper component proteinase (Hc-Pro) in enhancement of replication of many unrelated viruses. In one such report it was shown that Hc-Pro suppressed the PTGS of β-glucoronidase (GUS) reporter transcript on a highly expressed locus [92]. In another study, GUS silenced

Nicotiana tabacum plants were crossed with four independent transgenic plants expressing TEV-P1/HC-Pro and it was observed that silencing was reversed in the resulting offsprings [87]. These observations led to identification of P1/Hc-Pro as the first RSS.

In the same year, Brigneti and co-workers showed that PTGS of a green fluorescent protein (GFP) in *Nicotiana benthamiana* plants transgene is suppressed after infection with *Cucumber Mosaic Virus* (CMV). In an interesting experiment they expressed HC-Pro of PVY and 2b protein of CMV-encoded proteins in a PVX vector and demonstrated that both can act as RSS. They also demonstrated that HC-Pro acts by blocking the maintenance of PTGS in tissues containing established silencing, whereas the 2b protein prevents initiation of gene silencing in the newly emerging tissues [81]. Since then several RSS from plant, insect (like B2 protein of *Flock House Virus*) and animal (like NS1 encoded by *Influenza Virus*) viruses have been identified [87,93,94]. It was also shown that the RSS can suppress silencing in both animal and plant cells, regardless of their host preference due to the conserved nature of the silencing phenomenon [95]. These findings triggered the search for more RSS and since then, a number of viral encoded RSS have been discovered (Table 2). It emerges that the viruses employ RSS as a common strategy against one of the most potent induced defense system.

3.2. Identification of RNA silencing suppressors

The analysis of candidate viral proteins as potential RSS was enabled by the development of different screening systems, based on monitoring their role in reversing the RNA mediated silencing of reporter genes like GFP or GUS. The assays utilized different reporter constructs, such as partial or complete inverted repeats and this also provided an indication on the possible site of action of the RSS [96]. The most commonly used *in planta* assay is based on transgenic tobacco plants constitutively silenced for a reporter gene [97]. The RSS activity can be assayed by rummaging for localized reporter gene expression following transient expression of the virus encoded protein [98,99]. As a modification of this method the reporter gene and the putative RSS are co-infiltrated in wild type tobacco leaves followed by monitoring the reporter gene expression [86,100,101]. It is anticipated that in presence of RSS activity, the reporter gene expression will be retained to a high level or may even increase.

An alternative method involves generating two types of stable transgenics, one containing a silenced reporter gene and the other expressing the candidate viral RSS [87,92,102-104]. The two plants are crossed and the progeny is screened for reporter gene expression. However, this method is labour intensive and often over-expression of RSS in the plants affects seed formation and leads to developmental defects. Alternatively, the candidate RSS can be ectopically expressed from a heterologous viral vector, which is inoculated on to the silenced transgenic plants. PVX based vectors lack the ability to restore the reporter gene expression in such assays and thus serve as suitable vectors to test the viral genes for their RSS capability [81].

The affect of RSS on systemic movement of the silencing molecules can be assayed by grafting of a reporter gene silenced rootstock to a reporter gene expressing scion. The silencing signal systemically spreads from rootstock to scion to silence the reporter gene expression. The candidate RSS is introduced in the plant serving as the rootstock with the help of genetic crossing. In presence of RSS activity the spread of the silencing signal will be curtailed and there will be no effect on the reporter gene expression in the scion. This assay is not only time consuming but requires the production of transgenics as well as breeding experiments.

3.3. Mechanism of RNA silencing suppression

The RSS encoded by different plant viruses appear to suppress the silencing based virus defense pathway in different points [68,86,100,105-107]. They primarily act on the common biogenesis or functional components of the pathway causing suppression of the siRNA-mediated [108-113] pathways, resulting in breakdown of the host anti-viral defense response [96]. The common sites of action include:

3.3.1. Binding double stranded RNA

In the silencing pathway the long dsRNA acts as a major inducer and small dsRNA serves as a major effector molecule. The binding of RSS masks the long dsRNA and thus protects it from the DCL action, thereby preventing its processing into siRNA [114]. The binding to small dsRNA like siRNA duplex prevents their sorting into the AGO containing RISC complex and renders them functionally inactive. Binding to small RNA duplex is a common strategy for many of the viral encoded RSS [114] encoded by phylogenetically and evolutionarily divergent viruses like tombusvirus P19, closterovirus P21, carmovirus CP, pecluvirus p15, hordeivirus QB, potyvirus HC-Pro, CMV-2b [115-120].

The RSS also interfere with miRNA biosynthesis in plants and inhibit the cleavage and translational repression of target genes by specific miRNA in the plant developmental pathway [101,102-104,121-124]. In plants, virus-induced disease symptoms often result in developmental abnormalities resembling perturbation of miRNA-mediated function. Several studies have now shown that transgenic expression of RSS can alter the accumulation and/or functioning of miRNAs leading to developmental abnormalities related to the action of miRNAs [125]. Tombus virus encoded P19, *Beet Yellow Virus* encoded P21 and Potyvirus P1/HC-Pro bind to duplex forms of miRNAs [103].

The crystal structure of RSS further indicates that the mechanism of RNA binding also varies [126]. TAV2b recognizes siRNA (19 nt) duplex by a pair of hook-like structures and adopts a R-helix homodimer structure to measure siRNA duplex in a length-preference mode, whereas P19 protein uses an extended S-sheet surface and a small R-helix to form a caliper-like architecture for binding and measuring the characteristic length of siRNAs. Few RSS

proteins have the ability to bind both long and short dsRNA, like p14, FHV-B2 [120]. AC4 protein of ACMV is a unique RSS because of its ability to bind to single-stranded forms of miRNAs and siRNAs and thus interferes with both miRNA-mediated function and suppression of siRNA-mediated PTGS.

3.3.2. Binding to biogenesis components

Many RSS have been found to interact with and inhibit the activities of DICER/DCL and this directly affects the small RNA biogenesis. P38 of *Turnip Crinkle Virus* suppresses DCL4 activities [127]. The P6 protein of CMV suppresses the host DRB4 activity which is required to facilitate the activity of DCL4 enzyme [128]. Among the non-plant virus FHV-B2 interacts with the PAZ domain of DICER [120] and the Hepatitis C virus core protein also directly interacts with DICER to antagonize RNA silencing [126].

RDR6 is another important component mainly associated with sense gene mediated silencing and transitive siRNA biogenesis by generating dsRNA. MYMIV-AC2 has been found to be interacting with RDR6 to interfere with RNA silencing [129]. Potyvirus P1/HC-Pro also interferes with the HEN-1 mediated methylation of miRNA [103].

3.3.3. Interference with RISC

Suppression activity at RISC level is achieved by targeting the AGO protein. Polerovirus encoded P0 and CMV-2b suppresses RNA silencing by destabilizing the AGO1 [130,131]. Similar silencing mechanism has also been observed in case of MYMIV-AC2 [129]. P1/HC-Pro also inhibits miRNA-mediated cleavage of target mRNAs, but the exact mode of action of this protein in the silencing pathway is not known.

3.3.4. Interference with DNA methylation

Some RSS have the ability to reverse the small RNA mediated TGS. They can cause reversal of TGS by various mechanisms. It has been reported that the 2b protein of severe Shan-Dong (SD) isolate of *Cucumber* mosaic virus, suppresses RdDM by binding and sequestering siRNAs in a process involving AGO proteins in the nucleolus [132]. Another mechanism is displayed by the AC2 protein of Begomovirus and Curtovirus genera. They inactivate *Adenosine Kinase*, thereby reducing production of the methyl donor (SAM) and causing release of TGS [133,134]. The C1 protein of beta-satellite of *Tomato Yellow Leaf curl China Virus* inactivates *s-adenosyl homocysteine hydrolase*, an enzyme required for synthesis of SAM and thus reduces the level of cytosine methylation of viral DNA [135]. The C2 protein of *Beet Severe Curly Top Virus* increases the life-span of SAMDC1 and thus suppresses DNA-methylation mediated gene-silencing in Arabidopsis [136]. The AC2 protein of *Indian Cassava Mosaic Virus* up regulates RAV2, which acts as a transcriptional repressor, inhibiting transcription of

KYP, a histone methyl transferase.

4. Applications of RNA silencing to counter virus attack

The viral pathogens depend on the host's cellular machinery for reproduction; hence it is challenging to eliminate them without damaging the host plant. Therefore, most management strategies for diseases caused by plant viruses are directed at preventing infection. The RNA silencing based strategies to induce a highly specific antiviral state in plants have been extensively employed to raise virus resistant transgenic plants, even before the exact mechanisms were comprehended. This is evident from the commercial cultivation of *Papaya Ringspot Virus* resistant 'SunUp' papaya and virus resistant potato varieties "NewLeaf Plus" and "NewLeaf Y". The understanding of the VIGS and RSS helped to further refine the techniques. The targeting of the RSS, which play an important role in viral pathogenicity, emerged as efficient antiviral strategy. Ever since the anti-viral properties of RNAi have been tested in many other crops.

4.1. Pathogen –derived resistance

Sanford and Johnston [137] developed a simple concept of parasite or pathogen-derived resistance to explain the observations that a plant infected with one virus shows resistance to infection by same or a closely related virus strain.This concept was utilized in the early attempts to engineer resistance through the introduction of viral genetic material into the plant genome [138-140].

An excellent example is provided by the pioneering work of Robert Beachy's group in 1986 by providing coat protein (CP)-mediated resistance to TMV [141]. It was demonstrated that the over-expression of viral CP gene in transgenic plants could protect these plants from the infection by TMV and closely related tobamoviruses. It was shown that transgenically expressed CP interfered with the assembly of TMV particles due to hindrance with the inter-subunit interactions [141]. These results also indicated that plants could be genetically transformed for resistance to virus disease development and the trait of resistance could be stably transmitted to the next generation [142]. This phenomenon was referred as coat-protein-mediated resistance (CP-MR) and was found to be effective in a variety of host or virus combination. CP-MR thus became a choice for the researchers to develop transgenic plants against various viruses. The CP-MR was used against the *Rice Stripe Virus* by introducing its CP in japonica rice by protoplast electroporation followed by generation of transgenic plants [143]. CP-MR was also reported for *Potato Virus Y* in tobacco [144]; *Tomato Yellow Leaf Curl Virus* (TYLCV) in tomato (145); *Pea Enation Mosaic Virus* in pea [146]; *Potato Mop-Top Virus* in potato [147] and *Cucumber Mosaic Virus* 1B in tobacco [148].

In viruses the CP plays an important role in ssDNA protection, movement and trans-

mission of viruses. A CP-deficient ACMV-KE clone lost its systemic infection capacity in cassava plants and showed reduced functional interaction with its vector *Bemisia tabaci* [149]. The vector specificity determinant regions were also shown in the CP of *Abutilon Mosaic Virus* [150]. Expression of a mutated non-functional CP therefore appeared to be a potential strategy to impede the virus spread amongst its vectors. Later, it was shown that, mutant forms of TMV CP had stronger inter-subunit interactions and these were found to confer increased levels of CP-MR compared with wild-type CP.

The viral replication-associated protein (REP) also emerged as a strong candidate for pathogen-derived resistance. The genomes of plant single-stranded DNA viruses do not encode polymerases, but their replication requires interaction between the REP and host polymerases. The Rep protein by itself is not a determinant of disease or pathogenesis [151]. It was shown that over-expression of truncated *rep* gene (encoding for the N-terminal 210 amino acids of REP) showed resistance against *Tomato Yellow Leaf Curl Sardinia Virus* up to 15 weeks post virus inoculation [152,153]. Similar observations were reported for the expression of the N-terminal region encompassing the DNA binding and oligomerization domain of *Tomato Leaf Curl New Delhi Virus*. This also accorded resistance to heterologous ACMV, *Pepper Huasteco Yellow Vein Virus* and *Potato Yellow Mosaic Virus* [154].

The virus also encodes movement proteins (MP) that are required for cell-to-cell and long-distance movement. Tobacco plants expressing *Tomato Mottle Virus* (TMoV) encoded movement proteins, BV1 or BC1, showed a significant delay in infection to TMoV and CaLCV infection [155,156]. Non-functional MPs may compete for Nuclear Shuttle Protein; required for long distance movement in begomoviruses interaction or oligomerization and this could explain the resistance previously observed in mutated MP expressing plants [157]. However the overexpression of these proteins also had deleterious effects on plant development [158]. Over the years, other full-length or truncated viral genes, like RdRp, proteinase, satellite RNA, defective interfering RNA, and noncoding regions, have been extensively used to engineer virus resistance.

4.2. Antisense RNA

Regulation of gene expression by antisense RNA (asRNA) was first discovered as a naturally-occurring phenomenon in bacteria [159], however its effectiveness in eukaryotic cells was demonstrated as early as 1984 [160]. The antisense technology is based on blocking the information flow from DNA via RNA to protein by the introduction of an RNA strand complementary to the sequence of the target mRNA. It was hypothesized that the antisense RNA base-paired with its target mRNA thereby forming dsRNA duplex causing the blockage of mRNA maturation and/or translation [161]. It was later shown that the dsRNA is recognised as a substrate by the RNA silencing machinery resulting in its rapid degradation into siRNAs

which in turn cleave the target mRNAs in a sequence dependent manner. The antisense technology has potential as therapy to treat many genetic and metabolic disorders, for identifying gene functions and in crop development.

The application of artificial antisense RNA was demonstrated in plants by down-regulating the *chs* gene which is responsible for flavonoid biosynthesis [162,163]. The chs-antisense RNA elicited increased fungal disease susceptibility in *Arabidopsis* plants [164]. This technology was successfully used in Flavr Savr to delay tomato ripenining and rotting by introducing the antisense RNA for *Polygalactourodase* gene to inhibit the synthesis of the enzyme [165,166]. The expression of antisense RNA against *Potato Leaf Roll Luteovirus* CP triggered virus resistance in the transgenic plants [167]. Day and coworkers [168] used antisense AL1 transcripts of TGMV to engineer geminivirus resistance in tobacco plants. Resistance was engineered against the *Cotton Leaf Curl Virus* by using anti-sense constructs of Rep, REN and TrAP genes [169]. The antisense technology was also effective in confirming resistance against infection by viruses like PVX [170], TMV [171], CMV [171] and TYLCV in *Nicotiana benthamiana* [172] and tomato [173].

4.3. Hairpin RNA and double stranded RNA

The direct application of RNA silencing in plants was initiated by the use of intron looped self complimentary hairpin RNA (hpRNA) constructs [174]. The hpRNA constructs contained, 100-800 bp long fragments of the target gene cloned in sense and antisense orientations, separated by an intron sequence. When transcribed *in planta* the primary transcripts folded into a hairpin structure, which could be recognized and processed by DCLs into siRNAs [175]. The siRNAs then induced PTGS and repressed the target gene strongly. These were found to be more effective in silencing the target genes as compared to overexpression of antisense transcripts. The hpRNA have emerged as the reagents of choice for triggering specific RNAi against a variety of viruses in different plant species [176-178]. These constructs are delivered into the cells through agrobacterium or gene gun as plasmid or viral vectors where the get transcribed and processed into sRNAs. The hpRNA encoding constructs driven by a maize ubiquitin promoter bestowed immunity to transgenic barley against *Barley Yellow Drawf Virus* [179]. Transgenic plants resistant to *Cassava Latent Virus* were produced by introducing a tandem repeat of its subgenomic DNA B [180]. HpRNA construct with 425 bp conserved region of *Bean Golden Mosaic Virus* REP was used to generate virus resistant tobacco plants [181]. Recently it was reported that a spray application of a long noncoding dsRNA on barley plants was effective in targeting three fungal cytochrome P450 *lanosterol C-14α-demethylases*, required for biosynthesis of fungal ergosterol [182]. Similarly wheat plants pre-infected with BSMV containing antisense sequences against target genes of the fungus, *Fusarium culmorum* caused a reduction of corresponding transcript levels in the pathogen and reduced disease symptoms [183]. Similar efforts are ongoing to leverage the power of

RNAi in engineering effective, broad-spectrum and ecologically safe resistance against many viruses in different crops [184-193]. The major limitation of this technology lies in the generation of aberrant siRNAs, which result in silencing of the non-specific genes.

With the unveiling of the mechanism of RNA silencing it was clear that the dsRNA serves as the trigger for PTGS, so strategies based on introduction of dsRNA were also employed. The major bottleneck for the application of this technology lies in the proper designing and effective delivery of siRNAs molecules. Several online software sare now available to design the correct siRNAs [28]. The choice of delivery technique(s) is more or less governed by the preparation of the siRNA. Among the popular approaches for siRNA production are chemical synthesis, *in vitro* transcription, expression vectors and PCR expression cassettes. It is important to adapt the correct delivery strategies to facilitate better cellular accumulation of siRNAs and their release close to the respective targets site. It is generally believed that the siRNAs are passively endocytosed [184] however,the in vivo transmission of naked siRNAs in-vivo is limitated by inefficient cellular uptake, nucleolytic degradation and other problems like trapping in non-desirable cellular compartments. Hence direct application siRNA involves mixing these molecules with 'biocompatible' and 'genocompatible' formulations for appropriate delivery [185].

In plants, generally vectors are used to generate and deliver siRNAs to the target tissues instead of direct delivery. The use of viral vectors has been discussed in the section 2.1 under VIGS. The plasmid vectors can exist as episomes or integrate in the genome. The plasmids are used to express around 70 nt shRNAs or hpRNAs, which are transcribed into effective dsRNAs capable of silencing the target genes [186,187]. One of the excellent application is the use of specific dsRNA and siRNAs for HC-Pro region to block the replication and transmission of PVY through the potato plants [194].

The dsRNA also induces genomic methylation [195]. Methylation of the promoter sequence induces TGS, which unlike PTGS is stable and heritable. However if methylation occurs in the coding sequence, it has no apparent effect on the transcription of the locus, although silencing still occurs at the post-transcriptional level. The dsRNA carrying a sequence homologous to the promoter of the transgene can guide the methylation and TGS in plants. Methylation of a *Tomato Leaf Curl Virus* derived transgene promoter and consequent transgene silencing have been observed on infection [196] strongly suggesting that virus-derived siRNAs are also generated for the transcribed and non-transcribed intergenic regions of the viral genome [197]. The dsRNA-guided methylation of geminivirus bidirectional promoters may down-regulate the transcription of viral genes, resulting in inefficient virus replication [198].

4.4. Artificial miRNA

Artificial microRNA (amiRNA) technology is based on designing miRNA or engineering miRNA artificially by mimicking the intact secondary structure of endogenous miRNA precursors to utilize the natural silencing pathway to target desired transcripts [199,200]. It has been shown that altering several nucleotides within sense and antisense strands of miRNA has no bearing on its biogenesis and maturation, as long as secondary structure of its precursor remains unaltered. The amiRNA acts as a specific, powerful and robust tool that can be applied to study metabolic pathways, gene functions and for improving favourable traits. The amiRNA technology was first used for gene knock down in human cell lines [201] and later in Arabidopsis [202], where they were shown to effectively interfere with reporter gene expression. Subsequently, it was demonstrated that amiRNAs when expressed under constitutive or tissue-specific promoters can down-regulate a number of endogenous genes without affecting the expression of other unrelated genes [124,203].

The amiRNA sequences are designed according to the determinants of plant miRNA target selection, such that the 21 nt sequence specifically silences its intended target genes. They resemble the natural miRNAs in containing a Uracil residue at their 5' end; having an Adenine/ Uracil residue as their 10^{th} nucleotide and displaying 5' instability relative to their amiRNA* sequence [204,205]. The miRNA mediated gene regulation has also emerged as a second generation tool in the field of RNAi technology and having various applications in the field of agriculture, medicine and in the field of functional genomics studies [203, 206-211].

The amiRNA technology is being utilized to target the invading viral gene transcripts and the effective strategies employ targeting the viral encoded RSS transcripts [212]. The amiRNAs arising from ath-miR-159 backbone were effective in targeting viral sequences encoding RSS, P69 of *Turnip Yellow Mosaic Virus* and HC-Pro of *Turnip Mosaic Virus* [206]. Similarly, amiRNA sequences targeting 2b of *Cucumber Mosaic Virus* (CMV), a potent RSS, can confer effective resistance to CMV infection [208]. The amiRNA targeting overlapping regions of geminiviruses genes, AC1, AC2 and AC4 were used to generate transgenic tomato plants that could resist infection of ToLCV New Delhi variety [213,214]. These amiRNAs were also used for generating resistance against *Watermelon Silver Mottle Virus* in tobacco [215].

4.5. Artificial tasiRNA

Artificial tasiRNA technology has also been used to generate virus resistant plants. The strategy involves designing a binary vector incorporating the 5' and 3' binding sites of miR390 flanking the RSS sequence on each side, respectively. This vector when introduced in plants produces artificial tasiRNAs from the RSS encoding sequences. These tasiRNAs can inactivate the viral transcripts containing homologous sequences. The transgenics producing the artificial tasiRNAs were used to protect the plants against the invading ToLCVs [216]. Such

strategy could in principle be adopted to develop plants tolerant for other phytoviruses.

5. Figure

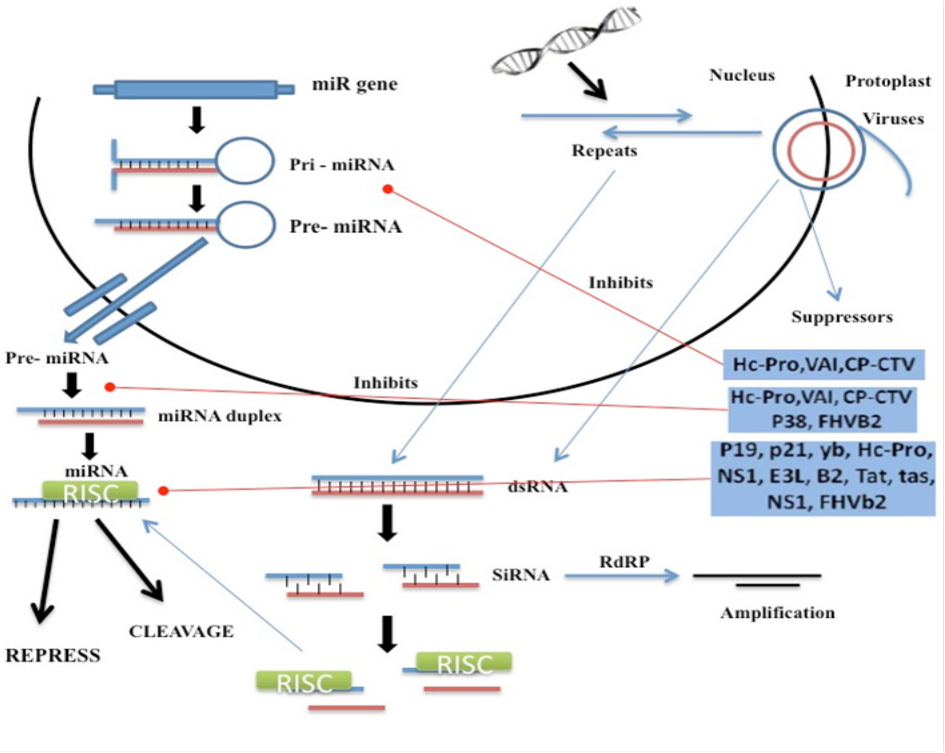

Figure 1: The different modes of interference of the known RNA silencing suppressors on miRNA and siRNA biogenesis pathway. The suppressors may interrupt the RNA silencing pathway at different steps from the beginning of biogenesis, small RNA maturation and loading into RISC.

6. Tables

Table 1: List of viruses used for the construction of VIGS vectors.

Virus Backbone	Reference
RNA Virus and their satellites	
Tobacco Mosaic Virus (TMV)	[43,52]
SatelliteTobacco Mosaic Virus (STMV)	[53]
Potato Virus X (PVX)	[37]
Tobacco Rattle Virus (TRV)	[44]
Barley Stripe Mosaic Virus (BSMV)W	[54,55]
Pea Early Browning Virus (PEBV)	[56]
Brome Mosaic Virus (BMV)	[57]
Bean Pod Mottle Virus	[58]
Cucumber Mosaic Virus (CMV)	[59]
Tomato Mosaic Virus (TMV)	[60]
DNA Virus	
Tomato Golden Mosaic Virus (TGMV)	[61]
Cabbage Leaf Curl Virus (CaLCV)	[62]
Tomato Leaf Curl Virus (ToLCV)	[51,63]
Tomato Leaf Curl Virus satellite	[64]
Satellite DNAβ of Tomato Yellow Leaf Curl China Virus (TYLCV)	[65]
Tobacco Curly Shoot Virus	[66]

Table 2: List of suppressor molecules identified from different plant viruses.

Genome genome	Virus	Suppressor protein	Type of silencing mechanism	Reference
DNA	African cassava mosaic virus	AC2 AC4	Local Systemic	(68, 73)
	Tomato golden mosaic virus	AL2	-	(74)
	Tomato yellow leaf curl virus-C	C2	Local and Systemic	(75, 76)
	Beet curly top virus	L2	-	(74)
RNA	Turnip crinkle virus	CP (P38)	Local	(77, 78)
	Beet yellows virus	p21	Local	(79)
	Citrus tristeza virus	p20	Local	(80)
		p23	Local and Systemic	(80)
		CP	Systemic	(80)
	Beet yellow stunt virus	P22	Local	(79)
	Cowpea mosaic virus	S coat	Local	(68)
	Cucumber mosaic virus	2b	Local* and Systemic	(81, 82)
	Peanut clump virus	p15	Local and Systemic	(83)
	Barley stripe mosaic virus	γb	-	(84)
	Beet western yellows virus	P0	Local and not systemic	(85)
	Potato virus X	P25	Systemic	(86)
	Potato virus Y	HC-Pro	Local and Systemic*	(81, 87)
	Rice yellow mottle virus	P1	-	(68)
	Tomato mosaic virus	126-kDa protein	-	(68, 88)
		130-kDa protein	Local	(89)
	Tomato bushy stunt virus	P19	Local and Systemic (binds siRNAs)	(68, 90)
	Cymbidium ringspot virus	P19	Local and Systemic (binds siRNAs)	(91)
	Turnip yellow mosaic virus	p69	Local	(50)

7. Conclusions

In plants, the RNA-dependent silencing of gene function serves as a key regulatory mechanism forming a crucial link between defense, development and adaptation to stress. In response to virus attack it encompasses the first line of protection for restricting accumulation or spread of invading viruses. The phenomenon of RNA silencing has enormous potential to be exploited as a tool to counter virus attack. As a counter defensive mechanism, the viruses encode the RSS proteins to replicate and establish in the host plants. The RSS have the ability to suppress the RNA silencing at different stages of the pathway andmutation in these proteins result in attenuated symptom development and mild disease manifestation. Thus, RNA silencing based strategies designed to inhibit the RSS activity can play a crucial rolein developing virus resistance. It is expected that further growth in knowledge will help in adapting more innovative designs to enhance the robustness of RNAi technology towards developing disease-free crop plants.

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9. References

1. Anderson JP, Daifuku R, Loeb LA. Viral error catastrophe by mutagenic nucleosides. Annual Review in Microbiology 2004;58:183-205.

2. Agrawal N, Dasaradhi P, Mohmmed A, Malhotra P, Bhatnagar RK, Mukherjee SK. RNA interference: biology, mechanism, and applications. Microbiology and Molecular Biology Reviews. 2003;67(4):657-685.

3. Sanan-Mishra N, Mukherjee SK. A peep into the plant miRNA world. Open Plant Science Journal. 2007;1:1-9.

4. Jorgensen RA, Que Q, Stam M. Do unintended antisense transcripts contribute to sense cosuppression in plants? Trends in Genetics. 1999;15(1):11-12.

5. De Haan P, Gielen JJ, Prins M, Wijkamp IG, van Schepen A, Peters D, et al. Characterization of RNA-mediated resistance to tomato spotted wilt virus in transgenic tobacco plants. Nature Biotechnology. 1992;10:1133-1137.

6. Lindbo JA, Dougherty WG. Untranslatable transcripts of the tobacco etch virus coat protein gene sequence can interfere with tobacco etch virus replication in transgenic plants and protoplasts. Virology. 1992;189(2):725-733.

7. Roth A, Breaker RR. Selection in vitro of allosteric ribozymes. In: Sioud M. (Ed) Ribozymes and siRNA Protocols: Methods in Molecular Biology, Springer; 2004. 252:145-164.

8. Hamilton AJ, Baulcombe DC. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science. 1999;286(5441):950-952.

9. Peláez P, Sanchez F. Small RNAs in plant defense responses during viral and bacterial interactions: similarities and differences. Frontiers in Plant Science. 2013;4:343.

10. Sanan-Mishra N, Varanasi SP, Mukherjee SK. Micro-regulators of auxin action. Plant Cell Reports. 2013;32(6):733-740.

11. Szittya G, Burgyán J. RNA interference-mediated intrinsic antiviral immunity in plants.In: Cullen BR. (Ed) Current Topics in Microbiology and Immunology. Springer;2013. 371:153-181 .

12. Roth BM, Pruss GJ, Vance VB. Plant viral suppressors of RNA silencing. Virus Research. 2004;102(1):97-108.

13. Enquist L. Virology in the 21st century. Journal of Virology. 2009;83(11):5296-5308.

14. Uprichard SL. The therapeutic potential of RNA interference. FEBS Letters. 2005;579(26):5996-6007.

15. Davies KM. Genetic modification of plant metabolism for human health benefits. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2007;622(1):122-137.

16. Gu L, Knipple DC. Recent advances in RNA interference research in insects: Implications for future insect pest management strategies. Crop Protection. 2013;45:36-40.

17. Sindhu AS, Maier TR, Mitchum MG, Hussey RS, Davis EL, Baum TJ. Effective and specific in planta RNAi in cyst nematodes: expression interference of four parasitism genes reduces parasitic success. Journal of Experimental Botany. 2009;60(1):315-324.

18. Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature. 2000;404(6775):293-296

19. Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes and Development. 2001;15(2):188-200.

20. Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ. Argonaute2, a link between genetic and biochemical analyses of RNAi. Science. 2001;293(5532):1146-1150.

21. Nykänen A, Haley B, Zamore PD. ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell. 2001;107(3):309-321.

22. Tang G, Reinhart BJ, Bartel DP, Zamore PD. A biochemical framework for RNA silencing in plants. Genes and Development. 2003;17(1):49-63.

23. Mateos JL, Bologna NG, Palatnik JF. Biogenesis of plant microRNAs.In: Erdmann VA and Barciszewski J (Eds) Non Coding RNAs in Plants: Springer; 2011. p. 251-268.

24. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. 1998;391(6669):806-811.

25. Wesley SV, Helliwell CA, Smith NA, Wang M, Rouse DT, Liu Q, et al. Construct design for efficient, effective and high-throughput gene silencing in plants. The Plant Journal. 2001;27(6):581-590.

26. Du TG, Jellbauer S, Müller M, Schmid M, Niessing D, Jansen RP. Nuclear transit of the RNA-binding protein She2 is required for translational control of localized ASH1 mRNA. EMBO Reports. 2008;9(8):781-787.

27. Schwab R, Voinnet O. miRNA processing turned upside down. The EMBO Journal. 2009;28(23):3633-3634.

28. Sanan-Mishra N, Karjee S, Mukherjee SK . Design and delivery of small RNAs for RNAi technology. Dynamic Biochemistry, Process Biotechnolgy and Molecular Biology 2010; 4(1):20.

29. Mateos JL, Bologna NG, Chorostecki U, Palatnik JF. Identification of microRNA processing determinants by random mutagenesis of Arabidopsis MIR172a precursor. Current Biology. 2010;20(1):49-54.

30. Vazquez F, Vaucheret H, Rajagopalan R, Lepers C, Gasciolli V, Mallory AC, et al. Endogenous trans-acting siRNAs regulate the accumulation of Arabidopsis mRNAs. Molecular Cell. 2004;16(1):69-79.

31. Pruss G, Ge X, Shi XM, Carrington JC, Vance VB. Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. The Plant Cell. 1997;9(6):859-868.

32. Ratcliff FG, MacFarlane SA, Baulcombe DC. Gene silencing without DNA: RNA-mediated cross-protection between viruses. The Plant Cell. 1999;11(7):1207-1215.

33. Ratcliff F, Harrison BD, Baulcombe DC. A similarity between viral defense and gene silencing in plants. Science. 1997;276(5318):1558-1560.

34. Voinnet O. RNA silencing as a plant immune system against viruses. Trends in Genetics. 2001;17(8):449-459.

35. Szittya G, Molnár A, Silhavy D, Hornyik C, Burgyán J. Short defective interfering RNAs of tombusviruses are not targeted but trigger post-transcriptional gene silencing against their helper virus. The Plant Cell. 2002;14(2):359-372.

36. Van Kammen A. Virus-induced gene silencing in infected and transgenic plants. Trends in Plant Science. 1997;2(11):409-411.

37. Ruiz MT, Voinnet O, Baulcombe DC. Initiation and maintenance of virus-induced gene silencing. The Plant Cell. 1998;10(6):937-946.

38. Baulcombe DC. Fast forward genetics based on virus-induced gene silencing. Current Opinion in Plant Biology. 1999;2(2):109-113.

39. Kurihara Y, Watanabe Y. Arabidopsis micro-RNA biogenesis through Dicer-like 1 protein functions. Proceedings of the National Academy of Sciences USA. 2004;101(34):12753-12758.

40. Voinnet O. Origin, biogenesis, and activity of plant microRNAs. Cell. 2009;136(4):669-687.

41. Burch-Smith TM, Schiff M, Liu Y, Dinesh-Kumar SP. Efficient virus-induced gene silencing in Arabidopsis. Plant Physiology. 2006;142(1):21-27.

42. Lindbo JA, Silva-Rosales L, Proebsting WM, Dougherty WG. Induction of a highly specific antiviral state in transgenic plants: Implications for regulation of gene expression and virus resistance. The Plant Cell. 1993;5(12):1749-1759.

43. Kumagai M, Donson J, Della-Cioppa G, Harvey D, Hanley K, Grill L. Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. Proceedings of the National Academy of Sciences USA. 1995;92(5):1679-1683.

44. Ratcliff F, Martin-Hernandez AM, Baulcombe DC. Technical advance: tobacco rattle virus as a vector for analysis of gene function by silencing. The Plant Journal. 2001;25(2):237-245.

45. Liu Y, Schiff M, Dinesh-Kumar S. Virus-induced gene silencing in tomato. The Plant Journal. 2002;31(6):777-786.

46. Burch-Smith TM, Anderson JC, Martin GB, Dinesh-Kumar SP. Applications and advantages of virus-induced gene silencing for gene function studies in plants. The Plant Journal. 2004;39(5):734-746.

47. Hileman LC, Drea S, Martino G, Litt A, Irish VF. Virus-induced gene silencing is an effective tool for assaying gene function in the basal eudicot species *Papaver somniferum* (opium poppy). The Plant Journal. 2005;44(2):334-341.

48. Wang X, El Naqa IM. Prediction of both conserved and nonconserved microRNA targets in animals. Bioinformatics. 2008;24(3):325-332.

49. Brigneti G, Martín-Hernández AM, Jin H, Chen J, Baulcombe DC, Baker B, et al. Virus-induced gene silencing in Solanum species. The Plant Journal. 2004;39(2):264-272.

50. Chen J, Li WX, Xie D, Peng JR, Ding SW. Viral virulence protein suppresses RNA silencing–mediated defense but upregulates the role of microRNA in host gene expression. The Plant Cell. 2004;16(5):1302-1313.

51. Pandey P, Choudhury NR, Mukherjee SK. A geminiviral amplicon (VA) derived from Tomato leaf curl virus (ToLCV) can replicate in a wide variety of plant species and also acts as a VIGS vector. Virology Journal 2009;6:152.

52. Lacomme C, Hrubikova K. Enhancement of virus-induced gene silencing through viral-based production of inverted-repeats. The Plant Journal. 2003;34(4):543-553.

53. Gosselé V, Faché I, Meulewaeter F, Cornelissen M, Metzlaff M. SVISS–a novel transient gene silencing system for gene function discovery and validation in tobacco plants. The Plant Journal. 2002;32(5):859-866.

54. Fitzmaurice WP, Holzberg S, Lindbo JA, Padgett HS, Palmer KE, Wolfe GM, et al. Epigenetic modification of plants with systemic RNA viruses. Omics: A Journal of Integrative Biology. 2002;6(2):137-151.

55. Holzberg S, Brosio P, Gross C, Pogue GP. Barley stripe mosaic virus-induced gene silencing in a monocot plant. The Plant Journal. 2002;30(3):315-327.

56. Constantin GD, Krath BN, MacFarlane SA, Nicolaisen M, Elisabeth Johansen I, Lund OS. Virus-induced gene silencing as a tool for functional genomics in a legume species. The Plant Journal. 2004;40(4):622-631.

57. Ding XS, Schneider WL, Chaluvadi SR, Mian MR, Nelson RS. Characterization of a Brome mosaic virus strain and its use as a vector for gene silencing in monocotyledonous hosts. Molecular Plant-Microbe Interactions. 2006;19(11):1229-1239.

58. Zhang C, Ghabrial SA. Development of Bean pod mottle virus-based vectors for stable protein expression and sequence-specific virus-induced gene silencing in soybean. Virology. 2006;344(2):401-411.

59. Sudarshana MR, Plesha MA, Uratsu SL, Falk BW, Dandekar AM, Huang TK, et al. A chemically inducible cucumber mosaic virus amplicon system for expression of heterologous proteins in plant tissues. Plant Biotechnology Journal. 2006;4(5):551-559.

60. Saejung W, Fujiyama K, Takasaki T, Ito M, Hori K, Malasit P, et al. Production of dengue 2 envelope domain III in plant using TMV-based vector system. Vaccine. 2007;25(36):6646-6654.

61. Peele C, Jordan CV, Muangsan N, Turnage M, Egelkrout E, Eagle P, et al. Silencing of a meristematic gene using geminivirus-derived vectors. The Plant Journal. 2001;27(4):357-366.

62. Turnage MA, Muangsan N, Peele CG, Robertson D. Geminivirus-based vectors for gene silencing in Arabidopsis. The Plant Journal. 2002;30(1):107-114.

63. Huang C, Xie Y, Zhou X. Efficient virus-induced gene silencing in plants using a modified geminivirus DNA1 component. Plant Biotechnology Journal. 2009;7(3):254-265.

64. Li D, Behjatnia SAA, Dry IB, Walker AR, Randles JW, Rezaian MA. Tomato leaf curl virus satellite DNA as a gene silencing vector activated by helper virus infection. Virus Research. 2008;136(1):30-34.

65. Tao X, Qing L, Zhou X. Function of A-Rich region in DNAβ associated with Tomato yellow leaf curl China virus. Chinese Science Bulletin. 2004;49(14):1490-1493.

66. Qian Y, Mugiira RB, Zhou X. A modified viral satellite DNA-based gene silencing vector is effective in association with heterologous begomoviruses. Virus Research. 2006;118(1):136-142.

67. Fofana IB, Sangaré A, Collier R, Taylor C, Fauquet CM. A geminivirus-induced gene silencing system for gene function validation in cassava. Plant Molecular Biology. 2004;56(4):613-624.

68. Voinnet O, Pinto YM, Baulcombe DC. Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. Proceedings of the National Academy of Sciences USA. 1999;96(24):14147-14152.

69. Shi B-J, Palukaitis P, Symons RH. Differential virulence by strains of Cucumber mosaic virus is mediated by the 2b gene. Molecular Plant-Microbe Interactions. 2002;15(9):947-955.

70. Hartitz MD, Sunter G, Bisaro DM. The tomato golden mosaic virus transactivator (TrAP) is a single-stranded DNA and zinc-binding phosphoprotein with an acidic activation domain. Virology. 1999;263(1):1-14.

71. Damirdagh IS, Ross AF. A marked synergistic interaction of potato viruses X and Y in inoculated leaves of tobacco. Virology. 1967;31(2):296-307.

72. Mlotshwa S, Verver J, Sithole-Niang I, Prins M, Van Kammen A, Wellink J. Transgenic plants expressing HC-Pro show enhanced virus sensitivity while silencing of the transgene results in resistance. Virus Genes. 2002;25(1):45-57.

73. Vanitharani R, Chellappan P, Pita JS, Fauquet CM. Differential roles of AC2 and AC4 of cassava geminiviruses in mediating synergism and suppression of posttranscriptional gene silencing. Journal of Virology. 2004;78(17):9487-9498.

74. Wang H, Hao L, Shung C-Y, Sunter G, Bisaro DM. Adenosine kinase is inactivated by geminivirus AL2 and L2 proteins. The Plant Cell. 2003;15(12):3020-3032.

75. Dong X, van Wezel R, Stanley J, Hong Y. Functional characterization of the nuclear localization signal for a suppressor of posttranscriptional gene silencing. Journal of Virology. 2003;77(12):7026-7033.

76. van Wezel R, Liu H, Wu Z, Stanley J, Hong Y. Contribution of the zinc finger to zinc and DNA binding by a suppressor of posttranscriptional gene silencing. Journal of Virology. 2003;77(1):696-700.

77. Qu F, Ren T, Morris TJ. The coat protein of turnip crinkle virus suppresses posttranscriptional gene silencing at an early initiation step. Journal of Virology. 2003;77(1):511-522.

78. Thomas CL, Leh V, Lederer C, Maule AJ. Turnip crinkle virus coat protein mediates suppression of RNA silencing in Nicotiana benthamiana. Virology. 2003;306(1):33-41.

79. Reed JC, Kasschau KD, Prokhnevsky AI, Gopinath K, Pogue GP, Carrington JC, et al. Suppressor of RNA silencing encoded by Beet yellows virus. Virology. 2003;306(2):203-209.

80. Lu R, Folimonov A, Shintaku M, Li W-X, Falk BW, Dawson WO, et al. Three distinct suppressors of RNA silencing encoded by a 20-kb viral RNA genome. Proceedings of the National Academy of Sciences USA. 2004;101(44):15742-15747.

81. Brigneti G, Voinnet O, Li WX, Ji LH, Ding SW, Baulcombe DC. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. The EMBO Journal. 1998;17(22):6739-6746.

82. Lucy AP, Guo HS, Li WX, Ding SW. Suppression of post-transcriptional gene silencing by a plant viral protein localized in the nucleus. The EMBO Journal. 2000;19(7):1672-1680.

83. Dunoyer P, Pfeffer S, Fritsch C, Hemmer O, Voinnet O, Richards KE. Identification, subcellular localization and some properties of a cysteine-rich suppressor of gene silencing encoded by peanut clump virus. The Plant Journal. 2002;29(5):555-567.

84. Yelina NE, Savenkov EI, Solovyev AG, Morozov SY, Valkonen JP. Long-distance movement, virulence, and RNA silencing suppression controlled by a single protein in hordei-and potyviruses: complementary functions between virus families. Journal of Virology. 2002;76(24):12981-12991.

85. Pfeffer S, Dunoyer P, Heim F, Richards K, Jonard G, Ziegler-Graff V. P0 of beet Western yellows virus is a suppressor of posttranscriptional gene silencing. Journal of Virology. 2002;76(13):6815-6824.

86. Voinnet O, Lederer C, Baulcombe DC. A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana*. Cell. 2000;103(1):157-167.

87. Anandalakshmi R, Pruss GJ, Ge X, Marathe R, Mallory AC, Smith TH, et al. A viral suppressor of gene silencing in plants. Proceedings of the National Academy of Sciences USA. 1998;95(22):13079-13084.

88. Ding XS, Liu J, Cheng N-H, Folimonov A, Hou Y-M, Bao Y, et al. The Tobacco mosaic virus 126-kDa protein associated with virus replication and movement suppresses RNA silencing. Molecular Plant-Microbe Interactions. 2004;17(6):583-592.

89. Kubota K, Tsuda S, Tamai A, Meshi T. Tomato mosaic virus replication protein suppresses virus-targeted posttranscriptional gene silencing. Journal of Virology. 2003;77(20):11016-11026.

90. Lakatos L, Szittya G, Silhavy D, Burgyán J. Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses. The EMBO Journal. 2004;23(4):876-884.

91. Szittya G, Silhavy D, Dalmay T, Burgyán J. Size-dependent cell-to-cell movement of defective interfering RNAs of Cymbidium ringspot virus. Journal of General Virology. 2002;83(6):1505-1510.

92. Kasschau KD, Carrington JC. A counterdefensive strategy of plant viruses: suppression of posttranscriptional gene silencing. Cell. 1998;95(4):461-470.

93. Delgadillo MO, Sáenz P, Salvador B, García JA, Simón-Mateo C. Human influenza virus NS1 protein enhances viral pathogenicity and acts as an RNA silencing suppressor in plants. Journal of General Virology. 2004;85(4):993-999.

94. Li W-X, Li H, Lu R, Li F, Dus M, Atkinson P, et al. Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing. Proceedings of the National Academy of Sciences USA. 2004;101(5):1350-1355.

95. Li H, Li WX, Ding SW. Induction and suppression of RNA silencing by an animal virus. Science. 2002;296(5571):1319-1321.

96. Karjee S, Minhas A, Sood V, Ponia SS, Banerjea AC, Chow VT, et al. The 7a accessory protein of severe acute respiratory syndrome coronavirus acts as an RNA silencing suppressor. Journal of Virology. 2010;84(19):10395-10401.

97. Elmayan T, Vaucheret H. Expression of single copies of a strongly expressed 35S transgene can be silenced post-transcriptionally. The Plant Journal. 1996;9(6):787-797.

98. Voinnet O, Baulcombe DC. Systemic signalling in gene silencing. Nature. 1997;389:553.

99. Voinnet O, Vain P, Angell S, Baulcombe DC. Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. Cell. 1998;95(2):177-187.

100. Llave C, Kasschau KD, Carrington JC. Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. Proceedings of the National Academy of Sciences USA. 2000;97(24):13401-13406.

101. Johansen LK, Carrington JC. Silencing on the spot. Induction and suppression of RNA silencing in the Agrobacterium-mediated transient expression system. Plant Physiology. 2001;126(3):930-938.

102. Kasschau KD, Xie Z, Allen E, Llave C, Chapman EJ, Krizan KA, et al. P1/HC-Pro, a viral suppressor of RNA silencing, interferes with Arabidopsis development and miRNA function. Developmental Cell. 2003;4(2):205-217.

103. Chapman EJ, Prokhnevsky AI, Gopinath K, Dolja VV, Carrington JC. Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. Genes and Development. 2004;18(10):1179-1186.

104. Dunoyer P, Lecellier C-H, Parizotto EA, Himber C, Voinnet O. Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. The Plant Cell. 2004;16(5):1235-1250.

105. Anandalakshmi R, Marathe R, Ge X, Herr J, Mau C, Mallory A, et al. A calmodulin-related protein that suppresses posttranscriptional gene silencing in plants. Science. 2000;290(5489):142-144.

106. Mallory AC, Ely L, Smith TH, Marathe R, Anandalakshmi R, Fagard M, et al. HC-Pro suppression of transgene silencing eliminates the small RNAs but not transgene methylation or the mobile signal. The Plant Cell. 2001;13(3):571-583.

107. Guo HS, Ding SW. A viral protein inhibits the long range signaling activity of the gene silencing signal. The EMBO Journal. 2002;21(3):398-407.

108. Carrington JC, Kasschau KD, Johansen LK. Activation and suppression of RNA silencing by plant viruses. Virology. 2001;281(1):1-5.

109. Li WX, Ding SW. Viral suppressors of RNA silencing. Current Opinion in Biotechnology. 2001;12(2):150-154.

110. Baulcombe DC. RNA silencing. Current Biology. 2002;12(3):R82-R4.

111. Moissiard G, Voinnet O. Viral suppression of RNA silencing in plants. Molecular Plant Pathology. 2004;5(1):71-82.

112. Roth A, Breaker RR. Selection in vitro of allosteric ribozymes.Ribozymes and siRNA Protocols: Springer; 2004. p. 145-164.

113. Silhavy D, Burgyán J. Effects and side-effects of viral RNA silencing suppressors on short RNAs. Trends in Plant Science. 2004;9(2):76-83.

114. Mérai Z, Kerényi Z, Kertész S, Magna M, Lakatos L, Silhavy D. Double-stranded RNA binding may be a general plant RNA viral strategy to suppress RNA silencing. Journal of Virology. 2006;80(12):5747-5756.

115. Vargason JM, Szittya G, Burgyán J, Hall TMT. Size selective recognition of siRNA by an RNA silencing suppressor. Cell. 2003;115(7):799-811.

116. Ye K, Malinina L, Patel DJ. Recognition of small interfering RNA by a viral suppressor of RNA silencing. Nature. 2003;426(6968):874-878.

117. Ye K, Patel DJ. RNA silencing suppressor p21 of Beet yellows virus forms an RNA binding octameric ring structure. Structure. 2005;13(9):1375-1384.

118. Lakatos L, Csorba T, Pantaleo V, Chapman EJ, Carrington JC, Liu YP, et al. Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. The EMBO Journal. 2006;25(12):2768-2780.

119. Goto K, Kobori T, Kosaka Y, Natsuaki T, Masuta C. Characterization of silencing suppressor 2b of cucumber

mosaic virus based on examination of its small RNA-binding abilities. Plant and Cell Physiology. 2007;48(7):1050-1060.

120. Singh G, Popli S, Hari Y, Malhotra P, Mukherjee S, Bhatnagar RK. Suppression of RNA silencing by Flock house virus B2 protein is mediated through its interaction with the PAZ domain of Dicer. The FASEB Journal. 2009;23(6):1845-1857.

121. Llave C, Kasschau KD, Rector MA, Carrington JC. Endogenous and silencing-associated small RNAs in plants. The Plant Cell. 2002;14(7):1605-1619.

122. Mallory AC, Reinhart BJ, Bartel D, Vance VB, Bowman LH. A viral suppressor of RNA silencing differentially regulates the accumulation of short interfering RNAs and micro-RNAs in tobacco. Proceedings of the National Academy of Sciences USA. 2002;99(23):15228-15233.

123. Park W, Li J, Song R, Messing J, Chen X. CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in Arabidopsis thaliana. Current Biology. 2002;12(17):1484-1495.

124. Alvarez JP, Pekker I, Goldshmidt A, Blum E, Amsellem Z, Eshed Y. Endogenous and synthetic microRNAs stimulate simultaneous, efficient, and localized regulation of multiple targets in diverse species. The Plant Cell. 2006;18(5):1134-1151.

125. Siddiqui SA, Sarmiento C, Truve E, Lehto H, Lehto K. Phenotypes and functional effects caused by various viral RNA silencing suppressors in transgenic Nicotiana benthamiana and N. tabacum. Molecular Plant-Microbe Interactions. 2008;21(2):178-187.

126. Chen HY, Yang J, Lin C, Yuan YA. Structural basis for RNA-silencing suppression by Tomato aspermy virus protein 2b. EMBO Reports. 2008;9(8):754-760.

127. Deleris A, Gallego-Bartolome J, Bao J, Kasschau KD, Carrington JC, Voinnet O. Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. Science. 2006;313(5783):68-71.

128. Haas G, Azevedo J, Moissiard G, Geldreich A, Himber C, Bureau M, et al. Nuclear import of CaMV P6 is required for infection and suppression of the RNA silencing factor DRB4. The EMBO Journal. 2008;27(15):2102-2112.

129. Kumar V, Mishra SK, Rahman J, Taneja J, Sundaresan G, Sanan-Mishra N, et al. Mungbean yellow mosaic Indian virus encoded AC2 protein suppresses RNA silencing by inhibiting Arabidopsis RDR6 and AGO1 activities. Virology. 2015;486:158-172.

130. Bortolamiol D, Pazhouhandeh M, Ziegler-Graff V. Viral suppression of RNA silencing by destabilization of ARGONAUTE 1. Plant Signaling and Behavior. 2008;3(9):657-659.

131. Ruiz-Ferrer V, Voinnet O. Viral suppression of RNA silencing: 2b wins the Golden Fleece by defeating Argonaute. Bioessays. 2007;29(4):319-323.

132. Duan CG, Fang YY, Zhou BJ, Zhao JH, Hou WN, Zhu H, et al. Suppression of Arabidopsis ARGONAUTE1-mediated slicing, transgene-induced RNA silencing, and DNA methylation by distinct domains of the Cucumber mosaic virus 2b protein. The Plant Cell. 2012;24(1):259-274.

134. Buchmann RC, Asad S, Wolf JN, Mohannath G, Bisaro DM. Geminivirus AL2 and L2 Proteins Suppress Transcriptional Gene Silencing and Cause Genome-Wide Reductions in Cytosine Methylation. Journal of Virology. 2009;83(10):5005-5013.

133. Trinks D, Rajeswaran R, Shivaprasad P, Akbergenov R, Oakeley EJ, Veluthambi K, et al. Suppression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes. Journal of Virology. 2005;79(4):2517-2527.

135. Yang X, Xie Y, Raja P, Li S, Wolf JN, Shen Q, et al. Suppression of methylation-mediated transcriptional gene silencing by betaC1-SAHH protein interaction during geminivirus-betasatellite infection. PLoS Pathogens. 2011;7(10):e1002329.

136. Zhang Z, Chen H, Huang X, Xia R, Zhao Q, Lai J, et al. BSCTV C2 attenuates the degradation of SAMDC1 to suppress DNA methylation-mediated gene silencing in Arabidopsis. The Plant Cell. 2011;23(1):273-288.

137. Sanford J, Johnston S. The concept of parasite-derived resistance—Deriving resistance genes from the parasite's own genome. Journal of Theoretical Biology. 1985;113(2):395-405.

139. Beachy RN. Mechanisms and applications of pathogen-derived resistance in transgenic plants. Current Opinion in Biotechnology. 1997;8(2):215-220.

138. Wilson TM. Strategies to protect crop plants against viruses: pathogen-derived resistance blossoms. Proceedings of the National Academy of Sciences USA. 1993;90(8):3134-3141.

140. Prins M, Goldbach R. RNA-mediated virus resistance in transgenic plants. Archives of Virology. 1996;141(12):2259-76.

141. Beachy RN. Coat-protein-mediated resistance to tobacco mosaic virus: discovery mechanisms and exploitation. Philosophical Transactions of the Royal Society B: Biological Sciences. 1999;354(1383):659-664.

142. Abel PP, Nelson RS, De B, Hoffmann N, Rogers SG, Fraley RT, et al. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science. 1986;232(4751):738-743.

143. Hayakawa T, Zhu Y, Itoh K, Kimura Y, Izawa T, Shimamoto K, et al. Genetically engineered rice resistant to rice stripe virus, an insect-transmitted virus. Proceedings of the National Academy of Sciences USA. 1992;89(20):9865-9869.

144. Farinelli L, Malnoë P. Coat Protein Gene-Mediated Resistance to Potato Virus Y in Tobacco: Examination of the resistance mechanisms-is the transgenic coat protein required for protection? Molecular Plant Microbe Interactions. 1993;6:284-292.

145. Kunik T, Salomon R, Zamir D, Navot N, Zeidan M, Michelson I, et al. Transgenic tomato plants expressing the tomato yellow leaf curl virus capsid protein are resistant to the virus. Nature Biotechnology. 1994;12(5):500-504.

146. Chowrira G, Cavileer T, Gupta S, Lurquin P, Berger P. Coat protein-mediated resistance to pea enation mosaic virus in transgenic *Pisum Sativum* L. Transgenic research. 1998;7(4):265-271.

147. Germundsson A, Sandgren M, Barker H, Savenkov EI, Valkonen JP. Initial infection of roots and leaves reveals different resistance phenotypes associated with coat protein gene-mediated resistance to Potato mop-top virus. Journal of General Virology. 2002;83(5):1201-1209.

148. Srivastava A, Raj SK. Coat protein-mediated resistance against an Indian isolate of the Cucumber mosaic virus subgroup IB in Nicotiana benthamiana. Journal of Bioscience. 2008;33(2):249-257.

149. Liu B, Preisser EL, Chu D, Pan H, Xie W, Wang S, et al. Multiple forms of vector manipulation by a plant-infecting virus: Bemisia tabaci and tomato yellow leaf curl virus. Journal of Virology. 2013;87(9):4929-4937.

150. Höhnle M, Höfer P, Bedford ID, Briddon RW, Markham PG, Frischmuth T. Exchange of three amino acids in the coat protein results in efficient whitefly transmission of a nontransmissible Abutilon mosaic virus isolate. Virology. 2001;290(1):164-171.

151. Hanley-Bowdoin L, Elmer JS, Rogers SG. Expression of functional replication protein from tomato golden mosaic virus in transgenic tobacco plants. Proceedings of the National Academy of Sciences. 1990;87(4):1446-1450.

152. Noris E, Accotto GP, Tavazza R, Brunetti A, Crespi S, Tavazza M. Resistance to Tomato Yellow Leaf Curl Geminivirus inNicotiana benthamianaPlants Transformed with a Truncated Viral C1 Gene. Virology. 1996;224(1):130-138.

153. Brunetti A, Tavazza M, Noris E, Tavazza R, Caciagli P, Ancora G, et al. High Expression of Truncated Viral Rep Protein Confers Resistance to Tomato Yellow Leaf Curl Virus in Transgenic Tomato Plants. Molecular Plant-Microbe Interactions. 1997;10(5):571-579.

154. Chatterji A, Beachy RN, Fauquet CM. Expression of the oligomerization domain of the replication-associated protein (Rep) of Tomato leaf curl New Delhi virus interferes with DNA accumulation of heterologous geminiviruses. Journal of Biological Chemistry. 2001;276(27):25631-25638.

155. Duan Y-P, Powell C, Webb S, Purcifull D, Hiebert E. Geminivirus resistance in transgenic tobacco expressing mutated BC1 protein. Molecular Plant-Microbe Interactions. 1997a;10(5):617-623.

156. Duan Y-P, Powell CA, Purcifull DE, Broglio P, Hiebert E. Phenotypic variation in transgenic tobacco expressing mutated geminivirus movement/pathogenicity (BC1) proteins. Molecular Plant-Microbe Interactions. 1997b;10(9):1065-1074.

157. Vanderschuren H, Akbergenov R, Pooggin MM, Hohn T, Gruissem W, Zhang P. Transgenic cassava resistance to African cassava mosaic virus is enhanced by viral DNA-A bidirectional promoter-derived siRNAs. Plant Molecular Biology. 2007;64(5):549-557.

158. Hou Y-M, Sanders R, Ursin VM, Gilbertson RL. Transgenic plants expressing geminivirus movement proteins: abnormal phenotypes and delayed infection by Tomato mottle virus in transgenic tomatoes expressing the Bean dwarf mosaic virus BV1 or BC1 proteins. Molecular Plant-Microbe Interactions. 2000;13(3):297-308.

159. Simons RW. Naturally occurring antisense RNA control—a brief review. Gene. 1988;72(1):35-44.

160. Izant JG, Weintraub H. Inhibition of thymidine kinase gene expression by anti-sense RNA: a molecular approach to genetic analysis. Cell. 1984;36(4):1007-1015.

161. Mol J, Van der Krol A, Van Tunen A, Van Blokland R, De Lange P, Stuitje A. Regulation of plant gene expression by antisense RNA. FEBS Letters. 1990;268(2):427-430.

162. Ecker JR, Davis RW. Inhibition of gene expression in plant cells by expression of antisense RNA. Proceedings of the National Academy of Sciences USA. 1986;83(15):5372-5376.

163. Van der Krol AR, Lenting PE, Veenstra J, van der Meer IM, Koes RE, Gerats AG, et al. An anti-sense chalcone synthase gene in transgenic plants inhibits flower pigmentation. Nature. 1988;333(6176):866-869.

164. Samac DA, Shah DM. Effect of chitinase antisense RNA expression on disease susceptibility of Arabidopsis plants. Plant Molecular Biology. 1994;25(4):587-596.

165. Sheehy RE, Kramer M, Hiatt WR. Reduction of polygalacturonase activity in tomato fruit by antisense RNA. Proceedings of the National Academy of Sciences USA. 1988;85(23):8805-8809.

166. Smith C, Watson C, Bird C, Ray J, Schuch W, Grierson D. Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants. Molecular and General Genetics. 1990;224(3):477-481.

167. Kawchuk L, Martin R, McPherson J. Sense and antisense RNA-mediated resistance to potato leafroll virus in Russet Burbank potato plants. Mol Plant-Microbe Interaction. 1991;4:247-253.

168. Day AG, Bejarano ER, Buck KW, Burrell M, Lichtenstein CP. Expression of an antisense viral gene in transgenic tobacco confers resistance to the DNA virus tomato golden mosaic virus. Proceedings of the National Academy of Science USA. 1991;88(15):6721-6725.

169. Asad S, Haris W, Bashir A, Zafar Y, Malik K, Malik N, et al. Transgenic tobacco expressing geminiviral RNAs are resistant to the serious viral pathogen causing cotton leaf curl disease. Archives of Virology. 2003;148(12):2341-2352.

170. Beachy RN, Loesch-Fries S, Tumer NE. Coat protein-mediated resistance against virus infection. Annual Review of Phytopathology. 1990;28(1):451-472.

171. Baulcombe DC. Mechanisms of pathogen-derived resistance to viruses in transgenic plants. The Plant Cell. 1996;8(10):1833.

172. Bendahmane M, Gronenborn B. Engineering resistance against tomato yellow leaf curl virus (TYLCV) using

antisense RNA. Plant Molecular Biology. 1997;33(2):351-357.

173. Yang Y, Sherwood T, Patte C, Hiebert E, Polston J. Use of Tomato yellow leaf curl virus (TYLCV) Rep gene sequences to engineer TYLCV resistance in tomato. Phytopathology. 2004;94(5):490-496.

174. Watson JM, Fusaro AF, Wang M, Waterhouse PM. RNA silencing platforms in plants. FEBS Letters. 2005;579(26):5982-5987.

175. de Felippes FF, Wang Jw, Weigel D. MIGS: miRNA-induced gene silencing. The Plant Journal. 2012;70(3):541-547.

176. Zrachya A, Kumar PP, Ramakrishnan U, Levy Y, Loyter A, Arazi T, et al. Production of siRNA targeted against TYLCV coat protein transcripts leads to silencing of its expression and resistance to the virus. Transgenic Research. 2007;16(3):385-398.

177. Reyes CA, De Francesco A, Peña EJ, Costa N, Plata MI, Sendin L, et al. Resistance to Citrus psorosis virus in transgenic sweet orange plants is triggered by coat protein–RNA silencing. Journal of Biotechnology. 2011;151(1):151-158.

178. Shekhawat UK, Ganapathi TR, Hadapad AB. Transgenic banana plants expressing small interfering RNAs targeted against viral replication initiation gene display high-level resistance to banana bunchy top virus infection. Journal of General Virology. 2012;93(Pt 8):1804-1813.

179. Wang MB, Abbott DC, Waterhouse PM. A single copy of a virus-derived transgene encoding hairpin RNA gives immunity to barley yellow dwarf virus. Molecular Plant Pathology. 2000;1(6):347-356.

180. Stanley J, Frischmuth T, Ellwood S. Defective viral DNA ameliorates symptoms of geminivirus infection in transgenic plants. Proceedings of the National Academy of Sciences USA. 1990;87(16):6291-6295.

181. Aragão FJ, Faria JC. First transgenic geminivirus-resistant plant in the field. Nature Biotechnology. 2009;27(12):1086-1088.

182. Koch A, Biedenkopf D, Furch A, Weber L, Rossbach O, Abdellatef E, et al. An RNAi-based control of Fusarium graminearum infections through spraying of long dsRNAs involves a plant passage and is controlled by the fungal silencing machinery. PLoS Pathogens.2016.12(10): e100590.

183. Chen W, Kastner C, Nowara D, Oliveira-Garcia E, Rutten T, Zhao Y, et al. Host-induced silencing of Fusarium culmorum genes protects wheat from infection. Journal of Experimental Botany. 2016. 67(17): 4979-4991.

184. Tenllado F, Barajas D, Vargas M, Atencio F, González-Jara P, Díaz-Ruíz J. Transient expression of homologous hairpin RNA causes interference with plant virus infection and is overcome by a virus encoded suppressor of gene silencing. Molecular Plant-Microbe Interactions. 2003a;16(2):149-158.

185. Tenllado F, Martínez-García B, Vargas M, Díaz-Ruíz JR. Crude extracts of bacterially expressed dsRNA can be used to protect plants against virus infections. BMC Biotechnology. 2003b;3(1):3.

186. Vanitharani R, Chellappan P, Fauquet CM. Short interfering RNA-mediated interference of gene expression and viral DNA accumulation in cultured plant cells. Proceedings of the National Academy of Sciences USA. 2003;100(16):9632-9636.

187. Abhary M, Anfoka G, Nakhla M, Maxwell D. Post-transcriptional gene silencing in controlling viruses of the Tomato yellow leaf curl virus complex. Archives of Virology. 2006;151(12):2349-2363.

188. Lennefors B-L, Savenkov EI, Bensefelt J, Wremerth-Weich E, van Roggen P, Tuvesson S, et al. dsRNA-mediated resistance to Beet Necrotic Yellow Vein Virus infections in sugar beet (Beta vulgaris L. ssp. vulgaris). Molecular Breeding. 2006;18(4):313-325.

189. Ramesh S, Mishra A, Praveen S. Hairpin RNA-mediated strategies for silencing of tomato leaf curl virus AC1 and AC4 genes for effective resistance in plants. Oligonucleotides. 2007;17(2):251-257.

190. Beale G, Hollins AJ, Benboubetra M, Sohail M, Fox SP, Benter I, et al. Gene silencing nucleic acids designed by scanning arrays: anti-EGFR activity of siRNA, ribozyme and DNA enzymes targeting a single hybridization-accessible region using the same delivery system. Journal of Drug Targeting. 2003;11(7):449-456.

191. Bumcrot D, Manoharan M, Koteliansky V, et al. RNAi therapeutics: a potential new class of pharmaceutical drugs. Nature Chemical Biology. 2006;2(12):711-719.

192. Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, et al. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes andDevelopment. 2002;16(8):948-958.

193. Yu JY, DeRuiter SL, Turner DL. RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. Proceedings of the National Academy of Sciences USA.2002;99(9):6047-6052.

194. Petrov N, Teneva A, Stoyanova M, Andonova R, Denev I, Tomlekova N. Blocking the systemic spread of potato virus Y in the tissues of potatoes by posttranscriptional gene silencing. Bulgarian Journal of Agricultural Science. 2015;21(2):288-294.

195. Hannon GJ. RNA interference. Nature. 2002;418(6894):244-251.

196. Seemanpillai M, Dry I, Randles J, Rezaian A. Transcriptional silencing of geminiviral promoter-driven transgenes following homologous virus infection. Molecular Plant-Microbe Interactions. 2003;16(5):429-438.

197. Akbergenov R, Si-Ammour A, Blevins T, Amin I, Kutter C, Vanderschuren H, et al. Molecular characterization of geminivirus-derived small RNAs in different plant species. Nucleic Acids Research. 2006;34(2):462-471.

198. Pooggin M, Shivaprasad P, Veluthambi K, Hohn T. RNAi targeting of DNA virus in plants. Nature Biotechnology. 2003;21(2):131-132.

199. Ossowski S, Schwab R, Weigel D. Gene silencing in plants using artificial microRNAs and other small RNAs. The Plant Journal. 2008;53(4):674-690.

200. Sablok G, Pérez-Quintero ÁL, Hassan M, Tatarinova TV, López C. Artificial microRNAs (amiRNAs) engineering–On how microRNA-based silencing methods have affected current plant silencing research. Biochemical and Biophysical Research Communications. 2011;406(3):315-319.

201. Zeng Y, Cullen BR. RNA interference in human cells is restricted to the cytoplasm. RNA. 2002;8(7):855-860.

202. Parizotto EA, Dunoyer P, Rahm N, Himber C, Voinnet O. In vivo investigation of the transcription, processing, endonucleolytic activity, and functional relevance of the spatial distribution of a plant miRNA. Genes and Development. 2004;18(18):2237-2242.

203. Schwab R, Ossowski S, Riester M, Warthmann N, Weigel D. Highly specific gene silencing by artificial microRNAs in Arabidopsis. The Plant Cell. 2006;18(5):1121-1133.

204. Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A. Rational siRNA design for RNA interference. Nature Biotechnology. 2004;22(3):326-330.

205. Mallory AC, Reinhart BJ, Jones-Rhoades MW, Tang G, Zamore PD, Barton MK, et al. MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5′ region. The EMBO Journal. 2004;23(16):3356-3364.

206. Niu Q-W, Lin S-S, Reyes JL, Chen K-C, Wu H-W, Yeh S-D, et al. Expression of artificial microRNAs in transgenic Arabidopsis thaliana confers virus resistance. Nature Biotechnology. 2006;24(11):1420-1428

207. Liang Z, Wu H, Reddy S, Zhu A, Wang S, Blevins D, et al. Blockade of invasion and metastasis of breast cancer cells via targeting CXCR4 with an artificial microRNA. Biochemical and Biophysical Research Communications. 2007;363(3):542-546.

208. Qu J, Ye J, Fang R. Artificial microRNA-mediated virus resistance in plants. Journal of Virology. 2007;81(12):6690-6699.

209. Kim Y-S, Ke F, Lei X-Y, Zhu R, Zhang Q-Y. Viral envelope protein 53R gene highly specific silencing and iridovirus resistance in fish cells by AmiRNA. PloS One. 2010;5(4):e10308.

210. Ai T, Zhang L, Gao Z, Zhu C, Guo X. Highly efficient virus resistance mediated by artificial microRNAs that target the suppressor of PVX and PVY in plants. Plant Biology. 2011;13(2):304-316.

211. Gao Y-F, Yu L, Wei W, Li J-B, Luo Q-L, Shen J-L. Inhibition of hepatitis B virus gene expression and replication by artificial microRNA. World Journal of Gastroenterology: World Journal of Gastroenterology. 2008; 14(29):4684–4689.. 2008;14(29):4684.

212. Tiwari M, Sharma D, Trivedi PK. Artificial microRNA mediated gene silencing in plants: progress and perspectives. Plant Molecular Biology. 2014;86(1-2):1-18.

213. Yadava P, Suyal G, Mukherjee SK. Begomovirus DNA replication and pathogenicity. Current Science. 2010;98(3):360-368.

214. Tien VV, Choudhury NR, Mukherjee SK. Transgenic tomato plants expressing artificial microRNAs for silencing the pre-coat and coat proteins of a begomovirus, Tomato leaf curl New Delhi virus, show tolerance to virus infection. Virus Research. 2013;172(1):35-45.

215. Kung YJ, Lin SS, Huang YL, Chen TC, Harish SS, Chua NH, et al. Multiple artificial microRNAs targeting conserved motifs of the replicase gene confer robust transgenic resistance to negative-sense single-stranded RNA plant virus. Molecular Plant Pathology. 2012;13(3):303-317.

216. Singh R, Rai N, Singh M, Singh S, Srivastava K. Selection of tomato genotypes resistant to tomato leaf curl virus disease using biochemical and physiological markers. The Journal of Agricultural Science. 2015;153(04):646-655.

Advances in Biotechnology

Chapter 5

A comparative in-vitro cytotoxicity study of biogenic and chemically synthesized metal (Ag and Au) nanoparticles

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Abstract

In the current scenario, the most frequently asked query regarding the metal nanoparticles is related to its toxic effects despite of their vast potential application in the field of health, pharmaceutical and medicine. Even though there are lots of studies carried out focusing on the toxicity of metal nanoparticles, a proper comparative study of biologically and chemically synthesized metal nanoparticles is lacking. The main aim of the study is a comparative morphological, biological and genotoxic studies of biogenic as well as chemically synthesized silver and gold nanoparticles on the cell line- African monkey kidney cells (Vero) and human spermatozoa. Chemically synthesized nanoparticles had significant cytotoxic activity leading to the cell death and the comet assay in the spermatozoa make us clear that it creates significant DNA damage too. The release of lactate dehydrogenase, nitric oxide and reactive oxygen species (ROS) after its exposure to the metal nanoparticles finally resulting damage to most biomolecules, including DNA and protein points to the effect of biogenic and chemically synthesized nanoparticles on different biological system.

Keywords: Toxicity; Metal nanoparticles; biologically synthesized; chemically synthesized

1. Introduction

Potential applications of metal nanoparticles due to their unique optical, thermal and antimicrobial properties made them the centre of attention during the recent years in the field of health, pharmacy, food and medicine industries. That being said, potential undesirable noxious effects of the same metal nanoparticles have been reported through many studies. Among the different metal nanoparticles, silver and gold were reported to be imperative because of their unique optical, thermal and antimicrobial properties with increased surface area and surface to volume ratio [1,2]

A different choice of biological systems like bacteria, fungi and plants were exploited and reported for the biosynthesis of silver and gold nanoparticles which theoretically claims that they are green synthesis and toxic free due to the biomolecules involved in the synthesis process. The toxicity of nanoparticles is mainly due to the hazardous chemicals involved in their synthesis process as reducing agents, which limits their usage. But the use of biological systems for the synthesis of nanoparticles which are comparatively green, offers several advantages such as ease in production, procedure, economical and environment friendliness. A proper study involving the comparison of chemically synthesized metal nanoparticles, biologically synthesized nanoparticles and their corresponding metal ion form is noble to counter such important queries relating the toxicity [3].

A comparative morphological, biological activity and genotoxicity study was carried out using myco-based as well as chemically synthesized silver and gold nanoparticles was carried out and analyzed. This is compared with corresponding metal in its ionic form which is considered to be more toxic. The IC50 value was calculated with respect to their cellular metabolic toxic activity using the African monkey kidney cells (Vero) and comet assay was performed to check the genotoxic activity of the silver and gold nanoparticles in human spermatozoa.

2. Materials and methods

2.1 Chemicals

Silver nitrate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium sulfate heptahydrate, ammonium sulfate and Muller-Hinton agar were procured from SRL (Mumbai, India).

2.2 Source of microorganisms

The fungus Trichoderma atroviride was obtained from the Culture Collection of CAS in Botany, University of Madras, India and maintained on potato dextrose agar (HiMedia, Mumbai, India) slants at 27˚C.

2.3 Production of biomass

To prepare the biomass for biosynthesis of silver nanopartices, the fungus was grown aerobically in liquid broth containing dihydrogen potassium phosphate (7g/L), dipotassium hydrogen phosphate(2g/L),magnesium sulfate heptahydrate(0.1g/L), ammonium sulfate (1g/L), yeast extract (0.6g/L), and glucose (10g/L). This was incubated on an orbital shaker at 27°C at 150 rpm, and the biomass were harvested after 72h of growth by sieving through a plastic sieve, followed by extensive washing with sterile double distilled water [3].

2.4 Biosynthesis of metal nanoparticles

The fungal biomass (20g wet weight) was mixed with 100 mL of sterile double distilled water and agitated on an orbital shaker at 150 rpm for 48h at 27°C. After incubation, the cell filtrate was filtered through Whatman filter paper no. 1. To 100mL of cell filtrate in Erlenmeyer flask, $AgNO_3$ and $AuCl_4$ was added and kept undisturbed for 24 hours in dark conditions to get an overall ionic concentration (Ag+ and Au+) of 10^{-3}M [3].

2.5 Chemical synthesis of metal nanoparticles

Silver and gold nanoparticles were chemically synthesized using chemical reduction method according to Asta et al., (2006) [4] and McFarland et al., (2004) [5] respectively.

2.6 Characterization of metal nanoparticles

Surface plasmon resonance of silver and gold nanoparticles was characterized using a UV–Vis spectrophotometer (Cary 300 Conc spectrophotometer) at a resolution of 1nm from 250 to 800nm. For transmission electron microscopy (TEM), the sample was prepared by placing a drop of colloidal solution on a carbon-coated copper grid and setting a completely dried drop by vacuum desiccators. The image of the sample was obtained using a transmission electron microscope (JEOL 2000 FX MARK II) equipped with an EDX attachment. X-ray diffraction pattern of dry nanoparticles was obtained using Philips MDL PW 1050 diffractometer with CuKα radiation (λ=1.5406 Å with Ni filter). The FTIR spectrum of the sample was recorded by Perkin-Elmer Fourier transform infrared spectroscopy; the spectrum ranged from 2000 to 1000cm-1 at a resolution of 4 cm-1 by making a KBr pellet with metal nanoparticles.

2.7 Toxicity study of metal nanoparticles in cell lines

Effect of biological and chemically synthesized silver and gold nanoparticles on African monkey kidney cells (Vero) were assayed by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide (MTT) assay [6]. The half maximal inhibitory concentration (IC50) of nanoparticles and the positive controls were found out by the experiment and were compared.

2.7.1 Lactate dehydrogenase (LDH) assay

LDH activity was determined by the linear region of a pyruvate standard graph using regression analysis and expressed as percentage (%) leakage as described previously by Ulmer et al., (1956) [7]. The amount of LDH released was expressed in percentage.

2.7.2 Nitric oxide (NO) assay

The amount of nitrite was determined by the method of Stueher and Marletta (1989) [8]. The pink colour developed was measured at 540 nm in a microquant plate reader (Biotek Instruments).

2.8 Toxicity study of metal nanoparticles on spermatozoa

Semen sample was collected from a healthy donor in 3 days interval and was used for the study.

2.8.1 Assessment of semen vitality and morphology

The vitality and morphology of the semen sample was analyzed by staining using eosin-nigrosin method [9] and examined under optical microscope.

2.8.2 Staining method (Eosin & Nigrosin method)

Twenty five micro litres of semen sample was mixed with 2 drops of 0.5% eosin solution in microcentrifuge tube and waited for 10 seconds. To the above mixture added 2 drops of 10% nigrosine stain were added and mixed gently. From the above mixture thin smears were made using another clean edged glass slide. The slides were air dried and were used for examining the morphology of sperms under oil immersion (100x magnification). Dead sperms took eosin stain and live sperms remained white in colour. The sperm vitality was expressed by counting the percentage of live and dead sperms. A large number of live but immotile sperm cells may indicate an abnormality in the axoneme.

Biologically and chemically synthesized silver and gold nanoparticles, silver nitrate and tetra choloroauric acid was added to the semen sample and was observed under normal optical microscope in 30 and 60 minutes interval at 40x and 100x oil immersion to study the sperm vitality as well as the morphology of the sample.

2.8.3 Comet Assay

The comet assay or single cell gel electrophoresis (SCGE) is a rapid, sensitive and relatively simple method for detecting DNA damage at the level of individual cells. DNA damage in the sperm suspension was analysed by following the methodology of Cabrita et al. (2005)

[10]. The lengths of migrated DNA (Comet tail) were measured using the CASP software and about 10-50 comets/point were scored.

2.8.4 Application of CASP software

The comets were analyzed using the CASP software. The images were used to estimate the DNA content of individual nuclei and to evaluate the degree of DNA damage representing the fraction of total DNA in the tail.

2.9 Statistical analysis

All the experimental data obtained in the present study were subjected to statistical analysis. Statistical Package for the Social Studies (SPSS) software, Version 11.5 for Windows was used to perform all the statistical analysis. Limits of significance for all critical ranges were set at $P<0.05$. For post-hoc comparisons the Least Significant Difference test (LSD) was employed.

3. Results and discussion

3.1 Silver and sold nanoparticles

Silver and gold nanoparticles were synthesized biologically as well as chemically and were used in the study. The characterization of biogenic silver and gold nanoparticles is shown in figure 1 and 2.

3.2 Toxicity study of metal nanoparticles in cell lines

3.2.1 MTT assay

Cell proliferation analysis results by MTT assay using different concentrations of metal nanoparticles is shown in figure 3 and 4. Results revealed that the complexes could reduce the cell viability of kidney cells (Vero) in a dose dependent manner. For tetra chloroauric acid alone, IC50 value was found to be 5µg/mL, for chemically synthesized gold nanoparticles it was 40 µg/mL and for biologically synthesized gold nanoparticles 80 µg/mL was the obtained IC50 value.

Along with the experiments of silver nanoparticles, Silver nitrate (AgNO3) in the same concentration was used as positive control and IC50 values for silver nitrate was found to be 10µg/mL, for chemically synthesized silver nanoparticles it was 20 µg/mL; and for biologically synthesized silver nanoparticles IC50 value was calculated to be 30 µg/mL [3].

3.2.2 Release of lactate dehydrogenase (LDH)

LDH leakage is routinely used as an indicator of damage to plasma membrane integ

rity and in assessing cytotoxic nature of a compound as dead cells release LDH into the culture medium. LDH is a cytosolic enzyme released into cell culture supernatant due to compromised membrane integrity, which is associated with cell death [11]. Figure 5 shows the percentage of LDH released from the vero cells after 48 hours of treatment with gold and silver nanoparticles respectively. There was a significant increase of LDH level in the culture supernatant of the cells that were treated with chemically synthesized nanoparticles compared to that of biologically synthesized. Nanoparticle induced cytotoxicity can be demonstrated by the increase in LDH leakage and associated loss of cell viability which may be due to the destabilization of membrane phospholipids.

3.2.3 Nitric oxide (NO) assay

The nitrite is the stable product of the nitric oxide released in response to oxidative stress. The amount of nitrite in the culture medium corresponds to the level of nitric oxide. Hence the level of nitrite was estimated to measure the nitric oxide produced after complex treatment [12]. Treatment of Vero cells with the nanoparticles resulted in enhanced Nitric oxide release than the untreated cells as shown in figure 6 for gold and silver nanoparticles respectively. Nitric oxide is a gaseous signalling molecule and a well-known, short-lived free radical which is produced non-enzymatically by iNOS and causes damage to most biomolecules, including DNA and protein.

Two major NOS-isoenzymes are present in cells: endothelial, membrane-bound, constitutively active NOS (eNOS), and cytoplasmic inducible NOS (iNOS) [13]. Previous studies showed that increased ROS generation lead to the activation of iNOS which in turn releases NO. RNS are by-products of Nitric oxide production in living cells. Up-regulated RNS production can cause cell damage or death, through nitration of biological target molecules such as DNA, lipids, and proteins [14]. Hence from the present study it is obvious that the nanoparticles could induce the Nitric oxide production in vero cells in which positive control ($AuCl_4$ and $AgNO_3$) and chemically synthesized gold and silver nanoparticles are being more potent in inducing Nitric oxide production. This confirmed that the biologically synthesized forms are less cytotoxic than other synthesized forms.

3.3 Toxicity study of metal nanoparticles in spermatozoa

3.3.1 Assessment of semen vitality and morphology

The vitality of the sperms was examined using eosin stain through optical microscope. Eosin could penetrate dead sperms and it did not occur in live sperms. This was taken as an indication to differentiate between live and dead sperm cells. Spermatozoa were found to be more damaged in positive controls, where $AgNO_3$ and $AuCl_4$ were used, than in chemically synthesized and least in biologically synthesized metal nanoparticles (figure 7a & b). The per

centage cell death rate of the spermatozoa after 30 and 60 minutes of exposure is illustrated in figure 8.

In case of normal spermatozoa, they are oval in shape with a smooth contour and an acrosome occupying 40-70% of the head having straight midpiece and tail. The abnormalities or differences are noticed in the head, midpiece, tail and acrosome cap. In the case of control and biologically synthesized silver and gold nanoparticles treated cells, the morphology of the cells was found to be normal. In the case of $AgNO_3$ and $AuCl_4$ treatments, most of the cells had taken eosin dye inside the cells, which confirmed the damage of the cell membrane. Many of the cells were devoid of the midpiece and tail due to effect of the chemically synthesized nanoparticles and metal ions. In many of the cells treated with chemically synthesized nanoparticles and metal ions, the damage of the cells was obvious with the intracellular matter coming outside which confirmed their cytotoxic effect.

3.3.2 Comet assay

Cells were analysed with the comet assay to measure DNA damage caused by the silver and gold nanoparticles and a comparative study under florescent microscope was carried out as shown in figure 9. The control DNA (untreated) was observed as round in shape, confirms an unaffected DNA, where as other DNA images shows significant damage and tailing of DNA. The detailed data obtained from the comet assay with the help of CASP software has been presented in table 1. The head and tail DNA damage was found to be higher in the case of silver and gold ions treated than in silver nanoparticles synthesized by chemical method and the least damage in biologically synthesized silver and gold nanoparticles.

As per the previous studies and findings, many engineered nanoparticles including gold and silver have been found to have genotoxic effects, such as DNA-strand breaks, point mutations and oxidative DNA adducts [15,16,17]. In our previous studies with different concentrations of biologically and chemically synthesized particles against fresh water fish, it was demonstrated the effect of metal nanoparticles in different tissues like muscle, liver and kidney in comparison with the intensity stress proteins (HSP 70) released after the exposure to nanoparticles [3]. Nanoparticles easily cross the nuclear membrane and they can therefore interact with DNA directly or indirectly even though the exact mechanism for this interaction is not well known. Some of these alterations or damage to DNA, when occurring in spermatozoa may cause spermatogenic defects that could eventually result in trans-generational defects and the genomic stability of sperm cells [18].

When the biological system is challenged with metal ions or nanoparticles, the chance of forming Reactive Oxygen Species (ROS) is more. Even though several metabolic processes may use ROS in a good way, many participate in an oxidative burst and act not only as direct

protecting agent against stress, but also as signals activating further reactions. Generally a biological system try to keep the concentration of ROS at the possible low level because they are more reactive than molecular oxygen (O_2) [19] and they react with almost every organic constituent of the living cell. The high reactivity of ROS is based on the specificity of their electronic configuration. ROSs is known to damage cellular membranes by inducing lipid peroxidation [20]. They also can damage DNA, proteins and lipids [21] leading to cell death, damage and viability. It may be concluded that a similar process had happen when the cells were exposed to nanoparticles leading to cell death.

4. Conclusion

A detailed comparative morphological, biological, and genotoxicity study of biogenic metal nanoparticles with chemically synthesized metal nanoparticles was carried out since a cost effective existing competitive alternatives for biologically synthesized metal nanoparticles are chemically synthesized ones. Regarding the half maximal inhibitory concentration, chemically synthesized nanoparticles have IC50 value less than that of their biogenic nanoparticles In the comparative genotoxicity study on the spermatozoa, the chemically synthesized metal nanoparticles caused more cell damage and DNA damage which was confirmed by Comet assay. The overall findings from LDH assay, nitric oxide assay, the photomicrographs and comet assay points to the effect of nanoparticles on different cells and it has been observed that the chemically synthesized nanoparticles could vigorously induce cell death, damage and viability more significantly than their biological counterparts.

5. Figures

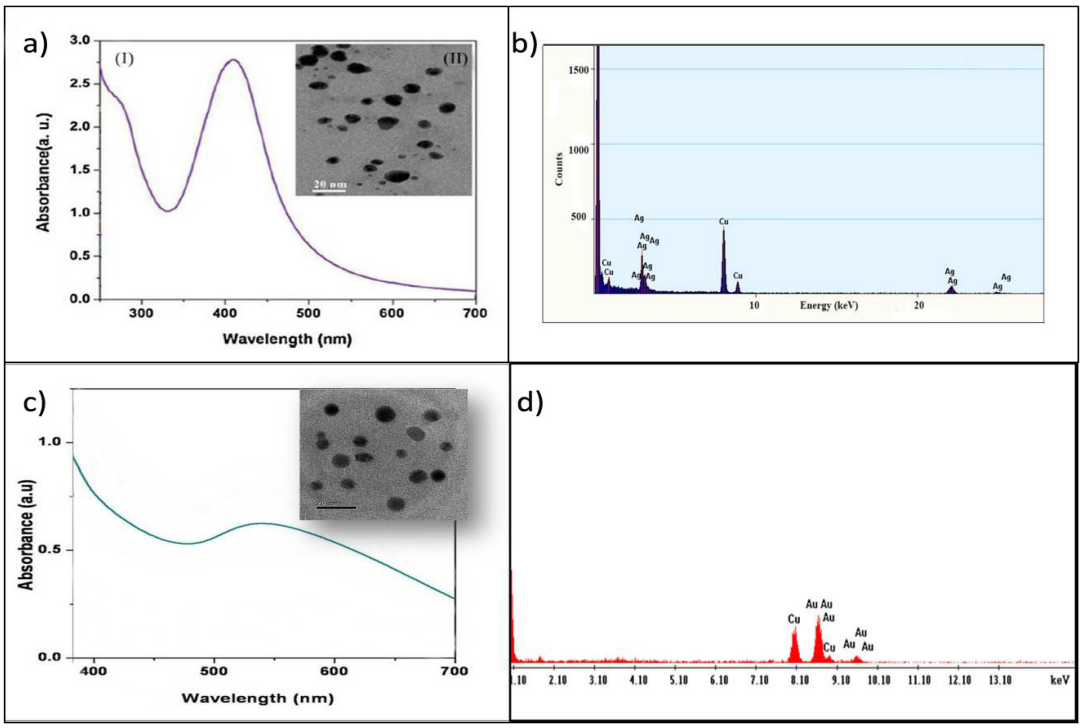

Figure 1: a. i) UV-Vis spectra recorded after the reaction of 1 mM AgNO3 solution with culture filtrate of fungal biomass. ii) TEM micrograph of biogenic silver nanoparticles

b. EDAX spectrum of silver nanoparticles

c. i) UV-Vis spectra recorded after the reaction of 1 mM AuCl4 solution with culture filtrate of fungal biomass. ii) TEM micrograph of biogenic gold nanoparticles

d. EDAX spectrum of gold nanoparticles

Advances in Biotechnology

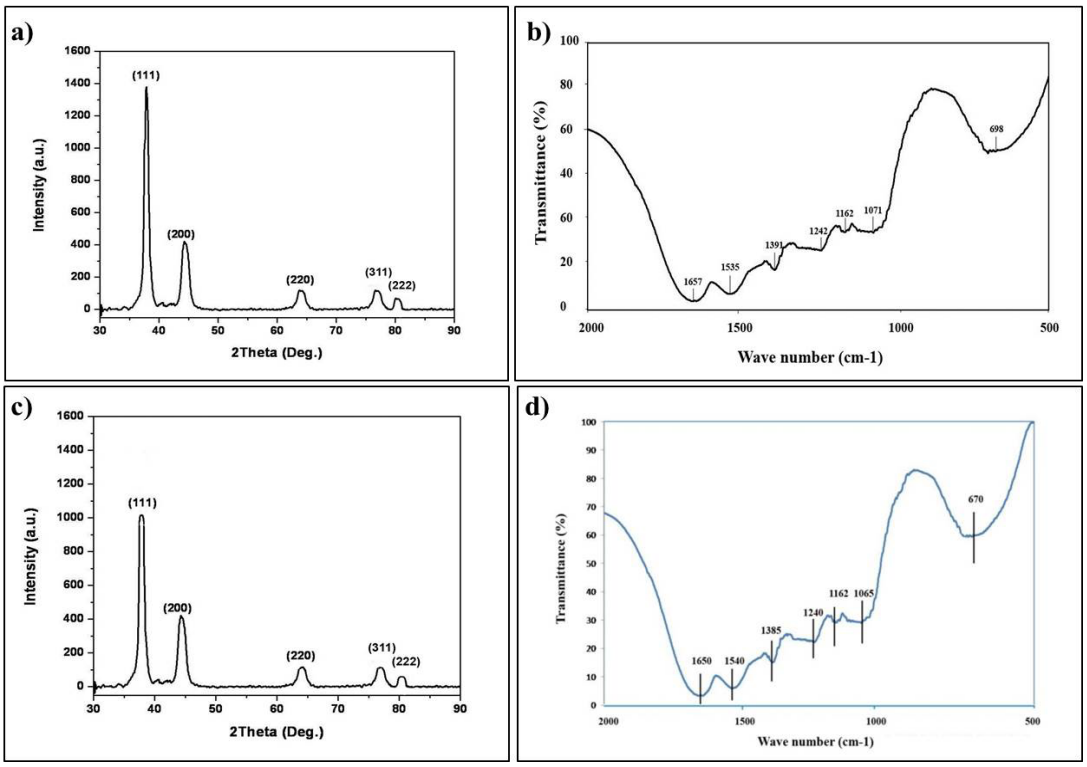

Figure 2: a) XRD pattern and b) FTIR spectrum of biogenic silver nanoparticles. c) XRD pattern and d) FTIR spectrum of biogenic gold nanoparticles

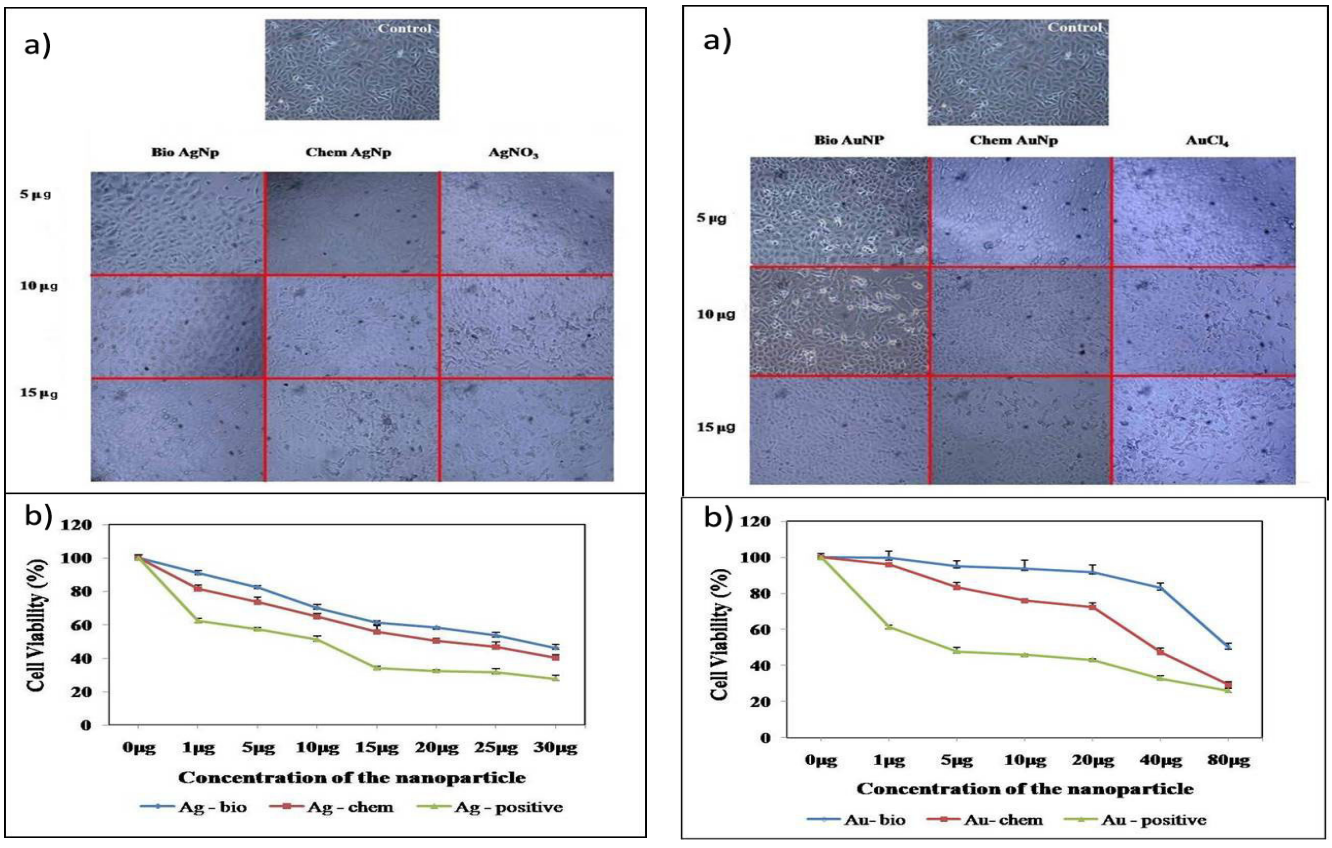

Figure 3: a) Optical microscope view and b) MTT assay performed on African monkey kidney cells (Vero) and exposed to different concentrations of biologically synthesized silver nanoparticles, chemically synthesized silver nanoparticles and silver nitrate

Figure 4: a) Optical microscope view and b) MTT assay performed on African monkey kidney cells (Vero) and exposed to different concentrations of biologically synthesized gold nanoparticles, chemically synthesized gold nanoparticles and tetra chloroauric acid

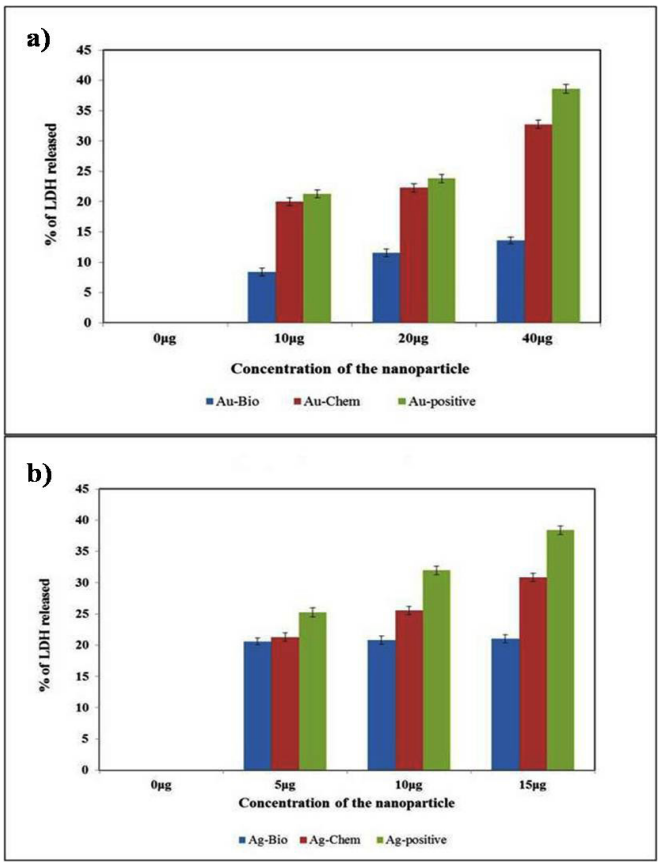

Figure 5: Lactate dehydrogenase assay performed on cell lines (a) with different concentrations of biologically synthesized gold nanoparticles, chemically synthesized gold nanoparticles and tetra chloroauric acid (b) with different concentrations of biologically synthesized silver nanoparticles, chemically synthesized silver nanoparticles and silver nitrate

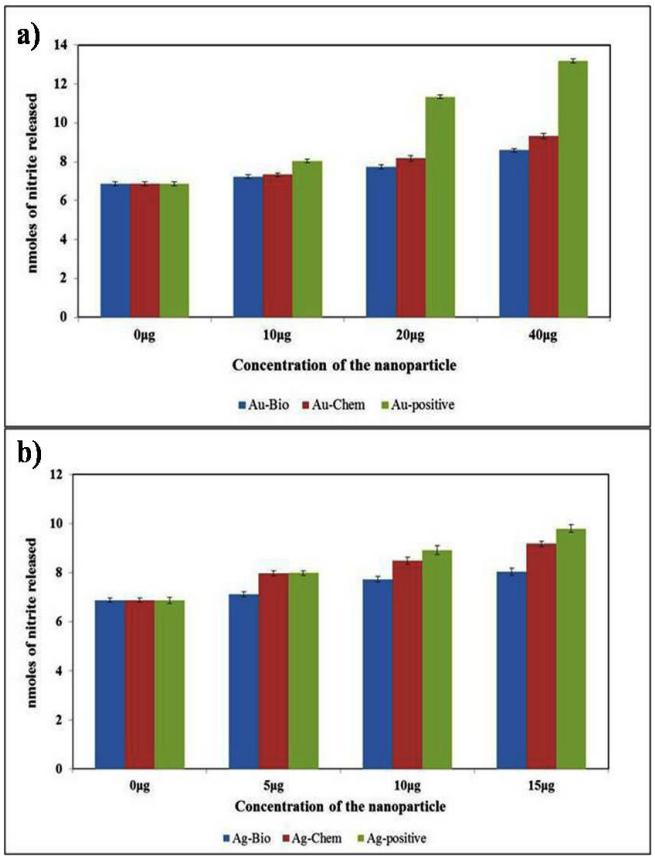

Figure 6: Nitric oxide assay performed on cell lines (a) with different concentrations of biologically synthesized gold nanoparticles, chemically synthesized gold nanoparticles and tetra chloroauric acid (b) with different concentrations of biologically synthesized silver nanoparticles, chemically synthesized silver nanoparticles and silver nitrate

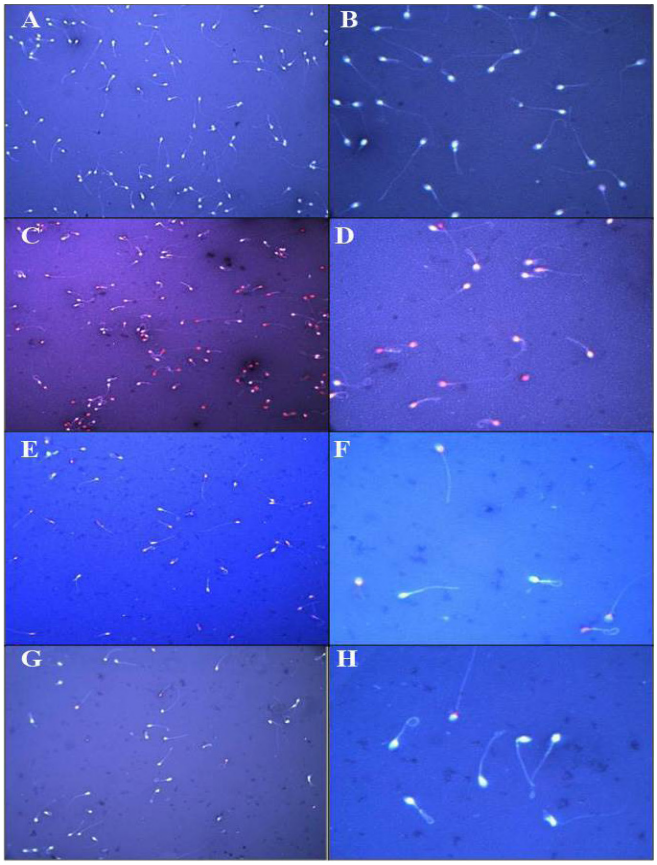

Figure 7a: Effect of silver nanoparticles on human spermatozoa after 30 min of incubation. Control cells after 30 min at (A) 40x and (B) 100x magnification, Cells treated with silver nitrate at (C) 40xand (D) 100x magnification, Cells treated with chemically synthesized silver nanoparticles at (E) 40x and (F) 100x magnification and Cells treated with biologically synthesized silver nanoparticles at (G) 40x and (H) 100x magnification

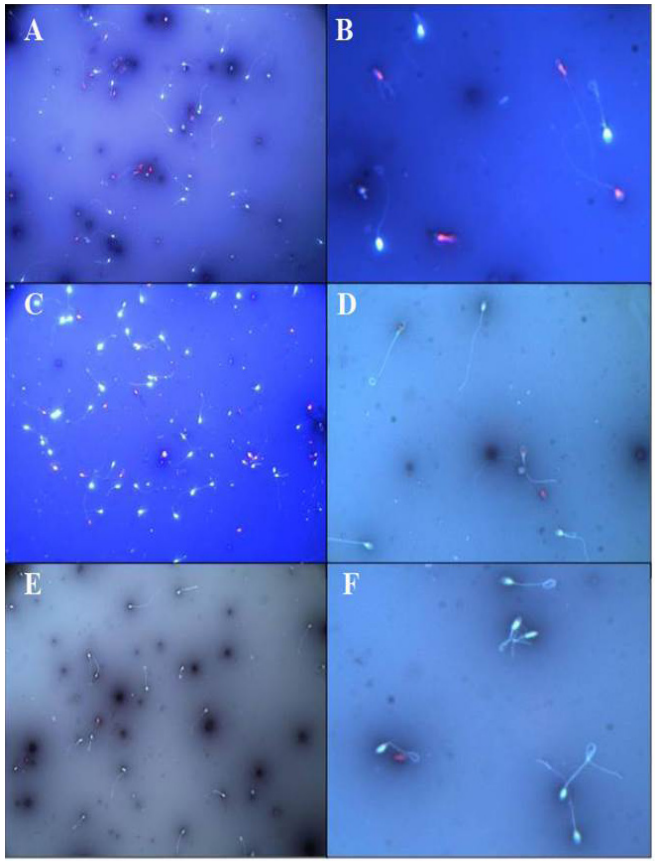

Figure 7b: Effect of gold nanoparticles on human spermatozoa after 30 min of incubation. Cells treated with gold chloride at (A) 40x and (B) 100x magnification, Cells treated with chemically synthesized gold nanoparticles at (C) 40x and (D) 100x magnification and Cells treated with biologically synthesized silver nanoparticles at (E) 40x and (F) 100x magnification

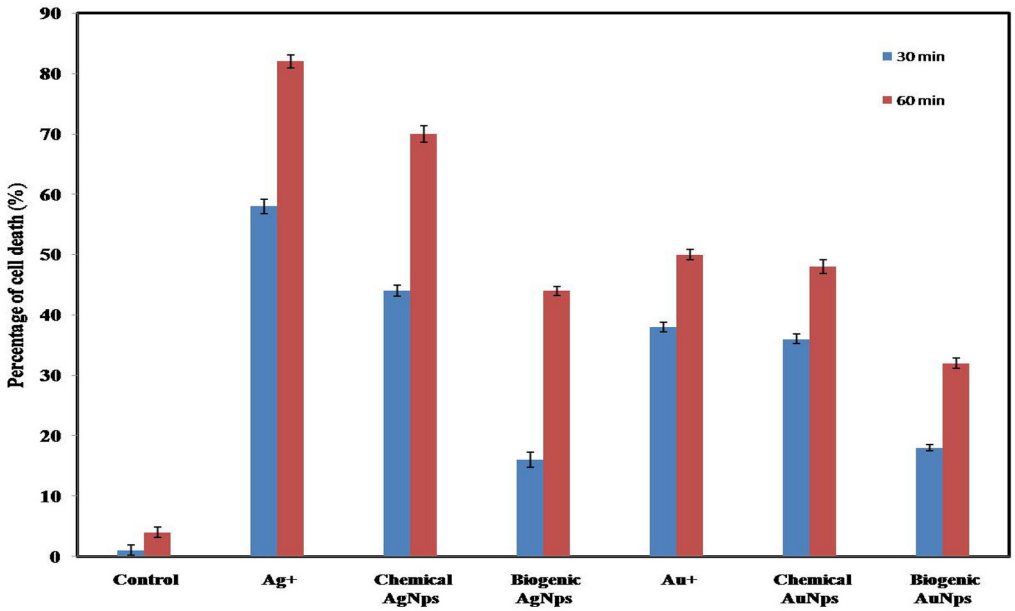

Figure 8: Percentage cell death rate of spermatozoa after 30 minutes and 60 minutes of exposure to different treatment

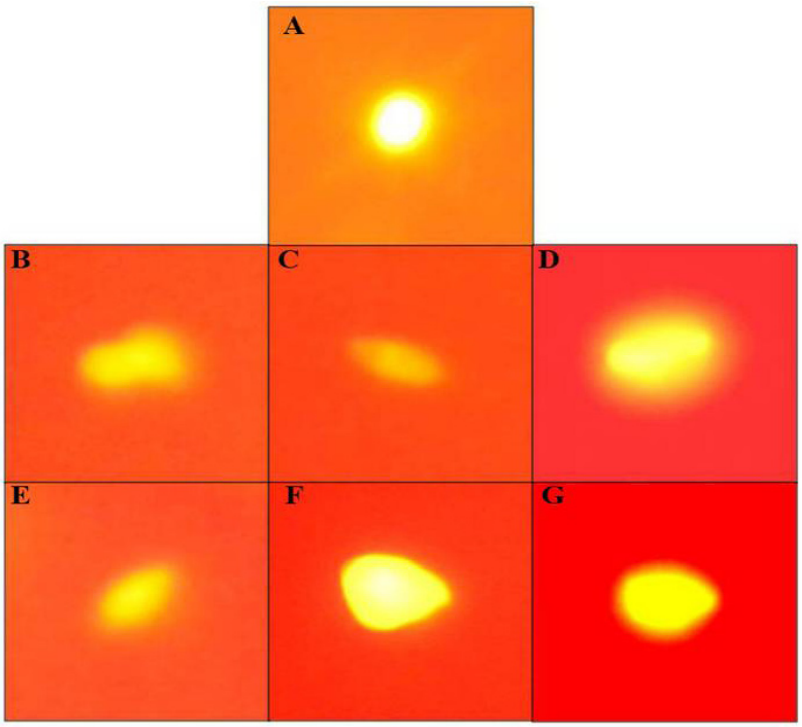

Figure 9: Fluorescent microscope view (100x) after Comet assay on human spermatozoa after different treatment with silver and gold nanoparticles

(A) Control, (B) Silver nitrate treated, (C) Chemically synthesized silver nanoparticles treated, (D) Biologically synthesized silver nanoparticles treated, (E) Tetra chloroauric acid treated, (F) Chemically synthesized gold nanoparticles treated and (G) Biologically synthesized gold nanoparticles treated

6. Table

Table 1: Comet assay analysis of human spermatozoa using CASP software

Treatment	Head DNA	Tail DNA	Tail Movement
CONTROL	99.956	0.044	0.001
$AgNO_3$	85.53	14.47	3.471
AgNPs (Biological)	90.83	9.17	1.375
AgNPs (Chemical)	87.55	12.45	1.493
$AUCl_4$	90.789	9.202	1.288
AuNPs (Biological)	96.848	3.152	0.347
AuNPs (Chemical)	96.199	3.801	0.342

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8. References

1. Girilal, M., Fayaz, A.M., Mohan, B.P., Kalaichelvan, P.T. Augmentation of PCR efficiency using highly thermostable gold nanoparticles synthesised from a thermophilic bacterium, Geobacillus stearothermophilus. Colloids and Surface B: Biointerface. 2013; 106: 165 – 169.

2. Fayaz, A.M., Girilal, M., Venkatesan, R., Kalaichelvan, P. T. Biosynthesis of anisotropic gold nanoparticles using Madhuca longifolia extract and their potential in infrared absorption. Colloids and Surface B: Biointerface. 2011; 88: 287–291.

3. Girilal, M., Krishnakumar, V., Poornima, P., Fayaz, A.M. and Kalaichelvan, P.T. A comparative study on biologically and chemically synthesized silver nanoparticles induced heat shock proteins on fresh water fish Oreochromis niloticus. Chemosphere 2015; 139: 461-468.

4. Asta, S., Igoris, P., Judita, P., Algimantas, J., Asta, G., 2006. Analysis of silver nanoparticles produced by chemical reduction of silver salt solution. Mater. Sci. 12: 287-291.

5. McFarland, D., Haynes, C.L., Mirkin, C.A., Van-Duyne, R.P., Godwin H.A., 2004. Color My Nanoworld. J. Chem. Educ. 81: 544.

6. Mossman, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Met. 1983; 65: 55-63.

7. Ulmer, D.D., Vallee, B.L., Wacker, W.E. Metalloenzymes and myocardial infarction. II. Malic and lactic dehydrogenase activities and zinc concentrations in serum. N. Engl. J .Med. 1956; 255: 450-456.

8. Stueher, D.J., Marletta, M.A. Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-gamma. J. Immunol. 1987; 139: 518-525.

9. Bjorndahl, L., Soderlund, I.,Kvist, U. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. Hum Reprod. 2003; 18: 813-816.

10. Cabrita, E., Robles, V., Rebordinos, L., Sarasquete, C., Herraez, M.P. Evaluation of DNA damage in rainbow trout (Oncorhynchus mykiss) and gilt head sea bream (Sparus aurata) cryopreserved sperm. Cryobiology. 2005; 50: 144-153.

11. Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P., Lipton, S.A. Apoptosis and necrosis: Two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc. Natl. Acad. Sci. 1995; 92: 7162-7166.

12. Jie, S., Xueji, Z., Mark, B., Harry, F. Measurement of nitric oxide production in biological systems by using griess reaction assay. Sensors. 2003; 3: 276-284.

13. McNaughton, L., Puttagunta, L., Martinez-Cuesta, M.A., Kneteman, N., Mayers, I., Moqbel, R., Hamid, Q., Radomski, M.W. Distribution of nitric oxide synthase in normal and cirrhotic human liver. Proc. Natl. Acad. Sci. U. S. A. 2002; 99: 17161-17166.

14. Yen, G.C., Lai, H.H. Inhibition of reactive nitrogen species effects in vitro and in vivo by isoflavones and soy-

food extracts. J. Agric. Food Chem. 2003; 51: 7892-7900.

15. Ahamed, M., Karns, M., Goodson, M., Rowe, J., Hussain, S.M., Schlager, J.J., Hong, Y. DNA damage response to different surface chemistry of silver nanoparticles in mammalian cells. Toxicol.Appl.Pharmacol. 2008; 233: 404-410.

16. Choi, J.E., Kim, S., Ahn, J.H., Youn, P., Kang, J.S., Park, K., Yi, J., Ryu, D.Y. Induction of oxidative stress and apoptosis by silver nanoparticles in the liver of adult zebrafish. Aquat.Toxicol. 2010; 100: 151-159.

17. Foldbjerg, R., Dang, D.A., Autrup, H. Cytotoxicity and genotoxicity of silver nanoparticles in the human lung cancer cell line, A549. Arch. Toxicol. 2011; 85: 743-750.

18. Lucas, B., Fields, C., Hofmann, M.C. Signaling pathways in spermatogonial stem cells and their disruption by toxicants. Birth Defects Res. Part C: Embryo Today: Rev. 2009; 87: 35-42.

19. Wojtaszek, P. Oxidative burst: An early plant response to pathogen infection. Biochem. J. 1997; 322: 681.

20. Ramadevi, S.,Prasad, M.N.V. Copper toxicity in Ceratophyllum demeresum l. (coontail), a free floating macrophyte: Response of antioxidant enzymes and antioxidants. Plant Sci. 1998; 138: 157.

21. Mittova, V., Volokita, M., Guy, M., Tal, M. Activities of SOD and the ascorbate-glutathione cycle enzymes in subcellular compartments in leaves and roots of the cultivated tomato and its wild salt-tolerant relative Lycopersicon pennellii. Physiol. Plant. 2000; 110: 45.

Advances in Biotechnology

Chapter 6

Bioprospecting of actinomycetes: Computational drug discovery approach

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Abstract

There is an urgent need for new drugs with increasing threat posed by multidrug resistant bacteria. Among the various sources of natural products actinomycetes hold prominent position due to their diversity and proven ability to produce bioactive metabolites. Generally, analytical instrumentation and chemical methods are widely used to identify and characterize potent compounds, however, of late genomic based approach and metabolomics tools are used for metabolite screening. This article deals with computer aided databases, genome based analysis and metabolomics tools to identify, mine and characterize natural products. A systematic approach including construction of natural product libraries and their crude extracts, dereplication, genome mining, bioinformatics, activation of silent gene clusters and increasing the active compound by precision engineering can lead to novel and potent drugs.

Keywords: marine ecosystem; actinomycetes; bioactive compounds; drug discovery; biodiversity

1. Introduction

The discovery of antibiotics in 19th century was a milestone in modern medicine and conferred one of the greatest benefits on mankind [1]. The availability of antibiotics from microbial products and industrial scale fermentation has allowed the successful treatment of many bacterial infections as well as the ability to perform invasive medical surgeries [2]. These “wonder drugs” categorized as antibiotics also have profound effect on livestock and

agricultural yield; as a result these antibiotics were referred as bioactive microbial metabolites. The continuous and overuse of antibiotics benefitted microorganisms to inherit genes from relative or can be acquired from nonrelatives on mobile genetic elements such as plasmids [3]. The horizontal gene transmission (HGT), spontaneous mutation, drug sensitive competitors' also benefitted bacteria to develop multiple mechanisms of resistance [4].

Another reason for the widespread resistance to antibiotics is the pervasive use of animal feeds to prevent infections. The extensive use of antibiotics in growth supplements for livestocks in both developing and developed countries contributes to increase risk of multiple drug resistant strains. The vicious cycle of antibiotic resistance keeps on repeating, by the use of antibiotics in food after ingestion by the animals it suppresses susceptible bacteria and allows antibiotic-resistant to flourish among the bacteria. The antibiotic resistant bacteria are transmitted to humans by consuming dairy and animal products which cause adverse health effects.

According to WHO (Fact Sheet, September 2016), each and every country contributes to antibiotic resistance. The infections caused by multiple drug resistant strains are at an increased risk of worse clinical outcomes and even death [5]. The centre for disease and control (CDC) has categorized various bacteria into urgent threat (*Clostridium difficile*, Carbepenem-resistant *Enterobacteriaceae* (CRE), Drug-resistant *Nisseria gonorrhoeae*), serious threat (Drug resistant *Campylobacter* sp., Fluconazole-resistant *Candida* sp.), Vancomycin resistant *Enterococci* (VRE), Drug resistant *Shigella*, Methicillin resistant *Staphylococcus aureus*, Drug-resistant *Streptococcus pneumoniae*, Drug-resistant *Mycobacterium* and concerning threat (Vancomycin-resitant *Staphylococcus aureus* (VRSA), Erythromycin-resistant Group A *Streptococcus*, Clindamycin-resistant Group B *Streptococcus*) of bacteria on the basis several factors including 10-year projection of incidence, transmissibility and availability of effective antibiotics, clinical, economic impact as well as barrier to prevention. The information and detailed report regarding alarming antibiotic resistant strains can be found at http://www.cdc.gov/drugresistance/threat-report-2013/pdf/ar-threats-2013-508.pdf. Looking at the present scenario of antibiotic resistance worldwide and incredible adaptability of microbes to adapt changes in environment due to their flexible metabolic power encourages researchers to develop therapeutic agents to combat antimicrobial resistance.

Historically, nature has been an enormous source of medicinal active compounds from plant and microbial sources for millennia. Microorganisms contribute majority of antibiotics which are clinically in use today. In golden era of microbial natural product screening, lots of efforts were put forward to screen potent microorganism from soil which has lead us to vast majority of microbial metabolites [6,7].

Actinomycetes are the most diverse microorganism which contributes to 45% of

antibiotics over fungi and unicellular bacteria. They are Gram-positive, spore forming and aerobic bacteria belong to the order Actinomycetales. The name "Actinomycetes" was derived from Greek "atkis" means ray and "mykes" means fungus, thus possessing the characteristics of both prokaryotes-bacteria and Eukaryotes-fungi [8]. This unique group can be characterized with aerial and substrate mycelium growth [9]. Actinomycetes have high GC content in their Deoxyribonucleic acid (DNA), free living microorganisms and are widely distributed in terrestrial and aquatic ecosystems, especially in soil forming as aerial mycelia [10].

The bioactive secondary metabolites produced by actinomycetes include antibiotics, antitumor agents, immunosuppressive agents and enzymes. These metabolites are known to possess antibacterial, antifungal, antioxidant, neuritogenic, anti-cancer, anti- algal, anti-helmintic, anti-malarial and anti-inflammatory [11,12]. Actinobacteria have made remarkable contributions to human life. The class Actinobacteria is especially notable for containing organisms producing diverse natural products, with members of the order Actinomycetales alone accounting for 10,000 such products [13].

2. Habitat of actinomycetes

Actinomycetes species are ubiquitous in nature and are most abundant in soil. They grow as hyphae like fungi responsible for the characteristically "earthy" smell of freshly turned healthy soil [14].They are primarily soil inhabitants [15] but have been found widely distributed in a diverse range of aquatic ecosystem, including sediments obtained from deep sea [16,17], even from greatest depth Mariana Trench [18,19]. Recently marine derived Actinomycetes are seeking attention as they produce novel antibiotic and anticancer agents with unusual structure and properties [20]. Maine Actinomycetes are the promising source for secondary metabolites [21]. It has been estimated that over past 10 years, 659 marine bacterial compounds have been isolated among which 256 compounds have been originated from actinomycetes [22,23]. Marine ecosystem harbours more than two third of the earth. In golden era of microbial natural product screening it was once thought that marine environment had unfavorable conditions for microbial natural products than terrestrial microbes but if we look at present scenario metabolites screening from terrestrial ecosystem leads to rediscovery of antibiotics whereas in the last 10 years research on unexplored marine ecosystem has accelerated in search of novel compounds to fight infectious diseases. Exploration of marine ecosystem attracts the interest of researchers based on four general aspects [24]:

- Biodiversity of microorganisms, especially isolated from unexplored or extreme environments;
- Structural diversity of secondary metabolites;
- Broad spectrum of active compounds; and

- Genetic engineering aimed at producing specific secondary metabolites and increasing the yields of important products.

Dr. Hans Peter- Fiedler Tubingen Germany, Professor Michael Goodfellow from the University of Newcastle Tyne and Professor Alan T. Bull from the University of Kent (UK) collaborated in year 2000-2013 to explore actinomycetes bioactive metabolites from unique terrestrial and marine habitats including sediments of deep-sea trenches located in the Pacific and Atlantic Oceans. All strains were taxonomically characterized to the genus level that permitted an individual submerged cultivation in the screening stage, applying genus-adapted cultivation media. The collaboration resulted in the discovery of various novel metabolites few of them are listed below:

3. Actinomycetes isolated from unique sources

- *Streptomyces sp.* $MECO_2$ was isolated from the stones of one of the Egyptian ancient tombs. Relatively high antitricophyton activity was attained with cultivation medium composed of (g/l): glucose, 5; casein, 0.0075; KNO_3, 0.05; NaCl, 2; K_2HPO_4, 6; $MgSO_4.7H_2O$, 0.05; $CaCO_3$, 0.02 and $FeSO_4.7H_20$, 0.01; pH 7 adjusted using phosphate buffer, inoculums size 2ml/50ml medium and agitation rate of 200 rpm at 30°C for 9 days incubation.

- A novel aerobic actinomycete, designated HA11110T, was isolated from a mangrove soil sample collected in Haikou, China. 16S rRNA gene sequence similarity showed that strain HA11110T belonged to the genus *Streptomyces*, most closely related to *Streptomyces fenghuangensis* GIMN4.003T (99.1 %), *Streptomyces nanhaiensis* SCSIO 01248T (98.8 %) and *Streptomyces radiopugnans* R97T (98.8 %). On the basis of phenotypic and genotypic data, strain HA11110T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces mangrovi* sp. nov. was proposed.

- Rare bioactive actinomycetes were isolated from unexplored regions of Sundarbans mangrove ecosystem and possess 93.57 % similarity with *Streptomyces albogriseolus* NRRL B-1305. The strain SMS_SU21, isolated from mangrove region possesses good antimicrobial and andantioxidant activity.

- Another research leads to discovery of a novel alkaloid, xinghaiamine A, from a marine-derived actinomycete *Streptomyces xinghaiensis* NRRL B24674T. Xinghaiamine A was identified to be a novel alkaloid with highly symmetric structure on the basis of sulfoxide functional group, and sulfoxide containing compound has so far never been reported in microorganisms. Biological assays revealed that xinghaiamine A exhibited broad-spectrum antibacterial activities to both Gram-negative persistent hospital pathogens (e.g. *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Escherichia coli*) and

- Gram-positive ones, which include *Staphylococcus aureus* and *Bacillus subtilis*. In addition, xinghaiamine A also exhibited potent cytotoxic activity to human cancer cell lines of MCF-7 and U-937 with the IC50 of 0.6 and 0.5 mM, respectively.

- Jiao [34] and Baskaran [35] isolated 42 actinomycetes isolates from mangrove sediments of Aandaman islands, India. Among 42 isolates, 22 species were found to possess antibacterial property against pathogenic microorganisms.

- Caerulomycin A- antifungal potential was isolated from marine invertebrate-associated *Actinoalloteichus* sp. using optimized medium and fermentation conditions by Marine invertebrate sample was collected from deep sea (Anjuna Beach, Goa, India) [36]..

- Prudhomme [37] findings underline the potential of secondary metabolites, derived from marine microorganisms, to inhibit *Plasmodium* growth. Salinosporamide A, produced by the marine actinomycete, *Salinispora tropica,* shows strong inhibitory activity against the erythrocytic stages of the parasite cycle.

4. Rare actinomycetes

Rare actinomycetes (eg *Nocardia* sp.) are referred to those actinomycete strains whose frequency is much lower than *Streptomyces* strains isolated using conventional methods [38]. The low occurrence of rare actinomycetes in contrast to diverse *Streptomyces* sp. is derived from the facts that they are hard to isolate from the environment and difficult to cultivate and maintain under conventional conditions [6]. Rare actinomycetes demands pre treatment of sample, appropriate isolation procedures and variety of different selection media including enrichment media [39,40] which makes more difficult for their cultivation and maintenance when compared to *Streptomyces* species. The spores of some rare actinomycete genera including *Streptosporangium* and *Microbispora* can withstand pre treatment with various chemicals [40]. They are widely distributed in terrestrial and aquatic ecosystems. Environmental factors such as soil type, pH, humus content, and the characteristics of the humic acid content of the soil affect their distribution [41]. Rare actinomycetes include some of these genera *Actinomadura, Actinoplanes, Amycolatopsis, Actinokineospora, Acrocarpospora, Actinosynnema, Catenuloplanes, Cryptosporangium, Dactylosporangium, Kibdelosporangium, Kineosporia, Kutzneria, Microbiospora, Microtetraspora, Nocardia, Nonomuraea, Planomonospora, Planobispora, Pseudonocardia, Saccharomonospora, Saccharopolyspora, Saccharothrix, Streptosporangium, Spirilliplanes, Thermomonospora, Thermobifida,* and *Virgosporangium* [42].

For the isolation of rare actinomycetes, variety of selective and enrichment media, addition of different antibacterial and antifungal antibiotics to the isolation media [39,43,44] use of xylose, chloride, collidine, bromide and vanillin [41] which act as chemo-attractants

for accumulating spores, chloramine treatment [43], as chlorination is known to suppress growth of contaminant bacteria, use of humic acid-vitamin enriched media [43] and different kinds of radiation [45] favor selective isolation of different actinomycetes genera resulted in successful cultivation of diverse and rare actinomycetes. If we go through previous published work, only 11 rare actinomycetes species producing altogether 50 bioactive compounds were known in 19th century [6]. Today the number of taxonomically described rare actinomycetes is close to 100, and due to recently developed advanced isolation techniques. Hayakawa et al., 1988 [41] investigated the distribution of rare actinomycetes various locations throughout Japan using a special isolation medium, HV agar. The distribution of rare actinomycetes genera in cultivated field soils (154 samples) was remarkable. *Dactylosporangium* and *Microtetraspora, Saccharomonospora*, and*Micromonospora* were most frequently isolated from mountainous forest soils, level-land forest or cultivated field soils, and pasture soils, respectively. [41] has listed out various physical, chemical and enrichment methods to isolate, cultivate and maintain rare actinomycetes. [46] explored Egyptian habitats which could lead to various rare actinomycetes isolates including Micromonospora (23 isolate, 65.71%), less commonly Actinoplanes (11 isolates, 31.43%) and rarely Dactylosporangium (1 strain, 2.86%) genera. In 2008, Eccleston et al. [47] reported the occurrence of *Microsmonospora* from the Sunshine Coast in Australia [47,48,49] reported the isolation of a rifamycin-producing *Micromonospora* from mangrove in South China Sea. Different genera such as *Brevibacterium, Dermabacter, Kytococcus, Microbacterium, Nesterenkonia*, and *Rothia* were isolated from mangrove sediments in Brazil [50]. In China, a number of rare actinobacteria including *Actinomadura, Isoptericola, Microbispora, Nocardia, Nonomuraea*, and *Rhodococcus;* were isolated from mangrove soils and plants [43]. Results from Ara et al. [51] showed that 17 different genera of rare actinobacteria were identified from a total of 241 isolates.

Biopropspecting and understanding of rare actinobacteria diversity led to the discovery of novel genus and species strains, such as "*Microbacterium mangrovi* sp. nov." [52], "*Mumia flava* gen. nov., sp. nov." [53] and *Sinomonas humi* sp. nov." [54] isolated from a mangrove forest in Tanjung Lumpur, Malaysia. The conventional approach to understand biodiversity of actinomycete isolates has come down new approaches including construction of environmental genomic libraries [55] the use of selective isolation media and phylogenetic analysis [56,57] culture-independent methods [58] and digital image analysis [59] have been extensively used to explore novel strains.

5. Approaches to effective drug discovery

With the discovery of Penicillin, millions of microorganisms have been screened for their bioactive potential from various soil samples worldwide. The bioactive metabolites produced are widely used as antibacterial therapeutics, such as erythromycin, streptomycin, tetracycline, vancomycin and chemotherapeutic drugs such as doxorubicin. Actinobacteria

have made remarkable contributions to human. Presently, researchers are hunting for novel strains from unexplored area such as marine ecosystem due to exhaustive culture dependent screening of actinomycete isolates from terrestrial environment leading to rediscovery of bioactive metabolites. There are different approaches which have been employed for novel drug discovery strains:

- Optimization of nutritional requirements to obtain maximum yield of metabolites, crude extracts or purified compounds were screened for biological activity without knowing the drug target. Once the potent compound has been identified, efforts have been made to analyze the target and mode of action of compound including the metabolic pathway. This approach to drug discovery can be categorized as bioactive-guided screening [60].

- Another approach to drug discovery includes the chemical screening of effective metabolite with bioactive potential. It implies sophisticated analytical instrumentation to elucidate the structural properties of metabolites such as high-performance liquid chromatography, mass spectrometry or nuclear magnetic resonance. The substances used in this particular approach were obtained from microbial sources. Nowdays, various metabolomics tools such as Cycloquest, GNPS-Genome to Natural Product Platform, NRPquest (Nonribosomal Peptide), PEP2 path are available to characterize BCG to improve metabolic profile of actinomycetes whereas compound databases including (CHEBI-Chemical Entities of Biological Interest, Chemspider, Novel antibiotics, PubChem, Antibiticome) are extensively used to obtain complete biochemical profile of metabolite.

- The target oriented screening is useful in identifying compounds that hit a known and validated molecular target. These targets represent a cellular and molecular structure involved in pathology of interest that the drug in development is meant to act on. High-throughput metabolic modeling tools allows generation of genome-scale metabolic models enables linking between genotype, metabolic phenotype and biosynthetic gene cluster of secondary metabolite producing organism. The metabolic modeling of genome in metabolomics (secondary metabolites) is very useful in predicting intracellular flux distribution of actinomycetes in specific environmental or genetic condition and gene manipulation targets for overproduction of secondary metabolites. MODEL-SEED is only known high throughput modeling tool which has been deployed to reconstruct multiple actinomycetes species for large scale metabolic studies [61].

6. Genome mining and identification of BCG's

A huge database of mass spectra for known bioactive compounds is available from

chemical libraries and can be efficiently used for dereplication [62]. The major drawback of using these programs is rediscovery of known compounds. To overcome this problem, Genome mining can be the answer. It is an alternative strategy which has become increasingly popular during the last decades [63]. This approach detects and analyses the biosynthetic gene cluster of the chemical compounds and subsequently connect those genes to molecules. The actinomycete genome, for example, contains approximately 8,000 genes coding for 20–50 proteins from secondary metabolite synthetic gene clusters [64]. Bioinformatics technologies allow the rapid identification of known gene clusters encoding bioactive compounds and to make computer predictions of their chemical structure based on genetic sequence information [65,66]. The chemical classes and the structure of encoded compound can be predicted using biosynthetic gene cluster approach. This information can be used to either guide a more targeted drug discovery technique (such as reactivity-guided isolation; [67] or peptide and glycogenomic approaches [68,69] or allow the heterologous expression in an optimized expression host (activation of silent gene cluster). There are web based comprehensive suite such as BAGEL-Bayesian analysis of Gene essentiality which can be utilized to identify the genes encoding precursor peptides whereas identifying BCG's with BLAST and HMMeR provide us insight of polyketides synthesized by type I and type II PKS, ribosomally and post translationally modified peptides (RiPPS).

Another approach to this problem can be microbial natural product library. The primary approach to discovery of novel natural products from extract is bioassay guided fractionation. The combined use of LC, solid-phase extraction (SPE) and NMR spectroscopy (LC-SPE-NMR) has been developed and continues to undergo refinement via expansion of the types of NMR analysis one can perform on samples once they've been separated, removed from LC mobile phase and placed into deuterated solvents [70-74,75]. Sophisticated instrumentation permits the rapid identification of minor and major secondary metabolites in natural product. However, cost and effort requirements, for the foreseeable future, limit the availability of such techniques to many academic natural product laboratories. Once a crude extract library has been established it can be used for target-based and whole-cell highthroughput screens related to infectious disease for the identification of active natural products [24].

Recently, various bioinformatics approaches have been developed to organize or interpret large sets of MS/MS fragmentation data. For example, solutions such as MAGMa (MS annotation based on in silico generated metabolites) allow matching of multistage fragmentation data against candidate molecules substructures and were successfully applied on complex extracts [76-78]. Among these new approaches, molecular networking (MN) is a particularly effective one to organize MS/MS fragmentation spectra. MN compares all MS/MS spectra in a given extract and groups them according to their similarity [79-82] The applications of these tools lead to the identification of novel compounds by avoiding re-isolation of known

compounds which could flourish the idea of developing novel drugs.

7. Future prospects

With the increase in population worldwide it is very difficult to combat emerging antibiotic resistance in pathogenic microorganisms due to their adaptive metabolism. Natural products and their derivatives historically have been an inevitable source of therapeutic agents. However from past decade microbial natural product research in pharmaceutical industry has declined due to lack of compatibility of traditional natural-product extract libraries with high throughput screening. Lack of required purity, availability and productivity of novel strains and pure compounds are few reasons for progressively decline in identification bioactive natural product with HTS.

- For novel natural products, microbes that are morphologically distinctive, taxonomically new, or isolated from ecologically unique region or sources which have not been screened for natural products can be lead to new active compounds.

- By implying high throughput screening programs, cell and target based assays is capable of detecting most representative natural products with new chemotypes [24].

- Genome mining for drug targets and computational based screening programmes revealed much bigger potential to synthesize natural products than have been isolated from conventional approaches. For example, GlaxoSmithKline has conducted studies with the antibiotic GKS299423 acting on topoisomerase II, in order to prevent the bacteria from developing resistance [83].

- High performance liquid chromatography bioassays and Liquid Chromatography-Mass Spectrometry for active fractions can easily detect dereplication of known natural products from new ones leading to their structure elucidation.

- Molecular approaches also act as key source in search of novel bioactive metabolites. Culture-independent molecular approach studies employ direct extraction of nucleic acids from the samples [58]. It often involves the amplification of DNA or cDNA from RNA extracted from environmental samples by PCR and the subsequent analysis of the diversity of the amplified molecules (community fingerprinting) [13].

- Metagenomic and metaproteomic technologies enable powerful new approaches to gene, genome, protein and metabolic pathway discovery [84. This new source of metabolic and chemical diversity will lead to important new basic knowledge and also contribute to ongoing drug discovery efforts against many disease indications. For example, Sherman and colleagues recently reported a model system for developing a meta-genomic approach to identify and characterize the natural product pathways

- from invertebrate-derived microbial consortia using the ET-743 (Yondelis) biosynthetic pathway[84].

- Another approach to overcome the problem of antibiotic resistance is the development of new drug combination or to develop the synergistic effect of drugs. Recently this approach has been proven to be successful in the treat of tuberculosis and HIV infected patients [63]

8. Table

Table 1: Novel metabolites produced by actinomycete isolates from unique sources

Antibiotic (isolated secondary metabolite)	Nature of antibiotic	Area explored	Name of the strain	Ref
Frigocyclinone	Angucyclinone	Antarctica	*Streptomyces griseus* NTK 97	[25]
Gephyromycin	Angucyclinone antibiotic	Antarctica	*Streptomyces griseus* NTK 14	[26]
Albidopyrone	Pyrone Antibiotic Inhibitory activity against protein-tyrosin phosphokinase 1B	North-Atlantic Ocean	*Streptomyces* strain NTK 227	[27]
Caboxamycin:	Benzoxazol antibiotic-Antibacterial, antitumor and phosphodiesterase inhibitory activities	Canary Basin at -3814 m	*Streptomyces* strain NTK 937	[27]
Abyssomicins B, C, D, G, H and atrop-Abyssomicin C	Polycyclic, Polyketide antibiotics Antibacterial activity against Gram-positive bacteria including MRSA and Vancomycin-resistant strains	Deep sea sediments	*Verrucosispora maris* AB-18-032	[28]
Atacamycins A–C	Macrolactone Antibioticsantitum and Phosphodiesterase inhibitoring activity	Hyper-arid soil from the Atacama Desert, North Chile	*Streptomyces* sp. C38	[29]
Dermacozines A–L	Phenazine antibiotics free radical scavenging activity, Antitumor and antiparasitic activities	Deep sea Sediments	*Dermacoccus abyssi* MT1.1 and MT1.2	[30
Proximicins A–C	Aminofuran antibiotics – strong antitumor activity	Deep sea Sediments	Marine strain *Verrucosispora fiedleri* MG-37	[31]
Pyrocoll	Diketopiperazine antibiotic Antibacterial, antiparasitic and antitumor active	Soil sample collected at Consett, County Durham, United Kingdom	Alkaliphilic *Streptomyces* strain AK 409,	[32]
Bendigoles A–C	Steroid metabolites	Activated sludge foam-Bendigo Biological Nutrient Removal Plant, Victoria, Australia	*Gordonia australis Acta* 2299	[31]
Langkocyclines A1, A2, A3, B1 and B2	Angucycline- Antibacterial and antitumor activities	Rhizosphere soil sample-Fine roots of the creeper *Clitorea* sp., growing on the sandy beach at Burau Bay, main Langkawi Island, Malaysia	*Streptomyces* sp. Acta 3034	[33]

9. Conclusions

By considering the present scenario of antibiotic resistance globally, there is no doubt that we will need antibiotics in future and we cannot decline the fact that natural products are most likely the best source. Among microorganisms, Actinomycetes are prolific producers of secondary metabolites and bioactive compounds contributing 70-80% of antibiotics commercially. However efforts have been made to cultivate rare and novel isolate from unexplored natural sources but steady decline in identifying novel compounds using conventional methods is nowhere leading us to discovery of pharmaceutical compounds. These challenges in microbial natural product discovery encourage researches to utilize computational, molecular and bioinformatics drug discovery programs to overcome the flaws in bioactive-guided screening, chemical screening, target oriented screening and high throughput screening. Genome mining approaches such as mining for biosynthetic genes, resistance genes, regulators, regulator guided activation, activation of silent gene clusters, target guided screening, bioactivity driven screening and chemical screening including real time mass-spectrometer, high-resolution mass-spectrometry can eliminate the rediscovery of known natural products from Actinomycetes isolates. These new approaches can lead us to successful novel compounds from unexplored regions or sources such as marine ecosystem.

10. References

1. Berdy, J. (2012): Thoughts and facts about antibiotics: where we are now and where we are heading. J Antibiot, 65: 385–395.

2. Hancock, R.E.W. (2001): Cationic peptides: effectors in innate immunity and novel antimicrobials. Lancet Infect. Dis, 1:156–164.

3. Read, A.F., Woods, R.J. (2014): Antibiotic resistance management. Evol Med Public Health, 28:147.

4. Davies, J., Davies, D. (2010): Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev, 74: 417-33.

5. The Review on Antimicrobial Resistance. Antimicrobial resistance: tackling a crisis for the health and wealth of nations London; The Review on Antimicrobial Resistance (2014) http://amrreview.org/sites/default/files/SECURING%20NEW%20DRUGS%20FOR%20FUTURE%20GENERATIONS%20FINAL%20WEB_0.pdf

6. Berdy, J. (2005): Bioactive microbial metabolites. J Antibiot, 58: 1–26.

7. Monciardini, P., Iorio, M., Maffioli, S., Sosio, M., Donadio, S. (2014): Discovering new bioactive molecules from microbial sources. Microb Biotechnol, 7: 209–220.

8. Das, S., Lyla, P.S., Khan, S.A. (2008): Distribution and generic composition of culturable marine actinomycetes from the sediments of Indian continental slope of Bay of Bengal. Chin J Oceanol Limnol, 26: 166- 77.

9. Lechevalier, H, Lechevalier, M.P. (1981): Introduction to the order Actinomycetales. In: Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG,Editors. The Prokaryotes. Germany: Springer-Verlag Berlin, 2: 1915-22.

10. Yoshida, A., Seo, Y., Suzuki, S., Nishino, T., Kobayashi, T., Hamada-Sato, N., Kogure, K., Imada, C. (2008): Actinomycetal community structures in seawater and freshwater examined by DGGE analysis of 16S rRNA gene fragments. Mar. Biotechnol. 10: 554–563.

11. Kekuda, T.R.P., Shobha, K.S., Onkarappa, R. (2013): Studies on antioxidant and anthelmintic activity of two Streptomyces species isolated from Western Ghat soils of Agumbe, Karnataka. J Pharm Res, 3: 26-9.

12. Ravikumar, S., Inbaneson, S.J., Uthiraselvam, M., Priya, S.R., Ramu, A., Banerjee, M.B. (2011): Diversity of endophytic actinomycetes from Karangkadu mangrove ecosystem and its antibacterial potential against bacterial pathogens. J Pharm Res, 4: 294-6.

13. Bull, A.T, Stach, J.E.M. (2007): Marine actinobacteria: new opportunities for natural product search and discovery. Trends Microbiol, 15: 491–499

14. Sprusansky, O., Stirrett, K., Skinner, D., Denoya, C., Westpheling, J. (2005): The bkdR gene of Streptomyces coelicolor is required for morphogenesis and antibiotic production and encodes a transcriptional regulator of a branched-chain amino acid dehydrogenase complex. J Bacteriol. 187: 664-71.

15. Kuster, E. (1968): Taxonomy of soil actinomycetes and related organisms. In: Gray S, Parkinson T,Editors. Ecology of soil bacteria. Liverpool: Liverpool University Press.

16. Walker, D., Colwell, RR. (1975): Factors affecting enumeration and isolation of actinomycetes from Chesapeake Bay and South eastern Atlantic Ocean sediments. Mar Biol, 30: 193-201.

17. Colquhoun, J.A., Mexson, J., Goodfellow, M., Ward, A.C., Horikoshi, K., Bull, A.T. (1998): Novel rhodococci and other mycolate actinomycetes from the deep sea. Antonie van Leeuwenhoek. 74: 27-40.

18. Takami, H., Inoue, A., Fuji, F., Horikoshi, K. (1997): Microbial flora in the deepest sea mud of the Mariana Trench. FEMS Microbiol Lett, 152: 279-85.

19. Pathomaree, W., Stach, J.E., Ward, A.C., Horikoshi, K., Bull, A.T., Goodfellow, M. (2016): Diversity of actinomycetes isolated from Challenger Deep sediment (10,898 m) from the Mariana Trench. Extremophiles, 10: 181-9.

20. Jensen, P.R., Williams, P.G., Oh, D.C., Zeigler, L., Fenical, W. (2007): Species-specific secondary metabolite production in marine actinomycetes of the genus Salinispora. Appl Environ Microbiol, 73: 1146-52.

21. Lam, K.S. (2005): Discovery of novel metabolites from marine actinomycetes, Currt Opn Microbiol, 9: 245-251.

22. William, P.G. (2008): Panning for chemical gold marine bacteria as a new source of therapeutics trends in Biotechnol 27: 45-52.

23. Sarkar, S., Saha, M., Roy, D., Jaisankar, P., Das, S., Roy, S., Rattan Gucchi, L.J., Sen, T., Mukherjee, J. (2008): Enhanced production of antimicrobial compounds by three salt tolerant actinobacterial strains isolated from Sundarbans in niche mimic bioreactor, Marine Biotechnology, 10: 518-526.

24. Liu, X., Bolla, K., Ashforth, E.J., Zhuo, Y., Gao, H., Huang, P., Stanley, S.A., Hung, D.T., Zhang, T. (2012): Systematics-guided bioprospecting for bioactive microbial natural products Antonie van Leeuwenhoek, 101: 55–66.

25. Bruntner, C., Binder, T., Pathom-aree, W., Goodfellow, M., Bull, A.T. , Potterat, O., Puder, C., Hörer, S., Schmid, A., Bolek, W., Wagner, K., Mihm, G., Fiedler, H.P. (2005): Frigocyclinone, a Novel Angucyclinone Antibiotic Produced by a Streptomyces griseus Strain from Antarctica. J. Antibiot. 58: 346–349.

26. Bringmann, G., Lang, G., Maksimenka, K., Hamm, A., Gulder, T. A.M., Dieter, A., Bull, A.T., Stach, J.E.M., Kocher, N., Müller, W.E.G., Fiedler, H.P. (2005): Gephyromycin, the first bridged angucyclinone, from Streptomyces griseus strain NTK 14. Phytochem. 66: 1366-1373

27. Hohmann, C., Schneider, K., Bruntner, C., Brown, R., Jones, A.L., Goodfellow, M., Krämer, M., Imhoff, J.F., Nicholson, G., Fiedler, H.P., Süssmuth, R.D. (2009): Albidopyrone, a new alpha-pyrone-containing metabolite from marine-derived Streptomyces sp. NTK 227. J. Ant. 62: 75–79.

28. Keller, S., Nicholson, G., Drahl, C., Sorensen, E., Fiedler, H.P., Süssmuth, R.D. (2007): Abyssomicins G and H

and atrop-Abyssomicin C from the Marine Verrucosispora Strain AB-18-032. J. Antibiot. 60, 391–394.

29. Nachtigall, J., Kulik, A., Helaly, S., Bull, A.T., Goodfellow, M., Asenjo, J.A., Maier, A., Wiese, J., Imhoff, J.F., Süssmuth, R.D., Fiedler, H.P. (2011): Atacamycins A-C, 22-membered antitumor macrolactones produced by Streptomyces sp. C38. J Antibiot (Tokyo), 64: 775-80.

30. Abdel-Mageed, W.M., Milne, B.F., Wagner, M., Schumacher, M., Sandor, P., Pathom-aree, W., Goodfellow, M., Bull, A.T., Horikoshi, K., Ebel, R., Diederich, M., Fiedler, H.P., Jaspars, M. (2010): Dermacozines, a new phenazine family from deep-sea dermacocci isolated from a Mariana Trench sediment. Org. Biomol Chem., 21: 2352-62.

31. Schneider, K., Graf, E., Irran, E., Nicholson, G., Stainsby, F.M., Goodfellow, M., Borden, S.A., Keller, S., Süssmuth, R.D. (2008): Bendigoles A~C, New Steroids from Gordonia australis Acta 2299. J. Antibiot. 61 :356-64.

32. Dietera, A., Hamm, A., Fiedler, H.P., Goodfellow, M., Müller, W.E., Brun, R., Beil, W., Bringmann, G. (2003): Pyrocoll, an antibiotic, antiparasitic and antitumor compound produced by a novel alkaliphilic Streptomyces strain. J Antibiot (Tokyo). 56: 639-46.

33. Kalyon, B., Tan, G.Y., Pinto, J.M., Foo, C.Y., Wiese, J., Imhoff, J.F., Süssmuth, R.D., Sabaratnam, V., Fiedler, H.P. (2013): Langkocyclines: novel angucycline antibiotics from Streptomyces sp. Acta 3034. J. Antibiot. 66: 609-16.

34. Jiao, W., Zhang, F., Zhao, X., Hu, J., Suh, J.W. (2013): A Novel Alkaloid from Marine-Derived Actinomycete Streptomyces xinghaiensis with Broad-Spectrum Antibacterial and Cytotoxic Activities. PLoS ONE 8: 75994.

35. Baskaran, R., Vijayakumar, R., Mohan, P.M. (2011): Enrichment method for isolation of bioactive actinomycetes from mangrove sediments of Andaman islands, India. Mal J Microbiol, 7: 26-32.

36. Ambavane, V., Tokdar, P., Parab, R., Sreekumar, E.S., Mahajan. G., Mishra, P.D., Lisette D'Souza, L., Ranadive, P. (2014): Caerulomycin A—An Antifungal Compound Isolated from Marine Actinomycetes Advances in Microbiology, 4,: 567-578.

37. Prudhomme, J., McDaniel, E., Ponts, N., Bertani, S., Fenical, W., Jensen, P. (2008): Marine Actinomycetes: A New Source of Compounds against the Human Malaria Parasite. PLoS ONE, 3: 2335.

38. Tiwari, K., Rajinder, K. (2012): Gupta Rare actinomycetes: a potential storehouse for novel antibiotics Critical Review Biotech, 32: 108–132

39. Qiu, D., Ruan, J., Huang, Y. (2008 Selective isolation and rapid identification of members of the genus Micromonospora. Appl Environ Microbiol,74: 5593–7.

40. Khanna, M., Solanki, R., Lal, R. (2011): Selective isolation of rare actinomycetes producing novel antimicrobial compounds. Int J Adv Biotechnol Res, 2: 357–75.

41. Hayakawa, M. (2008): Studies on the isolation and distribution of rare actinomycetes in soil. Actinomycetologica, 22: 12–9

42. Lazzarini, A., Cavaletti, L., Toppo, G., Marinelli, F. (2001): Rare genera of actinomycetes as potential producers of new antibiotics. Antonie van Leeuwenhoek, 78: 399–405.

43. Hong, K., Gao, A.H., Xie, Q.Y., Gao, H., Zhuang, L., Lin, H.P. (2009): Actinomycetes for marine drug discovery isolated from mangrove soils and plants in China. Mar Drugs, 7: 24–44.

44. Zhang, J., Zhang, L. (2011): Improvement of an isolation medium for actinomycetes. Mod Appl Sci, 5: 124–7.

45. Bredholdt, H., Galatenko, O.A., Engelhardt, K., Fjaervik, E., Terekhova, L.P., Zotchev, S.B. (2007): Rare actinomycete bacteria from the shallow water sediments of the Trodheim fjord, Norway: isolation, diversity and biological activity. Environ Microbiol, 9: 2756–64

46. Rifaat, H.M., Nagieb, Z.A., Ahmed, Y.M. (2005):Production of xylanases by Streptomyces species and their

bleaching effect on rice straw pulp. Appl. Ecol. and Environment. Res.4: 151–160.

47. Eccleston, G.P., Brooks, P.R., Kurtböke, D.I. (2008): The occurrence of bioactive micromonosporae in aquatic habitats of the Sunshine Coast in Australia. Mar. Drugs, 6: 243–261.

48. Xie, X.C., Mei, W.L., Zhao, Y.X., Hong, K., Dai, H.F. (2006): A new degraded sesquiterpene from marine actinomycete Streptomyces sp. 0616208. Chin. Chem. Lett, 17:1463–1465.

49. Huang, H.Q., Lv, J.S., Hu, Y.H., Fang, Z., Zhang, K.S., Bao, S.X. (2008): Micromonospora rifamycinica sp. nov., a novel actinomycete from mangrove sediment. Int J Syst Evol Microbiol, 58: 17–20.

50. Dias, A.C.F., Andreote, F.D., Dini-Andreote, F., Lacava, P.T., Sá, A.L.B., Melo, I.S. (2009b): Diversity and biotechnological potential of culturable bacteria from Brazilian mangrove sediment. World J Microbiol Biotechnol, 25: 1305–1311.

51. Ara, I., Bakir, M.A., Hozzein, W.N., Kudo, T. (2013): Population, morphological and chemotaxonomical characterization of diverse rare actinomycetes in the mangrove and medicinal plant rhizosphere. Afr J Microbiol Res, 7: 1480–1488.

52. Lee, L.H., Azman, A.S., Zainal, N., Eng, S.K., Ab Mutalib, N.S., Yin, W.F. . (2014c): Microbacterium mangrovi sp. nov., an amylolytic actinobacterium isolated from Tanjung Lumpur mangrove forest. Int J Syst Evol Microbiol 64: 3513–3519.

53. Lee, L.H., Zainal, N., Azman, A.S., Eng, S.K., Ab Mutalib, N.S., Yin W.F. (2014b): Streptomyces pluripotens sp. nov., a bacteriocin-producing streptomycete that inhibits meticillin-resistant Staphylococcus aureus. Int J Syst Evol Microbiol 64: 3297–3306.

54. Lee, L.H., Azman, A.S., Zainal, N., Yin, W.F., Ab Mutalib, N.S., Chan, K.G. (2015): Sinomonas humi sp. nov., an amylolytic actinobacterium isolated from mangrove forest soil. Int J Syst Evol Microbiol 65: 996–1002.

55. Donadio, S., Monciardini, P., Alduina, R., Mazza, P., Chiocchini, C., Cavaletti, L., Sosio,M., Puglia, A.M. (2002): Microbial technologies for the discovery of novel bioactive metabolites. J Biotechnol, 99: 187–198.

56. Jensen, P.R., Mafnas, C. (2006): Biogeography of marine actinomycete Salinospora. Enviornt. Microbiol. 8: 1881-1888.

57. Hozzein, W.N, Ali, M.I.A., Rabie, W. (2008): A new preferential medium for enumeration and isolation of desert actinomycetes. World J Microbiol Biotechnol, 24: 1547–52.

58. Mincer, T.J., Fenical, W., Jensen, P.R. (2005): Culture-dependent and culture-independent diversity within the obligate marine actinomycete genus Salinispora. Appl Environ Microbiol, 71: 7019–28.

59. Velho-Pereira, S., Kamat, N. (2010): Digital image analysis of actinomycetes colonies as a potential aid for rapid taxonomic identification. Nat Precedings, http://dx.doi.org/10.1038/npre.2010.4209.1

60. Lee, J.A., Uhlik, M.T., Moxham, C.M., Tomand, D., Sall, D.J. (2012): Modern phenotypic drug discovery is a viable, neoclassic pharma strategy. J Med Chem, 55: 4527–4538.

61. Weber, T., Kim, H. (2016): The secondary metabolite bioinformatics portal: Computational tools to facilitate synthetic biology of secondary metabolite production. Sys. Synth. Biotech, 1: 69-79.

62. Wohlleben, W., Mast, Y., Stegmann, E., Ziemert, N. (2016): Antibiotic drug discovery. Microb. Biotech. 9: 541-548.

63. Ziemert, N., Alanjary, M., Weber, T. (2016): The evolution of genome mining in microbes - a review. Nat Prod Rep, doi:10.1039/C6NP00025H.

64. Galm, U., Shen, B. (2006): Expression of biosynthetic gene clusters in heterologous hosts for natural product

production and combinatorial biosynthesis. Expert Opin Drug Discov, 1: 409–437.

65. Zazopoulos, E., Huang, K., Staffa, A., Liu, W., Bachmann, B.O., Nonaka, K. (2003): A genomics guided approach for discovering and expressing cryptic metabolic pathways. Nat Biotechnol, 21:187–190.

66. Farnet, C.M., Zazopoulos, E. (2005): Improving drug discovery from microorganisms. In: Zhang L, Demain AL (eds) Natural products: drug discovery and therapeutics. Humana Press, New York, pp 95–106.

67. Castro-Falcon, G., Hahn, D., Reimer, D., Hughes, C.C. (2016): Thiol probes to detect electrophilic natural products based on their mechanism of action. Acs Chemical Biology. 11: 2328-2336.

68. Kersten, R.D., Yang, Y.L., Xu, Y., Cimermancic, P., Nam, S.J., Fenical, W. (2011): A mass spectrometry-guided genome mining approach for natural product peptidogenomics. Nat Chem Biol, 7: 794–802.

69. Kersten, R.D., Ziemert, N., Gonzalez, D.J., Duggan, B.M., Nizet, V., Dorrestein, P.C., Moore, B.S. (2013): Glycogenomics as a mass spectrometry-guided genome-mining method for microbial glycosylated molecules. PNAS 110: 4407–E4416.

70. Larsen, T.O., Smedsgaard, J., Nielsen, K.F., Hansen, M.E., Frisvad, J.C. (2005): Phenotypic taxonomy and metabolite profiling in microbial drug discovery. Nat. Prod. Rep., 22: 672-695

71. Bobzin, S.C., Yang, S., Kasten, T.P. (2000): LC–NMR: a new tool to expedite the dereplication and identification of natural products. J Ind Microbiol Biotechnol, 25: 342–345

72. Wolfender, J.L., Waridel, P., Ndjoko, K., Hobby, K.R., Major, H.J., Hostettmann, K. (2000): Evaluation of Q-TOF-MS/MS and multiple stage IT-MSn for the dereplication of flavonoids and related compounds in crude plant extracts. Analusis 28: 895-906.

73. Gu, J.Q., Wang, Y.H., Franzblau, S.G., Montenegro, G., Timmermann, B.N. (2006): Dereplication of pentacyclic triterpenoids in plants by GC-EI/MS. Phytochem Anal, 17: 102–106

74. Konishi, Y., Kiyota, T., Draghici, C., Gao, J.M., Yeboah, F., Acoca, S., Jarussophon, S., Purisima, E. (2007): Molecular formula analysis by an MS/MS/MS technique to expedite dereplication of natural products. Anal Chem, 79: 1187–1197.

75. Lambert, M., Staerk, D., Hansen, S.H., Sairafianpour, M., Jaroszewski, J.W. (2005): Rapid extract dereplication using HPLC-SPENMR: analysis of isoflavonoids from Smirnowia iranica.

76. Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G.F., Chater, K.F. (2007): Genomics of Actinobacteria: Tracing the evolutionary history of an ancient phylum. Microbiol Mol Biol Rev, 71: 495-548.

77. Ridder, L., Van der Hooft, J.J., Verhoeven, S., De Vos, R.C., Vervoort, J., Bino, R.J. (2014): In silico prediction and automatic LC-MS annotation of green tea metabolites in urine. Anal Chem, 86: 4767–4774.

78. Allard, P.M., Péresse, T., Bisson, J., Gindro, K., Marcourt, L., Pham, V.C. (2016): Integration of molecular networking and in-silico MS/MS fragmentation for natural products dereplication. Anal Chem, 88: 3317–3323.

79. Watrous, J., Roach, P., Alexandrov, T., Heath, B.S., Yang, J.Y., Kersten, R.D. (2012): Mass spectral molecular networking of living microbial colonies. PNAS, 109: 1743–E1752.

80. Bandeira, N. (2011): Protein identification by spectral networks analysis. Methods Mol Biol, 694: 151–168

81. Liu, W.T., Lamsa, A., Wong, W.R., Boudreau, P.D., Kersten, R., Peng, Y. (2013): MS/MS-based networking and peptidogenomics guided genome mining revealed the stenothricin gene cluster in Streptomyces roseosporus. J Antibiot, 67: 99–104.

82. Fang, J., Dorrestein P.C. (2014): Emerging mass spectrometry techniques for the direct analysis of microbial colonies. Curr Opin Microbiol, 19: 120–129.

83. Jones, D. (2010): The antibacterial lead discovery challenge. Nat Rev Drug Discov, 9: 751–2.

84. Rath, C.M., Janto, B., Earl, J., Ahmed, A., Hu, F.Z., Hiller, L. (2011): Metageomic characterization of the marine invertebrate microbial consortium that produces the chemotherapeutic natural product ET-743. ACS Chem Biol, 6:1244–56.