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A NOVEL STRAIN OF *PANTOEA EUCRINA* ENDOPHYTE OF *MURRAYA KOENIGII* WITH SQUALENE CYCLASE ACTIVITY

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Abstract

Endophytic microorganisms are potential source of bioactive natural products and contain various enzymes involved in the production of secondary metabolites like cyclic triterpenes. In the present study, medicinal plants producing terpenes were analysed for the presence of the enzyme squalene cyclase. Crude leaf extract of six different medicinal plants were subjected to squalene cyclase assay and the enzyme product formed was subjected to TLC and FTIR analysis. The FTIR representation at 1643_{cm}^{-1} and 2949_{cm}^{-1} characteristic of the cyclic product, cycloartenol was observed in the TLC scrapped out spot of oxidosqualene treated hexane extract of Murraya koenigii. Further studies on the possible role of endophytes in the induction of squalene cyclase in the selected plant Murraya koenigii resulted in the selection of MKE15 from the four endophytic isolates MKE1, MKE5, MKE15 and MKE18. The PCR based screening for the squalene hopene cyclase (shc) gene was also done to select the potential endophytic strain. The selected endophyte was identified as

Pantoea eucrina and is being reported as an endophyte of Murraya koenigii for the first time. The conversion of squalene to the pentacyclic product hopene by the isolate was confirmed by TLC, HPLC, FTIR and GCMS analysis.

Keywords

Murraya koenigii, Endophyte, Pantoea eucrina, Squalene hopene cyclase, Oxido squalene cyclase, Hopenes, Polymerase chain reaction, HPLC, FT/IR, GC/MS

1. Introduction

Hopenes and related hopanoids are penta cyclic triterpenoids occurring in wide range of Gram-positive and Gram-negative bacteria. They integrate in the cytoplasmic membrane as integral membrane proteins. The key enzyme in the hopene biosynthesis is the squalene hopene cyclase (SHC). This enzyme catalyses the cyclisation of the linear triterpenoid squalene to the cyclic products hopene or hopanol. The enzyme can be a promising tool in synthetic chemistry in biotransformations and can be exploited for the synthesis of new drug derivatives. Eukaryotes have sterols instead of hopenes and the eukaryotic counterparts of SHC are oxidosqualene cyclases(OSC) which converts 2, 3-oxidosqualene to cycloartenol in plants and lanosterol in animals and fungi(Siedenburg and Jendrossek 2011).The chemistry and enzymology of the complex ring forming reaction catalysed by cyclases has attracted synthetic chemists. These enzymes are derived from a common ancestor and show common molecular features necessary for cyclisation.

Squalene hopene cyclase is a homodimer with a dimension of 50 by 70 Å (70 kDa per monomer). Each subunit consists of α- helical domains that build up a dumbbell shaped structure. Analysis of the crystal structure confirmed that SHCs are only partially integrated in the membrane and do not span the whole lipid bilayer (monotopic membrane protein). The membrane binding part of the enzyme is a nonpolar region that is encircled by positively charged amino acids enforcing the anchoring of the enzyme to the negatively charged surface of the phospholipid membrane. A protruding part in the center of this region contains a lipophilic channel and directs the substrate to the active-site cavity inside the protein (Wendt et al., 1999).

Diverse microbial flora can colonize internal plant tissues (endophytes), live on the surface (epiphytes) or in the soil surrounding the root system (rhizosphere micro biota) (Barea et al., 2005; Johnston-Monje and Raizada 2011). Endophytes are unique to the chemical environment within the host plants and they may interfere with expression of

certain genes within the host plants. Bacterial endophytes have been isolated from a wide variety of plants but their functional role is known with only limited isolates. Squalene hopene cyclases have been purified and characterised from several bacteria, *Alicyclobacillus acidocaldarius, Zymomonas mobilis, Rhodopseudomonas palustris, Bradyrhizobium japonicum, Methylococcus capsulatus* (Bath) (Ochs et al., 1992; Tappe 1993; Kleemann et al., 1994)and their nucleotide sequence has been determined. Prokaryotic squalene hopene cyclases shows structural resemblance to oxidosqualene cyclases from plants, animals and fungi and exhibit sequence similarity with them (Ourisson et al., 1987; Perzl et al., 1997).Bradyrhizobia are soil colonizers and contain a range of hopanoid derivatives and structurally related compounds (Perzl et al., 1997). The study on Bradyrhizobium shows the endophyte-plant interaction. There are many reports of endophyte induced regulation of secondary metabolites in plants but the mechanism involved has yet to be determined. Two fungal endophytes of *Catharanthus roseus Curvularia sp.* CATDLF5 *and Choanephora infundibulifera* CATDLF6 isolated from the leaves of the plant were found to enhance vindoline production by modulating the expression of key structural and regulatory genes of vindoline biosynthesis without affecting the primary metabolism of the host plant(Pandey et al., 2016). Plant endophyte metabolism can interact on any levels: (a) the endophyte induces host metabolism, (b) the host induces endophyte metabolism, (c) host and endophyte share parts of a specific pathway and contribute partially (d) the host can metabolize products from the endophyte and vice versa (e) the endophyte can metabolize secondary compounds from the host(Ludwig-Müller 2015). Studies on squalene hopene cyclases from different bacterial systems and its structural and functional aspects are being carried out across the globe. Squalene hopene cyclases from endophytes has not been reported yet in *Murraya koenigii.* The present investigation opens a new channel in triterpenoid synthesis of plant-endophyte system.

2. Materials and Methods

2.1 Selection and authentication of plants

Based on the presence of aroma and terpene production, six plants with both medicinal and secondary metabolite production properties were selected for the study. The selected plants include *Leucas aspera* (Lamiaceae), *Murraya koenigii* (Rutaceace), *Ocimum sanctum* (Lamiaceae), *Cymbopogan citratus* (Poaceae), *Chromolaena odorata (*Astereceae), *Catharanthus roseus* (Apocynaceae). Healthy plants were collected and from five different

geographical areas Alappuzha, Ernakulam, Pathanamthitta, Kottayam and Trivandrum identified at Department of Botany, St. Berchmanns College, Changanacherry, based on height, stem, leaf, root, inflorescence, habitat etc. The herbarium sheets of all the plants were prepared and deposited.

2.2 Selection of plants based on oxido squalene cyclase (OSC) activity.

Fresh leaves were collected from all six plants selected for the study, surface sterilized, dried and grinded with 5mM phosphate buffer. The extract filtered through powdered activated charcoal and the filtrates thus obtained were used for the assay of oxido squalene cyclase activity.

For analysis of enzyme activity, 200µL of the leaf extract was added to 200 µL of reaction mixture (containing 5 mM phosphate buffer (pH7.4), 0.5% Triton X 100 and 50 µM 2,3-oxidosqualene).The reaction mixture was incubated at 37° C for 2 hours with shaking. After incubation, equal volume of 15% ethanolic KOH was added to the reaction mixture for termination of the reaction. The mixture was then extracted twice with equal volume of diethyl ether. The organic layers were collected carefully and was evaporated to dryness and re-dissolved in 50 µL of diethyl ether. The extracts thus prepared were analysed using thin layer chromatography plate (Merck-GF254) using a solvent system toluene: acetone: formic acid (7:2:1). The developed plates were then sprayed with anisaldehyde reagent and were incubated at 100° C for 2 min and were visualised at 254nm. The scrap out which showed positive bands in TLC were also subjected to FTIR analysis using Shimadzu IR Prestige 21Spectroscope for the presence of products.

2.3 Isolation of endophytic bacteria

For the isolation of endophytic bacteria, healthy leaves of *Murraya koenigii* were collected from five different districts of Kerala viz. Alappuzha, Kottayam, Pathanamthitta, Ernakulam and Trivandrum. The leaves were then surface sterilised (Aravind et al., 2009) and final wash spread plated onto nutrient agar plate as control. The sterilized leaves were then macerated in sterile phosphate buffer saline (PBS) and serially diluted up to 10^{-4} dilution. The serially diluted samples were spread plated onto nutrient agar plates in triplicate. All plates including control were incubated at 30° C for 5 days and observed periodically for bacterial growth. Morphologically distinct colonies were selected and were purified by streak culture and used for screening studies. The isolates obtained from different geographical areas were designated as MKA (Alappuzha), MKE (Ernakulam), MKP (Pathanamthitta), MKK (Kottayam) and MKT (Thiruvananthapuram).

2.4 Screening of isolates for squalene hopene cyclase (SHC) activity.

For the screening of the isolates for squalene hopene cyclase activity, the obtained isolates were grown in 5 mL nutrient broth overnight at 28°C. After incubation, the supernatant were harvested by centrifugation at 12,000 rpm for 10 min. The supernatant thus collected was used for the screening of enzyme activity along with uninoculated broth as control.

Squalene cyclase activity were screened using the supernatant collected from the isolates using the procedure described by (Ghimire et al., 2009). For this, the reaction mixture was prepared which was having 200 µL of 1M sodium citrate (pH 6.8), 200 µL substrate (100 µM squalene, 1% (v/v)Triton X 100, 100mM sodium citrate pH 6.8), 300 µL collected supernatant of the isolates and 300 µL distilled water. The prepared reaction mixture was then incubated at 48°C for 4 hours. After incubation, the reaction mixture was extracted twice with 2000 µl hexane/2-propanol (3:2v/v) and the organic layer was collected. The collected organic layer was evaporated and was re-constituted in 50 µL hexane/2-propanol (3:2v/v). The crude extract thus obtained was used for the confirmation of the presence of SHC activity.

For the analysis of SHC activity, presence of hopene in the crude extract was checked initially using Thin Layer Chromatography (TLC). The extracts were spotted on to silica gel TLC plate (Merck GF254) and was developed using the solvent system hexane: ether (7:3). The developed plates were visualised under 254 nm in a TLC plate viewer. The scrapped out samples from TLC plate which showed positive results for hopene were subjected to Fourier Transform Infra-Red (FTIR) spectroscopy.

2.5 Identification of selected endophytic bacteria with squalene cyclase activity

For the identification of the isolates morphological, biochemical and molecular characterization were carried out.

2.5.1 Morphological and Biochemical characterisation

The colony morphology were identified based on the edge, surface, elevation and colour of the colony. Four isolates showing positive results for squalene cyclase activity MKE1, MKE5, MKE15 and MKE18 were identified based on biochemical properties and classified according to Bergeys Manual of Bacterial Classification.

2.5.2 Molecular characterisation by 16s rDNA sequencing

For molecular characterization, genomic DNA was isolated from the selected isolates which were confirmed positive for SHC activity. The selected isolates were grown in LB

broth and subjected to DNA isolation using Chromous Biotech Bacterial Genomic DNA Mini Spin Kit (RKT 17). Genomic DNA thus isolated was used as template for PCR amplification using the primer sequences 16SF (5'-AgA gTTTgA TCM Tgg CTC-3') and 16SR (5'-AAg gAggTg WTCCAR CC-3') (Jasim et al., 2016). PCR was carried out for 35 cycles in a Mycycler TM (Bio-Rad, USA)

Table-1: *PCR conditions for 16SrDNA amplification of the endophytic isolates*

Steps	Temperature	Time
Initial Denaturation	94°C	3 min
Denaturation	94°C	30 sec
Annealing	58°C	30 sec
Extension	72°C	2 min
Cycle repeated 34 times		
Elongation	72°C	7 min

The PCR products were subjected to agarose gel electrophoresis, purified and sequenced. The sequence data was checked by BLAST analysis (Zhang et al., 2000). Phylogenetic study of the 16SrDNA sequences of the isolates were performed with MEGA 5 using neighbor-joining method with 1,000 bootstrap replicates (Tamura et al., 2011).

2.6 PCR based screening for squalene hopene cyclase gene.

From the four isolates genomic DNA was extracted and used as template for the amplification of genes specific to **squalene hopene cyclase by PCR primers PA 1(5'-GAT GGT TCY TGG TAY GGT′-3′)** and **PA2 (5′ -CCC CAR CCR CCR TCY TCG TTC TG-3′).** PCR was carried out for 35 cycles in a Mycycler TM(Bio-Rad USA) with 50 µL reaction volume containing 50 ng of genomic DNA, 20 pico moles of each primer, 1.25 units of Taq DNA polymerase (Bangalore Genei), 200 µMol each dNTPs and 1× PCR buffer.

Table 2: *PCR conditions for amplification of squalene hopene cyclase gene*

Steps	Temperature	Time
Initial Denaturation	94°C	3 min
Denaturation	94°C	30 sec
Annealing	49°C	30 sec
Extension	72°C	2 min

Cycle repeated 34 times		
Elongation	72°C	7 min

The specific size of the product was confirmed by conducting agarose gel electrophoresis with standard molecular weight markers. The amplified product was further purified from the gel and subjected to sequencing using Big Dye Terminator Sequence Reaction Ready Mix (Applied Biosystem).

2.7 Confirmation of SHC activity in the selected isolate by HPLC and GCMS analysis

The level of utilisation of squalene (substrate) in the crude extracts was also analysed using High Performance Liquid Chromatography (HPLC) based method. For this, the crude extracts obtained were filtered through a 0.22 µm syringe filter and was analyzed using Shimadzu HPLC system (model LC 20 AP) using a C18 column (G 250 mm×4.6mm dimension; Particle size - 5µm; Pore size: 120Å). The elution was performed using 100% acetonitrile with a flow rate of 1 mL min^{-1}. The detection of the elution was performed using a UV detector at a wavelength of 195 nm. The reaction mixture containing squalene was kept as control.For the confirmation of the SHC activity of the isolates, the crude extracts of isolates found positive in the HPLC analysis were further subjected to GC-MS analysis (Shimadsu-QP2010S, Rxi-5Sil MS column) using helium gas and the oven temperature set from 250 to 320°C with temperature gradient of 4°C per minute increase followed by 20min at 320°C.

3. Result and Discussion

Oxido squalene cyclases (OSC) are enzymes which converts the linear substrate squalene to a pentacyclic product cycloartenol in plants. Based on the cyclisation events the cyclisation products are different in plants, either chair-chair conformation or chair-boat conformation. The products can be lupeol, amyrins, ursolic acid etc.(Haralampidis et al., 2001).

From the TLC analysis of hexane extracts of all six oxido squalene treated plant leaf extracts only three extracts showed visible bands after derivatization with anisaldehyde at 254nm.The different Rf values obtained were 0.56, 0.34 and 0.74 corresponding to different products obtained on treating oxidosqualene with the extracts of *Murraya koenigii* ,*Ocimum sanctum* and *Leucas aspera*, respectively. The Rf value 0.56 corresponds to the expected compound cycloartenol**.**

FTIR spectral representations at 1643cm^{-1}and 2949.6cm^{-1} corresponds to C-H bending or C-O stretching in unsaturated alicyclic 5 or 6 member ring. The presence of 5 or 6 membered unsaturated alicyclic representation with –C-O is characteristic of cycloartenol.

(Fig. 1) Based on the TLC and FTIR data analysis from the six plants selected for the study *Murraya koenigii*, was selected for the isolation of potential endophytic strains exhibiting squalene cyclase activity.

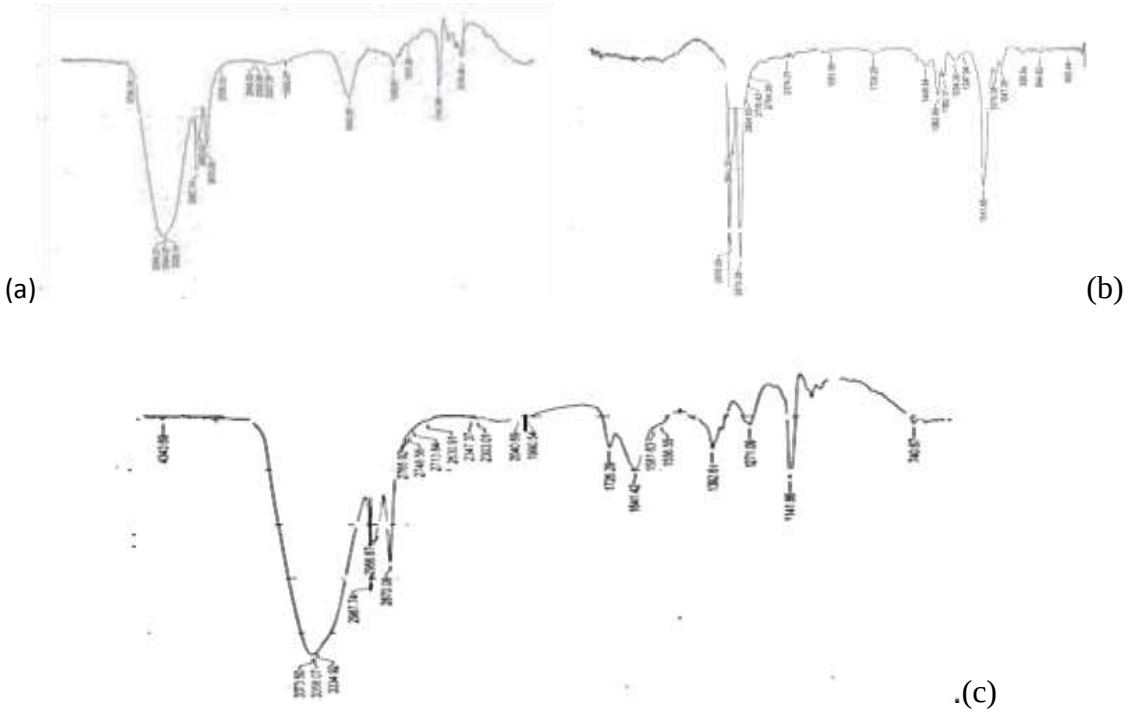

Figure 1: *FTIR spectrum of the hexane extracts of scrapped out spots from TLC positive for oxidosqualene cyclase activity :(a) Murraya koenigii (b) Ocimum sanctum (c) Leucas aspera*

Fresh and healthy leaf samples from five different districts in Kerala were used for isolation of endophytic bacteria from *Murraya koenigii*. The leaves were surface sterilized to remove the epiphytic microorganisms .There was no growth in control which indicated that the surface sterilisation was satisfactory. Required number of colonies were obtained from the nutrient agar plates inoculated with the leaves macerated with phosphate buffer saline.52 isolates were obtained from the plants collected from different regions. Based on the region of collection and the colony morphology the isolates were named as MKA, MKE, MKK, MKP, and MKT.

From the isolates obtained crude extracts of the isolates MKE1, MKE5, MKE15 and MKE18 gave positive results for squalene cyclase assay on TLC with hexane : ether(7:3) at

254nm.From the FTIR analysis representations of the four samples the representation at $1016_{cm}^{-1}$by the isolate MKE15 corresponds to a cyclic compound**(Fig. 2).**

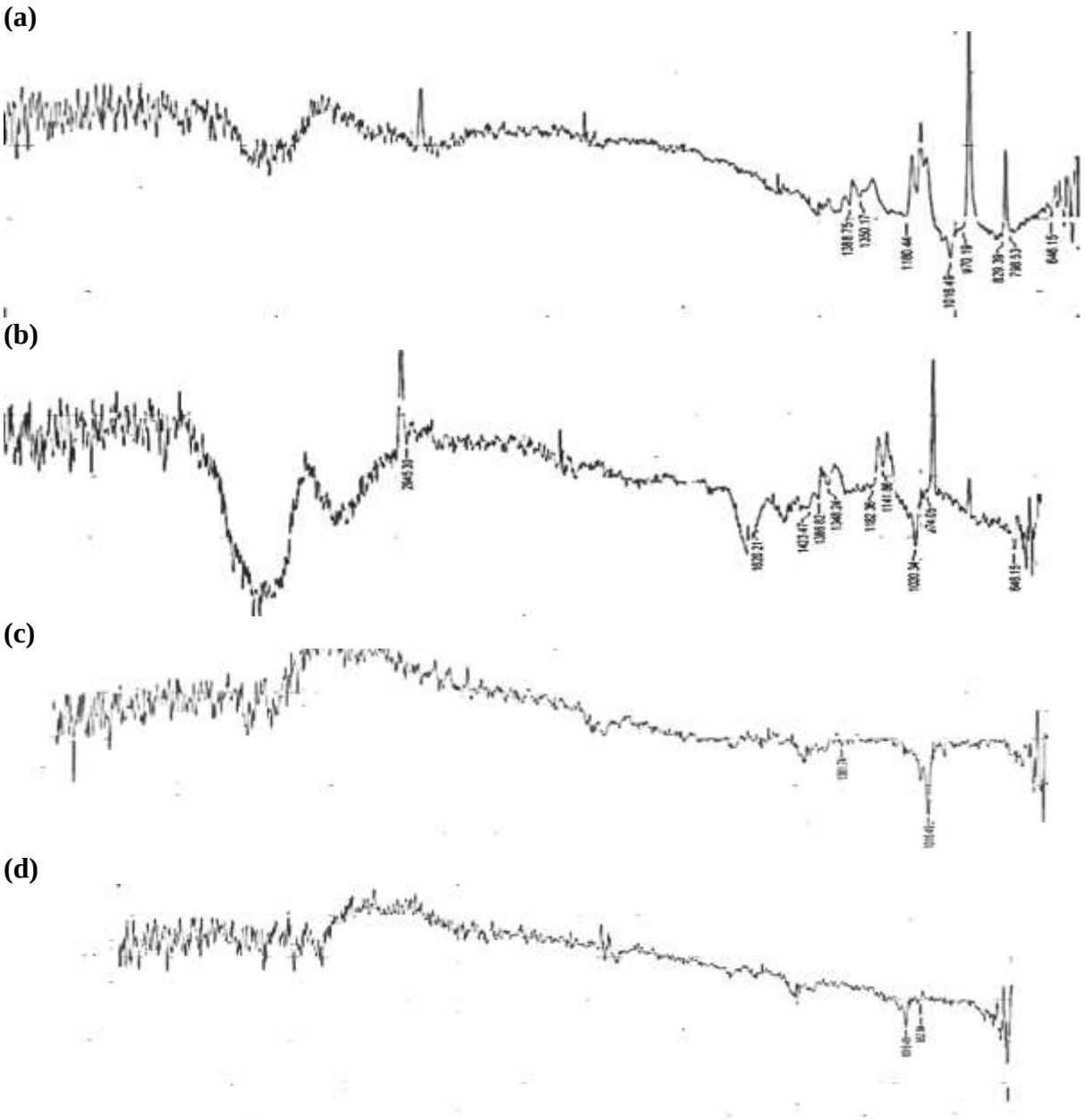

Figure 2: *FTIR spectrum of the scrapped out spots of TLC obtained for squalene treated with hexane extracts of the endophytic isolates of Murraya koenigii (a)MKE1 (b)MKE5 (c)MKE15 (d)MKE18.*

The 16S rDNA sequencing confirmed the molecular characterization of the endophytic isolates. The PCR amplified products of the 16S rDNA were confirmed by agarose gel electrophoresis which was then gel eluted and sequenced (**Fig. 3**). The BLAST analysis of the 16S rDNA sequence data of the selected endophytic isolates showed alignments with reported sequences in Gen Bank. The organisms can be grouped at subspecies level for the rapid identification of bacterial species. The highest sequence

similarities found with different bacterial genera and Gen Bank Accession numbers for the selected strains are summarized in (**Table: 3**).

Table 3: *Gen Bank accession numbers with alignment of sequences obtained with reported 16S rDNA gene sequences in Gen Bank and highest similarity with different species.*

Isolates	Similarity with		Gen Bank Accession Number
MKE1	*Pantoea agglomerans*	99%	**MF099410**
MKE5	*Pseudomonas mendocina*	99%	**MF099411**
MKE15	*Pantoea eucrina*	99%	**MF099412**
MKE18	*Enterobactersp:*	98%	**MF099413**

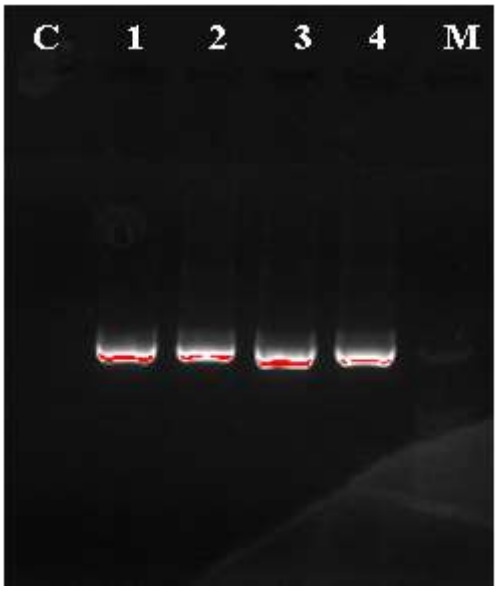

Fig 3: *PCR amplification 16S rDNA of the endophytic bacterial isolates from Murraya koenigii with squalene hopene cyclase (SHC) activity. (Lane C – Control; Lane 1 – MKE 1; Lane 2 – MKE 5; Lane 2 – MKE 15; Lane 2 – MKE 18 Lane M – Low range ruler (Merck Bioscience)*

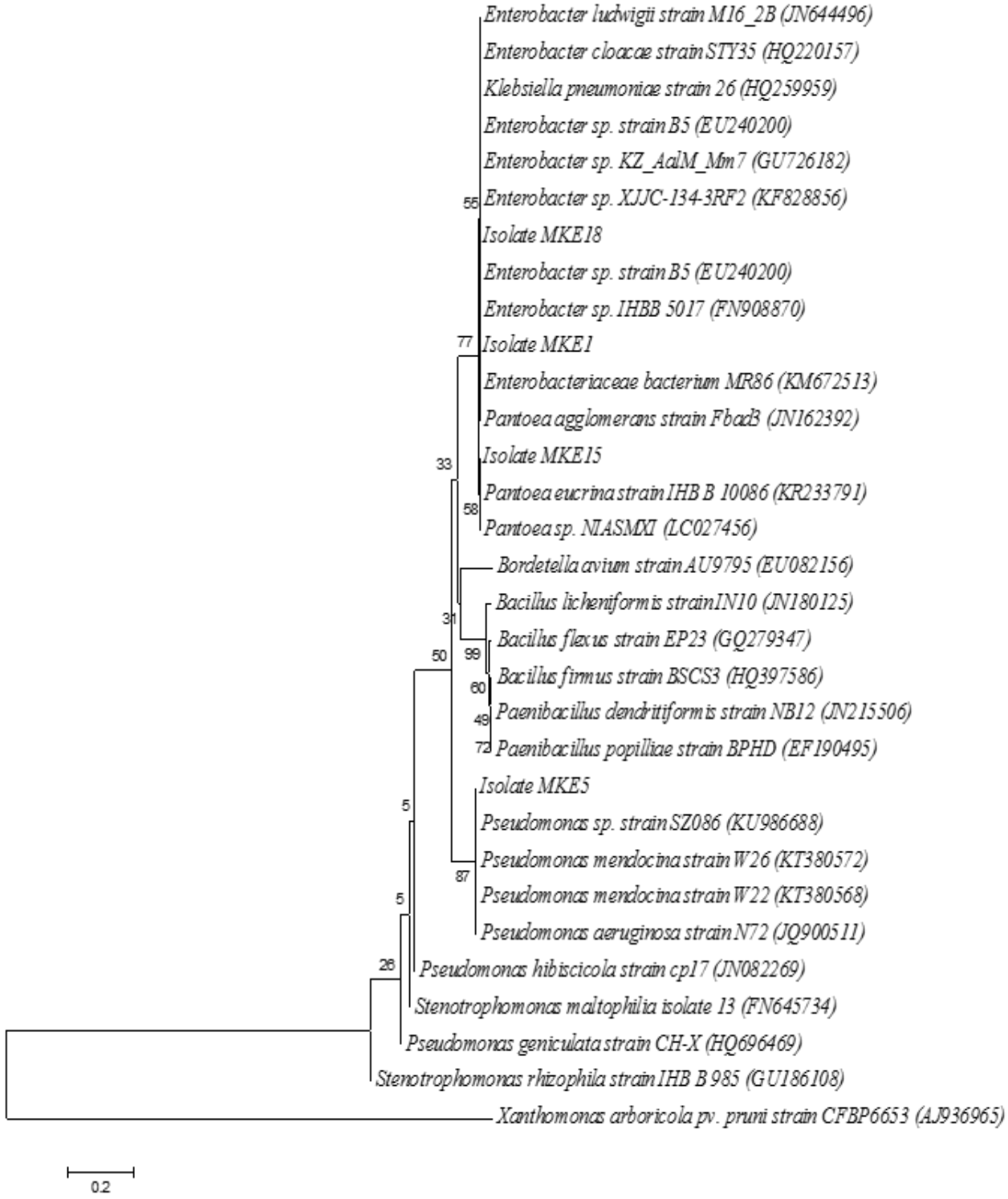

Figure 4: *Phylogenetic analysis of the endophytic bacterial isolates from Murraya koenigii with squalene hopene cyclase (SHC) activity*

The squalene utilization by the crude extracts of the four isolates after squalene cyclase assay was determined by HPLC analysis at 195nm. From the HPLC data, MKE5 and MKE15 showed maximum utilization of squalene. The presence of unique peak at retention

time of 8.5minutes and the reduced peak corresponding to squalene in the sample treated showed utilization of squalene **(Fig. 5).** The extracts of both the isolates which showed positive results for HPLC were subjected to GC/MS analysis to confirm the product formed. The extract of the isolate MKE15 showed the representation of the fragmentation pattern of the pentacyclic product hopene (retention time 19.492 min, m/*z* =410, base peak *m/z*=191.0) in the mass spectra**(Fig. 6 and 7).** This confirms the presence of the enzyme squalene hopene cyclase in the crude extract of the isolate MKE15.

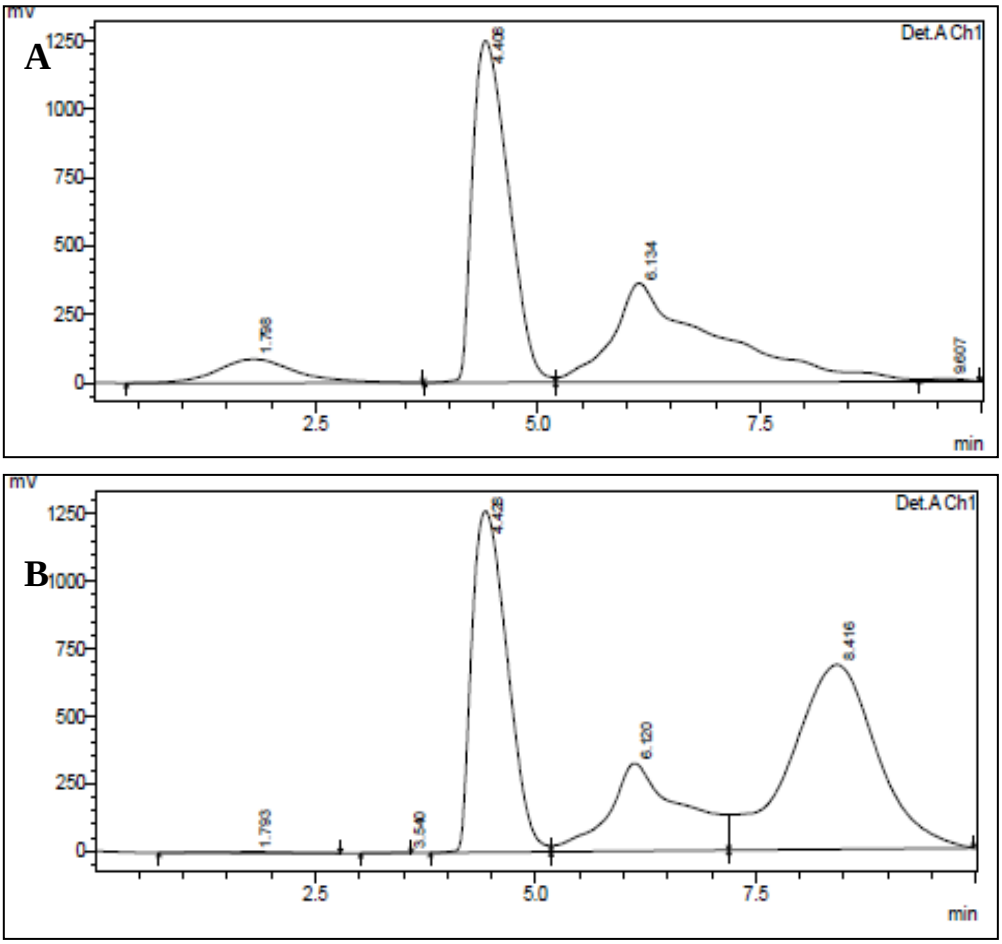

Figure 5: *HPLC profile of the isolate Pantoea eucrina (MKE15) showing squalene utilization at 195nm.*

(A) Chromatogram showing the peak of the substrate squalene in the control sample.

(B) Chromatogram showing the formation of a unique peak at retention time 8.5min after squalene utilization.

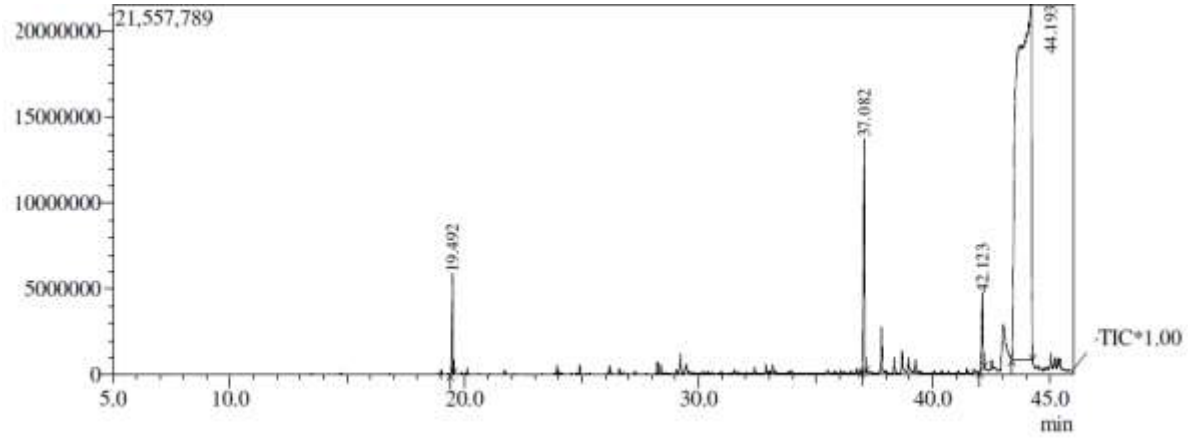

Figure 6: *Gas chromatogram of squalene treated hexane extracts of the endophytic isolate MK15 of Murraya koenigii*

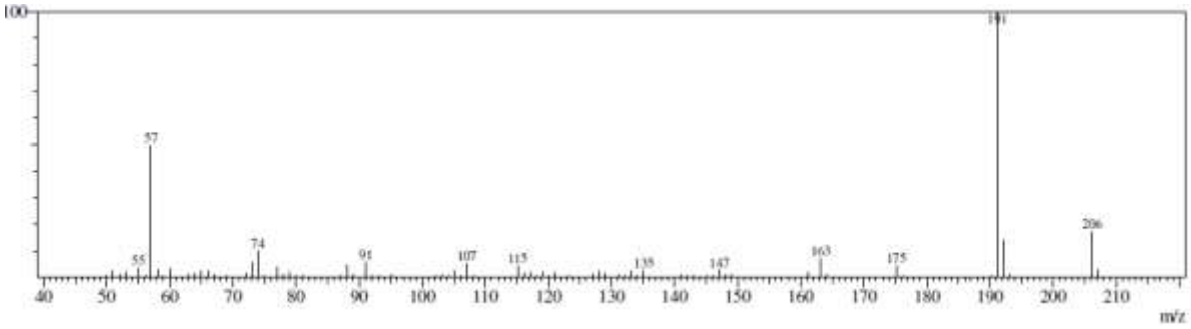

Figure 7: *Mass spectrum showing the representation of hopene (m/z 191) at retention time 19.492*

The genus *Pantoea* was first described by Gavini et al. with *Pantoea agglomerans* as the type species. The species of the genus *Pantoea* are Gram negative, facultatively anaerobic non spore forming rods and motile by means of peritrichous flagella. *Pantoea* has been isolated from various habitats like soil, water, food, plants, humans, animals etc. Only few species of *Pantoea* interact with plants and reside within plants as endophytes. *Pantoea* sp. has been reported as an endophyte from different plants capable of secondary metabolite production. *Pantoea alhagi*a novel endophytic strain with the ability to improve growth with multiple plant growth promoting factors and drought tolerance capacity in wheat has been isolated from the legume *Alhagi sparsifolia* Shap (Chen et al., 2017). Another strain *Pantoea stewartii* has been reported in *Scoparia dulcis* which exhibit squalene cyclase activity.

From the PCR based screening of the four isolates, MKE15 (*Pantoea eucrina)* showed amplification of the specific gene *shc* (squalene hopene cyclase) at 200 base pairs(bp) at 49°C thus confirming the presence of the gene in the endophyte **(Fig. 8)**. The

marker DNA is run in the first lane and the sample DNA in the second lane. The amplified gene can be cloned and expressed in a suitable vector. The endophytic control on the expression of the shc gene in the host plant *Murraya koenigii* need to be explored in detail. *Pantoea eucrina* is the first reported endophytic strain from the plant exhibiting squalene cyclase activity.

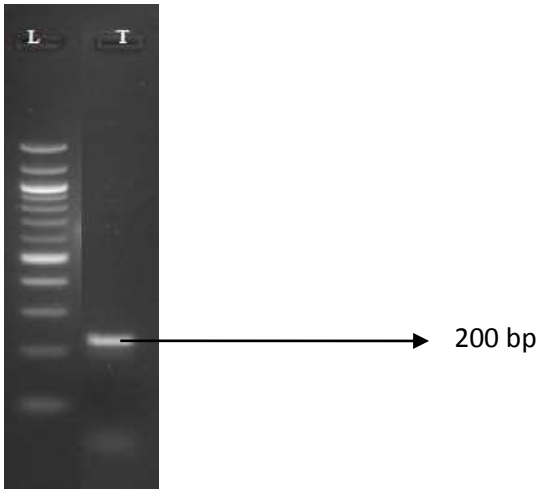

Figure 8: *PCR amplification of the gene coding squalene hopene cyclase (shc) in MK15. (Lane:1- Marker DNA and Lane-2 MKE15 DNA)*

Even though the studies on bacterial SHCs came into focus of the scientific community more than 40 years ago, the functional role of these enzymes in the endophytes and their effect on the synthesis or cyclisation of the substrate in the host plant as yet to be discovered. Research has to be focussed on the role of endophyte in the host plant, in producing the enzyme – whether it is by the induction of certain genes in the metabolic pathway or by mimicking the secondary metabolite produced by the host plant (Kumara et al., 2013).Squalene hopene cyclases can polycyclize substances in a one step reaction and hence they are interesting tools as biocatalysts in biotechnological processes for the synthesis of new pharmaceutical products. Moreover SHCs have broad substrate range which can be utilised for producing novel, unnatural cyclic products. Triterpene acids like betulinic acid, oleanolic acid and ursolic acid are active components in plant extracts of apple pomace, birch cork, clove flower, olive flower etc. and form an ideal starting material for pharmaceutical development (Jager et al., 2009). The diversity in the product formed from the substrate squalene in prokaryotes and oxidosqualene in eukaryotes by the cyclase enzyme makes this enzyme a promising tool in triterpenoid synthesis.

The limitation of purification of squalene cyclase in its host organism is its intracellular location and low concentration in cells. Detailed investigation into the structural aspects of

the enzyme is possible only when the enzyme is available in the crystallised form. Future research in the endophyte *Pantoea eucrina*, can be at the molecular level by expressing the squalene hopene cyclase coding gene in a suitable bacterial vector and its expression in a bacterial system. This will help in the large scale production of the enzyme for crystallising the protein and also for exploring the mechanism of enzymatic action.

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5. Conflict of interest

The authors declare no conflict of interest.

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