PCR-based Screening Approach: A Rapid Method to Detect the Biosynthetic Potential of Antimicrobials in Actinobacterial Strains

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Abstract

This study aimed to investigate the PCR-based screening strategy for the prediction of the antimicrobial biosynthetic potential of the selected *Streptomyces* strains originated from an extreme environment (Cholistan Desert, Pakistan). The biosynthetic potential was determined by using both molecular and culture-dependent screening approaches. The four biosynthetic genes clusters, including the *pks-1*, *nrps*, cyp P450 hydroxylase (*cyps*), and glycopeptide *oxy b* genes, were investigated in the selected strains by PCR amplification, sequencing, and by subsequent bioinformatics approaches. Among the 40 selected *Streptomyces* strains, 33 strains possessed the *nrps* gene, 17 strains carried the *pks-1* gene, four strains were found to have the *cyps* gene, and none of the strain carried *oxy b* gene. The *Streptomyces* strains including NR-1, NR-10, NR-14, and NR-15 were investigated for *in vitro* antifungal activity against *Fusarium oxysporum*, *Rhizoctonia solani*, and *Aspergillus* sp. The extracts were analyzed for chemical profiling (TLC and HPLC-UV), and a unique pattern of secondary metabolites was observed. The selected strains exhibited pronounced antifungal activity against the fungal test strains with the zone of inhibition up to 17, 18, and 19 mm, respectively. The study depicts that gene-based screening can be successfully applied to identify potentially bioactive strains by usin a single screening process. This PCR-based approach is rapid and can be used for sorting out and selecting the potential candidate among actinobacterial culture collections. Such a preselection or strain prioritization consequently decreases the time and efforts required for selecting the potential bioactive strain, which then can be subjected to the detailed chemical analysis.

K e y w o r d s: gene-based screening, polyene specific cytochrome P450 hydroxylase (CYP), *nrps*, *pks-1*, *Streptomyces*

Introduction

The gene-based screening allows the rapid detection of biosynthetic gene clusters in the isolated strains (Wood et al. 2007). In the latest years, genome mining has been focused on *Streptomyces* and has become a novel and rapid method to identify the previously unidentified gene clusters (Xu et al. 2019). Genes that are involved in the biosynthesis of secondary metabolites are mainly organized in the secondary metabolism biosynthetic gene clusters. With the progress of genomic sequencing technology, the mining of the organism's secondary metabolism biosynthetic gene clusters becomes possible (Bu et al. 2019; Xu et al. 2019). *Streptomyces* harbor over 20 secondary gene clusters encoding the biosynthesis of many cryptic metabolites that are not expressed under standard laboratory conditions. The genome of *Streptomyces* is genetically engineered to remove the non-essential genes and permit heterologous expression of genes encoding cryptic metabolites (Komatsu et al. 2010; Wu et al. 2017; Bu et al. 2019; Xu et al. 2019). In most of the cases, these gene clusters are silent or ordinarily expressed under the specified laboratory conditions (Ye et al. 2017). One of the essential features of the genome in the genus *Streptomyces* is the occurrence of biosynthetic gene cassettes (Hwang et al. 2014). The *Streptomyces coelicolor* and *Streptomyces avermitilis* contain more than 20 gene clusters for the production of secondary metabolites and innovative antibiotics (Busti et al. 2006). In the genome of

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Abbreviations

CYP – cytochrome P450 hydroxylase

NRPS – non-ribosomal peptide synthase
PKS-1 – polyketide synthase

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S. avermitilis there are 25 types of gene clusters for secondary metabolites. From the 25 genes clusters, eight are for type I polyketide, two for type II related polyketide, and eight gene clusters are involved in the biosynthesis of non-ribosomal peptide synthetases (NRPS) compounds (Omura et al. 2001).

The conventional method of natural drug discovery is based on the bioactivity-guided purification of compounds, which is laborious and led to re-discovery of compounds most often. However, most of the biosynthetic potential of microorganisms is not detected under laboratory conditions (Winter et al. 2011).

The biosynthetic gene clusters for polyenes showed the existence of cytochrome P450 hydroxylase. The cytochrome P450 hydroxylase (*cyps*) genes performed different types of oxidation processes in different organisms (Lamb et al. 2003). The polyene-specific cytochrome P450 hydroxylase (*cyps*) has been found in all the earlier categorized polyene gene clusters, such as for nystatin, amphotericin, pimaricin, and candicidin antibiotics (Lee et al. 2006). Glycopeptides are a significant class of antibiotics that inhibit bacterial cell wall synthesis (Sosio et al. 2003). Glycopeptide antibiotics biosynthesis gene cluster of balhimycin encodes the cytochrome P450 monooxygenases such as Oxya, Oxyb, and Oxyc that are responsible for three oxidation steps and convert the linear peptide into cyclized form to make them chemically active. Thus, these three oxygenases act in a stepwise manner in the order Oxyb, then Oxya, and Oxyc for the formation of glycopeptide antibiotics (Bischoff et al. 2001).

The genomic studies of actinomycetes indicated that non-ribosomal peptide synthetases and type I polyketide synthases (PKS-1) contribute about half of the biosynthetic systems that encode the genes for the biosynthesis of the secondary metabolites (Komaki et al. 2016). The PKS type I catalyzes the synthesis of macrolide antibiotics including erythromycin and tylosin (Le et al. 2014). The *pks-1* gene codes for at least three domains equivalent to a ketosynthase (KS), acyltransferase (AT), and an acyl carrier protein (ACP) that enable the condensation of different subunits. All the PKS I domains collaborate to form a new polyketide chain (Ayuso-Sacido and Genilloud 2005). The nonribosomal peptide includes clinically essential antibiotics, such as cyclosporins, bleomycin, vancomycin, and penicillins. A representative NRPS unit consists of three essential domains, such as an adenylation (A) domain, a peptidyl carrier protein (PCP), and a condensation (C) domain. New domains are continually evolving, as novel gene clusters for peptide biosynthesis are being categorized (Du et al. 2000).

The PCR-based screening approach sets the stage for the discovery of novel metabolites. This method helped to meet the medical severe demand for new drug candidates and enhance the acceptance of natural metabolic products as suitable drug candidates.

In this study, a PCR-based genome screening method was used for 40 independently isolated *Streptomyces* strains, and the detection of CYP specific polyene (cytochrome P450 hydroxylase), the glycopeptide *oxy b* gene (cytochrome P450 monooxygenase), type I polyketide synthase (PKS-1), and the non-ribosomal peptide synthase (NRPS) gene, based on the presence of the expected size of the PCR amplified DNA fragments, was performed. These results suggest that the PCR-based genome screening method is an efficient method for the detection of potentially valuable *Streptomyces*. The bioinformatics studies were also applied to confirm the presence of glycopeptide Oxyb, NRPS, and PKS-1 proteins, which play an important role in the antibiotics biosynthesis pathways. The functional analysis of the sequenced strains was performed by using different bioinformatics tools including BLASTn, BLASTp, EMBOSS TRANSq, and MEGA 6.0. The biological and chemical analyses were performed to confirm that the selected *Streptomyces* strains can produce the antifungal compounds (*cyps* genes) under the culture condition.

Experimental

Materials and Methods

***Streptomyces* strains and genomic DNA extraction.** A total of 40 *Streptomyces* strains were obtained from the collection of the Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan. The selected strains were previously isolated from Cholistan desert of Pakistan. The GYM-broth (glucose 10 g, yeast extract 5 g, malt extract 5 g, distilled water 1,000 ml) was prepared, and 40 ml broth was taken in a 100 ml flask and was inoculated with the fresh culture of *Streptomyces* strains in each case. The flasks were incubated at 28°C on a rotatory shaker for about 7 days. The culture broth was taken in the Eppendorf tube and centrifuged at 10,000 rpm for 2 minutes to get the cell pellet or mycelial mass. The cell pellet was further utilized for DNA extraction by using the tissue Genomic DNA Extraction Mini Kit (FavorPrep™).

PCR amplification of antibiotics biosynthesis genes. The PCR was performed (Primus 96 (PeqLab) thermal cycler). All amplifications contained a total volume of 50 µl with $0.5 \times$ Master Mix (25 µl) (Thermo Scientific), 10 pmol of each primer (3 µl) (1 st BASE laboratories), 100 ng of DNA template (3 µl) and 19 µl of deionized water. The gradient PCR was performed to identify the optimum annealing temperatures for each pair of primers. The PCR based screening of the non-ribosomal peptide synthase, polyketide synthase

Table I
PCR primers for the *nrps*, *pks-1*, *cyps*, and *oxy b* genes.

Genes	Primers	Sequence (5'-3')	Length	Tm	Product size	References
cyps	CYP-F	TGGATCGGCGACGACCGSVYCGT	23 bp	63.8	350 bp	Ayuso-Sacido and Genilloud 2005
	CYP-R	CCGWASAGSAYSCCGTCGTACTT	23 bp	56.6		
oxy b	GLY-F	CTGGTCGGCAACCTGATGGAC	21 bp	61.7	560 bp	Ayuso-Sacido and Genilloud 2005
	GLY-R	CAGGTACCGGATCAGCTCGTC	21 bp	61.7		
pks1	K1F	TSAAGTCSAACATCGGBCA	19 bp	48.4	1200–1500 bp	Ayuso-Sacido and Genilloud 2005
	M6R	CGCAGGTTSCSGTACCAGTA	20 bp	55.4		
nrps	A3F	GCSTACSYSATSTACACSTCSGG	23 bp	53.1	700 bp	Wood et al. 2007
	A7R	SASGTCVCCSGTSCGGTAS	19 bp	50.6		

1 (*pks-1*), polyene specific *cyp* P450 hydroxylase, and glycopeptide *oxy b* genes was accomplished using the primers given in Table I.

Sequencing of the amplified PCR product. The PCR products were purified by using MicroElute gel extraction kit (Favorgen) and sequenced by dye terminator chemistry using an automated sequencer, using the commercial facility of 1st BASE laboratories. The sequences were analyzed using the BLASTn search program at The National Center for Biotechnology Information (NCBI): http://www.ncbi.nlm.nih.gov/BLAST/. The BLASTn was performed to estimate the percentage homology with the reported gene clusters, and the sequences were submitted to NCBI, GenBank, to get the accession numbers.

Sequence analysis by bioinformatics tools. The sequences were analyzed by using various bioinformatics tools, such as the nucleotide sequence was translated by using EMBOSS transq (https://www.ebi.ac.uk/Tools/st/emboss_transeq/) into their resulted peptides sequence. The BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) of all the resultant translated frames was performed, to find the similarity index of the peptides based on the percentage similarity, the functional protein with the highest similarity, was selected from all the 6 reading frame. The translated sequence of *Streptomyces* proteins were selected for multiple sequence alignment by using the Clustal_W alignment tool built-in MEGA 6 (https://www.megasoftware.net/). The partial 16S rRNA sequences of the selected actinobacterial strains were compared using the BLAST tool available on NCBI. The sequences of closely related species were obtained from NCBI and aligned using the CLUSTAL_W program. The neighbor-joining phylogenetic tree was inferred using a Kimura's 2-parameters in software MEGA 6.0. Tree topologies were evaluated for branch support using 1,000 replications Fig. 1.

Preparation of cell extracts for biological and chemical screening. The cell extracts of actinomycetes were prepared by inoculating the actinomycetes cultures, in the 200 ml GYM-broth, the inoculated flasks were incubated for 6–7 days at 28°C on the rotatory shaker. The cultures were sonicated in the sonicating
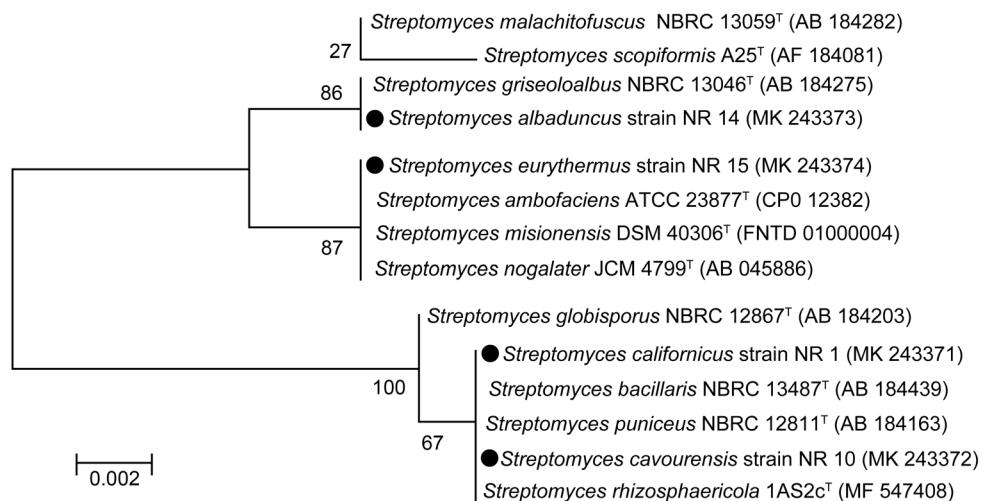

Fig. 1. Neighbor-joining tree based on 16S rRNA gene sequences of closely related type strains.
Evolutionary distance was calculated using Kimura 2-parameters with 1000 bootstrap value.

bath to break the cells, an equal volume of ethyl acetate was added, and the mixture was taken in a separating funnel and was vigorously shaken for 5–10 minutes. The separating funnel was kept un-disturbed; after some time, two distinctive layers appeared. The aqueous layer was separated from the organic layer carefully. The ethyl acetate was recycled on a rotary evaporator, and the extracts were obtained in methanol, and stored in clean vials at 4°C. These methanolic extracts were further used for *in vitro* antifungal activity and for chemical profiling, using TLC and HPLC/UV (Fatima et al. 2019).

Determination of the antifungal activity. The PDA (potato dextrose agar) plates were prepared, and the inverted side of the plate was marked from edge to about 2 cm from both sides. After marking, agar plugs were cut from well-grown cultures of selected actino-mycetes, and were placed on PDA plates about 2 cm from the edge, and the plates were incubated for about 2 days. After 2 days of incubation, similarly, agar plugs were cut from the fungal cultures, and were placed on the opposite side of the same plate that contained the actinomycetes agar plug. The plates were incubated for further 4 to 5 days at 27°C. After that the incubation zone of inhibition was measured.

In another method, the fungal test strains, including *F. oxysporum* (FO), *R. solani* (RS), and *Aspergillus* sp. (FN2) were streaked on SDA (Sabourad dextrose agar). The fresh cultures of fungal strains were swabbed with the help of a cotton swab on SDA plates, and the wells were made with the help of a sterile cork borer in the plates. Further, 60 µl of methanolic extracts were loaded on each well, and the plates were incubated at 28°C for 4–5 days. After incubation, the inhibition zones were measured in mm (Silambarasan et al. 2012).

Thin-layer chromatography (TLC). The metha-nolic extracts were spotted on the TLC plate with the help of a capillary tube. The spots were air-dried, before developing the plate with CH_2Cl_2/MeOH (10%) solvent system. The developed air-dried TLC plates were visual-ized under UV at 254 nm and 366 nm. The TLC plates were stained, by spraying with anisaldehyde/H_2SO_4, and Ehrlich's reagents, (Merck) individually.

High-performance liquid chromatography (HPLC-UV) analysis. The methanolic extracts of actinomycetes were analyzed, on the HPLC (Sykum HPLC system) by using the software clarity. The column used was Rp C18 with a 30 cm length. The Mobile phase was methanol and water (95:5), and the flow rate was adjusted to 1 ml/minute. The methanolic extracts were dissolved in HPLC grade methanol, and 20 µl of each extract were injected and were run for 20 minutes, the UV absorbance was measured at 254 nm. The peaks of each component were measured and were compared at different retention times (t_R) with standard UV absorp-tion data of secondary metabolites.

Results

About 40 selected *Streptomyces* strains were screened for the presence of cytochrome P450 hydroxylase (*cyps*) gene. Out of 40 strains (Table II), only four showed the presence of the *cyp* P450 hydroxylase gene, with the

Table II
Streptomyces sp. GenBank accession numbers of 16S rRNA genes.

S. No. Given Code of Strain	GenBank Accession No.	Identified as
NR-1	MK243371	*Streptomyces* sp.
NR-10	MK243372	*Streptomyces* sp.
NR14	MK243373	*Streptomyces* sp.
NR15	MK243374	*Streptomyces* sp.
NR11	MN912434	*Streptomyces* sp.
C2	MN912435	*Streptomyces pseudovenezuelae*
D6-3	MN912436	*Streptomyces flavogriseus*
H34A	MN912437	*Streptomyces* sp.
H34B	MN912438	*Streptomyces* sp.
NR28	MN912439	*Streptomyces flavoviridis*
NR1	MN912440	*Streptomyces* sp.
NR5	MN912441	*Streptomyces werraensis*
10M	MN912442	*Streptomyces* sp.
C3	MN912443	*Streptomyces fenghuangensis*
H32B	MN912444	*Streptomyces* sp.
B5K	MN912445	*Streptomyces fimbriatus*
H31A	MN912446	*Nocardioides* sp.
M19	MN912447	*Streptomyces albogriseolus*
MM5	MN912448	*Streptomyces Streptomyces griseus*
NR3	MN912449	*Streptomyces* sp.
M63	MN912450	*Streptomyces misionensis*
M32	MN912451	*Streptomyces* sp.
M12	MN912452	*Streptomyces steffisburgensis.*
MM7	MN912453	*Streptomyces fimbriatus.*
M13	MN912454	*Streptomyces niveoruber*
D3-1	MN912455	*Streptomyces bambusae*
M29	MN912456	*Streptomyces* sp.
M28	MN912457	*Streptomyces coerulescens*
NR24	MN912458	*Streptomyces silaceus*
NR22	MN912459	*Streptomyces* sp.
H26	MN912460	*Streptomyces steffisburgensis*
M43	MN912461	*Streptomyces rubrolavendulae*
NR12	MN912462	*Streptomyces neopeptinius*
NR6	MN912463	*Streptomyces coeruleoprunus*
D3-3	MN912464	*Streptomyces* sp.
D3-2	MN912465	*Streptomyces* sp.
M93	KM062032	*Streptomyces laurentii*
M71	KM062033	*Streptomyces vitaminophilus*
M54	KM062034	*Streptomyces hypolithicus*
M51	KM062035	*Streptomyces chartreusis*

Table III
Streptomyces sp. GenBank accession numbers
of the genes sequences.

S. No.	Isolates	Nucleotide length	GenBank Accession No.	% age homology	Genes encoding for
1.	NR-1	350 bp	98	CYP	MF279145
2.	NR-10	350 bp	100	CYP	MF279146
3.	NR14	350 bp	98	CYP	MK272790
4.	NR15	350 bp	98	CYP	MK272791
5.	NR-16	700 bp	100	NRPS	MF279147
6.	M13	700 bp	99	NRPS	MF279148
7.	NR-12	700 bp	98	NRPS	MF279150
8.	NR-6	1500 bp	98	PKS-1	MF279149

amplicon size of 350 bp. The strains NR10, NR15, NR14, and NR1 were detected for the presence of the *cyp* P450 hydroxylase gene. Out of 40 strains, about 33 strains showed the presence of the *nrps* gene. The *nrps* primer pair A3F/A7R amplified the band of approximately 700 bp. The strains including NR11, C2, D6-3, H34A, H34B, NR28, NR1, NR5, 10M, C3, H32B, B5K, H31A, M19, MM5, MM63, NR3, M12, MM7, M13, NR6, M29, M28, NR24, NR22, H26, M43, NR12, NR6, M29, M28, and NR24 were found to have the *nrps* gene. To determine the presence of the glycopeptide *oxy b* (P450 monooxygenase) gene in selected *Streptomyces* strains the PCR utilizing the primer pair Gly-F/Gly-R, which amplified the 560 bp fragment was carried out. Out of 40 strains, none of the strains exhibited the presence of the glycopeptide *oxy b* gene. The gene-specific PCR for *pks-1* utilized the KI-F/M6-R primer set; the amplified product of the primer was about 1200–1500 bp gene fragment. 40 strains were screened for the *pks-1* gene; among them 17 strains were found positive for the *pks-1* gene (Table III).

The PCR amplified gene fragments were sequenced, and sequence data was analyzed with the BLAST to check the percentage homology of the given genes such as the cytochrome P450 hydroxylase (*cyps*), glycopeptide *oxy b*, *pks-1*, and non-ribosomal peptide synthase (*nrps*) genes with the other genes that are present in NCBI GenBank. The percentage homology of the amplified gene fragments in different *Streptomyces* strains with the cytochrome P450 hydroxylase (*cyps*) gene was as follows: NR-1, NR-10, and NR14 (98%, 100%, and 100%). The percentage homology of the amplified genes in *Streptomyces* strains with non-ribosomal peptide synthase (*nrps*) gene are given as: NR-6, M13, M29, and NR-12 (100%, 99%, 100%, and 98%). The percentage homologies of *Streptomyces* strains with type 1 polyketide synthase (*pks-1*) were 98% for the strain NR-6 (Table III).

The sequences were submitted to NCBI GenBank (BANKit) as follows: strain NR-1 Accession No. MF279145, strain NR-10 Accession No. MF279146, strain NR-6 Accession No. MF279147, strain M13 Accession No. MF279148, strain NR-12 Accession No. MF279150, strain NR-6 Accession No. MF27914, strain NR-14 Accession No. MK272790, and strain NR-15 Accession No. MK272791 (Table II). The 16S rRNA gene accession numbers are given as follows: NR-1 Accession No. MK243371, NR-10 Accession No. MK243372, NR-14 Accession No. MK243373, and NR-15 Accession No. MK243374.

The nucleic acid sequence of strains NR-1, NR-10, NR-14, and NR-15 with the cytochrome P450 hydroxylase gene was translated using EMBOSS Transq. The similarity index of the translated nucleotide of strain NR-1, with cytochrome P450 hydroxylase protein from all six reading frames, were mentioned in Table IV. The EMBOSS_001_1 showed the highest percentage similarity of 98% with cytochrome P450 hydroxylase (CYP) protein, while the EMBOSS_001_4 indicated the lowest similarity with cytochrome P450 hydroxylase (CYP) protein. The EMBOSS_001_5 and EMBOSS_001_6 are non-functional proteins, and no significant similarity was found. The protein sequence that showed highest similarity index were further selected for alignment by using the MEGA 6.0. It might be possible the given antifungal activity of the *Streptomyces* strains, including NR-1, NR-10, NR14, and NR-15, was due to the different amino acid residues within the protein sequence. The bioinformatic studies confirmed the presence of cytochrome P450 hydroxylase), cytochrome P450 monooxygenase, non-ribosomal peptide synthase, and type I polyketide synthase proteins that plays a vital role in the antibiotics biosynthesis pathways. The biological and chemical screening results showed that the selected *Streptomyces* strains NR-1, NR-10, NR-14, and NR-15 can produce the polyene compounds under laboratory conditions.

The results of the agar plug method indicated that all the four strains carrying the *cyp* gene showed an

Table IV
The translated DNA sequence of NR-1 based on six reading frames and their percentage similarity with cytochrome P450 hydroxylase (CYP) protein.

Sequence translation (EMBOSS Transq)	% similarity with cytochrome P450 hydroxylase protein
EMBOSS_001_1	98
EMBOSS_001_2	50
EMBOSS_001_3	No significant similarity found
EMBOSS_001_4	45
EMBOSS_001_5	No significant similarity found
EMBOSS_001_6	No significant similarity found

Table V
Antifungal activity of the selected polyene producing *Streptomyces* sp. against different fungal strains
(*Fusarium oxysporum*, *Rhizoctonia solani,* and *Aspergillus* sp.).

The fungus strain tested	Zone of inhibition in mm					
	NR-1	NR-10	NR-14	NR-15	MM7	CHX
Fusarium oxysporum	17.0 ± 0.11	17.8 ± 0.18	14.7 ± 0.22	16.0 ± 0.25	5.1 ± 0.121	9.9 ± 0.26
Rhizoctonia solani	18.0 ± 0.32	12.2 ± 0.41	13.8 ± 0.45	16.6 ± 0.45	0.2 ± 0.11	10.9 ± 0.53
*Aspergillu*s sp.	22.1 ± 0.40	19.0 ± 0.12	18.8 ± 0.27	18.3 ± 0.38	1.7 ± 0.42	14.0 ± 0.18

inhibitory effect toward the tested fungal strains (Table V). The strain NR1 showed the most remarkable inhibitory effect on the tested fungal strains. The strains NR-1 and NR-14 have the most prominent inhibitory effect on the test strain FN2 (Fig. 2). While in the well-diffusion method cycloheximide (2 mg/ml) was used as standard, and the MM7 methanolic crude extract was used as the negative control. The crude extracts of strains NR-1, NR-10, NR-14, NR-15, and MM7 showed 17.0 ± 0.11 mm, 17.8 ± 0.18 mm, 14.7 ± 0.22 mm, 16.0 ± 0.25 mm, 5.1 ± 0.121 mm zone of inhibition, while CHX (cycloheximide) showed 9.9 ± 0.26 mm inhibitory zone against the *F. oxysporum* (FO). The selected crude extract showed the following zones of inhibition against the *R. solani* (RS): 18.0 ± 0.32 mm, 12.2 ± 0.41 mm, 13.8 ± 0.45 mm, and 16.6 ± 0.45 mm. The fungal strain *Aspergillus* (FN2) against which the antifungal activity was determined by
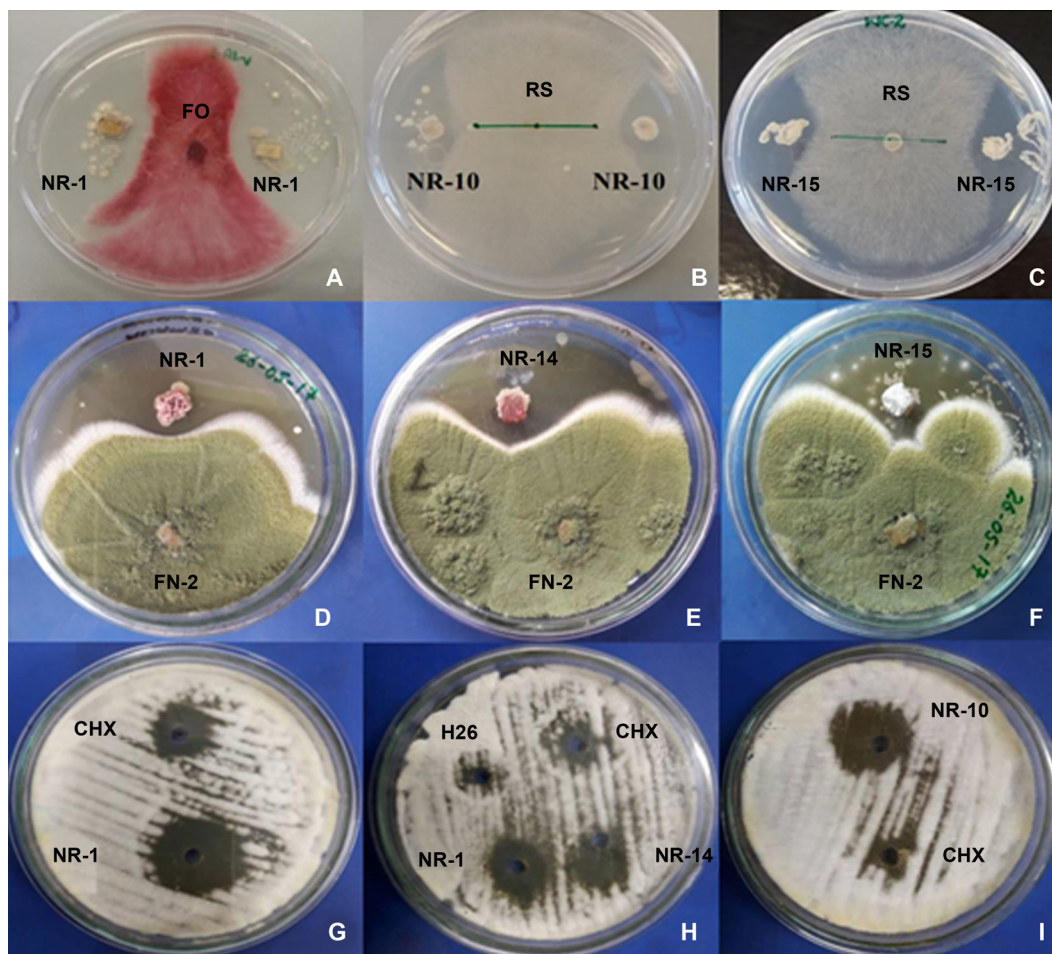

Fig. 2. Antifungal activity of the selected polyene producing *Streptomyces* sp. against different fungal strains tested (*Fusarium oxysporum* (FO), *Rhizoctonia solani* (RS), and *Aspergillus* sp. (FN2). (A), (B), (C) Anti fungal activity of NR-1, NR-10, and NR-15 by the agar plug method against *Fusarium oxysporum* (FO), *Rhizoctonia solani* (RS). (D), (E), (F) Activity of NR-1, NR-14, and NR-15 by the agar plug method against *Aspergillus* sp. (FN2). (G), (H), (I) Activity of NR-1, NR-14, NR-10, H26, and CHX (cycloheximide) by the well diffusion method against *Aspergillus* sp.
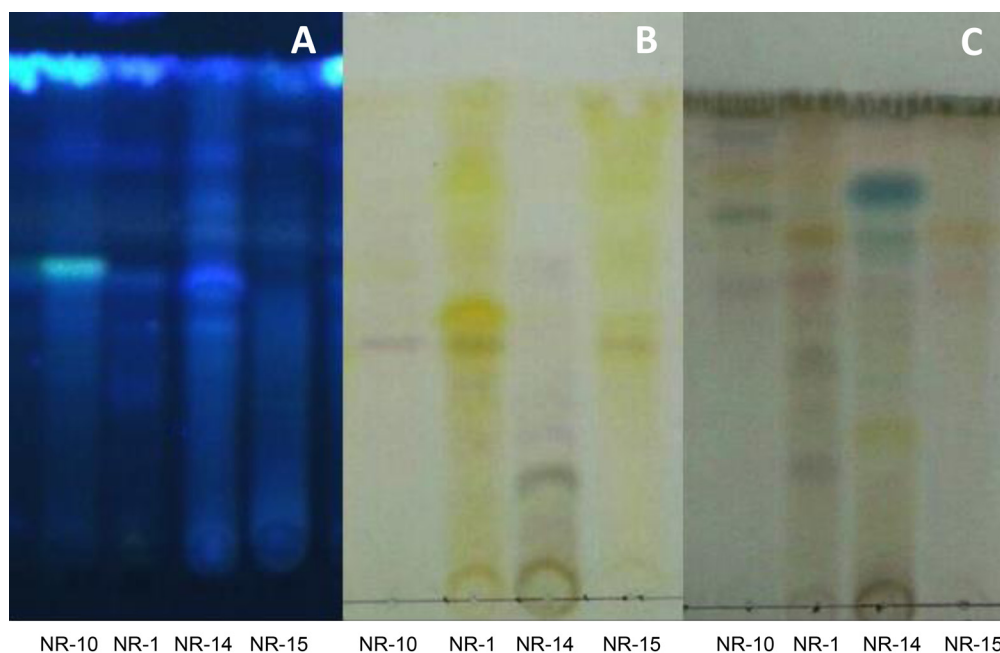
Fig. 3. Chemical profile of the selected Actinomycetes strains. (A) TLC plate at 366 nm.
(B) TLC plate after spraying with Ehrlich's reagent. (C) TLC plate after spraying with anisaldehyde reagent.

utilizing the crude extracts of NR-1, NR-10, NR-14, and NR-15 strains. The strain NR-1 showed the most prominent zone of inhibition which was 22.1 ± 0.40 mm, while NR-10, NR14, and NR-15 indicated 19.0 ± 0.12 mm, 18.8 ± 0.27 mm, and 18.3 ± 0.38 mm zone of inhibition, respectively. The MM7 (control) did not show any significant zone of inhibition compared to the tested *Streptomyces* extracts (Table V).

In a thin-layer chromatography, different biologically active components were analyzed in the crude extracts. The *Streptomyces* strains indicated various bands, which were of unique color. Many components of the crude extract displayed UV absorbance: however, most noticeable bands were observed in crude extracts of strains NR-14 and NR-1 (Fig. 3). NR-14 and NR-1 showed red, pink, and the most important green color band after spraying with anisaldehyde/H_2SO_4. The NR-10, NR15, NR 14, and NR-1 exhibited bands of yellowish, pink, and purple after staining with Ehrlich's reagent.

The biologically active strains, including NR-1, NR10, NR14, and NR-15 that showed the presence of polyene specific the cytochrome P450 hydroxylase (*cyps*) gene, were further analyzed on HPLC to indicate either these strains had peaks related to any polyene compounds when grown in culture. The strain NR-1 showed three peaks at different retention times; the most prominent peak was observed at t_R 2.95 minutes. The strain NR-10 showed two peaks, but the most prominent peak was detected at t_R 3.22 minutes. The other strains, which were analyzed on the HPLC chromatogram, included NR-14, which displayed two prominent bands at 2.94 minutes and 3.20 minutes

retention time (t_R). The strain NR-15 showed the most prominent peak at t_R 3.02 minutes (Fig. 4).

Each of the HPLC/UV chromatogram of methanolic extract of selected *Streptomyces* were compared with the standard nystatin HPLC/UV chromatogram (Hwang et al. 2007). The standard nystatin displayed the peak at a retention time of 3.03 minutes. After that comparison, it was confirmed that all the strains that showed the presence of polyene specific CYP P450, produced some polyene like antifungal metabolites.

Discussion

The current advances in the field of genomics, metagenomics, and high-throughput screening is beneficial for the natural product's detection (Genilloud et al. 2011). Due to the rediscovery of the already known metabolites, there is a strong need to explore the unique habitats and ecological niches, so that the probability of the discovery of novel metabolites with appropriate bioactivities could increase (Dhaneesha et al. 2017). The genome mining has thus delivered a comprehensive innovative tool, for the discovery of already identified, as well as previously unidentified natural metabolites, and the explanation of new biochemical revolutions and biosynthetic pathways (Chou et al. 2010).

Among the 40 tested *Streptomyces* strains only four strains indicated the presence of a predictable 350 bp PCR product for the polyene specific cytochrome P450 hydroxylase gene. The results indicated that polyene gene is a rare gene because only a low hit rate was found.
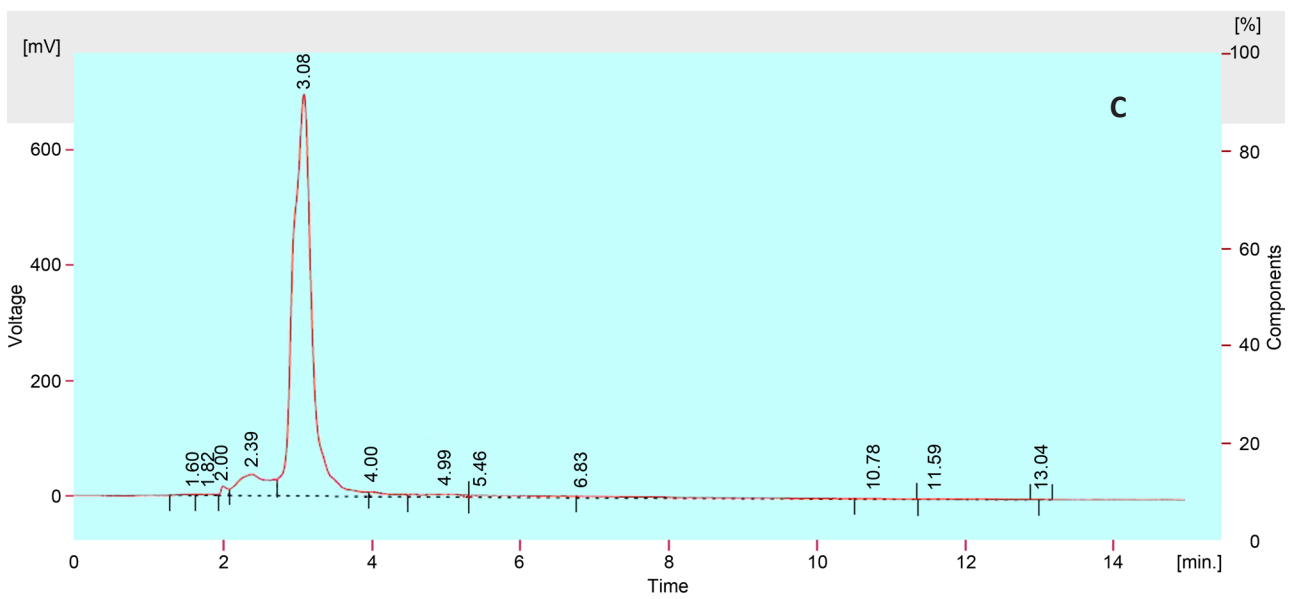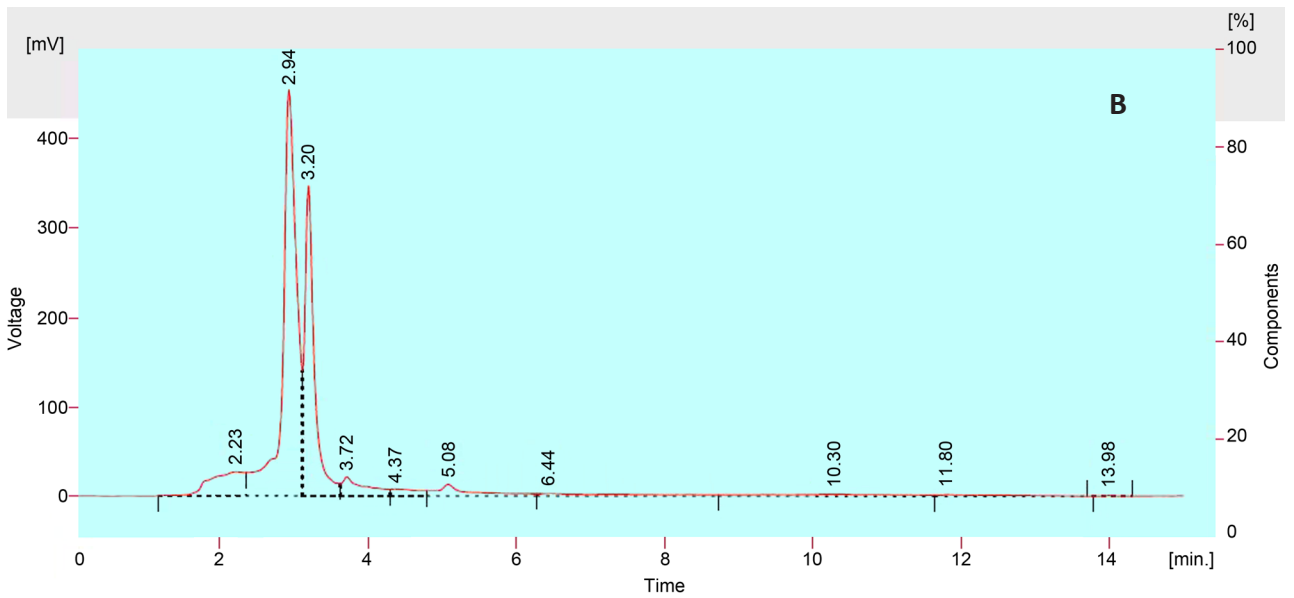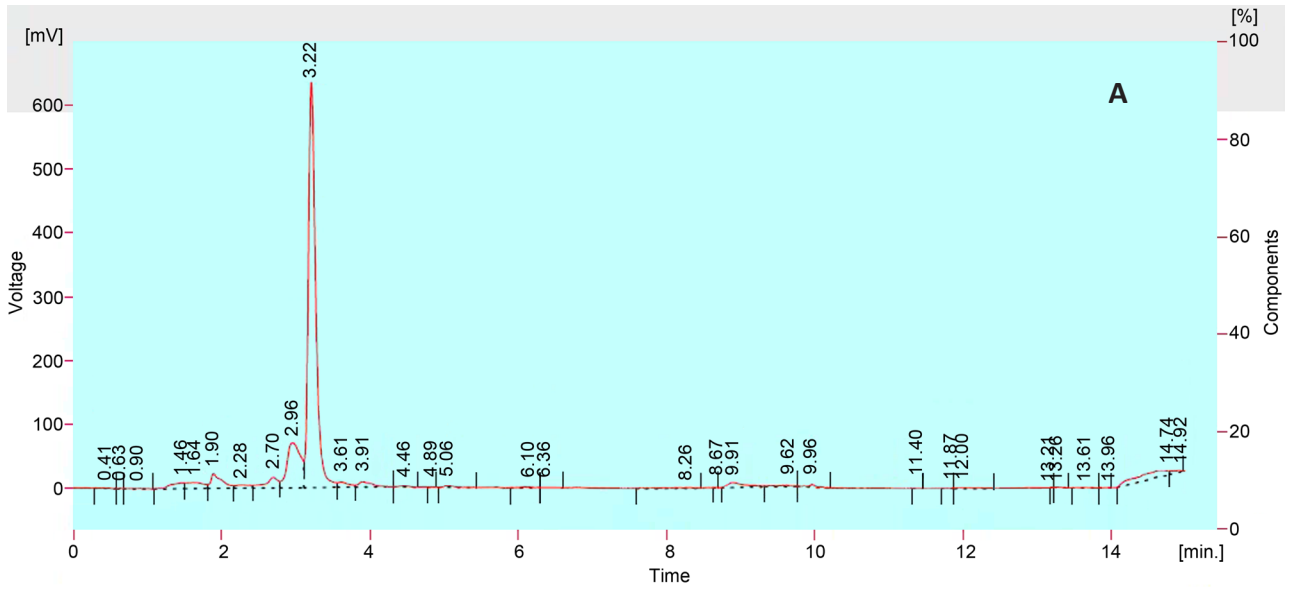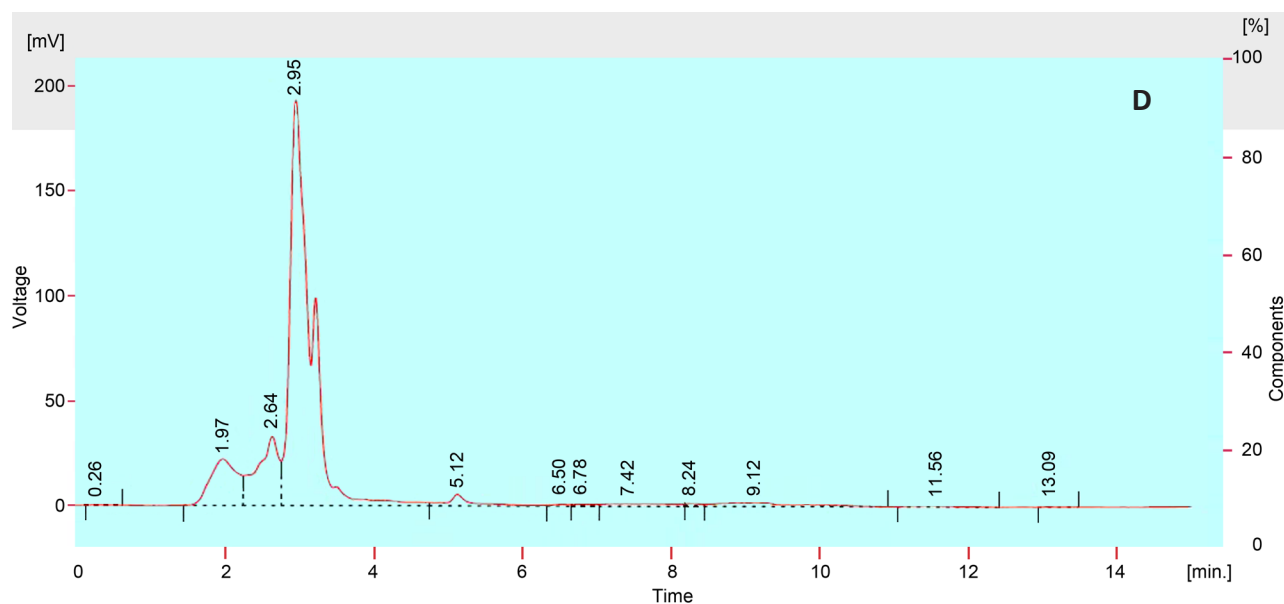
Fig 4. HPLC analysis of crude extracts of the polyene producing *Streptomyces* sp. (A) HPLC chromatogram of strain NR-1, (B) HPLC chromatogram of strain NR-10, (C) HPLC chromatogram of strain NR-14, (D) HPLC chromatogram of strain NR-15.

The use of fungal antibiotics is limited because of its high toxicity, so the genome-guided screening approach for the detection of new polyene antibiotics like compounds having improved pharmacological ability and less cytotoxicity is welcomed (Brautaset et al. 2002).

To screen the selected *Streptomyces* strains for the detection of the glycopeptide *oxy b* gene a PCR was performed. After optimization, not a single strain could be found positive for the desired gene; it might be because the glycopeptide gene cluster is rare. Encheva-Malinova et al. (2014) reported that almost all the strains possessed *pks-II* gene, and among the 11 strains screened for the *nrps* gene, six were positive for polyene, and four were positive for glycopeptide gene. The study confirmed that the glycopeptide antibiotics gene is rare among all four genes screened. In this study, for 33 strains amplification of the 700 bp fragment was successful that indicated the presence of the non-ribosomal peptide synthase gene. Gontang et al. (2010) reported that three primer sets for different domains of the *nrps* gene utilized for PCR based screening. The results of study showed that all domains were amplified in the isolated *Streptomyces*, and the biosynthetic domains were involved in the production of secondary metabolites. For the *pks-1* gene, the annealing temperature utilized in gradient PCR was $55 \pm 0.5°C$ for 2 minutes. The most appropriate PCR product of 1200–1500 bp was visualized at temperature 53°C.

The *in vitro* antifungal assays indicated that the selected *Streptomyces* have the potential to synthesize the polyene specific CYPs proteins when grown in culture conditions. The published literature also indicated that most of the secondary metabolites from *Streptomy-ces* are extracellular when grown under certain cultural conditions (Arasu et al. 2013).

Overall, this study revealed that a PCR-based screening approach that targets novel genes from biosynthetic gene clusters is a powerful tool for the rapid detection and identification of bioactive strains within the large culture collections. Moreover, in the recent past, the screening of large culture collections has led to the rediscovery of already known compounds; this greatly increases the usefulness of the PCR-based screening approach. Furthermore, in this study, a phylogenetic analysis of the amplified PCR products showed the exact prediction of the structural class of secondary metabolites being synthesized by an individual strain. The gene-based screening approach is helpful and can act as an additional pre-screening strategy for the selection of promising *Streptomyces* strains in a collection before cultivating the strains on a large scale for the purification and identification of the compounds. The relative abundance of the selected genes is shown in Fig. 5.
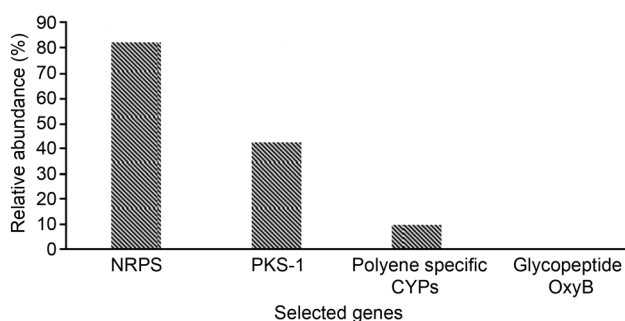

Fig. 5. The relative abundance of the *nrps*, *pks-1, cyps, oxy b* genes in the selected *Streptomyces* strains.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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