Analysis of co-culture of *Streptomyces sp*. VITGV156 with four different bacteria for enhancing secondary metabolites production

Ramesh Pooja and John Godwin Christopher*
Department of Biomedical Sciences, School of Bio Sciences and Technology, Vellore Institute of Technology, Vellore, 632014, INDIA
*godwinj@vit.ac.in

Abstract
Streptomyces plays a pivotal role in producing antibiotics, essential for treating diseases and enhancing human health. In this study, the endophytic novel Streptomyces sp. VITGV156 was used to create four different consortia with live bacteria Staphylococcus aureus, Bacillus subtilis, Escherichia coli and Pseudomonas aeruginosa to activate the production of secondary metabolites. The secondary metabolites produced in the medium were extracted with ethyl acetate. The antimicrobial activity of the extracts from the monocultures (VITGV156) and cocultures was tested. The zone of inhibition for the monoculture crude extracts ranged from 10mm-20mm. The antimicrobial activity of VITGV156 with E. coli ranged from 11mm-22mm and of VITGV156 with B. subtitles was 10mm-20mm. The coculture of VITGV156 with S. aureus yielded a zone of 13mm-21mm. Coculture of VITGV156 with P. aeruginosa showed a zone ranging from 12mm-21mm at a concentration of 100mg/ml.

Crude ethylacetate extracts were investigated for their antioxidant activity which was 77.67±0.2% for 156PA coculture followed by the other extracts. GC–MS analysis of these extracts revealed that five compounds were common in all four cocultures: benzeneacetic acid, benzoic acid, 2-amino acid, pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methyl propyl), N-acetyltyramine and cyclo-(l-leucyl-l-phenylalanyl). N-Acetyltyramine had a high percentage area. According to this study, all these cocultures produce bioactive secondary metabolites.

Keywords: Secondary metabolites, Antimicrobial activity, Antioxidant activity.

Introduction

Actinomycetes are a heterogeneous group of bacteria that are Gram-positive, form a branching network of filaments and produce spores. Actinomycete was first accurately described in 1877 by Harz and was isolated as *Actinomyces bovis* from cattle with lumpy jaws (actinomycosis). The term "actinomycetes" (ray fungi) was initially used by Gasperini (1890, 1892) to refer to microbes that are distinct from other bacteria in that they can create a mycelium made up of narrow hyphae, which rarely produces spores of comparable size[27]. According to Bergey's Manual of Systematic Bacteriology, the phylum *Actinobacteria* consists of the class *Actinobacteria*, to which member *Actinomycetales* belongs.

Actinomycetales are commonly referred to as actinomycetes. Actinobacteria encompass a well-defined clade of Gram-positive bacteria with high GC content in DNA and constitute one of the largest bacterial phyla, which was well described by Jagannathan et al[12]. They are found in various habitats including freshwater, marine systems and soil.

Actinomycetes play a pivotal role in the production of biologically active secondary metabolites such as antibiotics, biopesticide agents, plant growth hormones, immunosuppressants, chemotherapeutic medicines and antibacterial, antitumor, antifungal, antiviral and antiparasitic agents. It is well known that members of the *Streptomyces* genus can produce a wide variety of biologically active secondary metabolites. A single *Streptomyces* strain gene can produce more than 30 different secondary metabolites which include no ribosomal peptides, polyketides and many other classes of compounds[5].

Natural products from microorganisms are common sources of novel antibiotics. Novel microbial natural products (NPs) are considered important sources for the discovery of new generations of anti-infective agents. These compounds exhibit unique antibiotic potency and chemical diversity. Nearly 64% of the identified NP antibiotic groups are produced by filamentous actinomycetes, with the remaining 36% being produced by various fungi and bacteria. The recent discovery of novel antibiotic-producing strains from unexplored habitats, along with emerging technologies for genome mining and heterologous pathway expression, has re-energized the research interest in NPs[10].

The emergence of new diseases, occurrence of naturally resistant bacteria, increase in the rate of resistant infections and increase in the toxicity of existing chemicals have led to a pressing need for the search for new antibiotics. In 1995, penicillins, tetracyclines, cephalosporins, macrolides and quinolones were the top five antimicrobials sold on the US market[6]. In 2018, omadacycline, rifamycin, eravacycline, sarecycline and palzomicin were used. In 2019, cefiderocol, a combination of relebactam, pretomanid, imipenem and cilastatin were approved by the US Food and Drug Administration (FDA) as new antibacterial antibiotics[3].

Bacteria can adapt to environmental changes. Selective pressures within the human host including exposure to medical interventions such as antibiotics, interactions with innate and adaptive immune responses and competition with the commensal microbiota, all facilitate the progression of bacterial pathogens. Pathogenic bacteria can invade and damage a host species. Furthermore, they undergo additional genetic alterations, which can change their genome and alter their virulence traits[11]. Pathogenic mechanisms include contact between a pathogen and a new host, adherence of the pathogen to host cell receptors, local replication, spread to target organs and shedding of the pathogen into the environment[4].

Actinomycetes are well known to be inexhaustible sources of antibiotics. In the 1940s, 1% of the actinomycetes isolated from soil samples were involved in streptomycin production. Actinomycetes monocultures can produce many secondary metabolites, but it is agreed that under normal fermentation conditions, a significant proportion of microbial gene clusters get silenced. One strategy to activate a biosynthesis-related gene cluster is the use of a biological elicitor[1]. Several studies have demonstrated the use of mixed fermentation, also known as "cocultivation," for the production of novel active compounds from actinomycetes[20]. This cocultivation method has the unique characteristic of either increasing the production of biologically derived novel compounds or inducing silent gene clusters. Many recent coculture experiments have demonstrated the efficacy of this strategy, showing that cocultivation is a valid experimental approach for improving the metabolic diversity of organisms cultivated *in vitro*.

Some well-known bioactive compounds produced from the coculture of actinomycetes with different bacteria include biphenomycin C which was produced by coculturing *Streptomyces griseorubiginosus* no. 43708 with *Pseudomonas maltophilia* no. 1928[7]; alchivemycin A, which was produced by coculturing *Streptomyces endus* s-522 and *Tsukamurella pulmonis*[21] and Umezawamides a-b, which was generated by coculturing *Umezawaea sp*. Rd066910 and *Tsukamurella pulmonis* Tp[9] and tryptamine and bacillamides by coculturing *Streptomyces sp*. Cgmcc4.7185 and *Bacillus mycoides*[29].

The body produces antioxidants through a variety of pathways to combat oxidative damage. "Oxidative stress" refers to a change in the ratio of oxidants to antioxidants that favors oxidants[16]. Oxidative stress is caused by excessive levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Oxidative stress is defined as an imbalance between the body's enzymatic antioxidants and rates of free radical production.

This imbalance is responsible for several health problems including Alzheimer's disease, cancer, inflammation and cardiovascular disease[8]. It has been reported that the metabolites obtained from Streptomyces are effective in shielding neuronal cells from damage caused by oxidative stress[23].

This study aimed to increase the production of secondary metabolites by activating cryptic or silenced genes in the endophytic novel *Streptomyces sp.* VITGV156 cocultured with four different human pathogens—*Staphylococcus aureus, Bacillus subtilis, Escherichia coli and Pseudomonas aeruginosa*.

Material and Methods

***Streptomyces* VITGV156:** *Streptomyces* VITGV156 (MCC 4965) was purchased from MCC.

Monoculture and co-culture: *Streptomyces* VITGV156 was monocultured in ISP2 broth media (five sets of 500 ml conical flasks) and kept in an orbital shaker at room temperature at 150 rpm for 10 days to reach the exponential phase (control). *Streptomyces* sp. VITGV156 was grown to the log phase in four separate conical flasks (F1–F4), to which 1 ml of bacterial cultures of *Staphylococcus aureus* (SA), *Bacillus subtilis* (BS), *Escherichia coli* (EC), or *Pseudomonas aeruginosa* (PA) was added to 100 ml of each *Streptomyces* culture. A fifth flask (F5) that contained only *Streptomyces* sp. VITGV156 was used as a control. All these cultures were inoculated in an orbital shaker at 130 rpm for 5 days.

Extraction: After 5 days, the culture media in conical flasks F1 to F5 were centrifuged at 4000 rpm for 15 minutes at 4°C. The resulting supernatant was filtered through Whatmann filter paper. The above filtrates were mixed with ethyl acetate (1:2) and kept in a shaker for 1 day. The ethyl acetate mixture was separated with a separating funnel and the supernatant was discarded. The ethyl acetate with dissolved secondary metabolites was then condensed in a rotary vacuum evaporator.

Antibacterial activity: The antibacterial activity of all four cocultures and the control were assessed by the agar well diffusion method. Seven sets of Petri plates, each set containing four plates (A–D), were prepared using Mueller–Hinton agar. Thereafter, SA was added to the first set of plates, BS to the second set, EC to the third set and PA to the fourth set. All the plates were then inoculated under aseptic conditions. Then, wells 6 mm in diameter were cut using a sterile steel cork borer, onto which crude extracts at concentrations of 25, 50, 75 and 100 mg/ml were added to the wells. Then, tetracycline (positive control) and ethyl acetate (negative control)[22] were added. All the plates were then incubated for 24 hours at 37°C and the inhibitory zones (mm) formed were measured with a HiMedia antibiotic zone scale (PW297) ranging from 10 mm to 40 mm.

Antioxidant activity: The DPPH radical scavenging activity of the coculture extracts of the ethyl acetate extract of *Streptomyces* VITGV156 was determined. Methanol was combined with each crude extract at a concentration of 0.1

mg/ml, DPPH was generated at a concentration of 0.002% in methanol and ascorbic acid served as a reference. Test tubes were filled with two millilitres of DPPH solution, two millilitres of crude extracts of the VITGV156 monoculture and coculture containing EC, SA, BS and PA and two millilitres of ascorbic acid. The tubes were shaken well and placed in the dark for thirty minutes. Methanol served as a blank and the absorbance was measured at 517 nm using a UV–visible spectrophotometer[24].

GC–MS Analysis: The compounds present in the ethyl acetate crude extracts from monocultures and cocultures were separated and quantified by using GC–MS (Thermo Scientific, Waltham, MA, USA; 30 m × 0.25 mm × 0.1 mm thick TG-5MS fused silica capillary column and Trace GC Ultra and ISQ single quadrupole MS). The inert gas helium was used at a flow rate of 1 ml/min as the transport gas. Metabolite detection was carried out using an electron ionization system with 70 eV ionization energy. The temperatures were preprogrammed as follows: first, the temperature was raised to 50°C at a rate of 2°C/min, then to 150°C at a rate of 7°C/min and then further increased from 150–270°C at a rate of 5°C/min, followed by another increase at 270–310°C at a rate of 3.5°C/min at an increasing rate.

Results

Monoculture and co-culture: The VITGV156 monoculture incubated in a shaker at 130 rpm developed clumps. The rate of growth was slower at 130 rpm than at 150 rpm. Furthermore, an increase in cell biomass was observed at 150 rpm. *Streptomyces* VITGV156 cocultured with F1-F5 after the inoculation of four different bacteria and the resulting *Streptomyces* cells were dispersed and were turbid. All the cocultures showed uniform growth. F5 was maintained as a monoculture.

Antibacterial activity: The antibacterial activity of the crude extracts against SA, BS, EC and PA was examined with positive and negative controls at different concentrations of the coculture extracts. The samples showed an increase in zone diameter as the concentration increased. A maximum zone of inhibition of 33 mm was observed for 100 mg/ml of tetracycline treated with SA (Table 1) whereas the lowest zone of 23 mm was observed for 25 mg/ml with EC (Fig. 1).

Similarly, a maximum zone of 22 mm (100 mg/ml) in BS was observed for 156EC compared to those observed for SA and EC (both 20 mm (100 mg/ml)) and for PA [19 mm (100 mg/ml); Fig. 3]. A maximum zone of inhibition of 21 mm (100 mg/ml) was observed for 156SA for BS followed by 20 mm (100 mg/ml) for SA, PA and EC (Fig. 5).

In addition, 156PA also showed a maximum zone of 21 mm (100 mg/ml) compared to that of SA and BS and 20 mm (100 mg/ml) in EC and PA, as shown in fig. 6. Furthermore, 156BS showed an inhibitory zone of 20 mm (100 mg/ml) in BS followed by 19 mm (100 mg/ml) in EC, SA and PA (18 mm) (100 mg/ml), as shown in fig. 4. No zone formation was observed in the negative control (Fig. 7). The antimicrobial activity of the test organism is due to the presence of secondary metabolites which were analyzed via GC–MS analysis.

Antioxidant activity: DPPH radical scavenging assays revealed that the color changed from purple to yellow in the presence of antioxidant compounds. The absorbance at 517 nm revealed that the monoculture and coculture of VITGV156 had good radial scavenging activity compared with ascorbic acid at a standard of 97.98%. The highest antioxidant activity was recorded for the coculture of VITGV156 with PA [77.67± 0.2%] followed by the coculture with EC [75.82± 0.34%]. The lowest antioxidant activity [70.53± 0.27%] was recorded for the coculture with 0.1 mg SA (Fig. 8).

GC–MS Analysis: The secondary metabolites present in the crude extracts of the samples were analyzed using GC–MS. Table 2 shows the compounds identified that were similar between the monocultures and cocultures. Fig. 9 (a-e) showed the chromatograms of the ethyl acetate crude extracts with peaks, areas and retention times for both mono- and cocultures of VITGV156. The identified secondary metabolites and compounds were compared via a standard library search.

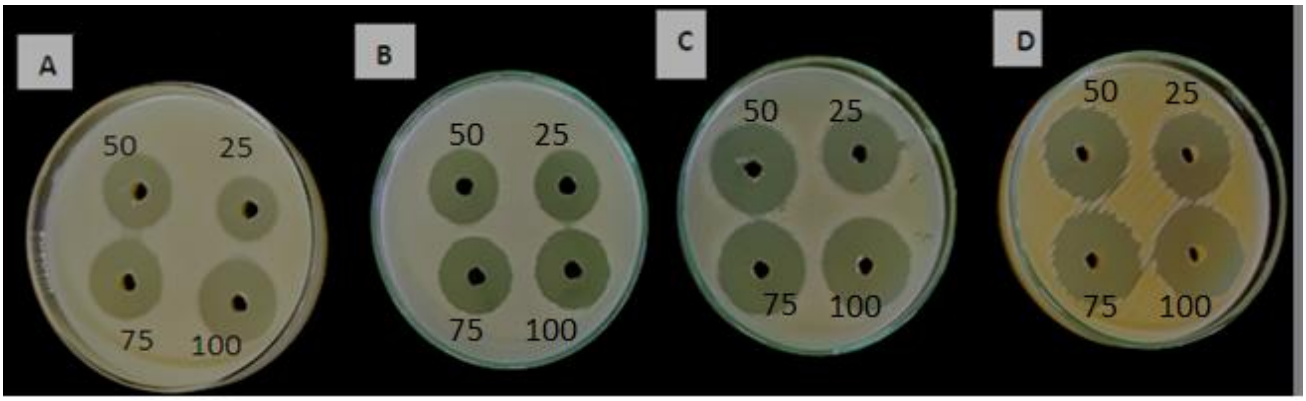

Fig. 1: Petri plates showing the inhibitory zone formation of tetracycline against Gram-positive and Gram-negative bacteria.

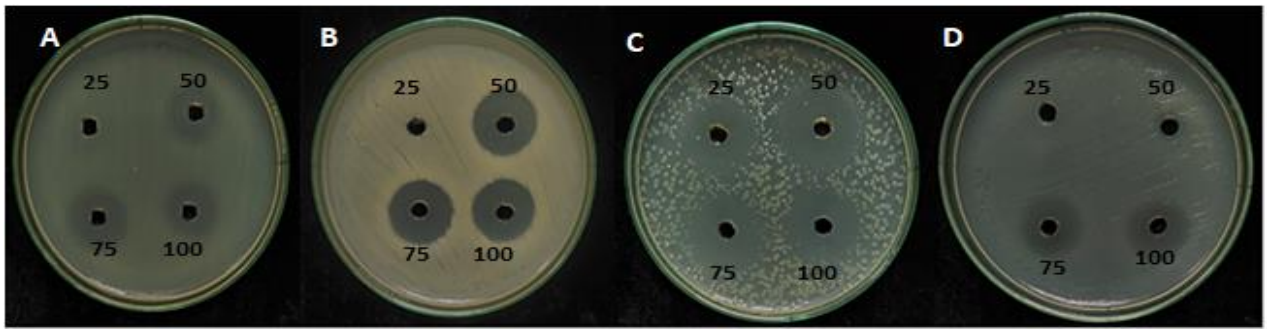

Fig. 2: Inhibitory zone formation observed for *Streptomyces* VITGV156 (monoculture)

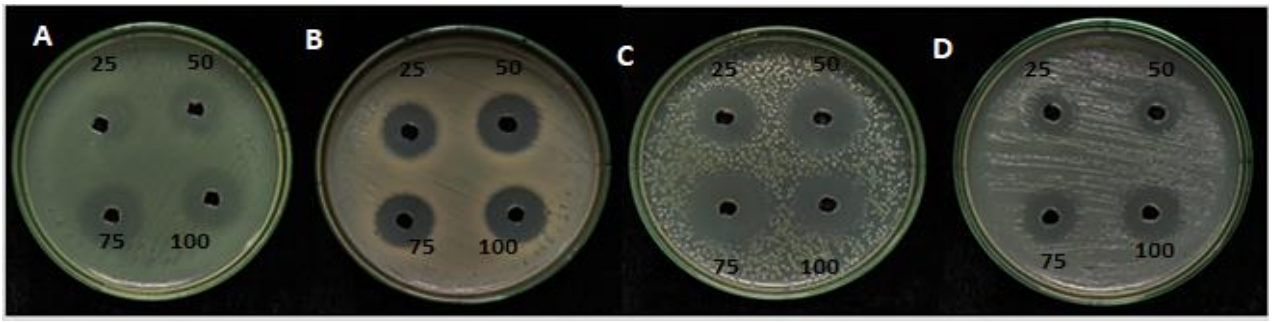

Fig. 3: Inhibitory zone formation observed for coculture with EC

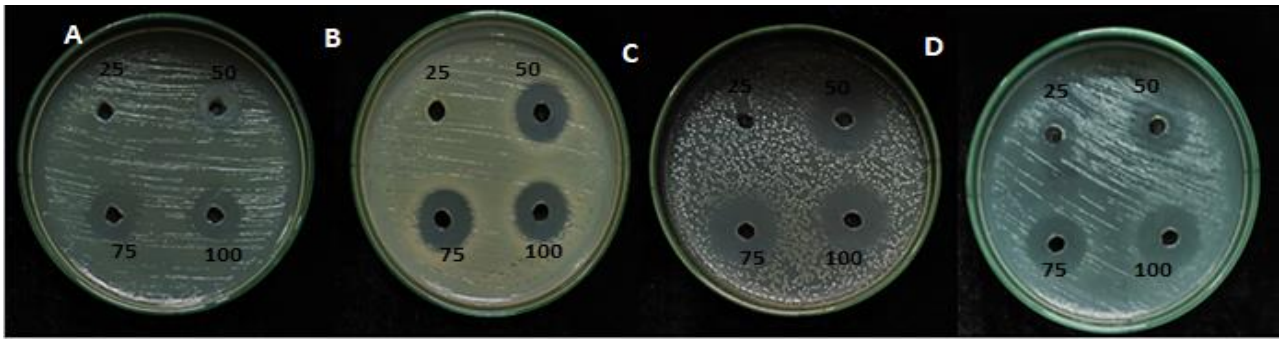

Fig. 4: Inhibitory zone formation observed for coculture with BS

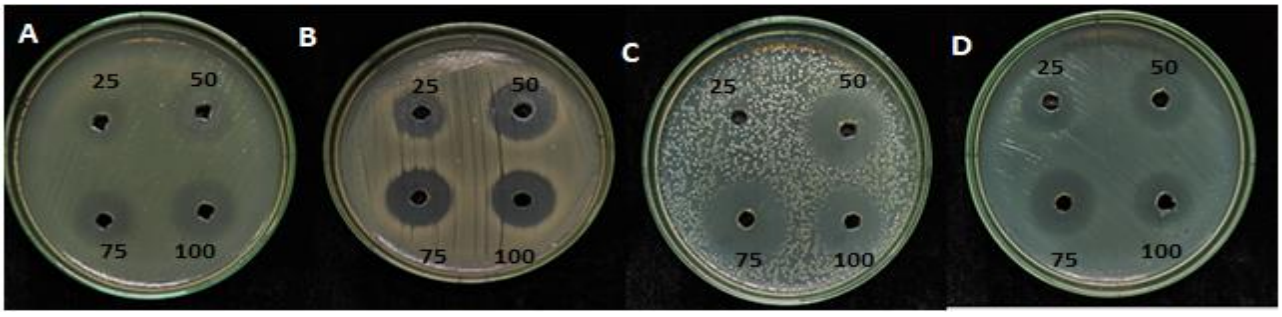

Fig. 5: Inhibitory zone formation observed for coculture with SA

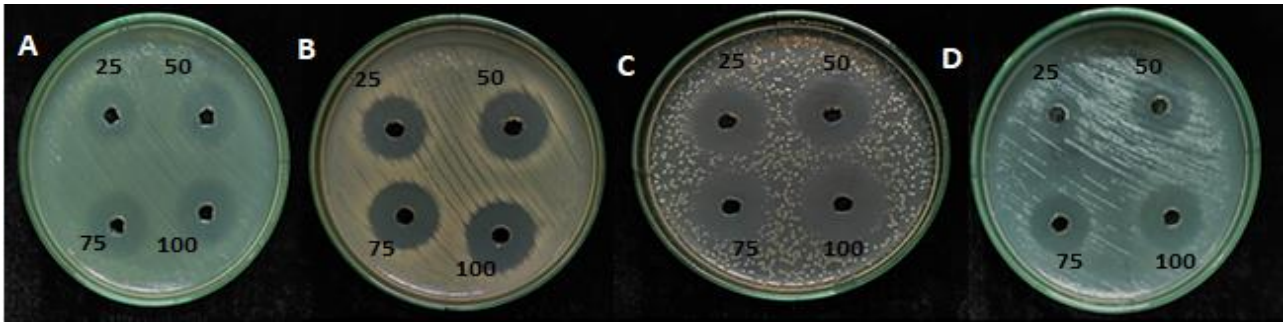

Fig. 6: Inhibitory zone formation observed for coculture with PA

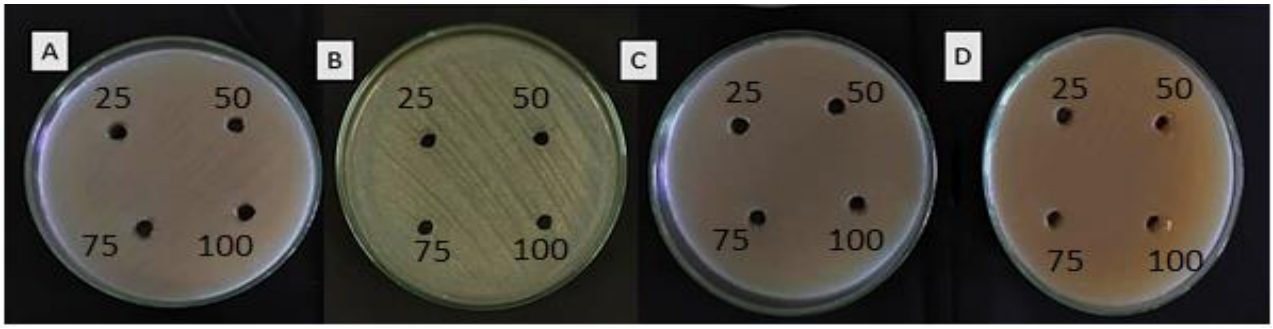

Fig. 7: Inhibitory zone formation observed for the negative control (ethyl acetate).
a. *Escherichia coli*, b. *Staphylococcus aureus*, c. *Bacillus subtilis* and d. *Pseudomonas aeruginosa*

Table 1
Zone of inhibition (mm) obtained for the four different bacterial strains

Zone of inhibition(mm)																
Sample	***E. coli***				***S. aureus***				***B.substilis***				***P. aeruginosa***			
Concentration (mg/ml)	**25**	**50**	**75**	**100**	**25**	**50**	**75**	**100**	**25**	**50**	**75**	**100**	**25**	**50**	**75**	**100**
Positive Control	23	24	27	27	27	30	32	33	24	25	26	27	27	29	30	31
156 M	-	15	16	18	-	18	19	20	16	18	19	20	-	10	17	18
156EC	11	14	17	20	17	18	19	20	19	20	21	22	13	14	17	19
156SA	13	15	17	20	15	18	19	20	-	18	19	21	14	16	17	20
156BS	-	16	18	19	-	16	17	19	10	18	19	20	-	12	16	18
156PA	14	16	18	20	18	19	20	21	18	19	20	21	12	16	19	20
Negative Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2
Comparative table showing the total number of compounds present in different culture extracts identified by GC–MS analysis

S.N.	Samples	No. of compounds
1.	156 M	60 compounds
2.	156EC	40 compounds
3.	156SA	40 compounds
4.	156BS	38 compounds
5.	156PA	45 compounds

Table 3
Common compounds found in all cocultures

S.N.	Compound	Co-Culture Groups	Retention Time (Min)	Area %	MF	MW (g/mol)	Activity
1.	Benzeneacetic Acid	156- PA	11.798	7.54	$C_8H_8O_2$	136.15	Anti-microbial activity
2.	Benzoic acid, 2-amino	156-SA	14.180	5.36	$C_7H_7NO_2$	137.14	Anti Inflammatory agents
3.	Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methyl propyl)-	156-EC	21.235	5.07	$C_{11}H_{18}N_2O_2$	210.27	Anti-oxidant activity
4.	N-Acetyl Tyramine	156-SA	20.153	16.33	$C_{10}H_{13}NO_2$	179.22	Antimicrobial activity
5.	Cyclo-(l-leucyl-l-phenylalanyl)	156-SA	28.533	3.92	$C_{15}H_{20}N_2O_2$	260.329	No reports Found

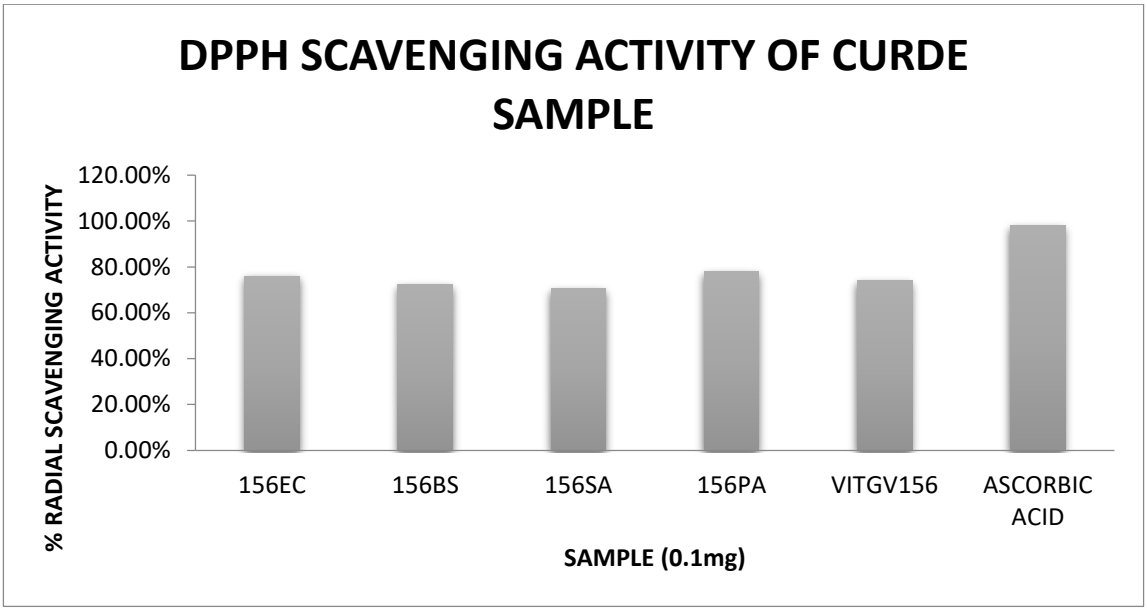

Fig. 8: Graphical representation of the DPPH radical scavenging activity of the ethyl acetate crude extracts

All four cocultures of *Streptomyces* VITGV156 expressed few common compounds. Five common compounds were found in all four bacterial coculture extracts: benzeneacetic acid, benzoic acid, 2-amino, pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methyl propyl); N-acetyltyramine and cyclo- (l-leucyl-l-phenylalanyl. The peak areas observed for various compounds produced by all four cocultures were as follows (Table 3): a peak area of 7.54% was observed for benzeneacetic acid produced by 156PA extract, a peak area of 5.36% was observed for benzoic acid and 2-amino acid produced by 156SA, a peak area of 5.07% was observed for pyrrolo[1,2-a]pyrazine-1,4-dione and hexahydro-3-(2-methylpropyl) produced by 156EC, a peak area of 16.33% was observed for N-acetyltyramine produced by 156SA and a peak area of 3.92% was observed for cyclo-(l-leucyl-l-phenylalanyl) produced by 156SA.

The compounds benzeneacetic acid, benzoic acid, 2-aminopyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- and cyclo-(l-leucyl-l-phenylalanyl) were all produced by both the coculture and monoculture strains. However, N-acetyltyramine was produced only by coculture strains. Table 4 shows that the compounds produced from 156SA (16.33%), 156EC (13.62%) and 13.46% from 156PA and 3-(4-hydroxyphenyl) propionic acid were categorized as low-, moderate- and high-area compounds respectively based on their area percentage. Trimethylsilyl ester accounted for 15.59% of the compounds produced from the coculture of 156BS. These compounds were the high-area compounds. Here, high amounts of N-acetyltyramine were produced in 156SA, which is commonly produced with a high quantity of area and has antimicrobial properties.

Discussion

In this research, microbial coculture was used for the production or activation of secondary metabolites, a promising tool for producing novel bioactive metabolites. The results showed that combined culture enhances the chemical defense mechanism between microorganisms that can activate their cryptic/silent genes to produce different secondary mechanisms. Here, a novel *Streptomyces* strain, VITGV156, which is an isolate from a healthy tomato plant, was cocultured with four different bacteria and analyzed for unique secondary metabolites. The extraction of secondary metabolites by ethyl acetate was found to dissolve more antimicrobial agents from the aqueous phase into the organic phase[18]. The ethyl acetate extracts from the strains *Streptomyces* AIA12 and AIA17 have high antibacterial and antioxidant effects[15].

According to Khadayat, the agar well diffusion method is the simplest and most effective way to assess the antimicrobial properties of crude extracts[13]. The ethyl acetate extract of VITGV156 showed good antibacterial activity against all the studied bacteria (Table 1). The crude extract of 156EC showed greater activity at 22 mm at a concentration of 100 mg/ml and a similar report on the zone of inhibition was reported for antimicrobial activity in *Streptomyces* VITAM-16[2] but was less active than tetracycline (23–31 mm), which had a greater zone of inhibition. However, here, the antimicrobial activity of the crude extracts was slightly lower against both Gram-positive and Gram-negative bacteria.

The free radical DPPH method is a quick, easy and affordable way to test the antioxidant potential of compounds. It is frequently used to assess the capacity of compounds to function as free radical scavengers. As previously reported, ascorbic acid served as a positive control (97.98%), similar to the findings of Lee et al[17] . The antioxidant activity of *Streptomyces* sp. VITGV156 cocultured with EC, BS, SA and PA was the highest at 77.67 ± 0.2% (Fig. 8). Similar results were reported in *Streptomyces speibonae*[25].

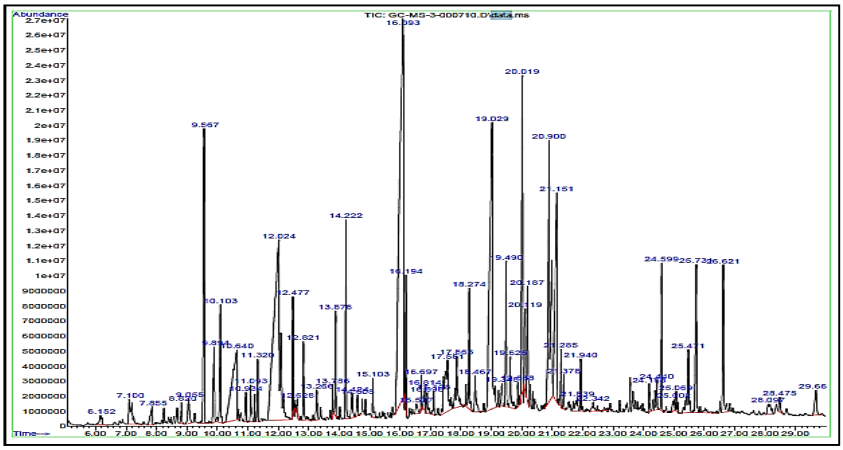

Figure 9a: GC–MS chromatogram of the ethyl acetate extract of the VITGV156 monoculture.

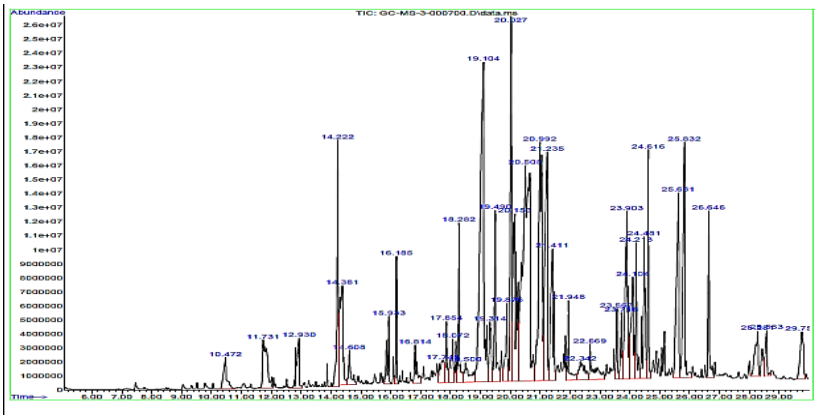

Figure 9b: GC–MS chromatogram of the ethyl acetate extract of VITGV156 cocultured with EC.

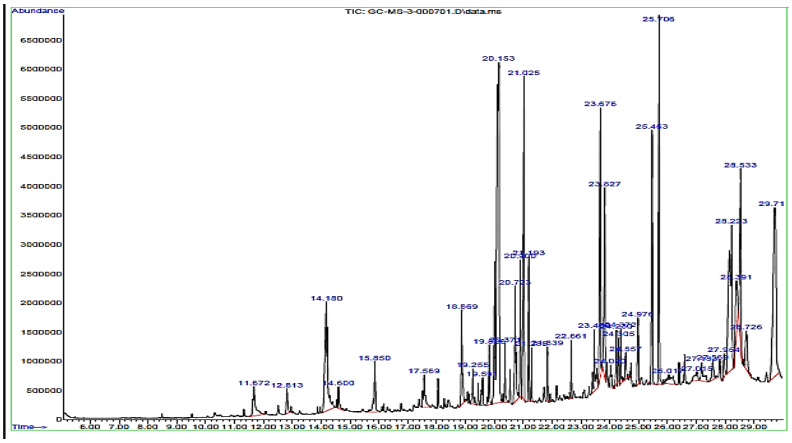

Figure 9c: GC–MS chromatogram of the ethyl acetate extract of VITGV156 cocultured with SA.

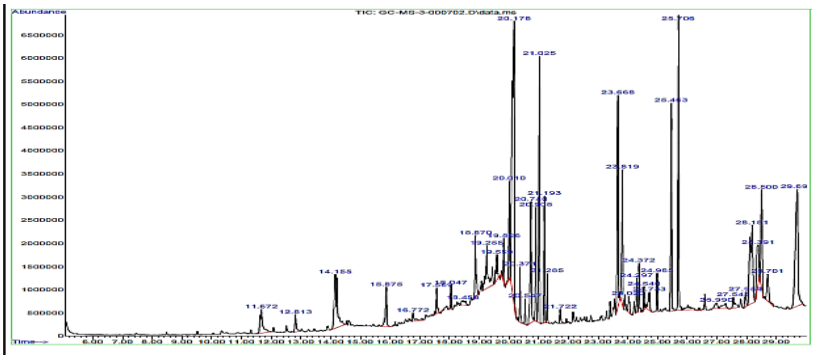

Figure 9d: GC–MS chromatogram of the ethyl acetate extract of VITGV156 cocultured with BS.

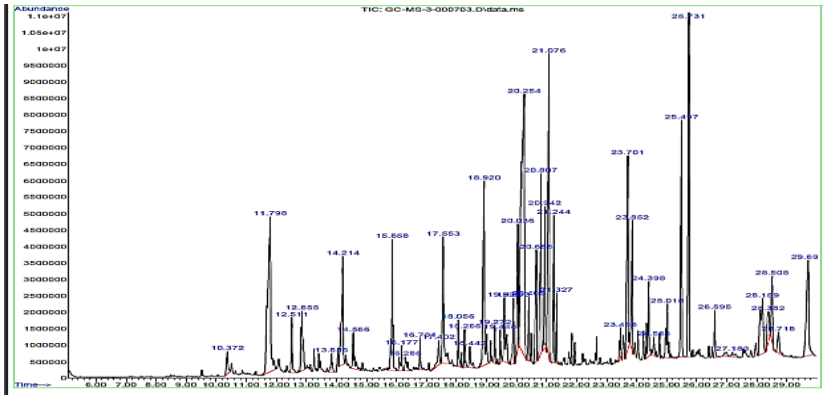

Figure 9e: GC–MS chromatogram of the ethyl acetate extract of VITGV156 co-cultured with PA

Figure 9: GC–MS analysis showing chromatograms of various peaks of secondary metabolites in the ethyl acetate extract and identifying compounds using a library search.

Table 4
Compounds separated based on the area (%)

AREA%	156EC	156SA	156BS	156PA	156 M
High	1	1	1	1	2
Moderate	2	4	4	3	2
Low	37	35	33	41	56

The GC–MS results of the coculture of VITGV156 with four different bacteria showed that five compounds were common in all four cocultures because they were expressed by *Streptomyces* VITGV156. These five common compounds support strong antimicrobial activities. Benzeneacetic acid, also known as phenylacetic acid, a compound that was identified from *Bacillus licheniformis* isolated from a Chungkook-Jang traditional Korean fermented soybean food that was reported to have antimicrobial activity, has been shown to have many activities in previous studies[14]. The analgesic and anti-inflammatory properties of the newly developed compounds were tested and they were compared with commonly used drugs such as phenylbutazone and aspirin. The most abundant antimicrobial compound in this extract is benzoic acid, 2-amino, also known as anthranilic acid, which is a major class of nonsteroidal anti-inflammatory agent.

The pyrrolo[1,2-A] pyrazine-1,4-dione hexahydro-3-(2-methylpropyl) reported in *Streptomyces* sp. VITMK1 is a nontoxic potential antioxidant agent capable of scavenging free radicals and the same compound is also available in VITGV156[19]. *Streptomyces sp*. YIM 67086 was isolated from *Dysophylla stellata and* produced the compound N-acetyltyramine which has antimicrobial activity as reported by Yang et al[28]. These compounds are known for their anti-inflammatory, antioxidant and antimicrobial properties. The 156EC batch showed a greater zone of inhibition (22 mm) with 19 antimicrobial compounds identified via GC–MS.

All cocultures of VITGV156 showed strong antioxidant activity. This activity is due to the presence of 12 antioxidant compounds identified by GC–MS. The strongest antioxidant agents detected in 156PA were pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- pentadecanoic acid, 2-propenoic acid, pentadecyl ester o-butyl O,O-diethyl phosphorothioate ethanol, 2-(tetradecyloxy)-; hydrocinnamic acid 13-docosenamide (Z)- and 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione. Slightly lower antioxidant activity was observed in 156SA, in which only 2 antioxidant compounds, indole and pyrrolo[1,2-a] pyrazine-1,4-di one, hexahydro-3-(2-methylpropyl) were detected[19,26]. However, for the other extracts, more than 4 antioxidants were detected. These are some known antioxidant compounds identified from GC–MS analysis.

This study clearly showed that *Streptomyces sp*. VITGV156 cocultured with all four different bacteria had activated cryptic/silenced genes. This herb contains antimicrobial and antioxidant compounds. These compounds should be checked for their antibiotic activities against multidrug-resistant pathogens.

Conclusion

The value of *Streptomyces*, which contributes to ecosystem balance and human well-being, across many fields is highlighted by its function in soil health, disease avoidance, bioremediation and commercial applications. Cocultivation is a new effective strategy for inducing the expression of poorly expressed or silent cryptic gene clusters. Co-culture of VITGV156 with other organisms has the capacity to produce potential secondary metabolites, especially antibiotics and antioxidants. However, further studies are needed to explore additional possibilities.

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