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Effects of different media and solvents on biological activities and secondary metabolites profiles of a coral-derived *Streptomyces* sp. RC4

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Abstract. *Rizky, Nofiani R, Rudiyansyah, Putri CD, Setiawan A, Wibowo DS, Setiyoningrum F. 2025. Effects of different media and solvents on biological activities and secondary metabolites profiles of a coral-derived* Streptomyces *sp. RC4. Biodiversitas 26: 739-747.* *Streptomyces* sp. RC4 demonstrates a potential source of secondary metabolites with various biological activities. Culture media and solvents for culture media influence the production of the metabolites. This study investigated the effect of different culture media and solvents on the biological activities and secondary metabolite profiles of *Streptomyces* sp. RC4. Seven different media, i.e., International *Streptomyces* Project (ISP) 1, ISP2, ISP4, A1, Starch Casein Broth (SCB), Potato Dextrose Broth (PDB), and PC-1, were used as growth media. Three different solvents, i.e., distilled water, 2.5% NaCl solution, and artificial seawater (ASW), were used to prepare each medium. The cultures were then shaken at 200 rpm for seven days, after which secondary metabolites were extracted with ethyl acetate. Each extract was tested for antibacterial activity and cytotoxicity using brine shrimp lethality assays (BSLT). The result showed that 11 of 21 extracts exhibited antimicrobial activity with varying levels of effectiveness. ISP2 extract (ISP2 dissolved in distilled water) demonstrated the largest inhibition zones, while SCB NaCl extract (SCB dissolved in 2.5% NaCl solution) inhibited the highest number of test microbes. Three extracts (ISP2, PC-1 ASW, and SCB NaCl extracts) were classified as highly toxic based on Clarkson's toxicity category (LC_{50} < 100 μg/mL). The most toxic extract was PC-1 ASW extract (PC-1 dissolved in ASW) with an LC_{50} value of 40 μg/mL. Metabolomic analysis revealed distinct chemical profiles for each extract, with secondary metabolites such as cyclo (Leu-Pro), cyclo (Pro-Val), *p*-cymene, o-cymene, and methoxy-phenyl oxime. Several compounds remained unidentified through molecular networking analysis, suggesting they might be novel. These findings indicated the diversity of secondary metabolites and biological activities of *Streptomyces* sp. RC4 is significantly influenced by medium and solvent compositions.

Keywords: Antimicrobial activity, metabolomic, molecular networking, *Streptomyces*, toxicity

Abbreviations: ASW: Artificial seawater; BGC; Biosynthetic Gene Custer; BSLT: Brine Shrimp Lethality Test; DHA: Docosahexaenoic Acid; GC-MS: Gas Chromatography-Mass Spectrometry; GNPS: Global Natural Products Social Molecular Networking; ISP: International *Streptomyces* Project; LC_{50}: median lethal concentration; LC-MS: Liquid Chromatography-Mass Spectrometry; LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry; *m/z*: mass-to-charge; NIST: The National Institute of Standards and Technology; OSMAC: One Strain Many Compound; PDA: Potato Detrose Broth; PDA: Potato Dextrose Agar; PDB: Potato Dextrose Broth; PCA: Principle Component Analysis; RT: Retention Time; SCB: Starch Casein Broth

INTRODUCTION

Streptomyces are Gram-positive filamentous bacteria with high G+C content. They are the largest genus of Actinobacteria phylum due to their abundance in almost all environments and the most prolific genus to produce various bioactive metabolites with diverse structural properties, such as antibiotics, pesticides, antitumors, antivirals, and antioxidants (Quinn et al. 2020; Liu et al. 2022; Zhang et al. 2024). However, *Streptomyces* is the predominant antibiotic producer, yielding around 5,000 known antibiotics, such as streptomycin, erythromycin, tetracycline, chloramphenicol, and rifampicin (Subramani and Aalbersberg 2012; Jelić and Antolović 2016).

Next-generation sequencing has extensively sequenced *Streptomyces* genus. Analysis of *Streptomyces* genomic data has revealed that *Streptomyces* sp. is rich in secondary metabolic biosynthetic gene clusters (BGCs) and typically encodes 25-70 secondary metabolic BGCs; for example, *Streptomyces* sp. RS2 contains 28 secondary metabolic BGCs (Belknap et al. 2020; Nofiani et al. 2023). Even though they are rich secondary metabolic BGCs, not all of these BGCs are active when cultivated under laboratory conditions. Only 10% of secondary metabolic BGCs are active in the biosynthesis of secondary metabolites under standard laboratory conditions (Liu et al. 2021).

One of the strategies to activate silent secondary metabolic BGCs is to use the One Strain Many Compounds (OSMAC) strategy. This strategy was carried out by altering simple culturing parameters such as media compositions, trace elements, and physical parameters (i.e., pH and temperature) (Zong et al. 2022; Martín-Aragón et al. 2023; Zhang et al. 2024). The ratio between carbon and nitrogen in media can influence secondary metabolite production. The lower or higher nitrogen content can produce specific

secondary metabolites for certain microorganisms. The reduced nitrogen content can increase Docosahexaenoic Acid (DHA) production in *Thraustochytriidae* sp. PKU#SW8 (Chen et al. 2020), whereas clavulanic acid production in *Streptomyces clavuligerus* F613-1 increases with a high nitrogen content (Fu et al. 2023). Trace metals added in the growth media also influence secondary metabolite production. For example, *Streptomyces griseorubiginosus* fermented under standard conditions (FA-1 medium) produces anthraquinone and anthrone derivatives; however, the use of SAF medium produces five different secondary metabolites such as N-acetyltryptamine, N-acetyltyramine, N-(4-hydroxyphenethyl)propionamide, JBIR-94, and terrestribisamide (Martín-Aragón et al. 2023). *Streptomyces globisporus* SCSIO LCY30 produces three angucyclines: mayamycin A, mayamycin B, and rabolemycin; two streptophenazines (streptophenazin O and M); and a macrolide dimeric dinactin using OSMAC strategy (Li et al. 2024). The other OSMAC strategy was co-culture, such as co-culture between *Streptomyces lividans* TK23 and *Tsukamurella pulmonis* TP-B0596 (a mycolic acid-containing bacterium) inducing *S. lividans* TK23 to produce red pigment (Onaka et al. 2011).

The high isolation of secondary metabolites from *Streptomyces* sp. often results in re-isolating previously identified or known compounds. Metabolomic analysis using instruments such as liquid or gas chromatography coupled with mass spectrometry (LC-MS or GC-MS) or Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) can overcome this issue and have advantages such as being highly sensitive, flexible, and widely applicable. LC-MS, LC-MS/MS, or GC-MS generate massive amounts of spectral datasets that can be used as queries to search for matching spectra in the GC-MS or LC-MS database. In addition, GC-MS or LC-MS/MS spectral data can be further analyzed using the online platform Global Natural Products Social Molecular Networking (GNPS) to identify compound structures present in a sample (Wang et al. 2016). Numerous programs, such as molecular networking, are available in the GNPS to search for a compound and its derivative by aligning MS spectral data with one another from GNPS spectral libraries to form nodes (precursor mass). For example, GNPS molecular networking identifies stenothricin derivatives from *Streptomyces* sp. DSM5940 extract (Wang et al. 2016). Unidentified MS spectral data in molecular networking can indicate potency to gain new compounds.

In a previous study, *Streptomyces* sp. RC4 showed high antimicrobial activity when the strain was cultivated on an ISP1 medium dissolved in Artificial Seawater (ASW) supplemented with the trace elements (Nofiani et al. 2020). Metabolomic analysis of *Streptomyces* sp. RC4 extract by molecular networking successfully identified a known compound, dactinomycin clusters (Nofiani et al. 2020). This study aimed to investigate the effects of the media and solvents on the secondary metabolite production of *Streptomyces* sp. RC4, the antimicrobial and cytotoxic activities, and secondary metabolite profiles using a metabolomic approach.

MATERIALS AND METHODS

Preparation of *Streptomyces* sp. RC4 isolate

The glycerol stock of *Streptomyces* sp. RC4 was defrosted, and one loop was taken using an inoculation loop and then inoculated on ISP2 solid media. Then, it was incubated at 30°C for 7-10 days or until spores appeared.

Production of *Streptomyces* sp. RC4 isolate and extraction of its secondary metabolites

One petri dish of *Streptomyces* sp. RC4 spores were resuspended with 3-5 mL of sterilized distilled water, then one mL of spore suspension was inoculated into 50 mL of ISP2 broth at pH 7 and shaken at 200 rpm, 30°C. After three days, one mL of the culture was centrifugated at 10,000 x g for 1 min, and the pellet was weighed. The pellet was resuspended in sterilized distilled water with a certain volume to obtain 0.1% w/v of the starter concentration. The starter (0.1% w/v) was inoculated into each medium and incubated at 200 rpm for seven days to obtain a bacterial culture. Seven broth media used in this study were ISP1 medium (tryptone 5 g/L; yeast extract 3 g/L), ISP2 medium (yeast extract 4.0 g/L; malt extract 10 g/L; dextrose 4 g/L), ISP4 medium (soluble starch 10 g/L; $MgSO_4{\cdot}7H_2O$ 1 g/L; NaCl 1 g/L; $(NH_4)_2SO_4$ 2 g/L; $CaCO_3$ 2 g/L; 1 mL of trace solution [$FeSO_4{\cdot}7H_2O$ 0.1 g; $MnCl_2{\cdot}4H_2O$ 0.1 g; $ZnSO_4{\cdot}7H_2O$ 0.1 g; 100 mL dH_2O]), A1 (soluble starch, 10 g/L; yeast extract, 4.0 g/L; peptone, 2.0 g/L); SCB media (soluble starch 10 g/L, casein 0.3 g/L, NaCl 2 g/L, KNO_3 2 g/L; K_2HPO_4 2 g/L; $MgSO_4{\cdot}7H_2O$ 0.05 g/L; $CaCO_3$ 0.02 g/L; $FeSO_4{\cdot}7H_2O$ 0.01 g/L), PDB media, and PC-1 medium (soluble starch 10 g/L, peptone 10 g/L; meat extract 10 g/L; sugar cane molasses 10 g/L)] (Shirling and Gottlieb 1966; Tangerina et al. 2021). Each medium was prepared in three different solvents: distilled water, NaCl 2.5 % solution, and ASW (NaCl 24.66 g/L; KCl 0.67 g/L; $CaCl_2{\cdot}2H_2O$ 1.36 g/L; $MgSO_4{\cdot}7H_2O$ 6.29 g/L; $MgCl_2{\cdot}6H_2O$ 4.66 g/L; $NaHCO_3$ 0.18 g/L), and then pH was adjusted to 7.

The secondary metabolites in each bacterial culture were extracted for each culture using ethyl acetate as a solvent with a ratio of 1 to 1 as much as three times. The organic layer was collected, and its solvent was evaporated using a rotary evaporator to obtain crude extract. The crude extract re-dissolved in methanol was de-fatted using n-hexane and then dried to get the extract. Finally, the extract was dissolved in methanol for antimicrobial and brine shrimp lethality tests and GC-MS analysis.

Antimicrobial test of *Streptomyces* sp. RC4 extracts

Antibacterial activity of *Streptomyces* sp. RC4 was tested using the agar well diffusion method against eight test bacteria (Nofiani et al. 2024). The test bacterial culture was prepared by inoculation of each test bacterium (*Bacillus subtilis* ATCC 6051, *Escherichia coli* ATCC 11775, *Pseudomonas aeruginosa*, *Staphylococcus aureus* ATCC 12600, *Salmonella enterica* ATCC 14028, *Streptococcus mutans*, *Salmonella typhi*) into nutrient broth (NB) medium and grown overnight at 200 rpm, 37°C. The test fungus (*Candida albicans* ATCC 10231) was inoculated on PDB and incubated at 200 rpm, 30°C for two days. 1 mL of each

bacterial or fungal culture mixed with 15 mL of warm nutrient agar (NA, for the test bacterium) or potato dextrose agar (PDA, for the test fungus) and plated in a Petri dish with a diameter of 9 cm. Then, it was punched into a hole using a sterilized puncher, and 20 µL the extract (25 µg/µL) was dispensed into the hole and then incubated at 37°C, 1-2 days for the test bacterium or 30°C for the test fungus. The formation of a clear zone was observed, and the diameter of the clear zone of the well was measured using a caliper.

Cytotoxicity of *Streptomyces* sp. RC4 extracts determined using the Brine Shrimp Lethality Test (BSLT)

The cytotoxic test was conducted using BSLT as described by Meyer et al. (1982). For two days, Nauplii was prepared by hatching brine shrimp eggs, *Artemia salina* Linnaeus 1758, into seawater at room temperature. Ten nauplii in 700 µL of seawater were added twenty µL of each extract with various concentrations (200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6 µg/mL, 3.125 µg/mL and 0 µg/mL), 20 µL methanol as solvent control and $K_2Cr_2O_7$ as a positive control, then re-added seawater until 1 mL and incubated at room temperature 24 hours. Finally, the dead nauplii were counted using a magnifying glass to obtain the median lethal concentration (LC_{50}) and counted by logit and probit equations with a 95% confidence level using IBM SPSS statistic 26. All the test was conducted in quadruplicate.

Analysis of secondary metabolite profiles of *Streptomyces* sp. RC4 extracts GC-MS

The GC-MS Shimadzu QP2010 Ultra installed with SH-Rxi-5Sil-MS column (0.25mm x 30.0 m, 0.25 µm) was used to analyze the selected *Streptomyces* sp. RC4 extracts. 1 µL (0.25 µg/mL) of each extract was injected at 240°C with split mode and 70°C column oven temperature, helium as carrier gas, and linear velocity as the flow control mode. The further detailed method is described in Table 1. The GC-MS raw data was deposited on MassIVE MSV000095999.

The National Institute of Standards and Technology (NIST) 20 Mass Spectral Library was used to predict volatile compounds detected on the GC-MS spectrums using the software in the machine. In addition, the GC-MS raw data were converted to mzML files using MSConvert software (Chambers et al. 2012) before metabolomic analysis using molecular networking from Global Natural Products Social Molecular Networking (GNPS). The integration and deconvolution of GC-MS data (mzML files) were processed to use MSHub-GC (Wang et al. 2016; Aksenov et al. 2021) and was deposited on https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=38348a13159d48f6a82e1ffbcd505068. Molecular networking of GC-MS data for all extracts was constructed using Molecular-Librarysearch-GC software from GNPS (Wang et al. 2016; Aksenov et al. 2021) and was visualized and annotated using Cytoscape version 3.10.1 (Shannon et al. 2003). The molecular networking result was available on https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=c8bb3abfcdc447edae35fe2216e6a9b7. Furthermore, each GC-MS raw data was extracted mass-to-charge (*m/z*) value using MZmine 3.9.0 software (Schmid et al. 2023) and then subjected to Principle Component Analysis (PCA) technique in the Unscrambler X statistical software to determine the correlation or discrimination among the extracts (Martens et al. 1987).

RESULTS AND DISCUSSION

Antimicrobial activity of *Streptomyces* sp. RC4 extracts

Each *Streptomyces* sp. RC4 extract was evaluated for antimicrobial activities using the test microbes. The test microbes used in this study were grouped into Gram-positive (*B. subtilis* ATCC 6051, *S. aureus* ATCC 12600), Gram-negative (*E. coli* ATCC 11775, *P. aeruginosa*, *S. enterica* ATCC 14028, *S. mutans*, *S. typhi*,) and fungi (*C. albicans* ATCC 10231). The results showed that not all extracts possess antimicrobial activity (Table 2). Ten of the twenty-one extracts that showed no antimicrobial activity were ISP1 NaCl, ISP2 NaCl, ISP2 ASW, ISP4, ISP4 NaCl, PC-1, PC-1 NaCl, A1, A1-NaCl, and PDB ASW extracts. The other extracts showed antimicrobial activity against various test microbes representing Gram-positive, Gram-negative, and fungi. For example, PDB NaCl extract could inhibit *B. subtilis*, *P. aeruginosa*, *S. entericca* ATCC 14028, *S. mutans*, and *C. albicans* ATCC 10231. SCB NaCl extract could inhibit all test microbes except *S. typhi* (Table 2). SCB NaCl extract showed the best antimicrobial activities based on the number of test microbes.

Table 1. GC-MS method used to analyze the selected *Streptomyces* sp. RC4 extract

Description	
Pressure	: 71.7 kPa
Total flow	: 26.7 mL/min
Column flow	: 1.13 mL/min
Linear velocity	: 39.0 cm/sec
Purge flow	: 3.0 mL/min
Split ratio	: 20.0
High-pressure injection	: Off
Carrier gas saver	: Off
Splitter hold	: Off
Oven temp. program	
Rate	: 4
Temperature (°C)	: 70
	: 300
Hold time (min)	: 2
[GC Program]	
Ionsourcetemp	: 230.00°C
Interface temp.	: 280.00°C
Solvent cut time	: 3.00 min
Detector gain mode	: Relative to the Tuning Result
Detector gain	: 1.00 kV +0.40 kV
Threshold	: 100
[MS Table]	
Start time: 3.00 min	: 3.00 min
End time: 65.00 min	: 65.00 min
ACQ Mode: Scan	: ACQ Mode: Scan
Event time	: 0.30sec
Scan speed	: 2,000
Start to end m/z	: 50.00-550.00

The diameter of the inhibition zone can be used to classify antimicrobial activity levels. Low, moderate, and high antimicrobial activity levels were classified by inhibition zone diameters of less than 5 mm, 5-10 mm, and greater than 10 mm, respectively (Nofiani et al. 2024). Eleven active extracts exhibited different levels of antimicrobial activity against eight tested microbes ranging from low to moderate levels. The ISP2 extract showed better antimicrobial activity than other extracts, with moderate antimicrobial activity against six tested microbes, but did not inhibit the growth of *S. typhi* and *C. albicans* ATCC 10231 (Table 2). Seven different media have different carbon, nitrogen, and mineral sources that play a significant role in microbial growth and secondary metabolite production. The carbon sources determine the balance between microbial growth and secondary metabolite production; nitrogen sources are responsible for amino acid biosynthesis, essential in the biosynthesis of polyketide and non-ribosomal peptide compounds (Das et al. 2023). Yeast extract, peptone, malt extract, meat extract, tryptone, and sugarcane molasses are among the seven media constituents with unknown chemical compositions, complicating ascertaining their function in the biosynthesis of secondary metabolites, especially antimicrobial chemical compounds. ISP1 containing yeast extract and tryptone) is categorized as a less complex medium than ISP2 (yeast extract, malt extract, and dextrose), while ISP4 and SCB are rich in minerals (Shirling and Gottlieb 1966; Tangerina et al. 2021). Malt extract in ISP2 can increase antimicrobial compound in *Streptomyces* sp. (Rammali et al. 2024).

The replacement of the distilled water in the ISP2 medium for cultivating *Streptomyces* sp. RC4 with the 2.5% NaCl solution or ASW solvents resulted in a loss of antimicrobial activity (Table 2). The NaCl in both media (the 2.5 % NaCl solution and ASW) in the ISP2 medium probably inhibited the biosynthesis of antimicrobial compounds in *Streptomyces* sp. RC4.However, the 2.5% NaCl solution as a solvent in the SCB medium might induce the synthesis of antimicrobial chemicals in *Streptomyces* sp. RC4 compared to distilled water and ASW. The SCB NaCl extract could still inhibit a test fungus and 5 test bacteria except for *S. typhi,* whereas the SCB and SCB ASW extracts could only inhibit four and one of the test microbes, respectively. The antimicrobial activity of the SCB NaCl extract was lower for most test microbes than the ISP2 extract. The low activity level of the SCB extract was probably caused by low concentration or less active antimicrobial compounds produced by *Streptomyces* sp. RC4.

Salinity can affect microbial growth. Optimal microbial growth occurs when the salinity and medium are fit in physochemical microbes. The inappropriate salinity and the medium can elevate osmotic pressure in the microbial cell system, resulting in cellular dehydration and disrupting microbial metabolic processes (Pan et al. 2019; Quinn et al. 2020). Microbes activate silent secondary metabolite gene clusters to biosynthesize specific secondary metabolites to restore the effect of salinity (Pan et al. 2019), including isoprenoids (and derivatives like terpenes), betaine, glutamate, polyols (mannitol), amino acids (proline) (Pardo-Esté et al. 2024). For example, *B. subtilis* LB7 produces more secondary metabolites in a medium with NaCl 1.5 M than 0.6 M NaCl (Pardo-Esté et al. 2024). Based on these results, compatibility between medium and solvent is crucial to induce the specific biosynthesis of antimicrobial compounds, particularly *Streptomyces* sp. RC4.

Table 2. Antimicrobial activities of *Streptomyces* sp. RC4 extracts prepared with different media and solvents

Extract name	Media	Solvent	Dose (µg/well)	Diameter of inhibition zone (mm)							
				BS	EC	PA	SA	SE	SM	ST	CA
ISP1	ISP1	Distilled water	500	3.9	0	5	0	5.3	5.3	0	6
ISP1 NaCl	ISP1	NaCl 2.5% solution	500	0	0	0	0	0	0	0	0
ISP1 ASW	ISP1	ASW	500	3.9	0	6	0	3.3	5.4	0	0
ISP2	ISP2	Distilled water	500	6.8	5.5	9	6	5.3	5.4	0	0
ISP2 NaCl	ISP2	NaCl 2.5% solution	500	0	0	0	0	0	0	0	0
ISP2 ASW	ISP2	ASW	500	0	0	0	0	0	0	0	0
ISP4	ISP4	Distilled water	500	0	0	0	0	0	0	0	0
ISP4 NaCl	ISP4	NaCl 2.5% solution	500	0	0	0	0	0	0	0	0
ISP4 ASW	ISP4	ASW	500	3.4	0	4.7	0	4.5	0	0	4.9
PC-1	PC-1	Distilled water	500	0	0	0	0	0	0	0	0
PC-1 NaCl	PC-1	NaCl 2.5% solution	500	0	0	0	0	0	0	0	0
PC-1 ASW	PC-1	ASW	500	6.1	6	4.4	0	4.5	3.2	0	1.4
A1	A1	Distilled water	500	3.1	0	4.1	0	3.4	5.4	0	3.7
A1 NaCl	A1	NaCl 2.5% solution	500	0	0	0	0	0	0	0	0
A1 ASW	A1	ASW	500	0	0	0	0	0	0	0	0
PDB	PDB	Distilled water	500	0	0	3.1	0	3.4	3.2	0	6
PDB NaCl	PDB	NaCl 2.5% solution	500	4.8	0	7.1	0	6.9	3.2	0	8.3
PDB ASW	PDB	ASW	500	0	0	0	0	0	0	0	0
SCB	SCB	Distilled water	500	3.1	0	0	0	4.1	4	0	7
SCB NaCl	SCB	NaCl 2.5% solution	500	6.4	3.9	4.1	3.8	3.7	4	0	7.6
SCB ASW	SCB	ASW	500	3.9	0	0	0	0	0	0	0
Tetracyclin (positive control for Gram-positive and Gram-negative bacteria)			10	20.8	30	24.5	20.8	19.1	18.7	13	0
Nystatin (positive control for fungi)			20 µL	0	0	0	0	0	0	0	14
Methanol (negatif control)			20 µL	0	0	0	0	0	0	0	0

Notes: BS: *Bacillus subtilis* ATCC 6051; EC: *Escherichia coli* ATCC 11775; PA: *Pseudomonas aeruginosa*; SA: *Staphylococcus aureus* ATCC 12600; SE: *Salmonella entericca* ATCC 14028; SM: *Streptococcus mutans*; ST: *Salmonella typhi*; CA: *Candida albicans* ATCC 10231

Cytotoxicity of *Streptomyces* sp. RC4 extracts

BSLT can be used as a preliminary test for various biological activities such as cytotoxic (Meyer et al. 1982; Omeke et al. 2018) and pesticide activities (Ullah et al. 2013). Tawaha (2006) also reports a positive correlation between brine shrimp toxicity and 9KB (human epidermoid carcinoma of the nasopharynx) cytotoxicity (p: 0.036 and Kappa: 0.56), while antitumor activity is confirmed with in vitro cytotoxicity and 3PS (in vivo P388 murine leukemia) activity (p: 0.033-0.0334) (Tawaha 2006).

LC_{50} values were different for each *Streptomyces* sp. RC4 extract ranging from 40 to 526 µg/mL (Table 3). Some researchers have classified the toxicity levels based on LC_{50} value. Tanamatayarat is classified as highly toxic (LC_{50}<10 mg/mL), moderately toxic (LC_{50}: 10-100 mg/mL), weakly toxic (LC_{50}: 100-1,000 mg/mL), and inactive (LC_{50}>1,000 mg/mL) (Tanamatayarat 2016), while Clarkson's classification is no toxic (LC_{50} above 1,000 µg/mL), low toxic (LC_{50} 500-1,000 µg/mL), medium toxic (LC_{50} 100-500 µg/mL), and highly toxic (LC_{50}: 0-100 µg) (Meena et al. 2020).

All extracts exhibited toxicity levels from high to medium based on Clarkson's category or moderate to weak based on Tanamatayarat's category (Table 3). Three extracts are classified as high toxicity according to Clarkson's category, namely ISP2, PC-1 ASW, and SCB NaCl. The lowest LC_{50} value was 40 µg/mL for PC-1 ASW extract, while the LC_{50} value for the $K_2Cr_2O_7$ (the positive control) was 10 µg/mL. Cyclophosphamide, a medication used to treat cancer, had an LC50 value of 16 µg/mL (Mbusi et al. 2024). Comparing the LC_{50} value of PC-1 ASW extract with that of the positive control and cyclophosphamide, classified as moderately toxic, indicated that PC-1 ASW extract has the potential for antitumor activity. Consequently, PC-1 ASW extracts are recommended for further study regarding antitumor or cytotoxic activities.

Three extracts of *Streptomyces* sp. RC4 had high cytotoxicity, namely ISP2, PC-1 ASW, and SCB NaCl extracts. The results indicated that the solvent used for preparing media affects biosynthesis or production of secondary metabolites. *Streptomyces* sp. RC4 cultivated on PC-1 medium dissolved in three different solvents (distilled water, NaCl 2.5% solution, ASW) exhibited varying cytotoxicity levels (Table 3). *Streptomyces* sp. RC4 cultured in PC-1 medium dissolved in the ASW showed the highest toxicity level compared to the other solvents, the distilled water and NaCl 2.5%. The ASW ingredients are rich in salts such as NaCl, KCl, $CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, $MgCl_2{\cdot}6H_2O$, and $NaHCO_3$, whereas the distilled water and 2.5% NaCl solution contains lower salts.

Aside from NaCl, certain salts in the ASW PC-1 might stimulate or enhance the production of secondary metabolites from *Streptomyces* sp. RC4 in the PC-1 medium, leading to a high nauplii mortality rate. The LC_{50} value of PC-1 dissolved in the distilled water or the NaCl 2.5% was higher than PC-1 in the ASW, which means the cytotoxicity was decreased drastically compared to that of the PC1-ASW. Trace metals such as Fe, Co, Cu, Mn, Ni, Zn, Mg, and Vanadium (V) can induce silent secondary metabolite biosynthetic gene clusters (Locatelli et al. 2016; Zong et al. 2022). Siderophore biosynthesis in the Streptomyces genus occurred under an iron-sufficient medium (Locatelli et al. 2016). In addition, $MgSO_4{\cdot}7H_2O$, KCl, K_2HPO_4, and KH_2PO_4 increase secondary metabolite production in *Streptomyces* sp. RUPA-08PR, particularly antimicrobial compounds (Ripa et al. 2009).

Table 3. LC_{50} value of *Streptomyces* sp. RC4 extracts prepared with different media and solvents

Extract name	Medium	Solvent	LC_{50}, µg/mL	Model logit	Toxicity level	
					Clarkson (Meena et al. 2020)	Tanamatayarat (Tanamatayarat 2016)
ISP1	ISP1	Distilled water	185	Log [p/(1-p)] = (-3.153) + 3.215 x	Medium	Weak
ISP1 NaCl	ISP1	NaCl 2.5% solution	367	Log [p/(1-p)] = (-2.256) + 2.099 x	Medium	Weak
ISP1 ASW	ISP1	ASW	154	Log [p/(1-p)] = (-3.916) + 4.068 x	Medium	Weak
ISP2	ISP2	Distilled water	60	Log [p/(1-p)] = (-1.727) + 0.029 x	High	Moderate
ISP2 NaCl	ISP2	NaCl 2.5% solution	294	Log [p/(1-p)] = (-2.739) + 2.622 x	Medium	Weak
ISP2 ASW	ISP2	ASW	375	Log [p/(1-p)] = (-2.496) + 2.349 x	Medium	Weak
ISP4	ISP4	Distilled water	259	Log [p/(1-p)) = (-3.075) + 0.012 x	Medium	Weak
ISP4 NaCl	ISP4	NaCl 2.5% solution	344	Log [p/(1-p)) = (-2.208) + 2.044 x	Medium	Weak
ISP4 ASW	ISP4	ASW	278	Log [p/(1-p)) = (-2.964) + 2.809 x	Medium	Weak
PC-1	PC-1	Distilled water	526	Log [p/(1-p)) = (-2.060) + 1.866 x	Medium	Weak
PC-1 NaCl	PC-1	NaCl 2.5% solution	337	Log (p/(1-p)) = (-2.540) + 2.418 x	Medium	Weak
PC-1 ASW	PC-1	ASW	40	Log (p/(1-p)) = (-2.496) + 2.658 x	High	Moderate
A1	A1	Distilled water	293	Log (p/(1-p)) = (-3.185) + 3.022 x	Medium	Weak
A1 NaCl	A1	NaCl 2.5% solution	323	Log (p/(1-p)) = (-2.540) + 2.418 x	Medium	Weak
A1 ASW	A1	ASW	294	Log (p/(1-p)) = (-2.739) + 2.622 x	Medium	Weak
PDB	PDB	Distilled water	400	Log (p/(1-p)) = (-3.100) + 2.836 x	Medium	Weak
PDB NaCl	PDB	NaCl 2.5% solution	372	Log (p/(1-p)) = (-3.783) + 3.446 x	Medium	Weak
PDB ASW	PDB	ASW	361	Log (p/(1-p)) = (-2.470) + 2.307 x	Medium	Weak
SCB	SCB	Distilled water	227	Log (p/(1-p)) = (-3.452) + 3.388 x	Medium	Weak
SCB NaCl	SCB	NaCl 2.5% solution	64	Log (p/(1-p)) = (-3.014) + 3.523 x	High	Moderate
SCB ASW	SCB	ASW	334	Log (p/(1-p)) = (-3.236) + 3.236 x	Medium	Weak
$K_2Cr_2O_7$ (a positive control)			10	Log (p/(1-p)) = (-3.986) + 3.908 x	High	Moderate

Streptomyces sp. RC4 cultured in the ISP2 medium, which dissolved using the ASW, had a lower toxicity level than that dissolved in 2.5% NaCl solution or distilled water. The ISP2 medium in NaCl also reduced the toxicity levels of *Streptomyces* sp. RC4. The SCB medium dissolved in a NaCl 2.5% solution increased optimal secondary metabolite production, which is responsible for cytotoxicity. The SCB medium in ASW caused NaCl content to increase from 2 g/L to 26.66 g/L, including the other minerals, and lower the cytotoxicity. Ripa et al. (2009) report that the antimicrobial compounds of *Streptomyces* sp. RUPA-08PR is optimum in the presence of 1% NaCl. The results indicated that a particular ingredient or interaction among ingredients in a medium or solvent can influence secondary metabolite profile characteristics on *Streptomyces* sp. RC4.

Secondary metabolite profiles of *Streptomyces* sp. RC4 extracts using GC-MS

The chemical profiles of the selected *Streptomyces* sp. RC4 extracts (ISP2, ISP2 NaCl, SCB, SCB NaCl, and SCB ASW extracts) were evaluated using GC-MS analysis. The correlation or discrimination of each extract calculated using PCA based on the GC-MS *m/z* values exhibited 60% and 25% total variability for principal components 1 and 2 based on the *m/z* values (Figure 1). Almost all the extracts showed no correlation except for ISP2 NaCl and SCB extracts (Figure 1). The smaller the angle formed between extracts, the stronger the correlation. The ISP2 and SCB ASW extracts had high toxicity levels, with comparable LC_{50} values of 60 μg/mL for the ISP2 extract and 64 μg/mL for the SCB NaCl extract (Table 3). However, their antimicrobial activity levels differed (Table 2). Both extracts showed no correlation based on the PCA score plot, indicating differences in secondary metabolite profiles (Figure 1). The GC-MS *m/z* values analyzed by PCA demonstrated a strong correlation or similarity in secondary metabolites between the SCB and ISP2 NaCl extracts. These results were consistent with the toxicity level classification of SCB and ISP2 NaCl extracts categorized as medium level (Table 3). Meanwhile, the SCB extract showed low antimicrobial activity against four test microbes, while the ISP2 NaCl extract exhibited no antimicrobial activity (Table 2).

The further GC-MS data from the selected extracts were used as a query against the GC-MS NIST 20 Mass Spectral Library to identify their compounds. The results were re-analyzed manually to eliminate background contaminating ions determined from the GC-MS data, such as polyethylene glycol, polypropylene glycol, phthalates, solvent modifiers, metal ions, tritons, tweens, and siloxanes. In addition, the compounds with a similarity value of ≥ 75% were presented in Table 4. All extracts contained aromatic, cyclic dipeptides, monoterpenes, and hydrocarbons (Table 4). ISP2 NaCl, SCB, SCB NaCl, and SCB ASW extracts except ISP2 extract were predicted to contain cyclo (Pro-Val) in varying quantities: 33% in ISP2 NaCl, 13% in SCB, 11% in SCB NaCl, and 35% in SCB ASW (Table 4). Cyclo (Pro-Val) isolated from various microorganisms, e.g., *Lysobacter capsici* AZ78 and *Nocardiopsis alba* DP1B, can inhibit phytopathogenic microorganisms (biopesticides) (Cimmino et al. 2021; Nofiani et al. 2024).

ISP2 NaCl extract also contained the predicted cyclo (Leu-Pro), reaching approximately 27% based on the area percentage. Cyclo (Leu-Pro) shows antimicrobial activity (Salman et al. 2022; Kim et al. 2023). ISP2 NaCl extract containing cyclo (Pro-Val) and cyclo (Leu-Pro) did not have antimicrobial activity, and the ISP2 extract that did not contain cyclo (Pro-Val) exhibited the best antimicrobial activities, indicating that cyclo (Pro-Val) or cyclo (Leu-Pro) was not responsible for antimicrobial activity. Meanwhile, all the extracts except ISP2 NaCl extract contained predicted methoxy-phenyl oxime. Methoxy-phenyl oxime has been reported to have an antitumor activity (Chipps et al. 2012). However, the methoxy-phenyl oxime might not be responsible for extract toxicity. Based on these results, unidentified compounds by GC-MS were probably involved in the antimicrobial activity and toxicity.

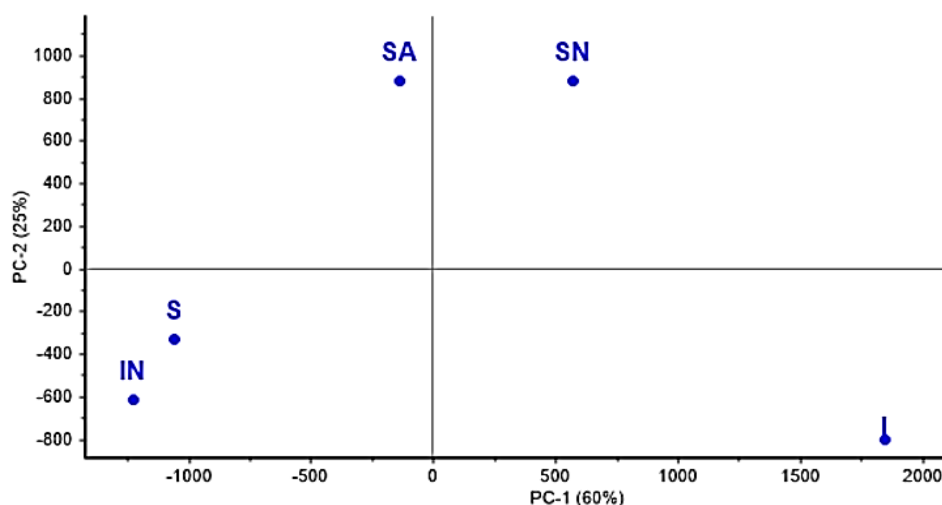

Figure 1. PCA score plot of *Streptomyces* sp. RC4 extracts based on the variability of GC-MS *m/z* value data. I: ISP2 media; IN: ISP2 NaCl extract. S. SCB; SN. SCB NaCl; SA. SCB ASW

Two monoterpenes, *o*-cymene, and *p*-cymene, were part of essential oils in ISP2 and ISP2 NaCl extracts, respectively. *P*-cymene has various pharmacological properties, including antimicrobial activity (Tian et al. 2018), antioxidant, anti-inflammatory, antiparasitic, antidiabetic, antiviral, and antitumor activities (Balahbib et al. 2021). *P*-cymene is a major compound (20%) in ISP2 extract based on the area percentage and probably contributed to antimicrobial activity

GC-MS datasets from five *Streptomyces* sp. RC4 extracts generated 24 clusters that composed 91 nodes (precursor mass), although only half of the total nodes were identified using the GNPS database through molecular networking analysis. The number of predicted compounds generated from searching with the GNPS database in the molecular networking was more and generally distinct from that of the GC-MS NIST 20 Mass Spectral Library (Table 4). The predicted p-cymene was discovered in both databases (Table 4, Figure 2), while cyclo (Pro-Val) and cyclo (Leu-Pro) were only recognized in the GC-MS NIST 20 Mass Spectral Library.

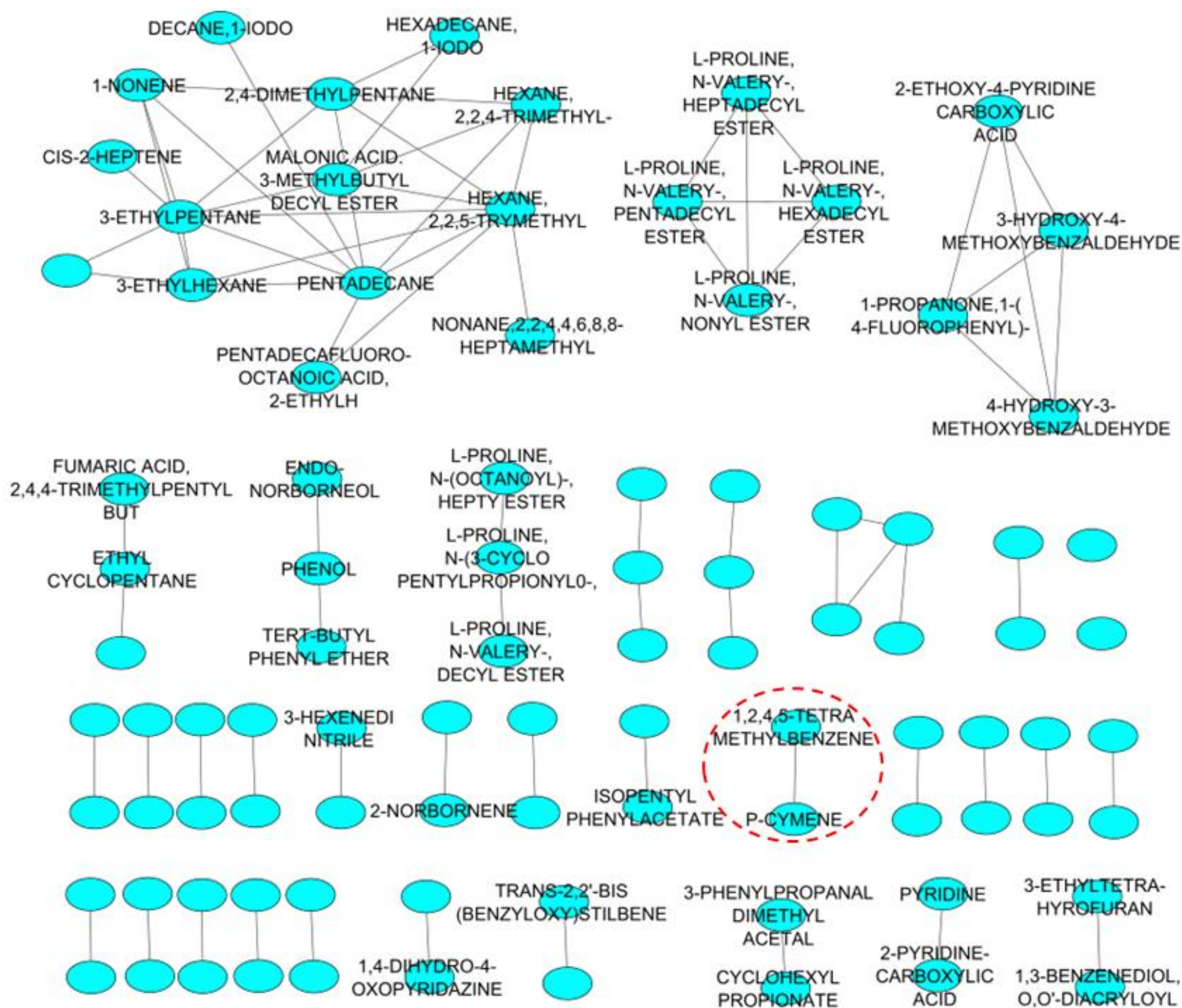

Figure 2. Molecular networking of GC-MS datasets from five *Streptomyces* sp. RC4 extracts. Tosca circle: node

Table 4. Predicted compounds of the selected *Streptomyces* sp. RC4 extracts cultured in different media and solvents based on comparisons of the spectral GC-MS data with the GC-MS NIST 20 mass spectral library

Peak No.	RT	Area%	Similarity	Base *m/z*	Predicted compound	Group
ISP2 extract						
2	3.962	6.94	79	133.05	Methoxy-phenyl oxime	Aromatic
3	4.521	4.92	86	108.10	Anisole	Aromatic
4	5.785	17.59	90	94.10	Phenol	Phenol
5	6.198	14.82	94	57.10	Decane	Hydrocarbon
6	6.950	20.05	93	119.10	*p*-Cymene	Monoterpene
ISP2 NaCl extract						
2	6.204	8.96	91	57.10	Decane	Hydrocarbon
3	6.957	3.16	83	119.15	*o*-Cymene	Monoterpene
7	30.493	32.67	91	70.10	Cyclo (Pro-Val)	Cyclic dipeptide
8; 9	32.909; 33.339	7.09; 16.51	86; 92	70.10	Cyclo (Leu-Pro)	Cyclic dipeptide
SCB extract						
3	3.947	9.91	86	133.10	Methoxy-phenyl oxime	Aromatic
4	4.121	5.21	87	55.10	Cyclohexanone	Cyclic ketone
5	6.199	10.31	89	57.10	Decane	Hydrocarbon
6	6.941	4.25	82	119.20	Benzene, tert-butyl-	Aromatic hydrocarbon
11	30.483	13.11	89	70.10	Cyclo (Pro-Val)	Cyclic dipeptide
SCB NaCl extract						
3	3.952	9.28	85	133.10	Methoxy-phenyl oxime	Aromatic
4	6.207	7.57	89	57.10	Decane	Hydrocarbon
5	7.001	12.81	94	57.10	1-Hexanol, 2-ethyl-	Alcohol
12	30.500	11.22	87	70.10	Cyclo (Pro-Val)	Cyclic dipeptide
SCB ASW extract						
2	3.954	10.69	84	133.10	Methoxy-phenyl oxime	Aromatic
4	6.201	13.93	92	57.10	Decane	Hydrocarbon
6	30.497	34.47	91	70.10	Cyclo(Pro-Val)	Cyclic dipeptide
7	45.319	4.65	50	77.05	Phosphorous acid, triphenyl ester	Aromatic ester

Note: RT: Retention time

In conclusion, the specific ingredient and the compatibility of the ingredients in the medium and solvent utilized to cultivation of *Streptomyces* sp. RC4 may influence secondary metabolite profiles, which can be evaluated through biological activities and metabolomic analysis. The ISP2 medium and SCB medium dissolved in a NaCl 2.5 % solution demonstrated the highest efficacy for antimicrobial compound biosynthesis, as evidenced by the diameter of the inhibition zone and the number of inhibited test microbes, respectively. The best medium for cytotoxic activity was PC-1 ASW, exhibiting an LC_{50} value of 40 µg/mL. The result indicated that the PC-1 ASW medium was suitable for production of cytotoxic compounds. Each extract also exhibited a different chemical profile based on metabolomic analysis using GC-MS analysis, such as cyclo (Leu-Pro), cyclo (Pro-Val), *p*-cymene, *o*-cymene, and methoxy-phenyl oxime that produced on different media and solvents. *P*-cymene in ISP2 extract might be partially responsible for antimicrobial activity. The molecular networking analysis revealed that certain compounds remained unidentified, indicating they were probably the new compounds.

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