

IN SILICO IDENTIFICATION OF SECONDARY METABOLITES FROM AQUATIC *STREPTOMYCES* ISOLATE WITH ANTIYEAST AND ANTICANCER ACTIVITIES

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Abstract

This study explored the potential of bioactive metabolites from *Streptomyces* strain WHD3007-4. Isolated from a Nile tilapia pond, aquatic *Streptomyces* sp. WHD3007-4 was cultured in four media (International *Streptomyces* Project-2 Medium (ISP2), 301, Starch Casein Broth (SCB), and Bennet's Broth (BN). Its secondary metabolites were extracted using ethyl acetate and acetone at a ratio of 15:2 (v/v). The cytotoxicity of all crude extracts on the human colorectal cancer cell line HCT-116 was assessed using the MTT assay. The percentage of viable HCT-116 cells was lowest with *Streptomyces* sp. WHD3007-4 in the SCB medium (36.20%). The antiyeast activity of the crude extract from *Streptomyces* sp. WHD3007-4 in SCB medium was assessed against *Candida tropicalis* (TISTR No. 5136) using the disk diffusion method. The crude extract effectively inhibited *C. tropicalis* growth at 2 mg. Known compounds in the crude extract from *Streptomyces* sp. WHD3007-4 in different media were identified using liquid chromatography-tandem mass spectrometry, with the data processed using MZmine 3 for molecular networking and spectral library searching on the Global Natural Products Social Molecular Networking (GNPS) web platform. Several candidate anticancer molecules were identified: kynurenic acid, brevianamide F, *N*-(2-hydroxyphenyl) acetamide, and decumbenone A. These results suggest that *Streptomyces* sp. WHD3007-4 is a potential source of antiyeast and anticancer agents.

Keywords: *Streptomyces*; Secondary Metabolites; Anticancer; Antiyeast

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Introduction

Streptomyces strains are recognized as prolific sources of diverse biologically active compounds with antibacterial, antifungal, antiviral, antioxidant, anticancer, and plant growth-promoting properties (Alam *et al.*, 2022). However, the potential of *Streptomyces* remains largely untapped due to the constrained expression of secondary metabolite biosynthetic gene clusters (BGCs) under laboratory cultivation conditions or their expression at levels that may not be readily detectable (Romano *et al.*, 2018). Therefore, stimulating the expression of silent genes within BGCs is an intriguing avenue for exploring novel bioactive secondary metabolites.

The One Strain Many Compounds (OSMAC) approach has revealed that a single strain of microorganisms can produce diverse molecules upon cultivation under different environmental conditions (Romano *et al.*, 2018). Tangerina *et al.* (2020) demonstrated the metabolite production of marine *Streptomyces* sp. BRB081 varied with the growth medium. Furthermore, the OSMAC and metabolomics approaches have been combined to comprehensively explore and discover novel bioactive compounds.

This study investigated the cytotoxicity of crude extracts of *Streptomyces* sp. WHD3007-4 on human colorectal cancer cells and their inhibitory effects on *Candida tropicalis* (TISTR No. 5136). It also employed liquid chromatography-tandem mass spectrometry (LC-MS/MS) and MS-based metabolomics tools to explore the metabolite production of *Streptomyces* sp. WHD3007-4 cultured in different media.

Materials and Methods

Fermentation and extraction of secondary metabolites

The aquatic *Streptomyces* sp. WHD3007-4 was obtained from previous studies, having been isolated from water in a Nile tilapia pond in Khlong Dan, Bang Bo District, Samut Prakan Province, Thailand (Euanorasetr *et al.*, 2021). This strain was cultivated in 100 mL of liquid media for 14 days at 28°C under shaking at 200 rpm. This experiment examined four liquid media: (1) International *Streptomyces* Project 2 (ISP2; 0.4% yeast extract, 1% malt extract, 0.4% dextrose, and 60% natural seawater; Shirling and Gottlieb, 1966), (2) 301 medium (301; 2.4% starch, 1% glucose, 0.3% peptone, 0.3% meat extract, 0.5% yeast extract, 0.4% CaCO₃, and 60% natural seawater; Euanorasetr *et al.*, 2010), (3) Starch Casein Broth (SCB; 1% starch, 0.03% casein, 0.2% KNO₃,

0.005% MgSO₄·7H₂O, 0.2% K₂HPO₄, 0.2% NaCl, 0.002% CaCO₃, 0.001% FeSO₄·7H₂O, and 60% natural seawater; Euanorasetr *et al.*, 2020), and (4) Bennet's Broth (BN; 0.1% yeast extract, 0.1% beef extract, 0.2% casein enzymic hydrolysate, 1% dextrose, and 60% natural seawater; Jones, 1949). After incubation, the culture medium was extracted with 100 mL of ethyl acetate and acetone at a ratio of 15:2 (v/v). Next, the supernatant was transferred to a separatory funnel to separate the solvent from the culture medium, retaining only the solvent phase. Then, the solvent was concentrated using a rotary evaporator at a water bath temperature of 35°C until completely dry.

MTT cell viability assay

The cytotoxic effects of crude extracts of *Streptomyces* sp. WHD3007-4 on human colorectal carcinoma cells (HCT-116) were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For this assay, HCT-116 cells were seeded at a density of 2×10^3 cells per well in 96-well plates and incubated at 37°C with 5% CO₂ for 24 hours. The crude extracts (10 µg/mL in dimethyl sulfoxide [DMSO]) were added to the culture plates, which were further incubated at 37°C with 5% CO₂ for 72 hours. After incubation, serum-free medium containing MTT was added, and the plates were further incubated at 37°C with 5% CO₂ for 3 hours. Next, the serum-free medium containing MTT was removed, and the purple formazan crystals were dissolved in DMSO. Finally, the absorbance of the colored solution was measured at 570 nm using a multi-mode microplate reader. Doxorubicin and DMSO were used as the positive and negative controls, respectively.

Evaluation of the antiyeast activity of the crude extracts

The antiyeast activity of the crude extracts of *Streptomyces* sp. WHD3007-4 in SCB medium was assessed using the disk diffusion method. A *C. tropicalis* (TISTR No. 5136) suspension, adjusted to 0.5 McFarland standard turbidity, was spread over the surface of potato dextrose agar (PDA) plates. Next, sterile paper disks with 20 µL of the crude extracts at concentrations of 0.1, 1, 10, and 100 mg/mL were placed on the agar surface. Amphoterin B (10 mg/mL) and DMSO were used as the positive and negative controls, respectively. The inhibition zones were measured after overnight incubation at 37°C. The experiments were performed in triplicate.

LC-MS/MS analysis and MS data processing

The crude extracts of *Streptomyces* sp. WHD3007-4 cultivated in four different media were analyzed using LC-MS/MS with a flow rate of 0.4 mL/min for 13.13 min and a 5 μ L injection volume. The mobile phase employed a gradient of 0.02% formic acid in water and 0.016% formic acid in acetonitrile. MS was measured via heated electrospray ionization using a sheath gas flow rate of 40 (arbitrary units), an auxiliary gas flow rate of 15 (arbitrary units), and a vaporizer temperature of 317°C. The scan range was monitored from 150 to 2000 m/z. Next, the LC-MS/MS data were converted into the .mzXML file format using MSConvert software (Chambers *et al.*, 2012). Then, the converted files were processed with MZmine 3 software (Schmid *et al.*, 2023): mass detection, chromatogram creation, isotope grouping, and feature alignment. Finally, the processed data were exported in the .mgf file format.

Molecular networking and MS/MS spectral library searching

The processed MS data were submitted to the Global Natural Product Social Molecular Networking (GNPS) web platform (<https://gnps.ucsd.edu>; Nothias *et al.*, 2020). Molecular networks were constructed using feature-based molecular networking (FBMN) and spectral library searching with default values used for all parameters. Finally, Cytoscape software (Shannon *et al.*, 2003) was employed to visualize the FBMN results.

Results and Discussion

Anticancer activity of crude extracts on HCT-116 cells

This study employed the OSMAC approach to explore secondary metabolites. It cultivated *Streptomyces* sp. WHD3007-4 in four different media to comprehensively understand its potential. The total yields of extracts from *Streptomyces* sp. WHD3007-4 cultured in ISP2, 301, SCB, and BN media were 20–30 mg per 100 mL. The anticancer activities of the crude extracts were assessed by their effect on the viability of HCT-116 cells. The percentage of viable HCT-116 cells was lowest with doxorubicin (6.16%), followed by the crude extract of *Streptomyces* sp. WHD3007-4 cultured in SCB medium (36.20%), as illustrated in Figure 1.

The observed inhibitory effects of all crude extracts on HCT-116 cells revealed that they could all inhibit cancer cells. The crude extract of *Streptomyces* sp. WHD3007-4 cultured in SCB medium exhibited the greatest toxicity to HCT-116

cells. This finding is consistent with previous reports, emphasizing the significant role of *Streptomyces* as a source of bioactive compounds with anticancer properties. One example is doxorubicin, which was initially isolated from *Streptomyces peucetius* var. *caesius* (Arcamone *et al.*, 1969) and has been widely used to treat various cancers, including breast, lung, gastric, ovarian, and thyroid (Thorn *et al.*, 2011).

Antiyeast activity of crude extracts on *C. tropicalis*

Since several anticancer compounds possess antiyeast activity, the crude extract of *Streptomyces* sp. WHD3007-4 cultured in SCB medium was assessed for antiyeast activity. Its antiyeast activity against *C. tropicalis* (TISTR No. 5136) was assessed using the disk diffusion method. The zone of inhibition observed with different concentrations of the extract against *C. tropicalis* (TISTR No. 5136) is shown in Figure 2 and Table 1. Antiyeast activity was observed at 2 mg of the extract and with amphotericin B, which was used as a reference compound.

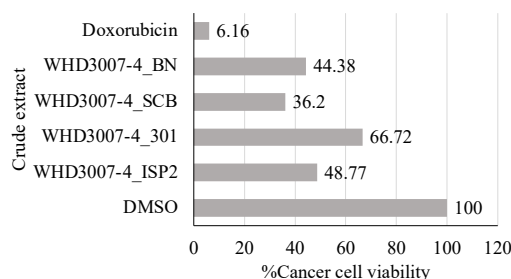


Figure 1. The cytotoxicity of *Streptomyces* sp. WHD3007-4 extracts on HCT-116 cells



Figure 2. Antiyeast activity of the crude extract of *Streptomyces* sp. WHD3007-4 cultured in SCB medium against *C. tropicalis* (TISTR No 5136). (A) 2 μ g of extract, (B) 20 μ g of extract, (C) 200 μ g of extract, (D) 2 mg of extract, (E) 200 μ g of amphotericin B, and (F) DMSO

Table 1. Zone of inhibition of different concentrations of the crude extract of *Streptomyces* sp. WHD3007-4 cultured in SCB medium against *C. tropicalis* (TISTR No. 5136)

Concentration of the crude extract	Diameter of the inhibition zone (mm)
2 µg	0
20 µg	0
200 µg	0
2 mg	10.77 ± 1.53 uc
Amphotericin B	9.67 ± 0.58

The diameter of the zone of inhibition includes the diameter of the disk (6 mm). Abbreviation: uc, unclear inhibition.

These results demonstrate that the crude extract of *Streptomyces* sp. WHD3007-4 cultured in SCB medium exhibited inhibitory effects against *C. tropicalis*. This *Candida* species is widely considered a highly virulent strain, ranking second only to *C. albicans* (Zuza-Alves *et al.*, 2017). Interestingly, 2 mg of the crude extract exhibited a zone of inhibition comparable to amphotericin B, a well-known antifungal agent. This finding underscores the importance of exploring *Streptomyces*-derived compounds with anti-*Candida* activity (Martins *et al.*, 2023).

In silico identification of compounds produced by *Streptomyces* sp. WHD3007-4

Molecular networking involves comparing all MS/MS spectra within an extract, grouping them based on similarity by establishing a cosine score. Since MS/MS spectra are linked to the chemical structure of fragmented metabolites, molecular networks can group molecules based on structural similarity (Allard *et al.*, 2016). The bioactive metabolites were further explored through metabolomics by performing molecular networking and comparing MS spectra with a library of MS/MS spectra in the GNPS platform. Several anticancer compounds were identified (kynurenic acid, brevianamide F, *N*-(2-hydroxyphenyl) acetamide, and decumbenone A) based on the cosine similarity

scoring of MS/MS spectra, as illustrated in Figure 3. The results also revealed that *Streptomyces* sp. WHD3007-4 mainly produced kynurenic acid when cultured in the 301 medium, with lower production observed in the BN and ISP2 media, respectively. *Streptomyces* sp. WHD3007-4 produced brevianamide F in all tested media, with the greatest production in 301 medium. *Streptomyces* sp. WHD3007-4 mainly produced *N*-(2-hydroxyphenyl) acetamide and decumbenone A in the ISP2 and 301 media, respectively.

This study employed high throughput dereplication of natural product MS/MS data within the GNPS platform, which involves querying newly acquired MS/MS spectra against the entirety of the accumulated reference spectra in the GNPS spectral libraries (Wang *et al.*, 2016). The application of this metabolomics tool revealed the presence of several bioactive compounds in the crude extract of *Streptomyces* sp. WHD3007-4. Among all the compounds annotated, kynurenic acid, brevianamide F, decumbenone A, and *N*-(2-hydroxyphenyl) acetamide had the largest cosine scores. Kynurenic acid has been reported to inhibit the proliferation of colon cancer cell lines HT-29, LS-180, and Caco-2 (Walczak *et al.*, 2011). Brevianamide F can be isolated from

Streptomyces sp. and is cytotoxic against the HCT-116, OVCAR-8, and SF-295 cell lines (Bojarska *et al.*, 2021). It has been reported to inhibit the growth of *C. tropicalis* and exhibit greater activity against *C. albicans* than amphotericin B (Nishanth Kumar *et al.*, 2014). *N*-(2-hydroxyphenyl) acetamide has been reported to exhibit potent activity against MCF-7 cells (Aziz *et al.*, 2019). Decumbenone A has been reported to exhibit cytotoxicity against SK-MEL-5, SK-MEL-28, and HCT-116 cancer cells (Zhuravleva *et al.*, 2012). Bioactive compounds could be discovered and purified through antiyeast- or anticancer-guided isolation, and molecular networking data could be useful for dereplication. Moreover, investigating genomic data could further facilitate their discovery (Schorn *et al.*, 2021).

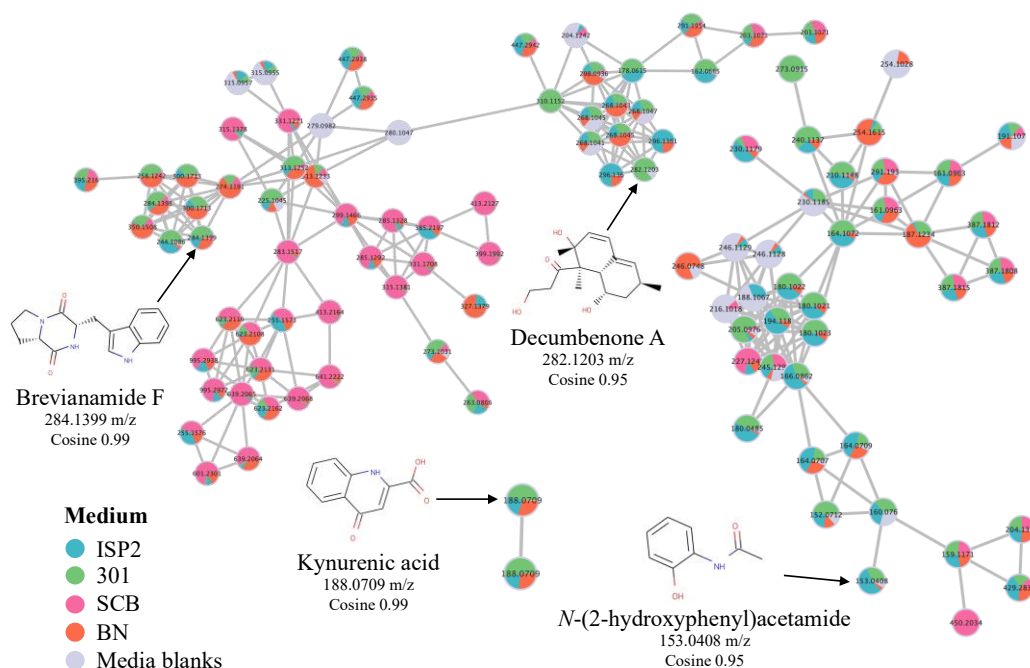


Figure 3. The molecular network created from the LC-MS/MS data of all crude extracts of *Streptomyces* sp. WHD3007-4. The compounds with a high cosine similarity score (≥ 0.95) and precursor mass are shown. The node color denotes the medium

Conclusions

Our *in vitro* investigations have demonstrated that crude extracts of *Streptomyces* sp. WHD3007-4 exhibit potential anticancer effects and can inhibit yeast cells. Employing an *in silico* approach for metabolomics-based compound identification using MS, this study has revealed the presence of several bioactive compounds produced by this strain. Several putative compounds were identified, such as kynurenic acid, brevianamide F, *N*-(2-hydroxyphenyl) acetamide, and decumbenone A, which have previously been reported for their anticancer or antiyeast properties. Future research should further purify these compounds to comprehensively explore their bioactivity.

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