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Evaluation of fermentation conditions triggering increased antibacterial activity from a near-shore marine intertidal environment-associated *Streptomyces* species

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ABSTRACT

A near-shore marine intertidal environment-associated *Streptomyces* isolate (USC-633) from the Sunshine Coast Region of Queensland, Australia, cultivated under a range of chemically defined and complex media to determine optimal parameters resulting in the secretion of diverse array of secondary metabolites with antimicrobial properties against various antibiotic resistant bacteria. Following extraction, fractioning and re-testing of active metabolites resulted in persistent antibacterial activity against *Escherichia coli* (Migula) (ATCC 13706) and subsequent *Nuclear Magnetic Resonance* (NMR) analysis of the active fractions confirmed the induction of metabolites different than the ones in fractions which did not display activity against the same bacterial species. Overall findings again confirmed the value of *One Strain–Many Compounds* (OSMAC) approach that tests a wide range of growth parameters to trigger bioactive compound secretion increasing the likelihood of finding novel therapeutic agents. The isolate was found to be adaptable to both marine and terrestrial conditions corresponding to its original near-shore marine intertidal environment. Wide variations in its morphology, sporulation and diffusible pigment production were observed when different growth media were used.

1. Introduction

Order *Actinomycetales* within the class Actinobacteria, have been the most prolific source of new antibiotics since the 1940s, providing 45% of the currently known antibiotics [1–3]. Members of the genus *Streptomyces* in particular, have produced 80% of the currently used antibiotics [1]. Despite the prevalence of *Streptomyces* species with over 500 currently described, it is estimated that just 1–3% of antibiotics produced in nature from this taxon have been discovered leaving the remaining 97–99% still unknown and indicating opportunities for natural product investigators to re-examine this prolific source with strategic and target directed approaches. Searches for the new streptomycete species from previously unexplored environments with the aid of selective pressures may result in the discovery of novel bioactive compounds [4,5]. In particular, relatively under-studied marine-dwelling species might be worthy of focused investigations in parallel with the advances in marine sampling techniques as well as the increasing metagenomics data revealing the occurrence of new species in these locations [6,7].

Marine inhabiting members of the genus *Streptomyces* are found in near shore marine sediments, mangrove sediments, deep-sea sediments ranging from 150 to 10,000 m below sea level, freely floating in seawater at various depths, and symbiotically associated with other marine organisms such as the marine sponges. Atmospheric deposition events also contribute towards their dissemination in the marine environments [8,9]. Diverse ecological niches occupied by marine *Streptomyces* species increasingly prove to be a prolific source for biodiscovery [10–13]. Examples include the detection of bioactive marine *Streptomyces* species, grouped within the "MAR4" cluster, which proliferate widely across different oceanic ecosystems. The members of this cluster have been a source of novel chlorine containing terpenoid dihydroquinone compounds

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that exhibit potent bioactivities [14]. Unique metabolic chemistry originating from "MAR4" cluster isolates can also be a further source of halogenating enzymes and biogenic organohalogens, in addition to producing antibacterial and anticancer agents [11]. The halogenations might result in the improved drug properties such as two chlorine substituent in vancomycin are necessary to induce the chemical conformation that produces the clinical effect. Accordingly, the notion that incorporation of a halogen atom (such as chlorine or bromine) is linked to biological activity of compounds deriving from marine-associated bacteria might strengthen in-depth exploration of these environments that are rich in ionic compounds [11].

Researchers attempting to maximise production of metabolites from various *Actinomycetales* species in order to discover novel chemical structures adapted an approach called *One Strain–Many Compounds* (OSMAC) [15–18]. The OSMAC approach allows the testing of many variables determining metabolite production simultaneously and multiple growth variables may be changed between tests to elicit antibiotic production [15,19]. The process is not entirely random and the approach build on the parameters used successfully by other investigators in the past that provide a starting point for the new tests [15]. The utilization of such accumulated experimental knowledge deriving from testing of varying growth parameters to increase the detectable levels of metabolic diversity in *Streptomyces* in the past, can also reveal the cryptic metabolic complexity possessed by this group of bacteria [15].

In the light of the above presented information a marine environment derived *Streptomyces* isolate (USC-633) was cultivated under a range of chemically-defined and complex media composition to determine optimal parameters resulting in the secretion of metabolites with antimicrobial properties against various antibiotic resistant bacteria.

2. Materials and methods

2.1. Streptomyces species (USC-633)

The test organism used in this study was a near-shore marine intertidal environment-associated isolate from the Sunshine Coast Region of Australia. The taxonomical position of the isolate in relation to its nearest relatives has recently been described by Kurtböke et al. [20]. Isolate belonged to the genus *Streptomyces* (Fig. 1a and b) (GENBANK accession number: KX379153) with 68% similarity to the node point where other "MAR4" cluster-associated *Streptomyces* species (CNS-689_SD06_MAR4 (EU214957) and MAR4 CNR925 PLO4 (DQ448742) were positioned [20]. Initial chemical studies on the isolate also revealed its ability to produce brominated and chlorinated compounds [20].

2.2. Solid and liquid defined media preparations

In defined solid media the carbon sources were tested singly (arabinose, fructose, glucose, raffinose, mannitol, sorbose, rhamnose, sucrose, xylose, galactose and lactose) with and without salt supplementation (16.5 g artificial sea salt (Ocean Nature, Australia)) as well as trace element additions [(1) $FeSO_4$ (0.01 g), (2) $CaCO_3$ (0.01 g), (3) K_2HPO_4 (0.25 g), (4) KNO_3 (0.1 g), (5) NaCl (0.15 g), (6) $MgSO_4$ (0.1 g), (7) Na_2HPO_4 (0.4 g), (8) $C_3H_5NaO_2$ (0.1 g), (9) $ZnSO_4$ (0.01 g), (10) $MnSO_4$ (0.01 g)] to each formula. Single amino acids were also incorporated into each above listed media containing artificial sea salts, as well as into the ones containing above listed trace elements. The amino acids singly used in each media were: (1) cysteine, (2) L-valine, (3) L-phenylalanine, (4) L-histidine, (5) L-methionine, (6) L-tyrosine, (7) L-threonine, (8) L-arginine, (9) glycine, (10) L-asparagine and (11) L-serine.

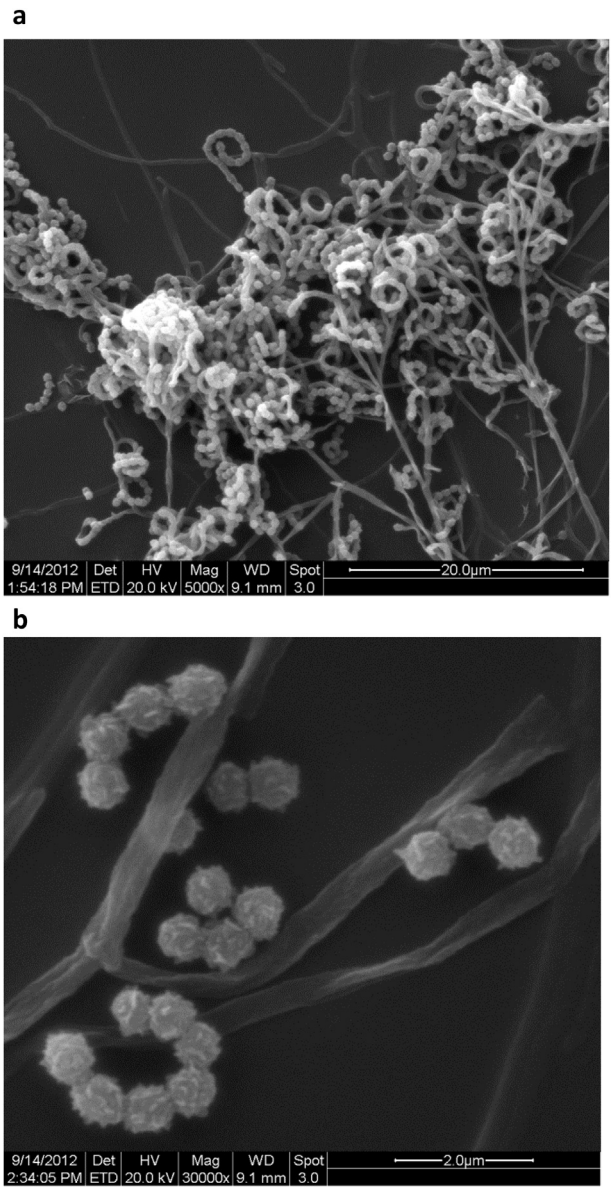

Fig. 1. Electron micrographs of the streptomycete isolate (USC-633) illustrating open spiral spore chains and spiny spores produced on an oatmeal agar.

Liquid defined media were also prepared in the same way with the exception of the incorporation of sorbose, xylose and agar.

2.3. Solid and liquid complex media preparations

Twenty three different formulations were used for the solid complex media. The artificial sea salt where incorporated was again from the Ocean Nature, Australia. Compositions per 500 mL were: (1) **Nutrient-poor sand extract agar:** mannitol (1 g), sterilised local beach sand extract (300) Soil and sand extracts were made by adding 200 g of solid to 400 ml of DI H_2O and autoclaving on liquid cycle twice. The liquid steep was poured off leaving heavy solids behind. (2) **Nutrient-rich mud extract agar:** mannitol (1 g), sterilised local mangrove mud extract (300 ml), (3) **Nutrient-poor bush soil extract agar:** mannitol (1 g), sterilised local bush soil extract (300 ml), (4) **Nutrient-rich potting soil extract agar:** mannitol (1 g), sterilised local brand of potting soil extract (300 ml), (5) **Minimal complex agar:** Glucose (1 g), mannitol (1 g), white

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refined sucrose (0.5 g) Yeast extract (1 g), Oatmeal (ground, 1 g), Soybean meal (1 g), Casamino acids (1 g), Artificial sea salt (16.6 g), (6) **Casein-soy-mannitol agar:** mannitol (10 g), Casein (5 g), Soybean meal (5 g), Artificial sea salt (16.6 g), (7) **Hemp-protein-sucrose aga**r: Brown unrefined sucrose (10 g), Peptone (2 g), hemp protein powder (Hempfoods, Australia, 10 g), Artificial sea salt (16.6 g), (8) **Starch agar:** Glucose (5 g), Potato starch (5 g), Corn starch (5 g), Yeast extract (3 g), Peptone (2 g), Beef Extract (2 g), Artificial sea salt (16.6 g), (9) **Potato Dextrose agar** (PDA) (OXOID, Australia), (10) **Tryptone-casein-sucrose agar:** White refined sucrose (12 g), yeast extract (1 g), casein (3 g), casamino acids (2 g), Tryptone (5 g), Artificial sea salt (Ocean Nature, Australia, 16.6 g), (11) **Fish Oil agar:** Glucose (10 g), yeast extract (2 g), Fish Oil (Blackmores 1000 brand, 15 g), Artificial sea salt (16.6 g), (12) **Beef extract-glucose agar:** Glucose (10 g), yeast extract (1 g), Peptone (1 g), Beef extract (10 g), Artificial sea salt (16.6 g), (13) **Brain Heart-sucrose agar:** Brown unrefined sucrose (10 g), Yeast (1 g), Peptone (1 g), Brain heart extract (15 g), Artificial sea salt (16.6 g), (14) **Mixed sugar-protein agar:** White refined sucrose (5 g), brown unrefined sucrose (5 g), yeast extract (2 g), casein (3 g), soybean meal (3 g), beef extract (3 g), Artificial sea salt (16.6 g), (15) **Difco Marine Agar**, (16) **Glucose-Yeast extract-Calcium chloride agar**: Glucose (5 g), Yeast extract (5 g), calcium chloride (10 g), (17) **Glucose-Yeast extract-Calcium carbonate agar:** Glucose (5 g), Yeast extract (5 g), Calcium carbonate (3.5 g), (18) **Chitin agar:** Chitin (4 g), Artificial sea salt (16.6 g), (19) **Cellulose-Yeast extract agar:** Cellulose (9 g), Yeast extract (2 g), (20) **Wickersham's Malt-Yeast Extract agar with trace elements:** Malt (3 g), Glucose (10 g), Yeast extract (3 g), Peptone (5 g), (21) **Wickersham's Malt-Yeast Extract agar with trace elements:** Malt (3 g), Glucose (10 g), Yeast extract (3 g), Peptone (5 g), $FeSO_4$ (0.01 g), $CaCO_3$ (0.01 g), K_2HPO_4 (0.25 g), KNO_3 (0.1 g), NaCl (0.15 g), $MgSO_4$ (0.1 g), Na_2HPO_4 (0.4 g), $C_3H_5NaO_2$ (0.1 g), $ZnSO_4$ (0.01 g), $MnSO_4$ (0.01 g), Artificial sea salt (16.6 g), (22) **Oatmeal agar:** Steel cut oats (20 g), Yeast extract (1 g), (23) **Oatmeal Salt agar:** Steel cut oats (20 g), Yeast extract (1 g), Artificial sea salt (16.6 g).

Complex liquid media used were: (1) **Wickersham's Malt-Yeast Extract:** Glucose (10 g), Yeast extract (3 g), malt (3 g), Peptone (5 g), (2) **Tryptone soya broth (TSB)** (OXOID, Australia): Tryptone soya broth, (3) **TSB with glucose:** Tryptone soya broth (30 g), glucose (10 g), (4) **Potato-Glucose:** potato starch (10 g), glucose (5 g), peptone (5 g), yeast extract (3 g), (5) **Corn-Glucose:** corn starch (10 g), glucose (5 g), peptone (5 g), yeast extract (3 g), (6) **Starch:** starch (Difco, 10 g), glucose (5 g), peptone (5 g), yeast extract (3 g). Formulae 1–6 were also used with the addition of trace elements: (1) $FeSO_4$ (0.01 g), (2) $CaCO_3$ (0.01 g), (3) K_2HPO_4 (0.25 g), (4) KNO_3 (0.1 g), (5) NaCl (0.15 g), (6) $MgSO_4$ (0.1 g), (7) Na_2HPO_4 (0.4 g), (8) $C_3H_5NaO_2$ (0.1 g), (9) $ZnSO_4$ (0.01 g), (10) $MnSO_4$ (0.01 g).

2.4. Detection of the biological activity deriving from different fermentation conditions of the Streptomyces species (USC-633)

(i) Time series test

TSB was used for the time series experiment to detect the peak of antibiotic production [21] and the inoculated flasks were incubated on a rotary shaker (Bioline Incubator Shaker, Australia) set at 30 °C and 120 rpm. After eight days of fermentation, flasks (in replicates for each different media used) were removed and spun at 10,000 rpm to remove cell matter and cease the fermentation. This step was repeated at days 16, 20 and 24 and the resulting fermentation broth was collected and refrigerated at −4 °C [22].

(ii) Agar plug tests

Agar plugs (10 mm) removed from all complex solid media (after 21 days of growth) and were tested against five reference ATCC strains of antibiotic resistant and human pathogenic bacteria (Table 1) to determine any antibiotic activity [23].

Bacterial lawns were prepared using 1 ml of the each reference culture (x10^8 CFU/ml) spread onto Mueller Hinton agar plates [24]. Each of the plugs were then placed onto the lawns of the previously inoculated different ATCC reference strains and zones of inhibition were recorded [25,26].

(iii) Spot-in-well and Paper disk diffusion assays

Two different methods were used to test the antibiotic activity produced by the isolate (USC-633) when cultivated in different liquid media. The first one was the “spot-in-well test”. Fermentation broth (1 mL) from each of the 16 defined liquid fermentation media (after 21 days growth) was pipetted aseptically into the well cut into the agar that was previously inoculated (x10^8 CFU/ml) of the ATCC reference strain (Table 1). The plates were then incubated at 37 °C for 24 h and zones of inhibition were observed [27–29]. The fermentation media resulted in inhibitory activity were further tested using both crude and fractioned metabolite extracts using the disk diffusion assay.

The crude and fractioned metabolite extracts (see section *HPLC analysis and fractionation of crude extracts*) were redissolved in DSMO and pipetted (1 mL) onto Whatman diffusion paper discs (10 mm), then vacuum desiccated [30]. The discs were then placed onto the agar plates, one in each quarter, which were inoculated previously with the reference test strains (see section *Agar plug test*) and then incubated at 37 °C for 24 h. Following incubation the clearance zones around the disks were recorded in millimetres.

2.5. Chemical studies

(i) Extraction of bioactive metabolites

For solid-state fermentation extractions, all of the agar contents of the fermentation media used were completely removed from the plates and soaked with ethyl acetate (Merck Millipore, Australia) in a sterile 500 mL beaker after 24 h the ethyl acetate fraction was decanted into a separate clean beaker through Whatman filter paper (Grade 1) capturing solids. Each beaker was then combined with sodium chloride (2 g) and sodium sulphate (2 g), vigorously

Table 1
Details of the reference strains used in the antibiotic susceptibility testing.

Strain code	ATCC 29247	ATCC 51575	ATCC 25922	ATCC 13706	ATCC BAA-1705
ID	*Staphylococcus aureus* (sbsp. *Aureus* Rosenbach)	*Enterococcus faecalis*	*Escherichia coli* (Migula)	*Escherichia coli* (Migula)	*Klebsiella pneumoniae*
Resistance	Ampicillin	Vancomycin, Gentamycin, Streptomycin	Beta-lactam	Nalidixic acid-resistant mutant	Carbapenems (Imipenem, Ertapenem)

Source: American Type Culture Collection https://www.atcc.org/.

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stirred and allowed to settle. The resulting clear liquid on top of the beaker was poured into a round bottom flask for connection to the rotor-vac apparatus (Buchi Rotavapor R-215 with Digital vacuum controller V-855). The rotavapor water bath was set to 40 °C and rotation to 120 rpm until the flask was completely dry [31]. After rotor-vac cycle was completed a small amount of di-chloromethane was added to the round bottom flask to re-dissolve the crude extract. The di-chloromethane crude extract mixture was pipetted into a 2 mL phenominex glass vial. The vials were placed under gentle N_2 needles for 30 min to evaporate the di-chloromethane and then stored at −4 °C [32].

For the liquid fermentation, the resulting broths were transferred from each conical flask evenly into two 50 ml aseptic centrifuge tubes. The tubes were centrifuged for 30 min at 7000 rpm. Both supernatant and the resulting sediment were extracted with 30 ml of ethyl acetate, followed by the addition of the sodium chloride (2 g) and sodium sulphate (2 g). The resultant mixtures were vigorously stirred and allowed to settle and the clear liquid on top was poured into a round bottom flask for connection to the rotor-vac apparatus (Buchi Rotavapor R-215 with Digital vacuum controller V-855). The above described procedures by Farnaes et al. [31] and Chaillan et al. [32] was again repeated.

(ii) HPLC analysis and fractionation of crude extracts

Crude extracts showing bioactivity were fractionated into 6 fractions using HPLC and the obtained fractions were re-screened to detect persistent bioactivity [33–36]. For the HPLC fractionation, all the crude extracts were dissolved in 600 μL DMSO, a Waters 600 pump equipped with a Waters 966 PDA detector, a Gilson 715 liquid handler and a Phenomenex Onyx Monolithic C_{18} column (4.6 × 100 mm) were used. Millipore Milli-Q PF filtered H_2O and HPLC grade solvents were used for chromatography analyses. A solvent system consisting in MeOH—H_2O with 0.1% TFA was used for the HPLC analyses. The HPLC gradient method was a linear gradient from 10% to 50% MeOH in 3 min at 4 mL/min flow, then a convex gradient to 100% MeOH in 3.5 min at 3 mL/min flow, held for 0.5 min at 100% MeOH at a flow rate of 3 mL/min, then for 1 min at 4 mL/min, a linear gradient to 10% MeOH is applied in 1 min at 4 mL/min flow, then held in the same conditions for 2 min. The total HPLC run was 11 min within six fractions were collected between 2 and 8 min.

(iii) NMR analysis of active fractions

Active fractions from extracts deriving from three different complex media (media #8, #17 and #21), with antibiotic activity against *E. coli* Migula (ATCC 13706) were selected for further NMR analysis as well as the medium #6, which resulted in abundant colony growth but failed to trigger antibiotic activity against *E. coli* Migula (ATCC 13706). ^{1}H NMR analysis was performed using a Bruker Avance III HDX 800 MHz spectrometer utilising a Triple (TCI) Resonance 5 mm Cryoprobe with Z-gradient and Automatic Tune & Match. For NMR fingerprint experiments, the samples were dissolved in 200 μL of DMSO-d_6 and run in a 3-mm NMR tube with nt = 128 scans. The ^{1}H chemical shift was referenced to the DMSO-d_6 solvent peak at δ_H 2.50 ppm.

3. Results

3.1. Growth on solid and liquid defined media

USC-633 was able to utilise a wide range of carbon sources when trace elements or sea salt were added into the growth media. Without the supplementation it only grew on raffinose as a sole defined carbon source (Table 2, Fig. 2a and b).

USC-633 also displayed minimal growth on six of the defined media with amino acids when supplemented with the artificial sea salt and on three of the defined media when supplemented with the trace elements. When there was no trace element supplementation it only grew on the agar in which L-Methionine was the only amino acid.

Table 2
Growth characteristics of the USC-633 on different defined media.

Solid defined media	Growth at 20 days			Colony Colour			Diffused pigment			Sporulation		
Presence of supplements	Plain	Trace	S. Salt	Plain	Trace	S. Salt	Plain	Trace	S. Salt	Plain	Trace	S. Salt
Carbon Source												
Arabinose (1)	−	−	−									
Fructose (2)	−	++	+++		G	b		−	+		−	+
Glucose (3)	−	++	+++		G	g/b		+	+			−
Raffinose (4)	+	++	+++	BB	BB	b	−	+	−		−	+
Mannitol (5)	−	+++	+++		Bk	G/g		+++	+++		−	+
Sorbose (6)	−	−	−									
Rhamnose (7)	−	++	+++		G	G/B		+	+		−	−
Sucrose (8)	−	+++	+++		BB	b		++	−		−	+
Xylose (9)	−	−	−									
Galactose (10)	−	+	+++		GG	b/g		−	+			−
Lactose (11)	−	+++	+++		BB	b/g		+++	−		−	+
Nitrogen Source												
L-Cysteine (1)	−	−	+			Wh			−			+
L-Valine (2)	−	−	−									
L-Phenylalanine (3)	−	−	−									
L-Histidine (4)	−	−	−									
L-Methionine (5)	+	+	+	Wh	Wh	Wh/b	−	−	−	−	−	−
L-Tyrosine (6)	−	−	−									
L-Threonine (7)	−	−	+			GG			−			−
L-Arginine (8)	−	+	+		Gr	Gr		−	−			
Glycine (9)	−	−	−									
L-Asparagine (10)	−	+	+		BB	BB		−	−			
L-Serine (11)	−	−	+			BB			−			

Growth density: (−) No growth, (+) very small colony formation/minimal growth, (++) Discrete colonies/Medium growth, (+++) Confluent/Heavy growth. **Colony colour:** (b) Light Brown, (B) Brown, (BB) Dark Brown, (g) Light Green, (G) Green, (GG) Dark Green, (Gr) Grey, (Wh) White, (Bk) Black.

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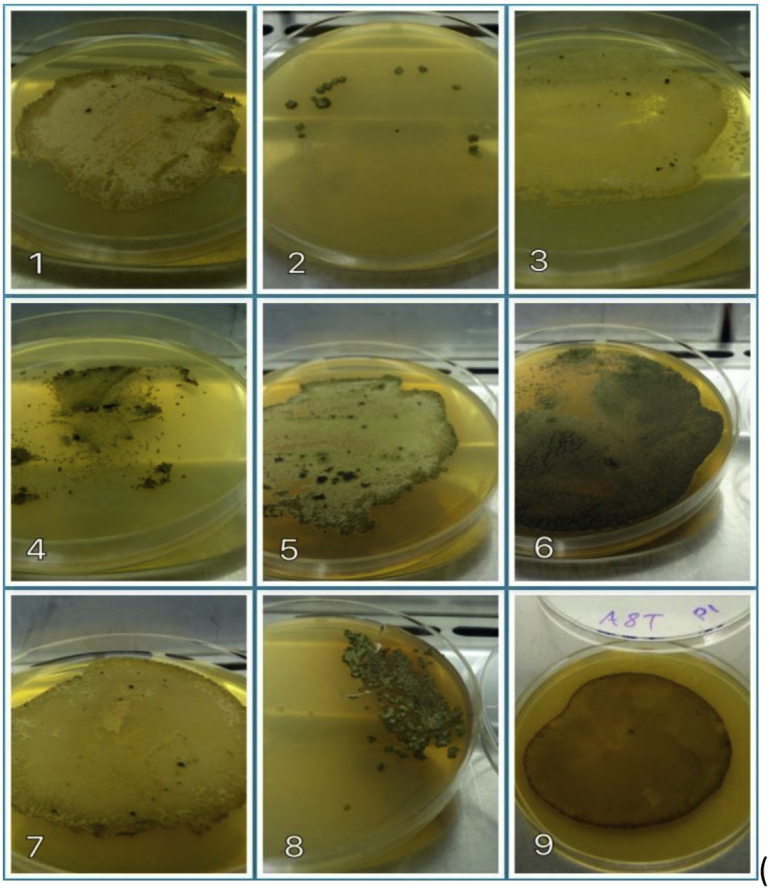

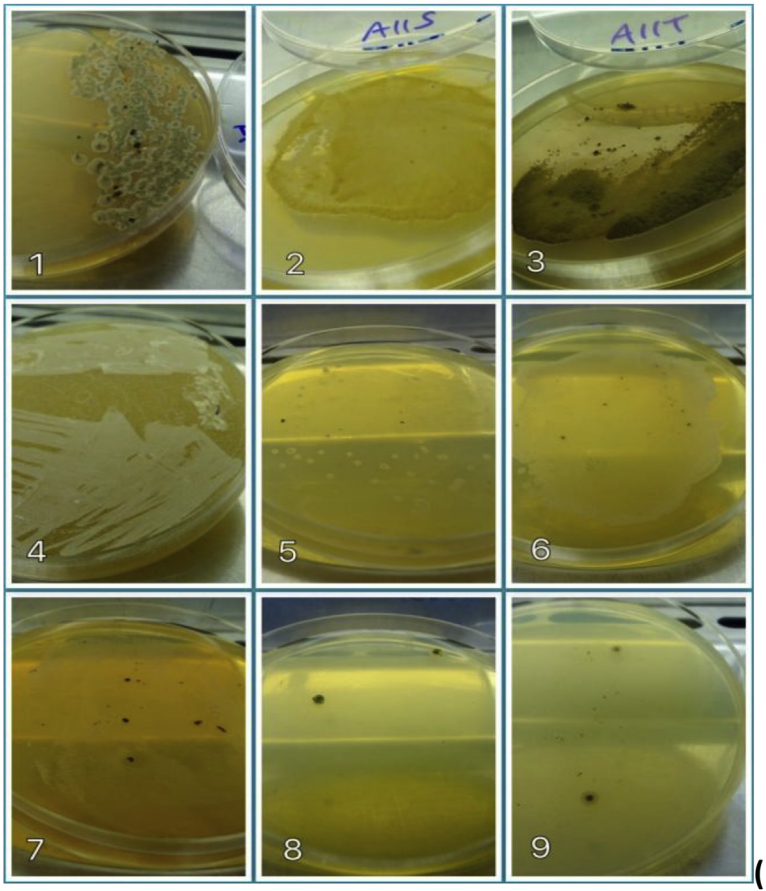

Fig. 2. Growth on media with (a) different carbon* and (b) different nitrogen** sources. **a**: 1: Glucose + artificial sea-salt, 2: Glucose-trace elements, 3: Raffinose + artificial sea-salt, 4: Raffinose-trace elements, 5: Mannitol + artificial sea-salt, 6: Mannitol-trace elements, 7: Rhamnose + artificial sea-salt, 8: Rhamnose + trace elements, 9: Sucrose-trace elements; **b**: 1: Galactose- + artificial sea-salt, 2: Lactose + artificial sea-salt, 3: Lactose-trace elements, 4: L-Cystine + artificial sea-salt, 5: L-Methionine, 6: L-Methionine + artificial sea-salt, 7: L-Methionine-trace elements, 8 &9: L-Serine + artificial sea-salt.

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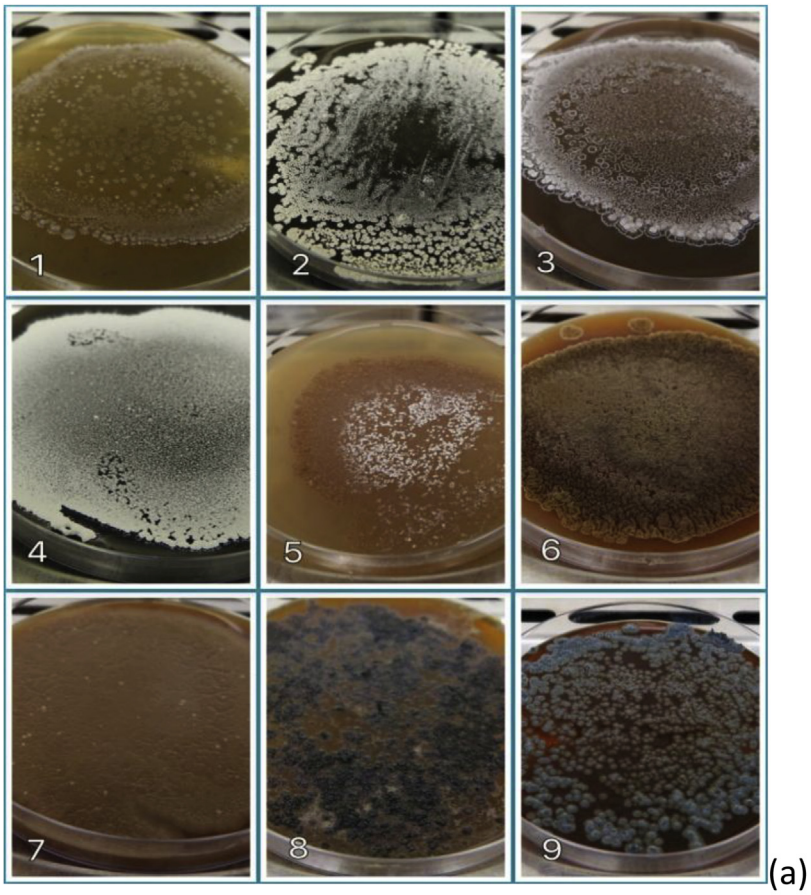
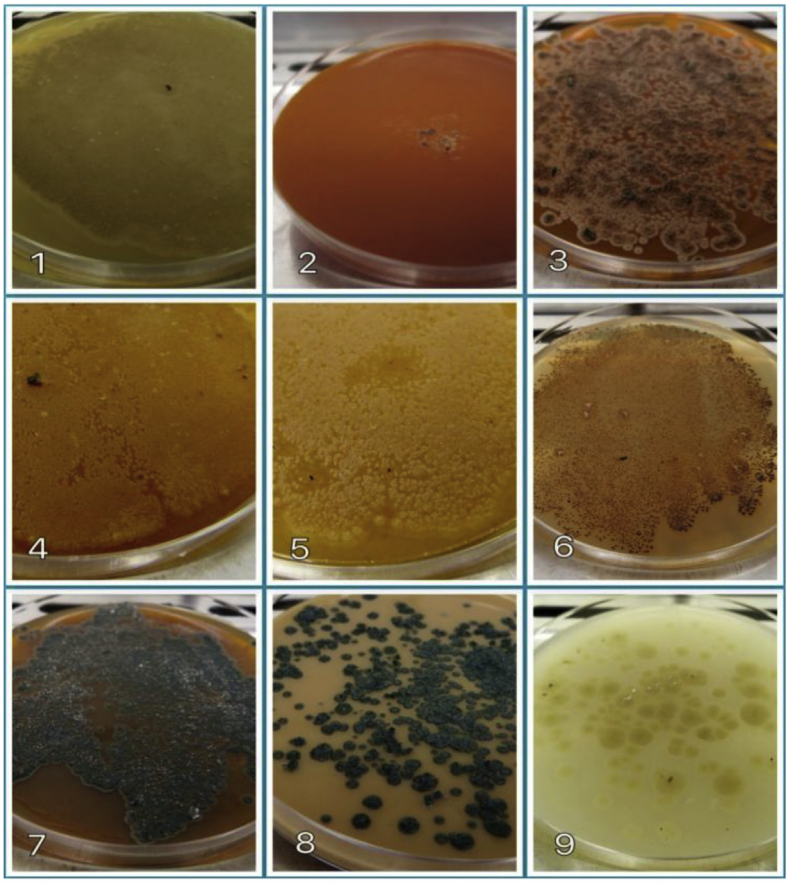


(a)



(b)

Fig. 3. Growth on solid complex media**a**: photo numbers corresponding to media type: 1: #1, 2: #2, 3: #3, 4: #4, 5: #5, 6: #6, 7: #7, 8: #8, 9: #9, **b**: 1: #10, 2: #11, 3: #12, 4: #13, 5: #14, 6: #15, 7: #16, 8: #17, 9: #18, **c**: 1: #19, 2: #22, 3: #23, 4: #20, 5: #21. Photos were taken after 21 days incubation.

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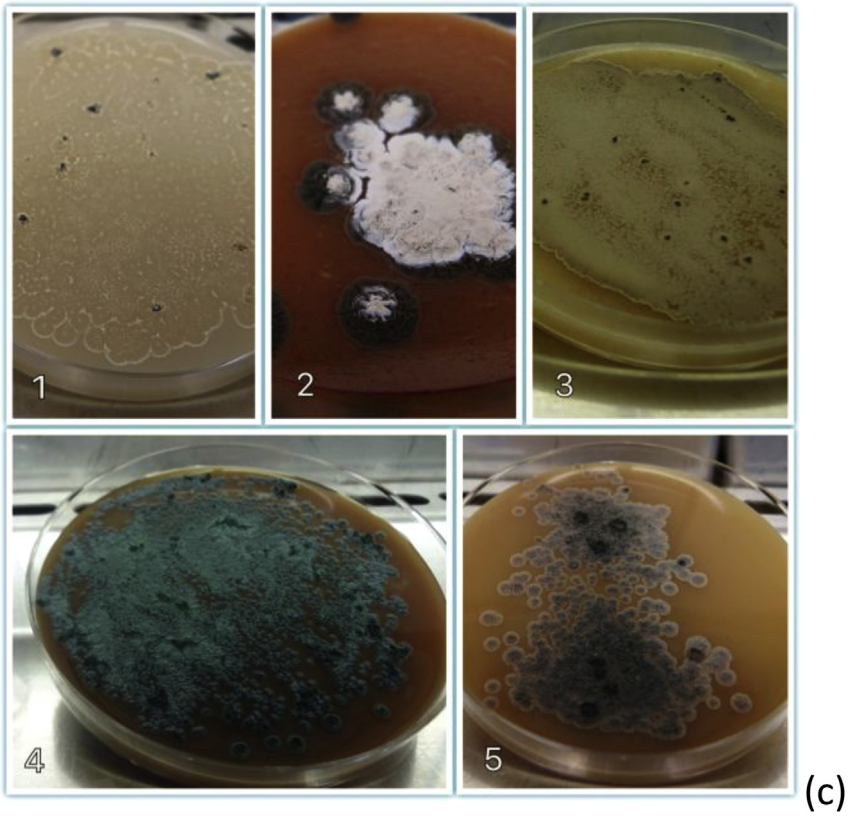

Fig. 3. (*continued*).

In liquid defined media USC-633 displayed medium to heavy growth levels on all of the defined carbon sources used when trace elements were added, with growth most favourable on glucose, raffinose, and mannitol. When liquid media was supplemented with the artificial sea salt the isolate failed to grow in the ones with arabinose and fructose. USC-633 also showed minimal growth on L-methionine and L-asparagine with the artificial sea salt supplementation. Growth improved when the medium containing L-serine, L-threonine, L-asparagine and L-arginine was supplemented with the trace elements.

3.2. Growth on solid and liquid complex media

Growth of the isolate on complex solid media was generally profuse with the exceptions of fish oil (medium #11), chitin (medium #18), cellulose-yeast extract (medium #19) and oatmeal/salt (medium #23) which supported minimal growth of the isolate. While growth was generally attained across different complex solid media and large differences in morphology including sporulation, colony and pigment colour were observed (Fig. 3a, b, c). USC-633 displayed a sporulation response when grown on both soil/sand extract growth media, minimal complex media, cellulose media, and minimal media supplemented with calcium. It also displayed pigment production when grown on complex starchy and glucose containing media (e.g. Fig. 3c, photo #4).

Abundant growth levels were restricted to fewer formulae when complex liquid growth media were tested. Profuse cell density and change in fermentation medium colour were observed in Tryptic Soy Broth (TSB), and the TSB supplemented with glucose and the trace elements.

3.3. Detection of the biological activity of the Streptomyces species (USC-633)

Agar plugs from complex media generated clearance zones against *E. faecalis* (ATCC 51575) and *E. coli* Migula (ATCC 13706) in both orientations tested, suggesting antibiotic compounds excreted were able to diffuse easily through the agar and were not just limited to colony surface-bound metabolites (Table 3). No antibiotic activity in the form of clearance zones was detected against the ATCC reference strains 29247, 25922, and 1705 with the agar plugs obtained from all different complex solid media (Table 3).

In the spot-on-lawn test when aliquots from liquid defined media were placed into the wells the only detected bioactivity was against *E. coli* Migula (ATCC 13706). The activity (8 mm of inhibition zone) originated from the liquid media incorporated with glucose and mannitol together with the trace elements. No antibiotic activity was detected against other ATCC reference strains when all other carbon and nitrogen sources were tested.

The time-series study also provided some indication that secondary metabolite production with antibiotic activity was present in the TSB fermentation medium from day eight onwards.

In the filter paper disk assay biological activity was only persistent against *E. coli* Migula (ATCC 13706) with the fractions obtained from the complex media #s 8, 9, 10, 12, 16, 17, 20 (Table 4). Fractions 4 and 5 were subjected to NMR analysis and compared with the fractions 4 and 5 obtained from the extracted fermentation of medium #6 which failed to produce antibiotic activity against *E. coli* Migula (ATCC 13706). The NMR spectra of the active fractions confirmed the induction of metabolites in agreement with the detected antibiotic activity as no similar peaks were recorded in the extracts obtained from the medium #6 which did not triggered the antibiotic activity (Fig. 4 a. b).

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Table 3
Antibiotic activity obtained on complex solid media.

Complex solid media	Zone of inhibition (mm)				
ATCC strain code	29247	51575	25922	13706	1705
Nutrient-poor beach sand extract agar (#1)	–	–	–	–	–
Nutrient-rich mud extract agar (#2)	–	–	–	–	–
Nutrient-poor bush soil extract agar (#3)	–	–	–	–	–
Nutrient-rich potting soil extract agar (#4)	–	–	–	–	–
Minimal Complex agar (#5)	–	25U, 20D	–	10U, 10D	–
Casein Soy Mannitol agar (#6)	–	16U, 10D	–	–	–
Hemp-protein sucrose agar (#7)	–	–	–	–	–
Starch agar (#8)	–	25U, 20D	–	15U, 13D	–
Potato Dextrose Agar (#9)	–	18U, 12D	–	18U, 15D	–
Tryptone-casein sucrose agar (#10)	–	22U, 28D	–	20U, 22D	–
Fish Oil agar (11)	–	–	–	–	–
Beef Extract-glucose agar (#12)	–	14U, 14D	–	12U, 10D	–
Brain Heart-sucrose agar (#13)	–	–	–	–	–
Mixed sugar-protein agar (#14)	–	–	–	–	–
DIFCO Marine agar (#15)	–	25U, 25D	–	–	–
Glucose-Yeast extract-Calcium chloride agar (#16)	–	15U, 15D	–	13U, 16D	–
Glucose-Yeast extract-Calcium carbonate agar (#17)	–	20U, 18D	–	18U, 14D	–
Chitin agar (#18)	–	–	–	–	–
Cellulose-Yeast extract agar (#19)	–	–	–	–	–
Wickersham's Malt-Yeast Extract agar (#20)	–	25U, 20D	–	25U, 18D	–
Wickersham's Malt-Yeast Extract agar with trace elements (#21)	–	25U, 25D	–	18U, 12D	–
Oatmeal agar (#22)	–	–	–	–	–
Oatmeal-salt agar (#23)	–	–	–	–	–

–: no activity, (U) when plugs were placed colony surface away from the host lawn, (D) when agar plugs were placed surface down onto the host lawn.

4. Discussion

After testing a wide range of complex and defined growth media conditions, the findings indicated remarkable evidence of the adaptability of the *Streptomyces* isolate (USC-633) to both terrestrial and marine specific growth conditions. Such degree of nutritional adaptability may contribute to its existence and survival in the near shore marine environment where daily intertidal fluctuations occur. USC-633 also displayed greater colony mass formation and morphological changes such as the pigment production on many starch and sugar media combinations, when supplemented with the trace elements. On some nutritionally minimal media such as chitin, scarce sporulation observed might be as part of a survival response to nutritional scarcity. Such sporulation might also be for tougher living conditions exist in its original habitat such as lower nutrient and oxygen levels associated with most marine intertidal and sand/sediment habitats [37–39]. No pigment production observed on chitin growth media, which is consistent with observations made by others investigating unrelated *Streptomyces* species [40]. As it was isolated from an near shore intertidal marine environment it is difficult to conclude whether it was a true marine inhabitant or was a transient terrigenous one, but the ability of USC-633 to thrive in salty conditions could suggest this strain is adapted, or has been in the process of adapting to the marine

Table 4
Antibiotic activity results for the "filter paper disk assay".

Fraction#	Gross quantity crude natural extract (mg)	ATCC 13706 inhibition zone diameters (mm)					
		1	2	3	4	5	6
Nutrient-poor beach sand extract agar (#1)		–	–	–	–	–	–
Nutrient-rich mud extract agar (#2)		–	–	–	–	–	–
Nutrient-poor bush soil extract agar (#3)		–	–	–	–	–	–
Nutrient-rich potting soil extract agar (#4)		–	–	–	–	–	–
Minimal Complex agar (#5)		–	–	–	–	–	–
Casein Soy Mannitol agar (#6)		–	–	–	–	–	–
Hemp-protein sucrose agar (#7)		–	–	–	–	–	–
Starch agar (#8)	65.6	–	–	–	36	22	30
Potato Dextrose Agar (#9)	297	–	–	–	38	24	36
Tryptone-casein sucrose agar (#10)	23.2	–	–	–	–	14	–
Fish Oil agar (11)		–	–	–	–	–	–
Beef Extract-glucose agar (#12)	36.6	–	–	20	–	18	27
Brain Heart-sucrose agar (#13)		–	–	–	–	–	–
Mixed sugar-protein agar (#14)		–	–	–	–	–	–
DIFCO Marine agar (#15)		–	–	–	–	–	–
Glucose-Yeast extract-Calcium chloride agar (#16)	75.6	–	–	–	42	24	26
Glucose-Yeast extract-Calcium carbonate agar (#17)	104	–	–	–	34	24	35
Chitin agar (#18)		–	–	–	–	–	–
Cellulose-Yeast extract agar (#19)		–	–	–	–	–	–
Wickersham's Malt-Yeast Extract agar (#20)	169	–	–	–	40	24	35
Wickersham's Malt-Yeast Extract agar with trace elements (#21)		–	–	–	–	–	–
Oatmeal agar (#22)		–	–	–	–	–	–
Oatmeal-salt agar (#23)		–	–	–	–	–	–

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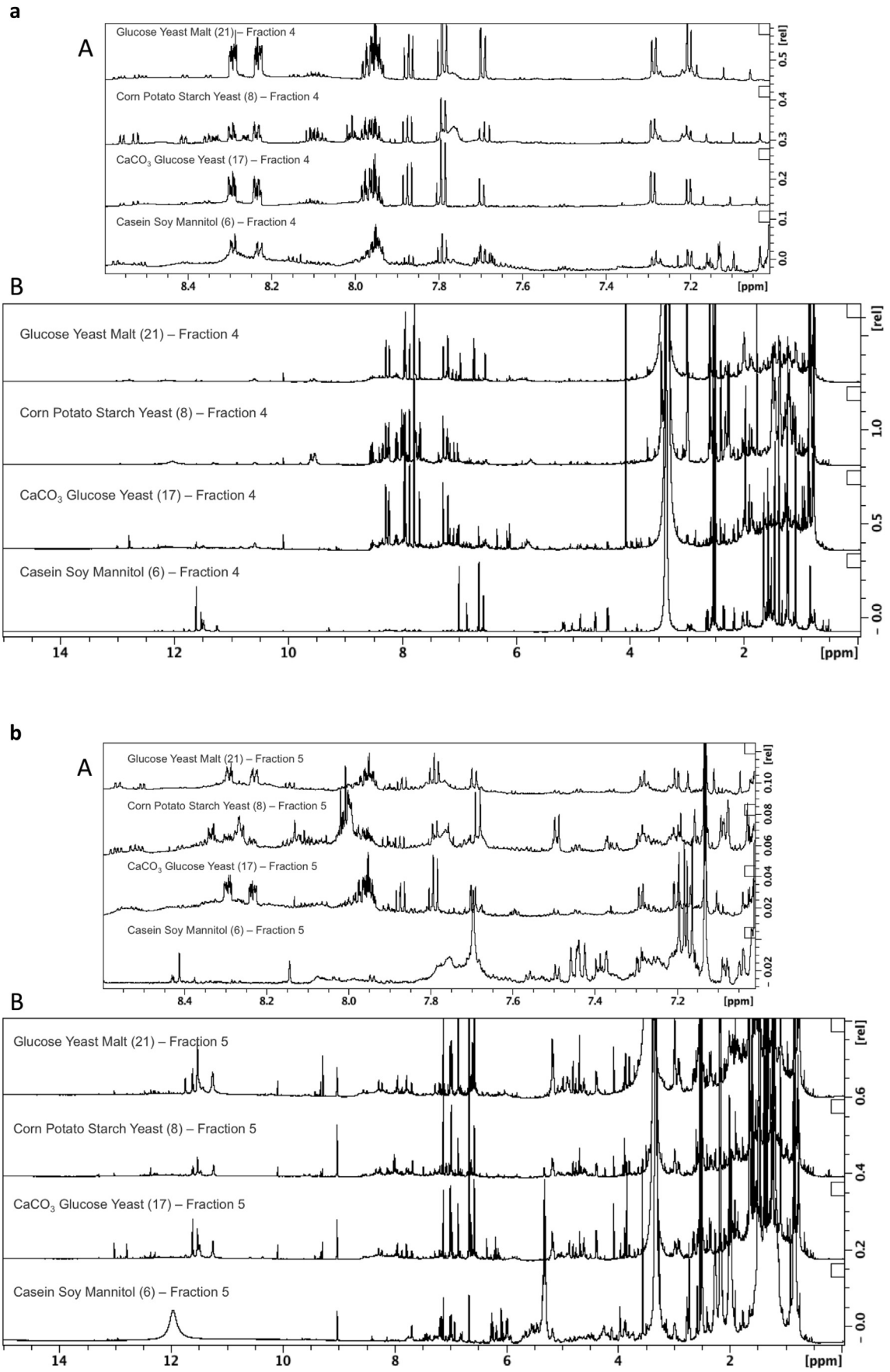

Fig. 4. **(a)** ^{1}H NMR spectra of fraction 4, and **(b)** fraction 5 generated from bioactive extracts of the complex solid media (#8, #17 and #21) against the inactive fraction 4 from the extract of the medium #6. A: chemical shift from 7.0 to 8.6 ppm, B: chemical shift from 0 to 15 ppm.

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conditions. Conversely the positive growth on soil extract agars, and starch/sugar media supplemented with trace elements might suggest that the species is still very capable of survival in terrestrial conditions with an ability to utilize a wide range of nutrients mostly found in terrestrial environments. Such adaptation characteristics might make the isolate a prime one for bioactivity investigations.

The results of antibiotic activity testing indicated that growth and antibiotic production media differed. Investigation using NMR suggested that USC-633 failed to produce antibiotic activity when grown on a medium containing casein, which agrees with early research by Williams and Davies [41] that determined *Streptomyces* species could be selectively grown and enumerated on casein based growth media, but that this growth media would not trigger antibiotic production.

Fascinating colour changes displayed by the isolate on variety of media used might also suggest pigment production might be occurring in response to nutritional variations [42]. Specialised cellular functions during production of antibiotics by *Streptomyces* colonies under different triggering conditions, such as during the transition from vegetative growth to sporulation, as an adaptive response to competition, and during starvation in a defensive response to a shortage of nutrients were reported [2]. The expression of antibiotic producing genes leading to the production of antibiotics in cells is modulated by a complex sensing ability that allows for expression of an antibiotic under multiple growth parameters [2,43]. This sensing ability which function part of the cellular machinery that interprets the supply of available nutrients while correspondingly initiating various other metabolic pathways that might include production of particular secondary metabolites. Growth of the producer organism may not always equate with reliable secondary metabolite production, and in some cases growth (in terms of increased colony mass) may be proficient without other metabolic pathways being activated.

A comparative test for antibiotic activity using the “paper disc” assay produced a slightly different result only showing activity against *E. coli* Migula (ATCC 13706). The loss of activity against *E. faecalis* (ATCC 51575) shown previously in the “agar plug” assay might indicate that either the active compound in the crude mix was lost or modified during the fractioning and disc impregnation processes. Further testing and refining of the protocol is necessary to determine if activity against *E. faecalis* (ATCC 51575) can be isolated to a fractioned product. The active fractions of USC-633 crude metabolite were collected during the non-polar phase of the HPLC protocol suggesting the compound of interest may be non-polar or have a very weak polarity.

The research results presented here might support further investigations that the secondary metabolites of USC-633, produced under specified growth conditions, could be investigated further as potential therapeutic agents due to the demonstrated antibiotic effects against nalidixic acid resistant mutant of *E. coli* Migula (ATCC 13706). In particular, the (non-polar) mid-fractions show higher antibiotic activity than the early fractions. Perhaps more crucially results agree with the earlier statements that the variation in growth parameters is an essential approach to natural product discovery because of the highly developed genetic sensing machinery in streptomycetes, species must be tested to the extreme limits of survivability as one means of maximising the genetic expression of metabolites [15]. Furthermore, findings underline the importance of marine bio-prospecting as a source of presumptive new antibiotic leads while validating the OSMAC design concept that seeks to unlock cryptic secondary metabolic pathways through variation of growth parameters [15–18].

The clinical success of natural product discovery since the 1940s over other drug development strategies from the members of the order *Actinomycetales* demonstrates the importance of uncovering new secondary metabolites from this group of bacteria. In particular, the marine associated ones might offer previously unknown chemical scaffolds. As findings of the presented study confirm again that the marine associated *Streptomyces* species are evidently a valuable source of potential new antibiotic drug leads. When compared to environments that have already been explored, the untapped marine habitats and diversity of microorganisms in these locations combined with their unique marine influenced secondary metabolite chemistry might result in the discovery of new therapeutic compounds effective in the fight against antibiotic resistant bacterial infections. This chemical diversity can be captured using NMR-fingerprinting of natural products in fractions, as they are less complex comparing to crude extracts. For further confirmations of the nature of compounds produced by the USC-633 the NMR-fingerprints can then be used to follow isolation focusing only on the new peaks that are in the active fractions and not in the inactive fractions. As NMR reveals all compounds containing hydrogen, it can be used to guarantee that all compounds within the active fraction are isolated.

Overall findings of the study again reveals the value of *One Strain–Many Compounds* (OSMAC) approach that can test a wide range of growth parameters to trigger bioactive compound secretion increasing the likelihood of finding novel therapeutic agents. In the special issue dedicated to Prof. Arnold Demain who pioneered industrial fermentation procedures, we again would like emphasize that all past studies should be utilized to form a sound platform from which advance molecular approaches can take the investigations further to reveal the true potential of the bioactive strains.

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