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The Search for new Antibiotics from *Microtetraspora gluaca* DEM 31097

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ABSTRACT

The rise in antibiotic resistance has led to the search of new antibiotic agents with new mechanisms of action from actinomycetes bacteria. Actinomycetes are slow growing Gram-positive bacteria which are known producers of antibiotic compounds. Therefore, the aim this study was to search for antibiotic compounds using understudied unusual actinomycetes obtained from the Demuris Ltd collection of over 1000 actinomycetes bacteria. The prescreening of 66 unusual actinomycete strains at Demuris Ltd led to the selection of the DEM 31097 strain based on its bioactivity. From taxonomic identification using 16S rRNA analysis of DEM 31097, the closest relative was found to be a *Micro*tetraspora *gluaca* with a 99.84% match. The growth of DEM 31097 was optimised by fermentation in a large scale 20 L bioreactor and the fermentation was monitored for contamination and onset antibiotic production. From the fermentation, the antimicrobial activity was detected after 159 hours 46 minutes. Using bioassay-guided fractionation, the bioactive secondary metabolite was identified as a possible gram-positive antibiotic. After a series of liquid-liquid extractions and isolation procedures were attempted the bioactive compoun.d(s) present in DEM 31097 appeared to be polar, pH independent, and either solvent or concentration dependent.

KEYWORDS: Antibiotic, *Microtetraspora glauca* DEM 31097, bioassay-guided fractionation.

1. INTRODUCTION

Natural products are defined as secondary metabolites and they can have a broad range of functions in many biological systems.[1] Traditionally herbal medicine has long utilised bioactive natural products, in the form of plant materials or extracts, in the treatment of diseases.[2,3] The earliest evidence for the use of natural products derived from plants in medicine was found on a Sumerian clay slab, thought to be about 5000 years old, on which were described 12 drug preparation recipes from over 250 plants.[4]

Bacteria are diverse in nature and are major sources of bioactive compounds.[5] The majority of these compounds come from bacteria that inhabit the soil, specifically the actinomycetes.[6] Actinomycetes are filamentous, Gram-positive actinobacterium mainly found in soil and freshwater.[6] Actinomycetes are rich sources of novel bioactive compounds, with many strains still unexplored.[5,7] Examples of antibiotic producing actinomycetes and their antibiotics include *Streptomyces* (e.g. tetracycline), *Amycolatopsis rifamycinica* (e.g. rifamycin) and *Actinomadura* (e.g. spirotetronate).[8,9]

Use of antibiotics over time led to the occurrence of antibiotic resistance by disease causing bacteria. Bacteria have developed several different mechanisms to resist the effects of antibiotics. These mechanisms led to the rise of multidrug-resistant strains of bacteria which cannot be controlled by existing antibiotics.[10]

To successfully continue the discovery of biologically active compounds, new approaches must be considered in order to reduce rediscovery of known compounds or analogues. These analogues tend to have similar mechanisms of action on bacteria as their known analogues.[11] New approaches such as research into the understudied genus of actinomycetes have shown promising results in the search for novel classes of antibiotics.[12] The chances of discovery of new novel antibiotics with different mechanisms of action are very high.

Therefore, the aim of this research is to search for new antibiotics from understudied *Microtetraspora gluaca* DEM 31097. Prior to this project, 66 actinomycete understudied strains were collected and prescreened for antibiotic activity against both Gram-positive and Gram-negative pathogenic strains at Demuris Ltd. From the 66 understudied strains DEM 31097 was selected for further studies based on the biological activity presented.

2. MATERIALS AND METHODS

2.1 Growth media and Disc diffusion assay

All procedures were done under sterile conditions.

2.1.1 Growth media

Growth media are prepared using distilled water in a sterile bottle and depending on which media is being used (Table 1) the pH is adjusted using 2 M NaOH or 50 % HCl at room temperature. The prepared media are autoclaved (sterilised) in an Astell autoclave at 121 °C for approximately 3 hours and allowed to cool to 60 °C in a hot water bath before use.

Table 1: Different growth media and their compositions

Medium	Components	pH
ISP2- (Glucose, yeast and malt) extract	4 g/L Glucose, 4 g/L Yeast Extract and 10 g/L Malt Extract.	7.0
Nutrient Broth	15 g/L Peptone, 6 g/L Sodium Chloride, 3 g/L Yeast Extract and 1 g/L glucose	7.0
Nutrient Agar	5 g/L peptone, 1.5 g/L yeast extract, 1.5 g/L beef extract, 5 g/L sodium chloride and 15 g/L agar.	7.4

2.1.2 Disc Diffusion Assay

Samples are prepared by dissolving in a minimum about of solvent (~ 1ml of solvent). For a 100 ml disc diffusion assay agar, the agar was prepared by pipetting 100 µL *Bacillus* reporter strain Table 2, 100 µL erythromycin and 100 µL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) each into a 100 mL autoclaved growth media. This impregnated media is poured onto an agar plate under lamina flow and allowed to set for about an hour.

The spotted discs were prepared by labelling and spotting each 5mm disc with 10 µL of samples, positive and negative controls and left to dry for about 30 minutes depending on the solvents used to prepare the samples. The spotted disc containing different samples are then mounted onto the set seeded agar plate and incubated in Sanyo Ltd MIR-262 incubator at 30 °C overnight. Diameters of clear zones of inhibition are recorded once the bioassay plate is removed from the incubator.

Table 2: Reporter strain used in assessing the mode of action of the bacterial strain

Bacillus reporter strain	Mode of action	Resistance	Positive control
YpuA	Cell wall synthesis	Erythromycin	Cefotaxime

2.2 Fermentations

10mL of liquid ISP2 medium were inoculated with a single loop of spores of the selected strain and incubated in an Innova®40 enclosed orbital shaker at 160 rpm for 3 days at a temperature of 30°C. A

10% inoculum (culture in 10 ml ISP2 media) was used to subculture 50mL, 200mL and 500mL of ISP2 medium sequentially. As the strains were growing exponentially from the seed culture only a single day of incubation was required for the 50mL, 200mL and 500mL cultures. Erlenmeyer flasks containing media to a quarter of their volume were used to give sufficient aeration. For the 500mL fermentations baffled 2L flasks were used to give additional aeration.

Large-scale fermentation was carried out using a 20 L glass fermenter from applikon Biotechnology with an ez-control. Temperature of 30 °C and a starting pH of 7 were maintained but not controlled during the fermentation process.

Dissolved oxygen was set at a minimum of 40 % which was controlled by the impeller speed and compressed air intake was kept constant. The impeller speed was at 250 rpm to reduce foaming; foaming was further controlled by the use of a few drops of PPG (1 ml L^{-1}) in the reactor and 50 % antifoam A in a sterile bottle controlled by the foam probe connected by a peristaltic pump.

Samples were taken three times a day and the OD_{450} was measured in triplicate. The samples taken were streaked on to nutrient and growth media plates then incubated overnight at 30 °C to monitor contamination.

2.3 Offline sampling

Several parameters such as glucose concentration, free phosphate concentration and optical density were measured after samples were collected from fermenter. The glucose concentration was measured using the SD Codefree blood glucose monitoring system.

2.4 Determination of phosphate concentration in the supernatant

1 mL of samples collected in 1.5 mL sterile tubes are centrifuged at 1000 rpm for 8 minutes. To separate 1.5 mL sterile tubes, 85 µL of the supernatant, the Pi standards, the liquid ISP2 media and reagent blank (distilled water) were each pipetted. To each of these 1.5 mL sterile tubes we added 1000 µL of ASA reagent and incubated for 90 minutes at 37 °C, after 90 mins blue colouration was observed. To stop the reaction, 400 µL of 2M H_2SO_4 was added to each of the 1.5 mL sterile tubes. The absorbance was measured at 820 nm against reagent blank and the values recorded. The absorbance of Pi standards was plotted against concentration to obtain a Trent line equation which was used to calculate the phosphate concentration of the samples.

Antibiotic activity was assessed by a disc diffusion assay, after the samples were taken.

2.5 HPLC-DAD

An Agilent Technologies© 1260 infinity HPLC with an integrated diode array detector was used to assess the purity of extracts throughout the purification processes. For a 200 µl dilution, samples were dissolved 20 µl of HPLC grade methanol (MeOH) and diluted with 180 µl of HPLC grade H_2O. A C18 phenomenex® column (150 x 4.60 mm, 4 µm) was used. The typical gradient performed was 0% acetonitrile (CAN) (0.001% formic acid (FA)) to 100% (FA) over 45 minutes at a flow rate of 1ml min^{-1}. Injection volume ranged between 40 to 200 µl depending on the volume of the diluted sample.

2.6 Extraction and purification: DEM 31097

DEM 31097 cultures obtained after fermentation was centrifuged at 8000 rpm for 10 minutes and cell pellet was collected and the supernatant discarded.

994 g of wet cell pellet collected was frozen before being freeze dried for 7 days, and weighed after 7 days to give 395 g of dry cell pellet. The dry cell pellet was soaked in acetone and stirred for 2 days at room temperature, filtered and washed twice more with acetone. The solvent (acetone) in the cell pellet extract was evaporated using a rotary evaporator to yield a 47 ml concentrated solution.

The next step was a liquid-liquid extraction of the cell pellet extract as described in Figure 1.

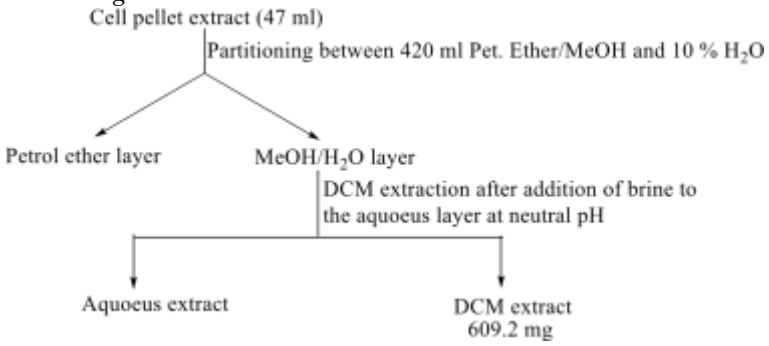

Figure 1: Flow diagram showing extraction procedure of DEM 31097 cell pellet

3. RESULTS AND DISCUSSION

3.1 Taxonomic identification of DEM 31097 strain

Under the microscope, DEM 31097 appeared to have spores in a chain of four spores which is characteristic of the *Microtetraspora* strain. A *Microtetraspora* has two mycelia, a vegetative mycelium which has a long, branching and wavy filament that penetrates the agar (Figure 2) forming tough and raised colonies and an aerial mycelium that appears on the vegetative mycelium and are scantly branched and straight.[13]

Using a 16S rRNA alignment on a Mega6™ software, the phylogenetic tree was plotted and a neighbouring tree was produced using *Actinomudura madurae* DSM 43067 as a root. On analysis, the closest neighbour was *Microtetraspora glauca* with a 99.84 % similarity (See Figure 3).

Figure 2: An agar plate showing DEM 31097/TW41a strain

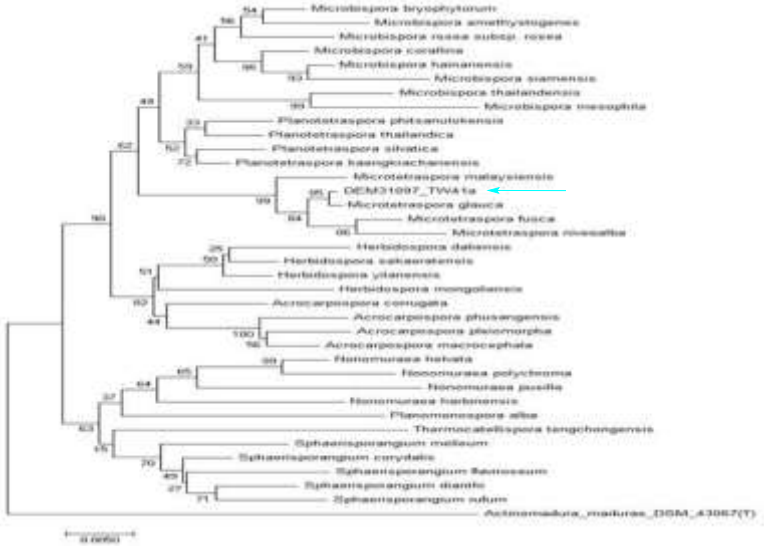

Figure 3: A 16S rRNA phylogenetic tree showing DEM 31097 and its relatives

Microtetraspora sp. is an understudied strain of actinomycetes in which no bioactive compounds with antibiotic activity have been reported from these species. Its neighbours on the 16S rRNA also are not known to produce antibacterial secondary metabolites. Therefore, this strain was chosen because the secondary metabolite causing the observed antibiotic activity against Gram-positive bacteria was likely to be a new novel bioactive compound.

3.2 Growth optimisation of DEM 31097 for the production of novel antibiotics

To investigate the bioactivity of DEM 31097 further, large-scale fermentation using a 20 L fermenter of the strain was required. From the shake flask fermentation, antibiotic production was observed approximately after 7 days, thus on a larger scale we estimated that at least 7 days of fermentation would be required.

DEM 31097 was taken from frozen glycerol stocks of spore suspensions from a -80 °C freezer, revived and streaked onto ISP2 agar plate and left to grow in an incubator for 3 days. Spores taken from the freshly grown plates were used to inoculate 10 mL liquid ISP2. The culture was grown exponentially by culturing into larger flasks each time using about 10 % of inoculum, before finally inoculating into a 20 L fermenter containing about 18 L of autoclaved ISP2 media.

We checked for contamination by using the samples collected throughout the fermentation process. To each daily sample collected, a sterile loop was used to collect and streak the bacteria samples onto two different agar plates. One agar plate contained solid ISP2 media and the other contained a solid nutrient agar. These inoculated plates were incubated at 30 °C for three days. After three days each plate was viewed for presence of any fast-growing bacteria such as *Bacillus* or *E.coli*.

Several parameters were used to monitor growth of the bacteria; these parameters were optical density, glucose concentration and free phosphate concentration (Figure 4).

The optical density is a representation of the bacterial growth. The optical density at (OD_{450nm}) for the first 100 hours was approximately 0.5 at OD_{450nm}, indicating the lag phase before it started rising quite quickly between 110 and 140 hours to about 2.7 at OD_{450nm}, indicating the exponential phase and stationary phase, before declining steadily from around 150 to 240 hours when the bacterial growth was in death phase.

Glucose concentration and free phosphate concentration both decreased during the course of fermentation. Free phosphate concentration from the graph appeared to be taken up much faster by the bacteria than the glucose, as its concentration decreased from about 2.5 mM to 1.0 mM in approximately 30 hours. However, both nutrients depleted once the bacteria appeared to have reached its stationary growth phase. It is during this nutrient starvation that bacteria start to compete for survival and start producing antibiotics as a defence mechanism.[14] This is observed in figure 4 when bacteria growth reached its stationary phase and started declining at approximately 159 hours.

The antibiotic activity test against Gram-positive bacteria was carried out on the samples collected, to determine the onset of antibiotic production. The samples collected were centrifuged at 4000 rpm for 15 minutes, filtered and the supernatants collected. The supernatants were concentrated by solid phase extraction using a C18 cartridge with MeOH as the eluent. The cell pellet was extracted with acetone. The cell pellet extracts, the supernatant and the SPE fractions collected from these extractions were tested for antibiotic activity using disc diffusion assay verses *B. subtilis ypua* (a Gram-positive bacterial reporter strain for cell wall synthesis activity).

After 159 hours, antibiotic activity was observed from the cell pellet extracts, however no activity was observed from SPE fractions at this time point. With the onset of antibiotic production obtained, the fermenter was kept running for 3 days to monitor any changes in antibiotic activity. On the 11th day the fermenter was partially harvested and the bacteria culture was centrifuged, filtered and the cell pellet was bioactive after being tested for antibiotic activity. Meanwhile the fermenter was refilled with more growth media to obtain sufficient bacterial culture for analysis, before the bacteria culture was finally harvested on day 21.

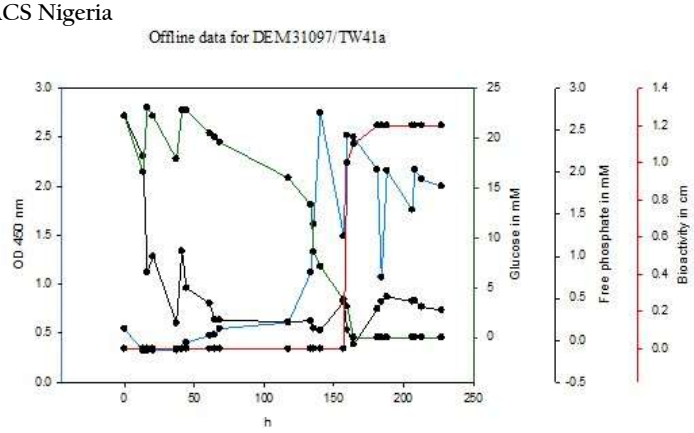

Figure 4: Offline data of DEM 31097/TW41a 18 L cultivation

3.3 Extraction and Purification of Bioactive Natural Products from Cell Pellet Extracts of DEM 31097

We therefore decided to attempt to isolate the secondary metabolite(s) responsible for the observed antibiotic activity that were present in the cell pellet. The first step in this process was to isolate the cell pellet material from both the fermenter runs. The cell pellet was frozen, then freeze-dried and extracted with acetone and concentrated to yield a crude extract. The crude extract was bioactive after being tested for antibiotic activity using disc diffusion assay.

After several extraction attempts using solvents of varying polarity, the addition of brine to the aqueous layer, during dichloromethane (DCM) extraction was successful with the antibiotic activity being observed from the organic layer. The next step was to examine suitable chromatography conditions on the DCM/brine extract, including normal phase chromatography and size exclusion chromatography. Our first purification attempt used normal phase chromatography.

Normal phase chromatography was performed by using three different solvents one after another, namely ethyl acetate (EtOAc), DCM and MeOH, a preparative reverse phase HPLC and Size-exclusion chromatography (SEC) using MeOH were all carried out on samples of the crude extract. The fractions were collected after each purification step was pooled together according to the solvents used and concentrated via rotary evaporation. A test for antibiotic activity using disc diffusion assay was carried out on the fractions obtained and the results were negative. We postulated that this might be due to the bioactive compounds either degrading as a result of instability during chromatography or being retained on the silica.

Due to the difficulties in isolating bioactive compounds from the cell pellets of the fermentations of DEM 31097, possibly due to compound instability, we decided to re-grow the bacteria in shake flasks to see if the previously observed biological activity in the supernatant could be recovered. We envisaged that the supernatant activity was due to the presence of an alternative antibiotic which might prove easier to isolate.

4. CONCLUSION

DEM 31097 has been taxonomically investigated and identified as a *Microtetraspora sp.* which poses antibiotic activity against Gram-positive bacteria. A suitable growth condition for DEM 31097 has been optimised and achieved in a large-scale fermenter. Interestingly, we observed that different growth condition seem to lead to the production of possibly different secondary metabolite(s) by DEM 31097. After a series of extraction and isolation procedures were attempted the bioactive compound(s) present in DEM 31097 appeared to be polar, pH independent, and either solvent or concentration dependent. Also, progress has been made in the design of a liquid/liquid extraction protocol for the purification of the cell pellet antibiotics. In shake flask fermentation, the isolation of the antibiotic compound present in the supernatant has begun, but these compounds appear unstable in methanol. Thus, we plan to develop an isolation protocol for extraction of secondary metabolite(s) present in the supernatant.

The difficulty encountered in the development of a new isolation protocol of bioactive compounds from understudied actinomycetes arises from the absence of limited literature and knowledge of the newly

isolated strain of actinomyctes [12]. Therefore, the use bioassay fractionation method allows for the steady screening of extracts based on their biological activity against disease causing and antibiotic resistant strains. As a result the potential use of understudied actinomycetes in the search for new antibiotic compounds with new and improved modes of action for combating antibiotic resistance is promising.

REFERENCES

(1). A. Demain and A. Fang, in *History of Modern Biotechnology I*, ed. A. Fiechter, Springer Berlin Heidelberg, Editon edn., 2000, vol. 69, pp. 1-39.

(2). R. Croteau, T. M. Kutchan and N. G. Lewis, *Biochem. Mol. Biol. Plants*, 2000, **24**, 1250-1319.

(3). R. N. Cavalcanti, T. Forster-Carneiro, M. T. M. S. Gomes, M. A. Rostagno, J. M. Prado and M. A. A. Meireles, in *Natural Product Extraction: Principles and Applications*, The Royal Society of Chemistry, Editon edn., 2013, pp. 1-57.

(4). B. B. Petrovska, *Pharmacognosy reviews*, 2012, **6**, 1.

(5). M. I. Hutchings, A. W. Truman and B Truman, *Current Opinion in Microbiology*, 2019, **51**, 7280.

(6). M. F. Traxler and R. Kolter, *Natural product reports*, 2015, **32**, 956-970.

(7). Y-G. Zhu, Y. Zhao, D. Zhu, M. Gillings, J. Penuelas, Y. S. Ok, A. Capon and S. Banwart, *Environment International*, 2019, **131**, 105059.

(8). M. D. Surette, P. Spanogiannopoulos and G. D. Wright, Acc. Chem Res., 2021, **54(9)**, 20652075.

(9). J. Euanorasetr, B. Intra, P. Mongkol, S. Chankhamhaengdecha, P. Tuchinda, M. Mori, K. Shiomi, T. Nihira and W. Panbangred, *World J Microbiol Biotechnol*, 2015, **31**, 391-398.

(10). K. E. Vivekanandan, P. V Kumar, R. C Jaysree and T. Rajeshwari, *Global Medical Genetics*, 2025, **12 (2)**, 100042.

(11). C. Pearce, P. Eckard, I. Gruen-Wollny and F. G. Hansske, in *Natural Product Chemistry for Drug Discovery*, The Royal Society of Chemistry, Editon edn., 2009, pp. 215-241.

(12). J. Parra, A. Beaton, R. F. Seipke, B. Wilkinson, M. I. Hutchings and K. R. Duncan *Current Opinion in Microbiology*, 2023, **76**, 102385.

(13). J. E. Thiemann, H. Pagani and G. Beretta, *J. Gen. Microbiol.*, 1968, **50**, 295-303.

(14). J. Martín and P. Liras, in *Reprogramming Microbial Metabolic Pathways*, eds. X. Wang, J. Chen and P. Quinn, Springer Netherlands, Editon edn., 2012, vol. 64, pp. 115-138.