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Original Article

PRODUCTION OF ANTIMICROBIAL AND ANTICANCER FOM FEATHER-KERATINOLYTIC *NOCARDIOPSIS SP*. 28ROR AS A NOVEL STRAIN USING FEATHER MEAL MEDIUM

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ABSTRACT

Objective: Production of bioactive secondary metabolites from a feather-degrading actinobacterial species using feather meal medium.

Methods: Protease producer actinobacterial isolates (22) recovered from farm soil, poultry farm soil and feather wastes were used to test the antagonistic effect on pathogenic bacteria and fungi, including *Staphylococcus aureus, Escherichia coli*, *Microsporum canis* and *Trichophyton mentagrophyte* using Mueller-Hinton agar and potato dextrose agar (PDA) media. The best isolate was used to produce the active metabolites in feather meal medium composed from (g/l) 10 feather meal, 5 sucrose and 0.3 l cement extract were dissolved in tap water, in addition to the standard medium composting of (g/l) glucose (1%), tryptone (1%), KH_2PO_4 (0.07%) and K_2HPO_4 (0.14%) were dissolved in distilled water, both of them at initial pH 9. The secondary metabolites were partially purified by Sephadex LH20 column and the antimicrobial activity and cytotoxic activity were assayed.

Results: 31.82% of isolates inhibited the growth of both bacterial and fungal test organisms and the best one was *Nocardiopsis sp*. 28ROR (GenBank: KC702802.1) at a significant level $P< 0.05$. It produced bioactive metabolites in both feather meal broth and the standard media. The partially purified metabolites inhibit the breast cancer cell line MCF-7 (51%) and normal hepatic cell line WRL-68 (9%), in addition to inhibiting *S. aureus* and*Trichophyton menta agrophyte* growth.

Conclusion: The actinobacteria has vast abilities to degrade very complex wastes and converted to simple constituents to reprocess in other industries. So the *Nocardiopsis sp. 28ROR* was a novel strain produced anticancer, antimicrobial substances using feather meal medium as a cheap waste medium.

Keywords: *Nocardiopsis*, Keratinase, Antimicrobial, Anticancer, Feather medium

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INTRODUCTION

The genus *Nocardiopsis* produced a vast variety of bioactive compounds such as pendolmycin (antifungal and tumor promoting agent), in addition to secreting extracellular enzymes like alkaline tolerant enzymes, amylases, alkaline serine proteases and keratinolytic proteases [1, 2]. *Nocardiopsis* species produced a novel antibiotic like naphthospirononones (antimicrobial and anticancer compounds) [3], apoptolidins (selective anticancer agents) [4], griseusins (antimicrobial and anticancer compounds) [5], thiopeptides (antibiotic) [6] and lipopeptides (surfactants) [7]. This actino-bacterium is thus of considerable biotechnological importance and can serve as potential sources of natural products and new metabolites in the future. On the other hand, some of actinobacterial species had ability to degrade the feather waste, which are the valuable sources for variety of amino acids, predominantly cystine, lysine, proline, and serine [8], because of these abilities of actinobacteria particularly *Nocardiopsis* it may be used to produce the antibiotics using feather waste degradation as a sources for carbon and nitrogen [9, 10]. So our objective of the study was using keratinase producing action bacteria to produce antimicrobial substances in a culture medium containing feather waste as a cheap medium.

MATERIALS AND METHODS

Materials

The materials were provided from Biotechnology and Genetic Engineering laboratory at the University of Babylon. Analytical grade solvents and Mueller-Hinton agar (HiMedia, India). Sephadex G-50 superfine gel and Sephadex G-25 superfine gel (Pharmacia Biotech). Glucose, tryptone, KH_2PO_4 and K_2HPO_4 (BDH, England).

Feather meal preparation

Feather meal was prepared from native chicken feathers as described by Saibabu *et al.* [11] with slight modifications. White feather waste was collected from poultry slaughterhouses, and it was washed several times with soap and water to eliminate blood and dirt remainder, and then dried for 24 h in sunlight. The feathers were cut with scissors into small pieces of 1-3 cm long. Defatting of feather pieces was performed by soaking them in a mixture of chloroform: methanol (1:1) for 2 d, followed by chloroform: acetone: methanol (4:1:3) for 2 d. The solvent was replaced every day. The feathers were finally washed several times with tap water to eliminate the solvent residual, dried using the oven at 40 °C for 24h, ground using an electric blender and used as feather meal.

Actinobacterial isolates

Twenty-two keratinase producing actinobacterial isolates were screened for antimicrobial production, which previously isolated from different sources such as farm soils, soils of a poultry farm and waste chicken feathers (unpublished data) in Biotechnology and Genetic Engineering Laboratory at the University of Babylon in Babylon Province.

Screening for antimicrobial activity

Preliminary screening for antibacterial activity was done by the cross-streak method [12], on potato dextrose agar (PDA) medium and Mueller-Hinton agar (MHA) using pathogenic Gram positive and negative bacteria as test organisms including *Staphylococcus aureus* and *Escherichia coli*. Whereas primary screening of antifungal activity was performed according to Crawford *et al.* [13] method using PDA medium and *Microsporum canis* and *Trichophyton menta agrophyte* as test pathogenic fungi. All pathogenic isolates were obtained from Biotechnology laboratory at Babylon University.

Also, the antimicrobial activities were assayed according to agar well diffusion method was performed [14] using the MuellerHinton agar medium for *S. aureus* and potato dextrose agar for *T. mentagrophyte*. The inoculum of test organisms was prepared separately by mixing a three microorganism colonies (1 ml) from the exponential phase

with 9 ml of sterile nutrient broth and compared the turbidity with that of the standard 0.5 McFarland solution which is equivalent to 106-108 CFU/ml. The sterile swab was dipped into suitably adjusted inoculum. The entire MHA and PDA surfaces were swabbed evenly by the cotton swab. The inoculated plates were left at room temperature for 3-5 min to absorb surface moisture before well formed. The wells (6.0 mm diameter, 2.0 cm apart) were formed in a medium using a sterile cork borer, and then 100 µl of filtered of *Nocardiopsissp.* 28 ROR broth was loaded into each well for the assay of antagonistic activity. The plates were pre-incubated at 4 °C for 2 h to allow uniform diffusion into the agar. Later, the plates were incubated in suitable conditions for test organisms' growth at 37 °C for 18h (*S. aureus*) and 28 °C for 3d (*T. mentagrophyte*). The antimicrobial activity was evaluated by the measuring of inhibition zone diameters.

Production and extraction of active metabolites

The antimicrobial metabolites were produced using two different media (100 ml), the first one as an optimal production medium for antibiotics from the 28ROR strain [15] was composed (g/l) from glucose (1%), tryptone (1%) KH_2PO_4 (0.07%) and K_2HPO_4 (0.14%) were dissolved in distilled water at initial pH 9. The second was a modified medium feather meal-cement extract medium composed from (g/l) 10 feather meal, 5 sucrose and 0.3 l cement extract, which were mixed well together and completed to 1 l by tap water after adjusted to pH 9. Both sterilised media were inoculated with 5% of culture suspensions (OD 0.5 at 600 nm) of the actinobacterial strain (28ROR) inoculum, and then incubated at 35° for 14-21d. Subsequently, the secondary metabolites were harvested by cooling centrifuge at 4 °C and 10 000 rpm for 15 min and the antimicrobial activities were assayed.

Partial purification of active secondary metabolites

The culture filtrate of both media was separately concentrated by rotary evaporator at 40 °C. The concentrated filtrates (50 ml) were fractionated by adding 40% saturation of ammonium sulfate, and 30 ml concentrated cooled acetone (-20 °C), mixed well by vortex, leave to stand for 15-30 min in ice-bath, then centrifuged at 4 °C and 10 000 rpm for 15 min, followed three phase formation, the salt phase (lower layer), acetone (upper layer) and the protein layer floated within acetone phase. Protein precipitate was separated by cooling centrifugation at 10 000 ×g for 10 min. This step to precipitate proteins and polypeptides and the antimicrobial metabolites were soluble in acetone phase. The protein precipitate dissolved in 20 ml Tris buffer and keratinase activity was assayed [16] and store in-24 °C. The acetone phases which containing the antimicrobial metabolite were evaporated by the oven at 35 °C. The concentrated solutions were partially purified by gel filtration column chromatography using two steps the first one by Sephadex G-50 superfine gel (fractionation range of Sephadex G-50: 1 500-30 000 Daltons), then Sephadex G-25 superfine gel (Pharmacia Biotech), which separated biological products within fractionation range 1 000-5 000 Daltons. The columns were prepared according to Pharmacia Biotech methods [16]. The gel filtration was performed by Sephadex G-50 column (D x l) 1.9 x 50 cm using 0.02 M phosphate buffer at pH8 as running buffer, 2 ml of concentrated sample was loaded onto the column, the fractions recovered from column at 60 ml/h flow rate and the fractions were collected at 3 ml size volume. After that, the absorbance of fractions was read at 280 nm and the antimicrobial activities were assayed [14]. The active fractions were combined, concentrated and loaded (2 ml) into a Sephadex G-25 column (1x62 cm), then it was eluted by phosphate buffer 0.02M at pH8 and collected fractions at flow rate 60 ml/h and 3 ml fraction volume. Subsequently, the absorbance of fractions at 280 nm was read, and the antimicrobial activities were assayed [14]. After that, the active fractions were combined and dried in a vacuum dryer, then known the weight of dried substances (100-1000 µg) were dissolved in DMSO (dimethyl sulfoxide) for further investigation. The antimicrobial activities of partial purified actinobacterial metabolites were assayed according to agar well diffusion method was performed [14] using the MHA medium for *S. aureus* and PDAfor *T. mentagrophyte*, each well loaded by 100 µl of 1 mg/ml of partially purified metabolites, as well as the cytotoxic activity was assayed.

Cytotoxic activity assay

MTT assay was performed according to the references [18, 19] at the Centre for Natural Products Research and Drug Discovery, Department of Pharmacology, Faculty of Medicine, University of Malaya/Kuala Lumpur, Malaysia.

The cell viability was determined by colorimetric assay using 3-[4,5–dimethylthiazoyl]-2, 5-diphenyltetrazolium bromide (MTT dye), two kinds of cells were used the human breast cancer cell line (MCF-7cell line) and the normal human hepatic cells (non-tumorigenic fetal hepatic cell line WRL-68). Briefly, 100 µl cell suspension was added to the flat-bottomed microculture plate wells, each cell line on a separate plate were treated with 100 µl microbial extract, incubated for 24 h, and centrifuged to remove the dead cells. An aliquot of 100µl from 2 mg/ml MTT dye was added to each well and incubation was continued for a further 4h, then 50µl of solubilization solution of DMSO was added to each well. The experiment was performed in triplicate. After complete solubilization of the dye, the absorbance of the coloured solution obtained from living cells was read at 620 nm with an ELISA reader. The mean absorbance for each group of replicates was calculated. The control was the non-treated cultures in all experiments that contained cells in the medium only.

Calculate percent viability by using formula [18]:

$$\% \text{ Cell viability} = \frac{\text{Live cell count}}{\text{Total cell count}} \text{x} 100$$

Data analysis

Data were analyzed using SPSS version 22 software Fisher's exact and T-test and ANOVA one way with a significant *P* value of<0.05.

RESULTS AND DISCUSSION

Primary screening of active metabolite production

The ability of produce antimicrobial metabolites from protease producing actinobacterial isolates were investigated, 59.00% of isolates appeared antifungal activity against both test organisms (*M. canis* and *T. mentagrophyte*), whereas 31.82% of the isolates showed antibacterial activity against *E. coli* and 22.73% against *S. aureus* (table 1). The best one (*P*< 0.05) was selected to produce antimicrobial substances (the isolate 28 ROR). These results in consensus with previous studies that documented the actinobacterial species are the main sources for producing antimicrobial substances and the same organisms had the ability to produce antifungal, antibacterial and anticancer substances [2, 3, 20-24].

The potential isolates 28 ROR which recovered from poultry farm soil, and identified depending phenotypes and physiological characteristics and molecular bases using partial sequencing of the 16S rRNA gene and the phylogenetic tree was constructed by the neighbor-joining method for comparison of the 16S r RNA gene sequences, indicated that the strain belonged to the genus *Nocardiopsis* (the accession number KC702802.1) and named *Nocardiopsis sp. 28ROR* (unpublished data).

The selected strain *Nocardiopsis sp. 28ROR* produced two types of keratinase (acid and alkaline) at different pH values (6 and 10) also the optimum activities of these enzymes at pH 6.0 and 10.0 (unpublished data). Also, the strain 28ROR had the ability to produce antibacterial, antifungal and anticancer in both standard and feather meal media.

From our preliminary results, we can conclude the modified medium (feather meal-cement extract) as a cheap medium gave a good result for the production of bioactive metabolite substances and could be optimized for over-production. Logically, the strain 28ROR produced keratinase enzymes that active within wide range of pH values and hydrolyzed completely feather meal to amino acids and small peptides after 14 d, this lysate (soluble portions) was rich in most of the amino acids like cysteine, alanine, glycine, serine and others, also it contained soluble amides [25] and it utilized as carbon and nitrogen sources for its growth, metabolisms, and produced the secondary metabolites like antimicrobial and anticancer substances. Also, the production media supplemented with basic minerals,

including buffer salts, to enhance bacterial growth, enzyme production and overcome the changing of the initial pH of media. In our study, cement extract was used as a source of minerals, and it acts as a strong alkaline buffer to prevent the initial pH value changes by the byproducts of microorganism. Because of the cement extract was rich with some alkaline minerals like calcium oxides and other metal oxides such as magnesium, ferric, aluminium and sulfurs [26].

Table 1: Antimicrobial antagonism of actinobacterial isolates

Isolates	Test organism (mm±SD)			
	M. canis	*T. mentagrophyte*	*E. coli*	*S. aureus*
1	22±0.11	11±0.05	0	0
2	23±0.11	12±0.05	13±0.17	0
3	0	0	0	11±0.05
4	0	0	0	0
5	0	0	0	0
6	12±0.05	11±0.05	32±0.17	0
7	0	0	0	0
8	0	0	0	12±0.11
9	0	0	0	0
10	22±0.11	10±0.05	21±0.17	0
14	0	0	0	0
16	13±0.11	12±0.15	0	0
17	13±0.11	31±0.11	22±0.05	0
18	22±0.05	12±0.11	0	11±0.11
20	23±0.11	11±0.05	0	0
21	0	0	0	12±0.11
25	23±0.17	22±0.17	0	0
26	31±0.11	23±0.11	0	0
27	11±0.17	21±0.11	12±0.05	0
28*	24±0.17	33±0.17	13±0.05	23±0.17
29	0	0	21±0.11	0
30	12±0.05	21±0.05	0	0
100%	59.00	59.00	31.82	22.73

Antagonism screening for antibacterial activity was done by the cross-streak method [12] on MHA medium, and for antifungal activity was done depending on Crawford *et al.* [13] method using PDA medium. Antagonism (mm±SD), SD Standrd Diveation and 0: no inhibition. * Statistically significant a level $P< 0.05$.

Production and extraction of active secondary metabolites

The secondary metabolites were produced from the strain 28ROR using two media, including optimal antimicrobial production medium as a standard medium and the second was modified feather meal-cement extract medium. Although, the product in standard media was higher than modifying medium ($P\leq 0.05$), but the modified medium was cheaper and available in an environment as a waste. The metabolite substances were extracted from both media using three-phase of extraction methods using ammonium sulfate and acetone and the active metabolites located within acetone layer, which was separated and concentrated using a rotary evaporator, and then they were partially purified using gel filtration chromatography [16] by two steps, Sephadex G-50 column (fig. 1), followed a Sephadex G-25 column (fig. 2). In the first step, the high molecular weight of polypeptides over 30 000 Daltons was separated from the low molecular weights of metabolite substances, which were eluted within the void volume of the column.

Subsequently, the active fractions were separated by a Sephadex G-25 column and the active fractions were investigated for antimicrobial activities and cytotoxic activity. These results in consensus with previous studies that documented the antimicrobial substances could be purified by many methods like chromatography methods depending on the physical characteristics, chemical structure and molecular weight of the antibiotics [27-29].

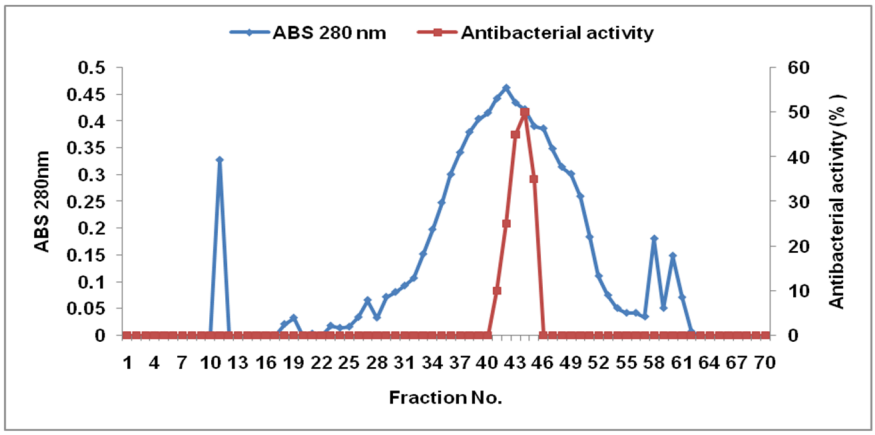

Fig. 1: Gel filtration chromatography of feather meal-cement broth filtrate using Sephadex G-50 columns

The gel filtration was performed using 0.02 M phosphate buffer at pH8, column (D xl) 1.9x50 cm, void volume (V_o)= 32 ml (fraction No.11), Total volume (V_t) = 140 ml (fraction No.47), sample volume = 2 ml, flow rate = 60 ml/h, fraction volume = 3 ml, fractionation range of Sephadex G-50: 1 500-30 000 Daltons.

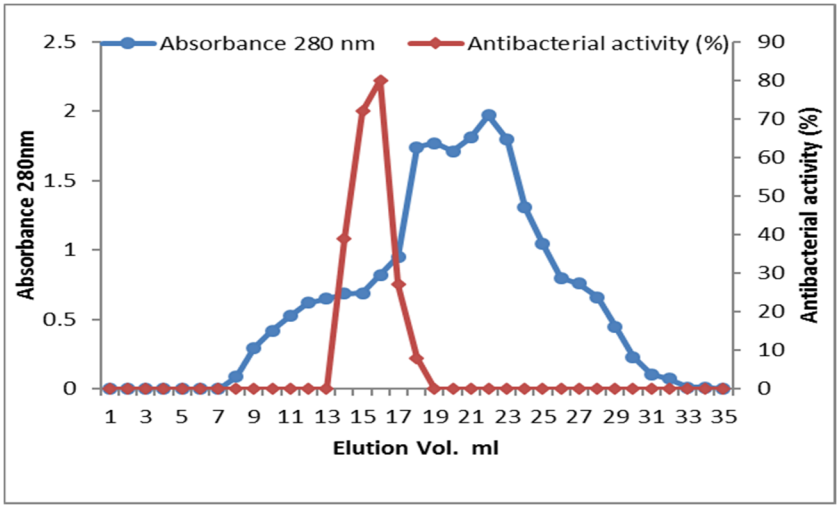

Fig. 2: Antibacterial substance separation by Sephadex G-25 column chromatography after Sephadex G-50 column from feather meal-cement broth filtrate

The gel filtration was performed using 0.02 M phosphate buffer at pH8, column (D x l) 1x62 cm, void volume (V_o)= 14.6 ml (fraction No.5), Total volume (V_t) = 49 ml (fraction No.17), sample volume = 2 ml, flow rate = 60 ml/h, fraction volume = 3 ml, fractionation range of Sephadex G-25: 1 000-5 000 Daltons.

In this experiment, the secondary metabolites were harvested after 21d because of these metabolites were accumulated in culture filtrate and keratinase enzymes were reduced about 90%. After that, the bioactive metabolites were extracted using the salt-solvent mixture to remove protein precipitate and recover only solvent soluble molecules of metabolites, amino acids and small peptides from acetone layer. Subsequently, the active products concentrated by evaporation and partially purified by gel filtration [17]. The recovered amino acids and small peptides could be used as a fertilizer or supplement to microbial media, in addition to the active metabolite substances having a low molecular weight less than 5000 Daltons, which used as antimicrobial and anticancer substances after further characterization and study the mode of actions.

The antimicrobial activity assayed against *S. aureus* and *T. mentagrophyte* according to agar well diffusion method. The results revealed that the strain 28ROR produced antimicrobial substances in both media after 7d and were increasing until 21d and the production of antibacterial substances was higher in the standard medium than modified medium (17 mm and 13 mm respectively), whereas the antifungal substances were relatively the same (12 mm).

Also, cytotoxic activity was assayed by MTT method using the human breast cancer cell line (MCF-7cell line) and the normal human hepatic cells (non-tumorigenic fetal hepatic cell line (WRL-68) and the results appeared the partially purified culture extract had cytotoxic activity as showed in the table (2). From our results the partially purified metabolite (400 µg/ml) had the ability to inhibit breast cancer cell line (MCF-7) at a percentage between 44-51 whereas it slightly inhibited normal hepatic cell line (WRL-68) at 9%, *P*= 0.0005). The above results indicate that the *Nocardiopsis sp.* 28ROR is a novel strain for production anticancer using feather waste and could be further purification and characterization, in addition to two types of keratinases and antimicrobial metabolites.

Many studies reported that the actinobacterial genera like *Nocardiopsis* species had the ability to produce a wide spectrum of antimicrobial and anticancer metabolites that had higher ability to inhibit cancer cell lines in comparison with another source like plant products having antioxidant activity and they inhibit cancer cell lines [1-7, 30-33].

Table 2: Cytotoxic activity of partially purified extract of *Nocardiopsis sp*. 28ROR against breast cancer cell line MCF-7 and normal hepatic cell line WRL-68

Partially purified extract (µg/ml)	MCF-7 mean±SD	WRL-68 mean±SD	*P-value*
Feather meal medium			
400	56.28±5.61	91.74±1.81	0.000
200	90.15±1.02	92.78±1.70	0.084
100	91.74±1.82	94.44±0.12	0.062
50	92.78±1.70	95.25±0.81	0.086
Control medium			
400	49.16±0.95	91.77±0.84	0.000
200	88.1±0.85	94.4±0.26	0.000
100	90.9±0.24	95.30±0.26	0.000
50	91.23±1.10	96.06±0.23	0.002

*All the values are the average of three readings, mean±SD, SD = Standard Deviation

CONCLUSION

In the present scenario over the world, academic and industrial research focuses mainly on recycling of waste by using micro-organisms that have the ability to degrade the waste like a chicken feather to prevent their accumulation in the environment. In the other hand, the degradable constituents can be reprocessed or utilised as nutrition sources to the same microorganisms to produce commercial substances such as pharmaceuticals or antibiotics after controlling the growth and production parameters for these microorganisms.

The *Nocardiopsis sp. 28ROR* was a novel strain produced anticancer, antimicrobial substances and two types of extracellular keratinizes (acid and alkaline) in cheap feather waste medium at a wide tolerance range of temperature and pHs.

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CONFLICT OF INTERESTS

We declare that we have no conflict of interest

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