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Production of antimicrobial agents from *Aspergillus fumigatus* isolated from local soil sample

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Abstract : Since the 1928, fungi have been used for the production of antimicrobial compounds as secondary metabolites. Fungiwas isolated from soil sample and identifiedas *Aspergillus fumigates* depending on morphological and molecular diagnostics. Purified *Aspergillus* isolate was screened for antimicrobial production activity and test the effect of fermentation media, pH, temperature, sugar concentration and duration on the productivity and optimized to determine the optimal conditions for antimicrobial metabolite production. The optimal production condition of antimicrobial metabolite was observed in Potato dextrose solid media under pH 6.5 and incubation temperature 30°C.Antimicrobial activity of fungal extract was tested against test pathogenic bacteria and fungi. The fungal extract was tested against ten pathogenic bacteria and ten pathogenic fungi. Antibiotic described using three chemical test (Fourier transformed infrared spectroscopy (FTIR), Nuclear magnetic resonance (NMR), Euro EA Elemental Analyzer (CHNS)) to detect the chemical forma for the extracted compounds.The aim of these researches to screening for antimicrobial producing fungi from local soil sample.

Key words: *Aspergillus fumigates*, antimicrobial compounds, optimal conditions, solid media.

1-Introduction

Antibiotics are antimicrobial compounds produced by living microorganismsas secondary metabolites, exhibit either anti-microbial, anti-fungal[1],anti-bacterial[2] , anti-tumour,anti-viral[3] and anti-protozoal activities[4]. More than 16500 antibiotics have been isolated before,where 29% of them produced by fungi [5] , but only fiftyhave attain wide use. The other antibioticcompounds failed to attain commercial importance for some reasons such as toxicity to human andanimals, effectiveness or high production costs[2].

Fungi considered as good natural sources for antimicrobial metabolite[6,7,8], mainly in molds of the order *Aspergillus*[9]. In 1928 Alexander Fleming, isolated *Penicillium notatum* producing a metabolite capable of killing Gram positive bacteria[10]. From that time, producing of antibiotics by molds is widely used in chemotherapy especially cephalosporin, penicillin and fusidic acid, which have both anti-bacterial and anti-fungal activity [11].

The ability of fungal strains to produce antimicrobial substances is influenced by different conditions such as fermentation media, pH , temperature, sugar concentration and duration , that play an important role in the production of antimicrobial metabolites[12].

Dueto an increase of microbial resistance to available drugs, fungi have been used for the production of antibiotics , hence, more fungi needs to be examined for new antimicrobial agents[13].

Current study aimed to determine the ability of fungi that isolated from soil sample to producing new antimicrobial metabolites and optimization of the optimal conditions for production.

2-Materials and methods

Fungal isolates

Soil samples were collected from different type of soil collected from AL-Hilla, Babylon province during the year 2015, brought to the laboratory and processed for fungal isolation. The soil samples were diluted for 1:10(g-ml) , in distilled water , 1ml were poured on potato dextrose agar (PDA) plates in triplicates . The plates were incubated at 28^0C for 7days. The fungal isolates were purified by pure culture techniques and refrigerated in potato dextrose broth with 20% glycerol for further studies.

2.1: Antimicrobial activity

2.1.1 Primary screening

Primary screening for antimicrobial activity was done by the cross-streak method [14], on potato dextrose agar (PDA) using phosphate buffer to maintain the pH 7. Purified fungi isolates were streaked across one side of the plates then incubated at 28°C for 3 days. Test pathogenic microbes (*Bacillus seraus, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Acinetobacter , Klebcilla pneumoniae , Streptobacilli, Streptococcus faecalis, proteusmeraplis,Staphylococcus epidermidis, Candida albicans ,C. tropicalis, C.glabrata, C. dubliniensis, C.krusei and C.parapsilosis,*were streaked perpendicular to the fungus growth, and the plates were further incubated at 37°Cfor24h. The antimicrobial activity was determined bymeasured inhibition zones in (mm)[15,16].

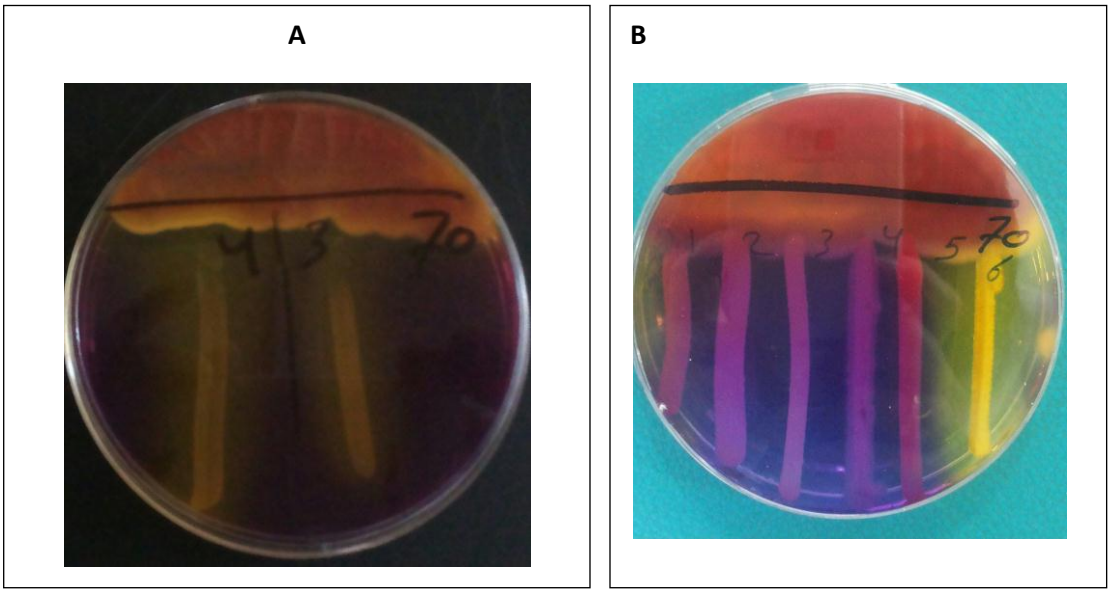

Figure 1: A-Primary screening of the isolate 70 against pathogenic bacteria. B-Primary screening of the isolate 70 against pathogenic yeast (without production).

Primary screening of antifungal activity was done with Crawford method[17]. On the PDA plates, the purified molds isolates were streaked onto one side of the plates then incubated at 28°C for 72 h, or when the colonies are visible. Then, an agar disc from 7day old culture of each test pathogenic mold (*Aspergillus flavus, Aspergillus niger , Trichoderma raprum, Penicillium chrysogenum*) was transferred onto the opposite side of fungal growth. Mold disc were also placed on inoculated PDA plates separately as a control. Plates were incubated at 28°C for 5 days[18]. The inhibitory effect was measured by calculating the difference between the radius of pathogenic fungus growth (y) in the test plate and the radius in the control plates (y0), where $\Delta y = y0 - y$.

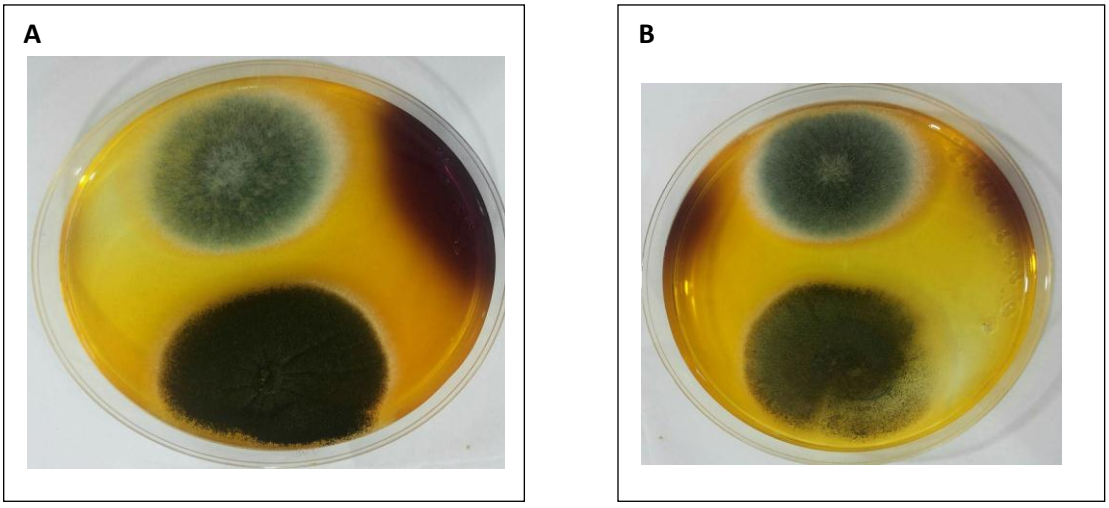

Figure 2: Primary screening of the isolate 70 against pathogenic molds A: *Aspergillus niger*, B: *Aspergillus flavus* (without production).

2.1.2 Secondary screening

The best antimicrobial producing mold isolate (70,104) war selected and appeared belongs to the genus *Aspergillus* based on the growth color on different media(PDA, CYA, MEA) and light microscopy. Based on the diameter of inhibition zone, secondary antimicrobial screening was done under optimal fermentation conditions by disc diffusion method[19]. Grown in liquid and solid media with pH 7 at 30°C for 7 days. The liquid media was prepared by adding 400g of potato pealsto 1000ml of distilled water ,boiled for 30 min then filtered used double layer of gauze,The volume was completed to 1000ml with distilled water[20].the solid state fermentation conditions described by[21]with modification were used Potato peals were dried in an oven at 70˚C for two days.CalibratedPH to 7with phosphate puffer for liquid and solid media then autoclaved at 121˚C for 20 min. Potato are very common in industrial production of antibiotics by molds [22].

In order to obtain the cell-free filtrate, with whatman filter paper No1. On the MHA, the pathogenic microorganisms were streaked , filter disks saturated with 15 µl fungal extract were put onto agar plates ,The discs were carefully dropped on to the surface of the Muller Hinton agar plates used sterile forceps. The plates were incubated at 37°C overnight for bacteria and at 30°C for 72 h for fungi. The antimicrobial activity was detected by measured inhibition zone diameter.

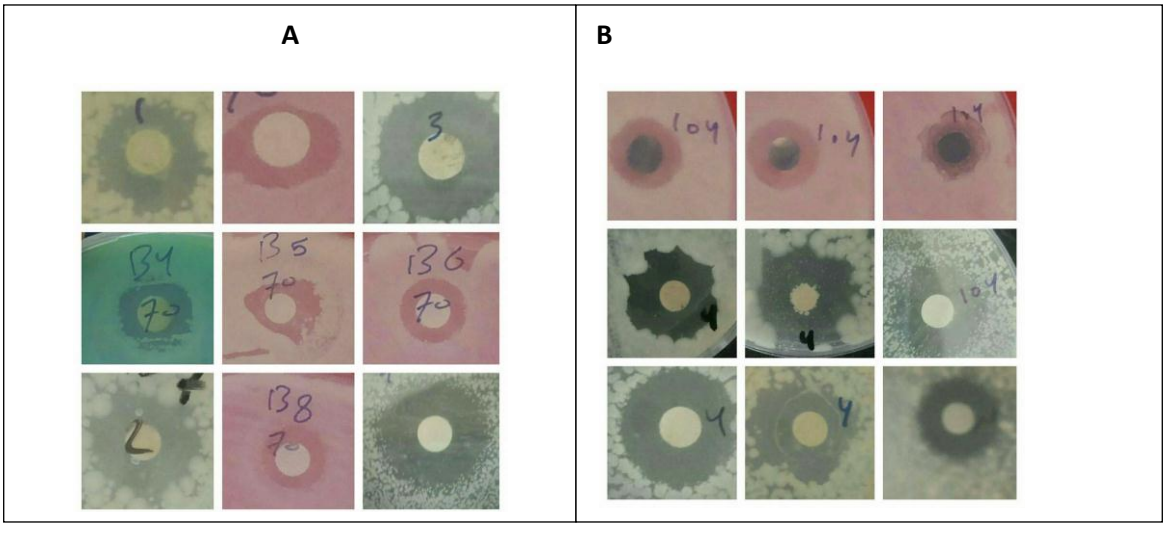

Figure 3: Antibacterial of antimicrobial metabolite for the A-isolate 70 and B- isolate 104 on test pathogenic bacteria .

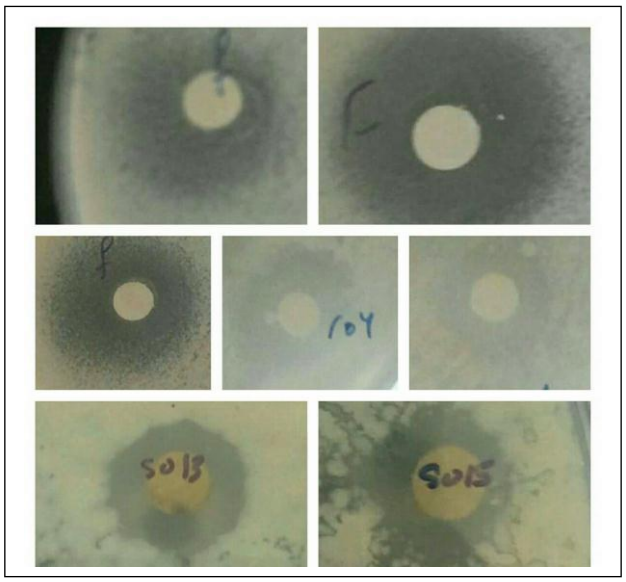

Figure 4: Antifungal activity ofantimicrobial metabolitefor the isolate 104 against test pathogenic fungi .

2.2 Optimization of pH, temperature and duration of antibiotic production

The pH levels of production media were adjusted from 6 to 9, by using phosphate buffer for pH (6, 7 and 8) and glycine buffer for pH (9 and 10) to study the impact of PH. Similarly, the optimum temperature for bioactive metabolite yield was measured by incubating the production medium at temperatures ranging from 25°C to 40°C. the optimum sugar concentration for bioactive metabolite yield was detected by using production medium with 1%,2% sugar concentration . While maintaining all other conditions at optimum levelsproduction media with the selected mold producer war tested for antibiotic production daily, for 15 days.

2.3 Molecular Identification of the selected active isolate

2.3.1 Genomic DNA extraction

The isolate was cultured on potato dextrose agar for 5 days at 30ºC. ZR Fungal/ Bacterial DNA MiniPrep™ DNA isolation Kit was used for DNA extraction and MEGA quick-spine™ Total Fragment DNA purification kit for purification.. The extraction procedure was followed according to the manufacturer instructions. The concentration of DNA and purity were measured using the Nanodrop. The extracted DNA was kept at -20ºC.

2.3.2 Polymerase Chain Reaction (PCR)

PCR reactions were conducted using iCycler Thermal Cycler (Bio-Rad, USA Laboratories, Inc.). ITS rRNA genes were amplified using primers ITS1 and ITS4 primer .

(ITS) region of fungal rRNA genes was amplified using the fungal universal primers ITS1 as a forward primer and ITS4 as a reverse primer. The cycle conditions, predenaturation at 95ºC for 10 min.; 35 cycles of (denaturation at 94 ºC for 1 min, annealing at 50ºC for 2 min and extension at 72ºC for 2 min); final extension at 72ºC for 10 min followed by holding at 4ºC. A 5µl portion of the amplicon was electrophoresed through a 1% agarose gel to check the band size.

2.4Antibiotic Description

For antibiotic description are used three chemical test to detect the chemical forma for the extracted compounds ,Fourier transformed infrared spectroscopy (FTIR), Nuclear magnetic resonance (NMR),Euro EA Elemental Analyzer (CHNS).

3. Results and Discussion

3.1 Antibacterial primary screening The results showed that 7 test pathogenic bacteria were inhibited on PDA by the isolate 70 and 8 test pathogenic bacteria were inhibited on PDA by the isolate 104 which agrees with what previously found that PDA is the best medium for antibiotic primary screening [23,24].

3.2 Antifungal primary screening Antifungal primary screening assays revealed that 5 pathogenic yeast and 2 pathogenic molds inhibited by the isolate 104on PDA.

3.3 Antimicrobial secondary screening

The results showed that potato peals broth media show optimal antagonistic activity against pathogenic microorganisms. While other tested broth media showed variable activity. The pathogenic molds were inhibited only by filtered supernatant of potato peals broth media with inhibition zone 2 cm for both isolates 70,104. At the same time, thepotato peals solid media gave the highest antimicrobial activity against test pathogenic bacteria with inhibition zone 2cm. This medium is more economic than potato dextrose broth that was used for antibiotics production[25]. In contrast, the lowest antimicrobial activity value against all pathogenic microorganisms was obtained at the rice shell broth media.

Table (3.1) : Inhibition zone diameter of indicator pathogenic bacteria in mm

	Diameter of inhibition zone (mm)	
Treatment	**70**	**104**
Bacillus seraus	22.57	21.57
Escherichia coli	-	11.76
Staphylococcus aureus	19.89	15.18
Pseudomonas aeruginosa	14.60	15.68
Acinetobacterbaumannii	18.68	18.84
Klebcillapneumoniae	15.14	20.85
Streptobacilli	18.97	24.07
Streptococcus faecalis	21.13	33.70
Proteus meraplis	10.22	10.52
Staphylococcus epidermidis	19.82	15.21

Table (3.2) :Inhibition zone diameter of indicator pathogenic fungi in mm

	Diameter of inhibition zone (mm)	
Treatment	**70**	**104**
C. tropicalis	9.07	12.51
C. albicans	-	11.98
C. glabrata	-	12.36
C. duplensis	-	-
C. kruzei	10.91	11.93
C. parapsilosis	-	12.25
Aspergillusflavus	-	-
Aspergillusniger	-	13.05
Trichodermaraprum	-	-
Penicilliumchrysogenum	-	14.27

3.4: Optimization of production condition

3.4.1:Optimization of production media

potato peals solid media gave the highest antimicrobial activity against test pathogenic bacteria with stability of production because of this technique supports controlled release of nutrients, best suited for

fermentation techniques involving fungi and microorganisms that require less moisture content and higher productivity[26,27].

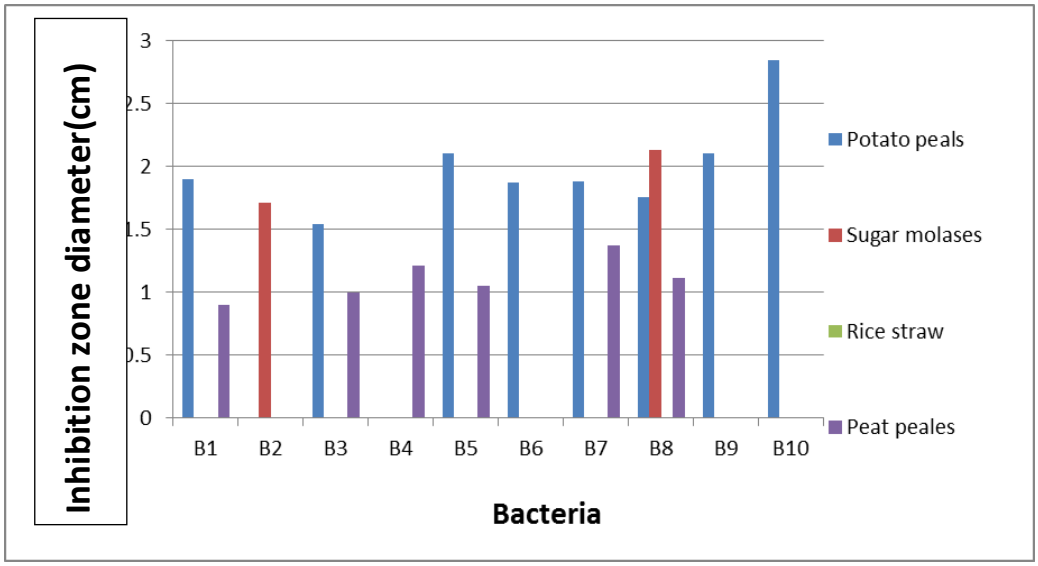

Figure 5: Antimicrobial secondary screening test for 70 and 104 isolate at different production media.

3.4.2:Optimization of production temperature

The optimal of antibiotic activity was recorded at 30°C, while the 25°C where recorded The lowest activity of antibiotic, the inhibition zone diameter. While there was no production at 40°C (Figure 7).

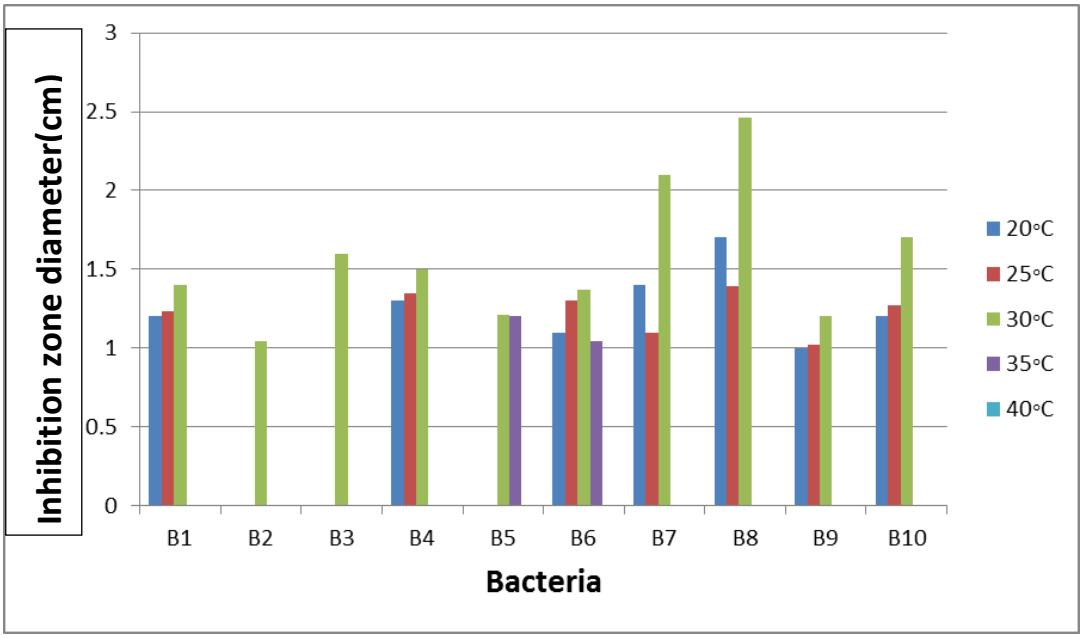

Figure 6: Antibiotic production at different temperatures.

3.4.3 Optimization of pH

The antimicrobial activity ranged from pH 6.5-7 and the highest activity was recorded at pH 6.5 with inhibition zone 3.5cm for S. aureus and 2.8cm for M. canis, but the activity decreased with high PH conditions and no activity was found with alkaline conditions (Figure 8).

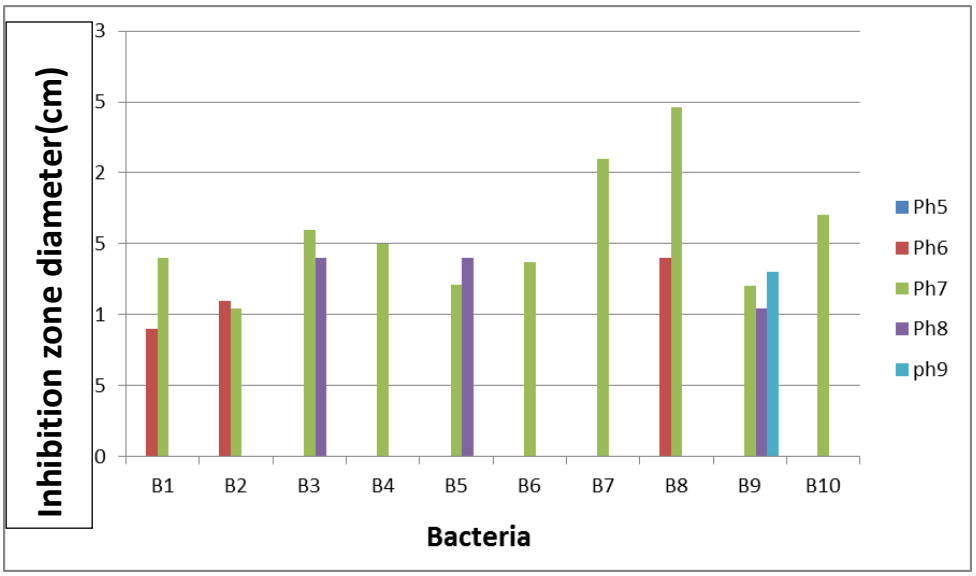

Figure 7: Antibiotic production at different PH.

3.4 Optimization of production period

There was no production for the first three days but the antibiotic was produced on the day 4 with moderate antimicrobial activity. On the fifth day of fermentation, the highest antimicrobial activity was achieved with inhibition zone diameter of 3.3 cm for S. aureus and 2.5 for M. canis and this continued till the fourteenth day (Figure 6).

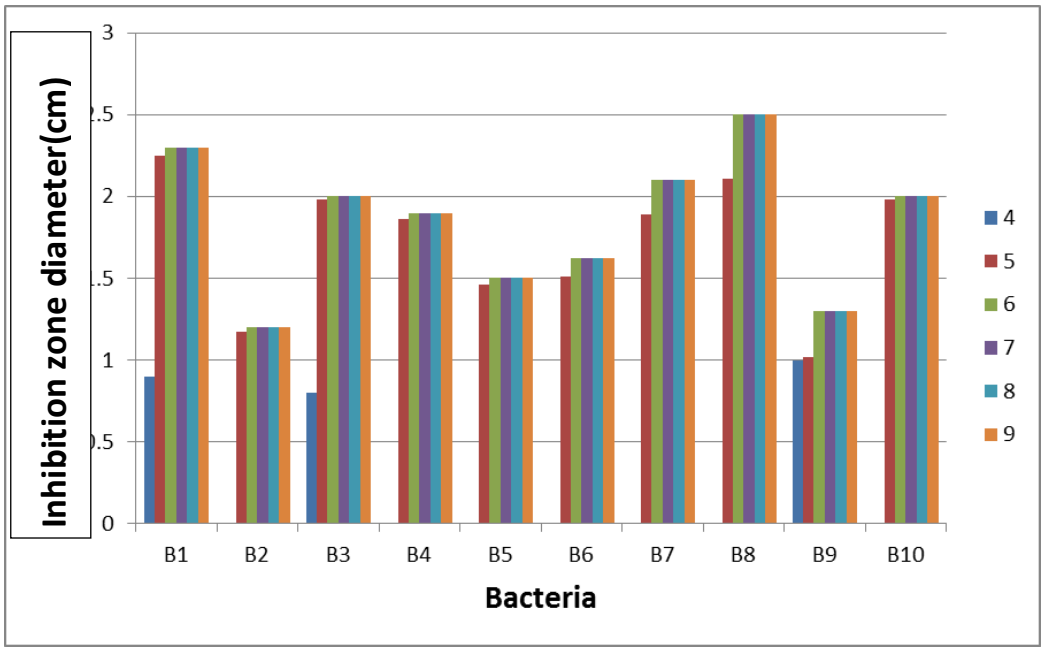

Figure 8 : Antibiotic production at different production time.

3.5 Sequencing analysis

The sequencing similarity illustrated that the isolates 70 and 104 belong to the *Aspergillus sp.*

Table (3.3) Molecular identification of antimicrobial producing isolates 70 and 104

Code	Species scientific name	Sequence ID
70	*A. fumigatus*	KX349472.1
104	*A. fumigatus*	HE864321.1

3.6 Antibiotic description

The antibiotics were diagnosed with Fourier transformed infrared spectroscopy(FTIR), Nuclear magnetic resonance(NMR) and Euro EA Elemental Analyzer (CHNS). The result illustrated in the table (3.4)

Table (3.4): Structural component of antimicrobial compounds for isolates70 and104

Compounds	70	104
Aromatic ring (C=C) cm^{-1}	1537.21	1465.9
(C=O) cm^{-1}	1759.08	1722.4
(O=H) cm^{-1}	3331.07	–
Aromatic (CH) cm^{-1}	2983.8	2974.2
Aliphatic (CH) cm^{-1}	2852.7,2926	2935.66
(N-H) cm^{-1}	3493,3562	–
(S-O) cm^{-1}	731.02	–
(S=O) cm^{-1}	1051.2	–
(O=C-H)	–	–

Table (3.5): CHNS analyses for the isolates 70 and 104

Sample	C%	H%	N%	S%
70	54.596	10.064	2.103	-
104	43.334	5.722	6.359	5.565

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