ISOLATION OF PLASMID DNA FROM *STREPTOMYCES SP*. BACTERIA AND *ESCHERICHIA COLI* PBR322 TRANSFORMATION

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ABSETRACT

The local *Streptomyces sp*. strain showed an ability to produce antimicrobial metabolite active against standard strains, in primary and secondary screening. The produced antibiotic was extracted, purified and identified as a peptide antibiotic produced about 1.4g/L in 7 days incubation period, and its LD_{50} was 5500.

There was an inverse effect for orange acridine dye on the grown colonies number of *S. sp.*, the 28 μg/ml dye concentration was chosen as the best concentration because it led to colonies killing by 95%. Plasmid DNA extracted from *S. sp.* and then transformed to *E. coli* pBR 322, the *E. coli* pBR 322 showed negative results against the standard strains in primary screening before plasmid DNA transformation, while transformed *E. coli* pBR322 showed positive results. The antibiotic produced by trans. *E. coli* pBR322 was extracted, purified and identified by the same ways, which gave the same antibiotic produced by *S. sp.* with an increase of 2.2 g/L in the quantity and shorter period of time (2 days).

INTRODUCTION

Most antibiotics have been isolated from cultures of bacteria particularly Actinomycetes, fungi, algae, higher plant, lower and higher animals, they were used as antibacterial, antifungal, antiviral, antiprotozoal, antialgal, antihelminthic, insecticidal, herbicidal, cytotoxic and anticancer antibiotic[1]. But, the major part of microorganisms that can produce antibiotics inhabits in the soil[2].Majority of antibiotics produce by Actinomycetes belong to genus *Streptomyces* [3]. Actinomycetes produce approximately two-thirds of all known antibiotic of microbial origin, including over 6000 different chemical structures[4] .

The emergence of multiply antibiotics-resistance human pathogens has resulted in an urgent need for new antibiotics,investigators search for new more efficacious preparations produced by various groups of organisms[4].

The aims of the present study.

1. Isolation and purification of antibiotic produced by local isolate of *Streptomyces sp*..

2. Improvement of antibiotic production by *Escherichia coli* pBR322 transformation.

MATERIALS AND METHODS

The Test Organisms:

1. Standard Strains :Include the following microorganisms:-

Bacillus subtilis (PCI 219)**,***Escherichia coli* (NCTC 5933),*E. coli* (pBR 322),*Klebsiella pneumoniae* (ATCC 10031),*Pseudomonas aeruginosa* (NCTC 6750),*Staphylococcus aureus* (NCTC 6571), they were received from biotechnology lab., biology department, college of science, Basrah university .

2. Local Clinical Isolate (clinical strain)

Staphylococcus aureus, received from bacteriology lab., biology department, college of science, Basrah university.

3. Antibiotic Producer Strain: *Streptomyces sp***.** [5]

Culture Media:

1.Commercial media: including, Actienomyces Isolation Agar **,** Antibiotics medium base**,** Mueller hinton agar**,** Nutrient agar**,** Trypton soy broth and Yeast malt extract**.** All media were prepared as recommended by the manufacturing company (Difico)**,** sterilized by autoclaving 121°C for 15 min, cooled to 50°C and poured into Petri dishes.

2. Prepared media

1. Activation medium: It was prepared according to [6,7].

2. Seed Media: It was prepared according to [8].

3. Fermentation medium it was prepared according to [9]**.**

4. Luria-Bertani broth: It was prepared according to [10]

5. Luria-Bertani agar: It was prepared according to [10]

Activation of *Streptomyces sp*. Bacteria:

The activation of *Streptomyces sp*. Performed according to the method of [6,7.]

Preparation of Spores Suspension:

The spores suspension of the isolate was prepared according to [11].

Preservation of the Recovered Isolate:

Spores suspension was stored according to [12].

Primary Screening of Antibiotic Activity for *Streptomyces* sp.:

The primary screening was carried out according to [13].

Determination of Antibiotic Production by *Streptomyces sp.* in Fermentation Media:

This procece was performed according to methode of [14]. .

Secondary Screening for Antibiotic Produced in Fermentation Media:

The ability of the antibiotic production in fermentation medium were determined against stander bacterial strains, *Staphylococcus aureus* (NCTC 6571), *Escherichia coli* (NCTC 5933), *Bacillus subtilis* (PCI 219) and *Klebsiella pneumoniae* (ATCC 10031), according to plate agar diffusion method[15].

Extraction and Purification of the Produced Antibiotic in Fermentation Media:

Extraction and Purification of produced antibiotic was performed according to method describe by [16].

Identification

The producer antibiotic was identify according to [17]; [18]; [19]; [20]; [21]and [22].

Determination of LD_{50}:

The LD_{50} of extracted antibiotic was studied with male mice type (Albino), mice were divided into 5 groups, 8 mice in each group, one of them used as control, all control group mice were injected intraperitoneally by 0.25 ml D.W. other mice in all groups were injected intraperitoneally by 0.25 ml antibiotic solution in different concentration (3000, 4000, 5000 and 6000) ml/kg, all animal were examined for 3 days, the results were analysed according to probate analysis method described by [23].

Plasmid Curing from *Streptomyces sp.*:

Curing experiments were performed by using acridine orange as curing agents according to [4].

Determination of Antibiotic Activity of Curing Cells:

To determine the colonies that lost the antimicrobial activity after treatement with acridine orange. All growing colonies which appeared at 28 □ g/ml concentration after 7 days incubation were inoculated on Petri dishes containing AIA and incubated at 28°C for 7 days [34], then plug method describe by [13] were used.

Extraction of Plasmid DNA:

Plasmids DNA were extracted from *Streptomyces sp*. bacteria according to method describe [25].

Agarose gel electrophoresis:

DNA samples were loaded on 1% agarose gel and run in TBE buffer (89mM tris base, 89mM boric acid and 2mM EDTA, pH adjusted to 8) at 4V/cm, and stained with ethedium bromide, DNA bands were visualized by UV illumination at 340nm [25].

Primary Screening of *Escherichia coli* pBR322:

E. coli pBR322 was screened for antibiotic production according to plug method [13.]

Preparation of Competent Cells of *Escherichia coli* pBR 322:

Competent cell were prepared according to [10].

Plasmid DNA Transformation:

Method described by [10] was carried out to plasmid DNA transformation.Determination of Transformed Cells *Escherichia coli* pBR322To determinate the trans. *E. coli* pBR322, the method of (Hotta *et al*)[26]. was used and the plug method described by (Tyler *et al*)[13] was used to determine which colonies producing antibiotic. Extraction and Purification of Antibiotic Produced by Transformed *Escherichia coli* pBR322:

The methods described in previous stepes were used for extraction, purification and identification of the antibiotic produced by trans. *E. coli* pBR322. Then compare with antibiotic produced by *S. sp*.

Testing Transformed *Escherichia coli* pBR322 Ability to Retain Antibiotic Production

After preservation period for 6 months the antibiotic was produced by trans. *E. coli* in F.M. at the same incubation conditions, and then extracted, purified and identified by the same methods mentioned above

RESULTS

Screening of Antibiotic Activity for *Streptomyces sp., Escherichia coli* pBR322 and Transformed *Escherichia coli* pBR322.

Primary Screening:

The antibiotic activity of *S. sp., E. coli* pBR322 and trans. *E. coli* pBR 322 were tested against the standard strains.The size of growth inhibition zone vary with theses microorganisms,(table1).The results of antibiotic activity of *E. coli* pBR 322 against the standard strains was negative, which means that the *E. coli* pBR 322 before transformation did not able to produce any biological active metabolites, while trans. *E. coli* pBR 322 showed a positive results*(* table1).

Table (1): Primary and secondary screening for *Streptomyces sp.* and transformed *E. coli* pBR 322

Standard strains	Inhibition diameter zone (mm)			
	Primary screening		Secondary screening	
	S. sp	Trans *E.coli*	*S. sp*	Trans *E.coli*
Staph. aureus **(NCTC 6571)**	**20**	**29**	**24**	**30**
B. subtilis **(PCI 219)**	**18**	**22**	**19**	**25**
E. coli **(NCTC 5933)**	**15**	**20**	**17**	**21**
K.pneumoniae **(ATCC 10031)**	**16**	**20**	**15**	**20**

Secondary Screening

The local strain *S. sp.* showed a great ability to produce active metabolite in F.M. Growing trans. *E. coli* pBR 322 on F.M.III produced active metabolite more than that produced by *S. sp.* in the same media (table 2).

Table (2): Inhibition zones diameters of antibiotic extract from different F.M. for *S. sp.* and trans *E. coli* against *Staph. aureus* (NCTC 6571)

Strain	F.M.	Diameter of growth inhibition zone (mm)
S. sp.	**F.M**	**24**
Trans *E. coli*	**F.M**	**30**

Extraction and Purification of the Produced Antibiotic:

The active metabolite was extracted from F.M. filtrates for *S. sp.* and trans *E. coli* pBR 322 after incubation conditions, table (3). The resultant was yellow to orange crystal molecule, moist if exposed to air, with characteristic odor, it is identified as a peptide.

Table (3): Antibiotic produced in optimum growth condition

Strain	F.M.	Rotation/ minute	Incubation period (day)	Incubation temperature (^{o}C)	Antibiotic yield (g/l)
S. sp.	**F.M.**	**180**	**7**	**28**	**1.4**
trans *E. coli*	**F.M.**	**180**	**2**	**37**	**2.2**

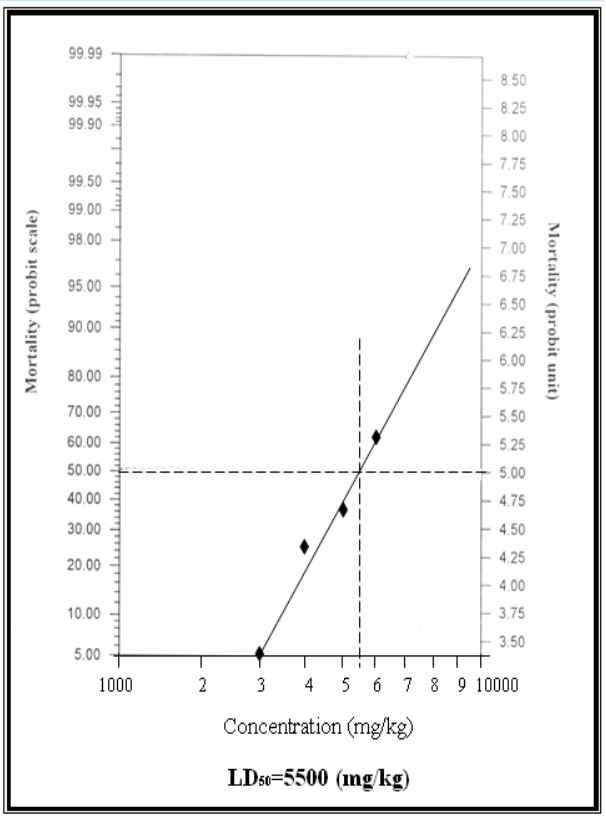

Fig. (1): Lethal dose 50 for antibiotic produced by *Streptomyces. sp.*

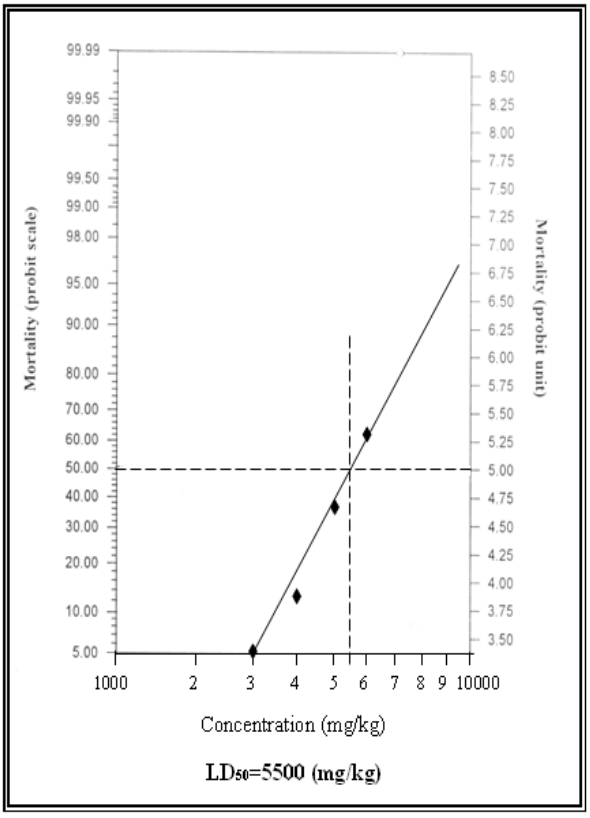

Fig. (2): Lethal dose 50 for antibiotic produced by transformed *E. coli* pBR322

The LD_{50} of the produced antibiotic by *S. sp*. and trans *E. coli* pBR322 showed an identical result (5500) mg/kg, figs. (1and 2).

Plasmid curing:

Fig. (3) showed an inverse effect for orange acridin dye concentration with the growth colonies number of *S. sp.* bacteria, the percentage of growing colonies was 28%, 5% and 0.4% when the dye was added in the concentration of 24, 28 and 32 μg/ml respectively. The 28 μg/ml dye

concentration was chosen as a best concentration because it leads to colonies killing percentage 95%.

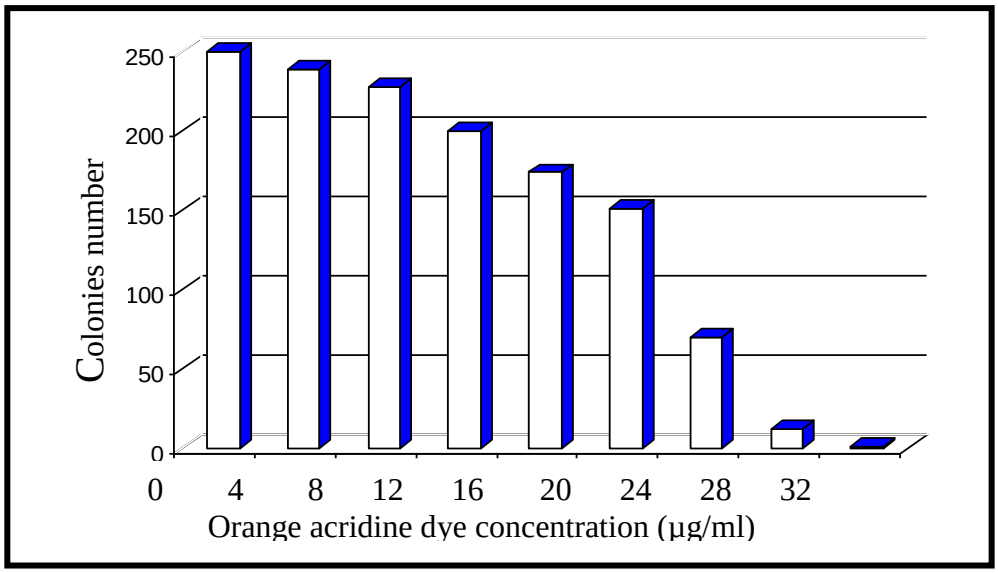

Fig.(3): Orange acridine dye effect on colonies number of

Only 5 colonies did not show antimicrobial activity from the colonies exposed to (28 µg/ml) acridine orange concentration

Extraction of Plasmid DNA:

Extracted plasmid DNA . from *S. sp*. give only one band on electrophoresis, fig. (4) a and b.

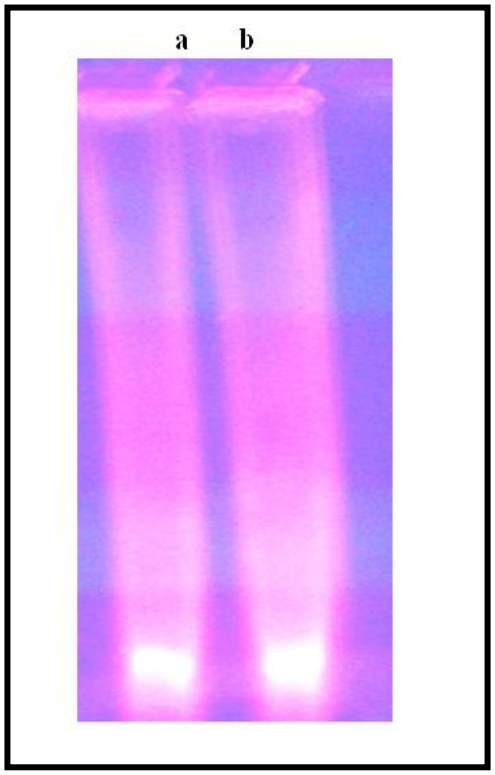

Fig. (4): Electrophoresis for extracted plasmid DNA from *Streptomyces sp.*

Estimation of Antibiotic Production Ability after Preservation Period

Trans. *E. coli* pBR322 has produced an equal amount of antibiotic after the preservation period and demonstrated the same diagnostic tests and biological properties **Fig. (5)**.

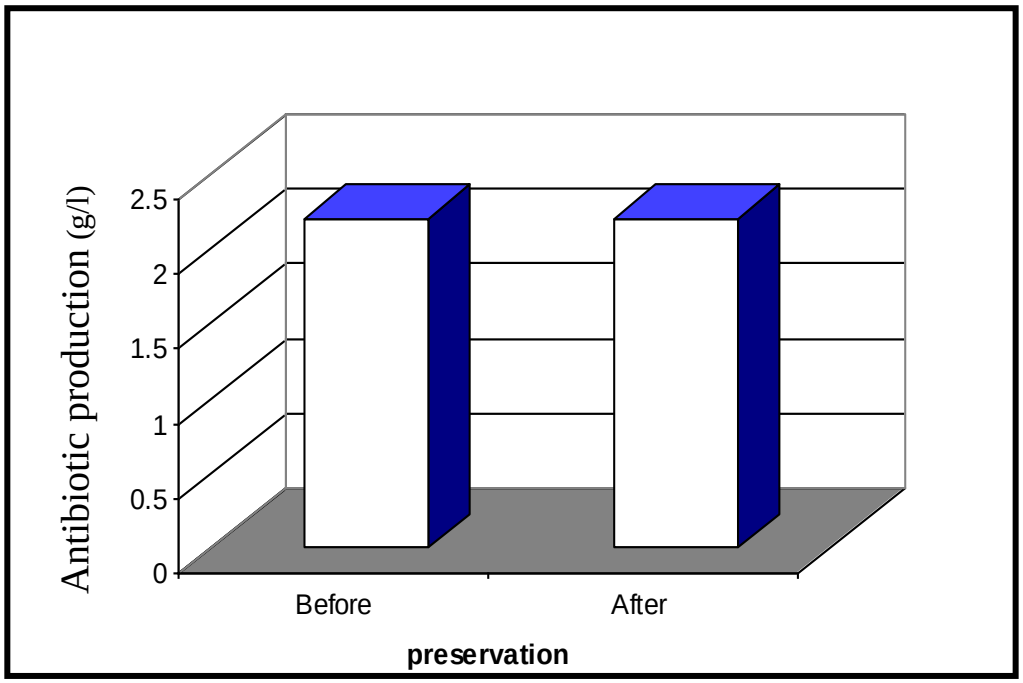

Fig. (5): Antibiotic production by transformed *E. coli* pBR322 before and after 6 months preservation

DISCUSSION

Plasmid Curing from *Streptomyces* sp: The removal of plasmid from bacterial cells lead to missing of certain characters of these cells such as antibiotic resistance or antibiotic production. In the present study the treatment of *Streptomyces sp*. with acridine orange (28 µg /ml) result in loosing of antibiotic production ability in (33.3%) for growing colonies and this results are in agreement with the results of [27] who reported that treatment of some species of *Streptomyces* bacteria with acridene orange dye in different concentration prevent antibiotic production by these microorganisms.

Preparation of Competent Cells:

The nonspecific acceptance of small soluble DNA fragment from the surrounding environment by a bacterial cell is termed transformation. Cells that are capable of accepting genetic material through this means are termed competent [28]. Competency of *E. coli* can be induced in this study by treatment with calcium chloride in the early lag phase of growth. The bacterial membrane is permeable to chloride ion but is non- permeable to calcium ions. As the

chloride ions enter to the cell water molecule accompany the charged particle. The influx of water causes the cell swell and is necessary for the uptake of the DNA [28]. Also calcium chloride induces structural alteration in the bacterial cell walls which help in the binding of DNA with cell wall and facilitate cell transformation [29].

Transformation: According to the ability of some *E. coli* pBR322 after plasmid DNA transformation to inhibit the growth of standard strains of Gram positive and Gram negative bacteria which was unable to inhibit them before transformation, we can say that the transformed *E. coli* pBR322 have the ability to accept a foreign plasmid DNA and express it, as antibiotic formation and production. These results are in agreement with [30], who reported that, the expression of *S.* genes coding for antibiotic biosynthesis in *E. coli* appears to occur, and with [31]; [32], who reported that genes coding for antibiotic biosynthesis appear to be clustered on the plasmid DNA of antibiotic produces in *Streptomyces* bacteria.

Not all *E. coli* pBR322 were transformed with plasmid DNA, this result is in agreement with [29]; [28], who reported that not all bacterial cells have been transformed with the plasmid and the potential for the plasmid not to propagate itself in all daughter cell. Therefore it is necessary to select bacterial cells that contain the plasmid. This is commonly performed using antibiotic selection. Marker genes can be used to determine if the gene has been taken up. Must they have some distinguishable characteristic for example antibiotic resistance [33]. In the present study antibiotic production genes was used as the marker genes for determination the transformed bacteria depending on growth inhibition activity against the test microorganisms.

In addition to the antibiotic production activity the trans. *E. coli* pBR322 acquired the resistance ability to the produced antibiotic, this result is in agreement with [34], who reported that the transfer of 60 kb conjugative plasmid pEJ97 from the bacteriocinogenic strain *Enterococcus faecalis* (EJ97) to *E. faecalis* (OG1X) conferred bacteriocin production and resistance on the recipient. This biological activity may be due to the resistance genes for the produced antibiotic which is found together with antibiotic production genes on the same plasmid. This result is in agreement with other studies as the study of [35], who reported that, the methylenomycin biosynthetic genes (*mmy*) found together with a resistance determinant (*mmr*), are all carried on large liner plasmid SCP1 in *Streptomyces coelicolor* A3(2), and the study of [35], who reported that a 70 kb liner plasmid pSV2 in *S. violaceoruber* SANK 95570 appeared not to be involved in methylenomycin production because (*mmr*) gene did not hybridize to it in spit of this plasmid carried the methylenomycin production genes (*mmy*), also the present results are in agreement with [36], result who detected the pSV1 plasmid by agarose gel electrophoresis and implicated it in methylenomycin production by its hybridization to the methylenomycin resistance (*mmr*) gene.

In comparative between the amount of the produced antibiotic by the two microorganisms *S. sp.* and transformed *E. coli* pBR322 we can notice the increase in antibiotic production by last bacteria in the same fermentation medium (F.M.III), 2.2 g/l during shorter period of time (2 days only), while 7 days are needed to produce 1.4 g/l in case of *S. sp.*. Therefore we can produce 7.7 g/l antibiotic by transformed *E. coli* pBR322 during the same period of time, these results are in agreement with [30], who reported that, the increasing antibiotic production by amplification of the genes coding for limiting enzymes in the biosynthetic pathways is possible, and it is an economic and commercial method to antibiotic production in a shorter time and same substrate (F. M.).

عزل الـ DNA البلازميدي من بكتريا *Streptomyces sp.* ونقله الى *Escherichia coli* pBR332

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الخلاصة

اظهرت العزلة المحلي *Streptomyces sp.* القدرة على انتاج مادة ذات فعالية مضادة للجراثيم القياسية في الغربلة الاولية والثانوية، تم عزل وتنقية وتشخيص المضاد المنتج وكان ذوطبيعة ببتيدية وامتلك نصف جرعة قاتلة 5500 ملغم/كلغم، وانتج بكمية محدودة 1.4غم/لتر في سبعة أيام. اظهرت نتائج المعاملة بالاكردين ان عدد المستعمرات النامية يتناسب عكسيا مع كمية الاكردين المضافة للوسط ، حيث بلغت 95% نسبة القتل عند تركيز 28µg/ml حيث تم اختياره كتركيز افضل لترحيل البلازميد. استخلص الـDNA البلازميدي من *S. sp.* ومن ثم نقل البلازميد الى بكتريا الـ*E. coli* pBR322 حيث اظهرت هذه البكتريا صفة انتاج مضاد بكتيري في الغربلة الاولية لم تكن تمتلكها قبل عملية النقل. ومن ثم تم عزل، تنقية وتشخيص المضاد المنتج من الـ*E. coli* pBR322 trans. بنفس الطريقة المستعملة لعزل، تنقية وتشخيص المضاد المنتج من *S. sp.* ، وكان هذا المضاد هو نفس المضاد المنتج من بكتريا *S. sp.* وبكمية اكبر2.2غم/لتر في فترة زمنية قصيرة (يومين).

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