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RESEARCH ARTICLE

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Biosynthesis, optimization and structure elucidation of antimicrobial agent produced by *Streptomyces clavuligerus* isolated from Egyptian soil

ABSTRACT:

The present study focused on antimicrobial compound production from *Streptomyces clavuligerus* isolated from Egyptian soil. The isolate had broad spectrum antimicrobial activity against *Escherichia coli NCTC 10416, Salmonella typhi* NCIMB 9331*, Bacillus subtilis NRRL B-543, Staphylococcus aureus ATCC 29213,* and *Candida albicans ATCC 10231.* The isolate was identified according to morphological and molecular techniques identification as *Streptomyces clavuligerus.* Different experiments were conducted to study the optimum conditions for the highest antimicrobial productivity of *Streptomyces clavuligerus*. Results indicated that, the optimum medium for the production of antimicrobial compound was YPSS- medium included starch as carbon source at concentration of 10 g/l and urea as nitrogen source at concentration of 6 g/l. The optimum incubation period was 7 days at an incubation temperature 30^{o}C if the optimum agitation speed was 150 rpm and the initial pH was 6.5 using phosphate buffer. Peel potato was the best carbon by product source while whey was the best nitrogen by product source. The extraction, purification and structure identification of the antimicrobial agent(s) produced by *Streptomyces clavuligerus,* were performed by UV analysis, IR and Mass spectroscopy which identified the active ingredient as Clavulanic acid and Holomycin.

KEY WORDS:
Molecular identification, Physiological parameters, Spectroscopic analysis.

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INTRODUCTION:

Biotechnologists are still searching for microbial metabolites to be used as antimicrobial compounds (antibiotics) to combat human, animal and plant diseases. Microorganisms constitute an inexhaustible reservoir of compounds with pharmacological, physiological, medical or agricultural applications (Smith, 1986). Among these microorganisms, Actinomycetes have special importance as potent sources of antibiotics and other biologically active substances of high commercial value and which have important applications in human medicine as anti-viral and anti-tumour compounds and in agriculture as herbicides, insecticides and anti-parasitic compounds (Watve *et al*., 2001).

The Actinomycetes are Gram positive, free living, non-motile bacteria that widely distributed in soil, water and can be found with greater or less frequency in most ecological niche (Takahashi and Omura, 2003). They produce numerous secondary metabolites essential for health including antibiotics (Chaudhary *et al*., 2013), enzymes and immunomodulators (Choi *et al*., 2015). Around the world there are 23000 bioactive secondary metabolites produced by

microorganisms have been reported and over 10000 of these compounds are produced by Actinomycete, 7600 derived from *Streptomyces* and 2500 from the so-called rare Actinomycetes (Berdy, 2005). Nowadays, about 130 to 140 microbial products and their derivatives uses in human medicine, mostly in chemotherapy and medicine. Furthermore, some of 15 to 20 compounds are used in agriculture mainly as pesticides, (plant protecting agent). Most of these compounds are also produced by Actinomycete (Moncheva *et al.*, 2002).

Clavulanic acid (CA) is a potent β-lactamase inhibitor β- lactamases are enzymes produced by a wide range of pathogenic bacteria, such as *Neisseria gonorrhoeae* and *Haemophilus influenzae* to inactivate β-lactam antibiotics, such as penicillin and cephalosporin. This inactivation occurs by cleavage of the C-N bond in the β-lactam ring of the antibiotic by the β-lactamase enzyme. The mechanism of β-lactamase inhibition is because of the structural similarity of CA to the natural substrate of the β-lactamase enzyme, which results in CA binding to the serine-hydroxyl group in the active site of the enzyme and causing irreversible enzyme inhibition. This irreversibly binding and inhibition of β-lactamase, leads to the formation of a stable acylated intermediate (Neto *et al*., 2005). Holomycin is related to a class of a natural product known as dithiolopyrrolone. It is thought that its mode of action is to inhibit the synthesis of RNA in whole cells or inhibit RNA polymerase chain elongation (Oliva *et al.,* 2001; Qin *et al*., 2013).

The aim of the present study was to isolate natural antimicrobial agents of biological origin and investigate the optimum conditions of the produced antimicrobial substances and extract, purify and identify the compound(s).

MATERIAL AND METHODS:

Screening of Actinomycetes antimicrobial activity:

Eleven Actinomycete isolates were previously isolated from various soil samples collected from different locations in Egypt using serial dilution method. The antimicrobial activity was determined against the tested bacterial and fungal pathogens *(Escherichia coli* NCTC 10416 and *Salmonella typhi* NCIMB 9331 as models for Gram-negative bacteria. *Bacillus subtilis* NRRL B-543 and *Staphylococcus aureus* ATCC 29213 as models for Gram-positive bacteria, *Candida albicans* ATCC 10231 as a model for pathogenic fungi) using the Agar plug diffusion method (Balouiri *et al.,* 2016) with a few modifications. An aliquot of 25.0 ml of the media (Nutrient agar for bacteria and PDA for fungi), was seeded with 20.0 µl suspension of the test microorganisms' suspensions and poured in 9.0 cm diameter plates. After solidification, 9.0 mm diameter wells were made using a sterile Cork-borer (duplicate for each sample). Then, 100.0 µl of each culture filtrate was poured in the prepared wells. Plates were kept in a refrigerator for one hour to permit homogenous diffusion of the antimicrobial agent. Then, the plates were incubated at 37°C for 24 hours for bacteria and at 28°C for 72 hours for yeast. The results were determined by measuring the diameter of inhibition zone**.**

Identification of the selected Actinomycete isolates:

Morphological characteristics of substrate and aerial hyphae, forms of spore chains and sporophores were studied on starch nitrate agar medium. This was done by the cover slip technique (Shirling and Gottlieb, 1966).

Molecular identification was conducted at Sigma scientific services company, Cairo, Egypt. DNA was extracted from 7 days old actinomycete culture grown on starch nitrate media. GenElute™ Bacterial Genomic DNA kit (Sigma, St. Louis, MO, USA) were used following manufacturing instructions. Primers for amplification of partial 16S rDNA were used. The forward primer was: 5’ AGA GTT TGA TCC TGG CTC AG 3’ and the reverse primer were: 5’- GGT TAC CTT GTT ACG ACT T 3’. The PCR products were used for the sequencing reactions at the Sigma Scientific Services Co., Cairo University. DNA sequencing reactions were carried out by cycle sequencing then analysed by a ABI Prism 3730XL DNA analyser (Model 3130, Applied Biosystems, Hitachi, Japan). Nucleotide sequences were compared with all accessible sequences in the NCBI databases using BLAST http://www.ncbi.nlm.nih.gov/blastn. The phylogenetic tree was displayed using the TREEVIEW program.

Optimization design of antimicrobial production:

Effect of different fermentation media on antimicrobial production:

Nine different fermentation media were used to investigate both the rate of growth and antimicrobial metabolites activity of *Streptomyces clavuligerus* according to Song *et al*. (2012). The production media used were: Starch nitrate medium (Waksman, 1961), Starch casein medium (Kumari *et al.*, 2013), ISP-4 medium (Shirling and Gottleib, 1966), YPSS-medium (VanderMolen *et al.*, 2013), MC- Beth Scales (MBS) medium (Teja *et al.*, 2014), Czapek Dox medium (Huang and Ling, 1973), Nutrient broth (APHA, 1985), Starch medium (Shirling and Gottleib, 1966), and ISP-2 medium (Pridham *et al*., 1957). An aliquot of 50 ml of each media was inoculated with a 10 mm disc size of actinomycetes and

after 7 days of incubation at 30ºC under shaking conditions (150 rpm) the growth and antimicrobial activity were recorded. All broth cultures were filtered and the antimicrobial activities of the filtrates against *Escherichia coli NCTC 10416, Staphylococcus aureus ATCC 29213,* and *Candida albicans ATCC 10231* were assayed using the Agar well diffusion method.

Chemical factors affecting on antimicrobial production:

Different nitrogen sources were added to YPSS- medium (control medium) as sole nitrogen source to test the ability of the selected isolate to utilize each of them and to produce antimicrobial metabolites. The used nitrogen sources were ammonium sulphate, potassium nitrate, urea, peptone, yeast extract, L-alanine, L-arginine, L-cystein, L-glutamine, L-leucine, L-valine, brewery yeast, soybean meal, and whey. Different concentrations of the optimum nitrogen source were added to (YPSS) medium as a basal medium.

Different carbon sources were added to YPSS medium as a basal medium. The carbon sources were: D-glucose, D-fructose, L-arabinose, sucrose, lactose, raffinose, starch, dextran, bagasse, peel carrot, peel potato, and white rice. Each source was separately added to YPSS medium (as a sole source of carbon) to give a final concentration of 14.0 g/l (w/v). After that, different concentrations of the optimum carbon source were added to (YPSS) medium as a basal medium.

Physical factors affecting on antimicrobial production:

Five physical factors affecting antimicrobial agent(s) production were tested. The harvest of flasks was carried out regular after 3, 4, 5, 6, 7, 8, and 9 days of incubation. Inoculums size ranging from ½ disc to 4 discs (diameter of disc was 10 mm) of *Streptomyces* were added to a flask containing 50 ml of the optimized production medium (YPSS). *Streptomyces* was cultivated using the optimized production medium (YPSS) at various incubation temperatures ranging from 25ºC to 40ºC. Flasks containing the optimized production medium (YPSS) inoculated with *Streptomyces clavuligerus* were incubated under shaking conditions at different agitation speeds, the growth rate was observed, and the antimicrobial activity was assayed using Agar well diffusion method.

Finally, the pH of the optimized production medium (YPSS) was adjusted in the range of pH 5.0 to pH 8.0 using phosphate buffer. The results of growth rate and antimicrobial activity were recorded after 7 days of incubation at 30º C and 150 rpm. The experiment was conducted in duplicate.

Extraction of the antimicrobial agent:

Streptomyces clear filtrate, containing the active metabolites, was used for the extraction process using different organic solvents. These solvents included chloroform, ethyl acetate, n-hexane and petroleum ether at 1:1 (v/v). The organic phase was collected using a separating funnel and evaporated using rotary evaporator (Labassco-Buchi) at 40 °C and 200 rpm. The crude residue was dissolved in a minimum amount of the extraction solvent and transferred to a dry and clean glass vial. The antimicrobial activity of the extract was monitored by the paper-disc diffusion method as reported by Balouiri *et al.* (2016).

Purification of the antimicrobial agent:

Using TLC the bands that gave antimicrobial activity were scratched and reconstituted in ethyl acetate solvent, filtered, then applied to a TLC Silica sheet and developed a solvent system of ethyl acetate: methanol (6:4 v/v) under saturated conditions to obtain the pure product. This process was repeated using TLC sheets several times to obtain an adequate quantity for further characterization. The chromatograms were detected by UV light (254 and 365 nm) (Stahl, 1969).

Structure elucidation of the antimicrobial agent:

Ultraviolet analysis (UV):

The UV analysis of the antimicrobial compounds was purified with a T80+UV/VIS Spectrometer, PG Instrument Ltd. Range: 190 - 1000 nm at National Research Centre.

Infrared spectrum (IR):

The IR spectrum of the antimicrobial compound was determined using a Fourier transform-infrared spectrophotometer (FTIR, Jasco 6100, and Model Japan). The sample was ground with spectroscopic grade potassium bromide powder and then pressed into a 1 mm pellet for FT-IR measurement in the frequency range of 4000 – 400 cm^{-1} (Mid infrared region) at National Research Centre.

Mass Spectrum:

The mass spectrum analysis was performed using an Agilent USA 6420 triple quad LC MS mass spectrometer, 1290 quat pump ,1290 sampler, 1290 TCC. Column acquity UPLC BEH shield RP 18 1.7 µm 2.1 x 150 mm at National Research Centre.

RESULTS:

Antimicrobial activity and Identification of the Actinomycete isolate:

The studied isolate had a broad-spectrum antimicrobial activity against *Escherichia coli NCTC 10416, Salmonella typhi* NCIMB 9331*, Bacillus subtilis NRRL B-543, Staphylococcus aureus ATCC 29213* and *Candida albicans ATCC 10231* with diameters

of inhibition zone (IZ) 25, 25, 20, 23, and 25 mm, respectively.

The organism produces greenish to greyish mass of spores on the aerial mycelium that completely covered the substrate mycelium. Brown diffusible pigment was also produced. Microscopic examination of the isolate illustrated that; it produces well developed, branched non-fragmented substrate mycelium that lacking spores.

The phylogenetic tree revelled isolate sequences between 900-1000bp fragment of the DNA region. The nucleotide sequence of partial 16S rRNA the isolate was identified as *Streptomyces clavuligerus* (Fig. 1).

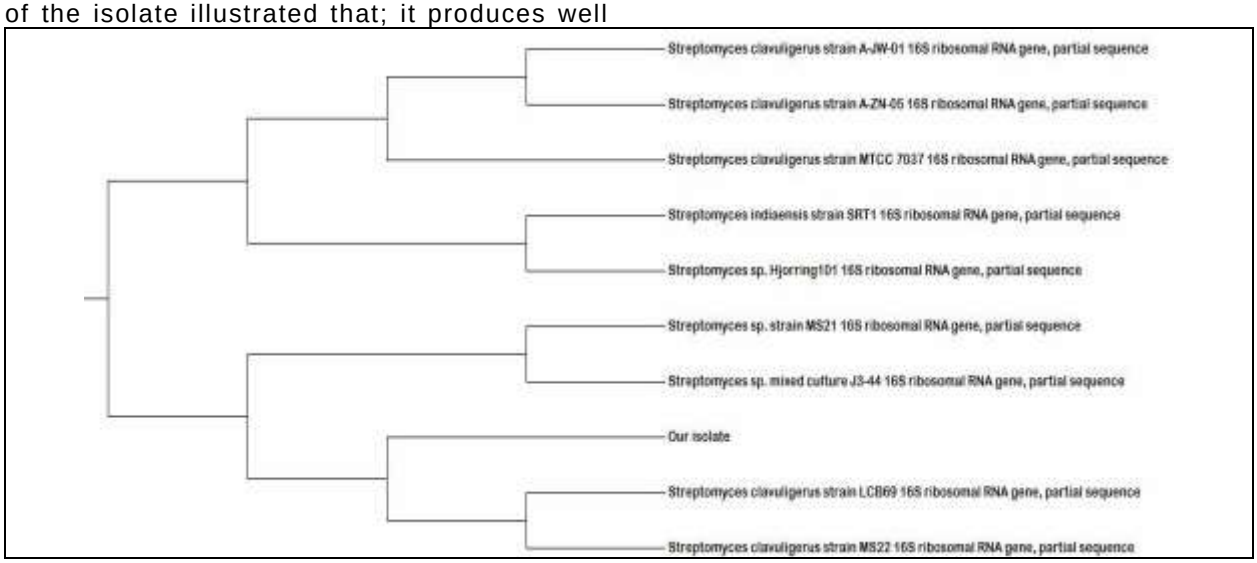


Fig. 1. The phylogenetic tree of isolate No. 2 compared to other *Streptomyces sp.* based on 16S rDNA sequences.

Optimization of antimicrobial agent(s) production by *Streptomyces clavuligerus*:

Effect of different fermentation media:

The antimicrobial activity of *Streptomyces clavuligerus* on different media revealed that, the maximum activity could be achieved using YPSS-fermentation medium, where the diameter of the inhibition zone reached 17, 16, and 18 mm when used against *E. coli, S. aureus* and *C. albicans* (Fig. 2), respectively. Inhibition zone 15, 14, and 18 mm could be obtained when *Streptomyces clavuligerus* was grown on starch casein medium. Less activity of the antimicrobial agent(s) was occurred when it was grown on starch nitrate medium, ISP-2, ISP-4, and Czapek dox medium.

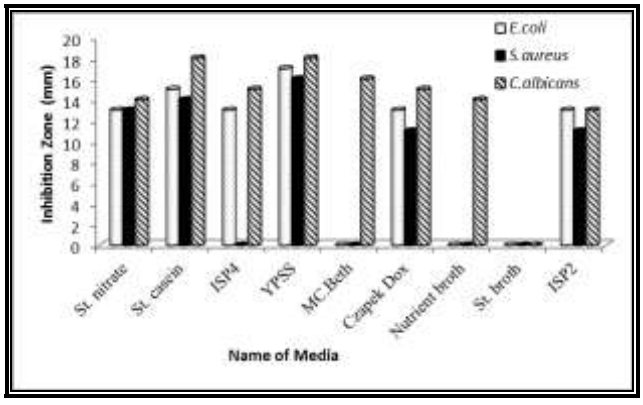


Fig. 2. Effect of different fermentation media on the activity of antimicrobial agent(s) produced by *Streptomyces clavuligerus* against *E. coli, S. aureus* and *C. albicans*.

Effect of different nitrogen sources:

Figure 3 shows that urea was the best nitrogen source for antimicrobial agent(s) production with inhibition zones reached 18, 17, and 19 mm diameter for *E. coli, S. aureus,* and *C. albicans*, respectively. Adding commercial nitrogen sources (brewery yeast, soybean meal and whey) had no effect on the antimicrobial activity. The dry weight of mycelia seemed to reach its maximum values of 5.94 g/l when leucine was used a sole nitrogen source. However, the supplement of the fermentation media with amino acids such as alanine, glutamine, valine and some natural nitrogen sources such as brewery yeast and soybean meal led to high production of mycelial dry weight. On the other hand, the lowest mycelial dry weight was recorded by the adding of whey and cysteine.

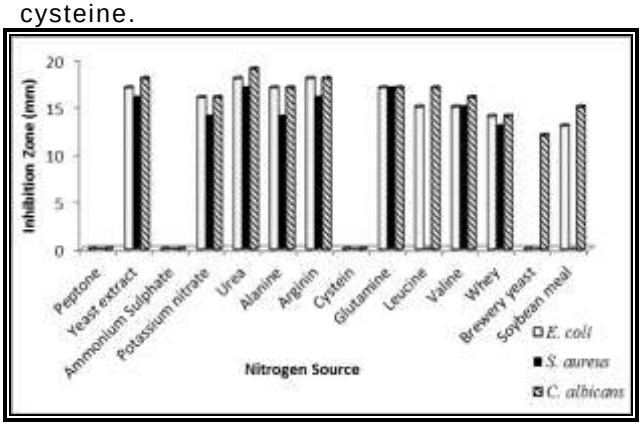


Fig. 3. Effect of different nitrogen sources on the activity of the antimicrobial agent(s) produced by *Streptomyces clavuligerus* against *E. coli, S. aureus* and *C. albicans*.

Effect of different concentrations of urea:

Figure 4 indicates that the antimicrobial activity increased with increase of urea concentration, reaching its maximum value

represented by 20, 17, and 20 mm diameter against *E. coli, S. aureus,* and *C. albicans* respectively at 6 g/l urea concentration. Further increase in the concentration of urea decreased of the activity of the antimicrobial agent(s) in all tested pathogens.

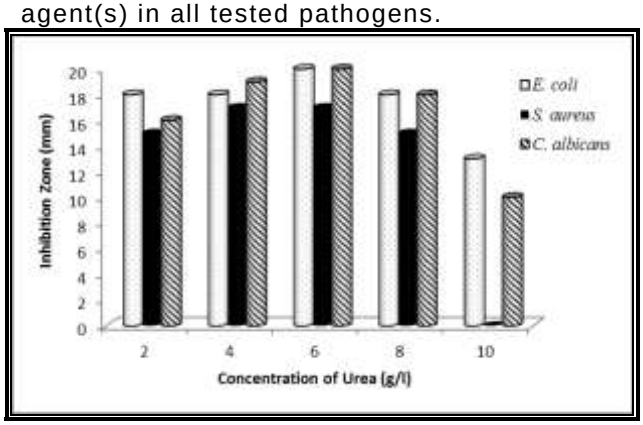


Fig. 4. Effect of different concentration of urea on the activity of the antimicrobial agent(s) produced by *Streptomyces clavuligerus* against *E. coli, S. aureus*, and *C. albicans*.

Effect of different carbon sources:

Figure 5 revealed that starch was the best carbon source to obtain the maximum activity of the antimicrobial agent(s) against the three test organisms used in this study. Starch gave inhibition zone diameters of 20, 15, and 17 mm with *E. coli, S. aureus*, and *C. albicans*, respectively. By using commercial carbon sources (peel potato, peel carrot, white rice, and bagasse) with *E. coli* had production of secondary metabolites was measured Bagasse had the least. Efficiency only peel potato had small antimicrobial activity while the other commercial compounds failed to achieve any activity with *S. aureus*. The natural sources had antimicrobial activity against *C. albicans* but, the highest activity obtained by using peel potato. Also, the results obtained that the mycelial dry weight seemed to reach its their maximum values of 3.66 g/l when sucrose was used a sole carbon source.

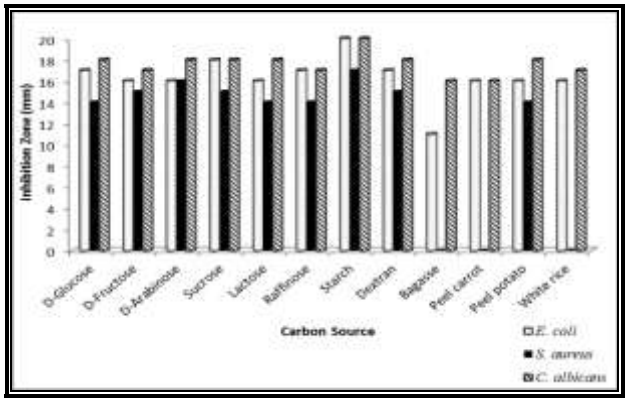


Fig. 5. Effect of different carbon sources on the activity of the antimicrobial agent(s) produced by *Streptomyces clavuligerus* against *E. coli, S. aureus*, and *C. albicans.*

Effect of different starch concentrations:

Figure 6 shows that starch increased the inhibition zone diameter of the antimicrobial agent(s) increase to a maximum of 20, 19, and 22 mm for *E. coli, S. aureus*, and *C. albicans*, respectively at 10 g/l. Further increasing the concentrations of starch decreased the inhibition zone of the antimicrobial agent(s) produced. According to these results, the concentration of the carbon source of the YPSS-medium could be replaced by 10 g/l starch and used as a sole carbon source.

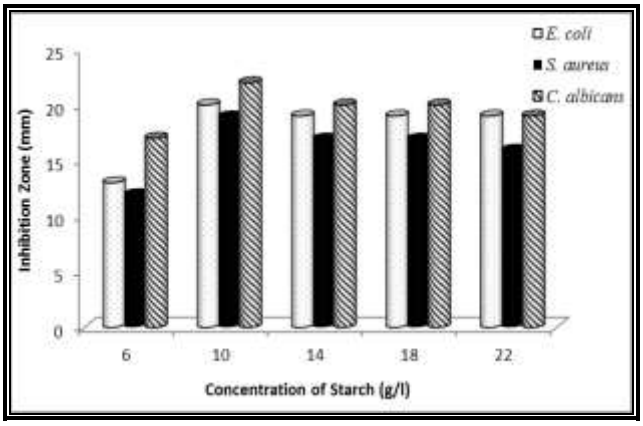


Fig. 6. Effect of different starch concentration on the activity of the antimicrobial agent(s) produced by *Streptomyces clavuligerus* against *E. coli, S. aureus*, and *C. albicans*.

Effect of different incubation periods:

Figure 7 indicated that, the activity of the antimicrobial agent(s) began after the fifth day of incubation and the maximum 20.0 mm inhibition zone diameter against *E. coli,* and *S. aureus* 23.0 mm diameter against *C. albicans* after seven days. Above these periods the activity remained nearby stable till the end of the incubation period. The mycelial dry weight increased as the antimicrobial agent(s) production increased and reached a maximum amount of 3.35 g/l after eight days of incubation.

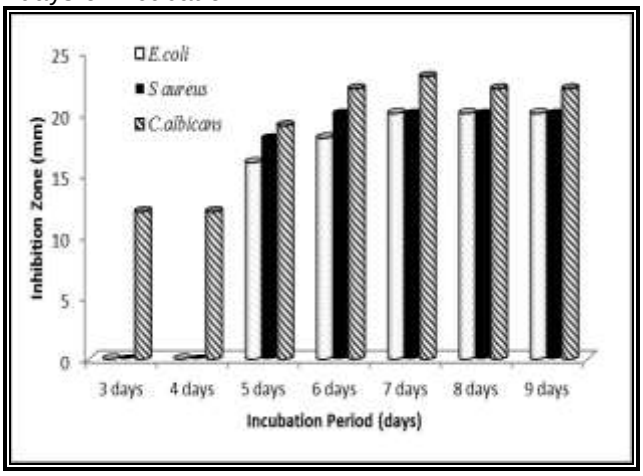


Fig. 7. Effect of different incubation periods on the activity of the antimicrobial agent(s) produced by *Streptomyces clavuligerus* against *E. coli, S. aureus,* and *C. albicans.*

Effect of different inoculum sizes:

The largest inhibition zones were 23, 22, and 24 mm diameter could be detected when 2 discs of vegetative cells were used against *E. coli, S. aureus,* and *C. albicans* (Fig. 8). Further increase in the inoculum

size had no effect on the antimicrobial activity. The mycelial dry weight increased with increase of the initial inoculum size and reached 3.80 g/l while the activity of antimicrobial agent(s) became stable against the three test organisms used in this work.

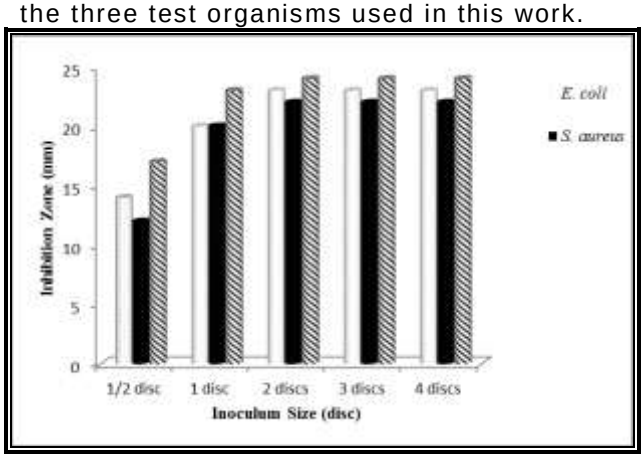


Fig. 8. Effect of different inoculum sizes on the activity of the antimicrobial agent(s) produved by *Streptomyces clavuligerus* against *E. coli, S. aureus,* and *C. albicans*.

Effect of different incubation temperatures:

Figure 9 shows that, the activity of the antimicrobial agent(s) was strongly affected by the variation in incubation temperature and increased as the temperature increased up to 30ºC which the maximum activity was represented by inhibition zones of 23, 22, and 23 mm diameter against *E. coli, S. aureus*, and *C. albicans*, respectively. Further increase of temperature 35ºC resulted in an obvious decrease in the activity of the antimicrobial agent(s) against the three test organisms.

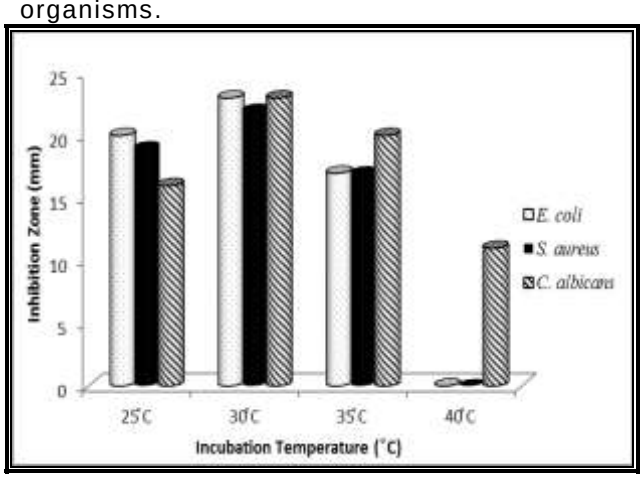


Fig. 9. Effect of different incubation temperatures on the activity of the antimicrobial agent(s) produced by *Streptomyces clavuligerus* against *E. coli, S. aureus,* and *C. albicans.*

Complete disappearance of the antimicrobial activity against *E. coli, S. aureus* and very weak antimicrobial activity against *C. albicans* were obtained at 40ºC. mycelial dry weight was maximum at 30ºC.

Effect of different agitation speeds:

Agitation speeds increased the activity of antimicrobial agent(s) increase (Fig. 10). The maximum value of the antimicrobial activity 23, 22, and 23 mm inhibition zone diameter against *E. coli, S. aureus,* and *C. albicans*, respectively at 150 rpm. Increasing the agitation speed up to 200 rpm, led to a relative decrease in the antimicrobial activity. The mycelial dry weight increased by the increase of the agitation speed to a maximum value of 3.42 g/l at 150 rpm. Any increase in the agitation speed led to a decrease in the total dry weight.

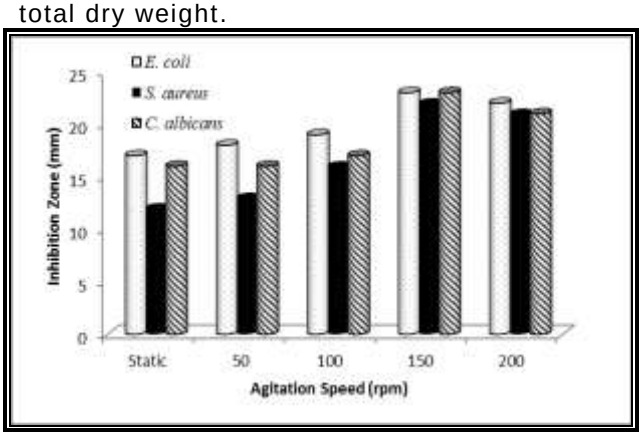


Fig. 10. Effect of different agitation speed on the activity of the antimicrobial agent(s) produced by *Streptomyces clavuligerus* against *E. coli, S. aureus,* and *C. albicans.*

Effect of different initial pH values of the fermentation medium:

Maximum antimicrobial activity was represented by inhibition zones of 24, 21, and 24 mm diameter against *E. coli, S. aureus*, and *C. albicans* at initial pH 6.5 (Fig. 11)., pH 5.0 and 6.0 also gave good results. An initial pH above pH 7.0 led to complete inhibition of the antimicrobial activity. The mycelial dry weight was also affected by the initial pH and reached its maximum of 4.76 (g/l) at pH 6.5.

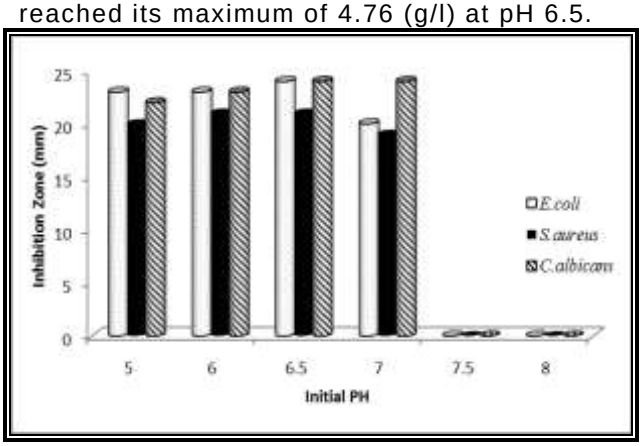


Fig. 11. Effect of different initial pH values on the activity of the antimicrobial agent(s) produced by *Streptomyces clavuligerus* against *E. coli, S. aureus*, and *C .albicans*.

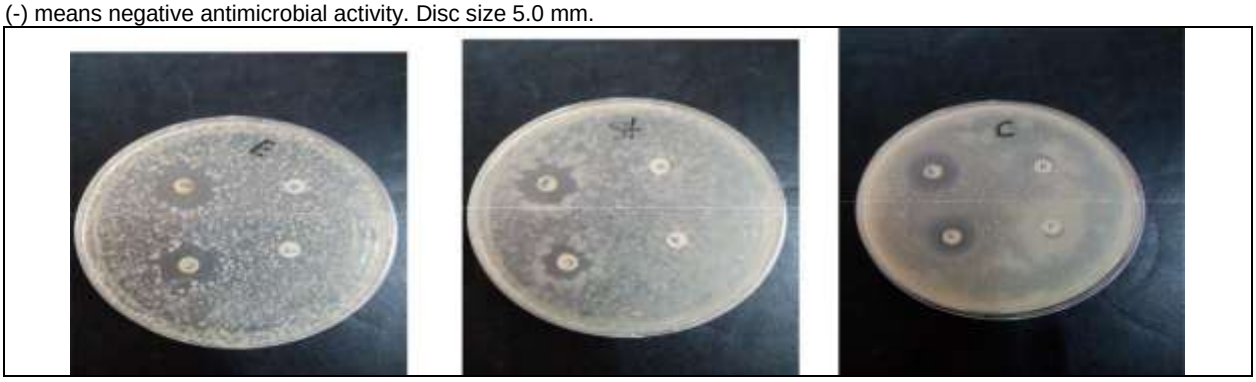
Extraction of the antimicrobial agent produced by *Streptomyces clavuligerus*:

Ethyl acetate was the best solvent for the extraction the antimicrobial metabolites from clear filtrate of *Streptomyces clavuligerus* (Table 1 and Fig. 12). Using chloroform also gave a satisfactory result. Hexane and petroleum ether failed to extract antimicrobial metabolites.

Table 1. Extraction of antimicrobial compound/s produced by *Streptomyces clavuligerus* using different organic solvents.

Organic solvent	Diameter of inhibition zone in (mm)		
	E. coli	***S. aureus***	***C. albicans***
Chloroform	18.0	14.0	19.0
Ethyl acetate	20.0	16.0	22.0
Hexane	0.0	0.0	0.0
Petroleum ether	0.0	0.0	0.0

(-) means negative antimicrobial activity. Disc size 5.0 mm.



A B C

Fig. 12. The diameters of inhibition zone obtained from extraction process using different organic solvents against *E. coli, S. aureus* and *C. albicans* respectively where E: *E. coli,* St: *S. aureus* and C: *C. albicans* while the discs refers to; E: Ethyl acetate, C: Chloroform, P: Petroleum ether and H: Hexane.

Separation and Purification of the antimicrobial fraction(s):

The separation of the active fraction(s) from the culture broth of *Streptomyces clavuligerus* was studied using different elution solvents. The maximum inhibition zone of 22 mm diameter against *E. coli*, *S. aureus,* and *C. albicans* was obtained from two overlapped bands using Ethyl acetate: Methanol (6:4) (Table 2).

Table 2. Bio autography of TLC sheets using extracted solution of *Streptomyces clavuligerus.*

Solvent systems (v/v)	Diameter of inhibition zone in (mm)		
	E. coli	***S. aureus***	***C. albicans***
(1) Ethyl acetate (10)	0.0	0.0	0.0
(2) Hexane: Ethyl acetate (5:5)	0.0	0.0	0.0
(3) Chloroform: Methanol (7:3)	15	15	15
(4) Ethyl acetate: Methanol (6:4)	22	22	22
(5) Methanol: Acetic acid (9:1)	0.0	0.0	0.0
(6) Hexane: Ethyl acetate (8.5:1.5)	0.0	0.0	0.0

(-) means negative antimicrobial activity

Structure identification of pure antimicrobial agent(s):

The ultraviolet spectrum (UV) analysis recorded peaks at 201, 273 and 373 nm is presented in figure 13.

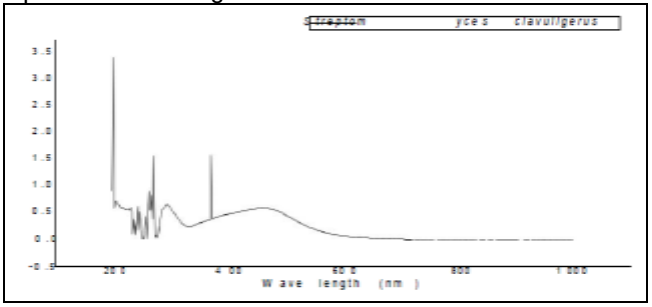


Fig. 13. Ultraviolet spectrum (UV) of the antimicrobial compound(s) produced by *Streptomyces clavuligerus.*

The IR spectrum data sheet of the sample is represented in figure 14. Comparing the IR spectrum of our sample and the IR library of BIO-RAD, we concluded that the sample contained alcohol and carbonyl group of ester group. The OH peak at range 3200-3400 with a broad peak of hydrogen, a primary alcohol peak C-O at 1000-1075, and A-CH_3 peaks; one CH peak at 2952-2972, another C-H at 2862-2882, a third C-H at 1435-1475 and finally a C-H at 1375-1380.

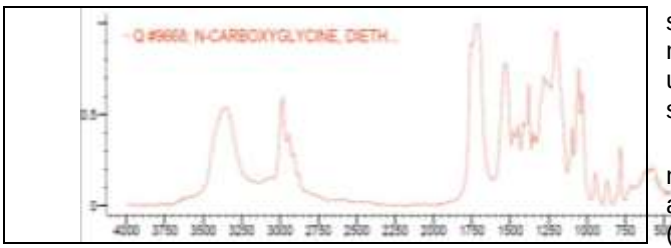


Fig. 14. Infrared spectrum (IR) of the antimicrobial compound(s) produced by *Streptomyces clavuligerus*.

The mass spectra showed clavulanic acid base peak at 218 m/z, clavulanic acid salt peak at 326.9 m/z, and finally clavulanic acid parent ion at 190.8 m/z. Another peak at 212.8 (M^+) and peak at 172.9 m/z (M^+-CH_2CO) were indicated for of holomycin.

The absorbance bands obtained by UV and the functional groups illustrated in the IR chart showed three estimated compounds with the following chemical formulas C_{16} H_{23} N O_9 with molecular weight 373.36, or C_{21} H_{25} N O_{10} S with molecular weight 483.4 or C_7 H_{13} N O_4 with molecular weight 175.18. Aiming to narrow estimations of possible antimicrobial agents produced by *Streptomyces clavuligerus*, mass spectrophotometry and preparative TLC were performed. It was revealed that the partially purified mixture contained clavulanic acid salt (potassium clavulanate) that is known to have a chemical formula $C_8H_8KNO_5$ with molecular weight of 237.25, and holomycin with formula $C_7H_6N_2O_2S_2$ and molecular weight 214.26 (Fig. 15).

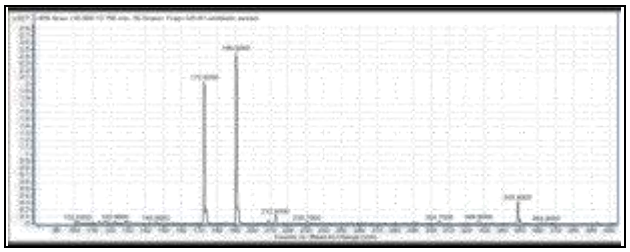


Fig. 15. The mass spectrum for the antimicrobial compounds produced by *Streptomyces clavuligerus*.

DISCUSSION:

A screening program conducted on 11 soil actinomycete isolates. Identification of the selected actinomycete isolate was carried out according to the key's given in Bergey's Manual of Determinative Bacteriology, Vol.4 (Locci, 1989). Phylogenetic characterization was mostly depend on the results of nucleotide sequences of the rRNA rather than on phenotypic structures (Garrity *et al*., 2005; Ludwig *et al*., 2012). The isolate was found to match the character of *Streptomyces clavuligerus* with the highest similarity (100%).

The different inhibition zones measured with fermentation media are in response to media composition. These results are in harmony with that of Kiers *et al*. (2000) and Palmqvist and Hahn-Hagerdal (2000) who stated that, the medium composition and suitable nutrients can promote the synthesis of metabolites, cell growth, antibiotic fermentation unit and antibiotics extraction process or suppress all these processes.

The fermentation medium by inorganic nitrogen and organic in supported the growth and the antimicrobial production. Bundale *et al*. (2015) stated that using of organic nitrogen compounds induced higher microbial biomass and antimicrobial metabolite production than inorganic nitrogen compounds. This may be attributing to its high content of nitrogen and easily solubility of urea in water and the fermentation medium (Marsh *et al*., 2005).

Upon studying the effect of different concentrations of the best nitrogen source, the maximum activity of secondary metabolites was attained at 6.0 g /l of urea for *Streptomyces clavuligerus* and above this concentration; a significant decrease was attained. It thought that, increasing the concentration of urea lead to liberating of high amount of ammonia that may alter the pH of the medium and thus disturbing of enzymatic activity that catalyse the metabolic reactions and also may also alter the permeability of the cell membrane as cited by Guimaraes *et al*. (2004).

The maximum activity was achieved by using starch as sole carbon source. It is thought that monosaccharaides are suitable for the growth of microorganisms while polysaccharides are associated with the production of the antibiotics as reported by Bundale *et al*. (2015). Moreover, da Silva *et al.* (2012) reported that starch and glycerol were the best carbon sources for the production of secondary metabolites by *Streptomyces sp.* Also, Holkar *et al*. (2013) showed that, celliobiose was the best carbon source followed by inositol for antibiotic production.

The optimum incubation period changes according to the species of the tested organism. In our study, the antimicrobial activity of *Streptomyces clavuligerus* was started at the 5th day, the optimum incubation period was found to be after 7 days. It is thought that; the production of the antimicrobial metabolites begins in the stationary phase which is characterized by slow growth rate and higher production of secondary metabolites as cited by Bundale *et al.* (2015). The present results agreed with that of Sharon *et al*. (2014) where *Streptomyces sp.* Kod 10 reached its maximum levels of antibiotic production after 7 days.The microbial behaviour strongly influenced by the size of the inoculum. As the inoculum size of vegetative cells increased, the activity increased. Further increase in the inoculum size lead to increase in the microbial dry weight but no significant change in the production of microbial secondary metabolites was recorded. Mukhtar *et al*. (2012) stated that, the quantity and quality of inoculation material play a crucial role in the bioprocess

results and a large inoculum may cause suppress the production of antimicrobial substances.

Complete loss of the antimicrobial compound(s) activity occurred at 40°C. This may be due to the raising the temperature may cause denaturation of these enzymes that involved in the production process. These results agree with Oskay (2011) who showed that, the maximum antibiotic production by *Streptomyces sp.* was obtained at 30°C. Adinarayana *et al.* (2003) also found that, maximum neomycin production by *Streptomyces marinensis* was obtained at 30°C.

Low antimicrobial activity obtained at static conditions and low agitation speed. Decrease of agitation rate might reduce dissolved oxygen level in fermentation that could affect antimicrobial metabolite production (Elattal *et al*., 2011). These results may be attributed to the increase of aeration of the culture medium and this could lead to enough supply of dissolved oxygen in the media and so it would increase the nutrient uptake by bacteria as cited by Beg *et al.* (2003).

pH values have a great effect on the enzymatic activities involves in the metabolic reactions, also can change the permeability of the cell membrane and cell morphology of the microbial cell (Bundale *et al*., 2015). The initial pH showed a significant influence on the maximum activity of the antimicrobial secondary metabolites and the growth of the producer microorganism. In our study, *Streptomyces clavuligerus* showed a maximum antibiotic activity at an initial pH of 6.0-6.5. These results are in harmony with Oskay (2011) who proved that, the antimicrobial productivity of *Streptomyces sp.* was optimum near neutrality.

The production, extraction and purification of the antimicrobial fraction(s) produced by *Streptomyces clavuligerus* were also studied. The results are in agreement with Parthasarathi *et al.* (2012) who stated that, the maximum inhibitory zones by *Streptomyces hygroscopicus BDUS 49* was observed when extraction took place using ethyl acetate. It is thought that, the differences in the composition and structure of the culture supernatants determined the difference in the choice of the most polar solvent for extraction and purification of the antimicrobial agents. The spectroscopic analysis of antimicrobial agent produced by *Streptomyces clavuligerus* isolate comes in harmony with Atta and Yassen (2014) who mentioned that the ultraviolet (UV) spectroscopic analysis of the purified β-lactamase inhibitor compound produced by their isolate of *Streptomyces clavuligerus*, showed a maximum absorption peaks at 285 nm.

The peaks of mass spectrum of the purified antimicrobial substance appeared at 190.8, 218, and 328, m/z. Also, the molecular weight of 200.0 suggested strongly that the product is clavulanic acid. This comes in accordance with Parag *et al*. (2006).

A peak of 212.8 of (M^+) molecular weight of holomycin and peak at 172 m/z (M^+-CH_2CO) indicated the presence of holomycin. Our results also come in agreement with another research conducted by Yin *et al.* (2012) who stated that the identification of holomycin purified in methanol by HPLC had m/z peak at 215. The primary characteristic fragment ions of holomycin obtained from MS-MS in a tandem mass spectrometer included peaks of 173 and 197.

The yellow colour of holomycin was clearly observed from the beginning of the cultivation and extraction procedures.

Clavulanic acid and holomycin were mentioned together in literature before; Mutants of *Streptomyces clavuligerus* with disruption in different genes for clavulanic acid biosynthesis produce large amounts of holomycin which explains the production of dark yellow colour, most probably that of holomycin, on the fourth day of incubation. A possible cross regulation of holomycin and clavulanic acid pathways was proved by De la Fuente *et al.* (2002).

CONCLUSION:

The optimum conditions required to achieve the maximum clavulanic acid and holomycin production from *Streptomyces clavuligerus* were, inoculating 2 discs of vegetative cells (10 mm) disc diameter/ flask containing 50 ml of production media (YPSS-medium) using urea as a sole nitrogen source at concentration of 6 g/l, using starch as a sole carbon source at concentration of 10 g/l, using for 7 days at 30ºC under 150 rpm agitation speed and pH 6.5 using phosphate buffer.

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تخليق وتعظيم انتاجية والتوضيح التركيبي لمركبات ضد ميكروبية من إنتاج *Streptomyces clavuligerus* المعزولة من التربة المصرية

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ركزت الدراسة الحالية على إنتاج المركبات المضادة للميكروبات من *Streptomyces clavuligerus* المعزولة من التربة المصرية. أوضحت النتائج أن العزلة لها نشاط مضاد للميكروبات واسع النطاق ضد E. coli NCTC 10416، Salmonella typhi NCIMB 9331، *Bacillus subtilis* NRRL B-543، *Staphylococcus aureus* ATCC 29213و *Candida albicans* ATCC 10231. وقد تم تعريف العزلة وفقا للتقنيات المورفولوجية والجزيئية واوضحت النتائج انها تعرف باسم Streptomyces clavuligerus. أجريت تجارب مختلفة لدراسة الظروف المثلى لتعظيم إنتاجية المركبات ذات النشاط الضد ميكروبى من *Streptomyces clavuligerus*. أشارت النتائج إلى أن الوسيلة المثلى لإنتاج المركب (المركبات) المضادة للميكروبات كانت المنبت لغذائي YPSS- . اشملت على النشا مصدر كربون بتركيز 10 جم / لتر واليوريا مصدر نيتروجين بتركيز 6 جم / لتر. بالإضافة إلى ذلك، كانت فترة الحضانة المثلى 7 أيام عند درجة حرارة الحضانة 30 درجة مئوية إذا كانت سرعة التحريك المثلى 150 دورة في الدقيقة وكان الرقم الهيدروجيني الأولي 6.5. من ناحية أخرى، كانت قشرة البطاطا هي أفضل أنواع الكربون رخيص، بينما كان مصل اللبن أفضل النيتروجين حسب رخيص. تم إجراء الاستخلاص والتنقية وتحديد هوية عامل (مضادات) مضادات الميكروبات التي تنتجها Streptomyces clavuligerus من خلال تحاليل الأشعة فوق البنفسجية، والأشعة تحت الحمراء والكتلة الطيفية التي أوضحت أن المركبات هي حامض Clavulanic و Holomycin.