Application Biotechnology of Recycling Agricultural Waste In Al-Khurmah Governorate For Production Antimicrobial Agent(S) By Actinomycetes Isolates Under Solid State Fermentation Condition

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ABSTRACT: Solid substrate cultivation (SSC) or solid state fermentation (SSF) is envisioned as a prominent bio conversion technique to transform natural raw materials into a wide variety of bioactive products. This process involves the fermentation of agriculture wastes (Rice straw, Wheat straw, Banana waste, Corn stover, Sweet potato Residue, Pearl barley and Peel apples) by actinomycete isolates in low moisture content. Eighty-two actinomycete strains were isolated from eighteen soil samples collected from Al-Khurmah governorate, kingdom of Saudi Arabia. Only one actinomycete isolate KH-2326-27 was found exhibited to produce wide spectrum antifungal activities by using Rice straw under solid state fermentation condition (SSF). The nucleotide sequence of the 16s RNA gene (1.5 Kb) of the most potent strain KH-2326-27 evidenced an 97% similarity with *Streptomyces antibioticus*. From the taxonomic features, the actinomycetes isolate KH-2326-27 matches with *Streptomyces antibioticus* in the morphological, physiological and biochemical characters. Thus, it was given the suggested name *Streptomyces antibioticus*. The parameters controlling the biosynthetic process of antifungal agent formation including: different pH values, temperatures, incubation period and different carbon and nitrogen sources were fully investigates. The active metabolite was extracted using n-Butanol (1:1, v/v) at pH 7.0. The separation of the active ingredient and its purification was performed using both thin layer chromatography and column chromatography techniques. The physico-chemical characteristics of the purified antifungal agent viz. color, melting point, solubility, elemental analysis and spectroscopic characteristics have been investigated. This analysis indicates a suggested empirical formula of $C_{14}H_{19}NO_4$. The minimum inhibition concentrations "MICs" of the purified antifungal agent were also determined. The purified antifungal agent was suggestive of being belonging to Anisomycin (Flagecidin) antibiotic produced by *Streptomyces antibioticus*, KH-2326-27.

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1. INTRODUCTION

Solid state (substrate) fermentation (SSF) is generally defined as the growth of microorganisms on (moist) solid material in absence or near – absence of free water [Shuler *et al.*, 2002]. In solid-state fermentation processes, the solid substrate not only supplies the nutrients to the culture, but also serves as an anchorage for the microbial cells. Amongst several factors that are important for microbial growth and activity, the most critical include substrate, particular size and moisture level [Lui and Tzeng 1999]. The aim of SSF is to bring cultivated actinomycetes in tight contact with the insoluble substrate and to achieve the highest nutrient concentration from the substrate for fermentation [Bhargav *et al.*, 2008].

Actinomycetes play an important ecological role in biodegradation, many are commercially important, either in the production of bioactive compounds such as antibiotics and enzymes or in useful biological processes such as biodegradation and waste treatment [Claessen *et al*., 2002]. *Streptomyces* spp. are soil bacteria with the ability to produce many biologically active products. Many of these compounds have important applications in human medicine (as antibacterial, antitumour or antifungal agents, immuno- modulators, etc.) and in agriculture (as growth promoters and agents for plant protection amongst others) [Colombo *et al.*, 2001]. Many antibiotics such as penicillin, cephamycin C, neomycin, iturin, cyclosporin A, cephalosporins are produced by SSF. Penicillin was produced by using *Penicillum chrysogenum* with substrates such as wheat bran of high moisture content (s = 70 %) and sugarcane bagasse [Dominguez *et al.*, 2000]. Cephamycin C is produced by a variety of microorganisms including *Streptomyces cattleya*, *Streptomyces clavuligerus* and *Nocardia lactamdurans*. Wheat raw supplemented with cottonseed-de-oiled cake and sunflower cake was used for production of cephamycin C using SSF

[Kota and Sridhar, 1999]. Wheat raw supplemented with raspberry proved to be optimum for production of neomycin by SSF. Some critical parameters considered to be optimum for production of neomycin are particle size of substrate, initial moisture content, inoculum volume, and incubation temperature [Ellaiah *et al.*, 2004].

Anisomycin is a pyrrolidine antibiotic produced by *Streptomyces* sp. that inhibits protein and DNA synthesis [Huang and Zheng, 2003]. Anisomycin has become an important tool in molecular biology [Borges *et al.*, 2006]. It also has been used for the treatment of *trichomonas vaginitis* and amebic dysentery [Jimenez and Vazquez, 2000] and as an agricultural fungicide [Korzybsky *et al.*, 1978]. More recently, it was reported that anisomycin showed high in vitro antitumor activity, and could be used in a synergistic fashion with a cyclin-dependent protein kinase inhibitor to kill carcinoma cells [Huang and Zheng, 2003]

In the present work were describe the isolation of an actinomycete strain from soil sample collected from Al-Khurmah governorate, KSA, which generates an antimicrobial compound. The identification of this strain, based on the Cultural, Morphology, Physiology, Biochemical characteristics and Numerical taxonomy of *Streptomyces* species program, as well as 16s rRNA methodology, is also reported. The extraction of bio-products and improve yields of the antifungal substances that demonstrated inhibitory affects against fungal pathogenic, by *Streptomyces antibioticus*, KH-2326-27 under solid state fermentation condition were reported. The bioactive substance was purified, spectroscopic analysis and biological activities were determined.

2. MATERIALS AND METHODS

2.1. Agriculture wastes: Rice straw, Wheat straw, Banana waste, Corn stover, Sweet potato Residue, Pearl barley and Peel apples. The collected agriculture wastes were used as a substrate for antimicrobial agent(s) production under solid state fermentation.

2.2. Microorganism: The actinomycete strain was isolated from soil sample collected from Al-Khurmah governorate, kingdom of Saudi Arabia. It was purified using the soil dilution plate technique described by [Williams and Davis, 1965].

2.3. Solid state fermentation: Five grams of solid substrate, in a 250 ml Erlenmeyer flask, were moistened 40 ml mineral salt solution containing the following ingredients (g/l): $NaNO_3$, 2.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5 and KCl, 0.5. thoroughly mixed and autoclaved at 121^oC for 15 min.

2.4. Test organisms

2.4.1. Bacteria: *Micrococcus luteus*, ATCC 9341*; Staphylococcus aureus*, NCTC 7447*; Bacillus subtilis*, NCTC 1040, *Bacillus pumilus*, NCTC 8214; *Escherichia coli*, NCTC 10416; *Klebsiella pneumonia*, NCIMB, 9111 and *Pseudomonas aeruginosa*, ATCC 10145.

2.4.2. Fungi: *Saccharomyces cerevisiae* ATCC 9763; *Candida albicans*, IMRU 3669; *Aspergillus niger* IMI 31276*; Aspergillus fumigatus* ATCC 16424 *; Aspergillus flavus* IMI 111023*; Fusarium oxysporum; Alternaria alternate; Botrytis fabae; Penicillium chrysogenum* and *Rhizoctonia solani*.

2.5. Screening for antimicrobial activity: The anti-microbial activity was determined by cup method assay according to [Kavanagh, 1972].

2.6. Taxonomic studies of actinomycete isolate: Morphological characteristics of the most potent produce strain KH-2326-27 grown on starch nitrate agar medium at 30 ºC for 5 days was examined under scanning electron microscopy (JEOL Technics Ltd.,). Physiological and biochemical characteristics: Lecithinase was conducted on egg–yolk medium according to the method of [Nitsh and Kutzner, 1969]; Lipase [Elwan *et al.*, 1977]; Protease [Chapman, 1952]; Pectinase [Hankin *et al*., 1971]; α-amylase [Cowan, 1974] and Catalase test [Jones, 1949]. Melanin pigment [Pridham, *et al.*, 1957]. Degradation of Esculin and xanthine [Gordon *et al*., 1974]. Nitrate reduction [Gordon, 1966]. Hydrogen sulphide production and oxidase test [Cowan, 1974]. The utilization of different carbon and nitrogen sources [Pridham and Gottlieb, 1948]. Cell wall was performed by the method of [Becker *et al*., 1964 and Lechevalier and Lechevaier, 1968]. The cultural characteristics were studied in accordance with the guidelines established by the International *Streptomyces* Project [Shirling and Gottlieb, 1966]. Colors characteristics were assessed on the scale developed by [Kenneth and Deane, 1955].

2.7. DNA isolation and manipulation: The locally isolated actinomycete strain was grown for 5 days on a starch agar slant at 30°C. Two ml of a spore suspension were inoculated into the starch- nitrate broth and incubated for 3 days on a shaker incubator at 200 rpm and 30°C to form a pellet of vegetative cells (pre-sporulation). The preparation of total genomic DNA was conducted in accordance with the methods described by [Sambrook *et al*., 1989].

2.8. Amplification and sequencing of the 16S rRNA gene: PCR amplification of the 16S rRNA gene of the local actinomycete strain was conducted using two primers, StrepF; 5.-ACGTGTGCAGCCCAAGACA-3. and Strep R; 5.ACAAGCCCTGGAAACGGGGT-3., in accordance with the method described by [Edwards *et al.*, 1989]. The PCR mixture consisted of 30 pmol of each primer, 100 ng of chromosomal DNA, 200 µM dNTPs, and 2.5 units of Taq polymerase, in 50 µl of polymerase buffer.

Amplification was conducted for 30 cycles of 1 min at 94°C, 1 min of annealing at 53°C, and 2 min of extension at 72°C. The PCR reaction mixture was then analyzed via agarose gel electro phoresis, and the remaining mixture was purified using QIA quick PCR purification reagents (Qiagen, USA). The 16S rRNA gene was sequenced on both strands via the dideoxy chain termination method, as described by [Sanger *et al*., 1977].

2.9. Sequence similarities and phylogenetic analysis: The BLAST program (www.ncbi.nlm.nih. gov/blst) was employed in order to assess the degree of DNA similarity. Multiple sequence alignment and molecular phylogeny were evaluating using BioEdit software [Hall, 1999]. The phylogenetic tree was displayed using the TREE VIEW program.

2.10. Optimization of the culture condition for antifungal agent production: The different physicochemical parameters to maximize the yield of antifungal agent by *Streptomyces antibioticus* KH-2326-27 under solid state fermentation were investigated. The optimized parameter was incorporated at its optimized level in the subsequent optimization experiments. The impact of initial pH values (4-9, adjusted with 1N HCl or 1N NaOH), incubation temperature (25-50°C), incubation period (2-8 days), size of inoculum on antifungal production using solid state fermentation of *Streptomyces antibioticus* KH-2326-27 was evaluated. Moreover, the effect of incorporation of additional carbon sources (starch, mannitol, sucrose, glucose, D-mannose, *meso*-Inositol and Galactose), additional nitrogenous compounds ($NaNo_3$, KNO_3, $(NH_4)_2SO_4$, $(NH_4)_2PO_4$, peptone, NH_4Cl and urea), to the production medium were studied.

2.11. Fermentation*:* The *Streptomyces antibioticus* inoculum was introduced aseptically into each sterile Baxter bottle containing 5 g dry weight of Rice straw supplied with 40 ml mineral salt solution containing the following ingredients (g/l): $NaNO_3$, 2.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5 and KCl, 0.5. The pH was adjusted at 7.2 before sterilization. After seven days of incubation at 30^OC, 50 ml of sterilized distilled water were added and shaken. Filtration was carried out through cotton wool and followed by centrifugation at 5000 rpm for 15 minutes. Only clear filtrates were tested for their antifungal activities.

2.12. Extraction*:* The clear filtrate was adjusted at different pH values (4 to 9) and extraction process was carried out using different solvents separately at the level of 1:1 (v/v). The organic phase was concentrated to dryness under vacuum using a rotary evaporator.

2.13. Precipitation*:* The precipitation process of the crude compound was carried out using petroleum ether (b.p 60-80 °C) followed by centrifugation at 5000 r.p.m for 15 min.

2.14. Purification by TLC*:* Separation of the antimicrobial compound into its individual components was conducted by thin layer chromatography using chloroform and methanol (24: 1, v/v) as a solvent system.

2.15. Purification by column chromatography*:* The purification of the antifungal compound was carried out using silica gel column (2.5 X 50) chromatography Chloroform and Methanol 10:2 (v/v), was used as an eluting solvent. The column was left overnight until the silica gel (Prolabo) was completely settled. One-ml crude extract to be fractionated was added on the silica gel column surface and the extract was adsorbed on top of silica gel. Fifty fractions were collected (each of 5 ml) and tested for their antifungal activities.

2.16. Physico-chemical properties

2.16.1. Elemental analysis: The elemental analysis C, H, O, N, and S was carried out at the microanalytical center, Cairo University, Egypt.

2.16.2. Spectroscopic analysis: The IR, UV and Mass spectrum were determined at the micro analytical center of Cairo University, Egypt.

2.17. Biological activity: The minimum inhibitory concentration (MIC) has been determined by the cup method assay [Kavanagh, 1972].

2.18. Characterization of the antifungal agent: The antibiotic produced by Streptomyces antibioticus was identified according to the recommended international references of [Umezawa, 1977 and Berdy, 1974, 1980a, b, c].

3. RESULTS

3.1. Screening for the antimicrobial activities under solid state fermentation condition (SSF): From screening program for the antimicrobial activities by using different agriculture wastes under solid state fermentation condition from actinomycete isolate, KH-2326-27 revealed that Rice straw is the best agriculture wastes were found exhibited various degrees of activities against unicellular fungi, *Saccharomyces cerevisiae* ATCC 9763 and *Candida albicans*, IMRU 3669 and filamentous fungi *Aspergillus niger* IMI 31276, *Fusarium oxysporum*, *Rhizoctonia solani*, *Aspergillus flavus*, *Alternaria alternate*, *Botrytis fabae*, *Aspergillus fumigatus* ATCC 16424 and *Penicillium chrysogenium* (Table 1).

3.2. Identification of the most potent actinomycete isolate:

3.2.1. Morphological characteristics :The vegetative mycelia grew abundantly on both synthetic and complex media. The aerial mycelia grew abundantly on Starch- nitrate agar medium; Inorganic salts starch agar medium (ISP-4) and Oatmeal agar medium (ISP-3) The Spore chains were spiral, and had a smooth surface (plate 1).

Neither both sclerotic granules and sporangia nor flagellated spores were observed.

3.2.2. Cell wall hydrolysate: The cell wall hydrolysate contains LL-diaminopimelic acid (LL-DAP) and sugar pattern not detected.

3.3. Physiological and biochemical characteristics: The actinomycete isolate, KH-2326-27 could hydrolyzes protein, starch, pectin, lecithin and casein, whereas lipid hydrolysis and catalase test are negative, melanin pigment is positive, degradation of esculin & xanthin was positive, nitrate reduction, citrate utilization and KCN utilization were positive, whereas, urea and production of H_2S are negative. The isolate KH-2326-27 utilizes mannose, mannitol, glucose, fructose, *meso*-inositol, galactose, Rhamnose, sucrose, starch, sodium malonate, valine, arginine, cyctein, histidine and glutamic acid, but do not utilize maltose, lactose xylose, and phenylalanine. Growth was detected in presence of up to (5%) NaCl. The actinomycetes isolate, KH-2326-27 utilizes sodium azid (0.01%), phenol (0.01%) and thallous acetate (0.001). Good growth could be detected within a temperature range of 25 ^{o}C to 50 ^{o}C. Good growth could be detected within a pH value range of 5 to 9. The actinomycete isolate KH-2326-27 not sensitive to Ampicillin (25ug/ml) Nalidixic acid (30 ug/ml) Cefoperazone (75ug/ml) and Fusidic acid (10 ug/ml, Gentamicin (10 ug/ml) and Kanamycin (30 ug/ml) (Table 2).

3.4. Color and culture characteristics: The actinomycete isolate shows the aerial mycelium is light gray; substrate mycelium is Light yellowish brown, and the diffusible pigment not produced diffusible (Table 3).

3.5. Taxonomy of actinomycete isolate: This was performed basically according to the recommended international Key's viz. [Buchanan and Gibsons, 1974; Williams, 1989; and Hensyl, 1994] and Numerical taxonomy of *Streptomyces* species program. On the basis of the previously collected data and in view of the comparative study of the recorded properties of actinomycete isolate in relation to the closest reference strain, viz. *Streptomyces antibioticus*, it could be stated that actinomycetes isolate is suggestive of being likely belonging to *Streptomyces antibioticus* (Table 4).

3.6. Amplification of the 16_S rRNA gene: The 16_S rRNA gene was amplified by polymerase chain reaction (PCR) using the universal primers. The primers that was used to 16S rRNA sequencing were 16F357 of the sequence strepF; 5'-ACGTGTGCAGCCCAAGACA-3' and strpR; 5'-ACAAGCCCTGGAAACGGGGT-3', the product of the PCR was analyzed on 1.5% ethidium bromide gel.

3.7. Molecular phylogeny of the selected isolate: The 16_S rRNA sequence of the local isolate was compared to the sequences of *Streptomyces* spp. In order to determine the relatedness of the local isolate to these *Streptomyces* strains. The phylogenetic tree (as displayed by the Tree View program) revealed that the locally isolated strain is closely related to *Streptomyces* sp., rather related to *Streptomyces* sp., rather than to *Streptomyces antibioticus* (Fig. 1). Multiple sequence alignment was conducted the sequences of the 16_S rRNA gene of *Streptomyces antibioticus*. Computer assisted RNA searches against bacterial database similarly revealed that the 16_S rRNA sequence was 97% identical *Streptomyces antibioticus* (Fig. 1).

3.8. Factors effecting on the biosynthesis of the antimicrobial agent: Maximum antifungal activity biosynthesis could be recorded that a incubation period for 7 days; pH 7.0; temperature 30ºC.; starch best carbon source and sodium nitrate best nitrogen source.

3.9. Fermentation, Extraction and Purification: The *Streptomyces antibioticus* inoculum was introduced aseptically into each sterile Baxter bottle containing 5 g dry weight of Rice straw supplied with 40 ml mineral salt solution containing the following ingredients (g/l): $NaNO_3$, 2.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5 and KCl, 0.5. The pH was adjusted at 7.2 before sterilization. After seven days of incubation at 30^{O}C, 50 ml of sterilized distilled water were added and shaken. Filtration was carried out through cotton wool and followed by centrifugation at 5000 rpm for 15 minutes. The clear filtrates containing the active metabolite (20 liters), was adjusted to pH 7.0 then extraction process was carried out using n-Butanol at the level of 1:1 (v/v). The organic phase was collected, and evaporated under reduced pressure using rotary evaporator. The antimicrobial compound was precipitated by petroleum ether (b.p. 60-80°C) and centrifuged at 4000 r.p.m for 15 minute. Its color is yellowish. Separation of antifungal agent into individual components was carried out by thin-layer chromatography using a solvent system composed of chloroform and methanol (24: 1, v/v). Only one band at $R_f = 0.6$ showed antifungal activity. The purification process through column chromatography packed with silica gel, revealed that the maximum activities could be recorded in fraction Nos. 24&25.

3.10. Physicochemical characteristics*:* The purified antifungal agent produced by *Streptomyces antibioticus* are produces characteristic odour, their melting points are 140°C. The compound is freely soluble in chloroform, ethyl acetate, n-butanol, acetone, ethyl alcohol, methanol and 10 % isopropyl alcohol, but insoluble in petroleum ether, hexan and benzene.

3.11. Elemental analysis*:* The elemental analytical

data of the antifungal agent produced by *Streptomyces antibioticus*, showed the following: The elemental analytical data of the antibiotic indicated that: C=63.4; H=7.1; N= 5.3; O= 24.2 and S= 0.0. This analysis indicates a suggested empirical formula of: $C_{14}H_{19}NO_4$.

3.12. Spectroscopic characteristics: The spectroscopic analysis of the purified of antifungal compound produced by *Streptomyces antibioticus*, the ultraviolet (UV) absorption spectrum of the antifungal agent recorded a maximum absorption peaks at 225, 279, 285 nm (Fig. 2). The Infra red (IR) spectrum of the antifungal agent showed characteristic band corresponding to 35 peaks (Fig.3). The Mass spectrum of antifungal agent showed that the molecular weight at 265.22 (Fig.4).

3.13. Biological activities of the antimicrobial agent: Data of the antifungal agent spectrum indicated that the agent is active against unicellular and filamentous fungi strains (MIC ranged from 15.62 to 62.5 µg/ml). The antifungal activity produced by *S. antibioticus*, KH-2326-27 showed maximum inhibitory activity against unicellular fungi *Saccharomyces cerevisiae* ATCC 9763 (15.62 µg/ml) and *Candida albicans*, IMRU 3669 (15.62 µg/ml) and maximum inhibitory activity was observed against filamentous fungi *Aspergillus niger Botrytis fabae* (52.7 µg/ml) *Aspergillus fumigatus* IMI 31276 (31.25 µg/ml) *Fusarium oxysporum* (52.7 µg/ml) *Rhizoctonia solani* (52.7 µg/ml), *Aspergillus flavus* (46.9 µg/ml), *Alternaria alternate* (31.25 µg/ml), ATCC 16424 (93.75 µg/ml), and *Penicillium chrysogenium* (62.5 µg/ml) (Table 5).

3.14. Identification of the antifungal agent: On the basis of the recommended keys for the identification of antibiotics and in view of the comparative study of the recorded properties of the antifungal agent, it could be stated that the antimicrobial compound is suggestive of being belonging to Anisomycin (Flagecidin) antibiotic [Umezawa, 1977 and Berdy, 1974, 1980a, b, c].

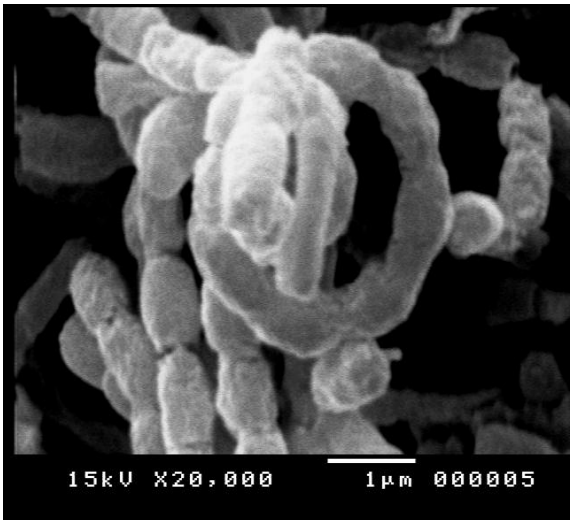

Plate 1. Scanning electron micrograph of the actinomycete isolate, KH-2326-27 growing on starch nitrate agar medium showing spore chain Spiral shape and spore surfaces smooth (X20,000).

Table 1. Antimicrobial potentialities of antibiotic-producing *Stresptomyces antibioticus* by different agricultural wastes under solid state fermentation condition [SSF].

*Organism number	* Mean values of inhibition zones (in mm) against															
	Bacteria							Fungi								
	S. aureus, NCTC 7447	*Bacillus subtilis*, NCTC 1040	*Bacillus pumilus*, NCTC 8214	*Micrococcus luteus*, ATCC 9341	*E. coli* NCTC 10416	*K. pneumonia*, NCIMB 9111	*P. aeruginosa*, ATCC 10145	*Candida albicans*, IMRU 3669	*S. cerevicea* ATCC 9763	*Asp. niger*, IMI 31276	*Asp. fumigatus*	*Asp. flavus*, IMI 111023	*Botrytis fabae*	*Fusarium oxysporum*	*Rhizoctonia solani*	*P. chrysogenum*
Rice straw	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**30.0**	**31.0**	**28.0**	**24.0**	**27.0**	**25.0**	**27.0**	**26.0**	**23.0**
Wheat straw	0.0	0.0	0.0	0.0	0.0	0.0	0.0	26.0	27.0	23.5	20.0	22.5	20.0	22.0	21.0	0.0
Banana waste	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Corn stover	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Sweet potato Residue	0.0	0.0	0.0	0.0	0.0	0.0	0.0	20.0	21.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pearl barley	0.0	0.0	0.0	0.0	0.0	0.0	0.0	22.0	23.0	20.0	13.0	19.0	14.0	18.0	17.0	0.0
Peel apples	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 2. The morphological, physiological and biochemical characteristics of the actinomycete isolate KH-2326-27

Characteristic	Result	Characteristic	Result
Morphological characteristics:		Mannitol	++
Spore chains	Spiral	L- Arabinose	+
Spore mass	Light Gray	*meso*-Insitol	+
Spore surface	smooth	Lactose	-
Color of substrate mycelium	Light yellowish brown	Maltose	-
Diffusible pigment	Not produced	D-fructose	+
Motility	Non-motile	Sodium malonate	+
Cell wall hydrolysate		**Utilization of amino acids:**	
Diaminopimelic acid (DAP)	LL-DAP	L-Cycteine	+
Sugar Pattern	Not-detected	L-Valine	+
Physiological and biochemical properties:		L-Histidine	+
Hydrolysis of:-		L-Phenylalanine	±
Starch	+	L-Arginine	+
Protein & Casein	+	L-Glutamic acid	+
Lipid	-	**Growth inhibitors**	
Pectin	+	Sodium azide (0.01)	+
Lecithin	+	Phenol (0.1)	+
Catalase test	-	Thallous acetate (0.001)	+
Production of melanin pigment on:		**Growth at different temperatures (˚C):**	
Peptone yeast- extract iron agar	+	10	-
Tyrosine agar medium	+	20	±
Tryptone – yeast extract broth	-	25-50	+
Degradation of**:**		55	-
Xanthin	+	**Growth at different pH values:**	
Esculin	+	3 - 4.5	-
H_2S Production	-	5-9	+
Nitrate reduction	+	9.5-12	-
Citrate utilization	+	**Growth at different concentration of NaCl (%)**	
Urea test	-	1-5	+
KCN test	+	7	-
Utilization of carbon sources		**Resistance to:**	
D-Xylose	-	Ampicillin (25ug/ml) and	+
D- Mannose	+	Nalidixic acid (30 ug/ml)	+
D- Glucose	+	Cefoperazone (75ug/ml)	+
D- Galactose	+	Gentamicin (10 ug/ml)	+
Sucrose	+	Kanamycin (30ug/ml)	+
L-Rhamnose	+	Fusidic acid (10 ug/ml)	+
Raffinose	+		
Starch	++		

+=Positive, - = Negative and ± = doubtful results, ++ = good growth.

Table 3. Culture characteristics of the actinomycete isolate KH-2326-27.

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
1-Starch nitrate agar medium	Good	**264-L.Gray** Light gray	**57-1.br** light brown	**58 m-br** moderate brown
2-Tryptone yeast extract broth (ISP-1)	No growth	-	-	-
3-Yeast extract malt extract agar medium (ISP-2)	moderate	**264-L.Gray** Light gray	**76-1-y-br** Light yellowish brown	-
4- Oat-meal agar medium (ISP-3)	Good	**264-L.Gray** Light gray	**76-1-y-br** Light yellowish brown	-
5-Inorganic salts starch agar medium (ISP-4)	Good	**264-L.Gray** Light gray	**76-1-y-br** Light yellowish brown	-
6-Glycerol – Asparagine agar medium (ISP-5)	No growth	-	-	-
7-Peptone yeast extract iron agar medium (ISP-6)	moderate	**264-L.Gray** Light gray	**57-1.br** light brown	**59-d.Br** Deep brown
8-Tyrosine agar medium (ISP-7)	moderate	**264-L.Gray** Light gray	**57-1.br** light brown	**59-d.Br** Deep brown

**The color of the organism under investigation was consulted with the ISCC-NBS color –name charts illustrated with centroid color.*

Table 4. Numerical taxonomy of *Streptomyces* species program (PIB WIN) (*Streptomyces* species) J. Gen Microbiol. 1989 13512-133 lang.

Characteristic	KH-2326-27	*Streptomyces antibioticus*
Diaminopimelic acid (DAP)	LL-diaminopimelic acid	LL-diaminopimelic acid
Sugar pattern	Not detected	Not detected
Spore chain rectiflexibles	-	-
Spore mass Spiral	+	+
Spore mass red	-	-
Spore mass gray	+	+
Diffusible pigment red/orange	-	-
Diffusible pigment yellow/brown	-	-
Melanin pigment:		
1-Peptone yeast extract-iron agar medium (ISP-6)	+	+
2-Tyrosine agar medium (ISP-7**)**	+	+
Active against of:		
1- B. subtilis and M.luteus	-	-
2- C. albicans and A.niger	+	+
Lecithinase activity	+	+
Lipolysis activity	-	-
Pectin hydrolysis	+	+
Nitrate reduction	+	+
H_2S production	-	-
Degradation of Xanthin	+	+
Growth at 45°C	+	+
Growth at NaCl 7% (w/v)	-	-
Growth with (% w/v): Phenol (0.1 % w/v) and Thallus acetate (0.001 % w/v)	+	+
Utilization of:		
L- Cysteine	+	+
L- Valine	+	+
L- phenylalanine	±	-
L- Histadine	+	+
Sucrose	+	+
meso-Inositol	+	+
Rhamnose	+	+
Raffinose	+	+

No.	Key	Source	Identification	ID Score
1	**KH-2326-27**	**KSA**	***Streptomyces antibioticus***	**0.97**

Table 5. Biological activities (MIC) of the antifungal agent by paper method assay.

Test organisms	MIC (µg/ml) concentration
1-Unicellular fungi:	
Candida albicans, IMRU 3669	15.62
Saccharomyces cerevisiae ATCC 9763	15.62
2-Filamentous fungi:	
Aspergillus niger IMI 31276	31.25
Aspergillus fumigatus ATCC 16424	62.5
Aspergillus flavus IMI 111023	46.9
Fusarium oxysporum	31.25
Rhizoctonia solani.	31.25
Alternaria alternata	31.25
Botrytis fabae	52.7
Penicillium chrysogenium	62.5

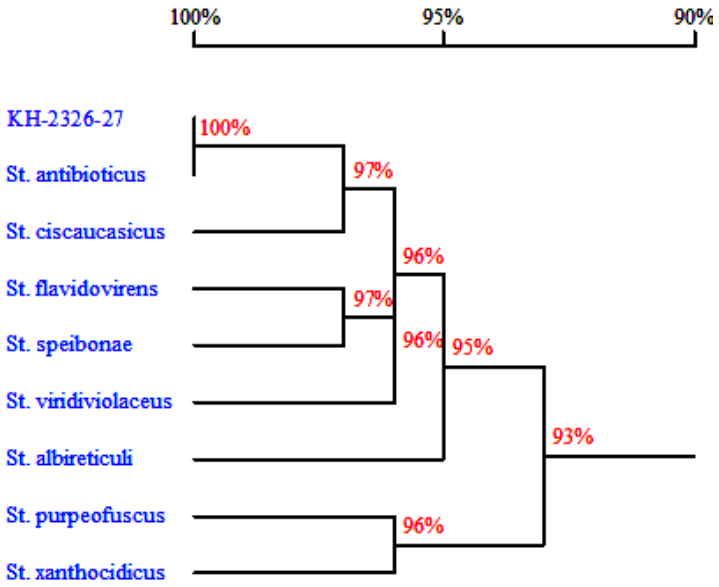

Fig. 1. The phylogenetic position of the local *Streptomyces* sp. strain among neighboring species. The phylogenetic tree was based on the pairwise comparisons of 16_S rRNA sequences.

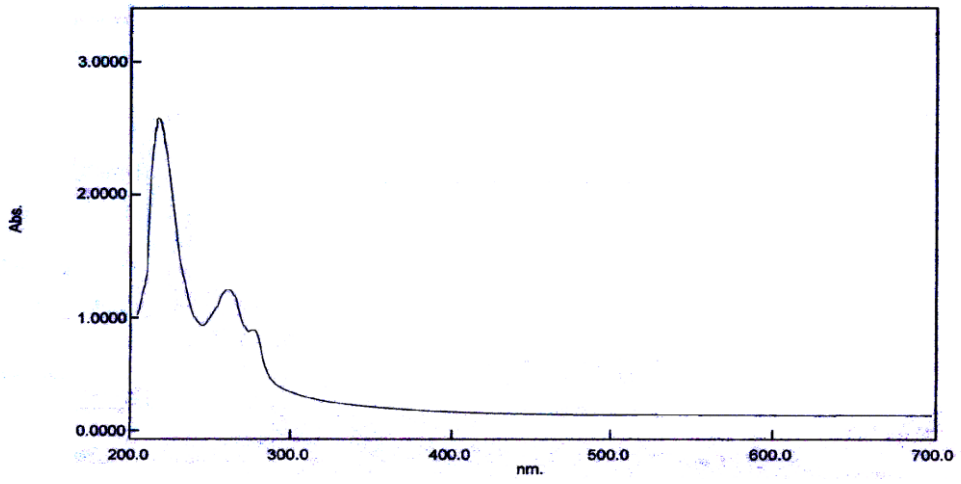

Fig. 2. Ultraviolet absorbance of antifungal agent produced by *Streptomyces antibioticus,* KH-2326-27

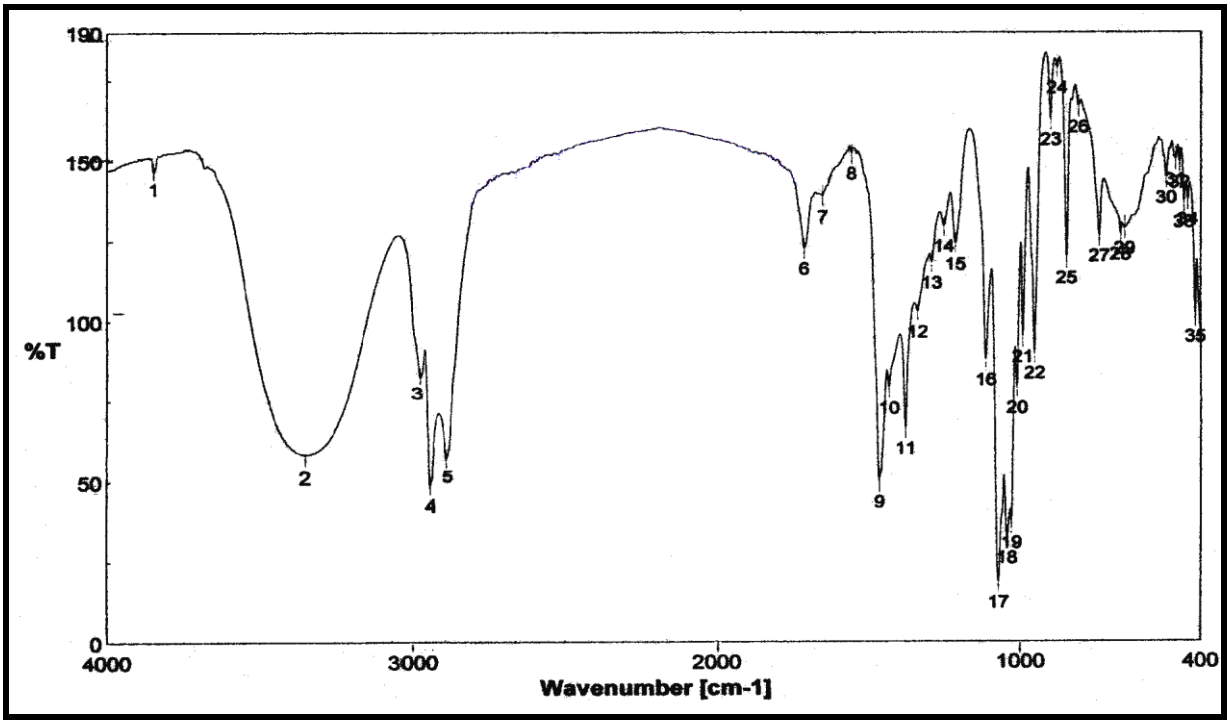

Fig. 3. FTIR spectrum of antifungal agent produced by *Streptomyces antibioticus,* KH-2326-27

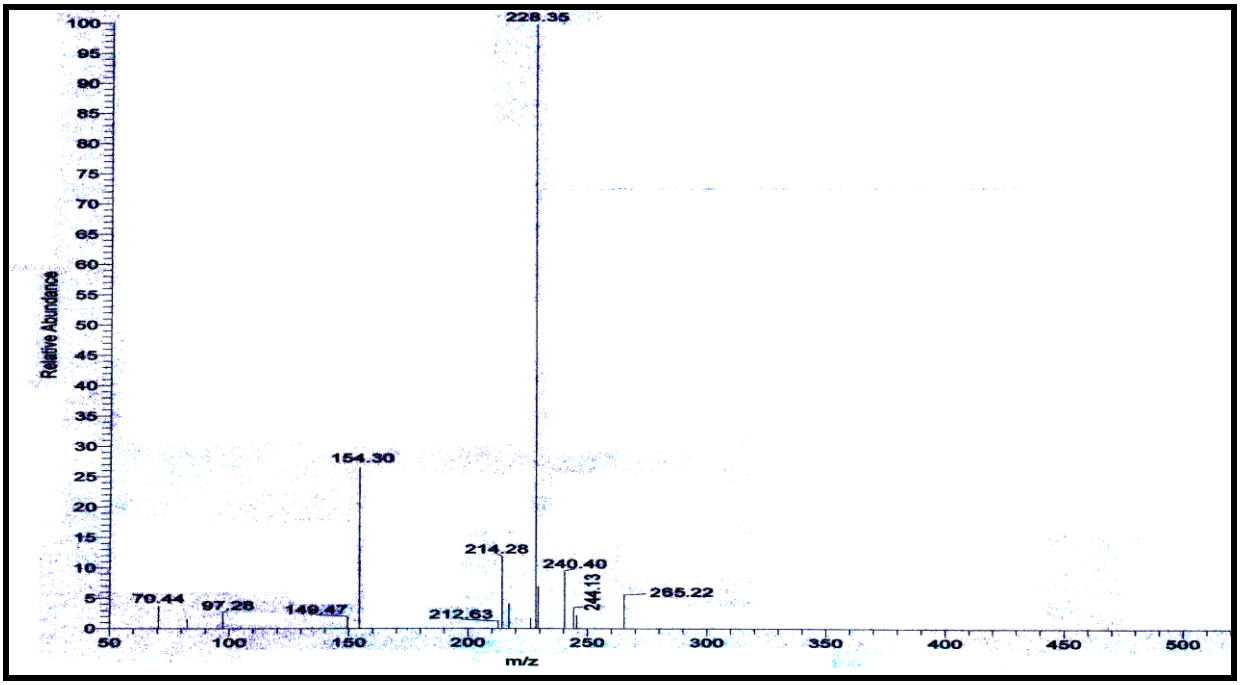

Fig. 4. Mass spectrum of antifungal agent produced by *Streptomyces antibioticus,* KH-2326-27

4. DISCUSSION

Antibiotics are traditionally produced by submerged fermentation, and their yields tend to be low due to the energy input [Tomasini, *et al* 1997]. The advantages of solid-state fermentation include (i) it is more competitive process, and it may be a viable option for the industrial production of secondary metabolites [Robinson, *et al*., 2001], (ii) It requires lower manufacturing cost by utilizing unprocessed and moderately processed raw materials (Adinarayana, *et al*., 2003), (iii) it is less sensitive to contamination when compared to submerged fermentation (Grohmann,

1993).

The *Streptomyces antibioticus* was isolated from Al-Khurmah governorate. The isolate was growing on starch nitrate agar medium for investigating its potency to produce antifungal agents. The actinomycete isolate, exhibited a wide spectrum antimicrobial agent [Kavanagh, 1972]. Identification process has been carried out according to [Williams, 1989, Hensyl, 1994 and Numerical taxonomy program, 1989]. For the purpose of identification of actinomycete isolate, the morphological characteristics and microscopic examination emphasized that the spore chain is spiral. Spore mass is light gray; while spore surface is smooth, substrate mycelium is light yellowish brown and no diffusible pigment was produced on ISP-media No. 3, 4 and 5. The results of physiological, biochemical characteristics and cell wall hydrolysate of actinomycetes isolate, exhibited that the cell wall containing LL-diaminopimelic acid (DAP) and sugar pattern of cell wall hydrolysate could not detected. These results emphasized that the actinomycetes isolate related to a group of *Streptomyces*. In view of all the previously recorded data, the identification of actinomycete isolate was suggestive of being belonging to *Streptomyces antibioticus*. The resulted sequence was aligned with available almost compete sequence of type strains of family streptomycetaeae. The phylogenetic tree (diagram) revealed that the local isolate is closely related *Streptomyces antibioticus*, similarity matrix is 97%.

From the data obtained it was evident that maximum antifungal activity biosynthesis could be recorded that a incubation period for seven days [Pandey, *et al.*, 2000]; pH 7.0 [El-Henawy, 2006 and Atta, 2010]; temperature 30ºC [Kharel *et al.*, 2004]; starch best carbon source [Adinarayana, *et al.*, 2002 and 2003 and Asagbra *et al.*, 2005]; $NaNO_3$ best nitrogen source [Khalifa, 2008 and Khaliq *et al.*, 2009].

The active metabolites were extracted by n-Butanol at pH 7.0 [Atta, 2010]. The organic phase was collected and evaporated under reduced pressure using a rotary evaporator. The extract was concentrated and treated with petroleum ether (b.p. 60-80°C) for precipitation process where only one fraction was obtained in the form of yellowish ppt. and then tested for their antifungal activity. Separation of antibiotic into individual components has been tried by thin-layer chromatography using a solvent system composed of chloroform and methanol (24:1, v/v) as developing solvent [Zhang *et al*, 2007 and Atta *et al.*, 2009]. The band with an R_f value at 0.6 which indicated that presence of one compound [Atta, 1999 and 2010]. For the purpose of purification process, the antibiotic were allowed to pass through a column chromatography packed with silica gel and eluting solvent was composed of chloroform and methanol (10:2 v/v), fifty fractions were collected and tested for their activities. The maximum activities could be recorded in fraction Nos. 24&25. Similarly, many workers used a column chromatography packed with silica gel and an eluting solvent composed of various ratios of chloroform and methanol [Criswell *et al.* 2006 and Sekiguchi, *et al.*, 2007].

The physico-chemical characteristics of the purified antibiotic revealed that, 140°C. The compound is freely soluble in chloroform, ethyl acetate, n-butanol, acetone, ethyl alcohol, methanol and 10 % isopropyl alcohol, but insoluble in petroleum ether, hexan and benzene; similar results were recorded by [Huang and Zheng, 2003 and Atta *et al.*, 2010].

A study of the The elemental analytical data of the antifungal agent produced by *Streptomyces antibioticus*, showed the following: The elemental analytical data of the antibiotic indicated that: C=63.4; H=7.1; N= 5.3; O= 24.2 and S= 0.0. This analysis indicates a suggested empirical formula of: $C_{14}H_{19}NO_4$. The spectroscopic analysis of the purified of antifungal compound produced by *Streptomyces antibioticus*, the ultraviolet (UV) absorption spectrum of the antifungal agent recorded a maximum absorption peaks at 225, 279, 285 nm. The Infra red (IR) spectrum of the antifungal agent showed characteristic band corresponding to 35 peaks. The Mass spectrum of antibiotic showed that the molecular weight at 265.2 [Huang and Zheng, 2003]. The MIC of antibiotic under study exhibited fairly active against unicellular and filamentous fungi strains (MIC ranged from 15.62 to 62.50 µg/ml). The antifungal activity produced by *S. antibioticus*, KH-2326-27 showed maximum inhibitory activity against unicellular fungi *Saccharomyces cerevisiae* ATCC 9763 (15.62 µg/ml) and *Candida albicans*, IMRU 3669 (15.62 µg/ml) and maximum inhibitory activity was observed against filamentous fungi *Aspergillus niger* IMI 31276 (31.25 µg/ml) *Fusarium oxysporum* (52.7 µg/ml) *Rhizoctonia solani* (52.7 µg/ml), *Aspergillus flavus* (46.9 µg/ml), *Alternaria alternate* (31.25 µg/ml), *Botrytis fabae* (52.7 µg/ml) *Aspergillus fumigatus* ATCC 16424 (93.75 µg/ml), and *Penicillium chrysogenium* (62.5 µg/ml)Similar investigations and results were attained by [Imnagaki *et al.*, 2006; Sekiguchi, *et al.*, 2007 and Atta, 2009]. Identification of antibiotic according to recommended international keys indicated that the antibiotic is suggestive of being belonging to Anisomycin (Flagecidin) antibiotic [Umezawa, 1967 and 1977 and Berdy, 1979 and 1980a, b & c and Huang and Zheng, 2003].

5. CONCLUSION

According to the previous studies and present study on production of antifungal substance, it is concluded that using SSF is more suitable than SLF to

produce of antibiotics by Actinomycetes. Some criteria were considered for this conclusion; increasing outcome, better control of solid state fermentation conditions and decreasing the cost. Also, since the content of fermentation is important, it is worthy to find a suitable content. There is no direct relation between antifungal production capacity of a strain in SSF and SLF conditions, and a strain's potential is the main factor of maximum antifungal production in SSF.

The present study mainly involved in the isolation of Actinomycetes based on the cultural, morphology, physiology and biochemical characteristics, as well as 16s rRNA methodology. Further work should be focused in most potent *Streptomyces antibioticus* for production the antifungal activities against unicellular and filamentous Fungi and studies parameters controlling the biosynthetic process of antifungal agent formation under solid state fermentation condition. The Anisomycin (Flagecidin) antibiotic produced by *Streptomyces antibioticus*, KH-2326-27 demonstrated obvious inhibitory affects against unicellular and filamentous fungi.

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