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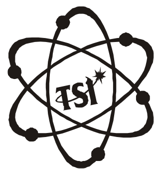

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Production and genetic improvement of novel antimycotic agent, saadamycin, against dermatophytes and other clinical fungi from endophytic *Streptomyces sp*. Hedaya48

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ABSTRACT

As a part of our ongoing efforts towards finding novel antimycotic and other bioactive agents from marine microflora of the Red Sea, recently secondary metabolites secreted by endophytes of some marine corals or sponges in Egypt were investigated. The new bioactive endophytic *Streptomyces sp*. Hedaya48 was isolated from the Egyptian sponge *Aplysina fistularis* and identified, which exhibited strong antimycotic activity against dermatophytes and other clinical fungi. Saadamycin [4-(hydroxymethyl)-5-hydroxy-2*H*-pyran-2-one] a new antimycotic compound, 5,7-dimethoxy-4-ρ-methoxylphenylcoumarin and vanillin were isolated from *Streptomyces sp*. Hedaya48. The producing strain was subjected to different UV irradiation doses and a mutant strain Ah22 with 10.5-fold higher saadamycin production was isolated (420mg/l as compared to 40mg/l produced by the parental strain). Production of saadamycin from mutant Ah22 was enhanced to 2.26 -fold (950mg/l) under optimized culture condition in batch culture and 2.38-fold increasing (1000mg/l) in bioreactor more than the yield obtained with the mutant strain Ah22 in normal production medium. Both saadamycin and 5,7-dimethoxy-4-ρ-methoxylphenylcoumarin were exhibited significant antifungal activities against several dermatophytes and other clinical fungal isolates as *Aspergillus fumigatus* and *Candida albicans*. © 2010 Trade Science Inc. - INDIA

KEYWORDS

Aplysina fistularis;
Endophytic;
Actinomycetes;
Dermatophytes;
Mutation.

INTRODUCTION

Dermatophytes are a group of fungi responsible for causing dermatophytoses in humans and there is evidence that the dermatophytes have acquired resistance to certain antimycotic drugs[23]. Fungi are eukaryotic and have machinery biosynthesis similar to that of higher animal and therefore agents that inhibit protein, RNA or DNA biosynthesis in fungi have greater potential toxicity to the host as well[9]. Therefore, the need for new effective and safe antifungal that selectively inhibit clinically important fungi without exhibiting any toxicity to humans is a major challenge to the pharmaceutical industry today, especially with the increase in opportunistic infections in the immune-compromised host[11].

Streptomycetes are the source of about 80 % of the antibiotics therapeutically used for the treatment of various human and animal diseases, i.e. aminoglycoside,

cephamycine, Polyene, tetracycline, macrolide, benzopyrones and Triazolopyrimidine antibiotic[5,7,28]. A variety of relationship exists between endophytes and their host ranging from mutualistic, symbiotic or antagonistic. Because of what appears to be their contribution to the host, the endophyte may produce a plethora of substances of potential use to modern medicine, agriculture and industry as novel antibiotics, immunosuppressant and anticancer compounds, which possess unique structures and great bioactivities representing a huge reservoir[22].

Induction of mutation followed by selection of the antibiotic production of mutants is still an important approach in all strains improvement programs[4,16]. Because of mutations affecting regulatory antibiotic genes, methods for broadening the range of mutagens useful in industrial processes are needed. UV irradiation is a fairly potent mutagen for many antibiotic producer microorganisms. The exposure of different species of *Streptomycetes* to mutagenic agents led to produce several highly producer antibiotic mutants as nystatin and antibacterial antibiotics production by *Streptomyces noursei* mutants[13].

2-Pyrone is a six-membered cyclic unsaturated ester, which is highly abundant in bacteria, plant, insect and animal systems and takes part in many different types of biological processes such as defense against other organisms, key biosynthetic intermediates, and as metabolites[21].

In the present study we reported in detail the taxonomy of the antibiotic saadamycin, producing strain, the improvement of antibiotic production by genetic tools *via* UV irradiation, optimization of the cultural conditions and by developing of a defined medium for the biosynthesis of this new antibiotic. Isolation; structural elucidation and biological activity of saadamycin, a novel pyrone derivative antibiotic from the mutant strain of *Streptomyces sp*. Hedaya48 were described.

MATERIALS AND METHODS

General experimental procedures

UV spectra were recorded with a Beckman DU 640 spectrometer and IR spectra with a JASCO FT/IR-430 instrument. The melting points were determined on a Buchi-540 melting-point apparatus. 1D and 2D NMR spectra were recorded on a Bruker DRX-500 instrument at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR. Low- and high-resolution mass spectra were recorded respectively on Shimadzu LCMS- 8000QPa and JEOL HX110A spectrometers.

Sponge material

Healthy specimens of Egyptian sponge *Aplysina fistularis* were collected from Sharm El-Sheikh, during the period of January to February, 2008. Taxonomic identification of sponge was performed by National Institute Oceanography and Fisheries Research Station, Hurghada, Egypt.

Isolation of the antibiotic producing strains

The endophytic *actinomycetes* strains were separated from the inner healthy tissue of *Aplysina fistularis* sponge according to the procedure described by Taechowisan and Lumyong[26] with slight modification. The samples were washed with distilled water and cut into small pieces of ca. 4×4 mm^2. Tissue pieces were rinsed in 0.1% Tween 20 for 1 min, then in 2.5% sodium hypochlorite for 15 min followed by washing in sterile distilled water for 5 min. Surface was sterilized with 75% ethanol for 5 min then rinsed in sterile water for three times. Finally the pieces were transferred to dishes of starch casein agar containing 100μg/ml nystatin and cycloheximide to inhibit the fungal growth until the mycelium or colony originated from the injury surface and incubated at 30°C for two weeks. Another segment of the same origin without surface sterilization was cultured as a negative control to check the presence of contaminated microbes on the sterilized segment surface. Single colonies were transferred periodically to the same medium and after 7 days, a pure culture of the *actinomycetes* strain were obtained and examined for anti-mycotic substances production.

Characterization of the selected hyper-producing actinomycete isolate

The hyper-producing strain Hedaya48 was identified by the analysis of phenotypic, chemotypic characteristics and the 16S rDNA sequence. Morphological and cultural characterization were done according to the diagnostic key of Szabo et al.[25], Williams et al.[30] and on the International *Streptomyces* Project (ISP)

Scheme as described by Shirling and Gottlieb[24].

Determination of the isomer of diaminopimelic acid (DAP) and the whole-cell sugar pattern were carried out as described by Hasegawa et al.[10] Fatty acid methyl esters were prepared by the trimethylsulphonium hydroxide method[1]. The base composition of genomic DNA of Hedaya48 strain was determined by the method of Mandel and Marmur[18].

16S rDNA sequencing

Genomic DNA was extracted and purified using the QIAGEN DNeasys Tissue Kit following the manufacturer's protocol for Gram-positive bacteria and animal tissue. Amplification of ribosomal DNA was performed using puReTaqTM Ready-To-GoTM PCR Beads (Amersham Biosciences). For amplification of the nearly complete 16S rRNA gene the eubacterial primers 27f and 1492r were used. The conditions for this PCR were: initial denaturation (2 min at 94°C) followed by 45 cycles of primer annealing (40 s at 50°C), primer extension (90 s at 72°C) and denaturation (40 s at 94°C), a final primer annealing (1 min at 42°C) and a final extension phase (5 min at 72°C). PCR products were checked for correct length on a 1% Tris-borate-EDTA (TBE) agarose gel (1% agarose, 8.9mM Tris, 8.9mM borate, 0.2m M EDTA), stained with ethidium bromide and visualized under UV illumination[27]. Sequence data were edited with Lasergene Software SeqMan (DNAStar Inc.) Next relatives were determined by comparison to 16S rRNA genes in the NCBI GenBank database using BLAST (Basic Local Alignment Search Tool).

Clinical target fungal isolates

Clinical fungal isolates of *Epidermophyton floccosum, Trichophyton rubrum, Trichophyton mentagrophytes, Microsporum gypseum, Aspergillus niger, Aspergillus fumigatus, Fusarium oxysporum, Candida albicans and Cryptococcus humicolus,* which provided by Tanta University Hospitals, Egypt. Fungi were maintained on (Sabouraud Dextrose Agar) SDA and yeasts on YMA (yeast malt extract agar) medium to be used as target organisms for this study.

Antibiotic bioassay

This was carried out using the paper-disc diffusion method[12]. The Sabouraud Dextrose Agar (SDA) for fungi or YMA agar medium for yeast was poured into sterile Petri-dishes (9 cm diameter) and allowed to solidify. 0.1ml fungal spore suspension (10^6 spores) of each test organism was inoculated into the agar surface. Sterile paper discs (6.0 mm diameter, Whatman antibiotic assay discs) were placed on the dried surface of the medium. Each disc received 20µl of the culture filtrate and Petri-dishes were kept in a refrigerator for 2 hours to allow for the diffusion of the antibiotic then incubated for 48 - 96 hours at 37°C. The inhibition zone diameter was measured in mm and the antibiotic concentration (µg/ml) was determined using a standard calibration curve using the purified antimycotic substance (saadamycin) produced by Hedaya48 strain.

Induction of mutation by UV- irradiation

Spores of Hedaya48 were gently scrapped from the surface of ISP-2 agar plates, washed with sterile normal saline (0.90%) and filtered through glass wool. Spore suspension was diluted to have a count of 10^4 spore / ml as determined by the viability observed on ISP-2 agar plate. Three ml of spore suspension was exposed to UV- light (Philips T-UV-30 W Lamp type number 57413 p/40) for different exposure time (5, 10, 15, 20, 25, 30, 35 and 40 min) placed about 25cm above from the liquid surface and gently swirled in a Petri dish. Following irradiation, spores were kept in dark at 4°C overnight to prevent photoreactivation. After incubation in dark, spores were plated on ISP-2 agar, incubated at 28°C and observed after 72 hrs. Mutant colonies with different morphology and expressing successively were selected. Both mutation and survival rates were determined after different exposure times to UV-irradiation and antibiotic production by mutants compared to the parent strain was studied.

Selection of wild and mutant strains for antibiotic production

For antibiotic production, a survey of fifty locally isolated *Streptomyces* strains as well as hundred mutant colonies after different UV-exposure times of the hyper antibiotic producer wild strain, Hedaya48, was carried out in shaken cultures using starch-nitrate broth

as a basal medium. It was composed of (g/l): starch, 10.0; $NaNO_3$, 2.5; K_2HPO_4, 1.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5, Seawater, 1.0 liter and pH was adjusted to 7.0.

Determination of dry weight

The cells were separated from the culture filtrate by centrifugation under 5000 rpm for 15 minutes, washed twice with distilled water and then dried at 60°C until reaching a constant weight.

Optimization of saadamycin production

In optimization of saadamycin productivity parameters, the parameter optimized earlier were incorporated in subsequent experiments. The optimization of anti-mycotic antibiotic production, saadamycin, was carried out in 50ml starch nitrate medium in 250ml Erlenmeyer flasks and monitored in terms of μg/ml.

Effect of different cultivation media

To know, which medium stimulates maximum antifungal activity, a slant culture of the hyperactive mutant, Ah22, on starch-casein agar was selected to inoculate 250ml Erlenmeyer flasks containing 50 ml each of the following media separately: medium 1, potato dextrose broth (PDB); medium 2, malt yeast peptone broth (MYPB); medium 3, glycerol asparagine; medium 4, starch nitrate; medium 5, composed of starch (1 %), yeast extract (0.4 %), peptone (0.2%) and medium 6 containing starch (1 %), glucose (0.3 %), malt extract (1 %), yeast extract (0.5 %) and flasks were incubated at 28°C and 180 rpm, then antibiotic activity of each cultivation medium was extracted with EtOAc and determined periodically after 3, 6, 9, 12 and 15 days of fermentation.

Typical time course of antibiotic production by Ah22 mutant and parent strain

Antibiotic production by the parent strain Hedaya48 and its mutant Ah22 were determined periodically during 14 days of fermentation using starch nitrate broth medium.

Temperature and pH

Seven 250ml Erlenmeyer flasks, each with 50ml starch nitrate medium were inoculated with Ah22 spores at a concentration of 10^6 spores/ml. Flasks were incubated at different temperatures viz., 20, 25, 30, 35, 37, 40 and 45°C on rotary shaker for 6 days.

The initial pH of the starch nitrate fermentation medium was adjusted to 3, 4, 5, 6, 7, 8 and 9 separately with 0.1N NaOH/0.1N HCl. All flasks were inoculated as mentioned above and incubated at 35°C on rotary shaker (180 rpm) for 6 days.

Effects of different carbon, nitrogen sources and metal ions on antibiotic production

The effect of different carbon, nitrogen sources and metal ions on antibiotic production was investigated. Starch of medium was replaced with different sugars at a concentration of 2%. Different nitrogen source and amino acids were used as sole nitrogen source for antibiotic production instead of $NaNO_3$ at a final concentration of 0.2%.

Extraction and isolation of saadamycin

The fermented broth of Ah22 mutant (5 L) was collected, extracted with ethyl acetate (1:1, v/v). The mixture was shaken over night and kept in stationary condition for 60 min to separate the solvent from aqueous phase. The organic extract was separated, dried over anhydrous sodium sulfate and concentrated under reduced pressure to yield 7.9 g as crude fungicidal extract. The extract was partitioned between hexane and 60% aqueous MeOH and then the fungicidal aqueous MeOH fraction was extracted with $CHCl_3$. Evaporation of $CHCl_3$ extract was done under reduced pressure to yielded a pale yellowish white solid (6.12 g), which was subjected to gel chromatography on a silica gel (60-120 mesh) column chromatography eluted with a linear gradient of hexane and ethyl acetate. Fractions eluted with hexane and ethyl acetate (7:3) were found to be pure fractions, and then collected to give compound **(1)** (vanilline, 10mg/l). Fungicidal fractions were combined, concentrated and further fractionated on a column of Sephadex LH-20 (40.0g) eluted with a linear gradient of CH_2Cl_2: MeOH. Fractions eluted with 0.1 and 0.4 MeOH in CH_2Cl_2 were found to be pure fungicidal fractions. The same pure fractions were combined and evaporated to yield, 5,7-dimethoxy-4-p-methoxylphenylcoumarin (80mg/l), and saadamycin (750mg/l) respectively as a major ingredient of the culture broth of the producing mutant.

Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of saadamycin against dermatophytes

The MIC values of saadamycin and reference antimycotic agent myconazole were determined by broth tube dilution procedure using two-fold dilution in sabouraud dextrose broth (SDB) at 37C for 96 h, MIC was determined as the lowest concentration of saadamycin showed no visible growth[2]. MFC values of saadamycin and myconazole were determined by sub-culturing 50 ml from tubes not visibly turbid and spot inoculating onto SDA plates. MFC values were determined as the lowest concentration that prevented growth on subculture[17].

RESULTS AND DISCUSSION

Isolation and screening of bioactive actinomycetes

Fifty endophytic actinomycete isolates obtained from sponge sample were tested for antimycotic subtances production, out of them ten isolates (20%) were produced the antimycotic metabolites. Among the producer isolates, the metabolites of the strain under the isolation number Hedaya48 was the potent active against dermatophytes, yeasts (*Candida albicans* and *Cryptococcus humicolus*) and other important clinical fungi (*Aspergillus niger, Aspergillus fumigatus* and *Fusarium oxysporum*) after 7 days of incubation at 30°C. Hedaya48 strain was selected as the hyper producer strain and then as the parent strain for further studies. Many authors suggested that Endophytes are rich sources of bioactive products as enzymes, antimicrobial, anti-cancer and anti-malarial agent[8]. Moreover, Taechowisan, and Lumyong[26] reported that secondary metabolites of endophytic actinomycetes of *Zingiber officinale* and *Alpinia galangal* were very active against phytopathogenic fungi.

Figure 1 : Spore surface ornamentation of *Streptomyces sp.* Hedaya48 strain

Taxonomic classification of hyper producer strain

The morphological and physiological characteristics of Hedaya48 strain, as well as its cell-wall type, whole-cell sugar pattern, fatty acid profile and the sequence of its 16S rRNA gene, are consistent with the characteristics of members of the genus *Streptomyces*. Strain Hedaya48 produced a blue–red to violet–red substrate mycelium and a blue spore mass composed of spiny spores in spirals-type spore chains as revealed by scanning electron microscopy (Figure 1). Whereas blue red, reddish blue, Pale blue to Dark blue, not pH-sensitive diffusible pigments are produced, melanin pigments were not produced.

Chemotaxonomic tests showed that the cell wall contained D-Alanine, Glycine and LL-DAP, indicating that it has cell-wall type I. Mannose, Galactose, Glucose and Ribose were detected as diagnostic sugars in the hydrolysates of whole cells. Fatty acid analysis showed that strain Hedaya48 contained a high proportion of saturated straight chain, iso- and anteiso-branched fatty acids: Iso- C14:0 (7.65%), C12:0 (6.39%), C15: 0 (23.12%), C18: 0 (8.74%), C18: 1 (10.24%), C18: 2 (22.42%), and anteiso-C16: 0 (21.44%). The G+C content of the genomic DNA was 71.2 %.

Strain Hedaya48 differs from other *Streptomycetes* that produce a blue spore mass as well as similarity by16S rRNA gene in several respects. Strains of *Streptomyces amakusaensis* (1451 bp 16S rDNA sequence by pair wise alignment of *S. amakusaensis* shows only 92% similarity to the strain Hedaya48) differs from strain Hedaya48 in producing smooth, blue spores in spirals spore chains and a pH-sensitive, yellow–brown substrate mycelium. This specie does not grow at 45°C and sensitive to penicillin G. It is unable to grow in the presence of 7% NaCl and sucrose and (+)-L-rhamnose are not used as sole carbon sources. L-Histidine can not be used as the sole nitrogen source[30]. *Streptomyces glaucescens* (showed 80% similarity with Hedaya48) produces a red–orange substrate mycelium and red-orange diffusible pigments as well as melanin were produced. It is sensitive to penicillin G and can

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TABLE 1 : Phenotypic and chemotypic characteristics of Hedaya48 strain

Cultural characteristics on ISP2	Growth luxury	Color of colony Deep blue	Soluble pigment Reddish blue
ISP3	Good	Violet blue	Pale blue
ISP4	Good	Deep blue	Reddish blue
ISP5	luxury	Deep blue	Light blue
ISP6	Good	Deep Reddish blue	Reddish blue
ISP7	Good	Deep pink blue	Light blue
Glucose-asparagine agar	luxury	Yellowish blue	Dark blue
Czapek's agar	Good	Deep blue	Blue red
Nutrient agar	Good	Medium blue	Pale blue
Physiological and biochemical characteristics		**Utilization of (1%)**	
Melanin production	-	Adonitol L-Arabinose	- +
H2S production	-	D-Galactose	+
Cell wall amino acids	D-Ala, Gly, 2, 6-	D-Mannose	+
Whole cell sugars	DAP	D-melibiose	+
Major fatty acids (%)	Man, Gala, Glu,	L-Rhamnose	+
Iso- C14:0	Rib.	L-Sorbose	-
C12:0		Mannitol	+
C15:0	7.65	N-acetyl glucosamine	+
Anteiso-C16:0	6.39	Lactose	+
C18:0	23.12	sucrose	+
C18:1	21.44	Maltose	+
C18:2	8.74	Raffinose	+
Characteristic phospholipids	10.24	Malate	+
DPG	22.42	Citrate	-
PI	+	Oxalate	+
PE	+	Utilization of	
PIMS	+	Casein	+
Aerobic reduction of $NaNO_3$	+	L-Histidine	+
Tyrosinase activity	+	L-phenylalanine	+
Hydrolysis of		**Sensitivity to antibiotics**	
Starch	+	Tetracycline	+
Cellulose	-	Streptomycin	-
Casein	+	Gentamycin	-
Gelatine	+	Erythromycin	-
Utilization of (1%)		**Chloramphenicol**	+
Glucose	+	Penicillin G	-
Glycerol	+	Growth at 45°C	+
Erythritol	-	Optimum NaCl for growth (%)	15-25
		Mol% G+C	71.2

not grow in the presence of 7% NaCl as well as raffinose or D-melibiose can not use as sole carbon sources by this strain[30]. *Streptomyces lomondensis* (showed 85% similarity with Hedaya48) produces warty to spiny, blue spores in rectiflexibiles or retinaculiaperti spore chains. The substrate mycelium is brick-red, rust-brown or straw-colored and a brown or pink diffusible pigment is produced. Melanin is also produced and casein is not degraded.

Streptomyces viridochromogenes (showed 87%

similarity with Hedaya48) produces green substrate mycelium and green, pH-sensitive diffusible pigments are produced. This specie produces melanin and it is sensitive to penicillin G and can not grow in the presence of 7% NaCl. It can not use sucrose as sole carbon source or L-phenylalanine as sole nitrogen source[30]. *Streptomyces tricolor and Streptomyces violaceoruber* (showed 84% similarity with Hedaya48) produce blue substrate mycelia, but differ from Strain Hedaya48 in producing smooth spores and spore mass not blue[30]. These differences support the other evidence that strain Hedaya48 is not a strain of *S. amakusaensis, S. glaucescens, S. lomondensis, S. viridochromogenes, S. tricolor or S. violaceoruber* and the results support that classification of strain Hedaya48 as a novel strain of *Streptomyces*. Additional data from the phenotypic characterization of the Hedaya48 strain is presented below (TABLE 1).

Induction of genetic variability in *Streptomyces sp.* Hedaya48

UV light induces a higher mutation frequency and a wider mutation spectrum than other mutagenesis. It is the most popular method used in induction of mutation[4,16]. Only those cells which survive can be screened as mutant based on phenotypic expression and the morphological mutants induced by UV light are shown in figure 2. Survival rate percentages decreased gradually by increasing exposure period from 5 to 20 min after that increased again when treated *Streptomyces sp.* Hedaya48 with 25 min UV exposure time and then decreased again after 30 and 35 min UV exposure times. While data in this figure showed an opposite trend, morphological variant percentages increased. The effect of different exposure times on both survival and morphological variant rates are shown in figure 3. Survival rate showed a characteristic curve shaped like a "saddle", it firstly decreased along with dose of 20 min exposure time, then increased in dose 25 min exposure time and finally decreased when dose surpassed 30 min of exposure. The down-up-down pattern (saddle shape) of survival due to UV implantation suggested that it had some obvious difference from the results of other traditional mutagens irradiation, such as NTG and X-ray. At the first "down" period (0-20 min), UV causes a series of DNA breaks and damage on the cell by etching of the cell wall, perforation of the membrane, destruction of the cell framework, etc. The degree of this damage to cell activity increases with increasing dose. With increasing dose to a certain value (25 min), the collision cascade results in a large number of vacancies in the genetic substance. Part of the vacancies in single strand DNA breaches induce an SOS reaction or other reparation and increase the survival rate of damaged cells. When the dose further increases (30 min), the cells are subject to serious damages, leading to a large quantity of double-strand breaks, which accumulate to an unrecoverable level, and the cell survival rate decreases again. These data are in line with those obtained by Khattab and El-Bondkly[13] by induction of nystatin and antibacterial activities at the same time in *Streptomyces noursei* NRRL 1714 by ultraviolet radiation, the survival percentages was decreased gradually by increasing exposure period, it decreased to 3.70% after 15 min exposure time.

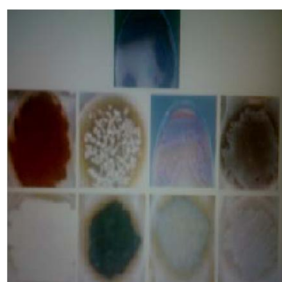

Figure 2 : Colony morphology and pigment coloration of UV-induced mutants of *Streptomyces sp*. Hedaya48 strain (in the first line)

After Hedaya48 strain was irradiated by UV light, obtained mutants, which differed from the original strain, not only in having a different yield of antifungal activity

TABLE 2 : Highest antifungal saadamycin induced mutants

Exposure time (min)	Mutant No.	Antifungal increasing (fold)
10	Ah22	10.5
	Ah25	2.4
	Ah30	3.5
	Ah32	5.1
15	Ah38	1.9
	Ah45	4.8
20	Ah53	1.6
	Ah60	3.8
	Ah62	2.3
30	Ah69	1.2

Original strain *Streptomyces sp*. Hedaya48 produced 40 µg / ml saadamycin (control)

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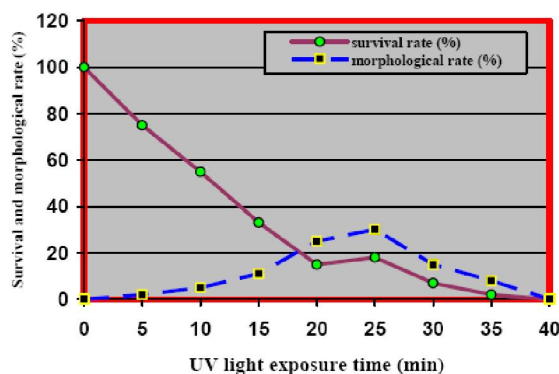

Figure 3 : Effect of UV exposure time on survival and morphological rate of Streptomyces sp.Hedaya48

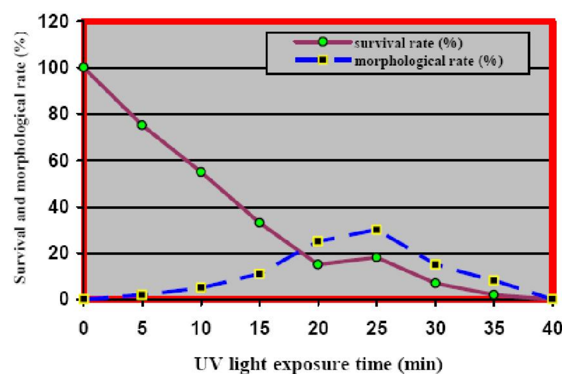

Figure 4 : Fermentation curve of antibiotic production by parent strain Hedaya48 and its mutant Ah22

but also by taxonomic properties such as color of mycelium, diffusible pigments, utilization of different carbon or nitrogen sources and nitrate reduction were obtained. Out of hundred surviving colonies some high antibiotic yield mutants with a wide variety of coloration on starch nitrate plates (TABLE 2 and Figure 2) were counted as follows: four mutants as a result of 10 min exposure time, i.e., Ah22, Ah25 and Ah30 and Ah32 showed 10.5, 2.4, 3.5 and 5.1-fold antifungal production more than the original strain, respectively. Moreover, two mutants as a result of 15 min exposure time, i.e., Ah38 and Ah45 produced 1.9 and 4.8 -fold antimycotic activity. Three mutants as a result of 20 min exposure time, i.e., Ah53, Ah60 and Ah62 produced 1.6, 3.8 and 2.3-fold antifungal higher than the parent strain, respectively and one mutant as a result of 30 min exposure time, i.e., Ah69 produced 1.2 -fold (TABLE 2). The average yield of antifungal saadamycin was dramatically increased from 40 mg/L in wild type to 420mg/L in Ah22 mutant and the antibiotic activity remained stable during twenty generations of flask culture. Accordingly, Ah22 was selected for further investigation as stable hyper producer mutant. Wieczorek and Mordarski[29] treated *Streptomyces olivaceus* with UV-light and isolated mutants differed from the original strain, in having a different spectrum of antimicrobial activity and taxonomic properties. Moreover, El-Gendy et al.[6] enhanced bioactive Pyrone derivatives secondary metabolites production in different marine *streptomyc*e species by genetics tools.

Time course of antifungal saadamycin production

The fermentation characteristics of the parental strain Hedaya48 and its mutant strain Ah22 in a starch-nitrate medium were monitored over a period of 14 days (Figure 4). Data in figure 4 demonstrate that the fermentation time of saadamycin antifungal is shortened from 10 days by the wild strain to 6 days by mutant Ah22, which provides great advantages in industrial production by increasing efficiency. Saadamycin production by Ah22 mutant strain was not detected during the first 24 h of fermentation; it is started at the second day (20µg/ml) to reach its maximum value at the 6 day (420µg/ml). Similar results were observed for secondary metabolism in actinomycetes as a new antibiotic, ayamycin production in batch cultures of *Nocardia sp.* Alaa2000[5].

Influence of some cultivation factors on the production of antibiotic

Production of antifungal has been known to be influenced by cultural conditions and media components, such as pH, temperature, carbon source, nitrogen source, metal ions and by the concentration of these components, which vary from organism to other. The production of the antifungal saadamycin by mutant Ah22 has been evaluated in respect to the culture conditions and the composition of the culture medium.

Effect of different fermentation media on antifungal production by mutant Ah22

Data in figure 5 showed that medium 4, starch nitrate broth and medium 6 (SGMY) were stimulates maximum antifungal activity by the hyperactive mutant Ah22 (420 and 380µg/ml respectively) after 6 days of fermentation at 30°C and 180 rpm.

Influence of carbon source

Optimization of antifungal production was carried out. Mutant Ah22 was able to grow in all tested carbon sources (TABLE 3). However, substitution of starch

TABLE 3 : Effect of different carbon sources on saadamycin production by mutant Ah22

Carbon source	Final pH	Dry weight (mg/ml)	Antifungal concentration (µg/ml)
Fructose	5.7	1.12	372
Glucose	7.8	2.12	400
Mannose	6.0	3.00	364
Galactose	7.2	0.9	10
Xylose	5.8	0.9	11
Lactose	6.4	1.0	16
Sucrose	6.8	2.12	86
Maltose	6.5	0.84	120
Starch	8.0	2.55	410
Glycerol	8.22	3.20	230
Mannitol	8.0	2.60	312
Raffinose	7.0	1.22	185
1% Starch+ .5% Glucose	8.0	3.14	450
1% Starch+ 1% Glucose	8.11	3.30	512
1% Starch+ 2 % Glucose	8.2	3.00	500

Ah22 mutant produced 420µg / ml saadamycin

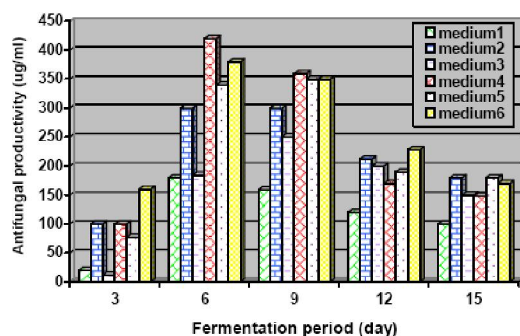

Figure 5 : Effect of different fermentation media on antifungal productivity by mutant Ah22

by a mixture of 1% glucose and 1% starch showed 21.9 % increase of antifungal production followed by cultures containing starch, glucose or mannose as sole carbon source. Cultures containing galactose, xylose or lactose yield the lowest amounts of saadamycin antifungal. The utilization of glucose and starch by Ah22 for growth and production of the antifungal indicates the presence of an active uptake system for these substrates. Starch and glucose were also found to be used as a sole carbon source by other *Streptomyces* species[3,5].

Influence of nitrogen source

The results (TABLE 4) revealed that the level of antifungal production greatly affected by the nature and

TABLE 4 : The role of nitrogen source on the production of antifungal saadamycin by mutant Ah22

Nitrogen source	Final pH	Dry weight (mg/ml)	Antifungal concentration (µg/ml)
$NaNO_3$ (control)	8.00	3.44	512
Casein	6.3	2.0	90
Peptone	6.3	3.1	250
Yeast extract	6.5	3.5	320
Tryptone	6.5	3.12	300
Soyabean	6.8	3.6	390
NH_4NO_3	5.7	1.8	80
$(NH_4)_2 SO_4$	5.40	2.32	44
KNO_3	8.12	4.12	500
Glutamic acid	7.0	2.5	140
Phenylalanine	6.62	2.4	549
Alanine	6.90	2.9	561
Valine	7.42	3.0	622
Tryptophan	6.18	2.64	22
Asparagine	6.9	1.48	30
Alanine + Phenylalanine+ Valine	7.9	4.00	650
$NaNO_3$+Alanine+Phenylalanine+Valine	8.5	4.22	786

concentration of the nitrogen source supplied in the culture medium. The antifungal production was increased by 21.48%, 9.57% and 7.23% in the cultures of Ah22 containing valine, alanine and phenylalanine, respectively as a sole nitrogen source (with 1% glucose and 1% starch as carbon source). However, cultures containing tryptophan, asparagine or ammonium salts showed the lowest antifungal activity. Cultures supplemented with a mixture of $NaNO_3$, Alanine, Phenylalanine and Valine showed 53.52% increase of antifungal production. The positive effects of DL-valine, alanine and phenylalanine may be due to their direct incorporation in the chromophore of saadamycin molecule as reported previously[19] for the enhancement of act-D production by up to 83% in *S. chrysomallus* with the addition of DL-valine in the production medium and for granaticin production in *S. violaceolatus*, respectively.

Influence of elements

The results given in TABLE 5 showed that $FeSO_4$, $MgSO_4$, NaCl, KH_2PO_4 and $CaCO_3$ play an important role in the promotion of saadamycin production. The antifungal yield in the presence of each of them in the culture medium separately was increased by 4.33, 5.47, 6.23, 6.62 and 10.05%, respectively. However,

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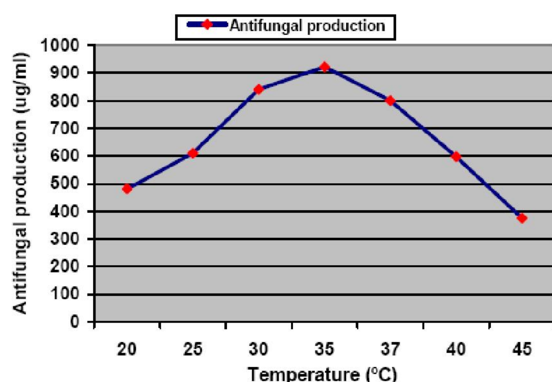


Figure 6a : Effect of temperature on saadamycin production by mutant Ah22

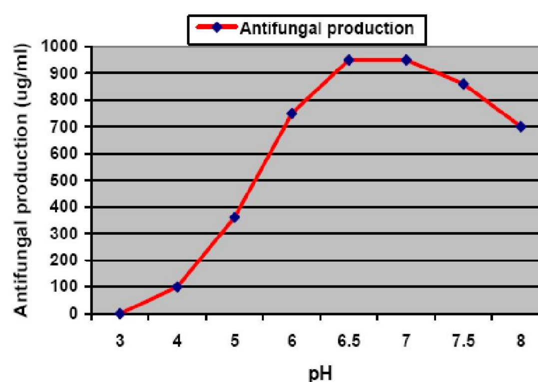
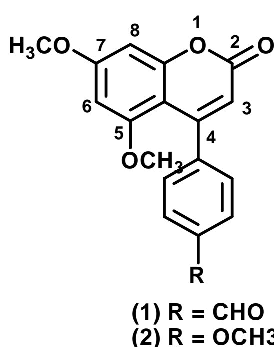


Figure 6b : Effect of intial pH on saadamycin production by mutant Ah22



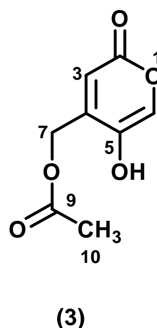


Figure 7 : Chemical structures of vanillin (1); 5,7-dimethoxy-4-p-methoxylphenylcoumarin (2) and saadamycin (3)

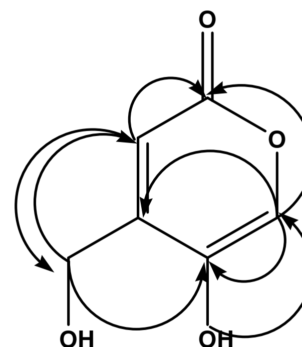


Figure 8 : HMBC correlations observed saadamycin antibiotic

using of a mixture of these metal ions lead to increase saadamycin production by14.5% compared to the control. These results are in agreement with that obtained for the importance of metal ions for the growth and antibiotic production by *Streptoverticillium rimofaciens*[14,15] and for granaticin production in *S. violaceolatus*[19]. We can concluded that, changes in the nature, type and concentration of carbon, nitrogen sources or elements contents of the culture greatly affect antibiotic biosynthesis in *Streptomycetes* as reported by many authors[3,5,7].

Influence of incubation temperature and initial pH value

Mutant Ah22 showed a wide range of incubation temperature for relatively good growth and antifungal production (Figure 6a). Maximum antibiotic production (922µg/ml) was obtained at 35°C. Higher incubation temperature (37-45 ºC) had an adverse effect on growth and antifungal production. On the other hand, the initial pH value of culture medium showed a significant effect on the growth and antifungal production of Ah22 strain (Figure 6b). The maximum growth and antifungal activity (950µg/ml) was obtained at an initial pH of 6.5-7.0. Similarly, different bioactive compound were produced extracellularly in *streptomycetes* cultures at pH values around 7[3].

Finally production medium containing (g/l): starch, 10; glucose, 10; $NaNO_3$,1.0; valine, 0.5; alanine, 0.25; phenylalanine, 0.25; KH_2PO_4, 1.0; $MgSO_4$, 0.05; $CaCO_3$, 2.0; NaCl, 1.0; $FeSO_4$, 0.1; Seawater 1L and pH 6.5, was recommended for saadamycin production by mutant Ah22. The optimized medium enhanced antifungal productivity to 126.19% (950µg/ml) in batch culture and 138.1 % increasing (1000µg/ml) in bioreactor more than the yield obtained with the mutant strain Ah22 in normal production medium (420µg/ml) (TABLE 6).

Structure elucidation of bioactive compounds

Chemical structures of vanillin (1); 5,7-dimethoxy-4-p-methoxylphenylcoumarin (2) and saadamycin (3) are presented in figure 7. Compounds **(1)**, vanillin, did not exhibit any antifungal activity and it was identified by comparison with authentic samples. Compound **(2)**, (5,7-Dimethoxy-4-p-methoxylphenylcoumarin

TABLE 5 : The role of trace elements on the production of antifungal saadamycin by mutant Ah22

Element	Final pH	Dry weight (mg/ml)	Antifungal concentration (µg/ml)
Control	8.39	4.00	786
$MgSO_4$	6.3	2.0	829
$FeSO_4$	6.62	3.1	820
$MnCl_4$	6.50	3.5	500
$ZnSO_4$	6.00	3.12	540
$CuSO_4$	6.51	3.6	340
KCl	6.70	1.8	770
NaCl	7.82	3.72	835
$CaCO_3$	6.90	3.64	865
K_2HPO_4	8.12	4.12	750
KH_2PO_4	7.82	3.50	838
KH_2PO_4+$MgSO_4$+$CaCO_3$+NaCl+$FeSO_4$	7.60	3.39	900

TABLE 7 : Physico-chemical properties of saadamycin antibiotic

Appearance	Yellowish white
Melting point	140-142°C
HRESI-MS *m/z*	141.0183 for (M-H)- calcd. 141.0188 for $C_6H_5O_4$
Molecular formula	$C_6H_5O_4$
UV λ_{max} (MeOH) (log ε)	213 (4.75), 273 (3.90)nm
IR (KBr) ν_{max}	3258, 3176, 1615, 1460, 1283cm^{-1}

C18H16O5, Figure 7) was a white powder: melting point, 150–152°C (EtOH); UV λ_{max} (MeOH)nm (log ε): 250 (4.07), 325 (4.29); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.20 (2H, d, J = 8.5 Hz, H-2′, H-6′), 6.87 (2H, d, J = 8.5 Hz, H-3′, H-5′), 6.50 (1H, d, J = 2.5 Hz, H-8), 6.22 (1H, d, J = 2.5 Hz, H-6), 5.96 (1H, s, H-3), 3.83 (6H, s, OMe-7, OMe-4′), 3.46 (3H, s, OMe-5); IR ν_{max} ($CHCl_3$) cm^{-1}: 1710, 1610, 1595, 1510, 1158, 1111, 1052, 952, 872, 860, 830; MS m/z (relative intensities): 312 $[M]^+$ (80), 284 $[M–CO]^+$ (100), 269 $[M-MeCO]^+$ (37), 241 $[M-43-CO]^+$.

The identification of this compound was confirmed by comparison with authentic sample.

Structure determination of the new antimycotic compound, saadamycin

The physico-chemical properties as well as ^{13}C NMR and ^{1}H NMR and HMBC data of Saadamycin were illustrated in (TABLE 7 and 8) and (Figures 7 and 8). Saadamycin antibiotic was obtained as yellowish white powder. High-resolution ESI-MS revealed a molecular ion peak at m/z 141.0183 for $[M-H]^-$ (calcd. 141.0188) corresponding with the molecular formula $C_6H_6O_4$ with four degrees of un-saturation. The IR spectrum absorptions at 1615 and 3176cm^{-1} indicated the presence of carbonyl and hydroxyl functional groups. The NMR spectra revealed the presence of one oxygenated aliphatic methylene carbon (δ_C 59.5), two olefinic methine carbons (δ_C 109.8, 139.2), one enolic carbon (δ_C168.1), one quaternary olefinic carbon (δ_C 145.7), and a carbonyl carbon (δ_C 173.9). These data indicated the presence of hydroxy-substituted 2-pyrone ring in saadamycin structure. ^{1}H and ^{13}C-NMR data of compound **(3)** were similar to those of 5-hydroxy-α-pyrone, which isolated and identified by Yasuyuki[31] except for the increase of hydroxymethyl group. The presence of the 4-hydroxymethyl was detected by the ^{1}H-NMR [4.29 (2H, s), 5.67 (1H, s)]. Additionally, HMBC correlations (Figure 8) from δ_H 6.34 (1H, s, H-3) to C-2, C-4, C-5 and C-7, from δ_H 8.03 (1H, s, H-6) to C-2, C-4 and C-5, from δ_H 4.29 (2H, s, H-7) to C- 3, C-5 revealed the structure of compound **(3)** as 4-(hydroxymethyl)-5-hydroxy-2H-pyran-2-one.

TABLE 6 : Effect of culture conditions on growth and antifungal production by the parent and mutant Ah22 strain

Culture condition	Dry weight (mg/ml)		Antifungal concentration (µg/ml)	
	Parent	Mutant Ah22	Parent	Mutant Ah22
Normal medium	3.22	2.89	40	420
Optimized medium	5.8	3.32	100	950
Optimized medium (bioreactor)	7	6.8	120	1000

TABLE 8 : ^{1}H- and ^{13}C-NMR, HMBC assignment of saadamycin in DMSO-d6, 500 and 125 MHz, TMS and δ in ppm)

Number	δ_H [J in Hz]	δ_C	HMBC
2	-	173.9 s	
3	6.34 (1H, s)	109.8 d	2, 4, 5, 7
4	-	145.7 s	-
5	-	168.1 s	-
6	8.03 (1H, s)	139.2 d	2, 4, 5
7	4.29 (2H, s)	59.5 t	3, 5
5-OH	9.07 (1H, s)	-	6
7-OH	5.67 (1H, s)	-	-

MIC and MFC values of saadamycin from mutant Ah22 against dermatophytes

The lower MIC (1 to 5.16µg/ml) and MFC (1.25

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TABLE 9 : Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of the bioactive compounds and myconazole against dermatophytes and other clinical fungi

Test organism	Saadamycin (µg/ml)		5,7-dimethoxy-4-ρ-methoxylphenyl coumarin (µg/ml)		Myconazole (mg /ml)	
	MIC	MFC	MIC	MFC	MIC	MFC
Trichophyton rubrum	5	10	7 5	100	6	12
Trichophyton mentagrophytes	1.5	1.75	90	90	3	6
Microsporum gypseum	1.25	1.25	100	150	3	6
Epidermophyton floccosum	1.0	1.5	50	66	6	12
Aspergillus niger	1.0	1.6	20	50	10	22
Aspergillus fumigatus	1.6	2.0	10	35	12	15
Fusarium oxysporum	1.2	2.0	22	49	8	12
Candida albicans	2.22	3.0	15	20	20	20
Cryptococcus humicolus	5.16	5.55	10	32	10	15.5

to10µgm/l) values of saadamycin antibiotic and MIC (10 to 100µg/ml) and MFC (20 to100µgm/l) values of 5,7-dimethoxy-4-pmethoxylphenylcoumarin (TABLE 9) compared to myconazole (MIC, 3 to 20 and MFC 6 to 22mg/ml) for all the dermatophytes tested indicating to the efficiency of these bioactive metabolites of Ah22 against dermatophytes. The new antibiotic, Saadamycin, showed greater potency against the dermatophytes than myconazole. On the other hand, 5,7-dimethoxy-4-pmethoxylphenylcoumarin showed good to moderate antifungal activity against dermatophytes and clinical fungi. El-gendy et al.[6] isolated bioactive pyrones compound from marine *Streptomyces* and it was potent antimicrobial agent against some of pathogenic bacteria and fungi. On the other hand, Previous reports indicated that 5,7-dimethoxy-4-methoxy phenylcoumarin was produced by numerous species of plants, including[20], but to our knowledge up till now there is no reports about its production from microbial origin as well as its antimycotic activity. Our study is the first in which saadamycin and 5,7-dimethoxy-4-p-methoxylphenylcoumarin isolated from culture filtrates of an endophytic *Streptomyces* species of Egyptian sponge *Aplysina fistularis*.

CONCLUSION

The results of this study conclude that saadamycin antibiotic is a major ingredient in the culture filtrate of *streptomyces sp*. Hedaya48 and it can play an important role in the inhibition of the dermatophytes and other fungal pathogens. Further investigations are necessary to determine the relationship between these bioactive compounds and host sponge.

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