



Veratraldehyde as a food additive produced by the marine isolate *Streptomyces diastaticus* LC360811

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ABSTRACT

Twelve actinomycete isolates were derived from 6 sediment samples collected from the Red Sea coast, Egypt. The marine isolate, RS10, the most potent candidate, proved its capability to produce antimicrobial compound(s) that strongly inhibit both Gram-positive and Gram-negative bacteria. RS10 was identified by 16S rRNA gene sequencing as *Streptomyces diastaticus* LC360811. Culture supernatant of *S. diastaticus* LC360811 was extracted using n-butanol and tested against bacterial indicator strains using the disc diffusion method. The crude extract was partially purified using column chromatography and assessed for antimicrobial activity. Fraction no. 35 showed the highest antimicrobial activity against *Acinetobacter* sp. (34 mm), *Pseudomonas aeruginosa* ATCC 9027 (30 mm), *Candida albicans* (26 mm), *Staphylococcus aureus* ATCC 47077 (25 mm), *Aeromonas hydrophila* (23 mm), *Streptococcus iniae* (22 mm) and *Bordetella* sp. (18 mm). This active fraction was then identified as veratraldehyde using spectroscopic techniques: GC-MS-MS and HPLC (diode array detector) and compared with the authentic reference. Veratraldehyde as a natural preservative proved to be a good candidate to preserve the flavor of juice, yoghurt and fermented milk and was able to control the growth of bacterial pathogens compared to the non-treated products till day fourteen.

INTRODUCTION

The discovery of new potent compounds and development of the currently known antibiotics are considered the most significant targets for competing the emerging pathogens in the 20th century. Although many antibiotics have been commercialized to compete various pathogens, however, bacterial resistance is spreading throughout the world, revealing the steadily decreasing potencies of relevant antibiotics (Kaur *et al.*,

2015). Microorganisms, especially actinomycetes, are still the main source of natural products with pharmaceutical and agricultural importance (**Hug *et al.*, 2018**).

Out of 22,500 well characterized biologically-active compounds reported in different sources, about 45% of them are produced only by actinomycetes (**Rahman *et al.*, 2011**). The first report about mycelium-forming actinobacterium from the marine sediments was conducted several decades ago (**Gebreyohannes *et al.*, 2013**). Recently, marine actinobacteria have been recognized as a source of novel antibiotics with unusual structures and properties, and have shown anticancer potential (**Manivasagan *et al.*, 2014**).

Marine actinomycetes have been considered as the origin of the several novel bioactive compounds, for instance: rifamycin from *Micromonospora*, salinosporamide-A, an anticancer metabolite from *Salinispora* strain and marinomycins from *Marinophilus* sp. (**Priyadarshini *et al.*, 2016**). In addition, abyssomicin-C is originated from *Verrucosispora* sp. and marinopyrroles from *Streptomyces* sp. (**Mohseni *et al.*, 2013**). *Streptomyces* are largely isolated from soil and marine sediments (**Tamreihao *et al.*, 2016**) and produce a large number of bioactive molecules with massive biosynthetic significance that remains unrivalled without a meaningful competitor among other microbial populations (**Igbinosa *et al.*, 2017**).

Synthetic food additives may cause many diseases such as; allergies, hyperactivity, asthma, hay fever and certain reactions such as skin rash, vomiting, headache, tight chest, hives and worsening of eczema (**Pandey *et al.*, 2012**). Veratraldehyde is naturally found in ginger, peppermint, raspberry and many others. It has several pharmacological activities like antibacterial, antifungal and anti-stress properties (**Kim *et al.*, 2014**; **Prashant *et al.*, 2014**). Moreover, it is used in perfume manufacturing, food preservative and flavoring agent in food industries (**Molleti & Yadav, 2017**) and are recorded as non-mutagenic compounds, non-carcinogenic and generally regarded as safe food additives (**Braga *et al.*, 2018**).

The present study was presented to explore the capability of novel marine actinomycetes isolated from the Red Sea coast, Egypt, to produce bioactive compounds to be used as natural food additives. It is extended to report the optimal conditions for maximum production of the bioactive compound(s) by the marine *Streptomyces diastaticus* using Plackett-Burman and Box-Behnken statistical designs.

MATERIALS AND METHODS

Sample collection and isolation

Six samples of sea water and sediments were collected at 20 cm to 2 m depth from EL-Ahyaa beach and Hurghada on the Red Sea coast in Egypt. The samples were maintained at 4°C till transported to the laboratory for further analysis. The isolation process was carried out using pour plate technique (**Subramani & Aalbersberg, 2012**). Starch casein agar medium (g/l; Casein powder, 1; Starch, 10; Agar, 20) was prepared

using filtered sea water. Amoxicillin (500 mg) and flucozole (150 mg) were respectively added to the medium to minimize the bacterial and fungal contamination. Inoculated plates were incubated at 28-30°C for 7-15 days. The selected actinomycete colonies were picked up and purified over starch casein agar medium (**Amin *et al.*, 2014**).

Tested bacteria

Twelve bacterial indicator strains were kindly provided by Pharmaceutical Microbiology Lab, Faculty of Pharmacy, Alexandria University and the Microbiology Lab, National institute of Oceanography and Fisheries (NIOF), Egypt. These strains included five Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538, *Staphylococcus aureus* ATCC 2923, *Listeria monocytogenes* ATCC 35152, *Staphylococcus epidermis*, *Streptococcus iniae*) and seven Gram negative-bacteria (*Escherichia coli* ATCC 8739, *Klebseilla pneumonia* ATCC 10031, *Pseudomonas aeruginosa* ATCC 9027, *Acinetobacter baumannii*, *Bordetella pertussis*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*) in addition to *Candida albicans*. Stock bacterial cultures were maintained on nutrient agar slants at 4°C with monthly transfer.

Screening for antimicrobial activity

In 250 ml Erlenmeyer flasks, 100 ml of ISP2 sea water medium (g/l: Yeast extract, 4; Malt extract, 10; Dextrose, 4) was inoculated with the purified actinomycete isolates and incubated under shaking condition at 30°C and 120 rpm for 7 days. Fermented cultures were centrifuged at 6000rpm for 45 minutes to separate the fermented broth from the mycelial balls. The clear supernatant was filtered by bacterial membrane filter and used for activity assay.

Screening was carried out using cup plate method (**Attimarad *et al.*, 2012**). Each well was loaded with 100 µl of cell free actinomycete cultures and allowed to diffuse about 2 hours in the fridge, then incubated at 37°C for 24 hrs. After incubation, the zone of inhibition was measured and expressed in mm diameter. Based on the results, the potential actinobacterial strain was selected for morphological and molecular characterization (**Thirumalairaj *et al.*, 2015**).

Molecular Identification

Genomic DNA was extracted from actinomycetes isolates according to protocol of GeneJet genomic DNA purification Kit (Thermo). Four µl from PCR mixture was loaded to examine the PCR product on 1% agarose gel against 1Kb plus ladder (Fermentas). The primers used were as follows:

F:- 5' AGA GTT TGA TCC TGG CTC AG 3'

R:-3' GGT TAC CTT GTT ACG ACT T 5'

Finally, sequencing of the PCR product was carried out on GATC Company (Konstanz, Germany) by using DNA sequencer (ABI 3730XL, Germany).

Plackett- Burman experimental design

Optimization of medium components to maximize the production of the bioactive compound(s) by marine *Streptomyces diastaticus* was carried out using Plackett-Burman experimental design model. A set of 12 experiments was constructed for ten factors including the following parameters: (1) incubation time, (2) temperature, (3) pH, (4) agitation rate, (5) volume of medium, (6) malt extract concentration, (7) yeast extract concentration, (8) dextrose concentration, (9) inoculum size, and (10) percentage of sea water. Each factor was tested at two levels (low and high), and statistical designs were expressed in coded values as -1 (low level) and +1 (high level). For -1 (low level) it was organised as follows: incubation time (7 days), temperature (20°C), pH (4), agitation rate (90 rpm), volume of medium (50 ml), malt extract concentration (5 g/l), yeast extract concentration (2 g/l), dextrose concentration (2 g/l) and percentage of sea water (0%). Whereas +1 (high level) was performed as follows: incubation time (17 days), temperature (40°C), pH (9), agitation rate (180 rpm), volume of medium (50 ml), malt extract concentration (15 g/l), yeast extract concentration (6 g/l), dextrose concentration (8 g/l) and percentage of sea water (50 %) (Table 1). The production was carried out in triplicates, and the average value was taken (**Rajendran *et al.*, 2012**). Statistical *t*-values for the two samples were calculated to determine the variable significance (**Al-Sarrani & El-Naggar, 2006**).

Table 1. Plackett-Burman design for 10 variables and twelve trials

Run	Inoculum size (ml)	Temp. (°C)	Initial pH	Agitation rate (rpm)	Volume of medium	Malt extract conc. (g/l)	Yeast extract conc. (g/l)	Incubation time (hr)	Seawater (%)
1	+	+	-	+	+	+	-	-	-
2	-	-	+	-	+	+	+	-	-
3	-	-	-	+	-	+	+	+	-
4	+	+	-	-	+	-	+	+	+
5	-	-	+	-	-	+	-	+	+
6	-	-	-	+	-	-	+	-	+
7	-	-	-	-	+	-	-	+	-
8	+	+	-	-	-	+	-	-	+
9	+	+	+	-	-	-	+	-	-
10	+	+	+	+	-	-	-	+	-
11	-	-	+	+	+	-	-	-	+
12	+	+	-	+	+	+	-	-	-

Optimization of the production of bioactive components using Box-Behnken design

Results of Plackett-Burman design showed that concentrations of yeast extract, malt extract and inoculum size are the three significant factors used to enhance the production of bioactive metabolite(s). The three components were studied at three different levels:

low (-), high (+) and base (0). The experiment was carried out following **Jose and Jebakumar (2013)** in 15 trials with two replicates, and the response values were the mean of two replicates.

Extraction of antimicrobial compounds using different solvents

The potential actinomycete isolate was inoculated into ISP2 broth, and incubated at 20°C in a shaker at 180 rpm for 17 days. Equal volume of various solvents (Petroleum ether, chloroform, ethyl acetate, n-butanol, ethanol, diethyl-ether and dichloromethane) was added separately to the culture supernatant to determine the best solvent for extraction. Crude extract, obtained from each solvent, was assessed for its activity against the tested bacteria using disc diffusion method. The antimicrobial activity was estimated by measuring the diameter of the inhibition zone after 24 hrs of incubation at 37°C (**Anuradha et al., 2014**).

Fermentation and preparation of crude n-butanol extract

Based on solvent screening, *Streptomyces diastaticus* isolate was subjected to fermentation conditions and monitoring of the antimicrobial agent production at the end of the fermentation period. Twenty liters of ISP2 broth was inoculated with the 7 days old pre-culture of *Streptomyces diastaticus* isolate and incubated for 17 days at 20°C and 180 rpm. At the end of the incubation period, the culture supernatant was harvested by centrifugation at 8,000 g speed for 15 min. The supernatant was extracted with equal volume of n-butanol (1:1 v/v) and vaporized to dryness in a rotary evaporator at 40°C. The crude extract was stored at 4°C for further studies (**Arulappan et al., 2012**).

Thin layer chromatography (TLC)

The n-Butanol crude extract was applied on silica gel sheets (Kieselgel 60, GF 254 of 0.25 thickness) with ethyl acetate: methanol: water (88:8:3 v/v) as solvent system according to **Aadhimani et al. (2015)** with some modifications to show different bands. The chromatogram was observed under ultraviolet (UV) light with short wave length 253 nm and long wave length 365 nm. Furthermore, spray P-anisaldehyde – sulphuric acid reagent was used for preliminary colored compounds detection. Based on the separation of bands the R_f value was calculated (**Wagner et al., 1984**).

$$R_f \text{ value} = \text{Distance travelled by solute} / \text{Distance travelled by solvent}$$

Column chromatography

Two grams of n-butanol crude extract were subjected to column chromatography using silica gel (100–200 mesh size, column 18 mm × 300 mm; Hi-media) packed and pre-equilibrated with petroleum ether. The column was first eluted with equilibration solvent i.e. petroleum ether (two bed volumes), followed by linear gradients of petroleum ether: ethyl acetate according to the method described by **Shetty et al. (2014)** with some modification, at a flow rate of 1 ml/min. The eluent is passed through the column by gravity. Different fractions of 10 ml each were collected and subjected to agar disc

diffusion assay and thin layer chromatography. Petroleum ether: ethyl acetate (10:90 v/v) was used as screening system to develop the chromatograms which were observed under UV light (254 and 365 nm). Similar TLC patterns with the same spot were mixed, and the solvent was evaporated on rotary evaporator. Thirty-five fractions were collected and screened against test microbes using disc diffusion assay.

Characterization of the metabolites

The fraction with the best antibacterial activity from column chromatography was further subjected to various spectroscopic analyses for characterization of the compound. Gas Chromatography Mass-Mass Spectrometry (GC-MS-MS) analysis was performed at the National Institute of Oceanography and Fisheries (NIOF), Alexandria, Egypt. GC-MS conditions were as follows: detector mass spectrometer voltage 70eV, and its source temperature was 300°C. The injection temperature was 240°C, and the split mode was 0.5 µl injection. The HP 55% dimethyl-95% diphenyl polysiloxane non-polar column was performed with length of 30 cm x 0.25 mm *i.d.*, coating thickness film 0.25 µm. The oven was adjusted at 80°C for 1 min, and the initial time was 1.5 min with 40°C which ended by a final temperature of 300°C and 4 min hold time, where the total run time was 15 min. The components were identified by comparing their retention times to those of authentic samples, as well as by comparing their mass spectra with those of Wiley 275 Library.

High performance liquid chromatography (HPLC)

HPLC was used for the identification and confirmation of the presence of veratraldehyde in the purified sample by comparing its chromatogram and its data (Retention time and the best wave length) with a standard using different wave lengths (260, 290 and 340 nm) according to the procedure described by **Tioosi *et al.* (2012)**.

Comparison between the active fraction and some commercial antibiotics

Five formulations of antibiotic-loaded discs were arranged according to their group: Aminoglycosides (Amikacin); Chloramphenicol (Chloramphenicol); Glycopeptides (Vancomycin); Quinolones (Ciprofloxacin) and Tetracycline (Tetracycline). They were used to compare their activities to the purified extract against the test organisms.

Active fraction as food additive

Pour plate method is usually the method of choice for counting the number of colony-forming bacteria present in a liquid specimen. Five µl/ml of purified compound was added to 10 ml of fruit juice (pomegranate, banana, orange, and guava juice) and dairy products (fermented milk and yoghurt). The Standard Plate Count (SPC) was used to determine the total number of aerobic bacteria present in the juice and dairy product (**Ismaili *et al.*, 2016**). Agar plates were incubated at 37°C for 24 hrs. Colonies were carefully counted and evaluated after 1st, 3rd, 7th, 10th and 14th days of storage.

RESULTS

Screening for secondary metabolites

Out of 12 marine actinomycetes strains, RS10 isolate strain collected from the sediments of Red Sea (Egypt) showed potential antibacterial activity against both Gram positive and Gram-negative bacteria, with average inhibition zone of 15.5 mm against Gram positive bacteria and 12.5 mm against Gram negative bacteria (Fig. 1).

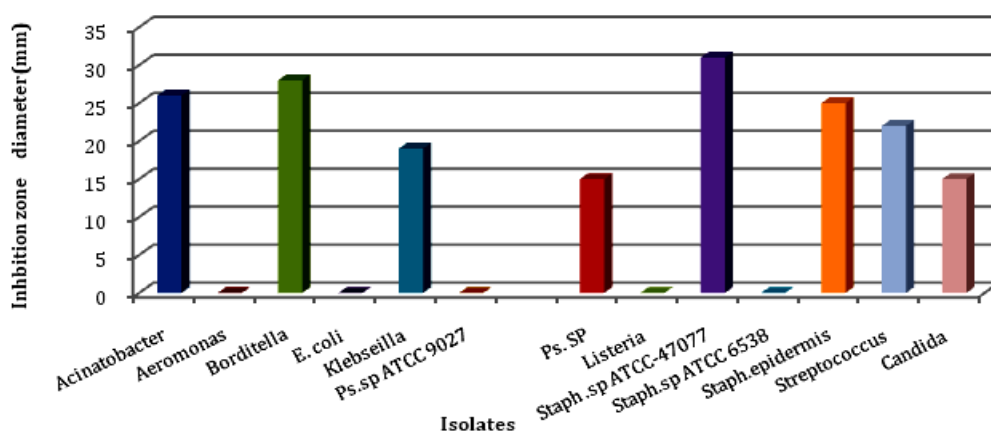


Fig. 1. Inhibition zone diameter of RS10 isolate against thirteen different isolates

Molecular identifications of Red Sea isolate (RS10)

The 16S rRNA gene sequences of RS10 isolate is compared to other sequences available in the NCBI database (Fig. 2). The sequence of RS10 isolate showed 97% similarity to *Streptomyces diastaticus* and was deposited in the NCBI database with the accession numbers of LC360811.

Plackett-Burman design

The effect of 10 variables as inducers for bioactive compound production from LC360811 isolate showed that the optimum conditions for maximum production of the bioactive compound are recorded in Table (2). Malt extract exhibited positive effect on the production of the bioactive compound as compared with the rest of factors (Fig. 3).

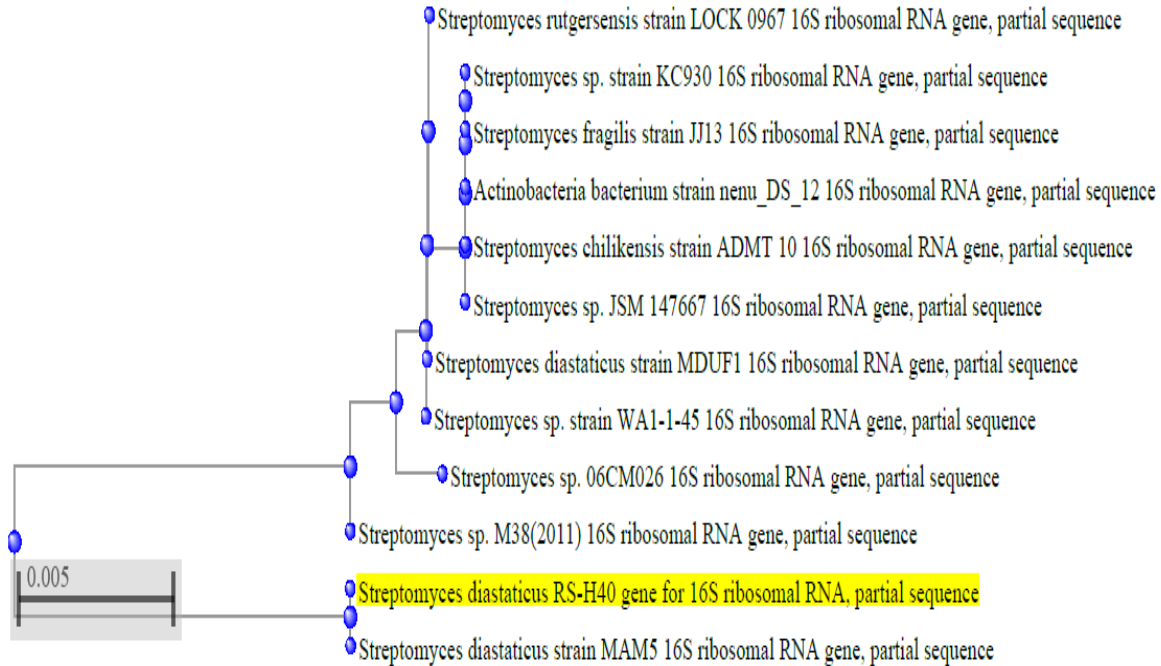


Fig. 2. Neighbor joining phylogenetic tree for the potential isolate RS10 identified as *Streptomyces diastaticus* with the closest NCBI (BLASTn) strains based on the 16S rRNA gene sequences.

Table 2. Statistical analysis of Plackett-Burman experimental statistical analysis of Plackett- Burman experimental results

Variables	Main effect	t-value*
Inoculum size	-19.54	-1.57
temp	-1.94	-0.14
pH	-18.2	-1.44
Agitation rate	2.28	0.16
Volume of medium	-7.42	-0.54
Yeast extract	-19.36	-1.55
Malt extract	25.72	2.28
dextrose	-4.94	-0.36
Incubation time	1.54	0.11
Sea water	-8.9	-0.65

*t- α = 0.1

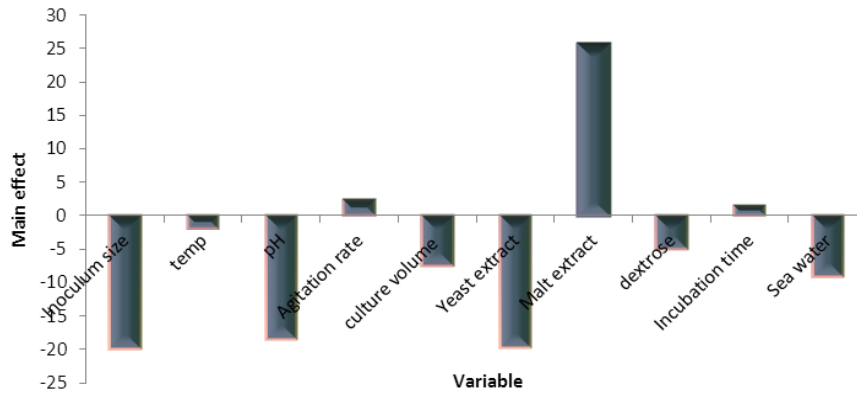


Fig. 3. Optimum condition using Plackett-Burman design for maximum activity produced by LC360811 isolate.

Optimization of the antagonistic activity with the most effective factors using Box-Behnken Design

Box-Behnken experiment was applied for maximum bioactive compound productivity; where yeast extract, malt extract and inoculum size had the three significant factors ($P < 0.05$) that influenced the productivity of the antimicrobial compound(s) as shown in (Figure 4) and its regression equation was as follows:

$$Y = -12.8771 + 1.6052X_1 + 7.065X_2 + 1.12X_3 - 4.4875X_1X_1 + 5.78947X_1X_2 + 3.25X_1X_3 + 7.674X_2X_2 - 1.11X_2X_3 - 20.144X_3X_3$$

Where Y is the antagonistic activity and X_1 , X_2 and X_3 are the coded values of yeast extract, malt extract and inoculum size. The value of R^2 is 0.953 and the regression analysis model is highly significant as evident from the calculated F -value (8.11).

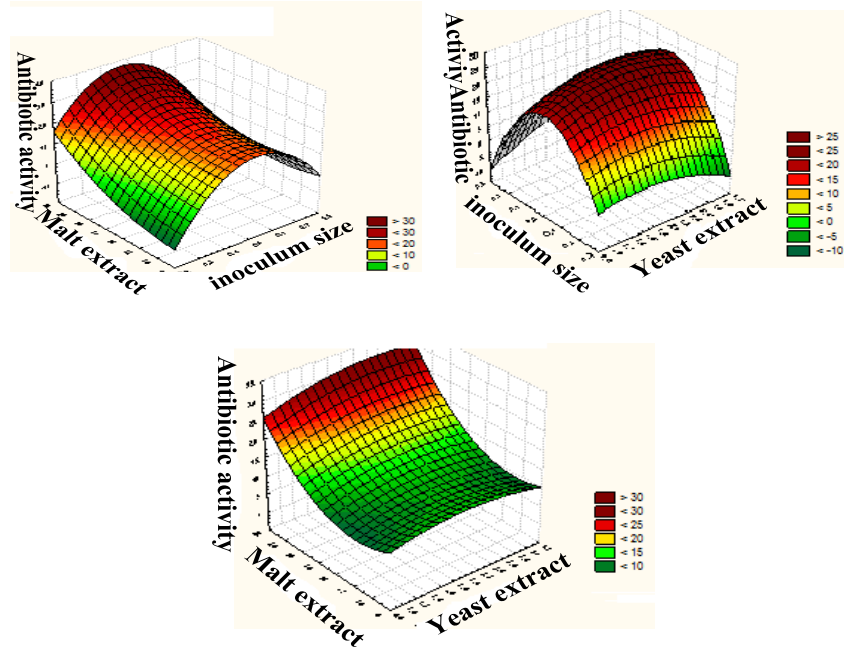


Fig. 4. Quadratic model of bioactive compound production from LC360811 isolate

Verification test of the model

Factors from Box experiments were verified practically. Antibacterial activity measured against *Streptococcus iniae* was 38 mm for *Streptomyces diastaticus* LC360811. The optimal level of the variables for the production of antibacterial metabolites by *Streptomyces diastaticus* LC360811 was as follows: incubation time (18 days), inoculum size (1.5 ml), yeast extract (5 g/l), temperature (20°C), agitation rate (180 rpm), malt extract (20 g/l), dextrose concentration (2 g/l), and initial pH (4)

Extraction of antimicrobial compounds using different solvents

Among the different solvents used for the extraction of the antimicrobial agents, n-butanol seems to be the potent one as it exhibited the highest average inhibition zone (12 mm) for all the tested bacteria. No inhibition zones were detected by dichloromethane or petroleum ether extracts. The secondary metabolites extracted by n-butanol showed different inhibitory activity against pathogenic bacteria, up to 20 mm with *Acinetobacter* sp. up to 17 mm with *Staphylococcus aureus* ATCC 47077, and no effect was detected on *E. coli* ATCC 8739 (Fig. 5).

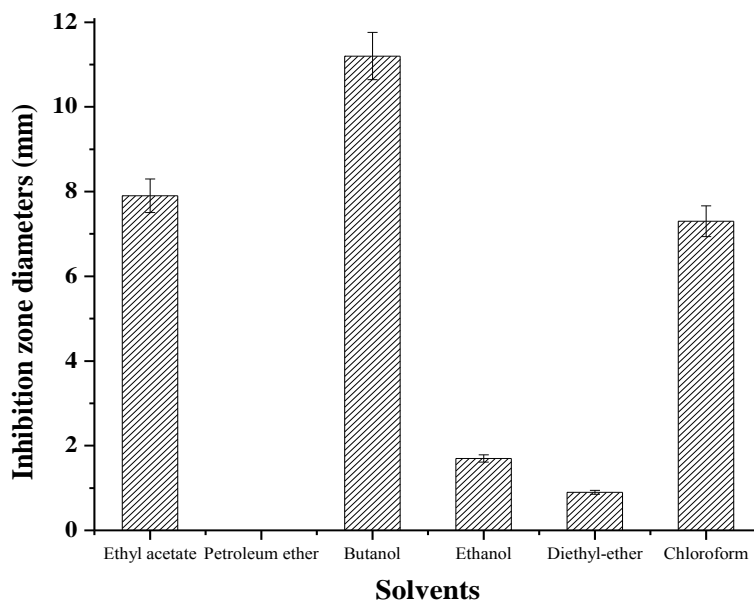


Fig. 5. Bioactivity of different organic solvents for the extraction of the bioactive compound(s) product by LC360811 strain. Bars are expressed as mean \pm SE; n = 5.

Purification of the bioactive compound using column chromatography

Out of 120 fractions, 35 sets of fractions were obtained from the column having different R_f values and antimicrobial activities against various pathogenic bacteria and yeast strains. Fraction number 35 showed the strongest antimicrobial activity and was further separated on TLC aluminum plates using the eluent system, hexane: dichloromethane: methanol (58:37:5) which succeeded to separate six different fractionated spots on TLC plate; only four spots were detected under UV light. All spots

were visualized using p-anisaldehyde spray reagent (Table 3). Bioautography test of TLC plate was carried out using *Acinetobacter* sp. as a pathogen indicator where R_f value of 0.26 exhibited a promising antibacterial activity against the tested pathogen.

Table 3. Bioautography and R_f of the active fraction (no. 35) against *Acinetobacter* sp

Fraction No.	R_f value	Inhibition zone diameter (mm)
1	0.07	-
2	0.26	26
3	0.32	-
4	0.68	-
5	0.91	-

(-)=No inhibition zone

Identification of bioactive compound using GC/MS-MS

Fraction number 35 was evaporated to dryness. A pale yellow residue was obtained and dissolved in DMSO. This residue was analyzed by GC/MS technique. Veratraldehyde was identified at retention time 9.10 with an illustrated mass spectrum and chemical structure using MS-MS analysis (Fig. 6). Further purification on TLC was carried out to scratch the active compound.

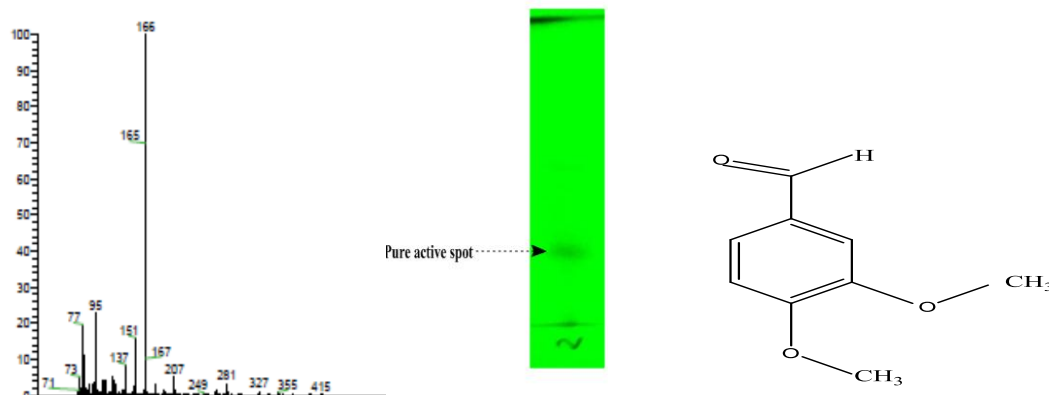


Fig. 6: Mass spectrum of bioactive n-butanol Veratraldehyde fraction (35) of *Streptomyces diastaticus*. Pure active spot under UV at 254 nm

Detection of Veratraldehyde using HPLC

Standard veratraldehyde sample was detected at wavelengths 4.088, 4.062 and 4.92 nm. Similarly, the purified sample was approximately detected at the same retention times; 4.119, 4.069 and 4.89 nm, as a confirmation that the sample purified from *Streptomyces diastaticus* isolated from the Red Sea was Veratraldehyde (Fig. 7).

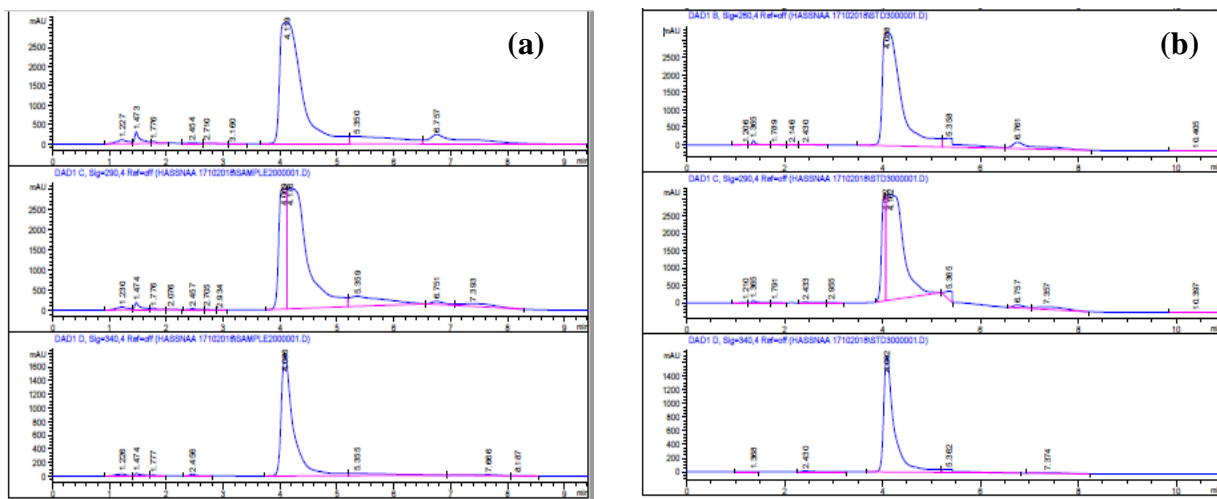


Fig. 7: HPLC chromatogram of purified Veratraldehyde (a) and its authentic sample (b) at three different wavelengths (260, 290 and 340 nm).

Comparison using some antibiotics

Commercial antibiotics were used to compare the antagonistic activity with those obtained from the purified veratraldehyde of *Streptomyces diastaticus*. The highest average inhibition by commercial compounds against Gram negative bacteria (28.6 mm) was recorded with Ciprofloxacin, while the lowest activity (9.4 mm) was detected with Tetracycline. Meanwhile, the inhibition of purified veratraldehyde from *Streptomyces diastaticus* (18.6 mm) was approximately similar to that of amoxicillin/clavulanic (17.8 mm). On the other hand, Ciprofloxacin caused the highest average inhibition gram-positive bacteria (32 mm) against 12.8 mm caused by veratraldehyde (Table 4). Minimum inhibitory concentration values of the veratraldehyde from *S. diastaticus* LC360811 was 26 mm against *S. iniae* and 25 mm against *S. aureus* ATCC 47077.

Table 4. Comparison between different commercial antibiotics and purified veratraldehyde

Antibiotic	Code	Inhibition zone diameter (mm)										
		Gram Negative					Gram Positive					
		<i>Aeromonas</i>	<i>K. pneumonia</i> ATCC 10031	<i>P.aeruginosa</i> ATC C 9027	<i>Bordetella sp.</i>	<i>Acinetobacter sp.</i>	Total average of G-ve bacteria	<i>St. aureus</i> ATCC- 2923	<i>St.epidermidis</i>	<i>L. monocytogenes</i> ATCC- 35152	<i>St. iniae</i>	Total average of G+ve bacteria
Amoxicillin/clavulanic	AUC	16	25	19	29	-	17.8	22	-	24	9	13.75
Chloramphenicol	C	35	20	14	37	20	25.2	40	22	20	26	27
Ciprofloxacin	CIP	42	33	15	28	25	28.6	32	29	37	30	32
Erythromycin	E	35	15	20	13	16	19.8	23	-	31	21	18.75
Tetracycline	TE	21	-	-	18	8	9.4	11	-	31	21	15.75
Purified veratraldehyde isolated from <i>St. diastaticus</i>		22	20	30	-	38	18.4	25	-	-	26	12.8

(-) indicated that no inhibition zone was detected

Purified veratraldehyde as food additive

The total bacterial count in all untreated samples with veratraldehyde increased gradually, attaining the maximum after 2 weeks with changing odor to offensive. Guava juice was fermented with the appearance of acid bubbles, while all the treated juice samples and dairy products maintained their fresh aspects and odor. In addition, the color of banana juice did not change in treated tubes, but it turned into brown color in untreated ones. On the 1st day, the bacterial count in fruit juices, treated by Veratraldehyde, decreased about 90.5% and 88.7 % in both pomegranate and orange juices, respectively, against 50 % and 43 % in banana and Guava juices, respectively. In the case of orange and banana juices, the preservative activity of the purified compound increased gradually with time reaching its maximum at the day 14 and kill about 90.9 % and 81 % of the total bacterial count in the two juices, respectively (Table 5). This activity decreased by the day 14 reaching only 11%, and slightly increased in case of guava juice (61.6 %). In case of dairy products, fermented milk and yogurt, bacterial counts decreased after the 1st day by about 85 % and 67.6 %, respectively, whereas the reduction in bacterial count were 70.3 % and 77.6 %, respectively, at day fourteen.

Table 5. Effect of the purified Veratraldehyde of LC360811 strain on the total bacterial count

DAY	CFU/ml for milk and Juice samples											
	milk		Yoghurt		Pomegranate juice		Guava juice		Orange juice		Banana juice	
	untreated	T	untreated	T	untreated	T	untreated	T	untreated	T	untreated	T
1	20	3	34	11	53	5	>300	150	71	8	223	127
3	22	3	98	10	>300	71	>300	249	98	12	>300	98
7	24	5	244	44	>300	143	>300	112	174	110	>300	77
10	84	81	>300	46	>300	277	>300	110	220	58	>300	54
14	54	16	>300	67	>300	267	>300	115	253	23	>300	57

(T) indicated treated samples

DISCUSSION

The incidence of multidrug resistant organisms is increasing and compromising the treatment of a growing number of infectious diseases. Therefore, there is an urgent need to develop new effective drugs against current antibiotic resistant pathogens (**Abad *et al.*, 2011; El-Naggar *et al.*, 2016, 2017; El-Moslamy *et al.*, 2018**).

The RS10 strain, isolated from Red sea sediment, showed positive activity against the tested microbes ranged from 10-30 mm. Although the potent isolate showed morphological variations on the three used culture media, RS10 displayed the substantial growth in starch casein agar medium. These results agree with **Gebreyohannes *et al.* (2013)** who attributed the excellent microbial growth of *Streptomyces* sp. in starch casein

agar medium to sufficient amount of nutrients in this medium, successful isolation of different marine *Streptomyces* sp. was performed with different morphological aspects on starch nitrate media with gray mass spore without diffusible pigment in media (Ye *et al.*, 2009; Al-Askar *et al.*, 2014)

The present study revealed that *S. diastaticus* LC360811 was able to inhibit *S. aureus* ATCC 2923, and had no effect on either *E. coli* ATCC 8739 or *P. aeruginosa* ATCC 9027. Similarly, Hunadanamra *et al.* (2013) stated that *S. diastaticus* SMP3-1 isolate was able to inhibit *S. aureus* ATCC 6538, whereas it showed weak antagonistic effect on *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853. Moreover, Subramanian *et al.* (2017) observed a broad antibacterial activity of *Streptomyces carpathicus* MK-01 against Gram positive bacteria.

Actinomycetes fermentation is a complex process; as it depends on its performance, fermentation medium, as well as the suitable environmental conditions (Singh *et al.*, 2017) such as: inoculum size, pH, incubation time, temperature and agitation rate. The statistical analysis of Plackett-Burman experimental results indicated significant value between malt extract (5 g/l) and the maximum productivity of *Streptomyces diastaticus* LC360811. Similarly, Bashir *et al.* (2012) found that glucose and yeast extract are the most suitable carbon and nitrogen sources, for the growth and production of the bioactive metabolites from *Streptomyces* PT1.

The results revealed that n-butanol extract exhibited the highest average inhibition zone (12) mm for *S. diastaticus* LC360811, with a maximum activity of 17 mm against *Staphylococcus aureus*. The n-butanol was reported the best solvent to extract antimicrobial compounds from *Streptomyces* sp. isolates (Torres *et al.*, 2017), and it is the solvent of choice with antibacterial activities against both *S. aureus* and *Candida albicans* (Amin *et al.*, 2016). Forar *et al.* (2007) referred to n-butanol as suitable for extraction of bioactive compounds from *Streptomyces* sp. In contrary, Vijayakumar (2012) observed that ethyl-acetate was the best solvent for the antimicrobial extract of the *Streptomyces* strain VPTS3-1 against *C. albicans* and *Pseudomonas mirabilis* with a maximum inhibitory zone of 20 and 19 mm, respectively. Moreover, in the present study, 1L of butanol extract produced approximately 20 mg sticky brown crude extract from *S. diastaticus* LC360811. This amount is considerably greater than that (1 mg) produced from 1.5 L of culture broth (Mercy & Krishnan, 2013).

TLC direct bioautography is a very sensitive assay that gives an accurate localization of active compounds (Balouiri *et al.*, 2016). Lomascolo *et al.* (1999) stated that veratraldehyde is a natural aromatic food additive, and identified it from white rot basidiomycetes. The present study might be unique to classify veratraldehyde from marine actinomycetes, perform extraction, and purify this compound from marine *Streptomyces* sp.

Lucera et al. (2014) reported that antimicrobials excreted from microbial sources find application in a number of food products such as milk, orange juice, and tomato juice, and in increasing the shelf life of chicken meat without changing the properties of the product. The purified veratraldehyde, isolated from *S. diastaticus*, increased the shelf life of fermented milk samples up to 21 days without any spoilage compared with untreated milk samples. Similarly, **Onyango et al. (2014)** reported that the incorporation of indigenous plants, *Olea europaea*, in milk samples extended the shelf life of fresh milk for 4 days and that of fermented milk up to one month without spoilage. In addition, in the present study, purified veratraldehyde extracted from *S. diastaticus* increased the shelf life for more than 14 days for orange juice and pomegranate juice more than banana and guava juice, without any observed change in the smell. Additionally, **Ali et al. (2016)** reported that supernatant of *Lactobacillus* increased the shelf life of orange juice than apple and pomegranate juices by 14 and 21 days of storage.

In conclusion, *S. diastaticus* LC360811 proved to be a good candidate for the production of bioactive antimicrobial compounds with potential role in maintaining the quality of fresh juice and milk as a food additive. Further studies are currently recommended to evaluate other activities of this product.

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