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FULL LENGTH ARTICLE

Bioactive phthalate from marine *Streptomyces ruber* EKH2 against virulent fish pathogens

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Abstract Marine *Streptomyces ruber* EKH2 isolated from sediments of Bardawil Lake, Egypt, was found as a promising strain for producing bioactive metabolite(s) working against some virulent fish pathogens. Some biochemical and morphological characterizations of marine *S. ruber* EKH2 were carried out. Cell free culture showed activities against the tested pathogens ranging from 15 to 30 mm. Optimized conditions for maximum activities were observed at neutrality and temperature 28 °C against the tested strains. Two grams of the ethyl acetate crude extract from 10 L culture supernatant was chromatographically separated into three fractions and bioassayed. One major antibacterial compound was separated exhibiting MIC average 12.5 μg/ml. Phthalic acid was structurally suggested on the basis of gas chromatography–mass spectrum (GC–MS) and infrared spectrum (IR). Phthalate activities were compared with known standard antibiotics used in fish therapy and found to be superior. A slight toxicity of phthalate against brine shrimp (LC_{50} = 2800 μg/ml) was observed. Dealing with pan-drug resistant bacteria in fish therapy, this study confirmed that marine *S. ruber* EKH2 is potentially used for extracting phthalic acid as a novel bioactive and non-toxic agent for treating bacterial fish infections.

Introduction

Over the last decades, world aquaculture industry has obviously grown. This development has been represented by a transition that involves a lot of work in farming methods which encourages an increased profitability (Food, 2012). Facing the fact, an increased threat of many diseases caused by a vast number of virulent microbes resulted in considerable economical losses. In order to develop rapid diagnostic tests and effective disease precautions, all research activities have been carried out with prevention strategies (Frans et al., 2013).

Recently, new therapeutic agents have entered the clinical area, with side effects (Rajan and Kannabiran, 2014). Side effects of existing drugs and drug resistance have become serious public health problems for both human and animals which require the development of new antimicrobial agents (Urban et al., 2003). Many scientists are working on new antimicrobial drugs, mainly of actinomicetal origin (Oskay et al., 2004; Paterson et al., 2004), where unique chemical structures obtained from actinomycetes are considered as

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antimicrobial, anti-parasitic, antiviral, antitumor activities and cytotoxic metabolites (Kekuda et al., 2010). Until recently, actinomycetes isolated from marine sediments, as the least explored resources, showed to be a source of bioactive compounds production; however, today it became one of the more promising sources.

Species of *Streptomyces* as versatile producers of new secondary metabolites from different biosynthetic pathways, originate from different ecological niches and could be used to hunt for novel bioactive compounds (You et al., 2013). Marine ecosystems with a large diversity of actinomycetes have the power of producing bioactive secondary metabolites (Olano et al., 2009a). Regarding secondary metabolic production from microorganisms isolated from the deep sea, it has been found to remain normally dormant or weakly expressed under laboratory conditions, which was critical for metabolite development prior to extensive chemical investigation (Ochi and Hosaka, 2013).

Bioactive compounds from marine actinomycetes of unique structure and obvious effect have been obtained; however, how to tap these treasurous compounds? (Li et al., 2013). Olano et al. (2009b) suggested that *Streptomyces* isolates from marine sediments are valuable for the production of antibiotics. Compounds synthesized by most *Streptomyces* species with broad spectrum features, including antibiotics, pesticides, herbicides, enzyme inhibitors and anti parasitic, are more importance to tend healthily and commercially effective drugs, and occupy approximately one third of the known isolated metabolites from *Streptomyces* (Balagurunathan et al., 2010).

Therefore, the purpose of this study is to evaluate, *in vitro*, the antagonistic activity of a bioactive metabolite from marine *Streptomyces ruber* EKH2 against some virulent fish pathogens. Moreover, the study would extend to separate, analyze and characterize the crude extract using thin layer chromatography, and be identified by gas chromatography–mass spectral (GC–MS) and infrared spectroscopic analysis.

Materials and methods

Isolation and characterization of S. ruber ekh2

Marine *S. ruber* EKH2 was isolated from Bardawil Lake sediment samples, Sinai Peninsula, Egypt, and identified by Xcelris Genomics labs in India. Luria–Bertani medium (LB), used for isolation, consisted of (g/l sea water); tryptone, 10; yeast extract, 5 and sodium chloride, 10, agar 20; pH adjusted at 7 and incubation temperature at 30 °C (Kandpal et al., 2012). Microscopic and morphological examinations were noted with respect to type of cells, aerial mycelium color, nature of colony and reverse side plate color.

Fish pathogens

The fish pathogens including *Aeromonas hydrophila*, *Edwardsiella tarda, Pseudomonas aeruginosa* and *Vibrio ordalii* employed for *in vitro* antimicrobial assay were kindly provided from the Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Alexandria University. The tested pathogens were stored in glycerol at −20 °C.

Fermentation condition and Bioactivity

One hundred milliliter of the production LB broth medium was inoculated by 2×10^7 CFU/ml spores suspension. Flasks were kept for 5 days at 120 rpm shaker speed and 30 °C (Deepika and Kannabiran, 2010). Fermented medium was collected and centrifuged from cell debris for further use as the crude extract.

Bioactivities were screened using disc diffusion method on tryptic soya agar (TSA) (Bauer et al.,1966). Twenty-five milliliter of sterilized TSA medium were mixed with 400 µl of over- night culture of tested organism (10^4 CFU/ml), then poured into three sterile Petri-dishes, as replicates, and allowed to solidify. Sterile plain discs (5 mm) were immersed in free cell filtrate of the cultivated *S. ruber* EKH2, and placed on the prepared plates then incubated at 30 °C overnight.

Extraction of bioactive compounds

For the extraction of the bioactive metabolites from fermented broth, different organic solvents (50 ml each) from polar (butanol, diethyl ether and ethyl acetate) to non-polar (benzene, hexane and petroleum ether) were tried for product recovery from 50 ml fermented broth in a 250 ml separating funnel. The mixture was shaken vigorously for 20 min and kept in stationary condition for another 20 min to separate the solvent from aqueous phase (Atta and Ahmad, 2009).

Minimal inhibitory concentrations (MIC) of the ethyl acetate extracted compound(s) were determined according to Richard et al. (2007). For maximum conditional activity, MIC was recorded at different temperatures (28, 37 and 45 °C) and pHs (6, 7, 8) in three replicates using representative response surface plot curves, STATISTICA (ver. 8, ed. 2006).

Thin layer chromatography

The culture broth (10 L) was extracted with ethyl acetate (1:1 v/v) stepwise and concentrated by rotary evaporator at 50 °C to yield 2 g of brown crude residue. The residue was chromatographed on silica gel preparative slides using different solvent systems: acetone, hexane and ethyl acetate, separately, or combined solvent systems; acetone:ethyl acetate (1:2), acetone:ethyl acetate. (2:1), hexane: ethyl acetate (1:2), and hexane: ethyl acetate (2:1). The starting crude spots were observed for migration and separation by the previously prepared mobile phases. R_f values of the obtaining colored and non-colored spots with the aid of visible and UV lamps were recorded (Usha et al., 2010). Using silica gel plates (20×20 cm dimensions and 0.50 mm thickness of 60GF254 fine grade), the active bands were gathered, dissolved in ethyl acetate and concentrated to dryness in the vacuum. Bioactivity of the selected bands was examined against the four pathogens using the broth dilution bioassay.

Spectral analysis

Gas chromatography–mass spectrum

The active fraction was analyzed using the SHIMADZU GC–MS-QP5050A with programm CLASS 5000 in the Central Lab of the Higher Institute of Public Health.