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In vitro, optimization of antibacterial activity of secondary metabolites produced by endophytic fungus *Stemphylium radicinum* in Iraq

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Abstract:

The aim of this study was to optimize cultural conditions for optimum bioactive metabolite production by endophytic fungus Stemphylium radicinum, isolated by surface sterilization method of Iraq. From root of calyptous plant .The fungus was identified as Stemphylium radicinum based on morphological characterization. Fungal secondary metabolites were carried out by ethyl acetate solvent. The antibacterial activity was tested against two strains of bacteria, Escherichia coli (ATCC 25922) and Staphylococcus aureus (NCTC 6571)by using a disc diffusion technique The effect of various carbon and nitrogen sources, pH, incubation period, and temperature on the bioactive metabolite production in a fixed volume of culture broth were studied. Bioactive metabolite production of endophytic fungus S. radicinum which exhibits a broad spectrum of in vitro antimicrobial activity against two strains of bacteria. Dextrose and yeast extract were found to be a best and most suitable carbon and nitrogen sources respectively, for the optimum production of bioactive metabolites. Maximum bioactive metabolite productions occur in pH of 6 and temperature at 25°C, incubation period was 10 days and NaCl showed positive influence on bioactive metabolites.

Key words: Agar disc diffusion assay, Antibacterial, Secondary metabolites, Optimize cultural**.**

1. INTRODUCTION

Fungi are considered as a good natural source for a production of bioactive secondary metabolites that contain different bioactive agents including antibiotics, anti-tumors, and antioxidants [1]. Endo-phytes are microbes that colonize living, internal tissues of plants without causing any harmful, overt negative effects [2]. Currently, endophytic fungi are viewed as an outstanding source of bioactive natural products because there are so many of them occupying literally millions of unique biological niches (higher plants) growing in so many unusual environments. Of the approximately 300 000 higher plant species that exist on the earth, each individual plant, of the millions that exist here, is host to one or more endo-phytes [3]. Secondary metabolism is regulated by carbon sources, nitrogen sources, phosphate, trace elements, precursors, induction of enzymes of secondary metabolism, catabolic repression and inhibition, feedback repression and inhibition and it controlled by auto-regulators [4]. Many of the microbes live in extreme environments such as high temperatures, high salt concentrations, low pH, and high radiation. Some of the physical factors also influence the fungal growth and metabolite productions. Usually the biotechnological production from microorganisms based on their special adaptations to their environment. Many new and interesting bioactive metabolites such as antibiotics, antiviral, anticancer and antioxidant compounds having pharmaceutical, industrial and agricultural importance are isolated and characterized from soil fungi [5]. Most of studies referred that the biosynthesis of fungal secondary metabolites is affected by different ecological factors and cultural conditions [6].

2. MATERIALS & METHODS

Sample collection, isolation and characterization of fungi

The roots of calyptus collected from Maysan city south of Iraq. Healthy roots were collected and processed separately within 48 h of collection. The root samples were surface sterilized by [7]. The surface sterilized roots segments were evenly spaced in Petri dishes containing potato dextrose agar (PDA) medium. The Petri dishes were sealed using Para-film TM and incubated at 26°C in alight chamber with 12 hours of light followed by 12 hours of dark cycles. The Petri dishes were monitored every day to check the growth of endophytic fungal colonies from the root segments. Identified of fungus was confirmed according to the available taxonomic literature.

Microbial target organisms

Two strains of bacteria, *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (NCTC 6571) were kindly provided by Microbiology Laboratory in the College of Science University of Maysan.

Fungal metabolite extraction

The fungal culture filtrate was extracted three times with 1:1 (volume) ethyl acetate using a separating funnel. The organic layer was collected and dehydrated with Na2SO4.

Optimization of culture conditions for the production of bioactive metabolite production

Basal medium

Potato broth medium with or without was used as a basal medium to determine the optimum conditions for antimicrobial bioactivity exhibits by *S. radicinum*. 500 ml conical flasks

containing 250 ml P broth supplemented with 1 % (w/v) of different carbon or/ nitrogen sources and sterilized.

Effects of carbon sources on production of bioactive metabolite production

Various carbon sources (Dextorse, Galactose, Glucose, Mannose, Maltose, Starch and Sucrose) were separately amended into TS broth medium at 1% (w/v) using 250 ml of medium in 500 ml conical flasks. Each flask was inoculated with three discs (5 mm diameter) taken from the fungal colony grown on PDA in Petri dish. Cultures were incubated at 25 ◦C for 10 days.

Effects of nitrogen sources on production of bioactive metabolite production

Different nitrogen sources (Asparagine, Peptone, Yeast extract (YE), Malt extract (ME), NaNO3, NH4Cl and (NH4)2 SO3 were separately amended into TS broth medium at 1% (w/v) using 250 ml of medium in 500 ml conical flasks. Each flask was inoculated with three discs (5 mm diameter) taken from the fungal colony grown on PDA in Petri dish. Cultures were incubated at 25 ◦C for 10 days.

Effect of pH on production of bioactive metabolite production

The effect of pH on bioactive metabolite production of the isolate was tested in the laboratory using liquid cultures containing different pH levels (4, 5,6,7,8 and9).

Effect of temperature on production of bioactivity metabolite production

The fungus was subjected to different temperature (15, 20, 25, 30 and 35°C) to study the optimum temperature required for bioactive metabolite.

Determination of incubation period

The fungus was Incubation periods ranging from7 to 26 days were used to determine the effect of incubation period on the active metabolite.

Effect of NaCl concentration on biomass and bioactive metabolite production

The effect of salinity on bioactive metabolite produced by the isolate *S. radicinum was* carried out by incubating in various NaCl concentrations, ranging from 3-7% with 1% of carbon and nitrogen source while other parameters were kept at optimum level. The bioactive metabolite production for each sodium chloride concentration were estimated and recorded.

Fungal metabolite extraction

The fungal culture filtrate was extracted three times with 1:1 (volume) ethyl acetate using a separating funnel. The organic layer was collected and dehydrated with Na2SO4 [8].

***In vitro* screening of antimicrobial activity**

To determine the antibacterial bioactivity of the fungal extract, a filter paper disc diffusion technique was employed. Petri dishes containing Muller Hinton Agar was prepared and bacterial suspension was made with normal saline containing 1 $x10^6$ cells per ml [9].

Minimal inhibitory concentration.

The minimal inhibitory concentration (MIC) values were determined by the standard serial dilution assay [10]. The inhibitory test was carried out on Muller-Hinton agar medium.

3 RESULTS & DISCUSSION

The production of antibacterial metabolites determined by disc diffusion assay method measuring the zone of inhibition against two strains of reference bacteria*, Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (NCTC 6571). Therefore, further experiments on optimization of cultural conditions for improved bioactive metabolite production. In the last decades more attention has been given to explore new bioactive compounds from fungi as natural sources [11]. In the present study, cultural conditions like pH, temperature, carbon and nitrogen sources were subjected to optimization as they influence bioactivity metabolite.

Effect of carbon source on antibacterial metabolite for *S. radicinum*. (Fig. 1) Among the carbon sources, Dextrose proved to be the best carbon source for antimicrobial metabolites production by the FUNGUS with inhibition zone 22.0, 24.0 mm against *E. coli* and *S. aureuse* respectively. Glucose also gave a similar pattern result followed by Sucrose, Starch and Mannose respectively. No antibiotic was produced when the medium was supplemented with galactose. Carbohydrates are known to have interference with the production of secondary metabolites [12].
Different types of nitrogen sources affected the production of secondary metabolite by *fungus*. (Fig. 2) Maximum antimicrobial activity was obtained when media was supplemented with Yeast extract with inhibition zone 20.5, 22.5 mm against *E. coli* and *S. aureus* respectively followed by (NH4)2SO3, NH4Cl, Asparagine, Peptone, Malt extract and NaNo3. Nonetheless, it has been stated that manipulating of nutritional factors would promote the biosynthesis of secondary metabolites by microorganisms [13].

The effect of pH on antimicrobial metabolites production by the fungus is presented in (Fig. 3). The optimum pH for antibacterial metabolite production was 6.0 with inhibition

zone 22.0, 20.0 mm against E. coli and S. aureuse respectively, [14] pointed out the most of the microorganisms have the ability to synthesis antimicrobial compounds at pH ranging from 5.5 to 8.5.

The *S. radicinum*, showed a narrow range of incubation temperatures for bioactive metabolite (Fig. 1). The increase of the incubation temperatures from 25 to 30C enhanced of bioactive metabolite. Maximum inhibition zone 22.0, 22.5 mm against *E. coli* and *S. aureuse* respectively was recorded at 25C. However, lowest inhibition zone was observed at low temperature of 15C (Fig. 4).

Effect of incubation period on the production of bioactive metabolites by *S. radicinum* was investigated. They observed that the production of metabolite commenced after 10 days (Fig. 5). [15] Observed that periodic production of culture flask is an essential parameter for optimum biosynthesis of antibiotic. NaCl concentration of 5 g/l was recorded as optimal for active metabolite production (Fig. 6)

Fig. 1. Effect of different carbon sources on bioactive metabolite production by *S. radicinum*

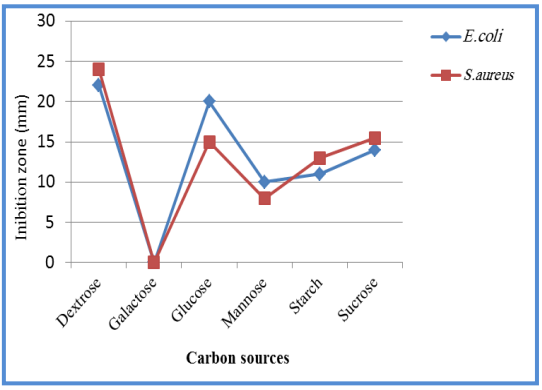


Fig. 2. Effect of different nitrogen sources on bioactive metabolite production by *S. radicinum*

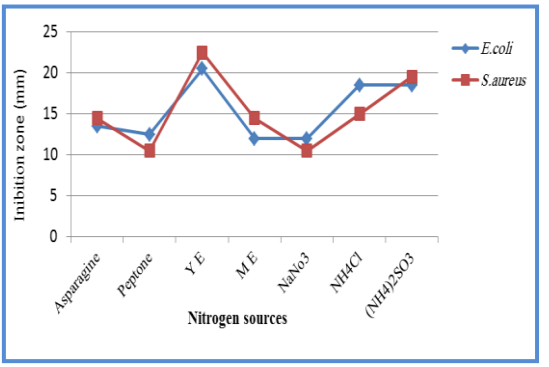


Fig. 3. Effect of pH of the medium bioactive metabolite by *S. radicinum*

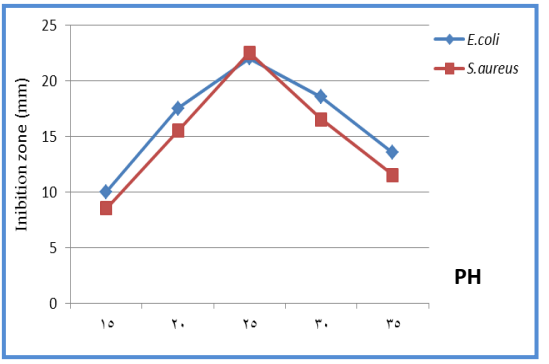


Fig. 4. Effect of temperature on bioactive metabolite by *S. radicinum*

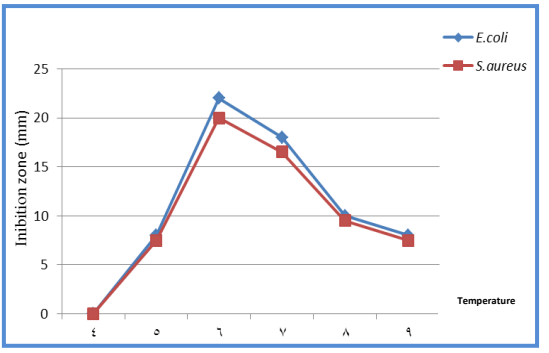


Fig. 5. Effect of incubation period on bioactive metabolite by *S. radicinum*

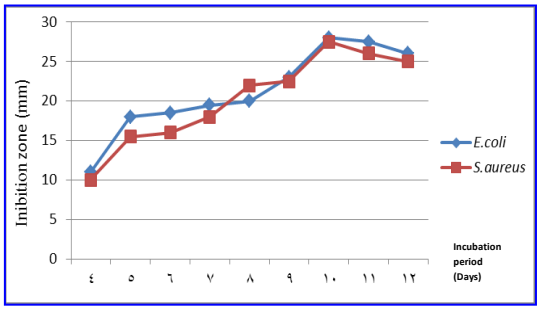
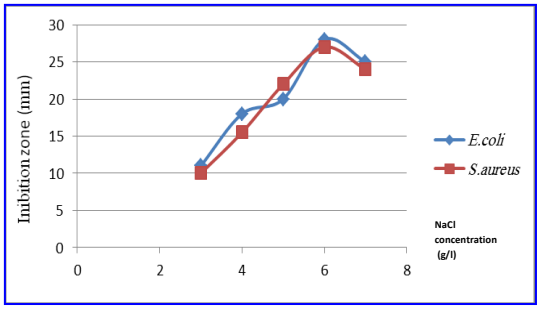


Fig. 6. Effect of NaCl concentration on bioactive metabolite by *S. radicinum*



5 CONCLUSION

In the present study, the antibiotics produced by *S. radicinum* grown under optimized conditions exhibited good antibacterial activity against gram positive, gram negative bacteria.

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