RESEARCH / INVESTIGACIÓN

Optimization of cultural conditions affecting improved bioactive metabolite production by endophytic fungus *Trichoderma harzianum*

Rashid Rahim Hateet[1*], Zainab Alag Hassan[2], Abdulameer Abdullah Al-Mussawi[2] and Shaima Rabeea Banoon[1]

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Abstract: The present study aimed to optimize cultural conditions for optimum bioactive metabolite production by endophytic fungus *Trichoderma harzianum*, isolated by surface sterilization method from the leaf of the eucalyptus plant. The fungus was identified based on morphological characterization. Fungal metabolites were carried out by ethyl acetate solvent. The antibacterial activity was tested against *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (NCTC 6571). Various carbon, nitrogen sources, pH, temperature, incubation period, and NaCl on the antibacterial metabolite production were studied. Bioactive metabolite production of *T. harzianum* exhibits a broad spectrum of in vitro antibacterial activity against two strains of bacteria. For the optimum production of bioactive metabolites, Dextrose and Glucose were found to be the best sources of carbon and the best sources of Nitrogen Yeast extract (YE) and (NH4)2SO. The maximum production of bioactive metabolites occurs at pH 7 and 25°C.; the NaCl showed a positive influence on bioactive metabolites.

Key words: Endophytic fungus, *Trichoderma harzianum*, Optimum conditions, bioactive metabolite, eucalyptus.

Introducción

The genus *Trichoderma* Pers. consists of species with agricultural, biotechnological, and industrial benefits[1]. The first complete genus description date back to 1969[2] and comprised nine species complexes grouping; species morphologically inseparable but genetically diverse. Recent advances in *Trichoderma* taxonomy have brought the present system of more than two hundred biologic species, supported by the molecular analysis of the variable genomic DNA regions with phylogenetic and taxonomic significance[3]. *Trichoderma* inhabits the root, soil, and foliar environments are highly interactive and free-living fungi and successfully used to control many crop pathogens in field trials[4]. Endophytes are microbes, which colonize plants' internal tissues without causing harmful, apparent adverse effects[5]. Complex interactions with host fungi and numerous endophytic fungi are beneficial for their hosts in many ways, including promoting host growth and nutrient gain and enhancing host resistance to phytopathogens, pests or abiotic stress[6]. *Trichoderma* spp. produce secondary metabolites with various biological activities affecting plant metabolism[7]. Endophytic fungi are now regarded as an outstanding source of bioactive metabolites because so many of them are cultivated in unusual environments, occupied by millions of unique biological niches (higher plants)[8,9]. Of the 300,000 higher plant species on the earth, one or more endophytes are present in each plant, of the millions which exist here[10]. Fungi are an excellent natural source of bioactive secondary metabolites production containing several bioactive agents, including antibiotics, antitumor, antidiabetic, and antioxidants[11-16]. The exploration of natural resources to new antimicrobial compounds has become more and more relevant.

Nevertheless, natural products provide important drug sources used in various fields of therapy[17]. Auto-regulators administer secondary metabolism by carbon, nitrogen, phosphate sources, trace elements, precursors, secondary metabolism induction enzymes, catabolism suppression and inhibition, and feedback suppression[18]. Numerous microbes live in extreme environments, such as high temperatures, high salt concentrations, low pH, and high radiation. Fungal growth and metabolite production also influence some physical factors. The biotechnological production of microorganisms usually depends on their specific environmental adaptations. The production of bioactive and antimicrobial agents, many new and exciting bioactive metabolites such as antibiotics, antivirals, and antioxidants have pharmaceutical, industrial and agricultural importance, isolated and characterized from soil fungi which affected by the physical and chemical parameters such as pH, temperature, incubation period, carbon and nitrogen and amino acid sources[8]. The majority of the study indicated a different ecological and cultural factor influencing the biosynthesis of secondary fungal metabolites[19]. This study aimed to assess the optimal cultural conditions for producing bioactive metabolites by *Trichoderma harzianum* endophytic fungus.

Materials and methods

Sample collection

The *Euclyptus camaldulensis* leaf from the southern province of Maysan in Iraq. 48 hours after collection, The surface sterilization of the leaf was based on (20).

Isolation and characterization of fungi

In Petri dishes containing medium potato dextrose agar (PDA), the segments of surface-sterilized leaf were evenly separated. In the light chamber with 12 hours of light and 12 dark hours, the dishes of Petri were sealed by Parafilm and incubated at 26°C. The Petri dishes were monitored every day to monitor the growth of endophytic fungal colonies from the leaf segments. Identification was made using Rifai's identification keys[2] and Bissett[21-23].

Microbial target organisms

Staphylococcus aureus (NCTC 6571) and *Escherichia coli* (ATCC 25922) standard test bacteria used in the current study.

[1] Department of Biology, College of Sciences, University of Misan, Amarah, Maysan, Iraq.
[2] College of Nursing, University of Basrah, Basra, Iraq.

Corresponding author: biorashed@uomisan.edu.iq

Cultivation was sub-cultured on a nutrient agar medium before the antibacterial test (Oxoid, England).

Fungal metabolite extraction

The filtrate for fungal culture was extracted three times using a separating funnel with 1:1 (Vol) ethyl acetate. The organic layer has been collected and dehydrated with Na_2SO_4.

Antibacterial activity

A filter paper disc diffusion technique[24] was used for determining the antibacterial bioactivity of the fungal extract. Petri dishes were prepared, and the bacterial suspension containing 1 x 10^6 cells per ml of Muller Hinton Agar[25].

Minimal inhibitory concentration.

The standard serial dilution analysis determined the minimum inhibitory concentration (MIC) values[26]. The inhibitory test was carried out on the Muller-Hinton agar medium.

Optimization of culture conditions for the production of bioactive metabolite

Basal medium

Potato broth medium with or without was used as a basal medium to determine the optimum conditions for bioactivity exhibited by *T.harzianum*. Erlenmeyer flasks (500 ml) containing 250 ml Potato broth supplemented with 1 % (w/v) of different carbon or/ nitrogen sources and sterilized.

Effects of carbon sources

Various carbon sources (Dextrose, Galactose, Glucose, Mannose, Starch, and Sucrose) have been amended separately into Tryptic Soy Broth medium at 1% (w/v) using 250 ml of medium in conical flasks. Each flask was inoculated with three discs (5 mm diam) taken from the fungal colony grown on PDA in a Petri dish. Cultures were incubated at 25ºC for 10 days.

Effects of nitrogen sources

Different nitrogen sources (Asparagine, Peptone, Yeast extract (YE), Malt extract (ME), $NaNO_3$, NH_4Cl, and $(NH_4)_2$ SO_3 were separately amended into Tryptic Soy Broth medium at 1% (w/v) using 250 ml of medium in 500 ml flasks. The three discs (5 mm diam) taken from the fungal colony grown on PDA in the petri dish were inoculated. Cultivations have been incubated for 10 days at 25°C.

Effect of pH

Effect pH for detecting the bioactive product metabolite has been tested in the laboratory with liquid cultures containing different pH levels (4,5,6,7,8 and 9).

Effect of temperature

The fungus has been exposed to different temperatures to investigate the best temperature required for the bioactive metabolite (15, 20, 25, 30, and 35°C).

Determination of the incubation period

The impact of the incubation on the active metabolite was calculated using incubation periods ranging from 7 to 26 days.

Effect of NaCl concentration

Incubation in various NaCl concentrations, the effect of salinity on the bioactive metabolite of *T.harzianum* isolate has been performed, from 3-7% to 1% of carbon and nitrogen sources, while other parameters have remained optimum. The production of bioactive metabolite has been calculated and recorded for each level of sodium chloride.

Results and discussion

The present study results showed a significant effect on the bioactive production of *T.harzianum* metabolites in different microbiological cultural conditions. The production of antibacterial metabolites was determined by the disc diffusion assay method measuring of inhibition zone against two strains of reference bacteria, *E. coli* (ATCC 25922) and *S. aureus* (NCTC 6571). Further experiments on cultural optimization conditions to enhance bioactive metabolite production are therefore underway. In the previous decades, more attention has been paid to new bioactive compounds from fungi that have become a natural source[27]. Cultural conditions such as pH, temperature, carbon, nitrogen sources, and NaCl concentration were optimized as they affect the metabolite of bioactivity in this study.

Effect of carbon source on antibacterial metabolite for *T.harzianum*. (Figure 1) Among the carbon sources, dextrose proved to be the best carbon source for antimicrobial metabolites produced by the fungus, with an inhibition zone 18.0 mm against *E.coli* and 20.0mm against *S.aureus*. Glucose also gave a similar pattern result followed by Sucrose, Starch, and Mannose, respectively, which agrees with (28). No antibiotic was produced when the medium was supplemented with galactose, and Carbohydrates are known to interfere with the production of secondary metabolites[29]. In addition to CO2, water, and energy, the production of intermediates, which produce primary and secondary metabolites is affected by simple carbohydrates like glucose and dextrose through metabolic pathways[30]. The addition of glucose resulted in the highest fungal growth but significantly reduced the production of bioactive metabolites. A higher concentration of glucose affects suppressively the production of bioactive metabolites in many fermentation processes[31]. It should be noted that the filamentous fungi are ubiquitous organisms able to obtain energy from very different substrates[32].

Figure 2; shows the effect of various nitrogen sources on *T. harzianum* production of bioactive metabolites. Maximum antimicrobial activity was obtained when media were supplemented with yeast extract with inhibition zone 18.5mm against *E.coli* and 20.5 mm against S.aureus followed by $(NH_4)_2SO_3$, NH_4Cl, Asparagine, Peptone, and $NaNO_3$. However, manipulation of nutrient factors has been stated to promote the biosynthesis by microorganisms of secondary metabolites[33].

The effect of pH on antimicrobial metabolite production by the fungus is presented in Figure 3. The optimum pH for antibacterial metabolite production was 7.0 with an inhibition zone of 18.0 mm against *E.coli* and 16.0 mm against *S. aureus*; (34) have noted that the most significant number of microorganisms can synthesize pH-based antimicrobial compounds between 5.5 and 8.5.

The influence of temperature is presented in Figure 4. The *T.harzianum*, showed a narrow range of bioactive metabolite incubation temperatures. The increase of the incubation temperatures from 25 to 30ºC enhanced bioactive metabolite. Maximum inhibition zone 20.0mm against *E.coli* and 20.5 mm against *S.aureus* was recorded at 25C. This indicates the stra-

in was strictly mesophilic for secondary metabolites production. However, the lowest inhibition zone was observed at a low temperature of 15ºC. These results are compatible with (14).

The influence of the incubation period on bioactive metabolite production of the isolate is presented in Figure 5. The production of metabolite increased and reached its maximum levels after 11 days and after that gradually decreased; this agrees with (14).

The influence of NaCl concentration on bioactive metabolite production of the isolate is presented in Figure 6. NaCl concentration of 6g/l was recorded as optimal for bioactive metabolite production.

Conclusions

This study showed good antibacterial activity against gram-positive and gram-negative bacteria produced by *T.harzianum* grown in optimized conditions.

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Conflicts of interest

The author declares that no conflict of interest exists.

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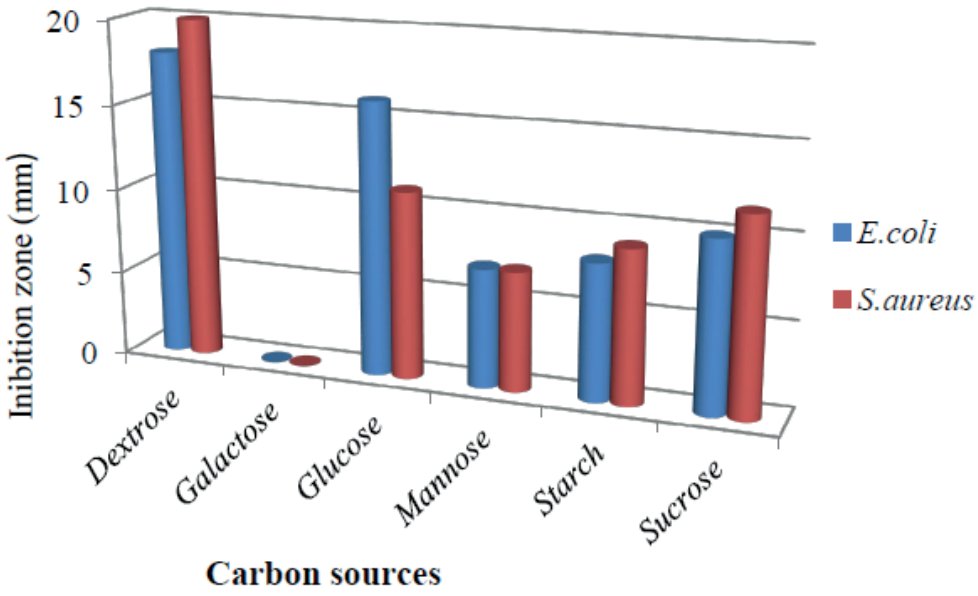

Figure 1. Effect of different carbon sources in the medium on bioactive metabolite by *T. harzianum*.

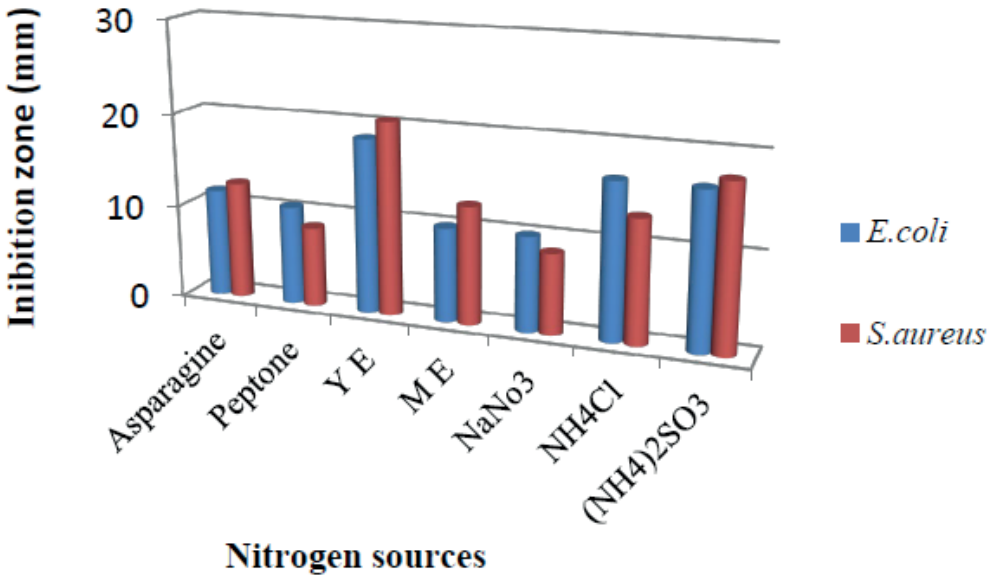

Figure 2. Effect of different nitrogen sources in the medium on bioactive metabolite by *T. harzianum*.

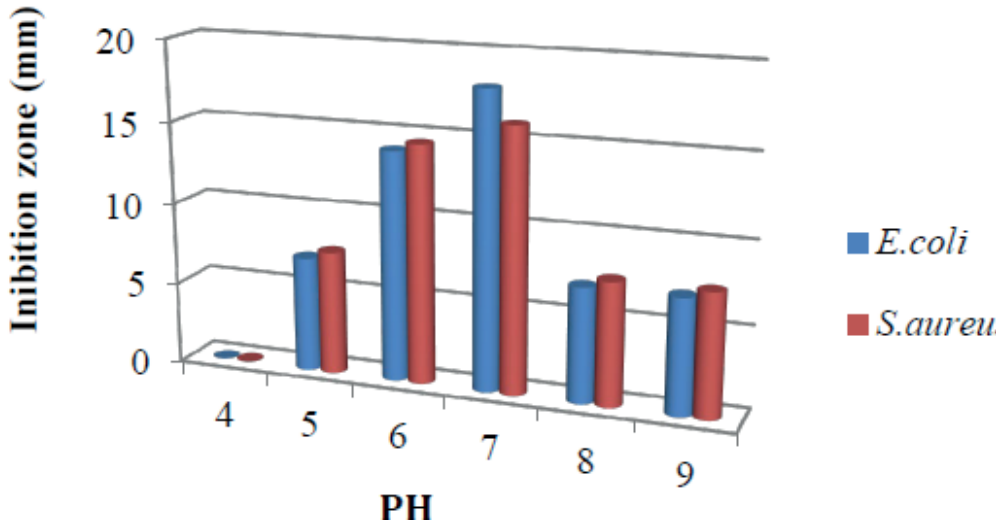

Figure 3. Effect of pH of the medium on bioactive metabolite by *T. harzianum*.

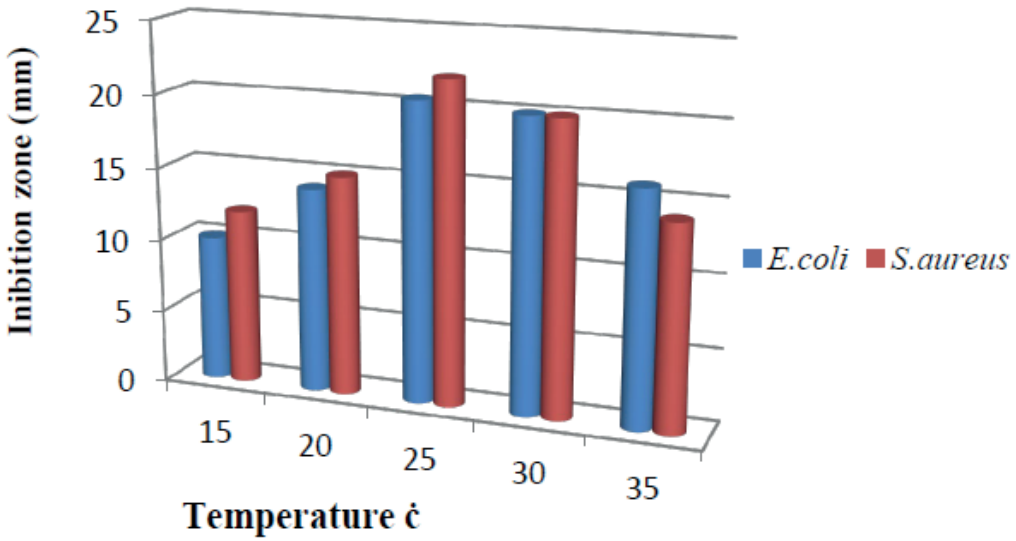

Figure 4. Effect of temperature on bioactive metabolite by *T. harzianum*.

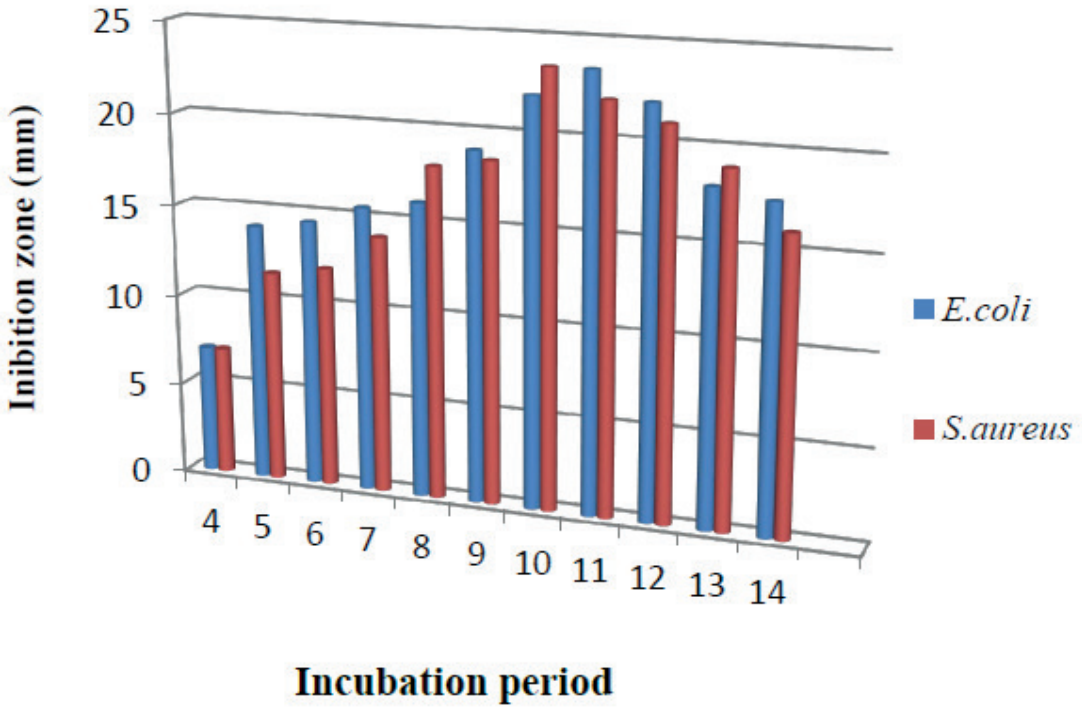

Figure 5. Effect of incubation period on bioactive metabolite by *T.harzianum*.

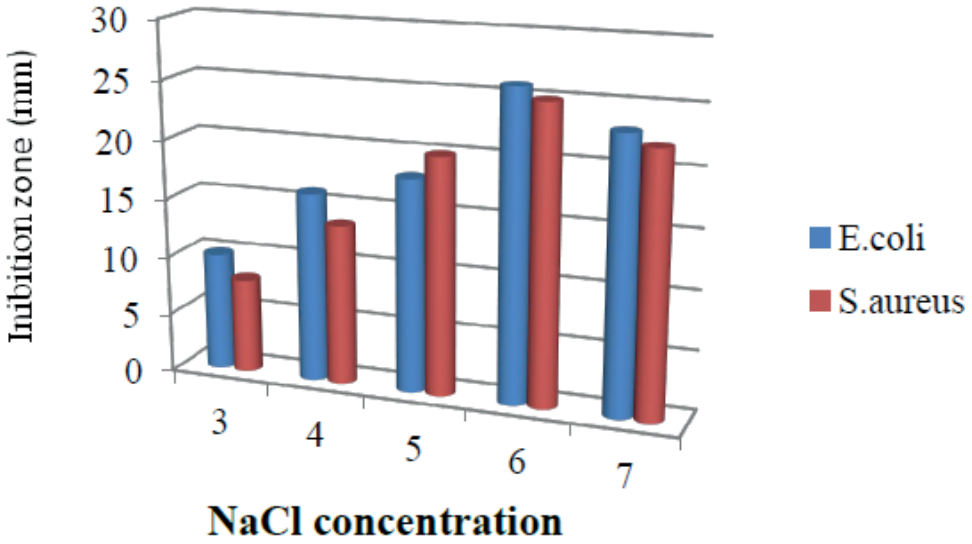

Figure 6. Effect of NaCl Concentration in the medium on bioactive metabolite by *T. harzianum*.

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