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***In vitro*, optimization of growth and bioactivity of antibacterial metabolite produced by *Rhizoctonia solani* Kuhn**

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Abstract

The aim of this study was to optimize the cultural conditions for optimal fungal growth and metabolic bioactivity of *Rhizoctonia solani* isolated from potato tubers in Basra, Iraq. Different culture media, temperatures, pH and carbon and nitrogen sources were used to elucidate their effects on the fungal growth response and bioactivity of the fungal metabolite against two bacteria strains *E. coli* and *S. aureus*. *R. solani* grew better on Trypto Soyo broth medium at pH 6 and temperature 30 C. Starch (C- source) and NaNO₃ (N-source) were most suitable for the fungus growth. Highest dry weight of fungal crude extract was obtained by using TS broth medium at pH 6 and incubation temperature 30 C. A disc diffusion technique was applied to examine the bioactivity of fungal crude extract against two strains of bacteria *E. coli* and *S. aureus*. The fungus exhibited higher antibacterial metabolic bioactivity (30 mm and 34 mm inhibition zones diam) against *E. coli* and *S. aureus*, respectively, by using TS broth medium amended with starch and (NH₄)₂SO₃ at pH 6 and 25 C temperature.

Key words: Antibacterial bioactivity, optimization of growth conditions, *Rhizoctonia solani*
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1. Introduction

There is an increase research interest to explore natural resources for new antimicrobial bioactive compounds. Nevertheless, natural products represent important sources of drugs used in different therapeutic fields [1]. However, fungi are considered as a good natural source for a production of bioactive secondary metabolites that contain different bioactive agents including antibiotics, antitumors, and antioxidants [2-4]. Most of studies referred that the biosynthesis of fungal secondary metabolites is affected by different ecological factors and cultural conditions [5-8]. Previous investigations reported that several antibacterial and antifungal bioactive compounds were isolated from different fungal species [9,10]. Nonetheless, most of the those investigations dealt with the Basidiomycetous fungal species that exhibit bioactive chemical components

against pathogenic bacteria and fungi [2,11-14]. Among the fungi, *Rhizoctonia solani* is widely distributed over the world and is considered as an important causative fungal pathogen of various plants hosts causing different plant diseases (seed decay, seedlings damping off, leaves foliar blight, root rot, and other plant diseases [15]. So far, as we aware from the literature, no data available regarding the production of bioactive metabolites by the *R. solani* with the exception of the report by Ma et al. [16] who isolated some bioactive compounds from endophytic species of *Rhizoctonia* as antibacterial agents against some strains of *Helicobacter pylori*. The aim of this study is to optimize the growth and bioactivity of metabolite produced by *R. solani* isolated from potato tubers against two strains of pathogenic bacteria.

2. Materials and methods

2.1. Fungal isolate

R. solani was isolated from infected potato tubers in Basrah (Iraq) and was grown on PDA medium (Oxoid, India) and maintained in slant tubes at 4 °C for further investigations.

2.2. Bacteria strains

Two strains of bacteria, *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (NCTC 6571) were kindly provided by the Microbiology Laboratory, College of Science, University of Basrah. They were stored in nutrient agar slants and kept at 4°C.

2.3. Fungal growth conditions

2.3.1. Effects of synthetic media on fungal growth

Five synthetic broth media were used including Potato Dextrose (PD), Czapeck's Dox (CD), Rose Bengal (RB), Trypto Soyo (TS) and Malt Extract (ME) to examine the effects of media on the growth response of *R. solani*.

250 ml of each broth medium was prepared in 500 ml conical flasks and each flask was inoculated with three discs (5 mm diam) taken from the fungal colony grown on PDA in Petri dish. Flasks were incubated at 25 °C for 10 days on a rotary shaker, then the mycelia were harvested by filtration on Whatman filter paper No. 1 and dried in an oven at 40 °C for 48 hr to determine the mycelia dry weight.

2.3.2. Effects of temperature on fungal growth

Growth of *R. solani* was examined at different temperatures (10, 15, 20, 25, 30, and 35 °C) using 250 ml TS broth medium in 500 ml conical flasks, each flask was inoculated with three discs (5 mm diam) taken from the fungal culture grown on PDA medium. Fungal cultures were incubated at 25 °C for 10 days on a rotary shaker and the mycelia dry weights were determined as above.

2.3.3. Effects of pH on fungal growth

Effect of different pH (4, 5, 6, 7, 8 and 9) on the growth of *R. solani* was examined. Fungal cultures were prepared as above, incubation was conducted at 25 °C for 10 days on a rotary shaker and the mycelia dry weights were determined.

2.3.4. Effects of carbon and nitrogen sources on fungal growth

Different carbon sources (Dextrose, Galactose, Glucose, Maltose, Starch and Sucrose) and nitrogen sources (Asparagine, Peptone, Yeast extract, 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 diam) taken from the fungal colony grown on PDA in Petri dish. Cultures were incubated at 25 °C for 10 days. Mycelia were harvested by filtration and the mycelia dry weight was determined. Three replicates were made for each treatment.

2.3.5. Fungal crude metabolite extraction

For each of the fungal growth treatment mentioned above, the fungal culture filtrate was extracted three times with 1:1 (vol) ethyl acetate using a separating funnel. The organic layer was collected and dehydrated with Na₂SO₄ then placed in Petri dishes and dried at room temperature. The dry weights of the fungal crude extracts were determined.

2.3.6. Optimization of bioactivity of fungal crude extract

Trypto Soy broth medium was used to determine the optimum nutritional and cultural conditions for maximum growth and antimicrobial bioactivity exhibits by *R. solani*. 500 ml conical flasks containing 250 ml TS broth supplemented with 1 % (w/v) of different carbon or/ nitrogen sources

separately. Each of the flasks was inoculated with three discs (5 mm diam) taken from fungal colony grown on PDA medium. Similarly, the bioactivity of fungal extract was determined using different temperatures (10, 15, 20, 25, 30 and 35 °C) and pH (4, 5, 6, 7, 8 and 9) using TS broth medium supplemented with the best carbon source with different nitrogen sources /or with the best nitrogen source and different carbon sources. All cultures were incubated for 10 days on a rotary shaker. Fungal filtrate from each treatment was extracted in 1:1 vol ethyl acetate by a separating funnel.

2.3.7. Antibacterial bioactivity assay

To determine the antibacterial bioactivity of the fungal extracts, a filter paper disc diffusion technique was employed. Petri dishes containing Muller Hinton Agar (Lab M limited ,UK) was prepared and bacterial suspension was made with normal saline containing 1 x10⁶ cells per ml. Each plate was inoculated with tested bacteria by swabbing onto the surface of agar medium. A disc (5 mm diam) was cut from the fungal colony grown on PDA and placed at the center of the agar medium. The plates were incubated at 37 °C for 24 h. The clear zones appeared around the discs indicating the growth inhibition and the inhibition zone diameter was measured. Triplicates were made and control was carried out.

2.3.8. Toxicity test

The toxicity of the fungal crude extract was tested using human blood following the described method [17].

2.3.9. Statistical Analysis

Data were analyzed using Analysis of Variance (ANOVA) between any pair of variables.

4. Results

Among the tested media, Trypto Soyo (TS) broth was the best medium that revealed a maximal growth (5 g dw) of *R.*

solani and rendered a highest metabolic crude extract production (0.28 g dw) (Fig.1).

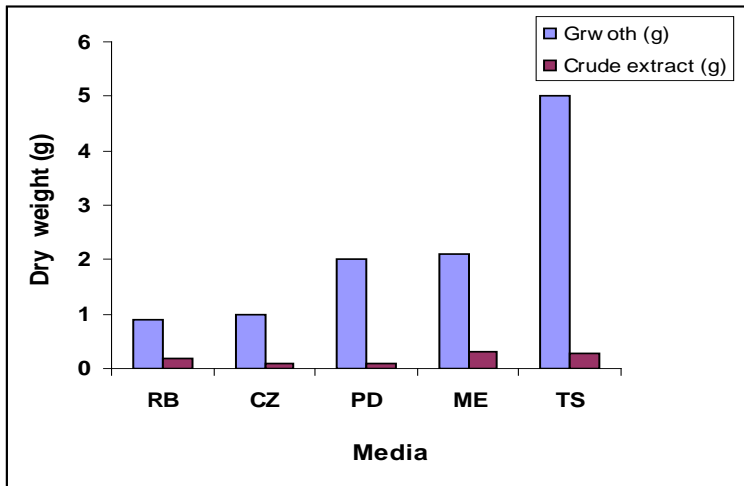


Fig.1. Effect of different broth media (Rose Bengal (RB), Czapeck's Dox (CD), Potato Dextrose (PD), Malt Extract (ME) and Trypto Soyo (TS)) on the growth and metabolite (crude extract) production of *R. solani* grown at 25 C for 10 days incubation.

Highest fungal growth (2.1 g dw) was obtained when starch (as a carbon source) was amended into the broth medium compared with other carbon sources (Fig.2),

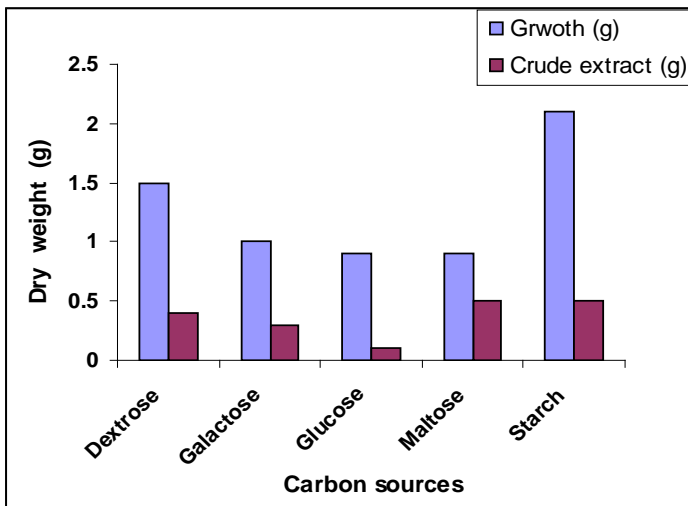


Fig.2. Effect of different carbon sources on the growth and metabolite (crude extract) production of *R. solani* grown in broth medium at 25 C for 10 day incubation.

While NaNO_3 was the most suitable for the fungal growth as compared with the examined nitrogen sources (Fig.3). Meantime, the amount of fungal crude

extracts production were maximum (0.5 g and 0.168 g dw) in broth medium supplemented with starch and NaNO_3 , respectively (Figs.2, 3).

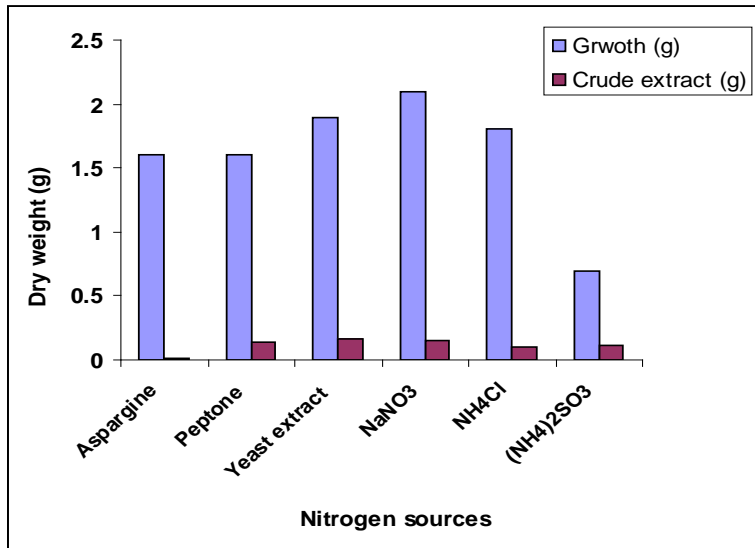


Fig.3. Effect of different nitrogen sources on the growth and metabolite (crude extract) production of *R. solani* grown in broth medium at 25 C and 10 days incubation.

R. solani exhibited higher growth and metabolite production at 30 C (Fig.4) and

pH 6 (Fig.5) when both starch and NaNO₃ were amended into the growth medium.

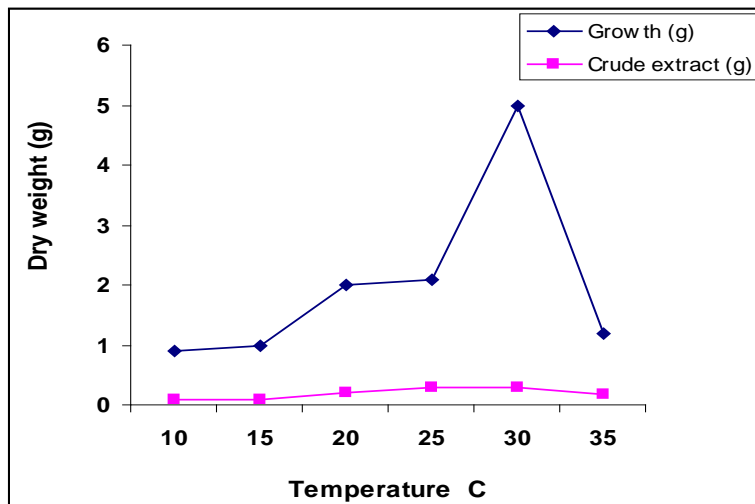


Fig.4. Effect of temperature on the growth and metabolite (crude extract) production of *R. solani* grown in broth medium amended with both starch and NaNO₃.

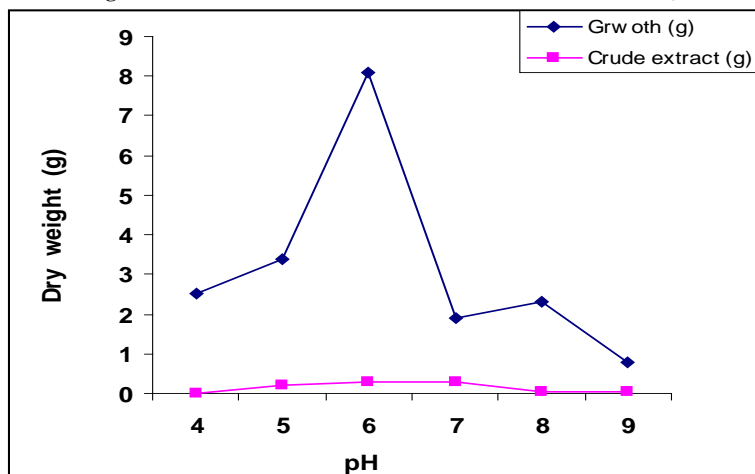


Fig.5. Effect of pH on the growth and metabolite (crude extract) production of *R. solani* grown in broth medium amended with both starch and NaNO₃.

The bioactivity assay revealed that the crude extract of *R. solani* grown in TS broth medium exhibited highest growth inhibition

(30 mm and 34 mm inhibition zone diam) against *E. coli* and *S. aureus* , respectively (Figs.6, 7).

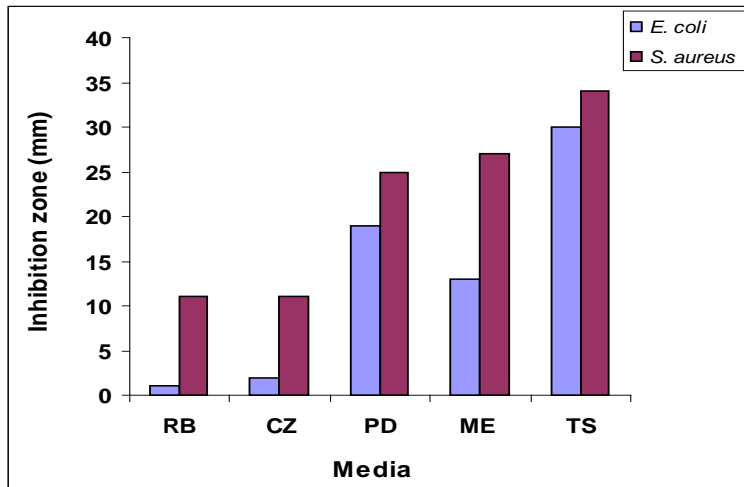


Fig.6. Effect of different media on the bioactivity of fungal crude extract of *R. solani* against two strains of bacteria.

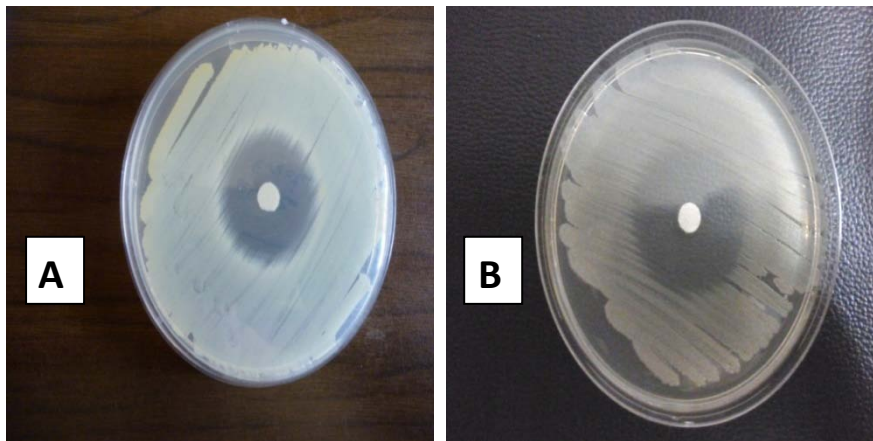


Fig.7. Inhibition zones exhibited by the crude extract of *R. solani* against *E. coli* (A) and *S. aureus* (B).

However, the bioactivity of fungal crude extract against the tested strains of bacteria was greater when starch (Fig.8) and $(\text{NH}_4)_2\text{SO}_3$ (Fig.9) were amended into the broth medium. The inhibition zones against both bacteria ranged between 25-30 mm diam under these conditions. Dextrose amended into the medium revealed a good bioactivity of the fungal crude extract against *S. aureus* (30 mm inhibition zone

diam) (Fig.8). Similarly, NaNO_3 supplemented into the growth medium gave a high inhibitory action of fungal metabolite against *S. aureus* (Fig.9). However, amendment of Asparagine into the growth medium did not show any bioactivity against *S. aureus*. Meantime, no bioactivity of crude extract against *E. coli* was detected when either yeast extract nor NH_4Cl were amended into the culture medium (Fig.9).

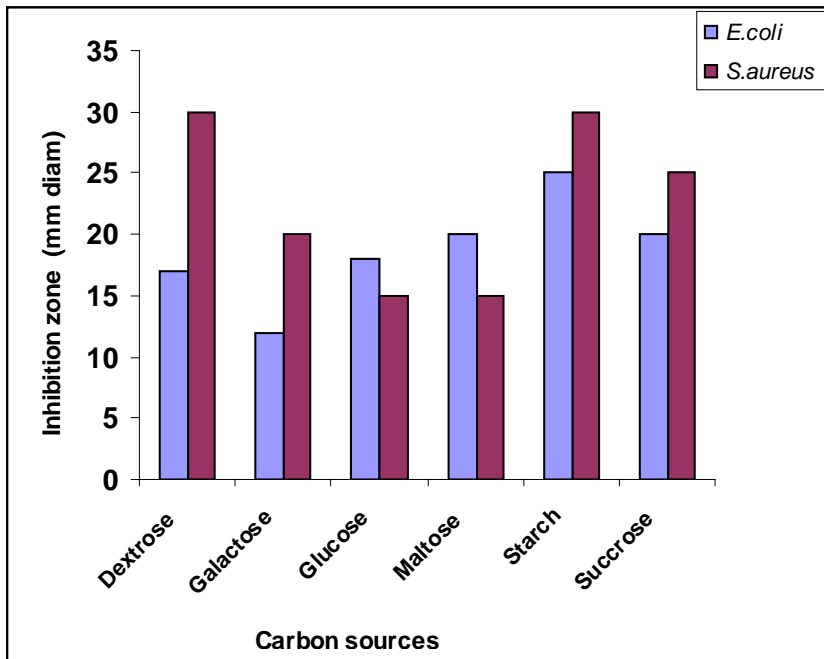


Fig.8. Effect of carbon source on the bioactivity of crude extract of *R. solani* against two strains of bacteria.

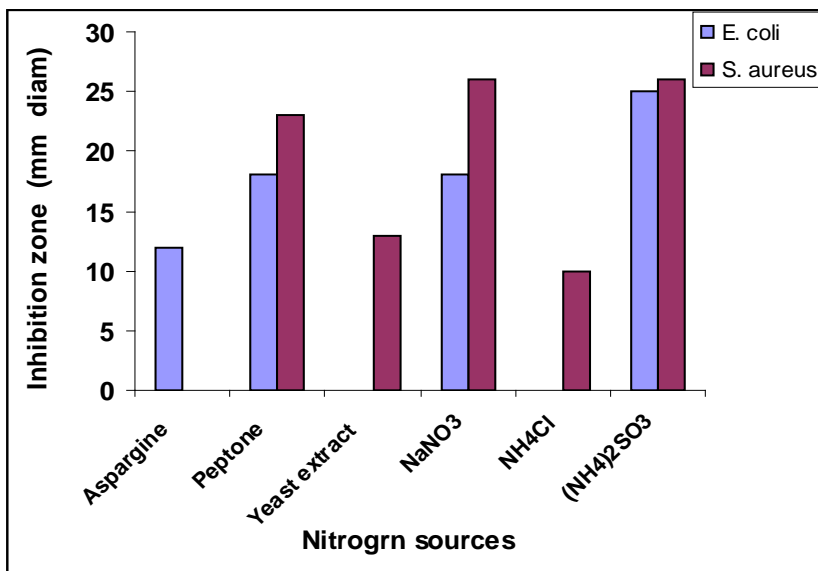


Fig.9. Effect of nitrogen source on the bioactivity of crude extract of *R. solani* against two strains of bacteria.

Nevertheless, the optimal temperature was 25 C and pH 6 for an optimal bioactivity (inhibition zones range 24- 30

mm diam) of fungal crude extract using broth medium incorporated with both starch and $(\text{NH}_4)_2\text{SO}_3$ (Figs.10, 11).

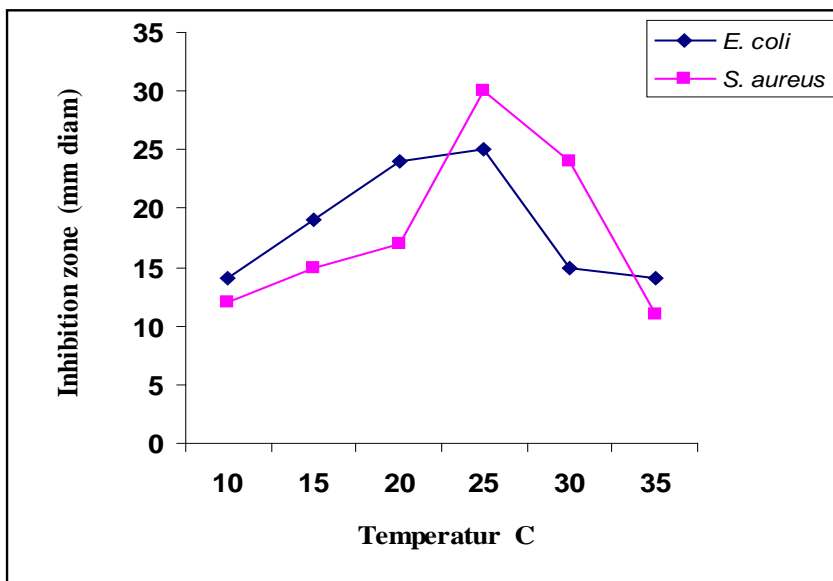


Fig.10. Effect of temperature on the bioactivity of crude extract of *R. solani* grown in broth medium amended with starch and $(\text{NH}_4)_2\text{SO}$

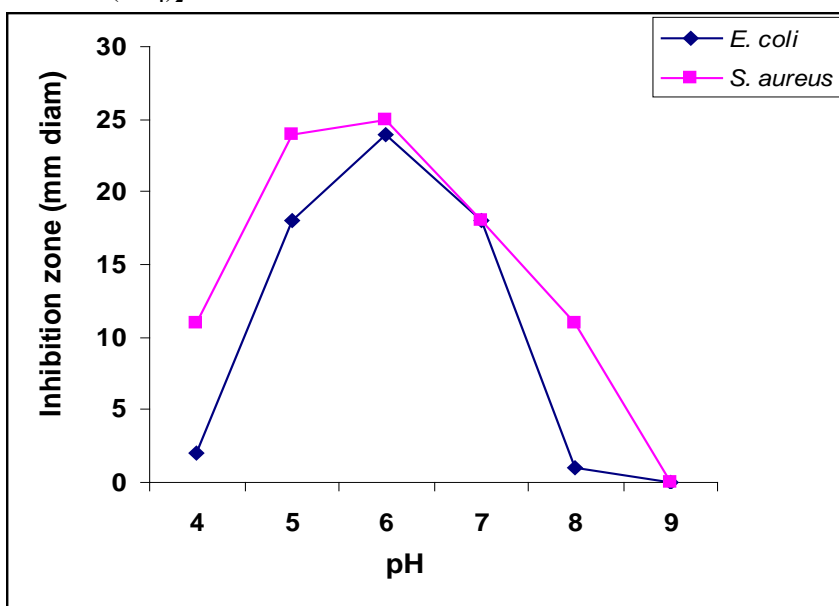


Fig.11. Effect of pH on the bioactivity of crude extract of *R. solani* grown in broth medium amended with starch and $(\text{NH}_4)_2\text{SO}_3$.

5. Discussion

In the last decades more attention has been given to explore new bioactive compounds from fungi as natural sources [18]. This is mainly due to the capability of fungi to synthesis secondary metabolites in culture media which contain various bioactive substances that can be used in pharmaceutical applications [2,3,4]. However, a production of fungal secondary metabolites is affected by different factors

[7,8], therefore, an optimization of fungal culture conditions must be considered for an exploration of antimicrobial bioactive metabolites. The present study elucidated that the fungus *R. solani* exhibited a better growth in Trypto Soyo broth medium compared with other tested media. This can be attributed to the chemical ingredients of this medium which enriched with nitrogen and carbohydrates and other minerals that

enhance the growth of this fungus. On the other hand, a production of antimicrobial metabolites by fungi is also influenced by nutrients mainly carbon and nitrogen sources [19]. In the present study, using of different nitrogen and carbon sources resulted in variation of the growth of *R. solani* as well as the crude extract production and bioactivity. It appeared that the metabolites produced by *R. solani* were varied due to variable carbon and nitrogen sources incorporated into the medium. Nonetheless, it has been stated that manipulating of nutritional factors would promote the biosynthesis of secondary metabolites by microorganisms [20]. Highest fungal growth and antibacterial bioactivity of crude extract exhibited by *R. solani* when starch as carbon source was amended into broth medium. A previous study reported that sucrose was the most suitable carbon source for the growth of some isolates of *R. solani* [7]. However, those workers did not use starch in their investigation. Other studies showed that an increased metabolic activity of *Mycena leptcephala* grown in a medium containing glucose and malt extract was observed [5]. Lowest level of growth and antifungal activity was reported when starch was used as carbon source and such observations are in contrary with the present study.

It can be speculated from the present results that *R. solani* possesses an enzyme system to break down the starch into sucrose and/or glucose and perhaps this process may provide a longer period during which conditions are suitable for optimal growth and production of bioactive metabolites as previously reported [21]. Meanwhile, other investigation reported that fructose as carbon source enhanced the antimicrobial activity of *Aspergillus* strains [8].

Apparently, carbon source is essential for a maximal yield of bioactive metabolite, however, the requirement of carbon source seems to be varied among different fungal species. This fact is also applicable for studied isolate of *R. solani*. The nature of the nitrogen source used in the present study has a remarkable effect on fungal growth, production and bioactivity of metabolite. NaNO_3 had a significant effect on the growth of *R. solani* while $(\text{NH}_4)_2\text{SO}_3$ showed a significant effect on the bioactivity of the fungal metabolite. A previous study showed that asparagine were the best nitrogen source for optimum growth and production of active metabolites by *Aspergillus* isolates [8]. On contrary, our study indicated that this nutritional source had less effect on growth and metabolite activity of *R. solani*.

Nonetheless, depending on the biosynthetic pathways exhibited by fungi, nitrogen sources may significantly affect the bioactivity of metabolites [22].

Fungal growth is often influenced by temperature and pH of the culture medium. The results showed that optimal growth of *R. solani* was at 30 C and the metabolic activity was at 25 C at pH 6. This is in concomitant with the study of Ritchie et al. [7] regarding the growth behavior of some isolates of *R. solani*. It has been stated that most basidiomycetes

grow over a wide pH and temperature range, although, many bioactive compounds are only stable within a narrow range of pH (5). In conclusion that the bioactive antibacterial metabolite produced by *R. solani* is significantly affected by nutritional and cultural conditions. Nevertheless, further purification and identification of the bioactive compounds from the crude extract of this fungus is needed.

6. Acknowledgment

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7. References

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مختبرياً، تحديد الظروف المثلى و الفعالية الحيوية ضد البكتيرية لأبيض الفطر *Rhizoctonia solani*

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الخلاصة

هدفت الدراسة الحالية لتحديد الظروف المثلى لنمو و الفعالية الحيوية لأبيض الفطر *Rhizoctonia solani* المعزول من درنات البطاطا المصابة في البصرة (العراق).
إستخدمت أوساط زرع مختلفة، درجات حرارة، حامضية، و مصادر كاربونية و نتروجينية لمعرفة تأثيراتها على نمو الفطر و فعالية أبيضه إتجاه عزلتين من البكتريا هما *S. aureus* و *E. coli*.
أوضحت النتائج بأن الوسط الزراعي Trypto soyo هو الأفضل لنمو الفطر، و كان أعلى إنتاجية للأبيض الفطري عند درجة حرارة 30 م و حامضية 6 بإستخدام النشأ كمصدر كاربوني و نترات الصوديوم كمصدر نتروجيني، و بإستخدام طريقة الإنتشار بالأقراص إتضح أن المستخلص الفطري الخام إمتلك فعالية إتجاه عزلتي البكتريا و كانت أقطار مناطق التثبيط هي 30 ملم و 34 ملم إتجاه *S. aureus* و *E. coli* على التوالي، عند درجة حرارة 25 م و حامضية 6 بإستخدام النشأ و كبريتات الأمونيوم.