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Influence of trace metals ions on growth and antifungal activity of *Bacillus subtilis* MHS15.

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Abstract

The effect of divalent metal ions in cultivation medium was studied to improve the growth and antifungal activity of *Bacillus subtilis* MHS15. Two metals salts namely FeSO4. 7H2O and MgSO4.7H2O at concentrations of 1 and 2 mM have stimulated the growth when the maximum optical density of 2.137 and 1.993 were achieved compared with 1.929 for control. Optical density has been suppressed using MnSO4.H2O, CuSO4.5H2O and ZnSO4.7H2O. The MIC against *Aspergillus niger* observed using 1 mM FeSO4.7H2O when the inhibition was 68.2% with an increase of 70.7% comparing with the maximum inhibition 48.2% by the control. Thus, the present study provides valuable information for enhancing growth and inhibitory effect of *B. subtilis* MHS15.

key words: *Bacillus subtilis* MHS15, ferrous swyati.

تأثير ايونات المعادن النزرة في نمو بكتريا *Bacillus subtilis* MHS15 وفعاليتها المضادة اتجاه الفطريات.

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الخلاصة

درس تاثير اضافة ايونات المعادن الثنائية في الوسط الزرعي لبكتريا *Bacillus subtilis* MHS15 بهدف تحسين النمو والنشاط المضاد للفطريات. من بين المعادن النزرة التي دعم بها الوسط الزرعي (TSB)، كبريتات الحديدوز وكبريتات الغنيسيوم المائية التي حفزت النمو للبكتريا المذكورة عند استخدامها بتراكيز 1 و 2 ملي مولار. اقصى كثافة نمو والتي كانت 2.137 و1.993 تم الحصول عليها باستخدام كبريتات المعادن الانفة الذكر مقارنتا مع 1.929 لمعاملة السيطرة. كثافة النمو ثبطت باستخدام كبريتات المنغنيز، النحاس والخارصين المائية. اقصى تثبيط 68.2% ضد العفن *Aspergillus niger* تم الحصول باستخدام كبريتات الحديدوز المائية بتركيز 1 ملي مولار بزيادة نسبتها 70.7% بالمقارنة مع نسبة تثبيط معاملة التثبيط البالغة 42.2%. لهذا الدراسة الحالية توفر معلومات ثمينة حول تحفيز النمو والفعل التثبيطي لبكتريا *Bacillus subtilis* MHS15.

الكلمات المفتاحية: بكتريا *B. subtilis* MHS15، ايونات كبريتات الحديدوز.

Introduction

For the time being, chemical pesticides in modern agriculture are being reduced for their hazardous impact to the natural environment and deteriorative effects on humans and are considered as safe status (2). Several strains of *Bacillus subtilis* have been reported that have potential for biological control of several plant diseases. For example, *B. subtilis* AU195 against *Aspergilla flavus* and *Bacillus subtilis* C9 against *Rizoctonia solani* (6; 12). They are considered as safe biological agents and their potential is regarded high (5) and a common soil inhabitant is now widely recognized as chemicals can also be lethal to the useful inhabitants of soil. *Bacillus subtilis* is a Gram-positive, spore forming bacteria, a powerful tool in biocontrol. As soil-living bacteria, naturally present in the closed zone of plant roots, *B. subtilis* is able to maintain stable contact with higher plants and support their growth. In addition, due to its wide host range, its ability to form endospores and produce varying antibiotics like iturin and surfactin with a broad spectrum activity, *B. subtilis* as well as other members of *Bacillus* genus are potentially useful biocontrol agents. Several mechanisms can clarify the enhancement of plant growth by bacteria existing in the rhizosphere. One of the main aspects of this stimulation is the inhibition of diseases caused by phytopathogens (16). It is known that metals are necessary for the normal metabolism of microorganisms as microelements, as well as for the production of antifungal agents such as lipopeptides. The regulatory effects of trace divalent metals on microbial secondary metabolism have been recorded for a variety of species and it was found that MgSO4, and FeCl2 have stimulated the growth and antifungal agents production of *Bacillus alvei* NRC-14. However, ZnCl2 and KCl showed negative effect on antifungal agents production each (14). This study was to determine the influence of various metal ions addition on the growth and antifungal activity of *Bacillus subtilis* MHS15**.** The majority of *Bacillus* species were nonpathogenic, because of this fact; many have been exploited for biotechnological and industrial applications (9).

Materials and Methods

Bacterial isolate:

Bacillus subtilis strain used in this study was identified at LGC Genomics Sequencing Service in Germany and given the code No: MHS15 (7). It has maintained on TSB slants at 4°C and subcultured each month intervals.

Fungal isolate:

Aspergillus niger has been obtained from the Department of Plant Protection/ College of Agriculture, University of Baghdad.

Growth density of *B. subtilis* on modified tryptone soya broth:

Method of Hung *et al* (4) has been applied. *Bacillus subtilis* MHS15 has been grown on Tryptone soya broth media supplemented within different concentrations of 0.5, 1, 2, 3 and 5 mM from stock solution of 50 m M of ferrous sulfate heptahydrate, manganese sulfate monohydrate, magnesium sulfat heptahydrate, copper sulfate pentahydrate, and zing sulfate heptahydrate. Each metal sulfate was added to Tryptone soya broth medium separately. The growth density (O.D at 600 nm) was determined using Bioscreen device after the wells of honeycomb plate being loaded with 300 µl from each sample and fixed at 37 ˚C and duration of the run 60 h and the interval 3 hrs continuous shacking.

Extraction of bacterial supernatant:

Each sample was grown in Tryptone soya broth medium at 37˚C for 60 h. The supernatants of cultural media of different treatments were separated by centrifugation at 10 000 g for 20 min followed by filteration (sterilization) through 0.22µm membrane filter to remove the bacterial cells. This supernatant considered as a crude extract containing antifungal substances and kept in sterilized plastic tube for determination of antifungal activity or further uses **(**15).

Determination of antifungal activity

The antifungal activity against *Aspergillus niger* was determined according to poisoned food technique according to method described by Grover and Moore (3).

Twenty five ml aliquots of sterilized potato dextrose agar media

which supplemented separately with 35%, v/v of sterilized bacterial supernatants as a crude antifungal agent extract. Media were poured in sterilized plates under aseptic conditions, allowed to cool and solidify. Six mm discs (from the edge) of six days old culture from the tested fungi were inoculated at the centre of PDA Petri dishes. The plates were incubated at 26°C for 5-7 days. The Petri dishes containing media free of the supernatant served as control. After incubation, the colony diameters for the fungi at each plate were measured in millimeter (13**)**.

The percentage inhibition of mycelial growth was calculated using the formula:

Percent of inhibition = C – T / C * 100

Where C = ml of mycelial growth in control plate.

T = ml mycelial growth in treatment plate.

Results and Discussion:

The biosynthesis of antifungal substances by spore forming *Bacillus* is not only highly dependent on medium composition but also on cultivation conditions as well such as incubation temperature and incubation period….etc. (1, 10). Metals are an integral part of metabolic process of all microorganisms and some of them are vital components of living systems and known as essential metal ions. Secondary metabolisms are affected by the presence or absence of these essential metal ions, as they may be responsible for activation of some of the biosynthetic pathways (11).

Optimized conditions were carried out to know the effect of divalent metal cations (FeSO4.7H2O, MgSO4.7H2O, MnSO4.H2O, CuSO4.5H2O and ZnSO4.7H2O) on growth density and antifungal activity of *B. subtilis* MHS15, using the basic media (TSB containing individual concentrations:1, 2, 3 and 5 mM of previous metal salts) Untreated culture (TSB free of metal salts) serving as control. Only two salts, FeSO4.7H2O and MgSO4.7H2O at concentrations of 1 and 2 mM were found to be cause a significant enhancement of growth density and antifungal activity, whereas the maximum optical density of 2.137 and 1.993 were obtained at 1 mM of FeSO4. 7H2O and MgSO4. 7H2O respectively compared with 1.993 for control. There was less increase of growth density when 2 Mm of both metal sulfates used (Fig 1 and 2). *Bacillus* exposed to various metal ions in their environment which are sometimes beneficial or detrimental depending on the chemical/

physical nature and oxidation state of the metal ion (8). Iron is an essential nutrient for living agents due to its noticeable activity in electron transport reactions in biological systems.

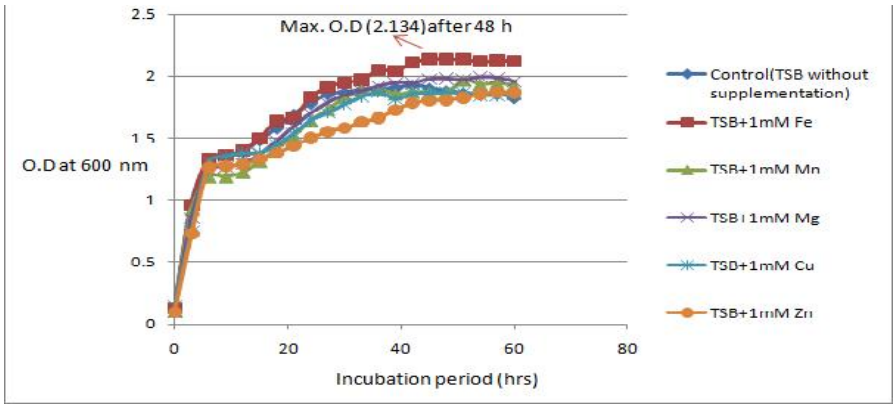

Fig (1): Effect of different metals at a concentration of 1 mM on growth of *Bacillus subtilis* MHS15. Each value represents the mean of three tests.

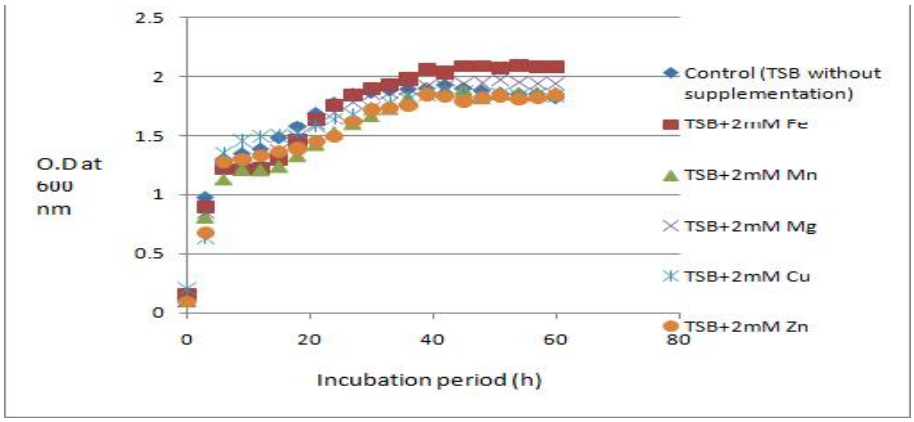

Fig (2): Effect of different metals at a concentration of 2 mM on growth of *Bacillus subtilis* MHS15. Each value represents the mean of three tests.

The higher concentrations of previous salts had no effect on cultural growth. In contrast, the salts ZnSO47H2O., CuSO4. 7H2O and MnSO4 suppressed growth of *B. subtilis* MHS15 (Fig 3 and 4). The maximum inhibition against *Aspergillus niger* observed using 1 mM FeSO4. 7H2O was 68.2% with an increase of 70.7% comparing with the maximum inhibition 48.2% by the control (Fig 5 and 6) and the best inhibition was noticed at stationary phase after 45 h of the growth.

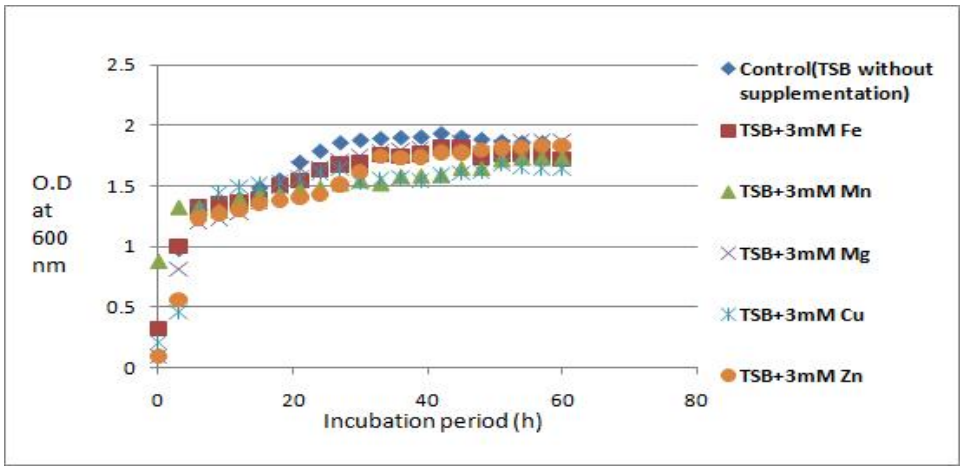

Fig (3): Effect of different metals at a concentration of 3 mM on growth of *Bacillus subtilis*. Each value represents the mean of three samples.

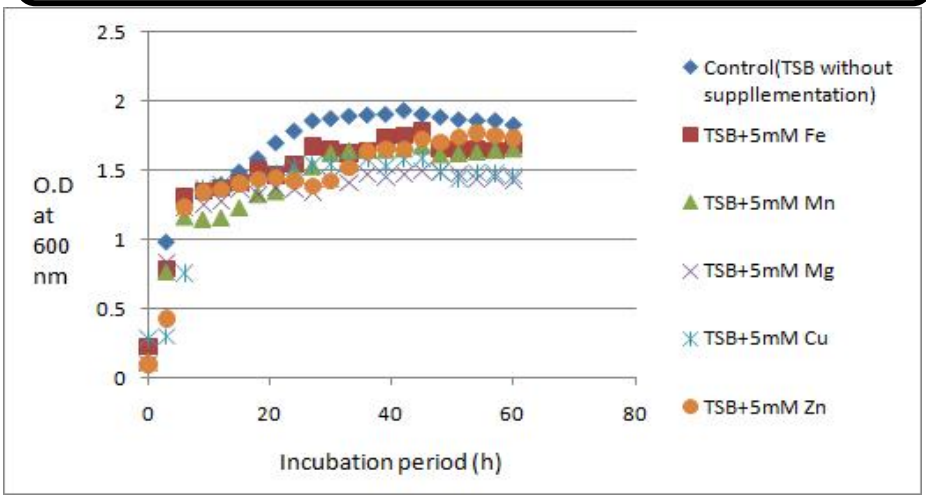

Fig (4): Effect of different metals at a concentration of 5 mM on growth of *Bacillus subtilis.* Each value represents the mean of three samples.

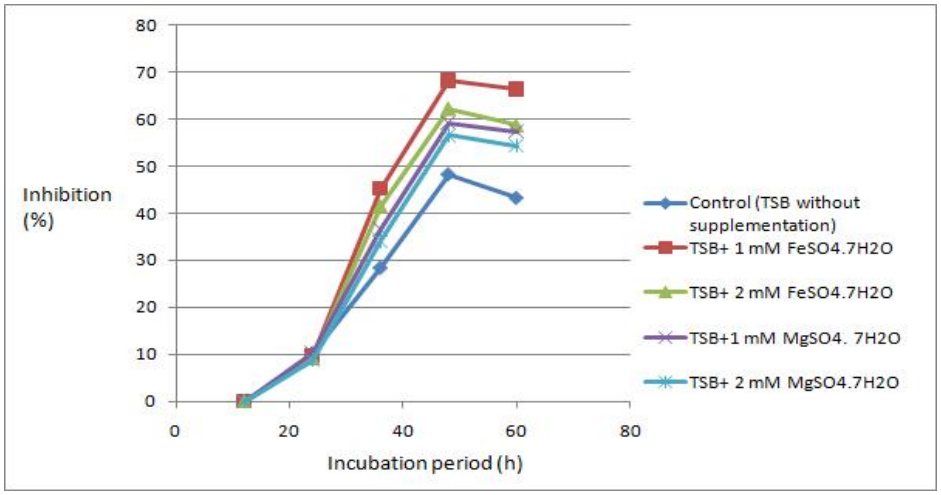

Fig (5): Comparison between inhibition % of *A. niger* by *Bacillus subtilis* MHS15 supernatant grown on TSB with FeSo4.7H2O or MgSo4.7H2O and without at 37˚C for 60 hours. Each value represents the mean of three samples.

Conclusions:

The finding of the present work is an important step towards selection of an optimum medium (TSB supplemented with 1 mM of FeSO4.7H2O and MgSO4.7H2O) which helps to gain the optimum growth and antifungal activity of antagonistic *Bacillus subtilis* MHS15.

References

1. Akpa, E.; Jacques, P.; Wathelet, B. Paquot, M.; Fucks, R.; Budzikiewicz, H. and Thonart, P. (2001). Influence of culture conditions on lipopeptide production by *Bacillus subtilis*. *Applied Biochemistry and Biotechnology*, 91:551-561. 4.

2. Food and Drug Administration. (1999). Code of Federal Regulations, title 21: food and drugs, chapter I: food and drug administration department of health and human services, part 184: direct food substances affirmed as generally recog- nized as safe. *US Government Printing Office*, Washington, DC.

3. Grover R. K., Moore JD. (1962). Toximetric studies of fungicides againstbrown rot organisms, *Sclerotia fructicola* and *S. laxa. Phytopathol*. 52: 876-880.

4. Hung Y. L.; Yerra K.; Wen S. W and Yew. M. T. (2007). Ferrous ion Enhanced Lipopeptide antibiotic iturin A., Production from *Bacillus amyloliquefaciens* B128, *Int. J. Appl. Sci. Eng*, 5(2): 123-132.

5. Kim PI and Chung KC. (2004) Production of an antifungal protein for control of *Colletotrichum lagenarium* by *Bacillus amyloliquefaciens* MET0908. FEMS *Microbiol Lett*, 234 (1): 177-183.

6. Moyne AL; Shelby R.; Cleveland T. E. and S. Tuzun, (2001) Bacillomycin D: an iturin with antifungal activity against *Aspergillus- flavus*, *Journal of Applied Microbiology*, 90: 622 ± 629.

7. Muhyaddin, M. O.; AL- Shareafi, H. R. and Allawi, S. S. (2015). isolation and identification of antifungal substances producer *Bacillus*. *Journal of the College of Basic Education*, 21(90): 33-44.

8. Neilands JB., (1974). Iron and its role in microbial physiology. In: Neilands J. B. (ed.). Microbial iron metabolism. *New York: Academic Press Inc*; 3-34.

9. Price, N. P. J.; Rooney, A. P.; Swezey, J. L.; Perry, E. & Cohan, F. M. (2007). Mass spectroscopic analysis of lipopeptide for *Bacillus* Strains isolated from diverse geographical locations. *FEMS Microbiol Lett*, 271(1): 83–89.

10. Rahman, M. S.; Ano, T. and Shoda, M. (2006). Second stage production of iturin A by induced germination of *Bacillus subtilis* RB14. *Journal of Biotechnology*, 125: 513–515.

11. Raza W., Yang X., Wu H., Huang Q., Xu. Y., Shen Q. (2010). Evaluation of fusaricidin- type antifungal compounds by *Paenibacillus polymyxa* SQR- 21. Bioresour. *Technol*. 101: 9264-9271.

12. Rezuanul Islam Md.; Yong Tae Jeong Yong Se Lee, and Chi Hyun Song. (2012). Isolation and Identification of Antifungal Compounds from *Bacillus subtilis* C9 Inhibiting the Growth of Plant Pathogenic Fungi. *Mycobiology*, 40(1): 59–66.

13. Satish, S.; Mohana, D. C.; Ranhavendra, M. P. and Raveesha, K. A. (2007). Antifungal activity of some plant extracts against important seed borne pathogens of *Aspergillus sp*. *Journal of Agricultural Technology*, 3(1): 109-119.

14. Shadia M. Abdel- Aziz1, Foukia E. Mouafi, and Abeer A. Keera1. (2013). Extracellular Metabolites Produced by a Novel Strain *Bacillus* alvei N. Heat- Shock Stress Induces Production of Antimicrobial Metabolites. *J. Basic. Appl. Sci. Res*, 3(12)410-420.

15. Tendulkar, S. R.; Saikumari, Y. K.; Patel1, V.; Raghotam M. T. K; Balaram P. and Chattoo1 B. B. (2007). Isolation, purification and characterization of an antifungal molecule produced by *Bacillus licheniformis* BC98, and its effect on phytopathogen *Magnaporthe grisea. Journal of Applied Microbiology*, 109: 2331-2339.

16. Toure Y.; Ongena M.; Jacques P.; Guiro A.; Thonart P. (2004). Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple. *Journal of Applied Microbiology*. 96(5):1151–1160.