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Isolation and identification of *Bacillus subtilus* as(probiotic) from intestinal microflora of common carp *Cyprinus carpio* L.

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Summary

The presence of *Bacillus* species Were investigated in the intestine of 50 fish specimens common carp *Cyprinus carpio* weigh range between 1400-1500g in weigh were obtained from commercial farms in north of Baghdad through period of September to December 2010.The result revealed presence of gram positive, rod shape bacteria grow in the mesosphilic temperature range. The optimal temperature was 25-35C° facultative aerobes and produce catalase an enzyme. The bacterial antagonist activity was tested with fish culture pathogenic bacteria. The modified antagonistic method results showed that *Bacillus subtilus* could inhibit *Aeromonas hydrophila* after 24 hours. The highest level of antibacterial substances of this *bacillus* was produced in 48 hours. The aim of this study is to isolate and identify the *Bacillus* spp. and study its efficacy as probiotic against A. hydrophila in a fish culture.

Key words: *bacillus, Aeromonas hydrophila*,probiotic

عزل وتشخيص بكتريا *Bacillus subtilus* (معززات حيوية) من مكونات امعاء اسماك الكارب الشائع *Cyprinus carpio* L.

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الخلاصة

فحصت امعاء 50نموذج من سمكة الكارب الشائع *Cyprinus carpio* بوزن يتراوح بين 1400 -1500غم ، جلبت من مزارع في شمال بغداد للمدة من ايلول الى كانون الاول عام 2010 . اظهرت نتائج الفحص وجود بكتريا عصوية الشكل وموجبة لصبغة كرام تنمو بحرارة mesophilic تتراوح بين 25-35 م° ، اختيارية النمو في الوسط الهوائي وتنتج انزيم الكاتليز , واظهرت نتائج اختبار الفعالية التضادية للبكتريا *Bacillus subtilus* ضد البكتريا المرضية بطريقة تضاد محورة ، ان البكتريا *Bacillus subtilus* تستطيع تثبيط نمو بكتريا *Aeromonas hydrophila* بعد 24 ساعة واعلى مستوى للمواد المثبطة ينتج كان بعد48 ساعة . هدفت الدراسة الى عزل وتشخيص بكتريا *Bacillus subtilus* وامكانية اضافتها كمعززات حيوية فعالة للسيطرة على البكتريا *Aeromonas hydrophila* في تربية الاسماك.

Introduction

The increasing in aquaculture is said to be paralleled with a corresponding increasing in the occurrence of infectious diseases resulting often from high stocking densities and stress conditions that favour the occurrence and spread of pathogens. Cultured fish suffer a wide variety of bacterial, viral, parasitic and fungal diseases (1). Examples include motile aeromonad septicaemia, vibriosis, columnaris, edwardsillosis and furunculosis .

Among these, the diseases caused by motile aeromonads particularly *Aeromonas hydrophila* are wide spread and affect a wide Range of mostly freshwater species. Motile aeromonads are implicated in a number of disease conditions including hemorrhagic septicemia, ulcerative conditions, abdominal distensions, fin/tail rot and exophthalmia (1).

Recently, disease management strategies were based mainly on chemotherapy (2). However, the emergence of drug resistance in pathogens, problems associated with drug residues in cultured fish, and awareness towards environmental pollution problems emanating from the use of chemotherapeutants have led to greater focus on alternative methods of disease management (3). In recent years, disease prevention by means of optimal husbandry and use of vaccines, immunostimulants and probiotics has been increasingly recognized (4, 5).

Probiotics may be considered as an alternative to antimicrobials in disease control strategies of cultured fish. Probiotics have been defined by different workers but the definition by Fuller (6) has attracted much attention. He defined probiotics as live microbial feed supplements which beneficially affect the host animal by improving its intestinal balance. Furthermore, FAO/WHO (7) referred to probiotics as live micro-organisms which confer health benefits on the host when administered in adequate amounts. Probiotics enhance the performance of the intestinal microbial flora by colonizing the gut and depriving pathogens of adhesion sites and nutrients (8). Research on probiotic in aquaculture focused initially on fish juveniles, but subsequently concern was shifted to fish larvae and shellfish (5) To date, most probiotics used in aquaculture belong to *Lactobacillus, Carnobacterium, Aeromonas, Bacillus , Pseudomonas* and *Vibrio* genera.

Probiotics such as the gram positive *Bacillus* offers an alternative to antibiotic therapy for sustainable aquaculture. Although many genera of bacteria were used as probiotic in aquaculture such as *Vibrio alginolyticus, Pseudomonas fluorescens* and *Alteromonas* sp. (9), *Bacillus* species offer several advantages over gram negative bacteria , including longer shelf life, because they produce end spores that are tolerant to heat and desiccation ,and the broad spectrum activities of their secondary metabolites (10). In *Vitro* production of inhibitory compounds toward known pathogens for the considered species has often been used in the selection of putative probiotics (5). In this study, the potential portions which were isolated from intestine of common carp were tested by focusing on competitive and inhibitive capabilities against some common pathogenic bacteria in aquaculture including *A. hydrophila*.

Materials and Methods

A total of 50 common carp were obtained from commercial fish in the north of Baghdad from September 2010- to December 2010, and maintained in aquarium filled with tap water at 21 $^{\circ}$C.

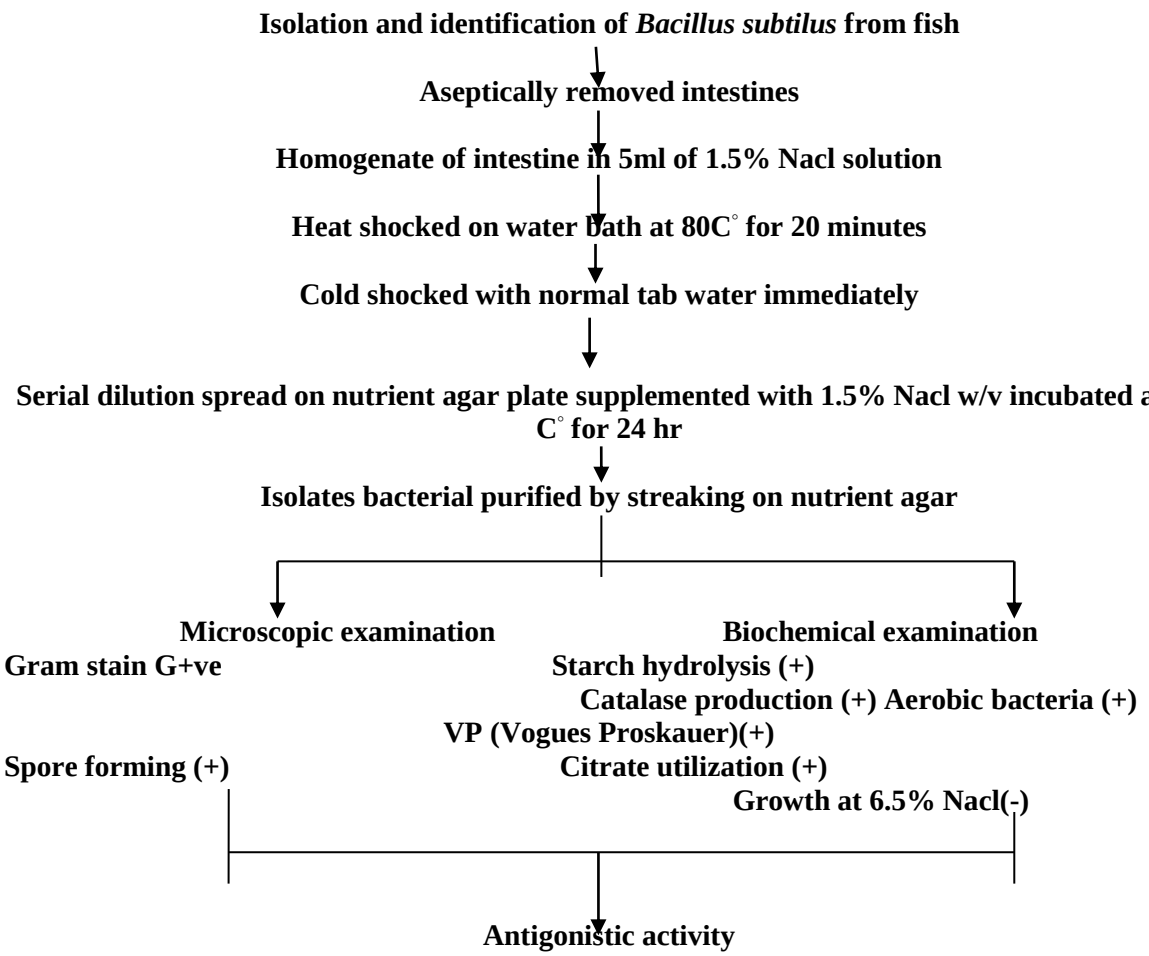
Analytical grade chemicals and dyes were obtained from Al-Kindi Company for production of veterinary vaccines and drugs, bacteriological media were obtained from oxoide UK which include set of biochemical media, blood agar base and Nutreint agar.

Bacillus spp. were isolated from the various samples of the common carp *Cyprinus carpio* of fish farm in the north of Baghdad (average between 1400-1500 g in weight).

They were brought to the laboratory alive and sacrificed. The abdomen surfaces were thoroughly scrubbed with an alcohol (70% ethanol) and aseptically dissected to remove the intestines , The Intestines were ground by homogenizer and dissolved in 5 ml of 1.5% NaCl per fish and the diluted 1.5% NaCl were heat shocked on water bath at 80 °C for 20 min followed by cold shock with normal tap water immediately (11).Then the intestine solution was spread on plates using spread plate technique on Nutrient agar (NA) supplemented with 1.5% NaCl (w/v) and incubated at 30 °C for 24 hours. Isolates were purified by streaking on NA supplemented with 1.5% NaCl (w/v). Catalase test were used for identifying *Bacillus* species. Identification based on their morphological and biochemical characteristics.

Pure cultures were kept in semisolid nutrient medium supplemented with 20 % (v/v) glycerol at -20^{o}C. Cultures were routinely grown on nutrient agar supplemented with 1.5 %(Oxoid) at 25^{o}C (graph1).

The bacterial isolate send to the collaborating laboratory (Central Health Laboratory/Baghdad /Ministry of Health). To confirm the identification of *Bacillus subtilus* that was isolated on Department of Pathology and Poultry diseases Laboratory / College of Veterinary Medicine/ University of Baghdad.



Graph (1) isolation and identification of *Bacillus subtilus*
Inhibition activities of *Bacillus subtilus*

This method was modified from (10). *Bacillus* from single colony was transferred to NA plate as three spots per plate and grown for 1 day, 2 days ,3days and 4days at 30°C. *A.hydrophila* was grown overnight in NB broth and 0.2 ml of the culture of *A. hydrophila* in NB was mixed with 20 ml NA agar (40- 45°C). This suspension was gently poured on top of the agar with the pregrown *Bacillus* isolates. After incubation for 24-48 hours at 30°C, the plates were inspected for growth inhibition zones on the lawn of *A. hydrophila*. The comparison between size of clear zone in the *Bacillus subtilus* Plate which were inoculated 1 day, 2 days,3 day and 4 days were determined.

Results

Isolation and identification of *Bacillus subtilus*

Biochemical characterization and identification of bacteria isolated table (1) had a strong gram-positive reaction. Cellular morphology suggests that it is a thick unicellular rod-shaped bacteria, capable of forming round, wavy, convex, rough, and opaque colonies. Cells are aerobic, endospore-forming, and motile while spores are ellipsoidal and central in position. Isolate bacteria was capable of utilizing glucose, xylose, sucrose, mannitol, arabinose, and fructose as carbohydrate sources for growth and grew at pH levels of 5, 8, 9, and 11 and salinities of 3%, 6%, and 8%, but not 10%. It did not decarboxylase the amino acids lysine, ornithine, and arginine. The isolate bacteria were positive for the Vogues Proskaeur reaction and negative for the methyl red test. It can produce catalase, oxidase, and in dole, but not hydrogen sulfide and can utilize citrate, reduce nitrate, liquefy gelatin, and hydrolyze starch and casein, but not urea.

Table (1) Identification of isolated *Bacillus subtilis* using morphological and biochemical characterization

Tests	Active ingredients	Reactions/Enzymes	results
Gram stain	+	Arginine	-
Cellulare morphology	Rod shape	Vogues proskaeur	+
Colony shape	Round, wavy convex,opaque	Methyl red	-
glucose	+	Citrate	+
xylose	+	Nitrate reduce nitrate	
sucrose	+	Gelatin	+
mannitol	+	Starch	+
Arabinose	+	Casein	+
Fructose	+	Urea	-
Growth at pH5.8.9 and 11	+	Catalase	+
Salinities 3%,6% and 8% and not 10%	+	Oxidase	+
Lysine	-	H_2S	-
Ornithine	-	Indol	+

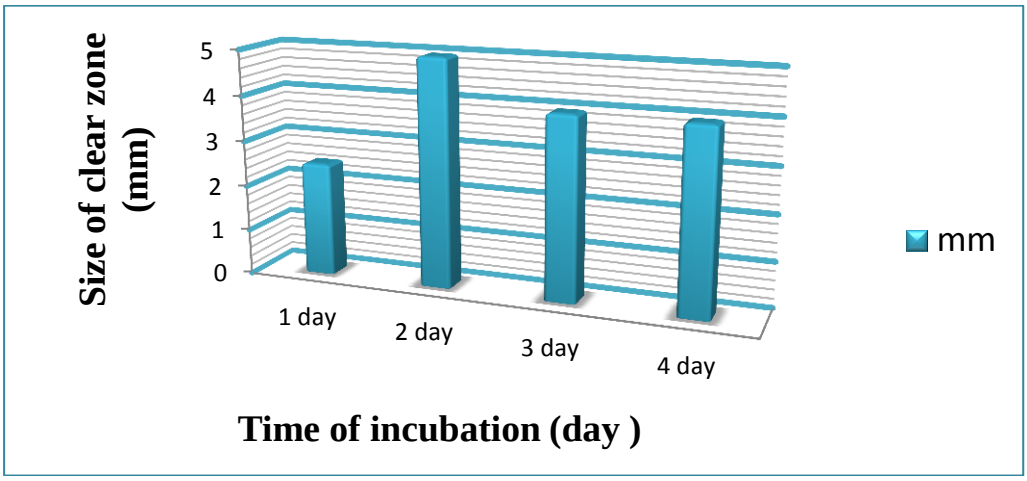
Inhibition activities of selected *Bacillus subtilis*

Bacillus subtilus showed inhibition effect to *A. hydrophila*. The results showed that *Bacillus subtilis* could produce antibacterial substance at highest level in 2 days that was 4.8mm (Fig 2**)** but there were no different from 3 and 4 days as measured by size of clear zone (Fig 3) and lower level in 1 day(fig 1) (Graph 2).

Fig(1) Inhibition zone of *Bacillus subtilis* against *Aeromonas hydrophila* after incubated *Bacillus* 1 day

Fig(2) Inhibition zone of *Bacillus subtilis* against *Aeromonas hydrophila* after incubated *Bacillus* 2 days

Fig(3) Inhibition zone of *Bacillus subtilus* against *Aeromonas hydrophila* after incubated *Bacillus* 3 ,4days



Graph (2) Size of clear zone of *Bacillus subtilis* when pre-cultured 1, 2 ,3 and 4 days showing inhibition effect against *Aeromonas hydrophila*

Discussion

Biochemical characterization and identification of bacterial isolated the morphological and physiological characteristics of bacterial isolated were comparison with (12) and (13) place this bacterium in the Bacillus genus and species *subtilis. Bacillus* is distinguished from other endospore-forming bacteria by being a strict or facultative aerobe, rod-shaped, and catalase-positive. *Bacillus subtilis* is distinguished from other members of the genus by its biochemical reactions, unicellular rod-shaped morphology, and ellipsoidal spore forming ability.

Bacilli are not commonly found in Gastro- intestinal tract but have been isolated from carps (14), coastal fishes (15), bivalves (16), shrimp culture ponds (17), and shrimp larvae-rearing medium (18).

Most *Bacilli* produce antibiotics such as difficidin, oxydifficidin, bacitracin, polymyxin, subtilin, mycobacillin, bacillin, gramicidin, or bacillomycin B and are antagonistic to pathogenic bacteria in both *in vivo* and *in vitro* conditions (19, 20).

Bacilli are not associated with aquatic organism pathology and are widely accepted and used as probionts (21). We focused only on genus *Bacillus subtilus* ,which showed antagonistic activity to pathogenic bacteria in aquaculture(*Aeromonas hydrophila)* in many studies (22; 17). *Bacillus subtilus.* are commonly found in marine sediments and therefore are naturally ingested by animals such as shrimps and fish that feed in or on the sediments (21). So, we isolated *Bacillus subtilus* from intestine of *Cyprinus carpio* and tested for antagonistic activity to show inhibition effect of *A. hydrophila*. The antibacterial substance was produced in highest level in 48 hours. The reduction of pathogen growth and cell density indicate that

extracellular bacteriolytic products produced by *Bacillus subtilus* were responsible for this inhibition. The *in vitro* production of compounds that inhibit known pathogens is often used in the selection of putative probiotic strains (18). In the present *in vitro* study, *Bacillus subtilus* inhibited *A.hydrophila* and hence was selected for further study as probiont. Also the results could explain higher levels of protection against *Aeromonas hydrophila* in the groups that was fed higher level of the probiotic contain *Bacillus subtilis.*

Many studies supported that *Bacillus subtilus* could reduce pathogenic bacteria in aquaculture. (17) reported *P. monodon* immersed in *Bacillus subtilis* BT23 at a density of 10^6 -10^8 -CFU/ml for 6 days showed 90% reduction in accumulated mortality when challenge with *Vibrio harveyi* at 10^3 - 10^4 CFU/ml for 1 hour.

(22) used microbial products, *Bacillus* spp., *Saccharomyces* sp., *Nitrosomonas* sp. and *Nitrobacter* spp. in fish and shrimp pond by immersion for 110 days, the results showed that *Bacillus* spp. were dominant in all ponds and the bacterial populations were changed use this probiotic.

It has been demonstrated that *Bacillus subtilus,* produced substances that could inhibit the growth of the pathogenic bacteria; *Bacillus subtilus* showed competitively exclude the pathogenic bacteria. The presence of this *Bacillus* spp. could protect the aquatic animals against the infection by pathogenic bacteria and might be applied as good probiotic in aquaculture.

References

1. Austin, B. and Austin, D.A. **(**2007**)**. Bacterial Fish Pathogens: Disease in Farmed and Wild Fish, 4th Ed. Springer-Praxis, Godalming.
2. Aoki,T.; Umeda,T.;Takami,K.;Kitao,T.;Chongthaleong,A.and Punyaratabandhu, P.(1992). Drug-resistant *Aeromonas hydrophila* in Thailand .in: proceedings of the second Asian , fisheries form (eds. R.Hirano and Hanyu),: 693-696.
3. Tonguthai, K. and Chanratchakool, P., 1992. The use of chemotherapeutic agents in Aquaculture in Thailand. In: Diseases in Asian Aquaculture Vol.1.(Sheriff, M Subasinghe, R. P. and Arthur, J. eds.). Fish Health Section, Asian Fish Society Manila, Philippines. Pp. 555-565.
4. Maroni,.(2000). Monitoring and regulating of marine aquaculture in Norway .Applied Ichthyology, 16:192-195.
5. Verschuere, L.; Rombaut, G.; Sorgeloos, P.andVerstraete ,W.(2000). Probiotic bacteria as biological control agents in aquaculture. Microbiology and Molecular Biol Rev., 64: 655-671.
6. Fuller, R.(1989). Probiotics in man and animals . Appl. Bacteriol., 66:365 – 378.
7. FAO/ WHO.(2001).Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Cordoba, Argentina. Report of a Joint FAO/WHO expert consultation. Available I www.fao.org/ESN/probio/report.pdf.
8. Gatesoupe, F.J. (1999).The use of probiotics in aquaculture. Aquaculture 180: 147-165.
9. Gram,L.;Lovod,T.;Nielsen,J.;Melchiorsen,J. and Melchiorsen,B.(2001).In vitro antagonism of the probiont Pseudomonas fluorescens AH2against Aeromonas salmonicida does not confer protection of salmon against furunculosis .Aquaculture ,199:1-11.
10. Jock, S.; Völksch,B.; Mansvelt,L. and Geider,K.(2002). Characterization of *Bacillus* strains from apple and pear trees in South Africa antagonistic to *Erwinia amylovora*. FEMS Microbiol.Lett**.**211: 247-252.
11. Watchariya, P,and Nontawit,A..(2007).Application of bacillus spp. Isolated from the intestine of black tiger shrimp (*penaeus monadon fabricius*)from natural habitat for control pathogenic bacteria in aquaculture . kasetsart.Nat.Sci.,41:125-132.
12. Sneath, P.H.A.**(**1986).Endospore-forming gram-positive rods and cocci.: 1104-1105.
13. Priest, F.G.**(**1993).Systematics and Ecology of *Bacillus*. : 3-16. In: A.L. Sonensheine, J.A.; Koch, R. and Losick .Bacillus subtilis and Other Gram-positive Bacteria: Biochemistry,Physiology and Molecular Genetics. Am. Soc. Microbiol., Washington DC, USA.

14. Kumar ,R.; Mukherjee, S.C.; Prasad, K.P. and Pal, A.K.(2006).Evaluation of *Bacillus subtilis* as a probiotic to Indian major carp *Labeo rohita* (Ham.). Aquaculture Research,37(12) :1215-1221.
15. Sugita, H.;Hirose ,Y.;Matsuo, N. and Deguchi,Y.(1998). Production of the antibacterial substance by Bacillus sp. strain NM 12, an intestinal bacterium of Japanese coastal fish. Aquaculture, 165(3-4):269-280.
16. Sugita, H.; Tanaami, H.; Kobashi, T. and Deguchi, Y.(1981).Bacterial flora of coastal bivalves. Bull. Jap. Soc. Sci. Fish., 47:655- 661.
17. Vaseeharan, B. and Ramasamy,P. (2003).Control of pathogenic *Vibrio* spp. by *Bacillus subtilis*BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*.Lett. Appl.Microbiol., 36(2): 83.
18.Rengpipat, S., Phianphak W., Piyatiratitivorakul S. and Menasveta, P. (1998). Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. Aquaculture, 167: 301-313.
19. Korzybski ,T.; Kowszyk, Z. and Kurylowicz,W. (1978). Antibiotics isolated from the genus *Bacillus* (Bacillaceae). 1529- 1661. In: Antibiotics - Origin, Nature andProperties. Vol. III, Am. Soc. Microbiol.,Washington DC, USA.
20. Zimmerman, S.B.; Schwartz, C.D.; Monaghan, R.L.; Pleak, B.A.; Weissberger B.; Gilfillan, E.C.; Mochales, S.;Hernande, S.; Currie, S.A.; Tejera, E. and Stapley,E.O..(1987). Difficidin and oxydifficidin: Novel broad spectrum antibacterial antibiotics produced by *Bacillus subtilus*. Antibiotics, 40(12):1677- 1681.
21. Moriarty,DJ.(1998).Control of luminous *Vibrio* species in penaeid aquaculture ponds.Aquaculture,164: 351-358.
22. Devaraja,TN.;Yusoff,F.M. and Shariff,M. (2002). Changes in bacterial populations and shrimp production in ponds treated with commercial microbial products. Aquaculture, 206: 245- 256.