Vietnam J. Agri. Sci. 2016, Vol. 14, No. 7: 1009-1015 Tạp chí KH Nông nghiệp VN 2016, tập 14, số 7: 1009-1015
www.vnua.edu.vn

ISOLATION, IDENTIFICATION, AND PRELIMINARY CHARACTERIZATION OF *Bacillus subtilis* WITH BROAD-RANGE ANTIBACTERIAL ACTIVITY FROM MUONG KHUONG CHILLI SAUCE

Nguyen Thi Thanh Thuy[1*], Vu Thi Huyen Trang[1], Vu Quynh Huong[1], Trinh Thi Thu Thuy[2], Nguyen Thi Lam Doan[1], Tran Thi Na[1], Nguyen Hoang Anh[1]

[1]Faculty of Food Science and Technology, Vietnam National University of Agriculture
[2]Faculty of Biotechnology, Vietnam National University of Agriculture

Email[*]: nttthuycntp@vnua.edu.vn

Received date: 24.05.2016 Accepted date: 10.08.2016

ABSTRACT

Bacillus subtilis is usually found in foods such as dry cured sausages, cheeses, traditional fermented milks, etc., in which they cooperate with other microorganisms during fermentation, release amylases, lipases and proteases… *B. subtilis* not only play with probiotic role but also produce antimicrobial substance. Muong Khuong chilli sauce is a naturally fermented product derived from Muong Khuong special peppers without heat treatment and is able to be preserved in ambient conditions for 1.5-2 years. Amongst 512 types of colonies, 48 *Bacillus* sp. strains were isolated from 80 chilli samples. Their ability against pathogenic bacteria was determined using the agar-well diffusion method through measuring the inhibition zone diameter. The four pathogenic bacteria strains tested were *Escherichia coli* RG1.1, *Salmonella typhi* GT4.3, *Listeria monocytogenes* MI2.6, and *Staphylococcus aureus* TS1.9. The results of 16S rRNA genotype sequencing for the two strains, TO43.13 and TO53.2, that had broad-range antimicrobial activity indicated that the TO43.13 sequence was homologous up to 95% with *B. subtilis* BcX1 (JX504009.1) and *B. subtilis* EPP2 2 (JQ308548.1). The TO53.2 strain sequence was homologous up to 94% with the two strains *B. subtilis* Pe-Lg-1 (FR687210.1) and *B. subtilis* YT2 (HQ143571.1). The optimal conditions for the growth and development of these two strains were with a 0.6% concentrations of NaCl, a pH of 7, and a temperature of 35°C.

Keywords: Antibacterial activity, *Bacillus subtilis*, Muong Khuong chilli sauce, pathogen bacteria.

Phân lập, định danh và sơ bộ xác định đặc tính của các chủng *Bacillus subtilis* có phổ kháng khuẩn rộng từ tương ớt Mường Khương

TÓM TẮT

Vi khuẩn *Bacillus subtilis* xuất hiện nhiều trong các thực phẩm như xúc xích khô, pho mát, sữa lên men truyền thống…, chúng kết hợp với các vi sinh vật khác trong quá trình lên men sinh ra các enzyme phân giải tinh bột, lipid, protein… *B. subtilis* không chỉ đóng vai trò như vi khuẩn probiotic mà còn sinh ra nhiều các kháng chất vi sinh vật gây bệnh khác. Tương ớt Mường Khương là sản phẩm được lên men tự nhiên từ giống ớt Mường Khương đặc biệt cay, không qua xử lý nhiệt và có thể bảo quản trong điều kiện thường từ 1,5-2 năm. 512 loại khuẩn lạc trong đó có 48 chủng *Bacillus* sp. được phân lập từ 80 mẫu tương ớt Mường Khương. Khả năng kháng khuẩn gây bệnh của chủng phân lập được xác định bằng phương pháp khuếch tán qua giếng thạch thông qua đo đường kính vòng ức chế. Bốn chủng kiểm định sử dụng là *Escherichia coli* RG1.1, *Salmonella typhi* GT4.3, *Listeria monocytogenes* MI2.6, *Staphylococcus aureus* TS1.9. Kết quả giải trình tự gen 16S rRNA cho thấy hai chủng có phổ kháng khuẩn rộng là TO43.13 và TO53.2. Chủng TO43.13 có độ tương đồng về kiểu gen tới 95% so với chủng *B. Subtillis* BcX1 (JX504009.1) và *B. Subtillis* EPP2 2 (JQ308548.1). Chủng TO53.2 có độ tương đồng về kiểu gen tới 94% so với chủng *B. Subtillis* Pe-Lg-1 (FR687210.1) và *B. Subtillis* YT2 (HQ143571.1). Điều kiện tối ưu cho sự sinh trưởng và phát triển của 02 chủng được xác định trong môi trường có nồng độ NaCl 0,6%, pH7 và nhiệt đô 35°C.

Từ khóa: Khả năng kháng khuẩn, *Bacillus subtilis*, tương ớt Mường Khương, vi khuẩn gây bệnh.

1. INTRODUCTION

Nowadays, using chemicals to prevent food from pathogenic bacteria is not encouraged. Hence, the research on antimicrobial substances produced from Generally Recognized as Safe (GRAS) bacteria, such as antibiotics, is an inevitable trend. Most of the studies have focused on lactic acid bacteria (LAB) and *Bacillus* ssp. The ability of *Bacillus* to produce antimicrobial compounds was reported more than 50 years ago (Stein, 2005). Some species that have the ability to produce antimicrobial substances are *B. polymyxa, B. licheniformis, B. pumilus,* and *B. subtilis*, among others (Bauzzi *et al.,* 2011).

Recently, more and more research projects about antimicrobial compounds produced by *B. subtilis* have been carried out. The study by Plyush *et al.* (2013) showed that two antimicrobial peptides produced by *B. subtilis* strain SK.DU.4 could inhibit Gram-positive bacteria. Maximum production of antimicrobial compounds produced by *B. subtilis* and *B. pumilus* against *Staphylococcus aureus* and *Micrococcus luteus* were observed at pH 8.5 and 5% glucose after 24 h of incubation at 30°C (Awais *et al.*, 2007). According to the study of Awais (2010), antibacterial activity produced by *B. subtilis* reached a maximum at pH 8 after 4 hours of incubation with various glucose concentrations.

In addition, *B. subtilis* also has antifungal properties. *B. subtilis* MTCC-8114 produces antifungal and antibiotic peptides that inhibit *Microsporum fulvum* and *Trichophyton* species (Kumar, 2009). According to the study of Oyedele *et al.* (2014), nine strains of *B. subtilis* isolated from the soil showed antifungal activity against indicator fungi such as *Aspergillus niger, A. flavus, Fusarium oxysporum,* and *Rhizopus stolonifer.*

Muong Khuong is located in a high mountainous district in the North of Vietnam and has harsh weather conditions. Chilli sauce is one of the special dishes from this mountainous area, and it is made from natural chilli peppers, garlic bulbs, fennel seeds, coriander seeds, cardamom, cinnamon, wine, salt, and water. Muong Khuong chilli sauce is well-known as a very special product because it is very spicy and does not undergo any heat treatment processing but it still can stay preserved for 1.5 to 2 years. A recent study has shown that microorganisms isolated from Muong Khuong chilli sauce are not only lactic acid bacteria but also bacteria from the *Bacillus* genus.

The objective of this study was to isolate, identify, and characterize *B. subtilis* bacteria from Muong Khuong chilli sauces to (i) obtain strains with broad-range capabilities against pathogenic microbes; (ii) diversify the microorganism sources from indigenous fermented foods; and (iii) orient to apply the pure cultures to traditional fermented foods in a controlled manner to ensure food safety.

2. MATERIALS AND METHODS

2.1. Materials

A total of 80 Muong Khuong chilli sauce samples were collected from different regions in Muong Khuong, Lao Cai province. The samples were taken aseptically and packaged in sterilized bags, then stored at 4°C. The isolation of *B. subtilis* was performed within 48 hours of being collected.

Test microorganisms *Escherichia coli* RG1.1*, Salmonella typhi* GT4.3*, Listeria monocytogenes* MI2.6*,* and *Staphylococcus aureus* TS1.9 were supplied from the collection of bacterial strains in the Faculty of Veterinary Medicine, Vietnam National University of Agriculture.

2.2. Methods

The isolation method for *B. subtilis* was modified based on the description of Yilmaz *et al.* (2006).

The identification methods for *B. subtilis* utilized taxonomy standards and biochemical reactions. Identification tests included Gram staining, motility, starch hydrolysis, catalase activity, spore formation, and methyl-red reaction. The DNA extraction process was performed using the CTAB method with some modifications. The PCR products were extracted via a PureLink^{TM} Quick Gel Extraction Kit according to the manufacturer's instructions. Extracts of PCR products were sequenced directly by PCR primers from the Macrogen company (South Korea). The sequences were identified after comparing them to previously published sequences available in the search software BLAST from NCBI (The National Center for Biotechnology Information).

Determination of the inhibitory effect of the isolates on test bacteria was carried out according to the agar-well diffusion method; the inhibition zone was measured using calipers (Reinheimer *et al.,* 1990).

The physiological characterizations of *B. subtilis* were investigated under different incubation conditions: NaCl concentration: 0.2, 0.4, 0.6, 0.8, and 1%; pH: 5, 6, 7, 8, and 9; and incubation temperature: 25, 30, 35, 40, and 45°C.

3. RESULTS AND DISCUSSION

3.1. Identification of *Bacillus* isolates from Muong Khuong chilli sauce

Bacillus was isolated from 80 Muong Khuong chilli sauce samples, which were collected from Muong Khuong, Lao Cai, Vietnam. More than 550 putative colonies were tested for morphology and biochemical reactions. The results are reported in Table 1.

Table 1 shows that most of the strains isolated from Muong Khuong chilli sauces were collected in the summer (80%). Although the number of samples collected in the summer and spring were approximately the same, the number of bacteria isolated in the summer was six times higher than in the spring. This can be explained by the fact that the temperature and moisture in the summer were suitable for the growth and development of these bacteria. The total kinds of colonies isolated reached 552.

Six tests, including spore formation, catalase activity, Gram staining, motility, starch hydrolysis, and methyl red reaction, were conducted to identify the *Bacillus* species. The results illustrated that less than 10% of the bacteria isolated were identified to be *Bacillus* species. Forty eight isolated colonies were *Bacillus* with positive results in all 6 tests. Interestingly, all of them were isolated from the summer samples. The 48 *Bacillus* isolates were preserved in glycerol-stock at -80^{0}C for continued testing for their abilities against pathogenic bacteria.

3.2. Selection and preliminary characterization of *Bacillus* isolates with broad-range antimicrobial activity

3.2.1. Antimicrobial activity of Bacillus isolates

The results of the antimicrobial activity of the isolated *Bacillus* colonies are shown in Table 2. It was observed that some strains of *Bacillus* had no inhibitory effects while others had an inhibitory effect on all four of the test bacteria. Among the 48 *Bacillus* isolates, there were 10 strains that had pathogen antimicrobial activity. It was noted that the pathogen antimicrobial activity of these strains was not from acidic conditions, as *Bacillus* did not produce lactic acid. Eight of the strains were able to inhibit the growth of *L. monocytogenes* MI2.6 (strains TO32.13, TO36.35, TO41.11, TO43.13, TO46.6, TO47.6, TO48.1, and TO53.2). The data clearly indicated that the antimicrobial activity of the above mentioned *Bacillus* strains had a greater effect on Gram positive bacteria than on Gram negative bacteria.

Table 1. Isolation of *Bacillus* sp. from Muong Khuong chilli sauce

Collecting season	Number of samples	Code of samples	No. of kinds of colonies isolated	No. of *Bacillus* sp. isolated
Spring	30	TO1 - TO30	72	0
Summer	34	TO31 - TO64	435	48
Autumn	16	TO65 - TO80	45	0
Total	80		552	48

Table 2. Results for the ability against pathogenic bacteria from isolated *Bacillus* species

No.	Code of strain	*E.coli* RG1.1	*S. typhi* GT4.3	*L. monocytogenes* MI2.6	*S. aureus* TS1.9
1	TO32.13	-	-	+	-
2	TO36.35	-	-	+++	-
3	TO41.11	-	-	++	-
4	TO43.13	+++	+++	+++	+++
5	TO44.1	+++	-	-	-
6	TO46.6	-	-	+	-
7	TO47.6	-	-	+++	-
8	TO48.1	-	-	+	-
9	TO53.2	++	++	++	++
10	TO56.3	-	-	-	++

Note: +: 2 mm ≤ Diameter of inhibited zone < 4 mm; ++: 4 mm ≤ Diameter of inhibited zone < 6 mm; +++: 6 mm ≤ Diameter of inhibited zone; - : no antimicrobial activity observed

Three strains of *Bacillus* sp., TO36.35, TO43.13, and TO47.6, showed the highest anti-*L. monocytogenes* MI2.6 activity. Besides, there were only 3 strains with anti-*E. coli* RG1.1 activity (TO43.13, TO44.1, and TO53.2), 2 strains with anti-*S. typhi* GT4.3 activity (TO43.13 and TO53.2), and 3 strains with anti-*S. aureus* TS1.9 activity (TO43.13, TO53.2, and TO56.3). These results showed that the *Bacillus* strains isolated in this study had higher resistance to Gram positive bacteria than Gram negative, which was different from the reports of Awais (2007) who stated that *B. subtilis* did not show any inhibition against *S. aureus*, a Gram positive bacteria. In addition, Perez *et al.* (1992) noted that the antimicrobial activity produced by *B. subtilis* MIR15 appeared to mainly be active against Gram negative bacteria including *E. coli.*

Two strains, TO43.13 and TO53.2, had broad-range antimicrobial activity and could inhibit all four food pathogenic bacteria tested. The results for the morphology and genotype sequencing are illustrated in Table 3.

It is clearly seen that each strain had different colony morphological characteristics. The colonies of *Bacillus* sp. TO43.13 were white, relatively large, concave in the middle like craters, and the surface was dry, wrinkled, and had a stench. Meanwhile, colonies of *Bacillus* sp. TO53.2 were white, flat, and had a dry surface and uneven border.

The results of 16S rRNA sequencing indicated that the genetic material of *Bacillus* sp. TO43.13 demonstrated similarities with the *B. subtilis* strain BcX1 (JX504009.1) and *B. subtilis* strain EPP2 2 (JQ308548.1) genotypes to 95%. Genetic sequencing of TO53.2 demonstrated similarities with the *B. subtilis* strain Pe-Lg-1 (FR687210.1) and *B. subtilis* strain YT2 (HQ143571.1) genotypes to 94%. In addition, based on the results of the colony, cell morphology, and biochemical tests, it can be concluded that TO43.13 and TO53.2 are *Bacillus subtilis* strains.

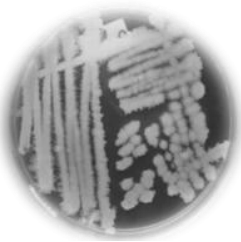
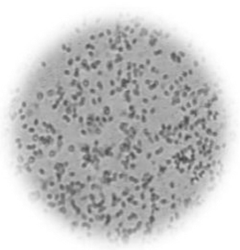
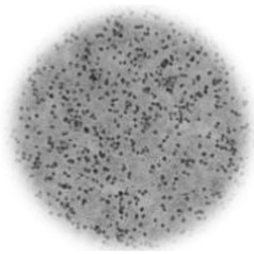
Hence, the two strains TO43.13 and TO53.2 were chosen for the physiological characterization study.

3.2.2. Physiological characterization of B. subtilis strains with broad-range antimicrobial activity

The growth of *B. subtilis* was studied under different incubation conditions, including NaCl concentration, pH medium, and incubation temperature.

As seen in the above figures, the growth and development of *B. subtilis* were affected by NaCl concentration, pH medium, and incubation temperature. Fig. 1a and Fig. 1b indicate that the two B. subtilis strains grew in NaCl concentrations from 0.2 to 1%, and a pH range of 6 to 9. However, the development of both strains was strongest in the medium containing NaCl at 0.6% and having a pH of 7. The strain TO43.13 exhibited its maximum growth after 22-24 h of incubation, while strain TO53.2 only needed 20 h to reach its maximum growth. At pH 5, the growth of both strains was inhibited. The range of temperatures from 30 to 40ºC was suitable for the growth of these two *B. subtilis* strains, but 35ºC was the optimal temperature. Notably, the growth of strain TO53.2 was inhibited at 25ºC, hence, it is clearly seen that strain TO53.2 was more thermal sensitive than strain TO43.13. This result was nearly similar with the report of Younis *et al.* (2010), which stated that the maximum growth of the *B. subtilis* KO strain was in the medium with a pH range between 6.5 and 7. Korsten *et al.* (1996) reported that the optimum incubation time for highest yield was achieved from a 24 h duration at 30-37ºC.

Table 3. Preliminary characterization of *Bacillus* sp. with broad-range antimicrobial activities

Code of colony	*Bacillus* sp. TO43.13	*Bacillus* sp. TO53.2
Colony morphology		
Cell morphology		
Result for 16S rRNA sequencing	95% ident *Bacillus subtilis* (JX504009.1, JQ308548.1) 95% ident *Bacillus amyloliquefaciens* (KT375322.1, KF964025.1) 95% ident *Bacillus* sp. (KF482852.1, KR258755.1) 95% ident *Bacillus methylotrophicus* (KC790325.1, KP851947.1)	94% ident *Bacillus subtilis* (FR687210.1, HQ143571.1) 94% ident *Bacillus pumilus* (FR687211.1, KM405294.1) 94% ident *Bacillus* sp. (KF863820.1, KT308215.1) 94% ident *Bacillus altitudinis* (JX475110.1, KU898276.1) 94% ident *Bacillus stratosphericus* (KR140182.1, KT072094.1) 94% ident *Bacillus aerophilus* (KC172027.1, KT719657.1)

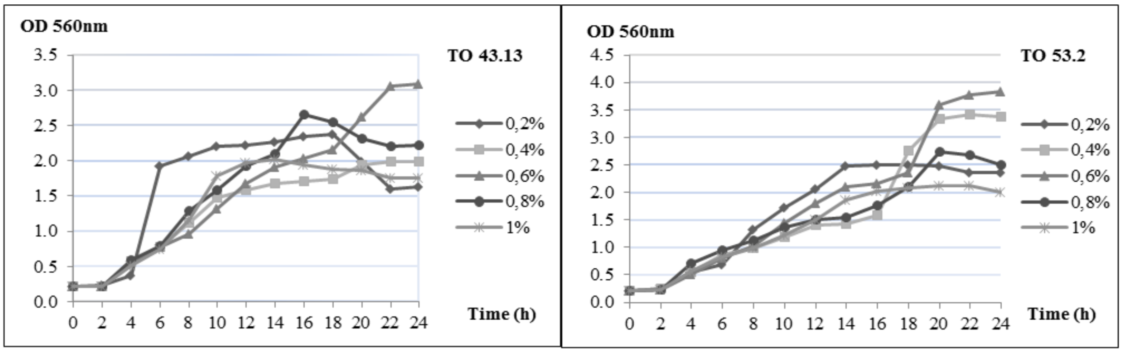


Fig 1a. Effect of NaCl concentration on the growth and development of *B.subtilis*

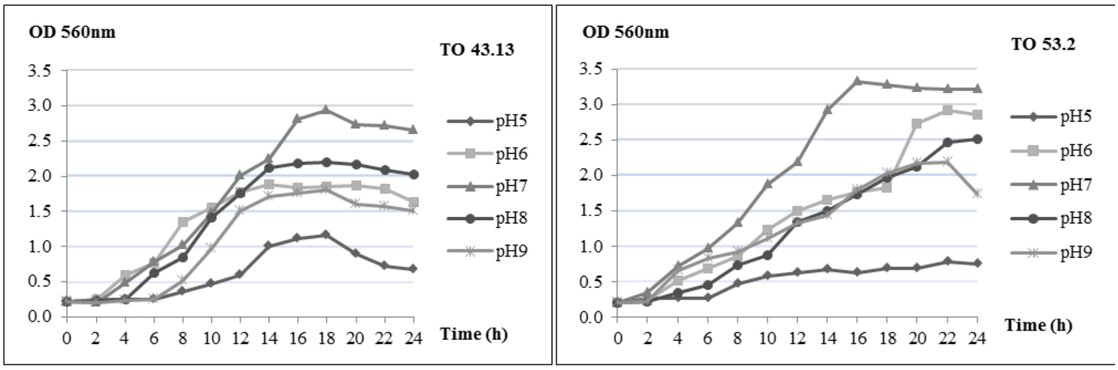


Fig 1b. Effect of pH medium on the growth and development of *B. subtilis*

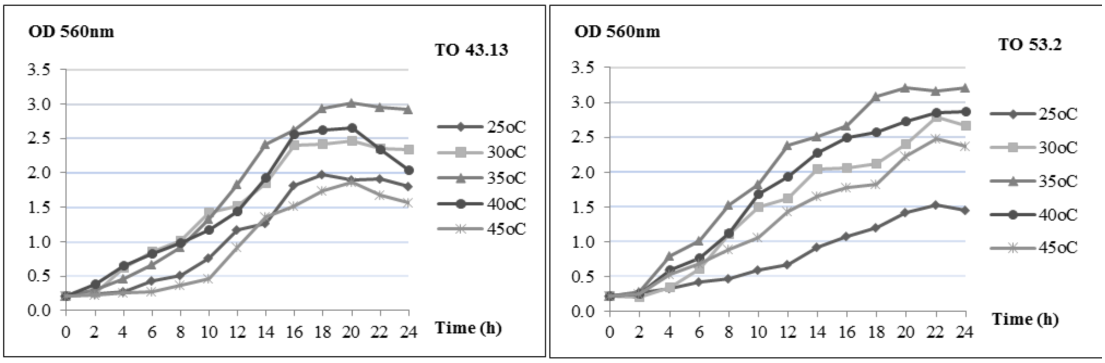


Fig 1c. Effect of incubation temperature on the growth and development of *B. subtilis*

It has been demonstrated that the strains TO43.13 and TO53.2, which can produce pathogen antimicrobial substances, have growth conditions close to other *B. subtilis* strains.

4. CONCLUSIONS

The results revealed a variety of *Bacillus* ssp. in chilli sauce samples collected in the different seasons from the Muong Khuong district with 48 *Bacillus* species collected in total. The present study discovered two *B. subtilis* strains with broad-range antibacterial activity. For these *Bacillus* populations, the genetic sequencing test was used to determine the identity of the two strains that had the highest antibacterial abilities. Moreover, preliminary characterizations of the *B. subtilis* strains, such as colony morphology and cell morphology, were observed. The incubation conditions were studied and realized that a NaCl concentration of 0.6%, pH of 7, and a temperature of 35ºC were the best conditions for the growth and development of these two strains.

Although the study indicated the successful optimized cultivation conditions for two *B. subtilis* strains with broad-range pathogen antimicrobial activities (TO43.13 and TO53.2) in the laboratory, further deep research needs to be done before applying these strains in food preservation.

AKNOWLEDGEMENTS

A special thanks to the Research and Teaching Higher Education Academy - Committee on Development Cooperation (ARES-CCD) for financial support. Furthermore, thanks to the Faculty of Veterinary Medicine, Vietnam National University of Agriculture for supplying the pathogenic bacteria strains.

REFERENCES

Awais M., A. Pervez, A. Yaqub, and M. M. Shah (2010). Production of antimicrobial metabolites by *Bacillus subtilis* immobilized in polyacrylamide gel. Pakistan J. Zool., 42(3): 267-275.

Awais M., A. A. Shah, A. Hameed, and F. Hasan (2007). Isolation, identification and optimization of bacitracin produced by *Bacillus* sp. Pak. J. Bot., 39(4): 1303 - 1312.

Baindara P., S. M. Mandal, N. Chawla, P. K Singh., A. K. Pinnaka, and S. Korpole (2013). Characterization of two antimicrobial peptides produced by a halotolerant *Bacillus subtilis* strain SK.DU.4 isolated from a rhizosphere soil sample. AMB Express, 3:2.

Baruzzi L., L. Quintieri, M. Morea, and L. Caputo (2011). Antimicrobial compounds produced by *Bacillus* spp. and applications in food. Science against microbial pathogens: Communicating current research and technological advances, 2: 1102-1111.

Korsten L. and N. Cook (1996). Optimizing Culturing Conditions for *Bacillus subtilis*. South African Avocado Growers’ Association Yearbook, 19: 54-58

Kumar A., P. Saini, and J. N. Shrivastava (2009). Production of peptide antifungal antibiotic and biocontrol activity of *Bacillus subtilis*. Indian Journal of Experimental Biology, 47: 57-62.

Oyedele A. O. and T. S. Ogunbanwo (2014). Antifungal activities of *Bacillus subtilis* isolated from some condiments and soil. African Journal of Microbiology Research, 8: 1841-1849.

Reinheimer J. A., M. R. Demkov, and M. C. Condioti (1990). Inhibition of coliform bacteria by lactic cultures. Aust. J. Dairy Technol., pp. 5-9.

Rončević Z. Z., J. A. Grahovac, D. G. Vučurović, S. N. Dodić, B. Z. Bajić, I. Z. Tadijan, and Z. M. Dodić (2014). Optimization of medium composition for the production of compounds effective against *Xanthomonas campestris* by *Bacillus subtilis*. BIBLID., 45: 247-258.

Stein T. (2005). *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Molecular Microbiology, 56(4): 845-857.

Yilmaz M., H. Soran, and Y. Beyatli (2006). Antimicrobial activities of some *Bacillus* spp. strains isolated from the soil. Microbiol. Res., 161: 127-131.

Younis M. A. M., F. F. Hezayen, M. A. Nour-Eldein, and M. S. A. Shabeb (2010). Optimization of cultivation medium and growth condition for *Bacillus subtilis* KO strains isolate from sugar cane molasses. American-Eurasian J. Agric & Environ. Sci., 7(1): 31-37.