M.BYAMBAJAV[1]

[1]Laboratory of Microbiology, Institute of Plant Protection, Graduate School, Mongolian University of Life Science

ABSTRACT

The products of Bacillus subtilis are commonly used against grown plants diseases in many countries. B.subtilis has antagonist activity and is spore forming, gram positive, rod shaped, widely spread in the environment. It produces over 66 types of antimicrobial compounds. We have identified B.subtilis strain from soil in the vicinity of Ulaanbaatar, Bogd mountain, named B.subtilis as MN99 *strain and tested antagonistic activity against plant disease including Alternaria panax, Alternaria spp fungus and investigated possibility to use on alternaria infection.*

B.subtilis MN99 *strain which we isolated has been defined as being B.subtilis of Bacillis type in terms of its physiological, biochemical, molecule genetic characteristics. Using antagonist activity of B.subtilis* MN99 *strain against A.panax fungus, the activity was 51.66% after 2 weeks and against Alternaria spp infect to cucumber, the result was 50.88% high after 2 weeks of inoculation, respectively. Based on the result of the above study, it is shown that the B.subtilis* MN99 *strain can be used against Alternaria disease in plants.*

Keywords:

"mailto:ardiu99@yahoo.com"

INTRODUCTION

Ehrenberg identified the bacterium *(current Bacillus subtilis)*, gave and published the original name *Vibrio subtilis* in 1835, but the bacterium renamed *Bacillus subtilis* by Ferdinand Cohn and Koch in 1872 [2,18,19].At the end of XIX century, researchers deleted *B.subtilis* from *Bacillus* genus by some of its characteristics, including motile.

Then researchers studied and identified its common similar characteristics with *Bacillus cereus* (Frankland and Frankland 1887), *Bacillus antracis* (Koch 1888) considering it is proper to include *B.subtilis* in one genus in terms of classification and included back in the *Bacillus* genus in earlier 1900s [18].*B.subtilis* is the best-characterized member of the *Bacillus* genus [13]. *B.subtilis* is commonly found in various ecological niches including soil, water and air [5]. *B.subtili s* aerobe, gram positive, motile, rod shaped and has main morphological characteristics that produces round oval spore in the center [18,19].

Preparations made of this bacterium are considered as having significant activity against diseases of grown plants and is widely used throughout the world. Lipopeptide antibiotic like compounds of several groups that is made synthetic by *Bacillus* has significant effect over many plant diseases [15,17]. Broggini and others (2005) identified antagonist activity of *B.subtilis* bacterium against plant fungus [4].According to Berdy.J (1974) *B.subtilis* makes 66 types of antimicrobial like compounds including surfactin, polymyxin, difficidin, subtilin, mycobacillin, bacitracin synthetic [3, 7].It has been estimated that 14.1% of total harvest or harvest of USD220 billion in the world every due to the plant diseases, only [1].Longer period of using chemicals for controlling plant diseases results in accumulation of chemicals in soil, plants and both human and animal bodies and becomes main conditions causing serious diseases or cancers [14].

Biological methods driven from microorganisms should be used for controlling plant disease instead of using powerful, chemical synthetic pesticide and fungicide. Using high dose chemical fungicide to prevent against and treat plant diseases for longer period will cause soil pollution and negative influences on human health. Biological control has advantages such as selected effect against the plant disease and requires no decomposition time after using [9].

The biological control of plant pathogenic fungi has received considerable attention as an alternative strategy [10]. Fungal plant disease is one of the major concerns to agricultural food production worldwide. Many strains of *B.subtilis* have been shown to be potential biocontrol agent against fungal pathogens [6]. *B.subtilis* has been widely studied as a potential biological agent against various plant diseases [8,20]. *Alternaria* is a fungus commonly spread in the soil and plants and becomes the causes of diseases in plants of various species, as well as a reason of dropping plant production rate. *Alternaria* produces toxins including alternariol, alternariol monomethyl ether, altenuen, tenuazoic acid, and altertoxins which lead to adverse impacts on food safety [11]

RESEARCH MATERIALS AND METHODS

Culture used for the research *B.subtilis* MN99 strain identified from soil in the vicinity of Bogd mountain, Ulaanbaatar (E=642652, N=5302748) has been used for the research. Pure culture of *A.panax* and *Alternaria spp* that caused plant disease as obtained from the Institute of Plant Protection, Laboratory of Microbiology and used for the research.

Method to culture *B.subtilis* MN99, *Alternaria sp* *B.subtilis* MN99 strain was inoculated in the potato and mineral broth and glucose containing medium with pH 7 to 7.5 and cultured

in shaking incubator at temperature 37^{0}C for 72 hours. *A.panax, Alternaria spp*-were grown on a PDA at temperature 25^{0}C for 7 days.

Detection and identification method of *B.subtilis* strain Morphological, physiological and biochemical characters of *B.subtilis* MN99 strain were identified as per commonly used method. Pure culture of *B.subtilis* MN99 strain which were grown under Nutrient agar medium was put in phosphate buffer solution, killed bacteria by boiling and isolated genomic DNA as per commonly used method.

Amplification of 16S ribosomal RNA gene fragment was made by use of fD1 and rP2 primers. Amplified fragment of 16S ribosomal RNA gene was purified from the gel by DNA purification kit.

PCR recipe (per unit:30 µL): 10x PCR buffer -2.5 µL, dNTPS -1.5 µL, $MgCl_2$ -2.0 µL, Taq-0.3 µL, Primer F (20 µM)-0.5 µL, Primer R (20 µM)-0.5 µL, tDNA (30-50 ng)-1.0 µL, dH_2O-21.7 µL

PCR heat exchange cycle: commencement stage 95°C -5 minutes, denaturation stage 94°C -1 minute, stage to annealing 59 (60)°C -1 minute, elongation stage 72°C -50 seconds, amplification stage 72°C -7 minutes, reaction was conducted under condition to conduct total of 34 cycles reaction and store 4°C. Genes sequence of 16S ribosomal 1364 pb product was identified, search was made by using BLAST (NCBI) with homologous sequence of Gen Bank and relationship was identified by drawing phylogenetic relationship by MEGA 7 software.

In vitro testing of antagonist activity of *B.subtilis* MN99 local strain In vitro testing of antagonist activity of *B.subtilis* MN99 strain has been conducted in accordance with dual culture method. Antagonist activity of *B.subtilis* MN99 strain was measured by comparing growth of control causative agent *Alternaria* with growth of *Alternaria* fungus grown in the center of *B.subtilis* MN99 on 5th and 14th days

$$antagonist\ activity\ \% = \frac{(a1 - a2)}{a1} * 100$$

a1-size of colony of fungi in control dish
a2- size of colony of fungi in a dish with *B.subtilis*

RESULTS OF THE STUDY

Some biological characteristics of *B.subtilis* MN99 strain *B.subtilis* MN99 local strain scattering or motile when growing it by sticking under semi – liquid agar condition.

Smear was prepared from *B.subtilis* MN99 strain which was grown for 24 hours, painted by Gram method and looked through microscope it was rod shaped, single or in short chains, Gram positive and produced spore in the center. *B.subtilis* MN99 had brighter colonies growing under NA medium and culture from 24 hour culture was taken by loop, put on the microscope slides and added hydro peroxide then there was bubble created or it had catalase activity.

Amylolytic activity of the isolates were measured by culturing on Starch agar medium for 7 days, followed by dropping iodine and then starch was hydrolyzed forming illuminated zone.

Identification of *B.subtilis* MN99 local strain species RNA was separated from culture grown from *B.subtilis* MN99 local strain under 36^{0}C NA medium for 48 hours as per methodology and conducted PCR using fD1 and rP2 primers. Sequence of 16S genes were identified in PCR product.

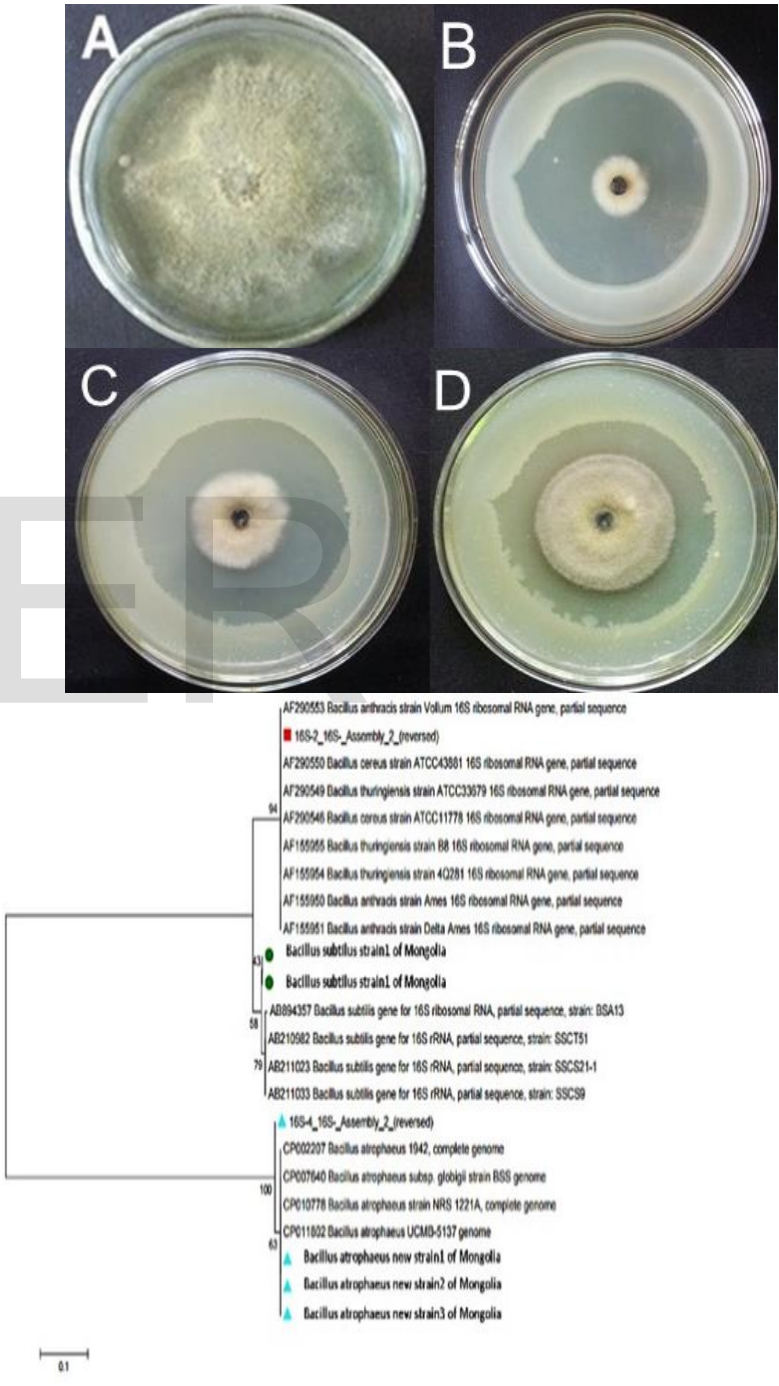

Figure 1. Comparison of phylogenetic relationship of *B.subtilis* MN99 strain Ribosomal 16S genes

Search of nucleotide sequence of 16S ribosomal RNA gene in *B.subtilis* local strain, made by using BLAST (NCBI)

with homologous sequence of bacterium belonging to the genus *Bacillus sp* stored in GenBank database and phylogenetic relationship comparisons by MEGA 7 software proved that its relationship was the closest to nucleotide sequences of *B.subtilis* gene. Accordingly, based on sequences of 16S ribosomal RNA gene, it is proven that the local strain is *B.subtilis*.

Estimation of antagonist activity of *B.subtilis* MN99 strain

Suspension of *B.subtilis* MN99 strain with $1x10^8$ titration, 0.05 ml aliquots was inoculated by spatula on PDA medium along the rim of Peter dish with width of 2 cm. After 24 hours of inoculation, the pure culture of causative agent was inoculated on 7 mm diameter area in the center of dish. Fungus *A.panax* started to grow on PDA medium at day 3 and growth rate increased at days 5 and 7, and the fungi covered fully the surface of nutrient medium at day 10. Results of testing antagonist activity of *B.subtilis* MN99 strain against fungus *A.panax* are shown in figure 2. According to the diameter measurement of growth rate of fungus *A.panax*, inoculate din the center of *B.subtilis* MN99 strain, fungus grew 3.93 cm on 5th day and 4.35 cm on 14th day in diameter.*B.subtilis* MN99 started to inhibit the growth of fungus *A.panax* from day 5 and fully stopped the growth from day 10. Measurements till day 28 revealed there was no any growth of the fungus or higher inhibition activity was found.

Figure 2. Testing of antagonist activity of *B.subtilis* MN99 strain against fungus *A.panax*. (A). 14 day growth of *A.panax*.(B-C). Growth pattern of *A.panax* in the center of *B.subtilis* strain. 5, 10 and 28 day growth.

Antagonist activity was estimated by comparative method through 6 repeats of 5 and 14-day growth and shown in Table 1.

TABLE 1.
Antagonist Activity Of *B.Subtilis* MN99 Strain Against *A.Panax*

Repetition	5 days			14 days		
	A.panax /control/, cm	*B.subtilis* MN99 + *A.panax*, cm	Antagonist activity , %	*A.panax* /control/, cm	*B.subtilis* MN99 + *A.panax*, cm	Antagonist activity, %
1	5.88	3.98	32.38	9.00	4.46	50.44
2	5.88	3.81	35.22	9.00	4.33	51.89
3	5.88	3.85	34.56	9.00	4.28	52.49
4	5.88	4.11	30.07	9.00	4.45	50.56
5	5.88	3.95	32.87	9.00	4.33	51.89
6	5.88	3.89	33.84	9.00	4.26	52.67
Average	5.88	3.93±0.11	33.16±1.83	9.00	4.35±0.08	51.66±0.95

According to Table 1, *B.subtilis* MN99 strain has antagonist activity of 33.16% on 5th day and 51.66% on 14th day against fungus *A.panax* which causes plant disease.

Test results of antagonist activity of *B.subtilis* MN99 strain against *Alternaria spp* are shown in Figure 3 and Table 2. Growth rate of fungus *Alternaria spp* inoculated in the center of *B.subtilis* MN99 strain was 3.75 cm on 5th day and 4.42 cm on 14th day in terms of diameter.

Measurements till day 28 revealed there was no any growth of the fungus or higher inhibition activity was found.

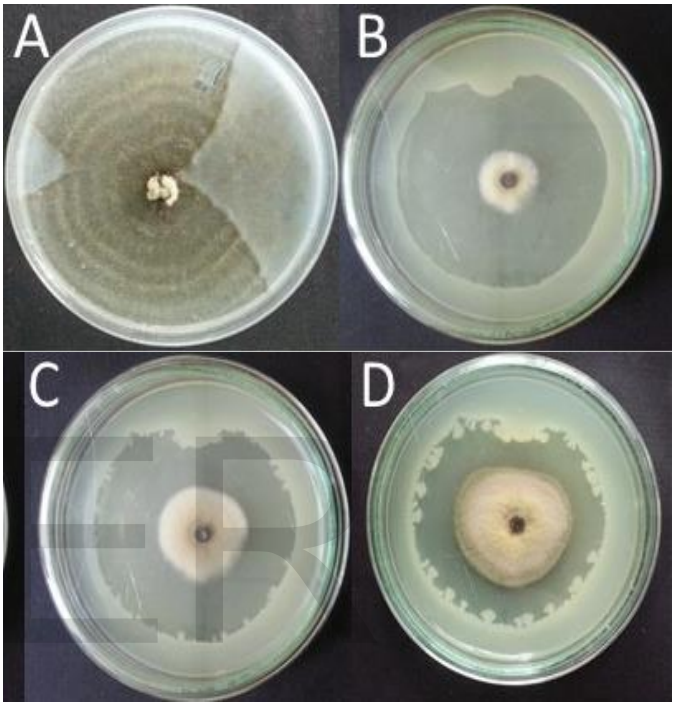

Figure 3. Testing of antagonist activity of *B.subtilis* MN99 strain against fungus *Alternaria spp* (A). 14-day growth of control *Alternaria spp* (B-D). 5th, 10th and 28th day growth of *Alternaria spp* in the center of *B.subtilis* MN99 strain.

Antagonist activity was estimated by comparative method through 6 repeats of 5 and 14-day growth and shown in Table 2

TABLE 2
Antagonist Activity Of *B.Subtilis* MN99 Strain Against-Alternaria-Spp

According to Table 2, *B.subtilis* MN99 strain has antagonist activity of 39.02% on 5th day and 50.88% on 14th day against fungus *Alternaria spp* which causes cucumber disease. Experimental errors were calculated by using SPSS-23 software. Antagonist activity of *B.subtilis* MN99 strain against the above disease causing fungus *Alternaria panax, Alternaria spp* and differences of control and trial groups were determined as 95% probability by one – way analysis of variance.

DISCUSSION

Historically, Ehrenberg named the bacterium as *Vibrio subtilis* in 1835, while Cohn renamed *Bacillus subtilis* in 1872. It has been informed that *B.subtilis* aerobic, gram positive, motile, rod shaped and has main morphological characteristics that produces round oval spore in the center.

Having studied some biochemical characteristics, it was defined as being catalase positive and hydrolyzes the starch [2,18,19]. *B.subtilis* MN99 strain which we have isolated from the soil as pure is same with those reported by othert researchers as its morphological and biochemical characteristics including Gram positive motile rods, occurs singly and in short chains, forms spore, has catalase activity, hydrolyzes the starch.

Results of measurement of growth rate of *A.panax*, grown on the center of *B.subtilis* were 3.93 cm on 5th day whereas, growth rate of *Alternaria spp* was 3.75 cm on 5th day. Antagonistic activity against *A.panax* was 33.16% and 39.02% against *Alternaria spp* on 5th day respectively, having average antagonist activity 36.09%. According to results of study by Kaltima, Phichai et al [12] antagonistic activity of *B.subtilis* against both *Alternaria spp* and *A.alternate* were 32.89% and 38.67% respectively having average antagonist activity of 35.78% and they are in agreement with those at days 5 to 7 in our study. According to our result, *B.subtilis* MN99 strain has antagonist activity of 51.66% against *A.panax*, 50.88% against *Alternaria spp*, having average antagonist activity of 51.27 % whereas, according to Moges.M.M et al [16] it antagonist activity of *B.subtilis* against fungus *Alternaria spp* was 43.79% in vitro condition on 14th day.

CONCLUSION

Repetition	5 days			14 days		
	Alternaria spp	*B.subtilis* MN99 + *Alternaria*	Antagonist activity, %	*Alternaria spp*	*B.subtilis* MN99 + *Alternaria*	Antagonist activity, %
1	6.15	3.83	37.79	9.00	4.56	49.33
2	6.15	3.78	38.60	9.00	4.38	51.39
3	6.15	3.74	39.27	9.00	4.45	50.58
4	6.15	3.76	38.93	9.00	4.24	52.87
5	6.15	3.69	39.93	9.00	4.55	49.44
6	6.15	3.71	39.61	9.00	4.35	51.67
Average	6.15	3.75±0.05	39.02± 0.76	9.00	4.42±0. 12	50.88±1. 37

1. *B.subtilis* MN99 local strain was determined as being *B.subtilis* species by its physiological, biochemical and molecule genetic characteristics and its antagonist activity was tested against fungus *Alternaria*.
2. *B.subtilis* MN99 local strain has antagonist activity of 33.16% on 5th day and 51.66% on 14th day against fungus *A.panax* which causes plant disease.
3. *B.subtilis* MN99 local strain has antagonist activity of 39.02% on 5th day and 50.88% on 14th day against *Alternaria spp* which causes cucumber disease.
4. It is has identified that *B.subtilis* MN99 can be used as a biological preparation for controlling *Alternaria* disease in plants further.

ACKNOWLEDGEMENT

The present study was performed by the funding of Governmental Scientific and technological foundation and we express our sincere thanks to our colleagues for their supports to this research.

REFERENCES

[1] J.N.Agrios., Plant pathology. Fifth edition. pp 414-426, 2004

[2] S.O.Alina, C.C.Petruta., "Biodiversity of Bacillus subtilis group and beneficial traits of Bacillus specie useful in plant protection". Romanian Biotechnological Letters Vol. 20, No.5, pp 10737-10750, 2015.

[3] J. Berdy., "Recent developments of antibiotic research and classification of antibiotics according to chemical Structure". Advances in Applied Microbiology.Volume 18, Pages 309-406, 1974.

[4] G. Broggini, B. Duffy, E. Holliger, H. Schärer, C. Gessler and A. Patocchi., "Detection of the fire blight biocontrol agent *Bacillus subtilis* BD170 (Biopro®) in a Swiss apple orchard". European Journal of Plant Pathology. Volume 111, Issue 2, pp 93–100, 2005

[5] D.W. Edgecomb, D. Manker., "Bacillus subtilis strain QST 713, bacterial disease control in fruit, vegetable and ornamental production". *Biocontrol of Bacterial*

Plant Diseases", 1st Symposium. No.408 pp.167-169, 2006

[6] O.I.M. Elhamshary and A.A. Khattab., "Evaluation of Antimicrobial Activity of Bacillus subtilis and Bacillus cereus and Their Fusants Against Fusarium solani". Research Journal of Cell and Molecular Biology, 2(2): pp 24-29, 2008

[7] B.B. Elsayed, S.M.H. Kamel, and M.M. Hassan., "Production of Antimicrobial Metabolites by *Bacillus subtilis* and their Applications". Biotechnology. Volume 12 (1): pp. 14-24, 2013.

[8] J.H.S.Ferreira, F.N.Matthee, A.C.Thomas., "Biological control of Eutypa lata on Grapevine by an antagonistic strain of Bacillus subtilis". Phytopathology, 81. pp. 283-287, 1991.

[9] K. Han-Soo, P. Jiyong, Ch. Sung-Won, Ch. Kee-Hyun, L. Gung Pyo, B. Soo Jung, L. Chang Hoo and K. Chung Sun., "Isolation and characterization of Bacillus strains for biological control". The Journal of Microbiology, Volume 41 (3) pp.196-201, 2003

[10] M. El R. Ilham, Susan, M.W. Asswah and A.A.Omaima., "Biocontrol of Some Tomato Disease Using Some Antagonistic Microorganisms". Pakistan Journal of Biological Sciences Volume 6 (4): pp.399-406, 2003

[11] N.V. Jovana, S.B.Jovana, I.B.Marija, F.B.Ferenc, B.S.Vera, N.C.Jelena, G.A.Milica, ALTERNARIA spp. ON SMALL GRAINS. Food & Feed Research.Volume 39, Issue 2 pp 79-88. 2012

[12] P. Kaltima., "Biological control of tomato leaf blight disease by high cell density culture of antagonistic *Bacillus subtilis"*. Khon kaen agr. J. 42 sup, pp 106-112, 2014.

[13] F. Kunst, N. Ogasawara, I. Moszer, A.M. Albertini, G. Alloni., "The complete genome sequence of the gram-positive bacterium Bacillus subtilis". Nature. 390(6657) pp 249-56, 1997

[14] Makoto shoda."Bacterial control of plant diseases". Journal of Bioscience and Bioengineering.Volume 89, Issue 6, pp 515-521, 2000

[15] .M.Ongena, P.Jacques., "Bacillus lipopeptides: versatile weapons for plant disease biocontrol"., Trends in Microbiology. Volume 16, Issue 3, pp 115-125, 2008.

[16] M.M Moges,. T Selvaraj., M.T. Jebessa.., "Influence of Some Antagonistic Bacteria against Early Blight (*Alternaria solani* (Ell. & Mart.) Jones & Grout.) of Tomato (Lycopersicon esculentum Mill.)"The African Journal of Plant Science and Biotechnology 6 (Special Issue 1) Global Science Book , pp 40-44, 2012

[17] K. Nagorska, M.Bikowski, M.Obuchowskji., "Multicellular behaviour and production of a wide variety of toxic substances support usage of *Bacillus subtilis* as a powerful biocontrol agen". Acta Biochimica Polonica. pp54:495–508, 2007

[18] .Paul De Vos George M. Garrity Dorothy Jones Noel R. Krieg Wolfgang Ludwig Fred A. Rainey Karl-Heinz Schleifer William B. Whitman.,. Bergey's " Manual of Systematic Bacteriology". Volume 3, The Firmicutes, pp 21-80, 2009

[19] P. H. A.Sneath, N. S.Mair, H. E.Sharpe., "Bergey's manual of Systematic Bacteriology". Volume 2, pp 1104-1139, 1986.

[20] S.B.Shinde, and M.D.Sadgir., "Efficiency of Pseudomonas fluorescens and Bacillus subtilis against Phytophthora spp. in citrus"., International journal of Plant Protection, volume 9, issue 1, pp 15-20, 2016