MORPHOLOGICAL AND MOLECULAR BASED IDENTIFICATION OF ANTIFUNGAL *BACILLUS LICHENIFORMIS*

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ABSTRACT

At present, plant diseases caused by soil borne plant pathogens have major constraints on crop production. Which include genera Fusarium spp, Phytophtora spp, Sclerotinia and Altenaria. Due to this reason, chemical fungicides are routinely used to control plant disease, which is also true in Mongolian case. However, use of these chemicals has caused various problems including environmental pollution with consequence of toxicity to human health also resistance of some pathogens to these fungicides are present. Fortunately, an alternative method to reduce the effect of these plant pathogens is the use of antagonist microorganisms. Therefore, some species of the genus Bacillus are recognized as one of the most effective biological control agent.
Our research was focused to isolate Bacillus licheniformis, with antifungal potential, from indigenous sources. In the current study, 28 bacterial cultures were isolated from soil and fermented mare's milk also named as koumiss. Isolated bacterial cultures were identified according to simplified key for the tentative identification of typical strain of Bacillus species. As a result 8 strains were positive and further screened for antifungal activity against Fusarium spp and Alternaria solani. Out of these 8 strains 5 strains are selected based on their high effectiveness against fungal pathogens and for further confirmation Polymerase Chain reaction run for effective bacterial strains using specific primers B.Lich-f and B.Lich-r.

KEY WORDS: *Bacillus lichenifornis*, indigenous source, antifungal effect, PCR

INTRODUCTION

Bacillus is one of the large genera of bacterial strains belonging to the family *Firmicutes.* It is a rod shaped endospore bearing bacteria which has diverse strains ranging from strictly aerobic to facultative anaerobic. [3]
Distribution and habitat of the genus *Bacillus* are very diverse and isolated from various sources such as soil, water, air and even food. Among this genus species like *Bacillus licheniformis* is excellent candidates for production of antifungal antibiotics [4], which play an important role in biological control of plant pathogens [5].
Since chemicals are being widely used on all kinds of crop plants [6], which bearing known and unknown side effects and world is shifting to biological control relied on antagonist microorganisms.
Especially in our country major crops like wheat and potato fields are affected by soil borne fungal pathogens caused by *Alternaria* spp and less popular fungi like *Fusarium* spp are widely distributed in tomatoes grown in green house of Mongolia.

Therefore, in our study different bacterial strains were isolated from soil and traditionally made mare milk or koumiss and screened for antagonist effect against entomopathogenic fungal cultures. Finally, effective bacterial strains were identified using conventional and molecular techniques.

MATERIALS AND METHODS

Isolation of bacterial culture

Several bacterial cultures were isolated from soil samples collected from forest regions located at Erdene and Batsumer soum of Tuv province. Soil samples (1.0g) were mixed into 100ml normal saline, afterward serially diluted from 10^{-1} – 10^{-6} ratio with normal saline [11]. 100µl of each diluted samples were inoculated in nutrient agar medium (Biolab) and incubated at 37°C for 24 hours.

10 ml from each koumiss samples were added to 90ml sterile physiological solution and mixed well with vortex. 1ml of these samples mixed into 9ml sterile distilled water repeated the same steps as soil samples. All strains were inoculated on nutrient agar and transferred further to different medium depending on identification method test.

Identification of bacterial strain

The identification of a selected bacterial strain was performed on the basis of morphological biochemical and molecular characteristics.

Morphological characterization

Morphological characteristics such as colony morphology (color, shape, margin, elevation, and surface) and cell morphology (shape, gram reaction, and arrangement) of the selected bacterial strain were studied for identification [7].

Biochemical characterization

The bacterial strain was subjected to simplified key biochemical tests including catalase test, methyl red (MR) and Voges Proskauer (VP) tests, anaerobic growth, growth in 7.0% NaCl according to standard protocol [7].

Antifungal activity assay

Bacterial strains which showed positive on above test are further experimented for antifungal activity against *Fusarium spp* and *Alternaria solani* strain grown on Sabouraud agar (SA) for a week in 25°C. 100µl bacterial cultures, grown on Nutrient broth, were inoculated in Potato Dextrose Agar (PDA) medium and spread out with spatula and placed in incubator with 28°C. One week old fungal strains were cut to 7mm diameter and placed in the middle of PDA with 24h old bacterial culture and placed back in 28°C.

In order to determine the antagonist effect of bacterial strain we used method developed by J.B.Sinclair *et al* with slight modification. In this method following formula is used to calculate percentage of bacterial antagonist effect:

$$\frac{a_1 - a_2}{a_1} x 100 = \%$$

a_1- Diameter of fungi grown on control

a_2 – Diameter of fungi grown on *B.lichenifromis*

The study had been run for 2 weeks with control and repeated for 3 times.

DNA preparation and PCR amplification

Genomic DNA extraction was performed for molecular based identification of isolates. The genomic DNA was extracted using the CTAB method with added Proteinase K [8].

The amplification was carried out in My Genie 32 Thermal Block (Bioneer). The PCR reaction mixture was prepared in a final 80µl of reaction volume consisting of 8.0µl PCR reaction buffer, 8.0µl of dNTPs mix, 8.0µl of $MgCl_2$, 16µl of 0.2 pM primers, 2.0µl genomic DNA template.

The PCR was cycled once at 95°C for 30 sec, 35 repetitions at 95°C for 30sec, 50°C for 30 sec, 72°C for 1.30 sec, and once at 72°C for 10 min.

The sizes of DNA fragments were estimated using a 100 bp+ DNA ladder (Solarbio). For the analysis of amplification products, 10µl of each PCR product was used. The extraction of DNA was confirmed by running in 1.0% (w/v) agarose electrophoresis gel containing ethidium bromide and visualized under UV transilluminator [9].

RESULTS

Several bacterial strains were isolated from soil samples collected from different soums of Tuv province and few strains from different provinces. (Table 1). Out of these strains 1-4 were from koumiss sample of Hujirt soum, Uvurhangai province, 5-11 were from koumiss of Mogod soum of Bulgan province, 12-18 were from soil samples taken from Batsumber soum and 19-28 were from soil samples taken from Erdene soum of Tuv province.

Table 1

Bacterial strains isolated from indigenous sources

S №	Bacterial strain	Sources	Location
1	A1	koumiss	Hujirt, Uvurhangai
2	A2	koumiss	
3	A3	koumiss	
4	A4	koumiss	
5	A5	koumiss	Mogod, Bulgan
6	A6	koumiss	
7	A7	koumiss	
8	A8	koumiss	
9	A9	koumiss	
10	A10	koumiss	
12	X1	soil	Batsumber, Tuv
13	X2	soil	
14	X3	soil	
15	X4	soil	
16	X5	soil	
17	X6	soil	
18	X7	soil	
19	X8	soil	Erdene, Tuv
20	X9	soil	
21	X10	soil	
22	X11	soil	
23	X12	soil	
24	X13	soil	

The identification of the selected bacterial strain was done on the basis of morphological, physiological and biochemical characteristics as well as PCR on genomic DNA. On the basis of morphology, physiology and biochemical tests, the selected strain was identified as *B. licheniformis*. Based on simplified key for identifying Bacillus species catalase activity, Methyl red Voges-Proskauer (MR-VP) test, anaerobic growth, growth in 50°C and ability to grow in medium with 7% NaCl were tested on selected strains. As a result strain A1, A4, A7, A10, X4, X9, X10, X13 were shown to have positive on above tests (Table2).

Table 2

Morphological, physiological and simplified identification reactions of bacterial strains

Cellular characteristics	
Gram staining	Positive
Morphology	Rod
Motility	Motile
Spore	Central, elipsoidal to cylindrical in shape
Size	0.6-1.0µm in length
Colonial morphology	
Nutrient agar	Finely wrinkled, dull, opaque, adherent colonies
Simplified identification reactions	
Catalase	Positive

Methyl red	Positive
Voges Proskauer	Positive
Anaerobic growth	Positive
50°C	Positive
7% NaCl	Positive

Bacterial strains were further tested for antifungal activities against *Fusarium spp* isolated from dried and shed coniferous tree and *Alternaria solani* isolated from infected wheat seed.

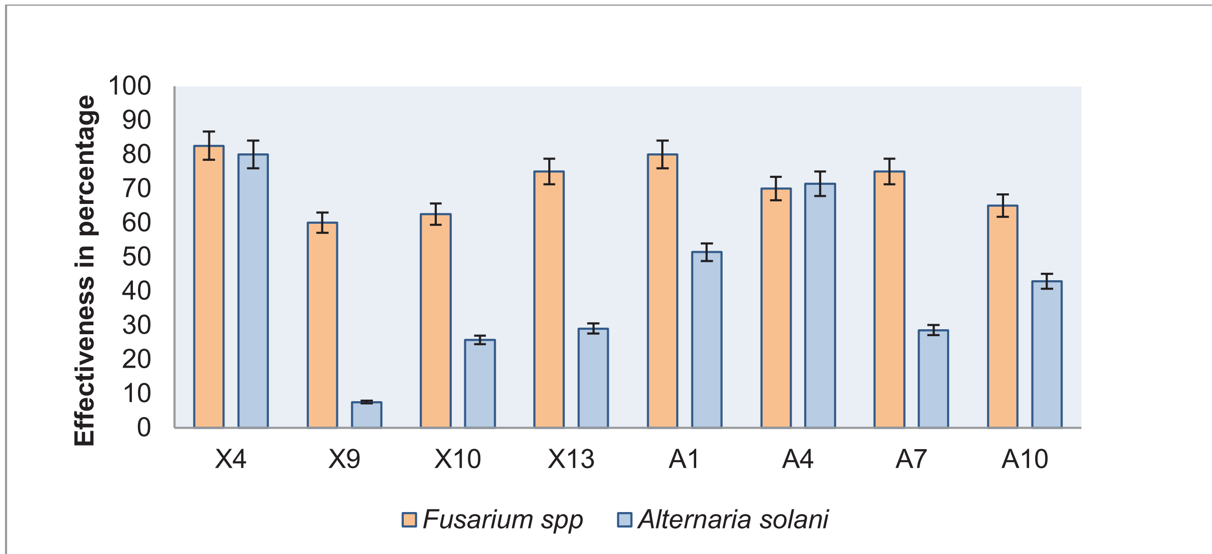

Figure 1. Effect of *Bacillus licheniformis* strain against fungal pathogens

Above figure illustrated 2 week results of antifungal activity of 8 strains of *Bacillus licheniformis* against *Fusarium* spp and *Alternaria solani*.

Strain X4 isloated from soil samples taken from Batsumber soum of Tuv province have showed 80-82.5% effective which was the highest antifungal activity against both fungal pathogens. Also strain A4 taken from koumiss samples originated from Hujirt soum of Uvurhangai province was 70-71.4% effective on both pathogens. Thus, 5 strains are selected based on their ability to inhibit 2 different types of fungal growth (Figure1).

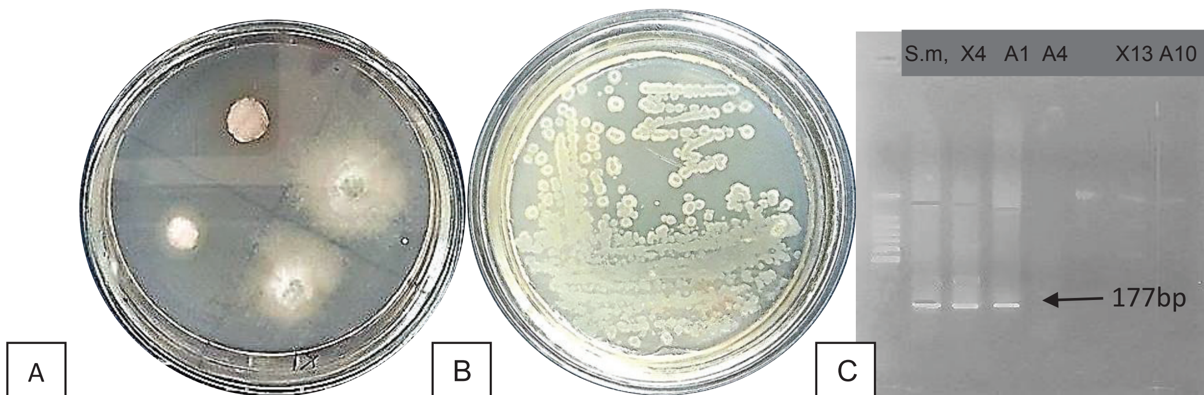

Figure 2. A, Result of antifungal activity of bacterial strain after 2 weeks. B, Pure culture of *B.licheniformis* used for DNA extraction. C, Result of agarose gel (1%) electrophoresis.

According to molecular identification PCR products X4, A1, A4 are given band in 177bp which matches with research done by Yue et al in 2014. Thus these three strains are proven to be *B.licheniformis*. (Fig 2-C).

DISCUSSION

B.licheniformis is widlely distributed in nature and found from various sources all over the world. Based on our study 2 strains of *B.licheniformis* are isolated from koumiss and one from forest soil taken from Batsumber soum of Tuv province. In 2014, Yue et al concluded that the size of the PCR product was 177bp for *B.licheniformis*. Based on our study 3 strains were given band on 177bp as well. This bacteria has antibiotic effect against fungal pathogens also synthesizes various ferments. In 2004, Rodica Mateescu et al have studied several strains of *B.licheniformis* which were effective against *Alternaria* spp. And strains isolated from koumiss and soil, were also effective against *Alternaria* spp according to our study.

CONCLUSION

Several bacterial strains were isolated from indigenous sources. On the basis of physiological, morphological and simplified biochemical tests 8 strain was identified as *B. licheniformis*. Out of these 8 strains 5 of them showed antifungal activity against fungal pathogens. DNA extraction and PCR analysis was performed to finalize the identification at molecular level and only 3 strains identified as a *Bacillus licheniformis* based on PCR amplification. 2 out of these three strains are isolated from koumiss which shows that bacterial strain contained in koumiss have higher antifungal effect than other strains found from soil samples.

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