



ISOLATION AND IDENTIFICATION OF BACTERIA CAPABLE OF BINDING TO AFLATOXIN

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ARTICLE INFO

Received:

03th Jun 2017

Accepted:

29th Nov 2017

Available online:

14th Dec 2017

Keywords: *Bio absorption, Coumarin, Aflatoxin, Spectrophotometry, Lactic Acid Bacteria*

ABSTRACT

Aflatoxin is one of the most dangerous mycotoxins produced under favorable conditions of relative humidity and high temperature by *Aspergillus* funguses. It rapidly disperses in the environment and causes a lot of damage to the food. Various microorganisms, including different types of Lactic Acid Bacteria, are capable of binding mycotoxins, such as Aflatoxins. Lactic Acid Bacteria are bound to the mycotoxins by polysaccharide and peptidoglycan components presenting in the cell wall, and lead to detoxicity and excretion of them. In the present study, Coumarin, due to its chemical structure similar to Aflatoxin, was used to isolate the bacteria with the ability to bind to mycotoxins. 20 isolates of *Lactobacillus* were isolated and purified using the enrichment and serial dilution techniques in the MRS (de Man, Rogosa and Sharpe) media. The ability of these isolates to bind to the Coumarin was evaluated using a spectrophotometer. Based on microscopic observations and biochemical tests, these isolates were classified as Lactic Acid Bacteria. 3 of 20 Lactic Acid Bacteria isolates had the highest ability to reduce 50 ppm of Coumarin after a week. The isolate *Enterococcus faecium* 2 showed the highest percentage of reduction in Coumarin (%19.6) than other isolates and the isolates *E. faecium* 1 and *E. casseliflavus* can reduce the Coumarin as much as %14 after a week. By examining Non-living biomass of bacteria, Non-living of the isolate *E. casseliflavus* showed the highest percentage of reduction in the Coumarin than other isolates. In addition, the isolates *E. faecium* 1 and *E. faecium* 2 reduced the Coumarin as much as %14 after a week. Based on 16S rDNA gene sequencing of the isolates with the ability to reduce Coumarin, two isolates of *E. faecium* and one isolate of *E. casseliflavus* were identified. Based on the results, it was observed that the samples collected from livestock and poultry farms can be a suitable source for isolating the bacteria which have the capability of decomposing mycotoxins in the environment, since these bacteria present in the digestive system of livestock and poultry, they can be used in the diet of them in order to reduce mycotoxins.

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To Cite This Article: Amir-Hossein Nazhand, Ghorban-Ali Nematzadeh, Ali Pakdin Parizi, Gholam-Ali Ranjbar, (2017), "isolation and identification of bacteria capable of binding to aflatoxin", *Pharmacophore*, 8(6S), e-1173211.

Introduction

Mycotoxins are of secondary metabolites of molds (*alternaria*, *claviceps*, *penicilium*, *aspergillus*, *fusarium*) and produced in reaction to stress conditions such as heat, cold and lack of oxygen and lead to contamination of food and feed [21]. The United

States Food and Agriculture Organization has reported that 25 percent of feeds of livestock and poultry are contaminated by mycotoxins and annually, it causes 1 billion dollars losses to livestock and poultry farms in the world [5]. In terms of public health and agriculture, the most important mycotoxins are including aflatoxin, trichothences, fumonisins, zearalenon, ochratoxin, patulin [5]. Fungal toxins (mycotoxins) existing in food cause serious problems in the health of humans and animals [3]. The main organ attacked by mycotoxins is liver. In human, they causes severe liver disorders and in animals, they cause disorders in digestive system, prevent the immune system's activity, reduce reproduction, increase the feed conversion ratio, reduce the production of milk and eggs, cause anemia, jaundice and growth retardation [11]. These toxins are found in milk, cheese, peanuts, corn, cottonseed, almonds, seasonings, figs, sorghum, dry bread and in various types of human and animal feeds [1, 4, 15]. Different chemical, physical and biological methods are used to control Aflatoxin in food and forage [16, 21]. Since physical and chemical methods, in addition to high costs, have few effects on the control of Aflatoxin, today, researchers' attention is drawn to the use of biological methods for the disposal of toxins and the maintenance of food quality [7, 13]. Among microorganisms, bacteria are very diverse and can be easily separated from different environments, which is why they are a good option to be used in biological systems against toxins [2]. Lactic Acid Bacteria are bound to the fungal toxins by polysaccharide and peptidoglycan components presenting in the cell wall, and lead to detoxicity and excretion of them [11]. A number of Lactic Acid Bacteria, including: *Lb. Plantarum* yo, *lb Plantarum* mw, *lb Fermentum* rs2, *Lactobacillus fermentum* oyb *Lb. Brevis* and *Lactococcus* rs3, reduce Aflatoxin by inhibiting vegetative growth and sporulation of Aflatoxin producing fungi [20]. Additionally, the bacterium *Lb.rhamnosus* gg have shown their high ability to absorb Aflatoxin B1 and increase intestinal resistance in response to mycotoxins [1]. Investigations performed on *Saccharomyces cerevisiae* and Lactic Acid Bacteria have shown that the mixture of these two microorganisms decreases the contamination in the food caused by these toxins by absorbing mycotoxins through their compounds in the cell wall [22]. The results of the studies on the maize seeds contaminated by mycotoxins showed that the strains of *Lb. acidophilus*, *Lb. brevis*, *Lb. casei*, *Lb. delbruekii*, and *Lb. plantarum* Binding to the toxins of contaminated seeds and prevent their spread in the environment [17]. Also probiotic bacteria *Lb. Rhamnosus*, *lb. Podococcus*, *Lb. Shermaniis* and *Lb. Freudenreichii* orentent the spread of toxins by placing on the maize seeds contaminated with fungal toxins [16]. The researchers have investigated the ability of lactic acid producing bacteria to absorb AFB1 in the digestive system of hens and showed the significant reduction in Aflatoxins due to the presence of these bacteria [6]. The researchers have conducted studies on the binding mechanism of aflatoxin to *Lb.rhamnosus*. They concluded that the main factor reducing Aflatoxins is their binding to sugary compounds, and some protein components of the cell wall of bacteria [15]. In addition, the suggestions have shown that binding of AFB1 to bacteria is a weak non-covalent bond, which is similar to the hydrophobic connections exiting in the surface of bacterial [18,19]. If the probiotic microorganisms are used as food supplement by animals, they can be excreted in the feces with toxins from the animals' bodies by binding to Aflatoxins [8].

Method

In order to isolate the bacteria with the capability of binding to mycotoxins, the samples were collected from the livestock and poultry farms located in the Mazandaran Province. MRS culture media was used for early screening of bacteria. Appeared colonies were purified and then cultured in the MRS culture media containing Aflatoxin as the only carbon source. Biochemical characteristics of the isolates were determined using conventional microbiological methods of gram and catalase tests and classified as lactic acid bacteria. To determine the ability of binding to mycotoxins by isolated strains, certain amounts of Coumarin were added to the liquid culture medium of each isolate and the specimens were kept at 37 ° C and after the end of storage time, microorganisms were isolated by centrifugation and after extraction of surplus Coumarin using chloroform, the amount of free Coumarin in the supernatant was measured using spectrophotometer at wavelength from 250 to 400 nm. 16S rDNA gene sequencing was used to identify the molecular properties of the strains of bacteria. In order to extract the DNA of the bacterial genome, CTAB method described by Sambroke was used. The results of sequencing were studied using BioEdit Software. Sequencing accuracy was checked using the same software and the similarity of the 16S rDNA gene sequence was performed for each isolate using the BLAST software in the NCBI database.

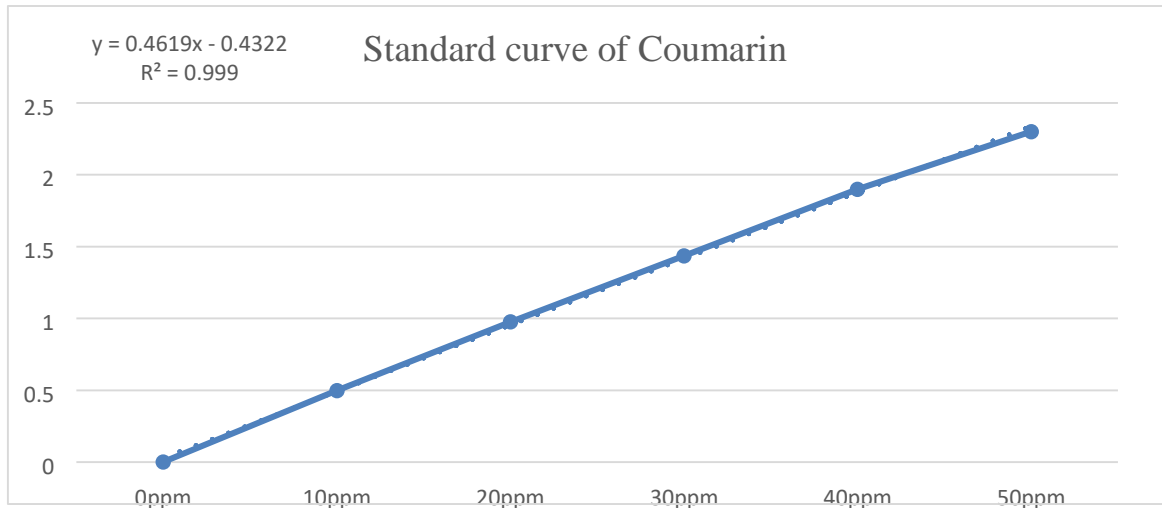


Diagram 1: Standard curve of Coumarin, maximum absorption at the wavelength of 274nm in a concentration of 50 ppm of Coumarin

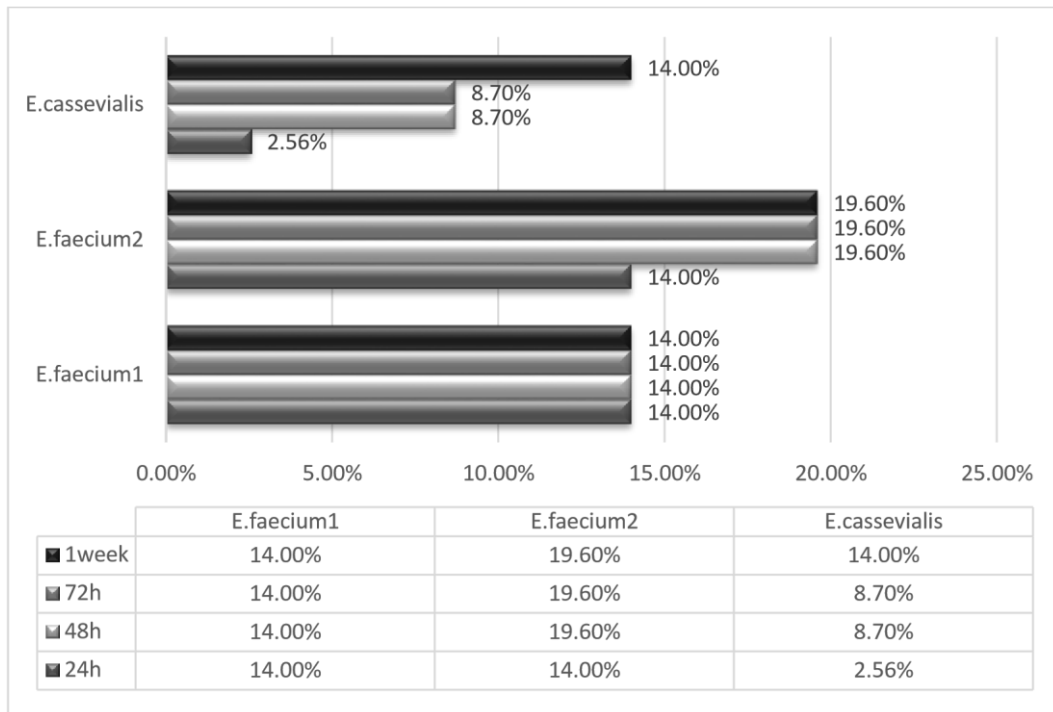


Diagram 2. Reduction in Coumarin by the live biomass of Lactic Acid Bacteria

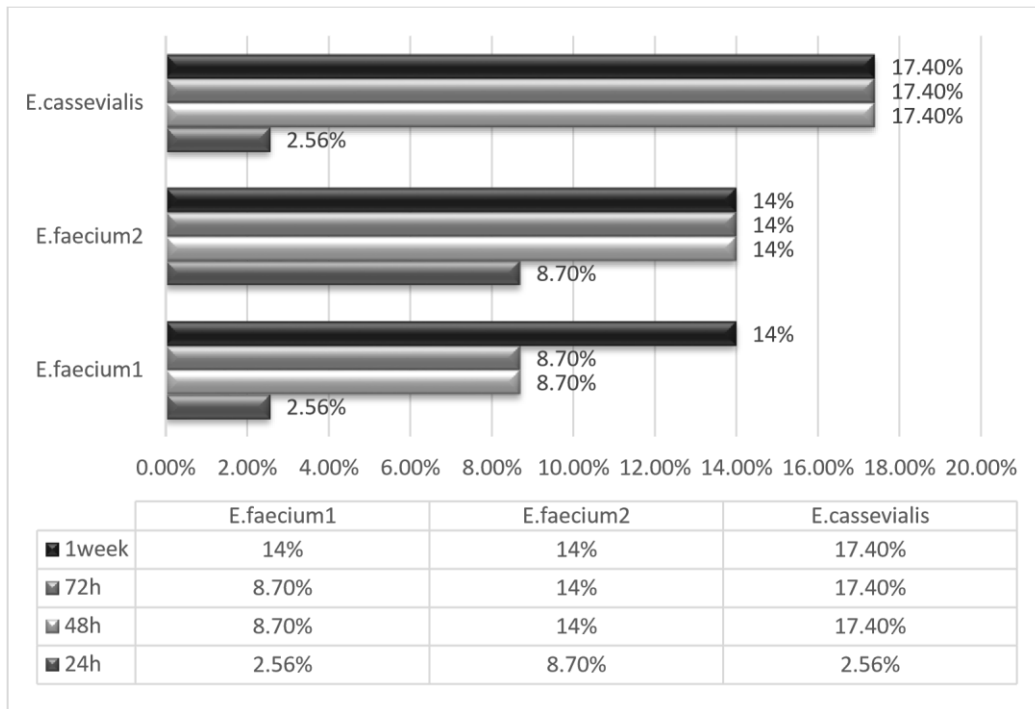


Diagram 3. Reduction in Coumarin by the Non-living biomass of Lactic Acid Bacteria

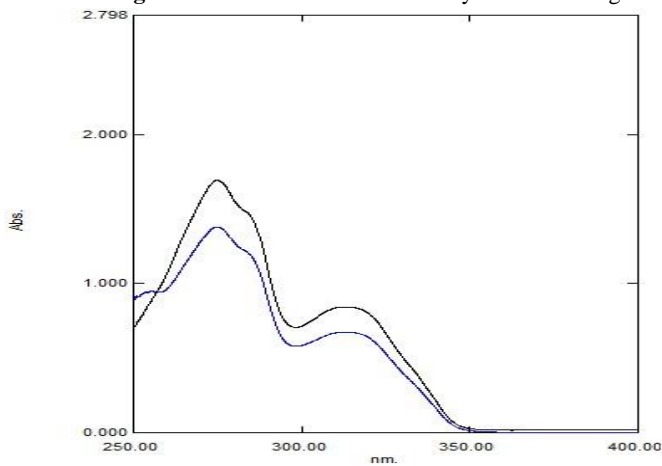


Diagram 4. The binding capability of Biomass of strain E. Faecium NDL2 (upper curve: Absorption curve of 50ppm Coumarin; lower curve: amount of Coumarin in the media of isolate E. faecium NDL2)

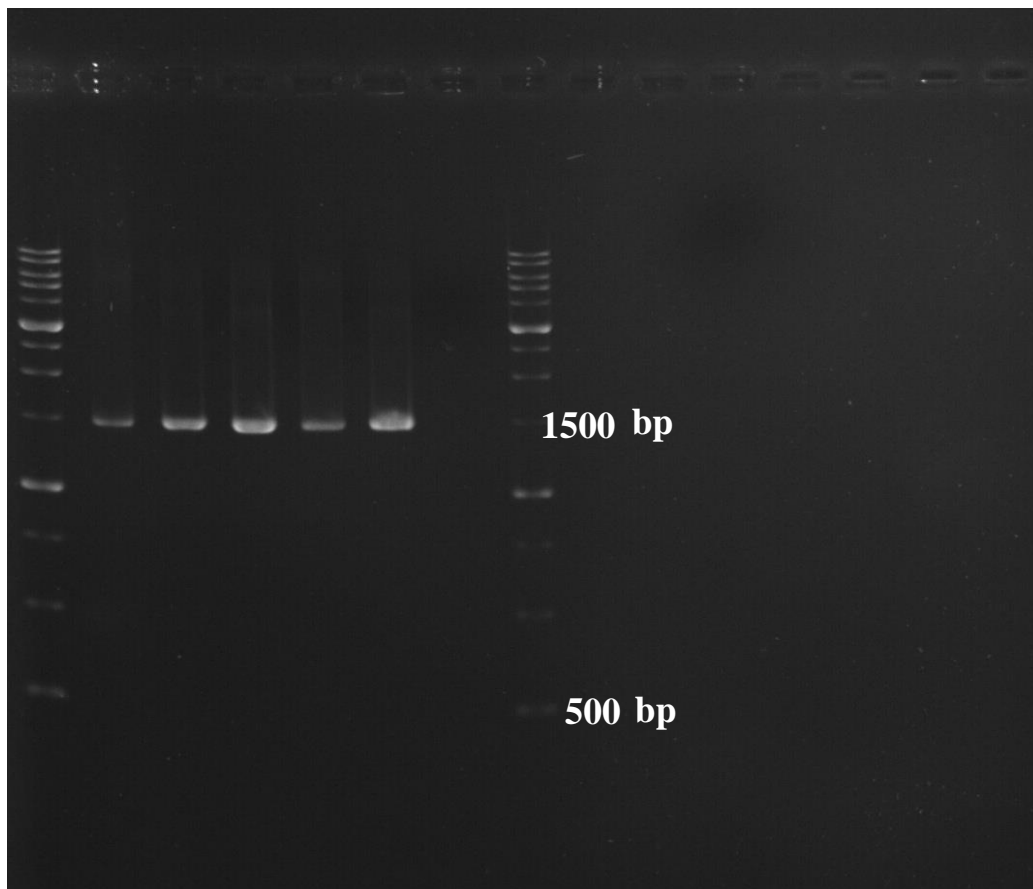


Figure1: Electrophoresis of PCR products on 1% agarose gel, from left to right: first well of DNA Ladder (1500 bp), other wells of PCR product of lactic acid-producing bacteria isolates

Results and discussion

72 colonies of soil specimens were isolated and purified and after early biochemical tests (Gram and Catalase), 20 isolates were identified as Lactic Acid Bacteria. The morphology of the isolates on MRS culture media is as very fine to medium size white colonies. Reduction in Coumarin was examined using biomass of Lactic Acid Bacteria. For each isolate, absorption of Coumarin was determined at the wavelength of 274 nm. The amount of removal of Coumarin from the media was calculated by substituting the absorbance number of each isolate in the equation obtained from the standard curve of Coumarin. The standard curve of Coumarin was drawn based on the concentrations of 0, 10, 20, 30, 40 and 50 ppm of Coumarin (Diagram 1). 3 of 20 Lactic Acid Bacteria isolates showed the highest ability to reduce 50 ppm of Coumarin in 24, 48, 72 hours up to a week. The isolate *E. faecium* 2 showed the highest percentage of reduction in Coumarin (%19.6) than other isolates (Diagram4) and the isolates *E. faecium* 1 reduced Coumarin as much as %14. After a week, the isolate *E. casseliflavus* could reduce the Coumarin as much as %14 (Diagram2). Non-living biomass of the isolate *E. faecium* 1 could reduce the Coumarin as much as %14 after a week (Diagram 3). Based on the studies on the reduction of Aflatoxin B1 by the bacteria *E. faecium*, it was reported that these bacteria reduce 19 to 30 percent of Aflatoxin by binding to it. Also, in (9), 50 to 100 ppm of Aflatoxin was used in the culture media of bacteria *E. faecium* and reduction in toxin as much as 17 to 27 percent was reported. Using genomic bacterial DNA, the size of the 16S rDNA gene in the PCR reaction is approximately 1500 bp, as shown in Figure1. Based on the results of the 16S rDNA gene sequencing, the isolates No. 20, 12 and 11 were identified of *Enterococcus* species and the similarity of 16S rDNA gene sequences of these isolates with the sequence of registered genes found in the NCBI database was 99%.

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