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Limosilactobacillus fermentum LAB212 mediated bio-control of Aflatoxin B1 produced by *Penicillium rubens* VBCA11

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The continuous increase in global population is constantly facing a challenge of food insecurity. A large portion of food, both raw and processed, is being spoiled during storage. Different fungal species are the main causes of spoilage by contaminating the foods and grains during storage.Some of them also produce mycotoxins. These mycotoxins are hazardous for human health. In the current study we had focused on the biocontrol of this mycotoxin by common contaminating spoilage fungi of gram seeds. *Penicillium rubens* VBCA11 a spoilage fungus was isolated from spoiled seeds. It was characterized, identified and later on screened for aflatoxin production. Thin layer chromatography, High performance liquid chromatography and Electronspray ionization-Mass spectrometric studies confirmed its ability for aflatoxin production. On the other hand *Limosilactobacillus fermentum* LAB212, isolated from natural fermented food product, was used to control this aflatoxin contamination. Different experiments were set up to check the efficiency of the bacteria in reducingor removing the toxin produced by VBCA11 from medium and contaminant gram seeds. HPLC spectrum of both the experiments shows complete removal of the toxin. Hence suitable formulation of this bacterium and its commercial application can provide us a safe preservation.

Key words: Aflatoxin, bio-preservative, chickpea, HPLC, lactic acid bacteria, mycotoxin, *Penicillium rubens*, spoilage

INTRODUCTION

Food safety of a country is dependent on the quality and consumer protection, which includes availability, utilization and constancy of food at that level where people can have physical and economic access, to reasonable, secure, and nutritious food to meet the requirement for an energetic and a healthy life.

When any one of these factorsgets weakened, the total community suffers from food insecurity and malnutrition. Spoilage and sometime mycotoxin contamination during pre- or post- harvest period causes food insecurity among most of the food chain (Udomkun *et al*., 2017). Food spoilage can be defined as the undesirable change in the food texture and appearance which is totally unfit for consumption (Lianou *et al*., 2016). During storage period different fungal species like *Fusarium* sp., *Aspergillus* sp., *Penicillium* sp., and *Candida* spp. with combination of physical and chemical environmental factors vigorously spoil both raw and finished products. Moreover some of the fungal members produce a varieties of toxingenic compounds which are potentially harmful for human health (Tournas*et al*. 2006., Casey and Dobson 2004., Mari *et al*. 2005; Kinay *et al.* 2005). Among the different type of mycotoxins, aflatoxins are the most important contaminant in the crops like maize, groundnuts, tree nuts, dried fruits and spices as well as in milk and meat products (Samson *et al.,* 2014). Aflatoxins are mainly produced as secondary metabolites by the genus *Aspergillus* and *Penicillium*. It has received great public health concern as it is highly toxic, mutagenic, carcinogenic

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and immunosuppressive agent and responsible for hepatic, extra-hepatic carcinogenesis (Hussein and Brasel., 2001). In addition, aflatoxin has a great contribution to alter and impair child growth (Turner *et al*., 2003). Overall, aflatoxin contamination has negative impacts on crop and animals which is not only wastage of natural resource, but also decrease market value which tends to economic losses. Due to these damaging effects, different countries and international organizations have established some strict rules and regulations in order to control aflatoxin contamination in food and feeds and also to prohibit the trade of spoiled contaminated food products (Juan *et al*., 2013). Now a day, scientists have developed several strategies to reduce the spoilage like sorting technology, treatments with electromagnetic radiation, ozone fumigation, chemical control agents, biological control agents etc. Physical control procedure is laborious and time consuming, on other hand chemical preservatives are not completely safe for health. So relying on biological control is only the safe alternative. Cottyand Mellon (2006)stated that increased number of nontoxigenic strains in the soil will compete with other toxigenic strains and outnumber them. Soil rich with nontoxigenicstrains has a carryover effect, which protects crops from contamination during storage period also (Atehnkeng *et al.,* 2014; Dorner and Cole, 2002). Another bio-control study by Anjaiah *et al*. (2006) says that some antagonistic strains of *Pseudomonas*, *Bacillus* and *Trichoderma* spp. resulted in significant reduction of mycotoxin in pre-harvest peanut seeds infected by *A. flavus*. *Acetobacter nigricans* AZT 54 can effectively control the fungal spoilage of maize, sorghum and wheat grains (Nagaraja *et al*., 2016). Among various natural biological antagonists, lactic acid bacteria (LAB) have been used for centuries as bio-preservative agent in many foods (Dalie *et al*., 2010) with their Generally Recognized as Safe (GRAS) status. The reason behind the inhibition of fungal growth is the production of different bioactive compounds such as organic acids, fatty acids, hydrogen peroxide and bacteriocins. These microorganisms are widely used for the production of fermented foods (Hammes and Hertel, 2003)which also extend the shelf-life of foods. Several authors reported that *Lactobacillus*, *Bifidobacterium*, *Enterococcus faecium*, and the yeasts *Saccharomyces cerevisiae* and *S.boulardii* shows antifungal activity as well as mycotoxin detoxification properties (Ahlberg *et al.,* 2015; El-Nezami and Gratz, 2011; Peltonen *et al*. 2001).

Therefore the purpose of our current study was to assess the potential of *Limosilactobacillus fermentum* LAB212, isolated from natural fermented food product,toremove the aflatoxin from gram seeds. The study will also establish the contribution of lactic acid bacteria regarding safety of foods and ensure food security.

MATERIALS AND METHODS

Microorganisms and culture conditions

L. fermentum LAB212 was cultured in Lactobacillus MRS medium (HiMedia, Mumbai, India) (de Man *et al.* 1960) and stored in 10% glycerol skimmed milk at 4°C. On the other hand the fungal isolates were maintained in malt extract (ME) agar medium (HiMedia, Mumbai, India) in slants at 4°C. For optimum toxin production the fungal isolate was cultured in SMKY medium (Sucrose-200 gm, Magnesium Sulphate- 0.5 gm, Potassium nitrate- 3 gm and Yeast extract- 7 gm/lit) (Davis *et al.*1966).

Isolation and identification of lactic acid bacteria

LAB strains were isolated from homemade curd samples. Each curd sample was serially diluted and spread on Lactobacillus MRS agar plates. Plates were then incubated at 28°C for 48 hrs. Initially five colonieswere selected based on their colony morphology and were checked for their antifungal potential against Penicilliumsp. by dual culture overlay method (Magnusson and SchnuÈrer 2001). Colonies showing prominent zones of inhibition were selected for further studies. The isolated potent strain LAB212 was identified by using 16S rDNA sequence homology and phylogenetic analysis.

Sampling and isolation of spoilage fungi

Spoiled gram seeds were(local variety B-115) collected from Durgapur, West Bengal. For isolation of spoilage fungi, at first seeds were thoroughly washed with sterilized water and then surface sterilized with 4% sodium hypochloride solutionfor two min and again washed with sterilized distilled water for 3-4 times. Now the seeds were placed on malt extract (ME) agar plates and incubated at 28°C for three days. All the plates were amended with streptomycin (150 µg/ml) for avoiding any bacterial contamination. After emergence of fungal mycelia, they were purified by repeated streaking and finally kept

in ME agar slants at 4°C for further studies.

Characterization and identification

The morphological character the spoilage fungal isolate was studied after staining the fungal mycelia with cotton blue andlactophenol and identified by using light microscope (Olympus CH2Oi).For detailed structure of the isolate, SEM study was performed. Spore bearing mature fungal mycelia were directly subjected to critical point drying (CPD)and finally gold coated using an ion sputter (Coater IB-2, Gike Engineering, Japan) and observed under scanning electron microscope (Zeiss).

The spoilage fungal isolate VBCA11 was identified on the basis of D1/D2 region of LSU (Largesubunit 28SrDNA) gene sequences using Veriti® 96 well Thermal Cycler (Model No. 9902). Forward and reverse DNA sequencing reactions were carried out with DF and DR primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 639 bp of D2 region of LSU gene was generated from forward and reverse sequence data using aligner software.The D2 region of 28S rDNA sequence was used to carry out BLAST alignment search tool of NCBI Gene Bank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program ClustalW. Distance matrix was generated using RDP database and the neighbor joining phylogenetic tree (Saitou and Nei, 1987) was constructed using MEGA5 (Tamura *et al.* 2011).

Study of aflatoxin production ability by the isolated fungus

To determine the toxin production ability of VBCA11 it was grown in SMKY broth and incubated at 28°C for 9 days. On the 10th day, mycelial mat was separated from the broth by filtering throughWhatman No.1filter paper. The culture filtrate was extracted twice with chloroform (1:1 v/v) in separating funnel. The chloroform portion was collected and passed through a bed of anhydrous sodium sulphate which was then evaporated to dryness using rotary vacuum evaporator at room temperature, weighed and stored at 4°C for further experiments.Presence of aflatoxinin in the extract was determined by thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and Electronspray ionization-Mass spectrometry (ESI-MS). For purification,the concentrated chloroform extract was separated and spotted on a 20 x 20 cm silica gel TLC plate. The glass plate was 0.25mm thick and was prepared by mixing 30g silica gel G (Merck, Mumbai, India) with distilled water. The plate was dried in oven at 110°C. Spotted chromatographic plates were developed by using diethyl ether: methanol: water (96:3:1) (Garber and Cotty, 1997) as running solvent and observed under UV light (360nm). All the bands were scraped and suspended in chloroform for 24 hr. Now these partially purified compounds were further analyzed using HPLC (Waters 1525 Binary system) equipped with Waters 2998 Photodiode Array (PDA) detector at 365nm. 25µl of partially purified compounds along with the aflatoxin standard mixture (Sigma-Aldrich) were filtered and injected into the HPLC system through a Rheodyne injection valve and separation was carried out by Symmetry C18 (5 µm 4.6 × 250 mm) column with Symmetry guard column using mobile phase consisting of a mixture methanol: acetonitrile: water (17:19:63 v/v) (Rohman and Triwahyudi, 2008) with a flow rate of 1.0 mL min-1. The purified compound was collected manually through Waters Fraction Collector III. The HPLC purified compound was then subjected to ESI-MS analysis for further confirmation.

Removal of aflatoxin by L. fermentum LAB212

To check the efficiency of *L. fermentum* LAB212 in removal of aflatoxinfrom the culture medium or from a system, this experiment was designed (Teniola *et al.* 2005). Overnight cultured bacterial cells (6×106 CFU/ml) were transferred to 100 ml MRS broth supplemented with 400µl of stock AFs at a concentration of 1mg ml -1 dissolved in sterilized water to attain the final concentration of 4 µg/ml. A control set was also prepared similarly without inoculating the bacteria. Both the flasks were incubated in 28°C for 48 h with agitation (100rpm). At the end of the experiment, after chloroform extraction the dried extract was subjected to HPLC assay for toxin analysis.

Degradation of aflatoxin by L. fermentum LAB212in gram seeds

Hull of the fresh gram seeds were removed and dried in oven at 60°C for 6hours. The dried seeds were grinded into fragments with mortar pestle and were distributed in two petridishes. Each petridish contained 20g of fragmented gram seeds supple

mented with aflatoxin (1mg/ml) solution. The first plate was used as the control set and the other plate was mixed with freshly cultured L. fermentum LAB212 cells (3×107CFU/ml). Both the plates were incubated in dark at 28°C for 72h. Toxin contained in each set was measured at 72h after suitable extraction.

RESULTS AND DISCUSSION

Isolation, characterization and identification of fungi

In the present study spoiled gram seeds were used for isolation of spoilage fungi. From such seeds two types of fungal strains with distinct colony morphologies were isolated and they were purified at pure culture single colony level. Morphological characterization under light-and scanning electron-microscope (Fig.1), revealed that one of them (VBCA11) belongs tothe genus *Penicillium* sp., and the other strain (VBCA9) remained unidentified due to lack of any reproductive structure.

PCR amplification and sequencing of D1/D2 region of 28S rDNA of the strain VBCA11 revealed a 700 bp nucleotide sequence which showed 99% pair wise similarity with Penicilliumrubens, strain CBS339.52 (Accession Number: MH868606.1). Neighbor joining phylogenetic tree also showed close similarity with this strain. On the basis of BLAST as well as phylogenetic analysis the strain VBCA11 was identified as Penicilliumrubens (Fig.2).

Isolation and identification of LAB

During LAB isolation, initially five colonies (LAB189, LAB211, LAB212, LAB214 and LAB217) were selected and among them one LAB strain (LAB212) showed good antifungal activity against *Penicillium* sp. Further morphological characterization indicated its Gram positive rod structures and biochemical properties matching with the genus *Lactobacillus.* PCR amplification of 16S rDNA sequence homology and phylogenetic analysis of LAB212 shows 99.92% pair wise similarity with *Limosilactobacillus fermentum* with NCBI accession number SUB9-818664 *Limosilactobacillus* MZ35-9838(our unpublished work).

Screening and confirmation of aflatoxin production by the fungal isolate

For determining different AFs producing ability of

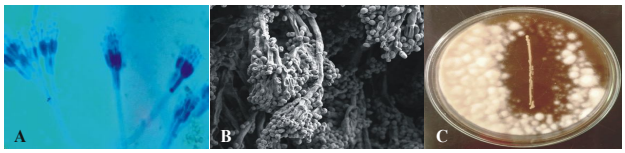

Fig. 1 : Light microscopic picture (10X magnification) (A); SEM image (B) of isolated spoilage fungus *Penicillium rubens* VBCA11; Inhibitory potential of *Limosilactobacillus fermentum* LAB212 against *P. rubens* VBCA11 by dual culture overlay assay (C).

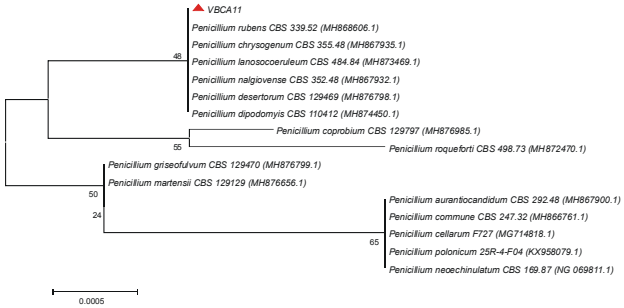

Fig. 2 : Phylogenetic tree showing the relative position of the isolated spoilage fungal strain VBCA11.

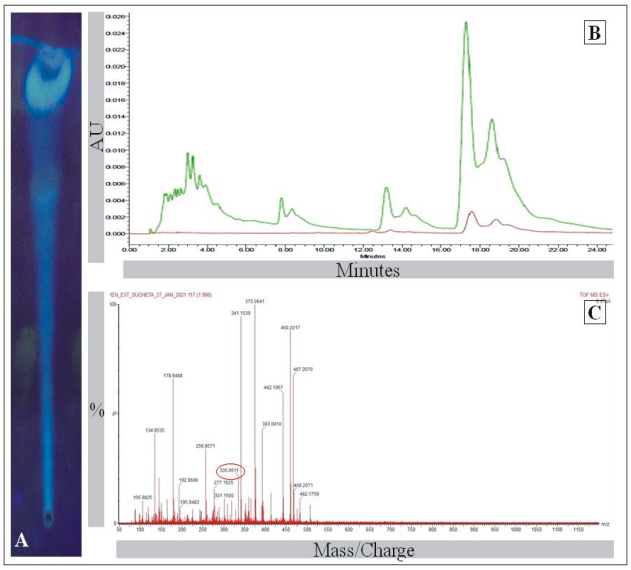

Fig. 3 : Aflatoxin production by VBCA11, TLC analysis(A); HPLC spectrum of aflatoxin mix (red) and chloroform extract of *P.rubens* VBCA11 (green) showing retention time at 17 min with maximum PDA absorption at 365 nm (B); ESI-MS spectrum of aflatoxin B1 showing the sodiated cation of aflatoxin B1: [M+Na]+ at m/ z = 335 from the HPLC purified extract of VBCA11(C).

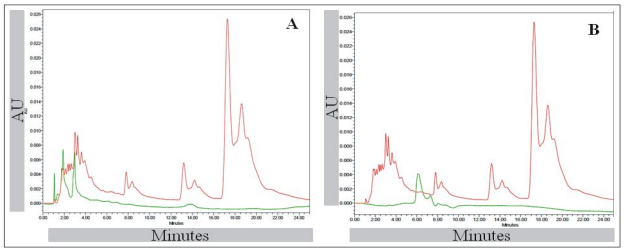

Fig. 4 : HPLC spectrum of control (red) and treated (green), Degradation of aflatoxin B1 from liquid media (A); Removal of toxin from gram seeds (B)

the fungal strain VBCA11, it was grown in SMKY medium and the spent culture was extracted with chloroform. Possible presence of aflatoxins and their detection has been monitored by TLC with suitable solvent system. Based on the color of the fluorescent band on TLC plate under UV light, it hasbeenpresumed that VBCA11 can produce aflatoxin (Fig. 3A). Further confirmation was done by performing HPLC analysis (Fig.3B). HPLC spectrum clearly showed the presence of aflatoxin in the extract of VBCA11, as the peak and retention time was found at 17 min with maximum PDA absorption at 365 nm which almost superimpose with the peaks ofstandardaflatoxin mixture. It was again reconfirmed with the aid of ESI-MS analysis. Presence of aflatoxinB1 was identified at m/z = 335 as sodiatedcation (Fig 3C). Several authors had reported that *P. viridicatum* secrets different mycotoxins like brevianamide A, citrinin, ochratoxins , patulin,penicillic acid and penitrem A, rubrosulphin and viopurpurin, viomellein and xanthomegnin, and viridicatin.

This is very important to find out the toxin producing ability of any fungal species infecting or contaminating different food materials. This will help in selecting or eliminating food materials before consumption. The ability of *Penicillium* sp. VBCA11 in production of aflatoxin B1 while growing on pulse grains is very important in terms of its further consumption. This knowledge will help consumers to accept such pulse grains or not. Removal of such kind toxins by any food grade microorganisms would help us in escaping the ill effects of toxins and save the precious agricultural produce from spoilage as the food grade LAB212 has proven to be a well efficient antagonist of the spoilage *Penicillium* sp. VBCA11 (Fig.1C)

Removal and degradation of aflatoxin

It is very essential to prevent contamination of food grains or pulse grains from toxigenic fungi. And again of the different methods to remove the already produced toxins in the food materials most of them are not suitable either from safety issues or from practical point of views. In the present study attempt has been taken to remove the already produced toxins by using food grade LAB strain. The efficiency of L. fermentumLAB212in degrading the toxin, both from the medium and from the pulses was studied (Fig 4). At end of both the experiments,it was found that the bacterial culture of the LAB straincan completely degrade or remove aflatoxinB1. When the bacterial cells were inoculated along with the aflatoxin,the degradation rate was very slowbut it was increased over time and ultimately at the end of the incubation the toxin wasremoved/degraded completely.Similar result was also observed by Guan *et al.* (2008). They have concluded from the linear correlation between time and degradation activity indicates that the degradation mechanism of these bacterial lysates is enzymatic.*B. subtilis* UTBSP1 could remove aflatoxinby 52.67% and 80.53% respectively at 24 and 48 hrs, from liquid culture (Farzaneh *et al.,* 2011).Kong *et al*. (2012) observed a 96% aflatoxinB1 biodegradation by *Rhodococc-useryth-ropolis*. The cell free extract of *R. erythropolis* and Mycobacterium fluorantheni-voranscan degrade aflatoxinwithin 8 h of incubation (Teniola *et al.*2005). Even different strains of lactobacilli can remove aflatoxins from aqueous solutions (Pierideset al., 2000). Peltonen *et al.* (2001) reported that two strains of *L. amylovorus* CSCC 5160 and CSCC 5197 and *L. rhamnosus* LC ¹/3 can efficiently bind with aflatoxinB1 and remove it from aqueous solution. They concluded that differences in aflatoxin B1 binding capability by the bacterial strains are probably due to various bacterial cell wall and cell envelope structures. Similar result was also observed by Haskard *et al*. (2000) during the study ofaflatoxin degradation by *L. rhamnosus* GG. Rastegar *et al.* (2017) reported that application of lemon juice and/or citric acid was able to reduce the level of aflatoxin B1 upto 49.2 ± 3.5% from roasted contaminated pistachio nuts. Pulse light treatment for 80 sec and 15 sec can also effectively degrade and detoxificify of aflatoxin B1 and B2 in rough rice and rice bran (Wang *et al.* 2016). Similarly the chromatogram of HPLC confirms the complete dimension of aflatoxin B1 in different substance from the parent compound as no similar peak was found in the HPLC chromatograms.

CONCLUSION

Penicillium rubens VBCA11, isolated from spoiled gram seeds,wasable to produce aflatoxin. Limosilactobacillus fermentum LAB212 a food grade lactic acid bacterium, showed very good antifungal activity against thistoxigenic fungal strain. The bacterium is involved in the aflatoxinB1-remediation process from the liquidculture as well as from the gram seeds. Suitable formulation and application of this bacterium can provide us a safe

preservation by acting asa bio-preservative agent and ultimately will lower down the aflatoxin related health issues.

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