

Research Article

DEVELOPMENT OF BIO CONTROL USING LACTOBACILLUS SPP. ISOLATED FROM PROBIOTIC FOR ASPERGILLOSIS IN ONIONS CAUSED BY ASPERGILLUS NIGER

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Received 08th August 2021; Accepted 10th September 2021; Published online 10th October 2021

ABSTRACT

Aim: To develop bio control method using Lactobacillus spp. isolated from probiotic for controlling aspergillosis in onions caused by Aspergillus niger. **Study Design:** Isolation of strains of Lactobacillus spp. from a commercial probiotic named 'ViBact' using DMRS agar – Growth in MRS broth- Isolation of Aspergillus niger on Sabouroud's agar from infected onion- Centrifugation of broth cultures of Lactobacillus spp. –Test using cell free broth of Lactobacillus spp. for inhibitory action on Aspergillus niger using agar gel diffusion technique- Application of cell free broth of Lactobacillus to infected onion and study of progression of symptoms. **Place and Duration of Study:** Department of Microbiology, H.P.T. Arts and R.Y.K. Science College, Nashik, Maharashtra, India. Duration: June 2021- October 2021 **Methodology:** Aspergillosis is a common post- harvest fungal disease of onions. Chemical preservatives used to prevent fungal diseases are hazardous to human health. Hence, an alternative method of preservation called 'biocontrol' using Lactic Acid Bacteria (LAB) was developed in this study. Aspergillus niger was isolated from infected onion on Sabouroud's agar. 10 strains of Lactobacillus spp. were isolated from a commercial probiotic named 'ViBact' using DMRS agar. Lactobacillus strains were grown in MRS broth for 48 hours in anaerobic jar, centrifuged and cell free broths were tested for inhibitory action on Aspergillus niger using agar well diffusion technique. Cell free broth was applied to infected onion and observed for progress of disease symptoms. **Results:** 10 strains of Lactobacillus spp. showing typical morphology of Gram positive rods were isolated from a commercial probiotic. 4 strains of Lactobacillus spp. showed significant inhibitory action on Aspergillus niger in agar well diffusion method. Large zones of inhibition were shown by strains 2 and 8. The largest zone of inhibition of 18 mm was shown by strain 2. When the cell free broth of this strain was applied to infected onion for 1 hour, further progression of symptoms of the disease was not seen whereas the infected onion without this treatment showed progression of typical symptoms after one week.

Keywords: Onion, Aspergillosis, Lactic Acid Bacteria(LAB), Bio control, probiotic, agar diffusion method.

INTRODUCTION

Aspergillus is a widely spread fungus in nature. Common species of Aspergillus causing infections in humans include Aspergillus fumigatus, Aspergillus flavus, Aspergillus terreusetc (Borkar *et al.*, 2020). Aspergillus niger is a common species which does not cause invasive disease but is associated with otomycosis (Araiza *et al.*, 2006), cutaneous infections in human beings (Loudon). Spores of Aspergillus niger are small sized, easily inhaled, act as allergen and are generally associated with lung infections in individuals with weak immune system. (Kierownik K., 1990). Aspergillosis is a common disease occurring at preharvest, harvest and post-harvest stages of agricultural products (Perrone *et al.*, 2007). It is a very common disease of onions caused by Aspergillus niger also known as 'black fungus'. India is the second largest producer of onions after China (FAO, 2012) and faces economic loss due to aspergillosis of onions. When onions get infected with Aspergillus niger, the fungus grows between the outer skin and the first fleshy scales of the onion. The black spore mass of the fungus can be seen on or between the outer scale of the onion bulbs, especially along the veins (Borkar *et al.*, 2020). Nagerabi and Ahmed in 2003 reported the spore load of Aspergillus Niger in red skin and white skin onions in Sudan. The incidence and sporulation of Aspergillosis in red skinned onion was 70.5 to 92.5% with 15.8 to 25.3 x 10⁶ spores load per gram of onion tissues, while in white skinned onion the incidence of Aspergillosis was observed on 51.5 to 65.0% onions with the spore loads of 12.6 to 20.6 x 10⁶ spores per gram of onion tissues. This spore load when released in to the surrounding environment in the form a spore

dust and spores or in the kitchen while cutting and using the onion, may serve as an inoculums to cause Aspergillosis in human beings. Aspergillosis can be effectively controlled by chemical treatment (Grinstein *et al.*, 1992). Benzoate, propionate, sorbate, nitrate, and sulfites are the common chemical preservatives used in food and agricultural industries (Silva and Lidon, 2016). Use of chemical preservatives have adverse effects on human health hence, alternative methods for preservation of agricultural and food products are looked for. 'Bio control' preservation is an alternative method to use of chemical preservatives. This method is being widely explored. The use of Lactic Acid Bacteria (LAB) to reduce levels or fully replace antifungal chemical preservatives is being explored as, these microorganisms are recognized as safe. They produce organic acid i.e. lactic acid and other metabolites such as phenyllactic, hydroxyphenyllactic, benzoic acids, fatty acids, volatile compounds (e.g., diacetyl, acetoin), cyclic dipeptides, hydrogen peroxide, reuterin, and/or proteinaceous compounds which have exhibited an antifungal activity (Crowley *et al.*, 2013; Leyva Salas *et al.*, 2017). Among Lactic Acid Bacteria, species of Lactobacillus have been shown to have antifungal activity. Lipinska et al in 2016 had used several Lactobacillus strains procured from culture collection centre and studied their antifungal activity against a wide range of fungi procured from the same source. Jeong-Dong Kim in 2005 had isolated several Lactobacillus species from household Kimchi and tested their antifungal properties against Aspergillus fumigatus. Variety of probiotics are available in market which are used to improve digestion, nutritional and microbial balance in the intestinal tract of human beings. They contain various types of bacteria. LAB are commonly used in probiotic preparations and their oral administration triggers both mucosal and systemic immune response (Naidu *et al.*, 1999). 'ViBact' is a commercially available probiotic which contains various genera of Lactic Acid Bacteria along with

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Clostridium butyricum (mentioned on the sachet). In current study this probiotic was used as a source of *Lactobacillus* spp. Antifungal activity of the isolates was tested on *Aspergillus niger* isolated from infected onion.

MATERIAL AND METHODS

Isolation of Lactic Acid Bacteria from commercial probiotic (ViBact)

ViBact (Figure 1) is a commercially available probiotic which contains various genera of Lactic Acid Bacteria along with *Clostridium butyricum*. *Lactobacillus* spp. was isolated from this probiotic sachet using sterile DeMan Rogosa and Sharpe medium (DMRS agar) with composition (gm/lit) Peptone:10, Meat extract: 8, Yeast extract: 4, Glucose: 20, Dipotassium hydrogen phosphate: 2, Tween 80:1, Manganese sulfate: 0.04, pH: 7 – 7.2, Agar:20 (De Man *et al.*, 1960). The contents of probiotic sachet were suspended in sterile saline. A loopful was streaked on sterile DMRS agar plates and incubated in anaerobic jar at room temperature for 48 hours. Isolated colonies were transferred individually to sterile DMRS agar slants, incubated in anaerobic jar (Figure 2) for 48 hours and after getting growth, preserved in refrigerator.



Figure 1: Probiotic sachet commercially available



Figure 2 : DMRS agar plate and slants incubated in anaerobic jar

Identification of the isolates

Slant cultures of isolates were subjected to Gram staining. Isolates showing morphology of Gram positive rods were selected (Typical morphology exhibited by *Lactobacillus* spp.). Identification of the isolates up to genus level was carried out using Bergey's manual of determinative bacteriology (Bergey *et al.*, 1994). For identification catalase test, homofermentative /heterofermentative test using sterile MRS broth incorporated with Durham's tube were carried out.

Isolation of *Aspergillus niger* from infected onion

Onions infected with black mold were selected (Figure 3). Black powdery spores of the mold were spread on sterile Sabouroud's agar with the following composition, (gm/lit) Dextrose: 40, Peptone: 10, pH: 5.6, Agar: 20, incubated for 4-6 days at room temperature. After sufficient growth, little growth was transferred to a drop of lacto phenol- cotton blue on a slide and with little teasing a wet mount was prepared and observed under binocular microscope for typical morphology of *Aspergillus niger*.



Figure 3: Onion infected with black mold-*Aspergillus niger*

Inhibition of *Aspergillus niger* by *Lactobacillus* spp.

Ten selected isolates of *Lactobacillus* spp. were grown in sterile MRS broth at room temperature in anaerobic jar for 48 hours. They were numbered as 1,2,3 ----10. After getting sufficient growth, they were centrifuged at 4000 rpm for 10 minutes. The supernatant was collected and tested for inhibitory action on *Aspergillus niger* by agar well diffusion method. In this method, spores of *Aspergillus niger* were harvested from 6 days old Sabouroud's agar plate cultures in sterile saline and dispersed evenly by shaking for 15 minutes. Spore count was adjusted to 1×10^6 spores/ml using improved Neubauer's chamber. 0.1ml spore suspension of *Aspergillus niger* was spread evenly on sterile Sabouroud's agar plate. With the help of flame sterilized cork borer (8mm diameter) a well was made in the centre of the plate. The well was filled with 0.1 ml of the supernatant of centrifuged, cell free MRS broth. Another well was filled with sterile distilled water as 'control' well. The plates were incubated for 6 days at room temperature. The plates were observed for a clear zone of inhibition of growth of the fungus.

Application of cell free extract of *Lactobacillus* spp. to infected onion

Two onions showing some initial signs of *Aspergillus niger* infection were selected. One onion was treated with the supernatant obtained

from broth culture of *Lactobacillus* spp. (strain 2), by immersing in it for 1 hour. The other onion was kept untreated. Both the onions were kept at room temperature and observed for next 7 days for the progress of symptoms.

RESULTS AND DISCUSSION

Isolation of lactic acid bacteria from commercial probiotic (ViBact)

Typical white colonies of Lactic Acid Bacteria were observed after 48 hours incubation in anaerobic jar at room temperature on DMRS agar (Figure 4). Colony characteristics were studied and individual colonies were transferred to DMRS agar slants. After 48 hours incubation in anaerobic jar, significant growth was observed on slants.



Figure 4: typical colonies of LAB on DMRS agar after 48 hours incubation at room temperature.

Identification of the isolates

When Gram staining of 30 isolates was performed, some of the cultures showed presence of Gram positive cocci. 10 Cultures which showed presence of Gram positive rods (Typical morphology of *Lactobacillus* spp.) were selected and numbered serially as 1,2 3, --- 10. (Figure 5).



Figure 5: Typical morphology of *Lactobacillus* spp-Gram positive rods

For identification of these isolates up to genus level, when catalase test was performed, no bubbling was seen in 3% hydrogen peroxide solution indicating negative test. The isolates were identified as homofermentative as there was no gas production in Durham's tube incorporated to MRS broth after 48 hours incubation at room temperature in anaerobic jar. From these observations they were identified as *Lactobacillus* spp. Similar method of identification using Bergey's manual of determinative bacteriology was used by Porwal et al in 2015 for identification of organisms isolated from activated sludge system.

Isolation of Aspergillus niger from infected onion

Typical white cottony growth was observed on Sabouroud's agar after 2 days incubation at room temperature. After 6 days the growth appeared blackish (Figure 6). Wet mount preparation of this culture showed typical morphology of *Aspergillus niger* under binocular microscope (Figure 7).



Figure 6: Growth of *Aspergillus niger*(black mold) isolated from infected onion on Sabouroud's agar.

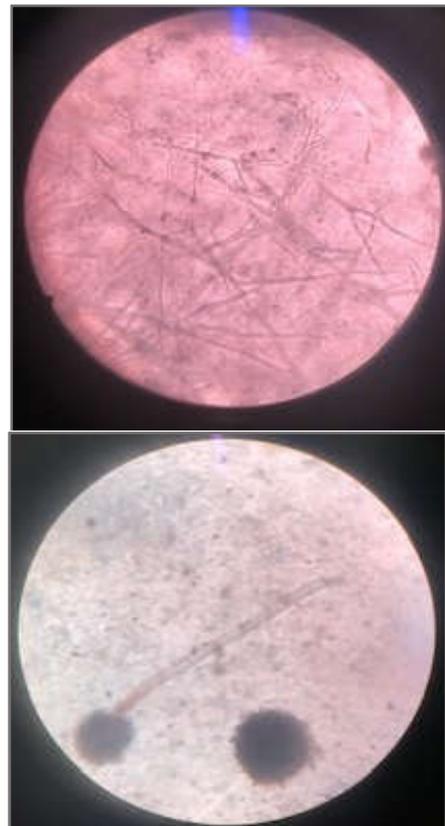


Figure 7: Typical morphology of *Aspergillus niger*

Inhibition of *Aspergillus niger* by *Lactobacillus* spp.

When Sabouroud's agar plates spread with spore suspension of *Aspergillus niger* and a central well filled with cell free broth of different strains of *Lactobacillus* spp were observed, a clear zone of inhibition of growth of *Aspergillus niger* was observed on 4 plates. Large zones of inhibition were obtained with strains 2 and 8. The largest zone of inhibition was 18mm obtained with strain 2. The 'control' well filled with sterile distilled water did not show any zone of inhibition (Figures 8 and 9). This indicated that some compound produced by *Lactobacillus* spp, present in broth had inhibitory action on growth of *Aspergillus niger*.



Figure 8: Inhibition of *Aspergillus niger* by cell free broth of *Lactobacillus* spp. Strain 2

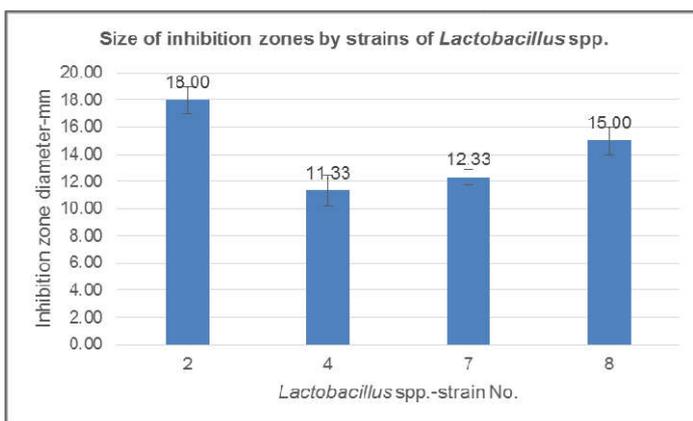


Figure 9: Size of zone of inhibition of *Aspergillus niger* by cell free broth of strains of *Lactobacillus* spp.

It has been mentioned by Delavenne et al in 2012 that the composition of MRS broth can strongly impact the expression of antifungal activity by LAB because it contains acetate, which may strengthen the antifungal activity of Lactic Acid Bacteria. In current study the same medium (MRS broth) was used to grow *Lactobacillus* spp. and the cell free broth has shown good antifungal activity. Sibi G. et al in 2013 mentioned that synthetic fungicides are toxic in nature and fungi can develop resistance to them. Hence, an alternative

method in form of extracts of various Indian spices were tested against black mold isolated from infected onion. In current study cell free broth of *Lactobacillus* spp. was used with the same intention of developing an alternative method for treatment of Aspergillosis of onions.

Application of cell free extract of *Lactobacillus* spp. to infected onion

When an infected onion was treated for 1 hour with cell free broth of *Lactobacillus* spp (strain 2) and observed for 7 days, there was no further progression of the disease. Whereas the onion which was not treated showed further progression of disease. Similar study was carried out by Saranya et al in 2017. They had used culture of *Aspergillus niger* grown on potato dextrose agar, onion seed were infected with fungus, treated with six different fungicides and efficacy of fungicides was determined.

CONCLUSIONS

Aspergillosis is a common post-harvest disease of onions. This disease causes economical loss of farmers and spores of *Aspergillus niger* growing on onions may cause allergies and Aspergillosis in human beings. Bio control of this disease using *Lactobacillus* spp. isolated from commercial probiotic proved to be an effective method. This method is helpful in avoiding use of chemical preservatives for onions as they are hazardous to human health and there is risk of resistance development in fungal pathogen. Application of solution of probiotic powder available commercially may prove to be an effective bio control method for Aspergillosis in onions.

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