LACTOBACILLUS BREVIS INHIBITION OF *ASPERGILLUS FLAVUS* AFLATOXIN PRODUCTION

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ABSTRACT

Lactic acid bacteria (LAB) are gram positive bacteria that produce lactic acid as their main product of metabolism. Studies showed that they are efficient against different species and can absorb aflatoxins from their environment. In this study, *Lactobacillus brevis* TMT-22 was tested for its ability to inhibit aflatoxin production and mycelial growth of *Aspergilius flavus* in liquid culture. Aflatoxin B1 biosynthesis and mycelial growth were inhibited in both simultaneous culture and individual antagonism assays, suggesting that the inhibitory activity was due to extracellular metabolites produced in cell-free supernatant fluids of the cultured broth of *L. brevis* TMT-22. In cell-free supernatant fluids of all media tested, MRS 1% yeast extract showed higher antiflatoxigenic activity. In this case, fungal growths, however, was not affected as measured by mycelial dry weight. The anti-aflatoxigenic metabolites from *L. brevis* TMT-22 were produced over wide range of temperatures between 25^{o}C and 37^{o}C. However, these metabolites were not thermostable since the inhibitory activity of the supernatant was inactivated within 30 minutes at 100^{o}C and 121^{o}C.

Keywords: Aflatoxin, metabolites, *Aspergillus flavus, Lactobacillus brevis*, inhibitory.

INTRODUCTION

Lactic acid bacteria (LAB) are Gram-positive, catalase and oxidase negative bacteria; they do not form spores and move spontaneously. They produce lactic acid as the main product of their metabolism. They grow anaerobically but are aero-tolerant (Walstra et al., 2006). With their involvement in food processing and their presence in the healthy microbiota of the human gastrointestinal tract, LABs are "generally recognized as safe" (GRAS) (Thipathi et al., 2012). They are widely used in food and feed, which have remarkable toxic properties including carcinogenic, mutagenic, teratogenic, immunosuppressive, and hepatotoxic effects in humans and animals (Alberts et al., 2006). Eighteen different types of aflatoxins have been identified so far, and aflatoxin B1 (AFB1) is the most prevalent and toxic metabolite produced by the fungi (Bhat et al., 2010). Several studies have demonstrated the efficiency of different species and strains of LAB in adsorbing aflatoxins from contaminated media (El-Nezami et al., 1998; Pierides et al., 2000; Oatley et al., 2000; Haskard et al., 2001; Peltonen et al., 2001; Azab et al., 2005; Fazeli et al., 2009; Bovo et al., 2013). In addition to the use of LAB as fermentative agents in food products, they are employed in recent times as aflatoxin decontaminating agents. Aflatoxins are mycotoxins produced by species of *Aspergillus flavus* and *Aspergillus parasiticus*, are postharvest pathogens of several important food crops including maize, peanuts and several tree nut crops (Farr *et al.*, 1989).

Many strains of *A. flavus* and nearly all strains of A. *parasiticus* are the major species of fungi producing aflatoxins (Klich and Pitt, 1988), which are known to be potent carcinogens (Squire, 1981) and hepatotoxinogenic chemicals and possess a severe carcinogen to animal and human health (Eaton and Callsgher, 1994). Therefore, international regulatory limits have been established for aflatoxinsin food and animal feed (Van Egmond, 1995).

These toxins are linked to fungal growth and the environment in which the grains/cereals are stored. Stored grains can be inhibited by physical methods such as aeration, cooling, modified atmospheres e.t.c or by fungistats of which the propionic, acetic and ascorbic acids are the most commonly used (Paster *et al.*, 1988*)*. In the storage agroecosystem, complex interactions occur between biotic and abiotic factors that have an impact on growth, toxin production and biological behavior. Then three main different factors such as physical, biological, and nutritional factors, affect aflatoxin production (Gourama and Bullerman, 1995). In nature aflatoxin producing fungi share the sane habitat with other microorganisms which can influence aflatoxin production. Many lactic acid bacteria such as *Lactobacillus spp.* were found to inhibit aflatoxin biosynthesis (Coallier-Ascah and Idziak, 1985; Karunaratne *et al.*, 1990). A silage inoculant mixture of *Lactobucillus* species was inhibitory to mycotoxin biosynthesis of *Penicillium* and *Aspergillus* species (Gourama and Bullerman, 1995; Karunaratne *et al,* 1990). In previous study, several *Lactobacillus* isolates were obtained from the mixture and screened for their inhibitory effect on mycelial production. In this paper, inhibition of aflatoxin production of *A. flavus* by *L. brevis* TMT-22, the effect of growth conditions of the bacterium on its antiaflatoxigenic activity and thermal stability of the inhibitory activity are presented.

MATERIALS AND METHOD

Bacterial and fungal strain

Bacterial culture of *L. brevis* TMT-22 isolated from Tomato obtained from a local market in Ibadan, Nigeria was maintained on malt extract agar slants at 4^{o}C and was transferred to 10ml sterile Modified Rogosa (MRS) broth to regenerate before use. *Escherichia coli* was grown at 37^{o}C in nutrient broth as positive control. Fully grown cell suspensions were stored at -80^{o}C in the presence of 90% glycerol as cryoprotectant, for long-term conservation. *Aspergillus flavus* producing aflatoxin B1 (AFB1) was obtained from contaminated culture tubes from the Growth Room of the Biotechnology Centre, Forestry Research Institute of Nigeria and aflatoxin B1 (AFB1) production during the growth of the fungal strain was closely observed. The fungus was grown on potato dextrose agar (PDA) slants at 25^{o}C and stored at 4^{o}C until further used and subcultured on a monthly basis.

Preparation of inoculum and growth media

Lactobacillus brevis TMT-22 was transferred to MRS broth and was incubated at 37^{o}C for 48h. The supernatant was prepared by centrifuging the *Lactobacillus* culture at 3000 x g for 15 min and sterilized by filtration with 0.45 µm pore size filter. The fungal spore suspension was prepared as described by Fan and Chen (1999). The fungus was grown on PDA petri dishes for 5-7 days at 25^{o}C until sporulation occurred. Spores of *A. flavus* incubated at 25^{o}C were collected from 10 day old PDA solid cultures in a sterile phosphate buffer solution containing 0.05% of Tween 80. The spores were loosened using a flamed wire loop. Mycelial debris was removed by filtration through sterile cheese cloth. This was further diluted to obtain a final spore suspension containing approximately 1.5 x 104 spores/ml determined using a Petroff-Hauser counting chamber (hemocytometer), spread plate technique on PDA plates and was used for all experiments.

Fungal growth determination

Fungal dry weight calculated according to Rasooli and Razzaghi-Abyaneh (2005) was considered as growth index. The fungal mycelia were separated from culture media using a separator funnel and then washed thoroughly with distilled water. The mycelial mats were collected by filtration through Whatman No. 4 filter papers, washed twice thoroughly with distilled water in an oven at 95^{o}C until constant weight. Mycelial dry weights were then determined.

Fungal growth inhibition (%) was calculated according to the following formula: Fungal growth Inhibition (%) = [(Total control weight – Total sample weight)/ Total control weight] x 100.

Inhibition assays

Both simultaneous and deferred antagonism assays were used. In the simultaneous antagonism assay, each isolate of L *brevis* was grown with *A. flavus* in 250 ml-Erlenmeyer flasks

containing 100 ml of sterilized MRS broth. The medium was inoculated with 2.5 ml of a spore suspension of *A. flavus* containing ca. 1.5 x 104 spores/ml, and 2.5ml of bacterial inoculum containing ca. 1x 107 cfu (colony forming units)/ml. Cultures were incubated at 25^{o}C and 100 rpm in an orbital shaker incubator for 10 days and then analyzed for aflatoxin production. Cell-free supernatant fluids were used in the deferred antagonism assay. Each bacterial isolate was grown at 30^{o}C and 100 rpm for 48 h in 100ml of MRS broth inoculated with 2.5 ml of bacterial inoculum containing ca. 1 x 107 cfu/ml. Then cell-free supernatant fluids were prepared by centrifuging the culture at 20, 000 xg and 4^{o}C for 15 minutes. The pH of the supernatant fluids was adjusted to 7.0 (pH of control MRS) by 0.1 N NaOH in order to rule out possible inhibitory effect due to lowered pH in the growth medium. Supernatant fluids were sterilized by filtration through a 0.45 µm pore size filter and 2.5ml were aseptically dispensed in 250 ml-Erlenmeyer flasks before inoculated with 2.5 ml of a spore suspension of *A. flavus* containing ca. 1.5 x 104 spores/ml. Cultures were incubated at 25^{o}C for 10 days and analyzed for aflatoxin production.

Determination of Aflatoxin B1 (AFB1) production

AFB1 concentration was measured in both mycelia and culture media using a TLC method. A known amount of fresh mycelia from each flask was processed for AFB1 estimation. After freezing of the fungal mat by liquid nitrogen, mycelia were homogenized by mortar and pestle in presence of chloroform as the extraction solvent. The culture media were extracted by chloroform after shaking in a separatory funnel. The chloroform extracts of both mycelia and media from each flask were separately collected and then concentrated near to dryness using a rotary evaporator. The sample extracts were spotted on Silica gel 60F precoated TLC plates along with the AFB1 standards and developed in a TLC chamber with chloroform:methanol (98:2, v/v) as mobile phase. The amount of AFB1 was calculated at 365 nm using CAMAG TLC Scanner 3 by comparison of under-curved areas of samples and standards.

Incubation temperature and time

Three incubation temperatures, i.e., 4, 25 and 37^{o}C, were tested for their effect on production of anti-aflatoxigenic activity. Potato dextrose broth (PDB) was used as the growth medium. The incubation was conducted at 100 rpm for a total duration period of 4 days. Supernatant fluids were prepared daily and tested for anti-aflatoxigenic activity.

Thermal stability of anti-aflatoxigenic activity

The thermal stability of the anti-aflatoxigenic activity was tested by exposing supernatant fluids to 50, 70, and 100 for 30 minutes in a water bath, and 121^{o}C for 30 minutes in an autoclave and quickly cooling tap water. The control medium was non-heated, filter-sterilized supernatant fluids. Stability of anti-aflatoxigenic activity at different pHs. The effect of pH on the stability of anti-aflatoxigenic activity was evaluated at pH 4, 6, 8, and 10 adjusted with 1 N HCl/NaOH. And it was placed at room temperature for 1 h. Cell-free supernatant fluids were sterilized by filtration through a 0.45µm pore size filter before using.

RESULTS AND DISCUSSION

Inhibition of aflatoxin and mycelium production of *A. flavus.*

The antifugal and antiaflatoxigenic activities of *L. brevis* TMT 22 were analyzed and the results are summarized in Table 1. *L. brevis* TMT 22 inhibited aflatoxin production and mycelial growth of *A. flavus* when both microorganisms were cultivated simultaneously in MRS broth.

Table 1: Inhibition of aflatoxin and mycelium production of *A.flavus* by *L.brevis* TMT-22

Isolate	AFB1 (µg/ml)	Mycelium dry weight (mg/l)
Control (*A.flavus*)	28.2 ± 1.2[a]	33.8 ± 1.6
L.brevis TMT-22 + *A.flavus*	0.3 ± 0.0	21.3 ± 0.7
E.coli + *A.flavus*	29.2 ± 1.7	22.1 ± 1.2

[a]Standard deviations.

Table 2: Effect of *L.brevis* TMT-22 cell-free supernatant on aflatoxin and mycelia production from *A. flavus*

Isolate	AFB1 (µg/ml)	Mycelium dry weight (mg/l)
Control	21.9 ± 1.2	39.4 ± 1.7
Cell-free supernatant	1.8 ± 0.1	27.6 ± 1.6
Cell-free supernatant + water (1:1, v/v)	18.4 ± 1.0	19.9 ± 0.7
Cell-free supernatant + MRS (1:1, v/v)	9.3 ± 0.3	25.5 ± 1.7

[a]Standard deviations

The aflatoxin production was reduced to 99.2% but the mycelium production was less inhibited with percentage inhibition of 34.4%. *A. parasiticus* and *A. flavus* were inhibited in their production of aflatoxin to 95% by *Bacillus Subtilis* (Kimura and Hirano, 1998), and *B. pumilus*, (Munimbazi and Bullerman, 1998), respectively. Also, the elimination of aflatoxin is a rapid process involving the removal of approximately 80% AFB1 in cases of *L. rhamnosus* (El-Nezami *et al., 1998*). Data shown in Table 2 indicated that aflatoxin production and mycelium growth were inhibited in supernatant fluids of cultured MRS broth. The levels of AFB1 produced in the Lactobacillus cell-free supernatant were 92% lower than the control (Table 2). When the supernatant was diluted two-fold with distilled water, higher amounts of AFB1 were obtained, however, the mycelial dry weight decreased (Table 2). Dilution of

Lactobacillus cell-free supernatant with MRS increased the amounts of AFB1 to 44%. In this case fungal growth, as measured by mycelial dry weight, was not affected. Influence of growth media on production of antiaflatoxigenic activity. In Table 3, all media tested for production of antiaflatoxigenic metabolites were related to growth of *L. brevis* and production of inhibitory metabolites.

Incubation temperature and time on the production of antiaflatoxigenic metabolites were tested at three different temperatures, 4, 25 and 37^{o}C, for 1 to 4 days of incubation time (Table 3). Antiaflatoxigenic metabolites were not produced when the bacterium was incubated at 4^{o}C since there was no evident bacterial growth in these cultures. The minimum temperature for growth of lactic acid bacteria is 5^{o}C (Dantigny and Molin, 2000; Gourama, 1997). Inhibitory activity was obviously detected in the supernatant fluids of cultures cultivated in 25 and 37^{o}C. AFB1 production was completely inhibited in supernatant fluids obtained from 3 and 4-day old bacterial cultures. Gournama (1997) reported that temperature had a considerable effect on production of antifungal activity by *L. brevis* with the great inhibitory effect against Penicillium and *Aspergillus* species occurring at 37^{o}C.

Thermostability of antiaflatoxigenic metabolites.

The thermostability of the inhibitory substance was tested at four different temperatures, namely, 60, 80,100 and 121^{o}C (Table 4). The Lactobacillus cell-free supernatant lost their inhibitory activity when heated to 100 and 121^{o}C, however heating the supernatant at 50 and 70^{o}C did not affect their antiaflatoxigenic activity.

Table 3: Effect of incubation time and temperature on antiaflatoxigenic activity by *L.brevis* TMT-22.

Incubation time	Incubation temperature					
	4^{o}C		25^{o}C		37^{o}C	
	AFB1 (µg/ml)	Mycelium dry weight (mg/l)	AFB1 (µg/ml)	Mycelium dry weight (mg/l)	AFB1 (µg/ml)	Mycelium dry weight (mg/l)
Control[a]	16.0 ± 0.6	6.5 ± 0.3	16.7 ± 1.7	16.7 ± 1.7	7.5 ± 0.9	17.0 ± 1.2
Day 1	14.4 ± 0.3	5.3 ± 0.2	3.1 ± 0.1	6.2 ± 0.4	0.5 ± 0.0	5.4 ± 0.3
Day 2	15.2 ± 0.7	4.9 ± 0.3	0.1 ± 0.0	3.0± 0.1	nd	2.6 ± 0.1
Day 3	15.8 ± 0.9	5.1 ± 0.1	nd[b]	2.9 ± 0.2	nd	2.2 ± 0.1
Day 4	16.1 ± 0.5	4.2 ± 0.2	nd	2.2 ± 0.1	nd	1.7 ± 0.1

[a]Control=potato dextrose broth without bacterial inoculums, [b]nd= none detected
[c]Standard deviations

The maximum aflatoxin level was obtained when the supernatant was heated to 100^{o}C in all media tested. At 100^{o}C, the antifungal and antiaflatoxigenic activity were affected. At 121^{o}C, aflatoxin production was not as high as 100^{o}C. This could be due to some changes in the chemical composition of the media at the higher temperature, which may interfere with the biosynthesis of aflatoxins. Hence, the effect of heating on the inhibitory activity suggested the

presence of protein compounds in the supernatant, thus the loss of the inhibitory activity upon heating at 100^{o}C was probably due to denaturation of the protein(s) or cofactors.

Table 4: Effect of Growth media on the antiaflatoxigenic activity of *L.brevis* TMT-22

Temperature	AFB1(*µg/ml*)	Mycelium dry weight (*mg/l*)
Control	16.4 ±1.4	29.7 ± 2.1
50^{o}C	0.0 ± 0.0	24.3 ± 1.5
70^{o}C	0.0 ± 0.0	16.3 ± 1.6
100^{o}C	21.5 ± 2.2	21.1± 2,1
121^{o}C	14.4 ± 1.1	22.4 ± 1.9

Conclusion

It can be concluded from the available results that antiaflatoxigenic substances are either acidic, proteinaceous compounds with wide pH range for activity of other pH-dependent organic metabolites. Temperature, pH and media components have roles they play in antiaflatoxigenic activity. It will be recommended that further studies on the purification, identification, chemical nature, and biological characteristics of these antiaflatoxigenic metabolites be carried out.

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