

Antagonistic Activity of Lactic Acid Bacteria Bioactive Molecules against Fungi Isolated from Onion (*Allium cepa*)

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Abstract

This study centered on the antifungal activity of Lactic acid bacteria (LAB) associated with onion. Lactic acid bacteria (LAB) and spoilage fungi were isolated from healthy and spoilt onion samples respectively. *Lactobacillus brevis* I, *Lactococcus lactis* I, *Lactobacillus plantarum* I and *Pediococcus pentosaceus* I were identified phenotypically from healthy onion bulbs while *Rhizopus stolonifer*, *Penicillium citrinum* *Trichoderma reesei*, *Aspergillus flavus* and *Aspergillus niger* were identified from the spoilt onion bulbs. The inhibitory activity of LAB metabolites was investigated against onion spoilage fungi. *Lactococcus lactis* had the highest inhibitory activity of 31 mm against decay fungi with the exception of *Trichoderma reesei* which was not inhibited by any of the metabolites produced by Lactic acid bacteria isolates. All the isolated lactic acid bacteria produced bioactive molecules; lactic acid, diacetyl and hydrogen peroxide with *Pediococcus pentosaceus* I produced the highest quantity of diacetyl (2.3 g/l) and lactic acid (4.1 g/l) while the highest quantity of hydrogen peroxide (2.4 g/l) was produced by *Lactobacillus plantarum*. Lactic acid bacteria produced bioactive molecules that are highly effective in the inhibition of onion spoilage micro-organisms and could prevent mycotoxins production.

Keywords: Lactic Acid Bacteria; Onion; Spoilage Organisms; Bioactive Molecules; Onion Based Products

Introduction

Onions (*Allium cepa*) are considered as an essential vegetable which is generally known for its nourishing and therapeutic value [1]. It has been shown that recurrent consumption of onions lessens the menace of stomach cancer and oesophageal tumour in trial animals [2]. Shinkafi and Dauda [3] defined onions to be susceptible to a number of diseases caused by fungi and bacteria. Allium vegetables contain alliin that has antibiotic properties that prevent the growth of ocular pathogenic organisms. Most common fungal disease of onions is pink rot (*Pyrenochaeta terrestris*), Fusarium basal rot (*Fusarium oxysporum*), botrytis bulb rot (*Botrytis* spp), black mould rot (*Aspergillus niger*) among others [4]. Onion juice has been found to be curative by reducing the pain caused by stings produced from honey bee and as an adhesive for outward application on insect bites and scorpion stings. The odour or the smell produced by onions causes insect to flee this is why they can be used as insect repellents [5].

In the establishment that are associated with food production, decay of onion by microbes is a herculean task [6]. Even though extensive and huge work had been conducted in this area without promising result, there is a need to prevent fungal contamination of these products and others in order to reduce wastage in terms of financial and economic loss and health hazards which may occur due to fungi poisons production [7]. Many species of fungi play a major role in the spoilage of onion by reducing the quality and the hygienic level of the food [8]. It is known that the most efficient way to prevent contamination of foods by fungi is to prevent the growth of spoilage fungi.

Unfortunately, due to very poor storage conditions, especially in West Africa countries, contamination of various food commodities with spoilage moulds is unavoidable [9]. Several techniques have been developed for the destruction of spoilage fungi in foods, these include: heating, treatment with ammonia and radiation [10]. However, these techniques have their shortcomings because they are expensive, impractical for application on a commercial basis and also lead to the destruction of important nutrient [11].

The method by which organism producing antimicrobial compounds is used to control pathogenic organisms has drawn much attention of researchers in this direction especially in the last few years [12]. Biological antagonists such as LAB have several potential applications which include anticarcinogenic and bile tolerance because of the production of antimicrobials compounds [13].

Lactic acid bacteria are gram positive, catalase negative, non-spore producer microaerophilic bacteria whose main fermentation product of carbohydrate is lactate. They are usually cocci or rods in shape and commonly found in foods [14]. As a result of their contribution to flavour, aroma and spoilage retardation, they are significant in food technology [15]. The antagonistic growth of spoilage organisms can be due to one or more antimicrobial substances, such as bacteriocin, organic acids, and hydrogen peroxide produce by this organism [16]. Lactic acid bacteria have been shown to display inhibitory activity against pathogenic organisms which make them to be beneficial for consumption [17].

Aim of the Study

The aim of this study is centered on the antifungal ability of lactic acid bacteria on some spoilage isolates obtained from the onion.

Materials and Methods

Equipment

Binocular Microscope Fisher Scientific Cat. No 12561328 Led Illumination D3012-2881-071, Incubator Ehrat GmbH 783 Emmendingen-Kollmarstreute Typ bk3, Haier Thermcool Refrigerator model -114-250N white, Microwa Swiss electric weighing machine (7730 model), Portable Autoclave Sterilizer Machine YX-280B China, Centrifuge (SE-CFTDZ-WS, Labkits, U-Therm International (Hong Kong) Limited), Pye-Unicam pH meter, cork borer, measuring cylinder, wire loop, bijoux bottle, screw cap bottle, Needle and syringe were used in the this research.

Sample collection

Fresh and spoilt onion bulbs were purchased from different markets in Ibadan Nigeria. The samples were collected in polythene bags and immediately transported to the laboratory for analysis.

Isolation of microorganisms

The culture media used for this research were MRS (De Mann Rogosa and Sharpe Oxoid, Basingstoke, England) agar, Malt extract agar, Potato Dextrose agar (Lab M) and their Broths. Fresh onion bulbs were aseptically blended to pulp and serial dilutions of the onion samples were prepared by using 1g of the healthy onion bulbs. This was done by mixing 1g each of the samples thoroughly with 9 ml of sterile distilled water to give 1:10 dilution. The dilution was made up to 10^{-6} . Using a sterile pipette of 1 ml, appropriate dilutions were plated out using the culture media stated above and incubated at 30°C microaerophilically for lactic acid bacteria (Harrigan and McCance) [18]. For the spoilt onion bulbs, the onion bulbs were stripped of their outer dry scales, and surface sterilized in 1% sodium hypochlorite solution for 60 seconds [19]. These were rinsed in distilled water and blotted dry with a sterile filter paper. Small segments of tissues (3 mm³) from the margins of rotted lesion were cut out with a sterile scalpel and plated on potato dextrose agar in 90 mm petri-dishes. The plates were incubated at room temperature (28 ± 3°C) for 7 days. Developing fungal colonies were sub cultured continuously on fresh PDA plates to obtain pure cultures of the isolates.

Identification of isolates

Fungal isolates were identified based on macroscopic and microscopic characteristics according to method of Barnett and Hunter [20].

Macroscopic examination of fungal isolates

The macroscopic characteristics of Fungal isolates; colour and surface texture of colonies on their respective plates were observed.

Microscopic examination of Fungal isolates

Lactophenol cotton blue stain was placed on a clean slide and a small piece of mycelium was carefully transferred into it with the aid of sterile inoculating needle. It was then gently smeared, covered with a clean slip and examined under high-power magnification (x 40) objective lens of the microscope. The presence of fruiting bodies like conidia and sporangiospores were examined.

LAB isolates were identified based on cultural, morphological, microscopic and biochemical characteristics such as gram stain, spore stain, catalase test and sugar fermentation tests according to the method of Sathe., *et al* [21].

Gram's staining

A drop of water was placed on grease -free slides, then a thin smear of a young bacterial isolates was made on the slide with a drop of water and was allowed to air dry. The prepared smear was flooded with crystal violet stain for 30seconds which was gently rinsed away under a slowly running tap. Afterwards, it was flooded with Grams iodine which was poured off after 60 seconds and gently rinsed with tap water. The slide was flooded again with 70% alcohol and was rinsed off with water immediately. Safranin was used to stain for 30seconds and then rinsed gently with water and allowed to air dry. It was examined under the microscope using oil immersion objective lens(x100). Gram positive cells appeared purple while Gram negative cells appeared pink [22].

Spore staining

Malachite green staining procedure was used. A thin smear of the pure culture was made on a clean slide and allowed to dry. The slide was flooded with malachite green and then heated for 5 - 10 minutes until steam rises and it start to bubble or boil. The stain was rinsed off with distilled water and counter stained with safranin for 15 seconds. This was then rinsed off with distilled water and allowed to dry. The slide was then examined under the microscope with immersion oil objective lens (×100), the spore was stained green and the bacteria cells stained red [23].

Catalase test

This test was done to detect the presence or absence of the enzyme catalase in each of the isolates. The enzyme catalase breaks down the hydrogen peroxide (H₂O₂) to release gaseous oxygen and water:



A smear of the isolates was made on a clean sterile slide; one drop of freshly prepared 3% hydrogen peroxide solution was added to each slide, positive test indicates evolution of gas while its absence indicates negative reaction [24].

Sugar fermentation

The fermentation patterns were determined using modified MRS medium from which meat extract and glucose had been omitted but containing 0.05% (w/v) Bromocresol purple indicator as basal medium this was then sterilized at 121°C for 15 minutes. The cultures to be tested were first grown in MRS agar plates for 18 hours. The culture cells were then aseptically transferred from plates, inoculated into tubes of the basal media (10 ml) containing the test carbohydrates and incubated at 30°C for 4 days. Tubes in which Bromocresol purple colour changed to yellow indicated growth and acid production and uninoculated tubes served as controls [25].

Antagonistic activity of LAB against spoilage fungi

The antagonistic activity of LAB isolates against fungi was determined using the overlay method of Magnusson and Schnurer [26]. LAB was inoculated in two lines of 2 cm long on MRS agar plates and incubated microaerophilically at 30°C for 48h. It was overlaid with soft agar (75% by weight, agar) preparation of PDA containing 9.5×10^4 spores/ml of fungal. The plates were then incubated aerobically at 30°C for five days. They were examined for clear zones of inhibition around the bacterial streaks.

Determination of lactic acid, diacetyl and hydrogen peroxide production by LAB isolates

For these measurements, the test organisms were grown in MRS broth. They were inoculated with 0.1 ml of a suspension of LAB species and incubated microaerophilically [27]. Incubation was for 96h at 37°C with the initial pH of 5.5. Cultures were centrifuged at 3000 rpm for 15 minutes. A known volume of the supernatant fluid was used for all the titrations at 24h interval except where otherwise stated.

Quantitative estimation of lactic acid

The production of lactic acid was determined by titration of the supernatant fluid of LAB with 0.1M NaOH and 1ml of phenolphthalein indicator (0.5% in 50% alcohol). Each milliliter of 0.1M NaOH is equivalent to 90.08mg of lactic acid. The lactic acid was then calculated according to the A.O.A.C method [28].

$$\text{Lactic acid} = \frac{0.1\text{M NaOH} \times N \text{ NaOH} \times \text{M.E} \times 100}{\text{Volume of Sample used}}$$

Where: 0.1M NaOH = Volume of 0.1 M of NaOH used; N NaOH - Normality of NaOH solution; M.E = Equivalence factor.

Determination of diacetyl production

Diacetyl production was determined by transferring 25 ml of the supernatant fluid of LAB into 100 ml flasks. Seven and half milliliters (7 ½ ml) Hydroxylamine solution was added to the flask and to a similar flask for residual titration. Both flasks were titrated with 0.1M HCl to a greenish yellow end point using bromophenol blue as an indicator. The equivalence factor of HCl to diacetyl is 21.5 mg. The concentration of diacetyl produced was calculated according to the method of A.O.A.C method [28].

$$\text{AK} = \frac{(b - s) (100E)}{W}$$

Where: Ak = Percentage of diacetyl; b = No. of ml of 0.1 M HCl consumed in titration of the sample; E = Equivalence factor; W = Volume of sample; S = No. of ml of 0.1M HCl consumed in titration of the sample.

Quantitative estimation of hydrogen peroxide

Hydrogen peroxide production was determined by introducing 25 ml of the supernatant fluid of LAB into the separate 100 ml flask. To each was added 25 ml of dilute H₂SO₄. This was then titrated with 0.1M potassium permanganate (KMnO₄). Each milliliter of 0.1M KMnO₄ is equivalent to 1.701 mg of H₂O₂. A decolorization of the sample was regarded as the end point. The volume of H₂O₂ produced was then calculated [28]

$$\text{H}_2\text{O}_2 \text{ Concentration} = \frac{0.1\text{M KMnO}_4 \times N \text{ KMnO}_4 \times \text{M.E} \times 100}{0.1\text{M H}_2\text{SO}_4 \times \text{Vol. of sample}}$$

Where: $0.1M\ KMnO_4 = \text{Volume of } 0.1M \text{ of } KMnO_4 \text{ used}$; $N\ KMnO_4 = \text{Normality of } KMnO_4$; $0.1M\ H_2SO_4 = \text{Volume of } 0.1M\ H_2SO_4 \text{ added}$; M.E = Equivalence factor.

Statistical analysis

Data were analyzed using Analysis of Variance (ANOVA) to determine significant difference between the means and these were expressed as mean ± standard deviation (SD). The level of significance was set at $p \leq 0.05$. The data were analyzed using SPSS version 17.0.

Results

Lactobacillus plantarum, *Lactobacillus brevis*, *Lactococcus lactis* and *Pediococcus pentosaceus* from fresh, healthy onion bulbs were identified with the percentage occurrence of 32%, 18%, 5% and 45%, respectively, while *Aspergillus niger*, *Aspergillus flavus*, *Rhizopus stolonifer*, *Penicillium citrinum* and *Trichoderma reesei* isolated from spoilt onion were identified with percentage occurrences of 36.2%, 10.3%, 25.9%, 12.1% and 15.5% respectively (Table 1 and 2).

Isolate	Number of occurrences	% occurrence
<i>L. plantarum</i> I	7	32
<i>L. lactis</i> I	1	5
<i>P. pentosaceus</i> I	10	45
<i>L. brevis</i> I	4	18
Total	22	100

Table 1: Percentage occurrence of LAB from fresh onions.

Name of Isolate	Number of occurrences	% occurrence
<i>Aspergillus niger</i>	21	36.2
<i>Rhizopus stolonifer</i>	15	25.9
<i>Aspergillus flavus</i>	6	10.3
<i>Penicillium citrinum</i>	7	12.1
<i>Trichoderma reesei</i>	9	15.5
Total	58	100

Table 2: Percentage occurrence of Fungi isolated from spoilt onion bulbs.

The result of antagonistic activity of LAB metabolites against spoilage moulds from onions is represented in table 3 and plate 1. *Lactococcus lactis* and *Lactobacillus plantarum* had the highest antifungal activity (31 mm) against *Aspergillus flavus* and *Penicillium citrinum* while *Lactobacillus brevis* I had the lowest antifungal activity (10 mm) against *Rhizopus stolonifer*. None of the LAB isolates produced metabolite that was inhibitory to *Trichoderma reesei*.

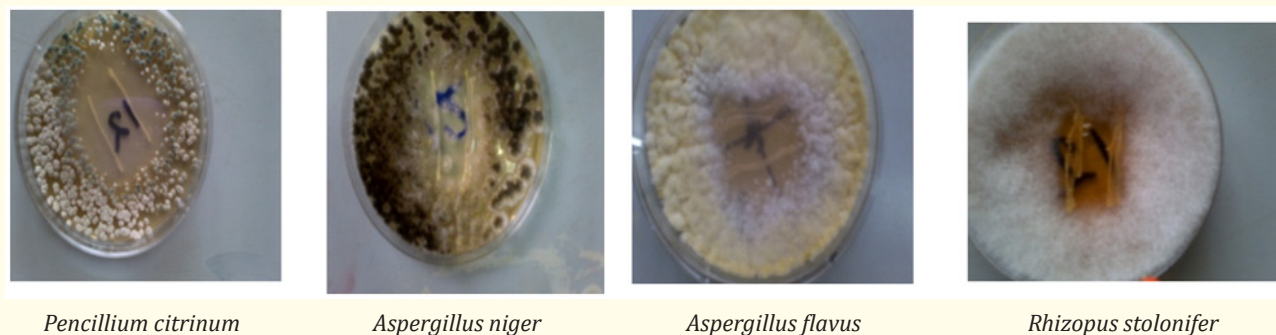


Plate 1: Antifungal activity of *L. plantarum*

LAB Metabolites	Fungi/ Zone of inhibition (mm)				
	<i>A. niger</i>	<i>A. flavus</i>	<i>P. citrinum</i>	<i>R. stolonifer</i>	<i>T. reesei</i>
<i>L. lactis</i>	24.00 ± 2.00 ^a	31.00 ± 1.00 ^a	26.50 ± 1.50 ^{ab}	18.50 ± 0.50 ^a	ND
<i>P. pentosaceus</i>	23.50 ± 1.50 ^a	24.50 ± 0.50 ^b	25.50 ± 3.50 ^{ab}	21.00 ± 1.00 ^a	ND
<i>L. plantarum</i>	22.00 ± 2.00 ^a	24.50 ± 0.50 ^b	29.00 ± 1.00 ^a	21.00 ± 1.00 ^a	ND
<i>L. brevis</i>	21.00 ± 1.00 ^a	26.50 ± 0.50 ^b	20.00 ± 0.00 ^b	10.00 ± 1.00 ^b	ND

Table 3: Antagonistic activity of LAB against fungi.

Key: ND: Not detected; Mean values with different superscript down the column are statistically significant using Duncan alpha ($p \leq 0.05$).

Quantity of antimicrobial compounds produced by LAB species is represented in table 4-6. Lactic acid, diacetyl and hydrogen peroxide were produced by all the LAB isolates. The quantity of metabolites produced increased as hours of incubation increased till 72 hours of incubation after which there was a decrease in the quantity of the metabolites. *Pediococcus pentosaceus* produced the highest quantities of diacetyl (2.3 g/l), while *Lactobacillus brevis* had the lowest quantity (0.6 mg/l). *Pediococcus pentosaceus* produced the highest quantity of lactic acid (4.1 g/l) at the 96 hours incubation period, while *Lactobacillus brevis* produced the lowest quantity (1.8g /l). Hydrogen peroxide was produced by the all LAB isolates. *Lactobacillus plantarum* produced the highest quantities (2.4 g/l) at 72 hours incubation period while *Lactococcus lactis* had the lowest quantity (0.8 g/l).

Isolate	Hours of Incubation/concentration of diacetyl (g/L)			
	24h	48h	72h	96h*
<i>L. plantarum</i>	1.6 ± 0.02 ^b	2.0 ± 0.04 ^a	2.1 ± 0.01 ^a	1.4 ± 0.06 ^{c#}
<i>L. brevis</i>	0.6 ± 0.01 ^c	1.3 ± 0.02 ^b	1.5 ± 0.03 ^a	1.0 ± 0.02 ^b
<i>P. pentaseus</i>	1.2 ± 0.05 ^c	2.1 ± 0.01 ^a	2.3 ± 0.02 ^a	1.6 ± 0.04 ^b
<i>L. lactis</i>	0.9 ± 0.02 ^c	1.5 ± 0.06 ^a	1.7 ± 0.01 ^a	1.2 ± 0.03 ^b

Table 4: Quantity of diacetyl produced by LAB.

Key: Hours of Incubation= *; concentration of diacetyl= #; Values are the means of triplicate determinations ± standard deviation; Mean values with different superscript down the column are statistically significant using Duncan alpha ($p \leq 0.05$).

Isolate	Hours of Incubation/ concentration of lactic acid (g/L)			
	24h	48h	72h	96h*
<i>L. brevis</i>	2.8 ± 0.05 ^c	3.1 ± 0.03 ^b	3.6 ± 0.02 ^a	1.8 ± 0.04 ^{d#}
<i>L. plantarum</i>	3.5 ± 0.02 ^b	3.8 ± 0.04 ^a	3.9 ± 0.01 ^a	2.3 ± 0.02 ^c
<i>P. pentaseus</i>	3.1 ± 0.06 ^c	3.6 ± 0.02 ^b	3.7 ± 0.02 ^b	4.1 ± 0.02 ^a
<i>L. lactis</i>	2.9 ± 0.01 ^b	3.3 ± 0.02 ^a	3.0 ± 0.00 ^a	2.4 ± 0.03 ^c

Table 5: Quantity of Lactic acid produced by LAB.

Key: Hours of Incubation= *; concentration of lactic acid = #; Values are the means of triplicate determinations ± standard deviation; Mean values with different superscript down the column are statistically significant using Duncan alpha ($p \leq 0.05$).

Isolate	Hours of Incubation/concentration of Hydrogen peroxide (g/L)			
	24h	48h	72h	96h*
<i>L. brevis</i>	1.2 ± 0.01 ^c	1.6 ± 0.03 ^b	2.2 ± 0.0 ^a	1.2 ± 0.01 ^{c#}
<i>L. plantarum</i>	1.7 ± 0.02 ^b	2.1 ± 0.04 ^a	2.4 ± 0.02 ^a	1.0 ± 0.03 ^c
<i>P. pentaseus</i>	1.3 ± 0.02 ^c	1.6 ± 0.02 ^b	2.0 ± 0.01 ^a	1.2 ± 0.02 ^c
<i>L. lactis</i>	1.0 ± 0.00 ^c	1.4 ± 0.02 ^b	2.0 ± 0.00 ^a	0.8 ± 0.01 ^d

Table 6: Quantity of Hydrogen peroxide produced by LAB.

Key: Hours of Incubation= *; concentration of Hydrogen peroxide = #; Values are the means of triplicate determinations ± standard deviation; Mean values with different superscript down the column are statistically significant using Duncan alpha ($p \leq 0.05$).

Discussion

LAB was obtained from fresh, healthy onion bulbs and was identified as *Lactococcus lactis*, *Pentosaceus pentaseus*, *Lactobacillus plantarum* and *Lactobacillus brevis*. According to Yi-sheng, *et al.* [29] many genera of LAB can be isolated from fresh vegetables. The presence of these organisms as microflora is attributed mainly to the surroundings of the vegetables [30,31]. The predominant LAB species was *P. pentosaceus*. Similar report has been observed in the research conducted by Emerenini, *et al.* [32] who reported that *P. pentosaceus* and *L. plantarum* had highest percentage occurrence from fruits and vegetables. The dominance of these organisms in food confers a lot of benefits to the food as these LAB species aid in protecting the food against spoilage [33].

The presence of *A. flavus* isolated from the spoilt onions is an indication that the onion bulbs were contaminated during long period of storage because of its seasonal nature. This study agrees with research conducted by Shehu and Mohammad [34], who reported *A. flavus* amongst other isolated fungi from spoilt onion. Tafinta, *et al.* [35] isolated *A. flavus* amongst other moulds from Sweet Orange (*Citrus sinensis*) fruits which had undergone long periods of storage.

The antagonistic activity of LAB against spoilage fungi isolated from spoilt onions showed that the fungi isolates were sensitive to the bioactive molecules produced by LAB (Lactic acid, hydrogen peroxide and diacetyl) isolates except for *T. reesei*, which was resistant to these metabolites. The antagonistic activity of LAB is attributed to the synergistic activity of the bioactive molecules produced. Asma, *et al.* [36] reported similar results where LAB inhibited some selected fungi.

Lactic acid, Diacetyl and hydrogen peroxide produced by the LAB as metabolites enhanced greatly at varying quantities as hours of incubation increases. This development is credited to the ability LAB to produce inhibitory substances as a strategy to survive and exclude other microorganisms in their environment. However, the quantity of metabolites produced, decreases after 72h with the exception of *P. pentosaceus* as the hours of incubation increases, which could be ascribed to the state of growth of the microorganism (Death phase). It was discovered that *P. pentosaceus* had a high quantity of lactic acid at 96 hours of incubation and this is in agreement with the similar findings of Hu, *et al.* [37] who reported acidic nature of *P. pentosaceus* that made the microorganism a very useful tool as a starter culture in the fermentation of most foods.

Consumption of contaminated onions with fungi or molds and mycotoxins as a secondary metabolite could cause adverse effects on humans and animals resulting in illnesses of different kinds and economic losses [38]. It also has an adverse effect and raised the toxic level in both human and animal reproductive system which could lead to infertility and cancer [39]. Therefore, in this study metabolites produced from LAB such as Lactic acid, Diacetyl and hydrogen peroxide were observed to inhibit the growth of contaminated fungi in onions as a vegetable commonly consumed both in developed and developing countries. Intake of LAB and its metabolites had been reported to be beneficial as observed by Adegoke and Ogunbanwo [16]. Hence, LAB and its metabolites could be used as a preservative of onions and other vegetable products for being safe and without harmful effect.

Conclusion

Onions are very important vegetables, with high medicinal and nutritional qualities. Due to their seasonal nature, they undergo long periods of storage, which exposes them to spoilage by fungi. It is therefore recommended that necessary hygienic precautions should be taken during storage in order to prevent invasion by spoilage fungi and subsequent contamination with spoilage moulds. LAB is known for the effective antagonistic activity of their bioactive molecules against spoilage microorganisms which has been shown in this research work. The metabolites produced by LAB isolate in this study could be used effectively for the inhibition of aflatoxigenic fungi and the improvement of shelf life of vegetables, especially onion based products such as onion paste or jam, dried onion, drinks, onion-supplemented bread and onion snack.

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