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Evaluation of Antibacterial Activity of Spices and Vegetables against Bacillus methylotrophicus strain Kharuss 0103

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ABSTRACT: *In this investigation the antibacterial activity of aqueous extracts of commonly used spices and vegetables were assayed against Bacillus methylotrophicus strain Kharuss 0103 isolated from poultry farm. Garlic (Allium sativum) extract showed maximum inhibitory effect on Bacillus methylotrophicus strain Kharuss 0103. Aqueous extracts of Zingiber officinale, Allium cepa, Beta vulgaris and Momordica charantia did not inhibit the growth of tested bacteria. Allium sativum were showing zone of inhibition of 30 mm and 24 mm using Agar well diffusion method and Agar disc diffusion method respectively against this strain. These results suggest that Allium sativum is a potential spice for inhibiting the growth of this bacterial strain isolated from poultry farm.*

KEYWORDS: *Antibacterial activity, Agar well diffusion method, Disc diffusion, Poultry farm bacteria, Spices extract, Vegetables extract.*

I. INTRODUCTION

In recent years food safety concerns have been focused on several pathogens. Man has been using natural products of animals, plants and microbial sources for thousands of years either in the pure forms or crude extracts [1]. Vegetables, herbs and spices are an important part of the human diet. They have been used for thousands of years to enhance the flavour, colour and aroma of food. In addition to this, vegetables and spices are also used for preservation and medical use. Naturally present antimicrobial substances have been recovered from various vegetables and spices. *Allium sativum* (Garlic) can be used as a spice in food and medicine [2]. A bioactive compound in garlic that has antibacterial activity is allicin, which is a volatile compound containing sulphur [3]. Aqueous extracts of garlic also had antibacterial activity against bacteria that was found for aquaculture products including *Citrobacter freundii*, *E. coli*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*[4]. *Zingiber officinale* (Ginger), belonging to the family, *Zingiberaceae* is widely used as a spice and medicine. Ginger has been used to treat digestive problems. Ginger has the capacity to eliminate harmful bacteria, such as *Escherichia coli*, responsible for most of the diarrhoea, especially in children [5]. *Allium cepa* L (Onion) belongs to the family *Alliaceae*. It is also known as 'garden onion'. Onions are effective against common cold, heart disease, diabetes, osteoporosis, coughs and sore throat [6]. Certain chemical compounds believed to have anti-inflammatory, anti-cholesterol, anticancer and antioxidant properties.The flesh part of the onion contains flavonoids. Polyphenols are the important component of onion which differentiates it from other *Allium* species. *Beta vulgaris* L (Beet root) juice is considered powerful to prevent infectious and malignant disease. Beet root is a potential source of valuable water-soluble nitrogenous pigments, called betalains. Betalains have been extensively used in the modern food industry [7]. Beetroot helps normalize the pH balance of the body and replenish the blood. *Momordica charantia* (Bitter gourd) often called 'bitter melon' belongs to the family *Cucurbitaceae*. The most important use of bitter gourd is, it reduces the blood sugar level and is very good for diabetic patients. *Momordica charantia* leaves are rich in phytochemicals which has free radicals scavenging activity. Bacteria present in the poultry farm are the causative agents of food poisoning, food spoilage, stomach pain, vomiting etc. Multi-drug resistant bacteria are present in chicken, apparently because of the use of antibiotics in poultry production, and are passing to people who work with, prepare or eat chicken, at some risk to their health. The present study was aimed to evaluate the potentiality of aqueous extracts of some of the commonly used spices and vegetables against *Bacillus methylotrophicus* strain Kharuss 0103 isolated from poultry farm.

II. MATERIALS AND METHODS

Sample Collection and isolation

Samples (surface soil) were collected from poultry farm of Guduvanchery, Tamilnadu (India).Soils were brought to the laboratory in aseptic condition. 1gram of surface soil sample was suspended in 9 ml of saline and mixed vigorously to make uniform suspension. After that soil samples were serially diluted up to 10^{-5} and 0.1ml of aliquots were spread over nutrient agar plates. The plates were incubated at 37ºC for 24hours. Pure

strains were picked out and purified by repeated streaking on nutrient agar slants. The culture was streaked on slants and kept in incubator at 37°C for 24 hours and were preserved in slants at 4±2°C.

Biochemical and Morphological Characterization

Purified isolate was characterized by biochemical analysis using the tests prescribed in Bergey's Manual of Systematic Bacteriology. The Tryptone broth, MR-VP broth, Simmon's citrate agar and Christensen's agar medium were used for Indole test, Methyl Red test, Voges Proskaver test, Citrate utilization test, Catalase test and Urease test. Gram staining and Motility test were performed under Morphological test.

Genomic DNA isolation

2 ml of bacterial culture were centrifuged at 6000 rpm for 5 minutes. The supernatant was discarded. 1 ml of UniFlex^{TM}Buffer 1 and 10 µl of RNase were added to the pellet obtained. Mixed well by pipetting and incubated for 30 minutes at 37ºC in a water bath. To the lysed sample 1 ml of 1:1 phenol:chloroform were added and mixed well. The sample was centrifuged at 10,000 rpm for 15 minutes at room temperature. The aqueous layers were separated in a fresh 1.5 ml vial. To the aqueous layer 1 ml of UniFlex^{TM}Buffer 2 were added and mixed well by pipetting. The mixture was centrifuged at 12,000 rpm for 15 minutes at room temperature. The supernatant was discarded. To the pellet 500 µl of 70% ethanol were mixed. Again it was centrifuged at 10,000 rpm for 10 minutes at 4ºC. The supernatant was discarded. The pellet was air dried for about 10-15 minutes till the ethanol evaporate. The pellet was resuspended in 50-100 µl of UniFlex^{TM}Elution Buffer. DNA was stored at -20ºC.

PCR amplification and sequencing

The 16S ribosomal RNA was amplified by using the PCR (ependorfep.Gradient) with *Taq* DNA polymerase and primers 27F (5`AGTTTGATCCTGGCTCAG 3`) and 1492R (5`ACGGCTACC TTGTTACGACTT 3`). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94°C for 4 min followed by 30 cycles at 94°C for 1 min, primer annealing at 52°C for 1 min and primer extension at 72°C for 1 min. At the end of the cycling, the reaction mixture was held at 72°C for 10 min and then cooled to 4°C. PCR amplification was detected by agarose gel electrophoresis and visualized by alpha image gel doc after ethidium bromide staining. The PCR product obtained was sequenced by an automated sequencer (Genetic Analyzer 3130, Applied Biosystems, and USA). The same primers as above were used for sequencing. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST available at http:// www.ncbi-nlm-nih.gov/. The DNA sequences were aligned and phylogenetic tree was constructed by using the Molecular Evolution Genetic Analysis (MEGA) software version 4.0. 16S rRNA sequence was then submitted to the GenBank, NCBI, USA.

Spices and Vegetables of interest

Most commonly used spices (*Allium sativum* and *Zingiber officinale*) and vegetables (*Allium cepa, Beta vulgaris* and *Momordica charantia*) were purchased from the local market in Guduvanchery, Tamilnadu (India).

Aqueous Extract preparation of spices and vegetables

Fresh garlic (*Allium Sativum L*) bulbs were peeled, weighed (10 g), and surface sterilized using 95% ethanol. The ethanol was allowed to evaporate in a sterile laminar flow chamber, and the garlic was homogenized aseptically with 10 ml of sterile double distilled water using a sterile mortar and pestle. The homogenized mixture was filtered through 8 layers of muslin cloth and centrifuged at 6000 rpm for 15 min. Supernatent was collected and kept at 4ºC for further use. Edible parts of fresh Onion (*Allium cepa)* were rinsed thoroughly in distilled water and air dried. 10 grams were then blended and homogenized with sterile double distilled water in 1:1 ratio. The juice was then filtrated and squeezed out of it. The extract was stored at 4°C. In the same way equal weight of edible part of Zinger (*Zingiber officinale*), Beet root (*Beta vulgaris*) and Bitter gourd (*Momordica charantia*[excluding seed]) were weighed and sterilized with alcohol. Homogenized with sterile double distilled water in equal volume using mortar and pestle. Filtrates were centrifuged and supernatant were stored at 4ºC for further use.

Test Microorganism

Bacillus methylotrophicus strain Kharuss 0103 (Accession no.-KC424493) isolated from poultry farm was used.

Antibacterial activity testing using Agar well diffusion assay

The bacteria was inoculated into 10 ml of sterile nutrient broth in conical flask, and incubated overnight at 37ºC in rotatory shaker. The culture was swabbed on the surface of sterile Mueller Hinton agar (Hi-media) plate using a sterile cotton swab. 5 agar wells were prepared with the help of sterilized cork borer with radius 5 mm. Using a micropipette, 100µl of spices and vegetables aqueous extracts (supernatant) were added to

the each well of the plate. The plates were incubated in an upright position at 37ºCfor 24 hours. The diameter of inhibition zones measured in mm and the results were recorded.

Antibacterial activity testing using Agar disc diffusion assay (Kirby-Bauer method)

The culture was swabbed on the surface of sterile Mueller Hinton agar (Hi-media) plate using a sterile cotton swab. For agar disc diffusion method, the disc (6 cm) was saturated with 25 µl of each of the spices and vegetables extract (supernatant). After that the disc was allowed to dry and introduced on the upper layer of the agar plate. The plate was incubated overnight in upright position at 37°C. Microbial growth was determined by measuring the diameter of the zone of inhibition.

III. RESULT

In the present study the organism isolated from poultry farm was identified as new strain of *Bacillus* species according to morphological (Table 1), biochemical characteristics (Table 2), 16SrRNA gene sequencing and phylogenetic tree (Fig-c). Antibacterial properties of spices and vegetables were studied against *Bacillus methylotrophicus* strain Kharuss 0103 isolated from poultry farm by Agar well diffusion method and Agar disc diffusion method (the two most commonly used method to determine antimicrobial susceptibility). Among all spices and vegetables, aqueous extract of *Allium sativum* were found to be active against *Bacillus methylotrophicus* strain Kharuss 0103. *Allium sativum* was showing zone of inhibition of 30 mm against this strain through Agar well diffusion method. *Bacillus methylotrophicus* strain Kharuss 0103 were found to be resistant against the aqueous extract of rest of the spices and vegetables[Table 3 and Fig. (a)]. Aqueous extract of *Allium sativum* was showing inhibitory effect against *Bacillus methylotrophicus* strain Kharuss 0103. *Allium sativum* was showing zone of inhibition of 24 mm against this strain through Agar Disc diffusion method. The strain was found resistant to rest of the spices and vegetables (*Zingiber officinale, Momordica charantia, Allium cepa* and *Beta vulgaris*)[Table 4 and Fig.(b)].The 16S rRNA sequence obtained was subjected to BLAST search using in the NCBI data base. BLAST search result showed that *Bacillus methylotrophicus* strain Kharuss 0103 has 99% similarity to the isolate *Bacillus sp.* A phylogenetic tree was constructed based on neighbor-joining method[Fig.(c)].

TABLE 1: Morphological characteristics of the microorganisms

CHARACTERISTICS	RESULT
Gram staining	+
Motility	+

TABLE 2: Biochemical test results of microorganisms

TESTS	*B. methylotrophicus* strain Kharuss 0103
Indole	_
Methyl Red	_
Voges Proskaver	_
Citrate utilisation	+
Catalase	+
Urease	_

TABLE 3: Measurement of zone of inhibition (in mm) by Agar well diffusion method

Bacteria	***Allium sativum***	***Zingiber officinales***	***Momordica charantia***	***Allium cepa***	***Beta vulgaris***
Bacillus methylotrophicus strain Kharuss 0103	30 mm	-	-	-	-

TABLE 4: Measurement of zone of inhibition (in mm) by Agar Disc diffusion method

Bacteria	***Allium sativum***	***Zingiber officinales***	***Momordica charantia***	***Allium cepa***	***Beta vulgaris***
Bacillus methylotrophicus strain Kharuss 0103	24 mm	-	-	-	-

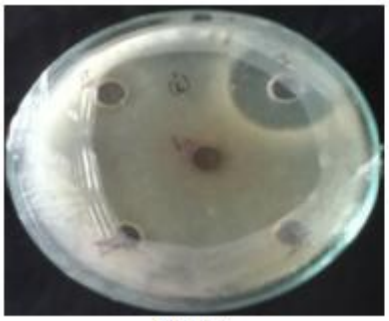

Fig: (a)

(i) =*B. methylotrophicus* strain Kharuss 0103

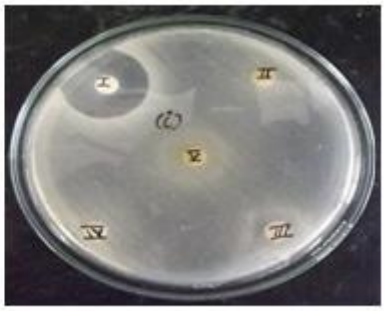

Fig: (b)

(i) =*B. methylotrophicus* strain Kharuss 0103

**(I = Allium sativum, II = Zingiber officinale, III = Momordica charantia, IV = Allium cepa, V = Beta vulgaris)*

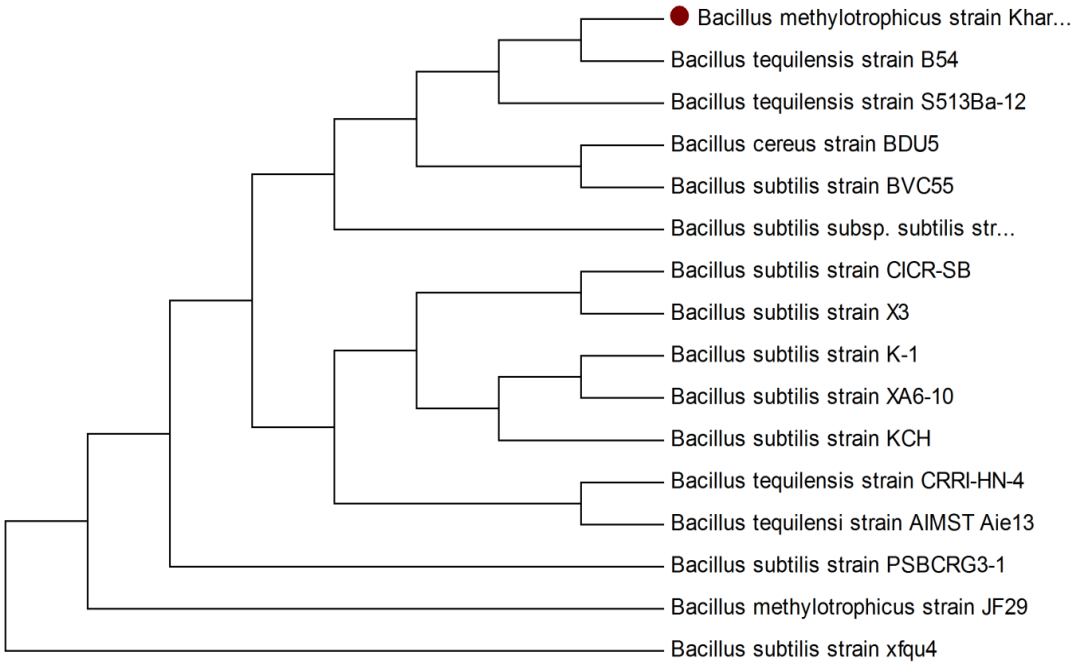

Fig: (c)- Phylogenetic tree obtained by Neighbor-joining analysis based on 16S rRNA gene sequences showing the phylogenetic position of strain Kharuss 0103.

IV. DISCUSSION

Srinivasan *et al* [14] reported that aqueous garlic extract (AGE) has the potential of a broad spectrum of activity against both Gram (+) and Gram (-) bacteria. In our study AGE has potential to inhibit the growth of poultry farm bacteria i.e. Gram (+) bacteria. The antibacterial activity of garlic is widely attributed to allicin. Allicin interferes with RNA production and lipid synthesis. All these things contribute to the bacteria can not grow in the presence of allicin or AGE. Onyeagba *et al* [16] reported that the crude extract of garlic and ginger applied singly and in combination did not exhibit any *in vitro* inhibition on the growth of test organisms (*Bacillus, Staphylococcus aureus, E.coli, Salmonella*). In our study aqueous extract of *Zingiber officinale* was found to be ineffective against *B.methylotrophicus* strain Kharuss 0103. Ranjan *et al* [18] reported that garlic can be used as food preservatives and thus the use of other chemical preservatives can be minimized, which could be beneficial for environment and consumer health, or a plastic for food preservation can be inverted using the antibacterial activity of garlic, the inner wall of the plastic coated with garlic. The onion bulbs contain numerous organic sulphur compounds including flavinoids, phenolic acids, saponins, cholesterol etc. The presence of these compounds may explain its antimicrobial activity. Benkeblia (2004) reported that in Algeria, red / purple onion exhibit better antibacterial activities as compared to yellow onion against *S. aureus* and *Salmonella enteritidis*. The zone of inhibition of extracts increased with increasing concentration of extracts[19]. In this study aqueous extracts of *Allium cepa* were showing no inhibitory effect on the bacteria even at high concentration (100µl). So research should be continued as its antimicrobial herb against the poultry farm bacteria. Leelaprakash *et al* [21] reported that *Momordica charantia* leaves are rich in phytochemicals which has free radicals scavenging activity. Recently researchers have found that *Momordica charantia* contains

several proteins that inhibit HIV *in vitro*, these proteins known collectively as ribosome inactivating proteins (RIPs) are alpha-momorcharin, beta-momorcharin and MAP-30 (Momordica anti-HIV protein) [22]. In this investigation, aqueous extract of edible parts of *Momordica charantia* were found to be ineffective against *B.methylotrophicus* strain Kharuss 0103. As research is still in progress, it is unclear which ingredients of this vegetable are having most antimicrobial activity. Extracts of beetroot showed some antimicrobial activity on *Staphylococcus aureus* and on *Escherichia coli* and also antiviral effect was observed[23,24]. In this study beet root was ineffective against the poultry farm bacteria *B.methylotrophicus* strain Kharuss 0103. A lot of research should be done to know about the compounds present in the beet root which has the antibacterial activity against the poultry farm microbes. Garlic extract, even at low concentration (25µl) is able to inhibit the growth of this strain, while other spices and vegetables are ineffective even at high concentration (100µl). From this investigation, it is clear that among all spices and vegetables tested, garlic has good antimicrobial property against the bacteria that have been isolated from poultry farm. Garlic has shown better activity against *B. methylotrophicus* strain Kharuss 0103 as compared to other aqueous extracts of spices and vegetables. As pathogenicity of *B.methylotrophicus* is unclear, but there is chance that this new strain may be pathogenic to human being. So garlic may be a good source for the treatment of the people working in the poultry farm who might be affected from this microbe. If garlic is provided to those people as a raw food in their diet, they can be cured from the infection of this microbe to some extent. The result of present study clearly indicates that the aqueous extract of garlic possess compounds with antimicrobial properties that can be further studied for their antimicrobial activity.

V. CONCLUSION

From our study and the earlier reports it is clear that garlic is a good antibacterial agent. Filtrates of fresh garlic can be used to inhibit the growth of this new strain of *Bacillus* species isolated from poultry farm. Fresh garlic filtrates may reduce the use of antibiotics in the poultry farm. Further research is required to know the physiological role and antibacterial activity of these spices and vegetables. Also another study should be continued to isolate and purify the active components of garlic and its inhibitory action against the bacteria present in poultry farm.

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