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RESEARCH PAPER

Isolation and Identification of Bacteria from Post-harvest Vegetables and Mechanism of Pathogenicity

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ABSTRACT

Vegetables are horticulture products having living tissues with continuing metabolism, i.e. respiration, water loss and cell softening throughout the post-harvest. Surveys of these post-harvest raw vegetables contaminated with various microorganisms. In the present study, the spoilage vegetables were purchased from the local market, Rajkot. Total eight bacteria isolates were obtained from spoilage vegetables and identified by morphological and molecular techniques. For identification of the strain, genomic DNA was isolated and amplified by universal primer and amplified DNA fragments were separated by electrophoresis. Bacteria were identified by *16S rDNA* gene sequencing. Purified PCR product was proceeded for cycle sequencing and sequenced on 3130 Genetic analyser (Applied Biosystem, USA). All the obtained sequences were submitted in the NCBI database. Further the bacterium was characterized by IAA secretion. Conditions for IAA production was optimized in a nutrient medium containing tryptophan and estimated by Salkowski reagent and characterized by TLC. The probable role of IAA secretion in the mechanism of pathogenicity is discussed.

HIGHLIGHTS

- Post-harvest vegetables remain metabolically active and prone to microbial spoilage.
- Eight bacterial isolates were obtained from spoiled vegetables in Rajkot.
- Identification was done using 16S rDNA sequencing and NCBI submission.
- IAA production was analyzed to study its role in pathogenicity.

Keywords: Bacteria, Postharvest, Vegetables, IAA (Indole acetic acid), pathogenicity

India is the second world largest major fruit and vegetable producer country of the world with 32 and 71 million metric tonnes, respectively (Mathur *et al*. 2014). In India, fruits and vegetables worth Rs. 13300 crores spoiled every year mainly during the post-harvest phase due to microbial diseases, disorder, transpiration and senescence which led to great economic loss (Tripathi *et al*. 2008; Bhosale, 2013). Postharvest decay of fruits and vegetables can be traced to fungi and bacterial infections that occur either between flowering and fruit maturity or during harvesting, subsequent handling and storage and marketing (Dennis, 1983; Droby, 2006). Perishable fruits and vegetables facilitate the easy attack of the micro-organism due to high water activity which spoiled rapidly. Microorganisms play an important role in spoilage of fruits and vegetables and can losses up to 50% production (Sudheer *et al.* 2007; Sharma *et al*. 2013).

Vegetables was contaminated with the microorganism and deterioration on fruits causes reduction in market values and nutritional qualities, and at

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times rendered the fruits non-fit for consumption (Muhammad *et al.* 2004). These microorganisms include a wide range of free-living as well as plant-associated bacteria, both symbiotic and phytopathogenic, have been documented to produce IAA (Tsavkelova *et al*. 2006; Gravel *et al*. 2007). IAA production plays an important role in numerous plant–pathogen interactions (Jameson, 2000). There have been several reports that the plant hormones produced by bacterial production of IAA is involved in the virulence of several interactions between microorganisms and plants (Suzuki *et al.* 2003). During infection, pathogens can elevate the host's normal auxin level either by synthesizing auxin themselves or by providing an effect to enhance plant-induced auxin synthesis. Using diverse mechanisms of action, including mimicking plant hormones, causing the pores in plant membranes or inhibiting host metabolic enzymes, these toxins can affect plants (Robert *et al.* 2006). In addition to elevating auxin levels, pathogens are known to disturb auxin signalling in their hosts, which is considered a major cause of plant damage. Several studies have shown that pathogen infection results in imbalances in auxin levels as well as changes in the expression of genes involved in auxin signalling. Taking this point into consideration, the aim of this study was to (i) isolation and identification of pathogens from the post-harvest spoilage vegetables (ii) Auxin production was determined from the all-isolated organisms to understand pathogenicity mechanism. Further it was confirmed by TLC analysis.

MATERIALS AND METHODS

Isolation of microorganism

The spoilage vegetables were collected from the local market of Rajkot. Bacteria were isolated from the different plants (Plate 1). The spoilage vegetables were washed under running tap water for 3 h and surface sterilized with 0.1% $HgCl_2$ for 15 min.

Plate 1: Postharvest spoilage vegetables

Further it was washed thrice with sterile distilled water. After surface sterilization, with help of sterile forceps and scalpel infected part of the vegetables was cut. The cut infected part was inoculated on N-agar medium under aseptic conditions and incubated at 37°C. Isolated bacteria were sub-cultured on nutrient agar medium at regular intervals, incubated at 37°C for 24 h and preserved at 4°C for further study. The colony characteristics of isolated bacteria like size, shape, pigmentation, margin, texture elevation and opacity were recorded (Table 1). Morphological examination of the isolates was done by Gram's staining techniques.

Table 1: Morphology and colony characteristics of isolated strain

Bacterial strain	Colony Characteristic	Morphology
SUB50	Round, raised, translucent, smooth, yellow	Gram negative
SUB51	Round, raised, translucent, smooth, creamy white	Gram negative
SUB52	Round, flat, transparent, smooth, greenish yellow	Gram negative
SUB53	Round, raised, opaque, smooth, Dirty white.	Gram negative
SUB54	Round, raised, opaque, smooth, creamy white	Gram positive
SUB55	Irregular, flat, opaque, gummy, creamy white	Gram negative
SUB56	Spindle, raised, opaque, gummy, yellow	Gram negative
SUB57	Round, raised, opaque, smooth, creamy white.	Gram negative

Identification of bacteria

Bacterial DNA was isolated according to (Chudasama and Thaker, 2012). The quality and concentration of the DNA was confirmed by measuring optical density 260/280 nm ratio. The *16s rDNA* gene was used for bacterial identification, amplified using universal primer. The amplified DNA fragments were separated by electrophoresis through 1.5% agarose gel. The purified PCR products were sequenced using a Big Dye Terminator V 3.1 Cycle Sequencing Kit using ABI 3130 genetic analyzer.

IAA production

Bacteria were allowed to grow in Nutrient broth containing L-tryptophan 200 mg/L for 10 days at 37°C in shaking condition. At regular time intervals (24 h) over a period of 10 day, IAA levels secreted into the culture media were measured. After every 24 h the broth was centrifuged at 3000 g for 20 min. The cell free supernatant was collected and used for estimation of IAA production. One ml supernatant was mixed with 2ml of Salkowski reagent (2ml of 0.5M $FeCl_3$ + 98ml 35% $HClO_4$) and tubes were incubated in dark. The intensity of red color developed within 30 min was measured at 530 nm. Uninoculated growth medium was used as a control. The whole assay was performed twice and readings were taken in triplicates.

Calibration curve of IAA

Indole acetic acid (Hi media) was used as a standard and calibration curve was prepared in a range of 10-100µg IAA/ml. Value of optical density of red color developed by test isolates was plotted on the curve and µg IAA produced/ml culture media by each organism was calculated.

Thin layer chromatography (TLC)

Ethyl acetate fraction of bacteria and standard were placed on TLC plates (Silica gel 60 F- f254, thickness 0.25 mm). TLC was run by using solvent system isopropanol: distilled water in 8:2 proportion and spots were detected by spraying Salkowski reagent. Rf value of the standard and IAA produced by the isolate was calculated.

RESULTS AND DISCUSSION

Spoilage generally causes undesirable changes in colour, odour, flavour, texture, and other characteristics of vegetables, and spoilage may occur during cultivation, transportation, or storage and lead to significant economic losses for farmers, the food industry, and customers worldwide. Postharvest disease is a major cause of spoilage of unprocessed fruits and vegetables (Nguyen and Carlin, 1994), and the contamination of fruit and vegetable products by spoilage bacteria and fungi are of great concern. Considering these, *in present study, total eight bacteria were isolated from different* spoilage *vegetables* and initially designated as SUB50, SUB51, SUB52, SUB53, SUB54, SUB55, SUB56 and SUB57. These bacteria were primarily differentiated on the basis of their colony characters and cell arrangement. The colony appearance of the isolates

varied significantly from each other (Table 1). Each isolate was further identified on the basis of DNA sequencing.

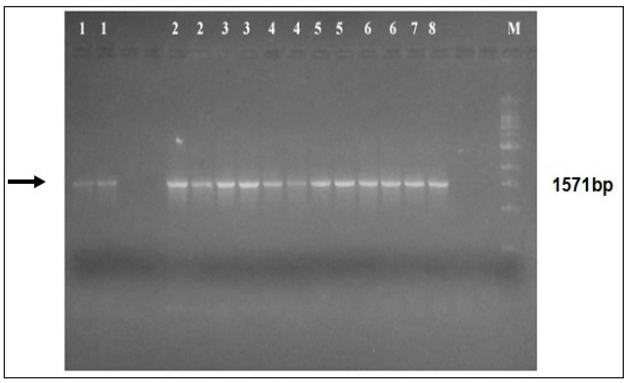

Fig. 1: Agarose gel electrophoresis of amplified 16s rDNA gene, 1517bp product using universal primer (lane 1, 2, 3, 4, 5, 6, 7, 8 and M contains: SUB50, SUB51, SUB52, SUB53, SUB54, SUB55, SUB56, SUB57 and marker, respectively)

In the present study, using the universal primer set, a 1517bp DNA fragment of the *16S rDNA* gene was amplified by PCR (Fig. 1). The 1517bp PCR amplified *16S rDNA* region was sequenced because it composed of both variable and conserved regions. The sequence obtained from SUB50 – SUB57 was compared to *16S rDNA* gene sequences available in the NCBI database by BLASTn homology search, as described by Altschul *et al.* (1997). The strain SUB50, SUB51, SUB52, SUB53, SUB54, SUB55, SUB56 and SUB57 showed similarity with *Stenotrophomonas maltophilia, Enterobacter asburiae*, *E. cloacae*, *E. cloacae*, *Exiguobacterium aurantiacum*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterobacter cloacae*, respectively. The similarity of reference sequence accession number and percentage identity is presented in Table 2.

Enterobacter and *E. coli* species are found in natural environment (water, sewage, soil and vegetables), especially *E. cloacae* is saprophytic microorganism frequently isolated from plant, flower, seeds and vegetables and from a wide variety of environmental sources, such as water, soil and foodstuff. *E. cloacae* cause disease of onions (Schroeder and Toit 2009). The genus *Pseudomonas* is the most heterogeneous and ecologically significant group of known bacteria and includes gram negative motile aerobic rods that are widespread throughout nature (Franzetti and Scarpellini, 2007). Earlier Lee *et al.* (2013) reported *Pseudomonas aeruginosa* plays a key role in spoilage of many vegetables. *Exiguobacterium* is a versatile gram-positive facultative anaerobes bacterium (Vishnivetskaya *et al.* 2009). This bacterium is a common inhabitant of soil and water confined to tropical and subtropical regions (Kumar *et al.* 2010). *Stenotrophomonas maltophilia* has been reported from a variety of microenvironments such as water, vegetables and soil (Valdezate *et al.* 2004).

IAA production

The IAA production from the *S. maltophilia* is presented in Fig. 2A. *S. maltophilia* was produced IAA in range 8-23.8 µg/ml for up to 10 days. IAA production in *E. asburiae* increased with day of culture inoculation and in a range of 14-371 µg/ml (Fig. 2B). *E. cloacae* strain SUB52 was produced with very less IAA and started at 6th day (Fig. 2C). *E. cloacae* SUB53 was produced in range (6-201 µg/ml) and gradually increased with day of inoculations (Fig. 3A). *E. aurantiacum* showed slowly increased in IAA production up to 5th day, declined thereafter and stabilized (Fig. 3B). It produced 2-15µg IAA/ml culture. The IAA production of *P. aeruginosa* was gradually increased with days of inoculation (Fig. 3C). *Escherichia coli* was produced the higher amount of IAA up to 8th day and then after it

Table 2: Bacteria isolated from infected plant material

No.	Strain	Host	Similarity	Accession no.
1	*Stenotrophomonas maltophilia* (SUB50)	*Trichosanthes dioica*	98%	MG451936
2	*Enterobacter asburiae* (SUB51)	*Cucumis sativus*	99%	MG451937
3	*Enterobacter cloacae* (SUB52)	*Solanum melongena*	99%	MW534691
4	*Enterobacter cloacae* (SUB53)	*Solanum melongena*	99%	MG451938
5	*Exiguobacterium aurantiacum* (SUB54)	*Vigna sinensis*	94%	MW534692
6	*Pseudomonas aeruginosa* (SUB55)	*Luffa aegyptiaca*	95%	MG451939
7	*Escherichia coli* (SUB56)	*Vigna sinensis*	100%	MG451940
8	*Enterobacter cloacae* (SUB57)	*Abelmoschus esculentus*	98%	MG451941

became stable at stationary phase (Fig. 4A). *E. cloacae* SUF57 was produced the maximum IAA at 3rd day and then after it became stable up to 10th day (Fig. 4B). The IAA extracted from the culture filtrate of *S. maltophilia, E. asburiae, E.cloacae, E. cloacae, E. aurantiacum, P. aeruginosa, E. coli* and *E. cloacae* showed a similar spot to that of the standard IAA in TLC analysis (Fig. 5).

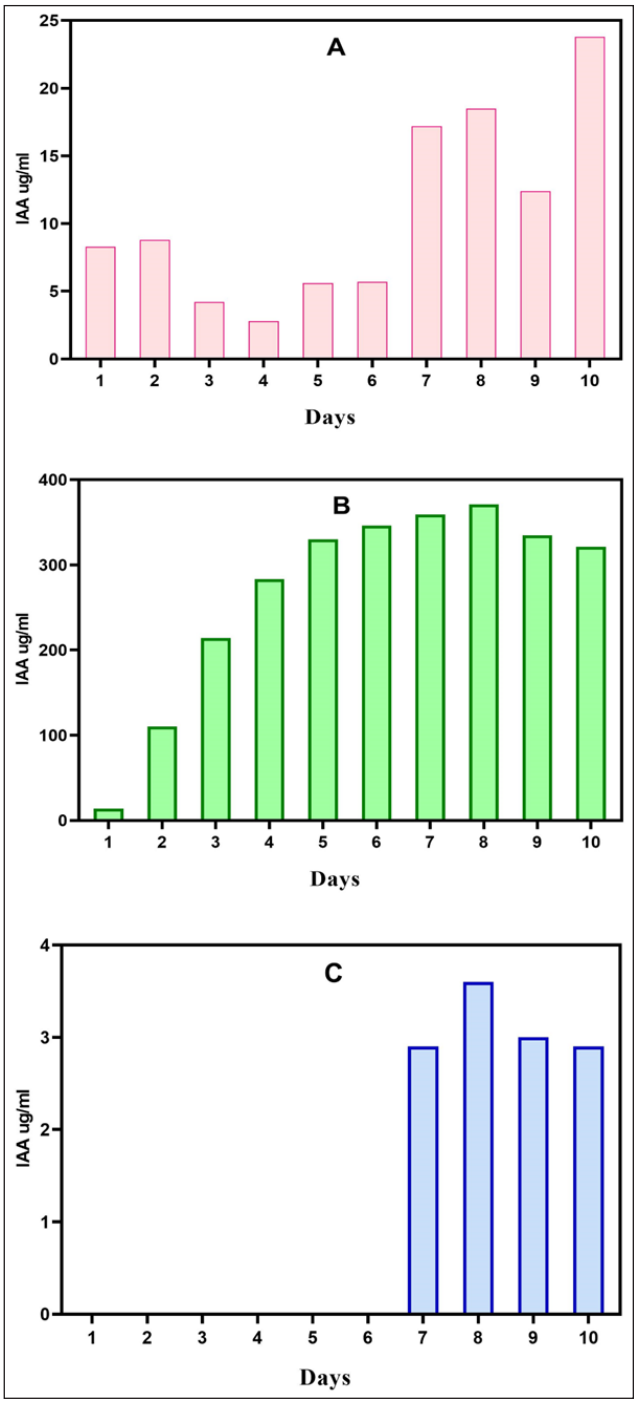

Fig. 2: IAA production with time changes by **(A)** *S. maltophilia* SUB50 **(B)** *E. asburiae* SUB51 **(C)** *E. cloacae* SUB52

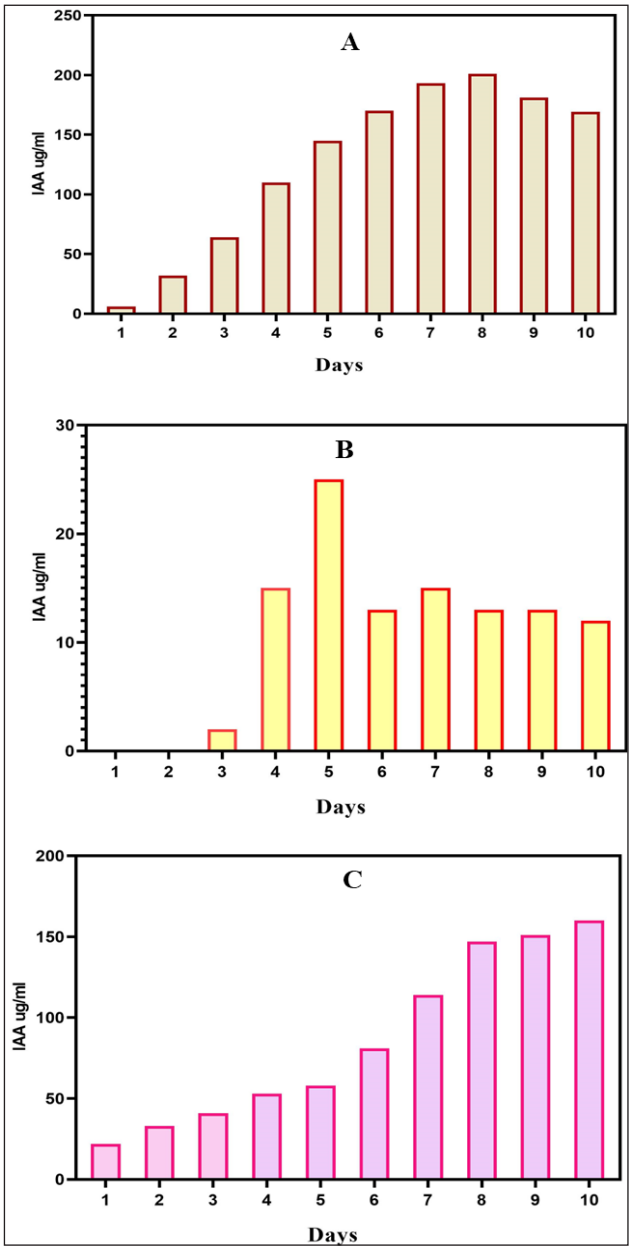

Fig. 3: IAA production with time changes by **(A)** *E. cloacae* SUB53 **(B)** *E. aurantiacum* SUB54 **(C)** *P. aeruginosa* SUB55

Many plant-associated microorganisms can synthesize auxins and it has been demonstrated to be a virulence factor in some pathogens (Costacurta and Vanderleyden, 1995). In the present work a nutrient broth with tryptophan was chosen for determining bacterial ability to synthesize IAA. The highest level of IAA accumulation in the bacterial culture was found during the early and late stationary growth phase. *Enterobacter asburiae* was produced a higher amount of IAA compared to other strains suggesting that it might have a gene for IAA synthesis which would express

during pathogenicity. *Pseudomonas sp*. and *E. coli* are synthesized the moderate amount of IAA and *Enterobacter cloacae* SUF52 produced in less quantity. These results showed that bacteria belonging to one genus produced different amounts of auxins. Previously Manulis *et al*. (1994) demonstrated that *Streptomyces scabies* has the ability to produce IAA, which was hypothesized to promote scab lesion formation through cell division during the development of wound periderm. Earlier Surico *et al.* (1985) reported that microbial IAA production has been shown to be important for pathogenicity of the *Pseudomonas syringae* pv. *savastanoi*, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* which induced galls or knots on stems and leaves of infected hosts.

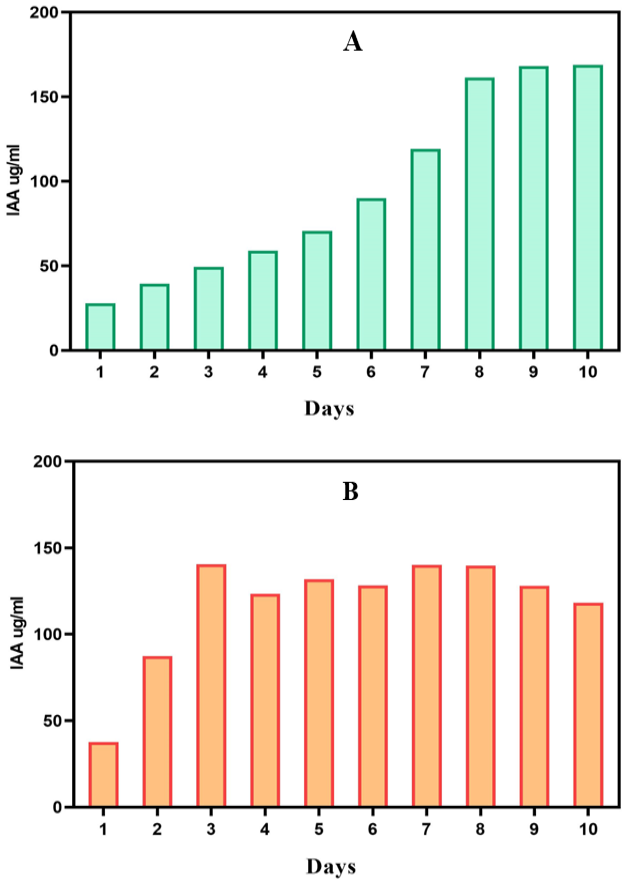

Fig. 4: IAA production with time changes by **(A)** *E. coli* SUB56 **(B)** *E. cloacae* SUB57

On the basis of results, it concluded that, in the present study eight bacterial strains are isolated from different spoilage vegetables as phytopathogens. This work revealed that *S. maltophilia, E. asburiae, E. cloacae, E. aurantiacum, P. aeruginosa,* and *E. coli,* are causative agent in deterioration of vegetables. These bacteria are also reported as phytopathogens in other systems. Deterioration of most vegetables is caused by bacterial infection. We therefore recommended timely spraying of the vegetables with natural pesticides to reduce the damaging activities of the bacterial pathogen and contamination with toxins and other related bacterial metabolites that might be hazardous to human health. From the results of IAA secretion, it is clearly indicated that all isolated bacteria from vegetables produced IAA in the tryptophan growth medium. So, auxin producing bacteria might have genes for IAA synthesis which would express during pathogenicity. It may be possible that at the initial stage of infection, pathogens generate IAA, hydrolyze plant cell wall polysaccharides and the cell wall saccharides that are released can serve as an ideal nutrition supply to pathogens for survival and later proliferation.

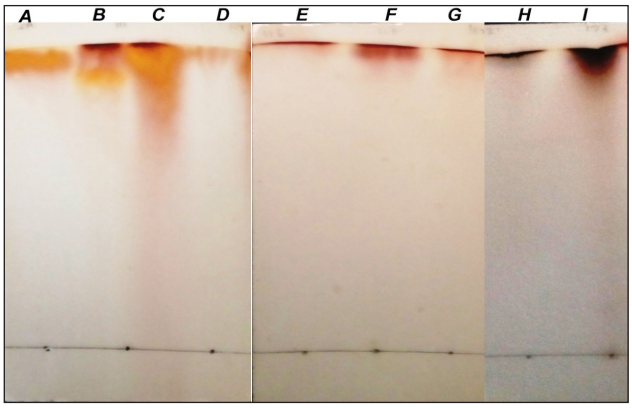

Fig. 5: Results of TLC **(A)** Standard **(B)** *S. maltophilia* SUB50 **(C)** *E. asburiae* SUB51 **(D)** *E. cloacae* SUB52 **(E)** *E. cloacae* SUB53 **(F)** *E. aurantiacum* SUB54 **(G)** *P. aeruginosa* SUB55 **(H)** *E. coli* SUB56 **(I)** *E. cloacae* SUB57

GENERATIVE AI DECLARATION

The author(s) confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript, and no images were manipulated using AI. The authors developed and verified all scientific content, data analysis, interpretation of results, and conclusions. The authors take full responsibility for the accuracy, integrity, and originality of the work presented, and no AI tool was listed as an author.

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