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Characterization of Bacterial Strains from Rotten Fruits Treated with Harmful Preservatives

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Abstract

Background: Fruits are beneficial to maintain good health, but microbes can spoil them. To avoid spoilage of fruits different harmful preservatives are being used that pose danger to human health and environment. This study was designed to isolate bacterial strains from rotten fruits preserved by using different preservatives.

Methods: Different rotten fruit samples were collected from different shops of Moon Market and Neelam block, Allama Iqbal Town, Lahore and used to purify bacterial cultures by growing on simple N-agar medium. Biochemical characterization was performed by different tests including gram staining, catalase, mannitol salt agar, glucose and fructose fermentation and nitrate reduction. Bacterial strains were further subjected to additional tests like HCN, H_2S production, metal resistance and antibiotic sensitivity.

Results: Nineteen bacterial strains were found positive for different tests and were characterized as *Bacillus* sp, *Staphylococcus aureus*, *Micrococcus varians*, *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*. Many bacterial strains were resistant to antibiotics and high doses of most metals specially mercury.

Conclusion: Due to the use of high doses of mercury for the storage of fruits, microorganisms have evolved resistance. It is an urgent need to take alternative measures for the storage of fruits for the safe lives.

SCAN ME

Introduction

Fruits play a vital role in human diet and provide different things like vitamins, minerals, carbohydrates, micronutrients, macronutrients and dietary fibers. Different nutrients like zinc, riboflavin, calcium, potassium and phosphorous are also supplied by fruits. Fruits consist of large quantity of compounds that are bioactive and show high antioxidant potential [1]. The people in the older times considered that fruits have magic and divine properties. Fruits are easily digested and have cleansing impact on the digestive tract and blood. People that eat fruits as a diet have good health.

According to World Health Organization (WHO) many diseases like heart diseases, micronutrient deficiencies, cancer and health conditions related to diet can be reduced by daily intake of five to eight portions of the fruits [2]. The fruit is available in two forms either fresh or processed form [3]. Fresh fruits are available for limited time span due to the senescence stage of the fruits. Collection and transportation of fruits is difficult after ripening. Therefore fruits are preserved through various methods like drying, freezing and canning for future consumption [4].

During the past few years, the infectious agents present in the fresh fruits were responsible for food borne illness [5]. Some methods are being used now a days to reduce the microbial population like SO_2, organic acids, sodium bisulfite, $CaCl_2$, acidified sodium chlorite and O_3 [6]. But the strong ionic water has various advantages e.g. cheap, harmless and applicable as compare to other sanitizing techniques to overcome microbial population.

Metals are also used for the preservation or to increase the shelf life of fruits. Mercury compounds e.g. methyl mercury and mercuric chloride are used to preserve the fruits and prevent the growth of microorganisms [7]. Both forms of mercury are carcinogenic for humans [8] and cause brain malfunctioning, kidney failure and immune dysfunction [9]. Antibiotics are the drugs that are used to treat the infections caused by bacteria. But bacteria show resistance towards antibiotics because it is a natural phenomenon of a bacterial cell to resist against antimicrobials. Excessive use and misuse of antibiotics in the field of medicine and agriculture confer bacterial resistance [10]. The trend to use the natural antimicrobial compounds has increased nowadays to treat the pathogenic microorganisms. The compounds that are biologically active show antimicrobial activity like essential oils, organic acids, aromatic compounds and alcohols and these compounds have no side effects [11]. In the environment, metal resistant strains can select the resistant mechanisms in which they are comparable to the variety of antibiotic resistant strains. The resistance genes of antibiotics and metals have common association because both genes are present frequently on the same mobile genetic elements [12]. The aim of this research is to check the resistance in bacterial strains against different metals and drugs in the rotten fruit samples. The bacterial strains showed high resistance towards heavy metals and drugs due to excess and misuse of them that cause danger to human health and environment. That's why use of natural compounds to preserve fruits are safe and pose no danger to the environment.

Methods

Sample collection

Rotten fruit samples were collected from different shops located at Moon Market and Neelam block, Allama Iqbal Town, Lahore. All samples were labeled properly and stored carefully in refrigerator at 4ºC in the sealed plastic bags. The pulp of rotten fruits was kept apart aseptically at -20°C for further processing.

Isolation and purification of bacterial strains from rotten fruit samples

Nutrient agar medium [13] was used to isolate bacterial species from different rotten fruit samples e.g. Mango bacterial strains (R-1, R-2, R-3, R-4, R-5), banana (R-6, R-7), guava (R-8, R-9), grape (R-10, R-11), peach (R-12, R-13, R-14), apple (R-15, R-16) and pomegranate (R-17, R-18, R-19). The pulp of different fruit samples was serially diluted in the Eppendorf. Serial dilution was prepared by adding 0.01g fruit pulp in 1ml autoclaved distilled water in the Eppendorf and labeled as 10^{-1}. Eppendorf was vortexed for proper mixing of pulp in the water. Series of two-fold dilutions were made up to 10^{-8} and 50 µl of dilutions from 10^{-4} to 10^{-8} were spread on N-agar plates under aseptic conditions. Inoculated plates were incubated at 37°C for 24 hours. Different colonies were selected according to the morphological features and streaked on N-agar plates by quadrant streaking method for purification. The purified cultures were then preserved at -20°C as 30% glycerol stocks to use further in the experiments.

Morphological and biochemical characterization of bacterial strains

Growth and colony morphology of different bacterial strains was examined according to the size, shape, margin, elevation, optical density and pigmentation. Biochemical characterization was performed for all bacterial isolates by different tests including Gram staining, catalase, mannitol salt agar, glucose fermentation, fructose fermentation, nitrate reduction, HCN and H_2S production by following methods described by [13].

Metal resistance test of bacterial strains

All bacterial strains were spread separately on L-agar plates and made six wells on each plate. Six different concentrations (20, 50, 70, 100, 120 and 150 µg/ml) of both metals (Cu, Hg) were prepared and 20µl of each metal was poured into each well. Plates were incubated at 37°C for 24 hours and metal resistance was determined by measuring the diameter of inhibition zones in mm [14].

Antibiotic susceptibility test

Antibiotic susceptibility test was performed for all bacterial strains against seven antibiotics; tetracycline, erythromycin, penicillin, chloramphenicol, streptomycin, ceftriaxone and cephalosporin (50 and 100µg/ml of

each). All bacterial strains were spread on L-agar plates and five wells were made on each plate. Antibiotics solution of 20µl volume was poured into each well aseptically and incubated plates at 37°C for 24 hours. Antibiotic sensitivity was observed by measuring diameter of inhibition zones in mm around the wells.

Statistical analysis

All experiments were done in triplicates and data were subjected to mean, standard deviation, analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) (P=0.01) by using SPSS V.20. Software.

Results

Morphological characterization of purified bacterial strains

Nineteen morphologically different bacterial colonies were selected from purified bacterial strains isolated from different rotten fruit samples. Colony morphology of different bacterial isolates was observed that include shape, margin, elevation, size, appearance, pigmentation and opacity. Most strains had circular shape, entire margin, raised elevation, shiny appearance, white to creamy pigmentation, opaque and translucent opacity. Size of colony varied from 1-4 mm in diameter only. R-1 strain had filamentous shape, rhizoid margin, flat elevation and dull appearance.

Strains	Biochemical tests						Expected bacterial strains
	Mannitol	Glucose	Fructose	Nitrate	HCN test	H_2S test	
R-1	----	----	----	----	+++	-	*Bacillus* sp.
R-2	----	+	----	+	-	+	*M. varians*
R-3	-	----	-	----	+	-	*S. saprophyticus*
R-4	+ (YC)	----	----	----	+	+	*Bacillus* sp.
R-5	+ (YC)	----	----	----	-	+	*S. aureus*
R-6	-	+	----	+	-	+	*M. varians*
R-7	-	+	----	+	+	+	*M. varians*
R-8	-	+	----	+	-	+	*M. varians*
R-9	+ (YC)	+	----	+	-	+	*S. aureus*
R-10	-	----	-	----	-	-	*S. saprophyticus*
R-11	+ (YC)	+	----	+	-	+	*S. aureus*
R-12	----	+	----	+	-	+	*M. varians*
R-13	+ (YC)	----	----	----	+	-	*Bacillus* sp.
R-14	- (PC)	----	+	----	+	+	*S. epidermidis*
R-15	+ (YC)	----	----	----	-	-	*Bacillus* sp.
R-16	----	+	----	+	+	+	*M. varians*
R-17	+ (YC)	----	----	----	-	-	*S. aureus*
R-18	- (PC)	----	----	----	-	-	*S. epidermidis*
R-19	- (PC)	----	+	----	++	-	*S. epidermidis*

Key: YC= yellow color, PC= pink color, + = positive, - = negative, ---- = not applicable

Table 1: Results of biochemical tests of bacterial strains isolated from rotten fruits.

Cellular and biochemical characterization

Different biochemical tests were performed to characterize and identify the purified bacterial isolates. Gram staining was performed in order to determine the cell shape, size (mm), type (gram positive or negative) and arrangement (single, pairs or chains) of all bacterial strains.

Fifteen out of nineteen bacterial strains were gram positive cocci and remaining were gram positive bacilli. After doing gram staining of selected bacterial strains, some other biochemical tests such as catalase, mannitol salt agar, glucose and fructose fermentation, HCN, H_2S production and nitrate reduction were performed. The selected strains were characterized as *Bacillus* sp, *Staphylococcus aureus*, *Micrococcus varians*, *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* (Table 1).

Heavy metal resistance test

Different concentrations of copper and mercury were used to determine the ability of bacterial strains to resist against heavy metals. By well plate assay, results were monitored and calculated the size of inhibition zones in mm. By using different concentrations of mercuric chloride; 20, 50, 70, 100, 120 and 150µg/ml, zone of inhibition size varied from 8-18 mm. R-4 strain was sensitive to all concentrations of mercuric chloride. R-5 strain showed resistance to 20µg concentration only and sensitive to all other concentrations. R-14, R-15, R-17, R-18, R-19 was sensitive to 100, 120 and 150µg concentrations. Remaining bacterial strains were resistant to all concentrations of mercuric chloride as shown in Fig 1.

All 19 bacterial strains showed complete resistance against copper sulfate. The study clearly reflects the level of environmental pollution polluted with the heavy metals and microorganisms have developed resistance against harmful level of mercury and copper (Figs 1 & 3).

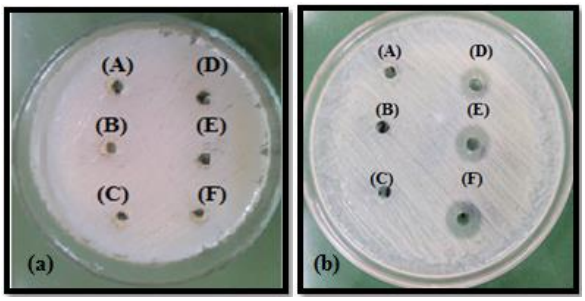


Figure 1: Bacterial resistance against heavy metals through well plate method A, B, C, D, E, F = 20, 50, 70, 100, 120 and 150ug/ml **(a)** $CuSO_4$ **(b)** $HgCl_2$ Clear zones around D, E and F indicate the sensitivity of bacterium to $HgCl_2$.

Antibiotic susceptibility testing

Two concentrations (50 and 100µg/ml) of seven antibiotics were used. Bacterial strains found sensitive to antibiotics were detected based on zone of inhibition around the wells in mm. Zone size varied from 8-26 mm. R-3 showed resistance against all antibiotics except tetracycline. R-4 strain was sensitive to almost all antibiotics. R-13 showed sensitivity towards antibiotics except streptomycin. All the bacterial strains showed resistance against penicillin except R-3 and R-17 but sensitive to tetracycline.

The level of sensitivity or resistance varied among all strains (Figs 2 & 4). Figure 4 clearly showed that (a) fifteen out of 19 bacterial strains 15/19 (b) 12/19 (c) 11/19 and (d) 11/19 bacterial strains were sensitive to 100 µg/ml ceftriaxone, streptomycin, erythromycin and chloramphenicol concentration respectively.

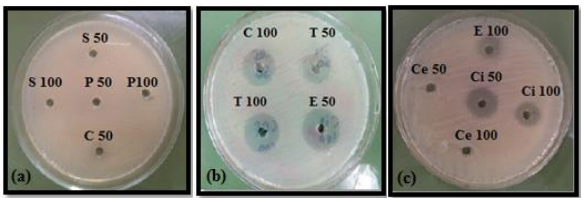


Figure 2: Antibiotic susceptibility testing (ug/ml) **(a)** R-10 bacterial strain showed resistance to streptomycin, penicillin and chloramphenicol **(b)** R-9 strain showed antibiotic sensitivity to chloramphenicol, tetracycline and erythromycin **(c)** R-1 strain showed sensitivity to erythromycin and ciprofloxacin and resistant to ceftriaxone **Key:** S= streptomycin, P= penicillin, C= chloramphenicol, T= tetracycline, E= erythromycin, Ce= ceftriaxone, Ci= ciprofloxacin

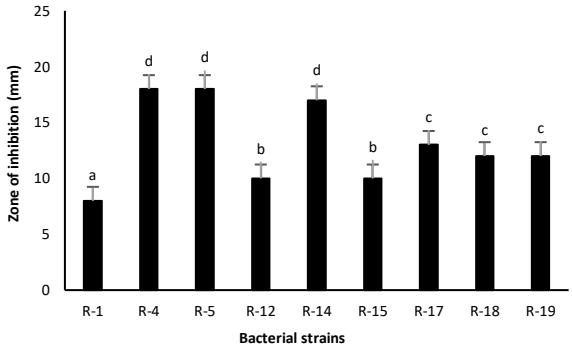
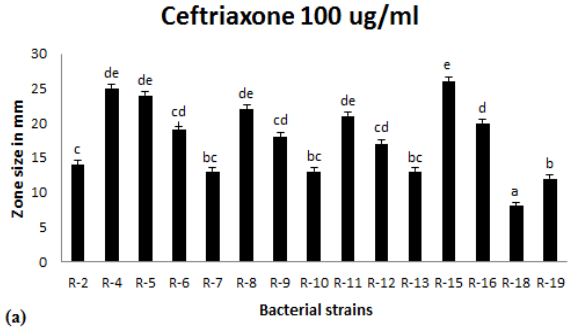
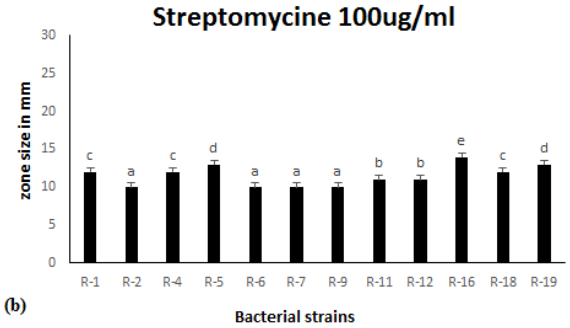
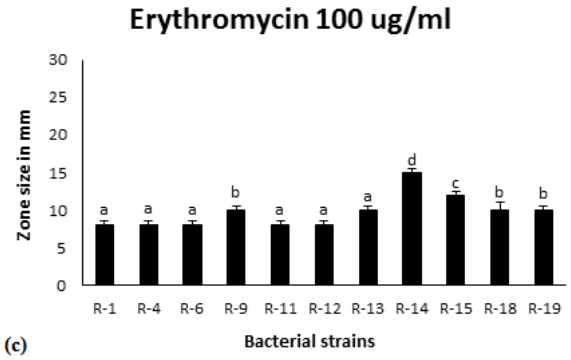


Figure 3: Diameter of inhibition zone ranged in 8-18 mm. Bars represents means ± standard deviation (S.D). Nine out of nineteen bacterial strains were sensitive.







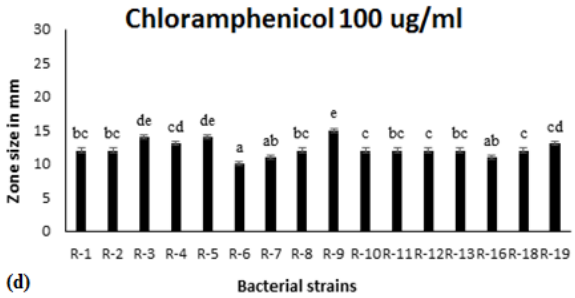


Figure 4: Diameter of inhibition zone ranged in 8-26 mm. Bars represents means ± standard deviation (S.D).

Discussion

Fruits are very important for human health because they provide nutrients, vitamins like A, C, B_6 and E, minerals and dietary fibers [15]. Fruits are easily digested and have cleansing impact on the digestive tract and blood. People that eat fruits as a diet have good health. Fruits are very useful to maintain level of moistness in one's body [16]. By regular intake of fruits like apples and berries as a healthy diet help in the prevention of chronic diseases and substitute good health [17]. Fruits have variety of microorganisms such as bacteria, fungi and yeast that may be responsible for spoilage. Spoilage microbes have demolishing influence on the product that is stored. To avoid spoilage of fruits, different preservatives are being used against the invading pathogens. Due to excess and misuse of heavy metals, environment became polluted and microorganisms have evolved resistance.

In this study, different rotten fruit samples were collected from different places and N-agar medium was used to purify bacterial strains. All bacterial strains were biochemically characterized. Some additional tests were also performed like HCN and H_2S production test. Mostly isolates showed positive results for H_2S production test and produced black colonies on lead acetate medium (Table 1). It was found that almost all strains which were resistant to mercury produce H_2S because it is already reported that bacterial strains resist mercury contamination by the formation of H_2S. It is already mentioned that H_2S aids in the volatilization of methyl mercury and reduces it to a highly toxic state to a detoxified state with the help of enzyme, mercuric reductase in the cell [18].

Due to excessive use of preservatives on fruits, bacteria evolved resistance against them that pose danger to human health and environment. It is reported that the pathogenic microorganisms like *S. aureus* is present in fruits [19]. It is reported that *S. epidermidis* is a skin wound opportunistic pathogen that causes many infections [20]. Human nervous system is very susceptible to all kinds of mercury. If we are constantly expose to high levels of mercury, then it causes damage to the kidney. Bacterial strains were subjected to antibiotic susceptibility testing against different antibiotics. Mostly bacterial isolates showed resistance against several antibiotics due to misuse of them. To overcome the problems created in the present scenario misuse of heavy metals and drugs should be avoided. Use of natural resources for preservation purpose is better than harmful preservatives for healthy and safe life because natural resources are ecofriendly and have no side effects.

Author Contributions

Roheen conducted the experimental work and drafted the manuscript. Zakia Latif planned and supervised the research work as well as formal analysis, funding acquisition, reviewed, edited and finalized the manuscript.

Conflicts of Interest:

There are no conflicts of interest.

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