Research Article

Microbes Associated with Freshly Prepared Juices of Citrus and Carrots

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Fruit juices are popular drinks as they contain antioxidants, vitamins, and minerals that are essential for human being and play important role in the prevention of heart diseases, cancer, and diabetes. They contain essential nutrients which support the growth of acid tolerant bacteria, yeasts, and moulds. In the present study, we have conducted a microbiological examination of freshly prepared juices (sweetlime, orange, and carrot) by serial dilution agar plate technique. A total of 30 juice samples were examined for their microbiological quality. Twenty-five microbial species including 9 bacterial isolates, 5 yeast isolates, and 11 mould isolates were isolated from juices. Yeasts and moulds were the main cause of spoilage of juices. Aspergillus flavus and Rhodotorula mucilaginosa were observed in the maximum number of juice samples. Among bacteria, Bacillus cereus and Serratia were dominant. Escherichia coli and Staphylococcus aureus were detected in few samples. Candida sp., Curvularia, Colletotrichum, and Acetobacter were observed only in citrus juice samples. Alternaria, Aspergillus terreus, A. niger, Cladosporium, and Fusarium were also observed in tested juice samples. Some of the microorganisms detected in these juice samples can cause disease in human beings, so there is need for some guidelines that can improve the quality of fruit juices.

1. Introduction

Unpasteurized fruit juice is defined as the product produced by pressing or squeezing of the fruits [1]. Consumption of fresh juices increased dramatically due to their freshness, high vitamin content, and low caloric consumption [2]. Extracted juices from fruits contain most substances which are found in the original ripe and sound fruit from which the juice is made. The high potassium and low sodium characteristic of most juices help in maintaining a healthy blood pressure. Vitamin C is naturally present in juices which are essential for the body to form collagen, cartilage, muscle, and blood vessels. It also helps in the absorption of iron [3].

Fruit juices contain a microflora which is normally present on the surface of fruits during harvest and postharvest processing which include transport, storage, and processing [4]. Many microorganisms such as acid tolerant bacteria and fungi (moulds, yeasts) use them as a substrate for their growth. Yeasts form the main flora of fruits before processing because of acidic pH. The major genera include Candida, Dekkera, Hanseniaspora, Pichia, Saccharomyces, and Zygosaccharomyces. Penicillium, Byssoschlamys, Aspergillus, Paeclomyces, Mucor, Cladosporium, Fusarium, Botrytis, Talaromyces, and Neosartorya are filamentous fungi most frequently isolated from fresh fruits and juices. Among bacteria, lactic acid bacteria and acetic acid bacteria have been isolated from fruit juices [5].

The critical factors affecting the spoilage of juices include juice pH, oxidation reduction potential, water activity, availability of nutrients, presence of antimicrobial compounds, and competing microflora. Among these factors, pH and water activity are the most influential factors affecting the spoilage of juices. The spoilage caused by microorganisms in juices includes cloud loss, development of off-flavours, CO2 production, and changes in colour, texture, and appearance resulting in degradation of product [6, 7]. The most
commonly reported bacterial genera include Acetobacter, Alicyclobacillus, Bacillus, Gluconobacter, Lactobacillus, Leuconostoc, Zymomonas, and Zymobacter. Among yeasts Pichia, Candida, Saccharomyces, and Rhodotorula are commonly encountered genera responsible for spoilage of juices [8]. Certain common moulds such as Penicillium sp., Aspergillus sp., Eurotium, Alternaria, Cladosporium, Paecilomyces, and Botrytis have also been reported in spoilage of fruit juices [5, 6].

Fruit juices have pH in the acidic range (<4.5) serving as important barrier for microbial growth. However, food borne pathogens such as E. coli and Salmonella survive in acidic environment of fruit juices due to acid stress response. Therefore, in the last two decades a number of food borne outbreaks associated with unpasteurized fruit juices have been documented in many countries [1, 9]. The source of outbreaks associated with unpasteurized fruit juices have been attributed to low pH values and high sugar content [19].

| Juices   | pH range | Mean |
|----------|----------|------|
| Orange   | 4.19–4.50 | 4.34 |
| Sweet lime | 4.70–5.47 | 5.08 |
| Carrot   | 5.76–6.03 | 5.89 |

2. Materials and Methods

2.1. Fruit Juice Preparation. Three juices commonly consumed in Kurukshetra such as orange (Citrus reticulata Blanco), sweet orange (Citrus sinensis), and carrot (Daucus carota) were selected for microbiological study. Sweet orange, carrot, and orange were purchased from the local markets of Kurukshetra from October 2011 to February 2012. Each sample was washed, peeled, and cut into pieces and juice was extracted through sterile hand blender and poured into sterile beaker.

2.2. Measurement of pH. The pH of juice samples was measured using a pH meter.

2.3. Microbiological Analysis. The microbiological study of fruit juices was done by serial dilution agar plate technique. Ten mL of juice sample was diluted with 90 mL of 0.1% sterile peptone water (1 g peptone, 1L distilled water) and plated on nutrient agar (pH 5.5) for enumeration of bacteria and PDA supplemented with antibiotic (pH 5.5) for enumeration of fungi in duplicates [4]. Uninoculated plates of PDA and NA were used as control. Mould and yeast isolates were purified on potato dextrose agar, bacteria on nutrient agar, and further subcultured for microscopic examination and identification.

2.4. Identification of Bacteria. For bacterial identification, 24-hour-old culture of bacteria was observed under microscope by gram stain method and further various biochemical tests were performed for the identification of bacteria such as catalase test, oxidase test, starch hydrolysis test, sugar fermentation test, IMViC test, and methods described in “Bergey’s Manual of Systematic Bacteriology” [14]. Further identification of bacteria was performed on the basis of methods described in “Compendium of methods for the microbiological examination of foods” [15, 16].

2.5. Identification of Yeasts. The methods adopted for identification of yeasts include morphological characteristics, fermentation of sugars, germ tube test and cycloheximide resistance test, and methods described in “Fungi and Food Spoilage” [17, 18].

2.6. Identification of Moulds. Moulds were identified on the basis of morphological and cultural characteristics such as colour of the colony, surface, appearance, presence, and absence of cross walls, and asexual and sexual reproductive structures. Further identification of moulds was carried out according to the methods described in “Fungi and Food Spoilage.” Moulds were cultured on Czapek yeast extract agar (pH 6.7), Malt extract agar (pH 5.6), and Glycerol nitrate agar (pH 7.0) at 25°C.

3. Results and Discussion

In the present study, 30 samples of freshly prepared juices (10 samples each of orange, sweet orange, and carrot) were examined for microbiological analysis. The pH range of juices is shown in Table 1. Factors which determine the colonization of juices by microorganisms include pH, redox potential, water activity, nutrients, structures, antimicrobial agents, temperature, relative humidity, and atmosphere [1]. In the present study the frequencies of occurrence of moulds and yeasts were more as compared to bacterial genera which is attributed to low pH values and high sugar content [19].

A total of 34 bacterial, 12 yeast, and 25 mould isolates were isolated from juices classified by grouping them into 9 bacterial species, 5 yeast species, and 11 mould species on the basis of phenotypic characteristics. Morphological and biochemical properties of bacteria were explained in Tables 2 and 3. Details of morphology and physiology of yeasts were described in Tables 4 and 5. Colonial and microscopic characteristics of various moulds were summarized in Tables 6 and 7.

Yeasts and moulds are capable of growth at pH values of 1.5 and at water activity values below 0.89. The minimum pH values allowing the growth of lactic acid bacteria (pH 2.9–3.5), acetic acid bacteria (pH 3.0–4.5), and enteric bacteria (pH 3.6–4.5) are higher than those for growth of yeasts and moulds [6].
### Table 2: Morphological characteristics of bacterial isolates of juices.

| Bacterial isolates       | Colour on nutrient agar | Configuration   | Margin | Elevation | Gram reaction | Shape of isolate | Endospore staining |
|--------------------------|-------------------------|-----------------|--------|-----------|---------------|------------------|-------------------|
| *Bacillus subtilis*     | White                   | Circular lobate | Irregular | Flat   | Positive       | Rods in chains   | Central spore     |
| *B. cereus*             | Off-white               | Circular        | Entire  | Convex   | Positive       | Rods in chains   | Central spore     |
| *Escherichia coli*      | Mucoid                  | Circular        | Entire  | Slightly raised | Negative         | Rods             | —                 |
| *Serratia*              | Mucoid                  | Circular        | Entire  | Umbonate | Negative         | Rods             | —                 |
| *Leuconostoc*           | Light yellow            | Circular        | Entire  | Convex   | Positive       | Coccis shape in tetrad | —                 |
| *Micrococcus*           | Bright Yellow           | Circular        | Entire  | Convex   | Positive       | Coccis shape in grapes like bunches | —                 |
| *Staphylococcus aureus* | Golden yellow colour    | Circular pin head colonies | Entire  | Convex   | Positive       | Coccis shape in grapes like bunches | —                 |
| *Lactobacillus*         | White                   | Circular        | Entire  | Raised   | Positive       | Rods             | —                 |
| *Acetobacter*           | Pale                    | Circular        | Entire  | Flat     | Negative       | Rods             | —                 |

—: absent.

### Table 3: Biochemical characteristics of bacterial isolates of juices.

| Bacterial isolates       | Catalase | Oxidase | Starch hydrolysis | Indole | IMViC test | Sugar fermentation |
|--------------------------|----------|---------|-------------------|--------|------------|-------------------|
|                          |          |         |                   |        |            | Glucose          |
|                          |          |         |                   |        |            | Lactose          |
|                          |          |         |                   |        |            | Mannitol         |
|                          |          |         |                   |        |            | Sucrose           |
| *Bacillus subtilis*      | +        | –       | +                 | –      | +          | +                |
| *B. cereus*              | +        | –       | +                 | –      | +          | +                |
| *Escherichia coli*       | +        | –       | –                 | +      | –          | –                |
| *Serratia*               | +        | –       | –                 | –      | +          | –                |
| *Leuconostoc*            | –        | –       | –                 | –      | +          | –                |
| *Micrococcus*            | +        | +       | +                 | –      | –          | –                |
| *Staphylococcus aureus*  | +        | –       | –                 | –      | –          | –                |
| *Lactobacillus*          | –        | –       | –                 | –      | –          | –                |
| *Acetobacter*            | –        | –       | –                 | –      | –          | –                |

+: positive; –: negative; A: acid; A + G: acid + gas.

### Table 4: Morphological details of yeast isolates of juices.

| Yeast isolates          | Colour on PDA | Configuration       | Margin      | Microscopic features                                      |
|-------------------------|----------------|---------------------|-------------|-----------------------------------------------------------|
| *Pichia*                | Off-white      | Hemispherical       | Irregular   | Ellipsoidal to cylindrical; reproducing by irregular budding |
| *Saccharomyces*         | Off-white      | Circular            | Irregular   | Spherical to subspheroidal; reproducing by irregular budding |
| *Candida kruzei*        | White          | Circular            | Irregular   | Ellipsoidal to long cylindrical; reproducing by irregular budding |
| *Rhodotorula*           | Pink           | Circular or spreading | Regular    | Ellipsoidal shape; reproducing by irregular budding         |
| *Candida parapsilosis*  | White to cream | Circular            | Regular     | Globose to ovoid budding                                    |

### Table 5: Physiological tests for yeasts isolates of juices.

| Yeast isolate          | Germ tube test | Cycloheximide resistance | Glucose | Sugar fermentation* |
|------------------------|----------------|--------------------------|---------|---------------------|
| *Pichia*               | –              | +                        | +       | +                   |
| *Saccharomyces*        | –              | –                        | +       | +                   |
| *Candida kruzei*       | –              | –                        | +       | –                   |
| *Rhodotorula*          | –              | –                        | –       | –                   |
| *Candida parapsilosis* | –              | +                        | –       | –                   |

+: positive; –: negative; * fermentation means production of gas independent of pH changes.
Table 6: Morphological details of mould isolates of juices.

| Mould isolate   | Colony colour on PDA on front side | Colony colour on PDA on reverse side | Microscopic features                                                                 |
|-----------------|-------------------------------------|-------------------------------------|--------------------------------------------------------------------------------------|
| *Aspergillus flavus* | Yellow green | Colourless                       | Conidiophores arise separately from foot cell, phialides uniseriate and sometimes biseriate; conidia globose to subglobose |
| *A. terreus*     | Brown       | Colorless                        | Conidiophore borne from surface hyphae, stripes long, and smooth walled; vesicles with densely packed, short, narrow metulae and phialides; conidia unicellular, spherical, and very small |
| *A. niger*       | Black       | Creamy                           | Hyphae septeate and hyaline, smooth walled conidiophores arising from foot cell; vesicles globose, whole vesicle fertile bearing two series of sterigmata; cætate conidia arranged in basipetal manner, unicellular, and globose |
| *Penicillium islandicum* | Ivy green  | Creamy                           | Short conidiophores bearing a compact verticil of metulae, phialides closely packed in clusters bearing cætate conidia arranged in basipetal manner, conidia elliptical, smooth, and hyaline |
| *P. digitatum*   | Green       | Colourless                       | Conidiophores borne from surface and aerial hyphae with thin smooth walls; bearing terminal penicilli; terverticillate but frequently biverticillate or irregular |
| *Alternaria*     | Black       | Colourless                       | Small to large sized conidia with beak; arising in chains in acropetal manner with both transverse and longitudinal septa |
| *Cladosporium*   | Black       | Colourless                       | Conidiophore tall, dark upright, and branched variously near the apex, conidia 1-2-celled ovoid to cylindrical shape |
| *Colletotrichum* | Cottony white to pale gray mycelium | Colourless                       | Aervulii disc shaped, typically with dark spines or setae at the edge of conidiophores; conidiophores simple, elongate conidia single celled, hyaline or brightly coloured, cylindrical or pointed, straight or curved |
| *Curvularia*     | Green to black | Black                           | Simple conidiophores bearing spores apically; Conidia dark, end cells, 3-5-celled; more or less fusiform, typically bent |
| *Fusarium*       | Wooly white | Colourless                       | Conidiophores slender and simple, short or branched irregularly or bearing a whorl of phialides; conidia hyaline, variable, principally of two kinds, macroconidia several celled slightly curved or bent at the point ends, microconidia 1-celled, ovoid or oblong, borne singly or in chains |
| *Geotrichum*     | White       | Colourless                       | Conidia borne solely by the breakup of hyphae to form arthroconidia |

Table 7: Colonial characteristics of different moulds’ isolates of juices on CYA, MEA, and G25N media.

| Mould isolate   | Colony colour on CYA Front side | Colony colour on CYA Reverse side | Colony colour on MEA Front side | Colony colour on MEA Reverse side | Colony colour on G25N Front side | Colony colour on G25N Reverse side |
|-----------------|---------------------------------|-----------------------------------|---------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| *Aspergillus flavus* | Yellow green                    | Colourless                       | Yellow green                    | Colourless                       | Yellow green                    | Colourless                       |
| *A. terreus*     | Brown                           | Dull brown                       | Brown                           | Dull brown                       | Brown                            | Dull brown                       |
| *A. niger*       | Black                           | Pale to bright yellow            | Black                           | Pale to bright yellow            | Black                            | Pale to bright yellow            |
| *Penicillium islandicum* | Greyish green               | Orange to rust brown central area | Greyish green                   | Orange to rust brown central area | Greyish green                   | Orange to rust brown central area |
| *P. digitatum*   | Greyish green to olive          | Pale or brown                    | Dull yellow green               | Pale or brown                    | Green olive                      | Pale                             |
| *Alternaria*     | Grey to black                   | Black                            | Grey to black                   | Black                            | Grey to black                    | Black                            |
| *Cladosporium*   | Olive to dark olive             | Grey                             | Olive                           | Grey                             | Olive                            | Black                            |
| *Colletotrichum* | Grey                            | Pale grey                        | Grey                            | Pale grey                        | Black                            | Grey                             |
| *Curvularia*     | Off-white to grey               | Grey                             | Off-white to grey               | Grey                             | Grey to black                    | Grey                             |
| *Fusarium*       | White to grayish rose           | Pale                             | White                           | Pale                             | White                            | Pale                             |
| *Geotrichum*     | White                           | Pale                             | White                           | Pale                             | No growth                        |                                  |
The frequency of occurrence of bacteria, yeasts, and moulds are summarized in Tables 8, 9, and 10, respectively. The occurrence of bacterial genera ranged from 10% to 56% (Table 8). *Bacillus cereus* and *Serratia* sp. were detected in a greater number of samples. *Bacillus cereus* was also observed in 64.91% of samples of unpasteurized street vended fruit juices [20]. *Leuconostoc* and *Lactobacillus* were also reported as important group of spoilage microorganisms in acidic products [21]. The presence of lactic acid bacteria more frequently occurs in unpasteurized juices [22]. These microorganisms produce acetic and formic acids along with ethanol and carbon dioxide which can alter the flavor of juice [23]. *Leuconostoc*, *Lactobacillus*, and *Acetobacter* were detected in tested juice samples (Table 8).

The presence of *E. coli*, *Salmonella*, and *S. aureus* in fruit juices is primarily concern because these pathogens were implicated in a number of outbreaks associated with fruit juices [1]. In our study, the presence of *E. coli* and *S. aureus* was detected in a smaller number of samples. The survival of pathogens in acidic environment of juices is attributed to their ability to regulate their internal pH and maintained at neutral pH by combination of passive and active homeostasis.
mechanisms [24]. The acid survival mechanisms of enteric bacteria are due to induction of enzymes that are involved in raising the internal pH and activation of enzymes devoted to the protection and repair of proteins and DNA [25].

Yeast genera responsible for spoilage of fruit juices include Candida, Pichia, Rhodotorula, Torulopsis, Saccharomyces, Zygosaccharomyces, Hansenula, and Trichosporon [26]. In our study, the dominant yeasts isolated from juices were Rhodotorula, Pichia, and Saccharomyces. Candida parapsilosis and C. krusei were only detected in orange and sweet orange juices, not detected in carrot juice. Ghenghesh et al. [9] also reported the presence of Candida sp. in 58% of orange juice samples. Rhodotorula, Pichia, Candida, and Saccharomyces have also been reported as spoilage causing organisms in pasteurized fruit juices [4, 27]. Yeast spoilage in fruit juices is characterized by formation of CO₂ and alcohol. Yeasts may also produce turbidity, flocculation, pellicles, and clumping. Yeasts also produce pectin esterases which degrade pectin causing spoilage; organic acids and acetaldehyde, which contribute to a “fermented flavor,” may also be formed [5, 6].

The dominant moulds recorded in fruit juices belong to Penicillium sp., Cladosporium sp., Aspergillus niger, A. fumigatus, Botrytis sp., and Aureobasidium pullulans. They produce mycelial mats and musty, stale off-flavours in juices [6]. Rhizopus and Mucor are also associated with spoilage of fresh fruits and vegetables [28]. In the present study, the most frequently encountered moulds were Aspergillus flavus, A. terreus, and Penicillium islandicum (Table 5). P. digitatum, Colletotrichum, and Curvularia were isolated from orange and sweet orange juices. Geotrichum was detected in orange and carrot juice. Spoilage by moulds in fruit juices is characterized by loss of juice cloud [6]. Among these, some moulds produce mycotoxins which are of great threat to human health. Major mycotoxins associated with fruit juices are byssochlaminic acid (Byssochlamys fulva, B. nivea), patulin (B. fulva, B. nivea, and P. expansum), ochratoxin (Aspergillus carbonarius), and citrinin (Penicillium expansum, P. citrinum) [29, 30].

4. Conclusion

Juices squeezed from fresh fruits and vegetables contain microorganisms which are potentially hazardous to public health. Juices were spoiled with high level of moulds and yeasts which is attributable to low pH of juices. The presence of pathogenic microorganisms in juices is clearly indication of food borne outbreaks. The selling and consumption of juices are never stopped on nutritional grounds as well as livelihood of street vendors. It is alarming situation for suitable agency to take some necessary action, make guidelines to prevent potential food poisoning from juices that contain pathogenic bacteria, and find natural antimicrobials from plants that control spoilage and pathogenic microorganisms in juices.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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