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MICROBIAL EXAMINATION OF MOULD AND YEAST IN FRUIT JUICES

Ayesha Alam[1], Shabnum Shaheen[1], Muhammad Ashfaq[2,*], Muhammad Ali[2], Javed Iqbal Watto[3], Mubarak Ali Anjum[1], Farah Khan[1], Sumaira Maqsood[1] and Muhammad Sajjad[4]

[1]Department of Botany, Lahore College for Women University, Pakistan; [2]Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan; [3]Department of Biotechnology, Faculty of Life Sciences, University of Central Punjab, Lahore, Pakistan; [4]Department of Environmental Sciences, COMSATS University Islamabad, Vehari campus 61100, Pakistan.

[*]Corresponding author's email: ashfaq.iags@pu.edu.pk

Fruit juices consists of water, carbohydrates, protein, lipids, acids, phenolics, dietary fibre and pigments that play a vivacious part in human nutrition, as they supply vitamins and essential minerals which are necessary growth factors in human diet and that can assist in maintenance of a good and healthy life. However due to their short shelf life these juices either glass bottles, plastic bottles, paper cups and plastic cups or unpacked can be contaminated by micro flora (especially mould and yeast) and can become a source of health hazard. Hence current study was focused to examine the diversity of mould and yeast contaminants in different types of packed and unpacked fruit juices (pH ranges from 3.35 to 4) of six different localities of Lahore such as Chouburji, Govt College of Science, Mozang, Muslim Town, Shadman and Iqbal town. The fungal contaminants (mould and yeast) isolated from their respective juices were cultured on malt extract agar as well as on potato dextrose agar under sterilized conditions. Serial dilution technique was used to compose the replicates in order to get viable count of micro flora in juices samples. Results indicated that in case of packed juices, the maximum fungal viable count was found in samples of Shadman ($10x10^{-4}$ to $14x10^{-5}$ cfu/ml) while minimum was found in Chouburji samples ($2x10^{-4}$ to $5x10^{-3}$ cfu/ml). The highest mould (803000 cfu/ml) in packed juices was found in locality of Mozang, but lowest value was observed in samples obtained from Muslim town i.e., 16000 cfu/ml. However, yeast contaminants were found in great concentrations in Shadman samples (71000 cfu/ml) and least count was found in locality of Iqbal town i.e., 200000 cfu/ml. For unpacked (fresh) juices the peak value of fungal viable count was observed in Shadman ($8x10^{-5}$ to $14x10^{-5}$ and $18x10^{-5}$) while minimum was found in GCS ($4x10^{-4}$ to $7x10^{-3}$). The uppermost value of mould count (1800000 cfu/ml was also observed in locality of Shadman and least mould (25000 cfu/ml) in unpacked juices was found in locality of GCS. Whereas maximum yeast i.e., 400000 cfu/ml was detected in locality of Shadman and least one 11000 cfu/ml was reported in Chouburji. Reported fungal species were *Aspergillus niger*, *Aspergillus terreus*, *Penicilium digitatum*, *Curvularia americanna*, *Fusarium moniliforme*, *Alterneria alternate* and *Saccharomyces cerevisae*. Results indicated that mould and yeast (fungal) count is much more standard tolerable range (10^2 to 10^3cfu/ml) hence this negligence and unawareness can lead to serious health damage of consumers.

Keywords: Microorganisms, microbial contaminants, spoilage yeasts, public health, fruit quality, consumer safety.

INTRODUCTION

Fruit juices are well recognized for their nutritive value, mineral and vitamin content (Sharma *et al*., 2013). Consumption of fruit and vegetable products has increased by more than 30% during the past few decades (Barth *et al*., 2009). They are very nutritive, invigorating and non-alcoholic beverages, which are liked throughout the world. In recent years these juices have been included significantly in diet of every person irrespective of age (Basar and Rahman, 2007). They are very scrumptious and palatable and they have most of the minerals like calcium, magnesium, phosphorus, and sodium as well as vitamins specially vitamin C (FDA, 1999). Processed juices contain mainly water, sugar, preservatives, color, fruit pulps and other additives as ingredients and must maintain sanitary standard (Levine, 1961).

The consumption of fruit juices could have both positive and negative effect on the consumers (Sharma *et al*., 2013). Freshly extracted fruit juices, have always considered as healthful drink, and may not always be safe owing to the heavy load of microbes (Kumari, 1995). Most fruit juices contain sufficient nutrients that could support microbial growth (Basar and Rahman, 2007). The major ingredients of the juice such as water, sugar, natural fruit pulp etc. may also carry some microbial contaminants (Rahman *et al*., 2010). Fruit juices contain various concentration of sucrose that constitutes a very important component of the medium for the growth of fungi (Palou *et al*., 1998). Microbial spoilage is a serious problem for the food industry as microbe can be any

contamination can occur during processing as well as handling of the end products (Koc *et al*., 2007).

Mould and yeast are sub classes of fungus and they are most notorious contaminants of fruit juices. Especially yeast spoilage has increased in recent years as a result of lower doses of preservatives and milder preservation processes, required for higher standards of food quality (Beuchat and Golden, 1989). Spoilage yeasts, such as *Saccharomyces cerevisiae*, *Candida lipolytica* and *Zygosaccharomyces* spp. can tolerate acidic environments (ICMSF, 1980). Moulds are generally considered to be the least important group of micro-organisms causing spoilage in fruit juice because of their limitation, inability to grow in the absence of air, with the exception of some moulds such as *Penicillium* and *Aspergillus* (Parish, 1991; Parish and Higgin, 1989). *Aspergillus niger* and *A. flavus* are the common allergens and may cause opportunistic invasive infections (Dehoog *et al*., 2000; Denning, 1998; Mau *et al*., 2002).

Juices squeezed from fresh fruits and vegetables are good for health but they may contain microorganisms which are potentially hazardous to public health. Maintaining the quality of processed fruit juices is important issue now because contaminated juices can transmit disease to people. Mainly moulds and yeasts are involved in juice spoilage. Hence there is need to explore which mold and yeast species are involved in juice quality deterioration so that specific sterilization procedure is followed to ensure juice quality. The specific aims and objectives of current study were to grow mould and yeast culture in different packed and unpacked juices, examine diversity of mould and yeast in studied juice samples, and highlight the conditions which enhance the mould and yeast proliferation in juices.

MATERIALS AND METHODS

***Sample collection*:** Various samples of packed and unpacked fruit juices were collected from different localities of the Lahore city (Chouburji, Govt College of Science, Mozang, Muslim Town, Shadman and Iqbal town) in months of February to May 2015.

Packaging of packed juices: Selected samples were packed in tetra packages, Glass bottles and canned juices.

***pH*:** pH of all collected samples was measured using pH meter and litmus paper. Almost all samples were alkaline in nature (Wang *et al*., 1992).

***Sterilization*:** Sterilizer (Memmert oven) was pre heated at 180°C for 20 minutes. Petri plates and pipette were put in the sterilizer for 2 hours.

***Media preparation*:** The preparation of different ratio of malt extract and potato dextrose media was shown in Table 1 and Table 2.

Table 1. Malt extract agar.

Sr.#	Chemicals	Weight
1.	Malt extract	2 grams
2.	Agar	2 grams
3.	Water	100 ml

Table 2. Potato dextrose agar.

Sr.#	Chemicals	Weight
1.	Potato dextrose agar	4 grams
2.	Water	100 ml

Four gram of potato dextrose agar was weighed and poured in to a beaker. 100 ml of water was added to potato dextrose agar and the solution was heated for 2-3 minutes. Continuous stirring was provided to the solution during heating to make homogenous mixture. After preparation of potato dextrose agar saturated solution of 10 ml was poured in a three test tubes. The test tubes were then covered tightly with cotton plugs. Liquid form media was autoclaved at about 15 lbs and 121°C.

***Serial dilution*:** For the observation of colony forming unit of fungal flora, 1 ml of juice sample was suspended in sterilized test tube containing 9 ml of sterilized distilled water. This test tube was well shaken which gave dilution of 1:10. Afterwards 1 ml of suspension from 1: 10 was transferred to second test tube which gave 1:100 dilutions. Similarly 1:1000, 1:10000 and 1:100000 dilutions were made.

***Inoculation*:** 1 ml aliquot from 1:1000, 1:10000 and 1:100000 dilutions were transferred to the sterilized petri plates and 10-15 ml of molten cooled agar poured containing 20,000 unit/liter penicillin and 200 mg/liter streptomycin. Petri dishes were incubated at 30±2°C for 5-7 days.

***Identification*:** The methods adopted for identification of fungi include morphological characteristics and methods described in "Fungi and Food Spoilage" (Pitt and Hocking, 2009).

***Quantification*:** Mould and Yeast colonies were analyzed quantitatively using methodology of (Webster and Weber, 2007) with slight modifications.

***Percentage contribution of each species*:** To find out percentage contribution following formula was used:

$$\%\,\text{contribution} = \frac{\text{Total No of cfu/ml of an individual specie}}{\text{Total no. of cfu/ml of all species}} x100$$

RESULTS AND DISCUSSION

Inoculated petri plates with fungal colonies can be seen in Figure 1. LM and SEM images of fungi can be seen clearly in Figure 2 and 3.

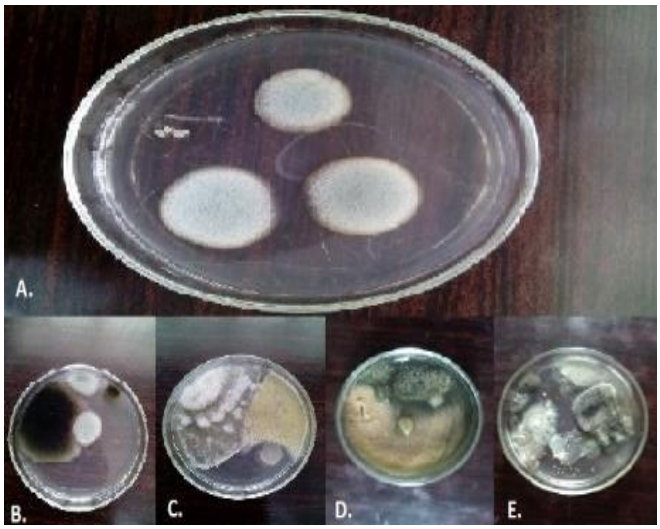

Figure 1. Media showing fungal colonies.

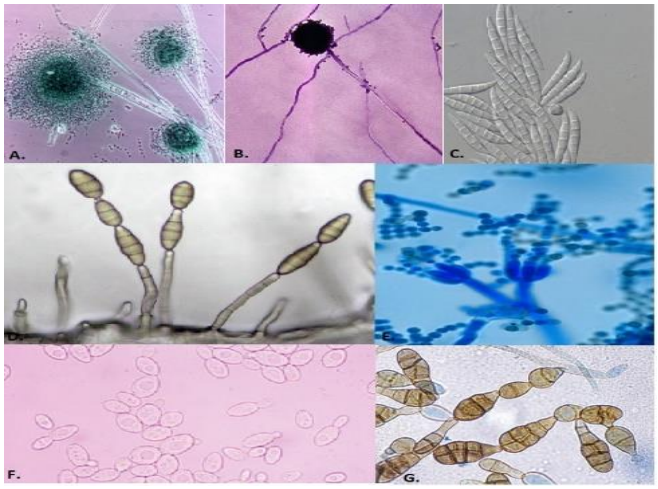

Figure 2. LM examination of mould and yeast.

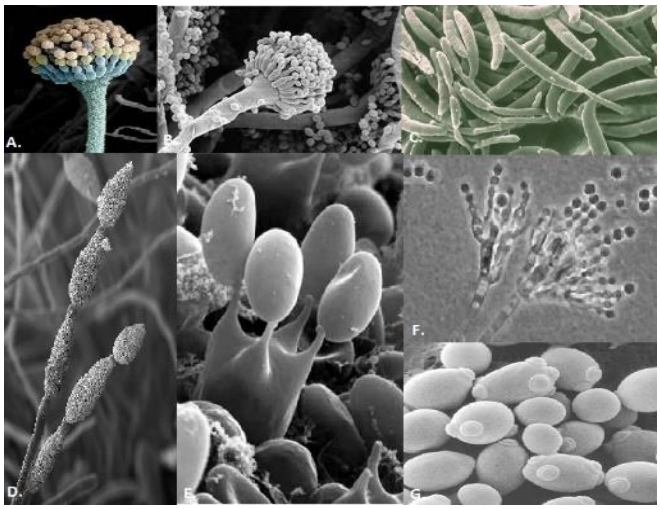

Figure 3. SEM Examination of mould and Yeast.

***Packed and unpacked juice samples of Chouburji*:** Total tested 4 packed and unpacked samples showed total viable count of $2x10^{-4}$ to $5x10^{-3}$ in packed juices whereas, $16x10^{-3}$ to $27x10^{-4}$ in unpacked juices. The highest fungal count was shown by unpacked juices. Results indicated maximum mould count was 260000cfu / ml and least mould count was 15000cfu / ml, whereas uppermost and lower most yeast count was 10000cfu / ml and 1000cfu/ml respectively. However, the results of packed juices demonstrated the mould contamination only. The peak mould count was observed as 2,0000cfu / ml and least value was calculated as 5000cfu / ml. There was no yeast count found in packed juice samples. The identified fungi were *Aspergillus niger, Alterneria alternate, Fusarium moniliforme, Pennicillim digitatum, Curvularia americana* and *Saccharomyces cerevisiae*.

***Packed and unpacked juice samples of Govt. College of Science*:** Total tested 4 packed and unpacked samples showed total viable count of $4x10^{-4}$ to $9x10^{-5}$ in packed juices whereas, $4x10^{-4}$ to $7x10^{-3}$ in unpacked juices. The highest fungal count was shown by packed juices. Results indicated maximum mould count was 600000cfu / ml and least mould count was 40000cfu / ml whereas yeast count was only 300000cfu / ml. The results of unpacked juices demonstrated the contamination as well. The peak mould count was observed as 20000cfu / ml and least value was calculated as 5000cfu / ml and reported peak yeast count was 20000cfu / ml and least one was 2000cfu / ml. The identified fungi were *Aspergillus niger, Aspergillua terreus, Alterneria alternate, Fusarium moniliforme, Pennicillim digitatum, Curvularia americana* and *Saccharomyces cerevisiae* Denning, 1998.

***Packed and unpacked juice samples of Mozang*:** Total tested 4 packed and unpacked samples showed total viable count of $10x10^{-5}$ to $4x10^{-3}$ in packed juices whereas, $64x10^{-3}$ to $3x10^{-5}$ in unpacked juices. The highest fungal count was shown by unpacked juices. Results indicated maximum mould count was 100000cfu / ml and least mould count was 16000cfu / ml whereas uppermost and lower most yeast count was 200000cfu / ml and 48000cfu / ml respectively. The results of packed juices demonstrated the contamination of mould and yeast. The peak mould count was observed as 800000cfu / ml and least value was calculated as 3000cfu / ml. The peak yeast count was observed as 200000cfu / ml and least value was calculated as 1000cfu / ml. The identified fungi were *Aspergillus niger*, *Penicillium digitatum* and *Saccharomyces cerevisiae* Nazim *et al*., 2008.

***Packed and Unpacked juice samples of Muslim Town*:** Total tested 4 packed and unpacked samples showed total viable count of $1x10^{-4}$ to $6x10^{-3}$ in packed juices whereas, $11x10^{-4}$ to $5x10^{-5}$ in unpacked juices. The highest fungal count was shown by unpacked juices. Results indicated maximum mould count was 400000cfu / ml and least mould count was 10000cfu / ml whereas maximum yeast count was 100000cfu/ml and minimum yeast count was 10000cfu / ml respectively. However the results of packed juices

Table 3. Qualitative and quantitative characters of fungal species (Mould).

Sr.	Fungal species	Shape and color of colony	Length and width of sporangia	Diameter of spore	Conidia	hyphae	Length and width of sporangiophore
1.	*Aspergillus niger*	Black	182.2 & 253.0 µm	15.8 µm	Basipetal	Septate	187.8 & 25 µm
2.	*Alterneria alternata*	Black	151.7 & 39.5 µm	10.2 µm	Beak like	Septate	144.5 & 12.5 µm
3.	*Penicillium digitatum*	Green	51.3 & 12.5 µm	7.9 µm	Basipetal	Aerial hyphae	122.6 & 10 µm
4.	*Curvularia americana*	Black to green	81.9 & 21.2 µm	16.8 µm	Dark and basal	Septate	198.6 & 14 µm
5.	*Fusarium moniliforme*	Wooly white	55.5 & 43.0 µm	16.5 µm	Small	Philades	387.5 & 17 µm
6.	*A. terreus*	Brown	63.9 & 80.5 µm	10 µm	Unicellular	Septate	222.6 & 10.6 µm

demonstrated the contamination as well. The peak mould count was observed as 10000cfu / ml and least value was calculated as 6000cfu / ml. There was no reported yeast in packed juice samples. The identified fungi were *Aspergillus niger, Aspergillus terreus, Penicillium digitatum, Curvularia americana* and *Saccharomyces cerevisiae* Olorunjuwan *et al*., 2013.

***Packed and Unpacked juice samples of Shadman*:** Total tested 4 packed and unpacked samples showed total viable count of $10x10^{-4}$ to $14x10^{-5}$ in packed juices whereas, $8x10^{-5}$ to $14x10^{-5}$ in unpacked juices. The highest fungal count was shown by packed juices. Results indicated maximum mould count was 700000cfu / ml and least mould count was 90000cfu / ml whereas peak yeast count was 700000cfu / ml and least yeast count was 10000cfu / ml respectively. However the results of unpacked juices demonstrated the contamination as well. The peak mould count was observed as 1100000cfu / ml and least value was calculated as 700000cfu / ml and reported peak yeast count was 300000cfu / ml and least one was 100000cfu / ml. The identified fungi were *Aspergillus terreus, Fusarium moniliforme, Pennicillim digitatum, Curvularia americana* and *Saccharomyces cerevisiae*.

***Packed and unpacked juice samples of Iqbal Town*:** Total tested 4 packed and unpacked samples showed total viable count of $1x10^{-5}$ to $5x10^{-5}$ in packed juices whereas, $18x10^{-5}$ to $2x10^{-3}$ in unpacked juices. The highest fungal count was shown by unpacked juices. Results indicated maximum mould count was 800000cfu / ml and least mould count was 200000cfu / ml whereas yeast count was only 100000cfu / ml. The results of packed juices demonstrated the contamination as well. The peak mould count was observed as 400000cfu / ml only and reported peak yeast count was 100000cfu / ml only. The identified fungi were *Aspergillus niger, A. terreus, Fusarium moniliforme,* and *Saccharomyces cerevisiae* (Oranusi *et al*., 2012).

Table 4. Qualitative and quantitative characters of fungal specie (Yeast).

Sr.	Fungal specie	Color of colony	Length and width of bud
1	***Saccharomyces cerevisiae***	Creamy white	32.5 & 16.8 um

The qualitative and quantitative characters of mould and yeast fungal species were shown in Table 3 and Table 4.

***Project scenario*:** Fruit juice consists of 100% pure juices and generally has no added ingredients (USDA, 2004). Fruits play a vital role in human nutrition by supplying the necessary growth factors such as vitamins and essential minerals in human daily diet and that can help to keep a good and normal health. Fruits are widely present in nature. One of the limiting factors that influence their economic value is the relatively short shelf-life period caused by pathogens (Al-Hindi *et al*., 2011). Pathogenic organisms can enter fruits and vegetables through damaged surfaces, such as punctures, wounds, cuts and splits that occur during growing or harvesting (Durgesh *et al*., 2008). Contamination from raw materials and equipment's, additional processing conditions, improper handling, prevalence of unhygienic conditions contribute substantially to the entry of bacterial pathogens in juices prepared from these fruits or vegetables (Victorian Government Department of Human Services 2005; Oliveira *et al*., 2006; Nicolas *et al*., 2007; Durgesh *et al*., 2008).

***Total viable count in packed juices*:** The present study illustrated the presence of seven different fungal isolates in different localities of Lahore such as Chouburji, GCS, Mozang, Muslim Town, Shadman and Iqbal town. The total viable count in packed juices was found as $2x10^{-4}$ to $5x10^{-3}$, $4x10^{-4}$ to $9x10^{-5}$, $10x10^{-5}$ to $4x10^{-3}$, $1x10^{-4}$ to $6x10^{-3}$, $10x10^{-4}$ to $14x10^{-5}$ and $1x10^{-5}$ to $5x10^{-5}$ cfu / ml in Chouburji, GCS, Mozang, Muslim Town, Shadman and Iqbal town respectively. The maximum fungal viable count was found in Shadman ($10x10^{-4}$ to $14x10^{-5}$ cfu/ml) while minimum was found in Chouburji ($2x10^{-4}$ to $5x10^{-3}$ cfu / ml). Among total viable count mould and yeast count was determined, and it varied from locality to locality. The total mould count in packed juices was reported as 25000, 640000, 803000, 16000, 790000, and 400000 in as Chouburji, GCS, Mozang, Muslim Town, Shadman and Iqbal Town respectively. The highest mould (803000 cfu / ml) in packed juices was found in Mozang, however lowest value of mould (16000 cfu / ml) in packed juices was found in Muslim town. Yeast content also contaminated the samples of different localities and in the study maximum yeast (200000 cfu / ml) in packed juices was found in Shadman and least count was found in Iqbal town i.e., 71000 cfu / ml (Figure 4 and 6).

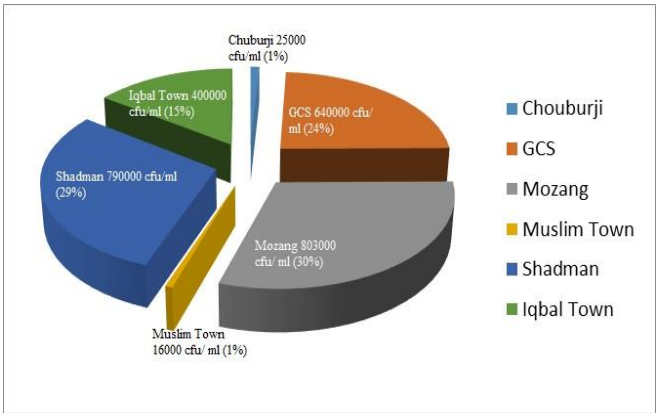

Figure 4. Comparison of total mould count in packed juices of different localities of Lahore.

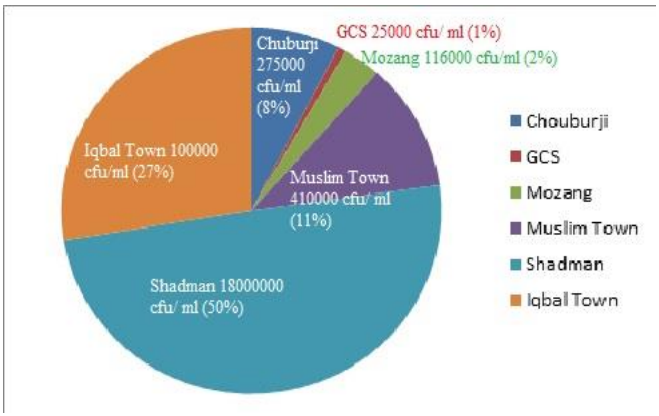

Figure 5. Comparison of mould value in total mould count in unpacked juices of different localities of Lahore.

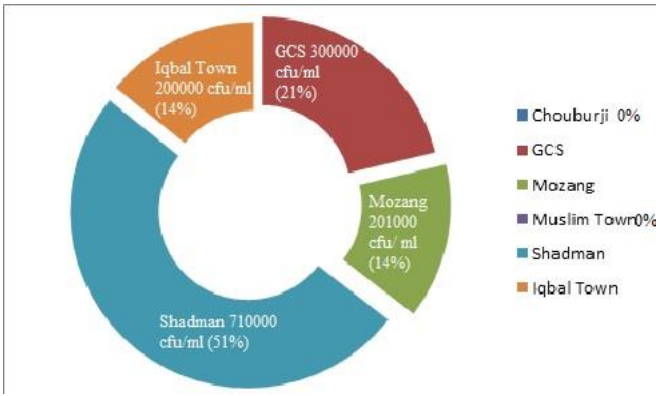

Figure 6. Comparison of total yeast count in packed juices of different localities of Lahore.

***Total viable count in unpacked juices*:** In case of unpacked juices the total viable count was estimated as $16x10^{-3}$ to $27x10^{-4}$, $4x10^{-4}$ to $7x10^{-3}$, $64x10^{-3}$ to $3x10^{-5}$, $11x10^{-4}$ to $5x10^{-5}$, $8x10^{-5}$ to $14x10^{-5}$ and $18x10^{-5}$ to $2x10^{-3}$ in Chouburji, GCS, Mozang, Muslim Town, Shadman and Iqbal Town respectively. The peak value fungal viable count was found in Shadman ($8x10^{-5}$ $14x10^{-5}$ $18x10^{-5}$) while minimum was found in GCS ($4x10^{-4}$ to $7x10^{-3}$). In unpacked juices the total mould count was 275000, 25000, 116000, 410000, 1800000 and 1000000 cfu / ml in Chouburji, GCS, Mozang, Muslim Town, Shadman and Iqbal Town respectively. The uppermost value of mould count (1800000 cfu / ml) in unpacked juices was found in Shadman and least mould (25000 cfu / ml) in unpacked juices was found in GCS locality (Fig. 5). In unpacked juices maximum yeast i.e., 400000 cfu / ml was found in shadman locality and least one 11000 cfu/ml was reported in chouburji locality (Figure 5 and 7).

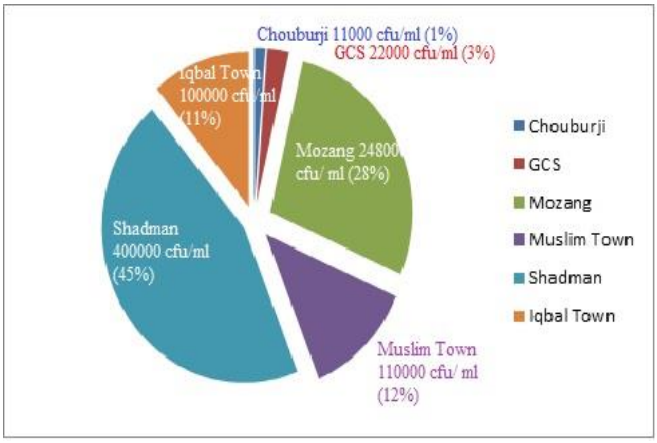

Figure 7. Comparison of total yeast count in unpacked juices of different localities of Lahore.

***Mould and yeast diversity in packed juices*:** The identified fungal and yeast isolates in packed and unpacked juices of all studied localities are reported as *Aspergillus niger, A.terreus, Penicilium digitatum, Curvularia americanna, Fusarium moniliforme, Alterneria alternata* and *Saccharomyces cerevisae.* Out of all seven fungal isolates *Penicilium digitatum, Aspergillus niger* and *Saccharomyces cerevisae* were present at their maximum peak in all localities. Maximum percentage of *Aspergillus niger* (42.85%) was found in locality of Muslim Town, *A. terreus* (33.33%) in locality of Iqbal Town, *Alterneria alternate* (14.28%) in locality of Chouburji, *Penicilium digitatum* (42.85%) in Chouburji, *Curvularia americanna* (15.38%) in GCS, *Fusarium moniliforme* (16.67%) in Iqbal Town and *Saccharomyces cerevisae* (33.33%) in localaties of Shadman and Iqbal Town (Figure 8).

***Mould and yeast diversity in unpacked juices*:** Mould and yeast in unpacked juices illustrated that maximum value of *Aspergillus niger* (51.16%) was found in Chouburji, *A. terreus* 45.45% in GCS, *Alterneria alternata* (25.58%) in Chouburji, *Penicillium digitatum* (22.72%) in Shadman, *Curvularia americanna* 9.09% in Shadman, *Fusarium moniliforme* and *Sacharomyces cerevisae* both are found to be 50% in Shadman and Iqbal Town, respectively (Figure 9).

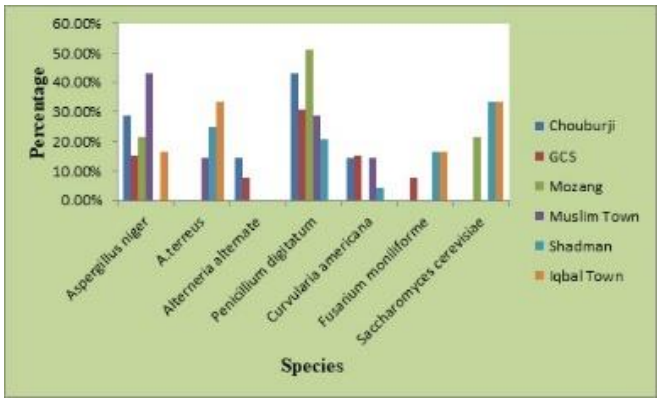

Figure 8. Comparison of percentage fungal isolates in packed juices of different localities of Lahore.

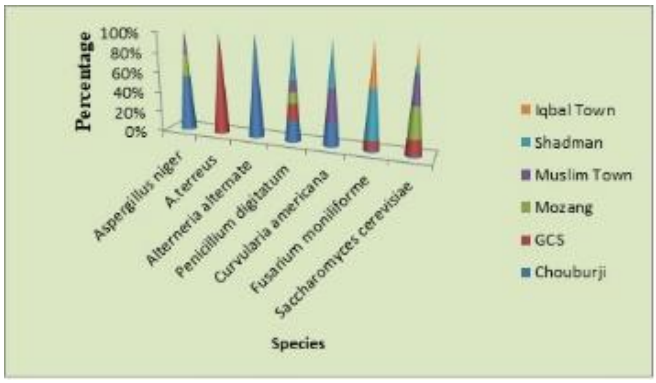

Figure 9. Comparison of percentage fungal isolates in unpacked juices of different localities of Lahore.

***Conclusion*:** According to FDA (2013), the acceptable value of mould and yeast in beverages and juices is 10^2 to 10^3 cfu/ml and the level when exceed would cause the rejection of the sample as it indicates potential health hazards and imminent spoilage respectively. The present study indicates much more value of mould (803000 cfu/ml to 1800000 cfu/ml) and yeast (200000 cfu/ml to 400000 cfu/ml) as compared to standard values in all the tested samples. None of the sample was found safe for consumption. The most frequently occuring fungal species are reported as *Aspergillus niger* and *Sacharomyces cerevisae* in packed juices while *Penicillium digitatum* and *Aspergillus niger* predominantly found in unpacked juices. This was due to poor handling, and temperature difference in localities. This is quite alarming situation for local community as the health of community is at risk. The microbiological quality of processed fruit juices is the most important aspect to be taken care of by the manufacturers and also the consistency of temperature while preservation and subsequent storage. Negligence in this area may result in serious contamination that ultimately represents a low quality product to the consumers, as these unwanted unhygienic conditions are usually due to the lack of knowledge and unawareness. Hence a considerable attention is required for sustainability of healthy community.

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