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Microbiological characteristics of bread during storage at room and refrigerator temperatures

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ABSTRACT

In the present study, bread samples were stored at room temperature (34°C to 36°C) for 5 days and refrigerator temperature (4°C to 8°C) for 28 days for determination of microbiological characteristics. Total fungal, bacterial counts, pH and moisture content of bread samples were determined before and after storage. The microorganisms were isolated by using serial dilution agar plate method. The molds (*Aspergillus luchuensis, A. flavus, A. terreus, A. niger, Drechslera hawaiiensis, Penicillium oxalicum, Scopulariopsis* sp. (*Deuteromycetes*) and *Rhizopus stolonifer* (*Zygomycetes*) and bacteria (*Bacillus psychrophilus, B. subtilis, B. megaterium* and *Staphylococcus aureus*) were isolated from bread samples stored at room temperature whereas at refrigerator temperature the molds (*Alternaria alternata, A. niger, Mucor* sp., and *Scopulariopsis* sp.) and bacteria (*B. sphaericus, B. polymyxa*, and *Bacillus* sp.) were isolated. Qualitatively, the bacteria and molds in bread stored at room temperature were found more than that of bread stored at refrigerator temperature. Quantitatively, the total fungal and bacteria counts were found to be more in bread stored at room and refrigerator temperatures respectively. The pH and moisture content of bread sample stored at refrigerator temperature ranged from 4.70 to 5.51 and 35.2% to 43.8% and at room temperature the pH and moisture content ranged from 4.94 to 5.60 and 32.1% to 39.5%. The moisture content in significantly affected the mold and bacterial counts of bread stored at room temperature whereas there was no effect of pH on microbial counts in bread samples stored at room and refrigerator temperature.

Key words: Bread, bacteria, mold, room and refrigerator temperature, pH and moisture content.

INTRODUCTION

Bread is a principal food for all categories of people, including children and it is considered unsafe for people when bread with a high microbial load is consumed. The production of bread and other bakery products has evolved from a primitive, cottage industry into a large scale, modern manufacturing industry, generating billions of dollars in revenue and employing thousands of personnel The ingredients of bread are supportive to growth of microorganisms and multiplication at different stages of bread production, slicing and wrapping (Smith *et al.* 2004; Khetarpaul *et al.* 2005).

The main types of microbial spoilage of bread are ropiness and moldiness. Ropiness has been described as a discolouration of bread ranging from brown to black with an unpleasant odour (Ogundare and Adetuyi 2003) and is caused mainly by *Bacillus subtilis,* but other species of *Bacillus* are capable of causing rope and these include *B. licheniformis, B. megaterium, B. cereus* and *B. pumilus* (Thomson *et al.* 1998; Sorokulova *et al.* 2003). The molds most frequently involved in the spoilage of bread are the so called "bread mold", *Rhizopus nigricans,* with other mold species like *Penicillium expansum, R. stolonifer, A. niger, Mucor* sp., *Monilia (Neurospora) sitophila,* (Gassem, 1999; Frazier and Westhoff 2003, Ogundare and Adetuyi 2003; Guynot *et al.* 2004, 2005, Rehman *et al.* 2007). The fungal species mainly *A. niger, A. flavus, A. glaucous. A. carnous, A. terreus, A. ochraceous, A. fumigatus, R. stolonifer, Trichoderma harrianua* and *T. roseum* have been isolated from the bread in India (Rai *et al.* 1990).

The shelf life of bread is mainly influenced by moisture content and its distribution in bread that influence the softness of the crumb, crispness of the crust, and the quality of bread. Water activity (a_w) and pH are also important factors that affect the microbial quality of food. The lower levels of water activity of microbial growth are approximately 0.90 for bacteria, 0.88 for yeasts, 0.80 for fungi, 0.65 for xerophilic fungi, and 0.60 for osmophilic yeasts (Karaoglu *et al.* 2005).

The aim of this study was to isolate and identify the microorganisms, and to see the effect of pH

and moisture contents on the microbial population in bread under two different conditions of storage.

MATERIALS AND METHODS

Isolation and enumeration of microorganism from bread

Seven packets of bread samples were purchased from local market in Kurukshetra, Haryana (India). The samples were then transferred to the laboratory in Department of Microbiology, Kurukshetra University, Kurukshetra. Six packets (packet marked 1 to 6) of bread samples were stored at room temperature (34 to 36^{0}C) for 5 days, while one packet of bread was stored in the refrigerator at 4 to 8^{0}C for 28 days for isolation and identification of microorganisms from bread. Serial dilution-agar plate method (also called viable plate count method) was used for the quantitative and qualitative determination of fungi and bacteria from bread (Aneja, 2003). In this method, 1g of bread samples were added into sterile 9 ml water blanks and further dilutions were made upto 10^{-4}. 0.1 ml aliquots from various dilutions were added into the sterile Petri plates. To these inoculated plates, plate count agar (PCA) for bacteria, potato dextrose agar (PDA supplemented with 2% wheat flour (Guynot *et al.* 2005)) for fungi were added. PDA was also supplemented with streptopenicillin to inhibit the growth of bacteria. The PCA plates were incubated at 37^{0}C for 24 to 48h and PDA plates were incubated at 25^{0}C for 3 to 7 days, in an inverted position. All the experiments were performed in triplicates. The isolated and purified fungi were maintained on PDA slants and bacteria on PCA slants for identification and further use. Quantitative estimation of bacteria and fungi was made at different storage conditions. Colony forming unit/s (CFU/s) were calculated by applying the formula:

$$\text{CFU(s)/ g} = \frac{\text{Number of colonies (mean) X dilution factor}}{\text{Volume plated (0.1ml)}}$$

Microbial Identification

Representatives from each colony type were selected from plates used for viable counts at each sampling time according to shape and/or colour. Isolated bacteria were identified accordingly by using manual and dichotomous key of Harrigan (1998); Doyle *et al.* (2001); Presscott *et al* (2005) and molds were identified using 7 days old cultures on potato dextrose agar following the manual of Gilman (1967); Ellis (1971); Domsch *et al.* (1980) and Frazier and Westhoff (2003).

Measurement of pH and moisture content

The pH of the each bread sample was determined by mixing one slice of bread in 100ml of sterile distilled water. The pH was recorded using µ pH System 361, serial no. 1037, Systronics (Thomson *et al.* 1998).

The moisture content of each bread sample (initial weight 5.0g) was estimated by drying of the bread sample in an oven at 105°C for 12h (Piazza and Masi 1995). The percent moisture content was calculated by applying the formula given below:

$$\text{Moisture content (\%)} = \frac{\text{Initial weight - final weight}}{\text{Initial weight}} \times 100$$

The mean values and standard deviation were determined using Pearson's coefficient. A significance level of 0.05 was assumed throughout.

RESULTS

pH and moisture content of bread samples during storage at room and refrigerator temperature

The pH and moisture content of each packet of bread sample during storage at room and refrigerator temperature are shown in table l and 3 respectively. Each packet of bread sample (1 to 6 packets) stored at room temperature (34 to 36^{o}C) ranged in pH from 4.94 to 5.60 with an overall average pH of 5.27. One packet of bread sample stored at refrigerator temperature (4 to 8°C) ranged in pH from 4.70 to 5.51 with an overall average pH of 5.28.

Moisture content of each packet (1 to 6 packets) of bread sample stored at 34 to 36^{0}C ranged between 32.1% and 42.6% with an overall average of 38.7%. Moisture content in the bread stored at 4 to 8^{0}C ranged between 35.2% and 43.8% with an overall average of 37.9%.

Microbial counts in bread stored at room temperature (34^0C to 36^0C)

The microbial (fungal and bacterial) counts, pH and moisture content of bread samples stored at room temperature are presented in the table 1 and 2.

Mold counts

A total of 8 molds (*A. luchuensis, A. flavus, A. terreus, A. niger, P. oxalicum, R. stolonifer, D. hawaiiensis* and *Scopulariopsis* sp.) were identified from bread stored at room temperature. No fungal growth was observed from the bread on the day of procurement of the bread from the market. Mold isolates and its counts varied in each packet of bread sample.

Aspergillus sp. were found on 1st, 3rd, 4th and 5th day and *Scopulariopsis* spp. was found on 2nd, 3rd, 4th and 5th day of isolation from bread stored at room temperature. Another fungus, *P. oxalicum* was isolated on the 2nd day. A zygomycetous fungus, *R. stolonifer* was isolated on the 1st and 4th day of isolation. The quantitative and qualitative changes of fungi in bread stored at room temperature as shown in the table 1. *A. terreus* and *R. stolonifer* were only found in bread on the 2nd day of isolation. *Scopulariopsis* sp. ($0.98x\ 10^1$ to $2.77xl0^4$) dominated in bread samples stored at room temperature (Table 1). The total fungal load in bread on PDA medium ranged between $1.91x10^1$ and $2.88\ x10^4$.The time period significantly affected the mold counts in bread stored at room temperature, these correlation were highly significant at the 0.05 level.

Bacterial counts

During study, the bacteria such as *Bacillus psychrophilus, B. megaterium, B. sphaericus* and *Staphylococcus* sp. were isolated from bread stored at room temperature. The total bacterial counts increased until 1st day, then decreased 2nd day, and thereafter increased until 5th day of isolation. *B. subtilis* was dominant in bread samples stored at room temperature. The bacterial counts (CFU/g), pH and moisture content (%) of bread samples during storage are presented in Table 2. Total bacterial load increased with the lowest counts of $1.0x10^1$ to $6.5x10^3$ (highest) of bread stored at room temperature (table 2). There was significantly high correlation between time period and bacterial counts. The negative correlation was found between pH and bacterial counts of bread stored at room temperature during the study. These variations were significant at the 0.5 level.

Microbial counts in bread stored at refrigerator temperature (4^0C to 8^0C)

The microbial (fungal and bacterial) counts, pH and moisture content of bread samples stored at refrigerator temperature are shown in the table 2.

Mold counts

Mold isolates identified mainly included *A. niger*, *Mucor* sp., *Scopulariopsis* sp. and *Alternaria alternata. A.* niger was found from bread on the day of procurement of bread from the market. *A. niger* was also found at different intervals 1st, 2nd, 4th and 21st day of isolation. No mold counts were observed at 3rd day of storage. *Scopulariopsis* sp. counts increased with time (5th, 14th, 21st and 28th day) but it was not found on the day of procurement of bread from the market, 3rd, and 4th day of storage. Number of mold genera increased until 2nd day, and then decreased. The mold counts did not show any correlation with pH and moisture content of bread stored at room temperature.

Bacterial counts

Bacterial isolates identified mainly included *Bacillus sphaericus* ($1.1x10^1$), *B. polymyxa* ($1.0x10^1$) and *Bacillus* sp. ($7.6\ x10^1$). The *B. sphaericus* and *Bacillus* sp. counts decreased on 2nd day and then increased on 3rd day, and thereafter decreased at 14th day. No bacterial counts were observed on the 21st day in bread sample during storage at refrigerator temperature and then recontaminated with *Bacillus* sp. on the 28th day. The total bacterial counts increased with the lowest counts of $2.1x10^1$ to $4.8x10^3$ (highest) of bread stored at refrigerator temperature (Table 4). The moisture content was moderately affected the bacterial counts in bread stored at refrigerator temperature. The negative correlation was found between pH and bacteria counts of bread stored at refrigerator temperature. Thus, these correlations were significant at the 0.5 level. The mean values and standard deviation of all parameters were shown in table 5 and 6.

On comparison, *A. luchuensis, A. terreus, Aniger, A. flavus, A. luchuensis, P. oxalicum, R. stolonifer, D. hawaiiensis* and *Sopulariosis* sp. were observed in bread stored at room temperature. *Aspergillus niger* and *Scopulariopsis* sp. were common to bread stored at room and refrigerator temperatures. *A.*

alternata and *Mucor* sp. were found only in bread stored refrigerator temperature (Table 7). *B. sphaericus* was found in both bread samples stored at room and refrigerator temperature. *S. aureus* was not found in bread sample stored at refrigerator temperature. Qualitative and quantitative analysis of bacteria in bread stored at refrigerator temperature was found to be more than that of bread sample stored at room temperature.

Table 7. Comparison of total fungal population at different storage condition in bread

S. NO.	Fungal isolates	Bread stored at	
		Room temperature (34- 36^{0}C)	Refrigerator temperature (4-8^{0}C)
1.	*Aspergillus luchuensis*	+	-
2.	*A. flavus*	+	-
3.	*A. terreus*	+	-
4.	*A. niger*	+	+
5.	*Alternaria alternata*	-	+
6.	*Rhizopus stolonifer*	+	-
7.	*Mucor* sp.	-	+
8.	*Drechsleria hawaiiensis*	+	-
9.	*Scopulariopsis* sp.	+	+
10.	*Penicillium oxalicum*	+	-

DISCUSSION

The seven genera and ten species of molds including *Alternaria alternata, A. luchuensis, A. flavus, A. terreus, A. niger, P. oxalicum, Drechslera hawaiiensis, R. stolonifer, Mucor* sp. and *Scopulariopsis* sp. were identified from the bread samples stored at room and refrigerator temperature. No yeast isolates were obtained during the study. The molds identified in this study were those common molds isolated from bread (Viljoen and von Holy 1997; Frazer and Westhoff 2003). The fungal species *A. niger, A. flavus, A. glaucous. A. carnous, A. terreus, A. ochraceous, A. fumigatus, R. stolonifer, Trichoderma harrianua* and *T. roseum* have been isolated from the bread in India (Rai *et al.* 1990). During a one year study of breads stored in plastic bags at 22°C for 5-6 days, *Penicillium* spp. were present in nearly all of the loaves, while *Aspergillus* spp. and *Cladosporium* spp. occurred on approximately half of the loaves (Legan and Voysey 1991).

In our study, *Aspergillus* spp. were found all most all bread samples during storage. Molds (*Penicillium* sp., *Rhizopus* sp., *A. niger, Alternaria* sp., *Mucor* sp. and *Fusarium* sp.) were associated with the fermented bread (khamir) produced from sorghum in Saudi Arabia (Gassem 1999). According to Ogundare and Adetuyi (2003), molds isolates included *Absidia corymbifera, P. frequentans westing, A. flavus, A. niger* and *P. citrinum* were obtained from freshly baked bread after 10 minutes.

According to Lund *et al.* (1996), *Penicillium roqueforti, P. corylophilum* and *Eurotium* species made up the important mycoflora associated with rye bread in Denmark. *P. decumbens, Paecilomyces variotti* and *A. flavus* were found more rarely, but were the major species found over a period of a few months. *P. commune, P. solitum, A. niger* and *Mucor* sp. were constant, but small, part of the total mycoflora of rye bread (Lund *et al.* 1996).

In the present study, *A. flavus* and *P. oxalicum* were observed in bread stored at room temperature while *Alternaria alternata* was isolated from bread stored at refrigerator temperature. These molds have been known to produce mycotoxins, which are both acutely and chronically toxic in animal and humans (Doyle *et al.* 2001; Jai *et al.* 2005). *A. flavus* has been recovered from flour, bread and bakery products (Pitt and Hocking 1985; Dragoni and Vallone 1997) and carcinogenic aflatoxin have also been found in bakery products (Pohland and Wood 1987) and rye bread (Filtenborg *et al.* 1996). *A. niger* was isolated from bread stored at room and refrigerator temperatures. It may be harmful as some strains are the producer of a heat stable toxin (ochratoxin A), which are hepatotoxic and nephrotoxic in nature (Heenan *et al.*1998; Jay *et al.*, 2005). Number of mold species and its population of bread stored at room temperature were higher than that of bread sample stored at

refrigerated temperature. Ogundare and Adetuyi (2003) reported that the fungal counts increased throughout the hour of storage of bread baked.

In this study, morphological, cultural and biochemical methods were used to characterize *Bacillus* and *Staphylococcus* species isolated from bread and found that the bread samples stored at room and refrigerator temperature were contaminated with *B. psychrophilus, B. subtilis, B. megaterium,* and *B. sphaericus, B. polymyxa, Bacillus* sp. and *S. aureus.* According to Collin *et al.* (1991); Baley and von Holy (1993), the bacteria species mainly *B. subtilis, B. licheniformis, B. cereus, B. firmus* and *B. clausii* are generally recognized as contaminants in bread.

Volavesk *et al.* (1992) stated that if a poor rope inducing *Bacillus* strain present with in a loaf produced an antimicrobial substrate then this could inhibit the growth of a vigorous rope-inducing strains also present within the loaf.

Quantitatively, *Bacillus* spp. were isolated from bread stored at refrigerator temperature with range 4.23x 10^3. According to Lund (1990), the level of *B. cereus* required to produce toxin is approximately 10^5 spores/g of food, while higher spore levels (10^1 to10^9 spores/g) are required for *B. licheniformis* and *B. subtilis.* Illness attributed consumption of bread with high number of *B. subtilis* or *B. licheniformis* has been reported (Thompson *et al.* 1998).

In the present study, *S. aureus* ($1.07x10^1$ to $5.84 x10^1$) observed in bread stored at room temperature. Leela *et al.* (1981) found that enterotoxigenic staphylococci in bakery products in India were always associated with cream and coconut filling. *S. aureus* producing enterotoxin A, B and E were found in cake, sweet puffs, vegetative puffs, and cream buns from five bakeries. Bread and buns from the same bakeries were negative for *S. xylosus, S. cohnii* and *S. aureus* (Sankaran and Leela 1983). According to Ogundare and Adetuyi (2003), *Staphylococcus* spp. were also found in bread baked with wheat flour.

In the present study, *B. subtilis* and *B. megaterium* were characterized as endospore former, cylindrical, aerobic and mesophilic. Our findings substantiate the findings of voysey (1989) who has been reported that mesophilic spore counts were not able to produce rope on bread. Ogundare and Adetuyi (2003) have given explanation for this decline which may be due to dehydration occurring in the bread because of loss of moisture to the environment. Acoording to Viljoen and von Holy (1997), *Bacillus* sp., *Aspergillus* sp. and *Penicillium* sp. were also predominantly found in bread.

The pH, water activity and temperature are the most important parameters that control the microbial growth responsible for the deterioration of food (Patriarca *et al.* 2001). These factors play an important role in spore germination and the growth of vegetative cells of *Bacillus* spp. during storage. In the present report the pH of bread samples stored at room and refrigerator temperature varied, finally decreased and the average pH was 5.27 and 5.28 respectively.

Kirchner and von Holy (1989) suggested that pH as an important controlling factor in the development of rope spoilage. According to the Cambell *et al.* (1991), freezing is the best storage method for breads containing no preservatives to prevent spoilage, whereas refrigeration enhances staling. Moisture content (%) of bread samples stored at room and refrigerator temperature varied during the study. This situation could be attributed to water loss in bread during the storage period (Ogundare and Adetuyi 2003). The large difference in moisture content had been observed in bread stored for 300 hours (Piazza and Masi 1995).

In this study, the mold counts in bread sample stored at room and refrigerated temperature generally increased as the storage period increased. Mold isolates such as *A. alternata, A. niger* and *Scopulariopsis* sp. were more common in bread stored at refrigerator temperature. *Scopulariopsis* sp. was predominant in bread samples stored at room and refrigerated temperature.

This is the first study in which *Scopulariopsis* sp. and *D. hawaiiensis* was found in bread sample stored at room temperature. *Scopulariopsis* sp. initially isolated from air, soil, straw, *Oryza* and *Ricinus* from Brazil, Europe, Hong Kong, India, Pakistan and USA (Ellis 1971). *Alternaria* species can cause spoilage of foods in refrigerated storage (Doyle *et al.* 2001).During the study, there was no yeast cell growth in bread samples stored at room and refrigerator temperature. According to Ogundare and Adetuyi (2003), the yeasts such as *Saccharomyces cerevisiae* and *Zygosaccharomyces bailli* were recovered throughout the hour of storage of bread baked with wheat flour from South western Nigeria.

The results of this study conclusively showed that the bacterial contamination comes from ingredients used and poor baking while mold contamination comes from environment. The breads which are expose

to air and dust from the environment, increase the microbial contamination and reduce the quality. In order to reduce the chances of contamination especially with unknown, mycotoxigenic molds and spore forming bacteria, hot bread should be allowed to cool, before wrapping and bread sellers should be advised not to expose the bread for a long period. Elimination of the contamination sources by improved cleaning and disinfection procedures quickly result in a significant reduction in the frequency of microbial growth in the packaged.

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