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Qualitative and quantitative analysis of microflora of Indian bakery products

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In the preasent endeavour, qualitative and qualtitaive analysis of microflora of bakery prodicts were carried out. The pH of bakery products was found to range between 4.94 and 6.00 while the moisture content was found to be higher in bread (43.8) and minimum in the biscuits (3.00). The bacterial and fungal flora of bakery products varied both qualitatatively and qualtitatively. The isolated bacteria such as *Bacillus subtilis, B. megaterium, B. shaericus, B. polymyxa, B. psychrophilus* and *Bacillus* sp. and molds *Aspergillus luchuensis, A. flavus, A. terreus, Alternaria alternata, A. Tenuissima, Penicillium oxalicum, Rhizopus stolonifer, Mucor* anf *Scopulariopsis* spp. were identified. Gram positive bacteria were found to be more prevalent than Gram negative bacteria. *Bacillus subtilis* and *Aspergillus luchuensis* were common to all the bakery products. Qualitatively, maximum number of molds were isolated and identified from breads followed by caked, pstries, patties and minimum in buns and biscuits while the maximum number of bacteria were obtained from bread followed by cakes, biscuits, patties and minimum in buns. These food associated bacterial and fungal isolates might responsible for food spoilage, food borne infection and intoxicationafter ingestion.

Key words: Bacteria, bakery products, bread, moisture content, qualitative, spoilage.

Introduction

Food spoilage is a metabolic process that causes foods to be undesirable or unacceptable for human consumption due to changes in sensory characteristics. Spoiled foods may be safe to eat if they may not cause illness because there are no pathogens or toxins present but changes in texture, smell, taste, or appearance cause them to be rejected (Smith *et al*., 2004; Doyle, 2007; Edward, 2007; Montville and Matthews, 2008). A food borne infection involves the ingestion of the microbial pathogens followed by growth in the

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host, including tissue invasion and release of toxins. Microbial growth in food products can result in a food intoxication in which symptoms are produced shortly after the food is consumed because growth of the disease causing microorganisms is not required. Toxins produced in the food can be associated with microbial cell or can be released from the cells. Some of the major bacterial genera which cause food borne infection and intoxication include *Staphylococcus, Bacillus, Escherichia, Shigella, Clostridium* and *Salmonella* (Madigan and Martinko, 2006; De Souza, 2008). Fungi are major plant and insect pathogens and profused growth of fungi on animal hosts produce the disease collectively called mycoses, while dietary, respiratory, dermal, and other exposures to toxic fungal metabolites produce the diseases collectively called mycotoxicoses. Mycotoxins are fungal metabolites that are present in a large part of the world food supply and bear potential threat to food safety (Qazi and Fayyaz, 2006). *Aspergillus, Penicillium* and *Fusarium* are known to produce mycotoxins in foods that result to cause mycotoxicoses after ingestion (Frazier and Westhoff, 2003). Some mycotoxins are mutagenic and carcinogenic and some display specific organ toxicity (Madigan and Martinko, 2006). Against this background, and relying on improved understanding and knowledge of the complexity of microbial interactions, recent approaches are increasingly directed towards possibilities offered by biological preservation (Rasooli, 2004). In lieu of the above justification, the objective of the present study was to analyze the qualitative and quantitative nature of microflora of bakery products.

Materials and Methods

Collection of bakery product samples

A total of 30 bakery product samples such as breads (10), biscuits (7), cakes (2), pastries (5), buns (3) and patties (3) were purchased from local market of Kurukshetra, Haryana, India and brought to the laboratory for isolation of bacteria and fungi.

Determination of pH of bakery products samples

The pH of the each bakery product sample was determined by mixing 15 g of a bakery material in 100ml of distilled water in 250ml Erlenmeyer flask and shaking on a magnetic stirrer for 15 to 20 minutes (Gassem, 1999). The pH of the semi liquid mixture was recorded by using a calibrated pH meter.

Determination of moisture content

The moisture content of each bakery product samples was determined by drying the sample (initial weight 5g) in an oven at 105°C for 12hrs in replicates of three (Piazza and Masi, 1995). The percent moisture content was calculated as given below:

$$\text{Moisture content (\%)} = \frac{\text{Initial weight of the sample - Weight of the oven dried sample}}{\text{Initial weight of the sample}} \times 100$$

Isolation of microorganisms by serial dilution agar plate technique

The serial dilution agar plate technique was used for the isolation of microorganisms (bacteria and fungi) from bakery products (Aneja, 2003; Jay *et al*. 2005). The Plate count agar (PCA), Potato dextrose agar (PDA) supplemented with 2% wheat flour (Guynot *et al*. 2005) and Yeast extract glucose agar (YGA) were used for bacteria, molds and yeasts respectively. In the serial dilution agar plate technique, 1 g of a bakery product was suspended and agitated in 9ml water blank (to make the total volume to 10ml) to form a microbial suspension. Serial dilutions of 10^{-2}, 10^{-3} and 10^{-4} were made by pipetting 1 ml into 9 ml water blanks (in triplicate for each dilution). To each of these inoculated plates, 15ml of sterile and cooled molten (45^0C to 50^0C) media (PCA for bacteria and PDA, supplemented with streptopenicillin for fungi) were poured and incubated at 37^0C for 24 hrs for bacteria and 25^0C for 3 to 7 days for fungi, in an inverted position. The plates were observed for the appearance of colonies and number of colonies produced on each plate of different dilutions were recorded. Number of colonies (CFUs/g) was calculated by multiplying plate count with the dilution factor as given below:

$$\text{CFUs/g} = \frac{\text{Number of colonies (mean) X Dilution factor*}}{\text{Volume plated (0.1ml)}}$$

*Dilution factor: Reciprocal of the dilution (e.g. $10^{-3}=10^3$)

Bacteria were purified by streak plate method on PCA and incubated at 37^0C for 24 hrs and transferred to PCA slants and maintained in refrigerator at 4^0C. Molds were purified by needle inoculation and disc transfer on PDA plates and incubated at 25^0C for 5 days and transferred to PDA slants and incubated at 25^0C for 5 days and maintained in refrigerator at 4^0C.

Identification of microflora of bakery products

Bacteria were identified following the dichotomous keys of Harrigan (1998), Doyle *et al*. (2001), Tserovska *et al*. (2002) and Oyetayo (2004). Fungal colonies were grown on PDA, CDA and MEA media at 25^0C for 7 days and following characteristics : colony characteristics (i. e. colour, exudates produced, growth of the colony), sporulating structures (conidial head, types of conidiogenous cells, arrangement of conidia, sporangial head, types of spores, pycnidia, accervuli, sporodochia, ascocarps etc.) were recorded and identified by following various manuals and monographs (Gilman, 1967; Ellis, 1976; Domsch *et al*., 1980; Kirk *et al*., 2001).

Results and discussion

Bacteria and fungi are known to cause spoilage of food stuffs not only in their storage on shelf but even during their storage under refrigerated conditions. Infact, bacteria, fungi and other microbes reside, grow, and sporulate on surface of the foods and cause the spoilage in due course when their number and mass is sufficient enough to secrete the enzyme which can break down the complex molecules structure of these food substances. In the present endeavour, a total of six bakery products such as bread, biscuit, cake, bun, patty and pastry were evaluated for microbiological and physico-chemical analysis. The pH of bakery product samples was found to be acidic that ranged between 4.94 and 6.00, indicating that there is more likelihood by the molds spoilage because molds love to grow in acidic environment. The pH and moisture content 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 bacteria. Kirchner and von Holy (1989) suggested pH as an important controlling factor in the development of rope spoilage.

During the present study, the bacterial isolates obtained from the bread were mainly species of *Bacillus* such as *B. subtilis, B. psychrophilus, B. megaterium, B. sphaericus* and *B. polymyxa* and *Staphylococcus aureus*. Our findings substantiate the findings of Thompson *et al.* (1998), Ogundare and Adetuyi (2003) and Pepe *et al*. (2003) who also isolated *B. subtilis*, *B. megaterium* and *Staphylococcus aureus* from the bread. Species of *Bacillus* such as *B. subtilis, B. megaterium* and *B. licheniformis* have been reported to be the major cause of rope spoilage in breads (Smith *et al*., 2004; Guynot *et al*., 2005). *Bacillus* spp. and *S. aureus* have been reported to be the major source of contamination of bakery products during post-preparation handling which may

lead to severe outbreak of food poisoning as a result of enterotoxin production by these bacteria, as reported by Smith *et al*. (2004).

The maximum population in bread obtained in the present investigation was that of *B. psychrophilus* ($6.0x10^1$ CFU/g) followed by *B. polymyxa* ($5\ 0x10^1$ CFU/g), *B. sphaericus* ($4.0x10^1$), *B. subtilis* and *B. megaterium* ($2.0x10^1$ CFU/g) and the minimum population was that of *S. aureus* ($1.5x10^1$ CFU/g). Our findings differ from that of Ogundare and Adetuyi (2003) who found lesser population of *Bacillus* spp. than that of *S. aureus* in bread. This may be due to two reasons owing to the poor hygienic conditions in the bakery and by not taking the proper care during post-preparation handling that might have resulted in the contamination of the bread (Ogundare and Adetuyi 2003).

Gram-positive bacteria were found to be more prevalent than Gram-negative bacteria in bakery products during the course of study. Of the various *Bacillus* spp. isolated from the six bakery products namely bread, biscuit, cake, bun, patty and pastry, *B. psychrophilus, B. sphaericus* and *B. polymyxa* were exclusively associated with breads. *B. megaterium* was present in bread, biscuit and cake samples but absent in pastries, patties and buns. The possible reasons for dominance of *Bacillus* spp. may be due to the common occurrence of endospores in flour and flour based products, as well as in the bakery environment. They are heat resistant and survive baking and under favourable conditions, grow to level associated with toxin production. The survival of spores during baking depends on the type of products, the internal temperature reached during baking, as well on the thermal resistance of the spores (Kaur, 1986).

The bakery products in the present study were found to have much lesser population of *Bacillus* spp., hence they are safe for human consumption. Lund (1990) reported that the level of *Bacillus* spp. required to produce toxin is approximately 10^5 to10^9 spores/g of food and the lower numbers denote to be safe for human consumption. Although a number of *Bacillus* species were isolated from the bread, the formation of rope was not visualized. Thus our findings are in conformity with the earlier workers (Van Holy and Allen, 1990; Thompson *et al.,* 1998) who worked on the detection of rope spoilage in bread by *Bacillus* and reported that not all isolates of *Bacillus* were capable of causing extensive rope in presence of the preservatives in the bread. There are reports that vinegar was more effective than calcium propionate at inhibiting rope in soft grain loaf (Nout, 1991). Thus the risk of food poisoning due to the presence of bacilli in bread could be minimal in our conditions. Moreover, absence of *Bacillus cereus* in bread indicates its safety for human consumption since *B. cereus* has been implicated in most of the food poisoning incidence (Parry and Gilbert, 1980).

In the present investigation, *Staphylococcus aureus* has been isolated from the two bakery products, cake and patty. *S. aureus* is ubiquitous in air, water, milk and on food contact surfaces. 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. have also been found in bread baked with wheat flour. Human harbouring *S. aureus* are a major source of contamination of products during preparation or post preparation handling. Ingredients may also be sources of high numbers of *S. aureus* (Sankran and Leela, 1983).

Occurrence of *Escherichia coli* in cake and patty in the present investigation is in conformity with the survey carried out in New Zealand which reported that *E. coli* was detected in 1.6% of cream filled bakery products at levels greater than 100CFU/g. Presence of *E. coli* in bakery products indicates possible insanitary conditions and warrants investigation of the condition of preparation and also, human contact may sometimes introduce *E. coli* (Adams and Moss, 2000).

The present study revealed the association of nine molds with bread that belonged to *Deuteromycetes* (anamorphic fungi) and *Zygomycetes*. Fungi were identified by using the manual of Gilman (1967), Ellis (1976), Domsch *et al.* (1980); Kirk *et al*. (2001) and Salar and Aneja (2007). A perusal of data in table 1 reveals that the fungal flora of six commonly available bakery products varied both qualitatively and quantitatively. Maximum number of molds were isolated from breads (9) followed by cakes (4) and minimum in the other products, i. e. biscuits, buns, patties and pastries. Of these nine molds, *Aspergillus luchuensis* was found to be associated with all the bakery products, indicating that it can grow on all the food stuffs irrespective of its variation in nutrient composition, moisture contents and pH. *Rhizopus stolonifer* was common to breads, biscuits, cakes, patties and buns, *Penicillium oxalicum* to breads, cakes, pastries and patties, *Alternaria tenuissima* to breads and pastries and *Mucor* sp. to breads and biscuits. However, *Aspergillus flavus, A. terreus* and *Scopulariopsis* have been exclusively isolated from the bread samples. The trend in variations in the fungal population (CFU/g) followed is similar to that of qualitative variations. The presence of four molds genera isolated in the present investigation namely *Aspergillus, Penicillium, Mucor* and *Rhizopus* are similar to those isolated earlier by Gaseem (1999) and Sharma and Tripathi (2006). *Scopulariopsis* sp. is a new addition to the earlier molds recorded in

bread. The isolation of three species of *Aspergillus* (*A. luchuensis, A. flavus* and *A. terreus*) from the bread in the present study is similar to the findings of Rai *et al.* (1990) who also recorded the domination of aspergilli in Indian bread samples. Qualitatively and quantitatively, the fungal flora of bread reported by Ogundare and Adetuyi (2003) from South Western Nigeria is different than reported from this place. The fungal counts have been found to higher than to Ogundare and Adetuyi (2003).

The molds that have been most frequently reported to be the cause of bread spoilage around the globe include *Rhizopus nigricans* (bread mold) and *Aspergillus niger*, *A. flavus, Monilia* (*Neurospora*) *sitophila, Absidia corymbifera, Penicillium frequentans, Penicillium expansum, P. citrinum, P. stolonifer, Mucor* sp. and *Cladosporium* sp. (Gassem, 1999; Ogundare and Adetuyi, 2003; Guynot *et al.*, 2005; Rehman *et al.*, 2007). The survey of literature reveals that *Alternaria alternata, A. tenuissima* and *Scopulariopsis* sp. have not been reported from bread and are thus new additions to the mold flora of bread in the present investigation. Since complete baking destroys all the bacterial cells, yeasts and molds spores survive except few species of bacteria (Frazier and Westhoff, 2003).

In our study, four fungal taxa *Aspergillus, Alternaria, Penicillium* and *Rhizopus* have been isolated from fresh cakes. However, only two molds *Aspergillus* and *Penicillium* were isolated by Abellana *et al.* (2001) from sponge cake in one study. The count of *P. oxalicum* have been found to be higher than the population of *Aspergillus luchuensis, Rhizopus stolonifer* and *Alternaria alternata.* In the present study, three fungal genera namely *Aspergillus, Alternaria* and *Penicillium* have been isolated from the pastry similar to those isolated from cake. However, the populations of *Aspergillus luchuensis* and *Alternaria tenuissima* were higher than that of *P. oxalicum*. Weiderborner *et al.* (2000) who studied the mold flora of pastries suggested that the mold growth in pastries may result in mycotoxin contamination. In the present study, the patties fungal flora included *Aspergillus, Penicillium* and *Rhizopus*. The maximum population was that of *P. oxalicum* followed by *A. luchuensis* and lowest of *R. stolonifer*. Two molds *R. stolonifer* and *A. luchuensis* were isolated from the buns. The population of *R. stolonifer* was higher than that of *A. luchuensis*. *A. luchuensis* and *Mucor* sp. were isolated from the biscuits. The population of *Mucor* sp. (1.5×10^2 CFU/g) was higher than that of *A. luchuensis* (1.0×10^1 CFU/g). Recording of lowest number of fungi in the biscuits can be correlated with very low moisture contents and is in conformity with the earlier findings of Frazier and Westhoff (2003), Jay *et al.* (2005).

Occurrence of higher number of molds in bread than to other bakery products may be due to water activity which is considered to be one of the important factors influencing microbiological spoilage of bakery products. Bread is a high moisture product of the various bakery products which allow the growth of all bacteria, yeasts and molds (Frazier and Westhoff, 2003; Jay *et al.,* 2005; Aneja *et al.,* 2008). The higher microbial counts of the bread samples have most probably been dependent on the flour containing high microbial count and/or from the bakery environment which might have contaminated the bread during the period of cooling (Ogundare and Adetuyi, 2003). The pH of a food is also critical because a low pH favours the growth of yeasts and molds. In neutral or alkaline pH foods, such as meats, bacteria are more dominant in spoilage and putrefaction. Thus our results clearly substantiate the findings of earlier workers as spoiled bread which is acidic in nature was showed the presence and dominance of molds (Frazier and Westhoff, 2001; Jay *et al.,* 2005).

Earlier, *Aspergillus flavus* has been recovered oftenly 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). *Alternaria* spp. have also been reported to cause spoilage of foods such as apple, tomatoes, blue berries, grains and other foods by producing mycotoxins namely alternariol, alternariol monomethyl ether, alternuene, tenuazonic acid and altertoxin I. *Penicillium oxalicum* is also a known producer of secalonic acid D which has significant animal toxicity (Doyle *et al.,* 2001). There are reports of occurrence of *Scopulariopsis* sp. from air, soil, straw, *Oryza* and *Ricinus* from India, Brazil, Europe, Hong Kong, Pakistan and USA and from whole and white wheat flour (Weiderborner *et al.,* 2000), but not from the bread, hence isolation of *Scopulariopsis* sp. is the first report of its occurrence from the bread.

It may be concluded from the present study that *Staphylococcus, Escherichia, Bacillus* (bacteria), *Aspergillus, Penicillium* and *Alternaria* (molds) are the common genera of molds generally isolated from the fresh bakery products- during the present investigation. These molds have been known to produce toxins, which are both acutely and chronically toxic in animal and humans. The need of hour is to control the microbial growth in bakery products by using preservatives from natural sources.

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Table 1. Types of microorganisms and their viable counts (CFU/g) in bakery products observed by serial dilution agar plate technique.

S. N.	Bakery product	pH	Moisture Content (%)	Fungi	(CFU/g)	Bacteria	(CFU/g)
1.	Breads	4.94-5.60	32.1-43.8	*Aspergillus luchuensis*	$4.5x10^1$	*Bacillus subtilis*	$2.0 x10^1$
				A. flavus	$1.2 x10^1$	*B. psychrophilus*	$6.0 x10^1$
				A. terreus	$1.0 x10^1$	*B. megaterium*	$2.0 x10^1$
				Alternaria tenuissima	$1.5 x10^1$	*B. sphaericus*	$4.0 x10^1$
				A. alternata	$1.0 x10^1$	*B. polymyxa*	$5\ 0 x10^1$
				Penicillium oxalicum	$4.0 x10^1$	*Bacillus* sp.	$1.0 x10^1$
				Rhizopus stolonifer	$5.0 x10^1$	*Staphylococcus aureus*	$1.5 x10^1$
				Mucor sp.	$1.0 x10^1$		
				Scopulariopsis sp.	$5.0x10^2$		
2.	Biscuits	3.20-4.50	3.00-5.00	*A. luchuensis*	$1.0 x10^1$	*B. subtilis*	$3.0 x10^1$
				Mucor sp.	$1.5 x10^2$	*B. megaterium*	$5.0 x10^1$
						Bacillus sp.	$3.0 x10^1$
3.	Cakes	5.30-5.60	41.0-43.0	*A. luchuensis*	$1.5 x10^1$	*B. subtilis*	$4.0 x10^1$
				P. oxalicum	$2.0 x10^1$	*B. megaterium*	$1.0 x10^1$
				Rhizopus stolonifer	$1.0 x10^1$	*Bacillus* sp.	$5.0 x10^1$
				A. alternata	$1.0 x10^1$	*S. aureus*	$1.0 x10^1$
						Escherichia coli	$1.0 x10^1$
4.	Pastries	4.00-4.50	40.0-43.0	*A. luchuensis*	$3.0 x10^1$	*B. subtilis*	$1.0 x10^1$
				P. oxalicum	$1.0 x10^1$	*Bacillus* sp.	$2.0 x10^1$
				A. tenuissima	$3.0 x10^1$	*E. coli*	$1.0 x10^1$
5.	Patties	5.00-6.00	35.0-40.0	*P. oxalicum*	$5.0 x10^2$	*B. subtilis*	$1.0x10^1$
				A. luchuensis	$5.0 x10^1$	*Bacillus* sp.	$1.2x10^1$
				R. stolonifer	$3.0 x10^1$	*S. aureus*	$1.5x10^1$
						E. coli	$1.5x10^1$
6.	Buns	4.94-5.50	30.0-43.0	*A. luchuensis*	$1.0 x10^1$	*B. subtilis*	$1.3x10^1$
				R. stolonifer	$6.0 x10^1$	*Bacillus* sp.	$2.0x10^1$

Table 2. Qualitative variation in bacteria on different bakery products.

Bacteria	Bread	Biscuit	Cake	Pastries	Patties	Buns
Bacillus subtilis	+	+	+	+	+	+
B. megaterium	+	+	+	ND	ND	ND
B. psychrophilus	+	ND	ND	ND	ND	ND
B. sphaericus	+	ND	ND	ND	ND	ND
B. polymyxa	+	ND	ND	ND	ND	ND
Bacillus sp.	+	+	+	+	+	+
Staphylococcus aureus	+	ND	+	ND	+	ND
Escherichia coli	ND	ND	+	+	+	ND

+ = Present; ND : Not detected

Table 3. Qualitative variation in fungi on different bakery products.

Fungi	Bread	Biscuit	Cake	Pastries	Patties	Buns
Aspergillus luchuensis	+	+	+	+	+	+
A. flavus	+	ND	ND	ND	ND	ND
A. terreus	+	ND	ND	ND	ND	ND
Alternaria tenuissima	+	ND	ND	+	ND	ND
A. alternata	+	ND	+	ND	ND	ND
Penicillium oxalicum	+	ND	+	+	+	ND
Rhizopus stolonifer	+	ND	+	ND	+	+
Mucor sp.	+	+	ND	ND	ND	ND
Scopulariopsis sp.	+	ND	ND	ND	ND	ND

+ = Present ND : Not detected

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