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Full Length Research Paper

Proteolytic and lipolytic activities of *Pseudomonas* spp. isolated from pasteurized milk

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Psychrotrophic bacteria have been recognized as a recurring problem in refrigerated storage and distribution of perishable food products. This study was performed to isolate, characterize and evaluate the prevalence of *Pseudomonas* spp. in pasteurized milk. Thirty five (35) pasteurized milk samples were collected aseptically from different dairy shops, supermarkets and groceries in different areas in Sadat City, Menuofiya Governorate, Egypt and analyzed for total viable count and *Pseudomonas* spp. occurrence and explain their proteolytic and lipolytic activities. The results showed that *Pseudomonas aeruginosa* was found to be the predominant from the *Pseudomonas* spp. followed by *Pseudomonas fluorescens*. The total viable count was (3.50 ± 0.085) × 10^4 cfu/ml. Out of 10 *Pseudomonas* spp. isolates that were examined for proteolytic and lipolytic activities; two isolates had proteolytic activity, while three had lipolytic activity and five isolates had both.

Key words: *Pseudomonas aeruginosa*, *Pseudomonas fluorescens,* proteolytic, lipolytic activities.

INTRODUCTION

Milk has many nutritious qualities that make it an important part of children's diet. To produce the best quality milk and to achieve all the nutritious benefits of it, the highest quality raw milk must be obtained. Pasteurization and shelf stable milk products through ultra high temperature (UHT) continuous flow sterilization are available (Goff and Griffiths, 2006). The conditions of heat treatment used for pasteurization depend on the final product; lower temperatures are used for refrigerated products and higher heat treatments are used for products stored at room temperature (USCFR, 2006). Psychrotrophic bacteria have been recognized as a recurring problem in the refrigerated storage and distribution of fluid milk, and perishable dairy products for several decades. So, the psychrotrophic have received increased attention by investigators during recent years, because modern developments in the handling and transportation of milk have resulted in milk being held for longer period at refrigeration temperature before processing, manufacturing or consumption.

Some psychrotrophic bacteria may grow at a temperature of 7℃ although their optimum temperature is h igher. Rapid cooling and cold storage of raw milk favor the growth of psychrotrophic bacteria in milk (Barbano et al., 2006). They become dominant microflora during cold storage of milk and their extracellular enzymes, particularly proteases and lipases, contribute to the spoilage of milk products (Hantis-Zacharov and Halpern, 2007). Microbial growth and metabolism shorten the shelf life of milk by producing undesirable changes in aroma and taste attributes that influence consumer acceptability of the products (Fromm and Boor, 2004).

Factors limiting milk stability are well established: bacterial contamination, inadequate packaging system and improper temperature control. Vulnerability of milk's fat and protein to physical-chemical alterations can also lead to deterioration, thus, reducing its quality. Cromie (1991) reported that the factors which influence the shelf

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life of pasteurized milk include the quality of the raw material, the binomial temperature/time pasteurization, resistant microorganisms to pasteurization (particularly Psychrotrophic), the presence and activity of post pasteurization contaminants, the packaging system and storage temperature post pasteurization which had the greatest impact on the stability of the product as shelf life of pasteurized milk. Extra cellular lipolytic enzymes produced from psychrotrophics can produce deteriorate effects as rancid flavours and odours in milk and dairy products that make a subsequent reduction of shelf-life and the products become unacceptable to consumers (Downey, 1980).

The proteolytic enzymes produced by psycrotrophics in milk are more powerful in its action on milk proteins than that naturally present in milk and that produced by leucocytes even if present by great amount (Grieve and Kitchen, 1985). *Pseudomonas* spp. produces a large number of extracellular toxins, which include phytotoxic factor, pigments, hydrocyanic acid, proteolytic enzymes, phospholipase and enterotoxins. Exotoxins are responsible for *Pseudomonas* spp. pathogenicity because it can produce leucopoenia, circulatory collapse, necrosis of liver, pulmonary odema, hemorrhage and kidney tubular necrosis. The enterotoxin produced is responsible for diarrhea disease.

High temperature for short times was less effective in destruction of the *Pseudomonas* spp. than that of temperature for long times because the condition created in the milk at high temperatures may response of injured cells (Cousin, 1982). Generally, they are considered incapable of surviving pasteurization and they may be post-processing contamination. Although, they can cause gastroenteritis if ingested in large number ($>10^6$ cell), but also the food would be clearly spoiled before such numbers would reached (Johnson, 1990). Moreover, its presence in milk and its products was considered as a possible indicator of fecal contamination. Also *Pseudomonas aeruginosa* has been implicated in epidemics of moderate to severe diarrhea in children in the form of enteric fever (Todar, 2002).

This study aimed to isolate, characterize and evaluate the prevalence of *Pseudomonas* spp. in pasteurized milk sold in Sadat City, Menuofiya Governorate, Egypt. Also, observation of the proteolytic and lipolytic behavior of it in the pasteurized milk was investigated.

MATERIALS AND METHODS

Thirty five (35) samples of pasteurized milk were collected randomly from various local milk supermarkets in different areas in Sadat city, Menuofiya Governorate, Egypt. Collected samples were examined for total viable cells and occurrence and behavior of *Pseudomonas* organisms. Psychrotrophic isolates were obtained from pasteurized milk by pouring plating appropriately diluted sample using nutrient agar. Incubation was done at 7℃ for 10 days (Cousin et al., 2001). The psychrotrophic isolates were subjected to biochemical characterization after activating the culture in nutrient broth at 37℃ for 24 h (Barrow and Feltham, 1993).

Identification of *Pseudomonas* isolates

The representative suspected colonies were purified and then identified according to Bergey's Manual of Systemic Bacteriology (1984) and API 20NE kit (England). *Pseudomonas* isolates were subcultured onto nutrient agar (NA) plates and incubated at 30℃ for 24 h. Pure cultures were inoculated into nutrient broth and incubated overnight at 30℃ prior to testing.

Oxidation fermentation test

Lactose negative, blue-black and greenish bright dark colonies were lactose positive as a result of incubation during 48 h at 30℃. Among these, continuous studies were carried out on the lactose negative ones and these colonies were applied on the oxidation-fermentation test. For *Pseudomonas* oxidative reaction, among the same samples of catalase (+), motility (+) and oxidase (+) activities, colonies were studied on growth at 4 and 41℃ which were first taken into consideration. So, *Pseudomonas*, which incubated into yeast extract medium were left at 4℃ for 7 to 10 days and at 41℃ for 24 h, respectively (Mickova et al., 1989).

Determination of the proteolytic and lipolytic activities of *Pseudomonas* spp.

Ten (10) strains belonging to *Pseudomonas* spp. isolated previously from pasteurized milk samples were investigated for their proteolytic and lipolytic activities as described by Harrigan and McCance (1976).

Proteolytic activity using skim milk agar

Overnight cultures were spot inoculated onto milk agar, standard plate count agar supplemented with 10% sterile skim milk. The inoculated plates were incubated at 20 and 4℃ for 10 days. The presence of transparent zones around the spots was recorded as positive strains referring to protease production, and subsequently flooded with 10% v/v acetic acid solution. Clear zone around the colonies after 1 min exposure were regarded as positive (Harrigan and McCance, 1976).

Lipolytic activity

Lipolytic counts (LP) were determined using NA containing tributyrin. The medium was prepared using 10 g of tributyrin and 28 g of NA. Plates were incubated at 20 and 4℃ for 72 h to determine viable LP. Lipolytic activity was determined by measuring clear zone around each colony (Harrigan and McCance, 1976).

RESULTS AND DISCUSSION

Incidence of *Pseudomonas* spp. among the examined samples

The samples showed an average of total viable count of $(3.50 \pm 0.085) \times 10^4$ cfu/ml. According to Prevention of Food Adulteration Act (PFA, 2007), total plate count of pasteurized milk should not exceed 30,000 cfu/ml.

Table 1. Prevelance and count of *Pseudomonas* spp. in examined pasteurized milk samples.

No. of sample	Positive samples		Minimum	Maximum	Mean ± SD
	No.	%			
35	10	28.57	1.5×10	2.5×10^3	$(1.5 \pm 0.01) \times 10^2$

Table 2. The bacteriological examination of *Pseudomonas* spp. in examined pasteurized milk samples.

No. of isolate	Catalase	Oxidase	O/F	Lactose fermention	Motility	MR	VP	Citrate utilization	Gelatin hydrolysis	Indol production	Nitrate reduction
1	+	+	O^+/F^-	-	+	-	-	+	+	-	+
2	+	+	O^+/F^-	-	+	-	-	+	+	-	+
3	+	+	O^+/F^-	-	+	-	-	+	+	-	+
4	+	+	O^+/F^-	-	+	-	-	+	+	-	+
5	+	+	O^+/F^-	-	+	-	-	+	+	-	+
6	+	+	O^+/F^-	-	+	-	-	+	+	-	+
7	+	+	O^+/F^-	-	+	-	-	+	+	-	+
8	+	+	O^+/F^-	-	+	-	-	+	+	-	+
9	+	+	O^+/F^-	-	+	-	-	+	+	-	+
10	+	+	O^+/F^-	-	+	-	-	+	+	-	+

However, it was observed that all the standard plate count were characterized by the predominance of typical small circular entire smooth and shining uncolored colonies. Furthermore, only this type of colony was evident in the plates used for taking psychrotrophic count (Table 1). The *Pseudomonas* spp. count was $(1.5 \pm 0.01) \times 10^2$ cfu/ml among different samples and the isolates were subjected to bacteriological examination as shown in Table 2.

The results showed that 10 samples were positive for occurrence of *Pseudomonas* spp., with a percentage of 26.7%. Identity of the isolates was confirmed by using API 20NE for detection and isolation of *Pseudomonas* spp.; 7 isolates *of P. aeruginosa* and 3 isolates *of Pseudomonas fluorescens* were detected. Many investigators indicated that some *Pseudomonas* spp. could survive heat treatment used in pasteurization of milk (Abad et al., 1993). Incidence of *P. aeruginosa* in pasteurized milk has been reported by Kumaresan and Annal Villi (2008). According to them, post processing contamination led to the predominance of heat sensitive *Pseudomonas* spp. in pasteurized milk.

Quality defects in pasteurized milk are most often the result of microbial contamination, growth and spoilage. Microbial defects usually become evident in the finished product through shelf-life evaluations or consumer complaints. Post-pasteurization contamination with psychrotrophic spoilage bacteria is most detrimental. In most cases, product contamination is the result of insufficient cleaning and sanitation of the processing equipment and plant environment. Practices that are being followed in the dairy processing unit would have singled out psychrotrophics as the major category of spoilage organism (Alatossava and Alatossava, 2007).

The proteolytic and lipolytic activities of *Pseudomonas* spp.

The high spoilage potential of *Pseudomonas* spp. is not only because of its ability to multiply at refrigeration temperatures but also because of their ability to produce thermostable proteases and lipases (Sorhaug and Stepaniak, 1997). The importance of proteases and lipases, which causes bacterial virulence, was proven in several studies. However, there was less study in the protease and lipase activities of *Pseudomonas* spp.

This study showed that the *Pseudomonas* spp. isolates did not show proteolytic and lipolytic activities at 4℃, while at 20℃ the *P. aeruginosa* and *P. fluorescens* isolates showed these activities as shown in Table 3. Two (2) isolates were positive for proteolytic; 3 isolates for lipolytic and 5 isolates for both proteolytic and lipolytic. This finding agrees with the findings of Dogan and Boor (2003) and Alatossava and Alatossava (2006).

Psychrotrophics were implicated in many defects in milk and dairy products which are a problem resulting from prolonged refrigeration storage (Swart et al., 1989; Garg, 1990) and distribution of perishable food products. *Pseudomonas* spp. have been implicated in the spoilage of processed milk kept under chilled condition because of their capacity to multiply under refrigeration with the production of thermostable proteases and lipases (Rajmohan et al., 2002) which plays an important role in

Table 3. Proteolytic and lipolytic activities of *Pseudomonas* spp. isolated from pasteurized milk samples.

Pseudomonas isolate	No.of tested isolates	Positive protyolytic isolates	Positive lipolytic isolates	Positive protyolytic and Lipolytic isolates
P. aeruginosa	7	1	2	4
P. fluorescens	3	1	1	1
Total	10	2	3	5

milk spoilage.

Conclusion

Raw milk deteriorates in only a few days even when stored under refrigeration temperatures. Moreover, pasteurized and refrigerated milk has a shelf-life of 7 to 10 days. In this study, the results cleared that after pasteurization, 10 samples from 35 samples were positive for occurrence of *Pseudomonas* spp., with a percentage of 28.57% that may be due to insufficient pasteurization or the post contamination by this genus and the temperature which milk and dairy products are exposed influences the type of microorganisms that will grow in them. As dairy equipment and utensils constitute the major source of many types of psychrotrophics in milk, so special attention should be considered in their cleaning and sanitation to produce milk of low bacterial count or even completely free of psychrotropics bacteria. The previous information indicated that psychrotrophics are still inevitable because they are widely distributed in nature, withstand sanitizers and can liberate heat stable enzyme causing spoilage of food and some of them considered as food borne pathogens. This knowledge increase attention toward the way by which the restriction of these microorganisms must be done.

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