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Effect of Temperature and Storage Period on the Constituents of Milk Inoculated with *Pseudomonas aeruginosa*

[1]Sanaa O. Yagoub, [1]Fardous A. Bellow and [2]Ibtisam E.M. El Zubeir
[1]Department of Microbiology and Molecular Biology,
Faculty of Science and Technology, El Neelain University,
P.O. Box 12702, Khartoum, Sudan
[2]Department of Dairy Production, Faculty of Animal Production,
University of Khartoum, Postal Code 13314, Khartoum North, Sudan

Abstract: The effect of incubation temperature and storage period on inoculated milk by *Pseudomonas aeruginosa* which well known as milk-spoilage microorganism was studied. Sterile milk sample were inoculated by 10^8-10^9 cfu mL^{-1} *P. aeruginosa* and stored at 0, 7, 12, 21, 37 and 45°C. The chemical analysis was conducted daily for all stored milk samples to investigate the effect of bacteria on milk constituents (fat%, protein% and acidity) and total bacterial count. The present results showed that milk which inoculated with *P. aeruginosa* and stored at different storage periods and temperatures, showed variations for fat, protein, acidity and bacterial count. Moreover, the inoculated milk samples showed a shelf life of 1-2 days at 37 and 21°C. However, the milk samples stored at 0, 7 and 12°C showed shelf life that ranged between 4-9 days. The present study concluded that the number of psychrotrophic bacteria significantly affected by both storage period and incubation temperature.

Key words: Dairy microbiology, *Pseudomonas aeruginosa*, milk spoilage, milk life span, milk constituents

INTRODUCTION

Fresh raw milk contains bacteria that grow well during storage at refrigeration temperature; these bacteria can overgrow between milk collections and consumption leading to milk deterioration (Doreene and Hyatt, 2000). Psychrotrophs, particularly, *Pseudomonas* sp. are known to be the main determinants of the shelf-life of pasteurized milk and refrigerated raw milk (Stevenson *et al*., 2003). The growth of *Pseudomonas* sp. is correlated with the occurrence of proteolytic activity in all food systems (Liu *et al*., 2006). *Pseudomonas* sp. secretes heat-stable extracellular enzymes (proteases and lipases) which survive pasteurization and even UHT heat treatments and degrade the casein and fat components of raw milk (Dunstall *et al*., 2005). Rajmohan *et al*. (2002), studied the productions of these enzymes. Nicodeme *et al*. (2005) and Vidyasagar *et al*. (2006) investigated the effect of growth conditions on proteolytic activity of *Pseudomonas* sp. while Koka and Weimer (2001) studied the influence of growth conditions on heat-stable phospholipase produced by *Pseudomonas* sp. Gugi *et al*. (1991) mentioned that the activity of extracellular proteases is maximal at growth temperature of 17.5°C. Deeth *et al*. (2002) referred the difference in spoiled behavior occurred by *Pseudomonas* sp. in skim milk and whole milk to proteases and lipases activities, respectively. The same authors added that *Pseudomonas* sp. in milk also contributes to the different flavors produce

Corresponding Author: Sanaa O. Yagoub, Department of Microbiology and Molecular Biology, Faculty of Science and Technology, El Neelain University, P.O. Box 12702, Khartoum, Sudan
Fax: 00249-83-776338

during cold storage of these milk types. Fajardo-Lira *et al*. (2000) investigated the effect of *Pseudomonas* sp. growth on the plasmin enzymatic system in casein and whey fractions of fresh milk. Generally, high levels of psychrotrophic bacteria in raw milk are required to contribute sufficient quantities of heat-stable proteases and lipases to cause breakdown of protein and fat after pasteurization (Barbono *et al*., 2006). The same authors added that sanitation, refrigeration and the addition of CO_2 to milk are used to control both total and psychrotrophic bacteria count.

In this study the effect of temperature and storage period on the constituents of milk that inoculated with *Pseudomonas aeruginosa* was recorded. The growth and count of *Pseudomonas aeruginosa* at different degree of temperature and storage period was also determined.

MATERIALS AND METHODS

Area of the Study

This study was conducted at El Neelain University, Faculty of Science and Technology during 2006.

Preparation of Milk Samples

About 70 mL milk were placed into sterilized glass bottles and plugged with foil paper. The milk was sterilized using the autoclave for 30 sec at 120°C and control samples of milk were analyzed for fat, protein and acidity as well as *Pseudomonas* sp. count.

Preparation of Inoculum's Organism

Pseudomonas aeruginosa that isolated from milk source was inoculated in nutrient broth medium and incubated at 37°C overnight. Serial dilutions were done and the total count of the organism mL^{-1} of the stock suspensions was determined by means of the surface viable counting technique (Richardson, 1985). About 10^8-10^9 colony-forming units mL^{-1} were used.

Storage of Milk Samples

Six batches of the inoculated milk samples were stored at different temperatures (0, 7, 12, 21, 37 and 45°C), inoculated milk were examined daily for 1-9 days to estimate the effect of *Pseudomonas* sp. on milk protein, fat, acidity. The total bacterial count was also estimated daily. The fat content was determined by Gerber's method (AOAC, 1990) and the protein was determined by Kjeldahl method (AOAC, 1990). Similarly the acidity of the samples was determined according to the method described by AOAC (1990). Total viable count was done according to standard methods described by Richardson (1985).

RESULTS

Inoculated milk samples that incubated above 12°C were spoiled at the second day and those incubated at 7 and 12°C were spoiled at day four and day seven, respectively (Table 1).

As shown in Table 1, fat% of inoculated milk was markedly affected by both temperature and storage period. These were appeared as reduction in values according to increasing of incubation temperature or reduction of the values at the same degree of temperature when storage period increased. Fat % also showed non significant changes at 0, 7 and 12°C but there were a significant changes in fat% values of milk samples that incubated at above mentioned degrees and those incubated at 21 and 37°C (Table 2). On the other hand, fat% showed non-significant changes within the first three days of the experiment otherwise, significant changes with increasing of storage period were detected as shown in Table 3.

Table 1: Effect of storage periods and temperature on constituents of milk inoculated by *Pseudomonas aeruginosa*

Period (Days)	Temperature (°C)	Fat (%)	Protein (%)	Acidity (%)	Total count cfu mL^{-1}
1	0	5.9±0.141	6.5±0.000	0.277±0.000	8.919±0.00
	7	5.3±0.212	5.9±0.000	0.344±0.000	9.342±0.00
	12	5.1±0.710	5.3±0.035	0.366±0.000	9.623±0.00
	21	2.9±0.141	2.3±0.000	0.688±0.000	9.690±0.00
	37	2.3±0.283	2.1±0.000	0.844±0.000	9.914±0.00
	45	-	-	-	-
2	0	5.1±0.0957	5.07±0.781	0.297±0.011	8.079±0.00
	7	4.9±0.141	4.40±0.000	0.333±0.000	8.591±0.00
	12	3.4±0.047	2.80±0.000	0.411±0.016	8.613±0.00
	21	-	-	-	
	37	-	-	-	
3	0	4.9±0.141	3.5±0.000	0.322±0.000	8.230±0.00
	7	4.5±0.000	3.8±0.071	0.355±0.000	8.591±0.00
	12	3.2±0.000	2.1±0.000	0.455±0.000	8.653±0.00
	21	-	-	-	-
4	0	4.3±0.000	2.6±0.000	0.333±0.000	9.230±0.00
	7	2.3±0.283	1.9±0.000	0.394±0.085	9.556±0.00
	12	-	-	-	-
	21	-	-	-	-
5	0	4.40±0.141	3.03±0.00	0.366±0.000	9.342±0.00
	7	3.05±0.071	2.10±0.071	0.500±0.000	9.690±0.00
	12		-	-	-
6	0	3.6±0.000	2.7±0.000	0.428±0.008	9.491±0.00
	7	1.9±0.071	1.5±0.000	0.661±0.008	9.756±0.00
7	0	2.9±0.071	2.2±0.000	0.466±0.000	9.810±0.00
	7		-	-	-
8	0	2.2±0.141	1.9±0.0141	0.501±0.008	9.763±0.00
	7	-	-	-	-
9	0	1.8±0.141	1.6±0.035	0.633±0.016	9.839±0.00
	7	-	-	-	-

-: Milk spoiled

Table 2: Variation of temperature and storage period on milk constituents

Temperature (°C)	Fat (%)	Protein (%)	Acidity (%)	Total count cfu mL^{-1}
0	4.000^{a}	3.527^{a}	0.398^{e}	9.225^{a}
7	4.0288^{a}	3.265^{b}	0.397^{e}	9.122^{b}
12	3.917^{a}	3.365^{b}	0.463^{d}	9.629^{c}
21	3.800^{b}	2.956^{d}	0.518^{c}	9.939^{b}
37	3.0200^{c}	2.606^{e}	0.852^{b}	10.206^{a}
45	2.650^{d}	3.175^{c}	1.2400^{a}	10.414^{a}

Mean within the same column bearing the same letter(s) are not significantly different ($p>0.05$)

Table 3: Effect of storage period on milk constituents

Period (days)	Fat (%)	Protein (%)	Acidity (%)	Total count cfu mL^{-1}
1	4.313^{a}	4.445^{a}	0.5059^{a}	8.763^{a}
2	4.395^{a}	4.102^{b}	0.4725^{b}	9.256^{b}
3	4.400^{a}	3.607^{c}	0.3828^{c}	9.081^{a}
4	3.728^{b}	2.547^{d}	0.4504^{c}	9.583^{b}
5	3.333^{d}	2.2033^{e}	0.5069^{a}	9.774^{b}
6	3.437^{d}	2.1413^{e}	0.4606^{b}	9.573^{c}
7	3.433^{d}	2.01167^{e}	0.4252^{d}	9.256^{d}
8	2.750^{e}	1.830^{f}	0.4738^{b}	9.656^{b}
9	2.350^{f}	1.508^{g}	0.5680^{a}	9.578^{d}
10	1.950^{g}	1.190^{h}	0.6968^{a}	9.832^{e}

Mean within the same column bearing the same letter(s) are not significantly different ($p>0.05$)

The protein % also showed reduction of values due to increasing in both incubation temperature and storage periods (Table 1). There were significant changes in protein % values at different incubation temperature except 7 and 12°C in which non-significant changes were detected (Table 2). Significant changes in protein % were also detected at different storage period except at days five, six and seven (Table 3).

The acidity % values of inoculated milk rose with increasing of both incubation temperature and storage period (Table 1). There were significant changes in the acidity values in the inoculated milk that stored at different temperature except at 0 and 7°C in which non-significant change was observed (Table 2). The acidity % was significantly affected by storage period (Table 3).

Pseudomonas aeruginosa count was affected by both incubation temperature and storage period (Table 1). Highest counts were detected at 12°C in all storage period except at the first day of the experiment in which 37°C showed the highest bacterial count followed by spoiled of all milk samples incubated at this temperature. The total bacteria count showed non-significant changes at 0 and 37°C, otherwise, significant changes were detected at different incubated temperatures (Table 2). As shown in Table 3 the total bacterial count was also significantly affected by storage period.

DISCUSSION

The study revealed that both temperature of incubation and storage period have various effects on the activity of *Pseudomonas aeruginosa* and its effect on fat and protein content of the milk. The reduction of fat % and protein % in the inoculated milk might be due the fact that to *Pseudomonas* sp. elaborate proteases and lipase enzymes, it is assumed that the production of these enzymes have significant effect on the growth kinetics of the organism, these supported the findings of Dunstall *et al*. (2005) and also the finding of Liu *et al*. (2006) who reported that the growth of *Pseudomonas* was correlated with the occurrence of proteolytic activity in all food systems. The continuous reduction of the values during storage period explained by the fact that the extracellular and periplasmic enzymatic activities of *Pseudomonas* sp. increased with 6 days incubation as mentioned by Kohlmann *et al*. (1991). In this study, it was clear that highest total bacterial count correlated with lowest fat % and protein % were observed at 12°C these support the findings of Gugi *et al*. (1991) who appointed 17.5°C as critical growth temperature of *Pseudomonas* sp. and the finding of McClements *et al*. (2001) who reported that the survival of *Pseudomonas* sp. grown at 8°C was more than those grown at 30°C, this result also confirm the findings of Labuza and Schmidl (1988) who reported that *Pseudomonas* sp. reduced fat and protein significantly at different temperatures. In this study the milk samples that inoculated with *Pseudomonas aeruginosa* and kept at above 37°C were spoiled on first day, while the milk samples kept at 7 and 0°C spoiled on the seventh and tenth day, respectively. This might be due to the ability of *Pseudomonas aeruginosa* to produce a high level of proteases and lipases on prolonged incubation as was reported earlier by Kohlmann *et al*. (1991) or due to fact that the ability of psychrotrophic bacteria both to survive for long periods and to produce high levels of proteases and lipases on prolonged incubation in milk as mention by Stead (1987). The acidity that caused milk spoiled arises from by-products of lipid and protein breakdown and it is finding to be affected by all *P. aeruginosa*, temperature and storage period.

We conclude that the *P. aeruginosa* is associated with milk spoilage as indicated by the change of milk constituents and shelf life. The effect of this bacterium is influenced by both variation of temperature and storage period.

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