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Influence of Milk Aeration on Growth of Psychrotrophic Pseudomonads

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ABSTRACT

The psychrotrophic microflora of raw milk from a Cornell University herd was examined and the three most frequently occurring isolates (*Pseudomonas* species) were subjected to oxygen concentrations of 1 to 12 ppm and temperatures of 3 to 9°C in growth studies in raw milk. At 3°C, a reduction in oxygen level from 9-12 to 1-3 ppm resulted in a 63% increase in generation time for *Pseudomonas fluorescens*. However, the reduction in growth temperature from 9 to 3°C at 9-12 ppm oxygen produced a 280% generation time increase for *P. fluorescens*. Similar observations were made for the other isolates. An analysis of variance revealed a significant interaction between the effects of oxygen and temperature on growth of the isolates.

Present-day milk handling methods involving the longer use of low-temperature holding and extensive pumping/agitation have influenced the spoilage patterns and rates of raw milk. Practices such as alternate-day pickup of refrigerated bulk milk, shipment of raw milk long distances, and shorter work weeks in processing plant operations have increased the aerated state and storage time of raw milk before processing. Consequently, growth of psychrotrophic microorganisms in raw milk represents a major quality control problem (*3,4,5*). Quality-deteriorating enzymes are produced by small numbers of these organisms (*1*). Speck and Adams pointed out that psychrotrophs probably are part of the flora of all normal raw milk since it is difficult to exclude them during production operations (*11*). Further, not only is the quality of market milk deteriorated by psychrotrophic growth but also important products such as cheese are adversely affected (*7,12*).

[1]*Growth on SPC agar in 2 d at 42°C, hydrolysis of gelatin within 7 d at 32°C, growth on cetrimide agar in 2 d at 32°C, production of brown pigment, anaerobic utilization of dextrose in 3 d at 32°C, production of alkaline conditions when grown on xylose in 3 d at 32°C, and susceptibility to penicillin G and polymixin B.*

Speck, in a review of possible approaches of controlling heat-resistant enzymes which are produced by the psychrotrophic flora of raw milk, mentioned the potential of diminished aeration levels and lower temperatures to reduce growth and metabolism by psychrotrophs (*11*). Adams et al., in a report on heat-stable proteinases produced by psychrotrophs, characterized their isolates as aerobic, motile, gram-negative rods of the genus *Pseudomonas* (*1*). Reduction of oxygen levels is effective in controlling the psychrotrophic flora of meat products at low temperatures (*6*); however, no data are available on the influence of aeration levels on these spoilage organisms in milk. This study reports the effect of aeration at different temperatures on the growth of psychrotrophic pseudomonads in raw milk.

MATERIALS AND METHODS

Psychrotrophic isolates

Raw milk samples, obtained from the herd of the College of Veterinary Medicine, Cornell University, were examined for psychrotrophic bacteria by the standard procedure which involves the use of the Standard Plate Count method except that plates were incubated at 7± 1°C for 10 d (*8*). The melted agar was tempered carefully to 44-46°C before pouring plates to prevent destruction of some psychrotrophic bacteria.

In addition, an unpublished selective medium, developed to enumerate *Pseudomonas* species (Naylor, H.B., personal communication), was prepared as follows: 5.0 g of peptone (Difco), 5.0 g of NaCl, and 23.5 g of Standard Plate Count Agar (SPC) were combined with a liter of distilled water, steamed to melt the agar, pH adjusted to 5.5 with HCl, and sterilized at 121°C for 15 min. Four ml of a 5% sterile triphenyl tetrazolium chloride solution were added aseptically to 96 ml of the sterile medium. Plates containing this medium were incubated at 22°C for 24 h.

Representative psychrotrophs were isolated from both media (only 2 of 50 isolates were from the selective medium) and maintained on SPC agar slants. The oxiferm minikit (Roche Diagnostics, Nutley, NJ) was used to assist in isolate identification. This system and other confirmatory tests[1] were used according to manual instructions (Roche Diagnostics, Nutley, NJ).

Preparation of inocula

Nutrient broth (BBL) was inoculated with selected isolates by loop transfer from a SPC agar slant and incubated at 30°C for 24 h. Inocula

were prepared by diluting the broth culture 1:100 with buffered dilution water used in the Standard Plate Count procedure (*8*). Initial levels of approximately 5×10^3 cells/ml in test milks were obtained by this procedure.

Determination of effects of different levels of oxygen and different temperatures on growth of selected isolates

Three organisms (*Pseudomonas fluorescens, Pseudomonas putida* and *Pseudomonas aeruginosa*) were selected from 50 isolates on the basis of the frequency encountered on plates involved in psychrotrophic counts. Raw milk was obtained aseptically from the Cornell Veterinary College herd and immediately cooled by placing the container in an ice-water mixture. One hundred and fifty ml were transferred to sterile 250-ml Erlenmeyer flasks (containing sterile magnetic stirring bars), inoculated with 2.2 ml of an inoculum prepared as described above, and placed in a 3°C incubator where oxygen level adjustments were accomplished by bubbling in compressed air or nitrogen filtered through a Millipore filter (0.45μ). The gasses were introduced into the samples through a sterile fritted glass gas dispersion tube and a Beckman Monitor II equipped with an Oxygen-Temperature Module was used to measure oxygen concentrations. Sample containers (250-ml Erlenmeyer flasks) were sealed with sterilized glass stoppers coated with silicone.

Plate counts of psychrotrophic bacteria (method was described earlier) were performed on uninoculated controls and inoculated samples every 2 d for 6 d and every day for 3 d on samples held at 3 and 9°C, respectively. Separate sample containers were used for each measurement. Samples were stirred for 1 min before plating. All trials were done in duplicate for each of the three isolates at 3 and 9°C. Oxygen measurements were made immediately after each sampling for enumeration of psychrotrophic bacteria. Oxygen levels were maintained throughout the incubation period within the ranges of 1-3 and 9-12 in inoculated milks held at 3°C. At the incubation temperature of 9°C, oxygen levels were maintained also in growth studies with *P. putida*. In trials with *P. fluorescens* and *P. aeruginosa* at 9°C, oxygen levels fell below the ranges at the end of the growth studies where the counts were high (approximately 6.8×10^7).

RESULTS AND DISCUSSION

In the initial phase of the study, the psychrotrophic flora of the raw milk supply, sampled at a point just before the farm's bulk storage tank, was examined by identifying colonies which developed in the psychrotrophic plate count procedure. Thirty-five of the 50 isolates were identified as species of *Pseudomonas; P. fluorescens, P. putida,* and *P. aeruginosa* were encountered most frequently. The isolation of psychrotrophic strains of *P. aeruginosa* was considered unusual. The literature indicates that this organism is normally a mesophile (*2*). Perhaps the *P. aeruginosa* strains isolated from the psychrotrophic plates were mutants that acquired the ability to grow at low temperatures.

The effects of different oxygen concentrations (1-3 and 9-12 ppm) at 3 and 9°C on growth rates of three most commonly occurring psychrotrophs in our samples were studied next; the data for *P. fluorescens* are shown in Table 1. The generation time at 3°C was extended from 19.0 to 31.0 h when the oxygen level was reduced from 9-12 to 1-3 ppm. At 9°C, the generation time was increased from 5.0 to 8.7 h when the oxygen concentration was reduced from 9-12 to 1-3 ppm. Although these increases are substantial, a greater influence on generation times was evident when the temperature was lowered from 9 to 3°C.

Data for *P. putida*, the second most frequent psychrotroph isolated, were similar (Table 2). The effect of temperature is larger than that of oxygen though both influences are significant. Similar growth patterns occurred in experiments with *P. aeruginosa* (data not shown).

An analysis of variance indicated that the incubation temperatures used as well as the oxygen levels studied had a statistically significant effect on generation times (Table 3). However, the three different *Pseudomonas* species reacted similarly to all oxygen/temperature conditions. A significant interactive effect between oxygen and temperature was evident. This interaction implies that the effect of temperature on generation times differs at different oxygen concentrations. This oxygen/temperature interaction was analyzed further statistically as follows: generation times were averaged, the means were recorded and a least significant difference test (LSD) (*10*) was performed on the significant effects revealed in the analysis of variance (Table 4). The anlysis indicates that at 9°C a significant reduction in growth rates of *Pseudomonas* species occurred as oxygen levels were lowered. However, at 3°C, the reduction in growth rates was much more pronounced. An increase in the mean generation time of 3.6 h (from 5.3 to 8.9) was recorded at 9°C with a drop in oxygen level from 9-12 to 1-3 ppm, while the increase in mean generation time at 3°C was 15.0 h (18.0 to 33.0 h) with approximately the same drop in oxygen concentration. A reduction in oxygen from 9-12 to 1-3 ppm resulted in a 68% increase in mean generation time at 9°C, while at 3°C the increase was 83%. Olsen and Jezeski (*9*) reported that the effects of temperature on the growth rate of a psychrotrophic strain of *P. fluorescens* were influenced by aeration as well as by the carbon source in the growth medium.

TABLE 1. *Effect of oxygen and temperature conditions on generation times*[a] *of* Pseudomonas fluorescens *in raw milk.*

Oxygen (ppm)	Temperature (°C)	Generation time (h)
1-3	3 ± 1	31.0
9-12	3 ± 1	19.0
1-3	9 ± 1	8.7
9-12	9 ± 1	5.0

[a]Means of two experiments.

TABLE 2. *Effect of oxygen and temperature conditions on generation times*[a] *of* Pseudomonas putida *in raw milk.*

Oxygen (ppm)	Temperature (°C)	Generation time (h)
1-3	3 ± 1	31.0
9-12	3 ± 1	16.0
1-3	9 ± 1	9.4
9-12	9 ± 1	5.4

[a]Means of two experiments.

The negative effect of reduced oxygen levels on growth rates of psychrotrophic *Pseudomonas* isolates is clearly evident; however, a reduction of storage temperature of raw milk causes a more marked decrease in growth rates. If milk storage temperatures increase (as indicated in our research at 3 and 9°C), limiting milk aeration is important in slowing the growth rates of psychrotrophs. In view of this, increased attention should be focused on sources of aeration in milk handling systems, such as dropline inlets of bulk tanks, leaky gaskets and improper operation of pumps. Milk handling systems which carefully control both temperature and aeration should extend the shelf-life quality of milk.

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TABLE 3. *Analysis of variance of the influence species of* Pseudomonas, *oxygen levels, and incubation temperature on generation times in raw milk.*

Source of variation	Degrees of freedom	Mean squares	F Value
Temperature	1	2127.60	340.3*
Oxygen	1	512.73	82.0*
Species	2	15.91	2.6
Temperature × oxygen	1	195.45	31.3*
Temperature × species	2	18.90	3.0
Oxygen × species	2	3.99	0.6
Temperature × oxygen × species	2	6.8	1.1
Error	12	6.3	

* Significant at the 0.01 level.

TABLE 4. *Least significant difference test on generation times*[a] *of Pseudomonas isolates at different oxygen and temperature conditions.*

Treatment	Number[b]	Mean	Difference between treatment means	LSD[c]
9°C, 9-12 ppm O_2	6	5.3		
9°C, 1-3 ppm O_2	6	8.9	3.6*	2.98
3°C, 9-12 ppm O_2	6	18.0		
3°C, 1-3 ppm O_2	6	33.0	15.0*	2.98
9°C, 1-3 ppm O_2	6	8.9		
3°C, 1-3 ppm O_2	6	33.0	24.1*	2.98
9°C, 9-12 ppm O_2	6	5.3		
3°C, 9-12 ppm O_2	6	18.0	12.7*	2.98

[a]Hours.
[b]Trials.
[c]Least significant difference.
* Significant difference at the 5% level, using T value of 2.1 based on error degrees of freedom of 12.