

Temperature Equilibration Times of Plate Count Agar and a Comparison of 50 Versus 45 C for Recovery of Raw-Milk Bacteria¹

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ABSTRACT

Sixty raw milk samples were plated using "Standard Methods" agar tempered to 45 or 50 ± 1 C. The standard plate count was significantly lower with the agar at 50 C. Tempering time (to 44-46 C) of a flask of agar in a water bath was about 5-10 min longer than that of a comparable flask of water. Time required to reach the desired temperature depended upon the volume of agar in the flasks, the number of flasks, and the volume of the water in the bath. Up to an hour of equilibration time may be necessary for newly autoclaved agar to reach the recommended temperature (44-46 C). Insufficient tempering time might cause an excessively high plating agar temperature which might cause a reduction in bacterial counts, especially of a heat sensitive psychrotrophic bacterium.

The extreme heat sensitivity of a psychrotrophic bacterium, *Pseudomonas fluorescens*, was recently shown by Gray et al. (2). Exposing cells to 36 C for 2 h resulted in an apparent 99% reduction in plate count. The count, however, increased after 25 C incubation due to repair of the heat-stressed bacteria. Vanderzant and Matthys (8) presented some evidence that an increase in pouring-agar temperature from 45 to 50 C resulted in decreased counts of pure cultures of bacteria isolated from dairy products. A 25% reduction in counts was found at 50 C with 6 of 9 cultures tested; however, they provided no statistical treatment for their data. Stapert et al. (7) compared Millipore-filtered water samples (receiving no extra heat treatment) to a water-gelatin solution heated to 45 C. The unheated water gave counts 3-4 times that of the heated solution. Comparison with the standard plate count (SPC) indicated that the higher

temperature of the agar was responsible for the decreased counts. Mossel and van der Moosdijk (6), on the other hand, found no differences between pour plate (agar at 44-46 C) and spread drop counts (agar at room temperature) of 42 food samples. Lawton and Nelson (5) used a temperature of 52 C for 7-9 min for partially inactivating cultures of pseudomonads. They observed reductions in counts of 2 to 4 logs. Heather and Vanderzant (3) found less than 0.1% survivors when a culture of *Pseudomonads putrefaciens* was heated to 50 C for 1 min.

Standard Methods for the Examination of Dairy Products (1) recommends that agar used for the SPC be tempered to 44-46 C before the plates are poured. In this method the temperature of the pouring agar is determined by checking the temperature of a similar flask of water subjected to the same conditions. Because of the possible difference in cooling times of water and agar due to differences in specific heat and viscosity, this method of temperature checking could lead to agar pouring temperatures high enough to destroy some of the heat sensitive bacteria. We chose the temperature of 50 ± 1 C for studying this effect and compared it to the *Standard Methods* temperature of 45 ± 1 C. We also compared the equilibration times of water and agar in identical flasks.

MATERIAL AND METHODS

Methods advocated by *Standard Methods* (1) were followed except that agar tempered at 50 ± 1 C was used in addition to the recommended 45 ± 1 C agar. The protocol for the experiments and the analyses were in general similar to those of a previous study (4). Sixty samples of raw milk were analyzed by nine analysts. In most instances replicates from the same dilution bottle were plated in duplicate plates. The temperature of the agars was determined by either inserting an alcohol-sterilized and dried thermometer directly in the pouring agar or by inserting a thermometer in a different flask of agar. Separate water baths were used for the two temperatures. A comparison was made of the time required for freshly autoclaved water or agar in similar vessels to reach the recommended temperature of 44-46 C. Temperature readings were made at intervals until the desired temperature was reached.

RESULTS

Statistical analyses of 45 vs 50 C agar

Results of the comparison of agar pour temperatures

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of 45 and 50 C on recovery of raw-milk bacteria are shown in Table 1. Arithmetic means of the plate counts

TABLE 1. Comparison of 45 and 50 C agar temperature for standard plate counts of raw milk

Analyst	Milk sample	45 C	50 C
A	1	137 ^a	141 ^b
	2	<u>29.5</u>	26.8
	3	<u>394</u>	363
	4	230	<u>236</u>
	5	<u>112</u>	101
	6	<u>93</u>	89
	7	<u>11.4</u>	11.1
	8	<u>324</u>	145
	9	<u>9.1</u>	8.5
	10	<u>50</u>	42
arith. mean		<u>139.0</u>	116.3
geom. mean ^c		4.326	4.187
B	11	<u>189</u>	134
	12	<u>3.7</u>	<u>7.1</u>
	13	16	16
	14	3.6	<u>4.3</u>
	15	5.4	<u>5.5</u>
	16	<u>50</u>	48
arith. mean		<u>44.65</u>	35.86
geom. mean		2.694	2.761
C	17	3.5	3.5
	18	22.5	<u>25.1</u>
	19	11.2	<u>11.8</u>
	20	18.2	<u>18.6</u>
	21	13.8	<u>13.8</u>
arith. mean		13.83	14.56
geom. mean		2.461	2.497
D	22	14.4	<u>16.4</u>
	23	<u>13.4</u>	11.9
	24	<u>63</u>	42
	25	3.5	<u>3.9</u>
	26	<u>5.0</u>	4.6
	arith. mean		<u>19.76</u>
geom. mean		2.445	2.361
E	27	109	<u>124</u>
	28	<u>247</u>	138
	29	<u>26.4</u>	22.8
	30	68	<u>72</u>
	31	148	<u>164</u>
	32	<u>97</u>	77
	33	<u>24.0</u>	19.2
	34	<u>8.0</u>	7.1
	35	<u>18.2</u>	14.5
	arith. mean		<u>82.74</u>
geom. mean		3.930	3.791
F	36	20.1	<u>20.6</u>
	37	<u>27.2</u>	25.6
	38	70	<u>72</u>
	39	<u>57</u>	51
	40	6.0	<u>6.6</u>
	41	6.6	<u>6.8</u>
	42	6.5	<u>7.0</u>
	arith. mean		<u>27.64</u>
geom. mean		2.876	2.884

G	43	<u>2.9</u>	2.3
	44	<u>5.8</u>	<u>6.0</u>
	45	<u>7.3</u>	6.8
	46	<u>2.8</u>	2.5
	47	<u>35</u>	25
	48	<u>7.4</u>	6.4
arith. mean		<u>10.21</u>	8.21
geom. mean		1.896	1.752
H	49	<u>10.7</u>	9.8
	50	<u>8.8</u>	6.2
	51	6.3	<u>6.8</u>
	52	<u>22.0</u>	12.8
	53	<u>18.3</u>	15.6
	54	<u>8.5</u>	8.8
	arith. mean		<u>12.42</u>
geom. mean		2.416	2.245
I	55	<u>9.8</u>	9.4
	56	<u>7.4</u>	6.6
	57	6.0	6.0
	58	<u>27.0</u>	13.3
	59	<u>18.6</u>	16.0
	60	<u>8.9</u>	9.4
arith. mean		<u>13.29</u>	10.09
geom. mean		2.451	2.249
All samples			
arith. mean		<u>40.73</u>	34.64
geom. mean		2.833	2.748

^aArithmetic mean of four values (2 replicates and 2 duplicate petri dishes); for actual counts multiply by 1000.

^bUnderlined figures denote the higher of the two means.

^cNatural log.

The temperature variation was ± 0.5 C at most and was checked by inserting thermometer into duplicate flasks of agar or by alcohol sterilizing and inserting in pouring agar.

were lower at 50 C for eight of the nine analysts. The geometric means (log e) were lower at 50 C with six analysts, higher for one (analyst B), and were about the same with the other two analysts. In 34 of 60 milk samples tested, a temperature of 50 C gave lower arithmetic mean counts than 45 C while four samples showed the same counts. Sixteen milk samples gave higher counts at 50 than at 45 C agar pour temperature. The percent differences between the two agar temperatures for each milk sample were calculated, transformed to an arcsin function, and a two-tailed t-test made on the mean of the differences. The results showed the 50 C counts to be significantly lower ($P < 0.05$) than the 45 C counts.

An analysis of variance of the logarithms of plate counts of raw milk samples is shown in Table 2. The highly significant difference between analysts ($P < 0.01$) may have resulted because analysts were from different geographical locations which might in turn have influenced the types of bacteria present in their particular raw milk samples. There was, as expected, a very great difference between samples, but no difference between replicates. The replicates for this experiment consisted of two aliquots from the diluted milk rather than two aliquots from the same raw milk samples. Analysis of the effect of agar pour temperature on plate

TABLE 2. Analysis of variance of plate count logarithms using 45 or 50 C agar pouring temperatures

Line	Source of error	df	Sum of squares	Mean square	F	Significant with P	
						<0.05	<0.01
A	Analysts	8	316.205	39.5256	4.59	yes	yes
B	Samples/analysts	51	439.143	8.61065	574	yes	yes
C	Replicates/samples	43	0.645017	0.015000	1.17	no	no
D	Temperatures	1	1.017969	1.01796	10.56	yes	yes
E	Temperatures × analysts	8	0.939072	0.117384	1.22	no	no
F	Temperatures × samples/analysts	51	4.91461	0.096365	7.52	yes	yes
G	Temperatures × replicate samples	43	0.550741	0.012808	<1.0	no	no
H	Residual (between plates)	206	3.22949	0.015677			
	Total	411	766.645				

F values calculated from ratios of lines A/B, B/C, C/G, D/F, E/F, F/G, G/H.

TABLE 3. Analysis of variance of variances of logarithms of plate counts between duplicate petri dishes with agar temperatures of 45 and 50 C

Line	Source of error	df	Sum of squares	Mean square	F	Significant with P	
						<0.05	<0.01
A	Analysts	8	16098.9	2012.36	2.26	yes	yes
B	Samples/analysts	51	45308.8	888.408	1.42	no	no
C	Replicates/samples	43	26853.2	624.493	0.48	no	no
D	Temperatures	1	3464.48	3464.48	3.96	no	no
E	Temperatures × analysts	8	15232.8	1904.10	2.18	no	no
F	Temperatures × samples/analysts	51	44570.2	873.925	0.67	no	no
G	Temperatures × replicate/samples	43	56301.8	1309.34			
	Total	206	207830.12				

F values calculated from ratios of lines A/C, B/C, C/G, D/G, E/G, F/G.

counts showed a highly significant difference between 50 and 45 C (50 was lower). Tests for interactions showed no consistent source of error between temperatures and analysts (line E), but there was a highly significant interaction ($P < 0.01$) between temperatures and milk samples within analysts (line F). However, there was no interaction between agar pour temperatures and replicates (line G). Table 3 shows the analysis of variance of the single-degree-of-freedom variances calculated for the pairs of petri dishes. These indicate homogeneous petri dish variances with each analyst. There was a significant error, however, between analysts.

Time required for agar to reach 45 C

Several analysts reported on their studies of the time required for pouring agar to reach the recommended temperature of 44-46 C. One such study was conducted with a Precision Scientific Co.⁹ bath, without agitation, having inside measurements of 10.5 inches by 13.5 inches and containing sufficient water to reach above the freshly melted agar in the flasks (200 ml agar in 250 ml narrow mouth Erlenmeyer flasks). With one flask of agar in the bath, 15-17 min were required to reach 44-46 C; with three flasks 26-28 min; and with five flasks 32-34 min.

Comparison of water and agar tempering times

A comprehensive study of the agar vs water temperature equilibration time was made using two different size water baths (Table 4). The larger one was 12 by 30 by 10 inches containing 32 liters of water with agitation; the smaller bath was 13¾ by 10¾ by 7 inches with 15 liters of water without agitation. Two volumes of agar and water were compared; one was 180 ml in 8-oz

⁹Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

TABLE 4. Time (minutes) for freshly autoclaved water and agar to reach 44-46 C

Type of fluid	Fluid ^a volume (ml)	Bath ^b size	Trial			
			1	2	3	Ave
Water	180	large	25	20	15	20
Agar	180	large	25	25	25	25
Water	180	small	45	35	40	40
Agar	180	small	50	50	50	50
Water	500	large	30	30	25	30
Agar	500	large	35	30	30	30
Water	500	small	50	45	45	45
Agar	500	small	50	50	50	50

^a180 ml volumes were in Brockway #12 (8 oz) medicine bottles; 500 ml volumes were in 1000 ml Erlenmeyer flasks.

^bLarge bath was Blue M Model MW 1130 A-1, measuring 12" × 30" × 10", and contained 32 liters of water with agitation by means of a paddle at 20 strokes per minute. Small bath was a Thelco Model 83, measuring 13¾" × 10¾" × 7", and contained 15 liters of water without agitation.

medicine bottles; the second was 500 ml in 1000-ml Erlenmeyer flasks. Only two flasks, one of agar, the other of water, were in each water bath at any one time. Temperatures were measured as accurately as possible. Three trials were made under each set of conditions with temperatures being measured as soon after autoclaving as possible (3 min) and at 5-min intervals thereafter. The three trials with bottles containing 180 ml water in the large bath showed that an average of 20 min were required to reach 46 C, while agar bottles required 25 min. The larger volumes (500 ml in 1000 ml Erlenmeyer flasks) in the larger bath cooled to 45 C in an average of 30 min for water and agar flasks. In the smaller bath, water bottles of 180 ml required an average of 40 min while the agar flasks required 50 min. In the bath, the large volumes required 45 min for the water and 50 min for the agar flasks.

Effect of degree of loading of bath on tempering time

One collaborator studied the tempering time of a water bath filled to capacity with 12 milk dilution bottles of agar or with two bottles. Twelve bottles required 50 min in one trial and 67 min in another while two bottles required 24 min to reach the recommended temperature.

Agar setting time

To determine the length of time the raw milk bacteria would be subjected to possibly deleterious temperatures, we determined the time required for the agar plates to solidify enough to prevent sliding when tilted. The average solidification time for 45 C agar was 3.2 min and for 50 C agar it was 4.4 min.

DISCUSSION

Our results showed a highly significant reduction in raw milk bacterial counts using an agar pouring temperature of 50 instead of 45 C. This underscores the need for carefully checking agar temperatures before plates are poured. Although the agar setting times indicated that the bacteria were in contact with the warm pouring agar for a maximum of about 3 min, it is possible that even then there was considerable damage to the psychrotrophs (2, 3). It would be best to check the temperature of each agar flask before pouring although this is inconvenient and might lead to contamination. As a guide, a tempering time of 1 h would appear to be adequate for proper temperature equilibration. Temperatures should be checked in one of the agar flasks although the differences in tempering times we observed were not in general very different from water flasks (10 min after the water flasks reached the desired temperature would insure that the agar flasks were also in the proper range). Reheating agar and water flasks at the same time in an autoclave would insure that each started out at nearly comparable temperatures before placing in the water bath. Some consideration in future

editions of Standard Methods should also be given to the volume of agar relative to the water bath volume as a guide to laboratories in preparing pouring agar.

CONCLUSIONS

(a). Use of a pouring agar temperature of 50 C resulted in lower standard plate counts, thus confirming reports of other workers.

(b). Water in flasks cools more rapidly than does agar in flasks although assurance that the agar is in pouring range may be had by using the agar no less than 10 min after the water in a flask reaches 45 C.

(c). The most important considerations in agar tempering are the size of the bath, the degree of loading, and agitation of water in the bath.

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