RESAZURIN REDUCTION TEST AS AN INDEX OF THE BACTERIOLOGICAL QUALITY OF FROZEN FOODS

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In the expanding field of the manufacturing of precooked frozen foods, it is recognized that there is the need for strict control of bacterial contamination. Bacterial contamination is most often determined by the enumeration of bacterial populations by a standard plate count method. However, the determinations require 2 to 3 days to complete; whereas, the use of a dye reduction test which has been introduced by several investigators offers the possibility of shortening the time to five to eight hours.

Proctor and Greenlie (*5*) were able to show a rough estimation of the number of bacteria present by the dye reduction test. Using resazurin they demonstrated that foods having a high bacterial plate count, i.e., over a million organisms per gram, had a reduction rate which produced a color change in the dye within 3 to 5 hours' incubation.

When resazurin and methylene blue tests were used on egg powder, Johns (*2*) reported difficulty in determining the end points of long reduction times. However, Scott and Gillespie (*6*) obtained a good correlation between the standard plate count and resazurin reduction times with egg pulp.

Straka and Stokes (*7*), using principally the same methods as Proctor and Greenlie, reintroduced a resazurin reduction test which permits the estimation of the number of bacteria in precooked frozen foods (poultry and meat pies) within 3 to 8 hours. They made a comparative study of approximately 77 poultry and meat pies in which both the dye reduction time and a plate count for each pie were determined. On the basis of their data, it was possible to subdivide the pies into several groups which covered a range from less than 35,000 bacteria per gram to more than 9,000,000 bacteria per gram.

Work by Ferguson, Yates, and Jones (*1*) showed that the rate of reduction of resazurin dye was not necessarily proportional to the number of aerobic, mesophilic bacteria present in frozen vegetables. The dormant bacteria present in the frozen vegetables influenced the rate of reduction of the dye. The number of bacteria in frozen vegetables cannot be estimated with any reasonable degree of accuracy by the resazurin reduction test.

Mallman, *et al.* (*4*) introduced an indicator reduction test using a Phytone-Nacconal medium which could be used for detecting insanitary plant operations as well as a possible means of predicting shelf-life of dressed poultry.

There is a need for a rapid test for determining the bacteriological quality of precooked frozen foods by the processors' quality control laboratories as well as other interested agencies. Since there has been some disagreement as to the usefulness of the resazurin reduction test in the estimation of bacterial populations for frozen foods, this investigation was undertaken in order to provide additional data.

MATERIALS AND METHODS

The resazurin reduction test and total bacterial counts were made on samples of frozen foods obtained from retail outlets in the city of Omaha, Nebraska.

A total of 123 samples of frozen foods which included meat pies, corn, beans, peas, peaches, blueberries, and cherries were examined for the total number of bacteria by a plate count and a resazurin reduction test.

A series of laboratory prepared unfrozen chicken meat pies were artificially inoculated with *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) in order to have high population levels of the organism. This bacterium was previously isolated from a routine bacteriological examination of chicken meat pies. The pies were then frozen. It has been our past experience that frozen meat pies under proper storage conditions, purchased at retail outlets have low total counts (*3*).

Samples were prepared for plating and resazurin reduction by adding four parts of sterile 0.1 per cent peptone water to one part of sample (*8*) in a sterile Waring blender and blending for three minutes. Fifty gram samples were used for testing the frozen vegetables. The frozen meat pies were sampled by removing the top crust aseptically and one-half of the internal contents of the pie plus the bottom crust placed into the Waring blender.

A 1:10 dilution for plating of one sample was made by diluting 50 ml of the 1:5 dilution of sample with 50 ml of sterile 0.1 per cent peptone water. Subsequent 10 fold dilutions were made by mixing 10 ml of the previous dilution with 90 ml of sterile 0.1 per cent peptone water. Tryptone glucose extract agar and

trypticase soy agar were used as the plating media. Plates were incubated for 48 hours at 37°C.

The dye reduction method used in this investigation was according to the procedure of Straka and Stokes (7).

Five ml of the 1:5 dilution of the blended sample were pipetted into a test tube containing five milli liters of trypticase soy broth and one-half milliliter of the standard resazurin solution. The contents were mixed slowly by inversion to avoid incorporation of air. The tubes were incubated in a 37°C water bath in the dark and read every half hour. When the contents of the tube had turned pink, readings were made at 15 minute intervals until the pink color disappeared and the lower four-fifths of the tube was colorless. This was considered to be the end point and the time of the final reading was recorded as the reduction time.

TABLE 1—THE RESAZURIN REDUCTION TIME IN HOURS AND BACTERIA PER GRAM FOR FROZEN VEGETABLES

Sample Number	Type of Sample	Plate Count Bacteria per Gram* Tryptone Glucose Extract Agar	Plate Count Bacteria per Gram* Trypticase Soy Agar	Resazurin Reduction Time in Hours	
1	Beans	740,000	770,000	7	
2	"	140	90	8	NC
3	"	420,000	460,000	7-1/2	
4	"	2,800	2,600	7-1/2	
5	"	36,000	37,000	8-1/2	
6	"	750,000	840,000	7	
1	Corn	1,300,000	1,400,000	4-1/2	
2	"	91,000	97,000	6-1/4	
3	"	80,000	86,000	6-1/4	
4	"	6,600	7,700	7-1/4	
5	"	73,000	64,000	7-1/4	
6	"	32,000	33,000	6-3/4	
7	"	26,000	28,000	7-3/4	
8	"	23,000	19,000	7-1/4	
9	"	47,000	65,000	6-3/4	
10	"	13,000	14,000	7-3/4	
11	"	49,000	67,000	7-1/4	
12	"	48,000	38,000	7-1/4	
1	Peas	41,000	46,000	7	
2	"	5,100	6,200	7-1/2	NC
3	"	6,300	6,900	7-1/2	
4	"	4,600	5,900	7-1/2	NC
5	"	77,000	100,000	7-3/4	
6	"	6,600	8,700	7-1/2	
7	"	1,500	1,500	8-3/4	
8	"	640	890	8-3/4	
9	"	1,500	1,400	8	
10	"	440	570	8-1/4	
11	"	650	660	8-1/4	
12	"	430	470	8-3/4	

NC = No Change
*Average of three determinations

TABLE 2—THE RESAZURIN REDUCTION TIME IN HOURS AND BACTERIA PER GRAM FOR FROZEN FRUIT PIES

Sample Number	Type of Sample	Plate Count Bacteria per Gram* Tryptone Glucose Extract Agar	Plate Count Bacteria per Gram* Trypticase Soy Agar	Resazurin Reduction Time in Hours	
1	Peach	320	340	8	NC
2	"	640	420	8	NC
3	"	350	610	8	NC
4	"	210	220	8	NC
1	Blueberry	250	400	7	NC
2	"	360	170	7	NC
3	"	310	300	7	NC
4	"	170	110	7	NC
5	"	220	150	7	NC
6	"	290	220	7	NC
1	Cherry	240	120	7-1/4	NC
2	"	600	160	7-1/4	NC
3	"	110	170	7-1/4	NC
4	"	240	270	7-1/4	NC
5	"	300	510	7-1/4	NC
6	"	390	520	7-1/4	NC

*Average of three determinations
NC = No Change

RESULTS AND DISCUSSION

The use of trypticase soy agar and tryptone glucose extract agar in determinations of total numbers of bacteria in the frozen vegetables, fruit pies, and in the inoculated chicken meat pies, has demonstrated little or no difference between these media. Both media can be used interchangeably and the use of tryptone glucose extract agar in the survey of frozen meat pies was a matter of the investigator's choice.

The resazurin reduction time in hours and bacteria per gram for frozen vegetables are recorded in Table 1. The examination of the results indicate that the highest plate count obtained on the samples examined was over one million bacteria per gram for corn. The resazurin reduction time for the corn sample was 4½ hours. Bean sample numbers 1, 3, and 6 had plate counts over 100,000 bacteria per gram and the resazurin reduction times were exceptionally long for such high total bacterial counts. The reduction times were seven hours or longer. This may have been due to a color interference caused by the blending of the bean samples.

Results of the examination of frozen fruit pies are tabulated in Table 2. These results indicate that the fruit pies contained a low total bacterial count; the resazurin reduction times were 7¼ hours or longer. Even after eight hours of incubation, no reduction of the resazurin had taken place in the peach pies.

The results of the survey of the retail frozen meat pies are tabulated in Table 3. The examination of the results shows the overall excellence of the bacteriological qualities of the frozen meat pies examined. There were no pies examined which had a total bacterial plate count over 100,000 bacteria per gram. All but one of the counts were under 60,000 bacteria per gram and the resazurin reduction times were five hours or longer. The exception, number 11 of the turkey pies, had a total count of 82,000 bacteria per gram and the resazurin reduction time was 5¼ hours. The limited survey of frozen meat pies did not show any sample with high bacteria counts. Therefore, in order to have frozen meat pies of high bacterial populations, a series of chicken meat pies were artifically produced with high counts of coagulase positive *Staphylococcus aureus*.

TABLE 3—THE RESAZURIN REDUCTION TIME IN HOURS AND BACTERIA PER GRAM FOR FROZEN MEAT PIES

Number	Producer	Type of Frozen Meat Pie	Plate Count Bacteria per Gram* Tryptone Glucose Extract Agar	Resazurin Reduction Time in Hours	
1	A	Turkey	9,800	7-½	
2	"	"	7,400	7-½	
3	"	"	6,100	7-¼	
4	B	Turkey	6,000	7-¾	
5	"	"	6,700	8	NC
6	"	"	1,700	7-¼	
7	C	Turkey	4,400	8	NC
8	"	"	2,400	8	NC
9	"	"	940	8	NC
10	D	Turkey	16,000	6	
11	"	"	7,700	5-¾	
12	"	"	16,000	6-¾	
13	E	Turkey	49,000	7-½	
14	"	"	13,000	8	NC
15	"	"	5,400	8	NC
16	F	Turkey	11,000	8	NC
17	"	"	17,000	7-¾	
18	"	"	18,000	8	
19	G	Turkey	6,300	6	
20	"	"	20,000	7-¼	
21	"	"	82,000	5-¼	
22	H	Turkey	350	8	NC
23	"	"	1,300	8	NC
24	"	"	1,100	8	NC
1	D	Tuna	130	8	NC
2	"	"	90	8	NC
3	"	"	70	8	NC
4	E	Tuna	1,000	8	NC
5	"	"	870	8	NC
6	"	"	5,700	8	NC
7	I	Tuna	50	8	NC
8	"	"	70	8	NC
9	"	"	180	8	NC
10	C	Tuna	70	8	NC
11	"	"	40	8	NC
12	"	"	60	8	NC
13	J	Tuna	530	8	NC
14	"	"	390	8	NC
15	"	"	990	8	NC
1	H	Beef	22,000	7-¼	
2	"	"	3,800	7	
3	"	"	2,800	7-½	
4	G	Beef	10,000	7-¼	
5	"	"	6,900	7	
6	"	"	3,700	7-¼	
1	B	Chicken	11,000	5-¾	
2	"	"	24,000	6	
3	"	"	16,000	6-¼	
4	A	Chicken	7,900	6-¼	
5	"	"	10,000	6-¼	
6	"	"	9,600	6-¼	
7	C	Chicken	9,100	6	NC
8	"	"	19,000	6	NC
9	"	"	56,000	6	NC
10	D	Chicken	33,000	6	NC
11	"	"	16,000	6	NC
12	"	"	7,500	6	NC
13	F	Chicken	16,000	7-¼	
14	"	"	34,000	7-¼	NC
15	"	"	31,000	7-¼	NC
16	H	Chicken	28,000	7-¼	
17	"	"	20,000	7-¼	
18	"	"	19,000	7-¼	

* Average of Three Determinations
NC=No Change

The results of this experiment are recorded in Table 4. The total bacterial counts varied from 640 million *S. aureus* per gram to 15 million organisms per gram and gave resazurin reduction times of 1-1/2 hours to 2-3/4 hours. This experiment demonstrated the effect of high populations of *S. aureus* in chicken meat pies on the resazurin reduction times.

The overall study demonstrated a correlation between the plate count and the resazurin reduction times in hours. However, it was noted that there was considerable overlapping of the number of organisms needed to reduce the resazurin in a given time. These variations are due to a great number of causes. One of the causes may be due to plant production where varieties of microorganisms are inadvertently introduced into the manufactured product. Each group or species of bacteria reacts differently according to its metabolic requirements and since not all bacteria respire at the same rate, varied resazurin reduction times are produced.

The color interference does give difficulty in deter-

TABLE 4—THE RESAZURIN REDUCTION TIME IN HOURS AND BACTERIA PER GRAM OF CHICKEN PIES INOCULATED WITH COAGULASE POSITIVE STAPHYLOCOCCUS AUREUS

Sample Number	Plate Count Bacteria per Gram Tryptose Glucose Extract Agar	Trypticase Soy Agar	Resazurin Reduction Time in Hours
1	640,000,000	610,000,000	1-½
2	560,000,000	490,000,000	1-½
3	630,000,000	650,000,000	1-½
4	270,000,000	290,000,000	1-½
5	27,000,000	25,000,000	2-½
6	22,000,000	21,000,000	2-¾
7	27,000,000	32,000,000	2-¼
8	15,000,000	17,000,000	2-¾

mining the end point of the resazurin reduction time. Observations from this study have indicated that samples of peas, beans, and beef pies have shown a color interference with the reduction test. This interference gives a longer resazurin reduction time than did other samples such as poultry and tuna pies.

The variations encountered in this study do not completely limit the usefulness of the resazurin reduction time test. Since the time needed for reduction of the resazurin generally decreases progressively with increased bacterial populations, its limitations in sensitivity should be recognized. The test can be used only in a broad classification of screening procedures in determining the number of bacteria per gram.

Straka and Stokes (7) recommended the use of three broad classes of numbers of bacteria present in a product as determined by the resazurin reduction time. Class one constituted a range of 0 to 100,000 bacteria per gram with a range of resazurin reduction time of five hours or longer. Class two constituted a range of 100,000 to 1,000,000 bacteria per gram with a range in reduction time of three to five hours. Class three included anything over 1,000,000 bacteria per gram with a reduction of resazurin in less than three hours. Our investigations showed similar results; however, we have used four classification groups rather than three. The results of both studies can be noted in Table 5.

Our past experience has demonstrated the need to have a rapid determination of total bacterial counts under 100,000 per gram. We were, therefore, interested in determining resazurin reduction times of bacterial numbers under 100,000 per gram.

The three classes of numbers of bacteria as recommended by Straka and Stokes (7) were found to be satisfactory for general bacteriological screening of some frozen foods.

SUMMARY

A total of 123 frozen food samples were examined bacteriologically by a resazurin reduction test as described by Stokes and Straka and for total numbers by using a plate count method. Examination of the results indicate that the total bacterial populations could be classified by total counts and resazurin could be classified by total counts and resazurin reduction times into several broad groups. The method and classification as recommended by Stokes and Straka in their investigations were found to be satisfactory and can be used with success for general bacteriological screening of some frozen foods. However, the results of our investigation could recognize four distinct classes. Class one which contained 0 to 10,000 bacteria per gram had a resazurin reduction time of eight hours or longer. Class two contained 10,000 to 100,000 bacteria per gram and showed a reduction time of six to eight hours. Class 3 which had 100,000 to 1,000,000 organisms per gram had a resazurin re-

TABLE 5—GROUP CLASSIFICATION OF BACTERIA PER GRAM AS DETERMINED BY THE RESAZURIN REDUCTION TIME

Straka & Stokes Recommendations*			Results of this Investigation		
Class	Reduction Time	Bacteria per gram	Class	Reduction Time	Bacteria per gram
1	5 hours or longer	Less than 100,000	1	8 hours or longer	0 - 10000
2	3 to 5 hours	100,000 to 1,000,000	2	6 to 8 hours	10,000 to 100,000
3	Less than 3 hours	More than 1,000,000	3	3 to 6 hours	100,000 to 1,000,000
			4	Less than 3 hours	1,000,000 to 500,000,000

* Robert P. Straka and J. L. Stokes (*See Reference No. 7*)
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duction time of three to six hours. Class 4 which contained over 1,000,000 to 500,000,000 or more bacteria per gram, exhibited a resazurin reduction time of three hours or less.

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