EFFECT OF TEMPERATURE AND pH ON GROWTH AND ENTEROTOXIN PRODUCTION BY *STAPHYLOCOCCUS AUREUS*[1]

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Abstract

Four strains of *Staphylococcus aureus* producing enterotoxin types A, B, C, and D were inoculated into buffered Brain Heart Infusion (BHI) broth at a concentration of 10^6 cells/ml and incubated with agitation at temperatures ranging from 7 to 50 C for intervals extending to 4 weeks. At 45 C, strains 265 (type A), 493 (type C), and 315 (type D) grew and produced enterotoxin, but there was a rapid decrease in viable cells and no enterotoxin produced by strain 243 (type B). In the range of 19 to 39 C, all strains grew and produced enterotoxin. At 13 C, strains 265, 243, and 493 grew but only strain 243 produced enterotoxin. Strain 315 did not grow at 13 C and none of the strains grew at 7 or 50 C. The population of *S. aureus* varied from 1.3×10^7 to 5.5×10^8 cells/ml when enterotoxin was first detected.

S. aureus strain 243 was inoculated at a concentration of 10^6 cells/ml into BHI broth with and without 0.2 M sodium phosphate buffer and incubated at 37 C for time intervals extending to 172 hr. The initial pH of the broth was adjusted to various values from 3.62 to 9.84. Growth occurred when the initial pH of the broth was 4.96 to 9.02 and a slight increase in population was noted at pH 4.76 and 9.40. Production of detectable amounts of enterotoxin was restricted to pH 5.15 to 9.02. Detectable enterotoxin was produced in 4 to 6 hr in non-buffered broth, but a minimum of 9 hr was required in buffered broth.

Ingestion of staphylococcal enterotoxin produced by *Staphylococcus aureus* may result in bacterial food poisoning. Since *S. aureus* may occur in low numbers in many foods *(1)*, it is important that food be handled to prevent multiplication of cells and production of enterotoxin. Several environmental factors such as water activity *(17)*, NaCl concentration *(10)*, atmosphere *(16)*, growth medium *(9)*, presence of other microorganisms *(12)*, temperature *(1, 4, 18)* and pH *(5, 13)* influence growth of staphylococci and may influence production of enterotoxin. Four immunologically different enterotoxins (A-D) have been purified and made available for study *(3)*. Enterotoxin A alone or in combinations with other enterotoxins is found in about 75% of the outbreaks of staphylococcal food poisoning *(3)*. Enterotoxin B is found in relatively few outbreaks but it has been studied more extensively than the other enterotoxins because relatively high concentrations are produced in laboratory media *(15)*. The objective of this investigation was to determine the effect of temperature on growth and enterotoxin production of four immunologically different strains of *S. aureus* and the effect of pH on production of enterotoxin B by *S. aureus* strain 243.

Materials and Methods

Media

Mannitol Salt Agar (MSA) was used to enumerate staphylococci and Brain Heart Infusion (BHI) broth was used to grow the cultures. BHI broth containing 0.2 M sodium phosphate buffer standardized to pH 7.0 after sterilization was used in all experiments involving effect of temperature in buffered broth. In experiments involving effect of pH, 0.2 M phosphate buffered BHI broth was prepared with the pH at various values between 3.62 and 9.84. When necessary, the pH of both buffered and non-buffered broth was adjusted with 6 N HCL or NaOH.

Cultures

Four strains of *S. aureus*, 265, 243, 493, and 315, which produce enterotoxins A, B, C, and D, respectively, were obtained from the late Dr. E. P. Casman of the Food and Drug Administration Laboratory in Washington, D. C. Each strain produces only one enterotoxin.

Culture propagation

The cultures were grown in BHI broth at 37 C and transferred daily for at least 3 days before use. Cultures in the stationary phase, incubated 12-24 hr, were used for inoculation. Sufficient inoculum was added to yield a population of approximately 10^6 cells/ml in 300 ml of BHI broth in 1-liter screw-cap erlenmeyer flasks. The flasks were incubated for time intervals extending to 4 weeks at selected temperatures in a rotary shaker operated at 175 rpm. Samples used to determine the effect of temperature were thermostatically controlled at 7, 13, 19, 26, 32, 37, 39, 45, and 50 C by the heating unit on the shaker and/or placing the shaker in a refrigerated room. The samples used to determine effect of pH were incubated at 37 C under the same physical conditions indicated above. At selected intervals a portion of the broth was removed aseptically to determine population, pH, and enterotoxin.

Determination of population

The broth samples were diluted as necessary in 0.01 M phosphate buffer. The populations of *S. aureus* were determined by the spread plate technique using pre-poured MSA plates prepared no more than 24 hr before use. Plates were incubated at 37 C for 48 hr and all colonies were counted.

Determination of pH

A Corning single electrode on a Beckman pH Meter (Model 1019) was used for all pH determinations. At each sampling period, approximately 10 ml of broth was placed in a 50 ml

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TABLE 1. POPULATION, pH, AND MINIMUM INCUBATION TIME ASSOCIATED WITH PRODUCTION OF MEASURABLE AMOUNTS OF ENTEROTOXIN FORMED BY FOUR STRAINS OF *S. aureus* WHEN INCUBATED WITH AGITATION IN BHI BROTH BUFFERED AT pH 7.0 WITH 0.2 M PHOSPHATE AT THE TEMPERATURES INDICATED

Incubation	*S. aureus* 265 (A Toxin)			*S. aureus* 243 (B Toxin)			*S. aureus* 493 (C Toxin)			*S. aureus* 315 (D Toxin)		
temperature (C)	Time (hr)	pH	Count* $\times 10^6$	Time (hr)	pH	Count* $\times 10^6$	Time (hr)	pH	Count* $\times 10^6$	Time (hr)	pH	Count* $\times 10^6$
7	(a)	6.92	<.0001	(a)	6.94	<.01	(a)	6.91	<.001	(a)	6.99	<.01
13	(b)	6.79	3	158	6.77	32	(b)	6.78	13	(b)	6.81	0.1
19	98	6.96	16	78	6.94	80	78	6.91	130	98	6.94	320
26	16	6.66	360	16	6.66	190	14	6.62	450	14	6.66	110
32	22	6.85	550	13	6.82	50	11	6.83	69	13	6.86	50
39	18	6.82	440	10	6.96	20	6	6.98	13	16	6.79	80
45	78	6.83	20	(c)	6.91	<.0001	38	6.78	120	38	6.82	15
50	(d)	6.90	<.0001	(d)	6.91	<.0001	(d)	6.90	<.0001	(d)	6.87	<.0001

*Counts were made on MSA. The initial inoculation was approximately 10^6/ml.
(a) No toxin detected after incubation for 673 hr.
(b) No toxin detected after incubation for 338 hr.
(c) No toxin detected after incubation for 122 hr.
(d) No toxin detected after incubation for 73 hr.

tube and centrifuged at 12,000 $\times$ *g* for 15 min at 2 C. The pellet was discarded. A small portion of the supernatant fluid was used for the pH determination, and the remainder of the fluid was stored at −30 C for enterotoxin assay at a later time.

Enterotoxin determination

The microslide gel-diffusion method *(2)* was used to determine the presence of staphylococcal enterotoxin. Purified staphylococcal enterotoxins A, B, C, and D, and corresponding antitoxins used as standards were obtained from Dr. Casman.

RESULTS AND DISCUSSION

Effect of temperature

Samples of broth taken immediately after inoculation did not contain detectable enterotoxin. All four strains of *S. aureus* grew over a temperature range of 13 to 45 C except strain 243 which failed to grow at 45 C and strain 315 which failed to grow at 13 C (Table 1). None of the strains grew at 7 or 50 C. When enterotoxin was first detected the population of *S. aureus* in the BHI broth varied from 1.3×10^7 to 5.5×10^8 cells/ml and the pH of the broth varied from 6.62 to 6.98. The temperature at which enterotoxin was produced was restricted to the range of 19 to 45 C, with the exception of *S. aureus* 243 which produced enterotoxin B over the range of 13 to 39 C. The incubation time required for enterotoxin production varied from 6 to 22 hr in the temperature range of 26 to 39 C and the time increased as the temperature varied above or below this range. Strains which produced enterotoxin at the incubation temperatures used did so within 98 hr, except strain 243 which produced enterotoxin B in 158 hr at 13 C.

Data in Table 1 show little difference among the four strains of *S. aureus* in the pH, population, or time required to produce measurable toxin during incubation at 26 C. At temperatures above and below 26 C the variation among strains in these categories was more apparent. There was a particularly abrupt increase in time required to produce toxin at 19 C. Since none of the strains grew at 50 C and only three of the four strains produced enterotoxin at 45 C, it is probable that 45 C is near the maximum temperature for toxin production. Likewise 13 C is near the minimum temperature for toxin production, since *S. aureus* 243 produced toxin at 13 C after 158 hr but the other strains did not produce enterotoxin during incubation for 2 wk at 13 C.

The incubation times and *S. aureus* populations associated with detectable amounts of enterotoxins A, B, C, and D in buffered BHI broth (Table 1) are similar to the times and populations reported by Donnelly et al. *(4)* for production of enterotoxin A in milk at 25-35 C. The minimum concentration of enterotoxin which can be detected by the microslide gel-diffusion technique is about 1 μg/ml *(2)*. Since about 20 μg of enterotoxin B, and considerably less enterotoxin A, may be toxic for man *(14)*, an enterotoxin concentration of 1 μg/g in an average serving of food is potentially hazardous. Data reported herein indicate that it is possible to predict the incubation time required at a particular temperature for cells of the four strains of *S. aureus* to produce measurable amounts of enterotoxin in buffered BHI broth, but it is not possible to predict the presence of enterotoxin in a particular sample by the population of *S. aureus* in that sample.

Genigeorgis et al. *(6)* used five strains of *S. aureus*, four of which were different from those used in the work reported here, and found statistically significant differences in growth rates among the strains. Under the conditions used in this investigation, no distinguishing differences were apparent among the four strains of *S. aureus*, except that each strain produces a different enterotoxin. Differences in growth rates

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TABLE 2. POPULATION, PH, AND MINIMUM INCUBATION TIME ASSOCIATED WITH PRODUCTION OF DETECTABLE AMOUNTS OF ENTEROTOXIN B BY S. *aureus* STRAIN 243 GROWN AT 37 C WITH AGITATION IN BHI BROTH CONTAINING 0.2 M PHOSPHATE BUFFER AT VARIOUS pH VALUES

Initial pH	Incubation time (hr)	Terminal pH	Terminal count on MSA[1] $\times 10^6$
3.62	ND[2]	3.61	<0.1
4.48	ND	4.50	<0.1
4.76	ND	4.92	1.2
4.96	ND	5.05	4
5.15	154	5.66	350
5.38	32	5.42	57
5.48	124	6.08	220
5.56	26	5.58	90
6.44	9	6.48	32
6.45	9	6.44	30
6.53	14	6.42	720
6.79	23	6.78	1700
6.94	19	6.79	570
7.03	12	6.97	25
7.20	23	7.17	1600
7.72	52	7.71	7
7.73	48	7.61	46
7.96	47	7.80	3000
7.97	58	7.75	800
8.14	56	7.89	1500
8.40	40	7.86	1200
8.61	52	7.71	400
8.67	36	8.02	680
9.02	24	8.89	3
9.40	ND		1.5
9.84	ND		<0.1

[1]Initial counts on MSA were approximately 10^6 cells/ml.
[2]ND = No detectable toxin produced within 172 hr.

of S. *aureus* exist but these differences do not appear to be related to differences in enterotoxin type.

Effect of pH

Detectable amounts of enterotoxin B were produced in phosphate buffered BHI broth with an initial pH of 5.15 to 9.02 and incubation times ranging from 9 hr at pH 6.44 to 154 hr at pH 5.15 (Table 2). In non-buffered BHI broth the incubation times required for production of detectable amounts of enterotoxin varied from 4 to 6 hr at pH 6.14 to 7.95 (Table 3).

Growth of S. *aureus* strain 243 occurred over a greater range of pH than toxin production. Gennison and Wadsworth *(8)* have calculated statistically that there is a 68% probability that plate count values determined experimentally will be within 6.6% of actual values. Therefore we must recognize the possibility of experimental error in the small increases in population indicated with *(a)* strain 265 at 13 C (Table 1); *(b)* strain 243 in buffered broth at pH 4.76, 9.02, and 9.40 (Table 2); and *(c)* strain 243 in non-buffered broth at pH 5.02 and 9.08. In buffered BHI broth, the incubation time required for production of a detectable quantity of enterotoxin varied considerably and was dependent upon the initial pH of the growth medium (Table 2). Less time was required for production of detectable toxin at pH 6.44 to 7.20 than at pH above and below these values.

The incubation time required to produce a detectable quantity of enterotoxin in non-buffered BHI broth varied little within the pH range in which toxin production occurred (Table 3). When detectable amounts of enterotoxin first appeared, the staphylococcal populations varied from 3.0×10^6 to 3.0×10^9 cells/ml in buffered BHI broth (Table 2) and from 7.5×10^6 to 1.2×10^9 cells/ml in non-buffered BHI broth (Table 3).

Morse et al. *(11)* and Peterson et al. *(13)* observed that the pH of the medium changes while the S. *aureus* culture is growing. If the pH of the medium is low there is a tendency for the pH to increase and if the pH is high there is a tendency for it to decrease. An increase in pH of the medium, an increase in offensive odor, and a darker colored pellet usually occurred in samples which produced toxin in the shortest incubation times.

Since the incubation time required to produce a detectable quantity of enterotoxin was less in non-buffered than in buffered BHI broth (Tables 2 and 3), it is reasonable to conclude that the phosphate buffer affected enterotoxin production. The 0.2 M sodium phosphate buffer delayed, but did not prevent, change in the pH of the growth medium. Phosphate has a high buffering capacity at pH 8 and in the range of 7.7 to 8.6 the incubation times required before enterotoxin could be detected in buffered BHI broth were considerably longer than the incubation times at higher or lower pH values. The differences in incubation times needed for measurable amounts of enterotoxin to be produced in BHI broth buffered at different pH values as well as the diff-

TABLE 3. POPULATION, PH, AND MINIMUM INCUBATION TIME ASSOCIATED WITH PRODUCTION OF DETECTABLE AMOUNTS OF ENTEROTOXIN B BY S. *aureus* STRAIN 243 GROWN AT 37 C WITH AGITATION IN NON-BUFFERED BHI BROTH AT VARIOUS INITIAL pH VALUES

Initial pH	Incubation time (hr)	Terminal pH	Terminal count on MSA[1] $\times 10^6$
5.02	ND[2]	5.00	2
6.14	6	5.34	500
6.62	6	5.70	750
7.13	4	6.78	7.5
7.55	6	6.62	1200
7.95	4	7.63	76
9.08	ND	8.90	3
9.86	ND	9.85	<0.1

[1]Initial counts on MSA were approximately 10^6 cells/ml.
[2]ND = No detectable toxin produced within 172 hr.

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erence between incubation times required for enterotoxin production in buffered and non-buffered BHI broth may be caused by stabilization of the pH of the medium.

In both buffered and non-buffered BHI broth, growth of S. *aureus* occurred over a wider range of pH than did enterotoxin production. It is theoretically possible, but frequently not feasible, to adjust the pH of food above or below the pH range for enterotoxin production. Few foods would be accepted by the consumer if the pH were above 9; although a number of acceptable foods naturally have a pH below 5. Genigeorgis et al. *(5)* reported that NaCl in the growth medium narrows the pH range in which enterotoxin production occurs. Genigeorgis et al. *(7)* also showed that growth of S. *aureus* occurred in meat in which they were unable to detect enterotoxin production. According to Reiser and Weiss *(15)*, the growth medium is known to affect the total amount of enterotoxin produced. The nature of the growth medium would also affect the incubation time needed for a measurable amount of enterotoxin to be produced. The pH ranges for growth and enterotoxin production by S. *aureus* are probably similar in broth and food. Assuming this is true, the data suggest that foods within a pH range of 5.1 to 9.0 which contain staphylococci may be considered to have a potential for staphylococcal food poisoning if other environmental conditions are favorable.

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