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Staphylococcal Enterotoxin B and Nuclease Production Under Controlled Dissolved Oxygen Conditions

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Enterotoxin B, nuclease, and total exoprotein production by *Staphylococcus aureus* strain S-6 was studied in a 0.5-liter fermentor system. While these extracellular products were elaborated over a wide range of aeration rates, maximal production occurred within the very narrow range of 125 to 150 cm^3 of air per min. The levels attained at the optimal aeration rate were not increased by maintaining a constant pH, although yield of enterotoxin:cell mass was highest at a constant pH of 7.0. During the growth cycle of the cultures, when aeration rate alone or aeration rate and pH were held constant, the dissolved oxygen (DO) levels, initially set at 100% of saturation, decreased to 5 to 10% 4 to 5 h after inoculation. The oxygen demand of the culture then maintained this level for an additional 4 to 6 h. This interval of low DO was characterized by maximal growth and exoprotein production. When the DO was controlled at a constant value throughout growth (by increasing or decreasing the airflow rate as appropriate), the culture demonstrated different optima for maximal growth and exoprotein production. A constant DO of 100% stimulated growth to extremely high densities, but the accumulation of toxin and nuclease was not observed. On the other hand, maintaining constant DO levels at 50 or 10% raised exoprotein levels higher than those achieved in a culture grown at the optimal aeration rate. Compared to the optimal aeration rate culture, the 10% DO culture yielded 20% more nuclease, 25% more toxin, and 40 to 50% more total exoprotein. These results indicate that it is the DO and not the aeration rate, per se, that is influential in controlling growth, toxin, nuclease, and total exoprotein production.

The production of staphylococcal enterotoxin is enhanced by vigorously shaking enterotoxigenic strains during growth (9, 11, 13, 14, 17, 19). Excessive shaking, while promoting growth, can actually decrease enterotoxin B secretion, however (4). A study on the staphylococcal nuclease has indicated that the production of this enzyme is also affected by aeration conditions during growth (6). Unlike the case of enterotoxin, the final nuclease yields were directly proportional to the cultural densities which were reduced 75% by anaerobiosis. The validity of this latter report has been questioned due to the results of a more recent investigation which indicated that the enzyme was not produced by cultures incubated anaerobically, although growth occurred (5).

Shake-flask experiments have yielded substantial amounts of information on the effects of aeration and media constituents on the production of nuclease, enterotoxin, and other exoproteins. This method, however, precludes the accrual of data on the effects of pH, airflow rates, and dissolved oxygen (DO) on growth and exoprotein production. Consequently, fermentor systems, which permit evaluation of these cultural conditions, are being utilized in staphylococcal research (1, 2, 10, 15).

To obtain maximal yields of enterotoxin B in a 50-liter fermentor, Metzger et al. (15) supplemented casein hydrolysate media with 0.2% dextrose and maintained the culture at a constant pH of 7.0 and a constant aeration rate of 10 liters per min.

A more extensive study indicated that whereas enterotoxin B production by S-6 in fermentors was optimized by pH control at 6.5 and 7.3, even higher titers were obtained in baffled shake-flasks (10). This same study showed that the conditions which were optimal for toxin production were not necessarily optimal for the synthesis and release of the other exoproteins. Nuclease production by fermentor cultures of S-6 grown without pH control

yielded levels 2.5 times higher than the levels achieved in baffled shake-flasks. It was also noted that a decrease in the gas flow rate from 2.0 to 0.2 liter per min caused a fourfold increase in the levels of enterotoxin C (strain 361) produced. This was the only exoprotein whose production was examined at a reduced aeration rate.

Our initial fermentor experiments indicated that an optimal airflow rate existed for enterotoxin B, nuclease, and exoprotein production, and that pH control at 7.0 did not increase the final yields of these products. Of far greater significance, we noted that the DO level of the cultures (aerated at a constant rate) varied between the extremes of 0 and 100% during growth; these changes most likely were caused by the metabolism of the growing culture. Subsequent experiments conducted under conditions of controlled DO revealed that these variations represented some type of oxygen conditioning, which apparently were necessary for toxin and nuclease production by strain S-6.

The results presented herein indicate that controlling the aeration rate, in effect, established the DO level in growing cultures, and that this is the parameter determining maximal production of staphylococcal exoproteins.

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MATERIALS AND METHODS

Organism. *Staphylococcus aureus* S-6 was obtained from M. S. Bergdoll (Food Research Institute, Madison, Wisc.) and maintained in the dry state on porcelain beads stored over Drierite (Hammond Co.) at 4 C.

Medium. The medium used for growth and toxin production was that described by Rosenwald and Lincoln (20): 4% N-Z amine type A (Sheffield), 0.4% yeast extract (Difco), and 0.1% K_2HPO_4 in distilled water, pH 6.8 to 7.0.

Cultural methods. The fermentor system consisted of a 1-liter Berzelius beaker containing 500 ml of medium to which was added 0.1 ml of antifoam A (Dow Corning Co., Midland, Mich.). Air was introduced into the media via glass tubing which was inserted through the headplate. The tubing in the medium terminated in four glass spargers arranged in an "X" configuration whose plane was parallel to the bottom of the beaker. The spargers were approximately 2 cm above the bottom of the beaker to accommodate a magnetic stirring bar. The gassing line contained a bacteriological filter (Balston, Lexington, Mass.). To prevent the release of infectious aerosols from the fermentor, the gas exit port was fitted with a bacteriological filter identical to the one used in the incoming air supply line. An oxygen controller (New Brunswich Scientific) controlled the aeration rates during experiments conducted at constant DO levels. A flowmeter (Fischer and Porter, Warminster, Pa.) was placed between the controller and the air filter.

The headplate was also fitted with a combination pH electrode (no. 808, Markson Science, Del Mar, Calif.) and a polaragraphic type oxygen electrode (Instrumentation Labs, Lexington, Mass.) placed within the medium to the level of the spargers. Probe data were accrued with a millivolt meter, automatic switch, digital printout system (Orion Research Corp. Cambridge, Mass.).

Automatic pH control (±0.1 U) was maintained by a titrator (Coleman model 32) modified for the Orion data recording system.

The fermentor, with media, headplate, oxygen probe, and the bacteriological filters in place, was sterilized in an autoclave at 121 C for 20 min. (The pH probe was sterilized by immersion in 3% H_2O_2 for 30 min. Before its insertion into the fermentor, the probe was rinsed with sterile distilled water.) After cooling, the fermentor was connected to the recording-control system, the media was equilibrated at 37 C, and a 1% inoculum from a 16 to 18-h shake-flask culture was introduced into the medium.

An incubation temperature of 37 ± 0.5 C was maintained by placing the fermentor in a stirred water bath. The culture was agitated at a constant rate of 600 to 625 rpm by a magnetic stirrer.

At specified intervals a 5-ml sample of the culture was removed. This was used to determine cultural turbidity and levels of extracellular proteins.

Assay procedures. Bacterial density was measured with a Klett-Summerson colorimeter with a no. 66 filter. When necessary, samples were diluted 1/10 in distilled water to obtain readings ranging from 20 to 250 Klett units.

Supernatant fluids were assayed for enterotoxin by the gel-diffusion technique of Weirether et al. (21). The assay tubes were incubated at 30 C for 21 h. Purified enterotoxin B, for the standard curve, was supplied by S. J. Silverman, Ft. Detrick, Md. Rabbit antiserum was purchased from Makor Chemicals, Jerusalem, Israel.

Nuclease activity was assayed by a method described by Chesbro and Auborn (3).

Extracellular protein was precipitated from the supernatant fluid with cold 5% trichloroacetic acid. The precipitate, harvested by centrifugation for 10 min at 10,000 × *g*, was washed once with 5% trichloroacetic acid and then resuspended in 0.05 N NaOH. The protein in this alkaline solution was determined by the method of Lowry et al. (12) with bovine serum albumin as a standard.

RESULTS

The optimal aeration rate for enterotoxin B, nuclease, and total exoprotein production. The growth of fermentor cultures of *S. aureus* at a number of constant aeration rates indicated that the production of the extracellular constit-

uents was optimal within the narrow range of airflow rates from 125 to 150 cm³ of air per min. Figure 1 depicts the yields of toxin, nuclease, and total exoprotein obtained at and near this optimum. The levels achieved at lower (50 cm³) or higher (500 to 1,000 cm³) airflow rates were less than 10% of the optimal yields. Final cultural turbidities, which were relatively constant in the 125 to 200 cm³ of air per min range, were approximately doubled at higher flow rates.

Analysis of the cultural productivity, defined as the increment of measurable toxin or other exoprotein divided by the average turbidity (Klett units) of an interval in the growth cycle, showed that the intervals characterized by peak toxin productivity did not necessarily coincide with peak nuclease- or exoprotein-producing intervals (Table 1). Further, although the 125 to 150 cm³ per min aeration rate yielded the highest levels of toxin, nuclease, and exoprotein (Fig. 1), higher productivity values were obtained during certain intervals at the other two rates. Plots of toxin, nuclease, and extracellular protein production against increasing cultural turbidity at different aeration rates are shown in Fig. 2. The exoproducts were, in general, secreted exponentially until maximal turbidity was achieved, and secretion continued during the subsequent interval of decreasing turbidity.

Growth and protein production patterns of a culture incubated at the optimal aeration rate are shown in Fig. 3. The course of two cultural parameters, DO and pH, is also depicted. (With minor variations, similar DO and pH patterns of the 75 to 90 cm³ of air per min and 175 to 200 cm³ of air per min cultures were obtained.) It can be seen that both of these conditions varied considerably during growth. The DO levels, initially at 100% saturation, were in the 5 to 15% range 4 to 5 h after inoculation. As the culture entered the stationary phase, the DO returned to the initial high values. After an initial decrease, the pH continued to increase to a maximum at 12 h. Toxin, nuclease, and exoprotein secretion continued, although at varying rates throughout growth. Since the variation of these parameters was observed consistently, the effects of controlling them at specific values were investigated.

The optimal pH for toxin, nuclease, and exoprotein production. To determine the effects of controlled pH, several cultures, grown at the optimal aeration rate, were held at constant

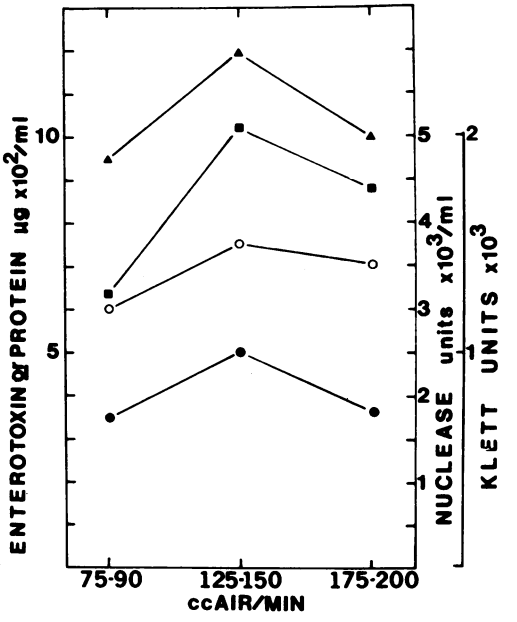

FIG. 1. *Turbidity and extracellular protein levels achieved by S. aureus S-6 grown at various constant airflow rates. Symbols:* ○, *turbidity (Klett units);* ●, *enterotoxin B;* ■, *nuclease;* ▲, *total exoprotein.*

TABLE 1. *Productivity[a] of S-6 grown at the indicated constant aeration rate*

Interval (h)	75 to 90 cm³ of air per min			125 to 150 cm³ of air per min			175 to 200 cm³ of air per min		
	Toxin	Nuclease	Exoprotein	Toxin	Nuclease	Exoprotein	Toxin	Nuclease	Exoprotein
2 to 4	2.83	38.94		3.38	66.18		2.62	54.76	16.67
4 to 5	5.02	90.69	16.19	7.58[b]	59.01	5.59	4.88	95.24	2.38
5 to 6	3.91	28.40	7.10				5.18	90.91	14.55
6 to 7	3.06	57.14	14.29	4.67[c]	42.22	10.67	7.00	37.35	5.45
7 to 8	4.72	22.64	6.60				10.18	34.91	11.64
8 to 13	2.55	18.04	4.94[d]	1.83	22.12	4.23	0.23	13.01	3.48
13 to 24	0.32	2.43	1.30	0.97	7.86	2.20	0.00	3.31	2.23

[a] Productivity is defined as the increment of measurable toxin, nuclease, or exoprotein divided by the average turbidity (Klett units) of an interval in the growth cycle.

[b] Represents 4- to 6-h interval.

[c] Represents 6- to 8-h interval.

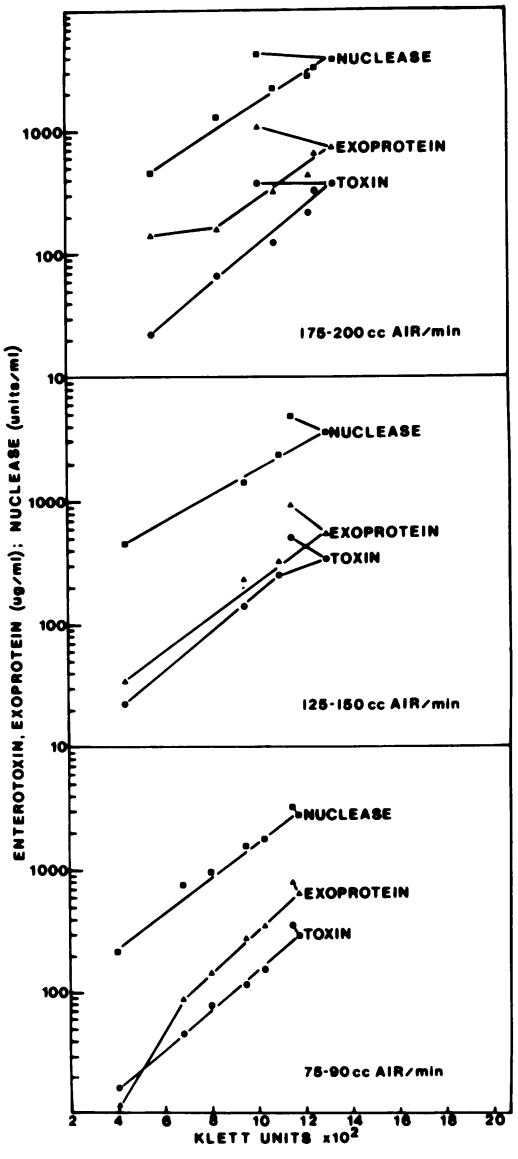

FIG. 2. *Extracellular protein production plotted against the increasing turbidity of cultures grown at the indicated constant airflow rates. Symbols: ●, enterotoxin B; ■, nuclease; ▲, total exoprotein.*

pH values from 6.0 to 7.5 (Fig. 4). A constant pH of 7.0 gave yields comparable to an uncontrolled pH culture, except the final cultural density which was lower. Examination of the productivity of the pH 6.5, 7.0, and 7.5 cultures (Table 2) indicated that constant pH 7.0 yielded cells that displayed higher productivities for toxin, nuclease, and total exoproteins than those observed in the other constant pH or constant aeration-rate fermentors. In particular, the productivity for toxin with a value of 10.75 was the highest toxin value observed in any of the experiments described in this study.

Under conditions of controlled pH, secretion did not occur at the same exponential rate throughout growth as in the cultures grown without pH control (Fig. 5). All of the exoprotein, at any of the constant pH values studied, were secreted at at least two rates. At PH 7.0 and 7.5, the secretion rate for all of the assayed products changed at the same point. At pH 6.5, however, the culture and/or conditions of incubation appeared to be exerting individual control over the secretion of each extracellular product, as none of the proteins changed secretion rates at the same time. In addition to exhibiting two secretion rates, constant pH cultures did not lyse following the attainment of maximal turbidity.

Incubation with pH control gave DO patterns similar to those of the cultures incubated without pH control (Fig. 3). Thus, the effects of controlling the DO during growth and extracellular metabolite production were determined.

Optimal DO levels for growth and for toxin, nuclease, and total exoprotein production. The effects of growing cultures at three specific DO levels is summarized in Fig. 6. At 100% DO, the culture exhibited the most rapid and profuse growth observed in any of the experiments described here. However, the supernatant fluid was void of toxin and nuclease throughout the experiment. The final level of total extracellular protein was only one-fifth of an uncontrolled DO culture.

An additional experiment was conducted to determine if the absence of enterotoxin B and nuclease was due to the actual lack of secretion of these proteins or to their secretion and subsequent denaturation due to the high aeration rate. A fermentor culture was grown at a constant aeration rate (125 to 150 cm^3 of air per min) from zero time through 4 h. This interval was characterized by decreasing DO levels (Fig. 3). At 4 h, the culture was then subjected to a constant DO level of 100% for the remainder of the experiment. To control the DO at this level, the aeration rate increased and then decreased during this interval. The final titers of toxin and nuclease were comparable to a culture grown at a constant aeration rate throughout the experiment. Furthermore, if protein denaturation did occur under the influence of 100% DO, then there would have been a decrease in the amounts of toxin and nuclease in cultures grown at constant aeration rates when the DO level of these cultures returned to 100% during the late stationary phase of growth.

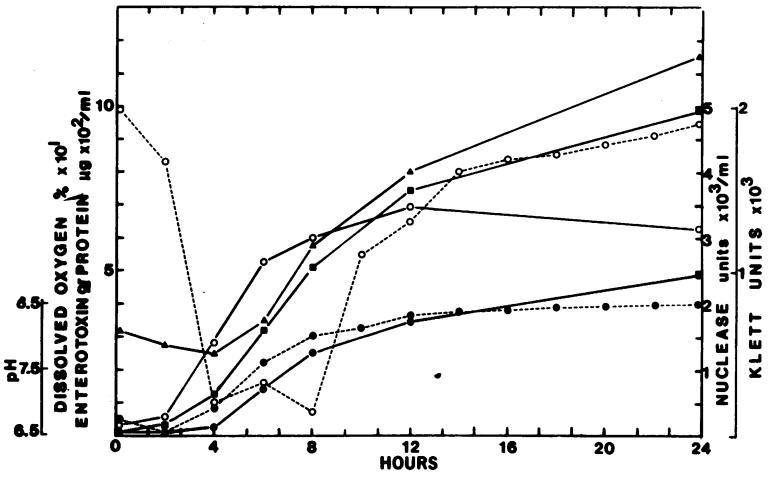

Fig. 3. *Growth, cultural conditions, and extracellular protein production of S. aureus S-6 grown at the airflow rate of 125 to 150 cm³/min. Symbols:* O——O, *turbidity (Klett units);* ●——●, *enterotoxin B;* ■——■, *nuclease;* ▲——▲, *total exoprotein;* O---O, *DO;* ●---●, *pH.*

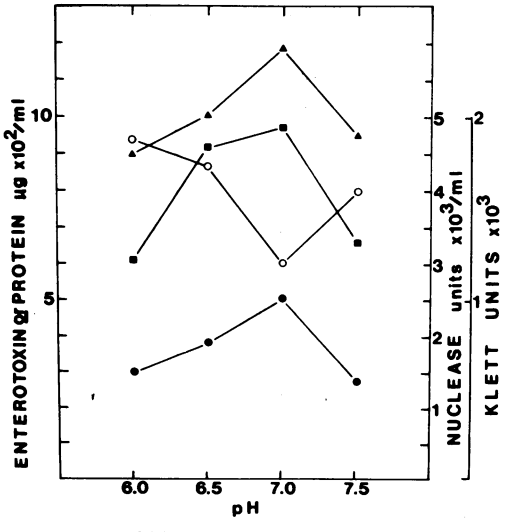

FIG. 4. *Turbidity and extracellular protein levels achieved by S. aureus grown at constant pH. For symbol explanation see Fig. 1.*

Cultures grown at the 50 and 10% DO levels yielded lower cultural densities but produced higher levels of toxin, nuclease, and total exoprotein. At 50% DO, the final yield of 650 µg/ml was 30% higher than that realized in the optimal pH or optimal aeration-rate cultures. Likewise, the yields of both nuclease and total exoprotein were increased by 25%.

Growth at a constant 10% DO level proceeded slowly; maximal turbidity was not reached until 36 h after inoculation. Under these conditions, however, the organisms produced the highest levels of toxin (725 µg/ml), nuclease (6,000 U/ml), and exoprotein (1,900 µg/ml) achieved in any of the experiments described in this report.

It was noted in Table 3 that control of the DO at 50% yielded a culture whose nuclease and total exoprotein values were the highest of the controlled DO, constant pH, or constant aeration-rate cultures. There were distinct differences among the controlled DO patterns during growth as shown in Fig. 7. At 50% DO, growth and production patterns resembled those of the constant pH experiments, i.e., two secretion rates and the absence of decreasing turbidity at the end of growth. The 10% DO culture, unlike the 50% DO culture, was not stable after it had attained its maximal turbidity, so that a 50% decrease occurred in 12 h. This characteristic, along with the constant secretion rate (in terms of Klett units) exhibited during growth, yielded patterns which resembled those of the cultures grown at constant aeration rates.

Manual cycling of the oxygen concentration in the milieu of a growing culture between high and low values further indicated that the DO concentration was most influential in determining the secretion of toxin, nuclease, and total exoproteins. By introducing the inoculum into a 0% DO media, the onset of toxin production, relative to growth, was delayed considerably (Fig. 8). In the fermentations previously discussed, a culture with a turbidity of 1,100 to 1,200 Klett units usually had a toxin titer in excess of 100 µg/ml. In the oxygen-cycled culture, there were only 25 µg/ml at this cultural density. Nuclease production, however, was

TABLE 2. *Productivity[a] of S-6 grown at the indicated constant pH*

Interval (h)	pH 6.5			pH 7.0			pH 7.5		
	Toxin	Nuclease	Exoprotein	Toxin	Nuclease	Exoprotein	Toxin	Nuclease	Exoprotein
2 to 4	2.86	21.14		3.86	81.82		0.98	13.99	1.40
4 to 5	2.65	58.28	7.95	9.76	123.81		3.00	62.54	7.82
5 to 6	3.76	64.52	5.38	10.75	98.11	15.09	6.28	65.70	14.49
6 to 7	9.33	48.69	7.11	5.08	84.75	27.12	2.53	33.76	1.27
7 to 8	8.61	33.71	1.50	4.17	35.27	7.36	6.39	72.18	3.76
8 to 12	1.28	31.41	4.49	0.87	6.93	1.89	0.91	10.20	6.69
12 to 24	0.09	2.56	1.95	0.48	−0.81	1.91	0.10	0.64	1.86

[a] Productivity is defined as the increment of measurable toxin, nuclease, or exoprotein divided by the average turbidity (Klett units) of an interval in the growth cycle.

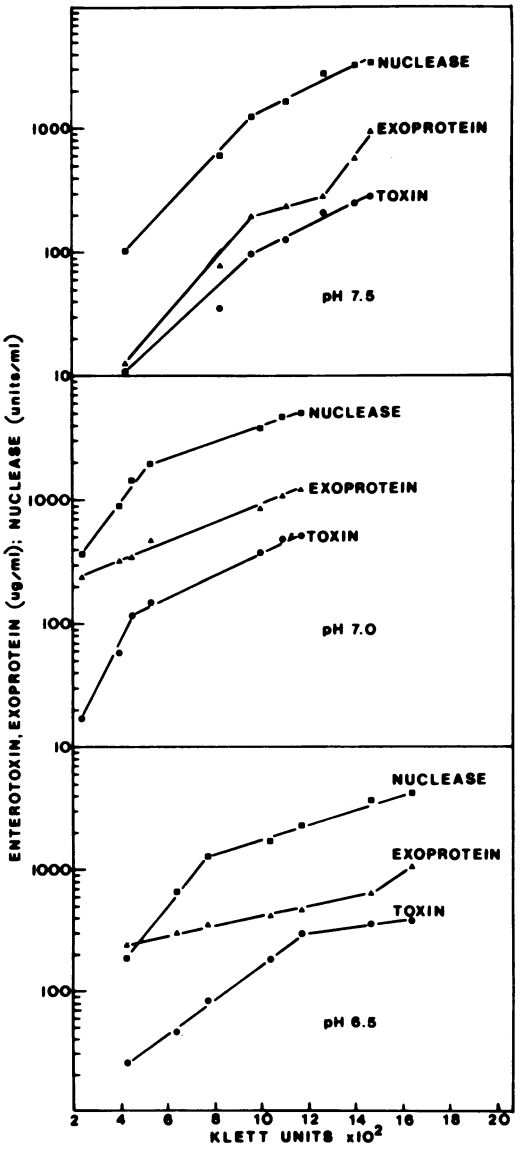

FIG. 5. *Extracellular protein production plotted against the increasing turbidity of cultures grown at the indicated pH. For symbol explanation see Fig. 2.*

enhanced by the low DO environment as maximal productivity was displayed during the initial low DO interval (Table 4). Likewise, the productivity of the total exoproteins was also maximal during this interval. Examination of the production of the extracellular constituents during each oxygen interval (Table 4) indicated that, at least during log phase growth, the nuclease was preferentially synthesized during periods of low DO, whereas the toxin and total exoproteins were produced during the high DO intervals. Most significantly, though, this apparent oxygen conditioning yielded a culture which, in stationary phase, produced substantial increases in toxin and nuclease in response to an interval of high DO which had been preceded by a period of low DO. This occurrence implied that oxygen appeared to strongly influence some function which regulated toxin, nuclease, and possibly overall extracellular protein production.

DISCUSSION

The production of enterotoxin B, nuclease, and the total exoproteins by fermentor cultures of *S. aureus* S-6 was influenced by several environmental factors, the most important and influential of which appeared to be DO. A constant aeration rate of 125 to 150 cm^3 of air per min appeared to be optimal for the production of toxin, nuclease, and total exoprotein.

This rate was quite similar to the 10 liters per min aeration rate which Metzger et al. (15) utilized for toxin production in the 50-liter fermentor. Others have also indicated that this aeration rate was the apparent optimum (2). For the sake of comparison, 1-liter shake-flasks with indentions were also utilized in this study (2). The levels of exoproteins attained were not

significantly different from those achieved in the aerated fermentor.

Contrary to these data, the recent report of Jarvis et al. (10) indicated that, in most instances, higher levels of enterotoxin and the other exoproteins were produced in well-aerated baffled shake-flasks, as compared to an aerated fermentor. A notable exception was nuclease whose fermentor levels were 2.5 times the levels achieved in the shake-flasks. This lack of correlation between the fermentor and shake-flasks, which these investigators noted, may be due to the apparant high gas flow rate established in their fermentor. The authors indicated that 2.0 liters per min was sparged into 2.0 liters of media.

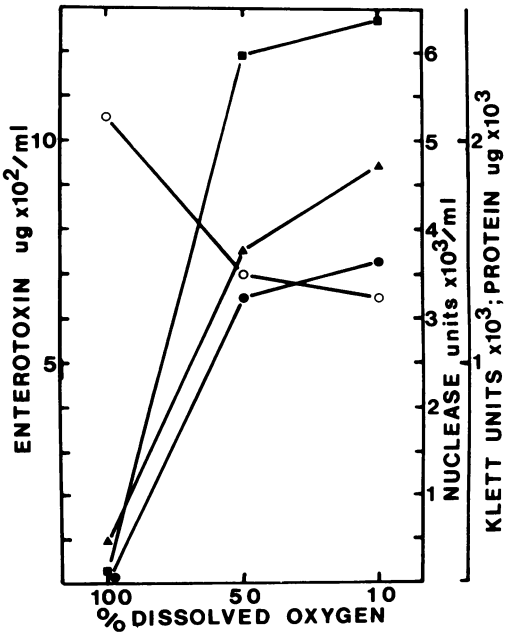

FIG. 6. *Turbidity and extracellular protein levels achieved by S. aureus S-6 grown at the indicated constant DO levels. For symbol explanation see Fig. 1.*

Our experience has indicated that a flow rate such as this caused a substantial decrease in the final yields of exoproteins. Maintaining a constant pH during the growth of S-6 at the optimal aeration rate did not increase the final yields of enterotoxin B, nuclease, or total exoproteins. However, we found that a culture grown at a constant pH of 7.0 did give results comparable to a culture grown at the optimal aeration rate without pH control. Metzger et al. (15) reported that the growth of their organism (a mutant of S-6) at pH 7.0, in the presence of 0.2% dextrose, yielded a 30% increase in the enterotoxin B levels. (This increase, which we did not note, was most likely due to the use of the mutant that had been selected for its ability to produce increased levels of enterotoxin B [J. F. Metzger, personal communication].) Jarvis et al. (10) also found maximal yields of fermentor-produced enterotoxin B at constant pH's of 6.5 and 7.3. The effect of added dextrose was not evaluated. As mentioned previously, these investigators noted that nuclease production was highest in fermentors without pH control, but the apparent optimal conditions for maximal total exoprotein production were established in baffled shake-flasks.

Arvidson and Holme (1) reported that pH

TABLE 3. *Productivity[a] of S-6 grown at the indicated constant DO level*

Interval (h)	100% DO			50% DO			10% DO		
	Toxin	Nuclease	Exoprotein	Toxin	Nuclease	Exoprotein	Toxin	Nuclease	Exoprotein
2 to 3	0	0	19.32	3.68[b]	73.53	25.00	0.42[d]	9.30	1.40
3 to 4	0	0	2,29						
4 to 5	0	0	−3.42	6.30[c]	151.85	39.26			
5 to 6	0	0	0						
6 to 8	0	0	0.20	7.65	122.95	19.13	0.71[e]	22.22	3.11
8 to 13	0	0	1.06	4.66	20.24	6.07			
13 to 24	0	0	−0.33	0.93	0	0.82	2.15	25.21	7.35
24 to 30							2.99	18.88	8.14
30 to 37							1.21	1.51	0.45
37 to 50							0.18	0	0.46
50 to 75							0.00	0	0

[a] Productivity is defined as the increment of measurable toxin, nuclease, or exoprotein divided by the average turbidity (Klett units) of an interval in the growth cycle.
[b] Represents 2- to 4-h interval.
[c] Represents 4- to 6-h interval.
[d] Represents 2- to 6-h interval.
[e] Represents 6- to 13-h interval.

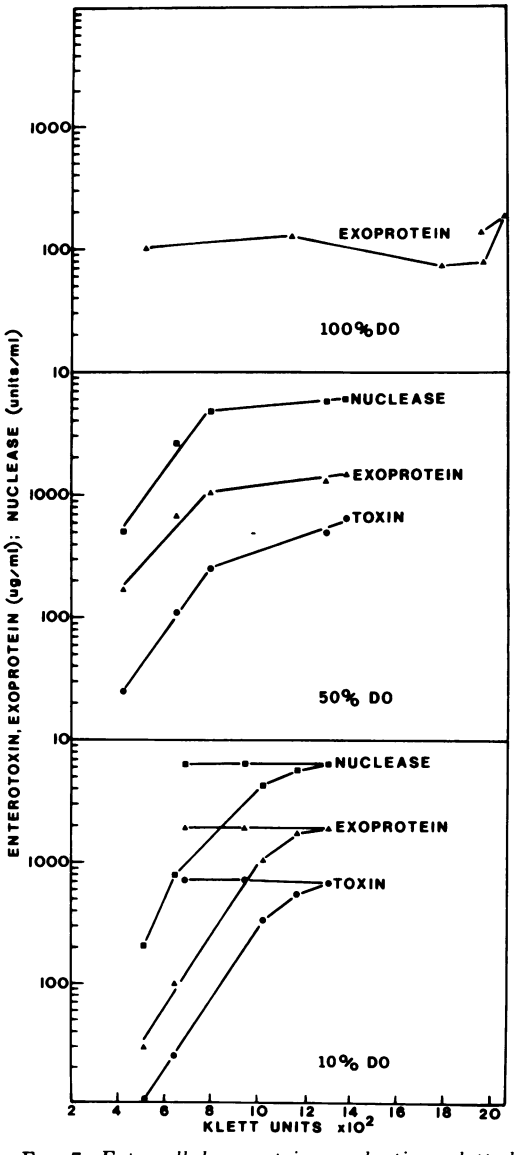

FIG. 7. *Extracellular protein production plotted against the increasing turbidity of cultures grown at the indicated constant DO level. For symbol explanation see Fig. 2.*

control at some value which gave the maximal yields of a particular toxin or enzyme was not necessarily the pH value that resulted in maximal yields of the same protein when produced by another staphylococcal strain. Analyses for enterotoxin were not conducted, but it was noted that pH control in the range of 7.0 to 7.5 was optimal for nuclease production by one strain, whereas strict control at pH 7.5 was optimal for the production of nuclease by another strain.

These reports, however, never mentioned the status of the DO during growth. Our experiments unequivocally indicated that a constant aeration rate never yielded a constant DO level. By and large, the DO level at any point during growth is apparently determined by the physiological state of the culture. Cultures grown at constant aeration rates, with and without pH control, consistently initiated rapid growth, enterotoxin, nuclease, and total exoprotein production only after the metabolism of the cells had decreased the DO to 5 to 15% from the initial 100% setting. The control of this parameter by increasing or decreasing the aeration rate, as appropriate, led to the elucidation of its importance as an apparent metabolic regulator. High DO levels appear to favor high yields of cells to the near exclusion of exoproteins; low DO levels favored high titers of toxin, nuclease, and total exoproteins, but lowered cell densities.

The similarities between the growth versus secretion patterns of the 10% DO cultures and the constant aeration rate cultures (Fig. 2 and 7) seem to indicate similar responses of the cultures to these conditions. Since the basic difference between the constant aeration cultures and the constant 10% DO cultures was the length of the low DO interval, it would appear that the DO was the crucial factor in determining the final exoprotein titers, i.e., the 10% DO culture was at the 10% DO level longer, thus higher levels were produced. The productivities associated with the 10% culture do not, at first glance, indicate that high titers could be realized. However, it was the long intervals associated with these productivities that yielded the very high titers.

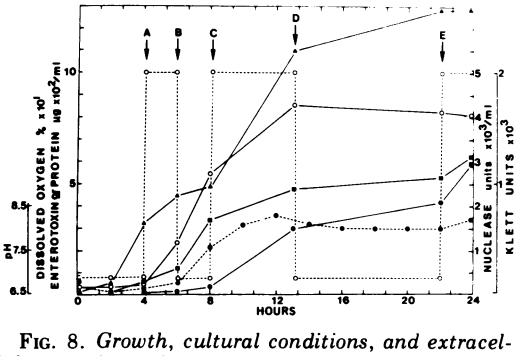

FIG. 8. *Growth, cultural conditions, and extracellular protein production of S. aureus S-6 grown under conditions of alternating DO levels. At points A, C, and E, the oxygen controller was set to maintain a DO level of 100%. At points B and D, the DO was set to be maintained at 5 to 10%. For symbol explanation see Fig. 3.*

TABLE 4. *Productivity[a] of S-6 grown under conditions of DO cycling*

Interval (h)	DO (%)	Toxin	Nuclease	Exoprotein
0 to 4	5 to 10	0	240.00	186.67
4 to 6	100	2.14	35.71	21.43
6 to 8	5 to 10	0.90	74.36	2.56
8 to 13	100	3.93	9.18	8.75
13 to 22	5 to 10	0.83	2.13	5.32
22 to 24	100	5.40	9.88	0.62

[a] Productivity is defined as the increment of measurable toxin, nuclease, or exoprotein divided by the average turbidity (Klett units) of an interval in the growth cycle.

The similarities between the patterns of the 50% DO culture and the constant pH cultures (Fig. 5 and 7) were probably due to the maintenance of a relatively favorable metabolic balance within the cells, which resulted from growth at the intermediate 50% DO level or at pH values close to neutrality. The constant pH 7.0 culture, although not yielding increased levels of exoproteins over the optimal aeration rate culture, did produce a population whose overall toxin productivity was the highest observed under any condition(s). Maximal productivities for nuclease and total exoprotein were observed in the culture that was cycled through high and low DO levels. The results of these manipulations also indicated that nuclease was preferentially produced during the low DO intervals, whereas the toxin and total exoproteins were secreted during the high DO intervals.

The basis for this oxygen effect may be founded in the observations made in other bacterial systems, not necessarily dealing with extracellular proteins (8). These reports have indicated that when the demands of a growing culture caused the DO (originally set at 100%) to fall below some apparent critical concentration (3 to 10%), cellular metabolism went through a transition phase. In *Klebsiella*, this stimulated the oxygen uptake rate by 30%. In *Pseudomonas*, oxygen affinity became higher below the critical oxygen concentration. The induction or derepression of enzyme systems did not appear to be involved, as the observed responses occurred within minutes. To determine what intracellular compounds, if any, mediated these transitions, fluorimetric methods were used to monitor the redox state of nicotinamide adenine dinucleotide within the culture of *Klebsiella* (7). The exhaustion of oxygen in the culture caused the rapid reduction of nicotinamide adenine dinucleotide; on resuming the airflow, the reduced nicotinamide adenine dinucleotide was immediately reoxidized. In conjunction with these changes, the levels of the adenosine phosphates in *Klebsiella* also varied in response to changing DO. The authors suggested that the overall energy balance appeared to be primarily controlled by the adenosine 5'-triphosphate level. This apparently was accomplished by the interaction of the adenosine 5'-triphosphate with the enzymes in the anabolic and/or catabolic pathways through feedback inhibition. Overall, it appears that the adenosine phosphates and the nicotinamide nucleotides may exert rapid, short term control over cellular metabolism.

In evaluating the observed effects that controlled DO has on *S. aureus*, similar controls may be functioning in the secretion of the toxin, nuclease, and total exoprotein. The aforementioned transition, which occurred in the metabolism of *Klebsiella* and *Pseudomonas* at low DO levels, could be the initiator and/or regulator of secretion in the staphylococci.

Insufficient oxygen tension during the constant DO fermentations at 10 and 50% may have caused an unfavorable energy balance with the cell, which the cellular machinery recognized through somewhat elevated levels of reduced nicotinamide adenine dinucleotide and/or depressed levels of adenosine 5'-triphosphate. Although the attainment of these abnormal levels may have served to aid in regulating cellular metabolism for short intervals, sustaining these levels may have led to the induction or derepression of the mechanisms responsible for increased toxin, nuclease, and total exoprotein production. In the presence of abundant oxygen (100% DO), the cells, presumably in a most favorable energy state, produced very little extracellular protein whereas they themselves proliferated substantially. These phenomena might possibly be explained by the observations made on the oxidative metabolism of glucose by *S. aureus* (18). The maximal amounts of energy generated from glucose in the presence of oxygen repressed enterotoxin secretion. However, anaerobic shock of these cells caused the differential rate of enterotoxin B production to increase. This increase could be eliminated by substituting an electron acceptor for oxygen (i.e., NO_3^-); thus, it appeared that the role of oxygen was limited to terminal electron acceptor. In discussing these results, the investigators did mention that "...it is conceivable that the repressive mechanism is associated with the energy state of the cell" and that "...repression probably was exerted in the presence of more available energy than was present under anaerobic conditions." In a later report (16), Morse

and Baldwin indicated that under no circumstances did oxygen serve as an inducer for toxin production. However, the results presented in that report, as well as the results contained herein, do not rule out the possibility that oxygen is indirectly regulating enterotoxin as well as nuclease and exoprotein production by means of the nicotinamide adenine dinucleotide-reduced nicotinamide adenine dinucleotide and adenosine 5'-triphosphate-adenosine 5'-diphosphate-adenosine 5'-monophosphate (energy) levels within the cell.

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