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## Studies on Sucrose-Induced Autolysis of Clostridial Cells

### Part 1. Induction of a Rapid Bacterial Autolysis by Sucrose Treatment

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Growing cells of *Clostridium saccharoperbutylacetonicum* NI-4 (ATCC 13564) were rapidly autolyzed in the presence of 0.3 to 0.5 M sucrose. This autolysis was apparently induced by the treatment with sucrose, and termed "sucrose-induced autolysis." The maximum effect was shown at 0.35 M sucrose, exhibiting about 50% loss of initial turbidity of the cell suspension. The rate of lysis depended on the age of culture. The most rapid lysis occurred in the cells of early-exponentially growing culture, but no lysis was observed on those of late-exponential and stationary phase cultures. The optimal pH was 6.0, and the optimal temperature 30°C. Lysis occurred either in the cultural medium or buffer solution (for example: sodium phosphate buffer). The lysis was accompanied by striking morphological conversion from original rod cells (3 - 6 x 0.4 - 0.6  $\mu\text{m}$ ) to spherical cells (1.0 - 1.5  $\mu\text{m}$  in diameter).

The same rapid autolysis was also observed on the relative strains of *C. saccharoperbutylacetonicum* and *C. sporogenes*. Cells of *C. butyricum*, *C. kaneboi* and *C. botulinum* were slightly autolyzed (10 - 15% loss of initial turbidity). However, no lysis occurred on the aerobes of several different genus and on the cells of some other species of genus *Clostridium* tested. The bacterial spheres developed during the lysis may be the protoplasts.

### INTRODUCTION

This study was started from an observation of unexpected premature lysis of the cells of *Clostridium saccharoperbutylacetonicum* in an usual penicillin lytic system designated to produce the protoplasts. It became clear by further investigation that the premature lysis was due to the concomitant sucrose added as the osmotic stabilizer for the developing protoplasts, and the lysis was regarded as a kind of the bacterial autolysis induced by sucrose treatment. Therefore, the lysis phenomenon was named "sucrose-induced autolysis". We reported a similar phenomenon of the rapid autolysis of *C. saccharoperbutylacetonicum* by treatment of hypertonic sodium chloride (Ogata and Hongo, 1973;

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1974). In this autolysis, the cells are completely destroyed. However, in the sucrose-induced autolysis, it is unique phenomenon that sucrose makes the cells converted morphologically from the rod cells to protoplast-like cells. Also, differently from sodium chloride, sucrose is one of well using hypertonic solutes to prevent a bacterium from the complete lysis. Moreover, our clostridia can utilize sucrose as carbone source for their growth and acetone-butan01 fermentation. There has not been found any report demonstrating a bacterial autolysis induced with sucrose or other simple compounds which can be utilized by the bacteria. It should be an interesting subject to understand how a simple compound of sucrose can induce a rapid autolysis of clostridial cells.

Bacterial autolysis has been known in various bacterial species (Galli and Huges, 1965; Schwarz *et al.*, 1969; Forsberg and Rogers, 1971), and has been used for various purposes including autolysin isolation (Kawata and Takumi, 1971; Shockman *et al.*, 1961; Fan, 1970) and protoplast formation (Mohan *et al.*, 1965; Kawata *et al.*, 1968; Joseph and Shockman, 1974). Ogata (1976) reviewed these works concerning autolysis, autolysin and protoplast. Throughout these works, autolysis is caused in neutral to alkaline buffer, and sucrose is only used as stabilizer to prevent the developed protoplasts from their destruction. In our work, sucrose is used as the inducer of autolysis and at the same time as stabilizer of developed protoplast-like cells. This phenomenon may provide a new method in the formation of protoplast of some *Clostridium* species without any requirement for extraneous supplements for the degradation of cell wall.

This study are carried out with the aims to clarify the lytic mechanism of the rapid autolysis of clostridial cells occurring in the hypertonic sucrose solution, and to establish a successful autolytic method to form protoplasts of clostridia. The mechanism of cellular autolysis occurring in the solution of sodium chloride is also clarified and compared with that of the former autolysis. We will prepare six following reports for this study.

## MATERIALS AND METHODS

### Bacterial strains

*Clostridium saccharoperbutylacetonicum* NI-4 (ATCC 13564) (Hongo and Murata, 1965) was used in this work unless otherwise mentioned. Various strains of *Clostridium*, *Bacillus* and other aerobic bacteria were also used.

### Media and cultural conditions

*C. saccharoperbutylacetonicum* and other *Clostridium* species except *C. acetobutylicum* were grown at 30°C under a reduced atmospheric conditions (5–10 mmHg) in TYA medium which contained glucose 40 g, Bacto-tryptone (Difco) 6 g, yeast extract 2 g, ammonium acetate 3 g, KH<sub>2</sub>PO<sub>4</sub> 0.5 g, MgSO<sub>4</sub>•7H<sub>2</sub>O 0.4 g, and FeSO<sub>4</sub>•7H<sub>2</sub>O 0.01 g per liter (pH 6.5). *C. acetobutylicum* was grown at 37°C under the same cultural conditions with other *Clostridium* species. Strain NI-4 was also

cultivated by using a modified TYA medium and a basal medium. The modified TYA medium contained 7 % (w/v) sucrose (sucrose-TYA medium) instead of glucose as the carbon source. The basal medium consisted of glucose 20 g, biotin 10  $\mu$ g,  $\text{KH}_2\text{PO}_4$  0.5 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.2 g,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.01 g and casamino acid 2 g per liter (pH 6.5). Aerobic bacteria were grown at 30°C or 37°C with shaking in the nutrient broth supplemented with 5g of glucose and 2g of yeast extract per liter (pH 7.0)

#### Preparation of exponentially growing cells

For the preparation of exponentially growing cells, initial optical density ( $\text{OD}_{660}$ ) at 660nm of culture was adjusted to 0.1 in a fresh medium and incubation was carried out until  $\text{OD}_{660}$  became 0.3. In the case of strain N1-4, this culture contained approximately  $1 \times 10^8$  cells/ml, and it was needed to cultivate 3 to 4 hr to reach. Cells were harvested by centrifugation (10,000 xg for 10 min at room temperature).

#### Measurement of turbidity and expression of lysis rate

Lysis and growth of bacterial cells were followed by measuring the  $\text{OD}_{660}$  with a photoelectric colorimeter (model 7A, Tokyo Koden Ltd.). The lysis rate or increased rate was expressed as the turbidity ( $\text{OD}_{660}$ ) of culture (or cell suspension) at given time against that of zero time.

#### Induction of a rapid bacterial autolysis

Cells were suspended in the medium or buffer solution containing 0.35 M sucrose unless otherwise mentioned. Lysis was induced by incubating the cells at 30°C with or without reduction of the atmospheric pressure. Aerobes were incubated at 30°C in the nutrient broth containing the same concentration of sucrose while shaking. Sucrose used in this experiment was a guaranteed grade of Ishizu Pharmaceutical Ltd.

#### Penicillin treatment

Cells were cultivated with or without penicillin G and sucrose. The drug was in the standard of medical use (100,000 units/ml) and purchased from Meiji Seika Ltd.

#### Inspection of cellular morphology

An aliquot of cell suspension was withdrawn before and after lysis. After fixation of the cells with 5 % (v/v) formalin for 10 min at room temperature, cellular morphology was inspected with a differential interference phase contrast microscope (Nippon Kogaku Kogyo Ltd.).

## RESULTS

#### Finding of a rapid bacterial autolysis

The cells of *C. saccharoperbutylacetonicum* were suspended in TYA broth which contain 100  $\mu$ g/ml of penicillin G with or without 0.35 M sucrose. Incubation was started under a reduced atmospheric condition. As shown in Fig.

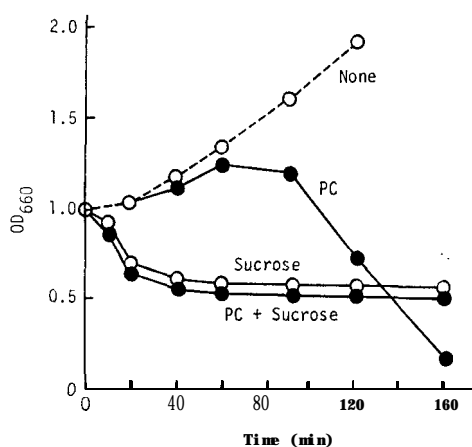


Fig. 1. Induction of a rapid cellular lysis of *Clostridium saccharoperbutylacetonicum* by penicillin-sucrose lytic system. Bacteria were cultivated in a fresh medium containing 100  $\mu\text{g}/\text{ml}$  of penicillin G (PC) and 0.35 M sucrose. Cultivation was carried out anaerobically by using Thunberg tubes.

1, the cells were sensitive to penicillin. The typical lysis began at about 90 min, when they were exposed to penicillin, and finished at 160min or more, exhibiting a 95 % loss of the initial turbidity. On the other hand, when sucrose was contained, the cells were lysed so rapidly that typical effect of concomitant penicillin was not exhibited. Moreover, no clear difference in lytic pattern was shown between the cultures which contained penicillin and the one did not. In the sucrose-containing cultures, the system did not lose about 50% of its initial turbidity even by continued cultivation for several hours. This result indicates that the premature lysis of the penicillin-containing culture was caused not by the penicillin but by sucrose. This was the first finding of sucrose-induced autolysis occurring on clostridial cells.

#### Sucrose-induced autolysis on actively growing bacteria

Experiments were carried out to understand the relationship between growing ability and lytic sensitivity of the bacteria.

##### 1) Lysis of the cells grown in different types of cultural media

Bacteria were grown respectively in TYA, sucrose-TYA, nutrient and basal media until their cultural turbidity ( $\text{OD}_{660}$ ) reached 0.3. They showed a vigorous growth in TYA and sucrose-TYA media, and the  $\text{OD}_{660}$  reached 0.3 after 3 to 3.5 hr of cultivation. In the nutrient broth and basal broth, they showed a lightly weaker growth than in the above media, and they needed 4 to 5 hr to reach  $\text{OD}_{660}$  of 0.3. As shown in Table 1, better lysis was observed on the cells grown in TYA and sucrose-TYA media than nutrient broth and basal medium. The cells grown in the basal medium showed only a slight lysis. This result indicates that sucrose-induced autolysis easily occurs on the cells grown actively. The type of cultural medium should give little affection

**Table 1.** Sucrose-induced autolysis on the cells grown in various cultural media. Bacteria were grown in various cultural media until they reached logarithmic growth phase. Harvested cells were suspended in fresh TYA medium containing 0.35 M sucrose, and autolyzed by incubation at 30°C under reduced atmospheric pressure. The growth rate indicates the average of turbidimetrical doublings of the culture per hour. The lysis rate indicates the per cent of decrease in turbidity after lysis for 60 min.

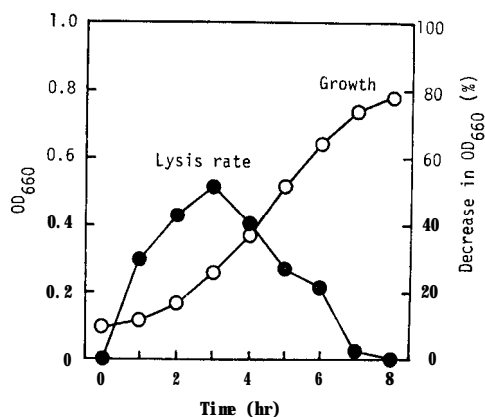
Medium	Growth rate (doublings/hr)	Lysis rate (%)
TYA	1.10	52
Sucrose-TYA <sup>1)</sup>	0.95	43
Nutrient	0.88	32
Basal	0.71	9

<sup>1)</sup> A TYA medium containing sucrose (7%) instead of glucose.

on the lysis, if bacteria can grow vigorously in the medium.

## 2) Sucrose-induced autolysis on various aged bacteria

To investigate the effect of cultural age of bacteria on the sensitivity of them to sucrose-induced autolysis, cells were harvested at different times of cultivation and autolyzed in the presence of sucrose. The cells grew exponentially between 1 to 7 hr after inoculation, and then their growth gradually slowed down to enter stationary phase. As shown in Fig. 2, the actively growing cells from the early-exponentially growing cultures were highly sensitive to the lysis, while the cells of late-exponential growth phase or stationary phase resisted the lysis. The most rapid autolysis occurred on the cells of the early to middle stage of exponential growth phase, cultivated for 2 to 4 hr. This result also indicates that sucrose-induced autolysis predomi-



**Fig. 2.** Sensitivity of various aged bacteria to sucrose-induced autolysis. Cells were harvested at different times of cultivation, and were autolyzed in fresh medium containing 0.35M sucrose. Other lytic conditions and estimation of the lysis rate were the same as Table 1.

nantly occurs on actively growing cells.

### Sucrose-induced autolysis under atmospheric pressure

It was apparent that lysis predominantly occurred under the conditions for the cells to grow actively. *C. saccharoperbutylacetonicum* is an absolute anaerobic bacterium, and requires for its growth a firm reduction of the atmospheric pressure (exhaustion of oxygen). However, the lytic system under reduced atmospheric pressure was accompanied with much inconvenience for handling. Therefore, lysis was carried out under atmospheric pressure to investigate whether or not lysis could occur under the given condition. As shown in Fig. 3, no significant difference, in the lysis rate and lysis pattern, was observed from each condition.

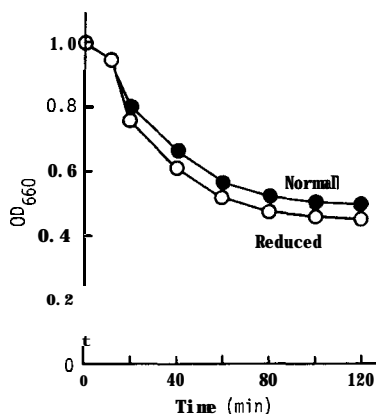


Fig. 3. Sucrose-induced autolysis under atmospheric (normal) and reduced atmospheric pressure (reduced). The atmospheric pressure was reduced to 5–10 mmHg by using Thunberg tubes and a rotary vacuum pump.

### Sucrose-induced autolysis in buffer solution

Experiments were performed to find out an appropriate buffer solution and optimal conditions for the occurrence of sucrose-induced autolysis in the buffer solution.

#### 1) Lysis with different buffer solutions

Lysis was carried out by using several different types of buffer solutions such as sodium phosphate, Tris-HCl and ammonium acetate which were frequently used in other bacterial autolysis. Buffer solutions were adjusted to neutral to weak alkaline pH. The most predominant lysis was evident in the sodium phosphate buffer solution among the tested solutions.

#### 2) Lysis with sodium phosphate buffer solutions of different molarities

To investigate the effect of the molarity of buffer solution on the progress of sucrose-induced autolysis, cells were autolyzed in the buffer solutions

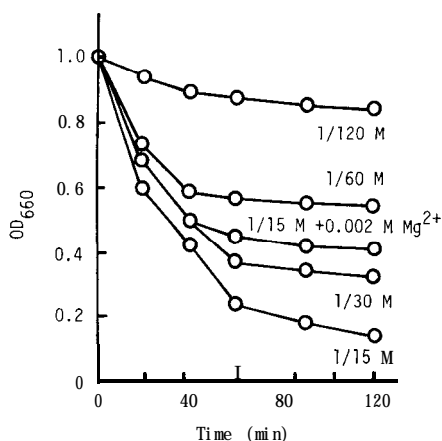


Fig. 4. Effect of the molarity of phosphate buffer solution on the progress of sucrose-induced autolysis. Lysis was carried out in sodium phosphate buffer solution (pH 6.5) of different molarities without reduction of atmospheric pressure.

of different molarities from 1/15 to 1/120 M (pH 6.5). As shown in Fig. 4, the higher the molarity of buffer solution, the more predominant the lysis became. In the solution of high molarity, the cells and the developed protoplast-like cells were destroyed. The lysis pattern in 1/60 M buffer solution or 1/15 M buffer solution containing  $Mg^{2+}$  was similar to that in TYA medium. The predominant lysis in the buffer solution of high molarity may be responsible to the high concentration of monovalent cations such as  $K^+$  and  $Na^+$ , because those cations also induced the cells to be rapidly autolyzed at the high concentration (Ogata and Hongo, 1973).  $Mg^{2+}$  are well known to stabilize bacterial protoplast (Joseph and Shockman, 1974; Weibull, 1953).

### 3) Effect of pH on buffer solution

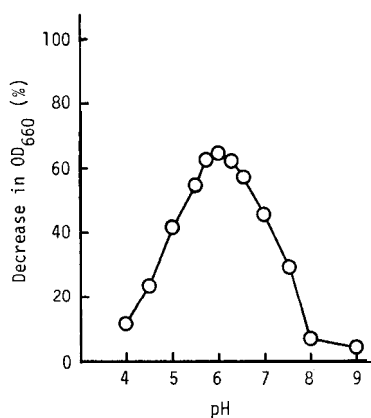
Cells were autolyzed in the buffer solution (1/60 M) containing sucrose and having various pH from 4.0 to 9.0. As shown in Fig. 5, rapid autolysis occurred at pH 5.5 to 6.5 with the optimum pH at 6.0.

From these results, it is clear that sucrose induces a rapid autolysis in buffer solution as well as in TYA medium. Sodium phosphate buffer solution (1/60 M, pH 6.0) was considered to be an appropriate fluid for induction of lysis in which it is easy to control the lysis.

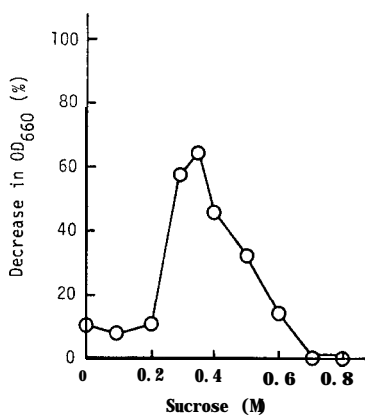
### Lysis with various concentrations of sucrose

To investigate the possible range of sucrose concentration to induce the rapid autolysis of cells, experiments were carried out with the buffered sucrose solution containing different concentrations of sucrose. As shown in Fig. 6, lysis occurred at 0.3 to 0.5 M sucrose, but no lysis occurred at both concentrations lower than 0.3 M and higher than 0.5 M. The maximum lysis observed at 0.35 M which exhibited about a 60 % decrease of the initial turbidity. When





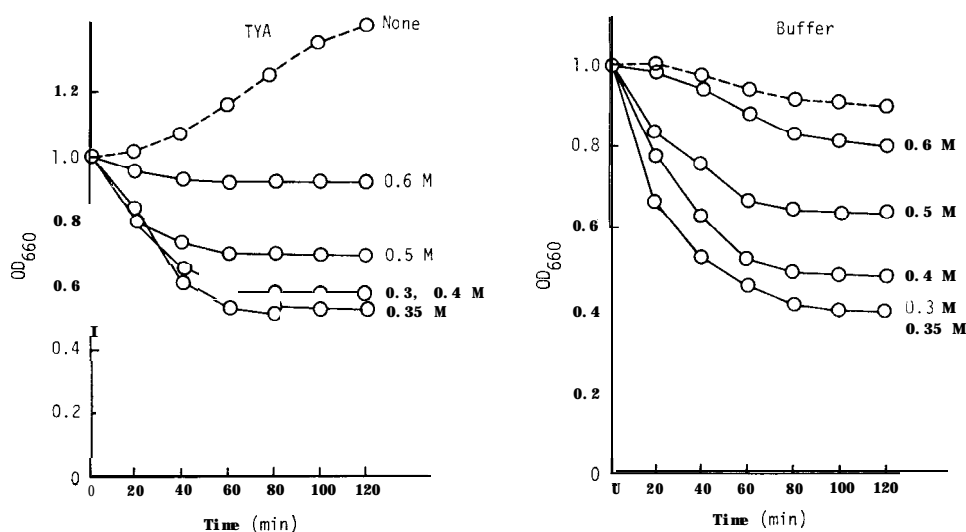
**Fig. 5.** Effect of pH of phosphate buffer solution on sucrose-induced autolysis. Cells were autolyzed for 2 hr in sodium phosphate buffer solution containing 0.35 M sucrose.



**Fig. 6.** Sucrose-induced autolysis at various concentration of sucrose. Cells were autolyzed in sodium phosphate buffer solution (1/60 M, pH 6.0) which dissolved various concentrations of sucrose. Lysis rate was represented as the per cent of decreased turbidity after 2 hr incubation.

TYA medium was used instead of the buffer solution, 50 to 55 % loss of initial turbidity was shown as the maximum at 0.35M. Lysis pattern with different concentrations of sucrose was shown in Fig. 7. These results indicate that sucrose-induced autolysis is depended entirely upon the concentration of inducer sucrose in the sense of its occurrence and lytic progress.

Another important observation in these experiments was that lysis progressed rapidly, and was levelled off at about 40min of incubation. Furthermore, no complete lysis was observed within several hours tested at any concentration of sucrose. We will explain this peculiar phenomenon later.



**Fig. 7.** Progress of sucrose-induced autolysis at various concentrations of sucrose. Cells were autolyzed in medium or sodium phosphate buffer (1/60 M, pH 6.0) containing sucrose (0.3–0.6 M). Other lytic conditions were the same as Fig. 4.

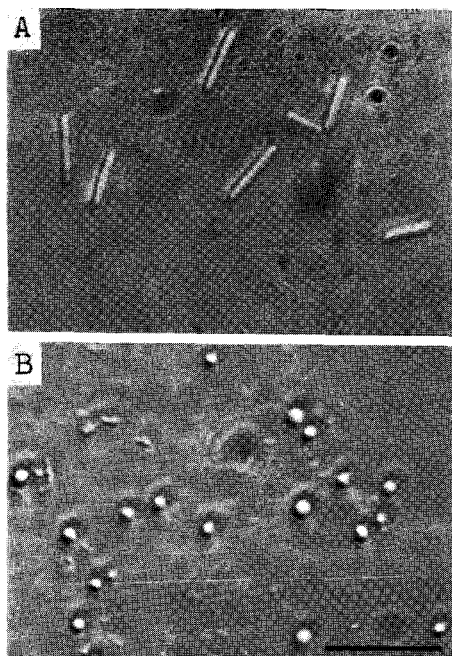
#### Conversion of cellular morphology

During sucrose-induced lysis, the original rod cells were converted to the spheres. Rate of morphological conversion from rods to the spheres was more than 99% for 60 min incubation, when the lysis was carried out in TYA medium containing 0.35 M sucrose. Figure 8 (A) shows the original rods ( $3.6 \times 0.4-0.6 \mu\text{m}$ ) before the lysis, and (B) shows the developed protoplast-like cells ( $1.0-1.5 \mu\text{m}$ ). The development of protoplast-like cells during sucrose-induced autolysis was apparently responsible for the incompleteness of lysis. It is the major difference of sucrose-induced autolysis from NaCl-induced autolysis in which clear lysis of cells are accompanied (Ogata and Hongo, 1973). Details of the morphological change of the cells and biophysical and biochemical properties of the protoplast-like cells shall be presented in following papers.

#### Behavior of various kinds of bacteria against sucrose treatment

The same experiments as performed on *C. saccharoperbutylacetonicum* were designed on various species of *Clostridium* and aerobic bacteria to know whether sucrose-induced autolysis was a characteristic phenomenon of *Clostridium* or not.

As shown in Table 2, lysis occurred on some strains of clostridia different in the species. Aerobic bacteria did not show any significant lysis. It is said that sucrose-induced autolysis is probably a specific phenomenon manifested by clostridial cells. Rapid lysis was evident on the cells of *C. saccharoperbutylacetonicum* and *C. sporogenes* among the clostridia tested. They showed a success-



**Fig. 8.** Bacterial morphologies before and after sucrose-induced autolysis. Photographs were taken with a differential interference phase contrast microscope after fixation of the cells with formalin. Photo. A: cells before lysis. Photo. B: cells after lysis for 60 min. Scale line indicates 10  $\mu$ m.

ful lysis in both medium and buffer solution exhibiting 50 to 60 % loss of their initial turbidity. On the other hand, other clostridia showed different lytic responses according to their species or suspension fluid of the cells. The cells of *C. butyricum*, *C. kaneboi* and *C. botulinum* were slightly autolyzed (10 to 15 % loss of initial turbidity) for early 30 min of incubation in the TYA medium with sucrose, but they recovered their initial turbidity by succeeding incubation for another 30min. When they were incubated in buffered sucrose solution, they showed apparently accelerated cellular lysis comparing with autolysis in the buffer solution without sucrose. The cells of *C. thiaminolyticum* was hardly autolyzed in the medium. However, they also showed accelerated lysis in buffered sucrose solution. No lysis was observed on the cells of *C. acetobutylicum* in both medium and buffer solution with sucrose. From these results, it is considered that sucrose-induced autolysis are the specific phenomena manifested by *Clostridium* species

#### DISCUSSION

The sucrose-induced autolysis of *C. saccharoperbutylacetonicum* must be dependent upon the lytic action of cellular autolysin, because the lysis occurred

**Table 2.** Different responses in various strains of bacteria against sucrose treatment. Cells of every bacterial strain were harvested during their early-exponential growth phase after cultivation as described in Materials and Methods, and they were incubated for 60 min at 30°C with 0.35 M sucrose to induce them to be autolyzed. Clostridia were incubated anaerobically in TYA medium or buffer solution. Aerobes were incubated in nutrient broth while shaking.

Organisms	Incubation of cells in	
	medium	buffer
<i>Clostridium saccharoperbutylacetonicum</i> NI-4 (ATCC 13564)	‡‡	‡‡‡
" NI-504 (ATCC 27022) <sup>1)</sup>	‡‡	‡‡
" No. 8 <sup>2)</sup>	‡‡‡	‡‡‡
<i>C. sporogenes</i> (IF6 12636)	‡‡	‡‡
<i>C. butyricum</i>	±	‡‡
<i>C. kaneboi</i>	±	‡‡
<i>C. botulinum</i> (IFO 3733)	±	‡‡
<i>C. thiaminolyticum</i> (IFO 3969)	-	+
<i>C. acetobutylicum</i>	-	-
<i>Bacillus megaterium</i> (IFO 3970)	-	NT <sup>3)</sup>
<i>B. subtilis</i> (IFO 12210)	-	NT
<i>B. cereus</i> (IFO 3015)	-	NT
<i>Staphylococcus aureus</i> (IFO 3060)	-	NT
<i>Mycobacterium smegmatis</i> (IFO 3082)	-	NT
<i>Escherichia coli</i> (IFO 13168)	-	NT
<i>Micrococcus luteus</i> (IFO 3333)	-	NT

<sup>1)</sup> A mutant strain from NI-4 resistant against infection of phages HM 2 and HM 3. <sup>2)</sup> A sensitive strain to bacteriocin clostocin 0 produced by NI-4. <sup>3)</sup> Not tested. - : no lysis, + : lysis. ± : lysis occurred within 30 of incubation, however, recovered the initial turbidity by succeeding cultivation. Lysis rate ; + : 1-20%, ‡ : 21-40%, ‡‡ : 41-60%.

without any supplement of lytic substances. There have been many reports on the methods for induction of bacterial autolysis (Ogata, 1976). Shockman (1965) hypothesized that bacterial autolysis is an appearance of unbalanced bacterial growth between biosynthesis and degradation of the cell wall, which can occur when a bacterium is exposed to some abnormal cultural conditions. Actually, some bacteria were rapidly autolyzed by cessation of aeration (Kawata *et al.*, 1961; Nomura and Hosoda, 1956), elimination of some specific nutrients from the cultural medium (Shockman *et al.*, 1961), salt treatment (Pooley *et al.*, 1970; Gilpin *et al.*, 1972; Ogata and Hongo, 1973), and cold shock (Ohmiya and Sato, 1975). Moreover, some bacteria were successfully autolyzed by incubation of them in an appropriate buffer solution without any specific treatment to induce the bacteria to be autolyzed (Joseph and Shockman, 1974; 1976; Helbeler and Young, 1975). A technique which inhibits the continuous synthesis of cell wall without inhibiting the action of autolysin may be useful to induce bacterial autolysis.

Sucrose has been used in wide variety of bacterial lysis to produce the protoplasts or spheroplasts (Weibull, 1953; 1963; Martin, 1963; Kruse and Hurst, 1972; Weise and Franser, 1973; Cho *et al.*, 1974; Calandra *et al.*, 1975). In these works, sucrose was used to prevent the developing protoplasts from

their further lysis. There has not been found any report demonstrating a rapid bacterial autolysis occurring by treatment with sucrose or other substances which are utilized by bacteria as the carbon source. Sucrose-induced autolysis of this study as well as NaCl-induced autolysis must be a new type of bacterial autolysis. We will report about the role of autolysin and sucrose on sucrose-induced autolysis.

*C. saccharoperbutylacetonicum* was used until 1960 for industrial production of organic solvents, acetone and butanol. The fermentation offering high concentration of sugar was tried to improve the productivity of the solvents. However, Hongo (1956) described that the fermentation did not undergo smooth with the broth containing above 10 % of sugar. This industrial trouble should depend upon occurrence of autolysis of the cells by which the fermentation underwent. There is a trend to revive the acetone-butanol fermentation process using clostridia. This study will also offer some valuable informations on this fermentation.

#### ACKNOWLEDGEMENTS

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