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Studies on Marine Bacteria Producing Lytic Enzymes-VI

Effect of Inorganic Salts on the Release of Lytic Enzyme from Bacterial Cells

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The effect of inorganic salts on the release of lytic enzyme was investigated using bacterial cells of strain V 37 grown for 6 or 8 hours in polypeptone-yeast extract medium containing 0.5 mole/l of NaCl.

No remarkable effect on the liberation of lytic enzyme was observed by 0.5 mole/l of inorganic salts used in this study.

Chloramphenicol at the concentration of 10 μg/ml did not cause inhibition of enzyme release by strain V 37.

Among various chemicals, triton X-100(0.1%) active to bacterial cell membrane seemed to be effective for the elution of lytic enzyme from cells of strain V 37.

Key words : marine bacteria, lytic enzyme

In the previous papers(SUGAHARA *et al.* 1978, 1980, 1981), strain V 37 isolated from coastal waters grew well in the medium without added inorganic salts, but could not extracellulary produce lytic enzyme. Among inorganic salts tested, NaCl or KCl was most effective for the production of lytic enzyme by strain V 37.

The production of extracellular enzymes may be mainly separated into two processes; the net synthesis of enzyme and the following liberation from bacterial cells. A number of papers have been reported on enzyme release from bacterial cells(KUSHNER and POLLOCK 1961, POLLOCK 1961a, 1961b, 1962, COLES and GROSS 1967a, 1967b, LAMPEN 1967, MALVEAUX and SAN CLEMENTE 1967, SARGENT *et al.* 1969, SARGENT and LAMPEN 1970). However, little work has been recorded on the release of lytic enzyme. According to POLLOCK(1961b), the liberation appeared to require an enzymic reaction, since it was temperature-dependent and did not occur at pH values less than 6.0, and the enzyme was not eluted from the cells of *Bacillus subtilis* by high concentrations of salts.

Although it has not been demonstrated whether the release of lytic enzyme from V 37 cells was enzymic or not, the present study was disignated to clarify the role of inorganic salts on the extracellular production of lytic enzyme by strain V 37.

Methods

Assay of bacteriolytic activity

Lytic activity was determined as described previously (SUGAHARA *et al.* 1976, 1978, 1979, 1980).

Culture of strain V 37 producing lytic enzyme

Culture of strain V 37 capable of producing lytic enzyme was carried out as described previously(SUGAHARA *et al.* 1978, 1980).

Release of lytic enzyme from bacterial cells

Cells of strain V 37 were grown at 30 °C for 6 or 8 hours with shaking in 500 ml Sakaguchi flasks containing 250 ml of polypeptone-yeast extract medium. Bacterial cells, harvested by centrifugation, were washed twice with solution to be tested and resuspended in the same solution to give about 1/2-1/4 of the original volume. For the release experiment, 10.0 ml of this suspension was incubated at 30 °C for 2 hours with shaking, and centrifuged at 13,000 × g for 20 minutes at 0 °C. The supernatant solutions, after washing the cells and from incubation mixture for release experiment, were assayed for bacteriolytic activity after dialysis against distilled water for 3 hours at 5 °C.

Results

Time course of release of lytic enzyme

In order to determine appropriate incubation time for the release of lytic enzyme from strain V 37 cells, cells grown for 6 and 8 hours were harvested by centrifugation, washed twice with distilled water, and then resuspended in distilled water. Cell suspension (10.0 ml) was incubated at 30 °C with shaking, and centrifuged to remove the bacterial cells at the times shown. The supernatant solutions obtained were assayed for bacteriolytic activity after dialysis against distilled water.

As shown in Fig. 1, in the case of the cells grown for 6 hours, the decrease in the optical density of cell suspension was observed during incubation: about 42.5% of the original cells seemed to be lysed for 4 hours of incubation. However, there was no appreciable difference in lytic activity released from cells for incubation time of 1 to 4 hours.

On the other hand, in the cells grown for 8 hours, the decrease in the optical density was very slight: about 5.3% for 2 hours and about 12.0% for 4 hours. Nevertheless, lytic activity liberated from cells did not increase for 4 hours of incubation.

It was likely that the increase in lytic activity released from cells was not directly associated with the decrease in optical density of cell suspension. Lytic activity may liberate from bacterial cells of strain V 37 in cell suspension within 1 hour under this condition.

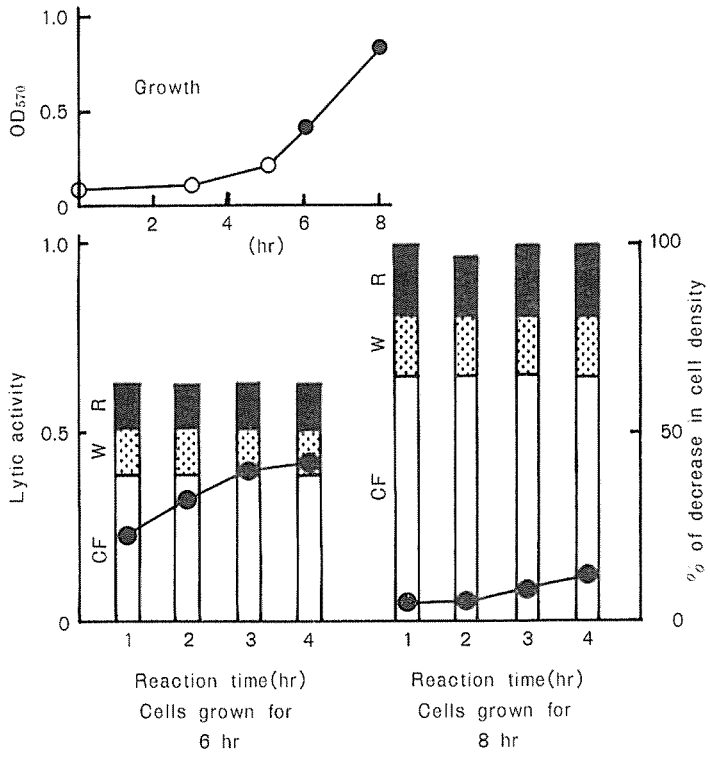

Fig. 1. Time course of release of lytic enzyme from V 37 cells grown in polypeptone-yeast extract medium containing NaCl (0.5 mole/l). CF;lytic activity of culture filtrate, W;lytic activity liberated from cells during washing, R;lytic activity released from cells during incubation.

Effect of temperature on the release of lytic enzyme

Strain V 37 cells grown for 6 and 8 hours were harvested separately by centrifugation, washed twice with distilled water and resuspended in distilled water. Cell suspensions (10.0 ml) were incubated for 2 hours at the following temperature: 0, 10, 20, 30, 40 and 50 °C.

Fig. 2 shows the effect of temperature on the liberation of lytic enzyme from strain V 37 cells grown in the medium containing 0.5 mole/l of NaCl. There was no remarkable difference in lytic activity released from strain V 37 cells grown for 6 hours, although the decrease in optical density of cell suspension increased with increasing temperature between 0 and 40 °C.

On the other hand, the liberation of lytic activity from cells grown for 8 hours increased with increasing temperature between 0 and 40 °C, and then decreased slightly at 50 °C. There may be different mechanism of enzyme liberation between cells grown for 6 hours and those for 8 hours.

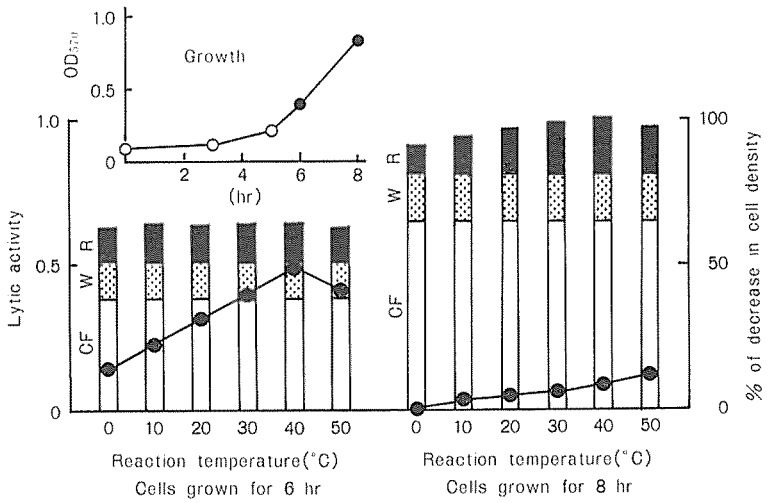

Fig. 2. Effect of temperature on the release of lytic enzyme from V 37 cells grown in polypeptone-yeast extract medium containing NaCl(0.5 mole/l). CF;lytic activity of culture filtrate, W;lytic activity liberated from cells during washing, R;lytic activity released from cells during incubation.

Effect of pH value on the release of lytic enzyme

In order to demonstrate the effect of pH value on the liberation of lytic enzyme from strain V 37 cells grown in the medium containing NaCl (0.5 mole/l), Tris-HCl

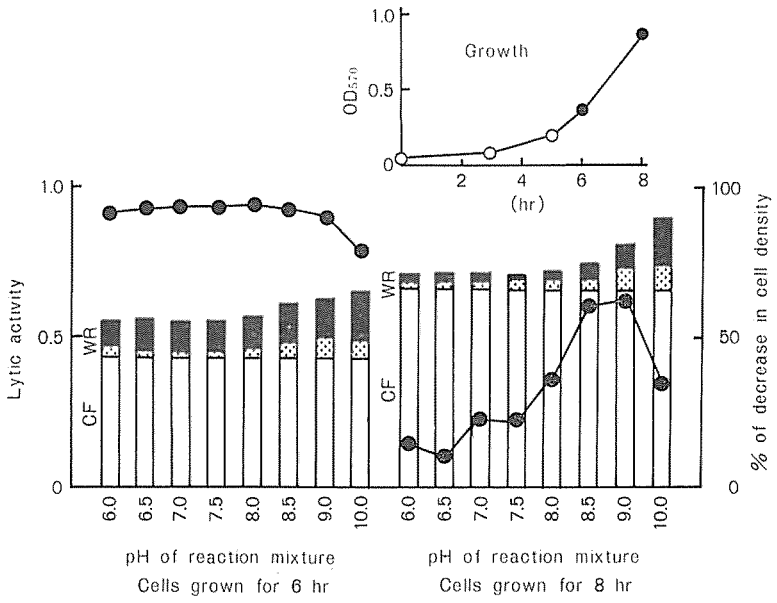

Fig. 3. Effect of pH value on the release of lytic enzyme from V 37 cells grown in polypeptone-yeast extract medium containing NaCl (0.5 mole/l). Tris-HCl buffer was used as pH solution. CF;lytic activity of culture filtrate, W;lytic activity liberated from cells during washing, R;lytic activity released from cells during incubation.

buffer was used for washing and suspending the cells(Fig. 3). No significant difference was observed in the lytic activity in washing solution and that released from cells at pH values between 6.0 and 8.0. Above 8.5 of pH value, the release of lytic enzyme was stimulated. The decrease in initial optical density of the cells grown for 6 hours was about 90–94% at pH values from 6.0 to 9.0.

Fig. 4 shows the effect of pH value on the release of lytic enzyme using Tris-HCl buffer containing 0.5 mole/l of NaCl. The decrease in initial optical density during 2 hours of incubation was about 38–96% in the cells grown for 6 hours and about 26–87% in the cells grown for 8 hours. The maximum liberation of lytic enzyme was observed at pH values from 9.0 to 10.0.

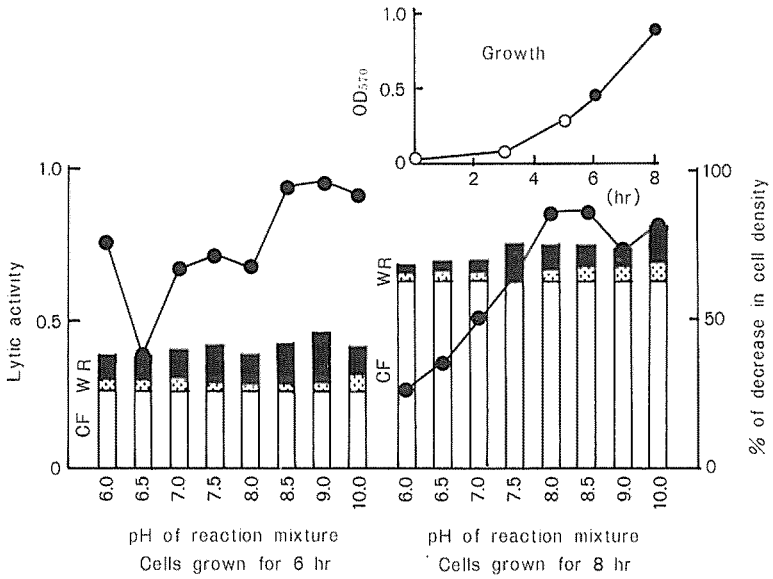

Fig. 4. Effect of pH value on the release of lytic enzyme from V 37 cells grown in polypeptone-yeast extract medium containing NaCl(0.5 mole/l). Tris-HCl buffer containing 0.5 mole/l of NaCl was used as pH solution. CF;lytic activity of culture filtrate, W;lytic activity liberated from cells during washing, R;lytic activity released from cells during incubation.

Effect of inorganic salts on the release of lytic enzyme

NaCl, KCl, artificial seawater; Fig. 5 shows the effect of inorganic salts on the liberation of lytic enzyme from strain V 37 cells. For washing and suspending solution, distilled water, artificial seawater, NaCl solution (0.5 mole/l) and KCl solution (0.5 mole/l) were used. Strain V 37 was grown in the media without added NaCl and containing NaCl(0.5 mole/l) for 6 and 8 hours.

In the case of cells grown in the medium without added NaCl, no significant lytic activity was observed in washing solution and in the supernatant of cell suspension

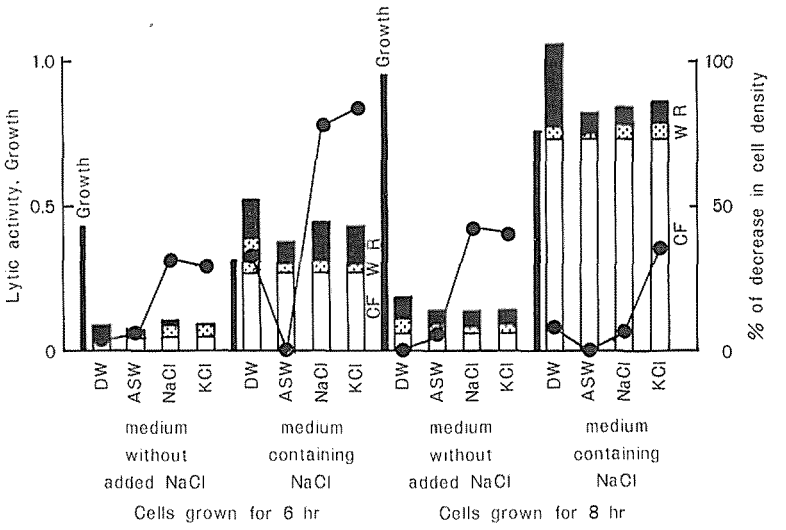

Fig. 5. Effect of inorganic salts (artificial seawater, NaCl and KCl) on the release of lytic enzyme from V 37 cells grown in polypeptone-yeast extract media without added NaCl and containing NaCl (0.5 mole/l). CF;lytic activity of culture filtrate, W;lytic activity liberated from cells during washing, R;lytic activity released from cells during incubation, DW;distilled water, ASW;artificial seawater.

using any one of distilled water, artificial seawater, NaCl and KCl solutions.

On the other hand, in the case of cells grown in the medium containing NaCl, the level of lytic enzyme released from cells was considerable in distilled water rather than in artificial seawater, NaCl and KCl solutions, although the decrease in optical density of cell suspension using distilled water was very slight: about 33% in the cells grown for 6 hours and only 8% in the cells grown for 8 hours. NaCl and KCl seemed to have no effect on the liberation of lytic enzyme from cells of strain V 37.

LiCl, $MgCl_2$, $CaCl_2$, Na_2SO_4, K_2SO_4, Li_2SO_4; As shown in Fig. 6, the decrease in optical density of cell suspension was negligible except for 78.8% of decrease for LiCl. Lytic activity released from cells grown for 6 hours was not so significant by the treatment of every inorganic solution to be tested. Cells grown for 8 hours also showed no change in optical density by the treatment of $MgCl_2$, $CaCl_2$, Na_2SO_4, K_2SO_4 and Li_2SO_4. Slight liberation of lytic enzyme was observed when $MgCl_2$ was used for washing and suspending the cells of strain V 37.

$MgSO_4$, NaH_2PO_4, $NaNO_3$, NH_4Cl; As shown in Fig. 7, the optical density of cell suspension decreased about 37–69% of original cell density in $NaNO_3$ and NH_4Cl solutions. The levl of liberation of lytic enzyme from cells by $NaNO_3$ was slightly high as compared with that by NaCl. No remarkable effect on the release of lytic enzyme was observed by the addition of $MgCl_2$ (0.5 mole/l) into NaCl (0.5 mole/l) solution.

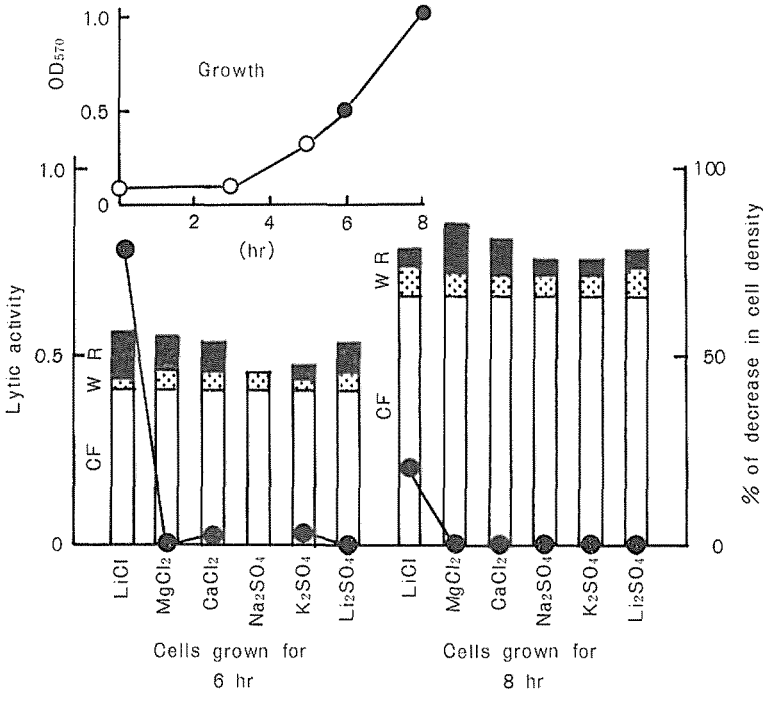

Fig. 6. Effect of inorganic salts(LiCl, $MgCl_2$, $CaCl_2$, Na_2SO_4, K_2SO_4 and Li_2SO_4) on the release of lytic enzyme from V 37 cells grown in polypeptone-yeast extract medium containing NaCl (0.5 mole/l). CF;lytic activity of culture filtrate, W;lytic activity liberated from cells during washing, R; lytic activity released from cells during incubation.

Effect of glucose on the release of lytic enzyme

As shown in Fig. 7, although the addition of glucose(3 g/l) showed the decrease in optical density of cell suspension at almost same level as compared with that without added glucose, the liberation of lytic enzyme was slightly inhibited.

Effect of chloramphenicol on the release of lytic enzyme

Addition of chloramphenicol (200 μg/ml) to a culture of strain V 37 inhibited the growth and lytic enzyme production almost completely. Chloramphenicol at a concentration of 10 μg/ml did not cause inhibition of enzyme release by strain V 37 (Fig. 7).

Effect of various chemicals on the release of lytic enzyme

As the chemicals active to the bacterial cell membrane, the following chemicals were used; triton X-100 (0.1%), tween 80 (0.1%) deoxycholic acid (0.1%), guanidine-HCl (0.4 M), trypsin (10 μg/ml), lysozyme (100 μg/ml) and EDTA-2Na (0.03 M). As shown in Fig. 8, the density of bacterial cells did not decrease considerably by the treatment

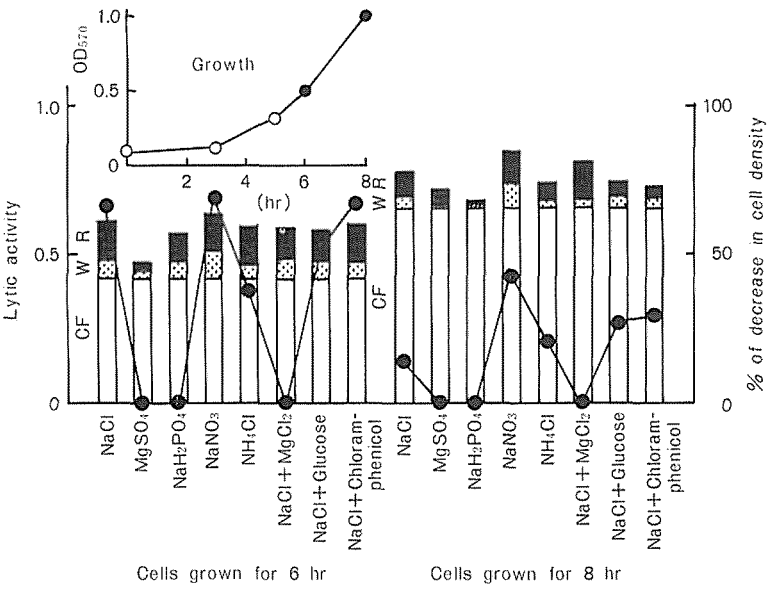

Fig. 7. Effects of inorganic salts(NaCl, $MgSO_4$ NaH_2PO_4, $NaNO_3$, NH_4Cl, $NaCl+MgCl_2$), glucose and chloramphenicol on the release of lytic enzyme from V 37 cells grown in polypeptone-yeast extract medium containing NaCl(0.5 mole/l).
CF;lytic activity of culture filtrate, W;lytic activity liberated from cells during washing, R;lytic activity released from cells during incubation.

of triton X-100, tween 80 and guanidine-HCl. Triton X-100 was most effective for the release of lytic enzyme from cells of strain V 37. However, no effect of NaCl addition to various chemicals was observed on the release of lytic enzyme.

Discussion

MALVEAUX and SAN CLEMENTE (1967) described that elution of loosely bound acid phosphatase from *Staphylococcus aureus* was a function of pH and ionic strength: The enzyme was maximaly eluted from log-phase cells with 1.0 mole/l of KCl at pH 7.5 and the amount eluted increased as a function of ionic strength up to 1.0 mole/l, but stationary-phase cells required twice the concentration of KCl. They inferred that the enzyme was associated with the cells through electrostatic interactions. COLES and GROSS (1967a, 1967b) observed that part of cell-bound exopenicillinase of *Staphylococcus aureus* may be liberated instantaneously by inorganic anions. Maximum liberation was achieved with either phosphate or arsenate. Polyanions and low concentrations(0.1 M) of organic anions were also effective in releasing penicillinase.

On the other hand, penicillinase of *Bacillus subtilis* was not eluted from cells by treating with high concentrations of salts (POLLOCK 1961b).

In this study, no remarkable effect on the release of lytic enzyme of strain V 37

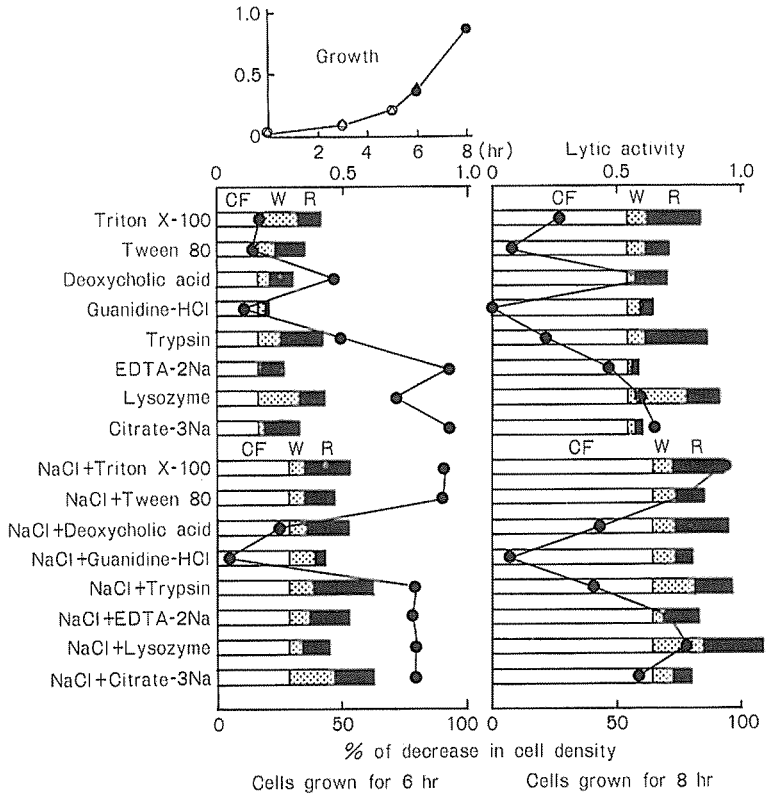

Fig. 8. Effect of various chemicals on the release of lytic enzyme from V 37 cells grown in polypeptone-yeast extract medium containing NaCl (0.5 mole/l). CF;lytic activity of culture filtrate, W;lytic activity liberated from cells during washing, R;lytic activity released from cells during incubation.

was observed by 0.5 mole/l of inorganic salts. A further study would be required to examine the effect of anions (phosphate, arsenate, organic anions etc.) on the liberation of lytic enzyme from strain V 37 cells.

The penicillinase liberation from *Staphylococcus aureus* by phosphate and arsenate showed marked pH-dependence (COLES and GROSS 1967a). Liberation of penicillinase by *Bacillus licheniformis* was also dependent upon temperature and pH value (LAMPEN 1967). POLLOCK (1961b) reported that penicillinase liberation from *Bacillus subtilis* did not take place at 0 °C and was nearly completely inhibited at pH values below 6.0. Penicillinase liberation increased further with temperatures above 35 °C, but at 45 °C this was always associated with considerable cell damage. MALVEAUX and SAN CLEMENTE (1967) also reported that maximal elution of loosely bound acid phosphatase from *Staphylococcus aureus*, presumably from the surface of cells, occured in the alkaline pH range.

These facts suggest that liberation may have an enzyme basis.

Chloramphenicol, at a concentration(40 μg/ml) which inhibited penicillinase synthesis, partially inhibited enzyme secretion of *Bacillus* sp. if added durirg the period of active synthesis (POLLOCK 1961b, LAMPEN 1967, SARGENT *et al.* 1969).

Release of lytic enzyme from strain V 37 was observed highly in the alkaline pH range(Fig. 3) and was not inhibited by chloramphenicol at a concentration of 10 μg/ml.

Sodium deoxycholate (0.05–1.0%) increased penicillinase liberation from *Bacillus subtilis* but rapidly let to cell damage (α-glucosidase leak) (POLLOCK 1961b), and at a concentration of 10 mg/ml was effective for rapid liberation of penicillinase from *Bacillus subtilis* (KUSHNER and POLLOCK 1961). About 0.5% of sodium deoxycholate, which disintegrated the membrane preparation, prevented release of free penicillinase of *Bacillus licheniformis* (LAMPEN 1967). EDTA, which presumably removed Mg^{2+} and other cations from the membranes, was also inhibitory for the release of free penicillinase of *Bacillus* sp. (POLLOCK 1961b, LAMPEN 1967). Trypsin(1 mg/ml) rapidly liberated a large proportion of penicillinase even in the presence of 0.05 M Mg^{2+} (KUSHNER and POLLOCK 1961).

However, deoxycholic acid(0.1%), EDTA-2Na (0.03 M) and trypsin(1 mg/ml) did not show remarkable effect on the release of lytic enzyme from strain V 37 cells, while triton X-100 (0.1%) was effective for the enzyme elution (Fig. 8). The decrease in cell density, that is cell damage, was significant under some conditions used for the release experiments. In this study, the measurement of α-glucosidase leak was not carried out as 'marker' of cell damage or cell lysis. Further studies on the problem of cell damage would be necessary in order to demonstrate the effect of inorganic salts and other chemicals on the release of lytic enzyme from strain V 37 cells.

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