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Ohba, Riichiro
Laboratory of Microbial Technology, Faculty of Agriculture, Kyushu University

Ueda, Seinosuke
Laboratory of Microbial Technology, Faculty of Agriculture, Kyushu University

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Optimum Medium Components and Culture Conditions for the Production of Intra- and Extra-cellular Pullulanase by *Aerobacter aerogenes*

Riichiro Ohba and Seinosuke Ueda

Laboratory of Microbial Technology, Faculty of Agriculture,
Kyushu University 46–09, Fukuoka 812

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Culture conditions for pullulanase production by *Aerobacter aerogenes* RS-1 were found to be quite different from those of other strains of the same species reported earlier. Among the components investigated, $(NH_4)_2SO_4$ and NH_4Cl produced pullulanase mostly inside the cells and the enzyme was not released from them. CH_3COONH_4 was the most effective nitrogen source for the production of extracellular pullulanase. Liquefied potato starch was found to be the most suitable carbon source in both cases of the intra- and extra-cellular pullulanase production. Mono- and di-saccharides could hardly produce pullulanase which may be an "induced enzyme". KC1 was not necessary for pullulanase production. $CaCO_3$ was an essential component for pH-control in the early stage of intracellular enzyme production. Peptone, $MgSO_4 \cdot 7H_2O$ and $FeSO_4 \cdot 7H_2O$ were found to play considerably important roles for pullulanase production and it became evident that the absence of each of them caused a decrease of 20–80 % of pullulanase production. K_2HPO_4 was the most important component since the bacterial growth was nil without it and consequently pullulanase could not be produced. Under optimum culture conditions, the periods for maximum intra- and extra-cellular pullulanase production were 48 and 96 hr respectively. Extracellular pullulanase production started later than intracellular one and then intracellular pullulanase began to secrete from cells into culture broth.

INTRODUCTION

The enzymes which hydrolyze starch are represented by a-amylase, β-amylase and glucoamylase. They have been investigated in detail for a long time. Afterward isoamylase, containing R-enzyme, (EC 3. 2. 1. 9) was first found from yeast (Kobayashi and Maruo, 1949; Maruo and Kobayashi, 1951) and broad bean (Hobson et al., 1951). The enzyme which hydrolyzes a-l. 6-glucosidic linkages has attracted special interest recently.

Pullulanase (EC 3.2.1.41, pullulan 6-glucanohydrolase) is an enzyme which hydrolyzes a-l. 6-glucosidic linkages not only in starch but also in pullulan (a kind of polysaccharide, Ueda ***et al.***, 1963), being named "debranching enzyme". Starch is hydrolyzed to amylose by an end-mechanism of action of pullulanase and pullulan is also cleaved at random finally to maltotriose (Ohba and Ueda, 1975). Direct production of maltose and maltotriose from starch and pullulan by an immobilized multienzyme of pullulanase and β-amylase has been at-

tempted (Ohba et al., 1978; Ohba and Ueda, 1980).

Since Wallenfels and Rached (1966) obtained crystalline intracellular pullulanase from *Aerobacter aerogenes*, substrate specificity (Abdullah *et al., 1966;* Abdullah and French, 1970), mechanism of action (Wallenfels ***et al., 1969;*** Drummond ***et al., 1969;*** Ohba and Ueda, 1975), molecular weight (Wallenfels ***et al.,*** 1969; Eisele ***et al.,*** 1972; Mercier ***et al.,*** 1972; Ohba and Ueda, 1973) etc. of intracellular pullulanase have gradually been studied by various workers. With regard to extracellular pullulanase, the authors (Ueda and Ohba, 1972; Ohba and Ueda, 1975) reported some chemical and enzymatic properties using crystalline enzyme.

Wallenfels *et al. (1966)* have reported on the mode of pullulanase formation from *A. aerogenes* which distinctly produced intra- and extra-cellular pullulanase by using maltose and glucose as carbon source. A strain of our *A. aerogenes* differed extremely in the mode of pullulanase formation from another one. That is, when $(NH_4)_2SO_4$ was used as a nitrogen source, enzyme was mainly produced inside the cells but when CH_3COONH_4 was used instead of $(NH_4)_2SO_4$, enzyme was mainly produced to the culture broth (Fujio ***et al.,*** 1970; Ueda and Ohba, 1972; Ohba and Ueda, 1973). However, it is required to investigate the effects of other components, their concentration and pH of the medium, in order to have a clear idea about this interesting mechanism of intra- and extra-cellular enzyme production. The present paper describes the effects of various medium components and the most suitable culture condition for the production of both intra- and extra-cellular pullulanase.

MATERIALS AND METHODS

Microorganism

Aerobacter aerogenes RS-1 (a mutant of *A. aerogenes No.* 105) which had been isolated and preserved in the authors' laboratory was used for pullulanase production.

Media and culture methods

The composition of slant agar medium for stock culture and precultivation was reported in the previous paper (Ueda and Ohba, 1972). The basal media, which were used conventionally by the authors, are listed in Table 1. A-medium was used for the production of intracellular pullulanase and B-medium for that of extracellular one. Table 2 shows the media used by Wallenfels *et al.* (1966) for the intra- and extra-cellular pullulanase production from *A. aerogenes.*

A loopful of the microorganism from a precultivated slant was inoculated into 100 ml medium contained in a 500ml Erlenmeyer flask and incubated at 30°C on a reciprocal shaker. Pullulanase activity was assayed after 48 hr culture in A-medium and 96 hr culture in B-medium unless otherwise specified.

Liquefied starch

Starch was treated with α-amylase (Neospitase K, Nagase Sangyo Ltd.) at 70–80°C for partial hydrolysis. The reaction was continued to proceed

Table 1. Basal medium for pullulanase production. Initial pH 7.6 (both media).

Component	Content (%, w/v)
A-medium	
Liquefied potato starch	1.0
$(NH_4)_2SO_4$	0.8
K_2HPO_4	0.1
$MgSO_4 \cdot 7H_2O$	0.05
KCl	0.05
Peptone	0.05
$FeSO_4 \cdot 7H_2O$	0.01
$CaCO_3$	0.5
B-medium	
Liquefied potato starch	1.0
CH_3COONH_4	0.8
K_2HPO_4	0.1
$MgSO_4 \cdot 7H_2O$	0.05
KCl	0.05
Peptone	0.05
$FeSO_4 \cdot 7H_2O$	0.01

Table 2. Medium used by Wallenfels et al. (1966) for pullulanase production. Initial pH 7.2 (both media)

Component	Content (%, w/v)
C-medium	
Maltose	0.8
$NaNO_3$	0.3
K_2HPO_4	0.1
$MgSO \cdot 7H_2O$	0.05
KCl	0.05
$FeSO_4 \cdot 7H_2O$	0.001
D-medium	
Maltose	0.4
Glucose	0.4
$NaNO_3$	0.3
K_2HPO_4	0.1
$MgSO_4 \cdot 7H_2O$	0.05
KCl	0.05
$FeSO_4 \cdot 7H_2O$	0.001

until the iodine coloration of the solution showed orange-red when a few drops of the reaction mixture was added to 5 ml of 0.001 N iodine-potassium iodide solution. Then the reaction was stopped by adjusting its pH at 2.0 with 1 N HCl. The filtrate obtained after filtration through a Toyo Roshi No. 101 filter paper was used as liquefied starch.

Assay of extracellular pullulanase

After cultivation, culture broth was centrifuged at 10,000 rpm for 10 min. The activity of the supernatant solution (crude enzyme solution) was used for extracellular pullulanase activity. The activity was expressed as pullulanase units (U) per ml of culture broth. Pullulan, which was prepared from *Pullularia pullulans* by the procedure of Ueda ***et al.*** (1963) with slight modification, was

used as a substrate of the enzyme reaction.

Pullulanase activity was assayed using a reaction mixture consisting of 1 ml of 0.5 % (w/v) pullulan and 0.5 ml of 0.5 M acetate buffer, pH 6.0. After preincubation of the reaction mixture at 40°C for a few minutes, 0.5 ml of suitably diluted enzyme solution was added followed by incubation at 40°C for 30 min. Reducing sugars released by enzyme action at 10 and 30 min were then measured by the Somogyi-Nelson method (Nelson, 1944; Somogyi, 1945) with maltotriose (Hayashibara Biochemical Lab. Inc.) as standard. One unit of pullulanase activity is defined as the amount of enzyme that liberates 1 μmole maltotriose at 40°C in 1 hr under the conditions described above.

Assay of intracellular pullulanase activity

After cultivation, 50ml of culture broth was centrifuged at 10, 000 rpm for 10 min. The cells were collected and washed with water. The washed cells were suspended in 50ml of water. The activity of the suspension was used as intracellular pullulanase activity. To prevent sedimentation of cells during the enzymatic reaction, a microspatula was put in the test tube (16×110 mm), and vibration of the shaker (amplitude, 20 mm; frequency, 160 times/min) caused it to rotate, keeping the cells in suspension. The other procedures were carried out by the same methods as described in the assay of extracellular pullulanase activity.

Determination of cell growth

Bacterial cell growth was measured using a spectrophotometer, as the optical density (O.D.) at 660 nm of the culture broth after diluting 20 folds with water. In the case of A-medium (usually containing $CaCO_3$), 0.01 N HCI was used to dilute instead of water, and residual $CaCO_3$ was fully neutralized.

Determination of residual sugars

Reducing sugars in each culture broth was determined as glucose equivalent by the Somogyi-Nelson method after hydrolyzing 1 ml of the supernatant with 2 ml of 1 N HCl for 3 hr and neutralizing with sodium hydrogen carbonate.

RESULTS AND DISCUSSION

Comparison of the basal media with media **used by Wallenfels et *al.***

The results of formation of intra- and extra-cellular pullulanase by ***A.*** aerogenes RS-1 using the basal media (A- and B-media) and the media used by Wallenfels et al. (1966) (C- and D-media) are summarized in Table 3. The intracellular pullulanase production was remarkably increased and extracellular one decreased in the A-medium, and conversely extracellular pullulanase production was significantly increased and intracellular one decreased in the B-medium.

On the other hand, in the C- and D-media, there was no intra- and extra-cellular pullulanase formation and the cells were poorly grown.

Accoring to Wallenfels ***et al.*** (1966), their ***A. aerogenes*** produced both intra-

Table 3. Comparison of the basal media with the media used by Wallenfels et al.

Medium	Cultivation period (days)	Final pH	Growth (O.D. at 660 nm)	Pullulanase activity	
				Intracellular (U/ml)	Extracellular (D/ml)
A		8.12	9.6	33.1	1.2
B	*4*	9.34	9.0	3.3	52.9
C	*2*	8.91	3.2	0	0.8
	4	9.08	3.2	0	0.7
D	2	8.94	3.2	0	0.9
	4	*9.04*	3.1	0	0.5

and extra-cellular pullulanase in the C-medium and only intracellular one in the D-medium. This investigation revealed that the mode of pullulanase formation by A. *aerogenes* RS-1 differed from that by bacteria used by Wallenfels et *al.* (1966), and showed its own characteristics, that is, intra- and extracellular enzyme could not be specifically produced by using carbon sources such as maltose and glucose.

Effect of nitrogen sources in the presence of calcium carbonate

Inorganic and organic nitrogen compounds (0.8%, w/v) containing especially ammonium group in most cases were added into the medium. Further, 0.5% (w/v) calcium carbonate sterilized in a hot air oven was added into the medium at the time of inoculation. After cultivation for 4 days, the culture broth was assayed.

The results of enzyme production using these nitrogen sources are shown

Table 4. Effect of nitrogen sources on pullulanase production in the presence of $CaCO_3$.

Nitrogen source	Final pH	Growth (O.D. at 660nm)	Pullulanase activity	
			Intracellular (H/ml)	Extracellular (D/ml)
$(NH_4)_2SO_4$	8.7	7.8	*44.* a	0.9
NH_4Cl	7.5	7.6	*37. 6*	0.1
$(NH_4)_2HPO_4$	7.7	5.8	*33.0*	24; 4
$(NH_4)H_2PO_4$	7.6	6.2	33; 7	27.1
$(NH_4)_2C_4H_4O_6$	8.9	9.0	5.8	10.8
CH_3COONH_4	8.9	11.2	8.5	41.0
$HCOONH_4$	9.3	8.4	33. a	16.0
NH_4NO_3	8.2	10.0	8.4	0
$FeSO_4(NH_4)_2SO_4$	7.8	10.8	6.1	0
$Fe(SO_4)_3(NH_4)_2SO_4$	7.9	9.0	0	6.4
$NH_4H_2C_6H_5O_7$	8.5	17.4	8.1	2.6
NH_2CONH_2	9.3	2.8	0	2.7
$NaNO_3$	8.9	5.2	8.6	11.3
KNO_3	9.2	5.4	0	10.3
$(NH_4)_2C_2O_4 \cdot H_2O$	9.3	2.2	0	0
H_2NCSNH_2	6.5	1.4	0	0
Peptone	7.6	11.4	0	0
Yeast extract	8.2	10.0	0	0
Malt extract	7.5	2.6	0	6.5
Tryptose	7.8	13.2	13.8	3.8
Soy bean	8.2	1.4	0	0
Casamino acids	8.8	11.4	12.6	5.8

in Table 4. The nitrogen sources produced high extracellular pullulanase were CH_3COONH_4, $(NH_4)H_2PO_4$, $(NH_4)_2HPO_4$ and $HCOONH_4$, in decreasing order. On the other hand, $(NH_4)_2SO_4$, NH_4Cl, HCOONH,, $(NH_4)H_2PO_4$ and $(NH_4)_2HPO_4$, also in decreasing order, acted as effective nitrogen sources for production of high intracellular pullulanase. However, $(NH_4)_2HPO_4$, $(NH_4)H_2PO_4$ and $HCOONH_4$ produced both intra- and extra-cellular pullulanase simultaneously, whereas particularly $(NH_4)_2SO_4$ and NH_4Cl produced pullulanase more abundantly in the cells and the enzyme was not released from them.

CH_3COONH_4 released largely pullulanase from the cells into the culture broth and accumulation of pullulanase in the cells was very small.

From the experimental results obtained above, productivity of pullulanase was not dependent on the final pH and cell growth using various nitrogen sources.

Effect of nitrogen sources in the absence of calcium carbonate

The effect of eight nitrogen sources selected on the basis of their effects on intra- and extra-cellular pullulanase production (Table 4), was investigated with the medium free from calcium carbonate (Table 5). The extracellular pullulanase productivity was found to be the highest in case of CH_3COONH_4 among the nitrogen sources investigated.

Intracellular pullulanase was accumulated in small quantity in the cells in *every* case of nitrogen sources.

It appears that if the pH for the cultivation of the bacteria is lowered below 5.0, the pullulanase production becomes increasingly difficult.

Table 5. Effect of nitrogen source on pullulanase production in the absence of $CaCO_3$.

Nitrogen source	Final pH	Growth (O.D. at 660nm)	Pullulanase activity	
			Intracellular (D/ml)	Extracellular (U/ml)
$(NH_4)_2SO_4$	4.0	1.8	0	0
NH_4Cl	4.0	2.8	0	0
$(NH_4)_2HPO_4$	5.3	9.2	0	10.0
$(NH_4)H_2PO_4$	5.2	9.4	0	3.5
$NaNO_3$	6.8	8.8	3.5	10.0
KNO_3	9.1	6.8	5.4	12.2
$(NH_4)_2C_4H_4O_6$	4.7	6.8	0	0
CH_3COONH_4	8.9	11.0	5.3	53.0

Effect of the concentration of nitrogen sources

It was revealed that when $(NH_4)_2SO_4$ or NH_4Cl was used as a nitrogen source of the medium, the cells accumulated much enzyme whereas a good amount of it was released from the cells in case of CH_3COONH_4. Therefore, A- and B-media are called as the intra- and extra-cellular enzyme producing medium respectively.

The pullulanase production was investigated by using 0 to 1.2 % (w/v) $(NH_4)_2SO_4$ in the intracellular enzyme producing medium. As shown in Fig. 1–A, the accumulation of the intracellular enzyme was increased by the increase of concentration of $(NH_4)_2SO_4$. Pullulanase was released in a very small quan-

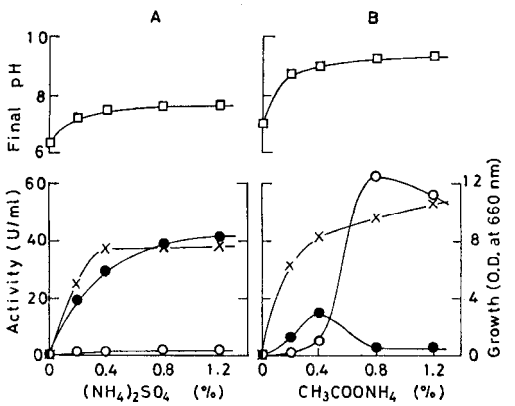

Fig. 1. Effect of the concentration of nitrogen sources on pullulanase production. A: intracellular enzyme producing medium. B: extracellular enzyme producing medium. ●: intracellular pullulanase activity. ○: extracellular pullulanase activity. x: cell growth. ❑ : pH.

tity in the medium in every case within the concentration investigated. Up to 0.4 % concentration, cell growth was poor and final pH was changed either in neutral or acidic side.

Then, the pullulanase production was investigated by using 0 to 1.2 % CH_3COONH_4 in the extracellular enzyme producing medium. As shown in Fig. 1-B, up to 0.5 % concentration, a small amount of pullulanase was produced and the quantity of intracellular enzyme was higher than that of extracellular one. The cell growth was also less. The optimum amount of CH_3COONH_4 was 0.8 %, the amount higher than this causing a decrease in extracellular pullulanase production. However, the cell growth and final pH were found to increase by the increase of concentration of CH_3COONH_4.

Effect of carbon sources

The effects of some mono-, di- and poly-saccharides were studied as carbon sources using the intracellular enzyme producing medium (Table 6). Liquefied potato starch showed the highest productivity of intracellular enzyme and then potato starch, though the cells could satisfactorily grow using all the carbon sources except dextran. There was no correlation between productivity of enzyme and final pH.

Pullulanase production was investigated using the same carbon sources in the extracellular enzyme producing medium (Table 7). The final pH was found to be below 6.0 and the cell growth was poor in case of glucose, galactose and fructose. All of these carbon sources did not cause extracellular enzyme production. Liquefied potato starch was also the most suitable carbon source for extracellular enzyme production. Liquefied starches acted as effective carbon source than raw starches. In these cases, there was correlation between productivity and final pH.

As pullulanase, which hydrolyzes α-1.6-glucosidic linkages, could be produced mainly from polysaccharides possessing α-1.4- and 1.6-glucosidic link-

Table 6. Effect of carbon sources on intracellular pullulanase production.

Carbon source	Final pH	Growth (O.D. at 660 nm)	Pullulanase activity	
			Intracellular (U/ml)	Extracellular (U/ml)
Soluble starch	7.1	7.5	7.1	14.7
Potato starch	7.6	10.7	28.2	1.1
Liquefied potato starch	8.1	9.6	34.1	1.3
Corn starch	8.2	8.8	11.8	1.4
Liquefied corn starch	8.2	7.2	23.1	1.5
Sweet potato starch	8.6	8.1	5.7	0.8
Liquefied sweet potato starch	8.3	5.8	6.2	0.4
Wheat starch	8.2	8.7	4.3	0.6
Liquefied wheat starch	8.3	5.5	4.4	0.4
Cassava starch	8.8	8.3	4.5	2.5
Liquefied cassava starch	8.2	6.9	24.1	2.8
Dextran	7.7	0.4	0	0
Pullulan	8.0	9.9	5.4	1.2
Maltose	7.9	10.3	7.5	0.6
Sucrose	8.1	9.8	0.5	1.1
Lactose	8.1	10.5	1.0	0.5
Glucose	8.0	11.3	5.0	0.4
Galactose	8.3	10.5	1.2	1.6
Fructose	8.0	11.1	0	0.5
Mannite	7.8	11.5	0.7	0

Table 7. Effect of carbon sources on extracellular pullulanase production.

Carbon source	Final pH	Growth (O.D. at 660 nm)	Pullulanase activity	
			Intracellular (U/ml)	Extracellular (U/ml)
-Soluble starch	9.1	9.5	2.2	34.8
Potato starch	9.2	12.1	9.5	4.6
Liquefied potato starch	9.3	10.7	3.2	58.9
Corn starch	9.3	11.7	11.7	0.7
Liquefied corn starch	9.2	10.0	1.7	45.7
Sweet potato starch	9.3	13.0	5.3	0.9
Liquefied sweet potato starch	9.4	8.9	1.5	29.8
Wheat starch	9.5	13.9	11.1	0.6
Liquefied wheat starch	9.4	8.1	1.3	22.9
Cassava starch	9.3	12.5	4.0	0.9
Liquefied cassava starch	9.4	9.7	2.4	57.6
Dextran	4.0	9.4	0	0
Pullulan	9.2	12.1	2.0	8.6
Maltose	9.3	11.4	1.2	9.7
Sucrose	9.3	10.3	0.5	0.9
Lactose	9.2	12.4	0.5	1.0
Glucose	5.5	0.4	0	0
Galactose	6.0	0.2	0	0
Fructose	5.7	0.6	0	0
Mannite	9.2	11.1	1.0	1.0

ages, it is considered that pullulanase is an "induced enzyme".

Effect of the concentration of liquefied potato starch

The effects of various concentrations of liquefied potato starch on the intra- and extra-cellular pullulanase production are shown in Fig. 2. The cell growth was increased by the increase of concentration of liquefied potato starch. The optimum concentration of liquefied potato starch for the intra- and extra-cellular enzyme production were found to be 1.25 and 1.5 %, respec-

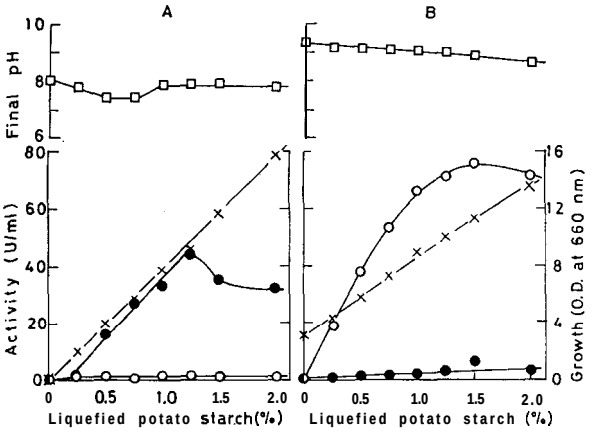

Fig. 2. Effect of the concentration of liquefied potato starch on pullulanase production. Symbols same as in Fig. 1.

tively (Fig. 2-A and B).

Effect of the concentration of dipotassium hydrogen phosphate

The results on the effect of concentration of K_2HPO_4 are shown in Fig. 3. Above 0.05 %, there was almost no variation of cell growth. The optimum concentrations of K_2HPO_4 for intra- and extra-cellular enzyme production were 0.1 and 0.2 %, respectively. At higher concentrations above 0. 1 %, the intracellular enzyme production was found to decrease very sharply (Fig. 3-A).

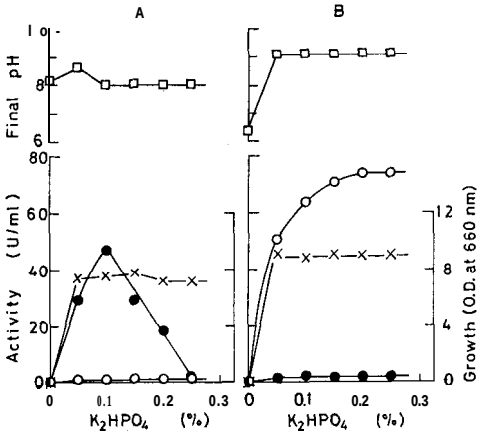

Fig. 3. Effect of the concentration of K_2HPO_4 on pullulanase production. Symbols same as in Fig. 1.

Effect of the concentration of magnesium sulfate

The results on the effect of concentration of $MgSO_4 \cdot 7H_2O$ are shown in Fig. 4. Above 0.025 %, the cell growth and final pH were found to be approximate-

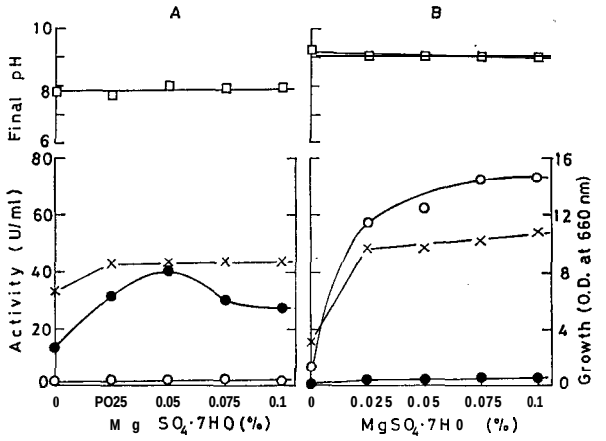

Fig. 4. Effect of the'concentration of $MgSO_4 \cdot 7H_2O$ on pullulanase production. Symbols same as in Fig. 1.

ly constant. The most favourable concentrations of $MgSO_4 \cdot 7H_2O$ for the intra- and extra-cellular enzyme production were 0.05 and 0.075 %, respectively.

Effect of the concentration of potassium chloride

As shown in Fig. 5, pullulanase production, cell growth and final pH did not vary within the concentrations investigated. Therfore, it does not seem to be an essential component for intra- and extra-cellular pullulanase formation.

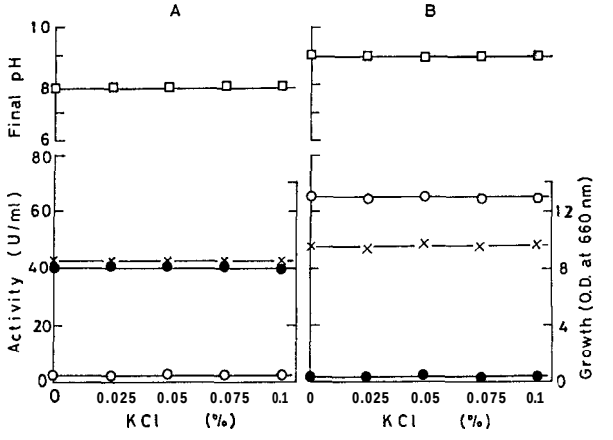

Fig. 5. Effect of the concentration of KC1 on pullulanase production. Symbols same as in Fig. 1.

Effect of the concentration of peptone

The results on the effect of concentration of peptone are shown in Fig. 6. Cell growth and final pH did not vary in case of intracellular enzyme produc-

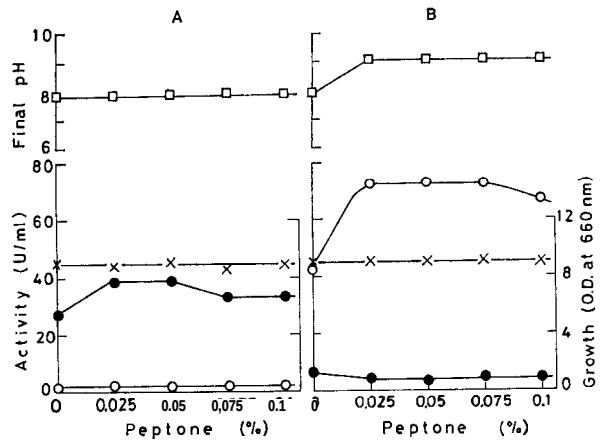

Fig. 6. Effect of the concentration of peptone on pullulanase production. Symbols same as in Fig. 1.

tion. The optimum concentration of peptone for the intra- and extra-cellular enzyme production were 0.025-o. 05 and 0.025-o. 075 %, respectively.

Effect of the concentration of ferrous sulfate

The pullulanase production was investigated using 0 to 0.04 % $FeSO_4 \cdot 7H_2O$ in the intra- and extra-cellular enzyme producing media. The results are shown in Fig. 7. In the intracellular enzyme producing medium, final pH was scarcely varied but the amount of intracellular pullulanase was fairly varied (Fig. 7-A). The most suitable concentration of $FeSO_4 \cdot 7H_2O$ was 0.02 %.

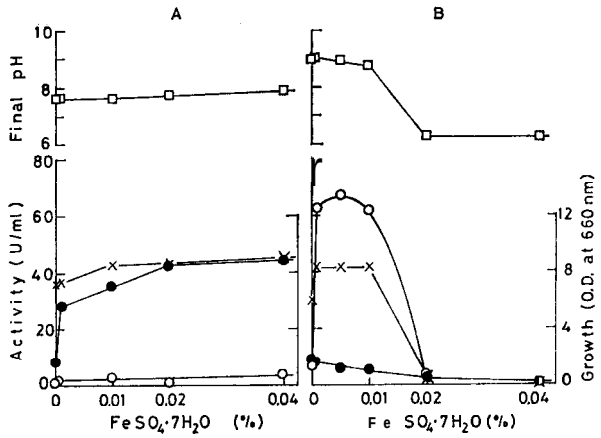

Fig. 7. Effect of the concentration of $FeSO_4 \cdot 7H_2O$ on pullulanase production. Symbols same as in Fig. 1.

In the extracellular enzyme producing medium, the productivity of extra-

cellular pullulanase, final pH and bacterial growth were greatly varied (Fig. 7-B). The optimum concentration of $FeSO_4 \cdot 7H_2O$ was 0.005 %. At higher concentrations above 0.02 %, the final pH decreased to acidic side, the cells could hardly grow and consequently pullulanase production did not occur. It seems that special attention should be paid to the content of $FeSO_4 \cdot 7H_2O$ particularly for the extracellular enzyme production.

From the facts described above, it is clear that the intracellular pullulanase production by this bacteria could not be converted into extracellular one and ***vice versa*** by using these components (liquefied potato starch, dipotassium hydrogen phosphate, magnesium sulfate, peptone and ferrous sulfate) at various concentrations.

Effect of the concentration of calcium carbonate

In this experiment, $CaCO_3$ was added to both media in the range of 0 to 1.5 % although $CaCO_3$ had not been used before in the extracellular enzyme producing medium. The results are shown in Fig. 8. When $CaCO_3$ was omitted from the intracellular enzyme producing medium, final pH and cell growth were decreased and the intracellular pullulanase production did not occur (Fig. 8-A).

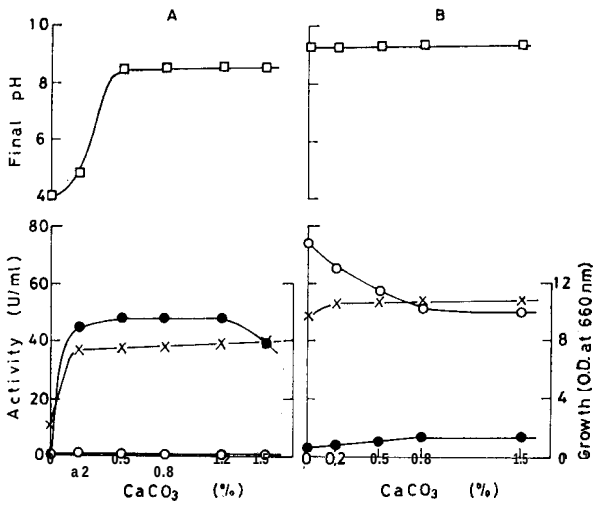

Fig. 8. Effect of the concentration of $CaCO_3$ on pullulanase production. Symbols same as in Fig. 1.

On the other hand, the extracellular pullulanase production was found to decrease gradually by the increase of concentration of $CaCO_3$ but the final pH and cell growth remained almost unchanged. In the absence of $CaCO_3$, the extracellular pullulanase was produced maximaly.

Effect of pH

The initial pH of the intracellular pullulanase producing medium in the presence of $CaCO_3$ was adjusted at various pH by adding 0.1 N HCI or NaOH.

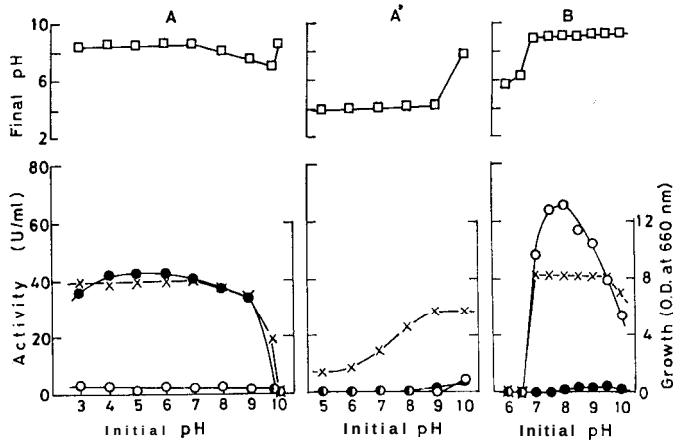

Fig. 9. Effect of initial pH on pullulanase production. A : intracellular enzyme producing medium in the presence of $CaCO_3$. In this case, initial pH was the value before adding of $CaCO_3$. A': intracellular enzyme producing medium in the absence of $CaCO_3$. B : extracellular enzyme producing medium. Other symbols same as in Fig. 1.

At the wide range between pH 3 and 9, the cell growth was rich and the intracellular pullulanase activity was high (Fig. 9-A). At pH 10, there was no growth and enzyme formation.

Then enzyme productivity was investigated in the intracellular pullulanase producing medium in the absence of $CaCO_3$ (Fig. 9-A'). Up to initial pH 9, the growth was poor, final pH became low at around 4 and there was no enzyme formation. At initial pH 10, both enzymes could be hardly produced although the final pH became at 8.0. The results suggested that the pullulanase production was not performed with the medium in the absence of $CaCO_3$ whatever initial pH was. A delicate pH-control *in vivo* during cultivation might be concerned in the biosynthesis of pullulanase. $CaCO_3$ was an essential component for the intracellular enzyme production.

On the other hand, in case of the extracellular enzyme production, when the initial pH was on acidic side, the bacterial growth was poor and final pH remained at acidic side (Fig. 9-B). The enzyme production was nil. If the initial pH was at alkaline side, the final pH of the culture broth always reached at about 8.5. The optimum initial pH for the extracellular enzyme production was 8.0.

Role of each component in pullulanase formation

On the basis of the experimental data, some essential components in the pullulanase producing media and their roles were considered in some detail. Fig. 10 shows the amount of pullulanase (activity), final pH and cell growth when only one component was lacked from the basal medium. In the intracellular pullulanase production, peptone was not concerned in the ceil growth but the amount of pullulanase was decreased by 30 % in the absence of this

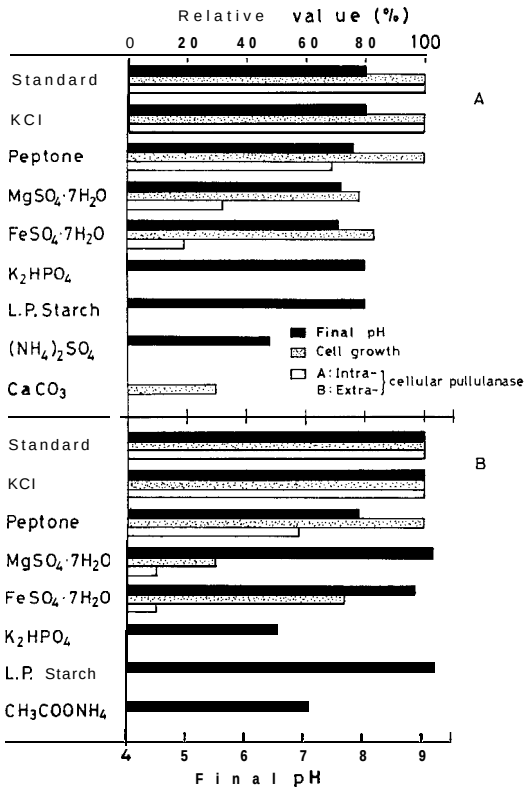

Fig. 10. Effect of lacking of medium component on pullulanase production. A: intracellular enzyme producing medium. B: extracellular enzyme producing medium.

compound (Fig. 10-A). The intracellular pullulanase production was found to be more decreased than cell growth by $MgSO_4$ and $FeSO_4 \cdot 7H_2O$. In the absence of K_2HPO_4, liquefied potato starch or $(NH_4)_2SO_4$, the bacteria could not grow at all and pullulanase was not formed. It was found that all other components except KC1 played important roles for pullulanase production.

The effects resulted from lacking of components for extracellular enzyme production were almost similar to those of the intracellular enzyme production (Fig. 10-B).

The optimum medium for pullulanase production and the time course of cultivation

The optimum media for intra- and extra-cellular enzyme production is given in Table 8. The time course of cell growth, pH and pullulanase production were investigated using the optimum media (Figs. 11 and 12). In the intracellular pullulanase producing medium, residual sugar of carbon source had been consumed within 18 hr (Fig. 11). The pH-value of the culture broth once decreased to 6.2, and then increasd gradually around 8.0. Intracellular

Table 8. The optimum medium for pullulanase production.

Component	Content (%, w/v)
Intracellular pullulanase producing medium	
Liquefied potato starch	1.25
$(NH_4)_2SO_4$	0.8
K_2HPO_4	0.1
$MgSO_4 \cdot 7H_2O$	0.05
Peptone	0.03
$FeSO_4 \cdot 7H_2O$	0.02
$CaCO_3$	0.5
Initial pH	7.0
Extracellular pullulanase producing medium	
Liquefied potato starch	1.5
CH_3COONH_4	0.8
K_2HPO_4	0.2
$MgSO_4 \cdot 7H_2O$	0.075
Peptone	0.03
$FeSO_4 \cdot 7H_2O$	0.005
Initial pH	8.0

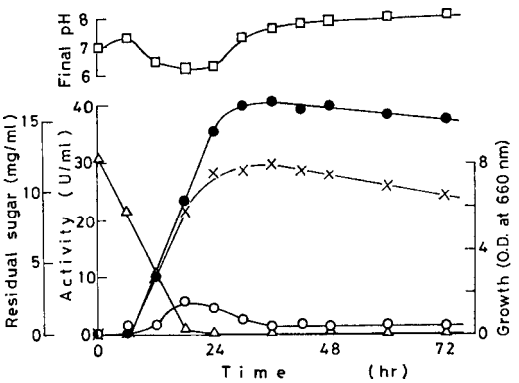

Fig. 11. Time course of intracellular pullulanase production.
△: residual sugar. Other symbols same as in Fig. 1.

pullulanase production was found to increase after 6 hr and became nearly parallel to the bacterial growth. The bacterial growth and intracellular pullulanase production occurred maximum at 36 hr, then these were decreased slowly and linearly. The extracellular pullulanase was hardly released into the medium during the cultivation.

In the extracellular enzyme producing medium, residual sugar were consumed within 36 hr (Fig. 12). The pH-value once decreased slightly and soon increased gradually up to 9. Pullulanase production was preceded by the bacterial growth. The pullulanase began to be accumulated in the cells after 24 hr and to be released soon outside the cells. The time of the maximum ac-

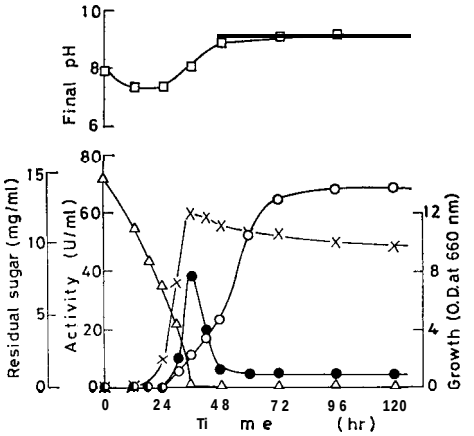

Fig. 12. Time course of extracellular pullulanase production. Symbols same as in Fig. 11.

cumulation of intracellular pullulanase almost corresponded to that of the maximum cell growth. As soon as the growth began to decrease, the release of intracellular pullulanase was speeded up rapidly. The maximum of extracellular pullulanase production occurred at 96 hr, and then its quantity remained the same.

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REFERENCES

Abdullah. M., B. J. Chatley, E. Y. C. Lee, J. Robyt, K. Wallenfels and W. J. Whelan 1966 The mechanism of carbohydrase action II. Pullulanase, an enzyme specific for the hydrolysis of alpha-l. 6–bonds in amylaceous oligo- and polysaccharides. Cereal *Chem.*, 43 : 111-118

Abdullah, M. and D. French 1970 Substrate specificity of pullulanase. Arch. ***Biochem. Biophys.*, 137** : 483-493

Drummond, G. S., E. E. Smith and W. J. Whelan 1969 Mechanism of action of pullulanase. ***FEBS Letters, 5 : 85-88***

Eisele, B., I. R. Rached and K. Wallenfels 1972 Molecular characterization of pullulanase from ***Aerobacter aerogenes. Eur. J. Biochem.. 26: 62-67***

Fujio, Y., M. Shiosaka and S. Ueda 1970 Isoamylase production by ***Aerobacter aerogenes (1)*** Effect of nitrogen source. ***J. Ferment. Technol., 48: 8-13*** (in Japanese with English summary)

Hobson, P. N., W. J. Whelan and S. Peat 1951 The enzymic synthesis and degradation of starch XIV. R-enzyme. ***J. Chem. Soc.*, 1951:** 1451-1459

Kobayashi, T. and B. Maruo 1949 Studies on the enzymic formation and degradation of starch Part I. Examinations on the amylosynthease (1) and (2). Nippon ***Nogei Kagaku***

Kaishi (J. Agr. Chem. Soc. Japan), 23: 115-123

Maruo, B. and T. Kobayashi 1951 Enzymic scission of the branch links in amylopectin. Nature, 167 : 606-607

Mercier, C., B. M. Frantz and W. J. Whelan 1972 An improved purification of cell-bound pullulanase from ***Aerobacter aerogenes. Eur. J. Biochem., 26: l-9***

Nelson, N. 1944 A photometric adaptation of the Somogyi method for the determination of glucose. ***J. Biol. Chem., 153: 375-380***

Ohba, R., H. Chaen, S. Hayashi and S. Ueda 1978 Immobilization of *Streptomyces flavochrogenes* pullulanase on tanic acid and TEAE-cellulose. ***Biotechnol. Bioeng., 20: 665-676***

Ohba, R. and S. Ueda 1973 Purification, crystallization and some properties of intracellular pullulanase from ***Aerobacter*** aerogenes. ***Agric.*** *Biol.* Chem., 37: 2821-2826

Ohba, R. and S. Ueda 1975 Some properties of crystalline extra- and intra-cellular pullulanase from ***Aerobacter*** aerogenes. Agric. *Biol.* ***Chem.. 39: 967-972***

Ohba, R. and S. Ueda 1980 Production of maltose and maltotriose from starch and pullulan by an immobilized multienzyme of pullulanase and β-amylase. ***Biotechnol Bioeng., 22*** : 1937-1954

Somogyi, M. 1945 A new reagent for the determination of sugars. *J. Biol.* ***Chem., 160: 61-68***

Ueda, S., K. Fujita, K. Komatsu and Z. Nakashima 1963 Polysaccharide produced by the genus *Pullularia* I. Production of polysaccharide by growing cells. *Appl. Microbiol.*, 11: 211-215

Ueda, S. and R. Ohba 1972 Purification, crystallization and some properties of extracellular pullulanase from ***Aerobacter aerogenes. Agric. Biol. Chem.. 36:*** 2381-2391

Wallenfels, K., H. Bender and I. R. Rached 1966 Pullulanase from ***Aerobacter aerogenes;*** production in a cell-bound state. Purification and properties of the enzyme. ***Biochem. Biophys. Res. Comm., 22: 254-261***

Wallenfels, K. und I. R. Rached 1966 Kristallisation und Stereospezifität der Pullulanase aus ***Aerobacter aerogenes. Biochem. Z.. 344: 524-526***

Wallenfels, K., I. R. Rached und F. Hucho 1969 Klassifizierung der Pullulanase als Exoenzyme mit Hilfe der Gaschromatographie. *Eur.* ***J. Biochem., 7: 231-233***