USING INORGANIC ELEMENTS TO CONTROL CELL GROWTH AND LIPID ACCUMULA- TION IN *LIPOMYCES STARKEYI*

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We studied how to control the ability of *Lipomyces starkeyi* cells to grow and accumulate lipid by adding inorganic elements. Adding Zn^{2+} , Mn^{2+} , and monopotassium phosphate at a stationary phase of growth caused rapid changes. However, adding these elements individually did not induce any marked changes in the cell growth and lipid accumulation. When these elements were added simultaneously, the yeast underwent a second logarithmic growth and the respiration rate increased. Concurrently, this addition stopped the normal increase in the amount of lipid in the culture (mg of lipid/ml of culture), and decreased the lipid content of the cells (mg of lipid/ 10^8 cells). The periods of second logarithmic growth and the cessation of lipid accumulation were shortened when monopotassium phosphate was not added. The function of monopotassium phosphate was replaced by another buffer chemical.

Artificial modification of the physiological conditions of growing microorganisms has often been used as a way to control metabolite production. In *Lipomyces starkeyi* grown in batch cultures, cell growth and lipid accumulation gradually change with the progress of the growth phases (1, 2). If the progress of the growth phases can be artificially controlled, cells with different abilities can be easily obtained. Development of such a control method enables us to study the metabolism of lipid accumulation in the yeast.

In our earlier report, we showed that the concentration of certain inorganic micro-ions seriously affect the cell growth and the lipid accumulation (3). That raised the possibility that these changes, which accompany the progress of growth phases, could be controlled artificially by changing the concentration of inorganic elements midway in the culture. In the present study, this possibility was examined.

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MATERIALS AND METHODS

Yeast and cultural conditions. *Lipomyces starkeyi* IAM 4753, a fat-producing yeast, was grown in aerobic conditions at 29.5°C with a reciprocal shaker as described previously (4).

Medium. A basal medium was used. It consisted of D-glucose, 100.0 g; $(\text{NH}_4)_2\text{SO}_4$, 5.3 g; KH_2PO_4 , 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g; NaCl, 0.1 g; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 2.42 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 18.0 μg ; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 18.8 μg ; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 3.2 μg ; KI, 13.0 μg ; H_3BO_3 , 42.9 μg ; and D-biotin, 0.2 μg ; in 1,000 ml of deionized and distilled water. The characteristics of the basal medium for lipid accumulation have been described in part (5). The chemicals were of the highest grade (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After adjusting the medium to pH 5.0, 100 ml of the medium was poured into a 500-ml shaking flask. The flask was autoclaved for 10 min at 115°C.

Addition of Zn^{2+} , Mn^{2+} , monopotassium phosphate, and/or sodium tartarate buffer. The yeast was initially grown in the basal medium. At a stationary phase of the growth (about 90 hr), the culture was divided into two parts. These were continuously shaken with and without addition of 0.23 mM Zn^{2+} (with $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), 0.40 mM Mn^{2+} (with $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$), 290.0 mM monopotassium phosphate (KH_2PO_4) and/or 100.0 mM sodium tartarate buffer (pH 3.1).

Analytical methods. The number of cells was counted with a hemacytometer (1). The amount of intracellular neutral lipid accumulated in oil globules was estimated by the method reported previously (6, 7). The pH of the medium was measured with a Hitachi-Horiba pH-meter Model F-7 (Hitachi, Ltd., Tokyo).

Oxygen uptake was measured with an oxygen electrode (YSI Model 533, Yellow Springs Instrument Co., Inc., Ohio, U.S.A.). The electrode was placed in a sample compartment (Gilson Water-Jacketed Cell, Gilson Medical Electronics, Inc., Beltline Middleton, U.S.A.), and was connected to a YSI model 53 Oxygen Monitor. The sample compartment was thermostatically maintained at 25°C by water circulation. The cell concentration in the sample compartment was adjusted with the fresh basal medium to about 1×10^7 cells/ml. Soluble protein in the cell-free extract was estimated by the method of LOWRY et al. (8), with bovine serum albumin as the standard. Cell-free extract was prepared by the method reported previously (9).

RESULTS

Simultaneous addition of Zn^{2+} , Mn^{2+} , and monopotassium phosphate

In the basal medium, the increase in the total cell number stopped at about 60 hr, but the amount of lipid in the culture (mg of lipid/ml of culture) and the lipid content of the cells (mg of lipid/ 10^8 cells) continued to rise until about 170 hr (Figs. 1A, 1B, and 1C).

When Zn^{2+} , Mn^{2+} , and monopotassium phosphate were added to the basal

Fig. 1. Effect of simultaneous addition of Zn^{2+} , Mn^{2+} , and monopotassium phosphate on total cell number (A), amount of lipid in the culture (B), lipid content of the cells (C) and pH of the medium (D).

L. starkeyi was initially grown in the basal medium (○). At the time indicated by the arrows, 0.23 mM Zn^{2+} , 0.40 mM Mn^{2+} , and 290.0 mM monopotassium phosphate were added simultaneously (●).

medium at 90 hr, logarithmic growth was resumed (Fig. 1A). The maximum number of cells in this medium was approximately 9×10^8 cells/ml. Concurrently, the increase in the amount of lipid in the culture stopped for about 50 hr after the addition (Fig. 1B). The lipid content of the cells began to decrease after the addition, and ultimately reached a minimum (less than $0.4 \text{ mg}/10^8$ cells) (Fig. 1C). The pH of the medium rose instantaneously from 2.6 to 3.6 when the elements were added, then the pH gradually decreased as growth progressed (Fig. 1D).

Figure 2 shows light-microscope pictures of the culture at 160 hr. The simultaneous addition of the three elements diminished both the cell size and the size of the intracellular oil globules.

Figure 3 shows the changes in the oxygen uptake rates of the cells grown in the medium with and without Zn^{2+} , Mn^{2+} , and monopotassium phosphate added together. The addition of these elements caused an increase in the respiration rate.

Fig. 2. Micrographs of *Lipomyces starkeyi*.

Micrographs were taken of the culture at 160 hr. A, the basal medium; B, the medium with Zn^{2+} , Mn^{2+} and monopotassium phosphate added simultaneously.

Fig. 3. Changes in oxygen uptake rates.

The culture started in the basal medium (○). At the time indicated by the arrow, 0.23 mM Zn^{2+} , 0.40 mM Mn^{2+} , and 290.0 mM monopotassium phosphate were added simultaneously (●).

Fig. 4. Effects of individual addition of Zn^{2+} , Mn^{2+} , and monopotassium phosphate on total cell number.

The culture started in the basal medium (○). At the time indicated by the arrow, 0.23 mM Zn^{2+} (Δ), 0.40 mM Mn^{2+} (□), or 290.0 mM monopotassium phosphate (×) was added independently to a different culture.

Fig. 5. Effect of simultaneous addition of Zn^{2+} and Mn^{2+} on total cell number (A), lipid content of the cells (B) and pH of the medium (C).

The culture started in the basal medium (○). At the time indicated by the arrows, 0.23 mM Zn^{2+} and 0.40 mM Mn^{2+} were added simultaneously (▲).

Fig. 6. Effect of adding sodium tartarate buffer with Zn^{2+} and Mn^{2+} on pH of the medium (A), total cell number (B) and lipid content of the cells (C).

The culture started in the basal medium ($\circ$). At the time indicated by the arrows, 100.0 mM (final concentration) sodium tartarate buffer (pH 3.1), 0.23 mM Zn^{2+} and 0.40 mM Mn^{2+} were added simultaneously ($\blacksquare$).

The limiting effect of Zn^{2+} , Mn^{2+} , and monopotassium phosphate on cell growth

To determine the effect of each element on cell growth, Zn^{2+} , Mn^{2+} , or monopotassium phosphate was added independently to a different culture (Fig. 4). Zn^{2+} was the most effective one of the three additives. However, none of these elements induced a marked increase in total cell number.

When Zn^{2+} and Mn^{2+} were added simultaneously, logarithmic growth, accompanied by a decrease in lipid content of the cells, occurred for a short period as shown in Figs. 5A and 5B (about half as long as the periods in Figs. 1A and 1C). The limited effect of Zn^{2+} and Mn^{2+} indicates that the addition of monopotassium phosphate prolongs physiological effects of these two ions.

Substitution of sodium tartarate buffer for monopotassium phosphate

In the medium with Zn^{2+} and Mn^{2+} added, the pH of the medium decreased continuously to about 1.8 (Fig. 5C). When monopotassium phosphate was added

with the two ions, the pH rose to about 3.7 then slowly decreased to about 2.4 (Fig. 1D). The period of logarithmic growth in the medium with Zn^{2+} , Mn^{2+} , and monopotassium phosphate added was longer than that of the medium with only Zn^{2+} and Mn^{2+} added (Figs. 1A and 5A). From this, we concluded that the buffer action of the monopotassium phosphate prolongs the cell growth advanced by Zn^{2+} and Mn^{2+} .

In order to verify our supposition, 100.0 mM (final concentration) sodium tartarate buffer (pH 3.1) instead of monopotassium phosphate was added with Zn^{2+} and Mn^{2+} (Fig. 6). The pH of the medium rose instantly to about 3.1 after this addition, then decreased finally to about 2.3 (Fig. 6A). The total cell number increased logarithmically and finally reached approximately 8×10^8 cells/ml (Fig. 6B). The lipid content of the cells decreased after the addition of Zn^{2+} , Mn^{2+} , and sodium tartarate buffer, and its minimum content was less than $0.4 \text{ mg}/10^8$ cells (Fig. 6C). These results were similar to those shown in Figs. 1A and 1C.

DISCUSSION

The C/N ratio in a medium is well known as a potent factor for cell growth and lipid accumulation in fat-producing yeasts (10–13). Our studies showed that the concentration of inorganic micro-ions is a more potent factor than the C/N ratio, at least in cell growth and lipid accumulation in *L. starkeyi* (1, 3, 5, 14). In the course of these studies, we frequently observed that the lipid accumulation of the yeast in a batch culture was governed by the progress of growth phases. In addition, the progress depended mainly on the initial concentration of inorganic elements in the medium. These results led us to expect that it is possible to control the progress of growth phases by adding some inorganic elements during the course of growth.

The simultaneous addition of Zn^{2+} and Mn^{2+} in the first stationary phase of growth, contributed considerably to the creation of a second exponential phase with a subsequent second stationary phase (Fig. 5). The progress from the first stationary phase to the second exponential phase is the reverse of the usual direction of progress of growth phases in a batch culture. The cessation of lipid accumulation, accompanied by the second logarithmic growth, suggests a certain change in the intracellular metabolism. This is supported by the results shown in Fig. 3. In general, the biosynthesis of fatty acid requires a large amount of citrate to be biosynthesized from glucose in the cells (15, 16). The increase in respiration rate implies that a large amount of citrate flows into the tricarboxylic acid cycle. This causes a decrease in the amount of citrate supplied to the fatty-acid biosynthesis pathway.

In the medium with Zn^{2+} , Mn^{2+} , and monopotassium phosphate added, the total cell number increased, but the amount of lipid in the culture did not increase (Figs. 1A and 1B). These facts suggest that the cells that proliferated after the addition did not biosynthesize neutral lipid, and that leads to the result shown in Fig. 1C.

The period of the second exponential phase induced by the addition of Zn^{2+} and Mn^{2+} only was shorter than that induced when monopotassium phosphate or sodium tartarate buffer was combined with these ions (compared Fig. 5 with Figs. 1 and 6). The addition of these buffer chemicals prevented the decrease in the pH of the medium (Figs. 1D and 6A). This suggests that the prevention of a decrease in the pH of the medium is a factor that prolongs the physiological effects of Zn^{2+} and Mn^{2+} on the cell growth and the lipid accumulation.

In the second stationary phase after the second exponential phase of growth, the amount of lipid in the culture increased (Fig. 1B). This suggests that an adequate supply of glucose remained in the medium. Therefore, retardation of the growth rate after the second exponential phase seems to be caused by the shortage of other physiological factors such as dissolved oxygen or inorganic elements needed for the cell growth.

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