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Hyperproduction of L-Glutamate Oxidase in Submerged Fermentation of *Streptomyces* sp. N1 with Culture pH Control and Calcium Addition

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Abstract

Production of L-glutamate oxidase (GluOx) by *Streptomyces* sp. N1 was investigated by controlling culture pH at 6.2, 6.7, 7.0, and 7.3 in a 5-L stirred fermentor. The corresponding GluOx activities obtained were 2.8, 4.2, 6.0, and 5.3 U/mL, respectively. Microbial growth was inhibited by increasing the medium pH from 6.2 to 7.0. The inhibitory effect was also observed in plate colony growth under incubation with a different initial pH value. The effect of calcium on GluOx production was also studied in the pH-controlled bioreactor. When the culture pH was controlled at 6.2 or 7.0, GluOx production could not be improved or was only improved slightly by initial addition of calcium to the medium. However, when the culture pH was kept at 6.7, initial Ca^{2+} addition (60 m*M*) conspicuously enhanced GluOx production up to 9.3 U/mL, which was about twofold of that without Ca^{2+} addition. The enzyme production level was the highest ever reported in the literature. During fermentation the inhibition of cell growth by Ca^{2+} addition was observed. For the morphological changes, the cells mostly existed as pellets in the medium without Ca^{2+} addition, whereas few pellets were found and almost all the cells were dispersed mycelia in the broth with Ca^{2+} addition.

Index Entries: L-Glutamate oxidase; submerged fermentation; calcium addition; pH control; morphological change; *Streptomyces* sp.

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Introduction

L-Glutamate oxidase (GluOx) can catalyze oxidative deamination of L-glutamate to produce H_2O_2, which can serve as an analytic reagent for the determination of L-amino acids. Likewise, the determination of L-glutamate using GluOx provides a new method for the analysis of L-glutamate in broth and food samples, and new possibilities for the estimation of serum glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) in clinical laboratories. For example, GluOx has been used to construct biosensors or enzyme reactors for the determination of L-glutamate concentration *(1)* or GOT and GPT activities *(2)*. Combined with other enzymes, GluOx has been used to construct biosensors for on-line monitoring of glutamine in animal cell culture *(3)* or to determine aspartame *(4)* and aspartate *(5)* in food or clinical samples. However, there are only a few reports on its isolation, purification, and production from different sources *(6–9)*.

Streptomyces sp. N1 isolated from soil samples can produce a large amount of extracellular GluOx in submerged culture *(7)*. During GluOx production in liquid fermentation in a shake flask, a very low GluOx production was detected when the culture pH was decreased to a low value. This result implies that control of the culture pH may be quite important to GluOx production by *Streptomyces* sp. N1. As previously reported, production of an antimicrobial protein leuconocin S by *Leuconostos paramesenteroides* at culture pH 7.0 was about 10-fold of that in the medium with pH 6.0 *(10)*, although the growth of the lactic acid bacteria at pH 7.0 was only slightly faster than that at pH 6.0. Also, production of cellulase by *Streptomyces reticuli* was strongly repressed at a low pH *(11)*, and peroxidase from *Streptomyces viridosporus* T7A was largely enhanced by addition of $CaCO_3$ to neutralize the culture pH *(12)*.

Calcium is considered an absolute cell growth requirement; an extracellular signal, trigger, or messenger; and a structure-former for many microorganisms *(13)*. There are reports regarding the effects of calcium on spore germination of *Colletotrichum trifolii*, aerial mycelium formation of many actinomycetes, and morphology of *Bacillus stearothermophilus (14,15)*. Calcium was found to inhibit the synthesis of some antibiotics; e.g., addition of 1.8 and 9.0 m*M* Ca^{2+} inhibited efrotomycin production by 50 and 100%, respectively, in *Nocardia lactamdurans* fermentation *(16)*. On the other hand, in fermentation of *Saccharomyces cerevisiae*, *S. bayanus*, and *Kluyveromyces maximum*, the positive effect of Ca^{2+} supplementation (0.75–2.0 m*M*) on ethanol production was observed *(17)*.

In this article, we examine the effects of both culture pH and Ca^{2+} on the growth and GluOx production by *Streptomyces* sp. N1 in a pH-controlled 5-L fermentor. In addition, we report the different morphologies of this strain in the bioreactor with or without addition of Ca^{2+}.

Materials and Methods

Microorganism

Streptomyces sp. N1 isolated from soil was used as described elsewhere *(8,18)*.

Slant and Plate Cultures

A medium (pH 7.0) containing 5% (w/w) wheat bran extract and 2% (w/w) agar was used. The cultures were incubated at 28°C for 7 d.

Mycelia Inoculum Preparation

A 250-mL shake flask with 30 mL of medium was used, which contained 3% (w/v) glucose, 0.6% (w/v) $(NH_4)_2SO_4$, 1.0% (w/v) corn steep liquid, 0.1% (w/v) $MgCl_2$, 0.1% (w/v) $CaCl_2$, and 0.07% (w/v) Na_2HPO_4. The medium pH was adjusted to 7.0 and sterilized at 121°C for 30 min. An inoculum (6-d old) of 10^7 spores was used. The mycelia inoculum was obtained after 12 h of cultivation at 28°C and 200 rpm.

Submerged Fermentation

Cultivation was carried out in a 5-L stirred tank reactor (B. Braun, Melsungen, Germany) with a working volume of 3 L. The medium was the same as in a shake flask. An inoculum (6-d old) of 10^9 spores was used. For the investigation on the Ca^{2+} effects, 60 m*M* Ca^{2+} was added to a fermentor at the beginning of fermentation and another fermentor was used as control. For all the fermentations, the cultivation temperature and aeration rate were maintained at 28°C and 2 vvm, respectively, and agitation speed was changed from 400 to 1000 rpm during the fermentation to maintain dissolved oxygen above 10% of air saturation. For controlling the pH value, 1 *N* NaOH solution was used. After 30 h of fermentation, 200 mL of sterilized medium containing 40 g of glucose, 4 g of $(NH_4)_2SO_4$, and 4 g of corn steep liquid were added to the fermentor.

Measurement of Dry Cell Weight

A sample of cell culture was centrifuged at 4000 rpm (i.e., 1432*g*) for 20 min, washed several times by deionized water, and then dried at 60°C in an oven until a constant weight was obtained.

Determination of GluOx Activity

The activity of GluOx was determined on the basis of the peroxidase-catalyzed chromogenic reaction. The reaction mixture was composed of 1 mL of 4-aminoantipyrine (2 m*M*), 2 mL of phenol (3 m*M*), 0.1 mL of horseradish peroxidase (60 U/mL), and 0.1 mL of sample. Reaction was carried out at 28°C and the absorbance was measured at 505 nm. One unit of GluOx activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of H_2O_2/min under the reaction conditions described here.

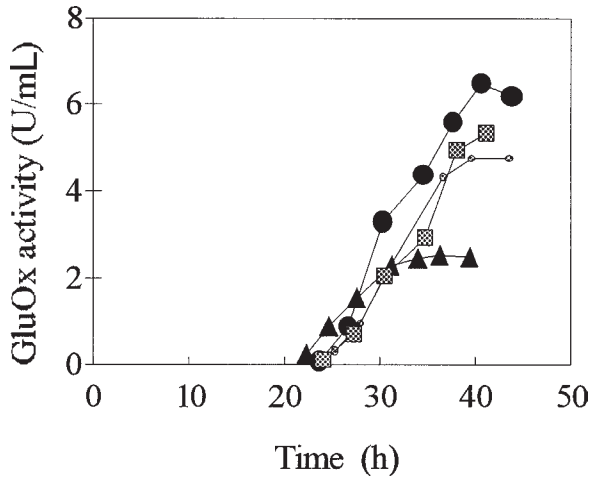

Fig. 1. Production of GluOx in a 5-L stirred bioreactor with pH controlled at 6.2 (▲), 6.7 (•), 7.0 (●), and 7.3 (▨), respectively.

Results and Discussion

Effect of Culture pH

Production of GluOx by *Streptomyces* sp. N1 at different culture pH values was examined by using a pH-controlled 5-L stirred fermentor. As shown in Fig. 1, at all the pH values investigated, GluOx activities appeared after about 24 h of fermentation, and increased to their maximum values around or before 40 h of fermentation. Both the length of the GluOx-producing period and the enzyme total production were increased with an increase of culture pH from 6.2 to 7.0. The highest GluOx activity (6.0 U/mL) was achieved in the fermentation at pH 7.0, which was more than twice that at pH 6.2. At an even higher pH of 7.3, enzyme production was decreased. In a shake flask in which cultured pH was not controlled, the maximum GluOx activity obtained was 1.0 U/mL *(7)*. Compared with that value, GluOx production in the fermentor was significantly improved (about six-fold) by controlling the culture pH at 7.0. Lower GluOx production in a shake flask is considered to be related with the culture pH. During shake flask fermentation, a typical change in culture pH was a gradual decrease from the initial value of 7.0 to about 6.4 during the first 21 h of culture and then a quick reduction to about pH 5.5 within the following 4–6 h, whereas GluOx was biosynthesized during h 26 or 36 of fermentation *(7)*.

In many cases, control of culture pH value is quite important for metabolite production because not only can a certain microorganism grow only at a certain range of pH, but also the syntheses of some metabolic products are quite sensitive to culture pH. For example, xylanase production by *Thermomyces lanuginosus* at a controlled pH of 7.5 was about 30% more than that in a pH-uncontrolled fermentation *(19)*. By cultivating a photosynthetic bacterium *Rhodobacter sphaeroides* at pH 6.8, the maximum

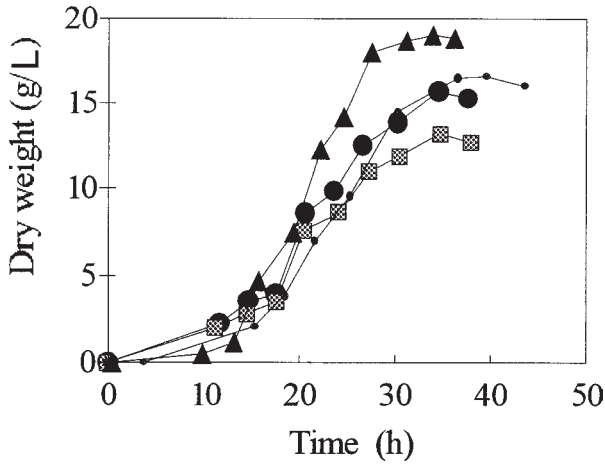

Fig. 2. The growth of *Streptomyces* sp. N1 in a stirred fermentor with pH controlled at 6.2 (▲), 6.7 (•), 7.0 (●), and 7.3 (▨), respectively.

activity of 5-aminolevulinic acid synthase was obtained, which was about 12 times that at pH 6.0, whereas the 5-aminolevulinic acid dehydratase reached its maximum when the bacterium was cultured at pH 8.0 *(20)*.

Figure 2 shows the growth curves of this microorganism at different culture pH values in the fermentor. The results indicate that the growth rate and maximum biomass obtained at different culture pH values were decreased with an increase in culture pH from 6.2 to 7.3. At pH 6.2, the maximum dry cell weight (19 g/L) was obtained after 30 h of fermentation, at which the biomass was about 20% higher and the fermentation period about 13 h shorter than those at pH 7.0. At an even higher pH of 7.3, the final production showed an even greater decrease (Fig. 2).

We also conducted agar plate incubation at initial pH values of 6.2 and 7.0, and by calculating the colony numbers, the inhibitory effect on the microbial growth by a relatively higher pH was also confirmed. Here, spores and mycelia were used as inocula. As shown in Fig. 3, for both types of inocula, the appearance of colonies and their maximum number obtained in agar plates with an initial pH of 7.0 was after 36 and 69 h of incubation, respectively, which were 21 and 33 h ahead of those in agar plates with an initial pH of 6.2, respectively (Fig. 3). However, the final numbers of colonies obtained in the plates with an initial pH of 6.2 were 1.3 and 3.2 times those in the plates with an initial pH of 7.0 when spores and mycelia were used as inocula, respectively (Fig. 3). This means that about 20% of spores and 70% of mycelia that could grow at pH 6.2 were inhibited at pH 7.0. The results indicate that this strain grew faster on the plates with an initial pH of 6.2 than with an initial pH of 7.0.

From these facts, compared with that at pH 6.2, the relatively higher GluOx production at pH 7.0 in the fermentor was supposed to be caused by the inhibition of the growth of low GluOx-producing mycelia by a rela-

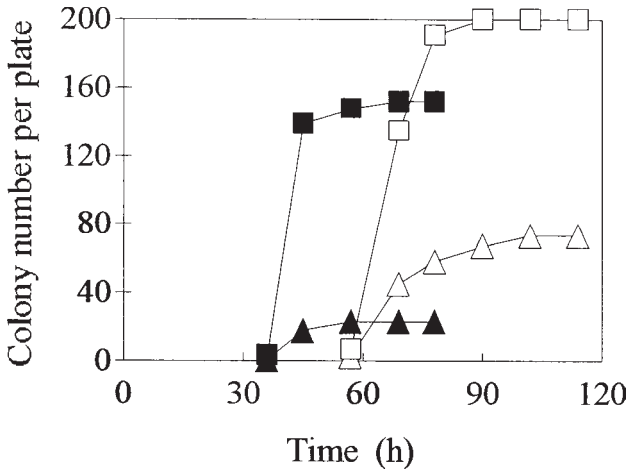

Fig. 3. Colony growth in plates with initial pH of 6.2 (open symbols) and 7.0 (solid symbols) using spores (squares) and 12-h old mycelia (triangles) as inocula. Each point represents the mean value of colonies growing on eight plates, and for all the data the standard deviation was <7%.

tively higher culture pH. Similarly, for the effect of L-glutamate, its high concentration could inhibit the growth and spore production of low GluOx-producing cells as previously observed *(18)*.

Effect of Ca^{2+} Addition

The effect of calcium on GluOx production was investigated by the initial addition of 60 m*M* Ca^{2+} to the submerged bioreactor fermentation with culture pH values controlled at 6.2, 6.7, and 7.0, respectively. As shown in Fig. 4, when the culture pH was controlled at 6.2 or 7.0, GluOx production could not be improved or was only improved slightly by calcium addition. However, when the culture pH value was kept at 6.7, GluOx produced with the addition of 60 m*M* Ca^{2+} was almost twofold that without Ca^{2+} addition. These results indicate that the improvement of GluOx production by Ca^{2+} addition was related to the culture pH. As shown in Fig. 5, at all three pH values, the cell growth was inhibited by the initial addition of 60 m*M* Ca^{2+} to the culture; the lag phase was delayed (for about 6 h) and the growth rate (data not shown) was reduced by Ca^{2+} addition.

The morphology of *Streptomyces* sp. N1 during the entire fermentation was observed in the medium with or without Ca^{2+} addition. Figure 6 shows the typical morphology of *Streptomyces* sp. N1 after 9, 12, and 32 h of fermentation with pH value controlled at 6.7. After 9 h of cultivation, small pellets, a central core with many extended annularly growing mycelia (Fig. 6A), appeared in the medium without Ca^{2+} addition. These small flocs became bigger and tidier to form pellets. Few mycelia were observed during the entire fermentation in the medium without Ca^{2+} addition. On the other hand, only mycelia were observed in the medium with Ca^{2+} addition

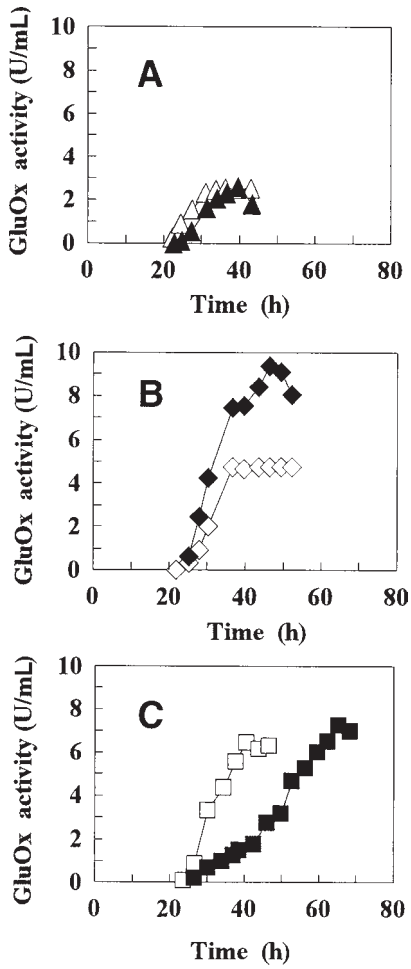

Fig. 4. GluOx production in a 5-L fermentor with (solid symbols) or without (open symbols) Ca^{2+} addition (60 m*M*) at a controlled pH of 6.2 **(A)**, 6.7 **(B)**, and 7.0 **(C)**.

after 9 h of culture, and these mycelia seemed to be finer than those in the medium without Ca^{2+} addition. During the following period of fermentation, few pellets were formed in the medium with Ca^{2+} addition (Figs. 6E and 6F). Similar phenomena were observed in the medium with the culture pH controlled at 6.2 and 7.0. Since GluOx production in the medium at pH 6.2 or 7.0 was not obviously improved by Ca^{2+} addition, the morphological change of *Streptomyces* sp. N1 by Ca^{2+} addition may not be directly related to GluOx synthesis.

In conclusion, the current work indicates that both pH and Ca^{2+} had significant effects on the GluOx production, cell growth, and morphology of *Streptomyces* sp. N1. Controlling the culture pH at 7.0 was optimal to the enzyme production (6.0 U/mL) by the cells in a 5-L stirred fermentor. When the culture pH was kept at 6.7 and initial Ca^{2+} addition (60 m*M*) to the

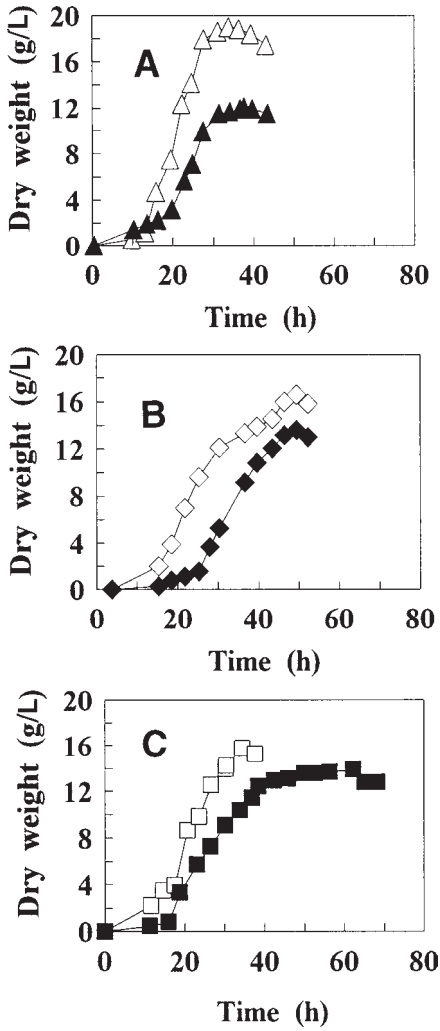

Fig. 5. The growth of *Streptomyces* sp. N1 in a 5-L fermentor with (solid symbols) or without (open symbols) Ca^{2+} addition at a controlled pH of 6.2 **(A)**, 6.7 **(B)**, and 7.0 **(C)**.

medium was conducted, a remarkable increase of GluOx production up to 9.3 U/mL was achieved, which is the highest level ever reported. We believe that the results reported here are quite useful for efficient large-scale production of GluOx by the microorganism.

To date, the mechanisms of culture pH and Ca^{2+} effects on the GluOx biosynthesis and morphology of *Streptomyces* sp. N1 are still unclear. Sixty millimolar Ca^{2+} in growth medium is apparently an unusually high concentration. The Ca^{2+} concentration dependence of GluOx production seemed to follow a nearly all-or-none pattern and the cell growth was gradually inhibited with an increase of medium Ca^{2+} level (data not shown). One speculation about this phenomenon is that many Ca^{2+} ions must bind

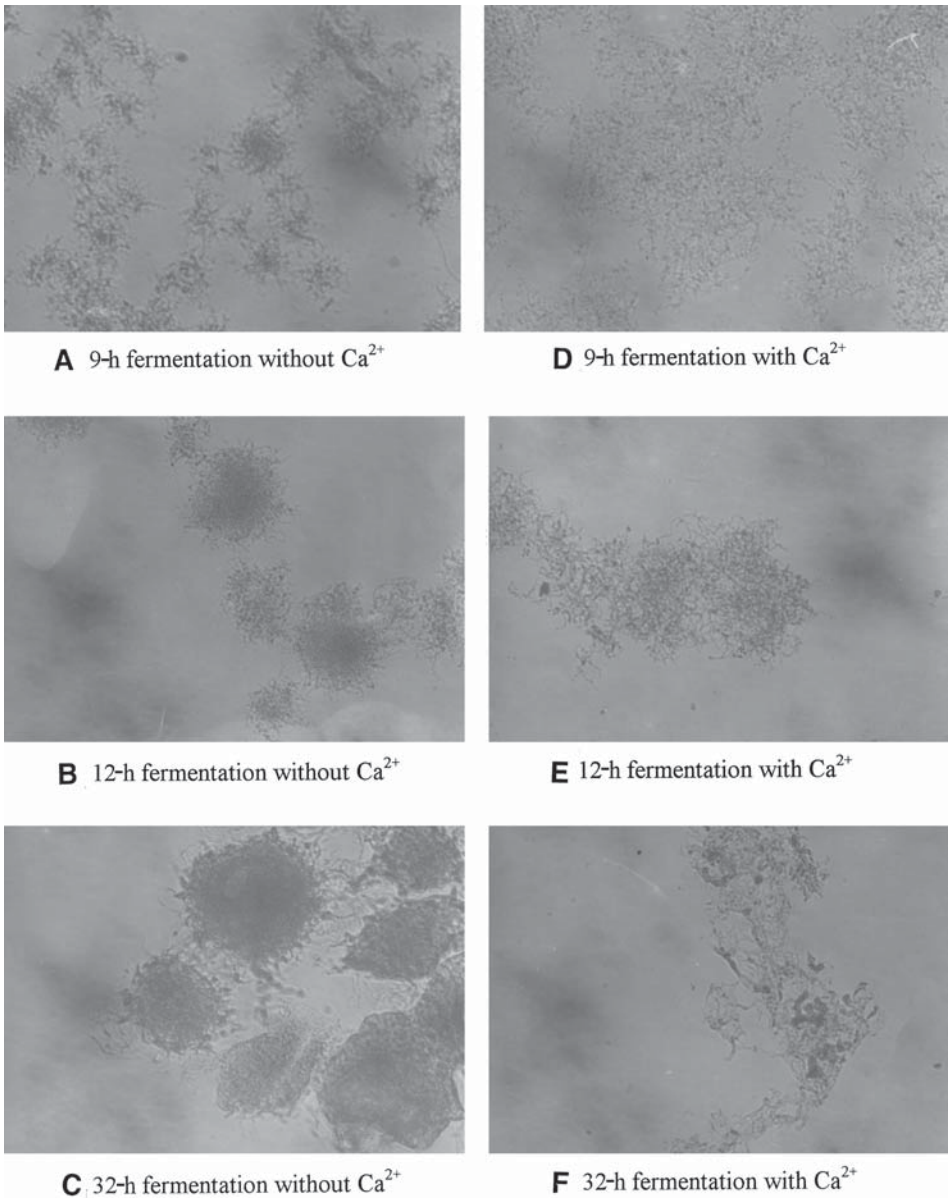

Fig. 6. Morphological changes of *Streptomyces* sp. N1 during bioreactor fermentation without (**A–C**) or with (**D–F**) initial Ca^{2+} addition under a controlled pH of 6.7 (original magnification ×200).

before calmodulin adopts its activating conformation, and the latter may initiate the transcription of the GluOx gene *(21)*. Also, some experimental data suggest a correlation between culture conditions that induce growth inhibition (pH 7.0 and 6.7 with 60 m*M* Ca^{2+}) and the improvement of GluOx production. A possible biochemical explanation is that when the growth was inhibited, the level of enzyme inhibitor(s) (e.g., product[s] of enzy-

matic reactions) was decreased, or the enzyme inducer(s) was accumulated, and thus the enzyme activity was increased. Related biochemical studies are necessary to verify these speculations and finally elucidate the molecular mechanisms of culture pH and Ca^{2+} effects on GluOx biosynthesis.

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