Songklanakarin J. Sci. Technol.
30 (Suppl.1), 65-71, April 2008

Songklanakarin Journal of Science and Technology
http://www.sjst.psu.ac.th

Original Article

Saccharification of cassava starch by *Saccharomycopsis fibuligera* YCY1 isolated from Loog-Pang (rice cake starter)

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Received 10 January 2007; Accepted 28 August 2007

Abstract

The main objectives of this study were to select amylolytic yeasts from Loog-Pang, a traditional starter culture for production of alcoholic foods and drinks in southern Thailand, and to optimize the saccharification of cassava starch to reducing sugar by the selected yeast isolate. Seventy-four yeast isolates were obtained from ten samples of Loog-Pang. The isolates were tested for amylolytic activity on Yeast-Peptone Cassava medium (YPC) contained 30 g/l of cassava starch. Only three isolates showed amylolytic activity which produced clear zone on the YPC agar. The best amylolytic strain with clear zone of 8 mm was identified by 26s rDNA as *Saccharomycopsis fibuligera*.

The optimum medium for saccharification by *Saccharomycopsis fibuligera*. YCY1 was only 50 g/l of cassava starch in distilled water without nitrogen sources added and pH adjustment. The optimal saccharification conditions were 200 ml cassava starch (50 g/l) in 500 ml Erlenmeyer flask, shaking at 100 rpm and 37°C. Under these conditions, the highest reducing sugar was obtained 46±0.53 g/l after 120 h cultivation (84% of the theoretical yield).

Keywords: Loog-Pang, rice cake starter, amylolytic yeast, cassava starch, saccharification, *Saccharomycopsis fibuligera*

1. Introduction

Cassava is an important commercial plant in north-eastern Thailand. It can be grown on very dry tropical lands (Laluce *et al*., 1988). Cassava could be utilized as food, feed and industrial raw materials. Two methods for starch hydrolysis are used for the conversion of starch to glucose either by acid hydrolysis (Benerjee *et al*., 1988; Agu *et al*., 1997) or by enzymatic hydrolysis with amylolytic enzyme from bacteria (Agrawal *et al*., 2005; Demirkan *et al.,* 2005) and fungi (Omemu *et al*., 2005; Konsula and Liakopoulou-Kyriakides, 2004). Acid hydrolysis of starch had widely use in the past. It is now largely replaced by enzymatic process since the hydrolysis occurs at milder condition than acid hydrolysis and is more friendly to an environment. However, the use of amylolytic enzyme increases the production costs (Altintas *et al*., 2002). Although, many kinds of microorganisms are able to produce amylolytic enzyme, the direct saccharification of cassava starch by yeast to produce glucose is very limited. Most studies used mold to hydrolyze cassava starch such as *Trichoderma reesei* (Opoku and Adoga, 1980) and *Rhizopus oligosporus* (Garg and Doelle, 1989).

Saccharomycopsis fibuligera has known to produce high amylolytic activity (Knox *et al*., 2004) and has been applied in food industry for producing sugar syrup (Sandhu *et al*., 1987), single cell protein (Lemmel *et al*., 1980; Clementi *et al*., 1980) and ethanol (Verma *et al*., 2000). Several advantages of using amylolytic yeasts for commercial applications have been described (Tubb, 1986). 50% saving in the glucoamylase concentration was achieved by using *Saccharomyces diastaticus* in the process for ethanol

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production from starch (Whitney *et al.,* 1985). The direct fermentation of starch into ethanol by a recombinant *Saccharomyces cerevisiae* strain YPG/AB, in YPS medium containing 30 g/l soluble starch resulted in reducing sugar and ethanol concentration of 4.22 g/l and 2.27 g/l, respectively (Ülgen *et al*., 2002).

Loog-Pang (rice cake starter) is a traditional starter used to produce alcoholic food in Thailand such as Kao-Mag (alcoholic sweetened rice), Lao (rice wine) and Num Som Saichu (vinegar). These traditional starters have various names such as marcha or murcha in India, ragi in Indonesia, bubod in the Philippines, Chinese yeast in Taiwan, nuruk in Korea (Tsuyoshi *et al*., 2005), banh men in Vietnam, koji in Japan and ragi tapai in Malaysia (Limtong *et al*. 2005). Microorganisms in Loog-Pang comprise of mold, bacteria and yeast. Most studies found *Saccharomycopsis fibuligera* as common yeast in Loog-Pang (Limtong *et al*. 2002).

In the present study, amylolytic yeasts were isolated from Loog-Pang in southern Thailand and the optimal conditions for cassava starch hydrolysis to produce reducing sugar by the selected yeast were evaluated.

2. Materials and methods

2.1 Sample collection

Ten samples of Look-pang (rice cake starter) were bought from different areas in the south of Thailand. All samples were stored at 4°C.

2.2 Isolation and selection of yeasts

One gram of sample was suspended in 50 ml of Yeast-Peptone Cassava medium (YPC medium; 0.5 g/l yeast extract, 0.5 g/l peptone and 30 g/l cassava starch) in 250 ml Erlenmeyer flask and incubated at 30°C on a rotary shaker at 150 rpm for 72 h. The culture broth was spreaded onto YPC agar plate and incubated at 30°C for 72 h. The yeast isolates producing amylolytic enzyme exhibited clear zone around colony when poured with Lugal's iodine solution and measuring the diameter of clarity of the clear zone. Colonies were purified by streaking three times on YPC agar plate. The pure colonies were selected and stored in YPC agar slant at 4°C.

2.3 Optimization of starch hydrolysis

The inoculum was prepared in YPC broth and incubated at 30°C on a rotary shaker at 150 rpm for 48 h. 5% v/v of the inoculum was added into 200 ml of the medium in the Erlenmeyer flask with various concentrations of cassava starch (10-50 g/l), nitrogen sources (yeast extract, peptone and ammonium sulfate), temperatures (25, 30 and 37°C) , pH (4.5, 5.5 and 6.5) and shaking speeds (100, 150 and 200 rpm). The inoculum size effect (3-10% v/v) was also studied after optimization of medium and environment.

2.4 Analytical methods

Cell growth was measured by the optical density of the culture broth at 660 nm. The protein concentration was determined by the method of Lowry *et al*. (1951). Reducing sugar was determined in the culture supernatant after centrifugation of yeast cells by dinitrosalicylic acid method (DNS method) (Miller, 1959). Starch concentration was determined colorimetrically at 620 nm in iodine solution using soluble starch as standard (Pintado *et al*., 1999).

The hydrolyzed cassava starch was analyzed by using thin-layer chromatography, following the method of Yang *et al*. (2004). The sample was spotted onto a Silica gel 60 F_{254} aluminium TLC plate (Merk Co., Germany) and was eluted with isopropyl alcohol-ethyl acetate-water (3:1:1, v/v/v) as the solvent system. After, the thin-layer chromatography plate was dried and visualized by dipping in a solution containing 0.3% w/v *N*-(1-naphthyl)-ethylenediamine and 5% v/v H_2SO_4 in methanol and heating on hot plate. A mixture of glucose (G1) and maltooligosaccharides (maltose (G2), maltotriose (G3), maltotetraose (G4) and maltohexaose (G6)) was used as standard.

Morphological and physiological characteristics of the yeast isolates were determined according to study assimilation ability of carbon compounds (Barnett *et al.*, 2000). The 26s rDNA of the selected yeast was determined by Faculty of Science, Mahidol University.

3. Results and Discussion

3.1 Isolation and selection of yeasts

Seventy-four isolates of yeasts were obtained from ten samples of Loog-Pang in the southern Thailand. Only three isolates showed a clear zones around the colonies on YPC agar plate. The best amylolytic strain (YCY1) showed clear zone of 8.0 mm (Figure 1). From 16 sources of carbon tested, the isolate YCY1 showed positive assimilation of only 5 carbon sources; D-glucose, D-Maltose, D-Saccharose, D-Cellobiose and Glycerol. The physiological characteristics of the isolate YCY1 are shown in Table 1. The 26s rDNA

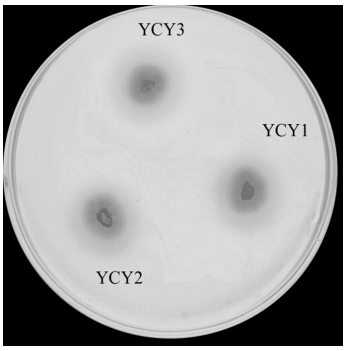

Figure 1. Hydrolysis of cassava starch by yeasts isolated from Loog-Pang grown on YPC agar.

Table 1. The physiological characteristics of the isolated yeast strain YCY1.

Color of colony	Cream, Smooth
Shape	Oval
Reproduction	budding yeast
Assimilation of carbon compounds	
D-Glucose	Positive
Glycerol	Positive
D-Cellobiose	Positive
D-Maltose	Positive
D-Saccharose (Sucrose)	Positive
Calcium 2-Keto-Gluconate	Negative
L-Xylose	Negative
D-Galactose	Negative
Xylitol	Negative
Inositol	Negative
D-Melezitose	Negative
Methyl-D-Glucopyranoside	Negative
N-Acetyl-Glucosamone	Negative
D-Lactose	Negative
D-Trehalose	Negative
D-Raffinose	Negative

analysis of the isolate YCY1 showed 97% homology with *Saccharomycopsis fibuligera*. So the isolate YCY1 was designated as *S. fibuligera* YCY1. This strain was also found in marcha, a traditional amylolytic starter in India (Tsuyoshi *et al*., 2005). In addition, most Loog-Pang samples comprised of *S. fibuligera,* which showed strong amylolytic activity (Limtong *et al*., 2002).

3.2 Optimization of starch hydrolysis

1) Effect of cassava starch concentration

Effect of difference concentrations of cassava starch on starch hydrolysis by *S. fibuligera* YCY1 was investigated when the culture was grown at 30°C and shaking at 150 rpm. Figure 2 shows that the reducing sugar concentration increased with increase in the initial cassava starch concentration from 10 g/l to 50 g/l. From this study, the optimum concentration of cassava starch for reducing sugar production by *S. fibuligera* YCY1 was 50 g/l and the highest reducing sugar was obtained 14.72 g/l (26% of theoretical yield) at 96 h cultivation.

2) Effect of nitrogen source

Effect of various nitrogen sources on growth and reducing sugar production was studied in the YPC medium with 1 g/l of different nitrogen sources, which the control medium had only 50 g/l of cassava starch. The results are shown in Figure 3. The medium with ammonium sulfate and the control medium (without nitrogen source) showed the

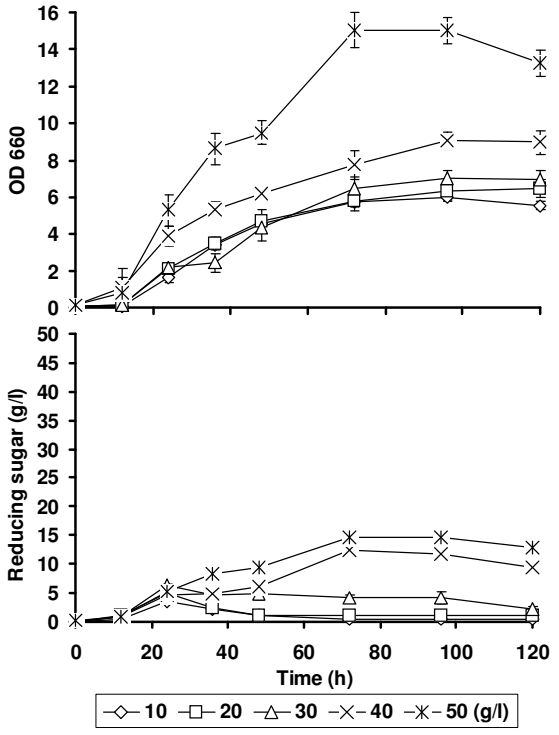

Figure 2. Effect of cassava starch concentration on growth and reducing sugar production by *Saccharomycopsis fiburigera* YCY1 at 30°C and 150 rpm.

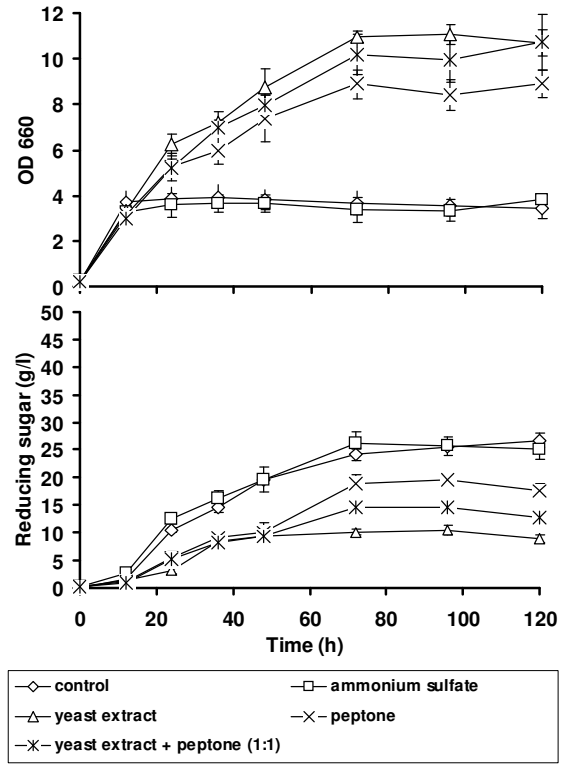

Figure 3. Effect of nitrogen source (1 g/l) on growth and reducing sugar production from cassava starch (50 g/l) by *Saccharomycopsis fiburigera* YCY1 at 30°C and 150 rpm.

highest reducing sugar concentration. There was less amount of reducing sugar in the medium with yeast extract, peptone or yeast plus peptone (1:1) as a nitrogen source. It was observed that cell growth in the medium with organic nitrogen sources was much higher than in the medium with ammonium sulfate and the control. So the yeast might consume the hydrolyzed sugar and other nutrients to stimulate growth. The result indicated that *S. fibuligera* YCY1 was able to grow directly in cassava starch and use only the nitrogen in the cassava starch for growth. Hence the medium without nitrogen source was used for further study.

3) Effect of temperature

The impact of the cultivation temperature on the cassava starch hydrolysis by *S. fibuligera* YCYI was conducted in 50 g/l of cassava starch without adding any nitrogen source by controlling the growth temperatures at 25, 30 and 37°C. It was observed that the reducing sugar increased as the temperature increased from 25 to 37°C. After 5 days at 37°C, the maximum reducing sugar concentration was 39.55 g/l (72% of the theoretical value) (Figure 4). The optimal temperature for glucoamylase and α-amylase of *S. fibuligera* is 40-50°C (Hostinová, 2002).

4) Effect of initial pH

The effect of initial pH on the saccharification of cassava starch by *S. fibuligera* YCY1 was carried out. The medium used in this study was prepared with 100 mM citrate-phosphate buffer with different pH values. Figure 5 shows that the highest reducing sugar production was obtained in the starch medium with uncontrolled pH (initial pH 6.6). The optimal pH for glucoamylase and α-amylase of *S. fibuligera* was 5.0-6.2 (Hostinova, 2002). In the medium with controlled pH at 6.5, the yeast produced a minimum reducing sugar concentration and cell growth. Current results revealed that starch concentration, pH and temperature affected both growth and saccharification by *S. fibuligera* YCY1.

5) Effect of inoculums size

This study aimed to shorten the time for starch hydrolysis. The results showed that increasing of the inoculum size from 3 to 10% did not significantly ($p>0.05$) increase the amount of reducing sugar produced after 5 days of cultivation (Figure 6). Therefore, the 3% (v/v) inoculum size was selected for the next experiment.

6) Effect of shaking speed

Effect of oxygen transfer rate was studied by varying

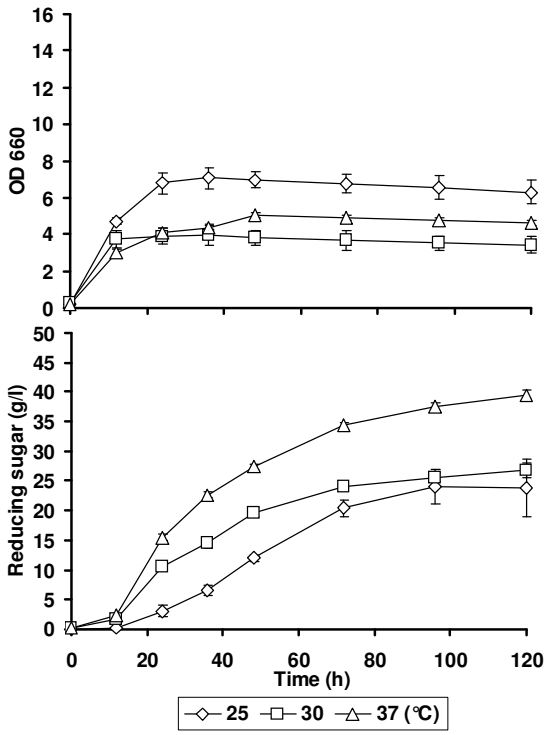

Figure 4. Effect of temperature on growth and reducing sugar production from cassava starch (50 g/l) by *Saccharomycopsis fiburigera* YCY1 at 150 rpm.

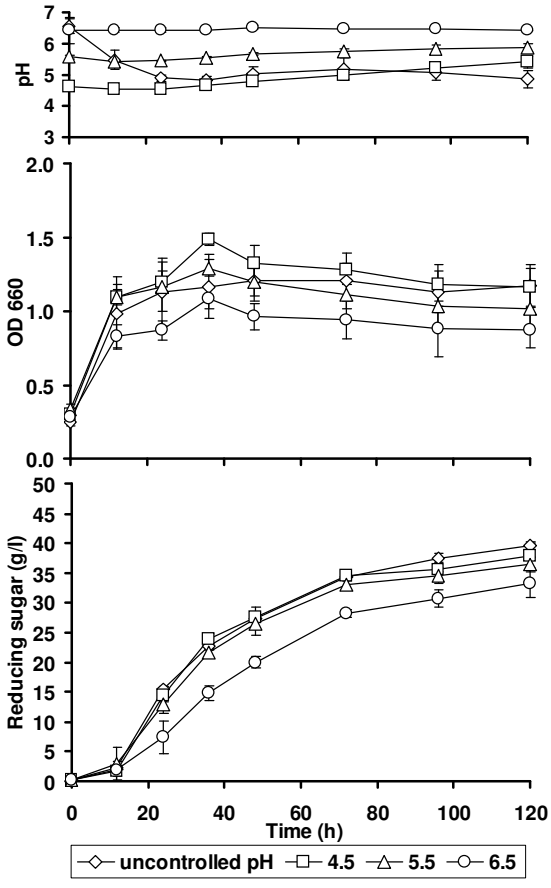

Figure 5. Effect of pH on growth and reducing sugar production from cassava starch (50 g/l) by *Saccharomycopsis fiburigera* YCY1 at 37°C and 150 rpm.

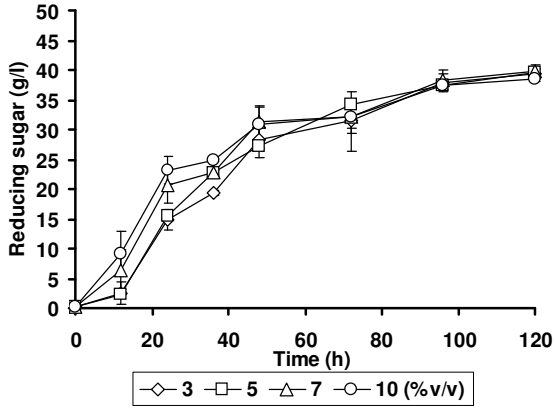

Figure 6. Effect of inoculums size on reducing sugar production from cassava starch (50 g/l) by *Saccharomycopsis fiburigera* YCY1 at 37°C and 150 rpm.

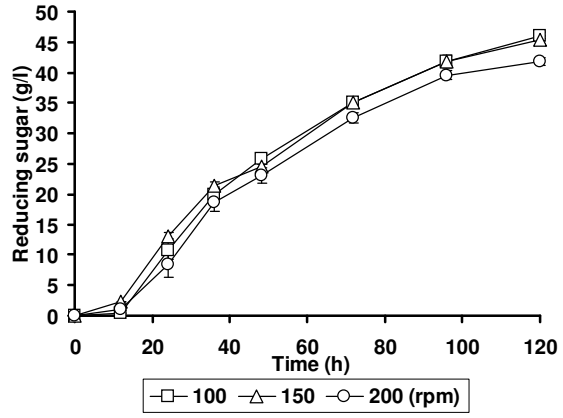

Figure 7. Effect of shaking speed on reducing sugar production from cassava starch (50 g/l) by *Saccharomycopsis fiburigera* YCY1 grown at 37°C.

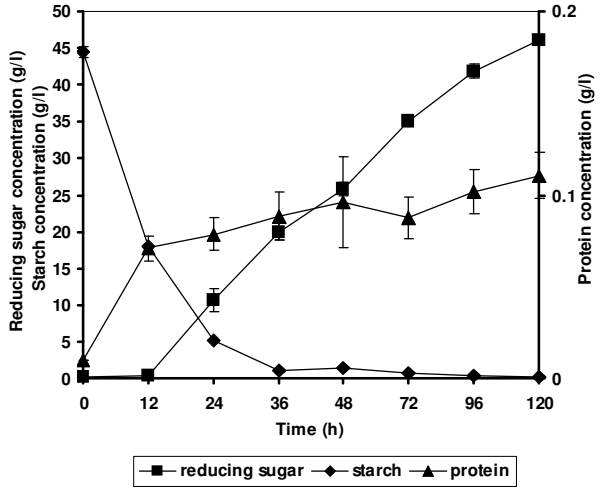

Figure 8. The time course of cell growth and reducing sugar production from cassava starch by *Saccharomycopsis fibuligera* YCY1 at 37°C and 100 rpm.

shaking speed from 100 to 200 rpm. The result shows that *S. fiburigera* YCY1 as cultivated in the starch medium with the shaking speeds of 100 and 150 rpm provided more reducing sugar than that cultivated at 200 rpm (Figure 7). During oxygen limitation, the yeast might consume less sugar for growth. Dostálek and Häggström (1983) found that *S. fiburigera* produced the reducing sugar at 200 rpm better than at 350 and 500 rpm.

3.3 The time course of cell growth and reducing sugar production

From the above studies, the optimum medium for saccharification by *S. fibuligera* YCY1 was 50 g/l cassava starch in distilled water without pH adjustment. The optimal cultivation conditions for reducing sugar production by *S. fibuligera* YCY1 in this medium were at 37°C and shaking speed at 100 rpm. Figure 8 shows the time course of starch hydrolysis and cell growth under the optimal conditions. Under these conditions, the highest reducing sugar was 46± 0.53 g/l (84% of the theoretical value) at 120 h of cultivation. Thin-layer chromatography analysis showed that the amylolytic enzyme of *S. fibuligera* YCY1 hydrolyzed starch to glucose and maltooligosaccharides. At 24 h of cultivation, the starch was converted to maltotetraose (G4) as a major product. After 24 h of cultivation, the maltotetraose decreased and the main products were maltotriose, maltose and glucose (Figure 9).

4. Conclusion

S. fibuligera YCY1 isolated from Loog-Pang (rice cake starter) could hydrolyze cassava starch with high reduc-

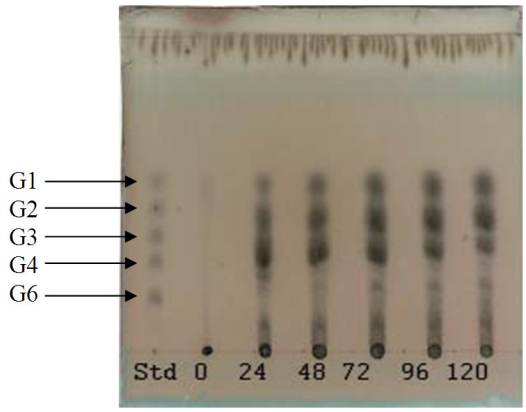

Figure 9. Thin-layer chromatographic analysis of the products from the hydrolysis of cassava starch by *Saccharomycopsis. fibuligera* YCY1. The solvent system contained isopropyl alcohol-ethyl acetate-water (3:1:1, v/v/v) and dipping reagent contained 0.3% w/v *N*-(1-naphthyl)-ethylenediamine and 5% v/v H_2SO_4 in methanol. A mixture of glucose and maltooligosaccharides was used as standards: glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4) and maltohexaose (G6).

ing sugar production. There is a potential to use this microorganism to saccharify starch for ethanol production.

Acknowledgment

The support by Faculty of Agro-Industry and the Graduate School of Prince of Songkla University is gratefully appreciated.

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