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The Dynamic Growth and Chemical Change of Mixed Cultures Inoculation on Tapioka Fermentation

MARIA ERNA KUSTYAWATI*, SRI SETYANI, AZHARI RANGGA, AND IRFA RISTA MUTIA

Department of Post Harvest Technology, Faculty of Agriculture, Universitas Lampung, Jalan S. Brojonegoro 1, Bandar Lampung, Indonesia.

Saccharomyces cerevisiae and *Lactobacillus plantarum* possess several extracellular and intracellular enzymes beneficial for cassava fermentation. Tapioka (cassava starch) has limited uses in food industries due to its low pasting properties, therefore, biomodification by the use of fermentation is needed. The research was aimed to monitor the growth of *S. cerevisiae* and *L. plantarum* during tapioca fermentation, and to evaluate the chemical change of the fermented tapioka. Mixed cultures was inoculated at the designed concentration into tapioca suspension and incubated at room temperature (30±2°C) in facultative aerobic condition for 0, 24, 48, 60, 72, 96, 120, and 144 h. The growth change of *S. cerevisiae* and *L. plantarum* was monitored, and the change of pH, residual sugar, and starch granule was investigated. The result showed that *S. cerevisiae* had longer lag phase as well as stationary than *L. plantarum* did*;* nevertheless*,* they both reached log phase at the same time. Co-inoculated mixed cultures did not affect the change on pH and reducing sugar but increased pronouncely protein content at stationary period. Besides, there was sign of erosion to the structure of cassava starch granules which was an indication of changes in the pasting property of the cassava starch.

Key words: chemical change, mixed culture co-inoculation, *Saccharomyces cerevisiae,* starch granule

Saccharomyces cerevisiae dan *Lactobacillus plantarum* memiliki berbagai enzim ekstraseluler dan intraseluler yang sangat mungkin memberikan manfaat pada modifikasi tapioka. Tapioka memiliki kegunaan terbatas pada industri makanan karena sifat pasting yang rendah, oleh karena itu, biomodifikasi dengan menggunakan fermentasi sangat dibutuhkan dalam upaya meningkatkan karakteristik tapioka. Kultur campuran pada konsentrasi tertentu diinokulasikan ke dalam suspensi tapioka dan diinkubasi selama 0, 24, 48, 72, 96, dan 120 jam pada suhu kamar (30±2°C) pada kondisi fakultatif aerobik. Pertumbuhan *S. cerevisiae* dan *L. plantarum* dimonitor, dan perubahan pH, gula residu dan granula tapioka diamati. Hasil pengamatan menunjukkan bahwa *S. cerevisiae* mempunyai fase lag dan fase stasioner yang lebih lama dibanding *L. plantarum*. Namun *S. cerevisiae* dan *L. plantarum* mencapai fase log pada waktu yang sama. Inokulasi kultur campuran *S. cerevisiae* dan *L. plantarum* tidak mengakibatkan perubahan terhadap nilai pH dan gula reduksi, tetapi meningkatkan protein secara nyata pada fase stasioner. Disamping itu, terdapat erosi pada struktur granula tapioka yang mengindikasikasikan adanya perubahan sifat pasta tapioka.

Kata kunci: fermentasi tapioka, inokulasi, kultur campuran, perubahan kimia, *Saccharomyces cerevisiae*

Saccharomyces cerevisiae has a very important role as a starter in the fermentation of various foods and beverages known as brewer's yeast, distillers yeast, and baker's yeast (Kurtzman and Fell 1998). In Indonesia, the use of yeast to produce traditional foods and fermented foods has not been so entrenched in comparison to fungi such as *Mucor* spp*., Rhizopus* spp*., Penicillium* spp., and *Aspergillus* spp., or the use of lactic acid bacteria *Lactobacillus casei, Lactobacillus lactis, Acetobacter xylinum, Acetobacter aceti,* due to lack of knowledge in the utilization as a starter or as an agent in the fermentation process. Yeast has amylolytic properties in starch degradation that is capable for producing amylase. Amylolytic yeast may have potential use in the food products as they contribute to the desired flavor (Schwan *et al*. 2007). The role of amylolytic yeast in producing yeast biomass from starch, and producing foods with low carbohydrates have much to do, such as production of amylase in fermentation of sticky rice, and cassava tape (Fleet 2001). Yeast great potential and still very necessary, especially in food diversification through a fermentation process to produce a new type of food or modification of existing products with better nutritional value, as well as aroma and texture adapted to the people's will.

Tapioca (cassava starch) has limited uses in the food industries due to its low in pasting properties. Researchers have focused on fermenting cassava with additional nutrients for improving the quality of cassava flour (Uboh and Akindahu 2005; Subagio

*Corresponding author: Phone/Fax: +62-721-783821, Email: maria.erna@fp.unila.ac.id

2006). However, a challenged method to improve the properties of tapioca has been attracting most scientists. One of the techniques was modification of physical, chemical, and pasting characteristic of tapioca by fermentation with the use of starter culture. Abriba (2012) investigated the microbial succession during the controlled fermentation of cassava tubers and isolated 8 potensial microorganisms, these were *Bacillus subtilis, Lactobacillus plantarum, Corynebacterium manihoti, Leuconostoc mesenteroides, Enterobacter aerogenes, Aspergillus niger, Geotrichum* sp., and *Saccharomyces cerevisiae*. Nevertheless, *L. plantarum* and *L.pentosus* were the dominat bacteria found in the beverage *cauim* produced from cassava and rice fermentation (Oguntoyinbo *et al*. 2010). Fermenting cassava with addition of with mixed cultures *Lactobacilus plantarus, Saccharomyces cerevisiae,* and *Rhizopus oryzae* produced the cassava flour having protein increased and reduced starct content (Gunawan *et al*. 2015). The addition of *Saccharomyces cerevisiae* during the fermentation of tapioca enriched the mineral and protein of the strach, as well as reduced the starch gelatinization temperature (Kustyawati *et al*. 2013). It was chalenged that inoculation of *S. cerevisiae* and *L. plantarum* can be used as starter cultures in submerged fermentation of tapioca slurry, since the mold such as *Rhizopus* sp. was most suitable to the solid fermentation. The objective of the work was to study the growth of *Saccharomyces cerevisiae* and *L. plantarum* during tapioca fermentation, to investigate the chemical change, and their effect on the pasting properties of the starch.

MATERIALS AND METHODS

Materials. Pure culture of *Saccharomyces cerevisiae* and *Lactobacillus plantarum* were purchased from the culture collection of Gadjah Mada University, broth Malt Extract broth, Malt Extract Agar (Difco™, Becton and Dickinson Company, Sparks, USA), saline (0.85 % NaCl), oxytetracycline and chloramphenicol, and reagents for chemical analysis were obtained from Sigma Chemicals Company (St.Louis, MO. White cassava tubers (Manihot utilisima var Kasetsart) were obtained from the Institute for Agricultural Research and Technology (BPTP) Bandar Lampung.

Culture Preparation. Single colonies from pure cultures of each species were inoculated separately into 5 mL of ME broth and MRS borth medium (Difco™, Becton and Dickinson Company, Sparks, USA) and then incubated at 25 °C and 32 °C for 24 h for *S. cerevisiae* and *L. Plantarum,* respectively. After this period, tubes were centrifuged, the pellets washed with sterile saline solution (0.9% NaCl, wt/vol) centrifuged and re-suspended again in sterile saline solution to obtain a concentration of about 7 log 10 CFU mL^{-1}.

Growth Medium Preparation. Fermentation experiments were carried out in submerged fermentation method of an extracted cassava. Briefly, 200 mL of extracted cassava slurry was placed into a 250 mL flask. The sugar concentration (10% glucose) was adjusted in distilled water and heated at 100 °C for 15 min to prevent sugar caramelization then added to the flask. The flask was independently inoculated with 50 µL of the yeast saline suspension to reach an initial concentration of inoculum of about 10.50±0.21 log10 CFU mL^{-1} for *S. cerevisiae*, and 10.36±0.32 log10 CFU mL^{-1} for *L. plantarum*. Flasks were incubated without shaken. Another flask was served as control without inoculation. The flasks were covered by cotton to create an microaerophilic condition then fermented at room temperature (30±2 °C) for 0, 24, 48, 72, 96, 120, 144, and 168 h. The filtrate was taken for analysis.

Chemical Analysis. Chemical analysis included pH, protein content, and reducing sugar. The pH of filtrate obtained from the fermentation was determined using Kent pH meter (Kent industry measurement limited, Surrey, England) model 7020 equipped with a glass electrode. Protein content expressed by soluble N was analyzed by the method of Kjedahl (AOAC 2009). Estimation of reducing sugar was done by Nelson-Somogyi method (Gusakov *et al*. 2011). Sugar that have the characteristic of being reducing sugars, as they contain functional groups capable of being oxidised and therefore causing reduction of other species under specific conditions. Structurally, reducing sugars must contain a free aldehyde or an alpha-hydroxy ketone capable of being oxidised.

Microbiological Analysis. Samples were taken from the fermentations daily. One mL of sample was taken from the flask and serially diluted to 10^{-4} to 10^{-6} with sterile distilled water into the test tubes. Following homogenizing the sample with the vortex, one mL of diluted sample was taken and spread plated onto petridishes with designated media that is malt extract agar (MEA) for growth of *S. cerevisiae*, and deMan Rogosa Sharpe agar (MRS) is for *L. plantarum*. Chloramphenicol 0.5% and Oxytetracyclin 0,5% were added to inhibit the bacteria. The plates were incubated aerobically at 29±2 °C for 24-48 h for *S. cerevisiae* and

37 °C for 48 h for *L. plantarum*. Counts were expressed as CFU mL^{-1}.

Effect of Mixed Culture Inoculation on Starch Granule and Pasting Properties. Structure change of starch granules was analized microscopically followed the method by McMaster (1994). Briefly, fresh sample of starch slurry (0,5% v/v) was taken and diluted into sterile aquades. After homogenizing an appropriate amount of diluted sample was dropped on to the *Haemocytometer* that has connection to the computer. Selested granules were then photographed. The pasting properties of native tapioca and modified tapioca (72 h fermentation) were observed and compared using the Brabender Micro Visco-Amylograph versoin 2.4.9 to evaluate gelatinization properties of starch during the process of cooking.

RESULTS

Microbial Growth. Four phases were detected in this experiment, they were adaptation phase (lag phase), growth phase (exponential phase), static phase (stationary phase), and mortality phase (death phase); even thogh, the growth phase of *L. plantarum* was not clear noted. The lag phase of *S. cerevisiae* was longer than *L. plantarum*; however, both of the cultures obtained the log phase at the incubation time of 72 h. The death phase of both *L. plantarum* and *S. cerevisiae* started at 120 h (Fig 1A).

pH Change. pH is one of the most important factors for maximizing growth of microorganisms. Co-inoculation of *S. cereviseae* did not affect the pH of the substrate. In the medium where either mixed cultures or *S. cerevisiae* alone was inoculated into, the pH decreased to 4 – 4.3 (Fig 1B). However, it decreased much lower than that of the other inoculated cultures when *L. plantarum* was co inoculated. Of all the measurements, the pH of the substrate started to decrease at 48 h fermentation, and stayed at the same value untill the fermentation was ending when it was co inoculated with *S. cerevisiae*.

Reducing Sugar. The ability of the mixed cultures to break down sugars is very little, of which it was also occurred to the control one (Fig 1C). Either control or mixed cultures started metabolizing sugar at the first 24 h, which was showed by sharply decreased the reduced sugar. However, the profile of reduced sugar was not variably changed at the following hours except at the first 72 h.

Protein Content. The protein content experienced to gradual augment with the fermentation time and that of more pronounced at 96 h of fermentation, thought as a stationary period. The contol had 1.32% and fermented one contained 1.82% at 96 h fermentation (Fig 1D).

The Change of Granule. Granules of native starch Hillum and lamellae of granules were noted in the native tapioca starch as seen in Fig 2A. They has hillum steadily stayed in the center of the granule and has bire fringence. Granules has birefringence characteristic under analyzes of polarization microcopic, and reflects the black and white color. The colors are as an indication of amilose structures in the starch. Where there was a notice of irregulated shape and cleaned appearance in the native strach, it could be due to the collition between granules during processing which could result in the breakage of granule structure. On the other hand, co-inoculated starch granules at 72 h incubation had irregulated shape (Fig 2B), lossed their birefringence characteristics, its hillum as well as lamellae was ruptured, and there was an indication of errosion on the periphere structur of granules.

DISCUSSION

Microbial Growth. Submerged fermentation of co-culturing *S. cerevisiae* and *L. plantarum* was applied in this study. The growth pattern of *S. cereviseae* and *L. plantarum* on cassava fermentation was shown in Fig 1. Four phases were detected, adaptation phase (lag phase), growth phase (exponential phase), static phase (stationary phase), and mortality phase (death phase). The lag phase of *S. cerevisiae* was longer than *L. plantarum*; however, both of the cultures obtained the log phase at the incubation time of 72 h. The environment condition such as the acidity of the substrate where the cultures were inoculated may comfort for the initial grow of *L. plantarum*. The long of lag phase experienced by *S. cerevisiae* indicated that the medium was unfavored. It was either lack of nutrition or excess of nutrient so that the cell needed the time to produce enzim suited to hydrolize the nutrients (Mahreni and Suhenry 2011). This may explain that *L. plantarum* had short time of the lag phase. At the stationary phase, wheather, *L. plantarum* or *S. cerevisiae* produced their metabolites products which stayed up to 120 h of incubation. This study was in agreement with Arroyo-Lopez *et al*. (2009) where yeasts had a very short lag period under the conditions included in the medium containing 50% glucose+50% fructose, ranged from 0.30 to 16.7 h. In correlation with the pH of the medium, it showed that the growth of *S.*

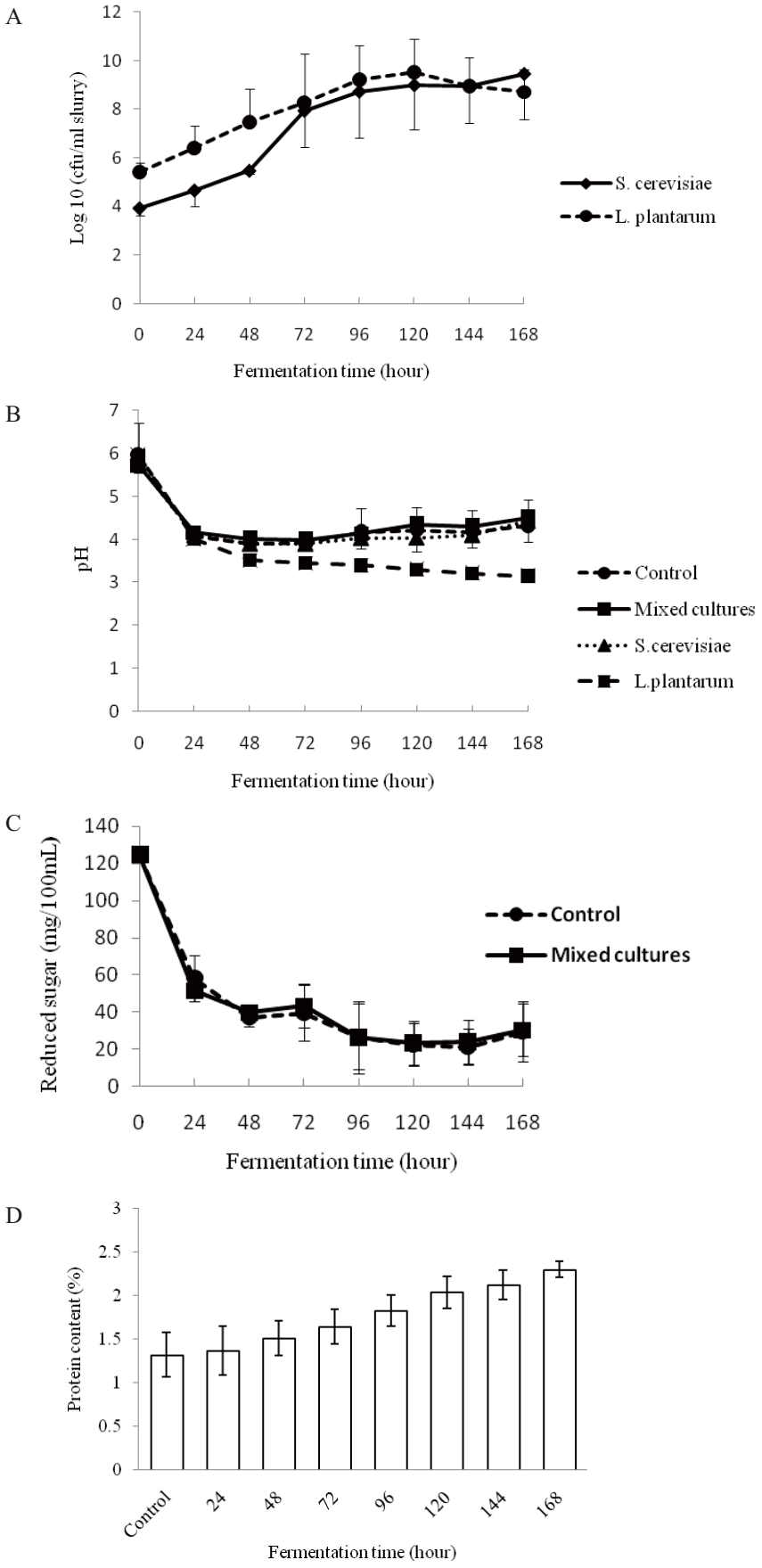

Fig 1 (A) Changes in the population of *Saccharomyces cerevisiae* and *Lactobacillus plantarum* during fermentation. (B) Effect of mixed culture co-inoculation on pH during fermentation. (C) Effect of mixed culture co-inoculation on reducing sugar during fermentation. (D) Effect of co-inoculated mixed culture on the protein content during fermentation.

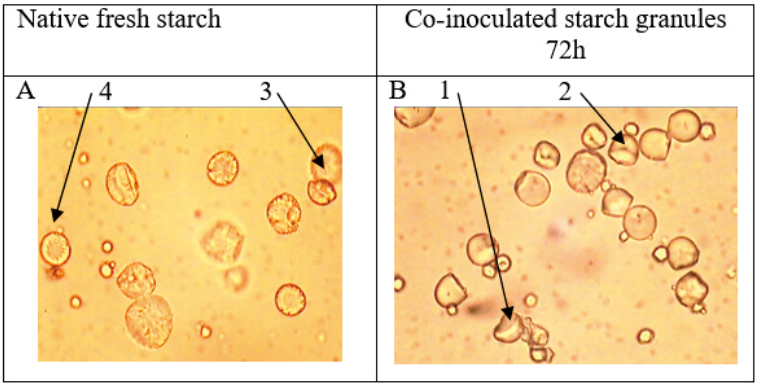

Fig 2 Effect of co-inoculated mixed cultures on the starch granules. Microscopic study at 100 magnification. 1. Signed of errosion, 2. Ruptured granules, 3. Hillus, 4. Lamellae.

Table 1 Amylograph properties of native tapioca and modified tapioca fermented for 72 h

Samples	Start gelatinization temperature (°C)	Maximum viscosisy (BU)	Breakdown (BU)	Setback (BU)
Native tapioca	71.2±0.15	1089±0.25	761±0.2	305±0.32
Modified tapioca	71.8±0.18	1150±0.15	624±0.15	588±0.15

cerevisiae may favor with low of pH 3-4 (Fig 1B). Wahono *et al.* (2003) found that optimum pH for *S. cerevisiae* was 4-4.5. pH affects the rate fermentation of *S. cerevisiae* of which the optimal value was 4-4.5 (Frazier and Westhoff 1978).

The Change of pH. It could be that the production of acid causing the decrease of pH was more carried out by *L. plantarum* than *S. cerevisiae*. The results agree with the other findings that the optimum pH levels for addition of *S. cerevisae* were from 3.5 to 6.0 (Manikandan and Viruthagri 2010; Polyorach *et al.* 2013). In correlation with the growth of bacteria (Fig 1a), it showed that the pH of the medium lowered as the increase of growth of *L. plantarum*. The metabolism products of the *L. plantarum* may greatly affect the pH of the fermentation media.

Reducing Sugar. This research showed that at the longer fermentation, reducing sugar increased. It was assumed that extracellular amylase activities was getting higher as the fermentation was longer resulting in more starch was hidrolysed which increased the reducing sugar content. This was in agreement with the research done by Kartikasari *et al*. (2016).

Protein Change. The protein content of the fermentation medium innoculated with mixed culture (1.82 %) was higher than that of the control one (1.32%). It was likely that yeast biomass contributed to the protein since there was no nitrogen source added to the fermentation medium. The microbial biomass product which was formed during the fermentation contributed to the protein increase since the cultures remained in the fermentation substrate. In addition, the production of microbial enzymes during the fermentation may also contribute to the increase of protein. This finding was egreement to the study done by Yuangklang and Wachirapakorn (2011) where crude protein of cassava pulp fermented by *S. cerevisiae* increased at the 5th day of fermentation, as a result of the production of single cell protein during the fermentation process.

The Change of Starch Granules. The analysis of starch granules was carried out to find out wheather the addition of mixed culture *S. cerevisiae* and *L. plantarum* was able to improve the pasting properties of cassava starch. Fig. 1C showed that the starch granules had lossed their bierefringence which were indicated by the ruptured of the granule lamellae and undetected hillus. The extracellular amylolytic enzyme produced by the cultures hidrolyzed the liberated starch granules especially in the granule surface and resulted in the formation of hole like looked at the granule surface that contributed to the possible liberation of starch from the granules. When the starch granules were degraded and the starch was released, the pasting properties of the strach such as starch gelatinization, viscosity, and other rheological properties of the starch could have been changed. The granules that losses their birefringence characteristic may have changes in their pasting properties due to the change in the amylose structure. The reasons beyond

this process could have been the enzymatic activity of cultures that hydrolyzed carbon backbone chain of the oligosaccharide in the starch. This study was agree with previous study done by Kustyawati *et al*. (2016) that an errotion occured in the starch granules fermented with *S. cerevisiae* only. The growth of lactic acid bacteria during cassava fermentation produces enzym hidrolyzing starch material resulting in the changes of its functional properties. The characteristic differences of such as granular shape, ratio amylose/amylopectin, molecular starch and the existence of other components influenced the ability of starch to form the final product characteristics which are desired (Copelan *et al.* 2009).

Pasting viscosity is an important characteristic of starch during heating of water-starch suspension as this was the basic thought when the starch was applied to food industries. In this experiment (Table 1), the increase in temprerature gelatinization of modified tapioca may be due to the changed of granule structure and the more complex compounds containing in the starch. The increase in protein and reducing sugar, and low pH may lead to more energy needed for gel formation. Breakdown is an indication of how easier the the rupture or breakdown the granule structure is (Varavinit *et al.* 2003). High breakdown value leads the strach to bear cohesiveness characteristic which less use in food industries. The modified tapioca had the low breakdown value meaning more aplicable to food industries. Another pasting property correlated to viscosity is setback. Setback value indicates the occuring of retrogradation or sineresis of the starch. The modified tapioca in this experiment had high setback value which meant it was easily undergo retrogradation. This may be influenced by high protein content in the starch. The presence of protein, lipid, ash, fiber, and as well amylose contribute to the retrogradation (Eliasson 2004). This finding was in agreemnet with Lindeboom *et al.* (2004) where starch with high protein and amylose will retrogade more because amylose entraps more water and undergoes recrystalization.

In conclusion, *S. cerevisiae* and *L. plantarum* were growth quite well during tapioca fermentation, and resulted in the chemical characteristic changes of modified tapioca. The increase in protein content and reducing sugar, as well as the occuring of granula erosion in the starch contributed to the improving of pasting properties of modified tapioca. *S. cerevisiae* and *L. plantarum* was important for improvement of tapioca pasting properties which was useful in food industries.

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