Investigation of the metabolic inhibition observed in solid-substrate cultivation of Clostridium thermocellum on cellulose

Vidya S.S. Dharmagadda, Sue E. Nokes, Herbert J. Strobel, Michael D. Flythe

Abstract

Metabolic inhibition of Clostridium thermocellum, when grown in a high solids environment, was investigated by comparing submerged fermentation (SmF), solid-substrate cultivation (SSC) and solid-substrate cultivation with media replacement by periodic flushing (FSSC). Cellulose conversion extent and end-product concentrations were measured over time. SmF converted ~65% of the cellulose in 240 h (10 days), whereas SSC converted 8% in the same period. FSSC converted approximately 25% and 47% of initial substrate after 240 h; 45% and 71% of initial substrate after 25 days, with media replacement every 24 and 12 h, respectively. The SSC experienced higher initial production rates for all fermentation products, but could not sustain production rates. When acetate concentrations reached a critical point, the acetate decreased the intracellular volume of C. thermocellum cell suspensions at pH values similar to those observed in SSC. Acids produced by fermentation exacerbated the already unfavorable osmotic condition of SSC, resulting in metabolic inhibition. Consistent with this finding, approximately constant amounts of ethanol, acetate and lactate were produced during each flush of the FSSC. Flushed solid-substrate cultivation maintained favorable growth conditions for C. thermocellum even up to 25 days, allowing more total product to be formed than in other cultivation methods.

1. Introduction

Solid-substrate cultivation (SSC) is an alternative production method to traditional submerged liquid fermentation (SmF). A number of advantages of the SSC process make it a promising technology for enzyme production (Chinn et al., 2006). Technical challenges which limit the implementation of many SSC processes include difficulty in removing metabolic heat, providing adequate aeration through the substrate, and controlling the substrate moisture content (Krishna, 2005). Nevertheless, SSC may be an attractive cultivation technique for thermophilic, anaerobic organisms because the heat and mass transfer problems encountered in SSC for mesophilic, aerobic organisms are not an issue (Chinn et al., 2006).

Clostridium thermocellum is a thermophilic, anaerobic bacterium known for its unusual ability to hydrolyze cellulose in lignin-containing materials (Lamed et al., 1983); for example lignocellulosic agricultural residues such as corn stover (Demain et al., 2005).

Unlike fungal cellulases, the C. thermocellum cellulase complex (cellulosome) is highly active on crystalline cellulose, with a specific activity reported to be around 50 times higher than Trichoderma sp. (Demain et al., 2005). The bacterium is also of interest because it has the potential for direct microbial conversion of the cellulosic biomass to ethanol by combining cellulase production, hydrolysis and fermentation in a single bioreactor (Lynd et al., 2002). While significant information exists on the growth and metabolism of C. thermocellum in SmF, there are only three previous reports on growth in SSC (Chinn et al., 2006, 2007, 2008). Chinn et al. (2006) screened nine thermophilic anaerobic bacteria in solid-substrate cultivation on four lignocellulosic substrates and observed twice the concentration of end-product (ethanol, acetate, and lactate) in SSC compared to SmF with some organism/substrate combinations. Chinn et al. (2006) also reported that end-product concentrations in SSC increased at a constant rate until day 6 when cessation of end-product formation occurred, even though sufficient substrate remained. Chinn et al. (2006) reported significant end-product formation from lignocellulosic substrates in SSC which demonstrated the potential of thermophilic, anaerobic bacteria to perform well in this unique environment. However, the reason for the observed inhibition in SSC was unclear, and elucidating this mechanism was the objective of the study herein reported (Chinn et al., 2007, 2008).
2. Methods

2.1. Cell culture preparation

*C. thermocellum* ATCC 27405 was obtained from the American Type Culture Collection (Rockville, MD). The organism was grown in basal medium that contained (per liter): 1530 mg Na₂HPO₄, 1500 mg KH₂PO₄, 500 mg NH₄Cl₂, 500 mg (NH₄)₂SO₄, 90 mg MgCl₂·6H₂O, 30 mg CaCl₂, 4000 mg yeast extract, 10 ml standard vitamins, 5 ml modified metals, 500 mg cysteine hydrochloride, 1 ml resazurin, and 4000 mg sodium carbonate. The medium pH was adjusted to 6.7 with NaOH unless otherwise indicated, and was maintained under a 100% carbon dioxide atmosphere. The vitamin solution contained (per liter 100 mg pyradoxamine 2 HCl, 200 mg riboflavin, 200 mg thiamine HCl, 200 mg nicotinamide, 200 mg CaD pantetheinat, 100 mg lipoic acid, 10 mg p-aminoazetic acid, 5 mg folic acid, 5 mg biotin, 5 mg cobalamin(Co B₁₂), 100 mg pyridoxal HCl, 100 mg pyridoxine. The modified metal solution contained (per liter): 500 mg Na₂EDTA, 200 mg FeSO₄·7H₂O, 10 mg ZnSO₄·7H₂O, 200 mg MnCl₂·4H₂O, 20 mg H₂BO₃, 20 mg CoCl₂·6H₂O, 1 mg CuCl₂·2H₂O, 2 mg NiCl₂·6H₂O, 3 mg Na₂MoO₄·2H₂O, 10 mg Na₂WO₄·2H₂O, 1 mg Na₂SeO₃.

2.2. Inoculum preparation

A bacterial culture from a ~80 °C stock was grown for 24 h in Balch tubes at 60 °C containing 10 ml of basal medium supplemented with 4 g per liter cellobiose. This initial culture was then used to inoculate 80 ml of cellobiose-containing medium. After 15 h of growth, this secondary culture was diluted with fresh basal media without cellobiose to prepare the standard inoculum stock (final optical density of 0.143 OD₆₀₀: ~0.07 g dry cells/l) for use in SmF, SSC, and flushed solid-substrate cultivation (FSSC).

2.3. Cultivation

All SmF experiments were carried out in 125 ml serum bottles containing 30 g/l microcrystalline cellulose (Avicel PH 101, FMC, Philadelphia, PA) as substrate. In the SmF cultivation, 46.5 ml of basal medium was amended with 3.5 ml of standard inoculum. In SSC, 1.5 g substrate was inoculated with 3.5 ml of standard inoculum and this provided an initial moisture content of 73% (wet basis). FSSC was carried out in 2.5 cm I.D. and 10 cm length glass columns loaded with 10 g of Avicel and inoculated with 23.4 ml standard inoculum yielding an initial inoculum of 0.156 mg dry cells/g substrate.

2.4. Sampling

Destructive sampling was carried out at 0, 24, 48, 60, 72, 96, 120, 144, 192 and 240 h for liquid and solid-substrate cultivation. The culture fluid in SmF samples was separated from cells and cellulose material by centrifugation (5000g, 10 min, 4 °C). Sampling of SSC bottles involved addition of 15 ml 100 mM potassium phosphate (pH 7) followed by centrifugation (5000g, 10 min, 4 °C) of this mixture. Aliquots of cell-free supernatant were stored at −20 °C for further analysis. The pellet was stored for dry weight determination, nitrogen analysis, and cellulose conversion measurements. In the FSSC, columns were flushed with 23.4 ml fresh media using one of two time intervals (12 or 24 h) for a period of 25 days with destructive sampling on day 10, 15, 20, and 25. The flushed media at each time interval was collected, centrifuged (5000g, 10 min, 4 °C) and aliquots were stored at −20 °C for end-product analysis.

2.5. End-product concentrations

Comparing product formation between liquid and solid-substrate cultivation is challenging, because the logical units for SmF are g/l, yet the logical units for SSC are g/g substrate. We chose to address this problem by reporting products from both cultivation methods in g/l of culture fluid. The SSC had additional buffer added to extract the end-products, however the concentration calculations were corrected back to the initial volume of liquid. We also include a comparison of the results in a suite of units at 10 and 25 days for comparison purposes.

2.6. Measurement of dry weight and cellulose conversion

The residual substrate from SmF and SSC bottles was washed with 50 ml distilled water and centrifuged twice at 5000g for 20 min. Dry weight was determined by drying the residual substrate (Avicel) at 100 °C until constant weight was achieved in at least 24 h. The fraction of cellulose converted (χ) was calculated using the equation:

\[
\chi = \frac{S_{\text{initial}} - S_{\text{final}}}{S_{\text{initial}}} \tag{1}
\]

where S denotes the dry weight of the substrate (g), either final or initial.

2.7. Nitrogen analysis

The washed pellets collected from incubations were analyzed for microbially-associated nitrogen according to method adopted AOAC 990.03 (AOAC, 1995) using a carbon–nitrogen analyzer (FP-2000, LECO Corporation, St. Joseph, MI, USA).

2.8. Carbon recovery

The carbon recovery was calculated as an index reflecting the extent to which consumed substrate was accounted for in fermentation products. Carbon recovery was calculated as: Carbon recovery = [3 × (E + A + L)]/[6 × S₀ − (S₁ − Reducing sugars)] which assumes stoichiometric production of CO₂ with acetate and ethanol. S₀ and S₁ denote initial and final substrate, E, A, L denote ethanol, acetate and lactate concentration (g/l), respectively.

2.9. End-product analysis

Cellulbiose, ethanol, glucose, and lactate in the supernatant were measured by enzymatic methods as previously described (Bergmeyer, 1963; Russell and Baldwin, 1978). Acetate concentrations were determined in acidified samples by gas chromatography, using a column (1.83 m long, 4 mm diameter) packed with SP-1000 (1% H₃PO₄, 100/120 mesh, Supelco). Nitrogen was used as a carrier gas and the inlet and detector temperatures were 185 and 190 °C, respectively. The system was programmed to maintain a constant oven temperature of 135 °C.

2.10. Intracellular volume

*C. thermocellum* ATCC 27405 was cultured in basal medium with cellobiose (4 g/l). Stationary phase cultures (16 h) were harvested by centrifugation (1500 g, 10 min) without exposure to oxygen. The cells were resuspended (400 mg cell protein ml⁻¹) in basal medium that was amended with sodium chloride or sodium ace-
tate (pH adjusted with HCl or NaOH as indicated). Cellobiose (4 mg ml⁻¹) was added, and the suspensions were incubated (60 °C, 20 min). The suspensions were anaerobically transferred to tubes containing [³H]–H₂O (50 μl ml⁻¹, 185 KBq μl⁻¹; Amersham Pharmacia Biotech Inc., Piscataway, NJ) or the extracellular marker, [³H]–taurine (0.5 μmol l⁻¹, 999 GBq mmol⁻¹; GE Healthcare, Buckinghamshire, United Kingdom). The tubes were mixed with a vortex, and incubated for an additional 5 min. When the tubes were opened, samples (0.9 ml) were transferred to microcentrifuge tubes that contained silicone oil (Barker and Kashket, 1977). The cells were separated from the supernatant by centrifugation (13,000g, 1 min). Supernatant samples were retained, and the tubes were frozen (−20 °C, 30 min). The cell pellets were clipped from the tips of the tubes with dog nail trimmers, mixed by vortex and permitted to dissolve in scintillation fluid (24 h). The pellet and supernatant samples were analyzed with a Packard Tri-Carb 1500 TR liquid scintillation counter (PerkinElmer, Waltham, MA). The intracellular volume was estimated from the ratio of water to the extracellular marker as previously described (Barker and Kashket, 1977; Flythe and Russell, 2007). The value of complete plasmolysis was determined with control cells, which were depleted by valinomycin and nigericin (5 μM each; Sigma–Aldrich, St. Louis, MO).

3. Results and discussion

Avicel was converted and microbial nitrogen increased in both SmF and SSC (Fig. 1). Approximately 65% of the Avicel was converted after 240 h in SmF, whereas the SSC converted 8% in the same period. Since Avicel is devoid of nitrogen, analysis of nitrogen in washed pellet samples was used as a measure of microbial growth. Nitrogen quickly increased in SmF until ~34 h. In contrast, the nitrogen content for SSC incubations did not change appreciably over time, indicating relatively little microbial growth in this treatment was measurable after 24 h. Avicel conversion continued even after net cell growth ceased. The pH of both SSC and SmF decreased over time with the pH of the SSC falling more rapidly in the initial 60 h of cultivation (Fig. 2).

SSC experienced higher initial production rates (Table 1) for all end-products. By 48 h SmF and SSC formed similar concentrations of ethanol and lactate; however acetate was twice as concentrated in the SSC, and reducing sugars were approximately three times as concentrated in the SSC than the SmF, predominately due to increased amounts of glucose, however SSC had consumed 1/10 of the Avicel as SmF. The carbon balance for the cultivations are reported in Table 1 as well.

The FSSC cultivations that were flushed every 12 h converted approximately 47% and 71% of the initial Avicel after 10 and 25 days, respectively. The cultivations flushed every 24 h converted 25% and 45% of the initial Avicel after 10 and 25 days, respectively (data not shown). The control incubations (solid-substrate cultivation without flushing) resulted in only 11% Avicel conversion after 25 days (data not shown). Cell-associated nitrogen increased throughout the entire period and the 12 h flushing treatment resulted in nitrogen values approximately 3-fold higher than the treatments flushed every 24 h. The pH of the media flushed from the cultures varied from 6.2 to 6.6 throughout the incubation time (Fig. 3).

Higher concentrations of ethanol, acetate and lactate were measured in the flushate for the 12 h flushing than the 24 h flushing (Fig. 4), resulting in a higher cumulative production of acetate and ethanol in the 12 h flushing FSSC cultivation method.

Fig. 5 compares the total end-product production rates for the different cultivation methods, showing that the SSC had the highest initial production rate (0–24 h), but SSC slowed considerably by 48 h. By the end of the fermentation period, only the FSSC method was still producing measurable end-products, and the 12 h flushing treatment exhibited a higher production rate than the 24 h flushing treatment.

Energized resting cell suspensions were used to determine the effect of acetic acid on C. thermocellum at the pH values observed in SmF and SSC. When the cells were incubated at neutral pH values, intracellular volumes of approximately 2 μl mg cell protein⁻¹ were observed (data not shown). The substitution of 80 mM sodium acetate for an equal concentration of sodium chloride had little effect at pH values near neutral. However, intracellular volume dropped to around 0.5 μl mg cell protein⁻¹ when the pH of the acetate suspensions was 5.9 or less, yet intracellular volume remained relatively constant in the sodium chloride solution at low pH.

We observed a significant decline in the rate of Avicel hydrolysis by C. thermocellum in SSC after 24 h, even though more than 90% of the substrate remained, and this observation is similar to the results of Chinn et al. (2006) on more complex substrates. Even in SmF, the metabolism of C. thermocellum ceased by 240 h even though ~35% of the initial Avicel remained. Indeed, cell nitrogen did not accumulate in SSC, and several other measurements indicated metabolic inhibition after the first 24 h of cultivation. The rate of product formation in SSC during the first 24 h was ~0.2 g l⁻¹ h⁻¹, but declined to ~0.04 g l⁻¹ h⁻¹ during the second 24 h. The pH decreased from 6.5 to 5.9 in the first 24 h, and 5.9 to 5.7 during 24–48 h. However, the cellulolytic enzyme system
appear to function throughout the fermentation because cellobiose and glucose continued to accumulate.

Cellobiose and glucose are the main products of cellulose hydrolysis by *C. thermocellum* (Ng and Zeikus, 1981). Johnson et al. (1982) reported a 35% inhibition of cellulase activity at 1 g/l cellobiose when microcrystalline Avicel was used as the substrate and complete inhibition of cellulase activity occurred at concentrations of 20 g/l. Note that *C. thermocellum*’s cellulosome contains the cellulase enzymes, and cellulase activity controls the rate at which cellobiose is available to the cell. *C. thermocellum*’s cellulosome was much less sensitive to glucose, as it required 60 g/l of glucose to produce 35% cellulase inhibition. In the present study, glucose concentrations never exceeded 13 g/l, therefore, glucose concentrations were not at inhibitory levels. Cellobiose accumulated to 3 and 1 g/l after 120 h, and 5.8 and 5.2 g/l at 240 h as detected in SSC and SmF, respectively (Table 1). Some inhibition to the organism’s cellulosome activity therefore may have occurred in SSC by 120 h, but the slowing rate of cellobiose availability by 120 h is not a plausible reason for the cells’ metabolism to essentially cease by 240 h in SSC.

Ethanol is also known to inhibit the growth of *C. thermocellum* under some conditions, which has been attributed to enzymatic feedback inhibition (Herrero et al., 1985). Herrero and Gomez reported that the growth of *C. thermocellum* was inhibited by 50% at 5 g/l ethanol (Herrero and Gomez, 1980). However, in the present study ethanol concentrations were <3 g/l in all cases, so ethanol is unlikely to be the cause of premature inhibition.

Regular flushing of cultures with fresh media in FSSC improved cellulose conversion when compared to SSC, and the improvement was more pronounced when the flushing was more frequent (12 h flushing).

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Table 1

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<th>24</th>
<th>48</th>
<th>60</th>
<th>72</th>
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<td>Avicel consumed (g)</td>
<td>0.05 ± 0.02</td>
<td>0.07 ± 0.04</td>
<td>0.16 ± 0.00</td>
<td>0.18 ± 0.02</td>
<td>0.19 ± 0.04</td>
<td>0.21 ± 0.06</td>
<td>0.23 ± 0.08</td>
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<td>Carbon recovery (%)</td>
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<td>94.61</td>
<td>85.69</td>
<td>80.49</td>
<td>75.85</td>
<td>72.02</td>
<td>68.19</td>
<td>64.36</td>
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**Solid-substrate cultivation (SSC)**

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<td>68.19</td>
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<td>60.54</td>
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A “–” indicates levels were below detection limits.

a Cellobiose and glucose were measured separately to determine if they were present at inhibitory levels.

b Carbon recoveries were calculated from ethanol, lactate, acetate, and soluble reducing sugar concentrations (which include cellobiose and glucose).
Increase in the nitrogen content, presumably cell mass, in the presence of solid substrate with two different flushing intervals showed that flushing the culture tubes with fresh media every 12 h maintained more favorable growth conditions for the bacteria. The products of cultivation in both 12 and 24 h flushing and the increase in percent nitrogen indicates that *C. thermocellum* was metabolically active even after 25 days which was not the case with the traditional SSC.

The SmF treatment exhibited the effects of inhibition between 48 and 60 h of fermentation, based on the nitrogen accumulation (Fig. 1). As seen in Table 1, Avicel was converted to cellobiose and glucose even when growth was inhibited. Again, this latter result indicates that the cellulose remained active through 240 h of fermentation. The rate of product formation decreased as the fermentation progressed, and the pH declined.

When fermentative bacteria grow in substrate-excess conditions, growth can be inhibited by the accumulation of fermentation end-products. In some cases, the cessation of growth is simply due to enzymatic feedback inhibition, which makes product formation energetically unfavorable (Moat and Foster, 1995). However, the accumulation of fermentation acids in the media is also accompanied by a decline in pH. Many bacteria maintain an intracellular pH value close to neutral, and the difference in pH across the cell membrane is called the ΔpH (Harold, 1986). Fermentation acids can pass through the cell membrane in the uncharged, protonated form, but when the proton dissociates in the more alkaline cytoplasm, the resulting anion is no longer membrane soluble (Russell and Diez-Gonzalez, 1998). Unless the cell responds, in-the-cell anions will accumulate in proportion to the ΔpH. For instance, when the extracellular lactate concentration is 0.1 M and the ΔpH is 1.0, the intracellular acetate concentration can be as great as 1 M (Flythe and Russell, 2006). Like all cytoplasmic molecules, the fermentation anions are associated with water in a shell of hydration (Csonka, 1989). This water contributes to the turgor pressure within the cell, thus; the effect on osmotic homeostasis can be substantial.

Bacteria counteract intracellular anion accumulation in a number of ways. *Streptococcus bovis* JB1 and *Escherichia coli* O157: H7 are highly resistant to fermentation acids (Russell, 1992; Diez-Gonzalez and Russell, 1997). These bacteria permit their intracellular pH to decline so that a large ΔpH is never formed, but this strategy requires enzymatic pathways that are tolerant of low pH. In contrast, *E. coli* K12 and *C. sporogenes* MD1 maintain a large ΔpH when the extracellular pH is acidic (Roe et al., 2002; Flythe and Russell, 2006). Fermentation acids accumulate, but the bacteria compensate by decreasing the concentrations of other cytoplasmic osmolytes. *C. sporogenes* MD1 responds to lactate accumulation by decreasing intracellular glutamate. This latter bacterium also expels intracellular potassium under acidic conditions, which causes a decrease in intracellular volume (Flythe and Russell, 2007). Given that the intracellular volume of *C. thermocellum* also decreased in the presence of acetic acid at low pH, a similar physiological response seems likely.

A decrease in intracellular water is advantageous when anion accumulation threatens to increase cellular turgor pressure, but SSC presents other osmotic challenges. SSC is an exceptionally hypotonic environment, which could desiccate cells by osmosis. Under hypotonic conditions, many bacteria increase the intracellular concentrations of potassium, glutamate, trehalose, and other osmolytes (Csonka, 1989). The osmolytes maintain isotonic intracellular conditions, and intracellular water is retained. However, large concentrations of fermentation acids were produced by *C. thermocellum* in SSC. It is clear that cells cannot concomitantly increase and decrease cell volume, which leads to the conclusion that the hypotonic and fermentation acid stress responses are incompatible. Indeed, a combination of fermentation acids and low water content has been used throughout history to prevent microbial growth in food (e.g. cheese, fermented sausage).

The results of the SmF and FSSC experiments were consistent with the hypothesis that a combination of osmotic and acid stresses inhibited the growth of *C. thermocellum* in SSC, because similar acetate concentration in SmF did not inhibit metabolism. Adding more liquid cultivation media (SmF) or flushing with media during cultivation (FSSC) prolonged metabolism, and demonstrated greater substrate conversion than SSC. Both of the FSSC treatments exhibited a greater rate of production at 240 h, with the 12 h flushing metabolizing at a faster rate than the 24 h flushing treatment. There are three plausible and mutually compatible mechanisms by which FSSC could promote metabolism and growth. Flushing with fresh media: (1) decreased the osmotic stress by adding water, (2) increased the pH, which decreased the ΔpH across the cell membrane, and (3) removed fermentation acids.

In spite of the challenges with growing bacteria in the solid-substrate environment, there may be some commercial advantages to this cultivation method. Table 2 compares products produced by

**Table 2**

<table>
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<th>g/l cultivation fluid</th>
<th>g/(g substrate used)</th>
<th>g/(g initial substrate)</th>
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<th>kg/m³</th>
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<td>0.29</td>
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<td>FSSC12 (25 days)</td>
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the four cultivation methods from several different angles. One of the more-often quoted advantages of SSC is that the products are more concentrated. This holds true for this experiment also, however if one wants to extract the product from the solid matrix, additional liquid is typically added and the advantage of a higher product concentration is lost. Another interesting way to compare techniques is to compare product/volume of reactor. FSSC12 has a slight advantage at 10 days, and a significant advantage if the fermentation proceeds to 25 days, which has implications when selecting production equipment.

4. Conclusions

Anaerobic, thermophilic bacteria may be ideally suited for solid-substrate cultivation because the heat and mass transfer challenges associated with aerobic, mesophilic organisms are alleviated. However *C. thermocellum* exhibited premature metabolic inhibition during solid-substrate cultivation on cellulose. The results of the SmF and FSSC experiments were consistent with the hypothesis that a combination of osmotic and acid stresses inhibited the growth of *C. thermocellum* in SSC, because similar acetate concentration in SmF did not inhibit metabolism. Therefore, in order to grow *C. thermocellum* in solid substrate culture, flushing on regular intervals with fresh media is suggested.

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