CHAPTER 29

PRODUCTION OF ETHANOL FROM XYLOSE BY *CANDIDA SHEHATAE* GROWN UNDER CONTINUOUS OR FED-BATCH CONDITIONS

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29.1 XYLOSE AS A FERMENTATION FEEDSTOCK

Xylose is a major component of angiosperm lignocellulosic residues. It is available from a number of different sources in the forest products industry, including fiberboard manufacture, sulfite waste liquors, production of dissolving pulp, and the hydrolysis of hardwood residues. Hydrolysis of wood for the production of liquid fuels, particularly ethanol, has been proposed and practiced at various times and especially during petroleum shortages such as those that occurred in World War II or the late 1970s and early 1980s.

In the past, conversion of wood sugars to ethanol has been limited to the hexoses because xylose was not fermentable; oxygen is required for growth of xylose-fermenting yeasts. For further information about the role of oxygen,[13] biochemistry,[15] microbiology,[14] cell physiology,[20] bioprocessing,[22] or other aspects of the yeast xylose fermentation[19,21] or for a more detailed treatment of growth kinetics and energetics in continuous fermentations: earlier reviews should be consulted. The present article deals with continuous and fed-batch fermentation of xylose by *Candida shehatae.*

Because of the prevalence of xylose in angiosperm lignocellulose, xylose is probably the second most abundant sugar in nature. It is also

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readily recovered. Xylans (and other hemicellulosic sugars) are more easily hydrolyzed by acid and enzymes than is cellulose, and the yields from wood are higher.[12,27] So, even though xylose comprises only 15% to 35% of the total sugars in wood, it comprises as much as 45% of the recoverable sugars.

Because xylose is such a large fraction of the total recovered sugars, its utilization is essential to the practical conversion of lignocellulosic residues. Any process attempting to ferment hexose sugars alone would encounter significant disposal costs for the resulting xylose if angiosperms were used as the feedstock. This is not a problem, however, because xylose recovered from enzymatic hydrolysate can be fermented to useful products, including ethanol and xylitol.

Ethanol has the largest single market. The present United States capacity for ethanol production is about 4.5×10^9 L/year. Essentially all the ethanol is processed from corn (maize). The wholesale price of ethanol (95%) is about $0.30/L in the United States, and about 65% of the production cost is in grain price. Because of various market considerations, chiefly the sale of by-product distiller's dried-grain solids, the maximum amount of ethanol that can be produced from maize is about 11×10^9 L/year. The potential demand for ethanol as a chemical feedstock or transportation fuel is significantly larger than this, so ethanol from wood sugars could be marketed.

Xylitol is a nonglucose sweetener. It exhibits excellent confectionery properties, it is noncariogenic, and noncarcinogenic, and it wholesales for about $20/lb. Xylitol is a by-product of xylose fermentation. A potential market exists for xylitol as a sweetener in chewing gums. Xylitol has been used as a sweetener in Finland for a number of years, and following extensive studies, the United States Food and Drug Administration has dropped objections to its domestic use (Reilly, personal communication; see also ref. 11).

29.2 SELECTION OF YEASTS FOR XYLOSE FERMENTATION

Most xylose-metabolizing yeasts do not produce ethanol. About half of all yeasts will grow on xylose under aerobic conditions, but very few of them will ferment xylose. An even smaller number will produce economically significant concentration of ethanol.[24] The best-studied organisms are *Pachysolen tannophilus, Candida shehatae,* and *Pichia stipitis.*

Numerous comparisons have been made among xylose-fermenting yeasts,[23] and *C. shehatae* is one of the best understood. It grows rapidly

on xylose under aerobic conditions, it ferments xylose under anaerobic conditions, and it produces relatively little xylitol. Strains of *P. stipitis* likewise possess many of these characteristics. These two yeasts are similar in many ways, but they differ in several respects from *P. tannophilus.* We have chosen to study *C. shehatae* because it metabolizes xylose almost as well as it does glucose and because it appears to have a relatively high ethanol tolerance.

29.3 BIOCHEMICAL LIMITATIONS TO XYLOSE FERMENTATION

The initial assimilation of xylose proceeds by a two-step mechanism (Figure 29.1). Once inside the cell, xylose is reduced to xylitol by aldose reductase coupled to either NADH or NADPH. An NADPH-specific enzyme is commonly found. In some organisms, the aldose reductase is nonspecific and can use either cofactor. If NADH is used in the first step, the resulting NAD^+ can be coupled to the oxidation of xylitol to xylulose by xylitol dehydrogenase in the second step. Overall, the energetics of these two reactions are slightly unfavorable, and xylitol can accumulate as a product. The reaction can be driven by providing excess NADPH (generated by the oxidative bypass) and by the phosphorylation of xylulose to xylulose 5-P by xylulokinase. All three of these assimilative enzymes are under inductive regulation. The 3, 4, 5, 6 and 7-carbon phosphorylated sugar intermediates making up the nonoxidative portion of the pathway are believed to exist more or less in a dynamic equilibrium, with erythrose 4-P present in the smallest concentration. Fructrose 6-P and glyceraldehyde 3-P can be drawn off either by the fermentative reactions of the Embden-Meyerhoff-Parnas pathway or by the oxidative bypass. Another alternative pathway is the phosphoketolase bypass leading directly from xylulose 5-P to the formation of acetic acid and glyceraldehye 3-P. If the oxidative bypass functions to drive the assimilative reactions, respiration is necessary to consume NADH and maintain the cellular redox balance.

29.4 RELATIONSHIP OF BIOCHEMICAL PATHWAYS TO CELL PHYSIOLOGY

Characteristics of the biochemical pathways dictate the cell physiology. Probably because of the need to provide a driving force for the first two redox reactions, the xylose fermentation rate is enhanced by aeration. This observation has been made repeatedly with all three of the

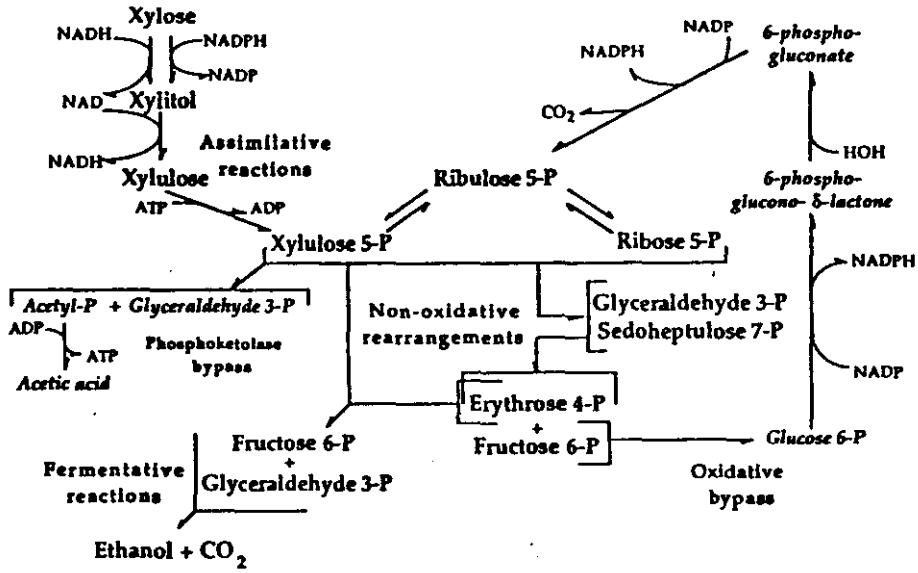

Figure 29.1: Proposed pathways for the fermentation of D-xylose 5-phosphate to ethanol by yeasts.

principal xylose-fermenting yeasts, so it is presumed to be a generalized phenomenon.[6-10,26]

In addition to stimulating ethanol production, oxygen is required for growth in xylose-metabolizing yeasts. The requirement of oxygen for growth appears to be a function of the cell physiology rather than of the sugar employed because xylose-metabolizing yeasts do not grow any better on glucose than they do on xylose under anaerobic conditions.[18] The requirement of oxygen for growth likewise has been generalized to several yeast species.[4,25]

An optimal low level of aeration must be maintained to obtain good xylose fermentation and growth. Oxygen-limited conditions are required to induce fermentative activity in xylose-fermenting yeasts.[16] However, too much oxygen is detrimental because xylose-fermenting yeasts appear to both produce and consume ethanol at the same time.[17]

These requirements create a complex interplay between oxygen and fermentation in xylose-fermenting yeasts such that if the yeasts are cultivated under fully aerobic conditions, they grow well, but do not ferment, and if they are cultivated under fully anaerobic conditions, they ferment well but do not grow. Moreover, if the conditions are fully anaerobic, in

some species, the rate of xylose utilization decreases dramatically and the product mixture shifts from ethanol to xylitol.

29.5 FERMENTATION IN CONTINUOUS OXYGEN-LIMITED CULTURE

Continuous culture requires continuous growth and hence a continuous supply of oxygen. Oxygen (in excess) is detrimental to ethanol production, so one might ask, "Why attempt continuous fermentation of xylose?" The simple answer is that continuous fermentation presents a challenge. More seriously, however, continuous culture is useful because it enables the investigation of cellular metabolism under steady-state conditions.

In batch culture, with cell biomass, substrate, and oxygen tension constantly changing, the enzymatic activities of cells are never fully adapted to the immediate conditions. Metabolic studies of cells grown in batch culture can be suggestive of biochemical changes, but they are not definitive, and they are hard to repeat.

Our objective in these experiments was to grow *C. shehatae* in continuous culture with oxygen as the limiting nutrient. By varying the dilution and aeration rates and the sugar feed concentrations, independently, we were able to attain different specific rates of aeration and growth for the cells under study.[3]

When cells were grown under fully aerobic (xylose-limiting) conditions, the dilution rate could be increased until cell washout at D = μ_{max} (= $0.28 h^{-1}$), where D is the dilution rate and μ_{max} is the maximum specific growth rate. Contrary to observations with respiration-limited yeasts such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe, C. shehatae* does not produce ethanol at high dilution rates as long as oxygen is present in excess.[4]

Several different methods can be used to shift from xylose limitation to oxygen limitation, but only a few are successful. If the xylose feed rate is increased (by increasing the xylose concentration) while keeping the oxygen transfer and dilution rates constant, the burst of growth leads to excessive foaming. If the xylose feed concentration and dilution rates are kept constant and the oxygen transfer rate is decreased, cells wash out and xylose accumulates in the effluent. Because of the cell washout, the effective specific aeration rate changes until a new, lower steady-state biomass concentration is attained (Figure 29.2).

The method of attaining oxygen-limited growth does not affect the eventual steady-state specific substrate utilization (Q_{xo}) or product for-

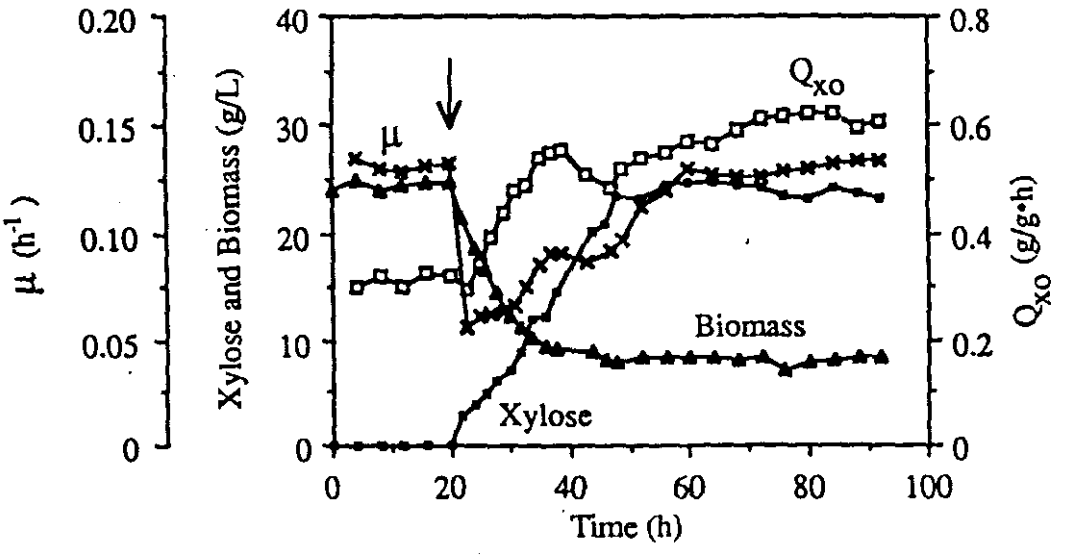

Figure 29.2: Release from xylose limitation by a reduction in the oxygen transfer rate. At the point indicated by the arrow, the oxygen transfer rate was reduced thereby shifting cells from continuous growth under xylose limitation to continuous growth under oxygen limitation. The dilution rate was $0.13h^{-1}$, xylose concentration 6%, temperature 30°C, and pH 4.5 throughout, μ, specific growth rate; reprinted with permissions

mation (Q_E) rates, but the loss of cell biomass leads to relatively low steady-state ethanol concentration.[3] To attain a high standing biomass under oxygen limitation, fully aerobic growth on a lower sugar concentration at a higher dilution rate must be shifted to oxygen-limited growth on a higher xylose concentration at a lower dilution rate. All three parameters - sugar concentration, aeration rate, and dilution rate - are changed at once. When this is done in a manner calculated to have minimal effect on the standing biomass, cells shift from sugar - limited growth to oxygen limited growth, with the consequential :accumulation of ethanol in the effluent. The specific carbon dioxide production rate (Qco_2) increases because of ethanol production, and the specific growth rate (μ) decreases in response to the new specific aeration rate (Figure 29.3). The resulting cultures are stable and can be maintained for several hundred hours. If cultures are shifted to fully anaerobic conditions, growth stops, and if the medium feed is continued, cells wash

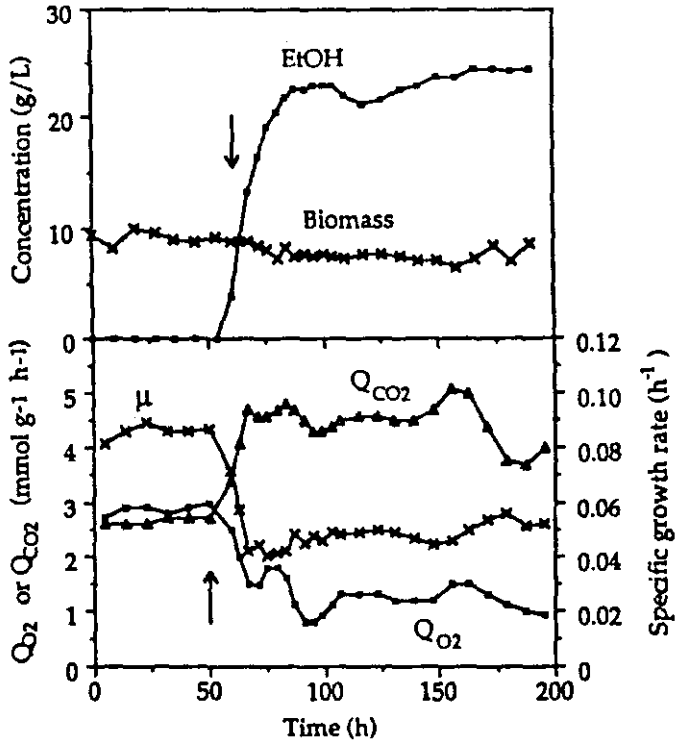

Figure 29.3: Q_{40}, specific xylose uptake rate response of a fully aerobic culture to the onset of oxygen limitation. At the point shown by the arrow, the oxygen transfer rate was changed from about 25 to about 15 mmol $L^{-1}h^{-1}$, the dilution rate was changed from 0.086 to 0.05 h^{-1}; and the feed xylose concentration was increased from 2% to 10%. Temperature and pH were the same as in Figure 29.2. Reprinted with permission.[3]

out. Details of continuous xylose fermentations, procedures, results, and analysis have been published.[1-5]

One principal objective of the experiments described here was to determine the biochemical characteristics of fermentative and nonfermentative cells. To this end, cells grown under fully aerobic and oxygen-limited conditions along with fermentative cells that had been held for an extended period under fully anaerobic conditions were harvested and examined for several enzymes critical to xylose metabolism. We found that the specific activity of alcohol dehydrogenase (ADH) increased although titers of other enzymes remained constant (Table 29.1)

Under fully aerobic conditions, no fermentation occurs, so all metabolism goes into cell growth. Under fully anaerobic conditions, no growth occurs and all metabolism goes into fermentation. At intermediate specific aeration and growth rates, different fractions of metabolized sugar go to either fermentation or growth. The fraction of metabolism

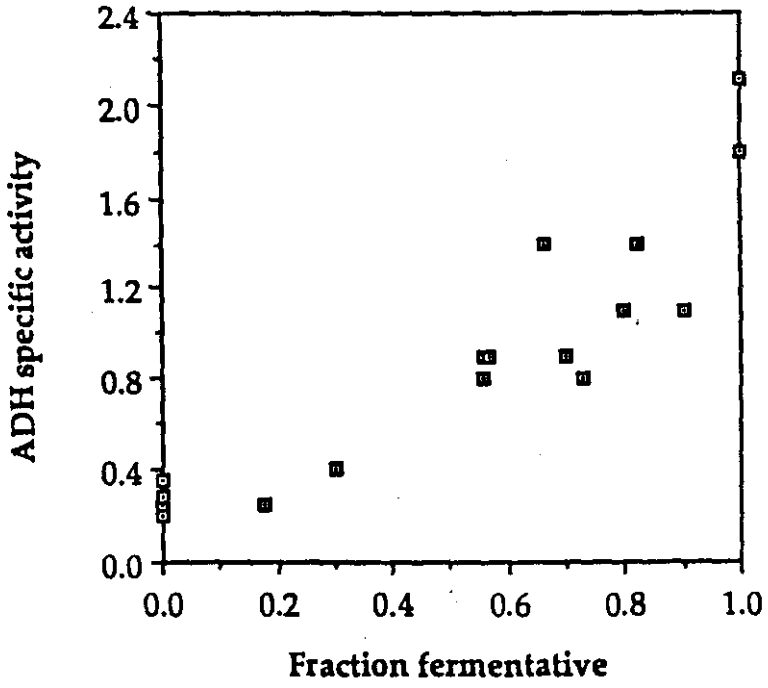

Figure 29.4: Qo_2, specific oxygen uptake rate correlation of the specific activity of alcohol dehydrogenase (ADH) with that fraction of cellular metabolism directed to fermentation. Reprinted with permission.[5]

that is fermentative can be defined mathematically and correlated with the specific activity of ADH in these cells. The ADH specific activity increases with the fermentative activity of the cells (Figure 29.4). These observations have led us to hypothesize that ADH plays a dominant regulatory role in determining whether cultures become fermentative."

Even though ethanol production can be sustained in continuous culture, ethanol never accumulates to very high concentrations, and the yields are less than maximal. This is attributable to several factors. First, to maintain continuous growth, a significant fraction of the sugar substrate must be diverted from ethanol production into cell growth. Second, if an attempt is made to attain higher ethanol concentrations by using lower dilution rates and higher sugar feed concentrations, the specific ethanol fermentation rate decreases because of ethanol toxicity.

The concentration of ethanol ultimately attained is proportional to the vigor, viability, and growth rate of the starting culture. This observation has led us to employ a two-phase process for ethanol production (Figure 29.5). In the first phase, we use continuous culture to generate a vigorous cell suspension. In the second phase, we initiate a fed-batch fer-

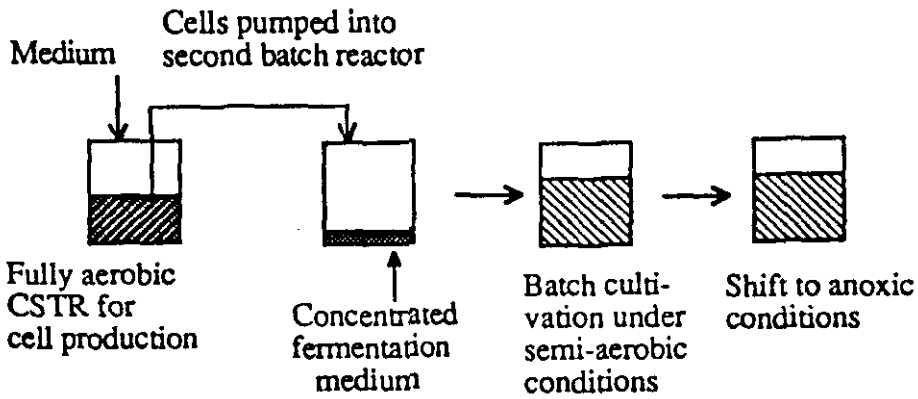

Figure 29.5: Scheme for two phase fermentation of xylose. Cells are grown under fully aerobic conditions and then shifted to a fed-batch fermentation.

mentation by pumping in a concentrated sugar feed under semi-aerobic conditions. The cells adapt to oxygen limitation by synthesizing ADH and ferment the xylose rapidly to ethanol. A significant amount of polyols (chiefly xylitol with some glycerol) is generated along with cell mass (Fig. 29.6). The cells can be rejuvenated or used as a by-product along with the polyols that are formed.

The ethanol concentration and formation rate shown are near records for yeast xylose fermentations in the absence of glucose. Although the products are still less than those routinely attained in glucose fermentations, they are technically recoverable. The economics of such a process would depend on many other unrelated factors such as feasibility. Fermentation of sugar mixtures obtained from hydrolysates presents additional problems and opportunities. Our current research in this area is focused on obtaining better yeast strains.

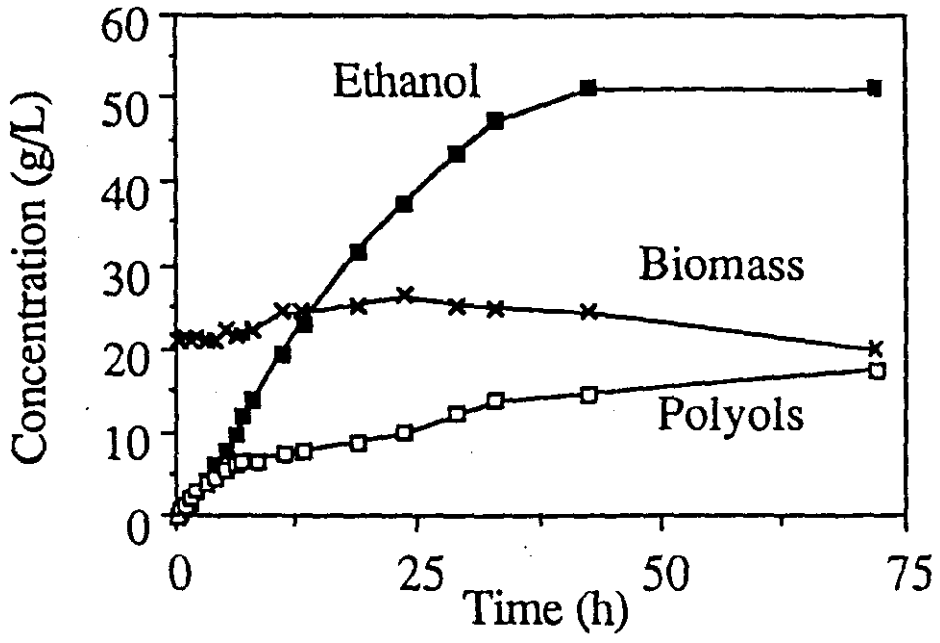

Figure 29.6: Product profiles in the second phase of fed-batch fermentation. Steady-state continuous growth was obtained on 4% D-xylose in basal medium under fully aerobic conditions. Efflux was then discontinued and a concentrated solution of xylose (66%) was pumped in over a 24-h period. At the same time, the oxygen transfer rate was lowered from about 50 to 25 mmol $L^{-1}h^{-1}$.

Nomenclature

μ	Specific growth rate	[h^{-1}]
Q_{xo}	Specific xylose uptake rate	[g xylose (g dry wt cells)$^{-1}h^{-1}$]
Qco_2	Specific CO_2 production rate	[mmol CO_2 (g dry wt cells)$^{-1}h^{-1}$]
QO_2	Specific oxygen uptake rate	[mmol O_2 (g dry wt cells)$^{-1}h^{-1}$]

	Specific Activity		
Enzyme	Fully Aerobic	Semi-Aerobic	Anaerobic
Xylose reductase			
NADPH-linked	0.28	0.31	0.33
NADH-linked	0.07	0.08	0.10
Xylitol dehydrogenase	0.15	0.17	0.24
G-6-P-dehydrogenase	1.3	1.1	0.9
Alcohol dehydrogenase	0.27	NA	1.8

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