COMMUNICATION TO THE EDITOR

Uncoupled Glycerol Distribution as the Origin of the Accumulation of 3-Hydroxypropionaldehyde During the Fermentation of Glycerol by *Enterobacter agglomerans* CNCM 1210

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Abstract: Batch fermentation of glycerol to 1,3-propanediol (1,3PPD) by *Enterobacter agglomerans* CNCM 1210 showed the lethal accumulation of 3-hydroxypropionaldehyde (3-HPA) when performed under initial substrate content higher than 40 g/L. Assigned to the inhibition by the NAD/NADH ratio of the 3-HPA converting enzyme: 1,3PPD dehydrogenase, intracellular assays were conducted in an attempt to identify the metabolic mechanisms involved in the increase of that ratio. An overflow metabolism through the 1,3PPD formation pathway was established, while a catabolic limitation in the oxidative branch at the level of glyceraldehyde-3-phosphate dehydrogenase occurred. Uncoupled activities of synthesis and consumption of reducing equivalents are thus suspected to provoke the increase of the NAD/NADH ratio and the subsequent accumulation of 3-HPA. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* **58:** 303–305, 1998.
Keywords: glycerol; *Enterobacter agglomerans;* 3-hydroxypropionaldehyde; catabolic limitation; inhibition

INTRODUCTION

As the glycerol deposit significantly increases due to the current increased biofuel production from colza oil, the bioconversion of glycerol to 1,3-propanediol has led to many works based on the utilization of various microorganisms, including *Klebsiella pneumoniae* (Streeskstra et al., 1987), *Citrobacter freundii* (Homann et al., 1990), and *Enterobacter agglomerans* (Barbirato et al., 1995) for the enterobacteria and species of the genus *Clostridium* (Forsberg, 1987). Glycerol dissimilation by these bacteria shows a dual pathway which consists in a reductive branch involving a glycerol dehydratase generating 3-hydroxypropionaldehyde (3-HPA), subsequently reduced in 1,3PPD by 1,3PPD dehydrogenase, with regeneration of NAD, and an oxidative branch implying glycerol dehydrogenase forming dihydroxyacetone (DHA) then phosphorylated by DHA kinase to dihydroxyacetone-phosphate (DHAP) (Magasanik et al., 1953; Rush et al., 1957).

When studying the metabolic flexibility of *E. agglomerans* by varying the initial glycerol content from 20 to 100 g/L at pH 7, an inhibition phenomenon occurred. Causing cessation of growth and of glycerol consumption, it was assigned to the accumulation of 3-HPA in the fermentation medium (Barbirato et al., 1996a). Such behavior was also observed during the fermentation of glycerol by *K. pneumoniae* and *C. freundii*. Investigated with *E. agglomerans*, the accumulation of 3-HPA was associated with an increase of the NAD/NADH ratio to 1.7, provoking a partial inhibition of 1,3PPD dehydrogenase (Barbirato et al., 1996b), which was supported by the competitive inhibition exerted by NAD with respect to NADH on the purified 3-HPA converting enzyme (Barbirato et al., 1997b). The effects on the central metabolism of *E. agglomerans* of the specific rate of glycerol consumption have been investigated by operating chemostat cultures at high growth and glycerol dissimilation rates. In this way, *E. agglomerans* showed a limiting step at the level of the glyceraldehyde-3-phosphate (GAP) dehydrogenase, characterized by an accumulation of

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triose-phosphate, DHAP and GAP, and of fructose-1,6-diphosphate (FDP) (Barbirato et al., 1997a). To minimize this catabolic limitation and enhance the glycolytic carbon flux, synthesis of 1,3PPD is favored to increase the availability as well as the rate of regeneration of the reducing equivalents (Barbirato, 1996, Ph.D. thesis), generating an overflow metabolism as defined by Neijssel and Tempest (1975).

Due to analogies regarding end-product profile and rates of consumption and production between the chemostat culture maintained at high glycerol rate of dissimilation and the batch fermentation performed at high initial substrate content, a limitation by GAP dehydrogenase, consecutive to an enhanced activity of the 1,3PPD metabolic branch, is suspected to occur under this latter condition. The purpose of this study is to investigate this scheme in order to explain the increase of the NAD/NADH ratio responsible for the lethal 3-HPA accumulation during batch fermentation.

MATERIALS AND METHODS

Microorganisms and Culture Conditions

The organism, *E. agglomerans* CNCM 1210 was used in the culture described below. Batch fermentation was performed in a 5 L reactor (Biostat B, B. Braun Biotech. International, Melsungen, Germany) regulated at 30°C and pH 7. Composition, preparation, sterilization, deaeration, and inoculation of the medium were as described previously (Barbirato et al., 1996b).

Analysis

Biomass concentration was determined by cell dry weight. Fermentation products were quantified by HPLC. All operating conditions were previously described (Barbirato et al., 1995).

Enzyme and Intracellular Metabolite Assays

Extraction and assay procedures for the determination of GAP dehydrogenase activity and NAD, NADH, DHAP, GAP, and FDP intracellular concentrations were previously reported (Barbirato et al., 1997a).

RESULTS AND DISCUSSION

Profiles of glycerol, 1,3PPD, and 3-HPA concentrations during the batch culture initiated at 60 $g.L^{-1}$ are given in Figure 1. Also illustrated are the specific rates of glycerol consumption and 1,3PPD production. These latter parameters increased jointly to 83 and 46 $mmol.g^{-1}.h^{-1}$, respectively, during phase I of the fermentation before decreasing drastically during phase II, simultaneously to the 3-HPA accumulation.

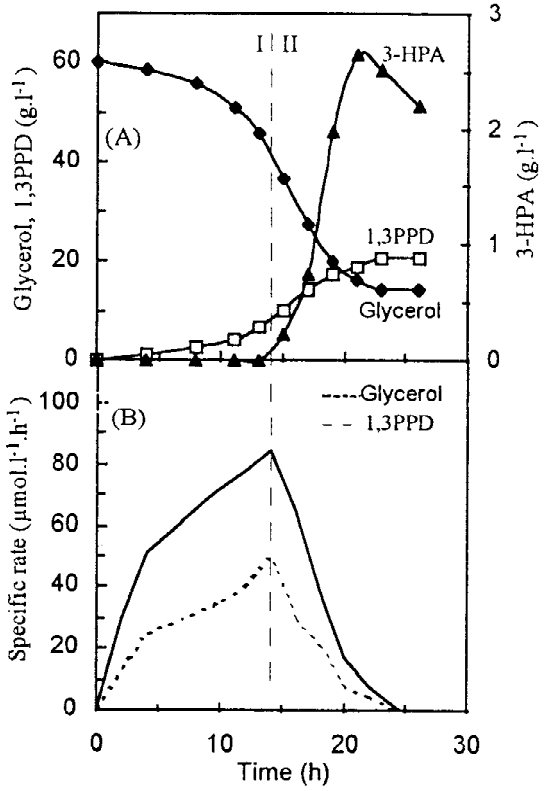

Figure 1. Batch fermentation of 60 g/L glycerol by *E. agglomerans.* (A) concentrations of glycerol, 1,3PPD and 3-HPA; (B) specific rates of consumption of glycerol and 1,3PPD during the two phases of the culture. Phase I and phase II correspond to the culture behavior before and after 3-HPA appearance, respectively.

A physiological approach, based on the evolution of DHAP, GAP, and FDP intracellular concentrations and of GAP dehydrogenase activity, was followed in addition to the NAD and NADH content profiles. Displayed in Figure 2, it showed that NADH concentration remained constant at 6.0 $\mu mol.g^{-1}$ during phase I of the fermentation, in contrast to NAD whose intracellular content increased from 8.3 to 10.4 $\mu mol.g^{-1}$, concommitantly to the increases of activities of glycerol dehydratase and 1,3PPD dehydrogenase to 2.0 and 1.3 $U.mg^{-1}$, respectively, as already mentioned (Barbirato et al., 1996b). In addition, while the level of activity of GAP dehydrogenase did not change significantly (about 2.2 $U.mg^{-1}$), DHAP concentration showed a continuous increase from 16.3 $\mu mol.g^{-1}$ to 31.3 $\mu mol.g^{-1}$. Fructose-1,6-diphosphate concentration evolution was similar to that of DHAP while GAP level remained low during phase I.

Phase II of the fermentation started as 3-HPA began to accumulate. It was marked by a significant decrease of the NAD and NADH concentrations, resulting in an increase of the NAD/NADH ratio over 1.7. GAP dehydrogenase activity exhibited first, a slight decrease, before decreasing quickly at the end of the fermentation. Also remarkable is the DHAP concentration which reached a maximum value of 35.0 $\mu mol.g^{-1}$ before diminishing slowly. Fructose-1,6-diphosphate content, increasing during phase I, suddenly decreased before remaining quite constant at 5.0 $\mu mol.g^{-1}$

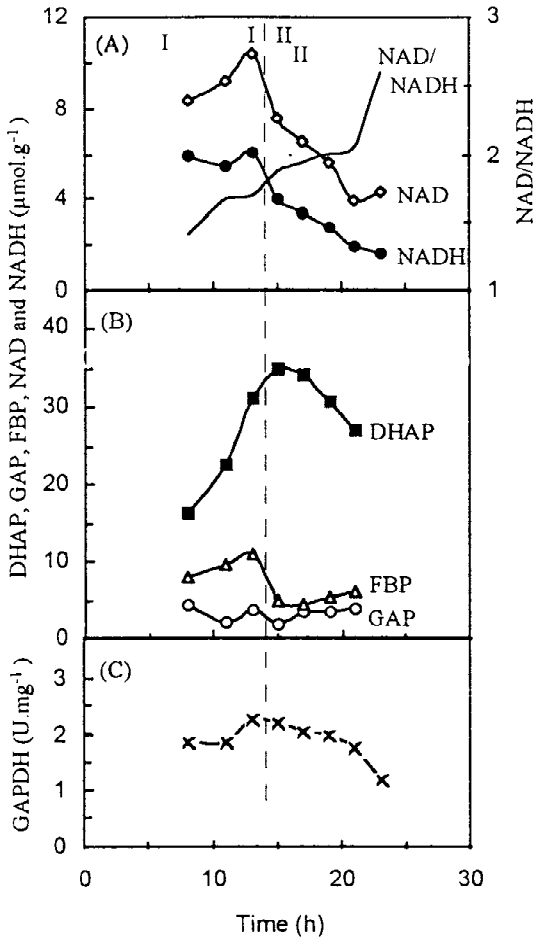

Figure 2. Intracellular concentrations of: (A) NADH and NAD, and NAD/NADH ratio; (B) glyceraldehyde-3-phosphate (GAP), dihydroxyacetone-phosphate (DHAP), and fructose-1,6-diphosphate (FBP); (C) activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) during the course of the fermentation of 60 g/L glycerol by *E. agglomerans.* Concentrations and enzymatic activities, resulting from four determinations by a single point, are given with a standard deviation of less than 15% and 12% of the value measured, respectively.

during phase II. GAP was still low, approximately 3.6 $\mu mol.g^{-1}$.

Several similarities between the results obtained during this batch fermentation and the behavior of the chemostat culture maintained at high dilution rate (Barbirato et al., 1997a) can be established. First, an excessive utilization of the 1,3PPD synthesis pathway, represented by a high 1,3PPD conversion yield and an increase of the NAD intracellular concentration, was common to both cultures. Second, a limitation by GAP dehydrogenase insufficiently induced, characterized by an accumulation of DHAP, was observed in each culture. A consequence of this overflow metabolism through the 1,3PPD metabolic pathway is that parallel mechanisms must dispose of the high rate of regeneration of the reducing equivalents (Rush et al., 1957; Streeskstra et al., 1987). These are solely dehydrogenases of the oxidative route of glycerol dissimilation, whose activities are more particularly limited by GAP dehydrogenase. As a result, while the reductive carbon flux increased, the oxidative one is thus limited. Uncoupled activities of synthesis and consumption of the reducing equivalent thus occurred, causing an increase of the NAD/NADH ratio subsequently followed by the partial inhibition of 1,3PPD dehydrogenase and the resulting accumulation of 3-HPA.

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