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Physiologic Mechanisms Involved in Accumulation of 3-Hydroxypropionaldehyde during Fermentation of Glycerol by *Enterobacter agglomerans*

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When grown in 700 mM glycerol within the pH range 6.0 to 7.5, anaerobic pH-regulated cultures of *Enterobacter agglomerans* exhibited an extracellular accumulation of 3-hydroxypropionaldehyde (3-HPA). This phenomenon, which causes fermentation cessation, occurred earlier when pH was low. In contrast, substrate consumption was complete at pH 8. Levels of glycerol-catabolizing enzymes, i.e., glycerol dehydrogenase and dihydroxyacetone kinase for the oxidative route and glycerol dehydratase and 1,3-propanediol dehydrogenase for the reductive route, as well as the nucleotide pools were determined periodically in the pH 7- and pH 8-regulated cultures. A NAD/NADH ratio of 1.7 was correlated with the beginning of the production of the inhibitory metabolite. Further accumulation was dependent on the ratio of glycerol dehydratase activity to 1,3-propanediol dehydrogenase activity. For a ratio higher than 1, 3-HPA was produced until fermentation ceased, which occurred for the pH 7-regulated culture. At pH 8, a value below 1 was noticed and 3-HPA accumulation was transient, while the NAD/NADH ratio decreased. The low rate of glycerol dissimilation following the appearance of 3-HPA in the culture medium was attributed to the strong inhibitory effect exerted by 3-HPA on glycerol dehydrogenase activity.

Enterobacter agglomerans has been recently described because of its ability to anaerobically convert glycerol to 1,3-propanediol (PPD) as the main product, and to acetic, formic, lactic, and succinic acids and ethanol as by-products (4, 7). PPD is a monomer of interest for the synthesis of biodegradable polyesters (12, 30). After glycerol enters the cell by diffusion, it can be metabolized via two parallel pathways comprising four enzymes encoded by the *dha* regulon (2, 19, 22, 23, 25). The first pathway is the oxidation of glycerol by glycerol dehydrogenase (Glyc DH) to dihydroxyacetone (DHA), which is then phosphorylated by DHA kinase to enter glycolysis. The second is the dehydratation of glycerol to 3-hydroxypropionaldehyde (3-HPA) by glycerol dehydratase, which is followed by the reduction of this metabolite by PPD dehydrogenase (PPD DH). The latter step is coupled to NADH consumption, which confers on the PPD formation pathway the physiological role of NAD regeneration.

Batch fermentation of glycerol by *E. agglomerans*, regulated at pH 7, revealed that growth and PPD production ceased after consumption of about 430 mmol, irrespective of the initial glycerol content. This phenomenon was the result of the accumulation in the fermentation broth of 3-HPA, whose inhibitory effect was demonstrated (5). Moreover, 3-HPA production during fermentation of glycerol at a high initial concentration was also noticed with *Klebsiella pneumoniae* and *Citrobacter freundii* and caused growth cessation and low product formation. Accumulation of this compound, which has broad-spectrum antimicrobial properties (3, 27), was thus generalized to all enterobacterial species producing PPD from glycerol (5).

In the present study, the parameters involved in the accumulation of this bacteriostatic metabolite were investigated with *E. agglomerans*. This was accomplished by monitoring the evolution of the levels of glycerol-catabolizing enzymes and of intracellular nucleotide pools (NAD and NADH) during batch fermentations performed under various culture conditions.

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MATERIALS AND METHODS

Bacterial strain and growth conditions. The bacterial strain *E. agglomerans* CNCM 1210 (Collection Nationale de Cultures de Microorganismes, Institut Pasteur, Paris, France) was maintained and cultivated before inoculation under anaerobiosis in a flask containing a nitrogen-gassed sterile rich medium consisting of 5 g of Bacto Peptone (Difco Laboratories, Detroit, Mich.) and 3 g of bacteriological meat extract (Biokar Diagnostic, Beauvais, France) per liter. When cultivated in 200 or 700 mM glycerol as a carbon source, the medium contained the following per liter of deionized water: 5 g of K_2HPO_4, 3 g of KH_2PO_4, 2 g of $(NH_4)_2SO_4$, 0.4 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of $CaCl_2 \cdot 2H_2O$, 4 mg of $CoCl_2$, 2 g of yeast extract (Biokar Diagnostic), 0.5 g of Bacto Peptone (Difco Laboratories), and 0.3 g of bacteriological meat extract (Biokar Diagnostic). This medium was gassed with a sterile nitrogen flux after being autoclaved (20 min at 120°C). Experiments were carried out in 1-liter glass reactors. The reactors were equipped with pH regulation (2300 Ingold transmitter controlling 5 M NaOH addition) and magnetic stirring. The temperature was maintained at 30°C by a thermostated water circulation system. For the fermentation carried out with an initial glycerol concentration of 200 mM, two samples were collected for the enzymatic assays during the last third of the fermentation and before the exhaustion of substrate.

For the biochemical study, a 5-liter reactor (Biostat B; B. Braun Biotechnology International, Melsungen, Germany) was used to collect, at several stages of the culture, fractions containing the same amount of cells (in dry weight) necessary for the enzymatic study. Culture conditions were the same as described above.

Analysis. Biomass concentration was determined by cell dry weight. Fermentation products were quantified by high-pressure liquid chromatography. All the operating conditions have been previously described (4).

Preparation of cells and cell extracts. About 25 to 30 mg of cells (dry weight) was harvested, transferred in a nitrogen-containing tube, and centrifuged immediately for 8 min at 8,000 × *g* and 4°C. After removal of the supernatant, the cell pellet was stored under a nitrogen atmosphere at −80°C until cell extract preparation.

Conditions of cell extract preparation were dependent on the enzyme activities being measured. For activities of Glyc DH and DHA kinase, harvested cells were washed with 5 ml of 100 mM potassium phosphate buffer (KPB) (pH 7.5). For PPD DH, the washing buffer consisted of 5 ml of 100 mM MOPS (morpho-

linepropanesulfonic acid; pH 7.5). After centrifugation for 12 min at 12,000 × *g* and 4°C, the washed pellet was suspended in 3 ml of the appropriate buffer. Cells were then disrupted at 4°C with a mechanical cell breaker containing 3 g of 0.1-μm-diameter glass beads for 8 cycles of 1 min each with 4-min cooling intervals. Cell debris and glass beads were removed by centrifugation at 15,000 × *g* for 15 min.

For glycerol dehydratase activity, anaerobic conditions were required because of the sensitivity of this enzyme to oxygen (10). A 100 mM oxygen-free glycine-KOH buffer (pH 8.1) containing 250 mM KCl and 1% (wt/vol) glycerol was used for washing and disrupting. Cells were also disrupted with the mechanical cell breaker according to the procedure described above but under anaerobic conditions which were maintained by nitrogen flux at each step of cell extract preparation.

Cell extracts were stored at 4°C until the assay procedure.

Enzyme assays. Enzyme activities were determined in their physiological directions at 30°C. All reagent solutions were prepared in deionized water and, if necessary, heated and gassed with nitrogen. A Uvikon 930 spectrophotometer (Kontron, Toulouse, France) was used. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate per min at 30°C.

Glyc DH (EC 1.1.1.6) activity was measured by monitoring, at 340 nm, the glycerol-dependent formation of NADH (24). The assay mixture contained 100 mM potassium bicarbonate (pH 9.0), 2 mM NAD, 30 mM $(NH_4)_2SO_4$, and 200 mM glycerol (ε_{340} = 6.22 mM^{-1} cm^{-1}).

DHA kinase (EC 2.7.1.30) activity was monitored in a coupled system in which the NADH-dependent reduction of the reaction product (DHA phosphate) to glyceraldehyde 3-phosphate was measured with a modified procedure based on that described by Johnson et al. (17). The assay mixture contained 50 mM potassium bicarbonate (pH 9.0), 2.5 mM ATP, 0.4 mM NADH, 15 mM $MgCl_2$, 2 mM dithiothreitol, 18 U of glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle, and 10 mM DHA. To prevent any secondary reaction (with Glyc DH), 15 mM α,α′-dipyridyl was added to the assay mixture (21).

PPD DH (EC 1.1.1.202) activity was determined at 340 nm by measuring the initial rate of 3-HPA-dependent NADH decrease. The reaction mixture contained 200 mM KPB (pH 7.8), 0.4 mM NADH, 0.4 mM $MnCl_2$, and 3 mM 3-HPA.

CoB_{12}-dependent glycerol dehydratase (EC 4.2.1.30) was assayed by the 3-methyl-2-benzothiazolinone hydrazone (MBTH) method of Toraya et al. (28). It is based on the ability of the reaction product (3-HPA) to react with MBTH generating azine derivatives which can be assayed by absorbance with a spectophotometer. The assay system, containing oxygen-free solutions, consisted of 5 mM KPB (pH 9.0), 50 mM KCl, 25 mM $MnCl_2$, 15 mM $(NH_4)_2SO_4$, 25 mM glycerol, and 0.5 mM ATP as enzyme activator (16). After 10 min of incubation at 37°C, 12 μM CoB_{12} was added to the assay mixture and the enzymatic reaction was stopped at 0, 1, and 2 min by transferring 200 μl of the assay mixture into a mixture containing 0.5 ml of 0.1% (wt/vol) MBTH and 1 ml of 100 mM potassium citrate (pH 3.6). After 15 min of incubation at 37°C, the 3-HPA formed was quantified from the A_{305} relative to a standard curve.

A determination of the effects of 3-HPA on enzyme activities was performed with crude extract by the initial addition of 3-HPA to the assay mixtures. To verify the reversibility of the effect, after a 10-min incubation in the presence of 10 mM 3-HPA, the metabolite was removed by gel filtration with a disposable G-25 M column (Pharmacia Biotechnology, Uppsala, Sweden) and activities were measured and compared to a nonincubated crude extract handled the same way.

Protein concentration was determined by the use of Coomassie brilliant blue G250 protein assay reagent supplied commercially by Bio-Rad (Hercules, Calif.), with bovine serum albumin as the standard.

Nucleotide extractions. Levels of NADH and NAD were measured after extraction from a culture broth sample as described by Vasconcelos et al. (29). NADH was extracted with KOH (pH 12.3). After a 10-min incubation at 30°C, the alkaline solution was centrifuged for 8 min at 5,000 × *g* and 4°C and the supernatant was collected. NAD was extracted with HCl (pH 1.3) by incubation for 10 min at 50°C. After centrifugation for 8 min at 5,000 × *g* and 4°C, the supernatant was progressively neutralized with NaOH before being stored at −80°C.

Nucleotide pool assays. Nucleotide pools were measured with a Hitachi (Tokyo, Japan) fluorimeter (model F-2000). Wavelengths of the fluorescence excitation and emission were 340 and 460 nm, respectively. Nucleotide concentrations were determined from a calibration curve elaborated with pure commercial NADH in a concentration range of 0.1 to 7 μM, in a mixture containing 80 mM triethanolamine buffer (pH 7.6), 0.8 mM EDTA, and 3 mM $MgSO_4$.

The NADH concentration was determined by measuring the fluorescence decrease linked to the conversion of pyruvate into lactate. The reaction mixture contained 100 mM triethanolamine (pH 7.6), 3 mM $MgSO_4$, 0.8 mM EDTA, 4 mM pyruvate, and 25 U of lactate dehydrogenase from rabbit muscle.

NAD pools were estimated by quantifying the increase of fluorescence consequent to the oxidation of ethanol. The assay system contained 150 mM pyrophosphate buffer (pH 8.8), 0.8 g of semicarbazide per liter, 10 mM ethanol, and 27 U of alcohol dehydrogenase from baker's yeast.

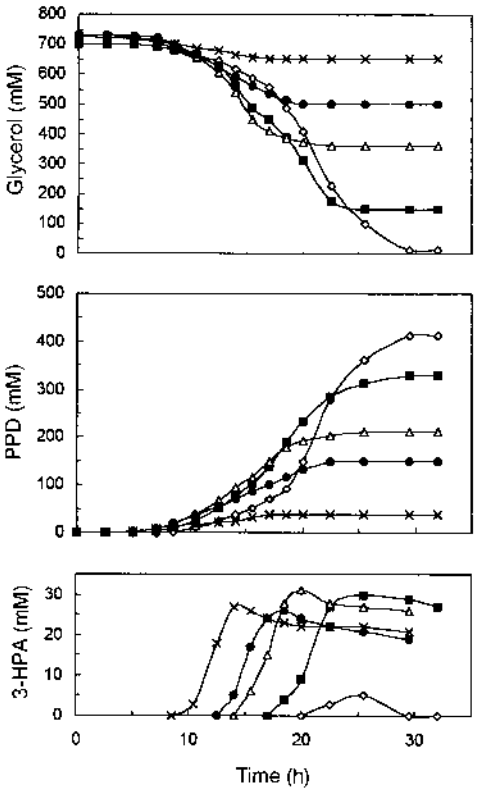

FIG. 1. Time course of *E. agglomerans* fermentation of glycerol. The effect of the culture pH on the kinetics of glycerol consumption, PPD production, and 3-HPA accumulation is indicated. The initial glycerol concentration was 720 mM. Symbols: ×, pH 6; ●, pH 6.5; △, pH 7.0; ■, pH 7.5; ◇, pH 8.0. Concentrations are given with a less than 3% standard deviation.

Chemicals. All chemicals, enzymes, and coenzymes were purchased from Sigma Chemical Co. (Saint Quentin Favalier, France). 3-HPA was synthesized from acrolein by the procedure described by Hall and Stern (15).

RESULTS

Effects of pH on fermentation behavior. Batch fermentations of glycerol at an initial concentration of 720 mM were carried out at pH values ranging from 6 to 8. As depicted in Fig. 1, the amount of glycerol consumed was dependent on the culture pH. Only 10% of the initial glycerol content was metabolized at pH 6; glycerol consumption increased with increasing pH and was complete at pH 8, and final PPD concentration was correspondingly high. Extracellular 3-HPA accumulation was noticed in each culture. Its concentration reached 30 mM except for the culture grown at pH 8. In that case, the maximum 3-HPA concentration was 4 mM. More relevant was the culture time when 3-HPA production began: the lower the pH, the earlier 3-HPA accumulated. Culture pH thus appeared as a parameter strongly influencing 3-HPA accumulation.

Biochemical study. Cultures of *E. agglomerans*, regulated at pH 7 and pH 8, were grown at an initial glycerol concentration of 700 mM. Levels of glycerol-catabolizing enzymes, as well as the nucleotide pools (NAD and NADH) were measured periodically in the cultures. No activities for the four enzymes specific for glycerol dissimilation were detected at the beginning of the cultures. As illustrated in Fig. 2, the pH 7-regulated culture exhibited an increase of glycerol dehydratase and PPD DH activities to 2.0 and 1.3 U mg^{-1}, respectively after 15 h of fermentation. In that first stage of the culture, glycerol dehy-

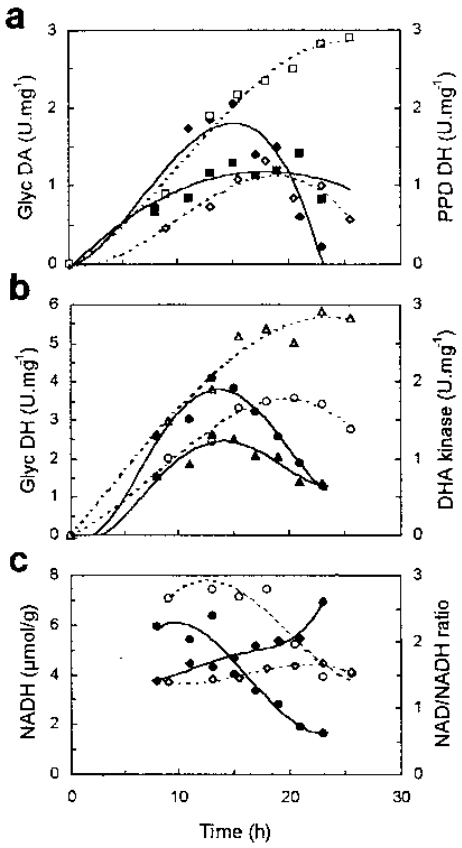

FIG. 2. Evolution of glycerol-catabolizing enzymes and nucleotide pools during the courses of *E. agglomerans* batch fermentations of 700 mM glycerol regulated at pH 7 and pH 8. Closed symbols and solid lines indicate the pH 7-regulated culture; open symbols and dashed lines indicate the pH 8-regulated culture. (a) ◆ and ◇, glycerol dehydratase (Glyc DA); ■ and □, PPD DH. (b) ▲ and △, Glyc DH; ● and ○, DHA kinase. (c) ● and ○, NADH; ◆ and ◇, NAD/NADH ratio. Standard deviations, calculated on four determinations by single point, are less than 15% of the measured activities.

dratase activity was continuously higher than that of PPD DH. The level of activity of glycerol dehydratase then dropped drastically, whereas the PPD DH level remained constant. The activities of enzymes of the oxidative route, Glyc DH and DHA kinase, showed a similar evolution: they reached maximum levels of 2.6 and 2.0 U mg^{-1}, respectively after 15 h of culture and then decreased. The NADH level was lower than that of NAD. The NAD/NADH ratio increased from 1.4 to 1.76 after 15 h of fermentation and up to 2.6 at the end of growth.

In the culture maintained at pH 8, glycerol dehydratase activity increased to a maximum level of 1.0 U mg^{-1}, which was then maintained. PPD DH activity was always higher than was that of glycerol dehydratase, and its evolution showed a continuous increase up to 3.0 U mg^{-1}, which was different from its evolution in the culture grown at pH 7. Levels of activity of Glyc DH and DHA kinase reached maximum values at the end of the fermentation. No drop in the activities of glycerol-catabolizing enzymes was thus noticed. NADH concentration stayed at 7.3 μmol mg^{-1} during the first 18 h of fermentation and then decreased in conjunction with the NAD level. The NAD/NADH ratio, initially at 1.4, increased slightly to a maximum of 1.68 before decreasing at the end of the fermentation.

To investigate whether the decreases of the enzyme levels were associated with 3-HPA accumulation, the effects of this metabolite on glycerol-catabolizing enzymes were quantified

TABLE 1. Influence of 3-HPA concentration on activities of various enzymes

Enzyme[a]	Activity[b] at the indicated 3-HPA concn (mM)		
	0.25	1.0	5.0
Glyc DH	60	33	20
DHA kinase	100	100	80
Glyc DA	100	100	n.d.[c]
LDH	100	100	100

[a] Glyc DA, glycerol dehydratase; LDH, lactate dehydrogenase.
[b] Activities are expressed as percentages of the reference activity measured without 3-HPA.
[c] n.d., not determined.

(Table 1). Glyc DH was strongly inhibited by 3-HPA added in low concentration. In comparison, the activity of DHA kinase was less inhibited by the metabolite and that of glycerol dehydratase was not affected by 3-HPA concentrations up to 1 mM. The effect of higher concentrations was not tested with the latter enzyme because of the range of accuracy of the assay procedure. Among the enzymes tested, Glyc DH thus showed the greatest sensitivity to 3-HPA, which explains the decrease of the specific rate of glycerol consumption observed as soon as 3-HPA accumulated, whatever the culture pH. When Glyc DH, DHA kinase, and glycerol dehydratase were treated for 10 min in the presence of 10 mM 3-HPA and separated from the metabolite by gel filtration, no changes in their activity were noticed. This showed the reversibility of the inhibitory effect of 3-HPA on the enzymes considered. In vitro activities measured during batch fermentations were thus not affected by 3-HPA and reflected real levels of enzymes.

Effects of pH on enzymatic levels. To investigate the effects of pH on the activities of glycerol-catabolizing enzymes, cultures with initially 200 mM glycerol were grown at pHs of 6.0, 6.5, 7.0, 7.5, and 8.0. With such a glycerol concentration, no 3-HPA accumulation was observed except in the pH 6.0-regulated culture, which was thus not considered for the enzymatic study. Levels of activity measured in the last phase of the fermentation are shown in Fig. 3. The level of activity of Glyc DH increased with increasing culture pH, while DHA kinase activity was independent of pH. Glycerol dehydratase showed an important decrease of activity with increasing pH. In contrast, PPD DH activity was higher when the cultures were

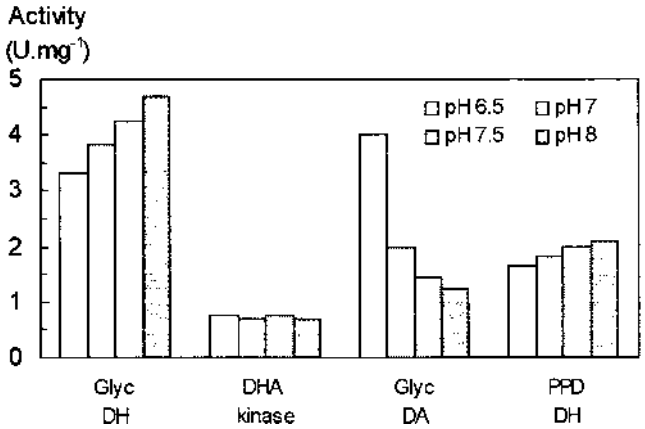

FIG. 3. Influence of pH on levels of activity of glycerol-catabolizing enzymes of *E. agglomerans* grown in 200 mM glycerol. Glyc DA, glycerol dehydratase. Standard deviations, calculated on four determinations by single point, are less than 12% of the measured activities.

TABLE 2. Influence of pH on the glycerol conversion yield of PPD (Y_{PPD}) and on the specific rate of PPD production (q_{PPD})[a]

pH	Y_{PPD} (mol/mol)	$q_{PPD_{max}}$ (mmol/g/h)
6.0	0.66	41
6.5	0.57	30
7	0.51	24
7.5	0.47	27
8	0.44	22

[a] Cultures of *E. agglomerans* were grown in glycerol at an initial concentration of 200 mM.

grown at an alkaline pH. The relative activities of glycerol dehydratase to those of PPD DH were about 2.4 at pH 6.5, 1.1 at pH 7.0, 0.7 at pH 7.5, and 0.6 at pH 8.0. Table 2 summarizes the conversion yields and specific production rates of PPD calculated for these same cultures. Important increases of both parameters were observed as culture pH decreased, while specific rates of glycerol consumption were nearly the same. Enzymatic levels measured correlate well with these observations, insofar as the level of Glyc DH was minimal at low pH whereas that of glycerol dehydratase was maximal. Therefore, the ratio between the first enzymes of the reductive and oxidative pathways probably determines the glycerol distribution between these two metabolic routes.

DISCUSSION

Fermentation of glycerol by *E. agglomerans* at high initial glycerol concentration revealed the accumulation of the only metabolite intermediate of the PPD formation pathway: 3-HPA. Provoking cessation of growth and of substrate consumption, the accumulation of this bacteriostatic compound, noticed for several enterobacterial species, probably reflects a metabolic imbalance. The biochemistry of the fermentation of glycerol has been mainly studied for the genus *Klebsiella* in connection with the control of the *dha* structural genes by a repressor (product of *dhaR*) and by catabolite repression and in connection with the posttranslational inactivation of Glyc DH during the switch from anaerobiosis to aerobiosis (9, 13, 14, 18, 20, 24, 26). Recently, Daniel and Gottschalk (11) have shown that the activity of glycerol dehydratase was growth temperature dependent since better expression of the corresponding genes was noticed at 28°C than at 37°C in a culture in which *Escherichia coli* expressed the genes of the *dha* regulon of *C. freundii*. However, no information regarding the effect of pH on the levels of activity of the enzymes involved in the anaerobic catabolism of glycerol was reported.

Cultures of *E. agglomerans* exhibited an accumulation of 3-HPA during fermentation of glycerol at initial concentrations up to 480 mM when culture pH was maintained at 7 (5). It was demonstrated that 3-HPA accumulated earlier when culture pH was low. By comparing enzymatic levels of the glycerol-reducing branch and the nucleotide pools at two culture pH values, several discrepancies were observed. Glycerol dehydratase activity was lower than that of PPD DH at pH 8. The reverse was noticed at pH 7, suggesting that pH is the determinant of enzyme levels. This was confirmed by experiments carried out within a pH range of 6.5 to 8.0 under conditions of low glycerol initial concentration at which 3-HPA does not accumulate. The lower the pH, the higher the relative activity of glycerol dehydratase to that of PPD DH. This is in accordance with the observation of Bouvet et al. (8), who reported a similar high relative activity for *K. pneumoniae* cultivated without pH regulation. This observation suggests that 3-HPA accumulation is more likely to occur at low than at high pHs. However, 3-HPA production in the culture medium was also noticed for the pH 8-regulated culture. This occurred although the level of activity of PPD DH was threefold higher than that of glycerol dehydratase. Attention was thus directed towards the nucleotide concentrations. In the culture grown at pH 7, the NAD/NADH ratio increased to a value of 1.76 as 3-HPA began to accumulate. Then, 3-HPA reached a final concentration of 30 mM concomitant with an increase of the NAD/NADH ratio to 2.6. Transient accumulation of 3-HPA, noticed for the pH 8-regulated culture, also correlated with the maximum NAD/NADH ratio of 1.68. In contrast to the pH 7-regulated culture, 3-HPA was then consumed while the nucleotide ratio dropped to 1.54. The sensitivity of the purified PPD DH from *E. agglomerans* to the NAD concentration has been previously investigated (6). The results showed that NAD behaves as a competitive inhibitor with a K_i of 0.29 mM. One can thus suggest that the NAD/NADH ratio is probably responsible for the partial inhibition of PPD DH activity, which causes 3-HPA accumulation. The critical value of 1.7 was reached and exceeded at pH 7, probably because of the unfavorable ratio of the two enzymes of the reductive branch. Even though the level of activity of glycerol dehydratase decreased subsequent to 3-HPA accumulation, it remained higher than that of PPD DH. At pH 8, however, 3-HPA did not accumulate further, and this observation was consistent with a decrease of the NAD/NADH ratio. Reasons for the increase of the NAD/NADH ratio during fermentation remain to be answered. A drop of the intracellular NADH concentration subsequent to a decrease of the carbon flow through glycolysis may be envisaged, as well as an overproduction of PPD generating an excess of NAD. Further investigations are needed to verify such hypotheses.

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