Lactate and ethanol dehydrogenase activities in continuous cultures of *Clostridium thermosaccharolyticum* LMG 6564

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The pattern of ethanol and lactate formation by continuous cultures of *Clostridium thermosaccharolyticum* LMG 6564 under glucose limitation is affected by culture conditions such as pH and dilution rate. NADH- and NADPH-mediated lactate dehydrogenase (LDH; EC 1.1.1.27) and alcohol (ethanol) dehydrogenase (ADH; EC 1.1.1.1 and EC 1.1.1.2) activities were measured in cell extracts from continuous cultures grown under different conditions. In conditions of high product formation, the NADH-mediated reaction was higher than the NADPH-mediated reaction for both LDH and ADH. LDH showed an absolute requirement for fructose 1,6-bisphosphate (FBP). Both NADH- and NADPH-linked LDH reactions were cytoplasmic, not sensitive to oxygen, and had a pH optimum of 6.0–6.5; the temperature optimum was 55–60 °C. The reverse reaction (lactate oxidation) could not be demonstrated. ADH activity was found in the particulate fraction of the cell lysate and was sensitive (not completely, but irreversibly) to oxygen. The temperature and pH optima were 43 °C, pH 7.0 and 45 °C, pH 8.8 for the NADH- and NADPH-mediated reactions, respectively. The production of at least two different ADHs is likely. LDH and ADH seemed to be regulated at the level of enzyme synthesis (direct correlation between the *in vitro* activities and the lactate and ethanol yields in the culture) with a second regulation of LDH by FBP at the reaction level.

Introduction

Several factors account for the biotechnological interest in enzymes from thermophiles (Zeikus, 1979). In the present work we report on the formation and regulation of alcohol dehydrogenase (ADH; EC 1.1.1.1 and EC 1.1.1.2) and lactate dehydrogenase (LDH; EC 1.1.1.27) in continuous cultures of *Clostridium thermosaccharolyticum*, a thermophilic saccharolytic bacterium. NADPH-linked ADH has been detected during the spore-forming phase of *C. thermosaccharolyticum* (Hsu & Ordal, 1970). As far as we know, this is the only report on these enzyme activities for this bacterium. More data are available for other thermophiles. For example, cell extracts of *Clostridium thermohydrosulfuricum* display both NADH- and NADPH-linked ADH activities (Lamed & Zeikus, 1981; Turunen *et al.*, 1987) as well as NADH-linked LDH activity (Turunen *et al.*,

1987). With another strain and under anaerobic assay conditions Hyun *et al.* (1985) discovered a NADPH-linked LDH. *Clostridium thermocellum* shows NADH-mediated ADH and LDH activities (Lamed & Zeikus, 1980b). *Thermoanaerobium brockii* contains NADH- and NADPH-linked ADH as well as NADH-linked LDH (Lamed & Zeikus, 1980a). All the LDH activities mentioned so far are under allosteric control by fructose 1,6-bisphosphate (FBP), as reported by Wolin (1964) for *Streptococcus bovis* LDH.

Limited information is available about the impact of these enzymes on the fermentation pattern of the thermophilic clostridia. This in contrast to our knowledge of the fermentation metabolism of the mesophilic clostridia. Indeed, during the last two decades, research on the fermentation metabolism of *Clostridium kluyveri* (Hillmer & Gottschalk, 1972, 1974; Lurz *et al.*, 1979), *Clostridium pasteurianum* and/or *Clostridium butyricum* (e.g. Jungermann *et al.*, 1973; Petitdemange *et al.*, 1976; Mitchell *et al.*, 1987), and certainly *Clostridium acetobutylicum* (see, for example, Jones & Woods, 1986; Freier & Gottschalk, 1987; Wiesenborn *et al.*, 1989a, b; Clark

Abbreviations: ADH, alcohol dehydrogenase; FBP, fructose 1,6-bisphosphate; LDH, lactate dehydrogenase.

et al., 1989; Rao & Mutharasan, 1989), has been encouraged, because of the importance of some of these organisms for the production of solvents (ethanol, butanol and acetone) or fuels (hydrogen gas and ethanol).

Pyruvate is the branch-point intermediate in the fermentation of glucose in the above-mentioned clostridia, including the thermophiles. The enzymes competing for pyruvate are LDH and pyruvate:ferredoxin oxidoreductase. When pyruvate is metabolized to products other than lactic acid, the latter enzyme plays a key role for the electron flow through the formation of acetyl-CoA and its subsequent conversion to acetate, ethanol, butyrate, butanol, acetone, etc. Furthermore, the electron flow from glycolysis is balanced by the production of hydrogen gas, ethanol, butyrate and/or butanol. The ratio of the metabolites depends on the fermentation conditions; an extreme example is the biphasic fermentation of *C. acetobutylicum*. During the initial growth phase (the acidogenic phase) the organisms produce acetic and butyric acids, carbon dioxide and hydrogen gas, resulting in a pH decrease, while during the second phase (solventogenic phase; when the cultures enter the stationary growth phase) they produce acetone, butanol and almost no hydrogen gas, while the volatile fatty acids are partly reassimilated, resulting in an increase in pH. There is a direct correlation between the levels of catabolic enzymes and the fermentation products formed (Andersch *et al.*, 1983). Also the properties of LDH of this *Clostridium* reflect the conditions under which lactate is produced by the cells (Freier & Gottschalk, 1987).

One of the aims of this study was to search for a possible correlation between LDH and ADH enzyme activities and the production of the respective metabolites, lactate and ethanol, during the *C. thermosaccharolyticum* fermentation. We have shown that when a continuous culture of *C. thermosaccharolyticum* LMG 6564 is run under glucose limitation, the ratio of the metabolites ethanol, lactate, hydrogen gas and carbon dioxide depends on the growth conditions (Vancanneyt *et al.*, 1990). With glucose as fermentable sugar in the pH range 7.0–5.0, lactate and ethanol are the main end-products. No lactate is formed at pH 7.0, when ethanol production is maximal; the opposite is true at pH 5.0. Furthermore, the ethanol:lactate ratio changes drastically with the dilution rate. Secondly, we wished to investigate the role of the co-factors NADH and NADPH in relation to ADH and LDH activity. It has been shown or suggested that both co-factors can be involved in catabolic reactions. Because the changes in ethanol and lactate production by *C. thermosaccharolyticum* were demonstrated with continuous cultures (Vancanneyt *et al.*, 1990), this culture technique was used in the present study.

Methods

Organism and growth conditions. *C. thermosaccharolyticum* LMG 6564 was grown anaerobically at 55 °C. It was maintained on the solidified basal medium (30 g agar l⁻¹) of Zeikus *et al.* (1979), supplemented with 3 g yeast extract, 10 g tryptone and 5 g glucose l⁻¹. Continuous culture experiments were performed in a 2.5 l fermenter (Biolafitte) with a working volume of 1.2 l or in a 0.5 l culture vessel of our own design (D. Raspoet, B. Pot, D. De Deyn, P. De Vos, K. Kersters & J. De Ley, unpublished results) with a working volume of 0.3 l. As liquid culture medium we used the basal medium of Zeikus *et al.* (1979) without Na₂S, but with the same amount of yeast extract and tryptone as above. The medium without the carbon source was autoclaved for 20 min (reactor) or 40 min (20 l medium reservoir) at 120 °C. Glucose (20%, w/v) and a silicone antifoam agent (Fluka AG; final concentration 250 µl l⁻¹) were autoclaved separately at 120 °C for 20 min. A freshly prepared sodium thioglycolate solution (final concentration 0.5 g l⁻¹) was filter-sterilized and added aseptically before starting. The Biolafitte fermenter was stirred mechanically, the reactor of our own design magnetically. Both fermenters were run under pH control with 4 M-NaOH. Anaerobic conditions were achieved by sparging the fermenter with N₂ (220 ml min⁻¹) through a membrane air filter (pore size 0.2 µm; Gelman Acro 50) for 30 min. The medium in the reservoir was flushed continuously with sterile N₂ at fivefold lower rate. A fixed culture level in the fermenters was achieved by an equilibrated in- and out-flow system with peristaltic pumps (Watson Marlow 501 U). The culture was checked daily for contamination by microscopic examination and by plating on nutrient agar (aerobic incubation at 55 °C) and on the medium of Zeikus *et al.* (1979) (anaerobic incubation).

Determination of glucose, fermentation products and cell dry weight. Glucose in reservoir media and culture supernatants was determined spectrophotometrically by the dinitrosalicylic acid method (Miller, 1959). The gas produced was captured under an inverted cylinder in water. Gas analyses were performed by gas chromatography as described previously (De Vos *et al.*, 1983). Lactate was determined by gas chromatography as described by Holdeman *et al.* (1977). Ethanol, butanol, and acetic and butyric acids were also quantified by gas chromatography (GC Intersmat 121; FID) as described previously (Vancanneyt *et al.*, 1987) with isobutyric acid (0.018 M) as internal standard. For the determination of cell dry weight, 5 ml of a grown culture was passed through a cellulose nitrate membrane filter (pore size 0.45 µm, diameter 47 mm), which was dried overnight under vacuum and weighed.

Preparation of cell extracts and enzyme assays. Cells were harvested by centrifugation of 0.5 l (Biolafitte fermenter) or 0.3 l (fermenter of our own design) culture fluid at 12000 g for 10 min at 4 °C and washed once with 25 mM-Tris/HCl (pH 7.4) plus 2 mM-dithiothreitol buffer (Turunen *et al.*, 1987). After another centrifugation, the cells were suspended in buffer as above (3 ml per g wet weight) and passed through a French pressure cell (1.5 × 10⁸ Pa). After centrifugation at 12000 g for 10 min at 4 °C, part of the supernatant (= crude cell extract) was stored at -20 °C; the rest was cleared of cell debris by ultracentrifugation at 85000 g for 4 h at 4 °C; the supernatant (= cytoplasmic extract) was also stored at -20 °C (Kersters & De Ley, 1975). All manipulations were performed either aerobically or anaerobically (under argon atmosphere) and standard enzyme assays were done at 50 °C.

The assays for LDH (cytoplasmic extract) contained, per ml, unless stated otherwise: 0.1 M-imidazole/HCl (pH 6.4), 0.375 mM-NAD(P)H, 2.5 mM-sodium pyruvate and 1 mM-FBP. The assays for ADH (crude cell extract) contained, per ml, unless stated otherwise: 0.1 M-Tris/HCl (pH 7.8), 2 mM-dithiothreitol, 0.375 mM-NAD(P)H and 15 mM-acetaldehyde. The reaction was started by adding 25 µl cell extract to

the mixture. Protein was estimated by the Lowry method with bovine serum albumin as standard. The enzyme activity was expressed as the initial rate [v ; min^{-1} (mg protein^{-1})] of substrate reduction calculated from $\Delta A \cdot d^{-1} \cdot \epsilon^{-1}$, where ΔA is the change in absorbance at 340 nm, a measure of the oxidation of NAD(P)H; d is the light path; and ϵ is the molar absorption coefficient of NAD(P)H at 340 nm. The change in absorbance was followed during 2 min. Measured enzyme activities were corrected for NAD(P)H oxidase activity.

Electrophoresis and gel staining. Discontinuous polyacrylamide gel electrophoresis on slab gels was done by a modification of the method of Davis (1964). We used an acrylamide/bisacrylamide mixture of 10% (w/v) ($T = 40\%$ and $C = 5\%$; T is the total percentage concentration of both monomers in the buffer, and C is the percentage concentration of N,N' -methylene bisacrylamide relative to T) in 1.00 M-Tris/HCl (pH 8.88) for the separating gel and 5% (w/v) in 150 mM-Tris/phosphoric acid (pH 7.40) for the stacking gel. LDH activity was located by the method described by Garvie (1969), modified as follows: for the direct staining, we used the method described by Turunen *et al.* (1987) and modified methods with different buffers (see below); for the indirect method, the gel was placed in a solution containing 1 mM-FBP, 10 mM-pyruvate and 1.4 mM-NADH in 0.1 M-imidazole/HCl (pH 6.2) for 15 min at 55 °C. The gel was removed from the reaction mixture and stained in a solution containing, per 100 ml, 0.1 M-Tris/maleate buffer (pH 8.3), 50 mg nitroblue tetrazolium and 2.74 mg phenazine methosulphate.

Results and Discussion

Lowering the pH and the dilution rate in a 0.5 l reactor favours lactate formation (Vancanneyt *et al.*, 1987, 1990). Table 1 shows data from analogous experiments with a larger culture vessel (1.2 l culture volume: Biolaftite fermenter). The effect of pH and dilution rate was slightly different from the previous results (Vancanneyt *et al.*, 1990). Again the highest ethanol production was found at the upper limit of the culture pH (7.0). However, with $D = 0.08\text{--}0.10\text{ h}^{-1}$, the amount of lactate produced per mol glucose remained almost constant within the pH range 5.4–7.0. When the dilution rate was lowered to 0.03 h^{-1} at pH 7.0, the lactate production decreased to 0.08 mol per mol glucose. When we used the smaller fermenter, no lactate was formed at pH 7.0 and $D = 0.09\text{ h}^{-1}$ (Table 2). This different result with the two reactors was confirmed by triplicate experiments. A different stirring mechanism and/or a different design can affect certain parameters such as the dissolved H_2 concentration in the culture fluid. This and/or the fact that one of the reactors contained an iron stirring axis probably explains the difference in fermentation pattern.

In vitro measurements of enzyme activities of aerobically and anaerobically prepared extracts from the fermentations are reported in Tables 1 and 2, respectively.

Enzymes involved in lactate and ethanol production

Tables 1 and 2 show that NADH-linked LDH activity was more than 20 times higher than the NADPH-linked

activity in lactate-producing cells. Crude cell extracts and cytoplasmic cell extracts both had the same rate of pyruvate reduction (data not shown), indicating that this enzyme system is not found in the particulate fraction of the cell lysate. Garvie (1980) also reported that all NADH-linked LDHs from the lactic acid bacteria are cytoplasmic. We could not demonstrate lactate oxidation in *C. thermosaccharolyticum* extracts, either spectrophotometrically or on polyacrylamide gels (direct staining). We tested different buffer systems (0.1 M-Tris/maleate, pH 8.3; 0.1 M-Tris/HCl, pH 7.0; 0.05 M-sodium carbonate, pH 9.2), different concentrations of DL-, L(+)- and D(–)-lactate, and addition of 25 mM- MgCl_2 and/or 1 mM-FBP. We also tested a coupled reaction system with glutamate to trap the pyruvate formed in the first reaction (see below):

(GPT, glutamate:pyruvate aminotransferase). It was the purpose of this coupled system to keep the pyruvate concentration lower so that the first reaction (1) is shifted to lactate oxidation. Turunen *et al.* (1987) showed the oxidation of L-lactate in *C. thermohydrosulfuricum* on polyacrylamide gel but not spectrophotometrically. On the other hand, Lamed & Zeikus (1980b) could not demonstrate lactate oxidation with FBP-activated L-LDH of *C. thermocellum* and *T. Brockii*, which was also true for L(+)-LDH of *C. acetobutylicum* (Freier & Gottschalk, 1987).

With aerobically prepared cell extracts, weak NADH- and NADPH-linked ADH activities were observed. The NADH-linked reaction is more sensitive to oxygen than the NADPH-linked reaction (data not shown), which was also true for ADH of *T. Brockii* (Lamed & Zeikus, 1980a). Furthermore, the reaction rate did not change after sparging the reaction mixture with argon for 15 min (data not shown). This means that oxygen affects the ADH activity irreversibly.

We found active ADH in the particulate fraction of the cell lysate only. This may be due to an association of these enzymes with membrane structures or, as was demonstrated for *C. kluyveri* (Lurz *et al.*, 1979) in solution with an ionic strength above 25 mM, to a precipitation of the dehydrogenase (complexes) in the form of polygonal bodies. The oxidation of ethanol with NAD^+ as electron acceptor could not be demonstrated; the NADP^+ -mediated reaction was not tested.

The ratio of the activities of the NADH-mediated versus NADPH-mediated ADH reactions was approximately 3:1 at pH 7.0 (1.10 mol ethanol per mol glucose) and 2:3 at pH 6.0 (0.20 mol ethanol per mol glucose)

Table 1. *Fermentation data for C. thermosaccharolyticum* LMG 6564 in continuous cultures (Biolaftite fermenter) under glucose limitation (50 mM-glucose input) at various dilution rates and pH values

Data represent means of three samples for each steady state. Standard deviations were within 3–5% of the mean. All steady states were repeated at least twice with the same results for the fermentation products formed (data not shown). The preparation of the cell extracts and the enzyme assays were performed aerobically.

D (h ⁻¹)	pH	Glucose consumed*	Ethanol*	Acetate*	Butyrate*	Butanol*	Lactate*	H ₂ gas†	Cell dry weight‡	v of LDH§	
										NADH- linked	NADPH- linked
0.10	5.4	50	13 (0.26)	17 (0.34)	17 (0.34)	7 (0.14)	15 (0.30)	1.72	1.56	1.32	0.035
0.10	6.0	50	24 (0.48)	16 (0.32)	8 (0.16)	8 (0.16)	20 (0.40)	1.36	1.11	1.53	0.064
0.08	6.8	50	36 (0.72)	14 (0.28)	1 (0.02)	4 (0.08)	19 (0.38)	1.04	1.77	1.79	0.075
0.08	7.0	50	47 (0.94)	20 (0.40)	2 (0.04)	6 (0.12)	20 (0.40)	1.04	1.05	2.20	0.077
0.05	7.0	50	49 (0.98)	19 (0.38)	0 (0.00)	5 (0.10)	15 (0.30)	1.00	0.81	1.34	0.067
0.03	7.0	50	52 (1.04)	28 (0.56)	2 (0.04)	6 (0.12)	4 (0.08)	1.22	0.81	0.56	0.037

* Expressed as mM and – in parentheses – as mol produced per mol glucose consumed.

† Expressed as mol produced per mol glucose consumed.

‡ Expressed as mg ml⁻¹.

§ Expressed as μ mol substrate reduced (mg protein)⁻¹ min⁻¹.

Table 2. *Fermentation data for C. thermosaccharolyticum* LMG 6564 in continuous cultures (0.3 l reactor) under glucose limitation (50 mM-glucose input) at D = 0.09 h⁻¹ and two different pH values

Data represent means from three samples for each steady state. Standard deviations were within 3–5% of the mean. All steady states were repeated at least twice with the same results for the fermentation products formed (data not shown). The preparation of the cell extracts and the enzyme assays were performed anaerobically.

pH	Glucose consumed*	Ethanol*	Acetate*	Butyrate*	Butanol*	Lactate*	H ₂ gas†	Cell dry weight‡	v of LDH§		v of ADH§	
									NADH- linked	NADPH- linked	NADH- linked	NADPH- linked
6.0	50	10 (0.20)	22 (0.44)	16 (0.32)	12 (0.24)	24 (0.48)	1.56	1.14	2.27	0.056	0.079	0.12
7.0	50	55 (1.10)	23 (0.46)	0 (0.00)	4 (0.08)	0 (0.00)	0.74	0.96	0.01	0.024	0.36	0.13

* Expressed as mM in the culture and – in parentheses – as mol produced per mol glucose consumed.

† Expressed as mol produced per mol glucose consumed.

‡ Expressed as mg ml⁻¹.

§ Expressed as μ mol substrate reduced (mg protein)⁻¹ min⁻¹ (the pH for measuring NADH-linked ADH was 7.2 and for NADPH-linked ADH 8.8).

(Table 2). This supports our view that the major part of the ethanol production is connected with the NADH-mediated reaction rather than with an NADPH-mediated activity. The NADPH-mediated ADH reaction in *C. thermosaccharolyticum* probably has only a limited catabolic function. This is not supported by the observation of Hsu & Ordal (1970) that NADPH-linked ADH is responsible for ethanol production in sporulating cells of *C. thermosaccharolyticum*. However, the role of the NADH-mediated reaction could have been overlooked because the cell extracts were manipulated in the presence of oxygen. Turunen *et al.* (1987) reported that the NADPH-linked ADH activity in *C. thermohydrosulfuricum* was tenfold higher than the NADH-linked activity. However, it is not clear whether all

manipulations for the preparation of the cell extracts were performed under anaerobic conditions.

The knowledge of ADH activities in thermophilic isolates is still very confusing. The present data suggest that strictly anaerobic conditions must be maintained throughout the whole preparation and measuring procedure to obtain reliable information on the function of this enzyme.

General catalytic properties of LDH and ADH of C. thermosaccharolyticum

The pH optima are shown in Fig. 1. The pyruvate reduction reaction (both NADH- and NADPH-linked) showed optimum activity between pH 6.0 and 6.5, which

Fig. 1. Effect of pH on the activities (expressed as % *v*: the percentage of the maximum activity of the particular experiment) of the catabolic enzymes tested. Cytoplasmic and crude cell extracts from continuous cultures (Table 2) at pH 6.0 and 7.0 were used for LDH and ADH measurements, respectively. ○, NADH-linked LDH; ●, NADPH-linked LDH; □, NADH-linked ADH; ■, NADPH-linked ADH.

Fig. 2. Effect of temperature on the activities (expressed as % *v*: the percentage of the maximum activity of the particular experiment) of the catabolic enzymes tested. Cytoplasmic and crude cell extracts from continuous cultures (Table 2) at pH 6.0 and 7.0 were used for LDH and ADH measurements, respectively. ○, NADH-linked LDH; ●, NADPH-linked LDH; □, NADH-linked ADH; ■, NADPH-linked ADH.

is somewhat higher than the pH 5.5–6.0 optimum for LDH from *C. thermohydrosulfuricum* (Turunen *et al.*, 1987). In the case of acetaldehyde reduction two pH optima were apparent: 7.0 for NADH- and 8.8 for NADPH-linked ADH. This supports our conclusion about the presence of two different enzymes for ethanol formation. The pH optimum for NADPH-linked ADH of *T. brockii* is 7.8 (Lamed & Zeikus, 1981). The pH optimum for the NADH-linked ADH of *C. thermosaccharolyticum* reflects the conditions for the highest ethanol production levels.

The effect of the incubation temperature on these enzyme activities is shown in Fig. 2. For NADH- and NADPH-linked LDH the *in vitro* optimum temperature under standard assay conditions was 55–60 °C, which is close to the optimal growth temperature (Vancanneyt *et al.*, 1987). For ADH activity the temperature effect was clearly different. In the first place, the optimum temperature (40–45 °C) was lower than for LDH. This corresponds well with previous observations (Vancanneyt *et al.*, 1987) that the ethanol yield with this strain is higher at 45–50 °C (definitely below the optimum growth temperature). Secondly, the temperature curves of NADH- and NADPH-mediated ADH were different. The former showed a rather broad optimum and the latter a sharp one. Again, this indicates that we are dealing with different enzymes, perhaps having different metabolic functions.

The effect of the substrate concentration on the activity of LDH (pyruvate) and ADH (acetaldehyde) was determined. In the case of ADH we determined K_m values of 2.3 and 2.0 mM for the NADH- and NADPH-linked reaction, respectively. For LDH, K_m values of 0.5 and 1.8 mM for the NADH- and NADPH-linked reaction, respectively, were found.

All LDH measurements were performed in the presence of 1 mM-FBP. In the assay conditions, (allosteric) stimulation of *C. thermosaccharolyticum* LDH by FBP is maximal with 1 mM-FBP. However, the required FBP level may be affected by the type of buffer used, its concentration, the pH, the pyruvate concentration and the bacterial strain (Garvie, 1980). Lamed & Zeikus (1980a) mentioned that in the case of *T. brockii* LDH the affinity of the enzyme for the activator FBP was pH dependent, although the maximum activity of the reaction was constant in the pH range 6.2–7.8. In general, FBP stimulation has been shown for LDHs of most streptococci, some lactobacilli (e.g. *Lactobacillus casei*) and several other mesophilic bacteria (Garvie, 1980). Freier & Gottschalk (1987) demonstrated FBP stimulation for LDH of *C. acetobutylicum*, a mesophilic member of the saccharolytic clostridia. Furthermore, the activities of all LDHs from thermophilic bacteria thus far studied (Lamed & Zeikus, 1980a, b; Ben-Bassat *et al.*, 1981; Clarke *et al.*, 1986; Turunen *et al.*, 1987) are FBP controlled. FBP activation leads to changes in the state of subunit associations (dimer or tetramer) at the time the affinity for pyruvate is increased and the tetrameric form of the enzyme is stabilized (Clarke *et al.*, 1986).

Correlation between LDH and/or ADH activity and metabolite formation in continuous culture

Data on LDH and ADH activities (Tables 1 and 2) clearly show the direct correlations between lactate and

Fig. 3. LDH activities on slab gels; reverse staining; electrophoresis time 16 h. Cell extracts (1.8 µg protein) from a culture in the Biolafitte fermenter (Table 1) was loaded on lanes 1 and 2 and from a culture in the fermenter of our own design (Table 2) was loaded on lane 3. Lane 1, $D = 0.08 \text{ h}^{-1}$, pH = 6.8; lane 2, $D = 0.03 \text{ h}^{-1}$, pH = 7.0; lane 3, $D = 0.09 \text{ h}^{-1}$, pH = 7.0.

alcohol production in the fermenter and the *in vitro* measured activities of the NADH-linked LDH and ADH. LDH activity was also visualized on polyacrylamide gels (Fig. 3). A broad slow-moving FBP-dependent LDH band in cell extract from *C. thermosaccharolyticum* was also observed in *C. thermohydrosulfuricum* extracts (Turunen *et al.*, 1987). The activity (broadness of the band) on the gel and the spectrophotometric measurements of LDH activity matched well with the corresponding lactate production levels in the cultures. These data at least suggest a regulation of LDH at the level of synthesis in *C. thermosaccharolyticum* cultures. This is in contrast with, for example, LDH of *Streptococcus mutans*. Yamada & Carlsson (1975) showed that intracellular LDH levels (measured as LDH activities of cell extracts) are of the same order of magnitude under homofermentative and heterofermentative conditions in continuous cultures of this organism. In *L. casei* (De Vries *et al.*, 1970) the specific activity of LDH likewise did not change when the culture conditions were different. For *T. Brockii* (Ben-Bassat *et al.*, 1981) a lower lactate yield on starch than on glucose was correlated with a lower FBP level as a consequence of a slower metabolic activity.

Apparently, the LDH activity is controlled by the intracellular FBP concentration. Although we did not measure the intracellular concentration of this activator, the absolute requirement of LDH for FBP strongly suggests that the LDH activity in *C. thermosaccharolyticum* is also under a similar control (probably allosteric), as has been demonstrated for other FBP-dependent LDHs (Yamada & Carlsson, 1975; Thomas *et al.*, 1979; Ben-Bassat *et al.*, 1981).

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