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Large Scale Production of D-Lactate Dehydrogenase for the Stereospecific Reduction of Pyruvate and Phenylpyruvate

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Summary. To initiate studies of the stereospecific reduction of pyruvate and phenylpyruvate to the corresponding D-2-hydroxyacids a limited screening was carried out for microorganisms possessing a high NADH-dependet D-lactate dehydrogenase activity. *Lactobacillus confusus* was found to produce the desired dehydrogenase, which showed also relatively high activity towards phenylpyruvate, so this strain was selected for large scale production of the enzyme.

A procedure for large scale purification of the enzyme starting with 24 kg wet cells is described including liquid-liquid extraction, ultrafiltration and chromatography on DEAE-cellulose, yielding a catalyst with specific activities of 216 U × mg^{-1} for pyruvate reduction and 15 U × mg^{-1} for phenylpyruvate reduction. A further tenfold purification can be achieved by affinity chromatography on Blue-Sepharose C-6B.

Parameters which are important for industrial application of the enzyme were determined: substrate specifity, pH and temperature optimum, temperature stability, stability at different pH-values, and the storage stability of the enzyme in crude extracts.

Introduction

Stereospecific isomeres of 2-hyroxyacids can obtained by reducing the prochirale 2-ketoacid to the corresponding chirale compound using a specific dehydrogenase as a catalyst (Wichmann et al. 1982). Usually the reduction equivalents needed come from the coenzyms NADH or NADPH. Industrial production of 2-hydroxyacids by this method would require an efficient coenzyme regeneration system, which we are currently developing using formate dehydrogenase for enzymatic NADH regeneration (Wichmann et al. 1981).

In this report we describe a NADH-dependent enzyme which catalyzes the reduction of pyruvate to D-lactate and phenylpyruvate to D-phenyllactate. Lactate dehydrogenases from mammalian tissues are typically L-specific, however D-specific lactate dehydrogenases are known from microorganisms as well (Buchanan and Gibbons 1974; Hensel et al. 1977). Data from literature on lactate dehydrogenase indicate that the substrate specifity is not absolute, but only the homologue 2-ketobutyric acid is reduced by known enzymes at a sufficient rate (Meister 1959). Reaction rates for phenylpyruvate reported are only 2% of the rates for pyruvate and are not attractive for a preparative application and had to be improved.

To find a lactate dehydrogenase with high activity towards phenylpyruvate as well as pyruvate or to find a specific phenyllactate dehydrogenase we first carried out a limited screening among strains of the genus lactobacillus. Clostridia are known to produce phenyllactate, but the enzyme involved is L-specific (Elsden et al. 1976; Simon et al. 1979). We concentrated our studies on enzymes working with NADH as coenzyme, since enzymes depending on NADPH or an allosteric effector like fructose-diphosphate (Heusel et al. 1977) are less desirable presently with regard to regeneration and the membrane reactor concept (Wichmann et al. 1981).

Materials and Methods

Microorganisms. Microorganisms in general as well as the production strain selected, *Lactobacillus confusus* DSM 20 196 were obtained from the German Collection of Microorganisms (DSM, Göttingen, W.-Germany).

Media and Growth Conditions. For the screening procedure each strain was cultivated in MRS-medium (De Man et al. 1960) at 30° C

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or 37° C, respectively. In order to develope a less expensive medium, different compositions of media were compared which are summarized in Table 1. 50 or 500 ml medium were placed in an Erlenmeyer flask without shikane and were sterilized at 121° C for 15 min in an autoclave. Flasks were inocculated immediately after cooling and placed on a reciprocal shaker operated at 50 rpm. Cells were harvested after 20 h by centrifugation, resuspended in 0.1 M phosphate buffer pH 7.0 and broken by treatment with a sonifier. Cell debris was removed by centrifugation. In the supernatant dehydrogenase activity with the substrates pyruvate and phenylpyruvate and protein content were measured. Bioreactors with 10 l medium and more were sterilized at 121° C for 30 min. *L. confusus* was grown under nitrogen at 30° C with slow stirring employing a turbine stirrer (150 rpm). The pH was followed and maintained later on at 5.0 by the addition of ammonia. The reactor was inocculated with an 8 h culture (1% of the final volume). Growth was followed by determining the optical density at 578 nm.

For large scale production of D-lactate dehydrogenase *L. confusus* was cultivated in a 5,000 l-bioreactor in a complex medium (M2) containing per liter glucose 20 g, yeast extract 10 g, meat extract 0.5 g, KH_2PO_4 2 g, $MgSO_4 \cdot 7\ H_2O$ 0.2 g, $MnSO_4 \cdot H_2O$ 0.05 g; pH 6.5. The culture was cooled down to about 15° C, 2 h after reaching pH 5.0, harvested by centrifugation in a Westfalia Chamber Centrifuge and stored frozen at −20° C until use.

Materials. PEG 1540 (pure grade) was obtained from BP-Chemie (Düsseldorf, W.-Germany). Potassium phosphate and potassium chloride used for the extraction of the enzyme were designated 'reinst'-grade and are products from E. Merck (Darmstadt, W.-Germany). DEAE-Cellulose (DE 52) for chromatography was obtained from Whatman (London, Great Britain). Blue Sepharose CL-6B was purchased from Pharmacia (Freiburg, W.-Germany). Romicon Hollowfiber Cartridge Type HF 26.5-43-PM 30 were obtained vom Amicon GmbH. (Witten, W.-Germany). MRS-medium and salts for cultivation were purchased from E. Merck (Darmstadt, W.-Germany), yeast extract from Ohly GmbH. (Hamburg, W.-Germany) and glucose for large-scale fermentation from Maizena (Krefeld, W.-Germany). NADH, NAD^+ and D-lactate (lithium salt) were purchased from Boehringer (Mannheim, W.-Germany), L-phenyllactate from Sigma (München, W.-Germany). $PEG_{20,000}$-NADH (Bückmann et al. 1981) was a gift from A. F. Bückmann, and formate dehydrogenase prepared according to Kroner et al. (1982) from K. H. Kroner, both GBF (Braunschweig, W.-Germany).

Analytical Methods. Assay during screening: D-lactate dehydrogenase was assayed according to (Biochemica Information, 1973) using a Zeiss PM4 photometer and a Vogel laboratory calculator LDC 277. The cuvette was thermostated and activity measured at a temperature of 25° C. The activity of the enzyme towards pyruvate and phenylpyruvate as substrates was determined by the reduction to lactate or phenyllactate, respectively. The assay mixture contained in 100 mM potassium phosphate buffer, pH 7.0, 0.6 μmol NADH, 2.27 μmol pyruvate or 19.6 μmol phenylpyruvate and limiting amounts of enzyme in a total volume of 3.00 ml. One unit of D-lactate dehydrogenase was defined as the amount of enzyme which catalyzes the consumption of 1 μmol of NADH min under the standard assay conditions.

The assay as carried out here does not distinguish between L- and D-Lactate dehydrogenase. The stereospecifity expected from literature (Buchanan and Gibbons 1974) for the enzyme from *L. confusus* was reconfirmed by the reverse reaction using D-Lactate as substrate and by independent measurements in the membrane reactor.

Optimized assay conditions for D-lactate dehydrogenase from *Lactobacillus confusus:* The temperature of the assay was raised to 30° C, the pH was set to 6.5, the assay mixture contained 0.6 μmol NADH, 16.3 μmol pyruvate or 45 μmol phenylpyruvate in 3.00 ml. To prevent loss of enzyme activity by dilution of samples with high specific activity 100 mM potassium phosphate buffer, pH 6.5 containing 2 mg/ml bovine serum albumine was used. The assay mixture for the NAD^+ dependent oxidation of D-lactate contained in 100 mM Tris/HCl-buffer, pH 10.0, 8.8 μmol NAD, 0.25 mmol D-lactate and limiting amounts of enzyme in a total volume of 3.00 ml. Protein concentrations were determined by the method of Bradfort (1976) using bovine serum albumine as a standard. Specific activity is expressed as units per mg of protein.

Acrylamide Gel Electrophoresis. Analytical gel electrophoresis was carried out according to Jovin et al. (1964). Activity staining for D-lactate dehydrogenase was done by the method of Lessie and Vander Wyk (1972) in a slightly modified version. After the electrophoresis the gels were incubated for 30 min at 30° C in 5 ml of 1 M Tris-HCl buffer, pH 9.0, containing 1 mg of 2,3,5-triphenyltetrazolium chloride, 250 μg of phenazine methosulfate, 2.5 mg NAD^+ and 4.8 mg D-lactate. The reactions were stopped by adding 0.5 ml of glacial acetic acid. The gels were removed and stored in 7% (v/v) acetic acid. Electrophoresis in the presence of sodium dodecyl sulfate was carried out according to Shapiro et al. (1967), using hemoglobin, chymotrypsinogen, pepsin, ovalbumin, and bovine serum albumin as marker proteins.

Liquid-Liquid Partition. The partition of the D(-)lactate dehydrogenase was investigated as follows: Variable amounts of stock solutions of PEG 1540 (50%) and salt were weight and placed in 10 ml graduated centrifuge tubes. The pH was controlled and fixed at certain values. Finally enzyme containing solution was added. Under appropriate conditions an aqueous two-phase system formed spontaneously (Albertsson 1971; Kula et al. 1982). The

Table 1. Composition of media for the cultivation of *Lactobacillus confusus*. Amounts are given as $g \cdot l^{-1}$; the pH-value of each medium was 6.5. Medium M1 is identical with the MRS-medium (De Man et al. 1960)

Constituent	M1	M2	M3	M4	M5	M6
Caseine peptone	10	–	–	–	–	–
Meat extract	5	0.5	0.5	–	0.5	0.5
Yeast extract	5	10	10	10	5	20
Glucose	20	20	20	20	20	20
Tween 80	1	–	–	–	–	–
Di-ammonia-hydrogencitrate	2	–	–	–	–	–
Sodium acetate	5	5	–	5	–	–
$MgSO_4 \cdot H_2O$	0.1	0.1	0.1	0.1	0.1	0.1
$MnSO_4 \cdot H_2O$	0.05	0.05	0.05	0.05	0.05	0.05
KH_2PO_4	2	2	2	2	2	2

partition studies were generally carried out at room temperture. After mixing for approximately 1 min on a Vortex mixer the two-phase system was separated by centrifugation for 5 min at 1,850 g in a swinging bucket rotor. The phase volumes were noted and enzyme activity was measured in both phases. The partition coefficient K of D(-)lactate dehydrogenase was determined by dividing the observed activity ($U \times ml^{-1}$) in the upper phase by the activity ($U \times ml^{-1}$) in the lower phase.

Results

Screening for D-Lactate Dehydrogenase

Out of 40 strains tested those with high activity to reduce phenylpyruvate are listed in Table 2. Comparing the relative rates with pyruvate as substrate, most strains reduce phenylpyruvate quite poorly in the range of 2% in accordance with Meister (1959). Seven strains however showed increased relative rates up to about 25% of pyruvate reduction under the assay conditions employed. From these strains *L. confusus* yielded maximum volume activity and was chosen for further studies.

Enzyme Production

Growth and enzyme production were followed in detail in a 10 l bioreactor using MRS-medium (Fig. 1). Enzyme activities were tested with both substrates (pyruvate and phenylpyruvate). As Fig. 1 demonstrates both activities take a parallel course, thus indicating that both substrates may be reduced by a single protein. Maximum specific and volume activities were found at the end of the exponential growth phase, about 10 h after inocculation. Two h before the enzyme showed maximal activity the pH had reached 5.0 and was maintained at this value. The further course of the fermentation indicated that 50% of the enzyme activity were lost after 13 h of incubation. After 16 h no activity could be detected anymore. The exact timing of the harvest is therefore of crucial importance for large scale production of the enzyme. For the large scale production of D-lactate dehydrogenase we tried to find a less expensive medium. As shown in Table 3 peptones could be successfully replaced by yeast extract and the amount of meat extract reduced to 0.5%. M2 is a suitable medium for the growth of *L. confusus* and without

Table 2. Lactate dehydrogenase activity in various microorganisms

Strain	DSM catalog no.	Isomere of lactate (Buchanan and Gibbons 1974)	Specific enzyme activity		Volume activity with phenylpyruvate ($U \times l^{-1}$)
			With pyruvate ($U \times mg^{-1}$)	With phenylpyruvate ($U \times mg^{-1}$)	
Lactobacillus confusus	(20 196)	D	12.3	2.1	630
Lactobacillus confusus	(20 194)	D	4.0	0.9	20
Lactobacillus fermentum	(20 052)	D	17.2	3.3	147
Lactobacillus brevis	(20 054)	D, L	32.2	5.4	280
Lactobacillus plantarum ssp. *arabinosus*	(20 205)	D, L	5.4	1.0	178
Leuconostoc paramesenteroides	(20 288)	D	16.7	1.2	40
Lactobacillus fructosus	(20 349)	D	7.5	1.8	110
Lactobacillus delbrueckii	(20 074)	D	34.0	0.9	40
Lactobacillus bifermentans	(20 003)		61.0	0	0
Lactobacillus leichmannii	(20 076)	D	28.3	0.8	258
Lactobacillus leichmannii	(20 355)	D	19.3	0.5	105
Lactobacillus cellobiosus	(20 055)	D	47.4	1.0	144
Lactobacillus buchneri	(20 057)	D, L	15.1	3.6	231
Lactobacillus xylosus	(20 175)	L	38.8	0.3	16
Lactobacillus bulgaricus	(20 081)	D	28.7	0.7	40
Lactobacillus acidophilus	(20 079)	D, L	13.8	0.7	28
Lactobacillus delbrueckii	(20 074)	D	37.1	0.2	8
Lactobacillus coryneformis ssp. *torguens*	(20 004)	D	1.9	0.6	52
Lactobacillus coryneformis ssp. *coryneformis*	(20 001)	D	8.3	1.3	260
Lactobacillus viridescens ssp. *minor*	(20 014)	D	24.4	0.8	39
Leuconostoc mesenteroides	(20 343)	D	47.0	0	0
Leuconostoc lactis	(20 202)	D	21.6	0.8	27

disadvantage for the production of the desired enzyme. The price is lowered from 2.93 to 0.83 DM per 1,000 U compared to the standard MRS-medium used during the screening.

Furthermore we checked whether a specific phenyllactate dehydrogenase can be induced by adding D,L-phenylalanin (0.1%) to the media. Our results (Table 3) indicate that the addition of the amino acid did not influence the level of enzyme activity. Using the cheaper medium M2 for cultivation of *L. confusus* in 5,000 l scale we obtained a yield of 24 kg wet cells with a total activity of 5.2×10^6 U (phenylpyruvate reduction). Compared to a yield of 692 U $\times$ l^{-1} in shake flask experiments the yield in the large scale production was 50% higher as expected. This higher yield may be a consequence of a better exclusion of oxygen during cultivation. The pH of the culture was successfully used as a signal to terminate the production. The culture was cooled down and harvesting started 2 h after the culture had reached a pH of 5.0.

Large Scale Purification of D-Lactate Dehydrogenase

Purification Steps

Crude Extract. 24 kg frozen *Lactobacillus confusus* cells were thawed in the cold room and suspended in 100 mM potassium phosphate buffer pH 7.0 containing 0.1% (v/v) 2-mercaptoethanol. The suspension was made up in a 100 l tank using a propeller stirrer (Ekato Type EMK 40) at 300 rpm. The total volume was 60 l representing a 40% cell suspension. The cells were disrupted by two consecutive passes through a continuously operating industrial agitator mill (Netzsch LME 20) (Schütte et al. 1983a, b). The grinding chamber was loaded with 85% packed volume of glass beads with 0.55–0.85 mm diameter. The mill was operated at a rotational speed of 1,200 rpm corresponding to an average tip speed of 8.8 ms^{-1}. The suspension was pumped through the mill with a Netzsch Mohno-pump (Type 2 NL 20 A)

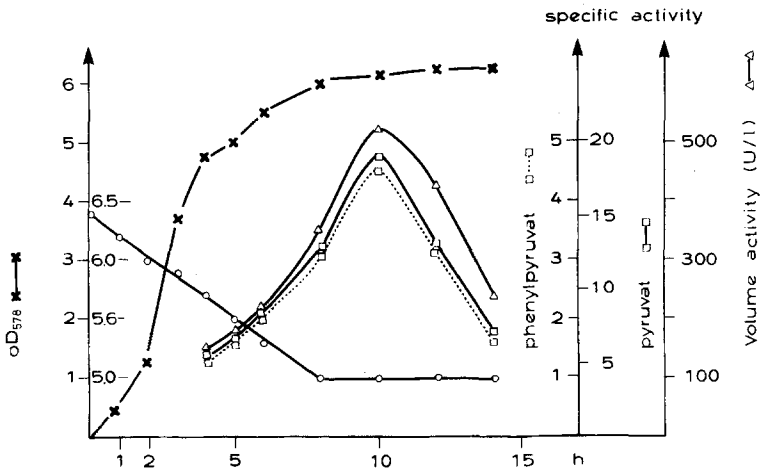

Fig. 1. Production of D-lactate dehydrogenase during growth in a 10 l bioreactor. ×———× absorbance at 578 nm; ○———○ pH; △———△ volume activity (phenylpyruvate reduction); □———□ specific enzyme activity (Pyruvate reduction); □- - - -□ specific enzyme activity (phenylpyruvate reduction)

Table 3. Media improvement for production of D-lactate dehydrogenase from *Lactobacillus confusus*

Medium	Dehydrogenase activity[a]		Cost index[b] (DM)	
	U × mg^{-1}	U × l^{-1}	1,000 l medium	1,000 U dehydrogenase[a]
M1	4.00	472	1,381	2.93
M2	3.89	468	389	0.83
M3	4.17	235	274	1.17
M4	2.02	229	334	1.52
M5	4.86	43	227	5.28
M6	0.99	34	378	11.12
M1 + 0.1% D, L-Phe	4.03	465		
M2 + 0.1% D, L-Phe	3.81	459		

[a] Phenylpyruvate reduction measured in the crude extract
[b] Calculations are based upon bulk prices per 10 kg (mineral salts) – 100 kg, at autumn 1982 in German marks (DM)

at a flow rate of 100 l × h^{-1}. The outer jacket of the agitator chamber and the seal was cooled with aqueous ethylene glycol solution at approximately −10° C so that the temperature of the cell suspension rose only from 4° C to 14° C during the two passes. The pH of the suspension dropped to 6.3 during the disintegration.

Removal of Cell Debris. To avoid time consuming solid-liquid separations in enzyme recovery the next two purification steps are based on liquid-liquid separation. The first partition step was designed to separate cell debris from the crude enzyme and utilized an aqueous two-phase system composed of polyethylene glycol and salt (Kula et al. 1982). The general procedure to find a suitable extraction system is outlined in "Methods". Polyethylene glycol 1,500 and potassium phosphate salts (pH 7.0 mixture) were weight and added to the cell homogenate (60 l). The mixture was brought to 108 l with deionised water corresponding to a 120 kg phase system containing 18% w/w PEG and 7% w/w salt. The dispersion was stirred for 2 h to ensure equilibrium of partition. Separation was carried out with the Gyrotester B from α-Laval (Poughkeepsie, New York) an open disc stack separator equipped with four screws of 13.5 mm length. The flow rate during separation was 60 l × h^{-1}.

Clean lower phase including the cell debris was discharged continuously through the underflow of the separator. The upper phase including the enzyme was collected through the overflow. Under these operat-

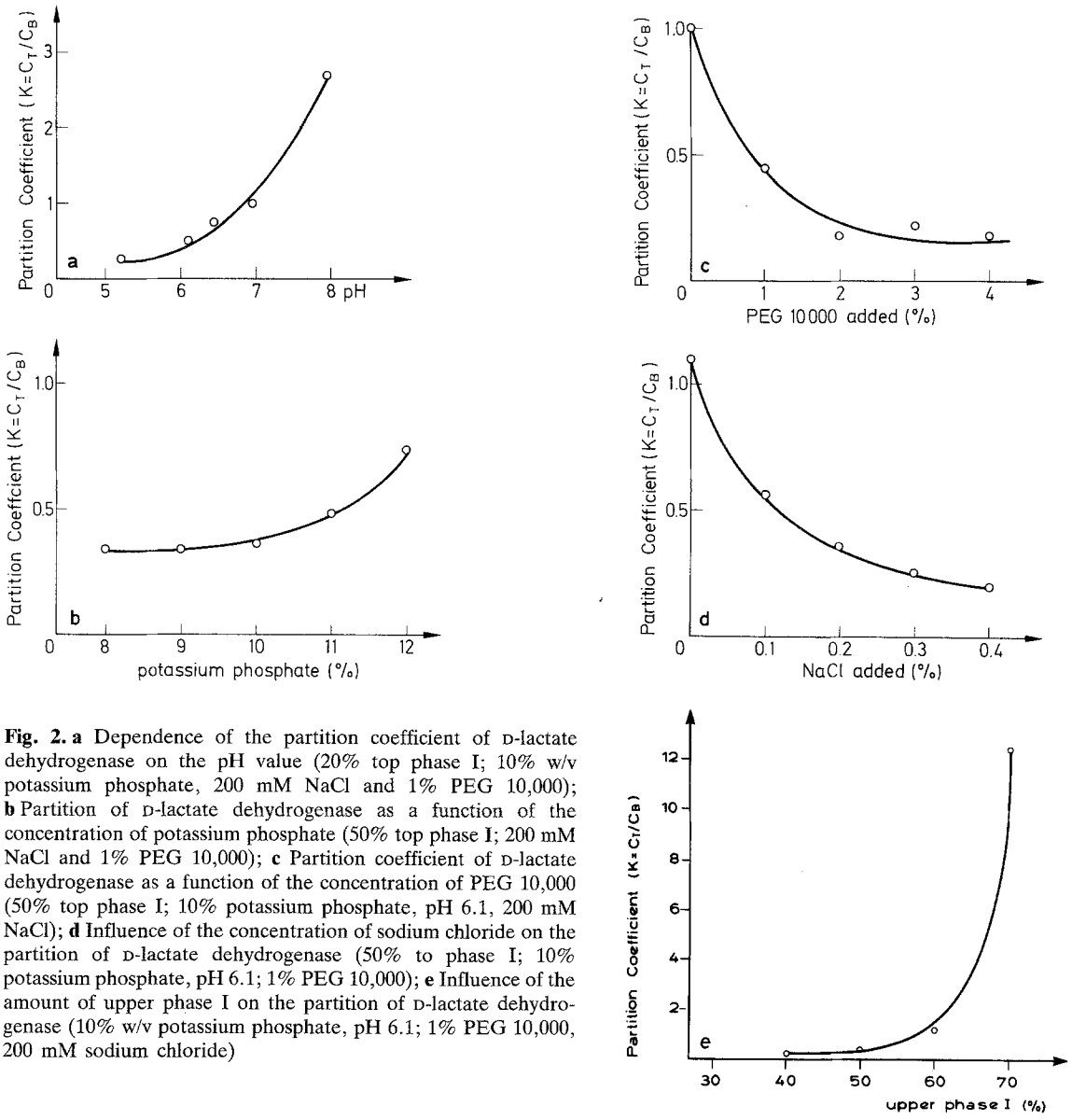

Fig. 2. a Dependence of the partition coefficient of D-lactate dehydrogenase on the pH value (20% top phase I; 10% w/v potassium phosphate, 200 mM NaCl and 1% PEG 10,000); **b** Partition of D-lactate dehydrogenase as a function of the concentration of potassium phosphate (50% top phase I; 200 mM NaCl and 1% PEG 10,000); **c** Partition coefficient of D-lactate dehydrogenase as a function of the concentration of PEG 10,000 (50% top phase I; 10% potassium phosphate, pH 6.1, 200 mM NaCl); **d** Influence of the concentration of sodium chloride on the partition of D-lactate dehydrogenase (50% to phase I; 10% potassium phosphate, pH 6.1; 1% PEG 10,000); **e** Influence of the amount of upper phase I on the partition of D-lactate dehydrogenase (10% w/v potassium phosphate, pH 6.1; 1% PEG 10,000, 200 mM sodium chloride)

ing conditions the upper phase still contained 1.5% of the lower phase which did not interfere with the following extraction step. 86 l of the polyethylene glycol rich upper phase were obtained extracting 95% of the D-lactate dehydrogenase activity from the cell homogenate.

Partition in a PEG/Salt System. The enzyme containing upper phase was pumped into a 200 l glass vessel with a diameter of 40 cm. PEG 10,000, potassium phosphate salt (pH 6.0 mixture) and sodium chloride were added to the upper phase, filled up with deionised water to 172 l and stirred for 2 h. The final composition of the phase system was 50% top phase (step 2), in addition 2% w/v PEG 10,000, 10% w/v potassium phosphate, 200 mM sodium chloride. A PEG/salt system formed and separation of the phases was allowed to proceed under gravity overnight. The D-lactate dehydrogenase activity was extracted in the lower salt rich phase (99 l).

Figure 2a–e analyze the dependence of the partition coefficient of D-lactate dehydrogenase on the pH-value, the concentration of potassium phosphate, the addition of PEG 10,000, the concentration of sodium chloride and the amount of the polyethylene glycol rich top phase optained in step 2 on the partition coefficient of the lactate dehydrogenase. From these investigations the optimal system employed for the large scale extraction of lactate dehydrogenase described above was deduced.

Ultrafiltration and Diafiltration. The enzyme containing lower phase of step 3 was concentrated using a Romicon Hollow Fiber Cartridge (Type HF 26.5-43-PM30). The hollow fiber cartridge with a nominal molecular weight cut off of 30,000 Dalton had an ultrafiltration area of 2.5 m^2 in the module and was operated with an average ultrafiltration rate of 15 l/h. The concentrated enzyme solution (20 l) was diluted several times with 10 mM potassium phosphate buffer pH 6.5 containing 0.1% (v/v) 2-mercaptoethanol and concentrated again until a conductivity of 5 mS was reached. The enzyme activity was almost completely recovered in a total volume of 13.6 l.

DEAE-Cellulose-Column-Chromatography. A column (10 × 90 cm) was packed with DEAE-cellulose under slight pressure and equilibrated with 50 mM potassium phosphate buffer pH 6.5, containing 0.1% (v/v) 2-mercaptoethanol. The enzyme solution obtained in the previous step was applied to the column at a flow rate of 900 ml × h^{-1}. The column was washed first with 15 l of equilibration buffer. Then the enzyme was eluted with a linear gradient produced from 20 l of 100 mM and 20 l of 400 mM

Table 4. Purification of D(−)-lactate dehydrogenase

Step	Volume (l)	Protein (g)	Pyruvate		Phenylpyruvate	
			Total activity (10^{-6} U)	Specific activity (U × mg^{-1})	Total activity (10^{-6} U)	Specific activity (U × mg^{-1})
Crude extract	60	1,518	66.5	43.8	5.22	3.3
Upper phase I	86	955	63.5	66.5	4.57	4.7
Lower phase II	99	713	60.8	85.2	3.79	5.4
Ultrafiltration	13.6	625	56.5	90.4	3.44	5.5
DEAE-cellulose chromatography	4.3	215	46.5	216.3	3.21	15.0

Table 5. Enzyme stability in the *Lactobacillus confusus* crude extract

Conditions	t_0 (%)	$t_{1\ month}$ (%)	$t_{2\ months}$ (%)	$t_{4\ months}$ (%)
Control	100	97	94	85
+ 0.01% 2-mercapto ethanol	100	99	99	83
+ 0.05% 2-mercapto ethanol	100	100	100	88
+ 0.1 % 2-mercapto ethanol	100	100	100	82
+ 0.2 % 2-mercapto ethanol	100	100	100	88
+ 0.5 % 2-mercapto ethanol	100	97	100	71
+ 1.0 % 2-mercapto ethanol	93	87	71	57
At pH 4.9	100	100	100	99
Preheated 50° C, 5 min	100	100	100	99
Preheated 55° C, 5 min	93	92	92	91

potassium phosphate buffer, pH 6.5. Fractions of 500 ml were collected and tested for D-lactate dehydrogenase activity. The enzyme is eluted with approximately 200 mM potassium phosphate concentration. The peak fractions were combined, concentrated by ultrafiltration to a final volume of 4,300 ml and stored at 4° C. The purification is summarized in Table 4.

Further Purification of D-Lactate Dehydrogenase by Affinity Chromatography on Blue-Sepharose CL-6B

An aliquot of the enzyme solution of the previous step was dialyzed against 20 mM Tris-HCl buffer pH 6.9, containing 5 mM $MgCl_2$, 0.4 mM EDTA and 0.1% (v/v) 2-mercaptoethanol (buffer A). The dialyzed enzyme solution was applied to a column (2.5 × 20 cm) packed with Blue-Sepharose CL-6B and equilibrated against buffer A. The column was washed with buffer A and elution was carried out with the same buffer containing 2 mM NADH. The flow rate was 30 ml × h^{-1} and 3 ml fractions were collected. The enzyme activity was located in the effluent, combined, concentrated by ultrafiltration and stored at −20° C after addition of 50% (v/v) glycerol. The specific activity of this enzyme was > 2,000 U/mg in the purest fraction. The overall recovery of the enzyme at this step was 76%. The purified enzyme gave a single band on disc gel electrophoresis that coincided with the D-lactate dehydrogenase activity. When the enzyme was subjected to gel electrophoresis in the presence of sodium dodecylsulfate only one protein band was observed (Fig. 3) with a molecular weight of 34,000 Dalton.

The molecular weight of the native D-lactate dehydrogenase was estimated to be 130,000 ± 10,000 by gelfiltration over Sephacryl S 300 superfine. (Blue dextran 2,000, ferritin, catalase, aldolase, bovine serum albumin, ovalbumin and chymotrypsinogen A were used for calibration.) These results point out that the native enzyme is composed of four subunits of equal size, which may be identical.

Effect of pH on the Enzyme Activity and Stability

The D-lactate dehydrogenase shows maximal activity in the pH range of 6.0−6.5 for the reduction of pyruvate and phenylpyruvate. The pH optima for the

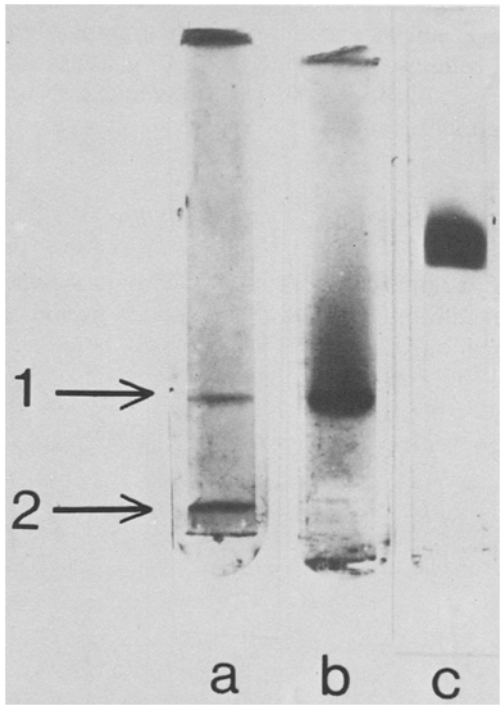

Fig. 3a–c. Acrylamide gel electrophoresis of the D-lactate dehydrogenase. **a** Analytical gel; 1 D-LDH, 2 tracking dye; **b** activity staining; **c** SDS-gel

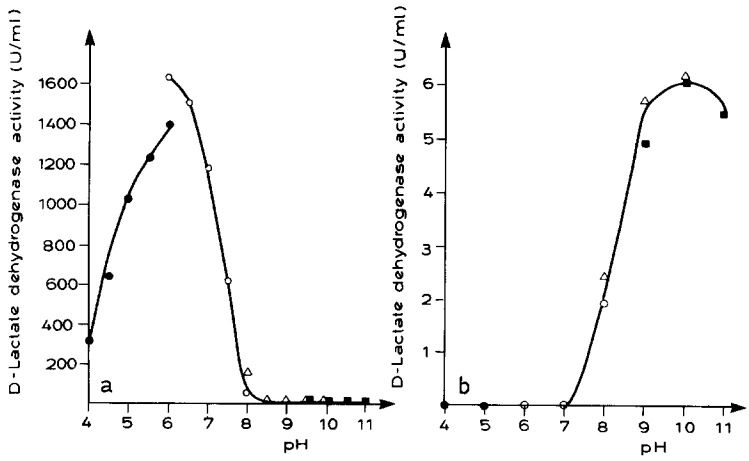

Fig. 4. a Effect of pH on the activity of D-lactate dehydrogenase. The reaction rate for the reduction of pyruvate was measured in a mixture containing standard concentrations of NADH and pyruvate and various buffer ions in 100 mM concentration at the pH value indicated. ● sodium citrate; ○ potassium phosphate; △ Tris-HCl; ■ glycine; **b** The reaction rate for the dehydrogenation of D-lactate was measured in the same buffers using a mixture containing standard concentrations of NAD^+ and D-lactate

dehydrogenation of D(-)lactate is observed between 9.0 and 11.0. The influence of pH for both reactions is shown in Fig. 4a and b.

The enzyme is very stable at pH 5.0–8.0 using sodium citrate-, potassium phosphate- and Tris-HCl buffers. The high stability of the enzyme at lower pH-range can be used also as a purification step for the D-lactate dehydrogenase. Using an aliquot of the concentrated lower phase obtained at step 3 of the purification procedure a pH-treatment at pH 4.8 increased the specific activity of the enzyme from 90 $U \times mg^{-1}$ to 153 $U \times mg^{-1}$ (substrate pyruvate, assay as described in Methods) with an overall recovery of 90% as shown in Fig. 5. This pH-treatment may be used instead of the chromatography step on DEAE-cellulose described above to increase the specific activity of the enzyme to acceptable levels for technical application.

D-*Lactate Dehydrogenase Storage Stability*

The storage stability of D-lactate dehydrogenase was followed at 4° C in the crude extract over a period of several weeks. Different amounts of 2-mercaptoethanol were added to aliquots of the crude extract and enzyme activity was measured twice a week. Results shown in Table 5 point out that the enzyme is very stable during storage. The presence of 2-mercaptoethanol apparently has no protecting effect and higher concentrations may be even disadvantageously. These results show that the customary measure to protect enzymes by addition of 0.1% mercaptoethanol to all buffers also taken here could be dispensed with in case of the D-lactate dehydrogenase from *L. confusus*. The enzyme was also found very stable in the crude extract adjusted with 10% phosphoric acid solution to pH 4.9. A 5 min treatment at 50° C or 55° C did not influence the storage stability of the enzymatic activity over the time period tested. The results obtained at low pH and preheated samples suggest that microbial contamination may account for the slight drop in activity observed after 4 month in the mercaptoethanol treated samples.

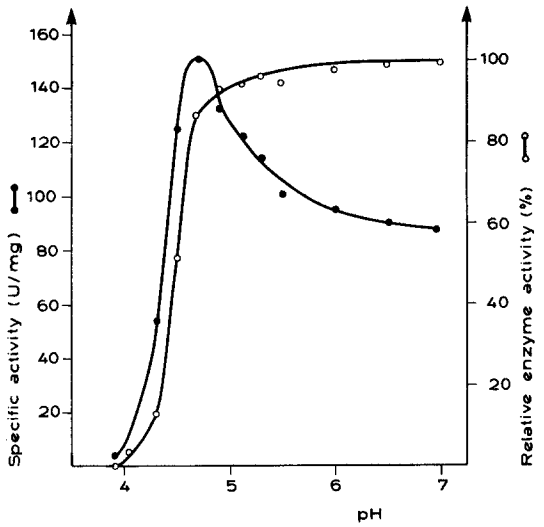

Fig. 5. pH-treatment of D-lactate dehydrogenase; specific activity (●———●) and recovery (○———○) of the enzyme are plotted as a function of the pH. Protein concentration and enzyme activity were measured after removing any precipitate formed during the pH-treatment

Table 6. Substrate specifity of D-lactate dehydrogenase

Substrate	Appearant V_{max} (μmol/min/mg protein at 30° C)	K_M-value (mM)
Pyruvate	2,144 (8 × K_M)	0.68
2-ketobutyrate	733 (5 × K_M)	7.2
Phenylpyruvate	253 (5 × K_M)	3.00

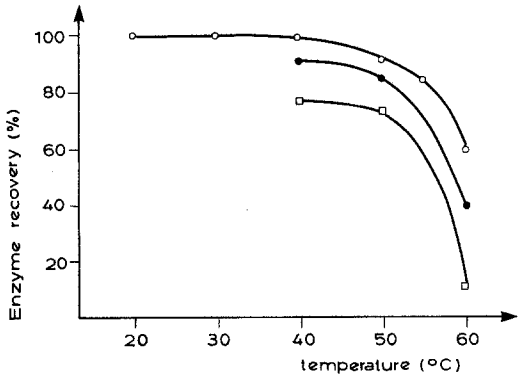

Fig. 6. Heat treatment of Lactobacillus confusus crude extract at various temperatures. D-lactate dehydrogenase activity was measured after 5 (○———○), 10 (●———●), and 15 min (□———□) heating time by the standard assay procedure

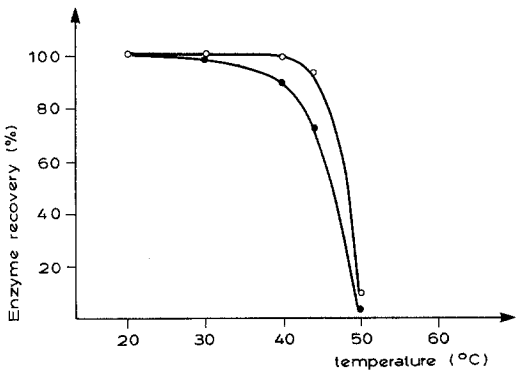

Fig. 7. The heat stability of purified D-lactate dehydrogenase was tested at various temperatures. The enzyme was incubated 15 min (○———○) and 30 min (●———●) in 100 mM potassium phosphate buffer, pH 7.0, containing 5 mg/ml bovine serium albumin at the temperatures indicated. Then the enzyme was cooled and immediately assayed under standard conditions

The heat stability of lactate dehydrogenase was tested with two preparations: the crude extract and a purified fraction of 188 U × mg^{-1} (pyruvate as substrate). Heat treatment at pH 7.0 at 40° C reduces the enzyme recovery already as shown in Figs. 6 and 7.

The initial reaction rate of D-lactate dehydrogenase increases to about 45° C (Fig. 8). At still higher temperatures thermal denaturation of the enzyme dominates indicating a low thermal stability of the enzyme. This is contrasted by the rather high stability towards low pH-values. The enzyme is also rather unstable at low protein concentrations as demonstrated in Fig. 9. Dilution of samples of high specific activity into buffer results in instantaneous loss of 40–50% of activity and a further linear decline with time. This can be prevented by including serum albumin in concentrations > 2 mg × ml^{-1} into the dilution buffer.

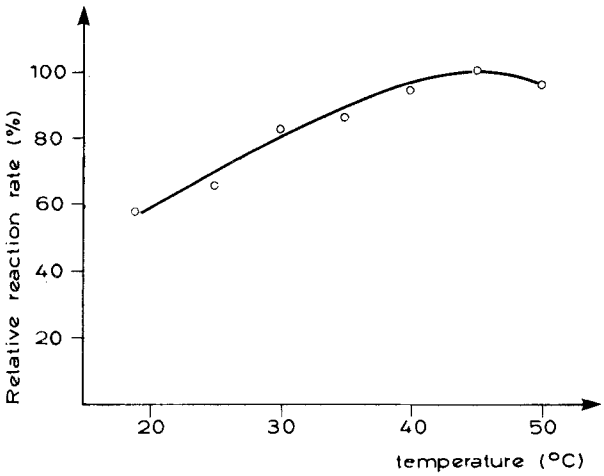

Fig. 8. Effect of temperature on the enzymatic activity of D-lactate dehydrogenase. The reaction rate of the enzyme was measured at various temperatures in 100 mM potassium phosphate buffer

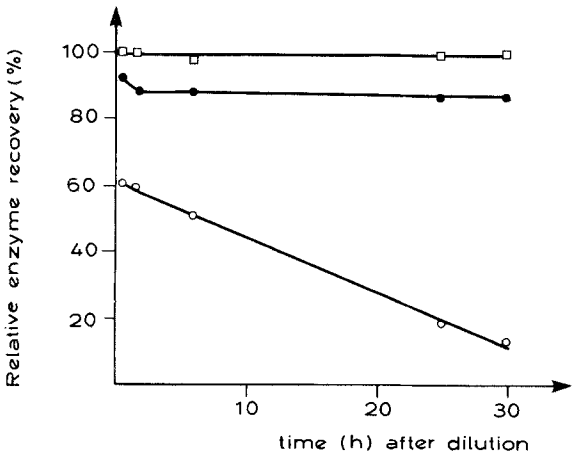

Fig. 9. Stability of D-lactate dehydrogenase at low protein concentrations. Purified enzyme was diluted 1 : 500 prior to activity measurements by the standard assay procedure. For dilution 100 mM phosphate buffer, pH 7.0; a) without BSA (○———○); b) containing 1 mg/ml BSA (●———●); c) containing 5 mg/ml BSA (□———□) was used. The activity of the diluted enzyme was measured after various times as indicated. The samples were stored at 5° C before assay

Substrate Specifity

Pyruvate as well as 2-ketobutyrate and phenylpyruvate served as a substrate for the D-lactate dehydrogenase from *L. confusus*. A weak activity was also detected for imidazole pyruvate as substrate but no activity was found towards 2-ketovalerate, 2-ketocaproate, 2-keto-3-methylbutyric acid, 2-keto-3-glutaric acid and indole-3-pyruvic-acid. The enzyme is specific for NADH as coenzyme. NADPH was tested over a range of concentrations up to 0.235 mM and found totaly inactive as a coenzyme. The Michaelis constant for NADH was determined to be 0.04 mM. The K_M-values and the apparent V_{max} for the reduction of the substrates by the D-lactate dehydrogenase are summarized in Table 6. The equilibrium of the reaction favours D-lactate formation. The reverse reaction, the NAD^+-dependent dehydrogenation of D-lactate to pyruvate proceeds with only 0.3% of the forward reaction rate under optimal conditions. The Michaelis constant were found to be 20 mM for D-lactate and 0.3 mM for NAD^+. When D,L-phenyllactate was employed as a substrate at concentrations up to 80 mM no NAD^+-dependent dehydrogenation could be observed at pH 10.0 during comparable measuring times. This may be either due to an inhibition by the L-isomer or to insufficient saturation of the enzyme by too low substrate concentration.

Determination of the Stereospecificty of D-Lactate Dehydrogenase from Lactobacillus confusus

To verify the stereospecifity of the enzyme we employed a simplified version of an enzyme membrane reactor as shown in Fig. 10. The reactor (10 ml final volume) was charged with 3.0 mmoles $PEG_{20,000}$-NADH, 30 U formate dehydrogenase (2 U × mg^{-1}) for NADH regeneration (Wichmann et al. 1981) and 80 U D-lactate dehydrogenase from *L. confusus* (86 U × mg^{-1} measured with pyruvate as substrate). The reactor was fitted with a YM5-membrane so that the modified coenzyme was kept in the reactor together with the enzymes. The reaction mixture was circulated with 30 ml × h^{-1} along the membrane. The substrate solution (6 mM pyruvate or 6 mM phenylpyruvate, respectively, in 0.3 M sodium formate, pH 7.0) was continuously fed for 20 h into

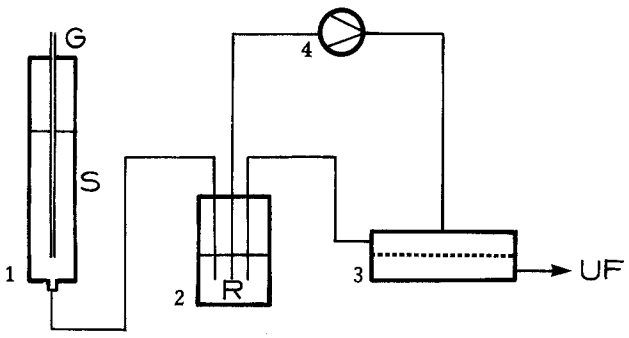

Fig. 10. Enzyme membrane reactor for the determination of the stereospecifity of the enzyme. *1* Reservoir with buffered substrate solution (S) (G = glass tube); *2* Thermostated reaction vessel; *3* Ultrafiltration membrane reactor (UF = ultrafiltrate); *4* pump

the reactor to replace the ultrafiltrate, which was collected in fractions of about 4.5 ml. The residence time was 3.3 h. The optical rotation of the ultrafiltrate was determined in a polarimeter (Perkin-Elmer 241) at 25° C and 436 nm. D-lactate and L-2-phenyllactate were used to obtain a set of calibration curves. From the sign and the value of the optical rotation of the ultrafiltrate the chiral position at C2 as well as the concentration of the hydroxyacids in the fractions could be evaluated. At steady state a reading of optical rotation of −0,016 for lactate produced and −0,097 for phenyllactate, respectively was obtained in the experiments. Our results demonstrate that the enzyme reduces both substrates to the corresponding D-isomeres. Without optimizing the process we observed 94% conversion to D-lactate and 97% conversion to D-phenyllactate, respectively under the reaction condition employed.

Discussion

Our screening revealed that several *Lactobacillus* strains possess dehydrogenases with a broad substrate specifity which appear to differ from the lactate dehydrogenases described so far. Under optimized assay conditions the reaction rate of D-lactate dehydrogenase for phenylpyruvate as substrate was about 8.5% of the reaction rate for pyruvate. This is a higher rate as we can find in the literature but not as high as measured initially in *Lactobacillus confusus* crude extract during our screening program. Comparing the substrate concentration in the screening assay with the K_M-values of the *L. confusus* enzyme (Table 6) reveals that especially pyruvate concentrations were too low to achieve saturation of the enzyme. This may hold also for other enzyme producers summarized in Table 2. Because of the high volume activity we selected *L. confusus* for the production of the desired enzyme and further studies.

To reduce the cost for the large scale cultivation of the organism we investigated other media compositions and could show that *L. confusus* grows without addition of the expensive casein peptone and with rather low levels of meat extract. In addition, the comparatively short cultivation time results in a good space time yield for the production of the enzyme. A simple and fast large scale purification of the D-LDH was developed based on liquid-liquid extraction (Kula et al. 1982). After two partition steps we obtained a crude enzyme free of cell debris with a specific activity of about 5.4 U × mg^{-1} with phenylpyruvate as substrate. A further threefold enrichment was possible by ultrafiltration and chromatography on DEAE-cellulose. Alternately an acid treatment at pH 4.8 can be used, to obtain the enzyme in sufficient purity to be used as an industrial catalyst. Final purification to homogeneous protein can be carried out by affinity chromatography using Blue-Sepharose as adsorbent and eluting the enzyme with NADH. During the purification procedure the relative activity with pyruvate and phenylpyruvate as substrate remained relatively constant, suggesting that both substrates are converted by the action of the same protein. This was also demonstrated by activity staining of highly purified samples. D-lactate dehydrogenase from *L. confusus* will accept modified, molecular weight enlarged PEG-NADH as a coenzyme and can be applied in a membrane reactor. The thermal stability of the enzyme is rather low but an unusual stability towards low pH-values was noticed. Suitably stored enzyme remains stable for several months.

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