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Pyruvate decarboxylase from *Zymomonas mobilis*. Isolation and partial characterization

S. Bringer-Meyer, K.-L. Schimz, and H. Sahm

Institut für Biotechnologie 1 der Kernforschungsanlage Jülich GmbH, Postfach 1913, D-5170 Jülich, Federal Republic of Germany

Abstract. Pyruvate decarboxylase (EC 4.1.1.1) from the ethanol producing bacterium *Zymomonas mobilis* was purified to homogeneity. This enzyme is an acidic protein with an isoelectric point of 4.87 and has an apparent molecular weight of 200,000 ± 10,000. The enzyme showed a single band in sodium dodecylsulfate gel electrophoresis with a molecular weight of 56,500 ± 4,000 which indicated that the enzyme consists of four probably identical subunits. The dissociation of the cofactors Mg^{2+} and thiamine pyrophosphate at pH 8.9 resulted in a total loss of enzyme activity which could be restored to 99.5% at pH 6.0 in the presence of both cofactors. For the apoenzyme the apparent K_m values for Mg^{2+} and thiamine pyrophosphate were determined to be 24 μM and 1.28 μM. The apparent K_m value for the substrate pyruvate was 0.4 mM. Antiserum prepared against this purified pyruvate decarboxylase failed to cross-react with cell extracts of the reportedly pyruvate decarboxylase positive bacteria *Sarcina ventriculi*, *Erwinia amylovora*, or *Gluconobacter oxydans*, or with cell extracts of *Saccharomyces cerevisiae*.

Key words: Pyruvate decarboxylase – *Zymomonas mobilis* – Purification – Molecular weight – Isoelectric point – Cofactor dissociation – Immunological studies

Thiamine pyrophosphate-dependent pyruvate decarboxylase (EC 4.1.1.1), which catalyzes the non-oxidative decarboxylation of pyruvate to acetaldehyde was first detected in yeast extracts by Neuberg and Karczag (1911). While this enzyme has been found widely distributed in plants (Vennesland 1951), it has only a very limited occurrence in bacteria (Scrutton 1971). Until now it was detected in *Zymomonas mobilis* (Dawes et al. 1966), *Sarcina ventriculi* (Bauchop and Dawes 1959), *Erwinia amylovora* (Haq and Dawes 1971; Haq 1984), and the acetic acid bacteria (DeLey and Schell 1962). In conjunction with alcohol dehydrogenase, pyruvate decarboxylase enables yeast and *Zymomonas* to produce nearly 2 mol ethanol per 1 mol glucose. In *Sarcina ventriculi* and in *Erwinia amylovora* pyruvate decarboxylase competes with other pyruvate decarboxylating enzymes; pyruvate-ferredoxin oxidoreductase leads to the formation of acetyl-CoA in *S. ventriculi* (Stephenson and Dawes 1971), and α-acetolactate synthase to α-acetolactate in *E. amylovora* (Haq and Dawes 1971). The aerobic organisms *Acetobacter* and *Gluconobacter* convert lactate to pyruvate, acetaldehyde and finally to acetate (DeLey and Schell 1962).

Pyruvate decarboxylases from the various sources contain Mg^{2+} and thiamine pyrophosphate (King and Cheldelin 1954; Morey and Juni 1968; Ullrich 1974, 1982; Haq 1984). The binding strength for thiamine pyrophosphate is pH-dependent and differs among the binding sites in the enzyme molecule (Morey and Juni 1968; Ullrich 1974). "Active acetaldehyde" (2-α-hydroxyethyl-thiamine pyrophosphate) was identified as a reaction intermediate of pyruvate decarboxylation (Holzer and Beaucamp 1961).

In recent years strains of *Zymomonas* have received much interest for ethanol production (Rogers et al. 1982; Bringer et al. 1984b). These bacteria efficiently convert glucose via the Entner-Doudoroff pathway to pyruvic acid which is then decarboxylated by pyruvate decarboxylase to acetaldehyde which is reduced by alcohol dehydrogenase to ethanol (McGill and Dawes 1971). Since pyruvate decarboxylase can be regarded as a key enzyme in the ethanol formation by *Z. mobilis*, this enzyme was isolated. In the present paper data on its molecular, catalytic and immunological properties are presented.

Materials and methods

Strains and cultivation. *Zymomonas mobilis* subsp. *mobilis*, strain ATCC 29191 and *Saccharomyces carlsbergensis*, strain DSM 70424 were cultured in a medium containing 50 g/l glucose as described previously (Bringer et al. 1984a). The cultures were inoculated with 0.15% (v/v) of a preculture and grown for 19 h at 30° C. *Erwinia amylovora*, strain DSM 50901 was grown according to Haq and Dawes (1971), *Sarcina ventriculi*, isolated from soil was cultured according to Finn et al. (1984). Cells of *Gluconobacter oxydans* subsp. *suboxydans*, strain DSM 2003, were obtained as described in the catalogue of the German Collection of Microorganisms.

Preparation of cell extracts. Cells of *Zymomonas mobilis* were harvested in the exponential phase of growth, washed once with 0.01 M Tris-HCl, containing 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM thiamine pyrophosphate, and 2 mM mercaptopropanediol, pH 7.0 ("Tris buffer"), and resuspended in the same buffer. At this stage the cells were concentrated about 50-fold (OD_{550nm} approx. 350). The cells

Abbreviations. Tris-buffer, 0.01 M tris-HCl buffer, containing 1 mM $MgCl_2$, 0.1 mM EDTA, 1.0 mM thiamine pyrophosphate, 2 mM mercaptopropanediol, pH 7.0

Offprint requests to: S. Bringer-Meyer

were disrupted in an x-press as described previously (Bringer et al. 1979) and after 6-fold dilution with Tris-buffer the extract was centrifuged at 48,000 × *g* and 5° C for 60 min. The supernatant containing about 5.5 mg protein/ml was used as cell-free extract.

Assay of pyruvate decarboxylase. The enzyme activity was measured at 30° C by determination of either acetaldehyde or carbon dioxide formed; with cell-free extracts of *Z. mobilis* the assays gave comparable results. Prior to activity determinations the enzyme samples were diluted with 0.1 M sodium citrate buffer, pH 6.0, containing 20 mM Mg^{2+} and 1.5 mM thiamine pyrophosphate. The formation of acetaldehyde was determined by the method of Ullrich (1970). The assay mixture consisted of: 105 µmol sodium citrate buffer, pH 6.0, 21 µmol $MgSO_4$, 18 µmol sodium pyruvate, 0.19 µmol NADH, 3.7 U alcohol dehydrogenase from yeast (Boehringer, Mannheim, FRG), and limiting amounts of enyzme in a total volume of 1.05 ml. The decrease in absorbance at 340 nm was followed. Since the NADH oxidase activity of the crude extracts (Bringer et al. 1984a) interfered with the NADH-dependent indicator reaction, controls were made with the omission of pyruvate and alcohol dehydrogenase. For the manometric assay of pyruvate decarboxylase, Warburg vesssels contained 300 µmol sodium citrate buffer, pH 6.0, 60 µmol $MgSO_4$, 51 µmol sodium pyruvate, limiting amounts of enzyme in a total volume of 3.0 ml. The reaction was started by addition of sodium pyruvate from the side arm of the Warburg vessel. One unit of enzyme activity was defined as either the formation of 1 µmol acetaldehyde/min or the liberation of 1 µmol CO_2/min.

For immunological assays, antibodies against the pyruvate decarboxylase from *Z. mobilis* were prepared as described by Bräu and Sahm (1986). Double immunodiffusion tests were conducted in 2% agarose gels with a 0.05 M Tris-HCl buffer, pH 7.5, containing 0.025% NaN_3 (Stollar and Levine 1963).

Isoelectric focusing. Polyacrylamide gel cylinders for isoelectric focusing were prepared by essentially following the procedure of Righetti and Drysdale (1971). The gels contained 6% (w/v) acrylamide, 0.24% (w/v) bisacrylamide, and 2% (w/v) carrier ampholytes ("Servalyt" from Serva, Heidelberg, FRG, and "Ampholine" from LKB Instrument, Gräfelfing, FRG). The electrode solutions were 50 mM NaOH (cathode) and 25 mM H_3PO_4 (anode) degassed prior to use. The gels were prefocused for 1 h and focused for 21.5 h (voltage held constant at 400 V). The gel rods were then fractionated into slices of 1 mm and eluted for 1.5 h in 0.5 ml H_2O for pH measurement. After addition of 0.5 ml of Tris buffer, 2-fold concentrated, the gel slices were further eluted overnight for the determination of enzyme activity.

Enzyme purification. Since it was found that SH-groups are essential for stability and activity of pyruvate decarboxylase from yeast (Ullrich 1982), mercaptopropanediol was added to the buffer solutions as a sulfhydryl protection reagent. Furthermore, 0.02% (w/v) NaN_3 was added to all buffers used for chromatographic purification steps. Pyruvate decarboxylase was precipitated in crude extracts with ammonium sulfate according to Green and Hughes (1955) at 55 to 80% saturation. The precipitate was re-dissolved with 10 mM potassium phosphate buffer, pH 6.8, containing 0.02% (w/v) NaN_3, and dialyzed against the same buffer.

This dialysate was applied to a high resolution hydroxylapatite (Calbiochem, Frankfurt, FRG) column (*d*: 2.5 cm, length: 19 cm), washed with the same buffer and eluted with a 500 ml gradient of 10 – 500 mM potassium phosphate, pH 6.8, with 0.02% (w/v) NaN_3. Maximum pyruvate decarboxylase activity was eluted at about 400 mM potassium phosphate. The active fractions of three runs were combined and the pyruvate decarboxylase activity was precipitated with ammonium sulfate at 80% saturation. The precipitate was re-dissolved with Tris-buffer and dialyzed against the same buffer.

This fraction was applied to a DEAE-cellulose (DE 52, Whatman, obtained from Vetter, Wiesloch, FRG) column (*d*: 2.5 cm, length: 50 cm) equilibrated with Tris-buffer. After washing the enzyme was eluted with a 500 ml gradient of 0 – 500 mM potassium phosphate, pH 6.8 in Tris-buffer. Maximum activity was eluted at about 200 mM potassium phosphate. The active fractions of three runs were combined and precipitated with ammonium sulfate (80% saturation).

This precipitate was re-dissolved with Tris-buffer and applied without further dialysis to a Sephadex G 200 (Pharmacia, Freiburg, FRG) column, which had been equilibrated with degassed Tris-buffer containing 0.1 M NaCl. The column was 2.5 cm in diameter and had a total volume of 418 ml. Elution of maximum activity was measured at 280 ml ($= V_e$; $V_o = 195$ ml). The active fractions were combined, concentrated by Amicon PM-10 (Amicon, Witten, FRG) ultrafiltration, mixed with an equal volume of 87% (v/v) glycerol and stored at −20° C.

Other methods. Protein was determined by the method of Bradford (1976). For an estimate of the molcular weight (Stockes' radius) of the native enzyme a Sephadex G 200 column (*d*: 2.5 cm, length: 94 cm) was calibrated with calibration kits from Pharmacia (Freiburg, FRG) and Boehringer (Mannheim, FRG). Electrophoresis in the presence of sodium dodecylsulfate (SDS) was performed according to Laemmli (1970). Calibration kits for SDS gel electrophoresis from Boehringer and Biorad (Munich, FRG) were used. Electrophoreses for the estimation of the purity of the enzyme were performed according to system 1 of Maurer (1971).

Results

Purification of pyruvate decarboxylase. After purification of the enzyme by differential $(NH_4)_2SO_4$ precipitation, and chromatographies on hydroxylapatite, DEAE cellulose, and Sephadex G 200 (Table 1), the enzyme preparation gave a single band upon polyacrylamide gel electrophoresis. The overall purification factor of 17 is significantly lower than that reported by Hoppner and Doelle (1983) who enriched the pyruvate decarboxylase from *Zymomonas mobilis* 447-fold to obtain a partially pure preparation. The main reason for this difference is the low specific enzyme activity of their cell free extract, 0.3 U/mg protein in comparison to 11 U/mg protein in the present work. These authors used cells from cultures which were inoculated with 10% (v/v) of a preculture and grown with an initial glucose concentration of 100 g/l for 9 – 12 h at 37° C. This may have resulted in stationary cells which tend to lyse in the presence of ethanol

Table 1. Purification of pyruvate decarboxylase from *Zymomonas mobilis*. One unit (U) of enzyme is the amount that catalyzes the production of 1 μmol acetaldehyde/min

Step	Total protein (mg)	Total activity (U)	Yield (%)	Specific activity (U/mg)	Purification (-fold)
Cell-free extract	1,045	11,408	100.0	10.92	1
$(NH_4)_2SO_4$ fraction 55–80% saturation	813	10,199	89.4	12.55	1.2
Hydroxylapatite	106	7,067	61.9	66.62	6.1
DEAE cellulose	50	5,816	51.0	116.81	10.7
Sephadex G 200	14	2,580	22.6	181.00	16.6

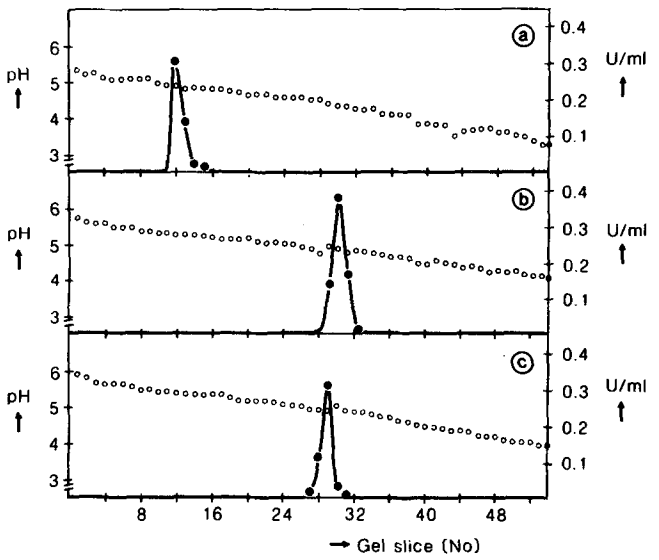

Fig. 1 a–c. Localization of pyruvate decarboxylase activity in polyacrylamide gel slices after isoelectric focusing. The carrier ampholytes used were **a** Servalyt pH 3–7, **b** Servalyt pH 4–6, **c** Ampholine pH 4–6.5

at the relatively high incubation temperature of 37°C (Lee et al. 1981; own results, unpublished.) When the purified enzyme was stored at −20°C in the presence of 50% (v/v) glycerol only negligible losses of enzyme activity occurred. The main purification step according to Hoppner and Doelle (1983), who reported about a 100-fold enrichment of pyruvate decarboxylase by chromatography with DEAE-cellulose, could not be successfully reproduced.

Molecular weight and subunits. The apparent molecular weight of the native pyruvate decarboxylase was estimated by gel chromatography (Sephadex G-200 column) to be 200,000 ± 10,000 which is slightly lower than the molecular weight of yeast pyruvate decarboxylase (230,000 – 250,000) (Ullrich 1982) and of the enzyme from wheat germ (300,000) (Balla and Ullrich 1980). When polyacrylamide gel electrophoresis was carried out in the presence of sodium dodecylsulfate, only one protein band with a molecular weight of 56,500 ± 4,000 was observed. These results suggest that the pyruvate decarboxylase from *Z. mobilis* is composed of four subunits of equal size, which are probably identical. The isoelectric point of the enzyme was determined by analytical isoelectric focussing to be 4.87 (Fig. 1).

Cofactor dissociation and enzyme reconstitution. During the purification of the pyruvate decarboxylase from *Z. mobilis* the specific activity decreased after ammonium sulfate precipitations. Activity could be restored by the addition of Mg^{2+} and thiamine pyrophosphate to the dialyzed enzyme or by dialysis buffer containing these cofactors. A similar behaviour was reported for the pyruvate decarboxylase of yeast which lost these cofactors during ammonium sulfate precipitation (Morey and Juni 1968; Ullrich 1970) and at pH values above 8.0 (Gounaris et al. 1975; Hopmann 1980). In order to determine the conditions for the reconstitution of the enzyme from *Z. mobilis* the dissociation and the association of the cofactors were studied (Table 2). Dialysis of an enzyme sample against a 0.08 M-glycine-0.2 M-phosphate buffer containing 0.1 mM EDTA, and adjusted to pH 8.9 with KOH (Ullrich 1970) resulted in a total decrease of enzyme activity. After dilution of this sample into the assay buffer, pH 6.0, at 30°C the activity was restored, time dependently, to 99.5% when both Mg^{2+} and thiamine pyrophosphate were present. The low reactivation of 2.6% of the initial enzyme activity which occurred with Mg^{2+} or thiamine pyroposphate alone could be due to traces of the cofactors in the dialyzed enzyme preparation.

Table 2. Conditions for the dissociation and association of the cofactors of pyruvate decarboxylase. TPP: thiamine pyrophosphate

Treatment	Incubation		Specific activity (U/mg protein)
	Mg^{2+} (20 mM)	TPP (1.5 mM)	
Before dialysis	–	–	7.58
	+	+	8.18
Dialysis pH 8.9[a]	–	–	0.00
Dilution into pH 6.0[b]	+	+	8.14
	–	+	0.21
	+	–	0.21

[a] 0.08 M glycine – 0.2 M phosphate with 0.1 mM EDTA
[b] 0.1 M sodium citrate

Affinity of the enzyme to the substrate and cofactors. As shown in Fig. 2 an apparent K_m value for pyruvate of 0.4 mM was determined in the presence of 20 mM Mg^{2+} and 1.5 mM thiamine pyrophosphate in sodium citrate buffer, pH 6.0. Hoppner and Doelle (1983) reported an apparent K_m for pyruvate of 4.4 mM for the pyruvate decarboxylase of *Z. mobilis*.The considerably lower apparent K_m value of 0.4 mM could in part be due to the buffer used in the enzyme assay.

The apparent K_m values of the apoenzyme for the cofactors Mg^{2+} and thiamine pyrophosphate were 24 μM

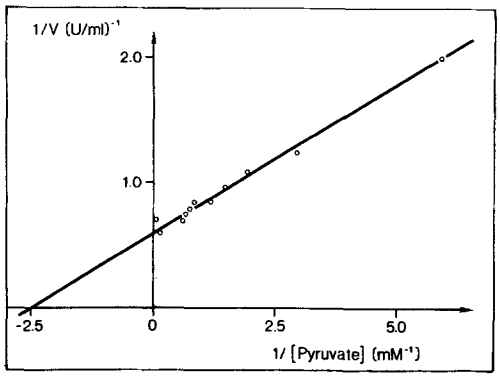

Fig. 2. Lineweaver-Burk plot for pyruvate decarboxylase from *Zymomonas mobilis* with pyruvate as a substrate. The concentrations of Mg^{2+} and thiamine pyrophosphate were 20 mM and 1.5 mM

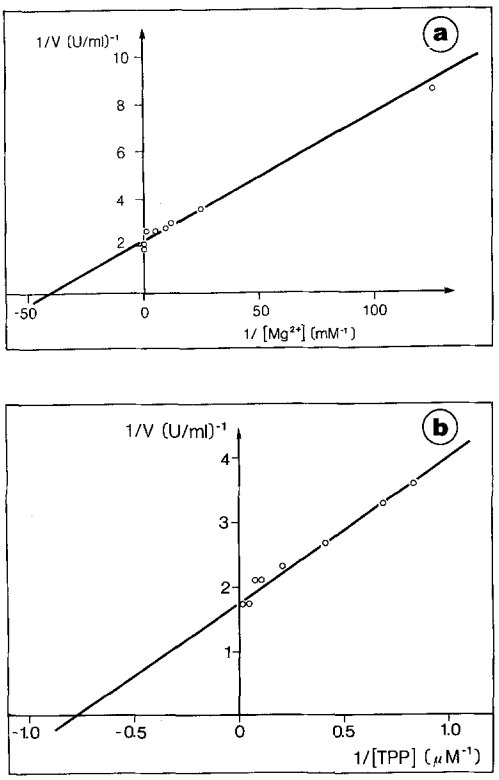

Fig. 3a, b. Lineweaver-Burk plots showing the reactivation of pyruvate decarboxylase as a function of $[Mg^{2+}]$ in the presence of 1.5 mM thiamine pyrophosphate (**a**), and as a function of [thiamine pyrophosphate] in the presence of 20 mM Mg^{2+} (**b**). The concentration of pyruvate was 17 mM

and 1.28 μM (Fig. 3). The apoenzyme was prepared by dialysis of an enzyme sample against the glycine-phosphate buffer described above. In order to reach association equilibria (Schellenberger et al. 1966) the samples were incubated for 30 min at 30° C and pH 6.0 prior to activity measurements in the presence of limiting amounts of either Mg^{2+} or thiamine pyrophosphate and saturating concentrations of the second compound.

Serological properties. The ability of antiserum prepared against the purified pyruvate decarboxylase from *Z. mobilis* to crossreact with pyruvate decarboxylases from other bacteria and from yeast was determined by Ouchterlony immunodiffusion tests. Surprisingly, no precipitation bands were formed with cell extracts of *Sarcina ventriculi*, *Erwinia amylovora*, or *Gluconobacter oxydans*, bacterial strains which are reportedly pyruvate decarboxylase positive (Scrutton 1971), nor with cell extracts of *Saccharomyces cerevisiae*. Therefore, it seems that the pyruvate decarboxylase from *Z. mobilis*, under the conditions used, is immunologically different from the other bacterial pyruvate decarboxylases and also from the yeast enzyme.

Discussion

Pyruvate decarboxylase, a key enzyme in the ethanol formation of *Zymomonas mobilis* channels 95% of the substrate carbon via acetaldehyde to ethanol and CO_2, as can be calculated from fermentation balances (Rogers et al. 1982). Correspondingly, this bacterium contains relatively high amounts of this enzyme which appears as a strong band when cell free extracts are electrophoretically separated in polyacrylamide gels (result not shown). It can be calculated from the purification factor that under the conditions of cultivation used during this work, pyruvate decarboxylase contributes 4–6% of the soluble cell protein. A comparison of specific pyruvate decarboxylase activities in different organisms is given in Table 3. However, restrictions have to be made with respect to the accuracy of pyruvate decarboxylase activity measurements with cell-free extracts. With the photometric assay pyruvate decarboxylase activity cannot be discriminated from that of lactate dehydrogenase when the cell extracts concomitantly contain alcohol dehydrogenase. Likewise, the manometric determination of carbon dioxide evolution can be erroneous in the presence of other pyruvate decarboxylating enzymes (e.g. acetolactate synthase, pyruvate-formate lyase in conjunction with formate-hydrogen lyase, pyruvate-ferredoxin oxidoreductase). All of these sources of error would lead to apparently higher pyruvate decarboxylase activities, so that the data given in Table 3 must be regarded as maximum values. With the exception of the pyruvate decarboxylase activity reported by Haq (1984) for *E. amylovora*, cell-free extracts of *Z. mobilis* have the highest specific activities of this enzyme. *Erwinia amylovora* and *S. ventriculi* produce ethanol with yields of approximately 50% of the theoretical maximum yield due to the formation of side products (Haq and Dawes 1971; Finn et al. 1984). In *Gluconobacter suboxydans*, an aerobic, acetate producing organism, pyruvate decarboxylase is involved in the catabolism of lactate (DeLey and Schell 1962). The activities of pyruvate decarboxylase reflect to some extend the efficiencies of the fermentative organisms to produce ethanol. In addition, the regulation of pyruvate decarboxylase and other pyruvate decarboxylating enzymes is decisive with respect to the pattern of fermentation products (Finn et al. 1984).

Although we could not detect an immunological crossreaction between the pyruvate decarboxylases of yeast and *Z. mobilis*, these enzymes are similar in their molecular weights and subunit numbers (Gounaris et al. 1975). Furthermore, the conditions for the binding of the cofactors, thiamine pyrophosphate and Mg^{2+}, appear to be similar, if not identical, for both enzymes (Schellenberger et al. 1966; Ullrich 1982). A comparison of properties of pyruvate decarboxylases is shown in Table 3.

Table 3. Comparison of properties of pyruvate decarboxylases from different sources. TPP: thiamine pyrophosphate

Organism	Molecular weight	Molecular weight of subunits	K_m app. (pyruvate) (mM)	Spec. activity of cell-free extracts[a] (U/mg protein)	References
Zymomonas mobilis	200,000	56,500	0.4	7.5 – 11.0	
Saccharomyces cerevisiae	230 – 250,000	60,000	1.1	1.5 – 2.5	Boiteux and Hess 1970 Gounaris et al. 1975 Ullrich 1970, 1982
Wheat germ	275 – 300,000	75,000	3.6	[b]	Balla and Ullrich 1980 Singer and Pensky 1952 Ullrich 1982

[a] Specific activities of cell-free extracts of *Erwinia amylovora*, *Sarcina ventriculi*, and *Gluconobacter suboxydans* were 1.1, 0.6, and 2.0 U/mg protein

[b] No photometrically determined values available; an estimated value from manometric data (Singer and Pensky 1952) is 0.02 U/mg protein

After cloning and expression of the pyruvate decarboxylase gene of *Z. mobilis* in *Escherichia coli* (Bräu and Sahm 1986), it will be of interest to study the sequence of this gene and compare it with the gene of *Saccharomyces cerevisiae*, which was recently analyzed (Hollenberg, personal communication). Furthermore, a comparison of the pyruvate decarboxylase of *Z. mobilis* with the E_1-protein of the pyruvate dehydrogenase of *Escherichia coli* (Stephens et al. 1983) could give additional support to the assumption of Swings and DeLey (1977) that the phylogenetic ancestors of *Zymomonas* were aerobic organisms.

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