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Chemically Defined Medium for the Accumulation of Intracellular Malate Dehydrogenase by *Streptomyces aureofaciens*

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A chemically defined medium was developed for the production of intracellular malate dehydrogenases by *Streptomyces aureofaciens* NRRL-B 1286. The composition of the medium (per liter) was as follows: 50 g of starch, 4 g of ammonium sulfate, 7.32 g of L-aspartic acid, 13.8 g of $MgSO_4 \cdot 7H_2O$, 1.7 g of K_2HPO_4, 0.01 g of $ZnSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.01 g of $MnSO_4 \cdot H_2O$, and 0.005 g of $CoSO_4 \cdot 7H_2O$. The pH of the medium was adjusted to 6.7 to 7.0 after sterilization. The activity of the intracellular malate dehydrogenases of the crude cell extract was greatest after 40 h of mycelium growth in a rotary shaker at 30°C. The best temperature for the enzyme reactions was approximately 35°C for NAD^+ activity at pH 9.7 and 40°C for $NADP^+$-linked enzyme at pH 9.0. The NAD^+ activity required Mg^{2+}, and both activities were sensitive to SH-group reagents. The $NADP^+$-dependent activity remained completely stable, and the NAD^+-dependent activity decreased to a very low residual level after 30 min at 60°C.

Enzymes acting on malate are widely distributed in nature. They are usually oligomeric enzymes and frequently are described as isoenzyme systems in the cells. The isoenzyme system of malate dehydrogenase (EC 1.1.1.37) is represented by two major forms, cytoplasmic and mitochondrial. The cytoplasmic form has been implicated in gluconeogenesis, as well as in the transfer of reducing power into mitochondria during aerobic glycolysis (17). Oxaloacetate-decarboxylating malate dehydrogenase (EC 1.1.1.40) plays an important role in cell metabolism as a donor of NADPH groups for biosynthetic processes. The literature describes several kinds of decarboxylating malate dehydrogenases (7, 9, 10, 12) which are localized in cytosol, as well as in mitochondria, and use NAD^+ or $NADP^+$ or both as a cofactor. NAD^+-dependent malate dehydrogenase derived from pig heart is used in biochemistry and food chemistry, mainly for glutamate-oxaloacetate transaminase and malate assays (1, 2). Some aspects of the malate dehydrogenases of *Streptomyces aureofaciens* were studied by Jechová et al. (5, 6) and Tinterová et al. (16) with a complex medium for microorganism growth.

This paper describes the development of a chemically defined medium for the submerged cultivation of *S. aureofaciens* with high accumulation of intracellular malate dehydrogenase activity. A chemically defined medium is useful for physiological and genetic studies. Some properties of the enzymes detected in the cell extract, resulting from growth in the medium developed here, are also described. Since malate dehydrogenase derived from pig heart is an important tool for enzymatic assays and the microbial enzyme is usually more stable, the development of an improved medium for the production of this enzyme is important from a practical, as well as a scientific, point of view.

MATERIALS AND METHODS

Dextrin, NAD^+, $NADP^+$, L-malate, maltose, amino acids, and organic acids were purchased from Sigma Chemical Co., St. Louis, Mo. Starch, glucose, and 2,4-dinitrophenylhydrazine were obtained from E. Merck AG, Darmstadt, Federal Republic of Germany, and the yeast extract was from Difco Laboratories, Detroit, Mich. Distilled water was used throughout. All other reagents were analytical grade.

***Streptomyces* cultivation.** The NRRL-B 1286 strain of *S. aureofaciens* which produces neither tetracycline nor chlortetracycline was the source of malate dehydrogenase. The culture medium used for inoculum preparation, the basic medium, was the starting point for subsequent medium improvements. The composition of the basic medium per liter was as follows: 50 g of sucrose (final concentration, 0.146 M), 4 g of ammonium sulfate (0.03 M), 3 g of yeast extract, 2 g of $CaCO_3$, 1.31 g of K_2HPO_4 (7.5×10^{-3} M), 0.2 g of $MgSO_4 \cdot 7H_2O$ (8.11×10^{-4} M), 0.01 g of $ZnSO_4 \cdot 7H_2O$ (3.47×10^{-5} M), 0.01 g of $FeSO_4 \cdot 7H_2O$ (3.59×10^{-5} M), 0.01 g of $MnSO_4 \cdot H_2O$ (5.91×10^{-5} M), and 0.005 g of $CoSO_4 \cdot 7H_2O$ (1.77×10^{-5} M). The initial pH was adjusted to 6.9 after sterilization. Growth was carried out at 30°C for 40 h in 125-ml Erlenmeyer flasks placed in a model G-25 Superohm rotary shaker (250 rpm, 3-cm-diameter circles) containing 20 ml of culture medium and 1 ml of inoculum. The inoculum preparation and mycelial growth were determined by measuring the dry weight as described previously by Laluce and Molinari (8). All experiments were carried out with 10 Erlenmeyer flasks for each medium or condition studied. The mycelial content of each flask was mildly disaggregated in a test tube by a rotating Teflon pestle and transferred to a bottle to form a pool from which samples were withdrawn for dry weight determination and enzyme extraction.

Preparation of crude cell extracts. Mycelial fractions (400 mg, dry weight) were collected by centrifugation (5,000 × *g*, 10 min, 6°C) and washed twice with a 0.05 M magnesium sulfate solution by centrifugation. The washed mycelia were frozen in a mortar and submitted to trituration at room temperature until completely defrosted. The trituration process was repeated three times. The disrupted mycelia were finally extracted during the last trituration with 10 ml of 0.1 M sodium phosphate buffer, pH 6.95. The mycelial debris was removed by centrifugation (20,000 × *g*, 20 min, 6°C), and the supernatant obtained was the crude cell extract.

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TABLE 1. Effect of modifications of basic medium on the formation of $NADP^+$-dependent activity[a]

Sugar (50 g/liter)	Supplement (0.05 M)	Growth (mg/ml)	Activity	
			Extract (U/ml)	Broth (U/ml)
Sucrose	L-Glutamic acid	6.5	20.2	3.3
Sucrose	L-Aspartic acid	6.1	51.8	4.2
Sucrose	DL-Alanine	8.1	8.0	1.6
Sucrose	Fumaric acid	3.3	37.7	3.1
Sucrose	Citric acid	3.7	29.2	2.7
Sucrose	L-Malic acid	3.7	47.4	3.2
Maltose	L-Aspartic acid	3.3	51.8	4.2
Dextrin	L-Aspartic acid	8.5	20.3	4.3
Glucose	L-Aspartic acid	5.9	6.5	9.5
Starch	L-Aspartic acid	13.5	100.2	27.2

[a] Growth for 40 h in the basic medium with the addition of 0.055 M magnesium sulfate.

Enzyme assay. The assay was based on the method of Robinson et al. (14). The composition of the reaction mixture was as follows: 0.1 to 1.0 ml of cell extract, 50 μg of NAD^+ or $NADP^+$, 500 μmol of potassium L-malate, and 200 μmol of potassium phosphate buffer, pH 7.0. A control was prepared for each assay in which the substrate was replaced by water. The final volume was 3 ml. The reaction was carried out at 35°C and started by the extract addition. After 60 min, the reaction was stopped by the addition of 1 ml of 30% trichloroacetic acid and the tubes were kept in an ice bath for 10 min. The precipitated protein was separated by centrifugation (3,000 × *g*, 5 min), and 3 ml of the supernatant was transferred to centrifuge tubes containing 1 ml of a 0.075% 2,4-dinitrophenylhydrazine solution prepared with 2 N hydrochloric acid. After 10 min of incubation at 35°C, the color reaction was developed by adding 2 ml of 4 N NaOH. Finally, the tubes were centrifuged to separate insoluble hydroxides before the A_{435} readings were taken. By the Ochoa procedure (13), one unit of enzyme activity was considered the amount of enzyme which caused an absorbancy increment of 0.01/min under the assay conditions. The activity was expressed in units per milliliter for enzyme extract and for broth (units of intracellular enzyme per milliliter of whole broth).

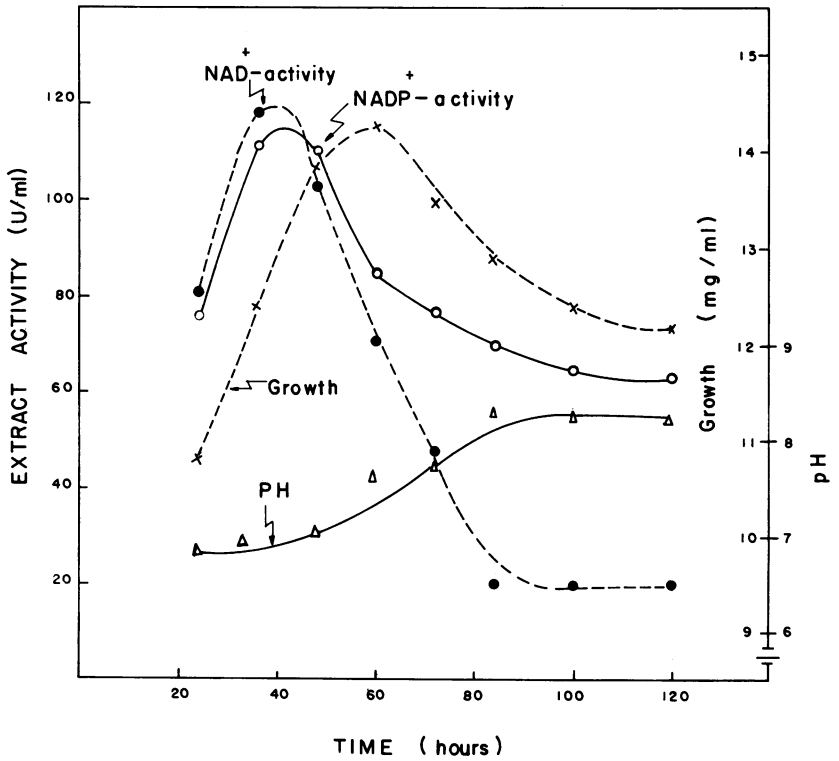

FIG. 1. *S. aureofaciens* growth, pH, and changes in intracellular NAD^+- and $NADP^+$-dependent activities for the best chemically defined medium. The composition of the medium (per liter) was as follows: 50 g of starch, 4 g of ammonium sulfate (final concentration, 0.03 M), 7.32 g of L-aspartic acid (0.055 M), 13.8 g of $MgSO_4 \cdot 7H_2O$ (0.055 M), 1.7 g of K_2HPO_4 (7.5×10^{-3} M), 0.01 g of $FeSO_4 \cdot 7H_2O$ (3.59×10^{-5} M), 0.01 g of $ZnSO_4 \cdot 7H_2O$ (3.47×10^{-5} M), 0.01 g of $MnSO_4 \cdot H_2O$ (5.91×10^{-5} M), and 0.005 g of $CoSO_4 \cdot 7H_2O$ (1.77×10^{-5} M). The pH of the medium was adjusted to 6.9 before inoculation.

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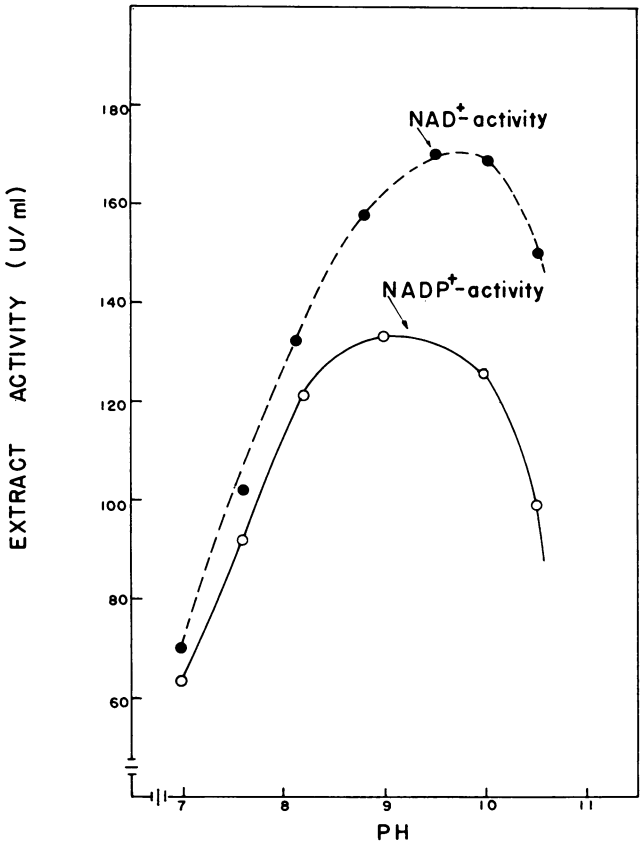

FIG. 2. Effect of pH on NAD$^+$- and NADP$^+$-dependent activities of the crude extract.

The activity of the cell extract of the mycelia grown in the best chemically defined medium obtained from this study may be also determined by measuring the A_{340} of NADH. The 1-cm cuvettes, kept at 28°C, contained the following reaction mixture: 0.045 M L-malate, 0.25 mM NAD$^+$, enzyme, and 0.01 M glycine-NaOH buffer, pH 9.7. The reaction was started by the addition of malate, which was replaced by water in the control cuvette. The extract must be diluted 100 times to obtain absorbance readings in the linear range of the standard curve (ΔA_{340}/min, 0.01 to 0.08). Specific activity was expressed as the number of micromoles of substrate consumed per minute per milligram of protein. Protein was assayed by the microbiuret method (4) with crystalline bovine serum albumin as the standard.

RESULTS

Medium improvement for accumulation of intracellular malate dehydrogenases. In preliminary experiments (data not shown), the substitution of L-glutamic acid for yeast extract in the basic medium and an increase in the concentration of magnesium sulfate were able to support reasonable growth and accumulation of NADP$^+$-dependent activity with a negligible amount of NAD$^+$-dependent activity. For this reason, all experiments for medium improvement were followed only by the assay of the NADP$^+$-dependent activity. The optimum initial concentration of L-glutamic acid and magnesium sulfate was 0.055 M, since higher concentrations inhibited enzyme activity formation. Replacement of L-glutamic acid by other amino acids and organic acids showed that L-aspartic acid was more suitable for enzyme production because it induced more activity per milliliter of broth (Table 1). Compared with sucrose or other sugars, starch was a better carbon source for the basic medium with L-aspartic acid and higher concentrations of magnesium sulfate (Table 1). Changes in the initial concentration of ammonium sulfate (results not shown) revealed that the concentration already used in the basic medium was satisfactory. Higher concentrations of ammonium sulfate inhibited growth and enzyme accumulation. The initial concentration of potassium phosphate in the original basic medium was the best for growth and enzyme accumulation. No potassium phosphate and concentrations above 0.0075 M drastically inhibited growth and enzyme formation. The presence of calcium carbonate did not affect growth and enzyme accumulation, and for this reason this compound was eliminated from the improved medium. The growth was not noticeably affected by the initial pH, in the range from 5.9 to 7.4, while the highest enzyme accumulation appeared at the initial pH of 7.3. The presence of NAD$^+$-dependent activity was assayed only in the final improved medium. Figure 1 shows the changes in pH, growth, and NAD$^+$- and NADP$^+$-dependent activities over time for the final improved medium. The maximum production of NAD$^+$- and NADP$^+$-dependent activities occurred at 40 h of growth.

Some enzyme properties. Sodium phosphate buffer at pH 7.0 used in all experiments was the best buffer for the extraction of NAD$^+$- and NADP$^+$-dependent activities. Tris hydrochloride buffer at pH 9.0 preferentially extracted the mycelial NADP$^+$-dependent activity (95% NADP$^+$ activity and 44% NAD$^+$ activity) while sodium acetate buffer extracted 75% of both. The pH optimum was approximately 9.7 for NAD$^+$ activity and 9.0 for NADP$^+$ activity (Fig. 2). The optimum temperature was approximately 35°C for NAD$^+$ and 40°C for NADP$^+$ (Fig. 3). There was a perceivable effect of magnesium ions on NAD$^+$ activity, and sulfhydryl groups participated in both activities but mainly for the NADP$^+$-linked enzyme (Table 2). The NAD$^+$-dependent activity was very unstable at 60°C but the NADP$^+$-dependent activity was quite stable (Table 3). The enzymatic extracts obtained with sodium phosphate buffer at pH 7.0 and Tris hydrochloride buffer at pH 7.2 retained all the NADP$^+$-dependent activity after 30 min at 60°C.

DISCUSSION

A non-tetracycline-producing strain of *S. aureofaciens* was used in this work. Hŏstálek et al. (3) found that the activities of the tricarboxylic acid cycle enzymes of a low-producing strain were higher than those of a high-yielding strain, with malate dehydrogenase being the most active of the enzymes.

In the best chemically defined medium developed here, the maximum production of NAD$^+$- and NADP$^+$-dependent activities appeared in the growth phase. The medium has 0.055 M L-aspartic acid and 0.055 M magnesium sulfate as stimulators of NADP$^+$-dependent activity. L-Aspartic acid was a better stimulant than L-glutamic acid was, and L-malic acid was also a good inducer of mycelial enzyme formation but growth was poor (Table 1). L-Glutamic acid and L-aspartic acid induced formation of a NADP$^+$-linked enzyme in a medium for *Escherichia coli* described by Murai et al. (12). The increase in concentration of P_i above 7.5×10^{-3} M showed a negative effect on the formation of NADP$^+$-dependent enzyme. The initial pH value of the chemically defined medium must be around 7, and the addition of

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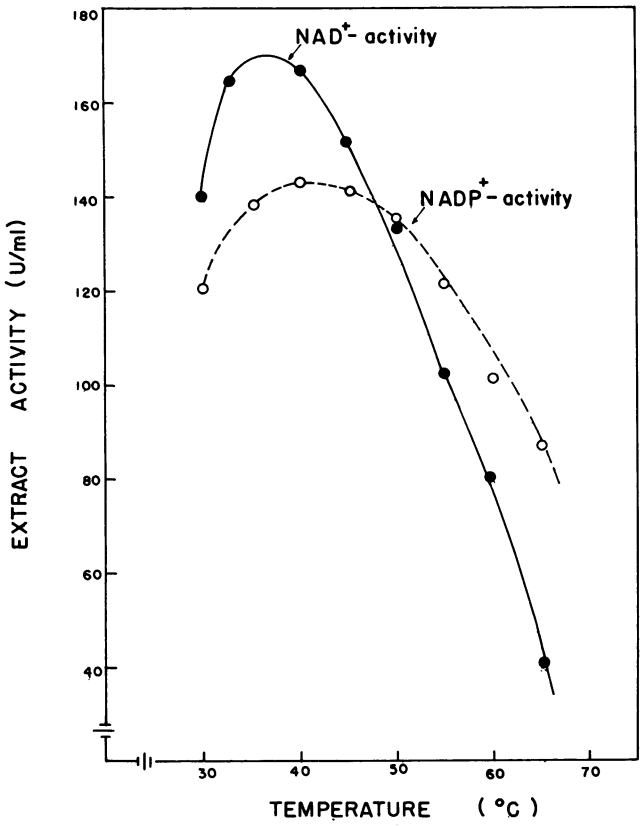

FIG. 3. Effect of temperature on NAD^+- and $NADP^+$-dependent activities of the crude extract. The enzymatic assays were performed at pH 9.7 for the NAD^+ activity and at pH 9.0 for the $NADP^+$ activity.

calcium carbonate had no effect on enzyme formation, as described in Results. Starch was a better carbon source than glucose, dextrin, sucrose, or maltose was.

Tinterová et al. (16) described two partly purified malate dehydrogenase (EC 1.1.1.37) isoenzymes isolated from *S. aureofaciens*. The isoenzyme activated by magnesium showed properties of the cytoplasmic malate dehydrogenase, and the other form inhibited by magnesium behaved like a mitochondrial enzyme. Jechová et al. (6) described, for the same microorganism, only one partially purified form of the decarboxylating malate dehydrogenase (EC 1.1.1.40), which was also activated by magnesium.

The $NADP^+$- and the NAD^+-dependent activities detected in the chemically defined medium of this paper showed pH optima of approximately 9.0 and 9.7, respectively, at 35°C. The NAD^+-dependent activity was probably the cytoplasmic malate dehydrogenase alone, because mycelia were washed with 0.05 M magnesium sulfate solution, which remained in the extract, inhibiting the other isoenzyme. Tinterová et al. (16) found a pH optimum of 9.0 at 28°C for the cytoplasmic malate dehydrogenase of *S. aureofaciens*, and Jechová et al. (6) found a pH optimum of 8.75 for the decarboxylating enzyme. The pH optima determined here were higher probably as a result of differences in assay conditions, mainly temperature. The NAD^+ activity is particularly magnesium dependent, and both were sensitive to sulfhydryl group reagents, agreeing with the results found by Jechová et al. (6) and Tinterová et al. (16).

TABLE 2. Effect of magnesium and sulfhydryl group reagents added to buffer on NAD^+- and $NADP^+$-dependent activities in the dialysis of the crude extract[a]

Supplement (5×10^{-3} M)	Relative activity (%)	
	NAD^+	$NADP^+$
None	100.0	100.0
Magnesium	140.0	112.4
Magnesium and mercaptoethanol	122.0	151.2
Magnesium and Iodoacetamide	92.0	50.6

[a] Crude extract (5 ml) was dialyzed for 12 h at 5°C against 500 ml of 0.1 M Tris hydrochloride buffer at pH 7.2 with mild stirring and buffer changes every 3 h.

TABLE 3. Effect of preincubation for 30 min at 60°C on NAD^+- and $NADP^+$-dependent activities of the crude extracts obtained with different buffers

Buffer (0.1 M)	pH of extract	Residual relative activities (%)	
		NAD^+	$NADP^+$
Sodium acetate-acetic acid	5.5	8.7	56.3
Na_2HPO_4-NaH_2PO_4	6.7	20.7	100.0
Tris hydrochloride	6.9	14.6	100.0
Tris hydrochloride	8.8	0.0	72.3

The pig mitochondrial malate dehydrogenase is more sensitive to thermal denaturation than is the cytoplasmic enzyme (15). The NAD^+-dependent activity produced by *S. aureofaciens*, growing in the chemically defined medium described here, showed a very low residual activity after 30 min at 60°C, but the $NADP^+$-dependent activity was completely stable in phosphate buffer at pH 7.0 and in Tris hydrochloride buffer at pH 7.2. The $NADP^+$-linked enzyme was more stable than was the pig cytoplasmic malate dehydrogenase, which has a half-life of 20 min at 55°C and is NAD^+ dependent (11).

The best medium described in this paper has the advantage of being chemically defined and also of producing a good yield of microbial malate dehydrogenases. A high enzyme level was confirmed by the UV assay (specific activity of 1.18). The colorimetric assay was used because of the very low enzyme activity detected in the preliminary experiments.

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