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Dihydroxyacetone kinase from a methylotrophic yeast, *Hansenula polymorpha* CBS 4732

Purification, characterization and physiological role

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Abstract. Dihydroxyacetone (DHA) kinase was purified to electrophoretic homogeneity from methanol-grown *Hansenula polymorpha* CBS 4732. The enzyme was a dimer with a molecular weight of 150,000, and had an isoelectric point of 4.9. The enzyme was active toward DHA, and D- and L-glyceraldehydes as phosphorylation acceptors, and only ATP served as a donor. ADP inhibited the enzyme at a physiological concentration. Magnesium ion was essential for the activity and stability. Some other divalent cations can substitute in part the magnesium ion. The DHA kinases found in cells grown on methanol and glycerol were immunologically identical, but were different from those of other methylotrophic yeasts as shown by immunotitration. A mutant (204D) derived from the yeast, which could not grow on methanol or DHA but could so on glycerol, was deficient in DHA kinase. Glycerol kinase activity was found in glycerol-grown 204D cells as well as the parent strain.

Key words: *Hansenula polymorpha* – Dihydroxyacetone kinase – Glycerol kinase – Methanol – Glycerol

The assimilation of methanol by yeasts proceeds through a unique pathway, the xylulose monophosphate cycle for formaldehyde fixation (van Dijken et al. 1978). This pathway begins with a special transketolase reaction (dihydroxyacetone synthase) (Kato et al. 1982) between formaldehyde and xylulose 5-phosphate, which leads to the formation of dihydroxyacetone (DHA) and glyceraldehyde-3-phosphate. This reaction is followed by that of DHA kinase (Hofmann and Babel 1982; Bystrykh and Trotsenko 1983). Since DHA kinase is known to be a key enzyme in glycerol and DHA dissimilation in some microorganisms (Lin 1976), much literature has dealt with the relationship between methanol and glycerol metabolism in methylotrophic yeasts (O'Conner and Quayle 1979; Babel and Hofmann 1982; Kato et al. 1986a; Tani and Yamada 1987a, b; Koning et al. 1987a, b). Recently, Koning et al. (1987b) reported on the phosphorylation as the initial step of the glycerol dissimilation in *H. polymorpha*.

In this work, DHA kinase was homogeneously purified from *H. polymorpha* CBS 4732 and characterized. Furthermore, the physiological role of the enzyme was discussed on the basis of the enzyme profiles for methanol- and glycerol-grown CBS 4732, and its mutant.

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Abbreviation: DHA, dihydroxyacetone

Materials and methods

Organisms and cultivation

Methanol-utilizing yeasts, which originated from the Centraalbureau voor Schimmelcultures, Baarn, Netherlands (CBS), were supplied by the Institute for Fermentation, Osaka, Japan (IFO). *Candida boidinii* No. 2201 (AKU 4705) (Kato et al. 1982) and *Hansenula polymorpha* DL1 (Levine and Cooney 1973) were also used. A mutant of *H. polymorpha* CBS 4732 (strain 204D), which is unable to grow on methanol and DHA, was isolated by the same procedures as described in the previous paper (Kato et al. 1986a). Cultivation of the yeasts was carried out at 30°C in a chemically defined medium (Kato et al. 1986a).

Preparation of a cell-free extract

Cell-free extracts were prepared by sonication, which were then dialyzed against 10^4-volumes of buffer A (Kato et al. 1982).

Enzyme assays

DHA kinase activity was assayed, with a standard reaction mixture containing 50 mM potassium phosphate (pH 7.0), 5 mM $MgCl_2$, 1 mM ATP, 1 mM DHA, 0.15 mM NADH, 3 units of glycerol-3-phosphate dehydrogenase (Boehringer Mannheim, FRG) per ml and enzyme, in a total volume of 1 ml, by following the decrease in absorbance at 340 nm at 30°C. For the control experiment, no ATP was added. Glycerol kinase activity was measured according to Koning et al. (1987b). DHA synthase (EC 2.2.1.3) was assayed by the published method (Kato et al. 1982). One unit of an enzyme was defined as the amount of the enzyme catalyzing the conversion of 1 μmol substrate per minute.

Analyses

Protein was measured by the Bio-Rad Protein Assay with bovine serum albumin as a standard. The molecular weight of DHA kinase was determined by high-performance liquid chromatography on G-3000SW (Toyo Soda, Tokyo) (Kato et al. 1986b). Acrylamide disc gel electrophoresis was carried

out in 5% polyacrylamide gels in Tris-glycine buffer (pH 8.3) (Kato et al. 1984). Sodium dodecyl sulfate (SDS) gel electrophoresis was performed in 12.5% polyacrylamide disc gels according to Laemmli (1970).

Purification of DHA kinase from methanol-grown cells of H. polymorpha CBS 4732

All manipulations were carried out at 0–5°C. Buffer B, consisting of potassium phosphate (pH 7.0), 5 mM $MgCl_2$, 1 mM EDTA and 0.024% phenylmethyl sulfonate, was used through the purification procedures. The concentration of potassium phosphate is noted in each case.

Step 1. Cell-free extract. The cells (100 g wet weight) was suspended in 500 ml of 10 mM buffer B and then disrupted for 4 h with an ultrasonic oscillator (19 kHz). Cell debris was removed by centrifugation at 16,000 × g for 30 min. By repetition of the ultrasonication, a total volume of 5.3 l of cell-free extract containing 27 g protein was obtained from 1.2 kg (wet weight) of cells.

Step 2. DEAE-Sephacel. The cell-free extract, adjusted to pH 7.0 with 1 M potassium phosphate (dibasic), was directly applied to a DEAE-Sephacel column (5 × 51 cm), previously buffered with 0.1 M buffer B and then washed with 5 column volumes of the starting buffer. The enzyme was eluted with a linear gradient of increasing NaCl concentration, between 0 to 0.3 M (total volume, 6 l). The active fractions (1,400 ml) were concentrated to 200 ml by ultrafiltration with an Amicon Hollow Fiber DH/H1 and then dialyzed twice against 5 l of 0.01 M buffer B.

Step 3. Phenyl-Sepharose CL-4B. The dialyzed solution, to which had been added solid NaCl to a concentration of 2.5 M, was applied to a Phenyl-Sepharose column (3 × 45 cm) equilibrated with 0.01 M buffer B containing 2.5 M NaCl. Elution was carried out with a gradient of decreasing NaCl concentration and increasing ethylene glycol concentration; the final concentrations were 0 and 50%, respectively, and the total volume was 2.4 l. The pooled fractions (564 ml) containing the activity were dialyzed three times against 5 l of 0.1 M buffer B.

Step 4. 2nd DEAE-Sephacel. The dialyzed solution was re-chromatographed on a DEAE-Sephacel column (1.6 × 30 cm) under the same elution conditions as in step 2. The active fractions (105 ml) were pooled and concentrated to 20 ml with a Diaflo membrane YM-10 (Amicon), and then dialyzed against 1 l of 0.01 M buffer B.

Step 5. Butyl-Toyopearl 650 M. To the dialyzed solution was added NaCl to a concentration of 3 M, and then the mixture was applied to a Butyl-Toyopearl 650 M column (1.6 × 30 cm) equilibrated with 0.01 M buffer B containing 3 M NaCl. The enzyme was eluted with a gradient of decreasing NaCl concentration, from 3 M to 0 (total volume, 440 ml). The active fractions (86 ml) were pooled and concentrated to 10 ml, and then dialyzed 5 times against 1 l of 0.01 M buffer B.

Step 6. Isoelectric focusing. Isoelectric focusing (pH 3 to 10) was performed on an LKB 110 ml column as described previously (Kato et al. 1984). The enzyme solution (53 mg protein) obtained in the preceding step was applied to the column. The active fractions were pooled and dialyzed 5 times against 1 l of 0.01 M buffer B, and then concentrated to 7 ml. The enzyme preparation showed the maximum specific activity obtained throughout the purification procedures, but gave one main protein band accompanied by two faint bands on polyacrylamide gel electrophoresis.

Table 1. Purification of DHA kinase from *H. polymorpha* CBS 4732

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification	Yield (%)
Cell-free extract	6,500	27,000	0.240	1	100
1st DEAE-Sephacel	7,030	1,040	6.76	28	108
Phenyl-Sepharose	3,770	205	18.4	77	58
2nd DEAE-Sephacel	4,190	137	30.6	128	64
Butyl-Toyopearl	2,620	72	36.4	152	40
Isoelectric focusing	1,964	53	37.0	154	30
Gel electrophoresis	561	17	33.0	136	8.6

Step 7. Preparative polyacrylamide gel electophoresis. A 0.5 ml aliquot of the enzyme solution (4 mg protein) was applied to a slab gel (16 × 14 cm, 2 mm thick; 5% polyacrylamide gel in Tris-glycine buffer, pH 8.3). Electrophoresis and enzyme extraction were carried out as described previously (Kato et al. 1984). The main protein band was extracted from the gel, and then the solution dialyzed against 0.1 M buffer B. The purified enzyme solution was concentrated and stored at 0°C.

Immunological experiments

Antisera were raised in a New Zealand white rabbit (Livingston 1974); 8 mg of the purified DHA kinase obtained in purification step 7 was used as the immunizing antigen. Immunodiffusion experiments were carried out according to the Ouchterlony double diffusion technique with some modifications (Garvey et al. 1977). Immunotitration experiments were carried out as follows: 0.2 units of DHA kinases from several yeasts, which were partially purified by the DEAE-Sephacel column chromatography described above, were incubated with various concentrations of the antiserum against DHA kinase, in a total volume of 0.3 ml, at 30°C for 10 min and then at 5°C for 16 h. After brief centrifugation, the residual DHA kinase activity in each supernatant was measured under the standard conditions.

Chemicals

DEAE-Sephacel and Phenyl-Sepharose CL-4B were products of Pharmacia Fine Chemicals (Uppsala, Sweden). Butyl-Toyopearl 650 M was purchased from Toyo Soda (Tokyo, Japan). All other chemicals were commercially available and of analytical grade.

Results

Purification of DHA kinase from H. polymorpha CBS 4732

DHA kinase was purified through the purification procedures described under Materials and Methods (Table 1).

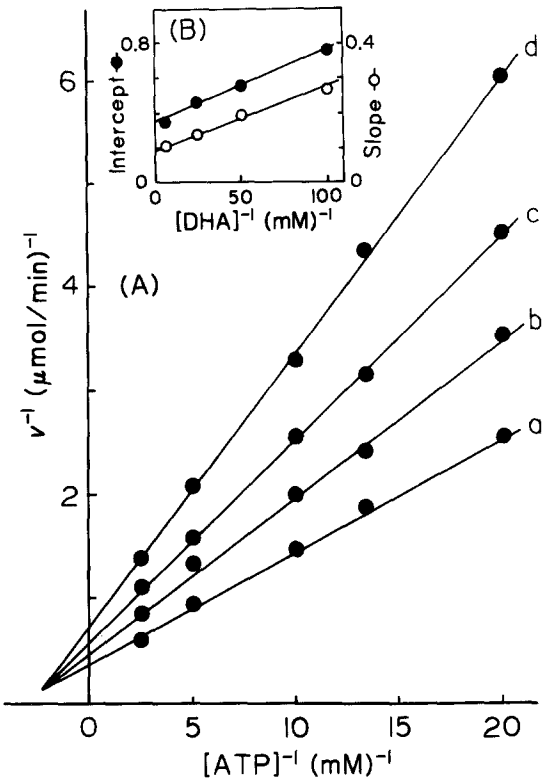

Fig. 1 A, B. Double reciprocal plots of the DHA kinase reaction. (A) Initial velocity versus ATP with various constant levels of DHA. (B) Replots of the intercepts and slopes versus the reciprocal concentration of DHA. DHA concentrations (mM): *a* 0.16; *b* 0.04; *c* 0.02; *d* 0.01. Each reaction mixture contained 0.04 mg of the purified enzyme

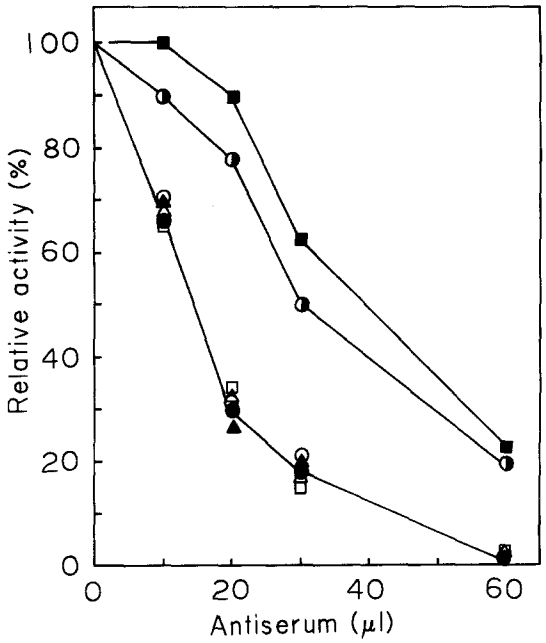

Fig. 2. Immunotitration of DHA kinases from various yeasts with antiserum against the DHA kinase of *H. polymorpha* CBS 4732. The conditions were given under Materials and methods. Purified DHA kinase from CBS 4732 (●), and cell free extracts of methanol-grown CBS 4732 (○) and glycerol-grown CBS 4732 (▲), and partially purified DHA kinases from methanol-grown CBS 1976 (△) and DL1 (□) and *C. boidinii* No. 2201 (■) and *Pichia pinus* CBS 744 (◑) were used

The purified enzyme gave one single band on polyacrylamide gel electrophoresis and on an SDS gel. On the isoelectric focusing, only one sharp protein peak, exhibiting constant specific activity, was seen, with a maximum at pH 4.9 (isoelectric point). The purified enzyme in buffer B could be stored at 0°C for at least 6 months without any loss of activity.

Molecular weight

The molecular weights of the native and SDS-treated enzymes were 150,000 and 72,000, respectively. The native enzyme is a molecule composed of two subunits with identical molecular weight.

Effects of pH and temperature on the enzyme activity

The enzyme exhibited maximum activity at pH 6.5 (potassium phosphate), 90% of the maximum activity at pH 6.0 and 7.0, and 67% at pH 8.0. Almost full activity was retained on standing for 1 h at 30°C in buffer B within the pH range of 6.0 to 8.0, and the activity was completely lost within 1 h at pHs below 4.0 (sodium acetate) and above 10.0 (sodium bicarbonate). The maximum activity was seen at 40°C in the standard reaction system. Half the activity was lost on incubation at 50° for 20 min.

Effects of metal ions on the enzyme activity

The enzyme required the magnesium ion for its activity, which was maximum at 5 mM $MgCl_2$. In the presence of a small amount of $MgCl_2$ (0.25 mM), the full activity was obtained on the addition of anyone of 5 mM $MnCl_2$, $CoSO_4$, $CaCl_2$ or $ZnSO_4$. When the enzyme was dialyzed against buffer B without $MgCl_2$, the activity was completely lost, and not restored on the addition of $MgCl_2$ (5 mM), indicating that the enzyme requires the magnesium ion for its stability as well as its activity.

Table 2. Enzyme activities of *H. polymorpha* CBS 4732 and mutant 204D grown on methanol and glycerol. Cell-free extracts were prepared from cells grown to the mid exponential phase

Organism	Growth substrate	Specific activity (units/mg)			
		Glycerol kinase	DHA kinase	DHA synthase	Alcohol oxidase
CBS 4732	Methanol	0.015	0.24	0.17	1.32
CBS 4732	Glycerol	0.064	0.074	ND[a]	0.26
204D	Methanol[b]	ND	ND	0.20	1.28
204D	Glycerol	0.048	ND	ND	0.18

[a] ND: not detected
[b] Cells were grown on ethanol and then incubated with 1% (v/v) methanol for 12 h for enzyme induction (Kato et al. 1986a)

Kinetics

Initial velocity studies on the purified DHA kinase were performed with respect to ATP, with a fixed concentration of DHA. As shown in Fig. 1, linear intersecting patterns were obtained, which also gave linear replots, indicating that the enzyme reaction proceeds via a sequential mechanism.

Table 3. Comparison of the properties of DHA kinases from various microorganisms

Organism	Specific activity (units/mg)	Molecular weight		Phosphorylation		pH optimum	References
		native	subunit	acceptor	donor		
		(kilodalton)					
Class 1							
Dunaliella salina	2.65	78–100	–	DHA	ATP	7.5	Lerner et al. (1980)
Klebsiella pneumonia	10	93	53	DHA	ATP	7.5–8.0	Johnson et al. (1984)
Schizosaccharomyces pombe	0.41	160	45	DHA	ATP	6.5	Marshal et al. (1986)
Class 2							
Candida methylica	37.6	–	–	DHA, DL-GA[a]	ATP, ITP UTP	7.5	Hofmann and Babel (1982)
Candida boidinii	5–10	139	71	DHA, DL-GA	ATP, ITP CTP, UTP GTP	7.2–8.2	Bystrykh and Trotsenko (1983)
Gluconobacter suboxydans	4.55	260	–	DHA, DL-GA	ATP, UTP	6.5	Tachiki et al. (1987)
Hansenula polymorpha	37	150	72	DHA, D-, L-Ga	ATP	6.5	this work

[a] GA: glyceraldehyde
–: not mentioned

The Michaelis constants were estimated from the replots to be 0.011 mM for DHA and 0.30 mM for ATP.

ADP was a competitive inhibitor with respect to ATP. The inhibition constant (K_i) was determined to be 0.21 mM for Dixon plots (Dixon and Webb 1979). No other nucleoside mono- or di-phosphates among AMP, GMP, UMP, CDP, GDP and UDP inhibited the enzyme activity at concentrations up to 4 mM.

Substrate specificity

The purified enzyme was also active toward D- and L-glyceraldehydes (1 mM each), with relative activities (as to toward DHA, 1 mM) of 13 and 45%, respectively. The apparent K_ms for D- and L-glyceraldehydes were 0.071 and 0.74 mM, respectively. As a phosphate donor for the DHA kinase reaction, only ATP was active, and no activity was seen when other nucleoside triphosphates, such as CTP, GTP, ITP and UTP, were used at concentrations of 0.2 to 4 mM in place of ATP.

Immunological experiments

In double immunodiffusion experiments, a single precipitin line was formed between the antiserum against DHA kinase and each of the cell-free extracts of *H. polymorpha* CBS 4731 grown on methanol or glycerol, and each extract gave a completely fused precipitin line when the purified enzyme was placed in neighboring wells. In addition, the immunotitration curve (Fig. 2) for each cell-free extract showed fair agreement with that for the purified enzyme. From these findings, it is concluded that the DHA kinases found in methanol- and glycerol-grown *H. polymorpha* CBS 4732 are immunologically identical.

DHA kinases from other methylotrophs, *Candida boidinii* No. 2201 and *Pichia pinus* CBS 744, were different from those of *H. polymorpha* strains (CBS 1976, CBS 4732 and DL1) as to immunotitration (Fig. 2). In the double immunodiffusion experiment, each of the DHA kinase preparations from the former two yeasts gave a precipitin line with a clear spur when the purified enzyme was placed in neighboring wells. The enzyme preparations from each pair of *H. polymorpha* strains gave a completely fused precipitin line. These results indicate that the DHA kinases from *C. boidinii* and *P. pinus* are distinguishable from the *H. polymorpha* enzymes.

Role of DHA kinase in the metabolism of methanol and glycerol

A mutant 204D derived from *H. polymorpha* CBS 4732 could not grow on methanol or DHA, but on glycerol. As shown in Table 2, no DHA kinase activity was detectable in the cells of 204 D grown on glycerol and incubated with methanol. Glycerol kinase activity was found in the glycerol-grown mutant and wild strains, indicating that the enzyme participate essentially in the glycerol dissimilation. In the glycerol-grown wild strain, a significant activity of DHA kinase was also detected.

Discussion

Some properties of microbial DHA kinases so far reported are listed in Table 3. These enzymes can be divided into two classes on the basis of their phosphorylation acceptor specificities: the first class is highly specific for DHA, and the second is active toward D-glyceraldehyde as well as DHA. The enzymes in the latter are not clearly distinguishable from triokinase (EC 2.7.1.28), which is another type of enzyme that catalyzes DHA phosphorylation. Although the DHA kinase from *H. polymorpha* was also able to phosphorylate both substrates, the affinity and activity toward DHA were much higher than those toward D-glyceraldehyde, furthermore, it showed no stereospecificity for glyceraldehyde. Thus, this enzyme should be classified as a DHA kinase (glycerone kinase, EC 2.7.1.29). Compared

with the enzymes from other methylotrophic yeasts, the molecular weight and subunit structure of the *Hansenula* enzyme are similar to those of the *C. boidinii* one, but the optimum pH and the phosphorylation donor specificity were different from those of the enzymes of *C. methylica* and *C. boidinii*. Furthermore, the *Hansenula* enzyme is immunologically distinguishable from the *Candida* enzyme.

The concentrations of ATP and ADP in yeasts are known to range from 0.3 to 1.0 mM (Polakis and Bartley 1966). The K_m for ATP (0.30 mM) and the K_i for ADP (0.21 mM) of the DHA kinase are within the ranges for such physiological concentrations, suggesting that the enzyme activity is regulated by the ATP/ADP concentration ratio during growth on methanol.

All DHA kinase-negative mutants of *H. polymorpha* CBS 4732 so far reported (O'Conner and Quayle 1979; Kato et al. 1986a; Koning et al. 1987a), as well as 204D in this work, could not grown on methanol or DHA, but on glycerol. From the immunological experiments, the yeast is assumed to have only one species of DHA kinase. Thus, the enzyme is concluded to play an essential role in the methanol assimilation. On the other hand, the enzymatic profiles of 204D and the wild strain grown on glycerol are consistent with the results of Koning et al. (1987b) that glycerol dissimilation in *H. polymorpha* is initiated by glycerol kinase. Although DHA kinase activity in the glycerol-grown yeast was about 30% of that in the methanol-grown cells, this activity could not be disregarded as compared to the level of glycerol kinase activity. An auxiliary role of DHA kinase in the glycerol metabolism remains to be examined.

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References

Babel W, Hofmann KH (1982) The relation between the assimilation of methanol and glycerol in yeasts. Arch Microbiol 127:119–124

Bystrykh LV, Trotsenko YA (1983) Purification and properties of dihydroxyacetone kinase from methylotrophic yeast, *Candida boidinii*. Biokhimiya 48:1611–1616

Dijken JP van, Harder W, Beardsmore AJ, Quayle JR (1978) Dihydroxyacetone: an intermediate in the assimilation of methanol by yeast? FEMS Microbiol Lett 4:97–102

Dixon M, Webb EC (1979) Enzymes, 3rd ed. Longman Group Ltd, London

Garvey JS, Cremer NE, Sussforf DH (1977) Methods in Immunology, a laboratory text for instruction and research, 3rd ed. W. A. Benjamin Inc, Massachusetts

Hofmann KH, Babel W (1982) Dihydroxyacetone kinase of methanol-assimilating yeasts. II. Partial purification and some properties of dihydroxyacetone kinase from *Candida methylica*. Z Allg Mikrobiol 21:219–224

Johnson EA, Burke SK, Forage RG, Lin ECC (1984) Purification and properties of dihydroxyacetone kinase from *Klebsiella pneumonia*. J Bacteriol 160:55–60

Kato N, Higuchi T, Sakazawa C, Nishizawa T, Tani Y, Yamada H (1982) Purification and properties of a transketolase responsible for formaldehyde fixation in a methanol-utilizing yeast, *Candida boidinii* (*Kloeckera* sp.) No. 2201. Biochim Biophys Acta 715:143–150

Kato N, Kobayashi H, Shimao M, Sakazawa C (1984) Properties of formaldehyde dismutation catalyzing enzyme of *Pseudomonas putida* F61. Agric Biol Chem 48:2017–2023

Kato N, Kobayashi H, Shimao M, Sakazawa C (1986a) Dihydroxyacetone production from methanol by a dihydroxyacetone kinase deficient mutant of *Hansenula polymorpha*. Appl Microbiol Biotechnol 23:180–186

Kato N, Yamagami T, Shimao M, Sakazawa C (1986b) Formaldehyde dismutase, a novel NAD-binding oxidoreductase from *Pseudomonas putida* F61. Eur J Biochem 156:59–64

Koning W de, Gleeson MAG, Harder W, Dijkhuizen L (1987a) Regulation of methanol metabolism in the yeast *Hansenula polymorpha*. Arch Microbiol 147:375–382

Koning W de, Harder W, Dijkhuizen L (1987b) Glycerol metabolism in the methylotrophic yeast *Hansenula polymorpha*: phosphorylation as the initial step. Arch Microbiol 148:314–320

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 227:680–685

Lerner HR, Sussman I, Avron M (1980) Characterization and partial purification of dihydroxyacetone kinase in *Dunaliella salina*. Biochim Biophys Acta 615:1–9

Levine DW, Cooney CL (1973) Isolation and characterization of a thermotolerant methanol-utilizing yeast. Appl Microbiol 26:982–990

Lin ECC (1976) Glycerol dissimilation and its regulation in bacteria. Annu Rev Microbiol 30:535–578

Livingston DM (1974) Immunoaffinity chromatography of proteins. In: Jakoby WB, Wilchek M (eds) Methods in enzymology, vol 34. Academic Press, New York San Francisco London, pp 723–731

Marshal JH, May JW, Sloan J, Vasiliadis GE (1986) Purification and properties of dihydroxyacetone kinase from *Schizosaccharomyces pombe*. J Gen Microbiol 132:2611–2614

O'Conner ML, Quayle JR (1979) Mutants of *Hansenula polymorpha* and *Candida boidinii* and in their ability to grow on methanol. J Gen Microbiol 113:203–208

Polakis ES, Bartley W (1966) Changes in the intracellular concentrations of adenosine phosphates and nicotinamide nucleotides during the aerobic growth cycle of yeast on different carbon sources. Biochem J 99:521–533

Tachiki T, Chigiri E, Tochikura T (1987) Purification and properties of dihydroxyacetone kinase from *Gluconobacter suboxydans*. J Ferment Technol 65:107–110

Tani Y, Yamada K (1987a) Diversity in glycerol metabolism of methylotrophic yeasts. FEMS Microbiol Lett 40:151–153

Tani Y, Yamada K (1987b) Glycerol metabolism of methylotrophic yeasts. Agric Biol Chem 51:1927–1933

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