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Biological Approaches Using Lichen-derived Cultures: Growth and Primary Metabolism‡

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Abstract

Activities and properties of malate dehydrogenase (MDH, EC 1.1.1.37), glutamate dehydrogenase (GDH, EC 1.4.1.4), and glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), enzymes involved in primary metabolism of lichen-derived cultures of *Cetraria* and *Umbilicaria* were determined. Effects of pH, temperature, carbon and nitrogen sources on these enzymes and on growth of lichen-derived cultures were also examined. The mycobiont of *Cetraria islandica*, a lichen occurring in nature on weakly acidic humus in the subalpine zone, grew well in 20°C cultures at pH 4.0 and, like *Usnea longissima*, grew better on mannitol and L-glutamine than on glucose or glycine. The growth of lichen-derived cultures could be correlated to specific conditions of their natural environments. The activities of GDH, MDH, and G6PDH did not differ significantly between the cultured mycobiont and photobiont of *C. islandica*,

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and mycobionts of *Umbilicaria caroliniana* and *U. pensylvanica*. Properties of enzymes from *C. islandica* did not reflect the habitat and locality of the lichen and were almost the same as those from non-lichens. Sugars and sugar alcohols gave no significant effects on the activity of the enzymes in the cultured *C. islandica* mycobiont. Among the amino acids tested, only L-glutamine inhibited GDH.

Keywords: lichen-derived culture, *Umbilicaria*, *Cetraria*, *Cladonia*, culture pH, carbon source, nitrogen source, culture temperature, enzyme activity, glutamate dehydrogenase, malate dehydrogenase, glucose-6-phosphate dehydrogenase

1. Introduction

Lichens are symbiotic associations of fungal (mycobiont) and algal (photobiont) partners. They grow slowly and can adapt to extreme environments. We considered whether these interesting characteristics of lichens were reflected in the properties and dynamics of their enzymes involved in primary metabolism.

Physiological studies of lichens, growth and adaptation, were summarized by Hale (1973), Kappen (1973), and Ahmadjian (1993). Carbon (Galun, 1988) and nitrogen metabolism (Rai, 1988), and the enzymology of lichens (Vicente and Legaz, 1988) were reviewed recently. However, enzymes involved in growth and adaptation of lichens have not been studied in detail.

We have successfully cultured lichen tissues and to date have accumulated approximately 700 lichen-derived cultures, as well as mycobiont and photobiont cultures derived from thallus fragments (Yamamoto and Yoshimura, 1992). To use these cultures for biological and chemical experiments is advantageous, because of their availability for successive experiments. In addition, the separation of symbionts may provide some indication of the contribution each symbiont makes to primary metabolism. The factors influencing growth of lichen-derived cultures have not been well investigated: only two earlier reports (Ahmadjian, 1961 and 1964) are available on growth factors of mycobiont cultures, while there is no previous report on the activities and properties of enzymes of primary metabolic pathways from lichen-derived cultures. We have previously investigated the growth of tissue cultures (Yamamoto et al., 1987 and Yoshimura et al., 1987) and in this paper, our objective was to further study factors affecting growth and to determine activities and properties of three enzymes involved in the primary metabolism, i.e., malate dehydrogenase (MDH, EC 1.1.1.37), glutamate dehydrogenase (GDH, EC 1.4.1.4), and glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) obtained from lichen-derived cultures.

2. Materials and Methods

Materials

We used the following cultures derived from thallus fragments by the Yamamoto method (1985): mycobiont and photobiont cultures of *Cetraria islandica* (L.) Ach. var. *orientalis* Asah. from tissue culture (strain no. CEIS0346-01C) from thallus (specimen no. E803-02) collected at Hokkaido Pref., mycobiont culture of *Umbilicaria caroliniana* Tuck. from tissue culture (Yoshimura et al., 1989), and mycobiont culture of *U. pensylvanica* Hoffm. from tissue culture (Yoshimura et al., 1989). Natural thalli for the enzyme investigations were collected at Nagano Pref. in 1992 and stored at $-80°$ C for 6 months. The effect of temperature and pH on growth in cultures and enzymes was studied in *C. islandica* and other aspects of the investigations involved all three species.

Subculture methods

Lichen-derived cultures were cut into segments and each segment was transferred to an agar-plate of Lilly-Barnett (LB) (1951) or malt-yeast extract (MY) (Ahmadjian, 1961) in Petri dishes (9 cm in diam.). These were cultured for three to four weeks under various conditions. Growth ratios (final fresh weight/initial fresh weight) were calculated. LB medium consisted of 1% (w/v) glucose, 0.2% (w/v) L-asparagine, 0.01% (w/v) K_2HPO_4, 0.005% (w/v) $MgSO_4 \cdot 7H_2O$, 0.2 ppm $Fe(NO_3)_3$), 0.2 ppm $ZnSO_4$, 100 ppb thiamine hydrochloride, 5 ppb biotin, and 2% (w/v) agar. Subculturing was also done onto LB medium modified as follows: a sugar or sugar alcohol (sucrose, mannitol, ribitol, glucose, or fructose) at 4% (w/v) instead of 1% (w/v) glucose; amino acid (D- or L-asparagine, L-glutamine, L-alanine, or glycine) at 0.2% (w/v) instead of 0.2% (w/v) L-asparagine. The pH of liquid LB medium was adjusted by NaOH or HCl. Liquid media with differing pHs were filtered through a membrane filter with a pore size of 0.22 μm without autoclaving. In the case of solid cultures autoclaved agar was added to liquid media, dissolved, and inoculated. Cultures on these media were maintained at 10, 15, 20, or 25° C.

Preparation of the enzyme extracts

About 1 g of fresh weight (FW) of each lichen-derived culture and 1 g of quartz sand were ground with a mortar and a pestle in 4 ml of 100 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride and 0.025% 2-mercaptoethanol. The homogenate was sonicated for 5 min and

then centrifuged at 8000 g for 10 min. The supernatant was used as an enzyme solution. Enzyme extractions from natural thalli were done without sonication. All steps in the enzyme preparation were carried out at 4° C.

Assays of the enzyme activities

The activity of malate dehydrogenase (MDH) was measured in 1 ml of a reaction mixture containing 40 mM potassium phosphate buffer (pH 7.5), 1 mM oxaloacetic acid, 0.16 mM NADH and 100 μl of each enzyme solution. The reaction mixture without oxaloacetic acid was pre-incubated at 30° C for 2 min, and the reaction was started by adding oxaloacetic acid. The activity of MDH was determined by measuring the decrease of absorbance at 340 nm after 10 sec. Experiments to determine the effects of pH or temperature on MDH activity were carried out at pH 5.5 to 8.5 and at 10° to 40° C. The relative activity (%) [(specific activity/specific activity under optimum conditions)$\times$100] was calculated.

The activity of glutamate dehydrogenase (GDH) was measured in 1 ml of reaction mixture containing 200 mM Tris-HCl buffer (pH 8.0), 260 mM NH_4Cl, 10 mM 2-oxoglutaric acid, 0.16 mM NADPH, 1 mM $CaCl_2$ and 100 μl of each enzyme solution. After preincubation at 30° C for 2 min the reaction was started by adding 2-oxoglutaric acid and after 10 sec the decrease of absorbance at 340 nm was measured. Experiments to determine the effects of pH or temperature on GDH activity were carried out at pH 7.0 to 9.5 and at 15° to 70° C.

The activity of glucose-6-phosphate dehydrogenase (G6PDH) was measured in 1 ml of reaction mixture containing 120 mM Tris-HCl buffer (pH 7.5), 3 mM glucose-6-phosphate, 0.3 mM $NADP^+$, 20 mM $MgCl_2$ and 100 μl of each enzyme solution. After preincubation at 25° C for 2 min the reaction was started by adding glucose-6-phosphate and after 10 sec the increase of absorbance at 340 nm was measured. Experiments to determine the effects of pH or temperature on G6PDH activity were carried out at pH 6.0 to 9.5 and at 15° to 55° C.

Measurement of protein content

The amount of protein in each enzyme solution was determined by the method of Bradford (1976) using a Bio-Rad protein assay kit.

Measurement of Km values

The activities of MDH, GDH, and G6PDH were measured using the following substrates and conditions: MDH, pH 7.5, 30° C, oxaloacetic acid

(0.02–1 mM) and NADH (0.02–0.32 mM); GDH, pH 8.0, 30° C, 2-oxoglutaric acid (2.5–50 mM) and NADPH (0.04–0.32 mM); G6PDH, pH 7.5, 25° C, glucose-6-phosphate (0.1–10 mM) and $NADP^+$ (0.02–1 mM). The Km values for above substrates of MDH, GDH and G6PDH in crude extracts were graphically calculated from Lineweaver-Burk plots.

3. Results and Discussion

Effects of culture conditions on growth

We suggested in our previous paper (Yamamoto et al., 1987) that the optimum growth temperatures of lichen tissue cultures reflected the conditions of their natural habitats. Similarly, we found that a cultured *Cetraria islandica* mycobiont isolated from a thallus collected at the subalpine zone grew well at low temperatures, i.e., 15° to 20° C (Fig. 1).

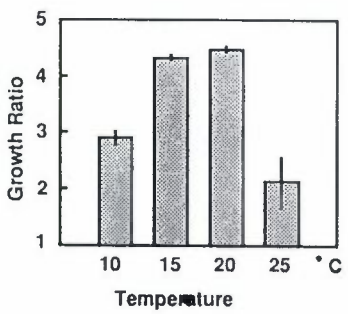

Figure 1. Effect of temperature on growth of cultured mycobiont of *Cetraria islandica*. The culture was maintained on 20 ml LB agar medium supplemented with 4% mannitol instead of glucose at 10°, 15°, 20°, or 25° C in the dark for 4 weeks. Means are SE of 2 replicates.

Natural *Cladonia vulcani* can grow on strong acidic soil near hot springs, and Yoshimura et al. (1987) reported that growth of cultured *C. vulcani* tissues was best at pH 4. Natural *Cetraria islandica* grows on weak acidic humus, and we found that growth of the *C. islandica* mycobiont was fastest at pH 4.0 (Fig. 2). This suggests that the cultured mycobionts, *C. vulcani* and *C. islandica*, reflected the acidic nature of their habitats.

Mannitol and glucose occupy significant positions in carbohydrate metabolism. Richardson and Smith (1968), Komiya and Shibata (1971), and Honegger et al. (1993) reported that some lichen mycobionts cultured in glucose-containing media accumulate mainly mannitol. In *Peltigera*, the carbon fixed by photosynthesis in photobionts is released as glucose to mycobionts, and there is converted to mannitol for storage (Smith and Douglas,

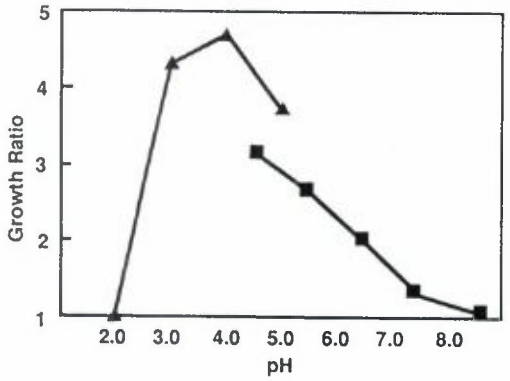

Figure 2. Effect of pH on growth of cultured mycobiont of *Cetraria islandica*. The solid culture (■) was maintained on 20 ml LB agar medium supplemented with 4% mannitol instead of glucose at various pHs at 15° C in the dark for 3 weeks. The liquid culture (▲) was maintained in 40 ml LB liquid medium supplemented with 4% mannitol instead of glucose at various pHs at 15° C in the dark for 4 weeks.

1987). Both mannitol (via mannitol-1-phosphate or fructose) and glucose (via glucose-6-phosphate) can be metabolized to fructose-6-phosphate (Vicente and Legaz, 1988). We proposed previously that lichen tissue cultures of species belonging to the same genus may differ with regard to their preference for either mannitol or glucose as a carbon source (Yamamoto et al., 1987). In this paper, we found that the *C. islandica* mycobiont was a mannitol-type that could utilize fructose better than glucose (Fig. 3).

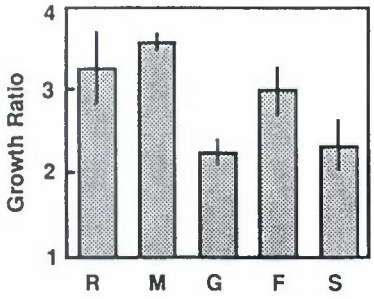

Figure 3. Effect of sugar and sugar alcohol on growth of cultured mycobiont of *Cetraria islandica*. The culture was maintained on 20 ml LB agar medium containing 4% ribitol (R), mannitol (M), glucose (G), fructose (F), or sucrose (S) instead of 1% glucose at 15° C in the dark for 3 weeks. Means are SE of 2 replicates.

No one has demonstrated which amino acids are essential for lichens or how they are metabolized. We proposed previously that lichen tissue cultures belonging to the same genus were grouped into three types based on their

relative performances in glycine and L-glutamine (Yamamoto et al., 1987). In this study, it was observed that the *C. islandica* mycobiont was an L-glutamine type and that cultures of its mycobiont thrived not only on L-glutamine but also on L-alanine (Fig. 4). Ahmadjian (1964) reported that L-alanine

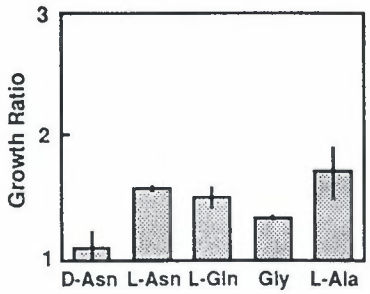

Figure 4. Effect of amino acid on growth of cultured mycobiont of *Cetraria islandica*. The culture was maintained on 20 ml LB agar medium with 0.2% L-alanine (L-Ala), L-asparagine (L-Asn), D-asparagine (D-Asn), L-glutamine (L-Gln), or glycine (Gly) instead of 0.2% L-asparagine at 15°C in the dark for 3 weeks. Means are SE of 2 replicates.

promoted growth of the two mycobiont cultures of *Cladonia cristatella* and *Acarospora fuscata*. Therefore, it is possible that these cultures belonged to the L-glutamine type.

Activities and properties of primary metabolic enzymes

Lichens grow very slowly as compared with other organisms. When the enzyme activity in cells is lower, turnover rate of metabolites may be low and consequently cell growth could also be slow. Low enzyme activity of the primary metabolisms in lichens could be the cause of slow growth.

Since lichens can grow in more extreme environments than many other organisms, two mechanisms of adaptation are possible: one concerns the regulation in the incorporation of substrates into cell walls and membranes, and the other concerns the adaptation of enzymes involved in primary metabolism.

The total MDH activity of the cultured *C. islandica* photobiont was 10 times higher than that in the natural thallus and cultured mycobiont, while relative activities of MDH in cultured bionts were 10 times higher than that of the natural thallus (Table 1). We found that the specific activity and coenzyme of GDH in natural thalli of *Peltigera aphthosa* (Rai et al., 1980 and 1981), *P. canina* (Rai et al., 1983), and *Pseudevernia furfuracea* (Jager and Weigel, 1978) and its properties in natural *Lobaria laetevirens* (Bernard and Goas,

Table 1. Malate dehydrogenase (MDH), glutamate dehydrogenase (GDH) and glucose-6-phosphate dehydrogenase (G6PDH) activities in lichen-derived cultures and other organisms

Organism	Ref.	MDH activity		GDH activity			G6PDH activity	
		Total*	Specific**	Coenzyme	Total*	Specific**	Total*	Specific**
Cetraria islandica								
cultured mycobiont	1	37.6	22.37	NADPH	5.72	3.40	2.22	1.32
cultured photobiont	1	332.0	20.44	NADPH	10.92	0.67	2.85	0.18
natural thallus	1	47.2	3.29	NADPH	4.44	0.31	0.14	0.01
Umbilicaria caroliniana								
cultured mycobiont	1	3.8	3.04	NADPH	2.90	2.32	0.40	0.32
Umbilicaria pensylvanica								
cultured mycobiont	1	7.0	5.71	NADPH	3.27	2.67	0.79	0.64
Lobaria laetevirens								
natural thallus	2	–	–	NADPH	–	0.37	–	–
Peltigera aphthosa								
natural thallus	3	–	–	NADPH	–	0.09	–	–
Peltigera canina								
natural thallus	4	–	–	NADPH	–	0.20	–	–
Bacillus subtilis	5	–	6.7	NADH	1.90	0.06	–	–
Bacherichia coli	6	–	–	NADPH	38.0	0.29	–	–
Aspergillus niger	7	–	–	–	–	–	2.90	0.30
Candida utilis	8	–	–	–	–	–	36.4	0.66
Chlorella sorokiniana	9	–	–	NADPH	47.0	1.30	–	–
Pea epicotyls	10	24.0	2.22	–	–	–	–	–
roots	11	–	–	NADH	0.32	0.06	–	–
Sweet potato	12	–	–	–	–	–	0.10	–
Beef heart	13	140.0	12.2	–	–	–	–	–
Rat liver	14	–	–	–	–	–	0.75	

*Units per g fresh weight of cells, **Units per mg of protein.

Ref. 1. Yamamoto et al., present paper, 2. Bernard and Goas, 1979, 3. Rai et al., 1981, 4. Rai et al, 1983, 5. Yoshida, 1965 and Kimura et al., 1977, 6. Veronese et al., 1975, 7. Jagannathan et al., 1956, 8. Engel et al., 1969, 9. Gronostajski et al., 1978, 10. Davies, 1969, 11. Pahlich and Joy, 1971, 12. Muto and Uritani, 1970, 13. Englard and Siegel, 1969, 14. Glock and McLean, 1953.

1979) are similar to those of the natural *C. islandica* thallus (Table 1). We also found no significant difference between the GDH total activities of cultured *Cetraria* and *Umbilicaria* mycobionts and those of natural thalli and cultured photobiont. However, the specific activities of mycobionts were 10 times those of photobionts and thalli (Table 1). The GDH activities in lichens were similar to those of other organisms, and NADPH-type GDH was commonly found not only in lichens, but also in other organisms (Table 1). We found that the G6PDH specific activity in the natural *C. islandica* thallus was much lower than in lichen-derived cultures or other organisms (Table 1). This result may indicate that the relative importance of alternative pathways of carbohydrate metabolism(s) differs in natural lichens.

Optimum pH and temperature of individual enzymes shown in Figs. 5 and 6 are not the same as optimal pH and temperature for growth of the cultured *C. islandica* mycobiont. Optimum and Km values of these lichen enzymes shown in Tables 2 and 3 were almost the same as those in other organisms,

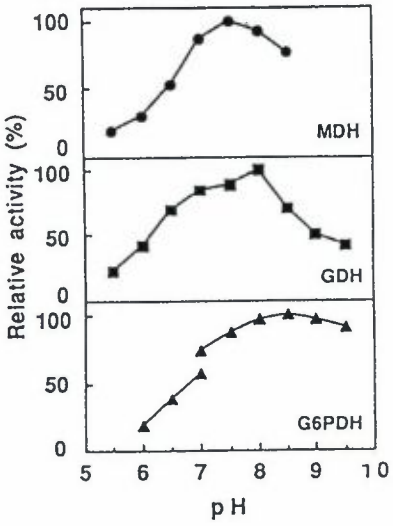

Figure 5. Effect of pH on the specific activities of malate dehydrogenase (MDH), glutamate dehydrogenase (GDH), and glucose-6-phosphate-6-dehydrogenase (G6PDH) from cultured mycobiont of *Cetraria islandica*.

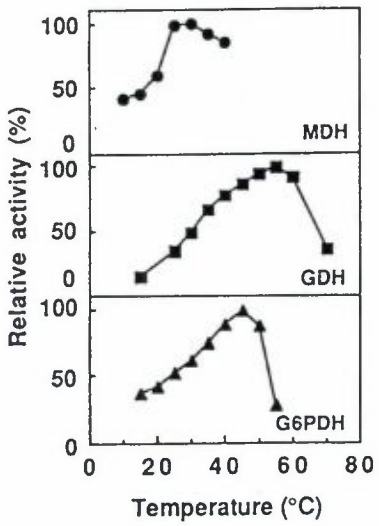

Figure 6. Effect of temperature on the specific activities of malate dehydrogenase (MDH), glutamate dehydrogenase (GDH), and glucose-6-phosphate-6-dehydrogenase (G6PDH) from cultured mycobiont of *Cetraria islandica*.

Table 2. Optimum pH and temperature of malate dehydrogenase (MDH), glutamate dehydrogenase (GDH) and glucose-6-phosphate dehydrogenase (G6PDH) in *Cetraria islandica* and other organisms

Organism	Ref.	MDH		GDH		G6PDH	
		pH	Temp.	pH	Temp.	pH	Temp.
Cetraria islandica							
cultured mycobiont	1	7.5	30	8.0	55	8.5	45
natural thallus	1	8.0	35	8.0	55	–	–
Lobaria laetevirens							
natural thallus	2	–	–	7.7	–	–	–
Bacillus subtilis	3	9.6	–	–	–	–	–
Thiobacills novellus	4	–	–	7.5	–	–	–
Escherichia coli	5	9.0	–	–	–	–	–
Candida utilis	6	–	–	–	55	9.1	–
Proteus inconstans	7	–	–	8.5	55	–	–
Yeast	8	–	–	–	–	8.5	–
Chlorella sorokiniana	9	–	–	7.2	–	–	–
Pea epicotyls	10	7.0	–	–	–	–	–
Beef heart	11	6.7	–	–	–	–	–
Rat liver	12	–	–	–	–	7.6	–

Ref. 1. Yamamoto et al., present paper, 2. Bernard and Goas, 1979, 3. Kimura et al., 1977 and Yoshida, 1965, 4. Lejohn and McCrea, 1968, 5. Murphey and Kotto, 1969 and Veronese et al., 1975, 6. Engel et al., 1969, 7. Shimizu et al., 1979, 8. Glaser and Brown, 1955, 9. Gronostajski et al., 1978, 10. Davies, 1969, 11. Englard and Siegel, 1969, 12. Glock and McLean, 1953.

Table 3. Km values of malate dehydrogenase (MDH), glutamate dehydrogenase (GDH) and glucose-6-phosphate dehydrogenase (G6PDH) in *Cetraria islandica* and other organisms

Organism	Ref.	MDH		GDH		G6PDH	
		OA* (μM)	NADH (μM)	2-OG** (mM)	NADPH (μM)	G6P (μM)	NADP (μM)
Cetraria islandica							
cultured mycobiont	1	148	82	11.6	68	440	60
natural thallus	1	110	190	11.8	110	–	–
Lobaria laetevirens							
natural thallus	2	–	–	2.2	28	–	–
Bacillus subtilis	3	61	27	–	–	–	–
Thiobacills novellus	4	–	–	7.4	77	–	–
Escherichia coli	5	50	–	–	–	–	–
Candida utilis	6	–	–	–	–	230	67
Proteus inconstans	7	–	–	2.3	3	–	–
Yeast	8	–	–	–	–	58	20
Chlorella sorokiniana	9	–	–	12	130	–	–
Pea epicotyls	10	90	120	–	–	–	–
Beef heart	11	34	52	–	–	–	–
Rat liver	12	–	–	–	–	13	13

*OA, oxaloacetic acid, **2-OG, 2-oxoglutaric acid

Ref. 1. Yamamoto et al., present paper, 2. Bernard and Goas, 1979, 3. Kimura et al., 1977 and Yoshida, 1965, 4. Lejohn and McCrea, 1968, 5. Murphey and Kitto, 1969 and Veronese et al., 1975, 6. Engel et al., 1969, 7. Shimizu et al., 1979, 8. Glaser and Brown, 1955, 9. Gronostajski et al., 1978, 10. Davies, 1969, 11. Englard and Siegel, 1969, 12. Glock and McLean, 1953.

Table 4. Effects of sugar and sugar alcohol on malate dehydrogenase (MDH), glutamate dehydrogenase (GDH) and glucose-6-phosphate dehydrogenase (G6PDH) activities in cultured *Cetraria islandica* mycobiont

		MDH activity		GDH activity		G6PDH activity	
Sugar	Growth***	Total*	Specific**	Total*	Specific**	Total*	Specific**
Ribitol	3.25	14.6	17.4	0.9	1.0	1.2	
Mannitol	3.60	15.5	20.1	1.1	1.4	1.1	1.4
Sucrose	2.35	23.0	26.3	1.0	1.2	1.6	1.9
Fructose	3.00	18.8	22.4	3.7	4.4	1.6	1.9
Glucose	2.25	17.2	22.4	2.8	3.6	1.7	2.2

* Units per g fresh weight of cells
** Units per mg of protein
*** Growth ratio=fresh weight after 3 weeks culture/initial fresh weight

Table 5. Effects of amino acid on malate dehydrogenase (MDH), glutamate dehydrogenase (GDH) and glucose-6-phosphate dehydrogenase (G6PDH) activities in cultured *Cetraria islandica* mycobiont

		MDH activity		GDH activity		G6PDH activity	
Amino acid	Growth***	Total*	Specific**	Total*	Specific**	Total*	Specific**
Glycine	1.36	11.8	19.9	2.1	3.5	1.1	1.8
L-Alanine	1.69	12.6	20.7	2.9	4.7	1.4	2.3
L-Glutamine	1.48	10.2	17.4	0.5	0.9	1.1	1.8
L-Asparagine	1.53	14.0	24.3	2.2	3.8	1.3	2.2
D-Asparagine	1.10	7.3	16.4	2.3	5.1	1.1	2.5

* Units per g fresh weight of cells
** Units per mg of protein
*** Growth ratio=fresh weight after 3 weeks culture/initial fresh weight

but this does not prove that MDH, GDH, and G6PDH are the same in all lichens or in lichens and other organisms.

Different sugars or sugar alcohols in the culture medium did not significantly affect the activities of MDH, GDH, and G6PDH in cultured *C. islandica* mycobiont (Table 4). Of amino acids tested so far, only L-glutamine inhibited GDH activity in the cultured mycobiont of *C. islandica* (Table 5).

The growth of lichen-derived cultures was definitely affected by their natural conditions, habitat (pH) and locality (temperature), whereas properties of MDH, GDH, and G6PDH were similar to those of other organisms, with no apparent property that might enable them to grow slowly and to adapt to more extreme environments. Lichens are unique organisms ecologically, but little is known of their primary metabolism. Lichens have metabolic adaptations that are not found in other organisms, and approaches using lichen-derived cultures may help to elucidate these.

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