Acetamide as potential C- and N-source of the extremely acid and heavy metal resistant filamentous fungus, *Bispora* sp.

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The extremely acid resistant filamentous fungus Bispora sp. (possessing two distinct growth optima at pH 1 and pH 7) is able to utilize metal complexed cyanides as N-source and to grow with acetamide as the only nitrogen and carbon source at both pH 1.0 and 7.0, whereas little growth is observed with formamide as N-source. The nitrogen content of the fungus grown with acetamide is similar to that of the control grown with NH_4^+ as N-source but higher than those cultures deprived of nitrogen. In the absence of appropriate substrates the fungus did not express amidases such, as urease, formamidase, acetamidase, whereas in the presence of formamide and acetamide, formamidase activity, but no acetamidase activity was observed. Since the fungus is able to grow with acetamide as the only C- and N-source, data imply the involvement of an enzyme in acetamide metabolism which is different from a normal acetamidase. The incorporation of ^{14}C-acetamide into the fungus and the subsequent utilization of the substrate, as analyzed by TLC, support the view that inducible enzymes related to C1-metabolism are of importance for the nutrition of Bispora sp. grown at pH 1.0 with acetamide as C- and N-source.

Abbreviations: AA, acetamide; FA, formamide; NHase, nitrile hydratase; FHL, formamide hydratase lyase; THF, tetrahydrofolate; TLC, thinlayer chromatography.

INTRODUCTION

Fe or Co containing nitrile-degrading enzymes [14] are widely dispersed in prokaryotes and lower orders of eukaryotes, but not in higher plants [21]. By means of hydro-lyases (nitrile-hydratases, NHases), some bacteria and fungi are able to detoxify cyanides (nitriles). E. g. cyanide hydratase (EC 4.2.1.66 = Formamide hydro-lyase, FHL) converts cyanide to formamide according to

$$(1)\quad HCN + H_2O \xleftrightarrow{FHL} HC\text{-}NH_2 \quad (O)$$

The resulting amides may be subsequently split by amidases into organic acids and ammonia. E.g.

formamide is into converted into formic acid and NH_3 according to

(2) $HC\text{-}NH_2 + H_2O \longleftrightarrow HCOOH\text{-}NH_3$

Subsequently a formate dehydrogenase (EC 1.2.1.2) may liberate CO_2 from formate

(3) $HCOOH + NAD^+ \xleftrightarrow{\text{Formate dehydrogenase}} CO_{2+}NADH + H^+$

In this case formamide can serve only as N-source but not as C-source. However, formate may be incorporated into N^{10}-formyl tetrahydrofolate (THF) [29, 30] according to

(4) $HCOOH + ATP + THF \xleftrightarrow{N^{10}\text{-formyl-THF-synthetase}} N10\text{-formly-THF} + ADP + Pi$

and subsequently used e.g. for methionine synthesis from homocysteine. In this case formamide can be used both as C- and N-source.

The two-step degradation of toxic cyanides to potentially useful substrates occurs in both bacteria [20] and fungi [4, 7, 8, 17, 31, 37]. In yeast, assimilation of amides can be used for systematic identification. Testing the hydrolysis of 23 different amides by 500 different yeasts and yeast-like strains shows that 10 of these amides are sufficient to differentiate seven genera and 19 species [22]. From the evolutionary view, the first step may present an adaptation to the chemical environment in the early history of the biosphere. However, a correlation between FHL activity and the phytopatho-genicity of some fungi to cyanogenic plants was also observed [10, 39]. The occurrence of amidases may reflect a secondary adaptation, following the evolution of NHases. It allows the utilization of the end products of NHase activities. Since nitrogen is often the limiting element in growth, the use of any amides present in the environment is of evolutionary advantage. Thus, it is not surprising that some microbes are able to live with cyanides as the only nitrogen source [3, 8, 16-18, 24] and additionally as the only C-source [7, 8, 18, 20, 23].

Considering the fact that many polluted soils, industrial wastes, and effluents contain considerable amount of various toxic cyanides, biodegradation of nitriles by microbes is of large interest from both the environmental and health points of view. Therefore the potential of microorganisms containing nitrile-hydratases and amidase for phytoremediation is studied with increasing intensity [2, 3, 6-8, 14, 23-25, 27, 32, 33, 35]. Recently it could be shown that the stress resistant fungal *Scytalidium,* which comprises thermophilic and acidophilic species [34] is able to grow on metal - complexed cyanides, typically found in former gasworks sites [2]. The genus *Scytalidium* is closely related to the recently isolated, extremely acid and heavy metal resistant filamentous fungus *Bispora* sp., which has been investigated also in this study. Stress physiology of the latter fungus was recently analyzed in our department [10-13]. Because of its strong acid and metal resistance, *Bispora* sp. may be a good candidate for phytoremediation of polluted soils, e.g. former gasworks sites and industrial wastes. Preliminary studies demonstrated that indeed *Bispora* sp. is able to grow with one metal-complexed cyanide as the only N-source. This prompted us to test whether this fungus can grow and utilize the amide products of NHase reactions by means of amidases. Results of these investigations are presented in this paper.

METHODS

Test organism. The acid tolerant filamentous fungus *Bispora* sp. (Collection of filamentous

fungi of CBS Netherlands, CBS number 335.97), isolated in our laboratory from non-sterile cultures of the unicellular acidophilic green alga *Dunaliella acidophila*, was cultured under axenic conditions either on agar or in suspension culture.

Agar culture. Agar plates were prepared using *D. acidophila* medium [1], complemented with 3 % agar (BACTO-agar, DIFCO) and 2 % glycerol (w/v) as carbon source and various N-sources. The standard N-source was ammonium. For other N-sources, see tables and figures. The pH was adjusted to 1.0 with sulfuric acid. In order to allow gelification of the agar at acid pH, double concentrated agar suspensions and double concentrated aqueous solutions of the medium (calibrated to pH 1.0) were sterilized separately and mixed together (1:1) shortly before gelification of the agar. For cultures at neutral pH the medium was calibrated to pH 7.0 (20 mM HEPES/NaOH buffer).

Suspension culture. The fungus was cultured axenically in 50 ml *D. acidophila* culture medium (pH 1.0) with NH_4^+ as N source and 2 % glycerol as carbon source [1]. The suspension mixture in 100 mL Erlenmeyer flasks were then placed in an incubator on a rotation shaker (20° C, dark, 130 rounds min-1, Multitron MT2, Infors HT). Under these growth conditions, fungal hyphae formed small black spheres with a diameter between 0.5 and 1 cm.

Determination of growth. To check the utilization of different N-sources the fungus was kept in suspension for at least 7 days in the absence of any external nitrogen source. Then fungal balls were transferred to agar plates with alternatively 20 mM NH_{4+}, $NaNO_3$, acetamide, formamide or 10 mM of metal complexed cyanides. For the determination of growth constants the time dependent enlargement of fungal colonies areas was measured using a computer controlled morphometric video system (DIAS, Delta Devices Ltd., Cambridge). Measured areas (accuracy O.5-1.5 % according to students t-test for 90% probability) were plotted semi-logarithmically as function of time. From the resulting lines apparent average growth constants (K_e) were calculated. Usually the increase of a colony area was followed over a period of 2 - 3 weeks.

Extraction of enzymes. For the *in vitro* determination of enzyme activities fungi pre-cultured at pH 7.0 were filtered, washed, and carefully ground in a mortar with liquid nitrogen. The ground samples were immersed in 2 ml of 0.1 mM HEPES/NaOH buffer (containing 1 mM DTT) to extract the enzymes and kept on ice or stored at -70°C until measurement.

Determination of amidase. Amidases in aqueous extracts of *Bispora* sp. (see above) were determined by monitoring the increase in electrical conductivity. Increase in electrical conductivity was detected when urease (EC 3.5.1.5) splits urea into NH_{4+} and dissolved inorganic carbon, formamidase (EC 3.5.1.9) splits formamide into formic acid and acetamidase (EC. 3.5.1.14) splits ace- tamide into acetic acid. The reaction was carried out at pH 7.8 (0.1 mM HEPES/NaOH buffer, 1 mM DTT) and at 20° C. The substrate concentrations that were added varied between 33 and 36 mM. The reaction volume of 20 ml contained fungal extracts corresponding to 0.74 - 1.5 mg protein. Rates of amidase activity were calculated as D µS mg^{-1} protein min^{-1}.

Incorporation of ^{14}C-labelled acetamide and formate into intact cells. Fungal balls pre-cultured with and without glycerol as C-source and with different N-sources (20 mM) (pH 1.0) were filtered, washed, and resuspended in 5 ml of culture medium without C-and N-sources. At zero time either 20µl of acetamide labelled with

^{14}C-iodoacetamide or 20 ml of formamide labelled with ^{14}C-formamide were added. The final concentrations of the labelled substrates were 0.1 mM and 0.085 mM, respectively. After 2 and 4 h fungal balls were filtered and washed three times with 20 ml of water. Then the samples were transferred into 5 ml of 80 % hot ethanol (70° C, 4h). Extraction was continued for 48 h at 0° C. After centrifugation 100, µl aliquots of the supernatant were taken for the determination of total radioactivity in the ethanol-soluble fraction by means of a scintillation counter. A zero time sample was prepared by adding radioactivity to the fungal ball with immediate removal of the medium, followed by the washing procedure described above.

The radioactivity of this sample, arising predominantly from external adsorption, was subtracted from all other samples in order to correct for radioactivity not taken up by the cells. The remaining supernatant was brought to complete dryness by evaporation under a stream of air and samples stored for TLC analysis at 0° C. The pellet fraction, consisting of cell wall material, lipids, and proteins, was washed two times with 5 ml of water to remove completely soluble radioactivity and dried for 48 h at 75° C, before it was ground to powder. Then samples were suspended in 1 ml of water and aliquots obtained were used for the measurement of radioactivity.

In vitro metabolism of ^{14}C-acetamide, ^{14}C-formate and ^{14}C-TF. Incubation of crude enzyme extracts of *Bispora* sp. with ^{14}C-iodo acetamide, ^{14}C-formate, and ^{14}C-TF was carried out in Eppendorf cups at 20° C in the presence and absence of a large variety of different metabolites and cofactors (ATP, glycine, glutamate, ribose-5-P, serine, folic acid). A final volume of 600 ml was obtained when these different reactants were mixed. Added to this mixture were 340 µl of HEPES/NaOH buffer (pH 7.8), 200 µl crude enzyme extract, and 10 µl of the labelled products, ^{14}C-iodoacetamide (0.1mM) or ^{14}C-formate (0.14 mM) or ^{14}C-methyl-THF (74 µM). The reaction was started by the addition of labelled compounds and terminated after 30 min by the addition of 1.5 ml hot 80 % ethanol. Samples were kept at 80°C for additional 30 min and then brought to complete dryness by evaporation and stored at -20°C until use. For TLC analysis residues were dissolved with 100 µl of 80 % ethanol and 50 µl aliquots were placed on TLC plates. Separation of the ethanol-soluble fraction was carried out by TLC, using cellulose as adsorbent (20 x 20 cm DC plates, Merck. Darmstadt, Germany) and the solvents used by Feige et al.[9]. For the analysis of total carbon, nitrogen and hydrogen fungal balls were washed in water, brought to complete dryness (24 h, 75°C), and then pulverized in a mortar. Aliquots of the dry powder, 5-20 mg, were analyzed in a CHN-O Rapid Analysator (Elementra-Heraeus, Hanau, Germany).

Data analysis. Growth experiments were repeated 4 times (duplicate samples for each treatment). Growth was monitored daily over a period of two weeks. Results from each set of data were summarized individually and apparent K_e growth constant were calculated. The K_e values from similar treatments (4 independent experiments) were averaged. Error bars as standard deviations (S.D.) were determined.

RESULTS

Growth and nitrogen status

The standard nitrogen source of *Bispora* sp. is NH_4^+. At pH 7.0 and with glycerol as C-source the growth constant K_e in the presence of this nitrogen source is close to 0.11 (Fig. 1).

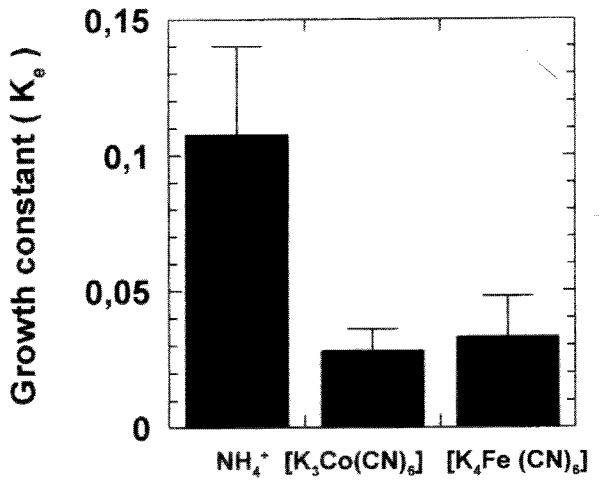


Figure 1. Growth of Bispora sp. (pH 7.0, glycerol as carbon source) with NH_4^+ and metal complexed cyanides as the only nitrogen source.

In the presence of nitrate instead of NH_4^+, growth at this pH was approximately similar to that of the control (Fig. 2), whereas at pH 1.0 growth with nitrate as N-source was slightly lower than that of the control. If NH_4^+ was replaced by the metallo cyanide complexes potassium hexacyanocobaltate or potassium hexacyanoferrate the growth constants dropped significantly, but still a growth of about 30 % of the control was observed (Fig. 1). This control growth encouraged us to investigate whether the putative products of NHase activitiy, namely short chain amides, can serve as growth substrates for *Bispora* sp. At both acid and neutral pH the fungus exhibited good growth with ammonium and acetamide as N-source (Fig. 2 and 3), whereas the growth was much lower with formamide. Growth with urea as the only nitrogen source generally was very low (Fig. 3), which can be explained by the fact that urease is a nickel containing enzyme. However, nickel is not a constituent of the growth media applied. Growth on acetamide was resumed only after a log period of several days (Fig. 3).

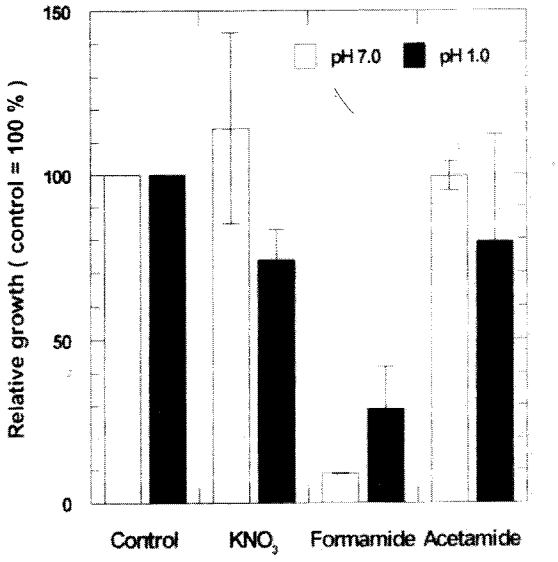


Figure 2. Growth of Bispora sp. as affected by the pH of the growth medium and the kind of N-source (glycerol as carbon source).

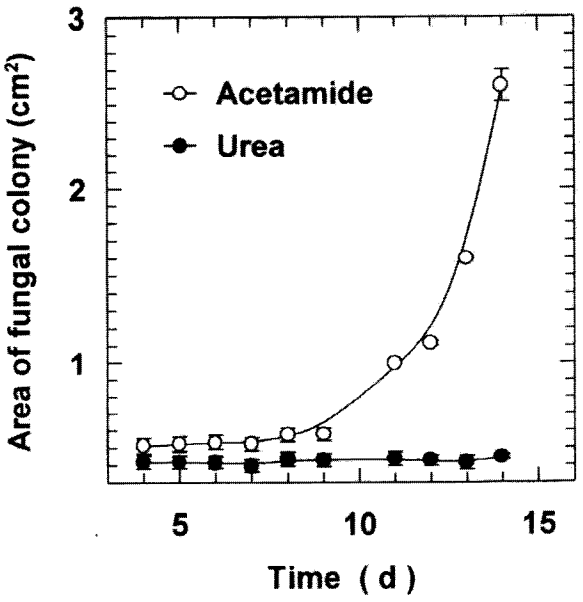


Figure 3. Growth of Bispora sp. in the presence of acetamide or urea as the only N- and C-source (pH 7.0).

The nitrogen content of control samples and of acetamide grown samples were relatively similar (53 - 62 g N kg^{-1} dry weight), whereas that of cultures deprived of nitrogen contained much less nitrogen (25 g N kg^{-1} dry weight) (Fig. 4 A). The corresponding values for cultures grown with formamide as only nitrogen source varied between 39 and 45 g N kg^{-1} dry weight. The molar ratio carbon/nitrogen were close to 10 in control cells and in cultures grown with acetamide, but was 2.5 fold higher in cultures deprived of nitrogen (Fig. 4 B). The average C/N ratios in formamide cultures were higher than those for control cells, but also lower than those cells deprived of N. Data indicate that *Bispora* sp. exhibits good growth and a reasonable N-status at both pH if cultured with acetamide as the only N-source and glycerol as C-source. With for- mamide as the only N-source, the N-status is comparable with that of the control but the growth rate is reduced. This may indicate that ammonia derived from a putative formamidase reaction is well used, but that C-supply is not adequate under these conditions.

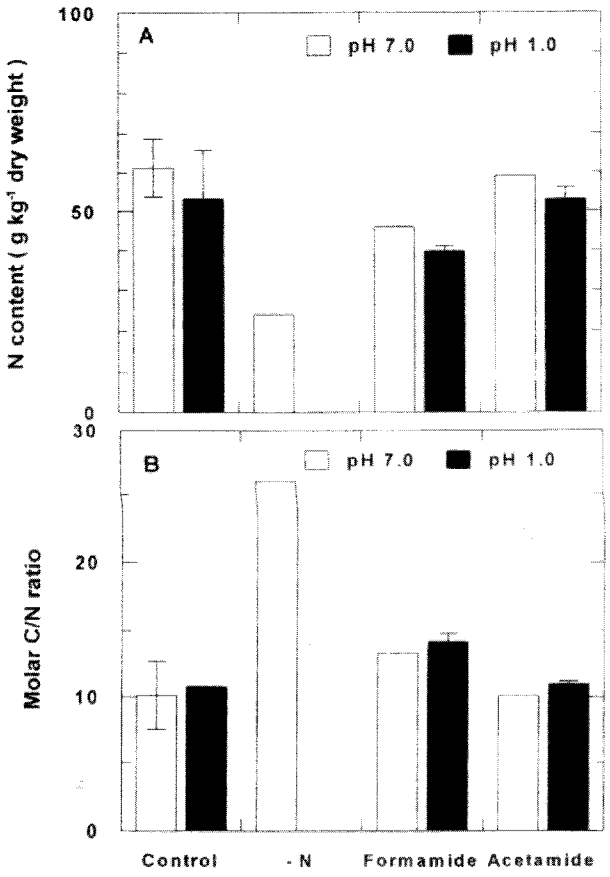


Figure 4. N-content (A) and molar C/N ratio (B) in Bispora sp. as affected by the pH of the growth medium and various N-sources (carbon source: glycerol).

Enzymes

When *Bispora* sp. is grown at pH 7.0 with NH_4^+ as N-source and glycerol as C-source it neither expresses urease (EC 3.5.1.5) nor formamidase (EC 3.5.1.9) or acetamidase (EC 3. 5. 1. 14 (Table 1). This agrees with the observation that NH_4^+ prevents the expression of these enzymes in filamentous fungi [46]. However, when cultured in the presence of formamide as N-source and glycerol as C-source a formamidase is expressed (Table 1) even though growth is low under such condition (Table 1, compare Fig. 2). When cultured with acetamide as N-source and glycerol as C-source which permits good growth (Fig. 2), neither urease nor acetamidase is produced but a formamidase is expressed (Table 1).

Incorporation and metabolism of ^{14}C-acetamide and ^{14}C-formate

The fungus takes up ^{14}C-acetamide into control cells and cells pre-cultured with acetamide, but much less into cells pre-cultured with formamide. This effect is observed both in cells pre-cultured with and without glycerol (Fig. 5A, B) as additional carbon source. Part of the radioactivity from ^{14}C-acetamide is incorporated also into the water insoluble, pelletable fraction of the fungus (not shown). TLC analysis reveals that products labelled from ^{14}C-.acetamide are glycine + serine, malate, alanine and Methyl-THF (?) (Fig. 7). Fig. 6 demonstrates that part of the ^{14}C from

Table 1. In vitro amidase and urease activity of Bispora sp. as affected by the N-source during the pre-culture and the substrate during the assay.

Experiment number:	Pre-culture of the fungus in:	Substrate during the measurement:	Amidase activity measured as change in conductivity ($\Delta\mu S\ h^{-1}\ \mu g^{-1}$ protein)
I	NH4+ (control)	formamide	0
		acetamide	0
II		urea	0
	formamide	formamide	0.064 + 0.42
		acetamide	0
	acetamide	formamide	0.121 + 0.008
		acetamide	0
	formamide	urea	0
	acetamide	urea	0
	formamide	formamide	0.046 + 0.006
	acetamide	acetamide	0
		formamide	0.033 + 0.003
		acetamide	0

Figure 5. Time-dependent incorporation of the label from ^{14}C-acetamide (final concentration 0.1 mM) into ethanol soluble fraction of Bispora sp. in the presence of different N-sources. A: Glycerol as carbon source. B: Acetamide or formamide as carbon source.

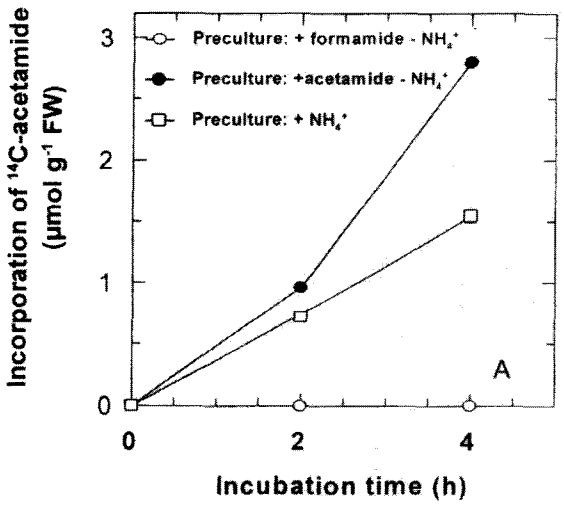
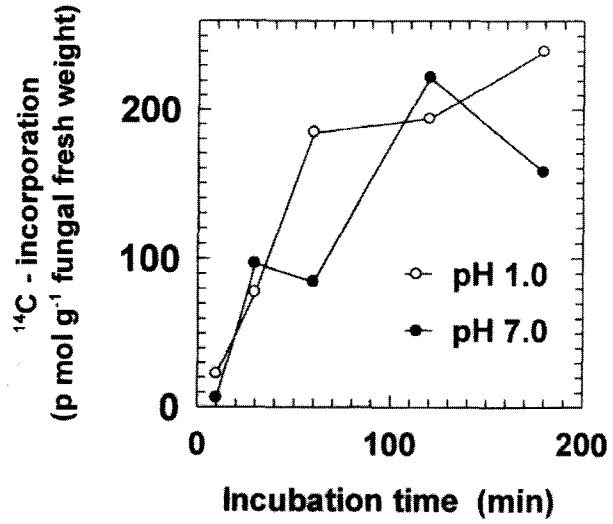
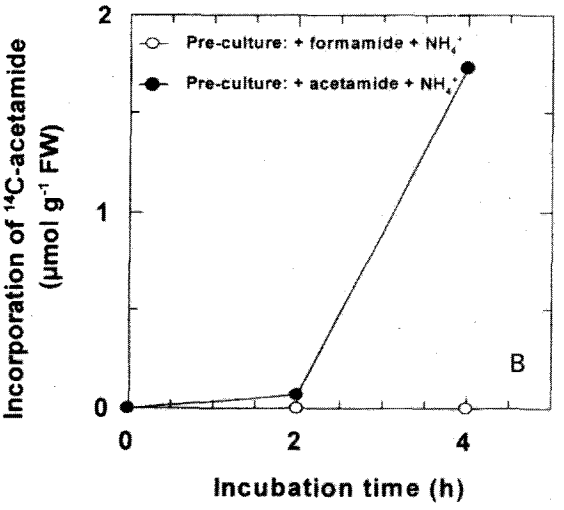


Figure 6. Time dependent incorporation of label ^{14}C-formate (final concentration 0.01 mM) into the insoluble, pelletable fraction of Bispora sp. at two different pH values of the growth medium.





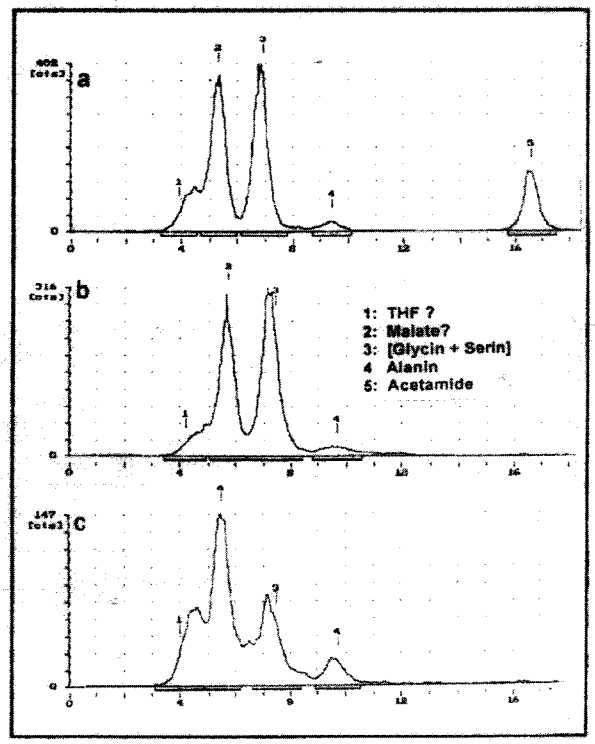
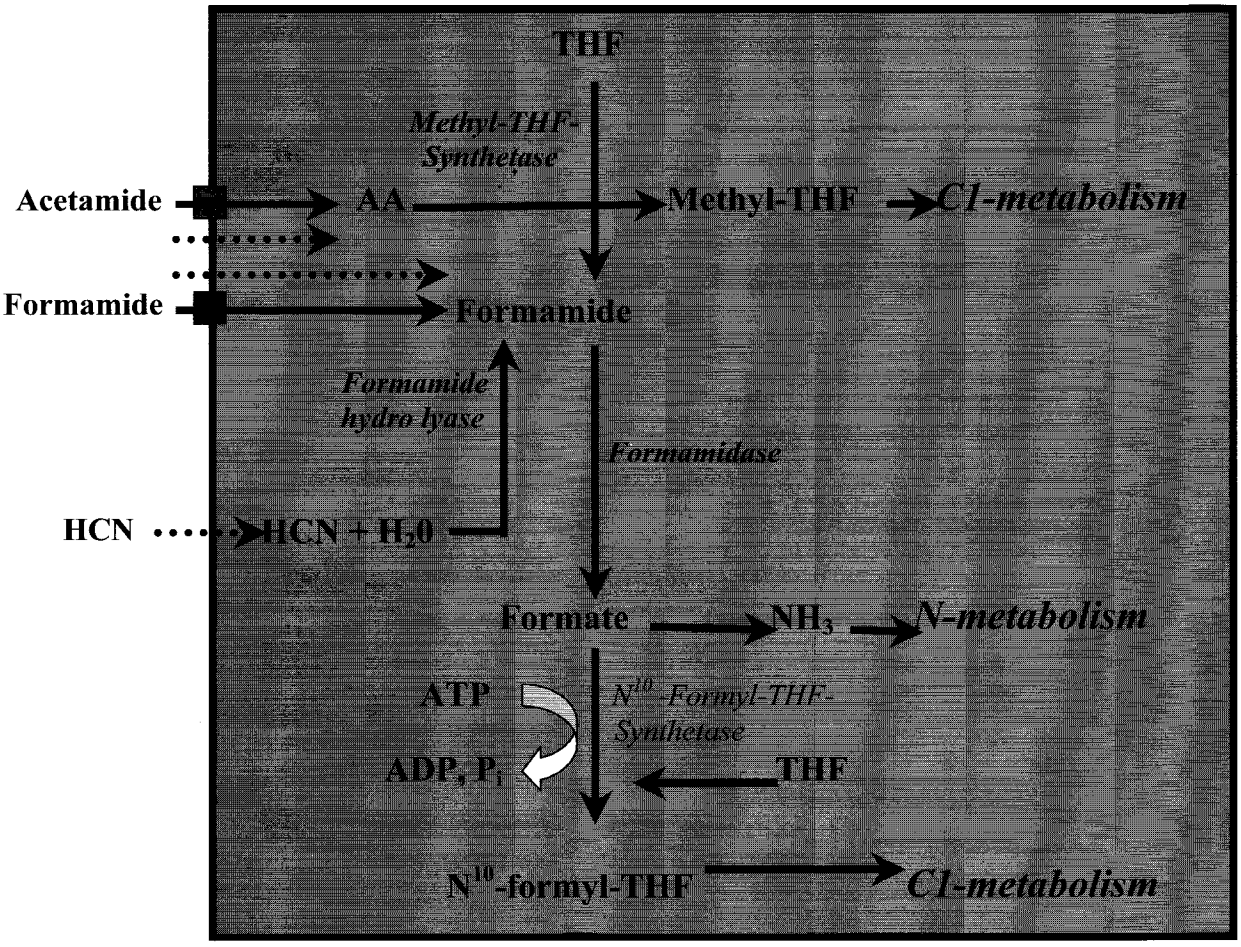


Figure 7. TLC analysis for the incorporation of the label from ^{14}C-acetamide (AA) (4 h) into ethanol soluble compounds of Bispora sp. (compare fig. 5). Pre-culture: a) + glycerol + AA - NH_4+; b) + glycerol + AA + NH_4+l c) – glycerol + AA + NH_4+. Compound 5 in a (AA) is by co-chromatography.

Figure 8. Putative schema of uptake and assimilation of acetamide, formamide, and metal complexed in the acid and heavy metal resistant filamentous fungus Bispora sp. and its relation to N-metabolism and C1-metabolism. Dotted arrows indicate diffusion and squares are possible sites of enzyme-linked transport.



^{14}C-formate is incorporated into the insoluble, pelletable fraction of *Bispora* sp. which is independent of the pH of the growth medium.

Labelling experiments with crude fungal enzyme extracts have yielded only preliminary results. However, they indicate a weak labelling of THF from ^{14}C-formate, a labelling of serine or glycine from ^{14}C-methyl-THF and a labelling of at least two non-identified compounds from ^{14}C-acetamide, very likely to be phosphate esters. Additional experiments have to be done for the identification of these compounds.

DISCUSSION

Results indicate that the acid and heavy metal resistant filamentous fungus, *Bispora* sp., can use acetamide as nitrogen and carbon source. A similar observation has also been made for bacteria [42].To a lesser extent than the bacteria, the fungus can assimilate also formamide and metal complexed cyanides, whereas it is totally unable to assimilate urea. The latter is likely related to the absence of nickel in the growth medium. Urease is known to contain nickel.

It is unknown whether acetamide and formamide are taken up by a catalyzed transport across the plasma membrane or by simple diffusion (Fig. 8). Both is thermodynamically possible. It was postulated that acetamide and formate are able to enter cells via aquaporines [19]. The reflection coefficient for acetamide and formamide was close to 0.91 and 0.80, respectively.

Once acetamide enter the cells, it is efficiently converted into other metabolites as shown by its minimum level in the cytoplasmic pool. This may also be true with formate. The supposed fate of both amides are in principal agreement with the data in literature (Fig. 8). However, an unusual phenomenon is the apparent absence of a classical acetamidase. Acetamide is supposed to be split into formamide and a C1-Unit. The latter is assumed to be fed into the C1-metabolism by a methyl-tetrahydrofolate synthetase which relates it to the metabolism of glycine, serine, and methionine [29,40, 41, 43-45]. It could be shown that the internal level of folic acid in *Bispora* sp. is about 0.5 µg per gram of fresh weight, corresponding to about 1.1 nmol per gram fresh weight [11]. This level is comparable to that of higher plants [29] and should enable the fungus to carry out an efficient C1-unit metabolism.

Formamide is split into formate and NH_3 by classical formamidase, causing a second entry into C1-metabolism by a formyl-tetrahydrofolate synthetase which connects to the synthesis of purines, glycine, serine, and methionine [40, 41, 43-45]. This occurs independent of whether it is taken up as growth substrate or as a product of acetamide assimilation.

If grown on metal complexed cyanides, a formamide hydro-lyase is supposed to produce formamide, which is metabolised as described above. Formamide hydro-lyases are high molecular weight proteins (native MW between 300 and 12100 kDa; subunits 41-45 kDa) [2-4, 7-8, 38,39]. Enzyme activity is generally found only under aerobic conditions but *Bispora* sp. being an obligate aerobe takes HCH but not CN^- as substrate. For this reason its activity is strongly dependent on the pH. It is reasonable to assume that the entry of the cyanides into the cells takes place in the lipophilic, protonated form. The assimilation of metal complexed cyanides by the acid and metal resistant fungus, *Bispora* sp., makes it a possible candidate for the remediation of industrial polluted soils.

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REFERENCES

1. Albertano, P., Pinto, G, Santisi, S., Taddei, R. Giorn. Bot. Ital. 115, 65-76 (1981).
2. Barclay, M., Hart, A., Knowles, C.J., Meeussen, J.C.L., Tett, V.A.. Enzyme Microbial Technol. 22, 223-231 (1998a).
3. Barclay, M., Tett, V.A., Knowles, C.J. Enzym. Microbial Technol. 23, 321-330 (1998b).
4. Brown, D.T., Turner, P.D., O'Reilly, C. FEMS Microbiol. Letters 134, 143-146 (1995).
5. Cluness, M.J., Turner, P.D., Clements, E., Brown, D.T., O'Reilly, C. J. Gen. Microbiol. 139, 1807-1815 (1993).
6. Cowan, D., Cramp, R., Pereira, R., Graham, D., Almatawah, Q. Extremophiles 2, 207-216 (1998).
7. Dumestre, A., Bousserhine, N., Berthelin, J. Compt. Rend. Acad. Sci. Ser. II A, 325, 133-138 (1997).
8. Dumestre, A., Chone, T., Portal, J.M., Gerard, M., Berthelin, J. Appl. Environment. Microbiol. 63, 2729-2734 (1997).
9. Feige, B., Gimmler, H., Jeschke, W.D., Simonis, W. J. Chromatogr. 41, 80-90 (1969).
10. Fry, W.E., Evans, P.H. Phytopathol. 67, 1001-1006 (1977).
10. Gimmler, H. Manila J. Sci. 3, 1-8 (2000).
11. Gimmler, H., Carandang, J. S. Acta Manilana 46, 1-7 (1998).
12. Gimmler, H.. Progress in Botany, 62, 194-214 (2001).
13. Gimmler, H., De Jesus, J., Greiser, A. Microbial Ecol. 42,87-98 (2001).
14. Kobayashi, M., Shimizu, S. Nature Biotechnology 16, 733-736 (1998).
15. Kunz, D.A., Nagappan, O., Silva-Avalos J., Delong, G.T Appl. Environ. Microbiol. 58, 2022-2029 (1992).
16. Kunz, D.A., Wang, C.S., Chen, J.L. Microbiol. 140, 1705-1712 (1994).
17. Linardi V.R., Dias J.C.T., Rosa C.A. Microbiol. Letters 144, 67-71 (1996).
18. Maier-Greiner, U.H., Obermaier-Skrobanek, B.M.M., Estermaier, L.M., Kammerloher, W., Freund, C., Wülfling, C., Burkert, U.I., Matern, D.G.H., Breuer, M.. Proc. Natl. Acad. Sci. USA 88, 4260-4264 (1991).
19. Meinild, A.K., Klaerke, D.A., Zeuthen, T. J. Biol. Chem. 273, 32446-32451 (1998).
20. Miller, J.M., Gray, D.O. J. Gen Microbiol. 128, 1803-1810 (1982).
21. Miller, J.M, Conn, E.E. Plant Physiol. 65, 1199-1202 (1980).
22. Mira, G.J., Garcia, M.P., Mira, G.A.J. Mycoses, 38, 101-106 (1995).
23. Narayanasamy, K., Shukla, S., Parekh, L.J. Indian J. Exp. Biol. 28, 968-971 (1990).
24. Nauter, G.C.C., Figueira, M.M., Linardi, V.R.. Revista Microbiologia 29, 12-16 (1998).
25. Padmaja, G., Balagopal, C. Can. J. Microbiol. 31, 663-669 (1985).
26. Palenik, B., Henson, S.E. Limnol. Oceanogr. 42, 1544-1551 (1997).
27. Pereira, P.T., Monteiro de Carvalho, M., Arrabaca, J. D., Amaral, C.M.T., Roseiro, J.C. Can. J. Microbiol. 43, 929-936 (1997).
28. Ramirez, F., Monroy, O., Favela, E., Guyot, J.P., Cruz, F. Appl. Biochem, Biotechnol. 70-72, 215-223 (1998).
29. Rebeille, F., Douce, R. In: Kruger N.J. (ed.) Regulation of primary metabolic pathways in plants. Kluwer Academic Press, Amsterdam, pp. 53-99 (1999).
30. Rhodes, D., Verslues, P.E., Sharp R.E. In: Singh B.J. (ed.) Plant amino acids. Marcel Dekker, New York, pp. 319-356 (1999).
31. Rissler, J.F., Millar, R.L. Plant Physiol. 60, 857-861 (1977).
32. Shah, M.M., Grover, T.A., Aust, S.D. Arch. Biochem. Biophys. 290, 173-178 (1991).
33. Shanker, R., Ramakrishna, C., Seth, P.K.. Arch. Microbiol. 154, 192-198 (1990).
34. Sigler, L, J.W., Carmichael, J.W. Can. J. Microbiol. 20, 267-268 (1974).
35. Silva-Avalos, J., Richmond, M.G., Nagappan, O., Kunz, D.D. Appl. Env. Microbiol. 56, 3664-3670 (1990).

36. Tonon, F, Priorde-Castro, C., Odier, E. Exp. Mycol. 14, 243-254 (1990).
37. Van der Walt, J.P., Brewis E.A., Prior, B.A. System. Appl. Microbiol. 16, 330-332 (1993).
38. Wang, P., Matthews, D.D., Vanetten, H.D. Arch. Biochem. Biophys. 298, 569-575 (1992).
39. Wang P., Vanetten H.D. Biochem. Biophys. Res. Comm. 187, 1048-1054 (1992).
40. Hanson, A.D., Gage, D.A., Shachar-Hill, Y. Trends Plant Sci. 5, 206-213 (2000).
41. Mouillon, J.M., Aubert, S., Bourguignon, J., Gout, E., Douce, R., Rebeille, F. The Plant J. 20, 197-205 (1999).
42. Pinechoty, F. C. R. Seances Soc. Biol. Fil. 182, 177-180 (1988).
43. Pasternack, L.B, Littlepage, L.E., Laude, D.A. jr., Appling, D.R. Arch. Biochem. Biophys. 326, 158-165 (1996).
44. Pasternack, L.B, Laude, D.A. jr., Appling, D.R. Biochemistry, 33, 74-82 (1994).
45. Pasternack, L.B, Laude, D.A. jr., Appling, D.R. Biochemistry, 33, 7166-7173 (1994).
46. Toton,F., Priorde,, C.C., Odier, E. Exp. Mycol. 14, 243-254 (1990).