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POLYMERIZATION OF RESORCINOL BY AN CRYPTOPHYCEAN EXOENZYME

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

R. SÜTFELD*

Max-Planck-Institut f. Limnologie, D-24302 Plön, Germany

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Key Word Index—*Cryptomonas spec.*; *Rhodomonas* spec.; Cryptophyceae; algae; exoenzymes; polymerase; resorcinol; dihydroxyphenols; polyphenols; allelopathy; detoxification; pollution

Abstract—In aqueous solutions exposed to sunlight, resorcinol, a *m*-dihydroxyphenol, does not undergo autoxidation as compared to catechol, an *o*-dihydroxyphenol. However, water taken from a lake (Schöhsee, Plön) caused a disappearance of resorcinol and enhanced catechol conversion. A screening involving species of various algae groups showed that resorcinol was being converted specifically by members of the Cryptophyceae family. The algae could be separated by ultrafiltration from the culture medium with full viability; the activity was found in the medium and could be characterized by protein precipitation and denaturation as a Cryptomonad exoenzyme with a M.W. $\cong 10^5$. The conversion rates of resorcinol increased exponentially during successive additions of substrate, pointing to a reaction on which the product functions as an increasing matrix for the addition of next substrate molecules. After seven resorcinol pulses of about 30 nmol/ml resorcinol to the same enzyme source, irregularly-shaped, water-insoluble crystals appeared as reaction products. The algae themselves, however, were severely affected after the third resorcinol application and did not survive subsequent addition. In the freshwater research, this result represents the complete sequence of an allelopathic interaction between resorcinol exuding aquatic macrophytes and surrounding microphytes; it may also contribute to the solution of open questions about the origin and turnover of phenolic DOM in aqueous systems. In environmental terms, it questions the role of industrial wastewater inputs, and on one hand the toxicity of resorcinol and on the other hand, the detoxification capability of natural waters. Lastly, it should be considered that this kind of enzymatic monophenol condensation could also participate in the synthesis of various polyphenolic compounds of higher terrestrial plants.

INTRODUCTION

Resorcinol (1,3-dihydroxybenzene; Fig. 1) has been reported as the main exudation product of axenically cultivated *Nuphar* seedlings [1]. Because of its general membrane-affecting properties [2], resorcinol was supposed to play a role as an allelochemical in interactions between this macrophyte and other organisms of the aquatic ecosystem. A first approach of its application in μ molar ranges to phytoplankton and zooplankton organisms showed no effects on Chlorophyceae or Cyanophyceae species but a toxic effect on a *Daphnia* (Cladocera) species. In the presence of the above mentioned organisms, the concentration of resorcinol remained almost constant over the incubation time. However, if applied to Cryptophyceae cultures, resorcinol concentration was reduced to zero within a few days, suggesting an uptake, an intra- or extracellular degradation or another form of a struc-

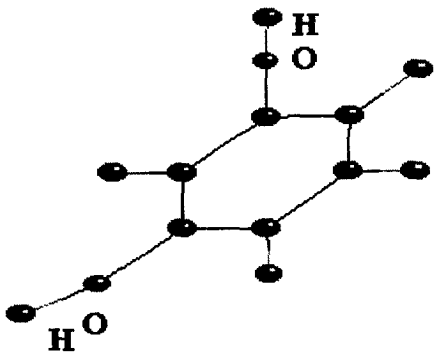

Fig. 1. Resorcinal (1,3-dihydroxybenzene).

* Author to whom correspondence should be addressed.

tural conversion caused by these algae. To date, biochemical degradation of resorcinol is only known from some heterotrophic microbial specialists which metabolize the compound in either aerobic [3, 4] or anaerobic reactions [5]. However, intermediates or reaction products as described there could not be detected using HPLC in the algae incubation assays. This study therefore, attempts to define a possible alternative route of resorcinol metabolism, as carried out by autotrophic Cryptomonads. Another experimental series was directed to extend current knowledge about the general physico-chemical properties of *m*-dihydroxylated phenols in water and lastly about their fate in natural freshwater systems. In this context, *o*-dihydroxyphenols, closely related to *m*-dihydroxyphenols, have already been intensively studied [6].

RESULTS

Lakewater experiments

A resorcinol solution was exposed in the summer to straight sunlight for ten days and remained stable in contrast to a catechol solution which lost about 50% of the compound and turned brownish (Figure 2). Under the same conditions, up to 60% of resorcinol, added to lake water (drawn from the Schöhsee in summer 1996), disappeared from the solution within ten days, whilst catechol showed a complete breakdown within five days. The same experiment, performed in winter 1995/1996 (cloudy sky, water taken from the frozen lake) showed no disappearance of resorcinol and only a slight (about 5%) loss of catechol.

Culture experiments

Essentially a degradation was observed if resorcinol was added to pure cultures of Chlorophyceae, Chrysophyceae or Cyanophyceae species. Slightly reduced amounts (Fig. 3) from Monorhaphidium (Chrysophyceae) suggested resorcinol conversion; however, this phenomenon turned out to be nearly the absorption of resorcinol on the cell surfaces, particularly in strains exhibiting fast reproduction rates. Only incubation assays containing Cryptomonad algae as *Cryptomonas* or *Rhodomonas* showed a significant disappearance of resorcinol from the nutrient solution. With most strains, the turnover of resorcinol needed about 5–7 days, and was characterized by a sigmoidal disappearance. In another experimental series, subsequent resorcinol pulses were given to the same algal culture always at the time when the concentration of previously given resorcinol had gone to zero. In the course of these steps, the reaction velocity increased dramatically so that finally the time for a complete turnover only required a few hours (Fig. 4). Non-axenic and axenic cultures yielded similar results.

Microscopic examination showed that the algal cells were strongly affected by resorcinol after the 3rd addition (Figs 5 and 6). After the next application, microphotographs indicated that obviously the outer cell membrane became fragmented and burst, and the entire inner compartments were released into the medium. This 'bursting' took place within a few

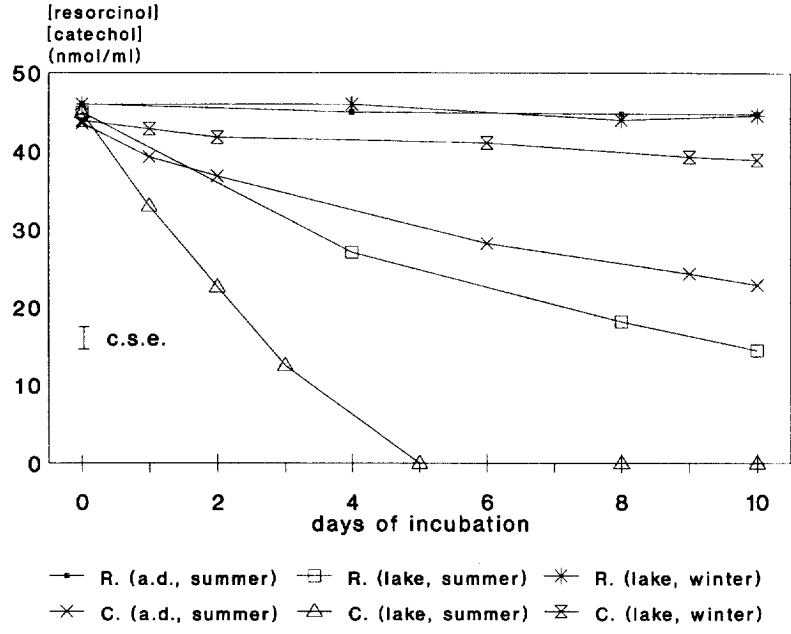

Fig. 2. Reaction of resorcinol (R.) and catechol (C.) if dissolved in distilled water or in summer and winter with lake water and exposed to summer or winter daylight, respectively. C.s.e., mean values ± common SE.

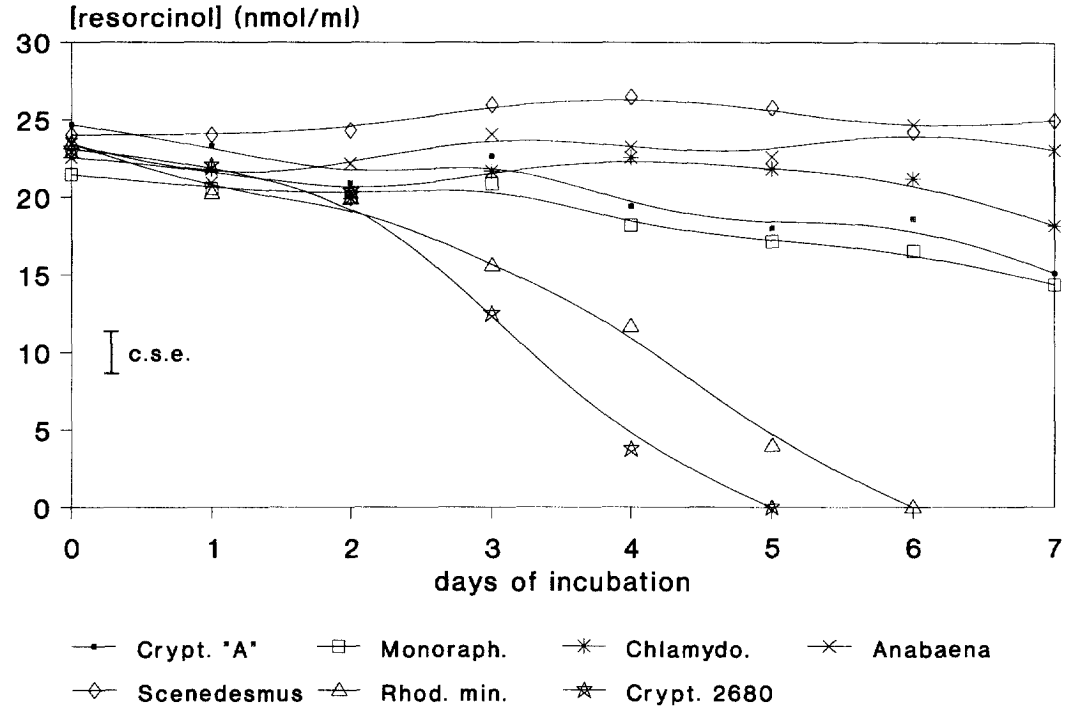

Fig. 3. Reaction of resorcinol if applied to algal cultures including members of the Chlorophyceae (Chlamydo., *Chlamydomonas* sp.; *Scenedesmus acutus*), Chrysophyceae (Monoraph.; *Monoraphidium* sp.), Cryptophyceae (Crypt. A, *Cryptomonas* sp. strain 'A'; Rhod. min., *Rhodomonas minuta*; Crypt. 2680, *Cryptomonas* sp. strain '2680') and Cyanobacteriae (*Anabaena* sp.) family. Cultures were adjusted to equal cell number before resorcinol addition at day zero. Statistics as given in Fig. 2.

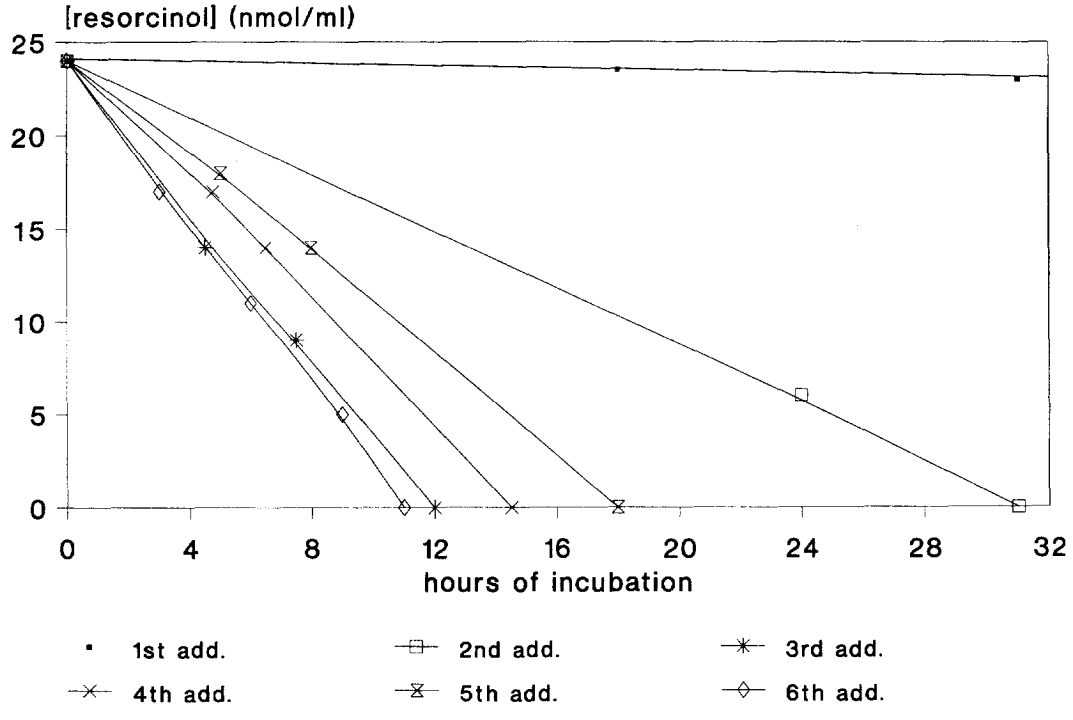

Fig. 4. Reaction of resorcinol during subsequent applications to a culture of *Cryptomonas* sp. strain '2680'. Each next application followed after complete turnover of the formerly applied substrate. Resorcinol content of the first application reached zero after 6 days of incubation.

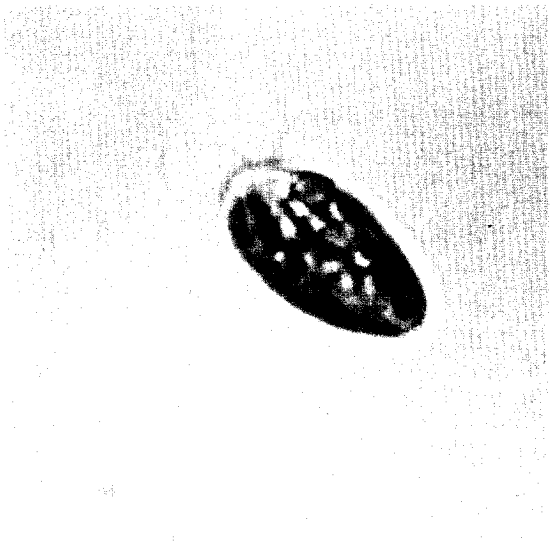

Fig. 5. *Cryptomonas* sp. strain 'A' before resorcinol application.

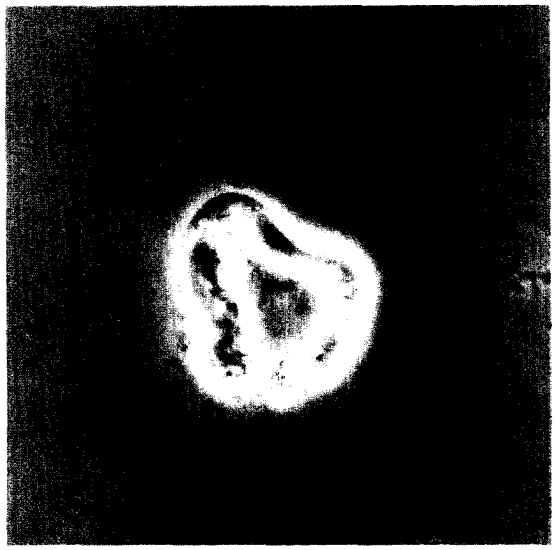

Fig. 7. *Cryptomonas* sp. strain '2680' (in mitotic stage) just after giving the 4th resorcinol pulse (phase contrast optics).

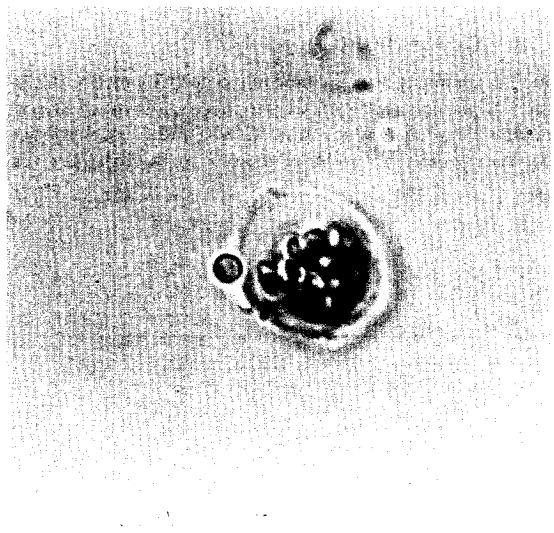

Fig. 6. *Cryptomonas* sp. strain 'A' after the 3rd resorcinol pulse.

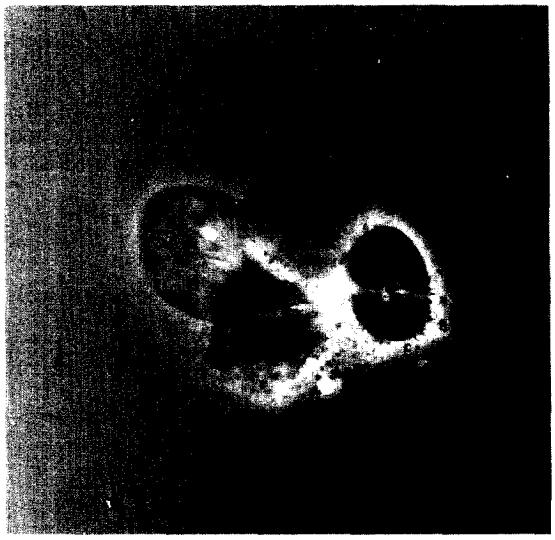

Fig. 8. Same object as in Fig. 7, 10 min later (phase contrast optics).

minutes (Figs 7 and 8). From this stage on, all parts of the organism disintegrated within a short time period.

By light microscopy, it was further proven that the Cryptophyceae retained their full viability if they were treated by the special ultrafiltration technique as described in the 'Experimental' section. Figure 9 shows that, after ultrafiltration (YM100), the main activity leading to resorcinol disappearance was located in the nutrient medium (filtrate), and that a residual activity only remained in the algal fraction (supernatant). Comparison of results of separations performed on XM50, YM100 and XM300 membranes (not shown here) suggested an apparent molecular mass of $\cong 10^5$ Da for the active enzyme. The YM100 ultrafiltrate exhibited an exponentially increasing specific activity if measured from one application step to the next (Fig. 10). At any stage, the reaction could completely be inhibited or terminated either by heating (microwave, 5 min) the sample, or by adding $(NH_4)_2SO_4$ (80% end concentration) or trichloroacetic acid (TCA, 5% end concentration), respectively, to the sample (Fig. 11).

After several resorcinol applications (usually, after 6 or 7 steps), the reaction mixture turned slightly white and turbid, showing a yellow-brownish fluorescence under neon light. Microscopical examination proved the existence of irregularly-shaped, crystal-like solids floating in the reaction mixture (Figs 12 and 13). These solids were insoluble in water, acids, alkali and organic

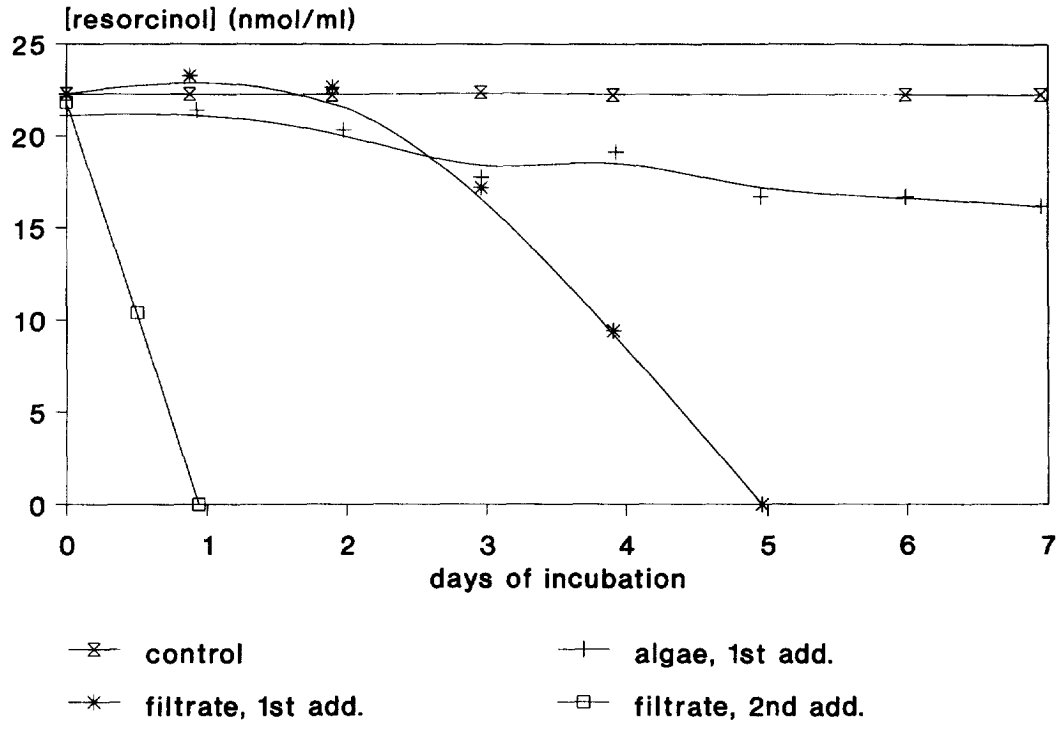

Fig. 9. Reaction of resorcinol applied to the ultrafiltrate (YM100 membrane) and to the residual fraction ('algae') of a *Cryptomonas* sp. strain 'M420' culture. Control: Culture medium without algae.

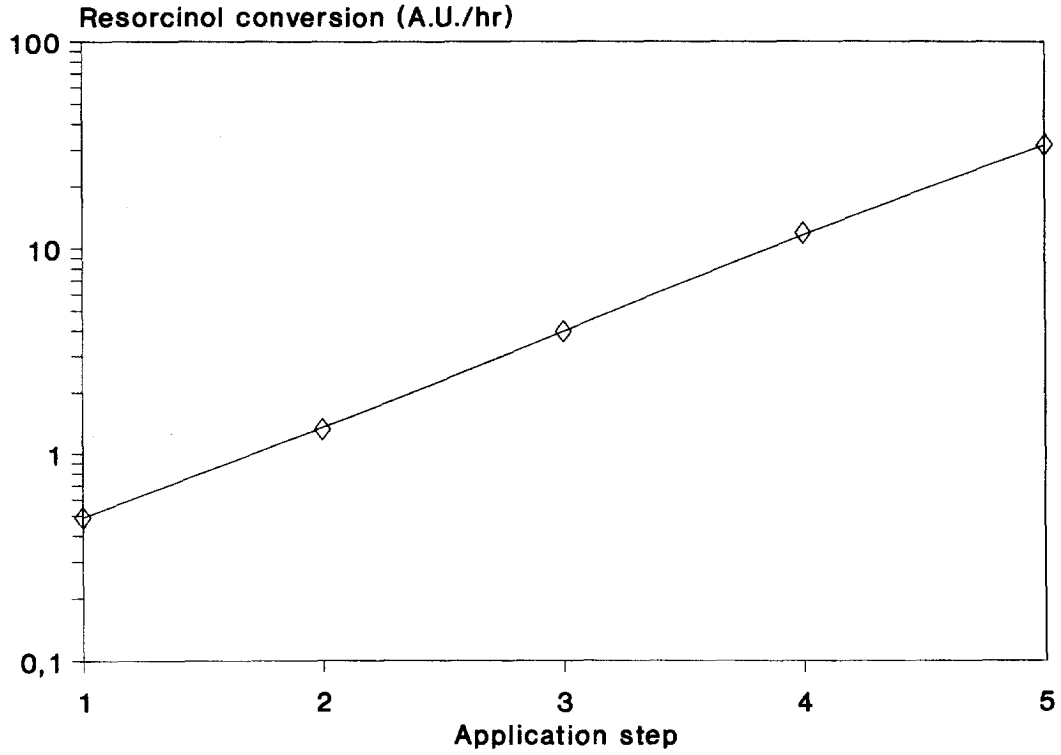

Fig. 10. Exponential increase of reaction velocity during successive resorcinol application steps. Enzyme source: Ultrafiltrate (YM100) of *C.* sp. strain 'M420'. A.U.: Arbitrary units (281 nm) from peak integration.

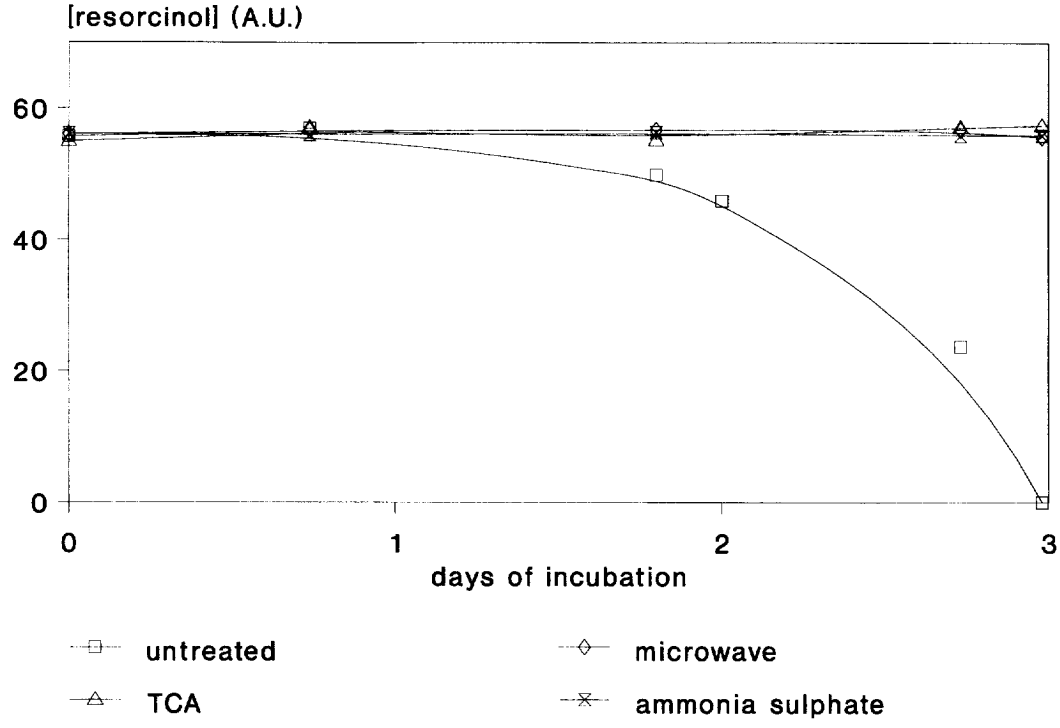

Fig. 11. Inhibition of resorcinol conversion by protein precipitation (ammonium sulphate) or denaturation (boiling, TCA-treatment).

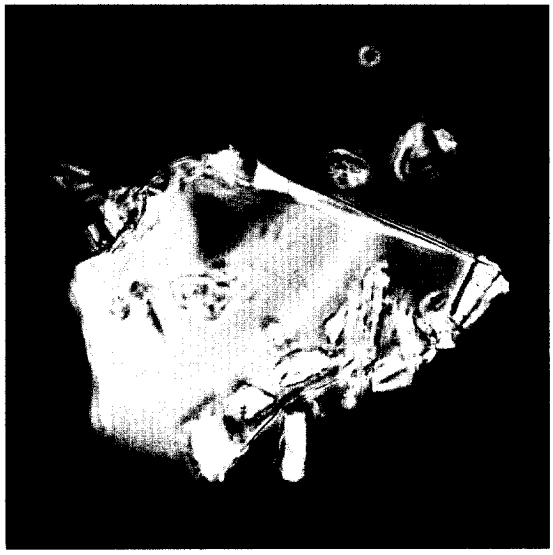

Fig. 12. Samples of polymer crystals formed by the algal exoenzyme after 7 resorcinol applications (phase contrast optics).

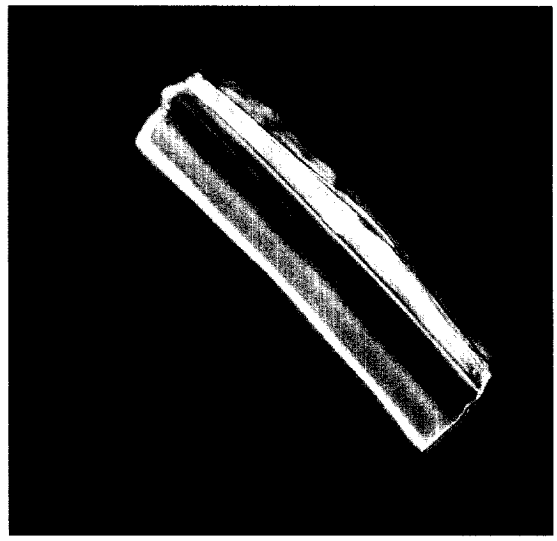

Fig. 13. Samples of polymer crystals formed by the algal exoenzyme after 7 resorcinol applications (phase contrast optics).

solvents. After seven resorcinol applications, crystals reached about the same size (8–10 μm) as *Cryptomonas* cells. No crystals were formed in control assays lacking either resorcinol or the enzyme source as well as in assays containing precipitated or denatured protein.

DISCUSSION

This study is the first report about the enzymatic condensation of a pure, water-soluble monophenol. This reaction is characterized by an exponentially increasing reaction rate if the substrate addition is being carried out in subsequent pulses. The end prod-

uct shows up in form of visible, water-insoluble solids. This result may stimulate new questions in the fields of aquatic and terrestrial plant biochemistry, physiology, ecophysiology and, last but not least, in environmental/industrial pollution research.

Very little information is available on the occurrence of unbound resorcinol and related monophenols in nature, see [1], probably because successful enrichment and separation of these compounds requires quite unusual chromatographic techniques [7, 8]. For this reason, unbound monophenolic compounds might be much more abundant in aquatic environments than commonly suggested. Dissolved organic matter (DOM) in freshwater environments has been said to contain about 40% of 'phenolic compounds' [9]. In lake systems, macrophytes are the major autochthonous source of DOM [10]. They release phenolics and other, unidentified compounds either by exudation as recently demonstrated [1, 8, 11], or by leaching caused by violation or decay. Terrestrial plants growing in surrounding wetlands or close to littoral zones which release phenolic DOM the same way [12] are an allochthonous source as well as DOM input of unknown origin from inlet waters. A third, as yet unpredictable allochthonous DOM source, which may be most harmful to the aquatic environment, is the anthropogenic input of resorcinol and/or resorcincylic compounds into lakes caused by waste waters produced by the wood- or adhesives industry or by oil shale spillings [13–15]. However, quantitative data about the range of this pollution are not available.

The combined results of the former [1] and this study supply novel information on a biochemical interaction course between an aquatic macrophyte (*Nuphar*) and a distinct group of microphytes, the Cryptophyceae. Obviously, *Nuphar* aims to protect its surface from colonizing epiphytic microorganisms or algae by exuding a membrane-affecting [2] toxin, resorcinol. In contrast to the ubiquitously occurring *o*-dihydroxyphenols like catechol which undergo spontaneous autoxidation in water [6], the *m*-dihydroxyphenol resorcinol turned out to be a more stable compound. Therefore, an enrichment with resorcinol would imply dangerous consequences for the natural ecosystem, particularly in shallow lakes with dense macrophyte populations. Indeed, the *Daphnia* experiment recently performed [1] demonstrated the deadly effect of minor doses of resorcinol on a zooplankton organism. However, the results from lake water experiments pointed to an alternative mechanism for resorcinol turnover which looked as if it were being mediated by microorganisms because of its seasonality. The algal culture experiments showed that Cryptophyceae exclusively bear the ability to remove resorcinol from the aquatic medium. Cryptomonad algae occur in almost all lakes, regardless of their trophic state. They regularly show an increased abundance following 'blooms' of other algal groups. These pulses in Cryptomonad abundance have been interpreted to be regulatory and stabilizing forces of the aquatic ecosystem [16]. Cryptophyceae cells contain, in addition to a huge nucleus, another DNA bearing compartment. This 'Nucleomorph' structurally looks like a nucleolus, and has been interpreted to be a residue of an eukaryotic endosymbiont that invaded into heterotrophic ancestors of this algae family [17]. For this reason, Cryptophyceae probably possess an exceptionally broad genetic potential when compared to other algal families.

The results obtained demonstrate that resorcinol reduction in aqueous systems is being catalyzed by an exoenzyme (M.W. $\cong 10^5$) of Cryptophyceae. With this, the detoxification capacity of a natural lake system should proceed concomitant to the seasonal changes in the density of Cryptophyceae populations. It seems unlikely that, by natural inputs only, resorcinol concentration in lakes would reach or exceed ranges of around 30 μmol/l as has been used here in the laboratory experiments. An industrial input in this range, however, could have the consequence that a natural Cryptophyceae population would probably withstand and be able to degrade just a single or another input of resorcinol within this range. However, a higher concentration or longer lasting input of resorcinol or resorcinylic compounds would certainly eliminate the Cryptophyceae population and could also eliminate the lake's detoxification capacity forever.

The enzymatic conversion of resorcinol is characterized by an exponentially increasing reaction rate. Such phenomenon may be imaginable if the product formed serves as matrix for the next substrate batch. In this case, the number of open link positions would increase simultaneously to the increasing size of the matrix; Inactivation of the enzyme or a lack of substrate terminates the reaction. The results obtained assume that the water-insoluble, crystal-like products are condensation polymers of resorcinol. However, final evidence of this will only be achieved by structural analysis of soluble oligomer intermediates or by the upscaling of incubations to gram quantities of resorcinol in order to examine the structure of the insoluble solid formed.

EXPERIMENTAL

Chemicals

Resorcinol was purchased from Merck in p.a. quality, catechol from Sigma Chemical Co. in 99% purity. Solvents used for HPLC were of HPLC grade.

Algal cultures

Samples were taken from strains routinely subcultured in the MPIL: Chlorophyceae: *Chlamydomonas* sp., cultivated in WC-medium [18] and *Scenedesmus acutus*, Z/4 medium [19]. Chrysophyceae (WC): *Monoraphidium sp.*. Cryptophyceae (WC): A greyish coloured strain *Cryptomonas* 'A', strain *C.* '2680' (yellow-green, DSMZ, Göttingen, Germany),

strain C. 'M420' (grey, axenic) and a reddish *Rhodomonas minuta* strain. Cyanophyceae (Z/4): *Anabaena* sp.. 100 ml batches of algae strains were cultivated in 250 ml Erlenmeyer flasks at 18°C and 20 μmol PAR/m^2 × s (15/9 hrs light/dark period, light supply by neon tubes). Before starting incubation series, cultures were adjusted to about equal quantity of individuals (approx. 0.8×10^6/ml) by dilution with nutrient solution. Estimation of average size and number of individuals per culture was done on a CASY 1 Cell Counter Vers. 2 (Schärfte System, Reutlingen, Germany) before and after incubations. Microphotographs were taken without any pretreatment of samples on a ZEISS Photomikroskop II equipped with phase-contrast optics.

Incubation series

Concentrated aqueous solns of resorcinol or catechol (final conc, 46 nmol/ml) were added to water samples drawn from the Schöhsee lake (Plön, Germany) in July 1995 and in February 1996. Samples were exposed to daylight for 10 days at room temperature. Compounds dissolved in distilled water served as controls. For algae and ultrafiltration experiments, a concentrated (1:100) aqueous resorcinol soln was added to the assays, yielding a final concentration of about 25 nmol/ml. Enzyme activity inhibition experiments were performed either by boiling (5 min microwave) the enzyme source before resorcinol application, or by adding $(NH_4)_2SO_4$ (80% final concn) or trichloroacetic acid (TCA, 5% final concn) to the enzyme source. In the latter cases, the pH of nutrient solns was corrected to the initial value by addition of dilute KOH. All incubation series included at least five replicates.

Samples pretreatment

Dependent on the size of algae, samples from incubation assays were filtered before application to HPLC using either Membrex 18 (Costar, Bodenheim, Germany) 0.2 m syringe filters or a sartorius (Göttingen) syringe filtration apparatus with 0.8 or 3.0 μm cellulose nitrate filter discs.

Quantitative estimations

HPLC was performed on a Beckman 'System Gold' solvent delivery system equipped with No. 126 pumps and a No. 168 diode array detector. Samples (1 ml each) were injected and fractionated on either 0.4 × 25 cm LiChrosorb RP18 or Spherisorb ODS2 (5 m each) columns (Merck; Phase Separations Ltd.), supplied with a 0.4 × 3 cm LiChrosorb RP8 (10 m) precolumn. Separations were run isocratically with 100% H_2O as mobile phase, and with an appropriate flow that kept the pressure between 14 and 18 MPa. Quantitation was performed by peak integration of arbitrary units (area$_{A.U.281}$), and calibration by using standard reference material [cf 1].

Separation of nutrient soln from algae

A 200 ml Amicon stirring cell was employed with either Amicon XM50, YM100 or XM300 membranes (0.5×10^5, 1×10^5 or 3×10^5 nominal molecular weight cutoff, respectively). Ultrafiltration was performed with gaseous CO_2 as propellant, max. pressure 10, 6 or 2 kPa, respectively. When the total sample was reduced to approximately 1/3 volume, the cell was decompressed as slowly as possible by careful reducing the excess pressure to atmospheric pressure.

Data processing

The 3D-structural formula of resorcinol was drawn using the molecule editor program MOLGEN (Math. Inst., Univ. Bayreuth, Germany). Statistical analysis of results was done as recently described [1].

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