Biodegradation **13:** 91–99, 2002.

Biodegradation of formaldehyde and its derivatives in industrial wastewater with methylotrophic yeast *Hansenula polymorpha* and with the yeast-bioaugmented activated sludge

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Accepted 23 April 2002

Key words: activated sludge, aerobic wastewater treatment, formaldehyde, *Hansenula polymorpha*, methylotrophic yeast, trioxane, urotropine

Abstract

Methylotrophic yeast *Hansenula polymorpha* were shown to cooperate with activated sludge from biological wastewater treatment stations, enhancing substantially its potential to biodegrade formaldehyde in industrial wastewater. After integration with yeast cells the modified sludge retained its original structure and activity whereas its resistance to elevated formaldehyde concentrations was significantly improved. The applicability of the yeast in the utilization of formaldehyde derivatives, as exemplified by urotropine and trioxane, was also investigated. The treatment of urotropine-containing wastewater with methylotrophic yeast was found to be effective at acidic conditions (pH below 5.5). Trioxane was not degraded due to the stability of an ether bond which made the molecule recalcitrant to oxidation via methylotrophic pathway reactions. It is concluded that the yeast species may be applied to treat wastewater containing formaldehyde and some of its derivatives as either monocultures or as an integrated, specialized element of the activated sludge biocenosis.

Abbreviations: COD – chemical oxygen demand; FD – formaldehyde; TRX – trioxane; URT – urotropine

Introduction

Formaldehyde (FD) is a very common chemical pollutant of water, air and soil (Walker 1964). It can be found in food and other consumer products as well as in industrial and municipal effluents. In the atmosphere, FD is a constituent of automobile exhaust and combustion processes. Strong reductive properties and high reactivity make formaldehyde an aggressive chemical agent that negatively affects cells and organs (Bardana & Montanaro 1991; Restani & Galli 1991; Chang & Gershwin 1992). A number of applications involving formaldehyde lead to the environmental release of this chemical at toxic concentrations, often exceeding maximum permissible levels. Such FD concentration limits are given as standards by environment protection agencies and are defined for both surface waters and treated wastewaters. In Polish regulations these levels are 0.2 and 2.0 mg/l, respectively (Instruction of the Polish Ministry 1991).

The total load of formaldehyde determined in wastewaters is a result of both the monomeric (free) form of this compound as well as of various FD derivatives such as ether, urea, phenolic condensates and composite complexes with resins, phenols, and many other chemicals. Most of the FD derivatives are generated in chemical industry processes as waste- or by-products and they are much less susceptible to biodegradation than free formaldehyde. The scientific research and practical studies on biodegradation of FD and its derivatives involve both the application of aerobic and anaerobic cultures of bacteria and fungi as well as activated sludge processes (Kaplan et al. 1979; Otake et al. 1995; Zijin et al. 1998; Omil et al. 1999; Fayolle et al. 2001; Hawari et al. 2001)

Formaldehyde can be utilized by bacteria and fungi that are capable of assimilating monocarbonic compounds (Michalik 1975), the so-called methylotrophic organisms. Methylotrophic yeasts metabolize single-carbonic compounds by means of a specialized enzymatic reaction pathway (Harder et al. 1987; Gleeson & Sudbery 1988a; Sibirny et al. 1988), as described in our previous paper (Kaszycki et al. 2001). In particular, the strain *Hansenula polymorpha* (Gleeson & Sudbery 1988b; Maidan et al. 1997) has proved to be effective in biodegradation of formaldehyde and methanol in artificial (Kaszycki & Koloczek 2000) and real (Kaszycki et al. 2001) industrial wastewater.

The aim of the present study was to further verify the applicability of methylotrophic yeasts used either to bioaugment activated sludge biocenoses or to treat industrial wastewater containing typical FD derivatives, as exemplified by urotropine and trioxane.

Materials and methods

Solutions and buffers were made using redistilled water. All of the chemicals were of analytical grade. Whenever required, fully sterile conditions were applied.

Wastewater and activated sludge samples

Three wastewater types from different technological fluxes of formaldehyde chemical industry plants were used. The samples were collected at the inlet of group biological treatment stations and then cooled to 4 °C. The specimens were analyzed immediately after collecting or they were stored at −20 °C before the experiments. Sample freezing was verified not to affect such wastewater parameters as the COD level, pH, and the content of particular xenobiotics. The characteristics of all the specimens used in the study are given in Table 1.

Similarly, the study included several independent activated sludge samples collected at aeration chambers of biological wastewater treatment stations of different chemical plants. As an additional reference in yeast integration experiments, the activated sludge from a typical municipal household wastewater treatment station was also used (wastewater type 4, lacking in FD and methanol). The activated sludge was applied in experiments within two weeks after collection. The sludge was stored at 4 °C and upon experiment, it was reactivated by placing 50–100 ml suspension in a 250 ml flask and by aerobically cultivating in a rotary shaker for 48 h at room temperature. Both the morphology and biological condition of each activated sludge sample were monitored by microscopic observations. In biodegradation studies, the sludge was preadapted to formaldehyde in a manner similar to methylotrophic yeast cultures (see below). Integration of the activated sludge with *H. polymorpha* was performed by adding the appropriate number of yeast cells to 20–50 ml of the sludge suspension and by cultivating the mixture under aerobic conditions for 24 hours at 25 °C.

In model experiments, a synthetic wastewater solution contained ammonium nitrogen, chloride and inorganic phosphorus at concentrations: 200 mg/l $(NH_4)_2SO_4$, 300 mg/l KCl, and 30 mg/l H_3PO_4 as well as a trace amount (0.0025%) of yeast extract to provide the cell cultures with a minimum amount of microelements, vitamins, and other factors. A defined concentration of a particular xenobiotic was added to the medium as a sole carbon source.

Yeast cell culture cultivation and preadaptation

Active-sludge integration and FD-biodegradation experiments were performed employing a prototrophic revertant of a *Hansenula polymorpha* NCYC 2309 (*Leu*$^-$) parental strain, obtained from the National Yeast Culture Collection, Norwich, U.K.

Cells were always grown on 2% (v/v) methanol in the absence of glucose so as to keep methylotrophic pathway enzymes induced. The optimal growth media as well as cell cultivation conditions were described in detail previously (Kaszycki & Koloczek 2000). At about 12 hours before the experiments in real wastewater environments, cells were preadapted to formaldehyde by adding 150–300 mg/l of this xenobiotic to the culture. Then, the yeast were centrifuged at 3000 g for 5 min, the optimal medium discarded, and the cells were resuspended in a given wastewater solution at a desired biomass. Yeast biomass was measured turbidimetrically as optical density at 540 nm (OD_{540}). OD_{540} =1 corresponded to the cell population of ≈9.2 × 10^6 cells/ml.

Yeast culture population and cell survival were monitored by a surface plating technique. Appropriate dilutions of the cell suspensions were plated onto Petri dishes with a Sabouraud/agar solid medium and incubated for 24 h at 37 °C. The number of countable cell colonies corresponded to the population of the viable cells in the original suspension tested. In experiments with the activated sludge, the number of cells was

Table 1. A list of the wastewater samples used in the study

Source	Sample no.	pH	COD (mg l^{-1})	FD total (free+bound) (mg l^{-1})	FD free (monomeric) (mg l^{-1})	Methanol (mg l^{-1})
Chemical synthesis plant:	1.1	7.4	2332	1128	1118	–
Raw wastewater inflow	1.2	7.1	1405	488	491	–
Chemical synthesis plant:	2.1	8.6	8784	1190	nd	367
Urotropine manufacturing department	2.2[b]	7.8	11309	2300	582[a]	2980[a]
Chemical synthesis plant:	3.1	5.0	1854	750	250	132[a]
Trioxane flux	3.2[c]	7.5	3214	850	185	250[a]
Municipal wastewater inflow	4	7.5	202	–	–	–

[a] Based on a gas chromatography analysis.
[b] Urotropine content 1675 mg l^{-1}.
[c] Trioxane monomer content 284 mg l^{-1}.

also determined in a hemocytometer by a direct cell counting with the use of a Thoma cell.

Xenobiotic biodegradation observations

In a typical experiment, a 15–20 ml wastewater specimen in a 250 ml flask was inoculated with $\approx 2.3 \times 10^6$ to 1.1×10^7 yeast cells/ml or with the yeast-enriched activated sludge. Whenever needed, OD_{540} of the culture was measured and some of the suspension was preserved for surface plating to check for cell viability. At each time interval during the course of a biodegradation experiment, a 1 ml sample was preserved for further determination of a xenobiotic content. Respective control samples, that is either uninoculated wastewater or the original activated sludge in the absence of cells, were always observed at identical experimental conditions.

Specimens for the determination of methanol, formaldehyde and FD-derivatives (1 ml) and for COD level (5–10 ml) were centrifuged at 15000 g for 3 min., and the supernatants kept frozen until the whole set was collected for analyses.

Analytical methods

Determination of methanol was done with a colorimetric enzymatic method as described previously (Kaszycki & Koloczek 2000). Formaldehyde and its chemical derivatives were determined by means of two independent colorimetric techniques. The concentration of the monomeric FD form ("free", unbound FD) was measured with a Nash reagent (Nash 1953), as described earlier (Kaszycki & Koloczek 2000). The total FD load, i.e., the sum of monomeric and chemically bound fractions, was determined with a simplified chromotropic method (Kaszycki et al. 2001), based on standard procedures (Polish Norms 1977, 1985) involving chromotropic acid (1,8-dihydroxynaphtalene-3,6-disulfonic acid, disodium salt) reagent in strong acid (H_2SO_4). For both methods, standard curves were generated using defined FD solutions after hydrolysis of paraformaldehyde. For urotropine determination, only a chromotropic method was used since the Nash reagent-based procedure proved to give unreliable results.

COD measurements were performed using a titrimetric technique with closed reflux and sodium dichromate digestion in sealed tubes, according to a standard method (Polish Norm 1987). Gas-chromatography analyses of wastewater samples were performed using a CHROM 5 gas chromatograph. Methanol and FD determinations were done using a HayeSep R column (150 × 3 mm), equipped with a TCD (thermal conductivity detector), at a temperature of 130 °C. Determinations of urotropine and trioxane were performed on a SILAR 5CP column (2 m × 3 mm) with a FID (flame ionization detector), at 190 °C.

Results and discussion

Integration of methylotrophic yeast with the activated sludge

The process of the adaptation of *H. polymorpha* cells to the activated sludge biocenosis was observed in several independent activated sludge samples from biological wastewater treatment stations of chemical plants (samples 1–3 in Table 1) as well as in a sample originating from a typical municipal wastewater treatment

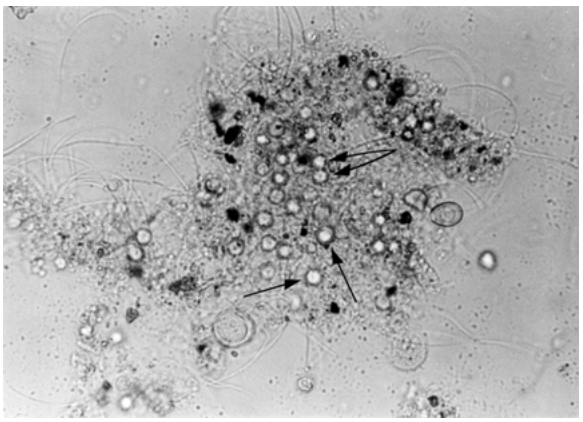

Figure 1. Microscopic image (magnification 300×) of the activated sludge floc fragment containing integrated *Hansenula polymorpha* cells (indicated by arrows), after 24 h of preincubation and another 24 h of biodegradation experiment, as described in Figure 2.

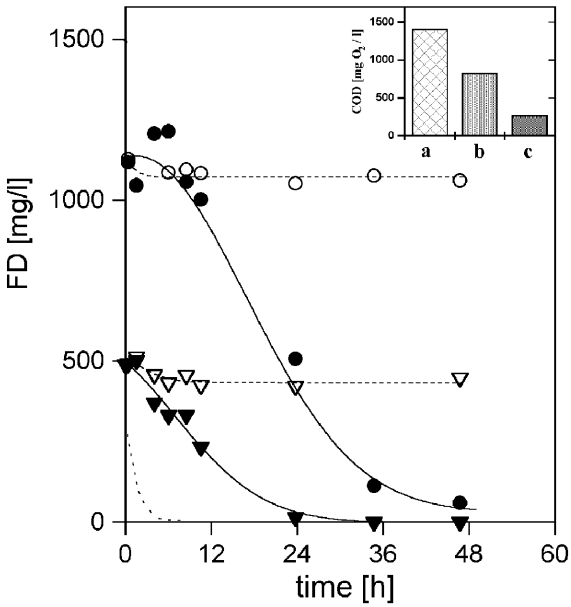

Figure 2. Formaldehyde biodegradation kinetics obtained in a wastewater type 1 at 25 °C with *H. polymorpha* cells (3×10^6/ml) integrated with the activated sludge (filled symbols) from a biological wastewater treatment station of a chemical syntheses plant. Circles and triangles represent experiments performed in samples 1.1 and 1.2, respectively. Open symbols (dashed lines) show the reference biodegradation activity of the original, unmodified activated sludge in both wastewater specimens. The dotted line represents biodegradation kinetics obtained either with the yeast-bioaugmented activated sludge or the activated sludge alone, at initial FD concentration of 300 mg/l. The insert shows the COD level determined for the wastewater sample before the experiment (bar a), and after a 24 hour treatment with the activated sludge alone (bar b) and the activated sludge integrated with methylotrophic yeast (bar c).

plant (sample 4). Microscopic morphological observations of yeast cells and of sludge flocs were performed along with *H. polymorpha* viability tests using yeast-selective media. The results indicate that in all the samples studied the methylotrophic yeast could integrate with activated sludge microorganisms and that the yeast remained physiologically active throughout the process. No significant changes in the structure of the flocs nor in the bacterial sludge content were observed in the presence of yeast cells. However, microscopic observations clearly showed that after 24 hours of incubation, most of the yeast cells were preferentially located within floc structures with only a few singular cells still present outside flocs (see Figure 1 for an example). The modified activated sludge retained its original sedimentation parameters, which is of great importance for effective clarification of the treated wastewater.

Yeast cell viability studies and hemocytometer population estimation in prolonged incubation with the activated sludge samples revealed a slow decrease in *H. polymorpha* cell number (down to 50% in 24 h, not shown). Yet, young and gemmating organisms could still be seen and the cells remained viable. The above facts imply that after introduction of yeast culture to the activated sludge suspension containing a number of various species, *H. polymorpha* was built into the trophic chain of a stabilized, mature sludge biocenosis and evidently it did not have a negative impact on the sludge condition. It should be mentioned here that other yeast species were also identified to be present as integral activated sludge microorganisms in the original samples taken from a municipal wastewater treatment plant (wastewater type 4). These yeasts, however, were found not to be methylotrophic and appeared at relatively low numbers (up to 10^4 cells/ml as determined by surface plating tests), and they revealed morphology which was substantially different from *H. polymorpha*.

An example of the biochemical activity of activated sludge augmented with methylotrophic yeast is presented in Figure 2. The biodegradation experiment was performed 24 h after the addition and preincubation with *H. polymorpha* cells. Both the original and yeast-augmented activated sludge revealed similar biodegradation kinetics at formaldehyde concentrations up to 300 mg/l (dotted line in Figure 2). However, at higher FD load in real wastewater (e.g., 500 mg FD/l in sample no.1.2, Figure 2, triangles) the activity of the original sludge was strongly hampered (Figure 2 ▽) whereas the sludge containing the integrated methylo-

trophic yeast remained effective up to about 1100 mg/l formaldehyde (Figure 2 ●, sample no. 1.1).

It should be pointed out that after the integration experiments, the yeast-bioaugmented activated sludge was applied to treat the original wastewater fluxes characterized by a very high FD content (Table 1). Normally, the wastewater inflow is diluted on entering group treatment chambers, resulting in significantly lowered effective FD concentration to which the biocenosis of the activated sludge must adapt. According to our data, the FD concentration exceeding 1000 mg/l had a significant negative impact on physiological activity and the structure of the activated sludge: such a high FD level led to floc fragmentation and disruption as well as to the decreased viability of several species (such as *Ciliata*). On the other hand, it is typical that the chemical content of industrial wastewater is highly variable and depends on particular technological processes applied as well as on seasonal changes in production. In the formaldehyde and methanol industry, the load of each compound determined in independent specimens of a particular wastewater type was found to vary several fold (cf. Table 1). Thus, it is not unusual that in wastewater treatment stations the content of xenobiotics may exceed the adaptational capabilities of the activated sludge. This further supports the idea of augmenting the activated sludge with FD-tolerant strains of methylotrophic yeast, which would make the active biocenosis even more resistant to seasonal changes in formaldehyde (and methanol) concentration.

Biodegradation of formaldehyde derivatives

In previous studies (Kaszycki & Kołoczek 2000; Kaszycki et al. 2001) we have shown that both monomeric formaldehyde and methanol could be effectively utilized in synthetic and real wastewater by methylotrophic yeast. We have also reported that *H. polymorpha* could physiologically adapt and then grow and proliferate in various industrial wastewater environments, containing free and bound forms of formaldehyde.

Formaldehyde derivatives often add much to the total load of this compound in industrial wastewaters, as seen by the chromotropic analysis. This technique allows the determination of both monomeric and chemically-bound FD and is recommended as the most reliable way to monitor formaldehyde pollution levels (e.g., Polish Norms 1977, 1985). Some of the complex FD derivatives that are soluble in water are

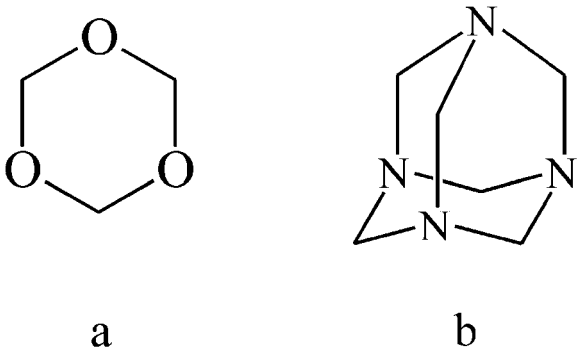

Figure 3. Chemical structure of formaldehyde derivatives present in wastewater samples used in the studies: (a) trioxane (trioxymethylene, TRX); and (b) urotropine (hexamethylenetetramine, URT).

especially difficult to decompose because they cannot be recycled or incinerated. In addition, their biological degradation is often ineffective which considerably prolongs the time required for wastewater treatment with activated sludge in aeration chambers. Thus, intensifying the biological process of utilization of such compounds before they reach surface waters or municipal waste treatment stations has great economic value.

So far, little or nothing has been done concerning the use of eucaryotic microorganisms in the treatment of wastewaters containing FD derivatives as well as a number of other xenobiotics. The applicability of yeast might be advantageous because of their physiological and metabolic elasticity, adaptation capabilities to extreme environmental conditions, genetic stability, sexual recombination, and finally, the possibility of obtaining highly specialized mutants using elaborate methods of selection and testing.

In the present work we have investigated biodegradation processes in industrial wastewater containing, apart from FD monomer and methanol, typical FD chemical derivatives: urotropine and trioxane (Figure 3).

Urotropine (URT) represents the FD-ammonia-type condensates used in food preservation and in various chemical syntheses (e.g., production of explosives). Trioxane (TRX), a cyclic alkylether FD derivative, as well as its water soluble oligomers, are used in synthetic materials production. TRX is not a toxic pollutant; however, it is barely biodegradable and adds to the total waste FD load as monitored by a chromotropic reaction.

The original wastewater samples examined were often found to be highly toxic, both to the activated

sludge and to methylotrophic yeast cultures. Normally, technological waste fluxes are diluted to less aggressive, non-lethal concentrations when they flow in and mix in group treatment station chambers. Since the research was made using the original raw fluxes, detailed toxicity tests were always performed and appropriate wastewater dilutions were used in further studies, as indicated in figure legends.

In the case of the wastewater with high urotropine content (wastewater samples 2.1 and 2.2 in Table 1), biodegradation with *H. polymorpha* was very poor at the original pH of 8.1 (see below). However, in acidic media (pH 4.5), as shown in Figure 4A, the total FD concentration clearly decreased during incubation with methylotrophic yeast suspension, as compared to the control FD level. It should be noted here that the URT content in wastewater no. 2. was approx. 80% of the total FD determined (cf. Table 1). Therefore, the 74% FD biodegradation efficiency observed in the 48 h incubation (Figure 5, bar f) implies that, apart from a complete degradation of monomeric FD, at least 50% of its bound form was utilized. Together with the decay of formaldehyde and oxidation of other compounds present in the specimen, the COD value decreased significantly during the course of the experiment (Figure 4B). Methanol present in URT-containing samples was always totally biodegraded (not shown).

In our opinion, the poor degradation of URT at pH 8.1 was caused by the stability of the molecule at basic pH. A slight decrease of FD level (about 9% in 48 h, Figure 5, bar e) was due to the effective degradation of a FD monomer. Bars a and b of Figure 5 prove that free formaldehyde could be effectively utilized at both acidic and basic environments. In the case of URT biodegradation, satisfactory results were obtained only at acidic conditions, upon lowering pH down to about 4.5 (Figure 5, bar f). This result can be explained by the decomposition of urotropine into easily assimilated substrates: FD monomer and ammonia. It is essential that the acidity of the environment be kept constantly low, since the increase in ammonia concentration may cause the medium to become more basic. This could in turn slow the process of disrupting URT molecules and thus make them less susceptible to biodegradation. Such an effect can be partially compensated by the acidification caused by the yeast metabolism (see below). Still, in order to keep the pH below 6.0, an additional titration of the medium with HCl was – in some cases – required.

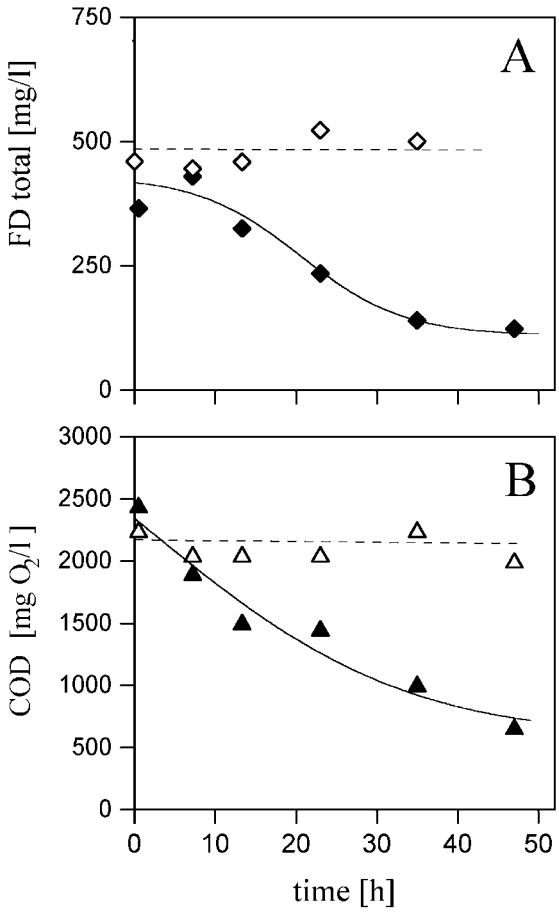

Figure 4. Biodegradation kinetics in a 5 times diluted wastewater sample no. 2, containing urotropine as a main constituent (approx. 80% of total formaldehyde), with *H. polymorpha* preadapted cell suspension (2.3×10^6 cells/ml) at pH 4.5. Section A represents the total FD content; section B – the COD decrease during the experiment. Open symbols (dashed lines) show respective control levels found for uninoculated wastewater.

Similar results of urotropine biodegradation in pure URT solutions were also obtained in a synthetic wastewater, that is in a minimal, defined medium, at low pH (Figure 5, bar d). In such a model system, at pH 5.3, *H. polymorpha* dense culture (at about 10^7 cells/ml) was able to biodegrade URT at initial concentrations up to 1600 mg/l (11.4 mM). Note that in URT biodegradation experiments presented in Figure 5, bars d (44%) and f (74%), show the percentage of total FD degraded within a particular (48 h) time. So, these conditions only represent some intermediate stages of biodegradation processes which were then completed within the next 2–3 days depending on the initial xenobiotic concentration. When the yeast cell

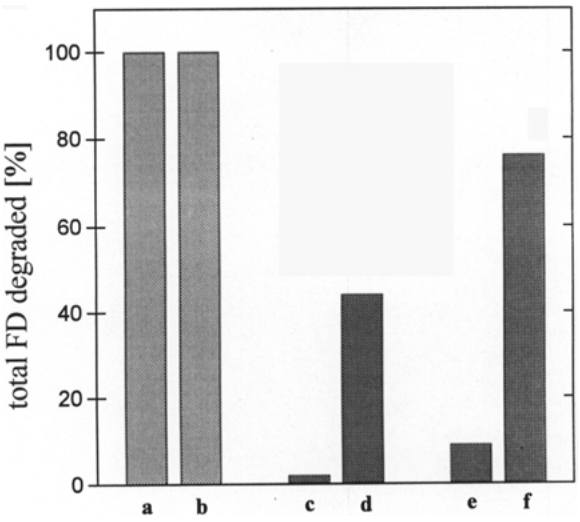

Figure 5. Formaldehyde biodegradation efficiency in wastewater samples containing monomeric formaldehyde (light gray bars) and urotropine (dark gray bars), incubated at 25 °C with *H. polymorpha* dense cell culture (≈10^7 cells/ml) at basic (bars: a, c, e) and acidic (bars: b, d, f) conditions. *Bars a and b*: degradation of FD obtained after 24 h in a real industrial wastewater (bar a), at initial concentration of FD monomer of 1750 mg/l, at pH 7.7, and in a model wastewater (bar b), at initial concentration 700 mg/l, at pH 5.3; *bars c and d*: 48 h degradation of urotropine (initial concentration 1600 mg/l) in a model wastewater, at pH 9.0 (bar c) and pH 5.3 (bar d); *bars e and f*: 48 h degradation in urotropine-containing industrial wastewater (approx. 80% of the total FD content) at pH 8.1 (bar e) and pH 4.5 (bar f).

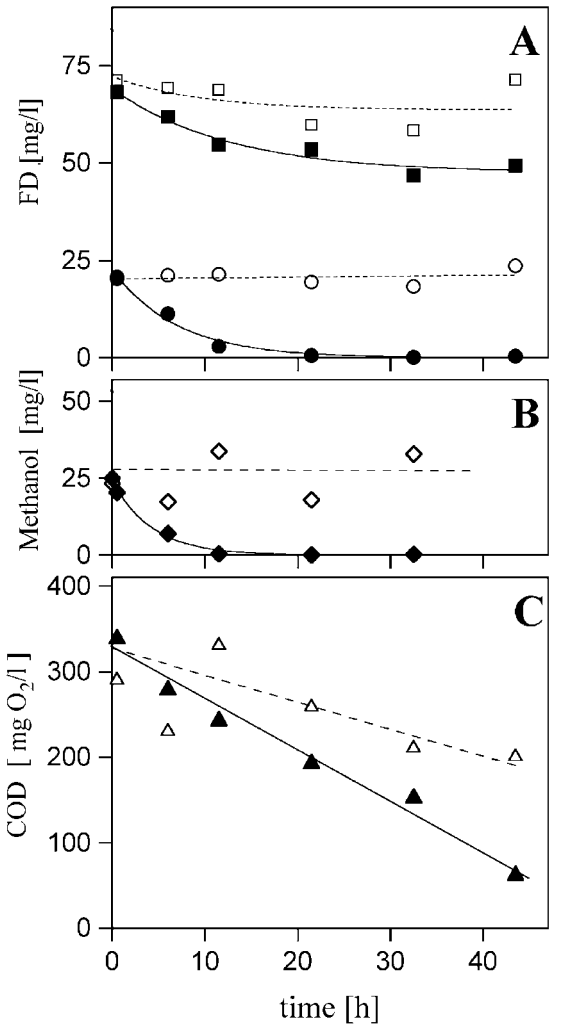

Figure 6. Biodegradation kinetics of formaldehyde (A) and methanol (B) with *H. polymorpha* preadapted cell suspension (9×10^6 cells/ml) at 25 °C, pH 9.0, in a 10 times diluted wastewater sample no. 3.2, containing monomeric FD (20 mg/l), trioxane and its polymers (50 mg/l), and methanol (25 mg/l). In section A, formaldehyde level is given as total FD (squares), and as free FD monomer (circles). Section C presents the COD decrease in the sample during the experiment. Open symbols (dashed lines) in all sections represent respective values obtained for the control sample (uninoculated wastewater).

culture was grown on URT as the only carbon source at pH 5.0, the observed biomass increase was comparable to that obtained in methanol-supplemented media (not shown), which indicates that in such conditions URT was indeed easily assimilated.

We note that Figures 4 and 5 show biodegradation kinetics obtained at 25 °C, that is at conditions typical of what occurs in wastewater treatment stations (Horan 1990). Even better results were obtained at physiologically optimal temperatures of 37 °C, at which the degradation rates were increased by up to about 30%. Likewise, at temperatures lower than 20 °C, the process of xenobiotic utilization was considerably slower. This temperature-dependent biochemical activity should be taken into account especially when the application of single methylotrophic yeast monocultures as biological filters is considered.

We suggest that the acidification of the environment may facilitate biodegradation of other formaldehyde complex derivatives which are unstable at low pH and thus are more susceptible to biological oxidations. Acidic medium would additionally conform to the yeast's pH optimum of 4 to 5 (Gleeson & Sudbery 1988a). Moreover, such an approach would also take advantage of the intense metabolism of *H. polymorpha* which leads to a spontaneous drop of pH in the cultivation medium due to the formic acid produced in the methylotrophic pathway (Sibirny et al. 1988; Maidan et al. 1997).

In TRX-containing wastewater (type 3 in Table 1) samples, as shown in Figure 6A, the monomeric FD component was easily utilized (Figure 6A ●), whereas the ether bond of the trioxane molecule was resistant to the enzymatic action of *H. polymorpha*. The comparison of FD degradation efficiency, as seen by two independent analytical methods, indicates that the level of total formaldehyde (Figure 6A ■) decreased by the same amount as did the monomer (approx. 25 mg/l). In Figure 6, typical kinetics of methanol biodegradation in the studied sample (section B) as well as the decay of the COD value (section C) are also presented. It should be mentioned here that the trioxane inflow is not being treated by a separate treatment station before entering the group wastewater tank, where all the technological fluxes mix. Since it is mixed down, the activated sludge-performed biodegradation of free and bound formaldehyde in trioxane-containing wastewater is made very slow. Hence, substantially prolonged aeration periods are required to lower the COD levels down to environmentally acceptable values.

To our knowledge, most of the data concerning biodegradation of ether linked environmental water pollutants collected to date are based on a few bacterial aerobic and anaerobic systems, although some ether-digesting activities were also reported for *Eucaryota* (Axelrod 1956; Ligocka et al. 1998). Much emphasis has been put on biodegradation of some naturally occurring ethers such as lignin, as well as on utilization of ether-based pharmaceuticals' herbicides and polyalkylene glycols (polyethers) (see Kawai 1987, 1995 for refs), and fuel oxygenates (Deeb et al. 2000; Fayolle et al. 2001). In biological oxidations of alkyl ethers, as discussed by Kawai (1987, 1995), monooxygenases play an important role. Thus, *H. polymorpha* could be useful in cocultures with bacteria producing monooxygenase activities, where the products of the TRX molecule cleavage (FD and methanol) would be ultimately degraded with the methylotrophic reaction pathway.

Conclusions

Hansenula polymorpha can be used to increase the effectiveness of treating formaldehyde-containing wastewaters with biological method by means of the enzymatic activities of methylotrophic pathway.

1. The yeast species proved to be active in various types of industrial wastewater as well as in activated sludge samples from chemical and household wastewater treatment plants.
2. The yeast-bioaugmented activated sludge revealed enhanced resistance to high FD concentrations and dramatically increased the biodegradation of formaldehyde.
3. At least some of formaldehyde derivatives, as exemplified by urotropine, could be biodegraded by means of treatment with the methylotrophic yeast, provided the process was being performed with an acidic pH and a temperature above 20 °C.
4. Trioxane was not utilized due to the lack of specific enzymatic activities which would enable the yeast to cleave the degradation-resistant ether bond into formaldehyde and methanol. However, methylotrophic yeast might be applicable as an integral part of the activated sludge biocenosis which reveals such biochemical activities.
5. Two alternative approaches to the treatment of formaldehyde-containing wastewater can be considered as optional and verified by appropriate technical tests:
   (a) the use of methylotrophic yeast monocultures in separate chambers as biological filters specialized in biodegradation of single-carbonic compounds. In such a case the cell biomass formed on assimilated xenobiotics may be further utilized in a variety of agricultural applications as a source of vitamins and protein.
   (b) directed modification of the activated sludge by the introduction of methylotrophic yeast and then subsequent integration of the yeast cells with the original sludge biocenosis.

Acknowledgements

We appreciate the help of Ms. M. Tyszka in handling activated sludge samples and in some of the measurements. This work was supported by Polish Research Committee Grant Projects no. 6 P04G 033 11 and 6 P04G 084 18.

References

Axelrod J (1956) The enzymatic cleavage of aromatic ethers. Biochem. J. 63: 634–641

Bardana EJ Jr. & Montanaro A (1991) Formaldehyde: an analysis of its respiratory, cutaneous, and immunologic effects. Ann. Allergy 66: 441–452

Chang CC & Gershwin ME (1992) Perspectives on formaldehyde toxicity: separating fact from fantasy. Regul. Toxicol. Pharmacol. 16: 150–160

Deeb RA, Scow KM & Alvarez-Cohen L (2000) Aerobic MTBE biodegradation: an examination of past studies, current challenges and future research directions. Biodegradation 11: 171–186

Fayolle F, Vandecasteele JP & Monot F (2001) Microbial degradation and fate in the environment of methyl tert-butyl ether and related fuel oxygenates. Appl. Microbiol. Biotechnol. 56: 339–349

Gleeson MA & Sudbery PE (1988a) The methylotrophic yeasts. Yeast 4: 1–15

Gleeson MA & Sudbery PE (1988b) Genetic analysis in the methylotrophic yeast *Hansenula polymorpha*. Yeast 4: 293–303

Harder W, Trotsenko YA, Bystrykh LV & Egli T (1987) Metabolic regulation in methylotrophic yeast. In: van Verseveld, HW & Duijne JA (Eds) Microbial Growth on C1 Compounds. Proceedings of the 5th International Symposium (pp. 139–149). Martinus Nijhoff Publ., Dordecht

Hawari J, Halasz A, Beaudet S, Paquet L, Ampleman G & Thiboutot S (2001) Biotransformation routes of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine by municipal anaerobic sludge. Environ. Sci. Technol. 35: 70–75

Horan NJ (1990) Biological Wastewater Treatment Systems. Theory and Operation. John Wiley and Sons, Chichester

Instruction of Polish Ministry of Environmental Protection, Natural Resources, and Forestry, dealing with water resources classification and conditions on wastewater offtake to ground and water (1991) Dziennik Ustaw RP no 116, pos. 503, pp. 1579–1583

Kaplan DL, Hartenstein R & Sutter J (1979) Biodegradation of polystyrene, poly(methyl methacrylate), and phenol formaldehyde. Appl. Environ. Microbiol. 38: 551–553

Kaszycki P & Kołoczek H (2000) Formaldehyde and methanol biodegradation with the methylotrophic yeast *Hansenula polymorpha* in a model wastewater system. Microbiol. Res. 154: 289–296

Kaszycki P, Tyszka M, Malec P & Kołoczek H (2001) Formaldehyde and methanol biodegradation with the methylotrophic yeast *Hansenula polymorpha*. An application to real wastewater treatment. Biodegradation 12: 169–177

Kawai F (1987) The biochemistry of degradation of polyethers. CRC Crit. Rev. Biotechnol. 6: 273–307

Kawai F (1995) Breakdown of plastics and polymers by microorganisms. Adv. Biochem. Eng. Biotechnol. 52: 151–194

Ligocka D, Sapota A & Jakubowski M (1998) The disposition and metabolism of 1,3,5-[U-14C]trioxane in male Wistar albino rats. Arch. Toxicol. 72: 303–308

Maidan NN, Gonchar MV & Sibirny AA (1997) Oxidation of exogenous formaldehyde in methylotrophic and non-methylotrophic yeast cells. Biochemistry (Mosc) 62: 636–640

Michalik J (1975) Methanol in the metabolism of microorganisms. Post. Mikrobiol. 14: 93–109 (in Polish)

Nash T (1953) The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochemistry 55: 416–421

Omil F, Mendez D, Vidal G, Mendez R & Lema JM (1999) Biodegradation of formaldehyde under anaerobic conditions. Enzyme Microbial. Technol. 24: 255–262

Otake Y, Kobayashi T, Asabe H, Murakami N & Ono K (1995) Biodegradation of low-density polyethylene, polystyrene, polyvinyl chloride, and urea formaldehyde resin buried under soil for over 32 years. J. Appl. Polymer Sci. 56: 1789–1796

Polish Norm PN-71 C-04593 (1977) Water and Waste Water. Determination of Formaldehyde Content. Polish Committee of Normalization and Measure

Polish Norm PN-74 C-04578/03 (1987) Water and Waste Water. Tests for Chemical Oxygen Demand and Organic Carbon Content. Determination of Chemical Oxygen Demand (COD) by Dichromate Method. Polish Committee of Normalization and Measure

Polish Norm PN-76 Z-04045/04 (1985) Determination of Formaldehyde in Work Places by Colorimetric Method with Chromotropic Acid. Polish Committee of Normalization and Measure

Restani P & Galli CL (1991) Oral toxicity of formaldehyde and its derivatives. Crit. Rev. Toxicol. 21: 315–328

Sibirny AA, Titorenko VI, Gonchar MV, Ubiyvovk VM, Ksheminskaya, GP & Vitvitskaya OP (1988) Genetic control of methanol utilization in yeasts. J. Basic Microbiol. 28: 293–319

Walker JF (1964) Formaldehyde, 3rd Edn. series no. 159. Reinhold Publ. Co., NY, London

Zijin L & Hegemann W (1998) Anaerobic toxicity and biodegradation of formaldehyde in batch cultures. Water Res. 32: 209–215