BIOCATALYSIS IN NON-CONVENTIONAL MEDIA

PT.2

C: GASEOUS AND (NEAR-)SUPERCRITICAL MEDIA

Biocatalysis in Non-Conventional Media, edited by J. Tramper et al.

The use of amylolytic and proteolytic enzymes in art restoration

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Abstract

Drawings on paper have very often been reinforced with another cellulosic support. As the paste (starch or collagen) becomes yellow and brittle with time, it is necessary to remove it. Proteolytic and amylolytic enzymes may help to hydrolyse the pastes. A knowledge of the physico-chemistry of papers and pastes helped us in the understanding of the diffusion of enzymes through papers and substrate accessibility. The use of a non ionic surfactant (Brij 35) to improve the diffusion rate and substrate accessibility and lower primary alcohols to stabilise pigments have been studied.

A new tool in art restoration

Works of art may be viewed as a technique (the overlying paint) and a support : canvas, wood panel, paper, cardboard and so on. The ethic in art restoration is to avoid as much as possible any interference with the technique itself whereas the support is very often the object of the restoration. Ever since paper was first used for drawings, it has generally been treated as a weak material and as so, it has been systematically reinforced with another support (paper cardboard or canvas) lined on its back. Linings were traditionnally made of starch paste and/or animal glue [1].

Pastes

The starch paste is basically a polymer of α-D anhydroglucopyranosid with α-1,4 bonds only for amylose and α-1,6 (4 to 6%) for amylopectin. The physico-chemical interactions of starch with water at different temperatures is not well known [2], but the adhesive properties have been used empirically for ages. The adhesive is obtained when starch is dispersed in hot water, and left to chill for retrogradation [3]. The pasting characteristics depend on several parameters including the vegetal origin of the starch, the concentration, the temperature and the duration of heating, the mixing conditions and so on.

Animal glues are prepared by extracting partially hydrolysed collagen from the skin or bones of mammals or fishes. Collagen is a very cohesive protein with a triple helicoïdal macrostructure composed of the repetition of the triplet PRO-X-Y where X is frequently Prolin and Y, Hydroxyprolin [4]. Solubilised fibers of collagen heated above 30°C become viscous and constitute a cohesive network due to hydrogen bonds. These hydrogen bonds may also occur between collagen and an organic support but the glueing power depends on the physico-chemical properties of the support like capillarity, pH, surface charges...[5].

As they get old, pastes and glues acidify and yellow [6] and the drawings become brittle. Distorsions (ridges and valleys on the paper) are observed between the drawing and its lining. In this case it is necessary to remove the lining as a first step of restoration. With fragile works of art, the removal tends to be time consuming and particularly delicate, requiring a very accurate tool. The reason why amylolytic and proteolytic enzymes are attractive in the hydrolysis of pastes as catalysts, is their narrow specificity which avoids interference with the technique. Whereas the usual removal methods only cause the pastes to swell, enzymes lead to their complete hydrolysis and removal can then be carried out with as less traction on the paper as possible. Since drawings lined on a cellulosic support represent a highly heterogeneous system, Michaelien enzymology is not suitable. The substrate is immobilized inbetween two papers which constrain the diffusion of the free enzymes : an heterogeneous medium in non-conventional conditions

Enzymes diffuse through the backing paper, then hydrolyse the paste and the product must be released quickly as a high local concentration will inhibit enzyme activity.

Papers

Papers are made of cellulosic fibers which constitute an overall heterogeneous macrostructure. Heterogeneity does not mean chaotic disposition of the fibers : a preferential orientation makes the paper have specific mechanical properties [7]. The physico-chemistry of paper depends on polyelectrolytes and acids or bases added to the paper but it is the weak chemical interactions (hydrogen bonds, Van der Waals interactions) which take place at crossings of the fibers which are responsible for the cohesivity of the paper [8]. The aggregates of cellulosic polymers could be interpreted in terms of percolation [9]. Since paper is a heterogeneous medium and there is such a wide variety in the different types of paper, it seemed necessary to study the diffusion of enzymes through the particular papers used. The effects of a non ionic surfactant on transport phenomenon were also assessed.

Paste hydrolysis in low water environment

For some techniques, a large amount of water could be disasterous (water colours, gouaches and some inks) because some pigments and some media are very fugitive in water. Fortunately some of these techniques are stable in organic solvents or water-miscible organic solvents. An aqueous solution of lower alcohols is satisfactory for pigment stability [10] but we must emphasize that there are no general rules for pigment stability. According to Cooper et al. many inks are stable in ethanol and 2-methoxyethanol or their aqueous

solutions. We decided to use the former alcohols in our experiments upon the stability and the catalysis in a low water environment.

Enzyme activity on pasted paper

The pasted papers were soaked in different enzymatic solutions for a fixed time period and a controlled temperature. A six channel recorder has been built to measure the length of removed paper versus time.

The area under the curve is called enzymatic work (force*time) and the ratio of the real enzymatic work and the theoretical work gives an efficiency of removal (E %) applied to the linings. In substracting the efficiency of the blanks, we obtain the difference of efficiency (DE %).

Diffusion of enzymes through papers

The paper is placed in between a two compartment diffusion cell. The diffusion of enzyme through the papers is measured by U.V. spectrophotometry (210 nm) for protein quantification.

Let "e" be the thickness of the paper, "S" the surface of diffusion, "V" the volume of a compartment, "D" the diffusion coefficient, "C_a" and "C_b" the protein concentrations in the compartments A and B.

$$C_o = C_a + C_b$$

The evolution of the protein concentration in the compartment B as a fonction of time is given by:

$$dC_b/dt = S(C_o - 2C_b)D / Ve$$

$$\text{Let} \qquad a = D S / V e$$

$$dCb / dt = a (C_o - 2C_b)$$

$$Ln (C_o/ (C_o - 2C_b)) = 2 a t$$

RESULTS

1-Diffusion

Diffusion coefficients of α-amylases through Canson and Johannot papers were 4,39 10^{-3} $cm^2.min^{-1}$ and 2,14 10^{-3} $cm^2.min^{-1}$ respectively whereas the diffusion coefficients of glucose were 0,74 $cm^2.min^{-1}$ and 0,31 $cm^2.min^{-1}$, approximately 150 times more. Diffusion of the enzymes appeared to be the limiting factor for the papers studied. The diffusion of α-amylase through lined papers was also assessed. The diffusion coefficient was similar to the one for α-amylase through Canson paper but a delay was observed at the beginning. This delay represents the diffusion time through Johannot paper and the hydrolysis of a small volume of the starch paste. It appeared to be simply proportional to the thickness of the starch paste (fig. 1).

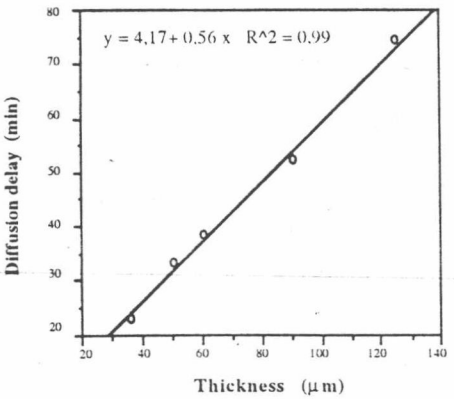

figure 1 : α-amylase diffusion delay is proportional to starch paste thickness

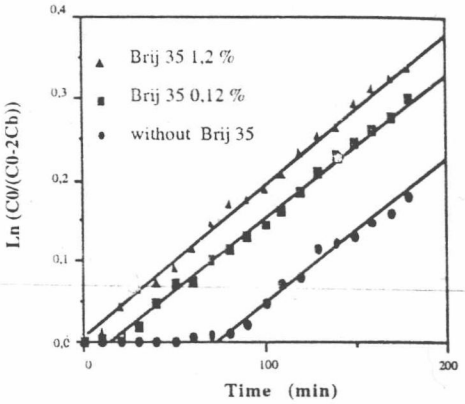

figure 2 : Brij 35 reduces the diffusion delay of α-amylase

Non ionic surfactants are known to preserve enzyme activity [11]. We used Brij 35 to improve the diffusion of amylase through papers and to give a better accessibility to the substrate (paste). Firstly, above the critical micellar concentration (CMC), Brij 35 slightly improved the diffusion coefficient of amylase through the papers. In addition to this a recent publication [12] gave evidence that cellulosic polymers have low affinity for non ionic surfactant.

The papers used were hydrophobic (Canson more than Johannot) because of their gelatin or starch surface layer. Presoaking both papers before our diffusion experiments partly solubilised the gelatin or starch on the surface of the papers and as a consequence we observed a poor improvement in the diffusion. Secondly, the initial delay in the diffusion was significantly reduced by increasing the concentration of Brij 35, showing that the main improvement when using Brij 35 is due to the better substrate accessibility (fig. 2).

The weak interactions of retrograded starch are responsible for its low solubility and partially crystalline structure [13]. For this the enzymatic hydrolysis is much slower on retrograded starch than the hydrolysis of soluble starch. An hypothesis has arisen that non ionic surfactants enhance enzyme desorption from insoluble substrates [14], which accelerates the hydrolysis.

The diffusion of proteases through animal glues has not been studied. However, it is well known that hydrophobic side groups of proteins in aqueous solutions tend to be folded to the interior of the protein molecule while polar groups are on the surface. The drying process is a complex phenomenon which leads to a rearrangement of the structure and finally lowers the solubility in water [6].

2-Enzyme concentration and removal

Whereas enzymes in solution display a proportionality between their concentration and their activity, the shape of our plots exhibits a threshold above which D.E. reaches a maximum. The threshold was reached for 20 mg/l of α-amylase and 100 mg/l for collagenase at 30°C (fig. 3).

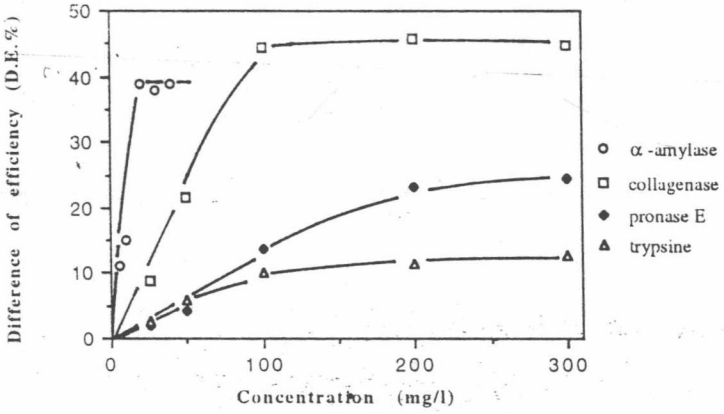

fig 3 : Enzyme concentration and removal

3-Temperature and removal

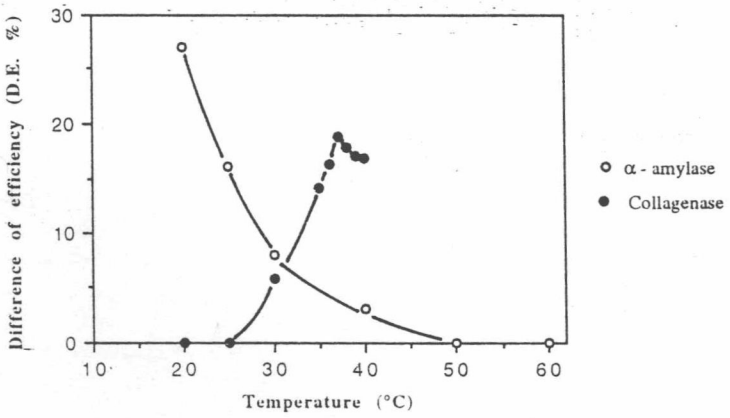

fig 4 : temperature and removal

D.E of removal using α-amylase on starch paste decreased with the temperature because the starch gels swell more rapidly with temperature than the α-amylase activity increases. The reverse phenomenon is observed for skin glues using collagenase. D.E. increases with temperature until it reaches the threshold corresponding to the temperature of solubilisation of the glue (fig. 4).

4- Removal with alcoholic solutions and non ionic surfactant

Alcohols and α-amylase activity

Aqueous solutions of ethanol and 2-methoxyethanol strongly inhibit α-amylase activity in solution. We checked that even above the CMC (0,0001 M or 0,1% W/V), Brij 35 did not modify α-amylase activity. For concentrations of Brij 35 above the CMC, the surfactant protected the enzyme from denaturation in a limited extent (fig. 5). Similar results were already shown for other enzymes in reversed micelles [11].

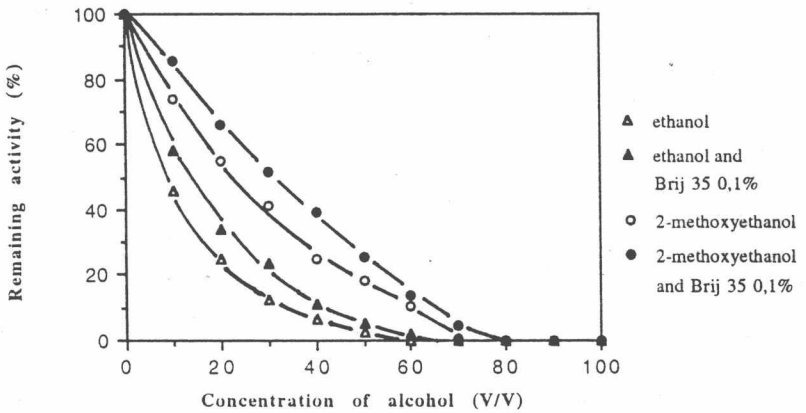

fig 5 : remaining activity of α-amylase in alcoholic solutions with and without Brij 35

The effect of Brij 35 on removal

We checked that, without the enzyme, the surfactant had only a limited effect on removal (less than 5% of D.E.). With the addition of 0,1% of Brij 35 in the enzymatic solution, the D.E. of the treatment dramatically increased at low temperature (25°C). The D.E. doubles with α-amylase for 30 minutes of treatment on the starch linings and, with collagenase, there is an increase by a factor of seven on rabbit skin glue (fig. 6). Even if the surfactant may enhance the solubilisation of some pigments and some media, the reduction in treatment duration could reduce this risk. However, the presence of Brij 35 could be satisfactory for most of old master engravings.

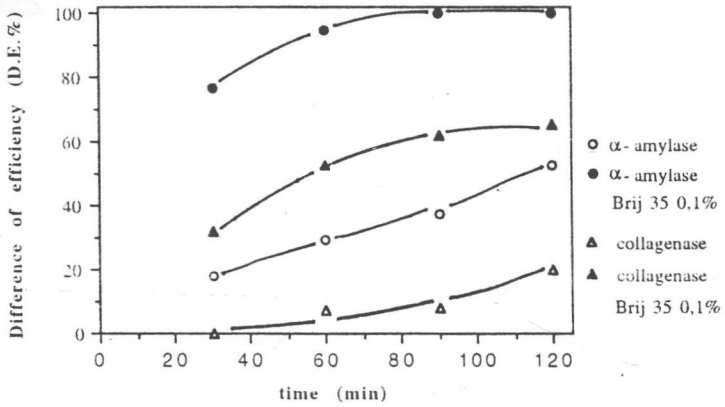

fig 6 : influence of Brij 35 on removal at 25°C

Removal with alcoholic solutions and surfactant

We previously discussed the problem of fugitive pigments and the possibility for using alcoholic solutions with a non-ionic surfactant to protect the enzyme from denaturation. We used a ternary solution of 2-methoxyethanol, Brij 35 and amylase in water to reach a compromise between the duration of the treatment, the efficiency and a possible reduction of pigment unstability (fig. 7).

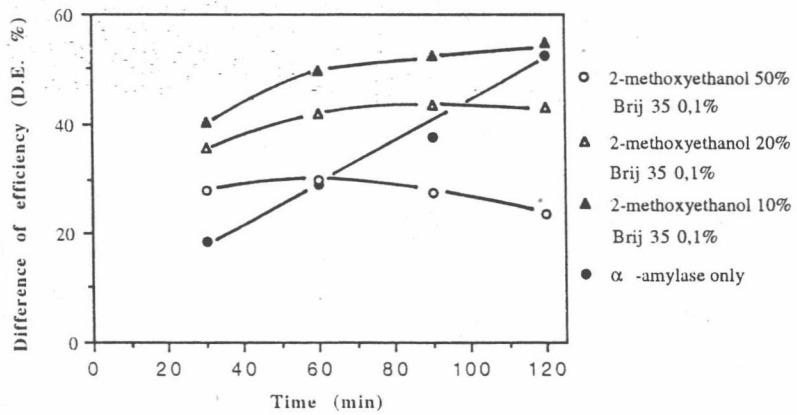

fig 7 : the use of ternary solutions (alcohols, Brij 35 and water) for paper removal

Conclusion

The medium that has been studied is highly complex and depends on many parameters which are not completely understood. Under the conditions of our work we have found evidence that efficient enzymatic treatments were obtained at low temperatures and with reduced enzyme concentration. The papers studied were hydrophobic because of their surface layer. Under these conditions the diffusion of enzymes through paper and paste mainly constrained the efficiency of removal. The diffusion is significantly improved when using a non ionic surfactant even below CMC concentration, but surfactants tend to increase pigment solubilisation whereas lower primary alcohols tend to stabilise them. The use of ternary solutions of surfactant, alcohol and water could maintain a good enzymatic activity, a good diffusion rate and a good pigment stability. Further studies concerning hydrolysis in low water environments to preserve pigment stability will be carried out. To this end we intend to use entrapped enzymes in reversed micelles in organic solvents [15], [16].

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Methyl Isobutyl and Methyl Ethyl Ketone Biodegradation in Biofilters

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Abstract

Microbiological waste gas treatment is a most promising new development in environmental biotechnology. Herein effective methyl ethyl ketone (MEK) vapour removal and effective methyl isobutyl ketone (MIBK) vapour removal from polluted air streams are demonstrated and various aspects of such treatment processes identified for subsequent investigation. Biofilters of the type employed provide an example of microbes functioning effectively in a non-conventional medium.

1. INTRODUCTION

Microbiological waste gas treatment represents a major development in environmental protection. Until some 30 years ago the concept of using microbes for pollutant removal from waste gases had not been subject to serious consideration [1]. However, increasingly stringent environmental legislation, together with its more effective enforcement during the past 15 years, has forced technologists to examine hitherto neglected possibilities for efficient and economic waste gas treatment.

The prospective biotreatment of waste gases has resulted in the development of two distinct types of bioreactor systems: biofilters and bioscrubbers. Biofilters are those systems where the resting or growing process culture is attached to a stationary solid support material such that direct contact occurs between the attached microbial film and the humid pollutant containing waste gas stream undergoing treatment. On the other hand, bioscrubbers are those systems where the pollutant containing waste gas stream is contacted with an aqueous solution in which either simultaneous or subsequent biodegradation of absorbed pollutants occurs as a result of the actions of growing microbes in or contacted

with the aqueous scrubbing solution. However, in the case of biofilters, filtration, as strictly defined, is not a pollutant separation mechanism. Pollutants present in waste gas streams can be either permanent gases or vapours. It has been shown that in biofilters pollutant biodegradation is directly associated with the aqueous solubility of the pollutant under consideration. Microbially mediated biodegradation on surfaces in contact with a humidified pollutant containing gas phase clearly represents an important example of biocatalysis in a non-conventional medium.

In the present communication the aerobic biodegradation of both methyl isobutyl ketone (MIBK) vapour as a single gaseous phase pollutant and when mixed with methyl ethyl ketone (MEK) vapour as a pollutant mixture in humid air in packed bed biofilters is considered. Both MIBK and MEK are widely used industrial chemicals with annual productions exceeding 250,000 and 600,000 tonnes, respectively. The nature of use of the two chemicals, i.e., in coatings and as solvents for adhesives, inks and paints, and in the case of MIBK, in the extraction of metal salts from solutions, results in their widespread presence in both concentrated and dilute waste streams as either single or multiple pollutants. In general, concentrated waste streams can be subjected to economic recovery processes, recycling or where a diverse spectrum of pollutants are present, treatment and disposal by incineration. Dilute waste streams frequently present problems with respect to their economic and efficient treatment. Gaseous waste streams, particularly those containing pollutant mixtures, are remarkably problematical in this latter context and, therefore, offer major challenges as far as waste gas biotreatment is concerned.

Fundamental factors relevant to the successful scaling-up and optimization of industrial scale biofilters include the definition of pollutant transfer mechanisms, questions of substrate and nutrient limitation, the rôle of the solid support material and effects resulting from its physical and chemical properties and, by no means least, the needs of the microbial process culture for specific nutrients. In this last matter, the question of the relative effectiveness of resting microbial cells and growing cells for efficient biodegradation requires resolution, but also effects resulting from death by lysis and subsequent "cryptic" growth [2] must be considered.

2. MATERIALS AND METHODS

A schematic diagram of the experimental system used is shown in Fig. 1.

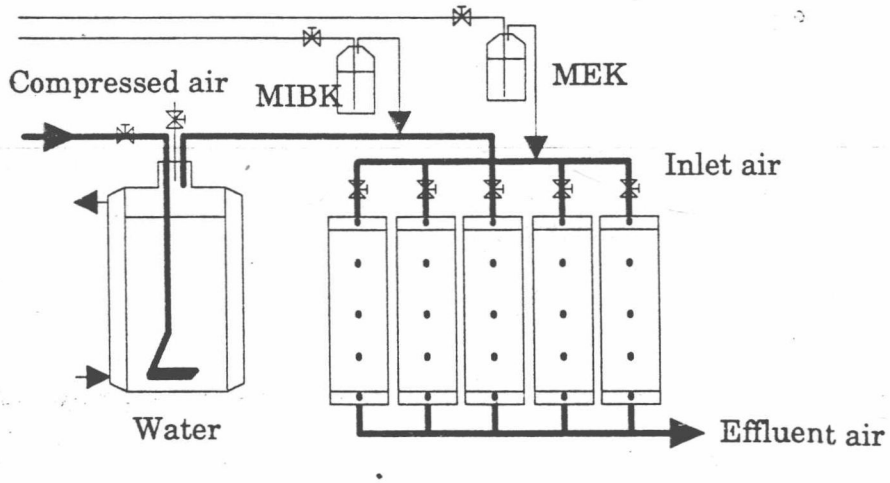

Fig. 1. Schematic diagram of the experimental system

Pollutant containing humid air stream. Compressed oil-free air is saturated with water vapour by sparging the air into a 50 L bottle containing deionized water and thermostated at 28°C. Two smaller compressed air streams were sparged into 0.5 L bottles containing either MIBK or MEK as required and subsequently mixed with the major humidified air stream. A metered flow of pollutant containing humid air stream was passed downwards through each of five vertical packed column biofilters operating under a range of selected parameters.

Biofilters and packing material. The biofilters were constructed from plexiglas tubing and were 1 m in length and 80 mm in internal diameter. The upper and lower 60 mm of each column was packed with expanded clay spheres and the remaining 880 mm with a commercially available biofilter packing (Bioton: ClairTech, Utrecht, NL), comprising an equivolume mixture of compost and polystyrene spheres. Acid neutralizing components are also present in the biofilter material. Before use the packing material was inoculated with a concentrated enrichment culture. Sample ports were located at 0, 25, 52, 78 and 100 percent of the active packing height in each column. Column temperatures were maintained between 21° and 25°C.

Inoculum. Solvent degrading enrichment cultures were grown on MEK and MIBK in shake flasks, with regular transfers, over a period of six months. The inoculum was prepared by concentrating 3 L of enrichment culture to 15 mL by centrifugation. This was sufficient to coat 1 kg of packing material prior to introduction into the columns.

Operating conditions. The active bed volume was 4.4 L and the mass of damp support material, comprising 60 percent water, was 950 g per filter. The void space in the bed was 50 percent. Gas flow rates of 200 to 300 L h^{-1} were used. This gave a surface loading of 40 to 60 m h^{-1} and a volumetric loading of 45 to 70 h^{-1}. The gas had a relative humidity greater than 95 percent and contained between 300 and 1200 mg m^{-3} solvent carbon. The pressure drop over the filter was less than 10 mm water column.

Analysis. The concentrations of MEK, MIBK oxygen and carbon dioxide in gas phase samples were determined by gas chromatography. In the case of MEK and MIBK, 1 mL gas samples were introduced into a Carlo Erba (Milan, I) type HRGC 5160 gas chromatograph fitted with a SE 54 column and operated isothermally at 70°C. The carrier gas used was 1.83 L h^{-1} hydrogen and detection was with a flame ionization detector. The detection limit was ca. 15 mg solvent carbon per m^3 of gas. Oxygen and carbon dioxide were determined by injecting 1 mL gas samples into a Shimadzu (Kyoto, J) type GC-8A gas chromatograph fitted with a combined 5Å molecular sieve / Porapak Q column and operated isothermally at 80°C. The carrier gas used was 2.64 L h^{-1} helium and detection was with a thermal conductivity detector. The detection limit was ca. 30 mg carbon dioxide carbon per m^3 of gas.

3. RESULTS

Results for the performance of a biofilter treating a mixed MEK/MIBK vapour in air are shown in Fig. 2, where the reduction in MEK/MIBK vapour concentration and carbon dioxide production and temperature relative to biofilter height are reported. Whereas an extremely high level of removal (ca. 98 percent) of the pollutant vapours is indicated, the measured production of carbon dioxide only corresponds to ca. 42 percent recovery of the carbon entering the system. The temperature profile in the biofilter showed a relatively slight gradient in the direction of air flow (ca. 1 C°). Several explanations exists for the low carbon recovery. Probably the most plausible is that the biofilter had only been started up 7 days prior to the experiment and the active biofilm of microbes was still being built-up. In similar biofilters where more prolonged operation had occurred,

up to 75 percent carbon recovery was observed. Neither volatile nor gaseous products, other than carbon dioxide or remaining traces of MIBK, could be found in the effluent gas stream. However, a fraction of the carbon dioxide produced was probably absorbed in water films within the biofilter and because of the neutralizing capacity of the packing, carbonate would be expected to be retained within the packing, thus accounting for a part of the missing carbon.

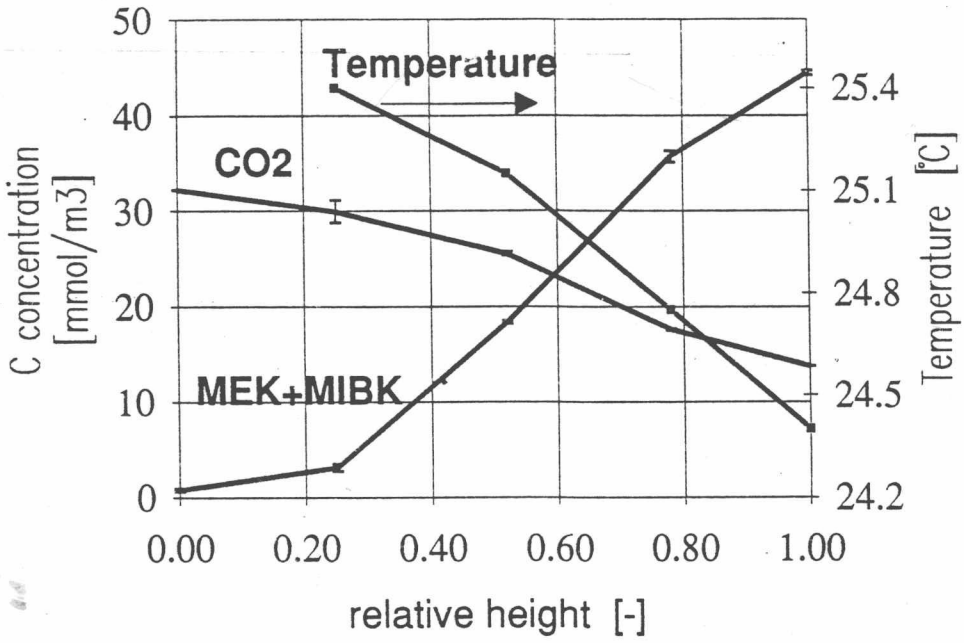

Fig. 2. Solvent, carbon dioxide and temperature profiles as a function of the relative height in the biofilter bed for MEK/MIBK removal from air. (inlet concentrations: MIBK=385 mg m^{-3}; MEK=383 mg m^{-3}; gas flow=284 L h^{-1}).

In Fig. 3, results are shown for a biofilter operating on an air stream containing MIBK alone, in one case, and a MEK/MIBK mixture, in the other case. In the former case, virtually the whole concentration of MIBK vapour is removed in the upper 20 percent of the biofilter. However, in the latter case, MIBK removal occurs throughout the upper 80 percent of the biofilter, but MEK removal only in the upper 60 percent of the biofilter. Clearly the presence of MEK in the air stream has a marked effect on MIBK removal. Both MEK and MIBK

removal were virtually complete and neither vapour could be detected in the effluent stream. Removal occurred simultaneously and in a parallel manner in the upper 60 percent of the biofilter. In shake-flask mixed cultures of enriched biofilter inocula, MEK was utilized as carbon and energy substrate by the cultures grown on MIBK and vice versa.

With respect to MIBK as a single pollutant in the air stream, the hourly biodegradation potential was 67 g MIBK per m^3 biofilter volume, whereas when MIBK was mixed with MEK, the hourly biodegradation potential was reduced to 21 g MIBK per m^3 biofilter volume. This suggests that the rate determining step in the overall process is most probably biological in nature, rather than a transfer limitation. This proposal will be confirmed by recirculating a fraction of the gaseous effluent stream, thereby establishing a differential reactor [3].

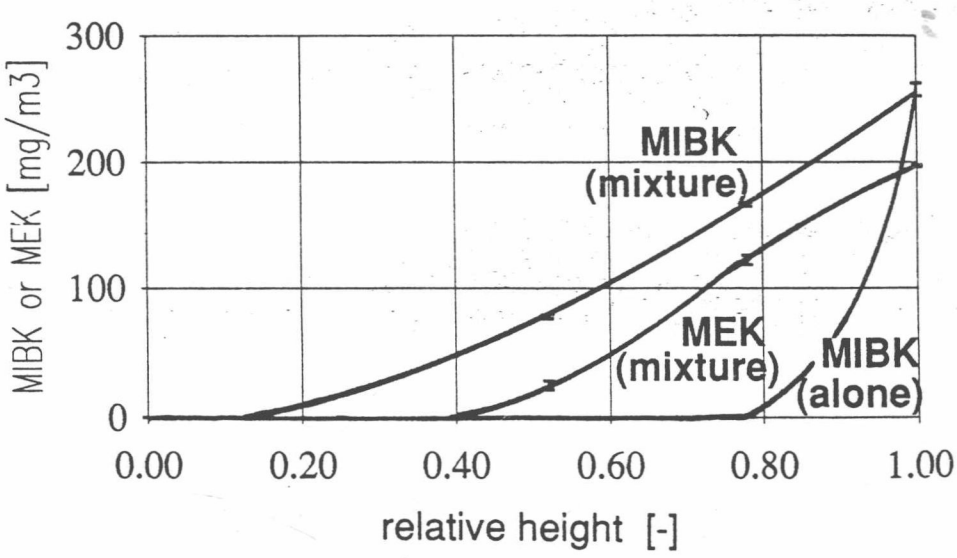

Fig. 3. Biodegradation profile of MIBK alone and of a MEK/MIBK mixture as a function of the relative height in the biofilter bed. (inlet concentrations: MIBK=385 mg m^{-3}; MEK=383 mg m^{-3}; gas flow=284 L h^{-1}).

4. CONCLUDING REMARKS

The present investigation demonstrates that biofilters of the type that have been constructed and operated here exhibit high efficiencies for the removal of both MEK and MIBK vapours from polluted air streams. However, the additional presence of MEK in an air stream polluted with MIBK markedly reduced performance with respect to MIBK removal potential. The poor carbon recoveries reported emphasize a need for further studies to elucidate the fate of the removed pollutants, although recoveries suggest that biodegradation is coupled with growth in the systems investigated.

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