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Growth rate influences reductive biodegradation of the organophosphorus pesticide demeton by *Corynebacterium glutamicum*

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Abstract

The organophosphorous pesticide, demeton-S-methyl was transformed by *Corynebacterium glutamicum* in co-metabolism with more readily degradable substrates. Glucose, acetate and fructose were tested as growth substrates, and the highest demeton-S-methyl biotransformation average rate (0.78 mg l^{-1} h^{-1}) and maximum instantaneous rate (1.4 mg l^{-1} h^{-1}) were achieved on fructose. This higher efficiency seems to be linked to the atypical behavior of *C. glutamicum* grown on fructose, characterized by a prolonged period of accelerating growth instead of a constant growth rate observed on glucose or acetate. More precisely, for growth rates in the 0.1–0.4 h^{-1} range, a direct coupling between the specific demeton-S-methyl consumption rate and the growth rate was demonstrated on fructose during batch –, steady state continuous – or continuous cultures with a controlled transient growth rate (accelerostat technology). The demeton-S-methyl biotransformation was more favoured during an acceleration phase of the growth rate.

Introduction

Microbial cometabolism, i.e., "transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound" is frequently observed for the transformation of xenobiotic compounds by individual microbial species (Janke & Fritsche 1985). A suitable growth substrate serves to induce enzymes, cofactors and metabolites required for the xenobiotic transformation and/or to support cell growth by generating energy and carbon. The growth substrate can be an analogue of the xenobiotic compound, (Adriaens & Focht 1991; Gilbert & Crowley 1997) often leading to a competitive inhibition between growth and non-growth substrates when they share the same enzymatic pathway (Aziz et al. 1999; Speitel et al. 1993), or a more conventional carbon source, like yeast extract (Nelson 1982), sugars (Wang & Loh 1999), or acids (Daugherty & Karel 1994; Ziegler et al. 1980). Wang & Loh (1999) have compared the cometabolic degradation of 4-chlorophenol by *Pseudomonas putida* using phenol or glucose as added growth substrates. These authors demonstrated that competitive inhibition phenomena leading to diminished rates of 4-chlorophenol degradation when using phenol as primary growth substrate could be avoided by using glucose.

In a recent report, we have demonstrated the aerobic biodegradation capacity of *Corynebacterium glutamicum* ATCC 13745 with regard to the P–S bond containing organophosphorus compound demeton-S-methyl (O,O-dimethyl-S-2-ethylthiolethyl phosphorothioate), a pesticide and a chemical warfare agent analogue, in co-metabolism with more readily transformable substrates (Girbal et al. 2000). The reaction mechanism of demeton-S-methyl biotransformation was shown not to be a hydrolysis but a reductive cleavage of a S–C bond leading to the accumulation of dimethyl thiophosphate in the culture medium. This reductive cleavage of the S–C bond involves

an unidentified dehydrogenase/oxido-reductase activity according to the reaction:

$$CH_3–CH_2–S–CH_2–CH_2–S–\overset{\overset{O}{\|}}{P}–(O–CH_3)_2 \rightarrow$$

$$CH_3–CH_2–S–CH_2–CH_3 + HS–\overset{\overset{O}{\|}}{P}–(O–CH_3)_2.$$

Among the growth substrates tested (glucose, acetate or fructose), fructose was shown to be the substrate favouring the most rapid rate of demeton-S-methyl consumption (Girbal et al. 2000).

In order to understand this higher efficiency of demeton-S-methyl biotransformation on fructose, the demeton-S-methyl consumption by *C. glutamicum* on fructose was characterized during batch, continuous cultures and continuous cultures with a controlled transient growth.

Materials and methods

Chemicals

Demeton-S-methyl and malathion were purchased from Cluzeau Info-Labo (Sainte Foy La Grande, France). Demeton-S-methyl was certified to be 97% and malathion 98.7% pure. All other chemicals were analytical grade and purchased from Sigma (Saint Quentin Fallavier, France).

Bacterial strain, media and growth conditions

The bacterial strain used in this study was *C. glutamicum* ATCC 13745. The growth medium used in the batch cultures was previously described (Girbal et al. 2000). For continuous cultures, the feed medium (prepared in 50-liter reservoirs and sterilized by filtration) used was previously described by Cocaign-Bousquet et al. 1996, except that glucose was replaced by fructose (15 g l^{-1}). Demeton-S-methyl (prepared in 100 mM Tris/HCl, pH 7.0 buffer) sterilized by filtration through cellulose acetate membranes (0.22 μm pore size) was added separately to the bioreactor to obtain 10 mg l^{-1} final concentrations. The cultures were grown in a 2-liter Discovery 100 Inceltech SGI bioreactor of working volume 1.5 liter for batch cultures and 1.0 liter for continuous cultures. The temperature was maintained at 27 °C, the pH was regulated at 7.0 by automatic addition of KOH (10 N) or HCl (3 N), and both aeration rate and stirrer speed were modified to avoid the dissolved oxygen concentration falling below 50% saturation. The inoculum medium was the same as the growth medium except that the KH_2PO_4/K_2HPO_4 concentration was increased to 30 mM to improve the pH-buffering capacity of shake-flask cultivation. The growth medium was inoculated (5%, by vol.) with an overnight culture (in exponential phase, absorbance at 650 nm = 3) grown in the presence of fructose but in the absence of demeton-S-methyl. In order to perform continuous cultures, the medium and demeton-S-methyl flows were started (in 9/10 and 1/10 ratios, respectively) after 15 h of batchwise growth. During continuous cultures, foaming was controlled by periodic additions of an antifoaming agent (Strucktol J633; Sofraret). The cultures were considered to be at steady state when all culture parameters (biomass, substrate, demeton-S-methyl and product concentrations) reached constant values. Steady state samples were taken over a period of several residence times. The controlled transient growth rate (accelerostat) experiments were realized by increasing (or decreasing) the dilution rate by 0.01 h^{-1} every half hour, while keeping the 9/10 and 1/10 ratios for the medium and demeton-S-methyl flows, respectively.

Analytical methods

Biomass, sugars and organic acid concentrations were determined as previously described (Cocaign-Bousquet et al. 1996). Demeton-S-methyl was extracted from the cell suspensions using a mixture of 50% *n*-hexane and 50% ethylacetate and its concentration determined by gas chromatography (Girbal et al. 2000), malathion (prepared in 100 mM Tris/HCl pH 7.0) was used as an internal standard. The demeton-S-methyl quantification threshold was 0.1 mg l^{-1}.

Results and discussion

During batch fermentations, the demeton-S-methyl consumption rates were higher on fructose than on acetate or glucose: an average rate of 0.78 mg l^{-1} was obtained during the growth phase with a maximum instantaneous rate of 1.4 mg l^{-1} (Girbal et al. 2000). The efficient biotransformation on fructose was correlated to the atypical behaviour observed on this substrate, characterized by a prolonged period

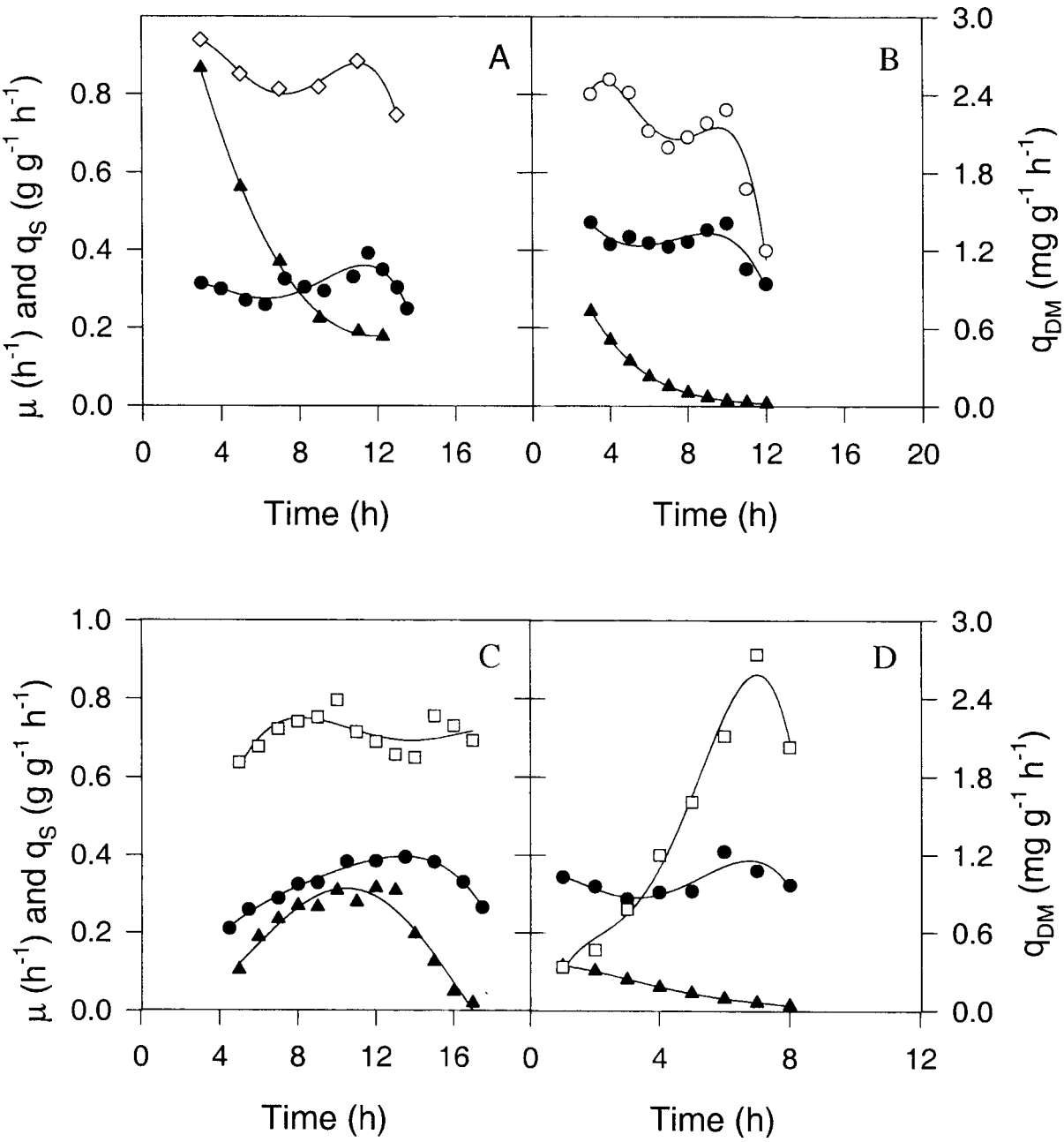

Figure 1. Specific growth and consumption rates in *C. glutamicum* cells grown on acetate (A), glucose (B) or fructose (C and D, the latter corresponding to the culture with a 10-fold increased biomass concentration in the inoculum) under batch conditions: the specific growth rate μ (●), the specific substrate consumption rates $q_{acetate}$ (◊), $q_{glucose}$ (○), $q_{fructose}$ (□), and the specific demeton-S-methyl consumption rate q_{DM} (▲).

of accelerating growth rather than a constant growth rate on either acetate or glucose (Figures 1A, 1B and 1C). On fructose, parallel evolution of the demeton-S-methyl consumption rate (q_{DM}) and the growth rate (μ) was seen indicating a direct coupling between these two rates. For a given growth rate, the lower q_{DM} obtained in the final decelerating phase could be linked to a possible limitation of the biotransformation by the very low residual demeton-S-methyl concentration measured at this point of the culture on fructose. The biochemical and stoichiometric analysis of fructose and glucose catabolism had revealed an increased NADH/NAD^+ ratio in *C. glutamicum* cells grown on fructose (Dominguez et al. 1998). Since the demeton-S-methyl cleavage involved a S–C reduction via a dehydrogenase/oxido-reductase enzyme (Girbal et al. 2000), fructose appeared to be the more suitable growth substrate to generate an adequate NADH supply and/or redox environment required for demeton-S-methyl biotransformation.

To check the positive role of the initial accelerating phase on the biotransformation efficiency, this phase was suppressed during a batch culture on fructose by using a higher initial biomass concentration (a 10-fold increase) in the inoculum (Figure 1D). A constant growth rate was present during the entire culture period and correlated to a decreasing evolution of q_{DM}, and a 3-fold decrease in the global pesticide consumption rate. This result seems to confirm the direct link existing between the growth rate and the efficiency of demeton-S-methyl consumption of *C. glutamicum* cells, an increasing growth rate being more favourable for pesticide biodegradation.

To understand the relationship between growth rate and demeton-S-methyl biodegradation in *C. glutamicum* cells grown on fructose, the pesticide consumption was studied during steady states of continuous cultures (chemostat) performed at dilution rates of 0.1, 0.2, 0.3 and 0.4 h^{-1}. The fructose, biomass and demeton-S-methyl concentrations versus the growth rate are presented in Figure 2A. All the cultures were fructose-limited with only low residual fructose concentrations being detected except at 0.4 h^{-1} (1 g l^{-1} of residual fructose). The culture established at the dilution rate of 0.4 h^{-1} is close to the critical dilution rate above which the culture would be washed out so the higher residual fructose concentration was to be expected. At low growth rate (0.1 h^{-1}), a high yield of fructose conversion into biomass (Rx/s = 0.85 g g^{-1}) was achieved while the residual demeton-S-methyl concentration was rather high (5.8 mg l^{-1}).

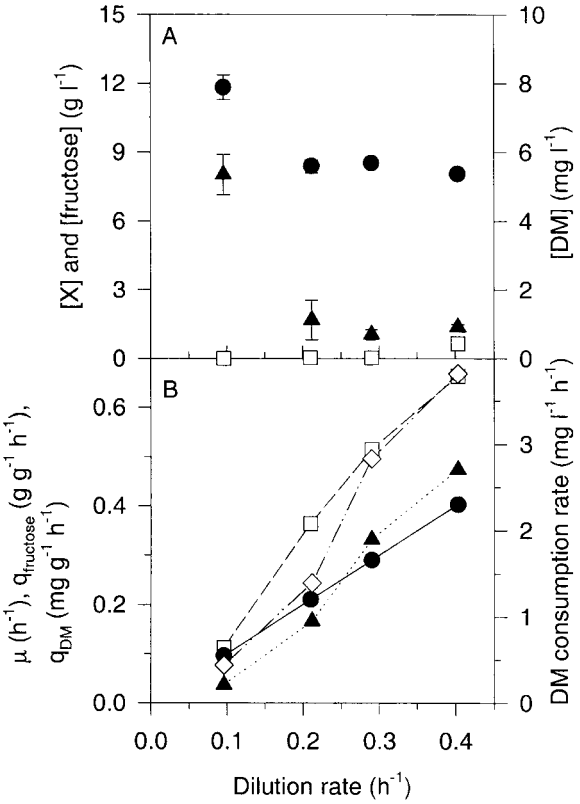

Figure 2. Steady state kinetics of *C. glutamicum* continuous cultures on fructose at various dilution rates. (A) Biomass concentration (×) (●), fructose concentration (□) and demeton-S-methyl concentration (DM) (▲). (B): growth rate μ (●), specific fructose consumption rate q_{fructose} (□), specific demeton-S-methyl consumption rate q_{DM} (▲) and the volumetric demeton-S-methyl consumption rate (◊).

It seems thus that at a low dilution rate, biomass production pathways were optimized at the expense of pathways involved in demeton-S-methyl biodegradation. At higher growth rates, the residual pesticide concentration was decreased, but remained in all cultures higher than 0.8 mg l^{-1}. The parallel profiles of μ and q_{DM} (Figure 2B) confirm the direct link between growth rate (or specific fructose consumption rate) and biodegradation efficiency. It is noteworthy that the maximum q_{DM} value (0.47 mg g^{-1} h^{-1}) obtained on fructose during a continuous culture at 0.4 h^{-1} was much lower than that observed (0.97 mg g^{-1} h^{-1}) at the same growth rate during batch cultures. This can probably be explained by the limitation of demeton-S-methyl consumption by the very low pesticide residual concentration (~0.8 mg l^{-1}) measured during the steady state at 0.4 h^{-1}. The steady state at 0.4 h^{-1} showed, nevertheless, the highest volu-

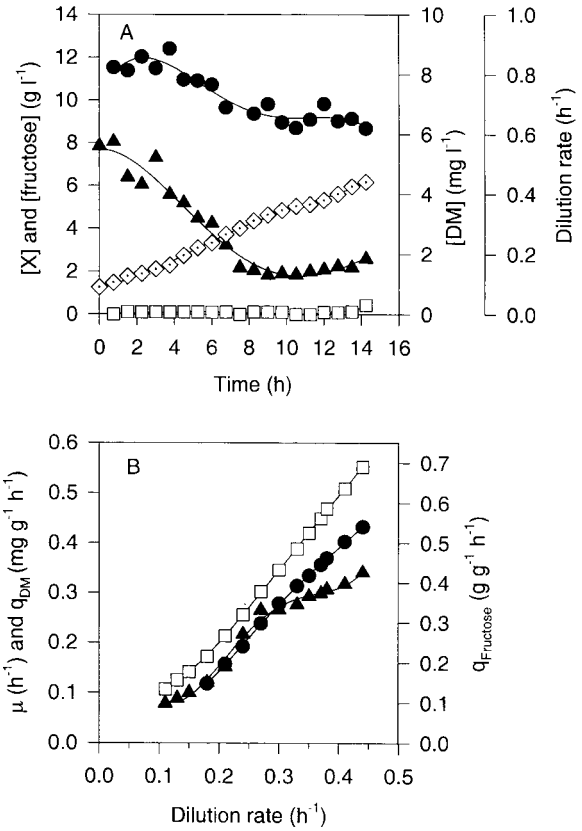

Figure 3. Kinetics of *C. glutamicum* continuous cultures on fructose during a linear increase of the dilution rate over the time. (A) Dilution rate (◇), biomass concentration [×] (●), fructose concentration (□) and demeton-S-methyl concentration [DM] (▲). The mean values and the standard deviations (error bars for $n = 9$) are presented. (B): growth rate μ (●), specific fructose consumption rate q_{fructose} (□), and specific demeton-S-methyl consumption rate q_{DM} (▲).

metric demeton-S-methyl transformation rate (3.8 mg l^{-1} h^{-1}, Figure 2B) obtained for either continuous or batch cultures.

In addition, demeton-S-methyl consumption was studied during a transient evolution of the growth rate in continuous cultures on fructose (accelerostat). The accelerostat has been used to study microbial metabolism (Kask et al. 1999; Paalme et al. 1997) since it offers an efficient manner to observe the behaviour of micro-organisms over a wide range of growth rates without the time delays associated with steady state chemostats. The controlled transient growth rate resulted in a linear increase of the dilution rate (acceleration rate of 0.02 h^{-2}), from 0.1 h^{-1} to 0.44 h^{-1} (Figure 3A). The culture was fructose-limited during the entire experiment indicating that the imposed rate of change was compatible with the capacity of *C. glutamicum* to

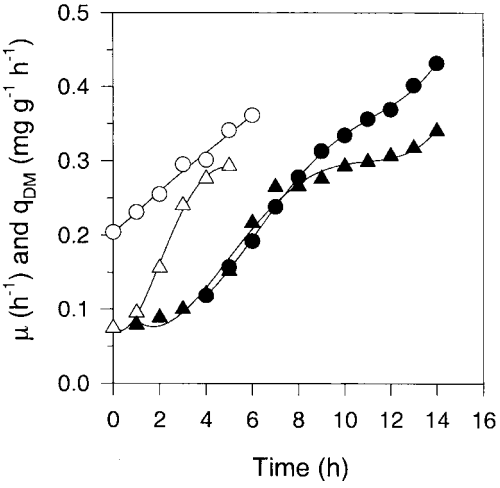

Figure 4. Specific demeton-S-methyl consumption rates (q_{DM}) and growth rate (μ) of *C. glutamicum* continuous cultures on fructose during controlled transient growth rate experiments: (▲) q_{DM} and (●) μ during an increase of the growth rate; (△) q_{DM} and (○) μ during a decrease of the growth rate.

increase its metabolic activity. The biomass and residual demeton-S-methyl concentrations were the highest at low dilution rates, as seen for steady state experiments, decreased until a dilution rate of 0.3 h^{-1} and remained constant thereafter at higher dilution rates. The linear increase of the dilution rate was accompanied by a quasi-linear increase of both μ and q_{fructose} (Figure 3B). At the same time, q_{DM} had a sigmoïdal evolution. After the growth rate of 0.24 h^{-1}, the slowing down of q_{DM} could be attributed to a possible limitation of the pesticide consumption due to the low residual demeton-S-methyl concentration (1.5 mg l^{-1}). A similar controlled transient growth rate experiment was performed except that the dilution rate was diminished from 0.38 h^{-1} to 0.21 h^{-1}. Figure 4 shows that the same relationship between the q_{DM} and the dilution rate was observed confirming the parallel evolutions of q_{DM} and μ seen in both batch and steady state continuous cultures.

Conclusion

It has been shown that the specific demeton-S-methyl consumption rate was coupled to the growth rate during fructose fermentation. This coupling was shown for growth rates in the 0.1–0.4 h^{-1} range, in experimental systems for which the evolution of the growth rate was under different constraints: (i) in batch cultures corresponding to a non-constrained evolution of

growth rate, (ii) in steady state fructose-limited continuous cultures in which growth rate was dependent upon inflowing medium supply, and (iii) in continuous cultures with a controlled transient (positive and negative) growth rate (accelerostat technology).

In the perspective of an industrial application of demeton-S-methyl biotransformation by *C. glutamicum* a high cell density (50 g l^{-1}) bioreactor process has been performed (results not shown). The maximum productivity of 3.8 mg l^{-1} h^{-1} was in the same range that the productivity observed for the steady state continuous culture at 0.4 h^{-1}. Thus, the increase of the biomass concentration did not compensate the lower specific pesticide degradation rate, result of the decelerating growth rate.

The net advantages offered by an accelerating growth rate can best be achieved using a sequencing batch reactor with controlled feeding of the primary growth substrate. Such a system would be expensive to operate on washed soil extracts and only justified in the case of localised heavy pollution. In more disperse pollution, *ex situ* bioremediation is probably not necessary as natural soil micro-organisms such as *C. glutamicum* will co-metabolise demeton-S-methyl. Unfortunately the scientific literature contains little information to confirm the extend and potential of *in situ* degradation.

References

Adriaens P & Focht DD (1991) Cometabolism of 3,4-dichlorobenzoate by *Acinetobacter sp*. strain 4-CB1. Appl. Environ. Microbiol. 57: 173–179

Aziz CE, Georgiou G & Speitel GE Jr (1999) Cometabolism of chlorinated solvents and binary chlorinated solvent mixtures using *M. trichosporium* OB3b PP358. Biotech. Bioeng. 65: 100–107

Cocaign-Bousquet M, Guyonvarch A & Lindley ND (1996) Growth rate-dependent modulation of carbon flux through central metabolism and the kinetic consequences for glucose-limited chemostat cultures of *Corynebacterium glutamicum*. Appl. Environ. Microbiol. 62: 429–436

Daugherty DD & Karel S (1994) Degradation of 2,4-dichlorophenoxyacetic acid by *Pseudomonas cepacia* DB01 (pRO 101) in a dual substrate chemostat. Appl. Environ. Microbiol. 60: 3261–3267

Dominguez H, Rollin C, Guyonvarch A, Guerquin-Kern JL, Cocaign-Bousquet M & Lindley ND (1998) Carbon-flux distribution in the central metabolic pathways of *Corynebacterium glutamicum* during growth on fructose. Eur. J. Biochem. 254: 96–102

Gilbert ES & Crowley DE (1997) Plant compounds that induce polychlorinated biphenyl biodegradation by *Arthrobacter sp.* strain B1B. Appl. Environ. Microbiol. 63: 1933–1938

Girbal L, Hilaire D, Leduc S, Delery L, Rols J-L & Lindley ND (2000) Reductive cleavage of demeton-S-methyl by *Corynebacterium glutamicum* in cometabolism on more readily metabolizable substrates. Appl. Environ. Microbiol. 66: 1202–1204

Janke D & Fritsche W (1985) Nature and significance of microbial cometabolism of xenobiotics. J. Basic Microbiol 25: 603–619

Kask S, Laht T-M, Pall T & Paalme T (1999) A study on growth characteristics and nutrient consumption of *Lactobacillus plantarum* in A-stat culture. Antonie van Leeuwenhoek 75: 309–320

Paalme T, Elken R, Vilu R & Korhola M (1997) Growth efficiency of *Saccharomyces cerevisiae* on glucose/ethanol media with a smooth change in the dilution rate (A-stat). Enzyme Microb. Technol. 20: 174–181

Nelson LM (1982) Biological induced hydrolysis of parathion in soil: isolation of hydrolysing bacteria. Soil Biol. Bioch. 14: 219–222

Speitel Jr GE, Thompsom RC & Weissman D (1993) Biodegradation kinetics of *Methylosinus trichosporium* OB3b at low concentrations of chloroform in the presence and absence of enzyme competition for methane. Water Res. 27: 15–24

Wang S-J & Loh K-C (1999) Facilitation of cometabolic degradation of 4-chlorophenol using glucose as an added growth substrate. Biodegradation 10: 260–269

Ziegler W, Engelhardt G, Wallnöfer PR, Oehlmann L & Wagner K (1980) Degradation of demeton S-methyl sulfoxide (metasystox R) by soil microorganisms. J. Agric. Food Chem. 28: 1102–1106