COMMUNICATION TO THE EDITOR

On-Line Monitoring of Bioreductions via Membrane Introduction Mass Spectrometry

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Abstract: Real-time and on-line continuous monitoring of reactants, intermediates, and final products for dicarbonyl compound bioreductions in a continuous plug flow reactor packed with baker's yeast (*Saccharomyces cerevisiae*) whole cells immobilized on calcium alginate beads was performed by membrane introduction mass spectrometry (MIMS) via selective ion monitoring.

Keywords: membrane introduction mass spectrometry; baker's yeast; alginate; bioreductions

INTRODUCTION

Membrane introduction mass spectrometry, MIMS (Johnson et al., 2000), is one of the simplest, fastest, and most sensitive techniques available for the determination of volatile (Alberici et al., 2002) and semi-volatile organic compounds (VOC; Mendes and Eberlin, 2000a) mainly in water (Alberici et al., 2001), but also in other matrixes such as air (Riter et al., 2001) and soil (Kostiainen et al., 1998) and other more complex or more viscous solid matrixes (Mendes et al., 2000b). In MIMS, direct introduction to VOC from a liquid or gas sample into a mass spectrometer results from selective transport through a membrane, most often a silicone polymer. The thin or ultra-thin membranes (Alberici et al., 1999) also work as a suitable interface with the proper mechanical resistance between the liquid or gaseous sample and the high-vacuum mass spectrometer. The hydrophobicity of the membrane and the permeability of the VOC permit rapid and simultaneous extraction, preconcentration, and sample injection (Moraes et al., 2000). The VOC are adsorbed from the solution onto the membrane, diffuse through the membrane, and evaporate from the membrane surface directly into the high vacuum ion-source region of the mass spectrometer. The ability to perform rapid and direct (without extraction or sample pretreatment) and quantitative analysis of VOC in a variety of matrixes is, therefore, one of the most attractive features of MIMS (Augusti et al., 2003; Hayward et al., 1990). The main disadvantage is the inability to readily distinguish and quantify stereoisomers.

Membrane introduction mass spectrometry has also been shown to be very suitable for real-time and on-line monitoring of a number of chemical processes such as (a) the major products and the volatile metabolites from fermentation by microorganisms (Hayward et al., 1990); (b) the conversion of chlorine to chloramines, an environmentally relevant reaction (Kotiaho et al., 1991); (c) the chlorination of phenol and related compounds, models of humic substances, by sodium hypochloride (Rios et al., 2000); (d) the oxidation of benzene derivatives by Fenton's reagent (Augusti et al., 1998); (e) the photolysis of aryl methyl ether in aqueous and aqueous-methanolic solutions (Wong et al., 1996); (f) the hydrolysis of epichlorohydrin, a significant compound in the polymer industry (Johnson et al., 1999); (g) the catalytic hydrodechlorination of aromatic chlorides, an important process to treat organochloro compounds (Lago et al., 2003); and to the study of the stability constants of small organic guest molecules into cyclodextrin hosts in aqueous medium (Burgos et al., 2003).

The reduction of diketone and α- and β-ketoesters was studied as model reactions (Scheme 1). Various NAD(P)H-dependent oxidoreductases may be involved in this kind of reduction by baker's yeast. Especially in redox reactions, the use of whole cells is attractive because of the in vivo regeneration of cofactors that are essential for continuous reaction. Regeneration of NAD(P)H must take place as yeast cells contain only a catalytic amount of NAD(P)H.

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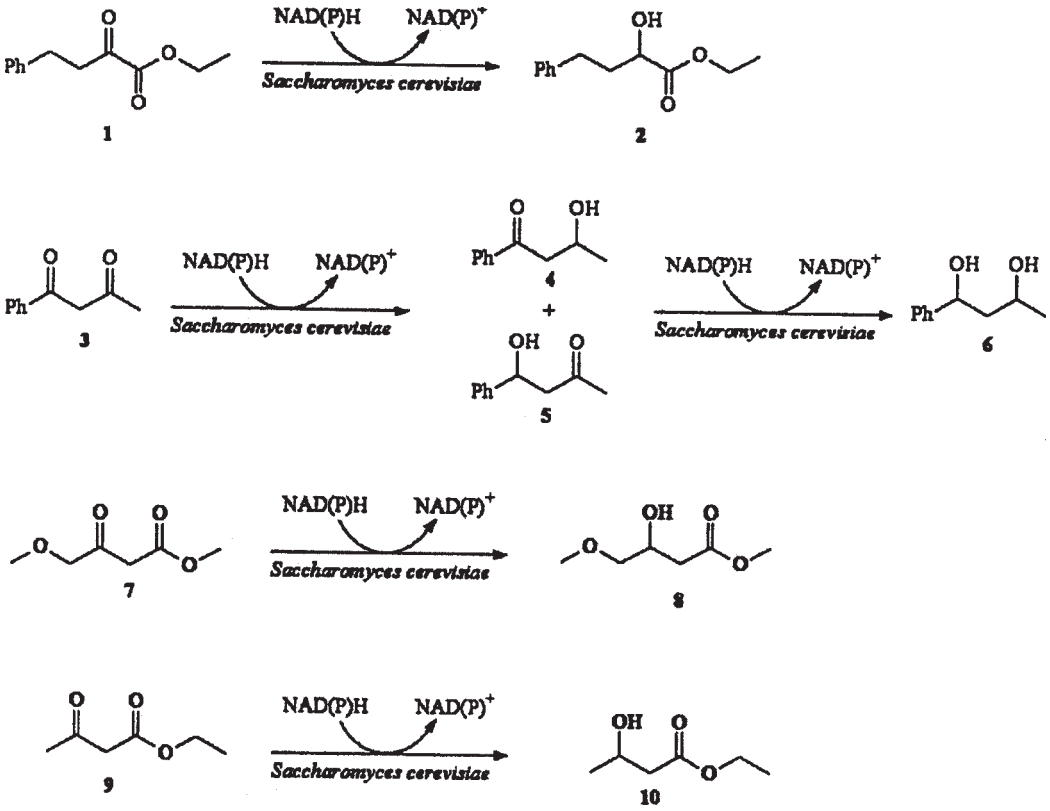


Scheme 1.

This is achieved by means of the metabolism of an electron donor, such as glucose or ethanol. This may involve NAD-dependent dehydrogenases of the hexose-monophosphate pathway, NADP-dependent isocitrate dehydrogenase, and NADP-dependent acetaldehyde dehydrogenase. Since the pathways in which these dehydrogenases participate reduce NADP as well as NAD, the produced NADH has to be re-oxidized by mitochondrial respiration. This explains the apparent paradox of an oxygen requirement for sustaining the overall reduction reaction (Chin-Joe et al., 2002).

Herein we report the use of MIMS to monitor a continuous process of bioreduction of the dicarbonyl compounds **1**, **3**, **7**, and **9** using baker's yeast whole cells immobilized on calcium alginate beads (Scheme 1). Membrane introduction mass spectrometry is used for real time on-line monitoring of the bioprocess with reactant (if not totally converted), intermediates, and product detection. Such monitoring otherwise requires time-demanding off-line sample collection and processing and chromatographic separation.

MATERIALS AND METHODS

Microorganism

Dry baker's yeast was purchased from Emulzint Ltd. (Belgium) and stored in a refrigerator (4°C).

Chemicals

1-Benzoylacetone (99%), ethyl 2-oxo-4-phenylbutyrate (97%), ethyl acetoacetate (99%) and methyl 4-methoxyacetoacetate (97%) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Sodium alginate was obtained from Vetec Co. (Rio de Janeiro, Brazil). The purity of all other chemicals was at least reagent grade.

Reduction Conditions

Continuous bioreductions with immobilized dry baker's yeast whole cells were carried out in a 180-mL bioreactor with 75 mL working volume. The reactor was packed with biocatalyst particles of 2.9 mm diameter operating in the up-flow mode (plug flow tubular reactor—PFTR). Immobilization of the biocatalyst on the alginate beads has been described elsewhere (Smidsrød and Skjåk-Bræk, 1990). A 200 mL aqueous 2% (w/w) sodium alginate solution, which was stirred for 3 h, was prepared for cell immobilization. Beads were left to harden in 600 mL aqueous 0.2*M* $CaCl_2$ solution for 1 h, filtered, washed extensively with distilled water and immediately used for the reduction process, or stored in 0.2*M* calcium chloride solution at 4°C overnight. Damage due to particle attrition was not observed. The reactor was sterilized (120°C for 15 min) prior to use. A total amount of 400 mL of an aqueous glucose/substrate solution was pumped continuously into the reactor with a peristaltic pump (ISMALTEC multichannel) at a flow rate of 1.2 mL/min (flow optimized from a previous study which gave 100% of substrate conversion, except for **7**). The reactor effluent was carried, via flow injection analysis (FIA) (Růžička and Hansen, 1986) to the mass spectrometer to be analyzed. After the addition of substrate, 400 mL of an

aqueous solution of glucose was fed into the reactor, until no more products could be detected by MIMS. The residence time in the reactor was 40 min.

Analytical Methods

First, we screened the compounds with CG-MS to identify the most abundant and/or different ions and then set the selective ion monitoring (SIM) for the MIMS experiment. The set-up for CG-MS analysis was DB1 column (polydimethylsiloxane) 30.0 m × 0.25 mm × 0.25 μm. The carrier gas was helium and the pressure was 100 kPa. The split ratio for injection of the sample (1.0 μL) was 60. The injector and detector temperatures were 230°C and 270°C, respectively. The temperature program was 80°C for 3 min, 12°C/min, 140°C for 10 min, 40°C/min, and 280°C for 5 min.

A single-quadrupole ABB Extrel (Pittsburgh, PA) mass spectrometer fitted with a high transmission quadrupole and equipped with a conventional MIMS probe (Mendes et al., 1996), with a 125 μm silicone sheet membrane (Silastic 500-3; Dow-Corning, Midland, MI) was used (Fig. 1). The membrane probe was directly connected to the mass spectrometer via a standard probe holder. The aqueous solution from the reactor at room temperature (24 ± 1°C) was pumped through the system via a peristaltic pump (ISMATEC multichannel) at the rate of 1.2 mL/min. At the T intersection, the sample stream was mixed with a water stream for on-line dilution before analysis.

RESULTS AND DISCUSSION

During the bioreduction of the α-ketoester **1**, MIMS monitoring showed no molecular ion $\mathbf{1}^+$ of *m/z* 206 (as well as none of its characteristic fragment ions of *m/z* 133 and 188), indicating the significant effectiveness of the bioreduction process, showing quantitative bioreduction of **1**. The single bioreduction product **2** was then monitored by

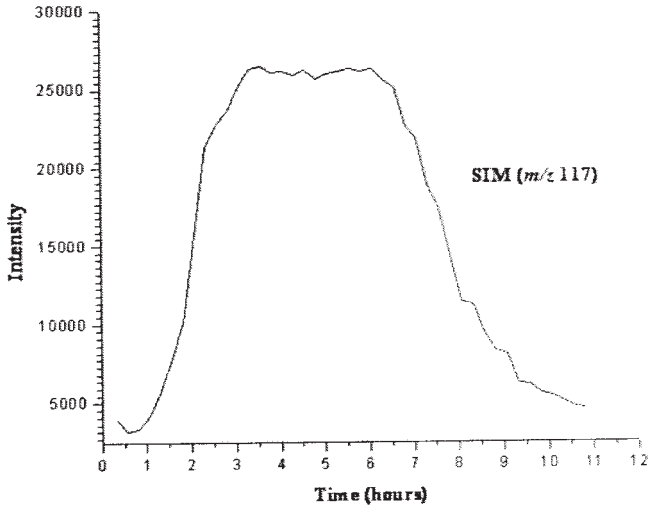


Figure 2. MIMS **1** → **2** bioreduction monitoring via SIM of the $\mathbf{2}^{+\cdot}$ characteristic fragment ion of *m/z* 117.

MIMS via selective ion monitoring (SIM) of its characteristic fragment ion of *m/z* 117. Figure 2 shows that the increase and then stabilization of **2** is conveniently monitored in real time and on-line by MIMS. Stabilization occurs owing to the steady state attained in the continuous bioreduction flow system after nearly 3 h of feeding substrate. The concentration decreases when the feeding of substrate **1** is interrupted and the bioreactor is flushed with water for cleaning.

The bioreduction of β-diketone **3** was also monitored by MIMS (Fig. 3). Bioreduction of **3** is challenging for MIMS monitoring using 70 eV electron ionization (EI) mass spectrometry since **4** and **5** display similar 70 eV EI mass spectra with the same set of ions. Therefore, whereas the final product **6** displays a characteristic ion of *m/z* 148 that was used for its MIMS monitoring via SIM, the isobaric intermediates **4** and **5** resulting from partial reduction of each carbonyl group of **3**, cannot be monitored via a single

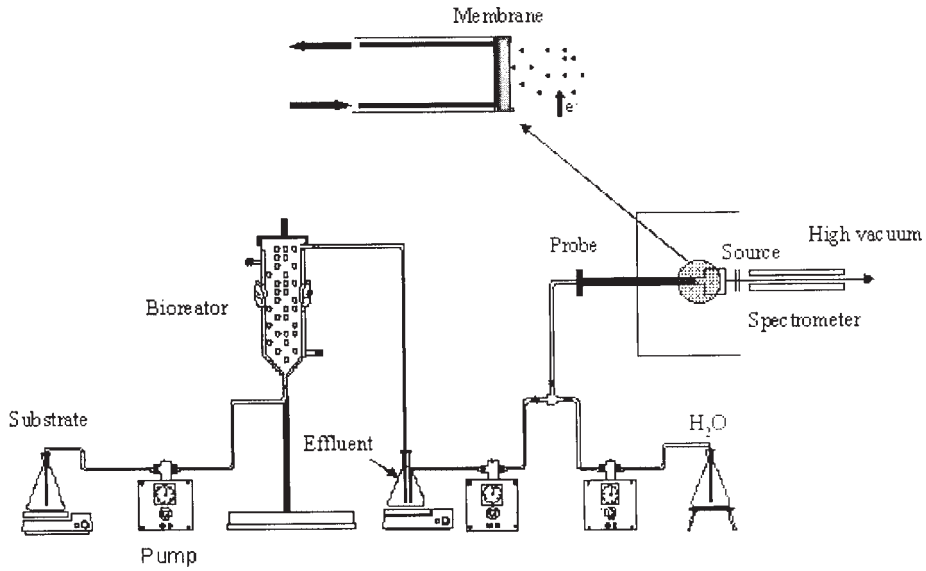


Figure 1. A schematic representation of the bioreduction-MIMS instrumental set-up.

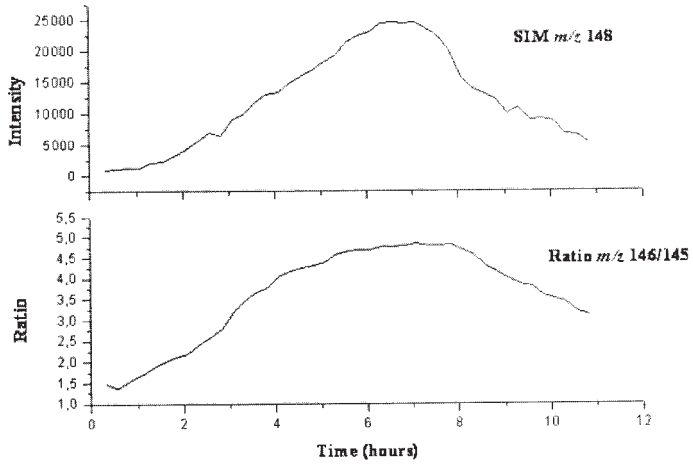


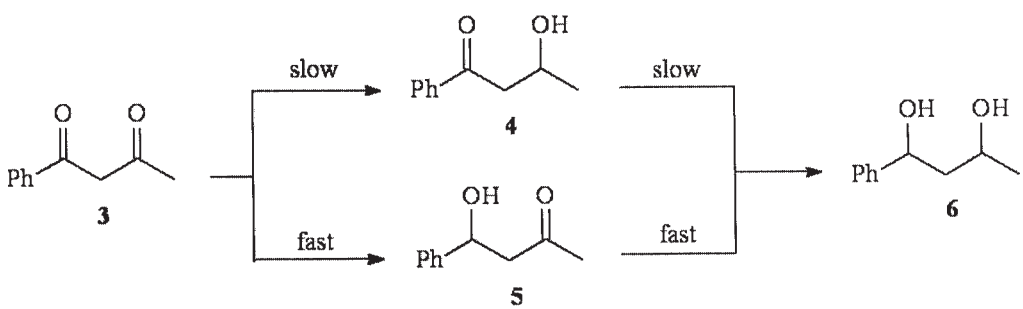
Figure 3. MIMS **3** → **4** + **5** → **6** bioreduction monitoring via (a) SIM of the $\mathbf{6}^{+}$ characteristic fragment ion of *m*/*z* 148, and (b) selective ion abundance ratio monitoring of the *m*/*z* 145: *m*/*z* 146 reveals the **4** + **5** mixture composition.

selective ion. Fortunately however, an examination of the respective mass spectra shows that the *m*/*z* 146 : *m*/*z* 145 ion abundance ratio is 1.5 for **5** and 4.5 for **4**. These ratios are therefore sufficiently distinctive to allow MIMS monitoring, not via SIM, but via selective ''ion-abundance ratio'' monitoring.

Figure 3 shows the MIMS monitoring of the bioreduction of **3** using SIM (*m*/*z* 148) for the final product **6** and selective ion abundance ratio (*m*/*z* 146 : *m*/*z* 145) for the intermediates **4** and **5**. A characteristic MIMS profile (Fig. 3a) for the final product **6** is observed, with an increase and then stabilization. From Figure 3b, we learn that the *m*/*z* 146: *m*/*z* 145 ion abundance ratio increases steadily as the bioreduction of **3** proceeds. At the beginning, this ratio is close to 1.5 indicating a predominance of intermediate **5**. After 6–8 h, the ratio stabilizes close to 4.5, indicating that now intermediate **4** dominates. We therefore conclude that **5** is formed and consumed by bioreduction much faster than **4** (Scheme 2). This trend likely results from the greater electrophilicity of the ''benzoylic'' carbonyl group of β-diketone **3**, as compared to its ''acetyl'' carbonyl group increasing susceptibility to the NADH type hydride attack that occurs during bioreduction. The bioreduction of **3** has been extensively studied and different results about the presence or absence of isomer **5** have been reported. Using nuclear magnetic resonance (NMR), **4** is the only isomer detected (Ahmad et al., 2004; Chênevert and Thiboutot, 1986). However, when the reaction is monitored by more sensitive techniques such as GC (Fauve and Veschambre, 1988), both isomers **4** and **5** are detected. Fauve and Veschambre had pointed that the two isomers **4** and **5** are very difficult to separate and characterize chromatographically (TLC rf values are almost identical and GC retention times are very close). The on-line MIMS monitoring described herein with selective ion abundance ratio presents therefore, time-resolved proof that both **4** and **5** are formed, and that **5** is formed and consumed faster than **4**.

The preferential formation and bioreduction of intermediate **5** over **4**, shown in real time by MIMS, was confirmed by off-line CG-MS. Samples were collected at 20 min intervals and the two intermediates could be separated using GC/MS. Under these conditions **5** eluted after 8.93 min and **4** after 9.15 min. At the beginning of the bioreduction process, the first sample showed a 90 : 10 ratio of **5** : **4**. For the last sample collected after 480 min of bioreduction, an inverse ratio was observed, that is 8 : 92.

The bioreduction of methyl 4-methoxyacetoacetate **7** and ethyl acetoacetate **9** was also monitored by MIMS via SIM (Figs. a and b, respectively, available in supplemental material) to show the versatility of this methodology. The bioreduction of **9** by baker yeast is a well known process and all substrate was reduced by the yeast giving **10**. Otherwise, the bioreduction of **7**, under the conditions used here, is slow and was not completed during the



Scheme 2.

monitoring time. In this case, part of **7** was not reduced and we could detect both **7** and **8** in the effluent.

CONCLUSIONS

Membrane introduction mass spectrometry is a suitable technique for rapid on-line monitoring of bioreduction processes, as shown here for the bioreduction of dicarbonyl compounds catalyzed by yeast encapsulated in calcium alginate. Immobilization of the biocatalyst, as herein performed onto alginate beads, is essential for MIMS monitoring, as it prevents leakage from the reactor and consequently the obstruction of the MIMS probe. When each reactant, intermediate, and final product displays a characteristic 70 eV EI molecular or fragment ion, selective ion monitoring can be used. In more complex cases, for which mass spectra with identical set of ions are observed, an alternative is to use selective ''ion-abundance ratio'' monitoring, as herein demonstrated for the ''proof-of-principle'' case of intermediates **4** and **5**. More gentle ionization techniques, such as chemical ionization coupled to tandem mass spectrometry, can also be used in the search for selective ions or ion-abundance ratios. The real time on-line continuous monitoring of bioreductions is very desirable, particularly for the nearly instantaneous detection of bioactivity reduction of selectivity loss, which is a serious concern in bioreduction processes. The continuous efforts to develop very simple and cost-effective miniature mass spectrometers dedicated to SIM of a single or a few characteristic ions, such as the cylindrical miniature ion traps (Riter et al., 2003), will certainly contribute to the effectiveness of MIMS real-time monitoring of bioreductions.

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