Bioresource Technology 174 (2014) 42–52

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

An innovative membrane bioreactor for methane biohydroxylation

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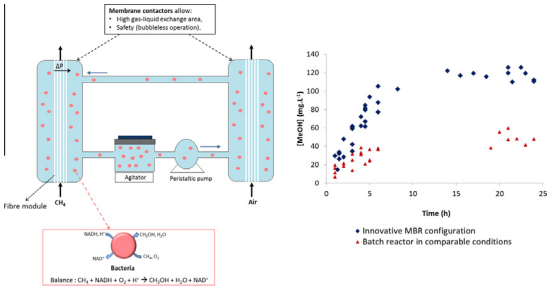
HIGHLIGHTS

- Methane biohydroxylation was done for the first time in a new MBR configuration.
- This new MBR couples two macroporous membrane contactors.
- This MBR exhibits a 2-fold enhanced mass transfer compared to a batch reactor.
- This MBR avoids gas bubbles and dangerous gas mixtures during operation.
- The obtained productivity is 35-fold higher than the only other MBR reported.

GRAPHICAL ABSTRACT



An innovative configuration of membrane bioreactor (MBR) for methane hydroxylation

ARTICLE INFO

Article history:
Received 12 June 2014
Received in revised form 26 September 2014
Accepted 1 October 2014
Available online 8 October 2014

Keywords:
Membrane bioreactor
Methane
Microbial hydroxylation
Methanol
Methylosinus trichosporium OB3b

ABSTRACT

In this study, a membrane bioreactor (MBR) was developed for efficient, safe microbial methane hydroxylation with *Methylosinus trichosporium* OB3b. This innovative MBR, which couples a bioreactor with two gas/liquid macroporous membrane contactors supplying the two gaseous substrates (methane and oxygen) was operated in fed-batch mode. The feasibility and the reproducibility of this new biohydroxylation process were first demonstrated. The mass transfer within this MBR was twice that observed in a batch reactor in similar conditions. The productivity reached with this MBR was 75 ± 25 mg methanol (g dry cell)$^{-1}$ h^{-1}. Compared to the literature, this value is 35 times higher than that obtained with the only other fed-batch membrane bioreactor reported, which was run with dense membranes, and is comparable to those obtained with bioreactors fed by bubble-spargers. However, in the latter case, an explosive gas mixture can be formed, a problem that is avoided with the MBR.

1. Introduction

Methane constitutes a large carbon feedstock that can potentially be used for the production of valuable oxygenated liquid products such as methanol or formic acid (Caballero and Perez, 2013). Developing processes that allow the selective hydroxylation of methane, i.e., its conversion into methanol, is thus of great interest. The high stability of its C–H bonds makes methane remarkably inert, however, and despite recent progress, its chemical activation remains unselective and highly energy consuming (Alvarez-Galvan et al., 2011; Mansouri et al., 2013). Microbial methane hydroxylation by aerobic methanotrophic bacteria has shown promise in this domain as this bioconversion is specific and takes place in mild physiological conditions. The conversion of methane into methanol is catalyzed by an MMO (Methane Mono-Oxygenase) in presence of oxygen according to the balance equation (Green and Dalton, 1989):

$$CH_4 + NADH + O_2 + H^+ \rightarrow CH_3OH + H_2O + NAD^+ \quad (1)$$

Isolated MMOs still exhibit limited activity and stability (Balasubramanian et al., 2010; Ito et al., 2014), which explains why whole-cell biocatalysts are currently preferred for methane biohydroxylation. The non-pathogenic bacterium *Methylosinus*

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http://dx.doi.org/10.1016/j.biortech.2014.10.001
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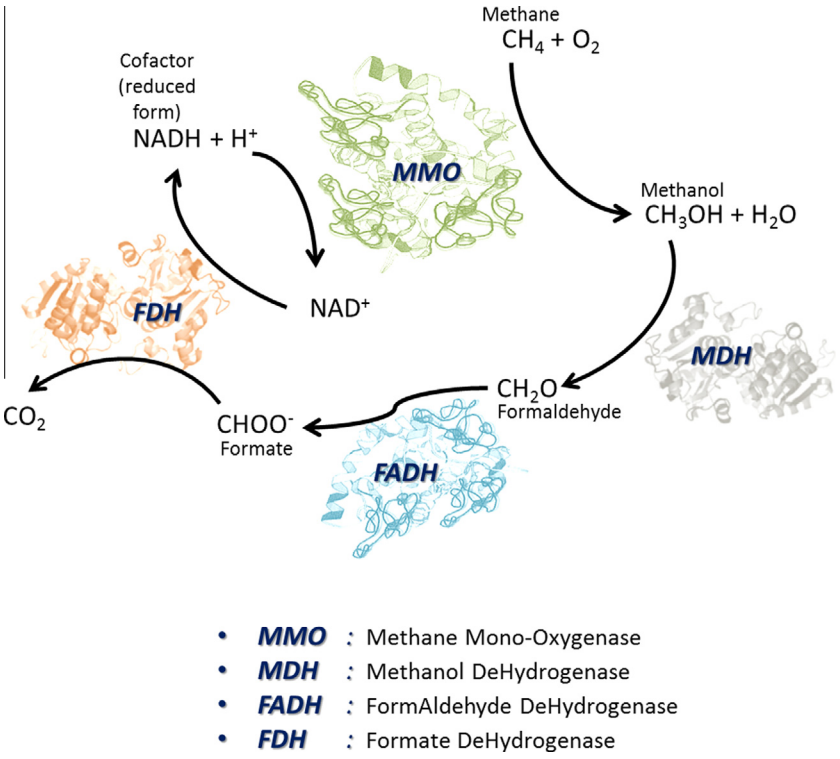


Fig. 1. Metabolic pathway used by methanotrophic bacteria for methane oxidation.

trichosporium OB3b has notably been identified as an efficient biocatalyst of this bioreaction (Duan et al., 2011; Kim et al., 2010; Lee et al., 2004; Mehta et al., 1991).

The metabolic pathway used by aerobic methanotrophic bacteria for methane assimilation is described in Fig. 1 (Ayala and Torres, 2004; Rojo, 2009; Tabata and Okura, 2008). As depicted, methane oxidation in whole cells does not stop at methanol but continues up to carbon dioxide, which points out the need to break the methanotrophic pathway after its first step. This problem can be solved by inhibiting the methanol dehydrogenase (MDH) activity with, for instance, the addition of phosphates and NaCl, and regenerating the NADH cofactor required for the hydroxylation reaction with the addition of sodium formate, which plays the role of electron donor (Duan et al., 2011; Kim et al., 2010; Lee et al., 2004; Mehta et al., 1991).

From the literature, it appears that the implementation of microbial methane hydroxylation in a bioreactor raises problems in terms of mass transfer efficiency and safety linked to the gaseous nature of the substrates (methane and oxygen). Regarding the mass transfer between the gas phases and the liquid containing the bacteria, it should be enhanced to avoid any substrate limitation. From the safety viewpoint, the risk lies in the fact that the mixture of the two gaseous substrates could be explosive: methane in air is indeed explosive between 5% v/v (LEL: Lower Explosive Limit) and 15% v/v (UEL: Upper Explosive Limit). To the best of our knowledge, batch bioreactors (Duan et al., 2011; Kim et al., 2010; Lee et al., 2004), fed-batch bioreactors (Kim et al., 2010; Lee et al., 2004; Markowska and Michalkiewicz, 2009) and a continuous bioreactor (Mehta et al., 1991) have been implemented to perform methane biohydroxylation.

In the case of batch reactors (Duan et al., 2011; Kim et al., 2010; Lee et al., 2004), the headspace is commonly filled with a substrate mixture containing a large methane excess to avoid explosion. In such reactors, the effective mass transfer area, which corresponds to the physical contact surface between gas and liquid, is relatively small and may limit the transfer.

This notably explains why continuously sparging the gaseous substrates directly into the bacterial suspension constitutes a usual way of feeding to prevent any substrate depletion (Kim et al., 2010; Lee et al., 2004; Markowska and Michalkiewicz, 2009). However, the use of bubble-spargers results in the formation of gas bubbles that may produce an explosive gas mixture.

To avoid this drawback, Duan et al. (2011) used dense silicone tubes to supply the substrates independently without any bubbles. However, the dense nature of the membranes used limited transport through the membrane to diffusion and, finally, was very penalizing for the mass transfer in comparison to spargers (Kim et al., 2010; Lee et al., 2004; Markowska and Michalkiewicz, 2009).

Membrane contactors, which involve macroporous membranes, are known to have a large surface area and have shown their ability to enhance gas/liquid mass transfer (Sanchez Marcano and Tsotsis, 2004). In particular, Coutte et al. (2010) demonstrated that membrane contactors with macroporous membranes could be used to efficiently oxygenate a bioreactor without forming any gas bubbles in the reactor.

In the present study, methane biohydroxylation was implemented for the first time in an innovative membrane bioreactor (MBR) configuration based on the coupling of a bioreactor and two macroporous membrane contactors (each contactor being fed separately with methane or air). In this MBR, the bacterial suspension circulates in a closed loop in the shell side of the contactors, thus allowing continuous feeding of the gaseous substrates into the reaction medium. In this work, the bacterium *M. trichosporium* OB3b was chosen as the biocatalyst and methane hydroxylation tests were first conducted in batch bioreactors to choose the reaction medium and study the hydroxylation performance in different conditions (biocatalyst concentration and the ratio of the gas headspace volume to the liquid volume). In a second part,

the biohydroxylation was implemented within the MBR, operated in fed-batch mode, to assess the feasibility and the reproducibility of the process. Finally, the influence of the biocatalyst concentration on the hydroxylation performance of this new bioreactor was studied.

2. Methods

2.1. Bacterial strain and cell cultivation

The bacterial strain *M. trichosporium* OB3b (NCIMB, 11131, England) was cultivated in a modified NMS (Nitrate Mineral Salts) medium (Kim et al., 2010) composed of a base medium (1.060 g/L KH_2PO_4, 4.340 g/L $Na_2HPO_4{\cdot}12H_2O$, 1.700 g/L $NaNO_3$, 0.340 g/L K_2SO_4 and 0.074 g/L $MgSO_4{\cdot}7H_2O$) enriched with usual minerals (0.570 mg L^{-1} $ZnSO_4{\cdot}7H_2O$, 0.446 mg L^{-1} $MnSO_4{\cdot}H_2O$, 0.124 mg L^{-1} H_3BO_3, 0.096 mg L^{-1} $Na_2MoO_4{\cdot}2H_2O$, 0.096 mg L^{-1} KI, 7.00 mg L^{-1} $CaCl_2{\cdot}2H_2O$) and also copper (1.25 mg L^{-1} $CuSO_4{\cdot}5H_2O$) and iron (11.20 mg L^{-1} $FeSO_4{\cdot}7H_2O$). The base medium was autoclaved while the usual mineral solution and the copper and iron solutions were prepared independently at a 1000× concentration and filtered on a 0.2-μm acetate cellulose membrane (Sartorius) before being added to the base medium in sterile conditions. The pH of the broth medium was adjusted to 7.0 ± 0.1 with NaOH, 0.1 M or HCl, 0.1 M.

The strain was maintained in liquid with a sub-culture (4% v/v into fresh medium) every 2 weeks over at most six months to avoid too great a loss of the strain's hydroxylating activity. Sub-cultures were performed in sealed 50 ml-vials containing a gas atmosphere composed of a mixture of methane and air (1:1 v/v). The total pressure of the gas mixture was atmospheric pressure and the liquid volume was 35 ml. The gas atmosphere of the vials was renewed 3 times per week, except for the first 3 weeks following the strain rehydration, during which the atmosphere was replaced daily. Vials were incubated at 30 °C on a rotary shaker (Unimax 1010, Heidolph) operated at 160 rpm.

Vial cultures aged 1 week were used as seed cultures to inoculate a 3 L-fermentor (Biostat A plus, Sartorius, France) allowing the production of larger volumes of bacterial suspensions. The working volume of this culture vessel was 2 L and the inoculum volume ratio ranged from 5% to 20% v/v depending on the desired culture time. The culture vessel was operated at 30 °C with gas flow rates fixed at 100 ± 5 ml min^{-1} for both methane and air (so that the headspace was composed of a 1:1 v/v mixture). Gases were sterilized by filtration at the inlet of the bioreactor using a 0.2-μm hydrophobic Teflon filter (Sartorius) and the rotation speed of the stirring blade was fixed at 110 rpm. The pH was measured during the culture and appeared to be constant (pH = 7.0 ± 0.1). The cultivation was stopped when the OD (Optical Density) at 600 nm reached 0.7–0.8.

A correlation between the suspension OD at 600 nm and the dry cell concentration was established by drying the bacterial pellets obtained from the centrifugation of the bacterial suspension at different stages of its growth with a desiccant balance (Humidity analyser HA60, Precisa). The correlation obtained was: [dry cell concentration] (g/L) = 0.322 × OD_{600nm} (−), with a linear regression coefficient R^2 = 0.9635, on the basis of six points, which were each reproduced twice.

2.2. Bioreactors for the methane hydroxylation

2.2.1. The membrane bioreactor (MBR)

Fig. 2 gives a technical description the new MBR for methane biohydroxylation.

Reaction took place in a 100-ml stirred tank containing two phases: the liquid reaction medium and a headspace filled with methane and oxygen with a gas volume (V_{gas}) of 90 ± 1 ml. The liquid, with a total volume (V_{liq}) of 150 ± 1 ml, was recirculated

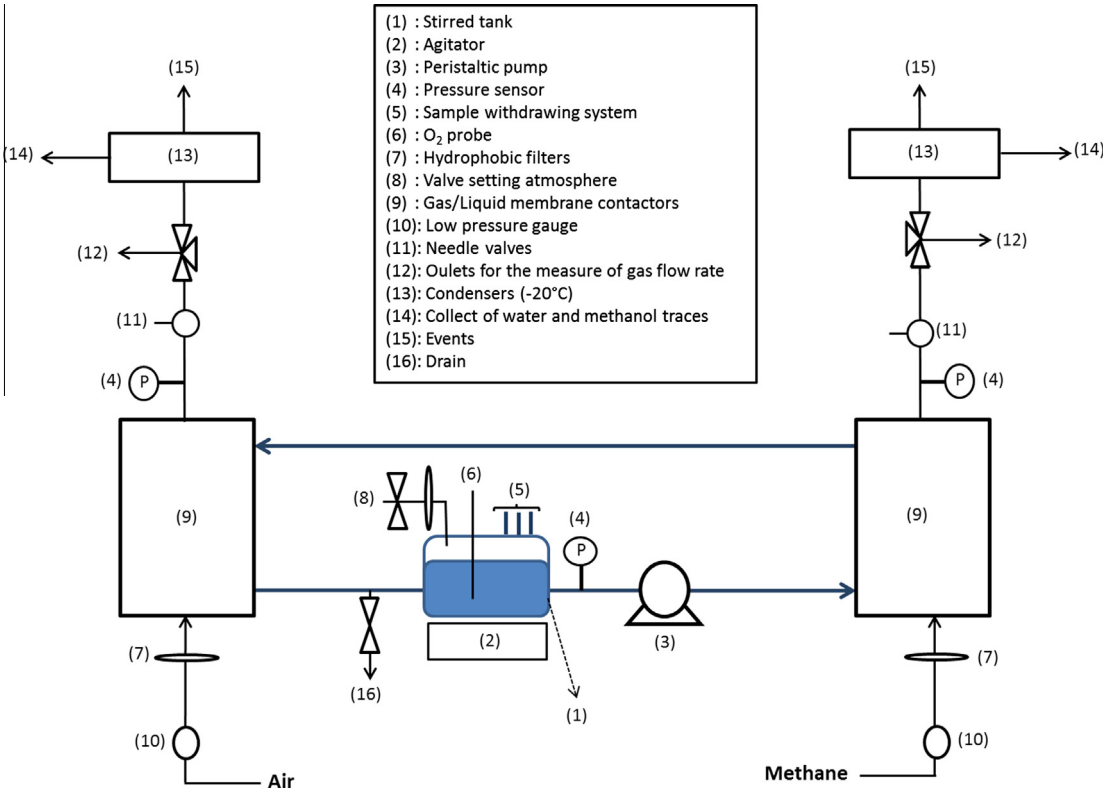


Fig. 2. Descriptive scheme of the MBR developed.

with a peristaltic pump (Masterflex L/S, Model 7518-00) at a flow rate of 37 ml min^{-1}. The volume ratio V_{gas}/V_{liq} in the bioreactor was consequently equal to 0.6. A pressure sensor was placed after the tank to measure the liquid pressure. The reaction tank was equipped with a sample withdrawal system, an O_2 probe (Oxi 340i, handheld meter) and an atmosphere setting valve.

Gases were continuously supplied to the system by means of membrane contactors connected to the circulation loop; the MBR was thus operated in a fed-batch mode. The membrane contactors were reusable modules (C75E-011-01N, Spectrumlabs) made of PolyEtherSulfone (PES) with a mean pore size of 0.45 μm and a membrane surface area of 500 cm^2. The total volume of each contactor was 41.2 ml and the fiber volume was 20.7 ml. Gases (CH_4 99.995% and air 99.999%) were supplied from bottles and sterilized on a 0.2-μm hydrophobic Teflon filter (Sartorius). The gas pressures were regulated by both low pressure gauges (placed before each contactor) and needle valves (placed after each contactor). These pressures were measured with pressure sensors inserted after the contactors. Pressures ranged from 12 ± 1 to $31 \pm 1 \times 10^3$ kPa and the associated gas flow rates were measured by means of an electronic flowmeter (Perkin Elmer). The outlet gas passed through condensers refrigerated at −20 °C so as to collect any traces of water and methanol. Condensate volumes were measured by weighing at the end of the experiment.

2.2.2. Batch reactors

Batch reactors were sealed vials with a total volume of 50 ml or 9 ml. The vial geometry was homothetically conserved whatever the volume. The vial headspace was filled with a sterile methane/air mixture (1:1 v/v) at atmospheric pressure. Two V_{gas}/V_{liq} volume ratios were implemented: 9.0 and 0.6 (where V_{gas} is the headspace volume and V_{liq} the liquid volume). The batch reactors were agitated on a rotator shaker (Unimax 1010, Heidolph) in an incubator. The sampling was carried out with a syringe.

2.3. Implementation of methane biohydroxylation in the bioreactors

2.3.1. Reaction medium and preparation of the stock resting cell suspension

Experiments were carried out with a freshly prepared, 0.2 μm-filtered reaction medium (pH = 7.0 ± 0.1), composed of 12.9 mM phosphate, 100.0 mM NaCl, 1.0 mM EDTA and 20.0 mM NaCOOH (Kim et al., 2010) in ultrapure water. At the end of the cultivation step (Section 2.1), the bacterial culture was centrifuged in sterile conditions for 20 min at 4500 rpm and 4 °C. The supernatant was removed and the bacterial pellets recovered were then suspended in the reaction medium and washed again by 20 min' centrifugation at 4500 rpm and 4 °C. The resting cell stock suspension was finally obtained by re-suspending the pellets once again in the reaction medium so that the OD at 600 nm was about 20 (determined from a dilution). This suspension could be stored at 4 °C for up to 48 h without any loss of hydroxylating activity.

2.3.2. Biohydroxylation in the batch reactor

Biohydroxylations were all performed at 30 ± 1 °C under stirring at 160 rpm. For each kinetics, a set of identical vials was filled with the same bacterial suspension and incubated. The reaction was stopped after different reaction times. When the volume ratio V_{gas}/V_{liq} = 0.6 was implemented, the CH_4/air (1:1 v/v) gas mixture was first bubbled into the reaction medium for 10 min as a preceding study had shown that 5 min were sufficient to saturate the reaction medium with oxygen in such bubbling conditions.

2.3.3. Biohydroxylation in the MBR

Biohydroxylations were carried out at room temperature (T = 25 ± 5 °C). The gas pressures were first adjusted to similar values ($12 \pm 1 \times 10^3$ kPa) for both methane and air and the reaction medium was put into circulation in the bioreactor with the required liquid flow rate. The reaction medium was saturated with methane for 10 min then with oxygen for 10 min (with gas pressures of $20 \pm 1 \times 10^3$ kPa) before the replacement of a 50-ml volume of reaction medium by an identical volume of bacterial suspension (with the desired biocatalyst concentration).

The experiments were then run for at least 24 h. Samples were regularly withdrawn and the total sampling volume did not exceed 15% of the bioreactor liquid volume. The first sample was taken after a delay of at least 3 times the reactor residence time (i.e., 4 min) to ensure that the bacterial suspension was sufficiently mixed. The methanol concentration was measured in each sample. At the beginning and end of each experiment, an OD_{600nm} measurement was also made to check the bacterial concentration.

After each experiment, gas overpressure ($20 \pm 1 \times 10^3$ kPa) was applied to the membrane contactors in order to desorb any bacteria that may have been adsorbed onto the fiber module. The bioreactor was then drained and washed with 5 times the reactor volume of water and disinfected with 0.5-L isopropanol (70%). The washings were performed in both inlet/outlet and outlet/inlet modes. Contactors were immediately dried with sterile air for at least 24 h and connected to hydrophobic air filters to maintain the sterility of the modules. The tank and the tubing were autoclaved and stored in sterile conditions.

All the experiments in the batch or membrane bioreactor were carried out at least 3 times in order to check the reproducibility of the results.

2.3.4. Methanol analysis by GC–MS

Liquid samples were analyzed by headspace GC–MS. Gas-chromatography (Clarus 580, Perkin Elmer) associated with a mass spectrometer (SQ85, Perkin Elmer) used a capillary column (Rt-Q-Bond Plot, Resteck). An automatic sampler (Turbomatrix HS16, Perkin Elmer) was coupled to the GC and sealed analysis vials of 22 ml were used. Elution was performed with helium as the carrier gas. In all cases, the method consisted of a 15-min sample thermostating step at 75 °C followed by a temperature ramp: 40 °C for 5 min, then 10 °C min^{-1} up to 150 °C and, finally, 150 °C for 15 min. A previous study had shown that gas/liquid equilibrium in the analysis vial was obtained after 5 min.

2.3.4.1. Analysis of the methanol produced in the liquid suspension. Samples were collected in 2-ml, locked Eppendorf tubes. Upon collection, samples were immediately centrifuged for 3 min at 4500 rpm and 4 °C in order to stop the reaction inside the sample by pelleting the bacteria. The supernatant was collected (1.5 ± 0.7 ml) and inserted into an analysis vial. An internal standard (absolute ethanol) was then added to the supernatant at a final concentration of 29.6 ± 0.2 mg L^{-1} before the vial was sealed for analysis. A preliminary study demonstrated that such sample treatment had no influence on the measured methanol concentration compared to a sample directly analyzed upon collection (the thermostating phase at 75 °C causing the reaction to stop).

Calibration curves were obtained with absolute methanol diluted in the reaction medium. Two calibration curves were plotted, for the upper (10–250 mg L^{-1}) and lower (0.5–9 mg L^{-1}) methanol concentration ranges.

2.3.4.2. Analysis of methanol in the condensate. The volumes of condensates collected (300–1000 μl) were too low to be analyzed with the previous headspace method. A headspace method by total sample evaporation was therefore used. Upon collection, 50 μl of each condensate sample was directly introduced into the analysis vial, which was subjected to the thermal cycle described above.

A calibration curve for methanol concentration ranging from 10 to 200 mg L^{-1} was established.

3. Results and discussion

3.1. Methane biohydroxylation in batch reactors

3.1.1. Choices of the culture medium (CM) and reaction medium (RM)

M. trichosporium OB3b is a methanotrophic bacterium that produces two forms of MMO, both of which catalyze methane hydroxylation: a soluble form (sMMO) and a membrane-bound particulate form (pMMO). The latter has been shown to actively participate in methane hydroxylation catalysis (Ayala and Torres, 2004; Balasubramanian et al., 2010; Ito et al., 2014). *M. trichosporium* OB3b is usually cultivated with an NMS medium (Whittenbury et al., 1970) and the growth medium composition has been shown to influence the production of these enzymes (Ayala and Torres, 2004; Fox et al., 1990; Murrell and Jetten, 2009). In particular, a modification of the NMS medium by an enrichment in iron(II) and copper(II) favors MMO production (Fox et al., 1990). It has been demonstrated that pMMO is mainly produced under conditions of copper sufficiency whereas sMMO is mainly produced under copper-limiting conditions (Ayala and Torres, 2004). In this work, aiming to perform methane biohydroxylation, a modified NMS medium (Fox et al., 1990) enriched with copper was preferred as the culture medium so that the cells would predominantly synthesize the pMMO form. The modified medium developed by Kim et al., 2010 (Kim et al., 2010; Lee et al., 2004) was consequently chosen as the culture medium (CM) for this study and bacteria were cultivated as described in Section 2.1.

The bacterial suspension used for the hydroxylation was then prepared according to Section 2.3.1 by pelleting the cultivated bacteria and suspending them in a reaction medium (RM) after a wash with this medium. The reaction medium (RM) was intended to inhibit MDH activity (Fig. 1) and keep the bacteria in a good physiological state during the reaction. Various reaction media have been employed (notably by Duan et al. (2011), Kim et al. (2010), Lee et al. (2004) and Mehta et al. (1991)) for methane biohydroxylation performed with the bacterium *M. trichosporium* OB3b. Table 1 summarizes the different operating conditions implemented in batch reactors in the literature and the results obtained.

Three research groups have developed different RM for methane hydroxylation: Mehta et al. (1991), Kim et al. (2010) and Duan et al. (2011). In their latest work, Kim et al. (2010) reported optimized RM giving good productivity (80 mg methanol (dry cell g)$^{-1}$ h^{-1}), which is higher than those reported more recently by Duan et al. (2011). The conditions used in the pioneering work of Mehta et al. (1991) resulted in the best productivities (280–285 mg methanol (dry cell g)$^{-1}$ h^{-1}) reported in the literature to date. However, the differences observed in the reported productivities may not be exclusively linked to the RM composition but may also depend on the origin of the strain, the culture medium used for cultivating the bacteria, the reaction temperature, the source of oxygen (air or pure oxygen) and the volume ratios V_{gas}/V_{liq} implemented. All three reported RM (Mehta et al., 1991; Kim et al., 2010; Duan et al., 2011 with or without sodium formate) were therefore tested in this study to choose the best RM for use in our experimental conditions and reactor configuration.

Preliminary experiments with these three different RM were carried out in a batch reactor with a volume ratio V_{gas}/V_{liq} fixed at 9, a gas mixture composed of CH_4:air (1:1 v/v) and a bacterial concentration of 73 ± 5 mg dry cell L^{-1} obtained from the same bacterial culture. The bacteria were washed with the reaction medium under investigation before being diluted in the same medium to reach the required mass concentration. The methanol productivities measured after one hour of reaction at 30 °C under 160 rpm agitation were (in mg methanol (dry cell g)$^{-1}$ h^{-1}): 15 (for an RM similar to the RM reported by Mehta et al. (1991)), 150 ± 20 (for an RM similar to the RM reported by Kim et al. (2010)), 15 ± 10 and 30 ± 15 (for an RM similar to the RM reported by Duan et al. (2011), respectively without and with sodium formate). The best productivity was clearly obtained for the reaction medium of Kim et al. (2010) and Kim et al. 2010). Controls performed without bacteria demonstrated that no spontaneous reaction occurred and thus that the methanol production was due exclusively to the bacteria. This reaction medium was thus chosen as the RM for the rest of this work.

Table 1
Batch reactor: maximum productivities (P_{max}) reported in the literature for methane biohydroxylation (in mg methanol (dry cell g)$^{-1}$ h^{-1}). Times (t_{pdvty}) for which the maximum productivities were measured are indicated. For each case, operating conditions: cell state ($cell_{state}$), temperature (T), composition of the reaction medium (RM composition), total production period (t_{prod}) and cell concentration ([cell]) are also given.

References	$cell_{state}$	V_{gas}/V_{liq} (−)	T (°C)	RM composition	t_{prod} (h)	t_{pdvty} (h)	[cell] (dry g/L)	P_{max} (mg g^{-1} h^{-1})
Mehta et al. (1991)	Free cells	5	35	100 mM phosphate 5 mM $MgCl_2$	–	–	0.6	**285**[a]
	Cells immobilized on DAE-cellulose			100 mM phosphate 5 mM $MgCl_2$	6	2.5	3.6	**280**[a]
				After adding 40 mM sodium formate at 6 h	14			
Lee et al. (2004)	Free cells	3	25	12.9 mM phosphate 20 mM sodium formate 200 mM NaCl	36	15	0.6	**25**
	Free cells				12	8		**75**
Kim et al. (2010)	Free cells with repeated methanol removal[b]	4	25	12.9 mM phosphate 20 mM sodium formate 100 mM NaCl 1 mM EDTA	24	8	0.6	**80**[b]
Duan et al. (2011)	Free cells	6	30	400 mM phosphate 10 mM $MgCl_2$	40	10	17.3	**4**[a]
				400 mM phosphate 10 mM $MgCl_2$ 20 mM sodium formate	40	20		**3**[a]

[a] Pure oxygen and methane used as substrates.
[b] Methanol removal repeated 3 times at 8 h intervals.

3.1.2. Establishing the biocatalyst concentration in the bioreactors

As described in Section 2.1, the strain was maintained in liquid form with sub-cultures in the culture medium (CM) since freezing of strain aliquots could damage the activity of the bacterial MMOs (Stirling and Dalton, 1979), leading to poor hydroxylating activity of the bacteria.

Moreover, this work evidenced that the intrinsic hydroxylating activity of the bacteria could vary with the age of the bacterial suspension (i.e., with the number of sub-cultures performed before the preparation of the bacterial suspension). In our case, a 4-fold reduction of the strain activity was notably observed over 6 months of cultivation. For this reason, we decided to report the catalytic concentration in u ml^{-1} in this study, where u designates a catalytic unit by analogy with enzymology, instead of the classical unit which is the bacterial mass concentration (mg dry cell ml^{-1}). The catalytic unit was defined here as the mass of bacteria required to form 1 μg of methanol in the RM within 1 h in a 50 ml-batch reactor incubated at 30 °C under stirring at 160 rpm and with a volume ratio V_{gas}/V_{liq} of 9. Thus, the catalytic concentration (u ml^{-1}) of a resting cell suspension corresponds to the methanol concentration (mg L^{-1}) measured in the conditions described above. Good reproducibility of the measurement of this catalytic concentration was evidenced by a low dispersion coefficient (7%).

For the different experiments carried out in this study, the biocatalyst concentration in the bioreactors ranged from 11 to 150 u ml^{-1}. These concentrations were adjusted to the required value by adequate dilution of the bacterial suspension.

3.1.3. Influence of the volume ratio V_{gas}/V_{liq} and the biocatalyst concentration on the hydroxylation performance in batch reactor configuration

Among the gaseous reactants, oxygen can be considered as the limiting substrate in this study because the gas mixture was composed of equal volumes of air and methane, corresponding to an oxygen/methane molar ratio of 1:4 while the solubility of methane in water is much higher than the oxygen solubility when oxygen is part of air ($2.42\ 10^{-5}$ mol L^{-1} for methane in water at 25.0 °C at 1.0 atm against 4.57×10^{-6} mol L^{-1} for the oxygen of the air in the same conditions; Perry's Chemical Engineers' Handbook, 1998). The volume ratio V_{gas}/V_{liq} was identified as a key parameter for methanol production because this factor is directly linked to the maximum quantity of oxygen available for the reaction and thus to a possible oxygen limitation.

Moreover, the biocatalyst concentration can be also identified as a key parameter for methanol production once there is no substrate limitation.

Concerning the oxygen limitation, the maximum methanol concentration ($[CH_3OH]_{max,th}$) that could theoretically be produced in the liquid phase of the batch reactor can be expressed on the basis of the maximum oxygen quantity available ($n_{O_2,max}$) according to the stoichiometry 1:1 in Eq. (1) and this concentration is a function of the volume ratio V_{gas}/V_{liq} as given in Eq. (3).

$$[CH_3OH]_{max,th} = \frac{n_{CH_3OH,max,th}}{V_{liq}} = \frac{n_{O_2,max}}{V_{liq}} = \frac{n_{O_2,max}}{V_{liq}} = \frac{1}{V_{liq}}\left(\frac{0.2V_{air}}{V_{m,g}}\right) \quad (2)$$

where $[CH_3OH]_{max,th}$ (mol L^{-1}) is the theoretical maximum methanol concentration, V_{liq} (L) is the liquid volume in the batch reactor, V_{air} (L) is the initial air volume introduced in the headspace, $V_{m,g}$ (L mol^{-1}) is the molar volume of ideal gases for a temperature of 30 °C. In this study, $V_{m,g}$ = 24.86 L mol^{-1}, $n_{O_2,max}$ (mol) is the maximum oxygen quantity available for the reaction, $n_{CH_3OH,max,th}$ (mol) is the maximum methanol quantity that could theoretically be produced in the liquid phase of the batch reactor.

Finally, as the initial air volume (V_{air}) is half of the total gas mixture volume (V_{gas}), we can write Eq. (3) as:

$$[CH_3OH]_{max,th} = \frac{V_{gas}}{V_{liq}}\left(\frac{0.2}{2V_{m,g}}\right)M_{CH_3OH} \quad (3)$$

where $[CH_3OH]_{max,th}$ (g L^{-1}) is the theoretical maximum methanol concentration, M_{CH_3OH} (g mol^{-1}) is the molar mass of methanol. M_{CH_3OH} = 32 g mol^{-1}.

In this work, two biocatalyst concentrations (11 ± 1 and 70 ± 5 u ml^{-1}) and two volume ratios V_{gas}/V_{liq} (9 and 0.6) were tested in the batch reactor. Fig. 3(a and b) gives the methanol production kinetics for these different operating conditions.

All kinetics exhibited two phases: one phase for which the methanol concentration increased linearly, meaning that production was maximum (up to 6 h) followed by a leveling off of the methanol concentration, when production no longer occurred.

The associated productivity calculated on the basis of the methanol concentration obtained at 3 h, taken as the middle of the maximum production phase, and the maximum methanol concentrations accumulated are also reported in Fig. 3c.

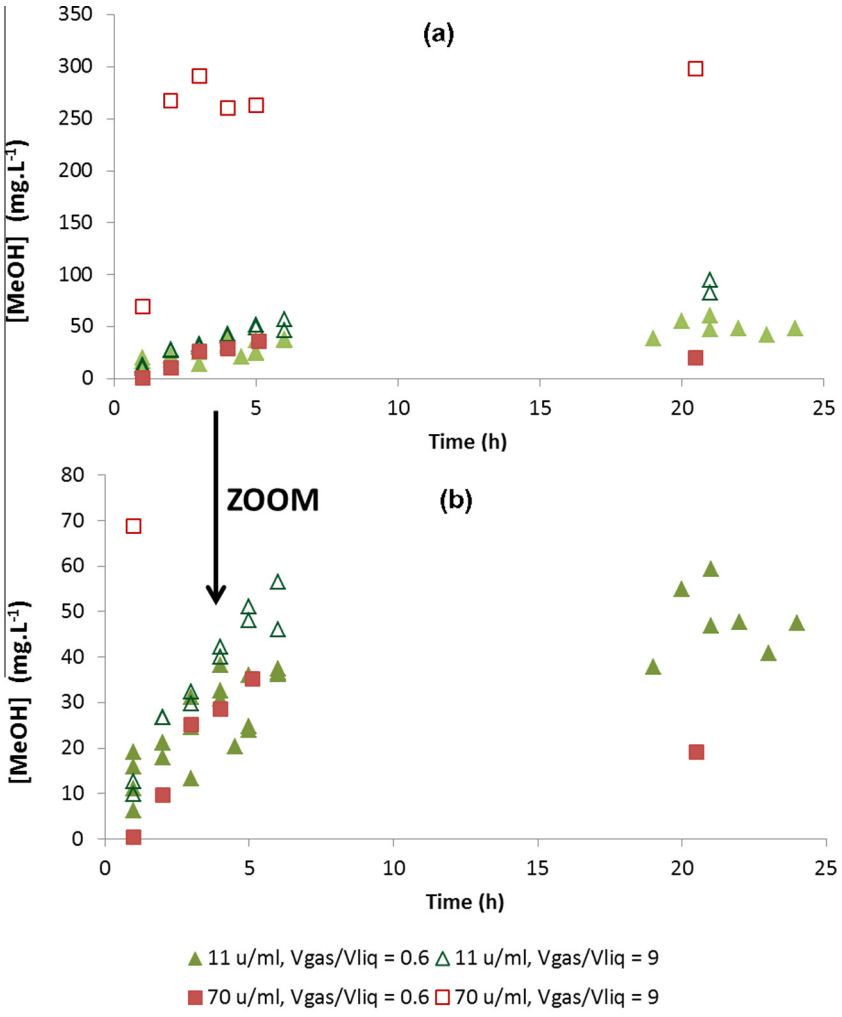
For the lowest volume ratio (V_{gas}/V_{liq} = 0.6), the methanol concentration profiles versus time and the maximum methanol concentrations did not show any significant differences for the two biocatalyst concentrations (11 and 70 u ml^{-1}). On the one hand, one explanation of the observed phenomenon may be a substrate limitation that occurred during the reaction. However, the theoretical maximum methanol concentration that could be produced in this case was 77 mg L^{-1} (determined from Eq. (3)), which is higher than the highest methanol concentration obtained (49 mg L^{-1}). This means that there was sufficient oxygen available for the reaction. On the other hand, the fact that the methanol production stopped for both biocatalyst concentrations at methanol concentrations of the same order of magnitude (35–49 mg L^{-1}) could be explained either by a reduced mass transfer or by methanol inhibiting the bacteria (as already observed by Kim et al. (2010) and Duan et al. (2011)).

Whatever the biocatalyst concentration, the productivity and the maximum methanol concentration accumulated were better for the highest volume ratio (V_{gas}/V_{liq} = 9) (Fig. 3c). In that case, the methanol production rates differed with the catalytic concentration but no proportionality coefficient was clearly identified between these rates and the biocatalyst concentration. Nevertheless, the highest productivity (1400 ± 170 MeOH Mu^{-1} h^{-1}, where u is the catalytic unit defined in this work) and the highest methanol concentration (290 ± 23 mg L^{-1}) were obtained for the highest catalyst concentration. However, this concentration remained much lower than the theoretical maximum methanol concentration that could be produced in this case, i.e., 1158 mg L^{-1} (determined from Eq. (3)). It could thus be reasonably assumed that no substrate limitation occurred for such volume conditions and the cessation of production evidenced after 3 h for the 70 u ml^{-1} concentration may have been due either to a mass transfer limitation or a methanol inhibition (Kim et al., 2010; Duan et al., 2011).

The best productivity (1400 ± 170 mg MeOH Mu^{-1} h^{-1}) was obtained for a biocatalyst concentration of 70 ± 5 u ml^{-1} and V_{gas}/V_{liq} = 9. It is worth noting that, when considered per unit mass of dry cell, this productivity (270 ± 50 mg MeOH (g dry cell)$^{-1}$ h^{-1}) appeared to be among the highest reported in the literature to date (Table 1).

3.2. Methane biohydroxylation within the new MBR

The interests of developing a new MBR configuration for methane biohydroxylation, such as the design presented here, lies in the large exchange area of the macroporous membrane contactors



(c)

Biocatalyst concentration ($u.ml^{-1}$)	V_{gas}/V_{liq} (-)	Productivity (mg MeOH.$Mu^{-1}.h^{-1}$)	$[CH_3OH]_{max,mes}$ ($mg.L^{-1}$)
△ 11 ± 1	9	950 ± 80	88 ± 6
▲ 11 ± 1	0.6	700 ± 270	49 ± 8
□ 70 ± 5	9	1400 ± 170	290 ± 23
■ 70 ± 5	0.6	120 ± 30	35 ± 4

Fig. 3. (a) Methanol production kinetics in a batch reactor for different biocatalyst concentrations (11 and 70 u ml^{-1}) and different volume ratios V_{gas}/V_{liq} (9 and 0.6); (b) zoom on the methanol production kinetics; (c) associated productivities calculated on the basis of the methanol concentration obtained at 3 h and maximum methanol concentrations accumulated in the batch reactors. Productivities are given in mg MeOH Mu^{-1} h^{-1} where u is the catalytic unit defined in this work and 1 Mu = 10^6 u; maximum methanol concentrations are given in mg L^{-1}.

combined with the operating safety offered by this innovative MBR. Hydrodynamic tests were first performed with the reaction medium devoid of bacteria to determine the operating parameters that allowed bubble-free, reproducible operation. A liquid flow rate Q_{liq} of 37 ± 1 ml min^{-1} (leading to a residence time of about 4 min) combined with gas flow rates (methane and air) of 47 ± 7 ml/min was shown to meet the required criteria. These gas flow rates correspond to the lowest gas pressures tested (12 ± 1 × 10^3 kPa), inducing the lowest gas consumption. Under these conditions, the dissolved oxygen concentration measured in the MBR versus time remained constant for 2 consecutive days and equal to the previously determined saturated concentration of dissolved oxygen in the reaction medium (i.e., 6.2 ± 0.1 mg L^{-1}).

3.2.1. Feasibility and reproducibility

A biocatalyst concentration of 11 u ml^{-1} was chosen to assess the feasibility of the methane biohydroxylation within this new MBR; this concentration being easily reproducible and requiring quite a small quantity of biocatalyst.

Fig. 4 shows the methanol production kinetics obtained in eight independent experiments. Among these, a shifted experiment was

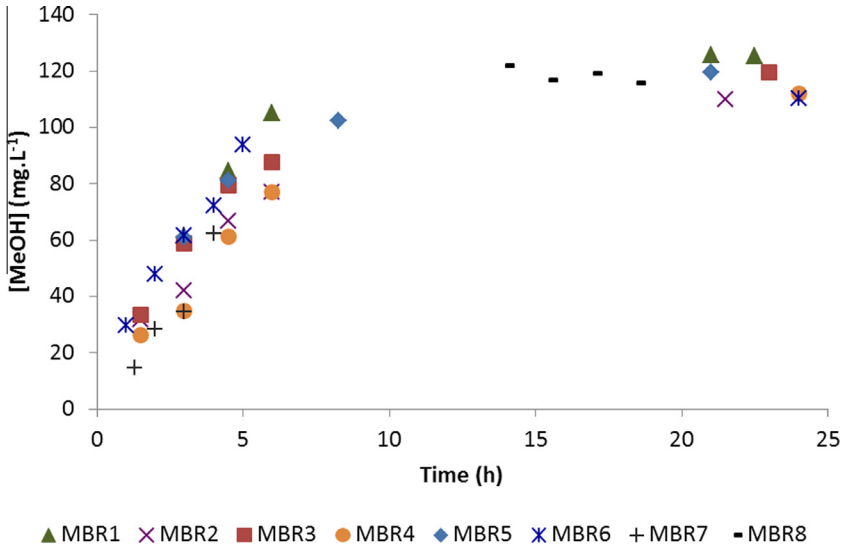


Fig. 4. Methanol production kinetics within the MBR (biocatalyst concentration: 11 ± 1 u ml^{-1} and V_{gas}/V_{liq} = 0.6).

performed to complete the methanol concentration profile between 14 h and 21 h.

The results demonstrate that high methanol production occurred in the MBR developed here. An average productivity of 1500 ± 400 mg MeOH Mu^{-1} h^{-1} was obtained on the basis of the methanol concentration measured at 3 h. The dispersion of the mean productivity (26%) pointed to relatively good reproducibility that could be induced first by a low dispersion on the biocatalyst concentration but was also influenced by the fact that the membrane area (0.1 m^2) was large in comparison with the suspension volume (150 ± 1 ml) and contributed locally to a homogenous substrate concentration.

As was observed in the batch reactor, the kinetics comprised two phases: a phase of maximum production (up to 6 h) followed by a plateau (since 14 h), at the beginning of which production stopped. The average methanol concentration accumulated in the bioreactor over 24 h was 120 ± 5 mg L^{-1}. The cessation of production may be attributed either to an inhibition of the bacteria by methanol (Duan et al., 2011) or to a substrate limitation. Monitoring the dissolved oxygen concentration in the reaction medium containing the bacteria at this concentration revealed a constant saturated oxygen concentration (6.2 ± 1 mg L^{-1}). Thus, there was no oxygen limitation for these conditions and consequently no methane limitation, as oxygen was known to be the limiting substrate.

It is worth noting that traces of methanol were recovered in the condensates collected over 24 h for each experiment. Nevertheless, total methanol quantities recovered in the condensate volumes ranged from 0.5 to 1.6 ± 0.1 mM and the average methanol quantity accumulated inside the bioreactor over 24 h was 571 ± 28 mM. So the proportion of methanol recovered in the condensates did not exceed 0.3% of the total methanol quantity accumulated in the MBR over 24 h. The methanol quantities collected in the condensates were therefore neglected in the rest of this work.

3.2.2. Comparison with batch reactors

Experiments were performed in a batch reactor and the MBR in similar conditions: V_{gas}/V_{liq} = 0.6 and biocatalyst concentration of 11 u ml^{-1} (Figs. 3 and 4). Productivities measured at 3 h (in mg MeOH Mu^{-1} h^{-1}) were 1500 ± 400 for the MBR and 700 ± 270 for the batch reactor, showing enhanced hydroxylation performance when the MBR was used.

The MBR differs from the batch reactor by its regular substrate feed. Its better performance could be due to a substrate limitation in the batch reactor or to enhanced mass transfer in the MBR. As discussed before (Section 3.1.3), there was no substrate limitation in the batch reactor and we assumed that the methanol concentration threshold observed in the batch reactor could be due either to a mass transfer limitation or to methanol inhibiting the bacteria.

In both systems (MBR and batch reactor), a methanol concentration threshold was observed although a significantly higher methanol concentration was reached for the MBR (120 ± 5 mg L^{-1}) than for the batch reactors (49 ± 8 mg L^{-1} for V_{gas}/V_{liq} = 0.6 and 88 ± 6 mg L^{-1} for V_{gas}/V_{liq} = 9, Fig. 3c), which contradicts the assumption of bacteria being inhibited by the methanol produced in the batch reactors.

A mass transfer limitation seems to be an interesting hypothesis. Thus, the better performance of the MBR compared to that observed in the batch reactors could be attributed to better substrate transfer toward the biocatalyst.

3.2.3. Comparison with the literature for continuous or fed-batch reactors

Table 2 summarizes the different operating conditions implemented in either continuous or fed-batch reactors reported in the literature together with the results obtained. The results of this work are also noted.

A mean productivity of 75 ± 25 mg methanol (g dry cell)$^{-1}$ h^{-1} was obtained with the new MBR developed in this study. This productivity was nearly 35-fold that reported by Duan et al., 2011 for a fed-batch bioreactor in which the gaseous substrates were fed through two dense silicone tubes immersed in the reaction medium. This result showed that macroporous membranes allowed much better gas/liquid transfer compared to dense membranes. In addition, the productivity reached with the MBR developed in this study appeared to be similar to the best productivities reported for either continuous or fed-batch bioreactors that were operated with bubble-spargers (75–80 mg methanol (g dry cell)$^{-1}$ h^{-1}) for the gas supply (Kim et al., 2010; Duan et al., 2011). However, by using bubbleless contactors, the MBR avoids the formation of bubbles and thus the possible formation of an explosive gas mixture. The MBR developed thus seems more attractive than the continuous reactors previously reported in the literature.

Table 2
Either continuous or fed-batch reactors: maximum productivities (P_{max}) reported in the literature and found in this work for methane biohydroxylation (in mg methanol (dry cell g)$^{-1}$ h^{-1}). Times (t_{pdvty}) for which the maximum productivities were measured are indicated. Operating conditions: operating mode and gas feeding system (mode), temperature (T), composition of the reaction medium (RM composition), total production period (t_{prod}) and cell concentration ([cell]) are also detailed.

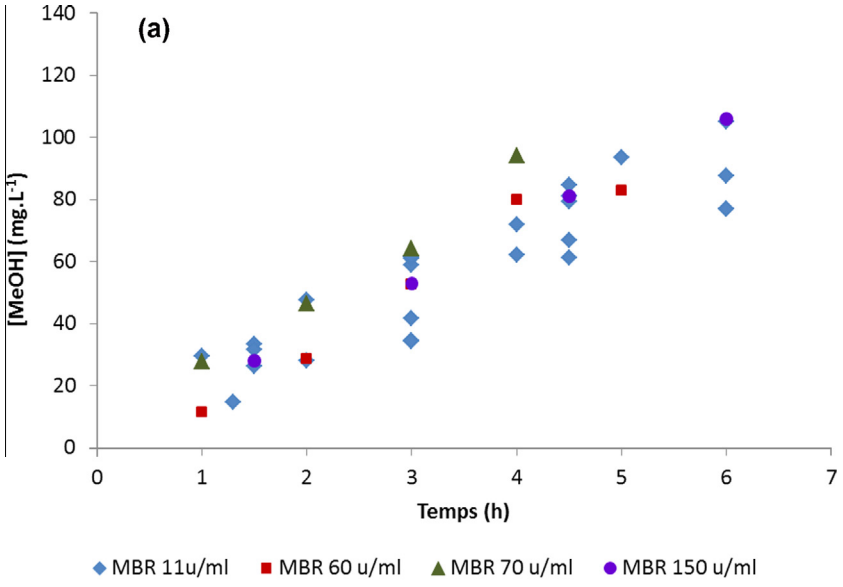
Reference	Mode	T (°C)	RM composition	t_{prod} (h)	t_{pdvty} (h)	[cell] (dry g/L)	P_{max} (mg g^{-1} h^{-1})
Mehta et al. (1991)	Continuous (sparger and immobilized cells)	35	100 mM phosphate 5 mM $MgCl_2$	11	5	2	**32**[a]
			After adding 4 pulses of sodium formate from 15 h	70	37		**80**[a]
Kim et al. (2010)	Fed-batch (sparger)	25	12.9 mM phosphate 20 mM sodium formate 100 mM NaCl 1 mM EDTA	16	8	0.6	**75**
Duan et al. (2011)	Fed-batch (dense membranes)	30	400 mM phosphate 10 mM MgCl2	40	20	17.3	**2**[a]
This work	Fed-batch (macroporous membranes)	25	12.9 mM phosphate 20 mM sodium formate 100 mM NaCl 1 mM EDTA	14	3	0.2 (eq. 11 u/ml)	75 ± 25 (eq. 1500 ± 400 mg MeOH Mu^{-1} h^{-1})

[a] Pure oxygen and methane used as substrates.

3.2.4. Influence of biocatalyst concentration on hydroxylation performance

As the biocatalyst concentration could have an influence on the methanol production, different catalytic concentrations were tested within the MBR: 60 ± 4, 70 ± 5 and 150 ± 11 u ml^{-1}. Fig. 5 shows the methanol production kinetics obtained with the MBR for these increasing biocatalytic concentrations over the maximum production period (Fig. 5a) and the respective productivities determined at 3 h (Fig. 5b).

The kinetics exhibited the same profiles during the first six hours with a maximum production phase followed by a significant slowdown (60, 70 and 150 u ml^{-1}) or even a stopping of production (11 u ml^{-1}) up to 24 h (data not shown). These types of behavior could probably be linked to limitation of a substrate, e.g. oxygen



(b)

Biocatalyst concentration (u.ml^{-1})	Productivity (mg MeOH.Mu^{-1}.h^{-1})
11 ± 1	1500 ± 400
60 ± 4	300 ± 80
70 ± 5	310 ± 80
150 ± 10	120 ± 30

Fig. 5. (a) Methanol production kinetics within the new MBR for different biocatalyst concentrations: 11 ± 1, 60 ± 4, 70 ± 5 and 150 ± 11 u ml^{-1} over the maximum production period and (b) associated productivities measured at 3 h.

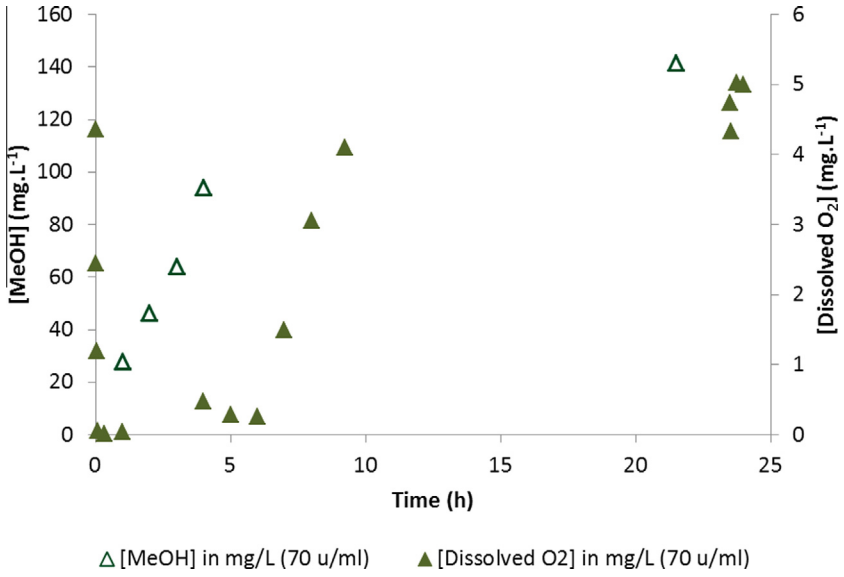


Fig. 6. Evolution of methanol concentration ([MeOH]) and dissolved oxygen concentration ([Dissolved O_2]) versus time in the MBR for a biocatalyst concentration of 70 ± 5 u ml^{-1}.

for biocatalyst concentrations higher than 11 u ml^{-1}. To check this assumption, the dissolved oxygen concentration was measured in the bioreactor during the methane hydroxylation for catalyst concentrations of 70 and 11 u ml^{-1}. For 11 u ml^{-1}, the dissolved oxygen concentration remained at the saturated oxygen concentration (i.e., 6.2 ± 0.1 mg L^{-1}) throughout the reaction, indicating that no oxygen limitation occurred (Section 3.2.1). Fig. 6 reports the evolution versus time of both methanol and dissolved oxygen concentrations for a biocatalyst concentration of 70 ± 5 u ml^{-1}.

The dissolved oxygen content decreased dramatically, falling almost to 0 during the first 5 min of the reaction, then increased very slowly up to 0.3 mg L^{-1} in the first 6 h (Fig. 6). After this first phase, the dissolved oxygen content increased continuously up to 5.0 ± 0.1 mg L^{-1} at 24 h. It is worth noting that this enhancement took place with a time-lag of approximately 5 h with respect to the methanol production. The oxygen content seemed to increase when methanol production reached its maximum.

This result shows an oxygen limitation during the first five hours. There could be a variety of reasons for this, such as very high substrate consumption or a dramatic decrease in mass transfer due to the plugging of membrane porosity by the adsorption of bacteria. The latter phenomenon has been already observed by Coutte et al., 2010 for surfactant production. When washing the modules, we noticed that bacteria were adsorbed on the surface of the hollow fibers and the observation was completed by OD_{600nm} measurements in the bioreactor. However, in our case, the OD_{600nm} of the suspension measured at 6 h was the same as the initial value. So the initial substrate limitation observed was certainly due to very rapid substrate consumption during the first hours of the bioreaction.

After 6 h, the dissolved oxygen concentration rose to 5.0 ± 0.1 mg L^{-1}, which is lower than the saturated oxygen concentration. The fact that the dissolved oxygen did not reach the saturated concentration (6.2 ± 0.1 mg L^{-1}) may be attributed to adsorption on the membranes as $33 \pm 4\%$ of the bacteria were adsorbed at the end of the experiment.

Moreover, with a lower biocatalyst concentration (11 u ml^{-1}), the dissolved oxygen concentration was equal to the saturation value even though a similar adsorption was observed ($35 \pm 4\%$ of the bacteria were adsorbed after 22 h). The fact that the catalytic concentration was 6.4-fold higher at 70 u m^{-1} than at 11 u ml^{-1} induced a 9-fold higher mass concentration in this case, which could explain why adsorption had an effect on the mass transfer at 70 u ml^{-1} and not at 11 u ml^{-1}.

In conclusion of this study on the influence of the biocatalyst concentration, as methanol production kinetics were similar for all the concentrations tested, the minimum biocatalyst concentration tested (11 u ml^{-1}) can be considered the most attractive since it limits the quantity of bacteria used.

4. Conclusion

This work studied microbial methane hydroxylation with *M. trichosporium* OB3b for different reactor configurations. An innovative MBR configuration coupling two membrane contactors to enhance the surface exchange was developed and resulted in an optimal mass transfer while avoiding the formation of bubbles or dangerous gas mixtures. Nevertheless, for all reactors studied, it was observed that methane biohydroxylation stopped, probably due to biological aspects that will be investigated further.

As a long-term perspective, this MBR could also be implemented for the biohydroxylation of other gaseous alkanes as bacteria strains are known to grow on C_2–C_4 gaseous alkanes.

Acknowledgements

Professors J.D. Lipscomb and I. Okura are gratefully thanked for providing the strain for our preliminary tests. We also thank Mrs. Loubna Karfane for her precious help in the follow-up of the MBR. Mrs. Susan Becker is thanked for reading the text.

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