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# Effect of Growth Conditions on the Expression of Soluble Methane Monooxygenase

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**Summary**

Methanotrophic-heterotrophic groundwater community (culture MM1) and the obligate type II methanotroph (strain Met1) isolated from the culture MM1 were found to express soluble methane monooxygenase (sMMO) enzyme system when grown in simple batch culture (shake flasks) under the determined conditions. To optimize conditions for the enzyme expression, effects of the essential constituents of nitrate mineral salts (NMS) medium on the growth and sMMO activity was studied. The obtained results confirmed that copper deficiency was essential for sMMO expression in both cases, *i.e.* when methanotrophic strain Met1 was grown in the community and as a single culture. Furthermore, under the experimental conditions the specific whole-cell sMMO activity of the strain Met1, measured by the naphthalene oxidation assay, showed no significant effect on the increased $KNO_3$ concentration from 10 to 20 mM. In contrast, when $KNO_3$ was replaced by $NH_4Cl$, both the growth of the strain Met1 and the sMMO activity were highly limited. The significant positive effect on the growth and sMMO activity was observed by increasing the initial $FeSO_4$ concentration in NMS medium from 12 to 52 μM. This suggested that $Fe^{2+}$ is an essential medium constituent, which can contribute the most to the optimization of shake flask system for sMMO expression.

*Key words:* soluble methane monooxygenase, type II methanotroph, methanotrophic-heterotrophic community

## Introduction

All methanotrophs are capable of expressing a particulate or membrane-bound MMO (pMMO). However, the capability to form soluble or cytoplasmic MMO (sMMO) has been observed only in some type II methanotrophs (*Methylosinus sporium*, *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M), the type X methanotroph *Methylococcus capsulatus* and the type I methanotroph *Methylomonas methanica* 68-1 (*1–3*). The pMMO and the sMMO differ not only by their locations within the cell, but also by the differences in their substrate specificity, oxygen and copper requirements, sensitivity to inhibitors, and NAD(P)H requirements, all of which reflect structural differences (*4–7*). The sMMO is expressed only under the conditions of copper limitation while in copper-sufficient media the pMMO is preferentially expressed. Several studies have demonstrated that copper availability is the most important individual factor governing either the location or the form of MMO enzyme in methanotrophic bacteria, although the mechanism through which copper regulates the expression of sMMO remains unknown as yet (*8–10*).

Although the primary function of the MMO enzyme system is to catalyze the oxidation of methane, sMMO is also responsible for the catalyzes of fortuitous oxidation

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of some hydrocarbons due to its unusual lack of substrate specificity. Those are *(i)* saturated, unsaturated, linear, branched, and cyclic hydrocarbons (up to approximately $C_8$ in size), and *(ii)* single- and double-ring aromatics, heterocycles, halogenated alkenes and ethers, all of them found oxidized by sMMO at a reasonable rate *(11–17)*. This capability of sMMO enzyme has attracted the interest of the scientists involved in developing biological methods for degradation of toxic chemicals (*18,19*). The capacity for oxidation and degradation of pollutants and the ready availability of methane as a growth substrate make those methanotrophs capable of expressing sMMO promising for bioremediation applications (*20,21*).

While the sMMO enzyme system has been purified and studied intensively during the last decade, the growth characteristics of microorganisms producing this enzyme have not been investigated and discussed in details. Therefore, the precise culture conditions influencing the intracellular location or the forms of the MMO enzyme are still unknown. The results reported so far only suggested that besides the requirement of copper limitation in culture medium, continuous cultivation in bioreactors provides much better conditions for the sMMO expression than culturing in batch system, where in general lower cell density can be achieved (*1,8,9*).

The objective of this work was to check and optimize the experimental conditions of simple batch system (shake flasks) for the expression of sMMO. A natural isolate identified as type II methanotroph (strain Met1), isolated from the methanotrophic-heterotrophic groundwater community, was used in this study. Intensive growth kinetic experiments presented in our previous papers (*22,23*) illustrated that, when cultured in shake flasks in nitrate mineral salts medium, the strain Met1 showed the preference for the conditions of low oxygen and excess methane tensions. Under these conditions the fastest growth was observed at 30 °C. The parameters considered in the present work include the concentrations of some key culture medium components such as copper, nitrate, ammonium and iron.

## Materials and Methods

### *Culture and growth conditions*

The methanotrophic-heterotrophic community (culture MM1) was enriched from groundwater aquifer solids in Moffett Field Naval Air Base (Mountain View, CA, USA). The details of the enrichment technique, culture conditions, morphological and physiological characteristics of mixed culture MM1 were described elsewhere (*13,16,22*). The culture MM1 was characterized as a stable consortium consisting of one methanotroph and, depending on growth conditions, four or five heterotrophs. The methanotroph (strain Met1), isolated from the culture MM1, was characterized as an obligate type II methanotroph. The culture MM1 and the strain Met1 were maintained as liquid cultures at 4 °C and periodically grown in Whittenbury mineral (NMS) medium (*24*) in shake flasks under a methane and air atmosphere (30:70) at 30 °C (*22,23*).

For the experiments presented in this work the culture MM1 and the strain Met1 were grown in 120-mL serum bottles containing 25 mL of mineral salts medium. Three series of experiments were performed. In the first series, the culture MM1 and the strain Met1 were comparatively cultured in NMS medium with or without copper addition, and the effect of $Cu^{2+}$ on the growth kinetics and MMO activity was studied. In further experiments, the culture MM1 and the strain Met1 were cultured in NMS medium without copper addition. The second series was performed to check the effects of nitrate concentration ($KNO_3$ was increased from 10 to 20 mM) and of ammonium salt addition ($KNO_3$ was replaced with $NH_4Cl$). The third series was performed to study the effect of $Fe^{2+}$ by increasing the concentration of $FeSO_4$ in NMS medium from 12 to 52 μM.

To achieve the initial concentration of approximately 25 % $CH_4$ and 15 % $O_2$ in the headspace, 30 mL $CH_4$ was injected in serum bottles after the withdrawal of an equal volume of air. The desired methane (10–25 %) and oxygen (5–15 %) tensions were maintained by repeated injections of 10–20 mL $CH_4$ and 5–10 mL $O_2$, respectively, every second day. The bottles were incubated on a rotary shaker (200 rpm) at 30 °C.

During cultivation the samples were withdrawn periodically with 2-mL sterile, disposable syringe, and biomass concentration, sMMO activity and protein concentration were determined.

### *Reagents*

Natural gas containing 98.5 % of methane was donated by INA Naftaplin (Zagreb, Croatia), air and oxygen were obtained from MG Croatia Plin (Zagreb, Croatia). All chemicals used for the growth media, colorimetric assay for sMMO detection and determination of proteins were of analytical reagent grade.

### *Biomass concentration*

Biomass concentration was determined on the optical density basis by measuring the absorbance at 600 nm on an UV/VIS-spectrophotometer (Model Cary 4, Varian, USA). The obtained data were converted to biomass dry weight (mg/L) by using calibration curves generated on dry weight of the culture of known absorbance (*22,23*).

Specific growth rates ($\mu$) of the strain Met1 for the exponential growth period were calculated from the equation:

$$\mu = \frac{1}{t_{n-1} - t_n} \ln \frac{x_{n-1}}{x_n} (\mathrm{d}^{-1})$$

where $x_n$ and $x_{n-1}$ are biomass concentrations in mg/L (dry weight) at times $t_n$ and $t_{n-1}$, respectively.

### *Colorimetric assay for sMMO activity*

Colorimetric assay is based on the capability of cells containing sMMO to rapidly oxidize the bicyclic aromatic hydrocarbon naphthalene to 1-naphthol and 2-naphthol. These products reacted with tetrazotized-o-dianisidine, resulting in the formation of purple diazo dye complex (*2,10,25*). The pMMO enzyme does not oxidize naphthalene to naphthol and is therefore unreactive in

the assay. In this work, the naphthalene oxidation assay as described by Brusseau *et al.* (*10*) was followed. The colored product was monitored on an UV/VIS spectrophotometer (Model Cary 4, Varian, USA) at 525 nm, and the specific activity of sMMO was expressed in nanomoles of naphthol formed per milligram of cell protein per hour (*2,25*). The protein content of the cell suspensions was determined by following Bradford microprotein procedure (*26*).

## Results and Discussion

Intensive growth kinetics studies presented and discussed in our previous papers (*22,23*) showed that the methanotrophic-heterotrophic community (culture MM1) originating from a groundwater aquifer and the methanotroph (strain Met1) isolated from the culture MM1 revealed good growth in simple shake flasks system under various methane and oxygen tensions. Furthermore, the obtained results suggested that the strain Met1 preferred the conditions of excess methane and low oxygen tensions ($CH_4$, 10–12 %; $O_2$, 3–5 %) and the temperature higher than those prevailing in its natural habitat (maximum growth rate was observed at 30 °C). Based on its cell morphology, the resting stages formed, the intercytoplasmic membranes and some physiological characteristics, the strain Met1 was characterized as type II methanotroph (*22*). This was confirmed by further characterization including the determination of fatty acid methyl ester profiles – FAME analysis and 16 S rRNA sequence analysis, which at the same time suggested the similarity of the strain Met1 with the most studied and characterized type II methanotroph *Methylosinus trichosporium* OB3b (*27*).

In order to check and devise experimental shake flask system for the expression of sMMO, effects of the essential constituents of nitrate mineral salts medium on the cell growth and enzyme activity were studied. The other experimental conditions maintained during the study (the headspace concentrations of methane and oxygen and temperature) were optimal for the growth of the strain Met1 (*22,23*). During cultivation sMMO activity was monitored by the naphthalene oxidation assay (*10*).

### *Effect of copper*

To check the effect of copper limitation on the expression of sMMO, the comparative growth kinetics study with the culture MM1 and the strain Met1 was performed. It is evident from Fig. 1 that, despite faster and more intensive growth of both the culture MM1

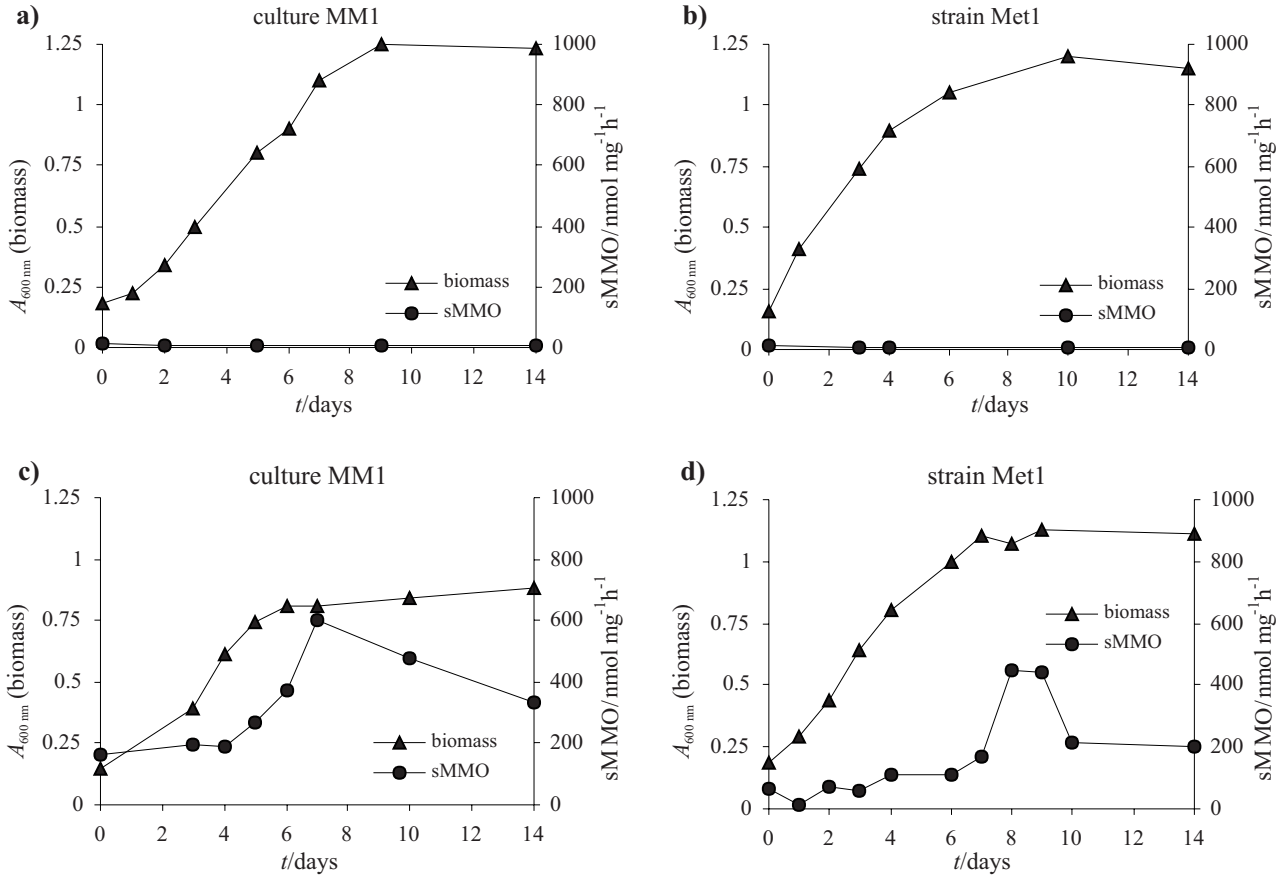


**Fig. 1. Growth (▲) and sMMO activity (●)** during the cultivation of the methanotrophic-heterotrophic community (culture MM1) and the obligate type II methanotroph (strain Met1), isolated from the culture MM1, in nitrate mineral salts medium containing 0.8 μM $CuSO_4$ (**a**, **b**) and in the same medium without $Cu^{+2}$ addition (**c**, **d**). The experiments were performed in shake flasks under a methane and air atmosphere ($CH_4$ 10–25 %; $O_2$ 5–15 %) at 30 °C. The data are means from triplicate flasks

and the strain Met1 in the original NMS medium (containing 0.8 μM $CuSO_4$) than in the same medium without $Cu^{2+}$ addition, sMMO activity was observed only in copper-free conditions. These results are in close agreement with previously published papers which showed that the $Cu^{2+}$ concentration as low as 1 μM was sufficient to shift the cellular MMO activity from soluble to the particulate form (*2,8,10,28*).

Furthermore, the results presented in Figs. 1c and 1d also illustrated that naphthalene oxidation rate, as a quantitative measure of the sMMO activity, was the highest in the early stationary phase and rapidly decreased after reaching the maximum value in both cultures. Similar observations were reported by Koh and collaborators (*2*) during shake flasks cultivation of *M. trichosporium* OB3b and *Methylomonas methanica* 68-1 in copper-free conditions. The authors presumed that copper is a specific inhibitor of the reductase component, one of the three sMMO proteins. The proposed mechanism is that copper interacts with FAD and $Fe_2S_2$, the prosthetic groups of this protein. It was also presumed that the synthesis of sMMO by some methanotrophs may be a survival mechanism in many environments where copper limits the growth of methanotrophs capable of expressing only pMMO. Thus, the habitats lacking readily available copper but abundant in methane and oxygen may provide a niche for sMMO-expressing methanotrophs (*1,3,5,8*).

### *Effects of the nitrogen source*

The effect of nitrate was studied by comparing the growth and sMMO activity during cultivation of the strain Met1 in NMS medium without $Cu^{2+}$ containing standard and twofold $KNO_3$ concentration. The obtained results showed that by increasing the $KNO_3$ concentration from 10 to 20 mM no significant effect on the growth of the strain Met1 was achieved (Figs. 2a and 2b). Furthermore, despite some oscillations in sMMO activity during the cultivation of the strain Met1 at higher nitrate concentration, the same value of naphthalene oxidation rate was achieved at the end of experiment in both cases. For illustration, Lee and collaborators (*29*) found that during batch cultivation in NMS medium with increased nitrate concentration (47 mM) the growth of the type II methanotroph *M. trichosporium* OB3b was highly inhibited. Growth inhibition was even more intensive at higher nitrate concentrations (94 and 200 mM). The authors concluded that at the concentrations higher than 20 mM nitrate could be partially reduced by nitrate reductase to nitrite, which might be toxic for the methanotroph. In those experiments sMMO activity was not monitored.

When the strain Met1 was cultured in modified NMS medium with the addition of $NH_4Cl$ instead of $KNO_3$, poor growth and no detectable sMMO activity were observed (Fig. 2c). This suggested that $NH_4Cl$, at the concentration of 10 mM, inhibited the whole-cell MMO enzyme system and suppressed the growth of the strain Met1. The most probable explanation for this observation was competitive inhibition between methane and $NH_4^+$ at the level of methane monooxygenase. The co-oxidation of ammonium salts by methanotrophs is due to the similarities in size and structure of $NH_4^+$ and $CH_4$ (*30,31*). Complete inhibition of sMMO activity and suppressed growth of the strain Met1 (Fig. 2c) suggested that during 12-day cultivation under the conditions of shake flask experiment, $NH_4^+$ almost completely excluded methane from the active site of MMO. This unexpected reduced growth of the strain Met1 may also be

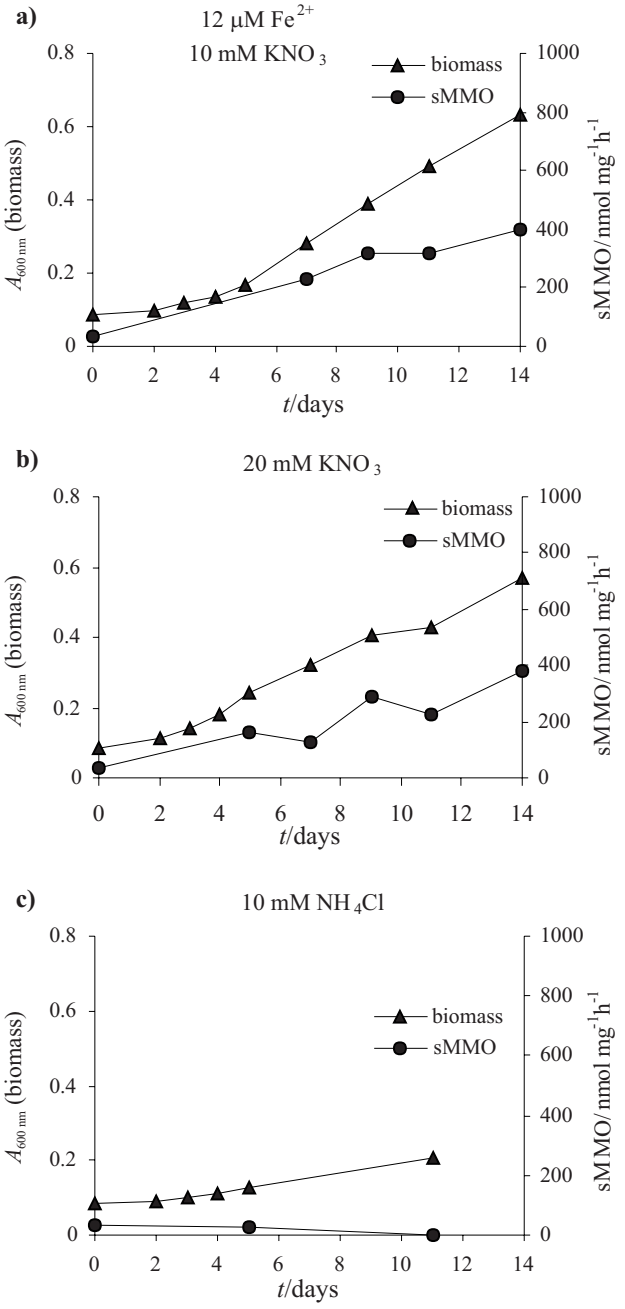


**Fig. 2. Effect of nitrate concentration and nitrogen source on the growth of the strain Met1 (▲) and the expression of sMMO (●); a)** The experiments were performed in nitrate mineral salts medium without $Cu^{+2}$ addition with standard $KNO_3$ concentration (10 mM) and **b)** with increased $KNO_3$ concentration (20 mM). **c)** For comparison, cell growth and the sMMO activity in mineral salts medium with $NH_4Cl$ (10 mM) instead of $KNO_3$ (10 mM) is also presented
Other experimental conditions were similar as in Fig. 1

Table 1. The effects of some essential medium constituents on the growth kinetics and sMMO activity during cultivation of type II methanotroph (strain Met1) and methanotrophic-heterotrophic community (culture MM1) containing the strain Met1. Experiments were performed in shake flasks system (120 mL serum bottles containing 25 mL of mineral medium) under methane and air atmosphere ($CH_4$, 10–25 %; $O_2$ 5–15 %) at 30 °C

| Mineral medium | μ | | sMMO | |
|---|---|---|---|---|
| | $day^{-1}$ | | nmol naphthol mg $protein^{-1}$ $h^{-1}$ | |
| | strain Met1 | culture MM1 | strain Met1 | culture MM1 |
| NMS | 0.50 ± 0.1 | 0.32 ± 0.08 | <5 | <5 |
| NMS[a] (without $Cu^{2+}$) | 0.18 ± 0.03 | 0.24 ± 0.05 | 400 ± 35 | 605 ± 40 |
| NMS[b] (increased $NO_3^-$) | 0.17 ± 0.03 | ND | 380 ± 50 | ND |
| NMS[c] ($NH_4^+$ instead $NO_3^-$) | 0.09 ± 0.01 | 0.12 ± 0.03 | <5 | ND |
| NMS[d] (increased $Fe^{2+}$) | 0.32 ± 0.04 | ND | 560 ± 45 | ND |

μ average specific growth rate (mean ± S.D. of three replicates) during the log phase
sMMO the maximum whole-cell activity (mean ± S.D. of three replicates)
NMS nitrate mineral salts medium (*24*) ($CuSO_4$ 0.8 μM; $KNO_3$ 10 mM; $FeSO_4$ 12 μM)
NMS[a] ($KNO_3$ 10 mM; $FeSO_4$ 12 μM)
NMS[b] ($KNO_3$ 20 mM; $FeSO_4$ 12 μM)
NMS[c] ($NH_4Cl$ 10 mM; $FeSO_4$ 12 μM)
NMS[d] ($KNO_3$ 10 mM; $FeSO_4$ 52 μM)
ND not determined

attributed to the additional non-competitive toxic effect of nitrite generated as an ammonium oxidation end product. Similar experiments performed in NMS medium with 1 mM $NH_4Cl$ and 9 mM $KNO_3$ (data not presented) supported this hypothesis since in this case no significant reduction of the growth nor sMMO activity were observed. Furthermore, the obtained results are in agreement with previously published papers which illustrate that many methanotrophs can oxidize ammonium salts to nitrite (*1,30,31*). The oxidation of $NH_4^+$ has been referred to as methanotrophic nitrification occurring only in the presence of methane. The mechanism of competitive inhibition of methane oxidation by $NH_4^+$ is not fully understood and there are no answers as yet which type of enzyme, the soluble or the particulate MMO is more affected by $NH_4^+$.

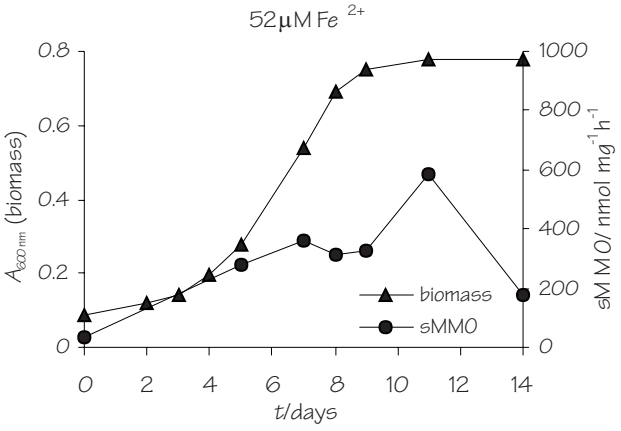


**Fig. 3. Effect of increased iron concentration on the growth of the strain Met1 (▲) and the expression of sMMO (●).** Experiments were performed in NMS medium without copper, containing standard concentrations of all other constituents (*24*) except $FeSO_4$, which was increased from 12 to 52 μM. For comparison see Fig. 2a

## *Effect of iron*

The effect of $Fe^{2+}$ as a limiting component of the culture medium was studied by comparative cultivation of the strain Met1 in NMS medium without $Cu^{2+}$ containing standard (12 μM), and increased (52 μM) $FeSO_4$ concentration. The obtained results showed that increased $Fe^{2+}$ concentration stimulated the growth of the strain Met1 (Figs. 2a and 3). In addition, the maximum value of sMMO activity was achieved faster than in the original NMS medium, after which a large drop of enzyme activity was observed. Based on the results of similar experiments Park and collaborators (*8*) suggested that the standard $Fe^{2+}$ level of 40 μM in Higgins nitrate salts medium without $Cu^{2+}$ was marginal for sustaining the maximum obtained sMMO activity during batch cultivation of *M. trichosporium* OB3b in bioreactor. Higher initial $Fe^{2+}$ concentration (80 μM) was found to increase both the final cell density and the sMMO activity. The large effect of $Fe^{2+}$ on the sMMO activity could be explained by the fact that Fe is an essential cofactor for the sMMO enzyme complex, which can represent more than 10 % of the total soluble protein in some methanotrophs (*3,8,29*).

## *Essential medium constituents for the sMMO expression*

Table 1 summarizes some of the obtained results of this and our previous shake flasks experiments in which the growth kinetics and sMMO activity were studied during cultivation of the type II methanotroph (strain Met1) under different conditions. A general observation was that in all cases except with increased $Fe^{2+}$ concentration, the specific growth rate of the strain Met1 was significantly higher in the presence of $Cu^{2+}$. On the other hand, the presence of $Cu^{2+}$ in the growth medium completely inhibited the expression of sMMO enzyme system. Results presented in Table 1 also illustrate that under the conditions of shake flask experiments (NMS medium without $Cu^{2+}$; $CH_4$ 10–25 %, $O_2$ 5–15 % in the headspace and at 30 °C) higher sMMO activity was achieved when the strain Met1 was grown in the community (culture MM1) than as a single culture. When comparing the values of maximum whole-cell sMMO activity for the strain Met1 grown as a single culture (400 ± 35 nmol naphthol mg $protein^{-1}$ $h^{-1}$) to those ob-

tained for *M. trichosporium* OB3b (75 nmol naphthol mg protein$^{-1}$ h$^{-1}$) and *M. methanica* 68-1 (220 nmol naphthol mg protein$^{-1}$ h$^{-1}$) when grown in similar conditions (2), it is evident that shake flask system used in this work was efficient for the sMMO expression.

The study of another important medium constituent, the nitrogen source, showed that standard nitrate concentration of 10 mM in NMS medium was sufficient to sustain good growth of the strain Met1 and the expression of the sMMO enzyme. When $KNO_3$ was replaced with $NH_4Cl$ no detectable expression of sMMO enzyme system was observed, and the specific growth rate was markedly suppressed.

In contrast to nitrate concentration, faster and more stable growth of the strain Met1 and significantly higher maximum whole-cell sMMO activity achieved at increased $Fe^{2+}$ concentration (52 μM) (Table 1) suggested that standard $Fe^{2+}$ concentration (12 μM) in NMS medium without $Cu^{2+}$ did not ensure optimum cell growth and sMMO expression under the conditions of shake flasks experiment. This further suggested that $Fe^{2+}$ is an essential medium constituent, which can contribute the most to the optimization of shake flask system for sMMO expression.

A general conclusion is that in addition to copper deficiency, availability of iron is an essential factor, affecting significantly the cell growth and the sMMO activity of the natural isolated type II methanotroph. It is recommended that the standard $Fe^{2+}$ concentration in NMS medium be increased to at least 50 μM for shake flasks experiments.

*Acknowledgment*

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# Utjecaj uvjeta uzgoja na sintezu citoplazmatske metan monooksigenaze

**Sažetak**

U ovom je radu utvrđeno da su metanotrofno-heterotrofna bakterijska zajednica (kultura MM1), podrijetlom iz vodonosnika podzemne vode, i metanotrofna bakterija tipa II (soj Met1), izolirana iz kulture MM1, sintetizirale citoplazmatsku metan monooksigenazu (sMMO) tijekom uzgoja u jednostavnom šaržnom sustavu (tresene boce) pod određenim uvjetima. Da bi se utvrdili optimalni uvjeti uzgoja za sintezu sMMO, proveden je niz pokusa u kojima je modificiran sastav mineralne podloge (NMS podloga), tako da je zamije-

njen izvor dušika (dodan $NH_4Cl$ umjesto $KNO_3$) te povećana koncentracija $KNO_3$ i $FeSO_4$. Rezultati usporednih istraživanja s kulturom MM1 i sojem Met1 potvrdili su da je prisutnost bakra najvažniji pojedinačni čimbenik o kojem ovisi da li će se MMO sintetizirati u citoplazmi ili membranski vezanom obliku. Dvostrukim povećanjem koncentracije $KNO_3$ (sa 10 na 20 mM) nije uočena značajna razlika u krivulji rasta metanotrofa Met1 niti u aktivnosti sMMO. Nasuprot tome, zamjenom $KNO_3$ sa $NH_4Cl$, rast kulture Met1 značajno je usporen, a aktivnost sMMO potpuno inhibirana. Povećanjem koncentracije $Fe^{2+}$ u podlozi sa 12 na 52 $\mu$M postignut je kudikamo brži rast i veća aktivnost sMMO. Postignuti rezultati pokazuju da je $Fe^{2+}$ važan sastojak podloge koji najviše može pridonijeti poboljšanju uvjeta za sintezu sMMO u šaržnom sustavu.