# The Influence of Abiotic Factors on the Activity of Methane-Oxidizing Bacteria

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**Abstract.** Methanotrophs are widespread bacteria and utilize a number of hydrocarbons under normal conditions. In this study, a model reaction of propylene oxidation by methane-oxidizing bacteria *Methylococcus capsulatus* (M) was considered. The influence of abiotic factors (the flow rate, exogenous metals) on methane-oxidizing bacteria has been studied. It was found that increasing the flow velocity slightly effect on the growth of bacterial biomass. Studies have shown that under the action of salts of the studied metals a significant decrease the propylene oxidation by bacteria is observed. Methanotrophs exhibit resistance to stress factors, which ensures their wide distribution in nature and provides biotechnological potential.

## 1 Introduction

An important component of the microbial community are methane -oxidizing bacteria – methanotrophs. Methanotrophs exist everywhere in nature and play an important role in the global carbon cycle and maintaining a safe level of methane [1]. By oxidizing a chemically inert molecule of methane, they produce carbon dioxide. The primary oxidation of methane by these bacteria is carried out by soluble and/or membrane-bound methanmonooxygenases (MMO). Along with methane, MMO catalyzes the oxidation of a number of other saturated hydrocarbons (ethane, propane, butane, pentane, etc.), unsaturated hydrocarbons (ethylene, propylene, butylene, butadiene, etc.), and aromatic compounds (toluene, phenol, etc.) [2]. There are two types of MMO: soluble (sMMO) and particulate or membrane-bound (pMMO) [3]. Membrane-bound MMO has narrower substrate specificity and greater activity compared to soluble MMO. Membrane-bound MMO, in contrast to the soluble form of MMO, has been found in the absolute majority of methane-oxidizing bacteria studied now. It is known that pMMO is composed of three polypeptides, 45 кDa (α -subunit), 27 kDa (ß -subunit), and 25 kDa (γ -subunit), with an $(\alpha\beta\gamma)_3$ tertiary structure [3].

The bacteria with the capacity to utilize one-carbon molecules as carbon and energy sources play an integral role in biotechnologies that are part of a sustainable, circular bioeconomy [4]. It means that methanotrophs are promising objects of biotechnology and bioengineering. Methanotrophs have enormous potential for the efficient biotransformation of methane to various bioactive molecules and useful substances [5]. The study of methanotrophs and their communities capable of consuming methane and natural gas with a

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satisfactory biomass yield remains an important scientific task. The most successful product of metanotrophic biotechnology is gaprin - feed protein. So it was evaluated the effects of increasing bacterial protein meal (BPM) (*M. capsulatus* Bath) as a fishmeal replacer in the diets of gift [6, 7]. However the number of highly productive strains which use is economically advantage is small. Another biological biotechnological processes using methanotrophic bacterial strains for methanol production are perspective but have some problems [8]. The studies on methanol production by methanotrophs are recommended to be carried out at a large scale under continuous fermentation and nonsterile conditions for the sustainable development of microbial cells [9]. For industrial technology we need found robust bacterial base. For this purpose biosynthetic potential and properties of methanotrophs needs to be investigated Thus, the purpose of this work is assess the external factors on methane-oxidizing bacteria *Methylococcus capsulatus* (M) in order to highlight their biotechnological potential.

## 2 Materials and methods

*Bacterial Growth.*

Cells of *M. capsulatus* (M) were grown in the flow cultivation regime in an Ankum 2M fermenter (Russia) on a standard mineral medium at 42 °C and pH 5.6 as described [10]. The flow velocity parameter was changed - D. Cultivation was carried out until the maximum cell concentration under these conditions was reached, which was measured by optical density at 560 nm.

The flow velocity is defined as D = F / V, where F is the flow rate of the medium $l*h^{-1}$, V is the volume of the fermenter.

The dry weight of the cells was determined by drying a fixed volume of cell culture until a constant weight was obtained.

*Membrane isolation.*

Cell suspensions were passed two times through a DKM-5 semiautomatic disintegrator (Institute of Problems of Chemical Physics, Russian Academy of Sciences). The cell lysate was centrifuged at 10000 g for 30 min to remove unbroken cells. Sufficient NaCl was added to the supernatant to bring the concentration to 0.5 M, to facilitate release of peripheral proteins from the membrane. The supernatant was then centrifuged at 45000 g for 60 min. The pellet was suspended in 50 mM Pipes buffer, pH 7.2. Membrane samples were frozen and stored in liquid nitrogen. The membranes were defrosted on air or under argon before the experiments. The samples were dialyzed against three changes of 0.25 M sodium-phosphate buffer, pH 7.2 at 4 ºC. This membrane fraction was frozen and stored in liquid nitrogen.

*Activity Assays.*

Enzyme activity was measured by monitoring the epoxidation of propylene with NADH or duroquinol as a reductant. The standard reaction mixture contained *M. capsulatus* (M) membranes, a reducing agent, and 0.02 M sodium phosphate buffer (pH 7.00). The reaction mixture was incubated with metal (1÷100×10-6 M) in a thermostated cell (42°C) with constant stirring for 30 min. The stock solutions of metal salts were prepared using deionized water and diluted accordingly. The reaction was initiated by injection of propylene. Samples from the gas phase were analyzed using an LHM-8MD gas-liquid chromatograph (model 5, Sevkavellectropribor, Russia) equipped with Porapak Q column. The content of propylene oxide in the sample was determined by a calibration curve constructed at different contents of propylene oxide.

*Electrophoresis.*

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [10]. The gel contained 7 % polyacrylamide. Protein

bands were visualized by staining with Coomassie brilliant blue R250, excessive dye was washed off with a solution containing 40% $C_2H_5OH$ and 10% $CH_3COOH$.

## 3 Results

Different methanotrophs have different physiological potentials and understanding how the microbiological communities shift in response to operational parameters might help to develop processes such as biomass. Metabolism of methanotrophs is highly reliant on the physiological conditions like temperature of cultivation, metal ions (copper and iron), media used (nitrate mineral salt), availability of substrates, humidity, incubation period, shaking conditions. Environmental conditions can vary in different ranges, which leads to different growth rates of methanotrophs. We carried out the cultivation of methanotroph in a bioreactor under non-sterile conditions and varied the flow rate. Thus, the growing regime leads to a minor different of increase in biomass (Table 1). When cultivating *M. capsulatus* (M) in the duct, the specific growth rate of the cell culture (μ) is equal to the velocity of the duct D.

**Table 1.** Biomass of *Methylococcus capsulatus* (M).

| **Biomass, $OD_{560}$, (dilution by 10 times)** | **D, $h^{-1}$** |
|---|---|
| 0.975 | 0.047 |
| 0.965 | 0.200 |
| 0.776 | 0.210 |

As can be seen from Table 1, when growing in the duct, the same maximum concentration of cellular biomass $OD_{560} = 0.97 \pm 0.01$ is achieved both at low (D=0.047 $h^{-1}$) and at high duct (D=0.200 $h^{-1}$). The increase in the biomass of methanotrophs does not significantly depend on the flow rate. It can be explained by the fact that at small amounts of biomass, the rate of absorption of methane and oxygen is lower than the rate of their mass transfer into the liquid phase, and the process is limited by the rate of biochemical reactions of methanotrophs. Further, due to the growth of biomass, the rate of absorption of methane and oxygen gradually increases and becomes higher than the rate of their absorption, and the process is limited by the rate of transport of gaseous substrates into the liquid phase.

The cultivation of methanotrophs is often plagued by a foreign microorganisms contamination which thrives on the byproducts of methanotroph metabolism [11]. We obtained membranes, the pMMO indeed constitutes the bulk of the proteins, subunits with molecular weights of 47, 27 and 25 kDa belong to pMMO. The polypeptide with a molecular weight of 60 kDa corresponds to one MDH subunit [10].

The sediment after centrifugation of destroyed cells is ICMs and possesses enzymatic activity, because it contains a pMMO. The supernatant fraction contains a large amount of proteins, but has no activity in the oxidation of propylene (Table 2).

**Table 2.** Isolation of bacterial membranes *M. capsulatus* (M).

| **Fraction** | **Specific activity, nmol of propylene oxide/(min•mg of protein)** |
|---|---|
| Whole cell | – |
| Washed membrane | 77.0 ±1.5 |
| Supernatant (45000 g) | 0 |

The activity of pMMO is decreased after dialysis (Table 3). The influence of metal removal with EDTA, and copper reconstitution on NADH- and duroquinol-dependent

methane hydroxylation activity of pMMO in the membrane fraction from *Methylosinus trichosporium* OB3b was investigated [12]. The NADH-driven activity was restored by exogenous copper ions. However the introduction of endogenous copper led to reconstitution activity of membrane fraction *M. capsulatus* (M) after dialized by 30 %. Probably that the degradation of enzymes and the loss of other components of the pMMO electron transport chain take place.

**Table 3.** Specific activity of membranes *M. capsulatus* (M).

| Fraction | Specific activity, nmol of propylene oxide/(min•mg of protein) |
|---|---|
| As-isolated washed membranes | 78±2.7 |
| 0.5 M NaCl salt-washed membrane | 74±2.5 |
| Washed membranes after defrosting on the air | 59±3.1 |
| Washed membranes after defrosting under argon | 74±1.9 |
| Washed membranes after dialysis | 31±2.1 |

We found that the defrosting of membranes *M. capsulatus* (M) in inert atmosphere leads to a more complete preservation of the enzymatic activity of pMMO (Table 3). It is can find application in the industrial production of useful substances from methanotrophs.

Microorganisms are in constant interaction with the external environment and are exposed to its various influences. Chemical factors affecting the vital activity of microbes include: the chemical composition of the nutrient medium, the reaction of the medium, the redox potential of the medium and the action of toxic (antiseptic) substances. Methanotrophs grow on a liquid nutrient mineral medium [13].The minimal nutrient media is prepared on tap water in which concentration of salts can vary (for example on season). Increasing the content of the main salts of the nutrient medium of methanotrophs leads to a decrease in their activity - to reduce the absorption of propylene (Fig. 1).

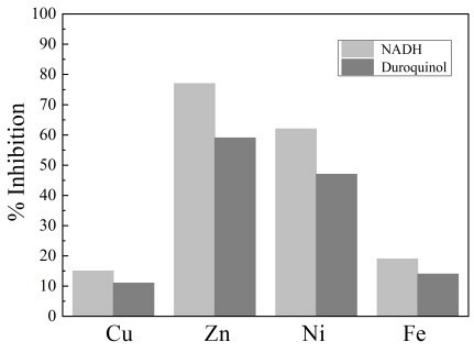


**Fig. 1.** Effect of exogenous metals on propylene oxidation activity of the membrane fraction *M. capsulatus* (M): Inhibition of exogenous of metals (100 μM).

All studied metals led to a decrease in the rate of propylene oxidation, catalyzed by pMMO. The greatest residual activity was observed for Cu and Fe (Fig. 1). At the same time, the activity of pMMO determined using duroquinol as a reducing agent was slightly lower compared to NADH. The same dependence was observed in the control experiment (in the absence of metals). Probably inhibition effect of metals on the *M. capsulatus* (M)

membranes was associated with inhibition of propylene transfer into the cell or with direct interaction of metals with components of the respiratory chain [13, 14].

It is known that NaCl is low-cost and stable inhibitor for MDH activity and found more feasible for biotechnology of methanol production [15]. We found that the introduction of 0.5 M NaCl does not lead to a statistically significant decrease in pMMO activity which confirms its effectiveness for industrial use (Table 3).

## 4 Conclusions

Obtaining new knowledge in methylotrophy would not only provide the necessary instruments for predicting activities of methylotrophs in environment, but will also enable more effective application of these organisms in industrial processes. Changing environmental conditions to a greater or lesser extent affects the living organism and causes it to actively counteract the changing influence. The main factors determining the vital activity of methanotrophs are the presence of methane and oxygen which characterized by relatively slow dissolution in an aqueous medium. Therefore, diffusion stages have a significant effect. There are many sources of methane in the biosphere. Our studies have shown that increase in the flow velocity in 5 times does not lead to a significant increase in biomass. Inhibitory effects on propylene oxidation were demonstrated by *M. capsulatus* (M) membranes amended with 100 μM of metals. Our studies have shown that pMMO is very sensitive to storage conditions.

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