Methanol production in the flow system with immobiblized cells *Methylosinus sporium*

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Introduction

Biomethanol is one of the perspective sources useful for direct application for the production of biodizel. Methanol is used in chemical industry in a variety of ways: it is a building block for other chemicals; it can be used directly as antifreeze and as a precursor to other compounds. The biomethanol is very attractive source for production of biofuel that can be from manufactured renewable raw materials.
The biological oxidation of methane by bacteria with methan monooxigenase activity is discussed in literature as one of approaches to obtaining of methanol under low enough temperature (25-35°C) and regular pressure [Xin, 2004, Mehta, 1991, Yu, 1998]. It is considered, that biological method of methanol production is more attractive as compared to chemical method of methanol production, realized at 350-400 °C and 8-10 MPa [Bahnisch, 2005; Lapkin, 2004].
Application of biotechnological approach to the methanol production should guarantee satisfied yields of target product and stability of functioning of biological systems with retaining of constant levels of productivity for a long period of time. At the same time, it is necessary to avoid the intensive growth of cells in the bioreactor, since the growth could base on the utilization of methanol by cells (Fig. 1). As a result the concentration of required product could decrease.

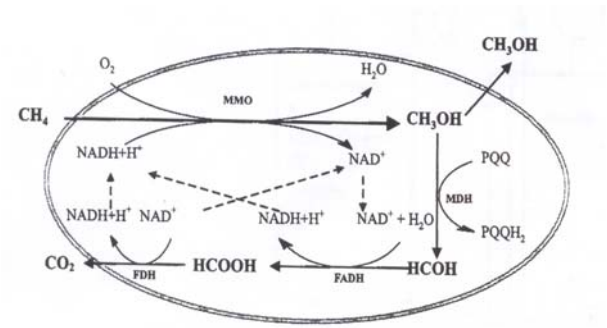

Fig.1. The pathway of methanol production and catabolism by bacteria of genius *Methylosinus* [Xin, 2004]

The effective realization of the process can be done via immobilization of cells producing methanol. It is known, that application of immobilized cells of microorganisms usually significantly simplify the use of cells in biotechnological processes due to removal of procedure of cell separation from reaction media, which is always necessary in the work with free cells. [Nedovic, 2005].
Now a narrow list of biocatalysts based on immobilized cells producing methanol is known, but to guarantee the effective transformation of methane to methanol by the immobilized biocatalysts, the constant introduction of various additives to the cultivation medium (phosphates, formats, ect.) is applied [Mehta, 1991, Yu, 1998]. Only application of the additives provides the effective functioning of the biosystems.
New biocatalyst was developed on the basis of bacteria of genius *Methylosinus*, immobilized into polymeric carrier. The main target of the work was to investigate the process of methanol production by developed immobilized cell biocatalyst under batch and continuous flow conditions.

Material and methods

Several strains of *Methylosinus sporium* cells (B-2119, B-2120, B-2121, B-2122, B-2123) and *Methylosinus trichosporium* (B-2117, B-2118) cells were tried in the work in immobilized form. To accumulate cell biomass for biocatalyst production, the cells were cultivated in the modified NMS-cultivation broth [Galchenko, 2001] under aerobic conditions at 28°C for 20 h on a shaker (Lab-therm, Adolf Kühner, Switzerland) with constant agitation (180 rpm). Cell biomass was separated from the cultivation broth by centrifugation (10,000 g, 20 min, Beckman J2-21 centrifuge, USA) and immobilized into polymer matrix according to previously patented procedure [Efremenko, 2006]. The aerobic process of methane oxidation to methanol was carried out at 28°C.
Composition of "gas-air phase" in reactor was determinated by gas chromatography (LHM 8, Russia) using a 2 m Porapac QS column. Argon was used as a carrier gas at 20 mL/min. The column temperature was 50°C, retention time was 185 sec for carbon dioxide. The injected volume was 0,2 ml. The concentrations of the mentioned compounds were determined using standards.
Methanol concentration in the reaction mixture was determined by gas chromatography using a ZB-FFAP column «Crystal 2000M» (30 m) (Russia). Conditions were following: temperature of detector was 45°C, He was used as a carrier gas, retention time was 171 sec.
The intracellular ATP concentration in immobilized bacterial cells was analyzed by bioluminescent method with luciferine-luciferase reagent (Lumtek, Russia).

Results and Discussion

To obtain active biocatalysts, the cells of seven strains of genus *Methylosinus* were concurrently immobilized by entrapment into porous polymer gel matrix. The immobilized biocatalysts were tested in the process of methanol production from methane under same batch conditions. The concentration of immobilized cell biomass in all experiments was the same and equal to 35 g/l. The initial ratio of methane and air in the gaseous phase used in the reactor was 1:3.5 at the beginning of the process. The replacement of spent nutrition medium with fresh one as well as gaseous phase in reactor was carried out after each 24 h of cell cultivation.
To confirm the possible multiple application of the immobilized biocatalysts three consequent working cycles were conducted. The viability of immobilized cells was controlled via determination of intracellular ATP concentration by bioluminescent method. The obtained data are performed in Table 1 and Fig.2.

Strain	**Cycle of cultivation**			
	0	**I**	**II**	**III**
B-2117	187.0	57.2	36.0	5.2
B-2118	190.9	66.3	22.2	9.6
B-2119	210.3	55.1	32.4	4.8
B-2120	195.1	48.2	28.1	10.0
B-2122	119.9	52.4	32.0	6.7
B-2123	202.5	45.1	32.1	6.2
B-2121	200.3	60.0	30.1	20.4

Table 1. Intracellular ATP concentration in immobilized cells (pmole/g biocatalyst) before and after their use in batch methanol production

Analysis of the data showed, that two strains B-2118 and B-2121 tried in immobilized form were characterized by maximal level of methanol production at the beginning of their exploitation (~60 mg/L), and notable differences in their characteristics appeared only after 72 h of their

cultivation (Fig.2). The strain *M. sporium* B-2121 retained up to 30% initial metabolic activity after the third working cycle (Fig.2). Same immobilized cells possessed the highest level of viability among all tried microorganisms after 72 h of their cultivation under batch conditions (Table 1). Since then, the preference was given to this strain in further experiments.
To optimize the parameters of batch process, the concentration of immobilized biomass in the reactor was varied in the range 35-70 g/L. The humidity of biocatalyst granules was 82-85%.
The ratio between methane and air in the gaseous phase of batch reactor also was varied and was taken as 1:8, 1:3.5 and 1:2.5. The results obtained after 72 h of cultivation of immobilized cells are presented in Table 2.

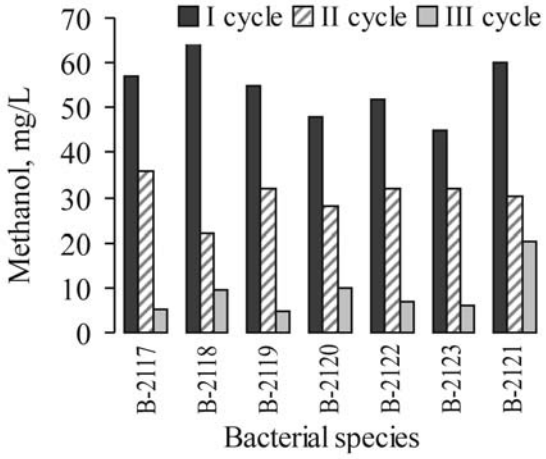

Fig.2. Concentration of methanol accumulated in the cultivation broth during the process of methane conversion by various strains of *Methylosinus* for three working cycles

$[CH_4]$, % vol.	$[CH_3OH]$, mg/L		$[CO_2]$, % vol.	
	Biomass, g/L			
	35	70	35	70
11	6.5	7.0	11.2	11.5
22	6.0	2.0	9.2	13.5
44	4.0	11.0	9.9	13.5

Table 2. The influence of concentration of immobilized cells in cultivation medium and methane concentration in gaseous phase on the accumulation of main products of the process

Obviously, the increase in concentration of cells in the reactor resulted in increase in velocities of accumulation of target product in the medium, but the increase in CO_2 accumulation also was observed.
The investigation of influence of methane concentration in the gaseous phase on the methanol production allowed to establish that 22% (vol.) is optimal concentration of the substrate for the system functioning.
The application of immobilized cells *M. sporium* B-2121 under continuous conditions in flow reactor was studied in the second part of the work. The thermostabled reactor (200 mL) was used in the experiments (Fig. 3). The content of gaseous phase was same as previously with ratio "methane : air" = 1:3.5. The flow rate of nutrition medium and gaseous phase was 0.1 mL/min and 5 mL/min, respectively.
The analysis of intracellular ATP concentration in immobilized cells before and after their use for 4 days in the flow reactor was $7{,}1 \times 10^{-10}$ mole/g biocatalyst and $5{,}05 \times 10^{-12}$ mole/g biocatalyst,

respectively. But the introduction of formate allowed to increase ATP level in immobilized cells and keep it for 7 days. The concentration of methanol in the effluent was 75±5 mg/L.

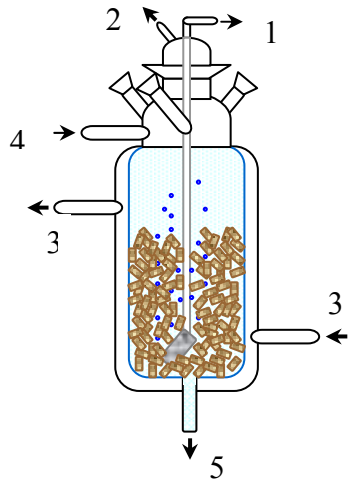

Fig. 3. The scheme of used bioreactor: 1, 2 – entrance and exit of gaseous phase, respectively; 3 – entrance and exit of thermostatted water, 4, 5 - entrance and exit of liquid phase, respectively

Conclusions

The results of biocatalyst usage under continuous flow conditions appeared to be more efficient as compared to batch process, since methanol was continuously removed from the working reactor and the general balance of the process shifted towards accumulation of final product. The possible continuous application of biocatalyst for methanol production was demonstrated.
Thus, it was established, that the process of conversion of methane to methanol organized with immobilized biocatalysts obtained on the basis of bacterial cells *M. sporium* looks very attractive to be further optimized and used.

Acknowledgments

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References

Bahnisch, H. (2005) *Method for catalytic production of methanol and a device for implementing said method*. Patent US No.6894081.

Efremenko, E., et al. (2006) Patent application RU No.2006130758.

Galchenko, V. (2001) *Methanetrophic bacteria*: M.: Geos, 500 p. (in rus).

Lapkin, S., et al. (2004) *Method for methanol production*. Patent RU No.2258691.

Mehta, P., et al. (1991) *Methanol biosynthesis by covalently immobilized cells of Methylosinus trichosporium: batch and continuous studied*. Biotech. Bioeng., 37, 551-556.

Nedovic, V., et al. (2005) *Applications of cell immobilization biotechnology*. Springer Pbsh. Ser.: Focus on biotechnology, 8B, 573 p.

Xin, J-Y., et al. (2004) *Production of methanol from methane by methanotrophic bacteria*. Biocatal. Biotransfor., 22 (3), 225-229.

Yu, C., et al. (1998) *Methanol biosynthesis by methanotrophic bacterial cells*. Enz. Engineering XIV Ann. N.Y. Acad. Sci., 864, 609-615.