Inoculum for the cultivation of Methylosinus sporium

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Abstract: This research assessed the effect of the amount of inoculum on the specific growth rate of *Methylosinus sporium* culture. Methanotrophs were inoculated in different quantities onto media of the same composition and volume. Seven different initial inoculum concentrations were obtained in duplicate. Samples were cultured for 31 hours, during which sampling and optical density measurements were carried out. In accordance with the fact that the optical density is directly proportional to the concentration of microorganisms in the sample, the specific growth rate was calculated. The research revealed an interval from 6.5 to 24 hours, during which the culture demonstrates a stable growth rate of 0.06 h-1, at an inoculation dose at which the optical density of the nutrient medium with the added inoculum is 0.030-0.121.

1 Introduction

Methanotrophic bacteria, or methanotrophs, are a subset of the physiological group of bacteria known as methylotrophs [1]. Aerobic methanotrophs are a group of gram-negative bacteria that use methane as a source of carbon and energy [2]. Methane oxidation occurs due to the action of methane monooxygenase (MMO) [3].

Methanotrophs have been intensively studied over the past 40 years, since these bacteria have significant metabolic potential for practical use in the biotransformation of a variety of organic substrates, bioremediation of pollutants (e.g., halogenated hydrocarbons), and single-cell protein production [4]. Methanotrophic bacteria play a vital role in the global methane cycle: there is evidence that such bacteria are an effective biological filter for the release of methane into the atmosphere from the places of its formation [4]. And thus, emissions and the greenhouse effect of methane on the Earth's climate are mitigated [2].

One of the most promising directions in this area is the production of methanotroph bioprotein, which is used as a feed additive and, in terms of nutritional value, can be compared with fish and soy protein [5].

In addition, such a biotechnological process can help solve the problems of recycling natural gas generated in coal mines [6] and reduce carbon dioxide emissions by approximately 50% [5].

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In this regard, the issue of selecting optimal conditions for cultivating methanotrophic organisms in industrial production is currently relevant.

The purpose of the research was to determine the effect of inoculum concentration on the specific growth rate of *Methylosinus sporium* culture.

2 Materials and methods

The object of the research was the culture of methanotrophic organisms *Methylosinus sporium* (Bowman et al. 1993). The strain was obtained from the All-Russian Collection of Microorganisms: B-2121 [7].

To begin the experiment, 16 Erlenmeyer flasks with partitions with a capacity of 750 ml were prepared (the experiment was carried out in 2 repetitions): 2 flasks (№ 1, 9) with nutrient medium 221, 150 ml each, 14 flasks (№ 2-8, 10-16) with nutrient medium 221 [7], 100 ml each. The flasks with the medium, water, dishes and a mixture of phosphates - NaH_2PO_4 * $12H_2O$ - 25 ml and K_2HPO_4 - 25 ml - were sterilized.

Flasks № 1 and 9 were used to obtain inoculum for further experiments. After sterilization, a mixture of phosphates was added to them, 3 ml each, under sterile conditions. 2 Petri dishes with the culture of *Methylosinus sporium* B-2121 were washed with sterile water (~ 50 ml). Inoculum material was added to flasks № 1, 9 in a volume of 15 ml each. Next, the flasks were closed with silicone stoppers with a gas supply tube. Cultivation was carried out on an orbital shaker with a gas mixture supplied through silicone hoses with a composition of methane:air 1:1 at a temperature of 30 °C for 19 hours.

Immediately after sowing, the optical density of the inoculum dissolved in water (15 ml per 150 ml of water) was measured using a KFK-3 photometer at a wavelength of 402 nm. The results are presented in Table 1.

Table 1. Optical density of seed material at the beginning and end of cultivation.

Time		№1		№9	
		OD402*1	OD402*3	OD402*1	OD402*3
Start of inoculum cultivation	17.07.2023 15:25:00	0.570	0.218	0.570	0.218
End of inoculum cultivation	18.07.2023 10:25:00	0.823	0.323	0.787	0.298

After 19 hours, a mixture of phosphates (2 ml each) was added to 14 flasks with medium 221 (№ 2-8, № 10-16) under sterile conditions. Next, 14 flasks from flasks № 1 and № 9 were inoculated.

Inoculation was carried out using the method of serial dilutions: 100 ml of inoculum was taken from flask № 1 using a Mohr pipette and added to 100 ml of nutrient medium in flask № 2. The contents of the flask were mixed and 100 ml of it was transferred into 100 ml of the nutrient medium of flask № 3, which in turn served as a source of inoculum for flask № 4. Next, the remaining flasks were also inoculated with each subsequent dilution of the inoculum by 2 times. From the last flask, 100 ml was taken and drained to achieve equal volume in all flasks. Thus, dilutions of 2, 4, 8, 16, 32, 64, 128 times were obtained.

The same thing was repeated with flasks № 9-16.

After seeding, the flasks are installed on an orbital shaker and connected to a gas source with a methane:air composition of 1:1 for further cultivation.

3 Research results

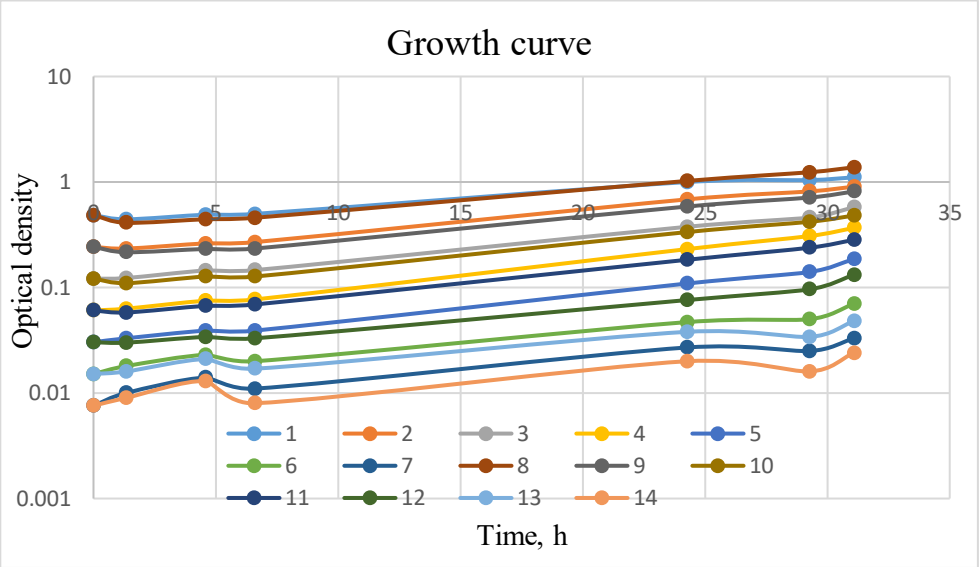
Cultivation was carried out for 31 hours, and the optical density was measured periodically in each flask. The final measurement results are presented in Table 2.

Table 2. Results of measuring optical density depending on cultivation time.

№ flask	Dilution degree	0	1.3333	4.5833	6.5833	24.25	29.25	31.083
1	2	0.4845	0.443	0.49	0.498	0.999	1.041	1.107
2	4	0.24225	0.234	0.262	0.269	0.684	0.813	0.903
3	8	0.121125	0.123	0.145	0.146	0.376	0.46	0.576
4	16	0.060563	0.063	0.075	0.077	0.231	0.307	0.367
5	32	0.030281	0.033	0.039	0.039	0.109	0.14	0.186
6	64	0.015141	0.018	0.023	0.02	0.047	0.05	0.07
7	128	0.00757	0.01	0.014	0.011	0.027	0.025	0.033
8	2	0.4845	0.411	0.444	0.458	1.023	1.236	1.377
9	4	0.24225	0.216	0.232	0.234	0.585	0.711	0.813
10	8	0.121125	0.11	0.127	0.127	0.335	0.419	0.481
11	16	0.060563	0.058	0.067	0.069	0.183	0.238	0.283
12	32	0.030281	0.03	0.034	0.033	0.076	0.096	0.132
13	64	0.015141	0.016	0.021	0.017	0.038	0.034	0.048
14	128	0.00757	0.009	0.013	0.008	0.02	0.016	0.024

Based on the measurement results presented in Table 2, growth curves were constructed - the dependence of optical density on the time of bacterial cultivation. The result is presented in Figure 1.

Based on the optical density, the coefficients a and b were calculated and based on them the specific growth rate was determined. The calculated coefficients a and b and the average specific growth rate are presented in Table 3.



1, 8 – dilution of inoculum by 2 times; 2, 9 – dilution of inoculum by 4 times; 3, 10 – inoculum dilution 8 times; 4, 11 – dilution of inoculum by 16 times; 5, 12 – dilution of inoculum by 32 times; 6, 13 – dilution of inoculum by 64 times; 7, 14 – dilution of the inoculum by 128 times.

Fig. 1. Growth curves of microorganisms in media with different inoculum concentrations.

Table 3. Coefficients a and b and the average specific growth rate of microorganisms depending on the dilution factor.

№ flask	Dilution degree	b	a	Specific growth rate
1	2	0.033042	0.409768	0.036
2	4	0.049415	0.196573	0.056
3	8	0.053748	0.102015	0.061
4	16	0.062569	0.050833	0.073
5	32	0.060502	0.025833	0.070
6	64	0.046766	0.014669	0.053
7	128	0.041889	0.008564	0.047
8	2	0.044527	0.342516	0.050
9	4	0.050201	0.16892	0.057
10	8	0.053732	0.089422	0.061
11	16	0.056279	0.047354	0.065
12	32	0.052246	0.022824	0.060
13	64	0.037986	0.013481	0.042
14	128	0.039761	0.006361	0.044

Table 4 presents the results of calculating the specific growth rate for each time period. The highest growth rate observed during the experiment is highlighted in red.

Table 4. Specific growth rate in different periods of time depending on the dilution factor.

№ flask	Dilution degree	Initial OD	Specific growth rate 0-1	Specific growth rate 1-2	Specific growth rate 2-3	Specific growth rate 3-4	Specific growth rate 4-5	Specific growth rate 5-6
1	2	0.485	-0.057	0.034	0.008	0.043	0.008	0.036
2	4	0.242	-0.024	0.038	0.014	0.060	0.038	0.066
3	8	0.121	0.012	0.057	0.003	0.061	0.045	0.167
4	16	0.061	0.032	0.061	0.014	0.073	0.066	0.124
5	32	0.030	0.076	0.058	0.000	0.067	0.057	0.229
6	64	0.015	0.180	0.091	-0.059	0.055	0.013	0.291
7	128	0.008	0.352	0.134	-0.089	0.058	-0.015	0.221
8	2	0.485	-0.091	0.025	0.016	0.051	0.042	0.068
9	4	0.242	-0.069	0.023	0.004	0.059	0.043	0.088
10	8	0.121	-0.060	0.049	0.000	0.063	0.050	0.091
11	16	0.061	-0.030	0.050	0.015	0.063	0.060	0.120
12	32	0.030	-0.007	0.042	-0.014	0.053	0.053	0.269
13	64	0.015	0.046	0.103	-0.081	0.051	-0.021	0.301
14	128	0.008	0.180	0.150	-0.133	0.059	-0.040	0.385

The highest specific growth rate is observed in the period 6.5 hours - 31 hours - this is the exponential phase of bacterial growth. Confidence intervals were calculated for this phase. The results are presented in Table 5 and Figure 2.

Table 5. Confidence intervals depending on the initial optical density.

Initial OD	Specific growth rate		Averagespecific growth rate	Standard deviation	Sampling	Confidence interval
0.485	0.036	0.050	0.043	0.006943	2	0.009622
0.242	0.056	0.057	0.056	0.000501	2	0.000694
0.121	0.061	0.061	0.061	1.04E-05	2	0.000015
0.061	0.073	0.065	0.069	0.004193	2	0.005811
0.030	0.070	0.060	0.065	0.005425	2	0.007518
0.015	0.053	0.042	0.047	0.005399	2	0.007482
0.008	0.047	0.044	0.045	0.001299	2	0.001801

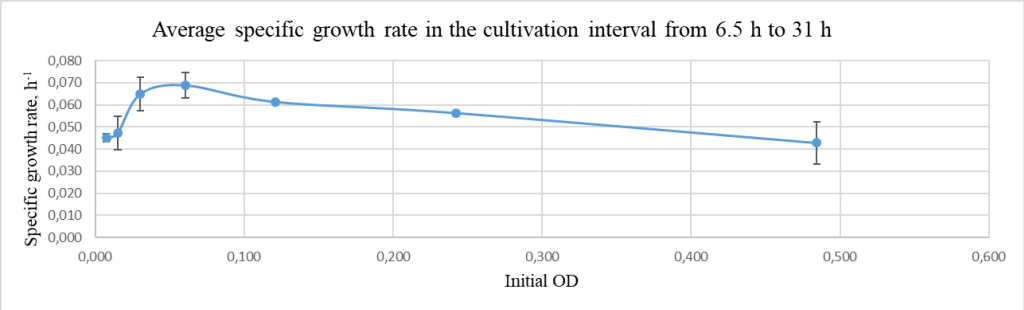


Fig. 2. Dependence of the average specific growth rate in the cultivation interval from 6.5 hours to 31 hours on the initial optical density in the flask.

4 Conclusions

The conducted studies showed that the culture of *Methylosinus sporium* B-2121 demonstrates a stable growth rate (more than 0.06 h^{-1}) in the range from 6.5 to 24 hours at an inoculum dose at which the optical density of the nutrient medium with the added inoculum is 0.030-0.121.

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