

Cultivation of *Methylocystis hirsuta* in Bubble Columns Packed with Pall Rings Utilizing Methane as Carbon Source

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Abstract

Methanotrophic bacteria use methane as a primary carbon source. The nutritional composition of the species is suggestive of its use as an alternative means of protein production. Furthermore, the production process under study is well within the context of clean technology. This work investigated the mass transfer coefficient, $K_L\alpha$, of oxygen and methane in a new bioreactor system, specially designed for cultivation of *Methylocystis hirsuta*, and the growth of this bacteria in different conditions and media compositions. The growth system consisted of three bubble column reactors (BCR) in series, packed with stainless steel Pall rings (1.5 cm), using methane as substrate. Methane and oxygen mass transfer to the liquid phase are critical phenomena in this process, and the new system mass transfer capacity was shown to compete with those of traditional bioreactors. Cell concentration achieved with a mineral medium was 1.1 g/L and 2.1 g/L with addition of supplements. The use of soybean oil, a tensioactive agent, increased cell concentration up to 4.5 g/L, showing the importance of an efficient mass transfer from the gas to the liquid phase. Clearly the process could be further improved by enhancing this transport, increasing the availability of oxygen and methane to the microorganism cells. The BCR system tested in this work is a promising new configuration of bioreactors for cultivation of methanotrophic bacteria, with a significant competitive advantage of lowering operational costs since mechanical agitation is no longer required.

Keywords: Greenhouse gas; Methanotrophs; Single cell protein; *Methylocystis hirsuta*; Pall rings; Bubble column.

Introduction

Worldwide population growth, along with its consequent increasing demand for food, is responsible for the current global protein scarcity, making necessary the search for new protein sources. Since the 1950s, the prospective uses of non-conventional resources, such as Single-Cell Protein (SCP), have been studied. SCP comprises either dry microbial cells or total protein extracted from a mixed or pure culture that can serve as feeding supplement for animals or even humans [1,2].

Compared to more traditional sources, the use of SCP presents some advantages: higher rates of protein synthesis in prokaryotes, high content of protein, lipids, carbohydrates, nucleic acids, vitamins, minerals and some essential amino acids, mainly lysine and methionine, and independence on seasonal factors [2,3]. A proper choice of microorganism for SCP must consider the product destination, as well as the unique properties of each microbial species [4].

Since microorganisms can use different types of substrates, a wide range of materials, residual or not, can serve as feedstock for SCP processes. Oil & Gas industry, for example, can provide natural gas (typically 94 mole % of CH_4) for SCP production [5-7]. In this case the reactor design should allow an efficient mass transfer of CH_4 to the liquid phase where microorganisms are suspended. This would require knowledge about microbial metabolism and composition, cell growth kinetics, culture media, aeration rate, temperature and pressure [8].

Although not many microbial species are capable of metabolizing CH_4 , there is a group of methanotrophic bacteria that exhibit this ability. These bacteria are able to assimilate methane through the action of the enzyme methane monooxygenase, which converts methane to methanol, followed by the action of methanol dehydrogenase, transforming methanol to formaldehyde. The generated formaldehyde can follow two distinct metabolic pathways to be converted in biomass. In methanotrophic bacteria Type I it follows the ribulose monophosphate (RMP) pathway and in Type II it follows the serine pathway. As examples of Type I families we have *Methylomonas* and *Methylobacter*, while as Type II it is possible to mention the families *Methylosinus* and *Methylocystis* [9-11]. Additionally, their cell composition is suitable for animal feeding after adequate treatments. These bacteria also have the potential to be applied in bioremediation, polyhydroxybutyrate (PHB) production and nitrogen fixation [11-13]. Some species of methanotrophs are currently used for animal feeding, fully approved by the European Union [14].

An important parameter widely used to evaluate system's capacity to transfer gas to liquid phase, is the volumetric mass transfer coefficient, $K_L\alpha$, which can be calculated by different methods [15,16]. This coefficient was originally introduced for oxygen, whose concentration in liquid phase can be easily measured by dissolved oxygen electrodes. It can be estimated for other gases, such as methane, allowing an assessment of the system's capacity to transfer CH_4 to the microorganism cells. $K_L\alpha$ values are related to several factors, such as the feed rate of the gas mixture, the residence time of the gas and the presence of fillings in the bioreactor, what increases the tortuosity of the gases, through the liquid column as well as their residence time in

the liquid phase. These parameters should be investigated to improve the efficiency of the mass transfer during the process, allowing a better use not only of oxygen but also of the substrate methane.

Considering all factors related to the cultivation of methanotrophs and the use of a new bioreactor system, some other parameters should be investigated in order to optimize cell growth and obtain higher yields in protein production. Culture media that use a single carbon source, such as methane, require supplementation with vitamins, cofactors and other nutrients, which are necessary to achieve higher yields and rates of growth. It is also important to investigate the use of substances that allow greater availability of gases to the liquid phase, such as tensoactives, establishing essential interaction between the hydrophobic and hydrophilic substances that are in the continuous and discontinuous phases.

These factors along with high mass transfer coefficients will make it possible to obtain higher cell density in smaller time intervals. The present work aims at estimating $K_L\alpha$ values for oxygen and methane as well as evaluating fermentation conditions for the methanotrophic bacteria *Methylocystis hirsuta*, so as to maximize the cell concentration for a new bioreactor design.

Materials and Methods

$K_L\alpha$ measurement by the gassing-out method

Assays were carried out using the gassing-out method (or degasification) which consists of bubbling N₂ through the system so as to remove all dissolved oxygen (DO) from the liquid phase. After that, airflow is initiated, and values of DO were measured every 15 seconds. The $K_L\alpha$ value for each system was calculated with the following classical equation based on the mass balance of oxygen:

$$\ln \frac{(C_S - C_{L,1})}{(C_S - C_{L,0})} = -k_L\alpha(t_1 - t_0)$$

where C_S is the saturation concentration of O₂, $C_{L,1}$ and t_1 are, respectively, the values of DO and time at the end of exponential phase and $C_{L,0}$ and t_0 are, respectively, the values of DO and time at point 0.

The reactor used to evaluate $K_L\alpha$ was a system of three interconnected glass bubble columns packed with Pall rings of stainless steel with approximately 1.5 cm of diameter and height. The packing was aimed at promoting turbulence in the liquid phase, breaking up bubbles and improving the mass transfer from the gas to the liquid. Efficiency of the fillings in increasing the value of $K_L\alpha$ of the system was observed when comparing the results with and without the use of Pall rings (Figures 1 and 2).

Considering that dissolved methane electrodes are not available commercially, values of $K_L\alpha$ for methane were estimated on the basis of $K_L\alpha$ for oxygen ($(K_L\alpha)_{O_2}$), using the following correlation proposed by Yu et al. [15] for pneumatically agitated reactors:

$$(K_L\alpha)_{O_2} = 1.125(K_L\alpha)_{CH_4}$$

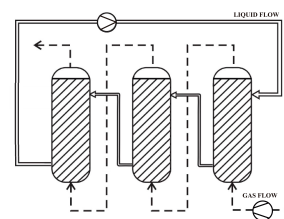


Figure 1: Schematic of the bubble column reactors (BCR) used during experiments. Dashed and continuous lines represent, respectively, gas and liquid flows between columns. Gas injected in fermentation assays was filtered by a 0.22 μ m Millipore filter.

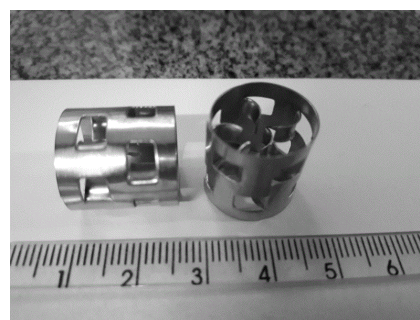


Figure 2: Pall rings used to fill the BCR columns. Each piece has 1.5 cm of diameter and height.

Fermentation assays

Culture medium used to evaluate *Methylocystis hirsuta* (ATCC BAA-1344) growth was a modified version of NMS 1306, recommended by the American Type Culture Collection (ATCC). Preliminary assays were carried out in shake flasks with controlled atmosphere to investigate fermentation conditions and possible influences in cell growth. The modified NMS 1306 composition is shown in Table 1.

Full scale assays were carried out in the previously described BCR system, consisting of three 1 L columns in series (total volume of 3 L) and using the modified NMS 1306. Assays with and without filling were done to evaluate the interference in cell growth. All subsequent experiments were done with the BCR system filled with Pall rings.

Previous experiments in shaken flasks indicated that yeast extract and casaminoacids are effective sources of metabolic supplements to *M. hirsuta*. This was fully confirmed in a second fermentation in the BCR system, using the modified NMS1306 supplemented with yeast extract and casaminoacids (0.2% (w/v) each).

Following the assays described above, soybean oil was used in order to speed up the solubilization of methane in water.

Solutions	Components	Mass
NMS (1000 mL)	MgSO ₄ · 7H ₂ O	1.0 g
	CaCl ₂ · 6H ₂ O	0.20 g
	KNO ₃	1.0 g
	KH ₂ PO ₄	0.272 g
	Na ₂ HPO ₄ · 12H ₂ O	0.717 g
	CuSO ₄	1.4 mg
	Traces element solution	0.5 mL
Traces element solution (1000 mL)	EDTA	500.0 mg
	FeSO ₄ · 7H ₂ O	200.0 mg
	ZnSO ₄ · 7H ₂ O	10.0 mg
	MnCl ₂ · 4H ₂ O	3.0 mg
	H ₃ BO ₃	30.0 mg
	CoCl ₂ · 6H ₂ O	20.0 mg
	CaCl ₂ · 2H ₂ O	1.0 mg
	NiCl ₂ · 6H ₂ O	2.0 mg
Na ₂ MoO ₄ · 2H ₂ O	3.0 mg	

Table 1: Modified NMS1306 composition.

Soybean oil acts as a tensioactive agent, increasing both the transfer rate of methane to the liquid as well as the availability of substrate for the microorganisms. Assay conditions were exactly the same as previous fermentations in BCR, except for the additional use of 1% (v/v) of soybean oil. In both cases, medium flow rates were such as to maintain the total volume of liquid, as well as the concentrations of supplements, at suitable levels for the microorganism growth.

The BCR system allowed for temperature monitoring only (around 30°C). The air-methane mixture flow rate was 4 L/min and its composition was maintained in 30% methane and 70% air. This proportion was optimized in a separate experiment, not reported here.

Results

K_La measurement by the gassing-out method

As expected, for both configurations the highest values were those for higher airflow rate, possibly related to the higher levels of turbulence/mixture in the columns. Calculated values of ((K_La)_{O₂}) were 166.1, 138.7, 111.2 and 79.9 h⁻¹ for the BCR filled with Pall rings and 96.9, 71.1, 51.9 and 29.6 for the BCR without fillings with 8, 6, 4 and 2 L/min of airflow rate, respectively. Results of ((K_La)_{O₂}) assays are shown in Figure 3.

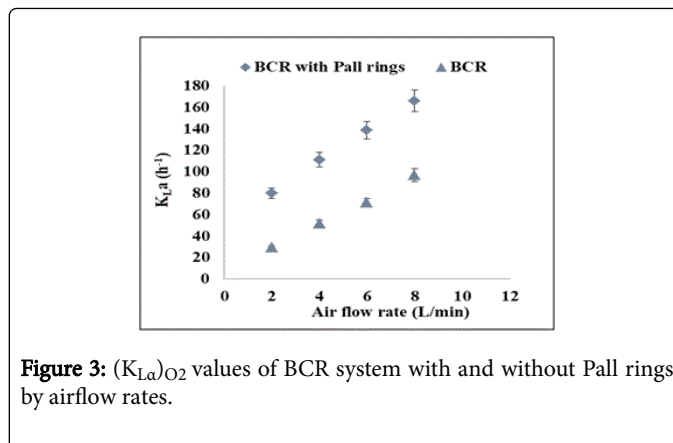


Figure 3: ((K_La)_{O₂}) values of BCR system with and without Pall rings by airflow rates.

Fermentation assays

Final cell concentration obtained with pure mineral media was only 1.1 g/L with fillings and 0.8 without the Pall rings. With supplements it increased by almost two fold (2.1 g/L) and with oil addition cell concentration achieved the highest value (4.5 g/L), a gain, respectively, of 91% and 300% as compared to the pure mineral medium with fillings. The “No methane” curve shows growth kinetics of the microorganism without methane supply, which confirms the use of methane as exclusive carbon source even in the presence of supplements. Figure 4 presents the growth kinetics of *M. hirsuta* using the BCR system.

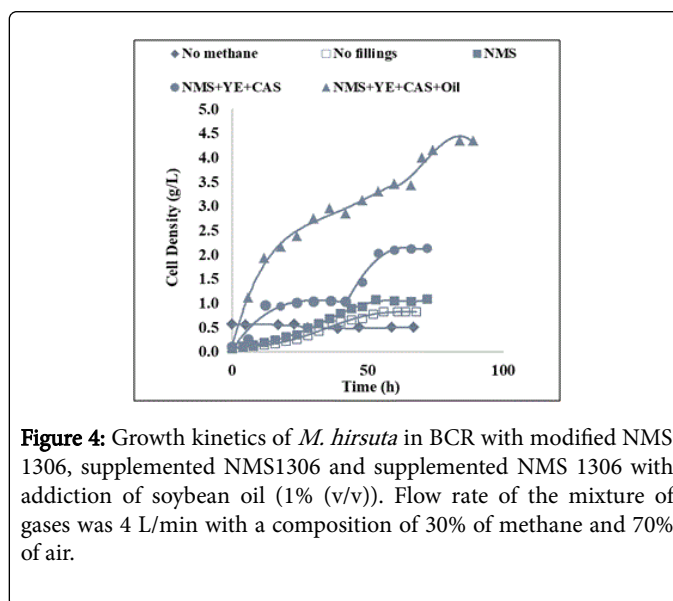


Figure 4: Growth kinetics of *M. hirsuta* in BCR with modified NMS 1306, supplemented NMS1306 and supplemented NMS 1306 with addition of soybean oil (1% (v/v)). Flow rate of the mixture of gases was 4 L/min with a composition of 30% of methane and 70% of air.

During supplemented NMS1306 assay, cell growth ceased possibly due to the depletion of supplements present in culture medium and cell concentration reached 1.0 g/L in 12 hours. After 30 hours without any further cell growth, the system was fed with supplemented medium to restore the initial levels of yeast extract and casaminoacids, and cell concentration rose to 2.1 g/L. On completion of fermentation, a sample of the culture medium was examined in a microscope (LEICA DSC295). Only small gram negative cells were present, confirming that the assay wasn't contaminated. Figure 5 shows the BCR system after 60 hours of fermentation.

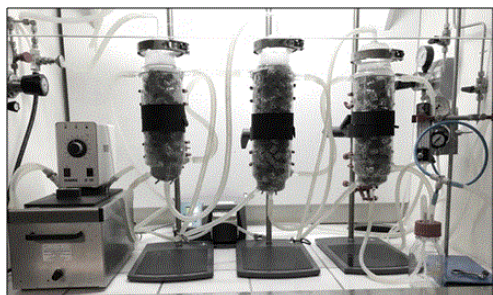


Figure 5: BCR system after 60 hours of growth of *M. hirsute*.

Discussion

The gain in KLa values using Pall rings reached 71, 95, 114 and 170% for 8, 6, 4 and 2 L/min which show the influence of the fillings in aeration efficiency, especially in situations of low turbulence and reduced air supply to the liquid phase. The presence of Pall rings is associated with tortuosity of bed passages and an increased gas hold up, with the gas evenly distributed across the bed section. Bubble coalescence, known to be detrimental for the process, was minimized while bubble breakage was promoted. Overall, the mass transfer from the bubble phase to the liquid improved as shown by the high values of. was estimated for the BCR filled with Pall rings using Yu et al. [17] correlation, and the following values were obtained: 147.6, 123.3, 98.9 and 71.1 for 8, 6, 4 and 2 L/min. These indicate an improved capacity for the developed bioreactor to efficiently supply oxygen and methane required for cell metabolism, actually making the new system a promising one for the cultivation of microorganisms in general.

In fermentation assays, observe the gains of adding supplements and oil to the NMS 1306 medium, either in relation to processing time or final concentrations of cells. Fermentation time to reach 1 g/L was reduced from 60 to about 20 and 12 hours, respectively, for the supplemented medium (with yeast extract and casaminoacids) and for supplemented medium plus soybean oil. Methane, a hydrophobic gas with very low water solubility, had its transference to the liquid facilitated by the presence of a tension active, increasing the contact area between gas (bubbles) and liquid, that is, improving its solubilization rate. The low cost of soybean oil in Brazil and its use in the food industry makes it an excellent choice of surfactant for SCP cultivation.

Traditional bubble column type reactors are known to be in disadvantage when compared to mechanically stirred and airlift reactors, which allow a more efficient circulation and homogenization of the culture medium. However, in this work, the use of packed bubble columns in series has increased the efficiency of methane transfer rate to the liquid, as seen in Figure 3, roughly in proportion to the increase in the gas flow rate. The increased methane residence time associated to the packing, provided a fermentation system of easy design, low operational cost compared to stirred reactors, and high efficiency in the mass transfer to the liquid phase.

Final cell concentrations achieved in BCR were much higher than those obtained in shake flasks (previously investigated, data not

shown). These results reinforce the utmost importance of enhanced gas mass transfer promoted by this system, resulting in an increased availability of methane (substrate) and oxygen in the liquid phase. Mass transfer of methane to the liquid phase stands as a critical aspect of this process, leaving open the development of techniques for even more efficient contact between gas and liquid. Cell concentrations obtained, in addition to high KLa values, makes the innovative BCR a promising new configuration of bioreactor for cultivation of methanotrophic bacteria, with the significant competitive advantage of lowering the operational costs since mechanical agitation is not required.

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