https://doi.org/10.17113/ftb.60.03.22.7372 original scientific paper

# Growth of *Methylobacterium organophilum* in Methanol Aiming at the Simultaneous Production of Single-Cell Protein and Metabolites of Interest

Running head: The versatile metabolism of *Methylobacterium organophilum*

Ana Cristina Pantoja Simões[1*], Rodrigo Pimentel Fernandes[1], Maysa Silva Barreto[2], Gabriela Bouça Marques da Costa[2], Mateus Gomes de Godoy[3], Denise Maria Guimarães Freire[2] and Nei Pereira Jr.[1]

[1]Laboratories of Bioprocess Development, School of Chemistry, Federal University of Rio de Janeiro, RJ 21941-972, Brazil
[2]Microbial Biotechnology Laboratory, Institute of Chemistry, Exact and Natural Sciences Center of the Federal University of Rio de Janeiro, RJ 21941-972, Brazil
[3]Laboratory of Biotechnology and Microbial Ecology, Institute of Microbiology, Federal University of Rio de Janeiro, RJ 21941-902, Brazil

Received: 11 June 2021
Accepted: 9 April 2022



## SUMMARY

*Research background*. This study aimed to monitor the growth of the methylotrophic bacteria *Methylobacterium organophilum* in a culture medium with methanol as a carbon source and to verify the production of unicellular proteins and other biomolecules, such as

*Corresponding author:
Phone: +552139387646
E-mail: anapantoja@eq.ufrj.br

carotenoids, exopolysaccharides and polyhydroxyalkanoates, making a compound more attractive as food intended for animal feed.

*Experimental approach*. Microbial growth was studied in shaken flasks to evaluate bacterial growth with different carbon: nitrogen ratios (C: N) and determine the best results in terms of cell volumetric productivity and substrate consumption rate. This condition was to be further applied in an in a fed-batch operating bioreactor system, which depicted the kinetic profile of cell growth, which concentrations were determined by dry cell weight, methanol consumption, measured by HPLC analysis, accumulation of carotenoids, which was analyzed by mass spectrometry, and the production of exopolysaccharide, which was characterized in terms of its chemical composition and submitted to chemical rheological analysis.

*Results and conclusions*. The best experimental condition was verified using an initial methanol concentration of 7 g/L in the culture medium. This same initial substrate concentration was used in the bioreactor in the fed-batch operation, and the biomass concentration was 5 g/L, after 60 hours of cultivation. The accumulation of carotenoids associated with cell growth was monitored, reaching a concentration of 1.6 mg/L at the end of the process. These pigments were then analyzed and characterized as a set of xanthophylls (oxidized carotenoids). In addition, two other products were identified during the fed-batch operation: an exopolysaccharide (EPS), which reached a concentration of 8.9 g/L at the end of the culture, and an intracellular granular structure which was detected by transmission electron microscopy (TEM), suggesting the accumulation of polyhydroxyalkanoate (PHA), most likely polyhydroxybutyrate.

*Novelty and scientific contribution*. *M. organophilum* demonstrated a unique ability to produce compounds of commercial interest, leading to a genuine understanding that this species has a metabolism distint. The use of *Methylobacterium organophilum* makes room for platform integration in the context of biorefineries as a result of its distinct metabolic diversity.

**Keywords:** single cell protein; methylotrophic; methanol

## INTRODUCTION

With alarming population growth, there has been a demand for protein production from alternative sources. In this context, the idea of protein production from unicellular organisms such as bacteria, the so-called “single-cell protein”, has reemerged. This process can be used for protein supplementation in a staple diet, replacing conventional sources such as soybeans, cattle and fishmeal. To reduce the operational costs of this process and consequently reduce the product's final price, the production of microbial protein from waste is a future possibility. The production of this alternative protein source would also offset the high costs of conventional feedstock (primary biomass), making food production less land-dependent and reducing the pressure on agriculture (*1*). Several microbial species already described can be used for this purpose (*2-5*).

Methylotrophic bacteria are a diverse group of microrganisms with a large number of specialized enzymes that allow them to grow on small carbon substrates without carbon-carbon bonds and use these substrates as energy and carbon sources (*6,7*).

A typical carbon substrate for many methylotrophic bacteria is methanol. While subgroups of these bacteria can use methane, other species use methylated sulfur, methylated amines and halogenated hydrocarbons, such as chloromethane, bromomethane and dichloromethane. Phylogenetically, most well-characterized methylotrophic bacteria are subclasses of Proteobacteria. Aerobic methylotrophic bacteria can use a $CO_2$-reduced carbon source. Methylotrophic genomes have been sequenced, and significant progress in elucidating the specific metabolism of such bacteria has been made (*8*).

This dissimilatory process occurs in several steps with formaldehyde as the central intermediate. Methanol conversion is initiated by methanol dehydrogenase, which oxidizes a C1 (single carbon) substrate to produce formaldehyde. This enzymatic step is catalyzed by a periplasmic enzyme-dependent quinone pyrroloquinoline (PQQ) in Gram-negative bacteria, which channels electrons to the oxidized terminal, or by an NAD(P)-enzyme-dependent enzyme in Gram-positive bacteria (*9*). A multitude of ways exist to convert formaldehyde to

$CO_2$ within an organism, which are involved in the efficient conversion of the highly toxic intermediate formaldehyde in microbial cells. This metabolic pathway was first discovered in *Pseudomonas extorquens* and is widespread among known methylotrophic bacteria (*10*).

Methanol is one of the building blocks in the chemical industry and can be synthesized from petrochemical or renewable resources such as biogas. Considering that methanol can be produced from the conversion of methane from natural gas, the price of methanol has tended to decrease, making it an attractive substrate in processes involving microorganisms for the production of metabolites that add value to the bioprocess. Whitaker *et al.* (*11*) mentioned that the yield of a product from methanol is similar to that from glucose on a carbon-carbon basis.

This is the case for the methylotrophic cells already mentioned. Methylotrophic bacteria bioprocessing technology has been studied in recent decades as it has been intended for the large-scale production of single-cell proteins. *Methylobacterium organophilum* is a Bacillus-shaped bacterium, denominated PPFM (pink pigmented facultatively methylotrophic), Gram-negative and classified as type II according to its membrane arrangement and assimilation of carbon compounds via the serine pathway. O'Connor and Hanson (*12*) reported that the main enzymes responsible for bacterial cell growth from one-carbon compounds are hydroxypyruvate reductase, serine glyoxylate aminotransferase, methanol dehydrogenase and glycerate kinase. The species *M. organophilum* has been studied for the production of biopolymers, such as polyhydroxybutyrate (PHB), exopolysaccharides, carotenoids, ectoin and vitamins (*13-18*).

Exopolysaccharides (EPSs) are macromolecules excreted in the culture medium by certain microorganisms. The possibility of using these compounds in the food, cosmetic and pharmaceutical industries has increased research in this field. EPSs can form highly viscous aqueous solutions, even at low concentrations, which is considered an attractive feature for industrial applications. The production of polysaccharides of microbial origin has emerged as an important raw material source for several industrial segments, including the food industry.

These macromolecules can even be used "in nature" as safe additives (*19*). Bacterial polysaccharides have already been applied industrially (*20*) and can be compared to the gums produced by other natural sources, such as seaweeds and plants (*21*). In microorganisms, polysaccharides can be part of the cell wall in the form of lipopolysaccharide (LPS). They can be attached to the cell wall as capsular polysaccharides (CPSs) or be secreted to the extracellular medium in the form of exopolysaccharides (EPSs) (*22,23*).

Xanthophyll-type carotenoids are pigments responsible for various colorations in many vegetables, flowers and plants in general, and many microorganisms are capable of producing them. Their beneficial effects on health have aroused the interest of the scientific community worldwide. The food, pharmaceutical and cosmetic sectors have invested in research on these important molecules and finding new sources and applications. Studies involving carotenoids also look for solutions for industrial processes, such as the elaboration of methods to maintain an adequate level of these substances in different foods so that their properties are not lost during processing and storage (*24*), which reaffirms the application of methylotrophic species as a possible animal feed producer, with the presence of carotenoids that provide additional nutritional value.

In regard to the production of biopolymers such as polyhydroxyalkanoates (PHAs), there are studies that have been associated with methylotrophic bacteria, including *M. organophilum*. PHAs are biodegradable and biocompatible biopolymers that can be produced from renewable sources (*25*). Depending on their composition, their properties vary, with approximately 150 monomers that have been reported in the literature, making this compost suitable for various applications (*26*).

Polyhydroxybutyrate (PHB) is one of these existing homopolymers, presenting properties similar to polypropylene (PP). PHB can be synthesized inside the cell in the form of granules located in the bacterial cytoplasm, which can occupy a good portion of the cytoplasm without changing the cell's osmotic pressure. It functions as an energy reserve in excess carbon conditions with limited nutrients (*26*).

Within this context, this paper exploits the metabolic diversity of *M. organophilum* grown on methanol as the sole carbon source in basic mineral medium. Different operation strategies and their implications in cell metabolism were evaluated, and the potential of this bacteria to produce high value-added molecules was evaluated.

## MATERIALS AND METHODS

### *Microorganism and medium composition*

The bacterium under study, *Methylobacterium organophilum* DSMZ – 18172, was acquired from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Activation was performed using both basic mineral media, as described in related works available in the literature, and as suggested by DSMZ for this species (*15*). The components of the mineral medium were $KNO_3$ (1 g/L), $MgSO_4.7H_2O$ (0.20 g/L), $CaCl_2.2H_2O$ (0.02 g/L), $Na_2HPO_4$ (0.23 g/L), $NaH_2PO_4$ (0.07 g/L), $FeSO_4.7H_2O$ (1 m g/L), $CuSO_4.5H_2O$ (5 µg/L), $H_3BO_3$ (10 µg/L), $MnSO_4.5H_2O$ (10 µg/L), $ZnSO_4.7H_2O$ (70 µg/L), $Na_2MoO_4.5H_2O$ (10 µg/L), and $CoCl_2.6H_2O$ (5 µg/L). After solubilization, the minerals in liquid medium were properly sterilized in an autoclave at 121 °C for 20 min. Methanol was added at a later stage as substrate in all tests after cooling the mineral medium to avoid loss by evaporation during the entire process. Bacteria were added after the flasks had all the necessary elements for cell growth.

### *Cell production in shake flasks*

The pre-inoculum added to all flasks took a bacterial growth time of 40 hours using a methanol concentration of 5.0 g/L. The inoculum it centrifuged at 10956 x*g* for 20 min, the supernatant removed, and the cells resuspended in a mineral medium. These aliquots it centrifuged, then removed the supernatant (residual methanol sent for analysis), and the pellet was resuspended in distilled water for optical density measurement in a spectrophotometer (UV-1800, Shimadzu, JAPAN). Optical density measurements were performed by drawing samples of 2 mL to measure the absorbance at a wavelength of 600 nm. Growth assays were

started by mixing 10 mL of the activated inoculum of the bacterium suspension with 190 ml of mineral medium (5 % *V*/*V*) containing methanol concentrations of 1, 4, 7, 12, and 18 g/L of methanol in 500 mL conical flasks. The bacterial cultures were incubated at 30 °C and 4 x*g* a rotary shaker (Innova 44, New Brunswick, USA).

*Cell production in an instrumented bioreactor by fed-batch operation*

A Biostat B bioreactor (B. Braun Biotech International, Germany) with automatic control was used for this assay. Cultivation started with 7 g/L methanol and your consumption was verified by liquid chromatography analysis. The dissolved oxygen was monitored online throughout bacterial cultivation with an electrode connected to the Biostat B bioreactor. The methanol added at the time intervals of the batch was diluted in a volume of mineral medium, always the same volume, 75 ml, of which 50 ml of mineral medium and 25 ml of methanol. This strategy circumvented the problem of methanol evaporation during its handling (given its significant volatility), providing both the substrate and the essential elements necessary for cell metabolism, maintaining a good carbon and nitrogen ratio, without limiting cell growth, and production of other compounds. The fed batch bioreactor conditions were as follows: temperature of 30 °C; stirring velocity at 450 rpm; pH=7; specific aeration rate: 9 L/min and the total bioreactor volume capacity was 10 liters (considering its headspace), which operated with a working volume of 6 liters. This strategy helps to control the foam that can be formed by the medium in the combination of aeration and mechanical agitation. The pH was maintained by adding solutions of sodium hydroxide (1M) and hydrochloric acid (1M), automatically in the bioreactor.

*Determination of cell concentration*

For monitoring cell growth kinetics, the cell concentration was determined gravimetrically as by means of a standard curve relating the dry cell weight and optical density at 600 nm. From this correlation, the calibration curve used in the tests to estimate the concentration of

*Methylobacterium organophylum* was based in an equation.

$$y = 1.56\ x + 0.03\ (R^2 = 0.998) \qquad /1/$$

*Methanol quantitation*

The methanol concentration in the samples was determined by high-performance liquid chromatography – HPLC - equipped with a refractive index detector (Waters 2414, Waters, USA). The column used was a PL Hi-Plex H8 micron, 300 × 7.7 mm. Sulfuric acid diluted in distilled water in the concentration of 0.005 mol/L was used as the mobile phase at a flow rate of 0.6 mL/min and a maximum pressure of 0.5 Pa. Methanol P.A. (Vetec Fine Chemicals, Brazil) was used as a standard. The column temperature was approximately 60°C, and the injection volume was 20 µL. The concentrations of the samples were calculated by comparison with the standard concentration of methanol calibration curve.

*Centesimal and essential amino acid analyses*

The protein content was determined by the *Kjeldhal* method (*29*) and calculated as a percentage (%) in each sample. Ash was determined using sample mineralization in a muffle after 6 hours at 550 °C and then cooled in a vacuum desiccator and weighed. The ash content corresponded to the difference between the mass of the sample contained in the crucible and the empty crucible (*29*). Carbohydrate contents were estimated using the Dubois method (*29*). The carbohydrate concentration was calculated using a standard glucose correlation curve. Amino acid analysis (an isocratic elution was used) was performed by high-performance liquid chromatography (Agilent 1260 Infinity HPLC System, USA) using a reversed-phase C18 column, 250 × 4 mm, 5 µm (flow 1.0 mL/min), Restek brand with mobile phase A as 0.05 M ammonium acetate (pH=6.8 adjusted with phosphoric acid) and mobile phase B as 0.1 M ammonium acetate (pH=6.8) in acetonitrile:methanol:water (44:10:46) Detection occurred by UV at 254 nm with a column oven temperature of 52 °C and an injection volume of 40 µL, with standards of 20 amino acids (alanine, arginine, asparagine, aspartic acid, tyrosine,

methionine, glutamic acid, histidine, glycine, isoleucine, leucine, lysine, serine, phenylalanine, proline, glutamine, threonine, tryptophan, OH-proline, valine) (*30*). For the preparation of standards, different masses (between 0.007 and 0.0210 grams) of each amino acid were weighed in individual 10 ml flasks (Tryptophan and OH-Proline were dissolved in water and prepared separately). After volumetric with 0.1N hydrochloric acid, the balloons were taken to ultrasound for approximately 10 min with the lid loosened; the flasks were shaken to homogenize the solution; aliquoted 100 µL of each amino acid solution into a small glass vial to make up the standard mix; with a subsequent 100 µL aliquot of the standard mixture into a glass container intended for use in the HPLC and following the analysis procedures for each sample.

*Carotenoid extraction, quantitation and identification*

An aliquot of 2 mL of the bacterial suspension was centrifuged (10956 x*g* for 10 min) and the supernatant was discarded, leaving the bacterial biomass as a precipitate. The cells were suspended in methanol (2 mL) and were then broken up by shearing with small-diameter glass beads (0.5-1 mm) in a vortex agitator for 10 min. The sample was centrifuged, and the supernatant was transferred. This process was repeated several times until complete removal of the pigment was achieved. Quantification of the carotenoids using spectrophotometry was described by Davies (*31*), associating the obtained values to the following general equation:

$$CT\ (\mu g.g^{-1}) = \frac{A.V.10^4}{E_{1cm}^{1\%}.W} \qquad /2/$$

A methanol absorption coefficient ($E_{1cm}^{1\%}$) of 2550 was reported by Davies (*31*); the absorbance (A) values using a wavelength of 450 nm were obtained from previous sample scans with a spectrophotometer (UV-1800, Shimadzu, Japan); V was the dilution volume (mL); and W was the weight of the dry cells in the sample used in the assay. This equation was used to calculate the mass of carotenoids per mass of cells, which was associated with the concentration in the bioreactor at the end of the fed-batch experiment that led us to determine the carotenoid concentration in the final fermentation media. Mass spectrometry analysis was

performed on a Nexera X2 Ultra-High Efficiency Liquid Chromatography System from Shimadzu coupled to a Max Impact mass spectrometer (APCI-Q-TOF configuration) from Bruker. Chromatographic separations were performed on a Hypersil ODS 150 × 2.1 mm column, 3 µm particle size, with a (Thermo) + Hypersil ODS precolumn (1 × 2.1 mm, 3 µm + 0.22 µm in-line filter). Water with 0.1 % formic acid (*V*/*V*) was used as mobile phase A, and methanol with 0.1 % formic acid (*V*/*V*) was used as mobile phase B. The oven temperature was 40°C, with an injection volume of 25 µL. The spectrometer was operated in scanning mode in the range of *m/z* 50-1200 with an APCI ionization source in positive ion mode. The acquisition mode was data-dependent (DDA/AutoMS), with isolation/fragmentation of 2 precursors per cycle. The mass values of the peaks and their fragments found were compared with databases available on PubChem (*32*).

*Chemical and rheological analyses of the exopolysaccharide*

After exopolysaccharide accumulation (36 hours), it was not possible to separate the cells from the polysaccharide using centrifugation only. Thus, to circumvent this problem, ice cold ethanol was added to the samples, which then was stirred in a vortex continuously before centrifugation. Two fractions were obtained: a gelatinous fraction containing the exopolysaccaride (supernatant) and bacterium cells (pinkish colored) which settled down to the bottom of the cuvette. As already described by Vijayendra et al (*ref. 19*), cold ethanol can be added and subjected to continuous stirring to separate the EPS. In this procedure, the cells are separated from the EPS after centrifugation and can be separated to measure their mass. The rosaceous characteristic of the cells makes it possible to visually verify this separation between cells and the more viscous compound produced by the cell.

The supernatant was separated and dried in an oven at 60 °C overnight, and the residual mass (EPS) after acid hydrolysis with 1 M $H_2SO_4$ at 80 °C for 2 hours was qualitatively and quantitatively analyzed for carbohydrate identification by liquid chromatography (Waters 2414, Waters, USA). A PL Hi-Plex H8 micron 300 × 7.7 mm column was used, and $H_2SO_4$ (0.005

mol.$L^{-1}$) was used as the mobile phase at a flow rate of 0.6 mL.$min^{-1}$ and a maximum pressure of 0.5 Pa. Rheological analysis of the EPS was performed in an AR-2000 Advanced Rheometer with an ETC oven (TA Instruments, USA) using approximately 3 g of dried sample, which was suspended to measure the shear rate and apparent viscosity. Oscillatory and steady-state measurements were performed at room temperature, which allowed determination of the elastic (*G'*), slimy viscosity (*G''*) and complex viscosity moduli as a function of angular frequency. In dynamic mode, the moduli of the materials are often measured. One such component is the elastic or storage modulus (*G'*), which is directly proportional to the energy stored in a deformation cycle. This feature is called the slimy viscosity or dissipative modulus (*G''*) and measures the energy dissipated or lost in the form of heat per deformation cycle. These properties provide essential information regarding the microstructure of the material and its predominant behavior.

*Transmission electron microscopy images*

Transmission microscopy was performed with JEOL 1200 EX (USA) equipment operated with an electron acceleration voltage of 80 kV with a Megaview III camera (12 bits) at 2 K resolution. In the sample fixation stage, 25 % glutaraldehyde in 0.2 M sodium cacodylate buffer was used for one hour at room temperature, followed by resolubilization with 0.1 M sodium cacodylate buffer solution (3 times) and Milli-Q water (Sigma, USA). In the postfixation stage, 1 % osmium tetroxide ($OsO_4$) with 0.1 M sodium cacodylate buffer (1:1) was used for one hour, with subsequent washing, followed by serial dehydration in acetone solutions (30, 50, 70, 90 and 100 %; 15 min each). The infiltration step was performed with the epoxy resin Epon. The inclusion part was carried out by transferring the sample to silicone molds and then including the Epon resin after removing all bubbles. After that, the samples were taken to an oven at 68 °C for 72 hours, and them observed at different increases.

*PHA staining by Sudan black*

Sudan black (VETEC, Brazil) was used as a 70 % (*V*/*V*) ethanol solution. A sample of the cell culture was deposited in a Petri dish in the form of a smear that was spread over and then dried for fixation onto the plate. Sudan black solution was then dripped all over the plate and left for 20 min. The plate was washed with distilled water, to remove the excess Sudan black, and then 90 % ethanol was dripped onto the plate, which was left for 5 min followed by washing with distilled water again until total removal of the used solutions (*25*). Sudan black has the appearance of a dark brown to black powder with maximum absorption between 596–605 nm and a melting point of 120–124 °C (*25*).

## RESULTS AND DISCUSSION

*Cell production in shake flasks*

To evaluate the ability of the strain to consume methanol as well as the potential inhibitory/toxic effects from its consumption, variations in the initial methanol concentration were assayed. These results are shown in Fig. 1 and demonstrate the distinct metabolic machinery of the bacterial strain used in this study regarding its ability to take up methanol at concentrations considered toxic to a large number of living organisms (*34*). The most important point to consider for the occurrence of methanol oxidation to formaldehyde in methylotrophic bacteria is the presence of methanol dehydrogenase (MDH), an enzyme that contains pyrroloquinoline quinone (PQQ). The presence of MDH in the bacterial periplasm probably reduces the typical toxicity of methanol when compared to its action in cells that do not have this enzyme. The high affinity with methanol makes its conversion into the cell faster (*9*).

This strain was able to consume methanol at concentrations as high as 17 g/L, despite not consuming it entirely at this concentration. In methanol concentrations up to 7 g/L, the bacterium was able to consume the entire methanol with the highest overall uptake rate (maximum of 0.25 g/L.h$^{1}$). Methanol was also totally consumed when its initial concentration was increased to 12 g/L; however, a longer cultivation time was required (84 hours). In the

medium with highest tested methanol concentration (17 g/L), only 48 % was consumed after 88 hours, when the fermentation was interrupted since the bacterial growth had ceased. Although it showed the ability to consume methanol, this bacterium was sensitive to the toxic effects caused by the increasing concentration of alcohol, which was reflected in the reduction of the methanol uptake rate as well as in the specific growth rate (above 7 g/L) and in the cell growth yield, which decreased as the methanol concentration increased (Table 1). The C:N ratios were calculated based on the concentrations of methanol and potassium nitrate since the medium contained only methanol as a carbon source in a solution of mineral salts, with $KNO_3$ as the only nitrogen source. The highest cell concentration in cell dry weight (approximately 5 g/L) was achieved with an initial methanol concentration of 12 g/L; however, the volumetric productivity of cells was approximately half of that obtained with an initial methanol concentration of 7 g/L. At the latter initial methanol concentration, the highest values for volumetric productivity was from 0.115 g/(L.h) and overall substrate consumption rate of the 0.250 g/(L.h) were reached. A study carried out by Jafari et al (*34*) using Methylobacterium showed that there is an optimum range of methanol concentrations (18 g/L) for cell growth, and above this concentration, methanol inhibits cell growth. Methanol concentrations in the range of 10 to 12 g/L generated higher cell concentrations. With a methanol concentration below this range, there was not enough carbon source to promote bacterial growth and at a methanol concentration above this range (20 g/L or more), the toxic effect of methanol inhibited growth (*34*).

Methylotrophic bacteria growing aerobically on single-carbon (C1) substrates produce formaldehyde as a central intermediate, which is assigned to impart toxicity to cells through its nonspecific reactivity with proteins and nucleic acids (*35*). A key challenge for these microorganisms is how to maximize the flux through formaldehyde while preventing the intracellular pool of free formaldehyde from accumulating to toxic levels (*36*). Methanol dehydrogenase (MDH) catalyzes the conversion of methanol to formaldehyde via an oxidation-reduction reaction that occurs in the periplasm. When formaldehyde crosses the

cytoplasmic membrane and enters into the cytoplasm, it reacts with either tetrahydrofolate ($H_4F$) or tetrahydromethanopterin ($H_4MPT$).

The first reaction occurs spontaneously (nonenzymatically) and enters into the serine pathway, and the former (the long route) has been demonstrated to be catalyzed by a formaldehyde-activating enzyme (*37*). However, the condensation rate and/or affinity for formaldehyde of these reactions are lower than the oxidation of methanol to formaldehyde by MDH (*38*). Thus, formaldehyde tends to accumulate in the cytoplasm, causing toxicity to the cells. Therefore, there should be a balance between the rate of methanol oxidation and the rate of formaldehyde assimilation to prevent or at least alleviate cell toxicity. The higher the oxidation rate of methanol, the greater the accumulation of formaldehyde, which in turn slows down cell metabolism. Table 1 also shows that as the methanol concentration increases, the C:N ratio also increases, but the cell yield factor decreases. This very likely occurs because high concentrations of carbon can lead to the accumulation of exopolysaccharides and other macromolecules in methylotrophic cells, such as polyhydroxybutyrate (PHB), as reported by Kim *et al.* (*39*), but which were not measured in the flask experiment of the present work. Additionally, as mentioned before, the highest volumetric productivity was achieved at a methanol concentration of 7 g/L, which corresponded to a C:N ratio of 19. Gowda & Shivakumar (*33*) reported that C:N ratios greater than 20 may be metabolically damaging to cells, and this was observed in the present work since the cell yield factor gradually decreased as the C:N ratio increased.

*Cell production in the instrumented bioreactor by fed-batch in mineral medium*

The simple initial batch started with 7 g/L of methanol, consumed in approximately 27 hours (Fig. 2), when the bioreactor began to receive additional volumes of methanol around a concentration close to 4.0 g/L. Measurements of methanol and dissolved oxygen were used as important parameters for methanol to be added to the bioreactor culture medium. Methanol and dissolved oxygen were monitored (by liquid chromatography measurements) to determine

the feeding time with the methanol medium. This was performed when the methanol was completely consumed and the oxygen concentration reached the set point (50% $O_2$ saturation), since the cellular metabolic activity ceased when the methanol was depleted in each period (Fig. 2). This justifies the decrease in the cell yield factor obtained in the fed-batch operation in the bioreactor. As foreshadowed, there was no inhibition by substrate the four feeding cycles, since the methanol was almost totally consumed from all cycles, except for the last one, even though this last cycle resulted in an appreciable consumption of methanol (85 %).

Maintaining the conditions of the fed-batch process for a total of 60 hours, with a temperature of 30º C, stirring at 450 rpm, the pH maintained around 7, with the addition of hydrochloric acid and/or sodium hydroxide and the aeration rate specific at 9 L/min, the maximum cell concentration was 5.05 g/L, with a maximum specific growth rate ($\mu_{xmax}$) of 0.11 $h^{-1}$, specific growth rate ($\mu$) of 0.06 $h^{-1}$, the percentage of substrate reduction of 97.3 %, cell growth yield was 0.24 g/g, overall substrate consumption rate ($Q_s$) and volumetric productivity ($Q_x$) were 0.34 g/(L.h) and 0.08 g/(L.h). At the end of the trial, a total of 1.6 x $10^{-3}$ (g/L) of carotenoids was obtained. As shown by Vila et al (*40*), the total carotenoid contents of the isolates were similar, ranging between 0.33-0.73 mg/g dry biomass, corroborating the fact that carotenoids are produced in lower amounts.

The production of EPS was confirmed in a strictly mineral medium containing methanol as the sole carbon source. EPS formation possibly occurs due to a deviation in the metabolic pathway related to oxygen limitations in the culture medium, showing a consequent decrease in cell growth. The EPS concentration in the system was estimated from sampling at the end of the process. A 25 mL aliquot was removed from the bioreactor and precipitated with 90 % cold ethanol as reported in the previous sections. The cells were separated from the supernatant, dried and weighed on an infrared scale. The dry weight was used to estimate an EPS concentration of 8.9 g/L in the bioreactor.

The hydrolysis of EPS followed by analysis by HPLC showed that the EPS was composed

of approximately 50 % carbohydrates, with approximately 15 % glucose and 35 % mannose. The late production of EPS by microorganisms is frequently related to secondary metabolism, often promoting growth during a subsequent shortage (in either of the substrates or other factors), providing some protection or barrier against an inhospitable environment and facilitating cell adherence to solid surfaces (*41*).

From the EPS rheological analysis, a decrease in viscosity was measured with an increase in the shear rate, suggesting pseudoplastic fluid behavior. Its viscosity was approximately 20 times greater than that of water. The elastic modulus is superior to the viscous modulus, confirming the characteristic of a non-Newtonian pseudoplastic (Fig. 3).

According to Becker *et al.* (*20*), two parameters are very important to evaluate the viscoelasticity of a fluid. They are the viscous modulus and the elastic modulus. The elastic or storage modulus (*G*') is an elastic measure of the material, that is, the material's capacity to store energy. The viscous or slimy loss modulus (*G"*) determines the fluid's ability to dissipate energy in the form of heat.

In an analysis performed by Choi *et al.* (*13*) with *Methylobacterium organophilum* (NCIB 11278 KC-1), the viscosity of the biopolymer studied was 0.018 Pa·s, which is approximately 10 times greater than the viscosity of xanthan gum and approximately 200 times greater than that of pullulan, a polysaccharide excreted by some species of bacteria. The result of this viscosimetric analysis was similar to that found in this work, whose value was approximately 0.019 Pa·s.

The extracted pigment was analyzed by liquid chromatography coupled to mass spectrometry, and the results were processed with MZmine 2.53 software (*14*). As demonstrated by Stepnowski *et al.* (*16*), this species can produce myxol-type pigments.

These authors named the pigment dihydroxylycopene (dihydroxy derivatives of oscillol) or myxol. Based on the known monoisotopic mass of certain carotenoids in general, a search was performed in the MZmine program (*14*), where the mass value was entered in a more specific way to find the *m/z* peaks (load/mass ratio) of each substance. Table 2 shows this list

with the mass, retention time, peak intensity and identification data of the molecules found in the sample from the comparison of the *m/z* ratio parameter.

ESI-MS analysis showed six fragments that could be identified by this method, with very high precision in the *m/z* values when compared to PubChem parameters (*32*): lutein or zeaxanthin (these compounds have the same molecular formula and mass but a different structural conformation), myxol, canthaxanthin, astaxanthin, spheroidene, and 1,1'-dihydroxylycopene. For example, the 1,1'- dihydroxylycopene pigment found in Stepnowski's work (*16*) with a monoisotopic mass of 572.4593 can be considered to be present in this analysis, as the monoisotopic mass value found here (*m/z* 572.4594) was very close to the mass found in the previous work. Further studies by high-resolution mass spectrometry and $^{1}$H NMR analysis are needed to confirm these structures.

*Centesimal analysis and essential amino acids*

According to the analysis of the centesimal composition, the most important components of *M. organophilum* are proteins, carbohydrates and lipids. Compared with yeast cells from breweries (*38*), which can be used, for example, as sources of single-cell protein, present a lower protein (39 %) and total lipid (0.5 %) content, *M. organophilum* is a good source for single-cell production due to its higher concentration of proteins and carbohydrates.

*M. organophilum* contains 54.1 % ± 2.9 de crude protein, 5.4 % ± 1.4 of lipids and 17.3 % ± 1.9 of carbohydrates. The percentage of nitrogen was 9.6 % ± 4.4 and the mineral material was around 7.8 % ± 0.2. The dry matter was 91.5 % ± 0.1 and the unquantified was 5.8 % ± 0.2.

Due to the presence of essential amino acids in its composition, *M. organophilum* biomass presents the potential for application in animal feed formulations and as nutritional supplementation for animal feed. The amino acids alanine, arginine, aspartic acid, tyrosine, glutamic acid, histidine, glycine, isoleucine, leucine, lysine, serine, phenylalanine, proline, threonine, tryptophan, and valine were identified. The amino acids methionine, cysteine, and

asparagine were not possibly identified due to limitations in the methodology used for analysis in the chromatograph, considering that the masses used as standard were in a range between 0.007 and 0.0210 grams. The Kuwait Institute for Scientific Research (KISR) initiated a research program for single-cell protein (SCP) production in 1977 (*42*), and four strains belonging to the genus *Methylophilus* were investigated to optimize the medium to improve the yield of bacterial biomass.

In studies on the biomass of *Rhodocyclus gelatinosus* for poultry, rearing presented photosynthetic pigments that are mainly responsible for the purple-red coloring generated in the cultivation of this bacterium (*43*), among which bacteriochlorophyll and carotenoids of the spheroidene series (spheroidene, hydroxysferoidene and spiriloxanthin) stand out. According to Khan *et al.* (*44*), the amino acids aspartic acid (17.82 ± 3.97 %) and leucine (15.90 ± 2.33 %) were identified in higher concentrations and methionine was not present, justifying its use as a possible protein for animal feed. A study with the carotenoid-producing photosynthetic bacterium *Rhodopseudomonas faecalis* by Patthawaro & Saejung (*45*) revealed that chicken manure was the best substrate for the production of single-cell protein by submerged fermentation using photosynthetic bacteria compared to pig, cow, and buffalo manure. The authors suggested this alternative form of SCP production for animal feed, as well as this strategy for the management of animal waste. In the present study, almost all amino acids were identified, and histidine was found at a higher concentration (Table 3).

Digestibility tests of protein preparations produced from SCP have previously been carried out, so that this type of supplementation could have greater acceptability on the market and with consumers in general. Works such as those by Zhang *et al.* (*46*) using *Corynebacterium glutamicum* and Jones *et al.* (*47*), showed that the use of this bacterium in pig feed (50 % traditional meal and 50 % SCP) and for the creation of aquatic cultures (replacing up to 52 % of fishmeal in salmon farming) does not present any damage to growth or the nutritional profile of the product, even though it is a preparation from different microbial sources, including microalgae, yeast, other fungi, and bacteria. All of these sources are actively being

investigated and commercialized and exhibit unique advantages and challenges.

Tlusty *et al.* (*48*) replaced up to 100 % and 55 % of the fishmeal in a compound feed for white shrimp and salmon, respectively, and tested their growth and consumer taste preference. In each of these tests, the animals performed equivalently when they were fed diets containing either the SCP from *Methylobacterium extorquens* or a standard aquaculture diet. These promising works, as well as a variety of others that can be found in the literature, increasingly encourage the development of technologies for the cultivation of SCP, mainly using innovative sources such as methanol. In short, the development of a feed obtained from a simple substrate, such as methanol, employing a pigmented bacterium that accumulates carotenoids adds another advantage: the production of biomass rich in protein nutrients and oxycarotenoid pigments, which can be used as a pigmenting ingredient in feed for different animals. The use of a meal with similar formulation characteristics for laying hens, as studied by Ponsano *et al.* (*43*), aimed to improve egg yolks and products containing eggs.

In animals, carotenoids exert various protective functions and can effectively destroy reactive oxygen species (free radicals) and inhibit their formation, functioning as antioxidants. In chickens, carotenoids can prevent encephalomalacia. Carotenoids appear to increase the cytotoxic activity of natural killers, decrease the growth rate of tumors and promote wound healing (*50-52*).

*Electron microscopy images and coloration with Sudan black highlighting the accumulated PHA*

Gowda & Shivakumar (*33*), who studied PHB production, reported that C:N ratios greater than 20 may be metabolically damaging to cells. Increasing the C:N ratio from 10:1 to 20:1 resulted in an increase of 1.6 g/L in cell biomass and caused a large reduction in PHB production (from 72.85 % to 2.5 %). The authors attribute these results to the rapid use of excess carbon, leading to increased growth due to physiological changes in cells and disfavored the production of PHB. This was also observed in the present work, since the cell

yield factor gradually reduced with the increase in the C:N ratio and, consequently, the specific growth rate. The cells were subjected to transmission electron microscopy analysis, which showed that the granules accumulated in the cytoplasm (Fig. 4).

Accumulation in the form of granules seems to distribute between the daughter cells during cell division. These granules are distributed heterogeneously among the cells, where some cells have larger granules than others. The accumulation in the form of a biopolymer shows that excess carbon was an important factor. The granules accumulate as an energy reserve and are protected by a lipid envelope, which can be degraded by the cell if necessary (*25*).

Sudan Black is commonly used as an auxiliary technique for suggesting the accumulation of polyhydroxyalkanoates (PHA) in different cells. Some authors relate the affinity of Sudan Black with the membranes surrounding intracellular biopolymers and others report the use of this dye specifically for indicating qualitatively the PHA accumulation (*26-28*). Sudan Black is a lipophilic dye that reacts with phospholipids, neutral fats and steroids to reveal the phospholipid membranes of PHB granules. These granules appear darkish when observed by light microscopy and in plate samples (Fig. S1).

## CONCLUSIONS

Methanol, a nonconventional substrate for microorganism growth, has many advantages when it is used as a single carbon substrate compared to methane, which, as a gas, has reduced solubility in the fermentation medium. Centesimal analysis of *Methylobacterium organophilum* cells showed a high percentage of crude protein and the presence of almost all essential amino acids, corroborating the indication of this microbial biomass for use as a single-cell protein. Beyond the possibility of producing protein from this sole carbon source, the production of carotenoids associated with *M. organophilum* growth makes this bioprocess commercially attractive. The biochemical production of carotenoids is very relevant due to the possibility of using a low-cost substrate under simple and controlled cultivation conditions.

However, further investigations should be carried out to increase the levels of expression of this important group of provitamins and antioxidants. Finally, there is the possibility of producing exopolysaccharides and polyhydroxyalcanoates, which accumulate through a deviation in the metabolic route, making the bioprocess even more attractive and diverse.

## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

## AUTHORS' CONTRIBUTION

Ana C P Simões carried out most of the experimental work, but also participated in work design, data analysis and writing of the manuscript. Rodrigo P Fernandes gave support to experimental work and was involved in data analysis and writing. Maysa S Barreto, Gabriela B M da Costa, Mateus G. Godoy, Denise M G Freire supported the experimental work, performing the analysis of amino acids profile of the bacterial species. N Pereira Jr was the main supervisor of all project tasks and the project coordinator.

## SUPPLEMENTARY MATERIALS

Supplementary material is available at: www.ftb.com.hr.

## ETHICAL STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors. The authors confirm that principles of ethical and professional conduct have been followed in this research and in the preparation of this article.

## ORCID ID

A.C.P. Simões https://orcid.org/0000-0001-8307-5494

R.P. Fernandes https://orcid.org/0000-0001-7691-0682

M.S. Barreto https://orcid.org/0000-0002-5922-5381

G.B.M. da Costa https://orcid.org/0000-0002-1973-7833

M.G. de Godoy https://orcid.org/0000-0003-2209-7993

D.M.G. Freire https://orcid.org/0000-0002-8298-5936

N. Pereira Jr. https://orcid.org/0000-0002-5900-9961

## REFERENCES

1. Anupama, Ravindra P. Value-added food: Single cell protein. Biotechnology. Advances. 2000; 18(6), 459–479. https://doi.org/10.1016/S0734-9750(00)00045-8

2. Adedayo MR, Ajiboye EA, Akintunde JK, Odaibo A. Single cell proteins: as nutritional enhancer. Advances in Applied Science Research. 2011; 2(5):396-409. Available from: https://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.463.5605&rep=rep1&type=pdf

3. Nasseri AT, Rasoul-Amini S, Morowvat MH, Ghasemi Y. Single Cell Protein: Production and Process. American Journal of Food Technology. 2011; 6:103-116. http://dx.doi.org/10.3923/ajft.2011.103.116

4. Ritala A, Hakkinen ST, Toivari M, Wiebe MG. Single Cell Protein – State of the Art, Industrial Landscape and Patents 2001–2016. Front. Microbiol. 2017; 8:2009. https://doi.org/10.3389/fmicb.2017.02009

5. Spalvins K, Ivanovs K, Blumberga D. Single cell protein production from waste biomass: review of various agricultural by-products. Agronomy Research. 2018; 16(2):1493-1508. http://dx.doi.org/10.15159/ar.18.129

6. Trotsenko YA, Murrell JC. Metabolic Aspects of Aerobic Obligate Methanotrophy. Advances in Applied Microbiology. 2008; 63:183-229. https://doi.org/10.1016/S0065-2164(07)00005-6

7. Zhang M, Yuan XJ, Zhang C, Zhu LP, Mo XH, Chen WJ, Yang S. Bioconversion of Methanol into Value-added Chemicals in Native and Synthetic Methylotrophs. Curr Issues Mol

Biol. 2019; 33:225-236.

https://doi.org/10.21775/cimb.033.225

8. Schrader J, Schilling M, Holtmann D, Sell D, Villela Filho M, Marx A, Vorholt JA. Methanol-based industrial biotechnology: current status and future perspectives of methylotrophic bacteria. Trends in Biotechnology. 2008; 27(2):107-115.

https://doi.org/10.1016/j.tibtech.2008.10.009

9. Anthony C. The Biochemistry of Methylotrophs. Introduction and carbon assimilation. 1982; Academic Press, London

10. Johnson PA, Quayle JR. Microbial growth on C1 compounds. Oxidation of methanol, formaldehyde and formate by methanol-grown *Pseudomonas* AM1. Biochem. J., 1964; 93:281-290.

https://doi.org/10.1042/bj0930281

11. Whitaker W, Sandoval NR, Bennett RK, Fast AG, Papoutsakis ET. Synthetic methylotrophy: engineering the production of biofuels and chemicals based on the biology of aerobic methanol utilization. Curr Opin Biotechnol. 2015, 33:165–175.

https://doi.org/10.1016/j.copbio.2015.01.007

12. O'Connor ML, Hanson RS. Enzyme Regulation in *Methylobacterium organophilum*. Journal of General Microbiology. 1977; 101:327-332.

https://doi.org/10.1099/00221287-101-2-327

13. Choi JH, Deok KO, Kim JH, Lebeault JM. Characteristics of a novel high viscosity polysaccharide, methylan, produced by *Methylobacterium organophilum*. Biotechnol Lett. 1991; 13:417-420.

https://doi.org/10.1007/BF01030993

14. T. Pluskal, S. Castillo, A. Villar-Briones, M. Orešič, MZmine 2: Modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data, BMC Bioinformatics. 2010; 11:395.

https://doi.org/10.1186/1471-2105-11-395

15. Hou CT. Methylotrophs: Microbiology, Biochemistry and Genetics. CRC Press, Florida, 1984, Apud: Green, PN. Methylobacterium. In: Dworkin M *et al.* (eds) The Prokaryotes. Springer New York. 2006; 257–265.
https://doi.org/10.1007/0-387-30745-1_14

16. Stepnowski P, Blotevoge K-H, Jastoff B. Extraction of carotenoid produced during methanol waste biodegradation. International Biodeterioration & Biodegradation. 2004; 53: 127-132.
http://dx.doi.org/10.1016/j.ibiod.2003.11.001

17. Stirling DI, Dalton H. Aerobic metabolism of methane and methanol. Apud: Bull AT, Dalton H (eds.) Biotechnology, Vol. 1. The Principles of Biotechnology: Scientific Fundamentals. Pergamon, Oxford. 1985; 379–408

18. Zúñiga C, Morales MS, Revah S. Polyhydroxyalkanoates accumulation by *Methylobacterium organophilum* CZ-2 during methane degradation using citrate or propionate as cosubstrates. Bioresource Technology. 2013; 129:686–689.
https://doi.org/10.1016/j.biortech.2012.11.120

19. Vijayendra, SVN.; Palanivel, G.; Mahadevamma, S.; Tharanathan, RN. Physico-chemical characterization of an exopolysaccharide produced by a non-ropy strain of *Leuconostoc sp*. CFR 2181 isolated from dahi, an Indian traditional lactic fermented milk product. Carbohydrate Polymers, Nova Iorque. 2008; 72:300-307.
http://dx.doi.org/10.1016/j.carbpol.2007.08.016

20. Becker A, Katzen F, Puhler A, Ielpi L. Xanthum gum biosynthesis and application: biochemical/genetic perspective. Appl. Microbiol. Biotechnology. 1998; 50:145-152.
https://doi.org/10.1007/s002530051269

21. Kumar AS, Mody K, Jha B. Bacterial exopolysaccharide – a perception. Journal of Basic Microbiology. 2007; 47:103-117.
https://doi.org/10.1002/jobm.200610203

22. Boels IC, Van K R, Hugenholtz J, Kleerebezem M, De Vos WM. Sugar catabolism and its

impact on the biosynthesis and engineering of exopolysaccharide production in lactic acid bacteria. International Dairy Journal. 2001; 11: 723-732.

http://dx.doi.org/10.1016/S0958-6946(01)00116-9

23. Sutherland IW. Novel and established applications of microbial polysaccharides. Trends Biotechnol. 1998; 16: 41-46.

https://doi.org/10.1016/S0167-7799(97)01139-6

24. Albermann C, Beuttler H. Synthesis of β-Carotene and Other Important Carotenoids with Bacteria. In: Vandamme EJ, Revuelta, JL (eds) Industrial Biotechnology of Vitamins, Biopigments and Antioxidants, Wiley-VCH, Weinheim. 2016; 227-263.

25. Cardoso, LOB. Produção de polihidroxibutirato (PHB) por bactérias metilotróficas. [MSc Thesis]. São Paulo, Brazil: Polytechnic School, University of São Paulo; 2017.

26. Akaraonye E, Keshavarz T, Roy I. Production of polyhydroxyalkanoates: the future green materials of choice. Journal of Chemical Technology Biotechnology. 2010; 85: 732-743.

https://doi.org/10.1002/jctb.2392

27. Balakrishna PA, Kumas JA, Thulasi K, Kumarapillai H. Evaluation of short-chain-length polyhydroxyalkanoate accumulation in *Bacillus aryabhattai*. Braz. J. Microbiol. 2017; 48 (3): 451-460.

http://dx.doi.org/10.1016/j.bjm.2017.01.005

28. Wei Y-H, Chen W-C, Huang C-K, Wu H-S, Sun Y-M, Lo C-W, Janarthanan O-M. Screening and Evaluation of Polyhydroxybutyrate-Producing Strains from Indigenous Isolate *Cupriavidus taiwanensis* Strains. International Journal of Molecular Sciences. 2011; 12(1):252-265.

https://doi.org/10.3390/ijms12010252

29. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Fred S. Colorimetric Method for Determination of Sugars and Related Substances. Analytical Chemistry. 1956; 28 (3): 350-356.

https://doi.org/10.1021/ac60111a017

30. Instituto Adolfo Lutz. Métodos Físico-Químicos para Análise de Alimentos, 4a ed.

Instituto Adolfo Lutz, São Paulo. 2005. Available from: http://www.ial.sp.gov.br/resources/editorinplace/ial/2016_3_19/analisedealimentosial_2008.pdf

31. Davies BH. Carotenoids in Higher Plants. In: Goodwin TW (Ed) Chemistry and Biochemistry of plant pigments. Academic Press, London. 1976; 38-15. https://doi.org/10.1007/978-3-642-66632-2_11

32. Kim S, Chen J, Cheng T, Gindulyte A, He J, He S, Li Q, Shoemaker BA, Thiessen P A, Yu B, Zaslavsky L, Zhang J, Bolton EE. PubChem in 2021: new data content and improved web interfaces. Nucleic Acids Res. 2021;49(D1):D1388–D1395. https://doi.org/10.1093/nar/gkaa971

33. Gowda V, Shivakumar S. Agrowaste-based Polyhydroxyalkanoate (PHA) production using hydrolytic potential of Bacillus thuringiensis IAM 12077. Brazilian Archives of Biology and Technology. 2014; 57(1). https://doi.org/10.1590/S1516-89132014000100009

34. Jafari P, Zamani M, Almasian F. Feed Stuff Production from Methanol by *Methylotrophic* microorganisms. Scientia Iranica. 2008;15(4):480-486. Available from: http://scientiairanica.sharif.edu/article_2938_1c057e5a5f93eb57ef7dcc0962913ddc.pdf

35. Feldman MY. Reactions of nucleic acids and nucleoproteins with formaldehyde. Progr. Nucleic Acid Res. Mol. Biol. 1973; (13):1-49. https://doi.org/10.1016/S0079-6603(08)60099-9

36. Marx CJ, Chistoserdova L, Lidstrom, ME. Formaldehyde-Detoxifying Role of the Tetrahydromethanopterin-Linked Pathway in *Methylobacterium extorquens* AM1. Journal of Bacteriology. 2003; 185 (24): 7160-7168. https://dx.doi.org/10.1128%2FJB.185.23.7160-7168.2003

37. Vorholt JA, Marx CJ, Lidstrom ME, Thauer, RK. Novel Formaldehyde-activating enzyme in *Methylobacterium extorquens* AM1 required for growth on methanol. Journal of Bacteriology. 2000; 182 (23), 6645-6650.

https://doi.org/10.1128/jb.182.23.6645-6650.2000

38. Hea H, Höpera R, Dodenhöfta M, Marlièreb P, Bar-Evena A. An optimized methanol assimilation pathway relying on promiscuous formaldehyde-condensing aldolases in *E. coli*. Metabolic Engineering. 2020; (60): 1–13.

https://doi.org/10.1016/j.ymben.2020.03.002

39. Kim SW, Kim P, Kim JH. Production of Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) from *Methylobacterium organophilum* by potassium-limited fed-batch culture. Enzyme and Microbial Technology. 1999; (24): 555–560.

http://dx.doi.org/10.1016%2FS0141-0229(98)00160-4

40. Vila E., Hornero-Méndez D., Azziz G., Lareo C., Saravia V. Carotenoids from heterotrophic bacteria isolated from Fildes Peninsula, King George Island, Antarctica, Biotechnology Reports. 2019; (21).

https://doi.org/10.1016/j.btre.2019.e00306

41. Belgrano FDS, Verçoza BRF, Rodrigues JCF, Hatti-Kaul R, Pereira Jr N. EPS production by *Propionibacterium freudenreichii* facilitates its immobilization for propionic acid production. Journal of Applied Microbiology. 2018; 125 (2): 480-489.

https://doi.org/10.1111/jam.13895

42. Yamada EA, Alvim ID, Santucci MCC, Sgarbieri VC. Centesimal composition and protein nutritive value of yeast from ethanol fermentation and of yeast derivatives. Brazilian Journal of Nutrition. 2003; 16(4):423-432

http://doi.org/10.1590/s1415-52732003000400006

43. Ponsano, EHG; Lacava, PM; Pinto, MF. Chemical composition of *Rhodocyclus gelatinosus* biomass produced in poultry slaughterhouse wastewater. Brazilian Archives of Biology and Technology. 2003; 46 (2):143 – 147.

https://doi.org/10.1590/S1516-89132003000200001

44. Khan FA, Bacha N, Sarzamin K, Haleem L, Waqas A. Optimization of Laboratory requirements through Experimental Design for the production of indigenous *Saccharomyces*

*cerevisiae* from orange peels. J. Sc. & Tech. Univ. Peshawar. 2016; 40 (2), 01-07.

45. Patthawaro S, Saejung C. Production of single cell protein from manure as animal feed by using photosynthetic bacteria. Microbiology Open. 2019; (8):e913. https://doi.org/10.1002/mbo3.913

46. Zhang HY, Piao XS, Li P. Effects of single Cell Protein Replacing Fish Meal in diet on Growth Performance, Nutrient Digestibility and Intestional Morphology in Weaned Pigs. Asian Australas. J. Anim. Sci. 2013; 26(9): 1320-1328. https://doi.org/10.5713/ajas.2013.13200

47. Jones SW, Karpol A, Friedman S, Maru BT, Tracy BP. Recent advances in single cell protein use as a feed ingredient in aquaculture. Current Opinion in Biotechnology. 2020; (61): 189-197. https://doi.org/10.1016/j.copbio.2019.12.026

48. Tlusty M, Rhyne A, Szczebak JT. A transdisciplinary approach to the initial validation of a single cell protein as an alternative protein source for use in aquafeeds. PeerJ. 2017; (5):e3170. https://doi.org/10.7717/peerj.3170

49. Banat IM, Al-Awadhi N, Hamdan, IY. Physiological characteristics of four *methylotrophic* bacteria and their potential use in single-cell protein production. World J. Microbiol Biotechnol. 1989; (5): 149-159. https://doi.org/10.1007/BF01741838

50.Bauernfeind JC. Carotenoid vitamin A precursors and analogs in food and feeds. J.Agric. Food Chem. 1972; 20 (3): 456-473. https://doi.org/10.1021/jf60181a003

51. Bauernfeind JC. Carotenoids as colorants and vitamin a precursors. Academic Press, New York. 1981. Available from: https://www.elsevier.com/books/carotenoids-as-colorants-and-vitamin-a-precursors/bauernfeind/978-0-12-082850-0

52. Latscha T. Carotenoids: their nature and significance in animal feeds. Roche Animal

Nutrition and Health, Switzerland. 1990.

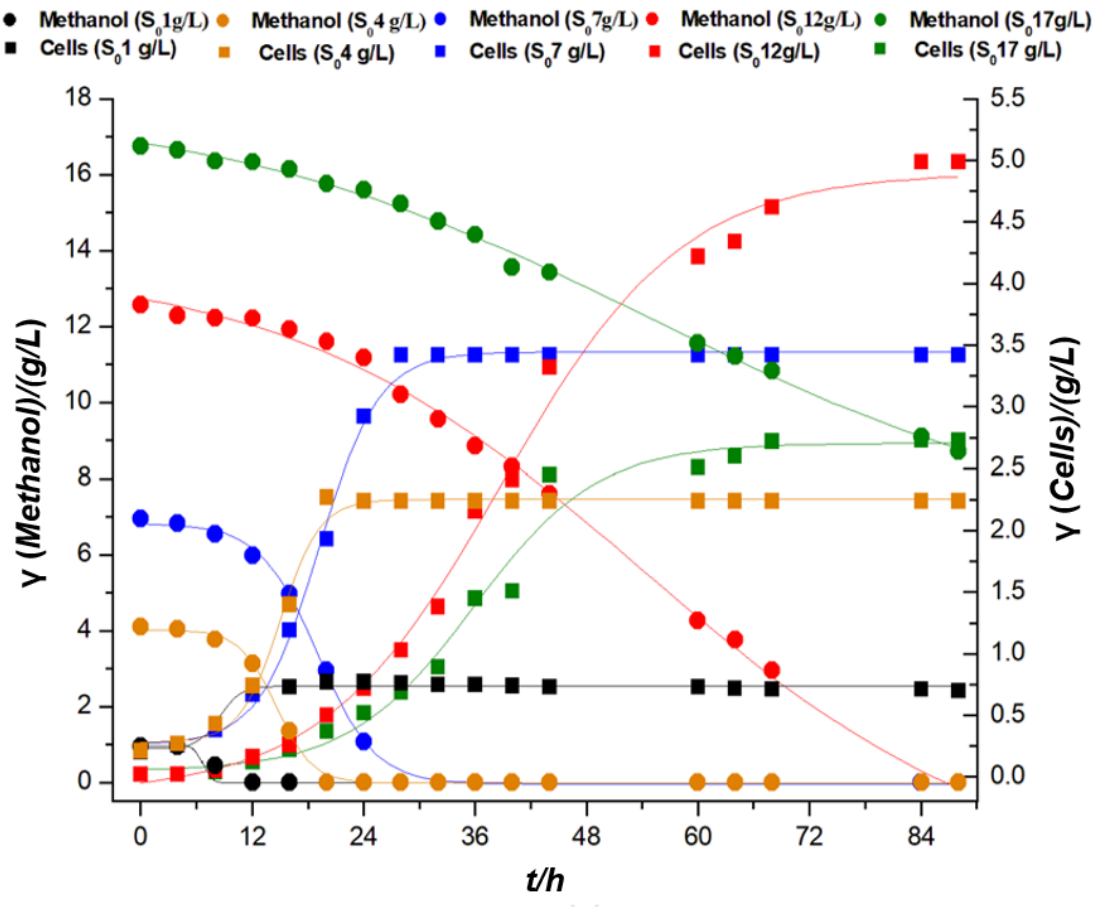


Fig. 1. Microbial biomass production under different methanol concentrations. Experiments were carried out in the following conditions: 30°C; 250 rpm; pH=7 and $0.10 < X_0 < 0.25$ g/L

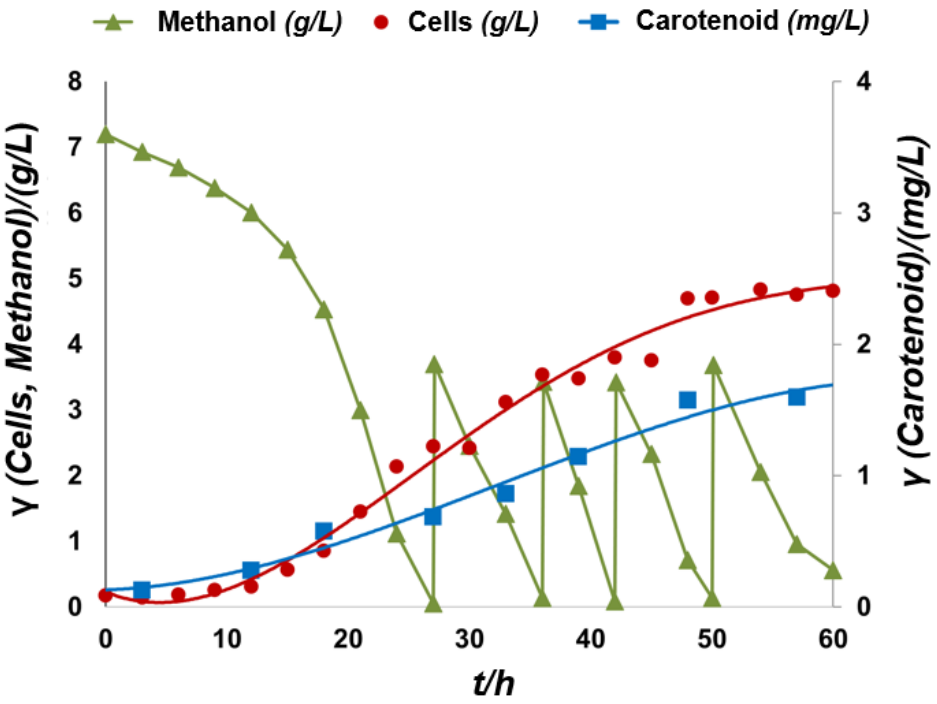


Fig. 2. Growth kinetic profile of *Methylobacterium organophilum* DSMZ 18172 in bioreactor by fed batch using methanol as the sole carbon source. Conditions: temperature: 30°C; agitation: 450 rpm; pH=7; specific aeration rate: 9 L/min

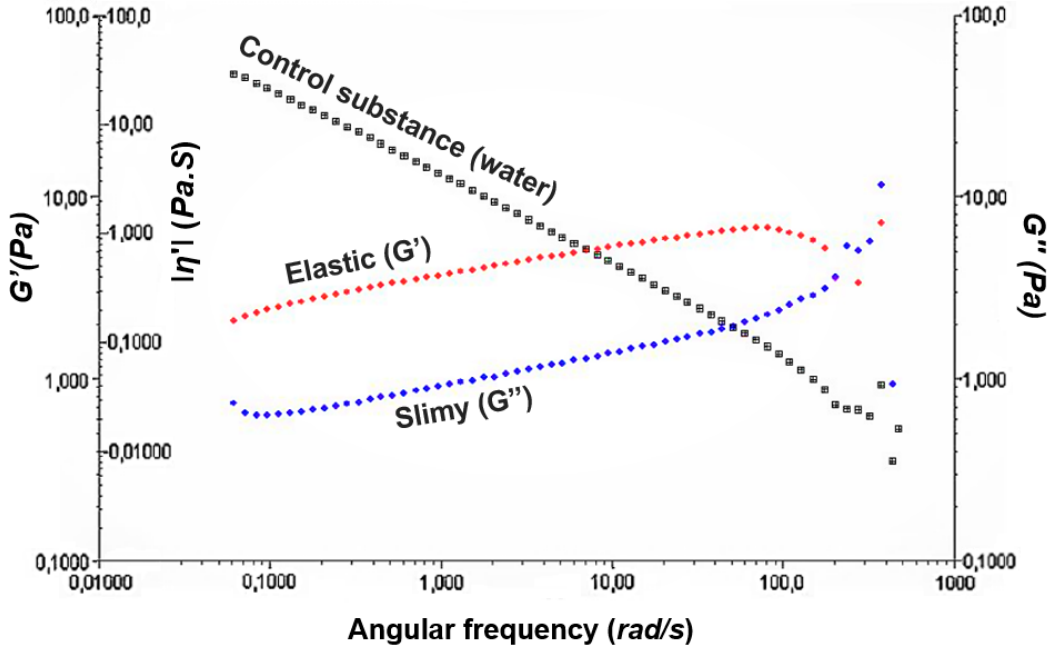


Fig. 3. Rheological analysis of exopolysaccharide. Abbreviations: *G'*: Mode Elastic; *G''*: Mode Slimy; *η'*: viscosity

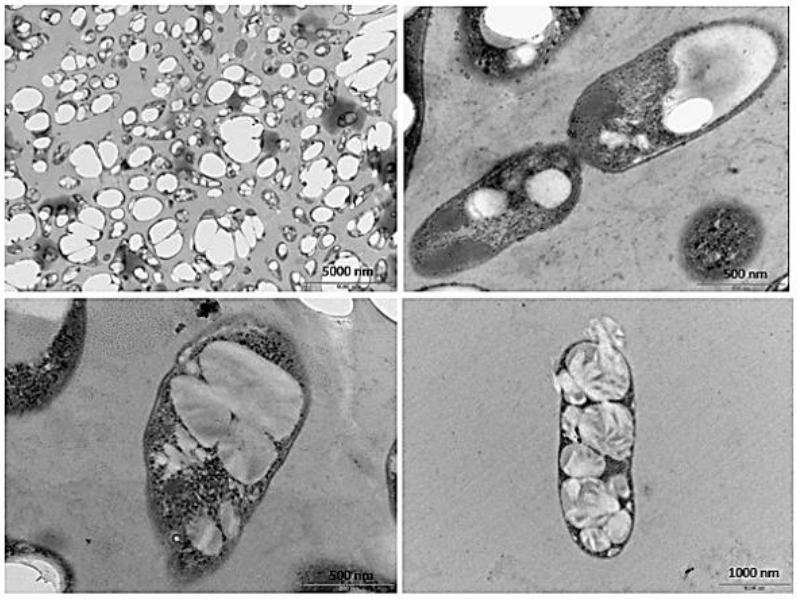


Fig. 4. Transmission electron microscopy (TEM) of a sample obtained from the fed-batch assay in bioreactor, showing accumulation of granules in the cells of *M. organophilum*

Table 1. Growth response variables for different concentrations of methanol in flasks

| γ (*methanol*)/ (*g/L*) | C:N | $X_{max}$/ (*g/L*) | $\mu_x$/ $h^{-1}$ | *SR*/ *%* | $t_{SR}$/ *h* | $Y_{X/S}$/ *g/g* | $Q_S$/ *g/(L·h)* | $Q_X$/ *g/(L·h)* |
|---|---|---|---|---|---|---|---|---|
| **1** | 3 | 0.77± 0.021 | 0.12± 0.007 | 100 | 12 | 0.57± 0.01 | 0.08± 0.001 | 0.06± 0.007 |
| **4** | 11 | 2.27± 0.049 | 0.14± 0.014 | 100 | 20 | 0.52± 0.01 | 0.20± 0.030 | 0.10± 0014 |
| **7** | 19 | 3.42± 0.021 | 0.10± 0.007 | 100 | 28 | 0.46± 0.02 | 0.25± 0.007 | 0.12± 0001 |
| **12** | 31 | 4.99± 0.007 | 0.08± 0.007 | 100 | 84 | 0.40± 0.01 | 0.14± 0.007 | 0.06± 0.014 |
| **17** | 45 | 2.73± 0.021 | 0.07± 0.007 | 48 | 88 | 0.32± 0.02 | 0.09± 0.008 | 0.03± 0.007 |

*C:N: carbon/nitrogen ratio; $X_{max}$: maximum cell concentration; $\mu_x$: specific growth rate; *SR*: percentage of substrate reduction; $t_{SR}$: time for substrate reduction; $Y_{x/s}$: cell growth yield; $Q_S$: overall substrate consumption rate; $Q_x$: volumetric productivity of cells

Table 2. Properties of the carotenoids found in the sample, compared with values by PubChem (*30*)

| PubChem 2.1 | | | (Present work) | | |
|---|---|---|---|---|---|
| Carotenoids | Standard Charge/Mass (*m/z*) | Molecular Formula | Charge/Mass (*m/z*) | Retention Time (min) | Intensity |
| Lutein or Zeaxanthin | 568.4280 | $C_{40}H_{56}O_2$ | 568.4269 | 22.29 | 2.3E2 |
| Myxol | 584.4229 | $C_{40}H_{56}O_3$ | 584.4234 | 22.28 | 1.5E2 |
| Canthaxanthin | 564.3967 | $C_{40}H_{52}O_2$ | 564.3977 | 24.84 | 2.0E2 |
| Astaxanthin | 596.3866 | $C_{40}H_{52}O_4$ | 596.3871 | 18.85 | 1.2E2 |
| Spheroidene | 568.4644 | $C_{41}H_{60}O$ | 568.4644 | 24.30 | 1.6E2 |
| 1,1'-dihydroxylycopene | 572.4593 | $C_{40}H_{60}O_2$ | 572.4594 | 21.83 | 1.6E2 |

Table 3. Amino acid composition for different protein sources

| Amino acids | a | b | c | d | e |
|---|---|---|---|---|---|
| Alanine | 1.3 0±0.07 | 5.4 | 6.98 | 13 | 1.44 |
| Arginine | 4.90±0.27 | 3.1 | - | 5 | 1.30 |
| Aspartic acid | 0.50±0.15 | 8.2 | 5.74 | 17.8 | 3.54 |
| Tyrosine | 0.70±0.09 | 2.9 | 2.9 | 5 | 0.87 |
| Glutamic acid | 0.40±0.06 | 7.2 | 6.85 | 4.5 | 2.14 |
| Histidine | 17.0±2.83 | 1.2 | 1.83 | 2 | 1.15 |
| Glycine | 1.30±0.04 | 2.9 | 4.18 | 3 | 1.42 |
| Isoleucine | 13.80±2.10 | 1.8 | 3.18 | 5 | 1.00 |
| Leucine | 1.60±0.16 | 4.9 | 6.80 | 15.9 | 2.05 |
| Lysine | 6.30±0.44 | 4.0 | 3.61 | 2.4 | 3.36 |
| Serine | 0.10±0.04 | 2.6 | 2.63 | 3.1 | 0.47 |
| Phenylalanine | 2.9±0.5 | 2.7 | 3.03 | 2 | 1.85 |
| Proline | 1.20±0.06 | 4.5 | 3.27 | 14 | 1.54 |
| Threonine | 0.50±0.12 | 2.6 | 3.52 | 4.3 | 1.40 |
| Tryptophan | 0.90±0.03 | - | 1.74 | - | - |
| Valine | 0.90±0.05 | 2.9 | 4.56 | 4.5 | 1.16 |
| Metheonine | - | 0.5 | 1.40 | - | 0.25 |
| Cysteine | - | 0.5 | 0.59 | - | - |
| Asparagyne | - | - | - | - | - |

a: *Methylobacterium organophilum* (This work); b: Five strains of the genus *Methylophilus*, Banat et al.[49]; c: *Rhodocyclus gelatinosus,* Ponsano et al.[43]; d: *Saccharomyces cerevisiae,* Khan et al.[44]; e: *Rhodopseudomonas faecalis,* Patthawaro & Saejung[45]

SUPPLEMENTARY MATERIAL

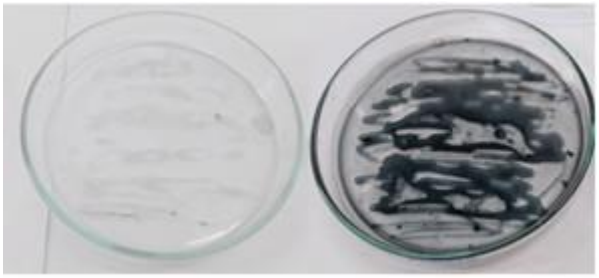

Fig. S1. Staining by Sudan Black of *M. organophilum* colonies. The first plate represents cells grown in shake flask by simple batch and the second plate corresponds cells grown in bioreactor by fed-batch operation