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DEVELOPMENT OF A BACTERIAL STRAIN COLLECTION RESISTANT TO SUPERCRITICAL CO_2

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ABSTRACT

Supercritical carbon dioxide (sCO_2) is an intermediate state between liquid and gas with valuable properties for industry. Its primary functions include serving as a solvent for extraction and separation processes, offering environmentally friendly solutions. Additionally, sCO_2 can act as a sterilizing agent against most microorganisms due to physiological stress (cytoplasm acidification, membrane permeabilization, etc.). Here, we present a collection of 58 bacterial strains isolated from cultures grown under sCO_2 conditions (35 °C, 100 bar), along with their morphological characterization and analysis of bioproducts secreted in oxygen-limited cultures. This study aims to characterize a collection of bacterial chassis resistant to sCO_2, potentially serving as platforms for the production of CO_2-soluble compounds. Such developments could significantly reduce both financial and environmental costs associated with bioprocesses.

Keywords: Supercritical CO_2. High Pressure. Bacterial Collection.

1 INTRODUCTION

Supercritical carbon dioxide (supercritical CO_2, sCO_2) plays a crucial role in modern industry due to its unique properties and versatility in various applications[1]. This special state of CO_2 occurs under conditions of temperature and pressure above the critical point, where it combines the characteristics of a gas and a liquid. In industry, supercritical CO_2 is valued for its ability to be an effective solvent in extraction processes, especially in the food and pharmaceutical industries, where it allows the production of pure extracts without leaving chemical residues[2]. Furthermore, it is used in purification processes and as a drying agent in heat-sensitive products, making it a sustainable alternative to traditional solvents due to its non-toxic and non-flammable nature. Thus, supercritical CO_2 not only contributes to industrial efficiency, but also to more sustainable and environmentally conscious practices[3].

Few studies explore the potential for microbial growth in environments exposed to sCO_2, as this is considered a sterilizing agent due to its harmful effects on microorganisms, such as cytoplasmic acidification and membrane destabilization[4,5]. Kyle and authors (2015) demonstrated that bacterial cultures isolated from geological CO_2 fixation sites were able to be grown in sCO_2 conditions, with *Bacillus* being the predominant genus. Freedman and colleagues (2018) were able to identify a strain of *Bacillus megaterium* capable of growing in sCO_2 conditions, producing acetate, lactate and succinate as final fermentation products and, after genetic engineering, producing isobutanol and isopentanol from 2-ketoisovalerate[8]. The use of *Bacillus* as a chassis has some advantages such as being one of the most trained genera with molecular tools available for gene editing, in addition to generally having low cultivation costs, tolerance to extreme environmental conditions and scalability.

2 MATERIAL & METHODS

Microbial screening: The microorganisms tested come from a *Bacillus* spp. germplasm bank isolated from soil and water from the Brazilian Cerrado and was kindly provided by external collaborators (Instituto Mato-grossense do Algodão). Cultures for morphological characterization were grown in LB (Lysogeny Broth) medium at 37 °C for 24 hours at aerobic conditions, evaluating colony aspects such as color, sheen, shape, margin, elevation, and size. Experiments under supercritical CO_2 conditions followed the protocol based on (Freedman et al., 2018). Briefly, inoculums (endospores) were cultivated in a modified M9 medium at 35 °C under 20, 60, or 100 bar with agitation. Samples were collected every 24 hours, with cell viability confirmed via heat shock (80 °C, 10 minutes) and colony-forming units (CFUs) counted on LB medium. Spore production and cellular morphology were observed using phase-contrast optical microscopy.

Bioproduct screening: Cultivations to identify bioproducts were carried out in two phases, the first for 24 hours in LB medium (35 °C – 150 rpm, aerobic) and the second in mineral medium, supplemented with a glucose (20 g/L) and ammonium sulfate (1 g/L) for 72h, 35 °C and 150 rpm. In the production phase, there was oxygen limitation using sealed flasks and occupancy of 40% of the bottle volume with culture medium. The supernatant and pellet were separated by centrifugation (8000 rpm, 10 minutes, 4 °C) for analysis of pH, dry mass and bioproducts. Organic acids and consumption of carbon sources were performed by high-performance liquid chromatography (ion exchange, Aminex HPX-87H BioRad, mobile phase 5 mM H2SO4, flow rate 0.5 mL/min, 55 °C) through light detection UV (214 nm) and refractive index, respectively. Minimum medium composition: KH_2PO_4 1,5 g/L, Na_2HPO_4 3,51 g/L, $MgSO_4.7H_2O$ 0,2 g/L, $CaCl_2.2H_2O$ 0,01 g/L, $C_6H_5Na_3O_7.2H_2O$ 0,153 g/L, $(NH_4)_2SO_4$ 1 g/L, glucose 20/L, trace elements 1 mL/L ($FeSO_4.7H_2O$ 20 g/L, $MnCl_2.4H_2O$ 0,03 g/L, H_3BO_4 0,3 g/L, $CuSO_4.5H_2O$ 0,01 g/L, $CoCl_2.6H_2O$ 0,2 g/L, $(NH_4)_6Mo_7O_{26}.H_aO$ 0,03 g/L, $ZnSO_4.7H_2O$ 0,03 g/L, $NiSO_4.7H_2O$ 0,03 g/L).

3 RESULTS & DISCUSSION

An initial collection of 170 strains of sporogenic bacteria isolated from water, soil and organic matter were used as a microbial bank. Morphological aspects of the colonies were evaluated in the cell multiplication process and their sporulation capacity. At the end of the process, the isolates were identified and divided into 9 large groups based on their phenotypic characteristics. Two aspects draw attention: the predominance of strains that synthesize extracellular polymers and the eventual production of pigments in liquid cultures. The first situation is already well described for *Bacillus subtilis*[9] and *Bacillus megaterium*[10] and is associated with the ability to attach, colonize surfaces and social interactions between microorganisms. Pigment production was only observed under some cultivation conditions (oxygen limitation, for example). It is known that *Bacillus* species produce pigments in their endospores to obtain photoprotection from UV radiation[11] and membrane stability (*B. megaterium*)[12].

Microbial screening was carried out in three gradual stages of increasing pressure. In the first cultivation, a consortium of endospores from all 170 strains was used as the initial inoculum and the bioreactor was pressurized in a range between 20 and 60 bar. Sampling began on the fifth day and continued daily until the twenty-first and in all of them it was possible to observe the presence of vegetative cells and endospores, which were confirmed as viable cells after colony counting (Figure 1).

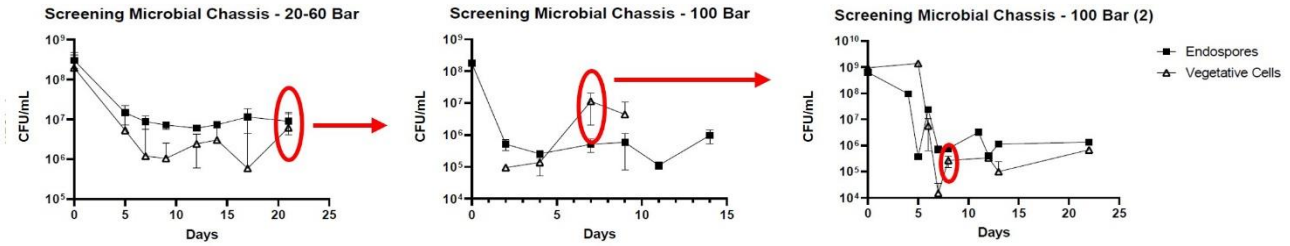

Figure 1. Microbial growth curve under supercritical CO_2 conditions. The red circle represents the microbial sample used to start the new fresh passage and/or isolate resistant bacteria.

The bacterial strains that survived 21 days of pressure (20-60 bar) were multiplied under ideal conditions and subjected to new cultivation but under supercritical CO_2 conditions (100 bar). With a previously enriched population, the growth curve showed a cell multiplication phase between days 3 and 7, which had not been observed before suggesting a cellular multiplication. The culture was maintained until complete sporulation on 14th day.

In the last enrichment step, the microorganisms isolated at the maximum growth peak (7 days, cultivation 2) were multiplied and challenged to the same previous conditions (100 bar). Once again, it was possible to identify a peak in cell growth between days 5 and 7, declining until the 21st day. To identify bioproducts secreted on the supernatant of mineral medium with limitation of oxygen. Axenic cultures were isolated from sampling points, 40 from day 2 (C2.1-C2.40), 3 from day 7 (C7.1-C7-3), 5 from day 8 (C8.1-C8.5) and 10 from day 9 (C9.1-C9-10), totaling 58 strains. Morphological analysis was carried out by evaluating aspects of the colony such as color, brightness, shape, margin, elevation and size. In total, 9 groups were formed (figure 2) and, notably, all strains are spore-forming microorganisms.

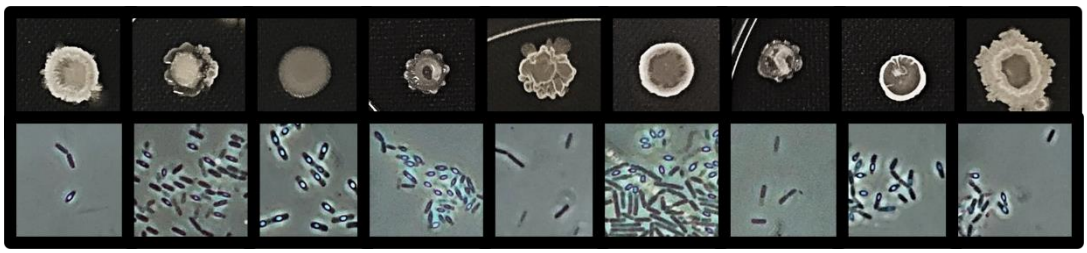

Figure 2. Nine groups with similar morphological characteristics.

To identify possible bioproducts formed in sCO_2, we initially evaluated the growth of these strains under oxygen-limited conditions. The culture supernatants were analyzed using liquid chromatography coupled to UV detectors at a wavelength of 214 nm, the detection range for organic acids. When overlapped, a diverse profile of products is observed among the bacterial strains (figure 3 illustrates the C2 strains). Among the available standards, we were able to identify a prevalence of lactic acid, acetic acid, formic acid and succinic acid as products.

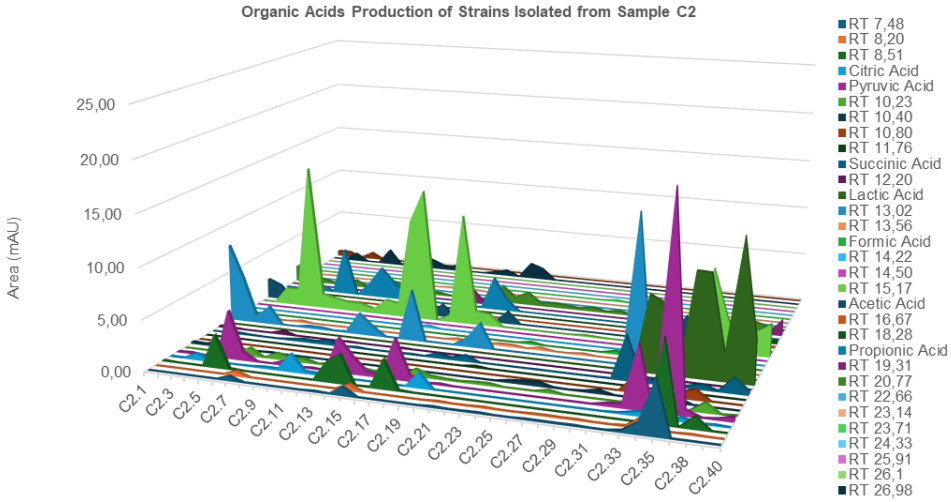

Figure 3. Bioproduct profile of strains isolated from sample C2. Analysis of area and retention time provided by HPLC (UV detection).

Among all the strains analyzed, one stood out for producing succinic acid and was studied in more detail for the development of a bioprocess. This bacterial strain, C2.31, has been shown to produce bioaromas that are soluble in sCO_2, such as acetoin and 2,3-butenediol. Future studies aim to associate biomass formation, production of these compounds and joint extraction methods in sCO_2.

4 CONCLUSION

There are bacteria capable of surviving in supercritical CO_2 conditions (35 °C, 100 bar) and can be used as a microbial chassis for the production of compounds soluble in carbon dioxide. Detailed studies of cultivation conditions and production levels must be carried out in the next stages of the study.

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