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PURPLE NON-SULPHUR PHOTOSYNTHETIC BACTERIA IN BREWERY WASTEWATER DURING ANAEROBIC DIGESTION

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ABSTRACT
Anaerobic digestion of brewery wastewater (BWW), under batch and constant light conditions, provided 70 mL of biogas with 80% CH_4 and a COD removal of 64%, corresponding to a yield of 372 m^3 biogas/kg COD removed. Additionally, a reddish pigmentation has developed throughout the process due to the growth of purple non-sulphur bacteria (PNSB). Based on morphological, physiological and cultural properties two strains of PNSB belonging to the genus *Rhodobacter* were isolated. The comparison of the nucleotide sequences of the 16S rRNA genes of the strains with the international databases (NCBI GenBank) made it possible to establish their taxonomical status as *Rhodobacter capsulatus* and *R. azotoformans* species.

Keywords: Brewery wastewater, anaerobic digestion, purple non-sulphur bacteria

INTRODUCTION
The brewery sector generates about 3–10 liters of high-polluted wastewater per each liter of produced beer [1]. The high pollution potential of these effluents is due to their organic load (sugars, soluble starch, ethanol and volatile fatty acids), suspended solids and the presence of nitrogen and phosphorus [2]. Anaerobic digestion (AD) has been used as a sustainable and environmentally friendly method for converting organic content waste into a renewable energy.
A ubiquitous group of anoxygenic photosynthetic microorganisms, the purple non-sulphur bacteria (PNSB), can be found in fresh, salt, acidic and basic waters, as well as in wastewaters [3]. The PNSB use various organic substances, including fatty acids, other organic acids such as succinate or malate, primary and secondary alcohols, carbohydrates and even aromatic compounds. Under phototrophic (anoxic/light) conditions, typical PNSB can grow under photoautotrophic conditions with H_2 or low levels of sulphide as electron donors [4, 5]. This biomass is not only rich in protein, fat and vitamin but also contains a significantly amount of useful carotenoids and biological co-factors [3, 6]. An increasing number of studies have been carried out to find antioxidant, anticancer, antimicrobial activities using microbial pigments. Researchers have been studying their potentiality for the health benefits in reducing the risk of cancer and neurodegenerative diseases [7].
This work offers to evaluate the brewery effluent treatment and isolate/characterize the population of anoxygenic purple bacteria that showed up in the liquid medium during the anaerobic process of BWW under lighting conditions. The main objective is to use the anaerobic digestion to treat the effluent and to produce biogas/methane and pigments, simultaneously, in a single technical step.

MATERIALS AND METHODS

Anaerobic digestion experimental set-up
Brewery wastewater (BWW) was collected from the *Sociedade Central de Cervejas e Bebidas brewery* (SCC, Vialonga, Portugal) after a primary treatment stage. Biological solids, collected from

an anaerobic digester plant (SIMLIS, Leiria, Portugal), were used as inoculum (I). The anaerobic digestion assay was carried out, under constant indoor light conditions, in glass vials of 71.5 mL total volume, using a substrate to inoculum ratio of 70%BWW+30%I (v/v). The experiment was conducted in triplicate, under mesophilic conditions (37±1ºC) for 34 days. A control assay was also provided using the same inoculum concentration but without substrate addition. During the anaerobic digestion process of BWW, the liquid medium became reddish and a sample was collected at the end of the experiment to further isolate microorganisms belonging to PNSB.

Analytical and chromatograph methods
Volatile solids (VS) and chemical oxygen demand (COD) were determined according to Standard Methods [8]. Biogas production was monitored daily and gas composition was analysed by gas chromatographic techniques according to ASTM Standard Method [9]. All gas volumes were adjusted to STP conditions (1 bar, 0°C).

Microbial isolation and Molecular identification
Microbial enrichment and isolation was performed using sterile Ormerod medium [10]. DNA isolation was carried out using benzyl chloride method [11]. To perform 16S rRNA gene amplification, 27F and 1492R universal primers were used [12, 13]. Sequencing of nucleotides of 16S rRNA gene amplified by PCR was determined in the «Macrogen» Inc. (Seoul, South Korea). Comparison of 16S rRNA nucleotide sequence was performed by BLAST algorithm (http:www.ncbi.nlm.nih.gov/blast). Phylogenetic analysis was performed using Geneious Prime (trial version) and MEGA 7 softwares [14, 15]. Alignment of nucleotide sequences was carried out using the ClustalW algorithm [16]. For the construction of phylogenetic trees, the Neighbor-Joining method was used [17]. Additionally, nucleotide sequences of closely related species of the phylogenetic *Rhodobacter* group deposited in the NCBI GenBank were included in the analysis.

RESULTS AND DISCUSSION

Anaerobic digestion of BWW
BWW is a very diluted and acidic effluent with low concentration of organic materials (7 g/L COD and 1 g/L VS). The digested BWW gave a biogas volume 10-fold higher than the produced by control, reaching a production of about 70 mL at the end of the experiment. Methane mean contents of 80% were obtained in biogas with the exception of units digesting only inoculum, where methane content reached an average value of about 70%.

Isolation and molecular identification of PNSB
Two PNSB candidates were isolated from the BWW sample. Morphologically, one important characteristics of the PNSB is the reddish pigmentation, which indicated the presence of the photosynthetic pigments, as well as colony and cells morphology, color, growth characteristics, Gram staining, spore forming ability and mobility (Figure 1).

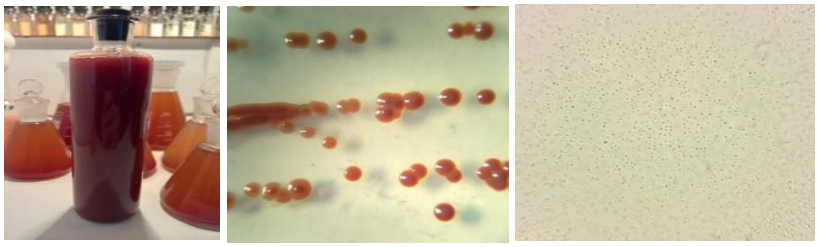

Fig. 1, Cultural peculiarities and morphology of red isolates of photosynthetic bacteria: growth in liquid medium, growth on agar, bacterial cells under microscope (1500x)

The obtained phylogenetic tree of sequences of 16S rRNA of the «unknown(Query_68871)» with 56% reliability form a single cluster and possess 97% sequence similarity with the corresponding nucleotide sequence of the *Rhodobacter capsulatus* strain XJ-1 (Accession number HM370064.1) from GenBank, and the query coverage is 97%. In a phylogenetic analysis of the fragment of the 16S rRNA gene, the «unknown(Query_68871)» was combined into one cluster with *R. capsulatus.*

The obtained phylogenetic tree of sequences of 16S rRNA of the strain «unknown(Query_158051)» with 95% reliability form a single cluster and possess 93% sequence similarity with the corresponding nucleotide sequence of the *Rhodobacter azotoformans* strain YLK20 (Accession number MG576210.1), and the query coverage is 98%. The analysis of the nucleotide sequences of the 16S rRNA of «unknown(Query_158051)» confirmed the belonging of the test strain to the species of *R. azotoformans*.

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