Journal of Environmental Management 270 (2020) 110825

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: http://www.elsevier.com/locate/jenvman

Research article

Effect of dibenzothiophene and its alkylated derivatives on coupled desulfurization and carotenoid production by *Gordonia alkanivorans* strain 1B

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ARTICLE INFO

Keywords:
Biodesulfurization
Dibenzothiophene and its alkylated derivatives
Gordonia alkanivorans strain 1B
Sulfur/carbon sources
Bacterial carotenoids

ABSTRACT

Nowadays, the production of green transportation fuels is essential for a healthy life and environment. Effective and complete removal of organosulfur recalcitrant compounds from fuel oils is crucial to meet the stringent requirements of sulfur standards. However, the industry's solution (Hydrodesulfurization, HDS) is not effective in the removal of complex sulfur heterocyclic hydrocarbons. Thus, the development of more efficient and eco-friendly/sustainable desulfurization methods is critical, as either an alternative or a complement to HDS, foreseeing the production of ultra-low sulfur fuels (ULSF). Among the desulfurization techniques available, microbial desulfurization of organosulfur hydrocarbons (biodesulfurization, BDS) is attracting great attention. BDS is carried out at mild operation conditions, making it energetically cheaper and more ecofriendly, since it does not require hydrogen and produces far less greenhouse gases emission than HDS. In this context, the behavior of *Gordonia alkanivorans* strain 1B, a desulfurizing bacterium and hyper-pigment producer, was evaluated in the presence of four sulfur sources common in fuel oils: dibenzothiophene (DBT); 4-mDBT; 4,6-dmDBT and 4,6-deDBT (single/mixed), in terms of both desulfurization rate and overall carotenoid production. Simultaneously, the influence of the carbon source used (fructose *vs* glucose) on the overall effectiveness of the coupled bioprocesses was also assessed. The results obtained highlight the potential of strain 1B to desulfurize all the tested recalcitrant compounds and simultaneously produce carotenoids. However, the highest BDS values were observed for 4,6-deDBT (5.75 μmol/g (DCW)/h) and for the mix of DBTs (5.20 μmol/g (DCW)/h), when fructose was used as carbon source. Indeed, when the mixture of DBTs ("model oil surrogate") was desulfurized by cells growing in fructose both desulfurization rate and total pigments amount were higher than those observed for glucose growing cells. Moreover, under these conditions, the strain 1B was able to produce high added-value carotenoids, namely astaxanthin, lutein and canthaxanthin. Hence, these results are promising when aiming to achieve a scale-up scenario. In fact, the inclusion of the production of high added-value products within a BDS process targeting ULSF may be a sustainable way to turn its scale-up economically viable.

1. Introduction

The use of fossil fuels results in the release of several pollutants to the atmosphere, of which the most known and widely studied is carbon dioxide. However, there are other equally harmful compounds that have deserved less attention, for example, the sulfur oxides. These are responsible for phenomena such as acid rain, which causes large damage to crops and buildings, as well as some types of cancer and respiratory diseases (Burns et al., 2016; Koenig, 2016; Lelieveld et al., 2015; Li et al., 2017).

To mitigate this problem, legislation was created to limit the maximum sulfur concentration allowed on transportation fuels. Currently, in order to comply with the stringent regulation of sulfur (S) level ≤10 ppm (EEA, 2019), refineries use physicochemical treatments, such as hydrodesulfurization (HDS), which combines high temperatures and pressures to remove the sulfur from fuels. This process requires large amounts of energy and the use of expensive metal catalysts leading to a greater carbon footprint and waste production (Naziri et al., 2014; Paixão et al., 2020). Moreover, HDS has reduced efficiency when dealing with complex sulfur molecules such as dibenzothiophene (DBT),

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https://doi.org/10.1016/j.jenvman.2020.110825
Received 6 March 2020; Received in revised form 14 May 2020; Accepted 22 May 2020
0301-4797/

and its derivatives, which have ethyl and methyl groups, such as 4-methyl dibenzothiophene, 4,6-dimethyl dibenzothiophene and 4, 6-diethyl dibenzothiophene (4-mDBT, 4,6dmDBT and 4,6-deDBT). These are very abundant in oil and especially recalcitrant, since they possess aromatic rings and alkyl groups which protect the sulfur atom, making its removal more difficult (Alves et al., 2015; Kampouraki et al., 2019).

Biodesulfurization is an alternative/complementary process of removing sulfur from fuels with the help of microorganisms. This technique, if correctly employed, can remove the sulfur from the most complex recalcitrant molecules, at ambient conditions, without the need for metal catalysts. This is one of the greatest advantages of biodesulfurization, however it does have several limitations. If the bacterial cells are exposed to multiple sulfur sources, which commonly occurs in oil, these can inhibit each other, leading to reduced desulfurization activity (Chen et al., 2008). So, when choosing a microorganism for biodesulfurization, it is important to assess its ability to desulfurize multiple compounds simultaneously.

Another problem resides on the cost of producing the biocatalyst, which, as in many other microbial dependent processes, represents a large percentage of the total costs. Different approaches have been taken to solve this problem, such as, increasing the activity of the biocatalysts by genetic manipulation; use alternative carbon sources; and use minimized culture media (Pacheco et al., 2019; Paixão et al., 2020). Another alternative for costs reduction is to exploit the production of secondary metabolites with high value-added, such as carotenoids.

Carotenoids are naturally occurring molecules, responsible for the red, orange, pink and yellow colors of many plants, animals and some microorganisms. Typically, they are composed of a 40-carbon linear skeleton, to which different modifications are applied to generate the several carotenoids and colors known. These modifications may include cyclization, or additions of functional groups (Britton, 1995). Depending on these groups, carotenoids can be divided into carotenes, if they are purely hydrocarbon, or xanthophylls if they contain, oxygen (Bhosale and Bernstein, 2005). Carotenoids are synthesized to fulfill important physiological functions (e.g. accessory light-harvesting on photosynthetic systems, antioxidant and photoprotective properties and regulation of membrane fluidity). Considering their properties, carotenoids have various industrial applications as dyes (textile, cosmetics, and fine chemical sectors), and due to their several beneficial effects for health, they have been exploited by the food and nutraceutical industries and recently, by the pharmacological industry (Cervantes-Paz et al., 2016; Esteban et al., 2015; Novoveská et al., 2019; Stange, 2016; Venil et al., 2014). Indeed, there is a vast market for carotenoids, which results in an estimated market value of about $2.0 billion in 2022 (McWilliams, 2018). For these reasons, there has been an increasing interest in finding new sources of carotenoids with novel characteristics applicable in commercial areas.

Gordonia alkanivorans strain 1B is a well-known desulfurizing bacterium with several works demonstrating its behavior with different commercial and alternative carbon sources (Alves et al., 2015, 2008; Alves and Paixão, 2014a; Paixão et al., 2016; Silva et al., 2013), as well as describing the costs associated with its production and further integration in a petroleum refinery (Alves et al., 2015). Nevertheless, recently, strain 1B was also described as a hyper-producer of carotenoids, namely astaxanthin, canthaxanthin and lutein, depending on the culture conditions (Fernandes et al., 2018; Silva et al., 2016). These works highlighted that the use of sulfate ($NaSO_4$) as sulfur source (S-source) significantly induces higher carotenoid production than DBT; however, it is also known that it greatly inhibits desulfurization, even at residual concentrations (Silva et al., 2013). So, in order to integrate carotenoid production within a fossil fuel desulfurization process, it becomes necessary to search for better S-sources.

In this context, based on the knowledge on the complex S-sources usually present on crude oil, the goal of this study was to evaluate the behavior of *G. alkanivorans* strain 1B in the presence of different S-sources: DBT, 4-mDBT, 4,6-dmDBT and 4,6-deDBT (single/mixed), in terms of both desulfurization rate and overall carotenoid production. Simultaneously, the influence of the carbon source (C-source) used (fructose vs glucose) on the overall effectiveness of the coupled bioprocesses was also assessed.

2. Materials and methods

2.1. Chemicals

DBT (99%) was obtained from Acros Organics, 2-hydroxybiphenyl (2-HBP) from Sigma and dimethylformamide (DMF) from Riedel-de-Haën. 4-mDBT (96%), 4,6-dmDBT (97%) and 4,6-deDBT (97%) were from Aldrich Chem. Co. The HPLC standards for astaxanthin (98%) and β-carotene (95%) were from Sigma, canthaxanthin (99%) was from Roche and lutein (90%) was from FloraGLO, Kemin. All other reagents were of the highest grade commercially available.

2.2. Microorganism and culture media

Gordonia alkanivorans strain 1B (Alves et al., 2005) was the desulfurizing bacterium used in this study and it is kept at a Culture Collection of Microorganisms (CCM at Bioenergy Unit/LNEG, Portugal, Lisbon). For the biodesulfurization (BDS)/carotenoid production assays, the microorganism was cultivated/maintained in sulfur-free basal salts medium as described in Alves and Paixão (2014b) and Silva et al. (2016). Filter sterilized fructose or glucose solutions (50% w/v) were added to the culture medium to an initial concentration of about 10 g/L, as the only carbon source. Stock solutions of the different S-sources (DBT; 4-mDBT; 4,6-dmDBT and 4,6-deDBT) were prepared in DMF with a concentration of 150 mM and each was added to the sterilized culture medium to obtain an initial concentration of 320 μM of S-source. A mixture of the four S-sources was also tested (to mimic a fuel oil composition ⇒ “model oil surrogate”), in which 80 μM of each compound was added to the culture medium. These concentrations were chosen to ensure excess of S-source.

Bacterial cultures were performed in 500 mL Erlenmeyer shake-flasks with 150 mL of culture medium, $pH_{initial} = 7.5$, in an orbital incubator (Unitron CH-4103, Infors AG, Bottingen, Switzerland), at 30°C, with a photoperiod of 13–14 h of light, and 150 rpm agitation. All the conditions assayed were performed in triplicates.

2.3. Carotenoids extraction

At the end of each set of assays, the remaining bacterial biomasses were harvested and separated from the supernatant through centrifugation (8600 g at 4°C, 20 min), after which they were stored at −20°C, kept from the light, until the pigment extraction was performed. Before the extraction, cells were slowly defrosted at 4°C, after which they were spread in glass petri dishes, no more than 5 g (wet weight) each, and set in an oven at 55°C, protected from the light, to reduce the water content to less than 60%. Then the pigments were extracted according to the procedure described in Fernandes et al. (2018) and Silva et al. (2016). After extraction, the samples were stored at −20°C, protected from light, in order to prevent carotenoid degradation until analysis.

2.4. Analytical methods

Cell growth was monitored by measuring dry cell weight (DCW), optical density at 600 nm (OD_{600}) and sugar consumption (fructose/glucose) using High Performance Liquid Chromatography (HPLC) as described by Fernandes et al. (2018).

Desulfurization was evaluated by measuring the end-product of the desulfurization of the different sulfur compounds. 2-HBP production was compared to a 2-HBP standard, while the concentrations of the other desulfurized DBT derivatives were calculated by comparison with the respective DBT. Extraction of these compounds was performed with ethyl acetate according to Silva et al. (2016). Samples were analyzed by gas chromatography (GC) in a gas-chromatograph (Model CP9001, Chrompack, Middelburg, The Netherlands) equipped with a flame ionization detector. An Alltech 10% SE-30 on 80/100 Chromosorb W-HP column was used with nitrogen as the carrier gas. The injector and detector temperatures were set for 250°C and 290°C, respectively. Depending on the compound analyzed different GC conditions were used. For DBT and 2-HBP the chromatograph oven start temperature was 210°C for 2 min and the end temperature 240°C maintained for 2 min (heating rate 10°C/min); 4-mDBT was used as internal standard to minimize variations. For 4-mDBT oven start temperature was 210°C for 4 min and the end temperature 250°C maintained for 2 min (heating rate 10°C/min), 4,6-dmDBT was used as internal standard. For 4,6-dmDBT oven start temperature was 210°C for 4 min and the end temperature 260°C maintained for 2 min (heating rate 10°C/min); 2-HBP was used as internal standard. For 4,6-deDBT oven start temperature was 210°C for 3 min and the end temperature 260°C maintained for 3 min (heating rate 10°C/min); 2-HBP was used as internal standard. When the mixture of the 4 compounds was tested, oven start temperature was 120°C for 3 min and the end temperature 260°C maintained for 3 min (heating rate 8°C/min); no internal standard was used.

For carotenoid characterization, the amount of total carotenoids extracted from each biomass was assessed by spectroscopy as described by Fernandes et al. (2018). The concentration of total carotenoids was calculated with the Lambert–Beer equation accordingly to Nobre et al. (2006), using the value of 2091.4 L/10 g/cm for the specific optical extinction coefficient at $\lambda = 477$ nm (wavelength of the maximum absorbance of canthaxanthin in ethyl acetate). Specific carotenoid identification and quantification was performed using HPLC, as described in detail by Silva et al. (2016). The pigment results are presented as percentage of mass per mass [% (g/g)], which correspond to g of carotenoid per 100 g of DCW. Results are the mean value of duplicates.

3. Results and discussion

3.1. *Bacterial growth and desulfurization: influence of S/C-sources*

G. alkanivorans strain 1B is a bacterium known to have diverse metabolic responses when exposed to different carbon and sulfur sources. Depending on its growth conditions, it has shown changes in growth rates, DBT desulfurization rate and carotenoid production. For example, the use of fructose as the carbon source results in higher growth and desulfurization rates (Alves and Paixão, 2014b), while the use of glucose benefits carotenoid production (Fernandes et al., 2018; Silva et al., 2016).

Most BDS studies with strain 1B rely on the use of a single S-source, mostly DBT; however, crude oil or its derivatives are rich in many different organosulfur compounds, some more recalcitrant than others. Thus, in order to evaluate how strain 1B responds to different complex sulfur sources, two sets of assays were designed with fructose or glucose as carbon source. The bacteria were grown with progressively more recalcitrant sulfur sources: DBT, 4-mDBT, 4,6-dmDBT, 4,6-deDBT (Alves et al., 2015; Mochida and Choi, 2004), and a mixture of the four different organosulfur compounds in equal parts.

Fig. 1 represents the time course profiles of the growth (Fig. 1A), sugar consumption (Fig. 1B) and desulfurization (Fig. 1C) of strain 1B using ≈10 g/L fructose, as the single carbon source (C-source) and 320 μM of S-source either DBT, 4-mDBT, 4,6-dmDBT, 4,6-deDBT, and an equimolar mixture of the four at 80 μM each. Table 1 summarizes several metabolic parameters determined for each tested condition.

In all conditions, strain 1B was able to grow (Fig. 1A), desulfurize (Fig. 1C) and fully consume the supplied fructose (Fig. 1B), but at different rates. The bacterial culture using 4,6-dmDBT started growing at 19 h, followed by the cultures using 4,6-deDBT or DBT, at 22 h. The results in Fig. 1A and Table 1 point out, that among the cultures using a single S-source, the culture with 4,6-deDBT presented the fastest growth and one of the highest growth rates (0.084 h^{-1}), attaining an OD_{600} of ≈15 within 84 h, with total fructose consumption. The growth profiles of the cultures with DBT and 4,6-dmDBT were similar to each other, ending their growth phase at about 90 h, with an OD_{600} of 13 and 12.3, respectively. However, the cells grown with DBT presented a higher growth rate (0.055 h^{-1}). In the assay using the mixture of DBTs, strain 1B presented a growth profile with a longer lag phase (up to 65 h) but attaining the highest growth rate ($\mu = 0.088$ h^{-1}) during its exponential phase. At the end of exponential phase (90 h), the growth slowed considerably, achieving a maximum OD_{600} of 12.9 after 157 h. At last, the bacterial growth using 4-mDBT as the sole S-source, presented the longest lag phase, with the exponential phase only starting within 96–119 h, with a growth rate of 0.027 h^{-1}. Complete carbon consumption was achieved within 180 h, with a maximum OD_{600} of 12.7.

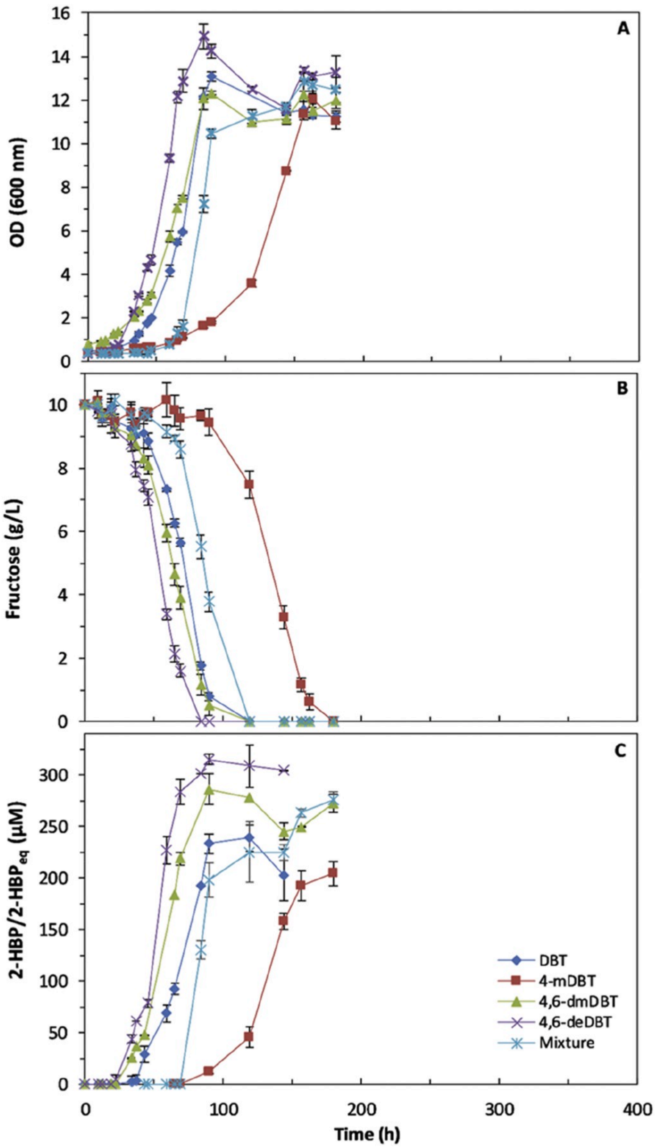

Fig. 1. Time course profiles for cellular growth (A), sugar consumption (B) and desulfurization curves (2-HBP or 2-HBP equivalent (eq) production), (C) of *G. alkanivorans* strain 1B in the shake-flask cultures in the presence of 10 g/L fructose as C-source, but different DBTs as S-source (320 μM), namely: DBT, 4-mDBT, 4,6-dmDBT, 4,6-deDBT and an equimolar mixture of these four organosulfur compounds. Standard deviation (n = 3) is represented as error bars.

The use of different DBTs (DBT or its derivatives), as S-source,

Table 1

Metabolic parameters for the desulfurization of different DBTs (DBT and its derivatives) as S-source by *G. alkanivorans* strain 1B, when grown in the presence of 10 g/L fructose as C-source.

Metabolic parameters	Fructose				
	DBT	4-mDBT	4,6-dmDBT	4,6-deDBT	Mix[a]
µ, Growth rate (h^{-1})	0.055	0.027	0.040	0.084	0.088
Maximum sugar consumption rate (g/L/h)	0.26	0.17	0.23	0.28	0.30
Maximum 2-HBP/2-HBP_{eq}[b] production (µM)	238.71	204.18	286.04	315.09	275.80
$q_{2\text{-}HBP}$, 2-HBP specific production rate (µmol/g (DCW)/h)	3.95	3.68	4.15	5.75	5.20

[a] Mixture of DBTs (DBT, 4-mDBT, 4,6-dmDBT and 4,6-deDBT).

[b] 2-HBP_{eq}: 2-HBP equivalent.

resulted in different desulfurization profiles as illustrated in Fig. 1. For 4,6-deDBT, the desulfurization started at 22 h, achieving the maximum concentration of the respective 2-HBP (2-HBP_{eq} = 315 µM) at 89 h, with the highest 2-HBP specific production rate ($q_{2\text{-HBP}}$ = 5.75 µmol/g (DCW)/h). The desulfurization of 4,6-dmDBT started at 33 h and finished at 89 h, producing 286 µM of 2-HBP_{eq}, with a $q_{2\text{-HBP}}$ of 4.15 µmol/g (DCW)/h. In the culture with DBT as the sole S-source, the desulfurization started at 33 h and attained its maximum within 89–120 h, with a 2-HBP production of 239 µM and the correspondent $q_{2\text{-HBP}}$ of 3.95 µmol/g (DCW)/h. The desulfurization profiles in the cultures with the mixture of DBTs or 4-mDBT presented longer lag phases. For the mixture of DBTs, strain 1B started desulfurization within 69–84 h achieving a combined $q_{2\text{-HBP}}$ of 5.2 µmol/g (DCW)/h, the second highest. After 89–119 h, when the C-source was depleted, the desulfurization of the remaining S-source continued up to 180 h, but at a much lower rate, attaining a combined 2-HBP production of 276 µM. Finally, for the growth assay with 4-mDBT the desulfurization by strain 1B was only observed after 89 h. In spite of a slower initial rate, the desulfurization rate increased to a $q_{2\text{-HBP}}$ of 3.68 µmol/g (DCW)/h, reaching a maximum of 204 µM of 2-HBP.

Fig. 2 represents the time course profiles of growth (Fig. 2A), sugar consumption (Fig. 2B) and desulfurization (Fig. 2C) of the strain 1B using ≈10 g/L glucose, as the single C-source and 320 µM of sulfur source either DBT, 4-mDBT, 4,6-dmDBT, 4,6-deDBT, and an equimolar mixture of the four at 80 µM each. Table 2 summarizes several metabolic parameters determined for each tested condition.

Once again, *G. alkanivorans* strain 1B was able to grow and desulfurize under all conditions assayed. In terms of growth profiles, Fig. 2A clearly shows different behaviors depending on the S-source tested, with a considerable lag phase (>4 days) observed under all conditions. The cultures grown with DBT or 4,6-deDBT as S-source achieved the highest values in terms of maximum OD_{600} observed within 16 days, 9.25 and 8.46, respectively, with growth rates of 0.019 h^{-1} and 0.018 h^{-1}, respectively. Moreover, only in the presence of these two S-sources a full consumption of glucose was observed within the time of the assays, namely at 312 h for DBT and 16 days for 4,6-deDBT (Fig. 2B). Considering the growth profiles obtained for 4-mDBT and 4,6-dmDBT, it can be stated that after a lag phase of about 96 h, the exponential growth started and the cultures achieved a maximal OD_{600} of 4.8 and 7, respectively. However, while an overall growth rate of 0.011 h^{-1} can be observed for the culture with 4,6-dmDBT along the whole exponential growth (96–264 h), in the case of the growth with 4-mDBT two growth rates can be distinguished: 0.014 h^{-1} (96–192 h) and 0.004 h^{-1} (216–336 h), but a mean value of 0.009 h^{-1} can be considered for the global growth. Indeed, this reduction of the bacterial growth in 4-mDBT is supported by the low consumption of glucose (Fig. 2B, 0.013 g/L/h), still remaining 6.2 g/L after 384 h. At last, the growth curve translating the behavior of strain 1B in the presence of the mixture of DBTs demonstrates that after a very long lag phase (240 h), the cells initiated a sharp exponential phase. The corresponding growth rate was 0.019 h^{-1}, similar to that for DBT when used as single S-source. At the end of the assay, after 384 h, a maximum OD_{600} of 6.3 was attained, but without complete consumption of the carbon source, clearly indicating the possibility of further growth.

In terms of desulfurization, the culture grown with 4,6-deDBT presented the highest rate, with a $q_{2\text{-HBP}}$ of 2.77 µmol/g (DCW)/h, reaching 266 µM of 2-HBP_{eq} by the end of the assay. The cultures grown with DBT

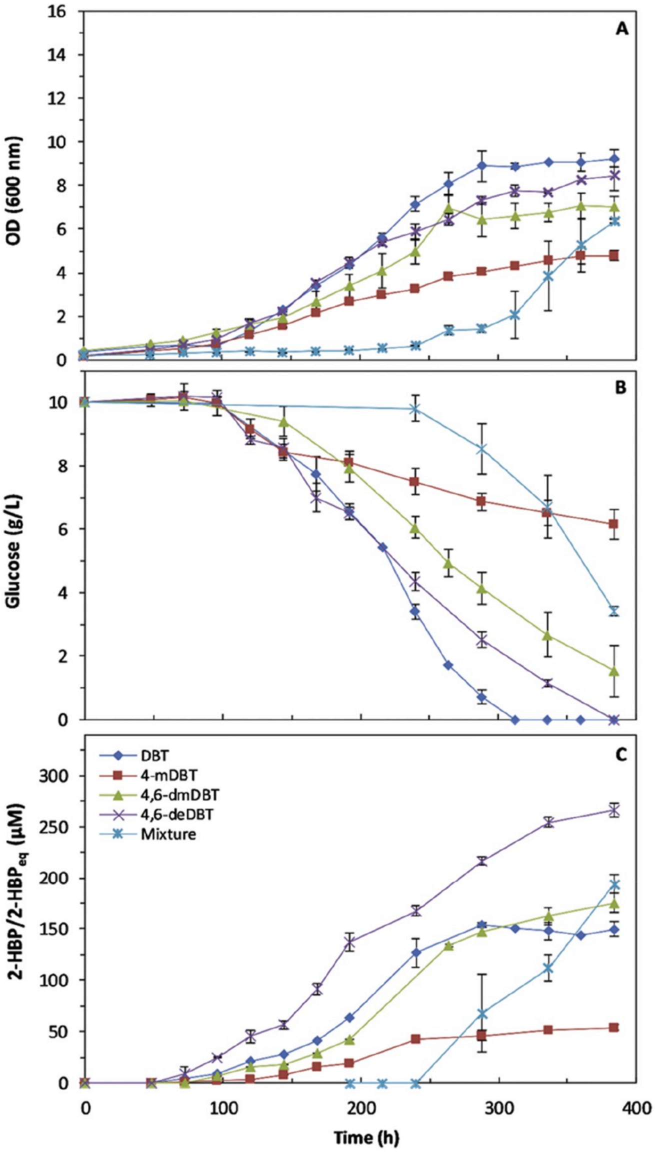

Fig. 2. Time course profiles for cellular growth (A), sugar consumption (B) and desulfurization curves (2-HBP or 2-HBP equivalent (eq) production), (C) of *G. alkanivorans* strain 1B in the shake-flask cultures in the presence of ≈10 g/L glucose as C-source, but different DBTs as S-source (320 µM), namely: DBT, 4-mDBT, 4,6-dmDBT, 4,6-deDBT and an equimolar mixture of these four organosulfur compounds. Standard deviation (n = 3) is represented as error bars.

Table 2
Metabolic parameters for the desulfurization of different DBTs (DBT and its derivatives) as S-source by *G. alkanivorans* strain 1B, when grown in the presence of 10 g/L glucose as C-source.

Metabolic parameters	Glucose				
	DBT	4-mDBT	4,6-dmDBT	4,6-deDBT	Mix[a]
μ, Growth rate (h^{-1})	0.019	0.009[b]	0.011	0.018	0.019
Maximum sugar consumption rate (g/L/h)	0.083	0.013	0.047	0.065	0.068
Maximum 2-HBP/2-HBP_{eq}[c] production (μM)	153.58	53.98	175.35	266.39	193.82
$q_{2\text{-}HBP}$, 2-HBP specific production rate (μmol/g (DCW)//h)	1.53	0.74	1.28	2.77	1.56

[a] Mixture of DBTs (DBT, 4-mDBT, 4,6-dmDBT and 4,6-deDBT).

[b] Mean value of the two growth rates exhibited by the culture (0.014/0.004 h^{-1}).

[c] 2-HBP_{eq}: 2-HBP equivalent.

and the mixture of DBTs presented similar $q_{2\text{-HBP}}$ (1.53 and 1.56 μmol/g (DCW)/h). With DBT, the cells started to desulfurize at 72 h and produced 154 μM of 2-HBP after 147 h. The cells grown in the mixture of DBTs started to desulfurize after 240 h and produced 194 μM of combined 2-HBP by the end of the assay. The cells grown with 4,6-dmDBT presented slightly lower results, starting desulfurization at 72 h, but taking 384 h to reach a final concentration of 175 μM of 2-HBP_{eq}. The 4-mDBT induced the lowest desulfurization rate (0.74 μmol/g (DCW)/h), with the cells reaching a maximum production of only 54 μM of 2-HBP_{eq}, by the end of the assay.

Comparing the results obtained for both C-sources, the fructophilic behavior of the bacterium is clearly evidenced, as expected (Alves and Paixão, 2014b). In fact, from Figs. 1A and 2A, it is possible to see much longer lag phases, and overall lower growth rates in glucose-based growths. As highlighted in Tables 1 and 2, from fructose to glucose set of assays there was a great reduction in all growth rates (55–78%). In fact, the overall growth time necessary for the cultures with fructose was less than half of that for the cultures with glucose. While with fructose the slowest growth took about 163–180 h (Fig. 1A, 4-mDBT), for glucose assays, three of the five cultures did not finish growing within 384 h, and could thus benefit from more time.

Within each C-source set of assays, the comparison of the results for growth and desulfurization for DBT and 4-mDBT (Tables 1 and 2; Figs. 1 and 2) clearly demonstrates the negative influence of the extra methyl group (-CH_3). The presence of this methyl group induced a significant reduction in terms of both growth rate and desulfurization ability (2-HBP produced; $q_{2\text{-HBP}}$). Indeed, the 4-mDBT was the most recalcitrant S-source tested, independently of the C-source used, for which the lowest desulfurization/growth values were obtained.

In contrast, when a second methyl group was present in the organosulfur compound used as S-source, as in the case of 4,6-dmDBT, the bacterial behavior was very different from that for 4-mDBT. In overall, higher rates for both growth and desulfurization were observed and, the maximum levels of the 2-HBP_{eq} produced were even higher than those obtained for DBT desulfurization (Figs. 1C and 2C; Tables 1 and 2). This behavior contrasts to what was observed using *Mycobacterium* sp. ZD-19 in which the specific desulfurization rate for 4,6-dmDBT was almost 3 times lower than that of DBT (Chen et al., 2008). In fact, *G. alkanivorans* strain 1B presented higher $q_{2\text{-HBP}}$ for both compounds highlighting its advantage as desulfurizing microorganism.

Moreover, when instead of two methyl groups (-CH_3), the S-source presented two ethyl groups (-CH_2CH_3), as the 4,6-deDBT, a pronounced enhancement of both growth and desulfurization rates was observed, independently of C-source used (Tables 1 and 2). In fact, in terms of single S-source tested, the 4,6-deDBT when combined with fructose, as C-source, resulted in the highest rates of growth, desulfurization and sugar consumption. However, when glucose was used as C-source, although the highest values for growth and sugar consumption were obtained with DBT, the desulfurization was much higher for the 4,6-deDBT. Thus, in both C-sources, the desulfurization rates of 4,6-deDBT and maximum 2-HBP_{eq} concentrations were the highest amongst the conditions tested (Tables 1 and 2).

Hence, in overall, these results seem to indicate that *G. alkanivorans* strain 1B benefits from the double methylation, and especially the double ethylation of the DBT molecule. Contrary to the expectations, the additional complexity and extra layers of protection of the sulfur atom, given by the two ethyl groups, stimulated both desulfurization and growth. Indeed, this effect could be due to several causes, namely: the different compounds could present different levels of microbial toxicity; the bacterium could be using different metabolic pathways for the more complex compounds; or there could be a higher affinity of the more complex compounds with the existent transport proteins. Very little is known on this field, however, there is some evidence that the uptake of DBT and its alkyl-derivatives may depend on active transport (Wang et al., 2011), as such, the affinity of the transporter proteins towards the compound could greatly influence its uptake and, consequently, its conversion rates and resulting bacterial growth.

Finally, when an equimolar mixture of DBTs (DBT and its three alkylated derivatives) was used as S-source for strain 1B growth (fructose/glucose), a long lag phase was observed (of 69 h/240 h, for fru/glu respectively), followed by high rates of both growth and desulfurization. After the prolonged adaptation period to the multiple DBTs, probably to synthesize the necessary BDS enzymes, the strain 1B attained the highest growth rates (0.088 h^{-1}/0.019 h^{-1} for fru/glu, respectively) and the second highest desulfurization rates (5.20 μmol/g (DCW)/h/1.56 μmol/g (DCW)/h for fru/glu, respectively) within each set of assays (Tables 1 and 2). This bacterial behavior is very advantageous since it indicates that the BDS process has a higher specificity than the HDS process. Indeed, strain 1B exhibited excellent results under conditions that are known to hinder the traditional physicochemical process.

3.2. *Carotenoid production*

3.2.1. *Total carotenoid analysis through spectrophotometry*

The next step was to analyze the overall carotenoid content present

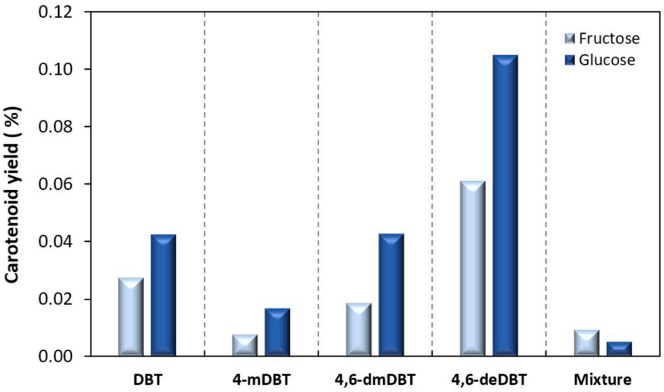

Fig. 3. Amount of total carotenoids (%, *i.e.* g/100 g DCW), assessed through spectrophotometry analysis, produced by *G. alkanivorans* strain 1B in the set of growth assays with different S/C-sources. (Note: Mixture = equimolar blend of DBT, 4-mDBT, 4,6-dmDBT and 4,6-deDBT).

on the extracts from the biomass samples of each condition tested, using an UV–Vis spectrometer (Shimadzu spectrophotometer UV-2401PC). The scanning profiles obtained revealed the presence of carotenoids with a maximum OD level at 472–475, depending on test conditions. Fig. 3 shows the carotenoid production (% *i.e.* g of total carotenoids per 100 g of DCW) and the influence of the culture conditions (S/C-sources) on the total pigment concentration. These results highlight that *G. alkanivorans* strain 1B produced the highest carotenoid concentration when cultivated with glucose and 4,6-deDBT (0.10%), followed by fructose and 4,6-deDBT, which is 40% lower (0.06%), and glucose with 4,6-dmDBT/glucose with DBT, which is 60% lower (both 0.04%). The biomass with the lowest carotenoid concentration was also obtained with glucose, when combined with the mixture of DBTs (0.005%), followed by the cultures obtained with fructose and 4-mDBT and fructose with the mixture of DBTs (0.008% and 0.009%, respectively).

These results are in accordance with previous data, described by Silva et al. (2016), obtained with single S-sources, in which the bacteria showed a clear increase in carotenoid production when glucose was used as C-source. However, this behavior seems to revert in the presence of the multiple complex S-sources (mixture of DBTs), in which case the fructose grown cells achieved a higher carotenoid concentration (0.005% in glucose *versus* 0.009% in fructose; Fig. 3). Contrary to what was observed in glucose, strain 1B was able of fully desulfurize the mixture of DBTs and consume all the C-source when grown in fructose. This fact may have contributed to the overall carotenoid content into biomass.

As stated before, when using multiple S-sources, the bacterial culture may need to produce multiple enzymes and/or transporters, which might result in a need to divert carbon that could otherwise be used in carotenoid production. Furthermore, Fernandes et al. (2018) and Silva et al. (2016) demonstrated that carotenoid production continues, and can even be induced after bacterial growth has ended and the C-source has been depleted. If the time of assay was extended, after the end of the growth phase, it might be possible to observe an increase in carotenoid concentration in both cultures grown with the mixture of DBTs.

In previous works, the light source was also proven to greatly influence carotenoid production by *G. alkanivorans* strain 1B (Fernandes et al., 2018; Silva et al., 2016). However, in this study all the assays were performed without control of the photoperiod, and with no direct lighting, maintaining the usual conditions described for most of BDS assays. Silva et al. (2016) report that cells grown with fructose and DBT in the absence of light and subsequently exposed to 3000 lux of light for 72 h produced 0.027% of total carotenoids, which is similar to the results obtained in this study by strain 1B cells grown with photoperiod (0.028%). However, when comparing the performance of cells grown with glucose and DBT under 3000 lux of direct light, from the same study, with that from this work, it is possible to note a reduction in the total carotenoid concentration from 0.07% to 0.04%, similar to the carotenoid production in the dark (glu/DBT/dark = 0.04%; Silva et al., 2016). These results may point out to the importance of the nature of the light source, since it was the most significant change between both studies. With DBT, as S-source, lower concentrations of carotenoids could be easily reachable without direct lighting, as observed in the culture with fructose; however, higher concentrations might need to be induced by a stronger light source, permanent lighting or different wavelengths.

Fernandes et al. (2018) reported carotenoid yields of 0.26–0.31% (corresponding to 2596–3100 μg/g DCW) by *G. alkanivorans* strain 1B from cultures with glucose and Na_2SO_4 in presence of direct light (400–3000 lux). Thus, even the highest yield obtained in this study, 0.1% (with glucose and 4,6-deDBT), was significantly lower than that obtained with glucose and Na_2SO_4. However, this result is the highest achieved using a complex organosulfur compound, and it could probably be increased if direct lighting was applied.

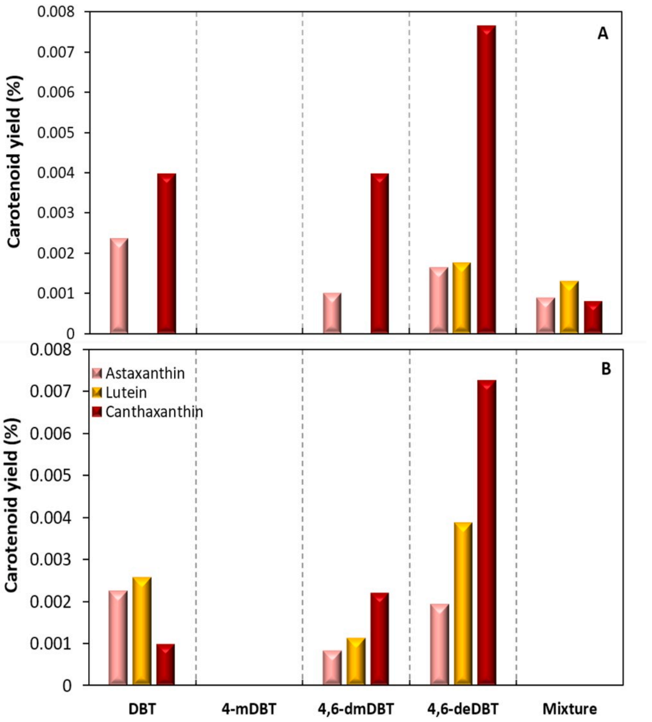

Fig. 4. Quantification of the main carotenoids (%, *i.e.* g/100 g DCW), through HPLC analysis, produced by *G. alkanivorans* strain 1B in the set of assays with different S-sources but using fructose (A) or glucose (B) as C-source. (Note: Mixture = equimolar blend of DBT, 4-mDBT, 4,6-dmDBT and 4,6-deDBT).

3.2.2. *Carotenoids characterization through HPLC analysis*

In order to identify the carotenoids present in each of the ten carotenoid extracts, they were further analyzed through HPLC, and the chromatograms obtained were compared with those for standards of astaxanthin, canthaxanthin, β-carotene and lutein. The results were interpreted based on retention times, peak profiles and bibliographic information. Fig. 4 describes the concentrations of the three carotenoids identified in the extracts from the biomasses grown with fructose (Fig. 4A) or glucose (Fig. 4B), with different S-sources. The results are shown in % (g of carotenoid per 100 g DCW) and translate the influence of S/C-sources, used during bacterial growth, in both nature and concentration of the carotenoids produced.

For extracts from biomasses grown in fructose as C-source (Fig. 4A), astaxanthin and canthaxanthin were detected in four of five conditions. The exception was for the extract from biomass obtained with 4-mDBT, which presented no identifiable carotenoids. Moreover, cells grown with 4,6-deDBT or the mixture of DBTs revealed the production of lutein (0.0018% and 0.0013%, respectively). The highest canthaxanthin production was detected for cells cultivated with 4,6-deDBT (0.008%), followed by DBT and 4,6-dmDBT grown cells, with half production (0.004%), and the cells grown with the mixture, with 10-fold less production (0.0008%).

When glucose was used as C-source to grow strain 1B (Fig. 4B), astaxanthin, canthaxanthin and lutein were detected in three conditions, but no identifiable carotenoids were detected when the bacterium was cultivated with 4-mDBT or the mixture of DBTs. Canthaxanthin presented the highest concentration (0.007%) in the biomass resulting from the use of 4,6-deDBT, followed by the biomass produced with 4,6-dmDBT (0.002%), less than a third, and the biomass grown with DBT (0.001%), seven times lower. Lutein also presented its highest concentration with 4,6-deDBT as the S-source (0.004%), with progressively lower concentrations when DBT or 4.6-dmDBT were used to grow the strain 1B (0.003% and 0.001%, respectively). Finally, astaxanthin was determined to have similar concentrations in biomasses from growths with DBT or 4,6-deDBT (0.0023% and 0.019%, respectively), and 0.0008% when *G. alkanivorans* strain 1B grew with 4,6-dmDBT.

In overall, Fig. 4 (A, B) points out for the 4,6-deDBT as the best S-source towards the highest amount of the three identifiable carotenoids (0.013%/0.011% with glu/fru, respectively). However, these yields represent only a small percentage of the total carotenoids produced (13.7%/22.9% with glu/fru, respectively; Fig. 3), with the large majority of the carotenoids still unidentified. The same is true for the rest of the extracts, especially for those from 4-mDBT grown cells (fru/glu) or mixture of DBTs grown cells (glu) in which no carotenoids were identified (Fig. 4 (A, B)).

Comparing these results with those from previous works (Fernandes et al., 2018; Silva et al., 2016), it can be observed that there are some variations in individual carotenoid concentration, which, as above referred, are most likely the result of the different light exposure. From the identifiable carotenoids produced using fructose, only astaxanthin benefited from the conditions tested in this study. Moreover, none of the test conditions assayed herein were able to produce biomass with higher pigment amount than that from the growth with glucose and Na_2SO_4, under permanent direct lighting.

Nevertheless, the overall results from this study highlight the potential of *G. alkanivorans* strain 1B to desulfurize several recalcitrant organosulfur compounds (single/combined), usually present in fuels, and simultaneously produce carotenoids. In fact, the inclusion of the production of high added-value products, such as carotenoids, within a BDS process aiming ultra-low sulfur fuels may be a sustainable way to turn its scale-up economically viable.

Carotenoids produced by microorganisms are nontoxic and the use of microorganisms (microalgae, yeasts, and bacteria) as pigment-producers have several advantages. Unlike plants, microorganisms have a short life cycle and are unaffected by the season/climate and geographical conditions, making it easier to produce carotenoids worldwide. Microorganisms can also produce color shades that are not found in plants, have high productivity, and are relatively easy to manipulate genetically towards improved fermentation and scale-up process. Moreover, microbial pigments are usually easy to extract (Calegari-Santos et al., 2016; Ramachandran et al., 2014; Venil et al., 2014). Thus, microbial pigments are in increasing demand, since they are a promising natural and safe alternative source for various industrial applications. To date, 1181 natural carotenoids have been described, of which 308 are produced by bacteria (Carotenoids database, 2019).

4. Conclusions

Currently, to meet the strict environmental legislation of the S-level, ≤10 ppm for transportation fuels, severe physicochemical treatment (deep HDS) of the recalcitrant organosulfur compounds present on fuels is usually required, which is associated with more environmental hazards and higher costs to the industrial process. The higher the complexity and number of additional alkylate groups in the organosulfur compound, the more protected the sulfur atom is from the action of the hydrogen atoms in the HDS process, and consequently harsher the treatment conditions must be in order to remove it.

In this scenario, BDS can emerge as an ecofriendly complementary technique. This study clearly highlights the potential of *G. alkanivorans* strain 1B to desulfurize several recalcitrant organosulfur compounds (single/combined), usually present in fuels, and simultaneously produce carotenoids. The highest BDS values were obtained in the presence of the most complex S-sources (4,6-deDBT: 5.75 μmol/g (DCW)/h and mix of DBTs: 5.20 μmol/g (DCW)/h), when fructose was used as C-source; however, in terms of total carotenoid yields, the best results, in general, were attained when glucose was used as C-source. Nevertheless, since the ultimate goal is to integrate BDS into a petroleum refinery, to improve the desulfurization of crude oil/fuels containing a mixture of organosulfur compounds, the best approach should be to use strain 1B growing cells in fructose. In fact, when the mixture of DBTs ("model oil surrogate") was desulfurized by cells growing in fructose both desulfurization rate and total pigments amount were higher than those observed for glucose growing cells. Moreover, in fructose/mix of DBTs culture condition, the strain 1B was able to produce high added-value carotenoids, namely astaxanthin, lutein and canthaxanthin, whose yields might be increased after optimization and under constant direct light.

In order to validate the promising results herein presented, demonstrating the industrial interest of *G. alkanivorans* strain 1B, further tests on real fuels must be carried out. Indeed, the inclusion of the production of high added-value products within a BDS process aiming ultra-low sulfur fuels may be a sustainable way to turn its scale-up economically viable.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Tiago P. Silva: Conceptualization, Investigation, Methodology, Data curation, Visualization, Writing - original draft. **Luís Alves:** Investigation, Methodology, Supervision, Validation, Writing - review & editing, Project administration, Funding acquisition. **Susana M. Paixão:** Investigation, Methodology, Supervision, Validation, Writing - review & editing, Project administration, Funding acquisition.

Acknowledgments

This work was financed by national funds through FCT (Fundação para a Ciência e a Tecnologia) in the scope of the project GreenFuel (PTDC/EAM-AMB/30975/2017). Tiago P. Silva also acknowledges FCT for his PhD financial support (SFRH/BD/104977/2014). In addition, this research was carried out within the Biomass and Bioenergy Research Infrastructure (BBRI) - LISBOA-01-0145-FEDER-022059, supported by Operational Programme for Competitiveness and Internationalization (P2020), by Lisbon Portugal Regional Operational Programme (Lisboa 2020) and by North Portugal Regional Operational Programme (Norte 2020) under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (ERDF).

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