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Optimization of high added-value pigments production by *Gordonia alkanivorans* strain 1B

Mestrado em Biologia Molecular e Genética

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"It's not about money... it's about sending a message."

Joker in *The Dark Knight* (2008)

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ABSTRACT

The actinomycetes of the genus *Gordonia* have been continually gaining interest, mainly due to their ability to degrade a variety of xenobiotics, environmental pollutants between others. They also have the capacity of synthesising organic compounds with commercial interest such as carotenoids.

Carotenoids are high added-value compounds, due to their wide application especially as colorants, food additives and also, considering some of their characteristics (antioxidant activity, scavenging, photoprotection, between others), they can be used as bioactive products in nutraceutical. Hence, the increasing interest on these valuable bioactive molecules has led to the search of alternatives, more cost-effective and with higher yields, towards their industrial production. In fact, microbial metabolism offers a promising option for pigments production. Herein, *Gordonia alkanivorans* strain 1B, a known desulfurizing bacterium, is exploited as a high carotenoid-producer microorganism.

The main goal of this work was the optimization of the carotenoids production conditions with further identification of the main type of carotenoids present. In this context, two parallel experimental designs (EDs) were carried out, one in the absence of light (ED1 – L_0) and the other at approximately 400 lux light (ED2 – L_{400}), to demonstrate the importance of light factor into carotenoids production even in the same growth conditions of C-source and S-source. Therefore, following the Doehlert distribution based surface response methodology, it was evaluated in both EDs the influence of two factors: % of glucose in a mixture glucose + fructose (0-100% glu in 10 $g.L^{-1}$), and sulfate concentration (7-37 $mg.L^{-1}$), for two responses assessed (biomass, total carotenoids). Moreover, two different times were studied to evaluate a possible influence of time: 72 and 216 h. Based on the different response surfaces obtained in both EDs, the best combination for carotenoids production by strain 1B was: 100% glucose and a sulfate concentration higher than 22 $mg.L^{-1}$ in the presence of light for $\geq$216 h. Results demonstrated that light, time period, glucose and sulfate content in the culture medium, played an important role towards the highest carotenoids production by strain 1B.

Next, the influence of time was studied in more detail, as well as, the influence of light performing an assay without light, with 400 lux and with photoperiod with a basal value of 3000 lux. In terms of growth, the culture with 400 lux had a higher growth rate than the others. The cultures with light had higher biomass (4.2 $g.L^{-1}$ instead of 3.5 $g.L^{-1}$). The highest value of carotenoid production described so far by *G. alkanivorans* strain 1B was achieved with this assay, reaching a value of 1608.6 µg (2596 $\mu g.g_{DCW}^{-1}$) in the culture with 400 lux and 21 days of growth. Higher light exposure or lower than the previous resulted in lower yields of carotenoids. An attempt to identify the type of carotenoids present was performed through further HPLC analysis of the different extracts. Only astaxanthin and lutein were identified, and these two pigments corresponded only to a percentage of 37 - 52% of the

total carotenoids extracted. This set of assays showed that carotenoid production increased through time in all light conditions, and as so, this is an important factor for this production. A supplementary assay using a stable value of 3000 lux without photoperiod was performed and resulted in the highest yield obtained so far, 0.31%, by a *Gordonia* species.

An evaluation of the cells physiology was made through flow cytometry analysis, considering that there is a set of studies describing the association between stress and carotenoid production. The results showed that the highest carotenoid producers (400 lux and 3000-7000 lux) were the ones with an overall higher population of stressed/dead cells (stressed:39-32% and dead: 8-12%), conversely, without light the healthy population was higher (81% instead of 49-60%), confirming this way the association between stress and carotenoid enhancement.

Lastly, considering the described properties of carotenoids, antioxidant and antimicrobial assays were performed. The antimicrobial activity showed no positive results. On the other hand, in the antioxidant activity assay, the extract with higher activity (culture without light) achieved 3.4% of DPPH$^{\bullet}$ (2,2-difenil-1-picril-hidrazilo) discoloration while the standard with similar concentration only had 1.8% of discoloration. Further testing should be performed using the purified pigments, since that in a crude extract other extractives may be present than pigments that can influence (positive or negatively) the overall properties of any bioactive compound present.

In conclusion, these promising results point out for the exploitation of *G. alkanivorans* strain 1B as a possible hyper pigment-producer microorganism that may be applied towards different industries, since carotenoids are valuable bioactive molecules for chemical, pharmaceutical, food and cosmetic sectors. Additionally, since strain 1B is known by its great potential to desulfurize fossil fuels, the simultaneous exploitation of this high added value product produced by this microorganism may benefit a future scale-up of BDS, aiming to its further application downstream of a petroleum refinery towards ultra-low sulfur fuels, turning the overall BDS process more cost-effective.

Keywords: *Gordonia alkanivorans* strain 1B; Experimental Design; Astaxanthin; Lutein; Carotenoids production

RESUMO

Com o aumento das restrições mundiais relativas à concentração de enxofre nos combustíveis fósseis, houve necessidade de estudar processos que removessem este composto. O processo implementado e que está ainda em uso é a hidrodessulfurização. No entanto, esta técnica representa elevados custos, devido às condições extremas que usa, nomeadamente, pressão e temperatura elevadas entre outros. Uma alternativa a este processo é a biodessulfurização que utiliza microrganismos como catalisadores, que usam o enxofre que removem, nas suas vias metabólicas. Este processo tem como principais vantagens relativamente à hidrodessulfurização o facto de ser mais ecológico, e de funcionar em condições amenas (temperaturas e pressão mais baixas). No entanto, os custos associados a este processo ainda continuam a ser um dos fatores limitantes à sua aplicação a nível industrial. Assim, têm vindo a ser feitas tentativas no sentido de minimizar os custos associados à biodessulfurização quer pela minimização do valor associado ao processo (meios de cultura, equipamento, fonte de carbono, entre outros), quer pela exploração de produtos resultantes da atividade dos catalisadores[1–5].

De entre vários microrganismos capazes de dessulfurizar estão espécies do género *Gordonia*, como por exemplo, *Gordonia alkanivorans* estirpe 1B[6]. Esta bactéria é aeróbia, gram-positiva e com uma cor rosa/alaranjada e com elevada capacidade de dessulfurização. Recentemente, Silva *et al.* descreveram a capacidade de produção de pigmentos, nomeadamente carotenoides, por esta estirpe[7]. Os carotenoides são usados principalmente como aditivos e corantes (na piscicultura, por exemplo) ou como suplementos nutricionais. Funcionalmente, são responsáveis por proteger as células de stress oxidativo devido à sua capacidade antioxidante e, como tal, estão associados à prevenção de diversos tipos de cancro. Desempenham também outras funções, tais como estabilização da membrana em bactérias, para além de serem responsáveis pela cor de muitos organismos. São portanto compostos com elevado valor económico[8,9].

Existem vários estudos que descrevem condições para otimizar/potenciar a produção de carotenoides, sendo que muitos referem a aplicação de condições de stress sobre os microrganismos. A formulação dos meios de cultura (composição em sais), temperatura de crescimento, oxigenação, fonte de carbono, exposição à luz e a diferentes comprimentos de onda de luz são algumas das condições testadas[10–13].

Neste contexto, e dando seguimento ao estudo desenvolvido por Silva *et al.*, este trabalho teve como principal objetivo otimizar as condições de cultura da bactéria *G. alkanivorans* estirpe 1B com o intuito de maximizar a produção de carotenoides[14].

Numa primeira fase, sabendo que a estirpe 1B é frutofílica[15], estudou-se qual a influência da combinação de fontes de carbono, nomeadamente da % de glucose numa mistura de glucose-frutose

(0-100% glucose) e da concentração de sulfato (7-37 mg.L^{-1}) na produção de carotenoides por esta bactéria. Para isso fizeram-se dois planeamentos experimentais (PEs) baseados na distribuição de Doehlert[16] para os dois fatores acima referidos, em diferentes condições de luz (400 lux de luz e sem luz). As respostas avaliadas nestes PEs foram a produção da biomassa e dos carotenoides totais, após 72 h e 216 h. Baseado nas diferentes respostas obtidas em ambos os PEs, a melhor condição para produção de carotenoides foi com 100% de glucose e concentração de sulfato ≥22 mg.L^{-1}, na presença de luz durante ≥216 h. Estes resultados mostraram que a luz, tempo, glucose e concentração de sulfato têm grande impacto na produção de carotenoides pela estirpe 1B.

Tendo por base a importância do tempo para a maior produção de pigmentos. Avaliou-se o comportamento da estirpe 1B durante o seu crescimento ao longo do período de 26 dias para a produção de pigmentos. Deste modo, a estirpe 1B foi cultivada em frasco agitado, com 10 g.L^{-1} glucose e 22 mg.L^{-1} sulfato, sob diferentes condições de luz (L_0 - escuro, L_{400} – 400 lux, L_{3000} – 3000 lux, com fotoperíodo variando entre 3000-7000 lux). Neste ensaio, L_{400} obteve a maior taxa de crescimento (0.0259 h^{-1}), seguida de L_0 (0.0235 h^{-1}) e por fim L_{3000} (0.0227 h^{-1}). Considerando a produção de biomassa, as culturas com luz obtiveram ~4.2 g.L^{-1}, enquanto que a cultura L_0 apenas atingiu 3.5 g.L^{-1}. Como esperado, L_0 obteve a produção de carotenoides mais baixa de todos os ensaios, com apenas 220 µg (447 µg.$g_{peso\ seco}^{-1}$), contrastando com o valor de 1609 µg (2596 µg.$g_{peso\ seco}^{-1}$) em L_{400} e 1455 µg (2359 µg.$g_{peso\ seco}^{-1}$) em L_{3000}. Os resultados das culturas com luz foram os mais elevados obtidos com a estirpe 1B descritos até ao momento (0.24 – 0.26%). Este rendimento, foi ainda melhor num ensaio adicional em que a cultura foi mantida a 3000 lux sem fotoperíodo, atingindo um valor de 0.31% (3100 µg. $g_{peso\ seco}^{-1}$), o melhor de entre outras estirpes de *Gordonia*. No ensaio, sem luz a produção de carotenoides parou assim que terminou a fase exponencial do crescimento da estirpe 1B, enquanto que, no ensaio com luz, mesmo após a fonte de carbono se ter esgotado continuou a existir produção ao longo do tempo. Os diferentes extratos foram também analisados por HPLC de modo a identificar-se os principais tipos de carotenoides produzidos por *G. alkanivorans* estirpe 1B. Apenas astaxantina e luteína foram identificadas nos extratos, mas estes carotenoides representam apenas uma parte dos pigmentos totais produzidos (entre 37 a 52%), havendo assim ainda muitos carotenoides por identificar.

O ensaio ao longo do tempo foi seguido também por citometria de fluxo de forma a avaliar o estado fisiológico das células, visto existirem diversos estudos que apresentam uma relação direta entre stress e aumento de produção de pigmentos. A maior percentagem de células stressadas/mortas foi obtida nas culturas com maior produção de carotenoides (culturas à luz) com 39%/12% e 32%/8% respetivamente, para L_{400} e L_{3000}. Por outro lado, L_0 apenas teve 18%/1% de células stressadas e mortas, no entanto e consequentemente, esta cultura foi a que obteve maior percentagem de células

saudáveis 81% *vs.* 49% em L_{400} e 60% em L_{3000}. Estes resultados estão assim de acordo com a literatura, no sentido em que existe uma relação entre elevado stress celular e maior produção de carotenoides. De facto, ao sujeitar as células a stress induzido por luz resultou num aumento da produção de carotenoides de 447 µg. $g_{peso\ seco}^{-1}$ para 2359-2596 µg. $g_{peso\ seco}^{-1}$.

Considerando as funções e possíveis aplicações dos carotenoides, o último passo passou por testar a atividade antioxidante e antimicrobiana destes extratos. Na atividade antimicrobiana não foram obtidos resultados positivos. Por outro lado, na atividade antioxidante, as amostras estudadas apresentaram resultados positivos. O melhor resultado foi obtido com o extrato da cultura sem luz, que obteve cerca de 3.4% de descoloração de $DPPH^{\bullet}$. Considerando que esta amostra tinha 478 $\mu g.L^{-1}$ de carotenoides e comparando com o padrão de ácido ascórbico de concentração semelhante (500 $\mu g.L^{-1}$), este último apenas teve 1.8% de descoloração, quase metade da atividade do extrato. No entanto, devem ser efetuados mais testes utilizando os pigmentos purificados, uma vez que nos extratos podem existir outros compostos que foram simultaneamente extraídos com os pigmentos e que podem influenciar (positiva ou negativamente) a atividade antioxidante observada.

Em conclusão, estes resultados apontam para o interesse em explorar a *G.alkanivorans* estirpe 1B como híper produtora de pigmentos que poderão ser aplicados em diversas indústrias devido ao seu valor elevado como moléculas bioativas. Torna assim real a possibilidade de aplicar estes compostos a diversas industrias dos mais variados sectores, como químico, farmacêutico, alimentar e cosmético. Não esquecendo que a estirpe 1B é conhecida pelo seu grande potencial para dessulfurizar combustíveis fósseis, a exploração simultânea deste produto de valor acrescentado poderá beneficiar, futuramente, o aumento de escala da BDS para uma aplicação nas fases finais de uma refinaria petrolífera, tornando assim o processo mais rentável.

Palavras-chave: *Gordonia alkanivorans* estirpe 1B; Planeamento experimental; Astaxantina; Luteína; Produção de Carotenoides

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ABREVIATION LIST

AA – Antimicrobial Activity

BDS – Biodesulfurization

CFDA – 5,6 Carboxyfluorescein diacetate

C-source – Carbon source

DBT – Dibenzothiophene

DMSO – Dimethylsulfoxide

DCW – Dry cell weight

DPPH$^{\bullet}$ – 2,2'-diphenyl-1-picrylhydrazyl

ED – Experimental Design

Fru – Fructose

Glu – Glucose

HDS – Hydrodesulfurization

L_0 – Culture grown without light

L_{400} – Culture grown with 400lux light

L_{3000} – Culture grown with a base value of 3000lux light with photoperiod

OD_{600} – Optical density at 600 nm

PI – Propidium iodide

SFM – Sulfur Free Medium

S-source – Sulfur source

μ_{max} – Specific maximum growth rate

$v.v^{-1}$ – Volume/volume

$w.v^{-1}$ – Weight/volume

1 - INTRODUCTION

1.1 - Sulfur problematic and the need for fossil fuels desulfurization

Pollution and its consequences to the environment are a focus of the public attention and the scientific community mainly to its effect in public health. Among others, atmospheric pollution is repetitively associated with causing and exacerbating diseases related to the respiratory system, and so, it represents a serious problem to public health[17].

The worldwide increase of fossil fuels consumption, mainly due to the exponential growth of industry, is a major contributor for this pollution. Fossil fuels lead to the creation of a large amount of waste products, arising from their combustion, due to their impurities. The most common products are particulates, gases as sulfur dioxide, nitrogen oxides and also some volatile organic compounds among others. Carbon, hydrogen and sulfur are the major components of crude oil and the higher its viscosity and density, the higher their sulfur content[18,19].

The combustion of high sulfur containing oils, lead to the formation of diesel particulate matter (DPM) which was classified as a carcinogenic by several regulatory and research agencies, such as the U.S. Environmental Protection Agency. DPM is related to bronchial irritation and asthma attacks in susceptible individuals. A solution to this escalating problem was crucial and so, worldwide environmental authorities imposed restrictions on the maximum amount of sulfur allowed in fossil fuels. This forced the implementation in refineries of a process whose purpose was to remove sulfur from fuels. The process implemented was the hydrodesulfurization (HDS), which it is still the industry's solution to accomplish the stipulated sulfur limits[1]. HDS brought a considerable decrease in the emission of dangerous compounds. Nevertheless, it has some disadvantages such as the fact that it requires hydrogen, extreme conditions, such as high temperatures and pressures, high residence times and sophisticated catalysts. Even though, in some cases HDS is not enough to meet the stringent environmental regulations due to the recalcitrance of heterocyclic sulfur compounds present in fossil fuels, being necessary to apply a deep HDS, which implies harsher conditions and consequently higher process costs. Thus, HDS or deep HDS, besides being highly expensive, may reduce the quality of the final product[1,2]. These problems led to the search of more sustainable alternatives towards ultra-low sulfur fuels.

An alternative/complementary process to HDS is biodesulfurization (BDS), which consists on the removal of sulfur by the action of microorganisms. These microorganisms use the removed sulfur in their metabolic pathways, which makes the process eco-friendlier. Moreover, bioprocesses for fuel upgrading do not require molecular hydrogen, are performed at mild operating conditions and produce far less greenhouse gas emission than thermochemical processes[3–5]. Additionally, the maintenance of the biological catalysts is also cheaper than the purchase or development of the chemical catalysts necessary to HDS. One of the limiting factors that still hinder the BDS industrial

scale-up is the lack of economic viability of this bioprocess[5,20]. Thus, attempts to make BDS economically competitive, i.e. minimizing every cost associated with the production of the biocatalysts without influencing the desulfurizing ability of the catalyst, include the use of a cost-effective culture medium (minimal medium using cheaper compounds as alternative carbon sources), and also the exploitation of high added value by-products from the catalyst activity, such as carotenoids or biosurfactants[7,21–23]. The preferred microorganisms for BDS are those that can selectively remove the sulfur from the recalcitrant compounds through the via 4S-pathway regulated by a *dsz* operon, in which the calorific value of the fuel is preserved. The 4S-pathway is widespread throughout several genera of microorganisms, such as *Arthrobacter*, *Agrobacterium*, *Brevibacterium*, *Klebsiella*, *Mycobacterium*, *Nocardia*, *Paenibacillus*, *Pseudomonas*, *Xanthomonas*, *Gordonia* and *Rhodococcus*, with these two last ones being the most promising[1,6,24].

1.2 - *Gordonia alkanivorans* strain 1B

Members of the *Gordonia* genus have many different metabolic pathways, which means that they have great potential for secondary metabolites formation[1,7]. Some of the anabolic products of the *Gordonia* species are biosurfactants (*G. amarae*, *Gordonia* sp. strain 321 and *Gordonia polyisoprenivorans*), Imidazol-2-yl amino acids (*G. rubropertincta* and *G. terrae*), gordonan (*Gordonia* sp. strain Y-102), gordonin and other glycosylated peptidolipids (*Gordonia hydrophobica*) and carotenoids (*G. jacobaea*, *G. alkanivorans*). These metabolites represent valid contributors to an added value to the process of BDS[25–27].

Gordonia alkanivorans was firstly isolated from tar-contaminated soil, and it was named due to its alkane-degrading function, useful for the biodegradation of organic contaminants and bioremediation of contaminated environments[26]. *Gordonia alkanivorans strain* 1B is an actinomycete that was originally isolated from oil-contaminated soil and has been described as having great desulfurization capacity[6]. It is an aerobic, gram-positive, catalase-positive, oxidase-negative and pink/orange-pigmented bacterium. Cells are short branched hyphae, which disintegrate to rods and coccus-like elements when visualized by phase contrast microscopy[6]. This bacterium has a fructophilic behaviour and has been described as capable of growing with alternative carbon sources, which may be an advantage considering their potential towards a decrease in the overall costs of the biocatalysts production in a scale-up scenario[5,15,28,29]. Moreover, recently, Silva *et al.*, described the ability of *G. alkanivorans* strain 1B to produce high added value compounds, namely carotenoids[7]. This discovery represents a new focus of research, moreover it can contribute to make, in the future, an economically viable scale-up of the BDS process using strain 1B as catalysts aiming its further integration downstream of a petroleum refinery.

1.3 - Carotenoids

Carotenoids are bioactive liposoluble compounds mainly synthesized by photosynthetic organisms and some non-photosynthetic organisms like bacteria, molds and yeasts. They are responsible for the color of plants, algae, some animals and microorganisms and can be yellow, orange or red pigments[30].

In nature carotenoids are normally C_{40} tetraterpenoids, formed by eight C_5 isoprenoid units joined head to tail and have a tail-to-tail linkage in the center, which results in a symmetrical structure. They have a central chain that alternates sequentially between double and single bonds, and can have a ring at one or both ends of the molecule, or be acyclic. They are all obtained from the isoprenoid pathway[7,31].

Carotenoids are divided into: a) carotenes, if their chain is mainly composed by carbon and hydrogen (β-carotene, torulene, α-carotene and lycopene) and b) xantophylls, if they have a more complex structure with different functional groups. In xantophylls, depending on the functional group there are different types of carotenoid. The possible functional groups are: hydroxyl groups (e.g. lutein and zeaxanthin), keto/oxo groups (e.g. echinenone, astaxanthin and canthaxanthin), epoxide groups (e.g. violaxanthin, antheraxanthin and neoxanthin) or methoxy groups (e.g. spirilloxanthin). Presently, there are over 700 carotenoids identified[8,32–35].

1.3.1– Functions and applications

Carotenoids are essential components of the photosynthetic apparatus and can be subdivided into primary carotenoids that act as auxiliary pigments in light harvesting during photosynthesis and thus are essential for survival, and secondary carotenoids that protect the photosynthetic machinery from stress like light excess by scavenging reactive oxygen species due to their efficient anti-oxidant activity[9–11,26,32,34].

In bacteria, carotenoids have an important role in species-specific coloration, photoprotection, reactive oxygen species scavenging, light harvesting and membrane stabilization. They can also be precursors to hormones and vitamins[7].

Carotenoids in humans protect the retina from light exposure namely from near-UV exposure and have a beneficial effect due to its antioxidant activity. They have a cytotoxic effect against cancer cells, and some are active compounds for example dihydroactinidiolide in the pheromones of insects[33,36,37]. Amongst the more than 750 existing carotenoids, only about 40 are present in a human diet and of these only 20 have been identified in the human blood and tissues. About 90% of the carotenoids in the human diet and body are β-carotene, α-carotene, lycopene, lutein and cryptoxanthin[38]. Some carotenoids are classified as pro-vitamin A carotenoids due to the fact that they can be converted into retinal by humans and animals because they have an un-substituted β-ionone ring like it happens in β-carotene, α-carotene, γ-carotene and β-cryptoxanthin. As an example, lutein and zeaxanthin accumulate in the macula of the eye, and protect the retina filtering high-energy blue light. Lycopene is a potent anti-oxidant and so it has been described as reducing the risk of coronary heart disease and

certain cancers. Astaxanthin has been described as having anti-inflammatory properties, ability to inhibit the oxidation of low-density lipoprotein and to produce animal pigmentation. Is regarded as a potential novel treatment for oxidative stress and inflammation in cardiovascular diseases[8,11,26,32,38–44].

The main applications of carotenoids are in the food industry as food colorants, as additives or even dietary supplements. They are used, for example, in the colouring of fish, squid and eggs. Some are used as fragrances and can also be used in the cosmetic industry and in nutraceutical. Currently a large set of clinical trials and associated patents are being performed in order to make use of this bio compounds in the pharmaceutical industry[8,9].

1.3.2- Production

The global market of carotenoids was of $1.2 billion in 2010 and $1.5 billion in 2014[9]. It's estimated to be around $1.8 billion in 2019 with a compound annual growth rate of 3.9%[32,45].

There are more than 750 isolated from natural sources and some may be obtained chemically like astaxanthin. The process of obtaining carotenoids is strictly regulated because of the resulting by-products with undesirable effects if consumed, and so their production by different natural sources has been gaining a lot of interest. Algae, yeasts and bacteria produce carotenoids, but only a few of these carotenoids, such as beta-carotene and astaxanthin are actually produced commercially by microorganisms, because of the high production costs associated with this process[9,30,32]. The major advantages of using microorganisms as sources for carotenoids are their short life cycle, compatibility to season and climate, production of different pigments with different colors and shades, and they're easier to scale-up. Furthermore, bacterial pigments nontoxic nature and easy separation from cell biomass are other interesting advantages. Some examples of pigment producing bacteria are *Flavobacterium* sp. (zeaxanthin), *Agrobacterium auranticum* (astaxanthin), *Micrococcus* sp. (various carotenoids), *Pseudomonas aeruginosa* (pyocyanin), *Serratia marcescens* (prodigiosin), *Chromobacterium* sp. (violacein) and *Rheinheimera* sp. (glaukothalin)[46]. One microorganism also described as a potential source for canthaxanthin was *Brevibacterium* KY-4313 and even with its production improved it still wasn't sufficient to meet the industrial demands[47].

Presently there are a considerable amount of commercial products based on lutein that are extracted from *Tagetes erecta* flower solely. Production from microorganisms is currently nonexistent. In the case of asthaxanthin there are already some products that are from microorganisms such as *Phaffia rhodozyma (Xanthophyllomyces dendrorhous)* and *Haematacoccus pluvialis*[8].

Unfortunately, only 2% of the global pigment production are from biological sources. Nowadays β-carotene, y-carotene, torulene and torularhodin are mainly obtained from yeasts from the genus *Rhodotorula sp., Rhodosporidium sp.* and *Sporobolomyces sp.,* and astaxanthin from strains of *Xanthophylomeyces sp.* β-carotene can also be produced by filamentous fungi such as *Gibberela sp.,*

Mucor sp., Blakeslea sp. and *Pleycomeyces sp.* In the case of bacteria their main pigment is canthaxanthin, and the genus associated with this production are *Pantoea sp., Corynebacterium sp., Micrococcus sp., Brevibacterium sp., Bradyrhizobium spp., Gordonia sp.* and *Dietzia sp.* Some microalgae also associated with commercial production of pigments are from the group *Chlorophycae* (*Chlorella sp., Dunaliella sp., Haematococcus sp.*)[33].

Although pigments can be produced synthetically, it has been demonstrated that the final pigment is relatively different from the natural version. Capelli *et al.* demonstrated that the natural astaxanthin is more powerful in terms of antioxidant activity than other commonly used antioxidants such as the synthetic form of astaxanthin. Another difference between these two molecules is that the natural astaxanthin contains supporting carotenoids and so it is more complex than the synthetic one, which may influence greatly its functions[48].

There are 8 type of carotenoids that are synthetically produced on an industrial scale, namely the C_{40}-carotenoids: lycopene, β,β-carotene, (3*R*,3'*R*)-zeaxanthin, canthaxanthin and astaxanthin, and three apocarotenoids: β-apo-8'-carotenal, ethyl β-apo-8'-carotenoate and citranaxanthin. They are mainly used as animal feed additives except lycopene, β-carotene and zeaxanthin, which are used as nutritional supplements[49].

1.3.3- Conditions for carotenoids production

Although pigments can be produced by microorganisms, the yield is low and still not able to compete economically with the synthetic production. Thus, there is a need to improve the performance of microrganisms and, consequently, increase the pigment yield[46].

There are several environmental and nutritional factors that have been described as influencing carotenoids production[32]. It has been described that, when subjected to several stress conditions (nitrogen and phosphate limitations, salt stress and high light intensity) a variety of unicellular green algae accumulate large amounts of carotenoids. For example, in *Dunaliella* species stress induces a huge accumulation of β-carotene and in *Haematococcus pluvialis* is astaxanthin[10].

Calegari-Santos *et al.* studied halophilic archaea and showed that the carotenoid production is salt dependent and the concentrations applied vary among the species according to their specific requirements. Other studies showed that a combination of salt and increased light resulted in high astaxanthin accumulation, and a strong induction of carotenoid biosynthesis genes in the green algae *Haematococcus pluvialis*. They have also demonstrated that the specific astaxanthin pathway and the general carotenoid biosynthesis are subjected to transcriptional control by light. Different light intensities seemed to be perceived via the redox state of components of the photosynthetic electron transport[10,41,50].

Oxygen supply is another factor that may influence the production of carotenoids. Liu *et al.* studied the relationship between oxygen transfer and carotenoid production in the yeast *Phaffia rhodozyma*

in shake-flask cultures. It was reported that there was a direct correlation between carotenoid yield and the oxygen transfer rate, namely an enhancement of carotenoid synthesis with an increase in the respiration activity of *P. rhodozyma*[12].

1.3.3.1- Gordonia genus carotenoids production

The first reported *Gordonia* that biosynthesized carotenoids was *G. jacobaea* MV-1. Amongst others, *G. jacobaea* MV-26 has been described as having a canthaxanthin production influenced by the culture conditions[13]. *Gordonia alkanivorans* SKF120101 has also been described as a light-induced carotenoid producer, and one of the possible produced pigments is dehydro-β-carotene[26]. In a more recent study, *G. alkanivorans* strain 1B carotenoid production was described as being influenced by light exposure and also by the carbon and sulfur source used. In the latter work, independently of the carbon source, when the bacterium was exposed to light it had a higher carotenoid yield. Another interesting result was that when glucose was used as carbon source the carotenoids yield was higher than with fructose despite this bacterium being described as having fructophilic behaviour. This productivity was even higher if sulfur was used as sulfur source in opposite to DBT (Dibenzothiophene). Among the total carotenoids produced, canthaxanthin, lutein and astaxanthin were identified, being canthaxanthin the major carotenoid produced[7].

Astaxanthin (3,3'-dihidroxy-β,β'-carotene-4,4'-dione) (**Supplementary Figure 1a**) is a highly-oxidized carotenoid responsible for the colours of some birds like flamingos, crustaceans and certain fish. It contains two keto groups on each ring structure, which is associated with its anti-oxidant activity[12,43,47,48,50,51].

Canthaxanthin (4,4'-diketo-β-carotene) (**Supplementary Figure 1b**) is a ketocarotenoid[47,52,53]. It is one of the carotenoids that does not have provitamin A activity, but it has anti-carcinogenic, immune-enhancing and antioxidant activity. It is described that canthaxanthin plays a role in enhancing the gap-junction cell to cell communication through upregulation of connexin, which is a gap-junction protein[43].

Lutein (**Supplementary Figure 1c**) is the most abundant carotenoid in all green vegetables and it is, with zeaxanthin, the major component of the macular pigments of the retina where they have protective roles. Lutein shares the carbon skeleton and bonding framework of α-carotene[8,43,54].

1.4 - Scope of the Thesis

Based on prior results obtained by Silva *et al.*, this study focused in better understand and exploiting the ability of *G. alkanivorans* strain 1B, a fructophilic desulfurizing bacterium, for carotenoids production.

In this context, the main goals of this work were:

- Optimization of the total carotenoids production conditions and identification of the different types of carotenoids present;

- Cell physiology evaluation within different culture conditions through flow cytometry and correlation to carotenoids production ability;
- Anti-oxidant and anti-microbial activities of the extracted carotenoids.

2 – MATERIALS AND METHODS

2.1 - Chemicals and Reagents

Dibenzothiophene (DBT, 99%) was obtained from Acros Organics (Geel, Belgium) and Sodium sulfate anhydrous (>99 %) from Merck (New Jersey, USA). 5(6)-carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) were acquired from Invitrogen (Massachusetts, USA). Astaxanthin (98%) was from Sigma-Aldrich, canthaxanthin (99%) from Roche and lutein (10%) from FloraGLO, Kemin. DMSO (99.9%), acetone (99.9%), ethyl acetate (99.8%) and methanol (99.9%) were obtained from CARLO ERBA Reagents (Val de Reuil, France). Ascorbic acid was from Sigma-Aldrich. The remaining reagents were of the highest grade commercially available. Stock solutions of glucose (glu) and fructose (fru) were prepared at 50% ($w.v^{-1}$), sterilized at 121°C, 1 atm and stored for further use as carbon source (C-source) in culture media. In the same way, a stock solution of Na_2SO_4 20 $g.L^{-1}$ was also prepared and autoclaved (121°C, 1 atm, 15 min) to be further used as sulfur source (S-source).

2.2- Microorganism and Culture Media

The microorganism used in this study was the bacterium *Gordonia alkanivorans* strain 1B, isolated in our laboratory[(6)]. The basal salts medium used for cultivation, maintenance and further for all of the growth/carotenoids production assays was a sulfur-free mineral (SFM) medium containing: NH_4Cl (1.22 g), KH_2PO_4 (2.55 g), $Na_2HPO_4.2H_2O$ (2.55 g), $MgCl_2.6H_2O$ (0.17 g) and 0.5 mL of a sulfur-free trace elements solution (TES) per litre of ultrapure water[(22)]. The final pH was adjusted to 7.5 prior to sterilization by autoclave (121°C, 1 atm, 15 min).

Prior to carotenoids production assays, filter sterile stock solution of carbon source (C-source) (fructose and/or glucose) was added to the culture medium, in aseptic conditions, to an initial concentration of 10 $g.L^{-1}$ of total sugar(s). Similarly, the stock solution of S-source (Na_2SO_4) was also added to obtain the desired final concentrations of 9.04 $mg.L^{-1}$, 22 $mg.L^{-1}$ and 34.99 $mg.L^{-1}$, depending on the assay.

The bacterial cultures were performed in 500 mL Erlenmeyer shake-flasks containing 150 mL culture medium, incubated in an orbital shaker (~150 rpm) within an acclimatization chamber (Fitoclima 14000E Walk-In, Aralab, Rio de Mouro, Portugal), at 30°C, in the presence/absence of light (0-400 lux). For all the assays, it was used as inoculum 2% ($v.v^{-1}$) of a *G. alkanivorans* strain 1B culture prior grown in SFM medium supplemented with a mixture of 5 $g.L^{-1}$ fructose and glucose (ratio 1:1) as C-source and 150 µM DBT as sulfur source (S-source), at 30°C for about 10 days.

Different assays were performed: i) two sets of experimental design (EDs) tests to establish the optimal conditions for the carotenoids production by *G. alkanivorans* strain 1B (C-source *vs.* sulfate concentration at dark/light); ii) assays for the evaluation of the Influence of growth time to the carotenoids production (assays at light: 400 lux and 3000 lux + photoperiod; and without light: 0 lux); iii) an assay with a stable value of 3000 lux for 19 days. All the assays were carried out at least in duplicate.

Biomass samples were collected during the course of the growth or at the end of the growth for the EDs. For the assay of evaluation of time a flask with culture was collected per time analysed. Aliquots of these samples were immediately analysed for: cell growth/biomass determination; sugar(s) consumption evaluation; and cell physiological state assessment through flow cytometry. The rest of each sample was centrifuged (8600 *g* at 4-5°C, 20 min in a refrigerated Sigma 2-16K centrifuge) and the respective cells were stored at -20°C until further pigment extraction and analysis.

2.3- Experimental Design Methodology

A surface response methodology, based on the Doehlert distribution for two factors[16], was used into two parallel experimental designs (EDs) towards optimal carotenoids production by *G. alkanivorans* strain 1B: one at approximately 400 lux light (TES-1330 digital light meter; TES Electrical Electronic Corp., Taiwan, R.O.C) and the other in the absence of light. These two EDs were performed to demonstrate the importance of the light factor in carotenoids production even in the same growth conditions of C-source and sulfur-source. In both EDs, the explanatory variables or factors studied and the respective experimental domains tested were: % of glucose in a mixture glucose + fructose of 10 $g.L^{-1}$ of total sugars ($X1$: 0-100% glu in the mix) and sulfate concentration ($X2$: 7-37 $mg.L^{-1}$ of sulfate). In each ED set of tests, fourteen experiments (7 conditions in duplicate) were carried out. The responses studied (Yi) in both EDs were: biomass and total pigments production by strain 1B, at 72 h and 216 h. The model used to express the responses was a second order polynomial model:

$$Yi=\beta_0+\beta_1X_1+\beta_2X_2+\beta_{12}X_{12}+\beta_{11}X_1^2+\beta_{22}X_2^2 \qquad \text{(Eq. 1)}$$

where: Yi - response from experiment i; β - parameters of the polynomial model; and X - experimental factor level[21,24].

2.4 - Carotenoids Extraction

The centrifuged biomass samples from the different assays was defrosted at room temperature and completely isolated from light exposure. Then it was distributed as uniformly as possible on a Petri dish using a loop, and further dried at 55°C for a period of 15 to 60 min. Portions of about 25 mg of the dried biomass (cells with ~60% humidity) were weighted into 1.5 mL Eppendorf microcentrifuge tubes for the extraction and another portion of 25 mg for dry cell weight (DCW) calculations.

To extract the carotenoids, 1 mL of Dimethyl Sulfoxide (DMSO) was added to each biomass in the microcentrifuge tubes and incubated on an orbital incubator at 50°C for 45 to 60 min. The tubes were

centrifuged at 14300 *g* for 5 min (Biofuge 15 centrifuge, Heraeus Sepatech, Germany) and the supernatant stored. The process was repeated with 0.5 mL of DMSO until the supernatant recovered from the biomass became colourless. The next step consisted in the extraction of the total pigment from the DMSO. Thus, the overall recovered supernatant was mixed with acetone, a NaCl solution at 20% $v.v^{-1}$ and ethyl acetate in a proportion of 1:1:6 respectively for each 4 mL of extracted supernatant. The mixture was gently shaken, left to rest (~1 h) for phase separation, and the coloured layer (ethyl acetate phase = top layer) was retrieved and placed at -20ºC overnight to promote effective phase separation. Finally, the top layer, containing the carotenoids, was collected and filtered through 0.22 µm Nylon - syringe filter and the final volume was measured. The samples were stored at -20ºC until further carotenoids analysis both by spectroscopy and high performance liquid chromatography (HPLC). Throughout the different steps of the extraction process, the samples were covered with aluminium foil to prevent carotenoids degradation due to light exposure.

2.5 - Analytical Methods

2.5.1 - Optical Density and Dry Cell Weight

Cell growth was monitored by the measurement of optical density of the culture at 600 nm (OD_{600}) (Thermo Electron Corporation Spetrofotometer, model Genesys 20, Madison, USA) and by the determination of dry cell weight (DCW). DCW was determined by centrifuging 1.5 mL of the bacterial culture broth and then drying the pellet at 100ºC overnight.

2.5.2- Sugar Consumption Evaluation

The concentration of the carbon source, fructose and/or glucose, was determined using HPLC instrumentation (LaChrom Merck/Hitachi, Germany), equipped with a differential refractive index detector. In this HPLC, a Waters SugarPak 1 column (6.5 x 300 mm, Bio-Rad Laboratories, CA, USA) was used, operating at 75ºC with Ca-EDTA at 50 $mg.L^{-1}$ as mobile phase with a flow rate of 0.5 $mL.min^{-1}$. The obtained data was analysed with Chromeleon software ver. 6.40 SP6 build 783 (1994-2003, Dionex).

2.5.3- Flow Cytometry Analysis for Cell Physiological Evaluation

The cells physiological state was evaluated by flow cytometry analysis. Data acquisition was performed in a FACSCalibur Flow Cytometer (BD Biosciencies, San Jose, CA, USA) equipped with an argon laser emitting at 488 nm. The data obtained was analysed using FCS Express 5 Flow Research Edition software (De Novo Software, 2016). Cells were stained with both aliquots of 10 mM CFDA (5,6 Carboxyfluorescein diacetate) in acetone solution (green fluorescence, FL1) and of 1.5 mM PI (propidium iodide) in distilled water solution (red fluorescence, FL3), accordingly to Silva *et al.* and Teixeira *et al.*[55,56]. Prior to the cytometry analysis the cells were centrifuged at 8500 *g* for 10 min (Biofuge 15, Heraeus Sepatech, Germany), resuspended in Tris-HCl Buffer (50 mM, pH 7.4) and sonicated for 10 seconds. The concentration of the cells suspension was adjusted to, approximately,

3000 events per second by flow cytometric analysis. Of the adjusted cell solution, 995 µL were incubated with 5 µL of CFDA solution for 30 min at 37°C protected from light exposure. After that, the cells were centrifuged at 8500 *g* for 10 min (Biofuge 15, Heraeus Sepatech, Germany), resuspended in 999 µL Tris-HCl Buffer and maintained in ice. To complete the double staining, 1 µL of PI solution was added and the flow cytometric analysis was immediately performed. Each test sample was collected in duplicate and the readings were performed six times total for each duplicate. To discriminate the cells from background noise and debris, instruments settings were selected for the forward and side scatter signals. Samples with only Tris-HCl buffer and no cells were analysed to assess the continuous presence of background noise and consequently confirming that this was not due to unstained cells. Cells harvested during the exponential phase of a culture with fructose as carbon source (5 $g.L^{-1}$) and 200 µM of DBT were used as the healthy cells control. For the dead cells control, healthy cells were incubated in ethanol at 70% ($v.v^{-1}$) for 1 min[55].

2.5.4- Pigments Analysis

The characterization of the pigments produced by *G. alkanivorans* strain 1B grown in the different culture conditions was performed by two different methodologies: spectroscopy analysis and HPLC. Firstly, to assess the amount of total carotenoids extracted from each biomass sample, UV–Visible spectrum (Shimadzu spectrophotometer UV-2401PC) was run, between 380 and 700 nm, and the concentration of total carotenoids was estimated based in the Lambert-Beer equation according to Nobre *et al.*, but using the value of 2091.4 $L.10g^{-1}.cm^{-1}$ for the specific optical extinction coefficient at λ = 477 nm (wavelength of the maximum absorbance of canthaxanthin in ethyl acetate)[7,57].

Further specific carotenoid identification and quantification was performed using HPLC (Agilent 1200 Series system, Agilent Technologies, Tokyo) equipped with a µ-Bondapack C18 (250/4.6 mm) column and a UV/VIS detector also from Agilent (λ = 477 nm), using methanol (with 0.2% water): acetonitrile (75:25) as eluent, the pigments were eluted for 90 min at a flow rate of 0.5 $mL.min^{-1}$. The identification of carotenoids in each extract from different biomass samples was carried out by comparing the retention time patterns with those obtained for individual standards, namely of asthaxanthin, canthaxanthin, β-carotene and lutein, using LC3D ChemStation software (Rev.A.10.02 [1757], 1990-2003, Agilent Technologies, USA). The pigment results were presented as percentage (%=g carotenoid/100g DCW), or in total µg (µg carotenoid per g DCW per 150 mL).

2.6- Determination of Antioxidant Activity

To evaluate the antioxidant activity of the carotenoids produced by *G. alkanivorans* strain 1B, the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical scavenging activity assay was performed, adapted from D'Angelo *et al*.[58]. Thus, for the $DPPH^{\bullet}$ scavenging assays, a stock solution of $DPPH^{\bullet}$ at 300 µM in methanol was prepared and then diluted to 100 µM. For each carotenoids extract in ethyl acetate, an aliquot of 0.2 mL of the test sample was added to 1.8 mL of the $DPPH^{*}$ 100 µM solution and absorbance

was immediately measured with a Kinetic analysis of the UV–Visible spectra (Shimadzu-UV-2401PC) at 517 nm for 10 min against a control (with ethyl acetate).

The radical scavenging/antioxidant acitivity is determined as the DPPH• decolorization percentage (% decolorization), calculated as the following equation[58]:

$$\text{DPPH}^{\bullet}\ \text{decolorization (\%)} = \left(1 - \frac{Absorbance_{\text{sample}}}{\text{Absorbance}_{\text{control}}}\right) \times 100 \qquad \text{(Eq. 2)}$$

where $\text{Absorbance}_{\text{sample}}$ and $\text{Absorbance}_{\text{control}}$ are, respectively, the absorbances in the sample and in the control, at 517 nm during the 10 min analysis.

Moreover, the scavenging activity of the carotenoids extract samples was compared with a set of ascorbic acid solutions with different concentrations (adjusted to the total carotenoids concentration present in each tested sample).

2.7- Determination of Antimicrobial Activity

The antimicrobial activity (AA) of extracts of the carotenoids produced by strain 1B, in different culture conditions, was determined for two different microorganisms: *Pseudomonas putida* and *Escherichia coli (LNEG Culture Collection of Microorganisms)*. Lysogeny broth (LB) or tryptone soya broth (TSB) are general-purpose culture media that may be used in the cultivation of a wide variety of fastidious and non-fastidious microorganisms towards the antimicrobial susceptibility testing. In this context, *Pseudomonas putida* was maintained on *Lysogeny broth* agar *(LBA)* and transferred to LB prior the antimicrobial activity assays at 30°C. *Escherichia coli* was maintained in tryptone soya agar (TSA, Oxoid) and grown in tryptone soya broth (TSB, Oxoid) during the assays, at 37°C.

For AA tests, aliquots of 0.1 mL of bacterial culture just grown in fresh liquid medium were transferred into Petri dishes (∅ = 90 mm), containing the respective solid medium, and uniformly distributed with sterile spheres. After the microorganism is embedded, sterile filter paper discs (∅ = 5 mm) were submersed into the samples to be tested, placed in the respective Petri dish and air dried for about 5 min (in order to evaporate the ethyl acetate for less toxicity). Control assays with ethyl acetate were carried out to assess the toxicity of the organic solvent with the pigments. The Petri dishes were incubated at 30 and 37ºC, respectively for *P. pudida* and *E. coli*, during 48-72 h. Antimicrobial activity was evaluated considering the inhibition halo formation developed around the disc, according with the Clinical Laboratory Standards Institute (CLSI) Guidelines[59]. The assays were carried out in duplicate plates.

3- RESULTS AND DISCUSSION

3.1- Experimental Designs

Silva *et al.*, studying the carotenoids production potential of *Gordonia alkanivorans* strain 1B, pointed out that the best condition for pigments production was culture growth with glucose and sodium

sulfate as carbon (C) and sulfur (S) source, respectively, in the presence of light (3000 lux), despite of the known fructophilic behaviour of strain 1B[7,15]. This work was a follow-up of this study in order to try to attain the optimal conditions for carotenoids production by strain 1B. In this context, two parallel experimental designs (EDs), one in the absence of light (ED1 – L_0) and the other at approximately 400 lux light (ED2 – L_{400}), were carried out to demonstrate the importance of light factor in carotenoids production even in the same growth conditions of C-source and S-source. So, in both EDs based on the surface response methodology accordingly to the Doehlert distribution[16] it was evaluated the influence of two factors: % of glucose in a mixture of glucose + fructose (10 $g.L^{-1}$ total sugars), ranging from 0-100% glu, and sulfate concentration, ranging from 7-37 $mg.L^{-1}$.

In each ED, fourteen tests were carried out corresponding to seven conditions tested in duplicate. The responses evaluated were the biomass ($g.L^{-1}$) and the total pigment production (μg), both after 72 h and 216 h. Total pigment values were calculated based on the spectrophotometric analysis of each carotenoids extract sample.

3.1.1 – ED1 (L_0)

Table 1 (APPENDIXES) shows the set of tests performed within the ED1, towards optimal conditions for carotenoids production by strain 1B in the absence of light (L_0), and the responses evaluated (biomass and total pigment production, both after 72 h and 216 h).

Analysing the data for 72 h in terms of biomass concentration, tests 1 to 6 showed the effect of varying glucose concentration for a constant sulfate concentration at the centre of the experimental domain (22 $mg.L^{-1}$). By increasing glucose from 0 to 5 $g.L^{-1}$ (50%), the biomass concentration was decreased by 13.7% (from 4.7 to 4.0 $g.L^{-1}$), and, when the concentration was increased to 10 $g.L^{-1}$ (100% of total sugars), the biomass was reduced to 15.5% of the original value (0.72 $g.L^{-1}$). Moreover, in tests 7, 8, 13 and 14, maintaining sulfate concentration at 34.99 $mg.L^{-1}$, and increasing the glucose/total carbon ratio from 25% to 75% resulted in a decrease of 13% of biomass (from 3.76 to 3.25 $g.L^{-1}$). Conversely, for lower concentrations of sulfate (9.01 $mg.L^{-1}$ in tests 9, 10, 11 and 12) the increase of glucose from 25% to 75% resulted in no relevant difference. Maintaining glucose at 25% and increasing sulfate from 9.01 to 34.99 $mg.L^{-1}$ (tests 9, 10, 13 and 14) resulted in a 54.5% biomass increase (from 2.43 to 3.76 $g.L^{-1}$). A similar tendency was observed on tests 7, 8, 11 and 12, in which glucose was maintained at 75% of total sugars and sulfate increased from 9.01 to 34.99 $mg.L^{-1}$, resulting in an 18.6% biomass increase (from 2.74 to 3.25 $g.L^{-1}$). These results highlighted the fructophilic nature of the strain 1B, as it was already described by Alves *et al.*[15]. The proportion of glucose had a high influence for biomass production. With higher concentration of glucose, the bacteria presented lower growth rates, obtaining reduced biomass values. However, for lower values of sulfate, under 22 $mg.L^{-1}$, glucose concentration became less important, indicating that sulfate might be at a limiting concentration.

Taking into account pigment production, at 72 h, tests 1 to 6 demonstrated that increasing glucose from 0 to 5 $g.L^{-1}$ resulted in an increase of 81% (from 36.1 to 65.4 μg). However, a further increment to 10 $g.L^{-1}$ led to a reduction to the original pigment levels. When sulfate concentration was 9.01 $mg.L^{-1}$ (tests 9, 10, 11 and 12), increasing glucose from 25% to 75% increased pigment accumulation by 217% (from 8.1 to 25.5 μg). When the same variation of glucose proportion was performed in a medium with 34.9 $mg.L^{-1}$ of sulfate, the pigment accumulation was improved by 176% (from 20.7 to 57.1 μg). When glucose was at a 25% proportion and sulfate was increased from 9.01 to 34.99 $mg.L^{-1}$, there was an increase of 157% (8.1 to 20.7 μg). In similar manner, when the growth was performed with glucose at a 75% ratio, the same increase in sulfate concentration resulted in an increase of 124% (25.5 to 57.1 μg) of pigment accumulation. These results show that both factors are equally important for pigment production at 72 h, also indicating that there is an equilibrium in the importance of both the sugars used for the growth. While glucose stimulates pigment formation, it results in slower biomass formation, so an intermediate value between fructose and glucose should be ideal.

Analysing the data obtained in the same conditions, after 216 h, the effect of varying the proportion of glucose (0 to 100% in the carbon mixture) while maintaining sulfate concentration at constant value (the centre of the experimental domain, 22 $mg.L^{-1}$) was observed. Tests 1 to 6 showed an increase of biomass concentration with the increase of glucose in the mix. When glucose was raised from 0 to 5 $g.L^{-1}$ and then to 10 $g.L^{-1}$, biomass increased 15.7% (from 3.15 to 3.65 $g.L^{-1}$) and 26.3% (from 3.15 to 3.98 $g.L^{-1}$), respectively. This behaviour was observed whenever glucose was increased, with greater differences for higher sulfate concentrations. An increase from 25% to 75% glucose at a sulfate concentration of 9.01 $mg.L^{-1}$ (tests 9, 10, 11 and 12) resulted in an increase 9.53% (2.16 to 2.38 $g.L^{-1}$), whereas, for sulfate at 34.99 $mg.L^{-1}$ (tests 7, 8, 13 and 14) biomass increased 18.6% (3.11 to 3.69 $g.L^{-1}$). The most significant differences were observed when varying the sulfate concentration. For a fixed value of 25% glucose, changing sulfate from 9.01 to 34.99 $mg.L^{-1}$, there was an increase of 44% of the biomass concentration (2.16 to 3.11 $g.L^{-1}$). While the same increase with 75% glucose results in a 55.4% increase in biomass (2.38 to 3.69 $g.L^{-1}$). In both cases, increasing the factor studied results in an increase of the response. However, increases in sulfate resulted in greater increases in biomass. Moreover, the correlation between the increase of glucose and the increase of biomass may contrast with the fructophilic nature of this bacterium[15]. This observation is due to the fact that in the assays with greater concentrations of fructose the strain 1B attains its maximum biomass value before 72 h and, thus, the value of biomass concentration noticeable at 216 h (3.15 $g.L^{-1}$) is already in decline. In fact, in a culture medium with higher glucose concentration, the cells presented lower growth rates, achieving the maximum biomass concentration at a later time, thus explaining the observed results.

Considering pigment production, the results showed a pattern similar to the biomass concentration. Tests 1 to 6 indicate that carotenoid production increases with the increase of glucose. Using sulfate at 22 mg.L^{-1} and changing the glucose proportion from 0 to 50% resulted in an increase of 45.9% (from 80.8 to 117.9 μg), while changing it from 0 to 100% led to a higher increase of 285% (80.8 to 311.1 μg). For sulfate at 9.01 mg.L^{-1} (tests 9, 10, 11 and 12) increasing glucose from 25% to 75% leads to a 59.1% pigment increase (32.8 to 52.2 μg), and, in these conditions, for sulfate at 34.9 mg.L^{-1} (tests 7, 8, 13 and 14), the pigment increase achieved a 108% increase (85.8 to 178.2 μg). Changing sulfate concentration from 9.01 to 34.99 mg.L^{-1} resulted in an increase of 161% (32.8 to 85.8 μg), when glucose presented only 25% of total sugars and 241% (52.2 to 178.2 μg) when glucose represented 75%. After 216 h of growth both factors seemed to affect pigment concentration. Sulfate concentration appeared to have greater influence, for lower concentrations of glucose and sulfate. While the proportion of glucose / total sugars presented a higher influence for greater sulfate concentration and higher glucose proportions.

Supplementary Figure 2 shows the response surfaces obtained for the variation of biomass (**Supplementary Figure 2a and 2b)** and pigment production (**Supplementary Figure 2c and 2d**) within the limits of the experimental domain for 72 and 216 h. Analysing the biomass response surfaces, at 72 h (**Supplementary Figure 2a**), for glucose values higher than 50% of the total carbon source, the increase of sulfate did not produce a high effect on the biomass production as seen by the presence of vertical lines. For glucose under 50%, the higher the sulfate concentration the higher the biomass production up to 19 mg.L^{-1}. The ideal condition for biomass production, at this time, was well represented in the upper left quadrant, where the value of 4 g.L^{-1} of biomass could be achieved, with a minimum of roughly 20 mg.L^{-1} of sulfate and a glucose amount between 15 and 40%. Thus, once more, this was representative of the fructophilic behaviour of strain 1B. Considering the response surface for 216 h (**Supplementary Figure 2b**), there was a clear change in the response, whereas, lower glucose values have lower biomass yields. It was also visible that for glucose under 40 %, sulfate loses some influence in the biomass production for values >20 mg.L^{-1}, conversely, sulfate concentrations lower than 20 mg.L^{-1} are the limiting factor in the biomass production, having more influence than the glucose concentration (lower quadrants). The best condition for biomass production seemed to be with glucose over 60%, achieving a value of 3.5 g.L^{-1} and values over 20 mg.L^{-1} of sulfate.

At 72 h, biomass production was higher with lower glucose concentrations, and at 216 h was the opposite, which is in accordance with the fructophilic behaviour of strain 1B. Nevertheless, in both cases, a minimum of roughly 20 mg.L^{-1} of sulfate was enough to achieve the higher biomass concentrations. Taking into account the pigment production at 72 h (**Supplementary Figure 2c**), it was possible to clearly have the ideal values of sulfate and glucose concentration at the centre of the response surface. A glucose concentration of 50 – 70% and the sulfate between 21 and 25 mg.L^{-1} were

the ideal towards the highest pigment production (60 µg). Moreover, in most of the response surface, sulfate and glucose concentration had equal importance in pigment production, as it was already described previously. The exception was for when glucose varied between 50 and 75%, in which the pigment production was mostly affected by the sulfate concentration. With 216 h, there was a change in the behaviour of strain 1B. For glucose below 35%, sulfate concentration >15 $mg.L^{-1}$ had no visible effect in the pigment production, but under this value there was decrease of pigment. For sulfate values under about 22 $mg.L^{-1}$ and glucose over 35%, the higher the glucose and sulfate the higher the pigment. In this quadrant, it seemed that both factors had equal weight for pigment production (lower right quadrant). For sulfate and glucose higher than 22 $mg.L^{-1}$ and 35%, respectively, sulfate lost influence in the pigment production, with higher glucose values leading to higher pigment (upper right quadrant).

Sulfate and glucose had high influence in pigment production for both the studied times, though a minimum sulfate concentration of approximately 22 $mg.L^{-1}$ was enough to achieve high pigment values in both cases. Taking into account the optimization of pigment production, one of the main goals of this work, there was no doubt that high concentration of glucose (100% ideally) and longer growth times were the ideal conditions.

3.1.2 – ED2 (L_{400})

Table 2 (APPENDIXES) shows the set of tests performed within the ED2, towards optimal conditions for carotenoids production by strain 1B at 400 lux of light (L_{400}), and the obtained responses (biomass and total pigment production, both after 72 h and 216 h).

Considering the culture at 72 h in terms of biomass concentration, tests 1 to 6 showed the effect of varying glucose concentration for a constant sulfate concentration at the centre of the experimental domain (22 $mg.L^{-1}$). Increasing glucose from 0 to 5 $g.L^{-1}$ (50%), the biomass concentration was decreased by 17% (from 2.85 to 2.36 $g.L^{-1}$). When the concentration of glucose was increased to 10 $g.L^{-1}$ (100% of total sugars), the biomass reduced 87% comparatively to 0% of glucose (2.85 to 0.36 $g.L^{-1}$). On tests 7 to 14, with a sulfate concentration of 9.01 or 34.99 $mg.L^{-1}$, increasing the glucose/total carbon ratio from 25% to 75% resulted in no relevant difference. Keeping glucose at 25% and increasing sulfate from 9.01 to 34.99 $mg.L^{-1}$ (tests 9, 10, 13 and 14) originated a 25% biomass increase (from 1.79 to 2.24 $g.L^{-1}$). On tests 7, 8, 11 and 12, with glucose maintained at 75% of total sugars and sulfate increased from 9.01 to 34.99 $mg.L^{-1}$, culminated in a 45% increase of biomass (1.80 to 2.61 $g.L^{-1}$).

Higher concentration of glucose, resulted in lower growth rates by the bacteria and consequently reduced biomass values. Moreover, lower values of sulfate, under 22 $mg.L^{-1}$, the glucose concentration became less important. For higher glucose concentrations, increasing sulfate led to an increment in

biomass production, achieving a value similar to the one obtained with 0% of glucose and 22 $mg.L^{-1}$ of sulfate.

Considering the pigment production, at 72 h, tests 1 to 6 showed that increasing glucose from 0 to 5 $g.L^{-1}$ resulted in a decrease of 25% (from 297.7 to 223.1 µg). Moreover, an increase to 10 $g.L^{-1}$ ended in a reduction of 69% comparatively to the original pigment levels (93.2 and 296.7 µg, respectively). With a sulfate concentration of 9.01 $mg.L^{-1}$ (tests 9, 10, 11 and 12), the increase of glucose from 25% to 75% had low influence in the pigment production (149.6 to 153.7 µg). When the same variation of glucose proportion was performed in a medium with 34.99 $mg.L^{-1}$ of sulfate, the pigment accumulation was improved by 31% (from 230.2 to 301.1µg). With glucose at 25% of the total, and sulfate increased from 9.01 to 34.99 $mg.L^{-1}$, there was an increase of 54% (149.6 to 230.2 µg). Similarly, when the growth was performed with glucose at 75%, the same increase in the sulfate concentration led to an increase of 96% (153.7 to 301.1 µg) of pigment accumulation.

Both factors are important for pigment production at 72h, and there is an equilibrium in the importance of both sugars used for the growth. With a constant concentration of sulfate (22 $mg.L^{-1}$), glucose has negative influence in pigment formation, with higher concentrations of glucose leading to lower concentrations of pigments. However, increasing the sulfate concentration with an increase of the glucose proportion may be a solution in order to achieve high pigment values.

Analysing the same conditions after 216 h, the effect of varying the proportion of glucose (0 to 100% in the carbon mixture) while maintaining sulfate concentration at constant value (the centre of the experimental domain, 22 $mg.L^{-1}$) was studied (tests 1 to 6). When glucose was raised from 0 to 5 $g.L^{-1}$, biomass had no relevant change (4.59 and 4.40 $g.L^{-1}$ respectively). Although, with an increase of the glucose to 100%, biomass decreased 19% relatively to the condition with 0%. Increasing glucose from 25% to 75% at a sulfate concentration of 9.01 or 34.99 $mg.L^{-1}$ (tests 7-14) resulted in no difference in the biomass production. The most significant differences were observed when varying the sulfate concentration. For both 25 and 75% of glucose, changing sulfate from 9.01 to 34.99 $mg.L^{-1}$ resulted in a 2-fold increase of the biomass. For biomass production, the factor that had a noticeable higher influence was the sulfate concentration. Higher sulfate concentration resulted in higher biomass production. Whereas, maintaining the sulfate concentration and varying the glucose amount, had little to none influence in the biomass. From this study, it was possible to assess the minimum concentration of sulfate, still capable of producing high values of biomass, which was 22 $mg.L^{-1}$.

Taking into account pigment production, using sulfate at 22 $mg.L^{-1}$ and changing the glucose proportion from 0 to 50% resulted in a decrease of 13% (from 477.9 to 418.2 µg), whilst changing it from 0 to 100% led to an increase of 66% (477.9 to 794.2 µg). For sulfate at 9.01 $mg.L^{-1}$ (tests 9, 10, 11 and 12) increasing glucose from 25% to 75% leads to similar pigment values, 196.7 and 201.0 µg, respectively. In these conditions, for sulfate at 34.99 $mg.L^{-1}$ (tests 7, 8, 13 and 14), the pigment achieves a 30%

increase (427.0 to 553.5 µg). When glucose represented only 25% of total sugars, changing sulfate concentration from 9.01 to 34.99 mg.L^{-1} led to an increase of 117% (196.7 to 427.0 µg), and when glucose represented 75% an increase of 175% (201.0 to 553.5 µg).

Similar to what occurred with the biomass production, the sulfate concentration had greater influence in the pigment production, than the glucose proportion. Lower values of pigments were a result of lower concentration of sulfate, even though there was a glucose variation, but the higher values of pigments were due to higher glucose. Though, in accordance to the biomass study, sulfate seemed to be the limiting factor for pigment production.

Supplementary Figure 3 shows the response surfaces obtained for the variation of biomass (**Supplementary Figure 3a and b**) and pigment production (**Supplementary Figure 3c and d**) within the limits of the experimental domain for 72 and 216 h. Analysing the biomass response surfaces, at 72 h (**Supplementary Figure 3a**), for glucose values higher than 50% of the total carbon source, the increase in sulfate did not produce a high effect on the production as seen by the presence of vertical lines. Decreasing the glucose in the medium conferred higher importance to the sulfate concentration. With glucose under 50%, there was a notable change in behaviour. Higher sulfate concentrations lead to higher biomass production, capable of achieving a maximum of 2.5 g.L^{-1}. An optimum value is also noticeable, with a minimum of roughly 21 mg.L^{-1} of sulfate and 20 to 25% of glucose. High glucose concentrations had a negative impact in the biomass production as already described previously. Thus, this was representative of the fructophilic behaviour of strain 1B. Considering the biomass at 216 h (**Supplementary Figure 3b**), there was a clear change in the response. Sulfate was the factor with higher influence in the biomass, represented by the horizontal lines in the response. In the left quadrants, sulfate is the most important factor for biomass production, for glucose values under 50%. With glucose over 50% sulfate gained more weight in the biomass production (lower right quadrant). The condition for high biomass production seemed to be attained with glucose under 50%, and a sulfate concentration over 22 mg.L^{-1}, achieving values of 4.5 g.L^{-1}. At 72 h, biomass production was higher with lower glucose concentrations, and at 216 h it was the opposite, which is in accordance with the fructophilic behaviour of strain 1B. Nevertheless, in both cases, a minimum of roughly 20 mg.L^{-1} of sulfate was enough to achieve higher biomass concentrations.

Considering the pigment production at 72 h (**Supplementary Figure 3c**), there was a visible equal influence of both factors. The increase of glucose resulted in a decrease of pigments, considering any fixed sulfate concentration. In the same quadrants, maintaining the glucose concentration and increasing the sulfate concentration resulted in an increase in pigment production. Even though, the higher pigment values were achieved with lower glucose proportions. At 216 h (**Supplementary Figure 3d**), there was a change in the behaviour of strain 1B. With glucose values between 50 and 25%, pigment production is only dependent of the sulfate concentration until a concentration of roughly

22 mg.L^{-1}, where there was no more change. For glucose under 60%, sulfate concentration played the factor with more influence in pigment production, where a higher concentration of sulfate led to higher pigment values (lower left quadrant). With glucose over 60%, until the 20 mg.L^{-1} of sulfate is achieved, both factors have equal influence in pigment production (lower right quadrant). Increasing the sulfate concentration from 22 to 30 mg.L^{-1} or decreasing the glucose concentration from 60 to 0% had little effect in the pigment production (upper left quadrant). For sulfate values over 22 mg.L^{-1} glucose was the factor with higher importance (upper right quadrant). The ideal condition for pigment production, was with 100% glucose and high sulfate concentration, over approximately 28 mg.L^{-1}, achieving a value roughly 3-fold higher than the one obtained in the best condition at 72 h.

Sulfate and glucose influenced the pigment production in both the studied times. Though, considering one of goals of the present work, the optimization of pigment production, there was no doubt that high concentration of glucose (100%) and higher growth time were the ideal conditions along with high sulfate concentration (over 22 mg.L^{-1}).

3.1.3 – Analysis of ED Factors

The data obtained from the experimental design was further used for a regression analysis and the polynomial model-derived parameters (β0 to β22) are shown in **Table 3** (APPENDIXES)**.** The β parameters of this polynomial model (when using coded units varying from -1 to +1) used to estimate the responses have the following meanings: β0 represents the centre of the experimental domain; β1 and β2 indicate the importance of the respective factors (glucose concentration in a fructose-glucose mixture and sulfate concentration, respectively) on the responses; the interaction parameter, β12, indicates how the effect of one factor is dependent of the level of the other factor. β11 and β12 values determine how the response surface folds downward (negative values) or upward (positive values) quadratically, more or less rapidly in accordance with the magnitude of the absolute value.

Considering first the ED1 (L_0), at 72 h, β1 (corresponding to the fraction of glucose in a fructose-glucose mixture) was negative for biomass, which means that its increase results in a decrease in biomass production opposing to what happened with β2. As for in pigment production this value is similar to that of β2 (sulfate concentration) and with β12 (interaction between β1 and β2), which means that increasing both the factors under study, individually or simultaneously, resulted in higher pigment yields. At this time sulfate was the factor with more influence in increasing biomass and pigment production. At 216 h, there was a change in the behaviour, with both factors β1 and β2 and their interaction (β12) increasing both biomass and pigment production with their increasing values. Here, for biomass production sulfate concentration was more relevant (β2 was 1.5 fold higher than β1), but for pigment production the concentration of glucose was more important with β1 being almost 2-fold higher than β2. Considering the interaction between β1 and β2 (β12), the effect on biomass and pigment production is lower than the one observed for the individual variation of each factor. In this

analysis it was showed that glucose and sulfate concentration have important influences for both biomass and pigment production. Model analysis by the coefficient of multiple determination (R^2) was also performed (**Table 3** (APPENDIXES)). This coefficient showed the amount of the sum of squares corrected for the mean that is accounted for by the residuals. For the data at 72 h this value was lower (0.72 for biomass and 0.78 for pigment production) than the one on the analysis for 216 h (0.98 for biomass and 0.94 for pigment production) meaning that the first had more exterior factors influencing the studied parameters.

Taking into account the ED2 (L_{400}), at 72 h, sulfate concentration was the factor with the highest influence for both biomass and pigment production, whilst the glucose concentration impacted negatively these productions (β1 and β12 lower than β2). At 216 h, for biomass production the glucose concentration (β1) resulted in a decrease, but considering pigment production it had almost the same influence as the sulfate concentration (β2). The interaction between the two studied factors was, for both biomass and pigment production, lower than the factor sulfate concentration. This data showed that sulfate concentration is the factor with highest impact in biomass and pigment production. Moreover, the values of R^2 where lower for 72 h (biomass-0.64 and pigment-0.61) than for 216 h (biomass-0.72 and pigment 0.91). Which means that at 72 h there were other influences other than the studied factors.

For both EDs, the F-test for the effectiveness of the factors applied to biomass and pigment concentration showed low levels of confidence. The same happened for levels of confidence of the F-ratio for the lack of fit. This means that, with a good level of confidence, it could be assumed that a significant amount of variance in the data was represented by the factors in the models, i.e. the factors, as they appear in the model, do have an effect upon the responses analyzed.

3.1.4 – Comparison between ED1 and ED2

In both EDs, ED1 in the absence of light and ED2 with 400 lux of light, the influence of the same two factors: % of glucose in a mixture glucose + fructose (10 $g.L^{-1}$ total sugars) and sulfate concentration were evaluated, towards the highest carotenoids production by *G. alkanivorans* strain 1B. The overall results obtained demonstrate the great influence of both factors studied on pigment production, despite their relative weight depend on the response evaluated (**Table 3** (APPENDIXES), $\beta 1$/ $\beta 2$). Moreover, the results highlight the crucial importance of light and time towards the highest pigments yield by strain 1B.

Taking into account the biomass production, both EDs showed that 72 h fermentation for strain 1B attain significant growth at 100% glucose, and thus for this response the factor % glucose in the mixture glucose + fructose has the greater weight (higher $\beta 1$). Indeed, the fructophilic behaviour of strain 1B was evident by the higher biomass production observed for the cultures grown with the lower

% of glucose. Besides, at light, the growth of strain 1B achieved a maximum biomass about half of that on dark (2.5 *vs.* 4.0 $g.L^{-1}$). But, for maximum biomass production in culture medium with lower % of glucose, the sulfate concentration must be >20 $mg.L^{-1}$ (**Supplementary Figure 2a and 3a**). At 216 h, a different behaviour was observed for ED1 (L_0) and ED2 (L_{400}) in terms of biomass and in this case the sulfate concentration was the factor with the major weight, mainly in ED2 (**Table 3** (APPENDIXES)). From **Supplementary Figure 2b**, at ED1 (L_0) and 216 h, it could be stated that highest biomass values ($\geq$3.5 $g.L^{-1}$) may be attained for >20 $mg.L^{-1}$ of sulfate and >60% of glucose. From **Supplementary Figure 3b**, at ED2 (L_{400}) and 216h, it could be stated that highest biomass values ($\geq$4.5 $g.L^{-1}$) may be achieved for >25 $mg.L^{-1}$ of sulfate and <50% of glucose. These results show that light exposure results in a different behaviour from strain 1B in terms of glucose and sulfate requirements for biomass production, specially changing the sugar consumption rate and consequently the growth rate. For higher % glucose, strain 1B needs more time to attain maximum growth and consequently higher pigment production, both in the presence and absence of light.

Focusing on pigment production, in overall the highest pigments production was obtained at light (ED2), both for 72 h and 216 h, as expected. From ED1 (L_0), the results for 72 h (**Supplementary Figure 2c**) pointed out for 50-70% of glucose and 21-25 $mg.L^{-1}$ of sulfate as the best conditions for pigment production. In contrast, the ED2 (L_{400}) results for 72 h point out for sulfate concentrations >30 $mg.L^{-1}$ and % glucose $\leq$55% (**Supplementary Figure 3c**) as the best conditions for higher production of carotenoids (>250 μg). But, considering the best carotenoids results after 216 h, despite the higher values have been obtained with light, both EDs pointed out for the highest pigments production being with culture conditions involving 85-100% of glucose and >20 $mg.L^{-1}$ of sulfate (**Supplementary Figure 2d and 3d**). Indeed the best pigment results achieved were those obtained when *G. alkanivorans* strain 1B was cultivated with 100% of glucose and 22 $mg.L^{-1}$ of sulfate for 216 h at 400 lux of light (~ 795 μg of total carotenoids, tests 3 and 4 of ED2, (**Table 2** (APPENDIXES)). In fact, both EDs show the influence of time, with the highest pigments production at 216 h. In ED1 (L_0), an increase of >400% of pigment production was observed from 72 to 216 h (**Supplementary Figure 2c and 2d**); whereas in ED2 (L_{400}) an increase of about 220% was recorded from 72 to 216 h (**Supplementary Figure 3c and 3d**).

Therefore, based on all these results, the best combination for carotenoids production by strain 1B seems to be 100% of glucose and a sulfate concentration $\geq$22 $mg.L^{-1}$ in the presence of light for $\geq$ 216 h. This is in agreement to Silva *et al.*[7]. The use of glucose for pigment production was also described for *G. jacobaea* MV-1 sp., where there was an increase in the carotenoid yield when the culture medium was supplemented with this carbon source[47].

3.2 - Influence of time

The evaluation of the influence of growth time in the carotenoids production by *G. alkanivorans* strain 1B was performed in shake-flask assays with light (400 and 3000-7000 lux, L_{400} and L_{3000}, respectively) and without light (L_0) during 26 days. The results of pigment production obtained in the experimental design assays pointed out for the use of glucose (10 $g.L^{-1}$) as the only C-source, sulfate ≥22 $mg.L^{-1}$ as S-source and presence of light as the best culture conditions towards higher carotenoids yield. However, it was still important to study the carotenoids production in the absence of light, either for comparison or considering its potential to make a downstream BDS scale-up scenario with strain 1B within a petroleum refinery towards zero sulfur emissions from burned fuels more cost-effective[7].

3.2.1 - Metabolic parameters

In **Fig. 1 (a,b,c)** are presented the growth and sugar consumption profiles of *G. alkanivorans* strain 1B with approx. 10 $g.L^{-1}$ of glucose and 22 $mg.L^{-1}$ of sulfate under different light conditions (L_0 - without light, L_{400} – 400 lux of light, L_{3000} – 3000 lux with photoperiod ranging from 3000-7000). Moreover, in **Table 4** (APPENDIXES) are described the metabolic parameters associated with these culture growths. From **Fig. 1a** results, it can be observed that in L_0 the culture ended its growth within 14 days, achieving a maximum of biomass of 3.5 $g.L^{-1}$. On the other hand, in presence of light, strain 1B grew faster, finishing its growth within 10 days for L_{400} and 12 days for L_{3000}, attaining about 4.2 $g.L^{-1}$ of biomass in both conditions. The higher biomass produced in the presence of light is in accordance with the higher OD_{600} values attained (**Fig. 1b:** 13.2 for L_{3000} and 14.1 for L_{400}).In the absence of light (L_0) the maximum OD_{600} attained was only about 10.6, which is also in agreement with the lowest biomass value achieved. In **Fig. 1c** can be observed the carbon source consumption profiles of strain 1B for the different light conditions. Glucose was completely consumed in all of the tested conditions, but at different times (10, 12, 14 days respectively at L_{400}, L_{3000} and L_0) and consequently at different rates

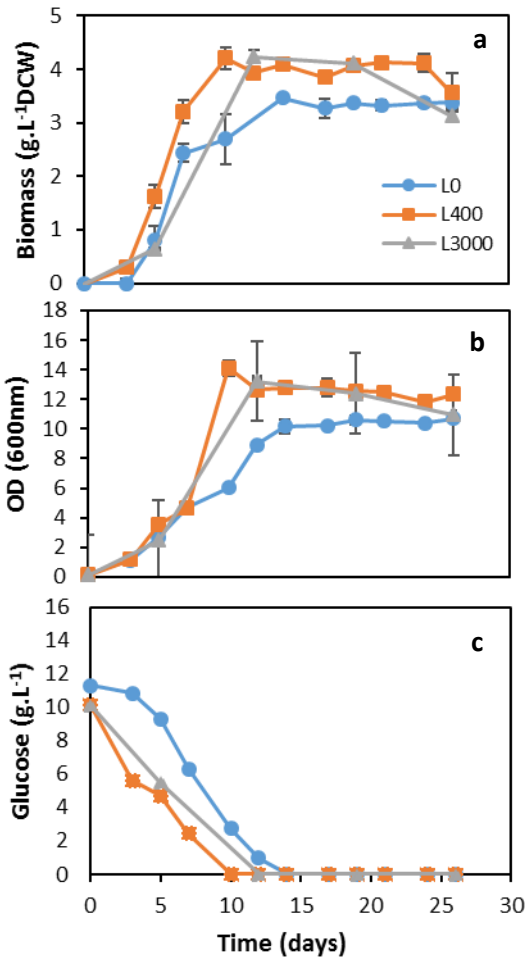

Figure 1 - Growth profiles, in terms of biomass production **(a)**, OD_{600nm} **(b)** and glucose consumption profiles **(c)** for *G. alkanivorans* strain 1B cultivated under different light conditions (L_0, L_{400} and L_{3000}) for 26 days. Results were obtained in triplicate and the standard deviation shown by the errors bars.

(**Table 4** (APPENDIXES)). The highest maximum growth rate (μ_{max} = 0.0259 h^{-1}) corresponding to the highest glucose consumption rate (0.0622 $g.L^{-1}.h^{-1}$) was observed for *G. alkanivorans* strain 1B grown at L_{400}.

These results point out to a different behaviour of strain 1B physiology when subjected to different light conditions, highlighting the 400 lux as the best light condition towards faster growth and higher biomass production (4.2 $g.L^{-1}$). The absence of light (L_0) and too much light (L_{3000}) decreased the sugar consumption rate (0.0541 $g.L^{-1}.h^{-1}$ for L_0 and 0.0382 $g.L^{-1}.h^{-1}$ for L_{3000}) and consequently decreased the μ_{max} (0.0235 h^{-1} for L_0 and 0.0227 h^{-1} for L_{3000}) in comparison with the corresponding culture metabolic parameters at L_{400} (**Table 4** (APPENDIXES)**).** The slightly lower growth rate observed in the culture at L_{3000} is in accordance with that observed by Silva *et al.*[14], confirming that too much light exposure may lead to a slower growth by strain 1B, possibly due to a photoinhibition.

3.2.2 - Carotenoids analysis

The next step was to analyse the carotenoid content of each time of each condition (L_0, L_{400}, L_{3000}). So, the results of the carotenoids analysis through spectrophotometry and HPLC analysis are shown in **Fig. 2** (**a** and **b**, respectively).

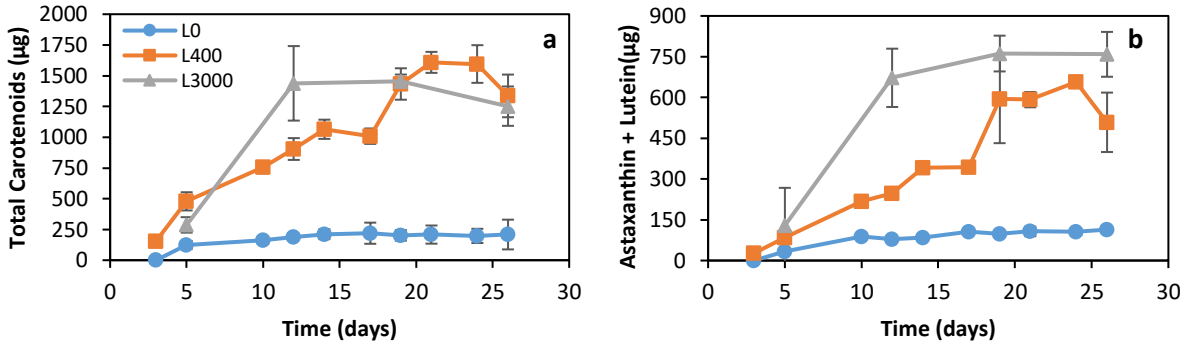

Figure 2 – Total carotenoids (µg of carotenoids per g DCW per 150 mL) produced by *G. alkanivorans* strain 1B in the assays with different light conditions (L_0, L_{400} and L_{3000}). **(a)** Amount of total carotenoids obtained through spectrophotometry analysis; **(b)** - Sum of astaxanthin and lutein, the carotenoids identified through HPLC analysis. Results were obtained in triplicate and the standard deviation is shown by the error bars.

The spectrophometry analysis in **Fig. 2a** shows that strain 1B had the lowest carotenoid content at L_0, achieving a maximum value between 212-220 µg at 14-17 days (corresponding to about 407-447 $\mu g.g_{DCW}^{-1}$). At L_{400} it had the highest carotenoid production with 1609 µg at 21 days (2596 $\mu g.g_{DCW}^{-1}$), succeeded with 1455 µg (2359 $\mu g.g_{DCW}^{-1}$) within 19 days at L_{3000}. The carotenoids production at L_0 seems to have been dependent of the growth profile of strain 1B (**Fig. 1b**), i.e. without light the production of carotenoids stopped at the 14th day which corresponds to the point where there was no more glucose available for growth (**Fig. 1c**). In contrast, this behaviour was not observed in the cultures grown with light (L_{400} and L_{3000}). At L_{400} or L_{3000}, even without any carbon source, strain 1B continued producing carotenoids (10 and 12 days, respectively, **Fig. 2a**), until the end of the assay

(26 days). This fact can be an indication that with light, strain 1B may use its energetic storage to produce carotenoids, which does not happen without light.

Further HPLC analysis (**Fig. 2b**) was carried out using different carotenoid standards available (canthaxanthin, astaxanthin, lutein, β-carotene) as an attempt to identify the type of carotenoids produced by *G. alkanivorans* strain 1B. In the tested samples, from the cultures grown at with the different light conditions, only astaxanthin and lutein were identified. The sum of these two carotenoids was higher in L_{3000} (astaxanthin + lutein = 762 µg at 19 days, corresponding to 1235 $\mu g.g_{DCW}^{-1}$) and successively decreased for L_{400} (astaxanthin + lutein = 657 µg at 24 days, corresponding to 1063 $\mu g.g_{DCW}^{-1}$) and L_0 (astaxanthin + lutein = 106 µg at 17 days, corresponding to 217 $\mu g.g_{DCW}^{-1}$).

Comparing the overall carotenoid profiles obtained from both methods, spectrophotometry (**Fig. 2a**) and HPLC (**Fig. 2b**), it could be highlighted the great discrepancy between the values obtained along the time for all the tested light conditions. HPLC analysis only expressed a small part of the total carotenoids, *i.e.* only a small amount of the total carotenoids extracted was identified as astaxanthin and lutein. Comparing the maximum values for carotenoids production obtained by HPLC analysis with the correspondent values obtained through spectrophotometry at the same times, it could be observed that the HPLC analysis accounted only for 40-48% of the total carotenoids for the culture grown at L_0, 37% of the total carotenoids for the culture grown at L_{400} and 52% of the total carotenoids for the culture grown at L_{3000}.

These results demonstrated that, for all cultures (L_0, L_{400} and L_{3000}), the production of carotenoids was time dependent, being observed a linear tendency until attained the maximum production. Thus, higher cultivation time lead to higher carotenoids content until strain 1B attained its maximum production (14-17 days for L_0; 19 days for L_{3000} and 21-24 days for L_{400}). In the case of the growth without light, contrary to the presence of light as already stated, the carotenoids production seemed to stop at the beginning of the stationary phase of strain 1B growth. Additionally, a longer incubation time may contribute for a decrease in the overall pigments content (see **Fig. 2**, profiles for L_{400} at 26 days). This effect may be due to a decrease in overall pigmented biomass, and/or to the carotenoids degradation.

Overall, the maximum carotenoids production by strain 1B was achieved in the late stationary growth phase (19–24 days) when the bacterium was cultivated in 10 $g.L^{-1}$ glucose and 22 $mg.L^{-1}$ sulfate with light. Without light, the carotenoids yield was very low in comparison with the ones obtained with light. Relatively to the overall carotenoids production on the different light intensity conditions (400 lux *versus* 3000 lux with photoperiod), in terms of spectrophotometry analysis, the maximum values were observed for L_{400}; however, this fact was probably due to the missed carotenoids content data within 12-19 days at L_{3000}. This should be further confirmed in future assays.

In **Fig. 3** (**a,b**) are presented the production time course profiles for the two carotenoids identified through HPLC analysis in the samples from cultures grown at L_0, L_{400} and L_{3000}, respectively astaxanthin (**Fig. 3a**) and lutein (**Fig. 3b**). Comparing both carotenoid profiles, there were considerable differences between the assays. The astaxanthin content was higher in biomass grown at L_{3000}, achieving a maximum of 443 µg at 19 days, followed by the biomass grown at L_{400} with 118 µg at 19 days and biomass grown at L_0 with 19 µg at 10 days. Contrary to the prior observations (**Fig. 2**), the production of astaxanthin by L_{400} culture did not increase through time as it happened with L_{3000} culture. Considering the lutein, the maximum values achieved were: 541 µg at 24 days by L_{400} culture, 397 µg at 26 days by L_{3000} culture, and around 106 µg at 26 days by L_0 culture. Thus, for the cultures grown with light, the lutein production increased through time and once more a linear correlation may be noted until the maximum values were attained. In the case of the L_0 culture, pigments production ceased when the growth stopped (14 days), as already observed in the overall net profiles (**Fig. 2a and b**). Moreover, for L_0 and L_{400} cultures the lutein maximum values obtained were higher than the maximum values of astaxanthin, whilst for L_{3000} culture the maximum astaxanthin content was higher than the maximum lutein content achieved. So, for L_0 and L_{400} cultures the major identified carotenoid was lutein, but for L_{3000} culture the main carotenoid produced was astaxanthin.

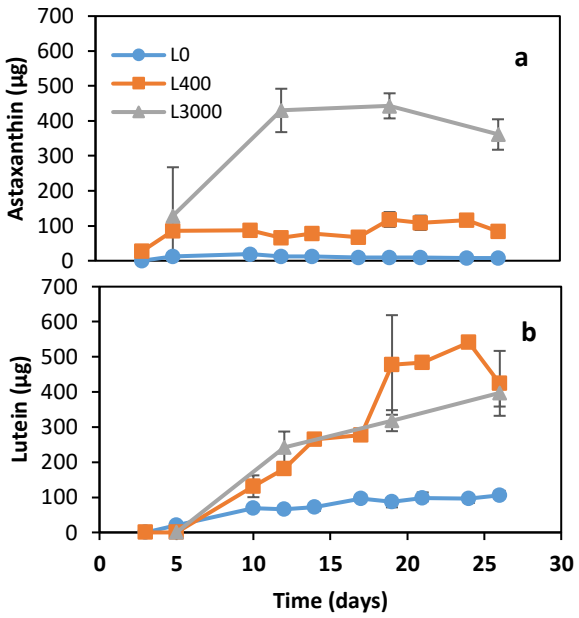

Figure 3– Amount of total carotenoids identified though HPLC analysis (µg of carotenoids per g DCW per 150mL) in the samples obtained in the assays performed with and without light (L_0, L_{400} and L_{3000}). **(a)** Astaxanthin content; **(b)** Lutein content. Results were obtained in triplicate and the standard deviation shown by the error bars.

Additionally, another assay was performed to evaluate the influence of the photoperiod on pigment production. Hence, strain 1B was further grown in 10 $g.L^{-1}$ glucose and 22 $mg.L^{-1}$ sulfate at constant 3000 lux of light, for 19 days. The result from spectrophotometry analysis obtained for the amount of total carotenoids production at 19 days was 1397 µg, which corresponds to about 3100 $\mu g.g_{DCW}^{-1}$ (*i.e.* 0.31%, where percentage is relative to g pigment per 100 g DCW). The higher overall pigment yield obtained in this assay, in comparison with that of the assay L_{3000} with photoperiod ranging from 3000 to 7000 lux (0.24% $\Rightarrow$ 2359 $\mu g.g_{DCW}^{-1}$), is mainly due to the lower value of total biomass achieved (3.1 g *vs.* 4.1 g). Besides, the net total pigments (µg) per 150 mL of culture medium were similar (1397 µg *vs.* 1455 µg $\Rightarrow \Delta$<4%). However, considering the HPLC value obtained for astaxanthin and lutein, 512 µg, a significant decrease (>30%) was observed in relation to the prior assay (762 µg, see

Fig. 2b). Thus, the photoperiod (3000-7000 lux) seemed to have induced the production of astaxanthin and lutein within the total pigments production by *G. alkanivorans* strain 1B.

In overall, the increase of carotenoids production with light exposure is in accordance to what Silva *et al*. 2016 described for strain 1B, and it has also been shown in other *G. alkanivorans* strains, such *G. alkanivorans* SKF120101[26]. In fact, the difference in the light conditions (0 to ≥3000 lux), used for the carotenoids production by *G. alkanivorans* strain 1B grown in the same optimal culture medium, greatly influenced the metabolism of the bacterium, which can be noted both by the results observed for metabolic parameters of the culture (**Table 4** (APPENDIXES)) and for the pigments profiles (**Fig. 2**). Thus, the divergence on the type and/or quantity of carotenoids produced by strain 1B seemed to be due to the overall culture conditions during the microbial growth. Probably, the total carotenoids production by strain 1B with higher astaxanthin content (L_{3000} culture) was due to an oxidative stress originated by the high light exposure (≥3000 lux). In fact, astaxanthin, among different carotenoids, is described as having a high antioxidant activity[48]. Still considering the light factor, besides the intensity, the wavelength used also influences the pigments production, type/quantity[60]. Moreover, other factor that sometimes is neglected but greatly influences the carotenoids production, especially the type of carotenoids, is the oxygenation of the culture. The carotenoids production by *Phaffia rhodozyma* was described as being highly influenced by the culture oxygenation, increasing with the enhancement of the respiration activity of this yeast. So, for higher astaxanthin production yield by *P. rhodozyma*, the increase of shaker speed and the decrease of liquid volume in the flasks are two important operating variables. Oxygenation is especially important for the formation of pigments with oxy-groups, such as astaxanthin and canthaxanthin[12].

Comparing the best results obtained for the total amount of carotenoids production by *G. alkanivorans* strain 1B in this study (2596 $\mu g.g_{DCW}^{-1}$ - 3100 $\mu g.g_{DCW}^{-1}$, corresponding to pigment yields of 0.26%-0.31%) with the best result reported by Silva *et al.* (2016), (2015 $\mu g.g_{DCW}^{-1} \Rightarrow$ 0.20%), it can be stated that some optimization was achieved (up to 35% of pigments increase), but further assays should still be carried out considering the influence of other important factors, such as oxygenation, photoperiod light/dark, light wavelength, extraction method, between others. However, relatively to the type/quantity of carotenoids identified in the overall pigments produced by strain 1B, through HPLC analysis, the results of this study differ significantly from those observed by Silva *et al.*[7]. This fact needs further confirmation with more HPLC analysis, nevertheless, it may be due to the difference in the strain 1B cultivation conditions, such as: amount of sulfate as S-source; light conditions (wavelength/intensity); oxygenation. In this study, the absence of detection of canthaxanthin may be also due to the fact that this pigment is hydroxylated towards astaxanthin, following a different production pathway in the biosynthesis of carotenoids[31].

Considering carotenoids production data reported for other species of the genus *Gordonia,* the most relevant includes *G. jacobaea,* whose wild-type produced 227 $\mu g.g_{DCW}^{-1}$ of carotenoids and its mutant 2500 $\mu g.g_{DCW}^{-1}$ [61]; and *G. ajoucoccus* A2T that produced 2900 $\mu g.g_{DCW}^{-1}$ of carotenoids in a bioreactor under optimized conditions with hexadecane[62]. These optimized results are very similar to the values obtained in this study for *G. alkanivorans* strain 1B (2596-3100 $\mu g.g_{DCW}^{-1}$), even with few optimizations towards maximum carotenoids production, highlighting the great potential of strain 1B towards its further exploitation as a hyper pigment-producer strain that may be applied into different industrial sectors.

3.2.3 - Evaluation of cell physiological state by flow cytometry

There are several studies describing the direct correlation between stress conditions and pigment production[10,11,50]. In this context, an evaluation of the cells physiological state during the time course of the growth and carotenoids production assays at different light conditions (L_0, L_{400} and L_{3000}) was performed using flow cytometry. This technique provides a quantitative measurement of individual cells per sample by the study of the light scatter and fluorescence emission properties[55,63]. Staining with different colorants allows to assess different physiological aspects of each cell. For this work two stains were used: CFDA, which allows the identification of the healthy cells (cells uptake the stain and convert it into its fluorescent form); and PI (an intercalating agent), that grants the identification of the dead cells because it bonds with the nucleic acids, and that only happens if the cell has its membranes compromised[64]. When there is simultaneous stain with both compounds, it represents the stressed cells, because even though cells are metabolically active they have membrane damage. The results obtained from flow cytometry analysis, showing the variation of the three different cell populations (healthy cells, stressed cells and dead cells) distinguished during the time course of each assay are presented in **Fig. 4**. These results show that the three cultures, from L_0, L_{400} and L_{3000}, have a similar overall

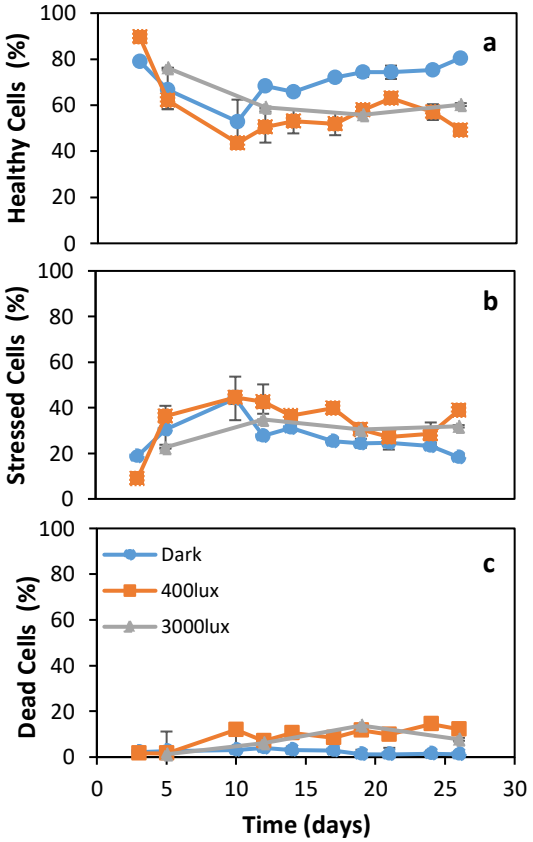

Figure 4 - Flow cytometry analysis through the time course of growth and pigments production of *G. alkanivorans* strain 1B with 10 $g.L^{-1}$ glucose and 22 $mg.L^{-1}$ sulfate with different light conditions (L_0, L_{400} and L_{3000}). **(a)** Represents the percentage of healthy cells; **(b)** the percentage of stressed cells; and **(c)** the percentage of dead cells. Results were obtained from duplicates and each analyzed 6 times. The populations of cells were assessed with the use of PI, which marked the dead cells; and CFDA, which allowed the assessment of the healthy cells. The cells marked with both compounds were labeled as stressed due to the fact that they are still metabolically active but have their membranes damaged.

behaviour, *i.e.* in the beginning, until the C-source was consumed (~10 days), there was a significant decrease in the % of healthy cells: from 79% to 53% in L_0 culture; from 90% to 44% in L_{400} culture; and from 76% to ≤56% (19 days, since there is no data for 10 days) in L_{3000} culture; with a simultaneously increase in the % of stressed and/or dead cells. After this period, the cultures seemed to improve their physiological state becoming healthier, achieving about 81%, 49% and 60% of healthy cells at 26 days, respectively in L_0, L_{400} and L_{3000} cultures (**Fig. 4a**). Also at 26 days, the correspondent % of stressed/dead cells observed were: 18%/1%, 39%/12% and 32%/8% respectively for L_0, L_{400} and L_{3000} (**Fig. 4b, 4c**). From these results, it can be stated that the highest % of dead cells was observed for L_{400} culture ($\Delta_{3\text{-}26\ days}$: 2 to 12%), followed by the L_{3000} culture ($\Delta_{3\text{-}26\ days}$: 1 to 8%). The culture grown at dark was the one with the lowest % of dead cells within 26 days ($\Delta_{3\text{-}26\ days}$: 2 to 1%).

Overall, it seems that the absence of light benefits the healthy state of the cells. This can be due to the fact that without light (L_0) strain 1B produces only little quantities of pigments (up to 447 $\mu g.g_{DCW}^{-1}$) and, therefore, the cells do not use their storage substances for that secondary metabolism. For this reason, the cells stay healthier. On the other hand, with light where the bacterium produced great amounts of pigments, even after they stopped growing, the use of cell reserves may influence its overall physiological state and, consequently, the cells become more stressed and/or dead in comparison with those of the culture grown at dark. From **Fig. 4b** and **4c** it could be observed that the highest values for stressed/dead cells were obtained for the cultures grown at light (L_{400} and L_{3000}). At the end of the assays (26 days), the % of healthy cells in L_0 culture was about 26 to 40% higher than in the L_{400} and L_{3000} cultures, respectively (**Fig. 4a**), the two cultures with the highest carotenoid content. However, after 26 days, there were still ≥54% of healthy cells of strain 1B for all conditions.

3.2.4 - Antioxidant activity

To evaluate the potential of each carotenoids extract, obtained from the *G. alkanivorans* strain 1B biomass grown with different light conditions (L_0, L_{400} and L_{3000}), for further application as antioxidant, the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical scavenging activity assay was performed. This assay is based on the reduction of the methanolic DPPH radical ($DPPH^{\bullet}$) in the presence of a hydrogen-donating antioxidant. A $DPPH^{\bullet}$ solution has an absorption band at 517 nm and an intense violet colour. When in presence of an antioxidant compound the absorbance of this solution decreases and the violet colour is lost. The scavenging capacity is inverse to the remaining $DPPH^{\bullet}$[58,65]. In this set of assays, since the ascorbic acid is one of the commonly used standards as antioxidant compounds (due to its high antioxidant activity and its human application), two concentrations of ascorbic acid, 500 $\mu g.L^{-1}$ and 1000 $\mu g.L^{-1}$, were prepared and used as positive controls for comparison with the antioxidant potential of different extracts obtained from cultures grown at dark, 400 lux and 3000 lux. The samples selected for antioxidant activity determination were those corresponding to the highest carotenoids production

in each condition, and presented the following concentrations of total carotenoids: 478 µg.L^{-1} in the extract from L_0 culture; 1050 µg.L^{-1} in the extract from L_{400} culture and 969 µg.L^{-1} in the extract from L_{3000} culture.

In **Fig. 5** are presented the results of the DPPH• method based assays for the three extracts and the two samples of ascorbic acid. These results show that the ascorbic acid at 500 µg.L^{-1} and 1000 µg.L^{-1} was able of a DPPH• discoloration of 1.8% and 7.0%, respectively, after 10 min. For the tested extracts, with a total carotenoids ranging from 500 µg.L^{-1} and 1000 µg.L^{-1}, values of % of DPPH• discoloration below 3.4% were achieved, after 10 min. Within the extracts, the extract from L_0 culture was the one that exhibited the higher antioxidant activity (3.4% after 10 min). However, in a crude extract may be present other extractives than pigments that can change the overall antioxidant potential of any bioactive compound present. Further studies must be carried out using using the purified pigments.

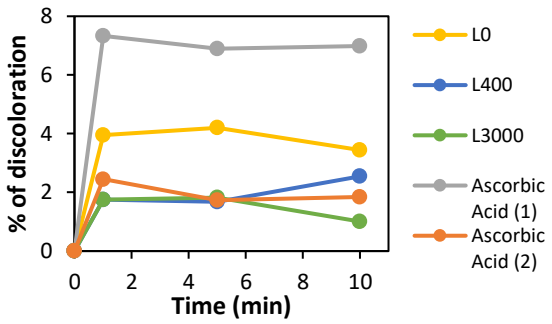

Figure 5 – Antioxidant activity of different carotenoids extract samples and standards represented by the percentage of discoloration of DPPH•. Extracts presented the following concentrations of total carotenoids: 478 µg.L^{-1} for the extract from L_0 culture; 1050 µg.L^{-1} for the extract from L_{400} culture and 969 µg.L^{-1} for the extract from L_{3000} culture. The ascorbic acid solutions presented the following concentrations: (1) – 1000 µg.L^{-1} and (2) – 500 µg.L^{-1}.

3.2.5 - Antimicrobial activity

In order to evaluate if the extracts of the carotenoids produced by strain 1B, in different culture conditions, present any bioactive compounds, for example compounds that could be used as antimicrobials, antimicrobial activity tests, based on the disc diffusion method, were performed for two microorganisms, *Pseudomonas putida* and *Escherichia coli*. Without any inhibition halo formation developed around the discs containing the three different carotenoids extracts, the results point out for the absence of antimicrobial activity. This could be due to the concentration of the bioactive compounds present in extracts, which may be insufficient to have an effect. Thus, further tests should be performed using more concentrated extracts.

4 – CONCLUSION

This main goal of this project was to optimize the biological production of carotenoids by *G. alkanivorans* strain 1B. The valorisation of this added-value product would make the overall BDS process more cost efficient.

The first step was an assessment of the ideal carbon source and sulfate concentration for pigment production. An experimental design was performed considering two variables: glucose concentration in a fructose-glucose mixture and sulfate concentration. The assay was performed without light and

under 400lux. It showed that, in both cases, the higher yield of pigment production was achieved in the longer fermentation assays (216 h) and using 100% and 22 $mg.L^{-1}$ of glucose and sulfate, respectively. Furthermore, the culture grown with light achieved the highest values of carotenoid production, demonstrating the importance of light. This assay demonstrated that time, glucose and sulfate concentration have great impact in the carotenoid production by strain 1B.

Based on the results obtained, the next step was to make an evaluation of the influence of time in the carotenoid production. Three assays were performed without light (L_0), 400 lux (L_{400}) and a base value of 3000 lux together with photoperiod (L_{3000}). L_{400} had higher μ_{max} than the ones grown with light, 0.0259 h^{-1} comparatively to 0.0235 h^{-1} and 0.0227 h^{-1} for L_0 and L_{3000}, respectively. Biomass production was higher in the cultures with light achieving a value of 4.2 $g.L^{-1}$ comparatively to the value of 3.5 $g.L^{-1}$ obtained by both cultures grown without light. As expected, L_0 had the lowest carotenoid yield of the three, achieving a value of only 212-220 µg. L_{400} and L_{3000} obtained 1609 µg and 1455 µg, respectively, these being the best results obtained so far with strain 1B. In terms of time assessment, carotenoid yield increased through time in the cultures with light, but this did not occur in L_0.

The carotenoids obtained were subjected to further analysis in order to achieve a possible identification. Of the carotenoids obtained only astaxanthin and lutein were identified, even though these represented only a low percentage of the total carotenoids extracted.

Considering the literature about stress potentiating carotenoid production, a determination of the physiological state of the cells was performed using flow cytometry. The major carotenoid producers (light grown) had higher percentages of stressed cells and dead cells than L_0, which might confirm the relation between stress and carotenoid production. The major difference was found in the healthy cell population where, for L_0, this population increased throughout time oppositely to the cultures grown with light.

Taking into account the properties of carotenoids, especially their antioxidant activity, the extracts of the previously described assays were used for an antioxidant activity assessment. The extract with higher activity was the one from L_0, which had higher activity than the ascorbic acid (standard) with similar concentration. The other extract had lower activities. The antimicrobial activity of the extracts was also tested and, the results showed no such activity.

In conclusion, these results are a reflection of the great potential of *G. alkanivorans* strain 1B to have an economically viable application in the BDS process on an industrial level, due to the high added value of carotenoids. This initial optimization, made possible to achieve the highest values of production of carotenoids so far, for this strain, which demonstrates the potential of strain 1B.

5 – FUTURE WORKS

Future perspectives for *G. alkanivorans* strain 1B are further optimization of the conditions to produce carotenoids, namely, the extraction process and testing other key factors that may have influence in its production (oxygenation, medium salts, different/alternative carbon sources, among others). It would also be important to further investigate the specific metabolic pathway of the carotenoid production in this microorganism. In the BDS context, it is fundamental to associate this process with pigment production, assessing the best condition for a high yield in both. Lastly, it is important to perform an upscale of the process, using a bioreactor in order to mimic the process on an industrial level.

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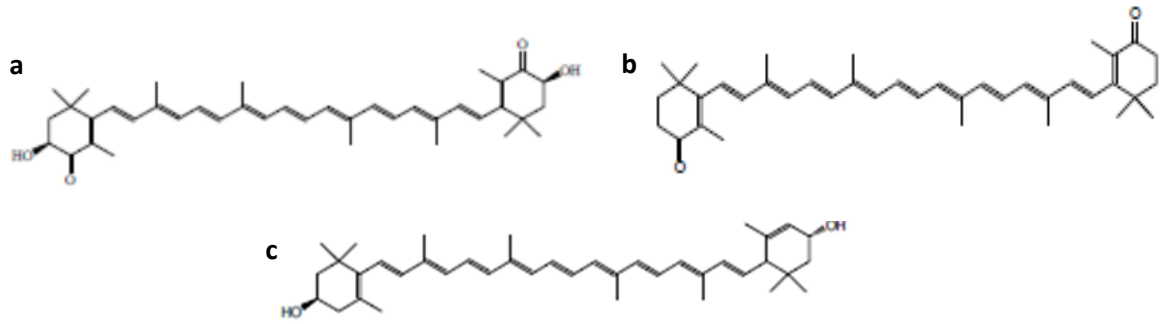
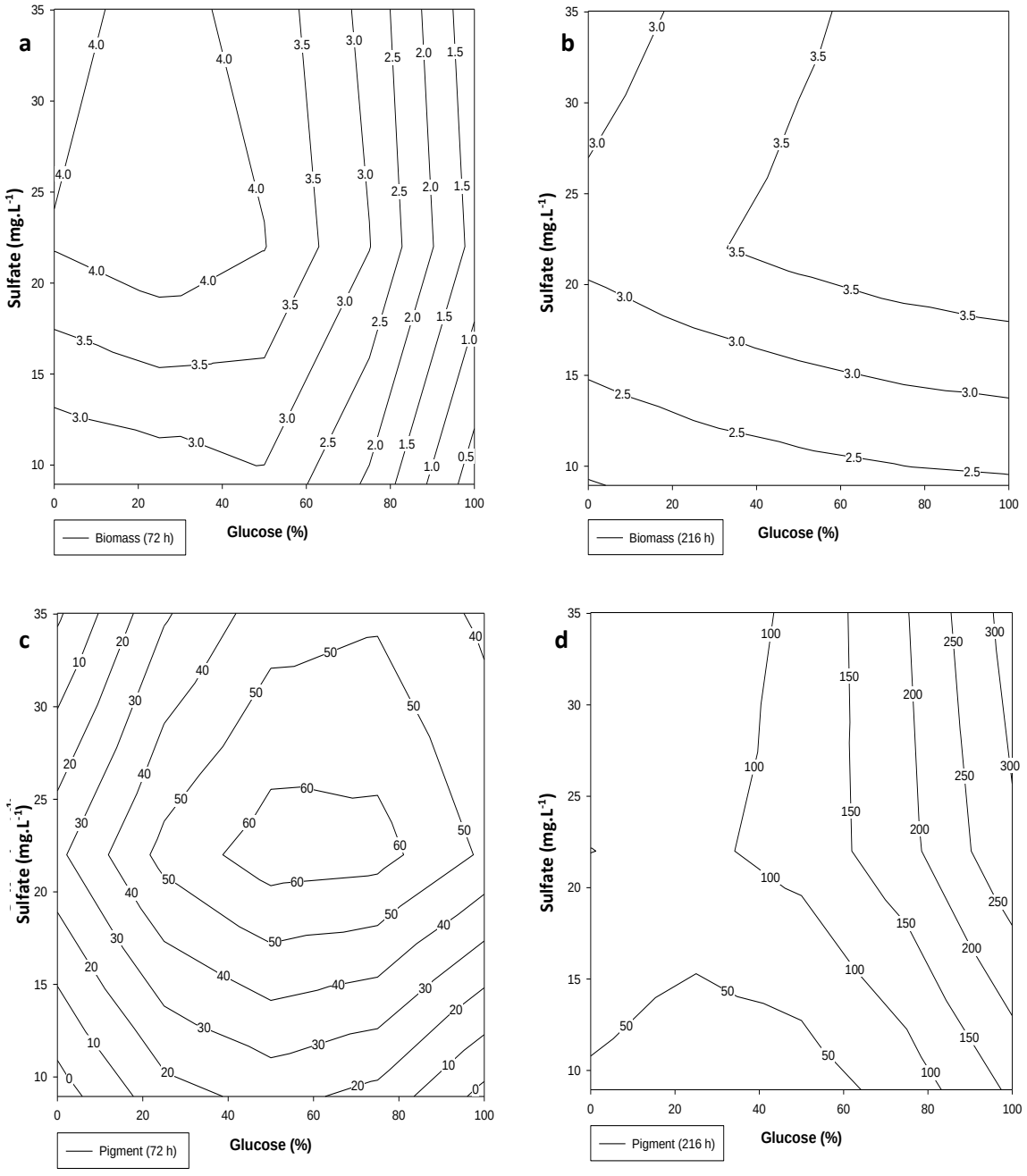
7 – APPENDIXES



Supplementary Figure 1 - Chemical structure of **(a)** Astaxanthin; **(b)** Canthaxanthin and **(c)** Lutein. Adapted from Tanaka *et al.* (2012)[i](43)[i].

Table 1- ED1 (L_0) according to a Doehlert distribution for two factors: % of glucose in a mixture glucose + fructose (0-100%) and sulfate concentration (7-37 $mg.L^{-1}$), and the responses evaluated (biomass and total carotenoids). Seven conditions were tested in duplicates (14 tests), for statistical analysis.

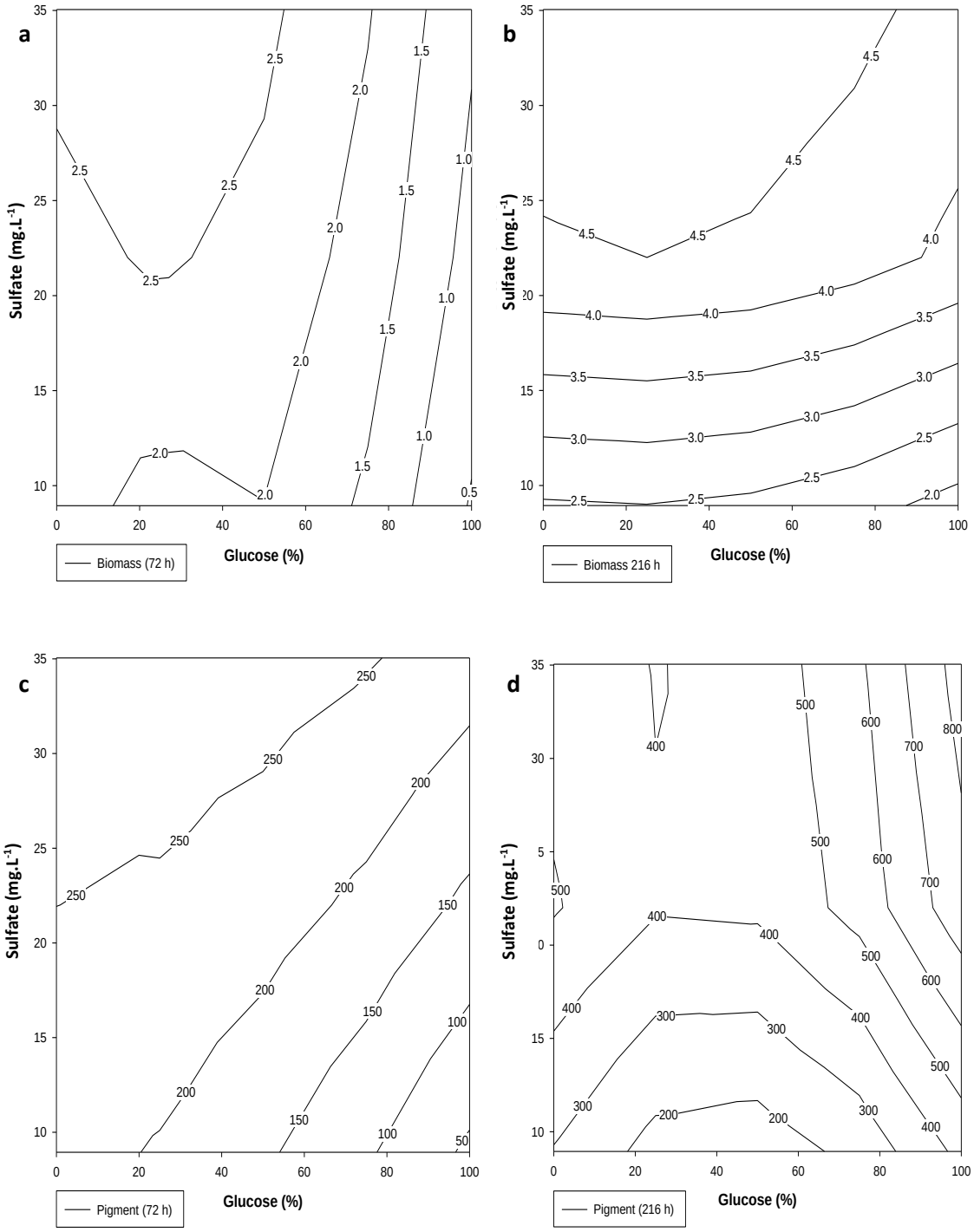
Test number	Glucose (%)	Sulfate ($mg.l^{-1}$)	72 h		216 h	
			Biomass ($g.L^{-1}$)	Total Carotenoids (µg)	Biomass ($g.L^{-1}$)	Total Carotenoids (µg)
1	**50**	**22**	3.87	74.6	3.53	110.2
2	**50**	**22**	4.17	56.2	3.76	125.5
3	**100**	**22**	0.81	40.6	4.06	336.8
4	**100**	**22**	0.63	39.7	3.90	285.3
5	**0**	**22**	4.06	31.8	3.25	92.6
6	**0**	**22**	5.25	40.3	3.05	69.0
7	**75**	**34.99**	3.39	52.8	3.79	185.0
8	**75**	**34.99**	3.11	61.3	3.59	171.4
9	**25**	**9.01**	1.99	5.1	2.19	34.0
10	**25**	**9.01**	2.87	11.0	2.13	31.6
11	**75**	**9.01**	2.55	19.4	2.45	58.7
12	**75**	**9.01**	2.93	31.6	2.30	45.7
13	**25**	**34.99**	3.52	20.3	3.23	84.4
14	**25**	**34.99**	3.99	21.1	2.99	87.1



Supplementary Figure 2- Response surfaces for the biomass production at 72 h **(a)** and 216 h **(b)**; and for the pigment production at 72 h **(c)** and 216 h **(d)**, obtained in ED1 (L_0) for the factors % glucose in a fructose-glucose mixture (0 – 100%) and sulfate concentration (7 – 37 $mg.L^{-1}$)

Table 2 - ED2 (L_{400}) according to a Doehlert distribution for two factors: % of glucose in a mixture glucose + fructose (0-100%) and sulfate concentration (7-37 $mg.L^{-1}$), and the responses evaluated (biomass and total carotenoids). Seven conditions were tested in duplicates (14 tests), for statistical analysis.

Test number	Glucose (%)	Sulfate ($mg.L^{-1}$)	72 h		216 h	
			Biomass ($g.L^{-1}$)	Total Carotenoids (µg)	Biomass ($g.L^{-1}$)	Total Carotenoids (µg)
1	**50**	**22**	2.42	230.8	4.20	400.5
2	**50**	**22**	2.30	215.4	4.65	435.8
3	**100**	**22**	0.32	103.7	4.17	912.7
4	**100**	**22**	0.40	82.6	3.27	675.6
5	**0**	**22**	2.86	290.2	4.55	462.0
6	**0**	**22**	2.84	303.1	4.63	493.8
7	**75**	**34.99**	2.42	286.8	5.00	592.5
8	**75**	**34.99**	2.80	315.4	4.57	514.4
9	**25**	**9.01**	1.83	104.0	2.39	136.3
10	**25**	**9.01**	1.75	195.2	2.30	257.0
11	**75**	**9.01**	1.81	151.6	2.43	204.2
12	**75**	**9.01**	1.79	155.8	2.27	197.7
13	**25**	**34.99**	2.48	225.0	4.71	427.0
14	**25**	**34.99**	1.99	235.3	4.71	427.0



Supplementary Figure 3 - Response surfaces for the biomass production at 72 h **(a)** and 216 h **(b)**; and for the pigment production at 72h **(c)** and 216 h **(d)**, obtained in ED2 (L_{400}) for the factors % glucose in a fructose-glucose mixture (0 – 100%) and sulfate concentration (7 – 37 mg.L^{-1}).

Table 3 - Parameters of the polynomial models representing the studied responses in ED1 (L_0) and ED2 (L_{400}). β0, response at the centre of the experimental domain; β1 and β2, parameters of the factors 1 and 2 (% glu and sulfate concentration), respectively; β12, parameter of the interaction of the factors 1 and 2; β11 and β22, self-interaction parameters of the factors 1 (% glucose) and 2 (sulfate concentration, $mg.L^{-1}$), respectively).

ENVIRONMENTAL CONDITIONS		DARK (L_0)				LIGHT (L_{400})			
	Growth time (h)	**72**		**216**		**72**		**216**	
	Model	**Biomass**	**Total Pigment**	**Biomass**	**Total Pigment**	**Biomass**	**Total Pigment**	**Biomass**	**Total Pigment**
PARAMETERS	**β0**	4.02	65.4	3.65	117.87	2.36	223.14	4.43	418.21
	β1	-1.34	10.33	0.41	95.39	-0.77	-55.33	-0.1	137.27
	β2	0.53	12.76	0.65	51.66	0.36	65.8	1.08	150.83
	β12	-0.47	10.91	0.21	42.17	0.21	38.59	0.65	105.36
	β11	-1.33	-27.3	-0.08	78.06	-0.76	-28.23	-0.27	217.83
	β22	-0.86	-41.01	-1.06	-66.86	-0.08	-9.94	-1.43	-191.01
FISCHER TEST	**Efectiveness of parameters**	4.04	5.64	73.95	25.50	2.89	2.47	4.17	15.88
	Significance level (%), F(5, 8)	0.04	0.02	0.001	0.001	0.09	0.12	0.04	0.001
	Lack of fit	23.68	17.25	0.02	17.17	93.51	35.82	3.29	0.78
	Significance level (%), F (1, 7)	0.001	0.004	>0.1	0.004	0.001	0.0005	>0.1	>0.1
R^2	**Coeficient of multiple determination**	0.72	0.78	0.98	0.94	0.64	0.61	0.72	0.91

Table 4 - Metabolic parameters for the different light culture conditions of *G. alkanivorans* strain 1B grown in shake-flask with 10 $g.L^{-1}$ glucose and 22 $mg.L^{-1}$ sulfate.

Metabolic Parameters	Condition		
	L_0	L_{400}	L_{3000}
μ_{max} (h^{-1})	0.0235	0.0259	0.0227
Maximum sugar consumption rate ($g.L^{-1}.h^{-1}$)	0.0541	0.0622	0.0382
Maximum biomass production rate ($g.L^{-1}.h^{-1}$)	0.0342	0.0333	0.0214