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RESEARCH PAPER

Influence of culture conditions towards optimal carotenoid production by *Gordonia alkanivorans* strain 1B

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Abstract With the increasing awareness on the toxicity of several synthetic dyes, demand for pigments from natural sources, such as microbial carotenoids, has gained interest as a promising safe alternative colour additive. In this study, a surface response methodology based on the Doehlert distribution for two factors [% of glucose in a mixture of glucose + fructose (10 g/L total sugars), and sulfate concentration] was used towards the optimal carotenoids production by *Gordonia alkanivorans* strain 1B in the presence of light (400 lx). Time influence on pigment production by this bacterium was also evaluated, as well as the cell viability profile during longer incubation periods at optimal conditions. Indeed, the highest carotenoid production (2596–3100 μg/g_{DCW}) was obtained when strain 1B was cultivated in the optimal conditions: glucose 10 g/L and sulfate ≥ 22 mg/L, in the presence of light for 19 days at 30 °C, 150 rpm. Flow cytometry showed that the highest production was somehow related with the cellular stress. These results highlight the great potential of strain 1B as a new hyperpigment producer to be exploited towards several applications.

Keywords *Gordonia alkanivorans* strain 1B · Surface response methodology · Doehlert distribution · Carotenoid production · Flow cytometry

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Introduction

Carotenoids are mostly known as nutritionally beneficial organic pigments naturally occurring in the plastids of photosynthetic organisms (plants, algae, and phototrophic microorganisms). However, these molecules can also be found in some non-photosynthetic organisms, such as bacteria, moulds, and yeasts, where their main function is to protect the cell from the damage caused by oxygen and light. Carotenoids are liposoluble pigments responsible for the colours of plants, algae, some animals, and microorganisms, ranging from yellow to orange, pink, or red according to their chemical structure [1–4].

Typically, carotenoids are C_{40} tetraterpenoids formed by eight C_5 isoprenoid units joined head to tail and have a tail-to-tail linkage in the centre, resulting in a symmetrical structure. These molecules 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 obtained from the isoprenoid pathway [2, 5]. Carotenoids can be divided into two main classes: (a) carotenes, which are purely hydrocarbons (β-carotene, torulene, α-carotene, and lycopene) and (b) xantophylls, which are similar, but present functional groups containing oxygen: 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) [2, 4, 6, 7].

Other than their colour and light harvesting properties, carotenoids have diverse bioactive and chemical properties. These compounds can present antioxidant activities, with specific roles or general health-promoting roles that reduce the risk or progression of diseases associated with oxidative stress. They can be precursors to vitamins, have

antitumor and antimicrobial properties, and act as oxygen transporters, etc. [2, 8–11]. Indeed, there is a vast market for carotenoids, generating interest in industries such as pharmaceutical, nutraceutical, food/feed additive, textile, cosmetics, and fine chemical sectors, which results in an estimated market value of about $1.8 billion in 2019 [12].

Carotenoid production, in sectors that directly or indirectly may influence the human health (e.g., food industry), is strictly regulated because of the potential toxicity of the synthetically derived pigments [3, 13]. Indeed, carotenoids produced by microorganisms are nontoxic and the use of microorganisms (microalgae, yeasts, and bacteria) as pigment-producers have several advantages. Unlike plants, microorganisms have a short life cycle and are unaffected by the season/climate and geographical conditions, making it easier to produce carotenoids worldwide. Microorganisms can also produce colour shades that are not found in plants; have high productivity; and they are relatively easy of genetically manipulation towards improved fermentation and scale-up process. Moreover, microbial pigments are usually easy to extract [3, 8, 11, 14]. Thus, microbial pigments are in increasing demand, since they are a promising natural and safe alternative source for various industrial applications.

More than 750 carotenoids, have been isolated and characterized, both from eukaryotes and prokaryotes, and novel carotenoids are still found every year [3, 8, 15, 16]. Of these, only a few, such as β-carotene and astaxanthin, are produced commercially through microbial cultivation, because of the high production costs associated with this process. However, much like in other biotechnological processes, these costs may be reduced by increasing the carotenoids yield, through the optimization of the production conditions for the microorganism, and/or lowering the costs of the culture medium using cheap alternative C-sources as nutrients (e.g., industrial byproducts and wastes).

Gordonia alkanivorans strain 1B is a widely known biodesulfurizing bacterium [17–21], which was recently described also as a carotenoid-producer, with canthaxanthin, lutein, and astaxanthin as the major identified carotenoids on crude extract [22]. Despite of the demonstrated frutophilic character of strain 1B [19], Silva et al. [22] pointed out for glucose and sodium sulfate as the best carbon (C) and sulfur (S) sources, respectively, for pigment production by this microorganism.

Hence, the present study was focused in the optimization of culture conditions towards the carotenoids production by *G. alkanivorans* strain 1B, using a surface response methodology based on the Doehlert distribution for two factors (% of glucose in a mixture of glucose + fructose (10 g/L total sugars) and sulfate concentration). Furthermore, time influence on pigment production by this bacterium was also evaluated as well as the cell viability profile during growth at optimal C- and S-sources conditions, but with different light exposure (0–3000 lux).

Materials and methods

Chemicals

Sodium sulfate anhydrous (> 99%) was obtained from Merck (New Jersey, USA). 5(6)-carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) were acquired from Invitrogen (Massachusetts, USA). DMSO (99.9%), acetone (99.9%), ethyl acetate (99.8%), and methanol (99.9%) were obtained from CARLO ERBA Reagents (Val de Reuil, France). The remaining chemicals were of the highest grade commercially available. Stock solutions of glucose (glu) and fructose (fru) were prepared at 50% (w/v), 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 was also prepared and autoclaved (121 °C, 1 atm, 15 min) to be further used as sulfur source (S-source).

Microorganism and culture media

The microorganism used in this study was *Gordonia alkanivorans* strain 1B [17], a bacterium previously isolated in our laboratory and kept at a culture collection of microorganisms (CCM at LNEG, Portugal, Lisbon). The basal salt medium used for cultivation, maintenance, and growth/carotenoid 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 [18]. The final pH was adjusted to 7.5 prior to sterilization by autoclave (121 °C, 1 atm, 15 min).

Thereafter, the carbon source (C-source) (fructose and/or glucose) was added sterilized to the culture medium, in aseptic conditions, to an initial concentration of 10 g/L 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, 22, and 34.99 mg/L, depending on the assay.

Carotenoid production assays

The bacterium was cultivated at 500 mL Erlenmeyer shake-flasks containing 150 mL culture medium, which were 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. Light intensity was measured using a TES-1330 digital light meter (TES Electrical Electronic Corp., Taiwan, ROC), and the shake-flasks were organized to ensure equal light

exposure. For all the assays, 2% (v/v) inoculum was used. This inoculum consisted on a strain 1B culture grown in SFM medium supplemented with a mixture of 5 g/L fructose and glucose (ratio 1:1) as C-source and 150 µM DBT as S-source, at 30 °C for about 10 days.

Different assays were performed: (1) a set of experimental design tests to establish the optimal conditions for the carotenoids production by *G. alkanivorans* strain 1B (C-source vs. sulfate concentration) under light (400 lux; (2) assays for the evaluation of the influence of growth time to the carotenoids production (assays at light: 400 lux and 3000 lux + photoperiod); (3) an assay with a stable value of 3000 lux for 19 days. All the assays were carried out at least in duplicate.

For the experimental design, two samples were collected, one during the exponential phase and one at the end of the growth (late stationary phase). However, for the assays evaluating the influence of time, a shake-flask was collected per each time analysed. This procedure avoid significant changes in overall culture volume, when an extend time period is tested. Aliquots of the samples were immediately analysed to evaluate: cell growth/biomass; sugar(s) consumption; and cell physiological state. The leftover 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.

Surface response methodology

A surface response methodology (SRM), based on the Doehlert distribution for two factors [23], was used towards optimal carotenoids production by *G. alkanivorans* strain 1B, in the presence of 400 lux light. In this experimental design, the explanatory variables or factors studied and the respective experimental domains tested were: % of glucose in a mixture glucose + fructose of 10 g/L of total sugars (*X*1: 0–100% glu in the mix) and sulfate concentration (*X*2: 7–37 mg/L of sodium sulfate). Fourteen experiments (seven conditions in duplicate) were carried out and the responses studied (Yi) were: biomass (g/L) and total pigments production (µg) by strain 1B, at 72 and 216 h. The model used to express the responses was a second-order polynomial model: $Yi = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_{12} + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2$, where: Yi—response from experiment *i*; β—parameters of the polynomial model; and *X*—experimental factor level [21, 24].

Carotenoid extraction

The centrifuged biomass samples from the different assays were thawed at room temperature and completely isolated from light exposure. Each biomass was distributed as uniformly as possible on a Petri dish and dried at 55 °C, for a period of 15–60 min, to reduce humidity to approximately 60%. Portions of about 25 mg of the dried biomass 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 sample in the microcentrifuge tubes and incubated on an orbital incubator at 50 °C for 45–60 min. The tubes were centrifuged at 14,300*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.

Then, the DMSO containing the pigment was mixed with acetone, a NaCl solution at 20% v/v and ethyl acetate in a proportion of 1:1:6, respectively, for each 4 mL of extracted supernatant. 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, 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 − 20 °C 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 − 20 °C until further carotenoids analysis by spectroscopy. Throughout the different steps of the extraction process, the samples were covered with aluminium foil to prevent carotenoids degradation due to light exposure.

Analytical methods

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 Spectrophotometer, 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 100 °C overnight.

Sugar consumption evaluation

The concentration of fructose and/or glucose was determined using HPLC (LaChrom Merck/Hitachi, Germany), equipped with a differential refractive index detector and a Waters SugarPak 1 column (6.5 × 300 mm, Bio-Rad Laboratories, CA, USA), operating at 75 °C with Ca-EDTA at 50 mg/L as mobile phase with a flow rate of 0.5 mL/min.

The data obtained were analysed with Chromeleon software ver. 6.40 SP6 build 783 (1994–2003, Dionex).

Flow cytometry analysis

The cell 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 were 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 Da Silva et al. [25] and Teixeira et al. [26]. 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 s. The concentration of the cell 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, instrument 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) and 200 µM of DBT were used as the healthy cell control. For the dead cell control, healthy cells were incubated in ethanol at 70% (v/v) for 1 min [25].

Pigment analysis

The characterization of the pigments produced by *G. alkanivorans* strain 1B grown in the different culture conditions was performed by spectroscopy analysis. Therefore, 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. [27], but using the value of 2091.4 L 10 g/cm for the specific optical extinction coefficient at $\lambda = 477$ nm (wavelength of the maximum absorbance of canthaxanthin in ethyl acetate) [22]. The pigment results were presented as µg of total carotenoids produced (µg carotenoids per g DCW in 150 mL).

Results and discussion

Surface response methodology

Aiming to define the optimal conditions for carotenoid production by strain 1B, an experimental design (ED), based on the SRM according to the Doehlert distribution [23], was carried out to evaluate the influence of two factors: % of glucose in a mixture of glucose + fructose (10 g/L total sugars) or glucose ratio (factor 1), and sulfate concentration (factor 2), on the biomass production and on the total pigment production. Table 1 shows the set of tests performed within the ED and the obtained responses (biomass (g/L) and total pigment production (µg), both after 72 h and 216 h).

Considering the culture biomass at 72 h, tests 1–6 showed the effect of varying glucose concentration for a constant sulfate concentration at the centre of the experimental domain (22 mg/L). Increasing glucose from 0 to 5 g/L (50% glu), the biomass concentration was decreased by 17% (from 2.85 to 2.36 g/L). When the concentration of glucose was increased to 10 g/L (100% glu), the biomass value was reduced about 87% comparatively to that of 0% glucose (i.e. from 2.85 g/L to 0.36 g/L). On tests 7–14, with a sulfate concentration of 9.01 or 34.99 mg/L, 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 (tests 9–10, 13–14) originated a biomass increase of 25% (from 1.79 to 2.24 g/L). On tests 7–8 and 11–12, with 75% glucose, the sulfate boost from 9.01 to 34.99 mg/L culminated in a biomass increase of 45% (1.80 to 2.61 g/L; tests 7–8, 11–12).

Higher % of glucose in culture medium have resulted in lower growth rates by the bacteria and consequently in reduced biomass values. Moreover, for lower values of sulfate (9.01 mg/L), 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 of sulfate.

Considering the pigment production, at 72 h, tests 1–6 showed that increasing glucose from 0 g/L (0% glu) to 5 g/L (50% glu) resulted in a decrease of 25% of total pigment (from 296.7 to 223.1 µg). Moreover, an increase to 10 g/L (100% glu) ended in a reduction of 69% of total pigment content, respectively from 296.7 to 93.2 µg. With a sulfate concentration of 9.01 mg/L (tests 9–10, 11–12),

Table 1 ED according to a Doehlert distribution for two factors: % of glucose in a mixture glucose + fructose (0–100%) and sulfate concentration (7–37 mg/L), and the responses evaluated (biomass and total carotenoids)

Test (#)	Factors		Responses-72 h		Responses-216 h	
	Glucose (%)	Sulfate (mg/L)	Biomass (g/L)	Total carotenoids (μg)	Biomass (g/L)	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

Seven conditions were tested in duplicates (14 tests), for statistical analysis

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 ratio was performed in a medium with 34.99 mg/L of sulfate, the pigment accumulation was improved by 31% (from 230.2 to 301.1 μg; tests 7–8, 13–14). With glucose at 25% of the total, and sulfate increased from 9.01 to 34.99 mg/L, there was an increase of 54% (149.6 to 230.2 μg; tests 9–10, 13–14). 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; tests 7–8, 11–12) of pigment accumulation.

Both factors are important for pigment production at 72 h and there is equilibrium in the importance of both sugars used for the growth. With a constant concentration of sulfate (22 mg/L), 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 ratio may be a solution to achieve high pigment values.

Analysing the same conditions after 216 h, the effect of varying the glucose ratio (0–100% in the carbon sources mixture) while maintaining sulfate concentration at constant value (the centre of the experimental domain, 22 mg/L) was studied (tests 1–6). When glucose was raised from 0 to 5 g/L, biomass had no relevant change (4.59 and 4.40 g/L, respectively). Despite of the increase of the glucose to 100%, the 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 (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 resulted in a twofold 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 (22 mg/L) still able of producing high values of biomass.

Taking into account pigment production response, using sulfate at 22 mg/L and changing the glucose ratio from 0 to 50% resulted in a pigment decrease of 13% (from 477.9 to 418.2 μg; tests 1–2, 5–6), whilst changing glucose ratio from 0 to 100% led to an increase of 66% (477.9 to 794.2 μg; tests 3–4, 5–6). For sulfate at 9.01 mg/L (tests 9–10, 11–12), the increase of glucose from 25 to 75% led to similar pigment values, 196.7 and 201.0 μg, respectively. On the contrary, for sulfate at 34.99 mg/L (tests 7, 8, 13 and 14), the increase of glucose from 25 to 75% contributed to an increase of 30% in total pigment produced (from 427.0 to 553.5 μg). Moreover, for glucose at 25%, the shifting of sulfate concentration from 9.01 to 34.99 mg/L led to a pigment increase of 117% (196.7 to 427.0 μg; tests 9–10, 13–14), and, in the same way, for glucose at 75%, this sulfate shifting led to an increase of 175% (201.0 to 553.5 μg; tests 7–8, 11–12).

Therefore, similar to what was observed for the biomass response, the sulfate concentration was the factor with the greatest influence in the pigment production. Lower values of pigments were a result from lower concentration of sulfate (9.01 mg/L), either for 25 or 75% of glucose. Indeed, the highest value of pigments was achieved when strain 1B was

cultivated with high glucose ratio (100%) but also with high sulfate concentration (≥ 22 mg/L). These results indicate that sulfate concentration may be a limiting factor for pigment production.

Figure 1 shows the response surfaces obtained for the variation of biomass (Fig. 1a, b) and pigment production (Fig. 1c, d) within the limits of the experimental domain for 72 and 216 h. Analysing the biomass response surfaces, at 72 h (Fig. 1a), 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 culture 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, achieving a maximum of 2.5 g/L. A maximal biomass value (2.5 g/L) is also noticeable for cultures with > 29 mg/L of sulfate and < 50% of glucose. High glucose

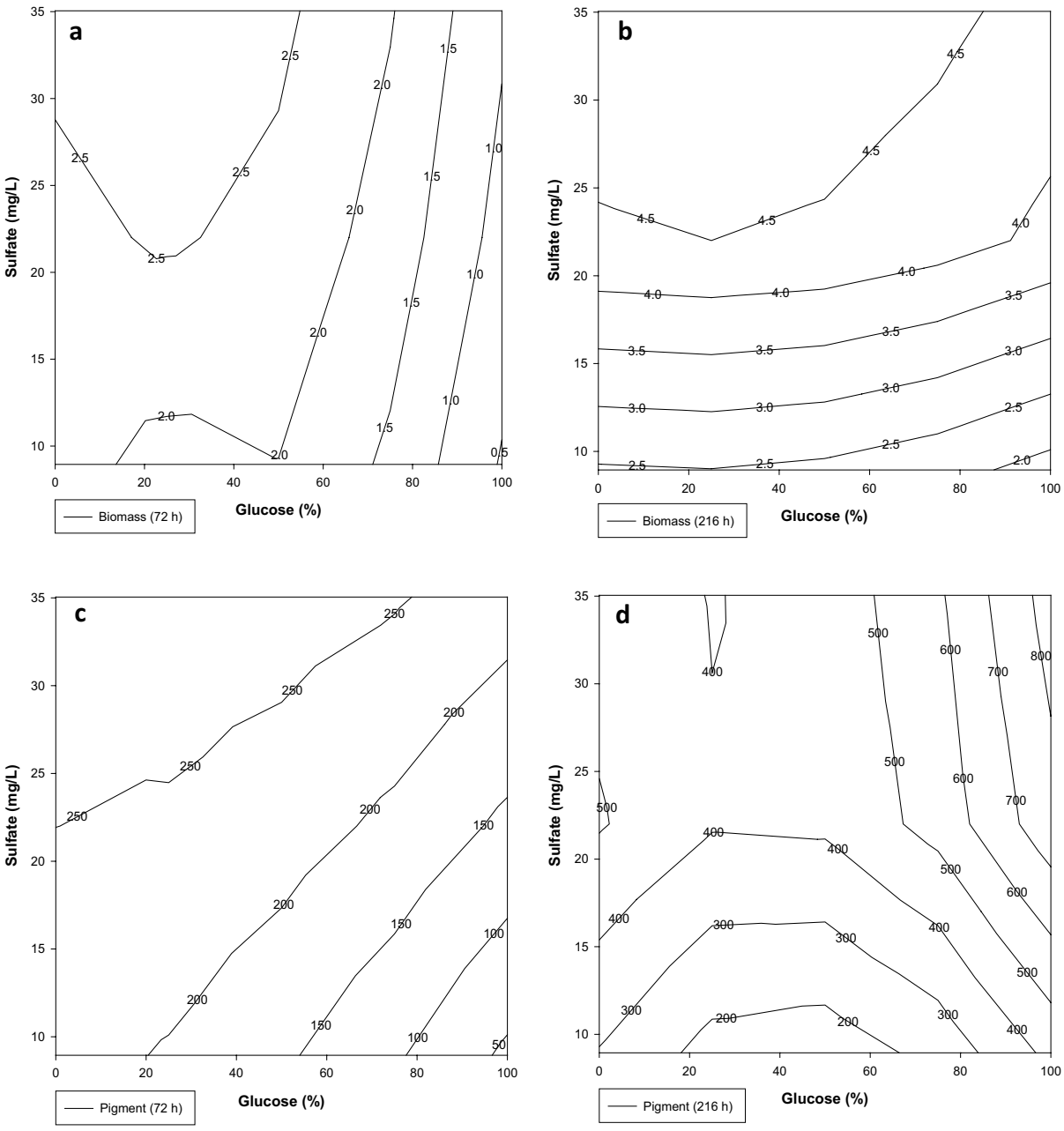

Fig. 1 Response surfaces for the biomass production (g/L) at 72 h (**a**) and 216 h (**b**); and for the total carotenoid production (μg) at 72 h (**c**) and 216 h (**d**), obtained in ED, under 400 lux of light (L_{400}), for the factors % glucose in a mixture of fructose + glucose (0–100%) and sulfate concentration (7–37 mg/L)

concentrations had a negative impact in the biomass production, highlighting the fructophilic behaviour of strain 1B. Considering the biomass at 216 h (Fig. 1b), 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 < 50%. However, for glucose > 50%, this factor loses some weight in the biomass production (lower right quadrant), despite still being the most influent factor towards biomass production. High biomass production seemed to be attained with glucose < 50%, and a sulfate concentration > 24 mg/L, achieving values of 4.5 g/L. Both at 72 and 216 h, biomass production was higher with lower glucose concentrations. Nevertheless, in both cases, a minimum of ~ 20 mg/L of sulfate was enough to achieve higher biomass concentrations.

Considering the pigment production at 72 h (Fig. 1c), 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. However, the higher pigment values were achieved with lower % glucose. Indeed, higher production of carotenoids (> 250 µg) may be achieved for sulfate concentrations > 30 mg/L and % glucose ≤ 55%. At 216 h (Fig. 1d), 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 ~ 22 mg/L, 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%, and up to 20 mg/L of sulfate, both factors have equal influence in pigment production (lower right quadrant). The increase of sulfate concentration from 22 to 30 mg/L for glucose concentrations from 0 to 60% had little effect in the pigment production (upper left quadrant). For sulfate values > 22 mg/L, glucose was the factor with higher importance (upper right quadrant). The best conditions for pigment production pointed out are 95–100% glucose and high sulfate concentration (> 22 mg/L). For sulfate > 28 mg/L, the pigment production achieved may be about threefold higher than the one obtained in the best conditions at 72 h (Fig. 1c, d). Indeed, the best pigment results achieved were those obtained when *G. alkanivorans* strain 1B was cultivated with 100% of glucose and 22 mg/L of sulfate for 216 h (~ 795 µg of total carotenoids, tests 3 and 4 in Table 1).

The overall results highlight the great influence of the studied factors, sulfate and glucose, on pigment production by strain 1B, and underline the crucial importance of time growth towards the highest pigment yield. Therefore, the best combination for carotenoid production by strain 1B seems to be 100% of glucose (10 g/L) and a sulfate concentration ≥ 22 mg/L in the presence of light for ≥ 216 h.

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 [28].

Analysis of ED factors

The data obtained from the experimental design were further used for a regression analysis and the polynomial model-derived parameters (β0–β22) are shown in Table 2. The β parameters of this polynomial model 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 (factor 1: % glucose in a mixture glu + fru or glucose ratio; and factor 2: sulfate concentration, respectively) on the responses; the interaction parameter, β12, indicates how simultaneous variation of both factors affects the response. 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 [24].

Considering the ED data for 72 h, the factor 1 (% glucose) presented the highest influence for biomass production (β1 > β2). β1 was negative, meaning that an increase of glucose ratio results in a reduction of biomass production. In terms of pigment production, sulfate concentration was the factor with the highest influence, with its augment leading to an increase in pigments; however, β1 is negative, indicating that under these conditions, at 72 h, an increase in % glucose results in a decrease in pigments.

At 216 h, biomass production is also negatively influenced by the % glucose, as illustrated by the negative parameter β1. However, this factor has much less influence in the response than factor 2 (β2 was almost tenfold higher than β1). In terms of pigment production, both factors have a similar positive influence, with factor 2 (sulfate concentration), having a higher importance (β2 > β1).

Fischer-statistical tests (*F* tests) were applied to evaluate the significance of the variances due to the effectiveness of the model fitting and due to the experimental error. The *F* test applied to the effectiveness of the model fitting to biomass and total carotenoids shows that the variance in the results is significantly accounted for by the values of the parameters both at the end of 72 h and 216 h at the *F* (5,8), approximately 0.1 significance level in both cases (Table 2). This test shows whether the source of variance in the residuals is due to the inadequacy of the models to reproduce experimental data. A second *F* test was performed to determine whether the origin of the

Table 2 Parameters of the polynomial models representing the studied responses in ED, under 400 lux of light (L_{400})

Environmental conditions	Light (L_{400})			
Growth time (h)	72		216	
Model	Biomass	Total carotenoids	Biomass	Total carotenoids
Model parameters				
β0	2.36	223.14	4.43	418.21
β1	−0.77	−55.33	−0.1	137.27
β2	0.36	65.8	1.08	150.83
β12	0.21	38.59	0.65	105.36
β11	−0.76	−28.23	−0.27	217.83
β22	−0.08	−9.94	−1.43	−191.01
Model validation (Fischer test)				
Effectiveness of parameters	2.89	2.47	4.17	15.88
Significance level (α), $F(5, 8)$	0.09	0.12	0.04	0.001
Lack of fit	93.51	35.82	3.29	0.78
Significance level (α), $F(1, 7)$	0.001	0.0005	> 0.1	> 0.1
R^2				
Coefficient of multiple determination	0.64	0.61	0.72	0.91

β0 response at the centre of the experimental domain, *β1 and β2* parameters of the factors 1 (% glucose) and 2 (sulfate concentration, mg/L), respectively, *β12* parameter of the interaction of the factors 1 and 2, *β11 and β22* self-interaction parameters of the factors 1 and 2, respectively

variance was due to the experimental error. The source of the variance contained in the residuals both at the end of 72 and 216 h was explained by the experimental error at the *F* (1,7) significance level of 0.001 and 0.1, respectively (Table 2).

Influence of growth time and light on carotenoid production

The ED demonstrated the influence of the incubation time on the total carotenoid production by *G. alkanivorans* strain 1B, indicating that longer incubation time induces higher carotenoid content in the culture. Consequently, a new set of growth assays was carried out towards detailed evaluation of the effect of this key factor on carotenoids production by this bacterium. Thus, *G. alkanivorans* strain 1B was cultivated during 26 days under the optimal conditions for carotenoid production: 10 g/L glucose as the only C-source and 22 mg/L sodium sulfate as S-source. In addition, three different light conditions were also tested: L_0—without light, L_{400}—400 lux of light, L_{3000}—3000 lux with photoperiod ranging from 3000 to 7000 lux to observe how both factors conjugate to influence pigment production.

During these three growth assays, the time-course profiles of different metabolic parameters (DCW, OD, glucose consumption, and total carotenoid production) were determined. Simultaneously, the physiological state of each culture during the growth time-course was also evaluated using flow cytomety.

Metabolic parameters

Figure 2a–c shows the growth and sugar consumption profiles of *G. alkanivorans* strain 1B under different light conditions. Moreover, Table 3 describes the metabolic parameters associated with these culture growths. From the results of Fig. 2a, 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. On the other hand, in the 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 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. 2b: 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. Figure 2c presents the carbon source consumption profiles of strain 1B for the different light conditions. Glucose was completely consumed in all 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 (Table 3). The highest maximum growth rate ($\mu_{max} = 0.026\ h^{-1}$) corresponding to the highest glucose consumption rate (0.062 g/L/h) was observed for *G. alkanivorans* strain 1B grown at L_{400}.

These results point out 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). The absence of light (L_0) and too much light (L_{3000}) decreased the

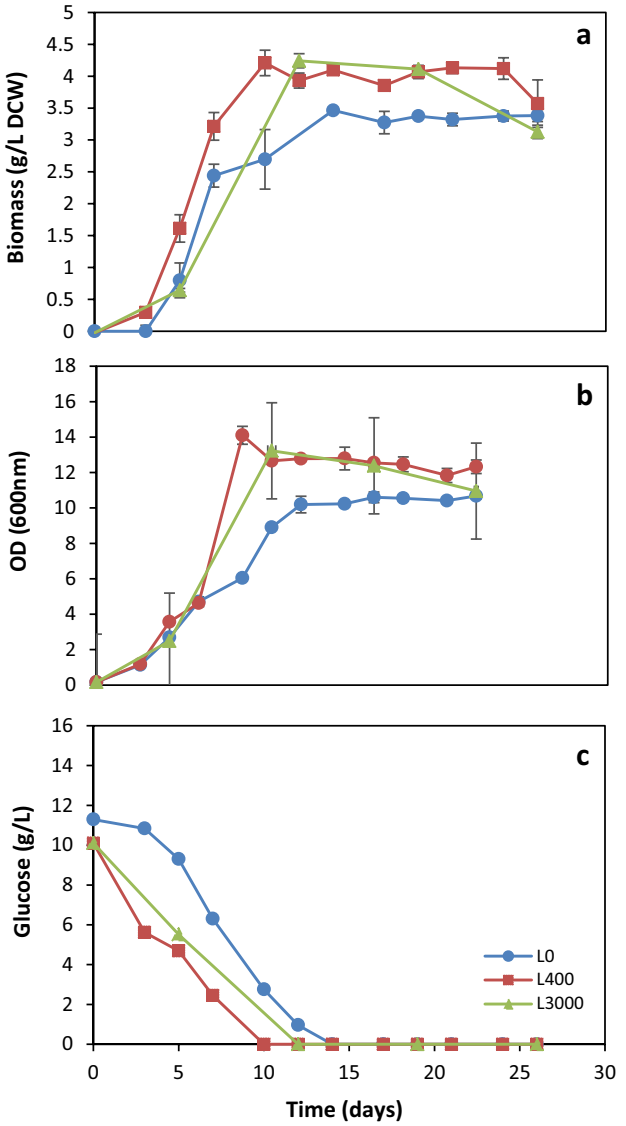

Fig. 2 Growth profiles, in terms of biomass production (**a**), OD_{600nm} (**b**), and glucose consumption profiles (**c**) for *G. alkanivorans* strain 1B cultivated under different light conditions (L_0, L_{400}, and L_{3000}) for 26 days. Results were obtained in triplicate and the standard deviation shown by the errors bars

Table 3 Metabolic parameters for G. alkanivorans strain 1B grown in shake-flasks with 10 g/L glucose and 22 mg/L sulfate under different light conditions

Metabolic parameters	Light exposure (lux)		
	L_0	L_{400}	L_{3000}
Specific growth rate, μ_{max} (h^{-1})	0.024	0.026	0.023
Maximum sugar consumption rate (g/L/h)	0.054	0.062	0.038
Maximum biomass production rate (g/L/h)	0.034	0.033	0.021

sugar consumption rate (0.054 g/L/h for L_0 and 0.038 g/L/h for L_{3000}) and consequently decreased the μ_{max} (0.024 h^{-1} for L_0 and 0.023 h^{-1} for L_{3000}) in comparison with the corresponding culture metabolic parameters at L_{400} (Table 3). The slightly lower growth rate observed in the culture at L_{3000} is in accordance with that observed by Silva et al. [22], confirming that too much light exposure may lead to a slower growth by strain 1B, possibly due to a photoinhibition.

Carotenoid analysis

The next step was to analyse the overall carotenoids content over time at each condition (L_0, L_{400}, L_{3000}), through spectrophotometry. The results obtained, presented in Fig. 3, show that strain 1B produced the lowest carotenoid content at L_0, achieving a maximum value between 212 and 220 µg at 14–17 days (corresponding to about 407–447 µg/g_{DCW}). At L_{400}, it had the highest carotenoid production with 1609 µg at 21 days (2596 µg/g_{DCW}), followed with 1455 µg (2359 µg/g_{DCW}) within 19 days at L_{3000}. The carotenoid production at L_0 seems to have been dependent of the growth profile of strain 1B (Fig. 2b), 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. 2c). 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. 3), 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.

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 the maximum

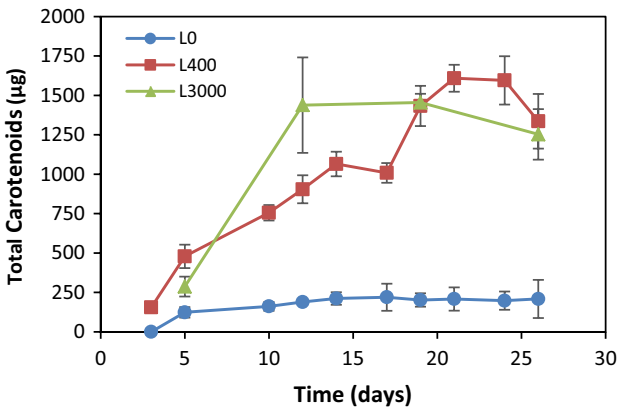

Fig. 3 Amount of total carotenoids (µg of carotenoids per g DCW per 150 mL), assessed through spectrophotometry analysis, produced by *G. alkanivorans* strain 1B in the assays with different light conditions (L_0, L_{400}, and L_{3000}). Results were obtained in triplicate and the standard deviation is shown by the error bars

concentration is attained. Thus, longer cultivation time led to higher carotenoid 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}). When grown without light (L_0), the strain 1B seems to stop the production of carotenoids at the beginning of the stationary phase, achieving a very low carotenoid content. In addition, too much long incubation time may contribute for a decrease in the pigment content (see Fig. 3, profile for L_{400} at 26 days). This effect may be due to a decrease in overall pigmented biomass, and/or to the carotenoids degradation.

In overall, the maximum carotenoid production by strain 1B was achieved in the late stationary growth phase (19–24 days) when the bacterium was cultivated in 10 g/L glucose and 22 mg/L sulfate with light. In terms of total carotenoid production on the different light intensity conditions (400 vs. 3000 lux with photoperiod), the maximum values were observed for L_{400}; however, this could be an artefact resulting from the missed carotenoid content data within 12–19 days at L_{3000}. This should be further confirmed in future assays.

In addition, another assay was performed to evaluate the influence of the photoperiod on pigment production. Hence, the strain 1B was further grown in 10 g/L glucose and 22 mg/L sulfate at constant 3000 lux of light, for 19 days. In these conditions, the amount of total carotenoids produced was 1397 µg, which corresponds to about 3100 µg/g_{DCW} (i.e., 0.31%, where percentage is relative to g pigment per 100 g DCW). This higher overall pigment yield observed, in comparison with that of the assay with photoperiod (0.24% ⇒ 2359 µg/g_{DCW}), it was mainly due to the lower value of total biomass achieved (3.1 vs. 4.1 g/L, respectively). Indeed, similar values of total pigments (µg) per 150 mL of culture medium were obtained in both assays (1397 vs. 1455 µg).

In overall, the increase of carotenoid production with light exposure is in accordance to the results described by Silva et al. [22] for strain 1B, and it has also been shown in other *G. alkanivorans* strains, such as *G. alkanivorans* SKF120101 [29]. 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. This can be noted both by the results observed for metabolic parameters of the culture (Table 3) and for the pigment profiles (Fig. 3). Thus, the divergence on the carotenoids produced by strain 1B seemed to be due to the overall culture conditions during the microbial growth.

Comparing the highest results obtained for the total amount of carotenoids production by *G. alkanivorans* strain 1B in this study (2596–3100 µg/g_{DCW}, corresponding to pigment yields of 0.26–0.31%) with the best result reported by Silva et al. [22], (2015 µg/g_{DCW} ⇒ 0.20%), it can be stated that a significant optimization was achieved (up to 35% of pigments increase). However, further assays should still be carried out considering the influence of other important factors, such as oxygenation, photoperiod light/dark, light wavelength, and extraction method, between others [30, 31].

Considering carotenoids production data reported for other species of the genus *Gordonia*, the most relevant includes *G. jacobaea*, whose wild-type produced 227 µg/g_{DCW} of carotenoids and its mutant 2500 µg/g_{DCW} [32]; and *G. ajoucoccus* A2T that produced 2900 µg/g_{DCW} of carotenoids in a bioreactor under optimized conditions with hexadecane [33]. These optimized results are very similar to the values obtained in this study for *G. alkanivorans* strain 1B (2596–3100 µg/g_{DCW}), even with a few optimizations for maximum carotenoid production. This highlights the great potential of strain 1B towards its further exploitation as a hyperpigment producer strain that may be applied into different industrial sectors. In addition, the potential of strain 1B to use different cheap alternative carbon sources, such as Jerusalem artichoke [34], carob pulp [24], recycled paper sludge [35], and sugar beet molasses [18], may be an advantage aiming to get an overall cost-effective pigments production process scale-up, which is one of the characteristics for an ideal pigment-producing microorganism.

Evaluation of cell physiological state by flow cytometry

There are several studies describing the direct correlation between stress conditions and pigment production [36–38]. In this context, an evaluation of the cells physiological state during the time-course of the growth and carotenoid 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 [25, 39]. Staining with different dyes allows assessing different physiological aspects of each cell. For this work, two staining agents were used: CFDA, which allows the identification of metabolically active cells (cells uptake the dye and convert it into its fluorescent form); and PI (an intercalating agent) that stains cells with a compromised membrane. PI works by binding with the nucleic acids inside the cell, but intact membranes are impermeable to this dye, so it only penetrates cells with compromised membranes [40]. Therefore, depending on the staining, cells were divided into three classifications: healthy (when stained only with CFDA), dead (when stained only with PI), and stressed (when there is a simultaneous stain with both compounds, 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, stressed, and dead) 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 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 are 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). In addition, 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, c). From these results, it can be stated that the highest % of dead cells was observed for L_{400} culture ($\Delta_{3-26\ days}$: 2–12%), followed by the L_{3000} culture ($\Delta_{3-26\ days}$: 1–8%). The culture grown at dark was the one with the lowest % of dead cells within 26 days ($\Delta_{3-26\ days}$: 2–1%).

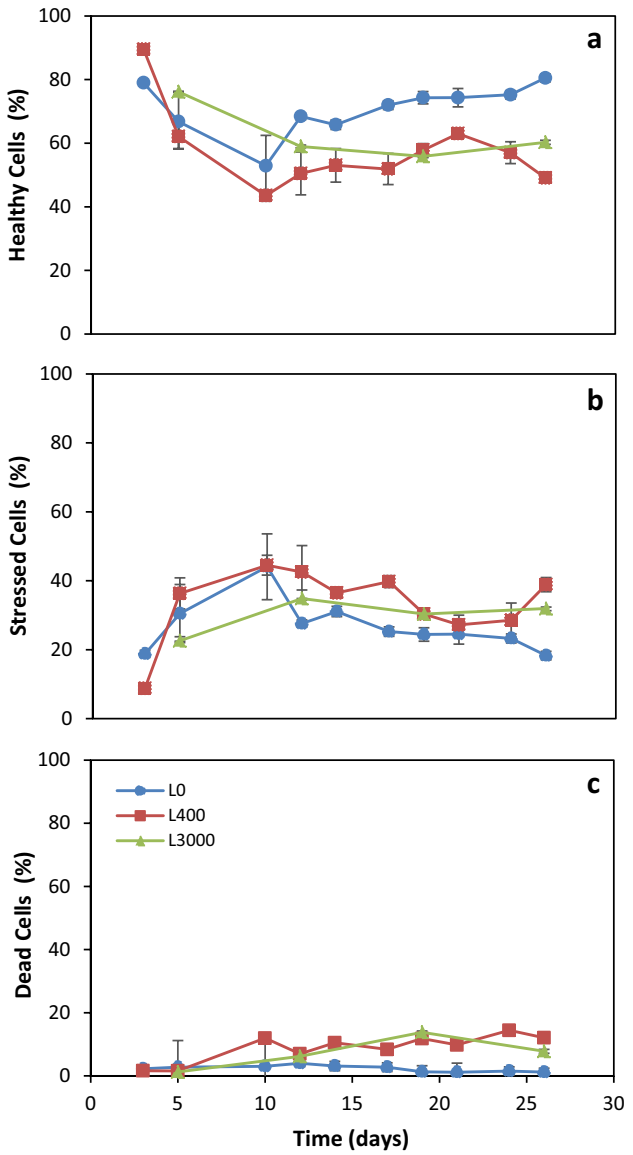

Fig. 4 Flow cytometry analysis through the time-course of growth and pigments production of *G. alkanivorans* strain 1B with 10 g/L glucose and 22 mg/L sulfate with different light conditions (L_0, L_{400} and L_{3000}). **a** Percentage of healthy cells; **b** percentage of stressed cells; and **c** percentage of dead cells. Results were obtained from duplicates and each analysed six times

In 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 μg/g_{DCW}) and, therefore, the cells do not use their storage substances for that secondary metabolism. On the other hand, with light where the bacteria 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, c, 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 carotenoids content. However, after 26 days, there were still ≥ 54% of healthy cells of strain 1B for all conditions.

Conclusions

In this study, an SRM based on the Doehlert distribution was used towards the optimization of carotenoid production by *G. alkanivorans* strain 1B, a well-known desulfurizing bacterium. The experimental design demonstrated that glucose 10 g/L as sole C-source and sulfate ≥ 22 mg/L as sole S-source are the best culture conditions for the highest carotenoids production by strain 1B. This work also highlighted the importance of incubation time length for the development of the pigments, demonstrating that longer running cultures, under the light, achieve higher carotenoid concentration. Indeed, under 400–3000 lux and after 19 days, a maximal carotenoids production of 2596–3100 μg/g_{DCW} was achieved by *G. alkanivorans* strain 1B. In addition, cell viability patterns established by flow cytometry showed that the highest carotenoid production was attained by the culture presenting the higher level of stress/dead cells. Further optimization of type/quantity of pigment can still be reached through the evaluation of other parameters (e.g., oxygenation, light wavelength, cheap alternative carbon sources, and green extraction solvents). But herein, it is highlighted the great potential of strain 1B as a new hyperpigment producer to be exploited towards several applications, since the

microbial carotenoids are valuable bioactive compounds attractive to different industries, such as chemical, pharmaceutical, cosmetics, and feed/food.

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