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Selection and characterization of a *Parachlorella kessleri* microalgal strain able to assimilate lactose, and grow on dairy waste

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ABSTRACT

One of the main limitations of commercial production of algae is the cost of cultivation, largely attributed to the cost of the nutrients; hence, finding cheap alternative substrates has been a significant line of research in this field. The dairy industry produces large amounts of wastewater that might be used as a cost-effective nutrient alternative, containing lactose as a main carbon source, as well as other essential nutrients like nitrogen and phosphate. Nevertheless, just a few algal species of commercial value can grow on any organic carbon sources, and even less are able to utilize lactose. In this work we have identified a *Parachlorella kessleri* strain capable of utilizing lactose for growth, and have characterized its ability to accumulate metabolites of commercial interest under heterotrophic growth conditions. *P. kessleri* was capable to utilize lactose from dairy wastewater, and to accumulate several amino acids, dicarboxylic acids, such as tartaric acid and succinic acid, and triacylglycerols in heterotrophic conditions. These metabolites have applications in the food, feed, and pharmaceutical industries, and in the green chemical industry for the production of bio-based green polymers and biofuels. The significance of these findings for future product development is discussed.

1. Introduction

The dairy industry, compared to other areas of the food industry, generates some of the largest volume of waste [1]. Up to 10 L of wastewater are estimated to be released per liter of milk processed [2]. In 2022 the yearly global milk production, and the global trade in milk products were estimated to be about 930 million and 85 million tons, respectively [3]. Unless properly valorized, the sheer volume of wastewater generated by the dairy industry needs to be treated for disposal, having significant cost implications and, if left untreated, significant ecological impact. Dairy wastewaters vary greatly in composition, nonetheless are generally characterized by high organic matter content, mainly attributed to the presence of lactose, elevated levels of total nitrogen and total phosphorus, high salt content, mainly in the form of K^+, Ca^{2+}, Na^+, and Cl^- ions, low alkalinity (thus low buffering capacity), and a wide pH range [1].

Of the approaches available to treat dairy wastewaters, biological methods are often preferred, where the contaminants, including lactose, serve as nutrients to prokaryotic and eukaryotic microorganisms, generally bacteria and fungi [4]. As lactose accounts for a significant fraction of the organic matter, it is important to choose species able to assimilate this disaccharide to reach an optimal waste treatment efficiency. To make it more sustainable, biological wastewater treatment may be coupled with the production of compounds having commercial value, e.g. enzymes [5], organic acids [6,7], or prebiotics [8].

Besides fungi and bacteria, eukaryotic microscopic algae (microalgae) and cyanobacteria able to grow on organic carbon sources have a great potential in biological treatment of dairy wastewaters. Indeed, the treatment of dairy waste streams by algae has been researched, either using them as single species, such as *Chlorella* sp. [9], or as part of a consortium [10]. However, the carbon substrates which can be assimilated by algae able to grow in a heterotrophic setting are species dependent [11], and to date, just a limited number of strains have been confirmed to actually utilize lactose for growth, a necessary trait to

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obtain efficient substrate utilization of dairy waste. For example, *Chlorella minutissima*, *Dunaliella tertiolecta*, *Nannochloropsis oculata* [12], *Tetradesmus obliquus* [13,14] and *Porphyridium purpureum* [15], have all been shown to utilize lactose, but there is very little information on the metabolic pathways involved in lactose assimilation in any of these algae. Therefore, it is of interest to identify and characterize new species of potential commercial value.

Algae, due to their great genetic diversity, have the potential to produce a wide range of metabolites, from phenolics and terpenoids, to fatty acids and polysaccharides, of interest for various industrial sectors, including the food, feed, nutraceutical, cosmetic and pharmaceutical industries [16], as well as in the green energy sector [17–19]. Certain metabolites, such as monounsaturated fatty acids and certain organic acids, can also serve as building blocks for the manufacturing of renewable plastics [20]. Coupling algal cultivation to the production of metabolites of industrial value or biofuels might significantly offset the costs of dairy wastewater treatment, making it economically sustainable and even profitable.

Among eukaryotic microalgae, *Parachlorella kessleri* has recently gained attention, primarily due to its capacity to accumulate high levels of neutral lipids for biofuel production. In fact, a lipid content of up to 60 % of the biomass dry weight was observed under nutrient deprivation [21], and/or when the alga was grown on glucose [22]. It was also found to produce compounds with nutritional and health benefits, such as exopolysaccharides (EPS) with potential anti-tumor effects [23], and bioactive compounds with antioxidant properties [24], including extracellular carotenoids [25]. Nevertheless, a detailed characterization of the metabolic profile of *P. kessleri* grown in autotrophic vs. heterotrophic conditions is currently not available; which might identify additional compounds of commercial interest produced by this species, besides the few that have been studied thus far.

In this work we isolated a *P. kessleri* strain in a screen aimed at the identification of microalgal strains able to assimilate lactose and grow on dairy wastewater. We have also characterized the metabolic profile of the selected strain when cultivated in heterotrophic conditions, compared to the same strain grown in autotrophic mode, to identify compounds of significant potential commercial value. The initial metabolomic profile would serve as a baseline for targeted research and development in the future to create industrial products from dairy waste.

2. Materials and methods

2.1. Screening on lactose and its monosaccharide components, and evaluation of the growth kinetics of the selected strain on lactose

2.1.1. Algae strains

The algae strains screened in this work were environmental isolates from the Imperial Valley and San Diego area (Southern California, USA); or were procured from public algae culture collections, namely the Culture Collection of Algae and Protozoa (CCAP), Oban, UK; the Microbial Culture Collection at the National Institute for Environmental Studies (NIES), Tsukuba, Japan; and the Culture Collection of Algae at the University of Texas at Austin (UTEX), Austin, Texas, USA. They were previously selected based on the ability of the strains to grow on glucose [26]. The specific strain characterized in this work (strain PK25, *P. kessleri*) was isolated from the University of California, San Diego (UCSD) field station [27].

2.1.2. Culture conditions

A first screening on agar plates was performed on modified Bold's Basal Medium with 5 g/l yeast extract as a nitrogen source [26] to which 10 g/l galactose or 10 g/l lactose was added as a sole carbon source. The plates were placed in the dark, or in the light under continuous illumination with fluorescent lights at 100 μmol photons/m^2s for 20 days, with visual observations every 5 days.

Subsequently, the selected strain, *P. kessleri* (PK25), was grown in shake-flask cultures on modified Bold's Basal Medium with 5 g/l yeast extract as a nitrogen source, and a starting concentration of 10 g/l glucose, galactose, or lactose. No sparging of air or air plus CO_2 was applied to the flasks. A working volume of 200 ml was used in 500 ml flasks. The estimated starting biomass concentration was 0.05 g/l. The experiment was performed for 15 days in triplicates in heterotrophic regime, and in mixotrophic conditions under fluorescent lights at 100 μmol photons/m^2s following a 16:8 h light:dark cycle. Control cultures in the light and in the dark did not contain external organic carbon source. A *C. reinhardtii* strain grown on 10 g/l lactose containing medium in the light and in the dark was used as a negative control.

2.1.3. Cell dry weight measurements

The cell dry weight was measured by centrifuging 1–3 ml of culture, and drying the pellet in a Savant SpeedVac SC100 system until constant weight was obtained. The empty tube weights and the weights after drying were measured and used for the calculation.

2.1.4. Lactose, galactose, and glucose measurements

Lactose, galactose, and glucose concentrations were measured enzymatically; lactose and galactose via the β-galactosidase/galactose mutarotase/β-galactose dehydrogenase protocol [28]; and glucose by the glucose oxidase/peroxidase (GOPOD) method [29], using the K-LACGAR and K-GLUC kits from Megazyme.

2.2. Evaluation of the growth kinetics of the selected strain on dairy wastewater

2.2.1. Origin of wastewater

The skimmed buttermilk waste was obtained from the cheese producer Capurso Azienda Casearia S.r.l. (Gioia del Colle, Italy); this represents one of the factory's two main waste lines. The dairy factory is manufacturing different types of products, such as ricotta cheese, mozzarella, and butter. The waste stream resulting from the production of mozzarella and ricotta cheese is skimmed resulting in butter and skimmed buttermilk. The waste is further treated and discharged to the municipal sewage system or used for biogas production [30].

2.2.2. Culture conditions

A preliminary evaluation to select the optimal wastewater:Milli-Q water or medium ratio was conducted in shake-flask cultures for 7 days using 50 ml flasks with 20 ml working volume in mixotrophic conditions with 16:8 h light:dark cycles under fluorescent lights at 100 μmol photons/m^2s. Subsequently, cultivation was performed for 15–20 days in triplicates in shake-flask cultures in heterotrophic regime, and in mixotrophic conditions under fluorescent lights at 100 μmol photons/m^2s following a 16 h:8 h light:dark cycle. No sparging of air or air plus CO_2 was applied to the flasks. A working volume of 40 ml was used in 100 ml flasks. The wastewater:medium mix cultures had an initial approximate lactose concentration of 1.6 g/l. Control cultures did not contain any added organic carbon. The estimated starting biomass concentration was 0.05 g/l. All experiments were conducted in sterile conditions. The wastewater was sterilized through a 0.22 μm pore size filter. The axenic nature of the cultures was confirmed at the end of the experiment through microscope observations.

2.2.3. Biomass growth measurement

Growth measurement during the preliminary evaluation was performed by cell counting using a Thoma counting chamber. During the biomass evaluation, cell dry weight was measured as described in Section 2.1.3.

2.3. Metabolomics

2.3.1. Untargeted polar metabolite analysis and lipidomics

Untargeted polar metabolite analysis and lipidomics on *P. kessleri* biomass heterotrophically grown on glucose, or autotrophically grown, was performed at the University of California Riverside Metabolomics Core Facility. Cultivation, sample preparation, and sample analysis were performed as described previously [26].

2.3.2. Carbohydrate analysis

Carbohydrate analysis of the autotrophically grown *P. kessleri* biomass was performed at the University of California, San Diego GlycoAnalytics Core. Cultivation, sample preparation and sample analysis were conducted as described previously [26].

3. Results

3.1. Strain selection on lactose

A set of 24 microalgal strains previously selected based on their ability to grow on glucose [26] were screened for their ability to grow on lactose or on galactose (the other monosaccharide constituent of lactose) as a sole organic carbon source (Table 1.). Our primary aim was to select a strain able to assimilate lactose in the dark; nevertheless, the test was performed also in the light, to gather more information useful for future possible process development based on mixotrophic growth conditions.

Of the tested strains, *Chlamydomonas pseudagloe* (CPA), the *Chlorella* species (WG, WG7, IV055), *Coelastrella* sp. (BFS32), *P. kessleri* (PK25), the *Chlorococcum* species (IV132, IV139), and four of the unidentified strains IV031, IV033, IV118, IV131 were able to grow on galactose in the light or in the dark. However, only one strain, *P. kessleri* (PK25), was able to assimilate lactose in mixotrophic and heterotrophic conditions. This strain is registered at the UTEX algae collection under accession number UTEX 3225.

We subsequently evaluated the growth kinetics of this isolate on lactose and on its two monosaccharide components. Growth in the presence of all tested sugars was significantly higher than when no organic carbon was added to the medium (Fig. 1.). More specifically: We

Table 1
Growth of selected algae strains on galactose and lactose in the light or dark.

Strain ID	Strain name	Origin	Galactose	Lactose
CCAP 11/41	*Chlamydomonas asymmetrica*	CCAP	–	–
NIES 2207	*Chlamydomonas asymmetrica*	NIES	–	–
UTEX 231	*Chlamydomonas debaryana*	UTEX	–	–
CPA	*Chlamydomonas pseudagloe*	SD	+	–
CPC	*Chlamydomonas pseudococcum*	SD	–	–
WG	*Chlorella* sp.	SD	+	–
WG7	*Chlorella* sp.	SD	+	–
BFS32	*Coelastrella* sp.	SD	+	–
DA25	*Desmodesmus armatus*	SD	–	–
PK25	*Parachlorella kessleri*	SD	+	+
IV006	*Chlamydomonas* sp.	IV	–	–
IV031		IV	+	–
IV033		IV	+	–
IV055	*Chlorella* sp.	IV	+	–
IV112		IV	–	–
IV113		IV	–	–
IV118		IV	+	–
IV131		IV	+	–
IV132	*Chlorococcum* sp.	IV	+	–
IV139	*Chlorococcum* sp.	IV	+	–
IV157		IV	–	–
IV233		IV	–	–
IV238		IV	–	–
IV241	*Desmodesmus* sp.	IV	–	–

SD: isolate form San Diego area; IV: isolate form Imperial Valley area, +: substrate utilization, -: no substrate utilization.

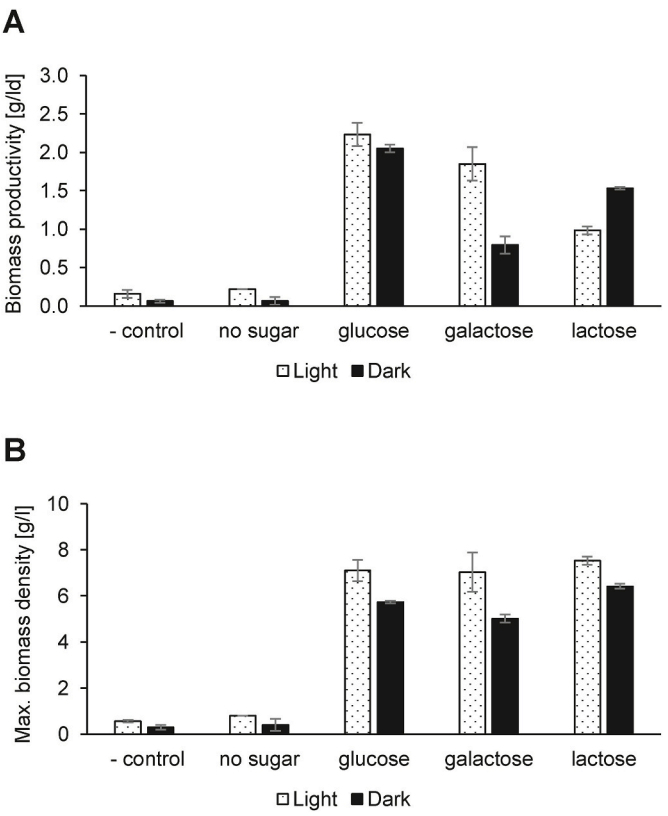

Fig. 1. Biomass productivity (Panel A) and maximum biomass concentration (Panel B) of *P. kessleri* grown on medium containing glucose, galactose or lactose as sole organic carbon source or without added organic carbon (no sugar) in the light (dotted bars) and in the dark (black bars). Negative control strain (control) was *C. reinhardtii* grown on lactose. Values are means of three replicates ± SD.

obtained a biomass productivity of 0.22 g/ld under autotrophic conditions. In the dark there was little growth (0.06 g/ld) in the absence of added sugars, likely due to residual organic carbon in the yeast extract supplemented to the medium as nitrogen source (Fig. 1., panel A). When we compared the biomass productivities on different sugars, we obtained the highest values on glucose in the light (2.23 g/ld) and in the dark (2.05 g/ld). The productivity on galactose in the light was somewhat lower but comparable to the value obtained on glucose (1.85 g/ld). In dark conditions, however, there was a significant drop in productivity when galactose was used as organic carbon source (0.79 g/ld). The productivity on lactose in the light was significantly lower than on the monosaccharides (0.98 g/ld), however, in the dark productivity was in between what was obtained for glucose and galactose (1.53 g/ld). As a comparison, the productivities of *Chlamydomonas reinhardtii* grown on lactose were comparable to what we obtained without any sugar addition to the medium: 0.16 g/ld in the light and 0.06 g/ld in the dark, indicating that this strain cannot use lactose for growth.

The maximum biomass densities obtained in the light for *P. kessleri* (PK25) were 7.10, 7.03, and 7.53 g/l on glucose, galactose, and lactose, respectively (Fig. 1., panel B). As for the dark regime, the corresponding values were 5.73, 5.02, and 6.42 g/l. On all sugars the maximum biomass concentrations were significantly higher than when there was no external organic carbon added to the medium: in autotrophic conditions we obtained a value of 0.80 g/l, and in the dark 0.40 g/l. These values were comparable to what we obtained for the negative control, *Chlamydomonas reinhardtii*: 0.57 and 0.30 g/l in the light and in the dark, respectively.

Fig. 2 shows the substrate (sugar) consumption in relation to the biomass growth when *P. kessleri* (PK25) was cultivated on lactose in the

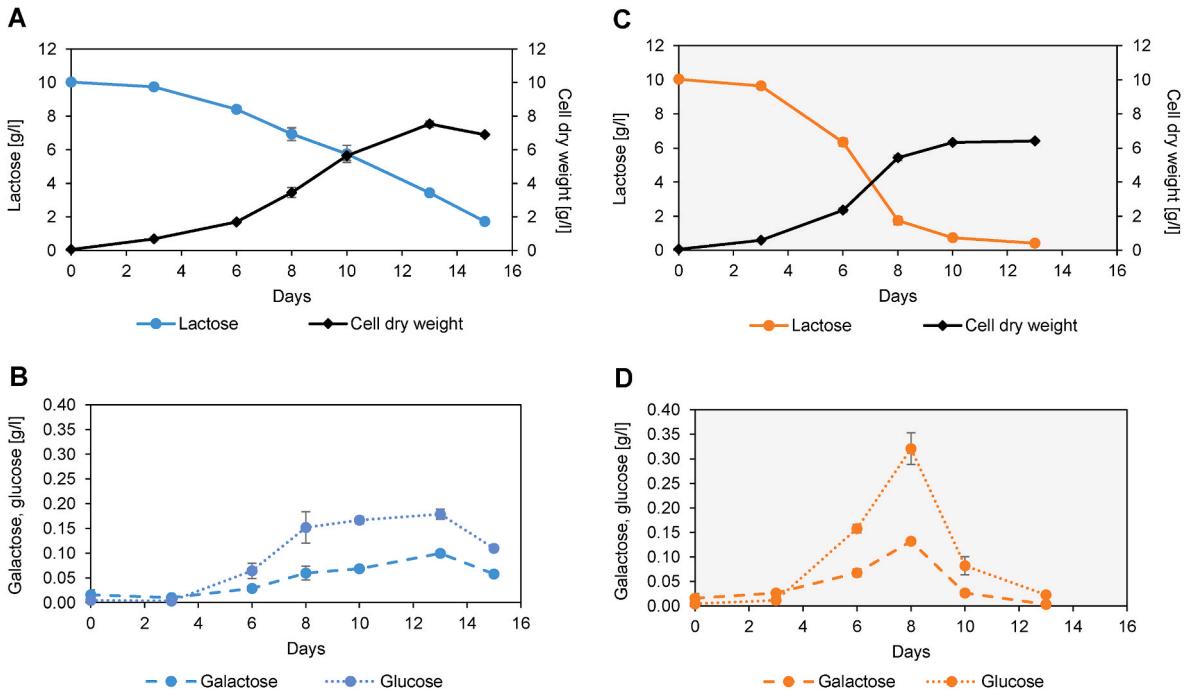

Fig. 2. Evolution of the lactose concentration vs. biomass growth in the light (Panel A) and in the dark (Panel C), and changes in the galactose and glucose concentration in the light (Panel B) and in the dark (Panel D) throughout the growth phase. Values are means of three replicates ± SD.

light and in the dark. In the light the cells reached the stationary phase (around day 13) when about 34 % of lactose was still present in the medium (Panel A). Lactose concentration continued to steadily decline during the stationary phase. As indicated on Panel B, there was a minor increase in the concentration of glucose and galactose throughout the exponential phase, likely as a consequence of enzymatic hydrolysis of lactose. Glucose and galactose concentrations peaked when stationary phase was reached, then started to gradually decline, indicating that they were taken up by the alga. Higher biomass productivity was observed in the dark than in the light (Panel C). When stationary phase was reached (around day 10) only 7 % of the lactose was remaining in the medium. As observed in the light there was some accumulation of the monosaccharide constituents throughout the exponential phase and a decline during the stationary phase (Panel D). The maximum concentrations of the monosaccharides were: 0.32 vs. 0.18 g/l for glucose, and 0.13 vs. 0.10 g/l for galactose in the dark and in the light, respectively.

3.2. Evaluation of the ability of the selected strain to grow on dairy wastewater

Since the selected *P. kessleri* strain appeared to be able to use lactose as sole carbon source, we also evaluated its ability to grow on a dairy wastewater rich in this disaccharide. More specifically, we cultivated the alga on skimmed buttermilk waste obtained from a cheese factory [30]. Fig. 3 shows the biomass productivities (Panel A) and maximum biomass densities (Panel B), in the light and in the dark, of *P. kessleri* (PK25) when grown on culture medium containing 30 % skimmed buttermilk (v/v), which was the wastewater dilution that yielded most significant algal growth during the preliminary study, in comparison with the medium with no added organic carbon source (Supplementary Table 1). The observations were made in a 15-day period. Panel C of Fig. 3 shows the time-course changes of the biomass concentration in the above-mentioned conditions in comparison with autotrophic conditions. We could observe a diauxic growth pattern both in the light and in the dark for the cultures on wastewater (Fig. 3. Panel C), probably due to the presence of different carbon sources in the complex substrate (Supplementary Table 2). The biomass productivities related to the first and the second phase ("Light 1" and "Light 2" on Fig. 3. Panel A) were 0.33 g/ld and 0.16 g/ld, respectively. In the dark the biomass productivities for the two phases ("Dark 1" and "Dark 2" on Fig. 3. Panel A) were 0.18 g/ld and 0.09 g/ld, respectively. In autotrophic conditions the biomass productivity was 0.08 g/ld, and in the dark, when no external organic carbon was added to the medium, there was a minor growth (0.04 g/ld), probably on the residual carbon present in the yeast extract used in the medium, as mentioned in Section 3.1. The maximum biomass concentrations (Fig. 3. Panel B) reached over the 15-day period were: 2.45 g/l and 1.37 g/l in the light, and 2.12 g/l and 0.38 g/l in the dark for the wastewater cultures and the cultures without added organic carbon, respectively. The lactose concentration in the heterotrophic wastewater culture in the dark was close to zero on day 15 of the experiment. In the light, the algae were monitored until day 20, while observing gradual biomass increase; even after 20 days of culturing, the lactose concentration in the mixotrophic culture was 0.74 g/l.

3.3. Metabolomics

To identify which molecules of potential industrial interest are produced by the selected strain, untargeted polar metabolite analysis and lipidomics analysis were performed on *P. kessleri* (PK25) biomass obtained after heterotrophic growth on glucose in comparison to autotrophic growth conditions. Glucose was chosen as an organic carbon substrate in heterotrophic conditions for the initial profiling, for reasons of comparability of the metabolite accumulation patterns to those of the strains of the initial selection [26], and as dairy wastewater is of variable composition, and can contain several types of carbon substrate.

Fig. 4 and 5. show the heatmap of the compounds with the highest relative polar metabolite and lipid abundances, respectively, in heterotrophic conditions on glucose, compared to autotrophic conditions. We observed higher accumulation of polar metabolites for *P. kessleri* (PK25) in heterotrophic than in autotrophic conditions (Fig. 4.). The relative abundances of certain proteinogenic amino acids, namely glutamic acid, aspartic acid, isoleucine, valine, threonine, phenylalanine, leucine, and lysine, were elevated in the dark; and most of the amino acids showed

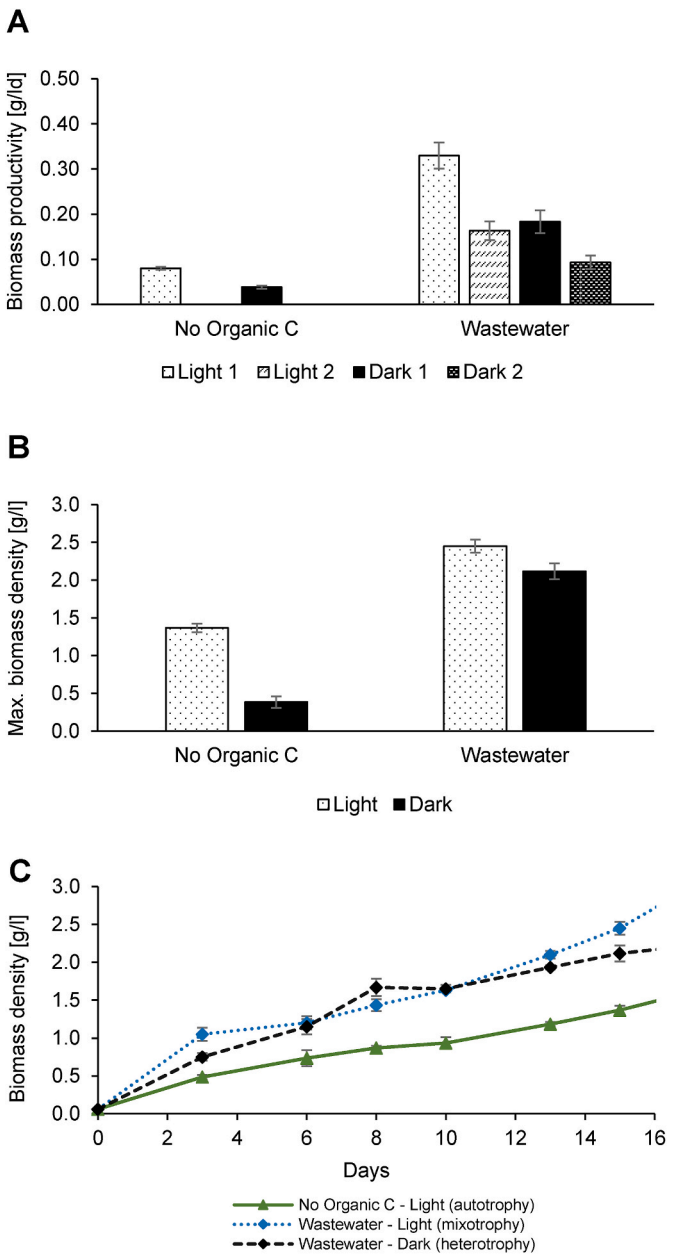

Fig. 3. Biomass productivity (Panel A) and maximum biomass concentration (Panel B) of *P. kessleri* when grown on filtered skimmed buttermilk, and without added organic carbon to the medium, in the light (Light 1 and Light 2, dotted and striped bars, respectively) and in the dark (Dark 1 and Dark 2, black bars and bars with trellis pattern, respectively). Panel C shows the time-course changes of the biomass concentration on wastewater in the above-mentioned conditions in comparison with autotrophic conditions. Values are means of three replicates ± SD.

more significant accumulation in the culture medium than in the biomass. Accumulation of certain amino acid degradation products and intermediates synthesized from amino acids was also observed, such as of indole-3-lactate, tyramine, O-acetylserine, and to a certain degree ethanolamine, resulting respectively from tryptophan, tyrosine, and from serine for the latter two compounds. Significant accumulation of the dicarboxylic acids succinic acid and tartaric acid was observed in heterotrophic conditions. As for the lipids, the most remarkable group of metabolites with significant accumulation in heterotrophic conditions in comparison with autotrophy is the group of triacylglycerols (Fig. 5.). The fatty-acyl moieties of highest abundance were C16:0, C18:1, C18:2, and C18:3.

Table 2 shows the monosaccharide composition of the exopolysaccharides produced by *P. kessleri* (PK25) in autotrophic conditions. The most abundant monosaccharide was galactose, followed by rhamnose, xylose, glucose, and mannose. The acid monosaccharide glucuronic acid was also detected in the supernatant.

4. Discussion

In this work we have screened a set of algal strains, previously shown to be able to assimilate glucose, for their ability to use lactose as a sole carbon source. The growth kinetics data obtained for the *P. kessleri* (PK25), indicate that this strain is able to assimilate lactose and utilize it for significant biomass production (Fig. 1 and 2.). To the best of our knowledge this is the first report of the ability of this species to metabolize lactose. Transient accumulation of glucose and galactose during the exponential phase both in the light and in the dark (Fig. 2.) suggests that lactose is not directly uptaken by the alga, but is subjected to extracellular hydrolysis, which would be in line with what was observed for other lactose-assimilating algal strains. Indeed, Zanette et al. [12] measured extracellular β-galactosidase activity for *D. tertiolecta*, *N. oculata*, and *C. minutissima*. The glucose and galactose concentrations were reported to be under 0.1 g/l during their experiments. Lactose is not expected to be found in the natural environment of *P. kessleri*, hence the role of the putative β-galactosidase secreted by this strain might be the hydrolysis of galactose residues from different polysaccharides found in the alga's surroundings, similar to the case of filamentous fungi [31].

Comparing the kinetics of lactose assimilation to the utilization of the monosaccharide constituents can provide some indications on the uptake mechanisms and metabolic routes involved. Glucose appears to be the preferred substrate for *P. kessleri* (Fig. 1.). The strain's behavior on galactose (Fig. 1.) is consistent with what was previously observed for *Chlorella vulgaris*, which utilizes glucose and galactose at comparable rates when grown in the light but assimilates galactose to a much lesser extent under heterotrophic conditions [32,33]. There is limited information available on the metabolic routes involved in galactose assimilation in algae, with some indication on the presence of the Leloir pathway in some species, such as *Galdieria sulphuraria* [34]. In some filamentous fungi galactose is metabolized through an alternative route, the oxidoreductive pathway [31,35], which requires NADPH (Fig. 6.). If this was the galactose metabolic route active in *P. kessleri*, that would explain why there is significantly lower biomass productivity on galactose in the dark than in the light, as photosynthesis also serves as a source of NADPH (Fig. 7.), possibly supporting conversion of galactose into biomass. Faster growth of *P. kessleri* in the dark than in the light on lactose (Fig. 1.) might be explained by the β-galactosidase expression regulation in this strain. Indeed, Stappler et al. [36] observed that glycoside hydrolase gene expression was affected by light in *Trichoderma reesei*, with a generally lower quantity of secreted proteins in the light. Identification of the metabolic routes involved in lactose metabolism, characterization of the expression of β-galactosidase and hexose transporter genes [37] in the light and dark would allow to better understand the lactose assimilation in *P. kessleri*, which could serve as a basis to design future strategies to enhance lactose utilization in this strain through genetic engineering.

P. kessleri (PK25) is able to utilize organic carbon from dairy wastewater, as shown on Fig. 3 by both biomass productivities and maximum biomass concentrations of the cultures grown on the waste material compared to when there was no organic carbon added to the medium. Even though the wastewater is a complex substrate containing lactose, and possibly other organic carbon sources as well, we also observed that, overall, there was more efficient lactose utilization in heterotrophic than in mixotrophic conditions, considering the lactose concentrations at the end of the experiment. Overall, our results indicate that *P. kessleri* could be a good candidate for dairy wastewater

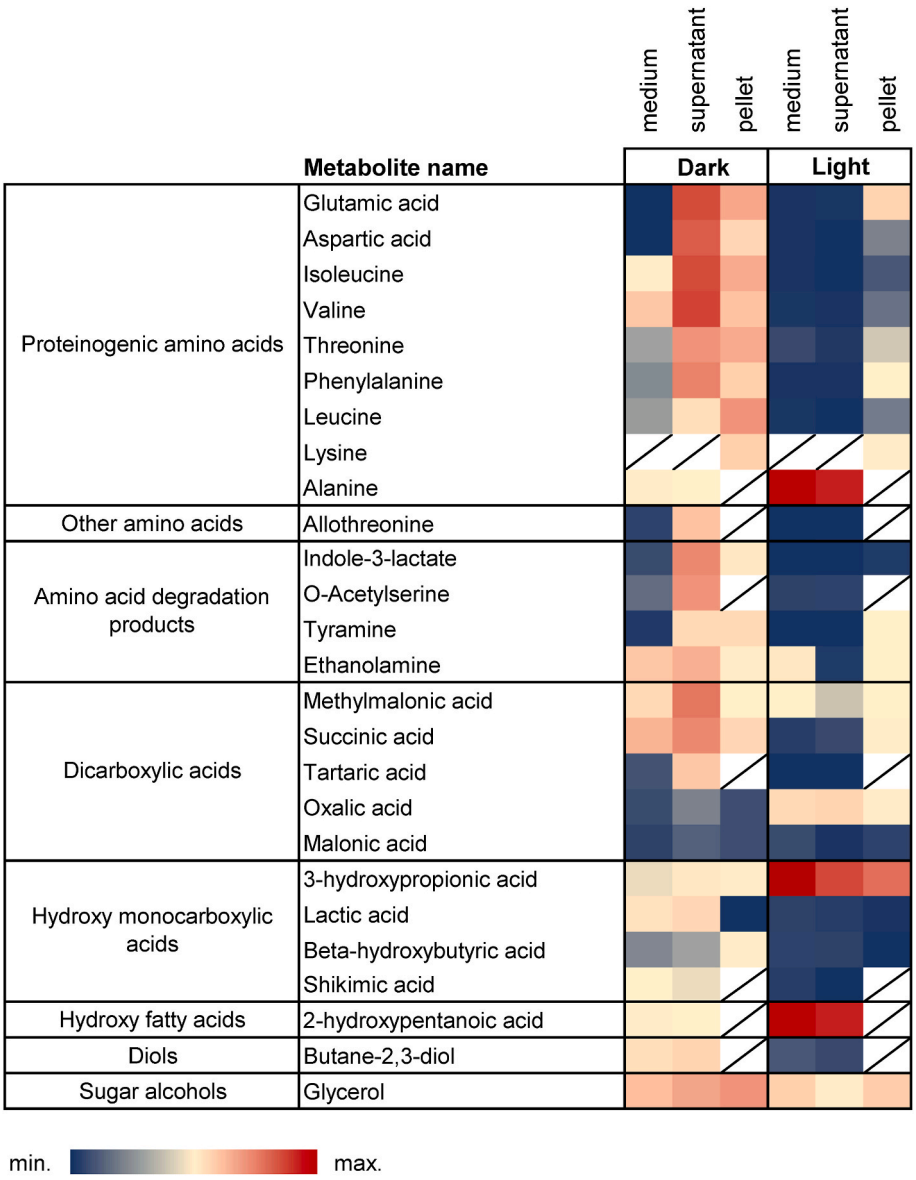

Fig. 4. Heatmap depicting the relative abundances of the relevant polar metabolites, normalized by the internal standard, for *P. kessleri* when cultivated in autotrophic and in heterotrophic conditions.

treatment, as it was shown to be able to reduce the organic matter load of the substrate. Further studies are necessary to quantify the nitrogen and phosphorus reduction, and to optimize the process. Moreover, in this work algae were grown under axenic conditions, using sterile techniques during media and equipment preparation and culture manipulation. Special attention should be paid in the large-scale process design to contamination control and to reduce costs associated to sterilization of substrates and equipment. A potential line of future research could be the study of the behavior of *P. kessleri* as part of a consortium containing lactic acid bacteria in non-sterile conditions.

Our ultimate long-term goal is to cultivate *P. kessleri* in heterotrophic conditions on dairy waste material to produce metabolites of commercial interest. Dairy wastewater may contain several carbon sources, hence we chose glucose, a commonly preferred monosaccharide by algae [38], and also a constituent of lactose, as the organic carbon substrate for the initial untargeted metabolic profiling for *P. kessleri* grown in heterotrophic conditions. Using this substrate also facilitates the comparability of the metabolite accumulation pattern to others strains, including the strains of our initial selection [26]. Future work may be dedicated to targeted metabolomics focusing on selected metabolites, using different carbon sources for cultivation.

The results of untargeted metabolomics performed on *P. kessleri* grown in heterotrophic conditions contrasted with the biomass grown in autotrophic mode indicate that several metabolites of commercial interest, such as amino acids, dicarboxylic acids, and triacylglycerols, were produced in high relative abundances in heterotrophic conditions on glucose in comparison with autotrophic conditions (Fig. 4 and 5.). High amino acid content on the organic carbon source, glucose (Fig. 4.), is in line with what was observed for the strains of the initial selection [26], i.e. *Chlorococcum* sp., *Desmodesmus* sp., and *Chlamydomonas debaryana*. The relative free amino acid pool sizes are a result of the tradeoff between amino acid and protein synthesis, and their catabolism [39]. The accumulation of amino acids could be the result of the carbon flux from glucose being directed towards *de novo* synthesis of these metabolites from intermediates of the Embden–Meyerhof–Parnas glycolytic pathway (phosphoenolpyruvate; pyruvate) and the tricarboxylic acid cycle (TCA) (oxaloacetate; α-ketogluratate) (Fig. 7.). Amino acids may also accumulate during protein degradation, for example in the process of redirecting the carbon flux towards other metabolic pathways, such as towards the generation of more significant levels of

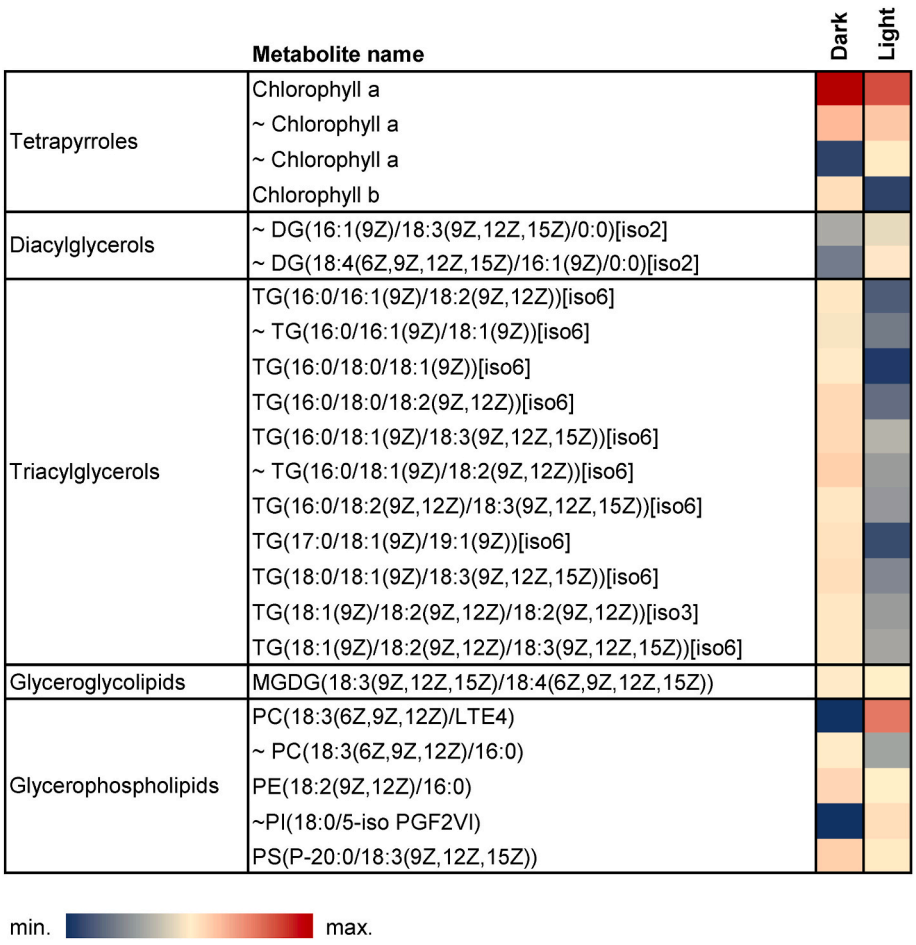

Fig. 5. Heatmap depicting the normalized relative abundances of the relevant lipid species (total iron current) for *P. kessleri* when cultivated in autotrophic and in heterotrophic conditions; ~: similar to.

Table 2
Monosaccharide composition of the exopolysaccharides produced by *P. kessleri* PK25 when cultivated in autotrophic conditions.

Monosaccharide name	w/w %
Arabinose	0
Rhamnose	21.6
Xylose	13.7
Mannose	10.3
Galactose	36.2
Glucose	14.2
Galacturonic acid	0
Glucuronic acid	4.1

storage compounds at late exponential/early stationary phase [40], when the samples were taken. Catabolic processes are suggested by elevated levels of amino acid degradation products (Fig. 4.). As these intermediates were also accumulated to a significant degree in the culture medium, extracellular amino acid pools may as well be the result of the degradation of secreted proteins by proteases [41].

Significant intracellular triacylglycerol accumulation by *P. kessleri* when grown on organic carbon in comparison with autotrophic conditions (Fig. 5.) without hindering biomass growth is in line with what was observed by other authors for other microalgae species [42]. At the end of the exponential phase there is also a tendency to shift the metabolic routes towards lipid storage compound synthesis (Fig. 7.) [40]. The major fatty acyl moieties in triacylglycerols and also in other abundant lipid species for *P. kessleri* (C16:0, C18:1, C18:2, and C18:3), were observed to be the most significant ones in the *Chlorophyceae* class [19]. Comparing the lipid accumulation pattern for *P. kessleri* to the strains of the initial selection [26], there was more significant triacylglycerol accumulation in heterotrophic conditions for *P. kessleri* than for *Chlorococcum* sp. and *Desmodesmus* sp., and it was comparable to what was observed for *Chlamydomonas debaryana*. There was a larger difference in triacylglycerol accumulation between heterotrophic and autotrophic conditions for *P. kessleri* than for the other species.

Succinic acid was observed to accumulate as a fermentation product under specific oxygen deficient conditions in some wild type algae [43] and mutant strains [44], as suggested, as a consequence of an active reverse, reductive TCA cycle branch, serving to maintain reducing power balance (Fig. 7.). Accumulation of succinic acid in our study under heterotrophic conditions (Fig. 4.) indicates that the availability of oxygen for *P. kessleri* may have been limited. High relative abundances of tartaric acid were observed for *P. kessleri* under heterotrophic mode (Fig. 4.). In plants, ascorbate is the precursor for the synthesis of tartaric acid (Fig. 7.), whereas there is limited information in the literature on the role of and the metabolic pathways leading to accumulation of this metabolite in algae.

Several metabolites identified in this study showing high relative abundances in heterotrophic conditions have applications in the food industry as flavor enhancers (glutamic acid), or sweeteners (aspartic acid, phenylalanine); in the feed industry, as quality enhancers (lysine, threonine); or as dietary supplements for athletes (isoleucine, leucine, valine) [26,45,46]. One of the applications of tartaric acid is in the manufacturing of pharmaceuticals, such as cough syrups or effervescent salts [47]. Succinic acid, besides other uses, can serve as a base

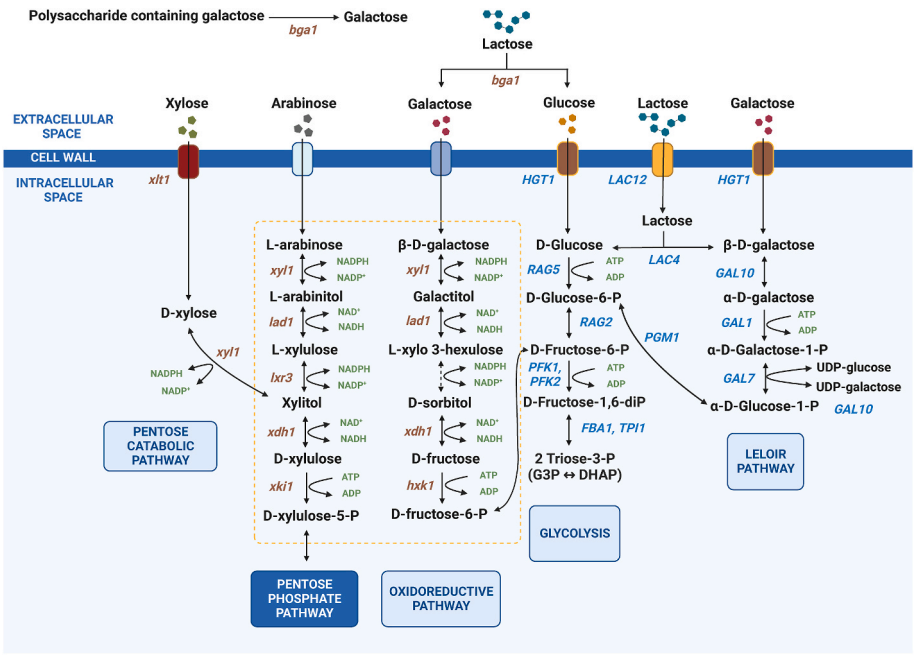

Fig. 6. Lactose catabolic pathways interconnected with L-arabinose and D-xylose catabolic routes in fungi. Genes relative to *Hypocrea jecorina* are shown in brown, and genes relative to *Kluyveromyces lactis* are represented in blue. Enzymes encoded by the genes: *bga1*: extracellular β-galactosidase; *xlt1*: putative xylose permease; *xyl1*: aldose reductase, D-xylose reductase; *lad1*: L-arabinitol dehydrogenase; *lxr3*: L-xylulose reductase; *xdh1*: xylitol dehydrogenase; *xki1*: xylulokinase; *hxk1*: hexokinase; *HGT1*: glucose/galactose transporter; *LAC12*: lactose permease; *LAC4*: intracellular β-galactosidase; *GAL10*: aldose 1-epimerase, UDP-galactose 4-epimerase; *GAL1*: galactokinase; *GAL7*: Gal-1-phosphate uridyltransferase; *PGM1/PGM2*: phosphoglucomutase; *RAG5*: hexokinase; *RAG2*: glucose-6 phosphate isomerase; *PFK1*, *PFK2*: 6-phosphofructokinase; *FBA1*: fructose-bisphosphate aldolase; *TPI1*: triosephosphate isomerase [31,35,51,52]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ingredient for the manufacturing of bio-based green polymers (polyurethanes) [20]. Triacylglycerols containing elevated amounts of saturated and monounsaturated fatty acyl moieties are suitable for biodiesel production [40]. Therefore, the selected *P. kessleri* strain has a good potential for industrial application. Genome sequencing and the development of genetic tools in the future will enable further improvement of this strain, as genetic transformation of *P. kessleri* has already been reported using different techniques [48,49]. Selected compounds identified as abundant in this work, or other metabolites of commercial interest showing low relative abundances in the initial metabolic profiling, e.g. oxalic acid, malonic acid, 3-hydroxypropionic acid, lactic acid, beta-hydroxybutyric acid, shikimic acid, or butane-2,3-diol (Fig. 4.), may be the focus of metabolic engineering aiming to enhance product yields.

Although a degree of heterogeneity of the EPS composition was found across the Chlorophyte species, the main monosaccharide was often observed to be galactose [50], which is consistent with our finding for *P. kessleri* (Table 2.). Further compositional and functional analysis of the EPS would be of great importance for this strain, with an outlook on commercial application, as these compounds were shown to have the potential to have significant health and nutritional benefits [23].

5. Conclusions

In the present work we have shown that *P. kessleri*, a microalga of potential commercial interest, is able to utilize lactose for significant heterotrophic growth. We have also found that the selected strain is able to grow on dairy wastewater and assimilate lactose from this complex substrate. Our findings are relevant from both the cost of commercial algae cultivation, as media cost is often quite significant; and from an environmental perspective, as dairy wastewater has a significant adverse ecological impact when left untreated. We have also observed, through untargeted metabolomics, the accumulation of several compounds of commercial value produced by *P. kessleri* (PK25) in heterotrophic growth conditions, including amino acids with primary application in the food, feed, and nutraceutical industry; tartaric acid, a valuable ingredient in the pharmaceutical industry; and succinic acid, which, amongst many other applications, can serve as a precursor for the manufacturing of bio-based green polymers. We have also confirmed that *P. kessleri* can accumulate, when grown on organic carbon, significant amounts of triacylglycerols, with a suitable composition for biofuel production. Future research may focus on characterizing the lactose assimilation pathway in *P. kessleri*; sequencing of the organism, and development of a genetic toolbox for this strain; and targeted metabolomics based on this initial profiling; with the ultimate goal to improve substrate assimilation and targeted product yield by process optimization and genetic engineering.

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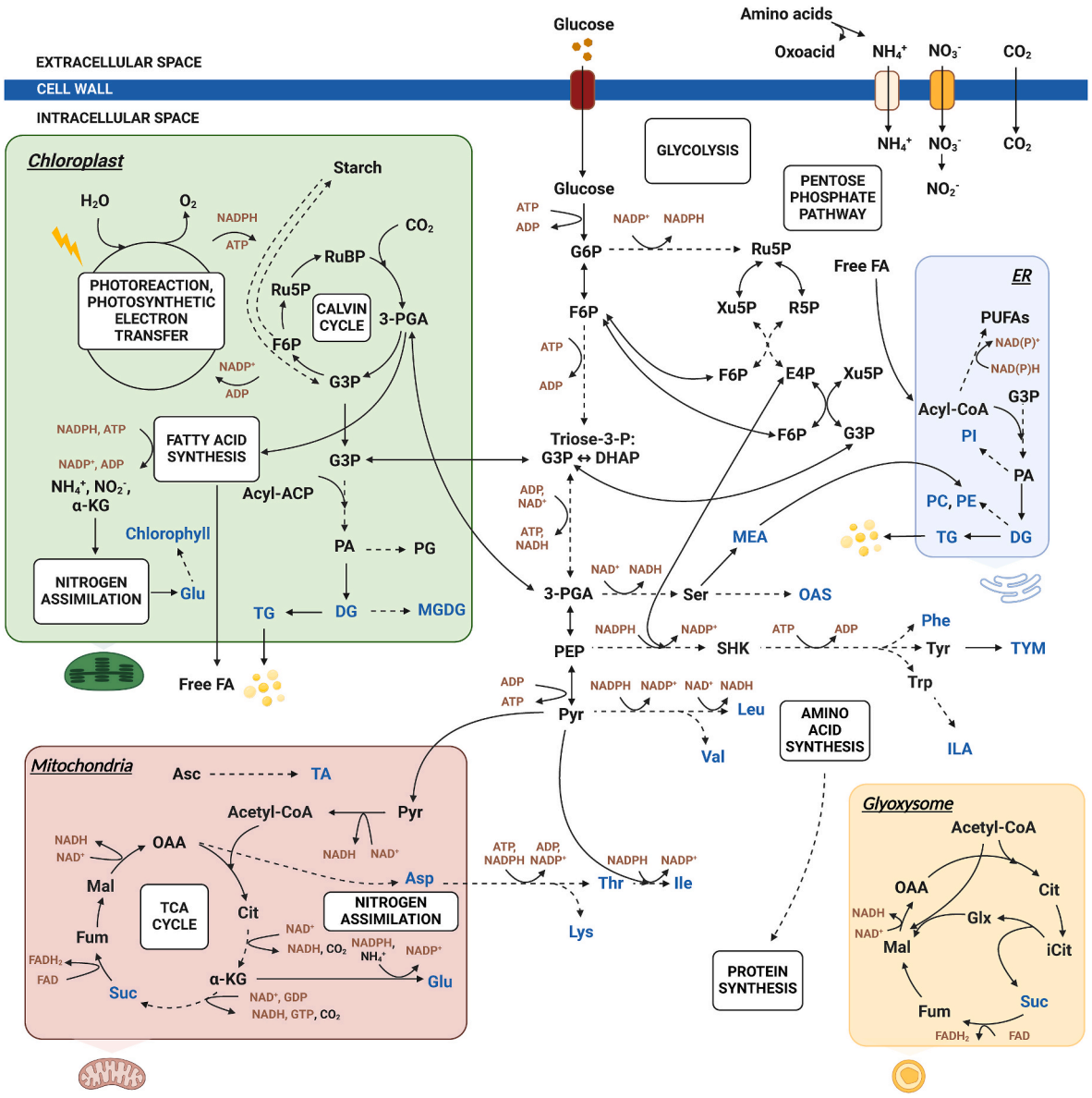

Fig. 7. Putative metabolic pathways involved in the accumulation of the main metabolites of high relative abundances, shown in blue, in *P. kessleri* cultivated in the experimental setup of this study under autotrophic conditions and under heterotrophic mode on glucose; MEA: ethanolamine; OAS: O-acetylserine; SHK: shikimate; TYM: tyramine, ILA: indole-3-lactate, TA: tartaric acid [19,38,53 54 55 56 57 58]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

European Union nor the European Commission can be considered responsible for them.

CRediT authorship contribution statement

Nora Hidasi: Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Amr Badary:** Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Hunter D. Jenkins:** Resources. **Francis J. Fields:** Resources. **Stephen P. Mayfield:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Simone Ferrari:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

SM is a founder and holds an equity position in Algenesis Materials Inc, a company that could benefit from this research. The other authors declare that the research was performed in the absence of any financial or commercial connection that may be interpreted as potential conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biombioe.2024.107344.

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