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Identifying *Chlorella vulgaris* and *Chlorella sorokiniana* as sustainable organisms to bioconvert glucosamine into valuable biomass

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ABSTRACT

Chitin is a major component of various wastes such as crustacean shells, filamentous fungi, and insects. Recently, food-safe biological and chemical processes converting chitin to glucosamine have been developed. Here, we studied microalgae that can uptake glucosamine as vital carbon and nitrogen sources for valuable alternative protein biomass. Utilizing data mining and bioinformatics analysis, we identified 29 species that contain the required enzymes for glucosamine to glucose conversion. The growth performance of the selected strains was examined, and glucosamine was used in different forms and concentrations. Glucose at a concentration of 2.5 g/L was required to initiate glucosamine metabolic degradation by *Chlorella vulgaris* and *Chlorella sorokiniana*. Glucosamine HCl and glucosamine phosphate showed maximum cell counts of about 8.5 and 9.0 log/mL for C. *sorokiniana* and *C. vulgaris* in 14 days, respectively. Enzymatic hydrolysis of glucosamine increased growth performance with *C. sorokiniana* by about 3 folds. The adapted strains were fast-growing and could double their dry biomasses during the same incubation time. In addition, adapted *C. sorokiniana* was able to tolerate three times glucosamine concentration in the medium. The study illustrated possible strategies for employing *C. sorokiniana* and *C. vulgaris* to convert glucosamine into valuable biomass in a more sustainable way.

1. Introduction

With the growing population and biotechnologies, waste generation is also increasing. The major proportion of the created waste contains high-value substances but remains underutilized. Chitin is the main component found in various wastes, including crustacean shells, insects, and fungal mycelia.[1–3] It makes up around 70 % of the dry shell waste of crustaceans and ranges from 15 to 45 % dry weight (DW) in fungal mycelia.[4,5] Approximately 80 % of the harvested crustaceans are processed into a variety of products, which generate a significant amount of solid waste.[6] Demand for seafood is expected to increase by 60 % as a result of an increase in the global population, which is expected to reach 9.8 billion people by 2050.[7] Similarly, with the current advances in biotechnology, filamentous fungi, and insects have been applied to produce various bioproducts including enzymes, organic acids and pigments, insecticides, herbicides, antibiotics, and other therapeutic metabolites, resulting in huge amounts of waste are produced annually, which continue to expand.[8–15] From the perspective of food security, these types of waste are considered to be a loss of value component.[16,17] In addition, potential environmental hazards might occur if waste is discarded without proper treatment.[18] Therefore, advancements in waste stream treatments are necessary to better shape the future of this industry.

The chitin-based wastes are often mechanically ground to reduce their size before going through a series of acid/alkali to remove minerals and proteins followed by chitin and chitosan hydrolysis to generate monomers such as glucosamine.[2,9,19,20] Enzymes such as chitinases, chitosanase, hexosaminidases, and B-glucosidases have been also used

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to depolymerization of chitin and produce glucosamine in a more eco-friendly approach.[21–25] Similarly, glucosamine was successfully derived by fermentation of chitin by several microorganisms. For example, Samant et al. (2019) used bacteria, including *Bacillus* sp., *Vibrio* sp, and *Brevibacterium* sp., to hydrolysis chitin and create glucosamine.

The majority of the generated glucosamine is used in pharmaceutical industries, especially in the context of osteoarthritis, however, the complexity of its purification in the downstream process is considered a crucial obstacle.[26–29] To further extend the generated glucosamine application, in a green way, we hypothesize that glucosamine can be reliable carbon and nitrogen sources for microorganisms including bacteria, fungi, and microalgae to produce valuable alternative protein biomass. Among them, microalgae are rich in proteins, lipids, ash, and other high-nutritive ingredients.[30] Furthermore, they are characterized by their capability to utilize a wide range of cheap nutrients and food waste.[31] These characteristics make them potential crop alternatives that might impact the current world's need for food in a more sustainable way to produce alternative and single-cell proteins compared to other microorganisms.[32]

In the last decades, green microalgae, including *Chlorella*, have been used as food, feed, biofuels, and to produce high value products like vitamins and pigments.[33,34] They are found in both terrestrial and aquatic environments and capable to utilize two types of nutrition, inorganic compounds such as CO_2, namely autotrophy, and organic compounds like sugars, called heterotrophy. Some green microalgae are capable of combining both nutrition, namely mixotrophic. The economic viability and biomass yield, however, remain crucial hurdles to their industrialization.[34,35] Additionally, the diversity of this group of microorganisms makes it difficult to identify the right candidates. In this study, we started by reviewing the literature and public databases to identify the key enzymes involved in the hydrolysis of glucosamine to glucose. Next, we used bioinformatics tools and resources to identify microalgae species/strains that produce those enzymes and, at the same time, are food-safe or generally recognized as safe organisms (GRAS). Our bioinformatics analysis identified 54 microalgae strains from 29 species. The list was further filtered using several parameters, such as the availability of the full protein sequence of the required enzymes in the databases, the availability of strain to acquire, and the suitability of the stain to the cultivation growth processes. Subsequently, the two selected strains, *Chlorella sorokiniana,* and *Chlorella vulgaris,* were subjected to different growth performance parameters and further investigated to improve glucosamine utilization. Our results indicate that the selected microalgae strains are ideal candidates for converting glucosamine into valuable biomass. Microalgae growth was improved after treating glucosamine with hydrolytic enzymes and by undergoing an adaptive laboratory evolution process.

2. Material and methods

2.1. Identifying glucosamine-utilizing strains

Related published data was surveyed to investigate metabolic pathways and processes that involve glucosamine in different organisms to identify the key enzymes involved in the glucosamine hydrolysis process. Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were used to identify the metabolic pathways with the key enzyme, glucosamine-6-phosphate deaminase (G6PD).[36,37] Next, the alternative names and IDs (15 different names/IDs) of the G6PD enzyme were retrieved from GeneCards database.[38] The enzyme names were used to perform a proteome-wide screening of all organisms in the UniProt database.[39] We used the taxonomy information of the UniProt database to select the spices/strains of the microalgae group possessing the enzyme, and the list was further filtered based on the availability of the full sequence of the enzymes in the databases.

2.2. Microalgal species and cultivation procedures

The shortlisted strains were obtained from different sources and were cultivated following procedures recommended by the suppliers (Supplementary Tables 1 and 2). To prepare the inoculum, isolated pure single colonies were picked up, and inoculated into 10 ml of BG11 (for fresh algae) and f/2 medium (for brackish/marine algae) and incubated for 10 days to form the mother stock culture.[40,41] All medium used in this study was adjusted to pH 7.5, incubated at photosynthetic photon flux density (PPFD) level of 100 mol m^{-2} day^{-1} (if light is required), at 25 °C under aerobic and 250 rpm agitation, except when otherwise stated. The pure culture was subcultured into 100 ml of appropriate media (containing 0.1 % glucosamine) grown in 250-mL Erlenmeyer flasks with an initial OD750 nm of 0.05 and maintained for up to 7–10 days to prepare the inoculums. D-glucosamine 3- sulphate (Sigma-Aldrich) was used in this study, except when otherwise stated. Cell growth was measured by the spectrophotometric method.[42,43] The biomasses in 5 mL were harvested using a centrifuge (Centrifuge 5810 R, Eppendorf, B. Braun, Melsungen, Hesse, Germany) at 12000×*g* for 15 min, at 4 °C, in triplicates. The harvested algae were washed with 0.9 % sodium chloride solution (NaCl–W) three times, then dried at 60 °C for 24 h for biomass analysis, and the reported results are the mean values of the final biomass dry weights. The specific growth rate (μ) was calculated $\mu = \ln(N2/N1)/(t\,2 - t\,1)$, where N1 and N2 are the biomass at time 1 (t 1) and time 2 (t 2).[44]

2.3. Effects of inducers on microalgae growth

Glucose, fructose, glycerol, and acetic acid at concentrations ranging from 0.1 to 2.5 g/L were explored, along with glucosamine at a concentration of 7.5 g/L (based on the next observation). Sterile glucose and glucosamine were dissolved in basal medium (the BG11 and f1 medium ingredients without carbon and nitrogen sources, mixotrophic regimen) and inoculated with *C. vulgaris* and *C. sorokiniana* and the inoculum size was adjusted to 0.05 at OD750 nm, each in triplicate and kept under the same incubation parameters.

2.4. Effects of glucosamine concentrations and salt types on microalgae growth

The growth capabilities of microalgae were evaluated against different concentrations and types of glucosamine. A variety of glucosamine concentrations, ranging from 0.5 to 12.5 g/L, with 2.5 g/L glucose, as an inducer, were prepared. The mixtures were inoculated with *C. vulgaris* and *C. sorokiniana* and the inoculum size was adjusted to 0.05 at OD750 nm, each in triplicate. Glucose (2.5 g/L) was prepared and dissolved in the basal medium without glucosamine, as a negative control. To examine the impact of different glucosamine salt types on microalgal growth, D-(+)-glucosamine hydrochloride, D-glucosamine 3-sulphate, and D-glucosamine 6-phosphate (Sigma-Aldrich, derived from multiple sources include *Aspergillus niger*, and plants), at a concentration of 7.5 g/L in the presence of 2.5 g/L of glucose were also subjected to microalgal cultivation under same incubation parameters (each in triplicate).

2.5. Enzyme degradation of glucosamine

To investigate further ways to improve the microalgal growth, glucosamine was subjected to enzymatic degradation using an enzyme cocktail of amyloglucosidase from *Aspergillus niger* and α-amylase enzyme from *B. subtilis*, and pectinase from *A. aculeatus* obtained from Sigma-Aldrich as a pre-treatment process before inoculations. The hydrolysis was performed at 60 °C, pH 5.5, for 4 h, with a concentration of 100 IU of each enzyme (individually or combined) per 1 g of the substrate. Sterile glucose (2.5 g/L) and the basal medium (BG11 medium component without carbon and nitrogen sources, supplementary

Table 2) were added to the resulting hydrolysates, adjust pH 7.5, and the mixtures were sterilized by filtration. Selected algae strains were inoculated and grew under the same incubation parameters mentioned above (each in triplicate).

2.6. Adaptive laboratory evolution of selected microalgae

The selected species were further subjected to a slow adaptation process over several months, aiming to improve their overall growth performance and glucosamine utilization. *C. vulgaris* and *C. sorokiniania* were first cultured in modified BG11 agar and incubated in the dark at 25 °C for up to 2 weeks or until heavy growth was observed. The modified BG11 agar contains the basal medium plus sterile glucose, which was gradually replaced with sterile glucosamine (without adding NaNO3). In more detail, the glucosamine level was first at a concentration of 0.5 g/L and glucose was at 9.5 g/L. Glucosamine levels were gradually increased and glucose concentrations were correlatively reduced, and the cultivation process was repeated until we reached 2.5 g/L glucose and 7.5 g/L glucosamine associated with heavy growth on the culture plates. To prepare inoculums from the evolved strains that grew on the plates, one pure colony was transferred into the glucosamine medium, and incubated at 25 °C in the dark under shaking at 250 rpm. The evolved strain inoculums were adjusted at a final concentration of 0.05 at OD750 nm into the glucosamine medium in the absence and presence of a concentration of 2.5 g/L glucose, and their growth was monitored and compared to the parent wild strains following the same methods above, each in triplicate. Protein contents were extracted using Lowry method and quantified by a UV–Vis spectrophotometer using bovine serum albumin (BSA) as a standard.[45]

2.7. High-performance liquid chromatography (HPLC)

For quantification of glucosamine, glucose, glycerol, and organic acids in the culture medium, cells were removed by centrifugation (12,000×*g*, 20 min), and the supernatant was analyzed using HPLC (Shimadzu) as described previously.[46] An Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) was used together with a Bio-Rad 125–0131 guard cartridge (Bio-Rad) and a refractive index detector (RID-10A, Shimadzu). The HPLC system was operated at 35 °C with 5.0 mM H2SO4 (flow rate, 0.6 mL/min) as the mobile phase. The concentration of glucosamine in the medium was quantified by comparing the retention times and peak areas of a standard curve of glucosamine at known concentrations.[22]

2.8. Statistical analysis

The statistical analysis of the growth rate and biomass recovery was performed with Microsoft Excel. For each studied condition, two independent experiments were performed. The results between the experimental groups were analyzed by one-way ANOVA and Tukey's *post hoc* analysis using the SPSS 12.0 statistical package program (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Database search and bioinformatics analysis shortlist microalgae candidates

From the literature review, we identified the glucosamine-6-phosphate deaminase (G6PD) enzyme as the potential enzyme, available in some algae strains, to be used for the conversion.[47,48] Further investigation of the enzyme in the KEGG database shows the role that the G6PD enzyme plays in the amino acid and nucleotide sugar metabolism pathways (Supplementary Fig. 1).[49] We obtained all alternative names of the G6PD enzyme from the Gene Cards database.[38] The database search resulted in 15 different names for this enzyme. Based on this result, we created a list of all names and used them to search the UniProt Knowledgebase[39] to get all the known organisms that code this enzyme. Our search results in over 27,500 organisms (including strains) that can produce this enzyme. Next, we used the taxonomy of those organisms to filter out all irrelevant organisms (bacteria, humans, model organisms) and keep the algae family alone. This step removed over 24,000 organisms. Next, we further filtered the remainder of the organisms based on the taxonomy of the algae, the groups that contain food-safe of GRAS organisms, and a complete sequence and data of the enzyme. This step resulted in the first shortlist of organisms (54 microalgae strains from 29 species) to be deeply investigated for their application potential in this application. Feeding a species that produces the G6PD enzyme in its cells with glucosamine as a primary feed will result in the production of excessive amounts of ammonia that might impact the growth of the microalgae by slowing it down or even killing the cells. Therefore, we investigated the ammonia hydrolysis capacity of the 19 organisms in the final list (Supplementary Table 1). This analysis resulted in the selection of seven species, including *Chlorella sorokiniana*, *Chlorella variabilis, Chlorella vulgaris, Micractinium conductrix, Monoraphidium braunii* (formerly known as *Monoraphidium neglectum*), *Nannochloropsis gaditana, and Chloropicon primus*, since they have both enzymes. Based on the growth performance in the presence of glucosamine, as shown in the results below, we select *C. vulgaris*, and *C. sorokiniana* for further investigation to improve their overall growth performances and biomass recoveries.

3.2. Effects of different inducers on microalgae growth

None of the microalgae could grow in the presence of glucosamine as the sole carbon source in the liquid medium. Therefore, it was hypothesized that adding suitable inducers might initiate the glucosamine metabolic pathway. Among the selected inducers, glucose showed significant growth with *C. vulgaris* with specific growth rate of 0.05 h^{-1} (Supplementary Table 3), while glucose and acetic acid were associated with significant growth with *C. sorokiniana* at a concentration of 2.5 g/L with specific growth rate (μ) of 0.03–0.04 h^{-1}, respectively (Fig. 1 and Table 1). The cell population of *C. sorokiniana* with the presence of glucose reached approximately 7.5 log cells/ml in 5 days before declining. A double time was required to reach the same density in the presence of acetic acid. *C. vulgaris* reached 8.1 log cells/mL in 5 days and gradually increased to 8.4 log cells/mL at the end, while acetic acid showed no significant growth. HPLC data showed, with *C. sorokiniana* gradual decreases in the concentration of glucose (2.8–0.65) and acetic acid levels (2.1–0.95) associated with a significant decline of glucosamine levels (7.3–2.3) and (7.4–4.0), respectively, during incubation, while no significant changes were detected with glycerol, fructose and the associated glucosamine (Table 1a–b). The values in brackets show the change in concentrations (g/L) from the initial day until day 13. For *C. vulgaris* glucose declined from 2.8 to 0.47 g/L at the end of culturing, and the associated glucosamine declined from 7.5 to 3.2 g/L in 13 days. Therefore, glucose was chosen as the standard inducer at a concentration of 2.5 g/L in the subsequent experiments.

3.3. Effects of different glucosamine types and concentrations on microalgae growth and biomass recovery

Gradual increase in the cell counts from 2.5 g/L to 7.5 g/L reaching about 8 log cells/mL with *C. vulgaris* at a glucosamine concentration of 5.0 g/L in 14 days, and about 9 log cells/mL at a glucosamine level of 7.5 g/L when extending the incubation to 20 days, but a long lag phase was observed. *C. sorokiniana* reached a maximum growth rate of 8.1 log cells/mL in 14 days when the glucosamine level was 7.5 g/mL without significant differences when extending the cultivation, (Fig. 2a). At 10 g/L glucosamine level, *C. vulgaris* still showed significant growth of 8.3 log cell/mL, but with a long lag phase. These counts agreed with the dried cell weights, which showed 0.6 g/L for *C. vulgaris* and about 0.3 g/

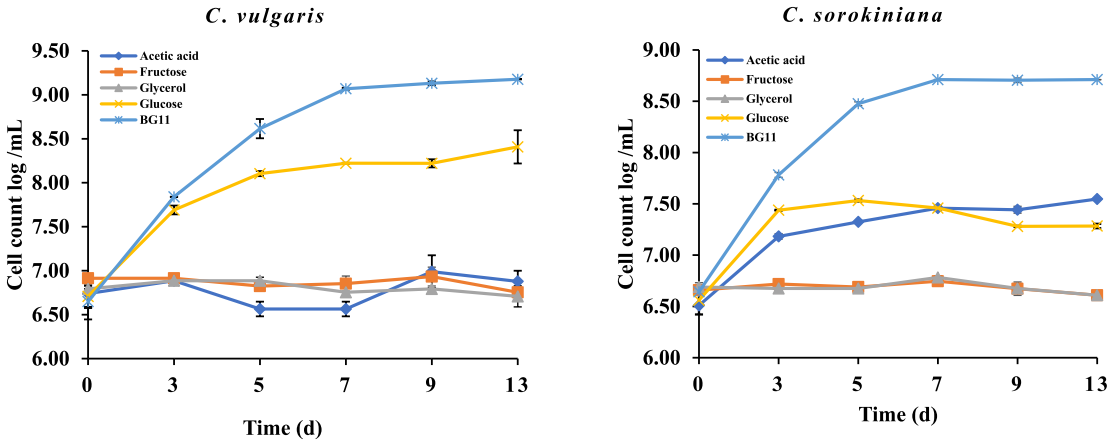

Fig. 1. The impact of different inducers on microalgae growth. The values reported are means of three replicates with error bars representing one standard deviation.

Table 1 (a)
Changes in inducer concentrations during *C. sorokiniana*, and *C. vulgaris* cultivations.

Days	Glucose		Glycerol		Fructose		Acetic Acid		BG-11 Glucose	
	C. S*	C.V**	C. S	C.V	C. S	C.V	C. S	C.V	C. S	C.V
0	2.83^{aA}	2.83^{aA}	2.61^{aA}	2.61^{aA}	1.93^{aA}	1.93^{aA}	2.14^{aA}	2.14^{aA}	8.09^{aA}	8.09^{aA}
5	1.63^{bA}	1.59^{bA}	2.52^{aA}	2.60^{aA}	1.89^{aA}	1.86^{aA}	1.99^{aA}	1.92^{aA}	6.77^{bA}	8.36^{aB}
7	1.71^{bA}	1.62^{bA}	2.43^{aA}	2.40^{aA}	2.00^{aA}	2.02^{aA}	1.99^{aA}	2.16^{aA}	4.03^{cA}	5.50^{bB}
9	1.76^{bA}	1.58^{bA}	2.44^{aA}	2.48^{aA}	1.99^{aA}	2.11^{aA}	0.99^{bA}	1.94^{aB}	ND $^{+ dA}$	3.86^{cB}
13	0.65^{cA}	0.47^{bcA}	2.65^{aA}	2.79^{aA}	2.03^{aA}	1.93^{aA}	0.95^{bA}	2.07^{aB}	ND^{dA}	2.09^{dB}

Table 1 (b)
Glucosamine concertation changes in the presence of different inducers.

Days	Glucosamine + glucose		Glucosamine + glycerol		Glucosamine + fructose		Glucosamine + acetic Acid	
	C.S*	C.V**	C.S	C.V	C.S	C.V	C.S	C.V
0	7.30^{aA}	7.53^{aA}	6.95^{aA}	7.25^{aA}	7.52^{aA}	6.99^{aA}	7.39^{aA}	7.35^{aA}
5	7.81^{aA}	7.55^{aA}	7.01^{aA}	7.69^{aA}	7.77^{aA}	7.03^{aA}	7.40^{aA}	7.09^{aA}
7	6.00^{bA}	6.10^{bA}	6.85^{aA}	7.52^{aA}	7.39^{aA}	7.09^{aA}	6.80^{bA}	6.92^{aB}
9	5.08^{cA}	4.38^{cB}	6.93^{aA}	7.01^{aA}	6.89^{aA}	6.85^{aA}	6.03^{bA}	6.87^{aB}
13	2.29^{dA}	3.21^{dB}	6.79^{aA}	6.73^{aA}	6.98^{aA}	6.97^{aA}	4.02^{cA}	6.99^{aB}

ND: not detected. The amount is expressed as mean (n = 3). The mean values in each column with the same letter indicate no significant difference at $\alpha \geq 0.05$, different lower-case letters (a–d) indicate statistical differences among the same inducers and mean values in the same row with different upper-case letters (A, B) indicate statistical differences between the two microalgae strains with the same inducers at $\alpha < 0.05$, by Tukey's multiple comparison One-way ANOVA test. C.S*. *C. sorokiniana,* C.V**. *C. vulgaris*.

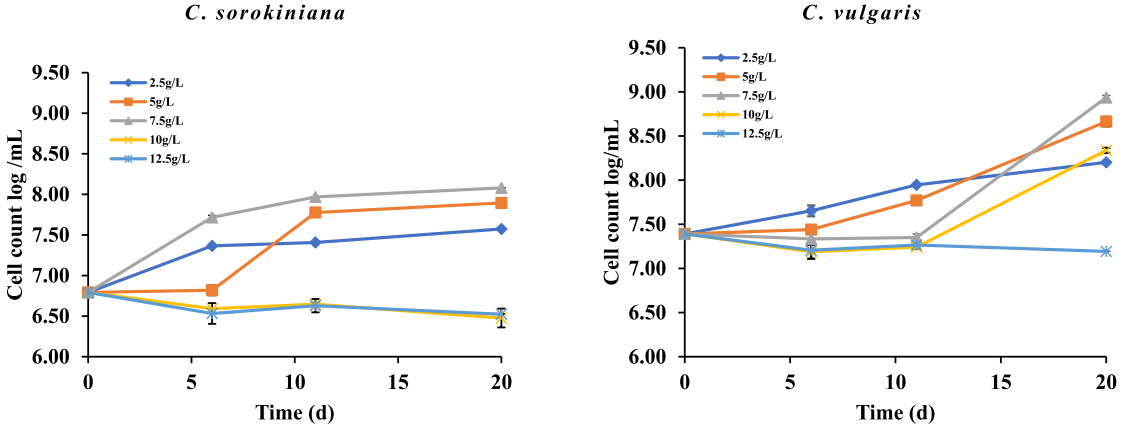

Fig. 2 (a). *Chlorella sorokiniana* and *C. vulgaris* growth behaviour under different glucosamine concentrations. Legend: 2.5 g/mL, 5.0 g/mL, 7.5 g/mL, 10 g/mL, 12.5 g/mL. The values reported are means of three replicates with error bars representing one standard deviation.

L of *C. sorokiniana* (Fig. 2b). Therefore, 7.5 g/L of glucosamine was used for the subsequent experiments with both microalgae species.

A maximum growth rate of 8.5 log cells/mL with *C. sorokinoiania* in the presence of glucosamine phosphate ($\mu = 0.06\ h^{-1}$), followed by glucosamine HCl and sulphate. *C. vulgaris* showed the highest growth with glucosamine sulphate at the initial stage reaching a maximum growth of 8.5 log cells/mL ($\mu = 0.04\ h^{-1}$) in 10 days, but sharp increases were observed by the end with glucosamine HCl and phosphate reaching a maximum growth of 9.0 log cells/mL in 2 weeks (Fig. 3a). Biomass dry weight was at a maximum of about 0.6 g/L in glucosamine HCl and phosphate with *C. vulgaris* followed by glucosamine sulphate. *C. sorokinoiania* achieved a maximum dry weight of 0.4 g/L in glucosamine phosphate followed by glucosamine HCl and glucosamine sulphate (Fig. 3b).

3.4. Effects of pre-treatment of glucosamine by enzymes on microalgae growth and biomass recovery

Amyloglucosidase and α-amylase compile were selected based on the preliminary observation (Supplementary Fig. 3) to shorten the overall process and reduce the upscaling cost to practical levels, and sterilization by filtration, assuming that it may preserve the hydrolysates without altering the physicochemical properties as shown with the autoclaving. The degraded sample contained sucrose, glucose, fructose, glycerol, acetic acid, and glucosamine, Table 2. Maximum growth of 8.0 log cells/mL was detected on day 7, treatment 1, which was significantly increased when adding 2.5 g/L glucose in the absence and presence of sodium nitrates (treatment 2 and 4) of about 8.2 log cells/mL and μ was about 0.08 h^{-1}. Maximum dry cell weight was obtained at 1.9 g/L when using 2.5 glucose and 7.5 degraded glucosamine (g/L), treatment 2, followed by the same treatment with the presence of sodium nitrates (treatment 4), Fig. 4. Two-fold increases in the dried biomass recovery were observed when using BG11 medium compared to the resulting enzymatic hydrolysates without adding glucose, which reduced to a one-fold difference when adding 2.5 g/mL glucose to the degraded glucosamine. The degraded compounds showed no significant changes in their concentrations during the first 5 days, except for glucose and acetic acid, which gradually declined in treatments 2 and 4 (Table 2). Sucrose and fructose were reduced to about 50 % of the initial concentrations by the end of incubation in treatment 1 and not detected with the other treatments. Glucosamine showed a similar pattern of a 2-fold reduction in treatment 1, while other treatments showed about 7-fold reductions.

3.5. Effects of laboratory evolution on kinetic growth parameters, and biomass recovery

The selected cultures were subjected to cycles of growth phases and nutrient modulation with sequential increases in glucosamine supplementation in a solid medium. Each cycle lasted between 10 and 14 days. The wild and adapted strains were subcultured in the broth medium containing the standard glucosamine medium (2.5 glucose and 7.5 glucosamine, Supplementary Table 2). The data showed that adapted *C. sorokiniana* reached a growth rate of 8.2 log cells/mL in less than 10 days, which required about 2 weeks with the wild strain, considering the higher initial inoculum of the wild strain as shown in Fig. 5 (μ were 0.06 and 0.05 h^{-1} with both adapted and wild strains, respectively). *C. vulgaris* reached about 8.6 log cells/ml in half the time required for the wild strain. Additionally, a significant increase was observed in the biomass recovery with both adapted species compared to the wild ones, which were double values, mainly with *C. vulgaris* (Fig. 5) (μ were about 0.04 h^{-1} with both strains). No growth was observed with both wild and adapted strains in the absence of glucose still. Both strains were cultivated under higher amounts of glucosamine at concentrations of 10, 12.5, 17.5, and 20 g/L. Glucosamine at a concentration of 17.5 g/L showed a maximum growth rate of 8.3 log cells/mL in 6 days followed by 12.5 and 10 g/L with the adapted *C. sorokiniana*. By cultivation completion, maximum growth of about 8.5 with 20 g/L showed a long lag phase during the first week, then significantly increased by the second week. *C. vulgaris* gradually increased from 8.5 to 8.9 log cells/mL at the concentration of glucosamines 7.5 g/L and 10 g/L, respectively, and then started to decline with increasing the levels of glucosamine. Longer lag phases were observed for all *C. vulgaris* strains, compared to *C. sorokiniana*, where significant growth started in the last quarter of incubation. The dried body weight gradually increased, with a maximum dry cell weight recovery of 1.6 g/L and a total protein content of 9.4 % found with *C. sorokiniana* grew in a medium containing glucosamine at 20 g/L, while *C. vulgaris* showed a maximum dry cell weight of 1.3 g/L and a protein content of 4.3 % at a glucosamine concentration of 10 g/L. Total organic carbon was measured and showed significant sugar was used in the adapted strain compared to wild types, *C. vulgaris* evolved strain consumed about 75–80 % of the sugar present in the medium, which was double the wild strain's capacity. *C. sorokiniana*, wild strains consumed about 30 % which improved to a maximum of 3-fold increases with the adapted strains (Fig. 6).

4. Discussion

According to KEGG metabolic pathways, the conversion of glucosamine to glucose in the cell is a complex process that involves several enzymatic reactions. Glucosamine is converted to fructose-6-phosphate through the glucosamine-6-phosphate isomerase enzyme. The fructose-6-phosphate then enters the glycolysis pathway, where it is converted to glucose-6-phosphate through a series of enzymatic reactions. Glucose-6-phosphate can then either be converted to glucose through the glucose-

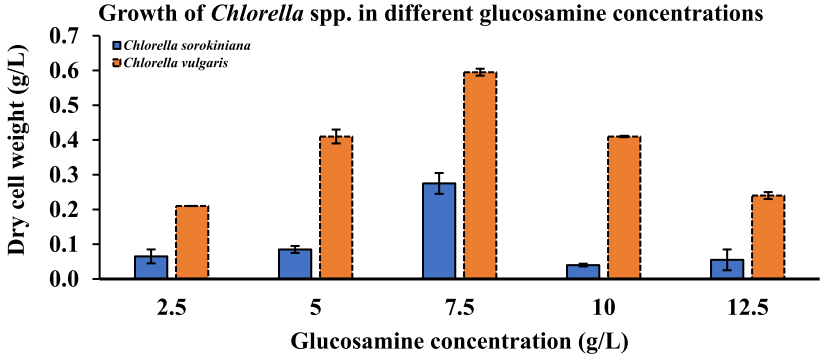

Fig. 2 (b). Dried cell weight (DCW) recovery of *Chlorella sorokiniana* (■) and *C. vulgaris* (■) at different glucosamine concentrations. The values reported are means of three replicates with error bars representing one standard deviation.

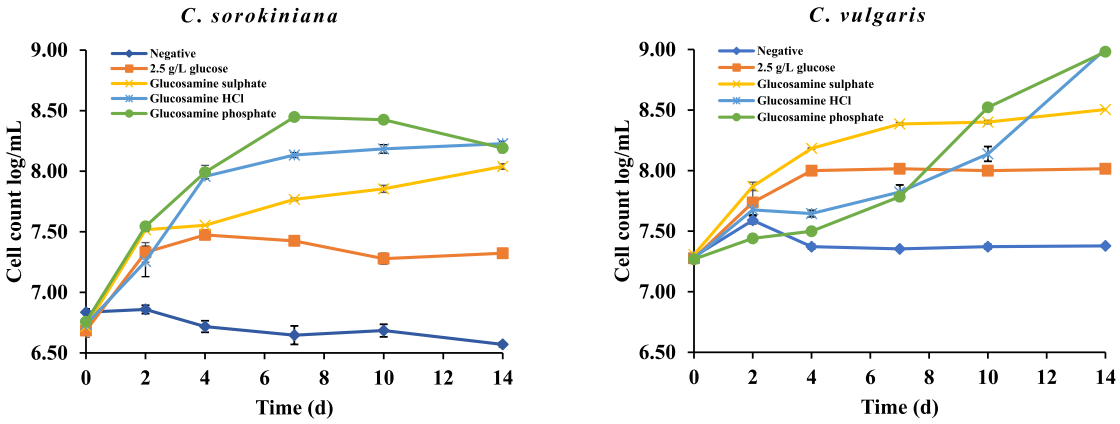

Fig. 3 (a). *Chlorella sorokiniana* and *C. vulgaris* growth behaviour under different glucosamine types. The values reported are means of three replicates with error bars representing one standard deviation. Legend negative control without sugar, glucose 2.5 g/L, glucose 2.5 and glucosamine sulphate 7.5 (g/L), glucose 2.5 and glucosamine HCl 7.5 (g/L), glucose 2.5 and glucosamine phosphate 7.5 (g/L).

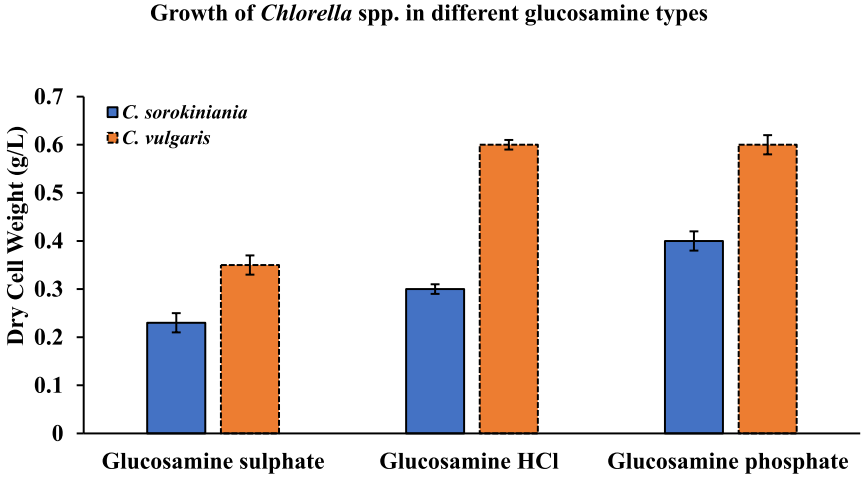

Fig. 3 (b). Biomass recovery of different glucosamine types of *Chlorella sorokiniana* (■) and *C. vulgaris* (■). Glucosamine levels were 7.5 g/L in all treatments. The values reported are means of three replicates with error bars representing one standard deviation.

Table 2
Chemical changes of enzymatic degraded glucosamine components during culturing *Chlorella sorokiniana*.

Treatments	Time (D)	Sucrose (g/L)	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Acetic acid (g/L)	Glucosamine sulphate (g/L)
Hydrolysate	0	0.25^{aA}	0.54^{aA}	0.66^{aA}	3.09^{aA}	3.51^{aA}	2.18^{aA}
(1) Hydrolysate	3	0.30^{aA}	0.86^{aA}	0.81^{aA}	3.70^{aA}	4.18^{aA}	2.34^{aA}
	5	0.29^{aA}	0.73^{aA}	0.81^{aA}	3.69^{aA}	3.46^{aA}	2.33^{aA}
	13	0.15^{bA}	0.27^{bA}	0.39^{bA}	1.48^{bA}	ND^{bA}	1.06^{bA}
	0	0.25^{aA}	3.04^{aB}	0.66^{aA}	3.09^{aA}	3.51^{aA}	2.18^{aA}
(2) Hydrolysate + glucose 2.5 g/L	3	0.30^{aA}	2.44^{bB}	0.80^{aA}	3.92^{aA}	3.97^{aA}	2.32^{aA}
	5	0.35^{aA}	1.76^{cB}	0.80^{aA}	3.63^{aA}	1.18^{bB}	2.33^{aA}
	13	ND^{bB}	0.17^{dB}	ND^{bB}	0.35^{bB}	ND^{cB}	0.36^{bB}
	0	0.25^{aA}	0.54^{aA}	0.66^{aA}	3.09^{aA}	3.51^{aA}	2.18^{aA}
(3) Hydrolysate + sodium nitrate 2 g/L	3	0.28^{aA}	0.77^{aA}	0.76^{aA}	3.49^{aA}	4.20^{aA}	2.60^{aA}
	5	0.27^{aA}	0.62^{aA}	0.76^{aA}	3.45^{aA}	3.28^{aA}	2.58^{aA}
	13	ND^{bB}	ND^{bC}	ND^{bB}	0.10^{bC}	ND^{bB}	0.21^{bB}
	0	0.25^{aA}	3.04^{aA}	0.66^{aA}	3.09^{aA}	3.51^{aA}	2.18^{aA}
(4) Hydrolysate + glucose 2.5 + sodium nitrate 2 g/L	3	0.20^{aA}	2.40^{bB}	0.74^{aA}	3.41^{aA}	3.47^{aA}	2.42^{aA}
	5	0.32^{aA}	0.79^{cA}	0.76^{aA}	3.28^{aA}	ND^{bC}	2.31^{aA}
	13	ND^{*bB}	ND^{dC}	ND^{bB}	0.25^{bB}	ND^{bB}	0.34^{bB}

ND: not detected. The amount is expressed as a mean of three replicates. The mean values in each column with the same letter indicate no significant difference at $\alpha \geq 0.05$, different lower-case letters (a, d) indicate statistical differences among the same treatments at a different time (day) and the different upper-case letters (A-C) indicate statistical differences between the different treatments at the same time, at $\alpha < 0.05$ by Tukey's multiple comparison One-way ANOVA test.

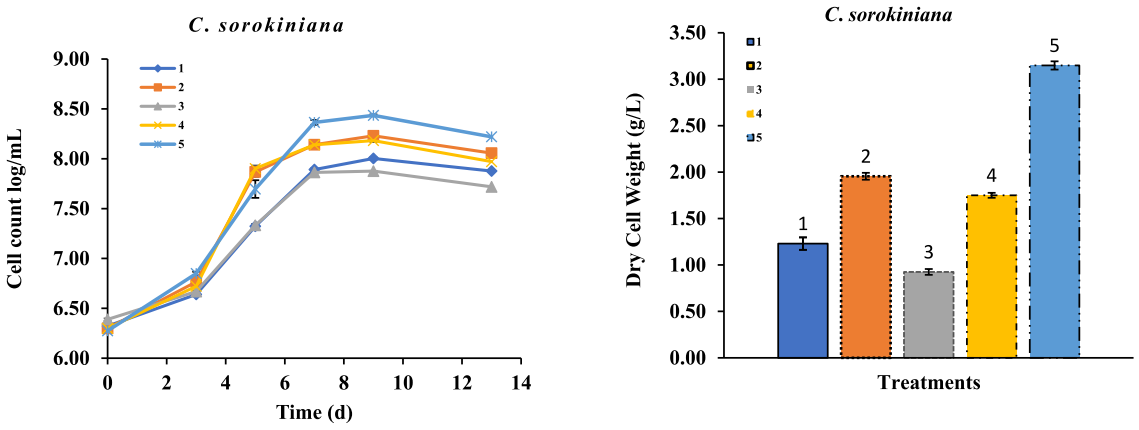

Fig. 4. *Chlorella sorokiniana* growth in enzymatically degraded glucosamine using Amylglucosidase, α-amylase, and dry cell weight recovery. The values reported are means of three replicates with error bars representing one standard deviation. Legend: hydrolysate (1), hydrolysate + 2.5 glucose g/L (2), hydrolysate + 2.5 sodium nitrate g/L (3), hydrolysate + 2.5 glucose and2.5 sodium nitrate g/L (4), BG11 (positive control, 5).

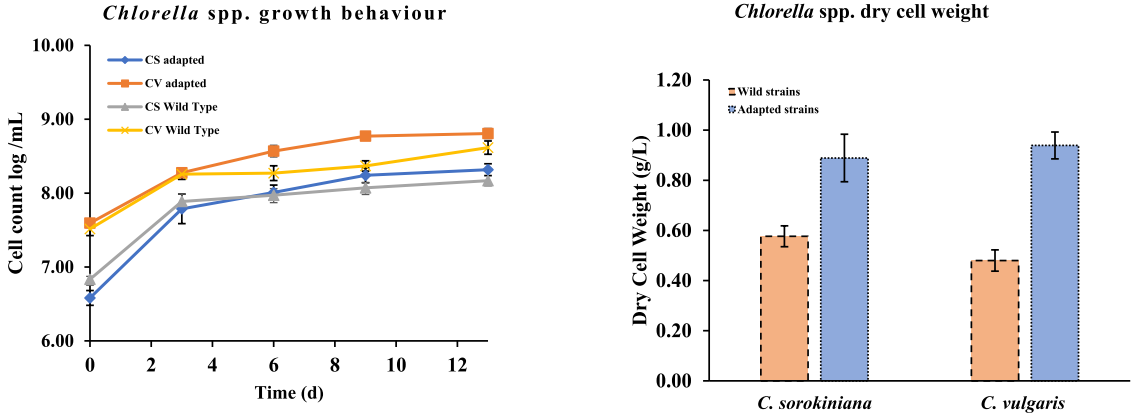

Fig. 5. Growth behaviour changes between wild and adapted *Chlorella sorokiniana* (CS) and *C. vulgaris* (CV) and their dry cell weight recovery (DCW) in glucosamine medium containing glucosamine at a concentration of 7.5 g/L and glucose 2.5 g/L. The values reported are means of three replicates with error bars representing one standard deviation.

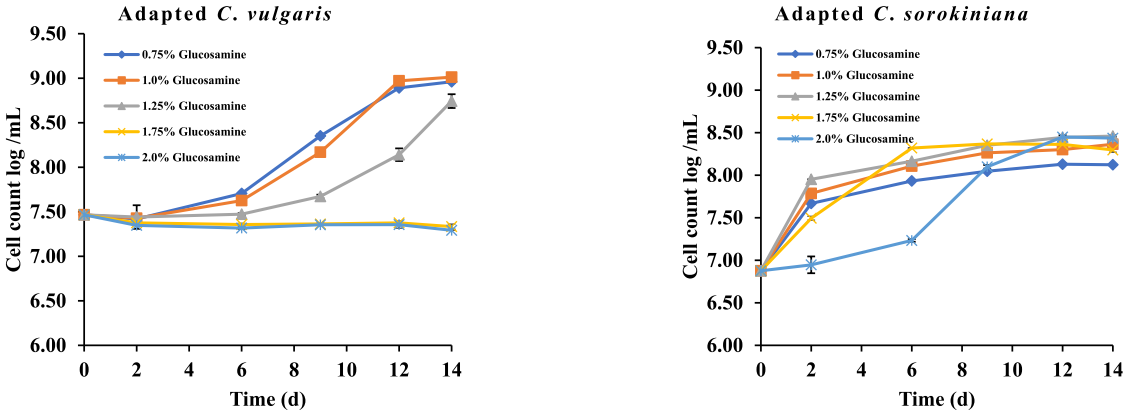

Fig. 6 (a). Growth behaviour changes of adapted *Chlorella vulgaris* and *C. sorokiniana* at different glucosamine concentrations.

6-phosphatase enzyme or enter the glycolysis pathway, where it is ultimately converted to pyruvate.[49] The enzymes involved in this process are tightly regulated, ensuring that the conversion of glucosamine to glucose occurs only when necessary. Thus, we started our search by identifying a glucosamine deaminase enzyme that mediates glucosamine to glucose conversion.

We identified the glucosamine-6-phosphate deaminase (G6PD) enzyme as the potential enzyme to be used for the conversion.[48] The

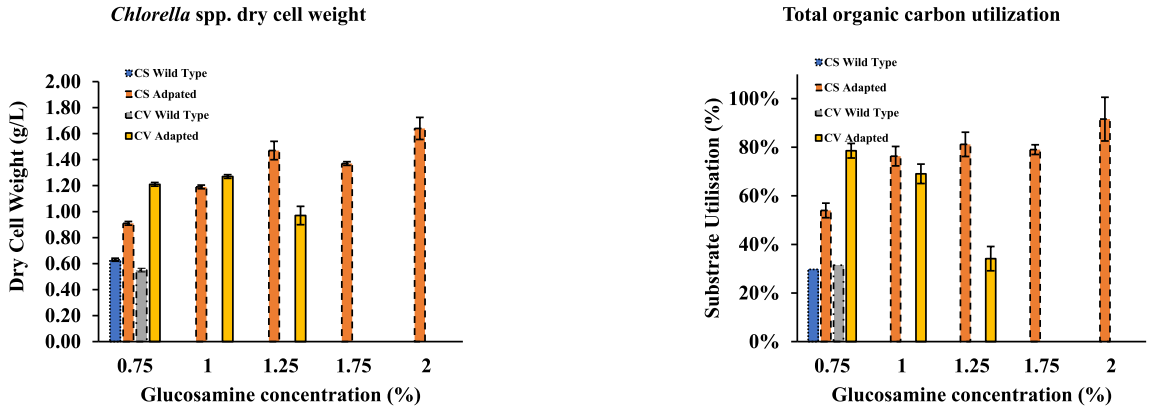

Fig. 6 (b). The correlated dry cell weight recovery and total organic carbon consumption at different glucosamine concentrations of wild and adapted *Chlorella sorokiniana* (CS) and *C. vulgaris* (CV) (—◆— 0.75 %, —■— 0.10 %, —▲— 1.25 %, —✕— 1.75 %, —✳— 2.0 %). The values reported are means of three replicates with error bars representing one standard deviation.

identification of the glucosamine-6-phosphate deaminase enzyme and its existence in some algae strains was made through an extensive literature review.[47] Further investigation of enzymes in KEGG databases shows the role that the G6PD enzyme plays in the amino acid and nucleotide sugar metabolism pathways.[49] Then we found that the enzyme exists in a wide range of organisms but with significant differences in its length and structure. According to the above review, feeding a species that produces the G6PD enzyme in its cells with glucosamine as a primary feed will result in the production of excessive amounts of ammonia that might impact the growth of the microalgae by slowing it down or even killing the cells. Therefore, we investigated the ammonia metabolism capacity of the 18 organisms in the final list (see results below). According to the KEGG database, ammonia metabolism is involved in 17 different metabolic processes, including some of the most major processes such as nitrogen metabolism and carbon metabolism.[49] We further investigated these two metabolic pathways to understand ammonia metabolism and use them to further enhance our species/-strain selection or ranking. Our investigations of the ammonia metabolism pathways show that the ammonia is converted to carbamoyl phosphate in the presence of carbon dioxide and the carbamoyl phosphate synthetase enzyme. Thus, we investigated our 19 strains to select those that could produce the carbamoyl phosphate synthetase enzyme. This analysis resulted in the selection of seven species.

The bioinformatics study confirmed the presence of the hydrolytic enzymes with 19 microalgae strains that might degrade glucosamine; however, when using glucosamine as a sole carbon source, even with the presence of a nitrogen source, they were not able to grow. This might be due to the enzyme levels being secreted in small amounts and insufficient to degrade the substrates. The enzyme activities were reported to be affected by several factors, including the concentration and types of carbon, nitrogen, and inducer agents.[50,51] It might also be due to the inhibitory effect of glucosamine. Glucosamine was reported to have strong antimicrobial activities at a concentration of 1 g/L against a wide range of bacteria, such as *Bacillus subtilis*, *Staphylococcus saprophyticus*, *Micrococcus luteus,* and *Pseudomonas fluorescens*[52], but no negative impact on yeasts at a concentration of 1 %, including *Candida albicans, C. krusei and C. glabrata*.[53] To the best of the authors' knowledge, no data was reported to explain the inhibitory degree of glucosamine in microalgae.

Carbon sources (glucose and acetic acid) showed to play essential roles in glucosamine expression at a particular concentration (2.5 g/L). Different carbon sources were essential to activate other hydrolytic enzymes. For example, glucose was vital to induce laccase enzyme activities in *Trametes pubescens* to degrade lignocellulosic substates,[54] cellobiose and glucose were found to increase the enzyme activity in a different study in the same isolate,[55] lactose and glycerol were vital for the enzyme activities in *Pseudotrametes gibbose*, *C. unicolor* and *F. fomentarius*.[55] The present study can be closely related to these later cases, which indicate the essential role of glucose as an inducer to improve the enzyme activities of glucosamine hydrolysis.

The microalgae growth rate and biomass productivity were monitored at different glucosamine concentrations, which showed low productivity at a high concentration, above 0.75 %. Bhattacharya et al. (2019) observed the attachment of N-acetyl-D-glucosamine on the surface of *Chlorella pyrenoidosa* using scanning electron microscopy (SEM) at about 2.2 % concentration of GlcNAc-induced surface changes that negatively affected its viability.

Acids, including HCl, phosphoric acid, and H_2SO_4, have been commonly employed to degrade chitin-based wastes and produce glucosamine.[56–58] These led us to examine the impacts of the different glucosamine forms on the microalgae growth performance. Glucosamine HCl and glucosamine phosphate were associated with better growth rates with *C. vulgaris* and *C. sorokiniana,* respectively, compared to glucosamine sulphate. We assume such differences might be related to the impacts of the different acids on the microalgae cell membrane permeability. The presence of acids, such as phosphoric, sulfuric, and hydrochloric acids, might impact the microalgae membrane permeability and is associated with potential implications for the uptake and regulation of some compounds. For example, related studies explained the various impact of dissolved organic acids, such as malic, humic, fulvic, and citric acids, present in the medium on the microalgae membrane permeability and the correlated uptake and regulation of essential compounds in the surrounding environment.[59,60] These findings may be useful when optimizing the upstream process of extracting glucosamine and producing the right chemical form that would be more suitable for selected microorganisms.

Amyloglucosidase, mixed with α-amylase, produces various metabolites, including sucrose, glucose, fructose, glycerol, and acetic acid. *C. sorokiniana* successfully showed uptake of these degraded compounds during growth, but not with *C. vulgaris*. *C. sorokiniana* is known as robust industrial microalgae that can use several carbon sources, including CO_2 and organic carbon such as glucose, sucrose, industrial glycerol, and acetic acid-rich wine waste.[61,62] Amyloglucosidase was reported to hydrolyze the external glycosidic bonds of the polysaccharides, while α-Amylase broke the internal glycosidic bonds resulting in high levels of reducing sugars in the resulting hydrolysates.[63,64] The capability of degrading glucosamine into smaller metabolites including organic acids, sugars, and glycerol using non-specific enzymes such as amyloglucosidase, α-amylase, and pectinases may light another pathway for future screening to other microalgae species or microbial groups that may

hydrolyze glucosamine by these non-specific enzymes, besides our former hypothesis of the specific enzyme, glucosamine-6-phosphate deaminase.

Evolved strains showed a shorter lag phase, reaching the same maximum growth rate as the wild strains in less time. Additionally, they proved to tolerate the high concentration of glucosamine, mainly for *C. sorokiniana*, which tolerated it about 3 times higher than the wild strain. The gradual adaptation process was successfully performed with *Desmodesmus* spp. that was slowly adapted from 10 % to 100 % unfiltered coal-fired containing 11 % CO_2, which typically causes acidification of the growth medium and initially could not be tolerated by the microalgae.[65] This approach is considered a powerful tool to improve the biological properties of microalgae with random genomic mutations, enjoys the advantage of low requirements, and is not considered genetically modified microorganisms that might raise public concerns.[66–68]

5. Conclusion and future perspective

In this work, we showed the capability of combining literature reviews and bioinformatic tools to identify potential candidate organisms for metabolic engineering for alternative protein applications. Our analysis recommended 19 microalgae species/strains that can potentially use glucosamine as a carbon and nitrogen source. In microalgae, the presence of glucose at a minimum concentration is essential to induce the uptake of glucosamine. We showed that different forms of glucosamine are associated with different growth behavior that may be relevant to the optimization of the glucosamine extraction process from chitin-based wastes. The slow growth of *C. sorokiniana* was further improved via the treatment of glucosamine by hydrolytic enzymes. Similarly, significant improvement was achieved when both strains were subjected to a laboratory evolution process that produced strains that could grow at high growth rates and tolerated higher glucosamine concentrations. The proof of concept has been achieved in the current study, however, further optimization of culture, enzyme, adaptation, chemical, and molecular analysis, as well as scaling up, will be required for future investigations and industrialization processes. Additionally, literature reviews and bioinformatic analysis showed that metabolic degrading enzymes might be present in other microalgae species. Further studies are required to search for possible ways to improve their growth performance.

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Author contribution

YC, YL, and KS conceived and supervised the research. HE, MH, SH, and SL conducted experiments and analyzed data. HE and MH wrote the manuscript. All authors read and approved the manuscript.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biotno.2024.01.003.

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