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Optimization of lipid production from seaweed mannitol by engineered *Yarrowia lipolytica* strains

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ABSTRACT

Brown algae represent an abundant and renewable marine biomass rich in carbohydrates, including mannitol, which can be a by-product of the industrial alginate extraction. This study explores the potential of valorizing seaweed-derived mannitol for lipid production using genetically engineered *Yarrowia lipolytica* strain. The modified strain AJD ΔEYD1 DGA1, lacking erythritol dehydrogenase (EYD1) and overexpressing diacylglycerol acyltransferase (DGA1), efficiently converted algal mannitol into single-cell lipids. Various pretreatment methods mimicking industrial alginate extraction were tested to assess their impact on mannitol recovery and yeast performance. Among tested conditions, soaking algae in 1% citric acid yielded the highest mannitol concentration (14.76 g/L) and supported efficient growth and lipid accumulation. The process was successfully scaled up to 1 L bioreactors. Fatty acid analysis revealed oleic acid (C18:1) as the predominant component (constituting on average 48% of the overall produced lipids), with monounsaturated fatty acids constituting over 60% of total lipids. The results confirm that engineered *Y. lipolytica* strain can efficiently utilise algal mannitol and convert it into lipids in a higher laboratory scale, offering a sustainable route for integrating alginate production with microbial oil bioprocesses. Further optimization of carbon-to-nitrogen ratios and continuous feeding strategies could enhance lipid yield and process scalability.

1. Introduction

Brown algae are a diverse group of marine organisms, with the estimated number of species reaching around 2 thousand. They are rarely found in freshwater systems and usually occur in colder, continental coast waters (Groisillier et al., 2014; Wehr, 2015). The production of seaweeds increases significantly, with Asia being the biggest exporter of algal biomass, accounting for more than 97% of the world production, which nearly all of it being produced artificially. Brown algae *Laminaria* and *Saccharina* alone account for more than 30% of the overall production for human use, but they are also utilized as fish fodder (Zhang et al., 2022). They are a source of many industrially important compounds, such as alginate, laminarin or fucoidan (Remya et al., 2022). The consumption of brown algae is the highest among other algae groups, consisting of 66.5% of the overall consumption by humans worldwide. That is mostly due to their complex composition, which may have therapeutic effects, including anti-inflammatory and antioxidant activities. Their abundance, ability to capture high quantities of CO_2, quick growth rate, number of species, low cost of cultivation and the fact that they do not compete for the agricultural land makes them not only an interesting source of food alternatives but also a promising research candidate (Kawai and Murata, 2016; Wei et al., 2013). Brown algae are considered a very important biomass, due to their high carbohydrate content, which can range from 4% to 70% (Zhang et al., 2022). Alongside carbohydrates, brown algae are known to produce lipids and their lack of lignin is important in the processing of the biomass for obtaining the compound of interest (Kawai and Murata, 2016).

The compound, which brown algae species can be utilized for on an industrial scale is alginate (Jayasinghe et al., 2022). Its multiple applications, from food additive to novel bandages, due to its non-toxicity and biocompability (Aderibigbe and Buyana, 2018) results in the constant increase of the demand. The extraction process for alginate is complicated and requires a number of pretreatment steps, which in turn

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result in different yields or qualities (Saji et al., 2022). The process is also heavily dependent on vast amounts of water. The differences between chemical composition of the species of brown algae used for this process results in a numerous procedures applied on an industrial scale, however, the process usually begins in soaking of the dry biomass of the seaweeds (Fig. 1). This step is conducted in various conditions, including low pH, addition of salts or thermal treatment (Bojorges et al., 2023). During this process, mannitol and other compounds are released into the rinse water, while alginate becomes insoluble alginic acid. Addition of sodium hydroxide or sodium carbonate leads to conversion to insoluble sodium alginate. The concentration of mannitol in the discarded liquid extracts depends on the species of brown macroalgae used and the conditions of the process itself. The water rich in mannitol, and other compounds, is not utilized, despite having a potential to sustain the growth of microorganisms able to produce value-added products. This is in part caused by the contamination of the rinse with salts, overall low pH due to the presence of non-organic acids and the fact that mannitol is not a preferred carbon source for growth by many yeast and bacteria species (Chujo et al., 2015).

Mannitol is a six carbon polyol, which is commonly used as an additive to chewing gums, an alternative for sugar (Zhang et al., 2022) or in medicine to lower the pressure build up in the skull (Kim et al., 2023). This polyol is a storage material for brown algae, and its concentration in the cells varies significantly based on the algae species, their cultivation area or the time of the harvest (Klindukh et al., 2011). The percentage of mannitol in dry biomass was reported as high as 30% for *Laminaria japonica* (Masura et al., 1993). The ease in which this compound can be extracted from brown algae was established. The potential of brown algae mannitol extracts and hydrolysates to sustain growth of microorganisms was also analyzed (Chades et al., 2018; Dobrowolski et al., 2022). Most of the focus on utilizing this carbon source was put on production of ethanol, but the recent study (Szczepańczyk et al., 2025) also suggested the possibility to use it as a base for production of single cell oils. The capabilities to utilize algal mannitol and production of fatty acids was confirmed on multiple laboratory scales, but the impact of additives used to increase the yield of alginate during the pretreatment steps has never been tested.

The process of alginate extraction from brown algae often requires the addition of strong acids or bases, while the rinse can also contain high concentration of salts. Low pH and high salinity are not a preferable for many microorganisms, but they do not impair the growth of *Yarrowia lipolytica* (Mirończuk et al., 2017; Rzechonek et al., 2017). This yeast species can withstand those harsh conditions and initiate production of value added products, such as erythritol (Mirończuk et al., 2016). Therefore, the pretreatment methods affecting the composition and parameters of the rinse containing mannitol may not be limiting to the growth and production of single cell oils.

In this study, the process of biomass and fatty acids production by *Y. lipolytica* on macroalgal mannitol extracts was scaled-up to analyze the possibility to implement this process on a larger scale. The potential pretreatment methods of the dry algae biomass for alginate extraction were also analyzed in terms of their implication for lipid synthesis and biomass production by *Y. lipolytica*. Additionally, the media obtained by rinsing the algae were supplemented to analyze the impact of second carbon source or impact of alternate conditions on the culture.

2. Materials and methods

2.1. Media used in this study and preinoculum preparation

Y. lipolytica strain AJD ΔEYD DGA1 (Szczepańczyk et al., 2025) was grown in YPD medium, containing 20 g/L of glucose, 20 g/L of peptone and 10 g/L of yeast extract (A&A Biotechnology) at 28 °C. The inoculum for lipid synthesis experiments were grown in YNB medium, supplemented with 20 g/L of glucose for 48 h with constant agitation of 200 rpm. Lipid synthesis was carried out in modified algal lipid synthesis medium, which was supplemented with ammonium sulfate to obtain the C/N ratio of 60, depending on the analyzed mannitol content, YNB without amino acids and ammonium sulfate, and 0.5% Yeast extract.

Additional changes to the lipid synthesis medium with algal mannitol were performed, such as supplementation of 20 g/L of glycerol or glucose - the C/N ratio was adjusted to value 60, taking into account the second carbon source in the medium. Similarly, the concentration of citric acid was also taken into consideration while optimizing the medium to limited nitrogen conditions. The medium was also supplemented with NaCl (3%). The addition of salt to the medium did not require adjustment of the amount of ammonium sulfate in the medium.

2.2. Mannitol extraction

The extraction of mannitol was performed in 2-L beakers, to which 100 g of dried *Fucus vesiculosus* (Dary Podlasia, Poland) was placed. Distilled water was used to soak the algae as described before (Szczepańczyk et al., 2025). After two hours of soaking under constant agitation *via* mechanic stirrer CAT R50 (Germany), the sample for HPLC analysis of mannitol content was collected. The remaining extract, without pieces of algae, was transferred to 50 mL tubes and centrifuged for 15 min at 2348 rcf at room temperature, to remove the small particles of the algae. Centrifuged extract was transferred to glass bottles and sterilized (121°C, 1.1 bar, 15 min). Additional changes to the extraction process included using HCl (0.1 N and 0.3 N) and citric acid

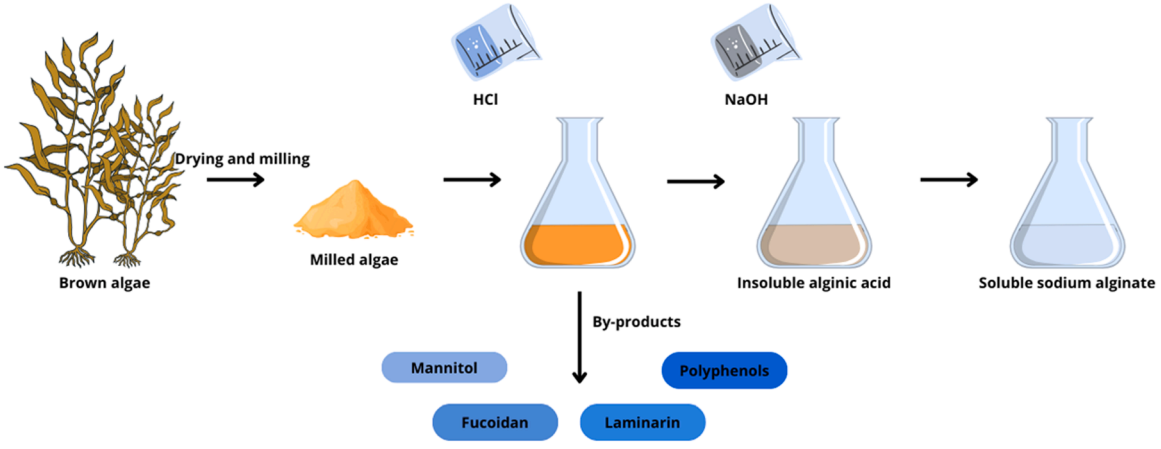

Fig. 1. Simplified scheme of alginate extraction process from brown algae (with the use of hydrochloric acid (HCl) and sodium hydroxide (NaOH) to soluble sodium alginate, with mannitol as one of the by-products of this process. The figure was created with Biorender.

1% (Bojorges et al., 2023) and 3% (Fawzy and Gomaa, 2021) solution for the soaking process were used. Artificial sea water was also used instead of distilled water to analyze the impact of high salinity on the process. The steps following the soaking of the dry algae biomass were the same regardless of the method of extraction.

2.3. HPLC analysis

The samples obtained from the bioreactor experiments were centrifuged in 15 mL tubes for 15 min at 21130 rcf. Supernatants were carefully transferred to new tubes, not to disturb the pellet. Supernatant post centrifugation was additionally centrifuged for 10 min at 21130 rcf. Samples from shake flask experiments were collected to 1.5 mL microcentrifuge tubes and centrifuged twice, as described above. Both types of the samples were then filtered using 0.22 μm syringe filters to the glass vials suitable for HPLC analysis. To characterize the concentration of mannitol and additional carbon sources, as well as citric acid in the medium, the samples were analysed by HPLC analysis using a HyperRez Carbohydrate H + column (Thermo Scientific), coupled to a UV detector (λ = 210 nm) (Dionex, USA) and a refractive index detector (Shodex, Japan), with the run time of 20 min. For the elution 25 mM trifluoroacetic acid with a flow rate of 600 μL/min was used, the column temperature was set at 65 °C.

2.4. Biomass analysis

10 mL of the media post-cultivation (bioreactor and shake flask studies) was collected to 15 mL tubes and centrifuged for 10 min (2348 rcf, room temperature) and washed with distilled water after discarding of the supernatant. The pellet was resuspended in water and run through the filtration system. To filter the biomass, nitrocellulose membrane 0.22 μm GSWP was used. The biomass post-filtration was dried on the nitrocellulose disc used previously for 24 h and then weight in drying scale at 105°C in RADWAG model MA 50/1.R.WH (Poland) scale.

2.5. Bioreactor studies

To perform the bioreactor studies the AJD ΔEYD1 DGA1 strain was grown in YNB medium with 2% glucose for 48 h in 0.25 L Erlenmeyer flasks (0.025 L working volume) on a shaker at 28°C and 180 rpm. The cultures were centrifuged (5 min, 2348 rcf) and washed twice and resuspended in distilled water. The bioreactor study was performed in 1 L bioreactor (Satorius), with 300 mL of the working volume containing various algal mannitol extracts media (as described previously). The pH was not adjusted or regulated during the cultivation. The initial air flow was set at 500 ccm and the temperature set at 28°C. Stirring was set at 600 rpm through the entire study. The initial OD_{600} of the culture was set at 1. 10 mL of samples for HPLC analysis were collected every 24 h. 200 μL of antifoam 204 A6426 (Sigma Aldrich) was added at the beginning of the experiment to avoid formation of the foam.

2.6. Lipid content analysis

Lipid synthesis was conducted in lipid synthesis medium containing: Yeast Nitrogen Base (1.7 g/L), Yeast Extract (5 g/L), mannitol and ammonium sulfate in C/N ratio of 60. The media were prepared with algal mannitol extracts, therefore the amounts of both mannitol and ammonium sulfate were adjusted to the values of mannitol obtained in the mannitol extracts from various of algal species shortly before sterilization. The lipid production analysis was conducted in lipid synthesis medium in 300 mL Erlenmeyer flasks with 30 mL of the working volume or 1 L bioreactors, with 300 mL of the working volume. The OD_{600} of the culture was set at 1.0. Analysis of the lipid content in medium with algal mannitol was carried out in 48-well plates in 3.5 mL of the working volume.

The biomass from the fermentation study was lyophilized for fatty acids production analysis. Approximately 10–20 mg of lyophilized biomass was mixed with 2 mL of 2.5% sulfuric acid in methanol, with internal standard of C17:0 (50 mg/mL). The extraction was carried out in glass tubes with Teflon caps. After 2 min of mixing, the samples were incubated for 90 min in 80°C with shaking. Extraction of fatty acids methyl esters was conducted after addition of 1 mL of hexane and then 0.5 mL of water. The organic phase with fatty acids appeared post mixing and centrifugation. This phase was then carefully transferred into glass vials for GC analysis on GC−2010 Plus apparatus) with a flame ionization detector (FID) and autoinjector (AOC−20i). The separation was performed on 70% cyanopropyl polysilphenylenesiloxane column.

3. Results

Genetic modification of yeast *Y. lipolytica*, resulting in deletion of erythritol dehydrogenase gene EYD1 *YALI0F01650g* significantly increases the capability of the strain to utilize mannitol and use it as a sole carbon source. Additionally, overexpression of gene *YALI0E32769g*, encoding diacylglycerol (DAG) acyltransferase allows for the conversion of mannitol into storage materials in form of single cell lipids (Szczepańczyk et al., 2025). In this study, the modified strain was grown on medium containing mannitol extracted from brown algae, which undergo multiple pretreatment steps (Bojorges et al., 2023). The multiplicity of possible protocols of alginate extraction process, alongside the need to further optimize the parameters of the culture conditions resulted in tests of the optimal conditions for biomass production and the single cell lipids synthesis by *Y. lipolytica.* Yeast strain *Y. lipolytica* AJD ΔEYD1 DGA1 was therefore tested on media obtained by mannitol extraction from algae with supplementation of other carbon source. Moreover, the impact of different possible pretreatment methods, as used in the alginate extraction protocols on an industrial scale, on mannitol concentration and subsequently yeast growth was analyzed (Table 1).

Firstly, to determine the influence of the aeration, the medium obtained by soaking brown algae *Fucus vesiculosus* in distilled water, as previously described (Szczepańczyk et al., 2025), was placed in shake flasks and baffled shake flasks. This experiment was carried out due to the observations by Mirończuk et al., (2017), in which the erythritol production and glycerol utilization was correlated with the aeration. Since the metabolic pathways of erythritol and mannitol are intertwined, this analysis was conducted as a prelude to the higher scale fermentations. Samples in this experiment were collected every 24 h, to analyze the mannitol utilization ratio based on the conditions. *Y. lipolytica* strain AJD ΔEYD1 DGA1 was able to utilize mannitol efficiently in both analyzed conditions. The mannitol was nearly depleted after 48 h, reaching 0.612 g/L for shake flasks and 0.618 g/L for baffled flasks (data not shown). The increased aeration can therefore have a very limited impact on a small laboratory scale. Due to the marginal difference the following experiments were carried out in shake flasks.

3.1. Scaling-up of the process

The effective production of lipids from algal mannitol after genetic engineering of *Y. lipolytica* strain AJD ΔEYD1 DGA1 lead to the most positive results in small laboratory scale (Szczepańczyk et al., 2025).

Table 1
Mannitol concentration and pH of the algal extracts depending on pretreatment soaking method of brown algae *F. vesiculosus*.

Pretreatment soaking method	Mannitol concentration [g/L]	pH
Distilled water	12.94	5.72
Artificial sea water	14.62	5.29
0.1 N HCl	13.79	3.16
0.3 N HCl	13.47	1.47
1% citric acid	14.76	3.79
3% citric acid	13.81	3.29

Thus here, in this study the process was up-scaled to 1 L bioreactors, with 300 mL of working volume. The utilization of mannitol in the bioreactor proved to be more effective, with almost the entirety (11.05 g/L) of the mannitol from the medium being utilized within 48 h as seen in Fig. 2. The experiment was stopped at this mark, with 1.85 g/L of residual mannitol left in the medium, to avoid degradation of storage lipids.

The biomass was analyzed at the end of the experiment and it reached on average 9.16 g/L. The lipid production and profile of fatty acids was established. Lipid production reached 9.96% of the total dry biomass which is significant, considering the overall low carbon content of the medium (Table 2). The fact that mannitol is not a preferable carbon source for *Y. lipolytica* additionally increases the importance of the findings.

The majority of the overall production was the oleic acid (C18:1), constituting 47.68%. Linoleic acid (C18:2) and palmitic acid (C16:0) and acid were also produced on the elevated rate, reaching 18.71% and 13.61% respectively. The predominant fraction of the fatty acids was mono unsaturated fatty acids (MUFA), reaching 59.87%. Saturated fatty acids (SFA) constituted 21.41% of the overall production, while poly-unsaturated fatty acids (PUFA) were produced mainly in a form of linoleic acid (Table 3). The lipid synthesis was on a similar level to the shake flask experiment with 30 mL of the working volume, where the lipid content reached 9.47%.

The ability to utilize algal mannitol, produce biomass and the overall lipid content in dry biomass was further tested in changed conditions, to optimize the process and characterize the impact of the changes on fatty acids production and their profile. The changes of the medium followed the possible pretreatment methods used in alginate extraction process.

3.2. Impact of pretreatment methods on mannitol concentration

Standard procedure for alginate extraction from brown algae requires soaking them in distilled water or weak acid solution. As the entire process is heavily dependent on water, the possibility of utilizing marine water instead of fresh water was tested. This would significantly reduce the negative impact on already depleting fresh water reserves. To obtain the algal mannitol medium, artificial sea water (ASW) was produced, by following the procedure by Nguyen (2018). Additionally, hydrochloric acid (HCl) solutions commonly used at industrial scale were tested. Due to the negative environmental impact of inorganic acids, the process of alginate extraction is also carried out with citric acid (Bojorges et al., 2023; Fawzy and Gomaa, 2021), therefore this pretreatment step was also taken into consideration in obtaining the mannitol from *F. vesiculosus*. The results of the mannitol concentration analysis and the pH of the extract are depicted in Table 1.

All of the tested pretreatment methods had a positive impact on mannitol concentration in the rinse, with the biggest difference observed for 1% citric acid, where the obtained mannitol increased by 14% as compared to distilled water. Additionally, the pH of all of the extracts, except for 0.3 N HCl, were suitable to begin the cultivation without the need to adjust the pH, as *Y. lipolytica* is able to withstand low pH around 3.0.

3.3. Lipid production on medium with high salt concentration

During the mannitol extraction process, in which artificial seawater was used, the final level of mannitol in the medium reached 14.62 g/L, which is a value 13% higher than that obtained in distilled water. It shows that the artificial sea water could be used in this process, but it could have limiting effect on further steps of the alginate extraction or its properties, which were not tested in this study. Moreover, the high concentration of salts could negatively impact the growth and lipid synthesis by *Y. lipolytica,* despite yielding slightly higher mannitol concentration in the final medium. This organism possesses the ability to withstand harsh environmental conditions, including high salinity, but the impact of those conditions on utilizing mannitol and production of lipids have not been analyzed yet. Moreover, the final concentration of salt in the medium could vary depending on the procedure.

The mannitol utilization process was therefore analyzed on algal mannitol obtained with ASW and addition of salt post extraction, to better control the final concentration of salts in the medium. The culture with mannitol extracted with addition of ASW was able to utilize the carbon source nearly completely within 72 h, with the initial OD of the culture set at 0.5. An average of 0.58 g/L of mannitol was still left in the medium at this time point, as seen in Fig. 3. The experiment was therefore stopped, as the residual mannitol protected the storage lipids from being degraded in carbon depleted medium. The analyzed biomass

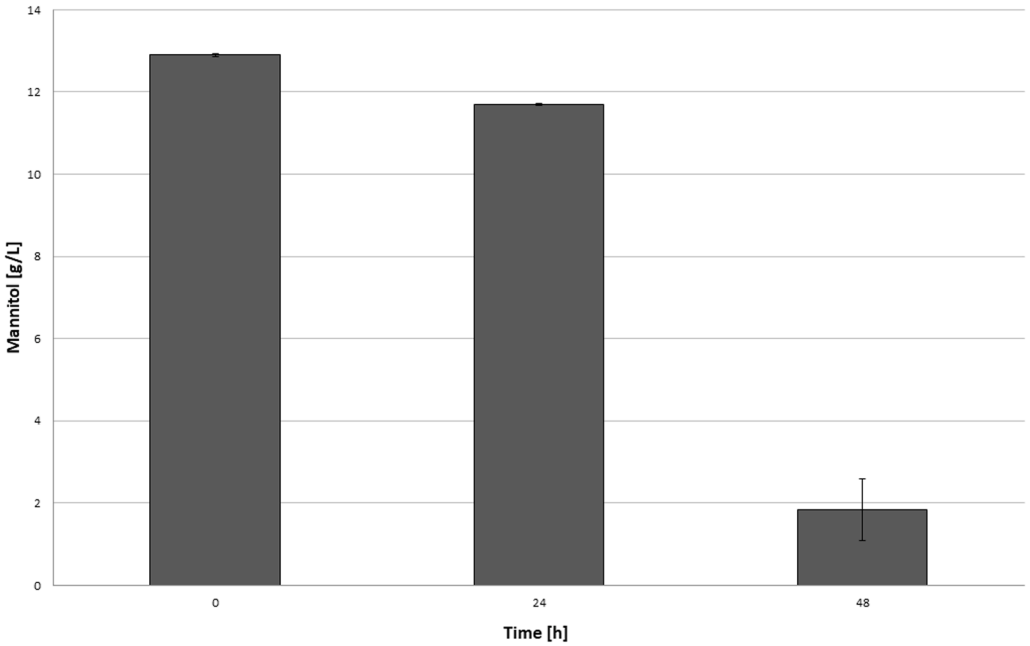

Fig. 2. Mannitol utilization by modified *Y. lipolytica* AJD ΔEYD DGA1 strain in 1 L bioreactors on algal mannitol medium without supplementation, obtained by soaking the algae in distilled water.

Table 2
Summary of the obtained results for biomass and lipid synthesis for the AJD ΔEYD1 DGA1 strain in tested conditions, scales and time of the culture.

Medium	Scale	Biomass [g/L]	Y of Biomass [g/g]	Q of Biomass [g/L/h]	Lipids [%]	Y of Lipids [g/g]	Q of Lipids [g/L/h]	Utilized mannitol [g/L]	Time of culture [h]
Algal rinse	Shake-flask	13.08 ± 0.18	0.93	0.27	9.47 ± 0.88	0.088	0.026	14.05 ± 0.38	48
Algal rinse	Bioreactor	9.16 ± 0.26	0.83	0.19	9.92 ± 0.65	0.082	0.019	11.05 ± 0.41	48
Algal rinse in ASW	Shake flask	13.25 ± 0.58	0.96	0.18	8.76 ± 0.92	0.084	0.016	13.79 ± 0.28	72
Algal rinse in 3% NaCl	Shake flask	11.92 ± 0.2	1.09	0.24	7.16 ± 0.31	0.078	0.018	10.97 ± 0.23	48
Algal rinse in 3% NaCl	Bioreactor	11.17 ± 0.62	0.92	0.23	8.68 ± 0.92	0.080	0.020	12.12 ± 0.8	48
Algal rinse in 1% Citric Acid	Shake flask	12.45	0.88	0.13	11.35 ± 0.32	0.100	0.015	14.13 ± 0.74	96
Algal rinse in 1% Citric Acid	Bioreactor	14.77 ± 0.79	1.21	0.20	12.07 ± 0.27	0.147	0.025	12.12 ± 0.43	72
Algal rinse with 2% glucose	Shake flask	21.62 ± 2.65	0.62	0.45	8.38 ± 0.14	0.052	0.038	14.79 ± 0.18	48
Algal rinse with 2% glycerol	Shake flask	24.90 ± 0.78	0.75	0.52	8.89 ± 0.27	0.067	0.046	13.18 ± 0.71	48
Mannitol with 1% Citric Acid	Shake flask	8.93 ± 0.4	0.62	0.19	11.69 ± 1.40	0.072	0.022	14.45 ± 0.65	48
Mannitol with 3% NaCl	Shake flask	6.66 ± 0.22	0.58	0.14	7.87 ± 1.45	0.046	0.011	11.41 ± 0.88	48

Table 3
Fatty acids profile in the tested conditions. C16:0– palmitic acid, C16:1– palmitoleic acid, C18:0– stearic acid, C18:1– oleic acid, C18:2– linoleic acid, SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids.

Medium % of lipids	**Algal rinse**	**Algal rinse (Bioreactor)**	**Algal rinse in ASW**	**Algal rinse in 3% NaCl**	**Algal rinse in 3% NaCl (Bioreactor)**	**Algal rinse in 1% Citric Acid**	**Algal rinse in 1% Citric Acid (Bioreactor)**	**Algal rinse with 2% glucose**	**Algal rinse with 2% glycerol**	**Mannitol with 1% Citric Acid**	**Mannitol with 3% NaCl**
16:0	8.62	13.61	12.35	9.42	12.56	10.33	10.44	9.06	9.79	6.51	7.24
16:1	15.33	12.19	12.01	15.52	11.96	13.25	12.45	14.41	15.54	13.71	14.60
18:0	5.35	7.80	7.70	7.28	7.74	6.11	6.56	6.37	7.60	4.01	4.56
18:1	55.50	47.68	45.68	55.31	45.57	48.14	48.25	52.99	52.79	61.31	55.49
18:2	15.20	18.71	22.26	12.46	22.17	23.08	22.30	17.17	14.27	14.45	18.10
SFA	13.97	21.41	20.05	16.70	20.30	16.44	17.00	15.43	17.39	10.52	11.80
MUFA	70.83	59.87	57.69	70.83	57.53	61.39	60.70	67.40	68.33	75.02	70.09
PUFA	15.20	18.71	22.26	12.46	22.17	23.08	22.30	17.17	14.27	14.45	18.10

reached 13.25 g/L.

Further analysis shifted towards the medium obtained with rinsing of algae in distilled water and adding the salt afterwards, to control its concentration in the medium. 3% of NaCl was selected as the condition of cultivation due to its proximity to the concentration of NaCl in ASW and the ability of *Y. lipolytica* to grow in this condition. The cultivation of the modified strain AJD ΔEYD1 DGA1 in the salt supplemented medium ended within 48 h, at which point no mannitol was detected in the medium. However, the initial concentration of mannitol was also lower, 10.97 g/L, as compared to 14.62 g/L for the medium obtained with ASW. The biomass was also lower, reaching on average 11.92 g/L.

Since the small-scale experiment using ASW and supplementing the medium with 3% (w/v) salt showed no significant impairment of mannitol utilization, the experiment was subsequently carried out in a bioreactor.

The cultivation was stopped after 48 h, as the concentration of mannitol dropped below 1 g/L, reaching on average 0.95 g/L. The addition of salt to the medium did not impair utilisation of mannitol significantly, moreover, the initial adjustment phase, in which this carbon source was not uptaken, was not observed. The AJD ΔEYD DGA1 strain was able to utilize on average 2.87 g/L of mannitol in NaCl supplemented medium within the first day of the experiment, as compared to 1.19 g/L for the medium with algal rinse obtained with distilled water. Moreover, the concentration of residual mannitol in the unsupplemented medium was also 0.9 g/L higher at the end of the experiment. The biomass analysis showed the rise of the overall dry biomass post-cultivation to 11.17 g/L, a 2 g/L increase from the bioreactor study with the control algal rinse medium. Lipid content, however, decreased to 8.68%, while the profile of the produced fatty acids was not altered significantly from the control conditions.

3.4. Lipid production on medium obtained with hydrochloric acid

Hydrochloric acid solution is one of the most commonly used pretreatment steps to extract mannitol, fucoidan, laminarin and polyphenols from the algal biomass in preparation for alginate extraction (Rustici et al., 2024; Savić Gajić et al. 2024). The addition of acid significantly decreases the pH of the rinse including mannitol, therefore the impact of this modification on *Y. lipolytica* AJD ΔEYD1 DGA1 strain growth was analyzed. The strain growth was totally restricted in the presence of hydrochloric acid.

To determine whether this effect was caused by soaking-derived by-products (which are not present in the distilled-water rinse) or by the acid itself, the experiment was repeated using a medium containing pure mannitol instead of the algal rinse. The analysis was performed again in 0.05 M HCl and YNB medium with 10% glycerol, but similarly to the lower concentration of the inorganic acid, the yeast strain did not grow as observed by the lack of both mannitol utilization and increase of optical density. This proves that the algal rinse obtained with hydrochloric acid is not optimal to support the growth and lipid synthesis of the modified *Y. lipolytica* strain. Further optimization of the medium would have to be required to use it as a basis for the lipid production, or

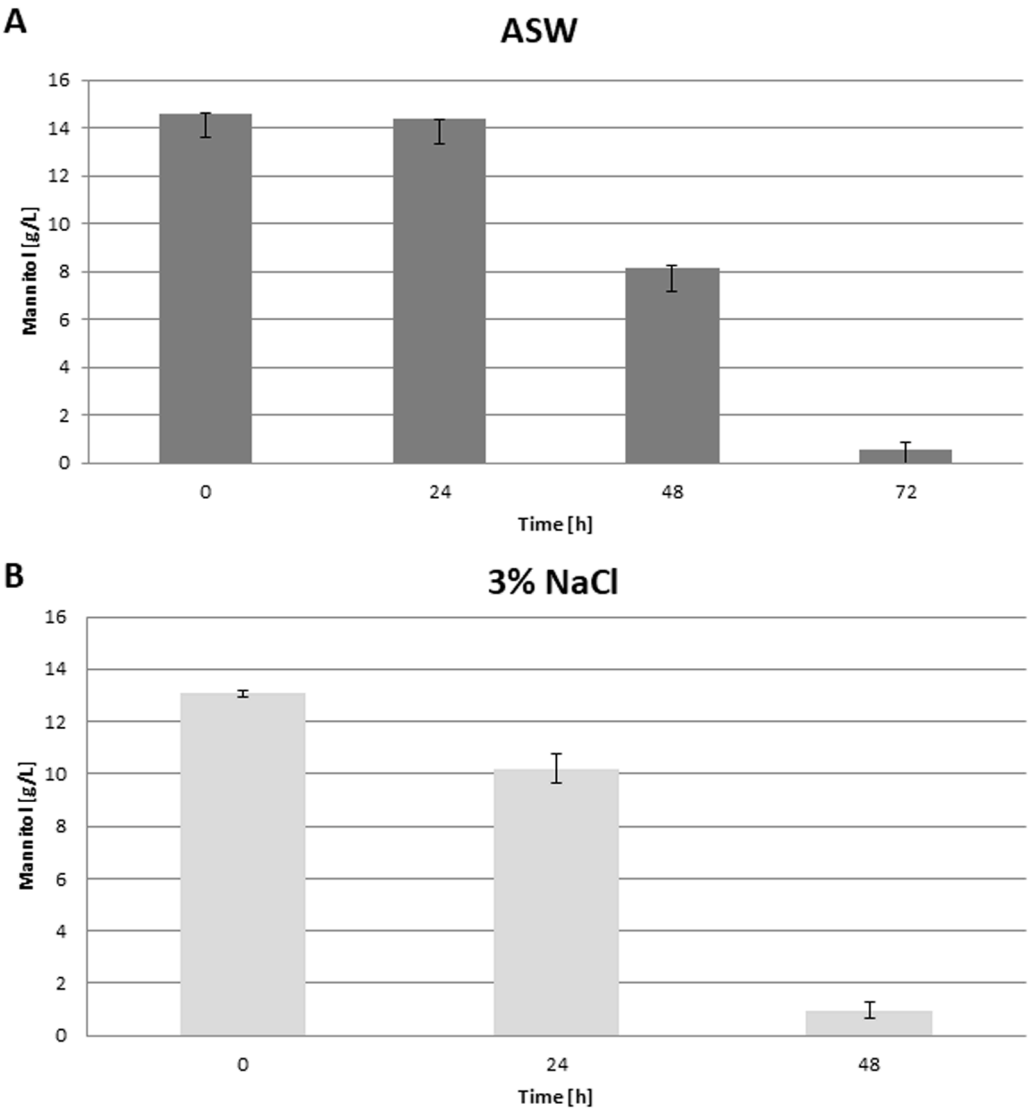

Fig. 3. **A -** Mannitol utilization by *Y. lipolytica* AJD ΔEYD1 DGA1 in algal rinse obtained with ASW, carried out in shake-flasks (dark grey). **B** - Utilization of mannitol by *Y. lipolytica* AJD ΔEYD1 DGA1 in bioreactor study with algal rinse medium supplemented with 3% NaCl during the extraction process (gray).

alternatively, the strain would have to be adapted to those limitation conditions, via adaptative laboratory evolution.

3.5. Lipid production on medium with citric acid

Citric acid is a compound, which can be produced on an industrial scale by *Y. lipolytica* (Rzechonek et al., 2019). This yeast not only possesses the ability to produce citric acid, but also utilize it as a carbon source. During processing of the brown algae with citric acid solution, 14.76 g/L of mannitol was obtained in lower concentration of the acid and 13.81 g/L for the higher concentration in the medium. The concentration of citric acid in the final medium was 14.64 g/L and 26.55 g/L for 1% and 3% solutions, respectively. The higher-than-initial citric acid concentration in the extract from 1% solution is likely due to the release of citric acid from brown algae itself, although this release appears to be very limited under most conditions. The addition of another carbon source in the medium had to be taken into consideration while calculating the amount of ammonium sulfate used to obtain the C/N ratio of 60. Additional modification of the soaking of algae did not result in a release of glucose, as its concentration in the medium did not exceed 0.01 g/L in the HPLC analysis. The modified AJD ΔEYD DGA1 strain was tested in the obtained media in shake flasks. As the *Y. lipolytica* possesses the ability to grow in low pH (Szczepańczyk et al., 2023), the medium was not initially adjusted to the previously checked pH conditions. The pH of the extracts was in both cases high enough to facilitate the proper growth of the yeast strain, as the citric acid is not a strong acid.

The total of 14.7 g/L of mannitol was nearly depleted within 96 h (Fig. 4), showing slowed consumption as opposed to the other tested media. Moreover, the citric acid was steadily consumed, reaching 2.18 g/L at the end of the experiment, with the highest dropped observed between 48 and 72 h. This also coincided with the drastic drop of mannitol in the medium. The biomass at the end of the experiment reached 12.45 g/L.

The experiment was also carried out in the bioreactor, in which the algal rinse with 12.66 g/L of mannitol and 14.61 g/L of citric acid was used. The process was concluded after 72 h due to the depletion of mannitol. Additionally, citric acid concentration dropped by more than 10 g/L, with residual citric acid left in the medium at the end of the experiment. The biomass analysis revealed increase in the overall dry biomass to 14.77 g/L, a 19% increase from the results obtained in shake flasks. The lipid profile analysis showed an increase in the overall percentage of lipid content in dry biomass to 12.07%, while the profile did not change significantly.

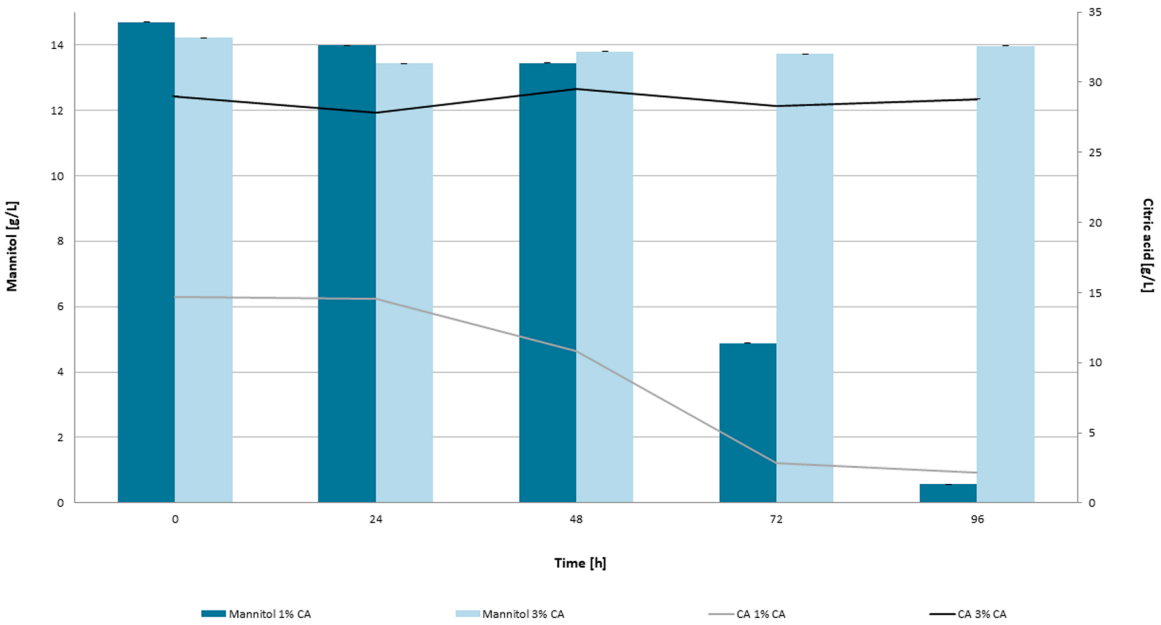

Fig. 4. Utilization of mannitol (bar chart) and citric acid (line chart) in shake flasks, with medium obtained by rinsing brown algae in 1% solution of citric acid (CA 1%) and 3% solution (CA 3%), carried out by the modified AJD ΔEYD1 DGA1 strain.

Additional analysis with the medium consisting of pure mannitol and citric acid was performed, to mimic the medium obtained from the algal rinse. This analysis was conducted to establish, whether there is a negative effect on the yeast growth caused by the rinsing of algae in the citric acid solution. In this case, citric acid was the carbon source consumed first, nearly depleted after 24 h of the experiment, while mannitol was consumed slower. Despite that, mannitol was not detected after 48 h of the experiment, showing the improvement over the algal rinse medium. The biomass in this case was lower, reaching 8.93 g/L, suggesting a shift towards metabolites, rather than the biomass. The lipid content was analyzed and showed no significant change as compared to the citric acid algal rinse, reaching 11.69%. Interestingly, this study yielded the highest overall production of MUFAs, reaching 75.02%.

The experiment was also carried out with higher concentration of citric acid. Despite the addition of an alternative carbon source to the medium, or rather because of it, the strain AJD ΔEYD1 DGA1 did not grow in this culture with the addition of 3% citric acid. During the course of the experiment the yeast did not consume either mannitol or citric acid. The pH of the medium during fermentation did not drop below the 3 value, therefore the impact of acidity was not the main cause of the highly impaired growth. The underlying issue with this pre-treatment method for mannitol extraction may be an increased level of other compounds produced by brown algae, which can have antifungal activities. On the contrary, the medium obtained with 1% of citric acid solution was proven to be effective for the growth of the modified strain (Fig. 4).

3.6. *Impact of additional carbon source in the lipid synthesis medium*

To determine the impact of additional carbon source, aside from citric acid used in the previous analysis, the experiment was performed with supplementation of glycerol in the medium since this species utilizes glycerol efficiently and shows preferability to it over glucose (Dobrowolski et al., 2016). Moreover, glycerol is a by-product of many industrial-scale processes and its stock value is lower than glucose, making its use more accessible and economically valid (Mirończuk et al., 2015). The addition of glycerol to the medium can further switch the carbon flux towards production of lipids by *Y. lipolytica,* so the impact of this carbon source on the process was analyzed. On the other hand, glucose is present in the algal hydrolysates (Dobrowolski et al., 2022), therefore the addition of glucose to the media was also analyzed in this study.

The supplementation of glucose or glycerol to the medium containing mostly mannitol as a carbon source was proven to be detrimental on mannitol utilization, however the strain in this study was able to uptake both carbon sources efficiently, as seen in Fig. 5. The biomass reached 21.62 g/L for the glucose supplemented medium and 24.9 g/L for the medium with glycerol. The experiment ended after 48 h, within which all of the mannitol was depleted. Interestingly, glucose was entirely utilized within 24 h of the experiment, while nearly 30% of glycerol was not consumed within this timeframe. However, in both cases the utilization of mannitol appeared simultaneously, which was questionable based on the preciously obtained data.

Lipid content results show that the addition of glucose or glycerol to the algal rinse medium did not increase the percentage of storage lipids significantly, with 8.38% and 8.89% values, respectively. This could, however, be caused by the breaking down of the lipids, as both glucose and glycerol, as well as mannitol, were not observed in the medium at the 48 h mark in which the samples were collected.

The summarization of the results allowed for the observation of changes in biomass and lipids yields, productivity and fatty acids profiles across all of the performed experiments in various media. Interesting results were obtained for the media with citric acid. Both algal rinse and the control medium with pure mannitol and citric acid caused the shift in the lipids profile in the modified *Y. lipolytica* strain, promoting unsaturated fatty acids, as the highest percentage of MUFAs was observed in the citric acid and mannitol medium. Additionally, in both laboratory scales, the algal rinse with citric acid yielded high percentage of PUFAs, which was not a consistent result for other media in both flask and bioreactor cultures. Citric acid, not only caused the shift in fatty acids profile, but also increased the yield of the lipids. It is important, as the mannitol rich rinses can be obtained on an industrial scale from alginate extraction, as opposed to the hydrochloric acid, which is currently in use. Moreover, the biomass in this type of medium was also the highest, excluding the supplementation of glycerol and glucose, as both of those carbon sources are easily utilized by *Y. lipolytica*.

The results indicate that the fatty acids profile is dependent on the carbon source used in the medium and the conditions of the culture. This fact is additionally proven by the fatty acids content in the biomass

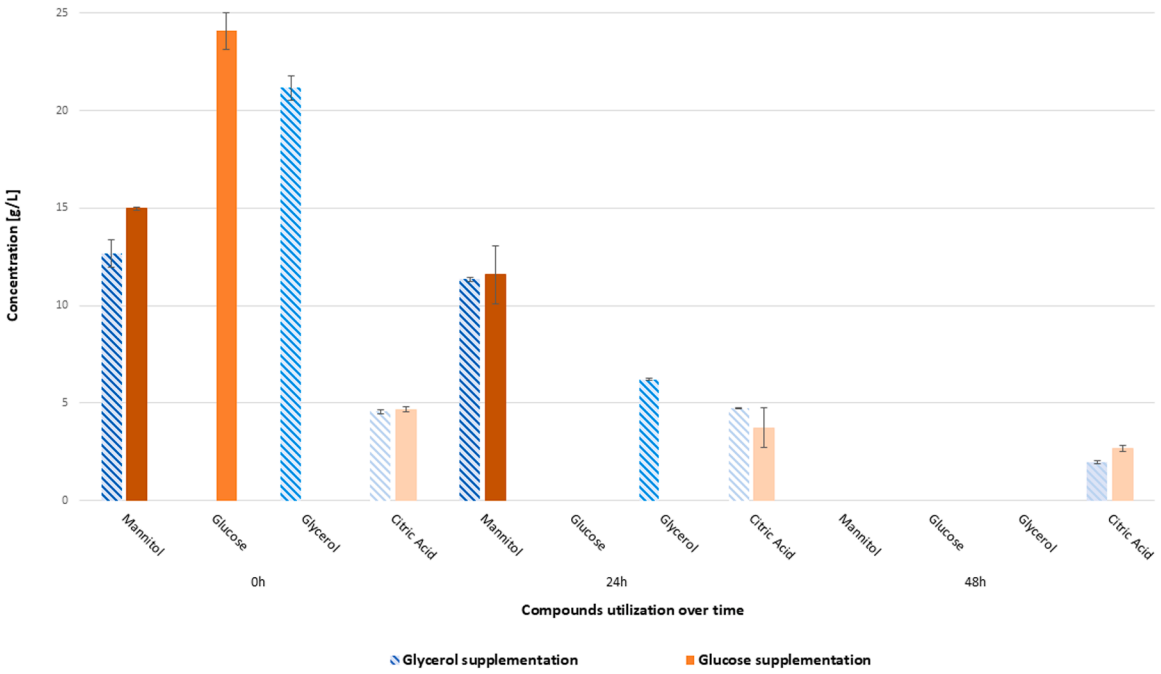

Fig. 5. Concentration of mannitol, glycerol, glucose and citric acid in media supplemented with glycerol (shades of blue, striped) and glucose (shades of red). The experiment was carried out in shake flasks cultures with the modified *Y. lipolytica* strain AJD ΔEYD1 DGA1.

grown in the media with sodium chloride. The capability to withstand harsh environmental conditions, including the high osmotic pressure, allow for *Y. lipolytica* to thrive. As the medium with salt addition did not impede the yeast's growth and lipid synthesis, it could be possible to use marine water in algal soaking pre alginate extraction. Therefore, the research should be conducted on the wastewater from the industrial process to provide more information about the optimization of the process and the impact of salts and additional compounds, which could be present in the industrial waste.

4. Discussion

Y. lipolytica strain AJD ΔEYD1 DGA1 was proven to be an effective way to utilize algal mannitol from brown algae rinse and to produce lipid from this waste material. The capability to utilize waste rinse, which is obtained as one of the steps of the alginate extraction is a promising path to facilitate the full potential of this waste. Due to the lack of universal protocol regarding this process, multiple factors were taken into consideration in this study. The analysis was therefore performed on artificially produced algal rinses, to sort out the possible sources of industry waste that can be included in the future studies, based on the obtained preliminary results.

Due to the previous promising results (Szczepańczyk et al., 2025), the production of lipids in a larger scale was conducted in the bioreactors. The results show that the process can be up-scaled with maintaining the high level of lipids produced, despite the low overall carbon content in the medium. This issue could be solved by the addition of other, easily utilizable carbon sources by *Y. lipolytica,* however, two different carbon sources can cause a lag phase, that is caused by the adaptation of the yeast to the new conditions. The medium for the cultivation can be further optimized when it comes to the nitrogen content. The rinse is rich in multiple different compounds, some of which can be used as a nitrogen, or carbon source. These compounds were considered when it comes to the analysis of carbon to nitrogen ratio. Therefore, the calculated C/N ratio of 60 was generalized based on the possible carbon sources present in the rinse, that could be detected thanks to the analytical methods available during the research. This, coupled with the low initial carbon content in the medium, could heavily affect the lipid production capabilities by the modified Y. *lipolytica* strain.

Interestingly, the shortening of the cultivation was observed in the bioreactor studies, in some cases, as a result of the lack of the initial lag phase, which was needed to adapt to the change of the main carbon source in the medium. On the one hand, it is useful when it comes to the economic viability of the process, while on the other, it can cause issues with pointing out the exact time, when the cultivation has to be stopped. This can cause the breaking down of the storage lipids, which in the presence of carbon source in the medium should not be utilized by the yeasts. It might be the reason why the production of lipids in the medium containing additional glucose and glycerol was not increased, on the contrary, it was lower than the one observed for the control medium without any changes. In those examples, the biomass production was also dramatically increased, so the low concentration of mannitol, which was present after 24 h of the experiment, could be too sparse to support the lipid accumulation. Additionally, higher biomass production correlates with the overall lower production of lipids, as the carbon flux is shifted towards the former process.

Glucose and glycerol in the medium were utilized first, while the mannitol was mainly utilized once the more conventional carbon sources were depleted. It is interesting from the standpoint of utilizing the algal enzymatic extracts, which contain high amounts of glucose, not present in the rinse. Previously conducted studies showed that glucose is easily consumed from the extract, while mannitol remains in the medium for much longer (Dobrowolski et al., 2022). In this study, the utilization of mannitol was simultaneous with glycerol, and most likely closely followed the uptake of glucose. More importantly, after glycerol of glucose were consumed, mannitol was depleted completely from the medium, which was not the case for the algal extracts, as presented by Dobrowolski et al. (2022). Moreover, despite glycerol being the preferred carbon source for *Y. lipolytica*, it was glucose that was utilized first from the algal rinses. That can be a clue as to what mechanisms are controlling the polyol utilization, especially in terms of their transport.

The overall differences in the mannitol content, which vary depending on the pretreatment method could be problematic in the standardization of the procedure. Despite the increase of mannitol release in the presence of salts, both with the artificial sea water and the

addition of 3% NaCl, the lower production of lipids in those conditions prove it to not be an efficient way to prepare the algal rinse to obtain value-added products by *Y. lipolytica*. It is specifically visible when it comes to the lipid synthesis, which is lower than that for the control conditions, while biomass is also lower. Those results are in correlation with the previously conducted study by Dobrowolski et al., 2022, in which both the control strain and the modified strain (Dobrowolski et al., 2022) (overexpressing DGA1) showed lower biomass and lipid content in glycerol supplemented medium, while similar results were also obtained for glucose. In this case only the WT strain was characterized by a higher biomass on ASW based medium. This data, alongside the result in this manuscript, prove that the addition of salt does impact the lipid synthesis negatively, no matter what the main carbon source in the medium is. On the contrary, the study on olive-mill wastewater supplemented with crude glycerol showed a slight increase in the lipid accumulation when the NaCl was added to the medium (Tzirita et al., 2019). However, the exact cause of the higher lipid production could be a combination of factors, such as the salt addition and the high phenolic composition. Nevertheless, the biomass was also decreasing with the sodium chloride supplementation, which correlates with the results of this study, while the time of the culture was increasing with the higher concentrations of NaCl (Tzirita et al., 2019).

The possibility to utilize algal rinse pretreated with the acids was also taken into consideration, both for the organic and inorganic acid. When it comes to the hydrochloric acid, the strain growth was restricted, despite the pH being in the range that does support the growth of *Y. lipolytica*. That rules out the industry waste in which the protocol requires the usage of HCl in the alginate extraction. On the contrary, citric acid, which is proposed as an alternative to inorganic acids, was proven to be a useful addition in the algal rinse medium. That is due to the fact that *Y. lipolytica* possesses the capability to not only produce, but also utilize this organic acid. The supplementation of this additional carbon source was visible when it comes to the produced biomass and the overall lipid content. Moreover, the metabolism of citric acid and lipids is intertwined, yet the synthesis of those products is affected differently by the conditions of the medium. While the production of citric acid is promoted in the alkaline environment, the lipids are synthesized more efficiently in acidic conditions (Zhang et al., 2019). Therefore, the possibility to utilize citric acid, and therefore affecting the pH of the medium, could limit the capability of the modified strain to produce lipids in algal rinse mediums obtained with citric acid.

It is worth noting that the production of lipids, despite not achieving high level of storage fatty acids, in most cases was obtain within 48 h of the experiment. Based on the presented results it seems that the biggest limiting factor is the initial biomass and the adaptation phase. This can be caused by the required change in the expression patterns, favoring those, related to mannitol metabolism, including the transport of this compound to the cells. However, despite years of research, the mannitol transporters, which can be the gatekeeper for the process to take place, remain unknown. The changes could however be induced by the adaptive laboratory evolution, that possibly could affect also the mannitol transport, which could likely be carried out by the symporters or non-specific transporters. The issue of mannitol transport can also impact the overall capability of the yeasts to utilize mannitol. The modification of the erythritol utilization pathway can be a cause of the change of the transport capabilities.

Since one of the biggest limitations of the proposed approach to utilize algal rinses is low concentration of carbon source in the medium, the chemostat strategy could be optimal. The limited mannitol concentration, depleted as quickly as 48 h, could be a main reason for the inhibition of further lipid production, and, what is worse, the potential breakdown of the already produced lipids. Constant feeding could minimise the risk of utilising storage lipids as carbon source and increase the overall yield.

5. Conclusions

The presented study demonstrated that *Yarrowia lipolytica* AJD ΔEYD1 DGA1 strain can efficiently utilize algal mannitol from brown algae rinse obtained by different pretreatment methods, as performed in the alginate extraction process. The method for obtaining the algal rinse has an impact not only on the overall mannitol content, but also the lipid synthesis by *Y. lipolytica*. The process was successfully scaled up in bioreactors, although lipid yields were influenced by low carbon content and an imbalanced C/N ratio. Optimization of medium composition, particularly nitrogen levels, could further enhance productivity and the usage of chemostat could further help to avoid lipid breakdown.

CRediT authorship contribution statement

Dorota A. Rzechonek: Writing – review & editing, Supervision. **Mateusz Szczepańczyk:** Writing – review & editing, Writing – original draft, Visualization, Investigation. **Adam Dobrowolski:** Writing – review & editing, Funding acquisition. **Elia Tomás-Pejó:** Writing – review & editing, Supervision, Funding acquisition. **Aleksandra M. Mirończuk:** Writing – review & editing, Supervision, Conceptualization.

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Declaration of Competing Interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

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