A novel process for enhancing oil production in algae biorefineries through bioconversion of solid by-products

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ABSTRACT

This paper focuses on a novel process for adding value to algae residue. In current processes oleaginous microalgae are grown and harvested for lipid production leaving

a lipid-free algae residue. The process described here includes conversion of the carbohydrate fraction into glucose prior to lipid extraction. This can be fermented to produce up to 15 % additional lipids using another oleaginous microorganism. It was found that *in situ* enzymes can hydrolyze storage carbohydrates in the algae into glucose and that a temperature of 55°C for about 20 hours gave the best glucose yield. Up to 75% of available carbohydrates were converted to a generic fermentation feedstock containing 73 g/L glucose. The bioconversion step was found to increase the free water content by 60% and it was found that when the bioconversion was carried out prior to the extraction step, it improved the solvent extractability of lipids from the algae.

Keywords: biorefinery; fermentation; carbohydrates; diatoms; lipids; microalgae

1. Introduction

There is a growing interest in biofuels as a sustainable and renewable option. Amongst these technologies oils produced by algae is a promising technology due to the amount of oils produced which is much higher than any other terrestrial crops used at the moment for biodiesel production (Schenk, 2008). According to Zeng et al. (2011) an average biodiesel productivity of 3.3 $kg.m^{-2}.yr^{-1}$ can be obtained from a microalgae plant. Algae not only grow and produce oils much faster than plants, but they also do not compete with food crops for land surfaces nor with fresh water; many species of microalgae can grow in brackish or seawater. They also fix atmospheric CO_2 into useful biomass. Currently, the microalgae industry is developing and efforts are being made to produce large amount of oils for biofuel production (Chisti, 2007).

In order to produce biofuels, microalgae species such as diatoms producing large amounts of lipids in short periods of time are of particular interest. They can accumulate lipids in the range 20-50 % of dry cell weight (Chisti, 2007). However, for the microalgal biofuel industry to develop, the algae residue remaining after lipid extraction needs also to be addressed. This biomass still contains a significant fraction of carbohydrates and proteins and is normally sold as fish meal (Thurmond, 2011). Unfortunately, a recent review has drawn attention to the fact that non-starch polysaccharides in these residues remain indigestible and cannot be used as an energy source by fish because they do not produce β-glucanases or β-xylanases (Sinha et al., 2011). Moreover, the addition of non starch polysaccharide to the diet of monogastric animals such as salmon has a negative impact on growth. It would therefore be

desirable to convert the carbohydrate fraction of the solid by-product and thereby enhance its value as fish meal.

It is known that microalgae can store both carbohydrates and lipids as food reserves for periods of starvation (Bacic *et al.*, 2009). Carbohydrates have been targeted for ethanol production because of the fast growth of algae. For instance, *Chlamydomonas* biomass can contain around 45 % starch, which can be hydrolyzed using commercial enzymes (α-amylase and amyloglucosidase). An ethanol fermentation based on this hydrolysis provided 235 mg ethanol/g algae (Choi *et al.*, 2010). In another study, *Chlamydomonas* biomass with 60 % carbohydrates (of which 35 % was starch) was hydrolyzed using 3 % sulphuric acid at 110°C for 30 min which released glucose at 58 % w/w and the ethanol fermentation provided a yield of 29 % from algal biomass (Nguyen *et al.*, 2009). Other papers have used anaerobic digestion or hydrogen fermentation to convert lipid-extracted microalgal biomass residues into methane and hydrogen, respectively. Methane yields in the range 0.2 – 0.5 m^3/ kg volatile solids (Briand, 1997; Morand, 1999) and hydrogen yield of 30 L/kg volatile solids (Yang *et al.*, 2010) have been reported in the literature.

While green algae store their carbohydrates mainly as starch, diatoms store chrysolaminarin instead, which is predominantly a (1,3)-β-D-glucan, with an average degree of polymerisation of 12-16 residues and a relatively low proportion of side-branching (Bacic *et al.*, 2009).

It is likely that the proportion of carbohydrates in oleaginous diatoms will be minimised as biofuel production processes are optimised for maximum oil production. As a result, ethanol production from residual carbohydrates in these processes would not appear to be a sensible target. It would be much more sensible to use glucose

released from these carbohydrates to produce more oil, using an oleaginous microorganism.

In this paper a process is proposed for the production of glucose from unextracted diatoms, which is then separated from the algae solids by filtration. The liquid fraction can be fed to a fermenter for the production of microbial oil using an oleaginous yeast, whereas the solid algae residue can be sent off for lipid extraction. The oil production from the yeast could ultimately be integrated with the algae oil in the existing downstream process. The aim of the paper is therefore to describe this novel process and to provide an insight into the optimum conditions to release sugars from diatoms.

2. Materials and Methods

2.1. Algae feedstock

The diatomaceous algae samples (from a few grams to several kg) were from different raceway ponds, fed with different nutrients and under different conditions, from different stages in the process (post-harvest, post-centrifugation and post-spray-drying, pre- and post-extraction) were tested. The algae were cultured in open raceways ponds (50 m long and ~4 m wide) agitated with paddle wheels (no replicate). Details of the samples referred to in this paper are given in Table 1. As the process being operated in the ponds was not an aseptic one, many of the samples received were contaminated with opportunistic micro-organisms. The extent of contamination in these samples was analyzed by spraying onto nutrient agar Petri

dishes and counting colonies after incubation at 30°C. Where saline conditions were required for experiments, a solution containing 35 g/L NaCl was used.

2.2 Lipids production using Rhodosporidium toruloides Y4

The oleaginous yeast *Rhodosporidium toruloides* Y4, which has previously been shown by Li et al. (2007) to be capable of high cell density culture was used throughout this study. It was kept at 4°C on Petri dishes containing 3 $g.L^{-1}$ malt extract, 10 $g.L^{-1}$ yeast extract, 10 $g.L^{-1}$ peptone, 10 $g.L^{-1}$ NaCl, 50 $g.L^{-1}$ glucose and 15 $g.L^{-1}$ agar.

2.3 Glucose production tests using in situ enzymes

In order to investigate the presence of *in situ* enzymes able to catalyze the degradation of chrysolaminarin into glucose, an aqueous mixture of algae sample L was treated in a French press (2000 psi) while keeping the mixture cold (ice water bath). The disrupted material (an aqueous suspension) was stored at 0 °C, and centrifuged the following day. The supernatant was serially passed through a qualitative filter then through a 0.45 μm filter. A final filter sterilizing step (0.22 μm) was applied, and the filtrated broth was stored at 0 °C. Then a β(1→4) bonded glucan (cellobiose) was added to the algal broth. Controls were run in parallel to control that cellobiose was not degraded by water or pH adjusted water.

2.4. Analytical methods

For the carbohydrates analysis, a known mass of dry sample was hydrolyzed in 1% H_2SO_4 at 130°C for 2 hours. The suspension was then centrifuged and carbohydrates in the supernatant were quantified using the phenol-sulphuric acid method (Dubois *et*

al., 1956). Glucose determination was carried out by applying the glucose oxidase method in triplicate samples, which were run in an automatic system (Analox GL6, UK). Free Amino Nitrogen (FAN) concentration was analyzed by the ninhydrin colorimetric method (Lie, 1973). Before lipid extraction a dry sample was ground three times in liquid nitrogen using a mortar and pestle. Lipids were extracted using an automatic solvent extraction unit (Soxtec-HT6 system, Höganäs, Sweden). A known mass of dry sample was placed in a thimble and placed in boiling methanol/chloroform mixture (1:1 by volume) at 140°C for 2 hours while the solvent was evaporated and condensed continuously. The cups collecting the lipids were weighed before and after the extraction to determine the lipid content by difference. The oil content was determined in triplicate.

The measurement of Total Nitrogen was carried out according to a TN kit manual from Hach-Lange GmbH using a persulfate digestion (Method 10072). Each sample was analyzed in triplicate. The coefficient of variance for ten identical samples was ± 5.5%. Moisture and total solid content were measured by drying a known mass of sample at 100-105°C until a constant weight was obtained according to Standard Methods (APHA, 1999).

The free water content of cell suspensions was measured by vacuum filtration of a known volume through a qualitative filter (Whatmann 1) until a constant volume was obtained. Viscosity of the suspensions was measured at 30°C using a Haake Rotovisco system. The stirring was performed by a Rotovisco RV20 stirrer and controlled by a rheocontroller RC20 connected to a computer.

2.5. Kinetic model of glucose production

Different kinetic models were employed to model glucose production over time, including the conventional Michaelis-Menten model. However, such models proved inappropriate. Two other kinetic models were tested: a non-linear regression (Equation 1) and the Boltzmann equation (Equation 2):

$$G(t) = G_{\max} \cdot \left(1 - e^{-Kt}\right) \quad (1)$$

$$G(t) = \frac{A_1 - A_2}{1 + \exp\left(\frac{t - x_0}{dx}\right)} + A_2 \quad (2)$$

Where $G(t)$ is the glucose concentration at time t (hr) and $G_{\max}$ is the maximum glucose concentration. A_1, A_2, x_0 and dx are coefficients determined using Excel Solver to obtain the best fit.

3. Results and discussion

3.1. The process

Like any other biorefinery process (ethanol production from sugar cane, biodiesel from rapeseed) the production of solid residue is a major challenge for algae based processes. However, in an integrated biorefinery the solid by-product represents an opportunity because it is a potentially cheap source of added value chemicals such as platform chemicals or speciality compounds.

Laboratory scale experiments have demonstrated high lipid content in algae (up to 70 % of dry cell weight), but large scale production suffers from non ideal growth conditions (light, temperature, pH, nutrients, dissolved carbon dioxide) as well as contamination issues. For 1 tonne of fresh material obtained after drying there is usually about 5-10 % residual moisture, 30-40 % lipids, 10-20 % carbohydrates, 10-15 % ash and the rest is protein. As a result, for every tonne of dried material, there

might be roughly 500 to 700 kg of extracted by-product. A commercial scale design would normally have a pond capacity of several thousand cubic meters, which means a daily output of several tons of dried material to be handled per day depending on the algae concentration in the ponds. A typical pilot scale algae batch production process is shown in Figure 1 where microalgae are grown in open ponds in which cell density ranges from 0.5 to 2 g dry biomass L^{-1} (Benemann and Oswald, 1996). The algae are then allowed to settle before being pumped to a sedimentation tank. The concentrated solids at the bottom of the tank are pumped to a centrifuge. This algae slurry is then dried and the powder is sent off for lipid extraction. Given the large volume to be processed on an industrial scale this process would normally be carried out continuously.

Figure 1 here…

3.2. Glucose production

Various strategies were tested to convert the chrysolaminarin in diatoms to glucose such as acid hydrolysis, French press, bead mill, sonication, autoclaving, microwave, fungal and bacterial hydrolysis and commercial enzymatic hydrolysis. However, the controls run alongside these preliminary experiments showed that glucose could be extracted directly from the algae when placing the dried cells into warm water. In a first attempt, several fermentation tests were carried out in flasks with isolated pure colonies (indigenous algae contaminants and from our culture collection), but none of them could hydrolyze the algal carbohydrates and produce glucose (See supplementary materials A1, A2 and A3). Further experiments showed that enzymes secreted by the algae and concentrated in the filtered broth (0.2 micron) could catalyze the degradation of a synthetic β–glucan (cellobiose) (see supplementary

material B). A third strategy consisted of inactivating, by autoclaving, the suspected "bioactive" broth extracted from algae. In this case, no glucose was produced in the autoclaved control flasks suggesting that the *in situ* enzymes were deactivated at high temperature. Another strategy revealed that glucose production from diatoms was very limited once the cells were treated with solvent to extract the lipids (See supplementary materials C1 and C2 on algae P and Q). Probably this happened because some enzymes are prone to solvent inhibition.

These preliminary tests, considered together, suggest that glucose production is the result of hydrolysis by *in situ* enzymes within the algae. This finding is consistent with that reported by Chiovitti et al. (2004) who demonstrated that glucose extracted from diatoms in hot water was derived from intracellular chrysolaminarin and not from extracellular polysaccharides. It is clear, then, that the indigenous microbial contaminants associated with the algae were, rather than hydrolysing chrysolaminarin, simply consuming the glucose produced by the algae themselves. Given the negative impacts caused by microbial contaminants, solvent and heat inhibition, these features emphasize the need to convert the carbohydrates before the lipid extraction step.

In subsequent experiments, glucose was produced and released into the medium by mixing a known mass of dry sample in 100 mL tap water at 30°C for 24 hours. Figure 2 shows the initial carbohydrate content and glucose yield from algae sampled at different stages of the process. A maximum carbohydrate content of 200 $mg.g^{-1}$ was measured in sample C while a maximum yield of 110 mg $glucose.g^{-1}$ was obtained for sample G, taken after the centrifuge step. Moreover, a maximum conversion of 74 % of carbohydrates to glucose was obtained with sample H. The lower carbohydrate contents and glucose yields in the samples collected after the drying step were correlated with a higher level of contamination. This was verified by spraying the

samples onto nutrient agar Petri dishes and counting colonies after 1, 2 and 5 days (See supplementary data D). The level of contamination was higher in the samples taken after the dryer, compared to the samples taken from the ponds after harvesting and those taken after the centrifugation step, indicating that contamination occurred during the post processing stages. In the existing process, the algae solids leaving the dryer are still moist and are left exposed to dry further which promotes contamination, further emphasising the need to process the glucose as soon as possible.

Figure 2 here…

3.3 The proposed addition to the existing process

Based on the results presented above, it is proposed to add a simple reaction step following the centrifuge, as shown in Figure 3, in order to produce and release glucose. It has been observed that the shear in the centrifuge can result in temperatures close to 40°C which is sufficient to release some glucose into the medium. Given this, the glucose left in a fresh algae paste will increase the risk of contamination in the process by fast growing aerobic microbes, which is why the glucose must be processed as soon as possible and certainly prior to extraction.

We have determined that the reaction step could take place in a simple insulated pipe acting as a plug flow reactor so that capital and operational costs remain low compared to other types of reactor. The addition of fresh water is avoided since the glucose extraction step is carried out in the initial process seawater. Another advantage of our process is that glucose can be extracted regardless of the age and lipid composition of the cells. The microalgae population coming from the ponds is a mixture of cells at various growth stages. Young cells may contain no lipids whereas

older cells are more likely to contain the expected levels of lipids associated with the residence time in the pond. It is, unfortunately, not possible to separate young cells from the older ones. Nevertheless, all the cells contain intracellular carbohydrates that can be used to provide glucose.

Our process can also provide an advantageous feature in the case of lower than expected oil production performance. If, for some reason, the lipid yield is lower than the design value (e.g. as a result of low ambient temperatures, lack of sunlight, low cell density etc.) the proposed process will still be able to make use of the carbohydrates to balance the loss of lipids, resulting in a reduced overall loss in the final oil yield.

Figure 3 here…

The glucose produced will then be separated from the algae solids using a solid/liquid separation step such as a rotary vacuum filter. This can be operated under a limited oxygen atmosphere to hinder the growth of aerobic microbes and reduce the need for sterilisation prior to any subsequent fermentation. The cake could be washed in order to recover as much glucose as possible. The liquid fraction can be fed to a fermenter containing an inoculum of an oleaginous microorganism such as the yeast *Rhodosporidium toruloides* that has been shown to grow on glucose and accumulate lipids (Li *et al.*, 2007), whereas the solid algae cake can subsequently be dried and sent off for lipid extraction.

The conversion of glucose into value-added product at an early stage in the process reduces the risk of contamination in subsequent units. We have observed that if the glucose remains available for any length of time, fast growing airborne microbes quickly take over. These decompose the algae residue, reducing its value as an animal

feed due to the degradation of proteins and liberation of nitrogen. In the proposed process, the total nitrogen content of the final residue will actually increase because it will no longer contain significant amounts of carbohydrates. This will add to its value as a feed material. In addition, the downstream processing of the algae stream would be cheaper because the chrysolaminarin is converted to glucose and removed. Less material is therefore sent for solvent extraction and thus less solvent will be required. Assuming lipid and carbohydrate contents of 30% and 20%, respectively, and assuming that around 75% of the carbohydrates are converted into glucose (maximum conversion obtained in our experiments), a glucose yield of 150 kg per tonne of algae would be obtained. Considering a glucose to lipid yield of 0.2 (Li *et al.*, 2007), up to 30 kg lipids could be obtained from yeast in addition to the initial 300 kg from the algae. Depending on the carbohydrate content of the diatoms (10 – 30 %), the overall lipid production could be increased by 5 to 15 %. In the following sections, the experiments relevant to the process steps are described.

3.4. Effect of temperature on glucose yield

A study was carried out to determine the optimal temperature for glucose production via *in situ* enzymes. The glucose yields were calculated as the mass of glucose obtained per gram of dry sample suspended in water and the results at various temperatures are shown in Figure 4. This shows that a temperature between 50 and 60°C was optimal to obtain the highest enzyme activity and glucose yield. We found that at lower temperatures, fast growing indigenous microorganisms would consume the glucose, while this was not the case at 55°C (See supplementary data E). On the other hand, at temperatures greater than 60°C the enzyme activity would be reduced

resulting in lower glucose yields. Therefore, a temperature of 55°C was chosen for the subsequent experiments.

Figure 4 here…

3.5. Effect of salinity, total solids concentration and glucose concentration on glucose production

Since the objective is to produce glucose from the algae cells after the centrifuge step, experiments were carried out to confirm that glucose could be produced in a seawater medium. We found that there was no significant effect of the salinity on glucose production (See supplementary material F). Parallel experiments were carried out to check that there was no adverse effect of the high solids concentrations leaving the centrifuge (glucose could be obtained from sample T having 33 % dry matter, see Figure 5). Since both the substrate (chrysolaminarin) and the *in situ* enzymes are in close proximity within each diatom cell, the presence (or absence) of interstitial liquid was not a problem and so slurry concentration had no noticeable effect.

3.6. Fed-batch production of glucose

A small scale, draw and fill, technique was used to determine the maximum glucose concentration that could be obtained, using the *in situ* enzymes, and to check that this glucose concentration was not inhibitory for the degradation of chrysolaminarin. The tests were performed in a 10 mL initial volume with 1 g dry algae sample being used each day for 3 days. At the end of each day the suspension was centrifuged, the solids discarded, and the supernatant used to suspend the next 1 g sample of algae. Using this technique, maximum glucose concentrations of 55 g/L and 73 g/L were obtained with algae samples C and G, respectively.

3.7. Effect of mixing on glucose production

The effect of mixing on glucose production from algae samples N and O was investigated in order to determine whether mixing was necessary, since the enzymes and substrate already coexist within each algal cell. It was found that continuous mixing for 24 hours on an orbital shaker at 200 rpm did not result in higher glucose concentrations. It was in fact observed that shaking was detrimental because mixing promotes oxygen diffusion and facilitates the proliferation of any fast growing aerobic microorganisms that are present in the algae samples and hence consumption of the glucose produced (see supplementary materials G1 and G2).

The effect of having no mixing was studied in a further experiment with contaminated algae sample R, in which duplicate reactors were blanketed with either air or nitrogen (anoxic environment). When the reactors were incubated at 55°C, similar results for glucose production were obtained in both sets (see supplementary material H). It was therefore unnecessary to exclude oxygen in order to reduce growth of the contaminants present in the sample, as long as the temperature was at an elevated level and no mixing was used.

However, maintaining a minimal gas headspace, with absence of oxygen, would still be an appropriate operational measure, as it is a simple and effective way to help minimise glucose loss.

3.8 Kinetics of glucose production

The kinetics of glucose production at 55°C is shown in Figure 5. Boltzmann's equation was fitted to the data and the coefficients A_1, A_2, x_0 and dx, representing the

best fit, are presented in Table 2. Based on this model, it can be predicted that approximately 21 hours incubation at 55°C will be required to reach 90 % of the maximum glucose yield. Interestingly, the curves in Figure 5 show that there was already some glucose present in the medium at time zero which was presumably due the temperature rise in the centrifuge. Because no mixing is required the glucose production step could easily take place in a simple insulated pipe acting as a plug flow reactor. Figure 5 also shows data for samples R and O obtained post-centrifugation and post-drying, respectively. In both cases, glucose production was much poorer than for sample T.

Figure 5 here…

3.9. Effect the reaction step on the viscosity

Viscosity plays an important role in mass and energy transfer in bioprocesses, and its variation is greatly dependent on temperature and other parameters. For this reason, we studied the effect of the reaction step (heating to 55°C) on the rheology of the algae slurry. In this experiment, a fresh algae slurry (Algae sample R) taken directly after the centrifuge step was used. The free water content was 28 % of the total volume (280 mL free water per litre of suspension). Interestingly, the free water content after 24 hours at 55°C increased to 44 % indicating that intracellular liquids diffused from the cells to the bulk. Probably, at the temperature of the reaction step, the cell wall is damaged as suggested by Chiovitti *et al.* (2004). As a consequence of the increased water content, the apparent viscosity of the slurry after the reaction step is lower than that before (see Figure 6). The slurry approximates to a Bingham plastic, behaving as a Newtonian fluid above a shear rate of around 100 s^{-1}. This has the benefit, in the process context, that it will not be more difficult to pump, post-reaction.

Figure 6 here…

3.10. Effect of the reaction step on lipid content

The lipid content in algae sample S was analyzed before and after the 55°C step in order to demonstrate that this temperature and the incubation period of 24 hours were not detrimental to the algae lipids. A lipid yield of 7.4 % (± 2) was found in the raw algae, while after the 55°C step it had increased to 13.5 % (± 0.6). Apparently, the heat treatment had enhanced the extractability of the lipids from the diatoms. In algae sample T a lipid content of 39.9 % was measured after the 55°C step. It is known that heat increases extraction yield (Cheung, 1999; Morrison and Coventry, 1989), and the higher lipid yield obtained is consistent with the higher free water content measured.

3.11. Effect of the reaction step on Total Nitrogen (TN) content

The algae residue after lipid extraction is usually used as fish meal and its value is related to its nitrogen content. The Total Nitrogen (TN) content of algae sample S before and after the 55°C step was analyzed in order to verify that no loss occurred during the reaction step. The TN content in the raw algae suspension was 20.3 (± 1.6) mg TN/g dry sample, while after the reaction step it was 21.3 (± 2.5) mg TN/g dry sample, indicating that there is very little change during the reaction. However, there may be some degradation of proteins, as separate analyses of Free Amino Nitrogen (FAN) showed, in one case (sample E) an increase from 1.9 to 2.8 mg FAN/g dry sample, though in samples I, J, K and N no significant effect was seen (See supplementary material I).

Because the proposed process includes a solid/liquid separation step after the 55°C step, the FAN dissolved in the liquid phase will go through the fermentation step resulting in approximately a 10 % loss (max. 2-3 mg N lost as FAN compared to a TN content of about 20-21 mg/g dry algae) in TN from the residual algae solids stream. However, this FAN will be taken up by the oleaginous microorganism and reappear as TN in the 'yeast cream' collected from the subsequent centrifugation step (Figure 2). After harvest, this yeast stream joins the algae solids and passes to the extraction unit, while any soluble FAN residue can be recycled back to the algae ponds.

3.12. Bioconversion of glucose to additional lipids

The yeast *Rhodosporidium toruloides* was chosen to test the proposed bioreaction step to convert glucose into additional lipids because it is relatively well known and non pathogenic. This yeast can accumulate lipids up to 70 % of its dry cell weight (Ratledge, 1991). Moreover, our experiments have shown that it can grow in a glucose medium with a wide range of salinity (0 – 55 g NaCl/L, see supplementary material J1, J2 and J3). The results described above can be used to show that the C/N ratio in the medium produced in our process will be in the range 20-30 which is known to be appropriate for the growth of a wide range of microorganisms. After the 55°C step a glucose concentration of 45 g/L glucose or 18 g C/L (1.5 moles Carbon) can be obtained. For the nitrogen, it was measured that 2.8 mg N as FAN/g dry sample was released in the liquid phase after the 55°C step. If we consider a typical algae suspension from the centrifuge with 22% solid content, then about 0.62 g FAN will be dissolved in 780 mL of liquid, so a concentration of 0.8 g FAN/L can be expected (0.057 moles Nitrogen). This gives a C/N ratio of 22.5 g C/g N (or 26.3 mol

C/mol N). Using this step, it is possible to increase the overall lipid production by 5 to 15 %.

4. Conclusions

A simple method, consisting of holding algae at 55°C for about 20 hours, has been developed to extract glucose from diatoms. *In situ* enzymes hydrolyze storage carbohydrates yielding a maximum of 110 mg glucose/g dry algae with up to 75% conversion. The glucose produced can be used to grow an oleaginous microorganism that will produce additional lipids. This concept can be applied by adding just a few units to a typical large scale process and could increase the overall lipid yield by 5 to 15% depending on the carbohydrate content of the algae.

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Table 1. Characteristics of algae used in this study.

[1] stage in the process where the algae was taken.
[2] state of the sample when received.
[3] was the sample solvent-extracted?

Sample	Stage[1]	State[2]	Extracted[3]	Contaminated
A	Post-harvest[4]	Dry	no	no
B	Post-harvest[4]	Dry	no	no
C	Post-harvest[4]	Dry	no	no
D	Post-harvest[4]	Dry	no	no
E	Post-centrifuge[5]	Dry	no	yes
F	Post-centrifuge[5]	Dry	no	yes
G	Post-centrifuge[5]	Dry	no	yes
H	Post-centrifuge[5]	Dry	no	yes
I	Post-dryer[6]	Dry	no	yes
J	Post-dryer[6]	Dry	no	yes
K	Post-dryer[6]	Dry	no	yes
L	Post-dryer[6]	Dry	no	yes
M	Post-dryer[6]	Dry	no	yes
N	Post-dryer[6]	Dry	no	yes
O	Post-dryer[6]	Dry	no	yes
P	Post-extraction	Dry	yes	yes
Q	Post-extraction	Dry	yes	yes
R	Post-centrifuge[5]	wet	no	yes
S	Post-centrifuge[5]	wet	no	yes
T	Post-centrifuge[5]	wet	no	yes

[4] Post-harvest samples were taken from the pond just before the paddle-wheel was stopped and dried within 1h.
[5] Post-centrifuge samples were taken after centrifugation (4-5 hours after harvesting).
[6] Post-dryer samples were dried in a ring-dryer. It can be up to 12 hours before they are dried.

Table 2. Boltzmann's coefficients for the kinetic model of glucose production

Sample	Boltzmann coefficients			
	A_1	A_2	x_0	dx
Algae O	-379.94	7.07	-23.42	5.53
Algae R	-289.46	24.48	-47.35	15.63
Algae T	-17.47	45.6	3.03	7.35

Figure Captions:

Figure 1. Large scale algae production process.

Figure 2. Initial total carbohydrate content of various algae samples and associated glucose yields when the algae were suspended in warm water (30°C) for 24 hours.

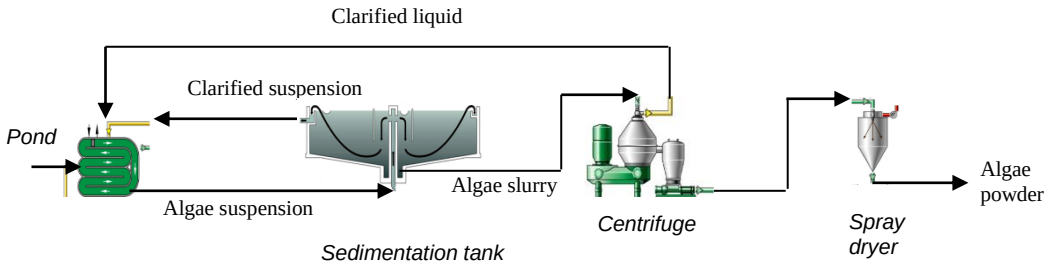
Figure 3. Large scale algae production process showing proposed additional steps (within the dotted box).

Figure 4. Effect of temperature on glucose production from algae by *in situ* enzymes.

Figure 5. **Glucose production kinetics at 55°C. The lines show the best fit to the Boltzmann equation**.

Figure 6. Rheological behaviour of an algae slurry before and after the glucose production step at 55°C.

Figure 1. Large scale algae production process.



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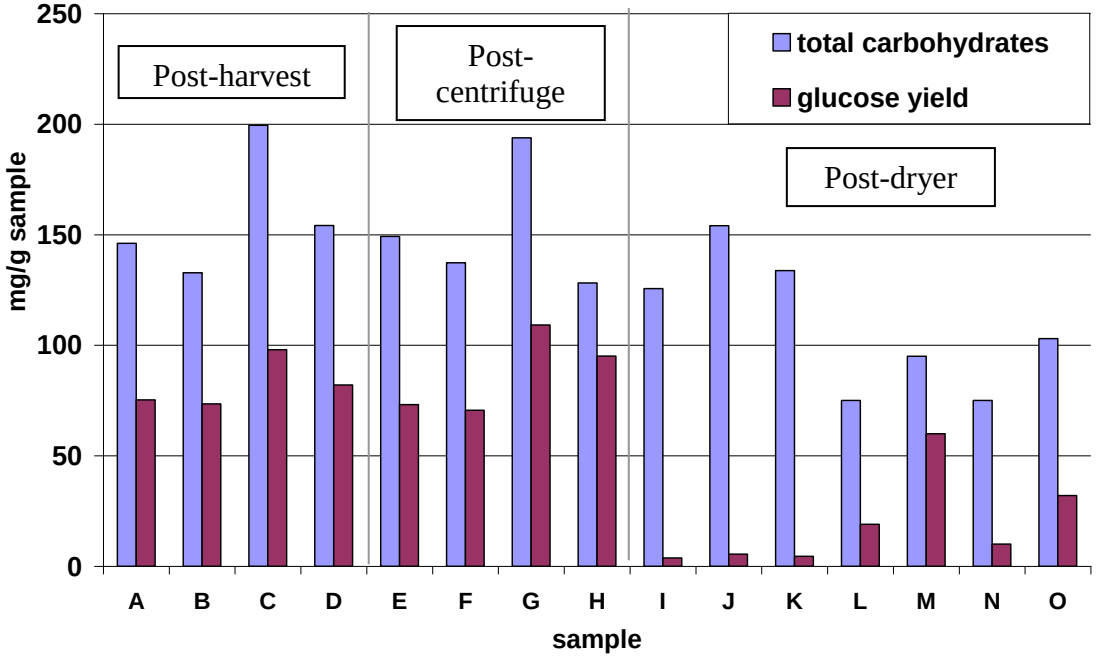
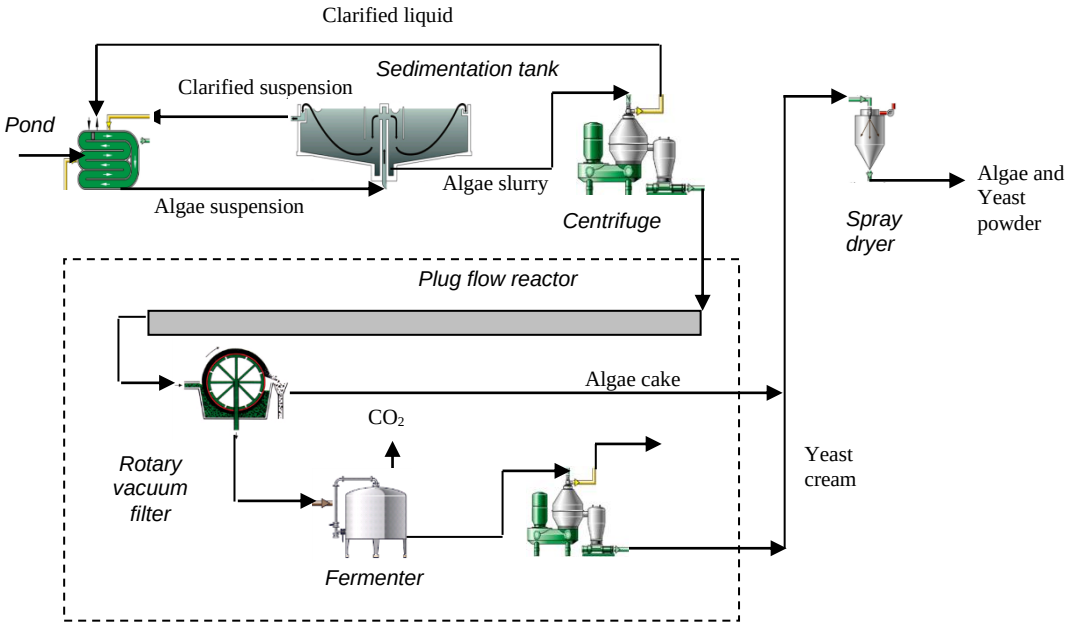


Figure 3. Large scale algae production process showing proposed additional steps (within the dotted box).



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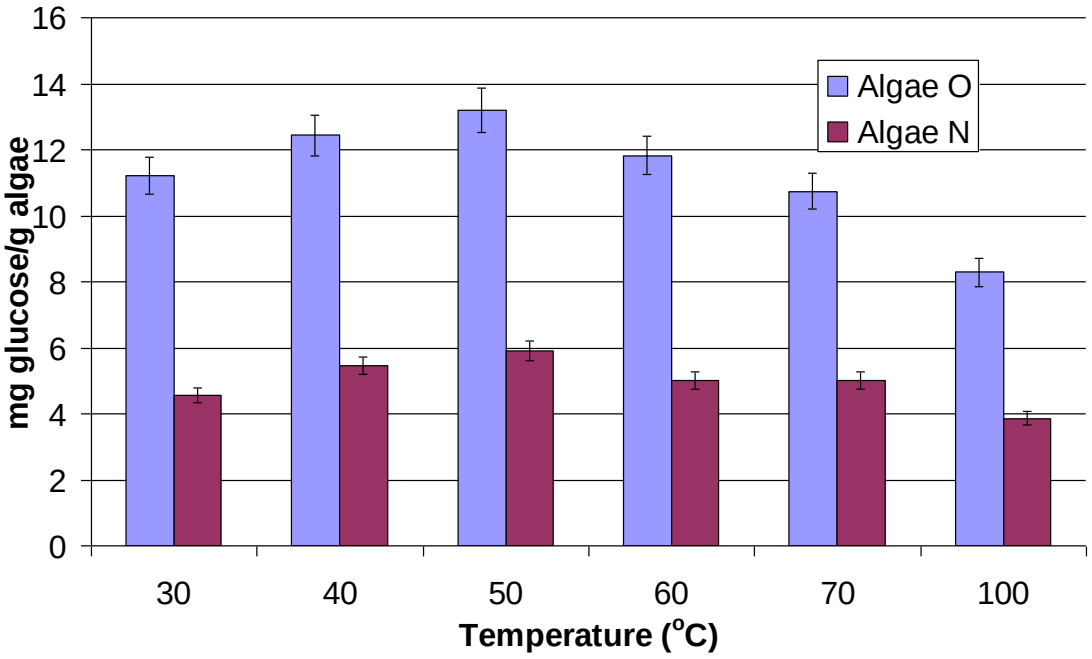

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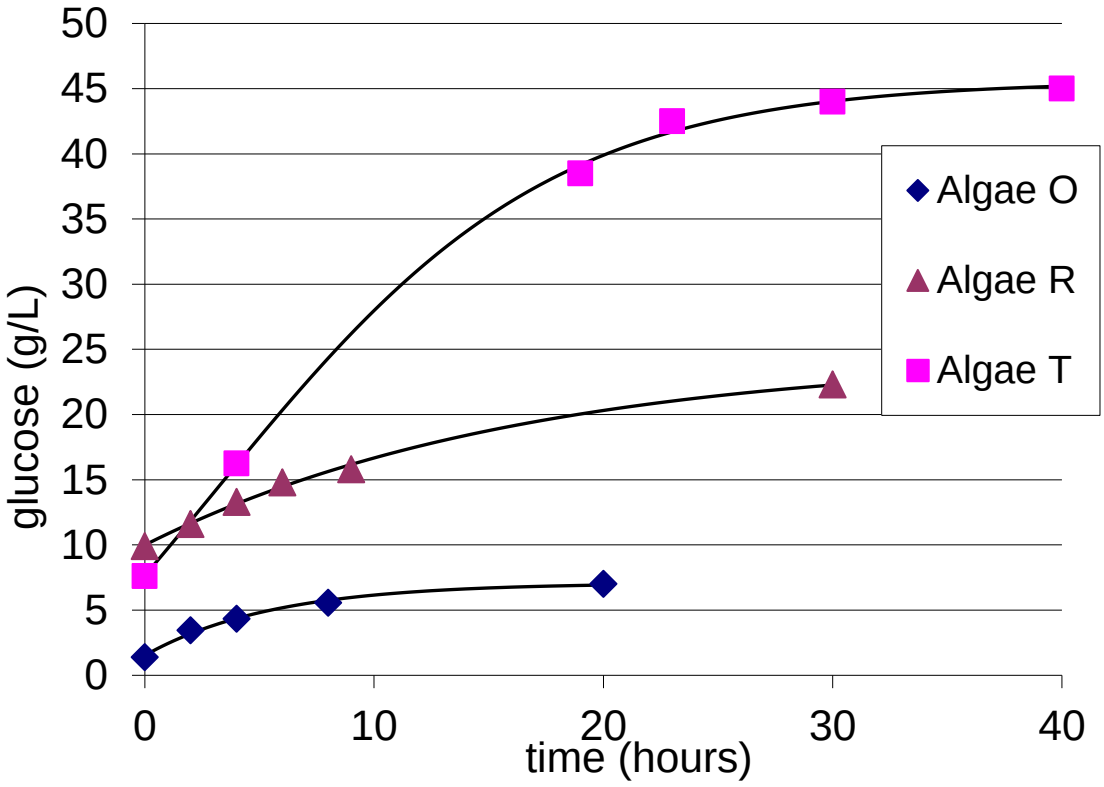

Figure 6. Rheological behaviour of an algae slurry before and after the glucose production step at 55°C.