EXTRACTION OF LIPIDS FROM FERMENTATION BIOMASS USING NEAR-CRITICAL DIMETHYLETHER

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The extraction of lipids from both wet and dry biomass produced by fermentation has been carried out using near-critical dimethylether (DME) as the extraction solvent. Fermentations were carried out from a shake flask to 300 litre scale using the micro-organism *Mortierella alpina*, and up to a 20 litre scale for *Phaffia rhodozyma* and *Agrobacterium tumefaciens*. The lipids extracted at a laboratory and pilot scale from the biomasses were enriched in arachidonic acid, astaxanthin, and co-enzyme Q10 respectively. Extractions were also performed on marine microalgae, produced by fermentation elsewhere, to obtain lipids rich in EPA. Lipids could be extracted from wet biomass using DME, which could result in improved process economics. However, water is co-extracted, which has to be separated from the lipid. The biomass shrunk considerably during packed bed extraction, leading to channelling. In contrast, the extraction of lipids from freeze-dried biomass was routine, and yields were substantially higher than using CO_2 or CO_2 + ethanol. DME also extracted polar lipids from both wet and dry biomass, leading to the higher yields. Further work was also performed on fractionation and/or chemical modification of the lipids that were extracted by DME.

INTRODUCTION

The fermentation of micro-organisms to produce biomass rich in high value lipids is a relatively recent industrial process. The production of a wide range of lipids is possible, including lipids rich in polyunsaturates; carotenoids; and co-enzyme Q10 (CoQ10) [1-3]. Specific micro-organisms that are harvested through a heterotrophic fermentation or photobioreactor process can produce lipids that are highly enriched in either GLA, EPA, DHA and/or ARA [1,4]. The production of lipids from specific micro-organisms via industrial fermentation has been commercialized by Martek and others, mainly for infant formula products using hexane as the solvent [4]. The extraction of lipids enriched in LCPUFA from micro-organisms appears to have only been investigated at a research scale using CO_2 or CO_2 + co-solvents, and has been reviewed by Mendes [5]. The limitations of the combined process package have been the high costs of fermentation and drying the biomass prior to extraction, and poor yields obtained using supercritical CO_2. Poor yields are due to a combination of factors, including difficulties in breaking open dry cell walls by grinding, and the lipids being a mixture of neutral and insoluble complex lipids, which are not soluble in supercritical CO_2 [5]. The extraction of carotenoids from biomass has been studied in some more detail, especially astaxanthin from *Phaffia rhodozyma* and *Haematococcus pluvialis* respectively [5-8]. While astaxanthin has a relatively low solubility in supercritical CO_2, it can be increased through the use of vegetable oil [6] or ethanol [7] as a co-solvent. More recently, the use of DME followed by CO_2 has been proposed for extracting astaxanthin from dry *Haematococcus pluvialis* biomass [8]. To overcome the high costs of drying, and maximise the total lipid yield we have pioneered a process using pressurized liquid

dimethylether (DME) at 313 - 333 K to extract a variety of wet biomass from fermentation processes [9]. Here, we present some fermentation and extraction results

EXPERIMENTAL

Fermentation and pre-preparation. The fermentation of the filamentous fungi *Mortierella alpina* (IRL 176, MA); the yeast *Phaffia rhodozyma* (IRL 4-4P9, PA); and the bacterium *Agrobacterium tumefaciens* (IRL 538, AT) was performed on-site at Industrial Research Limited. All strains of micro-organisms were from IRLs' collection, and were stored at -80 °C. The fermentation of MA was conducted in a 300 l bioreactor, with a secondary seed stage, using media containing glucose and soybean flour and operated in a fed-batch configuration. The fermentation was conducted over 6 days at 25 °C, 0.5 vvm aeration, 300 mbar backpressure and 0.5 m/s agitation. Additional glucose was added on day 4 and 5 and the fermentation broth harvested via filtration on day 7. The fermentation of PA was conducted in a 20 l bioreactor, with a secondary seed stage, using media containing glucose, HySoy and various salts with the addition of either glucose of sodium hydroxide during the fermentation to maintain a pH of 6. The fermentation was conducted over 7 days at 22 °C, 0.5 vvm aeration and 2.0 to 0.5 m/s agitation. The fermentation broth was harvested by centrifugation on day 7. The fermentation of AT was conducted in a 20 l bioreactor, with a secondary seed stage, using media containing sucrose, corn steep powder and salts and operated in a fed batch or continuous configuration. The fermentation was conducted over 4 days at 32 °C, 1 vvm aeration and 1 -2 m/s agitation. Additional sucrose was either added to maintain the concentration in the bioreactor at 10 g l^{-1} or added on day 1 and 3 and the fermentation broth harvested via filtration on day 7. The fermentation broth was harvested on day 4. Alga fermentation was performed by Photonz Corporation, and biomass was supplied either in heat-killed solution or as frozen solids after centrifuging. MA and AT biomass was split into batches, which were either frozen or freeze-dried. PR biomass was freeze-dried prior to extraction. Frozen MA was passed through an Urschel knife mill prior to extraction. Freeze-dried MA was passed through a Wiley knife mill prior to extraction

DME and CO_2 extraction.The extraction of all micro-organisms was performed in a packed-bed type apparatus. The scale of apparatus used depended on the type of biomass and ranged from a 500 ml lab scale to a 2 x 10 litre pilot scale plant. The extractions were performed on both wet and dry material for most of the organisms. Fermentation biomass was added to an extraction basket, which was placed in the extraction vessel. DME was then passed upwards through the vessel at the desired temperature and pressure, and then through pressure reduction valve(s) and into the separator(s). Lipid extract (and water) were recovered in the first separator when using only DME as the solvent, or were fractionated into two separators when using CO_2. Water was removed from the extract by rotary vacuum evaporation, and through simple phase separation when possible. DME or CO_2 was recycled back to the pump via a mass flow meter, water trap and condenser/subcooler to complete the cycle. Further lipid fractionation was performed using CO_2 at a laboratory scale. Crude lipid extract was added to an extraction basket, and supercritical CO_2 was passed upwards through the lipid mixture. Neutral lipid was collected in the separator, and complex lipid was recovered from the extraction vessel basket.

Lipid analysis. The fatty acid composition of the extracts was determined by converting the lipids to methyl esters and then analyzing by GC using an in-house method adapted from the literature. The astaxanthin and co-Q10 concentrations of PR and AT respectively (both lipid extracts and biomass samples from fermentation) were determined by HPLC, again using in-house methods adapted from the literature.

RESULTS AND DISCUSSION

Mortierella alpina. The pilot scale (20, 300 litre scale) heterotrophic fermentations of a wild-type strain of MR resulted in biomass with lipids containing around 28 % ARA. The change in major fatty acid profile as a function of the fermentation time is shown in figure 1 for the 300 litre scale fermentation. Of note is the decrease in linolenic acid (C18:2) and increase in arachidonic acid (C20:4) with fermentation time.

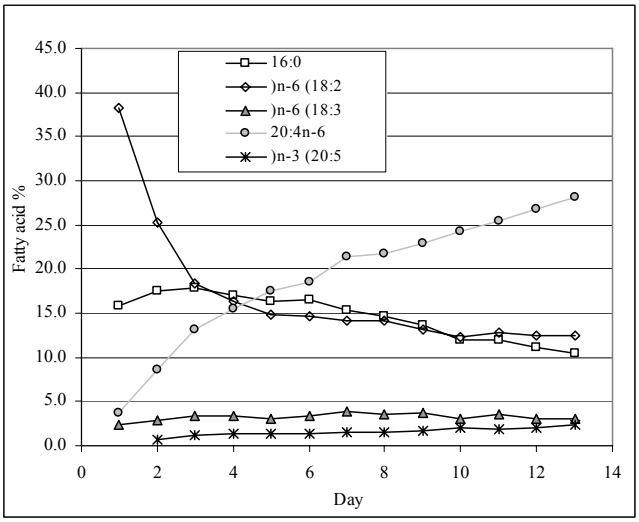

Figure 1: Fatty acid composition of *Mortierella alpina* fermentation biomass as a function of fermentation time

The lipids were extracted directly from wet biomass using liquid DME, and from freeze-dried biomass using either supercritical CO_2 or liquid DME. The fatty acid profiles of the extracts closely match those of the final biomass (~ 28 % ARA), showing that there is no denaturation of the polyunsaturates due to the extraction process. The extraction yields from both wet and dry biomass using DME are similar on a dry solids basis. DME extracts both neutral and complex lipids, and thus the yield is substantially higher than that of CO_2. The extraction of lipids from freeze-dried biomass was routine. The lipids are extremely soluble in DME, and can be extracted over a short time frame. In contrast, the extraction of lipids using CO_2 is solubility limited. The solubility is lower than a C18 triglyceride oil, due to the substantial concentration of C20 fatty acids in the lipid.

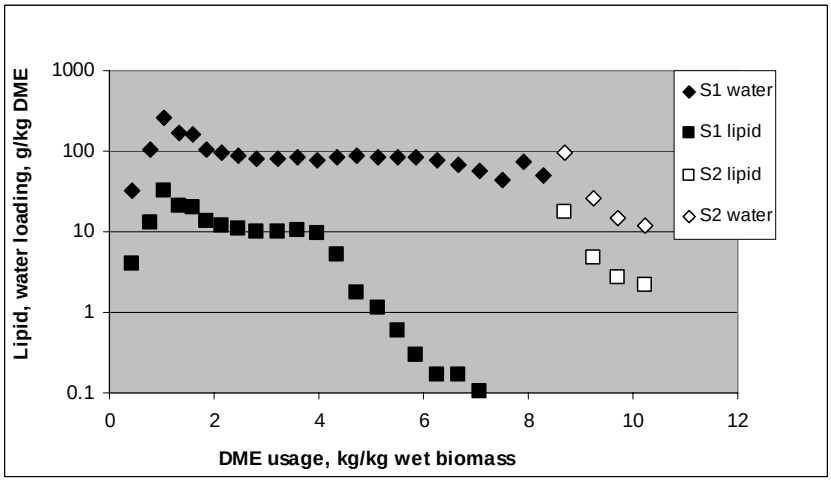

Figure 2 Lipid and water loading in DME as a function of DME usage for the extraction of wet *Mortierella alpina* biomass. Dark symbols first stage extraction; light symbols second stage extraction

The differential rate of extraction of water and lipid was examined for the pilot scale extraction of MA using DME at ~ 333 K. The solubility of water in liquid DME increases substantially with temperature, and is of the order 100g/kg at around 333

K [10,11]. Water is extracted at higher than its solubility limit in DME for the binary mixture during the initial stages of extraction, and then decreases to a steady state level. Similarly, the rate of extraction of lipid reaches a peak, settles to a steady state level and then drops to zero due to channeling and bed shrinkage. The estimated loadings of lipid and water are shown in figure 2. The bulk of the lipid was extracted during the early stage of extraction, which is a useful finding, as it means that the solids do not need to be extracted to a bone-dry state, and the amount of water to be separated from the lipid after extraction can be minimized. This finding matches our earlier results for dairy streams and liquid egg yolk streams [11,12], where lipids were able to be extracted whilst retaining most of the water in the raffinate stream. We found that the bed of extracted material was non uniform, with some areas of the bed bone dry, and others still obviously wet. The beds were generally remixed and repacked, leading to a short further extraction to recover the remainder of the water and non-extracted lipids. As the extraction does not require all the water to be extracted, and packed beds lead to channelling problems, we are now investigating stirred tanks for performing the extraction.

Phaffia rhodozyma. The fermentation of PR was carried out in a 20 litre bioreactor. Nitrosoguanidine (NTG) was used as a mutagenic agent to increase the astaxanthin content of PA. A range of concentrations (20, 40, 60, 80 µg/ml) and times (10, 20, 40, 60 minutes) were used on actively growing cultures. After mutagensis, intense red coloured colonies were selected from agar plates containing ß-ionone. Four mutagenic passages were conducted using the colonies with the most productive astaxanthin content from the previous passage as the starting material. Figure 3 shows the concentration of astaxanthin from a number of colonies that were subjected to NTG treatment.

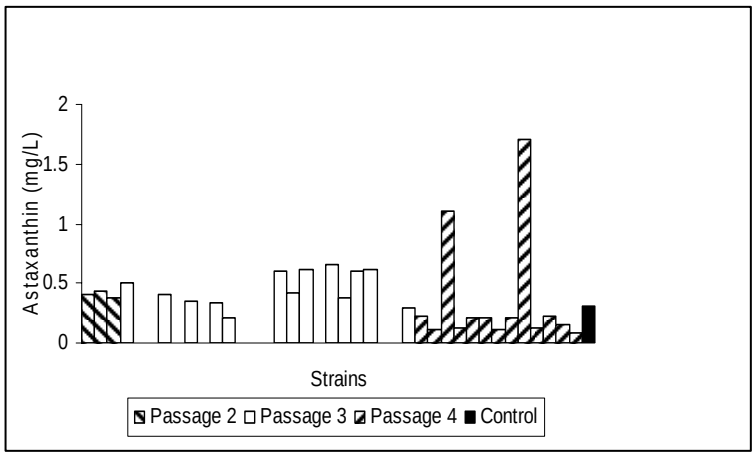

Figure 3. Astaxanthin content of fermentation culture strains as a function of the number of mutagenesis passages

The extraction of astaxanthin and other lipids from freeze dried biomass was performed using DME at a 2 litre scale. A total lipid yield of around 9 % by mass was obtained. The lipid was mostly triglycerides containing C18 fatty acids, with astaxanthin as the main pigment at a concentration of ~ 30mg/100g lipid. This matches previously reported yields from a variety of microorganisms [5]. The astaxanthin was present as a mixture of free astaxanthin, and astaxanthin mono-esters. The method of pre-treatment of the biomass prior to extraction was critical to the extraction yield. When the biomass was pH treated to attempt to lyse the cells prior to drying, the cell wall became impenetrable and no extraction of carotenoid was possible. Remarkably, the acid-treated dried biomass could be stored at room temperature for a number of months with no apparent decrease in colour, even though astaxanthin is highly unstable. In contrast, with no pH treatment of the cells, astaxanthin was easily extractable after freeze-drying, and the residual biomass after extraction was faintly pink in colour.

Agrobacterium tumefaciens. The fermentation of a wild type AT was carried out in a 20 l bioreactor. The sucrose content of the fermentation broth (left hand axis); co-enzyme Q10 broth concentration and packed cell volume (both right hand axis) are shown as a function of the fermentation time in figure 4. The fermentation broth became extremely viscous after 120 hours, and so the fermentations were halted and the biomass was recovered by centrifugation.The extraction of lipids rich in CoQ10 (ubiquinone) from AT was routine when the biomass was freeze-dried, but was more difficult from wet biomass. Polysaccharide was exuded from the biomass during fermentation and the heat kill step, and this resulted in sticky wet solids after centrifugation. These solids tend to agglomerate during the extraction process, giving rise to bypassing, which was exacerbated by bed shrinkage due to the co-extraction of the water. The solids had to be repacked and re-extracted twice to obtain a full yield of lipid. The total yield of lipid from the wet biomass was slightly higher than from dry solids. The total yield of lipid was low, at around 2 % by mass; and the yield of CoQ10 was around 0.1-0.4 g/kg on a dry biomass basis. The extraction of CoQ10 from fermentation biomass does not appear to have been reported before. The solubility of CoQ10 in CO_2 and CO_2 + ethanol; and extraction of CoQ10 from nutraceutical capsules [ref]; and from freeze-dried fish [ref] at a tenfold lower concentration has been reported.

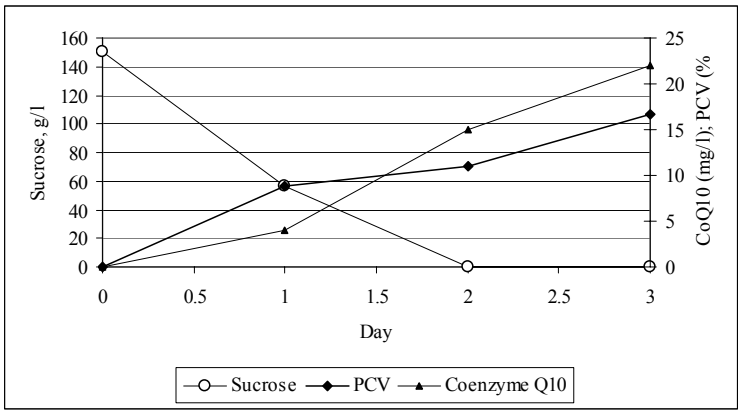

Figure 4: Sucrose usage, coQ10 broth concentration and packed cell volume (PCV) as a function of fermentation time.

Algae extraction. The wet marine algal biomass was extracted with DME. A mixed lipid product was obtained, that had an overall EPA content of between 9 -13 % by mass. The total lipid yield on a dry basis was in the range 28-45 %, depending on the fermentation conditions. An extraction was also performed on freeze-dried algal biomass, but the yield was substantially lower than from wet biomass, which may be due to the extent that the algae is crushed [5]. The differential rate of extraction of lipid and water was also determined in a manner similar to MA, by collecting the total extract over a certain time period and then evaporating the water from the

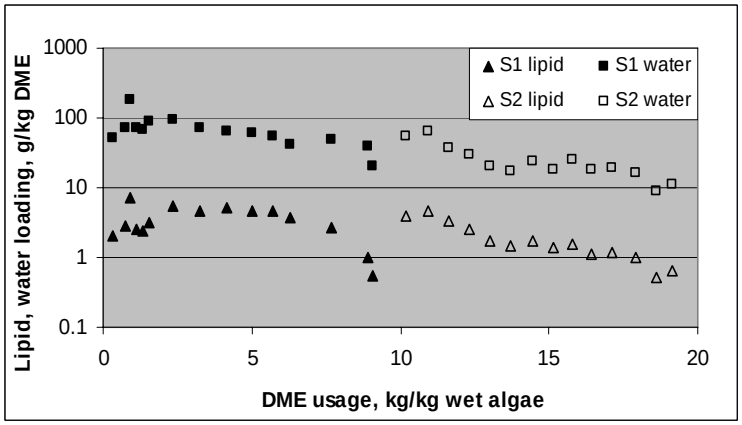

Figure 5: Lipid and water loading in DME as a function of DME usage for the extraction of wet algal biomass. Dark symbols first stage extraction; light symbols second stage extraction

extract mixture. The loading (note the log scale) of both lipid and water is shown as a function of the DME usage in figure 5 for both the first stage (S1) and re-extraction (S2). Here, we collected samples of total extract and then separated the water from the lipid. Again, the co-extraction of water resulted in bed shrinkage, giving bypassing as the experiment went on. The extraction rate of both lipid and water reached a peak not long after the extraction started, and then settled to a steady state value. The lipid loading then dropped to zero before the bed was repacked and re-extracted. The second stage extraction does not reach a steady state. The algal lipid mixture contained a variety of pigments, complex lipids and triglycerides. The complex lipid fraction was most enriched in EPA, but formed a relatively small proportion of the total overall lipids. We separated the neutral lipids from the complex lipids by re-extracting the bulk lipid extract with supercritical CO_2.

CONCLUSIONS

The fermentation of *Mortierella alpina, Phaffia rhodozyma*, and *Agrobacterium tumefacians* were performed at IRL to produce biomass rich in arachidonic acid, astaxanthin, and co-enzyme Q10 respectively. The extraction of lipids from all micro-organisms including algal biomass supplied from an external fermentation process was possible using DME from either wet or dry biomass. The lipid yield was usually superior from wet biomass, although two or more extractions were required because of shrinkage of the biomass due to loss of water. Water is extracted at its solubility limit in DME. The extraction rate of lipid was highly dependent on the water content of the feed and extent of extraction.

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