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Bulk Solvent Extraction of Biomass Slurries Using a Lipid Trap

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Abstract

Extraction of lipids and hydrophobic metabolites from microbial sources remains an obstacle in the production of these compounds at the laboratory and industrial scale. Analytical techniques for the total extraction of non-polar metabolites from biological material are well established, but rely on expensive and time consuming processes. This makes these techniques unsuitable for direct translation to continuous or large volume systems, unable to move beyond proof-of-concept studies, and leaves a major gap in the translation of new bio-products requiring a purified extract. Here we attempt to bridge that gap by demonstrating the use of a semi-continuous liquid-liquid extraction system capable of bulk lipid extraction from wet, untreated biomass, and simultaneous concentration of the unmodified extract in a lipid trap. A 1.8 L model was used to evaluate system dynamics with bacterial, fungal, algal, and plant feedstock, prior to scaling the system by an order of magnitude to demonstrate large-scale viability. Extraction efficiency was above 90% for each feedstock compared to standard Bligh and Dyer extraction. Following scale-up, extraction was performed on upwards of 4 kg of slurry (660 g dry weight), yielding an average efficiency of 96%, and allowing generation of a crude extract at a scale not previously possible in a laboratory setting. The resulting system allows for direct and high-throughput extraction of biomass sources without pretreatment, specialized instrumentation, or intensive user input.

Introduction

Intensive efforts in biotechnology have yielded organisms capable of producing a vast array of lipid-derived bio-products, whose major end-uses include plastics [1], surfactants [2], fuel replacements [3, 4], and pigments [5]. As the microbial and plant product profile has grown, so has the desire to investigate industrial production potential. In many cases, such as production of pharmacologically-active natural products like phytosterols [6], feeding studies with microbial-derived essential fatty acids [7], or the much-discussed general displacement of petrochemicals [8], extraction must be performed at a scale larger than analytical techniques are capable of, in order to generate sufficient material for downstream testing. This extraction step remains an obstacle limiting many studies to the laboratory scale, and is insufficient for successful translation of biotechnologies using microbial or plant derived lipid. The system described here overcomes the obstacle of bulk extraction, and enables process testing (i.e. bulk lipid isolation, characterization, and functionalization) at a scale informative of, and relevant to industrial production.

Lipid is an ambiguous term often used to describe biological fats and oils, but more broadly encompassing an array of hydrophobic metabolites including sterols, long chain alcohols, terpenes, essential oils, pigments, and carotenoids, among others [9, 10]. Current analytical techniques for solvent-based extraction of lipids and hydrophobic compounds from biomass typically rely on a mixture of a non-polar organic solvent and an alcohol. The widely used method of Bligh and Dyer [11] remains the standard for analytical-scale total lipid extraction from a wide variety of biomass sources, due to its simplicity, effectiveness, and widespread adoption. Other solvent-based lipid extraction techniques utilize variations on a similar theme, such as ethanol/hexane and isopropanol/hexane extractions [12, 13]. The fundamental difficulty in total lipid extraction is the effective removal of generally hydrophobic compounds with a broad range of polarities into a single phase. In the case of solvent-based extraction techniques, including the method of Bligh and Dyer, this separation is accomplished using centrifugation, an energy intensive, batch-wise, and time-consuming technique. As with most processes, direct scale-up is not feasible. Even when energy input is not an issue, centrifugation can still be prohibitive due to the lack of large instruments, the cost of continuous flow centrifuges for handling large volumes, and the difficulty of working with two phases in such instruments. In addition to challenges involving separation, the lipid constituent of a cell is often bound in an overall hydrophilic matrix of proteins, carbohydrates, and other cellular components, making single-pass total extraction with a non-polar organic solvent difficult since the solvent cannot access the shielded lipids [14].

Alternative extraction methods have been investigated in attempts to improve process efficiency, such as supercritical CO_2 [14], soxhlet [15], and accelerated solvent extraction [16], but like traditional solvent extraction, these systems can be difficult to scale up. Mechanical expression of oil is a common and high-throughput technique, but is inefficient when oil content is low (<20%), or water content high, as is typically the case with microbial feedstock. Methods for direct conversion of biomass to hydrocarbons or biofuels have also been developed, such as thermal treatment [17] and direct transesterification [18], but do not preserve the crude extract. Critical drying and grinding steps comprise another major challenge for soxhlet extraction, supercritical CO_2 extraction, thermal treatment, and mechanical pressing. These issues alone can rule out the use of these techniques when volumes exceed workable quantities. Despite this variety of techniques for analytical-scale extraction, few technologies have been scaled up due to challenges associated with process enlargement (Table 1). Industrial processes for the production

of edible oils from select plant feedstock (i.e. soybean) exist [19], as well as a variety of technologies for large-scale extraction of aromatic and medicinal compounds from plant material, including percolation, counter-current extraction, and distillation techniques [20]. However, these techniques also require a dry feedstock and in the latter case are less selective in the compounds they extract than techniques aimed specifically at the lipid fraction, making them impractical for use with aqueous slurries.

The ideal biomass extraction system must be able to handle a wet feedstock, be amenable to process enlargement and automation, require minimal pretreatment of the biomass, and produce a crude extract that has not been significantly affected by the extraction process. Continuous solvent extraction can meet all of these requirements. Devices for continuous liquid-liquid extraction have been constructed previously for a variety of purposes [21, 22], as well as automated [23], demonstrating feasible continuous operation. Recent studies have also focused on optimizing solvent choice and efficiency [24, 25], but a scalable system has yet to be developed. We have designed a straightforward, scalable, semi-continuous liquid-liquid extraction system, and demonstrated its effectiveness in generating an unmodified crude hydrophobic extract from a range of biomass slurries with no pretreatment, with the hope that this system will serve as a unit for processing a variety of natural metabolites regardless of the host organism. The described system utilizes readily available materials, equipment, and solvents, and can be scaled by orders of magnitude without changing the fundamentals or efficacy of the system.

Experimental methods

Biomass sources

The bacteria *Rhodococcus opacus* PD630 (*Ro*) was obtained courtesy of the Greenspan lab, University of California, San Diego, and cultured in 2 L flasks in LB media on a rotary shaker at 100 rpm at 30 ˚C. Conventional baker's yeast, *Saccharomyces cerevisiae* (*Sc*) was purchased dry from Red Star® and re-suspended in water. The yeast *Rhodosporidium toruloides* (*Rt*) was obtained from the Agricultural Research Service (NRRL) culture collection and cultured in 2 L flasks of YPD media on a rotary shaker at 100 rpm at 30 ˚C. Cultures of *Ro* and *Rt* grown in the laboratory were harvested via centrifugation. The microalgae *Scenedesmus dimorphus* UTEX 1237 (*Sd*) was obtained from the University of Texas at Austin culture collection and cultivated as described previously in outdoor ponds [26]. Biomass was harvested via settling and continuous flow centrifugation. Frozen, shelled soybeans, *Glycine max* (*Gm*) were purchased locally, thawed, and blended until homogenous (in order to obtain consistent dry weight measurements). All slurries were stored at -20 ˚C.

Analytical methods

Dry weight, lipid content, and lipid trap quantifications for each extraction experiment were measured gravimetrically, in quadruplicate, using an analytical balance readable to 0.1 mg. Extraction experiments were performed in triplicate. All solvents were reagent grade.

Dry weight percentage (g solids / g slurry) for each trial was determined by drying pre-weighed amounts of the slurry in aluminum dishes in an oven at 80 ˚C for twelve hours. Total mass of the solids in each extraction was determined by weighing the beaker containing the

slurry before and after addition to the extraction vessel, and multiplying by the solids percentage obtained via dry weight measurements.

Total lipid content of the slurry was determined by the method of Bligh and Dyer [11]. Total lipid content of the extract obtained using the lipid trap system was determined by evaporating the organic solvent from pre-weighed amounts of the crude extract in a bead bath at 80 ˚C. Total mass of the lipid extracted was determined by weighing the round-bottom flask containing the extract before and after removal of the extract, and multiplying by the lipid percentage obtained from dried sample measurements.

Thin layer chromatography (TLC) was performed using glass-backed silica gel 60 plates to visualize the lipid profile of the Bligh and Dyer and lipid trap extracts. 70:30:1 hexane - diethyl ether - acetic acid by volume was used as the solvent system [10]. Plates were visualized by immersion in a solution of 10% (w/v) $CuSO_4$, 4% (v/v) H_2SO_4, 4% (v/v) H_3PO_4 in MeOH followed by charring at 160 ˚C. Gas chromatography mass spectrometry (GC/MS) was run on an Agilent 7890A GC system connected to a 5975C VL MSD quadrupole MS (EI) following transesterification of the lipids to their methyl esters [27]. For transesterification, crude extracts were dissolved in 1 M HCl in methanol, incubated at 60 ˚C for 1 hour, then extracted twice with hexane. Samples were separated on a 60 m DB23 Agilent GCMS column using helium as carrier gas and a gradient of 110 ˚C to 200 ˚C at 15 ˚C/min, followed by 20 minutes at 200 ˚C.

Lipid trap system

The lipid trap system used for assessment of the method consisted of a 2 L glass reagent bottle used as an extraction vessel with a 1 L two-neck round-bottom flask serving as the “lipid trap” (Figure 1). The reagent bottle was fitted with a male 24/40 joint at the top of the straight wall, allowing connection to the round-bottom flask via a glass elbow. A Friedrich’s condenser was fitted to the top of the vessel. A generic magnetic stirrer hot plate with temperature control was used to heat and stir the vessel, and a heating mantle used to heat the lipid trap. A detailed schematic of the system is provided (Figure S1).

During each extraction, the extraction vessel was charged with the biomass slurry (550 mL) and isopropanol (650 mL). In the case of *Rt*, the slightly acidic slurry was neutralized using 6 M NaOH. After stirring had begun, hexanes (550 mL) were added to the vessel along with the condenser. The extraction vessel was then heated to 45 ˚C. If necessary, small additional amounts of isopropanol were added to the extraction vessel such that the organic phase remained sufficiently large to allow overflow without contamination of the aqueous emulsion phase. Hexanes (700 mL) were then added to the round-bottom flask serving as the lipid trap, along with boiling chips, and the flask heated to reflux at 68 ˚C. Temperature of the lipid trap was monitored during the entirety of each run using a standard thermometer readable to 1.0 ˚C. Each trial was run for 22 hours.

The scaled up 11 L system used for large extractions was identical in design, except a 13 L glass carboy was used as the extraction vessel, mechanically stirred using a 24 x 160 mm PTFE stirrer blade, and heated using a three inch wide flexible silicone band heater. A 2 L, two-neck round-bottom was used as the lipid trap. Temperature of both the extraction vessel and lipid trap was monitored during the entirety of each run using a standard thermometer readable to 1.0 ˚C.

Results and Discussion

Feedstock choice, extraction efficiency, and composition

Five sources of biomass were tested in the system to demonstrate its effectiveness in extracting the lipid fraction of both laboratory model and oleaginous production organisms. *Rhodococcus opacus* PD630, *Rhodosporidium toruloides* and *Glycine max* were chosen as model oleaginous bacterial, fungal, and plant feedstock, respectively. *Saccharomyces cerevisiae* was chosen as a readily available laboratory model organism. *Scenedesmus dimorphus* was picked specifically as a photosynthetic commercial-production organism, and because of its tough cell wall. *Sd* is highly resistant to complete dissolution, which can create problems for certain methods of extraction and digestion [28], but makes it an excellent test case for microalgae.

Despite major differences in size, cell membrane and wall composition, and total lipid content, a crude lipid extract was generated for each feedstock as efficiently as standard analytical techniques, but at a much larger scale. Following growth and harvesting, crude extracts of *Ro*, *Sc*, *Rt*, *Sd*, and *Gm* were generated at efficiency ratios of 0.93, 2.45, 1.09, 0.97, and 1.12 respectively, relative to Bligh and Dyer extraction (Figure 2). Overall, the relative degree of extraction compared to Bligh and Dyer varied little across all five organisms, with the exception of *Sc*. Total lipid content of *Sc* and *Gm* agreed well with values expected from literature [29], but was slightly lower than previous reports in the case of *Ro* and *Rt* [30, 31]. Literature values varied widely for *Sd* [32]. The large uncertainty in the measurements of *Ro* is most likely due to variable losses during a filtration step that was carried out on the crude lipid trap extract. This wash was performed only with the extract from *Ro* and was necessary due to the presence of insoluble non-lipid material. Similarly, the increased lipid/mass ratio of *Sc* is likely due to small amounts of insoluble material being extracted, since no filtration step was carried out, and the starting material was fully dried. Neither *Ro* or *Sc* showed variations in the profile of the extract. Lastly, it should also be noted that dry weights were determined as percent solids and include any residual salts and ash from the growth medium and processing of the biomass, so lipid percentages should not be taken as absolutes for each organism.

Timecourse experiments (Figure 2) using *Sd* and *Gm* revealed that the rate of extraction in the 1.8 L system varied on the order of hours between feedstock. Timecourse experiments were carried out with *Sd* and *Gm* specifically, since *Sd* contains a rigid cell wall and *Gm* has exceptionally high lipid content relative to the other feedstock tested. It was assumed differences in rate of extraction might be observed between the two organisms due to differences in cellulosic components, cell walls, and lignin content [33]. However, in both cases, the experiments revealed the bulk of extraction was completed after five hours. In this time, extraction was over 90% and 77% complete for Sd and Gm respectively. The rate of extraction is a combination of the rate of exchange of lipid from the aqueous to the organic phase and the rate of overflow (same as rate of reflux of the trap) of the organic phase in the extraction vessel. The fact that lipid accumulation in the organic phase of the extraction vessel was not observed with either organism, but the initial rate of lipid accumulation in the trap were nearly identical indicates that differences in composition had little affect on the rate of extraction, and the rate of extraction was proportional to the total amount of lipid present. Steady accumulation of lipid was observed in the trap, with the fatty acid profile of the extract remaining constant throughout extraction (Figure S2).

Lipid composition of the crude extracts was compared to Bligh and Dyer extracts using TLC and GC/MS. In all cases, both extracts showed identical composition (Figure 3).

Additionally, the fatty acid profiles highlight known and industrially relevant differences in triacylglyceride and fatty acid composition between the organisms. As expected, TLC and GC/MS profiling of extracts during the timecourse experiments revealed that no particular component of the lipid fraction was extracted more rapidly than another (Figure S2).

Mechanism of extraction, feedstock flexibility, and scale-up

The most important advantage of the semi-continuous solvent extraction system described here is the ability to generate an unmodified lipid extract from wet slurries, at a large scale, without pretreatment or the use of expensive instrumentation. The formation of a fine, stable emulsion phase at the isopropanol/water - hexane interface allows extraction and transport of the lipid fraction into the separated hexane phase, where it eventually overflows and is trapped. The degree of disintegration varied between the feedstock tested, and was observed via microscopy (Figure S3). In the case of *Rt*, *Sd*, and *Gm* intact cells and biomass clumps were visible following both Bligh and Dyer and lipid trap extraction, demonstrating that dissolution of the cell wall is not requisite (Figure S3). Regardless of complete or incomplete dissolution during the extraction process, all five biomass sources yielded crude extracts comparable to standard methods. None of the biomass sources required pretreatment or concentration following harvesting, and were used directly as obtained. Freezing was required to prevent lipid degradation during storage due to the quantities of biomass used. Solids content of the various feedstock used in the evaluation experiments varied from 1 – 16%. Minimum necessary water content was tested using lyophilized soybean slurry, and revealed that acceptable solids content of the incoming slurry can range from 1-70%, with the upper limit being set by the minimal water content necessary to obtain two phases. The fact that water with very low solids content functions fine in the system means the lipid fraction of dilute environmental samples with low solids content could also be extracted and concentrated.

Following scale-up of the system, triplicate experiments were carried out with *Sd*, resulting in an average efficiency of 96% compared to Bligh and Dyer extraction. Maximum solids content in a single extraction was over 650 g yielding over 140 g of crude extract.

Process considerations

Ease of construction, total capacity, and amenability to scale-up were important factors in design of the system. Both the small and large systems tested were constructed from readily available glassware and equipment, with minimal customization, making replication straightforward (Figure S1). The 1.8 L system used for evaluation can accept upwards of 50 g solid material in slurry form, with the limiting factor being the ability to stir the slurry magnetically. The 11 L system easily accepts many hundreds of grams, far exceeding the capacity of currently available extraction systems.

In both systems, temperature of the extraction vessel was maintained at 45 ˚C over the course of all extractions. Additionally, reflux was maintained in the lipid trap at 68 – 71˚C, and no major increase in temperature of reflux was observed during extraction. This was expected, as the overall concentration of lipid in the trap remained relatively low. If this concentration were to increase due either to extraction of a more oleaginous feedstock, or an increase in total feedstock

mass without exchange of the lipid trap solvent, it is expected that the temperature of reflux would increase.

We have tested the system in glass primarily for ease of construction. However, the system is fundamentally two vessels with a single connection, meaning scale-up beyond volumes workable with glass, as well as automation, and adaptation to a fully continuous system, would be straightforward. One advantage of glass is its amenability to teaching and demonstration.

Solvents chosen were inexpensive and commonly available. However, a safer replacement for hexane such as cyclohexane, toluene, or a terpene mixture could be used without issue. Isopropanol was chosen as the transfer solvent based on preliminary studies, but ethanol and acetone were tested as alternative transfer solvents (Figure S4), and showed nearly identical efficiency, demonstrating that the identity of the transfer solvent is less important than its ability to form a stable emulsion with the organic phase. This is likely due to the predominantly-hexane organic phase allowing only hexane-soluble molecules to overflow into the trap. Once extraction is complete, solvents can be recycled, since no solvent is lost during the extraction process. Sustainable options also exist for utilizing the delipidated biomass, with valuable options being aquaculture or animal feed [34]. Removal and disposal of residual solvent presents little challenge since no chlorinated hydrocarbon solvents or acids are used during the process, allowing straightforward recovery of the biomass after fractionation.

Precise engineering of the system was not carried out but could yield major improvements in energy efficiency at scales larger than discussed here. Improvements in heat transfer, extraction time, trap volume, and mixing would be critical, and the authors hope this work will be done.

Conclusion

Using a single, simple, and scalable system, crude lipid extracts have been generated from five distinct biomass sources without specialized pretreatment. In a scaled-up construction of the system, microbial biomass was extracted at a larger scale than ever previously reported in a laboratory setting. As the product profile of microbes and plant continues to grow, efficient systems like the one described here will serve a critical role in overcoming the obstacles of large-scale production and isolation of microbial-derived products, and advance the viability of sustainable production of bio-products.

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385 Table 1 (full page width)
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387 Brief literature survey of extraction trials in the last 15 years using microbial or plant slurries
388 with total solids content greater than 100 g.
389

Method	Organism	Scale (g)	Pretreatment	Preservation of Lipids	Possible in Lab Setting	Demonstrated Scalability	Reference
Solvent	*M. oleifera* (plant)	150	Drying and enzyme treatment	+	+	-	[15]
Thermal	*C. protothecoides* (algae)	160	Hydrolysis	-	+	+	[35]
SC - CO_2	*N.* sp. (algae)	180	Drying and grinding	+	+	-	[36]
SC - CO_2	*J. regia* (plant)	370	Pressing	-	-	-	[37]
Thermal	*S. cerevisiae* (yeast)	540	None	-	-	-	[38]
Lipid Trap	*S. dimorphus* (algae)	660	None	+	+	+	This manuscript

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Figure 1 (full page width)

Operational diagram of the lipid trap system as observed with microalgae as the feedstock. The extraction vessel (left) is initially charged with 550 mL of biomass slurry, 550 mL of hexane, and 650 mL of isopropanol as the transfer solvent, while the lipid trap is charged with 700 mL hexane (right) (1). The extraction vessel is heated to 45˚C to increase the rate of extraction, while the lipid trap is heated to reflux. Upon heating, extraction begins, and the condensed solvent from the trap causes the organic phase of the extraction vessel to overflow, carrying with it extracted lipid (2). As extraction continues, lipids become concentrated in the trap while extraction continues (3). Upon completion, the delipidated slurry remains in the extraction vessel, with concentration of the lipid fraction in the trap (4).

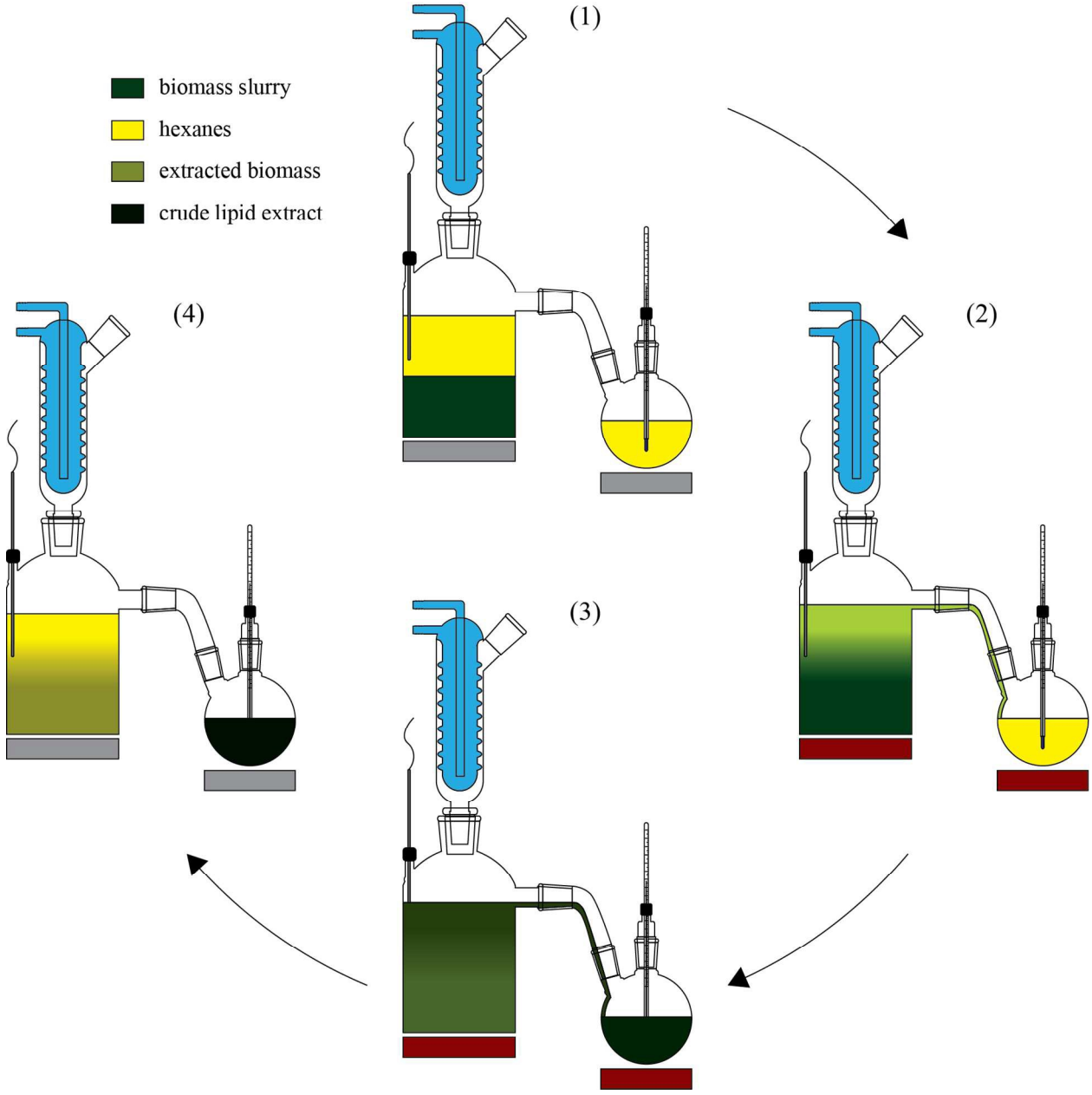

Figure 2 (full page width)

System dynamics. System capacity (A) was over an order of magnitude larger in the scaled-up lipid trap system, with the smaller 1.8 L system still providing a 10-fold increase in capacity over the analytical Bligh and Dyer method. Extraction efficiency (B) was comparable for each biomass source. Solid bars represent extraction efficiency using the lipid trap, hashed bars represent standard Bligh and Dyer extraction efficiency. Error bars represent the standard deviation of triplicate experiments. Timecourse experiments (C) revealed that extraction of *Gm*, the feedstock with the highest concentration of lipid, was over 80% complete after 10 hours, and extraction of less oleaginous feedstock (*Sd*) neared completion after 5 hours.

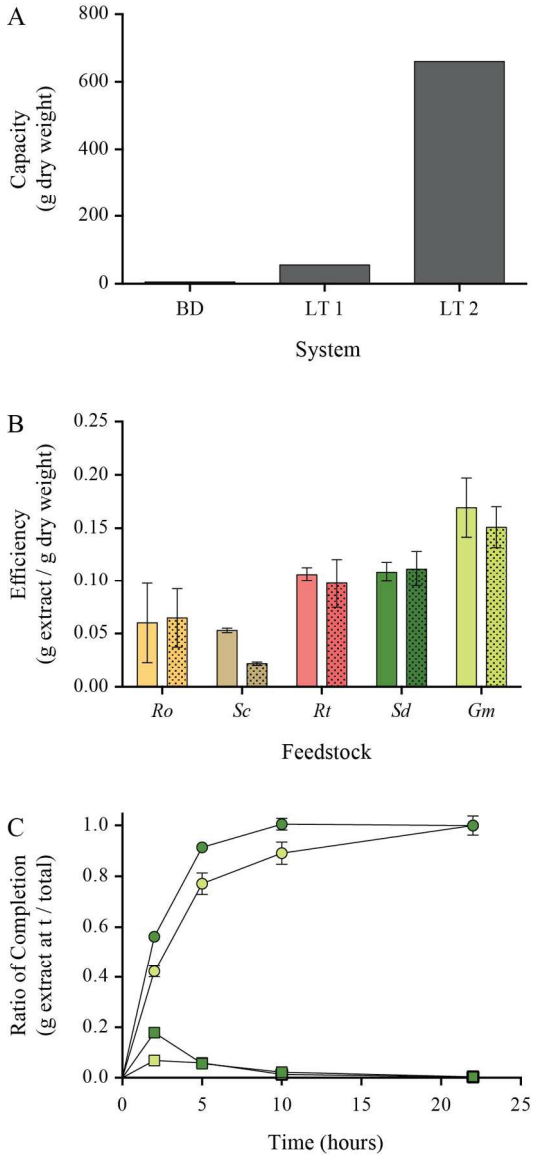

Figure 3 (full page width)

Compositional comparison using TLC and GC/MS of lipid trap (LT) extracts to Bligh and Dyer (BD) extracts. TLC of vegetable oil (VO) is shown for reference.