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Sipponen, Mika Henrikki; Pihlajaniemi, Ville; Vainio, Heidi; Essi, Palonen; Hokkanen, Sanna; Vahvaselkä, Marjatta; Pastinen, Ossi; Nyysölä, Antti; Laakso, Simo

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Published in:
Green Chemistry

DOI:
[10.1039/c5gc03008k](https://doi.org/10.1039/c5gc03008k)

Published: 01/01/2016

Document Version
Peer reviewed version

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Please cite the original version:
Sipponen, M. H., Pihlajaniemi, V., Vainio, H., Essi, P., Hokkanen, S., Vahvaselkä, M., Pastinen, O., Nyysölä, A., & Laakso, S. (2016). Integrating the opposites of biofuel production: Absorption of short-chain alcohols into oleaginous yeast cells for butanol recovery and wet-extraction of microbial oil. *Green Chemistry*, 18(9), 2775-2781. <https://doi.org/10.1039/c5gc03008k>

Article published in
Green Chemistry 18 (2016) 2775–2781

Accepted 18th January 2016

DOI: 10.1039/c5gc03008k

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Journal Name

ARTICLE

Integrating the opposites of biofuel production: absorption of short-chain alcohols into oleaginous yeast cells for butanol recovery and wet-extraction of microbial oil

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Recovery of two biotechnologically produced fuel components, butanol and microbial oil, is assessed by absorption of the six shortest 1-alcohols into oleaginous yeast cells. We show unexpectedly high extent of absorption of > C3 alcohols from water into *Rhodospiridium fluviale* cells with a lipid content of 69% of cell dry weight (CDW). Increasing carbon chain length of the alcohol boosts both the rate and the quantity of absorption of alcohol from water containing an initial ratio of 9.5 of CDW to alcohol. Under these conditions, 40% of butanol is removed from water, while methanol concentration remains unchanged in 48 h incubation with the oleaginous yeast cells. Lower absorption of alcohols into non-oleaginous baker's yeast cells as a reference suggests that a majority of the alcohols associates with the lipid droplets inside of oleaginous cells. Partition coefficient of the intracellular microbial oil to butanol exceeds those of oleyl alcohol and rapeseed oil by the factor of 4 and 16. The capacity of oleaginous yeast cells to absorb butanol reaches 13% of CDW from 48 g L⁻¹ butanol solution. Leakage of intracellular microbial oil occurs when the initial butanol concentration exceeds approximately 20 g L⁻¹. Butanol can be recovered after absorption from oleaginous yeast biomass, while microbial oil can be separated by subsequent wet-extraction with the alcohols as solvents. These results suggest that synergistic outcomes can be achieved by process integration both for the industry and the environment.

Introduction

Biotechnologically produced microbial oil and butanol have been key areas of interest for the production of biofuels.^{1,2} Besides productivity, the product recovery in either of these concepts is associated with fundamental, albeit partly contrastive problems that have obstructed commercial production. Extraction of oleaginous microorganisms and the energy-intensive distillation of butanol from dilute fermentation broths are the main threshold issues in terms of profitability.³

Intracellular lipids accumulate as an energy storage under growth-limiting conditions to comprise at least 20% of the cell

dry weight of oleaginous microorganisms,⁴ and values up to 80% have been reported for oleaginous yeasts that can grow on a broad range of carbon sources such as molasses or wood hydrolysates.^{5,6} In the recovery of microbial oil, the main challenge is the disruption of the fungal cell wall that protects cells from permeation of conventional extraction solvents. Moreover, significant content of intra- and extracellular water promote undesired formation of emulsions with common oil solvents and makes it difficult to break the thick cell walls. The problem cannot be overcome by drying the cells, and as a technical solution, wet-extraction of oleaginous microbes would be a more economical option.⁷ Partially water-soluble extraction solvents such as alcohols could be anticipated to perform better than water-immiscible solvents.

In acetone-butanol-ethanol (ABE) fermentation, the concentration of butanol should remain below the toxic threshold level of 13 g L⁻¹, which is a bottleneck for the product recovery by distillation.⁸ This toxicity results from the permeation of butanol into the microbial cells, a trait shared

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DOI: 10.1039/x0xx00000x

with shorter alcohols that have been utilized in the extraction of oleaginous microorganisms with solvent mixtures.^{5,9,10} These cell wall penetrating properties of butanol gave incentive to study interactions of alcohols and oleaginous yeast cells.

The objective of the current work is to assess feasibility of the absorptive recovery of butanol from dilute water solutions into oleaginous yeast biomass. Absorption kinetics are determined for the six shortest 1-alcohols to assess the effect of carbon chain length on the rate and extent of absorption. The data is compared to the liquid-liquid partition of the same alcohols in a cell-free biphasic oil/water mixture. The absorption capacity of the oily yeast to butanol is determined, and finally each alcohol is used separately in the wet-extraction step for the recovery of microbial oil. The target of these experiments is to provide an initial proof-of-concept for the combination of the recovery of intra- and extracellular products of aerobic microbial oil and fermentative butanol production processes.

The development of hybrid processes is an emerging field in biotechnology. For example, a synergistic combination of algal wastewater treatment with subsequent hydrothermal liquefaction has been proposed.¹¹ A recent study transferred ABE fermentation broth into a parallel bioreactor, and recovered 41–74% of the solvents from the condensate stream during aerobic cultivation of oleaginous yeast *Rhodotorula glutinis*.¹² However, the current work is the first report that addresses the interactions of alcohols and oleaginous yeast cells, and extends the scope to the recovery of microbial oil. Better understanding of these phenomena is important for further development of synergistic bioprocesses.

Experimental

Materials

The liquid chemicals used in this work were methanol (Rathburn), ethanol (Primalco), 1-propanol (LAB-Scan), 1-butanol (Merck), 1-pentanol (Merck), 1-hexanol (Fluka), and n-heptane (Riedel-de Haën), each with purity $\geq 98\%$. Additive-free rapeseed oil (Keiju, Finland), and commercial baker's yeast (Suomen Hiiva Oy, Finland) were used in partition and absorption experiments. Other chemicals were reagent-grade commercial products and were used as such.

Fed-batch bioreactor cultivation of oleaginous yeast

Rhodospiridium fluviale was grown in liquid medium containing (g L⁻¹) glucose 40, yeast extract 8, ammonium sulfate 2.5, magnesium sulfate 0.7, potassium dihydrogen phosphate 5.0, calcium chloride 0.1, and chloramphenicol 0.1. A 10% inoculum was used for cultivation in a 10 L stirred tank bioreactor (Sartorius) under conditions of 30 °C, maintaining pH 6 with 3 M sodium hydroxide, dissolved oxygen (DO) over 30% by stirring and 1 vvm aeration, and glucose concentration at 5–50 g L⁻¹. At the end of the cultivation, the biomass concentration and the lipid content were determined from the concentrated culture, which was transferred into a capped

container and stored in a refrigerated room at 6–7 °C until the absorption and extraction experiments were performed.

Determination of water/oil partition of alcohols

Partition coefficients of methanol, ethanol, propanol, butanol, pentanol, and hexanol was determined in a biphasic mixture of rapeseed oil and water (1:1, v/v). In duplicate experiments, the initial alcohol concentration in the water phase was 1–10 g L⁻¹. Glass tubes containing 4 mL of the mixtures were subjected to end-to-end agitation at 50 °C for 20 h. The tubes were centrifuged, allowed to stand at 50 °C for 1 h, and the concentration of the alcohol in the aqueous phase was determined by gas chromatography (GC).

Absorption of alcohols from water into yeast cells

Absorption of the six shortest 1-alcohols into oleaginous yeast *R. fluviale* was studied in 5 mL aqueous suspension in which initial concentrations of yeast cell dry weight (CDW) and alcohol were 37.8 g L⁻¹ and 4.16 g L⁻¹, respectively. The alcoholic yeast suspension was loaded into a 10 mL headspace vial closed with a Teflon gasket crimp cap, and subjected to 200 rpm agitation in an orbital shaker maintained at 50 °C. This temperature was chosen to avoid cell growth and metabolism during the absorption experiments. The incubation time was 2–48 h. Alcohol solutions (4.0 g L⁻¹) without yeast cells were incubated in parallel with the absorption experiments. The final alcohol concentrations were determined from the liquid phase (0.5 mL) mixed with 0.5 mL of 4 g L⁻¹ *iso*-butanol which served as an internal standard (IS) in GC analysis. As a reference, the above procedure was repeated for the yeast *Saccharomyces cerevisiae* with 24 h contact time. The absorption experiments were carried out in duplicate. The absorption phenomenon was assumed to follow first order kinetics, where the total mass flux of alcohol depends on the alcohol concentration (g L⁻¹) in water (c_{aq}) and in the absorbent (c_{abs}) according to the rate constants k_1 and k_{-1} (L h⁻¹), respectively (Eq. 1).

$$\frac{dn_{aq}}{dt} = -k_1 c_{aq} + k_{-1} c_{abs} = k_1 \frac{n_{aq}}{V_{aq}} + k_{-1} \frac{n_{tot} - n_{aq}}{V_{abs}} \quad (1)$$

The ratio of the rate constants is the dimensionless equilibrium constant of absorption, which should be equal to the observed partition of the alcohol between water and total cell dry weight (Eq. 2).

$$K = \frac{k_1}{k_{-1}} = \frac{c_{abs,eq}}{c_{aq,eq}} \quad (2)$$

Wet-extraction of oleaginous yeast with alcohols

The six shortest 1-alcohols were used as solvents in wet-extraction of oleaginous *R. fluviale* yeast cells. The aqueous cell suspension (1 mL, 189 mg CDW), harvested from the fed-batch cultivation, was extracted with 5 mL of methanol, ethanol, propanol, butanol, pentanol, or hexanol in a glass tube with a screw cap and a Teflon gasket. The tube was

subjected to end-to-end agitation for 2 h at 25 °C or 80 °C. The clear organic phase (5 mL) was transferred into a new glass tube and evaporated to dryness under nitrogen flow at 50 °C in a TurboVap LV Evaporator (Zymark, USA). Additionally, 1 mL of diluted cell suspension that contained 37.8 mg CDW was extracted in the presence of nonadecanoic acid methyl ester with 5 mL of heptane-butanol extraction solvent mixtures, where volume fraction of butanol was 0, 0.2, 0.4, 0.6, 0.8, or 1.0. The extraction time in free-fall agitation was 30 min at room temperature and in duplicate experiments.

Analytical methods

Total fatty acid (FA) analysis of freeze-dried yeast or microbial oil-containing extracts was carried out as previously described.¹³ In this procedure, the fatty acids were saponified with 3.7 M NaOH in 49% (v/v) methanol at 100 °C for 30 min and then methylated with 3.3 M HCl in 48% (v/v) methanol at 80 °C for 10 min. Extraction of the aqueous suspension with hexane/methyl-tert-butyl ether (1:1, v/v) isolated methyl esters in the organic phase, which was washed with aqueous alkali. Nonadecanoic acid methyl ester (Sigma) was added to the samples as an internal standard. Analysis of the fatty acid methyl esters was performed by a Hewlett-Packard model 6890 gas chromatograph using an HP-FFAP column (25 m, 0.2 mm i.d., 0.33 µm film thickness) with a flame ionization detector (FID). The column temperature was programmed from 70 °C to 200 °C at a rate of 25 °C min⁻¹ and the total run time was 30 min. The individual fatty acids were identified from their GC peak retention times relative to fatty acid methyl ester standards (Sigma).

The lipids were separated into the major classes of triacylglycerols (TAG), diacylglycerides (DAG), free fatty acids (FFAs), and phospholipids (PL) by thin-layer chromatography.¹⁴ Extraction of oleaginous yeast cells with dichloromethane/methanol (2:1) was conducted twice for 2 h in order to isolate the lipids. The extracts were combined and evaporated to dryness under nitrogen. A mixture containing known amounts of triheptadecanoin, dipentadecanoin, heptadecanoic acid, and dipentadecanoyl phosphatidylcholine (all from Sigma) was used as an internal standard. The separated lipid classes were scraped off from the thin-layer plates and used for fatty acid analysis as described above.

Methanol, ethanol, propanol, butanol, pentanol, and hexanol were analyzed by GC-FID relative to *i*-butanol as IS. The column used was a DB-WAXETR (30 m, 0.32 mm i.d., 1 µm film thickness) from Agilent Technologies, Inc. The injected sample (1 µL) was subjected to 10:1 split ratio in the inlet maintained at 200 °C. Helium was used as the carrier gas. The oven was initially maintained at 40 °C for 4 min, after which the temperature was increased to 200 °C at 30 °C/min. The FID was operated at 250 °C with hydrogen, air, and helium delivered at 45 mL/min, 300 mL/min, and 25 mL/min, respectively. Coefficients for the FID signal peak areas of methanol, ethanol, propanol, butanol, heptanol, and hexanol relative to the area of *i*-butanol at equal concentrations were 0.48, 0.68, 0.87, 0.98, 1.02, and 1.06, respectively.

Glucose and butanol were additionally analysed by high-performance liquid chromatography (HPLC). A Shodex SPO810 chromatography column maintained at 60 °C was eluted with deionized water at a flow rate of 0.7 mL/min. The column effluent was monitored using a type RID-10A refractive index detector (Shimadzu). Glucose or butanol in the injected sample (10 µL) were identified on the basis of the retention time, and quantified relative to external standard calibration.

Results and discussion

The objective of this work was to determine the critical factors for the absorption of butanol in comparison to a series of other short-chain alcohols from water using oleaginous yeast cells. These experiments were targeted to deliver information for the envisioned large-scale butanol capture process integrated to microbial oil production.

Lipid contents of oleaginous and non-oleaginous yeast cells

The oleaginous yeast cell suspension from the fed-batch cultivation was concentrated to a biomass concentration of 189 g L⁻¹ CDW, and found to have a lipid content of 69% analyzed as total FAs of CDW. The microbial oil comprised of 90.4% TAG, 3.0% diacylglycerols (DAG), 1.2% free fatty acids (FFAs), and 5.4% phospholipids (PL). Total FAs in *R. fluviale* consisted mainly of oleic acid (C18:1, 40.7%), palmitic acid (C16:0, 24.5%), and linoleic acid (C18:2, 22.2%), with lower contents of other FAs (Table 1). In contrast, the commercial baker's yeast *S. cerevisiae* contained only 4.3% of total FAs with higher relative proportion of palmitoleic acid (C16:1, 40.8%) than found in *R. fluviale* (1.7%). The FA distribution of *R. fluviale* is comparable to the values reported earlier for the oleaginous yeast *R. toruloides* at similar lipid content.¹⁵

Table 1. Lipid content (Total fatty acids as percentage of cell dry weight) and distribution of fatty acids (percentage of total FAs) in oleaginous yeast *R. fluviale* and baker's yeast *S. cerevisiae*.

Yeast	Lipid content (% of CDW)	Percentage of total FAs					
		C14:0	C16:0	C16:1	C18:0	C18:1	C18:2
<i>R. fluviale</i>	69.0 ± 0.0 ^a	tr. ^c	24.5	1.7	3.2	40.7	22.2
<i>S. cerevisiae</i>	4.3 ± 0.1 ^b	tr.	8.3	40.8	4.5	45.7	0.6

^a: mean value ± average deviation (N=2). ^b: mean value ± average deviation (N=4).

^c: tr., trace. Tetradecanoic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2).

Absorption of alcohols into oleaginous yeast

The absorption of the six shortest 1-alcohols alcohols from dilute water solution was investigated to assess oleaginous cells as absorbents for butanol. These experiments were carried out at the constant initial alcohol concentration of 4.16 g L⁻¹ to compare the effect of the carbon chain length on absorption. Either methanol, ethanol, propanol, butanol, pentanol, or hexanol was incubated with oleaginous yeast *R. fluviale* cells harvested from the fed-batch cultivation or with non-oleaginous baker's yeast (*S. cerevisiae*) as a reference. The 5 mL suspension contained 189 mg of CDW and 20 mg of

alcohol. The extent of absorption was followed by measuring the aqueous concentration of the alcohols as a function of time at 50 °C.

A notably higher absorption of butanol and the longer alcohols occurred into oleaginous *R. fluviale* compared to that of *S. cerevisiae* yeast cells (Fig. 1). The extent of absorption of butanol and hexanol was 4.2% and 9.1% as per the cell dry weight. The corresponding values of *S. cerevisiae* were 1.6% and 2.9%. These results pinpoint the positive impact of intracellular microbial oil on absorption of the alcohols, unconstrained by the yeast cell wall. The cell wall of the red yeasts including the genera *Rhodospiridium* consists of β -1,3-glucomannan among other polysaccharides, and presents a formidable barrier against permeation of some common extraction solvents.^{16,17} While the cell wall and the plasma membrane may prevent permeation of non-polar solvents, permeation of amphiphilic molecules into yeast cells occurs more readily. In fact, alcohols, such as methanol and butanol have been used for permeabilization of yeast cells to facilitate the recovery of intracellular enzymes, though the optimum concentrations in that usage were up to 100-fold higher than those in the absorption experiments of the present study.¹⁸

Figure 1. Comparison of absorbed amounts of alcohols after 24 h contact with oleaginous yeast *R. fluviale* or baker's yeast *S. cerevisiae*. The suspensions (5 mL) containing 20 mg of alcohol and 189 mg of yeast cell dry weight (CDW) were agitated at 50 °C in airtight headspace vials. The error bars indicate average deviations from the mean value (N=2).

The absorption kinetics showed faster reduction of concentration when the carbon chain length of the alcohol increased (Fig. S1). Methanol and ethanol were virtually unaffected by the oleaginous yeast cells, but the concentrations of propanol, butanol, pentanol, and hexanol decreased 11%, 40%, 65%, and 89% in 48 h. Rapid absorption occurred during the initial two hours with butanol, pentanol, and hexanol that absorbed into the oleaginous cells at rates (g alcohol/kg CDW/h) of 8.50, 18.5, and 35.6, respectively. The absorption of the alcohols was assumed to follow first order kinetics over a single barrier, which should theoretically describes a flux over a water-lipid interface or a membrane. The rapid initial removal of butanol, pentanol and hexanol from solution did not follow first order kinetics but the subsequent phases of absorption did (ESI, Figs. S2 & S3). This indicates that two separate major mechanisms contribute to absorption, and could be explained by rapid dissolution of a majority of the alcohol into the intracellular microbial oil, followed by slower absorption into less accessible sections.

Partition coefficients of the alcohols in a cell-free biphasic mixture of rapeseed oil and water were determined at 50 °C in order to assess the extent of absorption into oleaginous yeast cells at similar temperature (ESI, Fig. S4). The partition coefficient of butanol in rapeseed oil (0.82) is slightly lower than the value of 0.90 reported for methylated palm oil.¹⁹ The shorter alcohols are located clearly below and the higher alcohols above the butanol partition line. It is thus evident that among these alcohols butanol is the most mobile one in water and oil phases, which are also present in the oleaginous microbial cells. This biphasic solubility of butanol explains its known toxicity to microorganisms, which is a result of its partition into the cytoplasmic membranes and the consequential interference of cell function.²⁰ Extractive fermentations, steam stripping, and pervaporation among other techniques need further development to increase profitability of butanol production.² The target of the current research was to establish the information needed for a new absorptive recovery process of butanol from water. In order to assess effectiveness of this approach in comparison to simple liquid-liquid extraction method, the alcohol partition data was used to calculate the predicted alcohol concentrations in the intracellular oil phase of *R. fluviale* after 24 h absorption.

The oleaginous cells absorbed notably more alcohols than predicted from their o/w partition coefficients (Fig. 2). These differences cannot be solely explained by the different fatty acid composition of microbial oil compared to rapeseed oil.²¹ It should be noted that the absorbed concentrations of butanol and hexanol in the *R. fluviale* intracellular oil phase was 16.4-fold higher than suggested by the rapeseed oil partition coefficient. For comparison, the reported partition coefficient of oleyl alcohol to butanol is 3.30,¹⁹ which would still underestimate butanol absorption into intracellular microbial oil by a factor of 4. Because much lower absorption into baker's yeast was observed in Fig. 1, it appears that a majority of the absorption took place into the lipid droplets and their surrounding membranes. Absorption of the alcohols may have occurred also into other lipophilic sections such as plasma membrane and endoplasmic reticulum. Association of the alcohols with these membranes is influenced by their surface chemistry. Lipid droplets are 0.3–2.5 μ m in diameter and their membranes consist of a phospholipid monolayer also containing proteins.²² The working hypothesis is at present that the amphiphilic character of the alcohols facilitates their penetration through these polar membranes. The resulting accumulation of alcohols could disrupt the lipid droplet membranes and induce leakage of intracellular lipids. To study this hypothesis further, and in view of the desired recovery of butanol from fermentation broths, the effect of initial butanol concentration on absorption into the oleaginous yeast *R. fluviale* cells was investigated.

Figure 2. Partition of alcohols into intracellular microbial oil of the oleaginous yeast *R. fluviale*. Comparison of the values predicted from o/w partition of the alcohols to the values calculated based on the detected absorption of the alcohols into yeast cells.

The capacity of oleaginous yeast cells to remove butanol from water

A crucial property in the absorptive capture of alcohols from water is the corresponding absorption capacity of the oleaginous cells. The economical implementation of such recovery process at industrial scale should be conducted at moderate temperature. Therefore, the absorption capacity of oleaginous *R. fluviale* cells to butanol was determined at 25 °C. These experiments were carried out during 24 h at constant oleaginous yeast CDW concentration of 37.8 g L⁻¹, while the initial butanol concentration was varied. Results in Fig. 3 show that the percentage removal of butanol from water first increased to a maximum of 29% at 6 g L⁻¹ initial butanol concentration. Thereafter, the percentage removal decreased sharply to a level of 8–10% at initial butanol concentrations of 24–48 g L⁻¹. When calculated relative to the yeast CDW, the absorption capacity of the oleaginous cells showed an inverse sigmoidal curve, with a valley at 15–25 g L⁻¹ initial butanol concentrations (Fig. 3). This is an interesting concentration range because it parallels with the concentration of butanol above which few microorganisms can grow.²³ Bearing in mind that the total solvent concentration in ABE fermentation broths rarely exceeds 20 g L⁻¹, there appears to be a possibility for the synergistic combination of butanol recovery with the subsequent extraction of intracellular microbial oil. The optimum concentration of butanol would be 6–12 g L⁻¹ so that the maximum absorption is obtained without causing leakage of microbial oil from the oleaginous yeast cells. Maintaining butanol concentration below the critical 13 g L⁻¹ should be very beneficial to the viability of fermenting microorganisms. Previously, ABE fermentation has been improved by *in situ* partition of butanol into oleyl alcohol, methylated crude palm oil, polypropylene glycol, and oleyl alcohol/decane mixture, occasionally with the extractants packed in a semipermeable compartment.^{19,24,25} However, absorption of ABE solvents into semipermeable oleaginous microbial cells has not been reported so far. Recently, Cai and co-workers used aeration of a bioreactor for gas stripping of butanol admixed with yeast culture.¹² However, although the lipid content of the yeast *R. glutinis* did not exceed 24%, absorption of butanol into oleaginous biomass can explain the incomplete recovery of butanol regardless of the heavy aeration used.

Figure 3. Capture of butanol from water as a function of initial concentration in suspension containing oleaginous yeast *R. fluviale*. The percentage capture of butanol from water (black circles) and the amount of butanol absorbed per yeast cell dry weight (CDW) (white circles) are shown. Yeast suspension containing 37.8 g L⁻¹ CDW in 5 mL aqueous butanol suspension was agitated at 25 °C for 24 h. The errorbars indicate average deviations from the mean value (N=2).

One challenge of this microbial alcohol capture is the need to recover the absorbed alcohols from the cells. On the other hand, because the alcohol absorption is a passive process that involves cells cultivated close to their maximum intracellular microbial oil content, it is not required that the oleaginous cells remain alive. It was also confirmed that negligible cellular metabolism occurred with butanol present at 50 °C, since aqueous glucose concentration did not change during 48 h incubation (ESI, Fig. S5). As an initial concept validation, we achieved a complete butanol recovery by evaporation of the oleaginous yeast cells, which had been dialyzed in 7.2 g L⁻¹ butanol solution for 24 h (ESI, Table S1). Visual changes were not observed in the cells recovered after evaporation to dryness and rehydration in aqueous 0.9% (w/v) sodium chloride (ESI, Fig. S6). In the combinatory process, the oleaginous cells would be ultimately extracted with solvents after their alcohol absorption capacity has been reached. The absorbed alcohol could be recovered from the oil phase by evaporation or liquid-liquid extraction.

Wet-extraction of oleaginous yeast cells with alcohols

Alike plant oils, microbial oil separated from oleaginous biomass can be upgraded by catalytic hydrodeoxygenation to hydrocarbon fuels suitable to the current transportation infrastructure.²⁶ Hydrocarbon extraction solvents such as n-heptane are used in industrial oil isolation processes. However, the fungal cell wall and the associated water prevent direct contact of intracellular microbial oil with non-polar solvents. Absorption of butanol into oleaginous yeast might facilitate subsequent intracellular microbial oil recovery. To shed light on this, the yield of lipids from oleaginous yeast cells was first determined when the extraction solvent mixture contained varied proportions of butanol and heptane. The results revealed that pure butanol was the most effective extraction solvent, while heptane alone released only a negligible amount of lipids from the mechanically undisturbed cells (Fig. 4). The relative proportions of FAs in the butanol/heptane extracts were similar compared to the FA distribution of *R. fluviale* with respect to the total lipids (Table 1).

Figure 4. Wet-extraction of oleaginous yeast *R. fluviale* cells with butanol/heptane solvent mixtures in which volume fraction of butanol was varied. One mL of oleaginous yeast water suspension (38.9 mg CDW) was extracted with 5 mL of the solvent mixtures at room temperature (21 °C) during 30 min. Extraction yield was calculated as total FAs in the extract relative to the 69% total FA content of the oleaginous cells. The error bars indicate average deviations from the mean value (N=2). The inset figure shows the appearance of the organic phases passed through a 0.45 µm disc filter.

Finally, extraction of oleaginous yeast cells was tested with each pure alcohol separately. It turned out that the highest lipid extraction yields of 68% were obtained with methanol, ethanol, and propanol, while butanol, heptanol, and hexanol gave extracts with higher relative proportion of FAs (ESI, Fig. S7). This means that the three shortest alcohols extracted a higher proportion of compounds other than microbial oil from the yeast cells. In contrast, the lipophilic longer alcohols were more selective oil solvents that gave extracts with high content of microbial oil. Despite these differences, the FA composition of the extracts was unaffected by the extraction solvent or temperature of 25 °C or 80 °C (ESI, Table S2). One common feature among the alcohols was that the lipid extract contained red carotenoid pigments.^{27,28}

It is noteworthy that the cells were not subjected to any disruption treatment before the alcoholic extraction. Comparison of the extraction results to the literature values is not without complication since most of the procedures have been designed for analytical quantification of microbial oil. One previous work conducted extraction of oleaginous *R. toruloides* biomass with ethyl acetate after microwave- and enzyme-assisted pretreatment, and reported 97% yield of lipids.²⁹ However, this value was calculated based on gravimetric determination of the extract that can overestimate the yield due to the presence of non-lipid substances. In view of the development of a large scale extraction process the recovery and the reuse of the alcohols must be ensured. One option is to recover the alcohols from the water phase by extraction with organic solvent.^{30,31} In summary, alcohols enhance lipid separation from oleaginous yeast biomass, and this information should be taken into account when considering economical extraction solvent mixtures for oleaginous microbes.⁷

Conclusions

Absorption of a series of alcohols into yeast cells was investigated to assess the possibility of integrating the microbial oil production with the capture of butanol from

dilute water solution. This process would be implemented by two parallel bioreactor cultivations; one for the butanol fermentation and another for the production of microbial oil. In this setup, a continuous butanol recovery system would consist of a counter-current dialysis of oleaginous cells against butanol-containing cell culture. Recovery of butanol from oleaginous cells is achieved by dialysis against organic solvent or from the microbial oil-containing extract. The liquid medium at mitigated butanol concentration could be recycled into the first fermentation vessel. Promising absorption properties were found for the oleaginous yeast cells in view of this envisioned hybrid process. It turned out that oleaginous yeast cells do not consume, but instead absorb more than ten percent of their dry weight of butanol from water. Permeability of the cell wall does not limit the absorption, and the partition coefficient of oleaginous cells exceeds that of oleyl alcohol, one of the best known extraction solvents for *in situ* capture of butanol. Further research is needed to develop a continuous absorption process that utilizes oleaginous cells and allows for a synergistic product recovery and elevated fermentative butanol production at industrial scale.

Acknowledgements

The authors thank Ulla Åhman for experienced lipid analyses. Financial support from Neste Oil and the Finnish Funding Agency for Technology Promotion (Tekes) within the Microbial oil project is gratefully acknowledged.

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