Research Article

Received: 8 April 2021 Revised: 24 July 2021 Accepted article published: 29 July 2021 Published online in Wiley Online Library: 14 August 2021

(wileyonlinelibrary.com) DOI 10.1002/jctb.6871

Lipid reduction to improve clarification and filterability during primary recovery of intracellular products in yeast lysates using exogenous lipase

Sushobhan K Bandyopadhyay, Stephen A Morris, Darren N Nesbeth and Daniel G Bracewell*

Abstract

BACKGROUND: The yeast *Pichia pastoris* is a popular host organism for production of a range of biological products, several of which are intracellular. The disruption of yeast cells by homogenization also releases large quantities of lipids, which can foul the downstream membranes and chromatography matrices used for purification. This work examines lipid removal from yeast cells following homogenization by enzymatic degradation and its impact on the performance of the subsequent centrifugation and filtration.

RESULTS: Lipase treatment of cell homogenate at 37 °C for 2 h, followed by clarification using a scaled-down mimic of disc stack centrifugation, resulted in a 6.5-fold improvement in solids removal when compared to untreated feed material. The lipase-treated and untreated materials that had undergone initial centrifugation were then tested for filtration performance by passing the material through a 0.45 μm polyethylene sulfone membrane under constant flux. A 50% increase in throughput was observed in comparison to the untreated material.

CONCLUSION: These proof-of-concept data suggest enzymatic digestion of lipids, analogous to the widely performed DNA reduction using nucleases, could be a valuable process improvement strategy.

Keywords: bioprocessing; filtration; centrifugation; primary recovery

INTRODUCTION

Yeast expression systems are increasingly used to produce proteins of commercial importance, many of which are produced as an intracellular product. Consequently, the first step in purification of these products is cell lysis, often by means of high-pressure homogenization. This releases intracellular product along with other cellular material and debris. The first step in the downstream processing of this lysate is the removal of this debris by clarification. At scale this is usually accomplished by filtration. Large molecular components can cause membrane fouling and hence limit the efficiency of this process. One such component is cellular DNA. The working solution employed in many downstream processes is through incorporation of nuclease treatment to enzymatically digest large DNA molecules prior to filtration. A second, much less studied class of molecules which can cause significant fouling are cellular lipids. In this study we investigate whether an analogous approach of enzymatic digestion can be utilized to reduce membrane fouling and increase filtration efficiency.

Yeast cells contain a lipid storage compartment, referred to as a lipid particle, lipid droplet or oil body. They mainly consist of non-polar lipids and are used as building blocks for membrane lipid synthesis and are stored in a biologically inert form of fatty acids and sterol.[1] Yeast culture conditions change the accumulation of triacylglycerols (TAGs) in the cell.[2,3] A key step in this storage process is the hydrolysis of TAG to sterol esters (SEs) by lipases and hydrolases. These TAG lipases play an important role in lipid metabolism. These lipids classes have been quantified using various high-performance liquid chromatography (HPLC) techniques coupled with sensitive detection methods such as mass spectrometry.[4] Ejsing *et al*. used a shotgun approach incorporating HPLC coupled to electrospray mass spectrometry to identify and quantify as many as 250 species of lipids, which covered about 21 major lipid classes in *Saccharomyces cerevisiae*.[5] Using these

* *Correspondence to: DG Bracewell, Department of Biochemical Engineering, University College London, London WC1E 6BT, UK. E-mail: d.bracewell@ucl.ac.uk*

Department of Biochemical Engineering, University College London, London, UK

techniques, it is possible to identify the monoacetylated and diacetylated fatty acids and sophorolipids produced by yeast species.[6] Other methods using HPLC coupled with detectors such as charged aerosol detection (HPLC-CAD)[7-9] and evaporative light-scattering detectors (HPLC-ELSD)[10-12] are routinely used for the quantification of a large range of major lipid classes. For identification and quantification of lipid subclasses, more sensitive detection methods are needed, such as mass spectrometry.

Some studies have shown that lipid fouling of membranes and matrices occurs during downstream processing of intracellular products obtained from yeast host cells, and such fouling can have a major impact on downstream processing efficiency. In the case of membrane filtration, two major types of membrane filtration techniques are used in manufacturing of biological products, namely cross-flow filtration and dead-end filtration. Miller *et al*. carried out a study of constant-flux cross-flow filtration apparatus using a polyvinylidene fluoride membrane and soybean oil emulsion as the fouling agent. A major component of soybean oil is TAG, which gives an indication of the key components involved in lipid-based membrane fouling.[13,14]

Lipid fouling of the membranes and resins during downstream processing not only give rise to process-related issues such as back pressure and decreased flux rates, hence increasing the total process time, but also affect the dynamic binding capacities in chromatography resins. Being hydrophobic in nature, lipids could alter the resin surface chemistry properties or aggregate and cause pore blockage. This behaviour gives rise to inconsistent elution profiles, presenting a challenge to the reproducibility of the purification process. One of the most important product classes produced in yeast are virus-like particles (VLPs) used as vaccines.[15,16] Recombinant VLPs produced in fungal and bacterial systems have superior expression and affordability. When using a microbial system such as yeast to produce VLPs, they are produced in the endoplasmic reticulum (ER) of the cell. This ER becomes distended due to the formation of VLP subunits. This process of VLP formation and localization was described by Lünsdorf *et al*.[17] Disruption of the yeast cell is necessary to release intracellular products, and because of this localization of VLPs within cell organelles can be especially harsh. Consequently, impurities such as host cell DNA, lipids and cell organelles are also released into the process feed medium. Colloidal mixture of host cell lipid, DNA and protein impurities causes fouling of the downstream membranes and matrices. This fouling effect causes a major downstream issue during large-scale production and is found to be a rate-limiting step during primary recovery.[18,19] Lipids have been shown to reduce the binding of VLPs in hydrophobic interaction chromatography.[18] Lipid fouling can be improved by the addition of detergent to the yeast lysate before purification steps. However, the same study found that while 70% of the lipids responsible for fouling were removed the process also introduced 20% loss of VLPs.

The disruption of *Pichia pastoris*, used in this study as a model for the production of intracellular product such as VLPs,[20,21] is essential for extraction of the intracellular product; it also releases both lipid and host cell DNA impurities. Enzymatic approaches to target these DNA impurities are well established with nucleases such as Benzonase™, widely used in VLP manufacturing.[18,19,22] It has also been shown to be effective for cleaning ultrafiltration membranes.[23] Here, we study whether lipases can be used in an analogous fashion to enzymatically target lipid impurities for degradation to improve manufacturability.

In this proof-of-concept study, we examine lipid degradation using exogenous lipases for the removal of lipid impurities from yeast homogenates which are used for VLP synthesis in vaccine manufacturing processes. Commercially, lipases are used in various processes, including the production of detergents, paper, biodiesel, biopolymers, pharmaceutical derivatives and food processing.[24] Lipases are not currently used in biopharmaceutical manufacturing for the removal of lipid impurities; this contrasts with DNA impurities, which are often removed using nucleases. TAG lipases (EC 3.1.1.3) target the ester bond in TAG in the presence of water, giving rise to a diacylglycerol (DAG) and a fatty acid. Free fatty acids are soluble in aqueous medium; hence fatty acids on their own will have minimal impact on fouling of membranes in comparison to complex lipids such as TAGs. We propose that enzymatically reducing the lipid content in the feed material will improve performance downstream by reducing fouling.

To investigate this, we added a commercial lipase to *P. pastoris* homogenate for enzymatic degradation of lipids such as TAGs. This treated sample was then compared with the untreated homogenate for its filterability and throughput. Lipid was also extracted from the *P. pastoris* homogenate using the method described by Ejsing *et al*.[5] before and after treatment; we studied the reduction of TAG content using HPLC-ELSD techniques, showing the impact on filter performance. Filter fouling was further characterized using a constant flux filtration method.

MATERIALS METHODS

All chemicals, unless otherwise stated, were obtained from Sigma Chemical Co. Ltd (Poole, UK) and were of analytical grade.

Material generation and homogenization

Biomass generation was done using *P. pastoris* PPS9010 wild type strain obtained from DNA 2.0. The *P. pastoris* cells were cultivated in yeast extract peptone dextrose (YEPD) media, consisting of yeast extract (Cat. No. 103753, Merck Millipore, Watford, UK), peptone (Cat. No. DM941, Difco, BD UK) and dextrose (Cat. No. G8270, Sigma-Aldrich, UK). The cells were grown in 5 L shake flasks with a working volume of 1 L at 30 °C with 250 rpm shaking speed using a Kühner floor shaker (Kühner AG, Germany) for 50 h. The cells were harvested when the OD_{600nm} reached 60 and were centrifuged at 10 000 RCF for 30 min using a Beckman centrifuge (Beckman Coulter, USA). The cell pellet was stored frozen at −20 °C before further processing. Before use, the cells were thawed and resuspended by vortexing (10% wcw/vol) in 1× phosphate-buffered saline (PBS) made using 10× PBS tablets from Gibco (Cat. No. 18912014). The resuspended *P. pastoris* cells were homogenized at 500 bar pressure with five passes, as previously described by Bracewell *et al*.[25] The small-scale high-pressure homogenization for all the experiments was done using a Gaulin Lab 1000 homogenizer (APV Gaulin, Lubeck, Germany), which was operated at 500 bar and 4 °C.

Clarification of *P. pastoris* homogenate

The homogenized material was then clarified at multiple centrifugation speeds for 20 min at room temperature. The centrifugation speeds were in a range corresponding to operation in a large-scale continuous centrifuge, ensuring the possibility of scaling up the clarification process.

Sigma theory was applied to extrapolate to large-scale clarification conditions, using six different centrifugation speeds at laboratory scale, which corresponded to a possible flow rate range in a disc-stacked industrial-scale continuous centrifuge (pathfinder, PSC1), as shown in Table 1. In this study, the lowest flow rate,

Journal of Chemical Technology and Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

equivalent to 3.9 L h^{-1} has been used for clarification of the *P. pastoris* cell debris. The sedimentation capacity of the centrifuge was characterized in terms of an equivalent settling area (Σ_T). The centrifugation conditions were recorded in terms of volume and equivalent settling area time given by ($Vt\Sigma_T^{-1}$). The method for calculation of the laboratory-scale operating conditions (volume, time and rpm) to mimic industrial-scale centrifugation has been described elsewhere.[26] The sedimentation

Table 1. Centrifugation conditions for equivalent laboratory- to industrial-scale sedimentation performance

2 mL lab-scale centrifuge (Eppendorf 5415R; FA-45-24-11)			50 mL Lab-scale centrifuge (Eppendorf 5804R; FA-45-6-30)			Industrial-scale
$Vt\Sigma_T^{-1}$ lab	Time (min)	rpm	$Vt\Sigma_T^{-1}$ lab	Time (min)	rpm	continuous centrifuge (L h^{-1})
3.17E − 09	16	9800	3.10E − 09	20	8590	3.9
6.34E − 09	8	9800	6.20E − 09	20	6074	7.8
1.27E − 08	4	9800	1.24E − 08	20	4295	15.5
2.54E − 08	2	9800	2.58E − 08	20	2976	31.0
5.29E − 08	1	9800	5.17E − 08	20	2104	64.7
1.12E − 07	1	6600	1.07E − 07	5	2976	136.9

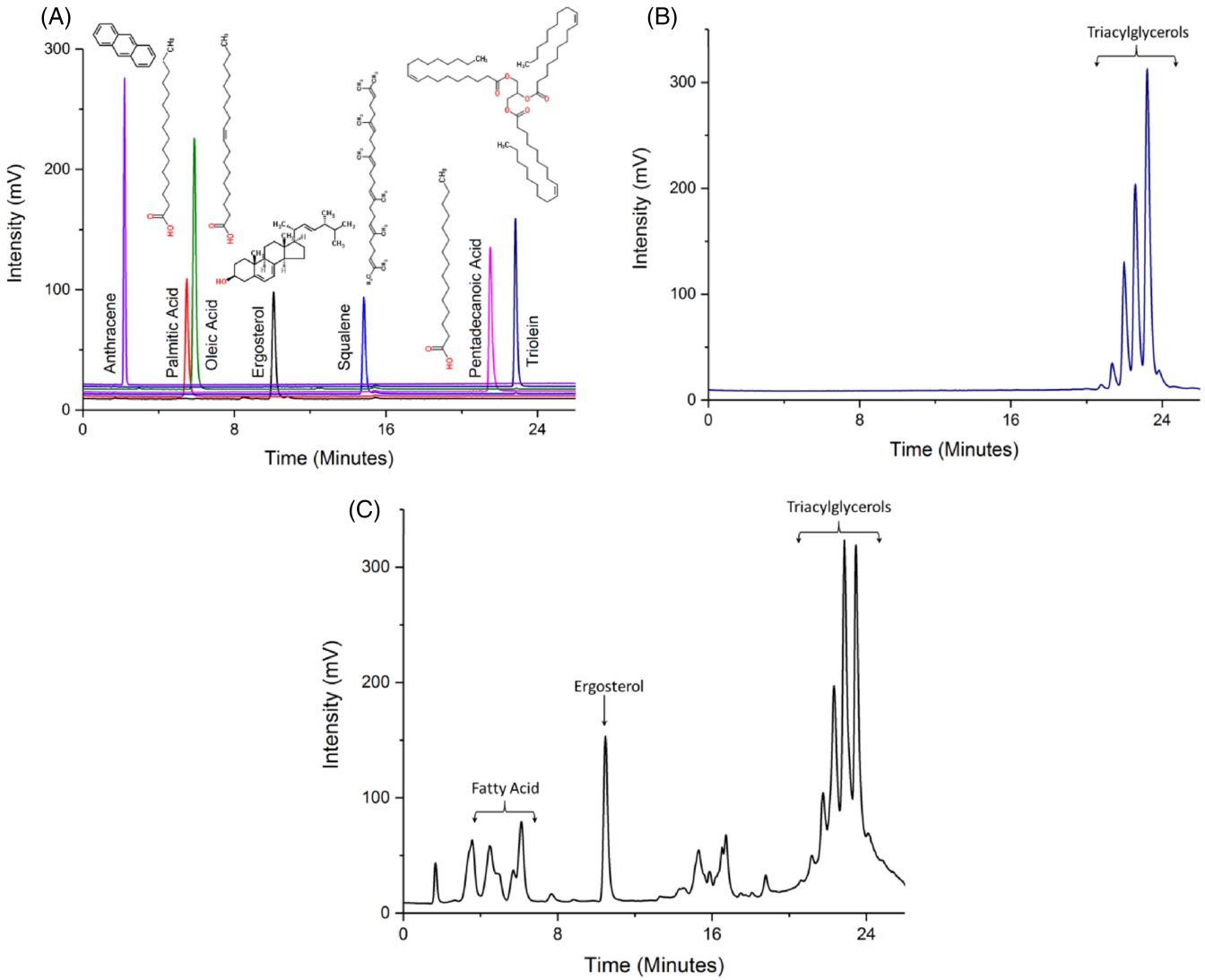

Figure 1. TAG profiles obtained from the HPLC analysis. (A) Overlay of standards (*y*-axis) analysed using RP-HPLC-ELSD. The commercial standards were run at 0.5 mg mL^{-1} concentration. (B) Triacylglyceride mixture from rapeseed oil eluted from the column near the same retention time as triolein. (C) A typical yeast homogenate where some peaks are identified as three major groups of lipids, fatty acid, ergosterol and TAGs. In each case lipids were eluted using a linear gradient at a flow rate of 0.34 mL min^{-1} and analysed using an ELSD detector. Identification was done based on comparison of retention time with the standards analysed using the same HPLC method.

Journal of Chemical Technology and Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

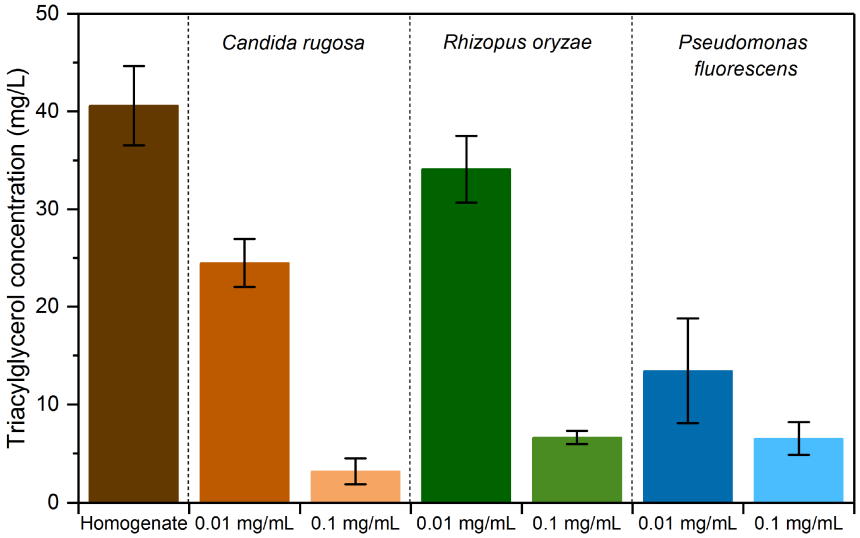

Figure 2. Concentration of TAG in *P. pastoris* homogenate after lipase treatment. *Pichia pastoris* homogenate was treated with lipases from different fungal and bacterial species to measure lipid degradation in yeast homogenate. The samples were incubated with 0.1 and 0.01 mg mL^{-1} with respective enzymes. The lipids from the samples were extracted and quantified using an HPLC-ELSD system. The error bars represent standard deviation for each data point (SD; $n = 3$). The sample treated with lipases from *A. niger*, *C. antarctica* and *Burkholderia* sp. did not show degradation of the TAG and are not included in this graph.

velocity distribution has been studied and modelled for industrial-scale applications by Clarkson *et al.* [27] The supernatants were collected and the pellets discarded. For all small-scale experiments, 2 mL sample was centrifuged using an Eppendorf 5415R centrifuge with FA-45-24-11 rotor using 2 mL centrifuge Eppendorf Safe-Lock tubes (Cat. No. 0030120094). For all 50 mL samples, centrifugation was done using an Eppendorf 5804R centrifuge fitted with an FA-45-6-30 rotor using 50 mL centrifuge tubes (Cat. No. 525-0155, SuperClear®, ultra high performance).

Lipase treatment

TAG lipases (EC 3.1.1.3) from *Aspergillus niger* (Cat. No. 62301), *Candida antarctica* (Cat. No. 02569), *Burkholderia* sp. (Cat. No. 75577), *Rhizopus oryzae* (Cat. No. 62305), *Pseudomonas fluorescens* (Cat. No. 28602) and *Candida rugosa* (Cat. No. L1754) were used. A stock solution of each lipase enzyme was made up at a concentration of 10 mg mL^{-1} in 1× PBS. Lipase treatment was done by addition of lipase enzyme to *P. pastoris* homogenate followed by incubation for 2 h at 37 °C under 180 rpm orbital mixing. For the filtration experiments *C. rugosa* lipase at 0.1 mg mL^{-1} concentration was used and compared with untreated (but still incubated under the same conditions) homogenate.

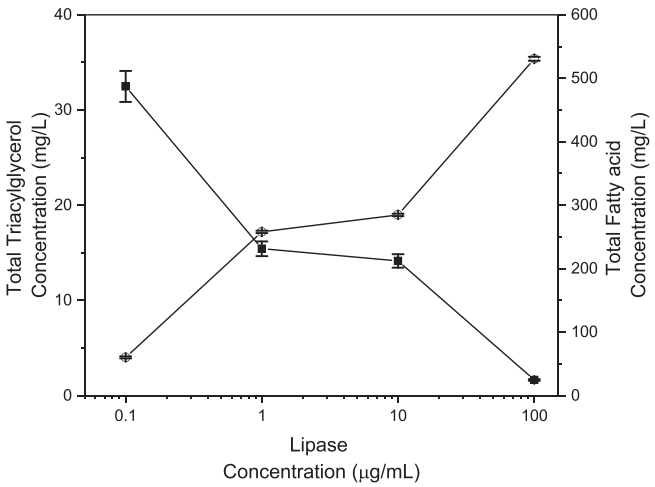

Figure 3. Degradation of TAG in *P. pastoris* homogenate when treated with *C. rugosa* TAG lipase. Yeast homogenate was treated with *C. rugosa* lipase (0.1–0.0001 mg mL^{-1}). The degradation of TAG and production of fatty acid were analysed using HPLC-ELSD. The total fatty acids (product) concentration increases with increasing concentration of enzyme. The error bars represent standard deviation for each data point (SD; $n = 3$).

Pichia pastoris *lipid analysis using HPLC*

For the quantification of lipids, HPLC-ELSD methods were used. Lipid was extracted from clarified homogenate of *P. pastoris* cells using the Bligh and Dyer method.[5,28] The supernatant was mixed with solvent in a ratio of 1:3 (17:1, chloroform:methanol, v/v) (850 mL (1266.5 g) chloroform with 50 mL (39.6 g) methanol)[5] in glass test tubes. The mixture was then vortexed using a vortex mixer for 5–10 min at 1800 rpm. The vortexed mixer was mixed with equal volumes of water. This was then centrifuged at 5000 × g for 5 min at room temperature. Two phases were generated due to the density difference between the aqueous phase and the alcohol phase. The lower layer was pipetted into a fresh glass tube and dried using a vacuum centrifuge (GeneVac, UK). The dried samples were redissolved in 200 µL solvent (1:2, chloroform:methanol, v/v) and 2 µL of this sample was injected into the HPLC-ELSD system for analysis. The lipid sample was analysed using a ZORBAX Stable Bond 300 C8, 300 Å, 3.0 × 100 mm, 3.5 µm HPLC column (Cat. No. 861973-306) and lipids were detected using an Agilent 1260 HPLC instrument fitted with Agilent 1200 Infinity series ELSD detector. The lipids were separated based on their polarity. The method for analysis was developed in house and was modified from a method described in the literature[29,30] for vegetable oil. The sample was injected and separated with a linear gradient having a decreasing concentration of methanol. The linear gradient conditions were set with buffer A (methanol–acetic acid–water; 750:250:4) and

Journal of Chemical Technology and Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

buffer B (acetonitrile–methanol–acetic acid–isopropyl alcohol; 500:375:4:122, v/v) at 5–70% in 14 min, 70–95% in the next 6 min, held for 3 min, and 95–5% B in 3 min. The ELSD settings were set with nebulizer at 50 °C, evaporator at 27 °C and gas at 1.4 SLM. The flow rate was kept at 0.34 mL min^{-1} throughout the run, for a total run time of 24 min. The profiles obtained from different classes of lipids and extracted lipids from *P. pastoris* homogenate were used to confirm identity of lipids derived from treated and untreated *P. pastoris* samples.

Dead-end filtration setup

To measure the degree of preliminary filter fouling and impact of lipase on fouling of clarified (centrifuged at 600 × g for 10 min), *P. pastoris* homogenate samples (with and without lipase treatment) were passed through a dead-end syringe filter using a syringe pump. Each run was done using a fresh membrane filter. *Pichia pastoris* homogenate samples were loaded on to the 10 mL syringe, which was then fitted to a syringe pump. Following this, a syringe pump was attached with PEEK tubing to a disc membrane filter holder. The connection between the disc membrane filter and the syringe pump was done using a T-junction. An absolute board-mounted pressure sensor (0–500 psi straight O-ring interface; Honeywell Cat. No. 40PC100G) was attached using a T-junction at the feed side of the filter. In the next step, the pressure sensor was attached to a digital recorder with the help of a data acquisition (DAQ) system. Obtained values from the DAQ system were calculated as the difference in pressure per unit time. The mass of filtrate passing through the filter was then measured using a calibrated weight balance. Filtration was performed at a constant flux. The feed pressure was measured using the absolute pressure sensor, and difference in pressure was calculated with retentate pressure assumed to be equal to atmospheric pressure. Feed samples were passed through a 0.45 µm polyethersulfone (PES) disc membrane fitted to a reusable polycarbonate syringe filter holder (25 mm; Cat. No. 16517-E, Sartorius) with an effective filtration area of 3 cm^2. The cut-off for the pressure build-up due to filter fouling was kept at 3 bar maximum. The increased pressure and total volume filtered were then used for the calculation of percentage throughput.

RESULTS AND DISCUSSION

The selection of a lipase for lipid degradation in yeast homogenate was based upon screening the catalytic activity of six candidate enzymes. Common commercially available TAG lipases, from *A. niger*, *C. antarctica*, *Burkholderia* sp., *R. oryzae*, *Ps. fluorescens* and *C. rugosa*, were considered. The lipase activities were

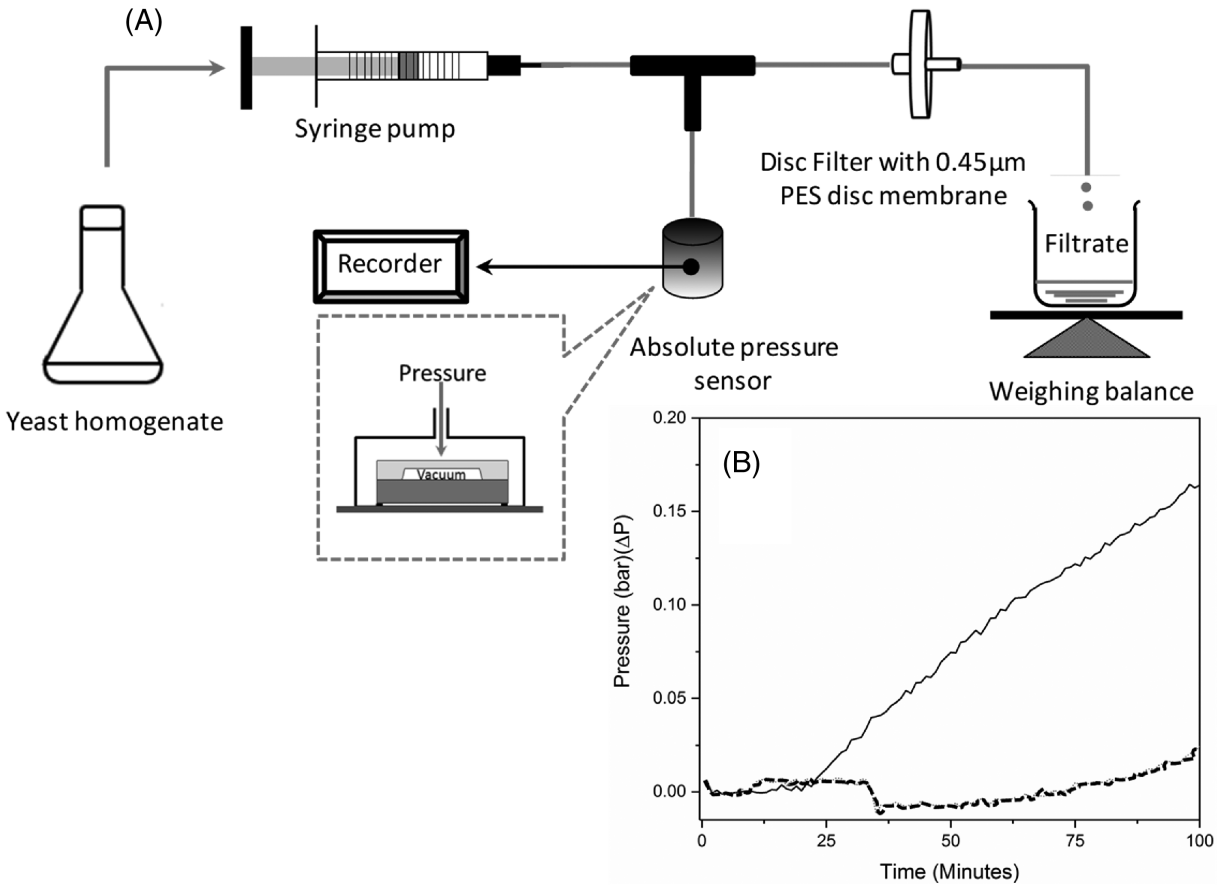

Figure 4. Schematic diagram for filtration set-up. (A) Schematic diagram of the filtration set-up using PES disc filter membrane (0.45 µm) to filter lipase-treated and untreated *P. pastoris* homogenate. 10 mL homogenate sample was pumped into the filter housing at 10 LMH (0.05 mL min^{-1}) using a syringe pump. A dead-end pressure sensor recorded the pressure readings using a DAQ system. The volume of filtrate was measured using a digital weighing balance. (B) The graph shows the inlet pressure reading obtained from the absolute pressure sensor. Pressure increases (solid line) when clarified untreated yeast homogenate was applied, whereas the clarified and enzyme-treated yeast homogenate shows marginal increase in pressure (broken line).

Journal of Chemical Technology and Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

measured in crude yeast homogenate. The selected lipase was then taken forward for comparative filter performance analysis between the enzyme-treated and control yeast homogenate feed materials.

Quantification of yeast homogenate lipids using HPLC

Lipid degradation was quantified using HPLC analysis.[29,30] For this analysis, lipids were first extracted using chloroform–methanol (17:1, v/v), for which the expected recovery measured using lipid

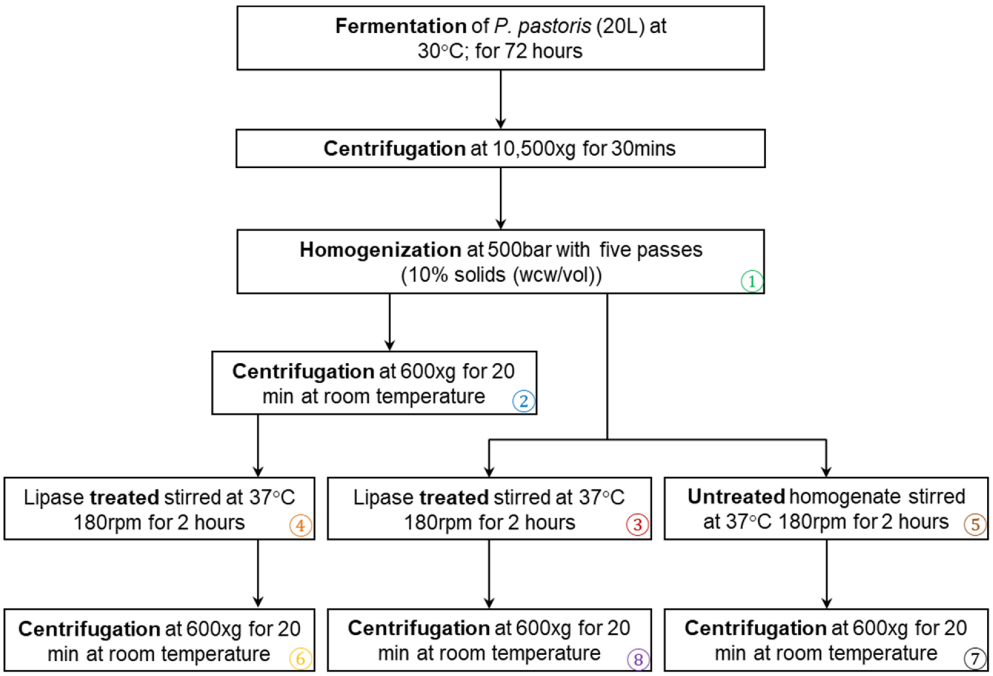

Figure 5. *Pichia pastoris* homogenate sample preparation for filter fouling test. The flow chart shows the sample preparation procedure before yeast homogenate was applied to dead-end filtration using a 0.45 μm PES disc membrane filter at 100 LMH. The lipase-treated and untreated samples were clarified before testing the filter by centrifugation at 600 × *g* (comparable to pathfinder continuous centrifuge at 60 L h^{-1}) for 20 min at room temperature. The five samples that were analysed are ① yeast homogenate, ② clarified homogenate, ③ *P. pastoris* homogenate treated with lipase, ④ clarified homogenate treated with lipase and ⑥ clarified homogenate treated with lipase and clarified again. Samples ⑤ *P. pastoris* homogenate untreated with lipase, ⑦ yeast homogenate untreated with lipase and ⑧ lipase-treated and clarified yeast homogenate where not analysed as they were difficult to filter, and the filter clogged quickly.

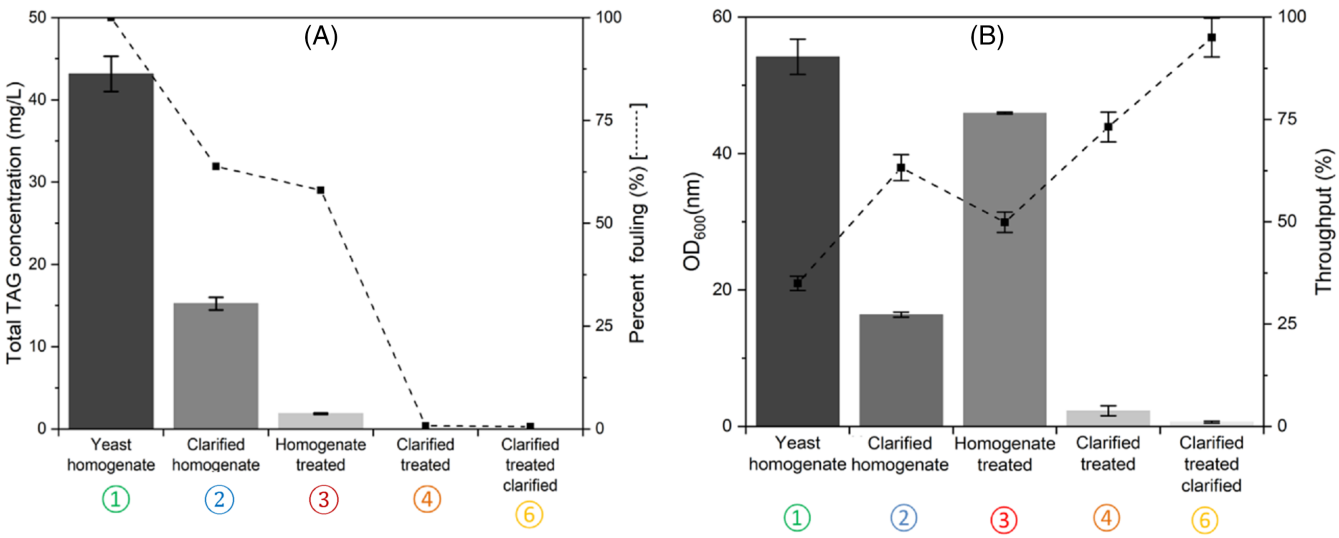

Figure 6. Lipase treatment on clarified and non-clarified *P. pastoris* homogenate. The coloured numbers in the graph are given as a reference to Fig. 5. (A) Lipase treatment was done on yeast homogenate and was analysed for total TAG. The percentage fouling (solid square connected with dash) occurred during dead-end filtration. Yeast homogenate shows maximum concentration of triacylglycerol (TAG). The yeast homogenate was centrifuged at 600 × *g* for 20 min and is denoted as clarified homogenate. Samples treated with *C. rugosa* lipase (0.1 mg mL^{-1}) before and after clarification showed reduced percentage fouling along with reduced TAG. The error bars represent standard deviation for each data point (SD; $n = 3$). (B) The samples were tested with solids remaining (OD_{600nm}) and corresponding relative percentage throughput (solid square connected with dash) obtained after filtration of the samples. Homogenate treated (not clarified) was tested, which gave the same amount of total TAG and percentage fouling and showed very little change in OD as well as percentage throughput obtained after filtration. The error bars on percentage throughput represent 5% standard error taken as for each data point ($n = 2$).

Journal of Chemical Technology and Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

standards was up to 80–99% of relative apolar lipids classes including TAG.[5] This method was selected for its high recovery of TAG lipid classes. The extracted and resuspended lipids were analysed using reverse-phase (RP)-HPLC and an ELSD detector. The TAGs and fatty acids were quantified using the total peak area in the corresponding regions of the chromatogram (see Fig. 1(C)) using appropriate standards (e.g., triolein; see Fig. 1(a,b)). Not all the peaks obtained from the crude yeast homogenate could be identified using this method. To separate and identify over 100 species[5] – all the classes of lipids from the crude homogenate – a detection method with higher sensitivity would be needed, such as two-dimensional or other techniques that can separate very closely related species.

Screening lipases for use in clarified yeast homogenate

To study the impact of different lipases (from *A. niger*, *C. antarctica*, *Burkholderia* sp., *R. oryzae*, *Ps. fluorescens* and *C. rugosa*) on *P. pastoris* host cell lipids present in the feed homogenate, their degradation was measured following incubation with the different enzymes.

Pichia pastoris cells were first homogenized using a Gaulin homogenizer (LAB 1000) with five discrete passes using a method previously described by Jin *et al.*[18] Enzymes were added to the homogenate, which was incubated and then analysed for host cell lipid content using RP-HPLC before and after subsequent clarification. Enzyme activities were compared at three different concentrations in the homogenate: 0.1, 0.01 and 0.001 mg mL^{-1}. The experiments were performed at pH 7. It should be noted that lipases endogenous to *P. pastoris* were considered as a part of the control sample, which showed little to no endogenous lipase activity. Hence the impact of endogenous lipase, if present, was negligible.

The enzymes having the highest activity, shown by the reduction in lipids from clarified yeast homogenate, were from the

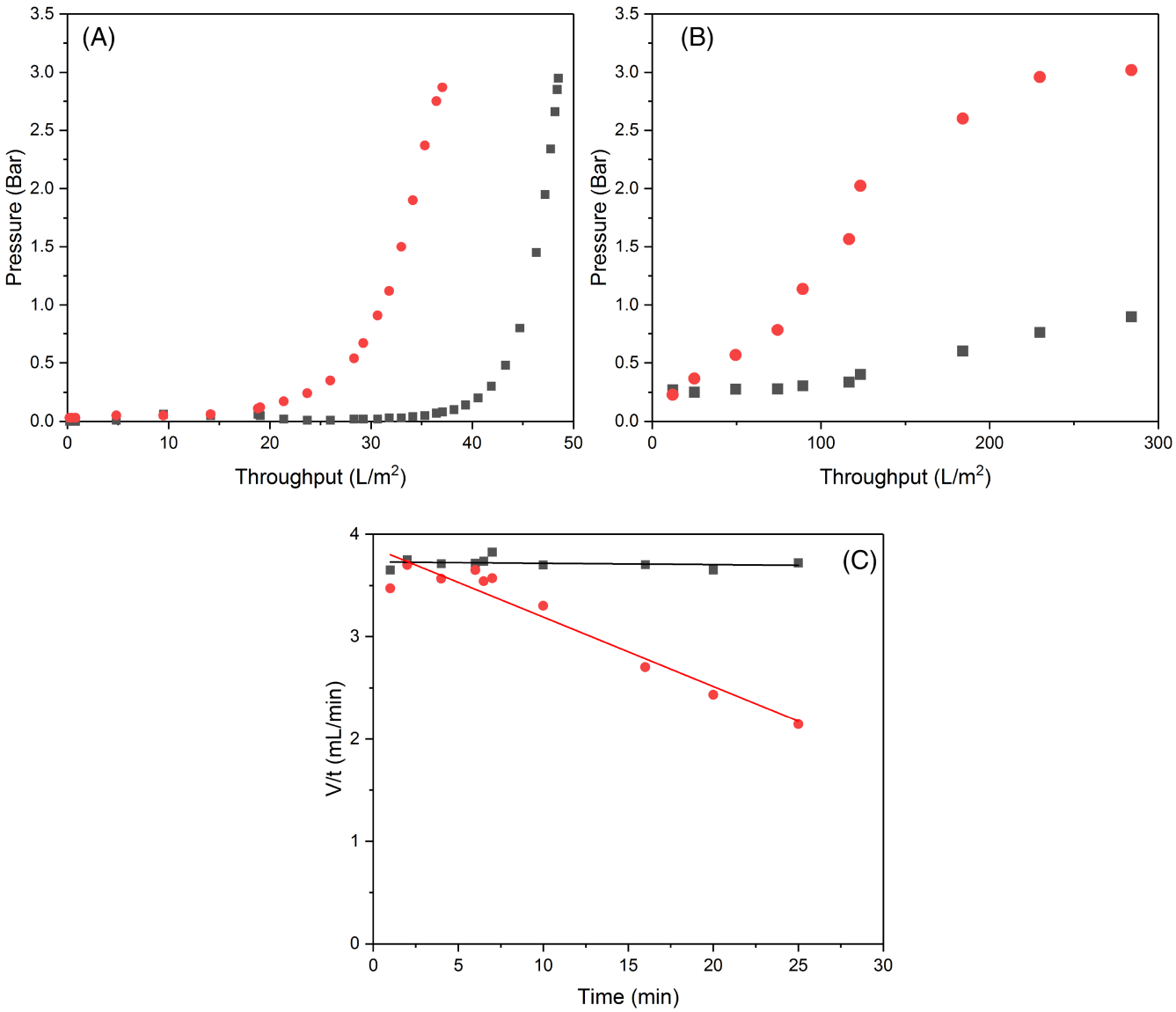

Figure 7. Dead-end filtration of clarified treated and untreated *P. pastoris* homogenate. (A) The pressure rise obtained when untreated homogenate (red solid circles) and clarified lipase-treated homogenate (black solid squares) were passed through a 0.45 μm PES dead-end filter. This is plotted against throughput obtained with single-step clarification. (B) The yeast homogenate is clarified again after treatment and shows an increase in throughput. (C) Data obtained from constant flow rate of 800 L m^2 h^{-1} is linearized by a standard fitting model.

Journal of Chemical Technology and Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

fungal species *C. rugosa* and *R. oryzae*, and the bacterial species *P. fluorescens*, as shown in Fig. 2. At the higher *C. rugosa* lipase concentration (0.1 mg mL^{-1}) the total TAG was reduced from 40.5 to 3.2 mg L^{-1} – a higher reduction than with any of the other tested lipases. The formation of fatty acids from the *P. pastoris* homogenate treated with *C. rugosa* lipase was also quantified, using the HPLC-ELSD method, as shown in Fig. 3. The lipase from *C. rugosa* lipase at 0.1 mg mL^{-1} degrades about 92% of the TAG from the *P. pastoris* homogenate. The sample treated with lipases from *A. niger*, *C. antarctica* and *Burkholderia* sp. showed little or no detectable degradation of TAG under the given reaction conditions, in comparison to *C. rugosa* lipase.

Impact of lipase on filter fouling

The filter performance of the clarified lipase-treated and untreated samples was measured by passing the samples through a single-layer dead-end filter (as shown in Fig. 4). The set-up represents a commonly used system for analysis of filter fouling during constant-flux dead-end microfiltration.[31] PES is a commonly used filtration medium and has been used elsewhere to show the fouling mechanism during filtration of material containing lipids, such as palm oil.[32] Considering the high lipid content in the yeast homogenate feed material, PES membrane was chosen for the filtration at low flux of 10 L m^2 h^{-1}. The lower flux rate is chosen to generate data needed to study filter fouling. Three technical repeats of the experiment were conducted, with similar results. The treated and untreated samples were both clarified using centrifugation (Fig. 5, steps ①, ②, ④ and ⑥, or steps ①, ②, ⑤ and ⑦). The effect of temperature and the enzyme was hard to separate as the effect of temperature would mask the effect of lipase treatment. The untreated sample was therefore also incubated under the same conditions as the lipase-treated samples. The treated and clarified samples showed reduced fouling and increased throughput during filtration. However, this effect cannot be completely attributed to the degradation of lipids due to the action of the enzyme. The filter used in this study was a 0.45 µm PES disc membrane. The open pore side was positioned towards the feed side and cut-off pressure was kept at 44 PSI (3.03 bar). It was found that the homogenate at higher concentration would foul the membrane before quantifiable data could be obtained. The pressure rise in the untreated and clarified homogenate was less than the homogenate sample that was not clarified. The *C. rugosa* lipase (0.1 mg mL^{-1}) treated and clarified homogenate was found to have much less pressure build-up, indicating clearer feed. This shows a positive impact on clarification on the yeast homogenate after the treatment of lipase (Fig. 6). As previously indicated, this experiment was done at lower flow rate of 10 L m^2 h^{-1} (0.05 mL min^{-1}) .

To determine the impact of lipase treatment on filter throughput, higher flow rates (100–800 L m^2 h^{-1}) were employed, as shown in Fig. 7. Centrifuged followed by lipase-treated and untreated homogenate samples were tested. Sample preparation was done using *P. pastoris* homogenate, which was centrifuged at 10 000 × *g* for 20 min before filter fouling evaluation. Briefly, the *P. pastoris* homogenate samples were processed identically and then clarified using centrifugation at 8590 × *g* for 20 min at room temperature and treated. A second centrifugation at the same speed was done to ensure that there was no cell debris which might contribute to the filter fouling and only suspended fatty material consisting of lipids remained in the solution, along with other cellular impurities. The filtration study was done at constant flow rate for comparison of treated and untreated samples, as shown in previously reported techniques in the literature.[33,34] The pressure, along with cumulative filtrate volume, was measured. The filtrate volume was noted for different flux ranges (50–800 L m^2 h^{-1}) (data not shown) until the filter is completely blocked. The total throughput was calculated at the given volumetric flux of 800 L m^2 h^{-1}. It was found that at lower constant flux the untreated sample and treated sample showed the same filterability and percentage throughput at the end of filtration after 25 min. With higher flux rate the fouling of the membrane becomes more evident, and a lower volume of homogenate filtrate is obtained at the end of filtration. At 800 L m^2 h^{-1} the untreated sample gave 50% less filtrate compared to the treated *P. pastoris* homogenate. This suggests that at a concentration of 0.1 mg mL^{-1} lipase can be used for the removal of lipids from the *P. pastoris* homogenate and has a positive effect at higher flux rate, which can be used for improvement for downstream processing of *P. pastoris*-based lipid-rich feed materials.

CONCLUSIONS

During the processing of intracellular recombinant proteins, host cell lipid impurities play a major role in downstream process fouling. A novel methodology to address this issue based on the use of exogenous lipase is demonstrated here. This is analogous to the use of nuclease (Benzonase®), which has become an established means to target nucleic acid impurities and their negative effects on downstream processing. The addition of exogenous lipase (commercially available *C. rugosa* at a concentration of 0.1 mg mL^{-1}) to clarified *P. pastoris* homogenate resulted in 80% removal of lipid impurities. This enzymatic hydrolysis of the lipids had a significant impact on the subsequent centrifugation and filtration steps. Improvement in throughput during filtration of clarified yeast homogenate is found at a flux of 800 L m^2 h^{-1}.

These improvements in downstream performance are particularly relevant for intracellular products such as VLP-based vaccine processes – e.g., hepatitis B,[18,19] where location within intracellular membrane compartments requires harsh homogenization conditions. By addition of lipase the lipid impurities will be removed and decrease the fouling of the membranes and matrices used in subsequent purification steps. *Candida rugosa* lipase was found to be the most suitable enzyme in comparison to five other microbial lipases tested on *P. pastoris* homogenate. Although the enzyme has a positive impact on lipid removal, it should be noted that a very high overall concentration (0.1 mg mL^{-1}) of lipase is needed to give meaningful removal of the lipid impurities from a complex mixture such as yeast homogenate. The *C. rugosa* lipase used in this study costs 0.012 EUR per milligram of the powdered enzyme. Consequently, to treat 1 L of material the equivalent cost would be 1.2 EUR. As all enzymes used in the bioprocessing industry are required to be GMP-grade materials, they are relatively expensive. However, the use of lipases should be of comparable cost to that of already commonly used enzymes such as Benzonase®. The removal of impurities may in turn help in the reduction of batch-to-batch variability, which occurs due to impurities that reduce the performance of downstream matrices.

The improvement in clarification due to lipase treatment has a positive impact on the process. The maximum volume of untreated homogenate that can be filtered using a PES membrane is 453 L m^{-2}. In comparison to the treated homogenate, the same filter can be used to filter 2678 L m^{-2} – a sixfold increase in filtration capacity.

J Chem Technol Biotechnol 2021; **96**: 3166–3174 wileyonlinelibrary.com/jctb
Journal of Chemical Technology and Biotechnology published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

Our findings illustrate the concept that inclusion of a lipase treatment to yeast homogenates prior to clarification will reduce fouling of downstream processes by endogenous lipids. This can be expected to result in improved process efficiency. Such a lipase treatment could become a standard part of purification protocols for products expressed in yeast, in much the same way that a nuclease treatment is used to prevent fouling by DNA. It is noted, however, that further work will be needed before this can be translated to an industrial process scale. One challenge may be the potential for proteolytic damage to some products during 37 °C incubation. The addition of protease inhibitors will help to minimize this. A more likely solution, however, would be the use of other lipases such as psychrotrophic *Bacillus sphaericus* cold-active lipase, having lipase activity at 15 °C and pH 8.0.[35] However, as this lipase is not commercially available at the time of study, it was not considered as a screening candidate. A complementary approach is the engineering of a lipase with higher catalytic activity to reduce the incubation times needed. Both approaches could be the basis of future studies.

ACKNOWLEDGEMENTS

The authors are pleased to acknowledge financial support from the UK Engineering and Physical Sciences Research Council (EPSRC) (grant numbers EP/L015218/1 and EP/P006485/1). Sushobhan K Bandyopadhyay gratefully acknowledges the Peter Dunnill Scholarship Fund at the UCL Department of Biochemical Engineering for providing his PhD studentship.

REFERENCES

1 Grillitsch K and Daum G, Triacylglycerol lipases of the yeast. *Front Biol* **6**: 219–230 (2011).
2 Grillitsch K, Tarazona P, Klug L, Wriessnegger T, Zellnig G, Leitner E *et al.*, Isolation and characterization of the plasma membrane from the yeast *Pichia pastoris*. *Biochim Biophys Acta Biomembr* **1838**: 1889–1897 (2014).
3 Koch B, Schmidt C and Daum G, Storage lipids of yeasts: a survey of nonpolar lipid metabolism in *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Yarrowia lipolytica*. *FEMS Microbiol Rev* **38**:892–915 (2014).
4 Shui G, Guan XL, Low CP, Chua GH, Goh JSY, Yang H *et al.*, Toward one step analysis of cellular lipidomes using liquid chromatography coupled with mass spectrometry: application to *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* lipidomics. *Mol Biosyst* **6**: 1008–1017 (2010).
5 Ejsing CS, Sampaio JL, Surendranath V, Duchoslav E, Ekroos K, Klemm RW *et al.*, Global analysis of the yeast lipidome by quantitative shotgun mass spectrometry. *Proc Natl Acad Sci USA* **106**:2136–2141 (2009).
6 Ribeiro IA, Bronze MR, Castro MF and Ribeiro MHL, Optimization and correlation of HPLC-ELSD and HPLC-MS/MS methods for identification and characterization of sophorolipids. *J Chromatogr B Analyt Technol Biomed Life Sci* **899**:72–80 (2012).
7 Khoomrung S, Chumnanpuen P, Jansa-Ard S, Ståhlman M, Nookaew I, Borén J *et al.*, Rapid quantification of yeast lipid using microwave-assisted total lipid extraction and HPLC-CAD. *Anal Chem* **85**:4912–4919 (2013).
8 Plante M, Bailey B, Acworth I and Clark D, *Analysis of Lipids by HPLC-CAD*. Dionex Company, Chelmsford, MA, USA (2011).
9 Moreau RA, Lipid analysis via HPLC with a charged aerosol detector. *Lipid Technol* **21**:191–194 (2009).
10 Donot F, Cazals G, Gunata Z, Egron D, Malinge J, Strub C *et al.*, Analysis of neutral lipids from microalgae by HPLC-ELSD and APCI-MS/MS. *J Chromatogr B Analyt Technol Biomed Life Sci* **942–943**:98–106 (2013).
11 Knittelfelder OL and Kohlwein SD, Lipid extraction from yeast cells. *Cold Spring Harbor Protoc* **2017**:408–411 (2017).
12 Kobayashi N, Noel EA, Barnes A, Rosenberg J, DiRusso C, Black P *et al.*, Rapid detection and quantification of triacylglycerol by HPLC-ELSD in *Chlamydomonas reinhardtii* and *Chlorella* strains. *Lipids* **48**:1035–1049 (2013).
13 Miller DJ, Paul DR and Freeman BD, A crossflow filtration system for constant permeate flux membrane fouling characterization. *Rev Sci Instrum* **84**:035003 (2013).
14 Demonstrator H and Melinek B, Membrane filtration. *Met Finish* **100**: 74–75 (2002).
15 Josefsberg JO and Buckland B, Vaccine process technology. *Biotechnol Bioeng* **109**:1443–1460 (2012).
16 Pattenden LK, Middelberg APJ, Niebert M and Lipin DI, Towards the preparative and large-scale precision manufacture of virus-like particles. *Trends Biotechnol* **23**:523–529 (2005).
17 Lünsdorf H, Gurramkonda C, Adnan A, Khanna N and Rinas U, Virus-like particle production with yeast: ultrastructural and immunocytochemical insights into *Pichia pastoris* producing high levels of the hepatitis B surface antigen. *Microb Cell Fact* **10**:48 (2011).
18 Jin J, Chhatre S, Titchener-Hooker NJ and Bracewell DG, Evaluation of the impact of lipid fouling during the chromatographic purification of virus-like particles from *Saccharomyces cerevisiae*. *J Chem Technol Biotechnol* **85**:209–215 (2010).
19 Burden CS, Jin J, Podgornik A and Bracewell DG, A monolith purification process for virus-like particles from yeast homogenate. *J Chromatogr B Analyt Technol Biomed Life Sci* **880**:82–89 (2012).
20 Ahmad M, Hirz M, Pichler H and Schwab H, Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. *Appl Microbiol Biotechnol* **98**:5301–5317 (2014).
21 Wang M, Jiang S and Wang Y, Recent advances in the production of recombinant subunit vaccines in *Pichia pastoris*. *Bioengineered* **7**: 155–165 (2016).
22 Jin, J. Lipid foulant interactions during the chromatographic purification of virus-like particles from *Saccharomyces cerevisiae*. Doctoral thesis, University of London (2011).
23 Allie Z, Jacobs EP, Maartens A and Swart P, Enzymatic cleaning of ultrafiltration membranes fouled by abattoir effluent. *J Membr Sci* **218**: 107–116 (2003).
24 Anobom CD, Pinheiro AS, de-Andrade RA, Aguieiras ECG, Andrade GC, Moura MV *et al.*, From structure to catalysis: recent developments in the biotechnological applications of lipases. *Biomed Res Int* **2014**:1–11 (2014).
25 Bracewell DG, Boychyn M, Baldascini H, Storey SA, Bulmer M, More J *et al.*, Impact of clarification strategy on chromatographic separations: pre-processing of cell homogenates. *Biotechnol Bioeng* **100**: 941–949 (2008).
26 Chatel A, Kumpalume P and Hoare M, Ultra scale-down characterization of the impact of conditioning methods for harvested cell broths on clarification by continuous centrifugation-recovery of domain antibodies from rec *E. coli*. *Biotechnol Bioeng* **111**:913–924 (2014).
27 Clarkson AI, Lefevre P and Titchener-Hooker NJ, A study of process interactions between cell disruption and debris clarification stages in the recovery of yeast intracellular products. *Biotechnol Prog* **9**: 462–467 (1993).
28 Bligh EG and Dyer WJ, A rapid method of Total lipid extraction and purification. *Can J Biochem Physiol* **37**:911–917 (1959).
29 Bullock S, Application note SI-01231 high resolution analysis of triglycerides in vegetable oils by HPLC with ELSD. *Polymer* **TB1066**:3–4 (2010).
30 Bullock S, Application note SI-01224 sensitive polar lipid analysis by HPLC using low temperature ELSD. *Polymer* **TB1058**:2–3 (2010).
31 Sun X, Kanani DM and Ghosh R, Characterization and theoretical analysis of protein fouling of cellulose acetate membrane during constant flux dead-end microfiltration. *J Membr Sci* **320**:372–380 (2008).
32 Chellam S and Xu W, Blocking laws analysis of dead-end constant flux microfiltration of compressible cakes. *J Colloid Interface Sci* **301**:248–257 (2006).
33 Field RW, Wu D, Howell JA and Gupta BB, Critical flux concept for microfiltration fouling. *J Membr Sci* **100**:259–272 (1995).
34 Hlavacek M and Bouchet F, Constant flowrate blocking laws and an example of their application to dead-end microfiltration of protein solutions. *J Membr Sci* **82**:285–295 (1993).
35 Joseph B and Ramteke PW, Extracellular solvent stable cold-active lipase from psychrotrophic *Bacillus sphaericus* MTCC 7526: partial purification and characterization. *Ann Microbiol* **63**:363–370 (2013).

