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Impact of flocculant addition in oil recovery from multiphasic fermentations

R.M. Da Costa Basto[a], M. Jiménez[a,1], R.F. Mudde[b], L.A.M. van der Wielen[a,c,*], M.C. Cuellar[a,2]

[a] Bioprocess Engineering, Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands

[b] Transport Phenomena, Department of Chemical Engineering, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands

[c] Bernal Institute, University of Limerick, Castletroy, Limerick, Ireland

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ABSTRACT

Emulsion formation is a major concern when dealing with multiphasic fermentations. Flocculants can be used together with other demulsification techniques to improve oil recovery in multiphasic fermentations. In this paper, the impact of adding flocculants during a multiphasic fermentation with 10 wt% dodecane, to destabilize the broth emulsion, improve creaming formation and enhance oil recovery is studied. Flocculants, $CaCl_2$ and $(NH_4)_2SO_4$ were shown to be the most promising flocculants. Flocculant addition, their time of addition, and its impact on multiphasic fermentations has been evaluated by comparing fermentation performance against reference fermentations and three oil recovery methods: gravity settling, gas enhanced oil recovery and centrifugation. When adding 75 mM of $(NH_4)_2SO_4$ during fermentation, the creaming rate during gravity settling increased 3-fold and the oil recovery by gas enhanced oil recovery was 35%, without altering fermentation performance. Addition of $CaCl_2$ during fermentation resulted in 88% and 67% oil recovery for early and late addition, which is a 4 and 3-fold increase in comparison with the reference. Yet, $CaCl_2$ deviated from standard fermentation performance when added immediately after second phase addition. In conclusion, flocculant addition during multiphasic fermentation can be used to destabilize microbial emulsions and potentially improve in situ oil recovery.

1. Introduction

Multiphasic fermentations, where an organic product phase is spontaneously formed or when an organic solvent is added for product removal, have been of investigated for the production of bio-based commodity compounds (Straathof, 2014) as well as speciality products, such as sesquiterpenes (Beller et al., 2015; Straathof and Cuellar, 2019). Sesquiterpenes are hydrocarbons with the chemical formula $C_{15}H_{24}$ that are produced via the terpenoid pathway in plants and microorganisms. The recent advances in microbial engineering have promoted the production of these compounds via fermentation. Products, such as, ß-farnesene, artemisinin and squalene, are already being produced at industrial scale (Benjamin et al., 2016; Kung et al., 2018) for applications ranging from fuels and lubricants, to pharmaceuticals, flavors and fragances.

* *Corresponding author at*: Bioprocess Engineering, Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands.
E-mail address: L.A.M.vanderWielen@tudelft.nl (L.A.M. van der Wielen).
[1] Current address: Bio Base Europe Pilot Facility, Ghent, Belgium.
[2] Current address: DSM Biotechnology Center, Delft, The Netherlands.

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The hydrophobicity of the organic phase creates an opportunity for in situ product removal (ISPR) at industrial scale, by integrating fermentation with a low cost recovery method improving its productivity, avoiding product degradation and/or product toxicity, and reducing downstream costs (Cuellar and Straathof, 2018; Dafoe and Daugulis, 2014). For simplicity, we refer to all these compounds as "oils" in this work. Unfortunately, during the fermentation, stirring and surface-active components (SAC's) present in the fermentation broth often create an oil-water (O-W) emulsion, instead of coalescence to separate phases (Heeres et al., 2014). Emulsion formation can lead to fermentations with higher viscosity and less efficient stirring and, consequently, lower mass transfer. Moreover, it can hinder product recovery after fermentation (Cuellar and Straathof, 2018). This will directly affect process costs and product price. A way to overcome this problem is to promote coalescence inside the bioreactor.

Emulsion stability is dependent on different factors such as stirring, droplet size, viscosity and the type of SACs (e.g.: salts, lipopeptides, phospholipids, glycolipids, proteins, cells and cells debris). From these, proteins are known to have a large impact in droplet stabilization (Delahaije et al., 2015; Furtado et al., 2015; Heeres et al., 2015; Singh and Ye, 2020). Proteins have three types of stabilization mechanisms: surface cohesion, steric and hydrostatic repulsion, and molecular flexibility. These mechanisms cause proteins to reshape their ternary structure, in order to maximize the number of interactions with their neighboring proteins (e.g.: hydrogen bonds, electrostatic bond, etc.) (Damodaran, 2005; McClements, 2004). An increase in the number of interactions equals to a more stabilized droplet interface and, consequently, higher emulsion stability.

Demulsification techniques, such as mechanical processes (e.g.: centrifugation, membranes, hydrocyclones) (Furtado et al., 2015; Heeres et al., 2014; Nylander et al., 2019; Tabur and Dorin, 2012), chemical methods (e.g.: addition of chemical de-emulsifiers, change in temperature, change in pH, addition of flocculants) (Dickinson, 2019; Heeres et al., 2014; Li et al., 2017; Setiowati et al., 2017; Van Hamme et al., 2006), biological transformation and additives (e.g.: decrease of cell content, type of substrate and nitrogen source and culture age, enzyme addition) (Liu et al., 2016; Nadarajah et al., 2002; Rocha e Silva et al., 2017), electrical methods and microwave irradiation (Cañizares et al., 2007; Zolfaghari et al., 2016) are widely reported for uses in food, petrochemical, waste water and pharmaceutical industries (SPE International, 2015). Large scale production of bio-based products commonly use centrifugation, de-emulsifiers and temperature or pH swings to break microbial emulsions (Huang et al., 2001; Renninger and McPhee, 2010; Renninger et al., 2011; Tabur and Dorin, 2012). Yet, most of these techniques cannot be integrated into fermentation, restricting ISPR, and can compromise product purity, which will infer in further purification steps.

Low-cost and mild alternatives for oil recovery during fermentation, such as gravity separation and gas enhanced oil recovery (GEOR), have recently been proposed and successfully tested in-line with a bioreactor at laboratory (Dolman et al., 2017; Heeres et al., 2016; Pedraza-de la Cuesta et al., 2017) and at pilot scale (Steinbusch, 2019). The in-line gravity separator proposed by Dolman et al. (2017) makes use of the density difference between product and medium to create a concentrated emulsion. By adding this separator during fermentation, there was a decrease in the bioreactor volumes and in fermentation time (Dolman et al., 2017). Still, this technology requires additional steps to recover the oil as a single phase, potentially incurring into higher costs. GEOR, on the other hand, relies on the affinity between oil droplets and gas bubbles and aims to generate a single oil layer (Heeres, 2016). Previous studies showed that one of the key factors for the oil separation is the creaming (defined as the rising of oil droplets against gravity) (Damodaran, 2005). It was observed that when an emulsion had the capacity to cream, oil recovery by GEOR could be achieved. However, the oil recovery achieved during the fermentation process is generally low (10–30% oil recovery) due to small oil droplet size, back-mixing and SACs (Da Costa Basto et al., 2019; Pedraza-de la Cuesta et al., 2017). In contrast, mechanical methods combined with de-emulsifiers and temperature swings, such as centrifugation, have been reported to recover 90% of the oil in fermentation broth emulsion (Tabur and Dorin, 2012). However, the use of centrifugation is more energy intensive, more costly and cannot be incorporated into a single equipment.

The use of flocculants to destabilize protein emulsions has been widely studied in literature. If protein interactions are weakened, droplet to droplet interactions can be increased and droplets can aggregate creating the phenomena known as flocculation (McClements, 2004; Tadros, 2013). This phenomenon allows to generate droplets aggregates, which rise easier to the top, promoting oil separation but also to promote coalescence of the oil droplets into a single phase. There are numerous chemical compounds with flocculant properties. These compounds can be divided in different groups dependent on their destabilization mechanisms (e.g.: reduce steric repulsion, reduce molecular flexibility, shielding electrostatic repulsion) and flocculant capacity (Choi et al., 2003; Damodaran, 2005; Dickinson, 2010; Nylander et al., 2019). Moreover, flocculants have also been widely used throughout the years in wastewater treatment. These compounds promote bacterial cell flocculation to enhance solid/liquid (S/L) separation. Studies have been performed on using chitosan as flocculant agent in *E. coli* fermentations, however, most of them led to cell damage and death (e.g., by breaking the cell walls) (Ojima et al., 2018; Rehn et al., 2013; Yang et al., 2014).

Based on a literature review, economic potential and environmental impact of each compound, it is observed that the most promising flocculants to be used during fermentation are inorganic salts. The advantage of using salts over polymers is that some of the salts with flocculant capacity are already incorporated in fermentation medium and can be easily adjusted to enhance droplet flocculation. The disadvantage is that most of the salts can be harmful to the environment. Although literature reports that di- and trivalent ions ($CaCl_2$, $CuSO_4$, $FeCl_3$) have higher flocculant capacity than monovalent ions ($(NH_4)_2SO_4$, NaCl, KCl) (McClements, 2005), NaCl, $CaCl_2$ and $(NH_4)_2SO_4$ are the ones depicting lower cost and lower environmental hazard (Table 1). A disadvantage is that in high concentrations (>0.2 mM) (PubChem, 2019), $(NH_4)_2SO_4$ can cause eutrophication in aquatic environment. Yet, this compound is vastly used in industry and standard wastewater treatment.

In this work, the impact of adding flocculants during a sesquiterpene fermentation to destabilize the microbial emulsion, improve creaming formation and oil recovery was studied. Two flocculants, $CaCl_2$ and $(NH_4)_2SO_4$, were used. Based on literature review, preliminary tests and environmental and economic impact, two concentrations were chosen for each flocculant: 10 and 20 mM and 50, 75 mM, respectively. The use of $(NH_4)_2SO_4$ was selected, since its monovalent ion (NH_4^+)

Table 1 – Summary of flocculants agents used to destabilize protein emulsions, their cost and environmental considerations based on the Globalized Harmonized System (GHS) (United Nations, 2017).

Type of flocculants	Description	Compounds	Cost ($/MT)	Environmental impact	Tested in fermentation	Tested in protein stabilized emulsions
Salts	Ionics compounds shield the charges surrounding the proteins, minimizing electrostatic repulsions. Di- or trivalent ions have the capacity of bridging proteins due to electrostatic interactions.	NaCl	50–150[a]	No environmental impact	(Salehizadeh et al., 2000)	(Azarikia et al., 2017; Rangsansarid and Fukada, 2007; Sarkar et al., 2016)
		KCl	220[b]	Harmful to aquatic life	(Salehizadeh et al., 2000)	(Keowmaneechai and McClements, 2002)
		$CaCl_2$	300[c]	No environmental impact	(Salehizadeh et al., 2000; Stewart, 2003)	(Azarikia et al., 2017; Keowmaneechai and McClements, 2002; Sarkar et al., 2016)
		$(NH_4)_2SO_4$	130–150[d]	Harmful to aquatic life with long lasting effect due to excessive ammonia.	(Stratford, 1992)	(Moelbert et al., 2004)
		$CuSO_4$	1725[e]	Very toxic to aquatic life	(Abolhassani and Astaraie, 2010)	(Silvestre et al., 1999)
Polymers and solvents	The most common mechanisms are bridging and depletion flocculation. The first one occurs when the polymer acts as a bridge between proteins, coating the droplet. The second occurs when two droplets are in close proximity and due to the polymer between droplets, a gradient in osmotic pressure is formed. Solvents also have the ability of displacing molecules from the interphase by establishing interactions with proteins.	Arabic Gum	5085[f]	No environmental impact	–	(Dickinson, 2003)
		Xantham Gum	1100–1600[g]	No environmental impact	–	(Krstonošić et al., 2015; Ye et al., 2004)
		PEG10 000	1500[h]	No environmental impact	–	(Losso and Nakai, 2002; Syrbe et al., 1998)
		Butanol	800–900[i]	No environmental impact but highly flammable	(Furtado et al., 2015)	–

Table 1 (*Continued*)

Type of flocculants	Description	Compounds	Cost ($/MT)	Environmental impact	Tested in fermentation	Tested in protein stabilized emulsions
Cross-linking agents	Polyfunctional molecules can create intermolecular bonds between proteins of different droplets and bridging flocculation can occur. Additionally, the creation of intermolecular bonds causes severe loss in protein flexibility which causes the droplet interface to rigidize making it more sensitive to shear.	Glutaraldehyde	2480–2550[j]	Highly toxic for aquatic life	(Wumpelmann and Mollgaard, 1990)	(Park et al., 2000; Sheldon, 2007)

[a] Langfang Huinuo Fine Chemical, Co., checked at March 2019 (Alibaba).
[b] Index Mundi, commodity prices, https://www.indexmundi.com/commodities/?commodity=potassium-chloride.
[c] Intratec solution, price at February 2015, www.intratec.us/chemical-markets/calcium-chloride-price.
[d] Zouping Boyi chemical industry Co., checked at March 2019 (Alibaba).
[e] Kemcore, flotation reagents, https://www.kemcore.com/copper-sulphate-pentahydrate-96.html.
[f] Nanjing Gemsen International Co., checked March 2019 (Alibaba).
[g] FoodChem International Corporation, checked March 2019 (Alibaba).
[h] Zibo Aojin Chemical Co., checked March 2019 (Alibaba).
[i] ICIS.
[j] Purex, focus on quality chemicals, checked March 2019 (Alibaba).

is already being used in the fermentation medium (15 mM) and water treatment is already in place. The oil recovery when adding flocculants was assessed by comparing three demulsification techniques (centrifugation, gravity settling and GEOR) used in microbial fermentation emulsions.

2. Materials and methods

2.1. Materials

The impact of flocculant addition on oil recovery was tested with two mixtures: a synthetic emulsion and a fermentation broth from a sesquiterpene fermentation operating with in situ solvent extraction of the product. Synthetic emulsion (Section 2.2.1) was prepared with MilliQ water (18.2 MΩ, Millipore systems), whey protein isolate (WPI) (Bulk Powders) and hexadecane (Sigma Aldrich, Reagent Plus) colored with Oil Red O dye (Sigma Aldrich). The emulsion from fermentation broth (section 2.2.2) was prepared with dodecane (Sigma Aldrich, Reagent Grade) also colored with Oil Red O dye. Flocculant stock solutions were prepared with MilliQ water and either $CaCl_2{\cdot}2H_2O$ (Merck, Reagent Grade) or $(NH_4)_2SO_4$ (Merck, Reagent Grade).

2.2. Emulsion preparation

2.2.1. Synthetic emulsion

The synthetic emulsion, used for testing different flocculants during preliminary tests, was prepared by adding 0.18 wt.% of WPI, 10 wt% of colored hexadecane and MilliQ water to a beaker. An external coil connected to a water bath (Eco Gold E4G, Lauda) was set around the beaker to keep a constant temperature of 30 °C throughout emulsion preparation. During the first 10 min, the WPI was stirred with water using a magnetic stirrer (RET Basic C, IKA). After that, the colored hexadecane was poured and stirred for 5 min. Finally, the mixture was homogenized with an Ultra-Turrax (IKA, Ultra-Turrax T25) at 24,000 rpm.

2.2.2. Emulsion from fermentation broth

The fermentation broth used in this work was obtained from 6 fermentations performed with a recombinant, sesquiterpene producing *E. coli* strain. Pre-culture I (with 50 mL Lysogeny broth (LB) medium, 10 g L^{-1} $C_6H_{12}O_6{\cdot}H_2O$ and 0.005 g L^{-1} of Carbenicilin) was inoculated with 0.5 mL of stock culture in LB medium with 25% v/v glycerol and grown in a rotatory shaker (Certomat BS-1, Sartorius) at 30 °C and 250 rpm for 15 h. After 15 h of incubation, Pre-culture II (with 200 mL LB medium, 10 g L^{-1} $C_6H_{12}O_6{\cdot}H_2O$ and 0.005 g L^{-1} of Carbenicilin) was inoculated from Pre-culture I in a sufficient amount to reach an initial OD600 of 0.2 and grown in the rotatory shaker at 37 °C and 250 rpm during 6 h. For all fermentations, the microorganisms were cultivated aerobically at 30 °C in fed-batch mode in a medium containing glycerol as carbon source and ammonium sulphate as nitrogen source. The general fermentation protocol followed the protocol described by Pedraza-de la Cuesta et al. (2017) with an aeration rate of 1 L min^{-1} and pH 6.3. The pH was controlled by adding phosphoric acid (3 M H_3PO_4). Foam was controlled by manual addition of 10% (v/v) pluronic L-81 (Sigma Aldrich). The batch medium was formed by: 1.3 g L^{-1} $MgSO_4{\cdot}7H_2O$, 4.2 g L^{-1} KH_2PO_4, 12 g L^{-1} K_2HPO_4, 2 g L^{-1} $(NH_4)_2SO_4$, 1.7 g L^{-1} citric acid, 0.008 g L^{-1} EDTA and 30 g L^{-1} of glycerol; The feed medium was formed by: 12 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.013 g L^{-1} EDTA and 200 g L^{-1} of glycerol. The fermentation conditions and general composition at the time of harvest are summarized in Table 2.

Table 2 – Fermentation conditions of all 6 fermentations used for testing the impact of flocculant addition on the emulsion stability.

Time of harvest[a] (h)	63.0
Time of addition of dodecane[a] (h)	22
Fermenter volume (L)	2
Power input (rpm)	1200
Average power input (kW/m^3)	6.4
Cell density[b] (g_X/kg_{broth})	20
Dodecane[b] (% v/v)	14
Air inflow (L/min)	1

[a] Being time 0 the start of the batch phase.
[b] At the time of harvest.

Once the OD600 reached a value of approximately 40, usually after 4 h of the starting of the constant feeding rate, the temperature of the reactor was reduced to 25 °C. After 1 h 0.1 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) solution was added to start induction. One hour after induction, dodecane was added as second phase in the reactor to reach 15% v/v at the time of harvest. Antifoam was added in three pulses after the addition of dodecane (fermentation time: 22, 23 and 40 h) to keep the antifoam concentration constant after feed dilution.

To assess the impact of $CaCl_2$ flocculant on the maximum growth rate and cell content during batch cultivation, a set of pre-cultures (with and without flocculant) with the compositions defined above, were grown in shake flask.

2.3. Flocculant addition

After preliminary tests, the selected flocculants were: 10 and 20 mM $CaCl_2$ and 50 mM and 75 mM $(NH_4)_2SO_4$. These flocculants and concentrations showed to have the highest oil recovery by centrifugation and gravity settling at smaller scale (results not shown). The flocculants were added to 4 independent fermentations and to the synthetic emulsion (see Table 3). For the fermentation, only the highest concentration of $(NH_4)_2SO_4$ was tested since there was not a great impact in the oil recovery for 50 mM of this flocculant with synthetic emulsion (see Sections 3.2.1 and 3.2.2). Moreover, for $(NH_4)_2SO_4$ the addition of flocculant had to be done from the beginning of the fermentation since this compound was already included in the medium recipe (see Section 2.2.2). For $CaCl_2$, the flocculant was only added after dodecane addition since the main goal was to study the flocculant impact in oil recovery. The addition of flocculant to the fermentation broth was made as injection pulses at different fermentation times to correct for the dilution caused by the feed. The different methods of addition and respective concentration are presented in Table 3.

2.4. Oil recovery experiments

Oil recovery experiments were performed using the different fermentation broth as described in Section 2.2.2. Three different methods were compared: centrifugation, GEOR and gravity settling.

For the centrifugation tests, 5 mL were transferred to a glass tube and centrifuged for 15 min at 4000 rpm (Heraeus, Multifuge 1 L-R). Three samples were used for the test (Table 4).

Table 3 – Flocculant addition to the fermentation broth and synthetic emulsion and their respective concentration.

	Flocculant and method of addition	Concentration (mM)
	Fermentation	
F1	Reference fermentation[a]	–
F2	Reference fermentation[a]	–
F3	Early addition of $(NH_4)_2SO_4$ at the beginning of the batch with an initial concentration of 140 mM	75
F4	Early addition of $CaCl_2$ immediately after dodecane addition.[b]	20
F5	Late addition of $CaCl_2$ as a pulse 1 h before harvest.	10
F6	Late addition of $CaCl_2$ as a pulse 1 h before harvest.	20
	Synthetic emulsion	
SE	Reference synthetic emulsion	–
SE1	$CaCl_2$	20
SE2	$(NH_4)_2SO_4$	50

[a] With an ammonium sulphate concentration at the beginning of the batch of 58 ± 7 mM.
[b] Additional pulses were made to correct for the dilution by the feed.

Table 4 – Fermentation samples used to test enhancement of oil recovery by centrifugation.

Sample	Time of sample	Fermentation time
S1	≈16 h after dodecane addition	38 h
S2	≈21 h after dodecane addition	43 h
S3	End of fermentation	63 h

After centrifugation, pictures of every tube were taken to quantify the amount of oil released.

For GEOR and gravity settling, oil recovery was only measured at the time of harvest (end of fermentation, 63 h). GEOR experiments used a similar set-up and protocol as described by Heeres et al. (2016). Gas sparging was generated by single orifice nozzles with 0.1 mm diameter nozzle (d_{nozzle}) and a superficial gas velocity of 0.1 cm/s. The air supply pressure was set at 3 bar and monitored using a manometer. An extra manometer was added between the mass flow controller and the column to warn in case of nozzle blockage. For the 6 fermentations, 150 mL of fermentation broth was added to the glass column. To the top of the mixture, 2.2 cm of uncolored oil was added (Da Costa Basto et al., 2019). After 2 h sparging, the gas flow was stopped, the mixture was left to phase-separate for 1 h and the volume of clear oil recovered was determined.

Gravity settling experiments were performed for the 6 fermentations by transferring 150 mL of the fermentation broth to a glass column (same column as for GEOR) and let it settle for 2 h. At the end, the concentrated oil layer formed was measured and the creaming rate (CR) could be calculated. For both GEOR and gravity settling experiments pictures were taken throughout the experiment, as described in Section 2.5.1.

2.5. *Analytic tools and data processing*

2.5.1. *GEOR and gravity settling pictures*

To monitor the amount of oil recovered during experiments, pictures were taken with a camera (EOS 200D, Canon) using a fixed set-up. In all cases, flash was used to preserve brightness of the pictures. Photos were taken every 1 min for the first 10 min, every 2 min for 10 min more, every 5 min for the next 15 min and after that, every 10 min until the end of the experiment (2 h).

2.5.2. *Creaming rate and oil recovery measurements*

Oil recovery was quantified by measuring the amount of clear oil layer (i.e. oil fraction $\varphi_{oil} = 1$) formed during experiments. For each experiment, the set of pictures was analyzed using the software *Image J* to measure pixel height. The conversion from pixels to cm (CF) can be done (Eq. (1)), since both column diameter ($d_{column} = 3.6$ cm) and centrifuge tube diameter ($d_{tube} = 1.5$ cm) are known. The creaming rate (CR) was estimated by Eq. (2). For both synthetic emulsion and fermentation broth, the cream volume (V_{cream}) was measured using the first picture at which the boundary between cream and broth (or water for the synthetic emulsion) was defined and the height of cream would not change in time (see Fig. 1). The oil recovery was attained with the area of the column (A_{column}) or of the centrifuge tube (A_{tube}) and the oil volume recovered can be calculated by Eq. (3). Where n is column or tube and i can be oil or cream. The oil fraction (φ_{oil}) for cream in fermentation broth is assumed to be 0.5 while in synthetic emulsion was measured to be 0.7 (Da Costa Basto et al., 2019).

$$CF(px/cm) = \frac{d_n(px)}{d_n(cm)} \quad (1)$$

$$CR(mL/min) = \frac{V_{cream}}{t_0 - t_{min}} \quad (2)$$

$$V_i(mL) = \phi_{oil} \cdot H_i(px) \cdot \frac{1}{CF}(cm/px) \cdot A_n(cm) \quad (3)$$

The percentage of oil recovery then becomes:

$$Recovery(\%) = \frac{V_{oil,recovered}(mL)}{V_{oil,total}(mL)} \cdot 100. \quad (4)$$

In case of fermentation broth, the fraction of antifoam (AF) added during fermentation was subtracted to obtain the percentage of oil recovery (Eq. (5)) (Da Costa Basto et al., 2019).

$$Recovery(\%) = \frac{V_{oil,recovered}(mL) - V_{AF(mL)}}{V_{oil,total}(mL)} \times 100. \quad (5)$$

2.5.3. *Microscopic pictures*

Microscopic pictures were taken of samples with and without oil and flocculant. In order to do that, a small drop of 10 μL was taken from homogenous broth and observed under the

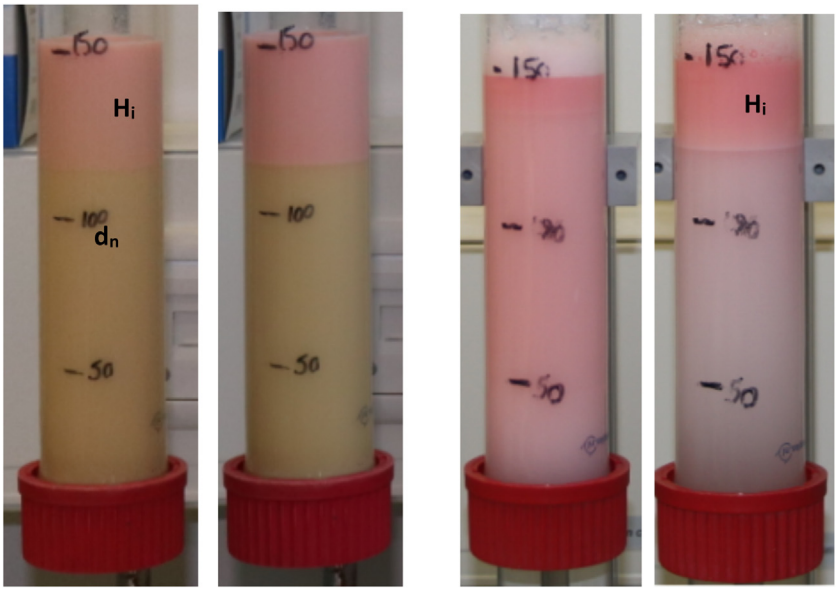

Fig. 1 – Measuring height of the creaming rate for fermentation broth (F3) and synthetic emulsion (SE) showing the boundaries and difference in cream height change.

microscope. To take the pictures, a camera with an adaptor for the microscope (Canon G12, Carl Zeiss 42126) was used.

2.5.4. *Fermentation performance*

In order to assess the effect of the flocculants in fermentation performance, two parameters were compared: biomass content (N_X) and CO_2 production during the fed-batch. The mass and carbon balances were calculated as in Pedraza-de la Cuesta et al. (2017). For F4 fermentation, the carbon balance could not be attained for lack of offline analysis.

2.5.5. *Online analyses*

The pH, dissolved oxygen (DO) and temperature were measured with online probes (Applikon). The feed rate and base addition were continuously monitored with a scale (Meter-Toledo; Sartorius). The CO_2 and O_2 concentrations in the bioreactor were analyzed by a continuous off-gas analyzer (NGA-2000 Fischer Rosemount). All sensors, off-gas analyzer and scales were connected to a control unit (Applikon) which recorded the broth parameters in a minute basis with a Multi Fermentor Control System (MFCS)/Win 2.1 Software (Sartorius Stedim Biotech S.A.).

2.5.6. *Cell dry weight*

Cell dry weight was measured by centrifuging 1.5 mL of broth at 13,000 rpm (Hearaeus, Biofuge Pico) for 5 min. Prior to centrifugation, the tubes with sample were weighted. After disposal of the supernatant, an additional washing step was made by adding 1 mL of MiliQ water and resuspending the pellet, followed by another centrifugation step as prior described. This extra washing step removed the salts from the pellet allowing a better quantification of cell dry weight. The tubes with the pellet were dried in an oven (Heraeus instruments) at 105 °C for 48 h. Afterwards the tubes containing the dry pellet were weighted. For fermentation F4 (see Table 3), the samples after flocculant addition could not be measured (see Section 3.1) and cell density at the time of harvest could not be confirmed by CDW measurements.

2.5.7. *Protein analysis*

A sample of 50 mL was centrifuged (Heraeus instruments, Stratos) at 17,000 rpm for 20 min and 4 °C. Afterwards, 25 mL of supernatant were added in a falcon tube for total nitrogen analysis (TNM-L, Shimadzu). From the same supernatant sample, 2 mL were taken to measure ammonia by a Hagh Lange Ammonium test (LCK303, Hagh Lange) in the range of 2–47 mg/L NH_4-N and following the protocol provided by the kit. The total organic nitrogen was obtained by subtracting the value of ammonia to the total nitrogen. Using a correction factor from literature (Mariotti et al., 2008) the protein concentration can then be obtained (Eq. (6)).

$$C_{prot}(mg/L) = C_{N,organic} \cdot 6.25 \quad (6)$$

2.5.8. *Statistical analysis*

The statistical significance between reference and mixture with flocculant addition, was evaluated for the synthetic emulsion and fermentation broth by a one-sample Student's t-test, where the mean of the reference fermentation was compared with a specified value of the fermentation with flocculant addition. The confidence interval was assumed to be 95%.

3. Results and discussion

3.1. *Impact of flocculants in fermentation performance*

In 5 independent fermentations, carbon balances were calculated and they closed with less than 3% gap. In F4 cell content could not be measured by the method in Section 2.5.4 due to cell flocculation in the cream. Without these data, balances could not be closed. Profiles, such as cell mass, CO_2 production (Fig. 2) and dissolved oxygen (results not shown) indicate that all fermentations, excluding F4, were comparable in terms of fermentation performance.

From the six performed fermentations, only F3 has flocculant from the beginning of the batch. For this case, the maximum growth rate (μ_{max}) at the beginning of the fed-

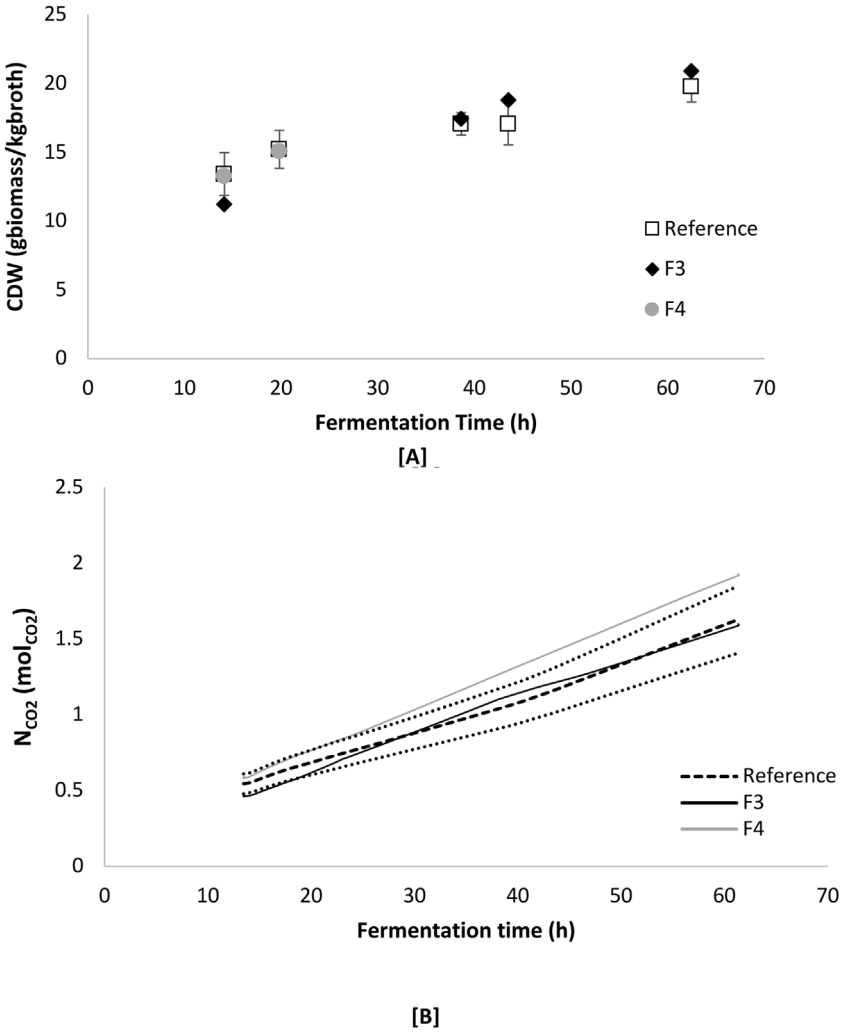

Fig. 2 – [A] Total biomass in the reactor and [B] CO_2 production for reference fermentations and fermentation with early addition of flocculants (F3 and F4). The error bars and dotted line represent the standard deviation of the reference fermentations.

Table 5 – Biomass present in the reactor at the beginning of the fed-batch (N_X (mol)) and average maximum growth (μ_{max}) between reference fermentations (F1, F2, F5 and F6) and fermentation/shake flasks with early addition of flocculants (F3 – $(NH_4)_2SO_4$ and $CaCl_2$).

Fermentation	N_X (mol)		μ_{max} (1/h)	
Reference	0.609 ± 0.028		0.442 ± 0.015	
F3	0.641[a]		0.437[a]	
Shake flasks	N_X (mol)	p-value	μ_{max} (1/h)	p-value
Reference	$0.0014 \pm 1E{-}4$	0.1	0.459 ± 0.003	0.4
$CaCl_2$ flocculant	$0.0013 \pm 0.7E{-}5$		0.462 ± 0.009	

[a] Only one fermentation performed.

batch was also tested. The results are presented in Table 5. It is observed that for F3, the addition of flocculant does not have an influence on the cell content and in the maximum growth rate of the microorganisms during batch phase. When adding $CaCl_2$, there is no statistically significant difference between the reference and the shake flask pre-culture for the biomass content (p-value = 0.1 > 0.05) and maximum growth rate (p-value = 0. 4 > 0.05) during batch phase.

The experimental profiles of F3 followed the same trend as the reference fermentations. Yet, for F4, the CO_2 production was higher than for the reference fermentation and F3. The correlation of the CO_2 profiles for F3 and F4 with the reference fermentation was assessed by calculating the correlation coefficient (R^2). For F3 and F4 the R^2 are 0.99 and 0.90, respectively. These results indicate that fermentation F4, although has a high correlation coefficient for the CO_2 profile, is influenced by the addition of flocculant, suggesting that it affected fermentation performance. This can also be observed by the increase in protein concentration (see Table 7). Still, fermentation F4 was used for oil recovery comparison with the other fermentations to better understand the impact of cell flocculation and protein concentration. $(NH_4)_2SO_4$ addition to the fermentation, at the concentrations tested, do not seem to affect its performance.

Table 6 – Creaming rate and standard deviation of fermentation broth with (F3) and without (F1 and F2) flocculant and synthetic emulsion with (SE1 and SE2) and without addition of flocculants. For fermentations where $CaCl_2$ was used (F4,F5 and F6), the creaming rates could not be measured.

Experiment	Time for cream formation (min)	Creaming rate [mL/min]	p-value
Reference	22	0.732 ± 0.07	
F3	14	2.8	–
Reference SE	60	0.509 ± 0.002	
SE1	60	0.513 ± 0.008	0.187
SE2	60	0.621 ± 0.003	0.016

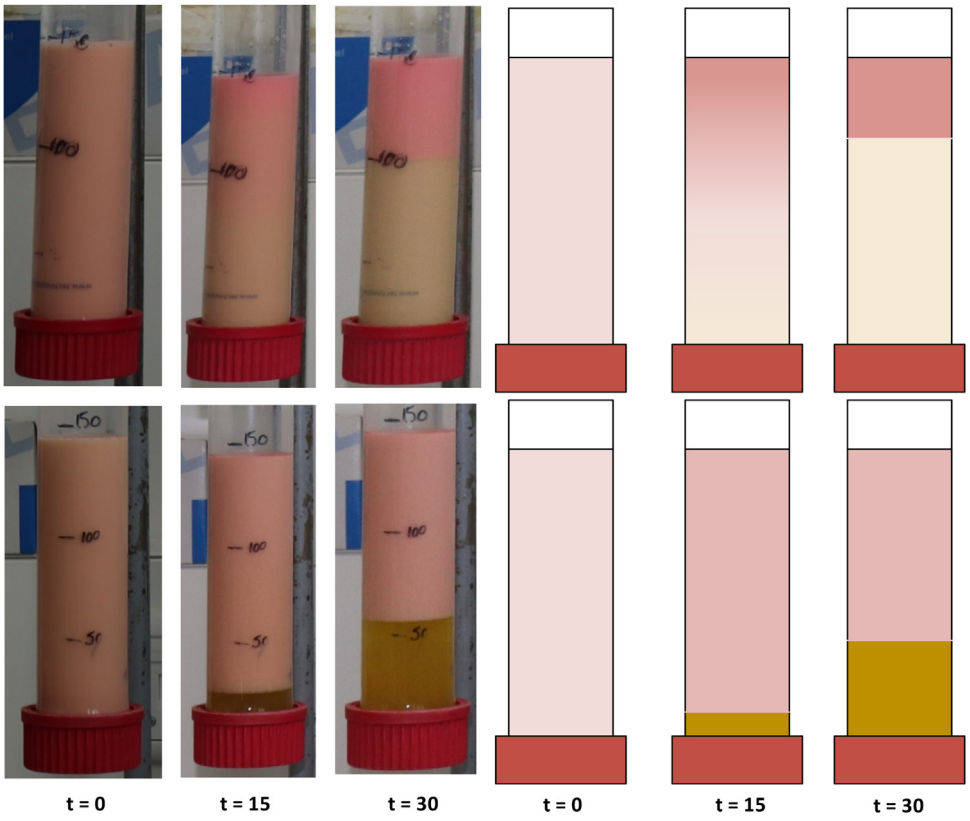

Fig. 3 – Oil recovery percentage achieved using GEOR for reference fermentation (F1 and F2), fermentation with early addition of $(NH_4)_2SO_4$ (F3), fermentation with late addition of $CaCl_2$ (F4, F5 and F6), reference synthetic emulsion (SE), and synthetic emulsion with addition of $(NH_4)_2SO_4$ (SE2).

3.2. *Enhancement of oil recovery by addition of flocculants*

3.2.1. *Impact on gravity settling*

The creaming rate for the synthetic emulsion and fermentation emulsion, with and without flocculant, is depicted in Table 6.

The synthetic emulsion containing $(NH_4)_2SO_4$ (SE2) displayed a creaming rate approximately 20% higher than the reference mimic emulsion (reference SE). In contrast, for the $CaCl_2$, the creaming rate did not change yet, the time for the cream to start being formed was almost instantaneous (1 min). When looking to the fermentation broth emulsion, it was observed that fermentation F3, where 75 mM $(NH_4)_2SO_4$ was used, displayed a creaming rate 3 times higher than the reference fermentation. Moreover, the time that it took for a cream layer to start being formed was almost doubled in the reference fermentation compared to F3. This shows the potential of using $(NH_4)_2SO_4$ to enhance oil recovery by gravity settling. For fermentation broths where $CaCl_2$ was added (F4, F5 and F6), the creaming rate could not be quantified. When adding the flocculant, there was no apparent difference between the broth inside the fermenter for the reference fermentation and the fermentation with flocculant. However, upon gravity settling, both broths behaved differently (see Fig. 3). For the reference fermentation, the cream started being formed at the top of column and increased with time. For the emulsion with $CaCl_2$, an emulsion de-watering was observed, where the cream layer also containing cells, started being formed at the bottom of the column and moved upwards. Although this change in behavior might not be beneficial for oil recovery by gravity settling, it shows that the $CaCl_2$ promotes cell flocculation.

When comparing fermentation broth emulsion with the synthetic emulsion, the creaming rate of the reference fermentation is higher than for the synthetic emulsion. This can be explained by the fact that the synthetic emulsion takes more time to form than the fermentation broth (see Fig. 1). However, in the synthetic emulsion, the only stabilizer are proteins whereas in fermentation broth there are other type of stabilizers components (Heeres et al., 2014). In the synthetic emulsion, more droplets rise to the top, taking more time to reach the final cream layer. Hence, the oil fraction in the final cream layer of the synthetic emulsion is higher.

Table 7 – Oil fraction, antifoam fraction and protein concentration for the reference fermentations and fermentation with addition of flocculant. For fermentation with late addition of flocculant (F5 and F6) the two first samples (S1 and S2) were used as reference fermentations since no flocculant was yet added. For the references fermentation the standard deviation is presented.

Fermentation	Oil fraction (g dodecane/g broth)			Anti-foam fraction (% w/w)			Protein concentration (g/l)		
Sample	S1	S2	S3	S1	S2	S3	S1	S2	S3
Reference	0.16 ± 0.006	0.16 ± 0.006	0.15 ± 0.001	0.22% ± 0.0005	0.22% ± 0.0005	0.22% ± 0.0004	4.18 ± 2.8	4.64 ± 2.9	2.67 ± 0.25
F3	0.16	0.16	0.15	0.19%	0.19%	0.18%	3.0	2.66	4.14
F4	0.15	0.15	0.14	0.26%	0.25%	0.23%	10.97	11.99	15.92
F5	Reference	Reference	0.14	Reference	Reference	0.17%	Reference	Reference	9.43
F6	Reference	Reference	0.14	Reference	Reference	0.18%	Reference	Reference	2.40

When $(NH_4)_2SO_4$ is added to the synthetic emulsion, it would decrease the electrostatic repulsion created by the proteins around the droplet and enable droplet coalescence and flocculation. However, in fermentation broth, the flocculant is not only interacting with the proteins but also with the other stabilizers around the droplets, which implies a larger change in creaming rate and time of cream formation.

3.2.2. *Impact of flocculants in oil recovery by GEOR*

The impact of flocculants in enhancing oil recovery by GEOR was assessed in the aforementioned fermentations and synthetic emulsion. The reproducibility of fermentations allows to exclude important parameters, such as oil fraction and biomass concentration, that can influence GEOR's performance and results (Heeres et al., 2016; Pedraza-de la Cuesta et al., 2017). The same cannot be held for fermentation F4. The oil recovery by GEOR for the fermentations and reference synthetic emulsion and SE2 are presented in Fig. 4.

During experiments with the synthetic emulsion, foam was produced and only for the experiments of reference SE and SE2, where $(NH_4)_2SO_4$ was added, the oil recovery could be quantified. The oil recovery doubled when adding the flocculant from 15% to 27%. This trend is even more clear in regard to the early addition of $(NH_4)_2SO_4$ in the fermentation broth (F3), where the oil recovery is three times larger than the reference fermentation, up to 35%. These results show that,

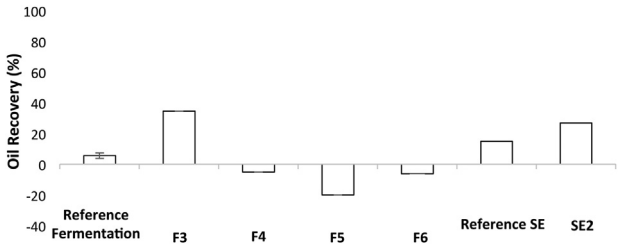

Fig. 4 – Oil on top for the different fermentations at the start of GEOR (a) and after 2 h of aeration and 1 h of gravity settling (b). From top to bottom: reference fermentation (F1), fermentation with late addition of 10 mM of $CaCl_2$ (F5) and fermentation with late addition of 20 mM $CaCl_2$ (F6).

as in the case of gravity settling, $(NH_4)_2SO_4$ has a positive impact in emulsion destabilization. When comparing these results with GEOR application in similar studies (Pedraza-de la Cuesta et al., 2017), a 3-fold higher oil recovery is reported here, showing the advantage of using flocculants for emulsion destabilization.

For the fermentations where $CaCl_2$ was added (F4, F5 and F6), the oil recovery was negative as the final oil layer measured was smaller than originally placed. This decrease can be explained by the oil back-mixing into the emulsion. Nonetheless, for fermentation F5 and F6, the color of the final layer

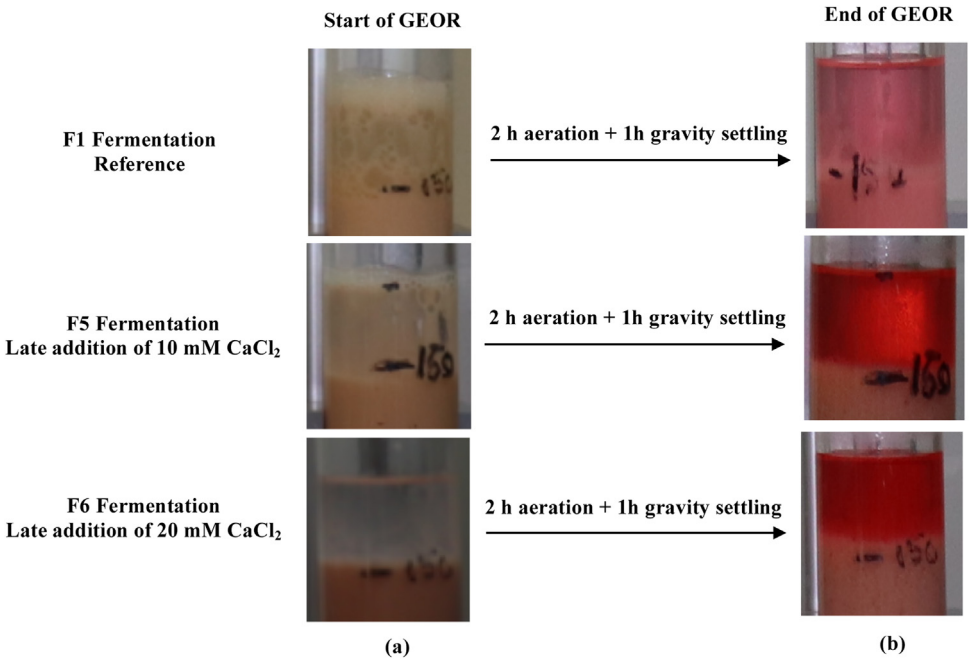

Fig. 5 – Oil recovery after centrifugation. Where the error bars represent 10% measurement error and the lines represent the standard deviation of the reference samples obtained by the independent duplicates.

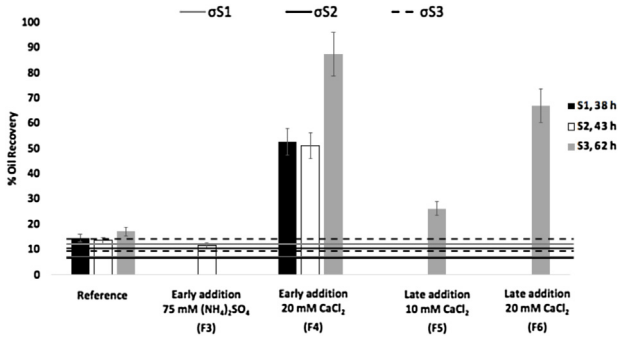

Fig. 6 – Microscopy pictures (10×) of fermentation broth with *E. coli*. (a) Sample without cream and oil with flocculant $CaCl_2$; (b) sample without cream and oil and without flocculant; (c) sample of homogeneous broth with oil and with flocculant $CaCl_2$; (d) sample of homogeneous broth with oil and without flocculant.

changed from uncolored to dark red colored (see Fig. 5. Only the emulsified oil is red colored (see Section 2.4) so it appears that demulsification did take place. Yet, clearly not good enough since recovery was negative.

From the difference in oil layer behavior together with the results shown in the previous section (see Section 3.2.1) it is possible to conclude that the addition of $CaCl_2$ creates an emulsion with different properties than the reference emulsions and emulsion with $(NH_4)_2SO_4$. The cream layer formed after aeration and gravity settling was denser and more viscous, which upon stopping the aeration led to an entrapment of the uncolored oil (fresh oil with no surfactant). This increase in apparent viscosity of the emulsion, due to $CaCl_2$ addition, has already been reported for oil-water synthetic emulsions (Azarikia et al., 2017). In order to avoid back-mixing of oil droplets, lower gas flow velocities could be used.

3.2.3. *Impact of flocculants in oil recovery by centrifugation*

Centrifugation is one of the methods most commonly used to break emulsions. However, for multiphasic fermentations this method only works together with temperature swing and demulsifiers. The shear force applied to the emulsion is such that the oil droplets separate as a clear oil layer, coarse cream ($\varphi_{oil} = 0.9$) or as a cream layer ($\varphi_{oil} = 0.5$), depending on the emulsion stability. Fig. 6 presents the clear oil layer formed after centrifugation of different fermentations with (F3, F4, F5 and F6) and without flocculant addition (reference). For the fermentation with late addition of flocculant (F5 and F6), the first two samples (S1 and S2) were used as duplos of the reference since no flocculant has yet been added to the fermenter. For fermentation F3, only one sample was used (S2) due to a technical failure. All samples were compared in terms of oil fraction, antifoam fraction and protein concentration (see Table 7).

All fermentations had the same oil fraction at each sample time. However, for fermentation F1 and F4 the antifoam fraction was higher which might have an influence in emulsion stability. Comparing F1 with the other reference fermentations, it is noticeable that at this antifoam concentration the emulsion stability does not seem to be affected (shown by the small standard deviation of the reference fermentations).

The highest oil recovery was observed for F4 (88% clear oil release), yet this cannot be fairly compared against the other cases, since the early $CaCl_2$ addition had an impact on fermentation performance and oil emulsification. Surprisingly, the results showed that F4 had higher protein concentration (see Table 7), which has been reported to increase emulsion stability (Heeres et al., 2014; Heeres et al., 2015; Pedraza-de la Cuesta et al., 2017). Studies reported $CaCl_2$ as a cross linking agent (Hariyadi et al., 2014; Ye and Singh, 2000) which can promote protein bridging making the emulsion more susceptible to the shear by centrifugation and promoting oil recovery. Furthermore, the oil recovery also increased for the fermentations with late addition of $CaCl_2$ (F5 and F6). The fermentation with higher amount of flocculant added (F6 – 20 mM) resulted in a higher recovery (67% – 3 times higher than the reference), showing that an increase in flocculant concentration is beneficial to the emulsion destabilization by centrifugation. On the contrary, the fermentation with early addition of $(NH_4)_2SO_4$ (F3), did not show any improvement in terms of oil release, since the oil recovery is inside the bandwidth of the reference. These results indicate that $CaCl_2$ makes the emulsion much more sensitive to the shear applied by centrifugation. However, for the oil recovery by centrifugation, $(NH_4)_2SO_4$ is not a useful flocculant.

3.2.4. *Comparison of three enhancement oil recovery methods when using flocculants during fermentation at lab scale*

In view of the results, Table 8 presents the best combination of flocculant and method of addition for the three oil recovery technologies (gravity settling, GEOR and centrifugation).

The early addition of $(NH_4)_2SO_4$ has shown to be the most beneficial to destabilize an emulsion from fermentation broth when using gravity settling and GEOR. Furthermore, this flocculant is very simple to apply during fermentation since it is already present in many fermentation media. In case of centrifugation, $CaCl_2$ has revealed to be the most helpful in emulsion destabilization. Its impact is closely linked with the time of addition, where early addition of this flocculant led to higher oil recovery but had an effect in fermentation performance. Clearly, follow-up research would benefit from larger data sets from fermentation with addition of flocculant. The extra data would allow to better understand the impact of flocculants into fermentation performance, to generate duplicates for gravity settling and GEOR experiments, as well as, to test different methods of flocculant addition and concentration in emulsion destabilization.

The beneficial effect in oil recovery observed by the addition of $CaCl_2$ in centrifugation and not in GEOR can be explained by the fact that the calcium ions not only shield electrostatic repulsions but can also create links between the proteins surrounding the oil droplets. The creation of flocs of biomass when using $CaCl_2$ during fermentation broth has been demonstrated for *Saccharomyces cerevisae* (Nayyar et al., 2017; Stewart, 2018), however, no literature was found regarding *E. coli* fermentations with $CaCl_2$ flocculant. This seems to be confirmed by the microscopic pictures shown in Fig. 7. Indeed, Fig. 7[A], shows the formation of cell flocs, in contrast with Fig. 7[B] where the cells are homogenously dispersed. When adding oil (Fig. 7[C] and [D]), the pictures suggest that cross-linking is happening not only with cells but also with oil droplets. This interaction can explain the change in fermentation performance, emulsion rheology and the characteristic creaming observed in Section 3.2.1. When the broth with $CaCl_2$ is under gravity settling, the cream formation retains a large fraction of the biomass present in the broth and oil cannot be separated. The same follows for GEOR, where the shear

Table 8 – Best combination between the flocculant tested and oil recovery technologies.

	Gravity settling	GEOR	Centrifugation
Enhancement of oil recovery	Early addition of $(NH_4)_2SO_4$ (75 mM)	Early addition of $(NH_4)_2SO_4$ (75 mM)	Late addition of $CaCl_2$ (20 mM)
Reasoning	Quantitative improvement relative to reference and easy adjustment for fermentation broth.		Quantitative improvement relative to reference.
Drawbacks	High concentration of ammonium which is toxic to the environment. Requirement of water treatment plants.		Create flocs of cells, potentially hampering cell recycle.

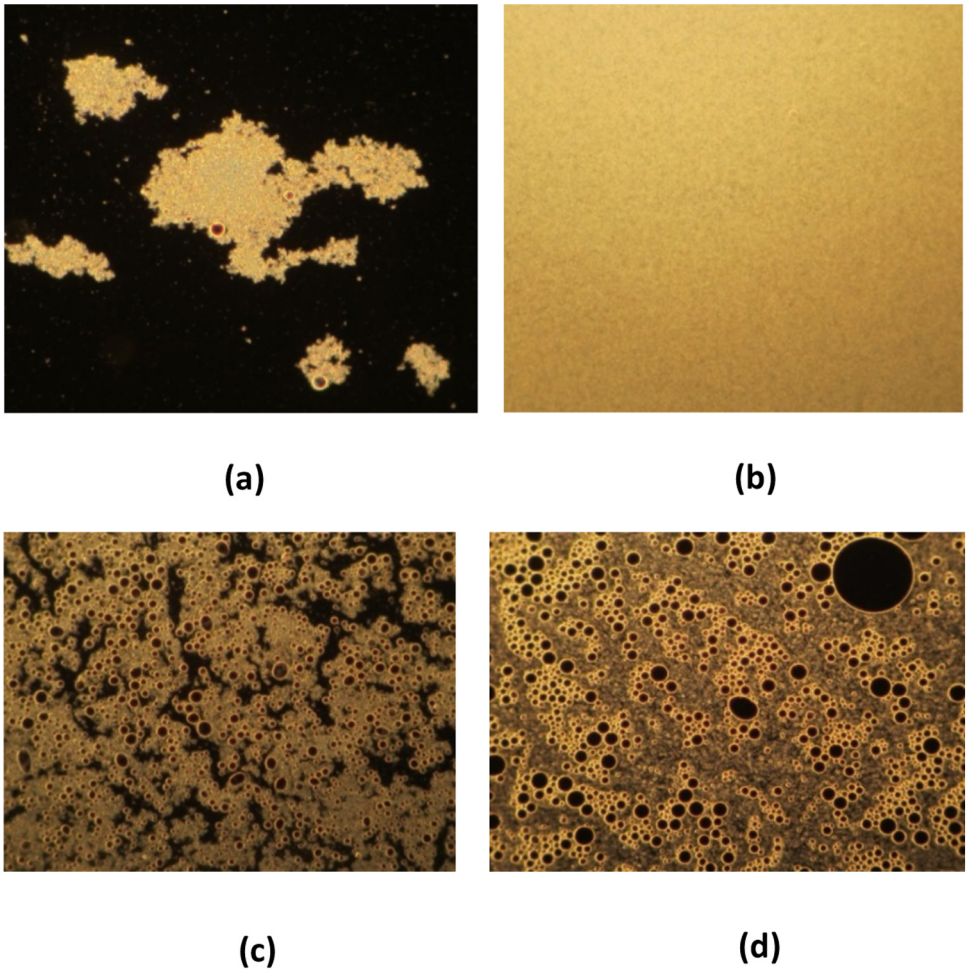

Fig. 7 – Microscopy pictures (10×) of fermentation broth with *E. coli*. (a) Sample without cream and oil with flocculant $CaCl_2$; (b) sample without cream and oil and without flocculant; (c) sample of homogeneous broth with oil and with flocculant $CaCl_2$; (d) sample of homogeneous broth with oil and without flocculant.

applied by the gas bubble at the conditions of this paper, helps to separate the emulsified oil but also traps the back-mixed uncolored oil into the cream. In regard with the effect of centrifugation, the extensive cross-linking reduces the molecular flexibility of proteins (Dickinson, 2019; Nylander et al., 2019). Therefore, the shear applied by centrifugation generates breaks in the droplets' protein coating, which promotes droplet coalescence.

Both the selected flocculants showed to have potential in terms of industrial applicability and oil recovery, not only due to their destabilization potential and fermentation compatibility, but also due to economic and environmental properties. $(NH_4)_2SO_4$ was able to improve oil recovery by gravity settling and GEOR. Moreover, it represents a great advantage for in situ oil recovery since this component can be used as base addition during multiphasic fermentations and its application would only require a change in medium composition. If a more traditional method as centrifugation would be used, then late addition of $CaCl_2$ would be preferred since early addition has an effect in fermentation performance. Addition of this compound in the later stages of a fermentation could help to minimize the steps needed for downstream processing of the emulsion as well as removing the necessity of temperature swings and chemical addition. However, the creation of biomass flocs can jeopardize cell recycle and fermentation performance. Furthermore, other technologies such as mechanical coalescers (van Aken et al., 2003) or centrifugal contactor separator (McFarlane et al., 2010; Oh et al., 2012) could be implemented together with the addition of $CaCl_2$ to improve oil recovery from multiphasic fermentations.

4. Conclusion

The impact of adding flocculants ($(NH_4)_2SO_4$ (75 mM) and $CaCl_2$ (10 and 20 mM)) to multiphasic fermentation in order to destabilize the microbial emulsion formed, has been pre-

sented. The addition of the flocculants into fermentation showed that they do not affect biomass content and CO_2 production. However, the early addition of $CaCl_2$ forms flocs of biomass which can impact fermentation performance and cell recycle. Three techniques for oil recovery were compared: gravity settling, GEOR and centrifugation. Creaming rate and oil recovery by GEOR improved by a factor 3 compared to the reference fermentation, when adding $(NH_4)_2SO_4$. Yet, there was no impact on oil recovery by centrifugation when using this flocculant. On the other hand, when using centrifugation after late addition of $CaCl_2$, 67% of oil recovery was obtained, resulting in a 3-fold increase compared to the reference fermentation. In conclusion, the flocculant, its time of addition, and its concentration can be tuned to the recovery method to improve in situ oil recovery of several technologies and by doing so, decrease production process costs.

Conflict of interest

Since summer 2018, Prof. Luuk van der Wielen and Dr. Maria Cuellar are (indirect and minority) shareholders in DAB BV, following the TU Delft regulations for staff inventors of intellectual property.

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