



Valorisation of cheese whey as substrate and inducer for recombinant protein production in *E. coli* HMS174(DE3)



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ABSTRACT

Every year worldwide around 190 million tons of cheese whey are generated resulting in a huge environmental burden. We recently published a study where we showed that *E. coli* strain HMS174(DE3) can be cultivated using only lactose as C-source and inducer. Motivated by the results we investigated using a concentrated whey feed instead of the lactose feed. Spray drying whey and dissolving the powder allowed preparation of a 40-fold concentrated whey containing 91% lactose and 81% protein of the original whey. Cultivations using the concentrated whey feed instead of a defined lactose feed revealed 39% higher growth rates, 24% higher biomass yields and even higher specific product titers for the model enzymes, flavanone 3-hydroxylase and chalcone 3-hydroxylase. Our strategy simultaneously provides a cheap substrate for large-scale production of technical enzymes and an excellent opportunity for cheese whey valorization, reducing the biological burden resulting from whey wastewaters.

1. Introduction

Currently, every year worldwide 180 to 190 million tons of cheese whey are generated as a byproduct whenever milk is coagulated for the production of cheese or curd (Yadav et al., 2015). Per 1 kg of cheese approximately 9 L of whey are generated, resulting in a huge environmental problem caused by the high volumes and the high organic content of this byproduct (Guimaraes et al., 2010). Dumping and disposal of whey are problematic as it negatively affects crop yields whenever applied on soil but also imposes danger to the aquatic life when released into waters as it reduces dissolved oxygen levels (Panesar et al., 2007; Yadav et al., 2015). The biochemical and chemical oxygen demand, as indicators for the assessment of the organic load of whey, are estimated between 30 and 50 g_{O₂}/L and 60 and 80 g_{O₂}/L respectively, for which lactose is largely responsible (Guimaraes et al., 2010). These values are far too high for channeling whey directly into waste waters as for instance in Germany, the discharge limit for sewage water from dairies lies at 0.11 g_{O₂}/L COD and 0.025 g_{O₂}/L BOD (Justice, 2004).

Current ways of cheese whey management include biological treatment, physicochemical treatment, direct land application or its use

as animal feed (Prazeres et al., 2012). Biological treatment mainly happens through anaerobic digestion (e.g. (Escalante et al., 2018; Lovato et al., 2019; Pagliano et al., 2018; Treu et al., 2019), fermentation to ethanol (Lawton and Alcaine, 2019; Zheng et al., 2019), hydrogen (Akhlaghi et al., 2017; Lopes et al., 2017; Pandey, 2017) or lactic acid (Garcia et al., 2017; Turner et al., 2017) as well as conversion to electricity by microbial fuel cells (Kondaveeti et al., 2019; Prazeres et al., 2012; Wenzel et al., 2017). Recently, also polyhydroxyalkanoate production from whey has been investigated, showing new trends in whey utilization (Bustamante et al., 2019; Koller et al., 2013). Another option is lactose hydrolysis (Ghosh et al., 2017; Liu et al., 2017), which is often performed as pretreatment, since many more organisms are able to metabolize glucose and galactose compared to those that are able to metabolize lactose directly (Prazeres et al., 2012; Siso, 1996). However, hydrolysis can be problematic as under acidic conditions undesirable byproducts are formed (Siso, 1996) and enzymatic hydrolysis can be difficult due to the formation of oligosaccharides (Gekas and Lopez-Leiva, 1985; Guy and Bingham, 1978) and/or lactose mass transfer limitations into the cells (Joshi et al., 1987). For all of these reasons direct utilization of lactose in whey seems desirable.

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We recently published a study where we showed that *Escherichia coli* strain HMS174(DE3) can recombinantly produce enzymes when fed with lactose, using the disaccharide as C-source and inducer simultaneously (Hausjell et al., 2018). We showed that similar biomass and product yields were reached using a lactose-only feed compared to a standard *E. coli* process using the BL21(DE3) strain, in a glucose fed-batch induced with IPTG (Isopropyl- β -D-thiogalactopyranosid). Motivated by these results we wanted to investigate the possibility of using concentrated whey instead of the defined lactose feed for recombinant protein production in *E. coli* HMS174(DE3). This would (I) provide an opportunity for direct whey valorization and (II) offer a cheap substrate for large scale production of technical enzymes. Usually lactose recovery from whey involves several filtration steps as well as ion exchange chromatography to separate the disaccharide from protein and lipids. This is followed by spray drying of the lactose (de Souza et al., 2019). Direct spray drying of the whey would circumvent all these steps resulting in a higher recovery of lactose and a cheaper C-source. Although the undefined, animal-derived feed source prevents its use for pharmaceutical purposes as it violates quality by design guidelines (Lawrence et al., 2014; Rathore and Winkle, 2009), this strategy could be highly attractive for white biotechnology applications.

To investigate our idea, we first looked into the preparation of a concentrated whey feed by either reverse osmosis or by dissolving spray dried whey powder in a 40-fold concentrate. For the latter we analyzed how much lactose and protein could be dissolved at a 40-fold concentration factor. In the concentrate we checked the necessity of trace element supplementation. We investigated several physiological parameters including the maximum growth rate and biomass yield of the *E. coli* HMS174(DE3) strains when grown on the concentrated whey feed. Finally, we analyzed productivity of *E. coli* strains and compared it to production on a defined lactose feed for two different model enzymes: flavanone 3-hydroxylase (FHT) from *Malus domestica*, expressed as soluble protein, and chalcone 3-hydroxylase (CH3H) from *Dahlia var. ibilis*, expressed as inclusion body.

2. Materials and methods

2.1. Strains and plasmids

Strains and plasmids were cloned and transformed as we described before (Hausjell et al., 2018).

2.2. Preparation of the whey feed

The whey feed was prepared by dissolving 300 g untreated spray dried whey powder kindly donated from NÖM (Niederösterreichische Molkerei, Baden, Austria) per 1 liter of distilled water and heating the solution to 80 °C for 5 min. Subsequently, centrifugation (20,000 g, 10 min, 21 °C) was performed to remove undissolved material. The solution was sterile filtered and supplemented with iron(III) citrate, cobalt(II) chloride hexahydrate, manganese(II) chloride tetrahydrate, copper(II) chloride dihydrate and sodium molybdate dehydrate to reach the same trace element concentrations as the standard DeLisa feed at 200 g/L sugar (DeLisa et al., 1999).

2.3. Fractionation and concentration of fresh whey

In parallel to the direct preparation of concentrated whey feed from spray dried whey powder, a concentration procedure for fresh whey with native lactose content was set up in order to mimic a direct industrial process without intermediate spray drying. For this purpose, fresh whey was prepared by dissolving 66.5 g of spray dried whey powder per 1 L of distilled water under stirring and heating to 80 °C for 45 min. Subsequently, suction filtering (10 μ m filter paper, 25 °C) was performed for solid particle removal.

An integrated membrane separation cascade has been selected to

increase the lactose content in the fresh whey consisting of an initial microfiltration step (MF) followed by ultrafiltration (UF) and reverse osmosis (RO) steps (Yorgun et al., 2008). Commercial flat sheet membranes from KOCH Membrane Systems Inc. based on polyethersulfone membrane material were applied for this purpose (MF: MFK-618 with 0.1 μ m pore size; UF: HFK-328 with 5 kDa molecular weight cut-off; RO: HR membrane with 99.6% NaCl rejection). Within this concept, MF was used for coarse particle removal while UF was used for protein and lipid removal in order to reduce the fouling tendency of the subsequent RO step (Cuartas-Urbe et al., 2009). As demineralization of lactose concentrate was undesired, RO with high salt rejection was chosen over nanofiltration and an additional ion exchange step. Opposed to literature, MF and UF steps were not operated in diafiltration mode as the major target of these first experiments was to maximise lactose content regardless of lactose recovery (Souza et al., 2010). Permeation experiments were conducted on a laboratory-scale membrane plant (OSMO MemCell OS-MC-01, 64 bar maximum pressure) equipped with a single 80 cm² flat sheet membrane module operated in cross-flow mode. The feed tank volume was limited to 1 liter necessitating a batchwise operation for processing a total of 4 liters of fresh whey. A piston pump provided feed flow with a volumetric cross-flow rate of 2.0 L/min for the membrane module while a pressure retention valve in the membrane retentate line allowed for manual adjustment of pressure on the high-pressure side of the membrane. Permeate was withdrawn at atmospheric pressure and collected in a weighed beaker for permeate flux calculation. Operation temperature of 10 °C was chosen as literature indicates that fouling tendency is significantly lower at low temperatures despite the lower fluid viscosity and increased turbulence on the membrane surface (Ng et al., 2018). Additionally, low temperatures are favourable for hygienic processing (Steinhauer et al., 2015a). Feed pressure was chosen individually for each process step for optimized performance (MF: 20 bar, UF: 5 bar, RO: 55 bar). Special emphasis was put on the analysis of reversible and irreversible permeate flux decline due to fouling and concentration polarization/gel layer formation. It has been reported in literature that fouling is mainly attributed to adsorption of proteins (Steinhauer et al., 2015b) and precipitation of minerals, in particular Calcium phosphate, on membrane surface and membrane pore structure (Ng et al., 2017). Nevertheless, fouling in general is highly dependent upon composition and history of whey and detailed analysis needs to be performed with directly fed fresh whey (Rice et al., 2009). The effect of fouling can be minimised by high cross-flow velocity and optimized operating pressure.

Permeate volume ratio PVR obtained by membrane separation steps MF and UF were calculated by the following equation:

$$PVR = \frac{V_P(t)}{V_0} \quad (1)$$

where V_0 is the initial feed volume and $V_P(t)$ is the permeate volume at time t . Volume reduction ratio VRR characterizing RO concentration step was calculated according to (Yorgun et al., 2008) by:

$$VRR = \frac{V_0}{V_0 - V_P(t)} \quad (2)$$

2.4. Cultivations

Cultivations were carried out as we described before (Hausjell et al., 2018) with minor modifications. The cultivations were performed in a DasBox Mini Bioreactor system (Eppendorf, Hamburg, Germany) with a volume of 250 mL. The temperature was set to 35 °C during the batch and un-induced fed-batch phase and 30 °C during induction. The reactors were aerated at 2 vvm and stirred with 2000 rpm. pH was measured with pH-Sensor EasyFerm Plus (Hamilton, Reno, NV, USA) and kept at 7.2 by addition of 12.5% NH₄OH, where the volume was monitored with DasGip MP8 Multipumpmodule (Eppendorf, Hamburg, Germany). Dissolved oxygen (dO) was measured with a fluorescence

dissolved oxygen electrode Visiferm DO425 (Hamilton, Reno, NV, USA) and kept above 30% of saturation by varying the ratio of oxygen and pressurized air. The exhaust gas was analyzed for CO₂ and O₂ content by DasGip GA gas analyzer (Eppendorf, Hamburg, Germany). The feed rates during fed-batch & induction phase were adjusted using a feed-forward control strategy, where the initial feed rate was calculated according to Eq. (3), with F being the feed inlet rate in g/h, q_s being the specific substrate uptake rate in g_{substrate}/g_{dry cell weight}/h, X being the biomass concentration in g_{dry cell weight}/L, V being the reactor volume in L and W being the amount of substrate per gram of feed in g_{substrate}/g_{Feed}.

$$F = q_s * X * \frac{V}{W} \quad (3)$$

All cultivations were carried out on DeLisa minimal medium (DeLisa et al., 1999) and consisted of a batch phase with 20 g/L glucose which resulted in 8 g_{dry cell weight}/L. This was followed by an 18-hour glucose fed-batch at a specific sugar uptake rate of 0.25 g_{substrate}/g_{dry cell weight}/h. After 14 h, a pulse with the lactose feed or whey feed was applied to a final concentration of 5 g/L lactose in the bioreactor, adapting the cells to lactose metabolism. At this time point the biomass concentration was approximately 30 g_{dry cell weight}/L. Four hours later, the glucose feed was changed to the lactose or the whey feed (from spray dried whey), both containing 200 g/L lactose, and the feed rate was set to 0.24 g_{substrate}/g_{dry cell weight}/h. The setpoint was slightly above the maximum specific lactose uptake rate determined in a previous study (Hausjell et al., 2018) to ensure that lactose was present in excess, as highest productivities were reached at the maximum growth rate (Hausjell et al., 2018). The fed-batch phase lasted for six further hours resulting in a total induction time of 10 h. Samples were taken at the beginning and end of each phase as well as every 2 h during induction. The samples were analyzed for dry cell weight, sugars and protein concentration in the cultivation supernatant as well as intracellular product levels.

2.5. Analyses

2.5.1. Dry cell weight

Dry cell weight concentrations were determined by pipetting 1 mL of cultivation broth into pre-dried and pre-weighted 2 mL plastic tubes and centrifuging them (4500 g, 10 min, 4 °C). The pellets were washed with 1 mL 0.9% sodium chloride solution and centrifuged again with the same parameters. Thereafter, samples were dried for at least 72 h at 105 °C.

2.5.2. Sugar analysis

Analysis of sugars was performed via HPLC on a Supelcogel column (Supelco Inc., Bellefonte, Pennsylvania, USA) as we described before (Hausjell et al., 2018).

2.5.3. COD and BOD analysis

The Chemical Oxygen Demand (COD) and Biochemical Oxygen Demand (BOD₅) are key parameters in wastewater management.

For the laboratory analysis of the COD the DIN 38409-H43 method was applied (DIN, 38409-43:1981-12). This is a short time method, which measures the oxygen equivalent of the amount of organic compounds oxidizable by potassium chromate. The method is applicable for a COD concentration range of 15–300 mg_{O₂}/L. For higher concentrations, the raw samples were diluted.

The BOD₅ is the amount of oxygen, consumed by aerobic microorganisms for oxidation of organic compounds in solution over 5 days. For determination of the BOD the DIN EN 1899-1 method (DIN, 1899-1998-05) with dilution and allylthiourea addition was applied. The method is applicable for a BOD concentration range of 3–6000 mg_{O₂}/L.

2.5.4. Analysis of protein concentration

Protein concentration was determined by Bradford assays where the

reagent was purchased from Sigma Aldrich (Sigma-Aldrich, Vienna, Austria). Assays were performed according to the manufacturer's instructions. Bovine serum albumin (Sigma-Aldrich, Vienna, Austria) dissolved at different concentrations was used as standard. The absorbance of the samples was measured on a Genesys 20 photometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.5.5. Trace element analysis

To determine the amount of trace elements in the sample solutions, inductively couple plasma mass spectrometry (ICP-MS) was used. Prior to analysis, the samples were digested with a 1/1 volumetric mixture of conc. nitric acid (65 mass%, EMSURE®) and H₂O₂ (30 mass%, EMSURE®). Therefore 1 mL of the sample were digested with 1 mL of the acid mixture in a water bath (70 °C) over night. Two dilution steps were executed, to match the element concentrations within the calibration range. A 1/10 v/v dilution with 1 vol% HNO₃ containing 0.5 µg kg⁻¹ indium (In) as internal standard was used to determine all elements except of Na and Mg. These elements were determined from a 1/10,000 v/v dilution due to their much higher concentration. All dilutions were prepared by using deionized water obtained by Barnstead™ Easypure™ II (18.2 M cm⁻¹), concentrated nitric acid (65 mass%, EMSURE®) and In ICP-MS standard (Certipure®, Merck, Germany). For signal quantification, an external calibration was used. To obtain a calibration function, calibration standards with different concentration levels covering 0.1 to 20 µg kg⁻¹ were prepared by mixing a multi element standard (ICP multi-element standard solution VIII, Certipure®, Merck, Germany) and Mo single element standard (Certipure®, Merck, Germany) with diluted nitric acid containing the internal standard.

Samples and standards were analyzed with iCAPQ ICP-MS instrument (ThermoFisher Scientific, Bremen, Germany) equipped with a quadrupole mass analyzer, a concentric PFA nebulizer and a peltier cooled quartz cyclonic spray chamber. Sample-uptake was achieved with the peristaltic pump of the instrument. The ICP-MS instrument was tuned on a daily basis with respect to a maximum of 115 In signal. The obtained data was processed using Qtegra software (ThermoFisher Scientific, USA). To minimize the influence of polyatomic interferences, the kinetic energy discrimination (KED) mode was used. Herein undesirable molecule ions are suppressed in the collision cell containing a mixture of helium with 7% hydrogen. Further instrument operation parameters can be found in Supplementary Table 1.

Observed signal intensities were normalized using the signal response for the internal standard (In), and finally converted into concentration units by means of external aqueous calibration. Derived In signals were constant over each measurement session (less than 5% relative standard deviation for the whole measurement period, indicating the absence of temporal trends), and no significant difference in In-response between samples and calibration standards was observed.

2.5.6. Product analysis

FHT was produced only as soluble protein while CH3H predominantly formed inclusion bodies. The concentration of soluble FHT was analyzed by IMAC as we described before (Hausjell et al., 2018). CH3H inclusion body content was measured by reverse phase HPLC techniques (Wurm et al., 2018).

3. Results and discussion

3.1. Fractionation and concentration of whey

Fractionation and concentration of fresh whey started with an initial feed volume of 4 L. MF for coarse particle removal was performed until PVR of 0.723 and UF for removal of proteins and lipids was performed until PVR of 0.899. Retentate losses, plant dead volumes and water flushing resulted in a drop of lactose content during these steps from an initial 37.7 g/L to 23.8 g/L after MF and 18.6 g/L after UF. The

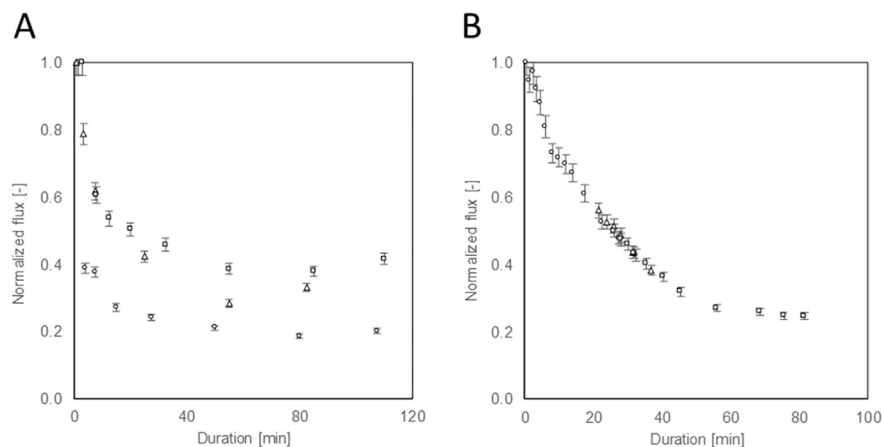


Fig. 1. Permeate flux decline during microfiltration MF (A) and ultrafiltration UF (B) fractionation steps. MF was performed in 3 independent batches at 20 bar with a mean initial permeate flux of 16.4 g/min. UF was performed in 3 consecutive batches at 5 bar with a mean initial permeate flux of 38.0 g/min. Error bars are calculated using error propagation assuming time measurement uncertainty of ± 1 s and weighing error of ± 0.01 g (scale accuracy class III).

subsequent concentration was performed with RO up to VRR of 9.61 (volume decrease by a factor of almost 10) resulting in a final lactose content of 86.4 g/L. Thus, the content of lactose was increased by a factor of 4.65 in the RO step. Again, lactose losses can be attributed to plant dead volumes and water flushing which become highly important considering the resulting low concentrate volume of 271 mL. Lactose losses to permeate can be neglected as no lactose was detectable in this stream using an HPLC method. General results of this process cascade are given in Supplementary Table 2.

Flux decline due to fouling, scaling and concentration polarization is a major topic in any membrane separation process and it was also clearly noticeable in the current process chain. Fig. 1 shows the decline of permeate fluxes in relation to the initial flux for MF and UF steps over time. A reduction of the permeate flux by 60 to 80% of the initial flux was recorded within the first 40 min of operation. This extent of flux decrease has also been reported in literature for MF and UF separation steps processing whey feeds (Rice et al., 2009; Steinhauer et al., 2015b). Nevertheless, it has also been reported that cleaning and regeneration of membranes using different cleaning agents is possible and leads to an almost complete restoration of initial flux (Yorgun et al., 2008).

Flux decline was also detected during RO operation but to a significantly lower extent compared to MF and UF as can be seen in Fig. 2. Two batches of 1300 mL each were concentrated using the same RO membrane sample. Permeate flux increased slightly (10%) during the first batch followed by a small decline at the end of this batch. Continuing with the second batch of identical composition led to a final flux decline of around 50% of initial permeate flux after a total of 182 min. We conclude that the preceding UF step was moderately successful in separating relevant fouling agents (mainly proteins are suspected). We expect that the complete separation of fouling agents combined with an automated retentate pressure control would allow for reaching lactose contents as high as 220 g/L in the whey concentrate at around 40 bar as already documented in literature (Marx and Kulozik, 2018).

3.2. Analysis of the concentrated whey feed

3.2.1. Protein and lactose recovery

Reconstituted whey by reverse osmosis only led to a lactose concentration of 86.4 g/L. In order to avoid biomass dilution in high cell density cultivations ($35 \text{ g}_{\text{dry cell weight}}/\text{L}$ after the fed-batch phase) at least a sugar concentration of 100 g/L is needed. Therefore another strategy to obtain a more concentrated whey feed was developed: The whey feed was prepared by dissolving 300 g/L of spray dried whey powder in distilled water. The undissolved material was then removed by centrifugation. The pellet, which was discarded and the supernatant, which was used as feed, were analyzed regarding their lactose and protein content. $91 \pm 4\%$ of the lactose and $81 \pm 9\%$ of the whey protein were dissolvable, leading to a strong reduction in the organic

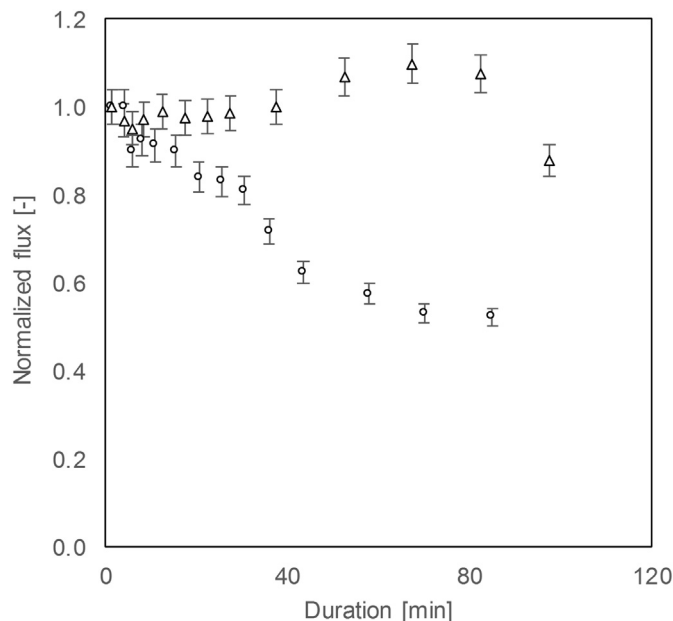


Fig. 2. Permeate flux decline during reverse osmosis RO concentration step. RO was performed in 2 independent batches at 55 bar with a mean initial permeate flux of 9.0 g/min. Triangles indicate the first batch and circles indicate the second batch over the same membrane sample. Error bars are calculated using error propagation assuming time measurement uncertainty of ± 1 s and weighing error of ± 0.01 g (scale accuracy class III).

content to be discarded and consequently the environmental burden caused.

3.2.2. COD and BOD analysis of the whey feed and residue

For further demonstrating the environmental benefits, COD and BOD of the dissolved whey powder as well as the supernatant (whey feed) and pellet (residue) were determined. Supplementary Table 3 summarizes the results. Dissolving the spray dried whey powder for preparation of the feed allowed the recovery of 87% and 75%, respectively, of the COD and BOD of the whey for valorization.

3.2.3. Trace element analysis

Motivated by the developed simple method for feed preparation, we wanted to check trace element levels to determine if supplementation with trace metals was necessary for use of the feed in high cell density cultivations. In order to determine trace element concentrations in the concentrated whey, ICP-MS analysis was performed. The concentrations of magnesium, iron, zinc, cobalt, manganese, copper, boron and

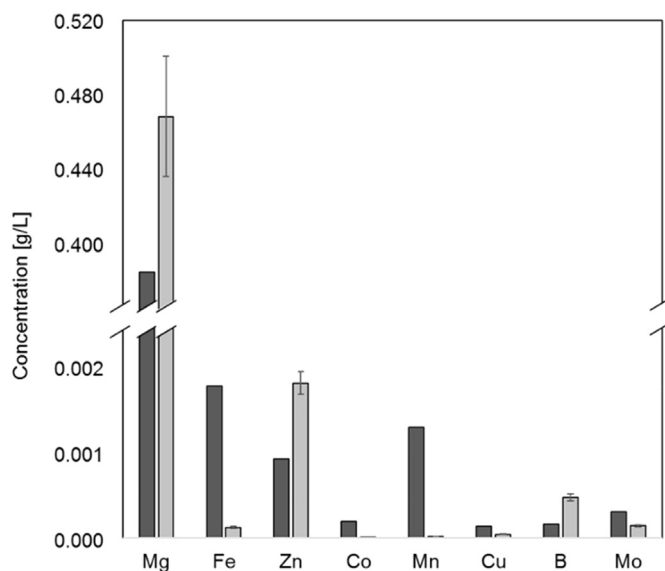


Fig. 3. Trace element levels. Trace element levels in the concentrated whey feed (light grey bars) compared to trace element levels in the standard DeLisa minimal medium feed (dark grey bars). Error bars of the light grey bars display the standard deviation calculated from four measurements. Dark grey bars indicate concentrations as found in the standard DeLisa recipe and therefore do not exhibit error bars. The concentrated whey feed was supplied with Iron, Cobalt, Manganese, Copper and Molybdenum to reach the same trace element concentrations as the standard DeLisa feed at 200 g/L sugar (DeLisa et al., 1999).

molybdenum were analyzed and compared to trace element concentrations in a standard DeLisa minimal medium (DeLisa et al., 1999). Results are displayed in Fig. 3.

The analysis showed that the concentrated whey feed only had to be supplemented with Iron, Cobalt, Manganese, Copper and Molybdenum to reach the same trace element concentrations as the standard DeLisa feed at 200 g/L sugar (DeLisa et al., 1999). Magnesium, Zinc and Boron were present at slightly higher concentrations in the concentrated whey feed.

3.3. Cultivations of *E. coli* strain HMS174(DE3) on the whey feed

3.3.1. Physiology

After supplementing the concentrated whey feed with trace elements, it was used for *E. coli* HMS174(DE3) cultivations. Most frequently strain BL21(DE3) is employed for recombinant protein production (Rosano and Ceccarelli, 2014). However, BL21(DE3) cannot metabolize galactose, resulting from cleaved lactose, making it difficult to perform cultivations using lactose as only C-source (Hausjell et al., 2018). Therefore HMS174(DE3), a strain without enzyme deletions in the Leloir pathway was employed. Alternatively also a BL21(DE3) strain that was made Gal⁺ could have been used (Menzella et al., 2003), however, we preferred the commercially available HMS174(DE3). Two strains, one expressing FHT as soluble protein, one expressing CH3H as inclusion body, were cultivated identically: First a batch and fed-batch phase for biomass generation were carried out on glucose. Then induction was performed either by a lactose fed-batch as described before (Hausjell et al., 2018) or by a fed-batch with the concentrated whey. Both fed-batches were conducted at the same specific lactose uptake rate of 0.24 g_{substrate}/g_{dry cell weight}/h. Physiological data during the lactose and whey fed-batches are displayed in Fig. 4. In Fig. 4A the specific growth rates during the induced fed batches are shown. For both strains the growth rate is clearly higher on the whey feed compared to the lactose feed. Keeping in mind that the same amount of sugar was fed per hour and per gram biomass, this elevated

growth rate must result from other metabolizable components present in whey such as proteins and lipids. This is also displayed in the biomass yields which are elevated on the whey feed, as shown in 4B. As a result of the higher biomass yield and higher growth rate, also the biomass concentration at the end of the cultivations was higher in those fed with whey. On average 60 g_{dry cell weight}/L was reached in the cultivations fed with concentrated whey as opposed to only 50 g_{dry cell weight}/L in those fed with lactose. All of this indicates that the cells grew better on the rich whey feed compared to the lactose feed.

In order to investigate if not only lactose but also the protein fraction from the whey feed was consumed by the cells, we analyzed the protein concentrations in the supernatant during the cultivations. As *E. coli* naturally secretes several proteins into the medium (Nandakumar et al., 2006), a certain protein concentration is always found also when the cells are grown on minimal media. Therefore, we compared the protein concentration in the supernatant during feeding with the whey feed to the protein concentration in the supernatant when we fed with the defined lactose feed. As whey protein not consumed by the cells, would accumulate in the culture supernatant, this would result in a higher protein concentration in the supernatant for cultivations using the whey feed. However, protein concentrations during induction increased from 0.47 mg/mL to 1.17 mg/mL similarly for both feeds as shown in Fig. 5. This led us to conclude that the cells consumed also the whey protein, which explains the higher growth rates and higher biomass yields. Further, this shows that consumption of the concentrated whey feed by the cells not only removes lactose but also whey protein, the other main organic component responsible for the high chemical and biological oxygen demand of whey (Guimaraes et al., 2010).

We further measured the COD in the filtered cultivation supernatant after the 10 h of induction. The COD value was determined as 19.94 g_{O₂}/L. At that time point the whey feed corresponded to 27.7% of the cultivation supernatant. As the whey feed had a COD value of 286.01 g_{O₂}/L (Supplementary Table 3), the total COD was reduced by 74.8%.

3.3.2. Produced enzyme titer

As the cells seemed to grow well on the concentrated whey we also investigated the final product titers for the two model enzymes. As shown in Fig. 6 the data for both enzymes follows the same trend: More product is found per liter cultivation broth in the whey fed-batches (5A). However, this is not just a result of the elevated biomass concentrations as there is even more product quantified specifically per gram dry cell weight (5B). A possible explanation for this could be the higher growth rate, in the whey cultivations as we previously found that product titers increased with higher growth rates for FHT and CH3H (Hausjell et al., 2018).

Although these results seem very promising also the limitations of using concentrated whey as C-source and inducer in *E. coli* bioprocesses have to be kept in mind. As the feeding source is a complex, undefined medium, derived from mammals, the use in biopharmaceutical processes is restricted due to Food and Drug Administration (FDA) regulations. Animal component containing media could introduce potentially harmful contaminants (Asher, 1999; Merten, 1999). Therefore, we recommend application of this concentrated whey feed in white biotechnology for the production of technical enzymes, where cheap substrates are needed. Variance in lactose, protein and trace element concentrations in whey from different sources or even different batches need to be kept in mind as they could definitely have an impact on the process performance. Therefore, we urge analysis of lactose and trace element content of the concentrated whey prior to application in *E. coli* cultivations. Lactose analysis can be performed via HPLC techniques, also in an online manner, where one measurement takes less than 15 min (Wurm et al., 2017). Analysis of trace elements must be carried out offline, since whey samples require digestion prior to measurement. Nevertheless, analysis is possible within a few hours in standard analytical labs.

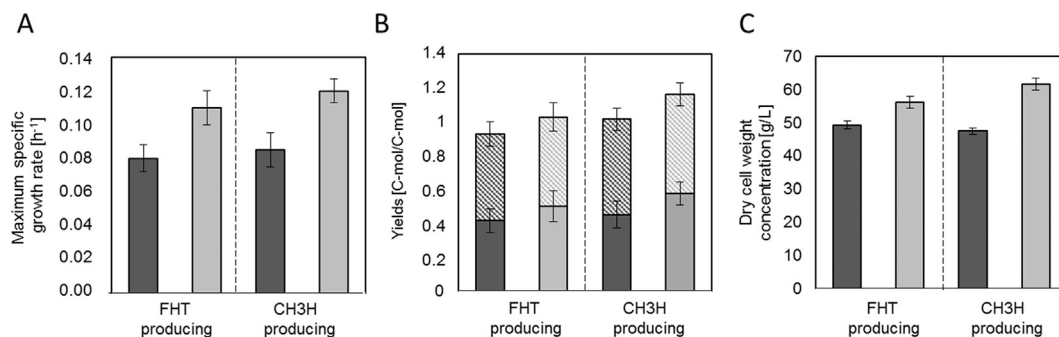


Fig. 4. Physiological parameters. Physiological parameters of HMS174(DE3) with two different plasmids for production of CH3H and FHT respectively, during growth on a lactose feed (dark grey bars) and the concentrated whey feed (light grey bars). (A) showing the maximum specific growth rates, (B) the biomass yields per C-mol of consumed lactose (full bars) and CO₂ yields per C-mol of consumed lactose (ruled bars) and (C) showing the reached biomass concentrations at the end of the cultivations. Error bars display the standard deviation calculated from three measurements.

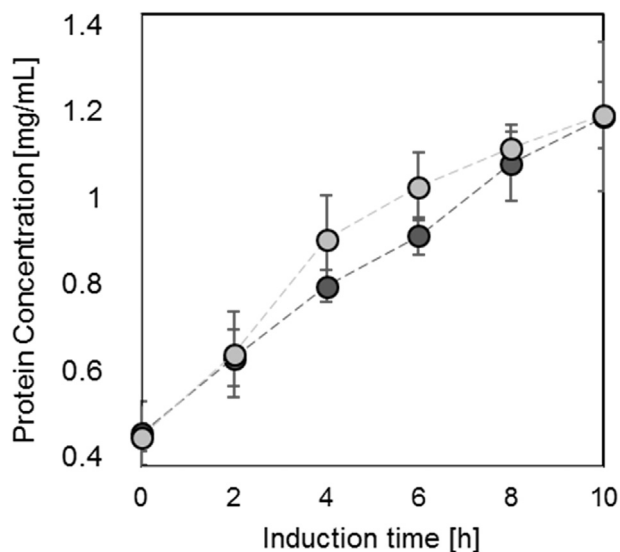


Fig. 5. Protein content in the supernatant. Protein content in the supernatant during cultivations over induction time shown for feeding with the concentrated whey feed (light grey) and the lactose feed (dark grey). Values are averaged from the FHT and CH3H producing cultivations. Error bars display the standard deviation calculated from three measurements.

In our study we used glucose for biomass generation in the batch and fed-batch phase. To reduce costs at large scales further, glycerol or cheaper C-sources could be used in *E. coli* cultivations, up to the point of induction.

Aside from *E. coli* fed batch cultivations, also continuous

cultivations using a whey feed could be an interesting approach. This might be especially attractive, as lower carbon concentrations are utilized and there would not be a need of concentrating the whey to lactose contents above 100 g/L.

4. Conclusions

We showed that it is possible to easily prepare a concentrated whey feed from spray dried whey powder, which allows recovering 91% lactose, 81% protein as well as 87% and 75% of the COD and BOD, respectively. This concentrated whey feed is applicable as C-source and inducer in *E. coli* HMS174(DE3)-cultivations, leading to higher growth rates and even higher specific product titers compared to a defined lactose feed. The developed strategy can be highly beneficial for the large-scale production of technical enzymes, as it provides a cheap substrate and a simple possibility of whey valorization.

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Declaration of competing interest

The authors declare that they have no conflicting interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biteb.2019.100340>.

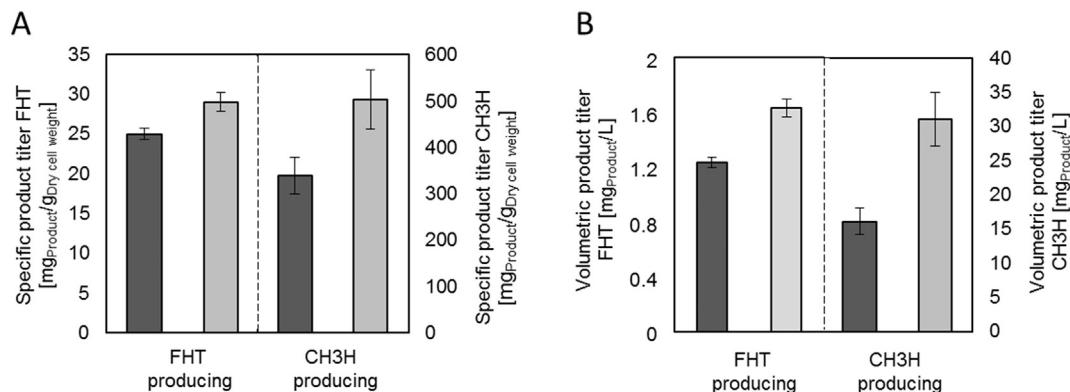


Fig. 6. Specific product titer analysis. Analysis of specific product titers on the lactose feed (dark grey) and the concentrated whey feed (light grey). (A) showing the volumetric product titers and (B) the specific product titers. Error bars display the standard deviation calculated from three measurements.

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