High-level production of industrially relevant oxidases by a two-stage fed-batch approach: Overcoming catabolite repression in arabinose-inducible *Escherichia coli* systems

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ABSTRACT

With the growing interest in enzyme applications there is an urgent demand for economic affordable and flexible enzyme production processes. In the present paper, we developed a high cell density fed-batch process for the production of two cofactor-containing oxidase, 5-hydroxymethylfurfural oxidase (HMFO) and eugenol oxidase (EUGO). The approach involved the arabinose inducible system to drive the expression while using mineral media. In order to overcome a major drawback of arabinose-inducible promoters, carbon catabolite repression, (CCR) by glucose, we developed a High Cell Density Culture (HCDC), two-stage fed-batch protocol allowing us to reach cell densities exceeding 70 g/L of Dry cell weight (DCW) using glucose as carbon source. Then, induction was achieved by adding arabinose, while changing the carbon source to glycerol. This strategy allowed us to obtain an 8-fold increase in recombinant HMFO titer when compared with a reference batch fermentation in Erlenmeyer flasks using terrific broth (TB), typically used with arabinose inducible strains. The optimized protocol was also tested for expression of a structurally unrelated oxidase, EUGO, where a similar yield was achieved. Clearly, this two-step protocol in which a relatively cheap medium (when compared to TB) can be used, reduces costs and provides a way to obtain protein production levels similar to those of IPTG-based systems.

Keypoints:

- Arabinose promoters are not well suited for HCDC production due to CCR effect
- This drawback has been overcome by using a two-stage Fed-batch protocol
- Protein yield has been increased by an 8-fold factor, improving process economics

INTRODUCTION

Industrial enzymes are widely used today in a variety of fields, ranging from classical biotechnology processes such as wine, beer or bread production, to use as additives in paper production, detergents or food and in synthetic chemistry for the synthesis of fine chemicals, drugs and drug metabolites (Kirk et al. 2002). The global enzyme market is predicted to reach a business value of more than 5 billion € by 2025 (FiorMarkets 2019).

The main sources for industrial enzymes are animal tissues, plant biomass and microbial fermentation. The last approach is the most favored way with 90% of the industrial enzymes coming from microbes (Saran et al. 2019). The use of recombinant DNA technology allowed the manufacturing of large quantities of heterologously expressed enzymes using submerged fermentation with known hosts (such as yeast, *E. coli or Bacillus spp*.), regardless the organism of origin of the enzyme (Demain and Vaishnav 2011). Besides, genetic techniques have made it possible to tune enzymes through enzyme engineering, allowing the design of enzyme variants with improved characteristics, like a higher thermostability (Wijma et al. 2014), broader substrate range (Reetz et al. 2005) or even new capabilities (Hyster and Ward 2016)

A popular workhorse for bacterial expression of recombinant proteins is *E. coli.* For this production host, the IPTG inducible promoter system is widely used and has been well described (Pardee et al. 1959; Gilbert & Müller-Hill 1966; Wurm et al. 2016). It allows the synthesis of recombinant proteins at a high yield thanks to its strong promoter, being tunable and compatible with high cell density culture (HCDC) protocols, maximizing the process yield (Lecina et al. 2013).

Nevertheless, two main drawbacks hamper the wide application of IPTG-based expression at industrial scale, a key step in order to obtain the required amounts of enzymes for industrial applications. First of all, the inducer, IPTG, is somewhat toxic to *E. coli* and thereby lowers the yield of biomass in fermentations (Rizkia et al. 2015). It is also toxic to humans, hindering large-scale usage and limiting its application for the production of human therapeutics (Darby and Hine 2005) . Furthermore, IPTG is relatively expensive and translates into relatively high costs of enzyme production (Tian et al. 2011; Einsfeldt et al. 2011; Lopes et al. 2019). Because of these drawbacks, various alternative expression systems have been developed to overcome these limitations (Lee and Keasling 2008; Valdez-Cruz et al. 2010; Jayaraman et al. 2016).

L-arabinose inducible systems based on pBAD promoters have also become popular in biotechnological research. It allows a more tightly regulated expression than IPTG inducible expression systems with little or no leakiness. The latter characteristic is quite useful for the expression of toxic proteins (Széliová et al. 2016). Besides, arabinose-inducible expression systems allow high-level expression at about the same levels as the IPTG-inducible expression systems (Guzman et al. 1995). When compared with IPTG, arabinose is cheaper and non-toxic. This makes this alternative expression system attractive for large-scale fermentations. Yet, when considering using a pBAD system, it is advised to use an *E. coli* strain that is devoid of arabinose catabolism. Several strains have been made for this purpose, such as *E. coli* TOP10 or BL21AI (Narayanan et al. 2011).

However, as in the case of IPTG, arabinose inducible systems have also constraints that prevented it widespread use in industry. The main disadvantage of this expression system comes from one of its advantages. The carbon catabolite repression (CCR) of pBAD promoters mediated by cAMP levels in the presence of glucose allowed a tightly regulated expression, but also

prevents the induction of the gene in presence of glucose (Lee and Jung 2007). Although the original pLac promoters also displays catabolite repression, the vast majority of IPTG inducible systems are mutant variants without CCR (Studier and Moffatt 1986), but no example of arabinose inducible promoter without CCR has been found by the authors in the literature up to date. Still, this problem can be easily overcome at lab scale by using complex culture media. But at large scale, mineral media are preferred due to the lower cost of its components and also the lesser batch-to-batch variability with respect to complex extracts and peptones (Gray et al. 2008). Because mineral media composition is defined and known more precisely than complex media composition, it allows the application of more advanced HCDC strategies like fed-batch, incrementing process yield. Nevertheless, mineral media are typically formulated using glucose as sole carbon source due to the better performance of *E. coli* cells growing on this carbohydrate, thus, hindering the application of HCDC protocols with arabinose inducible systems due to the aforementioned CCR effect (Delisa et al. 1999). The use of alternative sugars like fructose or sucrose would lead to the same effect with different intensities (Aidelberg et al. 2014) and alternative non-sugar carbon sources like glycerol or sorbitol would render the process inefficient because of the slow growth of the bacteria.

Among industrial enzymes, redox enzymes are attracting more attention due to the realization that they can be used for the manufacturing of chemical building blocks in green bio-based economy. An important class of redox enzymes are the oxidases. These biocatalysts catalyze highly chemo-, regio- and/or enantioselective oxidations. An example is the recently discovered 5-hydroxymethylfurfural oxidase (HMFO, EC 1.1.3.47) from *Methylovorus sp.* (Dijkman and Fraaije 2014). This bacterial enzyme contains a tightly bound FAD cofactor and is capable of converting HMF into furan-2,5-dicarboxylic acid (FDCA), a highly valuable building block for polymers. For the oxidation, the oxidase merely requires molecular oxygen. Because HMF can be prepared from biomass, this biocatalyst may develop as a promising tool for the synthesis of biomass-based polymers. Another interesting oxidase is eugenol oxidase (EUGO) which can be used for the conversion of phenolic compounds (Nguyen et al. 2016). This bacterial enzyme contains a covalently bound FAD as redox cofactor. EUGO is a bacterial homologue of the well-known vanillyl-alcohol oxidase from fungus *Penicillium simplicissimum* (VAO) and has a similar substrate scope, though it is expressed in higher yield (Jin et al. 2007). EUGO can be used in immobilized form for the biocatalytic synthesis of vanillin at the expense of molecular oxygen (García-Bofill et al. 2019). It can also be used for the synthesis of various other valuable phenol compounds (Nguyen et al. 2016).

In the present work, we developed an effective HCDC fed-batch protocol for the production of HMFO and EUGO using an arabinose inducible system in mineral media. Firstly, we compared enzyme production in batch cultures using TB (As standard reference for a typical process) and DM+Glycerol. Then, we implemented a two-stage fed-batch workflow in which (1) cells are grown on glucose up to high cell density, after which (2) the carbon source is switched to glycerol and protein expression is triggered by adding arabinose. Biomass and HMFO/EUGO enzyme titers were analyzed, showing that this protocol allows the production of recombinant enzymes in similar quantities to published IPTG-driven systems.

METHODS

Bacterial strain and plasmids

Plasmid carrying gene for the recently engineered stable variant of HMFO, pBAD-SUMO-7xHMFO (Martin et al. 2018) was used to transform *E. coli* BL21AI competent cells (ThermoFisher Scientific, USA).

Culture and feeding media

Terrific broth (TB) was prepared following the standard recipe: KH_2PO_4 9.4 g/L, K_2HPO_4 2.2 g/L, glycerol 5 g/L, yeast extract 23.6 g/L, tryptone 11.8 g/L. The composition of Defined Medium (DM) consisted in (Pinsach, 2009): KH_2PO_4 11.9 g/L, K_2HPO_4 2.4 g/L, NaCl 1.8 g/L, $(NH_4)_2SO_4$ 3 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, $FeCl_3$ 0.02 g/L, and trace elements at 0.72 ml/L. Trace elements solution: $CaCl_2 \cdot 2H_2O$ 1.44 g/L, $AlCl_3 \cdot 6H_2O$ 0.041 g/L, $ZnSO_4 \cdot 7H_2O$ 0.87 g/L, $CoCl_2 \cdot 6H_2O$ 0.16 g/L, $CuSO_4 \cdot 7H_2O$ 1.6 g/L, H_3BO_3 0.01 g/L, $MnCl_2 \cdot 4H_2O$ 1.42 g/L, $NiCl_2 \cdot 6H_2O$ 0.01 g/L, $Na_2MoO_4 \cdot 2H_2O$ 0.02 g/L. Riboflavin cofactor was added at a concentration of 0.05 g/L. Carbon source consisted in glycerol or glucose, depending on the experiment using 20 g/L for Glucose and 26 g/L for Glycerol. Glycerol was used at this concentration assuming a TOC content of 60% in order to get a 16 TOC/L in final media, similar to the calculated TOC of TB. In both media, ampicillin at 50 μg/mL was added as selection marker, and arabinose at 0.2 g/L was added for induction at the appropriate time. All reagents were purchased in Sigma-Aldrich, USA unless stated otherwise.

In fed-batch experiments, the initial base medium was the same defined medium used in the batch experiments, using glucose as carbon source, and two different addition media were used: one for a first fed-batch growth phase (growth phase medium) and another for the induction phase (induction phase medium). Growth phase medium consisted in: glucose (478 g/L), $CaCl_2 \cdot H_2O$ (0.089 g/L), $MgSO_4 \cdot 7H_2O$ (9.56 g/L), $FeCl_3$ (0.49 g/L), trace elements (same solution described before) (62.94 ml/L), Riboflavin (0.05 g/L) and ampicillin at 50 μg/ml. Induction medium had the same composition than growth medium with glycerol instead of glucose (at the same concentration of 478 g/L) and the addition of arabinose at 0.2 g/L.

Batch Fermentation with TB or DM+Gly

Bioreactor cultures were grown in a 2 L Biostat B bioreactor (Sartorius Stedim, Germany) equipped with pH, pO_2 and foam probes. The pH was maintained at 7.0 by adding NaOH (30%) and H_2SO_4 (2 M) for TB cultures, whereas for DM+Gly cultures NH_4OH (15%) was used as base and nitrogen source. An airflow of 1 vvm was applied, and oxygen levels were set to a Dissolved Oxygen (DO) setpoint of 30%, controlled through stirring, which ranged between 200 and 1100 rpm. Foam formation was controlled by means of automatic antifoam DF204 (20% [w/v]) addition. 1 mL/L of antifoam DF204 (20%) was added to the medium prior to sterilizing the bioreactor. Experiments were performed at 24 °C. Precultures were performed as follows: 10 mL cultures were inoculated with 50 μL of a frozen stock and incubated at 24 °C for 8 h, then 200 mL of culture were inoculated with the previous 10 ml and incubated overnight for 16 h. Preculture media were not supplemented with arabinose. This culture was used to inoculate the reactor at an initial OD_{600} of 0.2 for a total volume of 2 L. Both cultures were supplemented with arabinose at 0.02% (w/v) from the beginning of the culture. Glucose and glycerol concentrations were assessed using an automated Y15 analyser (BioSystems, Spain).

Fed-Batch Fermentation

Fed-batch experiments were performed in the same bioreactor than for batch cultures, and culture conditions were similarly set and controlled (see section 2.3), inlet air was mixed with oxygen after the maximum stirring was achieved in order to maintain DO setpoint after the maximum stirring was achieved. Precultures were prepared as follows (all steps at 37 °C and 135

rpm): 10 mL of DM were inoculated with 50 μL of frozen stock and incubated for 8h. Then they were transferred to 2 Erlenmeyer flasks of 250 mL with 50 mL of DM and incubated overnight for 16 h. After that, each 50 mL culture was transferred to a 500 mL Erlenmeyer adding an additional 50 mL of fresh DM medium, to reach a total of 100 mL of culture. The culture was inoculated at OD_{600} = 0.4 and consisted in a batch growth step for approximately 11 h. It was followed by a first fed-batch growth phase for 8 h at 37 °C, at a growth rate of 0.2 h^{-1}, in order to achieve high biomass levels. The medium feeding was performed through a pre-programmed exponential addition, using the following equation (Eq. 1):

$$F = \frac{\mu \cdot X \cdot V_0 \cdot e^{(\mu * \Delta t)}}{Y_{X/S} \cdot S_0} \quad (1)$$

Where F corresponds to the feeding flux (mL/min), μ to the set specific growth rate (h^{-1}), Δt to the time interval in which the feeding flux is applied (1 h), X to the predicted biomass concentration in the bioreactor at the end of the time interval, V_0 to the culture volume at the beginning of the time interval, $Y_{x/s}$ to the biomass/substrate yield (set at 0.35 g/g) and S_0 to the concentration of substrate (either glucose or glycerol) in the feeding medium. . This expression was deduced from substrate and biomass balance equations, as described by Pinsach et al. (2006) (Eq.2):

$$V_{ad}(t) = \frac{1}{S_0} \cdot \left(\frac{m_{sx}}{\mu_{fix}} + \frac{1}{Y_{X/S}} \right) \cdot X(t) \cdot V(t) \cdot (\exp(\mu_{fix} \cdot \Delta t) - 1) \quad (2)$$

After the first phase, a step of 1 h without feeding and with a linear decreasing of temperature to 24 °C was applied. This was followed by a second fed-batch phase, in which induction medium was fed as follows: first, a discrete amount of glycerol was added into bioreactor until a concentration of 5 g/L of glycerol was reached. Then, glycerol consumption was monitored by off-line analysis. When metabolic change from glucose consumption to glycerol was observed, induction was carried out by adding arabinose to a final concentration of 0.2 g/L (approx. 2-3 h after substrate change), and glycerol feeding was resumed maintaining a growth rate of 0.05 h^{-1} throughout the induction phase.

Product concentration determination: SDS-PAGE

The concentration of the enzyme in disrupted samples was determined by SDS-PAGE. 15 μl of Laemmli buffer (Bio-rad, USA) was added to 15 μL of each sample, the mixture was then incubated at 95 °C for 5 min and of which 15 μL was loaded onto a gel (MiniProtean TGX StainFree, Bio-rad, USA), which was run for 17 min at 300 V. 10 μL of molecular weight markers were also loaded on the gel. The concentration of the expressed protein in the extract was determined by densitometric analysis, comparing it to HMFO reference standard (GECCO Biotech, The Netherlands), using ImageLab software (Bio-rad). Images of gels and calibration can be seen in supplementary information.

Enzymatic activity test

HMFO activities were assayed using 5.0 mM vanillyl alcohol as substrate through an automated variant of the already published enzymatic assay (Martin et al. 2018) adapted to the Y15

automated analyser (Biosystems, Spain). Briefly, this assay follows the production of vanillin by using the extinction coefficient of 14 $mM^{-1}cm^{-1}$ at 340 nm.

RESULTS

Production of HMFO in Batch: Comparison between Complex and Mineral media.

To develop a more cost-effective fermentation, we considered *E. coli* BL21 strains, such as *E. coli* BL21AI, do not have any auxotrophy for any of the amino acids (Unlike TOP10 and derivatives) and therefore can be used in defined media. Interestingly, the *E. coli BL*21AI strain has been made to prevent leaky expression through the T7 RNA polymerase (T7 RNAP) by placing T7 RNAP into the araB locus of the araBAD operon. Therefore, and in contrast with other *E. coli* BL21 strains, *E. coli* BL21AI does not consume arabinose. Therefore, it was tested and compared to the *E. coli* NEB10β, typically used in arabinose inducible systems for both molecular biology and expression, but also devoid of arabinose metabolism. A two-fold increase in expression was observed when using *E. coli* BL21AI (776 U/L^{-1} in NEB10B vs 1227 U/L^{-1} in BL21AI). which prompted us to explore the use of *E. coli* BL21AI as expression host

Typical production processes using arabinose inducible strains at laboratory scale involve the use of complex culture media based on relatively expensive and ill-defined ingredients such as yeast extracts and protein hydrolysates. These lab scale processes are normally performed using Erlenmeyer flasks, although they are easily implemented in lab-scale bioreactors, obtaining some advantages in terms of biomass and final product titers (See Table 1 for comparison between Erlenmeyer flask fermentation and Bioreactor Batch in TB broth with BL21AI), thanks mainly to the controlled pH environment and the improved aeration. Nevertheless, these processes had little room for further improvement towards HCDC strategies and are costly to export to industrial scale, as previously mentioned. In order to develop a cost-effective and efficient HCDC, the feasibility for the production of HMFO in a cheaper mineral medium using arabinose as inducer had to be tested. As previously stated, arabinose driven expression systems are repressed when glucose is present the culture medium, so we tested glycerol as a sole carbon source in order to avoid expression interference and because it is a cheap industrial by-product, making it suitable for large-scale protein production. 2 L batches were performed comparing the performance for HMFO production in a bioreactor either in terrific broth (TB) or in mineral media with glycerol (MDG).

Figure 1 shows the monitored biomass and HMFO activity measurements throughout the fermentation. As depicted, there are two main differences between both processes. On one hand, *E. coli* grown on TB media only need 7 h to achieve 5 g of DCW whereas in glycerol-based DM media 25h is needed to reach the same biomass amount. The specific growth rate is, in accordance with these observations, 4 times faster in TB (0.47 h^{-1}) than in DM+Gly (0.12 h^{-1}). Nevertheless, despite achieving similar final biomass in terms of DCW (around 16-17 g of DCW) in both batches, the total enzyme activity is noticeably higher in DM+Gly. Overall, both processes are similarly productive when taking the time spent for reach the final amount of enzyme. Yet, the costs for the medium components are significantly lower for DM+Gly, which made the mineral media economically attractive for the amount of enzyme produced, as shown in Table 1.

Development of a two-stage fed-batch protocol for HMFO production

In order to achieve higher cell densities and maximize HMFO titers, a HCDC protocol using a two-carbon source two-stage fed-batch strategy was developed. Although glycerol can be used as a sole carbon source increasing enzyme titter, the total time for the process also increased markedly, as demonstrated in the previous batch experiment. As an alternative approach, glucose was used as a primary carbon source until a higher biomass was generated (about 80 gDCW). Then, glucose feeding was stopped, and glycerol was added to the bioreactor when glucose was depleted. Induction was performed with a discrete arabinose addition when the start of glycerol consumption was confirmed by off-line analysis.

As depicted in Figure 2, process yield is eight times higher in terms of biomass amount with a seven-fold increase in HMFO activity, compared to the reference process in complex media. Overall, the HCDC process is three times more productive than either the TB batch or DM+Gly batch approaches, for the same amount of time, without taking into account operative considerations like fermenter availability or cleaning cycles (see Table 1).

From an operational point of view, the batch and fed batch first phase (until induction) using glucose as a carbon source are quite straightforward. The induction, nevertheless, needs to be performed when glycerol began to be consumed by the cells, as described in the Material & Methods. With a tightly controlled feeding rate and process analytics, good enzyme titers can be obtained using this protocol.

As seen in Table 1, the two-stage fed-batch HCDC protocol represents the most economical way to manufacture a certain quantity of HMFO when considering the culture medium. Costs for culture media have a significant economic impact on the production of the enzyme: it represents a fourfold reduction in costs when compared with the complex medium approach, and a 25% reduction in costs when compared with the DM+Gly batch. This shows that the protocol developed is economically attractive.

Application of the protocol for EUGO expression

To test the applicability of the developed HCDC protocol to other relevant enzymes, we also tested the production of EUGO at high cell densities using the same two-stage fed-batch strategy with the same arabinose-driven promoter to induce expression. As shown in Figure 3, the obtained results are remarkably similar in terms of biomass produced and in final product titer achieved. The main difference between the two processes is the somewhat longer adaptation time for glycerol feeding in the case of EUGO. This change in adaptation time may be caused by the specific protein expressed and, thus, needs to be determined on a case-to-case basis.

DISCUSSION

Enzyme production methods at laboratory scale are rarely directly applicable to the manufacturing of the same enzymes at industrial scale. While costs play hardly any role when producing enzymes at milligrams scale, this becomes a showstopper when translating production to large scale enzyme production (Huang et al. 2012). In this paper, we report on a protocol that allows the cost-effective production of two biocatalytically relevant oxidases using an arabinose-driven expression system. Such expression system is often used at laboratory scale due to multiple advantages (Qiu et al. 2008), but often not considered applicable at industrial scale due to several drawbacks (Miyada et al. 1984). Our newly developed two-stage fed-batch strategy allowed us to perform HCDC while using arabinose as inducer. Furthermore, by a careful selection of the expression host, inexpensive mineral media could be used. This resulted in a process that is three times more productive than the one typically used with such expression

system and complex medium. Besides, the protein titers obtained are at the same range when compared with commonly used IPTG-driven expression systems (Sivakesava et al. 1999; Yazdani et al. 2004; Farrell et al. 2015; Kante et al. 2018).

The most important part in order to tune proper protein production within the developed protocol is a proper control of the inducer, arabinose, after switching the carbon source. When properly monitored, the *E. coli* BL21AI strain shows a good specific activity when compared to the other strategies (See table 1) when glycerol is used a sole carbon source, as demonstrated in the batch experiment (Fig. 1). The two-stage HCDC protocol allows boosting the productivity not only by a dramatic increase of biomass but also due to a limited total process time, due to the use of glucose in the first phase of the protocol. Still, this strategy leads to an inter-stage phase in which a non-adapted BL21AI strain needs to change its metabolism from glucose to glycerol consumption. Detection of glycerol consumption by proper analytics methods is critical to determine when induction by arabinose can be started. In our experience, premature induction, when the metabolism is not completely adapted to glycerol, can result in significantly lower productivities (data not shown). Despite the above considerations, once the inter-stage transition can be properly monitored through reliable glycerol analytics and feeding control, this two-stage protocol can become a very valuable tool for recombinant protein production using cheap culture media and inducer, with reproducible results for the expression of industrially relevant oxidases and other enzymes or recombinant proteins.

To conclude, in the present work we developed a two-stage fed-batch protocol for the production of HMFO and EUGO using an arabinose-inducible promoter. The workflow results in a 6-fold increase in productivity when compared with reference batch fermentations. The developed flexible protocol enables production of oxidases in a cost-effective manner, a crucial factor when considering industrial application of biocatalysts.

AUTHOR'S CONTRIBUTIONS

RR, NL and ACM conceived and designed the research. RR conducted the experiments. NL and MF provided the productive strains and purified proteins for analytical purposes. RR and NL analyzed data. RR, NL, ACM, MF and GG wrote the manuscript. All authors read and approved the manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

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Conflict of interest

All authors declare that they have no conflict of interest

Ethical approval:

This article does not contain any studies with human participants performed by any of the authors

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Figures

Fig. 1. Comparison between reference batch processes in terrific broth media (black) and defined media plus glycerol (red). Circles represent total biomass obtained from OD measurements whereas romboids represents glycerol and triangles depict enzymatic activity measurements in offline samples. Bars represent total enzyme obtained in each batch measured by SDS-PAGE densitometry.

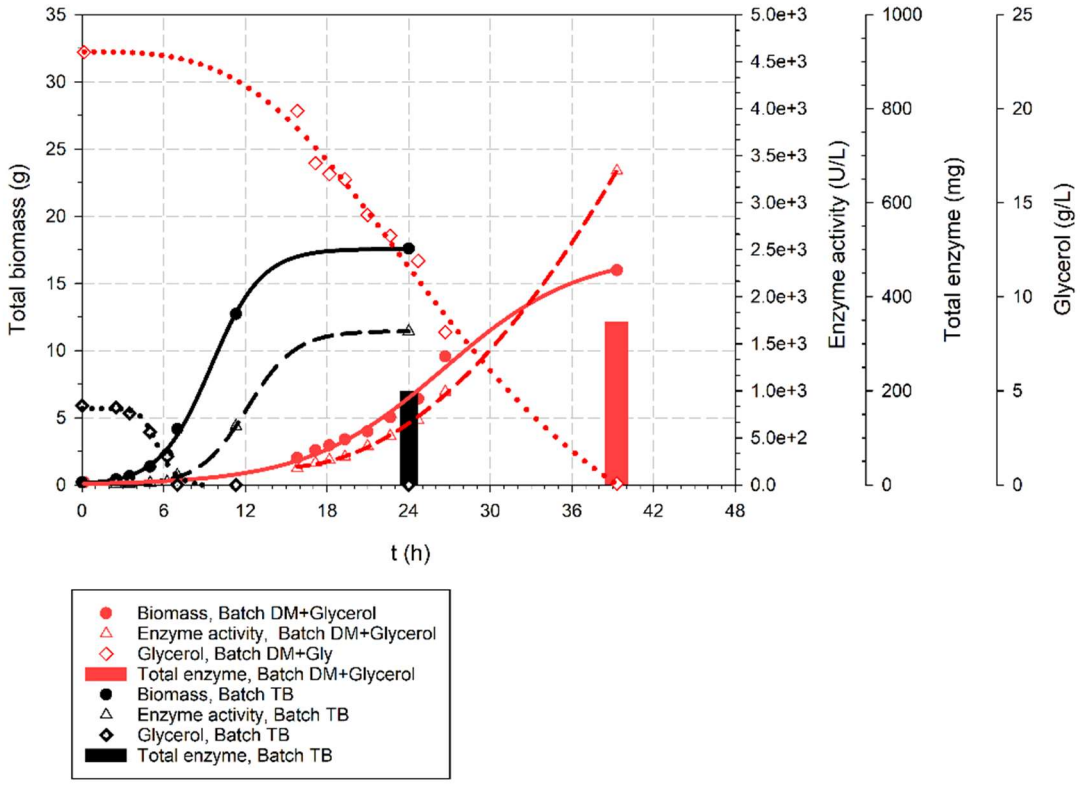


Fig. 2. Comparison between reference processes in terrific broth media (black) and two-stage fed-batch protocol (blue) for HMFO production. Circles represent total biomass obtained from OD measurements, romboids represents glycerol measurements, crossed circles represents glucose measurements and triangles depict enzymatic activity measurements in offline samples. Bars represent total enzyme obtained in each batch measured by SDS-PAGE densitometry. Arrows indicate the induction time in each case.

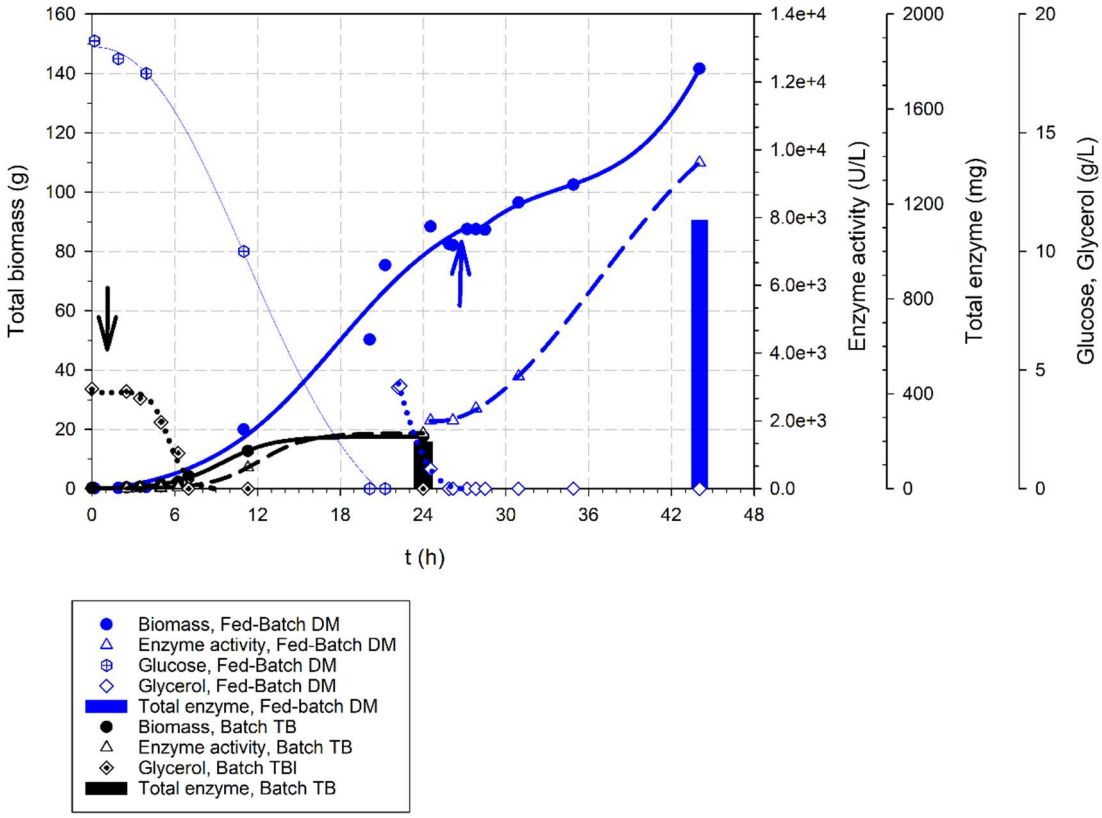
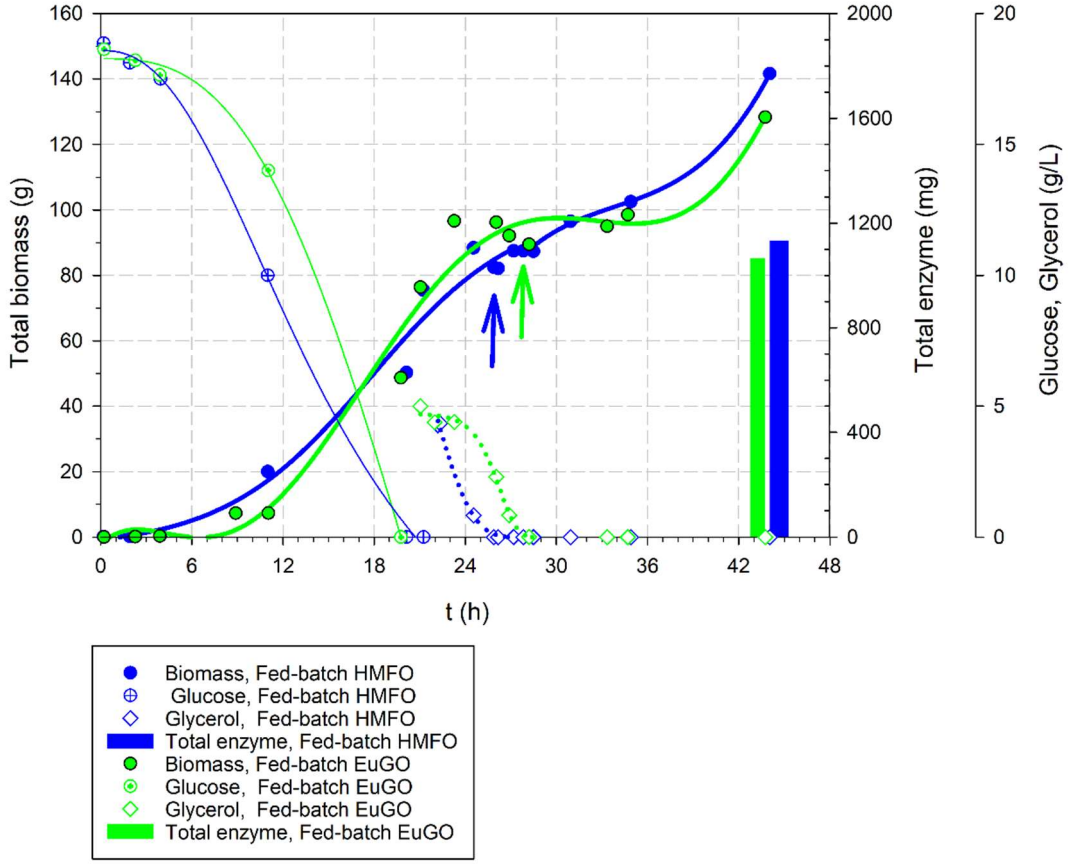


Fig.3. Comparison between two-stage fed-batch protocol for HMFO production (blue) and for EUGO production (green). Circles represent total biomass obtained from OD measurements whereas triangles depict enzymatic activity measurements in offline samples. Bars represent total enzyme obtained in each batch measured by SDS-PAGE densitometry. Arrows indicate the induction time in each case.



501 Table 1: *Relevant process parameters of each evaluated process at 2 L scale.*

	t (h)	DCW (g)	BIOMASS YIELD (gY/gS)	ENZYME ACTIVITY (U/L^{-1})	TOTAL ENZYME (mg)	μ_{MAX} (h^{-1})	SPECIFIC ACTIVITY ($U/gDCW^{-1}$)	VOLUMETRIC PRODUCTIVITY ($U/L^{-1}h^{-1}$)	PROCESS PRODUCTIVITY (mg/h^{-1})	CULTURE MEDIA COST ($€/L^{-1}$)	CULTURE MEDIA ECONOMIC IMPACT (€ OF MEDIA/g OF ENZYME)
ERLENMEYER TB	22	8.7	-	1227	148	0.47	300.0	55.8	6.72	5.76	77.8
BATCH TB	24	17.6	-	1634	200	0.47	180.4	68.1	8.33	5.76	57.6
BATCH DM+GLY	39	16.0	0.35	3340	347	0.12	405.0	85.7	8.82	3.23	18.6
TWO-STAGE HCDC	44	142	0.38 (Glu) 0.27 (Gly)	9625	1133	0.20 (Glu) 0.05 (Gly)	140.1	218.8	25.7	7.93	14.0

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Electronic supplementary material for

High-level production of industrially relevant oxidases by a two-stage fed-batch approach: Overcoming catabolite repression in arabinose-inducible *Escherichia coli* systems

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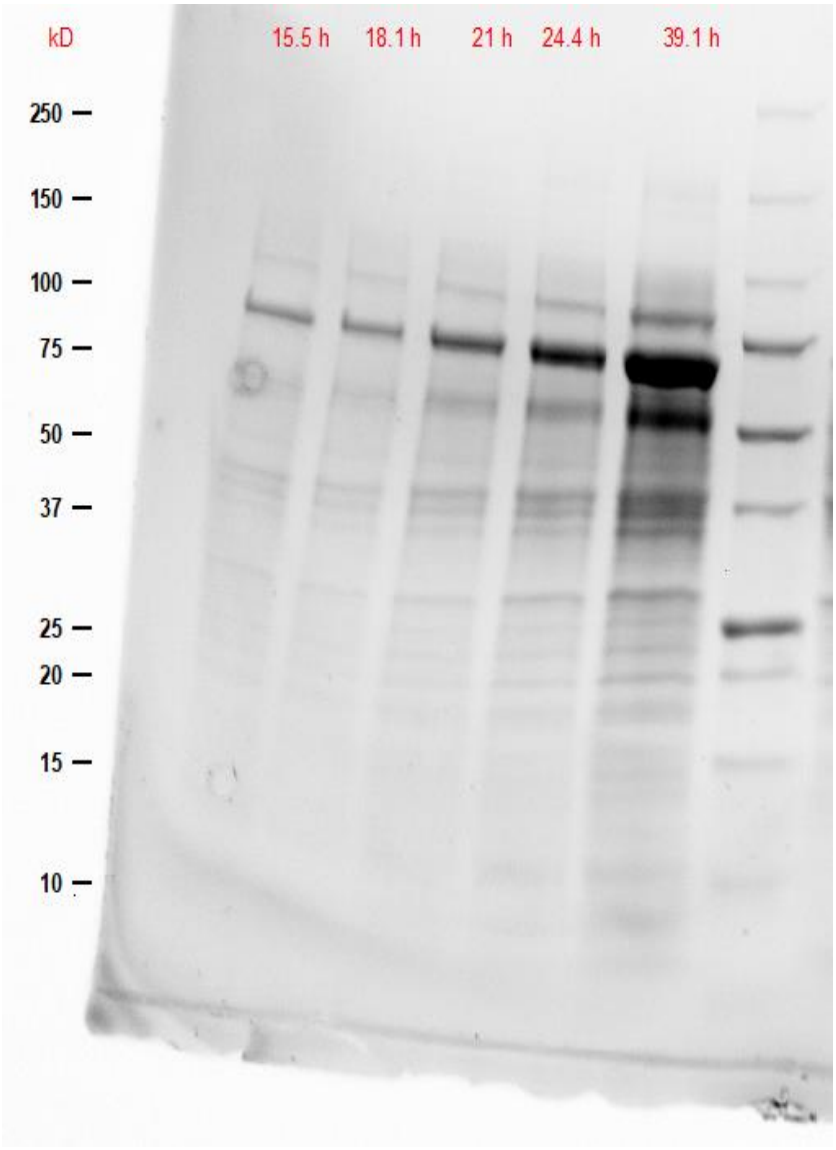


Figure S 1: Time course expression of SUMO-HMFO during DM+Gly Batch (Expected MW of 68kD). Molecular weight scale in kilodaltons (kD)

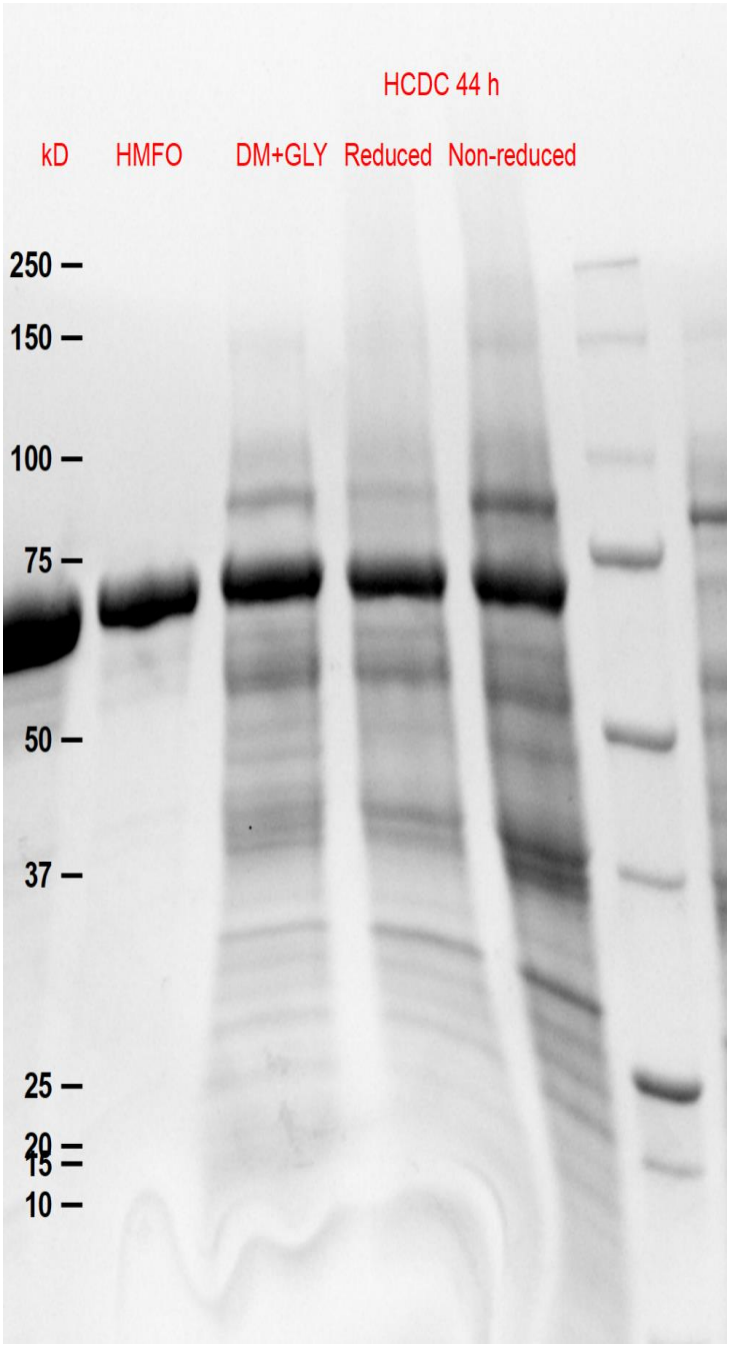


Figure S 2: Comparison of purified HMFO (first lane) with several fermentation samples (Dilution normalized by HMFO activity). Molecular weight scale in kilodaltons (kD)

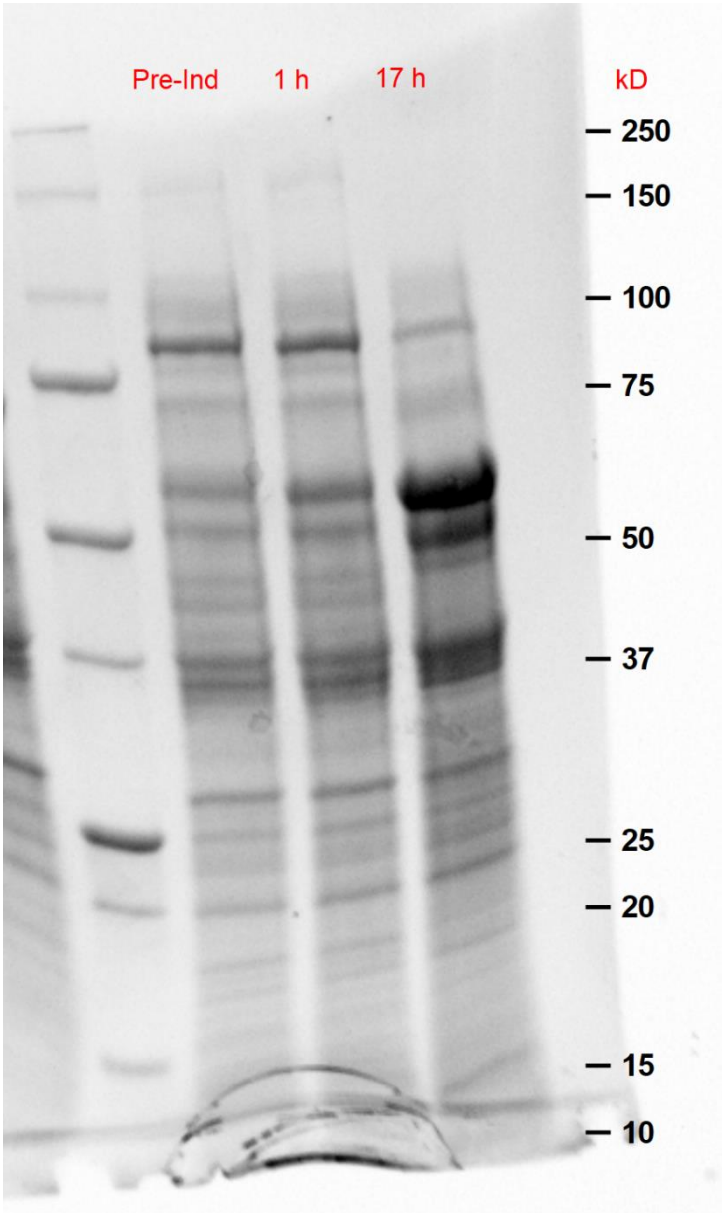
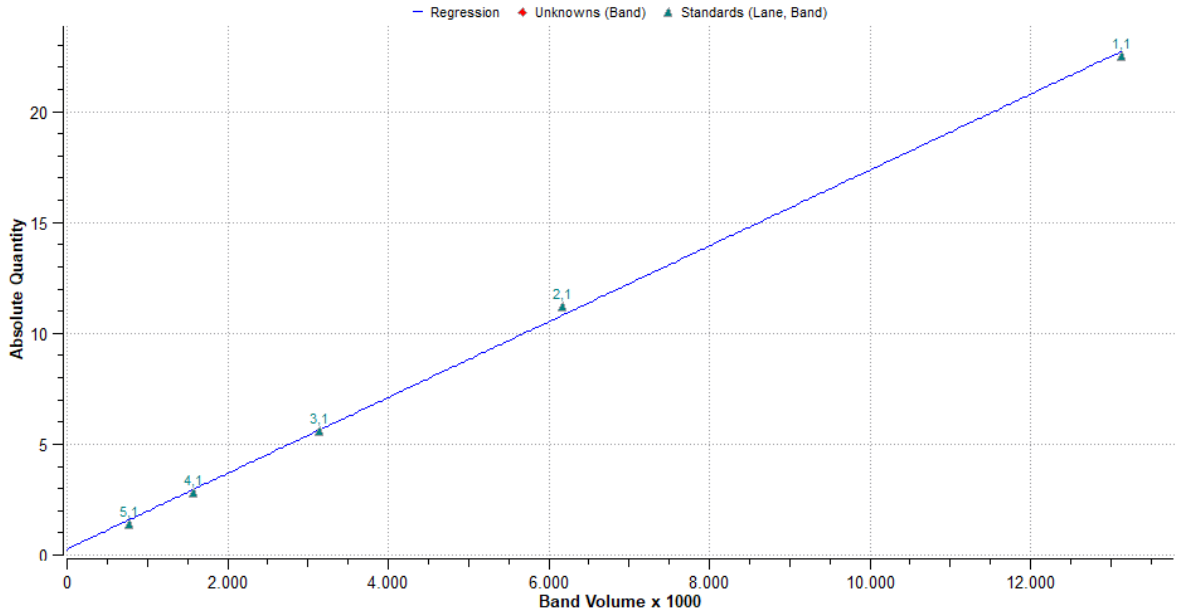


Figure S 3: Overexpression of EUGO during two stage HCDC Fed-batch (Expected molecular weight: 58Kda). Molecular weight scale in kilodaltons (kD)



Regression Method:	Linear
Formula:	y = 1.71E-06 * x + 0.253
R-Squared Value	0.999013

Figure S 4: SDS-PAGE calibration curve for purified SUMO-HMFO (Y-axis in micrograms)