

ARTICLE

Decoupling of recombinant protein production from *Escherichia coli* cell growth enhances functional expression of plant Leloir glycosyltransferases

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Abstract

Sugar nucleotide-dependent (Leloir) glycosyltransferases from plants are important catalysts for the glycosylation of small molecules and natural products. Limitations on their applicability for biocatalytic synthesis arise because of low protein expression (≤ 10 mg/L culture) in standard microbial hosts. Here, we showed two representative glycosyltransferases: sucrose synthase from soybean and UGT71A15 from apple. A synthetic biology-based strategy of decoupling the enzyme expression from the *Escherichia coli* BL21(DE3) cell growth was effective in enhancing their individual (approximately fivefold) or combined (approximately twofold) production as correctly folded, biologically active proteins. The approach entails a synthetic host cell, which is able to shut down the production of host messenger RNA by inhibition of the *E. coli* RNA polymerase. Overexpression of the enzyme(s) of interest is induced by the orthogonal T7 RNA polymerase. Shutting down of the host RNA polymerase is achieved by L-arabinose-inducible expression of the T7 phage-derived Gp2 protein from a genome-integrated site. The glycosyltransferase genes are encoded on conventional pET-based expression plasmids that allow T7 RNA polymerase-driven inducible expression by isopropyl- β -D-galactoside. Laboratory batch and scaled-up (20 L) fed-batch bioreactor cultivations demonstrated improvements in an overall yield of active enzyme by up to 12-fold as a result of production under growth-decoupled conditions. In batch culture, sucrose synthase and UGT71A15 were obtained, respectively, at 115 and 2.30 U/g cell dry weight, corresponding to ~ 5 and $\sim 1\%$ of total intracellular protein. Fed-batch production gave sucrose synthase in a yield of 2,300 U/L of culture (830 mg protein/L). Analyzing the isolated glycosyltransferase, we showed that the improvement in the enzyme production was due to the enhancement of both yield (5.3-fold) and quality (2.3-fold) of the soluble sucrose synthase. Enzyme preparation from the decoupled production comprised an increased portion (61% compared with 26%) of the active sucrose synthase homotetramer. In summary,

Abbreviations: GmSuSy, sucrose synthase from soybean (*Glycine max*); GT, glycosyltransferase; SEC, size-exclusion chromatography; UGT71A15, UDP-glycosyltransferase 71A15.

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therefore, we showed that the expression in growth-arrested *E. coli* is promising for recombinant production of plant Leloir glycosyltransferases.

KEYWORDS

growth-arrested *E. coli*, Leloir glycosyltransferase, protein quality, recombinant protein production, small-molecule glycosylation, synthetic biology

1 | INTRODUCTION

Plant metabolisms for natural product biosynthesis and detoxification involve an elaborate enzymatic machinery for attaching sugars onto noncarbohydrate small-molecule structures (Bowles, Lim, Poppenberger, & Vaistij, 2006). This glycosylation machinery comprises a large set of sugar nucleotide glycosyltransferases (GTs). The enzymes also referred to collectively as Leloir GTs, transfer glycosyl residues from sugar nucleotide donors to specific positions on acceptor substrates (Liang et al., 2015). Compared with other enzymes (e.g., glycoside hydrolases; glycoside phosphorylases) that are also able to glycosylate small molecules, the GTs often present a unique combination of high chemo/regioselectivity and relatively flexible substrate specificity (R. Chen, 2018; Desmet et al., 2012; Thuan & Sohng, 2013). The plant GTs are, therefore, promising catalysts for glycoside production (for reviews, see: Lim, 2005; Nidetzky, Gutmann, & Zhong, 2018). Glycosylated derivatives of small molecules (e.g., flavonoids, terpenoids, peptides) with important applications in the food, fragrance, cosmetic, and chemical industries are synthesized efficiently using GTs (De Bruyn, Maertens, Beauprez, Soetaert, & De Mey, 2015; Desmet et al., 2012; Hofer, 2016; Hsu et al., 2018; Kim, Yang, Kim, Cha, & Ahn, 2015; Nidetzky et al., 2018; Olsson et al., 2016; Schmölzer, Lemmerer, & Nidetzky, 2018; Schwab, Fischer, & Wüst, 2015; Schwab, Fischer, Giri, & Wüst, 2015; Xiao, Muzashvili, & Georgiev, 2014).

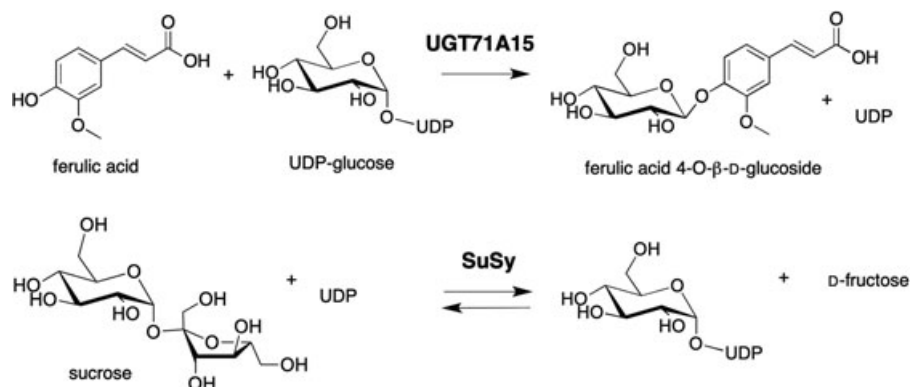
Limitation on the applicability of plant GTs arises from the fact that these enzymes are difficult to express in standard microbial hosts (Desmet et al., 2012; Lim, 2005; Nidetzky et al., 2018). *Escherichia coli* is most often used. In general, expression is low (≤ 10 mg/L; e.g., Arend, Warzecha, Hefner, & Stöckigt, 2001; Schmölzer, Gutmann, Diricks, Desmet, & Nidetzky, 2016) and poor (≤ 1 mg/L) in various instances (e.g., Cai et al., 2017; Welner et al., 2017). With notable exceptions (Arend et al., 2001; Dewitte et al., 2016; Priebe, Daschner, Schwab, & Weuster-Botz, 2018; Schmideder et al., 2016), the enzyme production has received relatively little attention for systematic process development. The main bottlenecks on the production efficiency thus remain largely unknown. Besides specific requirements an individual GT may have, it seems probable that there are also important factors of a more general, if not universal relevance. Discovery of such factors and process optimization along the lines thus suggested would present important advances in the biocatalytic application of GTs.

In a recent study of a bacterial Leloir GT (sucrose synthase from *Acidithiobacillus caldus*; Diricks, De Bruyn, Van Daele, Walmagh, &

Desmet, 2015) we showed that the constitutive expression in *E. coli* BL21 shifted the production of recombinant protein mainly to the stationary growth phase (Schmölzer, Lemmerer, Gutmann, & Nidetzky, 2017). Once the glucose carbon source had been depleted, the active enzyme was accumulated gradually to a substantial titer of ~ 350 mg/L of culture. This result gave rise to the working hypothesis of this study, namely, the expression in growth-arrested *E. coli* might constitute a general strategy for efficiency-enhanced production of (plant) GTs.

Here, we used a synthetic biology-based approach to decouple *E. coli* BL21(DE3) cell growth from the target gene overexpression (Mairhofer, Striedner, Grabherr, & Wilde, 2016). The underlying concept is built upon the Gp2 protein from the bacteriophage T7. Gp2 inhibits the *E. coli* endogenous RNA polymerase (Mekler, Minakhin, Sheppard, Wigneshweraraj, & Severinov, 2011) whereas it leaves T7 RNA polymerase unaffected (Mairhofer, Striedner et al., 2016). L-Arabinose-inducible expression of the Gp2 gene, thus, allows for the host RNA polymerase to be shut off and, hence, the cell growth to be arrested in a controllable fashion. Under the conditions managed by Gp2, the *E. coli* protein synthesis machinery is taken over for recombinant production of the target protein(s). The practical design embodied in enGenes technology involves genome integration of the Gp2 coding gene under control of the *araB* promoter inducible by L-arabinose. Within this strain background, a pET plasmid vector is used that contains the gene(s) of interest inducible by isopropyl- β -D-galactoside. This design provides flexibility and temporal control for the cell proliferation to be switched off and protein production to be induced (Mairhofer, Striedner et al., 2016; Mairhofer, Stargardt et al., 2016). It presents a new approach toward quiescent *E. coli* cells applied to recombinant protein production (for alternative approaches, see: C. Chen, Walia, Mukherjee, Mahalik, & Summers, 2015; Ghosh, Gupta, & Mukherjee, 2012; Mahalik, Sharma, & Mukherjee, 2014).

Here, we demonstrate the application of the outlined approach to individual and combined production of two representative GTs, the sucrose synthase from soybean (*Glycine max*; GmSuSy; Bungaruang, Gutmann, & Nidetzky, 2013) and the flavonoid GT UGT71A15 from apple (*Malus domestica*; Lepak, Gutmann, Kulmer, & Nidetzky, 2015). The GT reactions are shown in Scheme 1. Enzyme coexpression reflects the idea of a glycosylation cascade in which the sugar nucleotide donor substrate is formed in situ and continuously regenerated (Scheme 1; Nidetzky et al., 2018). We showed that the enzyme production in growth-arrested *E. coli* gives significant improvements in the amount and quality of the recombinant GT as compared with the exactly comparable production in the growing



SCHEME 1 The reaction of the glycosyltransferase UGT71A15 as used in this study and reaction of sucrose synthase (SuSy). Coupling of the SuSy reaction to the glycosyltransferase reaction allows for a glycoside synthesis with in situ regeneration of the uridine diphosphate-glucose as donor substrate (for review, see Nidetzky et al., 2018; Schmölzer et al., 2016; for coupled use of UGT71A15 and SuSy, see Lepak et al., 2015)

E. coli reference. We also showed transferability of the production strategy to a high-cell-density fed-batch culture of *E. coli* at 20 L operating scale and obtained up to 830 mg GT protein/L of culture in that way.

2 | MATERIALS AND METHODS

2.1 | Microbial strains

The *E. coli* strain referred to as enGenes-X-press is a BL21(DE3) derivative with the genotype *E. coli* B F⁻ompT gal dcm lon hsdS_B(r_B⁻m_B⁻) λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*] [*malB*⁺]_{K-12}(λS) attTn7::<araC-p_{araB}-gp2> Δ*araABCD*::CAT^R (Mairhofer, Striedner et al., 2016). The enGenes-X-press has the Gp2 coding sequence integrated into the genome at the Tn7 attachment (attTn7) site. The Gp2 gene expression is controlled by the L-arabinose inducible *araB* promoter and involves transcription by T7 RNA polymerase. The inserted sequence additionally involves a regulator (*araC*) and a chloramphenicol resistance gene. Furthermore, the strain features knockout of the complete L-arabinose degrading operon *araABCD*. The strain enGenes-X-press is proprietary material of enGenes Biotech GmbH. Its construction was reported elsewhere (Mairhofer, Striedner et al., 2016). The reference *E. coli* strain BL21(DE3) was from the Coli Genetic Stock Center (CGSC#: 12504). To compare the performance of the two *E. coli* strains, both were transformed with the relevant expression plasmids, as described below. It was shown (Mairhofer, Striedner et al., 2016) that in the absence of induction by L-arabinose, enGenes-X-press exhibits a growth behavior not different from that of *E. coli* BL21(DE3) and that the presence of L-arabinose does not affect the growth of the host strains as such.

2.2 | Enzyme expression

The previously described pET-STRP3 expression vector was used (Lepak et al., 2015). Enzymes are thus produced as fusion proteins

harboring N-terminal Strep-Tag II. For single enzyme expression, the coding genes were inserted via *NdeI* and *XhoI* restriction sites. The GmSuSy gene (GenBank: AF030231) was codon optimized for expression in *E. coli* (Bungarung et al., 2013). The UGT71A15 gene (GenBank: DQ103712) was used.

For enzyme coexpression, the vector referred to as pETduo was derived from pET-STRP3 by inserting the UGT71A15 gene into the plasmid harboring the GmSuSy gene. Suitable oligonucleotide primers (pDUO_Ins_fwd, pDUO_Ins_rev; Supporting Information) were used to amplify the introduced expression cassette by specific binding upstream of the promoter region and downstream of the terminator sequence. The receiving vector was linearized by amplification using pETduo_BB_fwd and pETduo_BB_rev (Supporting Information) as forward and reverse primer, respectively. The F1 ORI was eliminated in this step (Figures S1 and S2). The resulting DNA fragments were purified on agarose gel and fused by homologous recombination. The sequences of both GT coding genes were verified in the final expression vector.

Expression strains were obtained by transforming the respective expression vector into electrocompetent cells of the *E. coli* BL21(DE3) reference strain and the strain enGenes-X-press.

2.3 | Batch bioreactor cultivations

With exceptions noted, the strains *E. coli* BL21(DE3) and enGenes-X-press were cultivated under exactly comparable conditions. Each strain harbored the pET expression vector for production of GmSuSy, UGT71A15, or both. Precultures (250 ml in 1,000 ml baffled-shaken flasks) were inoculated from the glycerol stocks (100 μl) and incubated overnight at 37°C and 130 rpm (Certomat[®] BS-1; Sartorius, Göttingen, Germany). LB medium supplemented with sterile filtered antibiotics (*E. coli* BL21(DE3): 50 μg/ml kanamycin; enGenes-X-press: 50 μg/ml kanamycin; 34 μg/ml chloramphenicol) was used.

Bioreactor cultivations were performed, parallelly, in two Labfors III 3.6 L bioreactors from Infors HT Multitron (Bottmingen, Switzerland). A semisynthetic medium (Table S1) prepared from separately

autoclaved components was used. To this, 0.4 ml/L of a sterile filtered trace element solution was added (Table S2). Note: Glycerol was used as the carbon source. Glucose was not used in the batch cultivations because of the well-known effect of carbon catabolite repression by glucose on the induction of gene expression under control of the *araB* promoter and induced by L-arabinose (Brückner & Titgemeyer, 2002; Cagnon, Valverde, & Masson, 1991; Lee & Jung, 2007). A value of 40% air saturation was maintained using agitation and airflow cascade. Polypropylene glycol (PPG; 10%) was used for foam control. The pH was maintained at 7.0 using automated addition of 2 M KOH and 1 M H₃PO₄. Bioreactors were inoculated to an optical density (OD)_{600nm} of 0.5 at a temperature of 37°C. The temperature was lowered as per Table 1 before induction and initiated at an OD_{600nm} of 5. The *E. coli* BL21(DE3) strain was induced with filter-sterilized isopropyl β-D-1-thiogalactopyranoside (IPTG) solution to a final concentration of 0.1 mM. The enGenes-X-press strain was induced with IPTG (0.1 mM) and L-arabinose (100 mM). Samples (10 ml) were taken at certain times and analyzed for cell dry mass and glycerol.

Cell dry mass was determined from 10 ml samples. The cells were centrifuged, washed with 20 ml distilled water, resuspended, and transferred to a dried and weighed beaker, which was then dried at 105°C for 24 hr and reweighed. Cells were disrupted by ultrasonification (Supporting Information) to obtain the cell extract for measurement and purification.

2.4 | Fed-batch bioreactor cultivations at a larger scale

The strains *E. coli* BL21(DE3) and enGenes-X-press harboring the pET expression vector for production of GmSuSy were used. Fed-batch cultivations were performed in a Bioengineering AG bioreactor (Type NLF22; Wald, Switzerland) with 20 L total working volume (10 L batch volume). The bioreactor was equipped with standard computer-controlled units (Siemens Simatic S7; WinCC; Siemens AG, Munich, Germany). Semisynthetic medium was used (see the Supporting Information). Its composition was adjusted for a total production of 1,580 g dry cell mass. Note: As the cultivation was

done under carbon source-limited growth conditions, glucose could be used. This was not feasible in the batch cultivations.

Precultures (250 ml in 2,000 ml baffled-shaken flasks) were inoculated from cell bank vials (500 μl) and incubated at 37°C and 180 rpm (Infors HT Multitron) until an OD_{600nm} of 3.0–3.5 was reached. An amount of 1,000 OD_{600nm} units was transferred into 400 ml of 0.9% (by weight) NaCl solution and added to the bioreactor. Air saturation was kept at 30% through stirrer speed and aeration rate control. The O₂ and CO₂ contents in the outlet air were measured with a BlueSens (Herten, Germany) BlueInOne Gas Analyzer. The pH was controlled at 7.0 (±0.05) using 25% (by weight) NH₄OH solution. During the batch phase, the temperature was 37°C ± 0.5°C. PPG-2000 was added as antifoam (1.5 ml/L batch culture). Before starting the feeding, when the culture had reached stationary phase (after ~10 hr; ~8 g dry cell mass/L), the temperature was lowered to 30°C. Initially, an exponential substrate feed was used to provide a constant specific growth rate of 0.17 hr⁻¹ over 11 hr. Then, a linear substrate feed (9.54 g glucose/min) over 4 hr and another linear substrate feed (4.35 g glucose/min) over 15 hr were applied (see the Supporting Information). Substrate feed involved superimposed feedback control of weight loss in the substrate tank. Protein expression was induced after 15 hr of substrate feed. It included the addition of 31.6 mmol IPTG (=20 μmol/g cell dry mass) and 0.1 M L-arabinose based on the final volume of ~20 L. The induction solution contained 79 mM of (NH₄)₂SO₄ based on the end volume. PPG-2000 (500 ml in total) was used for foam control. Samples were taken at certain times and the cell growth was recorded as an increase in OD_{600nm}. The cells were harvested after 40 hr by centrifugation (30 min at 4°C and 4,420 g; Sorvall RC-5B; ThermoFisher Scientific, Waltham, MA) and stored at -20°C. They were disrupted by high-pressure homogenization (Supporting Information) to obtain cell extract for measurement and purification.

2.5 | Protein purification and characterization

2.5.1 | Strep-Tag purification

Prepacked 1 or 5 ml Strep-Tactin Sepharose columns (IBA Life Sciences, Göttingen, Germany) was used on an ÄKTA Explorer 100

TABLE 1 Glycosyltransferase activities from batch bioreactor cultivations of enGenes-X-press and the *Escherichia coli* BL21(DE3) reference

Glycosyltransferase	Construct	Induction temperature (°C)	Reference (U/L) ^a	enGenes-X-press (U/L) ^a	Reference (U/g) ^a	enGenes-X-press (U/g) ^a	Fold increase ^c
GmSuSy	single	30	285	700	25	115	4.6 (2.4)
UGT71A15	single	30	0.3	1.4	0.0	0.2	8.9 (4.7)
GmSuSy	single	25	8.7	16.7	1.1	4.2	3.9 (1.9)
UGT71A15	single	25	3.9	10.6	0.5	2.3	4.4 (2.7)
GmSuSy ^b	double	25	3.5	3.7	0.5	0.7	1.6 (1.1)
UGT71A15 ^b	double	25	1.8	3.3	0.2	0.6	2.6 (1.8)

Note. GmSuSy: sucrose synthase from soybean (*Glycine max*); UGT71A15: UDP-glycosyltransferase 71A15.

^aRecorded at the end of the bioreactor cultivation; results are from biological duplicates or triplicates and agree within less than 10% relative SD.

^bActivities were measured individually.

^cRefers to U/g data and (in brackets) U/L data.

system (GE Healthcare, Pasching, Austria) and operated at a flow rate of 1 and 5 ml/min, respectively. Tris/HCl buffer (100 mM, pH 8.0, 150 mM NaCl, 1 mM EDTA) was used. Alternatively, a column (0.5 cm inner diameter; 10 cm length) of Streptactin Sepharose High Performance (GE Healthcare) was used. The flow rate was 1 ml/min. Cell extract filtered through 1.2- μ m cellulose-acetate syringe filter was loaded onto the column. After loading, the column was flushed with buffer until the UV signal (280 nm) signal reached the baseline level. Elution was done with two column volumes of desthiobiotin (2.5 mM). The columns were regenerated with hydroxy-azo phenylbenzoic acid (1 mM) and equilibrated again with buffer.

2.5.2 | Size-exclusion chromatography

This was performed using a Superdex 200 increase 10/300 GL column (GE Healthcare) operated on ÄKTA Explorer 100 system. All runs were performed in 10 mM phosphate buffer (pH 7.4) containing 300 mM NaCl. The flow rate was 0.5 ml/min and UV detection (280 nm) was used. Strep-Tactin eluate (200 μ l) was applied to the column. For analytical SEC, a G3000SWXL column (300 \times 7.8 mm, inner diameter 5 μ m; Tosoh Bioscience, Tokyo, Japan) was used.

2.6 | Enzymatic activity measurements

Assays were performed at 30°C in 1.5 ml tubes on an Eppendorf (Vienna, Austria) Thermomixer comfort at 300 rpm. The enzyme was added as *E. coli* cell extract or in purified form to start the reaction. Product release was measured in samples (20 μ l) taken at four different times. Samples were diluted into acetonitrile (180 μ l) to stop the reaction. Precipitated protein was removed by centrifugation for 20 min at room temperature and 13,200 rpm. About 5–10 μ l of supernatant was used for further analysis. Enzymatic rates were determined from the linear time courses of product formation. One Unit (U) of enzyme activity is the amount of enzyme producing 1 μ mol product/min under the assay conditions.

2.6.1 | Sucrose synthase from soybean (*Glycine max*)

Sucrose (500 mM) and uridine 5'-diphosphate (10 mM) were used as substrates in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (100 mM; pH 7.5) containing 13 mM MgCl₂ and 0.13% bovine serum albumin (BSA; Bungaruang et al., 2013). The released uridine diphosphate (UDP)-glucose was measured by high-performance liquid chromatography (HPLC).

2.6.2 | UDP-glycosyltransferase 71A15

Ferulic acid (1 mM) and UDP-glucose (2 mM) were used as substrates in the HEPES buffer (50 mM; pH 7.5) containing 50 mM KCl, 13 mM MgCl₂, 0.13% BSA, and 2% dimethyl sulfoxide. The released ferulic acid-4-O- β -D-glucoside (Scheme 1) was measured by HPLC.

2.7 | Analytical HPLC methods

2.7.1 | Glycerol

This was analyzed on a Merck-Hitachi LaChrome HPLC System equipped with an Aminex HPX-87H column (BioRad, Richmond, CA), a Merck-Hitachi LaChrome L-7250 autosampler, and a Merck L-7490 RI detector. The system was operated at 65°C, using a flow rate of 0.6 ml/min with 5 mM sulfuric acid as the eluent.

2.7.2 | Reaction of GmSuSy

UDP-glucose and UDP were analyzed by reversed phase HPLC using a Kinetex[®] C18 column (5 μ m, 100 Å, 50 \times 4.6 mm; Phenomenex, Torrance, CA) in reversed phase ion-pairing mode. The analysis was performed at 35°C with a mobile phase of 87.5% 20 mM potassium phosphate buffer (pH 5.9) containing 40 mM tetra-*n*-butylammonium bromide and 12.5% acetonitrile. An isocratic flow rate of 2 ml/min was used and the detection was at 262 nm.

2.7.3 | Reaction of UGT71A15

Ferulic acid and ferulic acid-4-O- β -glucoside were analyzed by reversed phase HPLC using the above described Kinetex[®] C18 column in the reversed phase mode. The analysis was performed at 35°C using a gradient separation (mobile phase A: H₂O + 0.1% formic acid and mobile phase B: acetonitrile + 0.1% formic acid) at a flow rate of 1 ml/min. Gradient conditions were as follows: 0.00 min 10% B; 0.50 min 10% B; 4.00 min 70% B; 4.30 min 70% B; 4.31 min 10% B; 7.00 min 10% B. Detection was at 320 nm. Authentic standards were used for calibration.

3 | RESULTS AND DISCUSSION

The enzymes used are representative of plant GTs applied to biocatalytic glycosylation of small molecules (Nidetzky et al., 2018). UGT71A15 shows broad acceptor substrate specificity and has previously been used for the glycosylation of flavonoids (Lepak et al., 2015). It is a member of the GT family GT-1, a large enzyme family comprising numerous plant GTs involved in small-molecule glycosylation (Liang et al., 2015). UGT71A15 is a 50.2 kDa protein that functions as a monomer. GmSuSy belongs to the GT family GT-4. It is a functional homotetramer composed of 92.2 kDa subunits. GmSuSy has previously been used for UDP-glucose recycling in glycosylation reactions by coupled GTs, including UGT71A15 (Scheme 1; Lepak et al., 2015; Schmölzer et al., 2016; Schmölzer et al., 2018).

3.1 | Single gene expression for production of GmSuSy

We performed controlled batch bioreactor cultivations at 25 and 30°C to compare enzyme production in enGenes-X-press under

growth arrest to enzyme production in the normal growing *E. coli* BL21(DE3). The different temperatures were chosen to examine their effect on the enzyme production and cell growth. The results are shown in Figure 1 (panels A and B; 30°C) and in Figure S3 (25°C). At 30°C, enGenes-X-press and *E. coli* BL21(DE3) showed similar growth until the time of induction (8 hr). Whereas *E. coli* BL21(DE3) continued exponential growth afterward (Figure 1b), the growth of enGenes-X-press was reduced to a very small amount, hence, effectively switched off at this point (Figure 1a). Its growth arrest notwithstanding, the strain enGenes-X-press continued consumption of the glycerol carbon source similarly as the growing *E. coli* BL21(DE3) did (Figure 1a,b). The GmSusy production, measured as unit enzyme activity/g dry cell mass was far superior in enGenes-X-press as compared with *E. coli* BL21(DE3). Both strains started from a similar specific activity of 20 U/g at the time of induction. Whereas in *E. coli* BL21(DE3) the specific activity was constant in the induction phase, it increased almost linearly with time in enGenes-X-press and reached 115 U/g after 24 hr. This represents an approximately fivefold enhancement of specific activity compared with the reference. Cultivations at 25°C (Figure S3a,b) gave only poor enzyme production, with an activity of just ~4 U/g in enGenes-X-press and ~1 U/g in *E. coli* BL21(DE3). The strain growth was still switched off reliably in enGenes-X-press through induction but glycerol consumption was slow compared with *E. coli* BL21(DE3) in the postinduction phase. However, 25°C was not an

option for GmSusy production, we showed later that it was one for production of UGT71A15.

In Table 1 we summarize the production parameters of the different bioreactor cultivations performed. In terms of enzyme activity/culture volume, the production in enGenes-X-press was effective in improving (~2.5-fold) the reference production. However, decreased biomass formation by enGenes-X-press as compared with *E. coli* BL21(DE3) mitigated the effect of growth-arrested production on the cell mass-based specific enzyme activity. We showed later that enzyme production in high-cell density fed-batch culture could overcome this problem. Using the specific activity of the purified GmSusy of 4.5 U/mg protein, comparable to the earlier studies (Bungarung et al., 2013), we calculated that production in enGenes-X-press at 30°C gave an overall enzyme yield of 156 mg/L culture. The corresponding production in *E. coli* BL21(DE3) gave 63 mg/L. These values are used together with measurements of total protein content/g cell mass and activity/g cell mass to estimate that the recombinant GmSusy accounted for roughly ~5% and ~1% of the total intracellular protein in enGenes-X-press and *E. coli* BL21(DE3), respectively.

3.2 | Single gene expression for the production of UGT71A15

UGT71A15 was previously noted to be difficult to produce. In a shake-flask culture of *E. coli* BL21(DE3) about 1–5 mg protein/L was

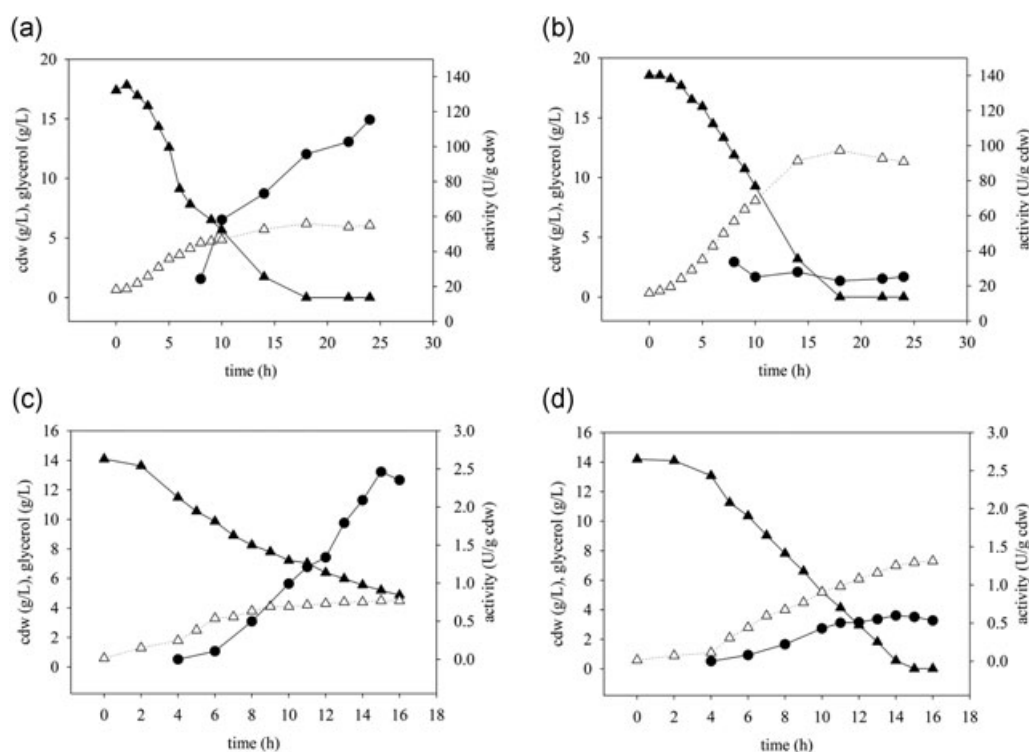


FIGURE 1 Time courses of growth, glycerol consumption, and enzyme formation in batch bioreactor cultivations of enGenes-X-press (a,c) and *Escherichia coli* BL21(DE3) (b,d) producing GmSusy (a,b) and the glycosyltransferase UGT71A15 (c,d). The induction temperature was 30°C for GmSusy production (a,b) and 25°C for UGT71A15 production (c,d). The symbols show cell dry mass concentration, open triangles; glycerol concentration, full triangles; volumetric enzyme activity, full circles. GmSusy: sucrose synthase from soybean (*Glycine max*); UGT71A15: UDP-glycosyltransferase 71A15

obtained (Lepak et al., 2015). The same series of bioreactor cultivations described above for GmSuSy were carried out with UGT71A15. The results are shown in Figure 1 (panels C and D; 25°C) and Figure S4 (30°C). Overall trends in biomass growth and glycerol consumption were similar as noted before. Production at 25°C was strongly preferred overproduction at 30°C, probably because UGT71A15 was not stable at the higher temperature. As observed for GmSuSy, the UGT71A15 was produced poorly in the growing *E. coli* BL21(DE3) (Figure 1d). There was only a small increase in specific enzyme activity in the phase after the induction. By contrast, UGT71A15 was formed efficiently in enGenes-X-press after the induction (Figure 1c). We noted that the arrest of growth of enGenes-X-press was less clear-cut at 25°C (Figure 1c) than it was at 30°C (Figure 1a). A marked slow-down of growth was, however, observed after the induction (Figure 1c). Nonetheless, the effect of growth reduction on enzyme production was pronounced, as evident from comparing panels C and D in Figure 1. Approximately, a fourfold increase in the specific activity was thus achieved. The activity of 2.3 U/g cell mass obtained for UGT71A15 was 50-fold lower than that obtained for GmSuSy, reflecting roughly the differences in specific activity of the two enzymes as purified proteins. The parameters of the enzyme production are listed in Table 1. The volumetric enzyme titer was 11 U/L culture when using enGenes-X-press, a 2.5-fold improvement was observed when compared with the reference strain. The specific activity of purified UGT71A15 is 0.5 U/mg. Translated into functionally expressed recombinant protein the production yield was 21 mg/L of enGenes-X-press culture, which can be compared with 8 mg/L of *E. coli* BL21(DE3) culture. In terms of abundance relative to total intracellular protein, values of ~1.0 and ~0.2% are calculated for enGenes-X-press and *E. coli* BL21(DE3), respectively.

3.3 | Gene coexpression for production of UGT71A15 and GmSuSy

Considering the temperature requirements of UGT71A15 revealed in the single gene expression studies, coexpression of the GmSuSy and UGT71A15 genes was performed at 25°C. Time courses from bioreactor cultivations of enGenes-X-press and *E. coli* BL21(DE3) are shown in Figure S5. The profiles of growth and glycerol consumption of both strains producing the two GTs (Figure S5) were highly similar as compared to their production of only UGT71A15 (Figure 1c,d). Upon induction, enGenes-X-press showed slow accumulation of both enzyme activities. The final specific activity (Table 1) was substantially lower (UGT71A15: fivefold; GmSuSy: eightfold) than the single gene expression cultures. Interestingly, the production of GmSuSy next to UGT71A15 was hardly detectable in *E. coli* BL21(DE3). Gene coexpression interfered with functional production of the individual enzymes and it did so in both enGenes-X-press and *E. coli* BL21(DE3). The volumetric titer of GmSuSy was decreased from 3.7 to 0.8 mg/L for enGenes-X-press and from 1.9 to 0.8 mg/L in the reference. For UGT71A15, titers dropped from 21.2 to 6.6 mg/L in enGenes-X-press and from 7.8 to

3.7 mg/L in the reference. Therefore, at this stage, enzyme production through single gene expression would be preferable. Irrespective of the possible incompatibility of GmSuSy and UGT71A15 for combined production in a single host, production under growth arrest in enGenes-X-press proved superior to production in growing *E. coli* BL21(DE3) under all conditions used (Table 1).

3.4 | Downstream processing and characterization of GmSuSy

Protein quality is key in recombinant protein production. Although activity is the quintessential parameter of functional expression of the enzyme, it alone is not sufficient to inform about the overall quality of the recombinant GTs production. We, therefore, isolated with Strep-Tactin affinity purification the GmSuSy produced in enGenes-X-press and *E. coli* BL21(DE3). The cell mass (~1 g dry matter each) from bioreactor cultures that run for 24 hr at 30°C (Figure 1a,b) was used. Target protein was recovered in a single sharp protein peak (Figure S6). The protein yield after purification was 26.7 mg/g for enGenes-X-press and 5.6 mg/g for *E. coli* BL21(DE3). The specific activity of isolated GmSuSy (enGenes-X-press: 5.1 U/mg; *E. coli* BL21(DE3): 4.8 U/mg) was in line with the previous report for GmSuSy produced in a shaken-flask culture of *E. coli* BL21(DE3; Bungarung et al., 2013). Analysis with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Figure S6) showed a low abundance of GmSuSy in the soluble fraction from cell disruption. On the basis of the semiquantitative densitometry, ~10% of GmSuSy was present in the soluble fraction in enGenes-X-press whereas it was only ~2% in the reference.

3.5 | Fed-batch cultivation for the production of GmSuSy at a larger scale

It was mentioned that if volumetric enzyme titer is the parameter used for evaluation, approximately, twofold lower biomass yield in enGenes-X-press cultivations as compared with *E. coli* BL21(DE3) cultivations reduces the benefit of enzyme production under conditions of arrested growth (cf. Table 1). We addressed this problem for the case of GmSuSy concomitantly with assessing the translatability of the enzyme production in enGenes-X-press to a high-cell-density fed-batch cultivation at one magnitude order larger scale (20 L). The important question was, whether under these conditions the advantage of approximately fivefold higher enzyme activity/g cell mass would be retained without compromising the total biomass formation. We recall that glucose was the carbon source used in the fed-batch cultivation. Glycerol was used in the batch cultivation to avoid the effect of carbon catabolite repression by glucose (Brückner & Titgemeyer, 2002; Cagnon et al., 1991; Lee & Jung, 2007).

The fed-batch protocol (Figure S7) gave a final biomass concentration of 56.0 g dry mass/L for enGenes-X-press and 67.5 g dry mass/L for *E. coli* BL21(DE3), a difference of just ~20% (Table 2). On the basis of the protein purified from cell material (~1 g dry

TABLE 2 Scaled-up production of GmSuSy in fed-batch bioreactor cultivation and recovery of the enzyme

<i>E. coli</i> strain	Biomass yield (g dry cells/L) ^a	Protein content(mg GmSuSy/g dry cells)	Protein recovery (mAU × ml) ^b	SEC active fraction/total active enzyme(%) ^c / -fold ^d
enGenes-X-press	56	14.8	673 ^e (80) ^f	61/12.5
BL21(DE3) reference	68	5.0	226 ^e (15) ^f	26/1

Note. GmSuSy: sucrose synthase from soybean (*Glycine max*); SEC: size-exclusion chromatography

^aFrom the end of the bioreactor cultivation. The corresponding volumetric yields of GmSuSy are 0.83 g/L using enGenes-X-press and 0.34 g/L using the BL21(DE3) reference

^bRelevant peak area (absorbance detection at 280 nm) in chromatography times the volume collected.

^cPercentage of enzymatically active protein in the eluate from the analytical SEC.

^dEnhanced production of the total active enzyme in enGenes-X-press as compared with the reference; that is: $80 \times 0.61 / (15 \times 0.26)$, from the table.

^eStrep-Tactin eluate, and

^feluate from the preparative SEC.

matter) thus received, we obtained 14.8 mg/g for enGenes-X-press and 5.0 mg/g for *E. coli* BL21(DE3), also qualitatively in line with SDS-PAGE (Figure S8). These numbers allow one to calculate a volumetric titer of GmSuSy, which was 830 mg/L for the enGenes-X-press culture and 337 mg/L for the *E. coli* BL21(DE3) culture. The recombinant GmSuSy thus accounted for 1.5% of the total intracellular protein in enGenes-X-press and only 0.5% in the reference. The specific activity of the GmSuSy isolated from fed-batch production was in the range 0.5–2.1 U/mg, between three and 10-fold lower than the specific activities of enzyme isolated from the batch bioreactor cultures.

We, therefore, subjected the enzyme obtained from Strep-Tactin purification to an additional step of size-exclusion chromatography (SEC) on a Sephadex 200 column, as shown in Figure 2. Samples from both productions showed a protein peak corresponding in molecular size (350–400 kDa) to the functional GmSuSy tetramer. However, there was also the material of a larger size that represented protein agglomerates of an undefined degree. The two samples analyzed differed considerably regarding the relative content of such agglomerates. Whereas in the sample from the *E. coli* BL21(DE3) culture, the agglomerated protein exceeded in abundance by far the protein in its native size. The

sample from the enGenes-X-press culture contained the GmSuSy tetramer as its main constituent.

As the protein peaks in the SEC of the *E. coli* BL21(DE3) sample were baseline separated, they were collected as fraction I and II and were further analyzed by analytical SEC, as shown in Figure S9. The fraction I did not elute from the column, probably because the agglomerates had already reached size limit for the SEC matrix. The analytical SEC of fraction II showed the expected tetrameric GmSuSy. We also determined the specific activity of the protein in each fraction and found none in fraction I and 4.7 U/mg in fraction II. By integrating the absorbance traces from the preparative SEC (Figure 2) we calculated, after normalization, the portion of native tetramer in the enGenes-X-press sample and found it to be 65% whereas that in the *E. coli* BL21(DE3) it was only 25% (Table 2).

Considering both the specific protein production/g cell mass and the quality of the protein thus made, we found that GmSuSy production in growth-arrested enGenes-X-press outperforms the reference production in growing *E. coli* BL21(DE3) by more than one magnitude order (12.5-fold; Table 2). In terms of improvement from batch to fed-batch culture, GmSuSy activity was increased from 285 to 390 U/L for *E. coli* BL21(DE3). In enGenes-X-press, however, the increase was far more significant (3.3-fold), 700 to 2,300 U/L, in

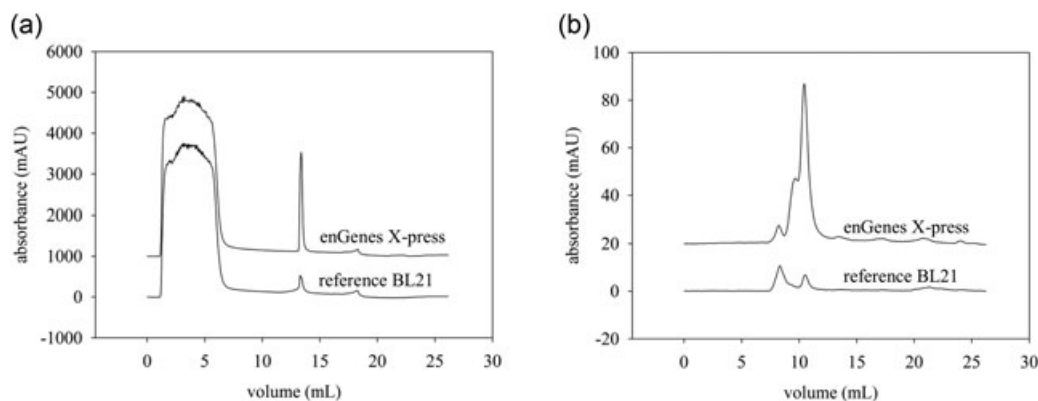


FIGURE 2 Protein quality analysis in GmSuSy produced in enGenes-X-press and *Escherichia coli* BL1(DE3) by high-cell density fed-batch cultivation. Absorbance traces of protein elution from Strep-Tactin affinity chromatography (a) and subsequent preparative SEC (b). The SEC trace reveals heterogeneity in both enzyme preparations, however, much less so in the preparation from production in enGenes-X-press. The peak at around 11 ml elution volume corresponds to the size expected for the native GmSuSy tetramer. GmSuSy: sucrose synthase from soybean (*Glycine max*); SEC: size-exclusion chromatography; UGT71A15: UDP-glycosyltransferase 71A15

consequence of the enhanced biomass formation in the fed-batch as compared with the batch culture.

4 | CONCLUSION

To advance biocatalysis for small-molecule glycosylation with plant GTs, better process technologies for the recombinant production of these enzymes are required (e.g., Priebe et al., 2018; Schmideder et al., 2016). The problem is difficult due to the involvement of multiple process variables, and the complex interrelationship these variables have with each other. A modularized approach interconnecting molecular engineering strategies at the levels of the gene, the protein, and the host organism is promising to make the development more predictable and faster. Focusing on *E. coli* as the production host, we showed here that gene expression under arrested cell growth was highly effective for GT synthesis as it enhanced yield and improved quality. Compared with the growing *E. coli* reference, the overall boost of production of the functional, high-quality enzyme in enGenes-X-press exceeded one order of magnitude. The total amount of functional enzyme (GmSuSy) produced in high-cell density fed-batch culture at 20 L scale was 830 mg/L, surpassing common titers of plant GTs in *E. coli* productions by 100-fold or more. The outstanding titer of 5.5 g/L for the glucosyltransferase from *Vitis vinifera* expressed in *E. coli* BL21(DE3) is clearly noted at this point (Priebe et al., 2018). With its effectiveness and scalability demonstrated in principle, the approach of enzyme production decoupled from cell growth might find further uses with different GTs and potentially other difficult-to-express proteins (see also: C. Chen et al., 2015; Gosh et al., 2012; Mahalik et al., 2014). For such proteins, HIV-1 protease can serve as a representative example (Mairhofer, Striedner et al., 2016). Practical realization of the approach in the strain enGenes-X-press thus represents a validated platform technology that offers flexible interconnection with gene and protein design strategies for enhanced recombinant protein production.

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CONFLICT OF INTEREST

J. M. is the CEO of enGenes Biotech GmbH and has an interest in the commercial exploitation of enGene-X-press technology for recombinant protein production.

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