https://doi.org/10.48048/tis.2025.9272

Optimization of Media, Expression Conditions and Extraction of SrUGT76G1 Glucosyltransferase and Sucrose Synthase for Glycoside Production

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Received: 6 November 2024, Revised: 8 December 2024, Accepted: 15 December 2024, Published: 30 January 2025

Abstract

The objective of this work was to produce the soybean sucrose synthase and SrUGT76G1 glucosyltransferase for glycoside production. Media, expression conditions, fermentation, and extraction were screened to gain high amounts of soluble enzymes from *E. coli* BL21(DE3). Lysogeny broth (LB) was identified as the best of 5 media tested. Then, Box-Behnken design was used to determine the suitable expression conditions, including temperature, lactose concentration, and time. Application of the expression conditions identified in flask cultures to the fermenter successfully increased the soluble enzymes from 7.3 to 14.1 mg/L for sucrose synthase, and from 14 to 17.7 mg/L for SrUGT76G1. To improve scalability, microfluidization was used for extraction and the condition of 25 g/L cell concentration and 69 MPa pressure gave 86 % disruption efficiency in a single pass, after which cell fragmentation was observed by scanning electron microscopy. The yields of sucrose synthase and SrUGT76G1 were 12.7 ± 1.2 and 18.4 ± 1.2 mg/L, respectively. This work demonstrates the benefit of surface response methodology in optimizing enzyme production for glycosylation reactions.

Keywords: Sucrose synthase, SrUGT76G, Glycosyltransferase, Box-Behnken design, Recombinant protein expression, Microfluidization

Introduction

The solubility of many bioactive compounds is low, which reduces their bioavailability and limits transmission to target cells. Several methods have been found to improve transport to cells, including liposomes, nanoparticles, drug glycosylation, etc. However, a traditional nano drug carrier such as liposomes presents a few disadvantages, such as poor stability, poor reproducibility, and low drug encapsulation efficiency [1]. Among the current techniques, glycosylation is a good strategy to improve the solubility of bioactive compounds by adding a carbohydrate moiety, which converts a drug compound to a prodrug, that can be converted to the drug by deglycosylation at the target cells. The carbohydrate helps with targeting the glycoside to cells due to the role of carbohydrates in cellular interactions and may improve pharmacokinetics and reduce toxicity and side effects.

In principle, glycosyltransferases, glycoside hydrolase and transglycosidases can be used to glycosylate bioactive compounds [2], but

glycosyltransferases tend to give the most complete conversions because the reaction that they catalyze is most energetically favorable. Among glycosyltransferases that have been used to glycosylate bioactive compounds, plant uridine diphosphate-sugar-dependent glycosyltransferases (UGTs) are of interest for their diverse specificities. In glucosyltransferase enzymes from plants, *Stevia rebaudiana* has been found to have 44 UGTs that may transfer glucose in the synthesis of steviol glycosides (SGs) [3]. Among those enzymes, SrUGT76G1 has been applied to produce bioactive glycosides [3-6], such as cannabinoid glycosides, rebaudioside A and rebaudioside M.

Sucrose synthase (SuSy) catalyzes the synthesis of sucrose from fructose and uridine diphosphate glucose (UDP-Glc), but under suitable conditions it can catalyze the reverse reaction to generate UDP-Glc from sucrose and uridine diphosphate (UDP) [7,8]. In terms of UDP recycling to UDP-Glc with glucosyl transfer from sucrose, Glycine max (soybean) SuSy (GmSuSy) was found to be more efficient than bacterial SuSy when coupled to a glycosyltransferase catalyzed glycosylation of phloretin to nothofagin [9] and was further applied to synthesis of calycosin-7-O-β-D glucoside [10]. GmSuSy was surprisingly stable at up to 50 °C [8,11]. Based on this characteristic of the GmSuSy enzyme, multiple enzyme reactions including SrUTG76G1 and GmSuSy can be applied to glycosylation with efficient regeneration of UDP-Glc from UDP and sucrose to gain more glycoside products.

For recombinant production of proteins, *Escherichia coli* host cells grow quickly, are simple to manipulate, and maintain genetic stability in large cultures, making *E. coli* one of the best hosts for producing recombinant proteins [12]. However, the production of recombinant intracellular protein is a disadvantage to use of *E. coli* on a large scale, since to extract the desired protein, the cells must be lysed. *E. coli* is a Gram-negative bacterium that is enveloped in an inner plasma membrane, a peptidoglycan cell wall, and an outer membrane [13]. The thin peptidoglycan wall is made up of glycan chains composed of repeating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNac) disaccharide units that are linked to those in adjacent strands by peptides.

The methods to open the cell envelope to release proteins are divided into mechanical and non-mechanical. Mechanical methods like bead milling, high pressure homogenization, and ultrasonication can be used to cause cell lysis. For non-mechanical methods like chemical or enzymatic lysis, digestion gently changes the cell envelope permeability, leading to an outflow of intracellular content. However, it still has drawbacks, such as using expensive and often toxic chemicals. In addition, the viscosity during the lysis process is affected by host cell DNA [14], resulting in the use of DNase I to reduce the viscosity during the lysis process. Currently, enzyme extraction methods using lysozyme are widely used, along with mechanical methods, including ultra-sonication, microfluidic or high-pressure homogenizers, bead milling, and cooled French presses [5,7,15]. In the lysis solution, some protease inhibitors are also added to protect enzyme activity. Nevertheless, the application of enzyme extraction methods on large scale is costly, so mechanical methods, such as ultra-sonication and microfluidization, are critical in biotechnology. Ultrasound sonication can cause the inactivation of many enzymes due to cavitation events, in particular, collapsing cavitation bubbles [16]. In comparison microfluidization or homogenization has many advantages over ultrasound sonication, such as decreasing particle size and improving homogeneity at low temperatures, which leads to less effect on enzyme activity. The use of high pressure to force sample fluids through a small opening over a short distance is the foundation of microfluidization. Moreover, the microfluidic extraction method can be applied without lysozyme and DNase, leading to savings on enzyme costs.

In this study, SrUGT76G1 and GmSuSy enzymes were produced by recombinant protein expression in *E. coli* BL21(DE3) from pET32a(+) and pET30a(+) expression vectors, respectively. Media were screened to select LB as a good medium for expression of both enzymes. In addition, the suitable expression condition was identified by Box-Behnken design with 3 factors (lactose inducer concentration, temperature, and time) and 5 center points. The best medium and suitable expression conditions were successfully applied in shake flask, 5-L fermenter, and 50-L fermenter cultures to produce SrUTG76G1 and GmSuSy for glycoside production. Microfluidic extraction successfully replaced the lysozyme extraction method in enzyme

extraction. Microfluidic conditions that increased the efficiency of extraction were also identified. This work resulted in efficient production of the enzymes necessary for economic production of glycoconjugates, such as cannabidiol glycosides.

Materials and methods

Overview of methods

An overview of the procedure for optimizing production of SrUGT76G1 and *G. max* sucrose synthase is shown in **Figure 1**. This summarizes our approach to recombinant enzyme production, including production of expression vectors and preliminary expression experiments, screening for appropriate media, optimization of expression temperature and time and lactose inducer concentration by Box-Behnken design, testing of optimal conditions in shake flask and 5 and 50 L fermenters and evaluation of enzyme extraction by microfluidization.

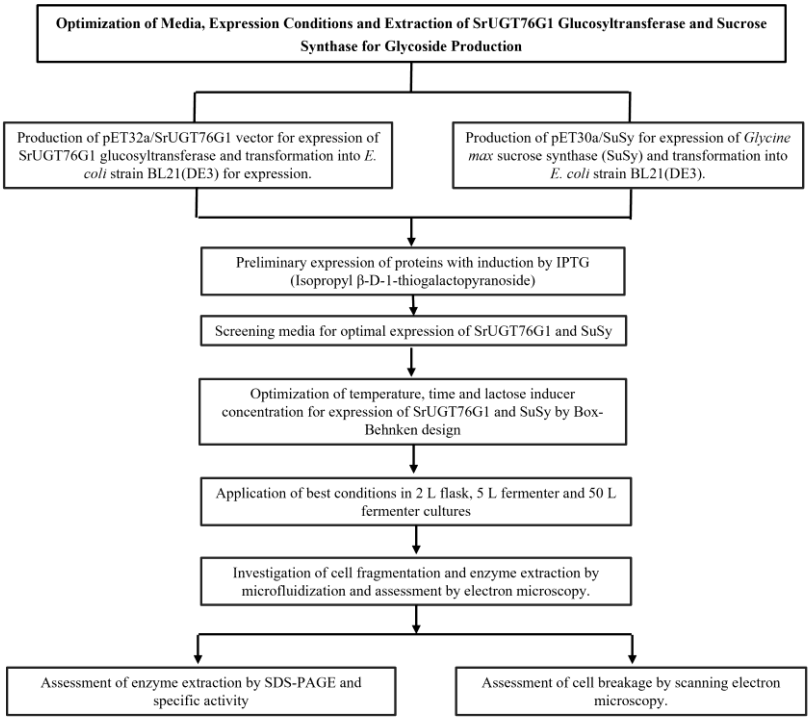

Figure 1 The flowchart of the methodology.

Strains and chemicals

Cannabidiol was extracted with 70 % ethanol, and the extracted material was clarified with micro, ultra, and nanofiltration to remove sediment. After that, an activated carbon column was used to remove chlorophyll, and then an evaporator was used to remove ethanol and concentrate the compound, which was then purified by short path distillation and preparative chromatography by AB Lab, Suranaree University of Technology. UDP sodium salt was purchased from Nanjing Duly Biotech Co., Ltd. (Nanjing, China). The UDP-Glo™ Glycosyltransferase Assay was purchased from Promega (Madison, WI, USA). The Amicon® Ultra-15 Centrifugal Filter 30K was acquired from Millipore-Sigma (Burlington, MA, USA). IMAC Sepharose™ 6 fast flow was purchased from GE Healthcare (Cytiva, Marlborough, MA, USA).

The gene encoding *Glycine max* sucrose synthase (designated SuSy from here on) optimized for expression in *E. coli* was synthesized and cloned into the *Nco*I and *Xho*I sites in pET30a(+) by Gene Universal Corp (Newark, DE, USA). All chemicals used in this study were of analytical grade. All measurements were made in triplicate, and standard deviations were calculated.

Cloning of SrUGT76G1

A gene optimized for *E. coli* expression of SrUGT76G1 (1465kb construct length) with 5' *Nco*I and 3' *Xho*I sites was synthesized by Twist company. The gene fragment encoding SrUGT76G1 was amplified by PCR with linker primers including forward 5'-GGAGGTACTGTTTCAGGGTC-3' and reverse 5'-ATTGGAACGATCTCGAGTCAC-3'. *Pfu* polymerase was used to amplify the gene with the temperature cycling parameters: 95 °C for 2 min, followed by 30 cycles of 95 °C 30 s, 55 °C 30 s, and 72 °C 210 s, followed by a final extension of 10 min, at 72 °C. The PCR product was gel-purified and cloned into the *Nco*I and *Xho*I sites of the pET32a(+) expression vector in-frame with the N-terminal thioredoxin and poly-histidine tags. The production of SrUGT76G1 was accomplished from this pET32a/SrUGT76G1 plasmid in *E. coli* strain BL21(DE3).

Screening media for both enzymes SuSy and SrUGT76G1

Five media including LB, Super optimal broth (SOB), Terrific broth (TB), modified M9 minimal medium (M9 modified), and FM auto-induction (FM) were used to evaluate the best medium for production of both enzymes (Supplementary material (SM) **Table S1**). The experiment was designed with cultivation in 500 mL flasks with 200 mL of medium, in triplicates (n = 3). The LB broth was used as medium for the starting culture, which was grown at 37 °C overnight. The starter culture was added at 1 % to the media and cultured for 7 h at 37 °C and 200 rpm. Then, 0.2 mM IPGT final concentration was added to the media except the MF auto-induction medium. The cultures were cultured overnight at 20 °C, with 200 rpm shaking. After that, the cells were collected by a Hitachi CR22GIII centrifuge at 4720×g for 20 min. The cell pellets were kept at –30 °C before extraction.

The SuSy and SrUGT76G1 enzymes were purified by immobilized metal affinity chromatography (IMAC) on a 1 mL cobalt-charged IMAC resin (Cytiva) column. The lysozyme method was used to extract enzymes with 20 mL lysis buffer and purification with IMAC as described below. During the experiment, the parameters final OD_{600}, wet weight of cells (WWC), amounts of enzymes, and enzyme activities were determined. TotalLab TL120 software was used to measure the amount of the SuSy (97 kDa) and SrUGT76G1 (69 kDa) bands on 10 % acrylamide SDS-PAGE gels loaded with 5 μg purified enzymes after a single IMAC purification by densitometry (SM, **Figure S1**; **Figure S2**). The gel was stained with Coomassie brilliant blue, and proteins were assumed to be equally stained to calculate the number of μg of the bands of interest based on the fraction of the total densitometric intensity found in those bands. Although proteins may stain differently, this gives a convenient relative parameter for comparison. The 1-way ANOVA test was used to analyze the results in SPSS software.

Investigating suitable expression condition for SuSy and SrUGT76G1 enzymes

The experiment was performed in 50 mL tubes with 10 mL of culture medium in triplication (n = 3). The experiment was designed based on Box-Behnken Design and included 17 runs (**Table 1**). The response was the densitometric amount of enzyme per 5 μg of total protein of cell extract supernatants. The amount was based on densitometric quantification with TotalLab TL120 software for 5 μg of protein from each supernatant run on 10 % acrylamide SDS-PAGE and stained with Coomassie brilliant blue (SM, **Figure S3**; **Figure S4**). The protein concentrations of the extracts were determined by the Bio-Rad (Bradford) protein assay.

Application of medium and suitable expression condition in 2-L flask, 5-L fermenter, 50-L fermenter for enzyme production

The medium that was best based on the media screening experiments for both enzymes was used as the medium for enzyme production. Media containing 50 μg/mL ampicillin or 30 μg/mL kanamycin were used for pET32a(+)/SrUGT76G1 and pET30a(+)/SuSy, respectively. A 1 % volume of LB broth feed starter was grown at 37 °C and 200 rpm orbital shaking for 16 h, and then transferred to the fermenter. After that, the cells were cultured at 37 °C at 200 rpm to reach an OD_{600} of 1 and then protein expression was induced based on the results of the expression condition experiment. The air flow was controlled at around 3 vvm for the 5-L fermenter and 10 vvm for the 50-L fermenter cultures. Samples were collected to determine OD, pH, DO% (only in the 5-L fermenter), and for SDS-PAGE to check

enzyme expression. Lactose was measured via HPLC with an Aminex® HPX-42A (300×7.8 mm^2) column and an RI detector. Chromatography was performed at a flow rate of 0.6 mL/min, with deionized water as the mobile phase, 20 µL sample injection, column temperature at 85 °C, and pressure at 185 psi.

Table 1 Box-Behnken design to identify suitable expression conditions.

Run	Factor 1 A: Temperature (°C)	Factor 2 B: Lactose (g/L)	Factor 3 C: Time (h)	Enzyme production response (µg/5 µg)			
				SuSy		SrUGT76G1	
				Experimental	Predicted	Experimental	Predicted
1	28.5	20	24	0.306	0.299	0.452	0.456
2	37	10	4	0.193	0.219	0.237	0.254
3	37	0	14	0.044	0.011	0.141	0.128
4	28.5	10	14	0.391	0.422	0.415	0.483
5	28.5	10	14	0.436	0.422	0.463	0.483
6	20	0	14	0.086	0.089	0.184	0.186
7	28.5	10	14	0.433	0.422	0.470	0.483
8	37	20	14	0.089	0.086	0.245	0.243
9	28.5	20	4	0.242	0.219	0.272	0.257
10	20	20	14	0.292	0.325	0.522	0.535
11	28.5	0	24	0.109	0.132	0.153	0.168
12	37	10	24	0.069	0.077	0.213	0.210
13	28.5	10	14	0.438	0.422	0.517	0.483
14	28.5	0	4	0.068	0.075	0.087	0.082
15	20	10	4	0.177	0.167	0.240	0.242
16	28.5	10	14	0.411	0.422	0.551	0.483
17	20	10	24	0.481	0.447	0.589	0.572

The cells were collected in a Hitachi CR22GIII centrifuge at 4720×g for 20 min. The cells were extracted by the lysozyme method (40 mL lysis buffer/1 L of culture) or microfluidic method (only for 50-L fermenter cell culture), as described in the enzyme extraction methods (below). Then, the soluble proteins in the supernatant were separated by centrifugation in a Hitachi CR22GIII centrifuge at 20,929×g for 15 min. Immobilized metal ion affinity chromatography (IMAC) was used to purify the proteins. After preparing the IMAC resin, the supernatant containing soluble protein was loaded to the column. Then, the column was washed with 5 column volumes (CV) of equilibration/wash (EQ) buffer (20 mM phosphate buffer, pH 7.5, 150 mM NaCl), 10 CV of equilibration/wash buffer containing 5 mM imidazole. The bound protein was eluted with 5 CV of elution buffer (20 mM phosphate buffer, pH 7.5, containing 500 mM imidazole). Samples from all steps were collected and evaluated by 10 % acrylamide of SDS-PAGE. Ultracell-30K filters were used to collect proteins by centrifugal filtration (Biofuge Stratos centrifuge at 3940×g for 40 min, washing 3 times with 20 mM Tris-HCl, 150 mM NaCl buffer, pH 7.5). The protein was collected and diluted for measuring with the Bio-Rad protein assay.

Enzyme extraction methods

From the 50-L-fermenter batch culture, 5 g of wet cells (equivalent to 2 L of culture) were weighed and used as samples to investigate the enzyme extraction methods. The experiment was done in triplicate and analyzed by ANOVA. In the lysozyme method, the sample was suspended in 80 mL lysis buffer containing 20 mM phosphate buffer pH 7.5 (EQ buffer), 150 mM NaCl, 0.4 mg/mL lysozyme, 1 % Triton-X 100, 1 mM

phenyl methyl sulfonyl fluoride (PMSF) 5 μg/mL DNase I, and 0.1 mg/mL soybean trypsin inhibitor for 30 min at room temperature. For microfluidization, the sample was suspended in 200 mL of a lysis buffer containing 20 mM phosphate buffer pH 7.5, 150 mM NaCl, 1 % Triton-X 100, 1 mM PMSF and 0.1 mg/mL soybean trypsin inhibitor and microflidization was done at 69 MPa at 10 °C for 5 passes by Hydraulic pilot M-110EH-30 microfluidizer® processor, (IDEX Material Processing Technologies, Westwood, MA, USA). The microfluidization, Eq. (1) from a book [17] was applied. The condition was controlled with the cell concentration (g/L) X = 25 g/L and pressure P = 69 MPa.

$$\ln (1/1 - D) = (0.0149 - 2.75\times10^{-5} X)\, N^{0.71} P^{1.165} \quad (1)$$

where N is the number of discrete homogenizer passes, P is the homogenizer operating pressure in MPa, and D is disruption efficiency with the fractional release of protein (the release of protein at a given moment divided by the highest release achievable).

Turbidity

The turbidity was determined by measuring the optical density of the sample at 750 nm in a UV218 VIS spectrophotometer [18]. The turbidity measurements were determined based on Eq. (2).

$$RT_I = 1 - \frac{T_i - T_0}{T_5 - T_0} \quad (2)$$

where RT_I was the relative turbidity after i passes, T_0 was the measured transmission of the cells that were resuspended in lysis buffer, T_i was the measured transmission for a suspension homogenized for i passes, and T_5 was the measured transmission for the suspension homogenized by microfluidic at 69 MPa for 5 passes.

Scanning *electron* microscopy

The *E. coli* BL21(DE3) cells expressing SuSy and SrUGT76G1 were observed after 0, 1, and 5 passes of the microfluidization process. The samples were fixed in 0.1 M phosphate buffer, pH 7.2, 2.5 % glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) overnight at 4 °C, and then washed 3 times in phosphate buffer, pH 7.2, at 4 °C, fixed in 1 % osmium tetroxide (Electron Microscopy Sciences) for 2 h, washed 3 times in distilled water (room temperature), and dehydrated in a graded acetone series (20, 40, 60, 80, and 100 % 3 times each). The sample was dried by critical point drying and covered with a layer of gold (Leica Sputter coater EM ACE600, Leica Microsystems, Wetzler, Germany) before being scanned with a scanning electron microscope (Carl Zeiss, Auriga, Germany) with an acceleration voltage of 3 kV.

Enzyme activity assays

DNS assay for SuSy enzyme

The SuSy *reaction* contained 20 mM sucrose, 2 mM UDP, 8 mM $MgCl_2$, 50 mM potassium phosphate buffer, pH 7.2, and 30 μg SuSy per 50 μL reaction. The reaction was incubated at 37 °C for 18 h. Then, 50 μL DNS reagent was added to each reaction tube, and it was boiled in water for 5 min. After that, the samples were centrifuged at 10,625×g for 10 min. Then, 90 μL sample was mixed with 360 μL water and 200 μL was transferred to a microtiter plate to read the absorbance at 540 nm. The A540 values were compared to a fructose standard curve to calculate the amount of fructose released from which enzyme specific activities were calculated. The blank was the reaction without enzyme.

UDP-Glo™ glycosyltransferase assay for SrUGT76G1 enzyme

SrUGT76G1 (5 μg/25 μL reaction) in 50 mM potassium phosphate buffer, pH 7.2, 9 mM $MgCl_2$, catalyzed the reaction of 1 mM UDP-Glc with 1 mM cannabidiol. The reaction was incubated at room temperature for 20 min. To the 25 μL reaction was added 25 μL UDP-Glo™ glycosyltransferase assay kit reagent and the mixture was incubated at room temperature for 1 h. After that, the luminescence signals were measured in a VarioskanTM LUX multimode microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Results and discussion

Screening media for production of the enzymes

Five media, including LB, TB, SOB, FM auto-induction, and modified M9, were screened to determine which was the best for SrUGT76G1 and SuSy production. Depending on the enzyme expression plasmid and host cell, enzyme expression varies in different media, as shown in **Table 2**. For example, beta-

mannanase was successfully expressed in M9 medium from pET28a in *E. coli* BL21(DE3) [19]. In another instance, compared to LB medium, TB medium was reported to occasionally produce higher enzyme expression [20].

In the current work, the enzymes can express well in LB, TB, and MF, but gave low expression in SOB and M9-modified media, based on SDS-PAGE of cell extract pellet and supernatant fractions (SM **Figure S1**; **Figure S2**). For SOB medium, the viscosity (20 g/L tryptone) may be higher than the other media, which may lead to the dissolved oxygen being lower than in the other media (data not shown). Therefore, SOB and M9-modified media were rejected as media for production of these enzymes. **Table 3** shows the properties of the enzymes in different media. The highest yields and specific activities of the enzymes were recorded in LB medium. The OD_{600}, reflecting the number of cells, was higher in all media containing simple carbon sources (glycerol, glucose), such as FM auto-medium, TB, and M9-modified, compared to LB medium. Yeast extract and tryptone, which are found in complex media like TB, LB, and FM auto-induction, are known to provide biosynthesis precursors such as vitamins and amino acids that enhance protein production in *E. coli* [21]. Additionally, the cellular stress during the synthesis of recombinant proteins may be decreased by yeast extract [21]. A synthetic medium comprising yeast extract and tryptone increased protein content in *E. coli* without increasing cell density, as seen in the current work using LB. Therefore, in this experiment, the LB broth medium was the best medium for SuSy and SrUGT76G1 production compared with TB, SOB, FM auto-induction, and M9-modified media. In previous studies, FM auto-induction medium was successfully used for high enzyme expression [15], but in our case, the activities of the enzymes expressed in FM auto-induction medium were lower than those from LB broth expression. Therefore, FM auto-induction medium was not used for SuSy and SrUGT76G1 enzyme production. However, in the FM auto-induction medium, the enzymes can express in lactose-induced conditions, which led to the idea for an investigation of the suitable expression condition with lactose as an inducer in place of IPTG.

Suitable expression condition for SuSy and SrUGT76G1 production

Since lactose could induce protein expression of the 2 enzymes in the autoinduction medium and has a number of advantages over IPTG, such as a hundredfold reduced cost, an impressive industrial advantage, non-toxicity, and a higher efficiency for expressing the target proteins [22], we investigated its use as an inducer. To initiate the T7 Lac promotor, galactosidase must convert the lactose present in the bacterial cell into allolactose. Therefore, lactose's inducing characteristics, such as dosage, temperature, time, and original bacterial concentration, must be clarified if it is to be used as an effective inducer.

To produce SrUGT76G1 and SuSy, the enzyme expression conditions were optimized to determine the ideal values of 3 independent variables (expression temperature, lactose concentration, and expression time). The results from each experimental run were described in a design matrix, and RSM based on the use of Box-Behnken design was carried out (**Table 1**). As seen in **Table 1**, experimental runs 4, 5, 7, 13, 16, and 17 for SuSy and 1, 4, 5, 7, 10, 13, 16, and 17 for SrUGT76G1 demonstrated high expression, with values ranging from 0.391 to 0.481 μg/5 μg total protein for SuSy and 0.415 to 0.589 μg/5 μg total protein for SrUGT76G1, respectively. The results showed a close similarity with the predicted results. The 2^{nd}-order model equation was fitted to the independent variables, and the goodness of fit was assessed. The applied equation's anticipated optimal values and the experimental amount of protein expression per 5 μg of total protein in the supernatant were used to validate the model's relevance. The predicted values plotted against experimental values showed that SrUGT76G1 and SuSy had R^2 values of 0.9948 and 0.9952, respectively (R^2 values greater than 0.75 show the suitability of the model) (**Figure 2**). This demonstrated that the values predicted by the models fit the experimental data well and ensured a suitable adjustment of the quadratic model to the experimental data.

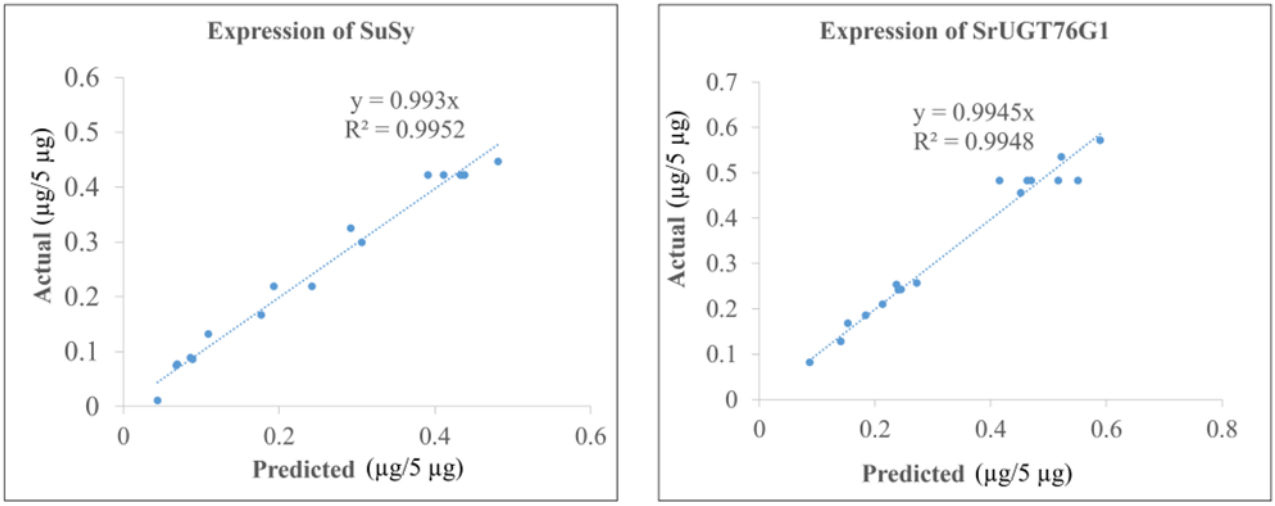

Figure 2 Predicted values plotted against experimental values for expression of SuSy (A) and SrUGT76G1 (B) enzymes. A linear regression line shows the fit of the data to a linear relationship with a slope of 0.993 for SuSy and 0.9945 for SrUGT76G1 showing that the average correspondence is very close to 1.00 (identical) and R2 values above 0.99 showing little variation from this high correspondence.

Table 2 Recently published recombinant protein production in *E. coli*.

Enzymes	Vector	Medium	Expression condition	Purification	Yield (mg/L)	References
GmSuSy- *Glycine max*	pET-STRP3	LB	25 °C, OD 0.8 - 1.0, 0.5 mM IPTG, overnight	Cooled French press, 100 bar IMAC 3 mL resin	12	[1,23]
AtSuSy- *Arabidopsis thaliana*	pET32a-AtSuSy	LB	16 °C, OD 0.6 - 0.8, 0.2 mM IPTG, 16 - 18 h	French press IMAC	-	[24]
GmSuSy- *Glycine max*	pXUN-SS	TB	25 °C, OD ≈ 0.8, 0.1 mM IPTG, overnight	HIC, AEX	11.6	[25]
GmSuSy- *Glycine max*	pET-STRP3-GmSuSy	LB	25 °C, OD ≈ 0.8 - 1, 0.2 mM IPTG, overnight	Cooled French press at 100 bar Affinity (strep-Tactin®)	12	[9,25]
GmSuSy- *Glycine max*	pET30a(+) GmSuSy in *E. coli* BL21(DE3)	LB	0.2 mM IPTG at 20 °C for overnight	Lysozyme extraction IMAC-Co-charged resin	3.73	In this study
GmSuSy- *Glycine max*	pET30a(+) GmSuSy	LB	27 °C, OD ≈ 1, 12 g/L lactose, 16 h	Lysozyme extraction IMAC-Co-charged resin	14.14	In this study
UGT76G1- *Stevia rebaudiana*	pRSFDuet-1-UGT76G1	LB	20 °C, OD ≈ 0.6 - 0.8, 0.1 mM IPTG, 36 h	Ultrasonic Cell Disruptor IMAC-Ni-charged resin	-	[26]
UGT76G1- *Stevia rebaudiana*	*E. coli* BL21(DE3) M/P-3-S32U-Stm3-	LB - flask	25 °C, OD ≈ 0.6 - 0.8, 0.01 mM IPTG, 24 h	Ultrasonication at 50 Hz for 5 min and	1.97 - flask	[5]

Enzymes	Vector	Medium	Expression condition	Purification	Yield (mg/L)	References
	UGT76G1 (Co-expression with *malK* and *prpD* gene)	synthetic medium-fed-batch		IMAC-Ni-charged resin	61.6 mg/L/h -fed-batch	
UGT76G1- *Stevia rebaudiana*	pET28a-CysQ-UGT, pET28a-EDA-UGT and pET28a-NusA-UGT	LB	30 °C, OD ≈ 0.5 - 0.6, 0.5 mM IPTG, 16 h	Sonifier and IMAC-Ni-charged resin	Increase 40 % soluble	[27]
Beta-mannanase	pET-28(+)	M9 medium	IPTG		1800	[19]
Lysostaphin- *Staphylococcus simulans*	pBADLys - *E. coli* Top 10	yeast extract 5 g/L, tryptone 10 g/L, $Na_2HPO_4{\cdot}7H_2O$ 7.10 g/L, KH_2PO_4 6.8 g/L, $MgSO_4$ 0.15 g/L, $(NH_4)_2SO_4$ 3.3 g/L, glucose 0.5 g/L) with 0.1 % (v/v) filter-sterilized arabinose solution	30 °C, 48 h, 0.1 % (v/v) filter-sterilized arabinose solution	Lysozyme + sonicator and cation exchange chromatography	184	[15]
SrUGT76G1 (*Stevia rebaudiana*)	pET32a(+) in *E. coli* BL21(DE3)	LB	0.2 mM IPTG at 20 °C for overnight	Lysozyme extraction IMAC with $CoCl_2$	9.9	In this study
SrUGT76G1 (*Stevia rebaudiana*)	pET32a(+) in *E. coli* BL21(DE3)	LB	14 g/L lactose at 20 °C for 22 h	Lysozyme extraction IMAC with $CoCl_2$	17.37	In this study

Table 3 Parameters of Susy and SrUGT76G1 production during media screening.

	Media	LB	TB	SOB	MF-auto	M9 modified
SuSy	OD final (OD_{600})	$2.30^{a} \pm 0.097$	$6.64^{d} \pm 0.29$	$3.58^{b} \pm 0.27$	$7.31^{e} \pm 0.074$	$4.53^{c} \pm 0.32$
	WWC (g)	$0.86^{a} \pm 0.061$	$1.57^{c} \pm 0.22$	$1.06^{ab} \pm 0.021$	$1.29^{bc} \pm 0.17$	$1.60^{c} \pm 0.091$
	Enzyme (mg)	$0.673^{e} \pm 0.023$	$0.627^{d} \pm 0.016$	$0.047^{a} \pm 0.007$	$0.277^{c} \pm 0.015$	$0.128^{b} \pm 0.015$
	Specific activity (Fructose mM/mg enzyme/min)	$3.09^{d} \pm 0.025$	$2.58^{c} \pm 0.12$	$0.034^{a} \pm 0.029$	$1.60^{c} \pm 0.029$	$0.214^{b} \pm 0.083$
SrUGT76G1	OD final (OD_{600})	$2.85^{a} \pm 0.12$	$5.30^{c} \pm 0.20$	$2.54^{a} \pm 0.018$	$6.31^{d} \pm 0.33$	$4.29^{b} \pm 0.20$
	WWC (g)	$0.787^{a} \pm 0.015$	$1.230^{b} \pm 0.070$	$0.950^{a} \pm 0.061$	$1.37^{b} \pm 0.056$	$1.59^{c} \pm 0.86$
	Enzyme (mg)	$1.001^{c} \pm 0.054$	$0.605^{b} \pm 0.012$	$0.181^{a} \pm 0.031$	$0.621^{b} \pm 0.019$	$0.197^{a} \pm 0.024$
	Specific activity (UDP µM/mg enzyme/min)	$19.3^{d} \pm 1.5$	$16.2^{c} \pm 1.7$	$4.34^{b} \pm 0.46$	$1.88^{b} \pm 0.57$	ND^{a}

Note: All values are presented as the mean ± SD (n = 3), $^{a, b, c, d, e}$ indicate significant differences, as numbers in the same row that do not share the same superscript are significantly different ($p < 0.05$) as determined by Turkey's range test (ND = Non detected).

Table 4 presents the findings of the 2nd-order response surface model as an analysis of variance (ANOVA). The significance of the regression is shown by Fisher's F-test and *p*-values. The F computer is higher than the F table, and the ANOVA tables show the overall significant effect of interaction terms on enzyme expression at the 5 % level of significance ($p < 0.05$). Low pure errors for SuSy expression (0.0017) and SrUGT76G1 expression (0.0110) suggest that the experimental data are well reproducible. The model is significant, as shown by the *F*-values for SrUGT76G1 expression (26.45) and SuSy expression (45.48). The likelihood of the model *F*-value being this high due to noise in the expression of both enzymes is 0.01 % at most. According to the calculated parameters and the accompanying *p*-values, all the factors had significant effects on the expression of both SuSy and SrUGT76G1 when considered as independent variables. However, due to the higher *p*-values for the time factor (**Table 4**), temperature and lactose inducer concentration were more significant factors impacting the expression of the enzymes than time of expression. The simplified quadratic models for the SuSy expression and SrUGT76G1 expression were built in terms of coded values after neglecting the impact of unimportant interacting factors from the broader quadratic model, and they are shown in Eqs. (3) and (4), respectively.

$$Y = 0.4218 - 0.0793\times A + 0.0778\times B + 0.0348\times C - 0.0403\times AB - 0.1053\times AC + 0.0058\times BC - 0.1235\times A^2 - 0.1705\times B^2 - 0.07\times C^2 \quad (3)$$

$$Y = 0.4832 - 0.0874\times A + 0.1158\times B + 0.0714\times C - 0.0585\times AB - 0.0933\times AC + 0.0285\times BC - 0.0657\times A^2 - 0.1445\times B^2 - 0.0977\times C^2 \quad (4)$$

Effect of temperature and lactose on enzymes expression

The production of proteins and the proliferation of cells are both greatly influenced by temperature. The rate at which substrates are consumed, the product's quality, and the length of the process are similarly impacted by this aspect. The increased rate of protein synthesis brought on by the temperature of the culture medium may not allow enough time for proper protein folding [28]. To enhance folding and solubility during the expression, the temperature range of 20 to 37 °C was identified as potentially good conditions for each protein (SrUGT76G1 and SuSy), as is shown in the contour plot in **Figure 3**. When the expression temperature was higher than 30 °C, the soluble proteins were rapidly reduced to no soluble expression. Besides that, the gene expression system's sensitivity to variations in inducer levels was assessed. Rising lactose concentrations led to a rise in the expression of recombinant protein, but this will reduce when over a certain limit [29]. That is why lactose was investigated in 0 - 20 g/L culture for SuSy and SrUGT76G1 expression. However, the temperature was the main factor affecting soluble enzyme expression because high-concentration lactose at high temperature led to decreased levels of soluble proteins likely due to poor folding of rapidly synthesized polypeptides [28]. From the contour of the RSM (**Figure 3**), SuSy had higher expression with a higher temperature than that of SrUGT76G1. Additionally, the amounts of lactose of 10 to 15 and 12 to 20 g/L can gain high expression for SuSy and SrUGT76G1, respectively.

Effect of time and lactose on enzymes expression

The results indicated that increasing lactose concentrations resulted in a significant rise in the amount of protein expression. However, from observation, regardless of the lactose concentration used, the expression remained constant or low. Therefore, from the design, the system responsible for protein production was fully active even at lower lactose concentrations. However, time and lactose have a relationship that is reciprocal. The cells required time for lactose induction of the lac operon. The minimum time for cell starting expression was around 9 h (**Figure 3**). The results indicated that the range of lactose 10 - 15 g/L and expression time 13 - 20 h was considered to give strong expression for SuSy. For SrUGT76G1, 12 - 16 g/L lactose and 18 - 22 h expression time were ideal. Understanding the impact of lactose concentration and expression time on enzyme expression is crucial for the development of efficient and effective recombinant protein production systems. By optimizing lactose concentrations and expression time, the scalability and cost-effectiveness of protein production ultimately benefits.

Table 4 Analysis of variance (ANOVA) for the quadratic models of the expression of SrUGT76G1 and SuSy enzymes from the results of Box-Behnken design.

Source	DF	Sum of squares	Mean square	*F*-value	*p*
SuSy					
Model	9	0.3873	0.0430	45.48	< 0.0001
A-Temperature	1	0.0502	0.0502	53.09	0.0002
B-Lactose	1	0.0484	0.0484	51.10	0.0002
C-Time	1	0.0097	0.0097	10.21	0.0152
AB	1	0.0065	0.0065	6.85	0.0346
AC	1	0.0443	0.0443	46.82	0.0002
BC	1	0.0001	0.0001	0.1397	0.7196
A^2	1	0.0642	0.0642	67.88	< 0.0001
B^2	1	0.1224	0.1224	129.37	< 0.0001
C^2	1	0.0206	0.0206	21.82	0.0023
Residual	7	0.0066	0.0009		
Lack of Fit	3	0.0050	0.0017	4.00	0.1067
Pure Error	4	0.0017	0.0004		
Cor Total	16	0.3940			
SrUGT76G1					
Model	9	0.4220	0.0469	26.45	0.0001
A-Temperature	1	0.0611	0.0611	34.45	0.0006
B-Lactose	1	0.1072	0.1072	60.47	0.0001
C-Time	1	0.0408	0.0408	22.99	0.0020
AB	1	0.0137	0.0137	7.72	0.0273
AC	1	0.0348	0.0348	19.62	0.0030
BC	1	0.0032	0.0032	1.83	0.2179
A^2	1	0.0182	0.0182	10.26	0.0150
B^2	1	0.0879	0.0879	49.58	0.0002
C^2	1	0.0402	0.0402	22.68	0.0021
Residual	7	0.0124	0.0018		
Lack of Fit	3	0.0014	0.0005	0.1745	0.9084
Pure Error	4	0.0110	0.0027		
Cor Total	16	0.4344			

Effect of temperature and time on expression of the enzymes

Similar to the relationship between temperature and lactose, the relationship between temperature and time also includes substrate consumption rate, proper protein folding, and particular growth rates. The temperature maintains a major role in soluble enzyme expression. The temperature should not be higher than 30 °C, the time for enzyme expression was started at 9 h for SuSy and 14 h for SrUGT76G1. The red zone with high SuSy expression is where the temperature is between 23 and 27 °C and between 14 and 24 h, and 20 to 24 °C and 16 to 24 h are red areas of strong expression for SrUGT76G1, according to the response contour (**Figure 3**).

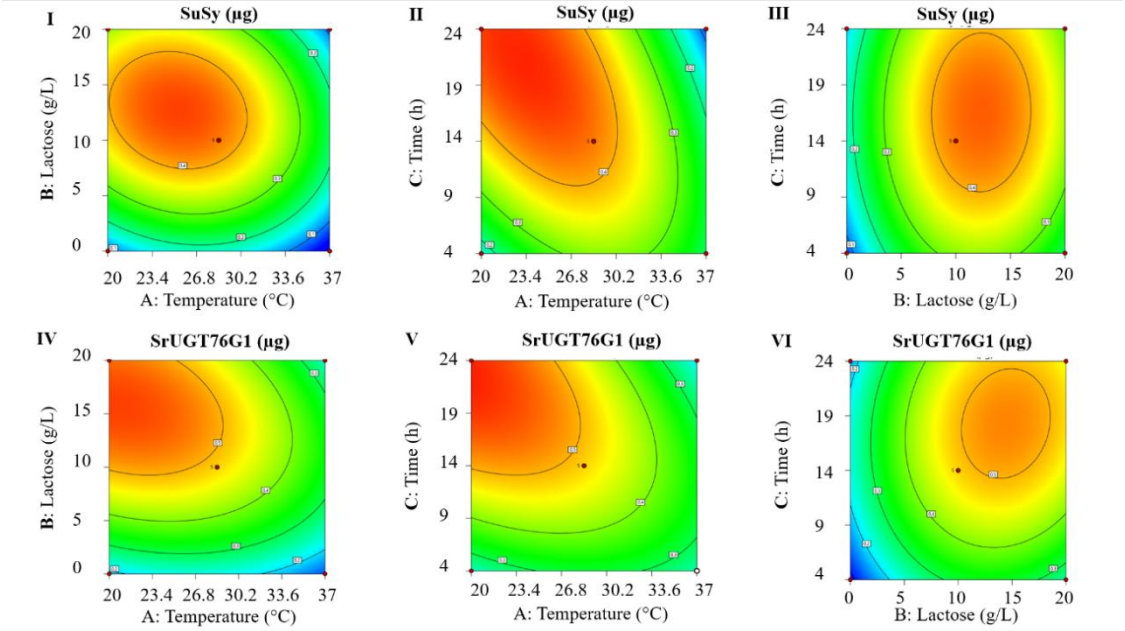

Figure 3 Contour plots of the combined effects of (I) SuSy-lactose and temperature, (II) SuSy-time and temperature, (III) SuSy-time and lactose, (IV) SrUGT76G1-lactose and temperature, (V) SrUGT76G1-time and temperature, and (VI) SrUGT76G1-time and lactose, respectively.

Optimization of SuSy and SrUGT76G1 expression conditions.

Expression temperature, lactose concentration, and time were all equally important factors in the numerical optimization technique used to optimize the process conditions for the SuSy and SrUGT76G1 synthesis by *E. coli* BL21(DE3) for appropriate expression conditions. Targeting high expression of soluble enzyme was the major criterion for optimization. The ideal operating parameters for SuSy expression were 27 °C, 12 g/L lactose, and a 16 h expression period in order to attain the desired 0.45 µg per 5 µg total protein. The expression setting condition for SrUGT76G1 was 20 °C, 14 g/L lactose, and 20 h expression time, targeted at 0.62 µg per 5 µg total protein. The target enzymes expression was quite close to the highest levels, but these target options were chosen to reduce time and amount of lactose inducer or increase temperature to reduce cooling and thereby decrease production costs. The experiment was run in a 2-L flask with 1 L medium, a 5-L fermenter with 3.5 L medium, and a 50-L fermenter with 35 L medium to validate the outcomes of optimization.

Application of suitable expression conditions for SuSy and SrUGT76G1 production in 2-L flask, 5-L fermenter, and 50-L fermenter cultures

There are 2 phases in the recombinant production of protein in *E. coli* BL21(DE3): Biomass accumulation without induction and protein expression induction for the production period. Nevertheless, a high cell density can frequently result in a number of serious issues, such as plasmid loss from *E. coli*, a large pH drop due to cell metabolites, and limited dissolved oxygen availability. These issues frequently lead to minimal or even no protein production while maintaining a high cell density. Therefore, lactose was added for the inducing period when the cell density reached an OD_{600} of 1. The expression conditions of SrUGT76G1 and SuSy were applied in a 2-L flask to examine the expression and activity of the enzymes. Then, the conditions for expression of the enzymes were also verified in 5-L and 50-L fermenter cultures. SuSy and SrUGT76G1 production could be successfully carried out under the optimized conditions, showing that the expression conditions were successful in the flask and fermenters. The yields of purified SrUGT76G1 were 14 mg/L for the flask, 15 mg/L for the 5-L fermenter, and 17.25 mg/L for the 50-L fermenter. For SuSy, the yields were 7.3 mg/L for the flask, 10.8 mg/L for the 5-L fermenter, and

14.1 mg/L for the 50-L fermenter, respectively. The specific activity of SrUGT76G1 and SuSy was not significantly different from different flask and fermenter cultures. Due to the rapid purification by a single IMAC step, the enzyme specific activities were affected by the ratio of enzymes to impurities. The enzyme expression in the 50-L fermenter for SuSy and SrUGT76G1 were higher than in the flask and 5-L fermenter. In the case of the fermenters, the densities of cells expressing SuSy were similar to each other, peaking at 3.16 ± 0.10 (**Figure 4**). However, in the 50-L fermenter culture, SrUGT76G1-expressing cell density was higher by 1 OD_{600} unit when compared with the 5-L fermenter culture. This may be caused by providing air around 10 vvm in the 50-L fermenter, which was higher than in the 5-L fermenter. This may also be the reason why there was more SrUGT76G1 enzyme in the 50-L than in the 5-L fermenter culture. Moreover, lactose serves as a carbon source for bacteria to encourage growth in culture, as previously reported [29]. This increases the amount of the target recombinant protein produced. However, the lactose concentration measured via HPLC remained unchanged over the expression period in the current study, suggesting that lactose digestion was not significant in this case with the low density culture of *E. coli* BL21(DE3).

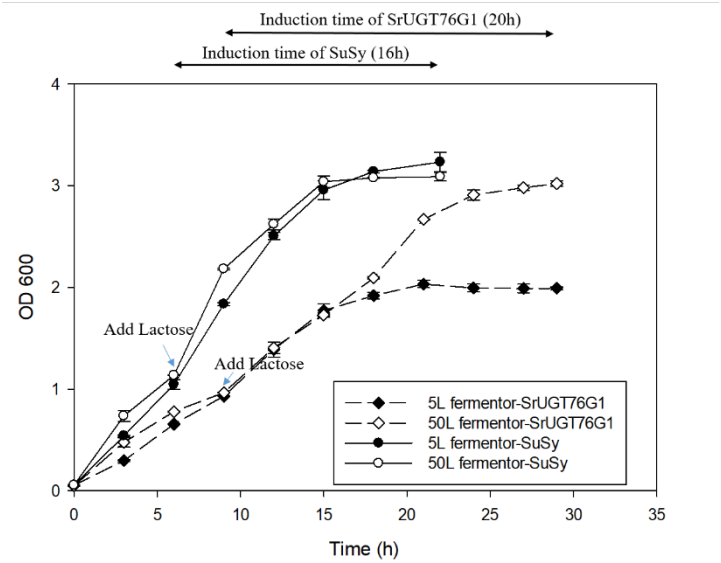

Figure 4 Growth curve of *E. coli* BL21(DE3) carrying the SrUGT76G1 and SuSy expression vectors for enzyme production in 5-L and 50-L fermenter cultures.

SDS-PAGE analysis of 5 µg of total protein in the cell lysate supernatant demonstrated that the expression of the enzymes increased with time in both 5-L and 50-L fermenters (**Figure 5**). When expression was induced with 0.2 mM IPTG in the shake-flask culture, the final enzyme yields were 9.9 mg/L for SrUGT76G1 and 3.75 mg/L for SuSy. Therefore, the suitable expression conditions for the enzymes with lactate induction were successful in increasing soluble enzyme yield compared with the IPTG inducer condition. Compared with other studies with IPTG as an inducer, the yields of enzymes were lower than others, such as 12 mg/L of SuSy in shake-flask cultivation [9,25] and 61.6 mg/L/h of SrUGT76G1 fusion protein with a solubility tag and fed-batch fermentation conditions [5]. However, this study was successful in screening for an effective medium and suitable expression conditions appropriate to apply in both shake-flask and fermenter to produce SuSy and SrUGT76G1 with lactose as an inducer. The yields were increased in the 50-L fermenter culture, which will support production of enzymes for bioactive glycosides production in future.

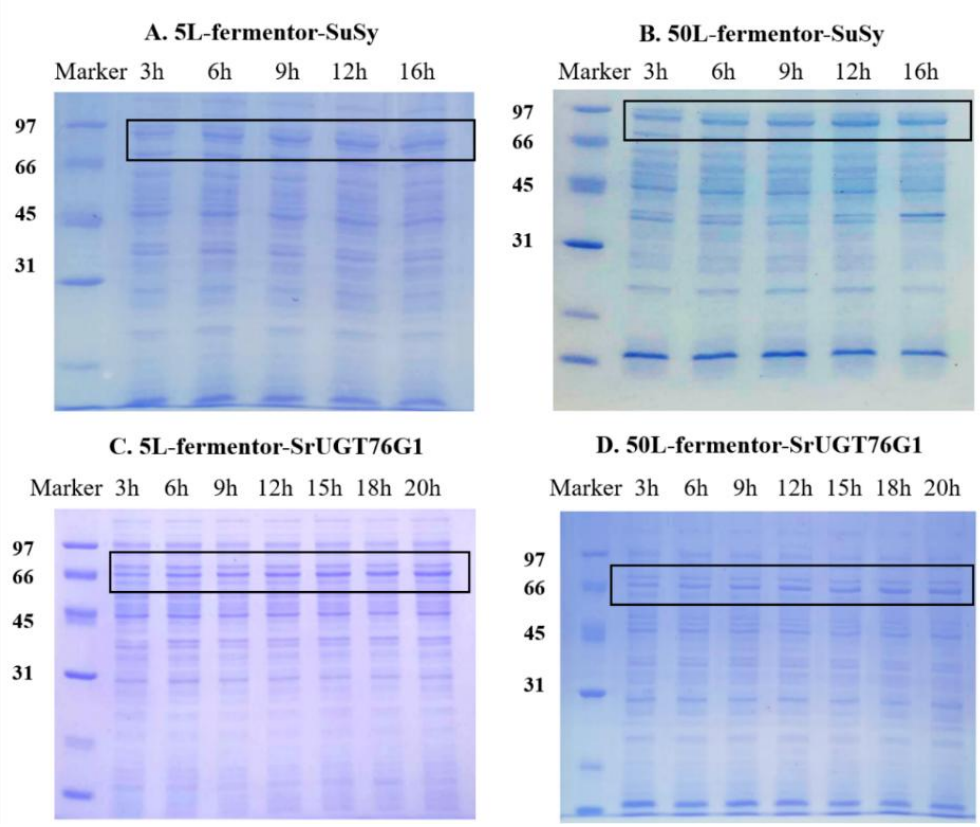

Figure 5 SDS-PAGE of 5 µg protein from supernatant during 16-h expression of SuSy and 20-h expression of SrUGT76G1 enzymes in 5-L fermenter and 50-L fermenter, respectively.

Lysozyme extraction method for SuSy and SrUGT76G1 enzymes

After cell collection, wet cells were kept at –30 °C at least overnight, resulting in a freeze-thaw cycle which supported the enzyme extraction. In the case of intracellular enzymes, the extraction of enzymes aims to degrade and rupture the cell walls of E. coli, releasing all the enzymes from the cytoplasm. Since E. coli is a Gram-negative bacterium, its cells are contained by 3 layers: An outer membrane, a peptidoglycan cell wall, and an inner plasma membrane [13]. The thin peptidoglycan cell wall is made up of glycan chains comprising repeated N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) disaccharide units linked to other chains by peptides. Lysozyme cleaves the β - (1,4)-glycosidic linkages at GlcNAc residues in peptidoglycan [30], which weakens the cell wall and thereby supports extracting enzymes from the cytosol. Triton X-100 was also added to help lyse cells by destabilizing the membranes [31]. According to **Table 5** the yields of SuSy and SrUGT76G1 enzymes were 14.1 ± 0.97 and 17.4 ± 1.4 mg/L, respectively. **Figures 6(A) - 6(B)** displays SDS-PAGE analysis of fractions from the purification process. The microfluidization extraction method was investigated as a potential replacement for the lysozyme extraction method in the event of large-scale enzyme extraction. Because lysozyme and DNase I are not included in the lysis solution for this method, it provides the benefit of reducing the materials used in the extraction process.

Table 5 Yields and specific activities of SrUGT76G1 and SuSy with different extraction methods.

Enzyme	Parameter	Microfluidic extraction	Lysozyme extraction
SrUGT76G1	Yield (mg/L)	$18.4^{b} \pm 1.2$	$17.4^{b} \pm 1.4$
	Specific activity (μM/min/mg)	$16.7^{b} \pm 2.6$	$17.7^{b} \pm 4.1$
Sucrose synthase (SuSy)	Yield (mg/L)	$12.7^{a} \pm 1.2$	$14.1^{a} \pm 0.97$
	Specific activity (mM/min/mg)	$2.7^{a} \pm 0.17$	$2.8^{a} \pm 0.09$

Note: All values were presented as the mean ± SD (n = 3). Numbers within the same row with different superscripts are significantly different ($p < 0.05$) as determined by Turkey's range test.

The microfluidization extraction method for SuSy and SrUGT76G1 enzymes

One piece of equipment that produces highly efficient emulsions or suspensions is high-pressure homogenization or microfluidization to decrease particle size and improve homogeneity. The use of high pressure to force sample fluids through a small opening over a short distance is the foundation of microfluidization. Pressure, cell concentration, and the number of microfluidic passes determine the disruption efficiency in the context of enzyme extraction [17]. In addition, the low temperature of microfluidic extraction reduces the possibility of inactivating enzymes; it breaks down cells without the need for DNase and lysozyme, which lowers materials extraction; and it is simple to use on a large scale for the enzyme industry.

The goal of this experiment was to determine the conditions under which *E. coli* BL21(DE3) would break down and release all its soluble enzymes. Due to cell breakdown, the differences between SrUGT76G1 clone cells and SuSy clone cells were compared based on the intact optical density of samples at 750 nm [18]. Despite the use of the same *E. coli* BL21(DE3) host cells, the homogenization of SrUGT76G1-expressing cells was faster than that of SuSy-expressing cells (**Figure 6(D)**). After the cells were broken down, the supernatant from each pass was measured to determine the total protein content and densitometric amounts of enzyme bands in 5 μg of total protein in SDS-PAGE (**Figure 6(C)**). Over the course of 5 passes, there was no discernible change in the total protein in the cell lysate supernatant after the 1st pass. Furthermore, **Figure 6(C)** demonstrates that there were no appreciable differences in the soluble protein bands between each pass. According to earlier research, cell breakdown is dependent on the number of microfluidic passes, cell concentration (g/L) ($0 \text{ g/L} \leq X \leq 150 \text{ g/L}$), and pressure (P) ($30 \text{ MPa} \leq P \leq 70 \text{ MP}$) [17].

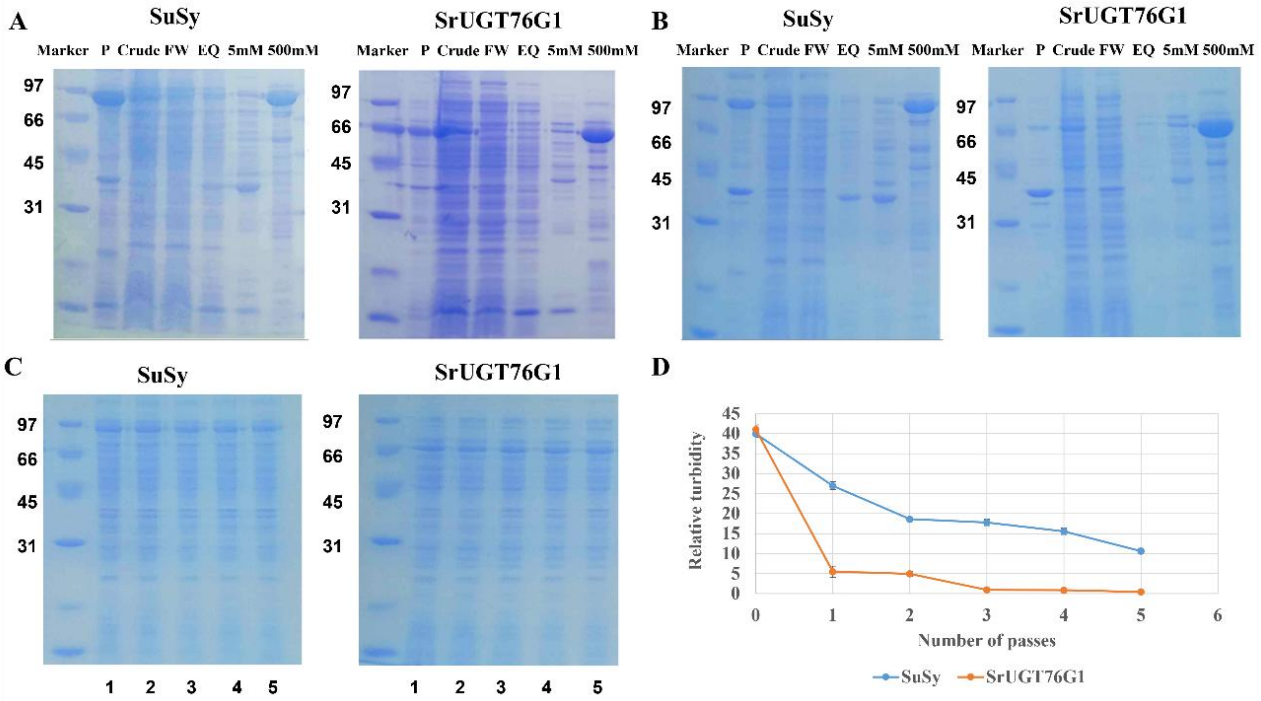

Figure 6 Purification of SuSy and SrUGT76G1 enzymes using different extraction methods. (A) SDS-PAGE of the fractions of SuSy and SrUGT76G1 purification after lysozyme extraction. P is the insoluble pellet after extraction and centrifugation, Crude is the supernatant, FW is the crude supernatant that flowed through the IMAC column, EQ is the wash of the column with equilibrium buffer, 5 mM is the wash with buffer containing 5 mM imidazole, and 500 mM is the elution of the protein with 500 mM imidazole. (B) SDS-PAGE of the fractions of Susy and SrUGT76G1 purification after microfluidic extraction. The fractions are as described in A. (C) SDS-PAGE of 5 μg total protein in crude supernatant in 5 passes of microfluidic extraction of SuSy and SrUGT76G1. The numbers indicate the number of passes through the microfluidic extraction process. (D) The turbidity of the suspension of *E. coli* BL21(DE3) after expression of SuSy and SrUGT76G1 in 5 passes of the microfluidic extraction process.

The disruption efficiency increased from 86 % for the 1st pass to 99.8 % for the 5th pass, following Eq. (1). On the other hand, the enzymes' solubility affects their extraction. With SrUGT76G1 and SuSy enzymes, nearly all of the soluble enzymes appeared to be extracted at 86 % disruption efficiency. Based on Eq. (1), it is possible to boost the cell concentration to 150 g/L at 69 MPa and achieve a disruption efficiency of 91.3 % over a minimum of 2 passes.

SEM was used to observe the cell morphology during disruption, by comparing cells before extraction, cells after 1 pass, and cells after 5 passes for the clones expressing each of the 2 enzymes, to learn more about the structural disruption of *E. coli* BL21(DE3) (**Figure 7**). Some *E. coli* BL21(DE3) cells were compromised in the sample before extraction because Triton X-100 disrupted the cell's lipid bilayer (**Figure 7(A)**). However, the SuSy and SrUGT76G1 expression clones of *E. coli* BL21(DE3) cells were shredded to a size of 400 - 600 nm during the 1st microfluidic pass (**Figures 7(B)** and **7(D)**). That explains why the soluble enzymes were consistent over the course of 5 microfluidic passes (**Figure 6(C)**). Although only the plasmids differed between the SrUGT76G1 and SuSy expressing cells, they underwent the same treatment with different results. The SrUGT76G1 expressing cells showed greater breakage in the lysis solution after microfluidic treatment, as seen in **Figure 7(E)**, while the SuSy-expressing cells appeared to be broken to smaller vesicles without complete rupture **Figure 7(C)**. After the 5th microfluidic pass, the SEM samples showed greater fragmentation than after a single pass, but this homogenization did not significantly affect the yield of enzyme extracted.

Based on all data, the microfluidic device can shred nearly 100 % of *E. coli* BL21(DE3) cells to a size

of 400 - 600 nm at 69 MPa and 25 g/L cell concentration in 1 pass. This results in an 86 % disruption efficiency. Based on 5 mL of IMAC-resin bound to cobalt, the enzymes were purified, yielding 18.4 ± 1.2 mg/L for SrUGT76G1 and 12.7 ± 1.2 mg/L for SuSy. This was similar to lysis with lysozyme, as shown **Table 5**, which shows that the difference in yields between these methods was not significant ($p > 0.05$). In conclusion, microfluidic extraction could replace lysozyme extraction based on the amounts and specific activities of the enzymes recovered.

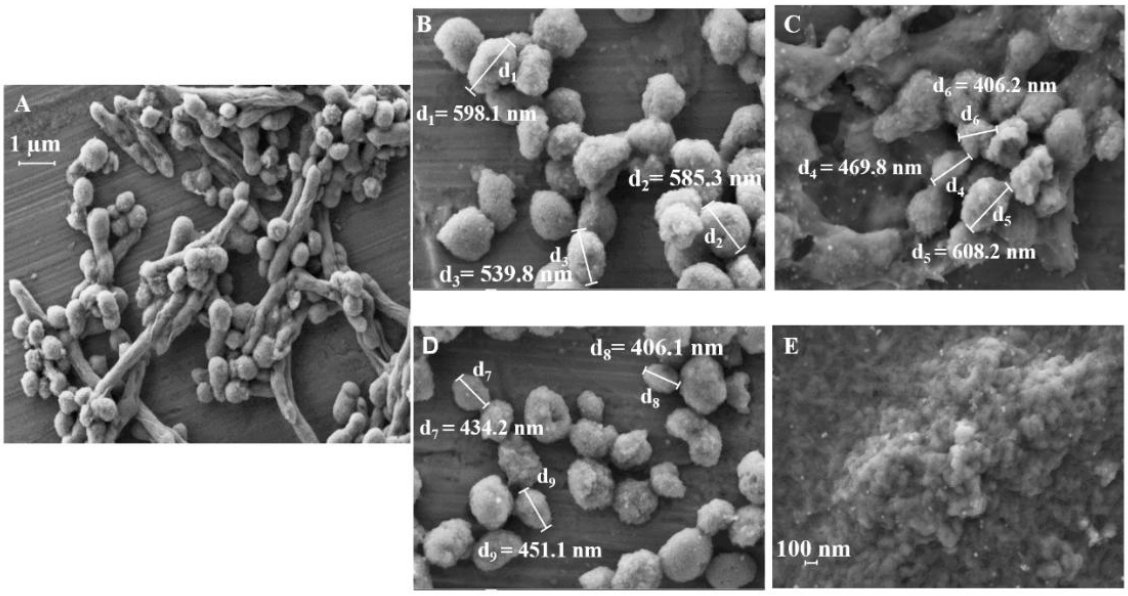

Figure 7 Scanning electron microscopy of the recombinant *E. coli* BL21(DE3) cells expressing SuSy and SrUGT76G1 enzymes during microfluidic extraction. (A) SuSy-expressing cells before microfluidic treatment, (B) SuSy-expressing cells after 1 pass, (C) SrUGT76G1-expressing cells after 1 pass, (D) SuSy-expressing cells after 5 passes, and (E) SrUGT76G1-expressing cells after 5 passes.

Conclusions

This study demonstrates the impact of media, temperature, inducer, and time on the expression of the enzymes SrUGT76G1 and SuSy in *E. coli*. For both enzymes, the appropriate expression conditions were successfully applied in shake-flask, 5-L-fermenter, and 50-L-fermenter cultures. The surface response methodology with Box-Behnken design could optimize expression conditions in a zone with strong enzyme expression. In addition, microfluidic technology was used to decrease cost, since it does not require lysozyme or DNase I and can substitute for enzymic extraction without affecting enzyme yield and activity. Future work on stabilization of the extracted enzymes for storage and shipping will enhance the value of this process for industrial production of these enzymes. Glycosides can be generated using the SuSy and SrUGT76G1 enzymes in conjunction with various enzyme systems, where SuSy recycles UDP-Glc from UDP. In the production of glycosides of interest, such as CBD glycosides [4], the use of these enzymes in a coupled system for generating glycosides can reduce the cost of production by reducing the need for UDP-Glc.

Acknowledgements

This research was funded by Suranaree University of Technology (SUT), Thailand and by the Office of the Higher Education Commission including an OROG scholarship. The study was supported by SUT, Thailand Science Research and Innovation (TSRI), and the National Science, Research and Innovation Fund (NSRF) (Project code 90464).

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Supplementary material

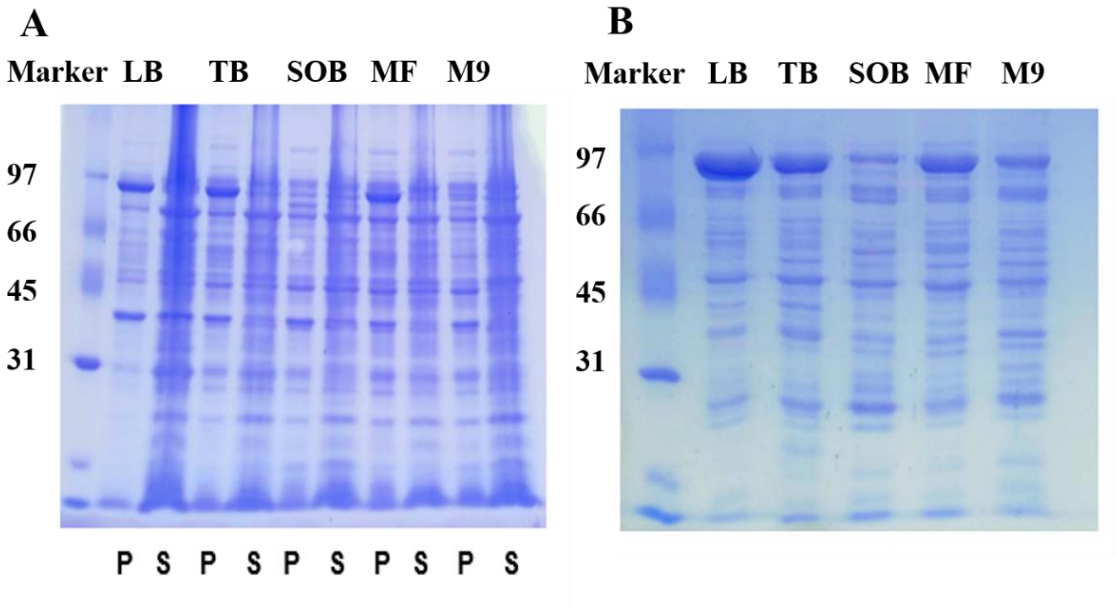

Figure S1 SDS-PAGE of pellet and supernatant of SuSy after enzymatic extraction (A) and SDS-PAGE of 5 µg total protein of Susy purified by a single IMAC step from different media (B).

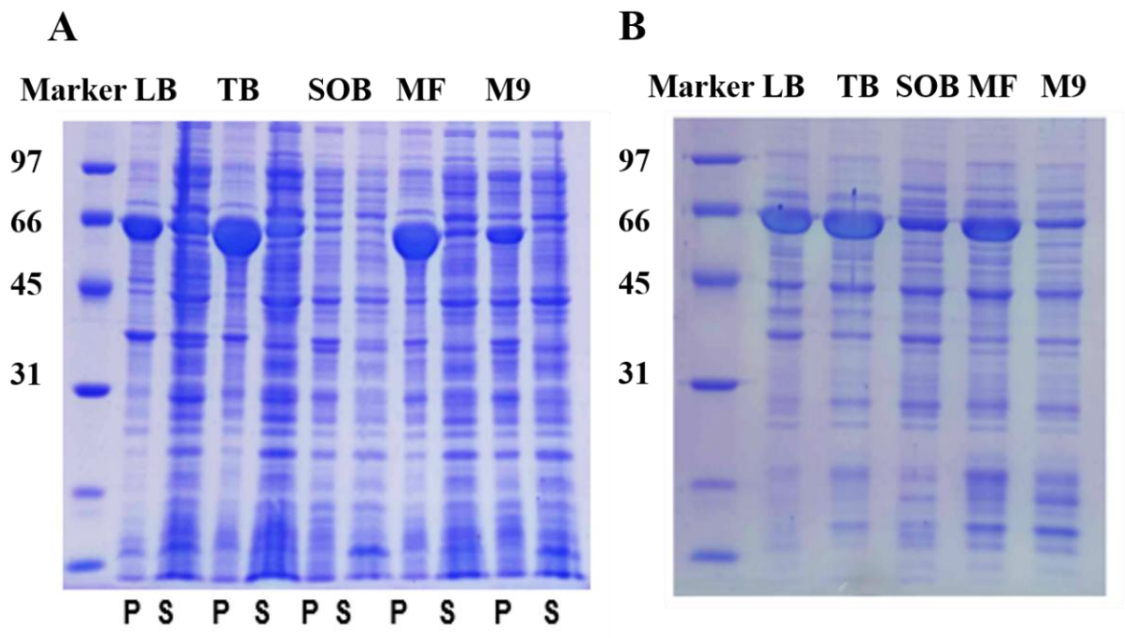

Figure S2 SDS-PAGE of pellet and supernatant of SrUGT76G1 after enzymatic extraction (A) and SDS-PAGE of 5 µg total protein of SrUGT76G1 purified by a single IMAC step from different media (B).

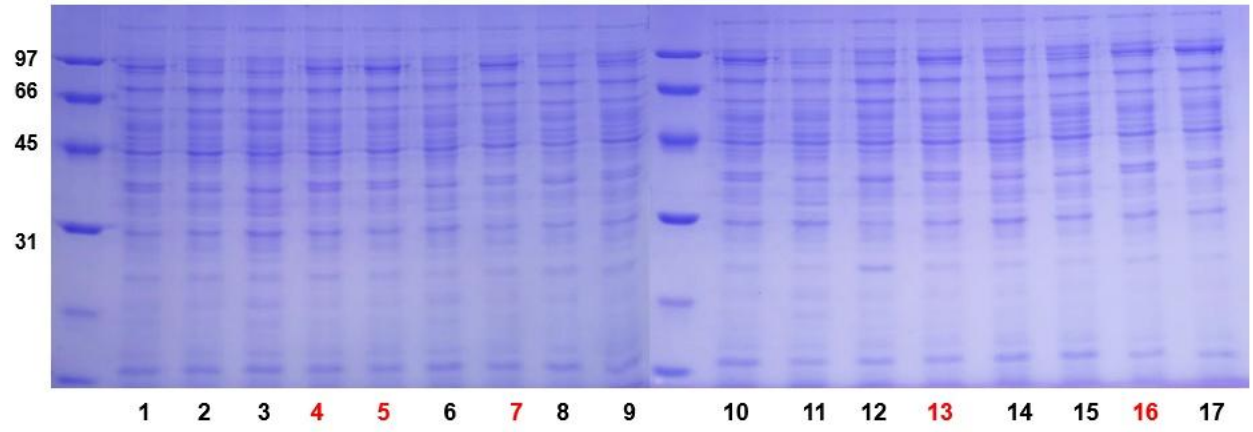

Figure S3 SDS-PAGE of 5 μg total protein from the extract supernatant of cells expressing SuSy from the 17 runs of the RSM experiment.

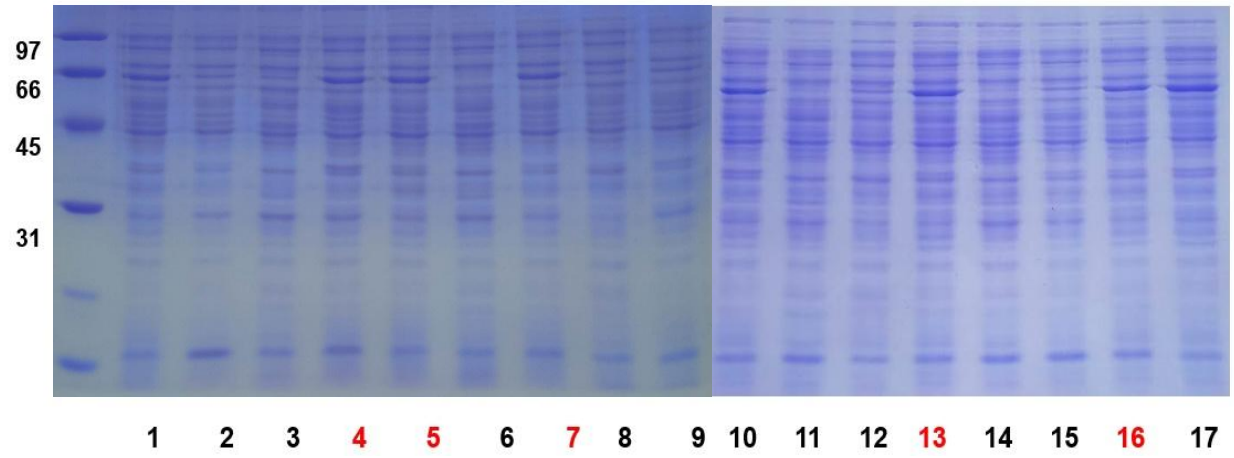

Figure S4 SDS-PAGE of 5 μg total protein from the extract supernatant of cells expressing SrUGT76G1 from the 17 runs of the RSM experiment.

Table S1 Compositions of the 5 media for *E. coli* BL21(DE3) cultivation.

Substance	**Unit**	**M9 modified**	**MF auto-induction**	**SOB**	**TB**	**LB**
Tryptone	g/L	-	10	20	12	-
Yeast extract	g/L	10	5	5	24	5
Peptone	g/L	-	-	-	-	10
Glycerol	mL	-	6	-	4	-
Glucose	g/L	10	1.5	3.6	-	-
NaCl	g/L	-	-	0.5	-	5
KH_2PO_4	g/L	7.5	6.8	-	2.31	-
K_2HPO_4	g/L	15	-	-	12.54	-
KCl	mM	-	-	2.5	-	-
$MgCl_2$	mM	-	-	10	-	-
$Na_2HPO_4.7H_2O$	g/L	-	10	-	-	-
$MgSO_4$	g/L	-	0.15	-	-	-
$(NH_4)_2SO_4$	g/L	2.5	3.3	-	-	-
$MgSO_4.7H_2O$	g/L	2	-	-	-	-
Citric acid	g/L	2	-	-	-	-
Lactose	g/L	-	20	-	-	-
Trace-element salts [21]	mL	1	-	-	-	-

(Trace-element salts (g/l in 1 M HCl): $FeSO_4 \cdot 7H_2O$, 2.8; $MnCl_2.4H_2O$, 2; $CoSO_4 \cdot 7H_2O$, 2.8; $CaCl_2 \cdot 2H_2O$, 1.5; $CuCl_2 \cdot 2H_20$, 0.2; and $ZnSO_4 \cdot 7H_2O$, 0.3). Bracketed numbers indicate references found in the main text document.