

Effect of Culture Conditions and Signal Peptide on Production of Human Recombinant *N*-Acetylgalactosamine-6-Sulfate Sulfatase in *Escherichia coli* BL21

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The production and characterization of an active recombinant *N*-acetylgalactosamine-6-sulfate sulfatase (GALNS) in *Escherichia coli* BL21(DE3) has been previously reported. In this study, the effect of the signal peptide (SP), inducer concentration, process scale, and operational mode (batch and semi-continuous) on GALNS production were evaluated. When native SP was presented, higher enzyme activity levels were observed in both soluble and inclusion bodies fractions, and its removal had a significant impact on enzyme activation. At shake scale, the optimal IPTG concentrations were 0.5 and 1.5 mM for the strains with and without SP, respectively, whereas at bench scale, the highest enzyme activities were observed with 1.5 mM IPTG for both strains. Noteworthy, enzyme activity in the culture media was only detected when SP was presented and the culture was carried out under semi-continuous mode. We showed for the first time that the mechanism that in prokaryotes recognizes the SP to mediate sulfatase activation can also recognize a eukaryotic SP, favoring the activation of the enzyme, and could also favor the secretion of the recombinant protein. These results offer significant information for scaling-up the production of human sulfatases in *E. coli*.

Key words: Morquio A, GALNS, signal peptide, sulfatase, recombinant enzyme, *E. coli*

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N-Acetylgalactosamine-6-sulfate sulfatase (GALNS; E.C. 3.1.6.4) is a lysosomal enzyme involved in the degradation of the glycosaminoglycans keratan sulfate (KS) and chondroitin-6-sulfate (C6S) [36]. Mutations in the *GALNS* gene produce an enzyme with null or reduced activity, leading to the accumulation of KS and C6S within the lysosome, producing mucopolysaccharidosis IV A (MPS IV A, Morquio A disease, OMIM 253000) [36]. MPS IV A has an estimated incidence of 1:200,000 and is characterized by systemic skeletal dysplasia, laxity of joints, hearing loss, corneal clouding, valvular heart disease, and pulmonary dysfunction [25, 36]. Although enzyme replacement therapy (ERT) [14, 35, 37] and gene therapy [1, 2, 17] are under evaluation, no corrective therapies currently exist for MPS IV A patients, and only supportive measures and surgical interventions are used to treat some manifestations of the disease [25, 36].

GALNS enzyme (UniProtKB/Swiss-Prot P34059) is synthesized as a 522 amino acid pre-protein, and maturation is carried out by crossing through the endoplasmic reticulum (ER), Golgi apparatus, and late endosome [20]. Enzyme maturation includes removal of the signal peptide (SP; cleavage site between Gly26 and Ala27: ASG-AP), linkage of oligosaccharides chains, and proteolysis [5, 32]. In addition, GALNS requires active-site activation by the formylglycine-generating enzyme (FGE), which catalyzes the formation of a formylglycine (FGly) residue from a cysteine [9, 10].

During the last years, we have worked on the production of iduronate-2-sulfatase and GALNS in *Pichia pastoris* and *Escherichia coli*, showing the feasibility to produce active forms of these human sulfatases [8, 22, 30, 33]. Recently, the production, purification, and characterization of an active recombinant GALNS enzyme in *E. coli*

BL21(DE3) were reported [26, 33]. The production of an active GALNS in *E. coli* suggests that glycosylation is not necessary for the production of an active form of the enzyme, and that bacterial formylglycine-generating enzyme can mediate the Cys-to-FGly conversion within mammal sulfatases. As a continuation of these studies towards the development of an ERT for MPS IV A patients, this study evaluated the effects of culture mode (batch versus semi-continuous), inducer concentration (isopropyl-beta-D-thiogalactopyranoside, IPTG), and presence of the signal peptide on GALNS production in *E. coli* BL21(DE3).

MATERIALS AND METHODS

Plasmids and Microorganisms

The pGEX-3X-GALNS plasmid was previously constructed by insertion of human GALNS cDNA (GenBank Accession No. NM_000512.4) into the pGEX-3X expression plasmid (GE Healthcare, USA) [33]. The human GALNS cDNA without the nucleotide sequence encoding for the SP was cloned from pGEX-3X-GALNS by using the primers GALNSnp-F 5'-ACGTGAATTCCCGCC CCGCAGCCCCCAACAT-3' and GALNSnp-R 5'-ACGTGAATT CCTAGTGGGACCAGAGGCA-3', in which the bold sequence represents the *EcoRI* recognition site, the underlined sequence is complementary to the GALNS cDNA, and the double-underlined sequence is the first codon after the signal peptide cleavage site. The PCR was carried out with KAPA HiFi HotStar ReadyMix (Kapa Biosystems, USA), under the manufacturer's instructions. The GALNS cDNA without the sequence encoding for the SP (*nspGALNS*) was inserted into the pGEX-5X-1 expression plasmid (GE Healthcare, USA; GenBank Accession No. U13856). This plasmid was selected because the restriction enzyme sites permitted to keep the gene in frame with the glutathione S-transferase peptide present in this plasmid, and it only differs with pGEX-5X-1 in the sequence of their multiple cloning site [3]. *E. coli* BL21(DE3) [*fluA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHI ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Anin5*; New England Biolabs, USA] chemical competent cells were transformed with pGEX-3X-GALNS or pGEX-5X-nspGALNS plasmids to produce the BL21/pGEX-GALNS and BL21/pGEX-nspGALNS strains, respectively. All procedures were carried out under standard molecular biology methods [4].

Microorganism Cultures at Shake Scale

BL21/pGEX-GALNS and BL21/pGEX-nspGALNS were cultured at shake scale under previously described conditions [33]. Briefly, microorganisms were cultured in 500 ml shake flasks with 100 ml of minimal growth medium (MGM) at 200 rpm and 37°C. After 12 h of culture, GALNS expression was induced with isopropyl-beta-D-thiogalactopyranoside (IPTG; Gold Biotechnology, USA), at 200 rpm and 18°C. IPTG concentration effect on GALNS induction was evaluated in a range from 0.5 to 1.5 mM, in which individual assays were monitored during 6 h and 5 ml samples were withdrawn every 2 h. *E. coli* BL21(DE3) without expression plasmid was used as a control and cultured as described without ampicillin supplementation. Each assay was done in triplicate.

Microorganism Cultures at Bench Scale

Batch mode. Culture conditions for GALNS in batch mode were as previously reported [33]. Briefly, microorganism culture was carried out in a Bioflo 110 reactor (New Brunswick Scientific, Co. Inc., Edison, NJ, USA), with a 3-L working volume at 200 rpm, 37°C, controlled pH 7.2, and an aeration rate of 2.5 standard temperature and pressure liters per minute (STPLPM). After 13 h of culture, GALNS production was induced through IPTG supplementation at 200 rpm and 18°C. Induction was monitored during 6 h, and 50 ml aliquots were withdrawn every 2 h. Inducer concentration effect on GALNS production was evaluated at 0.5, 1.0, and 1.5 mM IPTG. *E. coli* BL21(DE3) strain without expression plasmid was used as a control and cultured as described above without ampicillin supplementation. Each assay was done in duplicate.

Semi-continuous mode. To establish the effect of a higher biomass production and culture time on GALNS production, a semi-continuous process was conducted. This semi-continuous culture was carried out in a two-stage process beginning with a batch culture stage, conducted as described above, followed by a medium replacement stage. The operational conditions (*i.e.*, temperature, aeration, and agitation) of the batch stage were maintained along semi-continuous cultures. The medium replacement was done using a Pellicon XL Filter Module Durapore 0.45 μm, 50 cm² (Millipore, USA), which allowed recirculating of the biomass while removing the exhausted medium. Exchange of fresh and exhausted media was done at the same flow rate (1.2 or 2.4 ml/min), to keep constant the total culture volume. After the semi-continuous stage, GALNS was induced as described for batch cultures. Induction was monitored during 6 h and 50 ml aliquots were withdrawn every 2 h. IPTG concentration effect on GALNS induction was evaluated at 0.5, 1.0, and 1.5 mM. The *E. coli* BL21(DE3) strain without expression plasmid was used as a control and cultured as described above without ampicillin supplementation. Each assay was done in duplicate.

Crude Protein Extracts

Culture aliquots were centrifuged at 4,000 rpm for 30 min and 4°C. The supernatant (culture medium) was transferred to a new tube and stored at -20°C, while the pellet was washed 3 times with 1× PBS (composition per liter: 10.9 g Na₂HPO₄, 3.2 g NaH₂PO₄, 90 g NaCl, pH 7.2). The cell pellet was resuspended in lysis buffer (25 mM Tris, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 2% β-mercaptoethanol, 5% glycerol, 1% Triton X-100, pH 7.2) and lysated by sonication (Vibra-Cell, Sonics & Materials Inc., USA). The lysis supernatant (hereafter named soluble fraction) was stored at -20°C for further analysis, whereas the pellet, which may contain the inclusion bodies, was washed with 0.85% NaCl, solubilized with 1 ml of solubilization buffer (25 mM Tris, 2% β-mercaptoethanol, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 8 M urea, pH 7.0), and refolded by dialysis against 25 mM potassium acetate, pH 7.0. After dialysis, samples were centrifuged to obtain the solubilized and refolded inclusion bodies (hereafter named inclusion bodies fraction) [33].

GALNS Activity

GALNS activity was assayed using 4-methylumbelliferyl-β-D-galactopyranoside-6-sulfate (Toronto Chemicals Research, Canada) as substrate [39]. One unit (U) of GALNS activity was defined as the enzyme that catalyzes 1 nmol of substrate per hour, and specific

GALNS activity was expressed as U/mg of total protein. Protein concentration was determined by the Bradford assay. The enzyme activity was assayed in the soluble and inclusion bodies fractions and in the culture media. Owing to sampling restrictions, at shake scale the GALNS activity was measured only in the soluble fraction. Preliminary studies showed that under these conditions, the buffer used for sample preparation does not affect the GALNS enzyme activity assay.

Enzyme-Linked Immunosorbent Assay (ELISA) Quantitation

Ninety-six-well polystyrene microplates (Nunc Maxisorp) were coated with 10 µg/ml protein from the soluble or inclusion bodies fractions. The recombinant GALNS was recognized with a polyclonal rabbit anti-GALNS IgG antibody, produced against a mix of highly immunogenic human GALNS peptides located far away from the signal peptide region (Espejo J, Lizaraso L, Sosa A, Barrera A, unpublished data). An anti-rabbit IgG coupled with peroxidase (Sigma-Aldrich) was applied to the wells, and the experiment was developed with 3,3',5,5'-tetramethylbenzidine (TMB substrate; USA). The enzymatic reaction was stopped with 1N HCl solution, and the plate was read at 450 nm in an Anthos 2020 microplate reader (Biochrom, Cambridge, UK) [33]. A recombinant human GALNS enzyme produced in CHO cells, kindly donated by Dr. Shunji Tomatsu and Dr. Adriana Montaña, was used for the calibration curve.

Statistical Analysis

Differences between groups were tested for statistical significance using Student's t-test. A *p* < 0.05 values was considered statistically significant. All analyses were performed using Statgraphics Centurion v.16. All results are shown as the mean ± standard deviation (SD).

RESULTS

Evaluation of the effect of plasmid type on the microorganism growth did not reveal a significant difference (*p* > 0.05) in the growth rate (μ) of BL21/pGEX-GALNS ($0.461 \pm 0.047 \text{ h}^{-1}$ [33]) and BL21/pGEX-nspGALNS ($0.457 \pm 0.028 \text{ h}^{-1}$), suggesting that SP absence and use of a different expression plasmid did not have a significant effect on the bacterial growth. Furthermore, a successful processing of the SP from the enzyme produced by BL21/pGEX-GALNS was expected, since it was shown previously that the recombinant GALNS produced with this strain differs about 6 kDa from the one produced in CHO cells, which could be associated with the lack of glycosylations on the *E. coli* recombinant enzyme [26, 33]. On the other hand, BL21/pGEX-nspGALNS carried a GALNS cDNA in which the nucleotide sequence encoding for the SP was removed, then the synthesized protein lacked the SP from the beginning. In summary, both strains presented a similar growth rate, and it was expected to produce recombinant proteins that differ in the processing of the SP.

Enzyme Production at Shake Scale

The highest GALNS activity at shake scale was observed for BL21/pGEX-GALNS cultures. These activities varied from 0.10 to 0.28 U/mg (Fig. 1A), whereas the activities obtained with BL21/pGEX-nspGALNS varied from 0.01 to 0.10 U/mg (Fig. 1B). Although significant differences in GALNS activity were not observed among the evaluated IPTG concentrations, it was noticed that 0.5 mM IPTG favored the enzyme production in BL21/pGEX-GALNS (Fig. 1A). Strain BL21/pGEX-nspGALNS showed a different profile; the highest enzyme activity was observed with 1.5 mM IPTG, which was significantly different (*p* < 0.05) than that observed with the other IPTG concentrations. In all cases, the obtained enzyme activity with BL21/pGEX-GALNS was about 3-fold higher than those observed with BL21/pGEX-nspGALNS (Fig. 1). Enzyme activity in the culture media was not detected for any of the evaluated conditions and strains.

Enzyme Production at Bench Scale

The recombinant GALNS production by BL21/pGEX-GALNS and BL21/pGEX-nspGALNS in both soluble and inclusion bodies fractions was evaluated at 3 L scale. At this scale, the effect of two IPTG concentrations was evaluated,

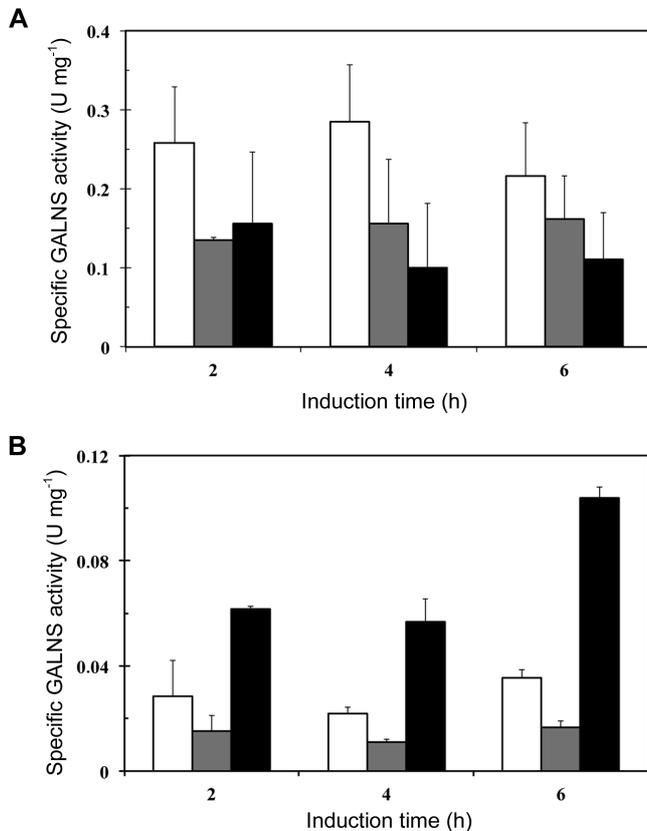


Fig. 1. GALNS production at shake scale by culturing of *E. coli* BL21/pGEX-GALNS (A) and *E. coli* BL21/pGEX-nspGALNS (B) strains after induction with □, 0.5; ■, 1.0; or ■, 1.5 mM IPTG.

(i.e., 0.5 and 1.5 mM), as well as the process operational mode, batch and semi-continuous. Aeration rate and agitation were settled to 2.0 STPLPM and 200 rpm, respectively, as previously reported [33].

Batch culture. Although the induction time is an important factor in the expression of recombinant GALNS, IPTG concentration showed a strong effect on GALNS production (Fig. 2). For both strains at the soluble fraction, it was observed that the highest specific enzyme activity was obtained with 1.5 mM IPTG (Figs. 2A–2B). However, each strain presented an independent performance. For

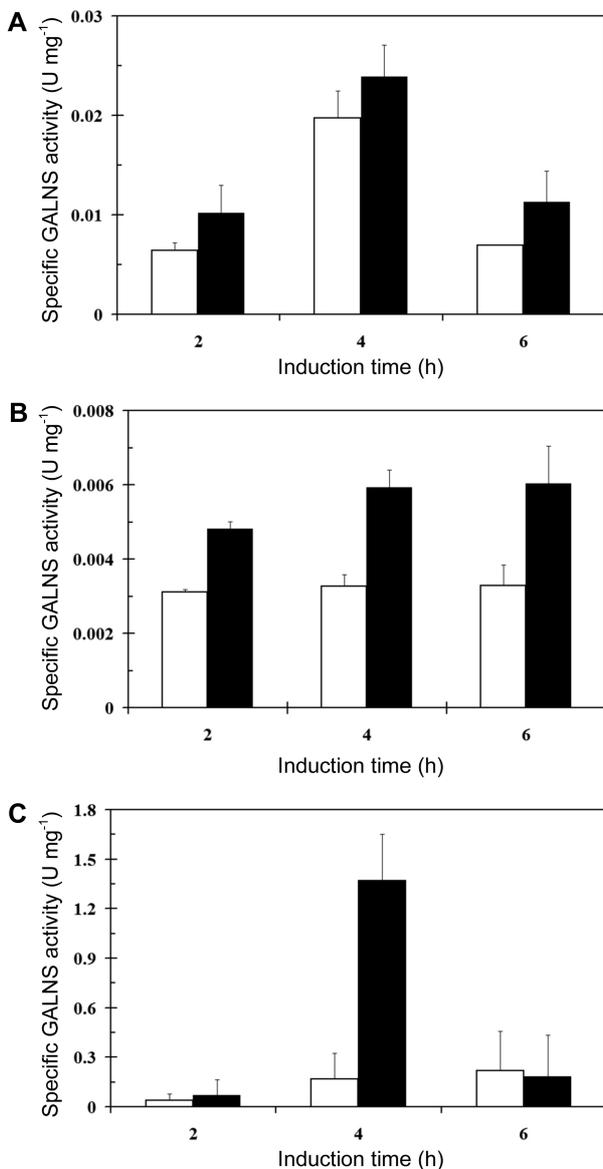


Fig. 2. GALNS production under batch culture conditions. Effects of IPTG concentration (□, 0.5; and ■, 1.5 mM) and induction time on GALNS activity in soluble fractions from batch cultures of *E. coli* BL21/pGEX-GALNS (A) and *E. coli* BL21/pGEX-nspGALNS (B) strains, and in inclusion bodies from *E. coli* BL21/pGEX-GALNS strain cultures (C).

BL21/pGEX-GALNS, the highest specific GALNS activities were 0.019 ± 0.002 and 0.023 ± 0.003 U/mg after 4 h of induction with 0.5 and 1.5 mM IPTG, respectively (Fig. 2A). In contrast, the highest enzyme activity for strain BL21/pGEX-nspGALNS was obtained with 1.5 mM of IPTG (0.0060 ± 0.0001 U/mg) after 2 h of induction, while a plateau at 0.003 U/mg was reached after 2 h of induction with 0.5 mM of IPTG (Fig. 2B).

Under the evaluated culture conditions, it was observed that removal of the SP (BL21/pGEX-nspGALNS strain) caused a notable reduction in the enzyme activity in the soluble fraction, which ranged from 3.1- and 7.6-fold lower than that obtained with the BL21/pGEX-GALNS strain. The GALNS activity was detected in all inclusion bodies fractions obtained from BL21/pGEX-GALNS cultures

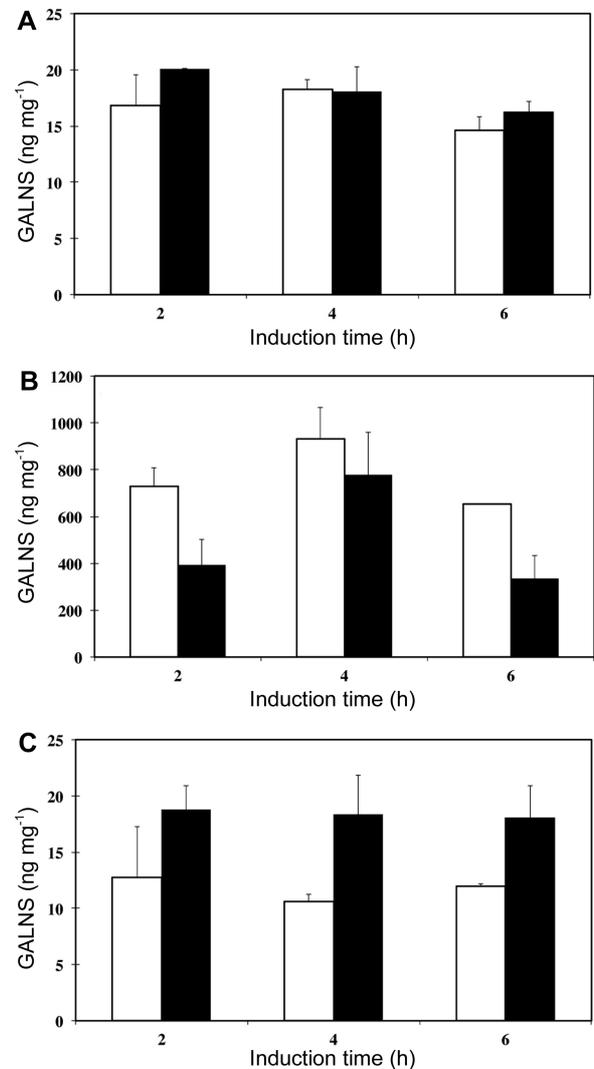


Fig. 3. ELISA quantitation of GALNS on soluble (A) and inclusion bodies (B) fractions from batch cultures of *E. coli* BL21/pGEX-GALNS, and soluble fraction from *E. coli* BL21/pGEX-nspGALNS (C) after induction with □, 0.5 or ■, 1.5 mM IPTG.

(Fig. 2C), whereas no inclusion bodies were recovered from BL21/pGEX-nspGALNS cultures. Nevertheless, no significant difference was observed among the GALNS activities from the inclusion bodies fractions at different induction times with 0.5 mM IPTG for the BL21/pGEX-GALNS strain (Fig. 2C). Using 1.5 mM IPTG, maximum activity was obtained after 4 h of induction (1.38 ± 0.28 U/mg), which was significantly higher than that observed at 2 and 6 h. Furthermore, this was the highest specific enzyme activity obtained in the batch assays. Enzyme activity levels in the inclusion bodies fraction at 0.5 mM IPTG were 5.8-, 8.5-, and 31.9-fold higher than in the soluble fraction after 2, 4, and 6 h of induction, respectively; whereas at 1.5 mM IPTG enzyme activity levels were 6.6-, 57.2-, and 15.9-fold higher than in the soluble fraction after 2, 4, and 6 h of induction, respectively. Finally, as it was observed at shake scale, enzyme activity was not detected in the culture medium for any of the evaluated strains.

ELISA quantitation results of GALNS from soluble and inclusion bodies fractions at batch culture conditions are shown in Fig. 3. For BL21/pGEX-GALNS batch cultures, it was observed that GALNS in inclusion bodies was 43.2-, 51.1-, and 44.8-fold higher at 0.5 mM IPTG and 19.0-, 43.0-, and 20.5-fold higher at 1.5 mM after 2, 4, and 6 h induction, respectively, than those levels observed in the soluble fractions (Figs. 3A and 3B). Contrary to the enzyme activity results in the soluble fractions, a significant difference for the GALNS concentration at the evaluated IPTG concentrations and induction times was not observed. However, in the inclusion bodies fraction, the highest GALNS concentrations was obtained after 4 h induction for both IPTG concentrations, 0.5 mM (932.5 ± 134.4 ng/mg) and 1.5 mM (776.7 ± 181.1 ng/mg) (Fig. 3B). The quantitation of GALNS from BL21/pGEX-nspGALNS cultures (Fig. 3C) showed that concentrations in the soluble fraction were similar or lower (up to 0.58-fold) than the levels observed for the BL21/pGEX-GALNS strain.

Semi-continuous mode. For this strategy, a batch culture was carried out for 13 h, after which the exhausted medium was removed by tangential filtration process, and fresh medium was added directly to the reactor. The OD_{620} was increased by 1.3- and 1.7-fold after 24 h of medium exchange at 1.2 and 2.4 ml/min turnover rates, respectively (Fig. 4). The initial glucose concentration was completely depleted during the batch stage, and increments on glucose concentration were not observed during the semi-continuous culture stage, regardless of the turnover rate (Fig. 4), showing that the glucose uptake rate by the cells was equal to or higher than the feeding and removal rates of medium. Despite that semi-continuous cultures of *E. coli* BL21/pGEX-GALNS showed a higher final cell density than that observed at batch cultures, a significant difference in soluble GALNS activity was not observed between both operational modes. Nevertheless, it was observed that the

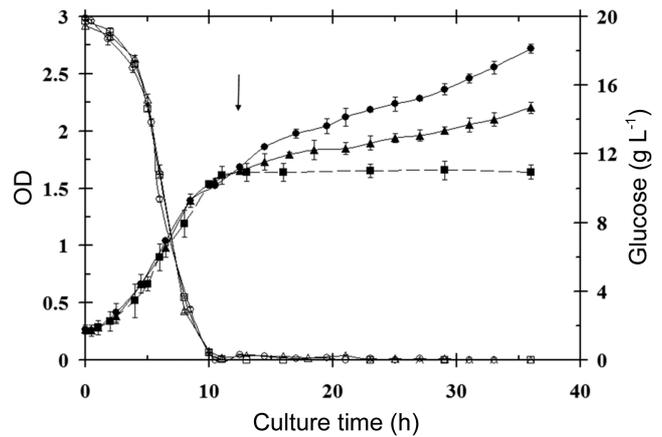


Fig. 4. Effect of medium turnover rate, 0 ml/min (batch mode, \square), 1.2 ml/min (\circ), and 2.4 ml/min (\triangle), on the cell density (closed symbols) and glucose consumption (open symbols). The arrow indicates the beginning of the semi-continuous stage.

higher the turnover rate, the higher the GALNS activity obtained in the soluble fraction (Fig. 5A). For this reason, a turnover rate of 2.4 ml/min was selected for subsequent experiments under this culture mode.

For the BL21/pGEX-GALNS strain, the highest GALNS activity in the soluble fraction was obtained with 1.5 mM IPTG after 4 h of induction, reaching an enzyme activity of 0.022 ± 0.002 U/mg (Fig. 5B). A reduction in GALNS activity in the inclusion bodies fraction was observed, which were 1.9- to 7.9-fold lower than the levels observed under batch culture conditions (Fig. 5C). The maximum GALNS activity in the inclusion bodies was 0.70 ± 0.04 U/mg after 4 h of induction with 1.5 mM IPTG. However, enzyme activity levels in the inclusion bodies fraction were between 1.0- and 48-fold higher than in the soluble fraction, as observed under batch culture conditions. Noteworthy, under this culture condition, GALNS activity was detected extracellularly, reaching up to 6.4 ± 1.1 U/mg (Fig. 5D).

For the BL21/pGEX-nspGALNS strain, the highest enzyme activity was obtained with 1.5 mM IPTG (Fig. 6). The GALNS activity in the soluble fraction was about 3.5-fold higher than those levels obtained in batch cultures with 0.5 and 1.5 mM IPTG, which contrast with the results obtained for the BL21/pGEX-GALNS strain. Longer induction times with BL21/pGEX-nspGALNS did not produce any significant increment in enzyme activity. In fact, after 30 h of induction, the enzyme activity was similar to that observed after 6 h (data not shown).

ELISA quantitation of GALNS on fractions from BL21/pGEX-GALNS under semi-continuous culture conditions showed a similar profile to that obtained for the enzyme activity. In the soluble fraction, the highest GALNS concentrations were observed after 6 h (4.94 ± 2.08 ng/mg) or 4 h (10.11 ± 1.77 ng/mg) of induction with 0.5 or 1.5 mM

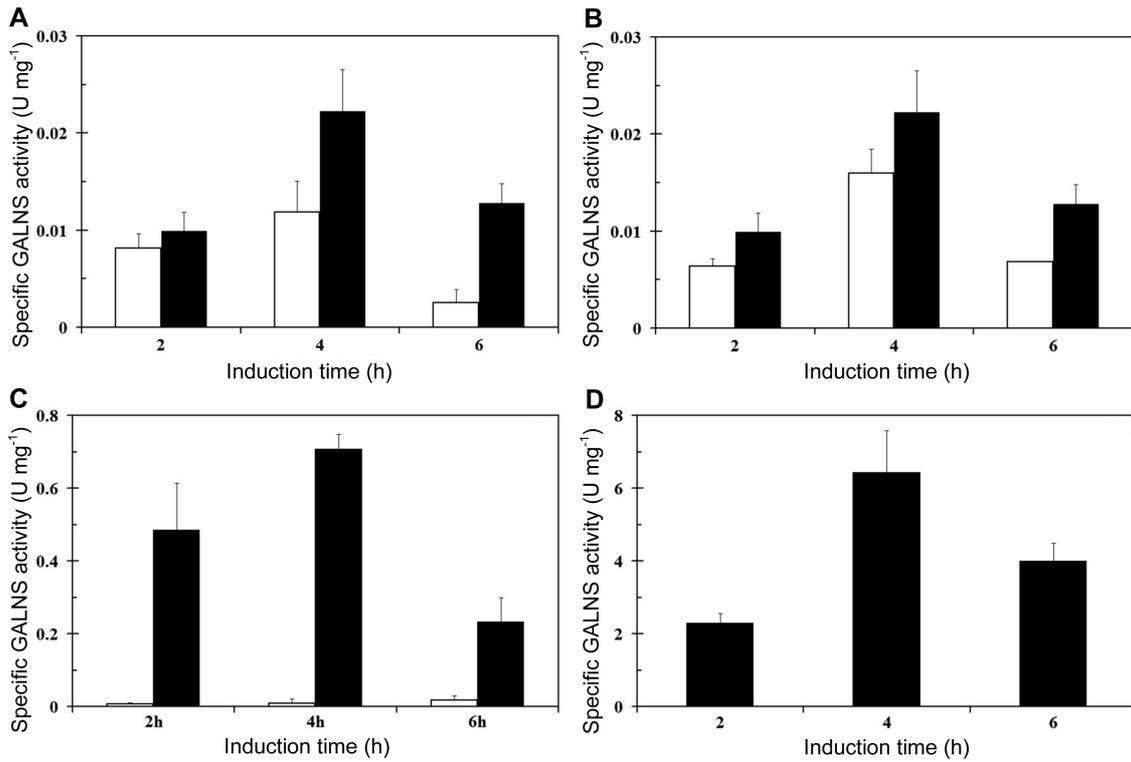


Fig. 5. GALNS activity under semi-continuous culture conditions of *E. coli* BL21/pGEX-GALNS. (A) Effect of culture medium turnover rate, 1.2 ml/min (□) and 2.4 ml/min (■), on soluble GALNS activity. (B) Effect of IPTG concentration, 0.5 mM (□) and 1.5 mM (■), on soluble GALNS activity at a culture medium turnover rate of 2.4 ml/min. (C) Effect of IPTG concentration, 0.5 mM (□) and 1.5 mM (■), on GALNS activity in inclusion bodies at a culture medium turnover rate of 2.4 ml/min. (D) GALNS activity in culture medium after culturing *E. coli* BL21/pGEX-GALNS in semi-continuous mode at a culture medium turnover rate of 2.4 ml/min.

IPTG, respectively. In the inclusion bodies fraction, there were not differences in the GALNS concentration, regardless of the IPTG concentration (Fig. 7A), which differs from the GALNS activity results obtained under batch culture conditions. However, as observed in batch cultures,

GALNS in inclusion bodies was 68.2-, 32.6-, and 25.8-fold higher at 0.5 mM IPTG and 50.4-, 13.6-, and 77.5-fold higher at 1.5 mM after 2, 4, and 6 h induction, respectively, than those levels observed in the soluble fractions (Fig. 7B). The GALNS concentration in both soluble and inclusion bodies fractions under semi-continuous conditions was lower than those obtained under batch conditions (between 1.7- and 7-fold), which agrees with the results of enzyme activity. As was noted for enzyme activity on soluble fractions from BL21/pGEX-nspGALNS, the highest concentration of GALNS determined by ELISA quantitation was obtained with 1.5 mM IPTG (Fig. 7C). Nevertheless, no significant difference was observed for GALNS concentration at the evaluated induction times, regardless of the IPTG concentration. Contrary to the results obtained with BL21/pGEX-GALNS, the GALNS amounts produced by BL21/pGEX-nspGALNS under semi-continuous mode were similar to the amounts obtained under batch mode.

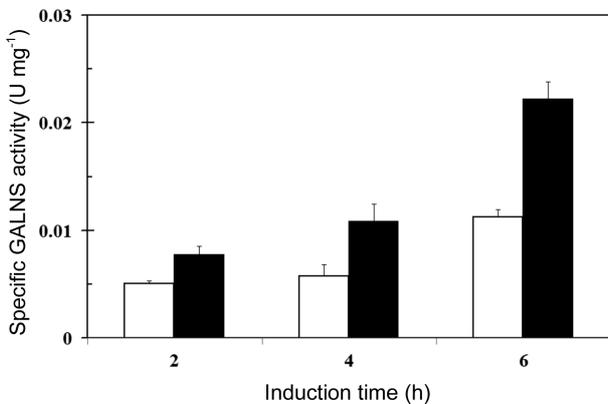


Fig. 6. GALNS activity for *E. coli* BL21/pGEX-nspGALNS cultured under semi-continuous mode. Effects of IPTG concentration 0.5 mM (□) and 1.5 mM (■) at a culture medium turnover rate of 2.4 ml/min.

DISCUSSION

Previously, the feasibility to produce an active recombinant GALNS enzyme in *E. coli* BL21(DE3) was demonstrated

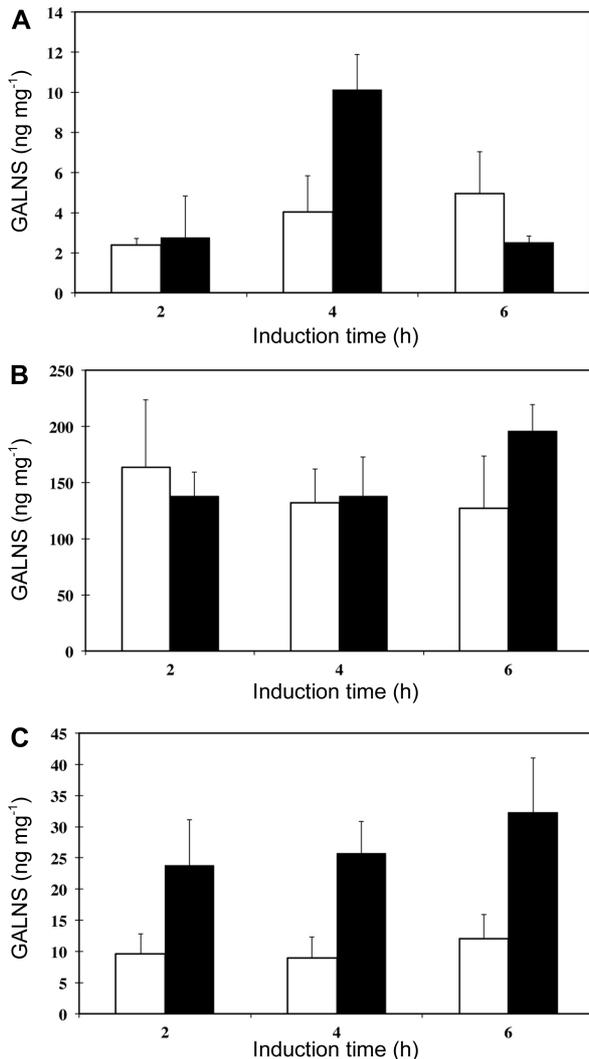


Fig. 7. ELISA quantitation of GALNS production under semi-continuous culture conditions on soluble (A) and inclusion bodies (B) fractions from *E. coli* BL21/pGEX-GALNS cultures, and on the soluble fraction from *E. coli* BL21/pGEX-nspGALNS cultures (C) after induction with □, 0.5 or ■, 1.5 mM IPTG.

[33]. In this study, the effects of IPTG concentration and the presence or absence of SP on GALNS levels at different scale levels and culture modes were evaluated. The results show that (i) optimal IPTG concentration depends on the strain and culture conditions and scale; (ii) removal of the signal peptide had a negative impact in GALNS activation and secretion, although it reduced the inclusion bodies formation, and (iii) secretion of recombinant GALNS was favored under semi-continuous culture conditions.

Selection of the IPTG concentration is an important task that needs to be considered during production of recombinant enzyme in *E. coli* [18]. The optimal IPTG concentration

depends on the strain, plasmid, cell density, and recombinant protein [18]. For example, the highest production of recombinant glycerophosphate oxidase was achieved with 0.005 mM IPTG by using *E. coli* BL21 and pET21b plasmid [42]; whereas for the recombinant eumelanin produced with *E. coli* strain W3110 and the pTrc99A vector, the highest enzyme production was observed with 1 mM IPTG [21]; and for the recombinant endoglucanase produced by *E. coli* BL21(DE3) with pET-28a(+) plasmid, the highest levels were observed between 0.2 and 0.4 mM IPTG [23]. In the present study, the optimal IPTG concentration varied depending on the strain, scale, and culture conditions. At shake scale, the highest enzyme activity was observed with 0.5 and 1.5 mM IPTG for BL21/pGEX-GALNS and BL21/pGEX-nspGALNS, respectively, whereas at bench scale both strains showed the highest enzyme activity after induction with 1.5 mM IPTG. These results correlate with those previously reported for BL21/pGEX-GALNS at shake and bench scales [33]. In summary, IPTG concentration should be optimized for each production system and recombinant protein.

At bench scale, excluding GALNS production with the BL21/pGEX-nspGALNS strain under batch mode, it was observed that a higher enzyme production was obtained after 4 h of induction for both soluble and inclusion bodies fractions, as well as extracellularly. However, ELISA quantitation showed that there was not a significant difference in GALNS concentration among the sampling times (2, 4, and 6 h) for each strain and the evaluated culture conditions. This performance could be associated with the activation process of the enzyme (*i.e.*, Cys-to-FGly conversion). Noteworthy, the highest intracellular enzyme activity level (1.38 ± 0.28 U/mg) was observed in the inclusion bodies fraction of the BL21/pGEX-GALNS strain cultured under batch culture, after 4 h of induction with 1.5 mM IPTG, which was significantly higher than those observed at 0.5 mM IPTG. However, the reason for this marked increase is not completely understood.

GALNS, as observed for all sulfatases, is characterized by the conversion of cysteine to FGly at the enzyme active site, mediated in eukaryotes by the formylglycine-generating enzyme [34]. This protein is present in the endoplasmic reticulum and catalyzes the Cys-to-FGly conversion after or at a late stage of the cotranslational protein translocation to the endoplasmic reticulum; that is, after removal of the signal peptide [11]. In prokaryotes, translocation of secreted proteins occurs as a posttranslational process [13]. Since the signal peptide is removed after protein translocation through the cell membrane [13], and prokaryotic FGly-forming enzyme is a cytosolic protein [24], it would be expected that the Cys-to-FGly conversion occurs before signal peptide removal. In fact, it was reported that activation of the prokaryotic sulfatase AtsA in *E. coli* was significantly reduced after AtsA signal peptide removal [24]. Although

important findings have been reported during the last years regarding prokaryotic FGly-forming enzyme [6], it is not completely understood how the signal peptide is involved in sulfatase activation in prokaryotes. In this sense, three different mechanisms have been proposed: (i) signal peptide may exert an effect on the protein folding state and modification competence; (ii) prokaryotic FGly-forming enzyme interacts with sulfatase through direct or indirect binding to the signal peptide, and this may be required for the FGly-forming enzyme activity; and (iii) the signal peptide, after binding to an adaptor, mediates the transport of the sulfatase to a cell location where FGly-forming enzyme is active [24]. Overall, the enzyme activity of GALNS in the soluble fraction was significantly reduced when the sequence coding for the signal peptide was removed. In this sense, GALNS activity in the soluble fraction was up to 7.6-fold higher with strain BL21/pGEX-GALNS than that observed with strain BL21/pGEX-nspGALNS. Since GALNS concentrations were relatively similar between cultures with and without SP, a significant reduction in enzyme activation was observed after removal of the SP. These results suggest that SP in *E. coli* could be necessary for the activation process of this enzyme. In addition, it is also shown that regardless of the mechanism by which the SP is associated with sulfatas activation in prokaryotes, this can also recognize a eukaryotic SP to mediate the activation of a human sulfatase, suggesting an evolutive conservation of this mechanism.

Protein aggregation (inclusion bodies) is a common issue during the production of heterologous proteins in *E. coli* [16]. As previously reported [26, 33], production of recombinant GALNS was higher in inclusion bodies than in the soluble fraction, regardless of the IPTG concentration or induction time. The aggregation of the recombinant GALNS depended on two factors: the enzyme sequence and the kinetics of the folding. In the first case, the absence of the SP avoided the production of inclusion bodies. Although the reasons for this result are not completely understood, we hypothesize that the absence of SP could avoid hydrophobic interactions that have been found to be one of the main determinants of inclusion body formation [38, 40]. Furthermore, the protein aggregation process depends on the kinetics of the folding, since changes in culture conditions (*i.e.*, semi-continuous culture) affect the rate of biosynthesis [16], leading to a reduction in the production of inclusion bodies.

An important result was that under semi-continuous culture conditions, recombinant GALNS was detected extracellularly. These results show the feasibility of obtaining an active extracellular GALNS enzyme, which could be used for enzyme purification [26], reducing the stages and cost of the downstream process. In this sense, SP may played a major role in the secretion of the GALNS, since enzyme activity in the culture media was only

detected in semi-continuous cultures of the BL21/pGEX-GALNS strain. The cotranslational transport of extracellular or membrane proteins is a highly conserved mechanism mediated by a ribonucleoprotein complex, the signal recognition particle (SRP), and the membrane-associated receptor (SR) [15]. Although there are some differences among the bacterial and eukaryotic SRP-SR systems, the bacterial system can replace their mammalian counterparts for protein translocation, suggesting the high conservation of this mechanism between bacteria and mammals [31]. In fact, prediction of SP cleavage sites by using SignalP 4.0 [29] showed that the GALNS signal peptide can be recognized at the same site (between Gly26 and Ala27) for both the artificial neural networks of eukaryotic and Gram-negative bacteria.

E. coli is one of the most used microorganism for the production of recombinant proteins, and it has been considered as a nonsecretory protein system [7]. However, several reports have shown that secretion of recombinant proteins may occur in commonly used *E. coli* strains through nonspecific mechanisms, depending on the strain and the culture methods [28]. For example, in the production of a polyester-cleaving hydrolase from *Thermobifida fusca*, it was observed that in a batch culture, the protein was mainly translocated to the periplasm, where it was completely released into the extracellular medium, whereas in fed-batch culture, most of the protein remained in the cytoplasm [12]. On the contrary, it was reported that the secretion of recombinant proteins is favored at high cell density cultures due to cell stress induced by environmental conditions [19]. The comparison of the *E. coli* strains BL21(DE3) and W3110 showed that the former one released more protein than W3110 strain, showing the potential of the BL21(DE3) strain for the production of extracellular recombinant proteins [41]. The release of proteins to the culture medium by different *E. coli* strains in a cell lysis-free manner has also been reported [19, 27].

In conclusion, different factors involved in the production of a recombinant GALNS in *E. coli* BL21(DE3), such as the presence of the native GALNS signal peptide, the concentration of IPTG, and operational mode (batch and semi-continuous), were reported. The results showed that the presence of SP has a positive effect on GALNS activation, since similar GALNS concentrations were observed with or without SP but lower enzyme activities were observed when the SP was removed. Although it is unknown how the mechanism of signal peptide recognition is linked to sulfatase activation, we have shown for the first time that this mechanism might also recognize a eukaryotic signal peptide to mediate the activation of a human recombinant sulfatase. In addition, the removal of the SP avoided the formation of inclusion bodies, which could be related with the reduction of hydrophobic interactions. The optimal IPTG concentration depended on the production

scale and the strain, since at shake scale the highest enzyme activity was observed with 0.5 and 1.5 mM IPTG for BL21/pGEX-GALNS and BL21/pGEX-nspGALNS, respectively, but at bench scale both strains showed the maximum enzyme activity with 1.5 mM, regardless of the operational mode. Another important result was that under semi-continuous mode, the presence of the SP favored the GALNS secretion, suggesting that the *E. coli* secretion machinery might recognize the human GALNS SP. However, further studies should focus on the characterization of the mechanism used for enzyme secretion and evaluation of the correct posttranslational modification of the recombinant enzyme. In summary, these results represent valuable information for the production of human sulfatases in *E. coli*, and highlight the role of the signal peptide in the secretion and activation of this enzyme in *E. coli*.

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