# Production and characterization of a human lysosomal recombinant iduronate-2-sulfatase produced in *Pichia pastoris*

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## Abstract

Hunter syndrome (Mucopolysaccharidosis II, MPS II) is an X-linked lysosomal storage disease produced by the deficiency of the lysosomal enzyme iduronate-2-sulfatase (IDS). Currently, MPS II patients are mainly treated with enzyme replacement therapy (ERT) using recombinant enzymes produced in mammalian cells. As an alternative, several studies have shown the production of active and therapeutic forms of lysosomal proteins in microorganisms. In this paper, we report the production and characterization of a recombinant IDS produced in the yeast *Pichia pastoris* (prIDS). We evaluated the effect of culture conditions and gene sequence optimization on prIDS production. The results showed that the highest production of prIDS was obtained at oxygen-limited conditions using a codon-optimized IDS cDNA. The purified enzyme showed a final activity of 12.45 nmol $mg^{-1}$ $H^{-1}$ and an apparent molecular mass of about 90 kDa. The highest stability was achieved at pH 6.0, and prIDS also showed high stability in human serum. Noteworthy, the enzyme was taken up by culture cells in a dose-dependent manner through mannose receptors, which allowed the delivery of the enzyme to the lysosome. In summary, these results show the potential of *Pichia pastoris* as a host to produce an IDS intended for a MPS II ERT.  Volume 65, Number 5, Pages 655–664, 2018

***Keywords:*** *culture conditions, iduronate-2-sulfatase, Hunter disease, Pichia pastoris, recombinant enzyme*

***Abbreviations:*** *CHO, Chinese hamster ovary cells; COS, CV-1 (simian) in origin, and carrying the SV40 genetic material cells; DMEM, Dulbecco's modified medium; DO, dissolved oxygen; ERT, enzyme replacement therapy; FGE, formylglycine-generating enzyme; GAG, glycosaminoglycan; GALNS, N-acetylgalactosamine-6-sulfate sulfatase; IDS, iduronate-2-sulfatase;* $IDS_{opt}$, P. pastoris *codon-optimized IDS cDNA;* $IDS_{wt}$, *wild-type human IDS; M6P, mannose-6-phosphate; Man, mannose; MPS II, Mucopolysaccharidosis II; prIDS, recombinant IDS produced in the yeast* Pichia pastoris*; rIDS, recombinant IDS; YNB, yeast nitrogen base.*

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# 1. Introduction

Hunter syndrome (Mucopolysaccharidosis II, MPS II; OMIM 309900) is an X-linked lysosomal storage disease produced by deficiency on the enzyme iduronate-2-sulfatase (IDS; 3.1.6.13). This deficiency leads to the lysosomal accumulation of the glycosaminoglycans (GAGs) heparan and dermatan sulfate [1]. Clinical manifestations of MPS II patients with severe phenotype include mental retardation, coarse facial features, short stature, skeletal deformities, joint stiffness, retinal degeneration, chronic diarrhea, progressive hearing impairment, and communicating hydrocephalus. On the other hand, patients with mild phenotype have somatic features similar to those observed in severe phenotype patients with a reduced progression rate and without central nervous system involvement [1].

Currently, MPS II patients are mainly treated with enzyme replacement therapy (ERT) [2, 3]. In addition, stem cells, bone marrow, or peripheral blood hematopoietic cells transplantations are also available [4]. Two recombinant enzymes are used for MPS II ERT: idursulfase (Elaprase®) and idursulfase-beta (Hunterasa®), which are produced in human and Chinese hamster ovary (CHO) cells, respectively [5]. The weekly infusion of these enzymes has led to the improvement in the 6-Min walked test, forced vital capacity, liver and spleen volumes, and a joint range of motion and urine GAGs levels [4, 6].

As an alternative to the production in mammalian cells, a growing number of studies have shown the possibility to produce active and therapeutic forms of lysosomal proteins in microorganisms [7]. For instance, we have reported the production of active forms of human rIDS and *N*-acetylgalactosamine-6-sulfate sulfatase (GALNS) and $\beta$-hexosaminidase A, B, and S in *E. coli* and/or *P. pastoris* [8–15]. Furthermore, recombinant $\alpha$-glucosidase [16], $\alpha$-galactosidase A [17], GALNS [18], and lysosomal acid lipase [19] produced in yeasts have shown successful cellular uptake both in cellular and animal models, and the reduction of the storage material. Taken together, these results support the feasibility of the use of recombinant proteins produced in yeasts for the development of ERTs for lysosomal storage diseases.

Culture conditions and yeast physiology have a significant impact on the final yields and productivity of the heterologous protein. Methanol, which is commonly used as an inducer of transgene expression in *P. pastoris*, has a high oxygen demand that leads to oxygen transfer problems [20]; reducing the expression of the recombinant proteins [21]. In this sense, it has been reported that high levels of recombinant protein can be obtained at limited oxygen conditions [22]. On the other hand, production at substrate (i.e., methanol) limited conditions allows to maintain a specific growth rate, the reduction of metabolic problems associated with the accumulation of methanol or its by-products, and the improvement in the reproducibility of the process [23].

As a continuation on the development of an ERT for MPS II using an enzyme produced in *P. pastoris* [8, 24–26], in this study we evaluated the effect of culture conditions (i.e., limited-oxygen and limited-substrate conditions) and codon optimization on the production of rIDS in *P. pastoris* (prIDS). Furthermore, the temperature pH and serum stability, and the cell uptake and intracellular trafficking for prIDS, were also evaluated.

# 2. Materials and Methods

## 2.1. Gene cloning and microorganism transformation

Wild-type human IDS ($IDS_{wt}$) cDNA (GenBank accession number nm_000202.6; Supplementary Material Fig. 1) was kindly donated by Dr. Shunji Tomatsu and inserted in pPIC9 plasmid (Thermo Fisher Scientific, San Jose, CA, USA) downstream of the $\alpha$-factor secretion signal from *Saccharomyces cerevisiae*. Furthermore, a *P. pastoris* codon-optimized IDS cDNA ($IDS_{opt}$, GeneArt®, Thermo Fisher Scientific; Supplementary Material Fig. 1) was also inserted in the pPIC9 plasmid. The constructs, pPIC9-$IDS_{wt}$ and pPIC9-$IDS_{opt}$, were linearized and used to independently transform competent cells of *P. pastoris* GS115. All procedures were carried out under standard molecular biology methods [27]. The Mut phenotype of *P. pastoris* clones was screened by culturing the clones in MM plates [1.34% (w/v) yeast nitrogen base, $4 \times 10^{-5}$% (w/v) biotin, and 0.5% (v/v) methanol] following the manufacturer's instructions (Thermo Fisher Scientific). Phenotype was also confirmed by PCR using the primers FW: 5′-GACTGGTTCCAATTGACAAGC-3′ and RW: 5′-GCAAATGGCATTCTGACATCC-3′, as previously reported [15, 28]. The strains *P. pastoris* GS115/$His^+$/$Mut^s$/HSA and *P. pastoris* GS115/$His^+$/$Mut^+$/B-Gal (Thermo Fisher Scientific) were used as $Mut^s$ and $Mut^+$ controls, respectively.

## 2.2. Shake flask cultures

Screening of the *P. pastoris* clones was done at 10 and 100 mL [12, 15]. Clones were grown in the YPD medium [yeast extract 1% (w/v); peptone 2% (w/v); dextrose 2% (w/v)] during 48 H at 28 °C and 250 rpm. Cells were harvested by centrifugation and resuspended in the BMG medium [potassium phosphate 100 mM pH 6.0 ± 0.2; yeast nitrogen base 1.34 % (p/v); biotin $4 \times 10^{-5}$ % (w/v); glycerol 1% (w/v)] and cultured for 24 H at 28 °C and 250 rpm. The cells were harvested and resuspended in the BMM medium [potassium phosphate 100 mM pH 6.0 ± 0.2; yeast nitrogen base 1.34 % (w/v); biotin $4 \times 10^{-5}$ % (w/v); glycerol 1 % (w/v); methanol 0.5 % (v/v)] and cultured for 120 H at 28 °C and 250 rpm. Methanol was added every 24 H to maintain a final concentration of 0.5% (v/v). Aliquots were taken every 24 H and stored at −20 °C until their use. The clones with the highest activity were selected for evaluation at the bioreactor scale. The cell density was determined by using a previously described calibration curve (g $L^{-1} = 0.528 \times OD_{600nm} \times 0.619$, $r^2 = 0.9870$) [8].

## 2.3. Bioreactor culture conditions

Bioreactor production of prIDS was done at a 1.65 L scale in a 3.7-L bioengineering reactor. Cultures were done in a modified fermentation medium FM22 (composition per liter: $KH_2PO_4$ 25.74 g, $(NH_4)_2SO_4$ 3 g, $K_2SO_4$ 8.58 g, $CaSO_4 \cdot 2H_2O$

0.6 g, glycerol 40 g, $MgSO_4 \cdot 7H_2O$ 7.02 g, biotin $4 \times 10^{-5}$% w/v, supplemented with *Pichia* trace minerals PTM4 1.0 mL) [29]. Protein production was done in two phases: (i) a batch culture with glycerol to achieve a 60-g $L^{-1}$ biomass and (ii) a fed-batch induction phase with methanol. At this scale, prIDS production was evaluated at standard conditions [8], and at substrate (i.e., methanol) or oxygen-limited conditions. The methanol concentration was maintained by using an ALCOSENS probe (Heinrich Frings, Rheinbach, Germany) with an automatic feed control. Cultures were done at 28 °C and pH 5.0 ± 0.2 during 96 H. The cell density was determined as described above.

### 2.4. Enzyme purification

prIDS was purified from culture medium due to the presence of the $\alpha$-factor secretion signal. Culture medium (~1.7 L) was filtered sequentially through Whatman® paper No. 1 and 42, and 0.45 and 0.22 $\mu$m polyether sulfone membranes (Pall, Port Washington, NY, USA). Permeate was ultrafiltered through a 30-kDa cutoff membrane (EMD Millipore, Billerica, MA, USA) up to reach a final volume of 20 mL. The retentate was diafiltered against 25 mM sodium acetate buffer (pH 5.0 ± 0.2) and concentrated up to 10 mL by using 10 kDa Amicon® Ultra Centrifugal filter (EMD Millipore Corporation). prIDS was purified by cation exchange chromatography with a Macro-Prep High S support column (Bio-Rad, Hercules, CA, USA) equilibrated with 25 mM potassium acetate (pH 5.0 ± 0.2) and eluted with a linear gradient of 0–0.5 M NaCl. Fractions with the highest activity were pooled, diafiltrated against 25 mM potassium acetate (pH 5.0), and stored at −80 °C for later use. Protein purification was monitored by sodium dodecyl sulfate-polyacrylamide gels electrophoresis (SDS–PAGE) under reducing conditions, Western blot, and IDS enzyme activity.

### 2.5. Enzyme activity assay

IDS activity was assayed by using 4-methylumbelliferyl-$\alpha$-L-Idopyranosiduronic acid 2-sulfate (Toronto Chemicals Research, North York, ON, Canada) as previously described [30]. One unit (U) was defined as the amount of enzyme catalyzing 1 nmol substrate per hour. Specific IDS activity was expressed as U $mg^{-1}$ of protein determined by the Lowry assay.

### 2.6. SDS–PAGE and Western blot

SDS–PAGE was performed as described by Laemmli [31] under reducing conditions. Protein samples (15 $\mu$L) were loaded on 12% acrylamide gels and 3% stacking gel. The molecular masses were determined by using Precision Plus Protein™ (Bio-Rad, 161-0363), including three reference bands (25, 50, and 75 kDa). The gels were stained with Coomassie blue R-250 (GE Healthcare Life Science, Pittsburgh, PA, USA). Equivalent volumes of purified recombinant proteins were loaded and run on a 12% (w/v) SDS–PAGE and electroblotted onto nitrocellulose membranes (Hybond C-Extra; Amersham Bioscience, Piscataway, NJ, USA). Membranes were blocked with a blocking buffer containing PBS 1× (composition per liter: 8 g NaCl, 0.2 g KCl, 1.44 g $NaH_2PO_4$, 0.24 g $KH_2PO_4$, pH 7.2 ± 0.2), 0.3% (v/v) Tween 20, and 5% (w/v) BSA (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 1 H. After blocking, all membranes were incubated overnight at 4 °C with primary chicken IgY anti-human IDS (1:500 in blocking buffer) [24], followed by incubation with a peroxidase-conjugated goat anti-chicken (1:2,000, Promega, Madison, WI, USA) for 1 H at room temperature. The specific bands were visualized using enhanced chemiluminescence (SuperSignal™ West Pico Chemiluminescent Substrate, Thermo Fisher Scientific). A leukocytes lysate from a human unaffected donor was used as positive control.

### 2.7. Proteases assay

Extracellular proteases were assayed as previously described [32], with some modifications. Briefly, 1 mL of centrifuged culture samples was mixed with 1 mL of 1% (w/v) casein solution. After 30 Min incubation at 37 °C, 1 mL of 15% (w/v) trichloroacetic acid (Sigma–Aldrich) was added to the mixture. The reaction was stopped by incubation at 4 °C during 5 Min. Finally, the mixture was centrifuged for 20 Min at 5,000*g* and 4 °C. The absorbance was measured at 280 nm in Eppendorf BioSpectrometer® spectrophotometer. One proteolytic unit (PU) was defined as the amount of enzyme required to increase the absorbance at 280 nm by 0.05 AU in 30 Min.

### 2.8. Recombinant IDS characterization

The effect of pH and serum on prIDS stability was assayed as previously reported [12, 14]. To evaluate the effect of pH on prIDS stability, 20 $\mu$L of the purified enzyme was incubated with 20 $\mu$L of 50 mM sodium citrate (pH 3.0 ± 0.2), or 50 mM sodium acetate pH 4.0, 5.0, or 6.0 ± 0.2, or 50 mM Tris–HCl (pH 7.0 or 8.0 ± 0.2), respectively. Samples were incubated at 37 °C for 1 H. To assess the stability of prIDS at 37 °C, 10 $\mu$L of the purified enzyme, spiked or not 20 $\mu$L of pooled human serum obtained from healthy donors, was incubated during 8 H at 37 °C. In all cases, after incubation under the respective conditions, the enzyme activity was assayed as described above. Kinetic parameters [elimination rate ($K_e$) and elimination half-life ($t_{1/2}$)] of IDS were calculated with GraphPad PRISM 6.0, assuming an exponential one-phase decay model.

### 2.9. Cellular uptake assay

The cellular uptake of the prIDS was assayed in HEK 293 cells (CLR1573; ATCC, Manassas, VA, USA), as previously described [12, 14, 15]. The cells were cultivated in Dulbecco's modified medium (Gibco, Carlsbad, CA, USA), supplemented with fetal bovine serum 15% (v/v), penicillin 100 U $mL^{-1}$, and streptomycin 100 U $mL^{-1}$, at 37 °C in a $CO_2$ incubator. Twenty-four hours before the experiment, $1 \times 10^5$ cells per well were seeded in 12-well plates, and the culture medium was replaced with fresh medium 2 H before the experiment. The purified enzyme was added to final concentrations of 0, 10, 50, and 100 nm [14, 33], with or without the presence of 2 mM mannose-6-phosphate (M6P; Sigma-Aldrich) or 2 mM methyl $\alpha$-D-mannopyranoside (Sigma-Aldrich). After 6 H of incubation, the culture medium was removed and the cells were washed three times with cold PBS 1×. Cells were lysed by

resuspension in 1% (w/v) sodium deoxycholate (Sigma-Aldrich). The enzyme activity was determined in the cell lysate and the culture medium as described above. All assays were performed in triplicate.

For the intracellular trafficking evaluation of prIDS, it was labeled with AlexaFluor 568 following the manufacturer's protocol (Molecular Probes, Thermo Fisher Scientific, San Jose, CA, USA). HEK293 cells were grown on coverslips treated with 0.01% ($^{W}/_{V}$) type II collagen (Sigma-Aldrich) and placed onto 12-well plates. Cells were seeded at a density of $2 \times 10^4$ cells per well as described above. Once the cells were confluent, the culture medium was exchanged for fresh medium 1 H before adding the fluorescent-labeled prIDS at a final concentration of 50 nm. After 12 H of incubation with the labeled enzyme, the cells were stained with LysoTracker® Green DND-26 (Molecular Probes, Thermo Fisher Scientific) following the manufacturer's protocol. Cells were then fixed using freshly prepared 4% paraformaldehyde in 1× PBS for 20 Min at room temperature, followed by 10 Min of permeabilization with 0.2% Triton X-100 in 1× PBS. Finally, the cellular nucleus was stained with 4′,6-diamidino-2-phenylindole dichydrochloride (DAPI; Thermo Fisher Scientific). Cells were imaged using an Olympus FV1000 confocal microscope equipped with 405, 473, and 559 nm laser lines using a 63 × /1.49 NA oil objective. DAPI (excitation 382–393, emission 417–477 nm), FITC (excitation 460–500 nm, emission 510–560 nm), and TRITC HyQ filter sets (excitation 530–560 nm, emission 590–650 nm) were applied to collect DAPI, LysoTracker® Green, and Alexa 568 signals, respectively. Images were processed by using NIH Image J software [34].

### 2.10. Statistical analysis

The results are shown as mean ± standard deviation (SD) and were analyzed by an ANOVA, followed by the Sidak *t*-test when appropriate. Differences between groups were considered significant when $P < 0.05$ on GraphPad PRISM 6.0.

## 3. Results and Discussion

### 3.1. Production at shake flask scale

Twenty and six clones were obtained after transformation of *P. pastoris* GS115 with the vectors pPIC9-$IDS_{wt}$ and pPIC9-$IDS_{opt}$, respectively. $IDS_{wt}$ and $IDS_{opt}$ cDNAs showed a 75.9% identity, without affecting the amino acid sequence. The Mut phenotype of all the clones was evaluated in the MM medium and by PCR, showing a $Mut^s$ phenotype (data not shown). At a shake-flask scale (100 mL), the highest volumetric and specific activities with pPIC9-$IDS_{wt}$ were 0.22 U $mL^{-1}$ and 1.0 U $mg^{-1}$, respectively. These activities remained relatively stable until the end of the culture (120 H), with enzyme activity levels of 0.21 U $mL^{-1}$ and 0.7 U $mg^{-1}$. A final biomass of 13.5 g $L^{-1}$ and an extracellular protein concentration of 0.28 mg $mL^{-1}$ were obtained. Similar results were observed with pPIC9-$IDS_{opt}$, with maximum volumetric and specific enzyme activities of 0.21 U $mL^{-1}$ and 0.51 U $mg^{-1}$. As observed, the specific enzyme activity in pPIC9-$IDS_{opt}$ was about twofold lower than that obtained with pPIC9-$IDS_{wt}$, which could be associated with the higher protein concentration observed with the pPIC9-$IDS_{opt}$ clone than observed with the pPIC9-$IDS_{wt}$ clone.

### 3.2. Production at the bioreactor scale

Production of prIDS at the bioreactor scale was evaluated at previously reported standard conditions [8] and at substrate-(methanol) or oxygen-limited conditions for the selected pPIC9-$IDS_{wt}$ and pPIC9-$IDS_{opt}$ clones 1 (Table 1 and Fig. 1). Production at oxygen-limited conditions for both pPIC9-$IDS_{wt}$ and pPIC9-$IDS_{opt}$ clones was carried out at dissolved oxygen (DO) lower than 1% (v/v); whereas production at substrate-limited conditions was carried out at methanol concentrations lower than 0.06% v/v.

At standard culture conditions [8], cell density, extracellular protein, and enzyme activity were 112 g $L^{-1}$, 1.34 mg $mL^{-1}$, and 0.36 U $mg^{-1}$, respectively, for pPIC9-$IDS_{wt}$, whereas for pPIC9-$IDS_{opt}$ the values were 149 g $L^{-1}$, 1.46 mg $mL^{-1}$, and 0.43 U $mg^{-1}$, respectively (Table 1 and Fig. 1). It is important to note that these results differ from previous studies of production of recombinant IDS in *P. pastoris* at standard conditions [8, 24–26], which showed higher enzyme activity levels than those reported in the present study. However, differences in substrate and enzyme activity assay conditions, limit the comparison among these studies.

At oxygen- and methanol-limited conditions, higher cell densities were obtained (between 188 and 316 g $L^{-1}$) than those observed at standard culture conditions. In contrast, oxygen- and methanol-limited conditions led to a reduction in the extracellular protein concentration (0.68–1.10 mg $mL^{-1}$). As observed at standard culture conditions, at limited culture conditions the use of $IDS_{opt}$ cDNA allowed to obtain higher enzyme activity values than those observed with $IDS_{wt}$ (Table 1 and Fig. 1). In addition, higher enzyme activities were obtained with the $IDS_{opt}$ cDNA at limited culture conditions (between 2.4- and 4.6-fold) than those observed with the same cDNA at standard culture conditions. In fact, the highest enzyme activity levels (1.58 U $mg^{-1}$) were observed with pPIC9-$IDS_{opt}$ at limited-oxygen conditions. In contrast, a marked reduction in IDS activity was observed with $IDS_{nat}$ both at oxygen- and methanol-limited conditions, with activity values lower than 0.04 U $mg^{-1}$ (Table 1 and Fig. 1).

In summary, these results showed that the highest production of prIDS was obtained by using the codon-optimized gene ($IDS_{opt}$) cultured at oxygen-limited conditions (Fig. 1). In addition, it was observed that the most sensitive variable for IDS production was the substrate (methanol) concentration, since, regardless of the gene sequence (wild-type or optimized), the enzyme activity values were lower than those observed at limited-oxygen conditions. These results agree with those reported by Charoenrat et al. [35], who showed that, during the production of a recombinant $\beta$-glucosidase in *P. pastoris*, higher enzyme activity levels were observed at oxygen-limited conditions (13 U $mg^{-1}$) than at substrate-limited conditions (8 U $mg^{-1}$). Similarly, Berdichevsky et al. [36] reported the

**TABLE 1** *Summary of cell density, protein concentration, enzyme activity, and protease activity during the production of prIDS using wild-type ($IDS_{wt}$) and codon-optimized ($IDS_{opt}$) human IDS genes, at standard and oxygen- and methanol-limited conditions*

| Gene | Condition | Cell density (g $L^{-1}$) | Protein (mg $mL^{-1}$) | Enzyme activity (U $mg^{-1}$) | Protease activity (PU $L^{-1}$) |
|---|---|---|---|---|---|
| $IDS_{wt}$ | Standard[a] | 112 | 1.34 | 0.36 | 1,039 |
| | Limited oxygen[b] | 296 | 1.10 | 0.04 | 1,634 |
| | Limited methanol[c] | 316 | 0.65 | N.D. | 1,635 |
| $IDS_{opt}$ | Standard[a] | 149 | 1.46 | 0.43 | 1,762 |
| | Limited oxygen[b] | 188 | 0.68 | 1.58 | 1,093 |
| | Limited methanol[c] | 240 | 0.70 | 0.87 | 1,161 |

N.D.: Not detected.

[a]As previously reported by Córdoba-Ruiz et al. [8].

[b]Dissolved oxygen lower than 1%.

[c]Methanol concentration was lower than 0.06%.

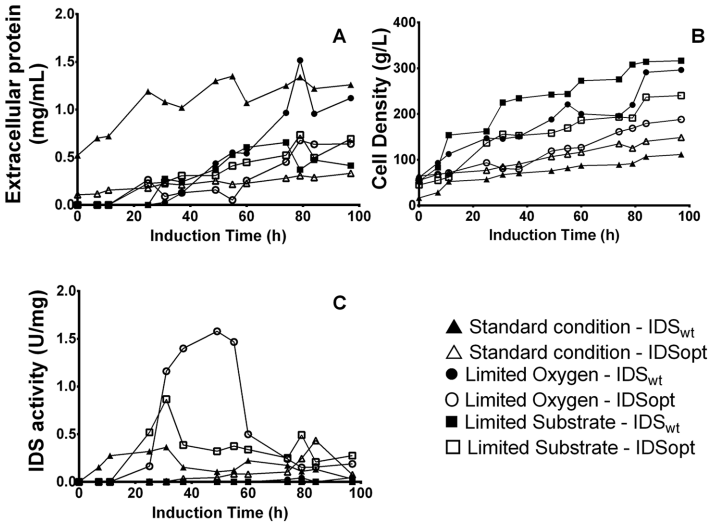


**FIG. 1** *Production of recombinant IDS at bioreactor scale. Human rIDS was produced at the bioreactor scale (1.65 L) at standard or substrate- (methanol) and oxygen-limited conditions using human wild-type ($IDS_{wt}$) or a P. pastoris codon-optimized IDS ($IDS_{opt}$) cDNAs. Cultures were followed up by the specific extracellular protein concentration (A), cell density (B), and IDS activity (C).*

improvement in the production of recombinant antibodies through the oxygen-limited cultivation of a glycol-engineered *P. pastoris* strain. They observed a reduction in the rate of maximum oxygen consumption, as well as an elongation of the induction phase leading to a 95% increase in the concentration of intact monoclonal antibodies.

Oxygen- and substrate-limited cultures of pPIC9-$IDS_{wt}$ showed that despite the high cell density values (up to 296 and 316 g $L^{-1}$, respectively), enzyme activity was lower than that obtained with the clone expressing the codon-optimized IDS gene. These results might suggest that human wild-type IDS gene cannot be properly expressed on *P. pastoris* or that high biomass densities could have a negative impact on the production of recombinant IDS. It is known that one of the main problems in the production of recombinant proteins in *P. pastoris* is the proteolytic degradation induced by proteases released into the culture medium [37]. In this sense, the high cell density observed in these cultures could favor the proteolytic degradation of prIDS by proteases released due to cell lysis. Extracellular proteases assay showed that pPIC9-$IDS_{wt}$ cultures at oxygen- and substrate-limited conditions (which showed the highest cell density) had higher protease activity (1,634 PU $L^{-1}$ at 96 H and 1,635 PU $L^{-1}$ at 55 H, respectively) than pPIC9-$IDS_{opt}$ cultured at the same conditions (1,093 PU $L^{-1}$ at 96 H 1,161 PU $L^{-1}$ at 95 H, respectively, Table 1).

### 3.3. IDS purification

prIDS was purified from the culture medium taking advantage of the presence of the $\alpha$-factor secretion signal at the N-terminal. After purification, the final enzyme activity was 12.45 U $mg^{-1}$ with a 276.7-fold of purification and a yield of 8.9% (Table 2), which suggest a production of 5.2 mg of prIDS per culture liter. The low yield was mainly associated with a protein loss during the ultrafiltration step, in which about 54% of the enzyme was lost. Although there is a report for the purification of rIDS produced in *E. coli*, there is not data about the final activity of the enzyme [38]. In this sense, this study represents the first report of the purification of an active rIDS enzyme produced in a microorganism. However, prIDS activity was significantly lower than that reported for the enzyme produced in mammalian cells, for which enzyme activity levels between 1.6 and 2.5 $\times$ $10^6$ U $mg^{-1}$ have been reported [5]. This marked difference in enzyme activity could be associated with the lack of formylglycine-generating enzymes (FGE) similar to

**TABLE 2** *Purification results of rIDS produced in P. pastoris GS115*

| *Fraction* | *Protein (mg)* | *Units (nmol $H^{-1}$)* | *Specific activity (U $mg^{-1}$)* | *Yield (%)*[a] | *Fold*[b] |
|---|---|---|---|---|---|
| Crude extract | 2,475.1 | 111.37 | 0.045 | 100.0 | 1.0 |
| Macro-Prep High S support | 1.3 | 12.36 | 9.510 | 11.1 | 211.3 |
| Sephacryl™ S-200 | 0.8 | 9.96 | 12.450 | 8.9 | 276.7 |

[a]The yield is based on the recovery of the activity after each step. This is calculated from the total activity (units).

[b]Refers to the increase in the specific activity.

those described in mammals or bacteria [39–42]. FGE catalyzes the formation of a formylglycine (FGly) residue from a cysteine at the active site of the enzyme [43]. However, the results of the present study, as well as those from previous reports of the production of human lysosomal sulfatases in *P. pastoris* [8, 9, 12, 25], suggest that this host should have a protein capable of catalyzing the Cys-to-FGly conversion, although with a lower efficiency than the mammalian counterpart. In this sense, the production of rIDS in *P. pastoris* might saturate the yeast Cys-to-FGly conversion system, avoiding the activation of the total produced protein. Similar results were observed during the production of recombinant sulfatases in mammalian cells, in which the overexpression of the human FGE was needed to increase the proportion of active enzyme [43, 44]. In addition, since prIDS was able to catalyze the hydrolysis of the fluorogenic substrate commonly used for MPS II diagnosis [30], the Cys-to-FGly conversion should be occurring within the sulfatase motif present in prIDS (PROSITE PS00523, AVCAPSRVSFLTG, in which underlined amino acid represents the catalytic residue).

An engineered recombinant IDS, fused with the heavy chain of a chimeric monoclonal antibody to the human insulin receptor, produced in **C**V-1 (simian) in **O**rigin, and carrying the **S**V40 genetic material (COS) cells, showed an enzyme activity of $51 \times 10^3$ U $mg^{-1}$ [45], which is 1,000-fold lower than that reported for an IDS produced in other mammalian cell lines. Noteworthy, the addition of 0.3 $\mu$g $mL^{-1}$ ($\sim$ 3,750 pM) of this engineered IDS to MPS II patient fibroblasts allowed an 84% reduction of GAGs accumulation. Similarly, *in vitro* evaluation of idursulfase and idursulfase-beta in MPS II patient fibroblasts led to a reduction of up to 40–60% of GAGs accumulations after addition of 5,000 pM IDS [46]. Taken together, these reports suggest that significant GAGs reduction can be observed with enzymes showing enzyme activities lower than those reported for idursulfase or idursulfase-beta. In addition, a recombinant GALNS produced in *P. pastoris*, which also have a significantly lower activity than the enzyme produced in CHO cells, showed a 60% reduction in the GAGs levels of Morquio A fibroblasts [47]. In this sense, it could be expected that prIDS allows a significant reduction of stored GAGs in a similar way than that produced by recombinant enzymes produced in mammalian cells.

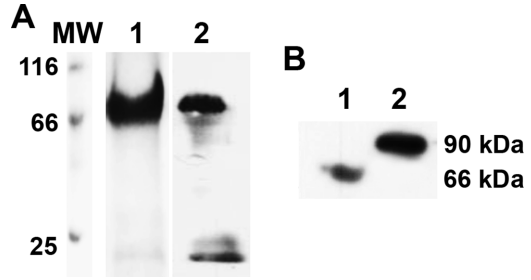


**FIG. 2** *(A) SDS–PAGE and Western blot analysis of purified prIDS. Lane 1: Purified protein was analyzed by SDS–PAGE and the gel was stained with Coomassie blue R-250. Lane 2: Western blot revealed with chicken anti-IDS antibody and visualized by chemiluminescence. MW: molecular mass marker. (B) Western blot analysis human leucocytes IDS (lane 1) and prIDS (lane 2). Proteins were revealed with chicken anti-IDS antibody.*

SDS–PAGE and Western blot analysis of prIDS allowed the identification of polypeptides with an apparent molecular mass of about 90 and 18 kDa (Fig. 2A). In addition, prIDS showed a higher molecular mass than that of IDS from human leucocytes (Fig. 2B). This difference in molecular weight could be associated with differences in the *N*-glycosylations produced by human cells and yeast [48, 49]. Nevertheless, a molecular mass of about 90 kDa has been reported for rIDS produced in CHO-K1 and COS cells [45, 50]; whereas for IDS purified from human liver, polypeptides with molecular masses of 42 and 14 kDa were reported [51]. In this sense, although high mannose *N*-glycans ($Man_{8\text{-}12}$) have been described for recombinant proteins produced in *P. pastoris* [52–55], in comparison with the low mannose *N*-glycans ($Man_5$) described for proteins produced in CHO cells [52], it seems that under the current culture conditions, prIDS might have similar posttranslation modifications to those carried out by mammalian cell. These results differ from previous reported of rIDS produced in *E. coli*, for which several bands were detected at the intracellular (62, 52, 49, and 40 kDa) and extracellular fractions (97, 49, 43, 41, and 40 kDa) [10], whereas a band at approximately 40 kDa was detected after purification [38]. Similarly, for the rIDS produced

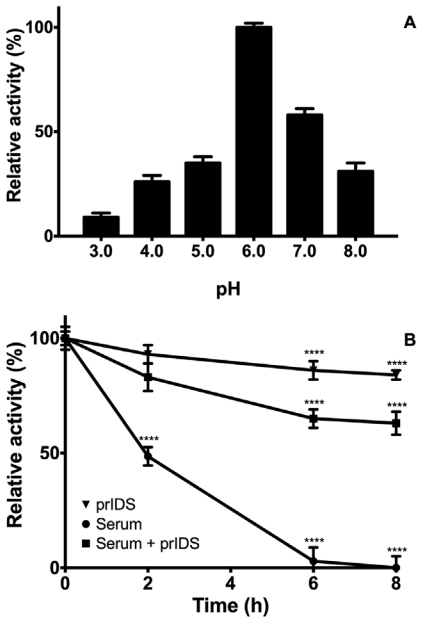


**FIG. 3** *pH and 37 °C stability. (A) Purified prIDS was incubated at pH 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 ± 0.2 during 1 H, after which enzyme activity was measured using the fluorogenic substrate. The activity is reported as relative activity against the enzyme activity values at pH 6.0 ± 0.2, which was the pH of maximum enzyme activity. (B) Stability of the purified prIDS was evaluated at 37 °C in the presence or absence of human serum from healthy donors. The enzyme was incubated during 8 H, after which enzyme activity was measured using the fluorogenic substrate. The activity is reported as relative activity against the activity at time 0 H. All assays were performed in triplicate.*

in *P. pastoris* GS115 at the shake flask scale (100 mL), fragments of 109, 92, 89, 82, 67, 49, and 40 kDa were reported at the crude extracellular fraction [9], suggesting that production under controlled conditions (i.e., bioreactor) might promote the correct protein maturation.

### 3.4. Recombinant IDS stability

The stability of the purified prIDS was assayed at different pH values and at 37 °C in the absence or presence of human serum (Fig. 3). The results showed that the highest stability of prIDS was observed at pH 6.0 ± 0.2. A marked reduction in activity was observed at lower pH values (<35% of the activity at pH 6.0), whereas at pH 7.0 and 8.0, the enzyme activity ranged between 58% and 31% of activity observed at pH 6.0. Similar results were previously reported for human liver IDS, depending of the substrate [51], as well as for other lysosomal recombinant enzymes produced in *P. pastoris* [12, 15].

At 37 °C, the prIDS showed high stability during the evaluated time (8 H), with a $K_e$ of 0.23 $H^{-1}$ and a $t_{1/2}$ of 3.1 H ($r^2 = 0.84$) (Fig. 3B). At the initial time, the enzyme activity on spiked human serum was sevenfold higher than that observed in nonspiked human serum. prIDS stability in human serum was lower than that observed for purified prIDS, showing a 25% reduction ($P < 0.001$) of the enzyme activity after 8 H evaluation in comparison of that observed for the purified prIDS (Fig. 3B). It is noteworthy that prIDS in human serum showed a significantly higher stability ($P < 0.001$) than that observed for the human IDS, with lower $K_e$ (0.26 $H^{-1}$) and higher $t_{1/2}$ (2.6 H, $r^2 = 0.93$) than those observed for nonspiked human serum ($K_e$ of 0.32 $H^{-1}$ and a $t_{1/2}$ of 2.1 H, $r^2 = 0.99$). In this sense, these results suggest that the prIDS could be stable under physiological conditions. These results agree with the stability observed for recombinant GALNS produced in *E. coli* and *P. pastoris* [11, 14], supporting the fact that differences in *N*-glycosylations (i.e., lack or structural changes) do not affect the stability of these type of lysosomal enzymes. Since prIDS was stable during 8 H, it is expected that this enzyme could have an *in vivo* half-life time that allows its therapeutic use. For instance, Xie et al. [56], for a recombinant human IDS produced in a human cell line (HT-1080), reported that after an intravenous infusion into *Cynomolgus* monkeys no enzyme was detected after 8 H of postinfusion. However, future studies should consider the pharmacokinetic evaluation of this prIDS enzyme.

### 3.5. Cell uptake and intracellular trafficking

Conventional ERT for lysosomal storage diseases is based on the capacity of the therapeutic enzymes to be taken up and delivery to the lysosome, where they catalyze the hydrolysis of the stored substrates [57]. Recombinant IDS produced in mammalian cells (idursulfase and idursulfase-beta) have highly sialylated and M6P-containing *N*-glycans, which allow their cell uptake in normal and MPS II patient fibroblasts [5, 46]. In addition, the cell uptake and lysosomal delivery of both idursulfase and idursulfase-beta is mediated by M6P receptors [46].

Addition of prIDS to HEK 293 cells showed a dose-dependent increase ($P < 0.001$) in the intracellular enzyme activity, suggesting an uptake of the recombinant enzyme (Fig. 4). Furthermore, coadministration of the prIDS with mannose or M6P to the cell cultures showed that the uptake was inhibited after addition of mannose, whereas significant cell uptake ($P < 0.001$) was still observed in the presence of M6P (Fig. 4), suggesting that prIDS is taken up through a mannose receptor-mediated process. Noteworthy, confocal laser scanning microscopy showed that the fluorescent-labeled prIDS colocalized with the lysosomal signal, suggesting an efficient delivery of the enzyme to the lysosome (Fig. 5). Taken together, these results show that prIDS is taken up through mannose receptors and delivery to the lysosome, without the need of any additional modifications of the enzyme or the *N*-glycans. These results are in agreement with previous reports showing that recombinant proteins produced in *P. pastoris* are taken up through mannose receptors and delivered to the lysosome [19], due to the presence of highly mannosylated *N*-glycans ($Man_{8\text{-}12}$) [52–55]. Similar results have been observed for human $\alpha$-glucosidase [58], lysosomal acid lipase

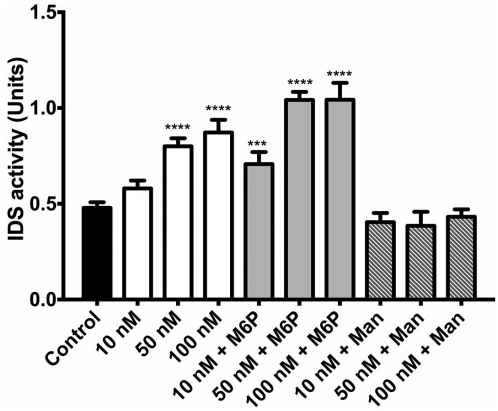


**FIG. 4** *Cellular uptake of prIDS. The cellular uptake of prIDS was assayed in HEK 293 cells. The purified prIDS was added to a final concentration of 10, 50, and 100 nm, with or without the presence of 2 mM M6P or 2 mM methyl α-D-mannopyranoside (Man). ***P < 0.001, ****P < 0.0001. In all cases the enzyme activity was assayed in the cell lysate after 6 H of incubation. HEK293 cells treated with PBS 1× were used as control. All assays were performed in triplicate.*

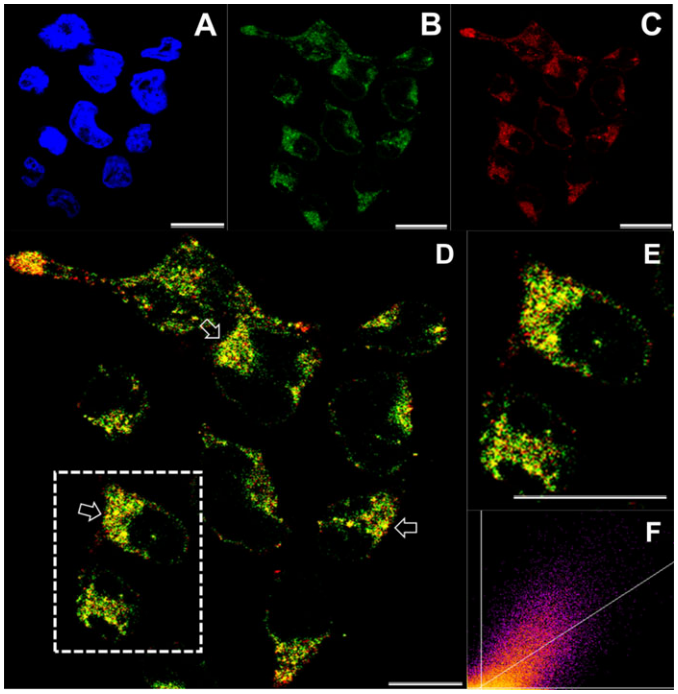


**FIG. 5** *Intracellular trafficking of recombinant IDS. The intracellular trafficking of prIDS was evaluated in HEK 293 cells. (A) DAPI staining, (B) LysoTracker® Green DND-26, (C) Alexa Fluor 568 labeled IDS, and (D) double fluorescence for LysoTracker® Green DND-26 and Alexa flour 568 labeled prIDS. Open arrows indicate different colocalization events. (E) Magnified image from the delimited region from (D), showing colocalization of IDS with the lysosomal marker. (F) Pearson correlation value was calculated by Fiji (Image J) with r = 0.7730 and 40.99% of colocalization volume.*

[19, 52], β-hexosaminidases [15], and GALNS [12] produced in *P. pastoris*, which showed a dose-dependent cell uptake without any additional processing of the enzymes. In addition, analysis of *N*-glycans from human recombinant GALNS produced in *P. pastoris*, under the same conditions described in the present study, showed the expected mannosylated *N*-glycans [59]. On the other hand, these results contrast with those of recombinant Hex-A [60, 61], α-glucosidase [62], and α-galactosidase A [63] produced in *Oryza minuta*, *Yarrowia lipolytica*, and *S. cerevisiae*, respectively, which required the treatment with a bacterial glycosidase to allows the cell uptake. Overall, these results continue to provide valuable evidence on the use of *P. pastoris* as a host for the production of lysosomal enzymes for therapeutic purposes. It is noteworthy that no further enzyme processing was needed to facilitate the cell uptake of the protein. However, recent glycoengineering studies in microorganisms have showed the possibility of producing proteins with human-like or tailored *N*-glycosylations that might have a positive impact on the stability, pharmacokinetics, or pharmacodynamics [48, 53].

## 4. Conclusions

In this study, we showed the production and characterization of a human rIDS in the methylotrophic yeast *P. pastoris* GS115. The results showed that the highest enzyme production was observed at limited-oxygen conditions and that the use of and optimized cDNA sequence favored the production of the recombinant enzyme. Although previous studies have reported the production of rIDS in microorganisms, this study shows for the first time the characterization of this recombinant enzyme. The enzyme showed it maximum stability at pH 6.0 ± 0.2 and high stability in human serum. Noteworthy, this prIDS was taken up by HEK 293 cells in a dose-dependent manner, without the need of any modification of the enzyme or the host. In addition, this prIDS was efficiently delivered to the lysosome. Overall, these results confirm the capacity of *P. pastoris* to produce active lysosomal enzymes and represent valuable information toward the development of an ERT for MPS II using this recombinant enzyme.

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## 6. References


[1] Neufeld, E., and Muenzer, J. (2001) In The Metabolic and Molecular Bases of Inherited Diseases (Scriver, C., Beaudet, A., Sly, W., and Valle, D., Eds.). 8th ed. McGraw-Hill, New York, 2001. pp. 3421–3452.

[2] Sohn, Y. B., Cho, S. Y., Lee, J., Kwun, Y., Huh, R., and Jin, D.-K. (2015) Mol. Genet. Metab. 114, 156–160.

[3] Muenzer, J., Beck, M., Eng, C. M., Giugliani, R., Harmatz, P., Martin, R., Ramaswami, U., Vellodi, A., Wraith, J. E., Cleary, M., Gucsavas-Calikoglu, M., Puga, A. C., Shinawi, M., Ulbrich, B., Vijayaraghavan, S., Wendt, S., Conway, A. M., Rossi, A., Whiteman, D. A., and Kimura, A. (2011) Genet. Med. 13, 95–101.

[4] Scarpa, M., Almassy, Z., Beck, M., Bodamer, O., Bruce, I. A., De, M. L., Guffon, N., Guillen-Navarro, E., Hensman, P., Jones, S., Kamin, W., Kampmann, C., Lampe, C., Lavery, C. A., Teles, E. L., Link, B., Lund, A. M., Malm, G., Pitz, S., Rothera, M., Stewart, C., Tylki-Szymanska, A., van, dP. A., Walker, R., Zeman, J., and Wraith, J. E. (2011) Orphanet. J. Rare Dis. 6, 72.

[5] Chung, Y. K., Sohn, Y. B., Sohn, J. M., Lee, J., Chang, M. S., Kwun, Y., Kim, C. H., Lee, J. Y., Yook, Y. J., Ko, A. R., and Jin, D. K. (2014) Glycoconj. J. 31, 309–315.

[6] Sohn, Y. B., Cho, S. Y., Park, S. W., Kim, S. J., Ko, A. R., Kwon, E. K., Han, S. J., and Jin, D. K. (2013) Orphanet. J. Rare Dis. 8, 42.

[7] Espejo-Mojica, Á., Alméciga-Díaz, C. J., Rodríguez, A., Mosquera, Á., Díaz, D., Beltrán, L., Díaz, S., Pimentel, N., Moreno, J., Sánchez, J., Sánchez, O. F., Córdoba, H., Poutou-Piñales, R. A., and Barrera, L. A. (2015) Mol. Genet. Metab. 116, 13–23.

[8] Córdoba-Ruiz, H. A., Poutou-Piñales, R. A., Echeverri-Peña, O. Y., Algecira-Enciso, N. A., Landázuri, P., Sáenz, H., and Barrera-Avellaneda, L. A. (2009) Afr. J. Biotechnol. 8, 1786–1792.

[9] Landázuri, P., Poutou-Piñales, R. A., Acero-Godoy, J., Córdoba-Ruiz, H., Echeverri-Peña, O. Y., Sáenz, H., Delgado, J., and Barrera-Avellaneda, L. A. (2009) Afr. J. Biotechnol. 8, 2871–2877.

[10] Poutou-Piñales, R. A., Vanegas, A., Landázuri, P., Sáenz, H., Lareo, L., Echeverri, O., and Barrera, L. A. (2010) Electron. J. Biotechnol. 13(3), https://doi.org/10.2225/vol13-issue3-fulltext-8Electronic.

[11] Rodriguez, A., Espejo, A. J., Hernandez, A., Velasquez, O. L., Lizaraso, L. M., Cordoba, H. A., Sanchez, O. F., Almeciga-Diaz, C. J., and Barrera, L. A. (2010) J. Ind. Microbiol. Biotechnol. 37, 1193–1201.

[12] Rodriguez-Lopez, A., Almeciga-Diaz, C. J., Sanchez, J., Moreno, J., Beltran, L., Diaz, D., Pardo, A., Ramirez, A. M., Espejo-Mojica, A. J., Pimentel, L., and Barrera, L. A. (2016) Sci. Rep. 6, 29329.

[13] Hernandez, A., Velasquez, O., Leonardi, F., Soto, C., Rodriguez, A., Lizaraso, L., Mosquera, A., Bohorquez, J., Coronado, A., Espejo, A., Sierra, R., Sanchez, O. F., Almeciga-Diaz, C. J., and Barrera, L. A. (2013) J. Microbiol. Biotechnol. 23, 689–698.

[14] Mosquera, A., Rodríguez, A., Soto, C., Leonardi, F., Espejo, A., Sánchez, O. F., Alméciga-Díaz, C. J., and Barrera, L. A. (2012) Process. Biochem. 47, 2097–2102.

[15] Espejo-Mojica, A. J., Mosquera, A., Rodríguez-López, A., Díaz, D., Beltrán, L., Hernandez, F. L., Alméciga-Díaz, C. J., and Barrera, L. A. (2016) Univ. Sci. 21, 195–217.

[16] Tiels, P., Baranova, E., Piens, K., De, V. C., Pynaert, G., Nerinckx, W., Stout, J., Fudalej, F., Hulpiau, P., Tannler, S., Geysens, S., Van, H. A., Valevska, A., Vervecken, W., Remaut, H., and Callewaert, N. (2012) Nat. Biotechnol. 30, 1225–1231.

[17] Sakuraba, H., Chiba, Y., Kotani, M., Kawashima, I., Ohsawa, M., Tajima, Y., Takaoka, Y., Jigami, Y., Takahashi, H., Hirai, Y., Shimada, T., Hashimoto, Y., Ishii, K., Kobayashi, T., Watabe, K., Fukushige, T., and Kanzaki, T. (2006) J. Hum. Genet. 51, 341–352.

[18] Rodríguez-López, A., Alméciga-Díaz, C. J., Sánchez, J., Moreno, J., Beltran, L., Díaz, D., Pardo, A., Ramírez, A. M., Espejo-Mojica, A. J., Pimentel, L., and Barrera, L. A. (2016) Sci. Rep. 6, 29329.

[19] Du, H., Schiavi, S., Levine, M., Mishra, J., Heur, M., and Grabowski, G. A. (2001) Hum. Mol. Genet. 10, 1639–1648.

[20] Arnau, C., Casas, C., and Valero, F. (2011) Biochem. Eng. J. 57 30–37.

[21] Daly, R., and Hearn, M. T. (2005) J. Mol. Recognit. 18, 119–138.

[22] Khatri, N. K., and Hoffmann, F. (2006) Biotechnol. Bioeng. 93, 871–879.

[23] Barrigón, J. M., Montesinos, J. L., and Valero, F. (2013) Biochem. Eng. J. 75, 47–54.

[24] Sosa, A. C., Espejo, A. J., Rodriguez, E. A., Lizaraso, L. M., Rojas, A., Guevara, J., Echeverri, O. Y., and Barrera, L. A. (2011) J. Immunol. Methods 368 64–70.

[25] Poutou-Piñales, R. A., Córdoba-Ruíz, H., Quevedo-Hidalgo, B. E., Landázuri, P., Echeverri Peña, O. Y., Sáenz, H., Vanegas, A., Acero, J., González, A., Herrera, J., Algecira, N., Caicedo, L., and Barrera, L. A. (2005) Univ. Sci. 10, 75–96.

[26] Rodríguez-López, A., Díaz, D., Beltrán, L., Sánchez, J., Moreno, J., Díaz, S., Pimentel, N., Mosquera, A., Soto, C., Pardo, A., Velásquez, O., Hernández, A., Leonardi, F., Espejo, A., Córdoba, H., Sánchez, O., Alméciga-Diaz, C., and Barrera, L. (2014) J. Inborn. Errors Metab. Screening. 2, 76.

[27] Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1999) Short Protocols in Molecular Biology, Wiley, Hoboken, NJ.

[28] Ayra-Pardo, C., Martínez García, C., and De la Riva, A. G. (1998) Biotecnol. Apl. 15, 173–175.

[29] Stratton, J., Chiruvolu, V., Meagher, M. (1998) In Methods in Molecular Biology: Pichia Protocols (Higgins, D, and Cregg, J., Eds.). pp. 107–120, Humana, Totowa, NJ.

[30] Tolun, A. A., Graham, C., Shi, Q., Sista, R. S., Wang, T., Eckhardt, A. E., Pamula, V. K., Millington, D. S., and Bali, D. S. (2012) Mol. Genet. Metab. 105, 519–521.

[31] Laemmli, U. K. (1970) Nature 227, 680–685.

[32] Ramakrishna, T., and Pandit, M. W. (1988) J. Biosci. (Bangalore) 13, 215–222.

[33] Dvorak-Ewell, M., Wendt, D., Hague, C., Christianson, T., Koppaka, V., Crippen, D., Kakkis, E., and Vellard, M. (2010) PLoS One 5, e12194.

[34] Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) Nat. Methods 9, 671–675.

[35] Charoenrat, T., Ketudat-Cairns, M., Stendahl-Andersen, H., Jahic, M., and Enfors, S. O. (2005) Bioprocess Biosyst. Eng. 27, 399–406.

[36] Berdichevsky, M., d'Anjou, M., Mallem, M. R., Shaikh, S. S., and Potgieter, T. I. (2011) J. Biotechnol. 155, 217–224.

[37] Potvin, G., Ahmad, A., and Zhang, Z. (2012) Biochem. Eng. J. 64, 91–105.

[38] Morales-Álvarez, E. D., Rivera-Hoyos, C. M., Baena-Moncada, A. M., Landázuri, P., Poutou-Piñales, R. A., Sáenz-Suárez, H., Barrera, L. A., and Echeverri-Peña, O. Y. (2013) J. Microbiol. 51 213–221.

[39] Sardiello, M., Annunziata, I., Roma, G., and Ballabio, A. (2005) Hum. Mol. Genet. 14, 3203–3217.

[40] Landgrebe, J., Dierks, T., Schmidt, B., and von Figura, K. (2003) Gene 316, 47–56.

[41] Almeciga-Diaz, C. J., Tolosa-Diaz, A. D., Pimentel, L. N., Bonilla, Y. A., Rodriguez-Lopez, A., Espejo-Mojica, A. J., Patino, J. D., Sanchez, O. F., and Gonzalez-Santos, J. (2017) Gene 634, 53–61.

[42] Bojarova, P., and Williams, S. J. (2008) Curr. Opin. Chem. Biol. 12, 573–581.

[43] Cosma, M., Pepe, P., Annunziata, I., Newbold, R., Grompe, M., Parenti, G., and Ballabio, A. (2003) Cell 113, 445–456.

[44] Fraldi, A., Biffi, A., Lombarda, A., Visigalli, I., Pepe, S., Setiembre, C., Nusco, E., Auricchio, A., Naldini, L., Ballabio, A., and Cosma, M. (2007) Biochem. J. 403, 305–312.

[45] Lu, J. Z., Hui, E. K., Boado, R. J., and Pardridge, W. M. (2010) Bioconjug. Chem. 21 151–156.

[46] Kim, C., Seo, J., Chung, Y., Ji, H. J., Lee, J., Sohn, J., Lee, B., and Jo, E. C. (2017) J. Hum. Genet. 62, 167–174.

[47] Rodríguez-López, A., Pimentel-Vera, L. N., Espejo-Mojica, A. J., Hidalgo, O. A., Tomatsu, S., and Alméciga-Díaz, C. J. (2018) Mol. Genet. Metab. 123, S123.
[48] Sola, R. J., and Griebenow, K. (2010) BioDrugs 24, 9–21.
[49] Demain, A. L., and Vaishnav, P.(2009) Biotechnol. Adv. 27, 297–306.
[50] Bielicki, J., Hopwood, J. J., Wilson, P. J., and Anson, D. S. (1993) Biochem. J. 289 (Pt 1), 241–246.
[51] Bielicki, J., Freeman, C., and Clements, P. R., Hopwood, J. J. (1990) Biochem. J. 271, 75–86.
[52] Du, H., Levine, M., Ganesa, C., Witte, D. P., Cole, E. S., and Grabowski, G. A. (2005) Am. J. Hum. Genet. 77, 1061–1074.
[53] Laukens, B., De Visscher, C., and Callewaert, N. (2015) Future Microbiol. 10, 21–34.
[54] Bretthauer, R. K. and Castellino, F. J. (1999) Biotechnol. Appl. Biochem. 30 (Pt 3), 193–200.
[55] Kesik-Brodacka, M. (2017) Biotechnol. Appl. Biochem. 65, 306–322.
[56] Xie, H., Chung, J. K., Mascelli, M. A., and McCauley, T. G. (2015) PLoS One 10, e0122453.
[57] Desnick, R., and Schuchman, E. (2012) Annu. Rev. Genomics Hum. Genet. 13, 307–335.
[58] Chen, Y., Jin, M., Egborge, T., Coppola, G., Andre, J., and Calhoun, D. H. (2000) Protein Expr. Purif. 20, 472–484.
[59] Rodríguez-López, A., Cardona, C., Pimentel, N., Espejo-Mojica, A. J., and Alméciga-Diaz, C. J. (2017) Mol. Genet. Metab. 120, S116.
[60] Tsuji, D., Akeboshi, H., Matsuoka, K., Yasuoka, H., Miyasaki, E., Kasahara, Y., Kawashima, I., Chiba, Y., Jigami, Y., Taki, T., Sakuraba, H., and Itoh, K. (2011) Ann. Neurol. 69 691–701.
[61] Srivastava, S. K., Wiktorowicz, J. E., and Awasthi, Y. C. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 2833–2837.
[62] Tiels, P., Baranova, E., Piens, K., De Visscher, C., Pynaert, G., Nerinckx, W., Stout, J., Fudalej, F., Hulpiau, P., Tännler, S., Geysens, S., Van Hecke, A., Valevska, A., Vervecken, W., Remaut, H., and Callewaert, N. (2012) Nat. Biotechnol. 30, 1225–1231.
[63] Chiba, Y., Sakuraba, H., Kotani, M., Kase, R., Kobayashi, K., Takeuchi, M., Ogasawara, S., Maruyama, Y., Nakajima, T., Takaoka, Y., and Jigami, Y. (2002) Glycobiology 12, 821–828.