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Production of tropane alkaloids by biotransformation using recombinant *Escherichia coli* whole cells

Alejandra B. Cardillo[a,b,*], María Perassolo[a,b], Mariela Sartuqui[a], Julián Rodríguez Talou[a,b], Ana M. Giulietti[a,b]

[a] Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Departamento de Microbiología, Inmunología y Biotecnología, Cátedra de Biotecnología, Buenos Aires, Argentina
[b] CONICET-Universidad de Buenos Aires, Instituto de Nanobiotecnología (NANOBIOTEC), Buenos Aires, Argentina

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ABSTRACT

Tropane alkaloids, such as hyoscyamine, 6β-hydroxyhyoscyamine and scopolamine, are secondary metabolites that were traditionally applied in medicine due to their anticholinergic activity. Hyoscyamine is converted into 6β-hydroxyhyoscyamine and scopolamine by Hyoscyamine-6β-hydroxylase (H6H). Nowadays, these bioactive compounds are obtained from natural producer plants due to the cost and complexity of their chemical synthesis. In the present work we explored the development of an alternative strategy for the production of the most valuable alkaloids, 6β-hydroxyhyoscyamine and scopolamine, using *Escherichia coli* harboring the H6H enzyme as biocatalysts. In addition, the protein extracts of the induced bacteria were assayed for the transformation of hyoscyamine into the more valuable alkaloids. For this purpose the *h6h*cDNA, previously amplified from *Brugmansia candida* total RNA preparations, was inserted in frame to the *trx* tag into the pET32a(+) vector. *E. coli* Origami strains were used as host for the expression. The strategy allowed us to produce enough quantities of a soluble and functional enzyme. Protein extracts and whole cells of the induced bacteria were able to transform hyoscyamine into the valuable products. In addition, we found that except from 2-oxoglutarate, no supplementation of the reaction mixture with the cofactors and co-substrates was needed. The process developed in this work is attractive since it could become an alternative to the traditional isolation of 6β-hydroxyhyoscyamine and scopolamine.

1. Introduction

Plant bioactive compounds, named secondary metabolites, are responsible for the medicinal properties and different health-improving effects of plant systems [1]. Frequently, these compounds have complex structures which are difficult to produce chemically and in a cost-effective process.

For this reason, many compounds of interest for the pharmaceutical industry are still isolated from the natural producer plants [2]. Even though many of them are commercially available, they are frequently expensive due to their low abundance in the nature [1].

This is the situation of tropane alkaloids, being hyoscyamine, 6β-hydroxyhyoscyamine and scopolamine the most relevant of the group. These metabolites are among the oldest drugs applied in medicine owing to their anticholinergic activity [3]. Hyoscyamine is converted into 6β-hydoxyhyoscyamine and scopolamine by two sequential reactions catalyzed by Hyoscyamine 6β-hydroxylase (H6H, EC 1.14.11.11) (Fig. 1) [4,5]. This 2-oxoglutarate-dependent dioxygenase is the final enzyme of the tropane alkaloid biosynthetic pathway. Previously, we isolated the enzyme sequence from immature anthers of *Brugmansia candida*, a South American native plant, and cloned in suitable cloning and expression vectors [6,7].

Tropane alkaloids can be synthesized chemically by 11 chemical steps [8], however, the chemical synthesis is complicated, costly and time consuming.

Biotechnological approaches are of increasing interest for the pharmaceutical production of medicinal compounds, in order to replace the isolation from the natural sources and/or chemical synthesis.

Biotransformation of organic compounds mediated by microorganisms is an economically viable strategy for the manufacturing of bioactive molecules [9]. There are several examples of the success-

* Corresponding author at: Cátedra de Biotecnología – Instituto NANOBIOTEC (UBA/CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 (CP 1113), Buenos Aires, Argentina.
E-mail address: acardillo@ffyb.uba.ar (A.B. Cardillo).

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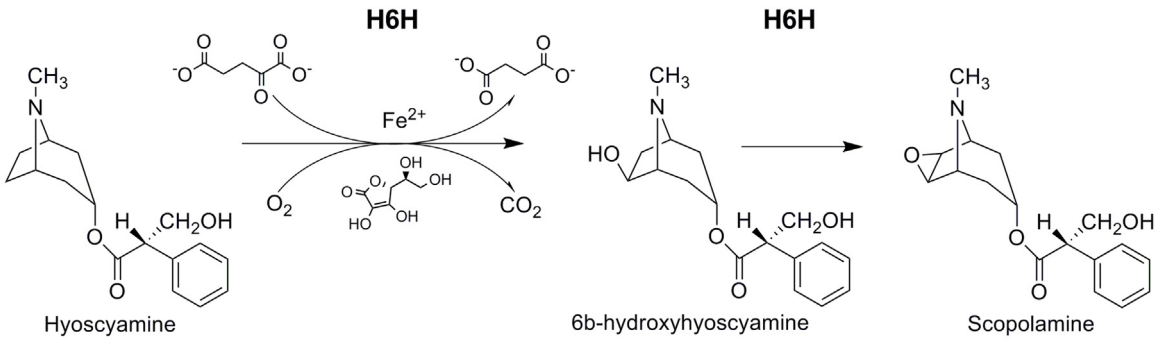


Fig. 1. Scheme of the reaction catalyzed by Hyoscyamine 6β-hydroxylase and the cofactors implicated in the transformation.

ful implementation of biotransformation using isolated enzymes or whole cells for the manufacturing of drugs applied in medicine [10,11].

In addition, this tool becomes the sustainable answer to the growing concerns of chemical industry according to the characteristics of these processes. Among the benefits of using microorganisms or enzymes for the production of Active Pharmaceutical Ingredients (APIs) we can mention the improvements in process productivity, the selectivity of transformations (chemo-, regio- and stereo-selectivity) and mild reaction conditions [12].

Scopolamine is the most valuable alkaloid, having a 10 times higher commercial demand than that of hyoscyamine and atropine [13–16]. This fact is related to its low abundance in many species used for the industrial production of these compounds and its preferred use for medical applications. In addition, in the last years several authors have described potential applications for 6β-hydroxyhyoscyamine, renewing the interest in tropane alkaloids [17–19]. It is worth to point out that several plant species that produce tropane alkaloids mainly accumulate hyoscyamine without reaching to the more valuable products, 6β-hydroxyhyoscyamine and scopolamine [20,21].

For these reasons, the production of the more valuable alkaloids by biocatalytic processes using genetically modified organisms appears to be a promising and attractive strategy for the pharmaceutical industry [22].

The aim of this work was to develop a recombinant biocatalyst in order to optimize a biotransformation approach for the production of tropane alkaloids, such as 6β-hydroxyhyoscyamine and scopolamine. For this purpose the plant enzyme H6H was cloned and expressed in *Escherichia coli*. Whole cells of this microorganism were applied as biocatalysts. In addition, the protein extracts of the induced bacteria were assayed for the transformation of hyoscyamine into the more valuable alkaloids.

2. Materials and methods

2.1. Chemicals

L-Hyoscyamine hydrobromide, Scopolamine hydrobromide, $FeSO_4$, 2-oxoglutarate, TRIS base and all media components were purchased from Sigma Chemical Co. (USA). Anisodamine (6β-hydroxyhyoscyamine) was kindly provided by Dr. László Kursinszki, Semmelweis University (Budapest, Hungary). L-Ascorbic acid and catalase were purchased from MP biomedicals (USA), PCR reagents by Invitrogen (USA), IPTG and restriction enzymes from Thermo Scientific (USA) and the cOmplete™, EDTA-free Protease Inhibitor Cocktail Tablets were supplied by ROCHE (Germany). Chloroform and methanol were purchased from Sintorgan S.A. (Argentina).

2.2. Strains, vectors and cloning strategies

The strains and culture media used in this work are listed in Table 1.

The *h6h*cDNA obtained in our lab as previously described by Cardillo et al. [6] was inserted in frame to the *trx* tag into the pET32a (+) vector from Novagen by a directional cloning between BamHI and XhoI sites.

The genetic constructions, named pH6H-trx(n) were confirmed for the insertion of *h6h*cDNA by PCR analysis using primers directed to the H6H sequence (H6H *Forward* 5′cgcggatccatggctacttttgtgtcgaac 3′; H6H *Reverse* 5′ ccgctcgagcggttattagacattgattttatatggc 3′) and to the vector (T7 promoter: 5′TAATACGACTCACTATAGGG 3′; T7 terminator: 5′GCTAGTTATTGCTCAGCGG 3′). Positive clones were verified by sequence analysis, carried out in Macrogen (Korea).

2.3. Expression of H6H in Escherichia coli *Origami strains*

Cell growth and induction of H6H expression was carried out as described in the pET system manual with minor modifications. Briefly, *E. coli* strains were grown at 37 °C in LB medium to an OD_{600} 0.6. At that time, IPTG 1 mM was added to the culture in order to induce the protein expression at 30 °C for 4 h. Induced cells were harvested by centrifugation at 4 °C and washed with breaking buffer (50 mM sodium phosphate buffer pH 7.4, 5% glycerol).

The cell pellet was resuspended in breaking buffer supplemented with the protease inhibitor cocktail and crude protein extracts were prepared by sonication. Regarding the sonication protocol, samples were maintained on ice during all the process. Short bursts were applied during 30 s followed by 30 s intervals for 5 min. Crude protein extracts were clarified by centrifugation for 5 min at 12000 rpm.

Protein concentration was determined according to Bradford [23].

2.4. SDS-PAGE and western blot analysis

The expression of the H6H enzyme was analyzed from different samples by SDS-PAGE using 15% polyacrylamide gels and Coomasie blue staining. In addition, samples were subjected to Western blot analysis as it was previously described [6]. Nitrocellulose membranes were incubated with polyclonal antibodies directed to the H6H (GenScript) and the protein was revealed by chemiluminescent ECL reagents (Pierce)

The soluble cytoplasmic fraction (SC), the total cell protein fraction (TCP) and medium fraction were evaluated. For the analysis, the cell pellet of induced bacteria was resuspended in Phosphate-Buffer Saline (PBS) to a 10X final cell concentration. For TCP, samples were diluted with 1 vol of 4X SDS-sample buffer [24], son-

Table 1
Strains used for cloning and expression of Hyoscyamine 6β-hydoxylase.

Strain	Genotype	Purpose	Culture media	Antibiotic resistance
E. coli DH5α	F-*rec*A1 *end*A1 *hsd*R17 (rk-, mk+) *sup*E44 λ-*thi*-1 *gyr*A96 *rel*A1	Cloning and plasmid amplification	Luria Bertani medium (LB) (Bacto-tryptone 10 g l^{-1}, bacto-yeast extract 5 g l^{-1}, NaCl 10 g l^{-1})[a]	
E. coli Origami(DE3)	Δ *ara–leu7697* Δ *lacX74* Δ *phoAPvuII phoR araD139 ahpC galE galK rpsL* F'[*lac* + (*lacIq*)*pro*] *gor522*:Tn*10* (Tc^R) *trxB*:kan (DE3)	Protein expression – Biocatalyst	Luria Bertani medium (LB) (Bacto-tryptone 10 g l^{-1}, bacto-yeast extract 5 g l^{-1}, NaCl 10 g l^{-1})[a]	Tetracycline (12.5 μg/ml) Kanamycin (15 μg/ml)
E. coli Origami B(DE3)	F – *ompT hsdSB*(*rB* – *mB* –) *gal dcm lacY1 ahpC gor522*:Tn*10* (Tc^R) *trxB*:kan (DE3)	Protein expression – Biocatalyst	Luria Bertani medium (LB) (Bacto-tryptone 10 g l^{-1}, bacto-yeast extract 5 g l^{-1}, NaCl 10 g l^{-1})[a]	Tetracycline (12.5 μg/ml) Kanamycin (15 μg/ml)
E. coli Origami(DE3)pLysS	Δ *ara–leu7697* Δ *lacX74* Δ *phoAPvuII phoR araD139 ahpC galE galK rpsL* F'[*lac* + (*lacI*^{q})*pro*] *gor522*:Tn*10* (Tc^R) *trxB*:kan (DE3) pLysS (Cm^R)	Protein expression	Luria Bertani medium (LB) (Bacto-tryptone 10 g l^{-1}, bacto-yeast extract 5 g l^{-1}, NaCl 10 g l^{-1})[a]	Tetracycline (12.5 μg/ml) Kanamycin (15 μg/ml) Chloramphenicol (34 μg/ml)

[a] For the selection and growth of the recombinants, media were supplemented with 100 μg ml^{-1} ampicillin and the corresponding antibiotics according to the strain used.

icated and heated for 5 min at 90 °C. For SC, samples were diluted with 1 vol of PBS instead of the SDS-sample buffer and processed as previously described [24].

The medium fraction was prepared by precipitating media samples with 100% (w/v) trichloroacetic acid (TCA). Samples were incubated for 15 min on ice and centrifuged. The protein precipitate was washed twice with acetone before resuspending in PBS as described above [24].

2.5. Biotransformation of hyoscyamine by protein extracts and whole induced cells

Crude preparations of the recombinant H6H were assayed for the enzymatic activity by measuring the transformation of hyoscyamine. The reaction mixture contained 50 mM Tris-HCl buffer (pH7.8 at 30 °C), 4 mM sodium ascorbate, 0.4 mM $FeSO_4$, 1 mM 2-oxoglutarate, 0.2 mM *l*-hyoscyamine hydrobromide, 500 μl catalase (MP biomedicals) [4,25].

In addition, whole induced cells were assayed as biocatalysts for transformation of hyoscyamine. Transformations were carried out in the reaction mixture described above and in 50 mM Tris-HCl buffer (pH7.8 at 30 °C) supplemented with 0.2 mM L-hyoscyamine hydrobromide.

The reaction was started by the addition of the crude protein extract (0.5–0.6 mg ml^{-1}) or whole induced bacteria (8.5–10 mg ml^{-1}) and was incubated at 30 °C, 250 rpm. Biotransformation was stopped by alcalinization of the samples with 1.2 M carbonate buffer pH 10.

2.6. Extraction and analysis of tropane alkaloids by HPLC

In order to extract the alkaloids from the reaction mixture described above, samples were made alkaline at pH 9 by the addition of 1 vol of carbonate buffer as it was described above [26]. Samples were extracted twice with 5 ml chloroform by vortexing and the organic phase was evaporated under gaseous N_2 [27,28]. The residue was dissolved in methanol-water (50:50 v/v) and filtered through a 0.45 μm pore nylon membrane.

HPLC analysis and quantification of the alkaloids was performed according to Cardillo et al. [26] on a Shimadzu LC-20AT system with a LiChro CART column 125-4 Lichrospher 60 RP-select B (5 μm), Merck (Germany). The elution was performed at 40 °C, isocratically with 0.01 M octanesulfonic acid pH3/methanol (65:35 v/v) at a 1 ml/min flow rate. The detection was performed at 220 nm.

2.7. Statistical analysis

The results presented were the means of three independent experiments. Sample variability was given as the standard deviation of the mean. When it corresponded, the statistical significance was evaluated by T-test or ANOVA (Analysis of variance) followed by the Tukey test. A difference between samples was considered statistically significant when the p value was <0.05. The analyses mentioned above were performed by the InfoStat software [29].

3. Results and discussion

3.1. Cloning and H6H expression by E. coli Origami strains

The *h6h*cDNA was cloned into the pET32a(+) vector in order to produce the enzyme in *E.coli* hosts. The cDNA was previously amplified from total RNA preparations from immature anthers of the South American native plant, *Brugmansia candida* [6].

Several colonies were obtained after the transformation protocol. Three of them were randomly chosen and analyzed by Colony-PCR using two different set of primers (Fig. 2). According to the bioinformatics analysis we expected an amplification of 1Kb fragment as a result of the Colony-PCR with H6H primers and a 1.7 Kb with T7 promoter and terminator primers (Fig. 2). Positive control for amplification with H6H primers was perform with a vector previously constructed in our lab that carries the gene sequence and for T7 amplification we used the pET32a (+) empty vector as DNA template that would allow us to amplified a 700 bp sequence.

The results shown in Fig. 2 corroborate the insertion of the *h6h*cDNA according to the size of amplified fragments for all the samples analyzed.

The genetic constructions were further confirmed by sequence analysis and used for transformation of the expression strains *E. coli* Origami(DE3), Origami B(DE3) and Origami(DE3)pLysS. The recombinant strains produced in this work are listed and described in Table 2.

It is well known that many recombinant proteins expressed in these hosts are often produced as aggregates. However, it is possible to find a proportion of the protein expressed in a soluble form in

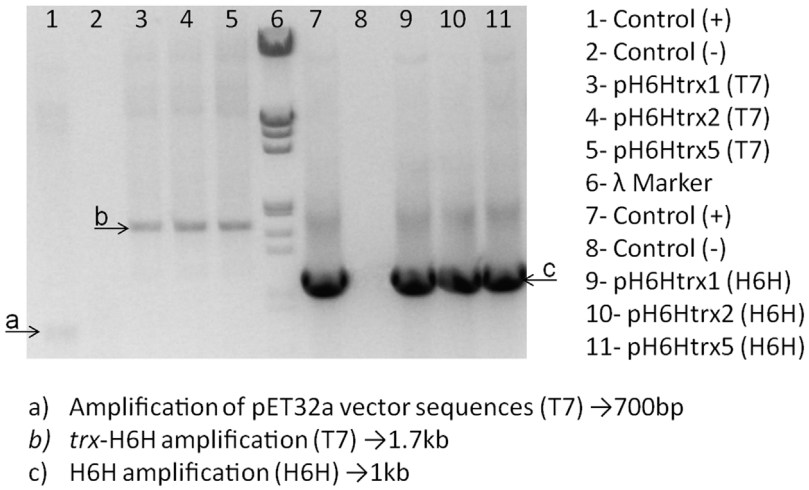


Fig. 2. PCR analysis of the genetic constructions using different sets of *primers*. (T7) *Primers* T7 promoter and terminator. (H6H) *Primers* H6H forward and reverse. The arrows indicate the size and the sequences amplified.

Table 2
Description of the recombinant strains obtained in this work.

Recombinant strain	Parental strain	Vector used for transformation
O—H6H	*E.coli* Origami(DE3)	pH6H-trx
O-pET	*E.coli* Origami(DE3)	pET32a
BL-H6H	*E.coli* Origami B(DE3)	pH6H-trx
BL-pET	*E.coli* Origami B(DE3)	pET32a
pL-H6H	*E.coli* Origami (DE3)pLys	pH6H-trx
pL-pET	*E.coli* Origami (DE3)pLys	pET32a

the cytoplasm of the cell. This fact is commonly observed with the high expression levels of systems such as the pET vectors.

For this reason, we analyzed the expression of the H6H enzyme in different cell fractions as we mentioned in Material and Methods section.

Figs. 3 and 4 show the analysis by western blot performed over the protein extract of the soluble cell fraction and the total protein cell fraction, respectively.

The results presented in Fig. 3 revealed that we succeeded in expressing this plant enzyme as a soluble protein using *E. coli* Origami strains as host for the expression.

We detected two bands of approximately 52739 and 35879 Da after incubation with antibodies directed to the H6H. This fact can be attributed to the presence of different populations of the recombinant enzyme. According to the molecular weight determined by comparing these bands to those of the marker we can assume that the H6H is present in two forms, one fused to de *trx* tag and the other in its native form, analogous to that produced in plants. It has to be considered that the ATG codon of the *h6h*cDNA was not removed from the nucleotide sequence. It is possible that the traduction started from the ATG of the H6H itself and not only from the *trx* tag. However, we won't discard the possibility of a cleavage as a consequence of the processing of the protein extracts.

We had observed this behavior previously (data unpublished) and the production of specific antibodies directed to the H6H enzyme was critical in order to explain the observations. The antibodies were produced in GenScript by immunization of rabbits with a synthetic peptide designed by analyzing the enzyme sequence.

In order to decrease the rate of protein synthesis and increase the amount of the target protein in the soluble and active form, cultures were induced at 30 °C instead of 37 °C. Even though the H6H was expressed soluble in the cytoplasm of the bacteria the results of the western blot analysis presented in Fig. 4 indicate that the protein is also present as inclusion bodies.

From a biotechnological point of view, this aspect needs to be optimized because there is a certain amount of energy consumed by the cell that is wasted, reducing the yield of the soluble (active) protein and thus increasing the cost of the productive process of the biocatalyst. Further modifications of the induction temperature and IPTG concentration could be evaluated in order to reduce the amount of insoluble protein.

Although reports of an *In silico* analysis of the H6H from *Hyoscyamus niger* postulate that the formation of disulfide bonds is not necessary for the proper folding and soluble expression of the enzyme [30], the strategy allowed us to produce sufficient amounts of the soluble protein. In addition, Liu et al. [31] produced the H6H from *Anisodus tanguticus* by a similar strategy, using the strain *E. coli* BL21*trx*B (DE3) as host for the functional expression of the protein. Even though the *in silico* analysis performed with the protein sequence of *B. candida* H6H about the probability of solubility when overexpressed in *E. coli* revealed that the enzyme should be insoluble in the bacteria [32], Kai et al. [33] reported the characterization of the H6H from *Anisodus acutangulus* by expressing the enzyme in *E. coli* BL21 and Cao et al. [34] the generation of mutants of the same enzyme. On the other hand, Li et al. [35] reported the characterization of *Atropa belladonna* H6H using the strain *E. coli* JM109 as host for its production.

Also, there are reports about the production of the H6H as a fusion to the maltose-binding protein (MBP) [5,36]. It is well known that MBP as well as *trx* tag enhance the solubility of many proteins when fused to them. Particularly for the *trx* tag, this protein not only enhances the solubility of the heterologous protein, but appears to induce the formation of disulfide bonds in the cytoplasm of the bacterial *trxB* mutants, such as the Origami strains used in this work [37].

Another fact that deserves to be discussed is the pre-induction. We found that for O-H6H and BL-H6H strains there is a basal expression of the heterologous protein. This behavior it was not observed for the pL-H6H strain (Figs. 3 and 4).

This fact is explained by the characteristics of the host. The strain Origami(DE3)pLysS carries an extra vector that allows the production of T7 lysozyme, the natural inhibitor of T7 RNA polymerase, which prevents the escape of the heterologous protein expression.

Nevertheless, we did not observe toxicity or interference in the normal functioning of the O-H6H and BL-H6H strains as a consequence of the H6H basal expression.

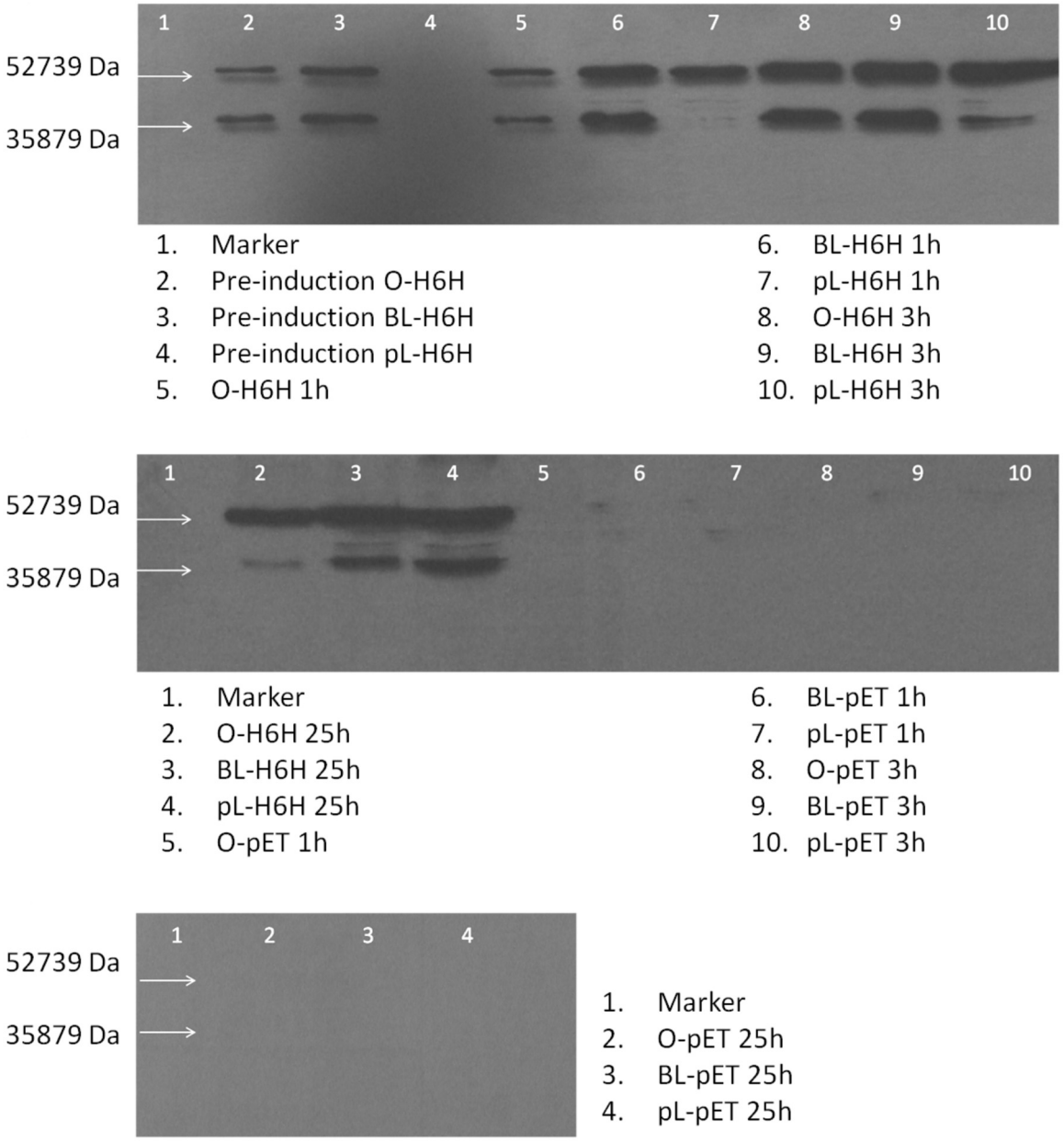


Fig. 3. Western blot analysis of the Soluble Cytoplasmic Fraction. O-H6H: *E.coli* Origami(DE3) + pH6H-trx, BL-H6H: *E.coli* Origami B(DE3) + pH6H-trx, pL-H6H: *E.coli* Origami (DE3)pLys + pH6H-trx, O-pET: *E.coli* Origami(DE3) + pET32 vector, BL-pET: *E.coli* Origami B(DE3) + pET32 vector, pL-pET: *E.coli* Origami (DE3)pLys + pET32 vector.

In order to complete the analysis of the expression system, we studied the medium fraction for the presence of the H6H (Fig. 5). The analysis was performed in order to evaluate the "leakage" phenomenon of the recombinant protein to the growth and induction medium as a consequence of cell damage.

According to the results presented in Fig. 5, we noticed that the protein is present in the medium. However, it is important to remind that when preparing these samples, the media fractions were precipitated and concentrated as it was described in Materials and Methods. For this reason, the sensitivity of the detection was highly improved and in consequence it does not represent a significant amount of protein. These findings were also confirmed when the media fractions were assayed as biocatalyst for the conversion of hyoscyamine since they were unable to transform the alkaloid (data not shown).

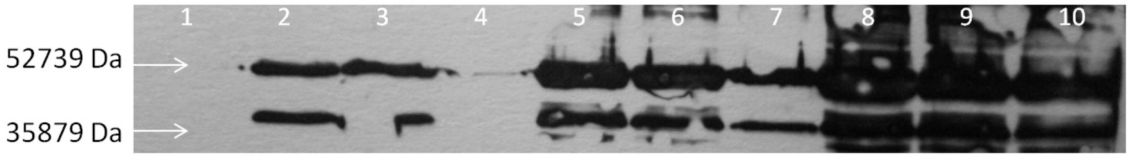
The fact that the heterologous protein was not detected in medium samples after 25 h-induction could be attributed to H6H degradation as a consequence of the prolonged exposure to culture conditions (Fig. 5B and D).

3.2. Biotransformation of hyoscyamine by crude protein extracts

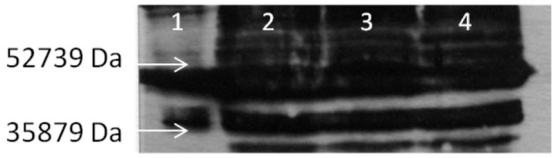
The cell-free crude protein extracts were evaluated as potential biocatalysts for the transformation of hyoscyamine into the valuable alkaloids, 6β-hydroxyhyoscyamine and scopolamine.

As mentioned in the introduction, the enzyme involved in the catalysis is a 2-oxoglutarate dependent dioxygenase that requires molecular oxygen and 2-oxoglutarate (2OG) as co-substrates. As a consequence of the transformation, 2OG is decarboxylated to succinate and CO_2, being the ferrous iron the cofactor of the reaction (Fig. 1).

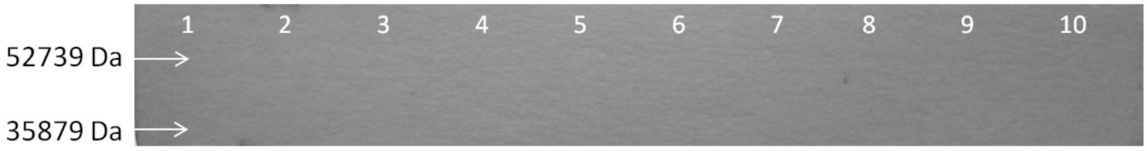
For this reason, the *in vitro* activity assays were performed supplementing the reaction mixture with the cofactors and co-



1. Marker
2. Pre-induction O-H6H
3. Pre-induction BL-H6H
4. Pre-induction pL-H6H
5. O-H6H 1h
6. BL-H6H 1h
7. pL-H6H 1h
8. O-H6H 3h
9. BL-H6H 3h
10. pL-H6H 3h



1. Marker
2. O-H6H 25h
3. BL-H6H 25h
4. pL-H6H 25h



1. Marker
2. O-pET 1h
3. BL-pET 1h
4. pL-pET 1h
5. O-pET 3h
6. BL-pET 3h
7. pL-pET 3h
8. O-pET 25h
9. BL-pET 25h
10. pL-pET 25h

Fig. 4. Western blot analysis of the Total Cell Protein (TCP) Fraction. O-H6H: *E.coli* Origami(DE3) + pH6H-trx, BL-H6H: *E.coli* Origami B(DE3) + pH6H-trx, pL-H6H: *E.coli* Origami(DE3)pLys + pH6H-trx, O-pET: *E.coli* Origami(DE3) + pET32 vector, BL-pET: *E.coli* Origami B(DE3) + pET32 vector, pL-pET: *E.coli* Origami (DE3)pLys + pET32 vector.

substrates that the H6H requires, and also with ascorbate and catalase for the improvement of the catalysis [4,38].

The results presented in Fig. 6A and B show the profile of the alkaloids transformed after 19 and 25 h by the crude protein extracts of strains O-H6H, BL-H6H and the corresponding controls (*E.coli* transformed with the pET23(a) empty vector).

According to the results presented in Fig. 6, it can be noticed that hyoscyamine was not consumed in control reactions (O-pET and BL-pET strains). These results allowed us to confirm the absence of secondary reactions as consequence of the enzyme background of the host.

After the incubation of the cell-free extracts in the presence of hyoscyamine for 25 h, this compound was almost completely biotransformed into 6β-hydroxyhyoscyamine and scopolamine (Fig. 6B). We determined that 6β-hydroxyhyoscyamine represented an (84.06 ± 0.62)% and scopolamine the (15.77 ± 0.85)% of the alkaloids recovered for O-H6H strain.

Regarding BL-H6H strain, 6β-hydroxyhyoscyamine represented an (81.47 ± 0.02)% and scopolamine an (18.05 ± 0.65)% of the alkaloids recovered.

No significant differences were observed in the bioconversion profile at 25 h between BL-H6H and O-H6H (Fig. 6B). However, we found that when the O-H6H reaction was stopped at 19 h there was a (50.68 ± 2.03)% of hyoscyamine untransformed. This behavior was not observed for BL-H6H samples where the precursor represented only a (3.18 ± 1.53)% of the alkaloids recovered (Fig. 6A). In fact, no significant differences were observed in the content of the three alkaloids for BL-H6H at 19 and 25 h. However, 6β-hydroxyhyoscyamine and scopolamine are significantly different at 25 h compared to 19 h for O-H6H ($p = 0.0457$ and $p = 0.0117$, respectively).

The extracts produced by the strain pL-H6H were not useful as biocatalyst for transforming hyoscyamine. We observed the degradation of the alkaloid added to the reaction mixture, suggesting the occurrence of secondary reactions (data not shown). Additional studies are needed to explain the mechanism of these side reactions. However, this issue remains an open question.

We have previously demonstrated that *Saccharomyces cerevisiae* was able to produce the H6H in a soluble and functional form [6]. The yeast cell-free protein extracts were also able to biotransform hyoscyamine into its products. However, for the production of yeast protein extracts it was imperative to process higher amounts of induced cells compared to the bacterial ones. The protein extract produced from a 50-ml culture of induced bacteria was enough to perform a complete set of biotransformation reactions in triplicate while a 500-ml culture of induced yeast was required for the same purpose. This fact can be related to the overexpression level of the different expression hosts.

In addition to the difficulties and complications of *S. cerevisiae* lysis, the yeast extracts required a concentration and desalting process for the proper catalysis of the recombinant enzyme [6]. This

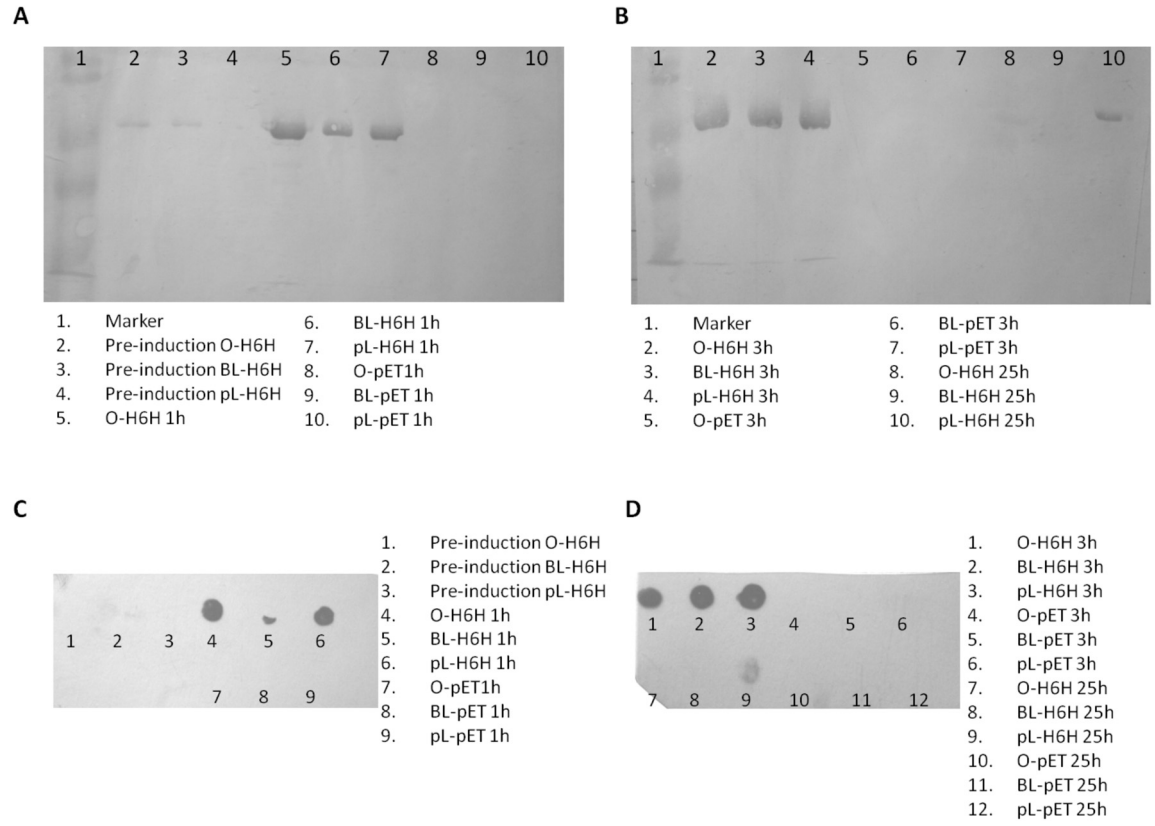


Fig. 5. (A) and (B) *Western blot* of the medium fraction. (C) and (D) *Dot blot* analysis of the medium fraction. O-H6H: *E.coli* Origami(DE3) + pH6H-trx, BL-H6H: *E.coli* Origami B(DE3) + pH6H-trx, pL-H6H: *E.coli* Origami (DE3)pLys + pH6H-trx, O-pET: *E.coli* Origami(DE3) + pET32 vector, BL-pET: *E.coli* Origami B(DE3) + pET32 vector, pL-pET: *E.coli* Origami (DE3)pLys + pET32 vector.

step was critical because an insufficient desalting of these preparations resulted in the inhibition of the reaction (data unpublished).

On the other hand, the protein extracts produced by *E.coli* were more stable and concentration or desalting of samples by changing the buffer was not necessary, thus resulting in a more robust and simple system for a biocatalytic application.

Moreover, this strategy turned out to be a cost-effective alternative to the yeast system. It is important to remind that the expression level is one of the crucial parameters in determining the cost contribution of the biocatalyst [39].

3.3. *Biotransformation of hyoscyamine by recombinant* E. coli *whole cells*

The possibility of biotransform hyoscyamine into 6β-hydroxyhyoscyamine and scopolamine using whole cells as biocatalyst is an interesting strategy to explore because of the unique advantages of this type of catalyst over the enzyme preparations [12,40]. Cofactor regeneration, stabilizing effects, ease of preparation are some of the advantages of these systems. In addition, it is interesting to note that whole cell catalysts represent the cheapest biocatalyst formulation being about 10-fold less expensive than purified enzymes [39,40].

For the reasons mentioned above, we analyzed the recombinant strains O-H6H, BL-H6H and pL-H6H as potential catalysts for the production of 6β-hydoxyhyoscyamine and scopolamine.

Because of the possibility that whole cells could provide the cofactors and co-enzymes or co-substrates required for catalysis [12,41], the assay was carried out in buffer or in the complete reaction mixture (supplemented with the compounds required for the catalysis) as it was described in Material and Methods section (Fig. 7A and B).

The behavior of pL-H6H and pL-pET strains was consistent to that reported for the cell free-protein extracts (data not shown).

O-H6H and BL-H6H were able to transform hyoscyamine in both media (Fig. 7A and B). However, both biocatalysts were more efficient in the complete reaction medium than the buffer (Fig. 7B). The data presented in Fig. 7 revealed that the whole cell is inefficient in providing sufficient amounts of all the cofactors and/or co-substrates needed for the bioconversion.

Regarding the transformation performed in buffer, the maximum production of the recovered alkaloids was a $(5.22 \pm 0.18)\%$ of 6β-hydoxyhyoscyamine and a $(0.67 \pm 0.39)\%$ of scopolamine (Fig. 7A).

However, the biotransformation rate was highly improved when the reaction was carried out in the reaction mixture getting a $(82.16 \pm 5.28)\%$ of 6β-hydoxyhyoscyamine and a $(12.82 \pm 4.28)\%$ of scopolamine (Fig. 7B). The amounts of scopolamine and 6β-hydoxyhyoscyamine produced in both media were significantly different ($p = 0.0153$ and $p < 0.0001$, respectively).

Control reactions carried out with whole cells transformed with the empty vector showed that hyoscyamine was not transformed, suggesting that no side products were formed as consequence of the host enzymes (Fig. 7A and B).

Previous works carried out in our laboratory with *S. cerevisiae* harboring the *h6h*cDNA, have shown that the yeast system was not suitable as whole cell biocatalyst. These findings were attributed to an insufficient uptake of the alkaloids by yeast cells [6].

On the other hand, the bacterial system not only resulted in a simple and robust alternative but the possibility of using the whole

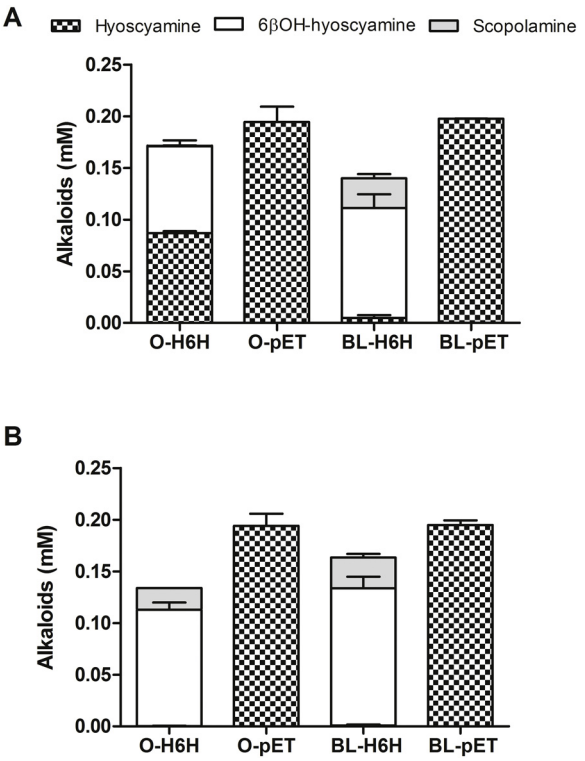


Fig. 6. Alkaloids produced at (A) 19 h and (B) 25 h of reaction with the protein extracts obtained from strains O-H6H, BL-H6H and the corresponding control strains. O-H6H: *E.coli* Origami(DE3) + pH6H-trx, BL-H6H: *E.coli* Origami B(DE3) + pH6H-trx, O-pET: *E.coli* Origami(DE3) + pET32 vector, BL-pET: *E.coli* Origami B(DE3) + pET32 vector.

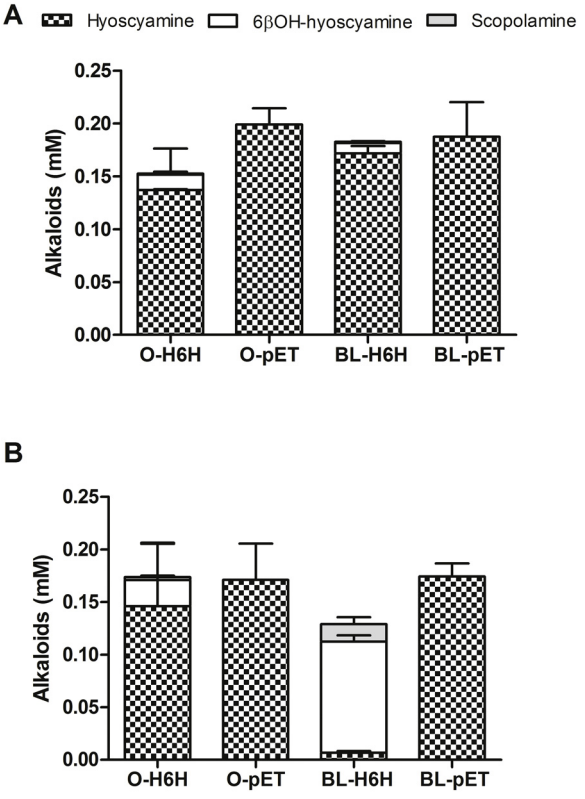


Fig. 7. Hyoscyamine biotransformation using whole induced cells as biocatalyst. (A) Buffer. (B) Buffer supplemented with cofactors. O-H6H: *E.coli* Origami(DE3) + pH6H-trx, BL-H6H: *E.coli* Origami B(DE3) + pH6H-trx, O-pET: *E.coli* Origami(DE3) + pET32 vector, BL-pET: *E.coli* Origami B(DE3) + pET32 vector.

cells also offered an additional advantage in view of the development of a biocatalytic process for tropane alkaloids production.

3.4. Analysis of the limiting cofactor

It is known that almost all organisms use peroxidase and/or catalase in order to maintain the H_2O_2 level below the limit of toxicity. Oxygen species are constantly produced within the aerobic organisms being one of the principal threats to these organisms [42,43]. *E. coli* is a recognized catalase positive microorganism and the determination of this enzyme activity is used for identification purposes.

According to the characteristics of the host, we decided to investigate if the catalase contribution of *E.coli* was enough to support the catalysis of the H6H.

Hashimoto et al. [4] reported that catalase omission from the reaction mixture resulted in a 12% of the maximum activity. Furthermore, we determined that catalase addition to the reaction mixture was critical in order to transform hyoscyamine efficiently by the H6H from *B.candida* [6].

For the reasons mentioned above, protein extracts from the strains O-H6H and BL-H6H were tested as biocatalysts for the transformation of hyoscyamine in absence of catalase. The reaction mixture was supplemented with all the other compounds required for the catalysis of the recombinant enzyme.

According to the results presented in Fig. 8, we concluded that the catalase present in the bacteria was enough to allow the efficient transformation of hyoscyamine by the recombinant H6H. In addition, control reactions performed with the strains harboring the empty vector confirmed the absence of secondary reactions.

Regarding the O-H6H strain, 6β-hydroxyhyoscyamine represented the (83.26 ± 1.35)% of the recovered alkaloids and scopolamine the (16.73 ± 1.25)%. On the other hand, when BL-H6H was used as biocatalyst 6β-hydroxyhyoscyamine represented the (80.08 ± 1.68)% and scopolamine the (19.91 ± 1.60)% of the recovered alkaloids.

No significant differences were found between strains, related to 6β-hydroxyhyoscyamine and scopolamine production when the catalase was omitted from the reaction mixture.

Due to these findings and the differences shown in Fig. 7A and B, we decided to analyze which cofactors were limiting for whole cell biotransformation in order to optimize the process (Fig. 9).

Different reactions mixtures were prepared by omitting one cofactor at a time. The reaction was carried out as described before by incubating whole induced cells (8.5–10 mg ml^{-1}) for 25 h at 30 °C. Further increases in biocatalyst concentration negatively affected the performance of the system (data not shown).

The biotransformation in the semi-complete medium allowed us to confirm the ability of the whole cells to convert hyoscyamine into 6β-hydroxyhyoscyamine and scopolamine in absence of catalase (Fig. 9). The results presented in Fig. 9 show that supplementing the reaction mixture with ascorbate or iron is not necessary for the catalysis with whole cells. In addition, the biotransformation was more efficient converting the entire precursor into its products than the semi-complete reaction ($p < 0.05$; Fig. 9). Of the alkaloids recovered in the reaction of ascorbate, 6β-hydroxyhyoscyamine represented the (90.35 ± 0.33)% and scopolamine the (9.64 ± 0.32)%. Regarding to Fe^{2+} reaction, 6β-hydroxyhyoscyamine was produced in a (86.75 ± 2.41)% and scopolamine in a (11.06 ± 1.39)%.

Previous reports from Hashimoto and co-workers emphasized the importance of supplementing the reaction with iron and ascorbate [3,4]. In addition, our experiences highlighted the need for their supplementation [6]. However, *E. coli* whole induced cells were able to provide enough quantities of these compounds required in the catalysis (Fig. 9).

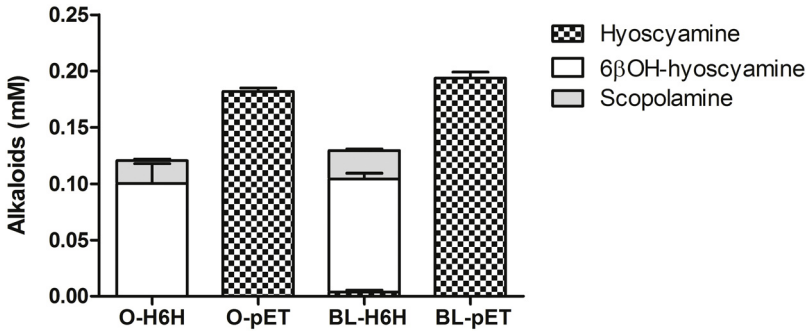


Fig. 8. Analysis of catalase limitation using crude protein extracts as biocatalysts. Alkaloids produced at 25 h by O-H6H and BL-H6H strains. O-H6H: *E.coli* Origami(DE3) + pH6H-trx, BL-H6H: *E.coli* Origami B(DE3) + pH6H-trx, O-pET: *E.coli* Origami(DE3) + pET32 vector, BL-pET: *E.coli* Origami B(DE3) + pET32 vector.

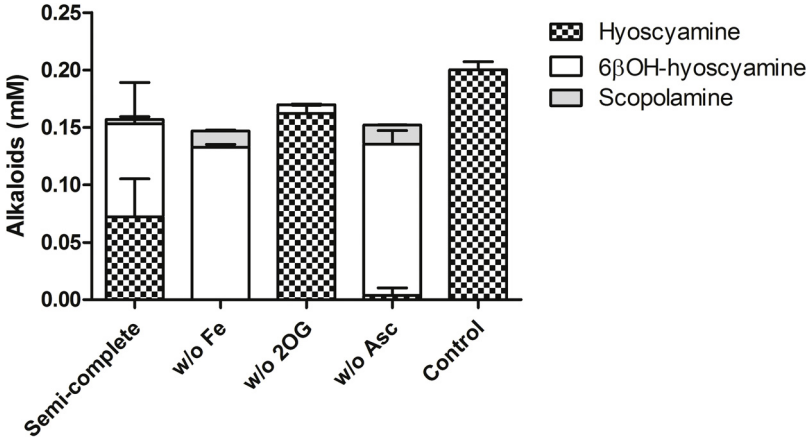


Fig. 9. Analysis of the limiting cofactor for the reaction using whole cells as biocatalysts. Alkaloids produced at 25 h with *E. coli* induced cells transformed with the pH6H-trx vector. Semi-complete: reaction media without catalase but all the cofactors; w/o Fe: reaction media without catalase and iron; w/o 2OG: reaction media without catalase and 2-oxoglutarate; w/o Asc: reaction media without catalase and ascorbate.

Nevertheless, we found that the 2OG provided by the cells was limiting for the biotransformation (Fig. 9). In this case around 95% of the alkaloid remained as hyoscyamine and only a (4.53 ± 0.16)% of 6β-hydroxyhyoscyamine was produced. This result highlights the requirement of the addition this compound to the reaction mixture.

The 2OG is an important metabolite of the intracellular pool that belongs to key metabolic pathways such as the Krebs cycle and is related to the aminoacid biosynthesis, among other functions. Regarding aminoacid biosynthesis, glutamate is formed by reductive amination of 2OG and is the precursor of arginine, proline and glutamine [44]. Although the bacterial cell produces 2OG, it is possible that the relevance of the functions mentioned above make this compound unavailable for the H6H reaction.

The results reported here about the analysis of the limiting cofactor are attractive because it was possible to reduce the reactants added to the reaction mixture, generating a simple and robust system to biotransform hyoscyamine with whole cells. In addition, the simplicity of the system has the advantage of reducing the cost of the process.

4. Conclusion

It was possible to produce the plant enzyme Hyoscyamine 6β-hydorxylase in *E. coli*. The cloning strategy, using the *trx* tag and the Origami strains allowed us to produce a soluble and functional enzyme due to its ability to transform the precursor into 6β-hydroxyhyoscyamine and scopolamine.

We observed the liberation of the enzyme to the culture medium. However, the leakage of the H6H was not significant and can be related to cell lysis.

Protein extracts and whole cells were efficient biocatalysts in the transformation of hyoscyamine. *E. coli* was able to provide all the cofactors and co-substrates of the reaction with the exception of 2OG. This compound must be included in the reaction for the proper activity of the biocatalyst.

The relevance of the results presented about the analysis of the limiting cofactor resides in the fact that it was possible to develop a biotransformation process of hyoscyamine with whole cells by supplementing the reaction medium with only 2OG. This fact allowed us to generate a simple and efficient system to increase the added value of this alkaloid with pharmaceutical applications with the advantage of reducing costs.

The process developed in this work is attractive since it appears to be an alternative to the traditional isolation of 6β-hydroxyhyoscyamine and scopolamine. In fact, the manufacturing of several drugs relies on biotransformation processes using isolated enzymes or whole cells [10,11].

The simplicity of the system, the reduced cost, the ease of the handling and culture of bacterial cells and the advantages of biotransformation as a tool for the synthesis of bioactive compounds turn this approach into a strategy that deserves to be analyzed for a commercial application.

Acknowledgments

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