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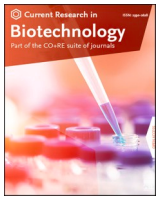

Production in *Nicotiana benthamiana* of a thermotolerant glucose oxidase that shows enzybiotic activity against *Escherichia coli* and *Staphylococcus aureus*

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ABSTRACT

Glucose oxidase (GOX) catalyzes the FAD-dependent oxidation of α-D-glucose to D-gluconolactone with production of hydrogen peroxide. This enzyme encounters many biotechnological applications from glucose sensors to applications in food, pharma and textile industries. For this purpose, recombinant GOX versions, usually derived from *Aspergillus niger*, are produced in fermentation systems, frequently in filamentous fungi because other production platforms such as bacteria or yeast have rendered meager results. We wondered whether *A. niger* GOX, more specifically a mutant version with superior thermotolerant properties, could be efficiently produced in *Nicotiana benthamiana* plants. To this aim, we used a tobacco mosaic virus-derived vector that is inoculated into plant tissues using *Agrobacterium tumefaciens*. Results exhibited the efficient production of the recombinant GOX in plants and the facile downstream purification when the recombinant protein is targeted to the apoplast, the space between plasma membranes and cell walls. The plant-made recombinant GOX displayed excellent catalytic properties in broad pH and temperature conditions. In addition to establishing a new strategy to produce recombinant GOX in plants as a green alternative to traditional fungal fermentation, we further investigated the potential application of this protein as an ezybiotic. Results exhibited a remarkable bacteriocide activity against *Escherichia coli* and *Staphylococcus aureus*.

1. Introduction

Glucose oxidase (GOX) is a FAD-dependent oxidase, typically produced by *Aspergillus niger* and other fungal species, that oxidizes α-D-glucose yielding D-gluconolactone and hydrogen peroxide. Structurally, GOX belongs to the glucose-methanol-choline (GMC) oxidase superfamily, characterized by the presence of an N-terminal FAD-binding domain, with an α-β fold, typical of dinucleotide binding proteins, and a C-terminal substrate-binding domain consisting of a β-sheet wrapped by α-helices (Hecht et al., 1993; Savino and Fraaije, 2021; Sützl et al., 2019; Wohlfahrt et al., 1999). GOX is an outstanding enzyme from an industrial point of view because of its multiple and important applications, a reason why it has been dubbed the enzyme "Ferrari" (Bauer et al., 2022). Although its use in biosensors for the measurement of glucose concentration in blood (Nery et al., 2016) is undoubtedly the most popular application, GOX has many other diverse and relevant uses in medicine, and in food, pharma and textile industries (Bauer et al., 2022; Dubey et al., 2017).

Industrial production of GOX is carried out using *Aspergillus niger* strains genetically engineered to boost productivity (Lambré et al., 2022). Different yeast species and *Escherichia coli* have also been assayed as production hosts, although with meager results so far (Bauer et al., 2022; Wang et al., 2022). GOX from *A. niger* is readily expressed in *Saccharomyces cerevisiae*, which makes possible to manipulate the enzyme in efforts to find engineered variants with improved properties. However, the yield is too low to allow industrial production (Marín-Navarro et al., 2015; Ostafe et al., 2014). The use of alternative hosts, which would provide increased versatility for traditional and new

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applications is therefore a relevant biotechnological goal. In this context, the use of plants as biofactory, and specifically *Nicotiana benthamiana* (Bally et al., 2018), represents an interesting alternative. This tobacco species grows fast in laboratory and greenhouse conditions producing a substantial amount of biomass in a reasonable amount of time; flowering and seed productions is not particularly lengthy. Remarkably, the so-called LAB strain of *N. benthamiana* is a natural RNA-dependent RNA polymerase 1 mutant with compromised immune response that makes the plant highly susceptible to pathogens such as *Agrobacterium tumefaciens* or many different plant virus species, which properly engineered can be used as gene delivery vectors. In addition, *N. benthamiana* leaves are particularly amenable to infiltration techniques that facilitate the delivery of solutions or bacterial preparations such as those of engineered *A. tumefaciens*.

Plants are arising as a reliable platform to produce recombinant proteins, in a technology known as molecular farming, based on price, scalability, biosecurity, and downstream processing (Kulshreshtha et al., 2022). Plants basically require sunlight and water to grow, and production can be easily scaled up; one plant can be considered a bioreactor unit that can be easily multiplied by growing more plants. No human and livestock pathogens have ever shown to infect plants, which alleviates biosecurity problems. In addition, if recombinant proteins are targeted to particular subcellular locations, such as the apoplast, the space between the cellular membrane and cell wall, which is particularly poor in endogenous proteins, purification of the recombinant protein by mechanical means is particularly straightforward (Talens-Perales et al., 2022).

The use of enzymes as antimicrobial agents is acquiring increasing biotechnological relevance. In many instances, enzymes can represent a complement or even a convenient alternative to conventional antibiotics. Enzymes with this activity have been designed as enzybiotics, a term originally coined to describe the antibacterial activity of lytic enzymes derived from bacteriophages that disrupt the bacterial cell wall (Nelson et al., 2001). However, the concept can also be applied to other enzymes, such as GOX, whose antimicrobial activity resides in the hydrogen peroxide production that is concomitant to its catalytic action (Bauer et al., 2022; Califano et al., 2021).

Improving GOX stability shall enhance its usability for different industrial applications. This is the rationale behind the isolation of mutated variants of the enzyme with increased resistance to thermal denaturation. In this work, we have used one such engineered GOX version (Marín-Navarro et al., 2015) to address two important biotechnological issues. Firstly, we have implemented an efficient alternative procedure for large scale production of the enzyme using *N. benthamiana* as a host. Secondly, we show remarkable antimicrobial (enzybiotic) properties of the plant-produced engineered GOX variant.

2. Materials and methods

2.1. GOX expression in N. benthamiana

A synthetic DNA coding for an engineered version of *A. niger* GOX, whose codon sequence was optimized for translation in *N. benthamiana* (IDT) (Supplementary Fig. S1), was assembled (Gibson et al., 2009) (Table S1) into intermediate plasmid pMTMVi-N (Shi et al., 2019), opened by digestion with *Age*I and *Xho*I (Thermo Scientific). The *Bsa*I (New England Biolabs) fragment from the resulting plasmid was assembled into pGTMV (Shi et al., 2019) (containing a TMV infectious clone, GenBank accession number MK087763.1) digested with *Nco*I and *Pfl*23II (Thermo Scientific) to build pGTMVΔCP-GOX (Supplementary Fig. S2).

A. tumefaciens (GV3101:pMP90), harboring the helper plasmid pCLEAN-S48 (Thole et al., 2007), was transformed with plasmid pGTMVΔCP-GOX. Liquid cultures were grown in lysogenic broth (LB) medium containing 50 μg/mL kanamycin for 24 h at 28 °C. Cells were pelleted by centrifugation and brought to an optical density (OD) at 600 nm of 0.5 in 10 mM MES-NaOH, pH 5.6, 10 mM $MgCl_2$ and 150 μM acetosyringone. Bacterial preparations were incubated for 2 h at 28 °C to induce the virulence genes, and used to infiltrate the leaves of 5-weeks old *N. benthamiana* plants. Preparations were infiltrated at the abaxial side of the leaves using a needleless 1-mL syringe. Plants were cultured in a growth chamber at 25 °C with a 16/8-h day/night photoperiod.

2.2. GOX purification and enzyme analysis

Infected tissues from *N. benthamiana* plants were harvested at 7 days post-inoculation (dpi) and infiltrated with phosphate buffered saline (PBS) buffer supplemented with 0.02 % (v/v) Silwet L-77. Infiltrated tissues were then introduced in a 50 mL syringe and squeezed to obtain an apoplastic liquid, which was finally clarified by centrifugation for 5 min at 13,000 rpm. GOX was further purified by ion exchange chromatography. The apoplastic fluid was dialyzed against buffer A (50 mM acetate/acetic buffer pH 5.5). The resulting sample was centrifuged at 12,000 *g* for 5 min and the supernatant was injected in a 1 mL ResourceQ column (GE-Healthcare) coupled to an ÅKTA-purifier system (GE-Healthcare). Flow-through was collected and the column was subsequently washed with 5 column volumes (CV) of buffer A and 5 CV of 10 % buffer B (50 mM acetate/acetic buffer pH 5.5, 1 M NaCl). Elution was carried out with a 5 CV linear gradient from 10 % to 50 % B, followed by a final wash with 5 CV of 50 % B and 5 CV of 100 % B. The fraction corresponding to purified GOX was quantified spectrophotometrically at 280 nm, using an extinction coefficient ($\varepsilon^{1\%}$) of 14.8.

GOX activity was determined as previously described (Marín-Navarro et al., 2015). Briefly, GOX samples were incubated at the indicated temperatures in a reaction mixture containing 85 mM glucose, 12 μg/mL horseradish peroxidase, and 0.17 mM O-dianisidine in the appropriate buffers for each pH (50 mM citrate pH 4 and 5; 50 mM phosphate pH 6 and 7; 50 mM Tris-HCl pH 8 or 50 mM borate-NaOH pH 9). Reactions were stopped by addition of 0.2 M HCl; absorbance at 400 nm was measured and the amount of oxidized glucose was determined by interpolation on a standard curve. One unit of activity was defined as the amount of enzyme oxidizing 1 μmol of glucose per minute at 37 °C and pH 7.0.

2.3. Antimicrobial assay

GOX antibacterial activity was assayed using previously grown bacterial cultures, either by using a disk impregnated with the enzyme on a solid medium or by incubation with the enzyme in a liquid suspension. For the disk diffusion test, *Staphylococcus aureus* and *E. coli* were grown at 37 °C to an OD (600 nm) of 0.6. Cultures were diluted 1/300 with buffer P (50 mM phosphate buffer pH 7) and 0.2 mL were spread on LB solid medium. Once plates were dried, 5 mm-diameter filter paper disks were placed on their surface. Four different solutions were prepared to impregnate the disks. Three of them containing 200 mM glucose in buffer P and GOX at different concentrations (0.7 to 12 U/mL). The fourth was a negative control without glucose, with GOX (2.8 U/mL). The filter paper disks were loaded with 12 μL of the different GOX treatments. For the liquid suspension test, *S. aureus* (CECT 794) and *E. coli* strain Nissle 1917 were inoculated in LB medium and grown at 37 ºC to an OD at 600 nm of 0.6 or to late stationary phase (24 h). Cells were collected by centrifugation at 12,000 *g* for 7 min, washed with 1 mL of buffer P and finally resuspended in 1.2 mL of the same buffer to an OD of 0.15. Aliquots (100 μL) were mixed with either 50 μL of GOX (5.5 U/mL) and 50 μL of 400 mM glucose in buffer P; 50 μL of GOX (5.5 U/mL) and 50 μL of buffer P; or 100 μL of buffer P, and incubated for 90 min at 37 °C. All these treatments were carried out in triplicate. Subsequently, these samples were inoculated into 300 μL of LB in a 96-multiwell plate with a final 1/50 dilution and growth was monitored by measuring OD at 600 nm every 30 min in a microplate reader (SPECTROstar Omega, BMG LABTECH). Relative number of viable cells after GOX treatment with or without glucose was estimated

from the inoculum size (N_0) of the growth curves. To this end, a classic logistic model was applied according to equation [1]:

$$N = \frac{b \cdot N_0}{N_0 + [(b - N_0) \cdot e^{-rt}]} \quad (1)$$

where N_0 and N are the cell densities at time 0 and at time t, respectively, b represents the maximum cell density in the medium, or carrying capacity, and r is the intrinsic growth rate of the culture. Parameters N_0, b and r were adjusted to fit equation [1] to experimental data through minimization of the sum of squared differences using the Solver complement within the Microsoft Excel software. Detection limit for this determination was calculated as the minimum N_0 value required to detect an OD increase of 0.1 units in 23 h.

2.4. Electrophoretic and mass spectrometry analyses

Preparations of apoplastic fluid and purified protein were analyzed by SDS-PAGE and gels were stained with Coomassie brilliant blue. Protein identification was carried out through liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. Gel slices containing the band of interest were digested overnight with 200 ng of sequencing grade trypsin (Promega) at 37 °C as described elsewhere (Shevchenko et al., 1996). The reaction was stopped with trifluoroacetic acid at a final concentration of 0.1 %. An aliquot (1 μL) of the digestion mixture was diluted to 20 μL with 0.1 % formic acid and loaded in an Evotip pure tip (EvoSep) according manufacturer instructions. LC–MS/MS was performed in an Evosep One system connected to a Tims TOF fleX mass spectrometer (Bruker). The sample loaded in the Evotip pure tip was eluted to an analytical column (PepSep C18 10 cm × 150 μm, 1.5 μm; Bruker) by the Evosep One system, and resolved with the 100 SPD chromatographic method defined by the manufacturer.

3. Results

3.1. Production of GOX in N. benthamiana

The main goal of this work was to analyze whether plants, and particularly the outstanding biofactory plant *N. benthamiana*, was an optimal platform to produce a thermoresistant version of *A. niger* GOX, an enzyme with multiple biotechnological and industrial applications. To this end, we used a TMV-derived vector that is delivered into *N. benthamiana* cells by means of *A. tumefaciens* (agroinoculation) (Shi et al., 2019; Talens-Perales et al., 2022). In this vector, GOX replaced most of viral coat protein (CP) coding region (Fig. 1), rendering a recombinant virus able to replicate and move cell-to-cell, but unable to move systemically through the plant. Using denaturing polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie brilliant blue staining, we analyzed protein accumulation in agroinoculated tissues and compared the protein content with those of control tissues. A differential band with a position within the range of the expected mobility of the recombinant GOX suggested efficient production of the heterologous enzyme in *N. benthamiana* at 7 dpi using the TMV vector (Fig. 2). The fact that this protein is indeed the recombinant GOX encoded by the *A. niger*-derived gene sequence present in TMVΔCP-GOX vector was conclusively demonstrated by the enzymatic assay of the purified protein (see next section). Three supernumerary proteins with estimated molecular masses of 34, 25 and 22 kDa (Fig. 2, P2 to P4) were also detected. These proteins were also observed in tissues agroinoculated with an empty plasmid (no TMV vector) and, to a lesser extent, in the mock-inoculated plants (i.e., infiltrated with buffer with no *A. tumefaciens*), but not in the non-treated control plants. Mass spectrometry analysis of these proteins allowed their identification, showing that they correspond to peroxidase N1, acidic endochitinase Q and pathogenesis-related protein R, respectively. Additionally, a noticeable protein of estimated 54 kDa (P1), corresponding to Rubisco large subunit, was also present in all samples (Table 1).

3.2. Purification and enzymatic analysis of GOX from N. benthamiana

The observed molecular weight of GOX (72 kDa) is lower than that reported for the enzyme produced by *A. niger* (86 kDa) (Ritter et al., 2013), but somewhat higher than that expected for the non-glycosylated monomer (63 kDa). Therefore, some type of post-translational modification, likely glycosylation, seems to have occurred. GOX was obtained after a single step of an anion-exchange chromatography with high

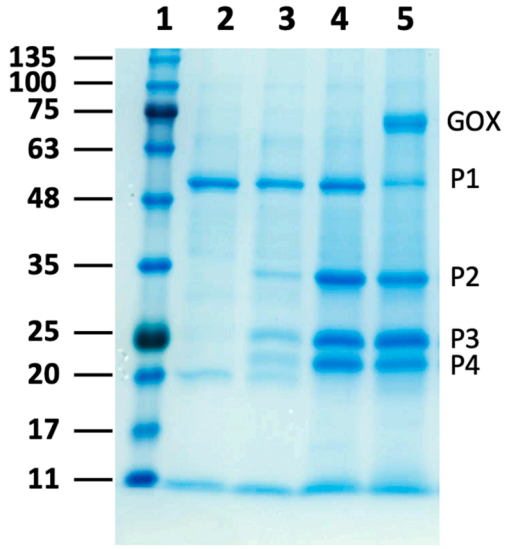

Fig. 2. Electrophoretic analysis of recombinant GOX production in *N. benthamiana* plants. Lane 1, protein standards; lane 2, non-infiltrated plant; lane 3, plant infiltrated with buffer; lane 4, plant agroinoculated with an empty plasmid; lane 5, plant agroinoculated with TMVΔCP-GOX. The positions of GOX and four supernumerary proteins (P1 to P4) are shown. Standard protein sizes in kDa are indicated in the left margin.

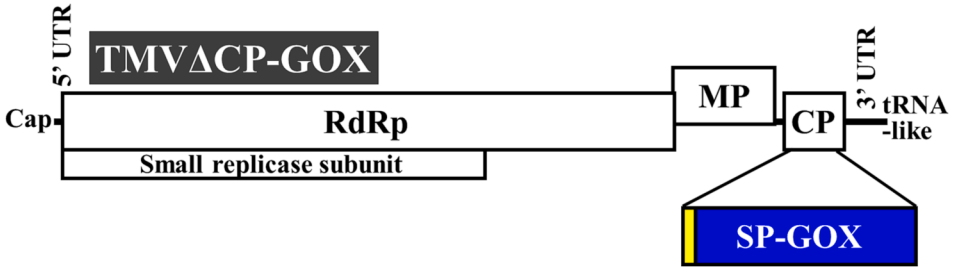

Fig. 1. Schematic representation of the TMV-derived vector to express GOX in *N. benthamiana* plants. 5′ UTR and 3′ UTR, 5′ and 3′ untranslated regions; RdRp, RNA-dependent RNA polymerase; MP, movement protein; CP, coat protein; SP, signal peptide, GOX, glucose oxidase. Other features such as a 5′ cap and 3′ tRNA-like structures are also indicated.

Table 1
Identification of proteins recovered from the apoplastic fluid of *N. benthamiana* plants agroinoculated with vector TMVΔCP-GOX.

	Protein band				
	GOX	**P1**	**P2**	**P3**	**P4**
Apparent molecular mass in SDS-PAGE (kDa)	72	54	34	25	22
Identification procedure	Enzymatic activity of the purified protein	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS
Protein description (best match)	Glucose oxidase. *Aspergillus niger*	Ribulose bisphosphate carboxylase large chain *Nicotiana debneyi*	Peroxidase N1 *Nicotiana tabacum*	Acidic endochitinase Q *Nicotiana tabacum*	Pathogenesis-related protein R major form *Nicotiana tabacum*
Expected mass (kDa)	?*	53	33	25	22
Uniprot accession	P13006	P48709	Q9XIV8	P17514	P13046
Physiological location in the plant cell	Alien	Chloroplast	Extracellular	Extracellular	Vacuolar

* Plant-made recombinant GOX apparently contains post-translational modifications.

purity (Fig. 3). The specific activity of the plant-made GOX was 110.8 ± 0.5 μmol·min^{-1}·mg^{-1} of enzyme at 37 °C and pH 7, which is similar to previously reported data (135 μmol·min^{-1}·mg^{-1} of enzyme at 40 °C and pH 5.5) for the wild-type version isolated from *A. niger* (Bhatti et al., 2006). This indicates that the triple mutation harbored in the GOX engineered version (Marín-Navarro et al., 2015) used in this work does not impairs enzyme activity.

The activity of plant-produced GOX was evaluated at different pH values and temperatures, as shown in Fig. 4. The results indicate that the recombinant GOX remains active over a broad pH range (4 to 8), with optimal activity at pH 6. Even under rather high acidic conditions (pH 4), the enzyme maintained around 50 % of its maximum activity, while under basic conditions (pH 9), it retained only about 10 % (Fig. 4A). Additionally, the enzyme exhibited maximum activity between 35 °C and 50 °C, with a gradual decrease in activity at lower and higher temperatures, falling to 50 % and 30 % of its peak activity, respectively (Fig. 4B). These findings suggest that plant-produced recombinant GOX is capable of functioning under optimal conditions across a wide range of pH and temperature.

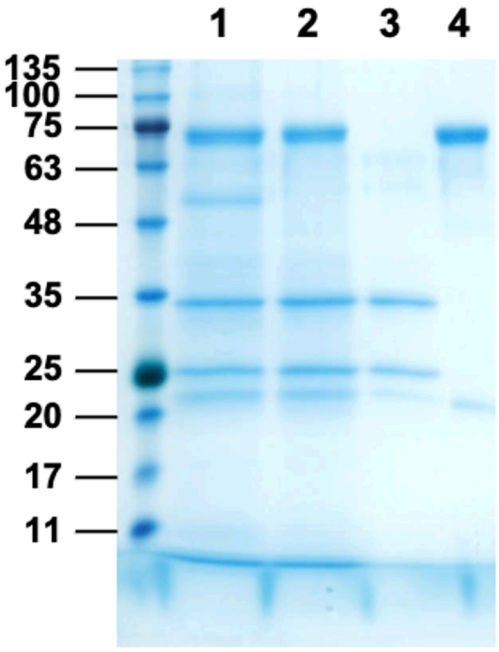

Fig. 3. Plant-made recombinant GOX purification. Aliquots of the preparations at different steps were separated by SDS-PAGE. Apoplastic fluid from *N. benthamiana* (lane 1) was dialyzed and centrifuged. The supernatant (lane 2) was injected in an anion exchange column. The flow through (lane 3) and the elution peak (lane 4) were collected. Size of standard proteins (left lane) indicated in kDa.

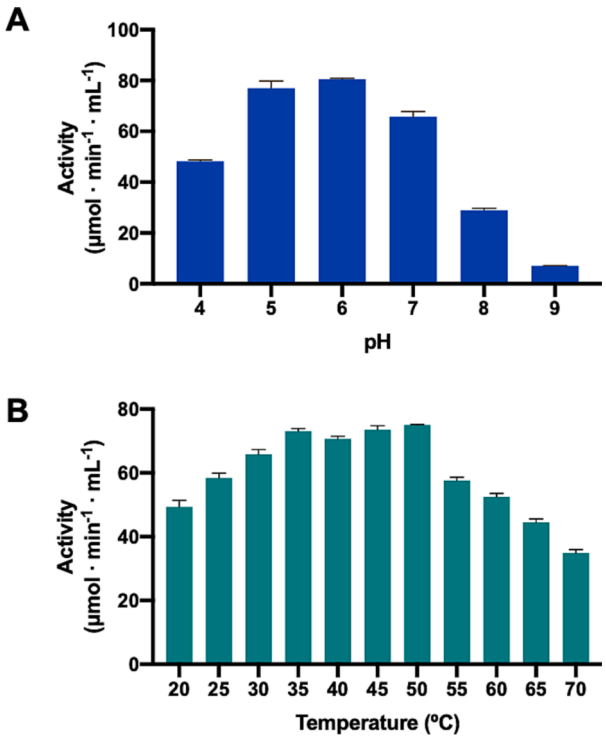

Fig. 4. Analysis of enzymatic activity of the plant-produced engineered GOX under different conditions of pH at 40°C (A) and temperature at pH 7 (B). Histograms represent average activity and standard deviation (n = 3) at the indicated conditions.

3.3. Antimicrobial activity of GOX produced in N. benthamiana against gram positive and gram-negative bacteria

The antimicrobial activity of the plant-made recombinant GOX was tested against a gram positive and a gram-negative bacteria, *S. aureus* and *E. coli*, respectively. Two different assays were performed. The first consisted on a disk diffusion assay carried out on a Petri dish whereas in the second the cells were incubated with the enzyme in liquid medium. Disk diffusion tests were assayed with increasing amounts of enzyme, which correlated positively with wider halo diameters resulting from growth inhibition (Fig. 5). Halo sizes were much higher for *S. aureus* than for *E. coli*, despite previous reports have described that gram-positive strains are usually less sensitive to GOX treatments. The thicker peptidoglycan of the gram-positive bacteria has been proposed to act as protective barrier to H_2O_2 and other reactive species but other

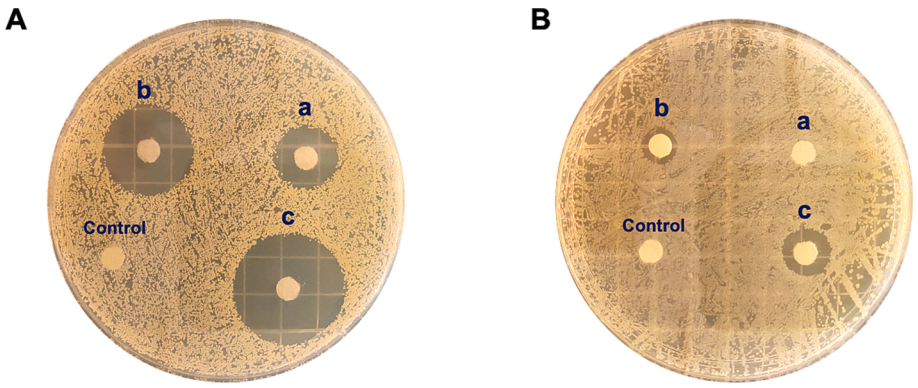

Fig. 5. Disk diffusion tests with *S. aureus* (A) and *E. coli* (B) were carried out with a reaction mixture containing 200 mM glucose and increasing amounts of GOX (a, 0.7 U/mL; b, 2.8 U/mL; and c, 12 U/mL). Control was performed with 2.8 U/mL of GOX without glucose.

enzymatic activities (catalase and superoxide dismutase) may also have this defense function (Califano et al., 2021; Mai-Prochnow et al., 2016). In particular, *E. coli* produces two inducible catalases (HPI and HPII). HPI expression is induced under logarithmic growth phase by H_2O_2 whereas both enzymes are overproduced during stationary phase compared to logarithmic phase (Schellhorn, 1995). Thus, HPI induction may explain the higher apparent resistance of *E. coli* strain in this assay. Therefore, a second test was carried out where cells were previously grown to logarithmic phase (i.e. not exposed to H_2O_2) (Fig. 6) or up to stationary phase (Fig. 7) and incubated in liquid media with GOX in the presence or absence of glucose. Samples were subsequently inoculated in fresh medium and culture growth was monitored to test cell viability.

When analyzing culture growth of log-phase treated cells (Fig. 6) a significant increase in the lag-phase was observed in the *S. aureus* cells incubated with GOX and glucose, compared to the controls, whereas no growth was detected after 24 h for *E. coli* cells subjected to the same treatment (Fig. 6A, C). This result is likely a consequence of the GOX-induced reduction in the number of viable cells. In both cases, cells treated with the enzyme in the absence of glucose behaved as the control, confirming that the reduction in cell viability is a specific consequence of GOX activity. An estimation of the reduction of the number of viable cells after enzymatic treatment, compared to the control without glucose, was obtained by fitting the logistic equation [1] to the experimental growth data (Fig. 6 B, D). According to these results, GOX treatment resulted in a 1.6 log reduction in *S. aureus* and at least a 13.6 log reduction in *E. coli*, estimation based on the detection limit ($N_0 = 1{\cdot}10^{-5}$) of the method. Compared to these results, cells grown to late stationary phase showed a higher sensitivity to GOX treatment in the case of *S. aureus* and a similar behavior in *E. coli* (Fig. 7).

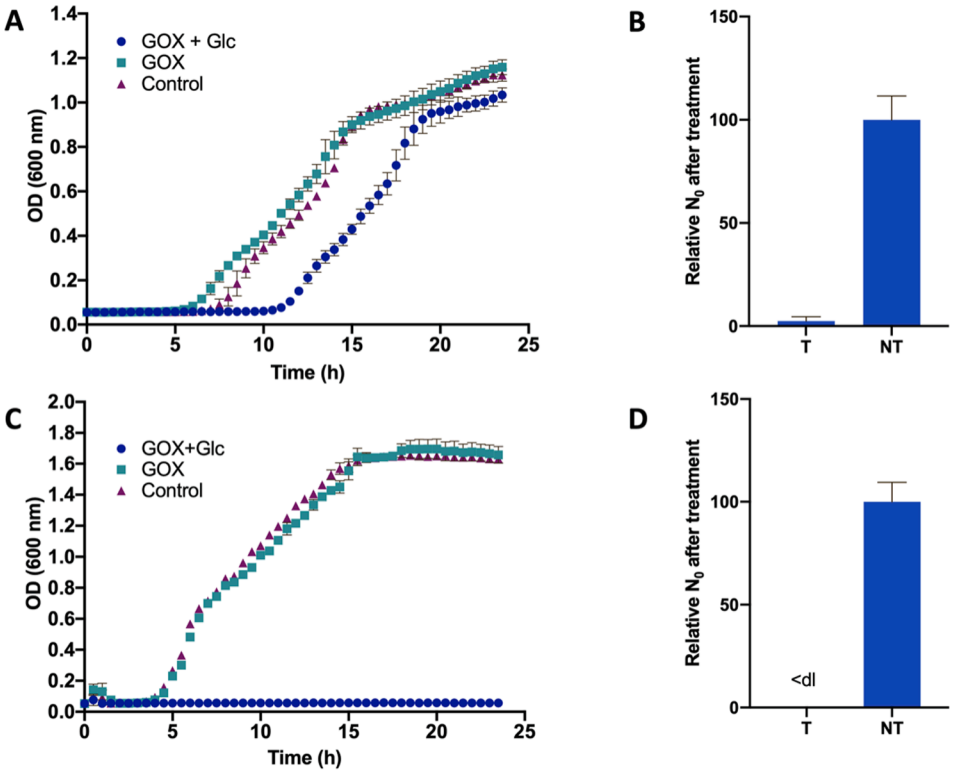

Fig. 6. Antimicrobial activity of the plant-made GOX against log-phase grown *S. aureus* (A, B) and *E. coli* (C, D). Culture growth (A, C) was monitored after incubation of liquid cell suspensions with the enzyme in the presence (GOX + Glc) or absence (GOX) of 100 mM glucose, or without any additive (control). Relative number of viable cells (N_0) after plant-made GOX treatment with (T) or without (NT) glucose for *S. aureus* (B) and *E. coli* (C); dl = 0.03 %.

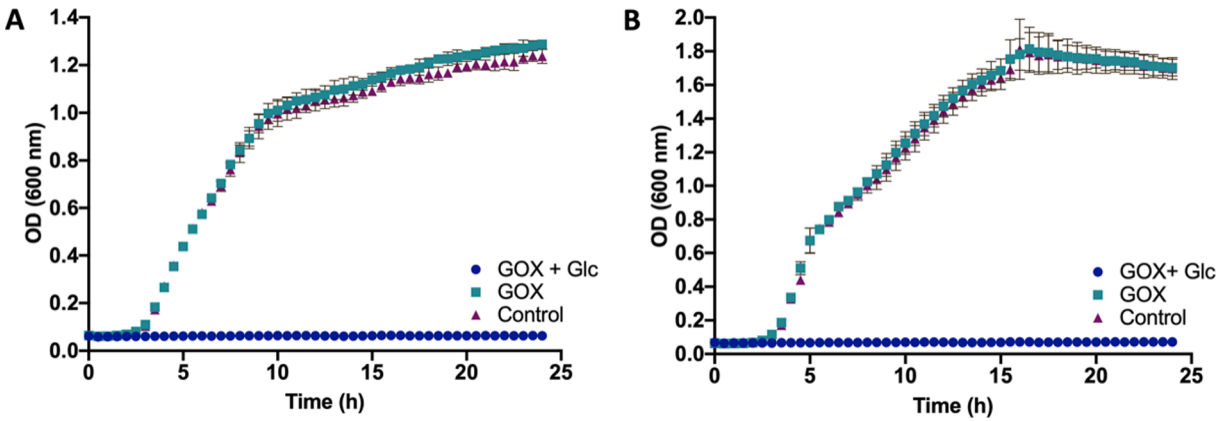

Fig. 7. Antimicrobial activity of the plant-made GOX against late stationary phase grown *S. aureus* (A) and *E. coli* (B). Culture growth was monitored after incubation of liquid cell suspensions with the enzyme in the presence (GOX + Glc) or absence (GOX) of 100 mM glucose, or without any additive (control). Plots represent the average (n = 3) optical density at 600 nm at different time points. Error bars represent standard deviation.

4. Discussion

Our results show that plants, and particularly *N. benthamiana*, are suitable hosts to produce GOX, a highly valuable industrial enzyme. A key feature of the system used here is gene expression driven by a TMV vector (Shi et al., 2019). GOX accumulation in plant tissues is based on RNA-to-RNA replication of the viral vector and efficient translation of the viral subgenomic mRNA in which the coding sequence of the recombinant protein is inserted. This subgenomic mRNA, which results from partial replacement of a viral coding region for that of the gene of interest, originally encodes the viral CP that reaches enormous amounts in infected tissues (Dawson, 2014), likely as a consequence of efficient translation regulatory signals at the 5′ and 3′ termini of the mRNA, in addition to the intrinsic stability of the viral protein. In our approach, the recombinant GOX is targeted to the export pathway by fusion of an amino-terminal peptide derived from *N. tabacum* (1–3)-β-endoglucanase (Diego-Martin et al., 2020), which efficiently targets the recombinant protein to the apoplastic space. This exclusive plant cellular localization, between the cytoplasmic membrane and the cell wall, is particularly poor in endogenous proteins (see Fig. 3). In addition, the apoplastic content can be mechanically separated from the rest of the plant tissues by a simple buffer infiltration followed by tissue squeezing using a hand operated syringe. Under this simple process, a protein preparation enriched in the recombinant protein of interest can be easily recovered. Production of the recombinant protein is also rather quick. Our previous time-course analysis using this same *N. benthamiana-A. tumefaciens*-TMVΔCP system with a extremophilic xylanase showed that 7 days is an optimal time point to harvest the tissues (Talens-Perales et al., 2022).

SDS-PAGE analysis of the apoplast fluids from *N. benthamiana* agroinfiltrated with exogenous DNA and, to a lesser extent in buffer-infiltrated tissues, shows the conspicuous presence of protein bands of 34, 25 and 22 kDa, corresponding to peroxidase N1, acidic endochitinase Q and pathogenesis-related protein R, respectively (Table 1). These proteins are not detected in the apoplast of non-infiltrated control plants. Additionally, a band corresponding to the large subunit of Rubisco was present in all preparations from infected, infiltrated with empty plasmid or buffer, and non-treated plants. The presence of Rubisco, an intracellular protein, in the apoplastic fluid is easily explained by the superabundance of this protein in the leaves, since even minor tissue damage can cause significant leakage to the apoplast fraction (Ishihama et al., 2022). The presence of the other three proteins in the apoplast of treated leaves in GOX expressing plants, but also in controls, can be explained by their known expression pattern. Peroxidase N1 is a secreted, defense-related protein, induced by stress conditions such as plant wounding (Hiraga et al., 2000). Acidic endochitinase Q has been reported to be induced by TMV infection (Payne et al., 1990). Pathogenesis-related protein R accumulates in the plant apoplast as a defensive mechanism against infection and is also a virus-induced protein (Pierpoint et al., 1987). Accumulation of these three endogenous proteins in the apoplast in our experiments seems to be a consequence of mechanical damage, rather than viral vector replication or *A. tumefaciens* infection, because they also accumulate in tissues infiltrated with just buffer. Interestingly, the plant response to tissue damage with expression of defense proteins does not prevent efficient GOX accumulation.

Antimicrobial activity of GOX has been well documented (Bauer et al., 2022; Califano et al., 2021; Wang et al., 2022). Our results, using an engineered GOX variant produced in *N. benthamiana,* confirm this property showing high bactericidal activity against *E. coli* and *S. aureus*, which was monitored in both cases by the decrease in viable cells (Fig. 6B, D). The relative higher resistance of *S. aureus* may be a consequence of the thicker peptidoglycan layer of the gram-positive bacteria, as previously suggested (Califano et al., 2021; Mai-Prochnow et al., 2016). The apparent higher resistance of *E. coli* to GOX observed in the disk diffusion assay (Fig. 5) turned out not to be so, as revealed by cell viability analysis. The apparent contradiction can be due to the induction of HPI catalase, promoted by the H_2O_2 generated by the GOX catalyzed reaction (Schellhorn, 1995). Furthermore, scavenging effects caused by *E. coli* cells highly reactive to H_2O_2 cannot be ruled out. Interestingly, cells grown to late stationary phase show similar or even higher sensitivity to GOX treatment than those grown to log-phase, even when catalase expression may be higher.

In sum, these results support that *N. benthamiana* is an optimal platform for the cheap, sustainable and scalable production of an engineered GOX and that the recombinant protein can be easily purified by mechanical means from plant tissues. Notably, in addition of well-known use in diagnosis and other biotechnological applications, our results highlight the potential use of plant-made GOX as an enzybiotic.

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David Talens-Perales: Conceptualization, Formal analysis, Investigation, Writing – review & editing. **María Nicolau-Sanus:**

Conceptualization, Formal analysis, Investigation, Writing – review & editing. **Julia Marín-Navarro:** Conceptualization, Formal analysis, Investigation, Writing – review & editing. **Julio Polaina:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. **José-Antonio Daròs:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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The proteomic analysis was performed in at SCSIE Universitat de València (Spain), a member of Proteored.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crbiot.2023.100148.

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