Recyclable thermophilic hybrid protein-inorganic nanoflowers for the hydrolysis of milk lactose.

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A B S T R A C T

Thermostable β-galactosidase (TmLac) has been immobilized as hybrid inorganic-protein nanoflowers using salts of Cu^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+} and Ca^{2+} as the inorganic component. The incorporation efficiency of enzyme into the nanoflowers was higher than 95% for a protein concentration of 0.05 mg/mL. The structure, activity and recyclability of the nanoflowers with different chemical composition were analyzed. Ca^{2+}, Mn^{2+} and Co^{2+} nanoflowers showed a level of lactase activity equivalent to their same content of free enzyme. Cu^{2+}nanoflowers showed only marginal enzyme activity in agreement with the inhibitory effect of this cation on the enzyme. TmLac nanoflowers provide an efficient methodology for enzyme immobilization and recyclability. TmLac-Ca^{2+} nanoflowers presented the best properties for lactose hydrolysis both in buffered and in milk, and could be reused in five consecutive cycles.

Keywords:

β-galactosidase; enzyme immobilization; lactose intolerance; milk products

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1. Introduction

Lactose, an essential ingredient in the diet of infants which is also present in an ample variety of food products, can also be a problem because of high rate of intolerance among adults [1-2]. Therefore, lactose-free products represent the fastest growing market segment in the dairy industry, with an expected market turnover of about 9000 million euros by 2022 [3]. Currently used procedure for industrial production of lactose-free milk and milk derivatives, consists in the addition of large amounts of fungal lactase. Generally, the enzyme is obtained from *Kluyveromyces* yeast strains. Although this practice satisfies the dairy industry because is cheap, alternative methods using immobilized enzyme could be cleaner and more practical in some cases. For instance: production of domestic devices for *in situ* hydrolysis of lactose. Although immobilization of lactase has been proposed and attempted since long ago [4], it has not been implemented, mainly because available lactases do not endure the manipulation required for immobilization and reuse, due to their structural liability. TmLac, the β-galactosidase (EC 3.2.1.23) produced by the thermophilic bacterium *Thermotoga maritima*, has emerged as an alternative tool for lactose hydrolysis at industrial level. This enzyme, which cleaves lactose efficiently, has an optimal temperature of 75 ^{0}C [5-7] that makes it suitable to immobilization by different procedures [6, 8-9]. The three-dimensional structure of TmLac at a resolution of 2.0 Å, obtained by cryogenic electron microscopy, has been recently reported [10].

Protein-inorganic hybrid nanoflowers [11] represent a novel concept of enzyme immobilization [12]. Originally, the nanoparticles were prepared by using copper (II) ions as the inorganic and BSA as the organic component. Scanning Electron Microscopy (SEM) pictures of the particles showed structures reminding flowers, hence the name. When an enzyme was

used as the organic component, the flowers exhibited enhanced activity compared with the free enzyme. Since its discovery, different enzymes and other biomolecules have been immobilized with this technique [13-14]. Metals other than copper have been successfully used for the formation of protein-inorganic hybrid nanoflowers, *e. g.* calcium, manganese, zinc, cobalt and iron [13-14]. The simplicity and low-cost of the procedure makes it very interesting for industrial applications. In this report the production and analysis of TmLac nanoflowers, using different metals as the inorganic part is described. Morphology, activity and recyclability were tested to stablish an adequate procedure to generate thermostable nanoflowers with efficient capability for lactose hydrolysis.

2. Materials and methods

2.1. Chemical and reagents

Salts used for nanoflowers formation ($ZnSO_4$, $CaCl_2$, $MgCl_2$, $MnSO_4$, $CoCl_2$, $CuSO_4$) and for buffer preparation (NaCl and $MgCl_2$) and lactose 1-hydrate were from Panreac Química (Spain). Chromogenic β-galactosidase substrate, *p*-nitrophenyl ß-D-Galactopyranoside (pNP-Gal) and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Sigma Aldrich. Protein in SDS-PAGE analysis was stained with BlueSafe Staining from NZYTech.

2.2 Production and purification of TmLac

Procedures used for the production and purification of TmLac have been previously described by Marin-Navarro et al. [6]. Cultures of *E. coli* Rosetta 2 strain, carrying plasmid pQE-80L in which the TmLac encoding gene had been cloned, were grown in 2XTY media (1.0 % yeast extract, 1.6 % tryptone and 0.5 % NaCl (w/v)), supplemented with 1 mg/L ampicillin and 34 mg/L chloramphenicol, at 37 ^{0}C with orbital shaking (200 rpm) up to an OD (600 nm) of 0.6. Then, the synthesis of histidine-tagged TmLac was induced by adding 1 mM IPTG followed by 5h incubation at 37^{0}C. The enzyme was purified from crude cell extracts in two stages. Firstly, it was subjected to thermal treatment at 85 ^{0}C during 15 minutes and recovered from the supernatant after centrifugation at 15,000 *g* during 30 minutes. Subsequently, TmLac was subjected to a second stage of purification carried out by nickel affinity chromatography, using 1 mL HisTrap FF crude column (GE Healthcare), equilibrated with buffer A (20 mM imidazole, 500 mM NaCl and 20 mM phosphate buffer, pH 7.4), in an ÅKTA-Purifier equipment (GE Healthcare). After injection of the sample, a washing step with 10 mL of 10 % buffer B (500 mM imidazole, 500 mM NaCl and 20 mM phosphate buffer, pH 7.4) was performed. Finally, elution was carried out with 10 mL of an increasing linear gradient of buffer B (from 10 % to 100 %) . Fractions containing TmLac (purity >90%) were dialyzed in 20 mM Tris-HCl buffer pH 7.4 with 50 mM NaCl and stored at -80 ^{0}C.

2.3 Production of TmLac-inorganic nanoflowers

Nanoflowers were obtained by the procedure described by Ge et al. [11] with some modifications. Stock solutions (120 mM) of different metallic salts: $ZnSO_4$, $CaCl_2$, $MgCl_2$,

$MnSO_4$, $CoCl_2$, $ZnSO_4$ and $CuSO_4$ were prepared. A stock solution of TmLac was prepared in phosphate-buffered saline (PBS: 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.4) at a final concentration of 0.05 mg/mL. To induce the formation of nanoflowers, 20 μL of the metallic salt solution were added to 1500 μL of the protein stock solution, in individual "incubation tubes"(2 mL eppendorf microcentrifuge tubes), which were incubated at room temperature for 24 h. After this time, nanoflowers precipitate from solution, appearing as a sediment.

Two complementary procedures were used to determine the TmLac load in the nanoflowers. Firstly, it was indirectly calculated by measuring the remaining (non-incorporated) TmLac activity in the supernatant of the "incubation tubes", once the nanoflowers had been separated by centrifugation (10 minutes at 15,000 *g*). For this analysis, *p*-nitro phenyl β-D-galactopyranoside (*p*NPGal) 5 mM, prepared in assay buffer (50 mM phosphate buffer, pH 6.5, 10 mM NaCl, 1 mM $MgCl_2$), was used as the substrate. The reaction was carried out at 75 ^{0}C by adding 20 μL of supernatant to 500 μL of the assay solution with the substrate. Reaction samples were incubated for different periods of time and terminated by adding 1 mL of 1 M Na_2CO_3. Enzyme activity was calculated as a function of *p*-nitrophenol released, measured spectrophotometrically at 400 nm. The alternative procedure consisted in the extraction and measurement of the TmLac protein contained in the nanoflowers. An "incubation tube" containing nanoflowers, was boiled with SDS (2%) for 5 min. The released protein was subjected to SDS-PAGE. The polyacrylamide gel was stained with BlueSafe (NZYTECH) and the resulting protein band (TmLac) was measured in a Proxima AQ-4 gel documentation system (Isogen).

2.4. Scanning Electron Microscopy (SEM)

Nanoflowers were visualized in a Hitachi SEM microscope (Hitachi S-4800) at an accelerating voltage of 10 kV and a working distance of 8.0 – 8.5 mm. Nanoflower samples were washed thoroughly with deionized water, dispersed and dried before being mounted on an 'Aluminum Specimen Mount'. They were fixed on the support using double-side adhesive tape and coated with a thin layer of gold-palladium sprayed on their surface.

2.5. Assay of lactase activity of nanoflowers

Determination of the enzymatic activity of nanoflowers was carried out in triplicate, in assay buffer, at the optimum pH (6.5) and temperature (75 ^{0}C) of the enzyme, with lactose as the substrate. The nanoflowers from two "incubation tubes" were collected, washed with PBS to remove any non-incorporated enzyme, and resuspended in either 2 mL of 5% (w/v) lactose in assay buffer (50 mM phosphate buffer, pH 6.5, 10 mM NaCl, 1 mM MgCl2) or 2 mL of skimmed milk. The reaction was incubated at 75 ^{0}C with gentle agitation. Samples (100 μL) were taken at different times and heated at 95 ^{0}C for 10 minutes to inactivate the enzyme. Equivalent samples of soluble enzyme were assayed in the same conditions as a control. Lactase activity was determined by measuring the amount of glucose released, using a glucose assay kit (Glucose (GO) Assay Kit, Sigma).

2.6. Nanoflowers recycling

The possibility of reusing the nanoflowers was tested by subjecting them to consecutive cycles of lactose hydrolysis. The nanoflowers collected from two "incubation tubes" were resuspended in 2 mL of 5% (w/v) lactose in assay buffer and incubated for 3.5 h at 75 ^{0}C with gentle agitation. After this time, the nanoflowers were recovered by centrifugation and the supernatant was saved for analysis. The nanoflowers were washed with 1 mL of assay buffer and placed again in 2 mL of assay buffer with lactose for the following reaction cycle. Remaining lactose in the supernatant of the reaction, after each cycle, was determined as a function of the amount of glucose measured with a Glucose (GO) Assay Kit (Sigma).

2.7. Statistical analysis

Statistical analysis of experimental data using one-way ANOVA with Dunnett's post test, using GraphPad Prism version 8 software, at 99% confidence level.

3. Results and discussion

3.1 Growth of TmLac nanoflowers

As explained in the experimental section, the formation of nanoflowers was induced by adding 20 μL of a metal salt solution to 1,500 μL of TmLac solution. Preliminary experiments were carried out to evaluate the optimal amount of protein to achieve an efficient

incorporation of TmLac into the nanoflowers. A range of protein concentrations between 0.1 and 0.01 mg/mL was explored. As shown in Figure 1, the use of TmLac at a concentration of 0.05 mg/mL assured its complete integration into nanoflowers obtained with different metals (Cu^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, and Zn^{2+}). This result is consistent to what has been reported for other proteins [15-17]. In all cases, the activity of incorporated enzyme, estimated indirectly by measuring remaining pNPG-ase activity in the buffer after the formation of the flowers, was over 95% (Table 1). This was corroborated by physical analysis by SDS-PAGE which showed that initially there was no detectable remaining protein in the incubation buffer whereas the flowers grown in this same buffer yielded an amount of protein equivalent to that used for their formation (Figure 1). It should be noted that TmLac-nanoflowers were not obtained when $MgCl_2$ was used (Table 1). Whereas the use magnesium has been reported effective for the production of nucleic acids (both DNA and RNA) nanoflowers it seems not to be effective for proteins [14]. The lower atomic mass of magnesium compared to that of the other metals tested could be the determinant factor. Although the chemistry behind the topology of nanoflower formation is not known in detail, metal ions interact with the amine and amide groups of proteins. Some of these interactions can act coordinately stabilizing the metal, at specific sites of the protein molecule, serving as nucleation points, driving the biomineralization process that yields the nanoflowers. Bioinformatic analysis has been used to predict potential nucleation sites for Cu^{2+}, Ca^{2+} and Zn^{2+}, in the structure of a thermostable fungal lipase [18].

3.2 Structure of TmLac nanoflowers

It is known that the morphology of hybrid protein-inorganic nanoflowers varies when different proteins are used. Protein Cu^{2+} nanoflowers prepared with BSA, β-lactoalbumin, laccase, carbonic anhydrase, or lipase, show different shape and size [10]. Figure 2 shows the TmLac nanoflowers obtained with different salts (Cu^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, and Zn^{2+}). Not surprisingly, size and shape of TmLac nanoflowers obtained with salt of different metals were quite different. Morphology is a result of the growth process. At an early stage, phosphate crystals are formed by the complexation of metallic ions bound to the protein molecules through coordination with amide and amine groups. At a following stage, agglomerates composed of proteins and primary crystals coalesce forming larger agglomerates, by interaction of ions presented in their surface. Finally, anisotropic growth proceeds yielding the observed structures [12, 19]. Final shape depends on the interactions stablished at the early stage, determined by the characteristics of the metallic ion (charge, size, etc.) and the spatial coordination of the ion with accessible protein residues [18]. For all salts tested, except for Mg^{2+}, the resulting nanoflowers reached a size between 2 and 10 μm (Figure 2). Cu^{2+} nanoflowers were the largest and best structured whereas those made with Co^{2+} did not show flower-like appearance, being more similar to scattered petals.

3.3 Activity of TmLac nanoflowers

Besides their different morphology, the activity of TmLac nanoflowers generated with different metallic ions also differed. Figure 3 presents results of lactase activity, using lactose as the substrate at about the same concentration (5% w/v) at which the sugar is present in

milk. After incubation for 30 minutes, TmLac Mn^{2+} and Ca^{2+} nanoflowers showed about the same activity that the equivalent amount of free enzyme. Compared to the control, Zn^{2+} and Cu^{2+}, showed marked decays of activity, around 2 and 10-fold, respectively. While copper has been widely used to produce nanoflowers with different enzymes, the use of this metal with TmLac severely affected the enzyme activity. It is known that Cu^{2+} is an inhibitor of TmLac [5] and also of other β-galactosidases such as *E. coli* [20], *Bifidobacterium longum* [21] and *Arthrobacter* [22]. Long-term incubation of nanoflowers made with the different metals in a lactose solution yielded similar profiles of lactose hydrolysis (Figure 4). Kinetics was somewhat delayed with Zn^{2+} nanoflowers due to lower specific activity of these structures (see Figure 3) but lactose hydrolysis was completed in about 3 hours in all cases, except for Cu^{2+} nanoflowers for which it did not progress beyond 5%. The capability of TmLac nanoflowers to hydrolyze lactose, not in buffer solution but in milk, was tested. Figure 5 shows that Ca^{2+} nanoflowers complete the hydrolysis of the amount of lactose contained in milk, indicated by the manufacturer (4.6 g/L).

3.4 Stochiometry of nanoflower formation

Reaction of PBS with $CaCl_2$ in the presence of a protein yields hybrid organic-calcium phosphates with different formulations. In the process of nanoflower growth, the formation of different types of inorganic crystals depends on the pH of the medium. Hybrid $CaHPO_4$-protein nanoflowers were obtained with PBS buffer at pH 6.8 [23-25], whereas $Ca_3(PO_4)_2$-protein nanoflowers were obtained when PBS buffer at pH 7.4 was used [15, 25]. Since TmLac nanoflowers in this study were grown at pH 7.4, it is assumed that TmLac-$Ca_3(PO_4)_2$ crystals were formed. Taking into account the low solubility of this salt ($Ca_3(PO_4)_2$ pKs ~ 25-29) [26], and the initial amounts of phosphate and calcium used in the experimental setting (4.8 and 1.5

mM, respectively), where calcium is the limiting reactant, it can be estimated that the amount of $Ca_3(PO_4)_2$ (molar mass 310.18 g/mol) precipitated as nanoflowers is 0.5 mM (0.16 mg/mL). Since the incorporation efficiency of TmLac (0.05 mg/mL), into nanoflowers is close to 100% (Table 1), the molar ratio of protein molecules to inorganic calcium centres can thus be estimated as one TmLac molecule (an octamer of ca. 1,000 kDa) per ca. 30,000 cation centres in the crystal. This is in agreement with the fact that the protein is mainly located in the core of the nanoflower, whereas petals are enriched in the inorganic component [11]. Based on these calculations, the amount of loaded protein should be around 250 mg per gram of nanoflowers. According with the results presented in Figure 4, which indicate that full hydrolysis of 5% (w/v) lactose is achieved in 3 hours with an enzyme concentration of 0.075 mg/mL, it can be estimated that the lactose contained in 200 mL (equivalent to a glass of milk) will be fully hydrolyzed by 1 g of TmLac-nanoflowers in 10 minutes. Similar results are expected with Co^{2+}, Mn^{2+} and Zn^{2+} nanoflowers

3.5 TmLac nanoflowers as a recyclable zymoactive material for lactose hydrolysis

Reutilization of nanoflowers for the hydrolysis of lactose was assayed by subjecting them to consecutive cycles of incubation for 3 hours, in lactose solution 5 % (w/v), at 75 ^{0}C. Figure 6 shows the results obtained for Mn^{2+}, Zn^{2+}, Ca^{2+} and Co^{2+}. The activity decreases markedly after the first cycle for Mn^{2+} and Zn^{2+} whereas Ca^{2+} nanoflowers maintain nearly full activity for three cycles. Differences in the capability of nanoflowers to retain its enzymatic activity can be attributed to different conformations adopted by TmLac within the protein-mineral network induced by each metal. In the process of nanoflower formation, protein nucleation sites, specific for each metal atom, play an essential role in the initial step, driving

the formation of the central scaffold to which the growing petals attach. The stability of Ca^{2+} nanoflowers suggests a stable conformation of the TmLac protein in its bonding to the mineral scaffold. Consequently, Ca^{2+} nanoflowers could be recycled with good efficiency, showing results similar to those obtained for TmLac covalently immobilized onto an epoxy support [6].

4. Conclusion

Hybrid protein inorganic nanoflowers produced with TmLac and Ca^{2+} salt showed good performance in terms of activity, stability and recyclability, better than those based on other metals (Mg, Cu, Mn, Zn and Co**).** The result obtained with the immobilization of TmLac into Ca^{2+} nanoflowers provide an efficient new methodology for the hydrolysis of lactose in milk. From these results, it can be estimated that one gram of TmLac-Ca^{2+} nanoflowers can complete hydrolysis of the lactose contained in a glass of milk, in about 10 min, and they can be reused at least three times. Application of this methodology for making lactose-free milk would be safe for human consumption.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Table 1. Fraction of TmLac protein incorporated in nanoflowers made with different metallic cations.

Metal	Protein incorporated (%)
Mg^{2+}	none[a]
Cu^{2+}	96.4±2.0
Mn^{2+}	96.5±1.7
Zn^{2+}	98.8±0.3
Co^{2+}	98.7±0.3
Ca^{2+}	95.5±2.4

[a]nanoflowers could not be obtained with Mg^{2+}

Figure Captions

Figure 1. Physical analysis (SDS-PAGE) of the presence of TmLac protein in the nanoflower growing buffer or incorporated into nanoflowers prepared with different metals. The initial protein concentration in the growing buffer was 0.05 mg/mL (C). After 24 h, the presence of protein was re-examined in the buffer (B) and in the nanoflowers (F).

Figure 2. SEM pictures (wide shot, left and nanoflower detail, right) of Hybrid TmLac inorganic nanoflowers obtained with Cu^{2+} (A), Mn^{2+} (B), Zn^{2+} (C), Co^{2+} (D) and Ca^{2+} (E).

Figure 3. Enzyme activity (glucose released from lactose) of TmLac nanoflowers obtained with different metals (*** $p < 0.01$).

Figure 4. Kinetic of lactose hydrolysis (measured as released glucose) by TmLac nanoflowers obtained with different metals and the free enzyme (control).

Figure 5. Kinetic of lactose hydrolysis from milk (measured as released glucose) by TmLac Ca^{2+} nanoflowers (NF) and the free enzyme (control).

Figure 6. Lactose hydrolysis by TmLac nanoflowers prepared with Mn^{2+} (A), Zn^{2+} (B), Ca^{2+} (C) and Co^{2+} (D) reused in successive cycles.

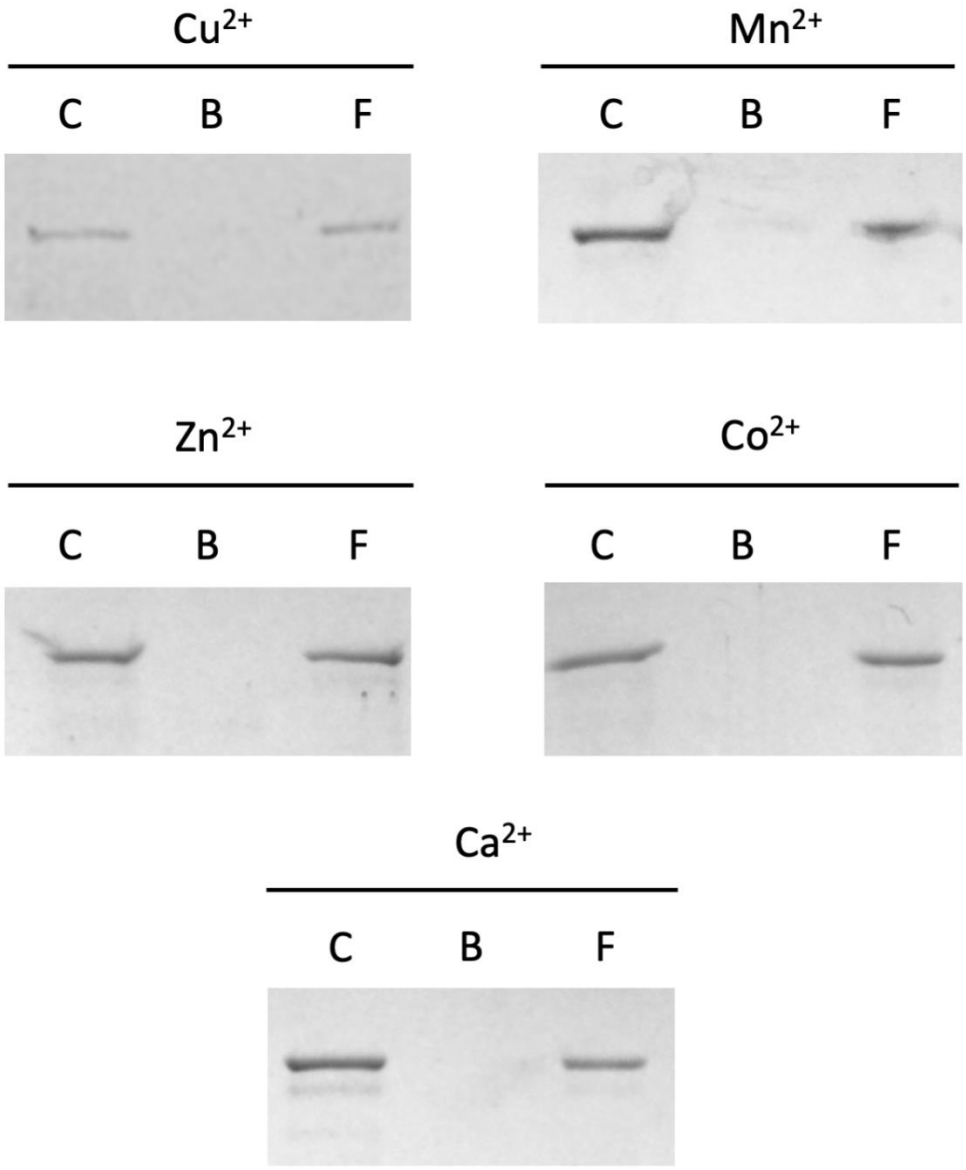

Figure 1.

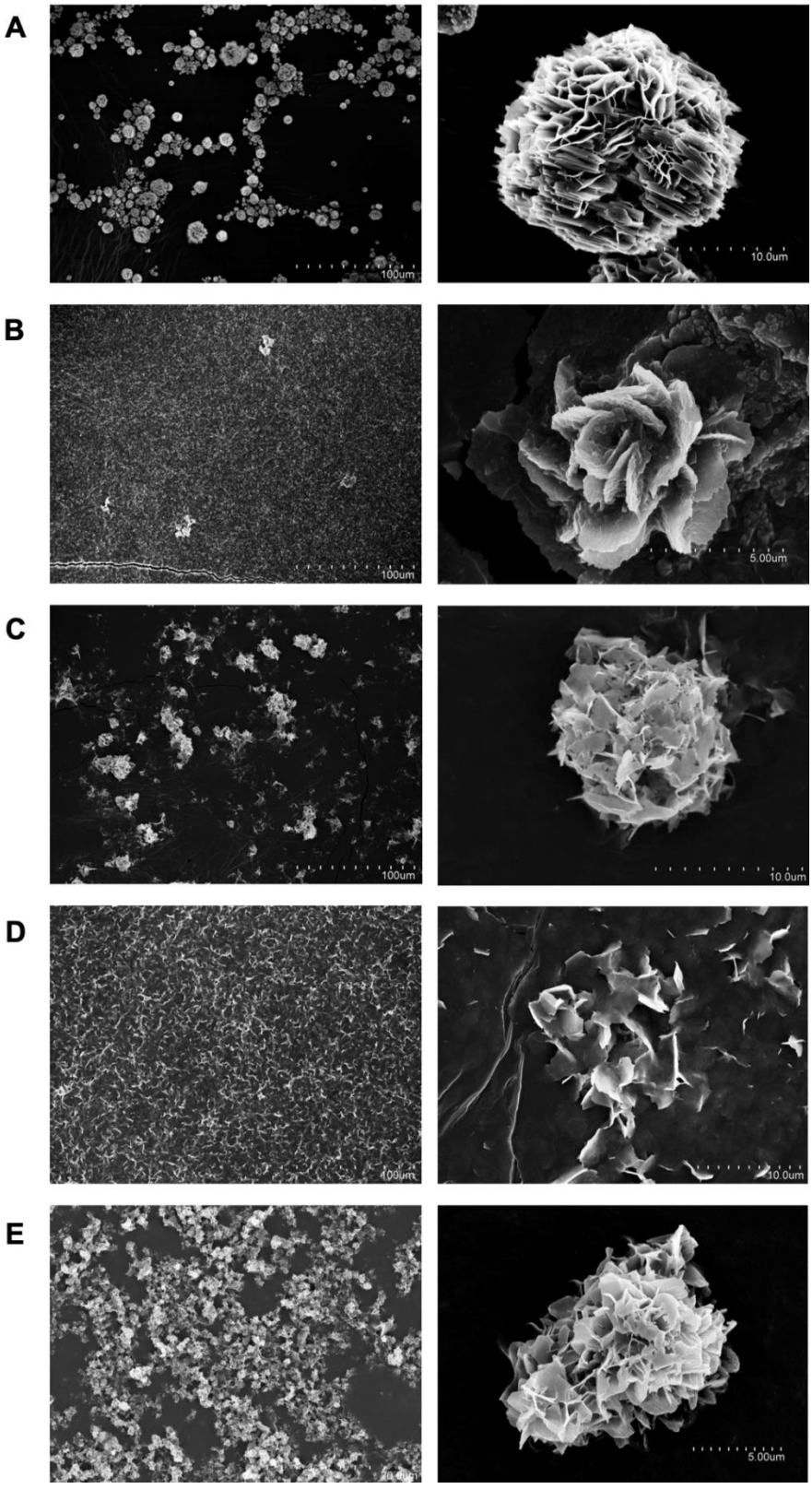
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Figure 2

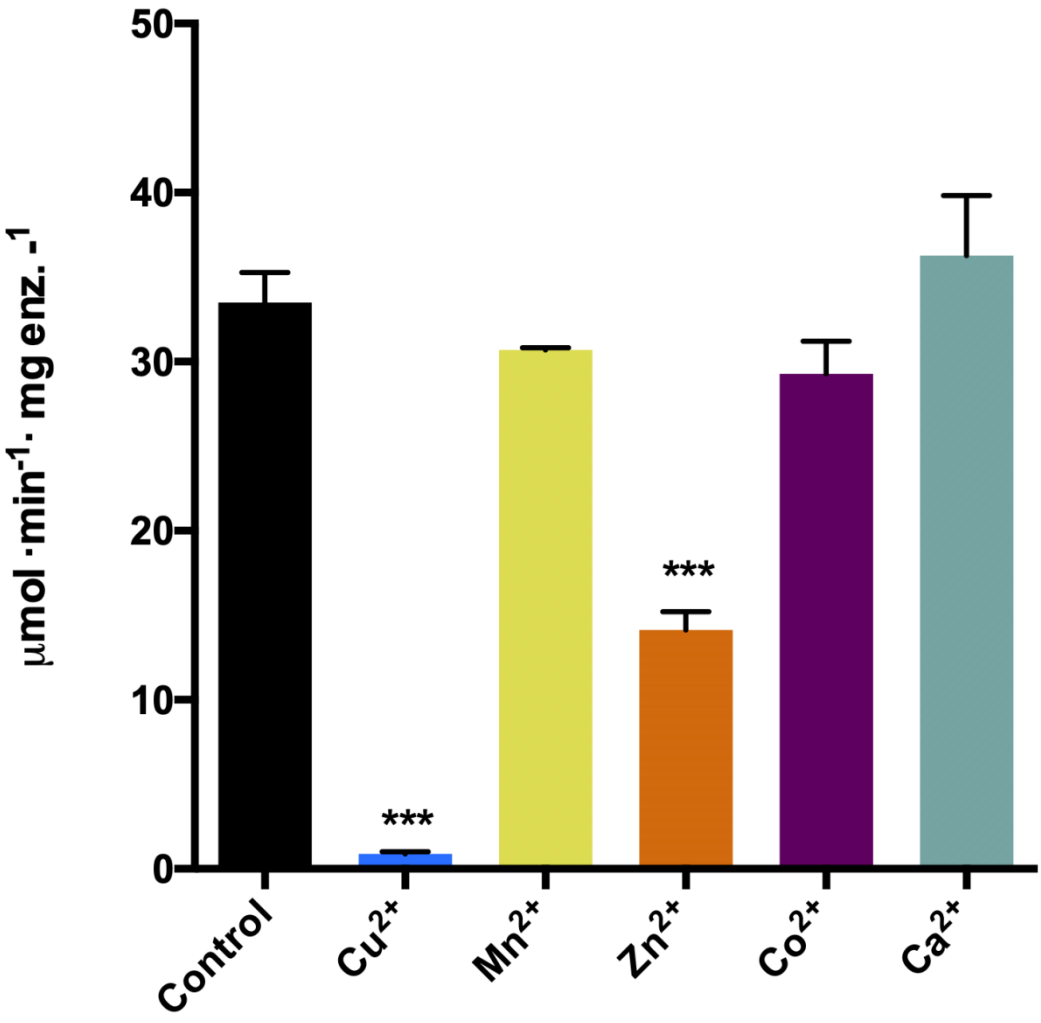

Figure 3

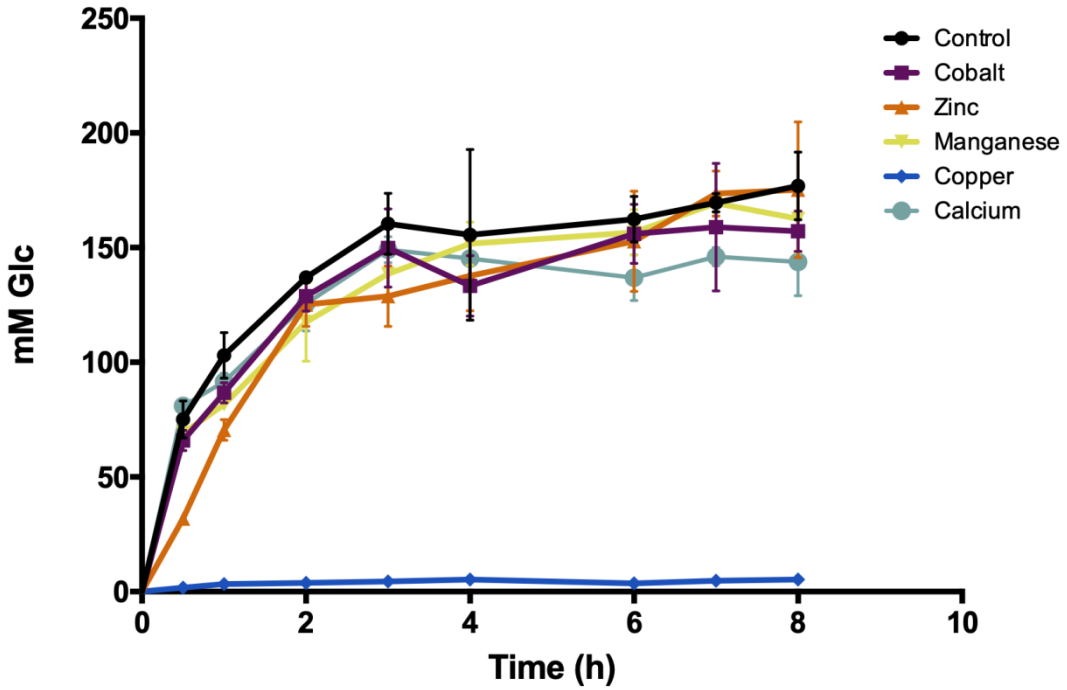
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Figure 4

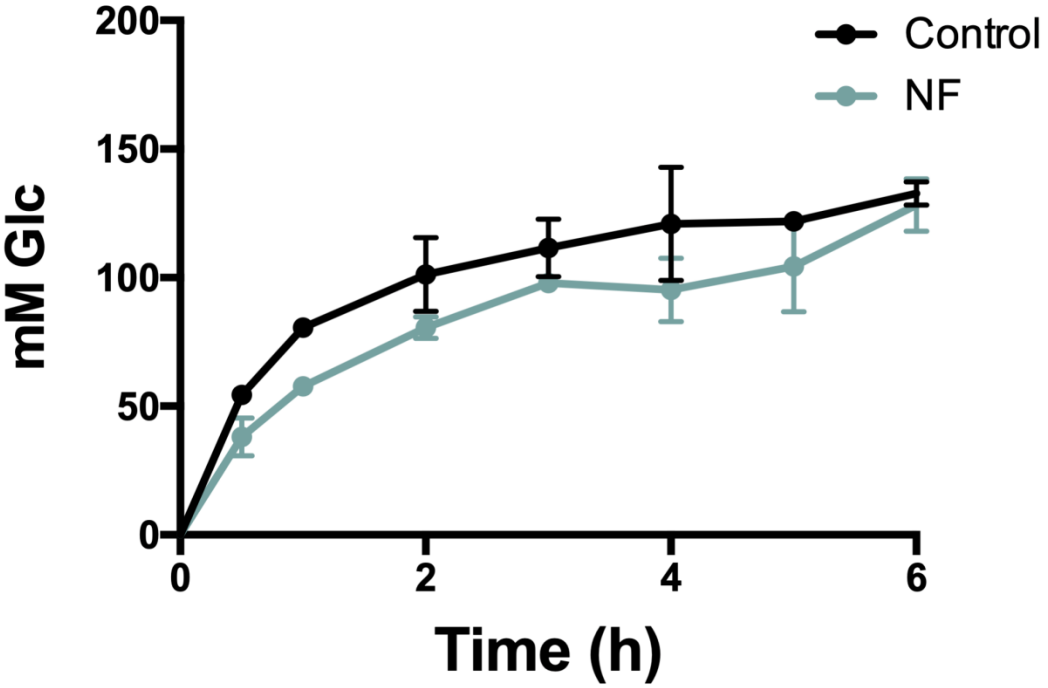

Figure 5

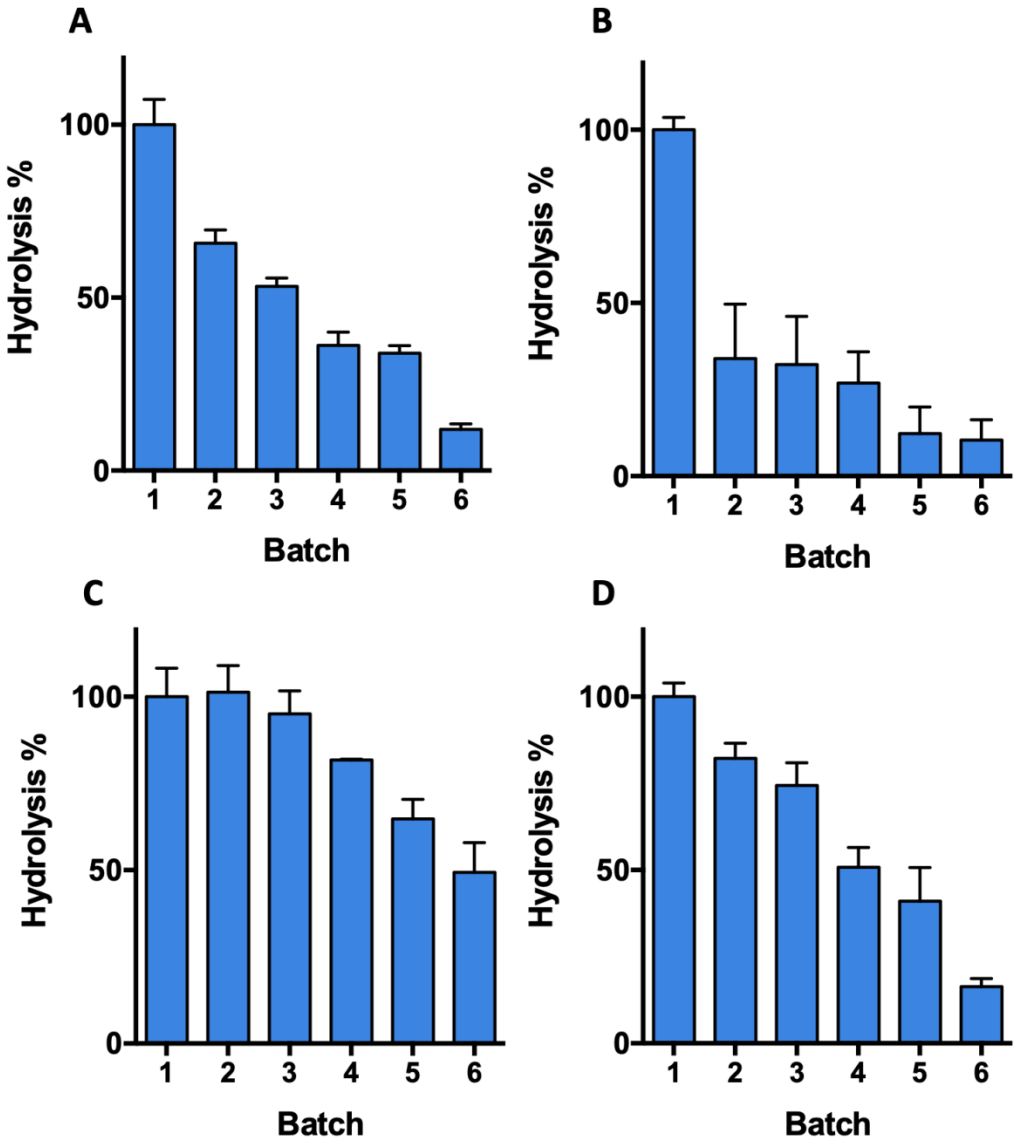

Figure 6