Surfactant-Free β-Galactosidase Micromotors for "On-The-Move" Lactose Hydrolysis

Roberto Maria-Hormigos, Beatriz Jurado-Sánchez, and Alberto Escarpa**

R. Maria-Hormigos. Prof. B. Jurado-Sánchez, Prof. A. Escarpa
Department of Analytical Chemistry
Physical Chemistry and Chemical Engineering
University of Alcalá
Alcala de Henares, E-28871 Madrid, Spain
E-mail: beatriz.jurado@uah.es; alberto.escarpa@uah.es

Prof. B. Jurado-Sánchez, Prof. A. Escarpa
Chemical Research Institute "Andrés M. del Río"
University of Alcalá
Alcala de Henares, E-28871 Madrid, Spain

Keywords: β-galactosidase, lactose, micromotors, milk, self-propulsion

Surfactant-free β-galactosidase micromotors are explored here as moving biocatalyst for highly efficient lactose hydrolysis from raw milk. The coupling of the hydrolytic properties of such enzyme with the efficient movement of carbon nanotube tubular micromotors results in nearly 100% lactose hydrolysis and two fold removal efficiency as compared with static conditions and with free enzyme. The incorporation of an inner Ni layer allows its reusability to operate in batch mode. The rough micromotor surface area allows the immobilization of a high loading of □-galactosidase and results in an increase in the enzyme affinity toward lactose. The new micromotor concept opens new avenues for the use of micromotors as moving immobilized biocatalyst to improve the technological process not only in food industry but also in other fields.

1. Introduction

Lactose is the most abundant disaccharide in milk and essential for the nourishment of newborn infants. It is hydrolyzed by the intestinal brush-border enzyme, lactase, into glucose and galactose.[1] Lack of the enzyme lactase in the small intestine resulted in heavy digestions and stomachache, being thus considered as a health threat worldwide.[2] To minimize such problems

the alimentary industry has developed different strategies to remove it from milk and derivative products.[3] Enzymatic hydrolysis using the enzyme β-galactosidase has proven to be a convenient way to broke lactose into glucose and galactose, which can be easily absorbed by the intestinal tract, avoiding such milk intolerance.[4] Biocatalyst immobilization onto nanosupports possesses several advantages over traditional chemical technologies such as higher specificity, facile product separation, and the possibility to work in continuous operation.[5] β-galactosidase has been successfully immobilized onto various materials for improved performance and stability as compared with "free-enzyme" alternatives.[6] In particular, the high surface-to-volume ratio offered by carbon nanomaterials resulted in an enhanced enzyme loading, improved activity and stability, allowing thus for a decrease of the biocatalyst cost in industrial biotechnology. Indeed, immobilization of β-galactosidase onto carbon-based nanosupports allows for multiple usage through chemical and physical treatment.[7] Herein we describe the use of surfactant-free carbon nanomaterial catalytic micromotors as moving nanosupports for the immobilization of β-galactosidase toward highly efficient lactose hydrolysis in continuous mode.

Self-propelled micromotors hold considerable promise as "dynamic supports" for the immobilization of enzymes for a myriad of applications.[8] Indeed, micromotors present several advantages compared to traditional materials employed in immobilized biocatalysts due to their capability to move in the reaction media eliminating substrate diffusion necessity.[9] Pioneering works on microtubular motors by Schmidt group demonstrated that the bubble propulsion mechanism imparted essential intermixing effect, which is extremely attractive for highly efficient enzymatic processes.[8c,d] Nevertheless, early attempts were devoted to explore the use of immobilized bio-catalyst—catalase—to achieve efficient propulsion[10] and for motion-based sensing protocols.[8e] Later on, lysozyme-based ultrasound-propelled motors were successfully employed as a highly efficient antimicrobial platform against gram-positive and gram-negative bacteria.[11] Self-propelled carbonic anhydrase functionalized micromotors are extremely useful for biomimetic carbon dioxide sequestration.[8f,h] The high towing force and large outer surface area of carbon allotrope based micromotors hold considerable promise for enzyme immobilization toward novel biotechnological applications.[12] Yet, the potential of such moving microreactors for biocatalyst immobilization toward applications into the biotechnological process remains unexplored. Indeed, only the Schmidt group reported a graphene-composite-sodium borohydride micromotor for value-added product synthesis.[13]

Herein we present, for the first time, the use of immobi- lized biocatalysts onto carbon tubular micromotors to remove a potentially intolerant substance like lactose by β-galactosidase

hydrolysis. The concept is illustrated in **Figure 1**. The micromotors are prepared by template electrodeposition using multiwalled carbon nanotubes (MW) as outer layer for further functionalization by surface carboxylic groups (-COOH) and nickel-platinum nanoparticles (Ni-PtNPs) for inner layer that allows efficient self-propulsion in milk an easy recovery from sample by magnetic separation.[12f] Enzyme β-galactosidase is immobilized by covalent chemistry leading to functional structure with potential to eliminate lactose in raw milk samples. The micromotors can propel in skimmed milk without the aid of any surfactant, simplifying the overall micromotor operation. The immobilized β-galactosidase activity and stability are evaluated under different temperature and pH conditions. Immobilized biocatalyst micromotors reusability by magnetic separation from sample and their performance in real skimmed milk are studied toward efficient operation. This is the first time that carbon allotrope based micromotors are used as novel support materials for biocatalyst immobilization.

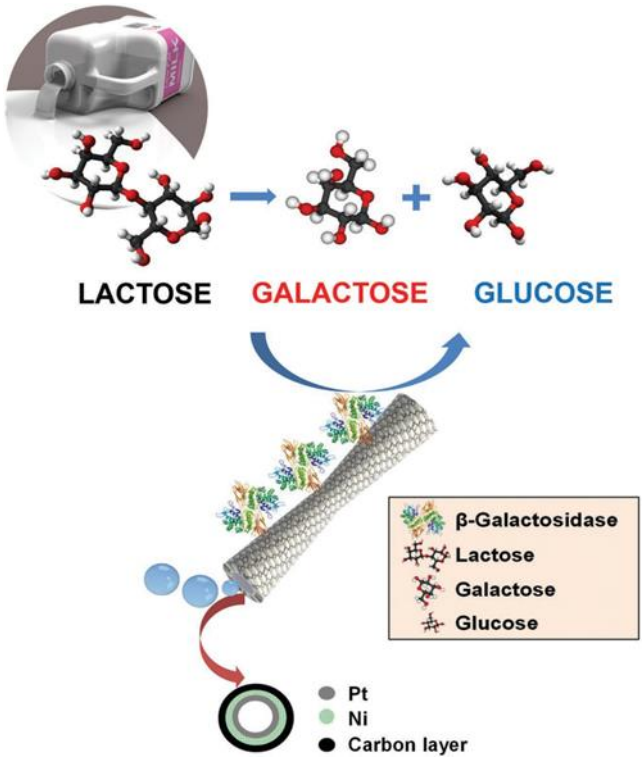

Figure 1. Surfactant-free β-galactosidase micromotors for lactose removal from milk.

2. Results and Discussions

Figure 2A illustrates a schematic of the fabrication protocol of MW-Ni-PtNPs micromotors and their functionalization with β-galactosidase enzyme to produce a moving immobilized biocatalyst. The large surface area of MW-based micromotors allows for the immobilization of a huge enzyme loading as compared with less rough counterparts.[7c,11] The nickel magnetic

layer allows straightforward separation of the immobilized biocatalyst for further reusability. β-galactosidase functionalization was carried out by the activation of the carboxylic (-COOH) surface groups in the micromotors by N-(3-dimethylaminopropyl)-N´-ethylcarbodiimide hydrochloride/N-hydroxysulfosuccinimide sodium (EDC/NHS) chemistry and later incubation with the enzyme. Scanning electron microscopy (SEM) images of **Figure 2B** show the well-defined conical morphology of the as prepared micromotors, which displays globular-like structures or flakes attached on its surface due to enzyme covering.

Interestingly, the micromotors can propel themselves in milk samples without the addition of any surfactant. The time lapse- microscopy images of **Figure 2C** (taken from Video S1 of the Supporting Information) illustrate the efficient propulsion of an MW-Ni-PtNP micromotor in skimmed milk (1% H_2O_2) with and without enzyme functionalization. A long tail of oxygen bubbles generated from the catalytic decomposition of H_2O_2 by the rough inner Pt inner layer is released from the rear large opening side of the micromotors, which propels efficiently atspeeds of 58 ± 12 and 16 ± 6 μm s^{-1} (1% H_2O_2) before and after enzyme functionalization, respectively. Such drastic speed decrease can be attributed to the enzyme immobilization steps (as reported in previous works) and catalyst fouling by milk proteins. This fact, however, did not hamper the practical application of our micromotors, as will be described later. As such, the micromotors can move at low fuel concentrations (1%) without the need for addition of an external surfactant because milk matrix actuates as surfactant itself. Indeed, native milk proteins such as casein are well known due to their surfactant properties, which thus aid for efficient bubble evolution and micromotor operation in raw samples. Hydrogen peroxide has been previously used for milk preservation, and some quantities are desirable to activate well-known lactoperoxidase against harmful bacteria.[14] Yet, as its use is limited in many countries, our system can be combined with the use of similar catalase-driven micromotors to deplete peroxide levels down to allowable levels.[15] While peroxide-driven motors are used here as proof-of-concept for the intended application, our strategy can be easily implemented using ultrasound-propelled or water-driven (magnesium) micromotors.

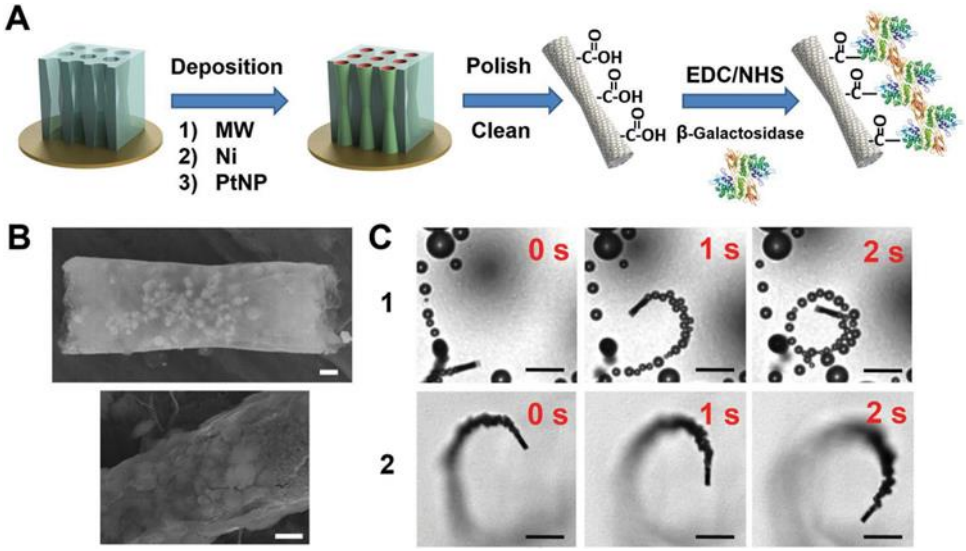

Figure 2. A) Schematic of the preparation of MW-Ni-Pt micromotors and its modification with β-galactosidase via EDC/NHS chemistry. B) Scanning electron microscopy images of the modified micromotors (scale bars, 1 µm). C) Time-lapse images (taken from Video S1 of the Supporting Information) of the efficient navigation of bare (1) and β-galactosidase (2) functionalized micromotors in milk samples containing 1% H_2O_2. Scale bars, 20 µm.

Prior to test the efficiency of our micromotor for dynamic lactose removal is essential to optimize critical enzyme operation parameters such as temperature, pH stability, and catalytic efficiency. To this end, different amounts of β-galactosidase enzyme (10–3000 U) were used for micromotor modification (76 000 motors mL^{-1}) to ensure optimal enzyme activity and assay reproducibility. The rate of activity of β-galactosidase micromotors was determined by the ortho-nitrophenyl-β-galactoside (ONPG) assay (see the Experimental Section and Supporting Information for further details). Enzyme activity increases as the amount of enzyme used to modify the micromotors increases up to 500 units (U). Greater amounts of enzyme (1000 U) do not produce any improvement in the micromotor performance. Over 2000 units of enzyme a loss of activity is noted possibly due to union and stacking between enzymes (data not shown). As such, 500 units of enzyme were chosen as optimal to modify the micromotors (76 000 motors mL^{-1}). Subsequently, the activity of free enzyme and β-galactosidase micromotors was evaluated under different temperatures (25–60 ºC) and pH (6–10) values. As shown in **Figure 3A**, pH exerts a strong influence in enzyme activity, with optimal enzyme activity observed at a narrow pH range (7.0–8.5) for immobilized enzyme. A slight decrease in the activity is noted for free enzyme at the same pH range. **Figure 3B** shows the influence of different temperatures on enzyme activity. As can be seen, a broad operational temperature interval is noted for the micromotors, with optimal operational temperature of 37 ºC. On the contrary, the activity of

free enzyme is more affected with temperature, losing 35% activity at 60 ºC (vs 10% loss of immobilized enzyme at the same temperature). Such reduction in β-galactosidase activity can be attributed to a change in the conformation of the qua- ternary structure in β-galactosidase active site, which is hampered when the enzyme is immobilized in the micromotor. Overall, the slight increase noted in pH and temperature stability of β-galactosidase micromotors spans its applicability as moving immobilized biocatalysts under larger experimental conditions.

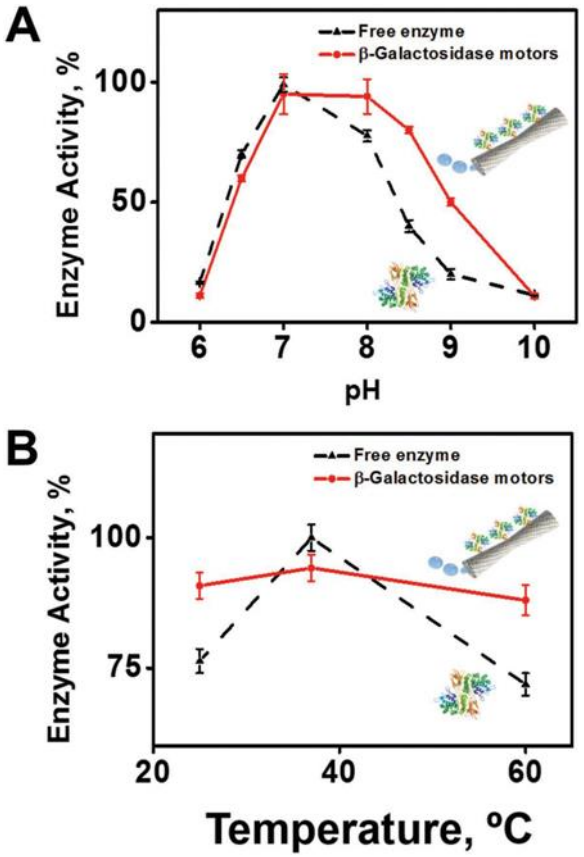

Figure 3. A) Temperature and B) pH stability of β-galactosidase immobilized micromotors and of free enzyme. Vertical bars represent standard error during experiments (n = 3). Percent enzyme activity was determined by using the following relationship: (enzyme activity at a given condition/maximum enzyme activity at 37 ºC and pH 7) x 100.

Under the optimized working conditions (37 ºC, pH 7.0), kinetic enzyme parameters were estimated by using ONPG (0.05–1 x 10^{-3} M) as substrate. **Figure S1** (Supporting Information) illustrates the Lineweaver–Burk plots for free and immobilized form of β-galactosidase. Affinity of free enzyme and β-galactosidase micromotors toward the substrate ONPG is expressed by the Michaelis–Menten constant (K_m) whereas the maximum reaction rate (V_{max}) reveals the turnover number of an enzyme, which is the number of substrate molecules converted into product by an enzyme molecule in a unit time when the enzyme is fully saturated

with substrate. Km value of β-galactosidase decreased about three times upon immobilization (5.5×10^{-3} M as compared with 16.4×10^{-3} M for free enzyme), while V_{max} also decreased (0.56 and 4.4×10^{-6} M min^{-1} for micromotors and free β-galactosidase, respectively). Despite such decrease in K_m and V_{max} after enzyme immobilization, the overall activity increase can be rationalized by the micromotor movement and enhanced fluid mixing.

The highly efficient self-propulsion capability of the functionalized micromotors, along with the corresponding bubble tail, results in a favorable hydrodynamic environment that increases the rate of the biocatalytic reaction between the modified micromotors and lactose (without external stirring), thereby offering a rapid lactose hydrolysis platform. **Figure 4** shows the use of β-galactosidase micromotors for lactose removal in skimmed milk samples. The process was monitored by determining lactose and its hydrolytic products, D-glucose and D-galactose (see the Experimental Section and Supporting Information for further details). Lactose removal was monitored on the basis of its selective reaction with methylamine in alkaline media, which generate a colored product that can be estimated by UV–vis. Similarly, glucose and galactose were determined by selective reaction with molybdate and generation of a blue product.[16] The corresponding calibration plots can be seen in **Figure S2** (Supporting Information). A lactose concentration of 46.7 ± 0.9 mg mL^{-1} was obtained for milk with a reference lactose concentration of 47 mg mL^{-1} on the packaging label. First, we optimize the amount of micromotors on the lactose removal efficiency in terms of time. As can be seen in **Figure 4A** the increase in the number of micromotors produces a decrease in time needed to remove lactose quantitatively. Thus, the navigation time decreases from 50 to 25 min as the amount of micromotors increases from 76 000 to 190,000 micromotors mL^{-1} and then remained almost constant. The latter number of motors was then selected as optimal for further experiments.

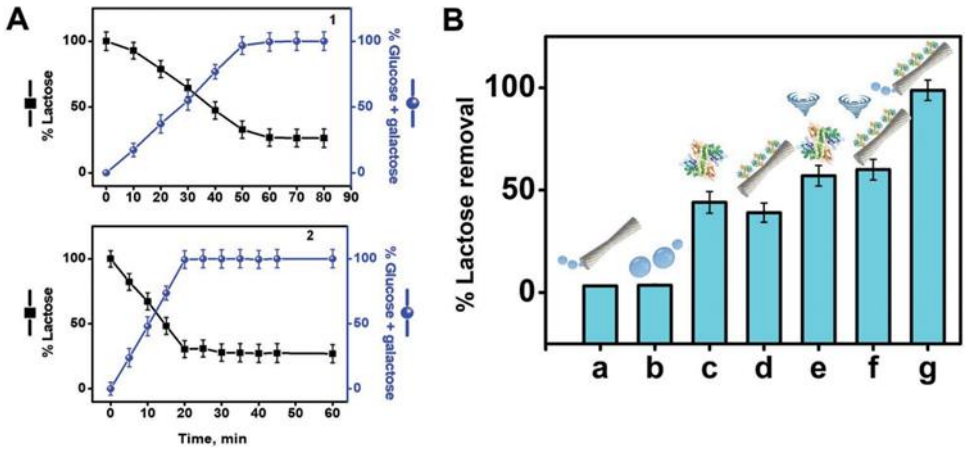

Figure 4. Lactose removal using β-galactosidase micromotors. A) Effect of time and number of β-galactosidase micromotors. (1) 76 000, (2) 190 000 micromotors mL^{-1}. B) Lactose removal efficiency under the presence of (a) swimming MW-Ni-Pt micromotors; (b) H2O2; (c) free

enzyme; (d) static MW-Ni-Pt-β-galactosidase micromotors; (e) free enzyme under mechanical stirring; (f) MW-Ni-Pt-β-galactosidase micromotors under mechanical stirring, and (g) swimming β-galactosidase-MW-Ni-Pt-micromotors. Conditions: Skimmed milk with lactose concentration, 46.7 ± 0.7 mg L^{-1}; time, 20 min; temperature, 37 ºC. Error bars represent the standard error during measurements (n = 3). Lactose removal was monitored by following the increase of galactose and glucose after treatment with the micromotors.

Lactose removal was estimated by monitoring the amount of glucose and galactose generated since the limit of the detection on the lactose assay hampers its application to detect concentrations below 20 mg mL^{-1}. As shown in **Figure 4B**, g, the micromotors result in quantitative lactose hydrolysis within 25 min treatment. In contrast, dramatically lower hydrolysis rates of 0%, 50%, and 48% were obtained using swimming MW-Ni-PtNPs, static β-galactosidase micromotors, and free β-galactosidase enzyme without stirring. In this context, as stirring fluids is a widely used strategy in industrial chemical processes, we compared the hydrolysis rate obtained with our micromotor with that using free enzyme and micromotors under stirring conditions (1000 rpm). As can be seen, dramatically lower percent removals of 60% were obtained in both cases. To get further insights into such moving phenomena and under the absence of similar studies in the literature, we compare the rate of lactose hydrolysis using free and immobilized enzyme under different stirring conditions (500 and 1000 rpm). As can be seen in **Figure 5**, both with free and immobilized enzymes, lactose removal increases with time, with almost complete hydrolysis after 40 min in both cases. For comparison, as shown in the graphics, quantitative lactose hydrolysis within 25 min treatment was achieved with swimming micromotors. This fact further reflects the high efficiency of our β-galactosidase-modified micromotors for lactose removal associated with the enhanced mixing and convection induced by its motion, enhancing mass transport and affinity toward lactose, and obviating the need for mixing or stirring industrial equipment for future real applications.

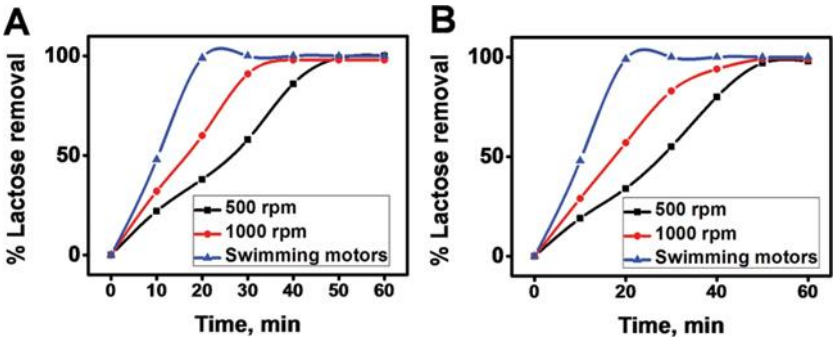

Figure 5. Effect of external magnetic stirring rate on lactose removal using A) free enzyme and

B) micromotor-immobilized enzyme. Conditions: Skimmed milk with lactose concentration, 46.7 ± 0.9 mg L^{-1}; temperature, 37 ºC.

As previously demonstrated, β-galactosidase functionalized micromotors are useful to address current demands of the world's biotechnological industries, i.e., enhancement in enzyme productivity and shelf life. Enzyme reusability is another important parameter for reducing cost while maintaining relatively high levels of enzyme activities. Our β-galactosidase micromotors are magnetic due to the intermediate nickel layer and can be easily separated from the milk sample for future reuse. The reusability of β-galactosidase micromotors was tested in repeated cycles of lactose removal-micromotors separation-washing-lactose removal (see **Figure 6A**). As can be seen in **Figure 6B**, two cycles were carried out with β-galactosidase micromotors with lactose removal of 93% during the first cycle and 82% on the second cycle. Such slight decrease in the micromotors performance during successive cycles can be attributed to a decrease in enzyme activity with time and/or to collisions between micromotors with vessel walls that produce the detachment of β-galactosidase and later withdraw during washing. However, complete lactose removal was achieved in 35 min at the second cycle, holding considerable promise for future use in the food industry.

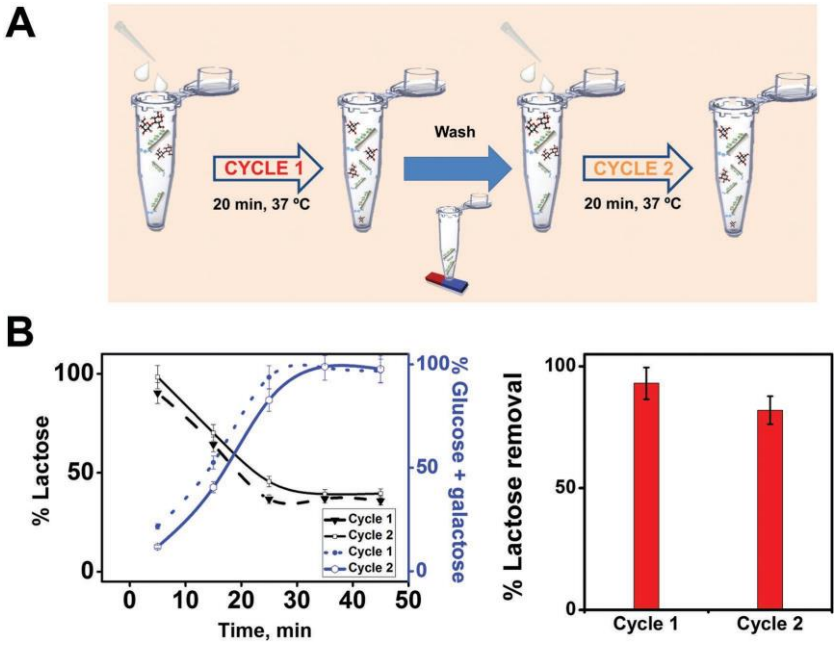

Figure 6. Reusability of β-galactosidase micromotors for lactase removal. A) Schematic of the reusability experiments. B) Graph showing the percent lactose removal and glucose - galactose generation over time during the different cycles. Plot on the right shows the total amount of lactose removed in every cycle at 20 min. Conditions: Skimmed milk with lactose concentration,

46.7 ± 0.9 mg L^{-1}; temperature, 37 ºC. Error bars represent the standard error during measurements (n = 3).

3. Conclusion and Future Directions

In conclusion, we developed here surfactant-free β-galactosidase functionalized micromotors as highly efficient moving platforms for greatly improved lactose hydrolysis. The micromotors can self-propel in milk samples at relatively low fuel concentrations without the need for addition of an external surfactant. Improved pH and temperature operation stabilities were observed after enzyme immobilization onto the micromotors as compared with the use of free enzyme. Dynamic micromotor movement and the associated fluid mixing enhanced the affinity of the enzyme toward lactose, resulting in two-fold lactose removal efficiency in 25 min as compared with static counterparts and free enzyme. The incorporation of an inner Ni layer allows for its reusability to operate in batch mode, with only an 11% loss of enzyme activity (in terms of lactose hydrolysis) in two successive cycles. In addition, our system can be easily combined with similar catalase-driven micromotors to deplete peroxide levels down to allowable levels. We have also demonstrated here, for the first time, that self-propelled micromotors are more effective than traditional means of stirring fluids, which are widely employed in industrial chemistry processes. To address future biocompatibility concerns, our group is working on replacing the inner Ni magnetic layer by incorporation of biocompatible Fe_2O_3 magnetic nanoparticles on the outer carbon nanomaterial layer. While peroxide-driven motors are used here as proof-of-concept for the intended application, our strategy can be easily implemented using magnetic, ultrasound-propelled or water-driven (magnesium) micromotors. In addition, hydrogen peroxide has been previously used for milk preservation, and some cases are desirable to activate the lactoperoxidase system against harmful bacteria. Yet, more efforts and extensive research are needed to translate the achievement and concept demonstrated in this study for practical lactose removal applications. As such, future efforts should be aimed at scaling up the protocol to treat higher volumes of milk and to investigate a more friendly propulsion mechanism such as magnetic propulsion. This is the first time that micromotors are used for a food-related agroalimentary process, opening thus new horizons in the field. The new protocol holds considerable promise for more efficient industrial processes in biotechnological industries.

4. Experimental Section

Reagents and Equipment: Multiwalled carbon nanotubes (Cat. No. 659258), chloroplatinic acid hydrate (Cat. No. 254029), nickel (II) sulfamate (Cat. No. 262277), nickel (II) chloride (Cat. No. N6136), boric acid (Cat. No. 15665), β-galactosidase from Kluyveromyces lactis (3180 U g^{-1}, Cat. No. G3665), lactose (Cat. No. L3750), 2-nitrophenyl β-D-galactopyranoside (ONPG, Cat. No. N1127), NHS (Cat. No. 56485), EDC (Cat. No. 03449), 4-morpholineethanesulfonic acid (MES, Cat. No. 69892), methylamine hydrochloride (Cat. No M0505), potassium hydrogen phthalate (Cat. No P1088), ammonium molybdate tetrahydrate (Cat. No 09878), sodium sulfite (Cat No. 71988), zinc acetate dihydrate (Cat. No. 96459), and phosphotungstic acid hydrate (Cat. No. P4006) were purchased from Sigma-Aldrich (Madrid, Spain). Glycine (Cat. No 141340) was supplied by Panreac (Madrid, Spain). Skimmed milk and whole milk were purchased from a local supermarket and used without any treatment in lactose removal assays.

Template electrochemical deposition of the micromotors was carried out using an Autolab PGSTAT 12 (Eco Chemie, Utrecht, the Netherlands). SEM images were obtained with a NovaNano FE-SEM 230 FEI instrument using an acceleration voltage of 10 kV. An inverted optical microscope (Nikon Eclipse Instrument Inc. Ti-S/L100), coupled with 10x and 20x objectives, and a Hamamatsu digital camera C11440 and NIS Elements AR 3.2 software, was used for capturing movies at a rate of 25 frames per second. Aqueous or milk 1% hydrogen peroxide solutions were used as chemical fuel. UV-visible experiments were carried out using a Perkin-Elmer Lambda 20 spectrophotometer at 405, 540, or 710 nm for ONPG, lactose, and galactose–glucose experiments, respectively.

Electrochemical Synthesis of Multiwall Carbon Nanotube Micromotors: Multiwall carbon nanotube micromotors (MW) were prepared by electrochemical reduction of the nanomaterial into the 5 μm diameter conical pores of a polycarbonate membrane (Catalog No. 7060-2513; Whatman, New Jersey, USA) following our previous work.[12e] The simultaneous electrochemical reduction and deposition of the MW were carried out using cyclic voltammetry (over -0.3 to +1.5 V vs Ag/AgCl, 3 m, at 50 mV s^{-1}, for ten cycles) from a platting solution containing 0.1 mg mL^{-1} of the nanotubes and 0.5 m of Na_2SO_4 in 0.1 m H_2SO_4. Subsequently, two metal tube layers were plated inside the reduced carbon layer. First, a nickel layer was galvanostatically deposited applying ten pulses (0.1 s) at -20 mA and followed by 300 s at -6 mA from a nickel platting solution (solution was prepared by dissolving 30 g of nickel (II) sulfamate, 1.06 g of nickel (II) chloride, and 3 g of boric acid in 100 mL of ultrapure water and adjusted at pH 4). Second, an inner PtNP layer was deposited by amperometry at +0.4 V for 750 s from an aqueous solution containing 4 x 10^{-3} M of H_2PtCl_6 in 0.5 M boric acid. The

sputtered gold layer was gently polished and the micromotors were released from the membrane by sequential treatment with methylene chloride (30 min, two times), isopropanol, ethanol, and ultrapure water (18.2 MΩ cm), with 3 min centrifugation following each wash. The template preparation method resulted in reproducible micromotors.

β-Galactosidase Immobilization: The micromotors were transferred to an eppendorf vial containing 100 µL of MES buffer (0.1 m, pH 5) and the coupling agents, EDC (20 mg) and NHS (20 mg) for 30 min. Then, the micromotors were rinsed twice with MES buffer. β-Galactosidase enzyme (10–3000 units) was incubated overnight at 4 ºC in MES buffer pH 5. Finally, the micromotors were washed three times with phosphate buffer (0.1 m, pH 7) to remove the excess of β-galactosidase and then suspended in phosphate buffer and stored at 4 ºC. This suspension can be stored for 4 d without any change in the enzymatic activity.

UV–Visible Experiments: ONPG was used as substrate for β-galactosidase rate activity experiments following the protocol described by Ansari et al.[7a] One unit (1.0 U) of β-galactosidase activity is defined as the amount of enzyme that releases 1.0 mol of O-nitrophenol (ε_m = 4500 L mol^{-1} cm^{-1}) per min under standard assay conditions (37 ºC, 15 min). Functionalized micromotors (76 000 motors mL^{-1}) were transferred to a 2 mL eppendorf vial containing 1.8 mL of phosphate buffer (0.1 m, pH 7, 1.5% NaCh, 1% H_2O_2) and 200 µL of a 2 x 10^{-3} M ONPG solution. The reaction was incubated at 37 ºC for 15 min. The same procedure was employed for two units (2 U) of free enzyme. Finally, the colorimetric intensity from O-nitrophenol was measured at 405 nm.

For lactose determination, 2.50 mL of blank, standard or milk test solution was mixed with 2.50 mL of glycine-NaOH buffer (0.03 m glycine, 0.33 m NaOH, and 0.03 m NaCl, pH 12.8), 0.250 mL of methylamine solution (5% methylamine-HCl in water), and 0.250 mL of sodium sulfite solution (1% Na2SO3 in water). The mixture was then heated in a water bath at 65 ºC for 25 min and cooled immediately in an ice-water bath for 2 min to stop the reaction. Color intensity was measured at 540 nm. Galactose–glucose detection was carried out by mixing 2.50 mL of phosphate–phthalate buffer (0.60 g KH_2PO_4 and 1.02 g K_2HPO_4 in 50 mL, pH 5.3) with 0.500 mL of blank, standard, or milk test solution. Next, 2.50 mL of ammonium molybdate solution (6% $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ in water) was added and the mixture was heated in a boiling water bath for 45 min. Next, the reaction was stopped in a cold-water bath for 5 min and color intensity was measured at 710 nm.[16]

Milk Sample Treatment for Sugar Determination: 1 mL of milk was mixed with 300 µL of a zinc acetate-phosphotungstic acid solution (25.0 g zinc acetate, 12.5 g phosphotungtic acid, and 20 mL of glacial acetic acid in 100 mL of water). After stirring for 10 min, the mixture was

filtered (with Whatman No. 7060-2512) and 0.5 mL of the filtrate was mixed with 5 mL of NaOH 0.1 M. Next, the solution was diluted to 10 mL with DI water and allowed to precipitate (15 min). Finally, 5 mL of the supernatant was diluted to 10 mL and used as sample.[16]

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

R.M.-H. acknowledges the FPI fellowship received from the Spanish Ministry of Economy and Competitiveness (BES-2015-072346). B.J.-S. acknowledges support from the Spanish Ministry of Economy and Competitiveness (Ramon y Cajal contract, RYC-2015-17558, co-financed by EU). A.E. acknowledges financial support from the Spanish Ministry of Economy and Competitiveness (CTQ2014-58643-R) and the NANOAVANSENS program (S2013/MIT-3029) from the Community of Madrid.

Received: ((will be filled in by the editorial staff))
Revised: ((will be filled in by the editorial staff))
Published online: ((will be filled in by the editorial staff))

References

[1] a) P. C. Pereira, Nutrition 2014, 30, 619; b) R. R. Mahoney, C. Adamchuk, J. Food Sci. Technol. 1980, 45, 962.

[2] R. Mattar, D. F. de Campos Mazo, F. J. Carrilho, Clin. Exp. Gastroenterol. 2012, 5, 113.

[3] H. Weetall, S. Yaverbaum, Corning Glass Works, 1974, US Patent 3852496 A.

[4] J. H. Hotchkiss, J. N. Tolbert, Cornell University, 2013, US Patent 8354259 B2.

[5] a) A. Axelsson, G. Zacchi, Appl. Biochem. Biotechnol. 1990, 24/25, 679; b) A. Dwevedi, A. M. Kayastha, J. Agric. Food Chem. 2009, 57, 682.

[6] a) B. Kamp, P. G. Rouxhet, Appl. Microbiol. Biotechnol. 1988, 27, 464; b) W. Chen, H. Chen, Y. Xia, J. Yang, J. Zhao, F. Tian, H. P. Zhang, H. Zhang, J. Dairy Sci. 2009, 92, 491; c) P. S. Panesar, S. Kumari, R. Panesar, Enzyme Res. 2010, 2010, 1; d) S. A. Ansari, Q. Husain, J. Mol. Catal. B: Enzym. 2010, 63, 68.

[7] a) S. A. Ansari, R. Satar, S. Chibber, M. J. Khan, J. Mol. Catal. B: Enzym. 2013, 97, 258; b) I. Shitanda, M. Itagaki, K. Asano, Chem. Lett. 2012, 41, 533; c) A. S. Campbell,

C. Deng, F. Meng, J. Hardinger, G. Perhinschi, N. Wu, C. Z. Dinu, ACS Appl. Mater. Interfaces 2014, 6, 5393.

[8] a) W. F. Paxton, K. C. Kistler, C. C. Olmeda, A. Sen, S. K. St. Angelo, Y. Cao, T. E. Mallouk, P. E. Lammert, V. H. Crespi, J. Am. Chem. Soc. 2004, 126, 13424; b) G. A. Ozin, I. Manners, S. Fournier-Bidoz, A. Arsenault, Adv. Mater. 2005, 17, 3011; c) Y. Mei, G. Huang, A. A. Solovev, E. B. Ureña, I. Mönch, F. Ding, T. Reindl, R. K. Y. Fu, P. K. Chu, O. G. Schmidt, Adv. Mater. 2008, 20, 4085; d) A. A. Solovev, Y. Mei, E. B. Ureña, G. Huang, O. G. Schmidt, Small 2009, 5, 1688; e) Y. Mei, A. Solovev, S. Sanchez, O. G. Schmidt, Chem. Soc. Rev. 2011, 40, 2109; f) J. Wang, Nanomachines: Fundamentals and Applications, Wiley-VCH, Germany 2013; g) J. Orozco, V. García- Gradilla, M. D'Agostino, W. Gao, A. Cortés, J. Wang. ACS Nano 2013, 7, 818; h) M. Uygun, V. V. Singh, K. Kaufmann, D. A. Uygun, S. D. S. de Oliveira, J. Wang, Angew. Chem., Int. Ed. 2015, 54, 12900; i) K. K. Dey, X. Zhao, B. M. Tansi, W. J. Mendez-Ortiz, U. M. Cordova-Figueroa, R. Golestanian, A. Sen, Nano Lett. 2015, 15, 8311; j) X. Ma, X. Wang, K. Hahn, S. Sanchez, ACS Nano 2016, 10, 3597; k) J. Katuri, X. Ma, M. M. Stanton, S. Sanchez. Acc. Chem. Res. 2017, 50, 2.

[9] a) J. Orozco, B. Jurado-Sánchez, G. Wagner, W. Gao, R. Vazquez-Duhalt, S. Sattayasamitsathit, M. Galarnyk, A. Cortés, D. Saintillan, J. Wang, Langmuir 2014, 30, 5082; b) D. Rojas, B. Jurado-Sanchez, A. Escarpa, Anal. Chem. 2016, 88, 4153.

[10] a) S. Sanchez, A. A. Solovev, Y. Mei, O. G. Schimdt, J. Am. Chem. Soc. 2010, 132, 13144; b) K. K. Dey, X. Zhao, B. M. Tansi, W. J. Mendez-Ortiz, U. M. Córdoba-Figueroa, R. Golestanian, A. Sen, Nano Lett. 2015, 15, 8311; c) X. Ma, A. C. Hortelao, T. Patiño, S. Sanchez, ACS Nano 2016, 10, 9111; d) L. K. E. A. Abdelmohsen, M. Nijemeisland, G. M. Pawar, G. J. A. Janssen, R. J. M. Nolte, J. C. M. van Hest, D. A. Wilson, ACS Nano 2016, 10, 2652.

[11] M. Kiristi, V. V. Singh, B. E. F. de Avila, M. Uygun, F. Soto, D. A. Uygun, J. Wang, ACS Nano 2015, 9, 9252.

[12] a) B. Jurado-Sánchez, S. Sattayasamitsathit, W. Gao, L. Santos, Y. Fedorak, V. V. Singh, J. Orozco, M. Galarnyk, J. Wang, Small 2015, 11, 499; b) V. V. Singh, A. Martin, K. Kaufmann, S. D. S. de Oliveira, J. Wang, Chem. Mater. 2015, 27, 8162; c) D. Vilela, J. Parmar, Y. Zeng, Y. Zhao, S. Sánchez, Nano Lett. 2016, 16, 2860; d) B. E. F. de Ávila, M. A. Lopez-Ramirez, D. F. Báez, A. Jodra, V. V. Singh, K. Kaufmann, J. Wang, ACS Sens. 2016, 1, 217; e) R. Maria-Hormigos, B. Jurado-Sanchez, L. Vazquez, A. Escarpa, Chem. Mater. 2016, 28, 8962; f) R. Maria-Hormigos, B. Jurado-Sanchez, A. Escarpa, Nanoscale 2017, 9, 6286.

[13] S. K. Srivastava, O. G. Schmidt, Chem. Eur. J. 2016, 22, 9072.

[14] FAO/WHO, Benefits and Potential Risks of the Lactoperoxidase System of Raw Milk Preservation, http://www.fao.org/docrep/009/ a0729e/a0729e00.htm (accessed: October 2017).

[15] N. H. Martin, A. Friedlander, A. Mok, D. Kent, M. Wiedmann, K. J. Boor, J. Food Prot. 2014, 77, 1809. T. A. Nickerson, I. F. Vujicic, A. Y. Lin, J. Dairy Sci. 1976, 59, 386.