SUPPORTING INFORMATION

Alteration of Enzyme Activity and Enantioselectivity By Biomimetic Encapsulation into Silica Particles. Stéphane Emond, David Guieysse, Severine Lechevallier, Jeannette Dexpert-Ghys, Pierre Monsan and Magali Remaud-Siméon

Experimental details

Table of contents

1- General

Bactotryptone and yeast nitrogen bases (without amino acids or ammonium sulphate [YNBww]) were purchased from Difco (Paris, France). Kanamycin and ampicillin were from Euromedex (Souffelweyersheim, France). Other chemicals were purchased from Sigma-Aldrich (USA). Recombinant Esterase I from *Pseudomonas fluorescens* was from Sigma-Aldrich. Lipase B from *Candida Antarctica* was from Codexis (formerly Biocatalytics). The R5 synthetic peptide was a kind gift from Dr. M.J. Broderick (University of Leeds, UK). Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs (Ipswich, MA, USA) and used according to the manufacturer's instructions. DNA purification was performed using QIAQuick or QIASpin purification kits from Qiagen (Basingstoke, United Kingdom). DNA sequencing was performed by Cogenics (Grenoble, France). The artificial genes were synthesized by GeneArt AG (Regensburg, Germany).

2- Strains and culture conditions

E. coli strain TOP10 (Invitrogen) was used for transformation and for amplification of recombinant plasmid DNA. *E. coli* strain BLR(DE3) (CalBioChem) was used for expression of recombinant proteins. *E. coli* cells were grown in LB medium (yeast extract 5 $g.L^{-1}$, tryptone 10 $g.L^{-1}$ and NaCl 10 $g.L^{-1}$) supplemented with 100 $\mu g.mL^{-1}$ ampicillin or 50 $\mu g.mL^{-1}$ kanamycin as appropriate. *Y. lipolytica* JMY1212 strain[1] was used for the transformation of expression cassette containing the *Preprolip2-CalB-R5* fusion gene. The yeasts were grown on YPD (yeast extract 10 $g.L^{-1}$, peptone 10 $g.L^{-1}$ and glucose 10 $g.L^{-1}$) or YTD (yeast extract 10 $g.L^{-1}$, tryptone 20 $g.L^{-1}$, glucose 50 $g.L^{-1}$, 50 mM phosphate buffer pH 6.8). Selection of Ura+ yeast transformants was performed on YNBcasaD plates (YNB with 2 g of casamino acids/L and glucose at 10 g/L).

3- Cloning in *E. coli* of PFE-I C-terminally fused to one to five R5 peptides

A synthetic gene encoding five repeats of the R5 peptide ($R5_5$, **Figure 1**) was cloned into the pMAL-c2x expression vector (New England Biolabs, USA) at the *Sac*I and *Xba*I restriction sites downstream from the *malE* gene, yielding plasmid pMAL-$R5_5$. A synthetic gene encoding N-terminally 6×His tagged wild-type PFE-I was then cloned in this plasmid at the *Nde*I and *Xma*I restriction sites, replacing *malE.* The resulting pPFE-$R5_5$ plasmid was used to construct pPFE-$R5_3$ and pPFE-R5 after digestion by *Eco*RI or *Bam*HI respectively, intramolecular ligation and transformation into *E. coli*. The three constructs were then assayed for expression. Protein expression was induced for 4 hours at 37°C in 20 mL LB-flask cultures with 1 mM IPTG (when OD600 = 0.6). Cells were harvested by centrifugation and re-suspended in 2 mL TALON buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl). After sonication, esterase activity was measured in cell lysates using *para*-nitrophenyl butyrate (*p*-NPB) as a substrate.

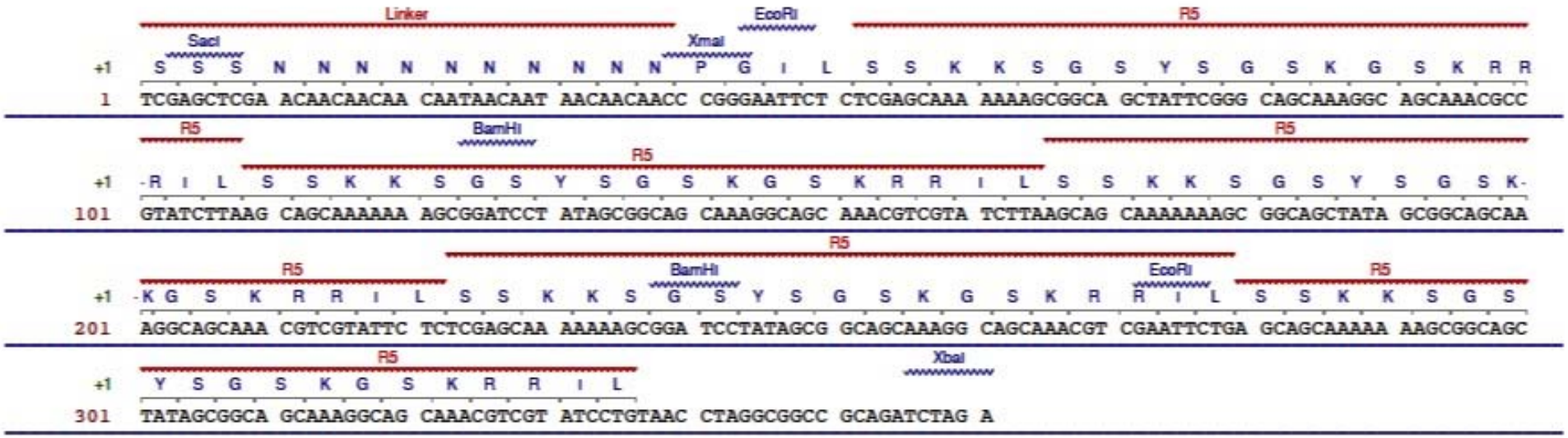

Figure 1. Sequence of the $R5_5$ synthetic gene.

4- Cloning of *Candida antarctica* lipase B fused to R5 in *Y. lipolytica*

A *Bam*HI-*Spe*I linker encoding the R5 peptide sequence (**Figure 2**) was cloned into plasmid JMP62-URA3Ex-TEF (containing the constitutive P-*TEF* promoter and the excisable *URA3*ex selection marker) at the *Bam*HI-*Avr*II sites to generate JMP62-URA3Ex-TEF-R5, which was transformed into *E. coli*. This plasmid was then used for the cloning of the *Bam*HI-*Avr*II fragment (containing the Preprolip2-CalB fusion gene) from JMP62-URA3Ex-CalB-6×His at the *Bam*HI-*Avr*II sites.[2] The resulting plasmid JMP62-TEF-URA3Ex-CalB-R5 was digested with *Not*I to excise the expression cassette flanked by zeta regions and composed of *URA3* marker, P-*TEF* promoter and *Preprolip2-CalB-R5* gene. This cassette was transformed into *Y. lipolytica* JMY1212 strain using the lithium acetate method.

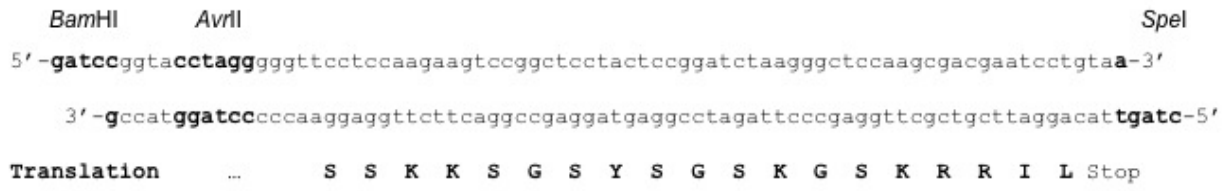

Figure 2. Sequence of the *Bam*HI / *Spe*I linker encoding the R5 peptide and cloned into JMP62-URA3Ex-TEF.

5- Production and purification of recombinant enzymes

Production of PFE-I-R5. For the production of PFE-I-R5, precultures of *E. coli* BLR(DE3) harbouring pPFE-R5 grown for 18 h in LB medium were used to inoculate 1 L LB-flask cultures at OD600 = 0.05. Protein expression was induced with 1 mM IPTG (OD600 = 0.6). After 6 hours of growth at 37°C, cells were harvested by centrifugation and re-suspended in 30 mL TALON buffer. After sonication, the extract was centrifuged (48,000g, 30 min, 4°C) and the supernatant was harvested for subsequent purification. The 6×His-tagged proteins were purified by affinity chromatography using 20 mL of TALON^{TM} Superflow resin (Clontech) on an FPLC system (ÄKTA^{TM} purifier, GE Heathcare). The proteins bound to the support were eluted by a step-gradient of imidazole with a major peak corresponding to PFE-I-R5 eluting at 50 mM imidazole (**Figure 3**).

Production of CalB-R5. For the production of CalB-R5, *Y. lipolytica* transformants expressing CalB-R5 were pre-cultured overnight at 28°C in YPD medium. For protein production and secretion, this pre-culture was used to inoculate 1 L of YTD medium in a 5 L-culture flask. After 55 h of growth, the supernatant was separated from the cells by centrifugation and 5-fold concentrated on a 10 kDa ultrafiltration membrane (Pall Filtron) and the medium buffer was exchanged to Tris-HCl 20 mM pH 7.8, ammonium sulfate 1 M. The recombinant protein was then purified by hydrophobic interaction chromatography using 20 mL of butyl sepharose resin on an FPLC system. CalB-R5 was eluted with a linear gradient of ammonium sulfate, 1–0 M. The purified protein was extensively dialyzed against Tris-HCl 50 mM pH 7.0 and concentrated to 3.8 $g.L^{-1}$ by ultrafiltration (10 kDa membrane, Centricon Millipore). For esterification assay with the free enzymes, proteins were lyophilized and stored at 4°C in the presence of molecular sieves (**Figure 4**).

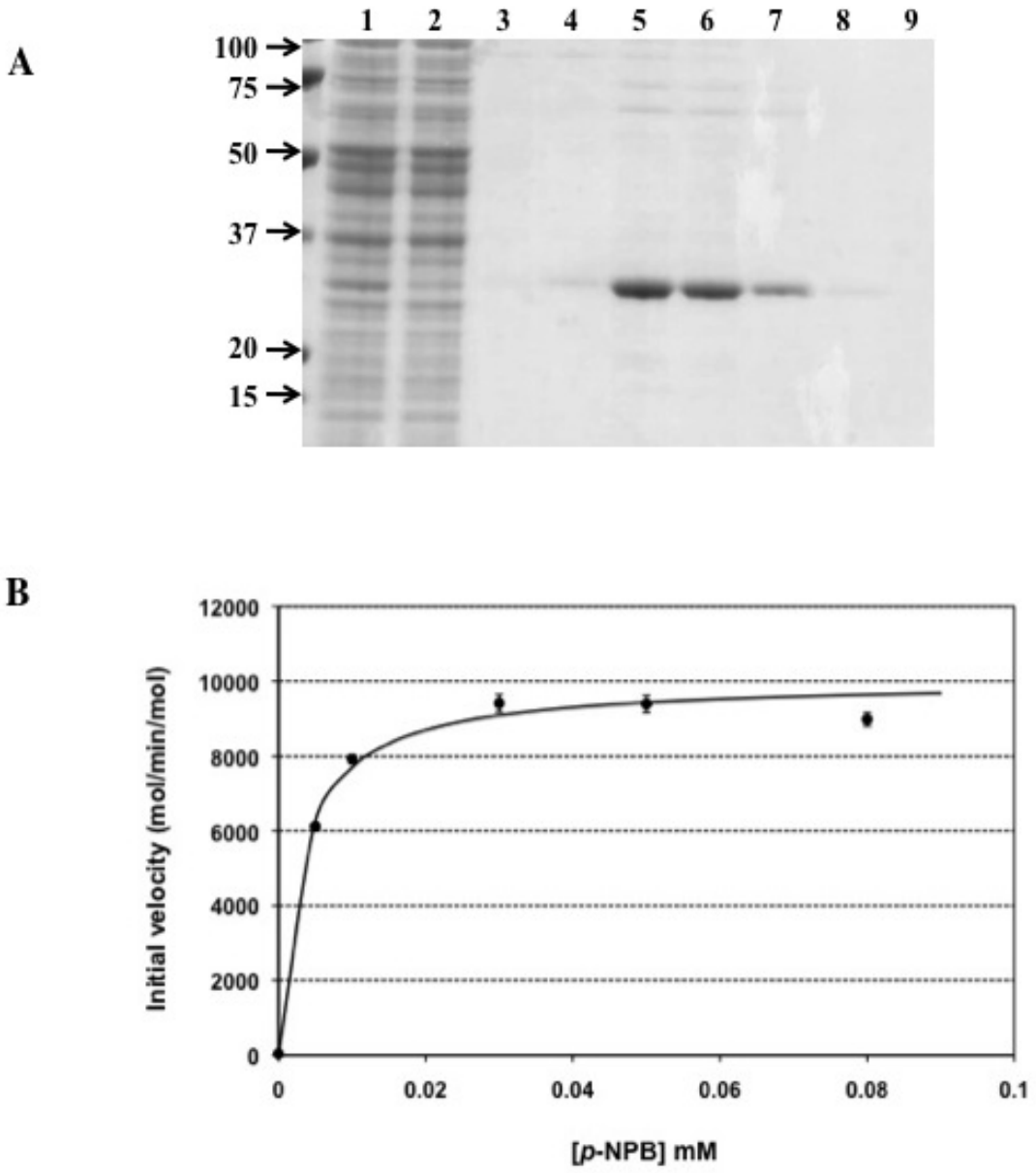

Figure 3. Production, purification and activity of PFE-I-R5. (A) SDS-PAGE. Lane 1: Total soluble protein extract; lane 2: flow-through; lane 3: wash; lane 4: Elution with 10 mM Imidazole; lane 5-6-7: Elution with 50 mM Imidazole; ; lane 8-9: Elution with 500 mM Imidazole. (B) Hydrolytic activity of PFE-I-R5 toward *p*-NPB. The assays (n=5) were performed using 0.01 µM enzyme in 100 mM Tris-HCl pH 7.5, 5% 2-Methyl-2-butanol.

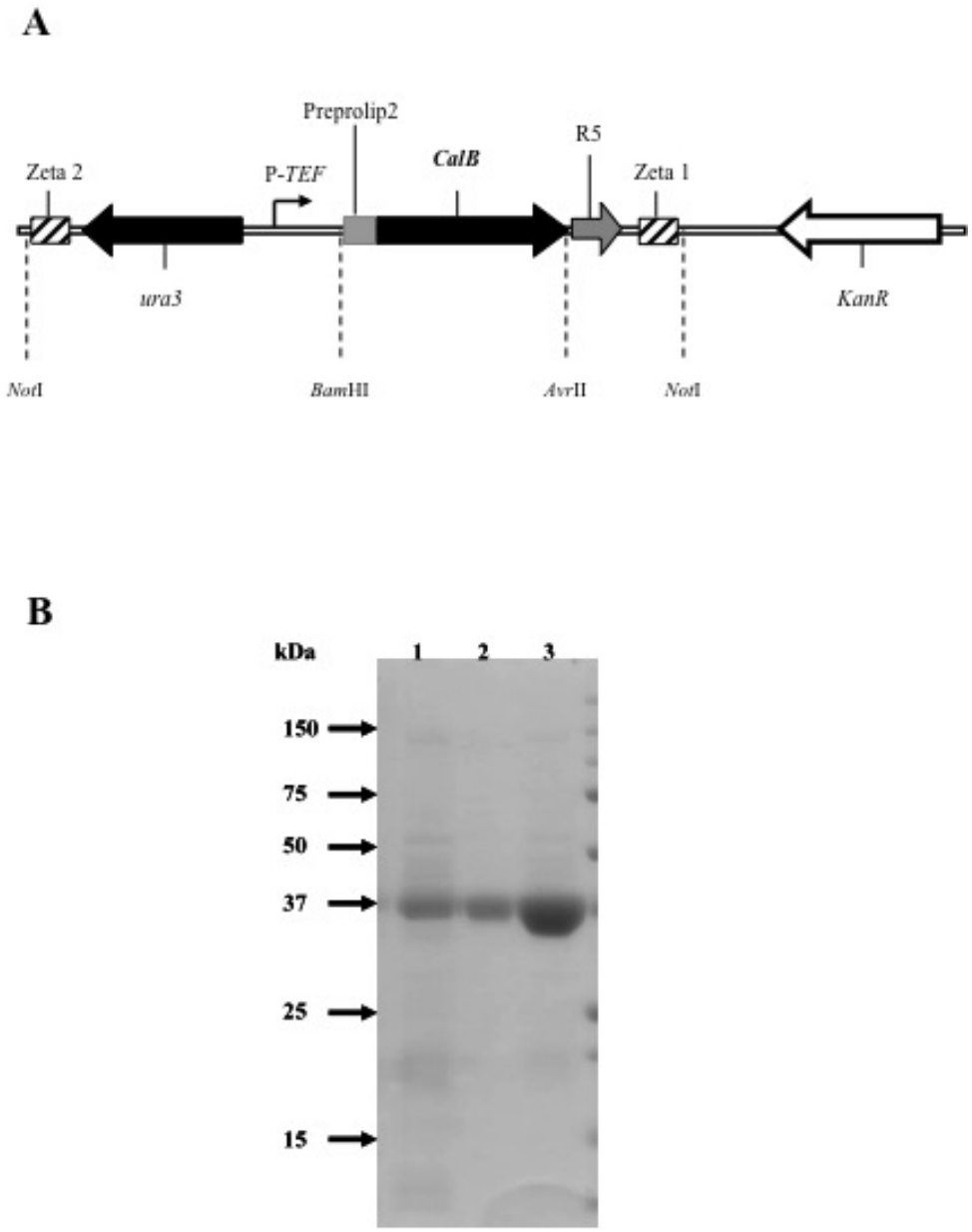

Figure 4. Production and purification and CalB-R5. (A) Schematic linear view of the JMP62-Ura3Ex-TEF-CalB-R5 plasmid. The cassette for expression of CalB-R5 in *Y. lipolytica* is released from the plasmid by *Not*I digestion and is flanked by zeta regions allowing single-copy insertion into the genome of *Y. lipolytica* JMY1212. (B) SDS-PAGE analysis of CalB-R5 secretion and purification by hydrophobic chromatography. Lane 1: *Y. lipolytica* culture supernatant; lane 2: purified CalB-R5 as eluted after hydrophobic interaction chromatography; lane 3: purified CalB-R5 after ultrafiltration.

6- Production and purification of MBP-R5$_n$ fusions

Pre-cultures grown for 24 h in LB medium were used to inoculate 1 L LB in a 5 L-culture flask. Protein expression was induced with 1 mM IPTG (OD600 = 0.6). After 6 hours of growth at 37°C, cells were harvested by centrifugation and re-suspended in 30 mL binding buffer (20 mM Tris HCl pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM DTT). After sonication, the extract was centrifuged (48,000 g, 30 min, 4°C) and the supernatant was harvested for subsequent purification. The protein fusions were purified by affinity chromatography using 20 mL of Dextrin Sepharose High Performance (GE Healthcare) packed in a XK16/20 column connected to an ÄKTA^{TM} purifier device (GE Heathcare). Following binding and extensive washing, the protein of interest was eluted using 10 mM maltose in the same buffer and analyzed by SDS-PAGE visualised with colloidal blue staining. Protein content was determined using the Bradford assay (Biorad). Prior to silica deposition, the proteins were concentrated to 3.8 g.L^{-1} by ultrafiltration using Amicon Ultra-15 centrifugal devices (Millipore) (**Figure 5**).

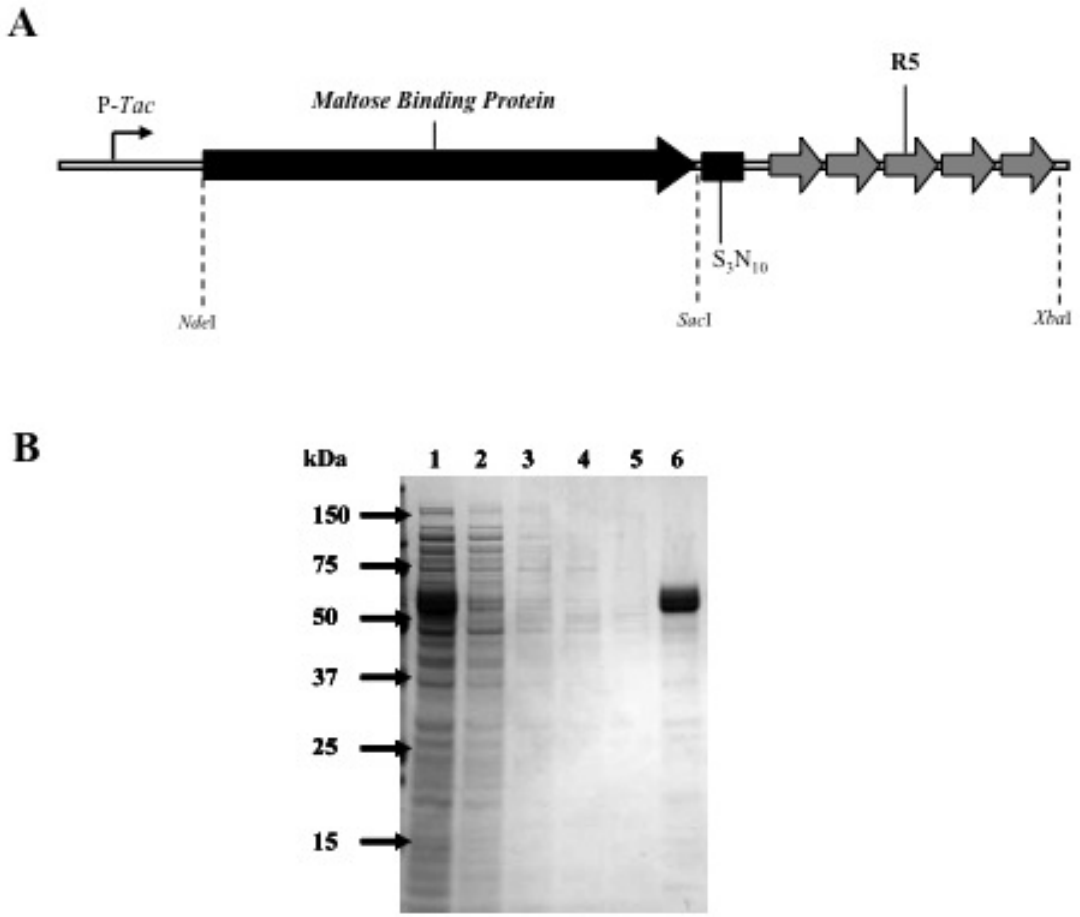

Figure 5. Production of MBP-R5$_5$ in *E. coli* and purification. (A) Schematic reprentation of MBP-R5$_5$ expression cassette. (B) SDS-PAGE analysis of MBP-R5$_5$ production and affinity purification. Lane 1: Total soluble protein extract ; lane 2: flow-through; lanes 3-4-5: washes; lane 6: Elution with 10 mM maltose.

7- Fusion enzyme encapsulation procedures

The proteins fused with R5 were mixed with gentle agitation at 4°C to a final concentration of 3 $g.L^{-1}$ with (1) the precipitation buffer (final concentration: 0.1 M KH_2PO_4 pH 7.0, 0.1 M NaOH) and (2) 0.1 M TMOS (1 M stock solution, hydrolyzed in 1 mM HCl). The mixture ratio was 8:1:1 (Protein:Buffer:TMOS). Silica precipitates were collected by centrifugation. Supernatants were analysed for protein content using the Bradford assay and for enzyme activity against *p*-NPB. Silica precipitates were washed twice with milli-Q water, dried by lyophilisation and stored at 4°C in the presence of molecular sieves.

8- Encapsulation procedures using PEI and R5

Proteins were mixed to a final concentration of 3 $g.L^{-1}$ with (1) the precipitation buffer (final concentration: 0.1 M KH_2PO_4 pH 7.0, 0.1 M NaOH), (2) 100 mM tetramethylorthosilicate (TMOS) (1 M stock solution, previously hydrolyzed in 1 mM HCl), and (3) 5 mM R5 peptide or PEI polymer (2 kDa). The mixture ratio was 7:1:1:1 (Protein:Buffer:TMOS:catalyst). The resultant silica particles were collected by centrifugation and then washed twice with deionized water. Supernatants were analysed for protein content using the Bradford assay.

9- Scanning electron microscopy, EDX and FTIR spectroscopies

Silica morphology was analysed by Scanning Electron Microscopy (SEM) using a JEOL 6700F microscope. Samples were prepared by depositing the dry powder on an adhesive carbon film. SEM-assisted energy dispersive X-ray spectroscopy (EDXS) was also performed on each sample. Chemical composition was further assayed by FTIR spectroscopy using a Perkin Elmer Spectrum 100 Series. Samples were prepared by mixing the powder with potassium bromide (1/100 by weight) in a pellet and spectra were recorded in the range of 4000-400 cm^{-1}.

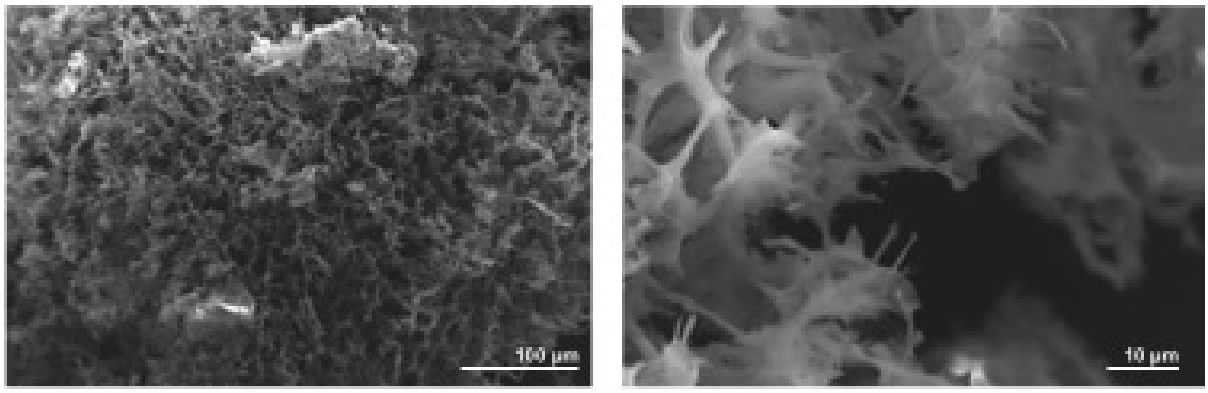

Figure 6. Scanning electron microscopy images of silica particles obtained using MBP-$R5_5$.

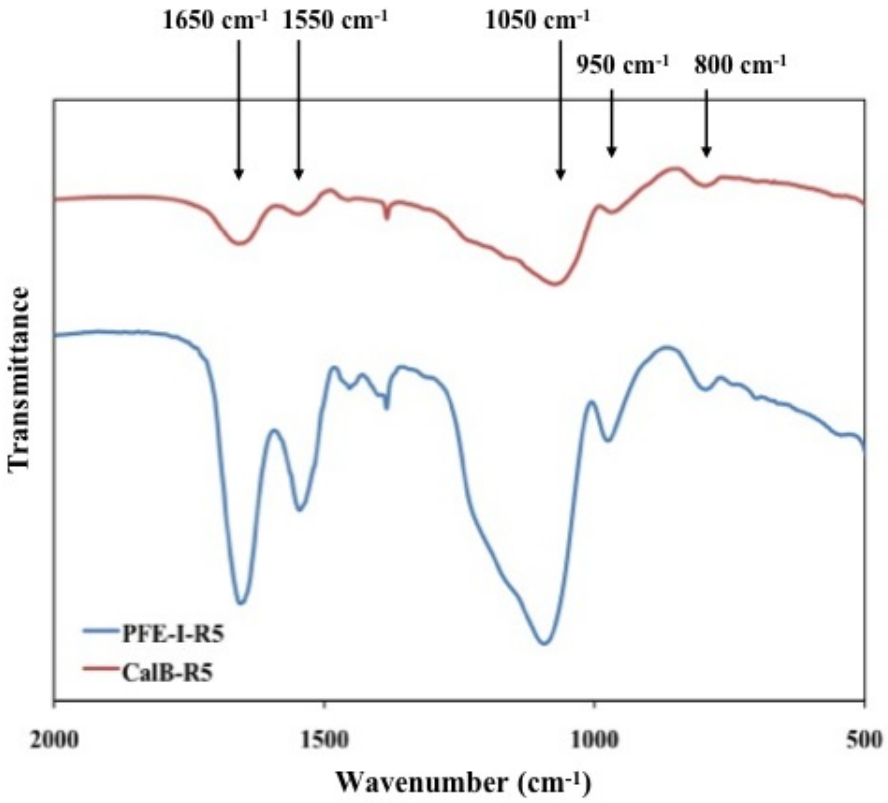

Figure 8. FTIR spectroscopy analysis of silica particles.

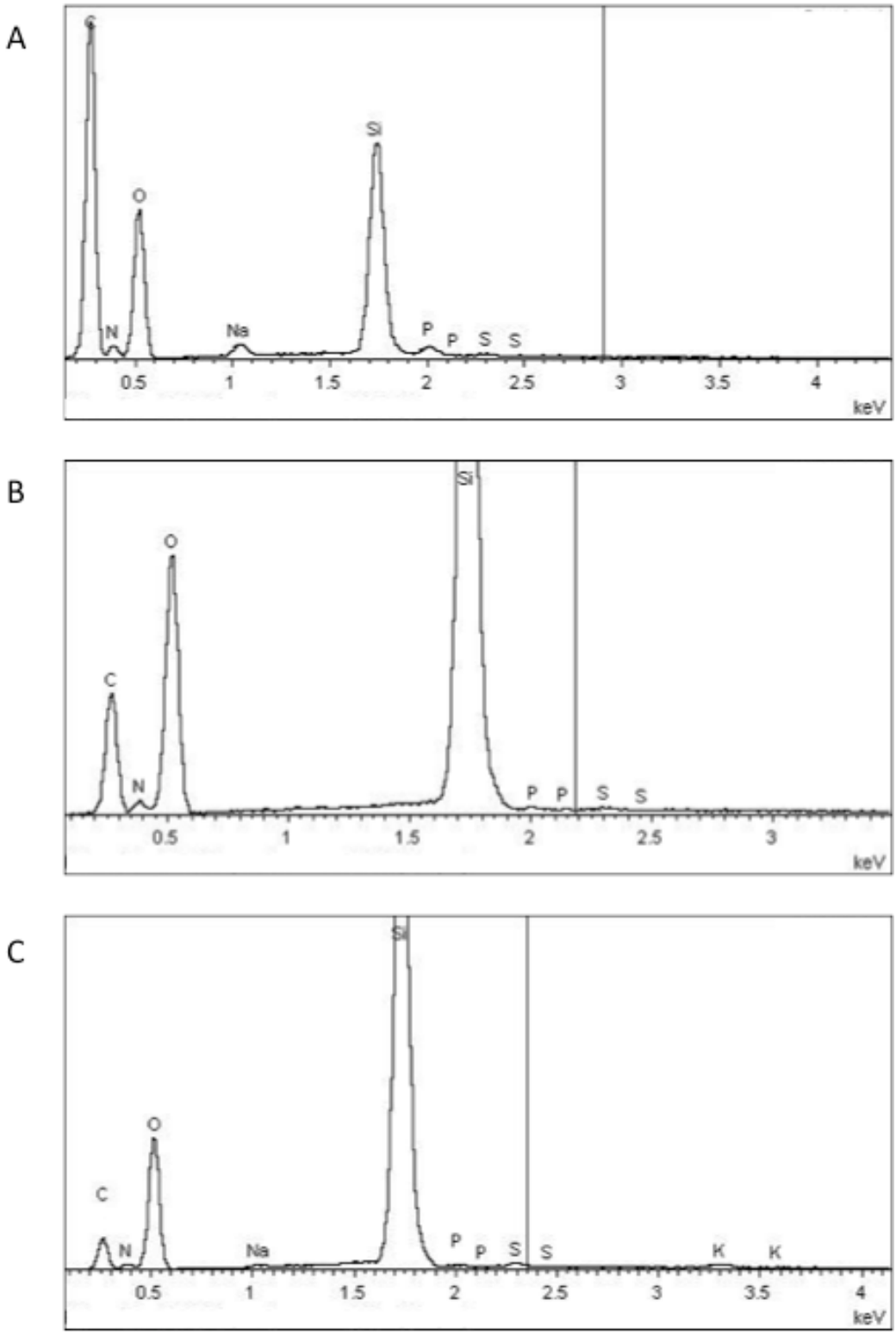

Figure 9. SEM-assisted energy dispersive X-ray spectroscopy on silica particles obtained using MBP-R5$_5$ (A), CalB-R5 (B) and PFE-I-R5 (C).

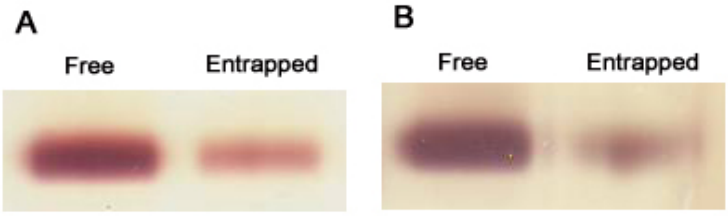

Figure 10. Silver-stained SDS-PAGE gel showing protein entrapment within silica matrix obtained using CalB-R5 (A) and PFE-R5 (B). Free refers to protein before encapsulation. Entrapped refers to protein collected after silica dissolution with 1 M NaOH at 37 °C.

Enzyme assays with *para*-nitrophenyl butyrate

When using free enzymes, reactions were performed in a 96-well microplate for 300 s at 25°C by mixing a 20 µl aliquot of enzyme extract with 175 µl of 100 mM Tris-HCl pH 7.5 buffer and 5 µl of *p*-NPB (40 mM in 2-methyl-2-butanol). For encapsulated enzymes, reactions (4 mL final volume) were performed in 100 mM Tris-HCl pH 7.5 with 1 mM *p*-NPB. Samples (200 µl) were withdrawn at regular time intervals. The absorbance at 405 nm was measured using a VersaMax tunable microplate reader (Molecular Devices Sunnyvale, CA) to monitor the release of *para*-nitrophenol. One unit of enzyme activity is defined as the amount of enzyme releasing 1 µmol of product per min at 25°C and pH 7.5.

11- Esterification of (R,S)-3-phenylbutanoic acid by ethanol

Reactions were performed by mixing the free enzymes or the enzymes immobilized in silica (*ca.* 15 mg) with (R,S)-3-phenylbutanoic acid (50 mM) and ethanol (150 mM) in n-octane (5 mL final volume) at room temperature and under agitation. The enantiomeric ratio (E) and the conversion rate were calculated using the endpoint method[3] from the enantiomeric excesses of both substrates and products determined by HPLC analysis using a chiralpak OJ column with UV detection at 254 nm.

12- References for Supplementary Information

1. F. Bordes, F. Fudalej, V. Dossat, J. M. Nicaud and A. Marty, *Journal of microbiological methods*, 2007, **70**, 493-502.
2. S. Emond, C. Montanier, J.-M. Nicaud, A. Marty, P. Monsan, I. Andre and M. Remaud-Simeon, *Appl. Environ. Microbiol.*, 2010, **76**, 2684-2687.
3. C. S. Chen, Y. Fujimoto, G. Girdaukas and C. J. Sih, *Journal of the American Chemical Society*, 1982, **104**, 7294-7299.