Simplifying Progress

Immobilized Enzymatic Reactors on Monolith Backbone for Rapid Plasmid Linearization

Maša Strašek[1], Špela Peršič Debenjak[1], Polona Skrt[1], Janja Skok[1], Urh Černigoj[1], Aleš Štrancar[1], Manuela Bartolini[2], Sebastijan Peljhan*[1]

[1] Sartorius BIA Separations d.o.o., Mirce 21, 5270 Ajdovščina, Slovenia
[2] Alma Mater Studiorum University of Bologna, via Belmeloro 6, 40126 Bologna, Italy

*Corresponding author: sebastijan.peljhan@biaseparations.com

1. Introduction

Immobilized enzyme reactors (IMERs) stand as innovative biotechnological constructs, seamlessly merging the catalytic proficiency of enzymes with the advantages of solid support matrices. Immobilized enzymes offer notable benefits such as improved stability, the potential to operate within a continuous system over extended durations, reusability of the enzyme, as well as reduced production costs and product purification steps. The aim of this study was to prepare a functional IMER on monolithic support for efficient pDNA linearization, that could be used in *in vitro* transcription reaction for messenger ribonucleic acid (mRNA) production.

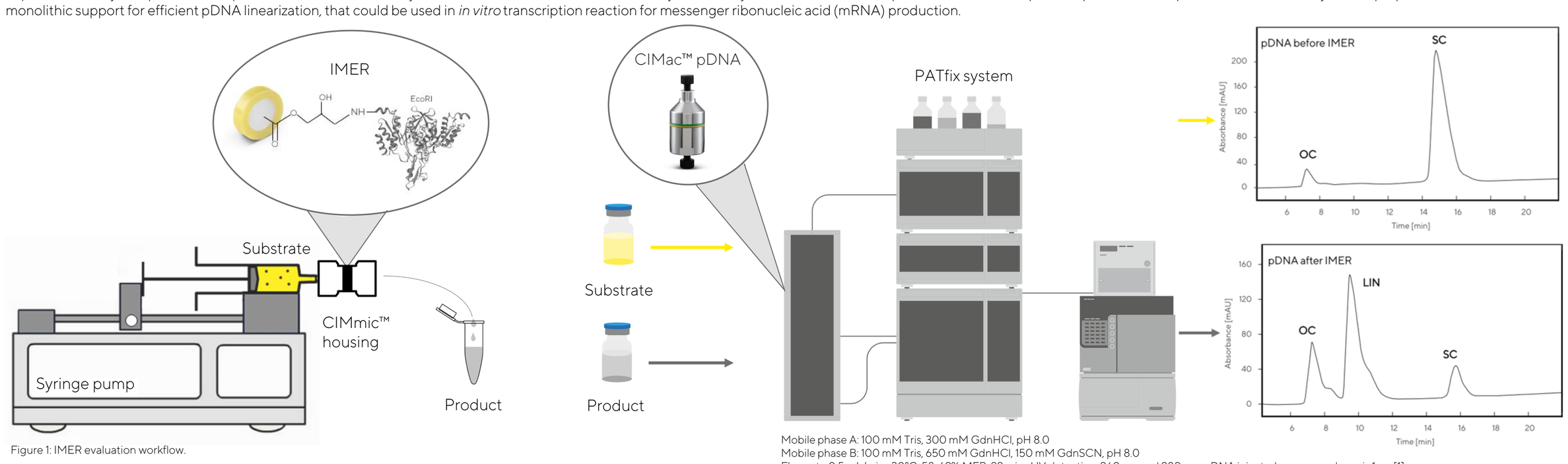

Figure 1: IMER evaluation workflow.

Mobile phase A: 100 mM Tris, 300 mM GdnHCl, pH 8.0
Mobile phase B: 100 mM Tris, 650 mM GdnHCl, 150 mM GdnSCN, pH 8.0
Flow rate 0.5 mL/min; 30°C; 58-69% MFB, 22 min; UV detection: 260 nm and 280 nm; pDNA injected mass on column is 1 µg [1]

2. Immobilization procedure

Aldehyde CIMmic™ ALD-0.1 Disk (Sartorius BIA Separations, Slovenia) with 6 µm channel diameter was chosen as a solid support for EcoRI enzyme coupling. EcoRI (New England Biolabs, USA) was covalently attached to the surface via imine formation, followed by reduction with sodium cyanoborohydride. General Sartorius BIA Separations immobilization procedure was used, where immobilization buffer, temperature, number of enzyme units, and immobilization time were optimized to prepare the IMER with the highest enzymatic activity.

3. Evaluation of IMER enzymatic activity

Enzymatic activity of EcoRI- IMER was determined:

- using model pDNA - plasmid pmFix6 (COBIK d.o.o., Slovenia) as substrate, previously purified using HIP2 Plasmid Process pack (Sartorius BIA Separations, Ajdovščina, Slovenia) by optimizing the manufacturer's protocol [2];
- substrate solution was prepared by dissolving pDNA in 1xNEBuffer™ (New England Biolabs, USA);
- reaction time: 15 minutes at 25°C;
- EcoRI-IMER activity was assessed off-line by chromatographic separation and quantification of pDNA isoforms in product using HPLC analytics performed on PATfix™ bio-chromatography system (Sartorius BIA Separations d.o.o., Ajdovščina, Slovenia).

4. Kinetics of IMER pDNA linearization

The kinetic parameters of the free enzyme in solution and the immobilized enzyme were investigated by building Michaelis-Menten plots (reaction velocity vs substrate concentration). From Figure 2, we can observe distinct dynamics for A) free enzyme and B) IMER, as the immobilization of the enzyme leads to alterations in both V_{max} and K_m values. Enzyme after immobilization exhibits different optimal reaction conditions in comparison to free enzyme in bulk. Using IMER, we can efficiently convert 50 µg of pDNA into approximately 37 µg of linear pDNA in just 15-minute reaction. Furthermore, across all kinetic reactions, performed in this experiment, IMER yields over 130 µg of linear pDNA, on the other hand free enzyme linearized 30 µg of pDNA.

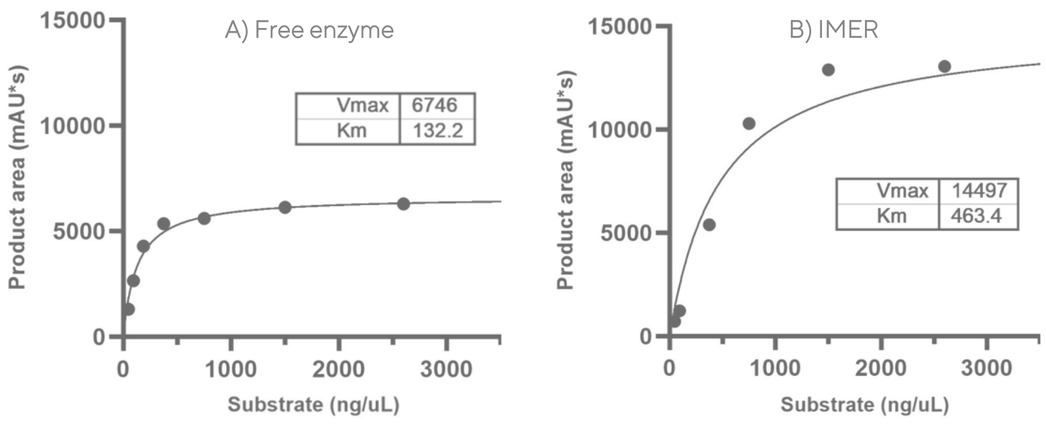

Figure 2: Michaelis-Menten plots for free EcoRI (A) and EcoRI-IMER (B). Vmax and Km values are reported in the inserts. Tested concentration range of substrate: 50-3000 ng/µL; reaction conditions for both assays were: room temperature (25°C), 15 minute-reaction time and for IMER 80 µL injection volume.

5. IMER stability studies

One of main advantages of immobilized enzyme over enzyme in solution is the reusability of enzyme, therefore additional attention was paid on preserving IMER functionality over extended period. Various parameters were tested to improve stability.

The experimental results indicate the following factors effecting the stability of IMER:

- IMER lifetime exhibits temperature dependency, with stability decreasing at elevated temperatures.
- IMER conversion performance shows enhancement as temperature rises.
- A balance between IMER lifetime and performance was set at 25 °C.
- Optimal storage conditions for short term IMER storage is 2 - 8 °C.

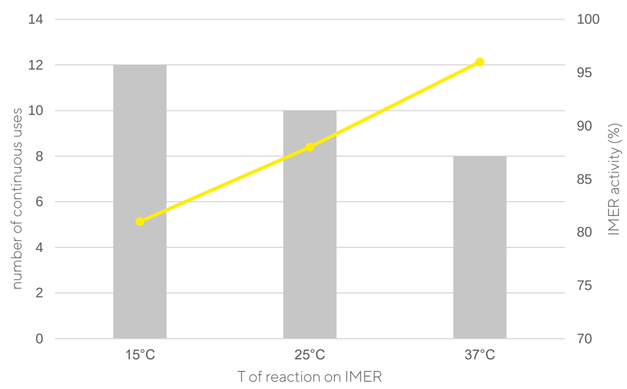

Figure 3: Impact of temperature (15°C, 25°C and 37°C) on EcoRI-IMER activity and stability over reaction cycles. EcoRI-IMER activity is given as percent of linear pDNA produced.

6. Use of IMER in IVT reaction for mRNA production

The practical IMER performance was verified with *in vitro* transcription mRNA production. One of main advantages of IMER uses is reducing additional purification costs – after linearization we obtain pure linear pDNA in reaction buffer, without a need to remove enzyme from solution.

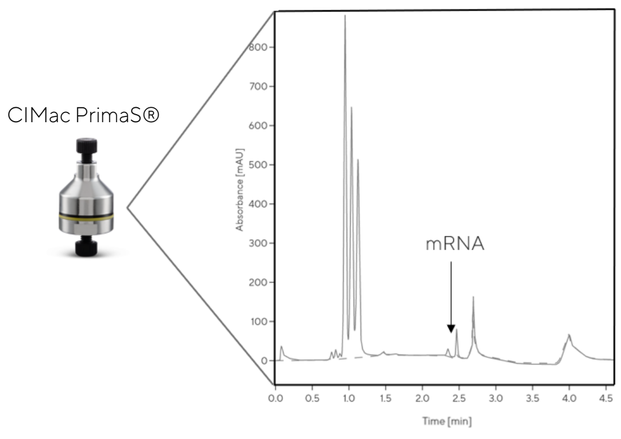

Figure 4: IVT analysis on CIMac PrimaS® (Sartorius BIA Separations d.o.o., Ajdovščina, Slovenia) analytical column, after 180 minutes of IVT reaction. Mobile phases: buffer A was 50 mM HEPES, pH 7.0; buffer B was 50 mM HEPES, 200 mM sodium pyrophosphate, pH 8.5; flow rate 2 mL/min; [3]

7. Conclusions

- We successfully immobilized restriction enzyme EcoRI on monolithic support.
- Kinetics on IMER and free enzyme were evaluated, where IMER proved to linearize higher amounts of pDNA at higher conversion rate compared to free enzyme.
- Finally, EcoRI-IMER was proven to be suitable for applications to IVT mRNA production.

References

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