



# Enzymatic hydrolysis of single-use bioplastic items by improved recombinant yeast strains

Marthinus W. Myburgh<sup>a,b</sup>, Willem H. van Zyl<sup>a</sup>, Michele Modesti<sup>c</sup>, Marinda Viljoen-Bloom<sup>a</sup>, Lorenzo Favaro<sup>b,\*</sup>

<sup>a</sup> Department of Microbiology, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa

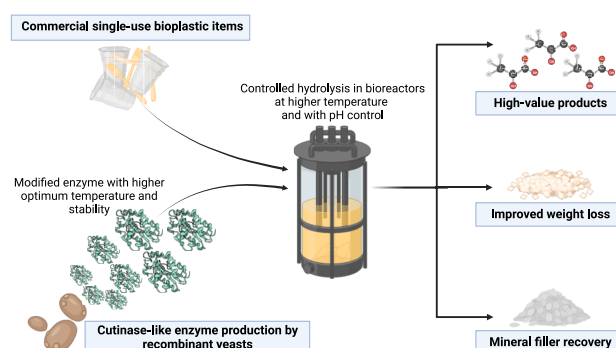
<sup>b</sup> Department of Agronomy Food Natural resources Animals and Environment (DAFNAE), Waste to Bioproducts-Lab, Padova University, Agripolis, Viale dell'Università 16, 35020 Legnaro, Padova, Italy

<sup>c</sup> DII, Department of Industrial Engineering, University of Padova. Via Gradenigo 6, 35131 Padova, Italy

## HIGHLIGHTS

- Recombinant yeast strains effectively hydrolyse single-use bioplastic items.
- rCLE1 is modified during yeast expression and resulted in increased hydrolysis.
- rCLE1 preferentially hydrolyses specific polymers in bioplastic blends.
- Bioplastic blends could be highly susceptible to enzymatic hydrolysis.
- Enzyme adsorption and product inhibition are bottlenecks to hydrolysis.
- Future work would integrate enzymatic hydrolysis in waste management settings.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Single-use bioplastic items pose new challenges for a circular plastics economy as they require different processing than petroleum-based plastics items. Microbial and enzymatic recycling approaches could address some of the pitfalls created by the influx of bioplastic waste. In this study, the recombinant expression of a cutinase-like-enzyme (CLE1) was improved in the yeast *Saccharomyces cerevisiae* to efficiently hydrolyse several commercial single-use bioplastic items constituting blends of poly(lactic acid), poly(1,4-butylen adipate-co-terephthalate), poly(butylene succinate) and mineral fillers. The hydrolysis process was optimised in controlled bioreactor configurations to deliver substantial monomer concentrations and, ultimately, 29 to 78% weight loss. Product inhibition studies and molecular docking provided insights into potential bottlenecks of the enzymatic hydrolysis process, while FT-IR analysis showed the preferential breakdown of specific polymers in blended commercial bioplastic items. This work constitutes a step towards implementing enzymatic hydrolysis as a circular economy approach for the valorisation of end-of-life single-use bioplastic items.

\* Corresponding author.

E-mail address: [lorenzo.favaro@unipd.it](mailto:lorenzo.favaro@unipd.it) (L. Favaro).

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## 1. Introduction

Plastics are ubiquitous in our everyday life, but as the urgency of their environmental impact becomes clear, a shift towards more sustainable alternatives is required. Bioplastics are part of a multi-pronged approach to reduce plastic pollution as they can be both biodegradable and produced from renewable resources. Although global trends are difficult to predict, growing market penetration by bioplastics is expected due to increased consumer demand, more competitive and cost-effective bioplastic production processes, as well as the implementation of regulatory frameworks that promote more sustainable plastic alternatives and extended producer responsibility (Teixeira et al., 2023).

Poly(lactic acid) (PLA) remains one of the main drivers in the continued growth of bioplastic production. However, bioplastics such as poly(1,4-butylene adipate-co-terephthalate) (PBAT) and poly(butylene succinate) (PBS) are also commercially available and expected to increase in production volumes (Rosenboom et al., 2022). Given its brittleness, PLA has limited applications in its neat form and is thus increasingly used as a blend with other more ductile bioplastics, such as PBAT and PBS (Qi et al., 2017; Su et al., 2019).

Currently, bioplastics certified as biodegradable are collected with biowaste and enter organic waste treatment facilities (Cucina et al., 2022). Reports have emerged that efficient bioplastic degradation requires thermophilic conditions and extended processing times, often beyond typical procedures (Bandini et al., 2022; Cucina et al., 2021). As a means to improve PLA degradation in small-scale composting applications, bioaugmentation with microbial consortia was recently successfully investigated and holds promise to treat bioplastic waste in organic waste streams (Mistry et al., 2023). However, efficiently recycling bioplastics appears necessary to maintain long-term sustainability (Aryan et al., 2021; Maga et al., 2019; Niaounakis, 2019; Scaffaro et al., 2019). For a fully circular recycling strategy, specialised chemical or biological processes are required to hydrolyse the polymers to their original monomers, e.g. PLA to lactic acid, which can feed back into production facilities to create a cradle-to-cradle process.

Microbial enzymes are promising platforms for developing improved bioplastic recycling systems based on their mild processing parameters, substrate specificity and ability to release monomers from bioplastic polymers. Various enzymes capable of bioplastic hydrolysis have been identified and characterised. Bacteria are the most well-known producers of extracellular polyester hydrolysing enzymes, with proteases, lipases, cutinases and esterases commonly associated with bioplastic degradative abilities (Lomthong et al., 2022; Mistry et al., 2022; Zhu et al., 2021). However, some of the most highly active polyester hydrolases are of fungal origin, with new candidates continually being discovered (Weinberger et al., 2020; Weinberger et al., 2017). One fungal enzyme candidate, the cutinase-like-enzyme (CLE1) from *Cryptococcus* sp. S-2, has shown high levels of activity on several bioplastic polymers, making it a good candidate for further development, especially in the recycling of bioplastic blends (Kawai et al., 2011; Masaki et al., 2005; Myburgh et al., 2023a).

Yeast expression systems are well-adept at producing enzymes from other eukaryotic organisms. For example, it was recently reported that recombinant production of CLE1 by an engineered *Saccharomyces cerevisiae* strain improved PLA hydrolysis (Myburgh et al., 2023a). However, further strain engineering is required to enhance the extracellular enzyme levels, with the choice of promoter being key in the expression system. Engineered promoters could improve recombinant protein levels, but the challenge is to find the best synergy between native or engineered promoters and the gene of interest, and several candidates have to be screened to identify the top performers (Myburgh et al., 2023b). One of the characteristics of *S. cerevisiae* is that it can hyperglycosylate recombinant proteins, resulting in their improved stability and activity at higher temperatures (Qin and Qu, 2014; Shirke et al., 2018). Enzymatic bioplastic hydrolysis at temperatures approaching the polymer's glass transition temperature could significantly improve

hydrolysis and the feasibility of enzyme-based recycling processes. Other constraints of enzymatic bioplastic hydrolysis include product inhibition, a decrease in pH with the release of organic acids and the inability to hydrolyse blends of different bioplastic polymers.

This study aimed to i) improve the recombinant expression of CLE1 in *S. cerevisiae* by evaluating several engineered and native yeast promoters, ii) investigate bioplastic hydrolysis at higher temperatures, and iii) identify critical bottlenecks in the enzymatic hydrolysis process.

## 2. Materials and methods

### 2.1. Media and cultivation

Unless stated otherwise, all media components and reagents were sourced from Sigma-Aldrich (Steinheim, Germany). Luminy L105 (poly(L-lactide)) and D070 (poly(D-lactide)) PLA substrates were kindly provided by Total Corbion PLA (Gorinchem, Netherlands). Commercial single-use bioplastic cutlery items (EI or SI cutlery), made through injection molding using two Mater-Bi® resins (abbreviated as EI and SI), and PLA cups were obtained from retail suppliers.

The *Escherichia coli* DH5α strain (Takara Bio Inc., Japan) was used for plasmid propagation. The *E. coli* transformants were maintained and selected on Luria Bertani (LB) agar and cultured at 37 °C in Terrific Broth, both containing 100 µg/mL ampicillin (Myburgh et al., 2020). The *S. cerevisiae* Y294 parental strain was cultivated at 26 °C on YPD agar. Recombinant strains were selected on SC<sup>URA</sup> agar (1.7 g/L YNB, 5 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 g/L glucose, 1.5 g/L amino acid mix without uracil and 20 g/L agar) and aerobically cultivated using 25 mL double-strength (2 × ) SC<sup>URA</sup> with 20 g/L glucose in 125 mL Erlenmeyer flasks on a rotary shaker (200 rpm) at 26 °C. Polyesterase activity was confirmed by spot-inoculation of 10 µL yeast cultures onto SC<sup>URA</sup> agar containing 0.2 % w/v Poly(ε-caprolactone) (PCL) (Mn 80 000, Sigma-Aldrich) (Molitor et al., 2019).

### 2.2. Strain construction

The genotypes of the microbial strains and plasmids used in this study are listed in Table 1. The CLEns fragment was isolated from yBBH-CLEns[TEFI] (referred to as yBBH-CLEns by Myburgh et al., 2023a) as an *EcoRI*, *XhoI* fragment and cloned in the corresponding sites of yBBH4-AteA[TDHi] (Myburgh et al., 2020), thus replacing the *ateA* gene to yield plasmid yBBH-CLEns[TDHi]. The latter was transformed into chemical-competent *E. coli* DH5α, isolated from the bacteria, and then into electro-competent *S. cerevisiae* Y294 (Cho et al., 1999). yBBH-CLEns[TDHi] and yBBH-CLEns[TEFI] were digested with *EcoRI* and *SacI* to remove both the promoter and *RPS25Ai* intron. Intronless promoter fragments were PCR-amplified from yBBH-CLEns[TDHi] and yBBH-CLEns[TEFI] using the respective TDH-F + TDHpCLEns-R and TEF-F + TEFpCLEns-R primer combinations (Table 1). PCR products were separated using electrophoresis (1 % w/v agarose gel), and the correct product was isolated with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). The digested vector was transformed into electro-competent *S. cerevisiae* Y294 together with the isolated PCR products to generate the intronless yBBH-CLEns[TDH] and yBBH-CLEns[TEF] constructs through yeast mediated ligation (YML).

### 2.3. Enzyme activity assays

A turbidity-based assay was used to evaluate PLA hydrolysing activity of crude supernatant from the recombinant *S. cerevisiae* strains cultivated in 25 mL 2 × SC<sup>URA</sup> medium. All assays were conducted per Myburgh et al. (2023a) using PDLA (Luminy D070) as substrate. The activity in the crude supernatant was also measured at 37, 42, 45 and 50 °C. The stability of the recombinant enzyme in the crude supernatant was evaluated by incubating the culture supernatant at these respective temperatures and measuring residual activity at 24-hour intervals. The

**Table 1**

Microbial strains, plasmids and primers used in this study.

Bacterial strains	Genotype	Reference
<i>E. coli</i> DH5α	<i>supE44 ΔlacU169 (q80lacZ&amp;M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Sambrook et al., 1989
yBBH-CLE1[TDHi]	<i>supE44 ΔlacU169 (q80lacZ&amp;M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 TDH3p-RPS25Ai-NatSecCLE -CLE1-ENO1<sub>T</sub></i>	This study
<b><i>S. cerevisiae</i> strains</b>		
Y294	<i>α leu2-3,112 ura3-52 his3 trp1-289</i>	ATCC 201,160
Y294[BBH]	<i>URA3 ENO1<sub>p</sub>-XYNSEC-ENO1<sub>T</sub></i>	Njokweni et al., 2012
Y294[AteA]-TEFi	<i>URA3 TEF1<sub>p</sub>-RPS25Ai-XYNSEC-ateA-ENO1<sub>T</sub></i>	Myburgh et al., 2020
Y294[CLEns]-TEFi	<i>URA3 TEF1<sub>p</sub>-RPS25Ai-NatSecCLE -CLE1-ENO1<sub>T</sub></i>	Myburgh et al., 2023a
Y294[CLEns]-TEF	<i>URA3 TEF1<sub>p</sub>-NatSecCLE -CLE1-ENO1<sub>T</sub></i>	This study
Y294[CLEns]-TDHi	<i>URA3 TDH3p-RPS25Ai-NatSecCLE -CLE1-ENO1<sub>T</sub></i>	This study
Y294[CLEns]-TDH	<i>URA3 TDH3p-NatSecCLE -CLE1-ENO1<sub>T</sub></i>	This study
<b>Plasmid name</b>		
yBBH4	<i>bla URA3 ENO1<sub>p</sub>-XYNSEC-ENO1<sub>T</sub></i>	Njokweni et al., 2012
yBBH4-AteA[TDHi]	<i>bla URA3 TDH3p-RPS25Ai-XYNSEC-AteA-ENO1<sub>T</sub></i>	Myburgh et al., 2020
yBBH-CLEns[TEFi]	<i>bla URA3 TEF1<sub>p</sub>-RPS25Ai-NatSecCLE-CLE1-ENO1<sub>T</sub></i>	Myburgh et al., 2023a
yBBH-CLEns[TEF]	<i>bla URA3 TEF1<sub>p</sub>-NatSecCLE-CLE1-ENO1<sub>T</sub></i>	This study
yBBH-CLEns[TDHi]	<i>bla URA3 TDH3p-RPS25Ai-NatSecCLE-CLE1-ENO1<sub>T</sub></i>	This study
yBBH-CLEns[TDH]	<i>bla URA3 TDH3p-NatSecCLE-CLE1-ENO1<sub>T</sub></i>	This study
<b>Primer name</b>		
TDH-F	<i>CATGATTACGAATTAATTCGAGCTCAGTTTATCATTATCAATACTGCCAT</i>	<b>Restriction site</b> SacI
TDHpCLEns-R	<i>CTAAAGCCAATGCTGAAACCAACATGAATTCGTGTGTTATTCGAAACTA</i>	EcoRI
TEF-F	<i>CATGATTACGAATTAATTCGAGCTCGCCGTACCACTTCAAAACACCCAAG</i>	SacI
TEFpCLEns-R	<i>CTAAAGCCAATGCTGAAACCAACATGAATTCCTTTGTAATTAACCTTAGA</i>	EcoRI

\*Underlined nucleotides indicate restriction enzyme sites while italics indicate regions of homology for yeast-mediated ligation.

effect of lactic acid on CLE1 activity was measured by either pre-incubating crude supernatant with increasing lactic acid concentrations, after which the residual activity was determined on emulsified PLA substrate or by adding increasing concentrations of lactic acid to the emulsified PLA substrate (Myburgh et al., 2023a) and conducting assays as reported previously. The pH was maintained at 7 for all substrates containing additional lactic acid.

## 2.4. SDS-PAGE

Recombinant *S. cerevisiae* strains were inoculated into 25 mL 2 × SC<sup>URA</sup> medium in 125 mL Erlenmeyer flasks and incubated for 72 h at 26 °C with constant shaking (200 rpm). The supernatant (200 μL) was subjected to acetone precipitation overnight (Denslow et al., 2001), followed by SDS-PAGE analysis (Myburgh et al., 2023a). In bioreactor experiments, supernatant samples were precipitated with trichloroacetic acid (TCA) and ice-cold acetone (Koontz, 2014) and dialysed against MilliQ H<sub>2</sub>O using a type-VS Millipore membrane (mean pore size = 0.025 μm, Millipore) to desalt samples before electrophoresis.

## 2.5. Small-scale hydrolysis trials

The recombinant *S. cerevisiae* strains were grown in buffered (0.1 M KH<sub>2</sub>PO<sub>4</sub> buffer, pH 7) 2 × SC<sup>URA</sup> for 72 h; the supernatant was harvested by centrifugation (2500 × g, 5 min) and used “as-is” in small-scale hydrolysis trials with 10 g/L L105 PLLA thin films (Myburgh et al., 2023a). Samples were collected daily to monitor lactic acid release with HPLC analysis. Similarly, small-scale hydrolysis trials were conducted on 20 g/L commercial bioplastic items, i.e. pieces of bioplastic cutlery and PLA cups. All small-scale hydrolysis trials were performed in triplicate. The commercial bioplastic items were mechanically pre-treated using a Waring 8010ES blender and sieved sequentially to collect a fraction with a particle size of < 2 mm.

## 2.6. Controlled hydrolysis in bioreactors

To ensure better control of the process parameters, constant mechanical mixing (200 rpm), pH and temperature control was implemented in 1-L bioreactors (Applikon Biotechnology, Schiedam, The Netherlands) with a working volume of 750 mL. The Y294[CLEns]-TDHi strain was aerobically cultivated in buffered 2 × SC<sup>URA</sup> for 72 h, and the

cell-free supernatant was added to 1-L vessels containing 10 g/L L105 PLLA thin films or 20 g/L commercial bioplastic substrates and 0.02 % sodium azide (to inhibit microbial growth). As the optimum pH of CLE1 is 7 (Masaki et al., 2005), the pH was maintained at 6.8 to 7 with 4 M KOH. The effect of pH control was assessed by comparing hydrolysis at 37 °C without pH control to hydrolysis at 37 °C with pH control. Increasing the processing temperature to 42 °C with pH control was also conducted. The pH was maintained between 6.8 and 7 with 4 M KOH. The BioXpert software (Version 1.13, Applikon Biotechnology) was used for data acquisition. Samples were collected daily for HPLC analysis and to monitor enzyme activity. Weight loss was determined at the end of the experiments (Myburgh et al., 2023a).

## 2.7. Molecular docking

Molecular docking experiments were performed using the AMDock graphical tool with AutoDock vina (Trott and Olson, 2009; Valdés-Tre-sanco et al., 2020). The CLE1 protein structure (Protein Data Bank (PDB); <https://www.rcsb.org>) ID 2czq) was used to prepare the protein receptor (Trott and Olson, 2009), while lactic acid (Zinc database ID: ZINC4658560) was used as ligand and was prepared for docking with the Gypsum-DL programme (Ropp et al., 2019). The first-order binding of lactic acid to CLE1 was visualised and analysed for contacts in PyMOL.

## 2.8. Analytical methods

Samples from the various hydrolysis trials were analysed using a Shimadzu Nexera HPLC system equipped with a RID-10A refractive index or UV detector (Shimadzu, Kyoto, Japan), with a Phenomenex Rezex ROA-Organic Acid H<sup>+</sup> (8 %) (300 mm × 7.8 mm) or Rezex RHM Monosaccharide H<sup>+</sup> (8 %) (300 mm × 7.8 mm) column as previously described (Myburgh et al., 2020). The concentration of each product was calculated using standard calibration curves from external standards.

FT-IR spectra were collected using a Thermo Scientific™ Nicolet™ iS™50 FT-IR spectrophotometer with a Smart ITR accessory and a diamond crystal. The analysis was performed in Attenuated Total Reflectance (ATR) mode and a spectral resolution of 2 cm<sup>-1</sup>.

A semi-quantitative composition analysis was also performed on select samples using an X-ray fluorescence (XRF) embedded to the

environmental scanning electron microscope (ESEM, Quanta 200 FEI).

### 3. Results and discussion

#### 3.1. Improved recombinant *CLE1* production

Promoters are key drivers of recombinant expression in *S. cerevisiae*, but the suitability of different yeast promoters for strong constitutive expression of heterologous genes is difficult to predict. This is especially true for engineered yeast promoters utilising promoter-proximal introns for enhanced expression (Myburgh et al., 2020; Myburgh et al., 2023b). The strength of intron-engineered promoters is influenced by several factors, including the distance of introns from important promoter regulatory sequences, intron–exon junction motifs and gene looping architectures (Dwyer et al., 2021; Hoshida et al., 2017; Myburgh et al., 2020). The genetic context in which engineered promoters are used can thus strongly influence promoter performance.

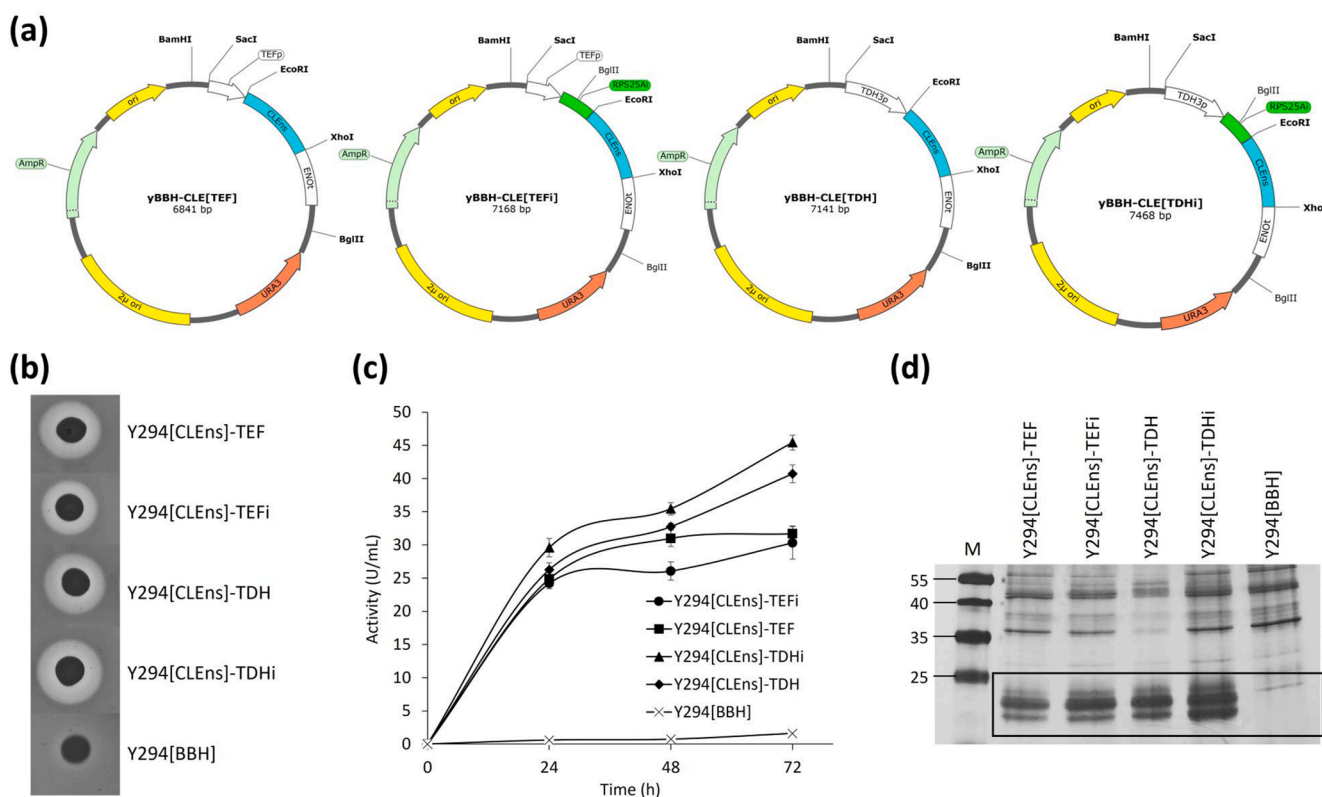
The recombinant *S. cerevisiae* strains were constructed to express the codon-optimised *CLE1* gene under the control of either the native or engineered yeast promoters (Fig. 1a). Spot-inoculation onto PCL emulsion agar plates showed different hydrolysis zone sizes for the recombinant strains (Fig. 1b). Preliminary evaluation indicates that the Y294 [CLEns]-TDH and Y294[CLEns]-TDHi strains, with variants of the *TDH3* promoter, displayed improved hydrolysis of the emulsified PCL substrate relative to the *TEF1* promoter variants. Quantitative activities on the emulsified PDLA substrate confirmed that both these strains significantly outperformed the *TEF1* promoter variants after 72 h of growth (Fig. 1c). This improved performance can be explained by the higher extracellular protein levels in the supernatant of the Y294[CLEns]-TDH

and Y294[CLEns]-TDHi strains (Fig. 1d), with Y294[CLEns]-TDHi that delivered the highest concentration of recombinant protein in the supernatant.

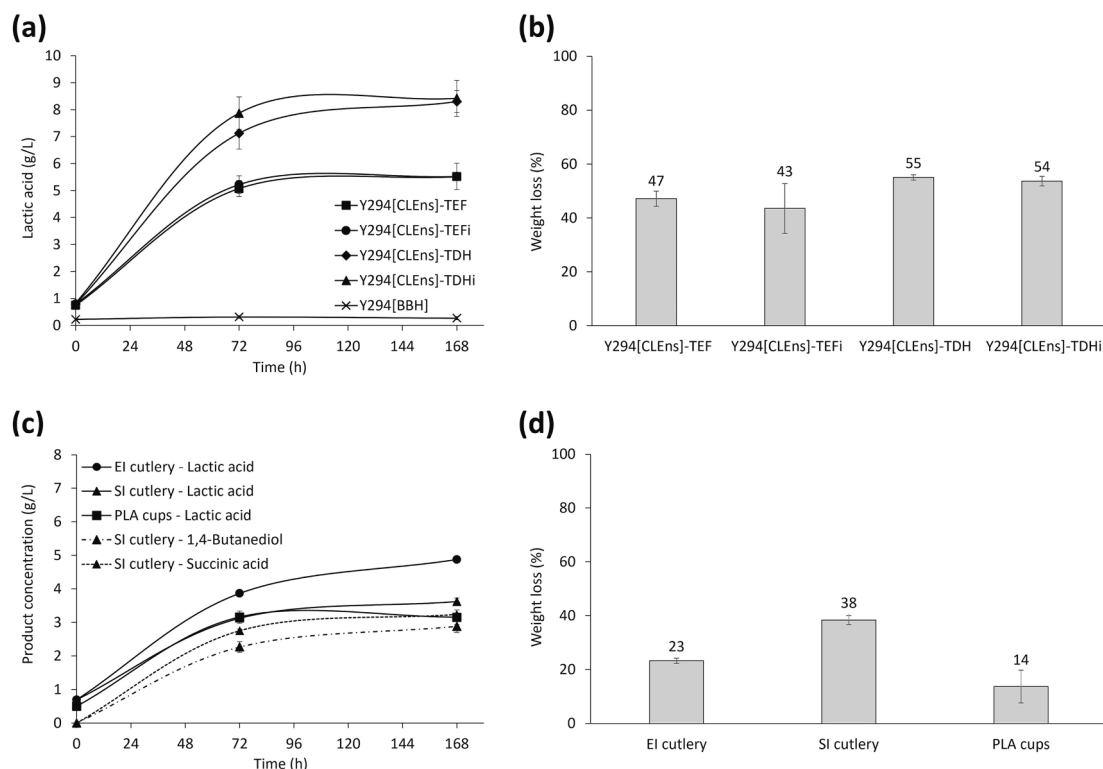
Previously, inserting the *RPS25Ai* intron proximal to the *TEF1* and *TDH3* promoters significantly enhanced, relative to the intron-less promoters, the expression of an amylase gene in *S. cerevisiae* (Myburgh et al., 2020). However, in the current study, enhanced expression of the *CLE1* gene was not so prominent for the intron-containing variants relative to the intron-less counterparts. Nonetheless, increased recombinant protein in the supernatant improved overall activity for the *TDH3* promoter variants compared to the *TEF1* promoter variants.

To confirm the improved performance of the Y294[CLEns]-TDH and Y294[CLEns]-TDHi strains, hydrolysis of solid PLA thin films was conducted at a small scale. Supernatant from the Y294[CLEns]-TDHi strain released 7.87 g/L lactic acid after 72 h of hydrolysis (Fig. 2a). The Y294 [CLEns]-TDH strain delivered very similar lactic acid concentrations, with both these strains significantly outperforming the *TEF1* promoter variants, and the *TDH3* variants releasing almost 3 g/L more lactic acid after 168 h. This increased release of lactic acid correlated to an increase in total weight loss of the PLA films (Fig. 2b), which also correlated with the higher protein levels observed for Y294[CLEns]-TDHi compared to the *TEF1* promoter variants (Fig. 1d).

The effectiveness of crude supernatant from the top-performing Y294[CLEns]-TDHi strain was evaluated for the hydrolysis of commercial bioplastic products at small scale (Fig. 2c, d). As expected, hydrolysis progressed slower on these commercial bioplastic items than on the thin films used for the screening of the different strains (Fig. 2a). Lactic acid was the main product released from EI cutlery pieces (Fig. 2c); a maximum of 4.88 g/L lactic acid was detected after 168 h, and a weight



**Fig. 1.** Improved expression of the fungal cutinase-like enzyme in *S. cerevisiae* using different native and engineered promoters. (a) Plasmid maps of the constructed yeast episomal expression vectors harbouring the respective promoters, expression enhancing promoter-proximal intron and codon-optimised *CLE1* gene with native secretion signal. (b) Hydrolysis zone formation of different recombinant *S. cerevisiae* strains on emulsified PCL agar plates after 72 h incubation at 30 °C. (c) Volumetric enzyme activity at 37 °C on emulsified PDLA substrate using supernatant collected at 24 h intervals from the different recombinant *S. cerevisiae* strains. Error bars represent the standard deviation from the mean of three biological replicates. (d) SDS-PAGE analysis indicates the production of extracellular rCLE1 by the different recombinant *S. cerevisiae* strains. The PageRuler™ Protein Ladder (Thermo Scientific) was used as a molecular weight marker (M), while the control Y294 [BBH] strain served as a benchmark.



**Fig. 2.** Small-scale hydrolysis of various bioplastic materials. (a) Lactic acid release from 10 g/L PLLA thin films during hydrolysis trials using crude supernatant from different recombinant *S. cerevisiae* strains. (b) Weight loss (%) of 10 g/L PLLA thin films after 168 h hydrolysis using crude supernatant from the different recombinant *S. cerevisiae* strains. (c) Product release during the hydrolysis of 20 g/L EI cutlery (circles) and SI cutlery (triangles) as well as PLA cups (squares) at 37 °C using crude supernatant from the *S. cerevisiae* Y294[CLens]-TDHi strain. (d) Weight loss (%) of 20 g/L bioplastic items after 168 h hydrolysis using crude supernatant from *S. cerevisiae* Y294[CLens]-TDHi strain. Error bars represent the standard deviation from the mean of three biological replicates.

loss of 23 % was observed after 7 days (Fig. 2d). Three unknown products were detected from the hydrolysis of EI cutlery pieces. By comparing chromatograms of neat PBAT hydrolysis products, it was confirmed that these unknown products were indeed the result of enzymatic PBAT hydrolysis (data not shown). Out of the three products, terephthalic acid (TPA) was determined to be the main constituent (see Supplementary Materials), while the other two unknowns could potentially be one of several hydrolysis products reported previously for PBAT hydrolysis by cutinases (Weinberger et al., 2020; Yang et al., 2023). To the authors' knowledge, this constitutes the first report of PBAT hydrolysis by recombinant CLE1 (rCLE1), which is particularly remarkable as minimal activity on PBAT has been reported for other fungal hydrolases (Weinberger et al., 2020).

As reported in Fig. 2c, three main products were detected from the enzymatic hydrolysis of SI cutlery pieces, namely lactic acid (3.62 g/L), succinic acid (3.24 g/L) and 1,4-butanediol (2.88 g/L). This points to a mixture of PLA and PBS in the commercial polymer resin that the rCLE1 in the crude supernatant can hydrolyse. It is known that CLE1 can hydrolyse neat PBS (Masaki et al., 2005), but this is the first report of hydrolysis of commercial blends of PLA and PBS polymers by rCLE1. A final weight loss of 38 % was observed for the SI cutlery pieces (Fig. 2d), significantly more ( $p < 0.05$ ) than for EI cutlery pieces (23 %). Only 3.15 g/L of lactic acid was released from 20 g/L PLA cups, with a final weight loss of only 14 % observed. The homogenous and highly crystalline nature of the PLA cups could potentially account for the slow rate of hydrolysis of this material.

Bioplastic items are increasingly constructed of polymer blends. Blends of PLA and PBS are known to have a higher wettability of polymer surfaces (Su et al., 2019), which could improve enzyme contact with the polymer surface and, therefore, their hydrolysis. Furthermore, once PBS particles in a PLA/PBS blend are exposed, water can penetrate in-between the PLA/PBS particle interface (Wang et al., 2016), which

could facilitate enzyme penetration into the bulk of the polymer and further enhance hydrolysis. FT-IR analysis of residual samples treated with rCLE1 indicated a shift of the complex carbonyl signature to lower wavenumbers for EI cutlery pieces (see Supplementary Materials). This points towards the preferential hydrolysis of the PLA fraction in the EI blend, thus exposing the PBAT fraction before its hydrolysis commences. This could be due to the specificity of rCLE1 towards PLA but could also result from differences in crystallinity between the blended materials. This would align with previous studies that showed that CLE1 preferentially hydrolysed amorphous regions of solid PLA films before moving to more crystalline areas (Kawai et al., 2011; Myburgh et al., 2023a). In contrast, a slight decrease in intensity of the low wavenumber signature in the carbonyl range was observed for SI cutlery treated with rCLE1 relative to the control-treated sample (see Supplementary Materials), meaning that there is not a strong preferential hydrolysis for PBS or PLA phases in this specific blend. Overall, the results suggest that polymer blends, such as those used for EI and SI cutlery manufacturing, could be more susceptible to enzymatic hydrolysis if appropriate enzyme candidates are selected. The current data indicates that rCLE1, with its broad-spectrum activity on various bioplastic substrates, is a suitable candidate for mixed bioplastic waste hydrolysis.

### 3.2. Glycosylation of CLE1 increases the optimum temperature for PLA hydrolysis

The yeast *S. cerevisiae* is known to hyper-glycosylate recombinant proteins during secretion. Glycosylation of the rCLE1 protein during extracellular production was previously observed (Myburgh et al., 2023a) and confirmed in this study (Fig. 1c). Predicting potential glycosylation sites with the *in silico* GlycoEP platform (Chauhan et al., 2013) showed a low likelihood of N-linked glycosylation for the CLE1 protein, while multiple O-linked sites were predicted. Deglycosylation of

the rCLE1 in the supernatant using the NEB Endo-HF kit (specific towards N-linked glycosylation) showed no difference in protein banding pattern (Fig. 3a), confirming that glycosylation of the recombinant protein is most likely O-linked. Glycosylation can change an enzyme's thermostability, significantly impacting its effectiveness for polymer degradation reactions (Shirke et al., 2018). This is due to the drastic increase in polymer hydrolysis as the reaction temperature approaches the polymer's glass transition temperature ( $T_g$ ).

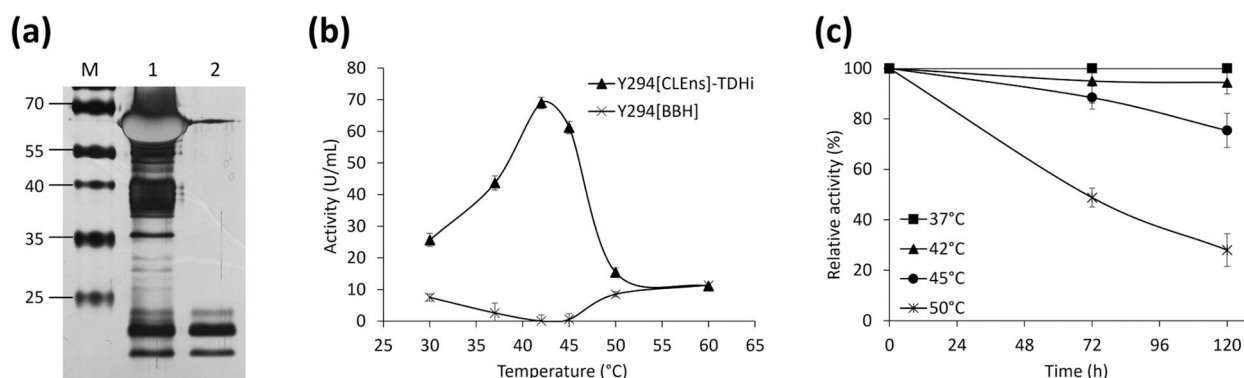
Previously, an optimum temperature of 37 °C was reported for CLE1 (Kamini et al., 2000). Since glycosylation was observed for the rCLE1, the activity in the supernatant from the Y294[CLEns]-TDHi strain was evaluated at higher temperatures on the emulsified PDLA substrate (Fig. 3b). Subsequently, a 5 °C increase in reaction temperature (42 vs 37 °C) resulted in a 58 % increase in extracellular activity for the recombinant strain. A 40 % higher activity was maintained at 45 °C relative to 37 °C, followed by a drastic decrease at 50 °C. Conducting longer hydrolysis reactions at higher optimum temperatures requires the recombinant enzymes to be stable for extended periods. The recombinant enzyme in the crude supernatant retained 94 % of its activity at 42 °C, 78 % at 45 °C and 28 % at 50 °C after 120 h (Fig. 3c). The increased activity towards PLA substrates at higher temperatures and the stability of the recombinant enzyme motivated further investigation under controlled conditions in laboratory-scale bioreactor experiments.

### 3.3. Improving bioplastic hydrolysis in bioreactors

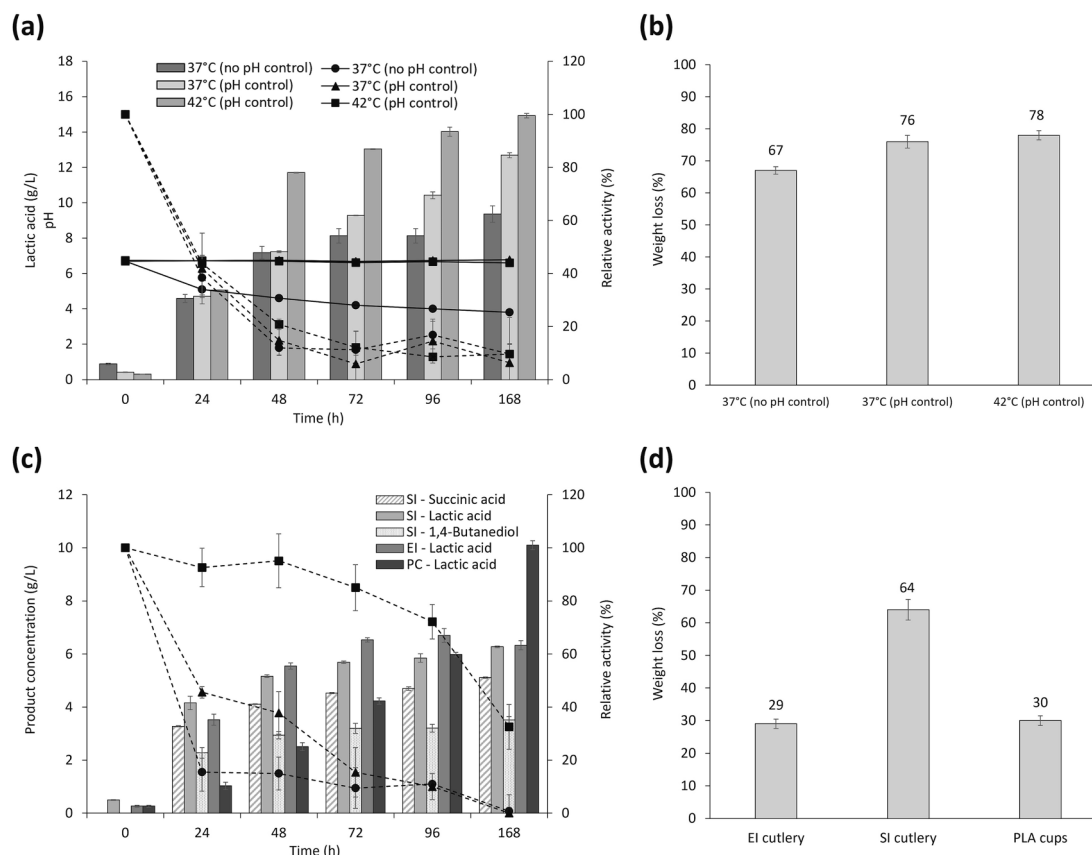
The enzymatic hydrolysis of bioplastics, such as PLA, PBS and PBAT, releases monomeric organic acids that acidify the reaction mixture. Since the optimum pH of the CLE1 enzyme is around pH 7, a decrease in pH could reduce enzyme activity and stability (Kamini et al., 2000). Thus, neutralising the reaction mixture during hydrolysis could improve product yields by mitigating pH-associated inhibition of the enzyme. Supernatant from the Y294[CLEns]-TDHi strain was used during the hydrolysis of 10 g/L PLLA thin films at 37 °C under non-neutralising and neutralising conditions, after which the process temperature was increased to 42 °C under neutralising settings (Fig. 4a). During the first 48 h at 37 °C, hydrolysis progressed similarly under neutralising and non-neutralising conditions, despite an almost 2 unit decrease in pH (4.6 after 48 h) under non-neutralising conditions. However, neutralising the reaction mixture improved PLA hydrolysis and lactic acid release from 72 h onwards, ultimately releasing 36 % more lactic acid. Maintaining pH control thus improved hydrolysis at extended incubation periods. The improved PLA hydrolysis from 72 h onwards resulted in a 9 % higher final weight loss under neutralising compared to non-neutralising conditions (Fig. 4b).

Increasing the process temperature to 42 °C released 73 % more lactic acid after 48 h (Fig. 4a). In fact, lactic acid concentrations released after 48 h at 42 °C were similar to those detected only after 168 h at 37 °C under neutralising conditions. This shortened time could be beneficial for developing an industrial process for enzymatic PLA recycling. Nonetheless, lactic acid release after 168 h at 42 °C was 18 % higher than at 37 °C under neutralising conditions and 59 % higher than under non-neutralising conditions. Interestingly, only a slight increase in total weight loss was observed at 42 °C (78 %) compared to 37 °C (76 %) under neutralising conditions. It is possible that both conditions resulted in a similar release of oligomers from solid PLA films, but that the higher enzymatic activity at 42 °C improved the hydrolysis of these oligomers to lactic acid. These results show significant improvements compared to small-scale hydrolysis trials both in lactic acid release (7.87 vs 13.03 g/L after 72 h) and final weight loss (55 vs 78 %). This also surpasses previous work reporting the extracellular recombinant production of CLE1 by *S. cerevisiae* and the use of culture supernatant for the hydrolysis of the same PLA substrate (Myburgh et al., 2023a). Furthermore, all three experimental conditions showed a similar profile for the decrease in enzyme activity despite the differences in product release, hydrolysis efficiency and conditions investigated. Considering that both processing temperatures did not significantly affect enzyme stability over time (Fig. 3c), the drastic decrease could result from product inhibition, or it could point to the adsorption of the enzyme to the substrate.

Given the benefit of neutralising conditions and a higher processing temperature, commercial bioplastic items were subjected to hydrolysis under these conditions (Fig. 4c). After 72 h, 6.53 g/L lactic acid was released from EI cutlery pieces which is 2.66 g/L more compared to that detected during small scale trials (Fig. 2c). Quantification of TPA release during hydrolysis showed very low concentrations after 24 h (62.71 mg/L), but this increased drastically over the next 24 h (383.73 mg/L) and finally reached a concentration of 613.18 mg/L after 96 h (see Supplementary Materials). This is more than the total PBAT hydrolysis products detected previously using a fungal strain isolated from environmental samples (Weinberger et al., 2020). Although a lower substrate loading (5 g/L) was used in the former, the total product release was measured after 21 days of hydrolysis compared to 4 days in the current study. The initial slow release of TPA could indicate preferential hydrolysis of the PLA fraction in the EI blend before PBAT hydrolysis commences more efficiently. This lag in product release correlated with a slower decrease in residual activity during the hydrolysis of the EI cutlery pieces, with 38 % residual activity remaining after 48 h, decreasing to 15 % after 72 h. These two observations further support the hypothesis that the decrease in residual activity may result



**Fig. 3.** Investigation of the characteristics of rCLE1 produced by the recombinant *S. cerevisiae* Y294[CLEns]-TDHi strain. (a) SDS-PAGE gel of Endo-HF treated and untreated crude rCLE1 samples (M - PageRuler™ Protein Ladder (Thermo Scientific) molecular weight marker, 1 - Endo-HF treated crude rCLE1, 2 - Untreated crude rCLE1). (b) Enzyme activity of crude supernatant from the top-performing recombinant Y295[CLEns]-TDHi strain at various reaction temperatures. (c) Stability of the rCLE1 in supernatant from the top-performing recombinant Y295[CLEns]-TDHi strain at indicated temperatures over an incubation time of 120 h. Error bars represent the standard deviation from the mean of three biological replicates.



**Fig. 4.** Hydrolysis of bioplastic materials in controlled bioreactor conditions using crude supernatant from the Y294[CLEns]-TDHi strain. (a) Hydrolysis of 10 g/L PLLA thin films at 37 °C without pH control (circles); 37 °C with pH control (triangles); 42 °C with pH control (squares). Bar graphs represent lactic acid release (primary axis) over time, while dashed and solid lines represent relative enzyme activity (secondary axis) and pH (primary axis), respectively. (b) Weight loss (%) of the PLA thin films was determined after 168 h of hydrolysis. (c) Hydrolysis of 20 g/L SI cutlery (circles), EI cutlery (triangles) and PLA cups (squares) at 42 °C with pH control. Bar graphs represent the respective product concentrations (primary axis) as quantified with HPLC analysis. Dashed lines represent relative activity (secondary axis) for the duration of the hydrolysis process. (d) Weight loss (%) of different commercial bioplastic items was determined after 168 h hydrolysis. Error bars represent the standard deviation from the mean of three replicates (triplicate samples analysed for each replicate).

from enzyme adsorption to the substrate as was previously observed in recent work on the enzymatic hydrolysis of synthetic fibres (Mihalyi et al., 2023). Overall, conducting the hydrolysis process in bioreactors at the improved conditions resulted in 29 % weight loss of EI cutlery pieces (Fig. 4d), 6 % higher than that observed during small-scale trials (Fig. 2d).

Hydrolysis of 20 g/L SI cutlery resulted in the release of 4.16 g/L lactic acid, 3.27 g/L succinic acid and 2.27 g/L 1,4-butanediol after 24 h. After 168 h of hydrolysis, lactic acid concentrations increased to 6.27 g/L, succinic acid to 5.11 g/L and 1,4-butanediol to 3.51 g/L. The total weight loss of the SI cutlery pieces reached 64 % after 168 h (Fig. 4d), which represents a notable increase of 26 % compared to small-scale hydrolysis of the same polymer at 37 °C and non-neutralising conditions (Fig. 2d). The higher processing temperature and neutralising conditions clearly resulted in improved hydrolysis of the commercial items.

An interesting observation was the release of clear crystals at the end of SI cutlery hydrolysis (see Supplementary Materials). The material was not solvable in most common solvents used to dissolve plastics (chloroform, dichloromethane, acetone, tetrahydrofuran, methanol, acetonitrile, ethanol, etc.). FT-IR analysis of these crystals indicated a prominent stretch vibration at roughly  $1000\text{ cm}^{-1}$ , which can be characteristic of Si-O-Si bonding and suggests that this could be some form of mineral filler used in the compounding of the commercial bioplastic items (see Supplementary Materials). In fact, semi-quantitative analysis of the composition of the crystals using X-ray fluorescence spectroscopy (XRF) indicated that the crystals are rich in talc, a common mineral filler

used in commercial bioplastic item production (Tolga et al., 2020). Therefore, hydrolysis of the available polyester in the commercial SI items may be close to complete even at a weight loss of only 64 %. The decrease in enzyme activity over time followed a similar trend to that of the PLA thin film hydrolysis, with less than 20 % residual activity after 48 h (Fig. 4c).

Hydrolysis of PLA cups using crude supernatant from the Y294 [CLEns]-TDHi strain at 42 °C and neutralising conditions showed a steady release of lactic acid. A final lactic acid concentration of 10.10 g/L and a weight loss of 30 % was obtained after 168 h of hydrolysis (Fig. 4c,d). This is more than double that observed in small-scale trials in terms of weight loss (Fig. 2d). In fact, lactic acid concentrations were almost four times higher at the end of the hydrolysis in bioreactors compared to small-scale trials (Fig. 2c). However, the lactic acid release was substantially slower than that from both SI and EI cutlery pieces. This could be explained by considering the relative enzyme activity during the hydrolysis of PLA cups, which remained above 95 % after 48 h and only decreased to 33 % after 168 h. This potentially shows poor adsorption of the enzyme with the unblended commercial PLA cup substrate and could be a result of the hydrophobicity of the substrate surface or its higher crystallinity. These results further corroborate the notion that blends of bioplastics could be more susceptible to hydrolysis when using appropriate enzyme candidates.

Given that residual activity decreased similarly under neutralising and non-neutralising conditions in the bioreactor experiments, except for the EI and PLA cup substrates, we sought to investigate product inhibition and enzyme adsorption to the substrate as the cause for this

observation.

### 3.4. Enzyme adsorption and not product inhibition results in reduced relative CLE1 activity

The effect of lactic acid on the activity of the rCLE1 was assessed (Fig. 5a). No significant ( $p < 0.05$ ) inhibitory effect was observed in the presence of less than 5 g/L lactic acid, which decreased activity towards the emulsified PDLA substrate by 16 %. In the presence of 10 g/L lactic acid, the activity decreased by 24 %. Therefore, product inhibition could be a limiting factor during the hydrolysis of PLA and PLA-containing bioplastic blends with rCLE1.

Molecular docking of lactic acid to the CLE1 protein was performed to identify binding modes for lactic acid and potential interactions with important amino acid residues in the CLE1 structure. The first-order binding mode of lactic acid to the enzyme was found in the substrate binding cleft of CLE1 (Fig. 5b). More specifically, the lactic acid molecule was closely associated to His180 and Ser85 of the catalytic triad as well as residues in the oxyanion hole of CLE1 (Fig. 5c). Two contacts between Ser85 and the lactic acid molecule at a cut-off distance of 3 Å was observed with another two between His180 and the lactic acid molecule at a distance of 4.5 Å (Fig. 5d). Ser85 functions as the nucleophile in the Ser85-Asp165-His180 catalytic triad of CLE1 and is essential for attacking the carbonyl carbon in the polymer backbone during PLA catalysis (Kawai et al., 2011). Contacts between the oxyanion hole within the substrate binding cleft of the enzyme and lactic acid were also detected. The polar contact was observed between the oxygen of the carboxylic acid group of lactic acid and the amide nitrogen of Gln54 (Fig. 5e). This amino acid (Gln54) is responsible for stabilising the Thr17 residue of the oxyanion hole and was previously suggested to play a significant role in the enzyme's catalytic activity (Kodama et al., 2009).

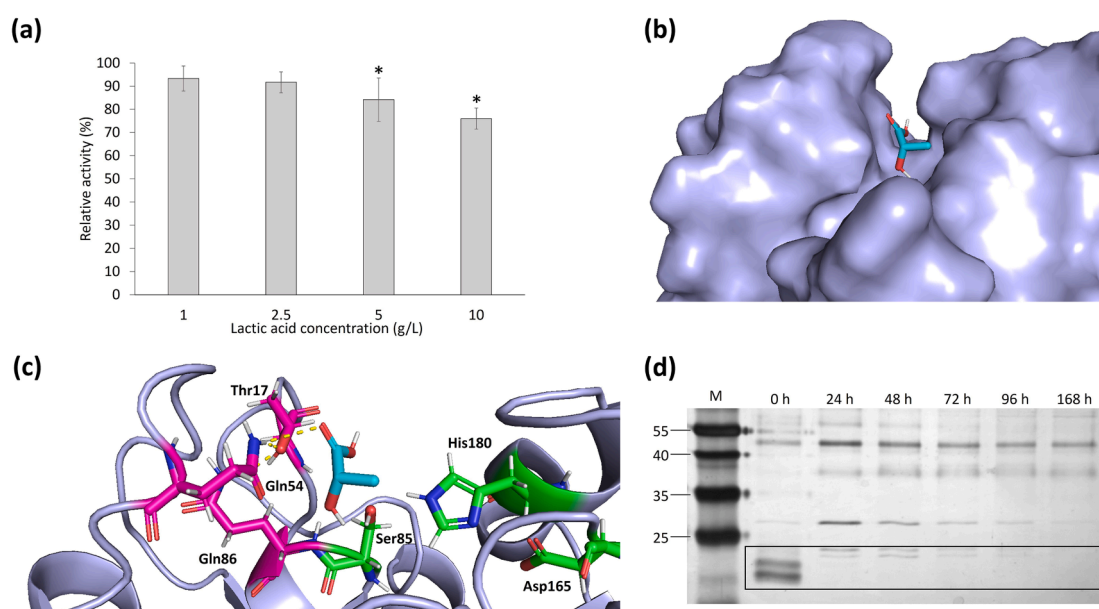
The results indicate that free lactic acid would associate with key residues in the CLE1 structure that could result in lower activity levels on the PLA polymer. Nonetheless, the binding affinity (estimated by the docking score) of the lactic acid molecule to CLE1 was only  $-3.8$  kcal/mol, implying that these interactions only result in a weak affinity of

lactic acid to the enzyme. Although a significant decrease in activity was observed and could be explained by the interactions with important residues in the catalytic domain of CLE1, these data do not adequately elucidate the drastic decrease in activity observed during the hydrolysis experiments conducted in bioreactors (Fig. 4).

SDS-PAGE was conducted on supernatant samples collected at 24 h intervals from the bioreactor experiments to determine whether the recombinant protein was bound to the substrate and thus removed from the supernatant (Fig. 5f). After 24 h of hydrolysis, recombinant protein could not be observed in the supernatant of PLA thin film bioreactor experiments which correlated with a significant decrease in the total protein concentration of the supernatant (data not shown). This points to its adsorption to the PLA films early in the hydrolysis, and thus a drastic decrease in activity is observed in bioreactor experiments (Fig. 4a). Therefore, considering the trends in the reduction of relative activity during bioreactor experiments on the commercial bioplastic items, the rCLE1 seems to associate better with SI than with EI and that the reduced adsorption of the enzyme with PLA cups could be the limiting factor causing slower hydrolysis rates and lactic acid release. The low residual enzyme content may limit further hydrolysis, and enzyme supplementation could be required for complete hydrolysis of the solid bioplastic substrates.

## 4. Conclusion

Enzymatic hydrolysis could benefit the future management of bioplastic waste. Improved production of recombinant CLE1 in yeast resulted in enzyme modifications that ultimately allowed for improved hydrolysis of single-use bioplastic items using controlled bioreactor settings. The results suggested that bioplastic blends (such as PLA and PBAT in the EI cutlery) may be more susceptible to enzymatic hydrolysis by rCLE1 than the homopolymer in pure PLA cups. Although product inhibition was observed, enzyme adsorption to bioplastic substrates could be a significant bottleneck. This work improved CLE1-mediated hydrolysis of bioplastics and moved enzyme-based management strategies closer to commercial viability.



**Fig. 5.** The effect of lactic acid on rCLE1 activity with molecular docking showing interactions of the organic acid with key residues within the substrate binding cleft of the enzyme. (a) Relative activity of rCLE1 in the presence of increasing concentrations of lactic acid. Error bars represent the standard deviation from the mean of triplicate assays. Significant differences ( $p < 0.05$ ) are indicated by asterisk. (b) Surface model of the first mode binding order of lactic acid to CLE1 shows binding in the substrate binding cleft of the enzyme. (c) Polar interaction between the oxygen of the carboxylic acid group of lactic acid and the amide nitrogen of the Gln54 residue in the oxyanion hole. (d) SDS-PAGE analysis of samples collected at 24 h intervals from controlled hydrolysis of PLLA thin films. The PageRuler™ Protein Ladder (Thermo Scientific) was used as molecular weight marker (M).

## CRediT authorship contribution statement

**Marthinus W. Myburgh:** Conceptualization, Investigation, Writing – original draft, Formal analysis. **Willem H. van Zyl:** Conceptualization, Resources, Supervision, Writing – review & editing. **Michele Modesti:** Resources, Formal analysis, Writing – review & editing. **Marinda Viljoen-Bloom:** Conceptualization, Resources, Supervision, Writing – review & editing. **Lorenzo Favaro:** Conceptualization, Resources, Supervision, 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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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biortech.2023.129908>.

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