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Supplementation of recombinant cellulases with LPMOs and CDHs improves consolidated bioprocessing of cellulose

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ABSTRACT

The increased demand for energy has sparked a global search for renewable energy sources that could partly replace fossil fuel resources and help mitigate climate change. Cellulosic biomass is an ideal feedstock for renewable bioethanol production, but the process is not currently economically feasible due to the high cost of pretreatment and enzyme cocktails to release fermentable sugars. Lytic polysaccharide monooxygenases (LPMOs) and cellobiose dehydrogenases (CDHs) are auxiliary enzymes that can enhance cellulose hydrolysis. In this study, four LPMO and two CDH genes were subcloned and expressed in the *Saccharomyces cerevisiae* Y294 laboratory strain. SDS-PAGE analysis confirmed the extracellular production of the LPMOs and CDHs in the laboratory *S. cerevisiae* Y294 strain. A rudimentary cellulase cocktail (cellobiohydrolase 1 and 2, endoglucanase and β-glucosidase) was expressed in the commercial CelluX™ 4 strain and extracellular production of the individual cellulases was confirmed by SDS-PAGE analysis. *In vitro* cooperation of the CDHs and LPMOs with the rudimentary cellulases produced by strain CelluX™ 4[F4–1] was demonstrated on Whatman filter paper. The significant levels of soluble sugars released from this crystalline cellulose substrate indicated that these auxiliary enzymes could be important components of the CBP yeast cellulolytic system.

1. Introduction

Bioethanol is considered a viable fuel extender and alternative to fossil fuels as its higher oxygen content generates better combustion and fewer greenhouse gas emissions [1]. It can be derived from sugar-rich resources such as corn or sugar cane, which may result in competition for food resources [2]. Therefore, it is important to use plant-based carbohydrates that are either non-food products (such as energy crops) or waste streams that are not used further downstream. While agricultural wastes provide options for second-generation biofuel feedstocks, paper sludge (a waste stream from the paper and pulp industry) and recycled paper are underutilised carbon sources suitable for bioethanol production. More than 500 000 tonnes of paper sludge is produced per annum in South Africa and is usually discarded in landfills [3]. Recycled paper and paper sludge can serve as an inexpensive feedstock provided that it can be efficiently hydrolysed into a fermentable carbon source.

Paper sludge consists mainly of cellulose that originates from plant cell walls and has a high energy potential for biofuels [2,4]. Cellulose is a polysaccharide consisting of linear D-glucose molecules linked via β-(1→4) bonds that require the action of a core set of enzymes (collectively referred to as cellulases) for enzymatic hydrolysis [3,5] (Fig. 1). These cellulases include cellobiohydrolases that remove cellobiose from the reducing and non-reducing ends of the cellulose chain [4]; endoglucanases that randomly hydrolyse internal glycosidic bonds to generate free chain ends and smaller oligosaccharides, and β-glucosidases that hydrolyse small oligosaccharides (including cellobiose) to glucose, which can be fermented to ethanol [6].

In the past decade, lytic polysaccharide monooxygenases (LPMOs) - also known as auxiliary activity (AA) enzymes - were identified as powerful tools for the degradation of cellulosic feedstocks as they boost the effect of several commercial hydrolase cocktails [7,8]. LPMOs cleave the glycosidic bonds of crystalline cellulose through hydroxylation at either the C1 or the C4 position of the sugar ring [9]. Cleavage at C1 yields aldonic acids, whereas cleavage at C4 creates a ketoaldose that spontaneously converts to a geminal diol [7]. Cleavage of the glycosidic

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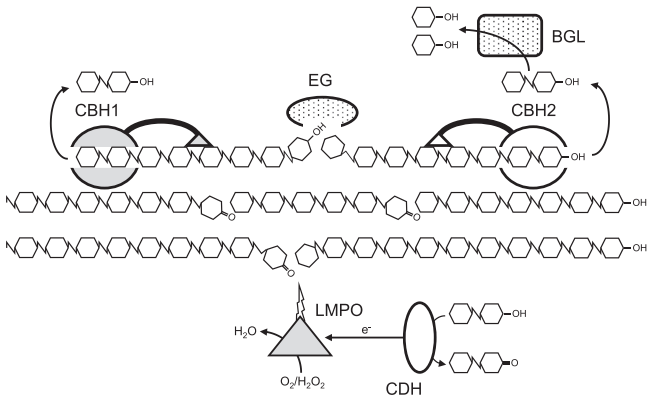

Fig. 1. A schematic representation of the actions of different cellulases and auxiliary enzymes during the hydrolysis of cellulose. Enzymes included are CBH1 = cellobiohydrolase 1, CBH2 = cellobiohydrolase 2, EG = endoglucanase, BGL = β-glucosidase, LMPO = lytic polysaccharide monooxygenase, CDH = cellobiose dehydrogenase.

bonds disrupts the crystalline cellulose structure (Fig. 1) and creates access points for other hydrolytic cellulases to further digest the biomass, thus increasing the conversion efficiency [10–12]. LPMOs can use both O_2 and H_2O_2 as co-substrates, but H_2O_2 was reported to exert higher catalytic rates [13]. Cellobiose dehydrogenases (CDHs) can support LPMO function by donating electrons to the copper centre in the LPMO active site through one-electron interprotein transfer [14,15]. CDHs also relieve product inhibition of cellulases through the oxidation of cellobiose to cellobiolactone.

The different industrial process configurations in which pretreated plant biomass can be converted to bioethanol include Separate Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Co-Fermentation (SSCF), Consolidated BioProcessing (CBP), Co-Treatment (CT) and Consolidated Bio-Saccharification (CBS) [16–18]. In CBP, saccharolytic enzyme production, saccharification and fermentation of plant biomass are combined in a single step, representing a promising strategy to improve process economics. Candidate organisms for cellulosic CBP are developed through either a native or recombinant cellulolytic strategy [19,20]. Engineering the expression of recombinant cellulases in the yeast *Saccharomyces cerevisiae* is a promising CBP strategy that leverages the yeast's superior ethanol production and relatively high process robustness. Several studies have reported the successful production of heterologous cellulases in recombinant yeast strains, but the complete conversion of crystalline cellulose by these strains remains elusive [16].

Given reports that AA family enzymes enhanced commercial cellulase activity on crystalline cellulose sources [10,12], LPMOs and CDHs could improve consolidated bioprocessing with recombinant yeast strains. This study aimed to produce functional recombinant LPMO and CDH enzymes in the *S. cerevisiae* Y294 laboratory strain. To evaluate their impact on the activity of a core set of cellulases, the LPMOs and CDHs were evaluated on a cellulose substrate together with a recombinant CelluX™ 4 industrial strain expressing the *Pccbgl1*, *Tecbh1-TrCBM-C*, *Clcbh2b* and *Treg2* genes, encoding a β-glucosidase (BGL1), two cellobiohydrolases (CBH1 and CBH2) and endoglucanase (EG2), respectively.

2. Materials and methods

2.1. Strains

All strains and plasmids used in this study are listed in Table 1. Detailed information on the genes used for plasmid constructions is provided in Table 2.

Table 1
Microbial strains and plasmids used in this study.

Strains and plasmids	Relevant genotype	Source
Strains		
E. coli DH5α	*supE44DlacU169 ([80lacZDM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 lacZ53*	Takara Bio Inc.
S. cerevisiae		
Y294	*a leu2–3112 ura3–52 his3 trp1–289*	ATCC 201160
Y294[BBH4]	*URA3 ENO1*$_P$*-XYNSEC-ENO1*$_T$	[36]
Y294[Pccbgl1]	*URA3 ENO1*$_P$*-XYNSEC-Pccbgl1-ENO1*$_T$	[36]
Y294[cbh1-TrCBM-C]	*URA3 ENO1*$_P$*-Tecbh1-TrCBM-C-ENO1*$_T$	[5]
Y294[EG2]	*URA3 PGK1*$_P$*-Treg2-PGK1*$_T$	[5]
Y294[C.l.cbh2b]	*URA3 PGK1*$_P$*-Clcbh2b-PGK1*$_T$	[29]
Y294[CDH2]	*bla URA3 ENO1*$_P$*-XYNSEC-CDH2-ENO1*$_T$	This laboratory
Y294[CDH3]	*bla URA3 ENO1*$_P$*-XYNSEC-CDH3-ENO1*$_T$	This laboratory
Y294[LPMO1]	*bla URA3 ENO1*$_P$*-XYNSEC-LPMO1-ENO1*$_T$	This laboratory
Y294[LPMO2]	*bla URA3 ENO1*$_P$*-XYNSEC-LPMO2-ENO1*$_T$	This laboratory
Y294[LPMO4]	*bla URA3 ENO1*$_P$*-XYNSEC-LPMO4-ENO1*$_T$	This laboratory
Y294[LPMO5]	*bla URA3 ENO1*$_P$*-XYNSEC-LPMO5-ENO1*$_T$	This laboratory
CelluX™ 4	*a/α bud5*	[35]
CelluX™ 4[F4–1][a]	*Sh Ble*; *ENO1*$_P$*-XYNSEC-Pccbgl1-ENO1*$_T$; *KanMX*; *ENO1*$_P$*-Tecbh1-TrCBM-C-ENO1*$_T$; *Hyg*; *PGK1*$_P$*-Clcbh2b PGK1*$_T$; *PGK1*$_P$*-EG2-PGK1*$_T$	This study
Plasmids		
YEp352	*bla URA3*	ATCC® 37673™
pBZD2-Bgl1	*bla* δ*-Sh Ble*; *ENO1*$_P$*-XYNSEC-Pccbgl1-ENO1*$_T$-δ	This study
pBKD2-Cbh1	*bla* δ*-KanMX*; *ENO1*$_P$*-Tecbh1-TrCBM-C-ENO1*$_T$-δ	This study
pBHD1-Cbh2	*bla* δ*-Hyg*; *PGK1*$_P$*-Clcbh2b-PGK1*$_T$-δ	This study
pBHD1-Eg2	*bla* δ*-Hyg*; *PGK1*$_P$*-Treg2-PGK1*$_T$-δ	This study
pCDH2	*bla URA3 ENO1*$_P$*-XYNSEC-CDH2-ENO1*$_T$	This laboratory
pCDH3	*bla URA3 ENO1*$_P$*-XYNSEC-CDH3-ENO1*$_T$	This laboratory
pLPMO1	*bla URA3 ENO1*$_P$*-XYNSEC-LPMO1-ENO1*$_T$	This laboratory
pLPMO2	*bla URA3 ENO1*$_P$*-XYNSEC-LPMO2-ENO1*$_T$	This laboratory
pLPMO4	*bla URA3 ENO1*$_P$*-XYNSEC*$_S$*-LPMO4-ENO1*$_T$	This laboratory
pLPMO5	*bla URA3 ENO1*$_P$*-XYNSEC*$_S$*-LPMO5-ENO1*$_T$	This laboratory

[a] Gene cassettes integrated into delta sites on genome using *KanMX*, *Sh Ble* and *Hyg* resistance markers.

Table 2
Recombinant genes used in this study.

Gene, enzyme	Origin	GenBank Acc nr	Secretion signal
Pccbgl1, BGL1	*Phanerochaete chrysosporium cbgl1*	AF036872	*XYNSEC*[a]
Treg2, EG2	*Trichoderma reesei eg2*	P07982.1	Native
Tecbh1, CBH1	*Talaromyces emersonii*	AAL89553	Native
Clcbh2b, CBH2	*Chrysosporium lucknowense cbh2b*	HH793136.1[b]	Native
CDH2, CDH2	*Myceliophthora thermophila cdh*	XP_003663382.1	Native
CDH3, CDH3	*Neurospora crassa cdh*	XP_956591.1	Native
LPMO1, LPMO1	*Podospora anserina gh61A*	XP_001911429.1	Native
LPMO2, LPMO2	*Podospora anserina gh61B*	XP_001907702.1	Native
LPMO4, LPMO4	*Myceliophthora thermophila gh61*	XP_003663414.1	Native
LPMO5, LPMO5	*Chaetomium thermophilum gh61*	XP_006692680.1	Native

[a] Secretion signal from *Trichoderma reesei xyn2*.
[b] European Molecular Biology Laboratory (EMBL)

2.2. Media and cultivation conditions

All media reagents and components were supplied by Sigma-Aldrich (Burlington, MA, USA) unless stated otherwise. The *Escherichia coli* DH5α strain (Takara Bio Inc., Kusatsu, Shiga, Japan) was used for plasmid propagation with transformants selected and maintained on Luria Bertani (LB) agar (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl and 20 g/L agar) containing 100 μg/mL ampicillin at 37 °C. The *S. cerevisiae* parental strains were maintained on YPD agar plates (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar), whereas the *S. cerevisiae* CelluX™ 4 transformants were selected on YPD plates containing 300 mg/L Geneticin G418, 300 mg/L Zeocin (Invitrogen, Waltham, Ma, USA) and 300 mg/L Hygromycin. The *S. cerevisiae* Y294 transformants were selected and maintained on Synthetic Complete agar plates lacking uracil (SC^{-URA}; 6.7 g/L yeast nitrogen base without amino acids (BD-Diagnostic Systems, Sparks, MD), 20 g/L glucose, 1.5 g/L synthetic dropout medium supplements and 20 g/L agar, pH 6). Aerobic cultivation of CelluX™ 4 strains was performed in 25 mL YPD broth (no antibiotics), whereas the recombinant *S. cerevisiae* Y294 strains were cultivated in double-strength SC^{-URA} ($2 \times SC^{-URA}$) broth containing 20 g/L glucose. All yeast strains were cultivated at 30 °C in 125-mL Erlenmeyer flasks on a rotary shaker at 200 rpm. An additional 10 mM $CuSO_4$ was added to the $2 \times SC^{-URA}$ medium for strains producing LPMOs.

2.3. Plasmid and strain construction

Four LPMO-encoding and two CDH-encoding genes (Table 2), codon-optimised for expression in *S. cerevisiae*, were prepared by GenScript using a proprietary algorithm (Piscataway, NJ, USA). The synthetic genes included *Pac*I and *Asc*I restriction sites at the 5' and 3' ends of the genes to enable cloning into the corresponding sites of pMU1531 [21] to create plasmids pCDH2, pCDH3, pLPMO1, pLPMO2, pLPMO4 and pLPMO5 (Table 1). The plasmids were transformed into *E. coli* DH5α, re-isolated [22] and their integrity was confirmed by restriction analysis. The episomal plasmids pCDH2, pCDH3, pLPMO1, pLPMO2, pLPMO4 and pLPMO5 were transformed into *S. cerevisiae* Y294 using electroporation [23] to generate the Y294[CDH2], Y294[CDH3], Y294 [LPMO1], Y294[LPMO2], Y294[LPMO4] and Y294[LPMO5] strains.

For the expression of the cellulases (*Talaromyces emersonii Tecbh1-TrCBM-C*, *Chrysosporium lucknowense Clcbh2b*, *Trichoderma reesei Treg2* and *Phanerochaete chrysosporium Pccbgl1*), the vectors were designed for targeted integration at the delta sequences through homologous recombination. The *Tecbh1-TrCBM-C* and *Pccbgl* genes were cloned into the *Pac*I and *Asc*I sites of pBKD2 and pBZD2, respectively, to generate pBKD2-Cbh1 and pBZD2-Bgl1. The *Clcbh2b* and *Treg2* genes were cloned onto pBHD1 to generate pBHD1-Cbh2 and pBHD1-Eg2, respectively. The CelluX™ 4 parental strain was transformed sequentially with pBKD2-Cbh1 (selection on 300 μg/L Geneticin) and pBZD2-Bgl1 (selection on 300 μg/L Zeocin). Thereafter, pBHD1-Cbh2 and pBHD1-EG2 were introduced simultaneously (selection on 300 μg/L Hygromycin), resulting in the new cellulolytic strain, CelluX™ 4[F4–1].

Colony PCR was used to confirm the presence of the gene cassettes using the Perkin Elmer Gene Amp R PCR System 2400 (Perkin Elmer) and TaKaRa Ex Taq polymerase (Takara Bio Inc.), with primers synthesised by Inqaba Biotec (Pretoria, South Africa). Genomic DNA was isolated from CelluX™ 4[F4–1] strain using the ZR Fungal/Bacterial DNA Kit (Zymo Research) and used as a template for PCR amplification of the gene cassettes containing the open reading frames of the synthetic genes.

2.4. SDS-PAGE analysis

The *S. cerevisiae* Y294 and Cellux™4 strains were cultivated in 20 mL $2 \times SC^{-URA}$ medium in 125-mL Erlenmeyer flasks for 24 h at 30 °C with constant agitation (200 rpm). Samples of 500 μL supernatant from 48-hour cultures were concentrated 25-fold with acetone precipitation [24] and the proteins were separated by 10% SDS-PAGE at 120 V for 90 min [25]. The protein species were visualised using the silver staining method [26]. The PageRuler™ protein ladder (Thermo Fisher Scientific, Waltham, Massachusetts, USA) served as the molecular mass marker.

2.5. Qualitative plate assays

The *S. cerevisiae* Cellux™ 4 strains were grown on SC^{-URA} plates containing 1% carboxymethylcellulose (CMC) at 30 °C for 72 h to confirm endoglucanase activity. The colonies were washed off and the plate was stained with 0.1% Congo Red for 15 min, followed by a brief destaining with 1% (w/v) sodium chloride [27]. The extracellular β-glucosidase (BGL) activity was confirmed on SC^{-URA} plates containing 0.1% esculin and 0.05% ferric citrate, incubated at 30 °C for 48 h. CDH activity was evaluated on SC^{-URA} agar plates containing 1% cellobiose and 1 mM 2,6-dichlorophenolindophenol (DCPIP) as an electron acceptor. The DCPIP was added to the molten agar after autoclaving; the plates were covered in foil and incubated at room temperature.

2.6. Quantitative liquid assays

The *S. cerevisiae* CelluX™ 4 transformants were inoculated to a cell density of 1×10^6 cells per mL in 20 mL YPD medium and aerobically cultivated for 72 h at 30 °C with agitation at 200 rpm. Total β-glucosidase activity (cell-associated and supernatant) was determined at 24-hour intervals using 10 mM *p*-nitrophenyl-β-D-glucopyranoside (pNPG; Sigma-Aldrich) as substrate, with a reaction time of 3 min at 50 °C [28]. The absorbance was measured at 400 nm using a FLUOstar Omega Microplate Reader (BMG LABTECH, Ortenberg, Germany) and compared with a *p*NP standard curve (0.075–1.25 mM). The extracellular CBH1 activity of transformants was evaluated on soluble fluorescent 4-methylumbelliferyl-β-D-lactoside (MU-Lac; Sigma-Aldrich) as described by Ilmén et al. [29] with a reaction time of 20 min at 37 °C and compared to a methylumbelliferone (MU) standard curve (0.63 μM to 20 μM). Endoglucanase activity was determined with the dinitrosalicylic acid (DNS) reducing sugar assay using 1% (w/v) carboxymethylcellulose, incubated for 30 min at 50 °C [27]. Glucose was used for a standard curve (0.5–1.5 mM) with absorbance measured at 540 nm. Dry cell weight (DCW) was determined as previously described [27]. Activities were expressed as units/g DCW, where one unit was defined as the amount of enzyme required to release 1 μmol of reducing sugar or equivalent per minute. Assays were performed in biological and technical triplicates and values are given as averages of these repeats with standard deviations indicated.

Avicel conversion was quantified using 2% (w/v) Avicel in 50 mM sodium acetate (pH 5.0), 0.02% (w/v) sodium azide, with or without 1 uL/mL Novozym188 (Sigma-Aldrich), with continuous stirring to ensure homogeneity. Equal volumes of the substrate mix and 72-hour yeast culture supernatant (final volume of 600 μL) were added to a 96-deep-well plate and incubated at 35 °C with shaking at 1 000 rpm in a Heidolph Titramax 1000 microplate shaker/incubator (Heidolph, Schwabach, Germany). Samples were taken at 0, 24 and 48 h and Avicel conversion was determined by measuring the amount of glucose released using a modified reducing sugar assay [30]. Avicel conversion was expressed as the percentage of substrate hydrolysed, based on the amount of glucose released during the assay.

CDH activity was determined as the time-dependent reduction of 300 μM DCPIP in 100 mM sodium citrate phosphate buffer (pH 5.5) containing 30 mM cellobiose [31]. The reaction was performed by adding 50 μL supernatant to 150 μL of the DCPIP-cellobiose solution in a microtitre plate well. The absorbance was measured at 520 nm ($\varepsilon_{520} = 6.8\ mM^{-1}\ cm^{-1}$) using a BioRad xMark™ Microplate Spectrophotometer (BioRad, Hercules, CA, USA) at time 0 and after 15 min of incubation at 30 °C. Sodium citrate phosphate buffer was prepared at different pH

values to determine the optimal pH of the CDH enzymes. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of reduced DCPIP per minute. Enzyme activity was calculated as follows:

$$Enzymatic\ activity\ (U/L) = \frac{\Delta A \times F \times V_{tot} \times (10^3)}{\varepsilon \times d \times V_s \times t}$$

where ΔA is the difference between the absorbance at the time final and the absorbance at time zero, F is the dilution factor, V_t is the total volume of the assay, ε is the molar attenuation coefficient, d is the pathlength, V_s is the sample volume of the assay, and t is the time duration in minutes.

Two assay methods were evaluated to determine LPMO activity. The first method measures the increase in absorbance at 469 nm over 300 s, which would indicate the conversion of 2,6-dimethoxyphenol (2,6-DMP) to coerulignone by active LPMOs [32]. The assay mix consisted of 860 μL of a 116 mM sodium phosphate buffer pH 6, 100 μL of a 10 mM 2,6-DMP stock solution and 20 μL of a 5 mM H_2O_2 stock solution. The peroxidase activity of LPMOs is based on the sample volume, enzyme dilution and the molar absorption coefficient of coerulignone ($\varepsilon_{469} = 53$, 2 M^{-1} cm^{-1}). The second method is based on the oxidation of reduced phenolphthalein (rPHP) to phenolphthalein in the presence of dehydroascorbate (DHA) as co-substrate to enhance the activity of the LPMOs [33]. For the buffer, 6 g $Na_2HPO_4{\cdot}7\ H_2O$ was dissolved in 200 mL Milli-Q water and adjusted to pH 7.25 with citric acid. The stop solution contained 5.25 g Na_2CO_3 and 4.2 g $NaHCO_3$ in 100 mL Milli-Q water, pH 10.3. The assay mix contained equal volumes of the assay buffer, 800 μM rPHP and 400 μM DHA; 50 μL of the LPMO supernatant was added to 150 μL of the assay mix and incubated at 40 °C while shaking at 450 rpm. After 30 min, 50 μL of the stop buffer was added to each sample and the spectrophotometric absorption was measured at 552 nm after 10 min (a pink colour would indicate a positive reaction).

2.7. Cellulose hydrolysis (Whatman paper)

The recombinant *S. cerevisiae* Y294 and CelluX™ 4[F-1] strains were cultivated for 3 days in 2 ×SC^{-URA} and 24 h in YPD medium, respectively, and the supernatant was harvested. The 10-mL assay mixture contained 50 μg Whatman® filter paper (Grade 1) in 0.05 M sodium citrate phosphate buffer (pH 5) containing 1 mL each of the CelluX™ 4 [F4–1] and different combinations of the recombinant *S. cerevisiae* strains secreting the LPMO and CDH enzymes. The *S. cerevisiae* Y294 [BBH4] strain lacking heterologous genes served as a control. Hydrolysis was done at 30 °C with gentle mixing at 30 rpm for 1 h and the amount of glucose and cellobiose released from the Whatman paper was quantified by HPLC analyses [34]. For the latter, a Dionex Ultimate 3000 system with a WPS-3000 T SL (analytical) autosampler, LPG-3400 AB pump, Coulochem III electrochemical detector (Esa, Inc., Chelmsford, MA, USA) with gold electrode was used together with a CarboPac PA1 (4 ×250 mm) analytical column coupled to a PA1 (4 ×50 mm) guard column (Thermo Fisher Scientific). Data analysis was done with Chromeleon version 6.80 software (Thermo Fisher Scientific).

2.8. Statistical analysis

For statistical analysis, *T*-tests and one-way analysis of variance (ANOVA) tests were performed. Significant differences between activities attained were investigated using a two-tailed *T*-test, assuming unequal variance. Differences with a $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Construction of recombinant S. cerevisiae strains

Strain CelluX™ 4 is a genetically modified *S. cerevisiae* strain originating from CelluX™ that has been developed for the cellulosic ethanol industry [35]. This strain can utilise xylose, displays inhibitors tolerance and resistance to stresses and maintains high cell viability during cellulosic ethanol fermentations. In this study, the CelluX™ 4 strain was transformed sequentially with the *Tecbh1-TrCBM-C*, *Pccbgl, Clcbh2b* and *Treg2* gene cassettes to obtain a strain expressing the four core cellulase enzymes, namely β-glucosidase (BGL1), two cellobiohydrolases (CBH1 and CBH2) and endoglucanase (EG2). To account for clonal variation, more than 20 transformants were evaluated based on their extracellular cellulase activities and the CelluX™ 4[F4–1] strain was selected for further study. The presence of the *Pccbgl*, *Tecbh1-TrCBM-C, Clcbh2b* and *Treg2* gene integrations in the CelluX™ 4[F4–1] strain was confirmed by PCR (data not shown).

Clear zones on CMC-plates after Congo Red staining would indicate endoglucanase activity, whereas a β-glucosidase would produce a black zone if it cleaved the glucoside group from esculin, releasing esculetin to react with ferric citrate [36]. The recombinant *S. cerevisiae* CelluX™ 4 [F4–1] strain produced hydrolysis zones on both CMC (Fig. 2A) and esculin plates (Fig. 2B), confirming both extracellular endoglucanase and β-glucosidase activities. As expected, the parental *S. cerevisiae* CelluX™ 4 strain was negative for both activities. SDS-PAGE analysis revealed four additional protein species in the CelluX™ 4[F4–1] supernatant relative to that of CelluX™ 4 (Fig. 2C). The four protein species of approximately 64, 68, 72 and 140 kDa in the supernatant of the CelluX™ 4[F4–1] strain correspond to the expected sizes of EG2, CBH2, CBH1 and BGL1, respectively. The SDS-PAGE analysis thus confirmed that all four enzymes were successfully secreted into the growth medium.

When the CelluX™ 4 strains were cultivated in liquid YPD in the absence of selective pressure, the CelluX™ 4[F4–1] grew slightly slower than CelluX™ at 48 h, but it matched the growth of the parental strain at 72 h (Fig. 3A). The *S. cerevisiae* CelluX™ 4[F4–1] strain displayed all the cellulase activities that were introduced, reaching an EG activity of 23.4 U/gDCW at 72 h (Fig. 3B), BGL activity of 34.4 U/gDCW at 72 h (Fig. 3C) and CBH1 activity of 0.31 U/gDCW after only 24 h (Fig. 3D). The volumetric CBH activity continued to increase, reaching 1.97 U/L at 72 h of incubation (Fig. 3E). However, since biomass accumulated at a faster rate, a decreasing U/gDCW was recorded (Fig. 3D). Avicel conversion of up to 0.52% per gram DCW in 48 h was observed for CelluX™ 4[F4–1] (Fig. 3F), while the addition of a commercial BGL (Novozym188) did not enhance Avicel conversion at these levels of crystalline cellulase hydrolysis.

The different LPMO and CDH gene cassettes were introduced on episomal plasmids into the laboratory *S. cerevisiae* strain Y294. The presence of the LPMO and CDH gene cassettes in the corresponding Y294 transformants was confirmed by PCR (data not shown). The Y294 [CDH2] and Y294[CDH3] strains displayed large clearing zones on DCPIP plates that are indicative of CDH activity (Fig. 4 A). DCPIP is a blue redox dye that becomes colourless when an active CDH donates an electron to it. The *S. cerevisiae* Y294[BBH1] control strain produced a small pink zone around the colony, possibly a result of non-specific enzymatic activity or acidification of the media due to the lack of CDH activity [37].

Two protein species (approximately 85 and 100 kDa, respectively) were observed in the supernatant of the *S. cerevisiae* Y294[CDH2] strain, whereas CDH3 was present as a faint species of ~85 kDa (Fig. 4B). The molecular mass corresponded with the predicted protein size of 88 kDa for the *M. thermophila* CDH and *N. crassa* CDH (https://web.expasy.org/compute_pi/). The LPMO1, 2, and 5 protein species displayed molecular masses of approximately 55, 45 and 40 kDa, respectively (Fig. 4 C). However, LPMO4 was present as a smear ranging from 45 to

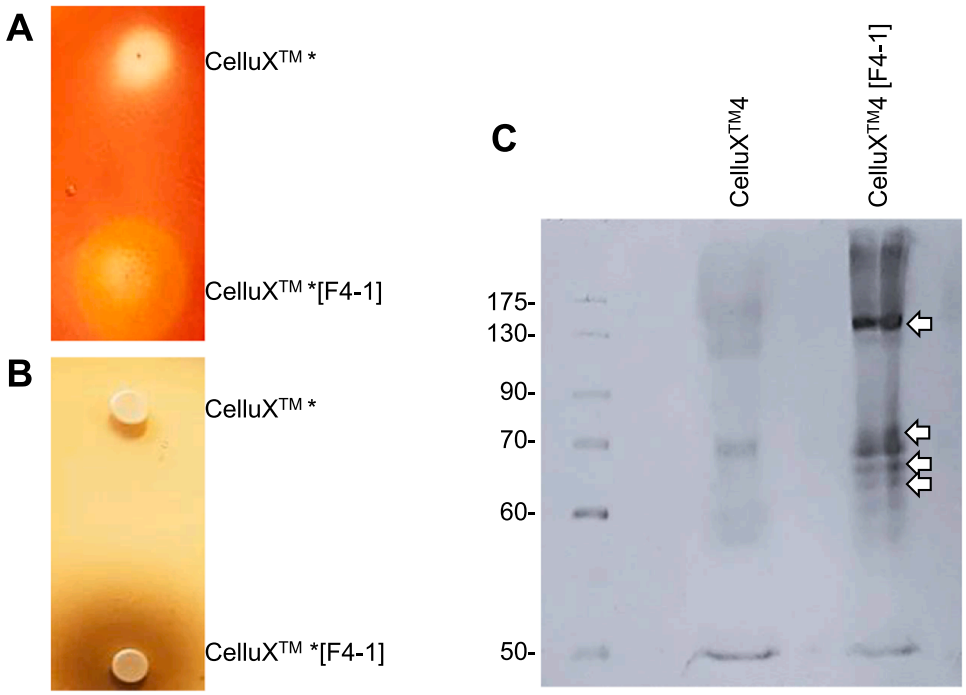

Fig. 2. The CelluX™ 4 and CelluX™ 4[F4–1] strains were spot-inoculated onto SC^{-URA} plates containing (A) CMC and (B) esculin for qualitative analysis of endoglucanase and β-glucosidase activity, respectively. (C) SDS-PAGE analysis of the supernatant from CelluX™ 4 and CelluX™ 4[F4–1], with additional protein species in the CelluX™ 4[F4–1] indicated by arrows. The molecular mass marker (with sizes in kDa) is depicted on the left-hand side.

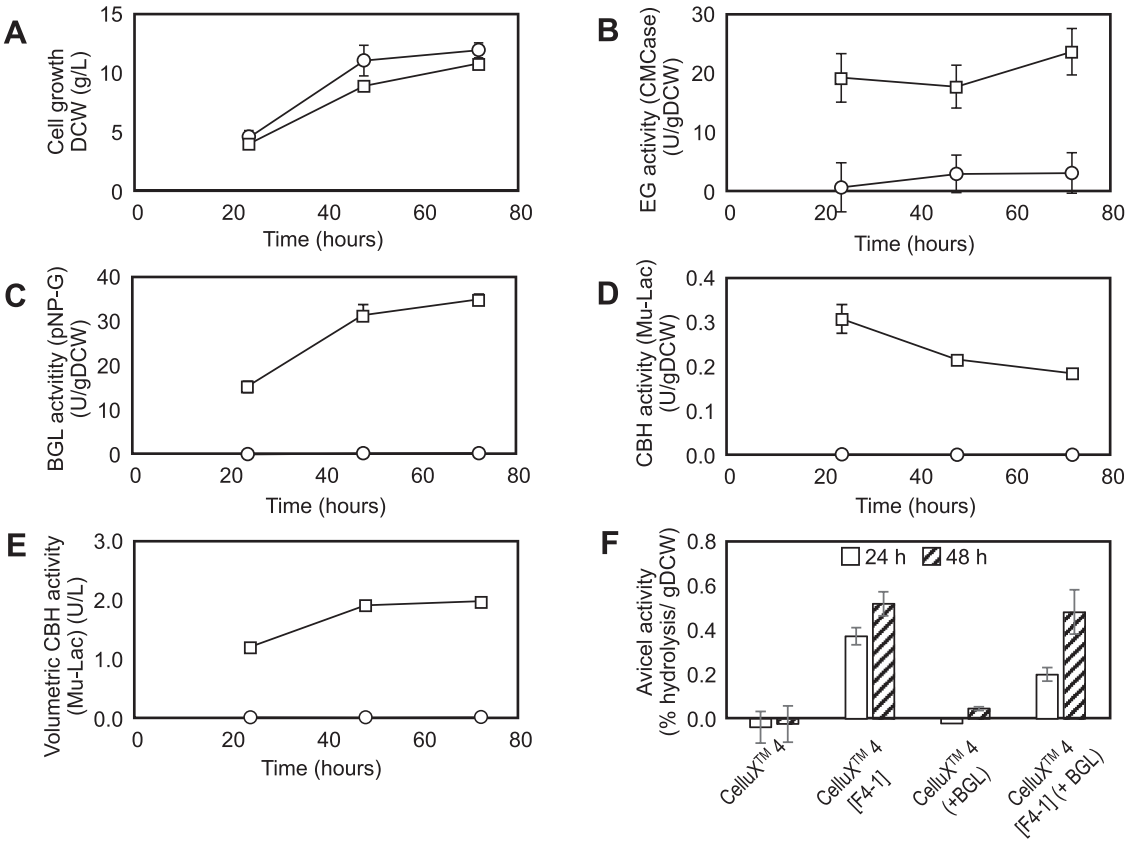

Fig. 3. The CelluX™ 4 (circles), CelluX™ 4[F4–1] (squares) strains were compared in terms of (A) Cell growth (gDCW/L); (B) endoglucanase activity on CMC; (C) β-glucosidase activity on *p*NPG; (D) specific cellobiohydrolase (CBH1) activity on Mu-Lac; (E) volumetric cellobiohydrolase activity (CBH1) on Mu-Lac and (F) activity on 1% Avicel with or without the addition of commercial BGL Novozym188. Values represent the mean of three repeats and error bars represent the standard deviations.

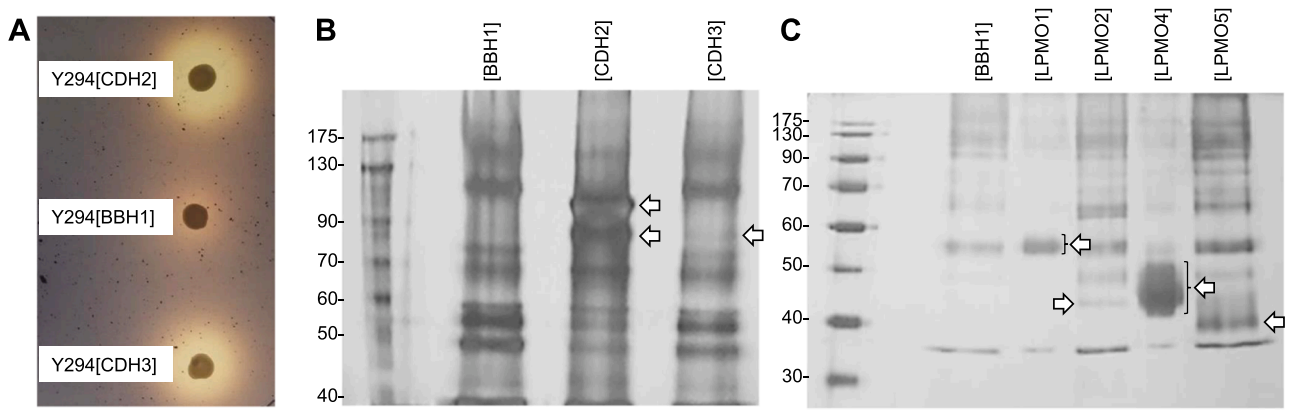

Fig. 4. (A) The *S. cerevisiae* Y294[BBH1], Y294[CDH2] and Y294[CDH3] strains were spot-inoculated onto DCPIP-containing SC^{-URA} plates for qualitative analysis of cellobiose dehydrogenase activity. SDS-PAGE analysis of the supernatants from *S. cerevisiae* Y294 strains expressing (B) CDHs and (C) LPMOs with relevant protein species indicated by arrows. The molecular mass marker (with sizes in kDa) is depicted on the left-hand side.

55 kDa, which is typical of a heterogeneously glycosylated protein. These protein species were slightly larger than their calculated molecular weights (32–35 kDa), which may be due to glycosylation often observed for recombinant proteins expressed in *S. cerevisiae* [38]. The SDS-PAGE analysis thus confirmed that all the CDHs and LPMOs were secreted into the medium and that their native secretion signals were recognised by *S. cerevisiae* Y294.

3.2. Cellobiose dehydrogenase activity of recombinant Y294[CDH] strains

The *S. cerevisiae* Y294[BBH1], Y294[CDH2] and Y294[CDH3] strains were cultured in 2 ×SC^{-URA} medium at 30 °C and the activity was monitored over time. The *S. cerevisiae* Y294[BBH1] strain generated more biomass than the Y294[CDH2] and Y294[CDH3] strains, suggesting that the expression of the CDHs resulted in additional stress or a metabolic burden on the cells (Fig. 5A). The Y294[BBH1] strain displayed a maximum activity of 5.5 U/L at 96 h, which is likely non-specific activity (Fig. 5B). The *S. cerevisiae* Y294[CDH2] and Y294[CDH3] strains displayed a steady increase in activity over time and reached maxima of 16.08 and 7.36 U/L after 96 h, respectively. The volumetric activities were lower than the 44.29 ± 9.48 U/L reported by Conacher et al. [39] for the *N. crassa* CDH expressed in *Komagataella phaffii* (*Pichia pastoris*), but exceeded that of the *M. thermophilum* CDH expressed in *K. phaffii* (3.70 ± U/L) in the same study. Expression in *K. phaffii* has typically resulted in more enzymes being secreted due to a higher biomass production than for *S. cerevisiae* in the same conditions [40]. The CDH2 and CDH3 displayed pH optima of 5 and 6, respectively (Fig. 5C).

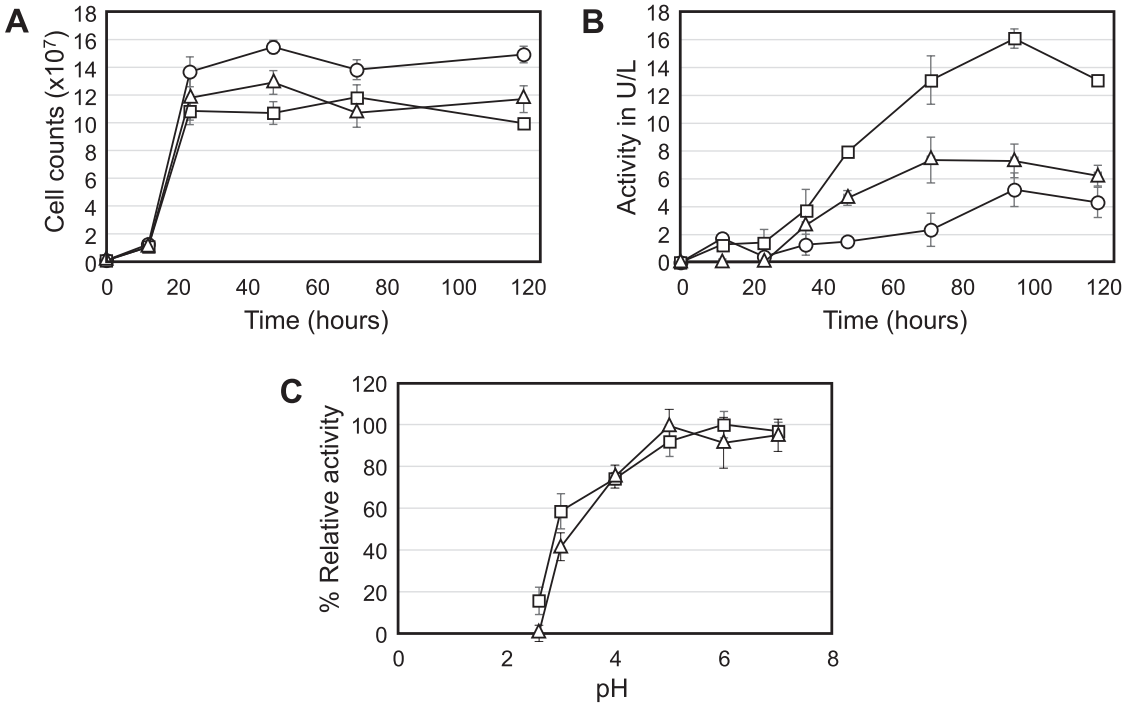

Fig. 5. The *S. cerevisiae* Y294[BBH1] (circles), Y294[CDH2] (squares) and Y294[CDH3] (triangles) strains were cultivated in 2 ×SC^{-URA} medium for 120 h and the (A) biomass and (B) CDH activity monitored daily. The supernatant of *S. cerevisiae* Y294[CDH2] and Y294[CDH3] cultures were used to determine the pH optimum of (C) CDH2 and CDH3, respectively. Values represent the mean of three repeats and error bars represent standard deviations.

3.3. Lytic polysaccharide monooxygenase activity of recombinant Y294 [LMPO] strains

The supernatant from the *S. cerevisiae* Y294[BBH1], Y294[LPMO1], Y294[LPMO2], Y294[LPMO4] and Y294[LPMO5] strains was collected daily for 7 days and tested for LPMO activity as described by Breslmayr et al. [32] and Brander et al. [33]. No activity could be detected at any time point by either method, despite confirmation of extracellular protein by SDS-PAGE (Fig. 4 C). It is possible that the activity displayed by the LPMO enzymes was below the detection limit of the assay methods or that the glycosylation of the proteins negatively affected enzyme activity [38]. Incorrect protein maturation may also have yielded proteins without the required N-terminal histidine or the methylation of this amino acid residue involved in copper binding [41]. Nevertheless, it has been shown that LPMOs can still be functional on their native polymeric substrates even when not showing activity on soluble substrates, although these polymeric activities may be challenging to detect [7,41].

3.4. Cellulase activity supplemented with LPMO and CDH on crystalline cellulose

A polymeric substrate containing both amorphous and crystalline regions is required to test the effect of the LPMOs and CDHs in combination with other cellulase enzymes. Grade 1 Whatman® filter paper is commonly used as a substrate to determine cellulase activity and has a high cellulose content without being too recalcitrant or too susceptible to enzymatic hydrolysis [42]. The hydrolysis of the Whatman paper was monitored in the presence of the complete set of core cellulases produced by CelluX™ 4[F4–1], and in the presence of different combinations of the supernatant from the different LPMO and CDH-producing strains. All the combinations that contained CDHs and/or LPMOs released more glucose than CelluX™ 4[F4–1] + BBH4, the supernatant from the Y294 negative control strain (Fig. 6 A). The addition of either CDH2 or CDH3 to the CelluX™ 4[F4–1] supernatant had a small, but significant impact, yielding 0.27 and 0.29 mg/L glucose after the 1-hour incubation, respectively. The addition of the LPMOs only improved glucose release for the CelluX™ 4[F4–1] + CDH2 + LPMO2 and CelluX™ 4[F4–1] + CDH3 + LPMO1 combinations (0.66 and 0.95 mg/L glucose, respectively).

Cellobiose levels of 7.06 mg/L were obtained for the CelluX™ 4 [F4–1] + CDH2 + LPMO1 combination, 3.88 mg/L for CelluX™ 4 [F4–1] + CDH2 + LPMO2 and 1.52 mg/L for CelluX™ 4[F4–1] + CDH3 + LPMO1 (Fig. 6B). The cellobiose accumulation could suggest inefficient β-glucosidase activity in CelluX™ 4[F4–1]. However, this seems unlikely as the results shown in Fig. 3F demonstrate that BGL activity was not a limiting factor in Avicel hydrolysis by the CelluX™ 4 [F4–1] strain. It is thus more likely that the cellobiose levels detected by HPLC represented cellobiolactone produced from cellobiose through CDH2 activity, which cannot be hydrolysed by a β-glucosidase.

The hydrolysis of Whatman paper indirectly confirms the activity of LPMO1 and LPMO2, despite the lack of activity detected with the colorimetric LPMO assays. It could therefore be concluded that the LPMO1 worked well in combination with CDH2 and CDH3, whereas the LMPO2 worked best in combination with CDH2. It remains unclear if LPMO4 and LPMO5 were indeed produced in an active form. Nevertheless, the additional glucose released by the CelluX™ 4[F4–1] cellulase mixture supplemented with CDH2 + LPMO1/2 and CDH3 + LPMO1 combinations suggests that additional ethanol could be produced from the cellulosic substrate.

Cellulases are known to work cooperatively to hydrolyse cellulosic plant material, but recalcitrant regions are unlikely to be efficiently hydrolysed in the absence of CDHs and LPMOs. The successful production of LPMOs and CDHs in *S. cerevisiae* and the positive indication of cooperation between these enzymes and the core cellulases is a positive step in the development of an *S. cerevisiae* strain that can hydrolyse plant material efficiently.

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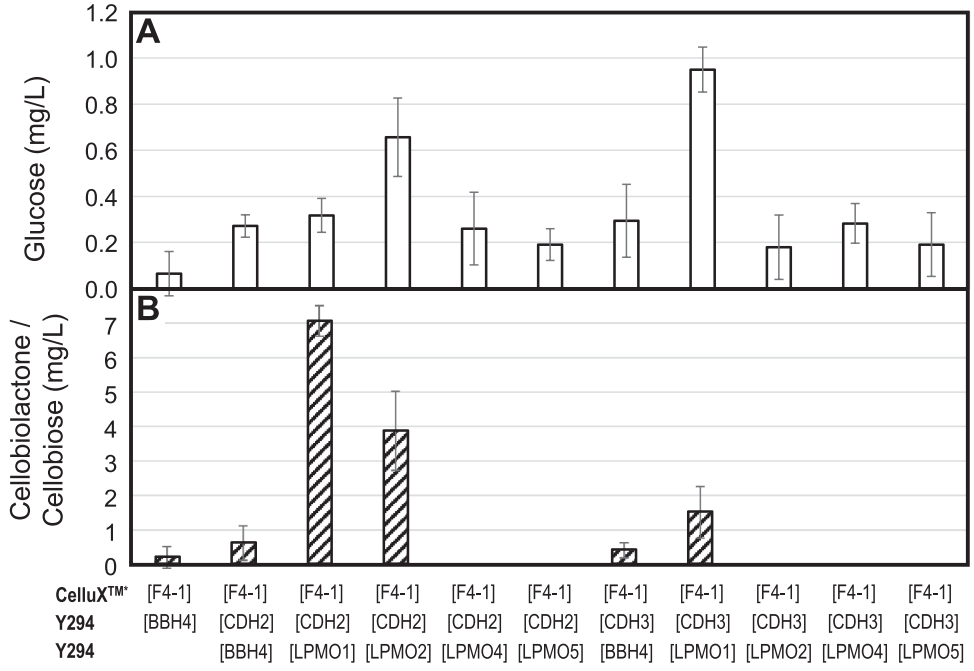

Fig. 6. (A) Glucose and (B) cellobiose/cellobiolactone released from Whatman paper when CelluX™ 4[F4–1] supernatant was incubated with that of the LPMO and CDH-producing strains. Values represent the mean of three repeats and error bars represent the standard deviations.

CRediT authorship contribution statement

All authors contributed to the conception and design of the study. Material preparation, data collection and analysis were performed by Ivy Smuts. Riaan den Haan selected and designed the genes encoding for auxiliary activities and determined cellulase activity. Nicole Blakeway screened the initial CelluXTM 4 CBP transformants that led to the selection of the CelluXTM 4[F4-1] strain. The first draft of the manuscript was written by Ivy Smuts and subsequent revisions were done by Shaunita Rose, Marinda Viljoen-Bloom and Willem Heber van Zyl. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no relevant financial or non-financial interests to disclose.

Data availability

No data was used for the research described in the article.

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