Rapid optimisation of cellulolytic enzymes ratios in Saccharomyces cerevisiae using in vitro SCRaMbLE

Elizabeth L. I. Wightman
Macquarie University Faculty of Science and Engineering

Heinrich Kroukamp (✉ heinrich.kroukamp@gmail.com)
Macquarie University Faculty of Science and Engineering https://orcid.org/0000-0002-9141-3112

Isak S. Pretorius
Macquarie University

Ian T. Paulsen
Macquarie University Faculty of Science and Engineering

Helena K. M. Nevalainen
Macquarie University Faculty of Science and Engineering

Research

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Abstract

**Background:** For the economic production of biofuels and other valuable products from lignocellulosic waste material, a consolidated bioprocessing (CBP) organism is required. With efficient fermentation capability and attractive industrial qualities, *Saccharomyces cerevisiae* is a preferred candidate and has been engineered to produce enzymes that hydrolyze cellulosic biomass. Efficient cellulose hydrolysis requires the synergistic action of several enzymes; with the optimum combined activity ratio dependent on the composition of the substrate.

**Results:** *In vitro* SCRaMbLE generated a library of plasmids containing different ratios of a β-glucosidase gene (*CEL3A*) from *Saccharomycopsis fibuligera* and an endoglucanase gene (*CEL5A*) from *Trichoderma reesei*. *S. cerevisiae*, transformed with the plasmid library, displayed a range of individual enzyme activities and synergistic capabilities. Furthermore, we show for the first time that 4,6-O-(3-Ketobutylidene)-4-nitrophenyl-β-D-cellopentaoside (BPNPG5) is a suitable substrate to determine synergistic Cel3A and Cel5A action and an accurate predictive model for this synergistic action was devised. Strains with highest BPNPG5 activity had an average *CEL3A* and *CEL5A* gene cassette copy number of 1.3 ± 0.6 and 0.8 ± 0.2 respectively (ratio of 1.6:1).

**Conclusions:** Here we describe a synthetic biology approach to rapidly optimize gene copy numbers to achieve efficient synergistic substrate hydrolysis. This study demonstrates how *in vitro* SCRaMbLE can be applied to rapidly combine gene constructs in various ratios to allow screening of synergistic enzyme activities for efficient substrate hydrolysis.

Background

Biofuels, made from renewable lignocellulosic biomass, are an attractive alternative to fossil fuels, however, more economic production methods are required. One strategy is to harness a consolidated bioprocessing (CBP) organism which would produce and secrete enzymes that hydrolyze cellulosic material, efficiently carry out fermentation, and be suitable for use at an industrial scale [1, 2]. The yeast *Saccharomyces cerevisiae* is a well-suited CBP candidate that fulfils the latter two requirements, however it does not produce the enzymes required for biomass hydrolysis. The conversion of cellulose into fermentable sugars requires the synergistic action of three main classes of enzymes including β-glucosidase (BGL), endoglucanase (EG) and cellobiohydrolase (CBH) which must be introduced to *S. cerevisiae* [3].

There have been continued efforts towards constructing CBP-ready *S. cerevisiae* strains by sourcing suitable genes from cellulolytic organisms such as *Trichoderma reesei, Saccharomycopsis fibuligera, Clostridium thermocellum* and *Aspergillus aculeatus* [4, 5, 6]. The construction of these strains has
mainly followed the strategies of either producing secreted enzymes or cell-wall tethered enzymes (both individually-bound and assembled in synthetic mini cellulosomes) (reviewed by [7]). As a result, S. cerevisiae strains capable of simultaneous saccharification and fermentation (SSF) on a range of simple cellulosic substrates are available [8, 9, 10], but the ratios of the enzymes required for optimal hydrolysis changes depending on the available substrate and mostly remains undefined [11]. Therefore, many efforts have now turned to the optimisation of enzyme ratios by harnessing different promoters [12], combining specific ratios of yeast strains displaying different enzymes [13] and manipulating gene copy numbers one by one [14].

There have been many rational engineering approaches to enhance cellulase production and secretion in S. cerevisiae (reviewed in [15]), however, since the best gene ratio to produce efficient hydrolysis for each substrate is not always known, techniques that generate large libraries of DNA and/or strains containing different gene ratios provide a promising approach. In previous work, Yamada et al. [16] produced a library of cellulolytic yeast strains using cocktail δ-integration. A. aculeatus CEL3A (encoding BGLI) and T. reesei CEL5B and CEL6A (encoding EGII and CBHII respectively) were randomly integrated into yeast chromosomes in one step, and strains with high enzyme activity on PASC (phosphoric acid swollen cellulose) were obtained. A strain was isolated that contained 1, 13 and 6 copies of CEL3A, CEL5B and CEL6A respectively, which achieved a PASC degradation activity of 64.9 mU per gram of wet cell weight. In highlighting the importance of optimal gene ratio rather than overexpression, this strain exhibited higher enzyme activity than the rationally engineered strain, despite having fewer enzyme-encoding gene copies. However, this approach may not give an accurate representation of the most ideal ratio of enzymes, as genes could be integrated in chromosome locations where expression is minimal (e.g. near transcriptionally repressed regions of the telomeres) [17].

A synthetic biology tool which could be used to develop CBP S. cerevisiae strains is SCRaMbLE (synthetic chromosome rearrangement and modification by loxPsym-mediated evolution) which facilitates accelerated genome evolution by initiating large-scale DNA recombination in vivo [18]. While “in vivo” SCRaMbLE is limited to SCRaMbLE-enabled synthetic chromosomes within the ‘Yeast 2.0’ initiative [18], the in vitro SCRaMbLE of DNA in a test tube generates diverse plasmid libraries that are not only compatible with Yeast 2.0, but can be transformed into any host including E. coli and non-synthetic yeasts [19]. In addition, host strains do not require DNA for expression of Cre recombinase, or loxPsym sites required for in vivo SCRaMbLE. Considering this and the fact that in vitro SCRaMbLEd DNA is intended to be maintained as plasmids in the cell, the host genome remains unaltered, retaining any valuable phenotypes of the strain, a feature of importance especially concerning industrial yeasts. In vitro SCRaMbLE has been used for S. cerevisiae in the optimisation of pathways producing β-carotene and violacein, revealing valuable genotype-to-phenotype relationships [19, 20].
Here, *in vitro* SCRaMbLE was applied to generate a plasmid library containing different ratios of the β-glucosidase gene *CEL3A* from *S. fibuligera* and the endoglucanase gene *CEL5A* from *T. reesei*. The DNA library was directly transformed into *S. cerevisiae* and synergistic enzyme activity of the recombinant strains was determined using BPNPG5 (Megazyme® K-CellG5-4V) as a substrate for the first time, enabling rapid screening of the transformants. By harnessing the strategy of *in vitro* SCRaMbLE, coupled with the screening method presented here, the copy number and activity ratio of CEL3A and CEL5A to confer the most efficient activity on BPNPG5 was determined.

**Results**

**Plasmid library generation by *in vitro* SCRaMbLE**

The *in vitro* SCRaMbLE strategy applied for engineering cellulolytic *S. cerevisiae* strains is summarized in Fig. 1). A library of plasmids was constructed by *in vitro* SCRaMbLEing loxPsym-flanked *CEL3A-HIS3* and *CEL5A-MET17* cassettes into acceptor plasmids (pAcceptor) containing loxPsym sequences and *hphMX4* conferring hygromycin resistance. The generated plasmid library was directly transformed into *S. cerevisiae* BY4741 without plasmid enumeration in *Escherichia coli*. All of the 160 randomly picked putative yeast transformants, selected on SC-*his*-met supplemented with 200 μg mL⁻¹ Hygromycin B agar plates, were confirmed using PCR, to contain the pAcceptor vector and at least one copy of the *CEL3A* and *CEL5A* expression cassettes.

**Cellulolytic activity of the *S. cerevisiae* transformants**

The individual and synergistic supernatant enzyme activity of Cel3A and Cel5A produced by the 160 randomly selected *S. cerevisiae* colonies were determined to obtain a comprehensive understanding of the diversity generated by the SCRaMbLEd plasmid library. A wide range of Cel3A and Cel5A enzymatic activities were observed for the evaluated strains, ranging between 0 - 115.86 ± 8.74 U/mL and 0 - 102.32 ± 5.20 U/mL respectively, with a subset of individual strains having both high Cel3A and Cel5A activity (Fig. 2). In general, strains exhibiting both high Cel3A and Cel5A activity also had high BPNPG5 activity (Fig. 2 – sphere diameter). Multiple regression analysis confirmed a strong correlation ($R^2 = 0.97$) between BPNPG5 activity and the combined activities of the Cel3A and Cel5A (Fig. 3). Twelve strains exhibiting the highest BPNPG5 activity were selected for further analysis. Enzyme activity data of these strains are shown in Table 3 and are indicated as green spheres in Fig. 2.

**BPNPG5 as a substrate to measure synergistic activity of Cel3A and Cel5A**

The synergistic action of incremental Cel3A and Cel5A activity changes on BPNPG5 hydrolysis, and the optimum activity ratio were determined (Fig. 4). No detectible pNP release were observed from Cel3A...
activity alone, with some activity detected in the presence of Cel5A only (0.002 ± 0.0009 U). At the respective lowest evaluated Cel3A and Cel5A ratios, namely 10:90 and 90:10, the presence of both enzymes resulted in significantly higher \( \rho \)-NP release than the sum of the individual actions of the enzymes on BPNPG5 (\( p < 0.001 \)). Maximum BPNPG5 hydrolysis was achieved with Cel3A:Cel5A ratios of 40:60 and 50:50 (enzyme activities using these two ratios were not significantly different, \( P > 0.8 \)) and the following equation was generated to model the action of Cel3A and Cel5A on the BPNPG5 substrate with an \( R \) squared value of 0.9658 with 156 degrees of freedom:

\[
Y = 8.305e-3 + 4.1e-5*A + 6e-6*B + 7.594e-6*A*B
\]

Where: \( Y \) = Synergistic enzyme activity of Cel3A and Cel5A (U), \( A \) = Cel3A activity (U), \( B \) = Cel5A activity (U), significance statistics are listed in Table 2. The ability to produce \( \rho \)-nitrophenol from BPNPG5 is highly dependent on synergy between Cel3A and Cel5A.

To determine how well the strain supernatant activities fits our model, measured BPNPG5 hydrolysis was compared with model-predicted BPNPG5 hydrolysis based on individual Cel3A and Cel5A activity of the 160 evaluated strains (Fig. 3). The measured supernatant BPNPG5 hydrolysis correlated strongly with the corresponding strain's predicted BPNPG5 hydrolysis (\( R^2 = 0.97 \)).

**Determining CEL3A and CEL5A cassette ratios conferring high BPNPG5 activity**

The ratio and copy numbers of CEL3A and CEL5A on *in vitro* SCRaMbLEd plasmids in *S. cerevisiae* were determined by qPCR (Fig. 5). Two sets of strains were analysed; the ‘high activity’ group contained 6 strains that showed the highest BPNPG5 activity and the ‘intermediate activity’ group which contained 6 strains exhibiting enzyme activities which had approximately the median BPNPG5 activity. The average ratio of CEL3A to CEL5A genes in strains with the highest BPNPG5 activity was ~1.6:1. This ratio was not significantly different to the ratio observed in strains from the intermediate activity group (\( p > 0.2 \)). However, there was a significant difference in gene copy number between the high and intermediate activity groups; The copy numbers of CEL3A and CEL5A in the high group were 1.3 ± 0.6 and 0.8 ± 0.2 respectively and in the intermediate group were 0.12 ± 0.10 and 0.26 ± 0.35 respectively. There was a significant difference in copy number for both CEL3A high and intermediate groups (CEL3A: \( p < 0.01 \)) and for CEL5A (\( p < 0.03 \)). There was no significant difference between the plasmid copy number per cell (represented by the *hphMX* cassette abundance, Fig. 5) between the high activity and intermediate activity groups (\( p > 0.2 \)).

**Discussion**

In the pursuit of economic bioproduct production from renewable lignocellulosic waste sources, consolidated bioprocessing is considered essential [2]. Significant progress has been made over the last
three decades by engineering yeast and other organisms with synthetic cellulolytic and hemi-cellulolytic capabilities (reviewed in [21]). With the major hydrolytic activities achieved in yeast independently, the focus in recent years has shifted to combinatorial cellulolytic enzyme expression and improving substrate unitisation efficiencies [10, 16, 22], with much interest in harmonising the synergistic action of the different enzymatic activities [11]. In this study we aimed to develop a rapid cellulase expression optimisation system in yeast, allowing selection based on the highest synergistic enzymatic activities.

The generation of expression vectors with randomized copies of CEL3A and CEL5A was achieved through a simple in vitro assembly strategy where loxP-flanked expression cassettes were combined with a suitable acceptor vector. Similar to random gene integration approaches, our in vitro SCRaMbLE strategy allows the evaluation of gene combinations with possible product interdependencies or synergies, but with an easily scalable DNA assembly step, with the capability to generate much greater combinatorial variations. In addition, the transformed in vitro SCRaMbLE library is not impacted by chromatin fluidity of the integration site which may impact gene transcription level variations. In our focussed screen within strains with high synergistic enzymatic activity, insertion of up to four expression cassettes was achieved (strain H4, data not shown). This is similar to a previous study which reported up to five insertions for β-carotene synthesis pathway assembly, using a similar methodology [19]. In the previous study and this work, only a single round of in vitro SCRaMbLEing was performed; the potential of combinatorial assembly of a wider range of gene cassettes or subsequent rounds of in vitro SCRaMbLE holds the promise of achieving even higher insertion rates.

To facilitate the efficient screening of the combinatorial vector library, a rapid method was developed utilising the BPNPG5 substrate to evaluate the synergistic enzyme activity of Cel3A and Cel5A. While BPNPG5 has previously been used to measure the activity of endoglucanase activity [23], this is to our knowledge the first time BPNPG5 has been used to assess synergistic Cel3A and Cel5A activity.

Our modelling results showed that maximum activity occurred at a Cel3A to Cel5A activity ratio of between 40:60 and 50:50 (Fig. 4), however not all library strains displaying this ideal activity ratio had high BPNPG5 hydrolysis. Up to a limit, the more total enzymatic activity that was present, in the ideal ratio, the higher the BPNPG5 hydrolysis was (Fig. 2). This observation is in line with the basic principles of enzyme kinetics. The impact of enzyme activity levels on BPNPG5 hydrolysis was clearly shown by the comparative BPNPG5 activities of the selected “high activity” and “intermediate activity” groups. Both groups had a similar Cel3A and Cel5A activity ratio (p>0.1), however the high activity group had significantly higher Cel3A and Cel5A activities (p<0.01, p<0.02 respectively) and thus represented library strains with both the ideal gene ratio and optimized enzyme levels.

Reflecting the higher enzymatic activities of the “high activity” group, significantly higher numbers of cellulase expression cassettes were detected per cell, than the corresponding “intermediate activity” group. Strains with the highest BPNPG5 activity had on average 1.3 ± 0.6 CEL3A and 0.8 ± 0.2 CEL5A cassettes per cell. Between the two selected groups, vectors with one to four cellulase cassette inserts were observed (corresponding to up to 21,158 bp), however this did not have a detectable negative
impact on plasmid copy numbers, with all strains having approximately two plasmids per cell (Fig. 5). Although no significant variation in plasmid copy numbers were observed between cells containing different vector sizes in our study, it is reasonable to expect that the incorporation of more DNA, and the metabolic burden of subsequent increased protein production, could impact the overall plasmid copy number per cell [24]. This burden might have been minimized by the unexpected, but previously observed [25], low episomal plasmid copy numbers per cell.

Many factors are at play that influences the optimal ratio of enzyme-encoding genes. The strategy presented here harnessing in vitro SCRaMbLE efficiently produces diverse libraries of randomized gene-copies in a standardized fashion that can be rapidly screened to uncover optimal ratios for substrates of different compositions. Up to date, rational engineering approaches to optimize enzyme ratios of cellulolytic strains have been limited due to the complexity brought about by (1) gene expression levels, (2) the specific activity and mode of action of enzymes from different origins, (3) the consortium of enzymes used and their relevant synergistic enzyme kinetics, and (4) whether genes will be integrated or maintained on expression plasmids. In a semi-rational screening approach, different optimal gene ratios were found using the cocktail δ-integration method. A S. cerevisiae strain with high activity on PASC contained 1, 13 and 6 copies of Aspergillus aculeatus CEL3A, T. reesei CEL5A and T. reesei CEL6A (Celllobiohydrolase II-encoding gene) respectively [16]. The differences in gene copy number (compared to the results described here) are not surprising, as a CEL3A from a different origin was used (from A. aculeatus), the ratio was optimized for activity on a different substrate (PASC) and the action of an addition enzyme was used (Cel6A). Additionally, δ-integration relies on targeting gene cassettes to random retro-transposon sequences throughout the yeast genome, allowing the insertion into genomic regions where expression might be limited or actively silenced (such as near telomeres) and thus might not indicate the ‘ideal’ cassette copy number required for re-engineering purposes. While two cellulase-encoding gene cassettes were used here in this proof-of-concept study, any enzyme-encoding genes could be added to the in vitro SCRaMbLE strategy where specific synergy optimisation between activities is required. In a CBP context, other genes such as those encoding celllobiohydrolases (CBH) or other supplementary activities could be added to enable the hydrolysis of more recalcitrant cellulose substrates such as Avicel. In the interest of uncovering precise ratios for efficient activity, the use of weaker promoters could allow smaller incremental changes and an even more gradual evaluation of expressed enzyme synergistic action.

Conclusions

The economic production of fuels or other chemicals from biomass requires the efficient conversion of all complex polysaccharides into fermentable sugars. Optimizing the ratios of the different hydrolytic activities of enzymes produced by recombinant yeast could achieve efficient release of sugars. In this study, we utilised BPNPG5 as a novel substrate to explore the synergistic action of an endo- and β-glucosidase produced in yeast. Using this substrate, we then proceeded to showcase in vitro SCRaMbLE as a novel synthetic biology tool to rapidly generate large combinatorial plasmid libraries for the co-expression of recombinant cellulases and ultimately demonstrated the capability to use this highly
tunable method to optimize, potentially any, enzyme system requiring synergy for direct transformation into suitable cell factories for screening.

**Materials And Methods**

**Strains and media**

The yeast strain used in this study was *Saccharomyces cerevisiae* BY4741 (MATa, *his3Δ1, leu2Δ0, met17Δ0, ura3Δ0*) [26]. Cultivation was carried out in liquid or solid (20 g L\(^{-1}\) w/v agar) YPD (10 g L\(^{-1}\) w/v yeast extract, 20 g L\(^{-1}\) w/v peptone, 20 g L\(^{-1}\) w/v glucose). Selection medium was 1.7 g L\(^{-1}\) yeast nitrogen base without amino acids and ammonium sulfate (Millipore), 1 g L\(^{-1}\) L-Glutamic acid monosodium salt monohydrate (Sigma-Aldrich), 20 g L\(^{-1}\) w/v glucose, 120 mg L\(^{-1}\) L-Leucine (Sigma-Aldrich), 20 mg L\(^{-1}\) Uracil (Sigma-Aldrich), 200 µg ml\(^{-1}\) Hygromycin B (Invivogen) and 20 g L\(^{-1}\) w/v agar. All yeast cultivation was done at 30 °C.

**Cellulase control strain construction**

Standard protocols were used for expression vector preparation [27]. Restriction endonucleases, T4 DNA ligase and Phusion DNA polymerase were purchased from Thermo Scientific and used as directed by the manufacturer. The Zymoclean Gel DNA Recovery kit (Zymo Research) was used according to the manufacturer’s instructions to elute digested DNA from agarose gels. For the construction of the episomal cellulase expression plasmids, the hygromycin B resistance (*hphMX4*) cassette was obtained from the pBHD1_SOD1 plasmid [28] by digesting with *Bam*H1 and *Spe*I and subsequent ligation into the pMU1531 [29], creating pHK112. The *Saccharomycopsis fibuligera* CEL3A and *Trichoderma reesei* CEL5A ORFs were amplified from the ySFI vector [30] and pLEGII vector [31] respectively, using primers containing restriction sites for PacI and Ascl. These PCR fragments were separated on an agarose gel, purified, digested with PacI and Ascl, and cloned into the corresponding sites of the pHK112 vector, yielding pHK112_S.f.CEL3A and pHK112_Tr.CEL5A. The integrity of the constructs were verified with Sanger sequencing (Central Analytical Facility, Stellenbosch, South Africa). The pHK112_S.f.CEL3A and pHK112_Tr.CEL5A vectors were transformed into BY4742 strains [26] using the LiOAc/SS carrier DNA/PEG method as described by Gietz and Schiestl [32] and recovered for 4-6 hours in liquid YPD medium at 30°C and subsequently plated out on YPD agar plates containing 300 µg/mL hygromycin B. The presence of the expression plasmid in putative transformants was confirmed with polymerase chain reaction (PCR) analysis using the following primers; 5’-GGATCCACTAGTTCTAGGGGTATACG-3’ and 5’-GACTGGCGCGCTTACAAACATTGAGTATAGTGGG-3’. Confirmed transformants harbouring the pHK112_S.f.CEL3A and pHK112_Tr.CEL5A vectors were referred to as BY4242[Cel3A] and BY4742[Cel5A] respectively.
**SCRaMble acceptor plasmid construction**

Detailed diagrams of all plasmid maps are provided in the supplementary material. The acceptor plasmid (pAcceptor) was constructed by replacing the cellulase expression cassette with two loxPsym sites in the backbone pHK112 vector. The loxPsym pair was PCR amplified from *S. cerevisiae* synthetic chromosome XIV (unpublished, from our laboratory). The 862 bp between the loxPsym sites contains no known coding sequences. A PCR was performed to amplify this sequence, with 200 µM dNTPs, 0.5 µM of “loxP pair SynXIV F” primer and 0.5 µM of “loxP pair SynXIV R” primer (Table 1), 50 ng of template DNA (gDNA from a yeast strain containing synthetic chromosome XIV), 1x Phusion® HF buffer (NEB) and 1 unit/50 µl Phusion® High-Fidelity DNA Polymerase (NEB) in a final volume of 50 µl, with the following program: 1 cycle of 98 °C for 30 sec, 30 cycles of 98 °C for 30 sec, 55 °C for 30 sec and 72 °C for 30 sec and 1 cycle of 72 °C for 5 min. The primers were designed to produce a PCR product with ends homologous to the plasmid backbone, as such, pAcceptor (Fig. S1) was constructed using Gibson assembly with the NEBuilder® HiFi DNA Assembly Master Mix (NEB) [33] according to the manufacturer’s instructions.

**SCRaMble donor plasmid construction**

The two donor DNA plasmids were chemically synthesized by GenScript, USA. pCEL3A-loxP contained the 2717 bp *S. buligera* CEL3A ORF encoding a β-glucosidase I, flanked by the constitutive TEF1 promoter and the homologous HXT7 terminator for transcriptional control, followed by HIS3 as an auxotrophic marker (Fig. S2). pCEL5A-loxP contained a *S. cerevisiae* codon-optimized 1194 bp *T. reesei* CEL5A encoding endoglucanase with an upstream 57 bp *T. reesei* XYN2 signal sequence. CEL5A was placed under the transcriptional control of the PGK1 promoter and the homologous HXT1 terminator followed by MET17 as an auxotrophic marker (Fig. S3). The entire cassettes on both pCEL3A-loxP and pCEL5A-loxP plasmids were flanked by loxPsym sites and reside in the multiple cloning site of a pUC57 cloning plasmid.

**In vitro SCRaMble**

The bottom-up *in vitro* SCRaMble strategy, described by [19], specifies that loxPsym-flanked donor DNA is SCRaMbled into the loxPsym sites in an acceptor plasmid, facilitated by Cre recombinase. *In vitro* SCRaMble was performed as previously described, with adjustments. Briefly, the 50 µl reaction was set up with 1 unit of Cre recombinase (NEB), 400 ng acceptor plasmid, 800 ng pCEL5A-loxP and 800 ng pCEL3A-loxP. Following 1 h incubation at 37 °C, the Cre recombinase enzyme was deactivated at 70 °C for 10 min.

**Transformation and of plasmid library**
The library of *in vitro* SCRaMbLEd plasmids was transformed into BY4741 *S. cerevisiae* [26] as described above. Following transformation, cells were recovered in YPD medium for 4 h, shaking at 200 rpm. Cells were selected on SC-\textsuperscript{-met}-\textsuperscript{-his} agar to isolate putative transformants which contained pAcceptor and at least one copy of *CEL3A* and *CEL5A*.

**Enzyme assays**

Supernatants from 160 *S. cerevisiae* cultures grown for 48 h in YPD supplemented with 200 μg mL\(^{-1}\) Hygromycin B were collected for measurement of enzymatic activity. CEL5A activity of culture supernatants was determined by DNS (dinitrosalicylic acid) assay. 10 μl of culture supernatant was incubated with 70 μl of 10 g L\(^{-1}\) w/v carboxymethyl cellulose (CMC) in 0.05 M sodium acetate buffer pH 5.0 at 50 °C for 10 min. The addition of 120 μl of DNS reagent [34] was added for determination of reducing sugars. Reactions were boiled and absorbance was measured at 540 nm. For CMC assays, glucose was used to draw a standard curve in the range of 3–50 mM from which the amount of enzymatic Units of each sample was calculated. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μM of reducing sugar per min. CEL3A activity was determined by the release of \(\rho\)-nitrophenol from \(\rho\)-nitrophenyl-\(\beta\)-glucoside (\(\rho\)NPG). 10 μl of culture supernatant was incubated with 1 μl of 1 mM \(\rho\)NPG and 89 μl of 0.05 M Sodium Acetate Buffer pH 5.0, at 50 °C for 10 min. The reaction was stopped with 100 μl of 1 M sodium carbonate and absorbance was measured at 400 nm. One unit of enzyme activity was defined as the amount of enzyme required for producing 1 μM of \(\rho\)-nitrophenol from the substrate per min. The synergistic activity of CEL5A and CEL3A was determined by the release of \(\rho\)-nitrophenol from BPNPG5 (4,6-O-(3-Ketobutylidene)-4-nitrophenyl-\(\beta\)-D-cellobiose) obtained from Megazyme. The Cellulase Assay Kit (CellG5 Method) (Megazyme® K-CellG5-4V) was used per the manufacturer’s instructions, except that the addition of \(\beta\)-glucosidase was substituted for 0.05 M acetate buffer. Culture supernatants were incubated with BPNPG5 for 10 min at 37 °C and absorbance was recorded at 405 nm. One unit of enzyme activity was defined as the amount of enzyme required for producing 1 μM of \(\rho\)-nitrophenol from the substrate per min. For \(\rho\)NPG and BPNPG5 assays a \(\rho\)NP standard curve in the range of 1.5–25 mM was used.

**Synergy evaluation and modelling**

To evaluate the suitability of BPNPG5 as a substrate to reflect synergistic enzyme activity of Cel3A and Cel5A, supernatants of BY4242[Cel3A] and BY4742[Cel5A] cultures containing either Cel3A or Cel5A, were prepared and the respective enzymatic activities determined using \(\rho\)NPG and DNS assays. Supernatants were mixed in different ratios and BPNPG5 hydrolysis evaluated, as described above. Keeping the total enzymatic units constant (at 100 U), the combined action of different ratios (increments of 10%) of Cel3A and Cel5A were determined. To model this synergistic relationship between Cel3A and Cel5A activity a multiple linear regression analysis (least squares) was performed using GraphPad Prism version 8.01 for
Windows, GraphPad Software, La Jolla California USA (www.graphpad.com). Based on this model, theoretical BPNPG5 activity were predicted for 160 yeast strains with in vitro SCRaMbLed plasmids, and compared with measured BPNPG5 activity.

**Extraction of DNA from yeast**

Total DNA from yeast transformants was extracted using the dilute sodium hydroxide lysis method. In short, yeast cells 100 μl of an overnight yeast culture, grown in YPD supplemented with 200 μg ml⁻¹ Hygromycin B, were pelleted by centrifugation at 4000 rpm for 2 min. The cell pellet was resuspended in 20 mM NaOH. Cell suspensions were incubated at 95 °C for 10 min. After pelleting cell debris by centrifugation at 4000 rpm for 2 min, cell lysates were directly used for quantitative PCR.

**Determination of gene copy number in plasmids**

Quantitative real time PCR (qPCR) was carried out using the Roche LightCycler® 480 instrument. Each reaction contained 5 μl of Agilent Technologies Brilliant II SYBR® Green QPCR Master Mix, 200 nM forward primer, 200 nM reverse primer, 1 μl of DNA template (supernatant from NaOH extractions) and nuclease-free water to obtain a final volume of 10 μl. Four sets of primers were designed to specifically amplify ~150 bp of the CEL3A, CEL5A and hphMX4 cassettes on plasmids and the native TAF10 from the genome (primers are listed in Table 2). Primer amplification efficiencies were determined using serial dilutions of total extracted yeast DNA. The cycling protocol used an initial denaturing step of 95 °C for 1 min, followed by 40 cycles of 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 30 sec. Fluorescence was recorded following each 72 °C cycling step. Crossing point (Cp) values were calculated using the Absolute Quantification software modules of the LightCycler® 480 Software. The number of plasmids per cell was determined as the relative hphMX4 copies per sample compared to the relative number of TAF10 copies of the same sample.

**Declarations**

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**Competing interests**

The authors declare no conflicts of interest.
Ethics approval and consent to participate

No ethics approval required.

Consent for publication

All authors consents to the publication of this manuscript.

Availability of data and materials

Plasmids and strains developed in this study will be made available on request to the corresponding author. Additional data and plasmid maps are provided as supplementary data.

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Authors' contributions

HK and HKMN conceived research project. HK, ITP, ISP and HKMN participated in the design, support and coordination of the project. ELIW conducted the experiments. HK and ELIW analysed data. ELIW wrote the manuscript. HK and HKMN edited the manuscript. All authors read and approved the nal manuscript.

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**Tables**

**Table 1** Primers used in this study.

| Primer Name          | Primer Sequence                      |
|----------------------|--------------------------------------|
| loxP pair SynXIV F   | 5'- CCACTAGTCTTCTAGAAAGCATTTTCCATGA -3' |
| loxP pair SynXIV F   | 5'- CAAAGAGGTTTAGACGGCCAGGCGTATTCTGATGA -3' |
| qPCR CEL5A F         | 5'- AGTGGTGTAAGGTTGCTGG -3'          |
| qPCR CEL5A R         | 5'- GCTGCATTTTGACCGATACCG -3'       |
| qPCR hphMX4 F        | 5'- CTTGATGTAGGAGGCGTG -3'           |
| qPCR hphMX4 R        | 5'- TCAGGCTCTCGCTGATTC -3'           |
| qPCR CEL3A F         | 5'- GTAGGCTCCAAGACGTCTGG -3'         |
| qPCR CEL3A R         | 5'- CTAATCGAAACCCATG -3'             |
| qPCR TAF10 F         | 5'- ATATCCAGGAGTCGGCTTCGATG -3'      |
| qPCR TAF10 R         | 5'- GTAGTCTTCTCATTCTGTTGATGTGTGTT -3' |

**Table 2** $P$-values of variable used in the multiple regression analysis

| Variable | $P$-value |
|----------|-----------|
| Intercept| <0.0001   |
| A        | 0.0526    |
| B        | 0.0016    |
| A*B      | <0.0001   |
Table 3 Enzyme activity of twelve *S. cerevisiae* strains that had the highest synergistic activity on BPNPG5

| Strain No. | Cel3A activity (U/mL) | Cel5A activity (U/mL) | BPNPG5 activity (mU/mL) |
|------------|-----------------------|-----------------------|-------------------------|
| 1          | 97.1 ± 8.7            | 81.8 ± 3.45           | 81.3 ± 3.69             |
| 2          | 115.9 ± 8.7           | 82.2 ± 5.41           | 80.9 ± 1.38             |
| 3          | 89.2 ± 1.9            | 100.9 ± 0.16          | 79.6 ± 1.90             |
| 4          | 113.2 ± 6.8           | 69.4 ± 2.13           | 70.7 ± 5.83             |
| 5          | 110.0 ± 1.8           | 70.8 ± 3.30           | 69.7 ± 3.01             |
| 6          | 108.5 ± 3.0           | 70.7 ± 0.905          | 68.9 ± 0.14             |
| 7          | 115.7 ± 11.4          | 63.1 ± 2.21           | 68.8 ± 0.41             |
| 8          | 85.9 ± 2.2            | 60.8 ± 2.35           | 65.0 ± 2.24             |
| 9          | 100.0 ± 4.0           | 61.8 ± 5.36           | 64.0 ± 0.24             |
| 10         | 103.8 ± 4.2           | 57.3 ± 2.64           | 62.6 ± 0.78             |
| 11         | 64.4 ± 7.3            | 87.6 ± 4.68           | 62.2 ± 0.61             |
| 12         | 91.6 ± 2.7            | 51.6 ± 2.45           | 61.7 ± 2.44             |

Figures

- **a**: Schematic diagram of the CRISP system
- **b**: In vitro SCRaMbLE
- **c**: Library transformed into *S. cerevisiae*
- **d**: Enzyme assays identified strains with the highest activity
- **e**: qPCR determined gene copy number
Figure 1

Schematic of the in vitro SCRaMbLE strategy to optimize the gene copy number and ratio of CEL3A and CEL5A for efficient synergistic enzyme activity. A DNA library was generated by SCRaMbLEing donor DNA (loxPsym-flanked sequences in pCEL3ALoxP and pCEL5ALoxP) in acceptor plasmids (pAcceptor) (a). The resulting library (b) contained plasmids with various copy numbers of CEL3A and CEL5A for direct transformation into S. cerevisiae (c) which conferred a range of synergistic cellulase activity, determined utilising the BPNPG5 substrate (d). The copy number of CEL3A and CEL5A genes which enabled the highest enzyme activity was determined by quantitative PCR (e).

Figure 2

The relationship between Cel5A and Cel3A activity compared to BPNPG5 activity in S. cerevisiae strains containing in vitro SCRaMbLEd plasmids. After 48 hours of cultivation, the culture supernatant of 160 strains containing in vitro SCRaMbLEd was used for enzyme assays; Cel3A activity was measured on
pNPG (y-axis), Cel5A activity was measured using a DNS assay on CMC (x-axis) and the synergistic activity of both enzymes was measured on BPNPG5 substrate (diameter of spheres). To highlight 12 strains with the highest synergistic enzyme activity, spheres were coloured green.

**Figure 3**

The predicted and actual (measured) BPNPG5 activity of *S. cerevisiae* strains producing Cel3A and Cel5A. All 160 randomly selected strains containing in vitro SCRaMbLED plasmids were cultivated for 48 hours and the culture supernatants were used for enzyme assays; Cel3A activity was measured on pNPG, Cel5A activity was measured with DNS assay on CMC and the synergistic activity was measured on BPNPG. Predicted synergistic activity was calculated using an equation generated by multiple linear regression analysis. Compared to the actual (measured) BPNPG5 activities, this model was highly effective in predicting BPNPG5 activity based on Cel3A and Cel5A (R2 = 0.97).
Figure 4

Supernatant enzyme activity on BPNPG5 using mixtures of Cel3A and Cel5A at different ratios. Enzyme mixtures were prepared using the culture supernatants of two S. cerevisiae strains producing Cel3A and Cel5A. Activity (U) of Cel3A and Cel5A was measured using p-NPG and DNS assays respectively, and mixtures of each were prepared using various ratios of Cel3A and Cel5A activity units from 0 to 1 in increments of 0.1 while the total number of activity units was 100 in all samples.

Figure 5

Average copy number of CEL3A, CEL5A and hphMX4 in populations of S. cerevisiae containing in vitro SCRaMbLEd plasmids. Strains H1-6 (high BPNPG5 activity group) exhibited the highest enzyme activity and strains I1-6 (intermediate BPNPG5 activity group) exhibited approximately the median enzyme activities. The box represents the 25th to 75th percentiles and the line across the box represents the median. The copy number per cell of CEL3A and CEL5A was significantly greater in strains H1-H6 (p<0.02, p<0.05 respectively) while there was no difference in the copy number of the hphMX4 marker (p>0.05), suggesting that plasmid copy number was consistent between groups. All gene copy numbers are reported per cell by comparison to a reference gene (TAF10) which occurs as a single copy in the haploid genome.

Supplementary Files

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