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ORIGINAL RESEARCH PAPER

Improved activity of the Cel5A endoglucanase in *Saccharomyces cerevisiae* deletion mutants defective in oxidative stress defense mechanisms

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Abstract
Objectives Developing a Saccharomyces cerevisiae system for optimizing the expression of recombinant eukaryotic proteins.
Results Two deletion mutants, which were hypersensitive to H_2O_2, were obtained by knocking out *CTT1* and *SOD2*, respectively. The mutation rate of the mutants was up to over 4000 times of the spontaneous mutation rate when treated with H_2O_2. Endoglucanase Cel5A was used as a model enzyme to evaluate the system for improving the expression of the recombinant protein. Sixteen mutants of the RDKY3615 (*ctt1*Δ) transformant and seven mutants of the RDKY3615 (*sod2*Δ) transformant had significantly high Cel5A activity, while none mutants of the RDKY3615 transformant had significantly high enzyme activity.
Conclusion The combination of deletion mutagenesis and H2O2 treatment greatly accelerate the generation of genetic variants and will be a useful tool in improving the heterologous expression in S. cerevisiae.

Keywords Catalase gene (*CTT1*) · Endoglucanase (Cel5A) · Hydrogen peroxide · Mutation rate · *Saccharomyces cerevisiae* · Superoxide dismutase gene (*SOD2*)

Introduction

Saccharomyces cerevisiae is widely used as a host for producing heterologous proteins of industrial or medical interest. It has particular advantages over bacteria in the production of eukaryotic proteins due, in part, to its ability to perform post-translational modification (Idiris et al. 2010). Many proteins have been expressed in *S. cerevisiae* (Celik and Calik 2012). However, in some cases, the expression of the recombinant protein failed (Hong et al. 2003) or the observed expression level or specific enzymatic activity was much lower than that of the wild type protein (Lynd et al. 2002).

Apart from its role as an important eukaryotic overexpression host, *S. cerevisiae* has been investigated regarding its capability to produce bioethanol and other

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commodities from lignocellulosic feedstock (de Jong et al. 2012; Hong and Nielsen 2012). Conventional *S. cerevisiae* is not able to assimilate cellulose directly and, order to produce ethanol from cellulosic biomass directly, many researchers have developed recombinant *S. cerevisiae* strains with the ability to produce cellulase (Den Haan et al. 2007; Matano et al. 2013; Nakatani et al. 2013). Ultimately, ethanol is produced from phosphoric acid-swollen cellulose with the recombinant yeast. However, the enzyme activity of the recombinant cellulase is normally lower than that from the original filamentous fungus and this has become a bottleneck in the one-step cellulose fermentation process.

Many of the approaches for improving the expression of the heterologous protein are difficult to apply as many interdependent factors are involved (Idiris et al. 2010). In this study, we developed a novel in vivo mutagenesis strategy with the deletion mutants of *S. cerevisiae* deficient in the defense of oxidative stress. The genes of catalase T (*CTT1*) and superoxide dismutase (*SOD2*) were deleted from the *S. cerevisiae* strain RDKY3615. The improved mutation rates of the deletion mutants were measured under H_2O_2 treatment. The strategy was further evaluated by improving the enzymatic activity of recombinant endoglucanase Cel5A. This novel method, which combines deletion mutagenesis and H_2O_2 treatment, could prove useful in improving the heterologous expression of cellulase in *S. cerevisiae*, as well as other kinds of proteins.

Materials and methods

Strains, plasmids and primers

Escherichia coli and *S. cerevisiae* strains and plasmids used in this study are listed in Table S1. The primers used are listed in Table S2.

Lysogeny broth (0.5 % yeast extract, 1 % tryptone, 1 % NaCl, 2 % agar for plates) was used for the growth of *E. coli* strains. *S. cerevisiae* were grown on YEPD medium (1 % yeast extract, 2 % Bacto-peptone, 2 % dextrose, 2 % agar for plates). Synthetic complete (SC) medium lacking respective amino acids was used for the selective growth of *S. cerevisiae* transformants.

Genes encoding catalase (Ctt1) or superoxide dismutase (Sod2) were deleted from the *S. cerevisiae* strain RDKY3615 using *trp1* as marker gene. Yeast transformations were performed as described (Yu et al. 2009). The successful gene deletion was confirmed by PCR using genomic DNA of the deletion mutants as templates.

The geneticin-resistance gene from the plasmid pFA6a-kanMX4 (kindly provided by Prof. Peter Philippsen of University of Basel, Switzerland) was amplified and inserted into pGADT7 (Clontech Laboratories, USA), yielding the plasmid pGADT7-km. An A-to-C transversion was then introduced into pGADT7-km with a site-directed mutagenesis kit (Promega, Q9280), yielding the resultant plasmid pGADT7-km^s. As a result of this transversion, the Asp198 of the gene product was substituted for Ala. Since the former Asp198 was located at the active site of the protein, kanamycin resistance activity of the enzyme was lost due to the mutation. The KanMX4 gene and its mutant were confirmed by DNA sequencing. The RDKY3615 transformant of pGADT7-km acquired resistance to Geneticin, and the transformant of pGADT7-km^s did not acquire resistance to geneticin.

Cell viability measurement and mutation rate assay

Mid-growth-phase RDKY3615, RDKY3615 (*ctt1*Δ) and RDKY3615 (*sod2*Δ) cells were resuspended in 0.9 % NaCl and treated with H_2O_2. The treated suspensions were then washed, diluted, and plated. After 72 h, the number of the colonies was counted, and the viability was calculated.

Plasmid pGADT7-km^s was transformed into RDKY3615, RDKY3615 (*ctt1*Δ) and RDKY3615 (*sod2*Δ). The cells of the transformants were treated with H_2O_2, and plated in triplicate in the presence or absence of Geneticin. The mutation rate was evaluated by calculating the reversion frequency of the kanamycin resistance gene.

Overexpression of Cel5A and mutagenesis of the RDKY3615 (*ctt1*Δ) and the RDKY3615 (*sod2*Δ) transformants

The Cel5A gene from *Hypocrea jecorina* (syn. *Trichoderma reesei*) was amplified and inserted into pGADT7. The pGADT7-Cel5A was transformed into RDKY3615, RDKY3615 (*ctt1*Δ) and RDKY3615 (*sod2*Δ). The successful overexpression was confirmed by SDS–PAGE.

The cells of the RDKY3615 (*ctt1*Δ) and the RDKY3615 (*sod2*Δ) transformants were treated with

2 mM H_2O_2 for 1 h, and were screened on two-layer plates with a cladding layer containing 0.5 % CMC (carboxymethylcellulose). The CMC around the colonies in the cladding layer was hydrolyzed by the Cel5A secreted by the colonies. Endoglucanase activity of the transformants was detected with the Congo Red staining method (Teather and Wood 1982). Three sets of screening experiments were carried out. In each set of experiments, three hundred plates (each plate contained about 100 colonies) of each of the RDKY3615, the RDKY3615 (*ctt1*Δ) and the RDKY3615 (*sod2*Δ) transformants were screened. The picked colonies were stored at −80 °C for further enzyme activity measurement.

Cel5A enzyme activity measurement and mutation evaluation

The mutants of the transformants were cultivated in shake-flasks. The endoglucanase activity of the fermentation broth was measured in triplicate using the dinitrosalicylic acid reagent method (Meinke et al. 1995). 23 mutants were selected for their high secreted enzymatic activity. The pGADT7-Cel5A plasmid was extracted from the selected mutants, and the Cel5A gene with its promoter and terminator was sequenced. The pGADT7-Cel5A plasmid extracted from the mutants of C15 and S5 was transformed into the reference strain RDKY3615, respectively. The enzyme activity of the transformants was measured. The C15 and S5 mutants carrying the pGADT7-Cel5A plasmid were cultivated in SC medium in absence of Geneticin. The strains which had lost the plasmid during the cultivation were confirmed by PCR. The plasmid pGADT7-Cel7A was constructed by inserting the Cel7A gene from *H. jecorina* (syn. *T. reesei*) into pGADT7, and was confirmed by DNA sequencing. The empty strains of the C15 and the S5 mutants were transformed with the reference plasmid pGADT7-Cel5A and the plasmid pGADT7-Cel7A. The Cel7A enzyme activity of the transformants was measured in triplicate using the *p*-nitrophenyl-β-D-cellobioside (pNPC) as the substrate (Wu et al. 2010).

Results

Cell viability of *CTT1* and *SOD2* deletion mutants

Two deletion mutants designated RDKY3615 (*ctt1*Δ) and RDKY3615 (*sod2*Δ), were constructed by knocking out *CTT1* or *SOD2* from RDKY3615, respectively. The growth of RDKY3615, RDKY3615 (*ctt1*Δ) and RDKY3615 (*sod2*Δ) were similar in YEPD medium (Fig. 1). The susceptibility of the three strains to H_2O_2 stress was monitored (Fig. 2). The survival rates of all the three strains decreased as the concentration of H_2O_2 increased. The two deletion mutants were more sensitive to H_2O_2 than RDKY3615. After treatment with 0.5 mM H_2O_2 for 1 h, 94.8 % of RDKY3615 cells were alive, indicating that most of the wild type cells could adapt to the stress caused by low concentration of H_2O_2. However, only 46.3 % RDKY3615 (*ctt1*Δ) cells and 21.7 % RDKY3615 (*sod2*Δ) cells survived under the same condition. After treated with 2 mM H_2O_2 for 1 h, 61.4 % RDKY3615 cells survived while only 17.9 % RDKY3615 (*ctt1*Δ) cells and 12 % RDKY3615 (*sod2*Δ) cells survived under the same condition.

Mutation rate assay under H_2O_2 treatment

The pGADT7-km^s plasmid was constructed and used as a reporter for the mutagenesis assay. Transformants of RDKY3615, RDKY3615 (*ctt1*Δ), and RDKY3615 (*sod2*Δ) carrying pGADT7-km^s were treated with H_2O_2. The mutation rates were assessed with the rate of reverse mutation of the pGADT7-km^s plasmid. As shown in Tables 1 and 2, the mutation rates of RDKY3615 (*ctt1*Δ) and RDKY3615 (*sod2*Δ) were

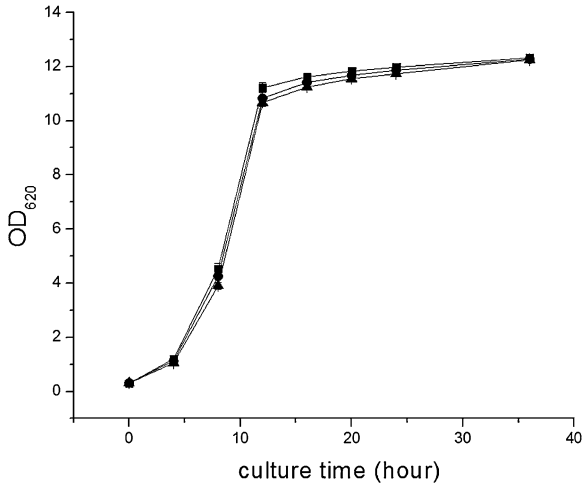

Fig. 1 The growth curves of RDKY3615 (*filled square*), RDKY3615 (*ctt1*Δ) (*filled circle*) and RDKY3615 (*sod2*Δ) (*filled triangle*) in YEPD medium. *Error bars* indicate ± SD, n = 3

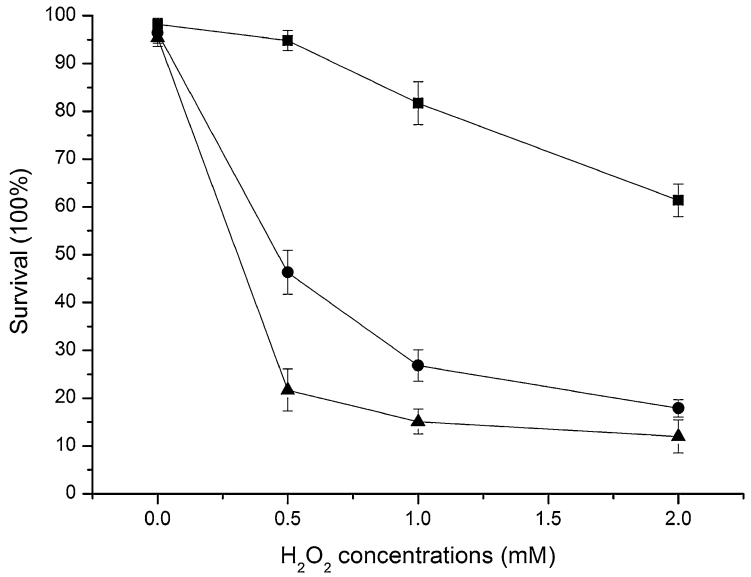

Fig. 2 The survival rate of RDKY3615 (*filled square*), RDKY3615 (*ctt1*Δ) (*filled circle*) and RDKY3615 (*sod2*Δ) (*filled triangle*) treated with H_2O_2 for 1 h. The survival (%) was calculated by dividing the number of the colonies under the H_2O_2 treatment with the number of the colonies without the H_2O_2 treatment. *Error bars* indicate ± SD, n = 3

Total number of coloines treated with H_2O_2/ total number of colonies not treated with H_2O_2

	0 mM H_2O_2	0.5 mM H_2O_2	1.0 mM H_2O_2	2.0 mM H_2O_2
RDKY3615	3048/ 3129	2814 /2958	2483/ 3006	1946/ 3124
RDKY3615(ctt1△)	2951/ 3061	1390/ 3173	800/ 2986	632/ 3026
RDKY3615(sod2△)	2943/ 3088	673/ 3145	476/ 2937	373/ 3079

higher than that of RDKY3615 under the same treatment conditions. The mutation rates of RDKY3615 (*ctt1*Δ) and RDKY3615 (*sod2*Δ) were about 11 and 10 times of that of RDKY3615 when treated with 2 mM H_2O_2 for 1 h (Table 1), respectively. Our result indicated that the deletion of *CTT1* and *SOD2* lead to increased mutation rates under the H_2O_2 treatments, and that RDKY3615 (*ctt1*Δ) and RDKY3615 (*sod2*Δ) were better than RDKY3615 with regard to the generation of in vivo mutagenesis under H_2O_2 treatment.

Overexpression of Cel5A and in vivo mutagenesis

The Cel5A endonuclease from *H. jecorina* (syn. *T. reesei*) was overexpressed in RDKY3615, RDKY3615 (*ctt1*Δ) and RDKY3615 (*sod2*Δ) with the plasmid pGADT7-Cel5A. The transformants were treated with H_2O_2 and were screened on plates containing 0.5 % CMC (Fig. S1). Three sets of screening experiments were carried out and 30 colonies with the largest hydrohaloes (a potential marker for high Cel5A enzyme activity) were picked for each of the RDKY3615, the RDKY3615 (*ctt1*Δ) and the RDKY3615 (*sod2*Δ) transformants in each set of experiments. The enzyme activity of the selected colonies was measured in flask experiments. Sixteen mutants of the RDKY3615 (*ctt1*Δ) transformant and seven mutants of the RDKY3615 (*sod2*Δ) transformant had significantly higher Cel5A activity ($P < 0.05$ with Student's *t* test) (Table 3; Fig. 3). However, none of the 90 picked colonies of the RDKY3615 transformant had significantly higher Cel5A activity (Table 3). The results indicated that RDKY3615 (*ctt1*Δ) and RDKY3615 (*sod2*Δ) were better than RDKY3615 with regard to obtaining the mutant with improved enzyme activity under H_2O_2 treatment.

Four mutants of the RDKY3615 (*ctt1*Δ) transformant, C1–1, C2–3, C3–3 and C3–4, had more than five times higher Cel5A enzyme activity than that of the control transformant. The relative enzyme activity of the mutant C3–4 was the highest among all the mutants, and was 8.5 times of that of the wild type transformant (Fig. 3). As for the mutants of the RDKY3615 (*sod2*Δ) transformant, S1–1 and S3–2 had more than five times higher enzyme activity than that of the control transformant. Mutant S3–2 exhibited 7.1-fold relative enzyme activity of the control, which was the highest among that of the RDKY3615 (*sod2*Δ) mutants (Fig. 3).

Table 1 Mutation rate of RDKY3615 (*ctt1*Δ) and RDKY3615 (*sod2*Δ) treated with H_2O_2 for 1 h

Strains treated with different H_2O_2 concentration (mM)	Mutation rate[a]	Relative mutation rate compared with the spontaneous mutation rate
RDKY3615		
0	$1.2 \pm 0.5 \times 10^{-8}$	1
0.5	$1.9 \pm 0.4 \times 10^{-8}$	2
2	$7.4 \pm 2.2 \times 10^{-7}$	62
5	$6.9 \pm 0.9 \times 10^{-6}$	570
RDKY3615 (*ctt1*Δ)		
0	$1.7 \pm 0.3 \times 10^{-8}$	1
0.5	$1.6 \pm 0.4 \times 10^{-7}$	13
2	$8.3 \pm 2.2 \times 10^{-6}$	690
5	$4.9 \pm 0.9 \times 10^{-5}$	4,100
RDKY3615 (*sod2*Δ)		
0	$5.4 \pm 0.3 \times 10^{-8}$	5
0.5	$2.1 \pm 0.4 \times 10^{-7}$	18
2	$7.1 \pm 1.6 \times 10^{-6}$	590
5	$2.3 \pm 0.2 \times 10^{-5}$	1,900

Values are presented as means ± SD (n = 3)

[a] The mutation rate was evaluated with the reversion frequency of the kanamycin resistance gene as calculated by dividing the number of the colonies in the presence of Geneticin with the number of the colonies in the absence of Geneticin

Table 2 Mutation rate of RDKY3615 (*ctt1*Δ) and RDKY3615 (*sod2*Δ) treated with 2 mM H_2O_2

Strain treated for different time (h)	Mutation rate[a]	Relative mutation rate compared with the spontaneous mutation rate
RDKY3615		
0	$1.2 \pm 0.5 \times 10^{-8}$	1
0.5	$2.2 \pm 0.08 \times 10^{-7}$	19
2	$3.3 \pm 0.8 \times 10^{-6}$	280
RDKY3615 (*ctt1*Δ)		
0	$1.7 \pm 0.3 \times 10^{-8}$	1
0.5	$2.7 \pm 0.08 \times 10^{-6}$	230
2	$2.9 \pm 0.8 \times 10^{-5}$	2,400
RDKY3615 (*sod2*Δ)		
0	$5.4 \pm 0.3 \times 10^{-8}$	5
0.5	$3.8 \pm 0.8 \times 10^{-6}$	320
2	$1.9 \pm 0.4 \times 10^{-3}$	1,600

Values are presented as means ± SD (n = 3)

[a] The mutation rate was evaluated with the reversion frequency of the kanamycin resistance gene as calculated by dividing the number of the colonies in the presence of Geneticin with the number of the colonies in the absence of Geneticin

To confirm the reliability of the plate screening, another 50 colonies with normal size of hydro-haloes were randomly picked for each of the transformants in the screening experiments. None of the colonies had significantly higher Cel5A activity. The results suggested that the screening with agar plates was reliable and that the colonies with larger hydro-haloes were more potentially with high enzyme activity.

The enzymatic activity of the mutants was measured again after 10 passages, and the elevated enzyme activity remained stable. The result indicated that the high enzyme activity was heritable. The plasmids of the 23 mutants selected were retrieved for sequencing. The plasmids extracted from the C15 and the S5 mutants were transformed into the reference strain RDKY3615, respectively. The enzyme activity of the two transformants was similar to the control transformant (Fig. 4). The empty strains of the C15 and the S5 mutants were obtained and transformed with the reference plasmid pGADT7-Cel5A. The enzyme activity of the C15 and the S5 transformants of pGADT7-Cel5A were similar to that of the C15 and the S5 mutants, respectively (Fig. 4). The results indicated that the strain is responsible for the improved enzyme activity.

Overexpression of Cel7A in the improved mutants

In order to investigate if the improved mutant hosts were able to produce other heterologous proteins at higher levels, the plasmid pGADT7-Cel7A was constructed and transformed into RDKY3615, the C15 and the S5 empty mutant strains. The Cel7A enzyme activity of the C15 and the S5 transformants was significantly higher ($P < 0.05$ with Student's t test), and was 1.6 and 2.3 times of that of the control transformant, respectively (Fig. 5).

Discussion

H_2O_2 causes DNA damage and induces mutagenesis (Moraes et al. 1990). It was used to accelerate the

Table 3 Number of the mutants of the RDKY3615, the RDKY3615 (*ctt1*Δ) and the RDKY3615 (*sod2*Δ) transformants with high Cel5A enzyme activity obtained in the screening experiment

Strains	Colonies with significantly high Cel5A enzyme activity[a]			
	Set 1	Set 2	Set 3	Sum
RDKY3615	0	0	0	0
RDKY3615 (*ctt1*Δ)	7	4	5	16
RDKY3615 (*sod2*Δ)	2	1	4	7

In each set of experiments, 30 colonies with larger hydro-haloes were picked from about 30,000 colonies for each of the RDKY3615, the RDKY3615 (*ctt1*Δ) and the RDKY3615 (*sod2*Δ) transformants. The secreted enzyme activity of the colonies was measured, and the abilities of the three stains for the improving the recombinant Cel5A activity were evaluated

[a] Student's *t* test was done to analyze the significance of the high Cel5A enzyme activity. The mutants with significantly higher enzyme activity than that of the control (with $P \leq 0.05$) were included

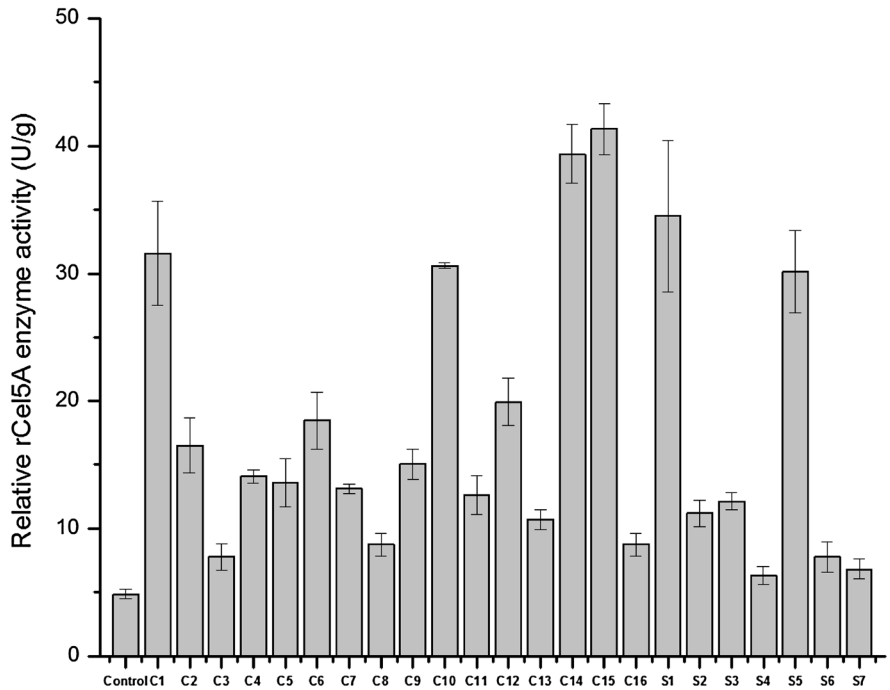

Fig. 3 Relative rCel5A activity of the mutants from the screening experiments. 23 mutants which had significantly higher (with Student's *t* test value $P \leq 0.05$) enzyme activity compared with that of the control are shown in the figure. C1–C16 were the mutants of the RDKY3615 (*ctt1*Δ) transformant; S1–7 were the mutants of the RDKY3615 (*sod2*Δ) transformant. *U* One unit of CMCase activity, defined as the amount of enzyme releasing 1 μmol of reducing sugar per min, *U/g* Cel5A enzyme activity per gram dry weight yeast cells. *Error bars* indicate ± SD, $n = 3$

generation of mutation in yeast cells in this study. Catalase T (Ctt1p) is involved in removing H_2O_2 and protecting *S. cerevisiae* cells against oxidative damage (Wieser et al. 1991). Superoxide dismutase (Sod2p) has been protects cells against O_2 toxicity (Unlu and Koc 2007). The deletion mutants of *CTT1* and *SOD2* were hyper-sensitive to H_2O_2, and were used for fast generation of mutants under H_2O_2 treatment in this study.

A prokaryotic strain, XL1-Red (*E. coli*, deletion of *mutS*, *mutD* and *mutT*, Stratagene) which is deficient in both DNA replication machinery and the major DNA repair pathways, has been developed and widely used for in vivo mutagenesis (Callanan et al. 2007; Lu et al. 2001). However, the *E. coli* host is not appropriate for the overexpression of some of the eukaryotic proteins which require post-translational modification. Our *S. cerevisiae* system is ideal for optimizing the expression of eukaryotic proteins. Moreover, certain mutation rates could be achieved by treating the cells with certain amounts of H_2O_2 for certain periods of time. The capability of adjusting

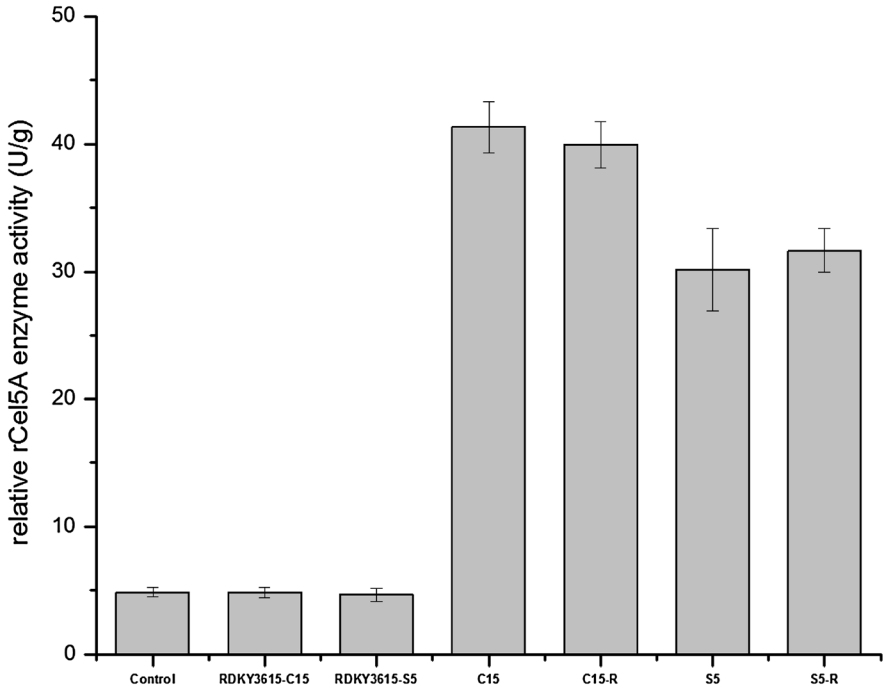

Fig. 4 Relative rCel5A activity of the RDKY3615, the C15 mutant and the S5 mutant tranformants of the reference pGADT7-Cel5A plasmid and the plasmids extracted from the C15 and the S5 mutant. The control was the RDKY3615 transformant of the reference plasmid pGADT7-Cel5A; RDKY3615-C15 and RDKY3615-S5 were the RDKY3615 transformants of the plasmid extracted from the C15 mutant and S5 mutant, respectively; C15 and S5 were the mutants obtained in the screening experiment; C15-R and S5-R were the C15 and the S5 empty host strain transformed with the reference plasmid pGADT7-Cel5A, respectively. *Error bars* indicate ± SD, n = 3

mutation rate is advantageous, as the optimal mutation rate for different mutagenesis experiments may be different.

The sequencing of the plasmids extracted from the mutants showed that the enhanced Cel5A enzyme secretion of the mutants was not due to a mutation/mutations on either the Cel5A gene or its promoter and terminator. The improved Cel7A enzyme secretion in the C15 and S5 mutants indicate that the two mutants were also able to produce another protein at higher levels. As the elevated enzymatic activity of both Cel5A and Cel7A was heritable, the mutation may reside on the *S. cerevisiae* genome. H_2O_2 has been found to induce oxidative damage to DNA, and the damage commonly lead to C-to-T transitions, G-to-T transversions, as well as single-base deletions (Lee et al. 2002). If the genes of *S. cerevisiae* involved in protein synthesis (e.g. post-transcriptional modification) and protein secretion are mutated due to the H_2O_2 treatment, the heterologous expression of Cel5A or Cel7A may be changed.

S. cerevisiae is an adequate host for the expression of many proteins. However, the expression sometimes fails completely (Hong et al. 2003). In some other cases, the recombinant enzyme has much lower expression level or specific enzyme activity than in the wild type host (Lynd et al. 2002). Our results showed that the combination of the gene deletion of *CTT1*/*SOD2* and the H_2O_2 treatment could significantly accelerate the generation of gene and/or host genome variants. The system could be used for improving the expression level and the specific enzyme activity of the recombinant protein.

In order to reduce the cost of ethanol production from cellulosic biomass, many researchers have developed recombinant *S. cerevisiae* with the ability to ferment cellulose directly (Den Haan et al. 2007; Nakatani et al. 2013). However, both the expression level and the specific enzyme activity of the recombinant cellulase are much lower than that in the original filamentous fungi (Qin et al. 2008; Wu et al. 2010). The decreased enzymatic activity of

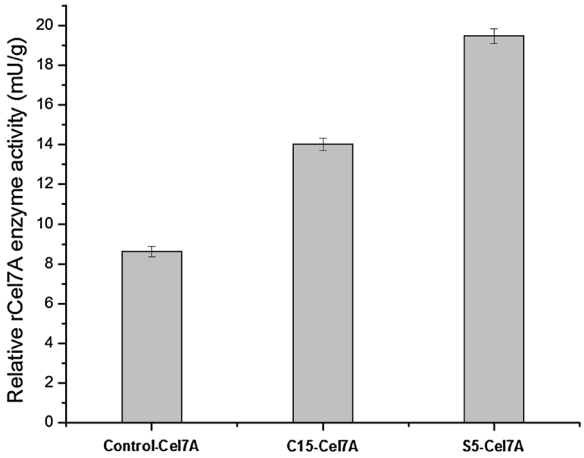

Fig. 5 Relative rCel7A activity of the RDKY3615, the C15 and the S5 transformants. Control-Cel7A was the RDKY3615 transformant of the plasmid pGADT7-Cel7A; C15-Cel7A and S5-Cel7A were the C15 and the S5 empty host strain transformed with the plasmid pGADT7-Cel7A, respectively. C15-Cel7A and S5-Cel7A had significantly higher (with Student's *t* test value $P \leq 0.05$) enzyme activity compared with that of control-Cel7A. *U* One unit of rCel7A activity, defined as the amount of enzyme releasing 1 μmol of pNP from the pNPC per min; *mU/g* Cel7A enzyme activity per g dry weight yeast cells. *Error bars* indicate ± SD, n = 3

cellulase has become one of the main problems in such studies, and a few strategies have been applied to overcome the problem (Van Zyl et al. 2014; Yang et al. 2014). The strategy we developed in this study successfully improved the enzyme activity of the recombinant Cel5A and Cel7A.

In conclusion, the deletion mutants of RDKY3615 (*ctt1*Δ) and RDKY3615 (*sod2*Δ) could accelerate the generation of in vivo mutagenesis under H_2O_2 treatment. 23 mutants with heritable high Cel5A activity and two mutants with heritable high Cel7A activity were obtained in this study. Our results suggest that the combination of the deletion of *CTT1*/*SOD2* and the H_2O_2 treatment is a promising approach for improving the heterologous expression of protein in *S. cerevisiae*. The potential value of the approach for use in one-step cellulose fermentation process warrants further investigation.

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Supporting information Supplementary Table 1—Strains and plasmids used in this study.
Supplementary Table 2—PCR primers used in this study.
Supplementary Figure 1—Screen of the mutants for high Cel5A activity on activity indicator plate. Each colony was formed from a single cell. CMC around the colonies was hydrolyzed by Cel5A secreted by the colonies. The size of the hydro-halo represented the secreted endoglucanase activity of the colony.

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