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Construction of recombinant industrial *Saccharomyces cerevisiae* strain with *bglS* gene insertion into *PEP4* locus by homologous recombination*

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Abstract: The *bglS* gene encoding *endo*-l,3-1,4-β-glucanase from *Bacillus subtilis* was cloned and sequenced in this study. The *bglS* expression cassette, including *PGK1* promoter, *bglS* gene fused to the signal sequence of the yeast mating pheromone α-factor ($MF\alpha1_S$), and *ADH1* terminator with G418-resistance as the selected marker, was constructed. Then one of the *PEP4* allele of *Saccharomyces cerevisiae* WZ65 strain was replaced by *bglS* expression cassette using chromosomal integration of polymerase chain reaction (PCR)-mediated homologous recombination, and the *bglS* gene was expressed simultaneously. The recombinant strain *S. cerevisiae* (SC-βG) was preliminarily screened by the clearing hydrolysis zone formed after the barley β-glucan was hydrolyzed in the plate and no proteinase A (PrA) activity was measured in fermenting liquor. The results of PCR analysis of genome DNA showed that one of the *PEP4* allele had been replaced and *bglS* gene had been inserted into the locus of *PEP4* gene in recombinant strains. Different *endo*-l,3-1,4-β-glucanase assay methods showed that the recombinant strain SC-βG had high *endo*-l,3-1,4-β-glucanase expression level with the maximum of 69.3 U/(h·ml) after 60 h of incubation. Meanwhile, the Congo Red method was suitable for the determination of *endo*-l,3-1,4-β-glucanase activity during the actual brewing process. The current research implies that the constructed yeast strain could be utilized to improve the industrial brewing property of beer.

Key words: *Endo*-l,3-1,4-β-glucanase (*bglS*), Gene replacement, Homologous recombination, *Bacillus subtilis*, *PEP4* gene, *Saccharomyces cerevisiae*

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INTRODUCTION

Barley β-glucans (1,3-1,4-β-D-glucans) are the principal constituent (70%) of barley endosperm cell walls (Palmer, 1989). β-glucan polymers originating from barley endosperm cell walls are one of the major concerns in the brewing industry. The amount and molecular weight of β-glucan in malt affect brewing house extract yield and wort and beer viscosities, as well as lautering, diatomaceous earth, and membrane filtrations. Barley β-glucans are also associated with beer hazes. The partially degraded β-glucans are particularly troublesome during brewing due to the sticky spent grains which retard wort separation at a lower permeability level, reduce beer filtration rate, and induce beer hazes (Palmer, 1989). And also the high viscosities (*Saccharomyces cerevisiae* cannot cleave the β-1,4 linkages of β-glucans) impede beer filtration in the presence of β-glucans. Therefore, an addition of commercial enzyme preparations is necessary. Alternatively, a heterologous gene encoding β-glucanase could be introduced into brewer's yeast. The latter option serves as an obvious task for metabolic engineering whereby the substrate range is extended to include β-glucans, and consequently the process performance of beer production may be improved (Jin *et al*., 2004).

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Microbial β-glucanases have been used to improve filtration, increase extract yield, and prevent β-glucan hazes. *Endo*-l,3-1,4-β-glucanases from barley and *Bacillus subtilis* have identical substrate specificities (Müller *et al*., 1998). Microbial *endo*-l,3-1,4-β-glucanase can be added during malting, mashing or fermentation procedures (Grujic, 1998; Jin *et al*., 2004), which can efficiently lower HMW (high molecular weight) β-glucan content in wort and beer (Todo *et al*., 1989; Kettunen *et al*., 1996), leading to improved beer filterability. The addition of 20×10^{-6} of β-glucanase at 0~2 °C for 2 d increases sevenfold the membrane filterability of bright beer (Sudarmana *et al*., 1996). The bacterial *endo*-l,3-1,4-β-glucanase hydrolyzes linear β-glucans containing β-1,3 and β-1,4 linkages such as cereal β-glucans and lichenan, with a strict cleavage specificity for β-1,4 glycosidic bonds on 3-*O*-substituted glucosyl residues (Gaiser *et al*., 2006) (Fig.1), playing an important biotechnological role in the brewing and animal feedstuff industries.

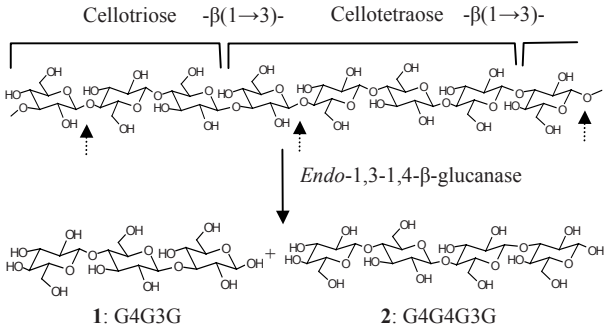

Fig.1 The degradation process of barley by *endo*-l,3-1,4-β-glucanase (Antoni, 2000)

Although the introduction of flocculence to brewer's yeast is a convenient method to separate the yeast from the brewing, beer filtration is still an important separation technique in the brewing industry. *Endo*-l,3-1,4-β-glucanases from *B*. *subtilis* (Cantwell *et al*., 1986), *Trichoderma reesei* (Panttilä *et al*., 1987a; 1987b), and barley (Olsen and Thomsen, 1989) have successfully been expressed in *S*. *cerevisiae*, and active enzymes were secreted. The production of β-glucanase did not affect beer quality, and furthermore, the β-glucans were efficiently degraded, resulting in an improved filterability (Panttilä *et al*., 1987a).

The appearance of a stable head of foam is a major consideration in beer quality assessment. As a consequence, many researchers have focused on foam-positive species, in particular, beer proteins, which have been implicated to present in the production and stabilization of foam. The decrease in foam stability caused by yeast proteinase A (PrA) in unpasteurized beer has been reported by several researchers (Muldbjerg *et al*., 1993; Yokoi *et al*., 1996; Cooper *et al*., 2000; Wang *et al*., 2005; He *et al*., 2006). PrA is coded by *PEP4* gene; construction of *PEP4* yeast strains would be helpful to keep foam stability (Wang *et al*., 2007a). Industrial brewing yeast is wild-type original strain in general and it cannot be replaced by laboratorial yeast in beer brewing; however, other researchers have not constructed industrial brewing yeast with *PEP4* gene deletion (Akada, 2002). In our previous research, it was reported that the PrA-deficient recombinant of industrial brewing yeast (WZ65/a) was constructed using polymerase chain reaction (PCR)-mediated gene disruption (Wang *et al*., 2007a), and self-cloning industrial brewer's yeast strains were constructed in the following study, in which the coding sequence of *PEP4* gene was deleted by *CUP1* gene and *GSH1* gene's replacement (Wang *et al*., 2007b).

MATERIALS AND METHODS

Microbial strains and plasmids

The host industrial brewing yeast, WZ65, was provided by China Lion Brewery Group. *B*. *subtilis* mutant ZJF-1A5 was isolated and bred to produce thermal stable *endo*-l,3-1,4-β-glucanase. *Escherichia coli* DH5α was preserved in the laboratory. Vectors, pUC18 and pUG6 (*KanMX*), were preserved in the laboratory.

Media and cultivation conditions

Escherichia coli was grown at 37 °C in Luria-Bertani medium supplemented with ampicillin (100 mg/L) when necessary. Yeast strains were grown at 28 °C in YEPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose). For selection for geneticin (G418) after yeast transformation, the YEPD plate was supplemented with G418 (200 mg/L).

Cloning of *Bacillus subtilis bglS* gene

Thermostable *bglS* gene from *B. subtilis* ZJF-1A5 was cloned by PCR with designed primers (forward primer, bg-F: 5′-GGGGGATCCATGCCTTATCT GAAACG-3′; reverse primer, bg-D: 5′-GGGAAG CTTATTTACAGAGGGGAGAA-3′). Amplification conditions were 94 °C for 4 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and finally 72 °C for 10 min. The PCR product was purified and sequenced by Shanghai Sangon Co., China.

Construction of recombinant pUC18-KPMBT vector

PGK1 promoter, the signal sequence of the yeast mating pheromone α-factor ($MF\alpha1_S$) and *ADH1* terminator ($ADH1_T$) fragments were amplified by PCR using *S. cerevisiae* WZ65 genome as the template with P1 & P2, P3 & P4 and P7 & P8 primers, respectively, adding relevant recognition sites to the 5′-end and 3′-end (Table 1). And the *bglS* gene for construction of the recombinant plasmid was amplified by PCR with P5 & P6 primers and *B. subtilis* as the template. Amplification conditions for the four fragments were 94 °C for 4 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and finally 72 °C for 10 min.

PGK1 promoter digested with *Bam*HI and *Xba*I was inserted into MCS (multiple clone site) of pUC18, generating pUC18-P. Then the $MF\alpha1_S$ was digested with *Xba*I and *Hin*dIII and ligated with the *Xba*I- and *Hin*dIII-cleaved pUC18-P, and the resulting plasmid was named pUC18-PM. The *bglS* gene fragment was cloned into pUC18 after both digested with *Hin*dIII and *Kpn*I, resulting in pUC18-B. Then pUC18-B was ligated with *Kpn*I- and *Eco*RI-digested *ADH1* terminator fragment and was designated as pUC18-BT. Fragment containing *PGK1* promoter/the signal sequence of *MFα1* factor fusion gene excised with *Nde*I and *Hin*dIII from the pUC18-PM was inserted into the same sites of pUC18-BT, and then pUC18-PMBT1 containing *Nde*I-*Eco*RI fragment of *bglS* expression cassette was constructed. The pUC18-PMBT1 was used as a template in PCR with P1 & P8 primers to amply PMBT fragment, and the PCR products were digested with *Sma*I and *Eco*RI and inserted into pUC18 to construct pUC18-PMBT. Finally, the *KanMX* cassette amplified by PCR using pUG6 as a template with P9 and P10 primers was cloned into the pUC18-PMBT at its *Bam*H and *Sma*I sites to produce final plasmid (pUC18-KPMBT).

Electro-transformation and screening of the recombinants

Using recombinant plasmid pUC18-KPMBT as the template, the fragment for replacement was PCR amplified with designed primers, P13 & P14 (Table 1). At their 3′-ends the oligonucleotides (majuscule)

Table 1 Oligonucleotides used in PCR-mediated gene disruption and verification

Primer	Sequence (5′→3′)	Primer site	Restriction site
P1	CCCCCGGGCTTCAACTCAAGACGCACAG	$PGK1_P$ upstream	*Sma*I
P1′	CCGGATCCCATATGCTTCAACTCAAGACGCACAG	$PGK1_P$ upstream	*Bam*HI, *Nde*I
P2	GGTCTAGATGTTTTATATTTGTTGTAAAAAGTAG	$PGK1_P$ downstream	*Xba*I
P3	GGTCTAGAAGAATGAGATTTCCTTC	$MF\alpha1_S$ upstream	*Xba*I
P4	GGCCAAGCTTCAGCCTCTCTTTTATC	$MF\alpha1_S$ downstream	*Hin*dIII
P5	GGAAGCTTCGGCTCAAACAGGTGGATCGTTTTTTG	*bglS* upstream	*Hin*dIII
P6	GGGGTACCGCATTATTTTTTTGTATAGCGCACCC	*bglS* downstream	*Kpn*I
P7	GGGGTACCGCGAATTTCTTATG	$ADH1_T$ upstream	*Kpn*I
P8	GGGAATTCGCATATCTACAATTGGG	$ADH1_T$ downstream	*Eco*RI
P9	GGGGATCCCAGCTGAAGCTTCGTACGC	*KanMX* upstream	*Bam*HI
P10	CCCCCGGGGCATAGGCCACTAGTGGATCTG	*KanMX* downstream	*Sma*I
P11	AGTAAAGAAGTTTGGGTAATTCGCT	Verification upstream	−
P12	AGTGTTCTATGTTTGCCTTGATTTC	Verification downstream	−
P13	5′-gtatttaatccaaataaaattcaaacaaaaaccaaaactaacatgCAGCTG AAGCTTCGTACGC-3′	Gene replacement upstream	−
P14	5′-atggcagaaaaggatagggcggagaagtaagaaaagtttagctcaGCAT ATCTACAATTGGG-3′	Gene replacement downstream	−

$PGK1_P$: *PGK1* promoter; $MF\alpha1_S$: Signal sequence of the yeast mating pheromone α-factor; $ADH1_T$: *ADH1* terminator

are homologous to the recombinant plasmid pUC18-KPMBT, while the oligonucleotides (lowercase) of the 5′-ends are homologous to the sequence of the left and right of the *PEP4* gene, respectively. The PCR product was purified and electro-transformed into industrial yeast *S. cerevisiae* WZ65. The electro-transformation conditions were: 7.5 kV/cm, capacitance 25 μF, parallel resistor 200 Ω, pulse lengths 5 ms. The transformants were selected on YEPD medium containing 200 mg/L G418. After incubation at 30 °C for 72 h, the recombinants were detected and identified.

The two-layer detection plate was used to isolate the transformed strains. The growth YEPD plate with the transformed strains was covered with 5 ml of 0.5% (w/v) agar solution including 0.1% (w/v) lichenan after the transformed colonies were inoculated onto a new YEPD plate to preserve. After incubated at 30 °C for 6 h, the agar-lichenan layer was stained with 0.1% (w/v) Congo Red solution for 30 min. The recombinants were preserved on YEPD slant after the clearing zone formed around the transformed colony in the stained plate.

Identification of the recombinant by PCR amplification

Using the genome of the recombinant as the template, three pairs of primers, P11 & P10, P12 & P5 and P11 & P12, were designed to verify the recombinant strains, with P11 & P12 located in the flanking region of the *PEP4* ORF (open reading frame), upstream 266 bp and downstream 321 bp, respectively (Table 1).

Determination of *endo*-l,3-1,4-β-glucanase activity

After the recombinant SC-βG was incubated in 10 ml YEPD medium at 30 °C for 24 h, 0.5 ml of culturing liquor was inoculated to 50 ml new YEPD medium. Samples were collected at different time points after inoculation. The culture supernatant of recombinant *S. cerevisiae* WZ65 strain was used as crude enzyme to assay the enzyme activity.

1. Congo Red method

The enzyme activity was assayed with barley β-glucan as substrate at 50 °C. Appropriate dilution of the supernatant was done using 0.05 mol/L citrate-phosphate buffer (pH 6.2). For every 900 μl substrate 100 μl supernatant was added. Aliquots (1 ml) were removed in duplicate at intervals, heated (100 °C) for 15 min and cooled to room temperature. Subsequently, 200 μl Congo Red (100 μg/ml) was added and the mixture diluted with buffer to 2 ml. Absorbency was measured at 540 nm (Wood *et al*., 1988). One unit of β-1,3-1,4-glucanase activity is defined as the amount of enzyme required to hydrolyze 1 μg β-glucan per minute in 1 ml fermenting liquor.

2. DNS (3,5-dinitrosalicylic acid) method

The activity of *endo*-l,3-1,4-β-glucanase was measured by a modified method described by Cantwell and McConnell (1983). The enzyme was appropriately diluted with phosphate buffered saline (PBS) (0.2 mol/L, pH 6.0). An aliquot of 0.1 ml enzyme solution prepared was added to 0.9 ml substrate solution (2 mg/ml lichenin) that was pre-incubated at 50 °C for 10 min, and then the mixture was incubated at 50 °C exactly for 10 min. The reaction was stopped by adding 1.5 ml dinitrosalicylic acid solution and boiling for 5 min. Then, the mixture was cooled immediately with cold water and added to 25 ml with distilled water. Absorbency was measured at 540 nm. The amount of reducing sugar was calculated by the absorption value at 540 nm. One unit of activity was defined as the amount of enzyme capable of producing 1 nmol reducing sugar per minute (using glucose as reference) in 1 ml fermenting liquor under the above conditions.

Proteinase A (PrA) activity assay

PrA activity was assayed using 1% (w/v) casein (pH 2.0) as the substrate and detecting products with modified Bradford (1976) method. One unit of PrA will hydrolyze 1 mg of insulin chain B (oxidized) per minute at pH 6.0 (25 °C) (Wang *et al*., 2005).

RESULTS

***bglS* gene cloning by PCR amplification**

To obtain the coding sequence of *bglS* gene, PCR amplification was performed with the designed pair of primers bg-F/bg-D. The PCR fragment was cloned and then sequenced by Shanghai Sangon. The amplified 849 bp fragment contained a single ORF 729 bp *bglS* gene (Fig.2). The putative protein consisted of 242 amino acids (AA), of which the first 28

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1    ATG CCT TAT CTG AAA CGA GTG TTG CTG CTT CTT GTC ACT GGA TTG TTT ATG AGT
       M  P  Y  L  K  R  V  L  L  L  L  V  T  G  L  F  M  S
55   TTG TTT GCA GTC ACT TCT ACT GCC TCG GCT CAA ACA GGT GGA TCG TTT TTT GAC
       L  F  A  V  T  S  T  A  S  A  Q  T  G  G  S  F  F  D
109  CCT TTT AAC GGC TAT AAC TCC GGT TTT TGG CAA AAA GCA GAT GGT TAT TCG AAT
       P  F  K  G  Y  N  S  G  F  W  Q  K  A  D  G  Y  S  N
163  GGA AAT ATG TTC AAC TGC ACG TGG CGG GCT AAT AAC GTA TCA ATG ACG TCA TTG
       G  N  M  F  N  C  T  W  R  A  N  N  V  S  M  T  S  L
217  GGT GAA ATG CGT TTA GCG CTA ACA AGC CCA GCT TAT AAC AAG TTT GAC TGC GGG
       G  E  M  R  L  A  L  T  S  P  S  Y  N  K  F  D  C  G
271  GAA AAC CGT TCT GTT CAA ACA TAT GGC TAT GGA CTT TAT GAA GTC AGA ATG AAA
       E  N  R  S  V  Q  T  Y  G  Y  G  L  Y  E  V  R  M  K
325  CCA GCT AAA AAC ACA GGG ATC GTT TCA TCG TTC TTC ACT TAC ACA GGT CCA ACA
       P  A  K  N  T  G  I  V  S  S  F  F  T  Y  T  G  P  T
379  GAT GGA ACT CCT TGG GAT GAG ATT GAT ATC GAA TTT TTA GGA AAA GAC ACA ACG
       D  G  T  P  W  D  E  I  D  I  E  F  L  G  K  D  T  T
433  AAG GTT CAA TTT AAC TAT TAT ACA AAT GGT GCA GGA AAC CAT GAG AAG ATT GTT
       K  V  Q  F  N  Y  Y  T  N  G  A  G  N  H  E  K  I  V
487  GAT CTC GGG TTT GAT GCA GCC AAT GCC TAT CAT ACG TAT GCA TTC GAT TGG CAG
       D  L  G  F  D  A  A  N  A  Y  H  T  Y  A  F  D  W  Q
541  CCA AAC TCT ATT AAA TGG TAT GTC GAC GGG CAA TTA AAA CAT ACT GCA ACA AAC
       P  N  S  I  K  W  Y  V  D  G  Q  L  K  H  T  A  T  N
595  CAA ATT CCG ACA ACA CCT GGA AAG ATC ATG ATG AAC TTG TGG AAT GGC ACG GGT
       Q  I  P  T  T  P  G  K  I  M  M  N  L  W  N  G  T  G
649  GTC GAT GAA TGG CTT GGC TCC TAC AAT GGT GTA AAT CCG CTA TAC GCT CAT TAT
       V  D  E  W  L  G  S  Y  N  G  V  N  P  L  Y  A  H  Y
703  GAC TGG GTG CGC TAT ACA AAA AAA TAA
       D  W  V  R  Y  T  K  K.  ***
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Fig.2 Nucleotide and deduced amino acid sequences of the *bglS* gene
The stop codon "TAA" was shown by asterisk at the end of the sequence

AA were predicted to be the signal sequence for the secretion of the protein to the extracellular medium and the rest 214 AA were the mature protein. The first 26 AA of the signal sequence region were deleted, and the rest of the sequence were fused to the signal sequence of *MFα1* factor to construct the *bglS* expression cassette in *S. cerevisiae*.

PCR amplification of *bglS* gene, *PGK1* promoter, *MFα1*$_S$, *ADH1* terminator and the *KanMX* cassette

The PCR products of the *PGK1* promoter, *MFα1*$_S$, *ADH1* terminator and the mature *bglS* coding sequence were 778, 285, 259 and 654 bp, respectively. *KanMX* cassette was 1652 bp in length (Fig.3).

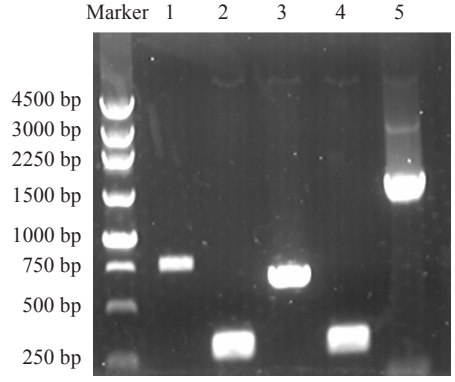

Fig.3 Agarose gel electrophoresis of PCR products
Lane 1: *PGK1* promoter; Lane 2: The signal sequence of the yeast mating pheromone α-factor (*MFα1*$_S$); Lane 3: *bglS* gene; Lane 4: *ADH1* terminator; Lane 5: *KanMX* cassette

Construction and identification of the recombinant vector pUC18-KPMBT

The five PCR-amplified fragments were cloned into pUC18 cloning vector to construct recombinant plasmid pUC18-KPMBT (*KanMX*-*PGK1*$_P$-*MFα1*$_S$-*bglS*-*ADH1*$_T$) according to the process described in the above section. The recombinant plasmid pUC18-KPMBT was shown in Fig.4.

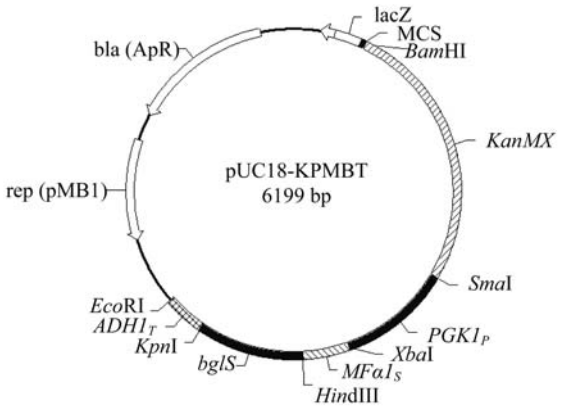

Fig.4 The recombinant plasmid pUC18-KPMBT

*Bam*HI and *Eco*RI were selected to digest the recombinant pUC18-KPMBT plasmid in order to identify the recombinant vector. The digestion results, the 2667 and 3614 bp fragments, were expected. The recombinant plasmid pUC18-KPMBT was also confirmed through PCR verification with P1 & P8 and P9 & P8 primers, resulting in 1976 and 3642 bp products,

respectively (Fig.5). Sequencing analysis of the recombinant plasmid by Shanghai Sangon Co. indicated that the constructed plasmid was correct.

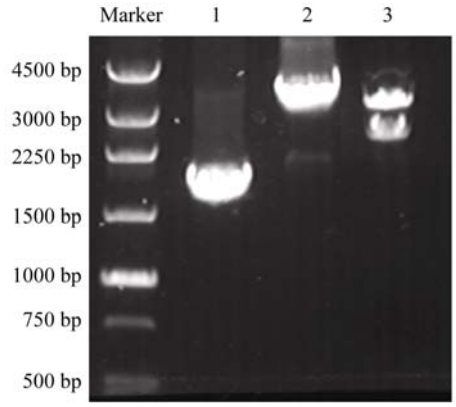

Fig.5 Identification of the recombinant plasmid
Lane 1: PCR product with P1 & P8 primers; Lane 2: PCR product with P8 & P9 primers; Lane 3: Recombinant plasmid pUC18-KPMBT digested with *Bam*HI and *Eco*RI

Yeast transformation and screening of the recombinants

PCR amplification of the fragment for gene replacement was pooled using P13 & P14 as primers and the recombinant plasmid pUC18-KPMBT as template in 50 μl volume containing 5 μl 10× PCR buffer, 1.2 mmol/L $MgCl_2$, 200 μmol/L dNTP, 20 ng of template plasmid, 0.2 μmol/L primers and 2.5 U Taq polymerase. The cycle conditions were 94 °C for 5 min followed by 30 cycles of 94 °C for 40 s, 58 °C for 1 min and 72 °C for 3 min, and finally 72 °C for 10 min. The purified PCR product (~5 μg) was electro-transformed into *S. cerevisiae* WZ65 strain. The transformation conditions and screening method for the recombinants were described in the previous section. The *S. cerevisiae* recombinants expressing *endo*-l,3-1,4-β-glucanase were detected by a clearing zone surrounding the yeast colonies after staining the lichenan-agar upper layer with Congo Red (Fig.6). The *endo*-l,3-1,4-β-glucanase activity was also detected in the supernatant of YEPD liquid cultures. PrA activity was also determined to be zero.

The recombinant *S. cerevisiae* WZ65 strain expressing *endo*-l,3-1,4-β-glucanase was identified by PCR amplification. Using the recombinant yeast genomic DNA as the template and P11 & P10, P5 & P12, P11 & P12 as primers, PCR amplification was used to verify the recombinant strains, resulting in 1914 and 1273 bp fragments with P11 & P10 and P5 & P12, respectively. The products with P11 & P12 were two fragments, the 4229-bp one for the KMBT and the 1830-bp one for the *PEP4* ORF (Fig.7). PCR analysis of the *bglS*-expressed yeast strain showed that the host strain was diploid, and one of the *PEP4* alleles was disrupted with the *bglS* expression cassette and the other was intact. The culture supernatant of recombinant *S. cerevisiae* WZ65 strain was also used as crude enzyme to determine PrA activity and *endo*-l,3-1,4-β-glucanase activity. The screened positive transformant was named as SC-βG.

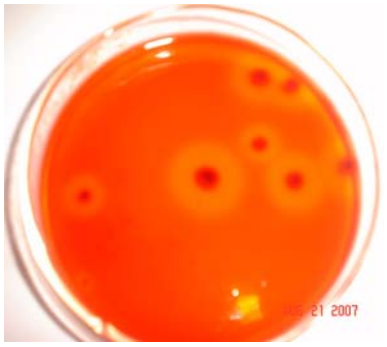

Fig.6 Detection of *endo*-l,3-1,4-β-glucanase activity in *S. cerevisiae* recombinants by cleavage of lichenan. The plate was then stained with Congo Red

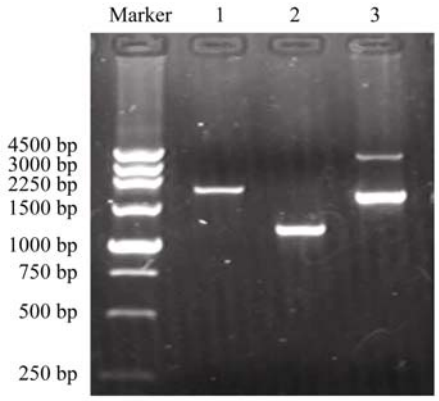

Fig.7 PCR verification of recombinant strain SC-βG
Lane 1: PCR product with P11 & P10 primers; Lane 2: PCR product with P5 & P12 primers; Lane 3: PCR product with P11 & P12 primers

In considering of high thermostability of *endo*-1,3-1,4-β-glucanase from *B. subtilis* compared with that from barley, the cloned *Bacillus bglS* gene was expressed in *S. cerevisiae* WZ65 strain to construct the recombinant brewing yeast with the capacity to hydrolyze β-glucans. The *Bacillus bglS* gene was very

lowly expressed in yeast under the control of its own promoter (Hinchliffe and Box, 1984) and increased when it was expressed and secreted under the control of $ADH1_P$ (*ADH1* promoter), $PGK1_P$ and signal peptides from yeast (Cantwell *et al*., 1986). In the current research, the $ADH1_P$ and $MF\alpha1_S$ were selected to improve the *B. subtilis endo*-1,3-1,4-β-glucanase expression efficiency in *S. cerevisiae*.

Endo-l,3-1,4-β-glucanase activity measurement of the recombinant

The activity of *endo*-l,3-1,4-β-glucanase secreted by *S. cerevisiae* WZ65 was determined by Congo Red method (Fig.8a). The glucanase activity of the recombinant strain increased quickly after cultivation for 24 h and reached the maximum of 69.3 U at 60 h. After then, *endo*-l,3-1,4-β-glucanase activity decreased slowly and was maintained around 50 U after incubation for 72 h. It was supposed that *endo*-l,3-1,4-β-glucanase expression was inhibited by metabolites (including alcohol, organic acids and other compounds) of SC-βG as cultivation period lasted; however, the deep reason underlined needs to be further investigated in the future research.

Furthermore, the β-1,3-1,4-glucanase activity was assayed by DNS method and the same trend as that of Congo Red method was observed (Fig.8b). It is relatively difficult to assay *endo*-1,3-1,4-β-glucanase activity by measuring the amount of reducing sugars. The problem arises due to the fact that there are only a few 1,4- linkages next to 1,3- linkages in barley β-glucan, allowing only a few sites where the enzyme can cut the glucan chain. This results in the production of only a few reducing sugar ends and if measured by the DNS method no effect was detected. Interestingly, although only were a few reducing sugar ends formed, a large clearing zone was formed during the plate assay with Congo Red. Results of the plate assays clearly show that the *endo*-1,3-1,4-β-glucanase only utilized barley β-glucan and lichenan, but not CMC (carboxymethyl cellulose), whereas the *endo*-1,4-β-lucanase hydrolyzed all three substrates (van Rensburg *et al*., 1997). Current results confirm that *endo*-1,3-1,4-β-glucanase only hydrolyzes β-1,4 linkages adjacent to β-1,3 linkages (Wolf *et al*., 1995), because CMC contains no 1,4- linkages next to 1,3- linkages. The two assay methods generated almost the same change curve for the enzyme activity (Fig.8).

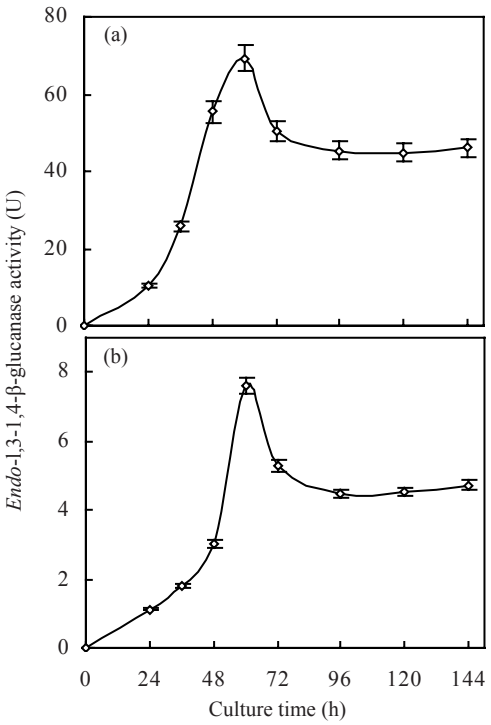

Fig.8 *Endo*-l,3-1,4-β-glucanase activity of the recombinant strain SC-βG assayed by Congo Red method (a) and DNS method (b)

DISCUSSION

In the current study, the recombinant industrial brewing yeast strain SC-βG with one of the *PEP4* alleles replaced with *bglS* expression cassette was constructed by PCR-mediated gene disruption and homologous recombination. The recombinant strain may have advantages in beer brewing, maintaining the beer foam performance especially to keep the foam stable in unpasteurized beer, improving beer filterability of brewing and increasing the stability of beer product.

PrA plays an essential role among vacuolar hydrolases (Rothman *et al*., 1986; Jones, 1991) in processing the mature forms of proteinases B (PrB) and carboxypeptidase Y (CPY) among proteases, alkaline phosphatase and RNase. An initial self-activation process of proteinase yscA is necessary for the activation of vacuolar zymogens (Rupp and Wolf, 1995). The inactive precursor molecules produced from the *PEP4* gene self-activate and subsequently activate other vacuolar hydrolases (Woolford *et al*., 1986; Ammerer *et al*., 1986). And

PEP4 is important for protein turnover after oxidative damage (Marques *et al*., 2006).

These roles of PrA are supported by the facts that the deletion of the structural gene of PrA certainly leads to the accumulation of the pro-forms of vacuolar proteases including proPrB and proCPY in the vacuole (Stevens *et al*., 1982). Mutations at the *PEP4* locus exhibit a dosage effect on the levels of some, but not all, of the enzymes whose expression requires the function of the gene. The *pep4* mutation results in a 90%~95% reduction in the levels of several vacuolar hydrolases in yeast, including PrA and PrB, CPY, RNase(s) and the repressible alkaline phosphatase (Jones *et al*., 1982; Zubenko *et al*., 1983; Stevens *et al*., 1982).

In the current work, the recombinant strain SC-βG was constructed for industrial production. Although the recombinant SC-βG yeast would secrete less PrA during beer brewing, the expressed glucanase would hydrolyze β-glucan theoretically. However, the effect of assumed reduction of expressed PrA on foam retention, the growth traits, the fermentative properties of the recombinant *S. cerevisiae*, and the hydrolytic capability of the recombinant β-glucanase to the β-glucan in the process of beer brewing need to be further investigated.

To improve the large-scale production of biotechnological products, it is very important to concentrate on engineering disciplines dealing with bioreactor design and optimization of fermentation technology, which may lead to an improved process performance, giving higher overall yields and productivities (Court *et al*., 2002). The focus on *S. cerevisiae* to fulfill several biotechnological purposes is still increasing. Since the sequence of the complete yeast genome is available, targeted genetic changes are easily obtained by recombinant DNA technology, which facilitates and accelerates metabolic engineering.

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