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Expression of aspartic protease from *Neurospora crassa* in industrial ethanol-producing yeast and its application in ethanol production

Zhong-peng Guo, Chong-yan Qiu, Liang Zhang, Zhong-yang Ding, Zheng-Xiang Wang, Gui-Yang Shi*

The Key Laboratory of Industrial Biotechnology, Ministry of Education, Center for Bioresources & Bioenergy, School of Biotechnology, Jiangnan University, Wuxi 214122, PR China

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ABSTRACT

In order to further improve the utilization rate of raw materials and increase ethanol productivity, the gene *Asp*, encoding aspartic protease in *Neurospora crassa* was cloned and expressed in industrial ethanol-producing yeast. To promote secretion of the acid protease, the gene was fused to signal sequence of the yeast α-factor gene and constitutively expressed under transcriptional control of the *Saccharomyces cerevisiae PGK1* promoter. The resultant recombinant enzyme was characterized with respect to pH and temperature optimum. Then, this acid protease was anchored to the yeast cell wall by fusing the mature protein to the α-agglutinin peptides. The resultant strain was evaluated in clarifying corn mash and very high gravity (VHG) raw starch fermentation. The present results demonstrated that expression of the acid protease increased both growth rate and viable yeast counts and the recombinant strain of *S. cerevisiae* exhibited a higher ethanol yield as compared to the parent strain.

1. Introduction

Bioethanol production by *Saccharomyces cerevisiae* is currently, by volume, the single largest fermentation process in industrial biotechnology [1]. Therefore, the development of cost-effective technologies for fuel ethanol production is a priority for many researchers. Cellulosic ethanol is a prospective option, but corn starch is still the most commonly used feedstock for ethanol production in many countries, including United State, Canada and China. The conventional procedure to produce ethanol from starchy materials requires two steps: hydrolysis of starch by amylolytic enzymes at first and then fermented by *S. cerevisiae*. In addition, to produce high yield of ethanol from cereal grains such as corn, the mash should be treated at 140–180 °C prior to amylolysis, and the energy consumed in this process results in high production costs. Thus, to save energy in cooking process, raw-starch-digesting amylase was developed to hydrolyze starchy materials [2,3] and the yeast strains able to ferment raw starch were also constructed by expression of amylolytic enzymes [4–8]. On the other hand, the production cost of ethanol can be reduced by improving the utilization rate of raw materials. Although corn starch contains relatively high protein content (10–13%), the yeasts used in fuel ethanol fermentation are unable to metabolize soluble proteins. However, so far the studies on acid protease in ethanol industry are insufficient and its positive effects on improvement of ethanol production are not well recognized in comparison to amylase.

In addition, very high gravity (VHG) fermentation technology on starchy materials is of commercial interest because of its potential to increase the final ethanol concentration and reduce processing costs in fuel ethanol production [9]. Under VHG conditions, it is critical that the medium contains adequate yeast-assimilable nitrogen (free amino nitrogen [FAN]) for maximum rates of sugar utilization and ethanol production. Meanwhile, with the realization of commercial production of an industrial scale of raw-starch-digesting amylase from fungi and bacteria in recent years, simultaneous saccharification and fermentation (SSF) on raw starch by *S. cerevisiae* becomes possible by addition of this amylase. Acid protease could promote saccharification by its lysing effect on the particles of the raw starchy material, and amino acids produced in this process are best nutrient sources and fermentation accelerator.

Neurospora crassa is the preeminent model organism among filamentous fungi, with a long history of seminal contributions, a wealth of basic information, and prospects for expanded use as the complete genome sequence become available. Its safety has been verified as it has been put to use in several human societies, either as a food or in processing foods and beverages [10]. This work is dedicated to evaluating the effects of expression of the aspartic protease from *N. crassa* on cell-surface of industrial ethanol-producing yeast in simultaneous saccharification and fermentation (SSF) on very high gravity (VHG) corn mash. The results suggested that the constructed yeast strain could grow faster and maintain a higher viable yeast counts during the fermentation process. Simultaneously, decreased fermentation time and increased ethanol yield were also observed for the recombinant strain as compared to the wild type. Expressing acid protease would greatly improve the productivity of ethanol industry.

* Corresponding author. Tel.: +86 510 85918229; fax: +86 510 85918229.
E-mail address: biomass_jnu@126.com (G.-Y. Shi).

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Table 1
Microbial strains and plasmids used in the present study.

Strain or plasmid	Relevant genotype	Source of reference
Strains		
S. cerevisiae	Wild type	CICIMY0086, JU
N. crassa	Wild type	CICIM F0028, JU
E. coli JM109	*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi* (*Lac-proAB*) F'[*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*M15	Strategene
Plasmids		
pMD18-T simple	*bla*	TaKaRa, Japan
pMG	*bla PGK1*$_P$-*PGK1*$_t$	This investigation
pMGKR	*bla kan* *PGK1*$_P$-*PGK1*$_t$-rDNA	This investigation
pMGKR-*Asp*	*bla kan PGK1*$_P$-*Asp*-*PGK1*$_t$-rDNA	This investigation
pMGKR-*Asp*-*AG*1	*Bla kan PGK1*$_P$-*Asp*-*AG* 1-*PGK1*$_t$-rDNA	This investigation

2. Materials and methods

2.1. Strains and media

The genotypes of the microbial strains and plasmids used in present study are summarized in Table 1. All strains used in this study were provided by Culture & Information Center of Industrial Microorganism of China Universities, JU. Yeast strains were routinely grown in 250-ml Erlenmeyer flasks containing 100 ml medium containing 2% peptone and 1% yeast extract supplemented with 2% glucose as carbon source (yeast peptone dextrose) at 30 °C, on a rotary shaker at 150 rpm. For selection of yeast transformants, G418 was added with the final concentration 500 μg/ml.

2.2. Construction of strains

PGK1 promoter and terminator were amplified by PCR from total chromosomal DNA isolated from *S. cerevisiae* with primers PF1 and PR2, TF1 and TF2, respectively. After digested by NcoI, the two fragments were ligated into pMD18-T to give pMG. Kanamycin resistance gene which confers resistance to Geneticin (G418) in *S. cerevisiae* was isolated from pPIC9K using primers KF1 and KF2. The primers used to amplify this fragment were designed to introduce 34-bp *loxp* site at the 5′ and 3′ ends. The PCR product was cut with NotI and inserted into the corresponding site of pMG. Then, a partial rDNA fragment of *S. cerevisiae* used as homologous integration site was cloned with primers RF1 and RF2 and ligated into the NdeI digested pMG to form pMGKR. According to the published genomic sequence of *N. crassa* in GenBank (http://www.ncbi.nlm.hih.gov/entrez/), the gene encoding aspartic protease (accession no. NCU00338), designated *Asp*, was cloned by PCR amplification from the strain *N. crassa* using primers APF1 and APR2. The amplified fragment including the entire coding region of aspartic protease was digested by EcoRI and inserted into the relevant site of pPIC9K, resulting in pPIC9K-*Asp*. Then, the fragment containing signal sequence of the yeast α-factor fusing with *Asp* was PCR-amplified from pPIC9K-*Asp* and this BamHI/NcoI digested fragment was inserted into the corresponding site of pMGKR. The resultant plasmid was named pMGKR-*Asp*. Finally, a NcoI digested fragment of C-terminal half of the yeast α-agglutinin gene (*AGα1*) [11] was inserted into the NcoI site of pMGKR-*Asp* to give pMGKR-*Asp*-*AG*1 (Fig. 1). All primers used in this study are listed in Table 2.

The plasmids pMGKR-*Asp* and pMGKR-*Asp*-*AG*1 were cleaved with SacII, purified and the linear DNA fragments were then introduced into the industrial ethanol-producing yeast by the lithium acetate method [12]. Transformants with the G418$^+$ phenotype were isolated. The genomic DNA analysis showed that the fusion gene was correctly incorporated into the rDNA locus of the chromosome of *S. cerevisiae* (data not shown).

2.3. Enzyme assay

Supernatant and cell-associated samples were obtained by centrifugation of crude samples at 8000 × *g* for 5 min. To determine the enzyme activity of intact cell pellets and cell-wall fractions, the cells were collected and washed twice with buffer (50 mM lactic acid/sodium lactate buffer, pH 3.0), and then resuspended with the same buffer. 1 ml of this suspension was vortexed with 1 g glass beads (0.5 mm diameter) for 15 min at 0 °C. Cell wall fractions were recovered by centrifugation of the homogenate at 8000 × *g* for 5 min, followed by washing with the same buffer. The reaction mixture containing 1.0 ml 1.0% casein, 1.0 ml supernatant sample or cell-wall fractions, or suspension of cell pellets was kept at 40 °C for 10 min. After that, the reaction was stopped by adding 2.0 ml of 0.4 M trichloracetic acid to the mixture and incubated under the same condition for 20 min. Then, 1 ml of the supernatant of the above mixture isolated by centrifugation at 8000 × *g* was mixed with 5 ml 0.4 M Na_2CO_3 and 1 ml Folin's reagent. Finally, the mixture was incubated for another 20 min at 40 °C. Tyrosine was quantified at 660 nm [13]. One unit of acid protease was defined as the amount of enzyme required to release 1 μg of tyrosine per minute from casein.

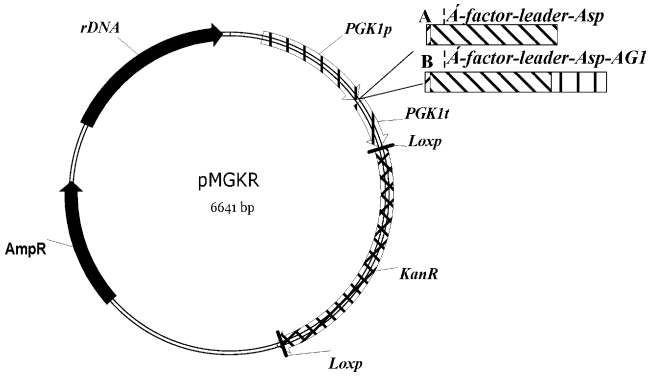

Fig. 1. Schematic summary of the construction of the different acid protease expression cassettes used in this study. (A) The expression cassette used for the production and secretion of the protease. (B) The expression cassettes constructed for anchoring the acid protease on the cell wall. The mature peptide sequence was fused at the C-terminus to the α-agglutinin (AGα 1) anchoring moieties. (*PGK*1 promoter, *S. cerevisiae* glyceraldehyde 3-phosphate dehydrogenase gene promoter; α-factor leader, 269 bp fragment encoding the α-factor signal sequence for secretion in *S. cerevisiae*; *Asp*, *N. crassa* aspartyl gene; *AG1*, half of the yeast α-agglutinin gene; KanR, Kanamycin resistance gene from Tn*903* which confers resistance to Geneticin in *S. cerevisiae* and kanamycin resistance in *E. coli*).

2.4. Immunofluorescence microscopy

Immunofluorescence microscopy was performed as reported previously [14]. Immunostaining was done as follows: Yeast cells were treated with the primary antibody, rabbit anti-aspartic protease, at a dilution rate of 1:200. After incubated at room temperature for 2 h, the cells were washed and sequentially reacted with the second antibody, FITC-conjugated goat anti-rabbit IgG, diluted 1:40 at room temperature overnight. Microscopic observation was performed according to method described previously [15].

2.5. Cultivation conditions

2.5.1. Ethanol fermentation in clarifying corn mash

Ethanol fermentation in clarifying solution was carried out in a 30-l automatic fermenter (Xinchang Deli Petrochemical Equipment, Zhejiang, China) as described previously [16]. Ground corn was dispensed in water with the ratio of ground corn to water equals to 1:3 (w/v) and the saccharification was performed according to Zhang et al. [16]. The clarifying corn mash was obtained by centrifuging the saccharified broth for 10 min at 10,000 × *g*. Nitrogen limited fermentations were carried out in clarifying corn mash containing 50 mg/l FAN and 118 g/l total sugar. Corn mash was

Table 2
The sequences of the oligonucleotide primers used in this study.

Primer names	Sequence (5′–3′) Restriction sites are italic/underlined	Restriction sites
PF1	ATTTTAGATTCCTGACTTCAACTC	
PR2	GCG*CCATGG*[a]*GGATCC*[b]TGTTTTATATTTGTTGTAAAAAGTAG	BamHI[a], NcoI[b]
TF1	CCG*CCATGG*[a]TTCTTTGGAATTATTGGAAGGTA	NcoI[a]
TR2	GCG*GCGGCCGC*[a]GAACGCAGAATTTTCGAGTTAT	NotI[a]
KF1	AG*GCGGCCGC*[a]*ATAACTTCGTATAATGTATGCTATACGAAGTTAT*[b]GCCCAGTAGTAGGTTGAGG	NotI[a], *loxp*[b]
KR2	AG*GCGGCCGC*[a]*ATAACTTCGTATAATGTATGCTATACGAAGTTAT*[b]TTGAAGTCGGACAGTGAGT	NotI[a], *loxp*[b]
APF1	ACAT*GAATTC*[a]ATGTCTTCGATT CTTTGTCAAGATGAAGAC	EcoRI[a]
APR2	GTGA*GAATTC*[a]TCACAACCCCAAAACCACC	EcoRI[a]
APF3	GGC*GGATCC*[a]ATGAGATTTCCTTCAATTTTTA	BamHI[a]
APR4	CCG*CCATGG*[a]TCACAACCCCAAAACCACC	NcoI[a]
AGF1	CCG*CCATGG*[a]AGCGCCAAAAGCTCTTTTATC	NcoI[a]
AGF2	CCG*CCATGG*[a]TTTGATTATGTTCTTTCTAT	NcoI[a]
RF1	CCG*CATATG*[a]CTCTATCCCCAGCACGA	NdeI[a]
RR2	CCC*CATATG*[a]GAGAAACGGCTACCACATC	NdeI[a]

[a] Restriction sit with corresponding restriction enzyme.
[b] Restriction sit with corresponding restriction enzyme.

fermented by recombinant strain CICIMY0086-2 and wild type supplemented with or without acid protease by inoculating at a rate of 1.02×10^8 CFU/ml. Experiments were carried out in triplicate.

2.5.2. Ethanol fermentation with raw corn starch

Fermentations were carried out under oxygen limited conditions in a 1 l shake flask containing 500 ml medium inoculated with a volume of overnight culture yielding a starting OD_{600} of 1.0. The medium contains 250 g raw corn starch and 500 ml deionized water with the ratio of ground corn to water equals to 1:2 (w/v). 100 U of raw-starch-digesting amylase was added with 1 g raw corn starch. During the fermentation process, the flasks were kept at 30 °C in a thermostatic chamber with no air supplied. The effects of expressing the acid protease on the cell surface of strain *S. cerevisiae* CICIMY0086 were studied under anaerobic growth conditions with raw corn starch medium. Fermentation experiments were performed in triplicate.

2.6. Analysis of product formation and determination of dry weight

The concentration of FAN in the supernatant of medium was determined colorimetrically using the EBC ninhydrin method [17] with glycine as the standard. Viable-cell counts were determined by the standard plate counting method. Amino acids were analyzed with a Biotronik LC 5001 amino acid analyzer. The concentration of glucose, ethanol, glycerol, pyruvic acid and acetic acid were measured by HPLC using a HP1100 column (Agilent, USA) as described previously [18]. The concentration of starch and total sugar were determined according to the previously described method [19,20]. The biomass concentration in the medium was measured gravimetrically as described earlier [21]. Briefly, three samples of cell pellets were washed twice with 0.9% (w/v) NaCl, kept at 110 °C for 24 h until constant weight was obtained. The cellular protein content was determined by a modified biuret method [22] using bovine serum albumin as a standard.

2.7. Measurement of viability

Acidification power test (APT) which has been widely used in the brewing was used to determine the viability of yeast and AP Value was measured according to Iserentant et al. [23].

3. Results and discussion

3.1. Secretive Expression of the acid protease in S. cerevisiae CICIMY0086

The acid protease activities of 10 transformant colonies were investigated. One of the recombinant strains, named APS, showed highest acid protease activity (6.8 ± 0.21 U/ml) was used in the following experiments. The recombinant acid protease showed highest catalytic activity at pH 4.0 and 45 °C (Fig. 2), whereas no detectable activity of acid protease was measured for the parent strain (The enzymatic activities were assayed in triplicate with the RSD < 5%). The results indicated that the heterologous gene had been successfully expressed in *S. cerevisiae*.

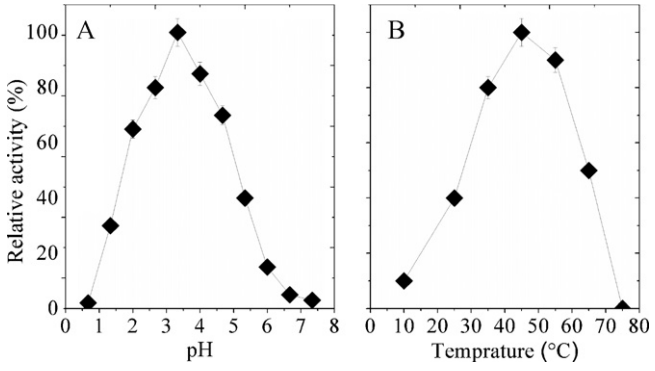

Fig. 2. Enzymatic characters of acid protease expressed by recombinant of *S. cerevisiae* APS. (A) Relation between recombinant acid protease activity and pH (filled diamond ♦); (B) Relation between recombinant acid protease activity and temperature (filled diamond ♦).

3.2. Characteristics of the strain secretively expressing acid protease in raw corn starch medium

The characteristics of the yeast strain secretive expression of acid protease were investigated under anaerobic growth conditions in raw corn starch fermentations. The results indicated that expression of acid protease in *S. cerevisiae* gave a higher ethanol production rate and the recombinant strain APS exhibited a $3.8 \pm 0.15\%$ improvement in the final ethanol concentration (relative to the amount of substrate consumed) as compared to the wild-type strain. The results suggest that the acid protease was successfully expressed and an improved ethanol yield was achieved. However, higher concentration of total residual sugar was detected at the end of fermentation for strain APS. The unpleasant fermentation may be due to the proteolytic effect of acid protease on amylase in medium. In order to avoid its negative effects on raw-starch-digesting amylase during the simultaneous saccharification fermentation, the acid protease was expressed on cell surface of yeast.

3.3. Construction of surface-engineered yeast displaying the acid protease

The acid protease activities of 30 transformant colonies were investigated. No enzymatic activities can be detected for the cell pellets, cell wall and culture supernatant for the wild-type strain. One recombinant strain (named *S. cerevisiae* CICIMY0086-2) was found to show apparently high acid protease activity was used in subsequent experiments (11.78 ± 0.23 U/g, 14.26 ± 0.19 U/g for cell pellets and cell wall, respectively, no activity could be detected for the cell supernatant).

3.4. Immunofluorescence microscopy

Immunofluorescent labeling of yeast cells was performed using the rabbit anti-aspartic protease antibody and FITC-conjugated goat anti-rabbit IgG. Cells harboring the control plasmid showed almost no labeling (Fig. 3a and b), while cells expressing acid protease exhibited green fluorescence localized at their surface (Fig. 3c and d). The results confirmed that the aspartic protease was displayed on the surface of *S. cerevisiae*.

3.5. Characteristics of the strain displaying acid protease on the cell surface in clarifying corn mash fermentation

3.5.1. Comparison of available FAN and the concentration of protein in the medium

It was reported VHG fuel alcohol fermentation could be stimulated when hydrolyzed wheat proteins were used as a source of FAN [24]. In case of the corn mash fermentation, FAN obviously played a role in growth and multiplication of yeast. Besides, in current high concentration mash fermentations, high pitching rate (more than 1×10^8 CFU/ml) was usually employed to decrease the preliminary lag phase. If available, the cells took up more FAN than was necessary for full fermentation of the sugar solutions. Although 250 mg FAN/1 was considered to be adequate for the full fermentation of normal gravity wort [25,26], VHG corn mash used in these experiments requires higher concentrations of FAN for rapid attenuation. In order to evaluate the effects of expression of acid protease, the initial concentration of FAN in corn mash was adjusted to approximately 50 mg/l, low enough for a fermentation to be sluggish but high enough for it to finish. Compared to the wild type, a higher concentration of FAN with a lower concentration of proteins in medium was observed for recombinant *S. cerevisiae* CICIMY0086-2 expression of aspartic protease and *S. cerevisiae* CICIMY0086 (wild type) supplemented with acid protease (Fig. 4a and b). Obviously,

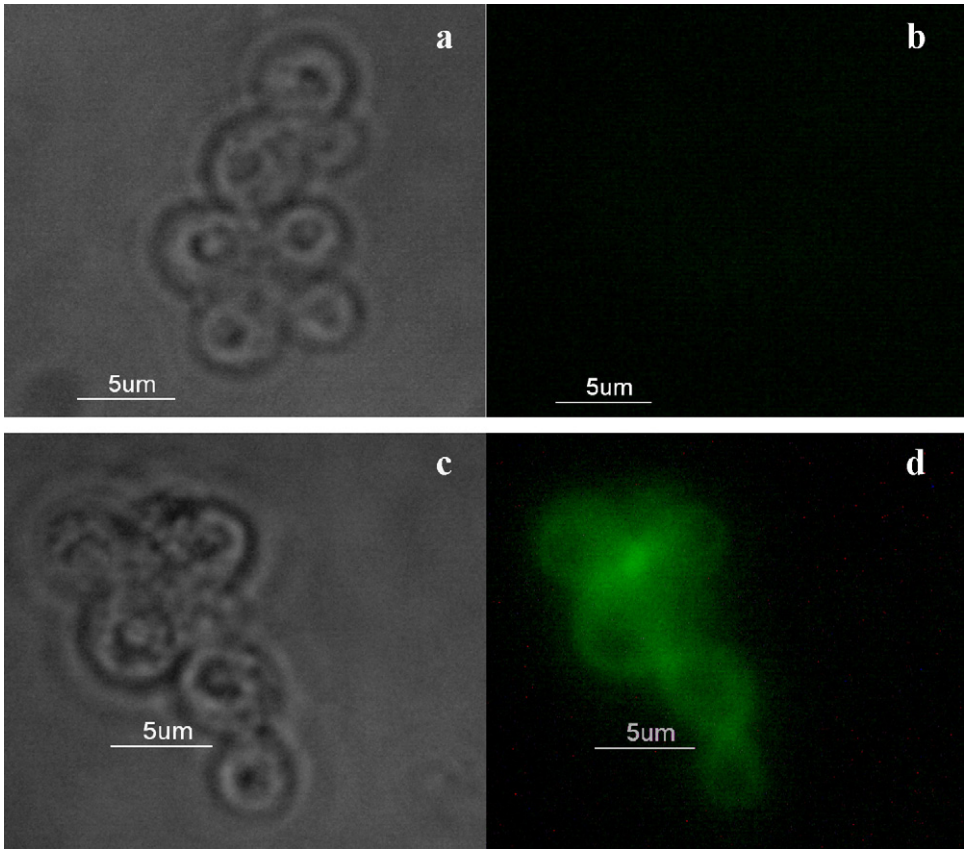

Fig. 3. Immunofluorescent labeling of transformed cells. Phase micrographs (a and c) and immunofluorescence micrographs (b and d). (a and b) *S. cerevisiae* CICIMY0086/pMGKR (control); (c and d) *S. cerevisiae* CICIMY0086-2/pMGKR-*Asp-AG*1 expressing the aspartic protease on cell surface.

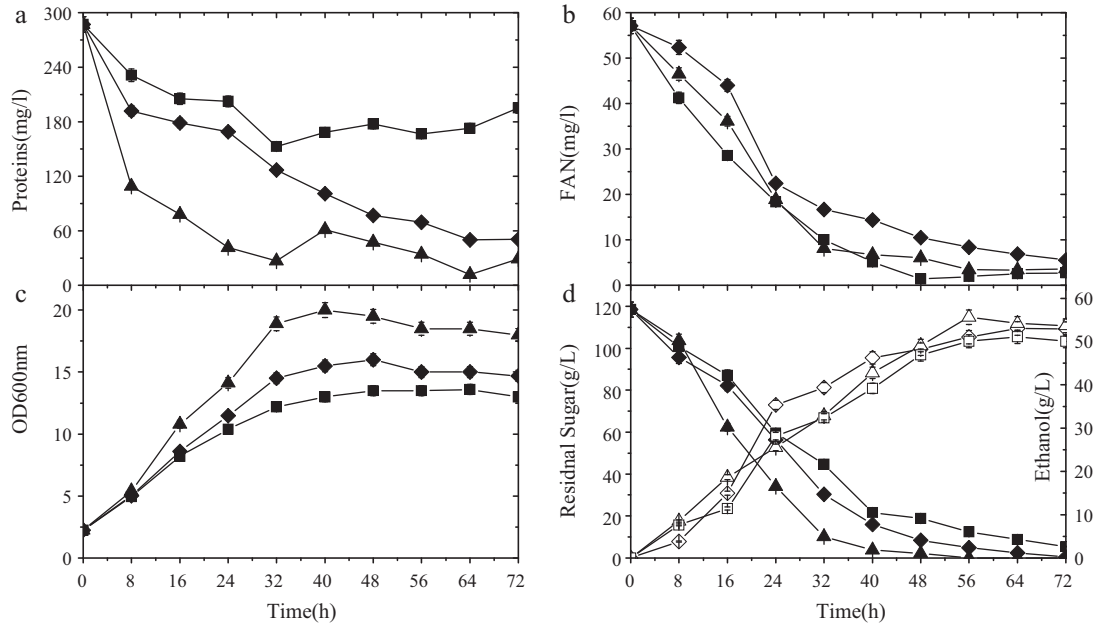

Fig. 4. Changes in measured parameters during anaerobic batch fermentation with clarifying corn mash. (a) Concentration of Protein of the medium of *S. cerevisiae* CICIMY0086 wild type (filled square ■), recombinant CICIMY0086-2 (filled diamond ♦) and wild type supplemented with acid protease (filled triangle▲), respectively. (b) Concentration of FAN of wild type (filled square ■), recombinant (filled diamond ♦) and wild type supplemented with acid protease (filled triangle▲), respectively. (c) OD_{600nm} of wild type (filled square ■), recombinant (filled diamond ♦) and wild type supplemented with acid protease (filled triangle▲„ respectively. (d) Concentration of residual sugar and ethanol of the medium of wild type (square ■, □), recombinant (diamond ♦, ◊) and wild type supplemented with acid protease (triangle▲ and △), respectively.

Table 3
Comparison of growth and compound yields of *S. cerevisiae* CICIMY0086 wild type with CICIMY0086-2, respectively, during anaerobic batch growth in clarifying corn mash.

Parameter	CICIMY0086[a]	CICIMY0086-2[a]	CICIMY0086 (supplemented with acid protease) [a]
Average fermentation rate (g glucose/h)	1.52 ± 0.05	1.83 ± 0.06	2.15 ± 0.08
Growth yield (g biomass/g glucose)	0.526 ± 0.02	0.564 ± 0.02	0.578 ± 0.01
Protein (g/100 g biomass)	53.28 ± 1.6	55.75 ± 1.8	57.32 ± 2.1
Ethanol yield (g ethanol/g glucose)	0.456 ± 0.02	0.496 ± 0.01	0.505 ± 0.02
Acetate yield (mg acetate/g glucose)	3.54 ± 0.15	3.52 ± 0.13	3.59 ± 0.16
Glycerol yield (mg glycerol/g glucose)	15.24 ± 0.63	12.16 ± 0.54	11.28 ± 0.48
Pyruvic acid (mg pyruvic acid/g glucose)	0.673 ± 0.03	0.678 ± 0.03	0.682 ± 0.02
Total residual sugar (g/100 ml)	0.87 ± 0.04	0.11 ± 0.01	0.06 ± 0.01

[a] Triplicate fermentations ± the standard deviation.

50 mg/l FAN was too low as the sole nitrogen source for VHG fermentation to be finished in our experiment. As a result, the final total residual sugar of wild type was higher and the duration of the fermentation was also prolonged compared to the recombinant strain and the control supplemented with acid protease (Table 3). However, when a non-deficient amount of nitrogen (300 mg/l) was used, the fermentation can be completed in less than 56 h (data not shown). Saita and Slaughter [27] previously attributed stimulation by nitrogen to its function as a substrate for protein synthesis. In our experiment, acid protease could stimulate corn mash fermentation by supplying with more available FAN, which would benefit the fermentation especially when a higher concentration of sugar was used.

3.5.2. Comparison of growth and fermentation rate

As reported by other researchers [28,29], excess assimilable nitrogen did not result in increase in numbers of cells or rates of cell division, but lead to increased cell mass accumulation, particularly when a small inoculum was used. In our experiments, the recombinant strain expression of acid protease on cell surface grew faster than the parent strain and its OD value could reach 14.0 in 24 h, whereas the wild-type strain supplemented with acid protease showed the highest growth rate and its maximum OD value could reach 20 in 36 h (Fig. 4c). Both the cells of the recombinant and wild-type strain in the acid protease-supplemented medium (Table 3) contained significantly higher cellular protein content at the end of fermentation than the control. Thus, it was likely that the larger cells with higher cellular protein content resulted in the higher OD value. Besides the quicker fermentation rate, rapid ethanol production and sugar consumption rate were also observed for recombinant and wild-type strain supplied with acid protease (Fig. 4d). As expected, the corn mash with no added extra nitrogen source did not ferment satisfactorily (Table 3). Supplement with acid protease significantly increased the rate of fermentation and the VHG fermentation was finished in 50 h with residual sugar less than 0.5 g/l, whereas 72 h needed to end fermentation in case of wild type. As for the recombinant strain, fermentation was ended in less than 64 h.

3.5.3. Comparison of product yields and total residual sugar

Table 3 shows the theoretical maximum amounts of ethanol could arise from wild type as the acid protease was added into the medium in these experiments. At 56 h, acid protease-supplemented corn mash contained significantly higher amounts of ethanol than did the control and yielded a maximum ethanol concentration of 50.2–50.5 g per 100 g of sugar. This was an extremely high yield nearly 98–99% of the theoretical maximum level; normal batch fermentations by yeasts optimally give rise to 93-95%of the theoretical value [30]. In contrast, the unsupplemented control medium gave rise to only 45.3–45.9 g/l ethanol, only 89%of the theoretical maximum. Besides, 8.7 g/l total residual sugar remained in the fermentation medium inoculated with the control strain, much higher than 0.1 g/l for recombinant strain and 0.06 g/l for wild type supplied with acid protease. The low yields of ethanol in un-supplemented medium and non-full fermentation on sugar for the wild type may resulted from the unfavorable VHG environment, indicating the importance of nutritionally optimizing these fermentations. In addition, among the evaluated three main byproducts, the production of glycerol was found to be higher in un-supplemented mash of wild type (Table 3). Since the respiratory chain does not function during anaerobic conditions and glycerol formation by yeast is to re-oxidize NADH formed in synthesis of biomass and secondary fermentation products, to NAD^+ [31], the reduced glycerol yield was likely due to the beneficial effects of more available FAN and essential amino-acids on synthesis of biomass. Meanwhile, the yield of acetate and pyruvic acid was indistinguishable for all the strains. The above results suggest that expression of acid protease can significantly increase the ethanol productivity in clarifying corn mash fermentations.

3.6. Characteristics of the strain displaying acid protease on the cell surface in raw corn starch medium

3.6.1. Comparison of growth rate, viable-cell counts and viability

The growth rate of the wild-type strain was 0.21 h^{-1} and a maximal yeast cell number of 2.7×10^8 CFU/ml was reached by 28 h. Thereafter the viable-cell counts, however, began to decrease. Expression of acid protease significantly increased the maximum specific growth rate (Table 4) and viable-cell counts (Fig. 5a) of recombinant strain. The viable cell accounts for the recombinant CICIMY0086-2 could reach 3.2×10^8 CFU/ml of medium by 20 h and the cell mass increased from an initial level of 0.6 mg/ml to a maximum of 16–18 mg/ml. Besides, improved biomass yield companied with higher cellular protein content for the recombinant strain was detected at the end of the fermentation. High growth and fermentation rate were critical for SSF with raw starch because the occurrence of contamination of other undesirable microorganisms was so common.

Table 4
Comparison of growth and compound yields of *S. cerevisiae* CICIMY0086 wild type with CICIMY0086-2, respectively, during anaerobic batch growth on 50% raw corn starch.

Parameter	CICIMY0086[a]	CICIMY0086-2[a]
Dry weight (g)	10.12 ± 0.21	10.88 ± 0.29
Growth rate (h^{-1})	0.24 ± 0.01	0.33 ± 0.01
Growth yield (g biomass/g glucose)	0.506 ± 0.02	0.544 ± 0.03
Protein (g/100 g biomass)	55.47 ± 1.5	56.95 ± 1.7
Ethanol concentration (v/v)	13.8 ± 0.12%	14.8 ± 0.21%
Total residual sugar (g/100 ml)	4.46 ± 0.14	3.57 ± 0.12

[a] Triplicate fermentations ± the standard deviation.

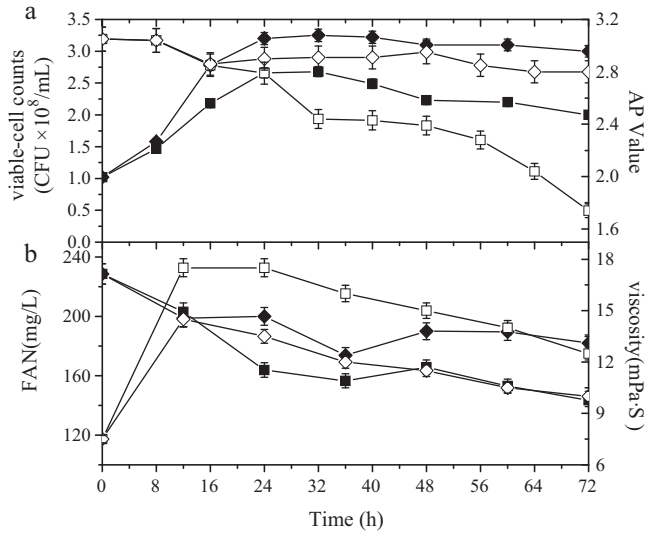

Fig. 5. Changes in measured parameters during anaerobic batch fermentation on raw corn starch. (a) Viable-cell counts and AP value of *S. cerevisiae* CICIMY0086 wild type (square ■, □) and recombinant CICIMY0086-2 (diamond ♦, ◊), respectively; (b) Concentration of FAN and viscosity of the medium of CICIMY0086 wild type (square ■, □) and recombinant (diamond ♦, ◊), respectively.

On the other hand, it was reported that the ability of yeast cells to excrete acidity through the action of membrane H^+-ATPase, H^+/K^+ exchange, and production of CO_2 and organic acids is a strictly controlled process that reflects very accurately the metabolic activity of the cells [32–34]. The AP test developed by Opekarová and Sigler [35] on the basis of these findings provided a fast and simple method of assessing the viability of yeast cells. As can be seen in Fig. 5a, with the increase of viable-cell counts in the exponential growth phase, AP value remained constant. With the consumption of available FAN, however, AP value of the wild type began to decrease and dropped below 1.8 at 72 h, whereas AP value of the recombinant strain could maintain at high level (>2.8) during the whole fermentation process. Since AP value below 2 indicated a reduced metabolic activity and AP value below 1.8 denoted low metabolic competences would result in sluggish fermentation [36], the cells with high viability can be harvested and used for further fed-batch fermentation.

3.6.2. Comparison of available FAN and the viscosity of the medium

Nearly 28% of the total 228.6 mg/l FAN was taken up by parent strain within 24 h. The initial concentration of FAN in the VHG raw corn starch fermentation was high, but assimilable nitrogen was not sufficient as can be seen from the high content of FAN at the end of fermentation for the wild type. The residual amounts may be un-utilizable forms of FAN such as higher peptides [≥4 amino acid residues] and soluble proteins [24]. However, higher available amount of FAN was observed for the recombinant strain (Fig. 5b). Analyzing the composition of the amino acids of medium showed that 14 out of the 17 tested amino acids were less than 10 mg/l and about 10 kinds of them cannot be testified after 24 h for wild type. On the contrary, although six kinds of amino acids were less than 5 mg/l for the recombinant strain after 48 h, their concentrations could maintain at this level until the end of the fermentation. The results confirmed the effects of expression of the acid protease to degrade the proteins in medium (Table 5). Previously study pointed out that arginine, serine, glutamate, threonine, aspartate and lysine were generally the most heavily utilized amino acids for wine yeast [37]. In our experiment, arginine, lysine, serine and threonine were deficient in medium. Obviously, expression of the acid protease could promote the fermentation by supplying with more assimilable essential amino acids. Meanwhile, maximum rates of sugar utilization and ethanol production could be achieved [38]. Limitation of nitrogenous nutrients had significant effects on most aspects of VHG wheat mash fermentation, including yeast growth and attenuation. Although available FAN could be increased by adding extra protease or other nutrients (yeast extract, amino acids, vitamin, etc.) as described in early research [38,39], the cost of production would also rise and fermentation process was made complicated. Furthermore, expression of acid protease could reduce the viscosity of the medium by degrading the proteins, resulting in less power consumption used in mixing (Fig. 5b).

3.6.3. Comparison of ethanol yields and total residual sugar

Expression of acid protease not only gave an increased rate of fermentation but also yielded greater amount of ethanol. There was no improvement of the ethanol production when $(NH_4)_2\ SO_4$ was added in a control experiment since the metabolic requirement of carbon source and ATP were not decreased (data not shown), while high amount of available FAN would enhance the cell growth and multiplication and reduce the amount of carbon source as well as

Table 5
Comparison of amino acids of *S. cerevisiae* CICIMY0086 wild type with CICIMY0086-2, respectively, during anaerobic batch growth on 50% raw corn starch.

Strain amino acid (mg/L)	CICIMY0086[a]				CICIMY0086-2[a]			
	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
Asp	14.05 ± 0.12	5.90 ± 0.13	1.396 ± 0.15	1.30 ± 0.13	13.67 ± 0.15	13.85 ± 0.18	5.64 ± 0.22	7.854 ± 0.25
Glu	44.03 ± 0.32	8.14 ± 0.32	9.00 ± 0.52	8.15 ± 0.25	44.11 ± 0.36	38.25 ± 0.45	5.56 ± 0.54	6.083 ± 0.51
Ser	8.73 ± 0.21	0.26 ± 0.09	ND[b]	ND	8.64 ± 0.22	5.26 ± 0.26	1.03 ± 0.19	1.18 ± 0.13
His	11.30 ± 0.08	0.09 ± 0.02	ND	ND	11.29 ± 0.14	8.66 ± 0.28	1.37 ± 0.22	1.03 ± 0.25
Gly	8.78 ± 0.10	4.97 ± 0.13	5.18 ± 0.24	4.60 ± 0.23	8.96 ± 0.15	10.93 ± 0.18	4.76 ± 0.31	4.05 ± 0.36
Thr	8.12 ± 0.13	1.17 ± 0.14	ND	ND	7.51 ± 0.17	3.33 ± 0.29	2.195 ± 0.21	1.43 ± 0.29
Arg	19.91 ± 0.18	0.18 ± 0.02	ND	ND	19.19 ± 0.19	13.63 ± 0.17	4.32 ± 0.26	3.21 ± 0.23
Ala	16.08 ± 0.21	1.13 ± 0.05	1.150 ± 0.23	ND	16.39 ± 0.25	17.84 ± 0.35	3.61 ± 0.18	3.40 ± 0.15
Tyr	9.31 ± 0.13	1.14 ± 0.09	1.98 ± 0.25	2.13 ± 0.34	9.60 ± 0.15	10.67 ± 0.18	2.02 ± 0.15	3.41 ± 0.45
Cys-s	4.08 ± 0.09	4.40 ± 0.11	4.120 ± 0.35	4.151 ± 0.29	4.02 ± 0.08	3.81 ± 0.09	3.913 ± 0.11	4.048 ± 0.36
Val	18.04 ± 0.25	4.14 ± 0.06	1.45 ± 0.26	1.36 ± 0.18	18.41 ± 0.30	19.09 ± 0.38	11.4 ± 0.21	3.68 ± 0.45
Met	3.99 ± 0.11	0.003 ± 0.005	ND	ND	3.46 ± 0.14	3.65 ± 0.07	1.08 ± 0.26	0.12 ± 0.01
Phe	19.98 ± 0.26	0.034 ± 0.01	ND	ND	18.84 ± 0.27	16.42 ± 0.56	1.04 ± 0.35	1.02 ± 0.28
Ile	12.05 ± 0.19	0.365 ± 0.08	ND	ND	12.49 ± 0.24	13.60 ± 0.17	1.75 ± 0.17	0.5 ± 0.12
Leu	46.30 ± 0.32	0.917 ± 0.12	ND	ND	47.10 ± 0.33	40.96 ± 0.81	1.12 ± 0.13	1.82 ± 0.14
Lys	34.41 ± 0.41	0.229 ± 0.15	ND	ND	34.02 ± 0.44	28.32 ± 0.29	11.1 ± 0.26	0.285 ± 0.10
Pro	9.77 ± 0.14	9.86 ± 0.52	9.54 ± 0.23	10.26 ± 0.77	10.07 ± 0.21	12.90 ± 0.33	11.00 ± 0.53	11.602 ± 0.21

[a] Triplicate fermentations.
[b] Not detectable ± the standard deviation.

ATP needed for protein synthesis. Consequently, a 7.2% increased ethanol yield for recombinant CICIMY0086-2 was achieved. Furthermore, full fermentation was completed in 62 h (compared with 72 h for the wild type) with the concentration of total residual sugar less than 0.2 g/l.

4. Conclusion

In this study, the surface-engineered yeast displaying acid protease had been constructed and evaluated in the VHG raw starch fermentation. The increased ethanol production of the novel strain was meaningful since the strain could be applied to industrial fermentation. Furthermore, lower viscosity and total residual sugar as well as a reduced duration of fermentation could decrease the cost of ethanol production even further. Future studies would be conducted to investigate the impact of the expressed protease on ethanol production from a metabolome level, including amino acids and sugars metabolism through fermentative and nitrogen metabolism.

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