Yeast
Yeast 2010; **27**: 1017–1027.
Published online 24 August 2010 in Wiley Online Library
(wileyonlinelibrary.com) **DOI:** 10.1002/yea.1811

Research Article

Improving the performance of industrial ethanol-producing yeast by expressing the aspartyl protease on the cell surface

Zhong-peng Guo, Liang Zhang, Zhong-yang Ding, Zheng-Xiang Wang and Gui-Yang Shi*
The Key Laboratory of Industrial Biotechnology, Ministry of Education, Center for Bioresources and Bioenergy, School of Biotechnology, Jiangnan University, Wuxi 214122, People's Republic of China

**Correspondence to:*
Gui-Yang Shi, The Key Laboratory of Industrial Biotechnology, Ministry of Education, Center for Bioresources and Bioenergy, School of Biotechnology, Jiangnan University, Wuxi 214122, People's Republic of China.
E-mail: biomass_jnu@126.com

Received: 27 May 2010
Accepted: 5 July 2010

Abstract

The yeasts used in fuel ethanol manufacture are unable to metabolize soluble proteins. The *PEP4* gene, encoding a vacuolar aspartyl protease in *Saccharomyces cerevisiae*, was either secretively or cell-surface anchored expressed in industrial ethanol-producing *S. cerevisiae*. The obtained recombinant strains APA (expressing the protease secretively) and APB (expressing the protease on the cell wall) were studied under ethanol fermentation conditions in feed barley cultures. The effects of expression of the protease on product formation, growth and cell protein content were measured. The biomass yield of the wild-type was clearly lower than that of the recombinant strains (0.578 ± 0.12 g biomass/g glucose for APA and 0.582 ± 0.08 g biomass/g glucose for APB). In addition, nearly 98–99% of the theoretical maximum level of ethanol yield was achieved (relative to the amount of substrate consumed) for the recombinant strains, while limiting the nitrogen source resulted in dissatisfactory fermentation for the wild-type and more than 30 g/l residual sugar was detected at the end of fermentation. In addition, higher growth rate, viability and lower yields of byproducts such as glycerol and pyruvic acid for recombinant strains were observed. Expressing acid protease can be expected to lead to a significant increase in ethanol productivity.

Keywords: industrial ethanol-producing strain; aspartyl protease; surface-engineered yeast; *Saccharomyces cerevisiae*

Introduction

Fuel alcohol is one of the most promising alternative liquid fuels among the primary renewable sources and energy. However, to make ethanol an economically feasible biofuel, the process has to be optimized in terms of yields as well as ethanol production rate (Casey and Ingledew, 1986). Very high gravity (VHG) fermentation technology is of commercial interest because of its potential to increase the final ethanol concentration and to reduce processing costs in fuel ethanol production (Ingledew, 1993). Under VHG conditions, it is critical that fermentations have adequate yeast-assimilable nitrogen (free amino nitrogen, FAN) for maximum rates of sugar utilization and ethanol production. Although the starchy materials used for ethanol production contain relatively high protein content (corn, 10–13%; feed barley, 15–18%), the yeasts used in fuel ethanol manufacture are unable to metabolize soluble proteins, making the materials of no value during fermentation. The intrinsic importance of assimilable nitrogen to yeast growth and metabolism is well known by the alcoholic beverage industries such as beer and wine, in which the medium contains both ammonium and amino acids (Beltran *et al*., 2005; Mäkinen *et al*., 1970), but few studies have investigated the influence of the nitrogen source on yeast growth and

product formation in the ethanol industry. It is already known that imbalance and, in particular, deficiencies in the supply of assimilable nitrogen compounds remain the most common cause of fermentation faults (Bely *et al.*, 1990; Ingledew and Kunkee, 1985). In the ethanol industry, nitrogen deficiencies would cause unpleasant effects on the growth and metabolism of yeast, resulting in stuck or sluggish fermentations. One way to solve these problems is by adding nutritional supplements, usually inorganic forms of nitrogen such as ammonium salts, to medium prior to fermentation (Bely *et al.*, 1990; Jiranek *et al.*, 1991; Monteiro and Bisson, 1992). However, without knowledge of the nitrogen content of the medium and the requirement for nitrogen by yeast, such additions are being made empirically. For the ethanol industry using cereal grains such as corn or wheat or economic crops such as cassava as fermentation mashes, which contain some protein, adding acid protease to the medium is another way to avoid problems. Amino acids produced in this process are the best nutrient substances and fermentation activating accelerator. However, simultaneous saccharification and fermentation (SSF) in VHG cannot be conducted due to the proteolytic effect of acid protease on other kinds of enzymes, specifically the raw-starch-digesting amylase. Although the materials can be treated by acid protease prior to fermentation and the activities of the enzymes could be lost by heating, the energy consumed during this process would definitely increase the cost.

In the present study, in order to improve the efficiency of material utilization and ethanol yield, as well as simplify the fermentation process, the *PEP4* gene, encoding a vacuolar aspartyl protease, was expressed secretively and cell-surface anchored in industrial ethanol-producing strain of *S. cerevisiae*. The two kinds of recombinant strains obtained, APA (expressing the protease secretively) and APB (expressing the protease on cell wall), were studied under ethanol fermentation conditions. This is the first report, to our knowledge, to express its own aspartyl protease on the cell surface of industrial ethanol-producing yeast and evaluate its effect on ethanol and single-cell protein (SCP) production. As the yeast *S. cerevisiae* has 'generally regarded as safe' (GRAS) status and no heterologous genes were introduced to the strain in our experiment, the novel strains would have practical use for the food, ethanol and feed processing industries with its characteristics of high viability and protein content.

Materials and methods

Strains and media

All strains used in this study were provided by the Culture and Information Center of Industrial Microorganisms of China Universities, JU. *Escherichia 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]} was used for recombinant DNA manipulation. Industrial ethanol production yeast *S. cerevisiae* CICIMY0086 (wild-type) was routinely cultivated in a medium composed of 1% yeast extract, 2% Bacto peptone and 2% glucose (YPD), and solid medium containing 2% agar. For selection of yeast transformants, G418 was added with a final concentration of 500 μg/ml. Incubation conditions were standardized on the rotary shaker at 30 °C with 150 rpm.

Construction of the plasmids

The plasmids used for yeast secretive and cell-surface anchored expression were constructed as follows: *PGK1* promoter and terminator were cloned by PCR amplification from *S. cerevisiae* with primers PF1 and PR2, TF1 and TF2. After digestion by *Sal*I, the two fragments were inserted into the pMD18-T simple vector (TaKaRa, Japan) resulting in plasmid pMG. The kanamycin resistance gene, which confers resistance to Geneticin in *S. cerevisiae*, was amplified from the plasmid pPIC9K using primer KF1 and KF2 containing a 34-bp *loxp* site on both ends, and was inserted into the *Not*I site of pMG. An rDNA fragment of the *S. cerevisiae* was cloned using primer RF1 and RF2 and inserted into the *Nde*I site of pMG1 used as homologous integration site; the resultant plasmid was named pMGKR. The *PEP4* gene, encoding aspartyl protease in *S. cerevisiae*, was cloned by PCR amplification using primers APF1 and APR2 containing *EcoR*I site on both ends. A 1218 bp PCR fragment including the entire coding region was obtained, after digestion by *Eco*RI; the fragment was inserted into the pPIC9K vector at the same site, resulting in plasmid pPIC9K-*PEP4*. Then the fragment containing signal sequence of the yeast α-factor fusing with

DOI: 10.1002/yea

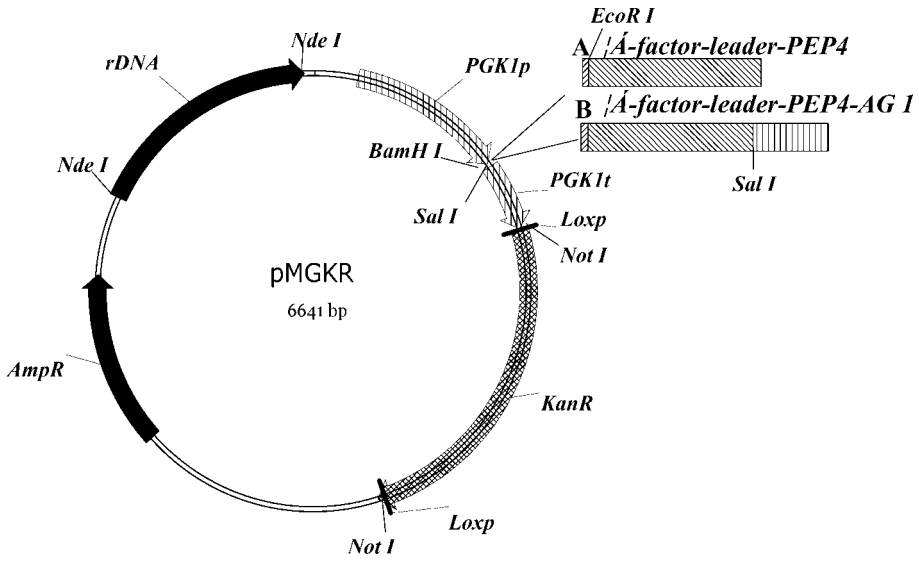

Figure 1. Schematic summary of the construction of the different acid protease expression cassettes used in this study. (A) The expression cassette used for the production and secretion of the protease. (B) The expression cassettes constructed for anchoring the acid protease on the cell wall. The mature peptide sequence was fused at the C-terminus to the α-agglutinin (AGα1) anchoring moieties. (*PGK1* 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; PEP4*, *S. cerevisiae* 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*)

PEP4 gene was obtained by PCR using plasmid pPIC9K-*PEP4* as template. The amplified fragment was digested by *Bam*HI and *Sal*I and then inserted into the plasmid pMGKR, resulting in plasmid pMGKR-*PEP4*. An anchor sequence of C-terminal half of the yeast α-agglutinin gene (*AGα1*; Van der Vaart *et al*., 1997), designated *AG*1, was prepared by PCR using primers AGF1 and AGF2. This *Sal*I digested fragment was inserted into the vector pMGKR-*PEP4*, resulting in plasmid pMGKR-*PEP4-AG*1 (Figure 1). All primers used in this study are listed in Table 1.

Measurement of enzyme activities

Supernatant and cell-associated samples were obtained by centrifuging crude samples at 10 000 rpm for 8 min. Cell pellets were washed twice with buffer (50 mM lactic acid/sodium lactate buffer, pH 3.0) in order to determine the intact cell pellet enzyme activity and isolate cell-wall fractions. The cells were disrupted by sonic dismembrator (VC750, Sonics, USA) with 30% of the total working energy for 5 min at 0 °C and the cell wall fractions were recovered by centrifugation of the homogenate at 10 000 rpm for 5 min and washed with the same buffer.

The substrate for acid protease reaction was prepared by adding casein to 50 mM lactic acid/sodium lactate buffer (pH 3.0) to give a concentration of 1.0%. After keeping 1.0 ml of the substrate at 30 °C for 2 min, 1.0 ml of cell-wall fractions or cell pellets suspended in the same buffer were added and the mixture was incubated at the same temperature for 10 min. The reaction was stopped by adding 2.0 ml of 0.4 M trichloracetic acid. After 20 min, 1 ml of the supernatant of the above mixture isolated by centrifugation at 10 000 rpm was mixed with 5 ml 0.4 M Na_2CO_3 and 1 ml Folin's reagent. After that, the mixture was incubated for another 20 min at 30 °C. One unit of acid protease was defined as the amount of enzyme required to release 1 μg of tyrosine/min from casein. Tyrosine was quantified at 660 nm (Lowry *et al*., 1951).

Immunofluorescence microscopy

Immunofluorescence microscopy was performed as reported previously (Kobori *et al*., 1992). Immunostaining was done as follows: yeast cells were

DOI: 10.1002/yea

Table 1. The sequences of the oligonucleotide primers used in this study

Primer names	Sequence 5′–3′	Restriction sites
PF1	ATTTTAGATTCCTGACTTCAACTC	
PR2	GCG*GTCGAC*[a] *GGATCC*[b] TGTTTTATATTTGTTGTAAAAAGTAG	*Sal*I[a], *Bam*HI[b]
TF1	CCG*GTCGAC*[a] TTCTTTGGAATTATTGGAAGGTA	*Sal*I[a]
TR2	GCG*GCGGCCGC*[a] GAACGCAGAATTTTCGAGTTAT	*Not*I[a]
KF1	AG*GCGGCCGC*[a] *ATAACTTCGTATAATGTATGCTATACGAAGTTAT*[b] GCCCAGTAGTAGGTTGAGG	*Not*I[a], *loxp*[b]
KR2	AG*GCGGCCGC*[a] *ATAACTTCGTATAATGTATGCTATACGAAGTTAT*[b] TTGAAGTCGGACAGTGAGT	*Not*I[a], *loxp*[b]
APF1	GGC*GAATTC*[a] ATGTTCAGCTTGAAAGCATTAT	*Eco*RI[a]
APR2	CGCA*GAATTC*[a] TCAAATTGCTTTGGCCA	*Eco*RI[a]
APF3	GGC*GGATCC*[a] ATGAGATTTCCTTCAATTTTTA	*Bam*HI[a]
APR4	CGC*GTCGAC*[a] TCAAATTGCTTTGGCCA	*Sal*I[a]
AGF1	CCG*GTCGAC*[a] AGCGCCAAAAGCTCTTTTATC	*Sal*I[a]
AGF2	CCG*GTCGAC*[a] TTTGATTATGTTCTTTCTAT	*Sal*I[a]
RF1	CCG*CATATG*[a] CTCTATCCCCAGCACGA	*Nde*I[a]
RR2	CCC*CATATG*[a] GAGAAACGGCTACCACATC	*Nde*I[a]

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

treated with the primary antibody, rabbit anti-aspartyl protease, at a dilution rate of 1 : 200. After incubating 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 : 100 at room temperature overnight (Kobori *et al*., 1992). Microscopic observation was performed according to method described previously (Kamasawa *et al*., 1996).

Cultivation conditions

Simultaneous saccharification and fermentation (SSF) was carried out in a 30 l automatic fermenter (Xinchang Deli Petrochemical Equipment, Zhejiang, China) as described previously (Zhang *et al*., 1999). Fermentation medium was prepared by dispensing the feed barley in deionized water with the ratio of barley powder to water equal to 1 : 3 (w/v). An aliquot of 100 U of raw-starch-digesting amylase was added with 1 g barley powder. Yeast cells were pre-cultured in 500 ml Erlenmeyer flasks at 30 °C in YPD medium until the OD value achieved 14.0. Then, this stationary phase culture was used to inoculate the bioreactor at a rate of 1.34×10^8/ ml. During the fermentation process, the temperature was kept at 30 °C with no air supplied. The agitation rate was kept at 100 rpm and the pH was kept constant at 5.0 by addition of 2 M NaOH. Glucose, 50 g/l, was supplemented to the medium for recombinant strains APA and APB at 56 h, and at 92 h for the wild-type. The culture broth was aerated with either 0.5 l/min airflow after the sugar was supplemented for 4 h in order to evaluate the utilization of FAN. Fermentation experiments were performed in triplicate.

The concentration of starch was determined according to the previously described method (Moraes *et al*., 1995). Total sugar concentration was determined by the phenol sulfuric acid method, as described previously (Dubois *et al*., 1956).

Measurement of FAN, amino acids and protein contents

Concentration of proteins in the medium was quantified also according to Lowry *et al*. (1951). Amino acids were analysed with a Biotronik LC 5001 amino acid analyser. The FAN concentration of the supernatant liquid was determined colorimetrically using the EBC ninhydrin method (European Brewing Convention., 1987) with glycine as the standard.

Analysis of product formation and determination of dry weight

The concentration of glucose, ethanol, glycerol, pyruvic acid and acetic acid in filtered samples withdrawn from the batch cultivations was determined by HPLC using an HP1100 (Agilent, USA) column eluted with 0.01 M H_2SO_4 at 50 °C. The biomass concentration in the medium and the specific growth rate of yeast was measured as described previously (Nissen *et al*., 1997). Cell counts were determined by the direct microscopic method (Thomas and Ingledew, 1990). Four

DOI: 10.1002/yea

samples were centrifuged at 5000***g*** for 10 min and the barley lees were removed by gradient centrifugation. The cells were washed twice with water and subsequently dried at 100 °C for 24 h and weighed. The other two cell samples were resuspended in 3 ml NaOH and total protein was determined by a modified biuret method (Verduyn *et al*., 1991) using bovine serum albumin as a standard.

Measurement of viability

The acidification power (AP) test, which is widely used in the brewing industry, was used to determine the viability of yeast. The AP value was measured according to Iserentant *et al*. (1996). The pH meter (WTW, Germany, model pH537) was recalibrated before each set of measurements. Samples were taken every 4 h during the fermentation process. The values presented are the means of two measurements; the standard deviation on the measurement was 0.05.

Results and discussion

Construction of the yeast expressing the aspartyl protease in two patterns

The plasmids pMGKR-*PEP4* for secretively expression and pMGKR-*PEP4-AG*1 for cell surface display of protease were cleaved by the restriction enzyme *Sac*II. After purified, the resultant linear DNA fragments were introduced into *S. cerevisiae* CICIMY0086 (wild-type) by the lithium acetate method (Ito *et al*., 1983). Transformants with the $G418^{+}$ phenotype were isolated. Correct insertion of the gene into the rDNA locus was verified by PCR. Then, the *cre*-recombinase expression vector pSH47 (Guldener *et al*., 1996) was transformed to the above recombinant strains and the excision of the kan^{r} gene was verified by PCR.

The acid protease activities of 100 transformant colonies were investigated. Two recombinants were used in the subsequent experiments, denoted APA for secretively expressing the protease and APB expressing the enzyme on the cell surface showed apparently high acid protease activities compared with the wild-type, which produced no detectable acid protease activity (Table 2). The recombinant strain APA protease showed the highest catalytic activity at pH 4.5 and 30 °C (Figure 2). The results are consistent with the previous study that showed that the initial product of the *PEP4* gene is likewise an inactive zymogen above neutral pH and is autocatalytically activated at acid pH values (Woolford *et al*., 1986).

Immunofluorescence microscopy

Immunofluorescent labelling of yeast cells was performed using the rabbit anti-aspartyl protease antibody and FITC-conjugated goat anti-rabbit IgG. Cells harbouring the control plasmid showed almost no labelling (Figure 3b), while cells expressing protease fusing to the α-agglutinin peptides exhibited green fluorescence localized at their surface (Figure 3d). The results confirmed that the aspartyl protease was displayed on the cell surface of *S. cerevisiae*.

Characterization of the strains expressing protease in barley mash fermentation

Barley mash was fermented by recombinant strains APA (secretively expressing the protease), APB (expressing the enzyme on cell surface) and *S. cerevisiae* CICIMY0086 (wild-type). Several parameters, including growth rate, FAN, biomass and protein content, were analysed.

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

Since 250 mg FAN/1 was considered to be more than adequate for the full fermentation with normal gravity wort (Ingledew, 1986; Jones and Rainbow, 1966), VHG corn mash fermentation requires higher concentration of FAN for rapid attenuation. In our experiments, the initial content of FAN in barley mash was about 287.9 mg/l, but assimilable nitrogen was insufficient, which can be seen by the high content of FAN at the end of fermentation and long-time attenuation for the parent strain. As shown in Figure 4b, the fast uptake of FAN for all three bath fermentations occurred during the initial 16 h, which was in accordance with the fast cell multiplication phase. Thereafter, the content of FAN for the wild-type became lower and lower and changed little at 32 h. However, the FAN content for the recombinant strains could be maintained at a higher level during the whole fermentation process. Meanwhile, in order to better evaluate the effects of expression of acid protease, the medium was supplemented with sugar

DOI: 10.1002/yea

Table 2. Distribution of aspartyl protease activity in wild-type and recombinants

	Enzyme activity		
Parameter	**Cell pellet[a]**	**Cell wall[a]**	**Culture supernatant[a]**
CICIMY0086	ND	ND	ND
CICIMY0086-APA	1.62 ± 0.54 (U/g)	0.48 ± 0.23 (U/g)	16.38 (U/ml)
CICIMY0086-APB	10.56 ± 0.15 (U/g)	13.34 ± 0.12 (U/g)	ND

[a] Triplicate experiments.
Cells were in exponential phase ($OD_{600\ nm} = 2$). CICIMY0086 = wild-type, CICIMY0086-APA and CICIMY0086-APB = recombinants secretively expressing protease and cell wall anchored expression of the enzyme.
ND = not detectable. Data ± the standard deviation.

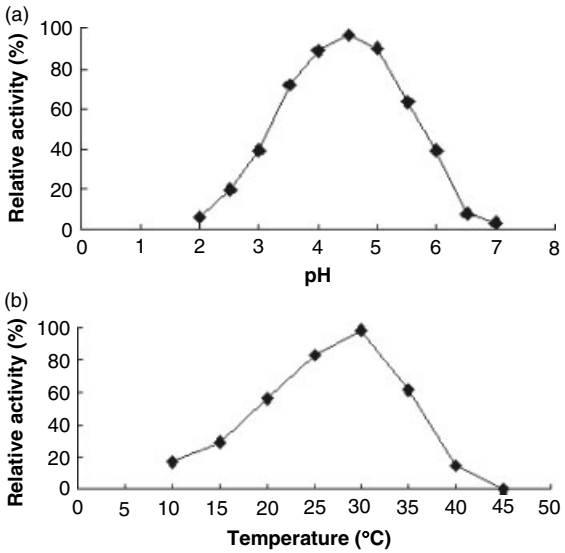

Figure 2. Enzymic characters of aspartyl protease expressed by recombinant of *S. cerevisiae* APA. (a) Relation between recombinant protease activity and pH (solid diamonds); (b) relation between recombinant acid protease activity and temperature (solid diamonds)

and airflow. As expected, cell growth accompanied by the consumption of FAN was observed for the recombinant strains, but not for the wild-type. Although 65.4 mg FAN/l could be detected in the medium for the wild-type at the end of fermentation, it was un-utilizable forms of FAN such as higher peptides (four or more amino acid residues) and soluble proteins (Thomas and Ingledew., 1990) and the source of FAN had become a limiting factor for fermentation to continue. On the other hand, a gradual decrease in content of protein in medium was observed for the wild-type, which might be due to the measurement methods, which were influenced by the presence of amino acids and peptides (Lowry *et al*., 1951; Figure 4a). As for the recombinant strains, a dramatic decrease in protein contents was observed during the initial 44 h, but a slight yet continuous increase of protein content, which might be the secretively expressed protease by the recombinant strain APA, was measured after 56 h. Enzyme detection showed that recombinant APA acid protease activity in the medium achieved 56.8 U/ml by this time. Analysis of the composition of the amino acids of medium showed that the amounts of arginine, serine, glutamate, threonine, aspartate and lysine, which were generally most heavily utilized amino acids for most wine yeast (Jiranek *et al*., 1995), were deficient in medium. Among them, arginine, glutamate, threonine and aspartate were the most consumed amino acids for all the strains. However, the concentration of these amino acids could stay at a higher level (more than 10 mg/l) during the whole fermentation process for the recombinant strains compared with the wild-type, for which most kinds of amino acids could not be detected after 36 h. Obviously, adequate amounts of necessary amino acids would promote growth and metabolism of the yeast. The above results confirmed the effects of expressing acid protease to degrade the proteins in medium.

Although the requirement for a nitrogen source by yeast in ethanol production with barley mash was unknown, in the high-concentration mash fermentations used, a high pitching rate (more than 1×10^8/ ml) was usually employed to decrease the preliminary lag phase of fermentation, which requires more FAN than when fewer cells were pitched. If available, the cells took up more FAN than was necessary for full fermentation of the sugar solutions. Saita and Slaughter (1984)

DOI: 10.1002/yea

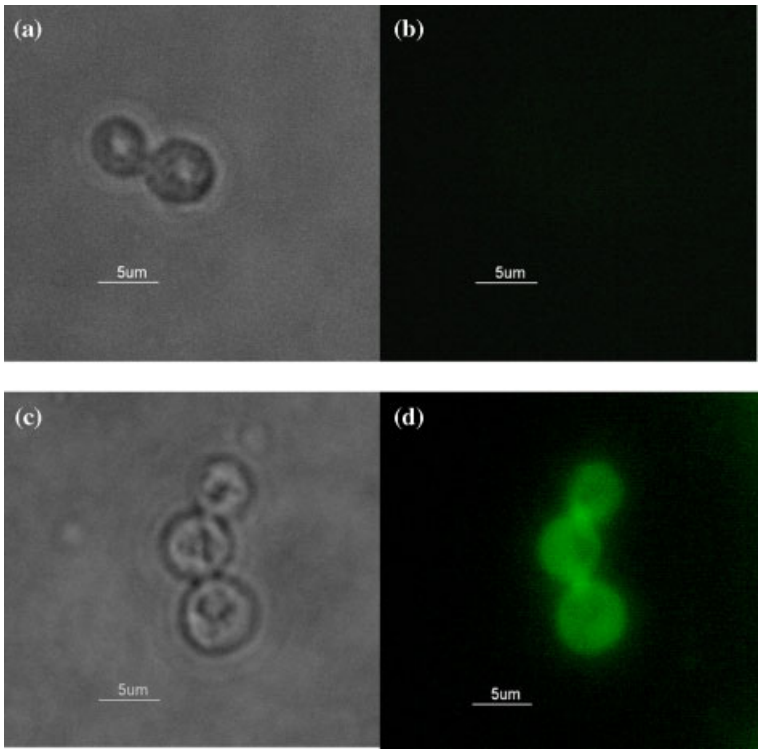

Figure 3. Immunofluorescent labelling of transformed cells. Phase micrographs (a, c) and immunofluorescence micrographs (b, d). (a, b) *S. cerevisiae* CICIMY0086/pMGKR (control); (c, d) *S. cerevisiae* CICIMY0086-APB/pMGKR-*PEP4-AG1* expressing the aspartyl protease on cell surface

previously attributed stimulation by nitrogen to its function as a substrate for protein synthesis. Although available FAN could be increased by adding extra protease or other nutrients (yeast extract, amino acids, vitamin, etc.), as described in early research (O'Connor-Cox *et al*., 1991; Reddy and Reddy, 2005), the cost of production would rise and the fermentation process would be made more complicated. The expressed protease could stimulate barley mash fermentation by supplying more available FAN.

Comparison of growth rate, fermentation rate, cell counts and viability

It is well known that VHG fuel alcohol fermentation can be stimulated when hydrolysed wheat proteins are used as a source of FAN (Thomas and Ingledew, 1990). In the case of the barley mash fermentation, FAN obviously plays a role in growth and multiplication of yeast. Higher maximum specific growth rate (Table 3) and cell counts (Figure 4c) were achieved by expressing acid protease. When sugar was supplemented to the medium, an increase in cell counts was observed for the recombinant strains. As for the parent strain, the specific growth rate was 0.37 ± 0.08/h and the maximal yeast cell number was 5.2×10^8/ ml of medium by 32 h, and there was a gradually decreasing tendency thereafter, while the recombinant strains exhibited a higher specific growth rate (0.51 ± 0.09/h for APA and 0.52 ± 0.07/h for APB) and a reduced initial fermentation lag phase. The cell counts for the recombinant APA and APB could reach 6.0×10^8 and 6.2×10^8/ ml of medium, respectively. The results differ from those of other studies (Agenbach, 1977; Monk *et al*., 1986; Vos and Gray, 1979) that excess assimilable nitrogen did not promote increased numbers of cells or rates of cell division might due to the fact that the assimilable nitrogen was not excessive for the recombinant strains in VHG fermentation supplemented with sugars. The ability of yeast cells to excrete acidity through the action of membrane H^+-ATPase, H^+/K^+ exchange

DOI: 10.1002/yea

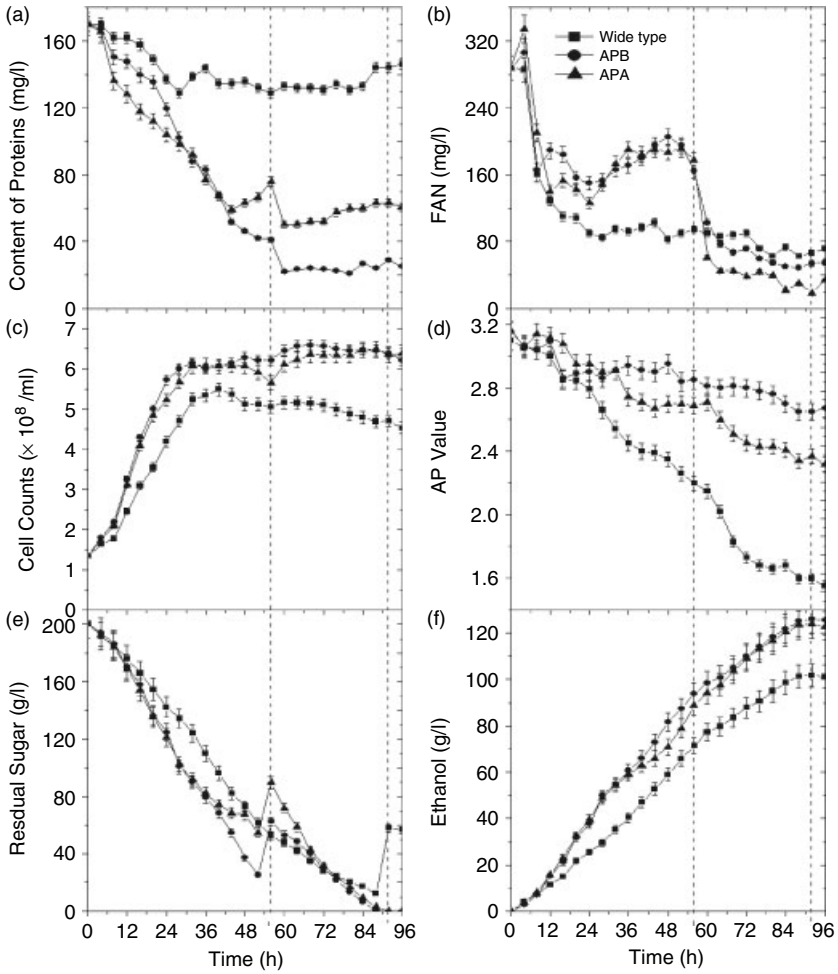

Figure 4. Changes in measured parameters during anaerobic batch fermentation with feed barley mash. (a) Concentration of protein of the medium for *S. cerevisiae* CICIMY0086 wild-type (solid squares), recombinant APA (solid diamonds) and APB (solid circles), respectively. (b) Concentration of FAN for wild-type, APA and APB. (c) Cell counts of wild-type, APA and APB. (d) AP value of the wild-type, APA and APB. (e) Concentration of residual sugar of the medium of wild-type, APA and APB. (f) Concentration of ethanol of the medium of APA and APB. The dashed line shows the time of glucose was supplementation

Table 3. Comparison of growth and compound yields of *S. cerevisiae* CICIMY0086 wild-type with recombinant strain CICIMY0086-APA and APB, during anaerobic batch growth in barley mash

Parameter	Wild-type[a]	APA[a]	APB[a]
Specific growth rate (1/h)	0.37 ± 0.08	0.51 ± 0.09	0.52 ± 0.07
Average fermentation rate (g glucose/h)	2.13 ± 0.45	2.69 ± 0.31	2.83 ± 0.25
Growth yield (g biomass/g glucose)	0.514 ± 0.10	0.578 ± 0.12	0.582 ± 0.08
Protein (g/100 g biomass)	52.13 ± 1.6	57.25 ± 1.8	57.82 ± 2.1
Ethanol yield (g ethanol/g glucose)	0.473 ± 0.05	0.502 ± 0.04	0.504 ± 0.06
Acetate yield (mg acetate/g glucose)	3.45 ± 0.11	3.47 ± 0.21	3.52 ± 0.12
Glycerol yield (mg glycerol/g glucose)	17.83 ± 1.23	13.46 ± 1.12	12.46 ± 2.10
Pyruvic acid (mg pyruvic acid/g glucose)	0.752 ± 0.05	0.456 ± 0.06	0.398 ± 0.05
Total residual sugar (g/ l)	34.13 ± 1.56	4.07 ± 0.12	2.36 ± 0.11

[a] Triplicate fermentations.
Data ± the standard deviation.

DOI: 10.1002/yea

and production of CO_2 and organic acids is a strictly controlled process that reflects very accurately the metabolic activity of the cells (Sigler *et al*., 1981a,b, 1983). The AP test developed by and Opekarová and Sigler (1982) on the basis of these findings provided a fast and simple method of assessing the viability of yeast cells. As can be seen in Figure 4d, with the gradual increase of cell counts in the exponential growth phase, the AP value changed little. After 16 h, with the consumption of available FAN and accumulation of the ethanol, the AP value of wild-type yeast began to decrease and dropped below 1.8 at 68 h. However, the ATP value of the recombinant strains was constantly maintained at a high level (above 2.5) during the whole fermentation process. Since AP values below 2 indicated a reduced metabolic activity and an AP value below 1.8 denoted low metabolic competences that could result in sluggish fermentation (Gabriel *et al*., 2008), the cells can be harvested and used for the further fed-batch fermentation.

Meanwhile, higher fermentation and ethanol producing rates for recombinant strains than the control were achieved by expressing the protease (Figure 4e, f). The observed stimulation of fermentation was apparently mediated through increased growth and cell multiplication as well as high yeast viability. As expected, the barley mash for the wild-type with no added extra nitrogen source had not fermented satisfactorily at the end of fermentation (Table 3). Expressing acid protease on cell surface significantly increased the rate of fermentation and the VHG fermentation was completed in 56 h with residual sugar less than 10.0 g/l before the glucose was added for APB. As for the recombinant APA, raw-starch-digesting amylase was added to the media after 44 h since the starch concentration did not change for 2 h before this time. This phenomenon might result from the proteolytic effect of acid protease on the raw-starch-digesting amylase in medium. Thus, anchoring the acid protease on the cell surface of yeast is a more practical choice in simultaneous saccharification/fermentation.

Comparison of product yields and total residual sugar

Expression of acid protease resulting in more available source of assimilable nitrogen not only increases the rate of fermentation but also yields higher amounts of ethanol. Such improvements resulted from the decreased need for carbon source and ATP used in protein synthesis. As shown in Table 3, the ethanol yield could reach 50.2 ± 3.72 and 50.4 ± 5.64 g per 100 g of sugar for APA and APB, respectively, which is significantly higher than the control maximum ethanol yield of 47.3 ± 4.51 g per 100 g of sugar. The comparatively low ethanol yield for the wild-type was due to the limiting amount of FAN in the medium, as can been seen from the fact that the final total residual sugar was relatively high (34.13 g/l). In addition, three main byproducts including acetate, pyruvic acid and glycerol were evaluated (Table 3). Fermented mash for the wild-type contained higher concentration of glycerol and pyruvic acid than that for the recombinant strains (APA and APB). During anaerobic conditions, the respiratory chain did not function and glycerol formation by yeast reoxidized NADH, formed in synthesis of biomass and secondary fermentation products, to NAD^+ (Ansell *et al*., 1997). Thus, the decreased production of glycerol might have resulted from the greater amount of available FAN benefitting the synthesis of biomass, leading to less essential amino-acids needing to be synthesized, while the production of acetate for recombinant strains and the wild-type were not found to be significantly changed. Meanwhile, an improvement of the biomass yield accompanied by higher cell protein content for recombinant strains expressing the protease were detected at the end of the fermentation. The yeast cells, also called SCP, were a high-quality nutrient additive and the barley lees could be treated appropriately for feed purposes.

Conclusion

In this study, acid protease was expressed in the industrial ethanol-producing yeast, either secretively or cell surface anchored. This shows that expression of the acid protease resulted in higher yeast cell counts with an increased rate of growth, and the recombinant strain of *S. cerevisiae* exhibited a higher yield of ethanol compared with the parent strain. Comparison of recombinant strains expressing the acid protease secretively and on the cell surface with the wild-type in barley mash fermentation showed that surface-engineered yeast is of practical use in ethanol and SCP production

DOI: 10.1002/yea

when a simultaneous saccharification/fermentation is employed. To our knowledge, this is the first study on expressing an acid protease on the cell surface of *S. cerevisiae*. Future studies will be conducted to construct starch-utilizing strain with high fermentation vitality, attempting to directly produce ethanol from uncooked starchy materials.

Acknowledgements

We acknowledge Professors Gui-yang Shi and Zheng-xiang Wang, Jiangnan University, for their valuable instruction. This work was financial supported by the Natural Science Foundation of China (20706024), the '863' Program (2006AA020101, 2007AA10Z359), Innovative Research Team of Jiangsu Province, the Open Project Program of the Key Laboratory of Industrial Biotechnology, Ministry of Education, China (KLIB-KF200701) and China Postdoctoral Science Foundation funded project (200801361).

References

Agenbach WA. 1977. A study of must nitrogen content in relation to incomplete fermentations, yeast production and fermentation activity. *Proc South African Soc Enol Vitic* 66–87.

Ansell R, Granath K, Hohmann S, Thevelein JM, Adler L. 1997. The two isoenzymes for yeast NAD^+-dependent glycerol 3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaption and redox regulation. *EMBO J* **16**: 2179–2187.

Beltran G, Esteve-Zarzoso B, Rozès N, Mas A, Guillamón JM. 2005. Influence of the timing of nitrogen additions during synthetic grape must fermentations on fermentation kinetics and nitrogen consumption. *J Agric Food Chem* **53**: 996–1002.

Bely M, Sablayrolles JM, Barre P. 1990. Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in enological conditions. *J Ferm Bioeng* **70**: 246–252.

Casey GP, Ingledew WM. 1986. Ethanol tolerance in yeasts. *Crit Rev Microbiol* **13**: 219–280.

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method of determination of sugars and related substances. *Anal Chem* **28**: 350–356.

European Brewery Convention. 1987. *Free Amino Nitrogen, Analytica-EBC*, 4th edn. Brauerei- und Getränke-Rundschau: Zurich; E141–E142.

Gabriel P, Dienstbier M, Matoulkova D, Kosar K, Sigler K. 2008. Optimized acidification power test of yeast vitality and its use in brewing practice. *J Inst Brew* **114**: 270–276.

Guldener U, Heck S, Fielder T, Beinhauer J, Hegemann JH. 1996. A new efficient gene disruption cassette for repeated use in budding yeast. *Nucl Acids Res* **24**: 2519–2524.

Ingledew WM. 1993. Yeasts for production of fuel ethanol. In *The Yeasts, Vol. 5: Yeast Technology*, Rose AH, Harrison JS (eds). Academic Press: New York; 245–291.

Ingledew WM, Kunkee RE. 1985. Factors influencing sluggish fermentations of grape juice. *Am J Enol Vitic* **36**: 65–76.

Iserentant D, Geenens W, Verachtert H. 1996. Titrated acidification power. A simple and sensitive method to measure yeast vitality and its relation to other vitality measurements. *J Am Soc Brew Chem* **54**: 110–114.

Ito H, Fukuda Y, Murata K, Kimura A. 1983. Transformation of intact yeast cells treated with alkali cations. *J Bacteriol* **153**: 163–168.

Jiranek V, Langridge P, Henschke PA. 1991. Yeast nitrogen demand: selection criterion for wine yeasts for fermenting low nitrogen musts. In *Proceedings of the International Symposium on Nitrogen in Grapes and Wine*, Ranz JM. (eds). American Journal of Enology and Viticulture: Davis, CA.

Jiranek V, Langridge P, Henschke PA. 1995. Amino acid and ammonium utilization by *Saccharomyces cerevisiae* wine yeasts from a chemically defined medium. *Am J Enol Vitic* **46**: 75–83.

Jones MO, Rainbow C. 1966. Simple methods of determining major nitrogenous compounds in worts and beers. *Am Soc Brew Chem Proc* 66–70.

Kamasawa N, Ohtsuka I, Kamada Y, Ueda M, Tanaka A, Osumi M. 1996. Immunoelectron microscopic observation of the behaviors of peroxisomal enzymes inducibly synthesized in an *n*-alkane-utilizable yeast cell, *Candida tropicalis*. *Cell Struct Funct* **21**: 117–122.

Kobori H, Sato M, Osumi M. 1992. Relationship of actin organization to growth in the two forms of the dimorphic yeast *Candida tropicalis*. *Protoplasma* **167**: 193–204.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275.

Mäkinen V, Haikara A, Enari TM. 1970. Influence of nitrogenous compounds on beer fermentation. *Soum Kemistilehti B* **43**: 443–447.

Monk PR, Hook D, Freeman BM. 1986. Amino acid metabolism by yeast. *Proceedings of the 6th Australian Wine Industry Technology Conference*, 14–17 July, Adelaide; 129–133.

Monteiro FF, Bisson LF. 1992. Nitrogen supplementation of grape juice. I. Effect on amino acid utilization during fermentation. *Am J Enol Vitic* **43**: 1–10.

Moraes LMde, Astolfi-filho PS, Oliver SG. 1995. Development of yeast for the efficient utilization of starch: evaluation of constructs that express α-amylase and glucoamylase separately or as bifunctional fusion proteins. *Appl Microbiol Biotechnol* **43**: 1067–1076.

Nissen TL, Schulze U, Nielsen J, Villadsen J. 1997. Flux distributions in anaerobic, glucose-limited continuous cultivations of *Saccharomyces cerevisiae*. *Microbiol* **143**: 203–218.

O'Connor-Cox ESC, Paik J, Ingledew WM. 1991. Improved ethanol yields through supplementation with excess assimilable nitrogen. *J Ind Microbiol* **8**: 45–52.

Opekarová M, Sigler K. 1982. Acidification power: indicator of metabolic activity and autolytic changes in *Saccharomyces cerevisiae*. *Folia Microbiol* **27**: 395–403.

Reddy LVA, Reddy OVS. 2005. Improvement of ethanol production in very high gravity fermentation by horse gram (*Dolichos biflorus*) flour supplementation. *Lett Appl Microbiol* **41**: 440–444.

Saita M, Slaughter JC. 1984. Acceleration of the rate of fermentation by *Saccharomyces cerevisiae* in the presence of ammonium ion. *Enz Microb Technol* **6**: 375–378.

Sigler K, Knotková A, Kotyk A. 1981a. Factors governing substrate-induced generation and extrusion of protons in the

yeast *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **643**: 572–582.

Sigler K, Kotyk A, Knotková A, Opekarová M. 1981b. Processes involved in the creation of buffering capacity and in substrate-induced proton extrusion in the yeast *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **643**: 583–592.

Sigler K, Pascual C, Romay C. 1983. Intracellular control of proton extrusion in *Saccharomyces cerevisiae*. *Folia Microbiol* **28**: 363–370.

Thomas KC, Ingledew WM. 1990. Fuel alcohol production: effects of free amino nitrogen on fermentation of very-high-gravity wheat mashes. *Appl Environ Microbiol* **56**: 2046–2050.

Van der Vaart JM, Te Biesebeke R, Chapman JW, Toschka HY, Klis FM, Verrips CT. 1997. Comparison of cell wall proteins of *Saccharomyces cerevisiae* as anchors for cell surface expression of heterologous proteins. *Appl Environ Microbiol* **63**: 615–620.

Verduyn C, Stouthamer AH, Scheffers WA, van Dijken JP. 1991. A theoretical evaluation of growth yields of yeasts. *Antonie Van Leeuwenhoek* **59**: 49–63.

Vos PJA, Gray RS. 1979. The origin and control of hydrogen sulfide during fermentation of grape must. *Am J Enol Vitic* **30**: 187–197.

Woolford CA, Daniels LB, Park FJ, Jones EW, Van Arsdell JN, Innis MA. 1986. The PEP4 gene encodes an aspartyl protease implicated in the posttranslational regulation of *Saccharomyces cerevisiae* vacuolar hydrolases. *Mol Cell Biol* **6**: 2500–2510.

Zhang KC, Shi GY, Xu R. 1999. A method for very high gravity ethanol fermentation. Chinese Patent, ZL96117189.8.