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Integrative Expression of Glucoamylase Gene in a Brewer's Yeast *Saccharomyces pastorianus* Strain

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Summary

The recombinant brewer's yeast *Saccharomyces pastorianus* strain was constructed by introducing the *ilv2*::*GLA* fragment released from pMGI6, carrying glucoamylase gene (*GLA*) and using the yeast α-acetolactate synthase gene (*ILV2*) as the recombination sequence. The strain was able to utilise starch as the sole carbon source, its glucoamylase activity was 6.3 U/mL and its α-acetolactate synthase activity was lowered by 33.3 %. The introduced *GLA* gene was integrated at the recipient genomic *ILV2* gene, one copy of *ILV2* gene was disrupted and the other copy remained intact. Primary wort fermentation test confirmed that the diacetyl and residual sugar concentration in the wort fermented by the recombinant strain were reduced by 65.6 and 34.2 % respectively, compared to that of the recipient strain. Under industrial operating conditions, the maturation time of beer fermented by the recombinant strain was reduced from 7 to 4 days, there were no significant differences in the appearance and mouthfeel, and the beer satisfied the high quality demands. That is why the strain could be used in beer production safely.

Key words: glucoamylase, α-acetolactate synthase, brewer's yeast, diacetyl, fermentation, gene disruption

Introduction

Beer has been a popular beverage for thousands of years and the main production steps include wort production, fermentation and maturation. During the barley malting and mashing stages in the production of wort, polysaccharides mainly from the malted barley are converted into malto- and isomaltooligosaccharides under the activity of enzymes. The maltooligosaccharides with lower molecular mass are further hydrolysed and converted into ethanol and carbon dioxide by industrial strains of *Saccharomyces cerevisiae*, whereas the higher oligomers remain in the fermented wort because the brewer's yeasts lack amylolytic activity and are unable to utilise these higher oligomers during the vegetative growth phase (*1*). Polysaccharides, in a partially degraded, non-fermentable form, are the major non-volatile components and will contribute to the high calorie value (*2*).

Nowadays, there is a rising trend among consumers to demand low calorie beverages due to the increased awareness of health, and the reduction of calories in alcoholic beverages, particularly beer, is of great commercial interest (*3*). Instead of adding an exogenous saccharifying enzyme during beer production, the reduction of calories could be achieved by introducing starch-hydrolysing gene into the brewer's yeast. Many researches have been carried out and α-amylase and glucoamylase encoding genes have been cloned from a number of sources including animals, vegetables and microorganisms. To achieve direct and efficient production of ethanol by fermentation from raw starch, anchor sequence was used

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to guide the *Rhizopus oryzae* glucoamylase and *Streptococcus bovis* α-amylase anchor to the surface of the yeast cells (*4*). But most researches on α-amylase and glucoamylase expression in yeast have been conducted with laboratory strains. Laboratory strains differ markedly from industrial strains in many respects. There are only a few reports on the application of the engineered amylolytic strains. Gundllapalli Moses *et al.* (*5*) showed that the genetic background of the engineered strains of *S. cerevisiae* plays a significant role in amylase production. The influence of the medium composition on biomass formation, ethanol production, amylase secretion, and plasmid stability of the engineered amylolytic strains under laboratory shake-flask experiment condition has been studied by Birol *et al.* (*6*) and Kang *et al.* (*7*). They constructed an amylolytic industrial baker's yeast by multicopy integration. More effort was put in studying amylase, but the function of glucoamylase is more important during the wort fermentation.

Diacetyl, which is formed from α-acetolactate, an intermediate in the biosynthetic pathways of valine (Val) and isoleucine (Ile) and produced by α-acetolactate synthase gene (*ILV2*), is a common off-flavour compound in beer and removed by maturation process, which increases the product cost. Acceleration of maturation, therefore, is economically important in the brewing industry and can be achieved by controlling the formation of diacetyl.

In this research, an integrating plasmid that carries glucoamylase gene (*GLA*), together with yeast 3-phosphoglycerate kinase 1 promoter ($PGK1_P$) and α-factor signal sequence (*MF*α1s), and the yeast α-acetolactate synthase gene (*ILV2*) as the homologous recombination site was constructed. An amylolytic brewer's yeast strain, free of bacterial vector sequence and drug-resistance markers, was developed by one-step gene replacement technique. The enzyme activities were determined and fermentation performances of the new strain were examined under the industrial brewing conditions.

Materials and Methods

Microorganisms and plasmids

A brewer's yeast *Saccharomyces pastorianus* Sc-33 was kindly provided by Shijiazhuang Xinle Brewery, PR China and used as the recipient for plasmid transformation. *Escherichia coli* DH5α was used for DNA manipulations. Plasmids YEp352 (*8*), YIp5 (*9*) and pBluescript II SK$^-$ (*10*) were used to construct recombinant plasmids. Plasmids pMαP and pLV2 (*11*), and pDBLeu (Life Technologies, USA) were used to provide phosphoglycerate kinase 1 promoter ($PGK1_P$), α-acetolactate synthase gene and alcohol dehydrogenase 1 terminator ($ADH1_T$), respectively.

Culture media and conditions

E. coli DH5α was grown in LB medium (5 g/L of yeast extract, 10 g/L of peptone, 10 g/L of NaCl, pH=7.0) supplemented with 50 μg/mL of ampicillin as required, at 37 °C. Yeast cells were grown at 30 °C on YPG medium (10 g/L of yeast extract, 15 g/L of peptone, 20 g/L of glucose). When selection for the presence of plasmid molecule was required, synthetic complete (SC) medium (containing 6.7 g/L of yeast nitrogen base (Difco), 5 g/L of soluble starch and 8 g/L of agar) was employed. Brewer's wort with an original gravity of 10 degrees Plato (°P) was kindly provided by Shijiazhuang Xinle Brewery, PR China. The sugar composition of the wort was as follows: maltose 46.7 g/L, maltotriose 14.6 g/L, glucose 8.3 g/L, non-fermentable sugars (mainly oligosaccharides) 25.3 g/L.

DNA manipulation and yeast transformation

DNA manipulation in *E. coli* DH5α was performed using standard methods (*12*). Isolation of yeast genomic DNA and transformation of the yeast strain was carried out using the methods described by Burke *et al.* (*13*). The lithium acetate transformation method was used as follows: a single yeast colony is inoculated on YPG slant and incubated at 30 °C for 48 h. A loopful of cells taken from the slant is inoculated to 2 mL of liquid YPG and cultured at 30 °C with shaking (200 rpm) for about 16 h. The cells are harvested at the end of logarithmic growth by centrifuging for 30 s at 5000 rpm, washed separately with water and 0.1 mM lithium acetate (LiAc). Then, the cells are resuspended with 500 mL of 0.1 M LiAc/0.01 M Tris-HCl (pH=7.5) solution, 5 μL of carrier DNA, 10 μL of vector and 700 μL of PEG 4000 are added and the Eppendorf tube is flipped until the cell pellet has been completely mixed, then cultured for 1 h at 30 °C. After that, the culture is heated in a water bath at 42 °C for 5 min and then cooled down to the room temperature. Finally, 200 μL of transformation solution are mixed with the SC medium on plates which are incubated at 30 °C for about 3–4 days to recover the transformants.

Cloning of glucoamylase gene

Using the genomic DNA of *Saccharomycopsis fibuligera* (provided by Microbiology Research Institute of Hebei Normal University, PR China) as template, the PCR amplification of glucoamylase-encoding sequence (GenBank accession number X58117) was carried out with primers: P1, 5′-TACGGATCCCTATGAGATTCGGTGTT-3′ and P2, 5′-GTGGTACCTTAAGCCAAAGCCTTGAC-3′. The *Bam* HI and *Kpn*I sites were introduced to facilitate the cloning of the glucoamylase gene. The PCR product (named *GLA*) was then purified and directly inserted into the *Bam*HI and *Kpn*I sites of YEp352, a yeast/*E. coli* shuttle vector, generating plasmid pLG6.

Construction of plasmids

The 1.56-kb *Bam*HI-*Kpn*I *GLA* coding region and a 0.56-kb *Kpn*I-*Sal*I fragment containing yeast alcohol dehydrogenase 1 terminator ($ADH1_T$) were inserted into the *Bam*HI + *Sal*I site of pBluescript II KS$^-$, resulting in vector pMGT. The 1.9-kb *Hin*dIII-*Pst*I fragment isolated from pMαP and the 2.11-kb *Pst*I-*Sal*I fragment released by digesting pMGT were ligated into *Hin*dIII + *Sal*I site of YIp5. The resulting plasmid was named YLG6. YLG6 encompasses $PGK1_P$-*MF*α1s-*GLA*-$ADH1_T$ fragment and the *GLA* gene was expressed under the control of yeast $PGK1_P$ and *MF*α1 signal sequence.

The 1.1-kb *ILV2*/*Bam*HI-*Aat*II and 0.5-kb *ILV2*/*Xba*I-*Sal*I from plasmid pLV2, together with 4.05-kb *Aat*II-*Xba*I fragment containing *GLA* expression cassette released from plasmid YLG6, were inserted into the vector pBluescript II KS$^-$ at *Bam*HI + *Sal*I site, generating the recombinant plasmid named pMGI6.

Enzyme assays

α-Acetolactate synthase activity was measured according to Park *et al*. (*14*) with modifications: 100 mM K_3PO_4 (pH=8.0), 5 mM $MgCl_2$, 1 mM TPP, 100 mM pyruvate, 0.2 mM FAD and 50 % cell extract (by volume) were cultured at 30 °C for 20 min. The reaction was terminated by adding 3.5 μL of 3 M H_2SO_4 per 200 μL of reaction mixture. The acidified reaction mixtures were heated for 15 min at 60 ºC and the α-acetolactate formed in the reaction was readily decarboxylated to acetoin or diacetyl. Both components were determined by gas chromatographic analysis, using a flame ionisation detector and a fused capillary column coated with DB-Wax (30 m×0.53 mm) and film thickness of 1.0 μm (Auto System XL, PE Company, USA). The carrier gas was nitrogen and flow rate was 10 mL/min. One unit of α-acetolactate synthase activity was defined as the amount of enzyme required to release 1 μmol of α-acetolactate per mg of protein per min under the assay conditions described above. Protein concentration was measured using a Bio-Rad protein assay kit with bovine serum albumin as the standard.

The glucoamylase activity was measured using the 3,5-dinitrosalicylic acid (DNS) method (*15*): a volume of 50 μL of centrifuged culture supernatant containing the secreted enzyme was incubated with 200 μL of 0.4 M sodium acetate buffer (pH=5.6) containing 2 % soluble starch for 30 min at 50 °C. The mixture was boiled for 10 min to stop the reaction. One unit of glucoamylase activity was defined as the amount of enzyme required to release 1 μmol of glucose per mL per min under the same culture conditions.

Genetic stability of amylolytic phenotype

The genetic stability of the yeast strain was determined by the method of Cha *et al*. (*16*). One colony of the yeast strain was cultured nonselectively in YPG medium in over 100 generations of cell multiplication (through successive inoculations). One hundred colonies on YPG medium were replicated onto SC medium. The number of tested colonies which showed amylolytic phenotype was calculated.

Purification of glucoamylase

The glucoamylase of the fermented supernatant was purified as described by Faridmoayer and Scaman (*17*) with some modifications. The fermented broth was centrifuged (12 000 rpm, 4 °C, 10 min) to remove yeast cells. Proteins in the supernatant were precipitated using 60 % ammonium sulphate saturation and isolated by centrifugation at 12 000 rpm and 4 °C for 30 min. The pellet was dissolved in 90-mL buffer 1 (20 mM sodium phosphate, pH=6.8) and dialysed against 4 L of the same buffer for approximately 24 h, with dialysing buffer changing every 12 h. The dialysed sample was applied to a DEAE-cellulose column (2.5×20 cm) equilibrated with buffer 1 at the flow rate of 1.5 mL/min. The unbound proteins were removed from the column by washing with five column volumes of buffer 1. Proteins were eluted using a stepwise gradient with 0.1 M and 0.2 M NaCl in buffer 1 at the flow rate of 1 mL/min. Fractions of 3 mL were collected with 0.2 M NaCl elution. Active fractions were pooled and applied onto the column (2.5×20 cm) of Sephadex G75. The active fractions were pooled and used for further SDS-PAGE analysis.

SDS-PAGE analysis

A sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 12 % polyacrylamide gels according to a standard protocol (*12*). The gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad) and the samples and protein marker were boiled for 7 min before loading.

Fermentation test

A single colony was transferred into 25-mL YPG broth in a loosely capped 100-mL flask and incubated for 18 h at 30 °C with shaking (160 rpm). This was used as seed culture for further experiments. The enlarged culture was carried out by inoculating 10 μL of the seed culture to 100 mL of wort with shaking at 25 °C. After 48 h, 900 mL of fresh wort were added and recultivated for additional 48 h at 20 °C. Then, the culture cells were harvested and inoculated in the fermentor at a density of approximately $1 \cdot 10^7$ cells/mL. The primary fermentation experiments were performed in a 5-litre bench-top fermentor with 3-litre working volume at 12 °C. Dissolved oxygen was controlled at 30 % of saturation with air using an adaptive control algorithm. The primary fermentation was completed after 7 days. At one-day intervals, samples were taken aseptically and analysed.

Industrial fermentation test

Industrial fermentation was carried out at a scale of 1000 kg according to the technique of Shijiazhuang Xinle Brewery, PR China, in which primary wort fermentation was performed for 5 days at 9 °C, followed by maturation at 13 °C for 7 days, and then the diacetyl content of the fermented broth was determined at different time intervals during the maturation time.

Analytical methods

Characterisation of the finished beer was performed by Alcolyzer Plus Beer (Anton Paar, Austria) and the concentrations of diacetyl were determined by gas chromatography as described above. To determine the content of diacetyl, beer samples were incubated for 30 min at 60 °C in a temperature incubator.

Results and Discussion

Introduction of GLA gene into brewing yeast

In order to disrupt the *ILV2* gene on the recipient genome, the recombinant plasmid pMGI6 (Fig. 1) was constructed and the construct validity of the recombinant expression vector pMGI6 was verified by cutting with restriction enzymes. The 5.65 kb fragment carrying the *ilv2*::*GLA* (5´-*ilv2*-$PGK1_P$-*MFα*1s-*GLA*-$ADH1_T$-*ilv2*-3´) gene was released upon cleaving pMGI6 by *Apa*I + *Not*I and used as a linear disruption cassette.

The 5.65-kb fragment was transformed into the brewer's yeast strain Sc-33 by lithium acetate method, and the transformants were screened on SC medium (containing starch as the sole carbon source), using the *GLA*

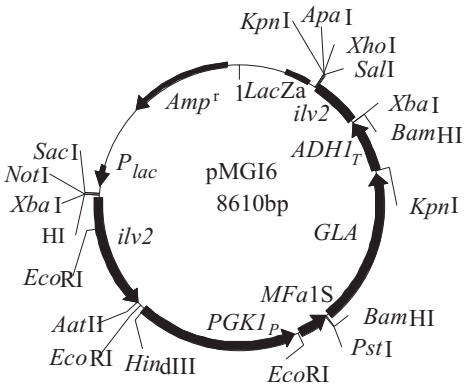

Fig. 1. The physical map of the recombinant plasmid pMGI6

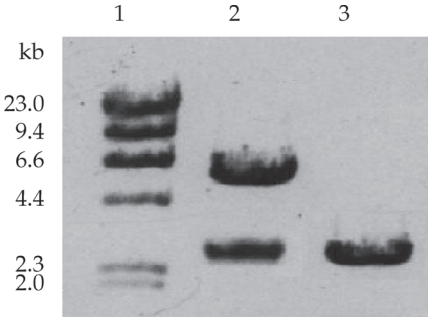

Fig. 2. PCR analysis of the genomic DNA by agarose gel electrophoresis. Lane 1: λDNA/*Hin*dIII marker, lane 2: transformant genomic DNA, lane 3: recipient genomic DNA

gene as a selective marker and further screened for those with low functional *ILV2* gene by measuring α-acetolactate synthase activity of yeast cells extract. As a result of this screening assay, two transformants were picked out.

Enzyme assays

The glucoamylase activity and α-acetolactate synthase activity of the screened transforments were determined (Table 1). Transformant 1 was selected for further examination and named ScG33. The α-acetolactate synthase activity of ScG33 was 66.7 % of that of Sc-33, and the glucoamylase activity of ScG33 was 6.3 U/mL. The results indicate that the *GLA* gene was expressed under the control of phosphoglycerate kinase 1 promoter, producing a functional protein. Compared to that of the recipient, ScG33 had reduced α-acetolactate synthase activity, therefore less intracellular acetolactate formed and decreased acetolactate level secreted into the culture medium.

The identification of the GLA gene integration and amylolytic phenotype stability

To determine whether a foreign gene had integrated into the genomic *ILV2* gene by double-cross homologous recombination, PCR of the genomic DNA was carried out using the primers which were used for PCR of α-acetolactate synthase gene (*11*). The agarose gel electrophoresis of PCR products showed that the transformant had two bands at about 2.9 and 5.6 kb and the recipient had a 2.9-kb band (Fig. 2). This indicated that the *GLA* gene had integrated at the recipient genomic *ILV2* gene and one copy of the *ILV2* gene was disrupted and the other copy remained intact. Considering the decreased α-acetolactate synthase activity, the disrupted *ILV2* gene should have no α-acetolactate synthase expression function and the gene disruption should lower the expression level of the α-acetolactate synthase enzyme.

The genetic stability of amylolytic phenotype of ScG33 was determined by the method described above and the genetic stability of ScG33 was 100 %.

Properties of glucoamylase expressed by ScG33

Glucoamylase expressed by ScG33 was purified by the methods mentioned above. The molecular mass of the purified enzyme was determined by SDS-PAGE and it had a band at about 62 kDa (Fig. 3). The physicochemical properties of the purified glucoamylase from the transformant ScG33 were in agreement with that of the glucoamylase encoded by *GLA*1 in *S. cerevisiae* (*18*). The enzyme gave a specific activity of 43 U/mg of protein, the optimal temperature for enzyme activity was 50 ºC and the optimum pH was 5–6. It could be concluded that ScG33 expressed the glucoamylase by introducing foreign *GLA* gene and the molecular mass of the glucoamylase protein was about 62 kDa. The transformant ScG33 had similar starch-hydrolyzing ability to transformants previously constructed by other researchers.

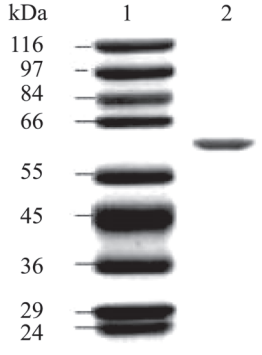

Fig. 3. SDS-PAGE analysis of purified extracellular glucoamylase protein from ScG33, lane 1: molecular mass markers, lane 2: sample from ScG33

Table 1. The activity of glucoamylase and α-acetolactate synthase

Enzyme	Activity	Transformant 1	Transformant 2	Sc-33
α-Acetolactate synthase	U/g	19.40	19.50	29.10
Glucoamylase	U/mL	6.30	6.21	0

The values are the means of results from triplicate experiments with standard deviation lower than 5 %

Primary wort fermentation

To investigate whether the disruption of the genomic *ILV2* gene could lower the diacetyl content and whether the *GLA* gene expression could reduce the residual sugar of the fermentation broth, primary wort fermentation was performed over 7 days at 12 °C. The fermentation broths were collected at different time intervals and diacetyl and sugar contents were determined (Fig. 4). At the end of the primary fermentation, more than 15.2 % sugar was metabolized by ScG33, the total residual sugar and diacetyl concentration in the fermentation wort of ScG33 were reduced by 34.2 and 65.6 % respectively, compared to that of Sc-33.

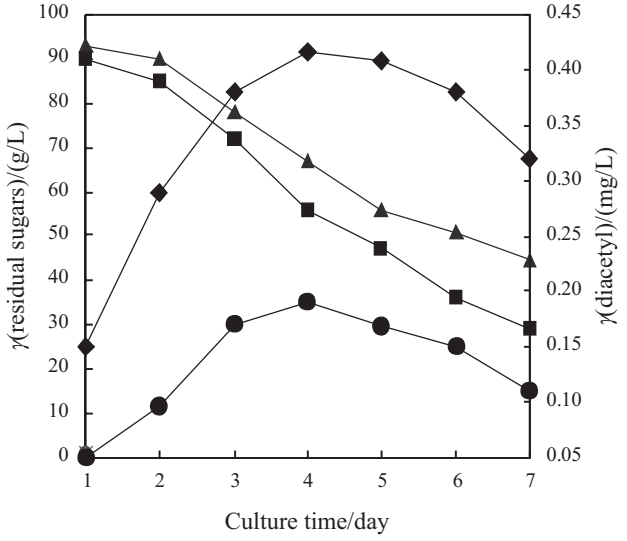

Fig. 4. Time courses of sugar utilisation and total diacetyl content of primary wort fermentation in a 5-litre bench-top fermentor under fermentation conditions (inoculation rate 10^7 cells/mL, working volume 3 L, agitation speed 160 rpm, temperature 12 °C, dissolved oxygen 30 %), ■ residual sugar of ScG33, ▲ residual sugar of Sc-33, ● total diacetyl of ScG33, ◆ total diacetyl of Sc-33. The values are the means of results from triplicate experiments with standard deviation lower than 5 %

This indicated that the formation of diacetyl was influenced by the disruption of *ILV2* gene and one copy disruption of *ILV2* gene was able to reduce the formation of diacetyl significantly, so the maturation time necessary to remove diacetyl from the fermented wort should be shortened. Sugar fermentation capacity of ScG33 strain was also improved by introducing the *GLA* gene and the strain gained the ability to hydrolyze oligosaccharides. Therefore, the residual sugar of the fermented broth was reduced.

Industrial wort fermentation

Industrial fermentation was carried out, and the diacetyl content of the fermented broth was determined at different time intervals during the maturation time. The diacetyl content in the ScG33 fermented wort was reduced to 0.1 mg/L after 4 days of maturation and the maturation time was shortened, while that of the recipient was 7 days. The characteristic parameters of the final beer such as ethanol, calories, original extract and dissolved CO_2 in bottles were determined (Table 2). The calorie value in beer fermented by the ScG33 was decreased compared to that of the Sc-33. With regard to the appearance parameters like clarity, colour and foam stability, the two kinds of beer were compared quite closely and there were no significant differences in mouthfeel. The beer quality fermented by the engineered strain ScG33 under the industrial conditions met the company standard. Therefore, it could be concluded that the beer produced using ScG33 had comparable quality to that using Sc-33, the expression of glucoamylase and the break of one copy of *ILV2* gene should cause no perceivable change to the beer and have little influence on the metabolism profile and routes concerning the aroma and flavour components such as esters and aldehydes.

Table 2. Results of the analysis of finished beer samples

Parameter	Strains	
	ScG33	Sc-33
Energy/(kJ/L)	1598	1947
Foam stability/s	264	263
γ(total soluble nitrogen)/(mg/L)	716	716
w(original extract)/%	10.0	10.0
Colour/EBC units*	3.6	3.7
φ(ethanol)/%	4.4	4.3
φ(total acidity)/%	1.60	1.56
Clarity/EBC units*	0.93	0.94
φ(dissolved CO_2 in bottles)/%	0.48	0.47
γ(diacetyl)/(mg/L)	0.098	0.100

*The parameters were measured in European Brewery Convention (EBC) units according to the EBC standard, based on spectrophotometry. The values of all the parameters are the means of results from triplicate experiments with standard deviation lower than 5 %

Conclusion

Considering the fermentation efficiency and production cost, this engineered strain should be suitable for industrial production of beer and might have a potential for use in low-calorie beer production. The transformant which is free of bacterial vector sequence and drug-resistance markers may improve the acceptance of the quality by the public. This study could contribute to constructing the recombinant industrial yeast strains used in alcoholic beverage production in order to avoid problems caused by transferring the bacterial antibiotic resistance marker to the pathogenic bacteria or fungi.

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