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Stable expression of glucoamylase gene in industrial strain of *Saccharomyces pastorianus* with less diacetyl produced

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Abstract - An integrating plasmid pMGI6 carrying glucoamylase gene (*GLA*) and using the yeast α-acetolactate synthase gene (*ILV2*) as the recombination sequence, was constructed from pBluescript II SK$^-$. The *ilv2*::*GLA* fragment released from pMGI6 was introduced into the brewing yeast *Saccharomyces pastorianus* and the resulting recombinant strain was able to utilise starch as the sole carbon source, its glucoamylase activity was 6.3 U ml^{-1} and its a-acetolactate synthase activity was lowered by 33%. Fermentation tests confirmed that the diacetyl concentration in wort fermented by the recombinant strain was reduced by 66% and the maturation time was reduced from 7 to 4 days. The beer fermented by the recombinant strain under industrial operating conditions satisfied the high quality demands and the strain could be used in beer production safely.

Key words: glucoamylase, a-acetolactate synthase, brewing yeast, diacetyl, fermentation, gene disruption.

INTRODUCTION

Beer has been a popular beverage for thousands of years and brewing is considered as the oldest biotechnological process. During the barley malting and mashed operation in the production of wort, the polysaccharides mainly from malted barley are converted into malto- and isomalto-oligosaccharides under the action of enzymes. The lower malto-oligosaccharides are further hydrolysed and converted into ethanol and carbon dioxide by industrial strains of *Saccharomyces cerevisiae* in the wort fermentation process, whereas the higher oligomers remain in the fermented wort because the brewing yeasts lack amylolytic activity and are unable to utilise these higher oligomers during the vegetative growth phase (Bamforth, 2002). Polysaccharides, in a partially degraded, non-fermentable form, are the major non-volatile components and will contribute to the high calorie value (Cortacero-Ramírez *et al*., 2003).

Nowadays, there is a rising trend for 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 (Nevoigt *et al.*, 2002). Instead of adding an exogenous enzyme during beer production, the reduction of calorie could be achieved by introducing starch-hydrolysing gene into brewing yeast. Many researches were 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 to guide the *Rhizopus oryzae* glucoamylase and *Streptococcus bovis* α-amylase anchor on the surface of the yeast cells (Shigechi *et al.,* 2004). But most researches on α-amylase and glucoamylase expression in yeast were conducted with laboratory strains. Laboratory strains differ markedly from industrial strains in many respects. Laboratory strains are haploid, whereas brewing yeast strains are polyploid, which are more stable, and their fermentation performance can be improved. There are only a few reports on the application of the engineered amylolytic strains. Gundlapalli Moses *et al.* (2002) have showed that the genetic background of the engineered strains of *S. cerevisiae* plays a significant role in amylase production and 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.* (1998) and Kang *et al.* (2003) have constructed an amylolytic industrial baker's yeast by multicopy integration. More efforts were on amylase, but the function of glucoamylase is more important during the wort fermentation.

Diacetyl, which is transferred from α-acetolactate, an intermediate in the biosynthetic pathways of valine (Val) and isoleucine (Ile), is a common off-flavor compound in beer and removed by maturation process, raising the product cost.

In this report, an integrating plasmid that carries glucoamylase gene (*GLA)*, together with yeast 3-phosphorglycerate kinase 1 promoter (*PGK1$_P$*) and α-factor signal sequence (*MF*α1s), and has the yeast α-acetolactate syn-

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thase gene (*ILV2)* as the homologous recombination site, was constructed and an amylolytic brewing 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 brewing yeast *Saccharomyces pastorianus* Sc-11 was kindly provided by local brewery and used as the recipient for plasmid transformation. *Escherichia coli* DH5α was used for DNA manipulations. Plasmids YEp352 (Hill *et al.*, 1993), YIp5 (Parent *et al.*, 1985) and pBluescript II SK$^-$ (Short *et al.*, 1988) were used to construct recombinant plasmids. Plasmids pMαP and pLV2 (Liu *et al.*, 2004), 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. *Escherichia coli* DH5α was grown in LB medium supplemented with 50 μg ml^{-1} ampicillin as required, at 37 °C. Yeast cells were grown at 30 °C on YPD medium (10 g l^{-1} yeast extract, 15 g l^{-1} peptone, 20 g l^{-1} glucose). When selection for the presence of plasmid molecule was required, synthetic complete medium (SC) (containing 6.7 g l^{-1} Yeast Nitrogen Base (Difco), 5 g l^{-1} soluble starch and 8 g l^{-1} agar) was employed. YPS (with 2% soluble starch instead of glucose in YPD) medium was used to assay the starch-utilising ability of the yeast transformants.

DNA manipulation. DNA manipulation in *E. coli* DH5α was performed using standard methods (Sambrook and Russell, 2001). Isolation of yeast genomic DNA and transformation of yeast strain were carried out by lithium acetate method (Burke *et al.*, 2000).

Cloning of glucoamylase gene. The PCR amplification of glucoamylase-encoding sequence (GenBank accession number X58117) from *Saccharomycopsis fibuligera* 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 1.55-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 *Eco*RI+*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*. (1995) with modification: 100 mM K_3PO_4 (pH 8.0), 5 mM $MgCl_2$, 1 mM TPP, 100 mM pyruvate, 0.2 mM FAD and 50% cells extract (v/v) were incubated at 30 °C for 20 min. The reaction was terminated by adding 3.5 μl of 3 M H_2SO_4 per 200 μl reaction mixture. The α-acetolactate formed in the reaction was readily decarboxylated to acetoin or diacetyl upon heating in acid solution. Both components were determined by gas chromatographic analysis. One unit of α-acetolactate synthase activity was defined as the amount of enzyme required to release 1 μmol α-acetolactate per mg protein per min under the assay condition 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 (Steyn and Pretorius, 1991): 50 μl centrifuged culture supernatant containing the secreted enzyme was incubated with 200 μl 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 glucose per ml per min at the same culture condition.

Purification of glucoamylase. The glucoamylase of the fermented supernatant was purified as described by Faridmoayer and Scaman (2004) with some modifications. The fermented broth was centrifuged (12 000 rev min^{-1}, 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 rev min^{-1} at 4°C for 30 min. The pellet was dissolved in 90 ml buffer 1 (20 mM sodium phosphate buffer, pH 6.8) and dialysed against 4 l of the same buffer for approximately 24 h, with dialysing buffer changed every 12 h. The dialysed sample was applied to a DEAE-cellulose column (2.5 x 20 cm) equilibrated with buffer 1 at the flow rate of 1.5 ml min^{-1}. 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^{-1}. Fractions of 3 ml were collected during the 0.2 M NaCl elution. Active fractions were pooled and applied onto column (2.5 x 20 cm) of Sephadex G75. The active fractions were pooled and used for further SDS-PAGE.

Fermentation test. A single colony was transferred into 25 ml YPD broth in a loosely capped 100 ml flask and incubated for 18 h at 30 °C with shaking (160 rev min^{-1}), which used as seed culture for further experiments. The enlarge culture was carried out by inoculating 10 μl of the seed culture to 100 ml wort with shaking at 25 °C. After 48 h, 900 ml fresh wort was added and recultivated for additional 48 h at 20 °C. Then, the culture cells were har-

vested and inoculated in the fermentor at a density of approximately 1 x 10^7 cells ml^{-1}. The primary experiments were performed in a 5-l bench-top fermentor with 3-l 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 was carried out at a scale of 1000 kg according to the technique of Shijiazhuang Xinle Beer Corporation, that primary wort fermentation was performed for 5 d at 9 °C, then maturation at 13 °C for 7 d, and 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). Concentrations of diacetyl were determined by gas chromatography, using a flame ionisation detector and a fused capillary column coated with DB-Wax (30 m x 0.53 mm and 1.0 µm film thickness (Auto System XL, PE Company, USA). The carrier gas was nitrogen and flow at 10 ml min^{-1} rate. To determine the content of diacetyl, samples were incubated for 30 min at 60 °C in a temperature incubator. During this incubation, a-acetolactate readily converted to diacetyl. Commercial corresponding compounds were used as a quantitative standard.

RESULTS AND DISCUSSION

In order to disrupt the *ILV2* gene on the recipient genome, the recombinant plasmid pMGI6 (Fig. 1) was constructed and the 5.65-kb fragment carrying the *ilv2*::*GLA* gene (5´-*ilv2*-*PGK1*$_P$-*MF*α1s-*GLA*-*ADH1*$_T$-*ilv2*-3´) was released upon cleavaging pMGI6 by *Apa*I+*Not*I and used as a linear disruption cassette.

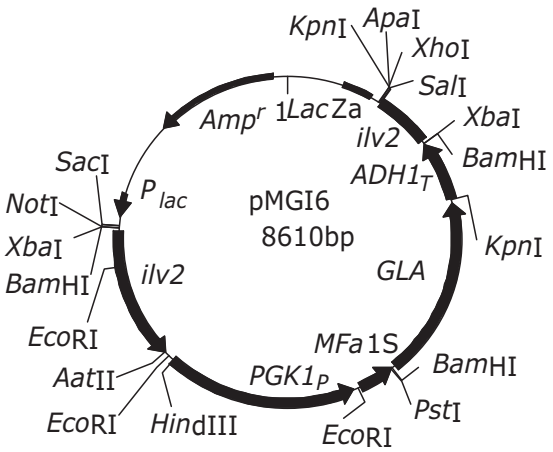

FIG. 1 - The physical map of the recombinant plasmid pMGI6.

The 5.65-kb fragment was transformed into the industrial yeast strain Sc-11 by lithium acetate method, and the transformants were screened on SC medium (containing starch as the sole carbon source) using the *GLA* gene as a selective marker and further screened for those with low functional *ILV2* gene by measuring α-acetolactate synthase activity of yeast cell extract. As a result of this screening assay, two transformants were picked out.

TABLE 1 - Determination of the autotrophy

Strains	SC	SC+Ile	SC+Val	YPD
Sc-11	–	–	–	+
Transformant 1	+	+	+	+
Transformant 2	+	+	+	+

TABLE 2 - The activity of glucoamylase and α-acetolactate synthase

Enzymes	Transformant 1	Transformant 2	Sc-11
α-Acetolactate synthase (U g^{-1})	19.4	19.5	29.1
Glucoamylase (U ml^{-1})	6.30	6.21	0

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

Slant cultures of the transformants were streaked onto fresh YPD plates and incubated for 2 d at 30 °C. A single colony of the transformant was transferred into 2 ml distilled water and cultured for 4 h, then restreaked onto a fresh SC plate with the starch as the sole carbon source and Val or Ile added respectively and incubated at 30 °C for 5 d (Table 1). The results showed that the transformants were able to use starch as the sole carbon source and the biosynthetic pathways of valine and isoleucine in the transformants were not interrupted. It could be deduced that one copy of *ILV2* gene on the recipient genomic DNA was disrupted and the other copy remained unchanged and functioned normally, and the transformant was an autotrophic organism.

The transformants were subject to assay of glucoamylase activity and α-acetolactate synthase activity (Table 2). One transformant was picked out for further examination and named ScG11. The α-acetolactate synthase activity of ScG11 was 67% of that of Sc-11 and the glucoamlase activity of ScG11 was 6.3 U ml^{-1}. The results indicated that the *GLA* gene was expressed under the control of phosphoglycerate kinase1 promoter and the use of the *MFα1* signal sequence was effective in guiding the glucoamylase secretion and the disrupted copy of *ILV2* gene could not express α-acetolactate synthase and only the unbroken *ILV2* gene had the probability to express α-acetolactate synthase, therefore lower expression level of α-acetolactate synthase activity was achieved and the acetolactate level secreted into the culture medium reduced compared with that of the recipient.

To study the capacity of ScG11 to utilise starch, the seed culture of ScG11 was inoculated in YPS medium at a rate of 10% (v/v) to carry out shake flask experiments. Fermentation samples were collected at different time intervals and the residual starch was determined. After 7 d fermentation more than 78% of starch was utilised by ScG11 (Fig. 2).

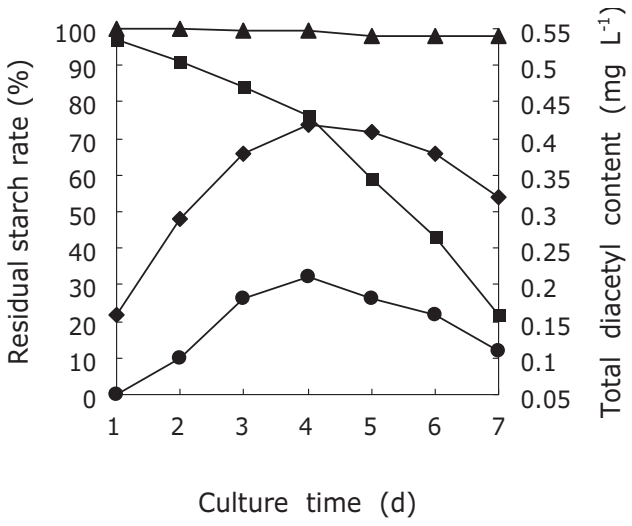

FIG. 2 - Time courses of starch utilisation and total diacetyl content in fermented broths. Symbols: squares, starch residual rate of ScG11; triangles, starch residual rate of Sc-11; solid circles, total diacetyl of ScG11; rhombuses, total diacetyl of Sc-11. The values are the means of results from triplicate experiments with standard deviation less than 5%.

To investigate whether the disruption of *ILV2* gene on genomic DNA could low down the diacetyl content of the fermenting broth, primary wort fermentation was performed over 7 d at 12 °C. The fermenting broths were collected at different time intervals and the diacetyl content was determined (Fig. 2). At the end of the primary fermentation, the total diacetyl concentration in fermenting wort of ScG11 was reduced by 66%, compared with that of Sc-11. This indicated that the synthesis of diacetyl was influenced by the disruption of *ILV2* gene, one copy disruption of *ILV2* gene was able to reduce the formation of diacetyl significantly and the maturation time necessary to remove diacetyl from the fermented wort by ScG11 was shortened.

Analysis of the purified glucoamylase sample of ScG11 was performed using SDS-PAGE according to Sambrook and Russell (2001) (Fig. 3). The transformant ScG11

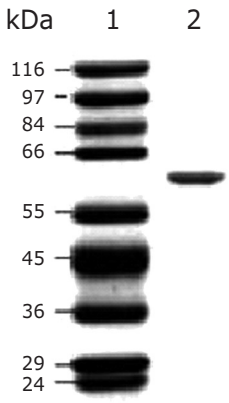

FIG. 3 - SDS-PAGE analysis of purified extracellular glucoamylase protein from ScG11. 1: molecular size markers; 2: ScG11.

TABLE 3 – Characteristics of the finished beers

Strains	Sc G11	Sc-11
Foam stability (s)	264	263
Clarity (EBC)	0.93	0.94
Total soluble nitrogen (mg l^{-1})	716	716
Colour (EBC)	3.6	3.7
Alcohol (% v/v)	4.4	4.3
Calories (kJ l^{-1})	1598	1947
Original extract (% wt/wt)	10.0	10.0
Total acidity (% v/v)	1.6	1.56
Dissolved CO_2 in bottles (% v/v)	0.48	0.47
Diacetyl (mg l^{-1})	0.098	0.1

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

showed a clear protein band at about 62 kDa and it could be concluded that ScG11 secreted glucoamylase protein into the culture medium and the molecular weight of the protein was about 62 kDa.

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 ScG11 fermented broth was reduced to 0.1 mg l^{-1} after 4 d maturation and the maturation time was shortened, while that of the recipient was 7 d. The characteristics of the final beers were determined (Table 3). The calorie value in beer fermented by the ScG11 was decreased compared with that of the Sc-11. With regard to clarity, colour and viscosity, the two kinds of beers compared quite closely and there were no significant differences in mouthfeel and the beer quality fermented by the engineered strain ScG11 under the industrial condition met the company standard. Then, it could be concluded the beer of ScG11 had comparable quality to that of Sc-11, and 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.

CONCLUSION

Considering the fermentation efficiency and production cost, this engineered strain should be suitable for industrial production of beer and might have the potentiality to be used in low-caloric beer production. The transformant was free of bacterial vector sequence and drug-resistance markers and should avoid the problems caused by transfer of bacterial antibiotic-resistance and improve the acceptance in the public opinion. This study could contribute towards constructing the recombinant industrial yeast strains used in alcoholic beverage production.

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