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Full Length Research Paper

Gene cloning of phenolic acid decarboxylase from *Bacillus subtilis* and expression in top-fermenting yeast strain

Xiaohong Cao[1]*, Yunqian Cui[1,2], Xinghua Liao[1], Guangtian Zhou[2] and Leo Thamm[2]

[1]College of Food Engineering and Biotechnology, Tianjin University of Science and Technology, Tianjin 300457, P. R. China.
[2]China-Germany Brewing Technical Service Center, Shandong Institute of Light Industry, Jinan 250353, P. R. China.

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Phenolic acid decarboxylase (*PADC*) gene, encoding phenolic acid decarboxylase, was cloned from *Bacillus subtilis* and ligated with a shuttle vector YEp352 to generate a novel plasmid YPADC. By analysis of sequencing and the restriction endonuclease digestion, the validity of construction was proved. Subsequently, the new vector was successfully transformed into wild-type top-fermenting yeast strain W303-1A; the mutant yeast strain W303+*padc* was obtained, which was tested on the laboratory-scale mashing and fermentation experiments. At the end of fermentation, the results showed an obvious increase of 4-vinylguaiacol content in top-fermented beers brewed with mutant yeasts. The final 4-vinylguaiacol concentration obtained with wild-type and mutant yeasts was 1.20 and 1.70mg/l, respectively. Additionally, the level of esters produced by the mutant strain was higher than that of the wild-type; there were therefore a marked clove-like and ester aroma in top-fermented beers brewed with the former. However, no evident differences were found in brewing characteristic between wild-type and mutant strains, especially the ability of utilizing fermentable sugar and reducing diacetyl. Taken together, these approaches indicated the possibility of cloning *PADC* gene and enhancing the concentration of 4-vinylguaiacol in top-fermented beers.

Key words: Clone, phenolic acid decarboxylase, top-fermenting yeast, 4-vinylguaiacol.

INTRODUCTION

Phenolic acids, also called substituted cinnamic acids, refer to several kinds of hydroxycarboxylic acids with phenolic hydroxyl groups. In cereal grain, they are mainly in the cell wall, esterified with arabinoxylans (Scalbert et al., 1985; Iiyama et al., 1990; Hartley et al., 1990), while in grapes, apples and other fruits, they mostly exist in the skin, stem and seed etc (Souquet et al., 2000; Cantos et al., 2002).

During the process of wine-making and beer-brewing, most phenolic acids are water-extracted and solubilised by hydrolases from the grape and malt into the juice and wort, respectively, which can be degraded by several enzymes (principally ferulic acid esterases, *p*-coumaroyl esterases or feruloyl esterases) to form 4-vinylguaiacol (4VG), 4-vinylphenol (4VP), 4-ethylguaiacol (4EG), 4-ethylphenol (4EP), vanillin, apocynol and so on (Smit et al., 2003; Vanbeneden et al., 2008). These compounds are helpful in enhancing the aroma of wine and top-fermented beers (e.g. Belgian white beers, German Weizen beers and Rauch beers) (Coghe et al., 2004; Vanbeneden et al., 2008). Especially 4VG, despite lower levels in wines and beers and historically being cataloged as a “phenolic off-flavor” (POF) (Thurston and Tubb,

*Corresponding author. E-mail: cuiyunqian@yahoo.com.cn. caoxh@tust.edu.cn. Tel: +86-22-60601428.

Abbreviations: 4VG, Vinylguaiacol; **4VP,** 4-vinylphenol; **4EG,** 4-ethylguaiacol; **4EP,** 4-ethylphenol; **POF,** phenolic off-flavor; ***PADC*,** phenolic acid decarboxylase gene*;* **HPLC,** high performance liquid chromatography; **YPD,** yeast peptone dextrose; **LB,** Luria Bertani; **PCR,** polymerase chain reaction; **PET,** polyethylene terephthalate.

Table 1. Primers designed for PCR[a].

Primer	Sequence (5'-3')
F-padc	5'-GCG TCTAGA ATGGAAAACTTTATCG-3'
R-padc	5'-GCG AAGCTT TTATAATCTTCCCGC-3'

[a]Primers include XbaI and HindIII sites (underlined nucleotides), respectively.

1981) in bottom-fermented beers, is still a well known characteristic aroma contributor to many types of wines and top-fermented beers, which is of vital importance for the overall flavor perception in these wines and top-fermented beers. Several bacteria and fungi contain phenolic acid decarboxylase (*PADC*) gene, encoding phenolic acid decarboxylase, which plays an important role in the biodegradation of a large amount of plants, for instance, *Pseudomonas putida* WCS358 (Venturi et al., 1998), *Acinetobacter* (Segura et al., 1999), *Pseudomonas fluorescens* (Narbad and Gasson, 1998) and *Bacillus subtilis* (Tran et al., 2008) etc.

In this study, the *PADC* gene was cloned from *B. subtilis*, expressed in *Escherichia coli* (Cavin et al., 1997, 1998; Zago et al., 1995; Smit et al., 2003; Tran et al., 2008) and subsequently transformed into the top-fermenting yeast strain, W303-1A. Over expressing the heterologous *PADC* gene in the mutant strain, the phenolic acid decarboxylase activity and its functions were gained. By means of laboratory-scale mashing and fermentation experiments, the concentration of 4VG in top-fermented beers was detected and the influence of the recombinant yeast strain on the aroma of top-fermented beers was determined.

MATERIALS AND METHODS

Materials and reagents

Easy Taq enzyme and 10 × buffer Taq without $MgCl_2$ were purchased from invitrogen (California, USA). Restriction enzymes, *XbaI* and *HindIII*, T4 DNA ligase and Wizard Purification Kit were purchased from Promega (Madison, Wisconsin, USA). Scarlett barley malt was provided by Zhongliang Malt Division (Dalian, China). Wheat malt was obtained from Weyermann (Bamberg, Germany). Hops were supplied from Barth-Haas Group (Beijing, China), which contained 4.9% α-acids (Analytica-EBC). High performance liquid chromatography (HPLC) grade 4VG (2-methoxy-4-vinylphenol) was purchased from Sigma-Aldrich (Shanghai, China). Other reagents were all of analytical grade.

Strains, plasmids and medium

B. subtilis, *E. coli* DH5α and plasmid YEp352 (shuttle vector for *E. coli* and Saccharomyces *cerevisiae*) were from our laboratory. Top-fermenting yeast strain W303-1A (*MATα,leu2-3/112,ura3-1, trp 1-1, his3-11/15,ade 2-1, can 1-100, GAL, SUC2, mal 0*) was obtained from Doemens (Munich, Germany). Yeast cells were cultured in yeast peptone dextrose (YPD) medium (10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose). The *E. coli* DH5α cells were cultured in Luria Bertani (LB) medium (5 g/l yeast extract, 10 g/l tryptone and 10 g/l sodium chloride). For the selection of *E.coli* transformants, 100 µg/ml ampicillin was included in the LB medium. For the selection of yeast transformants, SC^{-Ura} medium (6.7 g/l yeast nitrogen base without amino acids, 20 g/l glucose and 8.3 g/l of each of the growth factors Leucine, Histidine and Tryptophan) was used to provide auxotrophic pressure. Solid media contained 20 g/l agar. Bacteria and yeasts were cultured at 37 and 30°C, respectively.

Cloning and sequencing procedures

The polymerase chain reaction (PCR) method was used for the amplification of the *PADC* gene. Primers were synthesized (Table 1) by invitrogen (California, USA) and the genomic DNA from *B. subtilis* was used as the template to amplify the *PADC* gene. A thermal cycler (Bio-Rad, California, USA) was used and the PCR program was: Template denaturation at 94°C for 3 min, denaturation at 94°C for 30 s, primer annealing at 52.6°C for 30 s, primer extension at 72°C for 45 s (30 cycles) and final elongation at 72°C for 10 min. The volume of PCR-reaction mixture was 50 µl consisting of 5 µl 10 × Taq buffer, 4 µl 1.25 mM nucleotides (dNTPs), 1 µl of each primer, 1 µl template, 1 µl Easy Taq enzyme and 37 µl ddH_2O.

After PCR amplification, the PCR products were purified by Wizard Kit. Then the purified *PADC* gene and shuttle plasmid YEp352 were digested with *XbaI* and *HindIII*, separately. They were ligated with T4 DNA ligase to generate plasmid YEpPADC, which was transformed into *E.coli* DH5α to obtain the plasmid YPADC. The final construct YPADC was sequenced by invitrogen (California, USA) to ensure reliable identification.

Transformation into top-fermenting yeast strain W303-1A

Standard methods for yeast transformation were used (Adams et al., 1998). The new vector YPADC was transformed into top-fermenting yeast strain W303-1A and the transformants were selected on SC^{-Ura} plates. The new mutant strains were named W303+padc.

PCR analysis of mutant colonies

The *PADC* gene was confirmed by PCR colonies from the transformed strains on SC^{-Ura} plates. The PCR conditions and reaction system were as described in the procedure for amplifying the *PADC* gene above.

Phenolic acid decarboxylase activity assay of mutant strain

PADC activity assay of W303+padc was achieved using a modification of the protocol described by Smit et al. (2003). The control, W303-1A and mutant, W303+padc were incubated in 100 ml of SC^{-Ura} medium at 30°C, separately.

The *PADC* activity was assayed as previously described (Cavin et al., 1993, 1997; Harris et al., 2009), in which ferulic acid degradation and 4VG production were monitored by UV spectrophotometry; the former shows a peak at its maximal absorbance wavelength (285 nm) and the latter shows a peak at 258 nm. The disappearance of the 285 nm peak and appearance of a 258 nm peak indicate the degradation of ferulic acid and the formation of 4VG. The *PADC* activity was expressed as micromoles of substrate degraded per minute per milligram of protein.

Analysis of mutant stability

Selected transformants were inoculated into 10 ml YPD broth at

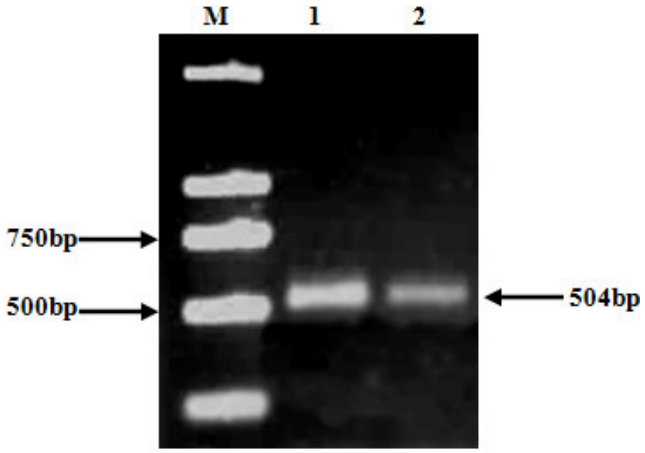

Figure 1. Amplification of *PADC* gene. M-D2000 DNA marker, 1,2-*padc* gene.

30℃. After incubating 10 generations, the colony was inoculated onto the SC and SC^{-Ura} plate separately, the genetic stability was calculated according to the following formula:

Mutant stability = (Colonies on SC^{-ura}/ Colonies on SC) X 100%

Laboratory-scale mashing and fermentation

After a stepwise propagation, the mutant strains were inoculated in brewer's wort in polyethylene terephthalate (PET) bottle and the wort was produced as follows: The ratio of barley malt and wheat malt was 70: 30; an automated mashing bath (LB8 Electronic mashing bath, Funke Gerber GmbH, Berlin, Germany) was used, the infusion mashing was adopted; the mashing in temperature was 45℃ (20 min), the proteolytic rest was 52℃ (40min), the amylolytic rest was 65℃ (70 min), the lautering temperature was 78℃; the boiling time was 90 min. The initial wort gravity was 11℃P. The main fermentation was carried out at 18℃. When the apparent extract was 4℃P, the open lid of PET bottle was closed until the diacetyl content of the green beer was reduced below 0.1 mg/l. The temperature was then decreased to 0℃ in a refrigerator and the secondary fermentation continued for more than 5 days. All mashing and fermentation tests were repeated three times, along with W303-1A strain as control.

4VG and volatile compounds analysis

The 4VG levels were determined by Shimadzu HPLC using a method described by Shinohara et al. (2000). The HPLC system consisted of a LC-10Avp pump, a 7725i Sample injector, a Shimadzu uv-vis detector and a N2000 data analyzer (Zhejiang University, Hangzhou, China). The samples were analyzed using a 15cm × 4.6 mm i.d. packed column (no. 6042278, C_{18}, Hitachi, Tokyo) eluted with water/acetonitrile/phosphoric acid (599/400/1,V/V) at a flow rate of 1mL/min. The UV detector was operated at 230 nm.

The volatile compounds (acetaldehyde, ethyl acetate, isobutanol, n-propanol, isoamyl alcohol, isoamyl acetate and ethyl caproate) were analyzed with a Perkin-Elmer (Shelton, CT) autosystem XL gas chromatograph. The methods used for analysis of aroma compounds were based on the proceedings of the European Brewery Convention (Thum and Back, 1999) with some modifications. A 5 ml sample was incubated for 30 min at 55℃ in a 20 ml glass vial prior to sampling the head space and n-butanol was used as an internal standard. The injector and detector temperatures were 225 and 250℃, respectively. Helium was used as carrier gas (1 ml/min) and nitrogen used as make-up gas (40 ml/min). The column was a CP-WAX-57-CB capillary column (0.25 mm i.d. × 60 m). The temperature program used began at 40℃ with a 12℃/min programmed increase to 100℃, followed by a 3.5 min hold at 100℃. A second program increase of 12 to 150℃/min was applied, followed by a 5.0 min hold at 150℃.

Sensory evaluation

A trained tasting panel was used for the evaluation of fresh top-fermented beers according to sensory analysis of attributes, including clove-like flavor, headache sense, ester aroma, taste, bitterness and aftertaste, in which the headache sense was evaluated in 30 min after tasting.

The rating scale ranged from 1 (very bad or very weak) to 5 (very good or very strong). Two blind samples brewed by the wild-type and mutant strain were evaluated and classified by the rating scale. Beers were kept at 5℃ for sensory evaluation.

RESULTS

Amplification of *PADC* gene

The PCR reaction was carried out, in which the genomic DNA from *B. subtilis* was used as the template and the F-padc and R-padc as primers to amplify the *PADC* gene. Using 1% agarose gel, the *PADC* gene was confirmed (Figure 1).

Analysis of restriction endonuclease of the constructed plasmid YPADC

The constructed plasmid YPADC was digested with restriction endonucleases *XbaI* and *HindIII* to identify the authenticity of construction and to further select the positive transformants. The theoretical sizes of the fragments that dealt with *XbaI* and *HindIII* are 5,181 and 504 bp and fragments of corresponding sizes were produced from the new vector YPADC (Figure 2). The analysis of the plasmid YPADC by restriction endonuclease digestion confirmed the validity of construction procedure.

Transformation into top-fermenting yeast strain W303-1A

After the new constructed vector YPADC was transformed into strain W303-1A, some white colonies emerged in 5 days on the SC^{-ura} plates, while no colony emerged on the control plates (Figure 3). This indicated that the new plasmid YPADC was successfully transformed into the strain W303-1A.

PCR analysis of mutant colonies

The insertion of the *PADC* gene was confirmed by PCR

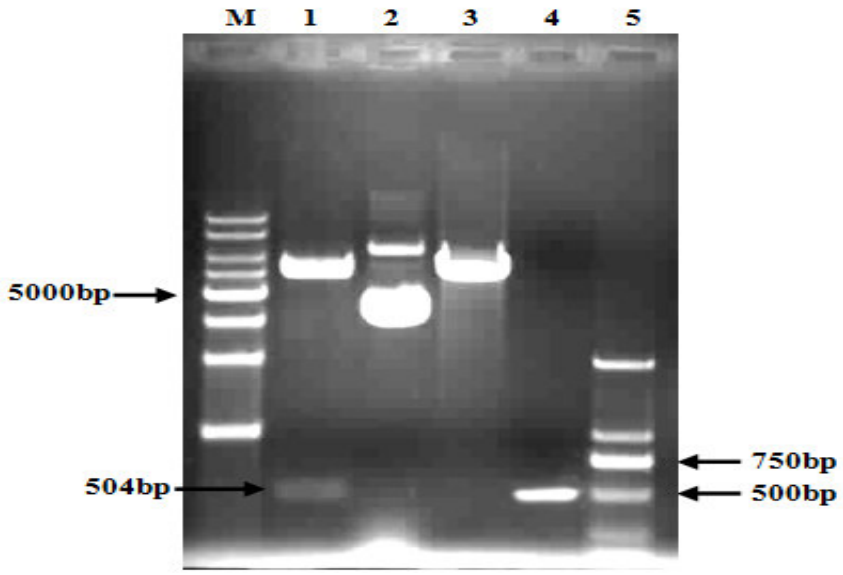

Figure 2. Analysis of restriction endonuclease XbaI and HindIII of the constructed plasmid YPADC.

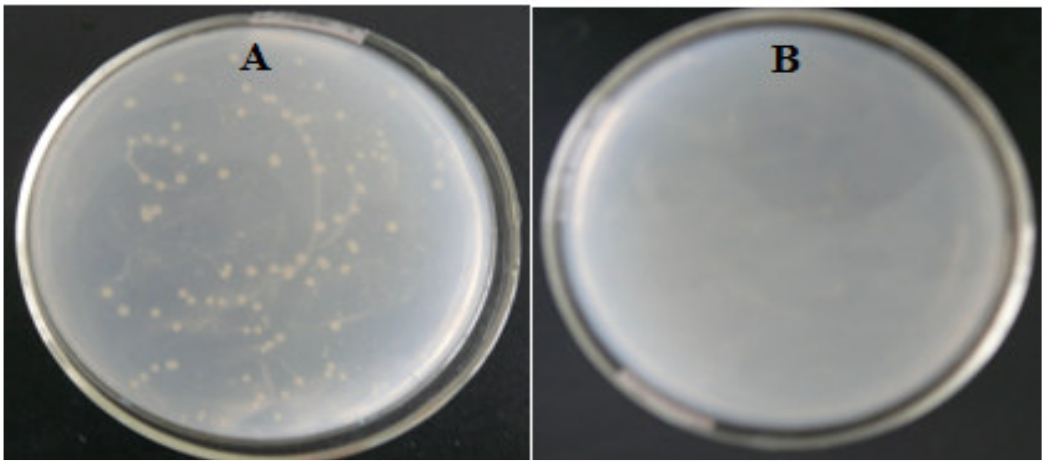

Figure 3. The mutant strain was screened by SC^{-Ura} plate. A: Mutant strain; B: control strain.

colonies from the transformed strains. The size of the W303+*padc* colonies PCR product was approximately 504bp in accordance with that of *PADC* gene fragment (Figure 4). This result showed that the *PADC* gene had been integrated into the appropriate chromosome of mutant strain. In contrast, this fragment was not produced from wild-type strain W303-1A.

Phenolic acid decarboxylase activity assay of mutant strain

Wild-type strain W303-1A and the mutant strain W303+padc were grown in SC^{-ura} medium supplemented with 0.3 mM ferulic acid; *PADC* activity measured by UV spectrophotometry is shown in Table 2. The *PADC* activity of mutant strain W303+padc was increased, which was 2.1-fold higher than that of wild-type strain W303-1A.

Analysis of mutant stability

After incubating 10 generations, the number of colonies on the SC and SC^{-Ura} plate was 62 and 33, respectively. By calculating according to the method described above, the transformants stability was 53.23%, which benefited for the continuous brewing beers with mutant strain.

M-1kb DNA Marker, 1- XbaI and HindIII digested recombinant plasmid YPADC, 2- recombinant plasmid YPADC, 3- XbaI and HindIII digested plasmid YEp352, 4-

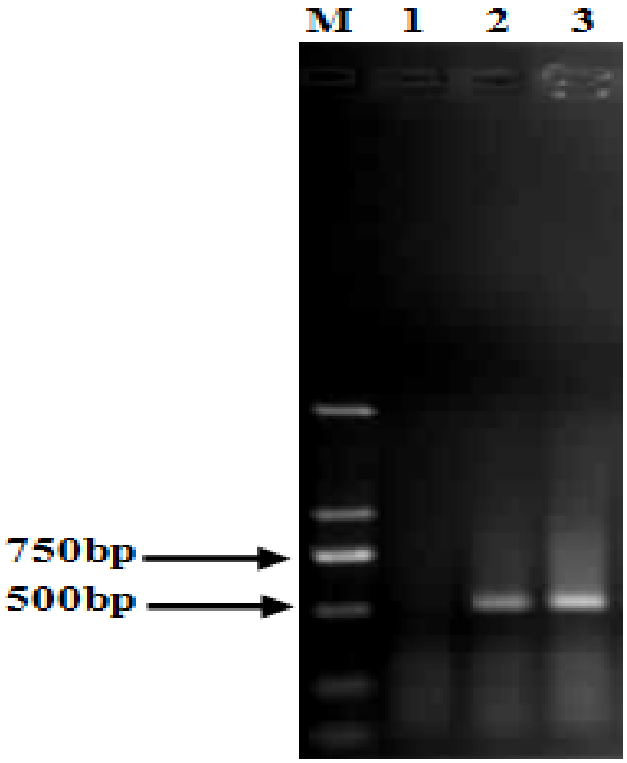

Figure 4. Colony PCR analysis of wild-type and mutant strain.

Table 2. *PADC* activity of wild-type and mutant strain.

Yeast strains	*PADC* activity (μmol/mg·h)
W303-1A	1.37 ± 0.06
W303+padc	2.88 ± 0.06

XbaI and HindIII digested PCR *PADC*, 5-D2000 DNA marker

The fermenting characteristic of mutant strain

In the course of fermentation with wild-type strain, W303-1A, and the mutant strain W303+padc, several critical brewing characteristics were focused on, such as variation of apparent extract and diacetyl. On the whole, the ability of utilizing fermentable sugar and reducing diacetyl was similar between the two strains, but the mutant strain W303+padc exhibited a slower performance (Figure 5).

The concentrationin of 4VG and other volatile compounds in top-fermented beers

The cloned plasmid YPADC was successfully transformed into industrial top-fermenting yeast strain W303-1A and integration was confirmed by PCR. A series of laboratory-scale mashing and fermentation experiments were carried out and the some volatile compounds were detected. In contrast to the wild-type strain W303-1A, the concentrations of 4VG and esters in the top-fermented beers brewed with mutant strains had notable increases (Figure 6, Table 3), Undoubtedly, which was good to enhance the aroma of top-fermented beers.

Sensory evaluation analysis

13 judges from a trained tasting panel evaluated the fresh top-fermented beers brewed with the wild-type and mutant strains; these were marked by a 5-point method. The score was obtained by calculating mean value results of 13 judges.

Sensory evaluation of fresh top-fermented beers brewed with the mutant strain revealed more clove-like flavor and ester aroma, which reflected the higher 4VG and ester levels (Figure 7). Except this, the headache sense of beer with the mutant was little stronger than that of the wild-type in which corresponding mean score was 3.9 and 3.5 rated by 13 judges, respectively (Figure 7). This was perhaps conduced by the higher alcohols in top-fermented beer with the mutant (Table 3). There were no distinct differences between the other three kinds of sensory analysis attributes. Lane M = D2000 DNA marker, lanes1 = W303-1A colony PCR and lanes 2 and 3 = W303+padc colony PCR.

DISCUSSION

The *PADC* gene has been successfully cloned from *B. subtilis* and transferred into the top-fermenting yeast strain W303-1A. Analysis of restriction endonuclease digestion, sequence, colony PCR and assay of phenolic acid decarboxylase activity showed that the recombination was valid and the *PADC* gene had been integrated into the chromosome of wild-type strain.

By the test of laboratory-scale mashing and fermentation, the brewing performance and corresponding volatile compounds of the wild-type and mutant strain were investigated. The results showed that their fermentation characteristics were very similar and the concentration of 4VG in top-fermented beers brewed with the mutant strain was enhanced from the control of 1.20 to 1.70 mg/l, as expected. To our knowledge, it was the first to enhance the 4VG concentration by expressing the *PADC* gene in the top-fermenting yeast strain. Previously, most reports on increasing the 4VG level was focused on brewing technology (Coghe et al., 2004; Vanbeneden et al., 2008). Nevertheless, compared with foreign top-fermented beers, the enhanced 4VG concentration was still not high enough in this study. Perhaps, expressing the *fdc* gene (encoding ferulic acid decarboxylase) from *B. pumilus* (Zago et al., 1995) will have more potential and

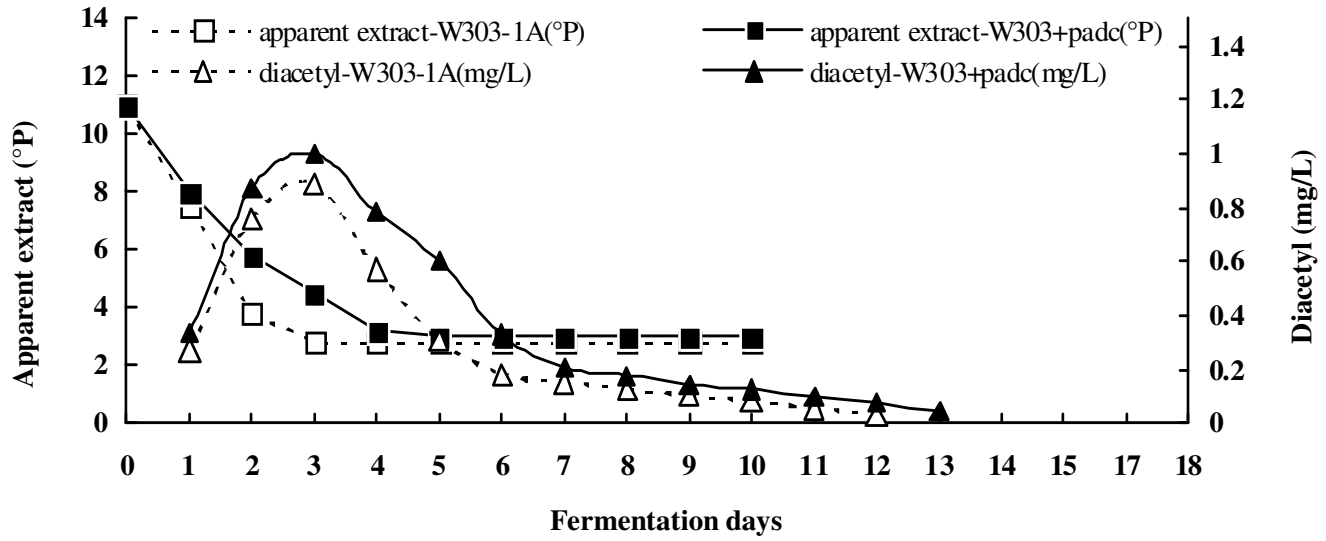

Figure 5. Variation of apparent extract and diacetyl during fermentation with wild-type and mutant strain.

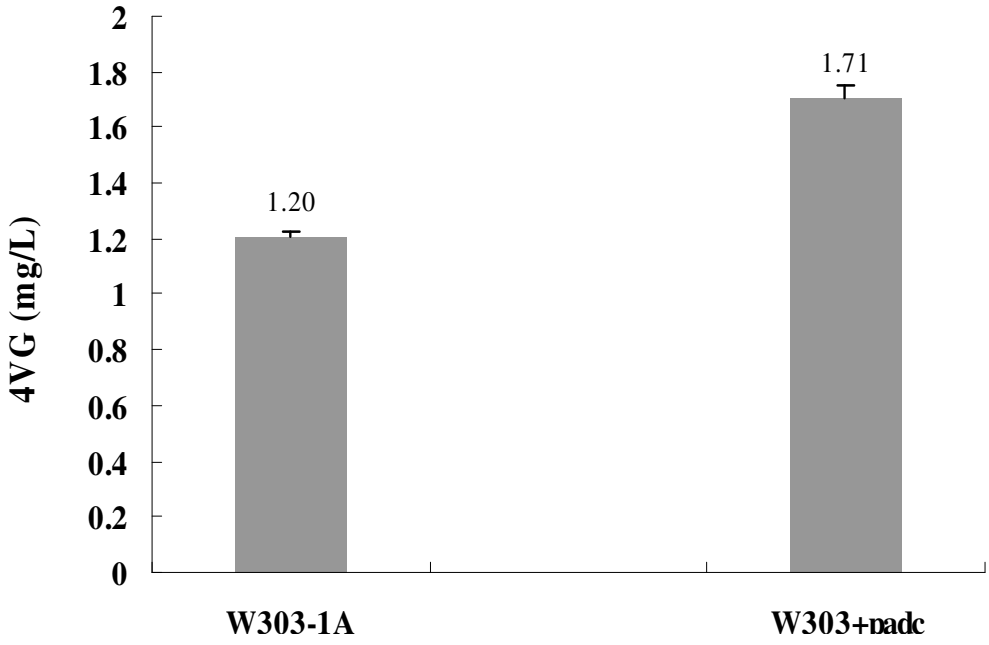

Figure 6. The concentration of 4VG from the wild-type and mutant strain.

pertinence to enhance the 4VG concentration in top-fermented beers, which is currently in progress, because ferulic acid is just the precursor of 4VG (Coghe et al., 2004; Vanbeneden et al., 2008).

To the top-fermented beers with the mutant strain, though the aroma of clove-like and ester had been improved on account of the higher level of 4VG and esters, respectively, the headache sense was pronounced due to the amounts of higher alcohols. The problem on reducing the level of higher alcohols produced by the mutant strain will be paid more attention in further research, in which the knockout of alcohol dehydrogenase (ADH) gene will be discussed in detail.

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Table 3. Volatile compounds in top-fermented beers brewed with wild-type and mutant strain.

Volatile compounds (mg/l)	Wild-type strain W303-1A	Mutant strain W303+padc
Acetaldehyde	42.266	48.634
DMS	0.012	0.047
Ethyl formate	0.288	0.305
Ethyl acetate	16.265	45.422
Isobutyl acetate	0.202	0.337
Isoamyl acetate	1.366	8.463
Ethyl caproate	0.059	0.166
Ethyl caprylate	0.059	0.149
N-Propanol	21.659	36.386
Isobutanol	35.107	41.533
Isoamyl alcohol	74.257	107.222

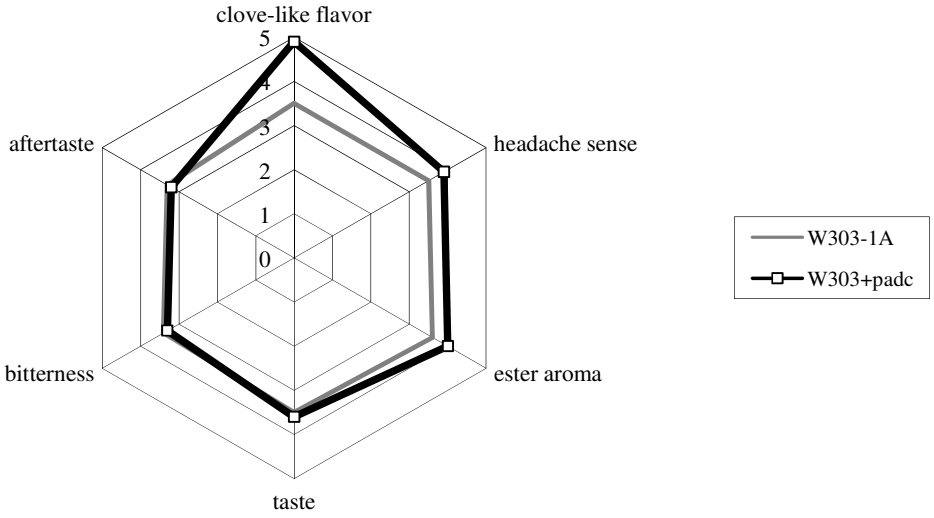

Figure 7. Sensory evaluation of fresh top-fermented beer brewed with wild-type and mutant strain.

Light Industry (Jinan, China) that ensures this study was smoothly accomplished.

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