Agr. Nat. Resour. 54 (2020) 48–54

AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Isolation and characterization of a new low-diacetyl-producing yeast for fermentation of rice beer using high- and low-gravity wort

Nattakan Keesod[a], Wilawan Sintuprapa[a], JinJing Wang[c], Qi Li[c], Ulaiwan Withayagiat[a,b,*]

[a] *Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand.*

[b] *Fermentation Technology Research Center, Faculty of Agro-Industry, Kasetsart University, Bankok 10900, Thailand.*

[c] *School of Biotechnology, Jiangnan University, Wuxi, Jiangsu, 214122, China.*

Article Info

Article history:
Received 5 December 2019
Revised 4 February 2020
Accepted 10 February 2020
Available online 28 February 2020

Keywords:
Brewing yeast,
Diacetyl,
High-gravity,
Rice beer

Abstract

Diacetyl is a buttery aromatic compound that is formed during beer fermentation as a by-product of amino acid synthesis. In particular, high-adjunct wort contains a low valine concentration that can stimulate diacetyl synthesis. The objective of this research was the selection of a low-diacetyl-producing yeast for brewing rice beer. Ninety-two yeast isolates were isolated from 55 yeast sources and were screened for their ability to produce ethanol in the rice wort. Three isolates (DG201, BNS04 and LPST01) were chosen as the maximum ethanol producers. LPST01 produced diacetyl (mean ± SE; l8.32 ± 1.63 mg/L in all-malt beer and 9.63 ± 0.78 mg/L in 70% (weight per weight; w/w) rice grit beer), and the levels were comparable to the values for the S-23 commercial yeast strain (7.80 ± 4.07 and 11.97 ± 4.08 mg/L, respectively). Sequence analysis of the divergence in the variable D1/D2 domain of the large subunit (26S) ribosomal DNA revealed that LPST01 was *Saccharomyces cerevisiae,* with a similarity of 100%. The strain was characterized for optimal fermentation temperature, flocculation property and diacetyl production in three worts prepared from three percentages of rice grit (30%, 50% and 70% (w/w)) under normal- and high-gravity conditions. At 20ºC, LPST01 had the highest specific growth rate (0.0626 ± 0.00 per hour) and was characterized as a non-flocculent yeast. LPST01 tolerated high-gravity worts. The diacetyl production and yield in high-gravity fermentations was slightly lower than that under normal gravity. These results suggested LPST01 was a promising strain for economical beer production, and that it could be used in high-gravity beer fermentation.

Introduction

The biological properties of yeasts have led to their widespread use in the field of biotechnology, with fermentation of sugars and alcoholic beverages being the oldest and most economically important application (Walker and Stewart, 2016). Many types of yeasts have been isolated for specific purposes and fermentation media; for example, yeasts isolated from fruits are used in the industrial production of ethanol (Tikka et al., 2013), in bakery products (Aruf et al., 2011) and in functional fermented beverage production (Amorim et al., 2018). Yeast isolated from cheese is used in industrial food and feed processes (Field et al., 2015). Brewer's yeasts are selected to ensure optimal yeast fermentation profiles, meaning that the final product will have the desired taste, color and alcohol content (Walker and Stewart, 2016).

* Corresponding author.
E-mail address: fagiulw@ku.ac.th (U. Withayagiat)

https://doi.org/10.34044/j.anres.2020.54.1.07

During beer fermentation, diacetyl (2,3-butanedione) and 2,3-pentanedione are vicinal diketones (VDK) formed by yeast cells as co-products of the amino acid synthesis pathway; both compounds impart a buttery or butterscotch aroma that may cause negative impressions of the beer depending on the types of beer product (Boulton and Quain, 2006). The diacetyl threshold concentration is normally 0.1–0.2 parts per million (ppm) for lager beer and 0.1–0.4 ppm for ale beer (Saison et al., 2009). Diacetyl formation is directly related to the valine synthesis pathway through insufficient valine available for yeast metabolism during fermentation, so that valine synthesis takes place in the mitochondria of the yeast cells (Ryan and Kohlhaw, 1974). The acetohydroxy acid synthase (AHAs) enzyme responsible for catalyzing the formation of α-acetolactate from pyruvate, will instead produce 2,3-dihydroxy-isovalerate, α-keto-isovalerate and subsequently valine and the reaction between α-acetolactate and 2,3-dihydroxy-isovalerate is rate-limiting (Suomalainen and Ronkainen, 1968). During fermentation and yeast growth, α-acetolactate is secreted through the cell walls into the wort, where it is converted to diacetyl (Krogerus and Gibson, 2013a). Thus, a brewer needs to control diacetyl synthesis during fermentation. The VDK content can be reduced by yeast during the final stage of fermentation, but the concentrations will not be lower than the threshold levels, necessitating a prolonging of the maturation process until the level is negligible in the final product. However, maturation processes are both energy- and time-consuming. Due to this hindrance, brewers seek to decrease the maturation process without affecting beer production by screening for low diacetyl production yeast strains via α-acetolactate production (Field et al., 2015).

The low diacetyl production that varies with the amount of α-acetolactate formed depends on transcription of the *ILV6* gene and overexpression of *ILV6* resulted in a two-fold increase in the diacetyl concentration (Duong et al., 2011).

There have been no previous studies concerning yeast performance and diacetyl concentration in rice beer. Hence, the objectives of this study were to select low diacetyl-producing yeast strains for brewing rice beer and to characterize the selected yeast isolates.

Materials and Methods

Samples for yeast isolation

Samples of fruits, flours, soils, water, local rice wine and local starter culture (called 'look-pang' in Thai) were collected randomly from Central, Northern, Southern, Eastern and Northeastern Thailand and kept at 4°C until needed.

Rice, malt, hops and yeast for rice beer brewing

Rice (Khao Dawk Mali 105), cultivated in Phichit, Thailand, was purchased from local farms. The pale malted barley was supplied by the Pathumthani Brewery Co., Ltd, Thailand. Tettnang hops (α-acid 5.0%) and crystal hops (α-acid 5.5%) (Hopsteiner, Germany) were purchased from local supplier. A bottom-fermenting yeast strain, Saflager S-23 (Fermentis, France), was selected as a low-diacetyl producing yeast.

Preparation of 70% (weight per weight) rice grit wort

The solid portions of rice, malt and soybean meal were 70%:20%:10% (weight per weight; w/w) and were milled to a particle size of less than 0.2 mm using a hammer mill (POLYMIX® System PX-MFC 90 D; U.S.A). Decoction mashing was applied for rice wort production. Rice and soy bean meal were mixed with water (a ratio of solid to liquid of 1:5). The Neutrase® enzyme (enzyme:rice, 400μL:50g; Novozymes; Danmark) was added and the temperature was raised to 52°C for 60 min. The mash was heated to 90°C for 20 min and then Termamyl SC® enzyme (enzyme:rice, 400μL:50g; Novozymes; Danmark) was added. After cooking, the mash was cooled to 62°C for mixing with another portion of malt mash prepared in the same ratio, and the temperature was held constant for 60 min. Afterward, the mixed mash was maintained at 72°C for 30 min and at 85°C for 5 min to stop enzyme activity. The wort was obtained by filtration through two layers of white cloth and was subsequently filtered through filter paper (Whatman No. 4).

Preparation of all malt wort samples

One kilogram of barley malt grits milled by the hammer mill and sieved using a 0.2 mm sieve pore size were mixed with water (a ratio of solid to liquid of 1:5). The temperature was raised to 52°C for 30 min, after which the mash was heated to 62°C for 60 min, then to 72°C and held for 30 min and finally to 85°C for 5 min. The wort was filtered in the same manner as for the rice wort.

Inoculum preparation

The yeast strain was prepared by inoculating one full loop in 10 mL wort and shaking at 150 revolutions per minute (rpm) at 30°C for 24 hr. Next, 10 mL of inoculum was added to 200 mL of wort and shaken at 150 rpm and 20°C for 48 hr. The starter was moved to 1 L of wort and shaken at 150 rpm and 20°C for 48 hr to reach a final cell concentration of 1×10^9 cells/mL. Cell pellets were collected using centrifugation at 2,147 × g for 10 min at 4°C (Eppendorf, model 5804 R, Germany). The initial cell concentration for beer fermentation was 1×10^6 cells/mL/°Brix of wort. The inoculum media was prepared in the same manner as the media for fermentation described above.

Local yeast isolation

One gram of crushed sample was placed in 4 mL of 0.8% (weight per volume; w/v) NaCl and was ten-fold serially diluted. An aliquot of 0.1 mL of diluted sample was plated on yeast malt extract (YM) agar mixed with 100 μg/mL of chloramphenicol (Pupchem; U.S.A.). The plate was incubated at 30°C for 48 hr and five colonies were selected from one plate to cross-streak on YM agar. A single colony of the yeast isolate was chosen, cultured in YM broth, and incubated at

30°C for 48 hr. Pure cultures of yeast isolates were mixed with 70% glycerol at a ratio of 1:1 and kept at -20ºC.

Screening of yeast by alcoholic fermentation ability

All yeast isolates were tested for alcoholic fermentation ability using rice wort. One colony of yeast isolate was added to a test tube containing 10 mL of rice wort and a Durham tube. The test tube was incubated at 30°C for 48 hr and the height of the gas in the Durham tube was recorded using a ruler once a day for two days (Cadavid et al., 2008). Any isolates for which the gas level in the Durham tube was higher than 2.5 cm (50% of total height of the Durham tube) were designated as a strong fermenting yeast and their ethanol concentration was analyzed using gas chromatography modified from EBC method no. 9.2.4 (European Brewery Convention, 1998a) as described below.

Two milliliters of samples or standards were diluted and mixed with 20 µL of 0.5% (volume per volume; v/v) n-butanol as an internal standard. Mixtures were filtered through a 0.45 µm nylon syringe filter to produce clear filtrates that were stored in 1.5 mL vials with screw caps. One microliter of the diluted sample was injected through a DB-WAX capillary column, 30 m × 0.32 mm × 0.15 µm (Agilent; U.S.A) in a gas chromatography apparatus (Agilent 7890B; U.S.A.). The injector temperature was set as 250°C and helium was used as the carrier gas. The oven temperatures were modified from the EBC method by setting at 40°C for 2.5 min and then increasing to 85°C at a ramp rate of 40°C/min, then to 250°C at 20°C/min and finally held at this temperature for 3 min.

Screening of yeast according to diacetyl production

Yeast isolates producing more than 1% (v/v) ethanol were selected to be tested for diacetyl production using all malt wort fermentation at 10ºC for 2 wk. Beer samples (3 mL) were collected every 24 hr for 2 wk and cells were removed using centrifugation at 2,147×g for 10 min at 4°C (Eppendorf, model 5804 R; Germany). The standard method of analysis of diacetyl in the beer samples according to EBC method no. 9.24.2 was used (European Brewery Convention, 1998b). Each 3 mL beer sample was saturated with 2.1 g of ammonium sulfate in a 10 mL amber vial and 30 µL of an internal standard of 2,3-hexanedione (0.5g/L) was added The vial was held at 35ºC for 40 min for sample/headspace equilibration. The volatile compounds in the headspace were absorbed by a DVB/CAR/PDMS 50/30 µm fiber and were injected into the gas chromatograph using a DB-WAX columnand an electron capture detector. Temperature programming was: 60ºC held for 1 min, then increased to 150ºC at 10ºC/min and held for 2 min, then increased to 250ºC at 10°C/min and held for 5 min. The injector and detector temperatures were 160ºC and 180ºC, respectively. Helium was used as a carrier gas at 3 mL/min and a split ratio of 20:1. The diacetyl concentration was calculated from an equation constructed from the linear regression of the ratio of the internal to external standards.

Yeast identification

Yeasts were identified using analysis of the D1/D2 region of the large subunit of the rRNA (LSU rRNA) gene sequence (Kurtzman and Robnett, 1998). DNA extraction was performed using a TIANamp Yeast DNA kit (Tiangen ®; China), and the diluted DNA samples for polymerase chain reaction (PCR) were prepared by adding 4 µL of genomic stocks to 1 mL of 0.1X TE buffer.

The D1/D2 domain of the LSU rRNA gene was amplified from yeast genomic DNA. The D1/D2 region of the LSU rRNA gene was amplified and sequenced with primers, NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (5'-GGTCCGTGTTTCAAGACGG) (Kurtzman and Robnett, 1998). Amplification was performed for 36 PCR cycles with annealing at 52ºC for 1 min, extension at 72 for 2 min and denaturation at 94ºC for 1 min. The PCR products were checked using 1.5% (w/v) agarose in 1X TAE buffer (0.04 M tris-acetate, 0.001 M EDTA [pH 8.0]) and stained with ethidium bromide. The purified products were submitted to Sangon Biotech Co. Ltd in China for sequencing. The sequences were compared pairwise using a BLAST search (Altschul et al., 1997).

Yeast characterization

Optimal fermentation temperature for yeast growth

The optimal fermentation temperatures for yeast isolates were evaluated using 10ºC, 15ºC and 20ºC for 48 hr. The fermentations were conducted by inoculation of 1×10^6 cells/mL/ºBrix into 1,600 mL of 70% (w/w) rice wort concentration at 12ºBrix. Five milliliters of the sample were collected for counting cell numbers under a microscope every 2 hr for 24 hr and every 4 hr after 24 hr, up to 48 hr.

Yeast flocculation

The yeast strain to be tested was cultured by inoculating one loop of yeast pellet in 100 mL of rice wort in a 250 mL Erlenmeyer flask. The cells were incubated at 20ºC on an orbital shaker at 150 rpm for 48 hr.

Flocculation ability testing was conducted as in the sedimentation test described by Dietvorst and Brandt (2008). Cell suspensions were inoculated in 12ºBrix rice wort (treatment) and rice wort with 100 mM EDTA added (control), and the turbidity adjusted to 1.5 at a wavelength of 600 nm (A600). One milliliter of sample was pipetted into semi-micro 10 mm path length cuvettes, size 1.5 mL volume and the optical density was read immediately and at 5 min intervals for 15 min, using a Shimadzu spectrophotometer UV-1800 (U.S.A.). The percentage of flocculated cells was calculated using Equation 1, and the strains were classified according to the flocculation percentage as non-flocculent (< 20%), high flocculent (>85%) or moderate flocculent (20-85%) according to American Society of Brewing Chemists (1996).

$$\text{Flocculation (\%)} = \frac{100 \times [\text{A600 (Control)} - \text{A600 (Sample)}}{\text{A600 (Control)}} \quad (1)$$

Effect of nitrogen content on diacetyl production in normal (12ºBrix) and high-gravity (18ºBrix) wort fermentation

In order to test the effect of nitrogen concentration on diacetyl production in normal (12°Brix) and high-gravity (18°Brix) worts, the rice percentage was varied (30%, 50% and 70% w/w), and the remaining portions were composed of barley malt and 10% defatted soybean meal (w/w). The worts were mashed in the same manner as indicated for the rice wort preparation and were fermented by the selected yeast isolate at a concentration of 1×10^6 cell/mL/ºBrix, at 20ºC for 5 d (normal gravity) and 7 d (high gravity).

Statistical analysis

The statistical analysis was carried out using the SPSS version 14 software (SPSS Inc.; USA). All chemical experiments were analyzed in triplicate. The analysis of variance and mean comparison by Duncan's multiple range test were used to determine differences between means at $p < 0.05$.

Results and Discussion

New local yeast isolates

The 55 samples from fruits, flours, foods, soils, water, local rice wine ('sato') and local starter culture ('look-pang') were collected randomly from the central, north, northeast and eastern areas of Thailand. Ninety-two yeast isolates were obtained, of which 73.7% were from fruits (dragon fruit, kiwi, banana, rambutan, guava, star fruit and mango), 5.3% from local rice wine, 5.3% from local rice wine starter culture and 15.7% from foods (steamed potatoes and potato soups). The top three isolates were DG201 (from dragon fruit), BNS04 (from banana) and LPST01 (from local rice wine) and they produced the highest mean (± SE) ethanol content (1.65 ± 0.53%, 2.08 ± 0.05% and 2.96 ± 0.03% (v/v), respectively). These isolates were tested for diacetyl production in all malt worts and were compared with a commercial strain (Saflager S-23) obtained from Fermentis, France. The diacetyl concentration was significantly increased with the intermediate fermentation time and decreased at the longer fermentation time (Fig. 1A and 1B). During the log phase, yeast assimilated free amino nitrogen (FAN) quickly and was able to synthesize deficient amino acids including valine, which shares an intermediate molecule (α-acetolactate) with diacetyl synthesis. The α-acetolactate is secreted into the fermenting wort and then transformed to diacetyl in the fermentation media (Okagbue, 1988). As the diacetyl presents a strong buttery off-flavor in lager beer, industrial strains suited for lager beer fermentation are selected for low diacetyl production or fast diacetyl reduction. Generally, laboratory strains or local strains cannot compete with industrial strains in terms of low fermentation capacity or levels of undesirable compounds (Stewart and Russell, 1981). However, some investigators have attempted to find new characteristic yeast strains that would be competitive with industrial strains, with the goal being to diversify yeast characters and thus widen the area of application (Van et al., 2000; Romano et al., 2003). In a study performed by Gibson et al., (2015), 14 different lager-brewing strains were tested with regard to their strength in producing diacetyl. One of the 14 strains produced a low amount of diacetyl, but did not otherwise differ from the others in fermentation performance, growth or flavor production.

The regulation of diacetyl by yeast cells acts via the gene encoding acetohydroxyacid synthase (AHAS) which catalyzes the formation of α-acetolactate from pyruvate. The enzyme is a specific character of each yeast strain, especially regarding the expression of the AHAS-coding genes (ILV2 and ILV6) responsible for diacetyl production (Lui et al., 2004). Diacetyl reduction depends on the enzyme ketone reductase, which activates a dehydrogenase enzyme active on both diacetyl and acetoin (Bamforth and Kanauchi, 2004). Yeasts take up diacetyl into the cells and convert it to 2,3-butanediol under low pH; this diol has a flavor threshold around 4,500 ppm and does not affect the aroma of the final beer (Hughes et al., 2011).

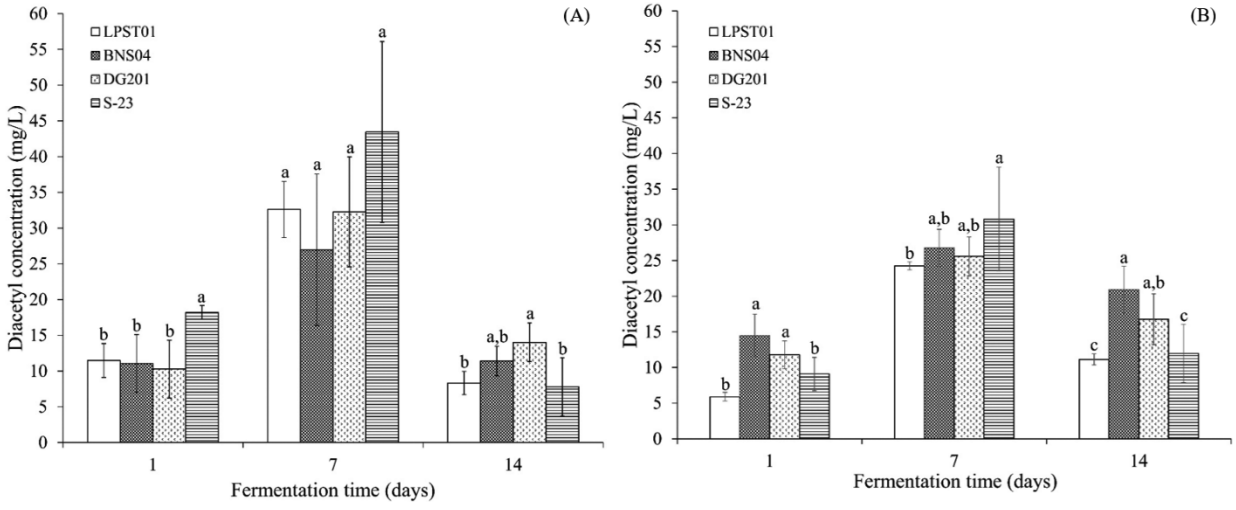

Fig. 1 Diacetyl content in fermentation flasks sampled from normal-gravity fermentation of all-malt wort (A) and 70% rice grit wort (B), with fermentation conducted at 10ºC for 14 d

Yeast identification and characterization

The selected yeast strains (LPST01, DG201 and BNS04) were identified using sequence analysis of the large subunit (26S) ribosomal DNA. Strain LPST01 showed high identity with that of *Saccharomyces cerevisiae*. Strains BNS04 and DG201 were identified as *Candida tropicalis* with each displaying 100% similarity. LPST01 as a strain of *Saccharomyces cerevisiae* has the ability to produce ethanol equivalent to that of the commercial yeast S-23.

LPST01 was analyzed for optimal fermentation temperature (10ºC, 15ºC and 20ºC) for 48 hr (Fig. 2). The mean (± SE) specific growth rate was highest at 20ºC (0.0626±0.00 per hour) and the maximum cell concentration was achieved within 16 hr. Optimizing the temperature is important for microbial growth (Siddiqui and Cavicchioli, 2006; Russell, 1990). In the present context, the optimal growth condition helps to guarantee a higher biomass for more vigorous fermentation and a proper flavor profile. The current results agree with those of Walker and Stewart (2016), who indicated that most strains of *S. cerevisiae* grow best in the temperature range 20–30°C and that the maximum biomass could be produced at 20°C.

The flocculation of cells during primary or secondary fermentation affects the fermentation speed, beer flavor, maturation rate and filtration rate (Jin and Speers, 1998; Stewart and Russell, 1981). In the current study, the percentage of flocculated cells of LPST01 was 1.721, indicating that it was a non-flocculent yeast (Fig. 3). For brewer's yeast, the flocculation characteristics of individual strains are an important indicator of the suitability of the strain for the relevant beer type, where a highly flocculent yeast at the end of fermentation will be easy to separate, which is convenient for harvesting the aggregated yeast cells in the fermentation tank. In contrast, cells that were too early flocculated could cause incomplete fermentation, whereas the non-flocculent yeasts and the delayed flocculated cells may cause difficulties during filtration, but they may be suitable for some turbid-style beers such as wheat beer (White and Zainasheff, 2010).

Effect of free amino nitrogen on fermentation performance of normal and high gravity worts using yeast strain LPST01

Varying initial concentrations of FAN and valine were prepared to study the effects on diacetyl formation in green beer fermented from normal-gravity and high-gravity-worts (Table 1). A minimum FAN concentration of 150 ppm has been recommended for optimal fermentation performance, while insufficient FAN may result in a slow rate of fermentation (Pierce, 1987). In this study, 70% rice grit provided sufficient FAN at 176.96 ± 1.63 mg/L, yielding 5.5% (v/v) ethanol in the green beer. This was not significantly different from the values for the medium and high FAN worts. In addition, the valine concentrations in the worts showed a tendency similar to FAN, but the concentration decreased with the increasing rice grit portion. All the valine present in the three normal-gravity worts was completely utilized, resulting in final concentrations of diacetyl in the green beer in the range 2.8–5.1 mg/L. These results indicated a greater initial valine concentration caused less formation of diacetyl, which agreed with those of Nakatani et al. (1984) and Cyr et al. (2007), in which an increased valine concentration in the wort significantly reduced the amount of diacetyl produced during fermentation. It has been reported that valine strongly inhibits the AHAS enzyme, resulting in less pyruvate being converted into α-acetolactate (Barton and Slaughter, 1992). Therefore, the more valine present, the less diacetyl formation. Krogerus and Gibson (2013b) investigated the amount of valine supplementation in a semi-synthetic wort and found that a concentration as high as 300 mg/L could reduce diacetyl in the final beer by 33% relative to the control using *S. pastorianus*. The current study found a 45% reduction in beer fermented from 30% (w/w) rice wort relative to 70% (w/w) rice wort. Furthermore, the current experiments with the high-gravity wort fermentation showed that LPST01 was an efficient yeast for the fermentation of a viscous wort (Table 2). The residual amount of valine (12 mg/L) in the final beer made from high-gravity wort prepared with 30% (w/w) rice grit indicated that approximately 126 mg/L of valine was utilized,

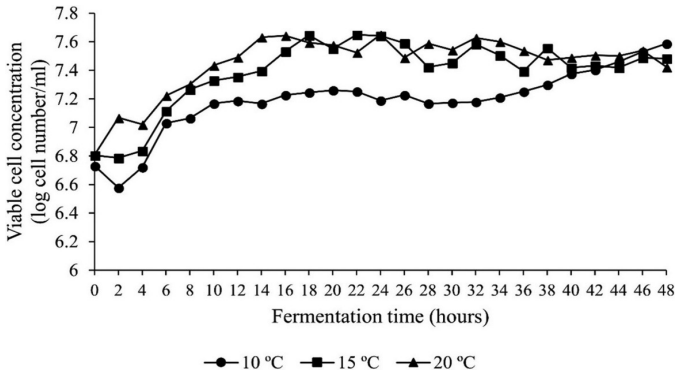

Fig. 2 Viable cells of strain LPST01 fermented with normal-gravity rice wort at 10ºC, 15ºC and 20ºC

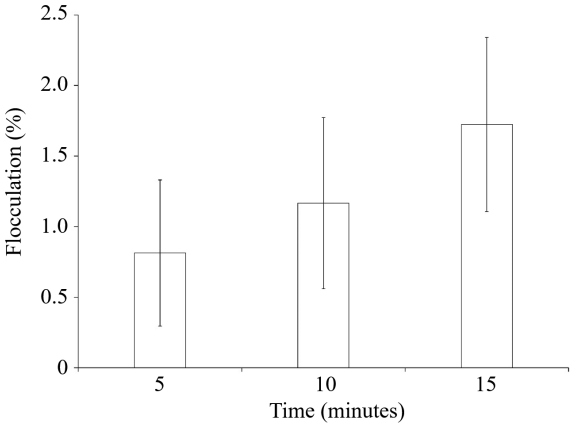

Fig. 3 Flocculation characterization of strain LPST01 cultured in the normal gravity rice wort, where error bars indicate ± SE

Table 1 Effect of free amino nitrogen (FAN) on diacetyl production by LPST01 fermented with normal-gravity rice wort (12 °Brix)

	Normal-gravity worts		
	30% Rice grits	50% Rice grits	70% Rice grits
Initial FAN concentration (mg/L)	226.13 ± 3.00[a]	193.93 ± 1.02[b]	176.96 ± 1.63[c]
Final FAN concentration (mg/L)	68.78 ± 3.91[a]	44.63 ± 1.92[b]	43.85 ± 4.43[b]
Initial valine (mg/L)	97.72 ± 1.2[a]	66.14 ± 0.4[b]	51.21 ± 0.1[b]
Final valine (mg/L)	nd	nd	nd
Ethanol (%)	5.7 ± 0.1[a]	5.6 ± 0.1[a]	5.5 ± 0.1[a]
Diacetyl concentration (mg/L)	2.8 ± 0.22[c]	4.3 ± 0.30[b]	5.1 ± 0.20[a]
Diacetyl/ethanol 5%(v/v)	2.49 ± 0.21[c]	3.84 ± 0.17[b]	4.64 ± 0.15[a]

nd = not detected.

Different superscript letters in the same column indicate significant difference ($p < 0.05$)

Values are shown as mean ± SE.

Table 2 Effect of free amino nitrogen (FAN) on diacetyl production by LPST01 fermented with high-gravity rice wort (18°Brix)

	High gravity worts		
	30% Rice grits	50% Rice grits	70% Rice grits
Initial FAN concentration (mg/L)	454.27 ± 4.76[a]	387.69 ± 0.36[b]	351.99 ± 1.42[c]
Final FAN concentration (mg/L)	127.09 ± 2.09[a]	116.87 ± 1.31[b]	116.85 ± 8.42[b]
Initial valine (mg/L)	137.98 ± 2.5[a]	107.94 ± 9.4[b]	84.12 ± 12.3[b]
Final valine (mg/L)	12.07	nd	nd
Ethanol (%)	8.3 ± 0.1[a]	8.1 ± 0.1[a]	7.9 ± 0.1[a]
Diacetyl concentration (mg/L	3.7 ± 0.61[b]	6.2 ± 0.65[a]	7.3 ± 0.70[a]
Diacetyl/ethanol 5.0%(v/v)	2.19 ± 0.37[b]	3.81 ± 0.46[b]	4.62 ± 0.44[a]

* nd = not detected.

Different superscript letters in the same column indicate significant difference ($p < 0.05$)

Values are shown as mean ± SE.

and this resulted in a 49% reduction of diacetyl relative to beer made from 70% (w/w) rice wort. However, there was no clear indication of the exact amount of valine needed to completely suppress diacetyl formation. In addition, the valine uptake rate is related to FAN content and amino acid profiles, which play important roles in the production of diacetyl and other flavor molecules (Krogerus and Gibson, 2013b; Pugh et al., 1997). Furthermore, the assimilation rate of diacetyl into the cells has been shown to be affected by membrane integrity, physiological conditions, fermentation parameters (oxygenation and temperature) and yeast flocculation. *S. cerevisiae* and *S. pastorianus* have different forms of the ketone reductase enzyme, and the lager yeasts have a greater number of ketone reductase enzymes compared with ale yeasts (Murphy et al., 1996). However, the higher fermentation temperature of ale beers enhances complex aromatic flavors; this can mask the presence of diacetyl, thereby raising the flavor threshold in ale beers to as high as 0.4 mg/L (Boulton and Box, 2003). In addition, the current study indicated that the diacetyl concentration of the standardized 5% (v/v) ethanol beers and the diacetyl yields produced from high-gravity worts were slightly lower than those found in beers made from normal-gravity worts (Table 2). These results indicated that osmotic stress might not influence the diacetyl content. LPST01 is a non-flocculent yeast, so there were no adjacent cells to retard the transportation of oxygen or amino acids through the cell walls.

S. cerevisiae LPST01 is a promising strain that was selected from 92 yeast isolates. LPST01 was able to conduct fermentation of high-adjunct beer under high-gravity wort conditions. The non-flocculent yeast displayed optimal growth at 20ºC, a temperature that was suitable for ale beer fermentation. The initial FAN and valine concentrations influenced the diacetyl content in the normal- and high-gravity beer fermentations. In the normal-gravity fermentation with 30% (w/w) rice grits, the yeast utilized more FAN and valine than in the 50% (w/w) and 70% (w/w) rice grit worts, and the same result was found under high-gravity fermentation. Moreover, the relative amount of diacetyl content in the final green beer (5% (v/v) ethanol) was inversely related to amino acid consumption, as the greater the FAN or valine consumption, the less the diacetyl formation.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgement

This research was supported by the Kasetsart University Research and Development Institute (KURDI), Bangkok, Thailand under grant no. 55.59 and by a Graduate Program Scholarship from the Graduate School, Kasetsart University. Special thanks are recorded to Dr Dale E. Taneyhill for editing the English in a draft version of this paper.

References

Altschul, S.F., Madden, T.L., Schaffer, J.Z., Zhang, J., Zhang, Z., Miller, W., Lipman, J.D. 1997. Gapped BLAST and PSIBLAST: A new generation of protein database search programs. Nucleic Acids Res. 25: 3389–3402.

American Society of Brewing Chemists. 1996. Yeast flocculation by absorbance method. J. Am. Soc. Brew. Chem. 54: 245–248.

Amorim, C.J., Piccoli, H.R., Duarte, F.W. 2018. Probiotic potential of yeast isolated from pineapple and their use in the elaboration of potentially functional fermented beverages. Food Res. Int. 107: 518–527.

Aruf, G.A., Asyikeen, N.Z., Sahilah, M.A., Khan, M.A. 2011. Leavening ability of yeast isolated from different local fruits in bakery product. Sains Malays. 40: 1413–1419.

Bamforth, C., Kanauchi, M. 2004. Enzymology of vicinal diketone reduction in brewer's yeast. J. Inst. Brew. 110: 83–93.

Barton, S., Slaughter, J. 1992. Amino acids and vicinal diketone concentrations during fermentation. Tech. Q. Master Brew. Assoc. Am. 29: 60–63.

Boulton, C., Quain, D. 2006. Brewing Yeast and Fermentation. Blackwell Science. Berlin. Germany.

Boulton, C., Box, W. 2003. Fermentation and disappearance of diacetyl during lager fermentation, in Brewing Yeast Fermentation Performance (Smart, K. Ed.), Blackwell Science. Berlin. Germany.

Cadavid, E.O., Lopez, C.C., Tofalo, R., Paparella, A., Suzzi, G. 2008. Detection and identification of wild yeasts in Champu s, a fermented Colombian maize beverage. Int. J. Food Microbiol. 25: 771–777.

Cyr, N., Blanchette, M., Price, S., Sheppard, J. 2007. Vicinal diketone production and amino acid uptake by two active dry lager yeasts during beer fermentation. J. Am. Soc. Brew. Chem. 65: 138–144.

Dietvorst, J., Brandt, A. 2008. Flocculation in *Saccharomyces cerevisiae* is repressed by the COMPASS methylation complex during high-gravity fermentation. Yeast. 12: 891–901.

Duong, C., Strack, L., Futschilk, M. 2011. Identification of Sc-type ILV6 as a target to reduce diacetyl formation in lager brewer's yeast. Metab. Eng. 13: 638–647.

European Brewery Convention. 1998a. *Analytica-EBC.* 5th ed. Section 9 Beer Method 9.2.4, Ethanol in beer: Instrumental Method, Fachverlag Hans Carl. Nürnberg, Germany.

European Brewery Convention. 1998b. Analytica-EBC. 5th ed. Section 9 Beer Method 9.24.2, Vinical Diketone in Beer: Instrumental Method, Fachverlag Hans Carl. Nürnberg, Germany.

Field, D., Eggert, L., Metzgar, D., Rose, R., Wills, C. 2015. Use of polymorphic short and clustered coding-region microsatellites to distinguish strains of *Candida albicans*. FEMS Immunol. Med. Microbiol. 15: 73–79.

Gibson, B., Krogerus, K., Ekberg, J., Monroux, A., Mattinen, L., Rautio, J., Vidgren, V. 2015 .Variation in α-acetolactate production within the hybrid lager yeast group *Saccharomyces pastorianus* and affirmation of the central role of the ILV6 gene. Yeast. 32: 301–316.

Hughes, P., Baxter, D., Hornsey, I. 2011. Beer: Quality, Safety and Nutritional Aspects, Royal Society of Chemistry. Cambridge, UK.

Jin, Y.L., Speers, R.A. 1998. Flocculation of *Saccharomyces cerevisiae*. Food Res. Int. 31: 421–440.

Krogerus, K., Gibson, B.R. 2013a. 125th anniversary review: Diacetyl and its control during brewery fermentation. J. Inst. Brew. 119: 86–97.

Krogerus, K., Gibson, B.R. 2013b. Influence of valine and other amino acids on total diacetyl and 2,3-pentanedione levels during fermentation of brewer's wort. Appl. Microbial. Biotechnol. 97: 6919–6930.

Kurtzman, P.C., Robnett, J.C. 1998. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Aaton. Leeuw. Int. J. G. 73: 331–371.

Lui, Z., Zhang, G., Liu, S. 2004. Constructing an amylolytic brewing yeast *Saccharomyces pastorianus* suitable of accelerated brewing. J. Biosci. Bioeng. 98: 414–419.

Murphy, C., Large, P., Wadforth, C., Dack, S., Boulton, C. 1996. Strain-dependent variation in the NADH-dependent diacetyl reductase activities of lager- and ale brewing yeast. Biotechnol. Appl. Biochem. 23: 19–22.

Nakatani, K., Takahashi, T., Nagami, K., Kumada, J. 1984. Kinetic study of vicinal diketones in brewing, II: theoretical aspect for the formation of total vicinal diketones. Tech. Q. Master Brew. Assoc. Am. 21: 175–183.

Okagbue, R.N. 1988. A note on the leavening activity of yeasts isolated from Nigerian palm wine. J. Appl. Bacteriol. 64: 235–240.

Pierce, J. 1987. The role of nitrogen in brewing. J. Inst. Brew. 93: 378–381.

Pugh, T., Maurer, J., Pringle, A. 1997. The impact of wort nitrogen limitation on yeast fermentation performance and diacetyl. Tech. Q. Master Brew. Assoc. Am. 34: 185–189.

Romano, P., Fiore, C., Paraggio, M., Caruso, M., Capece, A. 2003. Function of yeast species and strains in wine flavor. Int. J. Food Microbiol. 86: 169–180.

Russell, J. 1990. Cold adaptation of microorganisms. Phil. Trans. R. Soc. Lond. B. 329: 595–611.

Ryan, E., Kohlhaw, G. 1974. Subcellular localization of isoleucine-valine biosynthetic enzymes in yeast. J. Bacteriol. 120 : 631– 637.

Saison, D., Suchutter, D.D., Uyttenhove, B., Delvaux, F., Delvaux, R.F. 2009. Contribution of staling compounds to the aged flavour of lager beer by studying their flavor thresholds. J. Agric. Food Chem. 114: 1206–1215.

Siddiqui, S., Cavicchioli, R. 2006. Cold-adapted enzyme. Ann. Rev. Biochem. 75: 403–433.

Stewart, G.G., Russell, I. 1981. Yeast flocculation. Academic Press. New York, NY, USA.

Suomalainen, H. and Ronkainen, P. 1968. Mechanism of diacetyl formation in yeast fermentation. Nature. 220: 792–793.

Tikka, C., Osuru, P.H., Atluri, N., et al. 2013. Isolation and characterization of ethanol-tolerant yeast strains. Open Bioinf. J. 8: 421–425.

Van, D., Bauer, J., Brambilla, L., et al. 2000. An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. Enzyme Microb. Techno. 26*:* 706–714.

Walker, M.G., Stewart, G.G. 2016 *Saccharomyces cerevisiae* in the production of fermented beverages. Beverages. 30: 2–16.

White, C., Zainasheff, J. 2010. Yeast: The practical guide to beer fermentation. Brewers Publications, Colorado, U.S.A.