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Expression of a mutated *SPT15* gene in *Saccharomyces cerevisiae* enhances both cell growth and ethanol production in microaerobic batch, fed-batch, and simultaneous saccharification and fermentations

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Abstract The *SPT15* gene encodes a *Saccharomyces cerevisiae* TATA-binding protein, which is able to globally control the transcription levels of various metabolic and regulatory genes. In this study, a *SPT15* gene mutant (S42N, S78R, S163P, and I212N) was expressed in *S. cerevisiae* BY4741 (BSPT15-M3), of which effects on fermentative yeast properties were evaluated in a series of culture types. By applying different nitrogen sources and air supply conditions in batch culture, organic nitrogen sources and microaerobic condition were decided to be more favorable for both cell growth and ethanol production of the BSPT15-M3 strain than the control *S. cerevisiae* BY4741 strain expressing the *SPT15* gene (BSPT15wt). Microaerobic fed-batch cultures of BSPT15-M3 with glucose shock in the presence of high ethanol content resulted in a 9.5–13.4% higher glucose consumption rate and ethanol productivity than those for the BSPT15wt strain. In addition, BSPT15-M3 showed 4.5 and 3.9% increases in ethanol productivity from cassava hydrolysates and corn starch in simultaneous saccharification and fermentation processes, respectively. It was concluded that overexpression of the mutated *SPT15* gene would be a potent strategy to develop robust *S. cerevisiae* strains with enhanced cell growth and ethanol production abilities.

Keywords *SPT15* · *Saccharomyces cerevisiae* · Osmotic tolerance · Microaerobic fermentation · Simultaneous saccharification and fermentation

Yeong-Je Seong and Haeseong Park equally contributed to this study.

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Introduction

Saccharomyces cerevisiae is an alcohol yeast able to produce ethanol with high yield and productivity and has been used as a starter microbe in brewery and bakery manufacturing. Because of the long history of its safe usage in human life, *S. cerevisiae* was chosen as a host microorganism for production of biochemicals and biopharmaceuticals as well as ethanol fuel from renewable biomass. In order to meet such a wide range of its application, various advanced technologies have been used in order to engineer robust *S. cerevisiae* strains such as random knockout and overexpression, artificial transcription factor (e.g., zinc finger protein), ribosomal engineering, genome shuffling, adaptive laboratory evolution, genome editing by a CRISPR/Cas9 system, and global transcription machinery engineering, of which information was well summarized in some review papers (DiCarlo et al. 2013; Portnoy et al. 2011; Santos and Stephanopoulos 2008). As examples of resistance against osmotic stress, a MAP kinase of Hog1 is positively required for adaptation and survival of yeast at hyperosmotic condition, which was partially controlled by a global protein sumoylation level (Irqeba et al. 2014). A wine

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S. cerevisiae strain was engineered by adaptive evolution to be able to grow against high KCl concentration, of which mutants showed a metabolic shift from the ethanol pathway to the glycerol, succinate, and 2,3-butanediol pathways that were engaged in osmotic and salt stresses (Tilloy et al. 2014). Deletion of a RNA-binding protein of Pub1p increased enzymatic activities of glycerol-3-phosphate dehydrogenase and nicotinamidase, and enhanced stress tolerance of *S. cerevisiae* (Orozco et al. 2016). A new zinc cluster regulator of Znf1 directly controlled the genes belonging to gluconeogenesis, the glyoxylate shunt, and the tricarboxylic acid cycle and is related to tolerance to pH and osmotic stress (Tangsombatvichit et al. 2015).

Meanwhile, the global transcription machinery engineering (gTME) was designed by modulation of a TATA-binding protein encoded by *SPT15* in *S. cerevisiae* (Alper et al. 2006). The SPT15p protein is involved in the action of RNA polymerase (Eisenmann et al. 1989; Sanders et al. 2002) and known to bind DNA in order to control the specificity of most promoters in *S. cerevisiae* (Schultz et al. 1992). Some mutations of the regulatory domain of SPT15p resulted in pleiotropic changes in gene transcription levels (Cang et al. 1999; Eisenmann et al. 1989). In order to construct robust *S. cerevisiae* strains with improved physiological properties, a series of mutation of *SPT15* has been designed. For example, overexpression of a *SPT15* mutant (mutated *SPT15-300*) allowed engineered *S. cerevisiae* to grow at up to 100 g/L glucose while its control strain was unable to do (Alper et al. 2006). Mutation of *SPT15* in *S. cerevisiae* led to increased performance of xylose utilization and tolerance (Liu et al. 2010). Combinatorial mutation of SPT15p and TAF25p (a TATA-binding protein-associated factor) conferred higher tolerance against oxidative stress to *S. cerevisiae* (Zhao et al. 2014). Coexpression of the *SPT3* and *SPT15* genes in recombinant *S. cerevisiae* assimilating glycerol enhanced the intracellular contents of ergosterol and trehalose, leading to increases in stress tolerance and glycerol conversion to ethanol (Yu et al. 2012). Additionally, overexpression of a mutated *SPT15* (A101T) in recombinant *S. cerevisiae* increased the flux in the isoprenoid pathway and maximized the yield of a sesquterpene of α-farnesene (Wadhwa and Bachhawat 2016).

In our previous research, a library of *S. cerevisiae SPT15* variants was constructed by random mutagenesis and overexpression of the *SPT15-M2* and *SPT15-M3* mutant genes increased growth tolerance against 17.5% ethanol concentration (Yang et al. 2011). Recombinant *S. cerevisiae* L3262 expressing each *SPT15* mutant also showed higher performance of ethanol production in a simple aerobic cultivation. To further evaluate this positive effect of the *SPT15* mutation, in this study, the *SPT15-M3* mutant isolated previously was overexpressed in *S. cerevisiae* BY4741 (BSPT15-M3), of which effects on yeast cell properties were investigated in a series of fermentation processes. Batch fermentation was carried out in defined and complex media at oxygen-limited and microaerobic conditions. Effects of initial glucose concentration and additional glucose shock on the presence of high ethanol content were evaluated in microaerobic fed-batch cultures. Finally, simultaneous saccharification and fermentation (SSF) processes using cassava and corn starch were applied to confirm the enhanced fermentative ability by the *SPT15-M3* mutant overexpression in *S. cerevisiae*.

Materials and methods

Strains and plasmids

S. cerevisiae BY4741 (*MAT*a *his*3Δ1 *leu*2Δ *met*15Δ *ura*3Δ) and D452-2 (*MAT*a, *leu*2, *his*3, *ura*3, and *can*1) were used as yeast host strains. Plasmids pSPT15wt and pSPT15-M3 were derived from plasmid pRS316 with the *TDH3* promoter, *GAL7* terminator, and *URA3* genes, which contained the wild type and a mutant of the *SPT15* gene (*SPT15-M3*), respectively (Yang et al. 2011). The *SPT15-M3* gene encodes a mutated TATA-binding protein of SPT15p (S42N, S78R, S163P, and I212N). The plasmids were transformed into *S. cerevisiae* according to the Li-acetate method (Jo et al. 2016). Recombinant *S. cerevisiae* BY4741 strains transformed with plasmids pSPT15-M3 and pSPT15wt were named BSPT15-M3 and BSPT15wt, respectively. All strains and plasmids used in this study are listed in Table 1.

Batch and fed-batch cultures

Yeast cells were cultivated in a complex YP (1% yeast extract and 2% peptone), defined YNB-URA (0.67% yeast nitrogen without amino acid and 0.19% yeast dropout medium supplement without uracil) and 2× YNB-URA (two times higher concentration of YNB-URA) media with 185–480 g/L glucose (Samyang Genex Co., Incheon, South Korea). In batch cultures using a rotary incubator (Hankook Instrument Co., South Korea), 100 mL of the culture medium in a 500-mL baffled flask (Thermo Fisher Scientific, Waltham, MA, USA) was incubated at 30 °C, 80 rpm, and an initial pH of 5.5. Batch cultures were carried out in a 2.5-L scale bioreactor (KBT pw-250; KoBioTech Co., South Korea) containing the YP medium with 280–310 g/L glucose. Medium acidity was controlled at pH 5.5 by the addition of 2 N NaOH and 2 M HCl. Temperature was maintained at 30 °C during the cultivation. The agitation speed was set at 200 rpm, and air supply into the bioreactor varied as follows: no air supply (0 vvm), intermittent supplement at 0.1 vvm for 5 min in every 4 h, and continuous blowing at 0.1 vvm. For fed-batch culture, 1 L of the culture medium was composed of the YP medium and 200–295 g/L initial glucose. When glucose concentration in the medium reached a set point, 125 mL of 800 g/L glucose

Table 1 List of *S. cerevisiae* strains and plasmids used in this study

Name	Characteristics	Source
Strain		
BY4741	*MATa his3*Δ1 *leu2*Δ *met15*Δ *ura3*Δ	EUROSCARF[a]
D452-2	*MAT*a, *leu2*, *his3*, *ura3*, and *can1*	Jo et al. (2016)
BSPT15wt	BY4741 transformed with plasmid pSPT15wt	This study
BSPT15-M3	BY4741 transformed with plasmid pSPT15-M3	This study
Plasmid		
pRS316	*TDH3* promoter, *GAL7* terminator, *CEN6_ARS4*, *URA3*, amp^R	ATCC[b]
pSPT15wt	pRS316 + the *S. cerevisiae SPT15* gene	Yang et al. (2011)
pSPT15-M3	pRS316 + the *S. cerevisiae SPT15*-mutated gene (S42N, S78R, S163P, I212N)	Yang et al. (2011)

[a] European *Saccharomyces cerevisiae* Archive for Functional Analysis (http://www.euroscarf.de)

[b] American Tissue Collection Center (http://www.atcc.org)

solution was added one or two times. Culture conditions were set at the same as the batch culture. An agitation speed of 200 rpm and an aeration rate of 0.1 vvm were kept constant throughout the cultivation. All batch and fed-batch cultures were performed independently in duplicate or triplicate.

Simultaneous saccharification and fermentation

As carbon sources for SSF, commercially available corn starch powder (Samyang Genex Co., South Korea) and dried cassava given by the Changhae Ethanol Co. (Jeonju, South Korea) were used. To prepare cassava powder, the dried cassava was peeled and grinded with a mixer (HMF-995; Hanil Co., South Korea). After filtration using a sieve with 150 μm of pore size (Daihan Standard Test Sieve; Daihan Co., South Korea), the cassava powder was used for SSF. The corn starch or cassava powder (200 g) was suspended in double-distilled water, prior to liquefaction with 0.05% (*v*/*w*) of Liquozyme™ (Novozymes, Denmark) at 105 °C for 2 h. The culture medium of 1 L working volume was composed of 200 g/L of the liquefied starch and YP medium. For inoculation, a colony of each cell was added into the 10 mL YP medium with 20 g/L glucose (YPD20) and cultured for 20 h. After transferring the 10 mL culture broth to the 90 mL fresh YPD20 medium, the cells were cultured for 20 h and then centrifuged for collection of cell pellets. The cell pellets were suspended with a small amount of the YPD20 medium and used for inoculation into the main SSF medium. Culture conditions were maintained at pH 5.5, 200 rpm of agitation speed, and 0.1 vvm of aeration rate. To supplement more starch, 400 g/L soluble starch of corn or cassava was made by the same liquefaction process as described above and 125 mL of this starch solution was supplemented four times into the bioreactor. And 0.05% (*v*/*w*) of Dextrozyme™ based on the amount of supplemented starch was added four times. Liquozyme™ and Dextrozyme™ (Novozymes, Denmark) were gifts from the Samyang Genex Co. (South Korea).

Analysis

Cell growth was monitored by measuring an optical density at 600 nm of wavelength ($OD_{600\ nm}$) with a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The $OD_{600\ nm}$ was converted to dry cell mass by using a predetermined conversion factor, 0.8 g/L/$OD_{600\ nm}$. Concentrations of glucose, ethanol, glycerol, and acetate in culture broth were measured by a high-performance liquid chromatography (1200 Series; Agilent Technologies, Santa Clara, CA, USA) equipped with a Rezex ROA-organic acid H^+ column (300 × 780 mm; Phenomenex, Torrance, CA, USA) heated at 60 °C. A solvent of 5 mM H_2SO_4 was flowed at 0.6 mL/min. Detection was made by a RI detector (1200 Series; Agilent Technologies, USA). Dissolved oxygen (DO) content in culture broth was measured by an oxygen sensor (InPro6800; Mettler Toledo AG, Switzerland), where zero DO value was set with 5% Na_2SO_3 solution and 100% value was done by full aeration at a maximum impeller speed (Jo et al. 2016).

Results

Effects of medium types on cell growth and ethanol production at oxygen-limited condition

In the previous report, the iETS3 strain similar to *S. cerevisiae* BY4741/pSPT15-M3 (BSPT15-M3 strain) showed higher cell growth and ethanol production in a batch culture using a complex medium and at high aeration condition (Yang et al. 2011). To investigate the effect of medium types on ethanol production at oxygen limited condition, batch-type cultures of

recombinant *S. cerevisiae* BSPT15-M3 and BSPT15wt strains were carried out in a baffled flask containing two defined media (YNB and 2× YNB) and a complex medium (YPD) (Fig. 1). An agitation speed of 80 rpm was kept constant to maintain a low content of oxygen in the culture medium. In case of the YNB medium with 210 g/L glucose (YNB210), all parameters for cell growth, glucose consumption, and ethanol production were similar in each other. To increase the inorganic nitrogen source and glucose contents, a defined medium with two times higher concentrations of YNB components with 470 g/L glucose (2× YNB470) was used in the same batch culture. An increment of inorganic nitrogen content and glucose concentration enhanced both the glucose consumption rate and ethanol productivity of the BSPT15-M3 strain by 66 and 49%, respectively, relative to those of the BSPT15wt strain, whereas similar dry cell masses were obtained. Organic nitrogen sources of yeast extract and peptone were applied to assessment of the *SPT15-M3* gene expression in recombinant *S. cerevisiae*. As shown in batch cultures using the YPD medium with 190 or 320 g/L glucose (YPD190 or YPD320), the BSPT15-M3 strain showed about 35% higher dry cell mass and growth yield than the BSPT15wt control strain. However, kinetic parameters for glucose consumption and ethanol production in both YPD190 and YPD320 media were similar between the two strains or the BSPT15-M3 strain showed slightly higher values than the BSPT15wt strain. Similar trends of cell growth, glucose consumption, and ethanol production for the recombinant *S. cerevisiae* BY4741 strains were observed in the same batch cultures of *S. cerevisiae* D452-2 strains transformed with plasmids pSPT15-M3 and pSPT15wt (Fig. S1). As a result, the addition of organic nitrogen sources increased more the cell growth of the BSPT15-M3 strain at low air supply (80 rpm of agitation speed), not glucose consumption and ethanol production.

Effects of air supply at high glucose concentration

Reduction of air supply and high glucose concentration are favorable for ethanol production with a high yield. But, the expression of the mutated *SPT15* gene did not influence ethanol production at a low agitation speed as shown Fig. 1. To precisely investigate the effect of aeration on the glucose and ethanol metabolism at high glucose concentration, batch cultures in a bioreactor-containing YP medium with 280–310 g/L glucose were carried out at 200 rpm and three types of aeration (Fig. 2, Table 2). In all culture periods, dissolved oxygen content was kept at a basal level in the culture medium (data not shown), indicating that air supply in these conditions was a rate-limiting factor in carbon metabolism. As shown in Fig. 2a, b, both the recombinant *S. cerevisiae* strains showed similar profiles of cell growth and carbon metabolism in an oxygen-limited condition of no air supply. Meanwhile, changing of the oxygen-limited condition to microaerobic conditions enhanced both glucose consumption rate and metabolite production rates of the BSPT1-M3 and BSPT15wt strains. Interestingly, the BSPT15-M3 strain was more sensitive to

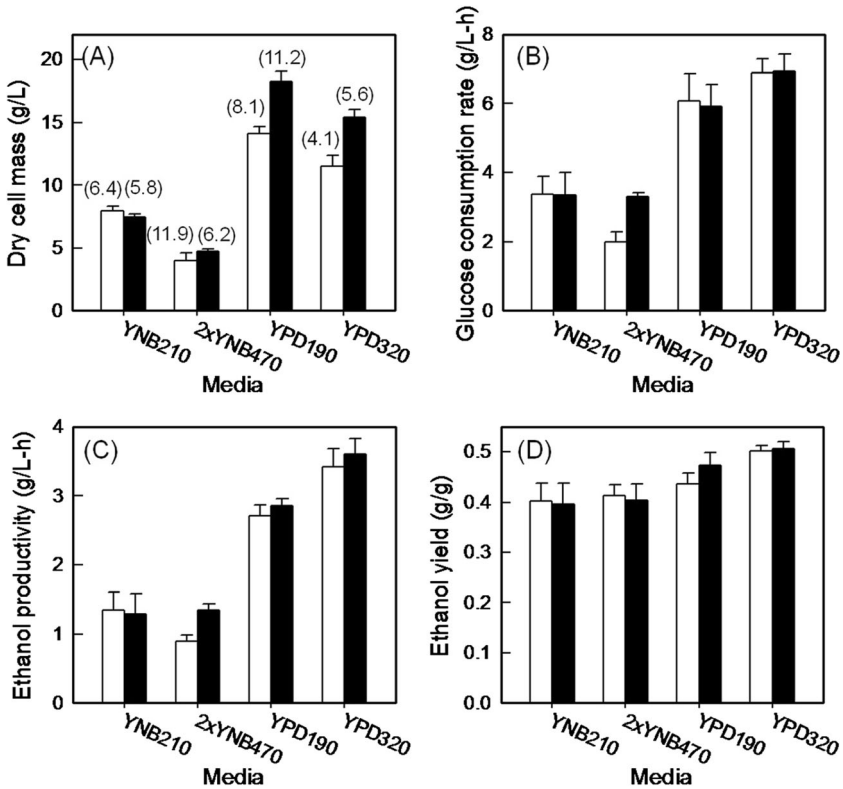

Fig. 1 Batch cultures of recombinant *S. cerevisiae* BSPT15wt (*white bar*) and BSPT15-M3 (*black bar*) strains in 100 mL of YNB210, 2× YNB470, YPD190, or YPD320 at 30 °C, 80 rpm, and an initial pH of 5.5. Each medium contained 210, 470, 190, and 320 g/L glucose in a row, and culture data were collected at 40, 48, 28, and 49 h after inoculation, respectively. For the 2× YNB470 medium, the data were calculated from 24 to 48 h of culture. The *number in parenthesis* indicates the growth yield based on glucose consumed. The batch culture was performed independently in triplicate

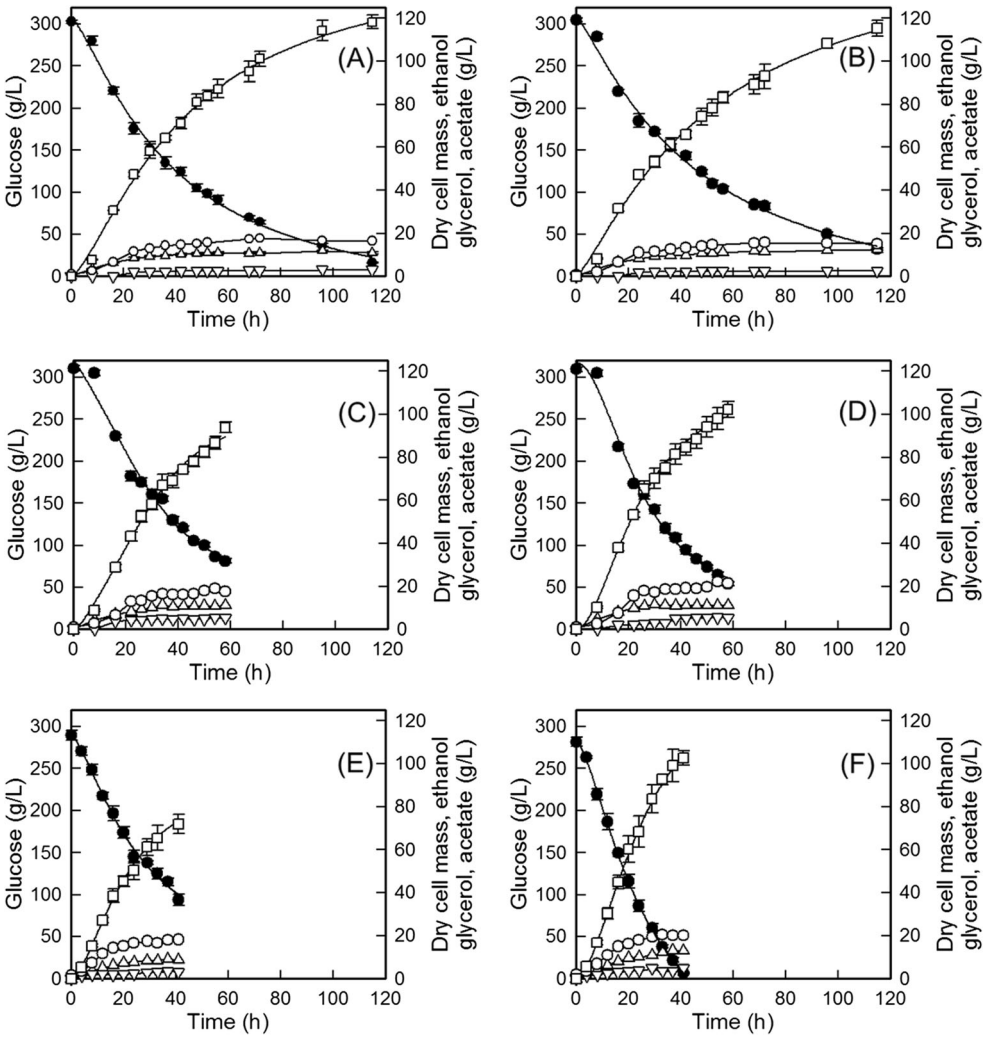

Fig. 2 Effects of air supply on cell growth and ethanol production in recombinant *S. cerevisiae* BSPT15wt (**a**, **c**, **e**) and BSPT15-M3 (**b, d**, **f**) strains. Batch cultures were performed in 1 L of the YP medium containing 280–310 g/L glucose at 30 °C, pH 5.5, and 200 rpm. Air was not supplied (**a**, **b**), supplemented intermittently at 0.1 vvm for 5 min in every 4 h (**c**, **d**), and blown continuously at 0.1 vvm (**e**, **f**) into the reactor. The *symbols* are denoted as follows: *open circle*, dry cell mass; *black circle*, glucose; *open square*, ethanol; *open upright triangle*, glycerol; *open inverted triangle*, acetate

aeration than the BSPT15wt strain. By increasing the aeration rate from 0 to 0.1 vvm, the BSPT15-M3 strain showed 2.83 and 2.50 times higher glucose consumption rate and ethanol production rate, respectively, whereas the BSPT15wt strain did show 1.92- and 1.71-fold increments only. Finally, the BSPT15-M3 strain consumed 275 g/L glucose in 41 h and produced 103 g/L ethanol with 6.71 g/L/h glucose consumption rate and 2.50 g/L/h ethanol production rate (Fig. 2f), which were about 1.4 times higher than those for the BSPT15wt strain at the same batch culture (Fig. 2e). More investigation of physiological properties of the BSPT15-M3 strain was carried out at a microaerobic condition of 0.1 vvm and 200 rpm.

Microaerobic fed-batch fermentations with glucose shock

To investigate the change of cell metabolisms by glucose shock in the presence of high ethanol content, fed-batch cultivations of the BSPT15-M3 and BSPT15wt strains were designed using 200 or 300 g/L initial glucose at the microaerobic condition. A concentrated glucose solution was added into the bioreactor at a set point of around 100 g/L glucose in culture broth, which value was chosen in the batch cultures (Fig. 2i, f) where the highest glucose consumption rate and ethanol production rate were shown. As illustrated in microaerobic fed-batch culture I (Fig. 3a, b), initial 300 g/L glucose was consumed, and cell and ethanol were produced as the same as their patterns in Fig. 2i, f. By supplementation of 100 g/L glucose at 24 h culture, the rates of glucose consumption (8.03 ± 0.98 g/L/h for the BSPT15-M3 strain and 6.86 ± 0.61 g/L/h for the BSPT15wt) and ethanol production (3.26 ± 0.33 and 2.78 ± 0.34 g/L/h) before the glucose addition were reduced by about two-fold. Even though the metabolic capacities of the two strains were reduced by the glucose shock, the BSPT15-M3 strain possessed almost 13.4 ± 6.8 and 9.5 ± 3.3% higher abilities of glucose consumption rate and ethanol productivity than the BSPT15wt strain irrespective of the glucose shock. Finally, microaerobic fed-batch culture I of the BSPT15-M3 strain using initial 300 g/L and additional 100 g/L glucose resulted in 9.5–14% higher performances of

Table 2 Summarized results of cell growth and ethanol production in microaerobic batch, fed-batch, and simultaneous saccharification and fermentations in recombinant *S. cerevisiae* BSPT15wt and BSPT15-M3 strains

S. cerevisiae strain	Culture type	Initial glucose concentration (g/L)	Final dry cell mass (g/L)	Total consumed glucose (g)	Ethanol concentration (g/L)	Ethanol productivity (g/L/h)	Ethanol yield (g/g)	Glycerol yield (g/g)
BSPT15wt	Batch[a] (no aeration)	303.0 (2.0)	16.5 (0.7)	287.0 (3.4)	118.2 (3.2)	1.03 (001)	0.41 (001)	0.038 (0.002)
	Batch[a]	290.0 (5.9)	18.1 (0.9)	196.5 (6.9)	71.9 (2.3)	1.75 (0.02)	0.37 (0.01)	0.044 (0.02)
	Fed-batch I[b]	289.0 (7.3)	15.3 (3.2)	283.7 (4.3)	99.6 (7.1)	1.91 (0.13)	0.39 (0.03)	0.14 (0.01)
	Fed-batch II[b]	198.9 (2.5)	13.5 (2.4)	341.7 (11.0)	109.6 (3.9)	2.11 (0.08)	0.40 (0.00)	0.12 (0.00)
	SSF[c]	–	–	–	104.2	1.07	0.39	0.06
BSPT15-M3	Batch[a] (no aeration)	305.0 (2.3)	15.1 (1.0)	272.9 (4.3)	115.2 (3.7)	1.00 (0.02)	0.42 (0.01)	0.045 (0.002)
	Batch[a]	281.8 (5.2)	20.0 (1.0)	275.2 (2.7)	102.5 (3.3)	2.50 (0.04)	0.37 (0.01)	0.047 (0.002)
	Fed-batch I[b]	294.3 (4.5)	17.5 (2.3)	321.5 (14.6)	109.1 (11.1)	2.10 (0.21)	0.38 (0.02)	0.12 (0.00)
	Fed-batch II[b]	197.9 (7.2)	19.5 (2.8)	374.0 (2.4)	123.2 (4.8)	2.37 (0.09)	0.41 (0.01)	0.09 (0.00)
	SSF[c]	–	–	–	108.3	1.12	0.41	0.07

All cultures were performed in a bioreactor containing the YP medium at 0.1 vvm and 200 rpm except for batch (no aeration)

The number in parenthesis indicates the standard deviation of the corresponding parameter

[a] Batch cultures were performed by using 280–310 g/L glucose

[b] These cultures were performed by using the initial 300 g/L and additional 100 g/L glucose for fed-batch I and the initial 200 g/L and additional 200 g/L glucose for fed-batch II

[d] In SSF, the liquefied corn starch solution of 400 g/L was used

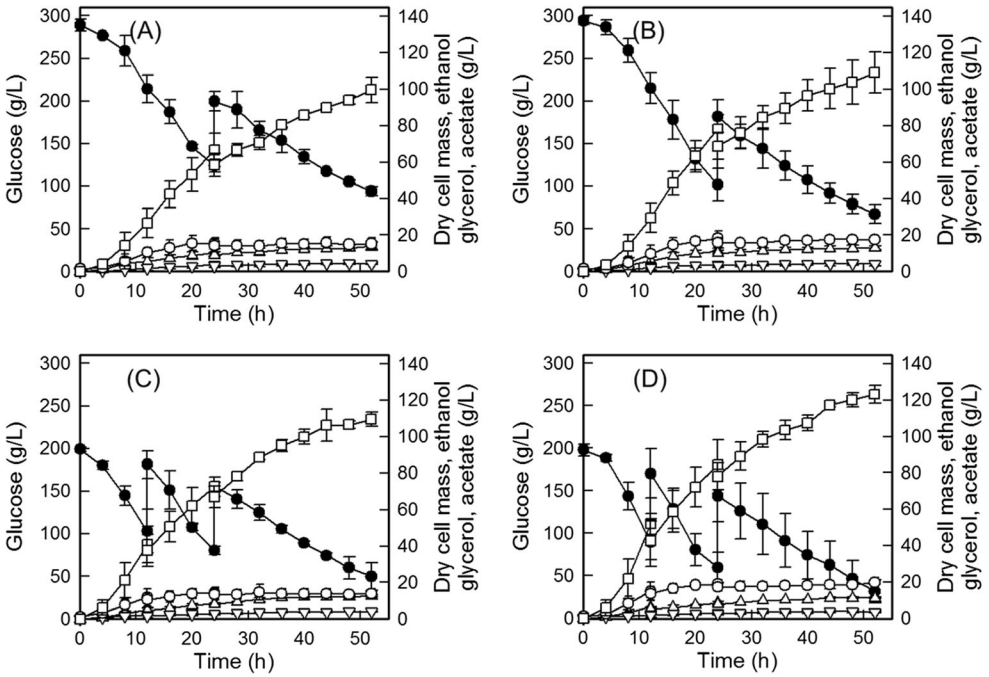

Fig. 3 Microaerobic fed-batch cultures of recombinant *S. cerevisiae* BSPT15wt (**a**, **c**) and BSPT15-M3 (**b**, **d**) strains in 1 L of the YP medium containing initial glucose concentrations of 300 g/L (fed-batch culture I, **a**, **b**) and 200 g/L (fed-batch culture II, **c**, **d**). After the batch-type culture (125 mL of 800 g/L glucose solution) was added one or two times into the bioreactor (*arrow*), culture conditions were set at 30 °C, pH 5.5, 0.1 vvm, and 200 rpm throughout the cultivation. The *symbols* are denoted as follows: *open circle*, dry cell mass; *black circle*, glucose; *open square*, ethanol; *open upright triangle*, glycerol; *open inverted triangle*, acetate

cell growth, glucose consumption, and ethanol production than the BSPT15wt strain (Table 2).

Another strategy of microaerobic fed-batch culture II was carried out by using 200 g/L initial glucose and adding 100 g glucose into 1 L culture broth two times in order to reduce the glucose shock at high ethanol levels (Fig. 3c, d). During the 24-h culture with one time addition of glucose, the BSPT15-M3 strain showed 7.9 ± 2.3 and 12.2 ± 1.1% higher glucose consumption rate and ethanol productivity, respectively (Fig. 3d), compared to the fed-batch culture I of the same strain provided above (Fig. 3b). Also, the same enhancement of ethanol production by the BSPT15-M3 strain was observed after 24 h of the culture. In comparison between the two strains in fed-batch II, the BSPT15-M3 strain showed 9.5 ± 0.6 and 12.4 ± 0.4% higher glucose consumption and ethanol production rates, respectively, than the BSPT15wt control strain (Table 2).

SSF of cassava and corn starch

To evaluate the ethanol producing-performance in simultaneous saccharification and fermentation (SSF), the BSPT15-M3 and BSPT15wt strains were cultivated in a bioreactor containing 200 g/L liquefied starch of cassava or corn, and at 200 rpm and 0.1 vvm (Fig. 4). The Liquozyme™ not only degrades the starch structure by endoglucanase activity but also releases glucose unit from glucan, so the initial glucose concentration in culture broth was around 12 g/L. Dextrozyme™ with exoglucanase activity was added intermittently into the bioreactor for more degradation of starch. As shown in the SSF of cassava starch (Fig. 4a, b), ethanol was produced linearly by consuming the released glucose of which concentration was maintained at around 7.5 g/L. Even though the liquefied cassava starch solution was supplemented two times at the final concentration of 100 g/L, ethanol productivity was kept constant in each case. Finally, the BSPT15-M3 strain produced 118.5 g ethanol with 1.98 g/h ethanol productivity in 60 h of the SSF using cassava starch, which corresponded to 4.5% increases in both parameters relative to the BSPT15wt strain. The final volume was 1.25 L. As the same as the cassava starch, the liquefied corn starch was subjected to SSF (Fig. 4c, d). For rapid release and additional supply of glucose, the Dextrozyme™ and liquefied corn starch solution of 400 g/L were added four times in culture broth. Finally, the SSF of the BSPT15-M3 strain resulted in 162.5 g ethanol concentration and 1.67 g/h ethanol productivity, which were 3.9% higher than the corresponding values for the SPT15wt strain. A similar ethanol yield of 0.40 g/g was obtained in all SSF processes.

Discussion

In our previous report, overexpression of the mutated *SPT15-M3* gene gave recombinant *S. cerevisiae* enhanced tolerance on high concentrations of glucose, salt, and sorbitol (Kim et al. 2013) as well as ethanol (Yang et al. 2011), which was verified in spot assay and aerobic fermentation with a complex medium. With respect to culture condition, batch cultures of *S. cerevisiae* iETS3 (L3262 strain) (Yang et al. 2011) and BYiETS3 (BY4741 strain)

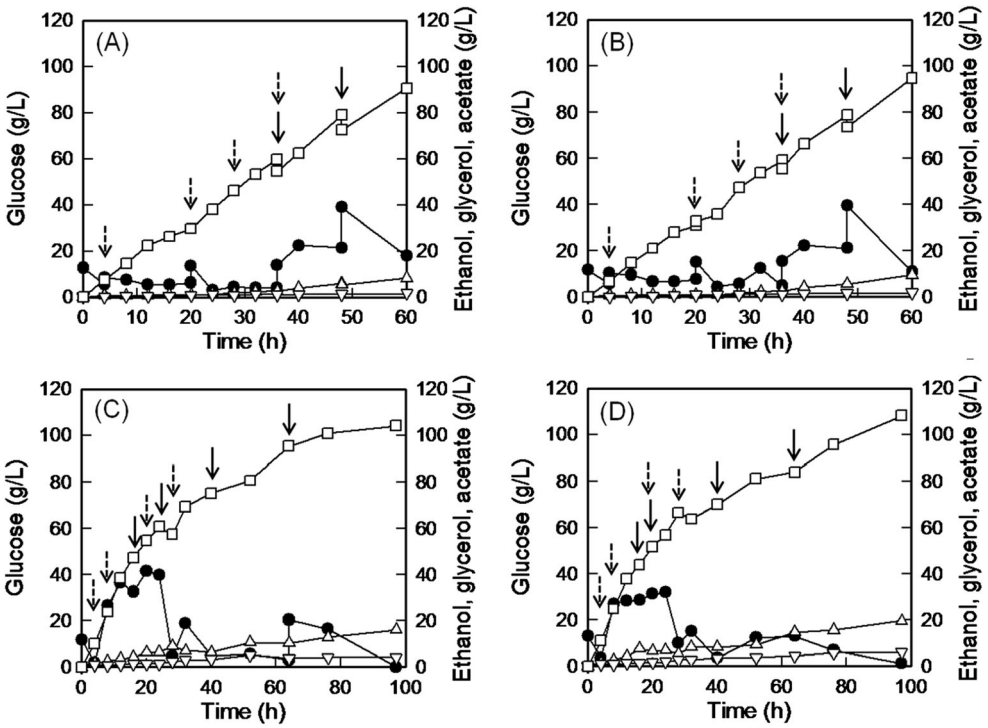

Fig. 4 Simultaneous saccharification and fermentation of recombinant *S. cerevisiae* BSPT15wt (**a**, **c**) and BSPT15-M3 (**b**, **d**) strains in 1 L of the YP medium with 200 g/L of liquefied cassava (**a**, **b**) or corn starch (**c**, **d**). Culture conditions were fixed at 30 °C, pH 5.5, 0.1 vvm, and 200 rpm. The *arrow with solid line* indicates the addition of 125 mL of 400 g/L liquefied cassava or corn starch, and the *one with dotted line* presents the supplementation of Dextrozyme™ at the final concentration of 0.05% (*v*/*v*). The *symbols* are denoted as follows: *black circle*, glucose; *open square*, ethanol; *open upright triangle*, glycerol; *open inverted triangle*, acetate

(Kim et al. 2013) with the *SPT15-M3* gene in each chromosome were carried out with high supply of pure oxygen at 0.4 vvm in a bioreactor. Also, recombinant *S. cerevisiae* BY4741 transformed with the *SPT15-300* mutant gene was cultured in a flask at 225 rpm (Alper et al. 2006) or without specified conditions (Baerends et al. 2009; Zhao et al. 2014). To precisely analyze the effect of the *SPT15-M3* expression on fermentative properties of recombinant *S. cerevisiae*, in this study, the BY4741 strain with the *SPT15-M3* gene (BSPT15-M3) was cultivated in a series of culture type at oxygen-limited and microaerobic conditions. Since oxygen (or air) supply could be a rate-limiting factor for central carbon metabolism and other cellular reactions in alcohol yeasts, the BSPT15-M3 strain was first cultured in a flask at 80 rpm, which was known to be an oxygen-limited condition (Maier et al. 2004). When oxygen content was limited, an increment of inorganic nutrients content from YNBD210 to 2× YNBD enhanced ethanol production along with glucose consumption without cell growth change (Fig. 1). By utilization of organic nitrogen sources, however, cell growth of the BSPT15-M3 strain was much higher than that of the SPT15wt without significant differences in glucose and ethanol production, of which phenomena were also observed in the same batch culture of recombinant *S. cerevisiae* D452-2 strains (Fig. S1). Considering the enhanced uptake of leucine by overexpression of the *SPT15-300* mutant gene (Baerends et al. 2009), overexpression of the *SPT15-M3* gene also might increase the consumption of amino acids in organic nitrogen sources. Meanwhile, elevation of air supply dramatically changed the cell metabolism. As shown in Fig. 2, the BSPT15-M3 strain showed higher performance of cell metabolism in microaerobic condition (0.1 vvm) than the SPT15wt strain (Fig. 2c), whereas the same fermentative properties between the two strains were observed at no air supply (Fig. 2a). These differences were also found in microaerobic fed-batch fermentations and SSF with corn and cassava starch. The reason of the enhanced cell growth and ethanol production caused by the *SPT15-M3* expression at more aerobic conditions was unclear. But, constitutive activation of Hog1 and low accumulation of reactive oxygen species were observed in the BYiETS3 strain similar to BSPT15-M3 (Kim et al. 2013), which suggested that the BSPT15-M3 strain might be more active at high air supply condition than the SPT15wt strain. Also, it was reported that mutation of the transcription factors of Spt15 and Taf25 resulted in high growth tolerance of *S. cerevisiae* in the presence of H_2O_2, which might be caused by modification of the cellular oxidation defense systems and hence improvement of the antioxidation ability of *S. cerevisiae* (Zhao et al. 2014). Meanwhile, the fed-batch fermentations of the BSPT15-M3 strain resulted in higher glucose consumption and ethanol production rates and lower yield of glycerol production than the SPT15wt control strain, when the glucose shock was imposed in the presence of 50 or 80 g/L ethanol (Fig. 3). Glycerol production is generally caused by redox balancing to reoxidize surplus NADH (Ansell et al. 1997; Tamas et al. 1999). In addition, expression of the *GPD1* and *GPD2* genes coding for glycerol-producing enzymes is induced by high osmotic pressure, of which a mechanism is mainly controlled by the high-osmolarity glycerol (HOG) pathway and different expression levels of about 150 genes (Krantz et al. 2004; Rep et al. 2001). However, the BSPT15-M3 strain showed about 11% lower yield of glycerol accumulation than the control strain. This result indicated that the BSPT15-M3 strain was more resistant on glucose shock without glycerol accumulation than the BSPT15wt control strain, which led to higher and faster cell growth and ethanol production. The similar properties were found in a report that co-overexpression of the *SPT3* and *SPT15* genes not only improved osmotic tolerance but also enhanced fermentation properties of recombinant *S. cerevisiae* HLH315 (Hou et al. 2009).

In conclusion, to exactly evaluate the fermentative abilities of recombination *S. cerevisiae* BY4741 overexpressing the *SPT15-M3* mutant gene, a series of culture was carried out with different nitrogen sources and at various aeration conditions. According to the air supply, the culture phenotypes were considerably different. More aeration was favorable for cell growth and ethanol production of the BSPT15-M3 strain, of which reason might be caused by being less sensitive to reactive oxygen species. Considering the resistance onto glucose shock in the presence of high ethanol contents and culture performance of SSF, the gTME mediated by the mutation of the *SPT15* gene could be a potent strategy in order to construct robust yeast strains.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals by any of the authors.

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