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Extra metabolic burden by displaying over secreting: growth, fermentation and enzymatic activity in cellobiose of recombinant yeast expressing β-glucosidase

Juanjuan Ding[1,2], Guohong Liang[2], Kun Zhang[1,3], Jiefang Hong[1], Shaolan Zou[1,2*], Haiyan Lu[2],Yuanyuan Ma[1], Minhua Zhang[1,4,5]

[1] Tianjin R&D Center for Petrochemical Technology, Tianjin University, Tianjin, 300072, China

[2] Key Laboratory of Systems Bioengineering, Ministry of Education, School of Chemical Engineering and Technology, Tianjin University, Tianjin, 300072, China

[3] School of Pharmaceutical Science and Technology, Tianjin University, Tianjin, 300072, China

[4] Key Laboratory for Green Chemical Technology of Ministry of Education, Tianjin University, Tianjin 300072, China

[5] State Key Laboratory of Engine, Tianjin University, Tianjin 300072, China

* Corresponding author. Tel: +86-22-27892069; Fax: +86-22-27892069.

E-mail address: slzhou@tju.edu.cn (Shaolan Zou).

Abstract

β-glucosidase was selected to be a reporter to study metabolic burden imposed by its expression in yeast. Cell growth, fermentation yield and enzymatic activity were used as indicators of the metabolic burden borne by 14 recombinant yeast strains. Various factors were found to affect metabolic burden, including *BGL*I gene source, gene dose, trafficking of the enzyme (either cell-surface display or secretion), and oxygen supply. While *BGL*I gene from *Aspergillus aculeatus* provided better performance for the host cells than that from *Saccharomycopsis fibuligera*, displaying β-glucosidase on the cell surface generally led to lower μ_m, total activity and ethanol titer, and longer lag period, lower (aerobic condition) or higher (anaerobic condition) biomass yield than that of secreting β-glucosidase. The negative effect on growth increased with gene dose level until a final failure to grow. This growth difference implies that displaying β-glucosidase on the cell surface imposes an extra metabolic burden. The molecular basis and mechanisms for this phenomenon need to further be investigated in order to develop better strategies for utilizing displayed and secreted enzymes in biotechnology and yeast breeding.

Keywords:

β-Glucosidase, Recombinant yeast, Displaying and secreting, Rate and yield, Metabolic burden

1. Introduction

Since the first report of surface display using a filamentous phage-coat protein, many new systems have been developed. The Baker's yeast *Saccharomyces cerevisiae* is among the most important organisms for cell surface display (Kondo and Ueda,2004; Teparic et al., 2010; Tanaka et al., 2012; Tanaka and Kondo,2015; Liu et al.,2016). Being one of the most widely used cell factories, *S.cerevisiae* is utilized in biotechnological processes ranging from many heterologous proteins to biofuels and chemicals production of medical or industrial interest (Fidan and Jixun, 2015; Jouhten et al., 2016). Display of a target peptide or protein on the yeast cell surface leads to numerous potential applications, including vaccine and antibody development, library screening, bioconversion, and biosorption (Kondo and Ueda,2004; Teparic et al., 2010; Tanaka et al., 2012; Tanaka and Kondo,2015; Liu et al.,2016).

Engineering yeast for the efficient production of complex proteins as functional entities still remains a major challenge. Although significant progress has been made, a deeper understanding of the mechanisms governing protein production in yeast will likely be crucial for developing more efficient protein production systems. The concept of metabolic burden has emerged from previous investigations in this area and has become a cornerstone in synthetic biology and metabolic engineering applications (Baumann et al.,2010; Klein et al., 2015; Gang et al.,2016) .This concept is also suitable for guiding efforts to implement cell surface display technology with *S. cerevisiae*. Many problems have been reported with attempts to optimize activity with cell-surface-displayed enzymes, such as variable cell-surface display efficiency,

activity/abundance and host-compatibility (van Der Vaart et al., 1997; Teparic et al., 2010; Zhang et al., 2012; Tanaka and Kondo,2015; Liu et al.,2016). Understanding the metabolic burden associated with surface display could be useful for elucidating the underlying issues.

β-glucosidase is an important enzyme in medicine and industry and has been isolated from a wide variety of sources, well studied, and recombinantly expressed in different hosts including yeast (Bhatia et al., 2002; Singhania et al., 2013,2017). The expression strategy in *S. cerevisiae* is to display enzyme on the cell surface, secrete enzyme into the medium, or produce intracellular β-glucosidase besides a transporter (Bhatia et al., 2002; Van Zyl WH et al., 2007; Wilde et al., 2012; Singhania et al., 2013; Liu et al., 2015; Eun Joong et al., 2017; Parisutham et al.,2017). In plenty of such studies, two β-glucosidase-encoding genes are typical and noteworthy. One is gene *BGL*1 from *Saccharomycopsis fibuligera* (Machida et al., 1988; McBride et al., 2005; van Rooyen et al., 2005; van Rensburg et al.,2012; Davison et al.,2016; Singhania et al., 2017), and another is gene *BGL*1 from *Aspergillus aculeatus* (Sakamoto et al.,1985; Van Zyl WH et al., 2007; Wang et al.,2013; Liu et al., 2015; Wang et al., 2016; Liu et al., 2017; Singhania et al., 2017). In fact, because of higher activity on cellobiose and / or cellooligosaccharides than that on other substrates, enzyme BGLI from those two sources was preferably applied in the cellulosic ethanol field. The catalytic activity of β-glucosidase is rate-limiting in the saccharification of cellulose, and it determines not only the rate but also the extent of cellulose hydrolysis by relieving end-product inhibition of endoglucanases and cellobiohydrolases (Wilde

et al., 2012; Singhania et al., 2017).

The catalytic activity of β-glucosidase on cellobiose allows recombinant strains that express β- glucosidase to utilize cellobiose directly for growth and ethanol production. It is expected that not only the enzymatic activity level but also the trafficking mode, either secretion or cell-surface display, of the enzyme would influence the growth and fermentation characteristics of recombinant yeasts in the presence of cellobiose. So in this study, enzymes BGLIs from *S.fibuligera* and *A.aculeatus* were selected as reporter proteins to investigate the effects of enzyme trafficking as well as gene dose and oxygen supply on activity, growth, and fermentation in cellobiose media for recombinant yeasts expressing β-glucosidase. This study therefore provides valuable information on the difference in metabolic burden between displaying and secreting, as well as their relationship and other key factors exerting metabolic stress on cell. In spite of the abovementioned reports on β-glucosidase expression in yeast, to date, there have been very few studies of this type.

2. Materials and methods

2.1. Strains, plasmids, media and growth conditions

The microbial strains used in this study are listed in Table 1 (Wang et al., 2013; Zhang et al., 2012). *Escherichia coli* Top10 was used for recombinant DNA manipulation. Recombinant plasmids were constructed and amplified in Top10 cultivated at 37 ◦C in Luria-Bertani liquid medium or on Luria-Bertani agar (1%

tryptone, 1% NaCl and 0.5% yeast extract, pH7.0). Ampicillin for selecting and proliferating resistant bacteria was added to a final concentration of 100 mg mL^{-1}. *S.cerevisiae* W303-1A was used as the transformation recipient. *S.cerevisiae* 7N1a and *Saccharomycopsis fibuligera* were used as the DNA donor strains. Yeast strains were generally cultivated at 30 □ in rich YPG or YPC medium (1% yeast extract, 2% peptone, 2% glucose or cellobiose), or basal CMG or CMC medium (6.7 g/L yeast nitrogen base without amino acids, 20 g/L glucose or cellobiose, and the appropriate amino acid and nucleic acid supplements) (Wang et al., 2013).

2.2. DNA manipulation, plasmid construction and yeast transformation

Standard molecular genetical techniques were used for nucleic acid manipulations (Sambrook and Russell, 2001).The primers were used and plasmids constructed were listed in Table 1. The information about expression elements used was shown in (Zhang et al., 2012) .The regulatory elements used in the aforementioned expression cassettes were same as those.

The primers P1 to P8 were used to construct the expression cassettes of β-glucosidase I enzyme encoding gene *BGL*1 of *S.fibuligera* by over-lapping PCR method, P*tpi-xyn*2s-Sf *BGL1*-cwp2-T*adh*I and P*tpi-xyn*2s-Sf *BGL1*-T*adh*I. The PCR products were first digested with restriction endonucleases *Hind*III + *Eco*RI or *Avr*II and then ligated into YCplac33, YEplac195 (Table 1) or integrating pGEM-Teasy-H1-*URA*3-H2 vector, respectively, and to obtain the six plasmids (Table 1). Among them the four YCplac33- and YEplac195-based plasmids were

transformed into *S.cerevisiae* W303-1A. The resultant strains were named as YC-Sf-C, YC-Sf, YE-Sf-C and YE-Sf, respectively. The other two plasmids, pGEM-Teasy-H1-*URA*- P*tpi-xyn*2s-Sf *BGL1*-cwp2-T*adh*I-H2 and pGEM-Teasy-H1-*URA*- P*tpi-xyn*2s- Sf *BGL1*-T*adh*I-H2, were first digested with *Not* I and the linearized DNA fragements were transformed into strain W303-1A cells to obtain two strains G-Sf-C and G-Sf.

The H1 and H2 sequences in pGEM-Teasy-H1-*URA*3-H2 were used as the homologous arms for integrating β-glucosidase genes into the W303-1A chromosome, corresponded to the nucleotide sequence between positions 81460-81959 and 81960-82459, respectively, in the *S.cerevisiae* S288c chromosome IV sequence (http://www.yeastgenome.org/). The PCR fragment H1-*URA*-H2 was amplified by over-lapping PCR method with the primers P9 to P14 and cloned into pGEM-T easy vector (Promega, USA) to lead to pGEM-Teasy-H1-*URA*3-H2.

Two kinds of expressing cassettes of *BGL*1 genes from *Aspergillus aculeatus* were obtained from pGEM-T easy-P*tpi-xyn*2s -Aa BGL1-*cwp*-T*adh*I and pGEM-T easy-P*tpi-xyn*2s -Aa BGL1-T*adh*I (Wang et al., 2013; Zhang et al., 2012) by digesting with *Pst*I + *Sca*I and then ligated with *Pst*I + *Sma*I digested- YCplac33 or YEplac195 vector fragment. Similarly, the two integrating plasmids and the resultant four shuttle plasmids led to six strains G-Aa-C, G-Aa, YC-Aa-C,YC-Aa,YE-Aa-C and YE-Aa, respectively (Table 1).

Transformation of yeast cells was carried out with lithium acetate method (Gietz et al., 1995). *URA*3 was used as a selective marker and yeast transformants were

screened in CMG solid medium absent of uracil. Integrating transformants were further identified by PCR with genomic DNA as template and the primers used were 5' ATACGTACCTGGGCTCCACCG 3' (Pup) and 5' TTGCGCGATAACCTAGTG TTT 3' (Pdown), which were all external to H1 and H2, respectively.

2.3. Growth, ethanol production and β-glucosidase activity evaluation of recombinant S.cerevisiae grown on cellobiose and glucose

After being precultivated in CMG medium for 24 h, the recombinant *S. cerevisiae* strains were grown aerobically in fresh medium for 24 h at 30 °C. The resultant cells were collected by centrifuga- tion, and these were washed twice with distilled water and then inoculated into 250 mL shake flasks containing 50 mL of YPG, YPC, CMG or CMC. The initial optical density at 660 nm (OD_{660}) of the medium was adjusted to 0.1. These cultures were allowed to grow aerobically or anaerobically at 30 °C and 190 rpm and sampled at regular time points for growth, β-glucosidase activity, and sugar and product analysis. Growth analysis was generally conducted by detecting the optical density of culture at 660 nm (OD_{660}). β-glucosidase activity was evaluated by using *p*-nitrophenyl- β-D-glucopyranoside (pNPG) as described before (Wang et al., 2013). Sugar and product analysis was carried out by HPLC method as described before (Wang et al., 2013).

2.4. Pretreatment of corn straw

Air-dried corn straw used in this study was harvested from the suburb of Tianjin,

China and was crushed into 20 - 80 mesh size granules. Corn straw was first treated with 2 % H_2SO_4 at a ratio of 100 g solid per 1 L liquid and kept 45 min at 121 □. Then the solid fraction was separated, washed with tap water until neutral and treated in 15 % (w/w) dilute aqueous ammonia at a ratio of 1:6 (solid / liquid, w/v) (Hong et al., 2015).

The resultant substrate was shown to contain 58.6 % cellulose, 3.6 % hemicellulose and 7.9 % lignin by dry weight (w/w), respectively, while the content of the three components in un-pretreated substrate was 33.6 %, 15.3 % and 17.0 %, respectively.

2.5. Simultaneous saccharification and fermentation of pretreated corn straws with the engineered strains

Simultaneous saccharification and fermentation (SSF) of pretreated corn straws was carried out in 100-mL Erlenmeyer flasks with a total working weight of 40 g. The slurry was diluted with sodium citrate buffer (50 mM, pH 4.8) containing nutrients of $(NH_4)_2HPO_4$ at 0.5 g/L, $MgSO_4.7H_2O$ at 0.025 g/L, yeast extract at 1 g/L to 10 % solid content by dry weight. The SSF experiments were performed at 30 □ under 150-rpm shaking for 96 h. Prior to the SSF process, pre-hydrolysis was performed at 50 □ with shaking at 150 rpm for 6 h. The cellulase Spezyme CP (Genencor International, Inc., 117 FPU/mL) was added at 20 FPU/g biomass, and β-glucosidase Novozyme 188 (Novozymes A/S, Denmark, 925 CBU/mL) was also used at 20 CBU/g cellulose if necessary. *S.cerevisiae* W303-1A and its recombinant strains

expressing β - glucosidase were grown aerobically in YPD and YPC medium for 24 h at 30 □, respectively. The resultant cells were collected by centrifugation and then inoculated. The yeast loading was 1 g dry weight /L. The samples were taken at regular time points for sugar and product analysis by HPLC method.

3. Results and Discussion

3.1. Cloning of β-glucosidase genes, construction of recombinant vectors and strains expressing β-glucosidase

In our previous work, we have isolated the β-glucosidase-encoding genes from *S.fibuligera* strain CGMCC 2.1626 and *A.aculeatus* stain CICC 2193 based on the sequence entries M22475 (for *S.fibuligera*) (Machida et al., 1988) and D64088 (for *A.aculeatus*) (Kawaguchi et al., 1996) available in GenBank (http://www.ncbi.nlm.nih.gov/entrez/). The sequencing results of recombinant plasmids YCplac33-P*tpi*- *xyn*2s-Sf *BGL*1-*cwp*2-T*adh*I and YCplac33-P*tpi-xyn*2s-Sf *BGL*1- T*adh*I showed that the insert fragment P*tpi-xyn*2s-Sf *BGL*1-*cwp*2-T*adh*I or P*tpi-xyn*2s-Sf *BGL*1-T*adh*I did not contain any mutations and the *S. fibuligera BGL*1 sequence has also been submitted into GenBank as accession HQ891006. Sequence alignment analysis of HQ891006 and M22475 showed their ORF sequences share 99 % homology. The sequencing results for the *A. aculeatus BGL*I cloning fragment have also been submitted into GenBank as accessions JN121996 and JN121997, respectively. Sequence alignment analysis indicated the *BGL*I gene of *A. aculeatus* has five introns and six exons, and the exon sequences share 95 % homology to the

known sequence of D64088.

Twelve recombinant plasmids (shown in Table 1) were constructed and used to transform *S.cerevisiae* W303-1A to uracil prototrophy (Ura+). The Ura+ transformants were further selected by their β-glucosidase activity in CMG media. PCR with total DNA from the respective transformants were also performed to confirm the presence of the recombinant plasmids. For integrating transformants G-Sf-C, G-Sf, G-Aa-C and G-Aa, the size of PCR products from genomic DNA by Pup and Pdown primers were 6830 bp, 6625 bp, 6806 bp and 6601 bp, respectively. For host strain W303-1A, it was 1101 bp.

3.2. Aerobic growth of three YC-based recombinant strains in cellobiose and glucose

It is well known that cello-oligosaccharide could be one of the important substrates for catalysis by enzymes with β-glucosidase activity. In fact, the conversion of cello-oligosaccharide to final product glucose via cellobiose is a limiting step in the enzymatic hydrolysis of lignocellulosic biomass, and β-glucosidase activity can often be a limiting factor (Singhania et al., 2013, 2017; Wang et al., 2013). Thus, cellobiose was selected as a substrate and a medium component for evaluating the growth and fermentation of the aforementioned recombinant strains. It is expected that there exists a direct relationship between the enzymatic activity level expressed and cellobiose utilizing ability.

In order to first decide on a kind of medium suitable for evaluating and comparing all of the strains, three YCplac33-based strains and five kinds of media

were selected and investigated. The growth data (Fig.1 and Table 2) indicate that there is a significant difference between the growth of strains YC-Sf –C and YC-Sf only in YPC or CMC media. This difference clearly indicates that when cellobiose is used as carbon source, the presence of an anchored peptide-encoding sequence (*cwp*2) caused β-glucosidase expression and cellobiose hydrolysis to lag, which prolonged the lag period and the time into stationary phase. The *cwp*2 anchor also imposed a metabolic burden that inhibited growth and led to a decrease in the maximum value of OD_{660}. Although the corresponding total enzymatic activities of the recombinant strains (data not shown) were found to vary greatly, independent of the *cwp*2 anchor, the growth of all strains in YPG or CMG media showed no significant difference. This typically showed that the constitutive expression of the recombinant BGL proteins did not significantly affect the growth of the strain. Thus, YPC media was chosen for the following experiments to allow more strains to display the difference with a greater diversity and width.

3.3. Aerobic growth of fourteen recombinant strains in cellobiose

The growth curves of fourteen recombinant strains aerobically in YPC medium are shown in Fig.2 and Table 3.Growth differences between the strains are readily apparent, demonstrating the complex and significant interaction between at least three factors: gene copy number, anchor moiety, and gene identity. Undoubtedly, copy number is most significant of these factors. From a single genome-integrated copy to several and dozens of gene copies, corresponding to shuttle vectors YCplac33 and

YEplac195, respectively, the lag period and the time into stationary phase was shortened, and the maximum values of μ_m and OD_{660} were increased. In addition, the *cwp*2 anchor had a negative impact, which again indicated its metabolic burden on the cell. Last, when the other parameters were kept the same, the growth performance from *A.aculeatus BGL*I was far superior to that from *S.fibuligera BGL*1, and the latter even led to an extreme growth defect, that is, only basic growth of strain YE-Sf-C in cellobiose. In fact, this growth defect likely came from the mutual action between three factors.

3.4. Anaerobic growth, ethanol production and β-glucosidase production of fourteen recombinant strains in cellobiose

It is well known that the oxygen condition greatly influences enzyme expression level, cell growth, and fermentation performance. Here, the anaerobic growth, ethanol production, and β- glucosidase production of the fourteen recombinant strains were further investigated in YPC media. The results are summarized in Figs.3, 4 and Table 4 and indicate significant mutual interaction among copy number, anchor, and gene. At the same time, the fourth factor, oxygen supply, also showed an obvious effect. First, Fig.3 and Table 4 clearly demonstrated that under anaerobic conditions the growth difference among those strains was greatly diminished compared to aerobic conditions. Three strains, YE-Sf-C, YE, and YC, still showed similar weak growth and the maximum OD_{660} value was (1.23 ± 0.064) ~ (1.42 ± 0.208). For the other eleven strains, the growth difference under the two different oxygen conditions

reflected more on the time to enter the stationary phase than the maximum OD_{660} value. While the maximum OD_{660} value was decreased from (12.75 ± 0.332) ~ (16.24 ± 0.629) under aerobic conditions to (7.41 ± 0.683) ~ (10.26 ± 1.438) under anaerobic conditions, the range of the ratio of the maximum to minimum OD_{660} value (Table 3 and 4) was (1.00 ~ 1.27) and (1.00 ~ 1.38), respectively. However, the time to reach the stationary phase of every strain was generally shortened by 12~36 h under anaerobic conditions compared to that under aerobic condition (Table 3 and 4). While the time into ethanol plateau closely matched the time to reach the stationary phase, the range of values for the maximum ethanol titer and yield was more limited than that of the growth parameters (Table 4).

In contrast with the growth and ethanol production, the difference in enzyme expression between strains shown by the enzymatic activity data (Fig.4 and Table 4) was more subtle. Therefore, the interaction among copy number, anchor, and gene was more fully presented by this parameter. While the other factors were same, *A.aculeatus BGL*I - expressing strains always showed higher total and extracellular activities than those of *S. fibuligera BGL*1 - expressing strains. Second, the effect of gene dose was shown to be significant and proportional to copy number of *BGL*I genes only for YCplac33-based and single - copy genome - integrated strains. Indeed, with the exception of YC-Sf-C and G-Sf-C, the YCplac33 vector could achieve total or extracellular activities of resultant recombinant strains several times higher than those of single - copy integrated strains. Finally, the *cwp*2 anchor sequence displayed its effect in two ways. One led to total activities that were lower than those without

*cwp*2 anchor sequence. The other resulted into high cell activities, as expected, and low extracellular activities, which was unexpected. The ratio of extracellular activity to total activity was 1.0 %- 6.7 %. Surprisingly, only 42.2 %- 50.1 % of the total activity was found in the supernatant of those strains carrying no anchor, which implied incomplete secretion of the enzyme.

3.5. Simultaneous saccharification and fermentation (SSF) of pretreated corn straws by recombinant strains

In order to further observe the effect of the *cwp*2 anchoring moiety on cell performance of the recombinant strains, SSF of pretreated corn straws by the strains YE-Aa-C and YE-Aa was carried out. The ethanol curves shown in Fig.5 indicate that during a 96 h fermentation time, the *cwp*2 anchor moiety led to a slight decrease in the ethanol titer, as expected. The maximum of ethanol titer and yield were 2.133 and 2.394 g /100 g, 64.2 % and 72.0 % of the theoretical value, respectively. On the other hand, β-glucosidase was shown to have a synergistic effect with commercial cellulase in the hydrolysis of pretreated corn straw, and the recombinant strain could save the β - glucosidase supplementation. The SSF result again implied that the difference in anaerobic fermentation performance was much less than the difference in the measured activity.

In spite of many reports on *A.aculeatus* or *S. fibuligera BGL*1 expression in yeast, this study provides the first direct comparison of them under the same conditions

(Machida et al., 1988; McBride et al., 2005; van Rooyen et al., 2005; van Rensburg et al.,2012; Davison et al.,2016; Sakamoto et al.,1985; Van Zyl WH et al., 2007; Wang et al.,2013; Liu et al., 2017). The results of the growth, ethanol production, and enzymatic activity evaluations all support the notion that *BGL*1 from *A.aculeatus* endow the host with a superior ability to utilize cellobiose and cellulose material. The inability of strain YE-Sf-C to utilize cellobiose to grow and ferment further confirmed the inferiority of *S. fibuligera BGL*1. The reason behind this performance difference can be attributed to their different nucleotide and amino acid sequences. It is reported in the UniProt database that the entries UniProtKB-P48825 (BGL1_ASPAC) and UniProtKB-P22506(BGL1_SACFI) correspond to the enzymes BGLI of *Aspergillus aculeatus* and *Saccharomycopsis fibuligera*, respectively. Sequence alignment analysis show that their amino acid sequences share 44 % homology.

Interestingly, a similar growth defect has also been reported with yeast expressing cell-surface-displayed cellulase, but the gene source did not include *S. fibuligera* (Bhatia et al., 2002; Van Zyl WH et al., 2007; Singhania et al., 2013; van Rooyen et al., 2005). R.van Rooen et al (van Rooyen et al., 2005) reported that the recombinant strains could not grow in media containing cellobiose (5.0 g/ L) under both aerobic and anaerobic conditions. The strains examined in that study contained a multi-copy-vector-based heterologous secreting expression system for *bgl*A (2526 bp) from *Aspergillus kawachii*, *bglB* (1767 bp) from *Candida wickerhamii* or *bgl1* (2139 bp) from *T.reesei*. Further, two cell-surface-display-expressing strains of *bgl*A could grow aerobically (μ_m as 0.06 and 0.07 h^{-1}, respectively) but could not anaerobically.

In this case, the anchors used were cell wall protein 2 (Cwp2) and α-agglutinin (AGα1) anchoring moieties, respectively.

The observed growth defect may stem from a combination of relatively low activity on cellobiose and the metabolic burden imposed by enzyme expression. In addition, it was supposed that the anchoring moieties may also contribute to the observed growth difference of the cell-surface- display-expressing strains of *bgl* A under both aerobic and anaerobic conditions. It is not clear whether or not a decrease in the gene dosage would lead to anaerobic growth.

In this study, all results clearly indicated a complex and significant interaction between four factors: gene copy number, anchoring moiety, gene source, and oxygen supply. This interaction also manifested itself as regular variations in growth, fermentation, and enzymatic activity in the presence of cellobiose (Figs.1-4, Tables.2-4). The level of enzymatic activity expressed was expected, and also determined the growth and fermentation performance of the resultant strain cells. However, a clear, quantitative relationship for all strains between BGL1 activity level, growth parameter (lag period, μ_m, OD_{660} value or biomass yield), and fermentation parameter (ethanol titer, rate or yield) remains elusive. Therefore an attempt to model such relation failed. In fact, the degree of difference of those parameters between all strains greatly varied with each other. The differences were maximum for activity level, high for lag period and μ_m, low for OD_{660} value, minimal for fermentation. In other words, the difference is reflected in more rate than the absolute level and yield to synthesize enzyme, hydrolyze cellobiose to release glucose, and thus produce

biomass and ethanol.

Similar to the typical effect of gene source, anchored versions generally showed a tendency, toward longer lag period, lower μ_m, lower total activity, lower ethanol titer, and either lower (aerobic condition) or higher (anaerobic condition) OD_{660} value compared to corresponding secretion ones. More importantly, these tendencies seemed to be irrespective of expression from a plasmid or a chromosomal integration, which is not consistent with a previous report (Wilde et al, 2012). Together, all of these phenomena imply that anchoring causes a metabolic burden effect that in extreme cases led the strain, for example strain YE-Sf-C, being unable to utilize cellobiose to grow and ferment.

It is generally agreed that two major expression strategies, displaying on the cell surface and secreting into the fermentation broth, each have their own advantages and disadvantages (den Haan et al., 2015; Liu et al., 2015). But identification of the preferred option remains problematic, due to insufficient quantitatively comparable data (den Haan et al.,2015). Of course, in some applications which require the reuse / recycling of enzymes or cells, for example, multi-batch fermentations, absorption in waste biorefinery and enzyme recovery, cell-surface display may be the better or even only choice (Matano et al., 2013; den Haan et al.,2015; Hong et al, 2015; Liu et al., 2015; Liu et al.,2016). As far as our case is concerned, it is necessary to more completely and quantitatively evaluate and balance their advantages and disadvantages. Our results clearly proved the existence of a metabolic burden directly imposed by an anchoring moiety at single, low, or high gene copy number.

In fact, metabolic burden is a longstanding problem in biotechnology (Gang et al, 2016). Proteins are the driving force for growth, but their production is a major consumer of energy and nutrients (Kafri et al., 2016). Approximately one-third of the yeast proteome enters the secretory pathway, and further approximately 15% of proteins that enter the secretory pathway are post-translationally modified on their C terminus by addition of a lipid-anchored glycosylphosphatidylinositol (GPI) moiety (Barlowe et al., 2013).Unfortunately, glycosylphosphatidyl- inositol (GPI) anchoring (of yeast cell surface proteins), which is one of the most important cell-surface-displaying methods used (Kondo and Ueda,2004; Teparic et al, 2010; Tanaka et al, 2012; Tanaka and Kondo,2015), is also reported to be the most complex and metabolically expensive of the lipid posttranslational modifications described to date (Orlean and Menon, 2007). Thus the extra cost of displaying protein on the cell surface by GPI-anchoring could be expected. On the other hand, displaying and secreting strategies have always been used in the cellulosic ethanol field, and β-glucosidase is among the most important enzymes in cellulose hydrolysis. Although the metabolic burden or growth defect of expressing cellulytic proteins destined for display- or secretion in yeast has been reported (Gorgens et al.,2001; van Rooyen et al, 2005; van Rensburg et al,2012), strict comparison of the metabolic burden between the displaying and secreting strategies has seldom been reported. In this study, our results confirm that the extra metabolic burden of displaying proteins over secreting them does indeed exist, increases with copy number, and is irrespective of plasmid-based or chromosomal expression. The inability of strain YE-Sf-C to utilize

cellobiose to grow and ferment provides an extreme example of this metabolic burden effect. Here the Cwp2 moiety used derives from a GPI-anchored protein and has been proved to obtain high anchoring efficiency (van Der Vaart et al., 1997; Teparic et al, 2010).

Much work still needs to be done to ensure a deeper, more precise and comprehensive investigation of the metabolic burden imposed by anchoring moieties. First, to obtain higher copy numbers, it is better to design an expression system in which all copies of the gene are chromosomally integrated in order to eliminate the consumption of resources for plasmid maintenance and replication. Second, more parameters need to be systematically investigated, including higher substrate concentrations. Last, the expression levels of genes related to the GPI-anchored protein transport and quality control systems in displaying and secreting strains should be measured to uncover the quantitative and qualitative changs behind the metabolic burden. Only after the molecular basis of the metabolic burdens associated with expression of proteins destined for display and secretion are better quantified and the mechanisms elucidated, can cell- surface display strategies be put to optimum use in biotechnology.

4. Conclusions

The evaluation of fourteen recombinant *S.cerevisiae* strains expressing β-glucosidase clearly indicated the complex and significant interaction between gene dosage, anchoring moiety, gene source, and oxygen supply. This interaction

manifested itself as variations in growth, fermentation, and enzymatic activity in the presence of cellobiose. The *BGL*I gene from *A.aculeatus* provided the host with better performance than the homologous gene from *S. fibuligera*. Compared to secretion, cell-surface display of the enzyme was found to impose an extra metabolic burden, which increased with copy number and was independent of plasmid-based or chromosomal expression. It is necessary to balance these considerations when using a cell-surface display strategy for the production of cellulosic ethanol.

Appendix A. Supplementary data

E-supplementary data for this work can be found in e-version of this paper online.

Acknowledgments

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Figure captions

Fig.1 The growth curves of three YCplac33-based strains aerobically in different media

Fig.2 The growth curves of fourteen strains aerobically in YPC medium

Fig.3 The growth curves of fourteen strains anaerobically in YPC medium

Fig.4 The beta-glucosidase activity curves of fourteen strains anaerobically in YPC medium (U/mL/OD660). (A) Total activity; (B) Extracellular activity.

Fig.5 Ethanol production during simultaneous saccharification and fermentation of three *S.cerevisiae* strains from pretreated corn straws. The 10 % solid content and cellulase Spezyme CP at the dose level 20 FPU / g biomass were used. After 6 h pre-hydrolysis, glucose and cellobiose concentration in the hydrolysate of pretreated corn straws were 0.554 ± 0.029 and 0.364 ± 0.008 g/100 g, respectively.

1 **Table 1 Plasmids and microbial strains used in this study**

2

Strains	Essential properties	Source or reference
Escherichia coli Top10	F- *mcr*A(*mrr-hsd* RMS-*mcr*BC)80 *lac*ZM15 *lac*X74 *rec* A1 *ara*139 (*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
S.cerevisiae W303-1A	*MAT*a *ade*2 *trp*1 *his*3 *can*1 *ura*3 *leu*2	In our lab
S.cerevisiae 7N1a	Wild type. Used for the donor of *URA*3 gene	In our lab
Saccharomycopsis fibuligera	Wild-type. Used for the donor of β-glucosidase I enzyme encoding gene *BGL*1 (named as Sf *BGL*1)	In our lab
YC-Sf-C	W303-1A (YCplac33-P*tpi-xyn*2s-Sf *BGL*1-*cwp*2-T*adh*I)	In this study
YC-Sf	W303-1A (YCplac33-P*tpi-xyn*2s-Sf *BGL*1-T*adh*I)	In this study
YE-Sf-C	W303-1A (YEplac195- P*tpi-xyn*2s-Sf *BGL*1-*cwp*2-T*adh*I)	In this study
YE-Sf	W303-1A (YEplac195- P*tpi-xyn*2s-Sf *BGL*1-T*adh*I)	In this study
G-Sf-C	W303-1A (H::*URA*-P*tpi-xyn*2s-Sf *BGL*1-*cwp*2-T*adh*I)	In this study
G-Sf	W303-1A (H::*URA*-P*tpi-xyn*2s-Sf *BGL*1-T*adh*I)	In this study
YC-Aa-C	W303-1A (YCplac33-P*tpi-xyn*2s-Aa *BGL*1-*cwp*2-T*adh*I)	In this study
YC-Aa	W303-1A (YCplac33-P*tpi-xyn*2s-Aa *BGL*1-T*adh*I)	In this study
YE-Aa-C	W303-1A (YEplac195- P*tpi-xyn*2s-Aa *BGL*1-*cwp*2-T*adh*I)	In this study
YE-Aa	W303-1A (YEplac195- P*tpi-xyn*2s-Aa *BGL*1-T*adh*I)	Wang et al.,2013
G-Aa-C	W303-1A (H::*URA*-P*tpi-xyn*2s-Aa *BGL*1-*cwp*2-T*adh*I)	In this study
G-Aa	W303-1A (H::*URA*-P*tpi-xyn*2s-Aa *BGL*1-T*adh*I)	In this study
YC	W303-1A (YCplac33)	Zhang et al.,2012
YE	W303-1A (YEplac195)	Wang et al.,2013

3 Sf *: Saccharomycopsis fibuligera*; Aa: *Aspergillus aculeatus*

4

Table 2 The growth parameters of three YCplac33-based strains aerobically in different media

strain	Time into stationary phase (h)	OD_{660} value into stationary phase	Lag period (h)	Specific growth rate (max), μ_m (h^{-1})
YC-Sf-C-YPG	~ 40	12.41	≤ 4 h	0.3617
YC-Sf-YPG	~ 40	13.56	≤ 4 h	0.3767
YC-YPG	~ 40	13.23	≤ 4 h	0.3700
YC-Sf-C-YPC	~ 88	10.90	52-64	0.0787
YC-Sf-YPC	~ 64	12.30	40	0.1021
YC-Sf-C-CMG	~ 28	4.32	4	0.3380
YC-Sf-CMG	~ 28	4.65	4	0.3440
YC-CMG	~ 28	4.43	4	0.3389
YC-Sf-C-CMC	36~40	3.52	16	0.1619
YC-Sf-CMC	28~ 36	3.96	12	0.2275

Table 3 The growth parameters of fourteen strains aerobically in YPC medium

strain	Time into stationary phase (h)	OD_{660} into stationary phase	Ratio to OD_{660} of strain G-Sf-C	Lag period (h)	Specific growth rate (max), μ_m (h^{-1})	Ratio to μ_m value of strain G-Aa-C
YE-Sf-C	—	1.31		—	—	
YE-Sf	~48	14.05	1.10	≤12	0.1377	2.01
YC-Sf-C	~90	13.15	1.03	54-60	0.0746	1.09
YC-Sf	~60	13.15	1.03	42	0.1142	1.67
G-Sf-C	~96	12.75	1.00	72	0.0685	1.00
G-Sf	~90	13.29	1.04	54-60	0.0700	1.02
YE-Aa-C	~42	15.62	1.23	≤12	0.1475	2.15
YE-Aa	~36	16.24	1.27	≤12	0.2005	2.93
YC-Aa-C	~(72-78)	13.97	1.10	48	0.0888	1.30
YC-Aa	~54	14.92	1.17	≤12	0.1283	1.87
G-Aa-C	~96	13.79	1.08	60	0.0796	1.16
G-Aa	~72	14.08	1.10	48	0.1054	1.54
YE	—	1.30		—	—	
YC	—	1.21		—	—	

Table 4 The growth, fermentation and enzymatic activity of fourteen strains anaerobically in YPC medium

strain	Growth			Ethanol production				Activity (max) U/ mL/OD_{660}	
	Time into stationary phase, h	OD_{660} into stationary phase	Ratio to OD_{660} of YE-Aa	Time into ethanol plateau, h	Ethanol titer (max) g /100 g	Ethanol yield [a]	Ratio to ethanol yield of G-Sf-C	Total	Extra-cellular
YE-Sf-C	—	1.42		ND[b]	—	—	—	—	—
YE-Sf	36	8.38	1.13	36	0.792	75.5	1.16	1.063	0.532
YC-Sf-C	60	10.16	1.37	60	0.714	68.1	1.05	0.230	0.013
YC-Sf	48	10.12	1.37	48	0.751	71.6	1.10	0.429	0.181
G-Sf-C	72	10.26	1.38	72	0.683	65.1	1.00	0.177	0.007
G-Sf	60-72	9.74	1.31	60-72	0.733	69.9	1.07	0.203	0.086
YE-Aa-C	24	8.06	1.09	24	0.729	69.6	1.07	1.167	0.027
YE-Aa	24	7.41	1.00	24	0.795	75.8	1.16	2.015	0.944
YC-Aa-C	24-36	9.97	1.35	24-36	0.723	69.0	1.06	0.959	0.021
YC-Aa	24-36	9.33	1.26	24-36	0.782	74.6	1.14	1.257	0.532
G-Aa-C	60	10.10	1.36	60	0.723	69.0	1.06	0.245	0.006
G-Aa	48	9.75	1.32	48-60	0.744	71.0	1.09	0.266	0.119
YE	—	1.33		ND	—	—	—	—	—
YC	—	1.23		ND	—	—	—	—	—

[a] Shown as % of the theoretical yield; [b] ND, not detected.

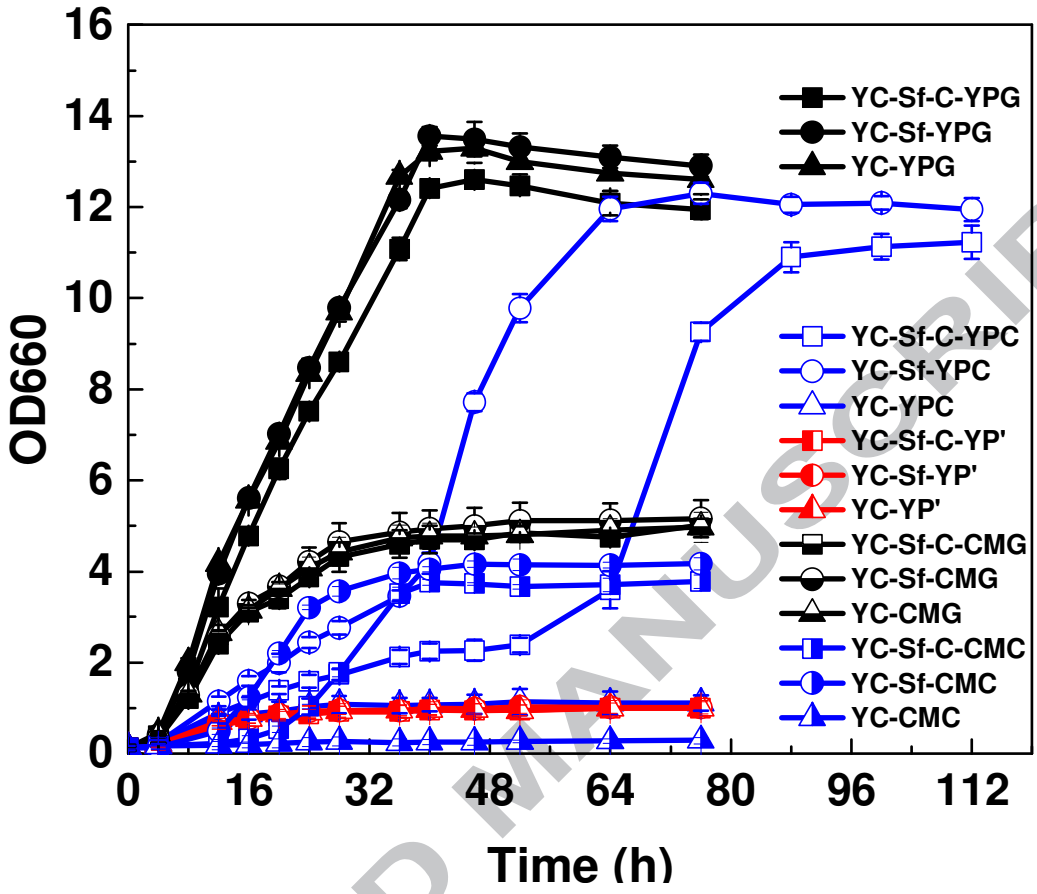

Fig.1 The growth curves of three YCplac33-based strains aerobically in different media

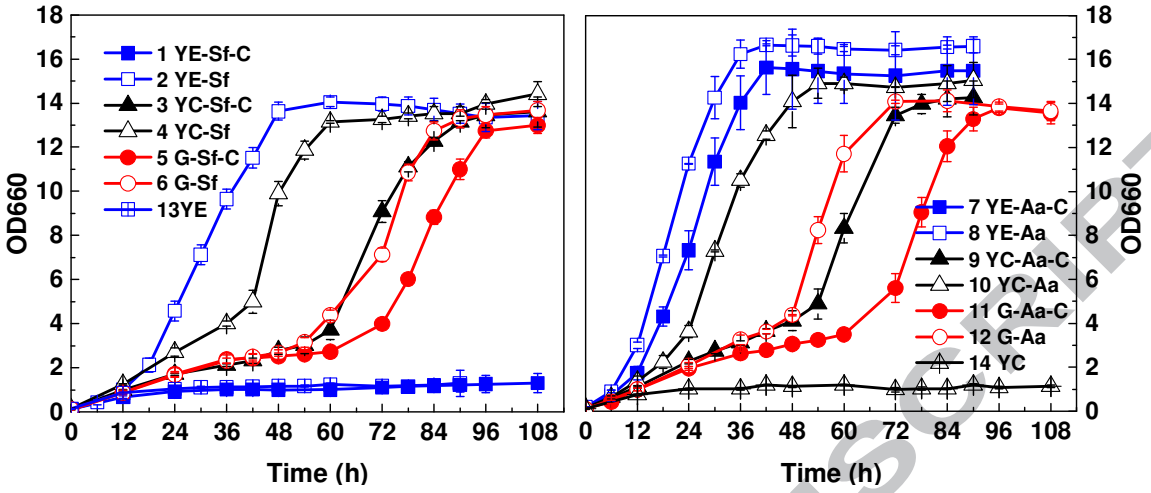

Fig.2 The growth curves of fourteen strains aerobically in YPC medium

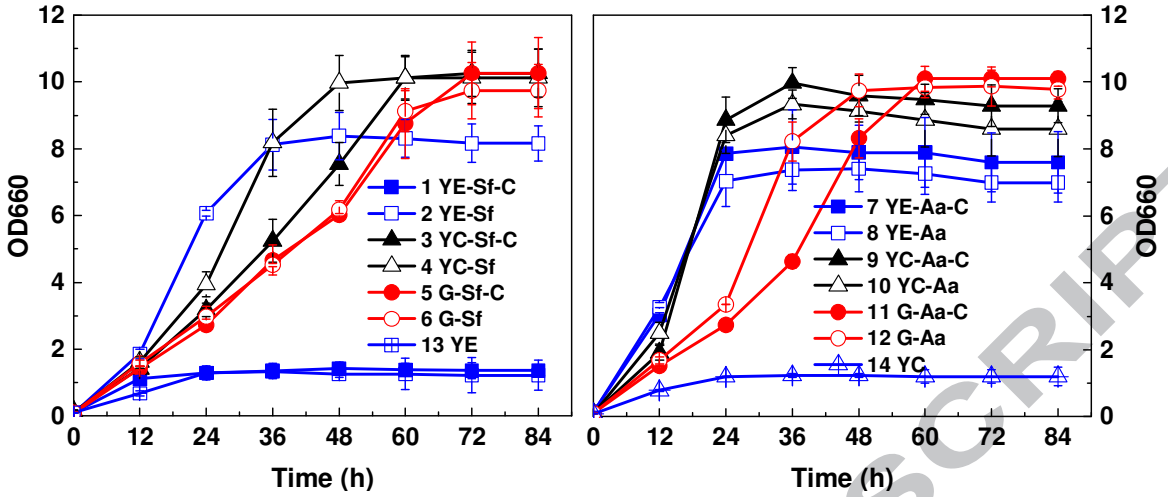

Fig.3 The growth curves of fourteen strains anaerobically in YPC medium

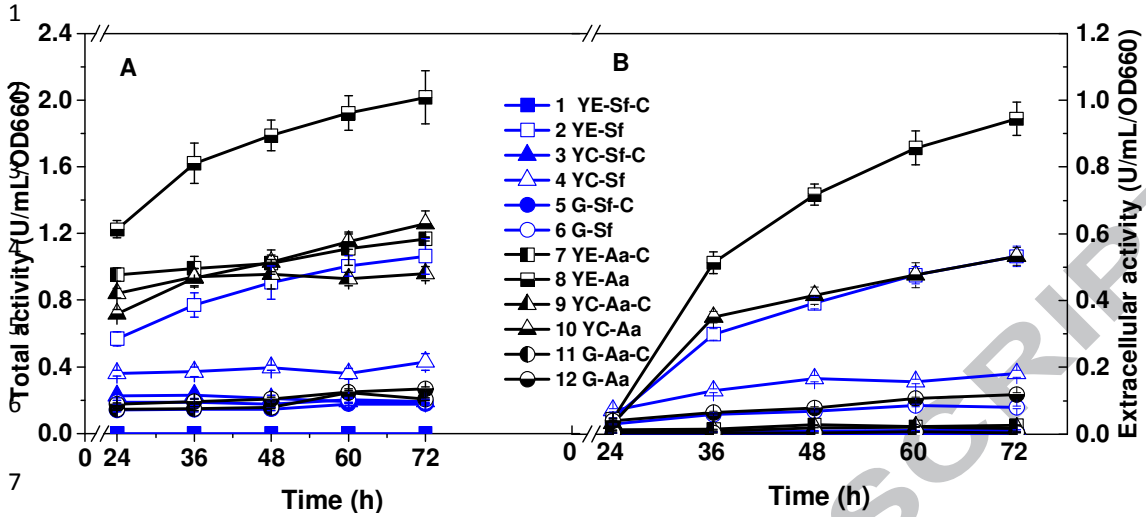

Fig.4 The beta-glucosidase activity curves of fourteen strains anaerobically in YPC medium (U/mL/OD660). (A) Total activity; (B) Extracellular activity.

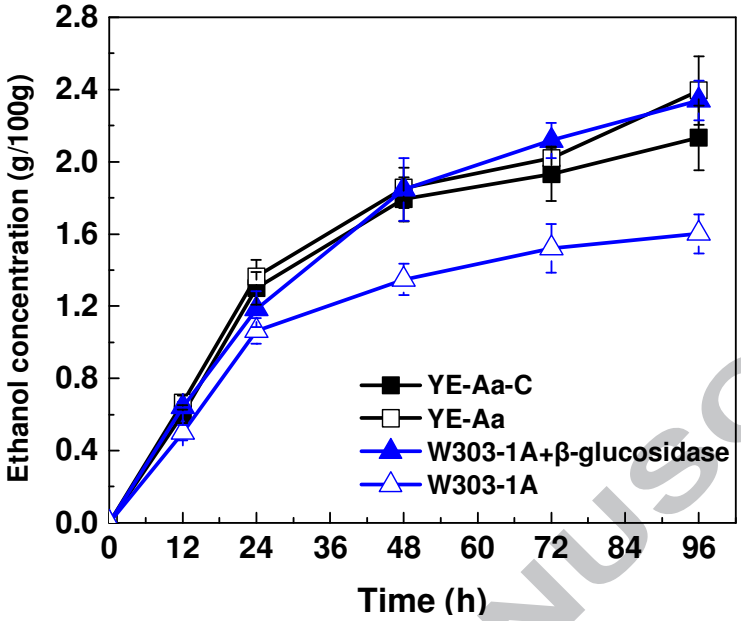

Fig.5 Ethanol production during simultaneous saccharification and fermentation of three *S.cerevisiae* strains from pretreated corn straws. The 10 % solid content and cellulase Spezyme CP at the dose level 20 FPU / g biomass were used. After 6 h pre-hydrolysis, glucose and cellobiose concentration in the hydrolysate of pretreated corn straws were 0.554 ± 0.029 and 0.364 ± 0.008 g/100 g, respectively.

Highlights (⩽ 85 characters)

- β - Glucosidase is an important enzyme in medicine and industry (cellulosic ethanol)
- Fourteen recombinant yeast strains were constructed and evaluated in cellobiose media
- Difference shown on activity; growth lag period, μ_m, biomass; ethanol titer, rate, yield
- Extra metabolic burden from displaying over secreting, Aa *BGL*I superior to Sf *BGL*I
- The study helps to optimize expressing strategy in biotechnology and yeast breeding