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RNA-Seq-based transcriptomic analysis of *Saccharomyces cerevisiae* during solid-state fermentation of crushed sweet sorghum stalks

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ABSTRACT

Bioethanol production based on solid-state fermentation (SSF) of sweet sorghum stalks has been demonstrated to have great potential due to the its low pollution and low cost. A novel *S. cerevisiae* strain TSH3 exhibited better SSF performance compared with BY4743 during SSF of sweet sorghum stalks. High-quality total RNA of *S. cerevisiae* was extracted from SSF mixture and the global gene expression profiles during SSF were studied using RNA-Seq. Compared with BY4743, TSH3's genes related to ribosome biogenesis, amino acid and coenzyme metabolism during early fermentation stage, secondary metabolite biosynthesis, metabolism in diverse environment during middle fermentation stage, and lipid metabolism during late fermentation stage were up-regulated; while the genes involved in fatty acid metabolism and peroxisome during early fermentation stage, ribosome biogenesis during middle fermentation stage, and mitotic cell cycle during late fermentation stage were down-regulated. Further dynamic analysis of TSH3's transcriptome reveals its three different metabolic stages: 1) ribosome biogenesis and respiration-fermentation transition; 2) biosynthesis of secondary metabolites and stress resistance; 3) plasma membrane related metabolism for stress resistance. These findings provided insight into the *S. cerevisiae* transcriptome during SSF of sweet sorghum stalks and suggest that TSH3 would be an ideal candidate for SSF-based bioethanol production.

1. Introduction

With the increasing deletion of fossil energy, bioethanol has become a transitional solution [1]. Sweet sorghum is considered to be a promising feedstock for biofuel production owing to its wide adaptability to land and climate and its high tolerance to adversity [2]. However, the crushing rate of sweet sorghum is low due to its sponge-like pith, which limits its application for ethanol production [3–5]. To solve this problem, a novel advanced solid-state fermentation (ASSF) technology has been developed to produce ethanol using crushed sweet sorghum stalks [6–9], demonstrating that solid-state fermentation (SSF) can be applied at industrial scale for bioethanol production [7].

In recent years, transcriptome sequencing (RNA-Seq) has become a powerful tool to obtain a snapshot of a cell's transcriptome and can thereby develop a deep understanding of its physiological state [10]. Although the transcriptomes of *Saccharomyces cerevisiae* have been studied under various conditions [11–14], these studies are based on liquid-state fermentation and the transcriptome during SSF is rarely studied. This is especially the case for SSF using sweet sorghum stalks as feedstock. During SSF, the diffusion of nutrients and metabolites and the movement of cells are limited as a result of the low level of free water, which may lead to local gradients of temperature, moisture, pH, nutrients and metabolites [10,15]. Therefore, the growth, gene expression and metabolism of yeast cells during SSF differ from those during liquid-state fermentation [10,16–18].

Recently, a novel wild-type *S. cerevisiae* strain diploid strain TSH3 has been isolated from the stalk surface of SO_2-treated sweet sorghum. This strain has shown remarkable thermotolerance, high ethanol productivity and great potential as a host for metabolic engineering modification [19], and therefore is considered as a candidate strain for ASSF-based ethanol production.

In the present study, RNA-Seq-based transcriptomic analysis was utilized to study the global transcriptional response of *S. cerevisiae* TSH3 during SSF of crushed sweet sorghum stalks. Since BY4743 is a commonly used diploid laboratory strain with a clear genomic background, it was chosen as the control strain in order to see the differences in gene expression between the potential industrial strain TSH3 and the laboratory strain BY4743. TSH3's advantages compared with BY4743 and the dynamics of TSH3's transcriptome between different stages of SSF were investigated. To our knowledge, this is the first study on the

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transcriptomic analysis of *S. cerevisiae* during solid-state fermentation of sweet sorghum stalks and can provide guidance for microbial metabolic engineering towards industrial production.

2. Materials and methods

2.1. Strains and media

Saccharomyces cerevisiae diploid strain TSH3, which was isolated from the stalk surface of SO_2-treated sweet sorghum [19], was used for fermentation experiments and RNA extraction all through this study. The diploid strain BY4743 (a/*αhis3Δ/his3Δ leu2Δ/leu2Δ* +/*lys2Δ met15Δ/*+ *ura3Δ/ura3Δ*) [9], which is commonly used in laboratory research, was used as the control strain. The seed suspension of both strains was grown in YPD medium (1% yeast extract, 2% peptone and 2% glucose) before solid-state fermentation. Sweet sorghum stalks were crushed into particles of 1–2 mm in diameter, and 3–20 mm in length as described by Li et al. [8] and the crushed sweet sorghum stalks were used as feedstock for solid-state fermentation.

2.2. Solid-state fermentation

The solid-state fermentation of crushed sweet sorghum stalks was conducted as described previously [9]. An exponential-phase yeast culture in YPD was washed with sterilized water and the cell density was adjusted to OD_{600} = 10.0. Then 100 ml cell suspension was mixed with 1 kg crushed sweet sorghum stalks to set the moisture content to be 70% in a shrunk rotary-drum fermenter, which was 0.25 m in length and 0.1 m in diameter, and then fully blended by rotating at 5 rpm in a 4 °C incubator for 20 min. 500-ml Erlenmeyer flasks were used for solid-state fermentation and each were loaded with 100 g blended substrates. The flasks were equipped with airlocks for oxygen-limited conditions, ensuring the exclusion of oxygen but allowing the release of other gases such as CO_2 (Fig. 1A). All the experiments were conducted at 30 °C with three biological replicates. The flasks were weighed at intervals of 2 h to calculate the weight loss, which is an indirect indicator of ethanol production.

In order to quantitatively investigate the SSF processes, the weight loss data of the two strains were fitted with logistic model [20]:

$$y = A_2 + \frac{A_1 - A_2}{1 + \left(\frac{x}{x_0}\right)^p}$$

where A_1, A_2, x_0 and p are parameters of logistic model.

Then the first-order derivative functions of weight loss-time functions were calculated to study the fermentation speed variations of the two strains.

2.3. Analytical method

The fermented sweet sorghum bagasse was sampled to measure the concentrations of residual sugar and ethanol. 30 g of sweet SSF mixture was stirred in a blender with 300 ml distilled water at room temperature and then sonicated for 15 min. The concentrations of ethanol and residual sugar in the supernatant were measured using high performance liquid chromatography (HPLC) with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA). The concentration of residual sugar (g/100 g substrate) is defined as the total concentration of sucrose (g/100 g substrate), glucose (g/100 g substrate) and fructose (g/100 g substrate). The metabolic yield (g ethanol/g residual sugar) is calculated by dividing the weight of ethanol produced by the weight of residual sugar consumed. The mass productivity (g ethanol/100 g substrate) is defined as the weight of ethanol produced from 100 g substrate per unit time.

2.4. Sample preparation for RNA-Seq

5 g of fermented sweet sorghum bagasse was added into 10 ml precooled LETS buffer (0.2 M LiCl, 0.02 M EDTA, 0.02 mM Tris·HCl pH 8.0, 0.4% SDS) and vortex mixed for 3–4 min. After centrifugation, 2 ml supernatant was used for total RNA extraction using EZNA® Yeast RNA Kit (Omega Bio-tek, Doraville, CA). The RNA samples were sent to SinoGene Scientific (Beijing, China) for further quality and quantity evaluation, cDNA library preparation, and sequencing.

2.5. RNA-Seq and bioinformatics analysis

The transcriptional profiles at different points of the SSF process were investigated using RNA-Seq. The read counts were adjusted by edgeR program package through one scaling normalized factor for each sequenced library. Differential expression analysis of two conditions was performed using the DEGSeq R package (1.20.0) [21]. The P values were adjusted using the Benjamini & Hochberg method. P value of 0.05 and $\log_2$(Fold change) of 1 were set as the threshold for significantly differential expression. Then the differentially expressed genes (DEGs) were selected for clustering analysis. Gene Ontology (GO) enrichment analysis of DEGs was implemented by the GOseq R package [22]. KOBAS software was used to test the statistical enrichment of DEGs in KEGG pathways [23]. GO terms and KEGG pathways with P values less than 0.05 were considered significantly enriched. The interactions of DEGs were predicted in the STRING database (http://string-db.org/) [24]. The FASTQ files containing the sequenced reads have been deposited in the EBI-ENA database under accession number PRJEB24828.

3. Results and discussion

3.1. SSF of crushed sweet sorghum stalks

In order to investigate the differences in SSF performance between TSH3 and BY4743, a mini batch SSF system was established using 500-ml flasks filled with crushed sweet sorghum stalks and sealed with airlocks (Fig. 1A). The weight loss of iTSH3 reached the maximum within 24 h, exhibiting much higher speed than BY4743 (more than 38 h) (Fig. 1C). That is to say that TSH3 produced gas more quickly and propagated better than BY4743. Meanwhile, the trends of ethanol production and sugar consumption were consistent with those of weight loss. The ethanol produced by TSH3 reached its maximum 5.71 g/100 g blended substrate within 18 h, while the highest ethanol concentration produced by BY4743 (5.81 g/100 g blended substrate) happened until 38 h (Fig. 1B, C). To further evaluate the performance of ethanol fermentation, the mass productivity and metabolic yield of each strain between 6 and 24 h were calculated. Even though TSH3 had a slightly lower metabolic yield (0.38 g ethanol/g sugar) than BY4743 (0.40 g ethanol/g sugar) between 6 and 24 h, its mass productivity (0.24 g ethanol/100 g blended substrate h^{-1}) was 51.4% higher than that of BY4743 (0.16 g ethanol/100 g blended substrate h^{-1}) (Table 1). To further investigate the SSF processes of TSH3 and BY4743, the weight loss data were fitted with logistic model and the results show that both sets of data fitted the model well ($R^2 > 0.99$) (Fig. 1C and Appendix A). Then the first-order derivative functions of weight loss-time functions were calculated to show the variations of fermentation speed as a function of time. The maximum fermentation speed of TSH3 appeared after around 8 h after inoculation, while that of BY4743 happened about 5 h later (Fig. 1D). All the above results suggest that TSH3 had significantly higher speed of ethanol fermentation than BY4743 when using crushed sweet sorghum stalks as the sole feedstock, which makes it a promising strain for using SSF-based ethanol production. It should be noted that the crushed sweet sorghum stalks were not sterilized prior to inoculation in order to simulate industrial SSF to the maximum extent. However, ethanol fermentation was not significantly affected by the microorganisms from the feedstock and the environment, just like