

## Medium Optimization for Improved Ethanol Production in Very High Gravity Fermentation\*

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**Abstract** An optimal medium (300 g·L<sup>-1</sup> initial glucose) comprising 6.3 mmol·L<sup>-1</sup> Mg<sup>2+</sup>, 5.0 mmol·L<sup>-1</sup> Ca<sup>2+</sup>, 15.0 g·L<sup>-1</sup> peptone and 21.5 g·L<sup>-1</sup> yeast extract was determined by uniform design to improve very high gravity (VHG) ethanol fermentation, showing over 30% increase in final ethanol (from 13.1% to 17.1%, by volume), 29% decrease in fermentation time (from 84 to 60 h), 80% increase in biomass formation and 26% increase in glucose utilization. Experiments also revealed physiological aspects linked to the fermentation enhancements. Compared to the control, trehalose in the cells grown in optimal fermentation medium increased 17.9-, 2.8-, 1.9-, 1.8- and 1.9-fold at the fermentation time of 12, 24, 36, 48 and 60 h, respectively. Its sharp rise at the early stage of fermentation when there was a considerable osmotic stress suggested that trehalose played an important role in promoting fermentation. Meanwhile, at the identical five fermentation time, the plasma membrane ATPase activity of the cells grown in optimal medium was 2.3, 1.8, 1.6, 1.5 and 1.3 times that of the control, respectively. Their disparities in enzymatic activity became wider when the glucose levels were dramatically changed for ethanol production, suggesting this enzyme also contributed to the fermentation improvements. Thus, medium optimization for VHG ethanol fermentation was found to trigger the increased yeast trehalose accumulation and plasma membrane ATPase activity.

**Keywords** medium optimization, very high gravity fermentation, trehalose, plasma membrane ATPase

### 1 INTRODUCTION

The finiteness of the global crude oil reserve and the excessive consumption of fossil fuels leading to environmental problems have stimulated considerable interest in alternative energy sources in recent years. Ethanol, both renewable and environmentally benign, is regarded as one of the best alternatives. Although lignocellulose (found naturally as agricultural waste, industrial waste, forestry residues and municipal solid waste, etc.) may finally become the cheapest and most abundant raw material to produce fuel ethanol, the mature technologies required for the efficient and economical conversion have not yet been realized [1]. Currently, the ripe technologies for ethanol production are crop-based, employing feedstocks such as sugar cane juice and corn starch. And ethanol yield based on the ratio of ethanol produced to sugar consumed has achieved as high as 90%–95% of the theoretical maximum under industrial conditions [2]. One of the further improvements can be attained by fermenting greater amounts of sugar by a process known as very high gravity (VHG) fermentation, a technology being defined as preparation and fermentation of mashes containing 27 or more grams of dissolved solids per 100 grams mash [3].

VHG ethanol fermentation can potentially attain higher final ethanol concentration in fermentation broth and consequently substantial energy savings can be achieved in the downstream distillation and waste distillate treatment, which is of economical importance since the energy cost is the second largest part in ethanol production [4]. However, as the sugar concen-

tration increases, the yeast is exposed to severe conditions such as high osmotic pressure, increased product inhibition and nutritional limitations, which seriously affect fermentation performance because of the problem of yeast growth and viability. To tackle the issue, many efforts have been made and various medium supplements have been employed to examine their influences on fermentation performance. For example, several investigators have improved VHG ethanol fermentation by externally supplementing certain additives such as yeast extract, ammonium, urea, amino acids, vitamins and horse gram [2, 5–8]. Similar investigations were performed in the case of wine with supplements such as inositol, catechin and SO<sub>2</sub> [9]. Nevertheless, these previous researches almost utilized “one-factor-at-a-time” approach, which is time-consuming and laborious and cannot address the interactions among different components. Published investigations aimed at understanding of the simultaneous effect of different nutrients being supplemented to VHG ethanol fermentation medium, a practical situation occurring in fermentation process, are rarely seen.

Hence, the purpose of this research was to evaluate the combined effect of different components in fermentation medium on VHG ethanol fermentation performance of self-flocculating yeast. In this work, the statistical approach of uniform design was employed to optimize the concentrations of medium constituents and establish a mathematical model for predicting the final ethanol level. Experiments were also conducted to understand physiological aspects linked to the significant improvements in fermentation performance by medium optimization.

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## 2 MATERIALS AND METHODS

### 2.1 Strain, media and culture conditions

The strain used in this study was a self-flocculating fusant of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* (termed fusant SPSC) [10], which was constructed by protoplast fusion technology.

Agar slope medium for culture maintenance was composed of ( $\text{g}\cdot\text{L}^{-1}$ ): glucose 10, yeast extract 3.85, peptone 3 and agar 20. Growth medium for yeast aggregates formation contained ( $\text{g}\cdot\text{L}^{-1}$ ): glucose 30, yeast extract 3.85 and peptone 3. Fermentation medium consisted of ( $\text{g}\cdot\text{L}^{-1}$ ): glucose 200, yeast extract 5 and peptone 3. Non-optimized VHG fermentation medium (used as the control medium) comprised ( $\text{g}\cdot\text{L}^{-1}$ ): glucose 300, yeast extract 5 and peptone 3.

To form yeast aggregates, a loopful of cells from agar slope medium were inoculated into a 250-ml flask containing 100 ml growth medium and cultivated on a rotary shaker ( $150\text{ r}\cdot\text{min}^{-1}$ ) at  $30\text{ }^{\circ}\text{C}$  for 18 h. To perform batch ethanol fermentation, the prepared yeast aggregates were inoculated into 200 ml fermentation medium in a 500-ml flask to a final cell density of  $(5\text{--}6)\times 10^7$  viable cells per liter and then cultivated at  $30\text{ }^{\circ}\text{C}$ .

### 2.2 Analytical methods

#### 2.2.1 Ethanol

Ethanol concentration was determined by gas chromatography (Agilent 6890A GC, USA, equipped with flame ionization detector) as described previously [10]. The temperature of the inlet and of the detector was  $160\text{ }^{\circ}\text{C}$  and  $230\text{ }^{\circ}\text{C}$ , respectively, and oven was operated isothermally at  $90\text{ }^{\circ}\text{C}$ . The carrier gas was  $\text{N}_2$  and *n*-butanol was used as the internal standard.

#### 2.2.2 Trehalose

The intracellular trehalose was measured basically according to anthrone method [11]. Yeast cells harvested by centrifugation at  $4\text{ }^{\circ}\text{C}$  were washed three times with ice-cold distilled water, weighed and frozen at  $-20\text{ }^{\circ}\text{C}$  until use. For trehalose determination, the frozen cells were thawed in approximately three times their volumes of  $500\text{ mmol}\cdot\text{L}^{-1}$  trichloroacetic acid, mixed vigorously and followed by incubation at room temperature for 35–40 min to extract trehalose from the cells. The extraction was repeated twice and the supernatants from the two extractions were combined for trehalose measurement. A  $250\text{ }\mu\text{l}$  aliquot of the obtained sample was supplemented with 1 ml anthrone reagent, mixed vigorously and then incubated in boiling water for 10 min. After that, the sample was cooled immediately in water and the absorbance at  $630\text{ nm}$  was measured.

#### 2.2.3 Plasma membrane ATPase

Plasma membrane ATPase activity was assayed based on the method of Serrano [12] with some modifications. Yeast cells were permeabilized by adding a mix-

ture of toluene : ethanol : 10% triton X-100 (1 : 4 : 1) to cells suspension containing  $100\text{ g}\cdot\text{L}^{-1}$  sorbitol and then shaking vigorously for 4–5 min. Cells collected by centrifugation were washed twice with  $100\text{ g}\cdot\text{L}^{-1}$  sorbitol and then suspended in sorbitol and kept in ice until use. The ATPase assay mixture with a total volume of 1.0 ml contained  $50\text{ mmol}\cdot\text{L}^{-1}$  2-(*N*-morpholino) ethane sulfonic acid,  $5\text{ mmol}\cdot\text{L}^{-1}$   $\text{MgCl}_2$ ,  $0.5\text{ mol}\cdot\text{L}^{-1}$  lactose,  $0.2\text{ mol}\cdot\text{L}^{-1}$  KCl,  $5\text{ mmol}\cdot\text{L}^{-1}$  sodium azide and  $100\text{ mmol}\cdot\text{L}^{-1}$   $\text{KNO}_3$ . The toluene-permeabilized cells (5 mg, fresh mass) were then added to the assay mixture and maintained at  $30\text{ }^{\circ}\text{C}$  for 5 min. The reaction was started by addition of  $20\text{ }\mu\text{l}$  of  $0.1\text{ mol}\cdot\text{L}^{-1}$  sodium ATP and after 10 min of incubation at  $30\text{ }^{\circ}\text{C}$  it was ceased with 2 ml of a solution containing 2% (by volume) sulfuric acid,  $5\text{ g}\cdot\text{L}^{-1}$  ammonium molybdate and  $5\text{ g}\cdot\text{L}^{-1}$  sodium lauryl sulphate. The phosphomolybdate was reduced with  $20\text{ }\mu\text{l}$  of  $100\text{ g}\cdot\text{L}^{-1}$  ascorbic acid and the absorbance at  $750\text{ nm}$  was recorded after 5 min. One unit of enzyme is defined as the amount of yeast cells (dry mass) required to liberate one nmol inorganic phosphorus per min under the conditions described above. Specific activity is expressed as units per milligram of dry cells.

### 2.3 Uniform design and data analysis

In this work, yeast extract, peptone,  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  were selected as the four independent variables with each factor at ten levels and supplemented to the fermentation medium according to uniform design (see the details in Results and discussion). Selecting  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  as factors was based on our previous experiments in which each ion was found to increase yeast ethanol tolerance and thus fermentation was improved [10, 13]. All the data analysis processes such as data regression and determination of the best combination of the factor values were conducted by the statistical software (DPS version 7.05), which is popular and commercially available in China [14].

### 2.4 Statistical method

The presented results are expressed as mean  $\pm$ SEM (standard error of mean) obtained from three independent experiments and error bars correspond to SEM.

## 3 RESULTS AND DISCUSSION

### 3.1 Optimization of medium components for VHG fermentation

Uniform design has been widely applied to multivariable optimization in many fields [14]. It is one of the best design techniques due to its advantage of requiring fewer experiments that lead to higher working efficiency [15]. In this work, to understand the combined effect of yeast extract, peptone,  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$

**Table 1** Factors and levels in 10 runs of a uniform design and the experimental results

| Run | Factor                    |                      |                           |                      |                 |                   |                       |                   | Ethanol<br>concentration/%<br>(by volume) |
|-----|---------------------------|----------------------|---------------------------|----------------------|-----------------|-------------------|-----------------------|-------------------|-------------------------------------------|
|     | $X_1$ (Mg <sup>2+</sup> ) |                      | $X_2$ (Ca <sup>2+</sup> ) |                      | $X_3$ (peptone) |                   | $X_4$ (yeast extract) |                   |                                           |
|     | level                     | mmol·L <sup>-1</sup> | level                     | mmol·L <sup>-1</sup> | level           | g·L <sup>-1</sup> | level                 | g·L <sup>-1</sup> |                                           |
| 1   | 4                         | 4.0                  | 6                         | 3.0                  | 1               | 3.0               | 4                     | 11.7              | 14.6±0.4                                  |
| 2   | 7                         | 7.0                  | 7                         | 3.5                  | 10              | 15.0              | 7                     | 18.3              | 16.3±0.3                                  |
| 3   | 9                         | 9.0                  | 4                         | 2.0                  | 2               | 4.3               | 8                     | 20.6              | 15.3±0.6                                  |
| 4   | 1                         | 1.0                  | 2                         | 1.0                  | 4               | 7.0               | 6                     | 16.1              | 15.6±0.5                                  |
| 5   | 10                        | 10.0                 | 9                         | 4.5                  | 7               | 11.0              | 5                     | 13.9              | 15.1±0.3                                  |
| 6   | 8                         | 8.0                  | 1                         | 0.5                  | 6               | 9.7               | 3                     | 9.4               | 15.2±0.4                                  |
| 7   | 3                         | 3.0                  | 10                        | 5.0                  | 5               | 8.3               | 9                     | 22.8              | 15.9±0.5                                  |
| 8   | 5                         | 5.0                  | 3                         | 1.5                  | 8               | 12.3              | 10                    | 25.0              | 16.1±0.2                                  |
| 9   | 6                         | 6.0                  | 8                         | 4.0                  | 3               | 5.7               | 1                     | 5.0               | 12.6±0.3                                  |
| 10  | 2                         | 2.0                  | 5                         | 2.5                  | 9               | 13.7              | 2                     | 7.2               | 14.2±0.2                                  |

**Table 2** Parameters for VHG ethanol fermentation (300  $\text{g}\cdot\text{L}^{-1}$  initial glucose) performed with optimal and control media respectively

| Fermentation parameters                                                 | Control     | Optimal     |
|-------------------------------------------------------------------------|-------------|-------------|
| final ethanol concentration/% (by volume)                               | 13.1±0.4    | 17.1±0.6    |
| ethanol production rate/ $\text{g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ | 1.23±0.05   | 2.24±0.08   |
| ethanol yield/ $\text{g}\cdot\text{g}^{-1}$ (based on glucose)          | 0.440±0.002 | 0.456±0.005 |
| glucose utilization/%                                                   | 78.3±2.8    | 98.5±0.1    |
| maximal biomass (dry cells)/ $\text{g}\cdot\text{L}^{-1}$               | 4.70±0.25   | 8.47±0.54   |

Note: Paired student  $t$  tests were performed with all  $P$  values <0.01, except  $P$  value for ethanol yield being 0.042.

which were simultaneously supplemented to the fermentation medium, a uniform design based on four factors with each factor at ten levels was employed. The detailed design and experimental results are shown in Table 1.

Utilizing the statistical software DPS version 7.05, a mathematical model was constructed by step-wise regression analysis for predicting final ethanol concentration:

$$\begin{aligned} \text{Ethanol concentration} = & 14.7 - 1.54X_2 - 0.429X_3 + \\ & 0.379X_4 - 0.00166X_1^2 + 0.254X_2^2 + \\ & 0.0286X_3^2 - 0.00855X_4^2 \end{aligned}$$

where  $X_1$ ,  $X_2$ ,  $X_3$  and  $X_4$  represent the concentration of  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ , peptone and yeast extract, respectively.

Meanwhile, the values of  $F$ ,  $P$  and  $R$  (multiple correlation coefficient) were determined to be 138.0, 0.0072 and 0.999, respectively. These data indicate that the above regression equation is significant at the 99% confidence level and there is a good agreement between the experimental and predicted values. An optimal supplementation for the VHG ethanol fermentation medium containing 300  $\text{g}\cdot\text{L}^{-1}$  initial glucose was then established as a mixture of 6.3  $\text{mmol}\cdot\text{L}^{-1}$

$\text{Mg}^{2+}$ , 5.0  $\text{mmol}\cdot\text{L}^{-1}$   $\text{Ca}^{2+}$ , 15.0  $\text{g}\cdot\text{L}^{-1}$  peptone and 21.5  $\text{g}\cdot\text{L}^{-1}$  yeast extract. Under the optimal condition, 17.1% (by volume) of final ethanol in the fermentation broth was obtained, representing over 30% increase compared with 13.1% (by volume) of the control level achieved under non-optimization condition (Table 2). The predicted value of final ethanol level was 17.4% (by volume), showing that there was a very good accuracy between the experimental and predicted data. Medium optimization also resulted in other improvements. The time required to end fermentation was shortened from 84 to 60 h (data not shown), and cells growth, substrate conversion and product productivity were increased by 80%, 26% and 82%, respectively (Table 2).

To improve VHG ethanol fermentation, many efforts have been made and media supplementations by single factor experiments have been frequently attempted. However, literatures regarding investigations into the combined impact of nutrients simultaneously supplemented to medium on VHG fermentation performance are still rarely found. In recent year, Wang and the co-workers [16] have reported a research leading to an increase in final ethanol level by around 20% via optimizing a medium. Many investigations performed in this area in the future may help accumulate more

knowledge to further improve VHG ethanol fermentation.

### 3.2 Physiological changes resulting from medium optimization

#### 3.2.1 Intracellular trehalose level

By optimizing the concentrations of medium constituents, the VHG ethanol fermentation performance was significantly improved. Then, further experiments to understand possible aspects involved in such enhancements were performed. Trehalose, a well-known storage carbohydrate for *Saccharomyces cerevisiae*, also functions as a stress protectant [17, 18]. Therefore, we investigated whether the content of this metabolite in yeast cells altered during VHG ethanol fermentation. As shown in Fig. 1, at the five points of time (12, 24, 36, 48 and 60 h), the amount of trehalose in the cells grown in the optimal fermentation medium was 17.9, 2.8, 1.9, 1.8 and 1.9 times that of the control, respectively. Furthermore, the amount of trehalose built up in the cells grown in the optimal medium reached its maximal level after 24 h of inoculation into fermentation medium, and at 24 h ethanol in fermentation broth was 10.4% (by volume) (Fig. 2),

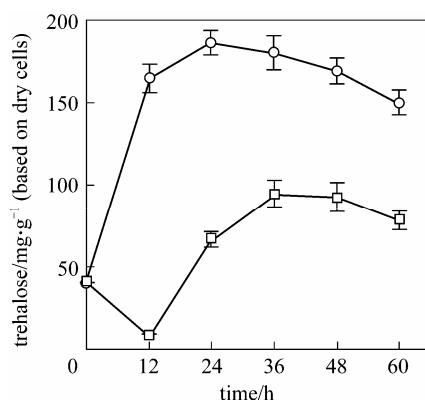


**Figure 1** Changes in intracellular trehalose content of cells in VHG ethanol fermentation process (Paired student *t* tests were performed with all *P* values <0.01)

□ control; ○ cells grown in optimal fermentation medium

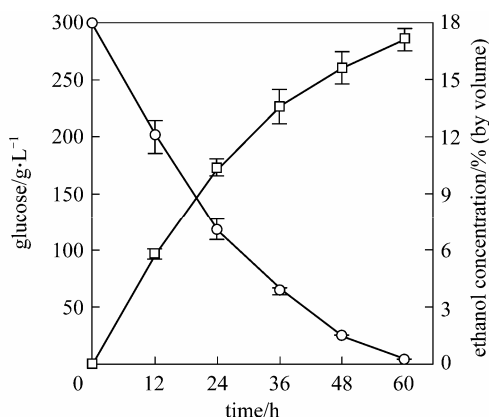


**Figure 2** Time course of VHG ethanol fermentation performed with optimal fermentation medium

□ ethanol; ○ glucose

accounting for over 60% of total ethanol (17.1%, by volume) finally obtained. By comparison, trehalose level in the control cells at 24 h was much lower, which corresponded to 4.3% (by volume) ethanol produced in fermentation broth (result not shown). Accumulating more trehalose in the cells grown in the optimal fermentation medium was persistently observed to the end of fermentation. On the other hand, once the control cells were inoculated into the fermentation medium, trehalose was rapidly degraded (although it increased again after 12 h) (Fig. 1). Nevertheless, this phenomenon was not observed in the cells grown in the optimal medium. In contrast, optimizing medium led to a sharp rise in intracellular trehalose at the early stage of fermentation (Fig. 1) when there was a considerable osmotic stress due to very high glucose level. Recently, it has been shown that up-regulation of trehalose biosynthesis proteins occurs during VHG ethanol fermentation [19]. Thus, it is suggested that the elevated accumulation of intracellular trehalose prompted by optimizing medium helps yeast cells relieve the osmotic and ethanol stresses imposed on them, thereby promoting VHG fermentation.

#### 3.2.2 Plasma membrane ATPase activity

The plasma membrane ATPase of *Saccharomyces cerevisiae* couples ATP hydrolysis to the expulsion of protons across the membrane, thus generating a trans-membrane proton electrochemical gradient which drives active transport of nutrients such as the uptake of amino acids [20]. The enzyme is also a major component of plasma membrane, which accounts for 15%–20% of the plasma membrane proteins and seems to play a significant role in maintaining the structural integrity of the cells [21]. It has been shown that plasma membrane ATPase activity of *Saccharomyces cerevisiae* is activated *in vivo* by ethanol, which is important to maintain the intracellular pH and secondary transport mechanisms [22, 23]. To survive severe conditions such as high osmotic and ethanol stresses encountered during VHG ethanol fermentation, the yeast should be able to cope with such challenges. Thus, it is of interest to understand any alterations implicated in yeast plasma membrane ATPase under VHG fermentation conditions, since this enzyme is essential to the yeast life [24]. As illustrated in Fig. 3, at the five points of time (12, 24, 36, 48 and 60 h), the plasma membrane ATPase activity of the cells grown in the optimal fermentation medium was 2.3, 1.8, 1.6, 1.5 and 1.3 times that of the control, respectively. The disparities in enzymatic activity between the optimal and control medium conditions were particularly conspicuous at 12, 24 and 36 h (Fig. 3). Interestingly, the three points of time were associated with dramatic changes in glucose concentrations for ethanol production (Fig. 2). Hence, it is suggested that a higher activity of this enzyme achieved with medium optimization is beneficial to propelling and accelerating fermentation process.

It is known that mutations influencing the plasma membrane ATPase of *Saccharomyces cerevisiae* induce

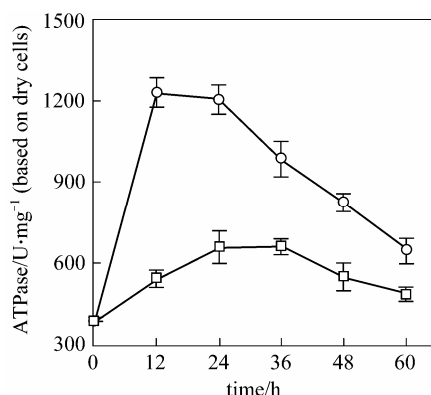


**Figure 3** Changes in plasma membrane ATPase activity of cells in VHGE ethanol fermentation process (Paired student *t* tests were performed with all *P* values <0.01, except *P* value for 60-h being 0.035)

□ control; ○ cells grown in optimal fermentation medium

an osmosensitive phenotype and its growth under high osmotic pressure is inhibited [21]. Moreover, plasma membrane ATPase activity under osmotic stress was proposed to be important for maintaining the proton gradient required to allow an elevated uptake of  $K^+$  ions to compensate high extraneous osmolality [25]. Interestingly, Trk1, the major yeast  $K^+$  importer, was found to be required for high-glucose stress tolerance in a recent report [26]. The importance of maintaining the plasma membrane ATPase activity under osmotic stress may be also attributed to its established trans-membrane proton electrochemical potential for the active transport of nutrients particularly the uptake of amino acids, since during VHGE ethanol fermentation the intracellular amino acid concentration was found to decrease in yeast cells due to the alterations exerted by osmotic stress at the level of amino acid transport via the plasma membrane, and the recovery of intracellular amino acids helped cells increase their stress tolerance [19]. Taken together, it is suggested that maintaining a relatively higher plasma membrane ATPase activity by medium optimization is another factor contributing to the improved performance of VHGE ethanol fermentation conducted in this work.

To date, no research has ever been reported on exploring yeast physiological changes triggered by medium optimization during VHGE ethanol fermentation. Such an understanding may benefit taking more rational strategies (*e.g.* metabolic engineering) to further improve fermentation performance. Thus our results presented here may assist in addressing such an issue.

## 4 CONCLUSIONS

VHGE ethanol fermentation was improved by uniform design optimization of a medium. With this medium, a final ethanol of 17.1% (by volume) was achieved within 60 h, representing over 30% increase in ethanol production and around 29% decrease in time required to end fermentation as compared with

those of the control, respectively. Other significant improvements such as cells growth and glucose conversion were also achieved. Moreover, throughout the fermentation process, the cells grown in optimal medium accumulated much more trehalose than the control, and the sharp increase of this metabolite coincided with the externally high osmotic stress. Meanwhile, the cells grown in optimal medium exhibited a noticeably higher plasma membrane ATPase activity than the control. Their differences in enzymatic activity were even larger when dramatic alterations in glucose concentrations for ethanol production happened. These data suggest that both trehalose and plasma membrane ATPase contribute to the fermentation enhancements.

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