Osmotic Pressure Regulation using KCl for Enhanced Erythritol Production using *Trichosporonoides oedocephalis* ATCC 16958

Liangzhi Li*, Lina Gu, Xin Ju, Cuiying Hu, Jiaolong Fu, Hongying Cheng and Pei Kang

School of Chemistry, biology, and material engineering, Suzhou University of Science and Technology, Suzhou, 215009, P. R. China

Received June 22, 2017 ; Accepted August 6, 2017

This study investigated the production of erythritol by *Trichosporonoides oedocephalis* in response to the varying osmotic pressure. Osmotic pressure was exerted by adding NaCl or KCl into the fermentation broth. It was demonstrated that appropriate raise in osmotic pressure enhanced erythritol production, wherein 5 g/L KCl addition increased erythritol yield by 44.21% compared to the control. The reason for the significant increase in the erythritol production showed that the 5 g/L KCl addition accelerated the glucose utilization in the late period of fermentation time. It was also revealed that 5 g/L KCl increased the activity of erythrose reductase (ER) in the same fermentation period. In bioreactor, the erythritol yield with initial 5 g/L KCl addition was up to 50.54 g/L, which was 47.69% higher than the control (34.22 g/L). The study provided a novel fermentation regulation, that is, economical addition of KCl made more effective production of erythritol when using *T. oedocephalis*.

Keywords: *Trichosporonoides oedocephalis*, osmotic pressure, erythrose reductase, erythritol, polyol

Introduction

Erythritol (1, 2, 3, 4-butanetetrol) is a four-carbon polyhydric alcohol, which exists naturally in fruits, mushrooms and also in fermented foods such as wine, cheese and soy sauce, and has inhibitory effects on the growth of *Streptococcus mutans* (Park et al., 2014; Mirończuk et al., 2014). Moreover, this polyol has 60 – 80% of the sweetness of sucrose in 10% (w/v) solution, enabling it to be an excellent natural sweetener (Jovanović et al., 2014). Erythritol has approximately 90% less energy compared to other sugar alcohols as it is absorbed in the small intestine with little amount reaching at the colon (Hashino et al., 2013). Furthermore, it also effectively reduces the possibilities of dental plaque and adherence of common streptococcal oral bacteria to the tooth surfaces, and consecutively shows better efficacy compared to other commercially available polyols like sorbitol and xylitol in the oral health maintenance and improvement (De Cock et al., 2016). More importantly, most erythritol ingested in the human body is not metabolized, but excreted in the urine without changing the insulin levels, which is considered to be safe for people with diabetes (Mirończuk et al., 2015). Due to aforementioned advantages, there is a growing demand for erythritol in the market year after year.

Accumulating evidence suggested that osmotic pressure impacts cell growth and its metabolism to a great extent (Gervais et al., 1992; Pratt et al., 2003; Liu et al., 2002). Yeast cells under hyperosmotic conditions with low water activity secrete compatible solutes such as glycerol and erythritol and protect itself from losing water to death (Hallsworth et al., 2003; Kobayashi et al., 2015). Moreover, in recent decades many researches had revealed the influence of osmotic pressure on carbon flux in the microorganisms, which in turn resulted in the diversified fermentation products and yields. For example, Yang et al in their study demonstrated that erythritol and mannitol distribution in the mixture of polyols were highly dependent on the osmotic pressure.
The high initial osmotic pressure increased erythritol production while reduced the yield of mannitol (Yang et al., 2014). Liu et al. reported that hyperosmotic stress had changed the cell physiology of Candida krusei greatly and disturbed the normal enzyme regulation. Consequently, the carbon flux towards glycerol-3-phosphate increased while that towards the tricarboxylic acid cycle decreased (Liu et al., 2006). Considering the non-ignorable influence of osmotic pressure, Kim et al. increased erythritol production by adjusting osmotic pressure to a low level during growth phase and to a high level during production phase in Trigonopsis variabilis, which gained the maximum yield of erythritol 46 g/L (Kim et al., 1997).

In general, the change in osmotic pressure is achieved by the addition of cheap and readily available salts such as NaCl and KCl. Takagi et al. suggested that addition of 0.5 or 1.0 M NaCl at mid-log phase or at the end of log phase with initial NaCl concentration being 1.0 M further increased the lipid content (70%) (Takagi and Karseno, 2006). Tomaszewska et al. reported that in the fermentation process with Yarrowia lipolytica, addition of NaCl to the medium improved erythritol production yield, and simultaneously inhibited mannitol formation (Tomaszewska et al., 2012). Some researches had proved that osmotic pressure played a key role in the cell growth and metabolism by affecting intracellular enzyme activity. Under hyperosmotic stress conditions, the expression of enzymes related to protein and nucleotide biosynthesis was inhibited drastically, while other crucial enzymes related to the biosynthesis of polyols, such as transketolase and triosephosphate isomerase were significantly induced (Yang et al., 2015). Furthermore, Yang et al. found that the specific activity of erythrose reductase (ER) was almost twice higher at high osmotic pressure compared to low osmotic pressure, but the specific activity of mannitol-1-phosphate dehydrogenase (M-1-PDH) exhibited the opposite trend (Yang et al., 2014). Another study conducted by Tomaszewska et al. demonstrated that the presence of NaCl contributed to the enhanced ER and transketolase activity at pH 3.0 (Tomaszewska et al., 2014). In addition, Park et al. found that the expression of ER in Candida magnoliae was up-regulated under osmotic pressure and salt stress conditions caused by a high concentration of KCl and NaCl (Park et al., 2011).

Trichosporonoides oedocephalis, an osmophilic yeast which tolerates up to 60% (w/v) glucose, makes it an ideal strain in the production of erythritol industrially. The existence of glycerol as a by-product in erythritol production not only decreased the yield of erythritol, but also complicated the process of subsequent separation and purification (Saburo and Toshiro, 1999). In our previous study, HOG1 (high-osmolarity glycerol) knockout mutants were constructed in T. oedocephalis, which showed a 144% increased erythritol production and 71.23% decreased glycerol production (Li et al., 2016). Furthermore, few optimization studies had demonstrated the ability of T. oedocephalis and its mutants in the production of polyols and mannanase (Li et al., 2012; Olaniy et al., 2013). However, the influence of osmotic pressure on erythritol production and its related enzyme activity in T. oedocephalis has never been reported so far.

Hence in this study, we initially investigated the impact of different concentrations of NaCl and KCl on erythritol production by culturing T. oedocephalis in shake flasks. Subsequently, comparative fermentations with and without 5 g/L KCl were carried out, and the activity of ER in erythritol production was also synchronously determined. Based on the above experiments, scale-up fermentations were conducted in a 5-L fermentor for the purpose of further enhancing erythritol productivity.

Materials and Methods

Strain and medium T.oedocephalis ATCC16958 strain purchased from American Type Culture Collection (ATCC, USA) was used in this study. The seed medium contained 20 g/L glucose, 10 g/L yeast extract, and 20 g/L peptone. The fermentation medium consisted of 200 g/L glucose, 10 g/L yeast extract, 0.5 g/L KH$_2$PO$_4$, 0.5 g/L MgSO$_4$. All reagents (analytical grade) were purchased from Aladdin, Shanghai, China.

Culture methods For seed culture, a loopful of T. oedocephalis from a fresh slant was inoculated into 50 mL of seed culture medium in 250 mL flask and then was incubated at 30°C and 200 rpm for 48 h. The seed inoculum was transferred into a 50-mL fermentation medium in 250 mL flask and was incubated at 30°C and 200 rpm for 120 h. Batch fermentations were carried out in a 5-L fermentor (Shanghai Baoxing Bio-Engineering Equipment Co., Ltd. China) containing 3 L working volume with a 10% inoculum level (v/v). The batch fermentations were performed at 30°C and pH value was maintained at 4.0 by the addition of 5 M NaOH. The aeration rate was controlled at 0.5 vvm and the agitation speed was controlled at 300 rpm. Samples were taken at 12 h intervals during the fermentation process. In this study, all determinations were made in triplicate.

Product analysis At the end of fermentation process, 10 mL fermentation broth was taken out, and was centrifuged at 14000 rpm for 20 min. The supernatant was then filtered through 0.22 µm membrane and was assayed for erythritol and glycerol by high-performance liquid chromatography (HPLC, Agilent Technologies Inc., Agilent 1260) using a NH2P-50 4E chromatography column (250 mm × 4.6 mm, Shodex, Japan) and detected with an evaporative light scattering detector (ELSD). For the sample analysis, the column was eluted at 30°C with acetonitrile: water (70: 30) at a flow rate of 0.7 mL/min. The drift tube temperature of ELSD was set at 35°C, and the nitrogen flow rate was 1.6 L/min.

Biomass measurement The biomass concentration was determined by measuring the dry weight of the cells. The aliquots (10 mL) of fermentation broth were sampled, centrifuged (14000 rpm, 20 min), and washed twice with deionized water. The
supernatant was carefully discarded and the cellular pellet was
dried in an oven (80°C) to a constant dry weight.

**Residual glucose determination** After appropriate dilution, the
residual glucose was determined by a bioanalyzer with glucose
oxidase membrane (SBA-40C, Institute of Biology, Shandong
Academy of Sciences, China).

**ER activity assay** For the determination of intracellular ER
activity, cells from the culture broth were harvested by
centrifugation at 14000 rpm for 20 min. After being washed twice
with 50 mM phosphate buffer (pH 7.0), the harvested cells were
resuspended in disruption buffer containing 50 mM Tris-HCl (pH
7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate,
0.1% SDS, and 1 mM phenylmethanesulfonyl fluoride. The cell
suspension was disrupted by grinding with acid-washed glass beads
that were 0.1 mm in diameter. Then the cells were centrifuged at
14000 rpm for 20 min at 4°C and the supernatants were used as cell
extracts. Total protein concentration in the supernatant was
determined with Bicinchoninic Acid assay using bovine serum
albumin as standard.

The ER activity was determined using 12 mM erythrose and
0.2 mM NADPH in 50 mM phosphate buffer (pH 6.5) by
monitoring the decreasing OD at 37°C for 2 min (Ookura et al.,
2005). One unit of ER activity was defined as the amount of
enzyme that catalyzes the oxidation of 1 μmol NADPH per min at
37°C. Specific ER activity was expressed as units of enzyme
activity per milligram of cellular protein.

**Results and Discussion**

**Effect of NaCl and KCl on the glycerol and erythritol
production** For studying the effect of osmotic pressure on glycerol
and erythritol production by *T. oedoecephalis* during the first stage,
different concentrations of NaCl and KCl (5 g/L to 50 g/L) were
added into the fermentation medium. As shown in Figure 1A,
glycerol production was increased in pace with the increasing
concentration from 5 g/L to 40 g/L regardless of NaCl or KCl. The
yield of glycerol with 40 g/L NaCl was 18.39 g/L, which was 190%
higher than that of glycerol with 5 g/L NaCl (6.34 g/L), the addition
of 40 g/L KCl resulted in the yield of glycerol of up to 30.97 g/L,
which showed an increase of 370% compared to 5 g/L of KCl
(6.59 g/L). Maybe it was because higher osmotic pressure
stimulated the strain to accumulate more glycerol, this in turn
stabilized the cellular osmotic pressure with the external
environment (O’Rourke et al., 2002). The data in Figure 1B
showed the maximum production of erythritol was at 5 g/L NaCl
and 5 g/L KCl. The concentration of these salts over 5 g/L caused
gradual decrease of erythritol production. It showed an appropriate
increase in osmotic pressure was beneficial to erythritol synthesis.
Similarly, Kim et al reported that the production of erythritol
reached maximal point at 0.4 M KCl or 0.3 M NaCl in investigating
the effect of osmotic pressure on the growth of Torula sp. and
erthyritol production (Kim et al., 1999). However, except the
difference between 40 g/L and 50 g/L NaCl for the glycerol
production, the glycerol and erythritol production was significantly
decreased when the salt concentration rose from 40 g/L to 50 g/L.
The sum of glycerol and erythritol production with 50 g/L KCl was
53.64 g/L, which was 14.34% lower than that of 40 g/L KCl
(62.62 g/L). This in turn demonstrated that high osmotic pressure

![Fig. 1. The effect of different concentrations of NaCl and KCl on polyol production and cell growth](image-url)
affected the metabolic pathway in *T. oedocephalis*, and ultimately hindered the synthesis of glycerol and erythritol. In addition, it was worth noting that the erythritol production achieved its maximal level at 43.55 g/L with the addition of 5 g/L KCl, which exhibited an up to 44.21% increase compared to the control (30.20 g/L). Simultaneously, glycerol production was 6.59 g/L with 5 g/L KCl while 6.10 g/L glycerol was generated without the introduction of this chemical. This illustrated that the addition of 5 g/L KCl did not increase the complexity of later erythritol separation. Meanwhile, the production of erythritol with the addition of KCl was higher than that of erythritol with the addition of NaCl at each experimental concentration. Particularly, the yield of erythritol was 36.22 g/L with 20 g/L KCl addition, which was 15% higher than that of erythritol with 20 g/L NaCl addition (31.48 g/L). Therefore, when compared to NaCl, KCl showed an advantage of erythritol promotion at the same mass concentration. This phenomenon may be attributed to the osmotic pressure enhancement caused by NaCl was higher than that by KCl under the same mass concentration according to the osmotic pressure calculation method (Hu et al., 2015). Furthermore, there was little difference in the cell dry weight between 40 g/L and 50 g/L salt concentration (Figure 1C). For example, by increasing the NaCl concentration from 40 to 50 g/L, the cell dry weight only decreased from 21.15 to 20.50 g/L. Moreover, from the concentration of residual glucose presented in Table 1, glucose without addition of salt remained at 22 g/L after 120 h of fermentation, while glucose was almost exhausted in all added concentration of salts. According to the analysis of residual glucose, it was clear that 5 g/L KCl accelerated the glucose consumption, and consequently eliminated the problem of residual substrate removal. In view of the fact that KCl was superior over NaCl and the high performance of 5 g/L KCl in erythritol enhancement and glucose utilization, we chose 5 g/L KCl to make the following comparative research.

**Comparative study with and without 5 g/L KCl** During the fermentation period, the related fermentation parameters of the substance sampled at 12 h intervals were measured. During the entire fermentation course (Figure 2A), the yields of erythritol with and without 5 g/L KCl were nearly the same before 84 h, and the corresponding yields at 84 h were 26.39 g/L and 25.29 g/L, respectively. However, the difference in erythritol production with and without 5 g/L KCl was increasing with the extension of fermentation time after 84 h. After 120 h of fermentation, the final concentration of erythritol with 5 g/L KCl reached 44.44 g/L, which increased by 39.40% compared to control (31.88 g/L). Besides, the ultimate yield of glycerol with 5 g/L KCl was only 6.43 g/L, which was conducive to the later separation and extraction for erythritol. On the other hand, as shown in Figure 2B, the glucose consumption rate with 5 g/L KCl was similar to that without 5 g/L KCl before 84 h, whereas glucose with 5 g/L KCl was continued to be utilized.

Table 1. The residual glucose when culture *T. oedocephalis* with different concentrations of NaCl and KCl

<table>
<thead>
<tr>
<th>salt concentration (g/L)</th>
<th>residual glucose (g/L)</th>
<th>NaCl</th>
<th>KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>22.00 ± 0.05</td>
<td>22.00 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.52 ± 0.03</td>
<td>0.56 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.26 ± 0.02</td>
<td>0.22 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.25 ± 0.01</td>
<td>0.24 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.22 ± 0.01</td>
<td>0.23 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0.21 ± 0.01</td>
<td>0.23 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0.20 ± 0.01</td>
<td>0.23 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Time course of polyol production by *T. oedocephalis* ATCC 16958 with and without 5 g/L KCl in shake flasks
(A) Glycerol and erythritol production
(B) Residual glucose
(C) Cell dry weight
Enhanced Erythritol Production

until it was depleted till the end of fermentation while the residual glucose without 5 g/L KCl remained almost unchanged in the later period of fermentation. In our study, erythritol was generated with glucose as the sole carbon source, so the difference in glucose utilization rate was possibly a reason that contributed to the improvement of erythritol yield with 5 g/L KCl. Under normal circumstance, the inhibition of cell growth was observed due to cell shrinkage, water flux out of the cytoplasm and a decrease in the turgor pressure when exposed to high osmotic pressure (Xu et al., 2010). Our experimental results demonstrated that high osmotic pressure such as 40 g/L and 50 g/L NaCl or KCl had a negative effect on the cell growth (Figure 1C). However, interestingly, the final dry weight of the cell with 5 g/L KCl was 27.43 g/L, which was 7.57% higher than that of without 5 g/L KCl (25.50 g/L). It illustrated that 5 g/L KCl was beneficial to cell growth (Figure 1C and Figure 2C). This could be another crucial reason for the enhanced yield of erythritol with the addition of 5 g/L KCl. In fact, it is reported that the production of erythritol is strongly related to the osmotic stress response system of Trichosporonoides species (Kobayashi et al., 2013). Moreover, a certain turgor pressure of the cell is also one of the necessary conditions for cell division (Hohmann, 2002). Therefore, osmotic pressure plays significant roles in cell growth and erythritol production. As far as we know, the osmotic pressure of the medium was calculated by the previous method, and the osmotic pressure of 5 g/L NaCl solution was (5/58.5) ×2×1000 = 170.94 mosm/L (Hu et al., 2015; Wright and Reed, 1988). In this study, the results showed that inorganic salts (NaCl or KCl) might be required as nutrients for erythritol production. It also played an important role in changes of osmotic pressure and in cell growth or production accumulation.

On the other hand, previous studies had reported that erythritol was synthesized via the pentose phosphate pathway (PPP) in eukaryotes (Kobayashi et al., 2013). The final step of erythritol formation was accomplished by NADPH-dependent ER for reduction of erythrose (Park et al., 2011; Jovanović et al., 2013). On account of the key role of ER in the erythritol synthesis, we determined its activity and also explored the underlying reason behind the raised productivity by 5 g/L KCl with T. oedoecephalis. The enzyme activity data (Figure 3) showed that there was a little gap in the activity of ER with and without 5 g/L KCl before 84 h, and the maximum was reached at 60 h simultaneously. The activity of ER with 5 g/L KCl was evidently higher than the control from 96 h. In particular, at 96 h culture, the activity of ER with 5 g/L KCl was 0.28 U/mg protein, which was 2.8-fold that of the control (0.10 U/mg protein). The result was consistent with the enhancement of ER activity in Candida in the presence of 0.5 M KCl (Park et al., 2011). In order to further optimize the promotion of 5 g/L KCl on the erythritol production, we chose the initial time (0 h) and fermentation metaphase (60 h) as two timepoints to introduce 5 g/L KCl. Similarly, the related fermentation parameters including glycerol and erythritol production (Figure 4A and 4B), residual glucose (Figure 4C) and cell dry weight (Figure 4D) were measured. What surprised us most were the extreme resemblance on the varying tendency and values between those two-additional ways. From the Figure 4, it seemed that there was almost no difference in polyols production, glucose consumption, and cell growth when the 5 g/L KCl was added in the fore mentioned two patterns. The phenomenon also confirmed that 5 g/L KCl played the role only in the later stage of fermentation. As a result, considering the lack of the necessity for the operation in the latter addition mode of the following scale-up fermentation study, we still selected the initial time as the better addition timepoints.

Scale-up fermentation in bioreactor On the basis of the above experiment, scale-up fermentations in a 5-L fermentor were conducted. Obviously, the trend in the glucose consumption in the fermentor was not same as that in shake flasks (Figure 5B). It demonstrated that the rate of glucose consumption in the shake flask was higher than that in fermentor during the early and middle stages of fermentation. This probably can be attributed to the difference in the external environment such as dissolved oxygen between shake flask and fermentor (Humphrey, 1998). Nevertheless, similarities still existed in these two types of scale fermentations. From the product analysis data (Figure 5A), we found that initial addition of 5 g/L KCl in the fermentor increased the synthesis of erythritol, and after 120 h of fermentation, the erythritol yield was up to 50.54 g/L, which was 47.69% higher than that of the control (34.22 g/L). At the same time, glycerol production was almost equal to that of control. Furthermore, the cell dry weight with 5 g/L KCl in the fermentor was 32.49 g/L (Figure 5C), which was a little higher than the control (31.26 g/L).

Conclusions In summary, these findings suggested that osmotic pressure exerted enormous influence on cell growth and metabolic pathways. The results revealed that the initial addition of 5 g/L KCl played an active role in increasing the erythritol production, and the maximum yield of erythritol in the 5-L fermentor was up to
50.54 g/L, which was 47.69% higher than the control (34.22 g/L). At the same time, the ER activity with 5 g/L KCl was higher than that without addition in the later fermentation period. This study provided guidance for further research regarding the function of osmotic pressure in *T. oedocephalis*, such as the possible change of carbon flux and its effect on the activities of other core enzymes in PPP pathway.

**Acknowledgements**  The authors are grateful for the financial support from the National Natural Science Foundation of China (Grant No: 21376156 and Grant No: 21676173). This study was also supported by Qing Lan Project of Jiangsu Education department. This work also supported by Natural Science Foundation from Education Department of Jiangsu Province (Grant No:311312310).

**Disclosure**  The authors declare no conflict of interest.

**References**


Enhanced Erythritol Production


Fig. 5. Batch fermentation with and without 5 g/L KCl (A) Glycerol and erythritol production (B) Residual glucose (C) Cell dry weight


