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ORIGINAL RESEARCH ARTICLE

# Biotin and $Zn^{2+}$ Increase Xylitol Production by *Candida tropicalis*

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**Abstract** In this study, the medium requirements to increase the production of xylitol by using *Candida tropicalis* (CT) have been investigated. The technique of single addition or omission of medium components was applied to determine the nutritional requirements. The addition of amino acids such as Asp, Glu, Gln, Asn, Thr, and Gly had no significant effect on CT growth. However, in the absence of other metal ions, there was a higher concentration of cell growth and xylitol production when only $Zn^{2+}$ was present in the medium. The analysis of various vitamins unveiled that biotin and thiamine were the only vitamins required for the growth of CT. Surprisingly, when only biotin was present in the medium as a vitamin, there was less growth of CT than when the medium was complete, but the amount of xylitol released was significantly higher. Overall, this study will increase the xylitol production using the single omission or addtion technique.

**Keywords** Biotin · Thiamine · Urea · Xylitol · $Zn^{2+}$

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## Introduction

Xylitol, a five-carbon alcoholic sugar, is popular in the food industry due to its non-carcinogenicity and lack of dependence on insulin [1–4]. People with diabetes and people with low glucose-6-phosphate dehydrogenase do not require insulin and glucose-6-phosphate dehydrogenase when consuming xylitol, which makes it a suitable alternative for these people [5, 6]. Xylitol is currently manufactured from xylose, the five-carbon sugar derived from hemicellulose hydrolysates, through a chemical process using $Ni/Al_2O_3$ as a catalyst [7–12].

The type of media utilized during fermentation greatly influences the chemical or nutritional environment, which in turn significantly influences the productivity and the economics of a fermentation process [13–18]. The media used to promote high productivity in commercial/industrial fermentation are mainly developed from complex sources of carbon and nitrogen [19]. However, the rate of growth and the activity of metabolic processes may be affected strongly by the type and ratio of nutrients provided to the culture. Due to this inherent inconsistency of natural origin, the fermentation performance may vary from lot to lot [20].

Yeast extract (YE) is used in the fermentation of xylitol, and thus xylitol is produced using microbial fermentation using YE as one of the medium ingredients. However, the variation yields have been observed due to unknown variations of YE. On top of that, the high price of YE hinders its uses in industrial applications. Moreover, the medium cost is one of the major factors in economic xylitol production [21–26]. Therefore, replacing YE with a synthetic defined medium (SDM) is required to lower the cost of medium and performance variability while maintaining the production yield [27]. Additionally, when producing xylitol, the performance consistency of synthetic-defined

media should be comparable to that of YE. When the concentration of nitrogen is high, the recovery and purification of xylitol production become very difficult. Xylitol purification is simplified because no additional contaminants are added to the media, leading to a lower production cost [28]. Hence, it's indeed crucial for the metabolic investigation to have a precise growth medium for the microorganisms, which really supports high yield and productivity. Therefore, SDM which enables exponential growth with high xylitol production while eliminating or adding the need for a single medium component, has been developed in the current study.

## Materials and Methods

### Microorganism and Media

*Candida tropicalis* KFCC-10690 was used in this study because it is an established member of the *Candida* genus [29]. Freezing of the cell stock was done at − 70 °C. This medium consists of 5 g/L YE and 20 g/L glucose. For fermentation, there were 5–33% xylose, 10 g/L YE, and 0–90 g/L glucose in a complex medium that had a concentration of 5 g/L $KH_2PO_4$. Finished materials were made up into separate batches of medium and dense components, which consisting of carbohydrates, basal salts, amino acids, vitamins, and metals [30–32]. The components of SDM were sterilized by a membrane filtration method [33] (Millex-GV filter; Millipore Corp., Bedford, Mass), and the working cultures of CT were propagated in SDM. Further, cultures were centrifuged and washed twice in 50 mM potassium phosphate and at the pH of 6.5 to elimination of carryover nutrients. For the inocula, 5% (vol/vol) exponentially growing cells were used.

### Fermentation Conditions

Inoculation was carried out on a 500 mL flask with 100 mL of culture medium for 10 h at 30 °C and 250 rpm. The resulting culture broth, diluted to a total volume of 10% (v/v), was transferred to a 500 mL flask and used to inoculate a 5-L jar fermenter, which was filled with 100 mL of production medium until it was 2.8–3.5 L of production medium (Kobiotech. Co., Republic of Korea). Complex media contains 200 g/L of xylose, 17 g/L of glucose, 1.3 g/L of $KH_2PO_4$, 2.5 g/L of $(NH_4)_2SO_4$, 0.13 g/L of $MgSO_4$, and 5 g/L of YE. SDM contains urea 3.1 g/L, xylose 200 g/L, glucose 17 g/L, $KH_2PO_4$ 1.3 g/L, $MgSO_4$ 0.13 g/L, biotin 16.5 g/L, folic acid 7.5 µg/L, thiamine 2.65 mg/L, boric acid 0.5 mg/L, copper sulfate 0.04 mg/L, potassium iodide 0.1 mg/L, ferric chloride 0.2 mg/L, manganese sulfate 0.4 mg/L, sodium molybdate 0.2 mg/L, and zinc sulfate 5.0 mg/L. Experiments in jar fermenters were conducted at 30 °C in a fed-batch mode controlling the pH at 4.8. The peristaltic pump (10–50 mL/h) continuously fed the solution of xylose or the mixture of xylose and glucose, which was aerated at 0.5 vvm. The agitation was increased from 250 to 750 rpm to maintain the percentage of dissolved oxygen above 20 until the cell mass reached 14 g/L; it was then decreased to 340 rpm to limit the concentration of dissolved oxygen.

### Enzyme Assay

Cultured cells were collected by centrifuging at 10,000 rpm for 15 min. Washing was carried out with 0.1 M Tris–HCl (pH 7.8), 0.5 mM EDTA, and 5 mM mercaptoethanol. Further, the cells were resuspended in a buffer [34] containing 20 mM Tris–HCl (pH 7.8), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulphonylfluoride. Glass beads of 0.5 mm in diameter were used for the suspension. (Sigma, USA). To determine the xylose reductase (XR) activity, a decrease in the absorbance at 340 nm was measured after the addition of D-xylose, a marker for NADPH oxidation (Sigma, USA) [35].

### Analytical Methods

A Bradford assay has been used to estimate protein concentration, and bovine serum albumin is being used as a standard [36]. To estimate the concentrations of xylitol, glucose, and xylose, HPLC coupled to an RI detector (Waters 410, USA) and a High-Performance Carbohydrate Column (4.6 mm × 250 mm, Waters, USA) were used. Acetonitrile/water (85:15 v/v) was used as a mobile phase at a 1.5 mL/min flow rate.

## Results and Discussion

### Influence of Nitrogen Source

A defined medium with a sole carbon source and a sole nitrogen source was designed in order to investigate the effect of the substrates on xylitol production. A shake flask system was developed to explore a range of inorganic and organic nitrogen sources in culturing a defined medium containing 200 g/L xylose and 17 g/L glucose as the carbon source. Ammonia, which is an important component of nitrogen metabolism in yeast, was also tested alongside two other common nitrogen sources, urea and nitrate, and CT grew on all of the nitrogen sources, indicating that they had been consuming it. With the exception of the ammonium acetate experiment, all experiments found that the glucose

supply had been depleted after 40 h. Although inorganic nitrogen sources like ammonium tartrate, ammonium nitrate, ammonium acetate, and sodium nitrate were consumed for biomass formation, the production of xylitol was poor after 60 h of cultivation. However, urea has been found to produce xylitol similar to the level achieved by complex media by YE.

### Effect of Amino Acids, Nucleic Acids, and Buffers

The cell growth and the amount of xylitol did not change regardless of whether single or multiple amino acids were added, and the same results have been observed when nucleic acids such as guanine, xanthine, adenine, and uracil were omitted from the growth medium. Moreover, no major changes were found in the growth and xylitol production of the strain when tenfold lower levels of buffers, such as phosphate, citrate, and acetate, were added. (Table 1).

### Effect of Metal Ions on the Growth and Xylitol Production

By excluding one metal ion at a time, the metal ion requirement of CT in SDM was determined. The strain grew well when $NiCl_2$ and $CoCl_2$ were omitted individually, and the cell growth was slightly inhibited when $FeCl_2$, $CoCl_2$, $H_3BO_3$, $Na_2MoO_4$, $MnCl_2$, and $CuCl_2$ were excluded. However, in the absence of $ZnCl_2$, the growth of CT was significantly decreased, and it appeared to be crucial for cell growth (Table 1). Thus, to determine the effect of $Zn^{2+}$ on cell growth and xylitol production, a 5-L jar fermenter was used. To that end, various concentrations of $ZnCl_2$ were tested in the range of 0 to 10 mg/L. The fermentation conditions were provided in the "Materials and Methods" section. Xylitol gave the greatest yield and productivity at a concentration of 5 mg/L of $ZnCl_2$ (Table 2). Further, an SDM mixture produced by the addition of optimal concentration of $ZnSO_4$ (5 mg/L) was as effective as the complete metal mixture of SDM has been observed to promote cell growth and xylitol production. XR activity of supernatants obtained from cultures grown without zinc were assayed with and without $ZnCl_2$ added to the samples, and no significant activities were found, even when the samples were incubated with zinc for 1 h at 37 °C before the assay. This suggests that zinc does not increase the level of XR activity by an enzyme mechanism. However, it seems to play a metabolic role and is needed during growth to induce significant protease production [37].

**Table 1** Nutrient requirements of *C. tropicalis* in synthetic defined medium investigated by addition or omission of a single medium component

| Added medium component | $OD_{600}$[a] | Omitted medium component | $OD_{600}$[a] |
|---|---|---|---|
| None | 14.4 | Phosphate | 11.2 |
| L-Alanine | 14.3 | $MgSO_4$ | 13.0 |
| L-Arginine | 14.7 | $H_3BO_3$ | 13.3 |
| L-Asparagine | 14.7 | $MnCl_2$ | 12.4 |
| L-Leucine | 14.4 | $ZnCl_2$ | 5.6 |
| L-Glutamic acid | 14.5 | $CuSO_4$ | 13.1 |
| L-Glutamine | 14.3 | $FeCl_2$ | 13.5 |
| Glycine | 14.4 | $NiCl_2$ | 14.6 |
| L-Lysine | 14.6 | $CoCl_2$ | 14.3 |
| L-Phenylalanine | 14.3 | $Na_2MoO_4$ | 13.5 |
| L-Proline | 14.3 | Biotin | 13.4 |
| L-Serine | 14.5 | Inositol | 15.1 |
| L-Tryptophan | 14.7 | Folic acid | 14.5 |
| L-Tyrosine | 14.7 | ρ-Aminobenzoic acid | 14.9 |
| L-Valine | 14.5 | Nicotinic acid | 14.4 |
| L-Histidine | 14.4 | Pantothenate | 14.8 |
| L-Cysteine | 14.6 | Pyridoxamine | 15.0 |
| Adenine | 14.8 | Pyridoxine | 15.2 |
| Guanine | 14.2 | Riboflavin | 10.5 |
| Uracil | 14.5 | Thiamine | 8.7 |
| Xanthine | 14.3 | | |

Values are the means ± standard deviations of triplicate measurements

[a]OD measurements were performed after 48 h of incubation

**Table 2** Effect of $ZnSO_4$ concentration on the cell growth and xylitol production

| | Conc. of $ZnSO_4$ (mg/L) | | | |
|---|---|---|---|---|
| | 0 | 1.0 | 5.0 | 10.0 |
| Cell conc. ($OD_{600}$) | 47.9 | 47.7 | 43.4 | 38.5 |
| Produced xylitol (g/L) | 186 | 260 | 252 | 246 |
| Yield (Volumetric, %) | 54.3 | 77.1 | 77.2 | 74.7 |
| Productivity (g/L h) | 2.10 | 3.31 | 3.63 | 3.32 |

## Effect of Vitamins on the Growth and Xylitol Production

In the absence of any individual vitamin, only riboflavin and thiamine were detected as essential nutrients for growth. In contrast, a similar OD was found when other vitamins were overlooked. Further, we found that folic acid, ρ-Aminobenzoic acid, pantothenate, inositol, niacin, and pyridoxine were unnecessary for cell growth. A single omission of the nonessential vitamins did not change the specific production of xylitol [37, 38], but when biotin was overlooked, the specific xylitol production was significantly decreased, and it emerged to be essential for xylitol production (Table 3). When CT was grown in a medium lacking vitamins except for riboflavin, biotin, and thiamine, the OD of the cultures was getting lower after 48 h. In contrast, the specific xylitol production was increased significantly (Table 3). Biotin limitation decreased the xylose consumption of CT, and the decrease became more significant as the initial concentration of biotin decreased. Biotin acts as a prosthetic group for carboxylases, and it is unclear why its limitation results in more xylitol accumulation.

## Xylitol Production Using a 5-L Jar Fermenter

Finally, a pH-controlled fed-batch culture experiment was carried out to compare the cell growth and xylitol production by CT in the complex medium and the SDM. The composition of the SDM met the nutritional requirements of the strains and took advantage of the beneficial effects in the downstream process. Fermentation of CT in complex medium containing is represented in Fig. 1. During the glucose consumption, pH decreased from 6.4 to 4.8; thereafter, pH increased to 6.8 until the end of the fermentation process. In the SDM, CT grew up with a minimal growth rate of 0.18 $h^{-1}$ and a final OD of 47.7 and produced 260 g of xylitol per liter with a conversion yield of 81.5% when grown in a pH-controlled fed-batch culture. On the other hand, in the complex medium, the growth rate was 0.23 $h^{-1}$, and the final OD was 47.1, while the xylitol production was 251 g $L^{-1}$ with the conversion yield of 78.1% (Table 4). Further, It has been observed that in both the complex medium and the SDM, xylitol production continued after growth had come to an end. Still, beyond the stationary growth phase, more xylitol production was observed in the SDM than in the complex medium. Moreover, the addition to SDM of the ten amino acids (Gln, Leu, Ile, Val, Met, His, Arg, Trp, Pro, and Phe) did not increase the xylitol production of CT.

**Table 3** Effect of individual and multiple omission of essential vitamins on the xylitol production by *C. tropicalis* in defined medium

| Omission | Xylitol (g/L) | Specific xylitol production (g/g of dry cell weight)[a] |
|---|---|---|
| None | 114 | 10.6 |
| ρ-Aminobenzoic acid | 117 | 10.8 |
| Biotin | 2.8 | 0.27 |
| Calcium pantothenate | 118 | 11.0 |
| Folic acid | 114 | 10.5 |
| Inositol | 112 | 10.2 |
| Niacin | 118 | 10.8 |
| Pyridoxine | 113 | 10.2 |
| Riboflavin | 108 | 10.1 |
| Thiamine | 103 | 9.90 |
| All essential vitamins[b] except riboflavin, biotin, and thiamine | 119 | 11.2 |

[a]The specific xylitol production was calculated from a standard curve of $OD_{600}$ against cell dry weight

[b]All essential vitamins: ρ-Aminobenzoic acid, biotin, calcium pantothenate, folic acid, inositol, niacin, pyridoxine hydrochloride, riboflavin, thiamine

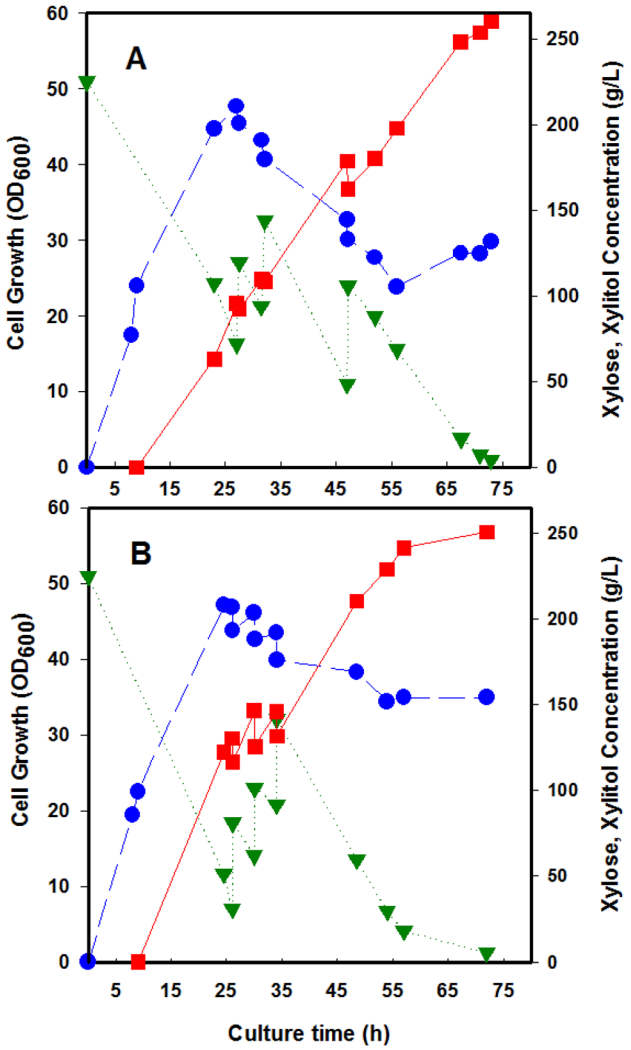


**Fig. 1** Xylitol production using SDM (**A**) and complex medium (**B**) containing urea and yeast extract as a nitrogen source, respectively. Blue circle: cell growth, green inverted triangle: xylose, red square: xylitol

## Conclusions

In this work, we developed a synthetic and cheap medium to allow reproducible xylitol production without variation in yields and productivity. The technique of single addition or omission of medium components revealed that the amino acids such as Asp, Glu, Gln, Asn, Thr, and Gly were slightly affecting the growth of CT. Further, we observed that the amount of cell growth and xylitol production was more significant when $Zn^{2+}$ ion was present in the medium and other metal ions were not. In addition, it has been observed that CT required only biotin and thiamine as individual vitamins. Surprisingly, when only biotin was present in the medium as a vitamin, the amount of xylitol production was significantly greater than in the complete medium.

**Table 4** Comparison of xylitol production between the SDM and complex medium containing urea and yeast extract as a nitrogen source, respectively

| | Medium | |
|---|---|---|
| | SDM | Complex |
| Initial pH of medium | 5.6 | 5.0 |
| Culture time (h) | 73 | 72 |
| Maximum cell conc. ($OD_{600}$) | 47.7 | 47.1 |
| Added conc. of xylose (g/L) | 300 | 300 |
| Final conc. of | | |
| Xylitol (g/L) | 260 | 251 |
| Xylose (g/L) | 4.3 | 5.2 |
| Glycerol (g/L) | 9.5 | 11.0 |
| Yield (Volumetric, %) | 81.5 | 78.1 |
| Productivity (Volumetric, g/L h) | 3.35 | 3.26 |

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