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ORIGINAL PAPER

Influence of metal addition on ethanol production with *Pichia stipitis* ATCC 58784

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Abstract Trace metals always act as cofactors or coenzymes in many cellular processes. Deficiency or excess of some metals will affect the fermentation of lignocellulosic hydrolysate. In order to make sure the deficient or excessive states of metals in culture medium, metal contents analysis was conducted in *Pichia stipitis* ATCC 58784 cells, synthetic medium, and diluted acid hydrolysate of rice straw. The results showed that Cu, Ni, and Co were deficient, and Al was a little excessive. So the influences of Cu^{2+}, Al^{3+}, Ni^{2+}, and Co^{2+} additions on the growth and ethanol production of ATCC 58784 were further researched. Low concentration additions of Cu^{2+} and Al^{3+} (<0.24 mM and <0.23 mM, respectively) improved biomass growth of ATCC 58784 by 34 and 13%, respectively; however, higher concentrations decreased biomass growth. On the other hand, addition of Cu^{2+} (0.39 mM) did not affect volumetric ethanol production significantly ($P = 0.05$) and addition of Al^{3+} (0.38 mM) showed no influence on volumetric ethanol production ($P = 0.68$). Addition of 0.074 mM Co^{2+} inhibited biomass growth of ATCC 58784 by 13% and volumetric ethanol production by 10%. The biomass growth and volumetric ethanol production of ATCC 58784 was arrested by the addition of 0.33 mM of Ni^{2+} by 53 and 65%, respectively.

Keywords Trace metal · Ethanol production · *Pichia stipitis* · Xylose

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Introduction

Ethanol production from lignocellulose is very attractive because of its low cost and abundance, and non-competition property against foodstuffs [1]. Economical production of ethanol from lignocellulosic hydrolysate requires both glucose and xylose efficiently [2]. However, the utilization efficiency of xylose is relatively lower than that of glucose and cannot meet the requirement of biofuels industry [3].

Pichia stipitis is one of the most outstanding xylose-fermenting yeast [2], which can convert xylose to ethanol at high yield [4]. Meanwhile, it is able to ferment most sugars in lignocellulosic hydrolysate including glucose, mannose, galactose, and cellobiose [5]. However, except for these merits, the ethanol production rate of *P. stipitis* is relatively low [1]. Oxygen, xylose concentration, pH and some growth factor can affect the performance of *P. stipitis* [6–9], in which metals have great influence on its performance [8].

Metals are known as coenzymes or cofactors in many cellular processes. Several transition metals, including manganese, iron, nickel, cobalt, copper, and zinc are required as catalysts in a variety of enzymatic reactions [10, 11]. However, excessive amounts of the same metal ions are toxic and even can damage the functions that they serve [10, 12–14]. Agbogbo and Wenger [15] pointed out that high concentration of calcium could increase cell growth while decrease ethanol production of *P. stipitis*. Mahler and Guebel [16] found that 4 mM of Mg was optimal for biomass growth and ethanol production of *P. stipitis* and that lower concentration of Mg lead to 49% of carbon flux to xylitol production. Slininger and Dien et al. [8] stated that the interaction of minerals with amino acids and/or urea was critical to the optimization of ethanol production by *P. stipitis* in both growing and stationary-phase cultures, and the addition of optimum mineral supplement including

Fe, Mn, Mg, Ca, and Zn could improve ethanol production from 24 to 54 g/l. The role of trace metals in the metabolic processes of microorganisms and higher organisms has become a special interesting field for further research.

Although the influences of some trace metals, e.g., Cu^{2+}, Al^{3+}, Co^{2+}, and Ni^{2+}, on some plants, yeasts and bacteria have been studied and summarized by some researchers [14, 17], their influence on *P. stipitis* is far from clear. In addition, whether lignocellulosic hydrolysate contained enough above-mentioned metals is still unclear. The objective of this study is first to find out the deficient or excessive states of metals in lignocellulosic hydrolysate compared with *P. stipitis* ATCC 58784 cells, and then to evaluate the effects of several deficient metal ions on the growth and fermentation activity of *P. stipitis* ATCC 58784.

Materials and methods

Microorganism

The yeast, *Pichia stipitis* ATCC 58784 was supplied by the American Type Culture Collection, which was preserved on agar slant at 4°C and stored at −80°C with 15% glycerol. The agar slant contained (per liter): peptone 5 g, yeast extract 3 g, malt extract 3 g, $CaCl_2$ 100 mg, KH_2PO_4 2.5 g, $MgSO_4{\cdot}7H_2O$ 500 mg, $(NH_4)_2SO_4$ 1 g, and xylose 10 g.

Dilute acid hydrolysate preparation

Dilute acid hydrolysate was prepared by hydrolyzing rice straw with 1.6% H_2SO_4 (1:10 solid/liquid ratio) at 121°C for 2 h [18]. The hydrolysate was separated through a Whatman GF/C filter paper and then neutralized by 23% ammonia water, sodium hydroxide or $Ca(OH)_2$ respectively. For some samples, boiling for 5 min or overliming to pH 11 with $Ca(OH)_2$ were carried out. The hydrolysate (after neutralization) was composed of 11.9 g/l of xylose, 3.3 g/l of arabinose, 9.8 g/l of glucose, 1.5 g/l of galactose, 0.56 g/l of 5-hydroxymethyl-2-furfural (HMF) and 0.12 g/l of furfural.

Pre-culture and fermentation

P. stipitis ATCC 58784 was first pre-cultured on basic medium (BM, Table 1) with 10 g/l of xylose for 24 h. Then, 0.2 ml of pre-cultured yeast was inoculated to 5 ml of BM for fermentation, and the samples were taken out after 48 h of cultivation. $CuSO_4{\cdot}5H_2O$ (0–0.39 mM), $KAl(SO_4)_2$ (0–0.38 mM), $NiCl_2{\cdot}6H_2O$ (0–0.33 mM), or $CoCl_2{\cdot}6H_2O$ (0–0.074 mM) was added respectively to BM to research their influences. For comparison, water addition was used as control.

The test tubes, sealed with porous silicon plugs and added with 5 ml of BM, were used as reactors. The cultivations were conducted on a shaking table (30°C, 110 rpm) and lasted for 48 h. The experiments were conducted in triplicate and the results were the average values under the designed conditions.

Table 1 The composition of basic medium (BM) for fermentation

Ingredients	Concentration (mg/l)	Ingredients	Concentration (mg/l)
$ZnSO_4{\cdot}7H_2O$	5.5	$(NH_4)_2SO_4$	1000
$MnCl_2{\cdot}4H_2O$	12.5	Yeast extract	3000
$CaCl_2{\cdot}2H_2O$	28	Peptone	5000
$MgSO_4{\cdot}7H_2O$	500	Malt extract	3000
$FeSO_4{\cdot}7H_2O$	50	Xylose	18000
KH_2PO_4	2500		

Samples for metal analysis

Accurate measurement of trace metals inside cells was a useful method to find out the fundamental requirement of trace metals for microorganisms [19]. In order to know the metal concentration in *P. stipitis* ATCC 58784 cells, 0.1 g of dried 1 day cultivated cells (cultivated on BM with additional salts, see Table 2) was digested with concentrated HNO_3 and $HClO_4$, and then was analyzed according to a previous research [19]. For the convenience of comparison, we presumed that the desired concentration of biomass in fermentation was 5 g/l and then the metals in cells were adjusted according to it.

At the same time, in order to find out if the trace metals required by ATCC 58784 were adequate in hydrolysate or synthetic media, metal levels in diluted acid hydrolysate and the synthetic media were also tested. The synthetic culture media (namely YPD) was composed by 5 g/l of peptone, 3 g/l of yeast extract, and 3 g/l of malt extract.

Determinations

The value of pH was measured with Twin B-212 pH meter (Horiba Company, Japan). Biomass was estimated from the optical density of culture at 655 nm (OD_{655}) measured with

Table 2 Additional salts added to BM for cultivating cells for metal analysis

Ingredients	Concentration (mg/l)
$CoCl_2{\cdot}6H_2O$	1.6
$NiCl_2{\cdot}6H_2O$	6
$CuSO_4{\cdot}5H_2O$	8
$(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$	5
$NaWO_4{\cdot}2H_2O$	0.5
$KAl(SO_4)_2$	0.5

Table 3 Metal levels (mM) in 5 g/l *P. stipitis* ATCC 58784 (namely In cells), dilute acid hydrolysate neutralized by ammonia water (namely NH_4OH), or by $Ca(OH)_2$ (namely $Ca(OH)_2$), or neutralized and overlimed by $Ca(OH)_2$ (namely Overliming), YPD media and basic medium (BM)

Metals	In cells	NH_4OH	$Ca(OH)_2$	Overliming	YPD	BM
Zn	0.015	0.048	0.049	0.006	0.007	0.026
Co	0.001	ND	ND	ND	ND	ND
Ni	0.004	0.001	0.001	ND	ND	ND
Cu	0.010	ND	ND	ND	ND	ND
Mo	4.08E-05	2.19E-04	2.34E-04	2.03E-04	ND	ND
W	ND	0.002	0.003	0.001	3.80E-04	3.80E-04
Fe	0.065	0.202	0.187	0.054	ND	0.180
Al	0.009	0.210	0.310	0.110	ND	ND
Mn	0.002	0.444	0.436	0.022	ND	0.063
Ca	0.016	0.444	37.7	28.2	0.084	0.274
Mg	0.508	2.18	2.75	1.83	0.126	2.156

ND not detected

a microplate reader BIO-RAD 550 (Nippon Bio-Rad Laboratories, Osaka, Japan), using the estimate that an OD of one is equivalent to 1.40 g/l of dry cells (data not shown). High-performance liquid chromatography (HPLC), equipped with Refractive Index (RI) detector (Jasco International Co., Tokyo, Japan), was used to analyze the contents of ethanol, xylose and xylitol in the culture after separated from biomass by filtering through a 0.2 μm filter. The analysis was carried on a Shodex RSpak KC-811 column (SHOWA DENKO K.K., Kawasaki, Japan) at 60°C with 1.0 ml/min eluent of water. Metals were analyzed by a plasma atomic emission spectrophotometer (ICAP-757, Nippon Jarrell-Ash, Kyoto, Japan), chemical pure salts and deionized water were used to prepare the standard samples.

Statistical analysis

One-way ANOVA was used to test the significance of the results which was carried out with Microsoft excel software at $\alpha = 0.05$. $P < 0.05$, $P < 0.01$, and $P < 0.001$ denote the significance levels of the difference.

Results

Metal levels in cells, dilute acid hydrolysate, YPD and BM medium

Metal levels in cells, dilute acid hydrolysate, YPD and BM medium were listed in Table 3. All the metals except W were detected in ATCC 58784 cells. Among these metals, Co^{2+}, Ni^{2+}, and Cu^{2+} were insufficient in the hydrolysate, YPD and BM medium. The concentration of Al^{3+} in the hydrolysate is relatively higher than that in cells. The concentration of Ca^{2+} in the hydrolysate neutralized by $Ca(OH)_2$ was about 2,400 times higher than that in cells. The result also indicated that overliming reduced the concentration of Zn, which is a cofactor for aldehyde dehydrogenate (ADH), from about 0.048 mM to 0.006 mM.

Since there was potential insufficiency of Cu^{2+}, Co^{2+}, and Ni^{2+} in the hydrolysate of rice straw, YPD and BM medium, which possibly affect the growth and ethanol production of *P. stipitis* ATCC 58784, the influences of Cu^{2+}, Co^{2+}, and Ni^{2+} additions were further researched. As Al^{3+} was suggested being toxic to plants and yeasts [20], the influence of Al^{3+} on *P. stipitis* ATCC 58784 was also studied.

Effects of metal on biomass growth

The influence of Cu^{2+}, Al^{3+}, Ni^{2+} or Co^{2+} addition on biomass growth of *P. stipitis* ATCC 58784 was depicted in Fig. 1. Less than 0.24 mM of Cu^{2+} addition stimulated biomass growth by 34%, however, biomass decreased when more Cu^{2+} was added. Low concentration of Al^{3+} slightly improved the growth of ATCC 58784 ($P < 0.001$), however, higher concentration of Al^{3+} (>0.23 mM) reduced its growth from 4.28 g/l to 3.70 g/l. Ni^{2+} remarkably inhibited biomass growth of ATCC 58784 ($P < 0.001$). When 0.33 mM of Ni^{2+} was added, the biomass concentration decreased from 3.8 g/l to 1.8 g/l. The results of Co^{2+} implied that low concentration of Co^{2+} (<0.045 mM) had little influence on biomass growth ($P = 0.39$), however, higher concentration of Co^{2+} arrested the growth of ATCC 58784 by 13% ($P < 0.001$).

Effects of metal on volumetric ethanol production

The influence of metal addition on volumetric ethanol production was depicted in Fig. 2. The results showed that Cu^{2+} addition (<0.39 mM) seemed to have less impact on volumetric ethanol production than on biomass growth of ATCC 58784 (Fig. 1, $P = 0.05$). Addition of Al^{3+} had little effect on volumetric ethanol production ($P = 0.68$). Addition of 0.074 mM Co^{2+} inhibited volumetric ethanol production

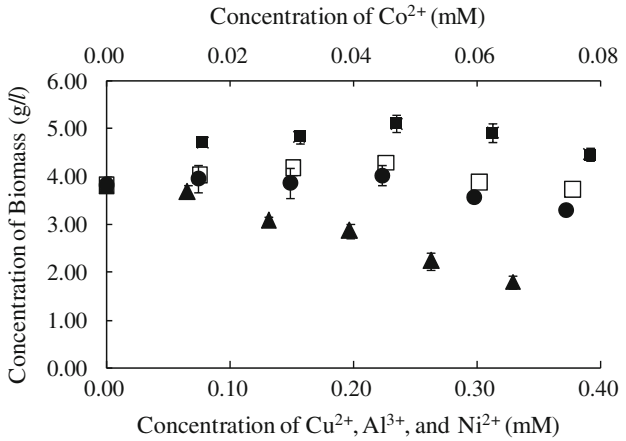

Fig. 1 Influence of metal addition on biomass growth of *P. stipitis* ATCC 58784. Cu^{2+}, *closed square*; Al^{3+}, *open square*; Ni^{2+}, *triangle*; and Co^{2+}, *closed circle* respectively

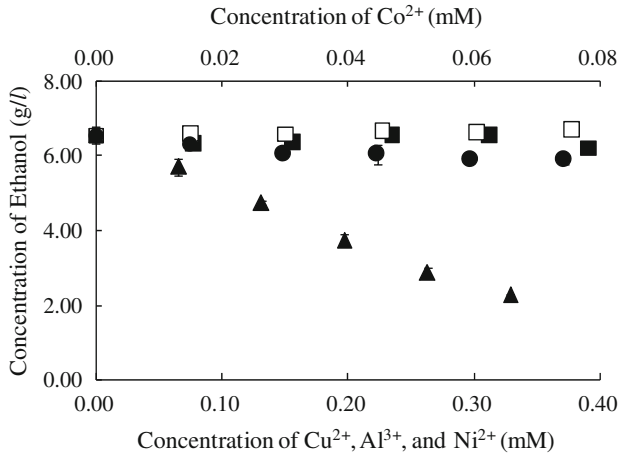

Fig. 2 Influence of metal addition on volumetric ethanol production of *P. stipitis* ATCC 58784. Cu^{2+}, *closed square*; Al^{3+}, *open square*; Ni^{2+}, *triangle*; and Co^{2+}, *closed circle* respectively

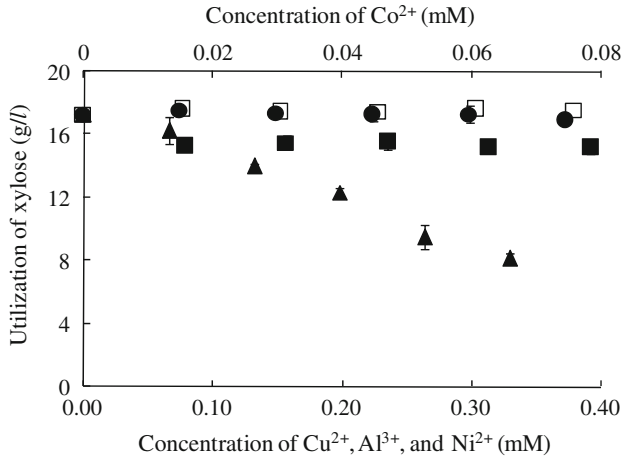

Fig. 3 Influence of metal addition on xylose utilization of *P. stipitis* ATCC 58784. Cu^{2+}, *closed square*; Al^{3+}, *open square*; Ni^{2+}, *triangle*; and Co^{2+}, *closed circle* respectively

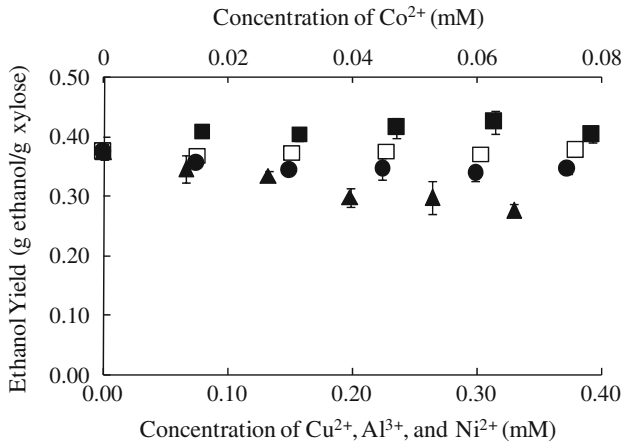

Fig. 4 Influence of metal addition on ethanol yield of *P. stipitis* ATCC 58784. Cu^{2+}, *closed square*; Al^{3+}, *open square*; Ni^{2+}, *triangle*; and Co^{2+}, *closed circle* respectively

of ATCC 58784 by 10% ($P < 0.05$). Ni^{2+} started to show an inhibition effect on volumetric ethanol production of ATCC 58784 at a very low level (0.066 mM), and 0.33 mM of Ni^{2+} arrested the volumetric ethanol production by 65%.

Effects of metal on xylose utilization

The influence of metals on xylose utilization was depicted in Fig. 3. Addition of 0.078 mM of Cu^{2+} reduced xylose consumption of ATCC 58784 from 1.72 g/l to 1.52 g/l ($P < 0.001$), however, this inhibition effect became less with the increase of Cu^{2+} dosage ($P < 0.05$). Addition of Al^{3+} and Co^{2+} had no influence on xylose consumption ($P = 0.59$ and $P = 0.41$, respectively). Ni^{2+} inhibited xylose consumption of ATCC 58784 significantly ($P < 0.001$). When 0.33 mM of Ni^{2+} was added, the xylose consumption decreased by 53%.

Effects of metal on ethanol yield

The influence of metals on ethanol yield was depicted in Fig. 4. Addition of Cu^{2+} increased ethanol yield of ATCC 58784 from 0.37 to 0.42 (0.31 mM of Cu^{2+} added) ($P < 0.05$) and addition of Co^{2+} decreased ethanol yield from 0.37 to 0.34 (0.060 mM of Co^{2+} added) ($P < 0.05$). Addition of Al^{3+} has no influence on ethanol yield of ATCC 58784 ($P = 0.64$). Ethanol yield decreased from 0.37 to 0.27 along with the addition of Ni^{2+} increased from 0 to 0.33 mM ($P < 0.001$).

Effects of metal on ethanol production per gram biomass

The influence of metal addition on ethanol production per gram biomass was depicted in Fig. 5. Addition of Cu^{2+} remarkably reduced the ethanol production per gram

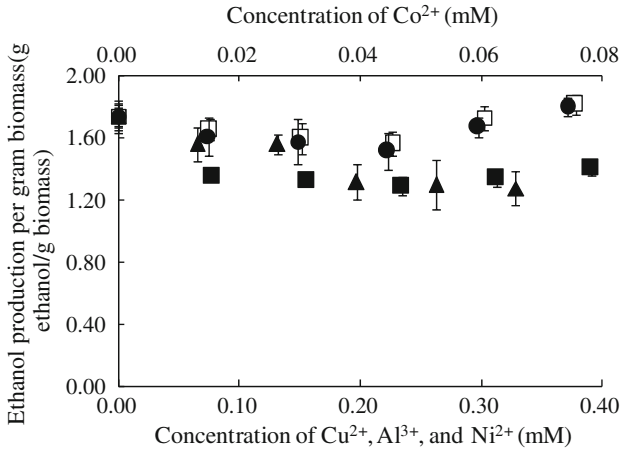

Fig. 5 Influence of metal addition on ethanol production per gram biomass of *P. stipitis* ATCC 58784. Cu^{2+}, *closed square*; Al^{3+}, *open square*; Ni^{2+}, *triangle*; and Co^{2+}, *closed circle* respectively

biomass of ATCC 58784 from 1.73 g/g to 1.29 g/g. However, when Cu^{2+} was further added from 0.08 mM to 0.39 mM, the ethanol production per gram biomass did not change greatly ($P = 0.18$). Addition of Al^{3+} (<0.3 mM) showed inhibitory tendency for ethanol production ($P = 0.08$), however, the significance of its influence was not very certain, because further addition of Al^{3+} restored and increased the ethanol production ($P < 0.05$). The ethanol production was significantly reduced by 27% when 0.33 mM of Ni^{2+} was added ($P < 0.001$). The influence of Co^{2+} on the ethanol production of ATCC 58784 was moderate in this research ($P = 0.08$).

Discussions

Cu^{2+} is the cofactor of cytochrome c oxidase [10] which plays a key role in the respiration metabolism of *P. stipitis* [21, 22]. Addition of Cu^{2+} might stimulate the respiration of ATCC 58784 and produce more energy for constructive metabolism. As a result, the biomass growth increased (Fig. 1). On the other hand, Cu^{2+} is also toxic to most organisms at higher concentrations (more than 0.094 mM for *Saccharomyces cerevisiae*) [10, 23]. And in this research, biomass of ATCC 58784 decreased when more than 0.24 mM of Cu^{2+} was added (Fig. 1). Furthermore, Cu^{2+} showed inhibitory effect on the ethanol production per gram biomass of ATCC 58784 (Fig. 5). Although Cu^{2+} remarkably influenced the growth of ATCC 58784, it did not affect the volumetric ethanol production greatly (Fig. 2). The similar effect of Cu^{2+} on *S. cerevisiae* was observed by Mrvčić and Štanzer et al. [23] who found that volumetric ethanol production and glucose utilization were not remarkably affected by addition of 0.4 mM of Cu^{2+}, although biomass decreased when the concentration of Cu^{2+} exceeded 0.094 mM. It suggested that the influence of Cu^{2+} on ethanol production activity was separated from that on constructive metabolism [15]. The increase of ethanol yield indicated that Cu^{2+} could improve xylose utilization efficiency (Fig. 3 and Fig. 4).

Al is the most abundant metallic element, constituting about 85% by weight of the outer crust of the earth [24]. To our knowledge, no nutrient role is known for aluminum; on the contrary, it has toxic effect on plants, microorganisms and human beings [20]. Low concentration of Al^{3+} was reported to enhance cell division and growth of yeast [25]. From the results obtained, we found that the growth of ATCC 58784 was improved when Al^{3+} lower than 0.23 mM, but decreased when Al^{3+} exceeded 0.23 mM. The results also indicated that ethanol fermentation and xylose utilization were not influenced by Al^{3+} addition though Al^{3+} affected the growth of ATCC 58784 (Figs. 1, 2, and 3). Little influence of Al^{3+} was observed on ethanol production per gram biomass, too (Fig. 5).

Ni^{2+} showed great influence on both growth and fermentation ability of ATCC 58784. Although Ni^{2+}-contained enzymes are involved in many metabolic processes [26], it is also known very toxic to many organisms, such as potentially inhibiting the synthesis of macromolecules (e.g., RNA and protein) [27], inhibiting the acetylation of histone H4 and damaging the heterochromatic regions of chromosomes [28], which reduces biomass growth in all (Fig. 1). Also, Ni could interfere with the roles of other trace elements such as Mg and Fe [27, 28], which might affect the ethanol production pathway and arrest the ethanol yield of ATCC 58784 (Fig. 2). The finding of Nishimura and Igarashi et al. [27] indicated that 0.1 mM of Ni^{2+} was enough for growth arrest of *S. cerevisiae*. In this study, 0.33 mM addition of Ni^{2+} resulted in 53% reduction of biomass growth and 27% decrease of ethanol production per gram biomass of ATCC 58784. This result indicated that Ni^{2+} should not be added when using *P. stipitis* ATCC 58784 to ferment xylose.

Co^{2+} plays a critical role in the synthesis of vitamin B_{12} [29–31]. However, excessive cobalt exposure can lead to various malfunctions for microorganisms, such as 'conditioned iron deficiency' [32], interference with the heme biosynthetic pathway [32], and increase of oxidative stress in cells. Co^{2+} can also mimic or replace ions, e.g., magnesium and calcium, in various essential reactions [30, 31, 33], and prevent the normal processing of the precursor of cytochrome c oxidase (COX) subunit 4 [31]. In this study, high concentration of Co^{2+} (>0.045 mM) would decrease the substrate oxidation rate, probably resulting in the decrease of biomass growth (Fig. 1). Co^{2+} was also reported to be able to substitute Zn in yeast alcohol dehydrogenase [34–36], which might reduce the ethanol production of ATCC 58784 (Fig. 2).

Conclusions

The influences of Cu^{2+}, Al^{3+}, Ni^{2+}, and Co^{2+} addition on the growth and ethanol production of *P. stipitis* ATCC 58784 was researched in this study. Low concentration of Cu^{2+} and Al^{3+} (<0.24 mM and <0.23 mM, respectively) could improve the biomass growth of ATCC 58784 by 34 and 13%, respectively, however, higher concentrations had opposite effects. Addition of 0.074 mM Co^{2+} inhibited the biomass growth of ATCC 58784 by 13% and volumetric ethanol production by 10%. Ni^{2+} is lethal to ATCC 58784. The growth and ethanol production of ATCC 58784 was nearly arrested by adding 0.33 mM of Ni^{2+}. In the future, the interaction of these metals, their interactions with the macro metals (e.g., Zn and Mg), and the optimum metal requirements for ethanol fermentation of rice straw hydrolysate should be investigated.

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