Bio-oil Treated by Cultivation of *Saccharomyces cerevisiae* (QH01)

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Biomass is a renewable and CO_2-neutral source of energy having the drawback of low energy density. The energy density can be augmented by the production of bio-oil through fast pyrolysis. The high content of water-extractable organic acids (oxygenates) in bio-oil is problematic in fuels. Cultivation of *Saccharomyces cerevisiae* for the consumption of these undesirable components can be used to upgrade the bio-oil. It was found that the bio-oil water phase can support the growth of *S. cerevisiae* at concentrations up to 20 vol. % under aerobic conditions. The oxygenates formic acid, acetic acid, and propionic acid had a promoting effect for the cultivation of *S. cerevisiae* in the following order: acetic acid > formic acid > propionic acid. However, phenol, *p*-cresol, and furfural inhibited the growth of *S. cerevisiae.* Kinetic analysis of the consumption of oxygenates showed that the regulation of *S. cerevisiae* was in accordance with a logistic function model.

Keywords: Bio-oil; Aerobic processes; Microbial growth; Yeast

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INTRODUCTION

Biomass, containing cellulose, hemicellulose and lignin, is a renewable and CO_2-neutral energy source. However, biomass has the drawback of low energy density, which restricts its applications. Bio-oil, which has a higher energy density, can be produced by pyrolysis of many biomass materials, such as barley straw (Mullen *et al.* 2009), corn stover (Mullen *et al.* 2010), rice husk (Tsai *et al.* 2007; Yan *et al.* 2010), wood (Agblevor *et al.* 2010; Kechagiopoulos *et al.* 2006), saw dust (Torri *et al.* 2010), and poultry litter (Mante and Agblevor 2010), *etc.*, which will decrease the transportation costs.

Bio-oil is a dark to brown organic liquid (Ayalur Chattanathan *et al.* 2012). The composition of bio-oils varies depending on the biomass sources, as well as the process conditions. Typically, it consists of organic compounds, including small organic acids (SOA), alcohols, ketones, aldehydes and phenolics (Sipilä *et al.* 1998). The main elemental constituents of bio-oil are carbon (C), hydrogen (H), oxygen (O), and nitrogen (N), and hence, its empirical chemical formula can be given as $C_nH_mO_kN_j \bullet xH_2O$ (Wang *et al.* 2007).

Bio-oil can be used for electricity production, for transportation fuels, and for production of chemicals such as phenol and oxygenates. However, bio-oil has a low pH

value and acidic instability due to its high oxygen content. Therefore, upgrading and purifying of bio-oil are required. The upgrading technologies are currently far from industrial utilization since they are expensive (Bridgwater *et al.* 1999). Therefore, an aquatic phase separation of the water-soluble compounds is needed (Pan *et al.* 2006), which makes it possible to upgrade the fractions separately.

The treatment of biomass for ethanol (Thomsen *et al.* 2009) and methane (Nizami *et al.* 2009) production *via* biochemical methods, and the upgrading of bio-oil *via* thermal processes have been applied. However, it is rare to combine the approaches of biochemical and thermal processes for the treatment of biomass. In our latest study, it was found that some aqueous components in bio-oil can be converted to succinic acid by *Escherichia coli* MG-PYC (Wang *et al.* 2013).

In this work, the aqueous phase of bio-oil was treated with Baker's yeast (*Saccharomyces cerevisiae*). *Saccharomyces cerevisiae* is a valuable microorganism with the virtues of both bacteria (living in single cell, growing fast, forming good flocs and suitable for various bioreactors) and fungi (high tolerance to acids, salts and elevated substrate concentration), so herein it was selected for cultivation test in bio-oil, which is a harsh environment for most of the microorganisms. This work is a part of our continuing research for using biological methods to upgrade bio-oil by eliminating some oxygenates and is the first step to use these oxygen containing compounds as sole carbon source to cultivate *S. cerevisiae*. Herein, the effects of different bio-oil model compounds on the growth of yeast cells were investigated, and the growth and consumption kinetics were modeled using logistic-type equations.

MATERIALS AND METHODS

Analytical Methods

The bacterial growth conditions were estimated from the optical density (OD_{600}) of the medium with a spectrophotometer (723N Scanning Visible Spectrophotometer, Shanghai Precision & Scientific Instrument Co. Ltd, China) operating at a wavelength of 600 nm. Formic acid, acetic acid, and propionic acid were analyzed by HPLC using a Bio-Rad Aminex HPX-87H ion-exchange column (7.8×300 mm) and a HP1200 HPLC working station (Agilent Technologies, USA) equipped with a UV detector (Agilent Technologies, G1315D) and a refractive index detector (Agilent Technologies, G1362A). The column was eluted isocratically at a rate of 0.6 mL min^{-1} with a 5 mM H_2SO_4 solution at 50 °C. The injection volume was 10 μL.

Phenol, *p*-cresol, and furfural were also analyzed by HPLC (Agilent) using an Agilent diode array detector (DAD) with an YMC-Pack ODS-A C18 column (5 μm, 4.6 × 250 mm, YMC, Japan). Elution was carried out using an acetonitrile-water mixture (50:50, vol. %) at a flow rate of 0.6 mL min^{-1} at 25 °C and DAD set at 210 nm. The injection volume was 10 μL.

Preparation of Aqueous Solution of Bio-oil

Raw bio-oil was produced from rice husk, which was obtained from Yineng Bio-energy of Shandong, China. Bio-oil and distilled water were mixed at a weight ratio of 1:20 and centrifuged for 25 min at 6225 g and 4 °C. After centrifugation, the liquid phase was obtained as an aqueous solution of bio-oil.

Microorganism

Saccharomyces cerevisiae (QH01) was obtained from Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences.

Seed Culture Preparation

The *S. cerevisiae* strain was maintained on yeast extract peptone dextrose (YPD) medium agar plates containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose, and 20 g L^{-1} agar at 4 °C. The inoculum medium was YPD composed of 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, and 20 g L^{-1} glucose. The cultures were grown in an orbital shaker at 220 rpm for 48 h at 30 °C, and 5 vol. % of the cultures was used for the inoculation of the growth media.

Growth Media

The effect of different carbon sources on the growth of *S. cerevisiae* was evaluated in batch cultures under aerobic conditions. These experiments were conducted in 100 mL of culture in 250 mL conical flasks. Cells were cultivated in a synthetic medium consisting of distilled water, 5 g L^{-1} $(NH_4)_2SO_4$, 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, trace elements (Table 1) and bio-oil or a single chemical as sole carbon source at 30 °C in orbital shaker at 220 rpm; the medium was adjusted to a pH of 5 with solid KOH. The carbon sources of bio-oil, formic acid, acetic acid, and propionic acid was directly added to the medium before sterilization, while phenol, *p*-cresol, and furfural that were filtered by 0.22 μm sterile filters were added to the medium after the medium was sterilized at 115 °C for 30 min.

Table 1. Content of Trace Elements in Synthetic Medium without Carbon Source

Trace elements	Concentration (mg L^{-1})	Inorganic salt	Concentration(mg L^{-1})
Biotin	0.05	$CoCl_2 \cdot 6H_2O$	0.3
Calcium pantothenate	1.00	$MnCl_2 \cdot 4H_2O$	1.0
Nicotinic acid	1.00	$CuSO_4 \cdot 5H_2O$	0.3
Inositol	25.00	$CaCl_2$	3.4
Thiamine HCl	1.00	$FeSO_4 \cdot 7H_2O$	3.0
Vitamin B6	1.00	$NaMoO_4 \cdot 2H_2O$	0.4
Para aminobenzoic acid	0.20	H_3BO_3	1.0
EDTA	15.00	KI	0.1
		$ZnSO_4 \cdot 7H_2O$	4.5

Experimental Method of *S. cerevisiae* Cultivation

Aqueous bio-oil solution and model compounds of the aqueous phase of bio-oil (formic acid, acetic acid, propionic acid, phenol, *p*-cresol, and furfural) in different concentrations were added to the synthetic medium. The concentrations of the reagents and the OD_{600} values of the media were measured at different stages. A blank medium without any carbon source was also tested for comparison. The changes in the OD_{600} values and the consumption of the carbon sources *versus* the cultivation time were investigated.

RESULTS AND DISCUSSION

Utilization of Aqueous Solution of Bio-oil as Sole Carbon Source

The OD_{600} values of the aqueous solutions (10 to 20 vol. % aqueous solution of bio-oil) *versus* the cultivation time were measured as shown in Fig. 1. The values were corrected by use of abiotic controls because the OD_{600} of aqueous bio-oil solution can change without microbial growth. Figure 1 shows that the OD_{600} of the medium without any carbon source (0 vol. %) changed little. OD_{600} with 20 vol. % aqueous bio-oil solution bio-oil increased with evolution time, while the highest increase happens on the sample with 10 vol. %. The results indicated that the bio-oil aqueous solution can support the growth of *S. cerevisiae*, while some inhibiting components may exist in the water phase of bio-oil, which need to be identified. To clarify the roles of the main components, some model compounds were individually investigated.

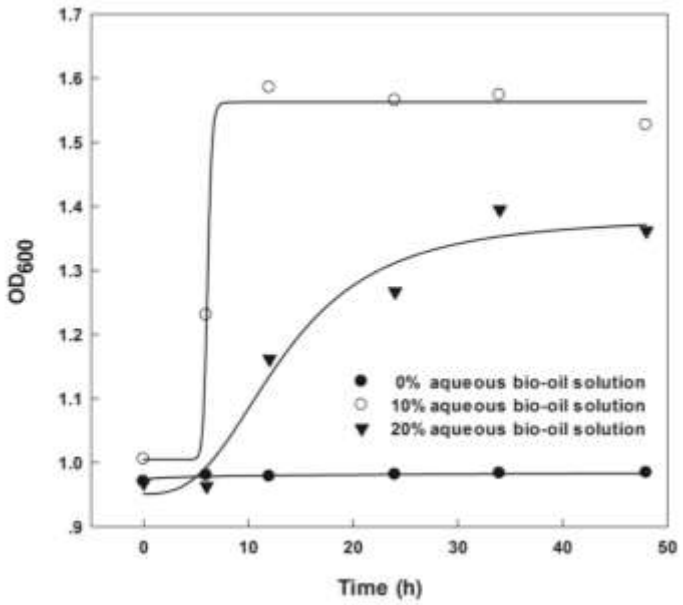

Fig. 1. Effect of aqueous bio-oil solution as sole carbon source for the growth of *S. cerevisiae*, as denoted by OD_{600}

Utilization of SOA as sole Carbon Source

Small organic acids (SOA), including formic acid, acetic acid, and propionic acid, were the major components in the aqueous bio-oil solution. These compounds were tested in the concentration range of 0 to 8 g L^{-1}. The OD_{600} values of the systems and the concentrations of the carbon sources versus cultivation time are shown in Figure 2.

It can be seen from Figs. 2A and 2B that the OD_{600} values of formic acid and acetic acid increased more than that without any carbon source, while the concentrations of formic acid and acetic acid simultaneously decreased. This indicated that formic acid and acetic acid can support the growth of *S. cerevisiae*. Comparatively, the changes of OD_{600} values with formic acid were smaller (from 1.4 to 2.8) than those with acetic acid (from 1.1 to 5.6). The OD_{600} values in both acetic acid and formic acid systems increased after a lag phase.

The consumption curves for formic acid and acetic acid were in accordance with the changes of the OD_{600} values. The formic acid of 1.9 g L^{-1} was 90% consumed, and

50% of the formic acid was consumed for the 7.8 g L^{-1} initial concentration, while almost 100% acetic acid was consumed for all initial concentration ranges examined. This indicated that *S. cerevisiae* is more tolerant to acetic acid than to formic acid in similar concentrations, which is consistent with the literature (Larsson *et al.* 1999). It is interesting to note that acetic acid is commonly thought to be a growth inhibitor to *S. cerevisiae* when glucose simultaneously is present in the medium (Palmqvist and Hahn-Hägerdal 2000).

Figure 2C shows that the OD_{600} values of the propionic acid systems did not increase with cultivation time, except for the lowest initial concentration of 2.0 g L^{-1} (OD_{600} increased from 1.6 to 2.4). The 2.0 g L^{-1} system showed an 80% consumption rate, while those at higher initial concentrations changed less than 5%. This indicated that *S. cerevisiae* cannot grow under higher initial concentrations of propionic acid, and that > 4.0 g L^{-1} propionic acid may even inhibit the yeast's growth.

Huang *et al.* (2011) reported that in Luria–Bertani (LB) media with glucose, formic acid showed a slightly negative effect on the growth of *S. cerevisiae* at low concentrations (1.0 to 4.0 g L^{-1}), and complete inhibition at high concentrations (5.0 to 8.0 g L^{-1}). The authors also reported that propionic acid showed the same effect and that a high concentration of acetic acid (6.0 g L^{-1}) resulted in significant growth inhibition. In this study, propionic acid at higher initial concentration showed a different level of growth inhibition of *S. cerevisiae*. Formic acid, acetic acid, and low initial concentration of propionic acid can be used as sole carbon source.

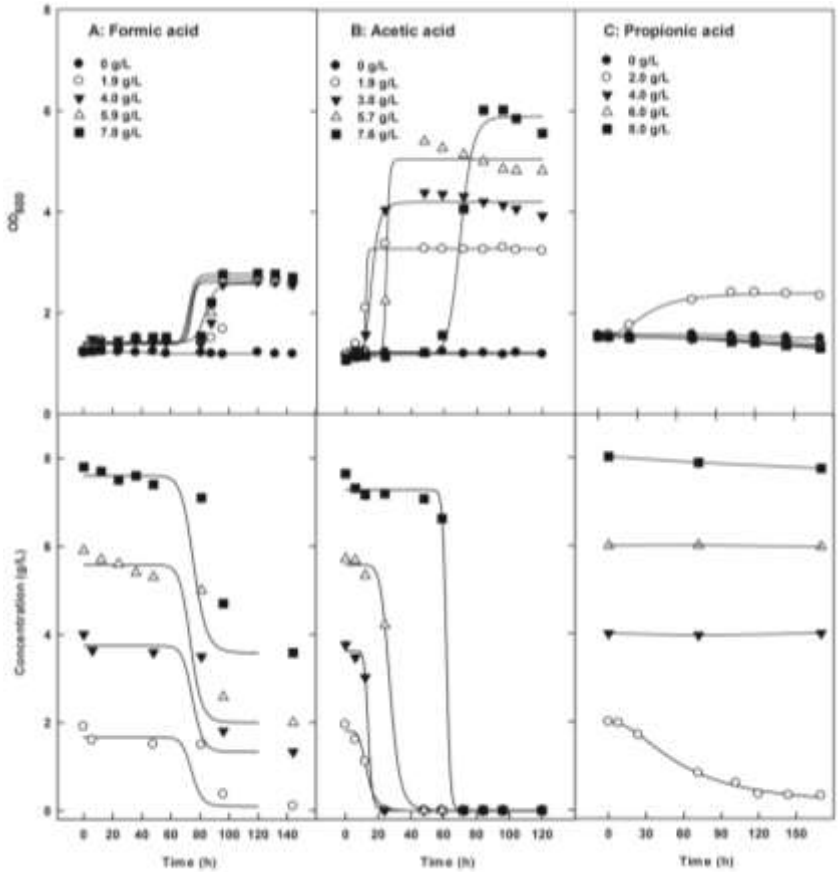

Fig. 2. Effect of SOA as sole carbon source on the OD_{600} value of *S. cerevisiae* (top), and the concentration of SOA (bottom): (A) formic acid, (B) acetic acid, (C) propionic acid

The difference between the two studies may relate to the presence of glucose; if a high concentration of glucose is present, such glucose is more preferred to be consumed by *S. cerevisiae*, and the SOA may have an inhibiting effect on the consumption of glucose, while if there is no glucose in the media, some SOA such as formic acid, acetic acid, and low concentration of propionic acid may be consumed for survival by adaptation.

Utilization of Phenolic Compounds as sole Carbon Source

The content of phenolic compounds in the bio-oil was 10 to 30%, and the main components were predominately phenol and *p*-cresol. Therefore, phenol and *p*-cresol in concentrations of 0 to 7.0 g L^{-1} were selected as model components for the *S. cerevisiae* cultivation test. The OD_{600} values and the consumption of phenol and *p*-cresol are shown in Figs. 3A and B, respectively.

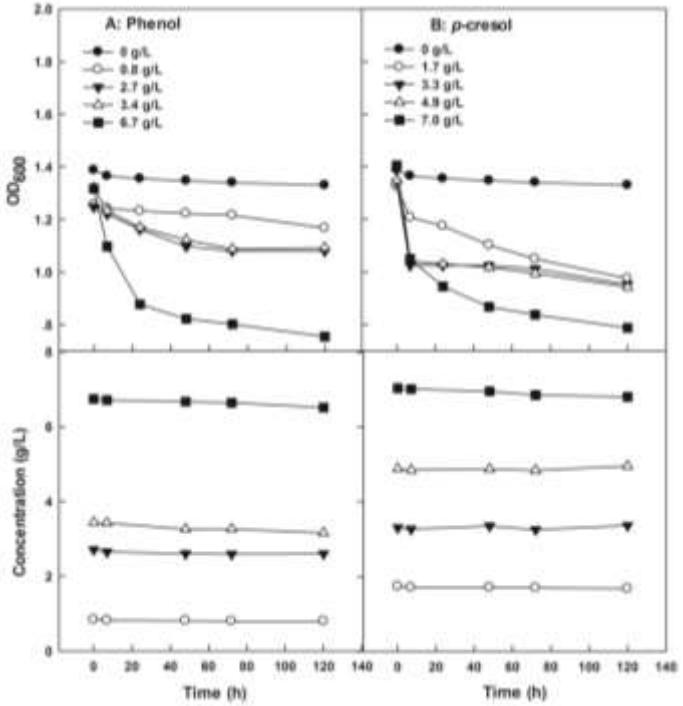

Fig. 3. Effect of phenolic compounds on the growth of *S. cerevisiae* (top) and the concentration change (bottom): (A) phenol, (B) *p*-cresol

Figure 3 shows that the OD_{600} values of phenol and *p*-cresol systems decreased with time. This indicated that phenol and *p*-cresol can inhibit the growth of *S. cerevisiae* and that this inhibition effect increases with increasing initial concentration. The decreased OD_{600} values and the constant concentrations of phenol and *p*-cresol demonstrated that *S. cerevisiae* cannot consume these phenolics as sole carbon source for growth.

Although QH01 cannot utilize phenol and *p*-cresol, there are still quite a few of microorganisms can consume phenolics. The bacterial strain EDP3 (Geng *et al.* 2006), isolated from an industrial activated sludge, could grow on phenol up to a concentration of 1000 mg L^{-1} in MP500 medium, while no growth was observed in an initial concentration of 1500 mg L^{-1}. It took nearly 60 h for the bacterial strain to consume 500

mg L^{-1} phenol to nondetectable levels and over 220 h for a 1000 mg L^{-1} initial phenol concentration. Similarly, the bacteria strain ASI (El-Sayed *et al.* 2003), isolated from an enriched phenol-activated sludge, could consume 100 mg L^{-1} phenol as sole carbon source to a nondetectable level in 30 h. Moreover, the bacterial strain WUST-C1 (Liu *et al.* 2012) isolated from activated sludge could grow on phenol at a concentration as high as 1600 mineral salt medium (MSM), and the phenol up to 1200 mg L^{-1} could be completely biodegraded within 36 h. Besides, *Acinetobacter* species has also been well-documented (Hoyle *et al.* 1995; Pessione and Giunta 1997; Abd-El-Haleem *et al.* 2002; Hao *et al.* 2002) in phenol degradation. *P*-cresol could be degraded effectively by a *pseudomonas* sp. (O'Reilly and Crawford 1989) isolated from creosote-contaminated soil immobilized in either calcium alginate or polyurethane. Generally, many microorganisms have been found capable in utilizing phenolics, which could be a hint for consumption of the phenolic components in bio-oil.

Utilization of Furfural as sole Carbon Source

Furfural is a major component of aldehyde in bio-oil and was therefore selected as a model compound to study its effect on the growth of *S. cerevisiae*. The OD_{600} values in the yeast cultivations and the consumption of furfural are shown in Fig. 4. It can be seen that the OD_{600} value decreased with the passage of time, which indicated that furfural inhibited the growth of *S. cerevisiae*. Furthermore, the inhibiting effect increased with increasing initial concentration of furfural. Meanwhile, the initial concentration of furfural decreased slightly, which also indicated that *S. cerevisiae* cannot consume furfural for growth. All the OD_{600} values in the media with 2.0, 4.1, 6.2, and 8.2 g L^{-1} furfural decreased with increasing evolution time, and the general reduced values were all around 0.3.

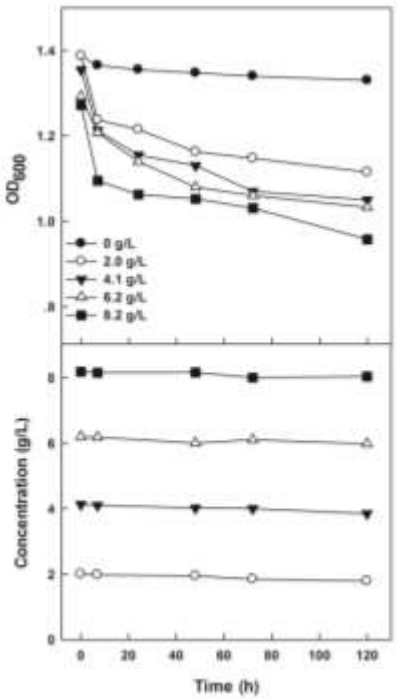

Fig. 4. Effect of furfural as sole carbon source on the growth of *S. cerevisiae* (top) and concentration change of furfural (bottom)

Furfural can be detrimental to downstream operations due to inhibition of enzymes (Tengborg *et al.* 2001) and lead to poor cell growth/function caused by the inhibition (Klinke *et al.* 2004). Despite this, the inhibition of furfural could be partly relieved by careful control of the culture condition (inoculum size, temperature, and initial pH) (Huang *et al.* 2011). For instance, furfural displayed a minimum inhibition at 10% inoculum size compared to 5% and 15% inoculum size. Interestingly, the effect of inhibition under combination of furfural and other aldehydes on cell growth was not augmented, on the contrary, the binary combination of furfural with the other three aldehydes (HMF, vanillin and 4-hydroxybenzaldehyde) exerted less inhibition than individual inhibitor itself (Huang *et al.* 2011). It seems that the inhibition of furfural in bio-oil cannot be avoided anyway, while its inhibition could be reduced by some selected furfural-tolerant microorganisms (Xia and Yuan 2008) or can be mitigated by combining furfural with some other aldehydes.

Comparison among SOA, Phenols, and Furfural

The above results illustrated that different components in bio-oil affected the growth of *S. cerevisiae* differently. To make an even clearer comparison among the components, the differences of OD_{600} (Δ-values of OD_{600}) between the final and the initial values were compared, as shown in Figure 5. The figure showed that initial concentrations of 1.9 to 7.8 g L^{-1} formic acid, 1.9 to 7.6 g L^{-1} acetic acid, and 2.0 g L^{-1} propionic acid can support the growth of *S. cerevisiae* (*i.e.*, positive Δ-values). Inhibition of *S. cerevisiae* and corresponding negative Δ-values were found for initial concentrations of 4.0 to 8.0 g L^{-1} propionic acid, 0.8 to 6.7 g L^{-1} phenol, 1.7 to 7.0 g L^{-1} *p*-cresol, and 2.0 to 8.2 g L^{-1} furfural; this indicated that *S. cerevisiae* cannot consume these compounds for growth. The general ability of the carbon sources in supporting the growth of *S. cerevisiae* can be ordered as acetic acid > formic acid > propionic acid. Figure 5 also shows that the promoting effect of SOA was more distinct than the inhibiting effect from phenols and furfural; thus, bio-oil can still be used as sole carbon source for *S. cerevisiae*.

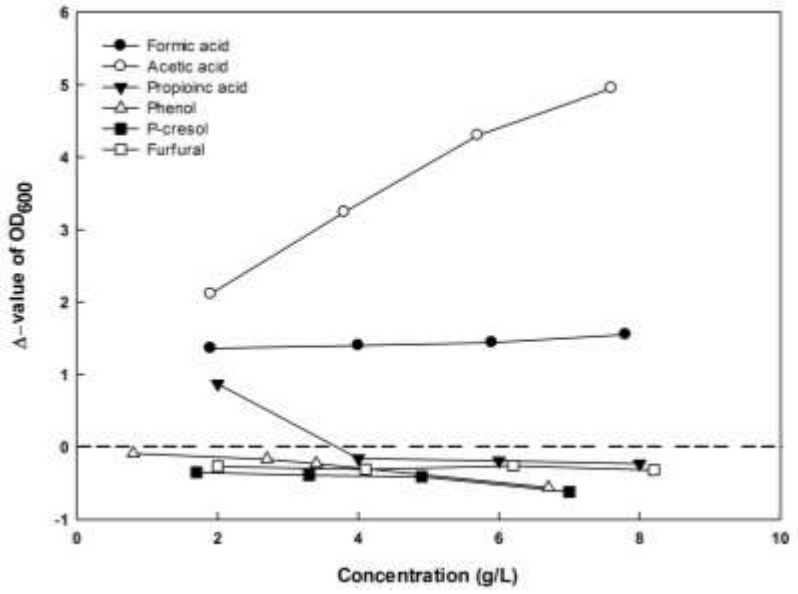

Fig. 5. Effect of different concentrations of carbon sources on growth of *S. cerevisiae* (Δ-value)

Kinetic Analysis of the Growth of Microbes and the Consumption of Carbon Sources

The logistic function model has been broadly used to describe the growth of microbes in many diverse biological systems (Sadkowski 2000); the integrated form of the equation can be written as Eq. 1. The substrate consumption rate can be expressed as Eq. 2 (Aghaie *et al.* 2012).

$$C = \frac{k_1 + k_2 (t / t_0)^a}{1 + (t / t_0)^a} \quad (1)$$

$$S = \frac{k_1 + k_2 (t / t_0)^q}{1 + (t / t_0)^q} \quad (2)$$

In these equations, C (g dry weight L^{-1}) is the real-time cell dry weight at a single point (1 OD_{600} = 0.51 g dry cell weight/L), S is the real-time carbon source concentration, t is time (h), a and q are power constants, and k_1 and k_2 are kinetic parameters. The kinetic parameters and model simulations were implemented *via* the software Origin (OriginLab Corp., Northampton, MA, USA).

Equations 1 and 2 were applied to fit the OD_{600} values and the consumption curves of the small organic acids (SOA), as shown in Fig. 2 with the drawn curves. It can be seen that the equations fitted the experimental data well, which indicated that the bio-conversion processes complied well with the kinetic regulations of logistic-type equations.

CONCLUSIONS

1. An aqueous bio-oil solution can be used for the cultivation of *S. cerevisiae*, and the aqueous bio-oil solution diluted by water to 10 vol. % was the optimal level.
2. Small organic acids including formic acid, acetic acid, and propionic acid showed positive influence on supporting cell growth, and the approximate sequence was acetic acid > formic acid > propionic acid. On the other hand, phenol, *p*-cresol, and furfural cannot support the growth of *S. cerevisiae* and even had an inhibiting effect.
3. Kinetic analysis of the growth and consumption of small organic acids was conducted, and the kinetic regulation was found to be in accordance with logistic equations.
4. It is very interesting that the aqueous phase of bio-oil can be utilized to support the growth of *S. cerevisiae via* a simple separation by adding water. It provides a potential method for the utilization of bio-oil to substitute for glucose as sole carbon source; in addition, the bio-oil can be upgraded due to the selective consumption of components by cultivation of *S. cerevisiae*. The consumption of small organic acids (SOA) is advantageous for reducing the acidity and for augmenting the heating value of the bio-oil.

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