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ORIGINAL RESEARCH PAPER

In vivo detoxification of furfural during lipid production by the oleaginous yeast *Trichosporon fermentans*

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Abstract In vivo detoxification of furfural by the oleaginous yeast, *Trichosporon fermentans*, under lipid-producing (i.e., nitrogen-limited) conditions was evaluated for the first time. During the initial fermentation phase, furfural was rapidly reduced to furfuryl alcohol, which is more toxic to *T. fermentans* than furfural. Furfuryl alcohol was subsequently oxidized to furoic acid which has low toxicity to *T. fermentans* and is the end product of the in vivo detoxification of furfural in this organism. These observations explain how *T. fermentans* can grow and accumulate lipids in medium containing furfural. They also indicate that strategies to minimize the transient production of furfuryl alcohol could further improve the capacity of the strain to produce lipids from furfural-containing lignocellulosic hydrolysates.

Keywords Detoxification · Furfural · Furfuryl alcohol · Furoic acid · *Trichosporon fermentans*

Introduction

The bioconversion of lignocellulose is mainly applied to the production of alcohols (especially ethanol) and methane (Hendriks and Zeeman 2009). Recently, however, lignocellulosic biomass has attracted attention as the feedstock for transformation by oleaginous microorganisms into lipids that can be used for biodiesel production (Huang et al. 2009; Zhao et al. 2008). A major barrier to the implementation of this technology is that, during hydrolysis of lignocellulosic biomass, besides fermentable sugars,

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various inhibitors such as aldehydes, organic acids and alcohol compounds are produced, and these inhibitors would prevent the growth and lipid accumulation of oleaginous microorganisms (Hu et al. 2009; Huang et al. 2011). Among such inhibitors, furfural is common in dilute acid-treated hydrolysates and its concentration can be as high as 2–3 g/l (Palmqvist and Hahn-Hagerdal 2000; Taherzadeh et al. 1997). Furfural causes accumulation of reactive oxygen species (ROS) and leads to damage to mitochondria, vacuoles, actin and nuclear chromatin when growing yeast cells are in contact with it (Allen et al. 2010).

Detoxification of furfural can be carried out by certain yeast strains via conversion to generally less toxic compounds such as furfuryl alcohol or furoic acid. However, most studies of the mechanism of inhibition and strategies to remove the inhibitors or overcome their toxicity have been tailored to ethanologenic processes using yeasts such as *Saccharomyces cerevisiae* (Almeida et al. 2007; Keating et al. 2006). In contrast to the anaerobic process that is used to produce ethanol, production of lipids by oleaginous organisms occurs under aerobic conditions and requires a medium containing an excess of carbon substrate and a limited amount of nitrogen source for efficient accumulation of lipids (Ratledge 2004). To date, there have been a number of reports showing the deleterious effects of various lignocellulose-derived inhibitors on the growth and lipid accumulation of oleaginous yeasts (Chen et al. 2009; Hu et al. 2009; Huang et al. 2011), but the possibility of using such microorganisms to detoxify the inhibitors in vivo remains unexplored.

Trichosporon fermentans is an oleaginous yeast strain that has potential for production of lipids from lignocellulosic biomass (Huang et al. 2009). Whilst growth and lipid production in *T. fermentans* are inhibited by furfural, it can grow and accumulate lipid with minimal inhibition in medium containing furfural at $\leq$2 mM (Huang et al. 2011). To further investigate the effect of furfural on *T. fermentans,* and to establish whether this strain could indeed detoxify this major inhibitor of lipid-producing microorganisms, we have examined the interaction of furfural and related compounds with this organism and have, for the first time, shown in vivo detoxification of an inhibitor during cell growth and lipid accumulation by an oleaginous yeast.

Methods and materials

Microorganism and chemicals

Trichosporon fermentans CICC 1368 was from the China Center of Industrial Culture Collection.

Furfural was from Sigma-Aldrich (USA). Furoic acid, and furfuryl alcohol were obtained from Alfa Aesar (UK). All other chemicals were from commercial sources and were of the highest purity available.

Medium, precultivation and cultivation

The precultivation medium contained: 20 g glucose and xylose (ratio 2:1, w/w)/l, 10 g peptone/l, 10 g yeast extract/l, pH 6.0. The fermentation medium (pH 6.5) contained: 100 g glucose and xylose (ratio 2:1, w/w)/l, 0.5 g yeast extract/l, 1.8 g peptone/l, 0.4 g $MgSO_4 \cdot 7H_2O$/l, 2.0 g KH_2PO_4/l, 0.003 g $MnSO_4 \cdot H_2O$/l, 0.0001 g $CuSO_4 \cdot 5H_2O$/l. Preculture was performed in a 250 ml conical flask containing 50 ml precultivation medium at 28 °C for 24 h in a rotary shaker (160 rpm). Seed culture (2.5 ml) was then inoculated into a 250 ml conical flask containing 47.5 ml of fermentation medium and cultivation was carried out in a rotary shaker (160 rpm) at 25 °C.

Effect of furfural and its derivatives on growth and lipid accumulation

Seed culture (2.5 ml) was prepared in precultivation medium as described above, and then it was inoculated into 47.5 ml fermentation medium containing furfural or one of its derivatives (furoic acid or furfuryl alcohol) and cultivation was carried out at 25 °C and 160 rpm for 7 days. All reported data are averages of experiments performed at least in triplicate.

Detoxification of furfural by *T. fermentans*

After precultivation, 2.5 ml seed culture was inoculated into 47.5 ml fermentation medium containing 7 mM furfural (0.67 g/l, the concentration at which 50 % of the cell growth of *T. fermentans* was inhibited) and cultivation was carried out at 25 °C and 160 rpm for 10 days. Fermentation broth (0.1 ml) was taken out at specific time intervals for inhibitor concentration analysis. Where necessary, samples were diluted with distilled water to bring them within

the appropriate range of concentrations before HPLC measurement. Concentrations of furfural and its derivatives were calculated by using a standard curve of known concentrations of each analyte. At the same time, the biomass, lipid content, lipid yield of *T. fermentans*, and the residual sugars concentration in the medium were measured.

Analytical methods

Biomass, lipid content and lipid yield were measured according to the procedures of Huang et al. (2009). Furfural and its derivatives were quantified by HPLC with detection at 220 nm. For the determination of furoic acid, an Aminex HPX-87H column at 65 °C was used and eluting with 0.005 M H_2SO_4 at 0.5 ml/min. Furfural and furfuryl alcohol were determined on an Xbridge C18 column (Waters Corp., USA) with water/methanol (75/25, v/v) at 1.0 ml/min.

Results and discussion

The toxicity of furfural to *T. fermentans* was initially compared with that of the two derivatives (furfuryl alcohol and furoic acid) that are commonly produced during its biological breakdown (Fig. 1). The reduction product, furfuryl alcohol, up to 16 mM had a slightly greater inhibitory effect on growth and lipid accumulation of *T. fermentans* than furfural, whilst the inhibitory effects of the oxidation product furoic acid were very much less than those of furfural. These results contrast sharply to the situation with an ethanologenic strain *Escherichia coli*, where furfuryl alcohol showed much lower toxicity than furfural or furoic acid (Zaldivar et al. 2000). The results with *T. fermentans* are similar to what was seen with the oleaginous yeast *Rhodosporidium toruloides,* where (as with *T. fermentans*) furoic acid was less inhibitory than furfuryl alcohol (Hu et al. 2009).

The main feature of the results with *T. fermentans* is, however, the fact that furoic acid showed only a very small toxic effect. In cultures of *R. toruloides*, furoic acid at 4 mM decreased the yield of biomass by more than 60 % and the lipid content by approx. 55 % (Hu et al. 2009). In contrast, the same concentration of furoic acid did not significantly alter the biomass yield of *T. fermentans* (Fig. 1a) and actually slightly increased the relative lipid content of the cells

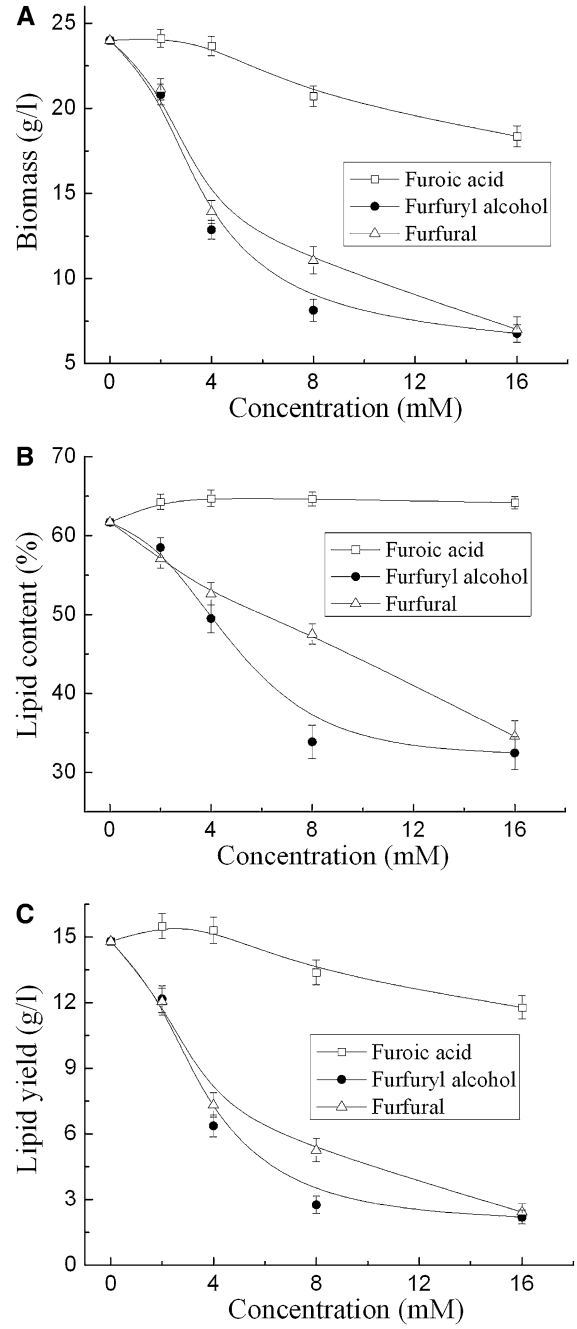

Fig. 1 Effect of furfural and its derivatives on the growth and lipid accumulation of *T. fermentans*: **a** biomass, **b** lipid content, **c** lipid yield. *Error bars* indicate standard error of experiments performed at least in triplicate

(Fig. 1b). When furoic acid was increased to 16 mM, the biomass of *T. fermentans* was still as high as 18.4 g/l (80 % of the uninhibited control), in contrast to 6.8 g/l and 7.0 g/l (28 and 29 % of the

control, respectively), in the presence of the same concentration of furfural and furfuryl alcohol. Moreover, the increase in relative lipid content of *T. fermentans* was sustained throughout the range of furoic acid concentrations tested (up to 16 mM; Fig. 1b).

To get a better understanding of the inhibitory effect of furfural and its derivatives on the growth and lipid accumulation of *T. fermentans*, we investigated the effect of these compounds on sugar consumption after fermentation for 7 days (Fig. 2). Furoic acid had no observable effect on the uptake of glucose by *T. fermentans*, which completely used up the glucose in medium to which 16 mM furoic acid had been added. In contrast, in medium containing furfural or furfuryl alcohol, glucose could not be utilized completely and the higher the inhibitor concentration was, the more glucose remained (Fig. 2a). Similarly, furoic acid exerted much less inhibition on xylose utilization (Fig. 2b) and total sugar removal (Fig. 2c) than furfural and furfuryl alcohol; indeed, in the presence of 2 mM furoic acid *T. fermentans* used significantly more xylose than the no-inhibitor control (Fig. 2b). These results are in general agreement with the above observation (Fig. 1) that furoic acid displayed the lowest toxicity among the inhibitors tested to cell growth and lipid accumulation of *T. fermentans*.

To investigate whether the oleaginous yeast *T. fermentans* could detoxify furfural in vivo while converting sugars into lipids, the concentrations of furfural and its two derivates were measured during lipid fermentation by this organism (Fig. 3). At the same time, the evolution of biomass, lipid content, lipid yield and residual sugars in the medium during the lipid fermentation were also evaluated (Fig. 4).

Starting with furfural at 7 mM (0.67 g/l), *T. fermentans* initially reduced furfural to the slightly more toxic furfuryl alcohol, so that essentially all the inhibitor was present as furfuryl alcohol after 12 h fermentation. There was no increase in the biomass of *T. fermentans* during this time. Afterwards, the concentration of furfuryl alcohol decreased while the concentration of furoic acid, the oxidation product of furfuryl alcohol, increased. Also, no furfural was detected anymore during this period. When the furfuryl alcohol was transformed into furoic acid, the biomass of *T. fermentans* increased as well (Fig. 4b) but the biomass was merely about 2.7 g/l after one day's fermentation, which was much less than that on

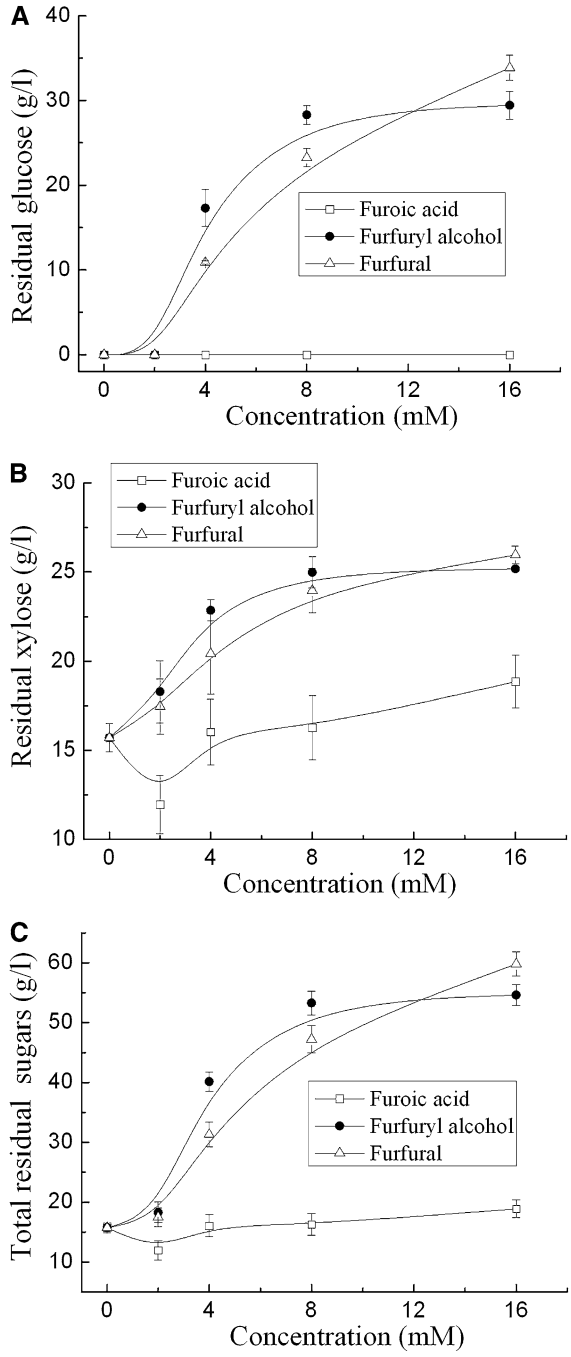

Fig. 2 Effect of furfural and its derivatives on sugar consumption by *T. fermentans*: **a** residual glucose, **b** residual xylose, **c** total residual sugars after seven days of fermentation. *Error bars* indicate standard error of experiments performed at least in triplicate

the medium with no inhibitor (Fig. 4a). The furfuryl alcohol concentration was as low as 1.25 mM by 72 h and completely removed by 216 h (9 days) of

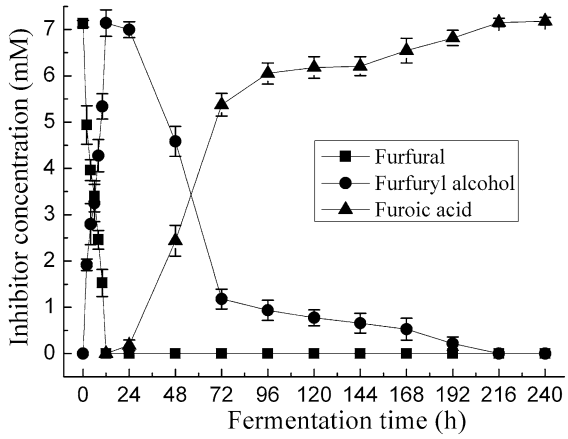

Fig. 3 Biotransformation of furfural during aerobic batch cultivation of *T. fermentans* under lipid-accumulating conditions. *Error bars* indicate standard error of experiments performed at least in triplicate

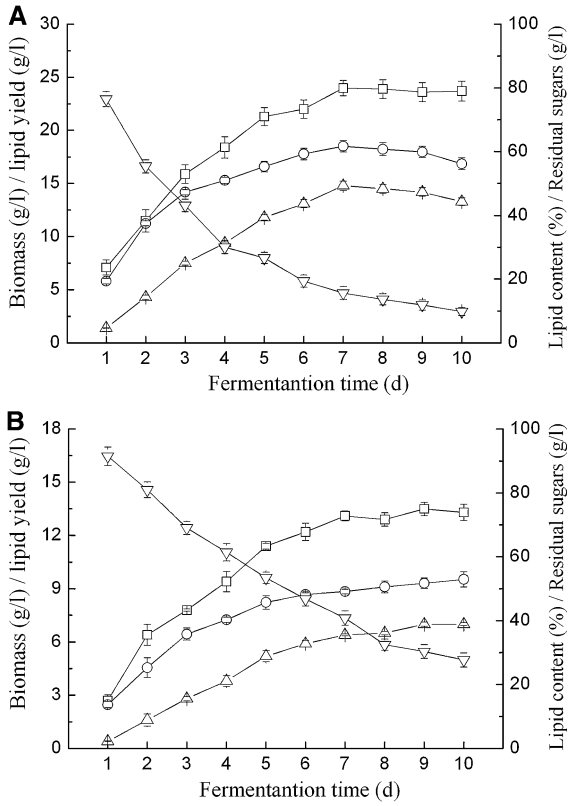

Fig. 4 Lipid fermentation of *T. fermentans* on the medium with **a** no inhibitor and **b** 7 mM furfural. *Square* biomass, *circle* lipid content, *upward triangle* lipid yield, *downward triangle* residual sugars

fermentation. The net impact of these changing levels of inhibitors on growth of the culture was that the biomass yield after 7 days was decreased by of about 50 % compared to the no-inhibitor control (Fig. 4). Also, this influenced the lipid accumulation and sugar consumption of *T. fermentans* obviously (Fig. 4). For example, after fermentation for 7 days, there were only 15.7 g sugars/l remaining in the medium with no inhibitor, while the residual sugar concentration was 40.8 g/l in the presence of furfural. However, *T. fermentans* could still consume sugars after 7 days with a steady rate although its consumption rate was slowed down by the inhibitor.

Generally during ethanologenic fermentations with yeasts, such as *S. cerevisiae*, owing to the anaerobic nature of the process, reduction is the main route of in vivo furfural detoxification and furfuryl alcohol is the main detoxification product (Palmqvist et al. 1999). In contrast, during steady state aerobic growth of *S. cerevisiae*, furfural is oxidized directly to furoic acid (Horvath et al. 2003). Here, in lipid-producing *T. fermentans* cultures, the toxic reduction product furfuryl alcohol accumulates transiently before oxidation to the essentially non-toxic furoic acid. Hence, although *T. fermentans* is inhibited in the early stages of the fermentation by furfural and furfuryl alcohol, it is able to overcome the inhibition and produce biomass and lipids by oxidation of the inhibitor to the less toxic furoic acid (Figs. 3, 4). As the initial concentration of furfural was increased, it was not until it exceeded 24 mM (2.3 g/l) that *T. fermentans* could no longer grow (data not shown).

In the experiment shown in Fig. 3, the conversion of furfural to furfuryl alcohol precedes observable growth and so it is likely that the reactions important for detoxification of the inhibitors are be performed by the cells present in the original inoculum. Consistent with this, we previously observed that a larger inoculum of *T. fermentans* could lead to increased biomass and lipid yields when furfural or other inhibitors were present although, intriguingly, an increase in the inoculum size from 10 to 15 % (v/v) of the culture volume actually decreased yields when furfural was present (Huang et al. 2011).

Conclusion

Trichosporon fermentans has been shown to detoxify furfural to furoic acid, which has much lower toxicity

to *T. fermentans* when compared with furfural and furfuryl alcohol. Furfural was detoxified in vivo by *T. fermentans* via the reduction to furfuryl alcohol and the subsequent oxidation of the latter to furoic acid. Hence, whilst *T. fermentans* shows the promising property of detoxifying furfural by converting it to the low-toxicity derivative furoic acid, the transient accumulation of the toxic furfuryl alcohol to some extent limits the ability of this organism to resist the presence of furfural in practice. Therefore, strategies to enhance the detoxification process and avoid the accumulation of toxic by-products, by manipulating culture conditions or by adaptation or genetic modification of the strain, are a focus for our on-going research.

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