# Inhibitor Tolerance: A Comparison between *Rhizopus* sp. and *Saccharomyces cerevisiae*

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Zygomycetes fungi are able to produce ethanol, and their biomass may hold a high market value, making them interesting microorganisms from a biorefinery perspective. In the present study, the inhibitor tolerance of the Zygomycetes fungus *Rhizopus* sp. was evaluated and compared with a flocculating strain of *Saccharomyces cerevisiae*. The inhibitors furfural, 5-hydroxymethylfurfural [HMF], acetic acid, and levulinic acid and the phenolic compounds catechol, guaiacol, and vanillin were applied in different combinations in a semi-synthetic medium. Glucose uptake and conversion of HMF in the presence of inhibitors were analyzed for the two organisms, and it appeared that the inhibitor resistances of *Rhizopus* sp. and *S. cerevisiae* were comparable. However, in the presence of catechol (0.165 g $L^{-1}$), guaiacol (0.186 g $L^{-1}$), and vanillin (0.30 g $L^{-1}$), the glucose uptake by *S. cerevisiae* was only 3.5% of its uptake in a medium without inhibitors, while under equal conditions, *Rhizopus* sp. maintained 43% of its uninhibited glucose uptake.

*Keywords: Rhizopus; Saccharomyces cerevisiae; Inhibitor tolerance; Phenolic compounds; Carboxylic acids; Furans*

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## INTRODUCTION

Pretreatment of lignocellulosic material at low pH and high temperature allows for efficient enzymatic decomposition of the cellulose into monomers, which subsequently can be fermented into ethanol. However, such pretreatment tends to produce inhibitory byproducts, reducing the product yields and also hampering the fermentation process (Taherzadeh *et al*. 1997a; Larsson *et al*. 1999b).

To improve the process economy of a biorefinery, compounds other than ethanol are produced (Demirbas 2009). As an ethanol-producing organism, the traditional choice, *Saccharomyces cerevisiae* (Baker's yeast), holds a relatively low value as a potential product in a biorefinery.

The utilization of xylose by *S. cerevisiae* requires the use of genetically enhanced strains, which is strictly regulated in many countries. Zygomycetes, however, are filamentous fungi that can efficiently produce ethanol from lignocellulosic hydrolysates and can utilize xylose under aerobic conditions (Abedinifar *et al*. 2009). Furthermore, *Rhizopus* species (Zygomycetes) are used in the preparation of the food "tempe" in Southeast Asia, which means that its mycelium biomass is suitable as, *e.g.*, fish feed (Bankefors *et al*. 2011), and this may be regarded as a valuable product in a Zygomycetes-based biorefinery (Ferreira *et al*. 2013). A Zygomycetes strain of *Mucor indicus* (formerly *Mucor rouxii*) may produce ethanol with yields of around 0.45 g $g^{-1}$ glucose, tolerating up to 73 g $L^{-1}$ ethanol in the medium (Millati *et al*. 2005). This fungus is also reported to tolerate the inhibiting liquid fraction of pretreated lignocelluloses and displays a good uptake of xylose under aerobic conditions (Sues *et al*. 2005; Karimi *et al*. 2006a,b). Certain members of the genus *Rhizopus* possess similar

properties in terms of ethanol production, uptake of sugars, and inhibitor tolerance (Karimi *et al*. 2006a).

*Rhizopus* sp. can be induced to form pellets, which are small spherical beads (*ca.* 1 to 10 mm) of intertwined hyphae. This can be highly advantageous from a process perspective; pelleted growth results in decreased broth viscosity, improving aeration, stirring, and heat transfer. Biomass in the form of pellets is also easier to separate and thus to recycle (Gibbs *et al*. 2000). However, due to mass transfer limitations in, *e.g.*, aerobic processes, only relatively small pellets are considered advantageous (Roa Engel *et al*. 2008). The morphology of the fungi also seems to influence, either directly or indirectly, their metabolism. For instance, while filamentous morphology was superior for the production of L-lactic acid by *Rhizopus arrhizus* (Marták *et al*. 2003), pelleted growth was optimal for the same process when the species *Rhizopus oryzae* was used (Bai *et al*. 2003). Zygomycetes pellets also can be utilized for immobilization of other microorganisms, *e.g.*, yeast, forming so-called biocapsules (Peinado *et al*. 2005). This type of co-fermentation has the potential of incorporating advantages possessed by both microorganisms.

The present study was aimed at establishing the level of inhibitor tolerance of *Rhizopus* sp. by performing batch trials in a semi-synthetic medium, testing different combinations of carboxylic acids (acetic acid and levulinic acid), phenolic compounds (catechol, guaiacol, and vanillin), and furans (furfural and HMF). Zygomycetes were used both as free cells and in a pelletized form, and a relatively inhibitor-tolerant, flocculating strain of *S. cerevisiae* was applied as a reference. Furthermore, hybrid cultures, comprising *S. cerevisiae* enclosed in pellets of *Rhizopus* sp. (biocapsules), were similarly investigated.

## EXPERIMENTAL

### Fungal and Yeast Strains

An isolated strain of *Rhizopus* sp. from Indonesian tempe, registered as CCUG 61147 at the Culture Collection, University of Gothenburg (Sweden), was used in all experiments. This strain was identified as RM4 in a previous publication (Wikandari *et al*. 2012). The fungus was grown on PDA plates containing 15 g $L^{-1}$ agar, 20 g $L^{-1}$ glucose, and 4 g $L^{-1}$ potato extract in an incubator at 30 °C for 4 days. To harvest the spores, 20 mL of sterile water were added to each plate, and the spores were released by agitation with a disposable spreader. A flocculating yeast strain of *Saccharomyces cerevisiae* CCUG 53310 was used as reference (Purwadi *et al*. 2007). The yeast was maintained on YPD plates containing 20 g $L^{-1}$ agar, 20 g $L^{-1}$ glucose, 20 g $L^{-1}$ peptone, and 10 g $L^{-1}$ yeast extract.

### Cultivation and Method for Evaluating the Effect of Inhibitors

*Rhizopus* sp. spores were transferred to 50 mL of medium in 250-mL Erlenmeyer flasks, sealed with cotton plugs, and placed in a water bath at 30 °C, with shaking at 150 rpm. The medium consisted of 20 g $L^{-1}$ xylose, 4 g $L^{-1}$ potato extract, 6 g $L^{-1}$ peptone, and 6 g $L^{-1}$ $CaCO_3$.

Biocapsules were prepared by transferring a loop full of yeast and *Rhizopus* spores ($1\times10^5$ per mL) to the xylose medium, allowing *Rhizopus* sp. to grow, but not the yeast. A Buerker counting chamber (depth 0.1 mm) was applied for measurements of spore concentrations by light microscopy. The spores were counted in 60 E-squares, each with a volume of 1/250 µL. The cultures were incubated for three days, after which they were cultured for one day in a sucrose medium (20 g $L^{-1}$ sucrose, 5 g $L^{-1}$ yeast extract), thus allowing *S. cerevisiae* to grow.

The same procedure was applied for the preparation of fungal pellets, but without yeast. Filamentous growth of *Rhizopus* was accomplished by omitting the calcium carbonate in the xylose medium. Flocculating yeast was prepared by incubating one loop full of yeast in sucrose medium for one day.

The effects of the inhibitors, carboxylic acids, phenolic compounds, and furans were evaluated using a $2^3$ full factorial design in duplicate for the four growth modes, in batch. The inhibitors were either absent (-1) or present (+1). The concentrations of inhibitors were as follows: 12.0 g $L^{-1}$ acetic acid and 23.2 g $L^{-1}$ levulinic acid (Fisher Scientific Sverige, Sweden); 0.165 g $L^{-1}$ catechol, 0.186 g $L^{-1}$ guaiacol, and 0.30 g $L^{-1}$ vanillin (Sigma-Aldrich Sweden AB, Sweden); and 1.5 g $L^{-1}$ furfural and 2.0 g $L^{-1}$ HMF (hydroxymethylfurfural) (Sigma-Aldrich Sweden AB, Sweden). These concentrations are identical to those used in a study by Westman *et al*. (2012).

The inhibitor tolerance experiment was commenced by transferring the biomass of the different growth modes to 100 mL of medium containing 30 g $L^{-1}$ glucose, 5 g $L^{-1}$ yeast extract, 7.5 g $L^{-1}$ $(NH_4)_2SO_4$, 3.5 g $L^{-1}$ $KH_2PO_4$, 0.75 g $L^{-1}$ $MgSO_4 \cdot 7H_2O$, 1.0 g $L^{-1}$ $CaCl_2 \cdot 2H_2O$, 10 mL $L^{-1}$ trace metal solution, and inhibitors according to the experimental design. The cultivations were carried out for 2 days in 250-mL cotton-plugged Erlenmeyer flasks in water baths at 30 °C, with shaking at 150 rpm. Samples were taken after 0, 2, 4, 6, 8, 10, 24, 29, 34, and 48 h.

## Analytical Methods

Samples from the cultivations were centrifuged for 10 min at 10,000×g and frozen until analysis.

The concentrations of glucose, metabolites, and inhibitors in the samples were analyzed by high-performance liquid chromatography (HPLC) (Waters 2695, Waters Corporation, Milford, USA) using a hydrogen-based column (HPX-87H, Bio-Rad, Hercules, USA) at 60 °C, with 5 mM $H_2SO_4$ at a flow rate of 0.6 mL $min^{-1}$ as the eluent. A refractive index (RI) detector (Waters 2414) and a UV absorbance detector (Waters 2487), fixed at 210 nm, were placed in series. The concentrations of the compounds (excepting furans and phenolic compounds) were determined from the RI chromatograms. Standards were made from chemicals purchased from Sigma-Aldrich Sweden AB of the highest available purity, with the exception of glucose which was purchased from Fischer Scientific Sverige. A minimum of four different concentrations were used for each compound.

## Statistical Analysis

Glucose consumption (g $L^{-1}$ $h^{-1}$; excluding the lag phase) and inhibitor concentrations were calculated for biocapsules, pellets, the filamentous growth form, and yeast, by means of a linear regression. Glucose consumption was subsequently employed as the response variable in a general, linear model, used to evaluate the effect of the inhibitors,

$$y = \mu + \alpha_i + \beta_j + \gamma_k + \delta_l + (\alpha\beta)_{ij} + (\alpha\gamma)_{ik} + (\alpha\delta)_{il} + (\beta\gamma)_{jk} + (\beta\delta)_{jl} + (\gamma\delta)_{kl} + (\alpha\beta\gamma)_{ijk} + (\alpha\beta\delta)_{ijl} + (\alpha\gamma\delta)_{ikl}$$

where $y$ = response variable, $\mu$ = total average, $\alpha$ = effect of the type of growth, $\beta$ = effect of furans, $\gamma$ = effect of carboxylic acids, and $\delta$ = effect of phenolic compounds. The statistical analysis was conducted using the software package MINITAB®, and factors were considered significant when p-values were less than 0.05.

## RESULTS AND DISCUSSION

The different inhibitors tested on *Rhizopus* sp. and flocculating *S. cerevisiae* in the present study, namely carboxylic acids (acetic acid and levulinic acid), phenolic compounds (catechol, guaiacol, and vanillin), and furans (furfural and HMF), may be considered to be representative of the three main groups of inhibitors that are known to be present in lignocellulosic hydrolysates (Larsson *et al*. 1999a). The flocculating strain of *S. cerevisiae,* chosen as a reference organism, is an industrial strain adapted to spent sulfite liquor, previously shown to be relatively tolerant to the inhibitory conditions in lignocellulosic hydrolysates (Dehkhoda *et al*. 2009; Westman, *et al*. 2012). From an industrial point of view, the flocculation of *S. cerevisiae* and the pellet formation of *Rhizopus* sp. are interesting properties, as both facilitate cell retention and separation of biomass. Formation of flocks may also be a contributing factor in tolerance to inhibitory compounds (Westman *et al*. 2012).

### Inhibitor Tolerance, General Trends

Glucose consumption of *Rhizopus* sp. and *S. cerevisiae* with and without inhibitors (furans or phenolic compounds) is displayed in Fig. 1. After a lag phase, the rate of glucose consumption was nearly linear for pelleted *Rhizopus* sp. as well as for flocculating *S. cerevisiae*. In Fig. 2, the results of the addition of carboxylic acids as well as the effects of combinations of inhibitors are included.

The uptake of glucose dropped to 0.053 g $L^{-1}$ $h^{-1}$ in *S. cerevisiae* after the addition of phenolic compounds, which is 3.5% of the uninhibited consumption, while in Zygomycetes, the uptake after the same treatment was 0.68 g $L^{-1}$ $h^{-1}$, comprising 43% of the uptake with no inhibitors present (Figs. 1 and 2). Because hydrolysis of lignocellulosic material produces a variety of phenolic compounds, this is an interesting observation. Vanillin, guaiacol, and catechol, used in the project, are only three of the about 20 identified phenolic compounds occurring in lignocellulosic hydrolysates. Phenolic compounds are often difficult to measure and are known to be toxic at very low concentrations (Larsson *et al*. 1999b; Martín *et al*. 2002).

The results were somewhat different for the other inhibitors. The glucose uptake was similar for the yeast and the fungus in the presence of carboxylic acids and furans, both in combination and when added separately. Organic acids (such as acetic acid) diffuse into *S. cerevisiae* in their undissociated form and are actively pumped out by the cells at the expense of intracellular energy (Pampulha and Loureiro-Dias 1989; Taherzadeh *et al*. 1997b). In anaerobic conditions this should theoretically lead to higher ethanol yields and consequently lower biomass and glycerol yields. Conversion of furans into their corresponding alcohols in *S. cerevisiae* has been suggested to depend on multiple gene-mediated aldehyde reduction (Liu *et al*. 2008) and to inhibit the intracellular generation of energy by fermentation (Taherzadeh *et al*. 1999; Modig *et al*. 2002). While the influence of furans and carboxylic acids on *S. cerevisiae* is relatively well elucidated, no detailed studies have been conducted on *Rhizopus* species. Although it remains to be elucidated exactly to which extent the mechanisms of inhibition in yeast are applicable to Zygomycetes, it was observed in this study that the effect of furans and organic acids on glucose uptake in *S. cerevisiae* and *Rhizopus* sp. was very similar for the strains used. It should be underlined, however, that significant differences were easily found between different strains of *S. cerevisiae* in terms of their tolerance to inhibitors (Martín and Jönsson 2003; Brandberg *et al*. 2004). Combining different types of inhibitors had the most severe impact on glucose uptake, which is in line with earlier research (Larsson *et al*. 1999a; Palmqvist and Hahn-Hägerdal 2000; Klinke *et al*. 2004).

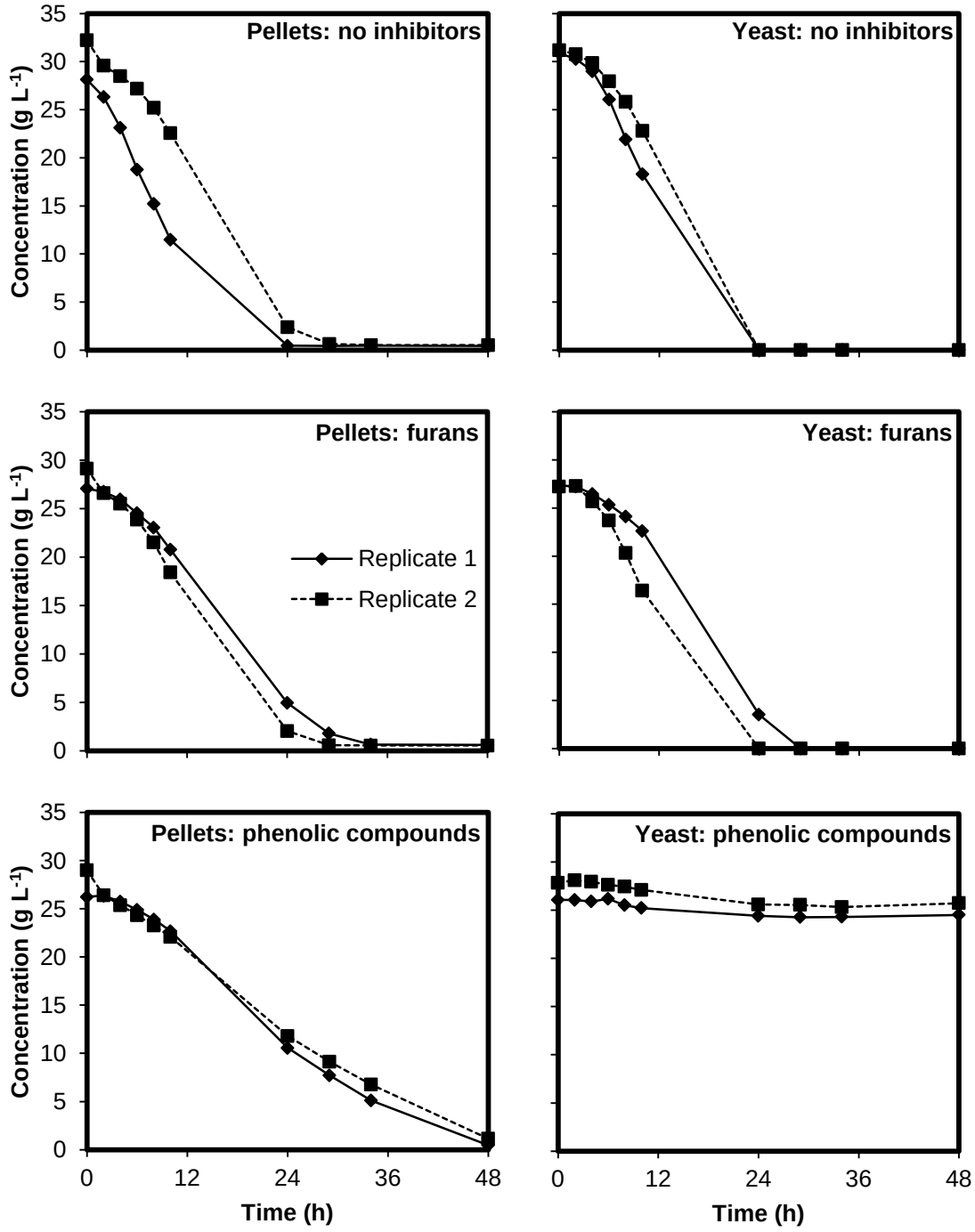


**Fig. 1.** Glucose concentration as a function of time during cultivation of pellets and yeast without the addition of inhibitors, with the addition of furans, and with the addition of phenolic compounds

## HMF Conversion

HMF (5-hydroxymethylfurfural) is metabolized by *S. cerevisiae*, which is a typical example of *in-situ* detoxification of lignocellulosic hydrolysates. Under anaerobic conditions, HMF is converted into 5-hydroxymethylfurfuryl alcohol (Nemirovskii *et al*. 1989; Nemirovskii and Kostenko 1991), which can be regarded as a sign of metabolic vitality. This conversion is slower than conversion of furfural into furfuryl alcohol (Taherzadeh *et al*. 2000). Table 1 shows the conversion of HMF after 10, 24, and 48 h of cultivation.

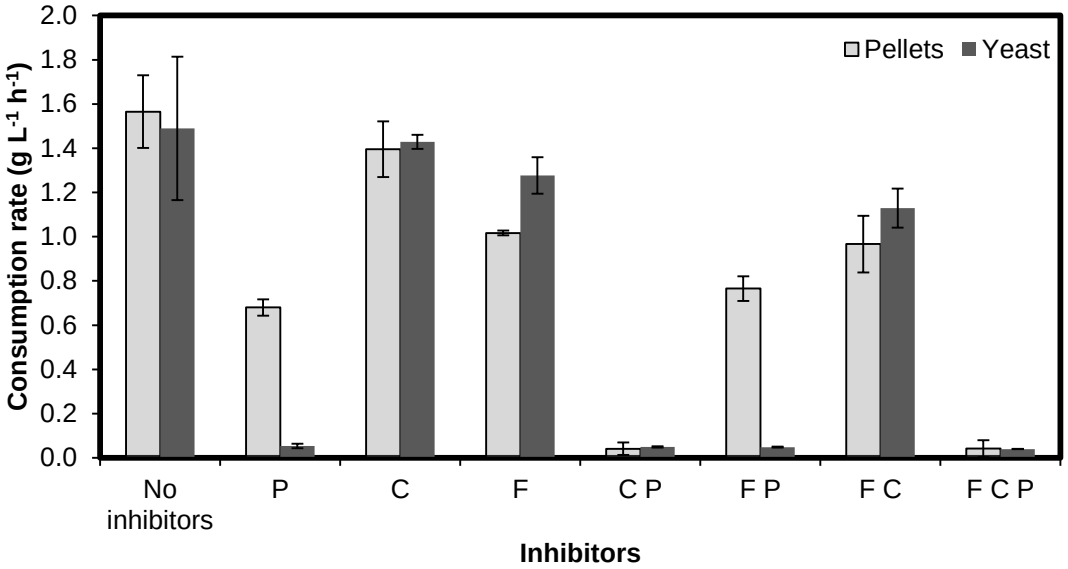


**Fig. 2.** Rate of glucose consumption in pellets and yeast in the presence of phenolic compounds (P), carboxylic acids (C), furans (F), and combinations of these. The error bars represent ± 1 std. dev.

The standard deviations reflect a variation in the duration of the lag phase. Nonetheless, it was concluded that in some cases, *Rhizopus* sp. was more efficient than *S. cerevisiae* in terms of HMF conversion. For instance, the conversion of HMF after 10-h exposure to phenolic compounds and furans averaged 91% in Zygomycetes, compared to 16% in flocculating yeast, confirming a higher tolerance to phenolic compounds in Zygomycetes. However, in the presence of carboxylic acids, HMF conversion by pelletized *Rhizopus* sp. tended to be slower than that of the yeast reference, although the high standard deviation should be noted. It may thus be deduced that *S. cerevisiae* is relatively tolerant to the organic acids used in the present study.

## Ethanol and Glycerol Yields

The association between ethanol production and glucose consumption was used to determine ethanol yield, allowing for yield estimations in experiments where the glucose was not entirely consumed. As expected, glucose consumption was very low in experiments where all inhibitors were present. Consequently, the estimation of the ethanol yield is rather inconclusive in these experiments (Fig. 3). The average ethanol yield (calculated for all experiments, with and without inhibitors) of 0.42 g $g^{-1}$ in flocculating *S. cerevisiae* was significantly higher than the ethanol yield of 0.34 g $g^{-1}$ in pelleted *Rhizopus* sp. (Table 2). These figures correspond to 82% and 66%, respectively, of the theoretical yield (0.51 g $g^{-1}$) of anaerobic ethanol production by *S. cerevisiae*.

The lower ethanol yield in *Rhizopus* sp. may to some extent be explained by a higher glycerol yield (determined in a manner similar to that used to determine ethanol yield). Glycerol is produced by *S. cerevisiae* under anaerobic conditions as a means to maintain the redox balance (Nordström 1966). With no inhibitors present, *Rhizopus* sp. in pellet form displayed a glycerol yield of 0.12 g $g^{-1}$ consumed glucose, compared with the yield of 0.06 g $g^{-1}$ for the yeast reference. With inhibitors present, glycerol yields were lower in absolute terms (reaching up to 0.08 g $g^{-1}$) and so was the average difference between yeast and Zygomycetes (between 0.0 and 0.04 g $g^{-1}$). As expected, the presence of furans generally reduced glycerol formation (data not shown). This was most likely a result of the furans being reduced, thus functioning as an alternative redox sink, preventing glycerol formation (Palmqvist *et al*. 1999; Sárvári Horváth *et al*. 2003). Notwithstanding, a difference in glycerol yield of 0.06 g $g^{-1}$ can explain no larger

difference in ethanol yield than 0.03 g $g^{-1}$ due to the principles of metabolism in *S. cerevisiae* (Nordström 1966; Zhang and Chen 2008).

**Table 1.** Conversion of HMF by *Rhizopus* sp. Pellets (P) and *S. cerevisiae* (Y) Using Various Inhibitor Combinations

| | | HMF conversion (%) | | |
|---|---|---|---|---|
| **Inhibitors** | | **10 h** | **24 h** | **48 h** |
| Furans | P | 97.0 ± 4.2 | 100.0 ± 0.0 | 100.0 ± 0.0 |
| | Y | 71.9 ± 28.1 | 100.0 ± 0.0 | 100.0 ± 0.0 |
| Furans, Phenolic compounds | P | 91.1 ± 5.5 | 100.0 ± 0.0 | 100.0 ± 0.0 |
| | Y | 15.9 ± 8.1 | 38.1 ± 38.4 | 47.3 ± 37.1 |
| Furans, Carboxylic acids | P | 23.7 ± 26.2 | 94.1 ± 8.4 | 100.0 ± 0.0 |
| | Y | 43.6 ± 36.2 | 100.0 ± 0.0 | 100.0 ± 0.0 |
| Furans, Carboxylic acids, Phenolic compounds | P | 9.3 ± 13.2 | 19.6 ± 25.2 | 42.2 ± 62.7 |
| | Y | 1.7 ± 3.3 | 6.1 ± 8.7 | 10.5 ± 14.1 |

Values are given as means ± 1 standard deviation

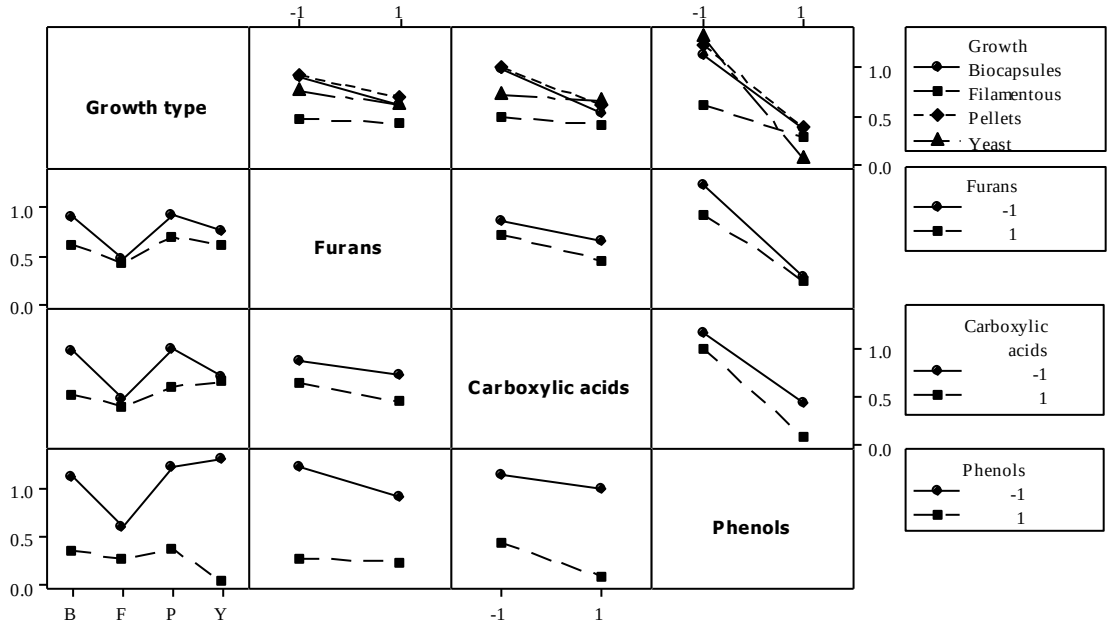


**Fig. 3.** Interaction plots of growth type (biocapsules, filaments, pellets, and yeast) and inhibitors (furans, carboxylic acids, and phenols). Effects were expressed as glucose consumption rate (g $L^{-1}$ $h^{-1}$). To interpret the figure, follow a factor (*e.g.*, furans) horizontally until reaching a vertical position of the other factor of interest (*e.g.*, phenols). The graph shows the mean rate of glucose consumption at each possible combination of the factors.

Another factor known to reduce ethanol yield is the presence of lactic acid-producing bacteria. However, significant amounts (> 0.1 g $L^{-1}$) of lactic acid were not detected in any experiment. A third possibility would be a higher respiration rate in *Rhizopus* sp., causing losses of carbon by the formation of carbon dioxide. Limited amounts of oxygen do indeed diffuse into the medium, but there is no reason for the diffusion being lower in the flasks with yeast. Besides, extensive respiration is not in harmony with the relatively high glycerol yields (Nordström 1966; Zhang and Chen 2008).

Addition of inhibitors generally reduced the production rate of ethanol, but the yield was not significantly affected. With no inhibitors present, the ethanol yield was in

fact almost identical to the calculated average yields. However, experiments where carboxylic acids were added, compared to the corresponding ones without carboxylic acids, on average increased the ethanol yield 10%, supporting the hypothesis (discussed above) that carboxylic acids might stimulate ethanol production (Taherzadeh *et al*. 1997b).

**Table 2.** Ethanol Yield from Biocapsules, Pellets, Yeast, and the Filamentous Growth Form in the Presence of Different Combinations of Inhibitors

| | Ethanol yield (g $g^{-1}$) | | | | |
|---|---|---|---|---|---|
| **Inhibitors** | **Biocapsules** | **Pellets** | **Yeast** | **Filamentous** | **Average** |
| No inhibitor | 0.35 ± 0.02 | 0.32 ± 0.06 | 0.40 ± 0.01 | 0.27 ± 0.04 | 0.34 |
| P | 0.37 ± 0.02 | 0.33 ± 0.01 | 0.44 ± 0.09 | 0.32 ± 0.01 | 0.36 |
| C | 0.41 ± 0.05 | 0.39 ± 0.00 | 0.43 ± 0.01 | 0.39 ± 0.02 | 0.40 |
| F | 0.33 ± 0.05 | 0.35 ± 0.03 | 0.41 ± 0.00 | 0.30 ± 0.08 | 0.35 |
| C P | 0.38 ± 0.07 | 0.38 ± 0.07 | 0.44 ± 0.04 | 0.30 ± 0.02 | 0.38 |
| F P | 0.36 ± 0.01 | 0.32 ± 0.06 | 0.38 ± 0.11 | 0.32 ± 0.01 | 0.34 |
| F C | 0.42 ± 0.06 | 0.35 ± 0.08 | 0.42 ± 0.02 | 0.39 ± 0.04 | 0.39 |
| F C P | 0.33 ± 0.08 | 0.24 ± 0.34 | 0.44 ± 0.14 | 0.35 ± 0.00 | 0.34 |
| Average | 0.37 | 0.34 | 0.42 | 0.33 | |

P = Phenolic compounds; C = Carboxylic acids; F = Furans

## Filamentous Growth *vs*. Pellets

With no inhibitors added, the filamentous form of *Rhizopus* sp. consumed glucose at a rate of 0.52 g $L^{-1}$ $h^{-1}$, which was only one-third of the consumption rate of 1.57 g $L^{-1}$ $h^{-1}$ in pelleted *Rhizopus* sp. under similar conditions. This rather conspicuous difference may be a result of the filamentous morphology. The filamentous fungi formed large aggregates of biomass in the shake flask, most likely slowing down diffusion of the carbon source. In addition to preventing the uptake of carbon, the aggregates may also to some extent protect part of the biomass from certain inhibitors.

Notwithstanding the relatively low baseline level of glucose consumption, the filamentous form of *Rhizopus* sp. turned out to be comparatively resistant to addition of inhibitors, even though in absolute terms, glucose uptake in the presence of furans and carboxylic acids was slower than in the pelleted form. Slow diffusion may again be the explanation, and one could speculate about the effect of "outer" layers of cells shielding the "inner" biomass, which is also relevant for other growth types in the present study and was not investigated further. Based on glucose consumption, the ethanol yields of filamentous and pelleted *Rhizopus* sp. did not differ, however, and were determined to be 0.33 and 0.34 g ethanol $g^{-1}$ glucose, respectively (Table 2).

From an industrial perspective, filamentous growth holds few if any advantages over pellets, but if the process conditions are not closely regulated, this may ensue. No consensus concerning what induces filamentous growth or pellets was found in the literature, and data appear to be strain-specific; see, *e.g.*, Papagianni (2004).

## Biocapsules

Hybrid cultures of *S. cerevisiae* and *Rhizopus* sp. (referred to as biocapsules) may theoretically possess advantages in terms of sugar uptake and inhibitor conversion due to the useful properties of the two organisms being combined. However, the hybrids in the present study, exposed to the same setup of inhibitors (Fig. 3) as in the other experiments, did not confirm this. No obvious advantage of biocapsules over pure

*Rhizopus* sp. (in pellet form) was discerned, *i.e.*, no enhanced inhibitor tolerance or conversion was observed. However, this may have been logical, considering that a pure culture of *S. cerevisiae* had few advantages over *Rhizopus* sp. in terms of inhibitor tolerance in the first place. The average ethanol yield of 0.37 g ethanol $g^{-1}$ consumed glucose in biocapsules (Table 2) fell between the corresponding yields of pure *Rhizopus* sp. pellets (0.34 g $g^{-1}$) and flocculating *S. cerevisiae* (0.42 g $g^{-1}$). Taking into account the results obtained in this study and that biocapsules are more laborious to produce than pure forms, their usefulness in an industrial context is probably limited.

### Statistical Analysis and Trend Validation

Table 3 shows the statistical evaluation of the effect of inhibitors on different growth types. The observations described in the present study were generally confirmed by repetitions of selected trials, such as pellets, biocapsules, and filamentous growth, cultivated with the addition of the two carboxylic acids and phenolic compounds. Experiments involving filamentous growth in cultivations with the addition of all inhibitors or without inhibitors were also repeated. In absolute terms, the sugar consumption was higher in the repeated experiments, indicating some systemic factor influencing the results. Nonetheless, assessing the results in a comparative perspective confirmed the observations above (data not shown).

**Table 3.** Significance (p-values) of the Effect of the Inhibitors on Different Growth Types Evaluated by Applying a General Linear Model Using the Software Package MINITAB

| Source* | P |
|---|---|
| GType | 0.000 |
| F | 0.000 |
| C | 0.000 |
| P | 0.000 |
| GType × F | 0.191 |
| GType × C | 0.001 |
| GType × P | 0.000 |
| GType × F × C | 0.789 |
| GType × F × P | 0.156 |
| GType × C × P | 0.020 |
| F × C | 0.535 |
| F × P | 0.003 |
| C × P | 0.020 |

*GType = Growth Type; F = Furans; C = Carboxylic acids; P = Phenolic compounds

## CONCLUSIONS

1. *Rhizopus* sp. in pellet form may function as a robust production strain in the process of fermenting liquids, resisting the inhibitors that are likely to be present in lignocellulosic hydrolysates.

2. A comparison with the reference used in the experiments, a flocculating strain of *S. cerevisiae* possessing high inhibitor tolerance, disclosed that the *Rhizopus* sp. in general had similar or better inhibitor tolerance.

3. *Rhizopus* sp. clearly showed a higher tolerance than *S. cerevisiae* (CCUG 53310) to the phenolic compounds used in this study.

4. Biocapsules produced no combined detoxification effect *in-situ* in this work.

## ACKNOWLEDGMENTS

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