Electronic Journal of Biotechnology 42 (2019) 1–5

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Effective diffusion coefficients and bioconversion rates of inhibitory compounds in flocs of *Saccharomyces cerevisiae*

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ARTICLE INFO

Article history:
Received 28 December 2018
Accepted 28 August 2019
Available online 04 September 2019

Keywords:
Bioconversion
Bioethanol
Biofuels
Detoxification
Diffusivity
Fermentation
Flocculation
Inhibitor tolerance
Saccharomyces cerevisiae

ABSTRACT

Background: Fermentation strategies for bioethanol production that use flocculating *Saccharomyces cerevisiae* yeast need to account for the mechanism by which inhibitory compounds, generated in the hydrolysis of lignocellulosic materials, are tolerated and detoxified by a yeast floc.
Results: Diffusion coefficients and first-order kinetic bioconversion rate coefficients were measured for three fermentation inhibitory compounds (furfural, hydroxymethylfurfural, and vanillin) in self-aggregated flocs of *S. cerevisiae* NRRL Y-265. Thièle-type moduli and internal effectiveness factors were obtained by simulating a simple steady-state spherical floc model.
Conclusions: The obtained values for the Thiéle moduli and internal effectiveness factors showed that the bioconversion rate of the inhibitory compounds is the dominant phenomenon over mass transfer inside the flocs.
How to cite: Landaeta R, Acevedo F, Aroca G. Effective diffusion coefficients and bioconversion rates of inhibitory compounds in flocs of *Saccharomyces cerevisiae*. Electron J Biotechnol 2019;42. https://doi.org/10.1016/j.rjbt.2019.08.001

1. Introduction

The production of bioethanol from lignocellulosic materials, also called second-generation bioethanol, is considered a promising strategy to increase worldwide production of this biofuel without affecting the food market. Therefore, many efforts have been directed in the last years to make a process technically and economically feasible for bioethanol production from these raw materials. One of the main problems to overcome is the presence of inhibitory and/or toxic compounds in the fermentation broth, generated during the pretreatment of the raw materials as a result of the use of high temperatures and chemicals such as acids, bases, organic solvents depending on the pretreatment [1,2]. These compounds mainly include acetic acid, furfural, hydroxymethylfurfural (HMF), and phenols like vanillin [3,4]. The presence of these compounds in the alcoholic fermentation increases the lag phase and decreases the yield and productivity of bioethanol [5]. A detoxification step before fermentation would increase capital and production costs and cause significant sugar losses depending on the method used. It has been shown that *Saccharomyces cerevisiae* is able to metabolize several inhibitory compounds to less toxic derivatives at the cost of an extended lag phase that decrease the productivity of the fermentation [6] and cause depletion of intracellular NAD(P)H pools, thereby affecting ethanol production and growth [7,8]. Several studies have been published on bioconversion and inhibitor tolerance by *S. cerevisiae*, mainly describing the molecular basis of tolerance [7,9,10,11].

The use of flocculating strains of *S. cerevisiae* has been proposed in the fermentation of inhibitor-containing wood hydrolysates because it has shown better performance in cultures containing inhibitors than with the use of nonflocculating strains [12]. This behavior relates to a physical isolation effect between the inhibitor compounds and the floc-forming biomass arising from mass-transfer diffusional restrictions [13]. Nevertheless, the distribution of floc sizes in a flocculating culture is not homogeneous, and there could be a significant fraction of yeast biomass exposed to the concentrations of inhibitory compounds found in the bulk liquid. It has been reported that hydrophobic compounds are more inhibitory than the hydrophilic ones [14], making bioconversion rate an important parameter to be studied along with diffusivity. In addition, an isolation effect to ethanol and amphotericin-B has been reported in flocs of *S. cerevisiae* [15,16]. Mathematical modeling of an encapsulated xylose-

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2019.08.001

Nomenclature

c	intrafloc inhibitor concentration, g/L
C_i	inhibitor concentration, g/L
C_S	inhibitor concentration on the floc surface (medium), g/L
C_t	inhibitor concentration at time t, g/L
C_∞	inhibitor concentration at equilibrium, g/L
D_e	effective diffusivity coefficient, m^2/s
k	bioconversión rate constant, 1/s
R	floc radius, m
η	internal effectiveness factor, adim.
ϕ	Thièle modulus, adim.

fermenting *S. cerevisiae* strain in the presence of furfural and HMF also points to an isolating effect, where inhibitor concentrations inside the cell pellet are negligible [17], but to date, the Thiele moduli and effectiveness factors, parameters used for indirectly describing the inhibitory phenomena inside a self-aggregated yeast floc, have not been described.

In this work, we report the determination of the effective diffusion coefficients and the bioconversion rate of three lignocellulosic-derived inhibitors, namely, furfural, HMF, and vanillin, in self-aggregated flocs of *S. cerevisiae* along with Thiele moduli and effectiveness factors to describe indirectly the mass transfer and bioconversion phenomena in the floc.

2. Materials and methods

2.1. Yeast strain and culture media

The yeast strain used in this study was *S. cerevisiae* NRRL Y-265, maintained in Petri dishes with YM-agar medium (10 g glucose/l, 5 g peptone/l, 3 g yeast extract/l, 3 g malt extract/l, 20 g agar/l). For liquid culture, the yeast was inoculated from stored plates to a 250 ml Erlenmeyer flask with 50 ml YM medium and cultivated in an orbital shaker at 30°C, initial pH of 4.5 and 200 rpm for 24 h.

2.2. Analytical methods for the determination of inhibitory compounds

Three inhibitors were chosen on the basis of the reported intrinsic detoxification ability of *S. cerevisiae*: furfural, HMF, and vanillin. The concentrations of these inhibitors were measured by HPLC (Infinity 1260, Agilent, U.S.A.) equipped with a Bio-Rad Aminex HPX-87H ion-exchange column and a UV detector (210 nm), using a mixture of 90:10 5 mM H_2SO_4: acetonitrile as the eluent with a flow rate of 0.6 ml/min and an oven temperature of 65°C.

2.3. Determination of effective diffusion coefficient

Spherical floc formation was induced by agitation using a tube roller (Stirrer Mixer SRT-2, Stuart Scientific, UK). To obtain yeast flocs with no biological activity, they were collected from a fresh culture, filtered, and washed under tap water and stored in a solution of ethylacetate (1:13 v/v) at 4°C for 24 h. After that, the biological deactivated flocs were washed under abundant tap water. Diffusion kinetics for each inhibitor were studied by injecting 500 µl of a solution containing the inhibitory compounds to a vial containing the suspension of flocs through a rubber stopper; the initial concentrations in the vial were 12.5 mM furfural, 4.75 mM HMF, and 3.3 mM vanillin (1.2 g furfural/l, 0.6 g HMF/l, and 0.5 g vanillin/l). The inhibitor concentration was further studied by taking samples of 50 µl at intervals of 0, 30, 60, 90, 120, 150, 180, 240, 300, and 600 s. Each experiment was conducted in duplicate.

For each of the above experiments, floc radius was measured by direct photography of flocs and further image analysis using FIJI for Windows (www.fiji.sc).

The effective diffusion coefficient (D_e) was determined by fitting the concentration kinetics of each inhibitory compound in the liquid phase of a suspension of yeast flocs with no biological activity to an expression that describes the diffusion of a solute in the transient state in an spherical object from a well-stirred solution in a limited volume [18], obtained from an analytical solution of Fick's law, [Equation 1]]. The model assumes spherical particles with a constant radius, with negligible external diffusional restriction and an initial zero concentration of solute inside the particle.

$$\frac{C_t}{C_\infty} = 1 + \sum_{n=1}^{\infty} \frac{2f(1+\alpha)}{f^2 + f^2\alpha + \alpha^2 q_n^2} \exp\left(-\frac{D_e q_n^2 t}{R^2}\right) \quad \text{[Equation 1]}$$

where q_n is the non-trivial root of $\tan q_n = 3q_n/(3 + \alpha q_n)$, f is the geometry factor (equal to 3 in this model, corresponding to a spherical geometry), and α is the liquid/solid volume fraction. The parameter α can also be expressed in terms of the final fractional uptake of solute by the flocs by the relation $M_\infty/VC_0 = 1/(1 + \alpha)$ [19], where M_∞ is the mass of solute taken by the floc at infinite time, and V is the volume of solution. The former relation is equivalent to $\alpha = (C_0/C_\infty - 1)$, where C_∞ is the equilibrium concentration of the selected inhibitor at $t = 600$ s. A nonlinear least-squares regression method was used for fitting the experimental values to [Equation 1]].

2.4. Bioconversion kinetics of inhibitory compounds

For each inhibitor, the bioconversion kinetics was determined in active cultures of *S. cerevisiae* NRRL Y265 grown in 500 ml Erlenmeyer flasks with 100 ml of defined medium (110 g glucose/l, 5 g KH_2PO_4/l, 2 g $(NH_4)_2SO_4$/l, and 0.4 g $MgSO_4 \cdot 7H_2O$/l) supplemented with a solution of vitamins (0.01 mg biotin/l, 2 mg calcium pantothenate/l, 0.01 mg folic acid/l, 10 mg inositol/l, 1 mg nicotinic acid/l, 1 mg p-aminobenzoic acid/l, 2 mg pyridoxine HCl/l, 1 mg riboflavin/l, and 2 mg thiamin/l). The pH of the medium was adjusted initially to 4.5 using NaOH solution. Stirring was done with magnetic bars at 500 rpm to avoid floc formation. The cultures were carried out for 18 h at 30°C in duplicate. The initial concentration of each IC in the medium was between 0.1 and 0.6 g inhibitor/l, and its concentration was followed by sampling the medium at intervals of 1 h.

The bioconversion rate coefficients were calculated by fitting each bioconversion kinetics to a first-order chemical degradation reaction model [20].

$$\ln C_i = \ln C_{i0} - kt \quad \text{[Equation 2]}$$

Statistical analysis of the diffusivity and bioconversion results (one-way ANOVA with Tukey HSD, level of significance 0.05) was performed in R v3.2.1.

2.5. Determination of Thiele moduli and effectiveness factor

If a yeast floc is considered as a spherical catalytic particle, and assuming no external diffusional restrictions, the inhibitor will diffuse from the floc surface toward the center; at the same time, cell biomass consumes the compound, modeled by a first-order chemical degradation reaction according to our assumption. The relative magnitude between these two phenomena can be analyzed using the Thièle modulus (ϕ), obtained from the effective diffusion coefficient (D_e), the bioconversion rate constant (k), and the particle radius (R). A further analysis can be made by calculating the internal effectiveness factor (η), defined as the fraction between the effective reaction rate and the intrinsic reaction rate with no internal diffusional restrictions (IDR); in the absence of IDR, η equals unity. The internal effectiveness

factor is related to the inverse of the Thiéle modulus: at $\phi > 5$, diffusion is the limiting step in the overall heterogeneous reaction, lowering the effectiveness factor; at lower ϕ values, reaction rate is not limited by diffusion, and η increases toward its maximum value [20,21].

Thiele moduli and internal effectiveness factors for each inhibitor were calculated using a steady-state model [20], which assumed simultaneous diffusion and first-order bioconversion reaction in the spherical particle and described by [Equation 3]], and its analytical solution is described using [Equation 4]].

$$D_e\left(\frac{d^2c}{dr^2}+\frac{2dc}{rdr}\right)-kc=0 \quad \text{[Equation 3]}$$

$$\frac{c}{C_s}=\frac{R}{r}\frac{\sinh\left(\frac{\phi r}{R}\right)}{\sinh(\phi)},\phi=\frac{R}{3}\sqrt{\frac{k}{D_e}} \quad \text{[Equation 4]}$$

The internal effectiveness factor for the above conditions is defined as

$$\eta=\frac{3}{\phi^2}(\phi coth\phi-1) \quad \text{[Equation 5]}$$

3. Results and discussion

The results of the floc measurements are presented in Fig. 1. The measured mean floc radius was 1.02 ± 0.65 mm, with most of the flocs (92.1%) measuring less than 2 mm in radius and almost 25% of the flocs less than 0.5 mm in radius.

Table 1 shows the effective diffusion coefficients and bioconversion rate constants determined for furfural, HMF, and vanillin, considering the former measured mean floc radius.

The yeast strain *S. cerevisiae* NRRL Y-265 showed significant differences in bioconversion rates ($p = 0.0249$) and diffusivity coefficients ($p = 0.0378$), where a higher bioconversion potential was found for furaldehyde inhibitors (furfural and HMF) than for the phenolic aldehyde vanillin. HMF exhibited higher diffusivity, followed by furfural and vanillin. Nevertheless, there was a statistically significant difference only between HMF and vanillin diffusivities ($p = 0.0334$). Remarkably, the measured values of furfural and HMF diffusion coefficients were similar to those assumed by Westman et al. for use in a mathematical model of a *S. cerevisiae* cell pellet inside an alginate capsule (in the mentioned study, the authors assumed that D_e is 25% of the diffusivity in water) [17].

Table 1
Effective diffusivity coefficients and bioconversion rate for each inhibitor. Values sharing the same superscript letter indicate that there are no statistical differences between them (Tukey HSD, significance level = 0.05).

Inhibitor	$D_e \times 10^{10}$ (m^2/s)	$\boldsymbol{k} \times 10^{10}$ (1/s)
Vanillin	4.23 ± 1.30^{a}	4.45 ± 0.56^{a}
HMF	11.34 ± 2.11^{b}	$5.93 \pm 0.22^{b,c}$
Furfural	$8.02 \pm 0.55^{a,b}$	7.44 ± 0.81^{c}

The D_e values obtained were related to the degree of hydrophobicity, as seen in Fig. 2, i.e., the lower the hydrophobicity, the higher was the D_e. $\log P_{octanol/water}$ values for vanillin, furfural, and HMF are 1.21, 0.41, and -0.37, respectively [22].

The corresponding Thièle moduli and internal effectiveness factors for two floc radii are presented on Table 2.

Experimental Thiéle moduli values for all inhibitors are under the critical value $\phi > 5$, and accordingly, internal effectiveness parameters are close to unity in all cases. Concentration profiles illustrated in Fig. 1 show the presence of all inhibitors at the center of the floc ($r = 0$) under steady-state conditions. Considering that experimental floc sizes at laboratory-scale cultures barely reach above 0.5 mm, these results could mean that the effect of diffusional restrictions on inhibitor tolerance by flocculating yeast strains is negligible.

The above results are in contrast with those of reports in which a protection from inhibitors has been shown in batch cultures of encapsulated *S. cerevisiae* [17,23], and it has been proposed that a spherical floc in flocculating yeast cultures acts as a physical barrier akin to alginate encapsulation, isolating cells from toxic compounds. However, it is possible that an alginate sphere and a spherical yeast floc have enough structural differences to invalidate the assumption of similar mass-transfer behavior. A steady-state yeast floc arises from the dynamic equilibrium between cellular aggregation and disaggregation [24] depending on the yeast strain, medium, and culture conditions, and a fraction of the biomass is expected to be present as free-floating cells. In view of the results shown in Fig. 1, most of the biomass present in a flocculating culture have a wide

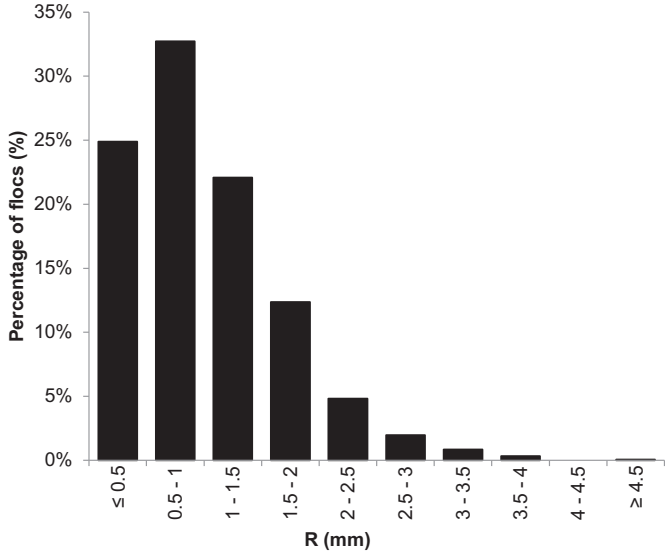

Fig. 1. Distribution of yeast floc radius under conditions used for the measurement of diffusion coefficients (n = 1865).

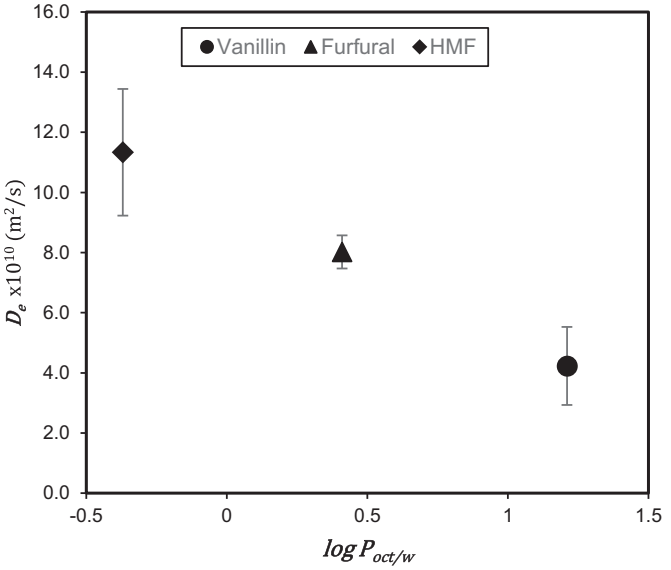

Fig. 2. Diffusion coefficients of inhibitory compounds on deactivated *S. cerevisiae* NRRL Y-265 flocs as a function of the partition coefficients on octanol–water (log P), e.g., hydrophobicity.

Table 2
Thièle moduli and internal effectiveness factors for two spherical floc radiuses.

Inhibitor	$R = 1$ mm		$R = 2$ mm	
	ϕ	η	ϕ	η
Furfural	3.21×10^{-4}	1	6.42×10^{-4}	1
Vanillin	3.42×10^{-4}	1	6.84×10^{-4}	1
HMF	2.41×10^{-4}	1	4.82×10^{-4}	1

range of floc sizes, making the smaller flocs more vulnerable to inhibitory effects. Alginate spheres are static and have better mechanical resistance thus being able to resist more severe culture conditions with no changes in their diameter and less cell leakage to the liquid medium.

Under certain conditions, similar diffusion coefficients have been found between yeast flocs and alginate spheres, but these values are dependent on measurement conditions and modeling assumptions [18]. Moreover, pore size is expected to be different between yeast flocs and alginate spheres; at least for alginate polymer membranes, solute diffusivity coefficients show an inverse correlation with pore size [25]. A yeast floc is homogeneous (composed of cells only), and its physical conformation approaches a bicubic packing, making floc porosity dependent mainly on the distance between individual cells. This distance is most likely dependent on the length of the peptidic bridges between cell walls, which is stated at 300 nm for the FloI flocculin protein [26]. In contrast, alginate spheres are heterogeneous (comprising a dense cell pellet, a liquid core, and the alginate wall) [17] and have a polymeric structure; their pore sizes are dependent on the crosslinking conditions, but the typical values are in the range of 5–7 nm [25].

An alternative explanation to the reported chemical tolerance on flocculating yeasts is that immobilization-related stress triggers changes in yeast metabolism, which increases resistance toward inhibitory compounds. Thus, the relationship between chemical stress tolerance and flocculation could arise as a result of genome-wide metabolism changes responding to immobilization stress, where stable flocculating yeast strains have better resistance to chemical stresses [12,15]. Lei et al. [27] reported an increased tolerance to ethanol shocks with bigger yeast flocs in *S. cerevisiae* with a maximum tolerance at $R = 0.15$ mm; basal levels of ergosterol, membrane sphingolipids, and ATPase activity were found to be proportional to ethanol tolerance. This behavior is similar to the cellular response in the presence of lignocellulosic inhibitors [28]. Results of basal gene-expression profile in flocculating *S. cerevisiae* have showed overexpression of genes related to carbohydrate, sterol, and lipid metabolism; gluconeogenesis; membrane transport; and stress-related transcription factors [15,29]. Similar profiles have been described for non-flocculating *S. cerevisiae* under encapsulation-related stress [30]. The differences between the obtained results in this work and those of previous reports could be explained by diffusion-related differences in the floc structure, a possible link between intrafloc diffusion and inhibitor hydrophobicity, and different metabolic profiles on immobilized yeasts.

4. Conclusions

The obtained values for the Thiéle moduli and internal effectiveness factors suggest that the intraparticle diffusional restrictions for inhibitory compounds are very low compared to the bioconversion rate of the inhibitory compounds. The bioconversion rate could change the inhibitory effect of these compounds inside the floc. More work must be performed to confirm and validate whether the bioconversion rate is the dominant effect of inhibitor tolerance by flocculating yeast cultures.

Financial support

Financial support granted to R. Landaeta by CONICYT's scholarship program (Comisión Nacional de Investigación Científica y Tecnológica) and Pontificia Universidad Católica de Valparaíso is gratefully acknowledged.

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