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EDITORA

SIMULTANEOUS ISOMERIZATION AND FERMENTATION OF XYLOSE AS A STRATEGY FOR HEMICELLULOSE VALORIZATION: INTRAPARTICLE DIFFUSIVE EFFECT OF IMMOBILIZED XYLOSE ISOMERASE

ISOMERIZAÇÃO E FERMENTAÇÃO SIMULTÂNEAS DE XILOSE COMO ESTRATÉGIA PARA A VALORIZAÇÃO DA HEMICELULOSE: EFEITO DIFUSIVO INTRAPARTÍCULA DA XILOSE ISOMERASE IMOBILIZADA

ISOMERIZACIÓN Y FERMENTACIÓN SIMULTÁNEAS DE XILOSA COMO ESTRATEGIA PARA LA VALORIZACIÓN DE LA HEMICELULOSA: EFECTO DIFUSIVO INTRAPARTÍCULA DE LA XILOSA ISOMERASA INMOVILIZADA

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ABSTRACT

Considering the context of biorefineries, the hemicellulosic fraction of biomass is still underutilized. However, for the production of 2G ethanol from biomass to be viable, it is necessary to establish a way to utilize this fraction. Saccharomyces cerevisiae yeast does not consume xylose, but it does consume its isomer, xylulose. In this context, the use of xylose from hemicellulose can be made feasible through Simultaneous Isomerization and Fermentation (SIF), using the enzyme xylose isomerase. To enable continuous or repeated batches operation, the enzyme can be co-immobilized with the yeast, providing a protective microenvironment against process conditions. However, since diffusive effects from the presence of immobilization supports can render the process unfeasible, characterization of this property is necessary. Thus, the present work evaluated the intraparticle diffusive effects of the xylose isomerase derivative immobilized on chitosan at different diameters, aiming to

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prove its viability in the SIF process. The IXI-Ch derivative obtained by immobilization in chitosan gel was found to be free of intraparticle effects, presenting kinetic parameters similar to those of the soluble enzyme. The use of this derivative co-immobilized with yeast in calcium alginate beads for the continuous production of 2G ethanol proved to be viable, since under the process conditions it showed an internal effectiveness of 94.5%.

Keywords: 2G Ethanol. Biorefinery. Simultaneous Isomerization and Fermentation. Heterogeneous Catalysts. Enzyme Immobilization.

RESUMO

Considerando o contexto das biorrefinarias, a fração hemicelulósica da biomassa ainda é subutilizada. No entanto, para que a produção de etanol de segunda geração (2G) a partir de biomassa seja viável, é necessário estabelecer uma forma de aproveitar essa fração. A levedura Saccharomyces cerevisiae não consome xilose, mas consome seu isômero, a xilulose. Nesse contexto, o uso da xilose proveniente da hemicelulose pode ser viabilizado por meio da Isomerização e Fermentação Simultâneas (IFS), utilizando a enzima xilose isomerase. Para possibilitar operações contínuas ou em bateladas repetidas, a enzima pode ser coimobilizada com a levedura, fornecendo um microambiente protetor contra as condições do processo. Contudo, como efeitos difusivos decorrentes da presença de suportes de imobilização podem tornar o processo inviável, é necessária a caracterização dessa propriedade. Assim, o presente trabalho avaliou os efeitos difusivos intrapartícula do derivado de xilose isomerase imobilizado em quitosana com diferentes diâmetros, visando comprovar sua viabilidade no processo de IFS. O derivado IXI-Ch obtido pela imobilização em gel de quitosana mostrou-se livre de efeitos intrapartícula, apresentando parâmetros cinéticos semelhantes aos da enzima solúvel. O uso desse derivado coimobilizado com levedura em esferas de alginato de cálcio para a produção contínua de etanol 2G mostrou-se viável, uma vez que, sob as condições do processo, apresentou uma efetividade interna de 94,5%.

Palavras-chave: Etanol 2G. Biorrefinaria. Isomerização e Fermentação Simultâneas. Catalisadores Heterogêneos. Imobilização de Enzimas.

RESUMEN

Considerando el contexto de las biorrefinerías, la fracción hemicelulósica de la biomasa aún está subutilizada. Sin embargo, para que la producción de etanol de segunda generación (2G) a partir de biomasa sea viable, es necesario establecer una forma de aprovechar dicha fracción. La levadura Saccharomyces cerevisiae no consume xilosa, pero sí consume su isómero, la xilulosa. En este contexto, el uso de la xilosa procedente de la hemicelulosa puede hacerse viable mediante la Isomerización y Fermentación Simultáneas (IFS), utilizando la enzima xilosa isomerasa. Para permitir operaciones continuas o en lotes repetidos, la enzima puede ser co-inmovilizada con la levadura, proporcionando un microambiente protector frente a las condiciones del proceso. No obstante, debido a que los efectos difusivos derivados de la presencia de soportes de inmovilización pueden hacer que el proceso sea inviable, es necesaria la caracterización de esta propiedad. Así, el presente trabajo evaluó los efectos difusivos intrapartícula del derivado de xilosa isomerasa inmovilizado en quitosano con diferentes diámetros, con el objetivo de demostrar su viabilidad en el proceso de IFS. El derivado IXI-Ch obtenido mediante inmovilización en gel de quitosano no presentó efectos intrapartícula, mostrando parámetros cinéticos similares a los de la enzima soluble. El uso de este derivado co-inmovilizado con levadura en esferas de alginato de calcio para la producción continua de etanol 2G demostró ser viable, ya que, bajo las condiciones del proceso, presentó una efectividad interna del 94,5%.

Palabras clave: Etanol 2G. Biorrefinería. Isomerización y Fermentación Simultáneas. Catalizadores Heterogéneos. Inmovilización Enzimática.

1 INTRODUCTION

Lignocellulosic materials have great potential as a carbon source for obtaining products of interest to society in biorefineries, since they consist mainly of cellulose and hemicellulose, which are composed of hexose and pentose monomers. In addition to being widely available and having low cost, they do not compete with food production (Worku et al., 2024). Among the products obtained in biorefineries from lignocellulosic biomass, great efforts have been made to develop robust processes to produce second-generation (2G) ethanol. However, the use of all sugars available in the biomass is an essential criterion in the process for it to be economically viable (Chandel et al., 2018). In this sense, the hemicellulose fraction of biomass is still underutilized, making the development of an efficient 2G ethanol production route from this fraction one of the major bottlenecks of this technology (Silva et al., 2012).

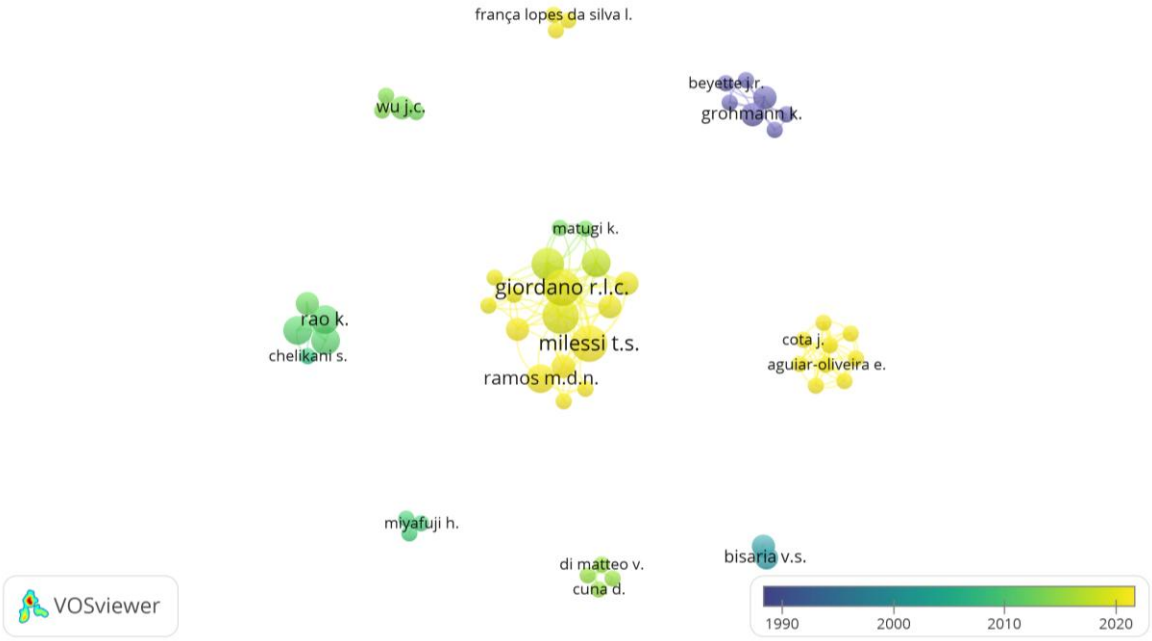
Currently, the most widely industrially used microorganism to produce ethanol from hexoses is the yeast *Saccharomyces cerevisiae*. However, its wild form does not possess the enzymatic system necessary for the metabolism of pentoses (Perez et al., 2022). On the other hand, this yeast is capable of assimilating xylulose into ethanol (Ferreira et al., 2026), so a viable alternative would be the isomerization of xylose to xylulose followed by fermentation to ethanol by *S. cerevisiae* (Silva et al., 2012).

The enzyme xylose isomerase (EC 5.3.1.5) is widely used industrially for the inversion of fructose to sucrose and also catalyzes the reversible isomerization of xylose to xylulose (Giordano et al., 2000). The isomerization of xylose to xylulose has an equilibrium ratio of 5:1 xylose/xylulose (Silva et al., 2012). To shift this equilibrium, continuous removal of xylulose by yeast can be used through a process of simultaneous isomerization and fermentation (SIF).

Xylose SIF is a technology that has been little explored in the literature, with the first studies dating back to the late 1980s and early 1990s (Lastick et al., 1990, 1989), However, after the development and maturing of recombinant DNA technology, the focus shifted to the expression of xylose isomerase in yeast (Demeke et al., 2013; Shin et al., 2019), so that SIF was deprioritized. This phenomenon is evidenced by the bibliometric analysis of the term SIF performed using VOSviewer 1.6.20 software (Figure 1). There is a temporal gap, in which the main collaboration clusters are represented by a purple cluster, associated with the early 1990s, and by three yellow clusters, corresponding to more recently published works, between 2025 and 2026.

Figure 1

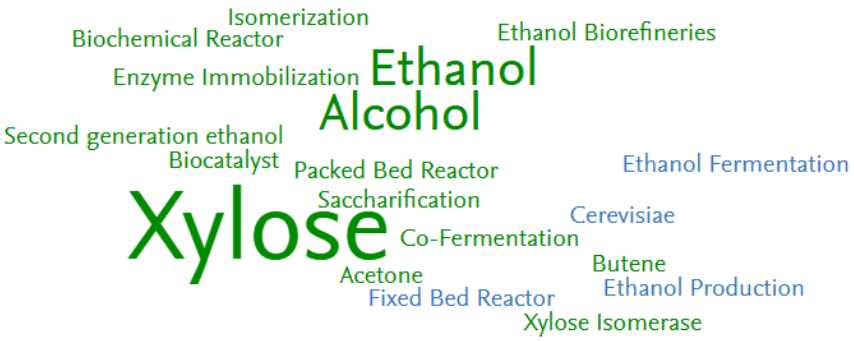
Collaboration network in Simultaneous Isomerization and Fermentation (SIF) performed using VOSviewer software



Due to this phenomenon, when searching the Scopus database using the terms "*Simultaneous Isomerization and Fermentation*" and "*Simultaneous Isomerisation and Fermentation*," only 20 papers are found, most of which were produced by Brazilian research groups, given the country's pioneering role in the field of biofuels. (Ferreira et al., 2026; Ramos et al., 2024; Sandri et al., 2023; Silva et al., 2012). Applying the SciVal bibliographic data processing tool to these papers, we obtain the word cloud shown in Figure 2, where it is clear that the development of SIF for the production of 2G ethanol is directly linked to the development of biorefineries, the concept of circular economy, and the direct application of process engineering in the design of bioreactors and the development of viable processes through biotechnology.

Figure 2

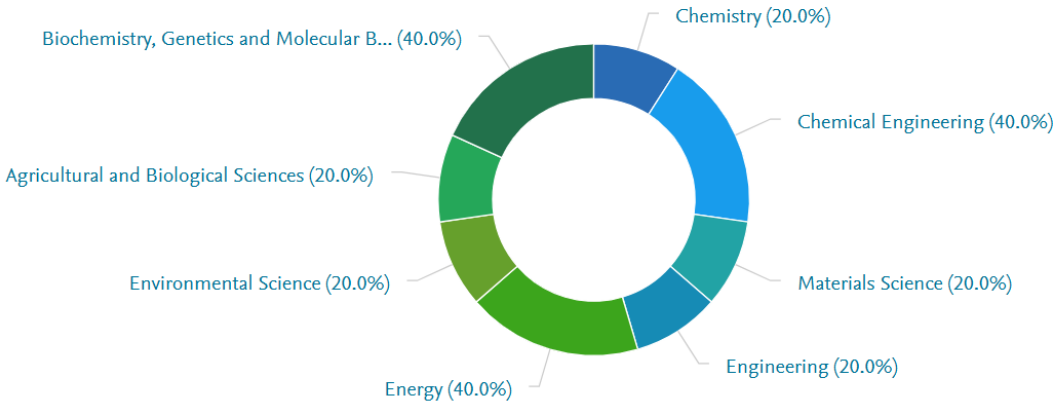
Word cloud generated by SciVal considering SIF articles obtained from the Scopus database



As illustrated in Figure 3, it can be observed that approximately 40% of the works available in the literature on SIF are in the field of Chemical Engineering. The development of the SIF process depends on the optimization of process factors, since enzymes and yeasts have different optimal conditions (pH and temperature) (Milessi et al., 2018). Additionally, considering the economic feasibility of the process, the use of immobilized enzymes in SIF becomes an interesting strategy, as it allows the reuse of enzymes in repeated cycles (Silva et al., 2012).

Figure 3

Distribution of SIF articles obtained from the Scopus database search in different areas of concentration according to the SciVal tool

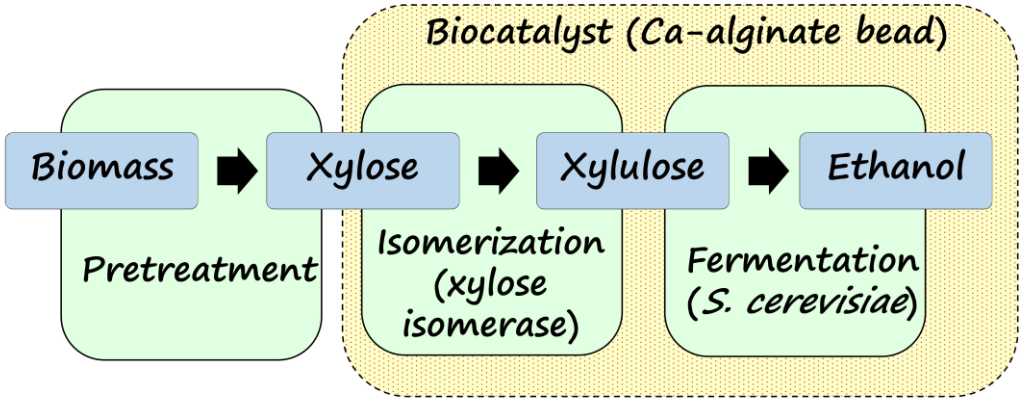


The immobilization of xylose isomerase also allows SIF to operate under conditions favorable to the yeast by creating a microenvironment within the immobilization support spheres, since xylose isomerase has low activity at pH below 5.0, the operating range of SIF (Jänis et al., 2008). In this context, the co-encapsulation of the enzyme and yeast in calcium

alginate spheres is explored in the literature as a strategy to overcome this limitation (Milessi-Esteves et al., 2019; Rao et al., 2008), forming a cascade process as shown in Figure 4.

Figure 4

Biocatalyst for SIF process composed of xylose isomerase and yeast co-immobilized on Ca-alginate beads



However, the presence of an immobilization support characterizes the system as heterogeneous catalytic, representing a physical barrier to the process and potentially causing mass transfer problems (Doran, 1995). Therefore, characterizing the biocatalyst in terms of its internal effectiveness based on inherent and apparent kinetic parameters is of great importance for optimizing the SIF process, especially considering the scarcity of studies that optimize SIF. Thus, the objective of this study was to evaluate the effectiveness of immobilized xylose isomerase present in a biocatalyst containing the enzyme co-immobilized with yeast, aiming to assess its viability in the SIF process.

2 MATERIALS AND METHODS

2.1 OBTAINING XYLOSE ISOMERASE IMMOBILIZED ON CHITOSAN (IXI-CH)

Initially, 2% (w/w) of chitosan powder was solubilized in 2% (v/v) acetic acid and homogenized for 30 minutes at room temperature. Next, 0.5M KOH was added in a 3:2 (v/v) ratio (volume of KOH/volume of solution) at 50°C and kept under stirring for 30 minutes (Silva et al., 2012). To verify possible diffusional effects resulting from the immobilization of xylose isomerase in chitosan, three different diameters of the derivative were prepared: P, which comprises microparticles obtained by gel coagulation under constant stirring; M (comprising beads with a diameter of 0.85 to 1.7 mm) and G (beads from 1.7 to 2.36 mm). The support

was activated by adding 5% (v/v) glutaraldehyde for 30 minutes, as described by Ramos et al. (2024).

Enzyme immobilization was performed immediately after activation of the support. An enzymatic solution of the commercial enzyme GENSWEET® SGI (3400 U/mL), a xylose isomerase from *Streptomyces rubiginosus*, kindly donated by DuPont ™ Genencor® (Palo Alto, CA, USA), was prepared at pH 7 (50 mM Tris maleate buffer) containing 5 mM $MgSO_4.7H_2O$ and 2.5 mM $CoCl_2.6H_2O$. The enzyme solution was added to the support at a support:suspension ratio of 1:10 (v/v). The suspension was kept under gentle agitation at 25°C for 20 h. At the end of the process, the derivative was reduced with sodium borohydride (1 mg/mL) for 30 minutes in an ice bath. (Ramos et al., 2024). The final derivative was washed with 200 mM Tris-maleate buffer, pH 7, distilled water, and 50 mM Tris-maleate buffer, pH 7, to remove residual enzyme and sodium borohydride, obtaining an enzyme derivative (IXI-Ch) at neutral pH.

2.2 ENCAPSULATION OF THE IGI-CH DERIVATIVE IN CALCIUM ALGINATE GEL

The encapsulation of IGI-Ch in calcium alginate gel was performed according to the co-immobilization methodology with *S. cerevisiae* yeast, as described by Milessi-Esteves et al. (2019). A sodium alginate solution 1% (w/w) in 5 mM phosphate buffer, pH 8, was prepared containing 15% w/w IXI-Ch. Calcium alginate gel pellets were formed by dripping this solution into a coagulation solution containing calcium chloride and magnesium chloride (0.25 M).

2.3 DETERMINATION OF KINETIC PARAMETERS

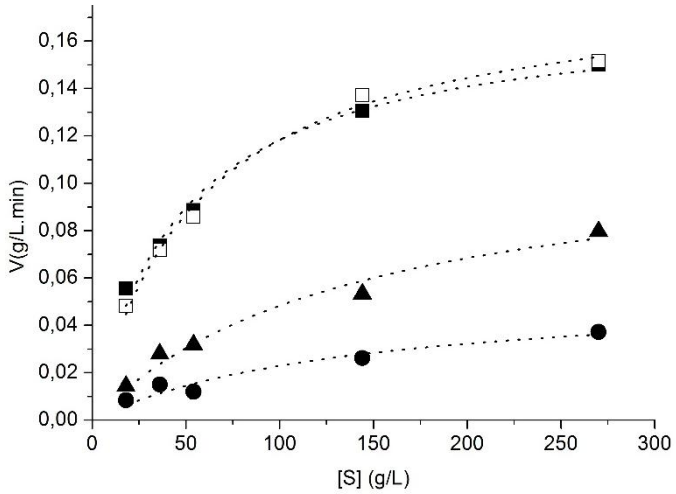
The intrinsic kinetic parameters of soluble xylose isomerase, as well as the apparent parameters of IXI-Ch, were determined by measuring the initial conversion rate of fructose to glucose at different substrate concentrations. Fructose solutions at concentrations of 0.1, 0.2, 0.3, 0.8, and 1.5 M were prepared in 50 mM Tris-maleate buffer (pH 7), containing $MgSO_4.7H_2O$ (50 mM) and $CoCl_2 \cdot 6H_2O$ (2.5 mM). The reaction was initiated by adding the enzyme to 5 mL of fructose solution. At regular reaction intervals, 100 µL samples were taken from the reaction medium and added to 100 µL of 20% HCl (v/v) to inactivate the enzyme. (Silva et al., 2012). The glucose concentration formed was then determined colorimetrically using a commercial enzyme kit containing glucose oxidase and peroxidase. Tests were performed at 60°C (optimum enzyme temperature), 50°C, 40°C, and 32°C (SIF process temperature) (Milessi et al., 2018). The kinetic parameters at each temperature were obtained by hyperbolic fitting of the velocity curves to the substrate concentration

3 RESULTS AND DISCUSSION

In order to verify the feasibility of using xylose isomerase co-immobilized with yeast for the production of 2G ethanol, mass transfer limitations caused by the presence of immobilization supports were investigated. The stability of the IXI-Ch derivative in the presence of ethanol and under the process conditions was previously studied by Silva et al. (2012), who demonstrated that the biocatalyst was suitable for the SIF process. However, since diffusive effects resulting from the presence of the support can render the process unfeasible, characterization of this property was necessary. To this end, the fructose-to-glucose isomerization rate curves were first obtained for the three different diameters of the enzyme derivatives studied and compared with the soluble enzyme (Figure 5).

Figure 5

Isomerization kinetics of fructose to glucose for soluble xylose isomerase (■) and different diameters of the IXI-Ch derivative, where (●) G corresponds to 1.7-2.36 mm, (▲) M = 0.85 to 1.7 mm, and (□) P are microparticles obtained by the technique proposed in this study



The IXI-Ch derivative obtained is highly catalytic, exhibiting behavior similar to the soluble enzyme at its smallest diameter (P). On the other hand, the other diameters tested (M and G) exhibited lower catalytic rates due to mass transfer limitations caused by the presence of the support. Since diameters M and G are larger, the diffusion path of the substrate to the biocatalyst is longer, thus evidencing the intraparticle diffusional effect. Giordano et al. (2000), studying the diffusional effect of a commercial immobilized xylose isomerase (Maxazyme®), determined that the ideal particle diameter for obtaining inherent parameters (i.e., equivalent to intrinsic parameters) was 0.15 to 0.35 mm, since it was small enough to eliminate intraparticle diffusional effects in fructose isomerization. The authors found that for diameters of 1.2 mm (equivalent to the M diameter in this study), the initial

velocity was significantly impaired, demonstrating the existence of intraparticle diffusive effects. Thus, the derivative proposed in this study proved to be viable for use in the SIF process since it did not present significant intraparticle diffusive effects.

Once the viability of the derivative was proven, we then sought to analyze the diffusive effects resulting from its co-immobilization with S. cerevisiae yeast encapsulated in calcium alginate gel. Intrinsic kinetic parameters (soluble GI) and apparent kinetic parameters (encapsulated IXI-Ch) were determined at different temperatures, which are presented in Table 1 and Figure 6.

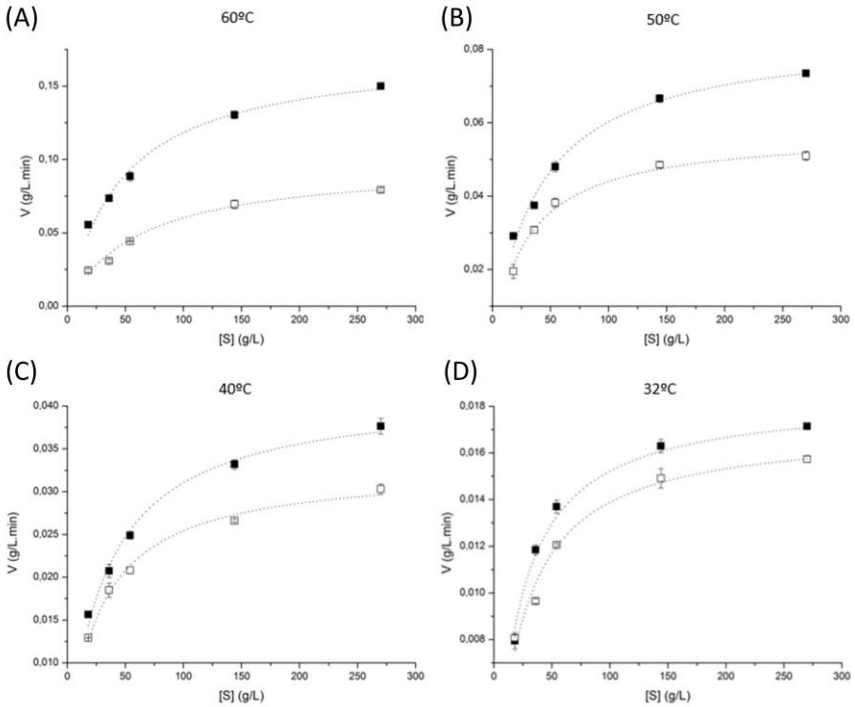
Table 1

Kinetics parameters and effectiveness of the fructose isomerization reaction catalyzed by xylose isomerase encapsulated in the alginate biocatalyst, intrinsic parameters (soluble enzyme) and apparent parameters (immobilized enzyme) at 32, 40, 50, and 60°C

Temperature	Soluble Enzyme		Immobilized Enzyme		Effectiveness
(ºC)	V_{max} (g/L.min)	Km (g/L)	V_{max} (g/L.min)	Km (g/L)	Intern
32	0.018	21.0	0.017	23.1	94.5%
40	0.042	34.3	0.033	28.9	78.7%
50	0.084	39.8	0.058	30.6	68.3%
60	0.174	46.8	0.098	61.8	56.2%

Figure 6

Effect of temperature on the kinetics of fructose isomerization to glucose by soluble xylose isomerase (■) and IXI-Ch encapsulated in calcium alginate gel (□) at different temperatures



A significant intraparticle diffusion effect is observed at 60°C (optimum enzyme temperature), with 56.2% internal effectiveness. However, this mass transfer limitation becomes less severe as the temperature decreases (Figure 6D), reaching an effectiveness of 94.5% at 32ºC (SIF process temperature). This is because the diffusion rate follows a linear trend with temperature, while the kinetic rate follows Arrhenius' law, which has an exponential relationship with temperature (Bouquerel et al., 2012). Therefore, with increasing temperature, mass transfer limitations become evident since the kinetic rate increases much more than the diffusion rate. However, at mild temperatures such as 32 °C, the co-encapsulation of IXI-Ch with *S. cerevisiae* yeast for the production of 2G ethanol has shown a promising biocatalyst, since internal effectiveness at this temperature is satisfactory, and the intraparticle diffusion effects present can be circumvented with the high productivity provided by the use of immobilized biocatalysts, such as conducting the process in continuous mode or in repeated batches, as well as operating with a high load of biocatalysts in the reactor.

4 CONCLUSIONS

Besides the temporal gap of works exploring SIF technology for ethanol production, this route is a potential strategy to xylose consumption and hemicellulose from biomass valorization. The IXI-Ch derivative obtained by immobilization in chitosan gel proved to be free of intraparticle effects, presenting kinetic parameters similar to those of the soluble enzyme. The use of this derivative in the biocatalyst containing co-immobilized xylose isomerase and yeast for the production of 2G ethanol proved to be viable, since under SIF process conditions it showed an internal effectiveness of 94.5%, which can be easily circumvented by conducting the process continuously or with high biocatalyst densities in the bioreactor.

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