catalysts

MDPI

Article

Immobilization of Alpha Acetolactate Decarboxylase in Hybrid Gelatin/Alginate Support for Application to Reduce Diacetyl Off-Flavor in Beer

Gustavo P. Costa, Leonardo B. Queiroz, Vitor Manfroi, Rafael C. Rodrigues * and Plinho F. Hertz *

Biotechnology, Bioprocess and Biocatalysis Group, Institute of Food Science and Technology, Federal University of Rio Grande do Sul, Av. Bento Gonçalves, 9500, P.O. Box 15090, ZC, Porto Alegre 91501-970, RS, Brazil
* Correspondence: rafaelcrodrigues@ufrgs.br (R.C.R.); plinho@ufrgs.br (P.F.H.); Tel.: +55-51-33087094 (P.F.H.)

Abstract: Beer production is the largest among alcoholic beverages. Its production process is complex and demands several steps. Lager beers commonly present an off-flavor of butter that is due to the presence of diacetyl, and to avoid such a problem, a long period of maturation (3–5 weeks) is required. Another way is the application of (α-acetolactate decarboxylase) ALDC to accelerate the process. The objectives of the present work were to develop a low-cost support using gelatin, a residue from capsules from the nutraceutical industry, to immobilize the ALDC enzyme. For this, the yield, efficiency and activity recovered, and the stability of free and immobilized enzymes at different temperatures and pH were evaluated. To evaluate the capacity of immobilized enzymes when applied directly to beer and their operational stability, three concentrations of glutaraldehyde (1%, 2.5% and 5%) were tested in distilled water as a cross-linking agent. The best results obtained were 95.6%, 27.0% and 23.6%, respectively, for yield, efficiency and activity recovery. Immobilization provided a high activity over a wide pH range. The immobilized enzyme showed greater stability at temperatures of 50 and 60 °C. The immobilized derivative showed adequate reuse capacity, and its dehydrated form had excellent activity after long periods of storage.

Keywords: ALDC; immobilization; alginate/alginate; gelatin residue

check for updates

Citation: Costa, G.P.; Queiroz, L.B.; Manfroi, V.; Rodrigues, R.C.; Hertz, P.F. Immobilization of Alpha Acetolactate Decarboxylase in Hybrid Gelatin/Alginate Support for Application to Reduce Diacetyl Off-Flavor in Beer. *Catalysts* **2023**, *13*, 601. https://doi.org/10.3390/catal13030601

Academic Editor: Luciana R. B. Gonçalves

Received: 26 February 2023
Revised: 8 March 2023
Accepted: 14 March 2023
Published: 16 March 2023

Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Beer is one of the world's favorite drinks. It has the highest consumption among alcoholic beverages and is third among all beverages, behind only the consumption of water and tea. The three largest beer producers are the US, China and Brazil. The global beer market size was valued at USD 605,246.1 million in 2020 and is projected to reach USD 816,847.7 million by 2030. Due to the high acceptance rate among consumers, lager beer dominates the market. It is estimated that the volume of production is equivalent to the sum of the other beer types (ale, stout, porter, etc.) [1–3].

The production of beer is complex and dependent on several stages. For example, for lager-type beers, the process is briefly described: the milling of the malt; mashing for conversion of starch to fermentable sugars; wort boiling and hop addition; cooling; fermentation (lager temperature); maturation (3 to 5 weeks, 0 to 5 °C); filtration; pasteurization; and packaging [4].

The off-flavor of butter reported in beers is due to the presence of diacetyl, a vicinal diketone, formed from the decarboxylation of α-acetolactate, the intermediate product of the synthesis of the amino acid valine during fermentation [5]. The reaction of decarboxylation of α-acetolactate in diacetyl occurs in the beer in the maturation phase, slowly and spontaneously, while the transformation of diacetyl into acetoin and subsequently into 2,3-butenediol occurs rapidly, catalyzed by reductions from yeast present in beer during maturation [6–8]. The spontaneous decarboxylation of α-acetolactate acts as a limiting factor in the optimization of the maturation time of beer. From the 1980s onwards, enzymatic

technology has been published to reduce the conditioning time, where the conversion of α-acetolactate to diacetyl is catalyzed by the enzyme α-acetolactate decarboxylase (ALDC) (EC 4.1.1.5) [9,10].

In this sense, the use of immobilized enzymes can be very useful for the beer industry. Enzyme immobilization is a technique that allows the preparation of a heterogeneous biocatalyst. There are several advantages of using immobilized enzymes such as the possibility of using continuous process, the separation of the enzyme from product and its reuse, and the possible increase in enzyme stability, among others [11–13]. There are several supports that can be used for enzyme immobilization. They can be based on inorganic materials, such as silica or magnetic particles [14–16], or from organic materials, such as chitosan, agarose, alginate or gelatin [17,18]. Additionally, metal–organic frameworks or amorphous coordination polymers can also be utilized to encapsulate enzymes [19–22].

Sodium alginate is a polysaccharide that can be used for the preparation of hydrogels by cross-linking with divalent cations (mainly Ca^{+2}). Several studies demonstrated the polyvalence of calcium alginate, when applying it to the production of films [23–25], immobilization of extracts [26], yeasts [27,28] and enzymes [29–31]. It is possible to form complex gels by adding other substances to change the characteristics of the alginate, such as gelatin. The alginate/gelatin complex has been studied for the encapsulation of betalains [32], microencapsulation of ascorbic acid (vitamin C) [33] and curcumin [34], for the preparation of films [25,35,36] and immobilization of enzymes [31,37,38]. The gelatin can be obtained by the residues of capsules discarded by the nutraceutical industry, since they consist of 48.4% gelatin, 30.0% water and 21.8% glycerol. Some studies demonstrated the efficiency of this residue in film production [39–41].

Thus, considering all these aspects, the objective of the present work was to develop an alginate/gelatin support, using nutraceutical industry capsule residue, for the covalent immobilization of ALDC, using glutaraldehyde as the cross-linking agent. For this, the immobilization yield was evaluated, as well as efficiency and activity recovery and parameters related to immobilization quality. The activity of the free and immobilized enzyme was tested at different pH, besides the thermal stability at different temperatures. The stability of immobilized, operational and storage ALDC was tested by applying it directly to the beer and quantifying activity during the storage period.

2. Results and Discussion

2.1. Enzyme Immobilization

The immobilization of ALDC in the alginate/gelatin particles was evaluated by changing the protein load and glutaraldehyde concentrations. The results of immobilization parameters and the support activity are presented in Table 1.

Table 1. ALDC immobilization on alginate/gelatin supports, evaluating the amount of enzyme offered and percentage of glutaraldehyde in the activation of support.

Offered Activity (U/g Support)	Glutaraldehyde	Yield	Efficiency	Activity Recovery	Activity (U/g Support)
5.0	1%	36.4% a	14.5% a	5.3% a	0.26
	2.5%	81.7% c	13.3% a	10.8% b	0.54
	5%	83.4% c	17.9% a,b	14.9% c	0.74
7.5	1%	52.6% b	22.0% b,c	11.6% b	0.87
	2.5%	79.3% c	26.6% c,d	21.1% d	1.58
	5%	95.6% d	21.9% b,c,d	21.0% d	1.58
10.0	1%	78.6% c	16.6% a,b	13.0% b,c	1.30
	2.5%	82.7% c	26.2% c,d	21.7% d	2.17
	5%	87.4% c,d	27.0% d	23.6% d	2.36

a, b, c, d Equal letters in the columns do not represent significant statistical differences ($p < 0.05$).

Immobilization yield represents the amount of protein that was attached to the support in theory, i.e., the enzyme that was not in supernatant at the end of the immobilization process. We can observe from Table 1 that the highest immobilization yields were obtained at the highest glutaraldehyde concentration (5%), for all enzyme loadings. It was the same for the other immobilization parameters (efficiency and activity recovery). Glutaraldehyde is the main agent for the support activation used for enzyme immobilization [42]. It has the capacity to react with primary amines, producing covalent cross-linking. The mechanism involved in glutaraldehyde enzyme immobilization can be explained by a first enzyme-support adsorption, ionic or hydrophobic, followed by covalent bonds, that can improve enzyme stability. Thus, the highest glutaraldehyde concentration might provide several points for enzyme interaction leading to better results.

The yield results found ranged from 36.4 to 95.6%, which is in accordance with other studies. Adriano et al. [43] evaluated supports of chitosan, chitosan/gelatin, chitosan/alginate and chitosan/carrageenan, obtaining a yield between 24 and 82% for chymotrypsin immobilization. In a work using β-galactosidase from *Aspergillus oryzae*, Tanriseven and Dogan [44] obtained an immobilization yield of 56% in alginate/gelatin cross-linked with glutaraldehyde, while Naganagouda and Mulimani [45] reached 71.8% for α-galactosidase mixed with gelatinous fibers of alginate and gelatin.

Thus, for the following experiments, a preparation using 10 U/g support and 5% of glutaraldehyde was selected. One important point that should be in mind when using glutaraldehyde as a cross-linking agent is its toxicity. Although glutaraldehyde promotes good cross-linking and mechanical properties for the particles [42], contradictory evidence has been highlighted on the cytotoxicity of glutaraldehyde cross-linked materials [46]. To reduce its effects, some strategies proposed lowering the concentration of the glutaraldehyde. Bigi et al. demonstrated that for gelatin films cross-linked with glutaraldehyde, there was no release of glutaraldehyde from films submitted to a concentration up to 1.5% [47]. In our case, although we chose 5%, the results using 2.5% were statistically equal. Nevertheless, the real toxicity for our particles should be tested to point out the real effect.

2.2. Effect of pH on ALDC Activity

The immobilization may generate alterations in the enzyme behavior in relation to the pH. Immobilization can change the optimal pH or even increase the pH range where the enzyme activity was close to the optimal. Figure 1 shows the results of the pH effect on the activity of the free and immobilized enzyme. The optimal activity observed was at pH 6.0 in both cases. It should be noted that the immobilized enzyme presented a pH range between 4.5 and 7.0, where the enzymatic activity was very close to optimal. It is important to remark that immobilized preparation presented a better activity than the free enzyme in a pH range usually found in beers (4.5–5.5). In the work of Qian et al. [16], a significant increase was not observed in the pH range of the ALDC action of *Bacillus brevis* immobilized on paramagnetic nanoparticles, in addition to finding the best pH conditions of 6.5 for free and immobilized enzymes.

2.3. Thermal Stability

The thermal stability was evaluated at different temperatures from 50 to 80 °C. However, temperatures above 70 °C affected the structure of the alginate/gelatin particle. Thus, the results obtained for thermal stability at 50 and 60 °C are presented in Figure 2. It can be observed that in both cases the immobilized preparation presented a higher stability than the free form. The half-life at 60 °C was 23 min for the free enzyme and 147 min for the immobilized enzyme, giving a stabilization factor of 6.4.

The results of the stabilization factor at a temperature of 60 °C were similar to those works using chitosan beads activated with glutaraldehyde for the immobilization of β-galactosidase [48], invertase [49], and chitosan activated with genipin for the immobilization of β-galactosidade [50]. In a study published by Pedroche et al. [51], the authors

suggested that the increase in thermal stabilization is associated with an increase in the number of covalent bonds between the enzyme and the support.

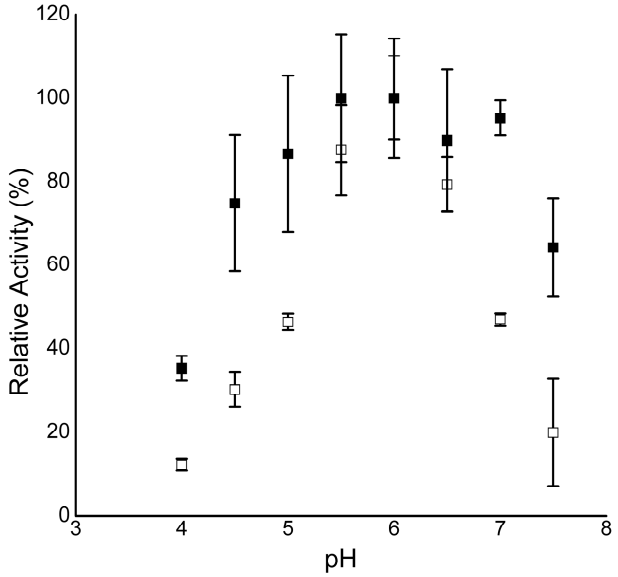

Figure 1. Effect of pH on the activity of free and immobilized ALDC at 37 °C. (□) Free and (■) immobilized enzymes. Error bars represent the standard deviation from the mean of three independent experiments.

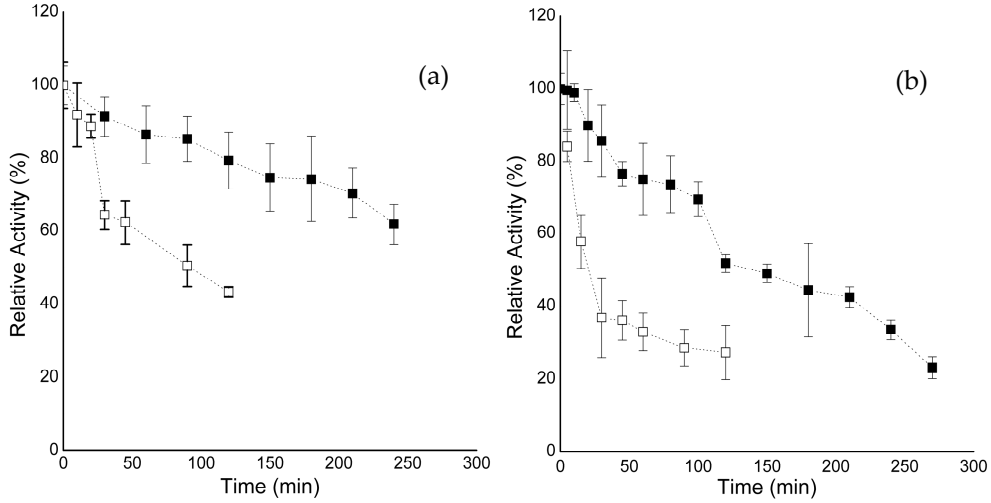

Figure 2. Thermal inactivation at different temperatures in pH 6.0 buffer, at (**a**) 50 °C and (**b**) 60 °C. (□) Free and (■) immobilized enzymes. Error bars represent the standard deviation from the mean of three independent experiments.

2.4. Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC)

Through the analysis of the graph of TGA, in Figure 3, we can observe three main bands of mass decay. In the first part of the graph between 100 and 240 °C, there is a decay of only 5%. The first significant decay occurs in the range of 240 to 300 °C with a further 20% reduction of the sample mass. From 300 to 400 °C, there is an additional 30% reduction, and in the last step from 400 to 550 °C, there is a further 15% reduction. Using gelatin films, Campo et al. [39] observed a great reduction in the range of 0 to 150 °C, suggesting that degradation or decomposition of low-molecular-weight protein and glycerol fractions occurred. The highest stability observed is possibly due to the cross-linking of glutaraldehyde with gelatin impregnated in the calcium alginate gel mesh. A similar effect was observed by

Danial et al. [52] when evaluating alginate/polyethyleneimine beads, and by Awad et al. [30], with alginate/cellulose (CMC)/polyethyleneimine, both activated with glutaraldehyde.

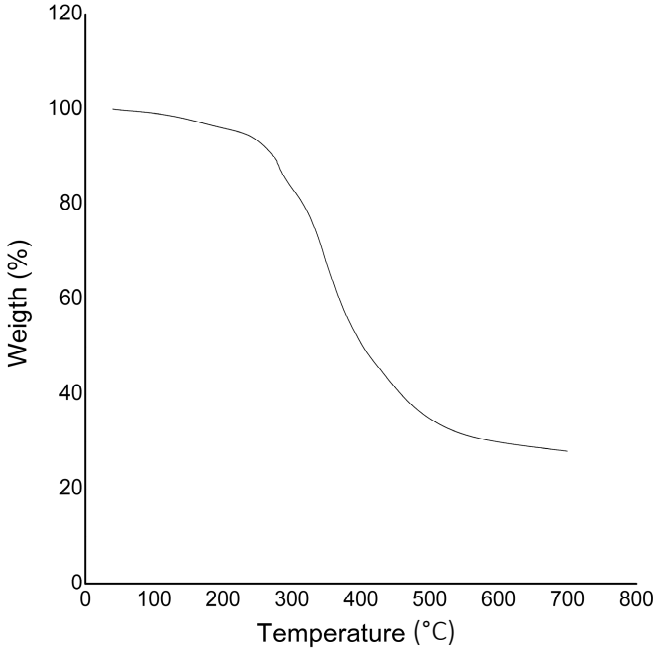

Figure 3. Thermogravimetric analysis (TGA) of alginate/gelatin spheres activated with glutaraldehyde.

The DSC thermograms, in Figure 4, evaluating the activated alginate/gelatin beads and ALDC immobilized on alginate/gelatin beads demonstrate subtle endothermic peaks at temperatures of approximately 75 and 78 °C. Such results may justify the loss of thermal stability of ALDC immobilized at temperatures equal to or greater than 70 °C. The glass transition temperature (Tg) of hydrated bovine gelatin determined by DSC was in the range of 70 to 90 °C [53]. Compared to films of cellulose nano-crystals (10%)/chitosan (30%) with starch (60%) or gelatin (60%), the Tg temperature was determined for gelatin as 75.8 °C [54].

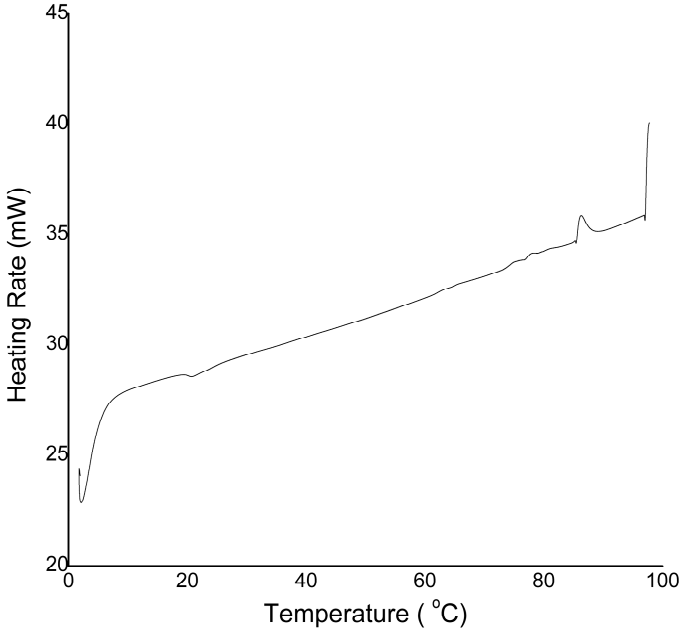

Figure 4. Differential scanning calorimetry (DSC) analysis chart of ALDC immobilized on alginate/gelatin beads.

2.5. Storage Stability

The storage stability was performed after lyophilization or vacuum drying of the immobilized preparations. Different than was observed for other polysaccharides beads, such as chitosan beads, the particle structure remained almost unaltered after the drying operation, and could be easily re-hydrated, returning to the original structure. The storage stability was monitored for 120 days, and the results are presented in Figure 5. The lyophilized particle presented greater stability, after 120 days of storage, maintaining approximately 91% of the initial activity, while the vacuum dried particles kept about 75% of their activity. These results are much higher than those found by Qian et al. [40], who found that ALDC immobilized on magnetic nanoparticles stored in buffer at pH 6.0, at 4 °C, for 14 days, maintained 70% of its activity.

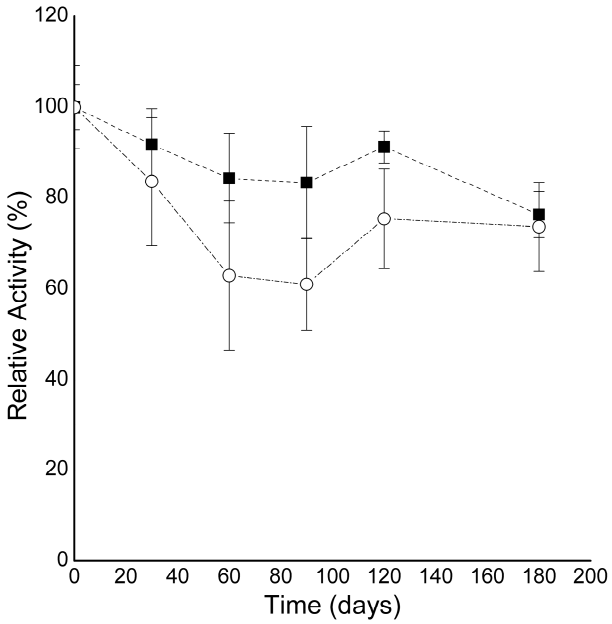

Figure 5. Evaluation of the quality of immobilized ALDC dehydrated during the storage of immobilized ALDC. (■) Lyophilized and (○) vacuum dried. Error bars represent the standard deviation from the mean of three independent experiments.

Considering the drying process, it can represent one of the main advantages of our hybrid alginate/gelatin material compared to other traditional polysaccharides used for enzyme immobilization, such as chitosan beads or alginate beads. Our particles could be dried and rehydrated, keeping their structure and activity. Chitosan beads and alginate beads lost their structure after drying and could not be rehydrated, losing functionality. For industrial and commercial purposes, it is interesting that this works with dried material because it is cheaper for storage and transport. In Figure 6, particles can be seen before and after the vacuum drying.

Figure 6. ALDC immobilized in alginate (**a**)/gelatin (**b**) particles before and after vacuum drying.

2.6. Operational Stability

The conversion capacity of α-acetolactate in beer was measured by applying immobilized ALDC according to item 3.10 and quantifying the acetoin formed, as described in item 3.9, for ten cycles. It is important to note that under the experimental conditions there was no spontaneous conversion of α-acetolactate into diacetyl in amounts detectable by the analytical methods applied. The reuse results are shown in Figure 7, where it can be observed that in the first three cycles, the relative activity was greater than 75%, and at the end of the ten cycles, the relative activity was about 25% of the initial one. In ALDC immobilized in $Ca_3(PO_4)_2$-ALDC@Alg microspheres, with its first reuse, it maintained 45% activity, and in the sixth, about 24% of the initial activity [29]. The losses of the enzyme activity could be explained by the presence of the substrate. The α-acetolactate solution was prepared with NaOH. Thus, the addition of substrate, in the long term, could lead to the loss of the calcium ion of the support structure through the interaction with sodium, and then the de-structuring of the alginate gel.

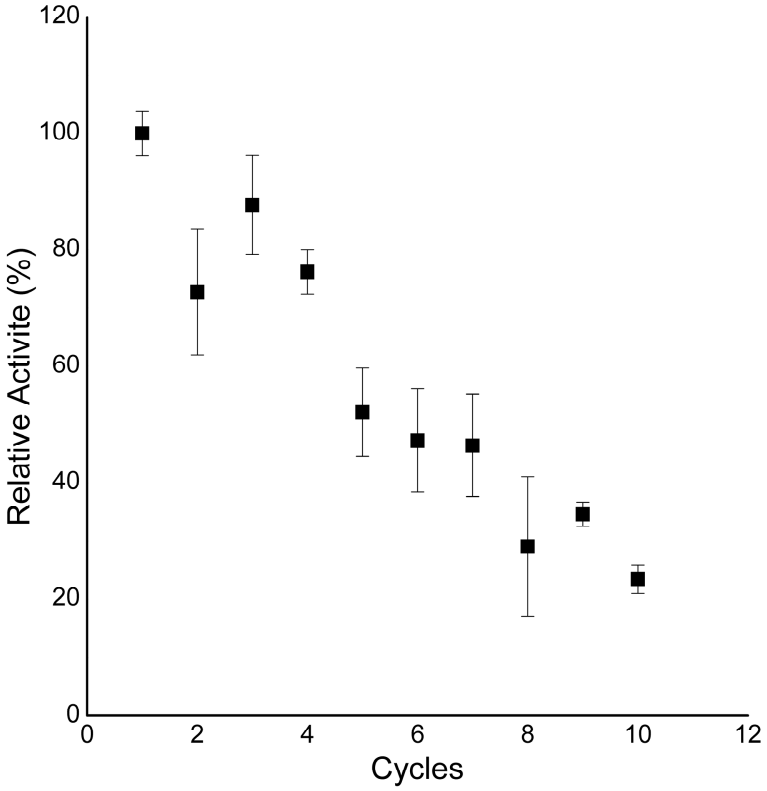

Figure 7. Relative activity of immobilized ALDC in the conversion of α-acetolactate to acetoin. Error bars represent the standard deviation from the mean of three independent experiments.

In order to verify the stability of the support particles in beer, the immobilized enzyme was incubated in degassed beer without substrate. The particles remained in contact with beer for 2 h, and then were filtered, washed and incubated in new fresh beer for 30 cycles. It can be observed in Figure 8 that after 30 cycles, the residual activity was 45%. Possibly, the losses could be due to hydrophilic and hydrophobic interactions of the proteins present in the beer with the support along the cycles, creating some substrate diffusional limitations when the activity was measured. However, due to the absence of sodium ions, the particles were more stable compared with the presence of substrate (Figure 8). Thus, the immobilized particles can be applied to the maturing beer process, since the α-acetolactate naturally present in beer is derived from microorganism metabolism and is free of sodium.

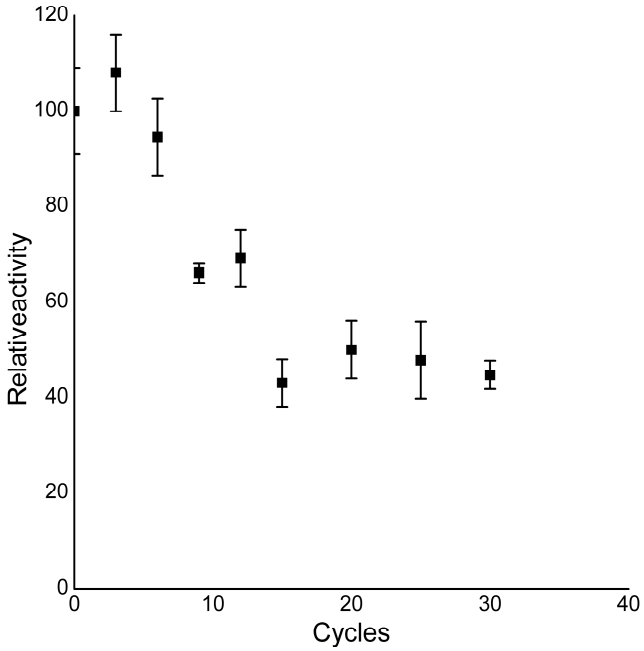

Figure 8. Evaluation of the stability of ALDC immobilized in beer during cycles of exhibition. Error bars represent the standard deviation from the mean of three independent experiments.

3. Materials and Methods

3.1. Reagents

The acquired 2000L Maturex from Novozymes is the commercial form of α-acetolactate decarboxylase enzyme (EC 4.1.1.5) from *B. brevis*; sodium alginate was obtained from Cromato Produtos Químicos Ltd.a (São Paulo, Brazil); capsule residue of gelatin was supplied by the Laboratório Químico Farmacêutico Tiaraju (Santo Ângelo, RS, Brazil); ethyl 2-acetoxy-2-methyl-acetolactate, anhydrous creatine, α-naphthol, acetoin (≥95%), ethyl acetate (>99.5%), diacetyl (>97%) and 2-pentanone (99.5%) were obtained from Sigma Aldrich (Brazil); glutaraldehyde (25%), citric acid, zinc chloride, calcium chloride dihydrate and sodium chloride were supplied by Nuclear (Brazil); sodium hydroxide, bibasic sodium phosphate and monobasic sodium phosphate were purchased from Neon (São Paulo, Brazil), and ethylene glycol was obtained from Synth (São Paulo, Brazil). All reagents used in this work were of analytical grade.

3.2. Support Preparation and Activation

The methodology for preparing the alginate/gelatin beads was adapted from those described by Callone et al. and Costa et al. [27,28], where 15% *w*/*v* capsule residue gelatin and 1.5% *w*/*v* sodium alginate were dissolved in distilled water at 50 °C. After homogenization, the mixture was kept in an ultrasonic bath for the elimination of air bubbles. The solution of alginate/gelatin was dripped, with the aid of a peristaltic pump (Engco, Piracicaba, Brazil), into a 0.1 M calcium chloride solution in an ice bath under mild stirring. After the complete gelatinization of the spheres, the calcium chloride solution was drained, washed 10 times with distilled water and stored at refrigeration until the next step. The support was activated using 5 mL of glutaraldehyde (1.0%, 2.5%, and 5.0%) solution in 0.02 M calcium chloride for 100 beads. It was kept at 37 °C in a thermostatic bath (Tecnal, Piracicaba, Brazil) for 3 h for complete activation, and washed 12 times with 5 mL of distilled water for the complete removal of non-carrier-attached glutaraldehyde.

3.3. ALDC Immobilization in Alginate/Gelatin Beads

Immediately after activation with glutaraldehyde, 100 beads were incubated with ALDC enzyme diluted in 4 mL of 0.02 M phosphate-citrate buffer, at pH 6.0 (immobilization buffer), plus 5 µM zinc chloride at 25 °C under slow agitation for 1 h. After this, the beads

were washed with the pH 6.0 immobilization buffer; immobilization buffer with 1 M NaCl; immobilization buffer with 30% *v*/*v* ethylene glycol; and finally with immobilization buffer. This sequence of washings aimed to eliminate enzymes that were not covalently bound to the support. After each washing, activity was measured in the washing solution to ensure that all proteins that were adsorbed were removed. The active alginate/gelatin beads with three concentrations of glutaraldehyde (1.0%, 2.5% and 5.0%) were tested with different concentrations of ALDC (5.0, 7.5 and 10.0 U/g support).

The immobilization parameters defined by Sheldon and Van Pelt [11] were calculated according to Equations (1)–(3):

$$\text{Immobilization yield (\%)} = 100 \times (\text{Immobilized Activity}/\text{Initial Activity}) \quad (1)$$

$$\text{Immobilization efficiency (\%)} = 100 \times (\text{Observed Activity}/\text{Immobilized Activity}) \quad (2)$$

$$\text{Activity recovery} = 100 \times (\text{Observed Activity}/\text{Initial Activity}) \quad (3)$$

3.4. ALDC Activity

The substrate solution (α-acetolactate) was prepared according to Kisrieva et al. [55]. For this, 10 µL of ethyl 2-acetoxy-2-methylacetoacetate was mixed with 1 mL of 0.1 M NaOH for 30 min at 37 °C, and then buffered with 1 mL of phosphorus phosphate (pH 6.0, 0.2 M). Free and immobilized ALDC activity were determined using 0.4 mL of phosphate buffer (pH 6.0, 0.2 M, with 5 µM zinc chloride) and 0.1 mL of α-acetolactate solution at 37 °C for 5 min. The reaction was stopped with the addition of 0.4 mL of 2.5 N sodium hydroxide solution.

One unit of α-acetolactate decarboxylase (U) is defined as the amount required to convert 1 µmol of α-acetolactate in acetoin per minute, at pH 6.0 and 37 °C [56]. The amount of acetoin formed was quantified using the colorimetric method proposed by Westerfeld [57], with modifications. For this, 1.0 mL of distilled water was added to 0.1 mL of the product of the enzymatic reaction, plus 0.4 mL of creatine solution (0.5% *w*/*v* in distilled water) and α-naphthol (5% *w*/*v* in sodium hydroxide 2.5 N) in a ratio of 1:1, and maintained at 37 °C for 45 min. The absorbance of the reaction product was measured at 525 nm. The quantification was performed using an external acetoin standard curve.

3.5. Activity in Different pH

The influence of pH on the activity of free and immobilized ALDC was determined by measuring the activity using different buffers: 0.2 M phosphate-citrate buffers at a different pH (4.0; 4.5; 5.0; 5.5 and 6.0), and 0.2 M phosphate buffer at pH 6.5, 7.0 and 7.5, plus 5 µM zinc chloride. For each case, the highest activity was considered as 100%.

3.6. Thermal Stability

The thermal stability of free and immobilized enzymes was measured at 50 °C and 60 °C at pH 6.0, with 0.02 M phosphate buffer plus 5 µM zinc chloride. Samples were collected at different times and cooled in an ice bath for 5 min. The relative activity was then determined from the residual activity after the heat treatment compared to the initial activity. The enzyme inactivation was described by a first-order equation, where the half-life time ($t_{\frac{1}{2}}$) is the time required to reduce the initial enzyme activity by 50% [49].

3.7. Storage Stability

For storage stability, the beads of immobilized ALDC were dried and the activity was determined for the period of 120 days. The beads were either vacuum-dried at room temperature or freeze-dried. For the quantification of activity, the beads were previously hydrated with 0.02 M pH 6.0 buffer for 4 h, and then drained and washed three times before measuring the residual activity.

3.8. Thermal Properties

The ALDC immobilized on alginate/gelatin beads was evaluated via thermogravimetric analysis (TGA), using the methodology described by Campo et al. [39], with modifications. Analyses were performed on Perkin Elmer equipment, TGA 6000 (Wellesly, MA, USA), where 10 mg of lyophilized samples were heated at 50 to 700 °C with a rate of 10 °C/min, using a nitrogen atmosphere with a flow rate of 20 mL/min. Differential scanning calorimetry (DSC) was used according to the methodology of Amadori et al. [58], using Perkin Elmer, DSC 8500 (Wellesly, MA, USA), calibrated with indium, with a heating curve of 0 at 100 °C, followed by cooling at 100 to 0 °C, at a rate of 10 °C/min, with a flow rate of 20 mL/min of nitrogen, where 5–10 mg samples were analyzed in hermetically sealed aluminum capsules.

3.9. Acetoin and Diacetyl Determination

Acetoin and diacetyl were simultaneously quantified using the method described by Kobayashi et al. [59], with adaptations, where 0.5 mL of the sample was added to 50 µL of 2-pentanone (1000 mg/L) as the internal standard and 0.5 mL of ethyl acetate, keeping the solution under vigorous stirring for 5 min, after centrifugation (3000× *g* for 2 min) and the organic phase was collected. The quantification was performed with a gas chromatograph coupled with flame ionization detector (GC-FID, Shimadzu, Kyoto, Japan) on a Shimadzu model GC-2010 Plus, using a RestekRTx®Wax polar column (30 m, 0.25 mm ID, 0.25 µm). The oven was kept at 30 °C for 10 min, after 20 °C/min up to 200 °C, and then maintaining the final temperature for 3.5 min. The temperature used in the injector was 240 °C and in the 250 °C detector, 1 µL of the sample was injected using splitless, with sampling time of 1 min, a total flow of 8.3 mL/min and a column flow of 1.06 mL/min.

The quantification of acetoin and diacetyl was performed using an external standard curve, with additions of the analytes in degassed commercial lager beer, obtained in a local market, produced by Heineken Brasil (Jacareí, São Paulo, Brazil), with 4.5% abv, which showed no acetoin and diacetyl sign. The internal standard 2-pentanone was added at a concentration of 100 mg/L, while acetoin and diacetyl were added in ten levels with concentrations between 10 and 400 mg/L and 8 and 380 mg/L, respectively. For acetoin, the limit of detection was 4 mg/L and the quantification was 8.2 mg/L, while for diacetyl, the limit of detection was 3.0 mg/L and 6.6 mg/L for quantification. The regression coefficients of the standard curve were R^2 = 0.9897 for acetoin and R^2 = 0.9879 for diacetyl.

3.10. Operational Stability of Immobilized ALDC Applied to Beer

The stability of immobilized ALDC in beer was determined by incubating the beads in degassed beer under stirring for 24 h, at room temperature. The beads were removed, washed with distilled water, and then drained and maintained at pH 6.0 in 0.02 M buffer for 10 min before measuring the residual activity.

The capability to convert α-acetolactate to acetoin in beer was evaluated by adding 1 U of immobilized ALDC per liter of degassed beer with the addition of α-acetolactate 5 mg/L. The solution was stirred for 1 h at room temperature. After this step, a liquid/liquid extraction was performed, followed by the gas chromatographic analysis. The beads were separated from the beer, washed and reused as described before.

4. Conclusions

Alginate/gelatin particles were successfully prepared, activated with glutaraldehyde and used for ALDC immobilization. The glutaraldehyde concentration appeared to be an important variable in the immobilization process, and the concentration of 5% was that which presented the best results among those tested. Moreover, the prepared immobilized particles were more stable to temperature than the soluble enzyme, as well as presenting a broader range of pH activity, and presenting a higher activity than the free form especially in the pH values found in beer. Additionally, the produced particles were able to be dried, either for lyophilization or vacuum, and re-hydrated, maintaining an enzyme activity close

to 90% of the initial activity. The immobilized enzymes were tested in beer and were stable for at least 30 cycles of 2 h each. The material prepared in this work presented interesting features for enzyme immobilization, and especially in the case of ALDC immobilization, to be prepared on a large scale, dried and used in the beer maturation stage to reduce the diacetyl off-flavor and maturation time.

Author Contributions: Formal analysis, G.P.C.; Investigation, G.P.C. and L.B.Q.; writing—original draft preparation, G.P.C.; writing—review and editing, P.F.H. and R.C.R.; supervision, V.M., P.F.H. and R.C.R.; All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, process 405418/2021-3), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS, Project number 17—/2551-0000939-8).

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors would like to thank to CNPq, CAPES and FAPERGS for supporting the research.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Kirin Kirin Beer University Report Global Beer Production by Country in 2017. Available online: https://www.kirinholdings.co.jp/english/news/2018/0809_01.html (accessed on 15 March 2023).
2. Baron, C. Beer Production Worldwide from 1998 to 2017 (in Billion Hectoliters). Available online: https://www.statista.com/statistics/270275/worldwide-beer-production/ (accessed on 28 January 2023).
3. Sinha, B. Beer Market by Type (Lager, Ale, Stout & Porter, Malt, and Others), Packaging (Glass, Metal Can, and Others), Category (Popular-Priced, Premium and Super Premium), and Production (Macrobrewery, Microbrewery, and Craft Brewery): Global Opportunity Analysis. Available online: https://www.alliedmarketresearch.com/beer-market (accessed on 7 February 2023).
4. Filho, W.G.V. *Bebidas Alcoólicas*, 2nd ed.; Blucher: São Paulo, Brazil, 2016; ISBN 9788521209553.
5. Krogerus, K.; Gibson, B.R. 125th Anniversary Review: Diacetyl and Its Control during Brewery Fermentation. *J. Inst. Brew.* **2013**, *119*, 86–97. [CrossRef]
6. Brányik, T.; Vicente, A.A.; Dostálek, P.; Teixeira, J.A. Continuous Beer Fermentation Using Immobilized Yeast Cell Bioreactor Systems. *Biotechnol. Prog.* **2008**, *21*, 653–663. [CrossRef]
7. Wainwright, T. Diacetyl—A Review. *J. Inst. Brew.* **1973**, *79*, 451–470. [CrossRef]
8. Bamforth, C.W.; Kanauchi, M. Enzymology of Vicinal Diketone Reduction in Brewer's Yeast. *J. Inst. Brew.* **2004**, *110*, 83–93. [CrossRef]
9. Godtfredsen, S.E.; Rasmussen, A.M.; Ottesen, M.; Mathiasen, T.; Ahrenst-Larsen, B. Application of the Acetolactate Decarboxylase from Lactobacillus Casei for Accelerated Maturation of Beer. *Carlsberg Res. Commun.* **1984**, *49*, 69–74. [CrossRef]
10. Godtfredsen, S.E.; Ottesen, M. Maturation of Beer with α-Acetolactate Decarboxylase. *Carlsberg Res. Commun.* **1982**, *47*, 93–102. [CrossRef]
11. Sheldon, R.A.; Van Pelt, S. Enzyme Immobilisation in Biocatalysis: Why, What and How. *Chem. Soc. Rev. J.* **2013**, *42*, 6223–6235. [CrossRef] [PubMed]
12. Rodrigues, R.C.; Ortiz, C.; Berenguer-Murcia, Á.; Torres, R.; Fernández-Lafuente, R. Modifying Enzyme Activity and Selectivity by Immobilization. *Chem. Soc. Rev.* **2013**, *42*, 6290–6307. [CrossRef]
13. Barbosa, O.; Torres, R.; Ortiz, C.; Berenguer-Murcia, Á.; Rodrigues, R.C.; Fernandez-Lafuente, R. Heterofunctional Supports in Enzyme Immobilization: From Traditional Immobilization Protocols to Opportunities in Tuning Enzyme Properties. *Biomacromolecules* **2013**, *14*, 2433–2462. [CrossRef]
14. Deon, M.; Carminatti Ricardi, N.; Carvalho De Andrade, R.; Hertz, P.F.; Nicolodi, S.; Costa, T.M.H.; Bussamara, R.; Benvenutti, E.V.; De Menezes, E.W. Designing a Support for Lipase Immobilization Based on Magnetic, Hydrophobic, and Mesoporous Silica. *Langmuir* **2020**, *36*, 10147–10155. [CrossRef]
15. Ricardi, N.C.; de Menezes, E.W.; Valmir Benvenutti, E.; da Natividade Schöffer, J.; Hackenhaar, C.R.; Hertz, P.F.; Costa, T.M.H. Highly Stable Novel Silica/Chitosan Support for β-Galactosidase Immobilization for Application in Dairy Technology. *Food Chem.* **2018**, *246*, 343–350. [CrossRef]
16. Qian, S.; Wang, C.; Wang, H.; Yu, F.; Zhang, C.; Yu, H. Synthesis and Characterization of Surface-Functionalized Paramagnetic Nanoparticles and Their Application to Immobilization of α-Acetolactate Decarboxylase. *Process Biochem.* **2015**, *50*, 1388–1393. [CrossRef]

17. Datta, S.; Christena, L.R.; Rajaram, Y.R.S. Enzyme Immobilization: An Overview on Techniques and Support Materials. *3 Biotech* **2013**, *3*, 1–9. [CrossRef]
18. Zdarta, J.; Meyer, A.; Jesionowski, T.; Pinelo, M. A General Overview of Support Materials for Enzyme Immobilization: Characteristics, Properties, Practical Utility. *Catalysts* **2018**, *8*, 92. [CrossRef]
19. Chen, G.; Huang, S.; Kou, X.; Zhu, F.; Ouyang, G. Embedding Functional Biomacromolecules within Peptide-Directed Metal–Organic Framework (MOF) Nanoarchitectures Enables Activity Enhancement. *Angew. Chem. Int. Ed.* **2020**, *59*, 13947–13954. [CrossRef] [PubMed]
20. Zhu, H.; Li, X.; He, Z.; Chen, Y.; Zhu, J.J. Metal Azolate Coordination Polymer-Enabled High Payload and Non-Destructive Enzyme Immobilization for Biocatalysis and Biosensing. *Anal. Chem.* **2022**, *94*, 6827–6832. [CrossRef] [PubMed]
21. Wu, X.; Yue, H.; Zhang, Y.; Gao, X.; Li, X.; Wang, L.; Cao, Y.; Hou, M.; An, H.; Zhang, L.; et al. Packaging and Delivering Enzymes by Amorphous Metal-Organic Frameworks. *Nat. Commun.* **2019**, *10*, 5165. [CrossRef]
22. Chen, G.; Huang, S.; Kou, X.; Wei, S.; Huang, S.; Jiang, S.; Shen, J.; Zhu, F.; Ouyang, G. A Convenient and Versatile Amino-Acid-Boosted Biomimetic Strategy for the Nondestructive Encapsulation of Biomacromolecules within Metal–Organic Frameworks. *Angew. Chem. Int. Ed.* **2019**, *58*, 1463–1467. [CrossRef] [PubMed]
23. Báez, G.D.; Piccirilli, G.N.; Ballerini, G.A.; Frattini, A.; Busti, P.A.; Verdini, R.A.; Delorenzi, N.J. Physicochemical Characterization of a Heat Treated Calcium Alginate Dry Film Prepared with Chicken Stock. *J. Food Sci.* **2017**, *82*, 945–951. [CrossRef]
24. Xiao, C.; Liu, H.; Lu, Y.; Zhang, L. Blend Films from Sodium Alginate and Gelatin Solutions. *J. Macromol. Sci. Pure Appl. Chem.* **2001**, *38 A*, 317–328. [CrossRef]
25. Rosellini, E.; Cristallini, C.; Barbani, N.; Vozzi, G.; Giusti, P. Preparation and Characterization of Alginate/Gelatin Blend Films for Cardiac Tissue Engineering. *J. Biomed. Mater. Res. Part A* **2009**, *91*, 447–453. [CrossRef] [PubMed]
26. Lupo, B.; Maestro, A.; Gutiérrez, J.M.; González, C. Characterization of Alginate Beads with Encapsulated Cocoa Extract Toprepare Functional Food: Comparison of Two Gelation Mechanisms. *Food Hydrocoll.* **2015**, *49*, 25–34. [CrossRef]
27. Costa, G.P.; Nicolli, K.P.; Welke, J.E.; Manfroi, V.; Zini, C.A. Volatile Profile of Sparkling Wines Produced with the Addition of Mannoproteins or Lees before Second Fermentation Performed with Free and Immobilized Yeasts. *J. Braz. Chem. Soc.* **2018**, *29*, 1866–1875. [CrossRef]
28. Callone, E.; Campostrini, R.; Carturan, G.; Cavazza, A.; Guzzon, R. Immobilization of Yeast and Bacteria Cells in Alginate Microbeads Coated with Silica Membranes: Procedures, Physico-Chemical Features and Bioactivity. *J. Mater. Chem.* **2008**, *18*, 4839. [CrossRef]
29. Zhao, F.; Wang, Q.; Dong, J.; Xian, M.; Yu, J.; Yin, H.; Chang, Z.; Mu, X.; Hou, T.; Wang, J. Enzyme-Inorganic Nanoflowers/Alginate Microbeads: An Enzyme Immobilization System and Its Potential Application. *Process Biochem.* **2017**, *57*, 87–94. [CrossRef]
30. Awad, G.E.A.; Wehaidy, H.R.; Abd El Aty, A.A.; Hassan, M.E. A Novel Alginate–CMC Gel Beads for Efficient Covalent Inulinase Immobilization. *Colloid Polym. Sci.* **2017**, *295*, 495–506. [CrossRef]
31. Rakmai, J.; Cheirsilp, B.; Prasertsan, P. Enhanced Thermal Stability of Cyclodextrin Glycosyltransferase in Alginate-Gelatin Mixed Gel Beads and the Application for β-Cyclodextrin Production. *Biocatal. Agric. Biotechnol.* **2015**, *4*, 717–726. [CrossRef]
32. Otálora, M.C.; de Jesús Barbosa, H.; Perilla, J.E.; Osorio, C.; Nazareno, M.A. Encapsulated Betalains (Opuntia Ficus-Indica) as Natural Colorants. Case Study: Gummy Candies. *LWT* **2018**, *103*, 222–227. [CrossRef]
33. Devi, N.; Kakati, D.K. Smart Porous Microparticles Based on Gelatin/Sodium Alginate Polyelectrolyte Complex. *J. Food Eng.* **2013**, *117*, 193–204. [CrossRef]
34. Sarika, P.R.; James, N.R. Polyelectrolyte Complex Nanoparticles from Cationised Gelatin and Sodium Alginate for Curcumin Delivery. *Carbohydr. Polym.* **2016**, *148*, 354–361. [CrossRef]
35. Dou, L.; Li, B.; Zhang, K.; Chu, X.; Hou, H. Physical Properties and Antioxidant Activity of Gelatin-Sodium Alginate Edible Films with Tea Polyphenols. *Int. J. Biol. Macromol.* **2018**, *118*, 1377–1383. [CrossRef]
36. Kazemi, S.M.; Rezaei, M. Antimicrobial Effectiveness of Gelatin-Alginate Film Containing Oregano Essential Oil for Fish Preservation. *J. Food Saf.* **2015**, *35*, 482–490. [CrossRef]
37. Stolarzewicz, I.A.; Zaborniak, P. Study on the Properties of Immobilized Biocatalysts with Lipase Activity Produced by Yarrowia Lipolytica in Batch Culture. *Chem. Biochem. Eng. Q.* **2017**, *31*, 251–259. [CrossRef]
38. Fadnavis, N.W.; Sheelu, G.; Kumar, B.M.; Bhalerao, M.U.; Deshpande, A.A. Gelatin Blends with Alginate: Gels for Lipase Immobilization and Purification. *Biotechnol. Prog.* **2003**, *19*, 557–564. [CrossRef]
39. De Campo, C.; Pagno, C.H.; Haas Costa, T.M.; De, A.; Rios, O.; Flôres, S.H. Gelatin Capsule Waste: New Source of Protein to Develop a Biodegradable Film. *Polímeros* **2017**, *27*, 100–107. [CrossRef]
40. Iahnke, A.O.E.S.; Costa, T.M.H.; De Oliveira Rios, A.; Flôres, S.H. Antioxidant Films Based on Gelatin Capsules and Minimally Processed Beet Root (*Beta Vulgaris* L. Var. Conditiva) Residues. *J. Appl. Polym. Sci.* **2016**, *133*, 43094. [CrossRef]
41. de Moraes Crizel, T.; de Oliveira Rios, A.; Alves, V.D.; Bandarra, N.; Moldão-Martins, M.; Hickmann Flôres, S. Biodegradable Films Based on Gelatin and Papaya Peel Microparticles with Antioxidant Properties. *Food Bioprocess Technol.* **2018**, *11*, 536–550. [CrossRef]
42. Barbosa, O.; Ortiz, C.; Berenguer-Murcia, Á.; Torres, R.; Rodrigues, R.C.; Fernandez-Lafuente, R. Glutaraldehyde in Bio-Catalysts Design: A Useful Crosslinker and a Versatile Tool in Enzyme Immobilization. *RSC Adv.* **2014**, *4*, 1583–1600. [CrossRef]
43. Adriano, W.S.; Mendonça, D.B.; Rodrigues, D.S.; Mammarella, E.J.; Giordano, R.L.C. Improving the Properties of Chitosan as Support for the Covalent Multipoint Immobilization of Chymotrypsin. *Biomacromolecules* **2008**, *9*, 2170–2179. [CrossRef] [PubMed]

44. Tanriseven, A.; Dogan, S. A Novel Method for the Immobilization of B-Galactosidase. *Process Biochem.* **2002**, *38*, 27–30. [CrossRef]
45. Naganagouda, K.; Mulimani, V.H. Gelatin Blends with Alginate: Gel Fibers for α-Galactosidase Immobilization and Its Application in Reduction of Non-Digestible Oligosaccharides in Soymilk. *Process Biochem.* **2006**, *41*, 1903–1907. [CrossRef]
46. Campiglio, C.E.; Negrini, N.C.; Farè, S.; Draghi, L. Cross-Linking Strategies for Electrospun Gelatin Scaffolds. *Materials* **2019**, *12*, 2476. [CrossRef] [PubMed]
47. Bigi, A.; Cojazzi, G.; Panzavolta, S.; Rubini, K.; Roveri, N. Mechanical and Thermal Properties of Gelatin Films at Different Degrees of Glutaraldehyde Crosslinking. *Biomaterials* **2001**, *22*, 763–768. [CrossRef]
48. Klein, M.P.; Nunes, M.R.; Rodrigues, R.C.; Benvenutti, E.V.; Costa, T.M.H.; Hertz, P.F.; Ninow, J.L. Effect of the Support Size on the Properties of β-Galactosidase Immobilized on Chitosan: Advantages and Disadvantages of Macro and Nanoparticles. *Biomacromolecules* **2012**, *13*, 2456–2464. [CrossRef] [PubMed]
49. Lorenzoni, A.S.G.; Aydos, L.F.; Klein, M.P.; Rodrigues, R.C.; Hertz, P.F. Fructooligosaccharides Synthesis by Highly Stable Immobilized β–Fructofuranosidase from Aspergillus Aculeatus. *Carbohydr. Polym.* **2014**, *103*, 193–197. [CrossRef] [PubMed]
50. Klein, M.P.; Hackenhaar, C.R.; Lorenzoni, A.S.G.; Rodrigues, R.C.; Costa, T.M.H.; Ninow, J.L.; Hertz, P.F. Chitosan Crosslinked with Genipin as Support Matrix for Application in Food Process: Support Characterization and β-d-Galactosidase Immobilization. *Carbohydr. Polym.* **2016**, *137*, 184–190. [CrossRef]
51. Pedroche, J.; del Mar Yust, M.; Mateo, C.; Fernández-Lafuente, R.; Girón-Calle, J.; Alaiz, M.; Vioque, J.; Guisán, J.M.; Millán, F. Effect of the Support and Experimental Conditions in the Intensity of the Multipoint Covalent Attachment of Proteins on Glyoxyl-Agarose Supports: Correlation between Enzyme-Support Linkages and Thermal Stability. *Enzym. Microb. Technol.* **2007**, *40*, 1160–1166. [CrossRef]
52. Danial, E.N.; Elnashar, M.M.M.; Awad, G.E.A. Immobilized Inulinase on Grafted Alginate Beads Prepared by the One-Step and the Two-Steps Methods. *Ind. Eng. Chem. Res.* **2010**, *49*, 3120–3125. [CrossRef]
53. Rahman, M.S.; Al-Saidi, G.; Guizani, N.; Abdullah, A. Development of State Diagram of Bovine Gelatin by Measuring Thermal Characteristics Using Differential Scanning Calorimetry (DSC) and Cooling Curve Method. *Thermochim. Acta* **2010**, *509*, 111–119. [CrossRef]
54. Noorbakhsh-Soltani, S.M.; Zerafat, M.M.; Sabbaghi, S. A Comparative Study of Gelatin and Starch-Based Nano-Composite Films Modified by Nano-Cellulose and Chitosan for Food Packaging Applications. *Carbohydr. Polym.* **2018**, *189*, 48–55. [CrossRef]
55. Kisrieva, Y.S.; Serebrennikov, V.M.; Zagustina, N.A.; Bezborodov, A.M. Isolation and Purification of Acetolactate Synthase and Acetolactate Decarboxylase from the Culture of Lactococcus Lactis. *Appl. Biochem. Microbiol.* **2000**, *36*, 109–114. [CrossRef]
56. Garmyn, D.; Monnet, C.; Martineau, B.; Guzzo, J.; Cavin, J.-F.; Diviès, C. Cloning and Sequencing of the Gene Encoding α-Acetolactate Decarboxylase from Leuconostoc Oenos. *FEMS Microbiol. Lett.* **1996**, *145*, 445–450. [CrossRef] [PubMed]
57. Westerfeldt, W.W. A Colorimetric. *J. Biol. Chem.* **1945**, *161*, 495–502.
58. Amadori, S.; Torricelli, P.; Rubini, K.; Fini, M.; Panzavolta, S.; Bigi, A. Effect of Sterilization and Crosslinking on Gelatin Films. *J. Mater. Sci. Mater. Med.* **2015**, *26*, 69. [CrossRef]
59. Kobayashi, K.; Kusaka, K.; Takahashi, T.; Sato, K. Method for the Simultaneous Assay of Diacetyl and Acetoin in the Presence of Alpha-Acetolactate: Application in Determining the Kinetic Parameters for the Decomposition of Alpha-Acetolactate. *J. Biosci. Bioeng.* **2005**, *99*, 502–507. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.