Miniaturized Platforms for High Throughput Biocatalysis

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Abstract Two immobilization methods were used in parallel throughout the work: covalent binding in Amberlite IRC-86 and entrapment in PVA, being the latter the one who showed better immobilization efficiency (94%) and overall performance. The optimal temperature and pH found for the enzyme immobilized in Amberlite IRC-86 was 55ºC and pH 4 whereas for PVA it was 65ºC and pH 4.5. No temperature shifts compared to the free form were observed, only higher tolerances to heat upon immobilization in both supports. With Amberlite IRC-86 a shift from pH 5 to 4 was observed upon immobilization. In the entrapped enzyme, the reaction showed signs of needing convective flow at the reactional volume of 1 mL, whereas for the covalently-linked enzyme the threshold occurred with 3 mL. The impact of the presence/absence of agitation in the reaction was found to be dependent of the immobilization method. Microtiter plates were used to evaluate the influence of geometric and dynamic parameters on the reaction and to establish them as a reliable and efficient high-throughput platforms for fructose production.

Key words: Inulin; Enzyme Immobilization; Amberlite IRC-86; PVA; Miniaturized Systems; Microtiter Plates

1. Introduction

The demand for fructose syrups has been increasing over the years, however its high cost has revealed to be limitative to its commercial diffusion[1]. Due to its sweetness, reported to be 1.3 to 2.0 times that of sucrose, and lack of the side effects linked with sucrose metabolism in humans, fructose is now emerging in the world of low caloric sweeteners[2]. Nowadays, fructose is being obtained by sucrose inversion thanks to the enzyme invertase or from hydrolyzed corn starch[3]. The latter is the main process used in industry today, but is characterized by a strong thermodynamic limitation as the values of the equilibrium concentrations of fructose and glucose are around 50% [3]. A different approach for fructose production relies in the enzymatic hydrolysis of inulin. Inulin occurs as an energy reserve in various plants and can be found in Jerusalem artichoke, dahlia, chicory, garlic, asparagus root and salsify[3]. This linear fructan-type polysaccharide consists of β-2,1 linked D-fructofuranose molecules, attached to a terminal glucose molecule through a sucrose-type linkage at the reducing end[4]. This reaction, catalyzed by inulinase, has been shown to be a promising process as one enzymatic step yields up to 95% of pure fructose[4]. Inulinases can have two distinct actions on inulin molecules: an endo-inulinase (E.C. 3.2.1.7) that has an internal action on the β-

1,2-fructan links, yielding a series of oligosaccharides of special interest for the food and pharmaceutical industry[5], and an exo-inulinase (E.C. 3.2.1.80), that has an endwise action that separates the first D-fructose molecule and proceeds until the last linkage within inulin is broken and D-glucose is released as a minor product[5]. When syrups with high fructose content are aimed, a synergetic action of both inulinases is explored to achieve total inulin hydrolysis.

The use of miniaturized devices in bioprocess development has been showing numerous advantages when compared to the traditional batch technology. One of the main goals associated with miniaturization is the need to develop cheaper, faster and more efficient reaction systems not only to achieve better biotransformation but also to increase its throughput and reproducibility. Microtiter plates (MTP) are examples of such systems that can be used as "small parallelized reactors". These MTP are available in a wide range of well number, geometries and volumes.

When considering enzyme kinetics, a rapid and efficient mixing upon solution addition is necessary to guarantee the reproducibility of the assays. The mixing process in shaken and stirred reactors can be characterized through dimensionless numbers, such as the Reynolds number (Re_f) (Equation 1).

$$Re_f = \frac{\rho\,\pi\,N\,D_w^2}{2\,\mu}\left(1-\sqrt{1-\frac{4}{\pi}\left(\frac{V_L^{\frac{1}{3}}}{D_w}\right)^2}\right)^2 \qquad \textbf{[Equation 1]}$$

Where D_w is the well diameter (m), V_L is the filling volume (m^3), ρ is the fluid density ($kg.m^{-3}$), N is the mixing frequency (s^{-1}), and μ is the fluid viscosity (Pa.s).

The Phase number (Equation 2) was adapted as an analogy of a partially filled rotating horizontal drum from liquid films to water-like solutions[6] and acts as an indicator of the operational conditions of the fluid flow regarding the inputted rotational movement in shaken reactors[7].

$$Ph = \frac{D_o}{D_w} \times (1 + 3\log_{10} Re_f) \qquad \textbf{[Equation 2]}$$

Where D_o is the shaking diameter, D_w is the diameter of the wells of the MTP and Re_f is the Reynolds number. When Ph > 1.26, together with the axial Froude number > 0.4, it is considered that we have in-phase conditions, meaning that the operating conditions are favorable and that the fluid follows the rotational movement. For Ph < 1.26 the operating conditions are out-of-phase, meaning that the fluid cannot follow the shaking movements, remaining at the bottom of the shake flask[8]. The assessment of this number constitutes a way to assure that the fluid flow is appropriate, as otherwise the mixing and the reproducibility of the operating conditions would be rather poor.

The axial Froude number (Equation 3), in this context (Fr_a > 0.4), means that the centrifugal acceleration is strong enough to project the fluid bulk against the surrounding walls of the wells.

$$Fr_a = \frac{u^2}{g\,D_o} = \frac{2\,(\pi\,N)^2\,D_o}{g} \qquad \textbf{[Equation 3]}$$

Where u is the fluid velocity ($m.s^{-1}$), N is the mixing frequency (s^{-1}), D_o is the shaking diameter (m) and g is the gravitational acceleration ($m.s^{-2}$).

The main goal of this project is to study the behavior and applicability of miniaturized

systems, such as small vessels and microtiter plates, in biochemical engineering and to establish them as robust platforms for high throughput biocatalysis and rapid process development. In order to do so, the importance of agitation in 1, 3 and 10 mL reactions will be evaluated as well as their influence on the enzyme kinetics'. The changes in the hydrodynamic environment upon the reaction scale up will also be evaluated and compared to the original system. Microtiter plates will be thoroughly tested on their high throughput capacity to study how parameters such as the agitation rate, the shaking diameter, the well shape and dimensions or the filling volume might affect the overall reaction rate. The enzymatic hydrolysis of inulin by inulinase was chosen as the model reaction.

2. Materials and Methods

2.1. Materials

Reagents The purified liquid Fructanase mixture (exo-inulinase and endo-inulinase in 50% glycerol and 0.02% Na azide) was acquired from Megazyme (Wicklow, Ireland). The substrate inulin, from *Dahlia tubers* and chicory root, were acquired from Acros Organics (Geel, Belgium). The immobilization support, Amberlite IRC-86 in hydrogen form, was acquired from Fluka Analytical and the reagents used in the immobilization protocol, polyethylenimine 50% aqueous solution and glutaraldehyde 24% (w/w) were obtained from Sigma-Aldrich and Acros Organics (Geel, Belgium), respectively. Polyvinyl alcohol (Lentikat liquid 250) was obtained from GeniaLab (Braunschweig, Germany). Polyethylene glycol, with an average MW of 600 (PEG 600) was acquired from Acros Organics (Geel, Belgium).

2.2. Immobilization procedures

Amberlite immobilization

Inulinase was immobilized onto Amberlite IRC-86 based on the method described by Obón and co-workers [9] and adapted by Rocha and co-workers for immobilization in IRC-50 [10]. First, 10 g of Amberlite IRC-86 were washed in 100 mL of distilled water for an hour and vacuum filtered. The same procedure was then applied but with acetate buffer 0.1 M pH 4.5 instead. The support modification procedure started with a 2-hour long incubation of 25 mL of polyethylenimine 10% with Amberlite IRC-86. The beads were recovered by vacuum filtration and activated by incubation with 50 mL of glutaraldehyde 10%, also during 2 hours. The support was recovered by vacuum filtration and incubated with 10 mL of diluted inulinase (10-fold in acetate buffer 0.1 M pH 4.5) at 4°C during 2 hours. Lastly, the support with the immobilized enzyme was vacuum filtered, washed with acetate buffer 0.1 M pH 4.5, vacuum filtered and stored at 4ºC until further use. All of the steps were performed under gentle magnetic stirring at room temperature, unless stated otherwise.

PVA immobilization

Inulinase entrapment in PVA was based on the methodology developed by Fernandes and co-workers[11]. Lentikat® liquid was heated up to 95ºC, until it was completely dissolved, and then allowed to cool down to 50ºC. 1 mL of a 5-fold diluted enzyme solution (in acetate buffer 0.1 M pH 4.5) was added to 5 mL of Lentikat® liquid and mixed through magnetic stirring in a 25 mL

jacketed vessel. After allowing the mixing of the solution, the preparation was extruded through a peristaltic pump into 50 mL of PEG 600, also under magnetic stirring, and left for a 2-hour long incubation. Afterwards the beads were collected, drained and washed with acetate buffer 0.1 M pH 4.5 for 30 minutes. Both supernatants were collected and assayed for protein quantification. PVA beads were then soaked in acetate buffer 0.1 M pH 4.5 and stored at 4ºC until further use.

2.3. Characterization of immobilized and free inulinase (pH and temperature)

The effects of pH and temperature on enzyme activity were evaluated in 30 minutes batch runs by incubating 15 mg of Amberlite IRC-86 or 3 beads (appx 70 mg) of PVA immobilized enzyme in 1 mL of inulin solution 50 $g.L^{-1}$ in acetate buffer 0.1 M in a pH range of 4–5.5 and in a temperature range of 45–70°C. For the free enzyme, 16.67 µL of inulinase diluted 100-fold in acetate buffer 0.1 M pH 4.5 were used similarly. Blanks with enzyme free solutions were performed likewise. Determination of enzyme activity was determined through the rate of reducing sugars formation in the reaction medium and converted in relative activity. All trials were performed in duplicate.

2.4. Biotransformation with the free and immobilized enzyme

Experiments with the free and immobilized enzyme were carried out in magnetically stirred (440 rpm) 1, 3 and 10 mL vessels. 50 µL of 10-fold diluted enzyme or 200 mg of Amberlite IRC-86 immobilized enzyme were added to 3 mL of *Dahlia* tuber's inulin 50 $g.L^{-1}$ prepared in acetate buffer 0.1 M pH 4 at 55°C. For the PVA immobilized enzyme, 50 µL of a 100-fold diluted enzyme solution or 210 mg of bead weight were added to 3 mL of chicory root inulin 50 $g.L^{-1}$ prepared in acetate buffer 0.1 M pH 4.5 at 50°C. Proportions were kept for the remaining volume trials. Samples were collected periodically, quenched in dinitrosalicylic acid (DNS) reagent and assayed for quantification of reducing sugars. The reaction rate was defined as the amount of reducing sugars formed over the course of the reaction and the calculations were based on the linear evolution of the measurements made, in general, in the first 30 minutes. All trials were performed in duplicate. Equivalent trials without magnetic stirring were also performed.

2.5. Microtiter plate assays

To study the reaction in a shaken environments, microtiter tests were performed by adding either 70 mg of PVA beads or 67 mg of Amberlite immobilized enzyme to 1 mL of 50 $g.L^{-1}$ *Dahlia* tubers inulin solution prepared in acetate buffer 0.1 M pH 4 at 50ºC or 55°C, respectively. Three different MTP were used: (A) – Square-shaped, round-bottomed 96-well plate (well length: 0.8 cm); (B) – Square-shaped, pyramidal-bottomed 24-well plate (well length: 1.7 cm); (C) – Round-shaped, flat-bottomed 24-well plate (well diameter: 1.6 cm). In order to keep the Froude number constant, trials in the MTP shaker (orbital diameter of 1.5 mm) occurred at 450 and 700 rpm while trials in the orbital shaker (orbital diameter of 2.5 cm) occurred at 110 and 170 rpm. Similar assays with a reaction volume of 3 mL were performed for plate B and proportions were kept the same. Samples were collected at 0 and 60 minutes, quenched in DNS reagent and assayed for quantification of reducing sugars.

2.6. Enzyme activity

The experimental set up for the determination of the kinetic parameters proceeded accordingly to the conditions described in 2.4. For *Dahlia* tubers inulin, the tested concentrations for the free and Amberlite immobilized enzyme were of 10, 20, 30, 50, 75 and 100 $g.L^{-1}$. Samples were collected at 0 and 20 minutes, according to the "sacrificial well" methodology[12]. The chicory root inulin concentrations for the activity for the free and PVA immobilized enzyme were of 10, 20, 30, 50, 75, 100 and 120 $g.L^{-1}$. Samples were collected at 5, 10, 15 and 20 minutes. Enzyme activity was determined through the rate of reducing sugars formation in the reaction medium and all trials were performed in duplicate. The initial reaction rates were plotted as a function of the substrate concentrations for the three reactional volumes.

2.7. Analytical methods

Quantification of reducing sugars was performed by the 2,4-dinitroslicylic acid (DNS) method [13]. Fructose was used for the establishment of a standard curve. The method was adapted to be performed in 96-well plates. For that, 10 µL of sample were added to 90 µL of distilled water and 100 µL of DNS reagent. The solutions were then incubated in a closed plate for 5 minutes at 100ºC and, allowed to cool down to room temperature. 500 µL of distilled water were added to the wells and, after mixing, 200 µL were transferred to a 96-well microplate. Absorbance was read at 540 nm. Blanks were either performed with distilled water or the substrate solution. Standard deviations did not exceed 10%.

3. Results and discussion

3.1. Characterization of immobilized and free inulinase (pH and temperature)

The influence of temperature and pH on the enzyme activity was assessed for both free and immobilized enzymes (Figure 1).

Regarding the reaction with inulin from *Dahlia* tubers, it is possible to see a shift in the optimal pH from 5 to 4 upon immobilization, suggesting that the immobilization favors the reaction to occur in more acidic environments. However this result is not as common as the many reported increases in the optimal pH when considering that the support used had an overall negative charge [10,14]. As for the temperature, 55ºC seems to be the optimal temperature for both the free and immobilized enzyme. Nevertheless, the immobilization contributed to a broader profile.

As for the chicory root inulin, the differences in the pH for the PVA immobilized and the free enzyme are basically non-existent in the pH ranges of 3.5–5, but at pH 5.5 it can be said that immobilization improved the reaction as the relative activity increased by 20%. Nonetheless the optimal pH falls within the pH range of 3.5–4. Regarding the temperature, the optimal values were obtained at 65ºC for both the free and immobilized enzyme. Similarly, the immobilized enzyme also seems to show increased tolerance to heat, yet the remaining experiments were carried at 50ºC due to the significant restrictions reported regarding the mechanical strength of the PVA beads and lens-shaped capsules at temperatures equal or higher than 55ºC [15].

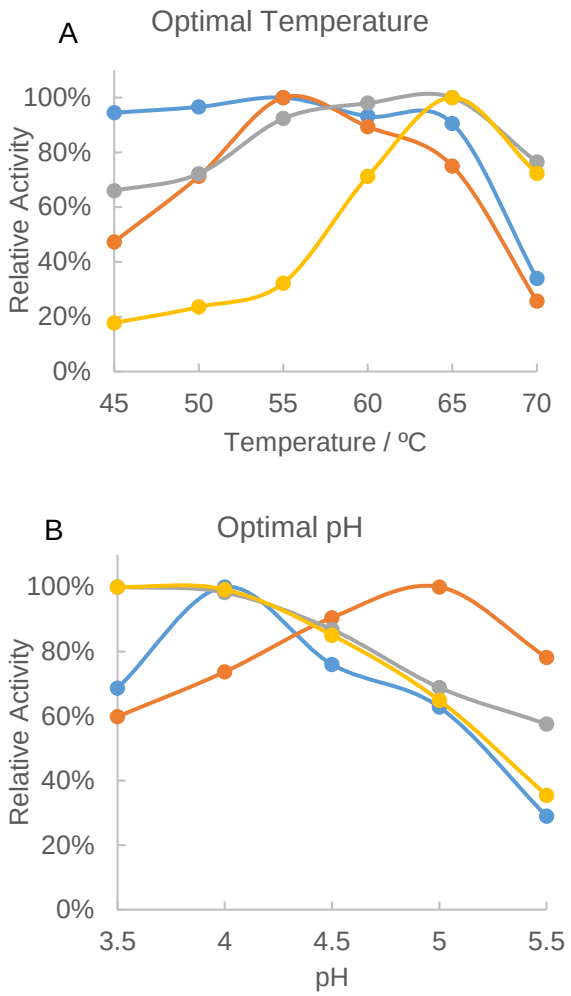

Figure 1 – Effect of the temperature (A) and pH (B) on *Dahlia* tubers inulin hydrolysis with Amberlite IRC-86 immobilized (—●—) and free enzyme (—●—) and on chicory root inulin hydrolysis with PVA immobilized (—●—) and free (—●—) enzyme.

3.2. Time courses of product formation of the free and immobilized enzyme

The effect of magnetic agitation in the different reaction volumes (1, 3 and 10 mL) was studied and the reaction rates of the Amberlite IRC-86 and PVA immobilized enzyme were compared to those of the free enzyme (Table 1).

For the free enzyme, in both agitated and non-agitated 1 mL reactions, no clear advantages in using, or not, agitation are noticed as the reaction rates are similar. However, at 3 and 10 mL, it is the non-agitated reaction that has the higher hydrolysis rate.

Regarding the enzyme immobilized in Amberlite IRC-86, the importance that agitation plays as the reactional volume increases is well evident as the linear evolution of product formation over the course of the reaction is gradually lost (data not shown). This is reflected in Table 1, as the more random and disperse measurements at 3 and 10 mL without agitation, suggestive of increasing heterogeneity and poor mixing, did not show any linear correlation. Interestingly, just like for the free enzyme, no differences in the hydrolysis rate at 1 mL with and without agitation were seen.

Table 1 – Inulin hydrolysis rates ($g.L^{-1}.min^{-1}$) at 1, 3 and 10 mL, with and without agitation (440 rpm) for the different immobilization methods tested and the free enzyme. In all cases the R^2 was higher than 0.94, except for Amberlite IRC-86 without agitation at 3 and 10 mL were no linear correlation was observed.

	1 mL		3 mL		10 mL	
	With agitation	**Without agitation**	**With agitation**	**Without agitation**	**With agitation**	**Without agitation**
Free	0.786	0.787	0.926	0.999	0.934	0.954
Amberlite IRC-86	0.149	0.149	0.158	–	0.242	–
PVA	0.229	0.206	0.230	0.236	0.197	0.221

These results seem to indicate need for convective flow in solid-liquid systems even for relatively low reaction volumes, unlike what is observed for homogeneous systems.

When the enzyme was immobilized in PVA, the overall diffusional effects related with the presence or absence of agitation were not as evident. Considering the 1 mL reactional volume, the presence of agitation only seemed to start being relevant after 30 minutes (data not shown). Higher hydrolysis rates were observed for the agitated reaction (Table 1). These results show to be in contrast with the observed with the free and Amberlite IRC-86 immobilized enzyme, where the intrinsic diffusional mechanisms in the non-agitated reactions seemed to be enough to keep up with the agitated reaction. At the 10 mL scale, just like the observed for the free enzyme the hydrolysis rates show slightly higher values for the non-agitated reaction. For the 3 mL reactional volume, the difference in the reaction rates with and without agitation is virtually negligible.

3.3. Microtiter plates assays

The influence of parameters such as the filling volume, the well geometry and dimensions, the agitation frequency and the orbital diameter of the agitator on the reaction's initial velocity were also tested (Figure 2). It was possible to observe that the systems showed very different behaviors accordingly to the immobilization support used.

No straightforward correlation regarding the agitation rate or the shaking diameter across the different plates could be made, however it was evident that under in-phase conditions increases in the shaking frequency at high shaking diameters slowed the reaction with PVA and had different effects with Amberlite IRC-86, depending on the plate's geometry. As for the remaining parameters, it seems that the enzyme immobilized in Amberlite IRC-86 benefited from the change from square to round wells whereas with PVA did not. The influence of the well dimensions was more significant for the covalently-bonded than for the entrapped enzyme.

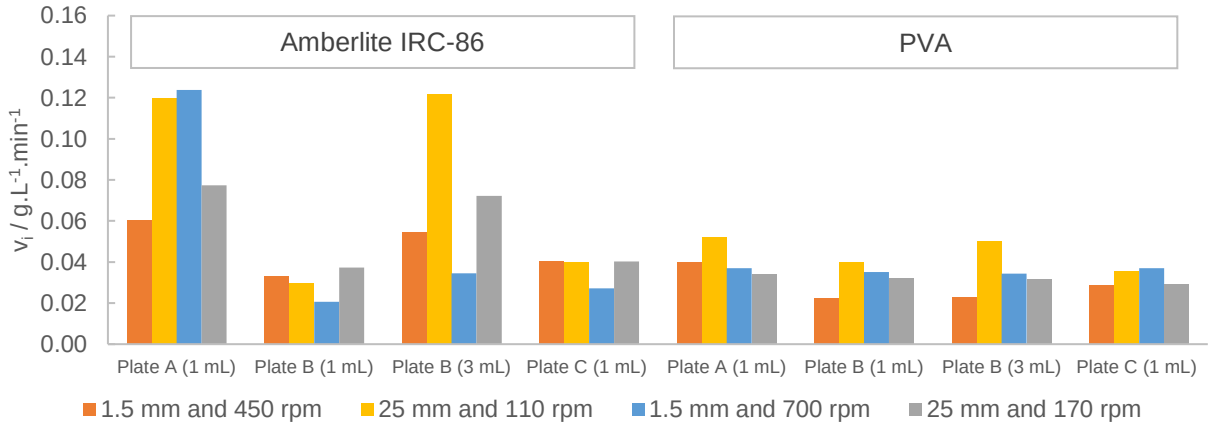

Figure 2 – Comparative studies of the reaction rate of inulin hydrolysis with both immobilized enzymes under various agitation speeds (110, 170, 450 and 700 rpm) and shaking diameters (1.5 and 25 mm). Different configuration MTP were used: Plate A – 96-square-shaped well, round-bottom; Plate B – 24-square-shaped well, pyramidal-bottom; Plate C – 24-round-shaped well, flat-bottom.

The fact that the same filling volume went from a smaller to a larger well, decreasing the height of the liquid column was expected to benefit the reaction, which did not occur.

Overall the Amberlite IRC-86 showed to be the most susceptible system to the alterations imposed while with PVA the alterations observed were always more discrete and at lower values, which might suggest that the reaction is slower due to intrinsic restrictions of the immobilization method itself.

3.4. Enzyme activity

In order to determine the kinetic parameters of the reaction (K_M, K_M^{app}, V_{max} and V_{max}^{app} and assess the immobilization effects on the kinetics of the enzyme, it was assumed that the enzyme followed a typical Michaelis-Menten kinetics.

The data obtained (Table 2 and Table 3) shows higher K_M^{app} values for both immobilized enzyme systems than the K_M values for the free enzyme, meaning that the reactions became less efficient upon immobilization. Such result is not unexpected as the immobilization process can affect the enzyme's catalytic activity, or even its affinity towards the substrate.

Regarding the PVA immobilized and free enzyme with chicory root inulin (Table 3), there is an increase of the K_M and K_M^{app} with the volume, and within a determined volume, it also tends to increase if the agitation is lost.

Table 2 – Summary of the kinetic parameters (V_{max} and K_M) for the free and Amberlite IRC-86 immobilized inulinase, with and without agitation in reactional volumes of 1, 3 and 10 mL.

FREE	**1 mL**		**3 mL**		**10 mL**	
	Agitated	**Non-agitated**	**Agitated**	**Non-agitated**	**Agitated**	**Non-agitated**
V_{max} / g.L^{-1}.min^{-1}	2.98	2.94	2.50	2.70	2.50	2.60
K_M / g.L^{-1}	80.62	70.89	63.31	74.53	60.00	70.00
AMBERLITE	**1 mL**		**3 mL**		**10 mL**	
	Agitated	**Non-agitated**	**Agitated**	**Non-agitated**	**Agitated**	**Non-agitated**
V_{max}^{app} / g.L^{-1}.min^{-1}	0.39	-	0.40	-	0.29	-
K_M^{app} / g.L^{-1}	127.80	-	135.90	-	125.60	-

Table 3 – Summary of the kinetic parameters (V_{max} and K_M) for the free and PVA immobilized inulinase, with and without agitation in reactional volumes of 1 and 10 mL. Values obtained through Hyper32® software.

FREE	**1 mL**		**10 mL**	
	Agitated	**Non-agitated**	**Agitated**	**Non-agitated**
V_{max} / g.L^{-1}.min^{-1}	0.19 ± 0.02	0.22 ± 0.03	0.23 ± 0.02	0.23 ± 0.02
K_M / g.L^{-1}	20.23 ± 7.80	22.98 ± 9.99	26.33 ± 6.34	28.23 ± 9.21
PVA	**1 mL**		**10 mL**	
	Agitated	**Non-agitated**	**Agitated**	**Non-agitated**
V_{max}^{app} / g.L^{-1}.min^{-1}	0.92 ± 0.11	0.55 ± 0.04	0.37 ± 0.02	0.42 ± 0.06
K_M^{app} / g.L^{-1}	26.95 ± 9.88	25.42 ± 5.63	39.95 ± 5.90	41.17 ± 14.17

These results suggest that the efficiency of the reaction tends to decrease with bigger non-agitated reactional volumes. As for the Amberlite IRC-86 immobilized and free enzyme with *Dahlia tubers* inulin (Table 2), the results are not that straightforward, but when considering only the 1 mL and 10 mL systems, the Michaelis constant tends to decrease when the volume is increased. As for the agitation, there is not any trending behavior that can be used to formulate conclusions.

In Table 2 it is possible to observe that V_{max} decreases upon immobilization in Amberlite IRC-86, which is also not surprising as after the immobilization procedures it is usual for the enzyme to lose some activity and thus to decrease the hydrolysis rate. On a different perspective, it is possible to infer that the V_{max} also tends to decrease as the reactional volume increases. However, when considering the same reactional volumes, when the agitation is lost the V_{max} tends to increase, which might suggest that the presence of agitation might not be that beneficial. In the case in which the substrate was inulin from chicory root, the results show that V_{max} actually increases (up from 2-fold to 5-fold for the 1 mL agitated system) upon enzyme immobilization in PVA**.** An increase in the reactional volume translates in a decrease of the V_{max} parameter, however the overall results show that the only reaction that seems to be truly affected and slowed down by the loss of agitation is with the immobilized enzyme at 1 mL.

4. Conclusion

Enzyme characterization was done for both immobilized forms. For the immobilization in Amberlite IRC-86 and PVA no major differences regarding the optimal temperature, 55ºC and 65ºC, respectively, were seen. Regarding the pH, enzyme entrapment led to practically no changes in the optimal pH range of 3.5–5, observed for both free and immobilized forms. As for Amberlite IRC-86, a shift from pH 5 to 4 was observed upon immobilization. Overall, immobilization proved to be a solid method of increasing enzyme's tolerance to heat and lower pH, a combination of special relevance in the industrial sectors to which this reaction can be applied.

The reaction with Amberlite IRC-86 was the one who struggled the most with the increasing volumes under non-agitated conditions. Still, whereas for Amberlite IRC-86 the need for agitation was only noticed for volumes higher than 3 mL, for the enzyme immobilized in PVA such occurred at 1 mL.

MTP were used to find the best conditions for inulin hydrolysis. The results varied greatly with the type of immobilized enzyme used. No correlation regarding the agitation rate or the shaking diameter across the different plates could be made. Round-wells showed to be the most benefic for Amberlite IRC-86 and squared-shaped for PVA. The increase in the well's dimensions decreased the reaction rate with Amberlite IRC-86, whereas when the filling volume was triplicated, the reaction rate almost doubled. With PVA no significant alterations were seen upon increasing well dimensions or filling volume.

The kinetic parameters showed an overall decrease in the enzyme efficiency ($K_M < K_M^{app}$) upon immobilization for both supports. With PVA a further decrease in the reaction efficiency was registered for increasing non-agitated reactional

volumes. Struggles to formulate conclusions on the behavior of Amberlite IRC-86 immobilized enzyme to volume increases and agitation loss arose. As for the V_{max}, the reaction slowed down upon immobilization in Amberlite IRC-86 and became faster with PVA.

The establishment of a set of optimal conditions for inulin hydrolysis in MTP with higher outputs than those of small vessels would be an interesting way to establish these miniaturized devices as crucial for bioprocess development.

5. References

1. Ricca, E., Calabrò, V., Curcio, S. & Iorio, G. The State of the Art in the Production of Fructose from Inulin Enzymatic Hydrolysis. *Crit. Rev. Biotechnol.* **27,** 129–145 (2007).
2. Fleming, S. E. & GrootWassink, J. W. Preparation of high-fructose syrup from the tubers of the Jerusalem artichoke (Helianthus tuberosus L.). *CRC Crit. Rev. Food Sci. Nutr.* **12,** 1–28 (1979).
3. Zittan, L. Enzymatic Hydrolysis of Inulin - An alternative way to fructose production. *Starch* **33,** 373–377 (1981).
4. Gill, P. K., Manhas, R. K. & Singh, P. Hydrolysis of inulin by immobilized thermostable extracellular exoinulinase from Aspergillus fumigatus. *J. Food Eng.* **76,** 369–375 (2006).
5. Bailey, J. E. & Ollis, D. F. *Biochemical Engineering Fundamentals*. (McGraw-Hill Book Co., 1986).
6. Nunes, M. A. P., Fernandes, P. C. B. & Ribeiro, M. H. L. Microtiter plates versus stirred mini-bioreactors in biocatalysis: A scalable approach. *Bioresour. Technol.* **136,** 30–40 (2013).
7. Walther, C. *et al.* Prediction of inclusion body solubilization from shaken to stirred reactors. *Biotechnol. Bioeng.* **111,** 84–94 (2014).
8. Klöckner, W. & Büchs, J. Advances in shaking technologies. *Trends Biotechnol.* **30,** 307–314 (2012).
9. Obón, J. M., Castellar, M. R., Iborra, J. L. & Manjón, A. β-Galactosidase immobilization for milk lactose hydrolysis: a simple experimental and modelling study of batch and continuous reactors. *Biochem. Educ.* **28,** 164–168 (2000).
10. Rocha, J. R., Catana, R., Ferreira, B. S., Cabral, J. M. S. & Fernandes, P. Design and characterisation of an enzyme system for inulin hydrolysis. *Food Chem.* **95,** 77–82 (2006).
11. Fernandes, P., Marques, M. P. C., Carvalho, F. & Cabral, J. M. S. A simple method for biocatalyst immobilization using PVA-based hydrogel particles. *J. Chem. Technol. Biotechnol.* **84,** 561–564 (2009).
12. Doig, S. D., Pickering, S. C. R., Lye, G. J. & Woodley, J. M. The use of microscale processing technologies for quantification of biocatalytic Baeyer-Villiger oxidation kinetics. *Biotechnol. Bioeng.* **80,** 42–49 (2002).
13. Miller, G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31,** 426–428 (1959).
14. Gupta, A. K., Kaur, M., Kaur, N. & Singh, R. P. A comparison of properties of inulinases of Fusarium oxysporum immobilized on various supports. *J. Chem. Technol. Biotechnol.* 293–296 (1992).
15. Cattorini, S. *et al.* Lentikat®-based biocatalysts: effective tools for inulin hydrolysis. *Chem. Biochem. Eng. Q.* **23,** 429–434 (2009).