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Short Communication

Optimization of fructose-rich syrups production from *Opuntia ficus-indica* inulin using immobilized inulinase on *Luffa cylindrical*

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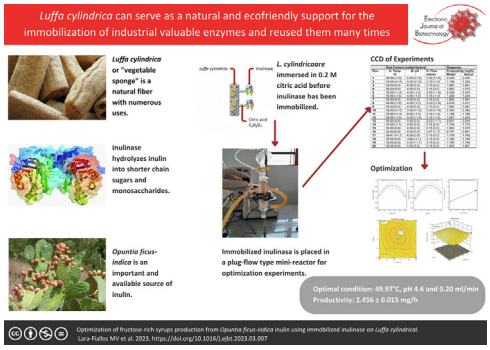
GRAPHICAL ABSTRACT



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ABSTRACT

Background: *Luffa cylindrica* has numerous domestic and industrial uses. For example, this natural fiber supports immobilization through the covalent bonding of an inulinase. In addition, fructose syrup from crude inulin obtained from prickly pear (*Opuntia ficus-indica*) can be obtained in a plug-flow mini reactor with this immobilized inulinase on *L. cylindrica*.
Results: A central composite design of experiments was used to maximize the enzymatic fructose production from crude inulin obtained from *Opuntia ficus-indica* in a plug-flow mini reactor. The experiments explore temperature (between 45 and 55°C), pH (4.0–5.0), and feed flow (0.1–0.2 ml/min). After verifying the adequacy of the quadratic model for productivity, it was maximized to find the optimal condition. It was at 49.97°C, 4.6 and 0.20 ml/min for the temperature, pH, and flow, respectively. Under the optimal condition, the quadratic model suggested a productivity of 2.456 ± 0.015 mg/h. Three validation experiments confirmed the validity of the model.
Conclusions: The results confirmed the suitability of *L. cylindrica* as support for the immobilization of inulinase.

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Production
Response surface methodology

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1. Introduction

Enzymes are catalysts of biological origin essential for life as we know it [1]. Most cellular metabolic processes involve enzymes. By 2015, around 3000 enzymes had been described, of which only 5% had an industrial application (only ∼170 enzymes) [2]. These values have been growing, estimating, in recent years, an appreciable expansion of the global market for industrial enzymes. By 2017, for example, the global value of the enzyme market was US$7.1 billion and is forecast to reach US$10.5 billion by 2024 [3].

The future bioeconomy foresees the general and widespread use of enzymes to partially hydrolyse the enormous volumes of plant biomass, which are made up of large chains of lignin hemicellulose and cellulose in fragments derived from these, easier to use and transform [4,5,6]. The enzymes can also perform more complex transformations to synthesise other valuable products for the chemical, pharmaceutical and cosmetic industries [7]. The future biorefineries will undoubtedly rest on the broader and more widespread use of these unique, organic molecules, called enzymes [6].

However, even today, many of the processes for obtaining bulk industrial enzymes with the required purity have prohibitively high costs, and this significantly limits their industrial use [8].

One of the ways that could constitute a solution to this problem is through the immobilisation of the enzymes, on or inside supports, which would allow the multiple reuses of the enzyme lowering its production cost [9]. To this end, numerous supports and procedures have been used, ranging from those that trap the enzyme [10], allowing it to preserve its original folding within the support, to others that fix, through covalent bonding, a part of the enzyme, usually far of their active site[11], with some specific and predetermined location of the support.

The support that is chosen, on the other hand, must have among its relevant characteristics their chemical stability, that it does not produce extractable substances that are incorporated into the reaction stream and even less that they intervene in the chemical transformations that take place [12]. In addition, it must be cheap and easily accessible. Numerous supports have been successfully used to immobilise or trap enzymes of industrial interest [1,13,14]. Many are synthetic supports, and some have been designed for this specific function [1].

Some supports of natural origin have also been used, among which are those from natural fibres, such as cotton, jute, flax, and luffa. The latter is from the *Luffa cylindrica* (commonly known as "vegetable sponge", "loofah sponge", or simply "loofah"), frequently used in household cleaning tasks and as a sponge for personal hygiene in bathrooms [15].

L. cylindrica has also been used as a percolating agent in the filtration of fuel spills [16] and other accidents [17].

Finally, *L. cylindrica* as support in immobilization has also been reported [18]. In this report, the performance of natural fibre from *L. cylindrica* as a support to immobilize an extracellular inulinase obtained from an *Aspergillus niger* NRRL3 strain for the fructose obtention was studied [18]. In addition, the production process using free and immobilized inulinase on *L. cylindrica* was compared [18].

In the present work, we will use the fibres of *Luffa cylindrica* to covalently immobilise an enzyme of industrial interest (inulinase) that allows its use during several operation cycles for fructose production. A central-composite design of experiments was performed to find the optimal condition of the process in a mini plug-flow enzymatic glass reactor using either commercial inulin or bulk-raw inulin (obtained from *Opuntia ficus-indica*) as raw material to produce fructose.

2. Experimental

2.1. Raw-bulk inulin extraction from Opuntia ficus-indica (white prickly pear)

The process begins with washing the *Opuntia ficus-indica* (white prickly pear), removing its spines with a steel brush, and peeling to separate the shell from the pulp. Next, the pulp of *O. ficus-indica* is chopped and weighed. Subsequently, water is added in a 4 ml H_2O/g prickly pear ratio to subject the mixture to a leaching process under mechanical agitation at 85°C for 45 min at 200 rpm in a thermostatic water bath. Next, the first filtration is carried out onto filtration paper (MN 615, Ø125 mm) to separate the biomass, leaving a broth with the presence of inulin [19].

Further, the broth is subjected to a carbonation process by adding $Ca(OH)_2$ up to 0.1 M, increasing the pH to 10.2. The carbonated mixture is allowed to stand for 15 min and neutralised with a 0.05 N HCl solution until it reaches pH 8.0. The latter causes the precipitation of impurities, which are removed through a second filtration.

The concentration of crude inulin from white prickly pear (*Opuntia ficus-indica*) was measured spectroscopically at 715 nm against a reference curve with inulin from chicory as a standard (Sigma-Aldrich I2255). Values of concentration of 25.3 ± 0.9 mg/ml (n = 6) with a purest above 90% were obtained [19].

2.2. Activation of the Luffa cylindrica for its use as an immobilisation support and the exo-inulinase immobilisation

Luffa cylindrica was cut into segments of approximately 20 cm in length to sterilise them at 121°C for 35 min. Subsequently, the *L. cylindrica* was cut again into smaller fragments 1 cm wide and 10 cm long before immersing the small pieces in a 0.2 M solution of citric acid at a ratio of 1:50 (m/v), shaking for 30 min, as reported elsewhere [20]. Next, it was drained and placed in an oven at 100°C for 60 min.

2.3. Preparation of the enzyme glass reactor and response surface methodology experiments

Subsequently, the activated *L. cylindrica* is submerged in 50 ml of an aqueous solution containing 25 I.U./mg of inulinase (Novozym® 960, Inulase, EC. 3.2.1.7, Sigma-Aldrich I6285) and left to stand for 30 min at room temperature before being placed inside the enzyme glass cylindrical mini reactor (Ø1.4 × 10.6, cm). Finally, the content of the mini reactor is completely drained by gravity, and then it is washed with three reactor volumes with distilled water at a flow rate of 0.2 ml/min.

The central composite design (CCD) of response surface methodology employed temperature (between 45 and 55°C), pH (4.0–5.0) and flow (0.1–0.2 ml/min) as controlled independent factors whilst absorbance was measured for each of the treatments,

leading to obtaining the values of concentration of fructose for each flow. Total productivity (in mg/h) was calculated by multiplying the concentration and flow of feeding to the glass – enzyme mini-reactor immobilized with inulinase.

After checking the suitability of the experimental quadratic model obtained, the total productivity was optimized to obtain, inside the design space of controlled factors the combination of these factors that maximises the total productivity.

2.4. Determination of fructose productivity

Once ten reactor volumes were fed to the enzymatic reactor, the steady state was assumed to be reached, and the hydrolysis reaction was stopped by adding the DNS (dinitrosalicylic acid) reagent [21]. After homogenizing the mixture, the absorbance was read in the UV–Vis spectrophotometer at 540 nm. Subsequently, it was calculated from a previously constructed calibration curve with fructose (C(mM) = 0.1716 + 1.4697·Abs@540, r^2 = 0.9954; for an absorbance, measured at 540 nm, and valid in a range of absorbance between 0.100 and 1.300), the concentration of fructose (mM) in the flow stream.

Finally, the total productivity of fructose (mg/h) was calculated multiplying the concentration of fructose obtained (in mg/ml) by the flow of the enzymatic reactor (in ml/min). So, the total productivity (in mg/h) remains as the unique response in this study.

3. Results and discussion

The factors under study and the responses obtained in the central composite design in the response surface methodology used are shown in Table 1.

The second-order models obtained for the concentration and productivity of fructose obtained by the enzymatic action of immobilized inulinase are [Equation 1]:

$$\begin{aligned}\text{Productivity} = {} & 1.86 - 0.003 \cdot A + 0.0143 \cdot B + 0.6065 \cdot C \\ & - 0.0034 \cdot AB + 0.0049 \cdot AC - 0.0031 \cdot BC \\ & - 0.0338 \cdot A^2 - 0.0338 \cdot B^2 - 0.0152 \cdot C^2 \\ & - 0.0104 \cdot ABC - 0.0206 \cdot A^2B - 0.0049 \cdot AB^2 \\ & + 0.0341 \cdot A^2B^2 \end{aligned} \quad (1)$$

All terms of Equation 1 are statistically significant (p value < 0.05), except those that were included to maintain the hierarchy of the chosen model. The values of the response variables obtained, as well as their corresponding real value, show an adequate correspondence with the experimental values measured, the maximum relative error of these being <0.6%, for total productivity (Table 1).

Total productivity model was statistically significant (p value < 0.0001) and, therefore, is suitable to explore, within the experimental space, possible maximum values for the total productivity.

The influence on the productivity of each of the three factors separately (A: temperature, B: pH, and C: flow) (Fig. 1a), as well as the contour and 3D graphs (Fig. 1b), show a maximum value of the productivity in the surroundings at the values of 49.97°C, 4.6 and 0.20 ml/min, for the temperature, pH, and flow, respectively. Under these conditions, the productivity of the model predicts a value of 2.456 ± 0.015 mg/h.

Three confirmation experiments were performed under the conditions of the optimal solution (at ∼50.0°C, pH 4.6, and 0.20 ml/min), showing values within the ranges predicted by the model (results not shown).

In a previous report [18], to achieve the covalent binding with the inulinase, *L. cylindrica* was first activated before immobilization with a 2.5% (v/v) glutaraldehyde solution for 2 h, according to the procedure reported elsewhere [22]. As a result, the immobilization yield reached 81% [18], whilst almost similar yield of 84% was reached here.

In such study an optimum temperature and pH of 55°C and pH 5.2, was obtained [18]. These values differ from those obtained here (50°C and pH 4.6), which could be due, among other things, to the origin of the enzyme used and to the activation procedure of *L. cylindrica* before immobilization.

Another study carried out with extracts of two Uzbek varieties of Jerusalem artichoke (*Helianthus tuberosus*), whose inulin contents were ∼12%, and performed in 500 ml T-flasks, a natural extract of *Aspergillus oryzae* was added. The presence of the enzymes secreted by the fungus, such as inulinase and extracellular proteases, transforms inulin into fructose. The experiment was carried out for 120 h, and the fructose contents and the enzymatic activity of inulinase and proteases in the medium were measured

Table 1
Results of the CCD of experiments. The independent variables (A: Temperature, B: pH, and C: Flow) and the real responses and the values obtained by the model of the dependent variables.

Run	Real Factors (coded factors)			Response	
	A: Temp	B: pH	C: Flow	Productivity (mg/h)	
	°C	–	ml/min	Model	Actual
1	45.00 (−1.0)	5.00 (+1.0)	0.20 (+1.0)	2.425	2.430
2	55.00 (+1.0)	5.00 (+1.0)	0.10 (−1.0)	1.196	1.202
3	50.00 (0.0)	4.50 (0.0)	0.15 (0.0)	1.860	1.861
4	50.00 (0.0)	4.50 (0.0)	0.15 (0.0)	1.860	1.870
5	55.00 (+1.0)	4.00 (−1.0)	0.20 (+1.0)	2.438	2.443
6	45.00 (−1.0)	4.00 (−1.0)	0.10 (−1.0)	1.228	1.234
7	50.00 (0.0)	4.50 (0.0)	0.15 (0.0)	1.860	1.861
8	45.00 (−1.0)	4.00 (−1.0)	0.20 (+1.0)	2.416	2.417
9	50.00 (0.0)	4.50 (0.0)	0.15 (0.0)	1.860	1.861
10	55.00 (+1.0)	5.00 (+1.0)	0.20 (+1.0)	2.392	2.392
11	55.00 (+1.0)	4.00 (−1.0)	0.10 (−1.0)	1.188	1.190
12	45.00 (−1.0)	5.00 (+1.0)	0.10 (−1.0)	1.207	1.209
13	50.00 (0.0)	4.50 (0.0)	0.23 (+1.7)	2.837	2.844
14	41.59 (−1.7)	4.50 (0.0)	0.15 (0.0)	1.769	1.775
15	50.00 (0.0)	4.50 (0.0)	0.15 (0.0)	1.860	1.870
16	50.00 (0.0)	4.50 (0.0)	0.07 (−1.7)	0.797	0.801
17	58.41 (+1.7)	4.50 (0.0)	0.15 (0.0)	1.759	1.765
18	50.00 (0.0)	3.66 (−1.7)	0.15 (0.0)	1.740	1.746
19	50.00 (0.0)	5.34 (+1.7)	0.15 (0.0)	1.788	1.794
20	50.00 (0.0)	4.50 (0.0)	0.15 (0.0)	1.860	1.861

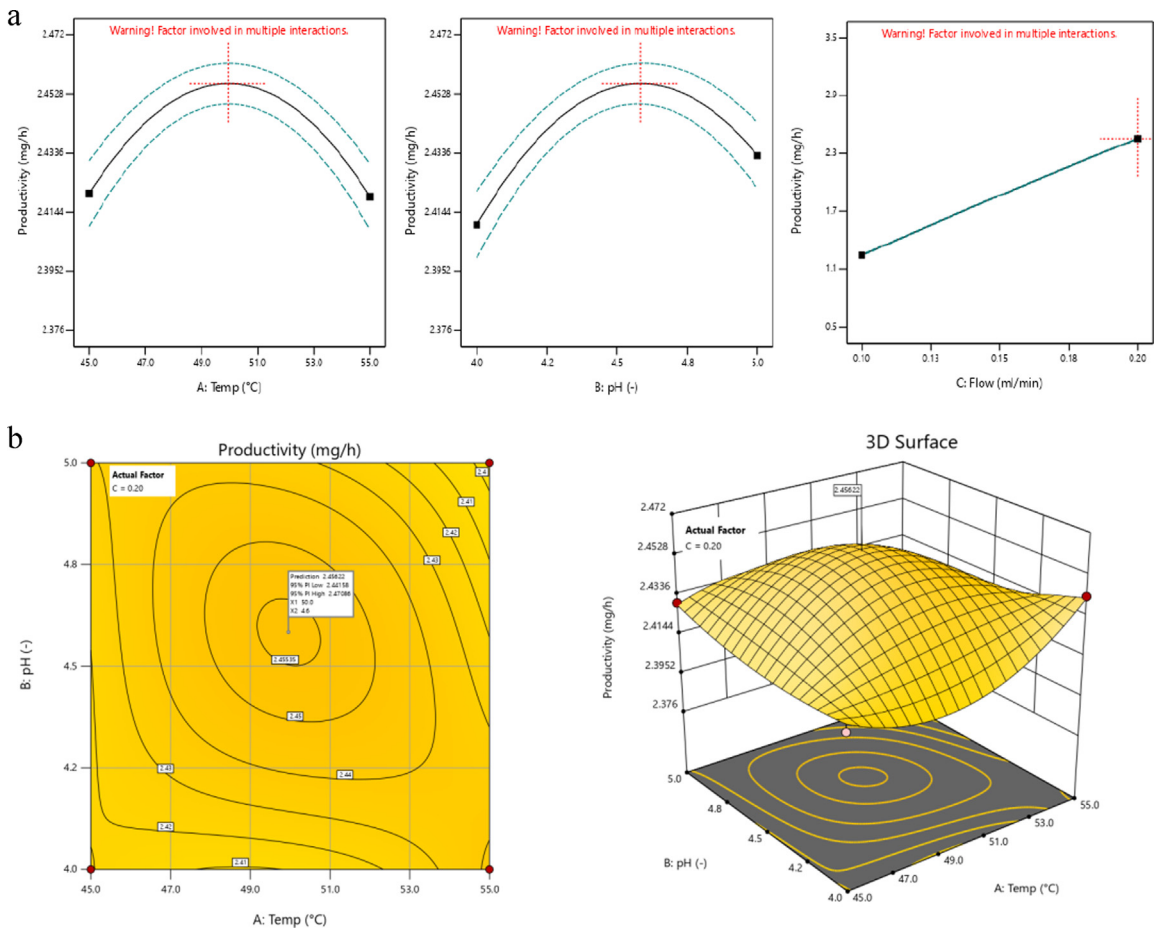

Fig. 1. Representation of one of the solutions found for numerical optimization and their relationship with the independent variables. (a) Influence on the productivity of each one of the factors separately. (b) Contour and 3D plots of productivity, as a function of temperature and pH, for a flow rate of 0.2 ml/min.

every 24 h. A maximum fructose concentration of 24 mg/ml was reached at 48 h, coinciding with the peak of inulinase activity. After that, fructose concentration decreased, presumably due to the decrease in inulinase activity due to the action of proteases, whose enzymatic activity values grew all the time [23].

In summary, *Luffa cylindrica* could produce fructose syrup from a crude inulin stream by an endo-inulinase covalent-immobilized on this natural fiber in a plug-flow enzymatic reactor. Moreover, CCD proves to be a helpful tool for undertaking optimization studies, as has been corroborated in this work. However, later studies must include higher flow values because, in this experimental assembly, the best value for the overall productivity could be higher.

Author contributions

- Study conception and design: MV Lara-Fiallos; E González-Suárez.
- Data collection: J Núñez-Pérez; DT Montalvo-Villacreses.
- Analysis and interpretation of results: MV Lara-Fiallos; RC Espín-Valladares; A Pérez-Martínez; E González-Suárez; JM Pais-Chanfrau.
- Draft manuscript preparation: MV Lara-Fiallos; JM Pais-Chanfrau.
- Revision of the results and approved the final version of the manuscript: MV Lara-Fiallos; DT Montalvo-Villacreses; RC Espín-Valladares; J Núñez-Pérez; A Pérez-Martínez; E González-Suárez; JM Pais-Chanfrau.

Conflict of interest

None of the authors have any financial or personal relationship that could inappropriately influence or bias the content of the research paper.

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