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Statistical Improvement of Batch Culture with Immobilized *Pichia pastoris* Cells for rPOXA 1B Laccase Production

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Abstract: Immobilized *Pichia pastoris* X33/pGAPZαA-*LaccPost*-Stop in Ca^{2+} alginate beads was employed for *Pleurotus ostreatus* rPOXA 1B laccase batch production. Sequential statistical improvement was achieved through Plackett-Burman (PBED), (PBED-T_{11}, 29.5±0.8 UL^{-1}), which allowed to increase activity by 2.36-fold (12.5±2.6 UL^{-1}) obtained in a preliminary study. Following, Box-Behnken Experimental Design (BBED) was implemented and obtained enzymatic activity in PBED-T_{11} was further increased by 33.5-fold (BBED-T_{12} 989.31±187.45 UL^{-1}). After BBED-T_{12} extrapolation to column, cell release remained high. To demonstrate laccase was not acting on Ca^{2+} alginate polymer, it was shown that both untransformed *P. pastoris* and *S. cervisiae* were able to be released from the alginate matrix and proliferate. Molecular docking evaluating interaction between rPOXA 1B and Ca^{2+} alginate, exhibited weak interactions between the active center and Ca^{2+} alginate polymer. Moreover, the active center conformation was not appropriate for ligand transformation. Immobilization conditions decreasing cell release (17.01±0.12 gL^{-1}) allowed for high enzymatic activity (1,453.93±0.43 UL^{-1}) with greater specific activity (18.33 Umg^{-1}). These conditions were: 4% Na^{2+} alginate (w/v) and 0.3 M $CaCl_2$, suggesting that Na^{2+} alginate and $CaCl_2$ concentrations can control cell release from this matrix.

Keywords: Immobilized Cell, Plackett-Burman, Box-Behnken, *Pleurotus ostreatus*, *Pichia pastoris*, Recombinant Laccase

Introduction

Laccases (E.C. 1.10.3.2, *p*-diphenol oxidase) are multi-copper oxidases capable of catalyzing oxidation of various aromatic compounds, particularly phenols. The reaction entails one electron abstraction with concomitant reduction of molecular oxygen to water in a four-electron transfer process. These enzymes, typically contain four copper atoms bound to three redox sites, where copper atoms act as co-factors. Laccases are present in plants, insects, bacteria and fungi (El-Batal *et al*., 2015; Niladevi *et al*., 2009; Rivera-Hoyos *et al*., 2013).

Laccases produced by fungi of the Basidiomycota *phylum* present a high redox potential in comparison with laccases from plants or bacteria. Therefore, fungal laccases have generated a great interest in biotechnological applications for industries such as,

textile, paper fabrication, food and pharmaceutical among others (El-Batal *et al*., 2015). Laccase has become an object for investigation, given it´s great stability and wide ranges of suitable substrates, which influences its ability to lignin degradation, toxic phenol extraction, synthetic dye degradation of azo-, indigo, triphenylmethane and anthraquinone types, cellulose pulp bleaching and pulping black liquor detoxification, juice and wine clarification, organic synthesis processes, residual water treatment and treatment of Polycyclic Aromatic Hydrocarbon (PAHs) contamination, among many others (Balan *et al*., 2012; Dwivedi *et al*., 2011; Gianfreda *et al*., 1999; Hautphenne *et al*., 2016; Morales-Álvarez *et al*., 2016; 2017; 2018; Popa and Cornea, 2015; Rivera-Hoyos *et al*., 2018). Laccases are useful, to develop different useful alternatives in industrial and bioremediation processes, without affecting the environment (Rivera-Hoyos *et al*., 2013).

Pleurotus ostreatus is a fungus of the Basidiomycota *phylum*. It has the capacity to synthesize ligninolytic enzymes, such as laccases and manganese peroxidase E.C. 1.11.1.13 (Hou *et al*., 2004). Laccase production from its native source has been reported to be scarce, thus isolation is limited for commercial purposes and non-profitable. Given these limitations, different strategies have been devised for laccase production. Examples include heterologous expression in yeast hosts, capable of high enzyme concentrations and activities. Additionally, production costs are low with high purity and quality (Rivera-Hoyos *et al*., 2013).

Pichia pastoris is an established protein expression host, employed for recombinant protein production. This yeast is easily genetically manipulated for intra- or extracellular protein production. In addition, it can yield high levels of protein with post-translational modifications (Cereghino *et al*., 2002; Gamboa and Trujillo-Roldán, 2009; Landázuri *et al*., 2009). Therefore, it has become a promising alternative, diminishing production costs and improving productivity (Kunamneni *et al*., 2008a; 2008b).

Likewise, laccase production has been carried-out using immobilized cells in polymeric supports, such as sodium alginate; which reduces production costs (Ting *et al*., 2013). Immobilized cell system is an attractive option for reusing yeast-alginate-beads and simply adding fresh media, eliminating the inoculum stage. Additionally, laccase production in reactor with immobilized cells could exceed some limitations present in the production itself. Moreover, immobilized cells can be easily separated from the reaction products (Fernández-Fernández *et al*., 2013).

The objective of this work was to statistically improve culture media, alginate concentration and Calcium Chloride ($CaCl_2$) for immobilization of a recombinant strain of *P. pastoris and* improve *P. ostreatus* rPOXA 1B laccase production using a batch culture of immobilized cells. Forthcoming application of this work is to implement columns for effluent treatment, where contaminated water would flow through alginate bead immobilized cells. In this sense calcium alginate beads, with entrapped microorganism would have an “adjuvant like effect”, since they would be releasing (for a long time) laccase producing cells, potentiating the desired effect. Before effluent is flowed through the column, retention time and contaminant load must first be determined.

Materials and Methods

Strains

We employed previously optimized *P. pastoris* X33/pGAPZαA-*LaccPost-Stop* (Clone 1) strain containing *POXA* 1B synthetic gene, coding for *P. ostreatus* POXA 1B laccase, previously maintained in a Master Cell Bank (MCB), (Poutou *et al*., 1994) in YPD-Z [2% peptone (w/v), 1% yeast extract (w/v), 2% D+glucose (w/v) supplemented with 20% (w/v) glycerol and 100 μg mL^{-1} Zeocin^{TM}] at -80°C (Rivera-Hoyos *et al*., 2015).

Free Cell Production

Once MCB vials were thawed, glass tubes containing 5 mL sterile YPD-Z media were inoculated and incubated at 30°C Overnight (ON) with agitation. Result from ON incubation was used to inoculate 30 mL YPD-Z in 100 mL Erlenmeyer flask. Flasks were incubated for 48 h. Last, 30 mL inoculate was cultured in 500 mL Erlenmeyer flask with 270 mL of YPD-Z and incubated for 60 h. This culture was used to produce cells for immobilization.

Calcium Alginate Immobilization

For strain immobilization, 1g of wet biomass was used per 34 mL dH_2O containing sodium alginate at 2.0, 2.5, 3.0 or 4.0% (w/v), depending on assay. To form alginate beads immobilization mixture was poured drop by drop into cold 0.1, 0.2 or 0.3 M $CaCl_2$ under constant agitation. Beads were kept in cold $CaCl_2$ (4°C) at the same molarity they were produced until used (Matiz *et al*., 2002). To eliminate yeast adhering to bead surface they were washed three times with 0.9% (w/v) NaCl (Bleve *et al*., 2008).

Statistical Improvement. Shake Flask Scale

Plackett-Burman Experimental Design (PBED)

The objective of this design was to determine the influence of media volume and culture media components on enzyme activity. Six factors were evaluated with two levels for each factor (Table 1).

Table 1: PBED factors and levels for laccase activity improvement

Code	Factor	Low level (-1)	Central point	High level (+1)
A	Culture media volume (mL)	150.00	225.00	300.00
B	$CuSO_4$ (mM)	0.10	0.55	1.00
C	Glucose (g L^{-1})	10.00	20.00	30.00
D	NH_4SO_4 (mM)	5.00	12.50	20.00
E	Peptone (g L^{-1})	10.00	15.00	20.00
F	Yeast Extract (g L^{-1})	5.00	7.50	10.00

To improve process efficiency under the same conditions all assays were performed in 500 mL Erlenmeyer flasks inoculated with one calcium alginate bead (2.5% w/v calcium alginate containing *P. pastoris* X33/pGAPZαA-*LaccPost*-Stop cells) per ml of culture media. Culture conditions were 30°C and 180 rpm for 168 h. Kinetic behavior follow-up of recombinant POXA 1B laccase immobilized cells was carried-out every 12 h until 168 h culture was completed. Assays were planed using Design Expert version 9.0 (2014) and fed with greatest enzyme activity observed.

The output for this design yielded 15 runs, composed by 12 treatments and one central point (assayed in triplicate) used to calculate the Standard Deviation (SD) for all assays. Cultures were assayed for bacteria contamination by Gram stain, total residual reducing sugar concentration (g L^{-1}) and total extracellular protein concentration (mg mL^{-1}). Response variables were enzyme activity (U L^{-1}), specific enzyme activity (U mg^{-1}) and productivity as a function of enzyme activity (U L^{-1} h^{-1}).

Box-Behnken Experimental Design (BBED)

For this design, results obtained from PBED were taken into account. The most significant factors and their percentage contribution on batch culture enzyme activity were considered. The design was performed using Design Expert V9.0 (2014). According to BBED the best treatment was repeated in triplicate, to obtain SD and calculate Coefficient of Variation (CV) among them. Assays were performed in 500 mL Erlenmeyer flasks at 30°C and 180 rpm for 168 h. Cultures were assayed for total residual reducing sugar concentration (g L^{-1}) and total extracellular protein concentration (mg mL^{-1}). Response variables were enzyme activity (U L^{-1}), specific enzyme activity (U mg^{-1}) and productivity as a function of enzyme activity (U L^{-1} h^{-1}).

Production of rPOXA IB Laccase with Immobilized Pichia Pastoris X33/pGAPZαA-LaccPost-Stop Cells. Column Scale

Improved BBED resulting media was used for column scale production. Three sodium alginate concentrations were evaluated and four columns assays were implemented (columns 0, 1, 2 and 3), general conditions were: 470 mL of media at 30°C and 0.0283 m^3 h^{-1} air supply, during 168 h. All columns were inoculated with 1 beads/mL. Samples were collected every 2 h during the first 12 h, followed by samples collected at 57, 96, 120, 144 and 168 h of culture. Differences among the columns were: column 0 (2.5% (w/v) sodium alginate for immobilization, dripped in 0.1 M $CaCl_2$), column 1 (4.0% (w/v) sodium alginate for immobilization, dripped in 0.1 M $CaCl_2$), column 2 (4.0% (w/v) sodium alginate for immobilization, dripped in 0.2 M $CaCl_2$), column 3 (4.0% (w/v) sodium alginate for immobilization, dripped in 0.3 M $CaCl_2$).

Cultures were monitored by OD_{600nm} readings to calculate *P. pastoris* X33/pGAPZαA-*LaccPost*-Stop dry biomass concentration (g L^{-1}) outside the alginate beads (Equation I), extracellular protein concentration (mg mL^{-1}), residual glucose concentration (g L^{-1}), enzyme activity (U L^{-1}), specific enzyme activity (U mg^{-1}) and productivity (U L^{-1} h^{-1}):

$$X_{\left(\frac{g}{L}\right)} = \frac{OD_{600nm}}{1.1726} \quad \text{(I)}$$

Computational Model for Calcium Alginate and Molecular Docking between Calcium Alginate and POXA 1B Lacccase

This assay was carried out to rule out laccase activity on calcium alginate degradation, resulting in cell release coming out of calcium alginate beads. Previously reported POXA 1B 3D laccase model was employed for this analysis (Rivera-Hoyos *et al*., 2015). Calcium alginate 3D structure (poly-α-L-calcium guluronate) was obtained from *http://www1.lsbu.ac.uk/water/hyalgh.html*; refined with Chimera software. Polar hydrogens were added to laccase receptor, followed by charge addition to these hydrogens, as well as to the ligand (alginate) using the Gasteiger method (Gasteiger *et al*., 2005), before AutoDock 4.2 (Morris *et al*., 2009) docking simulations were carried-out, to supply copper atom charges AutoDock suggested receptor coordinates modification. Grid was calculated locating it in the pocket where the bond takes place in this model, based on CASTp outcome (Dundas *et al*., 2006). Two different box dimensions were used for ABTS (403 and 503 points)

with a 0.375Å space. Additionally, molecular docking was performed without selecting a specific area to identify possible interactions sites between laccase and calcium alginate. Grid parameters and atomic affinity maps were calculated with AutoGrid 4. Each docking simulation was performed with a Lamarckian genetic algorithm with 2,500,000 energy evaluation with a population size of 150. Last, ligand molecular interactions with the model were determined and bond energies were registered as kcal mol^{-1}. Additionally, presence of a possible catalytic pocket for alginate lyase was identified (E.C. 4.2.2.3), selecting that originating from *Pseudomonas aeruginosa* periplasm, identified by 4OZV from PDB. Catalytic triad residues were located for this enzyme and for laccase for the primary-, as well as tertiary-structure using PyMol software for its comparison and analysis.

Analytical Techniques

Total Residual Reducing Sugar Concentration Determination

Total reducing sugar concentration was determined for each sample (in triplicates). To this end 3,5 dinitrosalicylic acid assay was used (Miller, 1959). A standard curve was prepared with D-glucose ranging from 0.1 to 2 gL^{-1} according to Equation II:

$$Subs_{\left(\frac{g}{L}\right)} = \frac{OD_{540nm} + 0.048}{0.6181} \quad \text{(II)}$$

Total Extracellular Protein Concentration Determination

Total extracellular protein concentration was determined by Biuret assay (Plummer, 1981) for each sample (in triplicates). A standard curve ranging from 0.5 to 5 mg mL^{-1} Bovine Serum Albumin (BSA) was prepared. Sample concentration was determining by using the following Equation III:

$$Prot_{\left(\frac{mg}{mL}\right)} = \frac{OD_{595nm} - 0.004}{0.0761} \quad \text{(III)}$$

Enzyme Assays

Laccase enzyme activity was monitored by 436 nm absorbance change (ξ_{436} = 29300 M^{-1} cm^{-1}) due to ABST oxidation in a 60 mM sodium acetate buffer (pH 4.5±0.2). 800 µL room temperature crude extract was added to 100 µL 600 mM sodium acetate buffer and 100 µL 5 mM ABTS. Green radical formation was evaluated spectrophotometrically during three minutes. A unit of activity is defined as the quantity of enzyme required for oxidation of 1 µmol ABST per minute. Blanc solution contained 800 µL distilled water, 100 µL 600 mM sodium acetate buffer solution and 100 µL 5 mM ABTS. Enzyme activity was expressed as U L^{-1} (Tinoco *et al*., 2001).

Specific activity was calculated by dividing enzymatic activity for each hour of culture by total extracellular protein concentration (Equation IV):

$$Spec.Act.\frac{Enz.Act.}{Prot.Conc.} \quad \text{(IV)}$$

Where:
$Act.Enz.$ = U L^{-1}
$Prot.Conc.$ = mg mL^{-1}

Productivity as a function of enzyme biological activity, expressed as U L^{-1} h^{-1} (Equation V) was calculated as follows:

$$P_{Enz.} = \frac{Enz.Act.}{Time} \quad \text{(V)}$$

Results

Pichia Pastoris X33/pGAPZαA-LaccPost-Stop Calcium Alginate Immobilization

After 60 h of culture *P. pastoris* X33/pGAPZαA-*LaccPost*-Stop (clone 1) cells were immobilized in calcium alginate matrix. Beads average diameter ranged between 0.3520±0.074 and 0.4979±0.041 mm, with an average weight of 0.3214±0.001 and 0.6737±0.001 g. Initial yeast cell count was 38×10^6 CFU mL^{-1} per 10 calcium alginate beads (Supplementary Material 1).

Shake Flask Statistical Improvement

Plackett-Burman Experimental Design (PBED)

This design was employed to evaluate the main effect of different factors assayed on extracellular enzyme activity detected for each treatment. Statistical analysis was performed at 168 h, where greatest enzyme activity was observed at previous assays.

Only PBED-T_2 and PBED-T_{11} treatments exceeded 12.5±2.6 U L^{-1} activity obtained from preliminary assay (Supplementary Material 2). Treatment PBED-T_{11} increased activity by 2.36 fold in comparison with the preliminary result (Fig. 1). Design results were statistically significant (Table 2).

Table 3 illustrates experimental results and prediction of the enzyme activity (U L^{-1}) in relation to treatments 2 and 11, including central points.

Table 2: Analysis of variance for laccase activity. ANOVA model adjusted to curvature and PBED factors effect on laccase activity

Source	Sum of squares	df	Mean Square	F –value	p-value ---------- Prob >F	Effect	Percent of contribution
Model	691.929	11	62.903	99.390	0.0015		
A- Culture media volume	81.640	1	81.640	128.996	0.0015	5.217	11.44
B- $CuSO_4$	46.454	1	46.454	73.401	0.0033	-3.935	6.51
C- Glucose	6.585	1	6.585	10.404	0.0484*	1.482	0.92
D- NH_4SO_4	16.728	1	16.728	26.432	0.0143	-2.361	2.34
E- Peptone	38.265	1	38.265	60.461	0.0044	-3.571	5.36
F- Yeast extract	148.143	1	148.143	234.075	0.0006	7.027	20.75
Curvature	71.203	1	71.203	112.505	0.0018		
Pure error	1.899	3	0.633				
Cor total	765.030	15					
R^2: 0.9044							
Adeq. Precision: 7.663							

*The only less-significant factor in this model

Table 3: PBED matrix for observed and predicted result (PBED-T_2 and PBED-T_{11}) comparison as consequences of factors influencing laccase activities

Treatment	Design point	Culture media volume (mL)	$CuSO_4$ (mM)	Glucose (g L^{-1})	NH_4SO_4 (mM)	Peptone (g L^{-1})	Yeast Extract (g L^{-1})	Observed Enz Act. (U L^{-1}) 168 h	Predicted Enz Act. (U L^{-1}) 168 h
T_2	**Factorial**	**150.00**	**0.10**	**10.00**	**5.00**	**10.00**	**5.00**	**18.6007**	**17.3827**
T_{11}	**Factorial**	**300.00**	**0.10**	**30.00**	**5.00**	**20.00**	**10.00**	**29.4653**	**28.2474**
	Central point	225.00	0.55	20.00	12.50	15.00	7.50	4.5933	8.2234
	Central point	225.00	0.55	20.00	12.50	15.00	7.50	5.5319	8.2234
	Central point	225.00	0.55	20.00	12.50	15.00	7.50	4.5696	8.2234

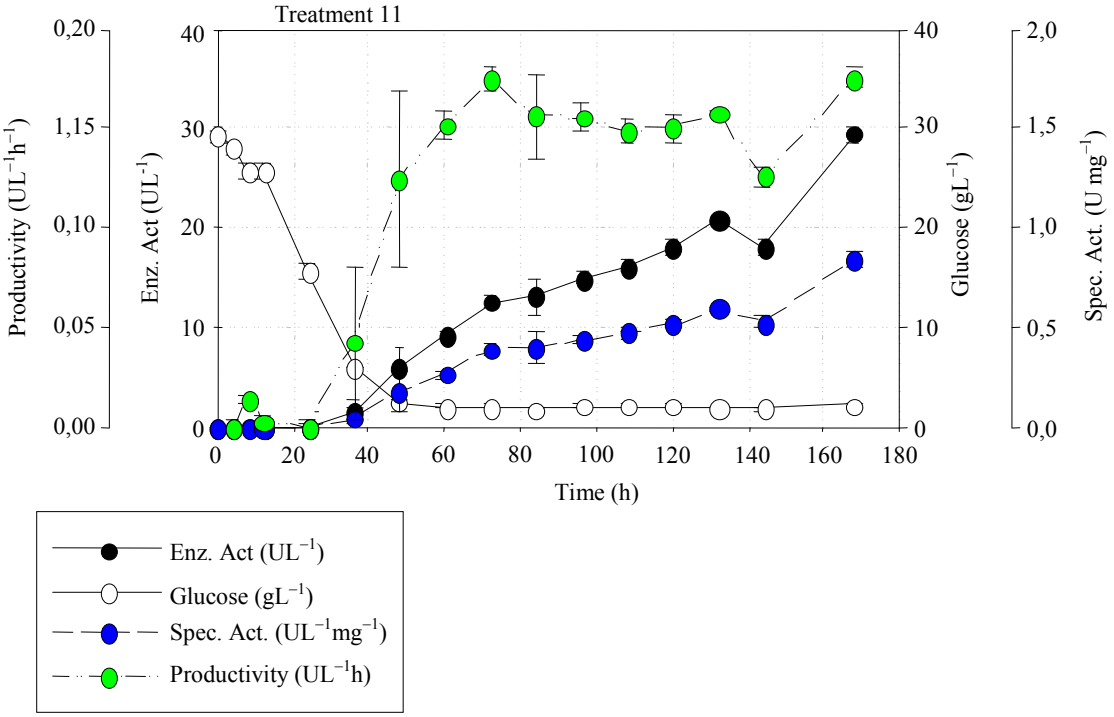

Fig. 1: Plackett-Burman Experimental Design (PBED). Treatment 11 results [**PBED-T_{11}**: 500 mL Erlenmeyer flask (300 mL media, 0.1 mM $CuSO_4$, 30 g L^{-1} glucose, 5 mM NH_4SO_4, 20 g L^{-1} peptone, 10 g L-500 mL ^{1} yeast extract), enzyme activity. 29.5 ± 0.80 U L^{-1} at 168 h of culture]

Box-Behnken Experimental Design (BBED)

This design was employed to optimize different factors from the previous assay (PBED) resulting in significant contribution on enzyme activity. Only two treatments did not exceed a PBED average enzyme activity (29.5±0.80 U L^{-1}), where BBED-T_3 and BBED-T_{12} were the treatments with greatest enzyme activities of 1,079.64±125.3 U L^{-1} and 1,300.34±125.3 U L^{-1}, respectively. Box-Behnken design was able to improve PBED-T_{11} (Fig. 1) by 44.1 fold with BBED-T_{12} (Fig. 2A), resulting in overall the highest BBED treatment. For all BBED assays cells were released too from beads and proliferated in the culture media. BBED data statistical analysis revealed significant differences (Table 4).

Table 5 exhibits observed and predicted enzyme activity (U L^{-1}) of treatments BBED-T_3 and BBED-T_{12}, including central points.

In light of these results optimization criteria was defined using Design Expert V9.0 (2014) software as follows: Independent variable "target" values, such as culture media volume (mL), yeast extract (g L^{-1}) and peptone (g L^{-1}) were established between ranges. In contrast, aimed dependent variable value, *i.e.* enzyme activity (U L^{-1}) at 168 h was set as a maximum value with importance of 5.

Maximum desirability established in the contour graphic was 0.876 and maximum optimized enzyme activity level was 1,130.84 U L^{-1} in the 3D surface after 84 optimization runs or factor combinations. Such output is discretely higher than BBED-T_{12} model prediction (1,105.81 U L^{-1}), however, lower than the observed activity (1,300.340 U L^{-1}) for the same treatment (Table 5).

Therefore, we decided to corroborate BBED-T_{12} acquired results and four new BBED-T_{12} replicas were carried-out at 96, 120, 144 and 168 h; obtained activity values were averaged with Box-Behnken replicas for the same treatment (Fig. 2B).

Hence, with these results BBED-T_{12} [BBED-T_{12}: [500 mL Erlenmeyer flask (350 mL culture media containing 0.05mM $CuSO_4$, 30 g L^{-1} glucose, 2.5 mM NH_4SO_4, 10 g L^{-1} peptone, 30 g L^{-1} yeast extract)] was replicated in column.

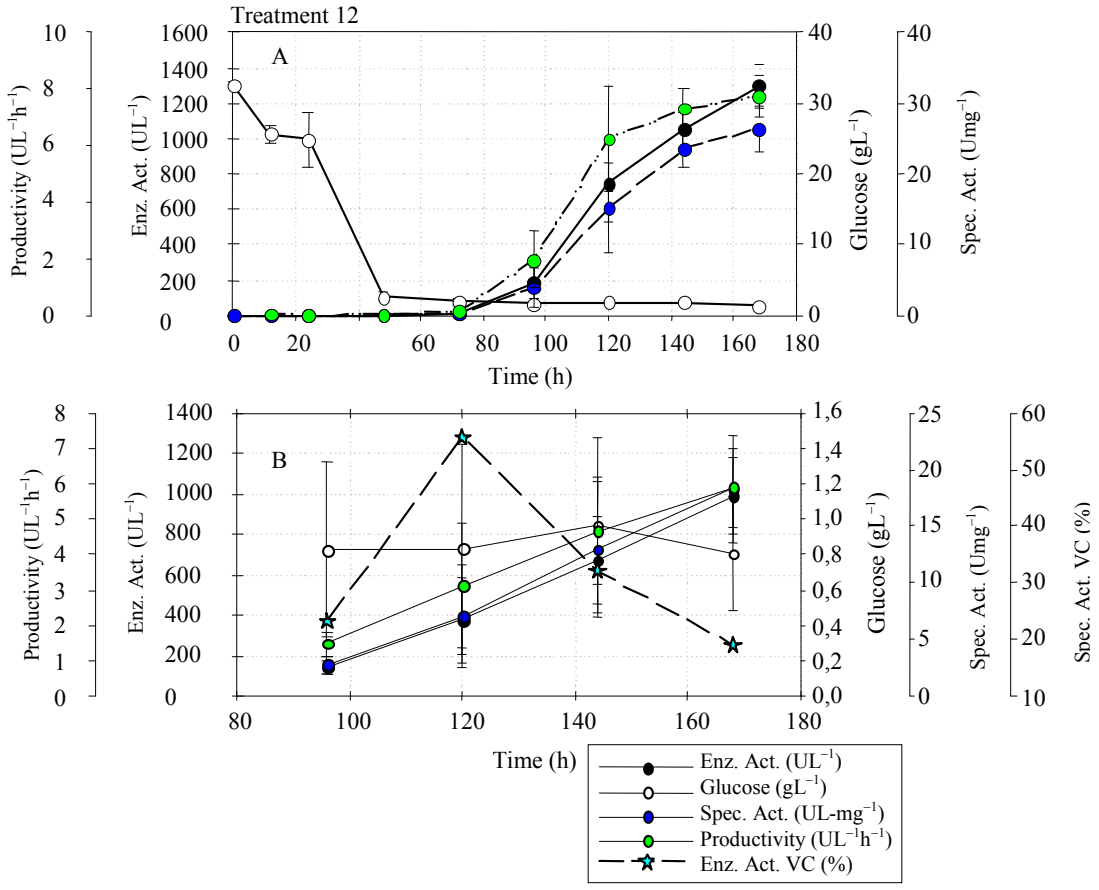

Fig. 2: **Box-Behnken Experimental Design (BBED). A.** Treatment 12 results [**BBED-T_{12}**: 500 mL Erlenmeyer flask (350 mL media containing 0.05mM $CuSO_4$, 30 g L^{-1} glucose, 2.5 mM NH_4SO_4, 10 g L^{-1} peptone, 30 g L^{-1} yeast extract), Act. Enz. 1,300.34 ± 125.3 U L^{-1} at 168 h]. **T_{12} production consistency analysis (five replics). B**. Enzyme activity at 168 h of culture variation coefficient (VC) was less than 20%

Table 4: BBED laccase activity ANOVA for response lineal model

Source	Sum of Squares	df	Mean Square	F-Value	p-value ---------- Prob >F
Lineal Model	2,09E+09	3	6,97E+08	51.45	<0.0001
A- Culture media Volume	5010.91	1	5010.91	0.37	0.5554*
B- Yeast Extract	2,02E+09	1	2,02E+09	149.18	<0.0001
C- Peptone	64839.07	1	64839.07	4.79	0.0512*
Residual	1,49E+08	11	13547.54		
Lack of Fit	1,26E+08	9	13957.19	1.19	0.5365*
Pure Error	23408.18	2	11704.09		
Cor Total	2,24E+09	14			
R^2: 0.9335					
Adj. R^2: 0.9153					
Pred. R^2: 0.8798					
Adeq. Precision: 19.720					

*Not significant factors

Table 5: BBED matrix comparison between observed and predicted results for BBED-T_3 and BBED-T_{12} as a consequence of factors influencing laccase activity (U L^{-1})

Treatment	Design point	Culture media volume (mL)	Yeast Extract (g L^{-1})	Peptone (g L^{-1})	Observed Enz Act. (U L^{-1}) 168 h	Predicted Enz Act. (U L^{-1}) 168 h
T_3	**Factorial**	**300**	**30**	**5**	**1,079.64**	**1,040.81**
T_{12}	**Factorial**	**350**	**30**	**10**	**1,300.34**	**1,105.81**
	Central Point	350	20	5	542.662	513.164
	Central Point	350	20	5	344.710	513.164
	Central Point	350	20	5	368.032	513.164

Table 6: Comparison among columns production of r*POXA 1B laccase*

Column #	Sodiun Alginate immobilization% (w/v)	Concentration of $CaCL_2$ for gel formation (M)	Enz. Act. (UL^{-1}) at 168 h	Dry Biomass (gL^{-1}) release from beads at 57 h
0	2.5	0.1	1,412.97±14.48	16.82±0.23
1	4.0	0.1	2,552.90±0.43	32.29±0.00
2	4.0	0.2	1,266.21±0.43	17.64±0.05
3	4.0	0.3	1,453.93±0.43	17.01±0.12

Production of rPOXA IB Laccase with Immobilized Pichia Pastoris X33/pGAPZαA-LaccPost-Stop Cells. Column Scale

In all columns, the end of the exponential growth phase of release cell from the beads was at 57 h (Fig. 3), but highest enzyme activity appeared at 168 h (Table 6).

Computational Modeling of Calcium Alginate and Molecular Docking between Calcium Alginate and POXA 1B Laccase

A previously reported model by our group (Rivera-Hoyos *et al*., 2015) was adapted to perform calcium alginate computational modeling. Three domains were identified D1, D2 and D3 and copper ions liganded to the catalytic nucleophile (Fig. 4A). Model evaluation revealed high quality by QMEAN, Ramachandran Plot and SuperPose (Rivera-Hoyos *et al*., 2015).

For molecular docking CASTp estimated 101 pockets on LaccPos surfaces. The pocket near the T1 active site was added, composed of 18 amino acids (Tyr154, Pro165, His166, Pro167, Asp207, Ser208, Asp209, Phe240, Ala241, Asp265, Ser266, Phe392, Ala393, Gly394, Pro395, Pro397, Ile455 and Trp457) occupying an area of 133.779Å^2. Most amino acids in this cluster forming the catalytic pocket were hydrophobic.

Out of the 10 different conformational outputs for each simulation laccase adopted with calcium alginate, none was found to be within the hydrophobic pocket. Never the less, according to potential energy values the most stable locations were selected to determine possible calcium alginate modifications due to POXA 1B laccase. After verifying each output it was evidenced interactions generated between protein and ligand were weak. Therefore, they didn't seem to produce ligand modification (Fig. 4B to 4E). As previously mentioned, it was shown alginate didn't ligate into the catalytic nucleophile, but did exert interactions with laccase at various nearby sites of the hydrophobic pocket. A possible hydrogen bond interaction to be highlighted was a calcium alginate sugar hydroxyl interacting with asparagine 326 in laccase. However, taking into account calcium alginate presents a great number of cyclic sugars in its structure; this hydrogen bond could be present at any nearby sugar hydroxyl group that would allow for this interaction.

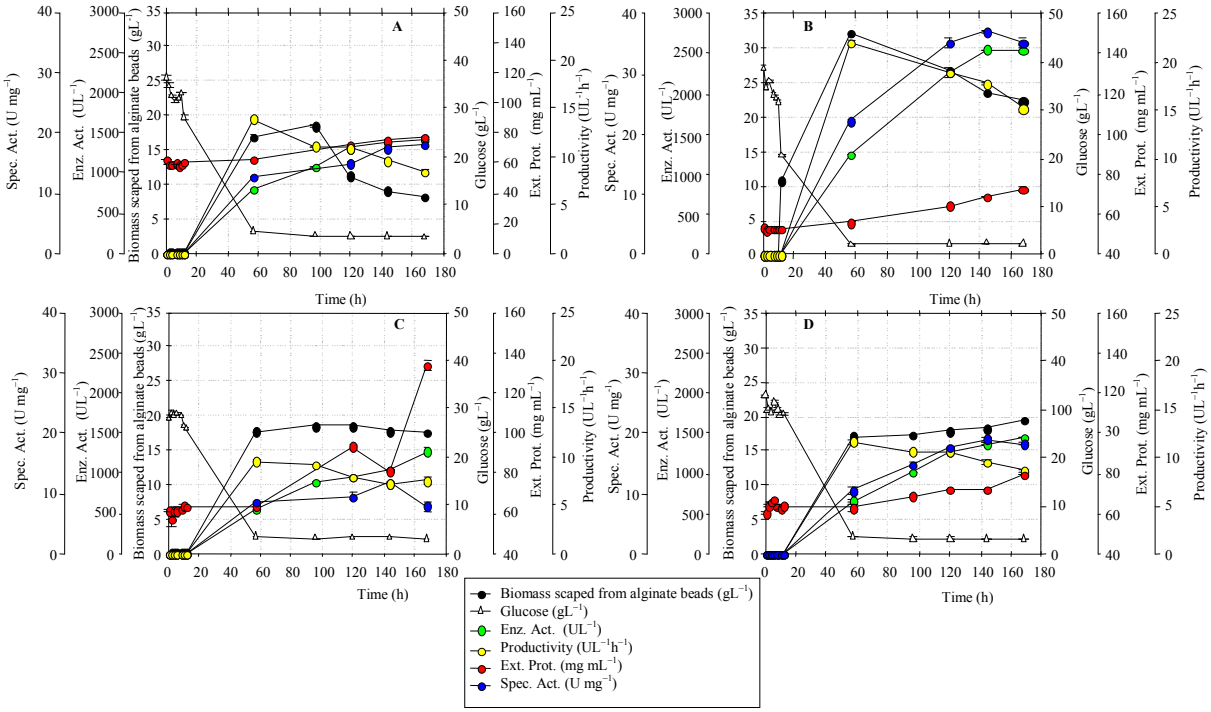

Fig. 3: ***Pichia pastoris X33/pGAPZαA-LaccPost*-Stop culture results. A.** *Pichia pastoris* X33/pGAPZαA-LaccPost-Stop immobilized in 2.5% (w/v) sodium alginate and 0.1M $CaCl_2$ (Column 0). **B.** Cells immobilized in 4% (w/v) sodium alginate and dripped into 0.1M $CaCl_2$ solution (Column 1). **C**. Cells immobilized in 4% (w/v) sodium alginate and dripped into 0.2 M $CaCl_2$ (Column 2). **D**. Cells immobilized in 4% (w/v) sodium alginate and dripped into 0.3 M $CaCl_2$ (Column 3)

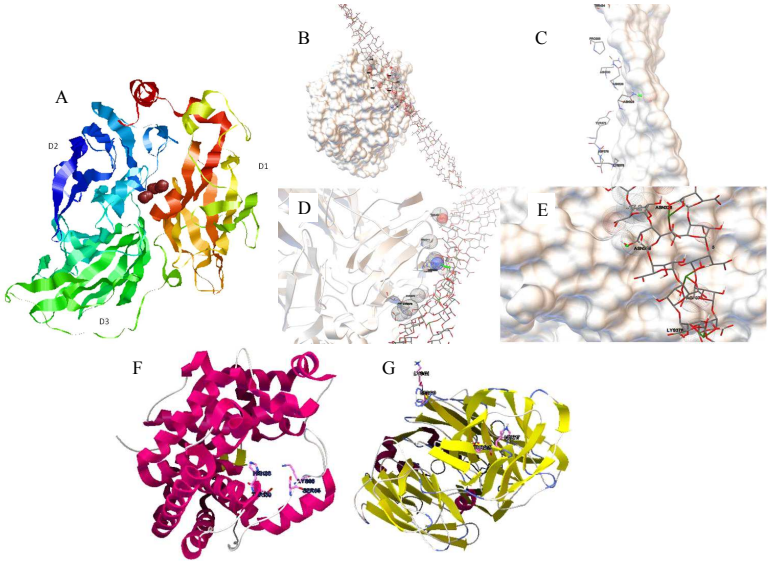

Fig. 4: POXA 1B laccase 3D structure, interactions between *Pleurotus ostreatus* POXA 1B laccase and calcium alginate (ligand), identification of residues required for alginate liase (E.C. 4.2.2.3) (4OZV) catalytic activity A. Representation of *Pleurotus ostreatus* POXA 1B laccase tertiary structure with β-sheets. Three domains (D1, D2 y D3) characteristic of laccases with copper atoms liganded to the catalytic nucleophile (Rivera-Hoyos *et al*., 2015). **B**. Interaction between ligand and receptor, with no structural modification of the ligand. **C**. Identification of laccase residue interacting with calcium alginate. **D**. Representation of *Pleurotus ostreatus* POXA 1B laccase β-sheets with domain interacting with calcium alginate. **E**. Approximation of the site for POXA 1B laccase and calcium alginate interaction. **F**. Representation of alginate lyase (4OZV) β-sheets, with identification of the four residues than form the catalytic pocket. **G**. Representation of *Pleurotus ostreatus* POXA 1B laccase β-sheets and corresponding residues with potential catalytic activity

In Fig. 4 F and G β-sheets diagrams depict 4OZV alginate lyase and POXA 1B laccase 3D structures. Despite alginate lyase having the same residues required for catalytic activity, laccase conformational location was not appropriate for catalytic activity.

Discussion

Pichia Pastoris X33/pGAPZαA-LaccPost-Stop Calcium Alginate Immobilization

Bead differences were observed for cells immobilized at different sodium alginate concentrations. Cells entrapped in sodium alginate dripped into 0.1M $CaCl_2$ exhibited increasing mechanical resistance to agitation with rising concentrations, where 2.5% (w/v) sodium alginate were less resistant, 3% (w/v) slightly more resistant and at 4% (w/v) did not display deformations or fissures, from the agitation process. Additionally, cells entrapped in 4% (w/v) sodium alginate dripped into 0.3 M $CaCl_2$ exhibited changes in diameter and weight (Supplementary Material 1).

The process of ionotropic gelation is responsible for sodium alginate bead formation. Sodium alginate is made up of two alternating monomeric units (β-D mannuronic acid and α-L guluronic acid). When two chains of α-L guluronic acid align, sites of coordination are formed, as a consequence of hairpin structures. Hairpin cavity size is adequate to accommodate Ca^{2+} ions, giving rise to network of mechanically stable alginate gels with calcium salts (Ting *et al*., 2013). Taking into account the chemical reaction between sodium alginate and $CaCl_2$, its effect on bead size and weight could have been the result of greater Ca^{2+} incorporation, augmenting ion concentration at coordination sites. Therefore, crosslinking could have become tighter, giving the bead more firmness, limiting cell release from within the bead. However, sufficient nutrient and product exchange was allowed with external media. Nevertheless, detailed molecular studies must be performed to prove this hypothesis, which was not the objective of this work.

Statistical Improvement. Shake Flask Scale

Based on preliminary results and to improve culture-media and conditions for P. ostreatus POXA 1B recombinant laccase production, it was decided to carry-out a statistical study in Erlenmeyer flasks. Results would then be corroborated and adjusted at column scale.

Plackett-Burman Experimental Design (PBED)

Model adjusted to curvature was significant ($p = 0.0015$), corroborated with an F-value of 99.39, indicating the model was significant relative to noise (Table 2). Furthermore, R^2 coefficient value specifies variability of predicted response compared to experimental results. Model predicted response becomes more reliable as R^2 approaches one. A $R^2 = 0.904$ was obtained; suggesting 90.4% of experimental data variability could be explained by adjusted model. Moreover, signal to noise ratio measurement is determined by adequate precision, where a ratio greater than 4 is desirable. An observed 7.663 ratio indicated an adequate signal, in addition to the possibility to navigate the design space for this model. All factors assayed were significant except glucose concentration.

Table 3, illustrates enzyme activity was different for each treatment. Only PBED-T_2 and PBED-T_{11} exceeded activity results from preliminary assay. Valuing results presented in Table 2, the following considerations were taken into account to define factors to be evaluated in the following BBED.

Media volume had an 11.44% positive contribution on enzyme activity (Table 2), suggesting greater volumes should be assayed. It is noteworthy to highlight Erlenmeyer flasks of the same brand and capacity was used for all assays, under the same parameters, in the same shaker. Therefore, demonstrating culture media volume was associated with oxygen transfer. Thus, greater volumes, *i.e*., 300, 350 and 400 mL were evaluated.

In contrast, copper sulfate had a 6.51% negative contribution (Table 2). Hence, lower concentrations should be appraised. Based on reports in the literature with promissory laccase activity results (Balakumaran *et al*., 2016), copper sulfate -1 concentration was reduced by 50% in the PBED to be established as a fixed factor (0.05mM) in the BBED.

A 0.92% positive contribution was determined for glucose (Table 2). This result implies increasing changes in glucose concentrations, would not have a substantial effect on enzyme activity. Therefore, PBED value of +1 (30 g L^{-1}) was proposed for BBED.

Ammonium sulfate had a 2.34% negative contribution to enzyme activity (Table 2). Thus, proposing lower concentrations should be assayed. Therefore, 50% of PBED -1 concentration, *i.e*. 2.5 mM was set for BBED. Concentration was not decreased to 0, in order to prevent inorganic nitrogen elimination from culture media.

Moreover, peptone also had a 5.36% negative contribution outcome on enzyme activity (Table 2), also suggesting a decrease in peptone concentration would be desirable. Hence, PBED -1 value (10 g L^{-1}) was used as +1 for BBED.

Last, yeast extract had a 20.75% positive contribution effect (Table 2). Given higher concentrations should be assayed, it was determined PBED +1value was used as the -1 BBED level. Table 7 illustrates final BBED.

Table 7: Proposal for BBED

Code	Factor	Low level (-1)	Level (0)	High level (+1)
A	Culture media volume (mL)	300.00	350.00	400.00
B	Yeast Extract (g L^{-1})	10.00	20.00	30.00
C	Peptone (g L^{-1})	0.00	5.00	10.00
	Fixed Factors	**Value**		
D	$CuSO_4$ (mM)	0.05		
E	Glucose (g L^{-1})	30.00		
F	NH_4SO_4 (mM)	2.50		

Box-Behnken Experiment Design (BBED)

An F-value of 51.45 was obtained; proposing a significant model with only 0.01% probability would be due to noise (Table 4). "Lack of Fit F-value" of 1.19 described this value was not significant or resulted from pure error. There was a 53.65% probability this large "Lack of Fit F-value" would be due to noise.

Additionally, a "Pred R-Squared" of 0.8798 was in reasonable agreement with the "Adj R-Squared" of 0.9153, with a difference less than 0.2. As previously described, "Adeq Precision" measures signal to noise ratio. A ratio greater than 4 was desirable, with an obtained lineal model ratio of 19.720, indicating an adequate signal. Therefore, this model was suitable to navigate the design space.

Predicted vs. obtained results are shown in Table 5. However, an attempt to graphically optimize culture media components resulted in slightly higher values compared to those obtained by and in agreement with the prediction. Never the less, Enzyme Activity (Enz Act.) values were lower compared to observed design values. Consequently, suggesting it would not be possible to improve enzyme activity with culture media components and assayed concentrations and conditions. Moreover, they marked the navigation boundary on the Response Surface Design. Subsequently, BBED-T_{12} replicas were carried-out to obtain Standard Deviations (SD) and corroborate if the assay were reproducible.

Figure 2B illustrates mean±SD (989.31±87.45 U L^{-1}) of five BBED-T_{12} replicas at 168 h of culture. This value was much closer to predicted model value (1,105.81 U L^{-1}) and resulting graphic optimization (1,130.84 U L^{-1}), than observed value in the design itself (1,300.34±125.3 U L^{-1}). Also, corroborating under experimental conditions the navigation limit on surface response had been reached. With validated BBED-T_{12} culture conditions column scale conditions were extrapolated.

Pichia Pastoris X33/pGAPZαA-LaccPost-Stop Immobilized Cell Production. Column Scale

Figure 3A (column 0) depicts rPOXA 1B laccase production throughout the assay. Exponential phase ended at 57 h, supported by residual glucose behavior, where it starkly decreased from 0 h to 57 h and then remained rather steady decreasing slightly up to 168 h. In addition, it was consistent with biomass kinetic behavior, corresponding to increased biomass, when cells were "released" from beads. Only at 12 h, free cells started to be detected in culture media. At the end of the exponential phase (57 h) dry biomass was 16.82±0.23 g L^{-1}, followed by stationary phase up to 96 h, to then proceed with cell death phase up to 168 h, when culture ended. Enzyme activity started to be detected from 57 h onwards, reaching a maximum 1,412.97±14.48 U L^{-1} value at 168 h. For this assay extracellular protein concentration was kept between 58 and 77 mg mL^{-1} throughout time. Productivity reached a maximum value at 57 h, followed by a decline. Last, specific activity steadily increased up to the end of the culture (168 h).

Extracellular proteins slightly increased during the 168 h of culture (Fig. 3A). This tendency was more evident after 96 h of culture, concurrently with cell death initiation and biomass decrease. This suggests in addition to culture media proteins, intracellular proteins were secreted into culture media by the cells. In addition, cell death resulting cell lysis, also added to extracellular proteins, contributing to total extracellular protein concentration. Despite a slight augmentation in extracellular protein concentration, enzyme activity was capable of increasing, favoring specific activity increase.

Column 0 assay was carried out to improve the process, since results reported by Dong *et al*. (2014) demonstrated alginate beads at sodium alginate concentrations less than 3.5% (w/v) were too fragile. Furthermore, the objective was to extrapolate previously obtained data. Moreover demonstrated BBED-T_{12} results were comparable, where an enzyme activity of 1,300.34±125.3 U L^{-1} was obtained at 168 h of culture in Erlenmeyer flask under agitation (Fig. 2), compared to column scale at 168 h with 1,412.97±14.48 U L^{-1} enzyme activity (Fig. 3A).

Figure 3B shows rPOXA 1B laccase production kinetics (column 1), where the residual glucose behavior supported exponential growth phase ended at 57 h. This result is in agreement with biomass kinetic behavior, which steadily increased with time as a result of cell release from alginate beads. Free cells were initially detected at 12 h in culture media. For this assay extracellular protein concentration remained between 52.37 and 72.51 mg mL^{-1} from 0 h to 168 h. Moreover, productivity reached a maximum value at 57 h, with a

decreasing tendency from then on. Last, specific activity presented a stark increase up to 144 h of culture and with a minor decrease at 168 h. Fig. 3B depicts extracellular protein increase through time, since biomass started to diminish from 57 h of culture, suggesting presence of proteins lysis. On the other hand, specific activity decreased, since enzyme activity was maintained constant to the end of the process, yet extracellular protein concentration continued to increase.

A study performed by Liu *et al*. (2009) evaluated different $CaCl_2$ concentrations to immobilize *S. cerevisiae* cells for ethanol production. That work reported beads at 1.0% (w/v) $CaCl_2$ (0.09 M) easily ruptured. At 2.0% (w/v) $CaCl_2$ (0.18 M), cells were flexible and resistant enough not to rupture during the process. At 4.0% (w/v) $CaCl_2$ (0.36 M) were stiff, beads formed with concentrations above this one were too stiff and presented low ethanol production, due to low nutrient transfer into the beads.

As was observed for columns 0 and 1, for column 2 kinetic behavior, residual glucose behavior supported exponential growth phase (from 12 h to 57 h) and remained constant afterwards (Fig. 3C). Biomass increased in time during the exponential phase and then continued stable. Exponential phase prolonged up to 57 h with dry biomass concentration of 17.64±0.05 g L^{-1}, followed by a stationary phase, which extended up to 168 h, when culture ended, given culture kinetics cell death phase was not observed. Moreover, as for 0.1 M $CaCl_2$ solution, enzyme activity was first observed at 57 h, increasing through time until the culture ended at 168 h. Compared with 0.1 M $CaCl_2$ solution enzyme activity was considerably lower (Table 6). For this assay extracellular protein concentration remained constant up to 60 h of culture. Extracellular protein concentration markedly increased from 144 h of culture up to 168 h. Productivity reached a maximum value at 57 h, followed by a discrete decrease. Last, specific activity was maintained rather constant up to 120 h of culture, reached its maximum value at 144 and then diminished slightly up to 168 h of culture.

As previously mentioned, a drastic increase in extracellular protein was observed at the end of the process (Fig. 3C). This result could be due to an experimental error or an interfering agent present in the sample, since biomass concentration was stable from 57 h to 168 h of culture. Therefore, this value could not have been the result of intracellular protein secretion from cell lysis. As a result of extracellular protein increase, specific activity decreased during this last phase of culture, despite enzyme activity was steadily increasing up to 168 h.

A reduction in cell release from alginate beads, in comparison with Column 1 could be due to a greater $CaCl_2$ concentration, as has been reported by other authors (Dong *et al*., 2014; Liu *et al*., 2009). Increasing $CaCl_2$ concentration makes beads more rigid and difficult to rupture, thus retaining cells within the beads. On the other hand, there was a reduction in enzyme activity in comparison with Column 1, likely due to limited oxygen and nutrient transfer (Dong *et al*., 2014). However, both Dong and Liu studies revealed the best $CaCl_2$ concentration for cell immobilization as at 0.18 M (2% (w/v)), a concentration similar the one employed in Column 2.

Based on Liu *et al*. (2009) and Dong *et al*., (2014) studies and with the objetive to control cell release from the beads to improve rPOXA 1B laccase production, a column 3 was assayed (Fig. 3D). In Fig. 3D, residual glucose decreased from the beginning up to 57 h of culture, in agreement to the columns 1 and 2. As can be observed, biomass increased at the end of the exponential phase (57 h). As was observed for column 2, biomass remained rather stable throughout the remainder of the culture, with slight increasing tendency towards the end of the culture. Exponential phase started at 12 h and lasted up to 57 h with a dry biomass of 17.01±0.12 g L^{-1}, followed by a stationary phase, which extended until the end of the culture (168 h). Enzyme activity was first recoded at 57 h of culture, reaching a maximum value at 168 h 1,453.93±0.43 UL^{-1}. For this assay extracellular protein concentration increased throughout the process, while productivity reached a maximum value at 57 h and then decreased. Last, specific activity was steadily increasing from 12 h up to the end of the culture.

Specific activity was continuously increasing, since enzyme activity and extracellular protein concentration were also incrementing with time (Fig. 3D). However, no important differences in enzyme activity or dry biomass values were observed at the end of the exponential phase in comparison to column 2. Consequently, 0.2 M $CaCl_2$ instead of 0.3 M $CaCl_2$ could be used to form calcium alginate beads. As has been described $CaCl_2$ solution at higher concentration results in more rigid beads. This effect would somehow limit nutrient transfer affecting cell metabolic activity (Dong *et al*., 2014; Liu *et al*., 2009), when the objective was to increase cell metabolism to promote enzyme activity.

On the other hand, it is important to highlight in addition to $CaCl_2$ concentration, sodium alginate concentration also has an effect on cell activity (Lee *et al*., 2011). As reported by Lee *et al*. (2011) study *S. cerevisiae* was immobilized at different sodium alginate concentrations used for ethanol production. Data revealed increasing sodium alginate concentrations had an effect on bead rigidity, with a repercussion on nutrient transfer. The authors also determined the best immobilization concentration was 2.5% (w/v), since it established equilibrium between organic compound production and cell stability.

The highest enzyme activity when comparing the four columns was observed for 4% (w/v) sodium alginate dripped into 0.1 M $CaCl_2$ solution (Fig. 3, Supplementary Material 3B). However, these conditions resulted in the assay with most cell release from alginate beads (Fig. 3, Supplementary Material 3A). Whereas under conditions for the other three column operation, *i.e.*, 2.5% (w/v) sodium alginate dripped into 0.1 M $CaCl_2$, 4% (w/v) sodium alginate dripped into 0.2 M $CaCl_2$ and 4% (w/v) sodium alginate dripped into 0.3 M $CaCl_2$, no important difference was observed for dry biomass in culture media at the end of the exponential phase (57 h), in addition to enzyme activity results (Fig. 3, Supplementary Material 3A). Regarding, 4% sodium alginate dripped into 0.2 M $CaCl_2$ solution effect on specific activity a notorious decrease was observed (Fig. 5, Supplementary Material 3C). As previously described, this assay presented a high extracellular protein concentration at the end of the culture (Fig. 3C).

Comparison of the four treatments (Columns 0 to 3) demonstrated cell release from alginate bead into culture media and cell proliferation was the result of having employed only up to 4% (w/v) sodium alginate dripped into 0.3 M $CaCl_2$ solution (Column 3).

In contrast to previous optimization media culture results in Erlenmeyer flasks, where a laccase enzyme activity of 1,300.34±125.3 U L^{-1} was obtained at 168 h of culture, when implementing a change to column scale with 0.0283 m^3 h^{-1} air, interesting results were obtained, depending on their respective conditions, column 0 has a 0.090 X increased enzyme activity, column 1, 0.96 X increased enzyme activity; column 2, 0.027 X decreased enzyme activity and Column 3 has a 0.12 X increased enzyme activity.

Taking into account that cells immobilized within 2.5 to 4.0% (w/v) sodium alginate beads were cultured in column scale and an important cell release was observed, the following question was established, would it be possible for rPOXA 1B laccase to act on calcium alginate polymer facilitating cell release?

The uncertainty was addressed through computational modeling. Hence, molecular docking was used to evaluate calcium alginate and rPOXA 1B laccase possible interaction.

Computational Modeling: Calcium Alginate and POXA 1B Laccase Interaction – Molecular Docking

According to Cambria *et al*. (2010), physico-chemical characteristics found in the pocket of LaccPost model are true catalytic pockets. For laccases they are essential to prevent metal ion contact with water. In addition, they facilitate hydrophobic substrate interaction with the center of the enzyme's catalytic site. This cavity is formed in D3 domain by an α-helix and a β-sheet (Cambria *et al*., 2010; Rivera-Hoyos *et al*., 2015).

Interactions were mainly due to the presence of polar residues in both structures (laccase and alginate), generating weak interactions that allow them to relate to each other (Fig. 4B to E). However, these types of interactions are not sufficient to cause any type of laccase catalytic activity on alginate. If this interaction is compared with that generated by enzymes of alginate lyase type (periplasmic, such as 4OZV identified by PDB (Fig. 4F, G), alginate lyase have a characteristic motif identified by four residues forming a hydrophobic pocket, where calcium alginate depolymerization can be catalyzed by hydrolysis of β 1-4glycosidic bond between two adjacent sugars, the same mechanism for β elimination (PubMed: 8370530, PubMed: 8335634, PubMed: 23215237). For these cases, prolonged calcium alginate digestion could generate dimeric or trimeric products.

Regarding its primary structure, residues involved in alginate lyase (for 4OZV: S-65, K-66, H-138 and T-139) are also present in laccase (S-130, K-131, H-217 and T-218). However, in their 3D structure these residues don't form a hydrophobic pocket. Therefore, for the evaluated laccase an enzymatic activity is very unlikely.

To validate the computational study and as a proof of concept to evaluate cell release wasn't related to possible enzymatic activity by rPOXA 1B laccase on calcium alginate polymer, from encapsulated alginate bead, a biological assay was performed using not-genetically modified *S. cerevisiae* and *P. pastoris* X33 immobilized, as previously described for column 0; since neither microorganism was capable of producing this enzyme (Supplementary Material 4).

Accordingly, it was proven cell release was not associated with laccase activity (Fig. 4, Supplementary Material 4). Additionally, these data validated molecular docking results. Computational docking model demonstrated residue interaction between laccase catalytic center and calcium alginate polymer was of very weak nature.

On the other hand, *P. pastoris* cell release from alginate bead was greater compared with *S. cerevisiae* (Fig. 4A). As observed by SEM analysis (Supplementary Material 4, Fig. 4C and D), *P. pastoris* diameter (5 µm – BNID 108942) was smaller in comparison with *S. cerevisiae* (6 µm – BNID 108943), (Milo *et al*., 2010). Cell diameter could be related to cell release from alginate bead. A smaller diameter allowed for cells to more readily be released from beads and proliferate within the culture media (Supplementary Materials 2 and 4).

Conclusion

Column preliminary assay produced 12.5±2.6 U L^{-1} of rPOXA 1B laccase activity at 156 h. Shake flask culture media statistical improvement resulted in PBED-T_{11} enzyme activity about 29.5±0.8 U L^{-1} at 168 h, whereas BBED-T_{12} greatly improved enzyme activity

to 989.31±187.45 UL^{-1} at 168 h of culture. Improved culture media composition and conditions were [500 mL Erlenmeyer flask (350 mL media containing 0.05mM $CuSO_4$, 30 g L^{-1} glucose, 2.5 mM NH_4SO_4, 10 g L^{-1} peptone, 30 g L^{-1} yeast extract) at 168 h of culture]. When these conditions were studied at column scale at 168 h greater enzyme activity was obtained 2,552.90±0.43 U L^{-1} (column 2). However, concomitant with this increased activity a greater release of cells from the alginate beads was detected. Moreover, results obtained with sodium alginate and $CaCl_2$ at different concentrations suggest cells released from calcium alginate beads are mainly responsible for enzyme activity. Never the less, immobilization conditions that reduced cell release (17.01±0.12 gL^{-1}), allowed for high enzymatic activity (1,453.93±0.43 UL^{-1}) and displayed higher specific activity (18.33 Umg^{-1}) were those evaluated in column 3 (4% (w/v) Na^{2+} alginate, 0.3 M $CaCl_2$); supporting the fact that combination of sodium alginate and $CaCl_2$ concentrations controlled cell release.

Molecular docking experiment and assay with native *P. pastoris* and *S. cerevisiae* (non-rPOXA 1B laccase producing cells), (Supplementary Material 4), demonstrated that interaction between laccase and calcium alginate polymer was not responsible for cell release, given very weak interactions between amino acids residues in the enzyme's catalytic center and calcium alginate polymer. In addition, conformation of the active center was inappropriate to generate a catalytic activity.

This work offered four conditions (columns 0 to 3) favoring rPOXA 1B active enzyme production, which is very valuable, considering its future purpose. Forthcoming application is to implement column scale for effluent treatment, where contaminated water would flow through alginate bead immobilized cells. In this sense, calcium alginate beads, with entrapped microorganism would have an "adjuvant like effect", since they would be releasing (for a long time) laccase producing cells, potentiating the desired effect. For this model before effluent is flowed through the column, retention time and contaminant load must first be determined. Undoubtedly this decontamination strategy would require subsequent treatment to lower residual microorganism load (recombinant yeast) after treatment, which is desirable regardless of Generally Recognized As Safe (GRAS) microorganism use. In addition, *P. pastoris* X33/pGAPZαA-*LaccPost-Stop* immobilization is better suited for contaminated water treatment than for rPOXA 1B production, since centrifugation step to remove biomass would not be eliminated. According to assay conditions, rPOXA 1B laccase production is still better when cultured as free cells.

Competing Interests

The authors declare no conflict of interests.

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Author´s Contributions

Raúl A. Poutou-Piñales: Conceived and designed the experiments, analyzed the data and wrote the manuscript.

Michelle Gouzy-Olmos, Luis M. Cháves-Tequia, María F. Rojas-Fajardo, Ángela M. Cardozo-Bernal and Luis D. Gómez-Méndez: Performed the experiments.

Eliana M. González-Neira and Aura M. Pedroza-Rodríguez: Analyzed the data.

Edgar A. Reyes-Montaño, Edwin D. Morales-Álvarez and Claudia M. Rivera-Hoyos: Performed the experiments, analyzed the data and wrote the manuscript.

Ethics

All authors read and approved the final version of this manuscript. There are not any ethical issues to declare that could arise after the publication of this manuscript.

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Supplementary Material

Supplementary Material 1

Average diameter and weight of calcium alginate beads containing recombinant *Pichia pastoris*. Yeast within beads were capable of producing POXA 1B laccase (n = 100).

Treatment	Diameter (cm) Mean ± SD	Weight (g) Mean ± SD
2.5% (w/v) Na alginate and 0.1M $CaCl_2$	0.3520±0.074	0.4002±0.020
3.0% (w/v) Na alginate and 0.1M $CaCl_2$	0.4979±0.041	0.6737±0.001
4.0% (w/v) Na alginate and 0.1M $CaCl_2$	0.3945±0.026	0.4659±0.001
4.0% (w/v) Na alginate and 0.2M $CaCl_2$	0.3986±0.022	0.3696±0.002
4.0% (w/v) Na alginate and 0.3M $CaCl_2$	0.3662±0.015	0.3214±0.001

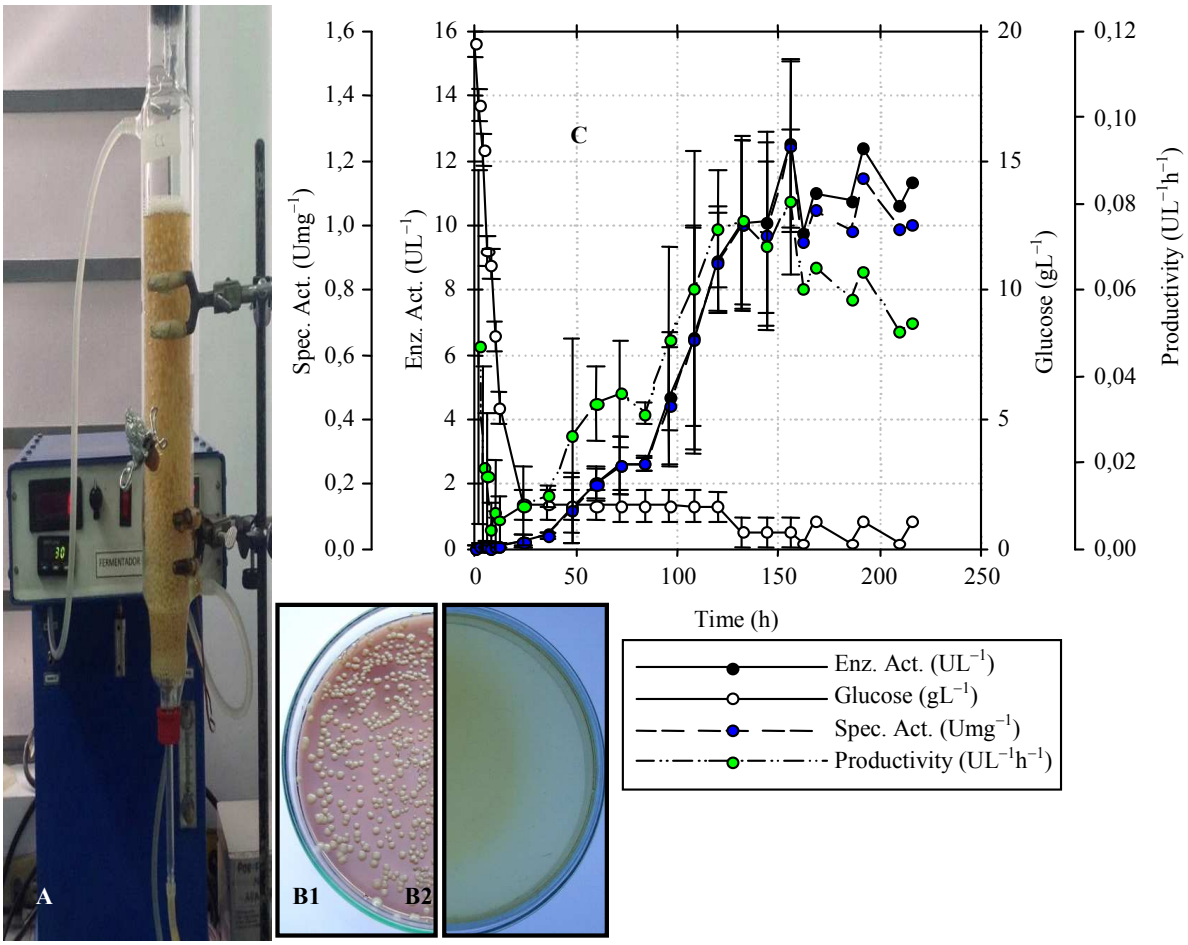
Mean average alginate bead diameter was determined using a Vernier calibrator. One-hundred beads were randomly sampled for each sodium alginate and calcium chloride concentration prepared. Additionally, mean weight was determined in groups of 10 units using analytical balance (Precisa XT220A).

For cell number determination in calcium alginate bead, ten calcium alginate beads containing immobilized cells were placed into phosphate buffer (0.2 M Na_2HPO_4, 0.2 M NaH_2PO_4, pH 7.0±0.2) at a ratio of 1 g beads/9 mL phosphate buffer. Beads were placed in sterile 15 mL tube incubated for 15 min at 30°C and vortexed for 10 min. Again they were incubated at 30°C for 24 h and vortexed for 10 min until beads were dissolved and cells were in suspension (Matiz *et al.*, 2002; Sarmiento *et al.*, 2003). To resulting cell suspension serial dilutions up to 10^{-5} were performed with phosphate buffer. 0.1 mL was seeded with Drigalsky spatula in agar Petri dishes containing YPD Agar (YPD + Agar-Agar 1.5% (w/v), supplemented with 0.1 mM $CuSO_4$ and 0.2 mM ABTS. Culture plates were incubated at 30°C for 72 h. Colonies were counted and reported as CFU mL^{-1}.

Supplementary Material 2

Column with immobilized *Pichia pastoris* X33/pGAPZαA-*LaccPost-Stop* cells, cell growth on YPG-ABTS agar and immobilized batch culture kinetics. A. Photograph depicting column geometry culture.B1. Petri dish demonstrating *Pichia pastoris* X33/pGAPZαA-*LaccPost-Stop* (clone 1) growth on YPGA agar supplemented with 0.1 mM $CuSO_4$ + 0.2 mM ABTS after 72 h of incubation at 30°C. B2. Culture media control, YPGA supplemented with 0.1 mM $CuSO_4$ +

0.2 mM ABTS before inoculating with recombinant strain. C. Kinetic behavior (average) between two biological replicas (columns A and B) of batch culture with immobilized *Pichia pastoris* X33/pGAPZαA-LaccPost-Stop (clone 1) for *Pleurotus ostreatus* POXA IB laccase production.



In this assay average velocity of glucose consumption was 1.06 g L^{-1} h^{-1}. It is important to highlight glucose was consumed during the first 19 h approximately and pH decreased from 7.0 to 5.0±0.2 (measured only at the beginning and at the end of culture). Average enzyme activity, specific activity and productivity "based on enzyme activity" behavior maintained the same tendency throughout the culture, with highest values of 12.5±2.6 U L^{-1},1.24±0.26 U L^{-1} and0.08 U L^{-1} h^{-1} at 156 h, respectively.

According to studies reported by Jorda *et al*., (2012), accelerated glucose consumption could be due to recombinant *P. pastoris* X33/pGAPZαA-*LaccPost-Stop* high maintenance requirements, associated with protein folding and conformational stress. Likewise high glucose consumption results in increased Krebs cycle activity (tricarboxylic acid (TCA)), since cells are not able to metabolize sufficient carbon from TCA cycle to compensate for high-energy demand derived from recombinant protein production (Heyland *et al.*, 2011; Poutou-Piñales *et al.*, 2010). (Jorda *et al.*, 2012)

A decrease in pH is characteristic of yeast metabolism; ruling out accelerated consumption derived from microorganism contamination. Likewise, to prevent bacteria contamination culture media was supplemented with 0.1 g L^{-1} chloramphenicol.

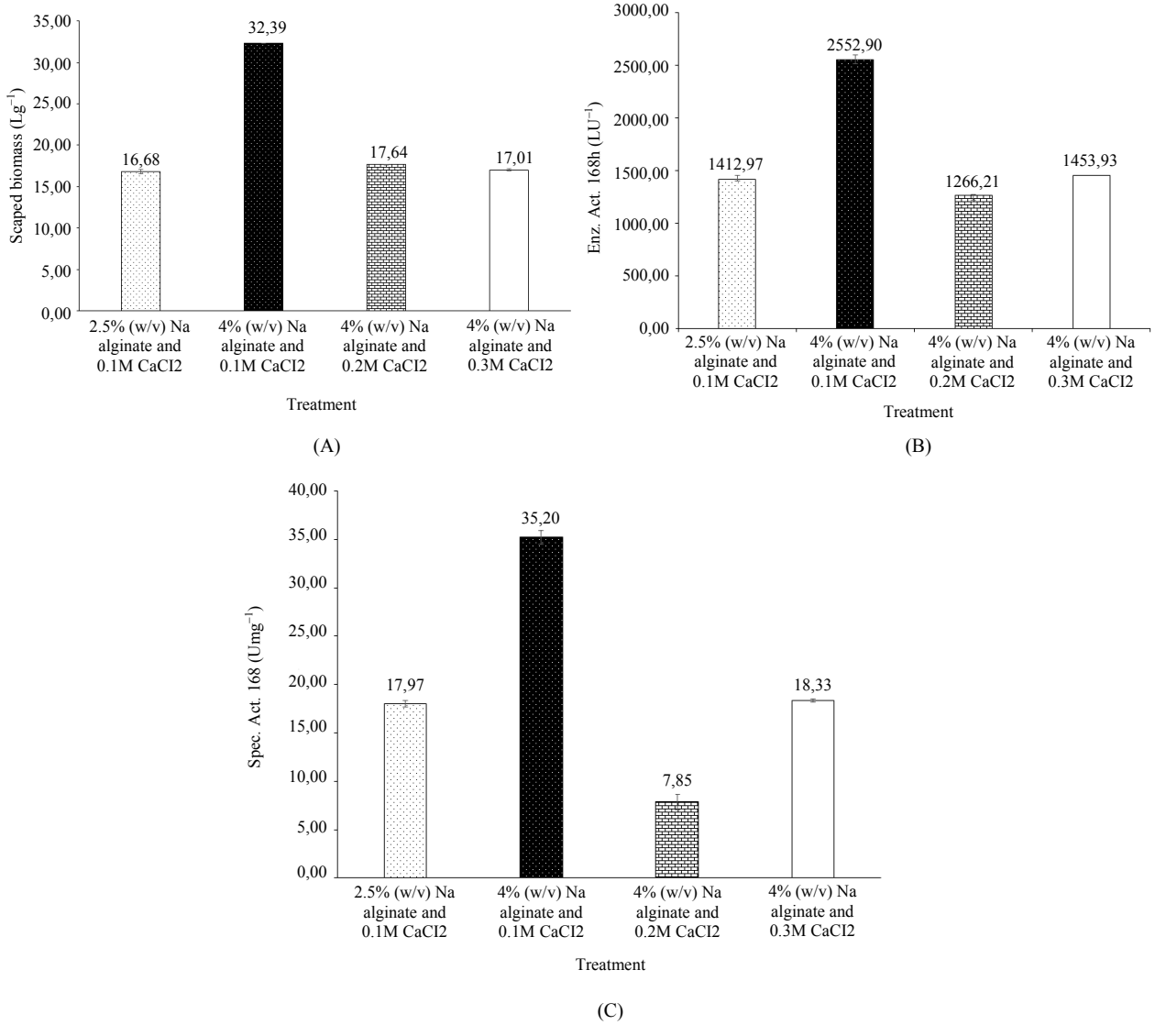
Figure shows specific activity had a similar behavior to enzymatic activity up to 168 h of culture. At this point specific activity decreased, establishing a slight difference from enzyme activity. Therefore, it was established all assays would be performed up to 168 h.

Comparing preliminary assay results with previously reported enzyme activity from the same clone cultured in 100 ml free cell culture (Rivera-Hoyos *et al.*, 2015), enzyme activity was starkly lower than 451.08 ± 6.46 U L^{-1} at 156 h. Suggesting, cell immobilization resulted in drastically decreased enzyme activity, nutrient and oxygen transfer, as

a function of crosslinking generated by w/v sodium alginate percentage, the reactor's geometry (Erlenmeyer flask vs. column) and pneumatic agitation (shaker vs. compressed air). In addition, it was also evidenced a considerable amount of yeast was released into the culture media. Therefore it was determined sodium alginate concentration should be increased from 2.0 to 2.5% (w/v) for the following assays, in an effort to prevent oxygen and nutrient transfer restriction.

Supplementary Material 3

Immobilized *Pichia pastorisX33/pGAPZαA-LaccPost*-Stop result tendencies for all treatments.A.Mean ± STD biomass (g L^{-1}) at the end of the exponential phase (57 h). B.Mean ± STDenzyme activity (U L^{-1}) at the end of culture (168 h). C.Mean ± STDspecific enzyme activity (U mg^{-1}) at the end of the process (168 h).



Supplementary Material 4

Non-Laccase Pichia Pastoris X33 and Saccharomyces Cerevisiae Erlenmeyer Flask Producers

For this assays *P. pastoris* X33 and*S. cerevisiae* cells immobilized in 2.5% sodium alginate (w/v) dripped into 0.1 M $CaCl_2$ were used. Previously improved BBED culture media was employed in three 500 mL Erlenmeyer flasks at 30°C and 180 rpm for each organism. Immobilized cells were inoculated at 1 bead/mL of culture media and sampled at 0, 12, 24, 48, 120, 144 and 168 h of culture. Cultures were monitored by OD_{600nm} readings to

calculate *P. pastoris* dry biomass concentration (g L^{-1}) outside the alginate bead (Equation I), extracellular protein concentration (mg mL^{-1}), residual glucose concentration (g L^{-1}), enzyme activity (U L^{-1}), specific enzyme activity (U mg^{-1}) and productivity (U L^{-1} h^{-1}):

$$X_{\left(\frac{g}{L}\right)} = \frac{0D_{60onm}}{1.1726} \quad \text{(I)}$$

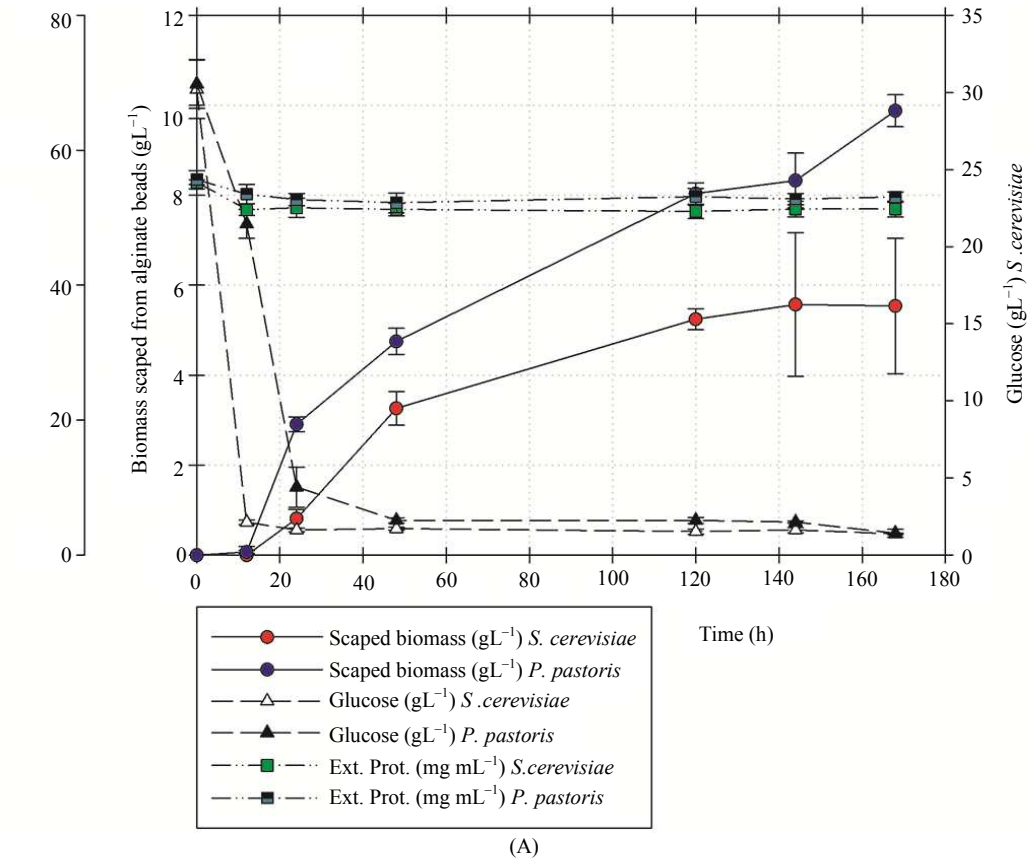
Cultures performed with *S. cerevisiae* were followed in the same manner by OD_{600nm} to calculate dry biomassoutside from calcium alginate bead (Equation II).

$$X_{\left(\frac{g}{L}\right)} = \frac{0D_{6onm} - 0.1835}{0.6151} \quad \text{(II)}$$

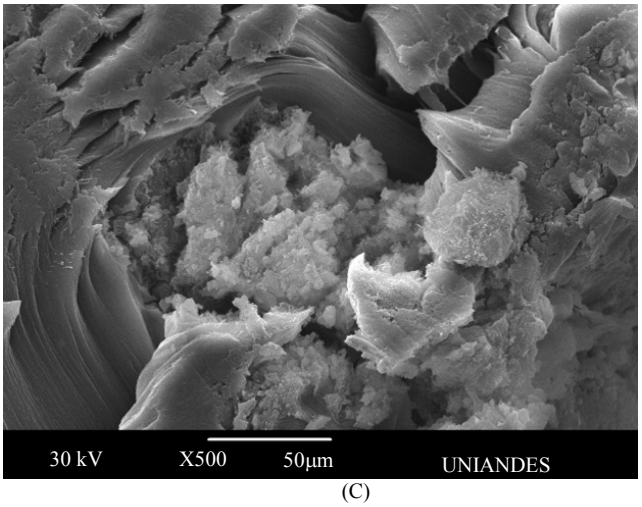
For both *rPOXA 1B* microorganisms after 168 h of culture, glucose and extracellular protein concentration had similar behaviors. Nevertheless, *P. pastoris* X33 cells release from calcium alginate beads presented a greater biomass concentration in comparison with *S. cerevisae* (9.9±0.36 g L^{-1} Vs. 5.54±1.5 g L^{-1}).

Scanning Electron Microscopy (SEM)

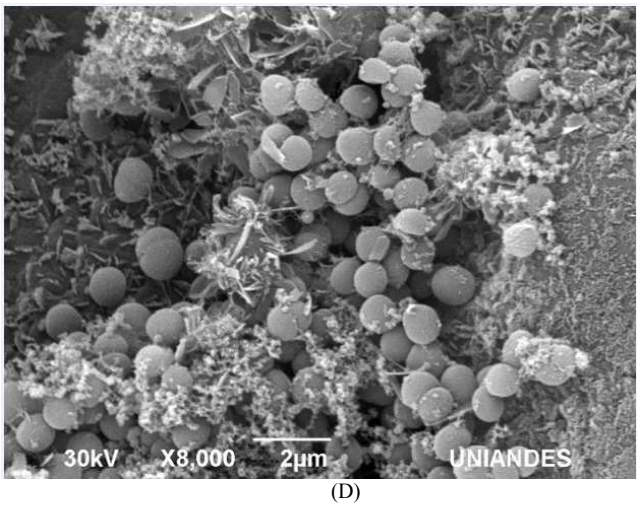
Immobilized *S. cerevisiae* and *P. pastoris* X33 in calcium alginate beads underwent fixation with 2.5% (w/v) glutaraldehyde at pH 7.2±0.2. Subsequently, beads were submitted to successive dehydration process in a series of ethanol solutions as follows: Once for 5 minutes in 70% (v/v) ethanol, twice for ten minutes in 95% (v/v) and three times for 20 minutes in 100% (v/v) ethanol. Beads were then dried-out in a critical point dryer (SAMDRI®-795), cut in half with a fine razor and covered with gold by sputtering (Dentom Vacuum Desk IV). Samples were observed at 500 X and 8,000 X magnification under high vacuum scanning electronic microscope (JEOL JSM- 6490LV) at 30 kV acceleration voltage (Romero de Pérez, 2003).



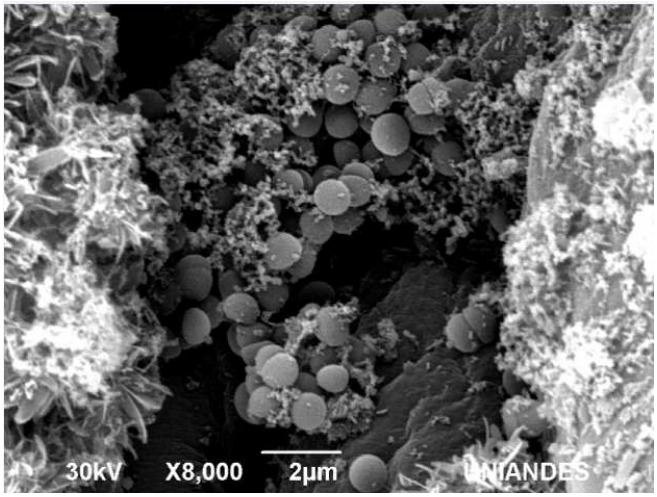
(A)



(C)



(D)



(D)

Pichia pastorisX33 and Saccharomyces cerevisiae immobilized cells in 2.5% (w/v) sodium alginate and 0.1M $CaCl_2$ A: Growth kinetics. B: Calcium alginate SEM at 500X. C: Scanning electron microscopy of *Saccharomyces cerevisiae*cells entrapped within calcium alginate beads at 8,000X. D: Scanning electron microscopy of *Pichia pastoris* X33 cells entrapped within calcium alginate beads at 8,000 X.

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