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Original article

KH_2PO_4 improves cellulase production of *Irpex lacteus* and *Pycnoporus sanguineus*

María Daniela Rodríguez *, Iván Mathías Alonso Paiva [1], María Lorena Castrillo, Pedro Darío Zapata, Laura Lidia Villalba

Laboratorio de Biotecnología Molecular, Instituto de Biotecnología de Misiones, Facultad de Ciencias Exactas, Químicas y Naturales, Universidad Nacional de Misiones, Posadas, Ruta Nacional N° 12, km 7, 5. CP 3300, Argentina

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ABSTRACT

The optimization of cellulase production by *Irpex lacteus* and *Pycnoporus sanguineus* was investigated. Fractional factorial design was conducted to determine significant variables and interactions. Response surface methodology was applied through Box-Behnken design to determine the optimum level of each factor on cellulase production. The optimal conditions of culture media were (g/L): for *I. lacteus* $CaCl_2.2(H_2O)$ 0.3, $MgSO_4.7(H_2O)$ 0.3 and KH_2PO_4 3, while for *P. sanguineus* was $CaCl_2.2(H_2O)$ 0.1, $MgSO_4.7(H_2O)$ 0.2 and KH_2PO_4 9. This optimized medium improved cellulase production, especially β-glucosidase activity values for *I. lacteus*. KH_2PO_4 was found in this work to be a useful mineral compound for cellulolytic production.

1. Introduction

The availability of fossil fuel resources and the increasing energy demand are the main driving forces in the search for alternative energy sources. The large-scale replacement of petroleum fuels by biofuels, such as bioethanol from lignocellulosic materials (bioethanol 2G) appears to be a powerful approach to meet the growing energy demands (Abril and Abril, 2009). Increasing the use of bio-fuels for energy generation purposes is of particular interest nowadays because they allow mitigation of greenhouse gases (Balat and Balat, 2009). Bioethanol 2G is particularly promising because it can use the power of biotechnology to reduce production costs, employ abundant and low cost raw materials, has a higher octane rating and is an environmentally clean product. Lignocellulosic residues from wood, grass, agricultural and forestry wastes are particularly abundant in nature and have a potential for bioconversion. Wood sawdust from pine and eucalyptus are the most abundant lignocellulosic residue in forest regions. The lignocellulosic material from softwood source contains about 44% cellulose, 21% hemicellulose and 28% lignin (Galbe and Zacchi, 2002), while hardwood has 40% cellulose, 17% hemicellulose and 21% lignin (Lima et al., 2014). Coniferyl alcohol is the principal component of softwood lignins, whereas guaiacyl and syringyl alcohols are the main constituents of hardwood lignins. The principal component of hardwood hemicellulose is glucuronoxylan, whereas glucomannan is predominant in softwood (Pérez et al., 2002).

Sawdust constitutes a renewable resource from which many useful biological and chemical products can be derived. Accumulation of lignocellulose materials in large quantities in places where agricultural and forest residues present a disposal problem results not only in deterioration of the environment but also in loss of potentially valuable material that can be used in biomass fuel production (Sánchez, 2009).

Ethanol production from lignocellulosic materials comprises the following main unit operations: pretreatment, hydrolysis of cellulose and hemicellulose, sugar fermentation and bioethanol separation (Alvira et al., 2010; Balat and Balat, 2009). To breakdown polymeric sugars in an environmental friendly process, it is necessary to decrease the cost of cellulases production involved in hydrolysis of cellulose, to increase volumetric productivity, to use cheaper substrates and to produce enzymes with high stability (Percival Zhang et al., 2006).

* Corresponding author.
E-mail address: inbiomis@gmail.com (M.D. Rodríguez).
[1] Present address: Instituto de Estudios de la Inmunidad Humoral (IDEHU), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, Buenos Aires CP 1113, Argentina.
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White-rot fungi have the ability to degrade most of wood components due to their capacity to synthesize hydrolytic extracellular enzymes that recognize the links between the components of both lignin (polyphenolic oxidases) and hemicellulose (hemicellulases). Potential applications of lignocellulolytic enzymes in industrial and environmental biotechnology require huge amounts of these enzymes at the lowest cost possible (Elisashvili et al., 2008). The enzyme cost is one of the factors determining the economics of a biocatalytic process and it can be reduced with optimum conditions for their production and low-cost substrates (Ayishal et al., 2015; Lynd et al., 2002; Prasad et al., 2014).

Irpex lacteus and *Pycnoporus sanguineus*, white-rot fungi native from Misiones (Argentina), have potential for cellulase production when growing on wood flour as a carbon source (Rodríguez et al., 2015). However, there is a need to develop strategies to achieve overproduction (Elisashvili et al., 2008). Optimization of growth conditions and the evaluation of effects and interactions to find optimal conditions, have been used successfully in improving cellulase production, and it can be used to optimize the culture medium with a minimum number of experimental trials (Dave et al., 2013; Hoa and Hung, 2013; Huang et al., 2015; Oberoi et al., 2014; Sadhu et al., 2014; Shajahan et al., 2017; Shashidhar et al., 2013; Singh and Kaur, 2012; Sukumaran et al., 2005; Trinh et al., 2013). Combinatorial interactions of medium components with the production of the desired compound are numerous and the optimum processes may be developed using an effective experimental design procedure (Hao et al., 2006). Response surface methodology is a common statistic method, which is very useful in the optimization of biotechnological processes (Govarthanan et al., 2015; Tan et al., 2016). The statistical design and the development of culture conditions for increase cellulase production is the key issue to improving the implementation of enzymes in bioconversion of biomass (Padilha et al., 2015; Valencia and Chambergo, 2013).

In this study, *I. lacteus* and *P. sanguineus* were utilized for cellulase production and the optimization of the mineral medium was investigated for the enhancement of cellulase production by submerged fermentation. This work focused on cellulases, and as the polyphenolic oxidases and hemicellulases were not evaluated, as a carbon source was used eucalyptus and pine flour as a 50:50 mix. Fractional factorial design was conducted to determine significant variables and interactions. Then, response surface methodology was applied to determine the optimum level of each factor on cellulase production.

2. Material and methods

2.1. Microorganisms

I. lacteus BAFC 1171 and *P. sanguineus* BAFC 2126 were provided by the Mycological Culture Collection of the Department of Biological Sciences, Faculty of Exact and Natural Sciences, University of Buenos Aires, Argentina. Strains were maintained on malt extract agar at 4 °C.

2.2. Raw material

Sawdust from *Pinus* sp. and *Eucalyptus* sp. were collected from sawmill near to Posadas (Misiones, Argentina). Both materials were air-dried until 10% moisture content, ground in a hammermill and sieved. *Pinus* sp. wood flour (PWF) and *Eucalyptus* sp. wood flour (EWF) were classified by screening on a 40 mesh sieve.

The raw materials were characterized following analytical methods described in a previous work (Rodríguez et al., 2015). PWF contained 65.17 ± 0.74 (%w/w) carbohydrates, 24.45 ± 1.31 (%w/w) lignin and 1.82 ± 0.29 (%w/w) extractives; while EWF contained 69.9 ± 2.11 (%w/w) carbohydrates, 12.01 ± 0.93, (%w/w) lignin and 1.27 ± 0.13 (%w/w) extractives.

2.3. Inoculum

Strains were first cultured on malt extract agar for 5 days at 28 °C. To prepare the inoculum, eight 28 mm^2 agar-plugs from each strain were cut and transferred to 500 mL Erlenmeyer flasks containing 100 mL of the following medium (g/L): ammonium sulphate 1.4, yeast extract 0.25, urea 0.3, glucose 5, $MgSO_4.7(H_2O)$ 0.1, $CaCl_2.2(H_2O)$ 0.1 and KH_2PO_4 5. The medium was adjusted at pH 5 and incubated at 29 °C in static conditions for 216 h.

2.4. Culture media and growth conditions

The inoculum was washed with sterile distilled water and homogenized with 50 mM acetate sodium buffer pH 4.8 and 0.1% (v/v) tween 80 until DO_{600} = 0.9. To each experiment, 14% (v/v) of inoculum was added to 250 mL Erlenmeyer flasks containing 50 mL of the following components (g/L): ammonium sulphate 1.4, yeast extract 0.25, urea 0.3, EWF 5, PWF 5.

$MgSO_4.7(H_2O)$, $CaCl_2.2(H_2O)$, KH_2PO_4, $ZnSO_4.7(H_2O)$, $CoCl_2.6(H_2O)$ and $FeSO_4.7(H_2O)$ were added according to each experimental design. Flasks were incubated in a reciprocal shaker at 80 rpm and 30 °C for 360 h. The culture was maintained for so long time to be able to detect each maximum enzyme activity, since these may or may not coincide over time. Samples of culture supernatants were stored at −20 °C.

2.5. Enzyme assays

The FPcellulase activity (FPA) and endo-1,4-β-glucanase activity (EGs – EC 3.2.1.6) were determined according to International Union of Pure and Applied Chemistry (Ghose, 1987). The cellobiohydrolase activity (CBHs – EC 3.2.1.91) was determined according to Wood and Bhat (Bhat and Wood, 1988). FPA was assayed by measuring the release of reducing sugars in a reaction mixture containing 0.1 mL of crude enzyme, 10 mg of Whatman No. 1 filter paper as substrate and 0.2 mL of 50 mM sodium acetate buffer (pH 4.8) at 50 °C for 60 min. EGs activity was assayed by measuring the release of reducing sugars in a reaction mixture containing 0.1 mL of crude enzyme and 0.1 mL of 2% (w/v) of carboxymethylcellulose solution in 50 mM sodium acetate buffer (pH 4.8) incubated at 50 °C for 30 min. CBHs activity was assayed by measuring the release of reducing sugars in a reaction mixture containing 0.1 mL of crude enzyme and 0.1 mL of 1% (w/v) of cellulose in 50 mM sodium acetate buffer (pH 4.8) incubated at 50 °C for 60 min and 125 rpm. Reducing sugars were assayed by dinitrosalicyclic acid method (Miller, 1959). One unit of FPA, EGs and CBHs activity were defined as the amount of enzyme required to liberate 1 μmol of glucose per min from the particular substrate under the assay conditions.

The β-glucosidase activity (BGLs – EC 3.2.1.21) was measured using p-nitrophenyl-β-D-glucopyranoside (Bailey, 1981). The release of p-nitrophenol was measured at 400 nm from a reaction mixture containing 0.9 mL of 0.03 M p-nitrophenyl glucopyranoside in 50 mM acetate buffer (pH 4.8) and 0.1 mL of suitably diluted enzyme, incubated at 50 °C for 15 min and 125 rpm. One unit of BGLs activity was defined as the amount of enzyme required to liberate 1 μmol of p-nitrophenol per min under the assay conditions.

2.6. Fractional factorial design

To optimize the concentration of the mineral medium for *I. lacteus* and *P. sanguineus*, the mineral sources that had a significant

Table 1
Factors and their levels in the 2^{6-1} fractional factorial design.

Variables [g/L]	Symbol	Coded factor level	
		−1	+1
$MgSO_4 \cdot 7\ H_2O$	X_1	0.1	0.5
KH_2PO_4	X_2	1	5
$FeSO_4 \cdot 7\ H_2O$	X_3	0	0.05
$CaCl_2\ 2\ H_2O$	X_4	0.1	0.5
$ZnSO_4 \cdot 7\ H_2O$	X_5	0	0.002
$CoCl_2 \cdot 6\ H_2O$	X_6	0	0.004

effect on cellulases activities were identified by a 2^{6-1} fractional factorial design (FFD). Experimental variables and levels in the FFD are shown in Table 1. In this design, six components were chosen from Mandels medium (Mandels and Reese, 1956). Each component was set into two levels: low (−1) and high (+1) level. The factors with a confidence level at or above 95% were selected to be optimized later.

2.7. Box-Behnken design

Box-Behnken design (BBD) was performed to optimize the statistically significant variables with positive influence on cellulolytic production from FFD results. The independent variables for each strain (*X1*, *X2*, *X3* and *X4*) were evaluated at three different coded levels: −1, 0 and +1 (Table 2).

The minimum and maximum ranges of variables were investigated. Response surface methodology (RSM) was used to analyse the results. Each enzyme activity was taken as the response (*Y*). The quadratic polynomial regression model was assumed for predicting response. The empirical formula to find the optimal cellulolytic yield was given by the Eq. (1):

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_4X_4 + A_5X_1X_2 + A_6X_1X_3 + A_7X_1X_4 + A_8X_2X_3 + A_9X_2X_4 + A_10X_3X_4 + A_11X_1^2 + A_12X_2^2 + A_12X_3^2 + A_14X_4^2 \quad (1)$$

where A0 was intercept; A1, A2, A3 and A4 were linear coefficients; A5, A6, A7, A8, A9 and A10 were interaction coefficients; A11, A12, A13 and A14 were quadratic coefficients. The fitness of the model was analyzed by the coefficient of determination (R^2). In addition, the response surfaces and contour plots were developed by using the fitted quadratic polynomial equation obtained from regression analysis, holding independent variables at constant value corresponding to the central point and changing the other variables.

2.8. Effect of KH_2PO_4 on cellulolytic production

Based on the Box-Behnken experimental design results, it was necessary to evaluate higher levels of KH_2PO_4 for *P. sanguineus*, so new concentrations were studied (6, 9 and 12 g/L). $CaCl_2.2(H_2O)$ and $MgSO_4.7(H_2O)$ were maintained constant at their optimum values (0.1 and 0.2 g/L, respectively).

These assays were performed in triplicate. The culture media and growth conditions employed were described above.

2.9. Statistical analysis

The statistical software package Statgraphic Centurion (StatPoint, Inc., version 15.2.05) was used for the experimental design matrix, regression analysis of the experimental data, and optimization procedure.

The analysis of variance (ANOVA) was used for data analysis, the least significant difference test (LSD test) was performed to establish differences among levels of a factor and standardized through Pareto charts. Differences were considered to be significant at p-value <0.05.

3. Results

3.1. Fractional factorial design

Initial screening of the most important mineral compounds ($MgSO_4.7(H_2O)$, $CaCl_2.2(H_2O)$, KH_2PO_4, $ZnSO_4.7(H_2O)$, $CoCl_2.6(H_2O)$, $FeSO_4.7(H_2O)$) and their interactions affecting cellulolytic production by *I. lacteus and P. sanguineus* was performed employing the FFD. Table 3 represented the FFD for thirty-five run experiments with two levels of values for each variable and the results with respect to cellulolytic production.

The effect of each mineral source and their interactions were standardized to determine the positive or negative influence on cellulolytic production, and the significant parameters (p-value <0.05) are shown in Table 4. The p-value was used as an indicator of analysis and is important for understranding the pattern of mutual interactions between the variables. A negative effect means that there is a decrease in the response parameter for every increase in the variable, and vice versa.

Table 4 shows that the cellulolytic production was positively influenced by KH_2PO_4; while $CoCl_2.6(H_2O)$, affected in a negative manner. For *I. lacteus*, FPA was positively influenced by $CaCl_2.2(H_2O)$ and $FeSO_4.7(H_2O)$, and negatively influenced by the interaction $MgSO_4.7(H_2O)$-$FeSO_4.7(H_2O)$ and $MgSO_4.7(H_2O)$-$CoCl_2.6(H_2O)$.

For *P. sanguineus*, EGs activity was positively influenced by the interaction KH_2PO_4-$FeSO_4.7(H_2O)$, whereas BGLs activity was negatively influenced. FPA and EGs activity were negatively influenced by KH_2PO_4-$ZnSO_4.7(H_2O)$. For *I. lacteus*, EGs activity was positively influenced by the interaction KH_2PO_4-$CaCl_2.2(H_2O)$. The EGs production by *P. sanguineus* was negatively influenced by $ZnSO_4.7(H_2O)$. Therefore, the optimization scheme was continued with KH_2PO_4, $FeSO_4.7(H_2O)$ and $MgSO_4.7(H_2O)$, without $ZnSO_4.7(H_2O)$ and $CoCl_2.6(H_2O)$. The optimization procedure included $CaCl_2.2(H_2O)$ only for *I. lacteus* because this factor and its interactions were significant (positively or negatively) only for this strain.

Table 2
Factors and their levels in the Box-Behnken design for experimental conditions.

Variable [g/L]	Symbol	*I. lacteus* Coded factor levels			*P. sanguineus* Coded factor levels		
		−1	0	+1	−1	0	+1
$MgSO_4\ 7\ H_2O$	X_1	0	0.3	0.6	0	0.2	0.4
KH_2PO_4	X_2	0.5	3	5.5	0	3	6
$FeSO_4\ 7\ H_2O$	X_3	0	0.05	0.1	0	0.05	0.1
$CaCl_2\ 2\ H_2O$	X_4	0	0.3	0.6			

Table 3
Matrix for 2^{6-1} fractional factorial experimental design and response data for determination of important variables for cellulolytic production.

Run	Variables						*I. lacteus* [U/L]				*P. sanguineus* [U/L]			
	X_1	X_2	X_3	X_4	X_5	X_6	EGs	CBHs	BGLs	FPA	EGs	CBHs	BGLs	FPA
1	−1	−1	−1	−1	−1	−1	481	18	58	86	122	16	156	138
2	1	−1	1	−1	−1	−1	421	24	77	86	113	14	62	79
3	−1	−1	1	1	−1	−1	549	26	68	81	148	18	58	72
4	1	−1	−1	1	−1	−1	542	18	60	75	207	22	110	101
5	−1	1	1	−1	−1	−1	169	18	66	44	233	51	207	103
6	1	1	−1	−1	−1	−1	588	44	70	96	219	40	207	124
7	−1	1	−1	1	−1	−1	507	37	79	94	220	38	211	110
8	1	1	1	1	−1	−1	361	22	51	59	233	22	134	93
9	−1	−1	1	−1	1	−1	308	24	64	106	152	10	116	62
10	1	−1	−1	−1	1	−1	219	28	73	100	187	16	160	86
11	−1	−1	−1	1	1	−1	204	18	57	76	204	23	125	123
12	1	−1	1	1	1	−1	300	37	77	78	154	34	65	80
13	−1	1	−1	−1	1	−1	657	51	53	81	230	58	225	117
14	1	1	1	−1	1	−1	355	41	61	82	226	33	209	111
15	−1	1	1	1	1	−1	427	29	62	45	224	34	181	109
16	1	1	−1	1	1	−1	293	40	79	94	202	38	203	113
17	−1	−1	1	−1	−1	1	381	24	67	78	196	19	120	86
18	1	−1	−1	−1	−1	1	211	19	63	31	131	25	149	51
19	−1	−1	−1	1	−1	1	239	29	84	79	176	6	126	62
20	1	−1	1	1	−1	1	505	31	67	81	167	9	57	66
21	−1	1	−1	−1	−1	1	411	49	49	75	67	0	44	43
22	1	1	1	−1	−1	1	167	20	47	28	204	36	177	116
23	−1	1	1	1	−1	1	204	42	67	73	202	42	182	118
24	1	1	−1	1	−1	1	259	43	49	95	207	34	109	112
25	−1	−1	−1	−1	1	1	338	30	89	93	206	32	195	92
26	1	−1	1	−1	1	1	599	34	64	82	215	22	85	95
27	−1	−1	1	1	1	1	818	43	69	82	163	20	107	76
28	1	−1	−1	1	1	1	499	36	54	83	183	17	98	73
29	−1	1	1	−1	1	1	396	55	73	94	267	6	209	101
30	1	1	−1	−1	1	1	960	44	71	80	261	39	217	108
31	−1	1	−1	1	1	1	503	35	75	98	224	45	195	119
32	1	1	1	1	1	1	170	43	52	98	263	29	155	99
33	0	0	0	0	0	0	720	44	63	119	222	34	136	101
34	0	0	0	0	0	0	714	46	62	100	256	37	184	103
35	0	0	0	0	0	0	740	45	64	94	222	31	154	98

$x1$: $MgSO_47(H_2O)$, $x2$: KH_2PO_4, $x3$: $FeSO_47(H_2O)$, $x4$: $CaCl_22(H_2O)$, $x5$: $ZnSO_47(H_2O)$, $x6$: $CoCl_26(H_2O)$.
−1 low level, +1 high level.

Table 4
Results from 2^{6-1} fractional factorial design.

Variable	*I. lacteus*				*P. sanguineus*			
	EGs	CBHs	BGLs	FPA	EGs	CBHs	BGLs	FPA
X_1	+						+	+
X_2	+	+	+	+	+	+	+	+
X_3				+		+		
X_4				+				
X_5					−			
X_6	−				−		−	−
Interactions								
X_5–X_3								
X_2- X_3		−			+		−	
X_1- X_6	−			−				
X_1- X_3	−			−				
X_2- X_5					−			−
X_4- X_2	+							
X_4- X_3	−							

$x1$: $MgSO_47(H_2O)$, $x2$: KH_2PO_4, $x3$: $FeSO_47(H_2O)$, $x4$: $CaCl_22(H_2O)$, $x5$: $ZnSO_47(H_2O)$, $x6$: $CoCl_26(H_2O)$.
+: positive influence, −: negative influence.

3.2. Optimization of mineral medium by RSM

Based on the above results, an optimization design was carried out. The mineral compounds $MgSO_4.7(H_2O)$, $CaCl_2.2(H_2O)$, KH_2PO_4 and $FeSO_4.7(H_2O)$ for *I. lacteus*; and $MgSO_4.7(H_2O)$, KH_2PO_4 and $FeSO_4.7(H_2O)$ for *P. sanguineus* resulted to be significant for cellulolytic production by the FFD and was optimized by the BBD to determine their effects and interactions on cellulase production.

The concentration of these components was varied to determine the optimal concentration of each one that produced maximum cellulolytic activities. Variables from the FFD that did not significantly influenced in the enzymatic activities were

maintained at the minimum level (Table 1). The experimental design matrix, with a total of 24 combinations and 3 replicates at the center points, was performed as shown in Table 5 for *I. lacteus*. The results showed that the mineral medium constituted in trial 22 gave maximal cellulase production.

For *P. Sanguineus*, the design resulted in 12 combinations with 3 central points (Table 6). Results showed that the combination number 2 yielded the highest cellulolytic production.

Results obtained from the BBD, were examined by analysis of variance and the effects were standardized in Pareto charts. For *I. lacteus*, the results are shown in Fig. 1. These Pareto charts show the variables in descending order of importance with the estimated effect. The bars that cross the vertical line indicate that these variables significantly influenced at the confidence level studied. For EGs no factor or interaction was statistically significant.

Results for *P. sanguineus* are shown in Fig. 2. For BGLs and FPA no factor or interaction was statistically significant.

The FPA, CBHs, BGLs (*I. lacteus*) and EGs activities (*P. sanguineus*) were positively influenced by X_2 (KH_2PO_4).

Equations describing the model obtained for each enzyme studied were obtained. The optimal medium can be achieved from the model. Eqs. (2–5) correspond to *I. lacteus*, while Eqs. (6–9) correspond to *P. sanguineus* were Y_1 and Y_5 represent EGs activity, Y_2 and Y_6 CBHs activity, Y_3 and Y_7 BGLs activity, Y_4 and Y_8 to FPA [U/L].

Table 5
Matrix for Box-Behnken design to optimize mineral concentrations for cellulolytic production by *I. Lacteus*; experimental and estimated results.

Runs	X_1	X_2	X_3	X_4	Experimental				Predicted			
					EGs	CBHs	BGLs	FPA	EGs	CBHs	BGLs	FPA
1	−1	0	0	−1	929	34	60	78	845	42	66	88
2	1	0	0	−1	527	36	58	88	573	36	49	89
3	−1	0	0	1	842	36	55	106	778	44	63	98
4	1	0	0	1	842	50	80	109	907	50	73	92
5	0	−1	−1	0	682	24	46	77	627	23	38	72
6	0	1	−1	0	421	41	64	101	507	45	60	95
7	0	−1	1	0	585	19	21	68	481	23	23	67
8	0	1	1	0	851	31	70	100	887	41	77	98
9	−1	0	−1	0	580	36	46	81	577	42	53	97
10	1	0	−1	0	739	36	50	93	816	42	62	99
11	−1	0	1	0	829	48	70	97	1004	40	66	100
12	1	0	1	0	368	47	49	101	623	40	51	94
13	0	−1	0	−1	450	22	27	65	509	23	32	68
14	0	−1	0	1	421	18	22	45	641	27	38	60
15	0	1	0	−1	617	49	72	85	650	39	65	79
16	0	1	0	1	592	53	78	97	785	51	81	103
17	−1	−1	0	0	559	26	40	65	604	16	27	49
18	1	−1	0	0	641	31	34	75	476	28	31	79
19	−1	1	0	0	759	53	76	114	690	48	72	108
20	1	1	0	0	954	33	57	60	675	36	62	74
21	0	0	−1	−1	525	43	46	91	562	39	49	83
22	0	0	−1	1	1044	62	89	114	901	51	79	112
23	0	0	1	−1	977	37	66	103	885	41	69	104
24	0	0	1	1	1084	48	70	83	813	45	60	89
25	0	0	0	0	726	44	49	94	845	49	53	96
26	0	0	0	0	935	56	55	88	845	49	53	96
27	0	0	0	0	874	46	55	106	845	49	53	96

X_1: $MgSO_47(H_2O)$, X_2: KH_2PO_4, X_3: $FeSO_47(H_2O)$, X_4: $CaCl_22(H_2O)$. Activities are expressed on U/L.

Table 6
Matrix for Box-Behnken design to optimize minerals concentrations for cellulolytic production by *P. sanguineus;* experimental and estimated results.

Runs	X_1	X_2	X_3	Experimental				Predicted			
				EGs	CBHs	BGLs	FPA	EGs	CBHs	BGLs	FPA
1	0	−1	−1	11	6	40	7	29	9	50	12
2	0	1	−1	133	18	94	31	120	17	94	27
3	0	−1	1	4	14	54	3	17	15	54	7
4	0	1	1	63	11	46	19	45	8	36	14
5	−1	0	−1	56	3	57	15	61	3	56	14
6	1	0	−1	56	3	80	19	46	2	71	19
7	−1	0	1	6	0	35	13	16	1	44	13
8	1	0	1	9	1	28	3	4	1	29	4
9	−1	−1	0	9	6	37	9	0	4	28	5
10	1	−1	0	2	3	42	9	0	2	41	4
11	−1	1	0	61	1	53	12	69	3	54	17
12	1	1	0	9	2	31	11	32	4	40	15
13	0	0	0	57	5	29	19	52	3	39	16
14	0	0	0	43	2	49	10	52	3	39	16
15	0	0	0	44	2	38	19	52	3	39	16

X_1: $MgSO_47(H_2O)$, X_2: KH_2PO_4, X_3: $FeSO_47(H_2O)$. Activities are expressed on U/L.

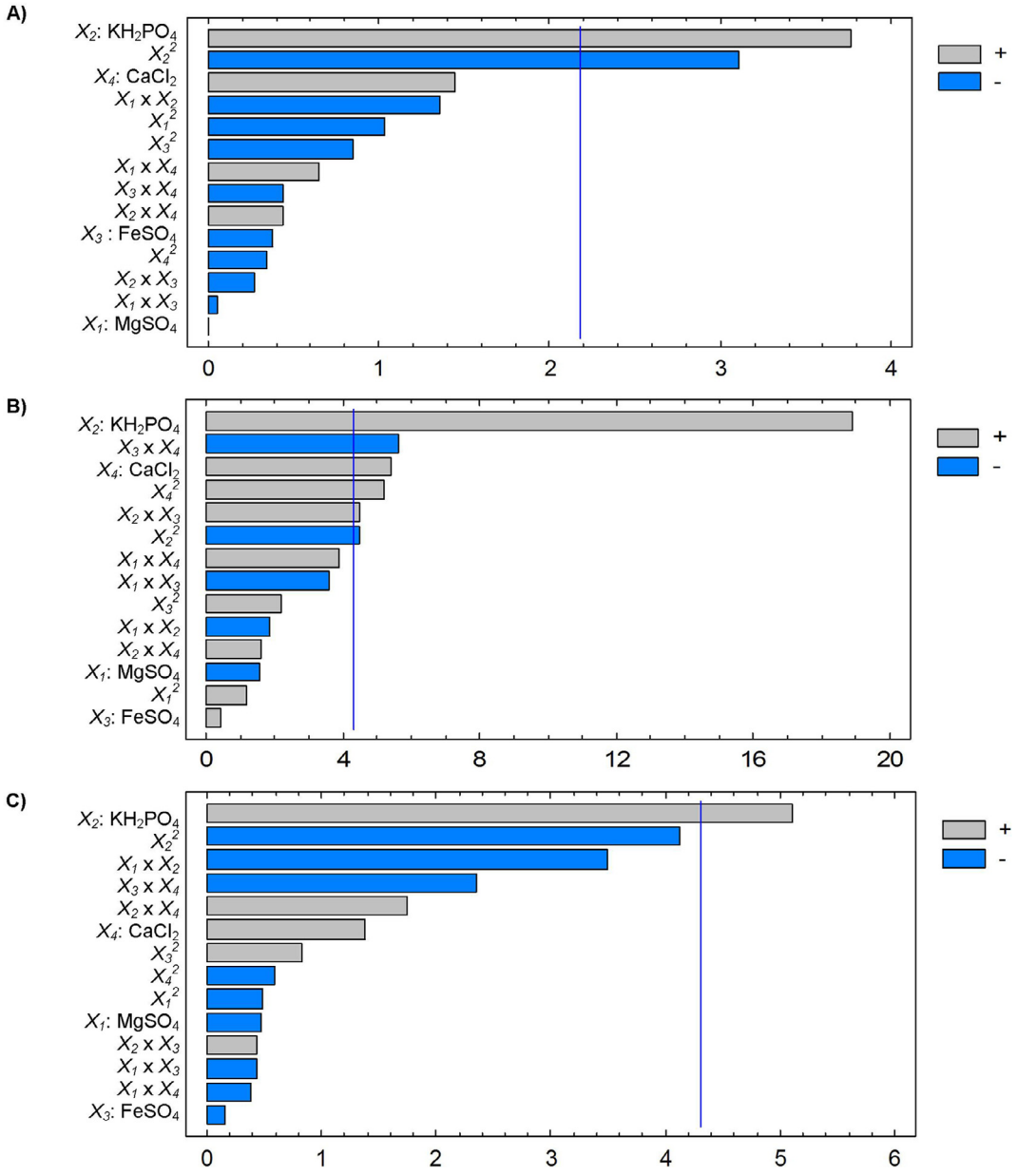

Fig. 1. Pareto charts with the estimated effect of the variables tested for (A) CBHs activity, (B) BGLs activity and (C) FPA in *I. lacteus*. The confidence level was 95%.

$$Y_1 = 845 - 35^*X_1 + 71^*X_2 + 59^*X_3 + 67^*X_4 - 52^*X_1^2 - 182^*X_2^2 - 38^*X_3^2 - 17^*X_4^2 + 28^*X_1^*X_2 - 155^*X_1^*X_3 + 100^*X_1^*X_4 + 132^*X_2^*X_3 + 1^*X_2^*X_4 - 103^*X_3^*X_4 \qquad R^2 = 93\% \tag{2}$$

$$Y_2 = 49 + 0.1^*X_1 + 10^*X_2 - 1^*X_3 + 4^*X_4 - 6^*X_1^*X_2 - 0.3^*X_1^*X_3 + 3^*X_1^*X_4 - 1^*X_2^*X_3 + 2^*X_2^*X_4 - 2^*X_3^*X_4 - 4^*X_1^2 - 12^*X_2^2 - 3^*X_3^2 - 1^*X_4^2 \qquad R^2 = 96\% \tag{3}$$

$$Y_3 = 53 - 1^*X_1 + 19^*X_2 + 0.4^*X_3 + 5^*X_4 - 3^*X_1^*X_2 - 6.25^*X_1^*X_3 + 7^*X_1^*X_4 + 8^*X_2^*X_3 + 3^*X_2^*X4 - 10^*X_3^*X_4 + 2^*X_1^2 - 7^*X_2^2 + 3^*X_3^2 + 8^*X_4^2 \qquad R^2 = 91\% \tag{4}$$

$$Y_4 = 96 - 1^*X_1 + 13^*X_2 - 0.4^*X_3 + 4^*X_4 - 16^*X_1^*X_2 - 2^*X_1^*X_3 - 2^*X_1^*X_4 + 2^*X_2^*X_3 + 8^*X_2^*X_4 - 11^*X_3^*X_4 - 2^*X_1^2 - 16^*X_2^2 + 3^*X_3^2 - 2^*X_4^2 \qquad R^2 = 92\% \tag{5}$$

$$Y_5 = 52 - 7^*X_1 + 30^*X_2 - 22^*X_3 - 11^*X_1^*X_2 + 1^*X_1^*X_3 - 16^*X_2^*X_3 - 26^*X_1^2 - 5^*X_2^2 + 6^*X_3^2 \qquad R^2 = 90\% \tag{6}$$

$$Y_6 = 3 - 0.1^*X_1 + 0.4^*X_2 - 0.5^*X_3 + 1^*X_1^*X_2 + 0.3^*X_1^*X_3 - 4^*X_2^*X_3 - 5^*X_1^2 + 5^*X_2^2 + 4^*X_3^2 \qquad R^2 = 94\% \tag{7}$$

$$Y_7 = 39 - 0.1^*X_1 + 6^*X_2 - 13^*X_3 - 7^*X_1^*X_2 - 7^*X_1^*X_3 - 15^*X_2^*X_3 - 3^*X_1^2 + 5^*X_2^2 + 15^*X_3^2 \qquad R^2 = 90\% \tag{8}$$

$$Y_8 = 16 - 0.9^*X_1 + 6^*X_2 - 4^*X_3 - 0.2^*X_1^*X_2 - 3^*X_1^*X_3 - 2^*X_2^*X_3 - 4^*X_1^2 - 2^*X_2^2 + 0.4^*X_3^2 \qquad R^2 = 87\% \tag{9}$$

The fitness of the model can be checked by the coefficient of determination (R^2). The R^2 value is always between 0 and 1. As R^2 reaches close to 1, the model becomes stronger and it predicts the response better (Jung et al., 2015). Results showed that R^2 were between 83 and 96%, which indicated that the model could explain these percentages of variability in the response. Regression

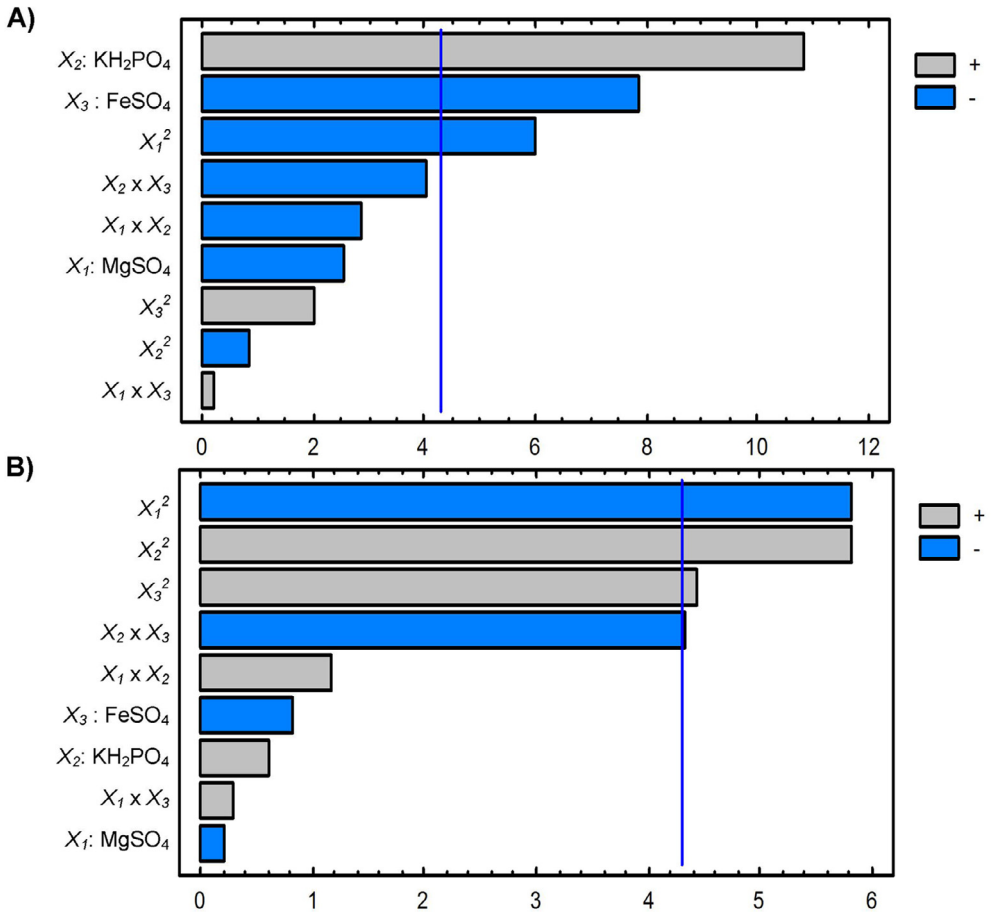

Fig. 2. Pareto charts with the estimated effect of the variables tested for (A) EGs activity and (B) CBHs activity in *P. sanguineus*. The confidence level was 95%.

equation can describe the real relationship between each variable and the response value. Eqs. (2–9) show that the cellulolytic production was positively influenced by KH_2PO_4.

The three-dimensional response surfaces were plotted to investigate the interaction between the variables and to determine the optimum concentration of each factor for maximum cellulase production. The contour plots of enzyme activities are shown in Figs. 3 and 4. The peaks and curvature indicated the maximum enzyme activity in the response surface plots. The shapes of the surfaces, circular (or) elliptical indicated remarkable interaction between the independent variables.

The optimum concentration of $MgSO_4.7(H_2O)$ coincided with the central point which corresponded to 0.2 g/L in *P. sanguineus* and 0.3 g/L in *I. lacteus*. For *I. lacteus*, the optimal concentration of KH_2PO_4 and $CaCl_2.2(H_2O)$ coincided with the central point corresponding to concentrations of 3 g/L and 0.3 g/L respectively, except for BGLs, where the optimum concentrations corresponded to +1 levels of both salts. For *P. sanguineus*, the highest level (+1) of KH_2PO_4 gave the maximum enzymatic activities. So, to find the optimum cellulolytic activities it was necessary to increase its concentration. The optimum concentration of $FeSO_4.7(H_2O)$ corresponded to −1 level (absence) for both strains.

3.3. Validation of experimental model

For *I. lacteus*, the maximum enzyme activities predicted by the RSM model were 1004 U/L for EGs, 51 U/L for CBHs, 81 U/L for BGLs and 112 U/L for FPA, while for *P. sanguineus* were 120 U/L for EGs, 17 U/L for CBHs, 94 U/L for BGLs and 27 U/L for FPA.

In order to verify the model, the optimal conditions were applied to three independent replicates for cellulolytic production. For *I. lacteus*, the average EGs activity was 808 U/L, for CBHs activity was 40 U/L, for BGLs was 82 U/L and for FPA was 87 U/L, whereas for *P. sanguineus* the EGs activity was 105 U/L, CBHs 10 U/L, BGLs 88 U/L and FPA was 29 U/L. The experimental values were in agreement with the predicted response.

3.4. Effect of KH_2PO_4 on cellulolytic production of P. Sanguineus

For *P. sanguineus*, the enzyme activities were positive influenced by KH_2PO_4 and the levels tested in the BBD were not high enough to find the optimum concentration, hence a new experiment was performed testing higher concentrations of this compound and the results are shown on Fig. 5.

Cellulolytic activities showed differences among the levels of KH_2PO_4 studied. In the case of EGs, CBHs and FPA, the activities values were similar for 9 and 12 g/L of KH_2PO_4, and higher than that produced with 6 g/L. For BGLs, the higher activities obtained corresponds to 9 g/L of KH_2PO_4 (155 U/L), so the appropriate concentration of KH_2PO_4 was 9 g/L.

The increase of 6–9 g/L of KH_2PO_4 involved, for example, an increase of 30 U/ L of BGLs (125–155 U/L). The gram of KH_2PO_4 costs 0.048 U$S, therefore, increasing from 6 to 9 g/L of KH_2PO_4 costs 0.144 U$S per liter of culture medium. 1 U of BGLs costs approximately 0.0245 U$S, so the increase of 30 U generated by the increment from 6 to 9 g/L of KH_2PO_4 corresponds to 0.651 U$S. Therefore, 0.144 U$S is invested to produce 30 U/L more, which in the market costs 0.651 U$S. The same occurs to the other cellulases.

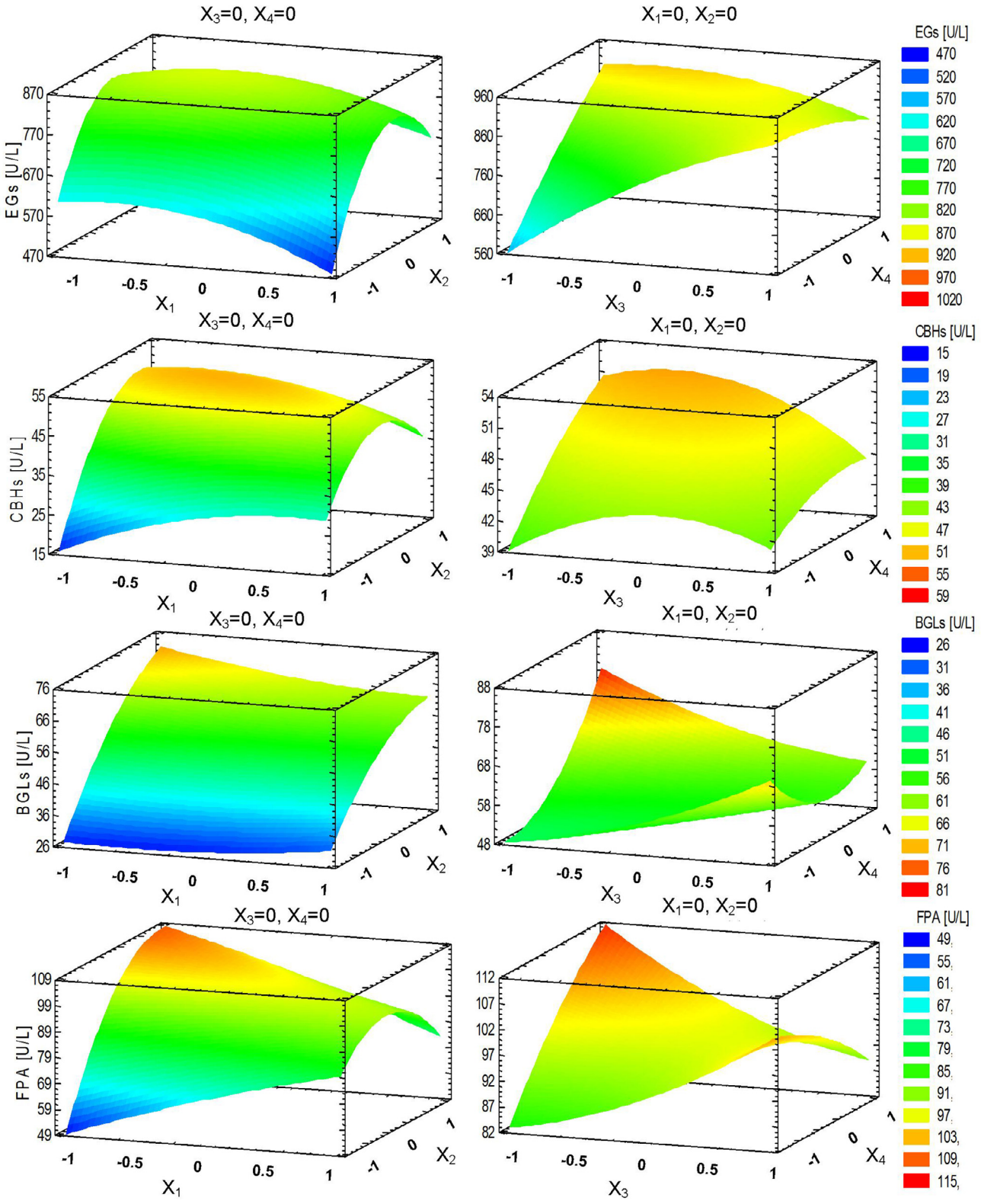

Fig. 3. Response surface plots of the Box-Behnken design to optimize the mineral medium for cellulolytic production by *I. lacteus*. X_1: $MgSO_47(H_2O)$, X_2: KH_2PO_4, X_3: $FeSO_47$ (H_2O), X_4: $CaCl_22(H_2O)$.

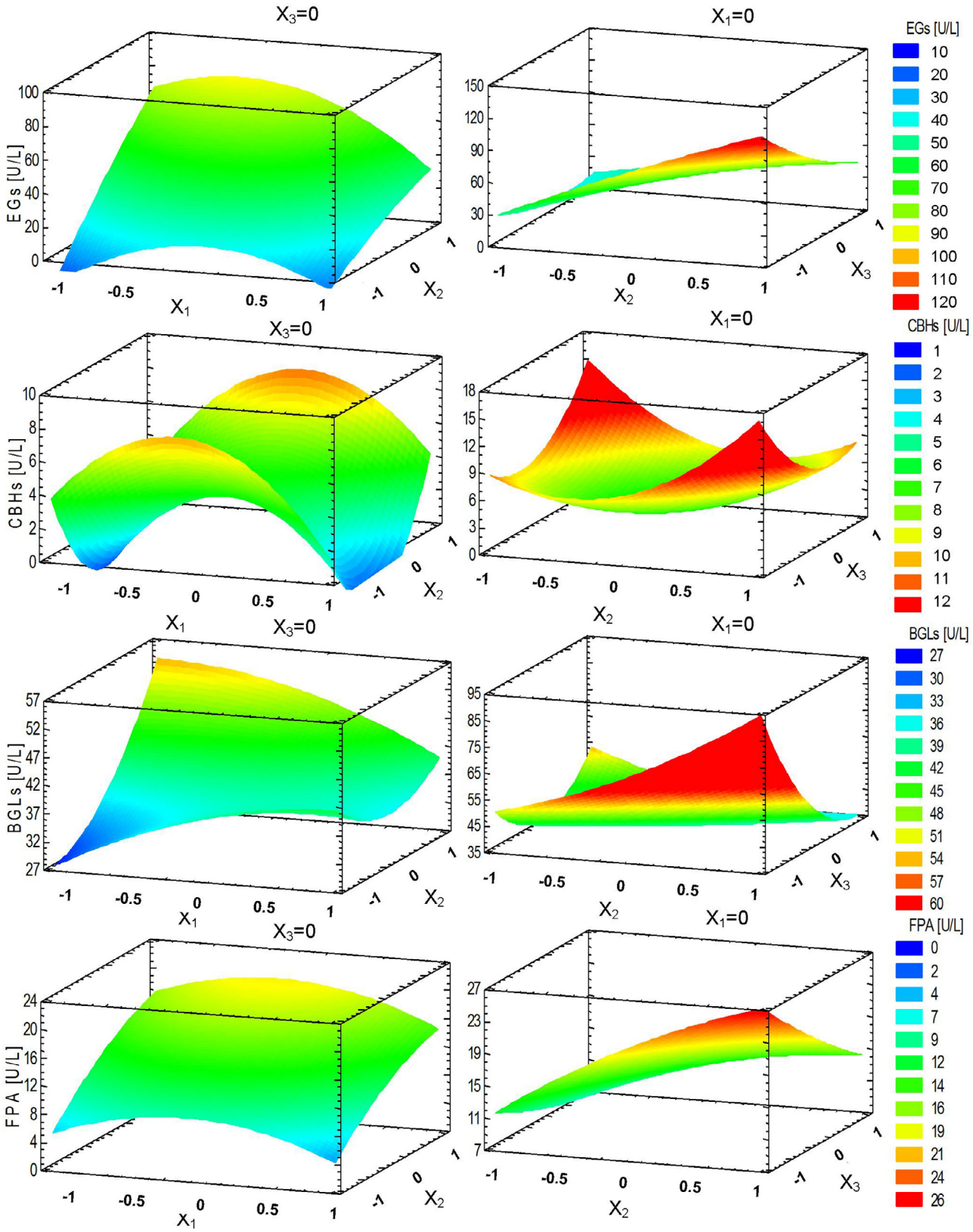

Fig. 4. Response surface plots of the Box-Behnken design to optimize the mineral medium for cellulolytic production by *P. sanguineus*. X_1: $MgSO_47(H_2O)$, X_2: KH_2PO_4, X_3: $FeSO_47(H_2O)$.

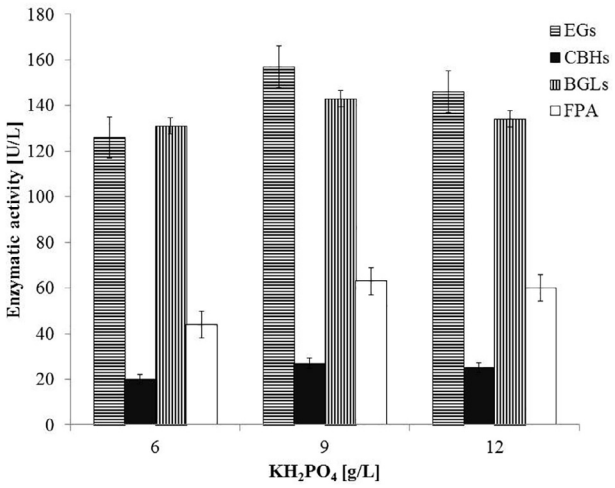

Fig. 5. Effect of KH_2PO_4 on cellulolytic production of *P. sanguineus.*

4. Discussion

4.1. Determination of significant variables for cellulolytic production by 2^{6-1} fractional factorial design

Analyzing the activity of each enzyme, it was found that the KH_2PO_4 positively influenced cellulolytic production on both strains. KCl had an increase of 12% in the production of EGs by *Peniophora* sp. (Trinh et al., 2013). Other authors have found that cellulase production by *Trichoderma reesei*, measured as FPA, was markedly reduced in the absence of KH_2PO_4 (Li et al., 2011; Wen et al., 2005). As regard to $FeSO_4.7(H_2O)$, a positive influence on CBHs activity of *P. sanguineus* and FPA of *I. lacteus* was observed. Other authors found that $FeSO_4.7(H_2O)$ at concentrations between 0.05 and 0.2% negatively influenced in EGs (Trinh et al., 2013) and CBHs production by *Trametes versicolor* (Shah et al., 2010). The $CoCl_2.6(H_2O)$ negatively influenced most of the cellulolytic enzymes (Table 4). For *T. reesei*, it was found that cobalt was not crucial for cellulolytic production (Wen et al., 2005). With respect to $CaCl_2.2(H_2O)$, its influence was positive, and its interaction with KH_2PO_4 was also positive in *I. lacteus*. $CaCl_2.2(H_2O)$ maximized the production of cellulases secreted by *Chaetomium* sp. (Kapoor et al., 2010) and *Bacillus licheniformis* NCIM 5556 (Shajahan et al., 2017). The $MgSO_4.7(H_2O)$ had a positive influence, but its interaction with other minerals was negative. The positive influence of $MgSO_4.7(H_2O)$ in the production of EGs is in agreement with similar behavior found by other authors (Singh and Kaur, 2012).

Complex substrates such as lignocellulosic residues induce the co-production of substrate degrading enzymes. For applications, improvement and modification of the complex molecular structure of lignocellulosic materials, the synergistic action of substrate degrading enzymes is required and crude enzymes can be more efficient than purified enzymes, decreasing total production cost considerably (Moteshafi et al., 2016; Yennamalli et al., 2013). For these reasons, it is important to study conditions in order to promote the production of cellulolytic cocktail.

4.2. Optimization of mineral medium using Box-Behnken design

After FFD experiments, the components of the mineral medium with significant influence on cellulolytic production were identified, and the concentration thereof was optimized by an optimization design.

For *P. sanguineus*, the optimal concentration of $CaCl_2.2(H_2O)$ was 0.1 g/L, value corresponding to an optimized composition for *T. reseei* using manure as the carbon source (Wen et al., 2005). For *I. lacteus*, the optimal concentration of $CaCl_2.2(H_2O)$ was 0.3 g/L. Regarding to $MgSO_4.7(H_2O)$, the optimal concentrations were 0.2 g/L and 0.3 g/L for *P. sanguineus* and *I. lacteus*, respectively. For *Penicillium echinulatum*, the optimum value of $MgSO_4.7(H_2O)$ and $CaCl_2.2(H_2O)$ were 0.375 g/L for each mineral, maximizing EGs, BGLs and FPA activities (dos Reis et al., 2015).

Through the BBD, it was found that the optimum concentration of KH_2PO_4 was 3 g/L for *I. lacteus*, but for *P. sanguineus* it was necessary a further experiment to find the right concentration of the mineral, corresponding to 9 g/L. For *T. reesei* the maximum production of EGs and total cellulases was achieved with 4 g/L, after optimizing the concentration of KH_2PO_4 (Hao et al., 2006).

Regard to each particular cellulase, higher EGs activities were obtained with *I. lacteus* (808 U/L). *Aspergillus niger* and *T. reesei* employing carboxymethylcellulose as carbon source produced 70 and 90 U/L respectively while *A. ochraceus* produced 225 U/L cultivated on rice straw (Lee et al., 2011). EGs activity was improved in both strains, with 800 U/L by *I. lacteus* and 145 U/L by *P. sanguineus* against 500 y 80 U/L in Mandels medium without optimization (Rodríguez et al., 2015).

Optimized medium improved cellulase production, especially BGLs activity values for *I. lacteus* (81 U/L), which were found to be 40-fold higher than conventional Mandels medium. For *P. sanguineus* the increase was 8-folds, 155 U/L in optimized medium against 19 U/L in traditional Mandels medium, however, this value was low compared with those reported by other authors using lignocellulosic substrates and synthetic medium (Lee et al., 2011; Yoon et al., 2013).

Regarding to FPA, increasing was not significant, however, for CBHs activity, in both strains were obtained 2-folds higher activity respect to traditional Mandels medium (27 U/L for *I. lacteus* and 14 U/L for *P. sanguineus*) (Rodríguez et al., 2015). The major CBHs activity corresponded to *I. lacteus* (40 U/L). This result was similar that produced by *P. sanguineus* employing corncob as carbon source (Falkoski et al., 2012). *I. lacteus* also produced the higher FPA (87 U/L) and this result was comparable as that obtained by *T. reesei* employing synthetic medium (Ezekiel et al., 2010).

5. Conclusions

Important variables of culture media for cellulase production were investigated by FFD. From results, the significant variables were investigated on BBD. Optimal conditions of culture media for cellulase production were (g/L): for *I. lacteus* $CaCl_2.2(H_2O)$ 0.3, $MgSO_4.7(H_2O)$ 0.3 and KH_2PO_4 3, while for *P. sanguineus* was $CaCl_2.2(H_2O)$ 0.1, $MgSO_4.7(H_2O)$ 0.2 and KH_2PO_4 9. KH_2PO_4 was found in this work to be a useful mineral compound for cellulolytic production. Therefore, the model was reliable for maximizing cellulase production of *I. lacteus* and *P. sanguineus*. Thus, this study demonstrated that RSM with appropriate experimental design can be effectively applied for the optimization of the variables of culture media in microbial fermentation. For these strains, however, remain to study the operational parameters such as culture temperature, pH and speed of agitation.

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