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POTASSIUM BIPHTHALATE BUFFER FOR pH CONTROL TO OPTIMIZE GLYCOSYL HYDROLASE PRODUCTION IN SHAKE FLASKS USING FILAMENTOUS FUNGI

P. dos S. Costa[1,2*], D. Robl[1,3], I. C. Costa[1], D. J. da S. Lima[1], A. C. Costa[2] and J. G. da C. Pradella[1]

[1]Brazilian Bioethanol Science and Technology Laboratory (CTBE), Brazilian Centre of Research in Energy and Materials (CNPEM), Rua Giuseppe Maximo Scolfaro 10000, Pólo II de Alta Tecnologia, CEP 13083-970, Campinas, São Paulo, Brazil.
[2]School of Chemical Engineering, State University of Campinas, Campinas, São Paulo, Brazil.
Tel.: +55 19 3518 3149; Fax: +55 19 3518 3104
E-mail address: patriciacosta.ufv@gmail.br
[3]Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil.

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Abstract – The optimization of culture medium with statistical methods is widely used in filamentous fungi glycosyl hydrolase production. The implementation of such methodology in bioreactors is very expensive as it requires several pH-controlled systems operating in parallel in order to test a large number of culture media components. The objective of this study was to evaluate potassium biphthalate buffer for pH control, which allows the optimization studies to be performed in shake flasks.The results have shown that buffering the culture medium with 0.1 M potassium biphthalate allowed pH control, resulting in a decrease of the standard deviation of triplicates for pH and activities of glycosyl hydrolase measurements. The use of this buffer allowed shake flask culture media optimization of enzyme production by *Trichoderma harzianum*, increasing the cellulase activity by more than 2 times compared to standard unbuffered culture medium. The same buffer can be used for culture media optimization of other fungi, such as *Penicillium echinulatum.*

Keywords: Potassium biphthalate buffer; pH control; enzyme production; glycosyl hydrolase; filamentous fungi.

INTRODUCTION

Cellulosic ethanol has been gaining increasing attention as an environmentally friendly biofuel with potential to compete with gasoline. Nevertheless, production costs are still prohibitive and the main costs are related to enzyme production and enzymatic hydrolysis of lignocellulosic material. The latter relies on the use of a plethora of glycosyl hydrolase enzyme complexes, which are mainly composed of cellobiohydrolase, endoglucanases and β-glucosidases in addition to a diversity of hemicellulases and other accessory proteins that help unlock the ligocellulosic complex arrangement (Berlin et al. 2005; Kumar et al. 2009). Cellulolytic filamentous fungi *Trichoderma, Penicillium, Aspergillus,* among others, have been used industrially and academically for glycosyl hydrolase enzyme complex production (Delabona et al., 2013b; dos Reis et al., 2013; Pereira et al., 2013; Dillon et al., 2006). Filamentous fungi such as *Trichoderma harzianum* and *Penicillium echinulatum* have emerged as potential candidates for increasing the efficiency of glycosyl hydrolase enzymes complex production.

*To whom correspondence should be addressed

Culture media and bioreactor process optimization (Delabona et al., 2013a; Delabona et al., 2013b; dos Reis et al., 2013; Pereira et al., 2013), as well as genetic improvements of these filamentous fungi (Dillon et al., 2006), have been studied to decrease production costs. Culture medium optimization by statistical methodologies is of great interest for filamentous fungi glucosyl hydrolase improvement (Delabona et al., 2013; Li et al., 2007). However, this methodology requires a great number of experiments and its implementation in bioreactors is very expensive and the shake flask submerged cultivation method is an attractive choice to access this information. It is known that the best pH for induction of cellulase for *P. echinulatum* S1M29 biosynthesis in a bioreactor is from 5.5 to 6.5 (dos Reis et al., 2014), while a pH from 5.0 to 6.0 is the best for cellulase induction for *T. harzianum* P1P49 in bioreactor cultivation (Delabona et al. 2012). On the other hand, the type and the concentration of culture media ingredients affect the pH during fermentation and it is difficult to predict pH fluctuations. In the case of high-throughput screening experiments carried out in shake flasks this fact may lead to inconclusive results as pH influences enzyme production. Therefore, an adequate control of this parameter during fungal submerged cultivation in shaking flasks is of paramount importance.

Potassium biphthalate buffer has a set of interesting physico-chemical properties that turns it into a potential candidate to stabilize the pH during microorganism growth. It covers a large range of pH in the proper range for cellulase production by fungus (3.4 to 5.9); it is not consumed, unlike citrate buffers; and it is physiologically compatible with filamentous fungi (Domingues et al., 2000; Ferreira et al., 2009).

The present work describes the utilization of potassium biphtalate buffers in shake flask cultivation of *P. echinulatum* and *T. harzianum* grown in complex culture media components (sugarcane bagasse submitted to different pre-treatments, cellulose, sucrose, soybean bran, wheat bran and yeast extract). The use of this methodology led to shake flask culture media optimization of enzyme production by *Trichoderma harzianum*, increasing the cellulase activity by more than 2 times compared to standard unbuffered culture medium. The same buffer can also be used for culture media optimization of *P. echinulatum.*

MATERIALS AND METHODS

Microorganism

The filamentous fungus *Trichoderma harzianum* P49P11, isolated in the Amazon Rainforest (Delabona et al., 2012), was obtained from Embrapa Instrumentation - São Carlos, SP, deposited in the collection of microorganisms of Embrapa Food Technology (Rio de Janeiro, Brazil), access number BRMCTAA115. The filamentous fungus *Penicillium echinulatum* S1M29 is a mutant (Dillon et al., 2011) provided by the University of Caxias do Sul, RS, Brazil. Microorganisms used were stored in glycerol solution (30 % v/v) at - 80 °C.

Submerged fermentation

All submerged fermentation experiments were conducted in a shaker using a 500 mL erlenmeyer flask containing 200 mL of the culture media at 200 rpm (Inova 44R, New Brunswick Scientific, USA) and incubated at 29°C as suggested by Pereira et al. (2013) and Machado et al. (2012) for *P. echinulatum* and Delabona et al. (2012, 2013a, 2013b) for *T. harzianum*.

The filamentous fungi were grown on PDA plates at 29 °C for 7 days. The spores were harvested with 20 mL of *Tween 80* solution (0.1 % v/v) and transferred to a pre-inoculum medium composed of Celufloc 200™ (CEL) (Celuflok Ind Com, Brazil) 10 g/L, glucose (GL) 10 g/L, peptone (PEP) 1 g/L, salt solution (Mandels & Reese, 1957) (SS) 100 mL/L and *Tween 80* 1 mL/L.The pre-inoculum was kept at 29 °C, 200 rpm for 72 hours. A volume of 20 mL of this pre-inoculum was transferred to a 500 mL *Erlenmeyer* flask containing 200 mL of culture media with composition varying according to the experimental design with and without potassium biphthalate buffer at pH 5.0 or 5.9, according to the studied conditions, and incubated in the same conditions described above. Experiments were designed and analysed using the DOE (Design of Experiments) package from STATISTICA 7.0 (Statsoft, USA). Mathematical modelling optimization used the backward elimination technique (Neto et al., 2010; Rodrigues & Iemma, 2005) and the embeded subroutine SOLVER (Excel 2013; Microsoft Inc. USA).

Buffer effect on enzymatic activity in different inductor carbon sources

Experiments were carried out in order to test the influence of potassium biphthalate buffer in fungal cultivation with different inductor carbon sources of glycosyl hydrolase measured as cellulase activity.

The influence of potassium biphtalate buffer was investigated for *P. echinulatum* S1M29 using different pretreated sugarcane bagasse at 10 g/L: steam exploded sugarcane bagasse (BEX), steam exploded sugarcane bagasse followed by hot NaOH delignification (BED), hydrothermal pretreated sugarcane bagasse (BH), and commercial micropulverized cellulose Celufloc200™ (Celuflok Ind Com, SP, Brazil) (CEL) supplemented with sucrose (SUC) 5.0 g/L, ammonium sulfate (SM) 1.4 g/L, soybean bran (SB) 2.0 g/L, wheat bran (WB) 5.0 g/L, yeast extract (YE) 0.5 g/L, *Tween* 80 1.0 mL/L, and salt solution (SS) 100 mL/L (dos Reis et al., 2013). These experiments were performed both in the presence and the absence of potassium biphthalate buffer (0.1M) at pH 5.9.

The influence of potassium biphthalate buffer on the cellulase activity was investigated for *T. harzianum* P49P11 in a culture medium composed of delignified steam exploded sugarcane bagasse (BED) 7 g/L, sucrose (SUC) 3 g/L, ammonium sulfate (SM) 1.4 g/L, peptone (PEP) 1 g/L, Tween 80 1 mL/L and salt solution (SS) 100 mL/L (dos Reis et al., 2013). These experiments were also performed both in the presence and the absence of potassium biphthalate buffer (0.1M) pH 5.0.

All the experiments were run in triplicate and results were presented as mean and standard deviation.

Experimental design

The experiments aimed to analyze the influence of potassium biphthalate buffer on pH control during the culture media optimization experiments using shake flasks, and pointed out the possible influence of these components on the pH variation.

Three experimental designs were carried out with *P. echinulatum* PS1M29 fungus: one Fractional Factorial Design (2^{5-1}) (FFD) and two Central Composite Rotational Designs, CCRD1 and CCRD2. The FFD (2^{5-1}) was performed in order to study five culture medium components (concentration range in parenthesis); SB (0-5 g/L), WB (0-10 g/L), YE (0-2 g/L), SUC (0-10 g/L) and salt solution SS (50-300 mL/L). The CCRD1 used high substrate concentrations of SB (5-15 g/L), YE (5-7.5 g/L) and BEX (10-30 g/L). The initial pH was set up at 5.9 using 0.1 M of potassium biphthalate buffer. The CCRD2 was performed by testing different amounts of SB (0-5 g/L) and YE (0-3 g/L) at initial pH 4.7 using 0.1 M biphthalate buffer. The culture media composition and the run number used in these DOE are displayed in Tables 1, 2 and 3.

Three statistical designs were carried out with *T. harzianum* P49P11 fungus: one Fractional Factorial Desing FFD (2^{6-1}) and two Central Composite Rotational Desings CCRD1 and CCRD2. The FFD (2^{6-1}) evaluated the influence of components SB (0-10 g/L), WB (0-5 g/L), SUC (0-10 g/L), YE (0-2 g/L), PEP (0-2 g/L) and SS (50-100 mL/L) on pH fluctuation. The CCR1 studied culture media composed of SUC (0-10 g/L), CEL (0-20 g/L) and SM (0-15 g/L), while the CCR2 studied culture media composed of SUC (0-10 g/L), CEL (0-20 g/L) and WB (0-15 g/L).

The culture media composition and the run number used in these DOE are displayed in Tables 4 and 5.

When not studied as above described other culture media components were fixed as follows: Celufloc (CEL) 10 g/L; ammonium sulfate (SM) 1.4 g/L, peptone (PEP) 1 g/L, Tween 80 1 mL/L and salt solution (SS) 100 mL.

For all the experimental designs, samples were collected at different fermentation times, centrifuged at 10.000 × *g* for 10 minutes at 10 °C and the supernatants had their pH measured. Activities were determined and the point of maximum activity was used as the response of the experimental design. Standard errors were calculated using central point replicates, as designed by the DOE package of Statistica 7.0.

Table 1. Culture media composition for *P. echinulatum* S1M29, FFD 2^{5-1}experiments.

Run	Soybean bran (g/L)	Wheat bran (g/L)	Sucrose (g/L)	Yeast extract (g/L)	Salt Solution (ml/L)
1	0.00	0.00	0.00	2.00	300.00
2	5.00	0.00	0.00	0.00	50.00
3	0.00	10.00	0.00	0.00	300.00
4	5.00	10.00	0.00	2.00	50.00
5	0.00	0.00	10.00	2.00	50.00
6	5.00	0.00	10.00	0.00	300.00
7	0.00	10.00	10.00	0.00	50.00
8	5.00	10.00	10.00	2.00	300.00
9	2.50	5.00	5.00	1.00	175.00
10	2.50	5.00	5.00	1.00	175.00
11	2.50	5.00	5.00	1.00	175.00
12	2.50	5.00	5.00	1.00	175.00
13	0.00	0.00	0.00	2.00	300.00
14	5.00	0.00	0.00	0.00	50.00
15	0.00	10.00	0.00	0.00	300.00
16	5.00	10.00	0.00	2.00	50.00
17	0.00	0.00	10.00	2.00	50.00
18	5.00	0.00	10.00	0.00	300.00
19	0.00	10.00	10.00	0.00	50.00
20	5.00	10.00	10.00	2.00	300.00

Table 2. Culture media composition for *P. echinulatum* S1M29, CCRD1experiments.

Run	Soybean bran (g/L)	Yeast extract (g/L)	Steam exploded sugarcane bagasse (g/L)
1	5	5	10
2	15	5	10
3	5	7.5	10
4	15	7.5	10
5	5	5	30
6	15	5	30
7	5	7.5	30
8	15	7.5	30
9	1.6	6.25	20
10	18.4	6.25	20
11	10	4.14	20
12	10	8.35	20
13	10	6.25	3.2
14	10	6.25	36.8
15	10	6.25	20
16	10	6.25	20
17	10	6.25	20
18	10	6.25	20
19	10	6.25	20
20	10	6.25	20

Table 3. Culture media composition for *P. echinulatum* S1M29, CCRD2experiments.

Run	Soybean bran (g/L)	Yeast extract (g/L)
1	7.2	1.44
2	17.8	1.44
3	7.2	3.6
4	17.8	3.6
5	5	2.5
6	20	2.5
7	12.5	1
8	12.5	4
9	12.5	2.5
10	12.5	2.5
11	12.5	2.5
12	12.5	2.5
13	12.5	2.5

Table 4. Culture media composition for *T. harzianum* P49P11, FDD 2^{6-1} experiments.

Run	Soybean bran (g/L)	Wheat bran (g/L)	Sucrose (g/L)	Yeast extract (g/L)	Peptone (g/L)	Salt solution (mL/L)
1	0	0	0	0	0	50
2	10	0	0	0	0	100
3	0	5	0	0	0	100
4	10	5	0	0	0	50
5	0	0	10	0	0	100
6	10	0	10	0	0	50
7	0	5	10	0	0	50
8	10	5	10	0	0	100
9	0	0	0	2	0	100
10	10	0	0	2	0	50
11	0	5	0	2	0	50
12	10	5	0	2	0	100
13	0	0	10	2	0	50
14	10	0	10	2	0	100
15	0	5	10	2	0	100
16	10	5	10	2	0	50
17	0	0	0	0	2	100
18	10	0	0	0	2	50
19	0	5	0	0	2	50
20	10	5	0	0	2	100
21	0	0	10	0	2	50
22	10	0	10	0	2	100
23	0	5	10	0	2	100
24	10	5	10	0	2	50
25	0	0	0	2	2	50
26	10	0	0	2	2	100
27	0	5	0	2	2	100
28	10	5	0	2	2	50
29	0	0	10	2	2	100
30	10	0	10	2	2	50
31	0	5	10	2	2	50
32	10	5	10	2	2	100
33	5	2.5	5	1	1	75
34	5	2.5	5	1	1	75
35	5	2.5	5	1	1	75
36	5	2.5	5	1	1	75
37	5	2.5	5	1	1	75
38	5	2.5	5	1	1	75
39	5	2.5	5	1	1	75
40	5	2.5	5	1	1	75

Table 5. Culture media compositions for CCRD1 (using Soybean Bran) and CCRD2 (using Wheat Bran) experiments for *Trichidermaharzianum*P49P11.

Run	Sucrose (g/L)	Celufloc (g/L)	Soybean Bran (g/L)	Wheat bran (g/L)
1	2.02	4.05	3.04	3.04
2	7.97	4.05	3.04	3.04
3	2.02	15.94	3.04	3.04
4	7.97	15.94	3.04	3.04
5	2.02	4.05	11.95	11.95
6	7.97	4.05	11.95	11.95
7	2.02	15.94	11.95	11.95
8	7.97	15.94	11.95	11.95
9	0.00	10.00	7.50	7.50
10	10.00	10.00	7.50	7.50
11	5.00	0.00	7.50	7.50
12	5.00	20.00	7.50	7.50
13	5.00	10.00	0.00	0.00
14	5.00	10.00	15.00	15.00
15	5.00	10.00	7.50	7.50
16	5.00	10.00	7.50	7.50
17	5.00	10.00	7.50	7.50
18	5.00	10.00	7.50	7.50

In all the experiments pH levels were measured using a pH probe and standard potentiometer (Mettler Toledo S220 model) and pH variation was calculated as the difference between the initial and final pH values. Supernatant cellulase activity was measured using the Filter Paper method with a reduction scale of 10 times (Ghose, 1987), from now on expressed as FPase activity and reported as FPU/mL. Reducing carbohydrate was measured by the DNS method (Miller, 1959), using glucose as a standard.

Schematic representation of the obtained results

A schematic representation of the present work is given in Figure 1.

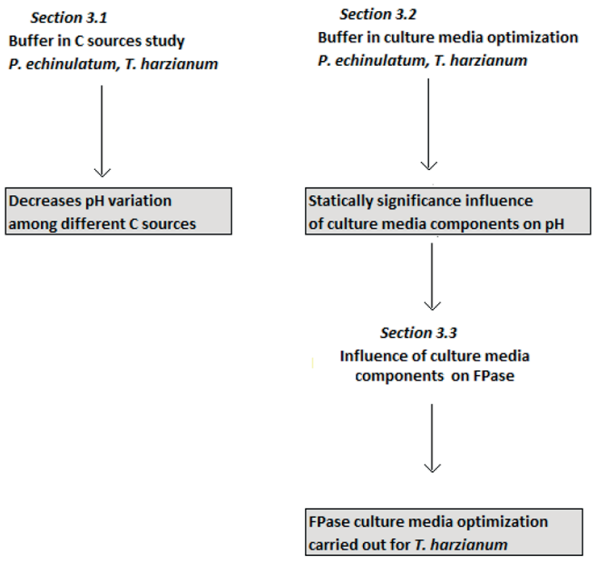

Figure 1: Diagram of experiments carried out with the main results (marked in gray) for shake flask cultivation of *P. echinulatum* S1M29 and *T. harzianum* P49P11 using potassium biphthalate buffer aiming at glycosyl hydrolase production.

RESULTS AND DISCUSSION

Buffer effect on pH variation and cellulase production using different inductor carbon sources

Sugarcane bagasse pretreated by different methods (BEX, BED and BH) or standard micronized commercial cellulose Celufloc (CEL) were used for *P. echinulatum* S1M29 and *T. harzianum* P11P49 as the glucosyl hydrolase inductor carbon source in shake flask experiments. The objective was to evaluate the influence of potassium biphthalate buffer (pH 4.7 to 5.9) on the FPase enzyme activity and culture broth pH variation over time.

Submerged fermentation experiments with *P. echinulatum* were run both in unbuffered and buffered condititions. The latter was conducted with a potassium biphatalate buffer at initial pH 5.9, known to be the optimum pH range for cellulase biosynthesis (dos Reis et al., 2014). The unbuffered assays displayed a large pH fluctuation over time for all tested carbon sources, decreasing from ~5.9 to 3.5 and returning to ~5.9 at the 144 h of fermentation (Fig 2a). On the other hand, buffered experiments decreased the pH fluctuation (Fig 2b) for all the tested carbon sources, with pH varying from ~ 6.0 to 5.0 in the first 72h and then rising to ~ 5.7 at the end of the experiment. Therefore, the use of biphthalate buffer accomplished its function to stabilize the pH in shake flasks cultivation and did not jeopardize the enzyme production in the tested conditions.

A previous study on the effect of various inductor sources carried out with *T. hazrzianum* P49P11 in controlled bioreactors demonstrated that delignified steam exploded bagasse (BED) was, among the tested sources, the most convenient for cellulase on-site production (Delabona et al. 2012), so experiments with bagasse submitted to the other pretreatments were not performed in the present work.

Cellulase production by *T. harzianum* P49P11using BED as inductor at pH 5.0 was quite different in buffered and unbuffered condition (Fig 3a and 3b). Stabilization of pH fluctuation over time was observed with the use of biphtalate buffer, with the pH maintained around 5.0, while the culture broth pH dropped to 3.5 in unbuffered conditions (Fig 3a). The maximum FPase activity attained 0.8 ± 0.02 FPU/mL from 72h to 96h when potassium biphthalate was used, while for the unbuffered culture this value never exceeded 0.4 ± 0.13 FPU/mL (Fig 3b). Clearly, a pH below 4.0 impaired glycosyl hydrolase biosynthesis in the latter experiments. This endorses the results of Delabona *et al.* (2012), who claimed that a pH range from 5.0 to 6.0 would enhance *T. harziamum* P1P49 FPase titers. Besides, the experiments carried out with *P. echinulatum* and *T. harzianum* demonstrated the efficiency of the proposed buffer system and corroborated the results of Ferreira et al. (2009), who first proposed the use of potassium biphtalate buffer in submerged shake flask cellulase production by *T. reesei*.

Culture medium components influence pH fluctuation

Culture media optimization is a key study to increase cellulose titers and therefore decrease the cost of the cellulolytic enzyme complex. However, culture media ingredients and their concentration may affect pH equilibrium during cell growth, jeorpardizing enzyme production if this parameter is not controlled. In order

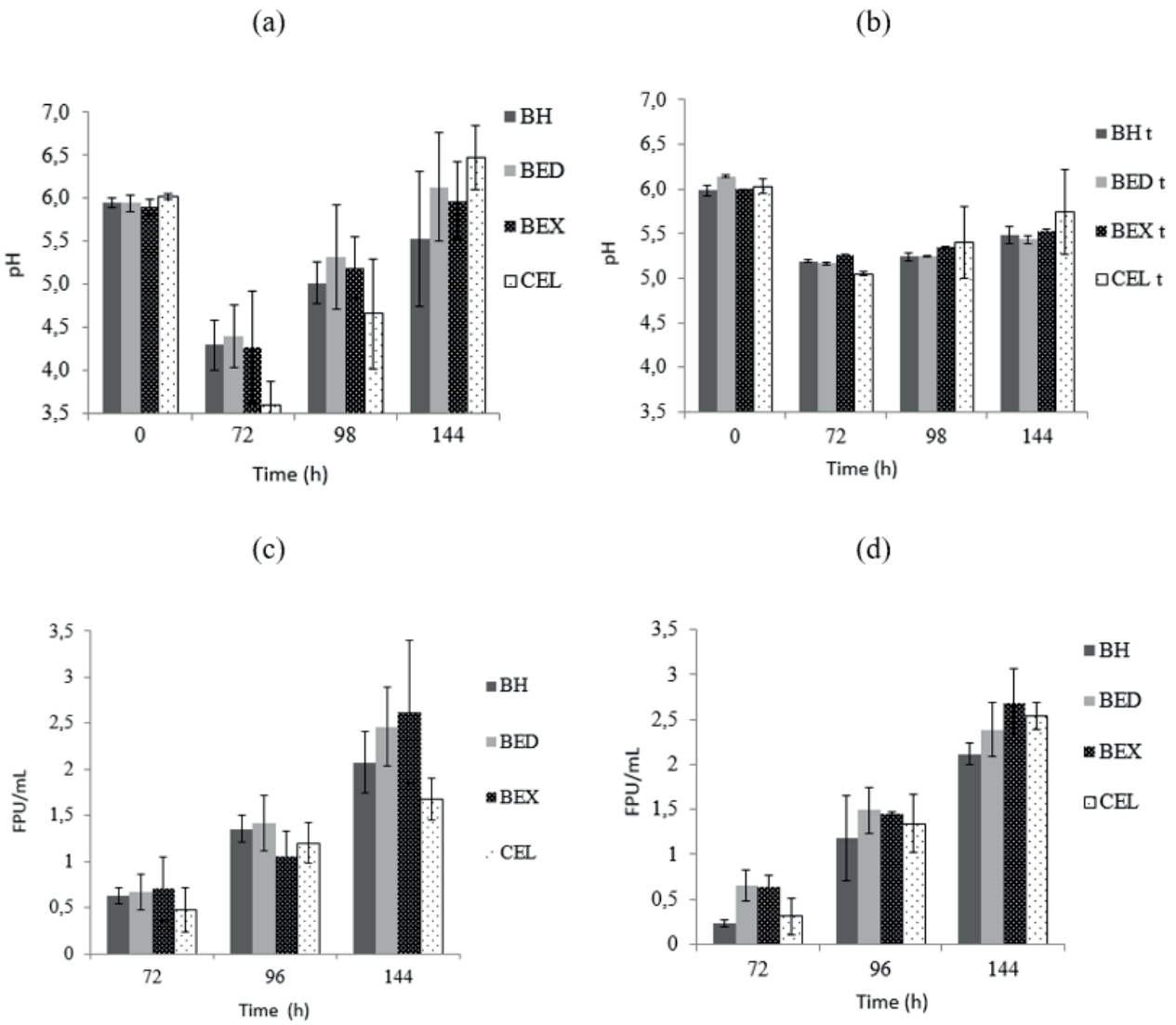

Figure 2: Time evolution of pH and FPase titer for *Penicillium echinulatum* S01M29 submerged cultivation in different carbon sources. (a) without pH control. (b) pH controlled by 0.1M potassium biphthalate. (c) FPase without pH control. (d) FPase controlled at pH 5.9 with 0.1 M potassium biphthalate buffer. Values of triplicates with their respective standard deviation.

to evaluate the potassium biphtalate buffer efficiency for pH control in shake flask cultivation experiments, several DOE were set up for the filamentous fungi *P. echinulatum* and *T. harzianum*. The culture media ingredients were selected based on previous glycosyl hydrolase production studies using *P. echinulatum* (Pereira et al., 2013) and *T. harzianum* (Delabona et al., 2013b).

P. echinulatum S1M29 case

Three sets of experiments were carried using *P. echinulatum* S1M29 with the potassium biphtalate buffer: a Fractional Factorial Design (FFD), which was set up to study the influence of five different culture media components, and two Central Composite Rotational Designs (CCRD1 and CCRD2) using a reduced number of culture media components, SB (soybean bran), YE (yeast extract) and BEX (delignified steam exploded sugarcane bagasse).

The assigned number of the run and the corresponding culture media compositions for the FFD, CCRD1 and CCRD2 are presented respectively in Tables 1 to 3. Figure 4 shows the values of ΔpH (ΔpH = final pH-intial pH) observed for the three sets of experiments. In Figure 4 the *x* axis stands for the number of the run presented in Tables 1 to 3. The statistical main effects of the considered medium components on ΔpH are summarized in Table 6.

The FFD carried out for *P. echinulatum* indicated that the influences of the studied culture medium components were not significant at 95 % of confidence level on pH variation, as p-values were much higher than 0.05 for all

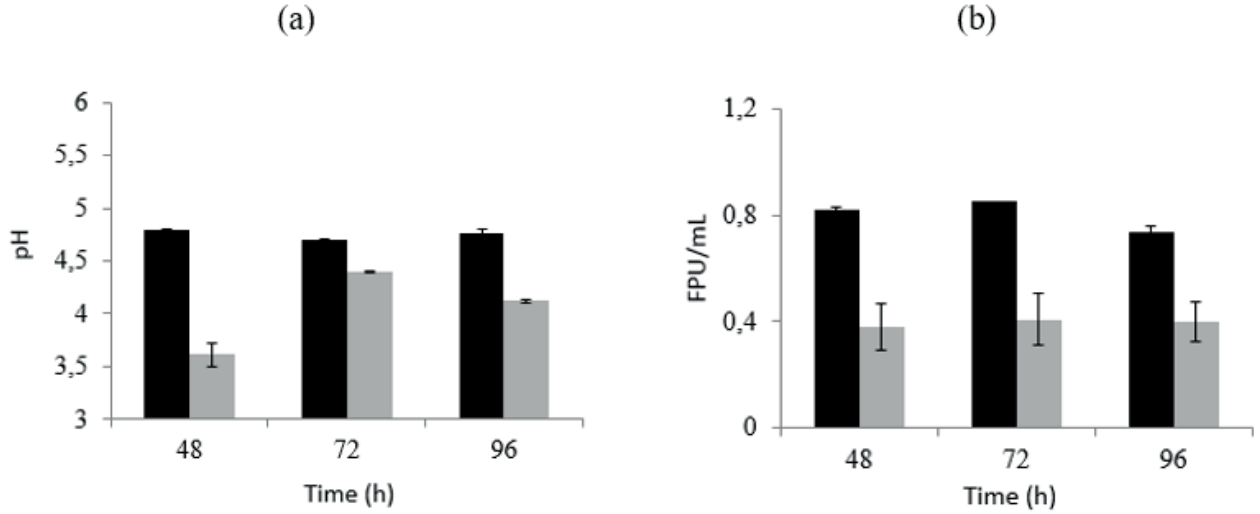

Figure 3: Time evolution of pH and FPase titer for *Trichoderma harzianum* P49P11 S01M29 submerged cultivation in culture medium, according to Delabona et al. 2013. (a) FPase in controlled pH 5.0 by 0.1 M potassium biphthalate buffer (black bar) FPase in uncontrolled pH (grey bar). (b) pH controlled by 0.1 M potassium biphthalate buffer (black bars) and without pH control (grey bar). Values of triplicates with their respective standard deviation.

the tested culture media components (Table 6). Figure 4a shows that pH fluctuation during cultivation was in the range of ± 1.0 around the initial value of 5.9. It was previously shown that the highest glycosyl hydrolase production was observed at pHs around 6.0 (dos Reis et al., 2014). Therefore, the potassium biphtalate buffer used was very efficient in maintaining pH at the optimum value for *P. echinulatum* glycosyl hydrolase biosynthesis, enabling the optimization studies for glycosyl hydrolase production to be performed in shake flasks.

To explore the possibility of the use of this methodology in a less mild environment, CCRD1 was run at much higher concentrations of soybean flour (SB) (5-15 g/L), yeast extract (YE) (5-7.5 g/L) and steam exploded bagasse (BEX) (10-30 g/L) (Table 2). In this circumstance, despite the use of potassium biphtalate buffer, the ΔpH variation was larger than ±1.0 and pH values as high as 8.5 were observed (Fig 4b). Effects analysis indicates that these values were probably due to high concentrations of SB and YE (Table 6). The BEX contribution was statistically significant to decrease culture broth pH. Mathematical modeling to study the influence of culture media on cellulase production was therefore not possible in shake flasks as the biphthalate buffer was not efficient to attain culture broth pH control.

To avoid this effect, CCRD2 experiments were run with lower SB and YE concentration levels, respectively 0-3 g/L and 0-5 g/L, maintaining the BEX concentration at 20 g/L. It was also anticipated that NH_4^+ secretion due to metabolism of SB and YE would increase the culture broth pH. To minimize this problem the initial pH values for the experiments with potassium biphthalate buffer were diminished to 4.7. Soybean bran and yeast extract increased the culture broth pH (Table 6), but a low pH variation was observed (Figure 4c).

Therefore, it was possible to maintain ΔpHs within quite a low range, which enabled us to carry out mathematical modeling studies to attain culture media optimization for glycosyl hydrolase production in shake flasks using the potassium biphtalate buffer.

T. harzianum P49P11 case

Three sets of experiments were performed with *T. harzianum* P49P11 using potassium biphtalate buffer: one Fractional Factorial Design (FFD) to verify the influence of five different culture media components, and two Central Composite Rotational Designs (CCRD1 and CCDR2) using a reduced number of culture media components.

The assigned number of the run and the corresponding culture media compositions for the FFD, CCRD1 and CCRD2 are presented respectively in Tables 4 and 5. Figure 5 shows the values of ΔpH (ΔpH = final pH-intial pH) observed for the three sets of experiments. The Figure 5 *x* axis stands for the number of the run presented in Tables 4 to 5. The main statistical effects of the considered medium components on ΔpH are summarized in Table 7.

Results from Table 7 show that in fact almost all the tested culture media components (except salt solution -SS, and wheat bran -WB) influenced the pH variation at the 95 % confidence level for all the DOEs carried out, demonstrating the high sensitivity of *T. harzianum* culture medium formulation to pH adjustment even with the use of the the proposed buffer.

Soybean bran (SB), yeast extract (YE) and peptone (PEP) exerted great influence on increasing the pH of the

Table 6. Main effects of culture medium components in the different DOE on pH variation (ΔpH = final pH - initial pH) in shake flasks *Penicillium echinulatum* S1M29 cultivation. The initial pH was 5.9 for the FFD and CCDR1 experiments, and 4.7 for CCRD2 experiments. Components that presented a significant effect at the 95 % confidence level are marked in gray (*see Tables 1, 2 and 3 for culture media compositions*).

Culture media components	FFD			CCDR1			CCDR2		
	Range	p-value	Effect	Range	p-value	Effect	Range	p-value	Effect
Soybean Bran (g/L)	0.00-5.00	0.2725	0.2612	5.00-15.00	0.000	+0.664	0.00-5.00	0.000	+0.39
Sucrose (g/L)	0.00-10.00	0.5806	0.1287	not evaluated	-	-	not evaluated	-	-
Wheat Bran (g/L)	0.00-10.00	0.5806	0.1262	not evaluated	-	-	not evaluated	-	-
Yeast Extract (g/L)	0.00-2.00	0.8755	0.0362	5.00-7.50	0.000	+1.58	0.00-3.00	0.000	+0.47
Salt Solution (mL/L)	50-300	0.1540	0.3462	not evaluated	-	-	not evaluated	-	-
Steam exploded sugarcane bagasse (g/L)	not evaluated	-	-	10.00-30.00	0.000	-0.884	not evaluated	-	-
R^2 adj	0 %			88.19 %			99.3 %		

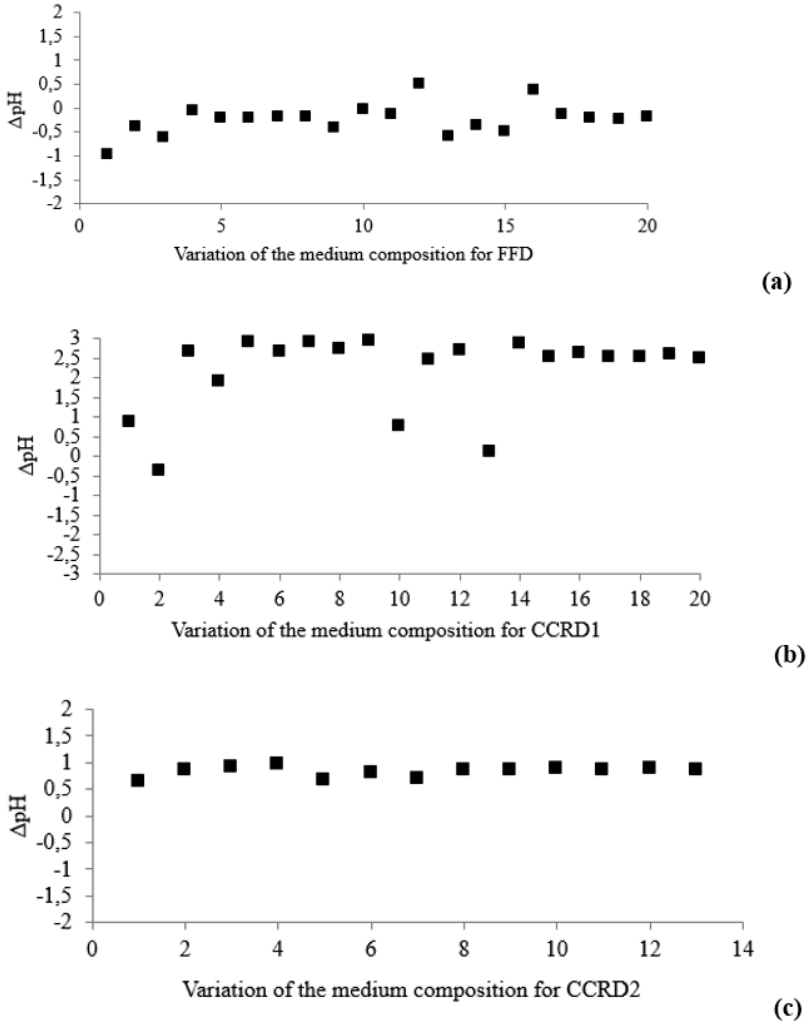

Figure 4: ΔpH for the different compositions of culture medium for *Penicillium echinulatum* S01M29 in submerged fermentation at 144 h (a) CCRD1 cultivation using potassium biphthalate buffer 0.1 M pH 5.9. (b) CCRD2 cultivation using potassium biphthalate buffer 0.1 M pH 4.7. (c) FFD cultivation using potassium biphthalate buffer 0.1 M pH 5.9.

culture at the 95 % confidence level, with p-values of *ca.* 0.000 for the three organic nitrogen sources, probably due to NH_4^+ acccumulation in the culture broth during assimilation of these components.

On the other hand, sucrose strongly decreased the pH of the culture medium at the 95 % confidence level for all DOEs carried out (p-value *ca.* 0.000, Table 7). This suggested an incomplete carbohydrate catabolism and probably an accumulation of carboxylic acids in the culture broth.

It was previously observed for *T. harzianum* P49P11 that pH variation within the range of ± 1.0 in the broth did not affect the activity of glycohydrolases if the pH was within the range of 5.0 to 6.0 (Delabona et al., 2013b). It can be observed from Table 7 and Figure 5a that the ΔpH values in the FFD were about 2.0 units greater than allowed, therefore invalidating the possibility of mathematical modeling studies for enzyme production optimization.

On the other hand, the CCRD1 and CCRD2, which used low component concentrations, led to ΔpH values within the range of ±1.0 (Figures 5b and 5c) , enabling mathematical modelling studies for glycosyl hydrolase culture media optimization in shake flasks using the potassium biphtalate buffer.

T. harzianum P49P11 culture media optimization

A mathematical model to describe FPase activity as a function of the medium components was therefore proposed based on CCRD2, which led to higher FPase activities when compared to CCRD1. The coefficients of the model are shown in Table 8.

The analysis led to the conclusion that only the linear effect of Celufloc (CEL) shows statistical significance at the 95% confidence level. Nevertheless, many of the other effects had p-values around 0.1, which could be statistically significantat at lower confidence limits. Thereby, the backward elimination technique was used to determine which terms should be maintained in the model.

Beggining with the complete model, the terms were eliminated one at a time, from the least significant to the most significant. The contribution of each of the eliminated effects on the model was evaluated by the adjusted R^2. The inclusion of terms in a model always raises the value of the coefficient of determination R^2, even when such terms do not influence the dependent variable. Thus, evaluating the value of R^2 is not enough to determine whether the model is good. The value of the adjusted R^2, on the other hand, only increases if the terms added to the model actually contribute to explain it and can be used to determine wich terms shold remain in the model.

Equation 1 shows the coded mathematical model obtained for FPase activity at 96h after the backward elimination procedure and recalculation of the model coefficients using the software Statistica. Table 8 shows the statistics from the analysis.

$$FPase\left(\frac{FPU}{mL}\right) = 0.52 - 0.02x_1 + 0.02{x_1}^2 + \\ +0.13x_2 - 0.03{x_2}^2 + \\ +0.02x_1x_2 - 0.04x_1x_3 + 0.04x_2x_3 \quad (1)$$

where x_1, x_2 and x_3 are the coded concentrations of sucrose (SUC), wheat bran (WB) and Celufloc (CEL), respectively.

Table 7. Main effects of culture medium components in the different DOE on pH variation (ΔpH = final pH - initial pH) in shake flasks *Trichoderma harzianum* P49P11 cultivation. The initial pH was 5.0 for all the experiments. Components that presented a significant effect at the 95 % confidence level are marked in gray (*see Tables 4 and 5 for culture media compositions*).

Culture media components	FFD			CCRD1			CCRD2		
	Range of concentration	p-value	Effect	Range of concentration	p-value	Effect	Range of concentration	p-value	Effect
Soybean Bran (g/L)	0.00-10.00	0.000	+0.968	0.00-15.00	0.000	+0.369	not evaluated	-	-
Sucrose (g/L)	0.00-10.00	0.000	-0.765	0.00-10.00	0.002	-0.259	0.00-10.00	0.023	-0.068
Wheat Bran (g/L)	0.00-5.00	0.780	+0.031	not evaluated	-	-	0.00-15.00	0.823	+0.003
Yeast Extract (g/L)	0.00-2.00	0.000	+0.544	not evaluated	-	-	not evaluated	-	-
Peptone (g/L)	0.00-2.00	0.000	+0.505	not evaluated	-	-	not evaluated	-	-
Salt Solution (mL/L)	50.00-100.00	0.128	+0.1769	not evaluated	-	-	not evaluated	-	-
Cellulose (g/L)	not evaluated	-	-	0.00-20.00	0.000	-0.372	0.00-20.00	0.000	-0.359
R^2 adj	80 %			88 %			95 %		

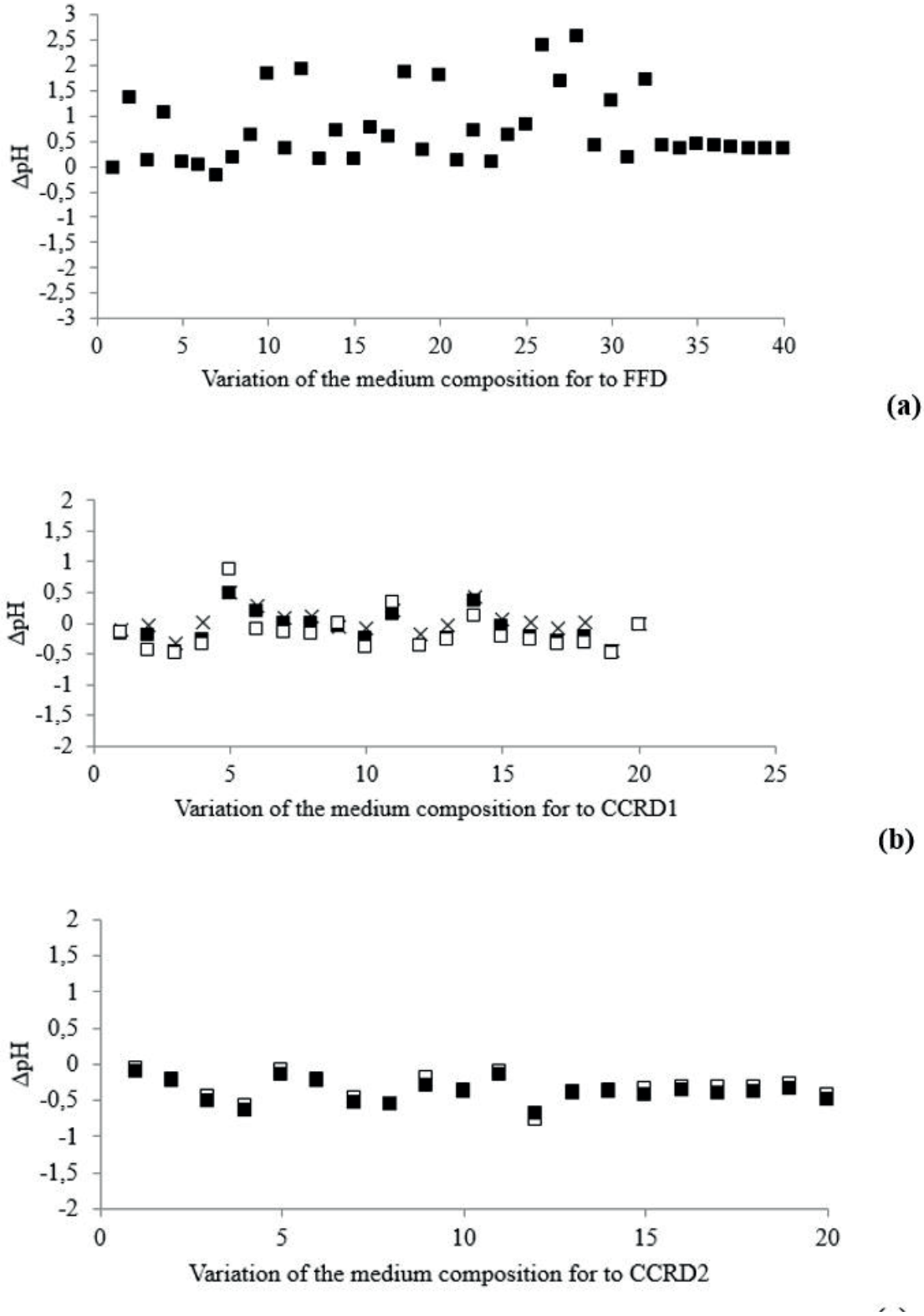

Figure 5: ΔpH for the different compositions of culture medium for *Trichoderma harzianum* P49P11 in submerged fermentation for cultivation using potassium biphthalate buffer 0.1 M pH 5.0. at 48 h (X), 72h (■) and 96 h (□). (a) CCRD1. (b) CCRD2. (c) FFD

The variables coding equations are

$x_1 = (SUC-5)/3$ (2)
$x_2 = (WB-10)/6$ (3)
$x_3 = (CEL-7.5)/4.5$ (4)

Table 8. Coefficients of the model determined to describe the influence of sucrose (SUC), wheat bran (WB) and Celufloc (CEL) on FPase activity in cultures of *Trichoderma harzianum* P49P11 in shake flasks buffered conditions (Equation1, coded units).

Factor	p-value	Coefficient
Media	**0**	**0.52**
1-SUC (L)	0.41	-0.02
SUC (Q)	0.10	0.02
2-CEL (L)	**0**	**0.13**
CEL (Q)	0.07	-0.03
1*2	0.10	0.02
1*3	0.14	-0.04
2*3	0.35	0.04
R^2	92.88 %	
R^2 adj	84.87 %	

Analysis of variance (ANOVA) was performed for this model and the F test of the regression was calculated as $F_{7,9}$ =13.07, which is higher than the tabulated value of 2.50. The F test of lack of fit was calculated as $F_{7,2}$ =2.64, which is lower than the tabulated value of 9.34, which shows that the model is significant at the 95 % confidence level.

Optimization of the FPase activity was performed using the subroutine SOLVER (Excel 2013, Microsoft Inc, USA), which predicted the FPase maximum activity to be 0.98 FPU/mL.That was achieved using culture medium with the following concentration: SUC = 0.0 g/L, WB = 15 g/L and C= 20.0 g/L. The optimal culture medium proposed by Eq 1 was afterwards validated in triplicate in additional shake flask experiments. The obtained mean value of these experiments was 0.92 FPU/mL ± 0.1 at 96h, similar to the maximum FPase activity predicted by Eq 1 (FPase predicted = 0.98 FPU/mL). The maximum experimental FPase value obtained with the potassium biphtalate buffered optimized culture medium was 2 times higher than the maximum titer of 0.4 FPU/mL found in shake flasks with standard culture medium (BED 7 g/L, SUC 3 g/L, unbuffered) proposed in the literature (Delabona et al., 2013b).

CONCLUSIONS

The pH control using potassium biphthalate buffer in submerged shake flask cultivation of *P. echinulatum* S01M29 and *T. harzianum* P49P11 proved to be efficient when the medium components and their concentration were properly selected. The present technique was applied in the culture media optimization and had a positive influence on the FPase activity of the two studied filamentous fungi.

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