OPTIMIZATION OF FERMENTATION MEDIA FOR THE GROWTH OF A FUNGUS USED AS BIOHERBICIDE

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ABSTRACT – In this work was optimized the composition of fermentation media for the growth of *Phoma* sp., which is a potential fungi to be used for the production of bioherbicide. For this purpose, the fungal growth was optimized, firstly, using synthetic media containing glucose, peptone and yeast extract. In a second moment, glucose was changed by sucrose food-grade and peptone and yeast extract by corn steep liquor and the growth optimized again. The optimized composition of synthetic media was glucose and peptone 20 $g.L^{-1}$, yeast extract 7.5 $g.L^{-1}$ and initial pH of 6.0. For industrial media, the optimized composition was sucrose 20 $g.L^{-1}$, CSL 8% and initial pH of 6.0. At these conditions, maximum fungal biomass were 22 and 33 $g.L^{-1}$, respectively. This study shows that industrial medium was better than synthetic medium and provides cost savings for a possible increase in production scale.

1. INTRODUCTION

Since the introduction of chemical herbicides some limitations as weeds that are resistant to herbicides, contamination of soil and water resources and chemical residues harmful to non-targets organisms (Li et al., 2003) are evident, motivating the search for new technologies or new molecules to overcome these problems. An alternative that has been used is the biological control, where the own micro-organism or a secondary metabolite produced by this microorganism can be employed as agent of control in a narrow field of application (Zhou et al., 2004).

Although the control of weeds using bioherbicides is attractive, research and commercialization of these products are yet low. Since the first reports of using bioherbicides from *Phytophthora palmivora* and *Colletotricum gloeosporioides* (Tebeest *et al.*, 1992), at least 11 products were commercialized (Bailey et al., 2011; Ash, 2010). Besides these commercialized products, Charudattan (2001) listed more than 50 examples of pathogens combination that present potential to be used as bioherbicide.

The search for biocontrol agents of weeds is increasing. Among the microorganisms that can be used, the fungal of genus *Phoma* sp. were reported as potential agent. Bailey *et al.* (2011) evaluated the action of *Phoma macrostoma* as bioherbicide, whereas Zhang *et al.* (2012) used *Phoma* sp. to obtain antimicrobial extracts. Parra *et al.* (2005) optimized the production of secondary metabolites of *Phoma* sp. with herbicide activity. Evidente *et al* (2001) used *Phoma exigua var. exigua* as a potential bioherbicide for the control of *Cirsium arvense*.

However, one of the key aspects on design of industrial fermentation for production of molecules with commercial interest, including bioherbicides, is the definition of substrate employed in the media formulation. The bulk fermentation broth is often considered as one of the most important component in the cost of the fermentation products, which usually can account to almost 50% of the whole production process (Li et al., 2013). Pure chemicals though idealistic would increase greatly the fermentation costs and would not be economically viable unless the fermentation product is high cost and low volume. Thus in order to lower the costs of production the search for the most cheapest and economical source of fermentation substrate will be the top most agenda in any proposed fermentation industry. Research on the selection of suitable substrates for fermentative processes has mainly been centered on agro-industrial residues because of their potential advantages (Li et al., 2013).

Based on these aspects, the main objective of this work was to optimize the media composition for the growth of *Phoma* sp. in shaken flasks. For this purpose, the fungal growth was optimized, firstly, using synthetic media containing glucose, peptone and yeast extract. In a second moment, glucose was changed by sucrose food-grade and peptone and yeast extract by corn steep liquor and the growth optimized again. Microbial kinetic for optimized conditions were determined. In addition, the fermented broth at optimized condition was applied for the control of target plant.

2. MATERIALS AND METHODS

2.1. Materials

Corn steep liquor (CSL) was obtained from Corn Products Brazil (Mogi Guaçu, SP, Brazil) and was used as received. Sucrose food-grade (Cristal) was purchase in a local market. All other chemicals, namely, $(NH_4)_2SO_4$, $FeSO_4.7H_2O$, $MnSO_4.H2O$, $MgSO_4$, glucose, peptone yeast extract were purchase from Sigma-Aldrich and were used as received.

2.2. Microorganism, inoculum and fermentations

The strain of *Phoma* sp. NRRL Y-7571 was obtained in the National Center for Agricultural Utilization Research – EUA (ARS). The culture was maintained in potato dextrose agar (PDA) at 4-6°C and subcultured every 15 days. Cell production for pre-inoculum was incubated on PDA in a petri dish for 8 days at 28ºC. Afterwards, the petri dish was washed with 5 mL of sterilized water and transferred to fermentation medium.

All fermentations were carried out in Erlenmeyer of 250 mL containing 50 mL of fermentation medium at 28°C, 120 rpm for 5 days. The micronutrient composition was maintained constant in all fermentations ($g.L^{-1}$): $(NH_4)_2SO_4$ 2.0, $FeSO_4.7H_2O$ 1.0, $MnSO_4$.H2O 1.0 and $MgSO_4$ 0.5 (Para et al., 2005; Zhang et al., 2012).

2.3. Optimization of synthetic medium for fungal growth

The composition of synthetic media was defined combining glucose, peptone, and yeast extract at different initial pHs. For this purpose, a Plackett–Burman design with 8 runs plus 3 central points (PB8) was used to determine the effects of independent variables on the growth of *Phoma* sp. The range of variables investigated was 5-15 $g.L^{-1}$ for glucose and peptone, 5-10 $g.L^{-1}$ for yeast extract and pH 5-7. Based on the statistical analysis of PB8, a central composite rotational design for two independent variables was proposed to optimize the concentrations of glucose and peptone. The response evaluated in both experimental designs was dry cell mass, which was expressed as gram of dry biomass per liter of fermentation medium.

2.4. Optimization of industrial medium for fungal growth

The formulation of industrial medium consisted in substituting glucose for sucrose food-grade and nitrogen sources (peptone and yeast extract) by corn steep liquor (CSL). In addition, it was evaluated the influence of micronutrient concentration on growth of *Phoma* sp. For this purpose, a central composite rotational design for three independent variables was proposed to optimize the concentrations of sucrose, CSL and micronutrient. The range of variables investigated was 15-35 $g.L^{-1}$ for sucrose, 10-30 wt% for CSL and 1-4 wt% for micronutrients.

Based on the analysis of first CCRD, a second one for two independent variables was conceived to optimize the concentrations of sucrose and CSL. The range of variables investigated was 15-25 $g.L^{-1}$ for sucrose and 5-15 wt% for CSL. The response evaluated in both experimental designs was dry cell mass, which was expressed as gram of dry biomass per liter of fermentation medium.

3. RESULTS AND DISCUSSION

3.1. Optimization of synthetic media for fungal growth

Table 1 presents the concentration of fungal biomass obtained in the eleven runs of the PB8 after 5 days of fermentation. The biomass concentration ranged from 4.09 (run 8) to 16.35 $g.L^{-1}$ (run 4). This variability in the results suggests that the independent variables present significant effects on fungal growth.

To check this, data of Table 1 were used to compute the magnitude and significance of these effects. Initial pH and yeast extract concentration did not present significant influence on fungal growth. By other hand, the effects for peptone and glucose were positive and statistically significant ($p<0.1$), indicating that the increase of concentration of both variables would lead to an increase in the concentration of fungal biomass in the fermentation.

Table 1 – Matrix of the Plackett–Burman design to evaluate the influence of synthetic media on concentration of fungal biomass after 5 days of fermentation

Run	pH	Glucose ($g\ L^{-1}$)	Peptone ($g\ L^{-1}$)	Yeast extract ($g\ L^{-1}$)	Fungal Biomass ($g\ L^{-1}$)
1	7 (1)	5 (-1)	5 (-1)	10 (1)	7.25
2	7 (1)	15 (1)	15 (-1)	5 (-1)	9.29
3	7 (1)	15 (1)	15 (1)	5 (-1)	14.77
4	5 (-1)	15 (1)	15 (1)	10 (1)	16.35
5	7 (1)	5 (-1)	15 (1)	10 (1)	9.18
6	5 (-1)	15 (1)	5 (-1)	10 (1)	5.85
7	5 (-1)	5 (-1)	15 (1)	5 (-1)	9.29
8	5 (-1)	15 (-1)	5 (-1)	5 (-1)	4.09
9	6 (0)	10 (0)	10 (0)	7.5 (0)	12.57
10	6 (0)	10 (0)	10 (0)	7.5 (0)	14.22
11	6 (0)	10 (0)	10 (0)	7.5 (0)	11.38

For this reason, a CCRD was used to evaluate the influence of glucose and peptone on concentration of fungal biomass, maintaining the concentration of yeast extract and pH at 7.5 $g.L^{-1}$ and 6.0, respectively. Table 2 presents the results obtained in the CCRD, where it is seen that the concentration of fungal biomass ranged from 11.74 (run 1) to 22.05 (run 8). The highest concentration of fungal biomass was obtained when glucose and peptone were maintained in the level +1 of experimental design. However, at the positive star points for peptone and glucose (runs 6 and 8, respectively), the increase in biomass was not verified. Meanwhile, the concentration of biomass increased considerably regarding the PB8, indicating that the strategy used was efficient to promote an increase in the fungal biomass.

The results presented in Table 2 were used to build a quadratic model expressing the concentration of fungal biomass in function of independent variables. Equation 1 presents the significant terms of the model ($p<0.1$).

$$FB_s = 18.68 + 2.53 \cdot G - 0.82 \cdot G^2 + 1.99 \cdot P - 0.70 \cdot P^2 \quad (1)$$

where FB_s is the concentration of fungal biomass obtained using synthetic media ($g.L^{-1}$), G and P are the coded glucose and peptone concentrations, respectively. The significant terms of models can be used to discuss the effects of each term on fungal growth. Both linear terms for glucose and peptone were statistically significant, but the effect of glucose was more accentuated than peptone. The negative quadratic terms concerning glucose and peptone indicate the presence of a maximum point in the system that is similar for both variables.

Table 2 – Matrix of the CCRD design to evaluate the influence of synthetic media on concentration of fungal biomass after 5 days of fermentation

Run	Glucose ($g.L^{-1}$)	Peptone ($g.L^{-1}$)	Fungal Biomass ($g.L^{-1}$)
1	10 (-1)	10 (-1)	11.74
2	20 (1)	10 (-1)	16.99
3	10 (-1)	20 (1)	16.51
4	20 (1)	20 (1)	22.05
5	8 (-1.41)	15 (0)	14.06
6	22 (1.41)	15 (0)	20.70
7	15 (0)	8 (-1.41)	15.47
8	15 (0)	22 (1.41)	19.78
9	15 (0)	15 (0)	18.19
10	15 (0)	15 (0)	18.92
11	15 (0)	15 (0)	18.92

The model was validated by analysis of variance (ANOVA). The calculated F-test for Equation 1 was about 8 times greater than the tabulated ones for significance at p=0.1, and the determination coefficients (R^2) was 0.9649. The high value for the determination coefficient indicate good fitting of experimental data, allowing the use of such model to predict the fungal biomass. The validated model was used to optimize the fungal biomass and the results obtained are presented in Figure 1a in the form of contour plots.

Maximum concentration of fungal biomass is obtained for glucose and peptone concentrations higher than 16 $g.L^{-1}$. Although is not fully delimited the optimum region in the contour plot, is possible to affirm that the optimized condition was found in this work that is glucose and peptone 20 $g.L^{-1}$, yeast extract 7.5 $g.L^{-1}$ and initial pH of 6.0. At this condition, maximum fungal biomass was 22 $g.L^{-1}$, which is higher than that obtained in the PB8, indicating that the strategy used to increase the fungal biomass was efficient.

3.2. Optimization of industrial media for fungal growth

The composition of synthetic media was based on peptone, glucose and yeast extract, which can be expansive for industrial applications, making unfeasible the operation at high volume bioreactors. For this reason, the constituents of fermentation media were changed for sucrose (carbon source) and corn steep liquor (nitrogen and micronutrients source), which are less expansive substrates. Table 3 presents the results obtained in the CCRD for two independent variables studied, namely, concentration of sucrose and corn steep liquor. The concentration of fungal biomass ranged from 33.29 (run 5) at 3.06 $g.L^{-1}$ (run 3). One aspect important to be pointed here is related to the concentration of biomass obtained, which was about 51% higher than for fermentations using

synthetic media. This is a resulted very expected, since the microbial growth was higher using a media considerably less expansive.

Table 3 – Matrix of the CCRD design to evaluate the influence of industrial media on concentration of fungal biomass after 5 days of fermentation

Run	Sucrose ($g.L^{-1}$)	CSL (%)	Fungal Biomass ($g.L^{-1}$)
1	15 (-1)	5 (-1)	19.00
2	25 (1)	5 (-1)	25.50
3	15 (-1)	15 (1)	3.06
4	25 (1)	15 (1)	4.46
5	13 (-1.41)	10 (0)	33.29
6	27 (1.41)	10 (0)	29.46
7	20 (0)	3 (-1.41)	15.43
8	20 (0)	17 (1.41)	5.50
9	20 (0)	10 (0)	26.09
10	20 (0)	10 (0)	25.49
11	20 (0)	10 (0)	30.83

In the same way that for fermentations using synthetic media, the results presented in Table 3 were used to build a quadratic model, expressing the concentration of fungal biomass in function of independent variables. Equation 2 presents the significant terms of the model ($p<0.1$).

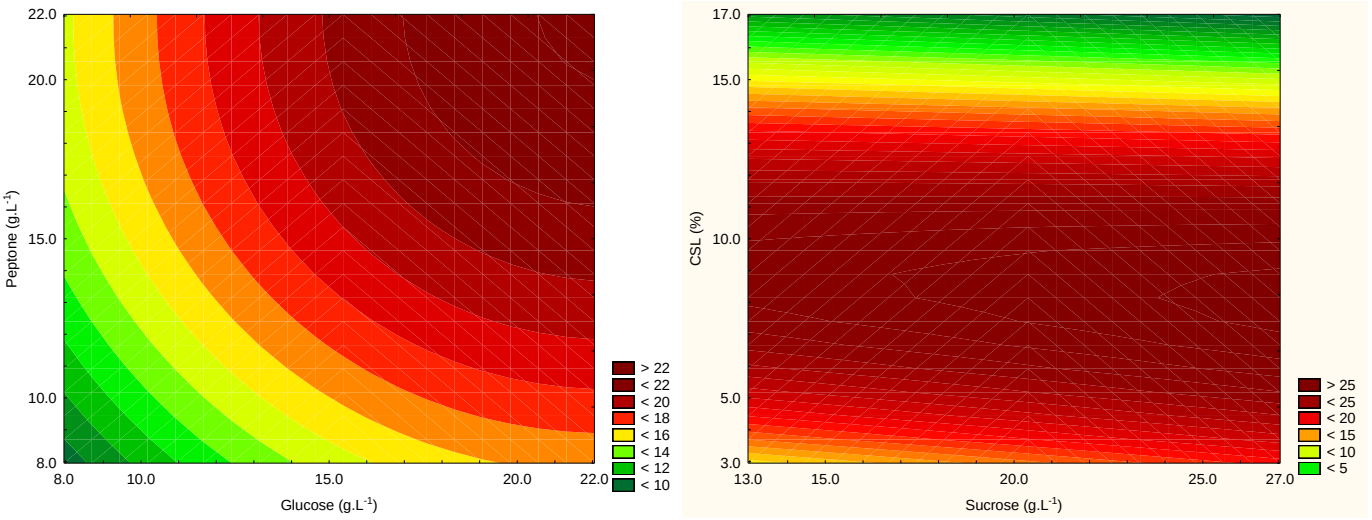
$$FB_i = 27.50 - 6.39 \cdot CSL - 10.55 \cdot CSL^2 \quad (2)$$

where FB_i is the concentration of fungal biomass obtained using industrial media ($g.L^{-1}$) and CSL is the coded corn steep liquor concentration. From the significant model parameters it is seen that only linear and quadratic terms referring to CSL concentration were significant in the range evaluated. This result is corroborating with data of Table 3, where one can easily visualized the influence of high concentration of corn steep liquor on the fungal growth, since for all fermentations carried out at concentrations above 15 wt%, the growth was very low.

The model was validated by analysis of variance (ANOVA). The calculated F-test for Equation 2 was about 2 times greater than the tabulated ones for significance at $p=0.1$, and the determination coefficient (R^2) was 0.8163, validating, in this way, the model generated. The validated model was used to optimize the fungal biomass and the results obtained are presented in Figure 1b in the form of contour plots.

Maximum concentration of fungal biomass is obtained for sucrose concentration higher than 17 $g.L^{-1}$ and CSL in the range of 8-10%. One aspect to be observed in Fig. 1b and deserve more detailing

is the fact that increasing the concentration of sucrose concentration, the optimal range for CSL also increase. By example, at sucrose 17 $g.L^{-1}$ the optimal range for CSL is between 8-9%, at sucrose 20 $g.L^{-1}$ between 7.5-9.5%, whereas for sucrose 25 $g.L^{-1}$ the optimal range is between 7-10%. From this finding is possible to conclude that the growth of fungus is dependent of sucrose concentration and, mainly, the increasing in the growth should be supported by increasing the nitrogen source in the media (in this work, CSL). The optimized condition was found in this work that is sucrose 20 $g.L^{-1}$ and CSL 8%. At this condition, maximum fungal biomass was 33 $g.L^{-1}$, which is 51% higher than that obtained using synthetic media.



(a) **(b)**

Figure 1 – (a) Contour plots expressing the fungal biomass in function of peptone and glucose concentration. (b) Contour plots expressing the fungal biomass in function of sucrose and CSL concentrations.

4. EVALUATION OF BIOHERBICIDE ACTIVITY

The effect of fermented broth without fungal biomass was evaluated after 21 days of application on the control the cucumber, which was the target plant used in this study. The fermented broth presented positive results, since some cucumber plants were severely affected by the herbicide. In fact, the results presented here are preliminary, but they show a promising future. Additional tests to identify and quantify the molecule (or molecules) with activity are necessary, besides definition of fermentation condition to maximize the production of molecule of interest.

5. CONCLUSION

In this work, the culture medium was optimized based on synthetic and industrial compounds for the growth of *Phoma* sp., which is a potential fungus for the production of bioherbicide. The optimized conditions for production of *Phoma* sp. by synthetic medium were glucose and peptone 20

$g.L^{-1}$, yeast extract 7.5 $g.L^{-1}$, and an initial pH of 6.0, yielding a maximum fungal biomass of 22 $g.L^{-1}$. For industrial medium, the optimized composition was sucrose 20 $g.L^{-1}$, CSL 8%, and initial pH of 6.0, obtaining maximum fungal biomass of 33 $g.L^{-1}$ that is about 50% higher than for synthetic medium.

6. REFERENCES

ASH, G.J. The science art business of successful bioherbicides. *Biological Control*, v. 52, p. 230-240, 2010.

BAILEY, K.L.; PITT, W.M.; FALK S.; DERBY, J. The effects of *Phoma macrostoma* on nontarget plant and target weed species. *Biological Control*, v. 58, p. 379-386, 2011.

BOYETCHOKO, S.M.; ROSSKOPF, E.N.; CAESAR A.J.; CHARUDATTAN, R. Biological Weed Control with Pathogens: Search for Candidates to Application. *Agriculture and Food Production*, v. 2, p. 239-266, 2002.

CHARUDATTAN, R. Biological control of weeds by means of plant pathogens: significance for integrated weed management in modern agro-ecology. *Biocontrol*, v. 46, p. 229-260, 2001.

EVIDENTE, A.; CIMMINO A.; ANDOLFI A.; BERESTSKIY A.; MOTTA A. Phomachalasins AeD, 26-oxa[16] and [15] cytochalasans produced by Phoma exigua var. exigua, a potential mycoherbicide for Cirsium arvense biocontrol. Tetrahedron 67, 1557-1563, 2011.

LI, Y.; SUN, Z.; ZHUANG X.; XU, L.; CHEN, S.; MINGZHI, L. Research Progress on microbial herbicides, *Crop Protection*, v. 22, p. 247-252, 2003.

Parra, R.; ALDRED, D.; MAGAN, N. Medium optimization for the production of the secondary metabolite squalestatin S1 by a *Phoma* sp. combining orthogonal design and response surface methodology. *Enzyme and Microbial Technology*, v. 37, p. 704-711, 2005.

RODRIGUES, M. I. E IEMMA, A. F. Planejamento de Experimentos & Otimização de Processos, 2005.

TEBEEST, D.O.; YANG, X.B.; CISAR, C.R. The status of biological-control of weeds with fungal pathogens. *Annual Review of Phytopathology*, v. 30, p. 637-657, 1992

ZHANG, L; WANG, S.Q.; LI, X.J.; ZHANG, A.L.; ZHANG, Q.; GAO. G.M. New insight into the stereochemistry of botryosphaeridione from a *Phoma endophyte*. Journal of Molecular Structure, 1016, 72–75, 2012.

ZHOU, L.; BAILEY K.L.; DERBY, J. Plant colonization and environmental fate of the biocontrol fungus *Phoma macrostoma*, *Biological Control*, v. 30, p. 634-644, 2004.