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STRATEGIES FOR FUNGAL SOLID-STATE CULTIVATION USING BY-PRODUCTS FROM COFFEE PRODUCTION AND PROCESSING

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ABSTRACT

Coffee production generates significant by-products such as coffee husk (CH) and coffee silverskin (CSS), posing environmental challenges due to their volume. Despite being nutrient-rich, CH contains compounds like caffeine and tannins, that limit its use. Fungi can degrade caffeine into xanthine, enhancing its utilization. Various fungal strains were assessed for caffeine degradation potential, with eight selected for experimentation. Initial tests on caffeine degradation showed modest results. Extrusion pretreatment was used to facilitate carbon metabolism, but negatively impacted fungal growth by 22.5% to 36.1%. An experimental design was employed to optimize conditions for caffeine degradation, showing promising results with 27.7% and 10.8% degradation within seven days. Growth kinetics revealed a peak at 72 hours, while enzyme production, particularly peptidase, peaked within 48 hours. Although high caffeine degradation conditions weren't achieved, fungal growth increased by 82%, setting parameters for future experiments aiming at improved degradation.

Keywords: *Coffea arabica*, Caffeine, Detoxification, coffee husk, enzyme.

1 INTRODUCTION

Coffee is a drink consumed throughout several continents and cultures. The global production is about 10 million tons to meet the high demand. In coffee production, there are two main processing methods: the wet and dry methods, the latter being the most used in Brazil. The dry method has, as the main by-product, the coffee husk (CH), while the subsequent roast produces a by-product known as coffee silverskin (CSS)[1]. Although not intrinsically polluting, the sheer amount of produced by-products turns them into environmental liabilities. To turn the coffee production chain eco-friendlier, the destination of such residues must change. Despite being nutritious, CH contains several components that limit its use as livestock food, such as caffeine and tannins.

Fungi produce *N*-demethylases, which convert caffeine into xanthine, thus making the caffeine's nitrogen available for further use. Several bioprocesses can be used with fungi, such as solid-state cultivation (SSC) and submerged cultivation (SmC). SSC with fungi can be used not only to achieve caffeine detoxification but also to enhance nutritional characteristics, such as crude protein content, amino acid profile, and non-fibrous carbohydrates.

CH contains high cellulose rates and very little available carbon. To make the present carbon easier to metabolize, extrusion was chosen as a pretreatment. Extruders disrupt the biomass by breaking the cellulose chain without chemical changes, thus without producing fermentation inhibitors[2]. Said disruption on create terminals to enzyme action, hence allowing the carbohydrates to be metabolized by the fungi.

Therefore, SSC with filamentous fungi was selected to reduce caffeine concentration. SSC can also produce hydrolases, such as proteases and cellulases, which have high market value and various applications.

2 MATERIAL & METHODS

Six different *Aspergillus* strains, two *Penicillium* strains, and one *Trichoderma harzianum* strain were selected and subjected to caffeine resistance assay to identify the ones more likely able to degrade said caffeine.

The caffeine resistance assay was conducted with Petri dishes containing potato-dextrose-agar media (PDA) with caffeine concentrations between 0g/L and 15g/L. All concentrations were incubated at 30°C and the colonies diameters measured daily for three days.

From that assay, eight fungi were selected for a caffeine degradation experiment. In order to evaluate caffeine degradation a SmC with adapted Czapek-Dox media (sucrose (20g/L), caffeine (1g/L), KH_2PO_4 (1.3g/L), $MgSO_4 \cdot 7\ H_2O$ (0.5g/L) e Na_2HPO_4 (0.12g/L), $(NH_4)2SO_4$ (0.14 g/L), pH 4.5)

From this experiment, four fungi were selected for SSC, namely *A. awamori* IOC 3914, *T. harzianum* IOC 4127, *A. tamarii* IOC 0397, and *A. wentii* IOC. CES was conducted with untreated CH, pretreated coffee husk (PTCH) (30-60 rpm, 40°C, 40-100 Nm, and CH:water ratio of 1:0 to 5:1), CSS, and a mixture of two or three.

In this project, we analyzed caffeine concentration using high-performance liquid chromatography (HPLC)[3,4] and the activity of hydrolytic enzymes of interest. Initial assays under different pretreatment conditions, supplementation, and waste mixtures did not have a significant impact on caffeine degradation.

To better understand fungal growth, assays with N-acetylglucosamine were performed accordingly with Aidoo[5].

To find the condition where the fungus degrades caffeine, a Plackett-Burman (PB) experimental design was developed (DOE) with twelve assays and three center points. The chosen factors were: Inoculum, molasses, moisture, ammonium sulfate, and CH/CSS ratio.

3 RESULTS & DISCUSSION

Among the *Penicillium* strains, *P. simplicissimum* was the most sensitive, with 65% inhibition at 72h with 1.5g/L of caffeine while the strain *P. chrysogenum* showed 60% inhibition (Figure 1). The *P. simplicissimum* strain was cut off furthers assays.

At 72h with 15g/L of caffeine, *T. harzianum* strain showed higher inhibition than any other *A. awamori* strain (IOC 3914= 73%, IOC 3915= 77% e IOC 0203= 66%) or the *A. tamari* strain (57%). Nevertheless the *A. niger* and *A. wentii* strains exhibited higher inhibition rates.

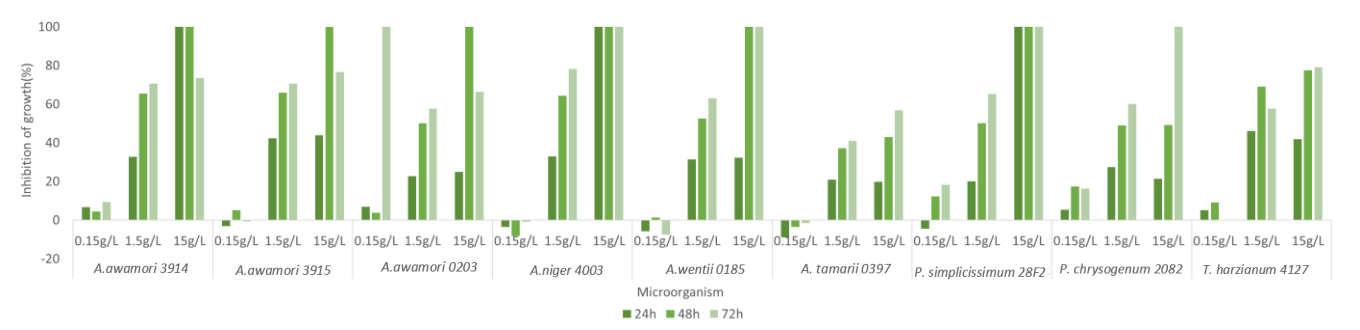

Figure 1 – Percentage of fungal growth inhibition on plates containing PDA medium added with caffeine at different concentrations. N=3

The degradation assay showed that none of the strains were capable of caffeine degradation (data no showed) under the tried conditions but was possible to identity the ones with better growth. Considering the strain *A. awamori* 3914 had one of the highest growths and had been previously described as able to degrade caffeine[6], said strain was selected for further trials.

Particle size distribution have a great impact on SSC[7]. Large-sized particles provide less surface area for fungal growth and lower water retention during cultivation, while small particle size causes media compression, hindering aeration and heat dissipation. In order to achieve ideal composition for fungi growth, three different mixtures of CH and CSS were tried. After four days of cultivation, none of them showed caffeine degradation (Figure 2) or visually a robust growth.

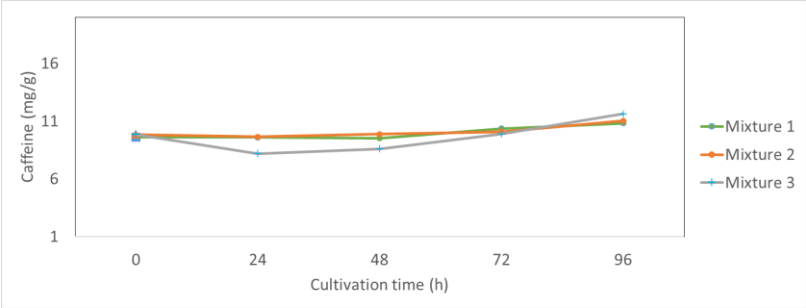

Figure 2 – *A. awamori* IOC 3914 solid-state cultivation with different mixtures of CH and CSS. All cultures used spore suspension inoculum ($1x10^7$ spores/g) and were conducted at 30°C for up to 96 hours. Mixture 1= 10% of raw CH, 75% of ground CH, 5% of raw CSS, and 10% of CSS; Mixture 2: 25% of raw CH, 65% of ground CH, 5% of raw CSS, and 5% of ground CSS; Mixture 3: 45% of raw CH, 45% of ground CH, 5% of raw CSS, and 5% of ground CSS.

In further experiments, extrusion was tested at 30 or 60 rpm, with different CH ratios and numbers of cycles. Various mixtures of CH and PTCH were then evaluated for fungal growth and caffeine concentration.

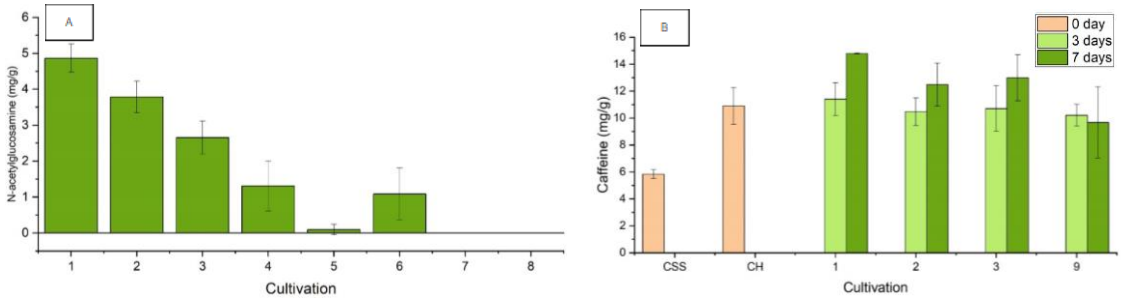

Figure 3 -A: Comparison of A. awamori growth in mixtures containing different proportions of PTCH and inoculum forms. B: Caffeine degradation. 1: 100% CH and $2x10^7$ spores/g; 2: 50% PTCH (CH:Water 1:0, 60rpm) + 50% CH and $2x10^7$ spores/g at 30°C; 3: 50% PTCH (CH:Water 1:0, 30rpm) + 50% CH and $2x10^7$ spores/g at 30°C; 4: 80% CH + 20% CSS, solid inoculum, 6.25% molasses at 30°C; 5: 20% CSS + 40% CH + 40% PTCH (CH:Water 3:1, 60rpm), solid inoculum, 6.25% molasses at 30°C; 6: 20% CSS + 40% CH + 40% PTCH (CH:Water 3:1, 60rpm), solid inoculum, 6.25% molasses at 25°C; 7: 20% CSS + 40% CH + 40% PTCH (CH:Water 5:1, 60rpm), solid inoculum at 30°C; 8: 20% CSS + 40% CH + 40% PTCH (CH:Water 5:1, 60rpm), solid inoculum, 6.25% molasses at 30°C; 9: 50% CH + 50% PTCH (CH:Water 3:1, 60rpm). Cultures 1-3 lasted 7 days, while cultures 4-9 lasted 4 days.

Extrusion negatively impacted fungal growth, reducing it to levels below detection (Figure 3.A). Regarding caffeine degradation, none of the assays showed improvement compared to the initial CH concentration (Figure 3.B), suggesting that extrusion under these conditions does not enhance degradation.

The DOE developed to improve caffeine degradation demonstrated that most variables significantly affected fungal growth (Figure 4). Additionally, some assays showed improved fungal growth compared to previous cultivations. Notably, two assays achieved caffeine degradation within seven days of cultivation, with degradation rates of 27.7% and 10.8%.

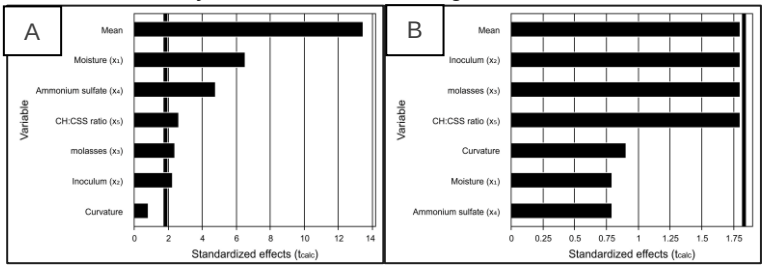

Figure 4 – A: Pareto chart of standardized effects from the experimental design for microbial growth over 6 days. p-value: 0.1. B: Pareto chart of standardized effects from the experimental design for caffeine degradation over 7 days. p-value: 0.1

Growth kinetics experiments revealed a peak at 72 hours, while enzyme production experiments indicated that peptidase production peaked within the first 48 hours, with low production of other enzymes likely due to high peptidase activity[8]. Although a high caffeine degradation condition was not achieved, fungal growth increased by 82%, establishing parameters for new experiments aimed at finding higher degradation rates.

The low degradation rate suggested that this strain has a limited capacity to degrade caffeine within a feasible time. Thus, conditions defined using the aforementioned PB were applied to a new strain isolated from coffee residues. This strain was subject to an SSC with 70% humidity, $1x10^7$ spores/g, 85% ground CH, 5% raw CH, 5% ground CSS, and 5% raw CSS. After seven days of cultivation, a 98.54% degradation rate was achieved. Further assays are required to verify if the strains produce mycotoxins.

4 CONCLUSION

In this work, it was concluded that raw coffee husk is excessively inhospitable for the growth of fungi of the genera *Trichoderma* and *Aspergillus*. Furthermore, the use of an extruder as a pretreatment to make the carbon present in cellulose available did not improve fungal growth, as nitrogen was found to be the limiting factor. Consequently, supplementation with ammonium sulfate promotes microbial growth, unlike molasses. The use of experimental design techniques led to an 82% increase in the growth of fungus *A. awamori* IOC 3914 when comparing cultivation with ground CH and cultivation with 70% humidity, $1x10^8$ spores/g, 2% ammonium sulfate, 55% ground CH, 5% raw CH, 35% ground CSS, and 5% raw CSS, indicating the best cultivation conditions for this strain. However, ammonium sulfate also limited caffeine degradation, necessitating the use of experimental design techniques to find the optimum level of supplementation that allows for both high caffeine degradation and the production of hydrolytic enzymes of interest. Although high caffeine degradation was not achieved within a reasonable cultivation time, a newly identified strain achieved significant caffeine degradation under optimized conditions, suggesting the need for further optimization of time and degradation conditions.

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