EVALUATION OF PROTEIN SECRETORY CAPABILITIES IN *ASPERGILLUS* SPECIES

Lana O. S. Silva[1], André F. Faria[1], Gabriela S. Alves[1], Gabriel B. Motta[1], Aryadne G. Rodrigues[1], Júlio V. Bernardi[1,2], Naiara H. Neuenfeldt[3], Liliana O. Rocha[3], Marcelo M. Brandão[2] & André Damásio[1]*

[1] *Department of Biochemistry and Tissue Biology, Institute of Biology, University of Campinas (UNICAMP), Campinas-SP, Brazil*
[2] *Center for Molecular Biology and Genetic Engineering (CBMEG), University of Campinas (UNICAMP), Campinas-SP, Brazil*
[3] *Department of Food Science and Nutrition, Food Engineering Faculty, University of Campinas (UNICAMP), Campinas-SP, Brazil*
** Corresponding author's email address: adamasio@unicamp.br*

ABSTRACT

Filamentous fungi, such as *Aspergillus* spp., are valuable cell factories for their capability to secrete high yields of proteins. However, some bottlenecks can hinder production levels. In *Aspergillus* spp., overproduction of recombinant proteins triggers the unfolded protein response (UPR) and induces the repression under secretion stress (RESS) mechanism. This mechanism reduces the cargo load of the endoplasmic reticulum, promoting cellular homeostasis. In this study, we compare the secretion capacity of two *Aspergillus* species in different sugar sources. We used the *Aspergillus oryzae* reference strain RIB40, a GRAS (Generally Recognized as Safe) strain, and three *Aspergillus flavus* strains, which do not produce aflatoxin, obtained from CFAM bank. The strains were cultivated in Yeast Extract media supplemented with 4% glucose (carbon catabolite repressor) or high maltose (inducer of amylase secretion) as carbon sources. We monitored sugar consumption, pH, protein secretion, and fungal biomass every 24 hours for 7 days. The strains that showed the strongest correlation between secreted proteins and fungal biomass were *A. flavus* CFAM 34 on glucose, and *A. flavus* CFAM 540 and 701 on high maltose. This comparison will contribute to selecting a strain with high secretion capabilities to be engineered as a chassis strain for recombinant protein production.

Keywords: *Aspergillus oryzae*. *Aspergillus flavus*. Protein secretion. Recombinant protein. Fungal cell factories.

1 INTRODUCTION

Filamentous fungi are known for secreting large amounts of proteins, making them valuable cell factories for the production of various industrially relevant products. Another advantage of filamentous fungi over other organisms is their efficient system for post-translational processing, which is essential for protein functionality. The most commonly used filamentous fungi cell factories are *Aspergillus* spp. and *Trichoderma* spp.. *Aspergillus* spp. are responsible for producing a wide range of products, including enzymes and organic acids [1,2]. *Aspergillus oryzae*, for example, is widely used in the food industry. It is considered a GRAS (Generally Recognized as Safe) organism and is used in fermentation and food processing industries [3].

Even though *A. oryzae* is established as an effective cell factory, some bottlenecks such as transcription, translation, protein folding, posttranslational modifications, transport, and secretion can limit production titer [4]. In *Aspergillus* spp. strains, the overproduction of recombinant proteins triggers the unfolded protein response (UPR), which induces target genes involved in this response, such as foldases and chaperones [5]. Moreover, this overproduction induces the repression under secretion stress (RESS) mechanism, which reduces the protein load in the endoplasmic reticulum, thereby promoting cellular homeostasis [6].

Another challenge during the production of recombinant proteins is the trade-off between fungal biomass formation and the production of the protein of interest. Overproduction of recombinant proteins has a high energy cost which can impair the balance of fungal metabolism by redirecting cellular resources from other cellular activities, such as biomass formation. The cell can adapt to this metabolic burden by reducing protein titer or fungal biomass formation [7]. In *Trichoderma reesei*, for example, high levels of secreted proteins were achieved at low growth rates with decreased biomass formation [8]. Therefore, for the biotechnology industry, it is preferable to use a cell factory that can efficiently produce the desired protein without accumulation of excessive fungal biomass.

Advances in molecular biology tools, including synthetic promoter, interference RNA (iRNA), and CRISPR/Cas9, enable the creation of optimized strains and prevent the challenges found during recombinant protein production [9]. Through genetic engineering is possible to design a strain capable of achieving high titers of secreted recombinant protein by adding multiple gene copies, optimizing codons, or deleting proteases, for example [10]. *A. oryzae* produces high amounts of proteases and deletion of some protease coding genes increased the yields of recombinant proteins in this fungus [11].

In this study, we compare the growth and secretory capacities of *A. oryzae* RIB40 and *A. flavus* (isolated strains) using different sugar sources. Our results will contribute to understanding the regulatory processes underlying efficient protein secretion in *Aspergillus.* The strain with the best secretome/fungal biomass correlation will be selected for genetic engineering and established as a chassis strain for recombinant protein production.

2 MATERIAL & METHODS

The *A. oryzae* reference strain RIB40 was acquired from the School of Pharmaceutical Sciences (FCFRP, Ribeirão Preto, SP), kindly provided by Dr. Gustavo Goldman. Three other strains, isolated in the Amazon rainforest (34, 540, and 701), were obtained from CFAM (http://cfam.fiocruz.br/index?catalogue).

The genome of CFAM strains was sequenced and annotated to investigate differences among them. CFAM strains were grown in a static liquid MMG (Minimum Media + 1% Glucose, pH 5.5) for 24 hours at 30°C, and then fungal biomass was dried and frozen in liquid nitrogen followed by maceration. DNA was extracted and samples were submitted for DNBseq sequencing. The sequenced data was submitted to the ByMyCell biotechnology company for genome assembly and annotation using RIB40 genome as a reference. The software BUSCO, antiSMASH and CAZymes (Carbohydrate-Active Enzymes) database were utilized to investigate genome assembly integrity, production of secondary metabolites and CAZymes content, respectively. Whole Genome Analysis was performed at CLC Workbench, using as entrance the following genomes: the three targeted CFAM strains, *A. oryzae* RIB40, *Aspergillus nidulans*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *T. reesei,* and *Neurospora crassa*. *A. oryzae* RIB40 was utilized as reference of annotations. After the alignment, we constructed the Average Nucleotide Identity Comparison (ANI), to show the similarity between these genomes. With the results from ANI method, a circular cladogram tree was made to determine the *Aspergillus* species closest to the CFAM strains.

Thin-Layer Chromatography (TLC) and Ultra-Performance Liquid Chromatography coupled to tandem Mass Spectrometry (UPLC-MS/MS) were performed to investigate the production of aflatoxins B1 and B2. In both analyses, the *A. oryzae* RIB40 was included as a reference strain and the aflatoxin producer, *A. flavus*, was included as a positive control.

For phenotypic analysis, 20 µL of spore solution was inoculated in triplicate onto the MMG agar media and spore macroscopic features were observed after 96 hours of growth, at 30°C.

To investigate protein secretion in different carbon sources, 10^6 fungal spores were inoculated into yeast extract (YE) supplemented with either 4% of glucose (carbon catabolite repressor) or high maltose (inducer of amylases secretion) carbon source. Secretome from fungal culture was collected every 24 hours, for 7 days by filtration, and secretome pH was measured. After secretome filtration, the fungal biomass was subjected to dry weight analysis. Sugar consumption was analyzed with Thermo Scientific™ Dionex™ ICS-6000 HPIC™ System. Protein concentration was determined through the Bradford method and the secretome profile was analyzed by SDS-Page.

3 RESULTS & DISCUSSION

The genome analysis indicated a high similarity between CFAM strains. According to genome assembly, the genome size (Mb) is almost identical (CFAM 34 and 701: 37.45 Mb, CFAM 540: 37.42 Mb). An analysis with BUSCO software to investigate the integrity of genome assembly, showed an identical pattern of single copies, multiple copies, fragmented and absent genes in the CFAM strains. The investigation of secondary metabolites with antiSMASH database showed a comparable number of gene clusters found in each secondary metabolite type. The CAZymes content in each CFAM strain was predicted with CAZy database. 199 CAZymes were predicted in 34 and 701 strains, and 200 CAZymes were predicted in strain 540. The Whole Genome Analysis showed a high similarity between CFAM strains and both *A. oryzae* and *A. flavus* species. The phylogenomic tree indicated that CFAM strains are clustered together and are closely related to *A. oryzae* and *A. flavus*. Most *A. flavus* strains produce toxins called aflatoxins and can cause human disease as an opportunistic pathogen [12]. According to the literature, *A. oryzae* is a domesticated strain from *A. flavus* that does not produce toxins [13]. We then analyzed if CFAM strains can produce toxins. Two different techniques were applied and both results indicated the absence of aflatoxins in CFAM strains. Even though CFAM and RIB40 genomes are very similar, they differ phenotypically. All three CFAM strains produce deep-green spores, similar to *A. flavus*[14], while *A. oryzae* RIB40 produces yellowish-green spores. Given the proximity of CFAM strains to *A. flavus*, the difference in spore color, and their isolation in the Amazon rainforest, these strains were classified as *A. flavus*.

The secretion capabilities of *A. oryzae* and *A. flavus* were analyzed using two different sugar sources, glucose and high maltose. In the glucose medium, all strains presented a similar pattern of sugar uptake, with most of the glucose being consumed after 72 hours of incubation. Conversely, the pH of the secretome of all strains decreased in the first 48 hours of growth and then increased after 72 hours. By day 7, the pH was sometimes higher than the initial level. When comparing glucose levels, it became apparent that the pH decreased as glucose was consumed. This is because of the fungal metabolism, which produces and secretes organic acids during sugar consumption[15].

In high maltose, the maltose was totally consumed after 48 hours of growth. In *A. flavus* CFAM 540 and 701 it is possible to observe an increase in glucose content in the first 72 hours, due to maltose breakdown, which is also rapidly consumed. The pH also decreases in the first 3 days of growth, probably due to the production of organic acids. The fungal biomass increased in the first 48 hours and remained stable throughout the 7 days. As expected, a higher protein secretion was observed in high maltose, since glucose is a non-inducible carbon source.

We also investigated the correlation between fungal growth and protein secretion in the 4 strains in the presence of glucose and high maltose. In glucose, we found that the *A. flavus* CFAM 34 strain showed a higher secretion/fungal biomass ratio during the first 4 days of incubation, with a significant correlation compared to *A. oryzae*. In high maltose, *A. flavus* CFAM 540 and 701 demonstrated the highest and most significant secretion/fungal biomass correlation for the first 5 days compared to *A. oryzae*.

This is a critical factor in strain selection, as we are prospecting fungal strains with a high proportion of protein secretion over fungal biomass synthesis.

Protein secretion in glucose was assessed using SDS-PAGE, revealing distinct patterns of secreted proteins between the *A. flavus* CFAM strains and *A. oryzae* RIB40. Furthermore, noticeable differences were observed among the *A. flavus* CFAM strains themselves. Specifically, *A. flavus* CFAM 34 predominantly secreted proteins smaller than 50 kDa, while *A. flavus* CFAM 540 exhibited a range of proteins larger than 30 kDa. In contrast, *A. flavus* CFAM 701 mainly secreted proteins with a molecular weight around 30 kDa. These findings indicate that the genomes of the *A. flavus* CFAM strains are highly flexible as they display similar genomic characteristics but divergent protein profiles.

4 CONCLUSION

The *A. oryzae* and *A. flavus* strains employed in this study are highly similar on a phylogenomic level, but the genomes of the *A. flavus* strains are nearly identical to each other. It is worth noting that the *A. flavus* strains used in this study do not produce aflatoxins. Growth of both *A. oryzae* and *A. flavus* on glucose was comparable; however, among the *A. flavus* CFAM strains, 34, 540, and 701 displayed a high correlation between secreted protein/fungal biomass, making them potential targets for genetic engineering efforts.

REFERENCES

1. Meyer V, Basenko EY, Benz JP, et al. Growing a circular economy with fungal biotechnology: a white paper. *Fungal Biol Biotechnol*. 2020;7(1):5. doi:10.1186/s40694-020-00095-z
2. Liu D, Garrigues S, de Vries RP. Heterologous protein production in filamentous fungi. *Appl Microbiol Biotechnol*. 2023;107(16):5019-5033. doi:10.1007/s00253-023-12660-8
3. Kobayashi T, Abe K, Asai K, et al. Genomics of *Aspergillus oryzae*. *Biosci Biotechnol Biochem*. 2007;71(3):646-670. doi:10.1271/bbb.60550
4. Jin FJ, Hu S, Wang BT, Jin L. Advances in Genetic Engineering Technology and Its Application in the Industrial Fungus *Aspergillus oryzae*. *Front Microbiol*. 2021;12. doi:10.3389/fmicb.2021.644404
5. Heimel K. Unfolded protein response in filamentous fungi—implications in biotechnology. *Appl Microbiol Biotechnol*. 2015;99(1):121-132. doi:10.1007/s00253-014-6192-7
6. Pakula TM, Laxell M, Huuskonen A, Uusitalo J, Saloheimo M, Penttilä M. The effects of drugs inhibiting protein secretion in the filamentous fungus *Trichoderma reesei*. Evidence for down-regulation of genes that encode secreted proteins in the stressed cells. *Journal of Biological Chemistry*. 2003;278(45):45011-45020. doi:10.1074/jbc.M302372200
7. Kastberg LLB, Ard R, Jensen MK, Workman CT. Burden Imposed by Heterologous Protein Production in Two Major Industrial Yeast Cell Factories: Identifying Sources and Mitigation Strategies. *Frontiers in Fungal Biology*. 2022;3. doi:10.3389/ffunb.2022.827704
8. Pakula TM, Salonen K, Uusitalo J, Penttilä M. The effect of specific growth rate on protein synthesis and secretion in the filamentous fungus *Trichoderma reesei*. *Microbiology (N Y)*. 2005;151(1):135-143. doi:10.1099/mic.0.27458-0
9. Martins-Santana L, Nora LC, Sanches-Medeiros A, Lovate GL, Cassiano MHA, Silva-Rocha R. Systems and Synthetic Biology Approaches to Engineer Fungi for Fine Chemical Production. *Front Bioeng Biotechnol*. 2018;6(OCT). doi:10.3389/fbioe.2018.00117
10. Ntana F, Mortensen UH, Sarazin C, Figge R. *Aspergillus*: A powerful protein production platform. *Catalysts*. 2020;10(9):1-29. doi:10.3390/catal10091064
11. Yoon J, Maruyama JI, Kitamoto K. Disruption of ten protease genes in the filamentous fungus *Aspergillus oryzae* highly improves production of heterologous proteins. *Appl Microbiol Biotechnol*. 2011;89(3):747-759. doi:10.1007/s00253-010-2937-0
12. KLICH MA. *Aspergillus flavus* : the major producer of aflatoxin. *Mol Plant Pathol*. 2007;8(6):713-722. doi:10.1111/j.1364-3703.2007.00436.x
13. Payne GA, Nierman WC, Wortman JR, et al. Whole genome comparison of *Aspergillus flavus* and *A. oryzae*. *Med Mycol*. 2006;44(s1):9-11. doi:10.1080/13693780600835716
14. Zulkifli NA, Zakaria L. Morphological and Molecular Diversity of *Aspergillus* From Corn Grain Used as Livestock Feed. *Hayati*. 2017;24(1):26-34. doi:10.1016/j.hjb.2017.05.002
15. Ochsenreither K, Fischer C, Neumann A, Syldatk C. Process characterization and influence of alternative carbon sources and carbon-to-nitrogen ratio on organic acid production by *Aspergillus oryzae* DSM1863. *Appl Microbiol Biotechnol*. 2014;98(12):5449-5460. doi:10.1007/s00253-014-5614-x

ACKNOWLEDGEMENTS

This study is financially supported by the São Paulo Research Foundation (FAPESP), Thematic Project grant - 2022/05731-2, with two associated fellowships: LOSS with the grant number 2023/12218-2, and GBM with the grant number 2024/03892-4. NHN was awarded with FAPESP fellowship with the grant number 2022/14246-0. This work was also financed by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brazil (CAPES) – Finance Code 001 – with AFF, GSA, AGR and JVB fellowships.