Characterization of RNA modifications from *Cladosporium sphaerospermum*

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Introduction

Research for this semester was focused on improving the methods of isolating genetic materials from the species *Cladosporium sphaerospermum* (*C. sphaerospermum*). This fungus, because of its expression of melanin, has been shown by previous researchers to impede current methods used for RNA isolation.[1] Further, this research focused on investigating tRNA modifications that are present in *C. sphaerospermum,* as data from post-transcriptional modifications can help shed light on how this organism can reprogram the protein synthesis pathways following the receipt of environmental clues. The tRNA modifications were to be analyzing using LCMS analysis following complete isolation from samples cells and digestion of conjugate nucleosides.

Investigating this topic is important because certain melanin producing fungi, like *C. sphaerospermum,* are able to thrive in high gamma-level radiation zones, like the 30km Exclusion Zone around nuclear plant near the city of Pripyat in the Ukrainian Soviet Socialist Republic, where the species has been noted to grow well.[2] Studies into the metabolic role of melanin indicated a 4-fold increase in the reduction of NAD+ to NADH upon irradiation by gamma rays, compared to the non-irradiated pigment. This increased supply of energy manifesting as reducing power accelerates growth by stimulating metabolism.[3]

Methods

Due to fungal cell wall being rich in chitin, it was hypothesized that by incubating samples in a concentrated solution of chitinase (a protein that is naturally produced by bacteria and fungi that is able to destabilize chitin by means of glycosidic cleavage) would aid in obtaining a higher quantity of RNA after homogenization and isolation. The relationship between chitinase and RNA isolation have never been investigated prior to this study. Chitin is highly resistant to moderate shearing forces, these same shearing forces, however, causes the mechanical breakdown of RNA causing samples to degrade. The experimental methods for this experiment were as follows:

1. The CBS1 strain of *C. sphaerospermum* was grown on potato dextrose (pH6.5) agar plate at 25°C to its log growth phase (~8 days) and harvested.
2. The fungus was incubated with 5 units of chitinase (Sigma Aldrich) for 72 hours at room temperature.
3. The cell suspension was homogenized, and RNA was isolated using a Qiagen RNeasy Plant Mini Kit.
4. The tRNA was purified using Nucleobond AX100 anion-exchange column. The purified tRNA was enzymatically hydrolyzed to ribonucleosides.[4]
5. The hydrolysate was analyzed by LC-MS[4] and data processed by Xcalibur to identify the resident nucleoside modifications.

Between steps 4 and 5, the samples were tested for purity using a 1% agarose gel in order to verify RNA viability and purity before the samples were digested for LC-MS analysis.

Results

The results for this experiment examined the amount of viable RNA that was obtained and the total purity of *C. sphaerospermum* samples that were incubated in chitinase versus the control samples that were directly lysed following harvesting. The total RNA reclaimed from both data sets was similar, with both the chitinase incubated and control samples yielding about 20µg of total RNA/solution. Concentrations and therefore total quantity of RNA samples were analyzed using a NanoPhotometer, and the results of which are provided in supplemental information. An interesting result came from analyzing the purity of the samples, depicted below, which shows that the chitinase samples yielded a much cleaner sample of RNA versus the control sample.

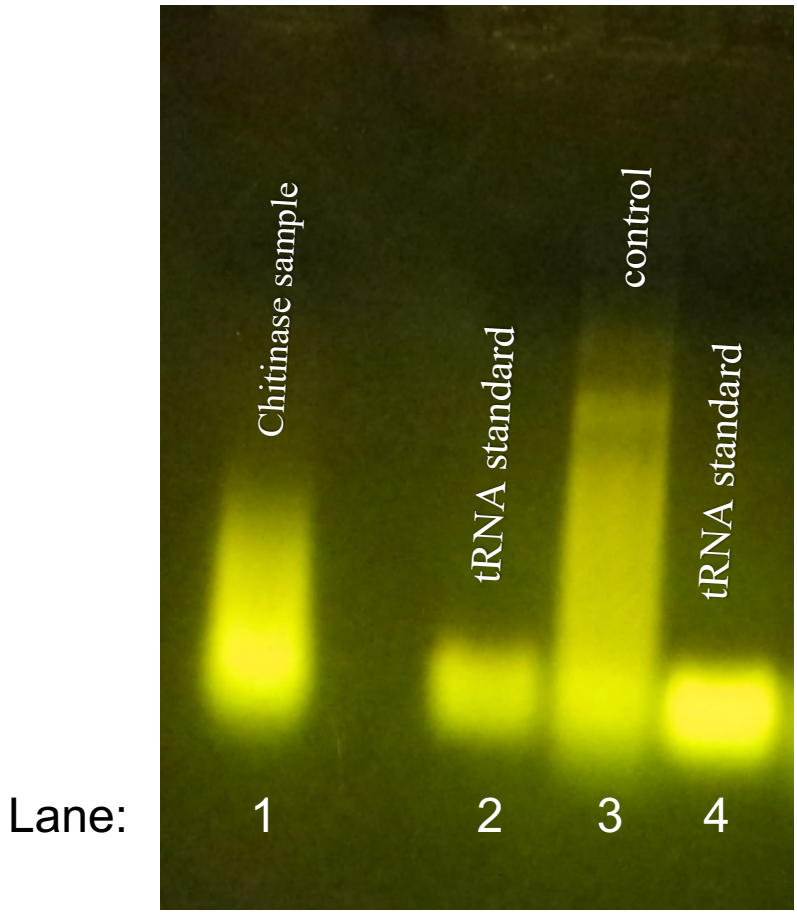

Figure 1.

The above image shows the resultant bands from the samples and controls that were processed in this experiment. Lane 1 shows the sample that was incubated for 72 hours in chitinase, lanes 2 and 3 show tRNA standards that were isolated from *B. subtilis,* and lane 3 shows the non-incubated sample of *C. sphaerospermum*. Resultant bands show that tRNA reclamation for both samples was non-degraded, but the higher bands of lane 3 show that there was tRNA contamination in this sample. Both lanes 1 and 3 showed moderate smearing, which is likely the result of high salinity in the samples, which is a common consequence of using box kits for RNA isolation.

Following verifying the viability of the samples, the chitinase incubated sample of purified tRNA was enzymatically hydrolyzed into ribonucleosides in order to prepare the samples for analysis by means of LC-MS. The resultant data was computed using Xcalibur (Thermo-Fischer Scientific) to identity the post-transcriptional modifications that were present in the sample.

Y	D	m4C	m5C
Cm	Um	m3U	m5U
I	Am	m2A	m1A
m1I	ac4C	hm6A	m2G
Gm	m7G	ncm5U	i6A
io6A	QtRNA	t6A	

Table 1.

Ribonucleoside modifications of *C. sphaerospermum* tRNA detected by LC-MS. The dection of queuosine, a predominantly bacterial modification in *C. sphaerospermum* is surprising. Its detection suggests similarities of genomic decoding mechanism between bacterial species and *C. sphaerospermum.*

Conclusions and future works

QtRNA is a tRNA modification that is highly conserved amongst bacterial species but has only been limitedly shown to be present in fungi. Especially when considering that this species has never had an RNA analysis, the ramifications of this investigation could have far reaching effects to the understanding of the inner-workings of melanized fungi and their evolutional history. Further, this research has potential to be studied more to show how pioneer species like *C. sphaerospermum* have such an innate ability to reclaim land that has been deemed by higher organisms as unsuitable. In regard to role of chitinase in RNA extraction, the results indicated a positive effect of the use of the protein, especially in regard to sample purity. However, although overall purity was shown to be enhanced, the total RNA for both the chitinase-incubated sample set and the control samples were similar, meaning that the relationship between this using this enzyme to maximize RNA recollection rates should be further investigated to harmonize incubation time to aid in maximizing reclamation.

Future work includes mapping of these modifications to specific tRNAs and understand their abundance in the overall tRNA pool. Moreover, it would be interesting to see how

tRNA modifications are affected by ionizing radiation to see how environmental modification this affects protein synthesis, especially when considering how studies into the metabolic role of melanin indicated a 4-fold increase in the reduction of NAD+ to NADH upon irradiation by gamma rays, compared to the non-irradiated pigment.[4]

The identification of such differences could also aid in development of antimycotic (antifungal) medications by targeting the translational machinery of pathogenic fungal cells, because this species has been shown to be an irritant to immunocompromised patients.

Works Cited

[1] Bloomfield BJ, Alexander M. Melanins and Resistance of Fungi to Lysis. Journal of Bacteriology. 1967;93(4):1276-1280. Web. 24 Feb 2018.

[2] Zhdanova, N. N., Zakharchenko, V. A., Vember, V. V., & Nakonechnaya, L. T. (2000). Fungi from Chernobyl: mycobiota of the inner regions of the containment structures of the damaged nuclear reactor. *Mycological Research*, *104*(12), 1421-1426.

[3] Dadachova, Ekaterina, et al. "Ionizing Radiation Changes the Electronic Properties of Melanin and Enhances the Growth of Melanized Fungi." PLoS ONE, vol. 2, no. 5, 2007. Web. 24 Feb. 2018.

[4] R. Ross et al., Methods (2016), 107:73.

Supplemental Information

Sample	concentration (ug/uL)	(A260/A280)	(A260/A230)
1	1.044	2.039	0.695
2	0.168	2.154	0.695
A	0.021	1.893	1.233
B	0.021	1.857	0.732

Table 2.

A representative Data set of purity for chitinase incubated samples versus a controlled data set. Samples 1+2 were control samples, whereas A+B were chitinase incubated. Sample 1 was shown by to be degraded when it was loaded to an agarose gel. Even though sample 2 had a high relative concentration, when further examined it was shown to have high levels of rRNA contamination which interfered with this reading.