An Roinn Talmhaíochta, Bia agus Mara
Department of Agriculture, Food and the Marine

Food Institutional Research Measure

Final Report

Developing yeast a s a factory for the production of the antioxidant ergothioneine ERGOYEAST

DAFM Project Reference No: 13/F/463

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End Date: 31/12/2015

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Collaborating Research Institutions and Researchers: Prof. Sean Doyle and Dr. David Fitzpatrick, both Maynooth University

Please place *one* "x" below in the appropriate area on the research continuum where you feel this project fits

Basic/Fundamental ⟶ Applied ⟶ Pre Commercial

1	2	3	4	5 X	6	7

Please specify priority area(s) of research this project relates to from the National Prioritisation Research Exercise* (NRPE) report;

Priority Area (s)	**Food for Health**

Key words: *(max 4)*
Antioxidant, yeast, cell-factory, ergothioneine

1. Rationale for Undertaking the Research

Using antioxidants in food products has implications for product stability, texture and shelf-life, additionally conferring health benefits for consumers. Ergothioneine (mol. mass = 229 Da) is a naturally occurring amino acid derivative, which is biosynthesised by certain bacteria and fungi, but not by *Saccharomyces cerevisiae* (Baker's yeast). Figure 1 shows ergothioneine is actually a modified version of histidine, whereby the imidazole ring is sulphurised, and the amino group is trimethylated. Unlike other cysteine-based thiols such as glutathione (GSH), ergothioneine is mainly found in the thione tautomer rather than a thiol form (Figure 1) at physiological pH (Fahey, 2013). At neutral pH, ergothioneine exhibits a higher redox potential (E'_0 = -60 mV) than GSH (E'_0 = -250 mV), which means that ergothioneine is less susceptible to auto-oxidation, and consequently more stable in an aerated aqueous solution (Hand and Honek, 2005; Hartman, 1990).

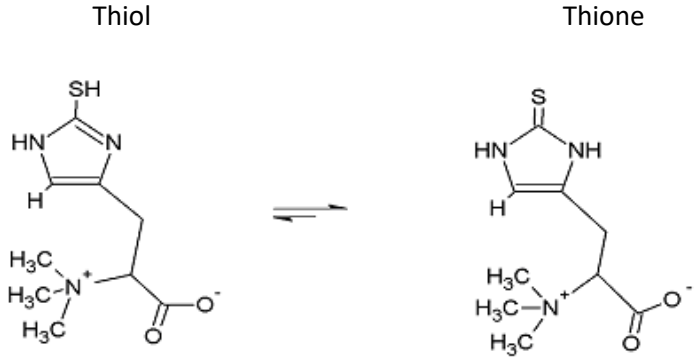

Figure 1: Ergothioneine exists in equilibrium between the thiol and thione forms.

Plants cannot bioynthesise ergothioneine and obtain it from soil microorganisms. Animals only get ergothioneine from their diet, and humans, specifically, acquire the modified amino acid via ingestion of plant or animal-based food containing ergothioneine. Animals possess a specific ergothioneine transporter (Gründemann *et al*., 2005), and concentration of ergothioneine occurs in specific tissues such as seminal fluid, erythrocytes, kidney and liver (Melville *et al*., 1954; Shires *et al*., 1997). Ergothioneine is found naturally in basidiomycete fungi and a number of companies promote the antioxidant benefits of constituent ergothioneine (http://www.monaghan-mushrooms.com/health-tips). However, the lack of standardisation of ergothioneine levels in mushrooms, along with limited knowledge regarding the timing of its biosynthesis, precludes precise assessment of its dietary benefits. Currently, the only way to manufacture ergothioneine is via a complex chemical synthesis, which is subject to a US patent (Pharmatech International Inc., 2010). Thus, a natural source of ergothioneine, which could be added to foodstuffs in precise physiologically-relevant amounts, would represent a quantum improvement in both manufacturer, and consumer, access to this potentially beneficial antioxidant.

Utilising our expertise in fungal biology (Davis *et al*., 2011; O'Hanlon *et al*., 2012; Fitzpatrick *et al*., 2011, Truman *et al*., 2012) and ergothioneine biochemistry (Gallagher *et al*., 2012), allowed the modification and optimisation the safe and effective cell factory, *S. cerevisiae*, to produce ergothioneine.

2. Research Approach

The project employed a variety of cutting-edge bioinformatics, molecular biology and biochemical techniques. A plethora of computational methods were used to assess the evolutionary history of the genes required for ergothioneine biosynthesis. This work was entirely novel as no such phylogenetic history of these genes existed at the time. Our work confirmed the bacterial origin of this gene family and the presence in specific lineages of the fungal kingdom. This work was published in the journal

Gene. The genes for biosynthesis of ergothioneine in *Aspergillus fumigatus* were identified and cloned into yeast expression vectors. This process allowed the *Aspergillus* genes to produce functional proteins in the non-ergothioneine producing yeast species, and allowed assessment of whether yeast can now make ergothioneine itself. The key point being that yeast is a biotechnologically safe and well-utilised organism that can be used to produce and harvest this important antioxidant. Gene expression of the *Aspergillus* genes in yeast was confirmed and also confirmed protein production for the key gene called *EgtA. EgtA* is the primary ergothioneine biosynthetic gene. Following confirmation of gene expression. Using HPLC and MS unequivocally identified ergothioneine being produced in yeast cells that carried the *EgtA* gene. Additionally, the antioxidant being produced was being excreted by the yeast cells. These points are significant as they relate specifically to the rationale behind the whole project. Being able to produce ergothioneine in yeast and that the organism readily excretes the antioxidant, provides a proof in principle for the approach taken and now provides a solid basis for further manipulation of the system to maximise production of this commercially important antioxidant.

3. Research Achievements/Results

The key and major finding from this work is that the biotechnologically well-utilised and safe organism baker's yeast, can be engineered to produce the commercially important antioxidant ergothioneine. We have unequivocally proven this can be achieved. Additionally, the finding that yeast readily excrete the antioxidant into the surrounding media provides an added benefit for development and optimisation of extraction procedures. Summary of research findings from the project tasks include:

a) First publication defining the molecular evolutionary history of genes required for biosynthesis of ergothioneine.
b) Production of range of yeast expression vectors harbouring *Aspergillus* genes required for ergothioneine biosynthesis.
c) Developed engineered yeast strains that produce ergothioneine.
d) Defined methods based on HPLC and MS for the detection of ectopically produced ergothioneine in yeast.

An important point to note from this project is that although the production of ergothioneine in natural biological processes and environments appears to require more than one gene, we are able to produce ergothioneine in yeast by expressing the single *Aspergillus* gene *EgtA*. This is important as this allows for the minimal genetic manipulation of yeast [do not need more foreign genes introduced into the organism!] to produce ergothioneine and means that yeast itself possesses the genetic [gene] or biochemical means to carry out the final steps of ergothioneine biosynthesis.

4. Impact of the Research

The impact of this project can be contextualised as both short term and long term. From a scientific standpoint the work has provided the first detailed evolutionary history of the gene required for ergothionieine biosynthesis. This work was published in 2014 in the journal Gene and has currently been cited 12 times. The information contained within this article will allow researchers working in the area of genetic manipulation of sulphur-containing biosynthetic pathways to better design experiments. Additionally, the wet-lab part of this project has produced an array of biological reagents for the expression of ergothioneine biosynthetic genes in yeast. Importantly, a proof of concept has been provided showing that yeast can be engineered to produce this important antioxidant. Importantly, we have also demonstrated that the extraction and recovery of ergothioneine from yeast is feasible and could be improved, scaled up and potentially be cost effective. Thus, the project has scientific impact in the short term in providing high quality research publications in the research field and also an array of

important biological reagents. For potential long-term impact the proof of concept and development of a methodology for ergothioneine recovery, provide a strong basis for further study and development of yeast to scale up procedures and produce significant amounts of ergothioneine with this newly developed production system.

In addition to the impact from results accumulated during the project, this work has also contributed to maintaining and enhancing the Irish scientific skills base. The project resulted in the training of a MSc student in advanced molecular biology and yeast/fungal genetics skills. The student produced a high-quality MSc thesis (awarded June 2016) and graduated from Maynooth University in September 2016. Directly from working on this project the student was offered employment at Alltech Ireland [Dunboyne, Meath] in brewing science [hence, directly utilising the skills acquired during this project]. The student currently has a permanent position with Alltech Ireland.

4(a) Summary of Research Outcomes

(i) Collaborative links developed during this research

N/A

(ii) Outcomes where new products, technologies and processes were developed and/or adopted

- Novel yeast reagents for the expression of ergothioneine biosynthetic genes.
- Genetically modified yeast that produce ergothioneine.
- Methodology for recovery of ergothioneiine for yeast.
- First published evolutionary history of ergothioneine biosynthetic gene family.

(iii) Outcomes with economic potential

- Long-term potential for upscaling of proof of concept of yeast that yeast can be engineered to produce the important antioxidant ergothioneine.
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(iv) Outcomes with national/ policy/social/environmental potential

N/A

4 (b) Summary of Research Outputs

(i) Peer-reviewed publications, International Journal/Book chapters.

- Jones G.W, Doyle S, Fitzpatrick D.A. (2014) The evolutionary history of the genes involved in the biosynthesis of antioxidant ergothioneine. Gene 549:160-170.

(ii) Popular non-scientific publications and abstracts including those presented at conferences

- Developing budding yeast as a cell factory for production of the antioxidant ergothioneine. Daragh Cuskelly, David Fitzpatrick, Grainne O'Keeffe, Sean Doyle and Gary W. Jones. Irish Fungal Meeting, Trinity College Dublin, June 20/21, 2015.
- Developing budding yeast as a cell factory for production of the antioxidant ergothioneine. Daragh Cuskelly, David Fitzpatrick, Grainne O'Keeffe, Sean Doyle and Gary W. Jones. Cell Stress

Society International, 7th International Congress on Stress Responses in Biology and Medicine. Huangshan City, China, 19-23 September 2015.

(iii) National Report

N/A

(iv) Workshops/seminars at which results were presented

N/A

(v) Intellectual Property applications/licences/patents

N/A

(vi) Other

- Daragh Cuskelly, Using yeast as a factory for the production of the antioxidant ergothioneine. MSc thesis September 2016, Maynooth University.

5. Scientists trained by Project

Total Number of PhD theses: 0

Total Number of Masters theses: 1

Mr. Daragh Cuskelly, MSc awarded in June 2016 from Maynooth University,
Title: "Developing budding yeast as a cell factory for production of the antioxidant ergothioneine"

6. Permanent Researchers

Institution Name	Number of Permanent staff contributing to project	Total Time contribution (person years)
Maynooth University	3	0.35
Total		**0.35**

7. Researchers Funded by DAFM

Type of Researcher	Number	Total Time contribution (person years)
Post Doctorates/Contract Researchers		
PhD students		
Masters students	1	2.0
Temporary researchers		
Other		
Total		**2.0**

8. Involvement in Agri Food Graduate Development Programme

Name of Postgraduate / contract researcher	Names and Dates of modules attended
N/A	

9. Project Expenditure

Total expenditure of the project:	€97,437.08
Total Award by DAFM:	€100,100
Other sources of funding including benefit in kind and/or cash contribution(specify):	€0

Breakdown of Total Expenditure

Category	Maynooth University	Total
Contract staff		
Temporary staff		
Post doctorates		
Post graduates	40,961.92	40,961.92
Consumables	31,274.73	31,274.73
Travel and subsistence	2,714.95	2,714.95
Sub total		
Durable equipment		
Other		
Overheads	2,714.95	2,714.95
Total	**97,437.08**	**97,437.08**

10. Leveraging

N/A

11. Future Strategies

Importantly, this work has provided a proof of concept that yeast can be engineered [minimally] to produce the antioxidant ergothioneine. The work also developed methods to recover the antioxidant being produced. To develop the project further the methodologies now need to be up scaled and optimised to allow for the maximum levels of ergothioneine production using this new and novel system. This will require further funding, which the PIs are considering for applying. Complicating factor is the relocation of Dr Jones to Leeds Beckett University UK and deciding on which funding sources offer the best opportunities for taking the project forward.

Additionally, the experimental results from this project are significant and of publication quality. Dr Jones and Prof. Doyle are in the process of drafting a scientific manuscript for submission to an applied microbiology journal.