Version June 2020

Sustainable Energy Authority of Ireland

National Energy Research, Development & Demonstration Funding Programme

FINAL REPORT TEMPLATE

SECTION 1: PROJECT DETAILS – FOR PUBLICATION

Project Title	A novel technology to maximise biofuel efficiency
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Report Submission Date	2/10/2023

	Name	**Organisation**
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Collaborators		

Project Summary (max 500 words)
Bioethanol is a potentially sustainable alternative to fossil fuels; however, challenges such as substrate shortages, low ethanol yield and high production costs all contribute to its relatively high price. It was recently discovered that by exploiting the hormetic effect of compounds such as H_2O_2, the reproductive ability and ethanol tolerance of biofuel producing organisms can be increased. Hormesis is a biological phenomenon characterised by beneficial results from exposure to low doses of a chemical that would be toxic at higher doses. *A3IS is a novel compound created by Nektr Technologies and its key characteristic is the slow and sustained release of low levels of H2O2 in a controlled manner. All components of A3IS are Generally Recognised As Safe (GRAS), thus it could be added to the fermentation medium without process changes.* *The aim of this project was to investigate the potential of A3IS to improve ethanol production in fermentation of Saccharomyces cerevisiae and other yeasts. The addition of A3IS to growing batch cultures of S. cerevisiae resulted in a longer lag phase, but pre-exposure of the inoculum to A3IS resolved this, demonstrating classical hormetic behaviour. Exposing the yeasts to A3IS improved tolerance to 25 % (v/v) ethanol in comparison to cultures that were not exposed, with up to 3-fold more cells surviving in the A3IS-treated cultures. Most interestingly, ethanol production also improved by approx. 12.5 % after exposure to A3IS. As A3IS is a biological*

system, timing is crucial and various testing parameters have to be brought in line to produce repeatable levels of ethanol in each experiment.
In conclusion, results obtained by this project indicate that A3IS has the potential to increase ethanol output and further research is warranted.

Keywords (min 3 and max 10)	Hormesis, A3IS, Ethanol, Biofuel, Yeast, Fermentation

NB – Both Section 1 and Section 2 of this Final Report will be made publicly available in a Final Technical Report uploaded online to the National Energy Research Database.

In the following Section, please provide a clear overview of your project, including details of the key findings, outcomes and recommendations. The section headings below are provided as a guide, please update or add to these as best suits your project.

By submitting this project report to SEAI, you confirm you are happy for Section 1 and Section 2 of this report to be made publicly available. If you wish to request edits to this section in advance of publication, please contact SEAI at EnergyResearch@seai.ie.

SECTION 2: FINAL TECHNICAL REPORT – FOR PUBLICATION (max 10 pages)

2.1 Executive Summary

Bioethanol is a potentially sustainable alternative to fossil fuels; however, challenges such as substrate shortages, low ethanol yield and high production costs all contribute to its relatively high price. It was recently discovered that by exploiting the hormetic effect of compounds such as H2O2, the reproductive ability and ethanol tolerance of biofuel producing organisms can be increased. Hormesis is a biological phenomenon characterised by beneficial results from exposure to low doses of a chemical that would be toxic at higher doses.

A3IS is a novel compound created by Nektr Technologies and its key characteristic is the slow and sustained release of low levels of H2O2 in a controlled manner. All components of A3IS are Generally Recognised As Safe (GRAS). The aim of this project was to investigate the potential of A3IS to improve ethanol production in fermentation of Saccharomyces cerevisiae and other yeasts.

The key findings were in increased ethanol resistance in S. cerevisiae and other biofuel producing microbes after exposure to A3IS which follows the principles of the classical hormetic effect. An 12.5% increase in ethanol production was also achieved when an in-house brewing yest was treated with A3IS. The results of this pilot project indicate that A3IS is a potential biofuel production enhancer and further research is warranted. This research project has been funded by the Sustainable Energy Authority of Ireland under the SEAI Research, Development & Demonstration Funding Programme 2021, Grant number 21/RDD/623.

2.2 Introduction to Project

Due to the current global challenges of providing energy security while ensuring environmental safety, the demand for an alternative and eco-friendly energy source is growing substantially [1]. The burning of fossil fuels contributes to greenhouse gas emissions as well as global warming causing rise in sea levels, climate change, loss of biodiversity and urban pollution [1-3].

Biofuel produced from various renewable sources is regarded as one of the most promising alternatives to fossil fuels. Many countries, such as USA, Brazil, China, Canada and several EU member-states have already invested interests in biofuel programs as the commitment to reduce the dependence on fossil fuels continues [1]. Bioethanol is a promising energy source which reduces greenhouse gas emissions by 19-48% (corn-based) and up to 100% (cellulosic feedstock) when compared to common gasoline while enhancing its performance when blended [4]. However, due to a shortage in substrate availability, low fermentation yield and relatively high production costs, there has been a considerable debate about the sustainability of biofuels [5-7].

It was recently discovered that hydrogen peroxide (H_2O_2) plays a crucial role in the induction of hormesis, a biological phenomenon by which a beneficial effect results from exposure to low doses of an agent that is toxic when given at higher doses [8]. The induction of mild stress can improve an organism's biological function and result in the development of resistance to either higher doses of the same stressor or other disturbing factors [9, 10]. The phenomenon has been observed in various organisms: from bacteria to humans. Current as well as early research suggests that exposure to low doses of H_20_2 induces cell growth in yeast cultures as well as reduced ethanol sensitivity [10-12]. In a study by Semchyshyn et al. [11], the tested Saccharomyces cerevisiae yeast strains grown in glucose and fructose medium demonstrated the peak hormetic response at 25mmol/L and 50mmol/L H_2O_2, respectively. At the hormetic concentrations, the yeast strains showed 130%-155% of the initial reproductive ability. A study carried out in 2014 [10] set the hormetic zone for several strains of S. cerevisiae at a hydrogen peroxide concentration of < 0.4mmol. The authors also showed that yeast

exposed to hormetic doses of H_2O_2 subsequently developed an increased resistance to ethanol exposure. Colony growths of 134% and 118% was observed when yeast was pre-incubation with 0.25mmol hydrogen peroxide and then challenged with 15% and 20% ethanol [10].

The proposed research project sets out to investigate an innovative solution to increase the market competitiveness, sustainability, and efficiency of biofuel production. A unique, patented technology (A3IS) owned by Nektr Technologies and developed in the Atlantic Technological University will be explored. The technology's key characteristic is the sustained and slow release of hydrogen peroxide at safe and controlled levels which is a distinct advantage over highly unstable pure hydrogen peroxide. All components of the novel technology are on the Generally Recognised as Safe (GRAS) Index with minimal environmental impact. During initial trials, the hormetic effect induced by A3IS was recorded and published in the patents protecting this technology.
Nektr Technologies hypothesises that the addition of A3IS to the fermentation process will increase biofuel production and raise its sustainability and efficacy while reducing the production cost. The continuous slow release of hydrogen peroxide will induce the hormetic effect, increasing fermentation microorganisms' reproductive ability while developing a higher resistance against ethanol.

2.3 Project Objectives

1. *Screen and quantify the hormetic effect on biofuel producing microorganisms for example Saccharomyces cerevisiae.*
2. *Establish if the hormetic dose increases the resistance against ethanol.*
3. *Establish if the hormetic dose increases biofuel production.*
4. *Investigate enzymatic activity of the A3IS hormetic effect in yeast*

2.4 Summary of Key Findings/Outcomes

This was the first trials assessing the potential of A3IS as a biofuel production enhancer. Previous research indicated the ability of H_2O_2 to induce an hormetic effect on biofuel producing microbes increasing reproductive ability and ethanol tolerance. However, the impact of those improvements on biofuel production was never assessed before. The project yielded positive results indicating the potential of the novel technology as a fermentation enhancer. However further research is needed to optimise the process and investigate the inclusion of A3IS at industrial scale manufacture.

Address each innovation in a bullet point below. Add as many bullet points as you need:

- *Innovation 1: Positive effect of A3IS on ethanol tolerance in S. cerevisiae*

  S. cerevisiae cultures were treated with different concentrations of A3IS and then exposed to 25 % ethanol for 1 hour, the surviving cells were counted. Significant improvement of surviving cell counts of 33-185% were noted depending on the A3IS concentration used.

- *Innovation 2: Increased ethanol production*

  The production of ethanol in an in-house brewing yeast strain was established in YPD cultures that were supplemented with 20 % glucose and grown for 5 days. Trials recorded a 12.5 % improvement in ethanol production in the yeast cultures.

- *Innovation 3: A3IS gives a hormetic effect with several biofuel producing yeast species such as S. cerevisiae, C. albicans, S. stipitis, E. aerogenes and a brewing yeast.*

  A3IS treated cell cultures showed a consistent increased in ethanol resistance.

2.5 Project Impact

As previously stated, this is the first time the effect of sustained hydrogen peroxide release on fermentation-based ethanol production was investigated. Although alternatives to fossil fuels are

needed, the sustainability of biofuels was questioned due to substrate shortages, low fermentation yield and relatively high production costs [5-7]. A3IS has shown to increase ethanol resistance and ethanol production when added to the fermentation process. This is a first step in the d evelopment process of a fermentation additive which will increase biofuel production, raise its sustainability and efficacy while reducing the production cost.

The application of the novel technology will support the Irish government in the transition towards a climate neutral energy system through the development of low-carbon technologies in a fast and cost competitive way. It will also support the SEAI in the transformation of Ireland to a society based on sustainable energy structures, technologies, and practices.

From a research aspect, the successful completion of the project is a first step in the development plan of A3IS as a biofuel production enhancer. The completion of the development plan will grow Ireland's national capacity to access, develop and apply international class energy RD&D by enhancing its international credibility in the field of renewable energy.
Regarding national guidelines and action plans, the development of Nektr's novel technology as a renewable energy technology will support a vast number of the outlined objectives:

- *Support the objective of supplying 15% of electricity demands from renewable sources.*
- *Provide for the increased use of biofuels to contribute to the growth of renewable energy and reduction of greenhouse gas emissions in the transport sector.*
- *Support the use of biomass to increase the level of renewable energy in the heat sector.*
- *Support the biofuel blending objectives: Statutory target at 1 1.1 1 1% from 1 January 2019 and 12.360% from 1 January 2020. Blending levels to reach E 10 and B12 by 2030 with statutory blend increasing incrementally. Increases in line with the overall Renewable Energy Source (RES) trajectory set out in the Energy Union Governance Regulation.*
- *Electricity Generation: 55% Renewable Energy Source-Electricity (RES-E) achieved in 2030 and maintained.*

2.6 Recommendations

This first assessment of A3IS as an ethanol production enhancer yielded very positive results. A3IS is a biological system and therefore many influencing factors have to be investigated and controlled for maximum output. It is recommended that further research is carried out to better understand and augment the impact of A3IS in the biofuel production sector. Furthermore, research is needed to incorporate A3IS in the industrial biofuel production process.

2.7 Conclusions and Next Steps

Results obtained by this project indicate that A3IS has the potential to increase ethanol output during the fermentation process. Further research is warranted and needed to maximise A3IS' impact on biofuel production and for the integration of A3IS in the industrial manufacturing process.