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Deuteration of proteins for neutron scattering and proton tunnelling research

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Abstract

Biodeuteration is the process of incorporating deuterium into biological molecules. Deuterated biomolecules are of interest to the users of ISIS Neutron and Muon Source as they provide contrast variation, which enables the selective visualisation of parts of multi-component biological complexes. Hence, we would like to develop a robust pipeline for deuterated protein production in-house. During this investigation we aimed to optimise the growth of protiated and deuterated *Botyrococcus braunii* microalgae cultures in flasks, produce an algae lysate as a carbon source for *Escherichia coli*, and optimise the conditions for recombinant protein expression in *E. coli*. We optimised the conditions for growing protiated and deuterated *B. braunii* cultures in-house in our environmental incubator (BINDER KBWF240), and those were 25°C with 16H:8H light: dark cycles and shaking at 60 rpm. We produced two different types of protiated and deuterated lysates (via autolysis and acid hydrolysis), which we used as a carbon source to evaluate expression in minimal media and find optimum recombinant protein expression conditions. We used an mNeonGreen-mCherry protein as a model to investigate the impacts of the different lysates, IPTG concentrations, and the additive solution on recombinant expression in *E. coli*, with the aim of achieving expression comparable to that of nutrient-rich LB media. The highest expression was found in samples grown in protiated ModC1 with either protiated or deuterated acid lysate, with 100 µM of IPTG, at 25°C gradually reducing to an undisclosed T1, and supplementation with an additive solution. Protein samples grown under these conditions were purified and analysed, revealing a deuteration level of 5.5%. Further investigations into using deuterated additive solution components were carried out and it was concluded that glucose is important for the mNeonGreen-mCherry expression yield. The recombinant proteins expressed under various conditions in deuterated ModC1 supplemented with deuterated lysate are yet to be purified and analysed for their deuteration level.

Abbreviations

Abbreviation	Full form
SANS	Small-angle neutron scattering
NR	Neutron reflectometry
MNG-MC	mNeonGreen-mCherry
H	Hydrogen
D	Deuterium
FRET	Förster Resonance Energy Transfer
FLIM	Fluorescence lifetime imaging microscopy
ESS	European Spallation Source
LB	Luria Broth (Miller)
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Lys1	Overnight autolysate
Lys2	Acid lysate
H-MNG-MC	mNeonGreen-mCherry produced in H-ModC1 with H-Lys2
D-MNG-MC	mNeonGreen-mCherry produced in H-ModC1 with D-Lys2

1. Introduction

ISIS Neutron and Muon Source is a facility that uses a high energy particle accelerator to produce neutron and muon beams. It is home to instruments that use these beams to characterise materials in varying fields of study, including biology (ISIS Neutron and Muon Source, n.d., Teixeira et al., 2008). Neutron scattering techniques are an effective tool for probing structural biology questions, making them highly valuable to our users in life science research in academia and industry. In the research fields of crystallography and reflectometry, neutrons offer a range of advantages over X-rays for studying biomolecular structure (Lakey, 2009). In comparison to X-rays, neutrons are less destructive yet can easily penetrate biological material (Ashkar et al., 2018). In principle, X-ray scattering depends on the number of electrons on the sample atoms, which makes it difficult to visualise hydrogen atoms as they have one electron. Neutrons, however, interact with the sample atoms' nuclei allowing clearer visualisation of hydrogen atoms (Lakey, 2009). Given the high prevalence of hydrogen in biomolecules and their roles in enzyme active sites, neutron scattering can be a valuable tool for investigating substrate-binding and enzymatic activity (Koruza et al., 2024). Additionally, neutrons can differentiate between the protium (H) and deuterium (D) isotopes of hydrogen (Ashkar et al., 2018). Protium contains one proton in the nucleus whilst deuterium has a proton and neutron (Tshilongo et al., 2026). The ability of neutrons to make this distinction makes biomolecule deuteration useful for structural biology (Lakey, 2009) and our users.

Deuterated molecules offer a variety of uses across a range of neutron scattering techniques. Hydrogen has more incoherent scattering than deuterium. This enables a greater signal to noise ratio in deuterated molecules (Meilleur et al., 2005), allowing the use of a smaller crystal to obtain the same amount of data as compared to a protiated molecule (Duff, 2019, Hazemann et al., 2005). Deuteration also provides contrast variation for neutron scattering. It can be utilised in conjunction with techniques such as small-angle neutron scattering (SANS) and neutron reflectometry (NR) to selectively enhance or silence deuterium-labelled components in multi-component systems (Koruza et al., 2024) by altering the D_2O to H_2O composition of the solvent. (Whitten & Trewhella, 2009).

Contrast variation with SANS is useful for investigating two-component systems such as protein-nucleic acid or protein-protein complexes (Chaudhuri, 2015). NR with contrast variation is a valuable tool for studying the structure of membranes (Caselli, Nylander & Malmsten, 2024). Hence, deuterated biomolecules are valuable for investigating biological systems.

Biodeuteration is the synthesis of deuterated compounds, for example proteins, using biological systems, such as bacteria, insect, yeast and mammalian cells. These cells must be grown in deuterated conditions, which enables deuterated proteins and complex metabolites to be harvested. Commonly used expression systems include bacteria, usually *Escherichia coli* (*E. coli*), or yeast due to their ability to adapt to deuterated environments well (Haertlein et al., 2016).

Current methods of deuterating proteins are dependent on the level of D-incorporation required and the resources available. Hydrogen-deuterium exchange can be used after the expression of proteins in protiated conditions. However, this only replaces labile hydrogens with deuterium (Blakeley, Hasnain & Antonyuk, 2015). Due to deuterated carbon sources such as D-glycerol being expensive (Duff, 2019), 65% to 85% deuterium incorporation into proteins could be achieved by expressing proteins in a minimal deuterated media with a protiated carbon source (Koruza et al., 2024). For perdeuteration (fully deuterated molecule), both a deuterated minimal media and a deuterated carbon source are needed (Meilleur, Weiss & Myles, 2009).

Botryococcus braunii (*B. braunii*) is a microalgae species which is rich in exopolysaccharides (Lupi et al., 1994), carbohydrates (Fernandes et al., 1989) and lipids (Metzger & Largeau, 2005). Its extract has previously been used as a carbon source for deuterated recombinant protein production (Koruza et al., 2024). *B. braunii* can successfully grow in highly deuterated media. They utilise photosynthesis to incorporate CO_2 and deuterium from D_2O-based media into organic molecules and metabolites without needing a deuterated carbon source (Koruza et al., 2024). The incorporation of D_2O directly into metabolites from the media is elucidated by the following photosynthesis equation, adapted for deuterated conditions (Stirbet et al., 2020):

$$6CO_2 + 12D_2O \rightarrow C_6D_{12}O_6 + O_2$$

B. braunii algae extract has been obtained by Koruza et al., (2024), by autolysing the culture at 50°C over 24 hours. It has then been used as a carbon source along with deuterated glycerol in the recombinant protein production of human carbonic anhydrase II in *E. coli* (Koruza et al., 2024). Additionally, studies using the *Tetradesmus obliques* algae strain compared a similar overnight autolysis protocol to acid hydrolysis using 1 M HCl. This investigation found that an acid lysate of algae showed better recombinant protein yield than an overnight autolysate (Fujiwara, Adachi & Sugimoto, 2025).

Bacteria tend to grow better in rich media as opposed to minimal media due to increased nutrient availability (Tao et al., 1999 and Elbing & Brent, 2019). However, for the high incorporation of deuterium into proteins, the bacterial cultures need to be grown in a deuterated minimal media supplemented with a deuterated carbon source (Li & Byrd, 2022). Unlike rich LB media, there are much fewer and more controlled protiated components that could be metabolised and incorporated into the recombinantly expressed protein, which is crucial for isotopic labelling (Cai et al. 2016).

The goal of this project is to establish a protocol for a deuterated protein production pipeline at the ISIS Neutron and Muon Source for the neutron beamlines. The first protein in this pipeline, which will be used to refine and optimise it, is an mNeonGreen-mCherry protein (hereafter referred to as MNG-MC) with a 3.3 nm linker designed at ISIS by the previous placement student Sian Pritchard. This protein is of interest to our collaborators at the Central Laser Facility, who would like to investigate the effects of deuteration of on Förster Resonance Energy Transfer (FRET) using Fluorescent Lifetime Imaging Microscopy (FLIM). Since MNG-MC fluoresces, it is convenient to measure protein expression without needing to purify it, for

example with the use of a fluorescent plate reader. The fluorescence is also visible to the naked eye, hence qualitative visual observations can provide reassurance on the observed trends.

This investigation aims were to (I) establish growing culture of protiated and deuterated *B. braunii* in flasks as opposed to bioreactors (which are significantly more expensive and time-consuming), (II) optimise a method to produce a rich deuterated lysate from *B. braunii* that supports recombinant protein expression in *E. coli*, (III) optimise the expression conditions to produce MNG-MC in protiated conditions, which can then be applied to deuterated conditions, (IV) express MNG-MC in deuterated conditions, and (V) purify and analyse the proteins using SANS, SDS-PAGE, and mass spectrometry.

2. Materials and Methods

2.1. Culture conditions of *B. braunii*

The *B. braunii* species utilised was of the Showa variant and Berkeley strain (Nomomura, 1988), and the protocol adapted from Koruza et al., (2024). The *B. braunii* was provided by Dr. Zoe Fisher at the European Spallation Source (ESS), from cultures grown in deuterated media for 10 years. The cultures were inoculated into a sterile, altered version of Bold's 3N media (Koruza et al., 2024): $NaNO_3$ at 0.740 g/L, $CaCl_2$ at 0.02 g/L, $MgSO_4$ at 0.036 g/L, K_2HPO_4 at 0.074 g/L, KH_2PO_4 at 0.174 g/L, NaCl at 0.024 g/L, Vitamin B12 at 0.135 mg/L, Biotin at 0.11 mg/L, Thiamine at 10.0 mg/L, Na_2EDTA at 4.5 mg/L, $FeCl_3$ at 0.584 mg/L, $MnCl_2$ at 0.25 mg/L, $ZnCl_2$ at 0.03 mg/L, $CoCl_2$ at 0.012 mg/L, Na_2MoO_4 at 0.025 mg/L, $CaCO_3$ at 0.2 mg/L. The components were dissolved in deionised H_2O or 95% recycled D_2O before filter sterilisation under a laminar flow hood (Koruza et al., 2024). The recycled D_2O was provided by Dr. James Tellam from the ISIS Deuteration Labs.

The *B. braunii* stocks (received frozen on wet ice and stored at -20°C) were thawed and inoculated into 100 mL of protiated modified Bold's 3N media and cultured in 1.8 L borosilicate Fernbach culture flasks. After two weeks, 25 mL of this culture was spun down at 6000 xg for 15 minutes (Koruza et al., 2024), and the pellet was resuspended in 100 mL of 95% recycled D_2O Bold's modified 3N media. This culture was grown at 23°C, in the BINDER KBWF240 environmental incubator, shaking at 60 rpm (adapted from Koruza et al., 2024).

2.2. Measuring and maintaining the growth of *B. braunii*

The growth of the deuterated and protiated cultures were measured weekly by centrifuging 1 mL culture aliquots and measuring the pellet wet weight as *B. braunii* biomass in mg/mL. When the biomass reached 18-25 mg/mL density, the cultures were diluted to 10-12 mg/mL with fresh media, adding the appropriate amount of filter-sterilised vitamin stock.

To ensure adequate aeration and light exposure, when the cultures reached 500 mL, half the volume was spun down at 6000 xg for 15 minutes at 4°C (Koruza et al., 2024), and the pellets and supernatant stored at -20°C. The culture was then diluted accordingly to 10-12 mg/mL with vitamin stock additions.

2.3. *B. braunii* overnight autolysate preparation

This protocol was adapted from Koruza et al. (2024), which was based originally on Kightlinger et al. (2014). The original protocol involved centrifuging the deuterated *B. braunii* culture at 6000 xg for 15 minutes. Every 100 g wet weight of the *B. braunii* pellet was resuspended in 400 mL 99% recycled D_2O, followed by 24 hours of incubation in a water bath at 50°C to allow autolysis. Post-autolysis, the samples were centrifuged at 10,000 xg for 30 minutes, and the resultant supernatant was aliquoted and stored at -80°C. For this investigation, this protocol was modified using conditioned *B. braunii* culture supernatant to resuspend the pellet instead of fresh D_2O. Additionally, protiated version of this lysate was also prepared using the protiated *B. braunii* culture, and its conditioned media for resuspension.

2.4. *B. braunii* acid lysate preparation

A second method was tested to prepare a rich *B. braunii* lysate. This protocol was an adaptation of the acid hydrolysis described in Hirs, Stein & Moore, (1954), and Fujiwara, Adachi & Sugimoto, (2025). The *B. braunii* culture suspension was aliquoted into 50 mL Falcon tubes and autolysed overnight as described above. Concentrated HCl or DCl, depending on whether protiated or deuterated lysate was being made, (approx. 12 M) was added to a final concentration of 1 M. The sample was heated in round-bottomed flask to 110°C, at 350 rpm for 48 hours under nitrogen gas to enable acid hydrolysis. The resulting acid lysate was allowed to cool to room temperature on the bench overnight. Conditioned media from the *B. braunii* culture was used to top the lysate up to the volume it was before acid lysis. It was then spun down at 10,000 xg for 10 minutes, and the supernatant was collected as acid lysate, pH 1.0. It was then processed to make it compatible with the *E. coli* physiological pH of 7.0 (Tuttle, Trahan & Son, 2021). The resulting acid lysate was aliquoted and stored at -80°C

2.5. MNG-MC construct

The MNG-MC construct was designed in-house by a previous placement student with the objective of expressing deuterated and protiated samples. The construct contains a rigid alpha helix linker of 3.3 nm between the mNeonGreen C-terminus and the mCherry N-terminus. The linker consists of a repeated motif of A(EAAAK)nA. The construct also contains a 6x His-Tag at the C-terminus. The protein sequence was produced in a pET-29b+ vector, by Twist Bioscience.

E. coli Rosetta (DE3) pLysS Competent cells (genotype: F-*ompT hsd*S_B($r_B^-m_B^-$) *gal dcm* (DE3) pLysSRARE (Cam^R)) (Sigma-Aldrich: 70956-M) were chosen as the expression system. The (DE3) label specifies the presence of a chromosomal T7 RNA polymerase gene with a *lacUV5* promoter, allowing compatibility with pET vectors and induction using IPTG (Studier & Moffatt, 1986, and Novagen, n.d.). The pLysS designation indicates the presence of a plasmid encoding chloramphenicol resistance and a T7 lysozyme. This inhibits T7 RNA Polymerase, reducing basal gene expression before induction (Studier, 1991 and Zhang & Studier, 1997). The Rosetta strain is developed to better support the expression of sequences with codons often found in eukaryotic cells, which MNG-MC contains, by containing genes encoding the compatible tRNAs (Novy et al., 2001) (Novagen, n.d.).

2.6. Recombinant protein expression

The MNG-MC protein was expressed in protiated or deuterated ModC1 minimal media (Koruza et al., 2024), or rich Luria Broth Miller (LB) media (Catalogue number 1280-1660 Fisher Scientific) to determine the optimal conditions.

The ModC1 media was supplemented with *B. braunii* overnight autolysate or acid lysate as the carbon source for *E. coli* (Koruza et al., 2024 and Fujiwara, Adachi & Sugimoto, 2025). ModC1 (Duff et al., 2015, and Middelberg et al., 1991): NH_4Cl at 2.58 g/L, KH_2PO_4 at 2.54 g/L, Na_2HPO_4 at 4.16 g/L, K_2SO_4 at 1.94 g/L, $FeSO_4$ at 20.0 mg/L, Trisodium citrate at 88.0 mg/L, $MnSO_4$ at 5.0 mg/L, $ZnSO_4$ at 8.6 mg/L, $CuSO_4$ at 0.76 mg/L, $MgSO_4$ at 670 mg/L, Vitamin B12 at 0.11 mg/L, Thiamine at 8 mg/L, and Biotin at 0.08 mg/L. These components were dissolved in deionised H_2O or recycled D_2O followed by autoclaving or filter-sterilisation, respectively.

Various culturing condition parameters were tested to optimise the expression of MNG-MC in protiated conditions, before expression was attempted in deuterated conditions.

The protocol for expression in minimal media was adapted from (Koruza et al., 2018) and (Koruza et al., 2024). Overnight cultures of the transformed *E. coli* cells were established by inoculating 80 mL of LB media with a 1 mL glycerol stock, supplemented with 30 µg/mL kanamycin and 34 µg/mL chloramphenicol (Novagen, 2002). They were incubated overnight at 37°C, shaking at 180 rpm. After at least 17 hours, the OD_{600} of the cultures were measured and they were diluted using LB media to an OD_{600} of 4.5 (late-log phase determined from preliminary growth curve experiments, data not shown). 10 mL of this culture was aliquoted into labelled Falcon tubes, which were then centrifuged at 6000 xg for 10 minutes at 20°C.

These pellets were resuspended in either LB media or ModC1 media. For ModC1 samples, either overnight autolysate (hereafter referred to as Lys1) or acid lysate (hereafter referred to as Lys2) was added as the carbon source. Kanamycin was added at a final concentration of 30 µg/mL (Novagen, 2002). IPTG was added to a final concentration of 100 µM (Lozano Terol et al., 2004 and Ojima et al., 2025), and cultures were incubated at temperature 'T1', shaking at 180 rpm. After a few hours, a solution of additives in millimolar concentrations was added (Ojima et al., 2025, and Callejo-López et al., 2023). The bacteria were grown in final culture volumes of 5 mL.

2.7. Quantification of expression using a fluorescent plate reader

100 µL of each sample was loaded onto a fluorescence microplate. The MNG-MC expression levels were measured by recording the fluorescence emission at 520 nm and 620 nm after excitation at 485 nm and 584 nm, for mNeonGreen (Shaner et al., 2013) and mCherry, respectively (Shaner et al., 2004) using the BMG Labtech FLUOstar Omega fluorescent plate reader. The OD_{600}, reflecting the cell number, of each sample was measured with at least three repeats using a NanoDrop, and cell number was calculated using their mean average.

No statistical analysis was possible as not all the experiments were repeated due to time constraints, as each experiment lasted one week. Additionally, the data between the different experiments was not always comparable as the gain settings on the fluorescent plate reader

was adjusted for each experiment. However, in some experiments that were repeated, similar general trends were observed for the results.

2.8. Purification of MNG-MC

The 5 mL cultures were harvested by centrifuging at 6000 xg, for 10 minutes at 20°C. The resultant pellets were either processed or stored at -80°C.

Affinity chromatography with a HisTrap FF crude 1 mL Ni-NTA column on the ÄKTA pure™ chromatography system was used to purify MNG-MC from the pellet. The buffers used for the binding and elution of the protein are as follows (Cytiva, 2020). Binding buffer: NaH_2PO_4 at 20 mM, NaCl at 0.5 M, and Imidazole at 20 mM, pH 7.4. Elution buffer: NaH_2PO_4 at 20 mM, NaCl at 0.5 M, and Imidazole at 0.5 M, pH 7.4.

The following protocol for preparation of the clarified cell lysate sample to load onto the column was adapted from the Novagen User Protocol TB245 Rev. F 1108. The pellet was resuspended in 2.5 mL of BugBuster Protein Extraction reagent per gram of pellet wet weight. cOmplete Mini Protease Inhibitor cocktail (Roche, Category number 11 836 153 001) was added to 2X of the working solution concentration suggested in the user manual (Roche, 2020). 5 µL Benzonase nuclease was added per gram pellet wet weight. The solution was gently mixed and then centrifuged at 16,000 xg for 20 minutes at 4°C. The supernatant was collected, and an equal volume of Binding buffer was added it. The resultant solution was used as the clarified sample and loaded onto the HisTrap FF crude 1 mL column. The column was washed with 20 column volumes of the Binding buffer. The protein was eluted using a gradient elution over 5 columns of Elution buffer. The eluted protein was collected in a 96-well fraction collector in 0.5 mL fractions.

The fractions likely containing MNG-MC according to the elution profile were analysed using SDS-PAGE. These fractions were pooled together and concentrated to approximately 1 mg/mL using a 50kDa concentrator (Thermo Scientific Pierce Protein Concentrator 88540). Final concentrations were confirmed using NanoDrop measurements.

The concentrated samples were then analysed using neutron scattering on the LOQ instrument at ISIS Neutron and Muon source, and mass spectrometry at the Research Complex at Harwell.

2.9. Analysis of MNG-MC

To study MNG-MC using FLIM at the Research Complex at Harwell, preliminary data collection was done using a one-photon and two-photon excitation Time-Correlated Single Photon Counting set-up. The one photon excitation used a 488 nm light at 39 MHz, obtained using a Super K Extreme NKT-SC 390-2,000 nm super continuum laser and a SuperK SELECT (NKT, Photonic Solutions, UK). To eliminate laser scatter, a 525/39 bandpass filter and an FL515 long-pass filter (Thorlabs) were utilised as emission filters.

For the two-photon excitation, a mode-locked tuneable laser, 660 – 1320 nm (Chameleon Discovery NX, Coherent Lasers), provided laser light at a wavelength of 950 nm with 100 fs

pulse width at 80 MHz. Two BG39 filters (Comar Optics, UK) were used to eliminate the near infra-red light.

For both one and two-photon excitation a custom build system constructed around a Nikon Ti2-E microscope equipped with a 60×, NA 1.27 water immersion objective was utilised. Photon detection was performed using a hybrid photomultiplier tube (HPM100-40, Becker & Hickl), linked to a single photon counting module (SPC830 and SPC-QC-104 (Becker and Hickl, GmbH)).

3. Results and Discussion

3.1. Optimising the growth of *B. braunii* in protiated and deuterated conditions

The *B. braunii* received from our collaborator Dr. Zoe Fisher had the recommended growth conditions of 23°C, with 12:12 hour light: dark cycle, and shaking at 60 rpm (Koruza et al., 2024). The environmental incubator used by Koruza et al., (2024) was a Multitron Pro INFORS HT, which has varying lighting settings that do not translate directly to the BINDER KBWF240 model used in this investigation. *B. braunii* growth can be monitored visually by observing colour and density. We also visually evaluated the condition of the algae cells by observing the liquid culture using a stage light microscope.

The *B. braunii* culture when first inoculated into fresh protiated modified Bold's 3N media was grown as described in the Material and methods. After the initial few days post-inoculation, where the culture was green, it began turning yellow, as seen in the left and centre panels of Figure 1. Yellowing of *B. braunii* is often seen when the culture moves to the stationary phase (Tonegawa et al., 1998, Lozoya-Gloria, Morales-de La Cruz & A. Ozawa-Uyeda, 2020). Due to this observation occurring shortly after inoculation, when the culture is expected to be able to grow without additional media supplements for 14 to 28 days (Koruza et al., 2024), it was unlikely that nutrient unavailability led to stress. The inoculated culture was non-axenic, and an overgrowth of symbiotic environmental bacteria was previously observed under these conditions in-house, suggesting this may have occurred this time. To boost growth, the culture was then incubated at 25°C, which resulted in a dark green, healthier-looking culture, as seen in the right panel in Figure 1. This observation is supported by the fact that this is within *B. braunii*'s optimal temperature range (Dayananda et al., 2007, and Nazloo et al., 2024).

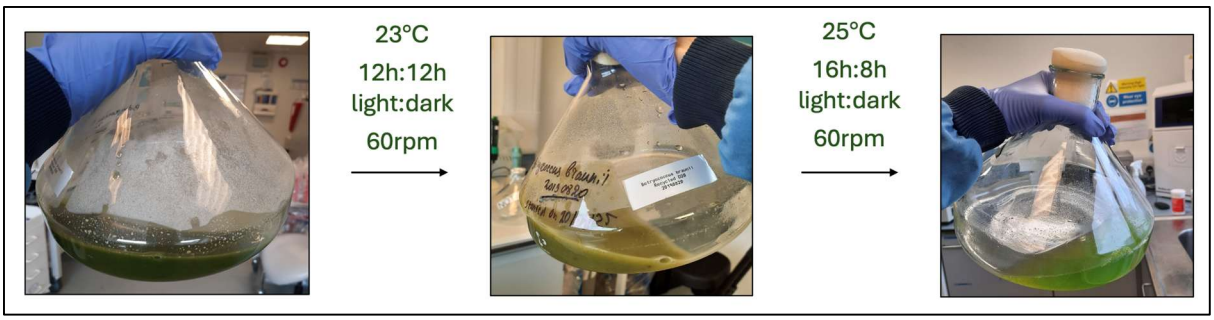


Figure 1: Visual changes in *B. braunii* cultures in response to variations in culturing conditions

Figure 2 represents the optimisation of *B. braunii* cultures in varying conditions, by displaying their growth. These data points were obtained using the wet pellet biomass measurements from 1 mL of culture. Given that the culture is non-homogenous, with cells clustering in clumps, these measurements may not be entirely representative of the actual biomass and growth. However, since cell clumping makes optical density an unreliable method of measuring algae growth, unlike OD_{600} with bacteria, (Koruza et al., 2024) this provided a simple way to monitor growth quantitatively.

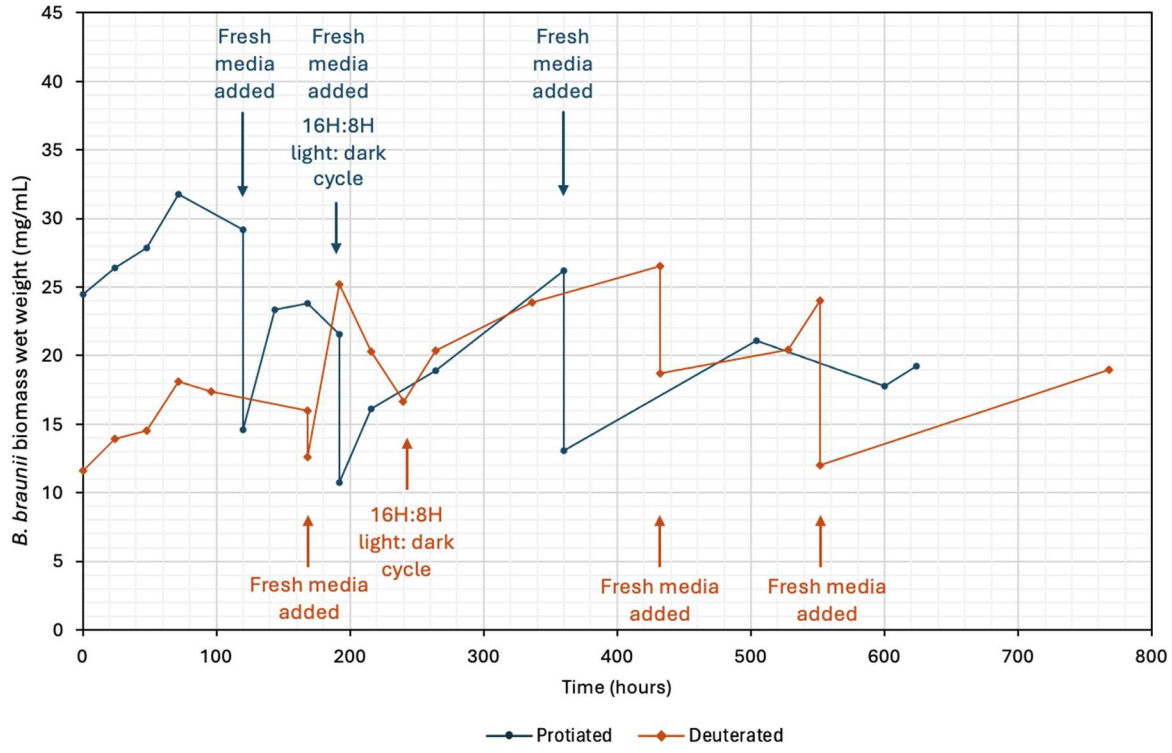


Figure 2: Protiated and deuterated *B. braunii* culture biomass (mg/mL pellet wet weight) as a function of time (hours). Arrows indicate time points where fresh media, supplemented with fresh vitamins was added to the culture.

Some papers reported good biomass growth with 16H:8H light: dark cycles, hence this change was introduced (Dayananda et al., 2007). Figure 2 shows an increase in protiated and deuterated culture biomass following this, although the protiated culture increase may also be due to the addition of fresh media and vitamins. These were added to prevent nutrient scarcity or stress conditions when growth stagnated, or the biomass density reached 25 mg/mL. As seen in Figure 2, *B. braunii* respond favourably as each addition is immediately followed by a biomass increase. The vitamins (thiamine, biotin and cobalamin) function as cofactors in *B. braunii*'s metabolic pathways, which may help boost growth (Croft, Warren & Smith, 2006).

B. braunii is slow growing, with a doubling time of 3 to 7 days (Cheng et al., 2019), which is reflected in Figure 2, though doubling time in this case was difficult to calculate due to the lack of sufficient data points as it was not the main purpose of the project. The protiated culture does not seem to consistently grow to a much higher cell density than the deuterated

culture, likely due to the strain being adapted to D_2O for many generations (Koruza et al., 2024).

Even prior to the temperature and lighting adjustments, the culture lasted longer than it has during previous attempts in the ISIS Biolab. One of the factors contributing to the poor growth previously observed may have been the lack of a CO_2 source present in the culture. Given algae's photosynthetic nature, bubbling CO_2 in low concentrations through the culture sometimes boosts growth (Cheng et al., 2018, and Nazloo et al., 2024). However, we were advised against doing this by our collaborator Dr Zoe Fisher, as it had previously killed this deuteration-adapted strain. The commonly accepted formulation for Bold's 3N media includes a soil water additive with $CaCO_3$ (Schwartz, 1975) which was not used by Koruza et al., (2024). It has been discussed that carbonate can react with H^+ ions from *B. braunii*, stabilising pH and releasing CO_2 (Miura et al., 2022). Hence, in this investigation, a $CaCO_3$ solution as per the original media formulation (Schwartz, 1975) was included. Given the survival of the culture, it is likely that this addition has been beneficial to algae growth.

It is a possibility that cultures in other labs survived without bubbling or $CaCO_3$ addition due to the potential use of water containing traces of limescale, which contains $CaCO_3$, for the media preparation.

3.2. Recombinant protein expression

The overall aim of the project was to express MNG-MC in deuterated minimal ModC1 media, supplemented with deuterated *B. braunii* lysate as a main carbon source. To achieve a high expression yield, the optimal culturing parameters were first investigated in protiated media with protiated lysate, with the aim of then applying these conditions to deuterated protein production.

To begin with, the expression of MNG-MC in nutrient-rich LB media at 24 and 48 hours after induction with varying concentrations of IPTG was tested.

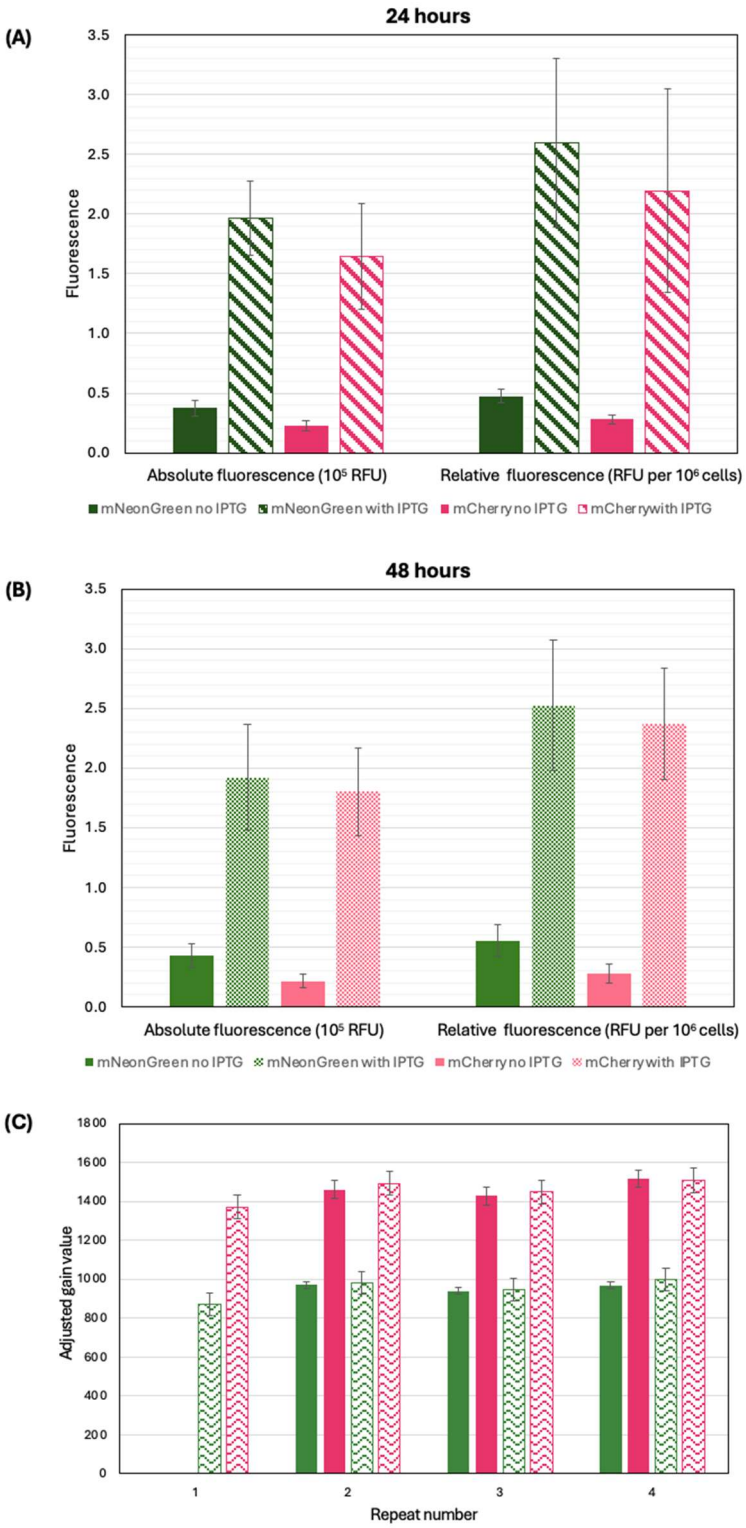


Figure 3: Absolute fluorescence (10^5 RFU) and relative fluorescence (RFU per 10^6 cells) of *E. coli* Rosetta pLysS cultures suspended in LB media, with and without induction with 100 µM IPTG at (A) 24 hours and (B) 48 hours. (C) Gain adjustment settings for each of the 4 repeats of this experiment at 24 and 48 hours. Error bars indicate standard deviation. Experiment 1 does not have 24H data as there was an error with the microplate reader and the data could not be collected.

Figure 3 displays the absolute and relative mNeonGreen and mCherry fluorescence recorded at 24 and 48 hours in LB media with and without IPTG. Whilst absolute fluorescence indicates the overall presence of MNG-MC in 100 µL, the relative fluorescence uses cell number calculated from OD_{600} to calculate MNG-MC expression per cell (indicated as per 10^6 cells for ease of interpretation).

As seen in Figure 3A and B, both absolute and relative fluorescence in LB without IPTG is much lower than in LB with IPTG. This indicates little basal expression of the construct, as expected from a pLysS strain (Studier, 1991, and Zhang & Studier, 1997). The recorded fluorescence in the uninduced sample could be attributed to the cells' autofluorescence (Surre et al., 2018).

The standard deviation error bars in Figures 3A and 3B are quite large across the values, due to the fluorescence values of the third replicate being a bit higher than the others, which are close in range. This could be attributed to slight environmental variations which are difficult to entirely avoid in batch cultures (Pouresmaeil & Azizi-Dargahlou, 2023). As the data was collected mostly with 4 repeats, there isn't a large enough sample size to confidently disregard a higher reading. Additionally, the gain values between each reading were adjusted for this set of repeats, which also could account for the large error bars. The variation in the gain adjustment settings across the repeats is found to be rather low, as shown in Figure 3C. Additionally, since the objective of this set of experiments was to assess the trends in expression levels rather than the ultimate quantification of the fluorescence, the averages were calculated and depicted in Figure 3A and B despite gain adjustments.

For the following analysis, relative fluorescence data was used to investigate the impact of varying conditions on individual cell expression, not considering their impact on overall growth. Additionally, as depicted in Figure 3, there was a general increase in fluorescence from 24 to 48 hours, hence all the subsequent experiments were run over 48 hours. The following data analysis compares the fluorescence of samples at 48 hours as this is when the cultures were harvested.

To optimise recombinant protein expression in these cells, various concentrations of IPTG were tested, as displayed in Figure 4.

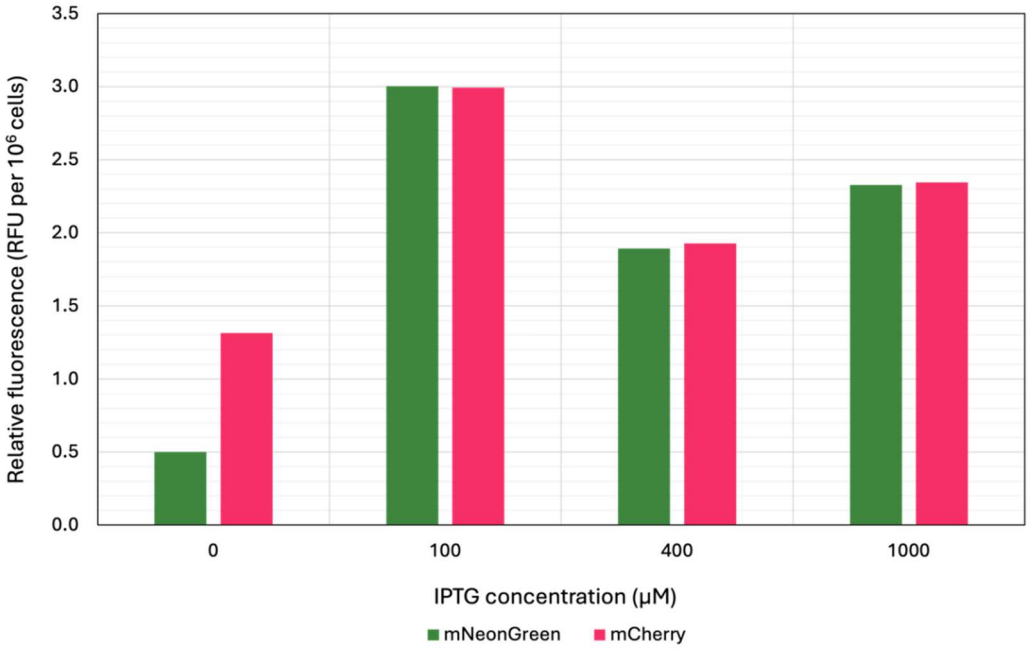


Figure 4: Relative fluorescence (RFU per 10^6 cells) of *E. coli* Rosetta pLysS cultures at 48 hours post-induction with 100, 400, and 1000 µM of IPTG in LB media. No standard deviation error bars as the experiments were not repeated in triplicate.

A range of IPTG concentrations have been recommended in the literature for protein expression in *E. coli* cells. The User Protocol TB055 Rev. C 0611JN suggests using 1 mM to induce expression in cells containing the pET29b+ vector as it has a T7 *lac* promoter, which offers tighter control on basal expression levels (Reischl, 1998). However, other literature investigating a range of IPTG concentrations at differing induction times has observed optimal expression from 0.05 to 0.1M IPTG despite the recommended 1 M concentration (Mühlmann et al., 2017). Three IPTG concentrations were trialled, 100 µM based on the findings of previous literature (Lozano Terol et al., 2004 and Ojima et al., 2025), 400 µM and 1000 µM based on the User Protocol TB055 Rev. C 0611JN recommendations for classic T7, and T7 *lac* promoters, respectively (Novagen, n.d.).

As seen in Figure 4, 100 µM displays highest expression in LB media. This is likely due to the increased metabolic burden of protein expression at a higher IPTG concentration (Mühlmann et al., 2017).

Hence, going forward all the experiments were induced with 100 µM of IPTG.

3.3. Evaluation of experimental conditions in protiated ModC1 media

The expression conditions for MNG-MC in protiated ModC1 media were optimised before expression was attempted in deuterated conditions. The main parameters investigated were the impact of the type of lysate (overnight autolysate (Lys1) or acid lysate (Lys2)), the additive solution, and post-induction temperature conditions on MNG-MC expression.

For the autolysate preparation, additional attempts to enrich the lysates were evaluated. These were heat-inactivation, sonication, and the resuspension of *B. braunii* pellets in

conditioned media (preliminary experiments, data not shown). Most of these steps did not make much of a difference. There was marginally better expression when the *B. braunii* pellets were resuspended in conditioned media as opposed to fresh H_2O. Hence, the only modification made to the original protocol was the resuspension of the *B. braunii* pellet in conditioned media from the first centrifugation rather than in fresh H_2O/D_2O.

The impact of the two types of lysates (Lys1 and Lys2), as well as supplementation with the additive solution was tested, and the results are displayed in Figure 5.

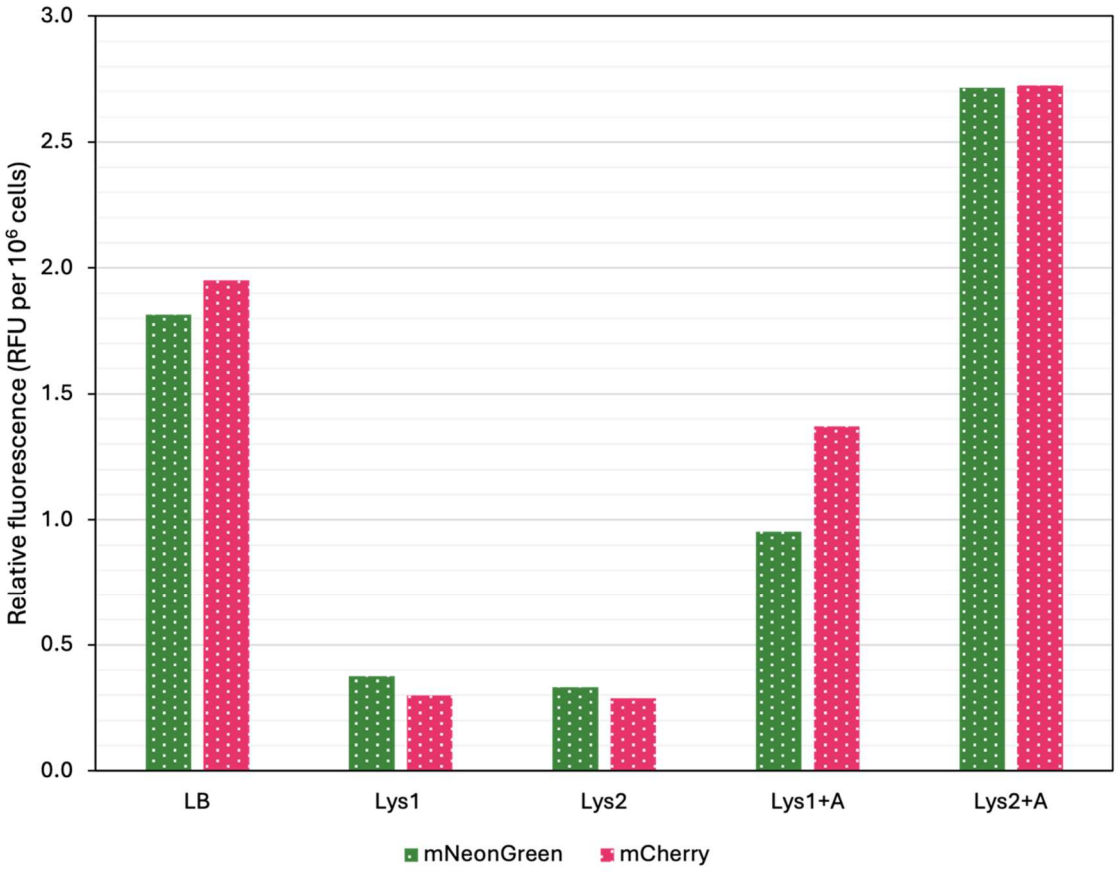


Figure 5: The relative fluorescence (RFU per 10^6 cells) of MNG-MC when supplied with LB media, Lys1, or Lys2 as a carbon source with and without the additive solution added a few hours post-induction. '+A' indicates the supplementation with the additive solution. Fluorescence readings were taken at 48 hours post-induction. Green bars indicate the fluorescence of mNeonGreen, whilst pink bars indicate the fluorescence of mCherry. Samples supplemented with Lys1 and Lys2 were grown in protiated ModC1, with both lysates being protiated. No error bars displayed as experiment was not repeated in triplicate.

Figure 5 indicates that the highest expression is observed when Lys2 is used as a carbon source in protiated ModC1 media with the additive solution. This agrees with the literature that compares the efficacy of overnight autolysis to acid hydrolysis of an algae lysate for recombinant protein expression (Fujiwara, Adachi & Sugimoto, 2025). The increased expression with Lys2 could be due to the presence of additional compounds for the *E. coli* to metabolise as the acid hydrolysis may have broken down the *B. braunii* biomass more than

the autolysis (Fujiwara, Adachi & Sugimoto, 2025). Hence, it is possible that the ModC1 media supplemented with Lys2 was richer than Lys1 and even LB. Recombinant protein expression increases the cell's metabolic burden and causes it to utilise its amino acid and energy resources (Donovan, Robinson & Glick, 1996 and Mühlmann et al., 2017). This may also lead to a stress response, which may be exacerbated in media with fewer nutrients, such as Lys1. Stress responses could increase the levels of proteases in the *E. coli*, thus resulting in lowered expression (Donovan, Robinson & Glick, 1996). Lys2 likely provides more biomolecules than Lys1, reducing the need for the *E. coli* to synthesise them (Elbing & Brent, 2019) and hence lowering the metabolic burden and stress responses associated with recombinant protein expression.

As seen in Figure 5, much lower expression was observed in the ModC1 cultures that were not supplemented with the additive solution, with little difference between the Lys1 and Lys2. This can be explained by the Rosetta pLysS strain used for expression. This strain enables the tight control of basal expression as it contains a chloramphenicol-resistant plasmid that expresses small quantities of a T7 lysozyme that inhibits the T7 RNA polymerase (Moffatt & Studier, 1987 and Studier, 1991), preventing MNG-MC expression. This is beneficial as it ensures that in deuterated conditions, little to no MNG-MC is expressed before the *E. coli* is supplied with the deuterated lysate and IPTG, which is essential for the deuterium-labelling. However, if the strain is cultured in a media lacking glucose, cyclic adenosine monophosphate (cAMP) levels in the cells increase and activate the chloramphenicol acetyl transferase promoter, which is sensitive to catabolite repression (Novy et al., 2001), according to the User Protocol TB055 Rev. C 0611JN (Novagen, n.d.). This may lead to higher levels of T7 lysozyme being expressed, which would inhibit the T7 RNA polymerase promoter and lower MNG-MC expression levels. The additive solution contains millimolar quantities of glucose, which likely prevents the increased lysozyme levels, enabling greater expression. This explains the increased in expression observed upon the addition of this solution. The disadvantage of this is that the *E. coli* would likely preferentially metabolise glucose instead of the biomolecules provided by the lysates (Moses & Prevost, 1966). Hence, very small quantities of glucose have been used, but there is still the possibility of a lower protein deuteration level due to the presence of a protiated carbon source.

In addition to carbon sources, two temperature conditions under 37°C were tested to assess their impact on MNG-MC expression, as displayed in Figure 6. Lower *E. coli* culturing temperatures have been shown in literature to improve recombinant protein expression and their solubility (Kataeva et al., 2005 and Shirano & Shibata, 1990). This is due to cellular processes occurring at a slower rate (Francis & Page, 2010) and the reduced activity of certain heat-shock proteases that tend to be upregulated during recombinant protein overexpression (Spiess, Beil & Ehrmann, 1999, Hunke & Betton, 2003, and Sahdev, Khattar & Saini, 2007).

The cultures were grown overnight in LB at 37°C and were incubated at a lower temperature T1 post-induction with IPTG. They were placed in the incubator either directly at T1 or at 25°C post-induction, which was gradually reduced to T1 as the incubator cooled.

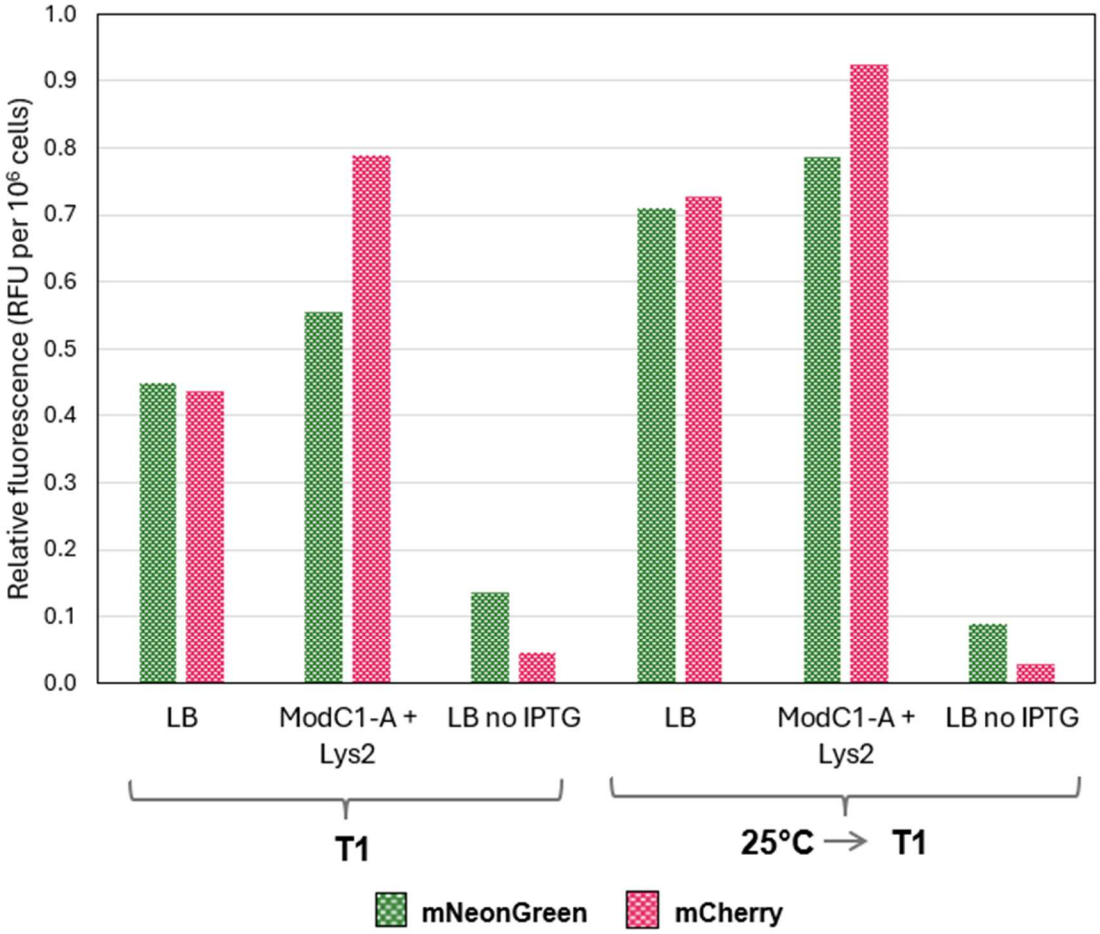


Figure 6: **The relative fluorescence (RFU per 10^6 cells) of MNG-MC at two temperature conditions in LB and in ModC1 supplemented with Lys2 and additives.** 'ModC1-A' indicates ModC1 supplemented with the additive solution. Fluorescence readings were taken at 48 hours post-induction. Green bars indicate the fluorescence of mNeonGreen, whilst pink bars indicate the fluorescence of mCherry. Samples supplemented with Lys1 and Lys2 were grown in protiated ModC1, with both lysates being protiated. No error bars as experiment was not repeated in triplicate.

Figure 6 illustrates much higher expression in *E. coli* grown both in LB and in ModC1 with Lys2 when the temperature is gradually reduced. This is speculated to be because the *E. coli* were spun, handled, and resuspended at ambient temperature, hence having a step-reduction in temperature may have led to an increased stress response. Studies on other strains of *E. coli* have observed the reduced expression of genes involved in protein synthesis when the cells were exposed to abrupt reductions in temperature (King et al., 2014). It is possible that the sudden change accompanied by the shift from rich to minimal media, and the addition of IPTG led to an increased stress response in comparison to the gradual temperature reduction, which may have allowed for more adjustment.

Based on these optimisation experiments in protiated conditions, the following culturing parameters were deemed the most effective and were used for the deuterated protein expression attempt. The *E. coli* was grown overnight at 37°C, spun down, and resuspended in

ModC1 media supplemented with 10% v/v Lys2. The cultures were induced with 100 µM IPTG. They were incubated at 25°C which was gradually reduced to T1 post-induction, and the additive solution was added a few hours post-induction.

3.3. Deuterated protein production

Deuterated protein production was attempted using the above protocol. Samples with both protiated (H-Lys2) and deuterated Lys2 (D-Lys2) were set up, in both protiated (H-ModC1) and deuterated ModC1 (D-ModC1). Additionally, for samples in D-ModC1, a wash-step was added before resuspension in D-ModC1 (Koruza et al., 2024). The pellets were first resuspended in 5 mL of conditioned 95% D_2O *B. braunii* media before being spun down again and resuspended in the appropriate volume of D-ModC1. This was to ensure little to no protiated LB media remained in the culture.

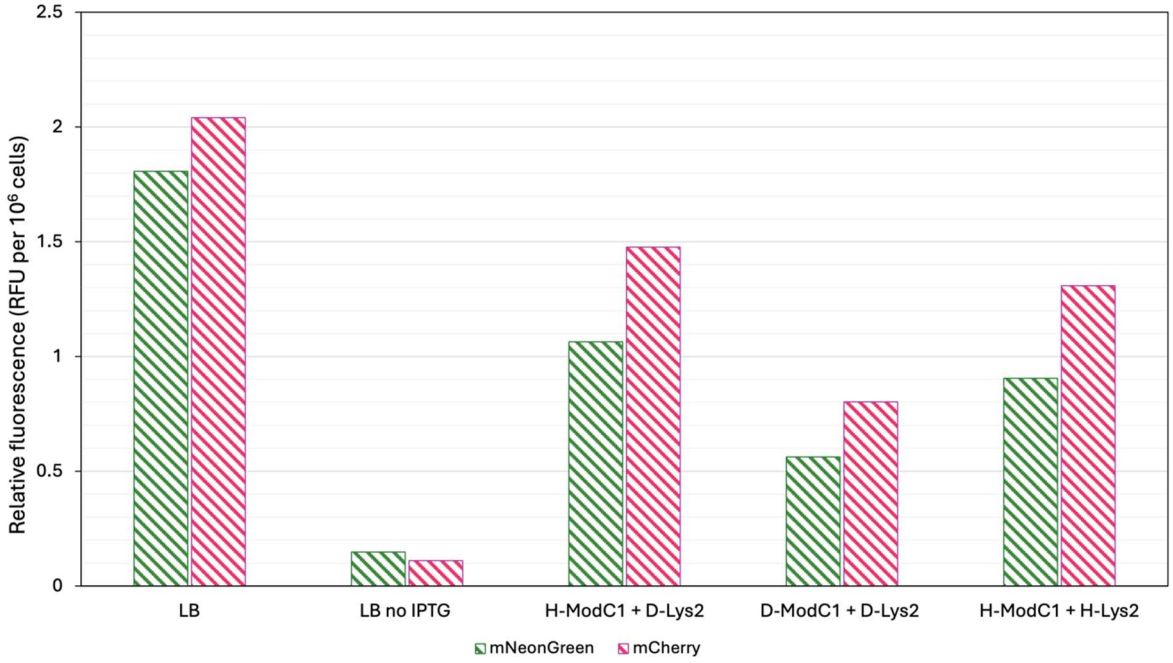


Figure 7: Relative fluorescence (RFU per 10^6 cells), of MNG-MC when grown in LB, H-ModC1 or D-ModC1, supplemented with H-Lys2 or D-Lys2. 'H-' or 'D-' before the ModC1 or Lys2 indicates whether they are protiated or deuterated, respectively. Green bars represent mNeonGreen and pink bars represent mCherry relative fluorescence. The additive solution has been added in equal quantities to all ModC1 samples a few hours post-induction.

Figure 7 shows the highest expression in LB in comparison to the other samples. The highest expression amongst the ModC1 samples was observed when protiated ModC1 was used with deuterated Lys2, followed closely by the protiated ModC1 supplied with protiated Lys2. The sample grown in deuterated ModC1 with deuterated Lys2 shows much lower expression.

This can be explained by the impact of deuteration on *E. coli* cellular processes and survival. Culturing *E. coli* in deuterated media has often resulted in lowered or erratic growth rates, particularly when the *E. coli* contain recombinant plasmids (Paliy et al., 2003). Proteomic studies have observed a shift in the proteome of *E. coli* cells in deuterated minimal media,

with lowered expression of translational proteins, which could explain the lowered MNG-MC levels (Opitz et al., 2019).

Unlike the previous experiments (see Figures 4 and 5) which displayed a higher expression of protiated MNG-MC in the Lys2-supplemented samples compared to LB, Figure 7 does not follow the same trend. This may be since the H-Lys2 used in this experiment was not the same batch as the Lys2 used in Figure 6. Since the lysate is made from *B. braunii* biomass harvested every two weeks or so, there may be slight variations in the biomolecular composition of each batch that are difficult to avoid. Across all the samples, mNeonGreen displays a lower fluorescence than mCherry. This could be due to the degradation of the mNeonGreen portion of some of the expressed protein. Given that it is observed regardless of the media or carbon source used, it may have been an issue with this stock of cells.

To improve the expression of deuterated MNG-MC, an additional step was added to the protocol. The *E. coli* was resuspended in ModC1 media, supplied with the lysate, and then left to incubate at 25°C with shaking at 250 rpm, to hopefully enable some acclimatisation to the new media conditions and increase aeration of the media, which is usually achieved by bubbling oxygen through the media or increasing shaking speed. After an hour, IPTG was added at 100 µM, shaking was reduced to 180 rpm, and the temperature was then gradually decreased to T1. Additionally, the cultures were grown in at 10 mL and in 100 mL autoclaved glass flasks to try and improve aeration of the media. This experiment was run twice, and the mean averages of the repeats are displayed in Figure 8A. Standard deviation was not calculated as the experiment was not repeated thrice.

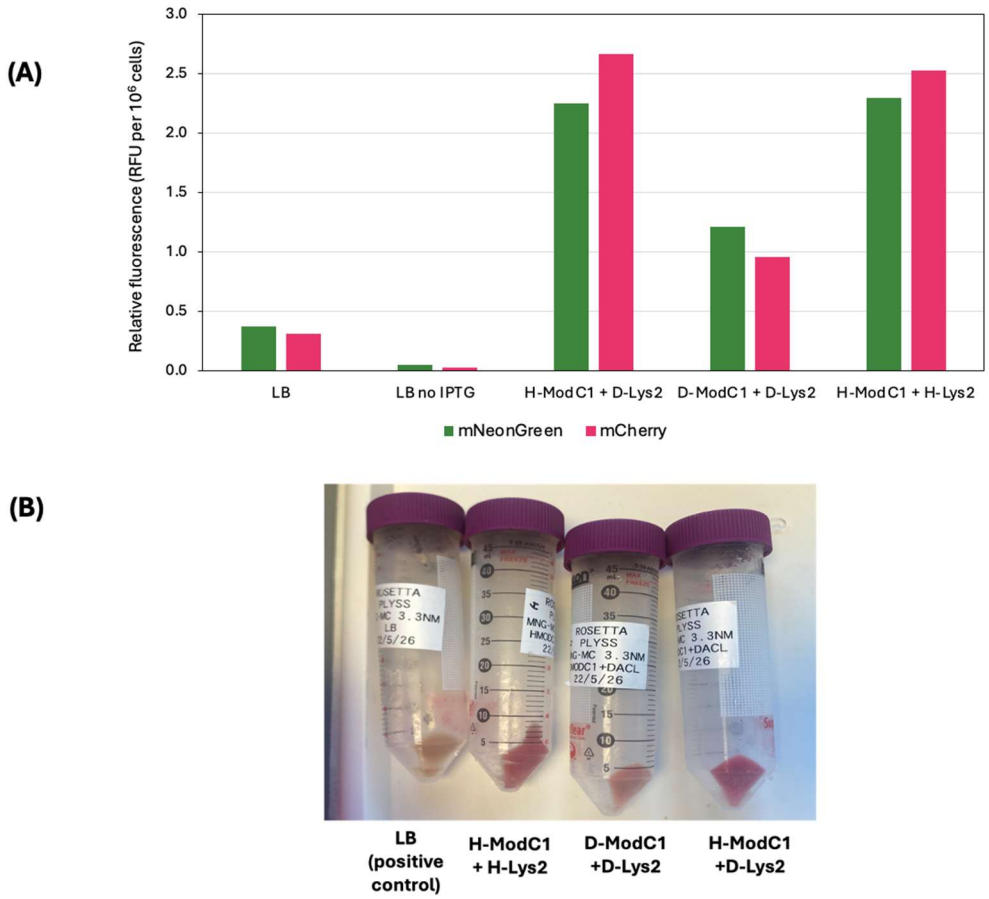


Figure 8: (A) Relative fluorescence (RFU per 10^6 cells), of MNG-MC when grown in LB, H-ModC1 or D-ModC1, supplemented with H-Lys2 or D-Lys2 after changes to expression protocol. 'H-' or 'D-' before the ModC1 or Lys2 indicate whether they are protiated or deuterated, respectively. Green bars represent mNeonGreen and pink bars represent mCherry relative fluorescence. The additive solution has been added in equal quantities to all ModC1 samples a few hours post-induction. **(B) *E. coli* pellets of the culture once spun down at 48 hours post-induction with IPTG.** Labels below indicate the media and carbon source used for the corresponding culture.

As seen in Figure 8A, an increase in expression across all the ModC1 samples can be observed, which suggests that the changes to the protocol may have been effective in boosting expression. The fluorescence (pink colour) is also visible across all the ModC1 samples in Figure 8B, and their intensity visually agrees with the data in Figure 8A.

The samples with protiated and deuterated lysates in protiated ModC1 have greater expression than the deuterated ModC1 sample. These results agree with those in Figure 7, which makes sense given the impact of deuterated conditions on *E. coli* physiological processes previously described (Paliy et al., 2003 and Opitz et al., 2019). The LB sample in these two experiments had lower expression. This may have been an issue with the media itself, but more repeats of this experiment would have to be conducted to investigate this.

Overall, a protocol resulting in the expression of some deuterated MNG-MC was successfully established. It is important to acknowledge that some of these graphs (most except the deuterated protein expression graphs, Figure 7 and 8) cannot be quantitatively compared to one another, as the gain values of the microplate reader were adjusted for each experiment, which was an error in the methodology. Within the individual graphs, the different data points can be compared, and the above analysis was done bearing this in mind. Additionally, the reliability of the above data could be increased with more repeats. Each of the experiments was only done one to two times due to time constraints, as each experiment takes nearly a week to complete and only six samples including controls can realistically be handled in one experiment. If more repeats of each experiment were conducted, statistical analysis of the data could also be carried out.

3.4. Purification and analysis of MNG-MC recombinant protein

Affinity chromatography with a Ni-NTA column (HisTrap FF crude 1mL) was used to purify MNG-MC, which contains a 6x His-Tag. Initially, MNG-MC expressed in protiated ModC1 with protiated Lys2, as well as MNG-MC expressed in protiated ModC1 with deuterated Lys2 were purified. This was done to investigate the deuteration level that could be achieved using protiated media and deuterated lysate, as these conditions achieved the highest yield.

For the sample expressed in protiated media with deuterated Lys2 (hereafter referred to as D-MNG-MC), the 0.2 g of pellet produced 1 mL of clarified sample after processing. Due to the column loading restrictions, the sample was loaded and eluted in five rounds. The elution profile of one of these rounds is displayed in Figure 9. The protiated sample (hereafter referred to as H-MNG-MC) was also purified in a similar manner.

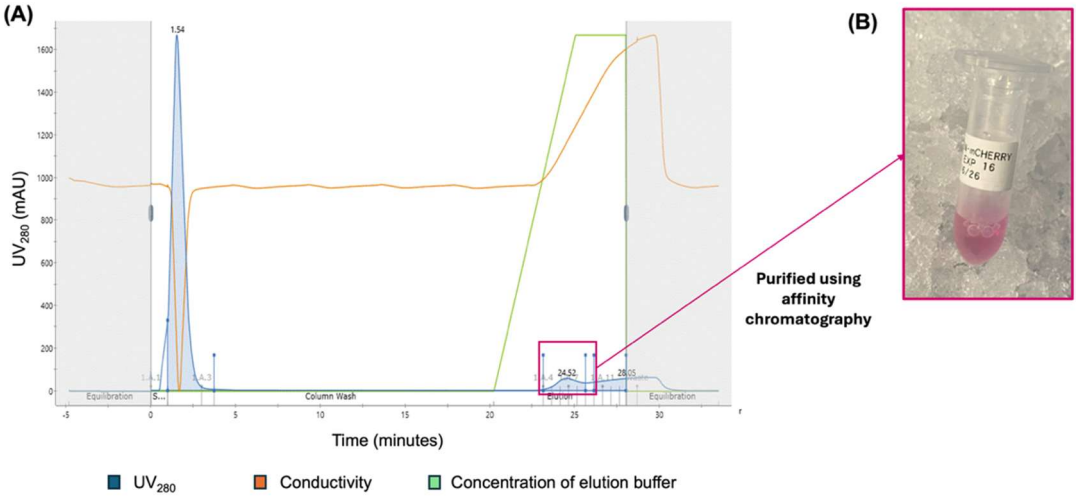


Figure 9: (A) Elution profile of one round of the affinity chromatography purification of D-MNG-MC. The blue line indicates UV_{280} as a representation of protein content. The orange line indicates conductivity. The green line indicates concentration of the elution buffer in the column. The pink box indicates the D-MNG-MC elution peak collected in 0.5 mL fractions. **(B) Final sample of all the eluted fractions pooled together.**

In Figure 9A, the largest, 24.52 min peak (we were only interested in pink fractions as it is the colour of our full-length protein) shows all the other cellular proteins in the sample that elute with the Binding buffer containing 20 mM imidazole. It also shows that each elution round resulted in about four fractions, 0.5 mL each, which contain the eluted protein. 15 µL of each of these fractions were run on SDS-PAGE, the results of which are shown in Figure 10.

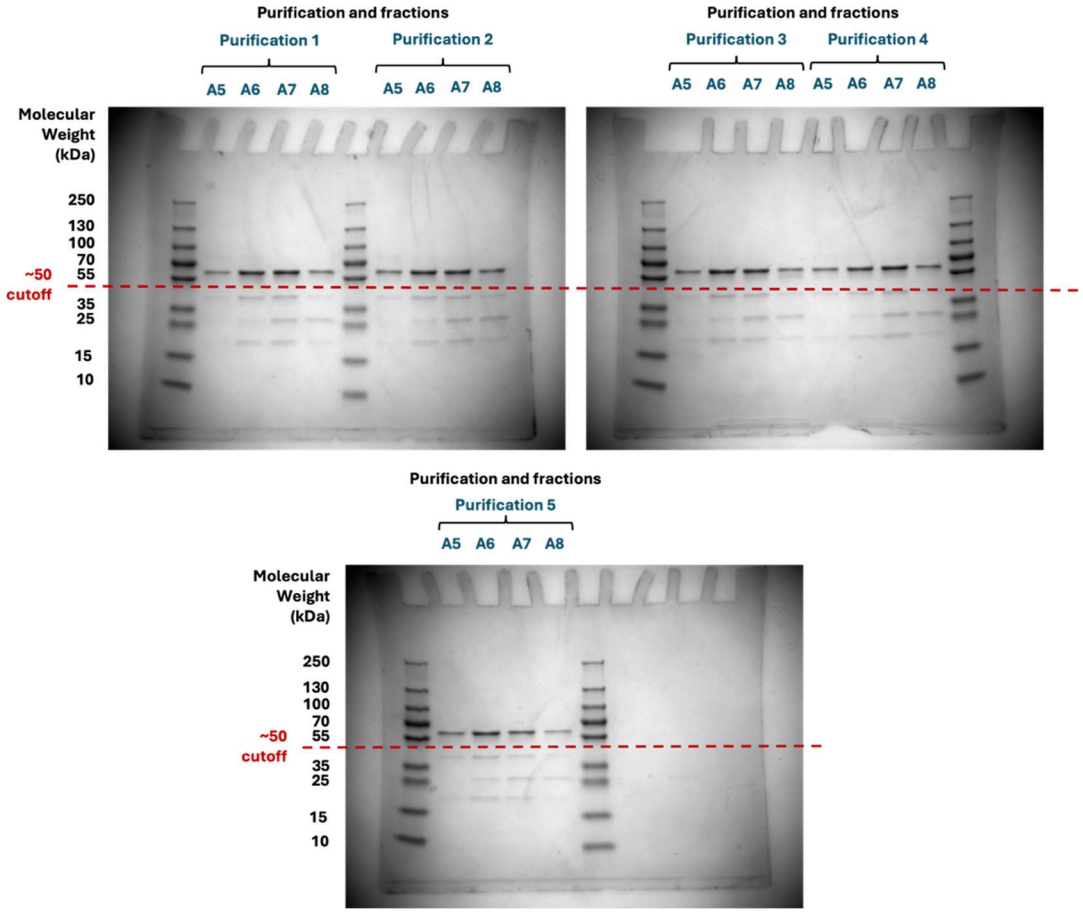


Figure 10: **SDS-PAGE results of all the fractions of D-MNG-MC purification containing the eluted protein.** Red dotted line indicates the molecular weight cutoff of the concentrator used in comparison to the components in the sample. The molecular weight ladder used was PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific™: 26619). The gels were stained with Coomassie Blue and imaged using white light.

Figure 10 evidently displays a major protein band between 55 and 70 kDa on the molecular weight ladder, which corresponds to the MNG-MC molecular weight of around 59.7 kDa, across all the fractions. There are some contaminants visible, and these could potentially be smaller fragments of the protein that were truncated or degraded but still contain the His-tag or histidine residues that may have interacted with the column. The fractions were pooled together and added to a 50 kDa concentrator (Thermo Scientific Pierce Protein Concentrator 88540) to try and remove the smaller impurities and wash off the imidazole.

1X PBS in H_2O was prepared and the sample was washed at least four times with it, by spinning at 2000 xg for 5 minutes at 4°C. The concentration of the MNG-MC in the concentrator was

measured using a NanoDrop, and the sample was further spun until a concentration of 1 mg/mL was achieved.

This was repeated with the protiated sample (hereafter referred to as H-MNG-MC). The resultant samples were not run on a gel to conserve as much as possible.

The D-MNG-MC sample was further divided into two samples. 200 µL of the sample was loaded into a Slide-a-Lyzer dialysis cassette and left to dialyse at 4°C in 1X PBS in fresh D_2O over 48 hours to ensure any labile hydrogens exchange with deuterium. The protein was concentrated in the 50 kDa concentrator until it was at around 1 mg/mL.

Thus, the following three samples were prepared for analysis using mass spectrometry at the Research Complex at Harwell: H-MNG-MC, D-MNG-MC (D_2O-dialysed), and D-MNG-MC (in H_2O-PBS buffer). The molecular weight comparison between H-MNG-MC and D-MNG-MC would provide a greater insight into how deuterated the protein sample is.

The mass spectrometry results are displayed in Figure 11.

As seen in Figure 11, all three samples have two main observed peaks. The peak around 59 kDa represents the MNG-MC protein. The difference between the molecular weights of D_2O-dialysed D-MNG-MC and H-MNG-MC is 224.8 Da, which corresponds to a 5.5% deuteration level. The difference between the D_2O-dialysed D-MNG-MC and D-MNG-MC in H_2O buffer is 1.2 Da. This indicates that very little change in the deuteration level of the protein after 48 hours of dialysis in D_2O buffer. Hence, it is likely that the 5.5% deuteration level achieved is on the backbone of the protein, rather than labile hydrogens.

The smaller peak observed is a lower molecular weight than the 50 kDa concentrator cut-off, indicating that the component should have filtered out due to its lower molecular weight. This suggests that it may interact with MNG-MC. It is speculated to be a truncated version of MNG-MC that may form dimers with the full-length protein under native conditions. A potential method of separating the proteins could be using urea, a common denaturant (Wingfield, 1995), or others, to separate the two proteins in the sample before running them through the concentrator again. The resultant sample could be dialysed against an appropriate refolding buffer to try and renature the MNG-MC protein (Yamaguchi & Miyazaki, 2014).

The protein produced in D-ModC1 with D-Lys2 has been purified but not processed further due to time constraints. Moving forward in the project, this sample will also be processed and studied using mass spectrometry.

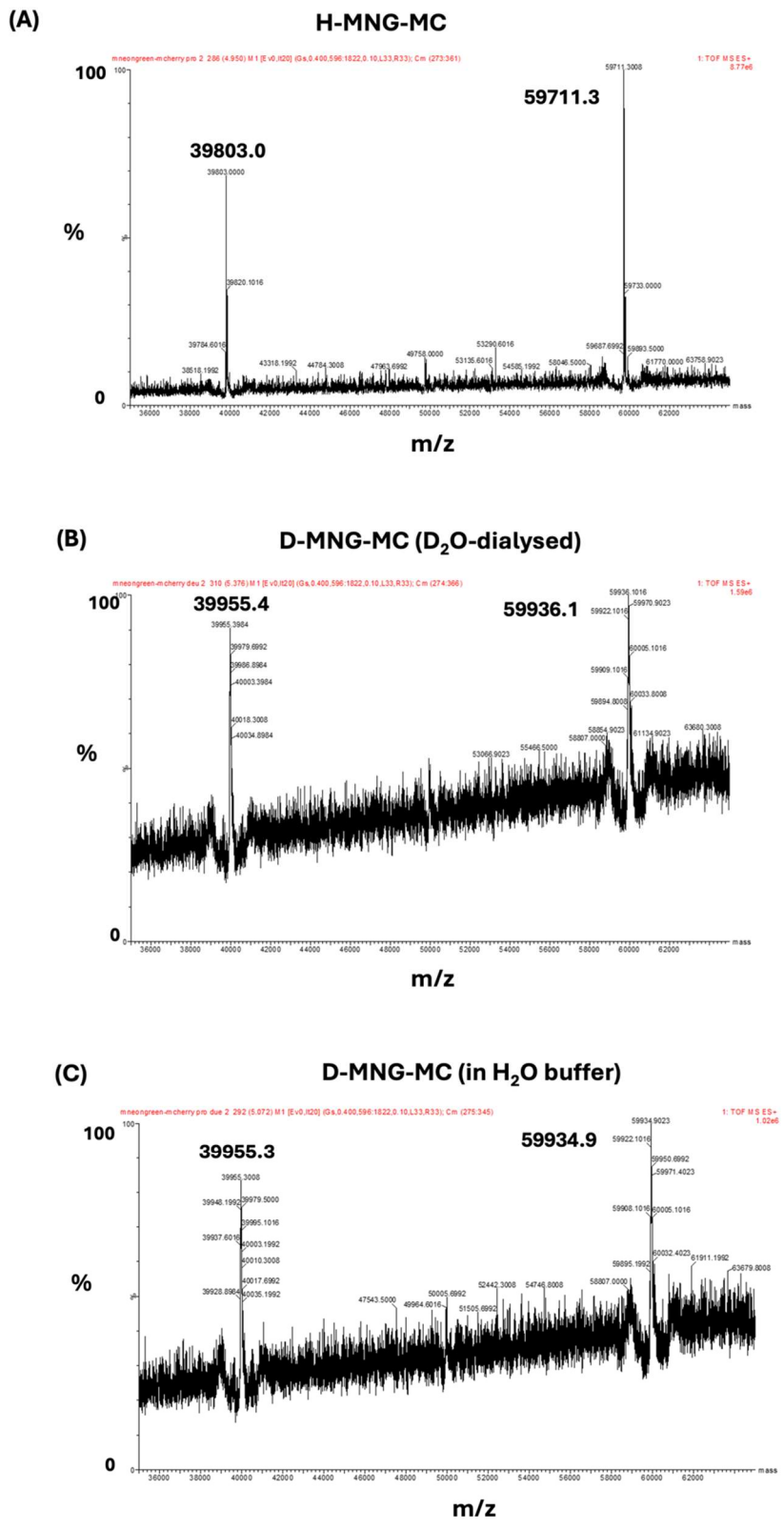


Figure 11: Mass spectrometry results of (A) H-MNG-MC (B) D-MNG-MC (D_2O-dialysed) (C) D-MNG-MC (in H_2O PBS buffer) samples. 10 μL of sample was run on the mass spectrometer. The black labels indicate the molecular weights of the two main peaks observed in Da.

The protein produced in D-ModC1 with D-Lys2 has been purified but not processed further due to time constraints. Moving forward in the project, this sample will also be processed and studied using mass spectrometry.

3.5. Evaluation of deuterated protein production in deuterated media

The mass spectrometry data indicates that when grown in protiated media, even with the deuterated lysate, only 5.5% of MNG-MC is deuterated. However, given that the replaced hydrogens are likely to be non-labile, this shows that some biomolecules from the lysate may have been metabolised by the *E. coli* and incorporated into MNG-MC during expression. The additive solution contained protiated glucose and millimolar amounts of another protiated carbon source. They were included in small quantities to support growth and expression based on the results from Figure 5. However, they were likely preferentially metabolised by the *E. coli* compared to the lysate, leading to lower deuteration levels. Hence, an experiment investigating replacing part of the additive solution with deuterated versions of the components and altering the glucose levels was carried out. MNG-MC was expressed in D-ModC1 using D-Lys2 as a carbon source (at either 20% v/v or 10% v/v), and with the additive solution containing no, normal or lower quantities of glucose. A protiated LB sample was also run as a positive control.

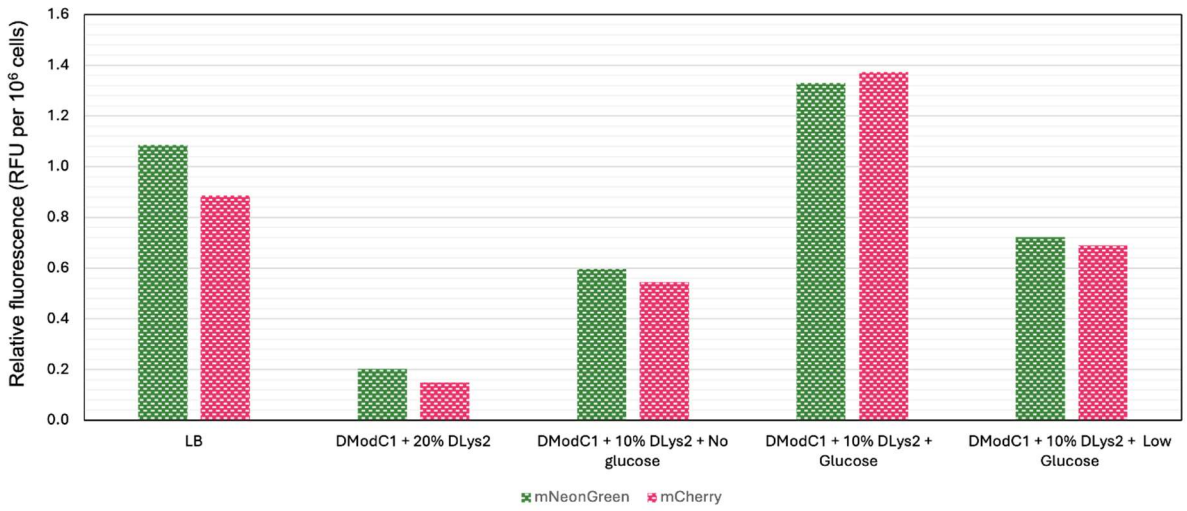


Figure 12: Relative fluorescence (RFU per 10^6 cells) of MNG-MC when grown in LB or D-ModC1, supplemented with D-Lys2 and variations of the additive solution, measured at 48H post-induction. 'H' or 'D' before the ModC1 or Lys2 indicate whether they are protiated or deuterated, respectively. Green bars represent mNeonGreen and pink bars represent mCherry relative fluorescence. All components in the additive solution apart from the glucose are deuterated.

As seen in Figure 12, the acid lysate on its own, even at higher quantities in the culture does not boost the expression of MNG-MC as much as when there is the additive solution present. Additionally, the comparison between the varying levels of glucose within the additive solution shows that the presence of glucose does greatly impacts the MNG-MC expression, hence reducing the amount of protiated glucose in the media would greatly reduce yield. This

is likely due to the use of the pLysS strain as previously described (Novy et al., 2001). Moving forward, a different *E. coli* strain could be trialled, to explore whether the protein yield in D-ModC1 supplemented with deuterated lysate increases when protein expression is not as tightly controlled (Novagen, n. d.). Moreover, one could attempt to adapt an *E. coli* strain used for expression to deuterated media to try and boost survival and expression upon induction, as other studies have done this before (Paliy et al., 2003).

There was not enough time to process these samples and compare their deuteration level to that of the H-MNG-MC and D-MNG-MC already analysed. Moving forward, the next objective of this investigation would be to purify and analyse these samples for their deuteration level as well.

3.6. SANS and FLIM

Two of the samples from Figure 11 (D-MNG-MC (in H_2O PBS buffer) and D-MNG-MC (D_2O-dialysed)) were put on the neutron beamline to be studied using small-angle neutron scattering on LOQ. Results of this experiment have not been displayed as they are preliminary data, collected by Dr. Najet Mahmoudi at the ISIS Neutron and Muon Source. The results were largely inconclusive with regards to the structure of the protein, but indicated low quantities of deuterium incorporation, which agrees with the mass spectrometry data shown in Figure 11. A higher protein concentration of the sample would need to be prepared to attain more conclusive structural data.

Using FLIM, the lifetime of mNeonGreen alone was previously recorded on the described set up to be 3.1 + 0.15 ns. The lifetimes of both the D-MNG-MC (D_2O-dialysed) and H-MNG-MC samples were found to be 2.7 + 0.15 ns, indicating fluorescence energy transfer from mNeonGreen to mCherry fluorophores.

There was little difference between the lifetimes of the two samples when observed using a multi-photon system. (Preliminary and data was collected jointly with Prof. Stan Botchway at the Octopus Central Laser facility, Harwell). This is an expected result due to the low deuteration levels of D-MNG-MC. Notably, the single-photon system also gave a two-component fitting, likely due to the presence of a contaminant protein, which was observed in the mass spectrometry results in Figure 11. Further purification of this sample, likely using denaturing conditions, would be required to be able to collect data using single-photon FLIM.

4. Conclusion

Through this investigation, *B. braunii* cultures were successfully grown in protiated at deuterated conditions. Cultures were successfully maintained at over 12 months at 25°C, shaking at 60 rpm, with a 16H:8H light: dark cycle in a BINDER KBWF240 environmental incubator. The cultures grew well enough to harvest biomass and produce two different types of lysates – the overnight autolysate and the acid lysate, both protiated and deuterated. The effects of the lysate used, IPTG concentration, temperature, and delayed supplementation with an additive solution were investigated, and the optimal expression conditions for MNG-MC expression in protiated conditions were established. The final conditions used to express the deuterated protein were using 10% v/v deuterated acid lysate as a carbon source,

allowing the *E. coli* to adjust for an hour to 25°C before adding 100 µM of IPTG and letting the temperature gradually drop to T1, then adding the additive solution.

Expression samples in D-ModC1 with D-Lys2 displayed the lowest fluorescence, likely due to the impact of deuterium on cellular functions. Purification and analysis of the proteins expressed in protiated ModC1 media with a deuterated lysate showed that the deuterium incorporation was still low, at 5.5%, even though those were likely backbone hydrogens. However, this was the first sample ever analysed by this lab. Further samples were produced in deuterated ModC1 media with deuterated lysate and varying amounts of protiated glucose, but to much lower yields. These samples are yet to be purified and analysed, but these next steps would provide greater insight to how the level of glucose and the presence of other deuterated components in the additive solution impacts MNG-MC deuteration.

Moving forward, this would inform which components would need to be deuterated to optimise deuterium incorporation, enabling the balance of this with yield and cost. Additionally, experimentation with adapting *E. coli* to D_2O, as well as using different pET vectors and *E. coli* strains with less tight expression could help investigate whether this could help boost expression in deuterated conditions.

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