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Effect of post-harvest conditions on antioxidant enzyme activity in *Dunaliella tertiolecta* biomass

Uttam K. Roy[1, 2*], Birthe V. Nielsen[2] and John J. Milledge[2]

[1]*Loughborough University, School of Architecture, Building and Civil Engineering, Epinal Way, Loughborough, Leicestershire, LE11 3TU*

[2]*University of Greenwich, Algae Biotechnology Research Group, Faculty of Engineering and Science, Central Avenue, Chatham Maritime, Kent, ME4 4TB*

[*]Corresponding author Uttam Roy (u.u.roy@lboro.ac.uk)

Abstract

Microalgae biomass is considered a sustainable feedstock to produce high-value compounds including enzymatic and non-enzymatic antioxidants, which are widely used for food, pharmaceuticals, textile, leather, and in the chemical industries. Suitable post-harvest storage conditions to minimise deterioration of enzyme activity is therefore crucial. In this study, the effect of storage temperature over time on antioxidant enzyme activity (catalase (CAT), superoxide dismutase (SOD) and peroxidase (POD) in four D. tertiolecta samples (whole wet cells, freeze-dried whole cells, crude extract and freeze-dried crude extract) were evaluated. Antioxidant enzyme activities in freshly harvested whole cells or in the crude extract stored at -20˚C were retained only for up to one month but when stored at -80˚C, both CAT and SOD activities remained unchanged for four months. Moreover, in the freeze dried whole cell samples, CAT and SOD activities were retained for 8 months at -20˚C, however, when a crude extract was prepared and freeze dried, enzyme activities decreased. Therefore, careful control of storage temperature could prevent unfavourable changes to antioxidant activity in harvested

cells thereby increasing shelf-life and the value of this type of biomass. This study recommends a suitable post-harvest storage temperature for preservation of Dunaliella biomass for both short-term and long-term stability of antioxidant enzyme activities. Our results strongly indicate that fresh whole cell extracts should be used in antioxidant enzyme assays, but if storage is necessary, whole fresh cells should be freeze dried and stored at -80˚C. Crude extracts prepared should be used in antioxidant enzyme assays within 24 h of preparation.

Keywords: Antioxidant enzyme activity, storage temperature, whole wet cells, crude extract, freeze-dried whole cells, *Dunaliella tertiolecta*.

1 Introduction

Antioxidant enzymes are used commercially in a range of food processes (to maintain colour and flavour and to increase shelf life), pharmaceuticals (bactericidal disinfectant, therapeutic agents), textile (fibre colourant), leather and chemical industries (stabiliser and skin tissue protector) (Mohsenpour et al., 2012; Jegannathan and Nielsen, 2013; Godic et al., 2014). The ability to produce catalase (CAT), superoxide dismutase (SOD), peroxidase (POD) makes microalgae a potential natural source of these enzymes (Mogharabi and Faramarzi, 2016). Microalgae-derived enzyme production could be made more economical by the optimisation of downstream processing (cell disruption, extraction, harvesting, possibly fractionation, and optimal post-harvest storage). Extensive studies have been carried out to assess cell harvesting, cell disruption, and extraction processes of microalgae-derived biomolecules including enzymes (Kim et al., 2013; Milledge and Heaven, 2013; Sharma et al., 2013; Gangl et al., 2015; Maadane et al., 2015; Trung et al., 2016; Khanra et al., 2018; Marrone et al., 2018). Nonetheless, little information exists on the effect of post-harvest storage conditions (temperature and time) on the stability of antioxidant enzymes. Optimal post-harvest storage

could increase the shelf life of biomass by minimising the loss of enzyme activity and could increase profits and reduce waste.

Cold storage is a widely used technology for the preservation of chemical, nutritional and sensory/physical properties of processed cells, as respiration and other metabolic reactions are slowed down (Gao et al., 2015). Biomass is prepared for cold storages either by a dry or a wet route. Wet biomass is usually immediately chilled or frozen whereas during dry storage, microalgae biomass is either freeze-dried or air-dried then stored cold (< 4°C). Freeze-drying is a better method to remove water compared to air-drying and is generally recommended for heat-sensitive antioxidant compounds (Aktas *et al.*, 2012; Goulas, Orphanides and Gekas, 2013; Youssef and Mokhtar, 2014; Mphahlele *et al.*, 2016; Nguyen, Van Tang and Le, 2018). It extends the shelf life by preventing microbial growth and by reducing lipid oxidation (Shofian *et al.*, 2011). Drying the biomass also reduces the weight and volume, making the material more convenient and cheaper to transport and store. However, drying equipment is expensive and requires high energy input resulting in increased downstream processing costs. Most industry processes work on freeze dried biomass, however some studies claim that this drying method is not compatible with antioxidant activity as these enzymes lose stability and therefore activity. Direct use of microalgae-derived extracts or enriched enzyme fractions might therefore be needed. In addition, antioxidant enzymes need to be extracted from the biomass prior to a purification process to obtain specific enzymes and at times the crude extract may require to be temporarily stored or transported before processing either due to malfunctioning of equipment or limitations in downstream processing facilities in large-scale biorefineries. For these reasons, it is critical to determine the stability of antioxidant enzymes in both whole wet cells and in the crude extract.

The stability of antioxidant activity rely on the type of antioxidant (astaxanthin, β-carotene, tocopherol, ascorbic acid, glutathione, phenolic, CAT, SOD, POD), nature of the biomass, length of storage time, storage temperature, and drying method (Lester *et al.*, 2004; Ryckebosch *et al.*, 2011; Andersson *et al.*, 2015; Gangl *et al.*, 2015; Napan *et al.*, 2015; Trung *et al.*, 2016). Although studies have examined antioxidant enzyme activity during storage, these have so far only been conducted for vascular plant materials and fruits. Moreover, some of these studies were carried out only over a few months: Available data on the effect of storage time, temperature and drying process on antioxidant activity are listed in Table 1. Antioxidant enzyme activity varies with time and storage temperature. For example, fresh biomass (*Xanthosoma sagittifolium* leaves) stored at low temperature (5°C) retained antioxidant enzyme activity up to 20 days; however, some cells (*Thalassiosira pseudonama*) needed to be stored below freezing (-20°C) to retain activity (Table 1). Conversely, prolonged storage (300 days) at -18°C was found to increase antioxidant activity, which may be as a consequence of induced oxidative stress (Oliveira *et al.*, 2011). Storing of kiwi fruit < 5°C increased antioxidant enzyme activity in fresh material, but at -20°C, the activity initially decreases, then increases over time (Gao, Yan and Chen, 2015).

Drying fresh biomass can retain antioxidant activity in cells (Mediani *et al.*, 2014; Tomsone and Kruma, 2014). However, most studies seem to be in general agreement that drying biomass in an oven or spray drying – both processes that require high temperatures,
de-stabilize enzyme activity. Freeze drying on the other hand, have been shown to retain activity in certain biomass. For example, freeze drying spinach leaves did not alter CAT, SOD or POD activity (Lester *et al.*, 2004), antioxidant activity after freeze drying, and storage of *Cosmos caudatus* at -20°C for 90 days, also was not observed to change total antioxidant activity (Mediani *et al.*, 2014), however, Moura *et al.*, (2016) suggested that fresh sugarcane

leaves stored at -80°C retained antioxidant enzyme activity for up to two months, whereas activity in the freeze-dried leaves was reduced. Mixed data in the literature (Table 1) therefore suggest that antioxidant stability after freeze drying might be biomass dependent, and that stability during storage depends on both time and temperature.

No information is available on the stability of antioxidant enzyme activity in microalgae biomass and biomass-derived crude extract during storages. Studies are needed to investigate the most suitable range of temperatures and storage times for post-harvest microalgae biomass or biomass-derived crude extract to preserve antioxidant enzyme activity.

The aim of this research was to evaluate the effect of storage time and temperature on the stability of antioxidant enzyme (CAT, POD and SOD) activities in D. tertiolecta biomass (wet whole cells and freeze-dried whole cells) during post-harvest storage. Storage stability of antioxidant enzyme activity in biomass-derived crude extract and freeze-dried crude extract was also evaluated. In addition, hydrogen peroxide and lipid peroxide contents were determined to assess oxidative stress at different temperatures during storage.

2 Material and methods

Microalgae species and reagents

D. tertiolecta (CCAP 19/30) was obtained from the Culture Collection of Algae and Protozoa (CCAP, Scotland, UK). Modified Lowry assay kit (Lowry reagent, BSA standard, and 2 N Folin-Ciocalteu (FC) reagents) were obtained from Thermo Scientific (UK). All other reagents and enzymes were purchased from Sigma Aldrich (UK).

Culturing of cells

D. tertiolecta (1 x 10^5 cells mL^{-1}) were inoculated into Modified Johnson medium (J/I) (pH 7.5) containing NaCl (87.66 g L^{-1}) (Sathasivam *et al*., 2013) and grown in glass flasks (2.5 L) placed in static incubator at (20°C ± 2) under cool white fluorescent light with a light: dark cycle (12:12 h) and a light intensity (100 µmol photons m^{-2} s^{-1}).

Experimental design

From one batch of culture, cells (10 mL culture in each tube) were harvested by centrifugation (3000xg, 15 min, 4°C) and divided into two groups: (i) whole wet cells (ii) lyophilised whole cells followed by freeze-drying (Thermo Scientific, USA). After harvesting another batch of culture, fresh cells pellet-derived crude extract (concentrated) was split into two groups: (iii) whole cells derived crude extract, and (iv) lyophilised crude extract followed by freeze-drying. Samples were stored at different temperatures and periods (Table 2). Antioxidant activity was assessed at different time points with storage up to 28 days defined as 'short-term' and up to 247 days defined as 'long-term'. All experiments were carried out in triplicate (n = 3).

Crude extract

Extracts for enzymes assay (CAT, POD and SOD) were prepared from fresh cell cultures of *D. tertiolecta* by a modified version of the method described by Tian and Yu, (2009). After re-suspending whole wet cells and freeze-dried wet cells pellets in an extraction buffer (1 mL, 50 mM potassium phosphate buffer (0.1 mM EDTA, 0.1%, Triton X-100, 1% PVP, and pH 7.5) were transferred to sterilized Eppendorf tubes (1.5 mL). The homogenate was vortexed (5 min) in the presence of silica beads (0.2 g), and the supernatant was separated after centrifugation (13,000xg, 30 min, 4°C). Crude extract samples (group iii and group iv) were prepared from whole wet cells pellet (10 g) by extracting (magnetic stirring) in extraction buffer (100 mL,

4°C, 4 h). The supernatant was used in the subsequent analysis for the antioxidant enzyme (CAT, POD, and SOD) activity.

Determination of antioxidant enzyme activity

2.1.1 Catalase (CAT) activity assay

Extract (0.05 mL) were transferred to a quartz cuvette (l = 0.1 cm) containing ice-cold phosphate buffer (1 mL, 50 mM, pH 7.00) (Zhang *et al.*, 2015). The reaction mixture was equilibrated (25°C, 5 min) and H_2O_2 (0.05 mL, 858 mM) was added (final concentrations H_2O_2 0.045 mM and phosphate buffer 39 mM). After mixing (gentle inversion), decrease in optical density (ΔOD) was recorded (every 10 sec, 5 min, 240 nm) against a blank sample containing dH_2O in the reaction mixture instead of extract (Lambda 365 UV/Vis Spectrophotometer, Perkin Elmer, US). The specific CAT activity was calculated as units per milligram of protein, Eq.1.

$$\text{CAT activity (U mg}^{-1}\text{ protein) in extract} = \frac{(\text{OD min}^{-1}) \text{ x total volume (mL) x df}}{0.0436 \text{ x Sample volume (mL) x protein (U mg}^{-1}) \text{ x length (cm)}} \quad \text{.........................Eq.1}$$

0.0436 = millimolar extinction coefficient of H_2O_2 at 240 nm, df = Dilution factor

2.1.2 Peroxidase (POD) activity assay

ABTS (2, 2' azino bis (3 ethylbenzothiazoline-6-sulfonic acid) solution (0.964 mL, 9.1 mM) in phosphate buffer (100 mM, pH 5.00) and extract (0.01 mL) was transferred to a quartz cuvette (l = 0.1 cm) and placed inside the spectrophotometer (Lambda 365 UV/Vis Spectrophotometer, Perkin Elmer, US). After equilibration (25°C, 5 min) of the reaction mixture, H_2O_2 (0.036 mL, 88.5 mM) the change in optical density (ΔOD) was recorded (every 10 sec, 3 min, 420 nm)

against a blank sample containing dH_2O in the reaction mixture instead of extract. The specific POD activity was calculated as units per mg of protein, Eq.2

$$\text{POD activity (U mg}^{-1}\text{ protein) in extract} = \frac{(\text{OD min}^{-1}) \times \text{total volume (mL)} \times \text{df}}{36.8 \times \text{Sample volume (mL)} \times \text{protein (U mg}^{-1}) \times \text{length (cm)}} \quad \text{.............................Eq.2}$$

36.8 = millimolar extinction coefficient of ABTS, df = Dilution factor

2.1.3 Superoxide dismutase (SOD) activity assay

Phosphate buffer (0.667 mL, 75 mM, pH 7.00), dH_2O (0.06 mg mL^{-1} of protein), xanthine (0.1 mL, 150 mM), and NBT (0.1 mL, 1.5 mM) was transferred into a quartz cuvette (d = 0.1cm). After equilibration (25°C, 5 min) of the reaction mixture, xanthine (0.023 mL, 0.4 U mL^{-1}) oxidase was added to it and the optical density (ΔOD) was recorded (per second, 3 min, 560 nm) against a blank sample containing dH_2O in reaction mixture instead of xanthine oxidase. After 3 mins, the extract (0.05 mL) was added into the reaction mixture and the decrease in optical density (ΔOD) was recorded up to 7 min. The specific SOD activity was calculated as units per mg of protein using the following formula, Eq.3 - 4

$$\text{Percent inhibition (\%)} = \frac{(\Delta OD_s\text{ min}^{-1} - \Delta OD_o\text{ min}^{-1}) \times 100}{(\Delta OD_s\text{ min}^{-1})} \quad \text{.............................Eq.3}$$

Increase in absorbance (Uninhibited) per min (ΔOD_s min^{-1}) at 560 nm

Inhibition of absorbance per min (ΔOD_o min^{-1}) by the sample at 560 nm

$$\text{SOD activity (U mg}^{-1}\text{ protein) in extract} = \frac{\text{Percent inhibition} \times \text{df}}{50\% \times \text{Sample volume (mL)} \times \text{U mg}^{-1}\text{ protein}} \quad \text{................................ ....Eq.4}$$

Total protein assay

A calibration curve was prepared using ovalbumin (0.5 - 128 µg). Standard protein or algae samples (0.2 mL) from each treatment were mixed with Lowry reagent (1 mL) and incubated (10 min, room temperature). Folin-Ciocalteu reagent (0.1 mL, 1 M) was added and the samples were incubated (30 min, room temperature) (Redmile-Gordon *et al.*, 2013). The absorbance was recorded at 750 nm (UV-visible spectrophotometer, Jenway 6305, UK) against a blank sample containing extraction buffer instead of sample extract.

Estimation of hydrogen peroxide (H_2O_2) content

Samples (whole cells pellet) were homogenised in a 1.0 mL reaction mixture (TCA, 0.25% w/v; potassium iodide (KI), 25 mM; phosphate buffer, 2.5 mM, pH 7.0). After mixing (3 min, vortex) the homogenate was transferred to a sterile Eppendorf tube (1.5 mL) and incubated (20 min) on ice bath in the dark. After centrifugation (12000xg, 4°C, 15 min) the supernatant was incubated (20 min, room temperature) and the absorbance was recorded at 350 nm against blank prepared by adding dH_2O (0.5 mL) instead of KI into the reaction mixture (Junglee *et al.*, 2014). A calibration curve for quantifying H_2O_2 content was prepared using standard solutions of H_2O_2 (0.06 - 0.310 nM) and estimated as above.

Estimation of lipid peroxides (MDA content)

Samples (whole cells pellet) were homogenised in 1.4 mL reaction mixture (Thiobarbituric acid (TBA), 0.3% w/v; TCA, 3.9% w/v). After mixing (3 min, vortex) the homogenate was transferred to a sterile Eppendorf (1.5 mL) tube and heated (30 min, 95°C). The mixture was incubated (10 min) on ice. After centrifugation (12000xg, 4°C, 10 min) the absorbance of the supernatant was recorded (532 and 600 nm) using a spectrophotometer (Jenway 6305, UK). A blank sample containing 1.4 mL reaction mixture (TBA, 0.3% w/v; TCA, 3.9% w/v) was also

prepared. In this assay, total lipid peroxide content was quantified as MDA levels (nmole 10^{-6} cells) and calculated using the following formula (Eq.5) (Hodges *et al.*, 2015; Shah *et al.*, 2001).

$$\text{MDA equivalents (nmol mL}^{-1}) = \frac{(A_{532} - A_{600}) \times 10^{-6}}{(155000)} \quad \ldots\ldots\ldots\ldots\ldots\text{Eq.5}$$

Where 532 nm represented the maximum absorbance of the TBA-MDA complex

600 nm represented the correction for non-specific turbidity

155000 (M^{-1} cm^{-1}) = the molar extinction coefficient of MDA

Statistical Analysis

All statistical analyses were performed in IBM SPSS 20 using one-way, two-way analysis of variance (ANOVA) using a post hoc Turkey's HSD test (for multiple comparison) with $\alpha < 0.05$. Time and temperature as independent variables and antioxidant enzyme activity as dependent variable were considered here. A t-test (non-equal variance) was also performed when comparing two means. All experiments were carried out in triplicate (biological replication, n = 3).

3 Results and discussion

The effect of three different temperatures and freeze-drying on antioxidant enzyme activity at different time points (28 days defined as 'short-term' and up to 247 days defined as 'long-term') was evaluated to obtain the optimal storage conditions of antioxidant enzymes in *D. tertiolecta* whole cells and in cells-derived crude extracts. Determination of H2O2 and MDA was carried out to determine the extent of oxidative stress induced by temperature and time in the cells during storage.

3.1 CAT, SOD and POD activity in whole wet cells

Storage conditions (time and temperature) were found to be critical parameters on retaining antioxidant enzyme activity in freshly harvested *Dunaliella* whole wet cells (Table 4). The results illustrate that CAT and SOD, but not POD, activity in whole wet cells stored at 4°C or -20°C for one day (24 h) did not change significantly ($p > 0.05$). At -80°C this was also the case for POD and SOD activity. However, a significant ($p < 0.001$) reduction in CAT activity was found.

At 4°C there were no significant changes in CAT and SOD activity for the first seven days of storage, however a significant increase in activity (CAT 140%, SOD 25%) was observed in the samples when assessed after 28 days. Conversely, POD activity significantly ($p < 0.05$) decreased (46%) after the initial 24 hours of storage at 4°C. At -20°C both CAT and SOD activity remained unchanged for 120 and 132 days, respectively, whereas POD activity decreased (45%) significantly after 7 days of storage. POD activity remained unchanged during storages at -80°C, which was also the case for SOD activity up to 132 days and for CAT activity up to 165 days of storage though after an initial reduction in activity (24 h, 27%).

Increased CAT and SOD activity at 4°C after 28 days of storages suggests an antioxidant enzymes defence response in whole wet cells to reduce oxidative stress induced by the temperature (4°C). SOD provides the first-line defence against ROS by catalysing the dismutation of $O_2^{\cdot -}$ to H_2O_2 and O_2, CAT directly converts H_2O_2 into H_2O and $O_2^{\cdot -}$. H_2O_2 content was significantly (320%) increased and MDA content (100 pmol 10^{-7} cells) significantly ($p = 0.042$) decreased by 42% (Table 3) at this time indicating that cells were still experiencing oxidative stress and the SOD dismutation of superoxide. In addition, the enhanced level indicates that H_2O_2 cannot be detoxified completely by CAT alone. The

enhanced SOD activity also protects the cell against lipid peroxidation which is correlated with a lowered MDA level. This finding is in agreement with the results of Yang *et al.*, (2012), who reported a higher CAT and SOD activity in kiwifruit after 30 days storage at 0°C. Similar result was found in a study by Jin *et al.*, (2011), who reported an increase in CAT and SOD activity, and decrease in APX and glutathione activity in strawberry fruit stored for seven days at 5°C. An increased level of CAT activity was also found for papaya fruit stored for 15 days at 5°C (Rivera-Pastrana *et al.*, 2014). A study by Dama *et al.*, (2010) reported an increase in SOD activity and decreased POD activity in mushrooms after 10 days of storage at 5°C, which is also in line with our finding.

A two-way ANOVA suggests a statistically significant ($p < 0.001$) interaction between time and temperature impacting CAT and POD activity for short-term storage, whilst this interaction was not statistically significant ($p = 0.264$) for SOD activity for the same time period. This result suggests that both time and temperature need to be considered to optimise short-term (28 days) post-harvest storage to retain CAT and POD activity, whereas one factor (either time or temperature) can sustain SOD activity in stored whole cells.

Though CAT and SOD were reduced in whole cells stored at -20 and -80°C for 247 days, POD activity was retained for the same time period when stored at -80°C. A significant enhanced H_2O_2 (98%) and MDA content (103%) in storage cells at -20°C after 247 days (Table 3) indicate the generation of oxidative stress even at this temperature. Compared to the H_2O_2 increase at 4°C, the increase was less at -20°C; MDA content is indirectly related to SOD activity. An increased MDA level indicates the decreased SOD activity since $O^{2.-}$ are no longer detoxified by reduced SOD and excess $O^{2.-}$ could therefore damage the cells membrane by lipid peroxidation thus causing an enhancement in MDA levels. At -80°C, no significant change in

H_2O_2 and reduced MDA levels compared to the day of processing indicate the adaptability of cells during long-terms storage.

Intracellular glycerol and free amino acids (glycine and glutamate) (Hosseini Tafreshi and Shariati, 2009), carbohydrate (Dai *et al.*, 2010), and cysteine residue have been suggested to prevent the formation of ice crystals and therefore stabilise and protect enzymes from surface denaturation at -80°C (Arakawa *et al.*, 2001; Costa *et al.*, 2002; Nita *et al.*, 2007; Kumar *et al.*, 2012). A two-way ANOVA demonstrates a significant ($p < 0.01$) interaction between time and temperature for storage time (0 - 247 days) and storage temperature (-20°C, -80°C) on the stability of CAT and POD activity. This result demonstrates that both time and temperature need to be considered to optimise long-term whole wet cells storage conditions to retain a CAT and POD activity. However, the interaction between time and temperature did not have a significant ($p = 0.142$) impact on SOD activity for the same range of time and temperatures. Moura *et al.*, (2016) investigated the effect of storage temperature on antioxidant enzyme activity and reported that sugarcane leaves stored at -80°C for 53 days did not significantly change CAT, SOD, and POD activity, which is consistent with the presented results. The finding is also in agreement with the study by Lester *et al.*, (2004), who observed 56 days retention of antioxidant enzyme activity in honeydew fruit and spinach leaves when stored at -80°C.

3.2 CAT, SOD and POD activity in freeze-dried whole cells

CAT (7.87 U mg^{-1} protein), POD (0.024 U mg^{-1} protein) and SOD activity (6.28 mg^{-1} of protein) after freeze-drying of whole cells demonstrate that freeze-drying does not have a significant effect ($p > 0.05$) on antioxidant enzyme activities relative to whole wet cells (Table 6). Enzymes can be stabilised during freezing and thereby retaining their activity as

intracellular molecules such as glycerol, amino acids, phenol, and sugar can serve as "water substitute" and form hydrogen bonds to the dry substrate (Kandil and Soda, 2015). In addition, intramolecular disulphide bond formation through cysteine residues in enzyme molecules have been suggested to provide additional stability (Kumar *et al.*, 2012; Kandil and Soda, 2015). Also, no significant changes of H_2O_2 and MDA content between whole wet cells and freeze-dried cells (Table 5) indicate the adaptability of whole wet cells to freezing temperature-induced oxidative stress during the freeze-drying process. These findings are consistent with a study by Martysiak-Zurowska *et al.*, (2017), who observed no changes in CAT activity between lyophilised and fresh human milk. Tan *et al*., (2013) investigated the effect of different drying methods on total antioxidant activity and reported freeze-drying as the most effective method to retain antioxidant contents (hydrophilic and lipophilic fractions) in fresh bitter gourd. Similarly, freeze-drying of fresh honeydew fruit and spinach did not have significant impact on CAT, SOD and POD activity, which is consistent to the findings here. However, a significant loss of antioxidant enzyme (CAT, SOD, and POD) activity was found in freeze-dried sugarcane leaves in a study by Maura *et al*., (2016). This could be due to the different intracellular composition or less cryo-protective components in sugarcane leaves compared to *Dunaliella* cells. This, however, remain to be determined.

Freeze-dried whole cells stored at -20°C for 247 days retained CAT and SOD activity, whereas POD activity significantly ($p = 0.001$) decreased (by 58%). Glycerol have been suggested to play a vital role in retaining CAT and SOD activity during storage of freeze-dried cells by stabilising the dry enzymes (Arakawa *et al.*, 2001). Freeze-dried whole cells stored at -20°C for 247 days did not show a significantly change in H_2O_2 (1.59 pmol 10^{-7} cells) and MDA (293 pmol 10^{-7} cells) content relative to whole wet cells, which indicates unstressed cells. A study by Chong and Lim, (2012), suggested that freeze-dried herbal tea retained its total antioxidant

activity for 30 days. Another investigation on the storage stability of *Cosmos caudatus* demonstrated that freeze-dried material did not lose its antioxidant activity when stored for 120 days (Mediani *et al.*, 2014). These studies are consistent with the findings presented here. However, a contradictory result was found in the study by Gebczynski *et al*., (2017), who obtained a decreased antioxidant activity in freeze-dried *Pyrus communis L.* when stored at 2°C for 240 days. This may be due to the high storage temperature.

In summary, though some studies show that enzyme activity is not retained after freeze drying, this study suggests that freeze-drying could be used to dry freshly harvested whole wet *D. tertiolecta* cell as antioxidant enzyme (SOD, CAT and POD) activity is retained. The data also suggest that compared to wet cells, freeze drying will be the best option in order to ensure long term stability however, less so for short term stability.

3.3 CAT, SOD and POD activity in crude extracts

Freshly harvested *D. tertiolecta* cells pellet-derived extract was kept at three different temperatures (4, -20, and -80°C) and CAT, POD, SOD activity was determined at different time points between the initial day of processing and 119 days (Table 7). Compared to whole cell samples, CAT activity was significantly higher in the crude extracts at all temperature tested, however this was not the case for POD or SOD. The reason for this increase is unknown and cannot be attributed to sample concentration during the extraction process, as this would have been reflected in the POD and SOD samples as well. In addition, CAT activity (29.48 U mg^{-1} protein) was significantly ($p < 0.0001$) enhanced in crude extract when stored for two days onwards at 4°C. Likewise, POD and SOD activity also showed trends of increasing but only after 14 days of storages. This increase in activity was also seen for whole wet cells when stored under similar conditions. Antioxidant enzyme activity continued to increase with storage

time with a threefold increase in CAT (64.94 U mg^{-1} protein), a one-and-a-half-fold increase in POD (0.033 U mg^{-1} protein) and a two-and-a-half-fold increase in SOD (17.26 U mg^{-1} protein) activity after 14 days of storage (Table 7). Further stored for 37 days at 4°C a fivefold increase in CAT activity (110 U mg^{-1} protein), a significant ($p < 0.001$) decrease (by 40%) in POD activity, and no significant change in SOD activity was observed. Compared to enzyme activity at the day of processing, a significant ($p < 0.01$) reduction of SOD activity (by 65%), no detectable POD activity, and no significant change of CAT activity was found when crude extracts were stored at 4°C for 119 days. A study by Kolahi-ahari, (2006) reported that an increased SOD activity after 21 days storage of kiwi fruit extract (supplemented with protease cocktail) at 4°C, with the activity retained for 30 days when stored at -20°C, which is consistent with the findings presented here. In addition, an increase in POD activity in a fresh-cut *Cucumis melo L.* after 12 days (Oms-Oliu *et al.*, 2008), and in *Picea abies L.* crude extract after 30 days (Has-Schon *et al.*, 2005) were observed when stored at 4°C, which are consistent with the presented result. The extraction buffer used to prepare the crude extract contained compounds that have previously suggested to impact enzyme activity. These include PVP, which could participate it the formation of PVP-CAT bio-conjugation thereby stabilising the enzyme resulting in increased activity (Michler, 2017). Likewise, the presence of EDTA in the extraction buffer has been shown to also improve CAT activity by inhibiting metalloproteases activity (Gong *et al.*, 2000), and PMSF have been suggested to act as protease inhibitor thereby enhancing SOD activity (Kolahi-Ahari, 2006).

Moreover, more dissolved oxygen (DO) at lower temperatures could result in enhanced antioxidant enzyme activity. Pashova *et al.,* (1999) reported that an increasing in DO from 20 to 60% resuted in significantly increased antioxidant enzyme activity (Pashova *et al.*, 1999).

At -20°C and -80°C neither CAT nor SOD exhibited similar increases in activity seen at 4°C. On the other hand, CAT activity remained significantly unchanged until day 37 where after activity dropped (37%) and, SOD activity did not significantly reduced after 37 days of storages. For both enzymes, storages at -80°C retained activity for the entire time period tested (119 days) with a decreasing trend for CAT activity (Table 7). The result is in strong agreement with a study of Gong *et al*., (2000), who investigated the stability of enzyme activity in crude extracts derived from apple-flesh tissue, and reported the retention of CAT activity in 14 days storage with 80% activity remaining after 28 days of storages at -80°C. Since CAT and SOD activity did not change significantly at -80°C for up to 119 days, this temperature would be the optimal for long-term storages of crude extracts.

Oliveira *et al.*, (2011) investigated the effect of low temperature (-18°C) on APX activity and found a decrease in APX activity in six clones of *Malpigia emarginta* purees after 150 days of storage, which is consistent with our result. No significant change in total antioxidant activity in blueberry extract was observed after 30 days of storage at -20°C and, thereafter, significantly decreased activity was found (Srivastava *et al.*, 2007). A study by Samad *et al.*, (2016) also reported no significant increase in antioxidant activity after five-week of storage of two *Phoenix dactylifera* extracts (Mabroom and Mariami) at -20°C, whilst other two extracts (Safawi and Ajwa) showed a decrease in CAT activity. This indicates that antioxidant activity during storage depends on the sample material.

A two-way ANOVA (interaction between time and temperature on antioxidant enzymes activity) indicates that the interaction effect (time and temperature) have a statistically significant ($p < 0.05$) impact on CAT, POD, and SOD activity in stored crude extract. Therefore, both time and temperature need to be considered to obtain optimal storage

conditions to retain antioxidant enzyme activity in crude extract. However, if storage is needed, we recommend storing the unprocessed cells rather than a crude extract (Table 4 and Table 7) if POD or SOD are the enzymes of interest. However, initially processing might reduce need the volume and energy input during freezing.

3.4 CAT, SOD and POD activity in freeze-dried crude extract

Though freeze drying of whole wet cells did not have significant impact on antioxidant enzyme activity (Table 6), crude extract followed by freeze-drying significantly ($p < 0.05$) decreased POD activity (by 26%) and SOD activity (by 42%) but had a no significant effect on CAT activity. The reason for this difference between freeze dried whole cells and freeze dried crude extracts remains to be determined, however, for the preparation of the crude extract, a buffer is used and this buffer and the conditions (temperature) used during the extraction of antioxidants might not have been sufficient enough to also extract protective compounds knows as 'cryo-protective' compounds; Intracellular cryo-protective molecules (such as polysaccharides and phenolics) can be extracted by high temperature (Zhang *et al.*, 2010) or acid hydrolysis (Jain and Karibasappa, 2017) or with organic solvent (Jerez-martel *et al.*, 2017). Without these 'cryo-protectors, enzymes are desaturated during the drying process reducing their activity in the crude extract. For this reason, we recommend that crude extracts should be used in antioxidant enzyme assays within 24 hours of extraction.

Freeze-dried extract stored at -20°C for 30 days significantly ($p < 0.05$) decreased CAT activity (by 31%), POD activity (by 43%), and SOD activity (by 39%) (Table 8). To explore the reason for decreases in antioxidant enzyme activity in crude extract during storage, further studies are required.

This result strongly indicated that fresh whole cell extracts should be used in antioxidant enzyme assays, but if storages is necessary, whole fresh cells should be freeze dried. One limitation of this study is that storages of freeze-dried material was only assessed at -20°C. Perhaps, the dried material would have stored equally well at higher temperatures.

Extraction followed by freeze-drying of harvested cells is not a suitable route for post-harvest processing of antioxidant enzymes unless down-stream processing is optimized to possible include the addition of stabilisers in order to retain enzyme activities in the crude extract. It is therefore recommended that crude extracts prepared should be used in antioxidant enzyme assays within 24 hours of preparation.

4 Conclusions

The effect of storage conditions (time and temperature) on the stability of antioxidant enzyme (CAT, POD, and SOD) activity in *D. tertiolecta* was assessed in four samples: whole wet cells, crude extract, freeze-dried whole cells, and freeze-dried crude extract. Antioxidant enzyme activity in whole wet cells was retained for five months at -80°C. Whole wet cells can be stored at -20°C for up to four months without any loss of CAT and SOD activity and up to one month without any loss in POD activity. Likewise, CAT and SOD activity remain unchanged in the crude extract for four months when stored at -80°C. However, if stored at -20°C, CAT and POD activity remains unchanged for only one month, whilst SOD activity remains unchanged for two. Crucially, CAT and SOD activity increased significantly in both whole wet cells and the crude extract if stored at 4°C for more than 7 days with the activity peaking at around 28 days. In freeze-dried whole cells samples, CAT and SOD activity remain unchanged for at least eight months at -20°C, whereas as a crude extract and freeze-dried, the activity of all enzymes in the samples decreased with time. For this reason, we recommend freeze drying whole cells

rather than processed samples prior to storages. This finding could have implications for algal biomass production sites that process *Dunaliella* biomass for the purpose of harvesting antioxidants. Whether these recommendations apply to other types of algal biomass remains to be confirmed in further studies. Moreover, the result suggests the need for the development of cryo-proctors or other small molecules in microalgae-derived crude extract to stabilise antioxidant enzyme activity, which will prolong the shelf-life.

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