



ACCELERATED OXIDATION TEST OF FREEZE-DRIED *PSEUDOMONAS FLUORESCENS* BTP₁, BB₂ AND PI₉ STRAINS

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ABSTRACT

Pseudomonas fluorescens strains are very susceptible to damage during freeze-drying and storage. It would be useful to have longer lasting viable powders. Membrane oxidation is one of the factors responsible for damages suffered by *P. fluorescens* strains during storage. Singlet oxygen (¹O₂) is a highly reactive form of molecular oxygen that may harm living systems by oxidizing critical cellular macromolecules. We exposed freeze-dried powders of three strains *P. fluorescens* (BB₂, BTP₁ and PI₉) to singlet oxygen produced by photosensitisation of Methylene Blue/ light during 2 hours. We observe a significant loss in survival rate, a pronounced oxidation of polyunsaturated fatty acids, protein oxidation (carbonyl groups) and protein insolubilisation. This study enables better predictions of types of packaging suitable for freeze-dried powders of *P. fluorescens*.

Keywords: Accelerated oxidation test (AOT), Carbonyl group, Freeze-drying, Oxygen singlet, *Pseudomonas fluorescens*, Reactive oxygen species (ROS).

Contribution/ Originality

This study is one of very few studies which have investigated on *Pseudomonas fluorescens* strains. This study has objectives to develop a method for to stabilise and storage freeze-dried bacteria gram negative which is very sensitive to all form of drying.

1. INTRODUCTION

Freeze-dried *P. fluorescens* is usually used in agriculture and in the microbiological industry, but it very susceptible to damage during freeze-drying and subsequent storage and it would be useful to have longer-lasting cultures [1]. However, freeze-drying also renders the powder vulnerable to Reactive Oxygen Species (ROS) attack during storage [2, 3]. Lipids are major

targets during oxidative stress. Free radicals can directly attack polyunsaturated fatty acids in membranes and initiate lipid peroxidation [3]. These highly reactive free radicals can oxidize lipids, proteins and DNA, contributing to a variety of important types of cellular damage. In biological membranes, lipid peroxidation is frequently a consequence of free radical attack [4, 5]. A primary effect of lipid peroxidation is a decrease in membrane fluidity, which alters membrane properties and can disrupt membrane-bound proteins significantly. This effect acts as an amplifier in which more radicals are formed and polyunsaturated fatty acids are degraded into a variety of products. Some of these, such as aldehydes, are very reactive and can in turn damage other molecules such as proteins [4, 6]. The main protein modifications observed are: loss of catalytic activity, amino acid modifications, carbonyl group formation, decreases in thermal stability, changes in viscosity, changes in fluorescence, fragmentation, formation of protein-protein cross-links, formation of disulfide bridges, and increased susceptibility to proteolysis [5]. Subsequent to the finding that some amino acid residues (including lysine, arginine, proline and threonine) can be oxidized to carbonyl derivatives [7], several methods to detect the carbonyl content of proteins were developed and used to measure protein damage [8].

The aim of this work is to determine the damage caused by ROS on freeze-dried powders of three *P. fluorescens* strains. To do this, freeze-dried *P. fluorescens* powders were exposed to singlet oxygen produced by the action of light on Methylene Blue (MB/ light). In this paper, we examined overall viability as well as specific oxidative damage to fatty acids, proteins and DNA in freeze-dried *P. fluorescens* powders after exposure to Methylene Blue/light.

2. MATERIALS AND METHODS

2.1. Organisms and Cultivation

The strain used in our study is *P. fluorescens* BTP1 from the Wallon Center of Industrial Biology laboratory (CWBI), *P. fluorescens* BB2 and PI9 from Faculty of Agro-Vets and Biological, Saad Dahlab of Blida University Algeria [9]. The strain was grown in a 20 L bioreactor (Biolafitte) containing 16 L of 863 medium for 20 hours and then concentrated 20-fold by centrifugation (Sorval RC 12 BP) at 4700 rpm. Afterward, the pellets were dried in a freeze-drier (LOUW KOELTECHNIEK BVBA) using a standard program in which the temperature was gradually increased from -25°C to 25°C at 0.9 mbar pressure during 48 hours [1].

2.2. Photosensitisation

Samples were exposed to 40 µM of Methylene Blue (MB) in the presence of light. Methylene Blue produces singlet oxygen when exposed to an incandescent bulb [4].

2.3. Water Activity (a_w) and Survival Rate

The water activity (a_w) of freeze-dried samples was measured at 25°C using a water activity meter (Gbx-meter, Paris, France). Standard salt solutions (Gbx) of known water activity were

used for calibration of the sensor at the measuring temperature. Percentage survival of strains during exposure was expressed as follows:

Survival (%) = $\frac{N}{N_0} \times 100$, where N_0 is the CfU/g of the sample at the end of freeze-drying and N is the CFU/G of the freeze-dried sample at a given time. Each result was the geometrical mean of at least three counts [10].

2.4. Protein Carbonyl Content

The protein carbonyl content was determined spectrophotometrically using the described 2,4-dinitrophenyl hydrazine (DNPH) labelling procedure of Fleming, et al. [8] with modifications. Cells were lysed by sonication and collected by centrifugation (1500 rpm, 10 min at 4°C). Each sample (0.2 ml) was then incubated with 0.8 ml 0.2% DNPH in 2.5 M HCl for 1 h at room temperature. The protein hydrazone derivatives were sequentially extracted with 10% (w/v) trichloroacetic acid, treated with ethanol/ ethyl acetate, 1:1 (v/v), and re-extracted with 10% trichloroacetic acid. The resulting precipitate was dissolved in 6 M guanidine hydrochloride. A blank was run through a parallel procedure with 2 M HCl alone instead of the DNPH reagent. Results are expressed as nmol/ carbonyl/ mg of protein as determined by absorption at 360 nm [11, 12].

2.5. Fatty Acid Analysis by Gas-Liquid Chromatography

Total lipids were extracted overnight from dried cells (1 g) with a chloroform-methanol (2:1 v/v) mixture. Saline solution (0.88% (w/v)) was added in order to obtain 2 phases and shaken overnight using a modified Folch method [3]. The lower organic phase contains all lipids except gangliosides. The chloroform-methanol extracts were pooled, filtered, and then evaporated and concentrated under reduced pressure at 35°C. Fatty acid methyl esters (FAME) were prepared from the concentrate with a 14% (w/w) solution of boron trifluoride (BF₃) in methanol as reagent (Sigma, St Louis, MO, USA). After heating at 70 °C in a water bath for 90 min, 0.5 ml of saturated NaCl, 0.2 ml of sulphuric acid (10%) and 0.5 ml of n-hexane were added. The methylated fatty acids were taken from the upper phase after decanting. Gas chromatographic analysis of the methyl esters was carried out on an HP 6890 (Hewlett Packard) gas chromatograph equipped with a flame ionization detector at 250 °C [10, 11]. A capillary column (30 x 0.25 mm) was used. Helium was used as the carrier gas (2.4 ml/minute) and the injection volume was 1 µl. Injection was done at 250 °C in splitless mode for 1 min. The oven temperature was held at 50 °C for 1 min, then increased by 30 °C/min to 150 °C, and then from 150 °C to 240 °C at 4 °C/min with a final hold of 10 min at 240 °C. Fatty acid methyl esters were identified by comparing their retention times with a standard mixture FAME MIX 47885U (Supelco, Bellefonte, USA). The relative fatty acid content was estimated as a percentage of the total peak area using a DP 700 integrator. The relative content (%) of each fatty acid was normalized by expressing it as a ratio of the relative content (%) of palmitic (C_{16:0}) acid for two reasons: Berlett

and Stadtman [1] C_{16:0} had the highest proportion at most points relative to other fatty acids, [2] C_{16:0} did not change significantly during storage ($p>0.05$) (data not shown) [13].

One gram of *P. fluorescens* BTP1 powder was washed twice by centrifugation (14000 g, 30 minutes at 4°C) in 10 ml of Tris 50mM, pH 8.8. The cells were then lysed for 3 minutes by sonication (Bandelin Sonoplus HD 2070) in Tris-EDTA (50 mM, pH 8.8). After sonication, centrifugation was performed (24,000 g, 15 minutes at 4°C) to remove cells and crushed cellular debris. The supernatant containing soluble proteins was assayed with the Bicinchoninic Acid Protein Assay kit from Sigma [14, 15].

2.6. Statistical Analysis

Data from three replications were analyzed by using analysis of variance to determine if significant difference ($P\leq 0.05$) existed between mean values (origin 6.1 system function).

3. RESULTS

Figures 1 to 4 show the respective evolution in survival rates, membrane fatty acid composition, soluble protein and carbonyl groups in the different powders during exposure to methylene blue. Figure 1 shows the survival rate for the three *P. fluorescens* strains. During 90 minutes exposure to Methylene Blue /light, we observed a pronounced decrease in the survival rate after 30 first minutes of exposure. As seen in Figure 2, the BB2 and PI9 strains have a great polyunsaturated fatty acids rate than BTP1; and we observed a strong decrease in polyunsaturated fatty acids (C_{18:2} and C_{18:3}) of all strains during the exposure. Whereas Figures 3 and 4 show changes in the concentrations of soluble proteins and carbonyl groups, which are indicators of protein oxidation, we observed a protein insolubilisation during exposure and increased concentration of carbonyl group, demonstrating the presence of oxidative phenomena.

4. DISCUSSION

In a previous work [9] *P. fluorescens* were produced in a 100 L bioreactor, with or without the addition of cryoprotector (2% glycerol) and freeze-dried. The storage stability of the freeze-dried samples was then studied by monitoring several parameters such as: loss in viability, protein insolubilisation and changes in membrane structure (precisely C_{18:2} and C_{18:3}). To be able to improve long term storage of bacteria requires knowledge of the phenomena such as oxidation (lipid, proteins and DNA) responsible for sample degradation over time [2]. We know that ¹O₂ can be generated in cells, such as under conditions of oxidative stress, decomposition of lipid peroxides, or by spontaneous dismutation of superoxide [16]. In our study, three strains of *P. fluorescens* were used: a psychrophilic strain, *P. fluorescens* BTP1, from the Walloon Center of Industrial Biology (CWBI) and thermophilic, BB2 and PI9, sent from Algeria to Saad Dahlab of Blida University [2, 9]. After freeze-drying, the powders were conditioned in glass tubes for an

accelerated oxidation test. The accelerated oxidation test was monitored by losses in cell viability, changes in carbonyl groups, protein concentration, DNA oxidation and lipid oxidation (by profiling fatty acids) during a 90 minute exposure of the powders to Methylene Blue/ light. The activity water (a_w) of all powders was measured before exposure and samples were taken every 30 minutes during exposure for analysis. The water activity of all powders was high; e.g., 0.33 for the BTP1 strain 0.29 for the PI9 strain and 0.39 for the BB2 strain.

The percentage of survivors is expressed by logarithmic values of the surviving fractions after 90 minutes exposure. We observe that the PI9 strain shows a higher survival rate after exposure than the BTP1 and BB2 strains. The decrease in survival rate observed after 30 minutes exposure to Methylene Blue/light is more pronounced for the BB2 strain (25%) compared to BTP1 and PI9 (60%), but after 60 minutes exposure the decrease is more pronounced with all strains. This shows that the singlet oxygen produced has a considerable impact on the viability of the freeze-dried powders. Lipids are also affected by this exposure; as with survival rates, levels of polyunsaturated fatty acids (C18:2 and C18:3) also decrease. This decrease is more pronounced with the BB2 strain, which has a greater value of water activity (Figure 2). Of all the strains, *P.fluorescens* BTP1 from the Walloon Center of Bio-Industry showed a lower percentage of polyunsaturated fatty acids (C18:2 and C18:3) [10] than BB2 and PI9 from the Algerian Republic. Lipid oxidation of membrane fatty acids has been deemed responsible for cell death during storage [11, 17]. The main relative percentages from all the strains before or after exposure are presented in Fig. 2. This decrease shows that our three strains are very sensitive to Methylene Blue /light exposure. Protein oxidation mediated by reactive oxygen species is accompanied by the conversion of proline, lysine, arginine and histidine residues into carbonyl derivatives, thereby providing a convenient assay for oxidative modification [17]. As shown in (Figure 3 and Figure 4) protein oxidation and protein insolubilisation were significantly increased in three strains during exposure to Methylene Blue /light. However, the BB2 strain showed significantly higher levels of carbonyl groups and protein concentration compared to the BTP1 and PI9 strains during this exposure. Oxidative damage to membranes results in increased membrane fluidity, compromised integrity, and inactivation of membrane-bound receptors and enzymes [17]. The decrease in protein concentration shows that proteins are being altered and insolubilized by the oxygen singlet produced by exposure to Methylene Blue/ light. Protein denatured is also pronounced after 30 minutes exposure, reflected in the decreased concentrations of soluble proteins. This decrease is more pronounced for the BB2 strain but it is still constant after 30 minutes for the BTP1 and PI9 strains. Carbonyl groups increase more for BB2 strain after a 30 minute exposure, whereas most of the increase for BTP1 and PI9 began after 60 minutes. This shows that the BB2 strain is the first strain to undergo protein oxidation, and in fact BB2 has a high water activity.

5. CONCLUSION

The results presented here provide experimental support for the hypothesis that Oxygen is one key element responsible for the mortality of *Pseudomonas fluorescens* during storage of the freeze-dried powder. These results indicate that water activity and lipid peroxidation represent important intermediary steps in the process of oxygen radical-induced genetic damage during storage of the powder. The differences observed in survival rate, fatty acids composition, proteins and carbonyl content for the three strains are due in nature of strain (BTP1 is mesophilic, BB2 and PI9 are thermophilic), because thermophilic strains have a great polyunsaturated fatty acids content. This test allows us to understand the mechanisms responsible for loss of viability in freeze-dried powders during storage. Our results show that exposure of powders to singlet oxygen produced by photosensitisation with Methylene Blue /light (MB/light), leads to membrane oxidation. This oxidation is responsible for the loss in viability during storage of freeze-dried bacteria powders. To reduce oxygen damage during storage, the protective compounds must be used before freeze-drying and powder packed in a bag impervious to light and with oxygen.

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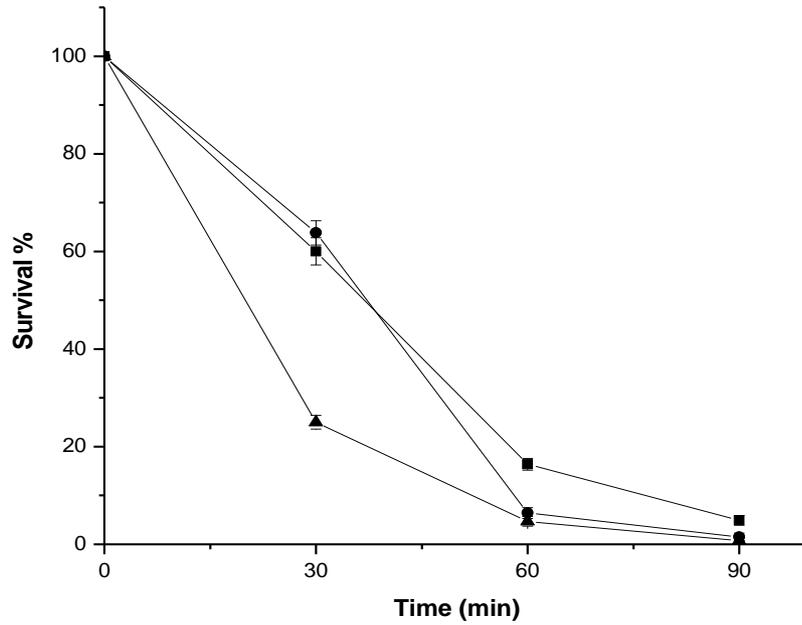


Fig-1. Effect of MB/light on viability of *Pseudomonas fluorescens* strains (■: PI9; ●: BTP1; ▲: BB2) over 90 minutes.

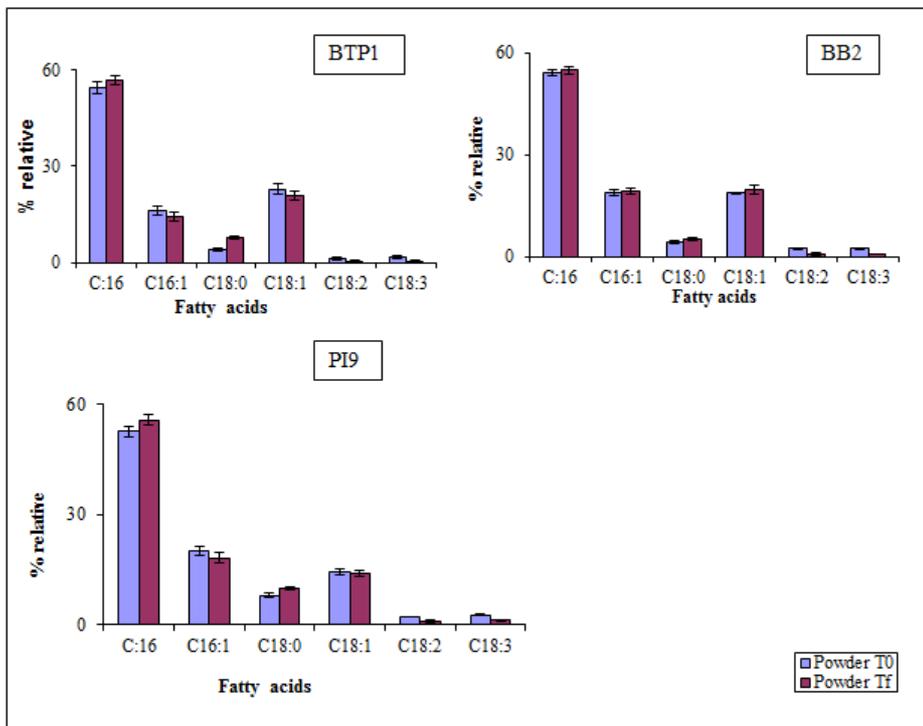


Fig-2. Fatty acid evolution after Methylene Blue/ light exposure of *P. fluorescens*.

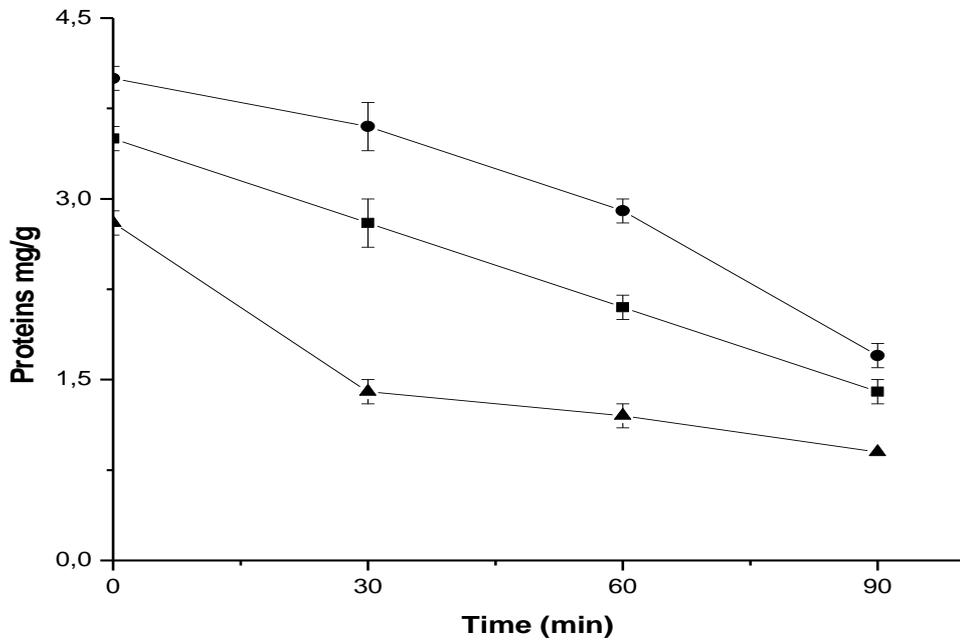


Fig-3. Protein content of *P. fluorescens* strains (■: PI9; ●: BTP1; ▲: BB2) during a 90 minute exposure to 25 mM MB/ light.

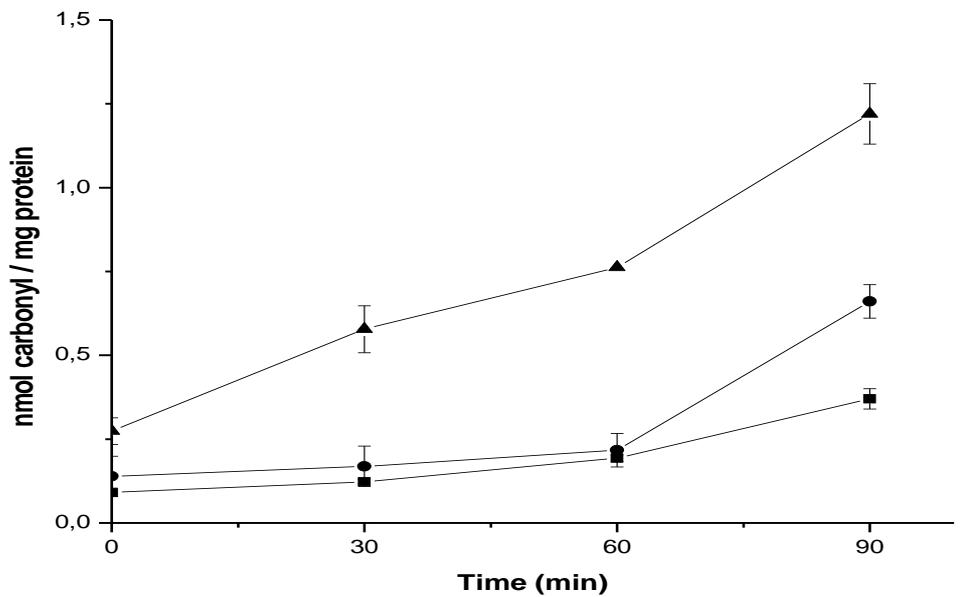


Fig-4. Protein carbonyl content of *P. fluorescens* strains (■: PI9; ●: BTP1; ▲: BB2) during a 90 minute exposure to 25 mM MB/ light.

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