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Impact of Protective Compounds on the Viability, Physiological State and Lipid Degradation of FreezeDried Pseudomonas Fluorescens BTP1 during Storage

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Abstract

The drying of bacteria remains a major alternative in order to keep them long term. After centrifugation, the bacterial pellet of *Pseudomonas fluorescens*BTP1 was divided in two fractions one with protecting compounds (2% glycerol or 5% maltodextrine) and one without and freeze-dried. After freeze drying, powders were sealed in aluminium bag under vacuum and stored at 4 or 20°C. The parameters such as viability, the conductivity and the ratio of polyunsaturated fatty acids/saturated fatty acids were used to investigate the viability of freeze-dried powders during storage. For example cell concentration of powder with glycerol (PG) at CFU/g before storage is 4.10^9 and after 7 month 2.10^8 at 4°C and $3{,}5.10^7$ at 20°C). The ratio of polyunsaturated fatty acids/saturated fatty acids decrease in function of time (e.g. at 4°C the ratios of C18:3 and C18:2 by C16:0 decreases respectively of 0,013 to 0,001 and 0,05 to 0,03 after 60 days of storage). In the present study, flow cytometric analysis was applied to evaluate the state in which the cells are at the end of storage time. We compared the survival results of bacteria obtained by plate count with the flow cytometric analysis results.

Introduction

Pseudomonas fluorescens are commonly used as bioherbicides replacing chemical

herbicides in agricultural, but it requires some formulation (liquid or powder) for his transport and or his use.[1, 2)] Freeze-drying is a commonly used method to preserve bacteria, in research as well as in industry. This technique is suitable for production of concentrated bacterial cultures, with the advantage that the dried material can be stored at ambient temperature and easily transported.[3-6)]

However, this technique brings about undesirable side effects, such as modifications in the physical state of membrane lipids and in the structure of sensitive proteins, causing the decrease of cell viability during processing as well as during subsequent storage.[6-8)]Consequently, some compounds such as polyols, polysaccharides, disaccharides, amino acids, proteins, vitamins, and various salts have been examined for their potential role to improve the survival of bacteria throughout freeze drying process[9-12,13)] reported that lipid oxidation of membrane fatty acids were responsible for cell death during storage for *Lactobacillus plantarum*.

In living organisms, lipids, particularly polyunsaturated fatty acid (PUFA) components of cell membranes, are described as extremely subjected to environmental stress. Loss of viability was related with decrease of the ratio unsaturated/ saturated fatty acids during storage.[13, 14)] Different species display different degree of freeze-drying survival, Gram-negative bacteria often showing lower survival than Gram-positive bacteria.[6)] Therefore, understanding the effect of these protecting compounds and their formulations is important to develop viable freeze-dried *Pseudomonas fluorescens*BTP1.

The aim of our work is to study and understand the factors responsible for cell death during freeze-drying and during storage at 4 and 20 °C. In this work, the viability of the freeze-dried *Pseudomonasfluorescens*BTP1 was evaluated by measuring conductivity, viable cell on PCA and by flow cytometric, membrane fatty acids composition before and after the drying process and during the storage. The effects of the temperature, glycerol and maltodextrine concentration during rehydration of the freeze-dried strain were also investigated.

Materials and Methods

Organisms and cultivation

The strain used in our study is *Pseudomonas fluorescens*BTP1 of Wallon Center of Industrial Biology laboratory (CWBI).[15)]

Production and freeze-drying

*Pseudomonasfluorescens*BTP1 was grown in 100 L bioreactor (Biolafite) containing 60 liters of 863 medium, for 20 hours and then concentrated 20 times by centrifugation at 4700 rpm. After centrifugation, the pellet was divided in three parts, one with and one without cryoprotectants respectively (2% w/w glycerol or 5% w/w maltodextrine) [3, 4)] and freeze-dried in a low freeze-drier (LOUW KOELTECHNIEK BVBA) with a standard program by increasing the temperature gradually from -25°C to 25°C at 0,9 mbar pressure during 48h.[16-18)]

Conductivity

For the analysis, 1 g of powder was soaked in Milli Q water (10 ml) for 4 h at room temperature. The conductivity (ms/cm.gdw) of the solution was measured using a conductivity meter (EC 215) by the method describes by.[19)]

Measurement of cultivability

Cultivability of cells was evaluated by plate counts and compared with cytometric results at days 210. After serial dilutions in peptone water, cells were plated onto solid 868 agar and incubated at 30°C for 24 h.[20)] Each result was the geometrical mean of at least three counts. Survival percentage was calculated as $100 \times N/N_0$, where N is the CFU/g DW at a given time and N_0 is the CFU/g dw at the end of freeze-drying.[3)]

Fluorescent probes and staining protocols

Carboxyfluoresceindiacetate (cFDA) was used to assess *Pseudomonas fluorescens*BTP1 viability, whereas the nucleic acid dye, propidiun iodide (PI), makes it possible to quantify damaged and dead cells. Before staining, cells suspensions of *Pseudomonas fluorescens*BTP1 were diluted in peptone water; at 0,2 optic density to 590 nm, centrifuged 2 minutes at 12000 rpm, and the pellets were resuspended in 1ml PBS buffer.[21)]

Live/dead assays were done by dual staining of the sample to differentiate viable, dead and stressed cells. The diluted suspension was first incubated with 10 µl of PI and 10 µl of cFDA for15 minutes at 37°C. After centrifugation and rinsing three times (12000 rpm for 1 minute) in 1 ml of PBS buffer, they are analyzed by flow cytometer.[20, 21)]

Fatty acids analysis by gas-liquid chromatography

Total lipids were extracted overnight from dried cells (1 g) with ethanol–ether (3:1 v/v) mixture. Ethanol/ether 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 14% (w/w) solution of boron trifluoride 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 a HP 6890 (Hewlett Packard) gas chromatograph equipped with a flame ionization detector at 250 °C.[3)]

Statistical analysis

Data from three replications were analysed by using analysis of variance to determine if significant differences ($P \leq 0.05$) existed between mean values and using Duncan multiple range test to compare treatments CFU counts and flow cytometric were transformed to their base 10 logarithms.

Results and Discussion

Viability of *Pseudomonas fluorescens*BTP1 during storage at 4 and 20°C

Drying bacteria remains a major alternative to keep them long term. After centrifugation, the bacterial pellet of *Pseudomonas fluorescens*BTP1 was conditioned with or without protecting compounds (2% glycerol or 5% maltodextrine) and freeze-dried. After freeze drying, powders were sealed in aluminium bag under vacuum and stored at 4 or 20°C. The storage stability of freeze-dried powders was studied by parameters such as loss of viability on the Plate Count Agar (PCA), electrical conductivity and evolution in membrane composition by measuring the ratio of unsaturated/saturated fatty acid. Viability (%) and concentration (Cfu/g) of bacteria during storage at 4 or 20°C with aw = 0,32 were determined using a procedure published by.[22)]These powders samples were analyzed singly (three plates per treatment) in experiment that were duplicated. In the present study, flow cytometricanalysis was applied to evaluate cellular injury sites affected by storage. Furthermore, it was attempted to relate data on the survival of bacteria, as obtained by plate count, with flow cytometric analysis data *table 1*[23)].

Table 1 shows the concentration of *Pseudomonas fluorescens*BTP1 after 270 days storage at 4 or 20°C respectively. The concentration of survival is expressed by the logarithmic values. Higher survival rates were obtained for the powder freeze-dried with maltodextrine. For example, at 4°C PS decreases from $1{,}9.10^{10}$ to $1{,}3.10^{7}$; PG decreases from 4.10^{9} to $1{,}5.10^{8}$ and PM decreases from $4{,}7.10^{10}$ to $1{,}5.10^{8}$ whereas at 20°C PS decrease $1{,}9\ 10^{10}$ to 5.10^{6}; PG decrease $4\ 10^{9}$ to 7.10^{7} and PM decrease $4{,}710^{10}$ to 3.10^{7}. By comparing both protecting compounds, glycerol has given the best viability rate during storage.

Table 1: Concentration of *Pseudomonas fluorescens* BTP1 (Log Cfu/g) after month 7 storage at 4°C and 20°C

Time (days)	4°C			20°C		
	PS	PG	PM	PS	PG	PM
0	10,2±0,1	9,8±0,2	10,7±0,3	10,2±0,1	9,8±0,2	10,7±0,3
15	9,5±0,1	9,8±0,2	9,9±0,1	9,1±0,1	9,4±0,1	9,4±0,1
30	9,5±0,1	9,6±0,2	10,1±0,1	8,7±0,2	8,5±0,1	8,5±0,1
45	8,6±0,1	9,5±0,2	9,9±0,1	7,7±0,1	8,0±0,1	8,0±0,1
60	7,9±0,2	9,1±0,1	9,8±0,1	7,3±0,2	7,2±0,3	7,3±0,4
160	7,8±0,1	8,9±0,1	8,8±0,1	7,2±0,1	7,2±0,2	7,4±0,2
210	7,1±0,0	8,8±0,0	8,8±0,1	6,3±0,3 **(7,5)**	7,2±0,2 **(7,7)**	7,5±0,2 **(7,9)**

* Brackets the flow cytometric values after 210 days storage at 20°C.

Figure 1 shows that the loss of cell viability for freeze-dried powders during storage at 4 or 20°C is related to the increase of electrical conductivity. The loss of viability is faster at 20°C than at 4°C; but it seems that, as for Gram +bacteria, loss of viability during the first 10 days of storage is very important. This result shows that storage temperatures affect the survival of *Pseudomonas fluorescens*BTP1. Mortality rate get higher with the storage temperature. It is now established that protecting compounds are almost essential when drying and storage microorganism.[3, 8)]

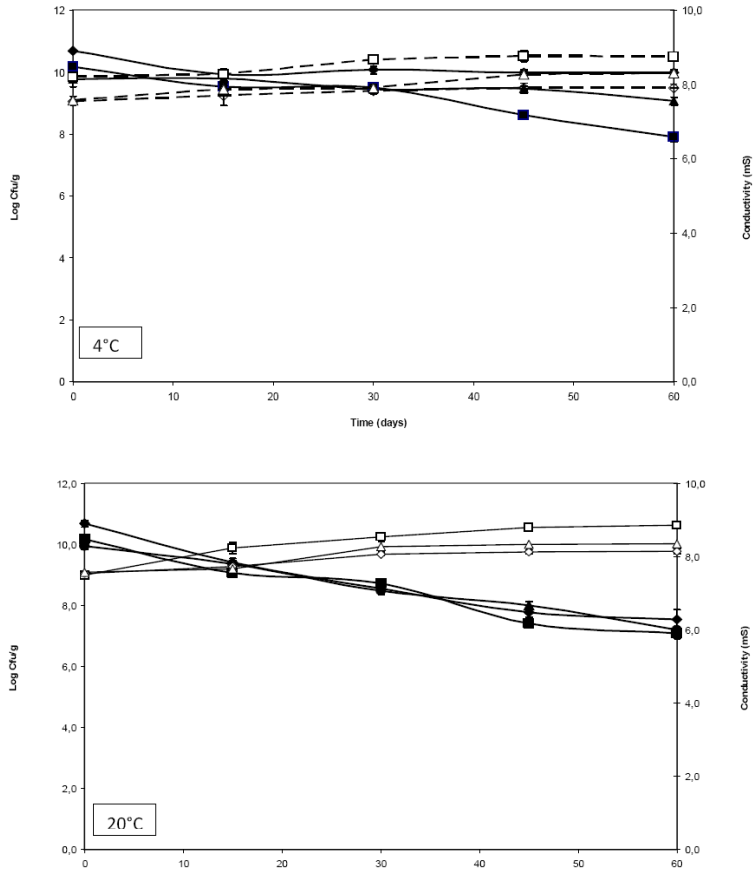

Figure 1: ■ survival Cfu/g [powder without protecting compounds (PS), ♦ powder with 5% maltodextrine (PM), ▲ powder with 2% glycerol (PG)] and electricalconductivity [□powder without protecting compounds (PS), ◊ powder with 5% maltodextrine (PM), Δ powder with 2% glycerol (PG)].

The viability of freeze-dried *Pseudomonas fluorescens*BTP1, stored for seven months at 20°C, was assessed on plat count agar (PCA) and compared to flow cytometric results tab. 1. This technique allows the determination of relative percentages of viable cells, dead cells and viable but non-cultivable cells after double staining by the PI and cFDA.[24)]For example in Fig. 2, we observe that the majority of cells are in an intermediate state (90.5% of viable but not cultivable cells). As shown

in fig. 1 that freeze-drying and storage leads to a decline in the percentage of viable cells, but an increase in the number of viable but non cultivable cells. As a consequence, it was demonstrated that freeze-drying and storage affected the membrane of *Pseudomonas fluorescens*BTP1. This result shows that many cells are in an intermediate state but not dead.[20)]Our results on freeze-drying and conservation of *Pseudomonas fluorescens*BTP1 show better viability rate than those found by [2)] on the strain of *Pseudomonas fluorescens*BRG100.

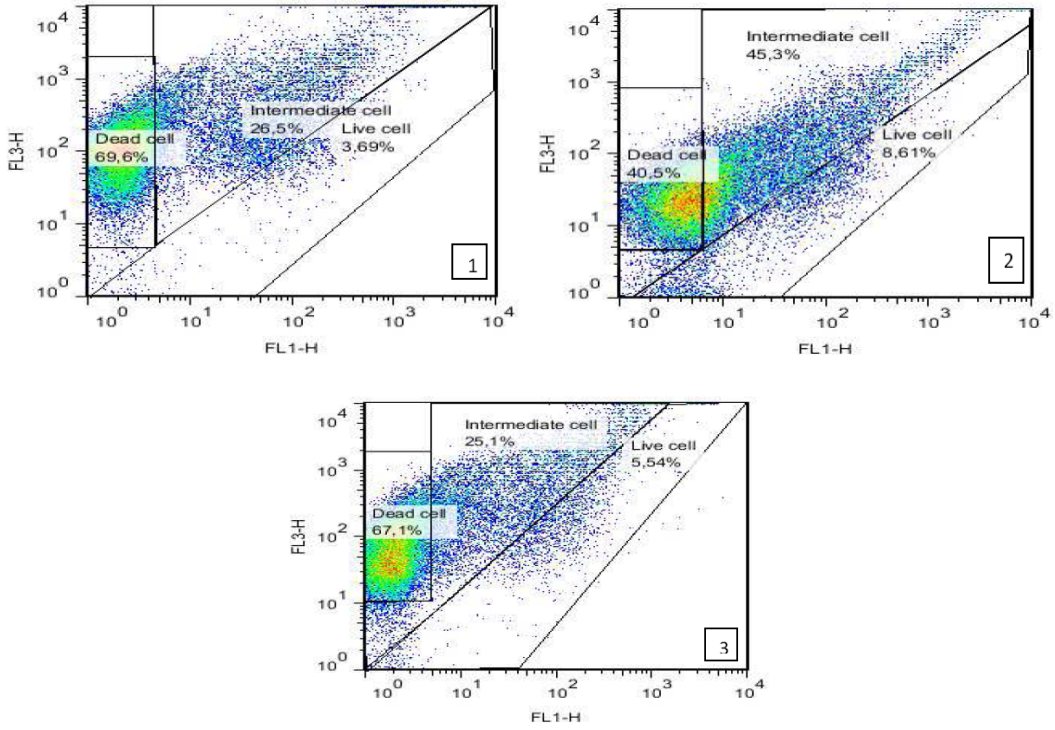

Figure 2: 1. Powder without protecting compounds (PS) 2. Powder with 5% maltodextrine (PM) 3. Powder with 2% glycerol (PG)

Cellular fatty acid composition

Freeze-drying causes damages in cytoplasmic membrane of *Pseudomonasfluorescens*BTP1 as shown by increased electrical conductivity and cytometric analysis. Membrane lipids play an important role in cytoplasmic membrane permeability, assuming that a large portion of the cellular fatty acids are present in the membrane. Like gram+ bacteria, the membrane damages cause changes in the lipids profile.[3)]Lipid oxidation of membrane fatty acid was deemed responsible for cell death during storage.[14)]

Table 2 shows the fatty acids profiles of *Pseudomonas fluorescens*BTP1 before and after drying. The relative percentages of the six main peaks of *Pseudomonas fluorescens*BTP1 cellular fatty acids are presented in this table. Those main peaks

were identified in relation with their retention times compared with standards as: palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic (C18:3). 4 of these 6 fatty acids have been previously identified in the cellular membrane of *Pseudomonas fluorescens.*[25, 26]We observe in fig. 3 that the polyunsaturated fatty acids of the powder without cryoprotectant (PS) are the most affected by freeze-drying (example 1,6% C18: 2 before freeze-drying to 0,7% after freeze-drying; 2,7% C18: 3 before freeze-drying to 1,5% after freeze-drying).

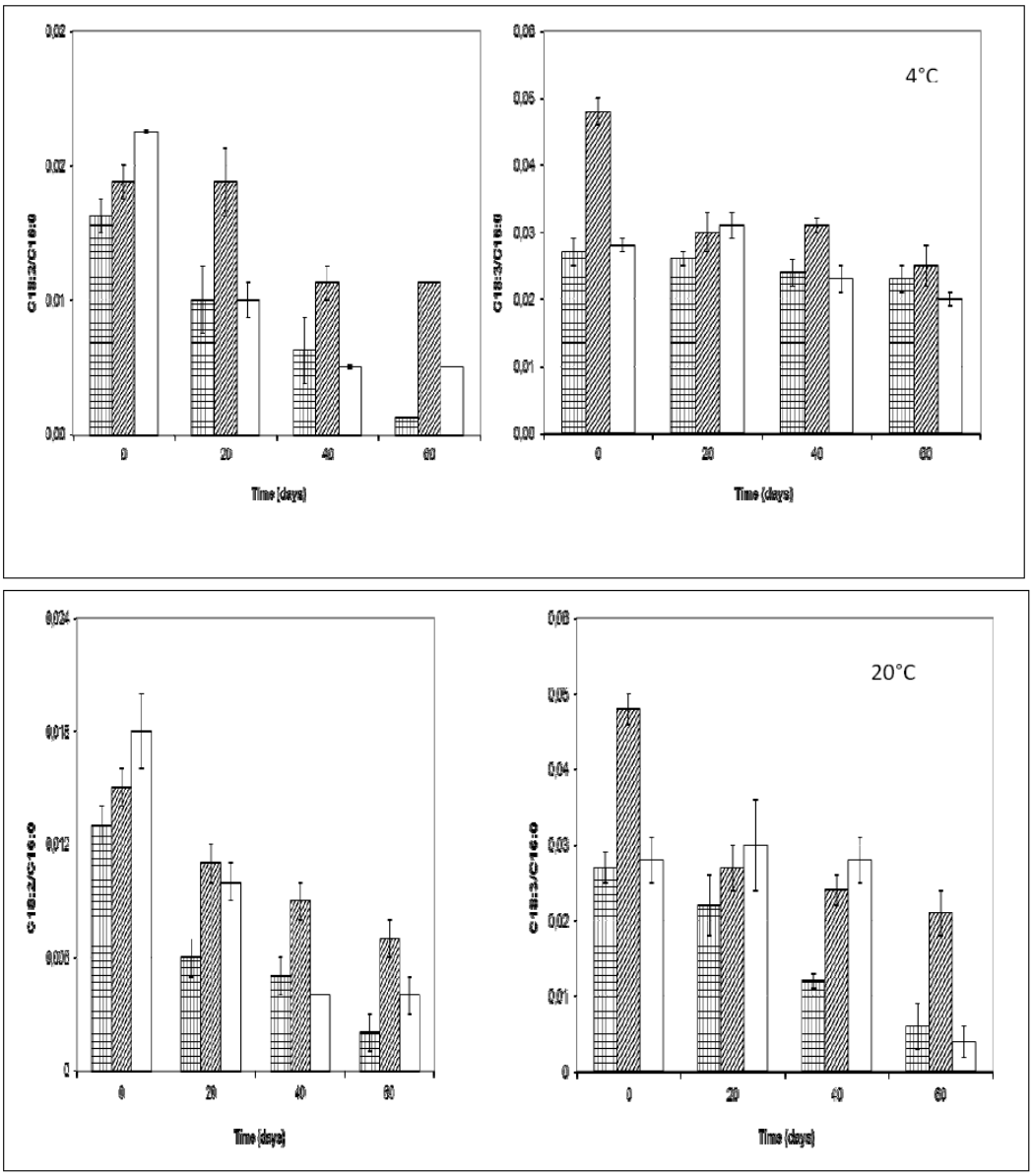

Figure3. : Powder without protecting compounds (PS) : Powder with 5% maltodextrine (PM) : Powder with 2% glycerol (PG)

Table 2: Cellular fatty acid composition of *Pseudomonas fluorescens* before and after freeze-drying

Treatment		Relative content (%)					
		C16 :0	C16 :1	C18 :0	C18 :1	C18 :2	C18 :3
Before freeze-drying	PS	54,2±0,1	16,3±0,8	4,5±0,4	20,7±0,2	1,6±0,4	2,7±0,3
	PG	55,2±0,6	17,4±0,2	3,3±0,5	19,9±1,0	1,4±0,0	2,8±0,6
	PM	55,9±0,4	18,1±0,9	3,6±0,9	19,3±0,7	1,3±0,2	1,8±0,5
After freeze-drying	PS	53,8±0,7	17,0±0,8	5,5±0,1	21,5±1,0	0,7±0,1	1,5±0,1
	PG	54,5±1,3	19,9±0,3	3,0±0,5	19,2±0,3	0,8±0,3	2,6±0,2
	PM	56,2±1,1	16,0±0,9	4,1±0,2	21,1±0,3	1,0±0,1	1,6±0,4

The six main fatty acids are palmitic (C16:0), pamitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3).

Changes in membrane structure are often displayed by measuring the ratio of polyunsaturated/saturated fatty acid. This ratio decreases during storage fig. 3, indicating that freeze-drying induces lesions in the cellular lipid-containing structures. Similar results were obtained for gram+ by.[14, 27)]This decreasing of unsaturated fatty acids due to oxidation was quantified on basis of ratio between each polyunsaturated fatty acid peak area and the palmitic acid peak area, since saturated fatty acids are not altered by oxidation .

Figure 3 shows evolution of the polyunsaturated/saturated fatty acids ratio after freeze-drying and during storage. We observe that the polyunsaturated/saturated fatty acids ratio decrease at 4°C and at 20°C for all storage conditions. This decrease is more pronounced for the powder without protecting compounds, which leads us to say that cryoprotectants slowed the oxidation phenomenon during freeze-drying and during 60 days of storage.

Conclusion

The results presented here provide experimental support to the hypothesis that freeze-drying and storage cause damage to *Pseudomonas fluorescens*membranes. The loss of viability during storage at 4°C or 20°C was associated with electrical conductivity increase and decrease in C18:2/C16:0 and C18:3/C16:0 ratios. Our results confirm the importance of protecting compounds to minimize loss of viability and its impact on C18:2/C16:0 and C18:3/C16:0 ratios during freeze-drying and storage. These data support further the view that PUFAs (linoleic and linolenic acids) play a key role in

determining cellular susceptibility to oxidative and/or heat stress. Flow cytometric show that after storage 210 days at 20°C the greater part of the cells is in intermediate state (viable but no cultivable). The flow cytometric associated with the plate count method, could be used as a predictive test for the description of physiological state of bacteria.

Acknowledgements

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Abbreviations

cFDA, carboxyfluorescein diacetate;
Cfu, colony forming unit;
DW, dry weight;
FAME, fatty acid methyl esters;
PCA, plate count agar;
PG, powder with 2% glycerol;
PI, propidium iodide;
PM, powder with 5% maltodextrine;
PS, powder without protecting compounds;
PUFA, polyunsaturated fatty acid

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