Electron Microscopic Studies on Spray-Dried and Freeze-Dried *chlorella Powders*

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The morphological changes that occurred on the *Chlorella* powders, after being spray-dried, and freeze-dried, were observed under scanning electron microscope. The spray-dried sample resulted in spherical particles and the inside of each particle was being occupied by a hole in the center. Each spherical structure consisted of more than a few thousand single *Chlorella* cells being piled up or stuck together. - By either increasing the heating. temperature or extending the heating duration time, the spherical form would instead result in a shrunken collapsed form. Freeze-dried *Chlorella* powders were found to be non-spherical and showed linear structure. Using the bacteriological and chemical analysis of spray-dried and freeze-dried powders, we found that the total chlorophyll content was higher than that in the freeze-dried powders, and the coliform counts showed a negative result in the case of spray-drying; the pheophorbide content was slightly higher in the freeze-dried one. These two different microstructures may reflect the physical properties of the dried-powders. By using SEM, the results are useful for estimating the manufacturing of reasonable *dried Chlorella* powders and for the parameters of its quality controls. In conclusion, at present spray-drying is the most commonly used method for the dehydration of *Chlorella* cells on a commercial scale.

INTRODUCTION

In the Orient, *Chlorella* cells are produced by mass cultivation by using mixotrophic or heterotrophic growths. The ultrastructural cytology of this cultivated *Chlorella* was described by Lin, *et al.*([1,2]) At present, spray drying is most commonly used for the dehydration of *Chlorella.* In our previous reports([3],[4],[5]), we reported the preliminary microstructure of spray-dried *Chlorella* powders, emphasizing on the cryotechniques which we have developed. Morris and Clarke([6]) described the method of cryopreservation of *Chlorella,* and they reported that there was no obvious correlation between freeze-fracture ultrastructure and cellular viability for *Chlorella.*

Since the photosensitization troubles which occurred in 1976([7]), total annual production of spray-dried *Chlorella* powders has been dropped from 1,290 *tons*([8]) to 350 *tons* in 1980 (as reported on Health Foods News, April 20, 1981, Tokyo, Japan). Only one-tenth of the total annual products are used for feeds; the rest are used in health foods. Little is known about the correlation between the different kinds of drying and bacteriological and chemical contents, although there has been some works done on the freeze drying and survival((,[6,9],[10],[11],[12]). In this investigation the comparative ultrastructural studies were carried out on the spray-dried and freeze-dried *Chlorella* powders. Moreover, the safety inspection -of the powder for foods was of more concern. We have also performed the preliminary concurrent bacteriological and chemical analysis on the dried powders for comparing their qualities.

MATERIALS AND METHODS

Algal culture: The unicellular photosynthetic green algae used in this experiment is*Chlorella pyrenoidosa,* which was cultivated in the open pool; the cells then were collected through centrifugation and subsequent washing([2]).

Drying: The spray-dried powders were made by applying the Kochiwa Spray Dryer by means of a centrifugal atomization *(10,000-12,000 rpm),* at an inlet temperature of 130-135^{o}C and an outlet temperature of 80-85^{o}C. The powder was formed within about 6-10 see after being injected into the chamber. Two kinds of procedures were used for freeze-drying, namely: slow freezing and rapid freezing. Samples (50ml) of the suspended *Chlorella* cells were placed in 300ml screw-cap bottles (Vir Tis Co., Gardiner, N.Y.). Cells were then frozen by rotating the bottles for I min in methanol maintained at -

60°C Upon removal from the freezing bath the bottles were immediately attached to VirTis freeze dryer (Model 10030), and a vacuum of 60M Torr of Hg was maintained for a *12hrs* drying period.

Electron Microscopy: - In order to observe the ultrastructure, the dried *Chlorella*powders were attached on double-stick adhesive tape on SEM stubs, and were then sputter-coated with a conducting layer of gold (about 200 to 400 4) by Eiko Ion Coater of Model IB-2 type. The specimens were kept in the desiccator until placement in the SEM chamber. Specimens were being observed with the Hitachi S-550 at 15 or 25 *KV,* and photomicrographs were taken with 6 x 7 cm negative films (ASA 125) under the initial magnification of 100 x to 5,000 x. TEAL micrograph was prepared according to the previous paper([2]). In order to observe the inside structure, the spray-dried powder encapsulated in an agar layer, then fixed in 1%, osmium tetroxide and freeze-fractured in the liquid nitrogen (as shown in Fig. 1).

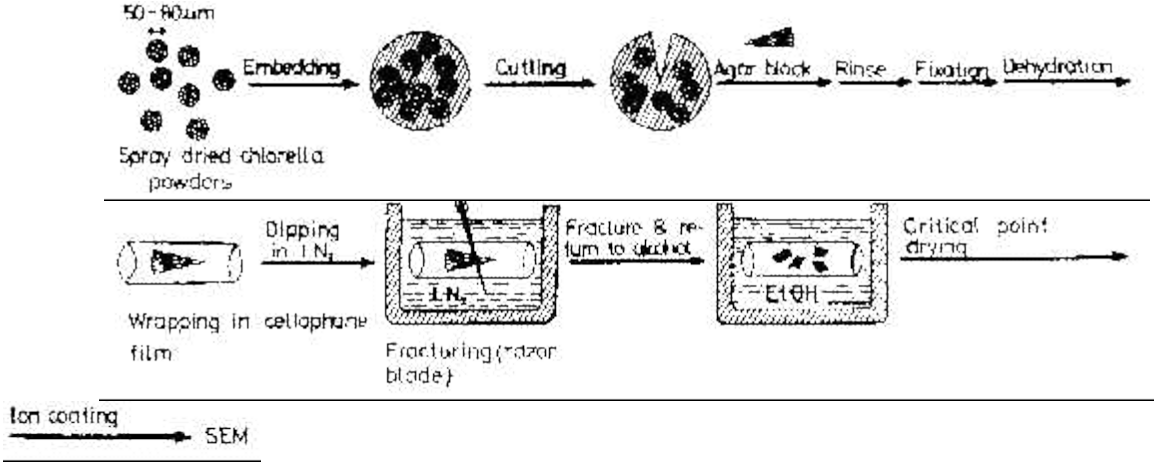

Fig. 1. Schematic diagram of a cryotechniques process of fracturing of spray *dried Chlorella* powders.

Chemical Analysis: The content of water was determined by drying at 105°C for 12 hours. The content of chlorophyll a was determined by the methanol extraction method . as described by Mackinney([13]). The relative activities of chlorophyllase were determined by the method as described by Tamai, *et al.([14])* The content of pheophorbide was estimated according to Wickliff and Arnoff([15]) and Brown([16]).

Bacteriological Analysis: The total count of bacteria was estimated by the conventional plate count method([17]) and coli-form bacteria were counted by using the deoxycholate media according. to the Difco manual([18]).

RESULTS AND DISCUSSION

The fine structures of mixotrophically grown *Chlorella* on glacial acetic acid cells are depicted in Fig. 2. As comparing the other report, the cells show a unique green algal character possessing well developed organelles([19]). Fig. 3 shows a SEM micrograph of cells, showing the spherical entities and their smooth surfaces. *The Chlorella* cells dried by sunlight, heating or vacuum evaporation, when being observed under the microscope, were known to have hundreds of cells aggregated into a clump.

Spray drying, involving the fine dispersion of droplets in the hot gas, is widely used for liquid food products([20]). The temperature of the droplets remains below the wet bulk temperature of the drying gas until almost all the water has been removed, due to the high evaporation rate, and consequently high temperature can be utilized.

The spray dried *Chlorella* sample resulted in a spherical particle (Fig. 4) being formed and the inside of each particle had a hole in the center (Fig. 5). This result coincided with the work on spray dried milk powder([21]). The matrix structure of the shell of each spherical particle consisted of more than a few thousand single *Chlorella* cells, with cells piling up or sticking together (Figs. 4 & *5).* Each granule's diameter showed approximately 50-80,um and

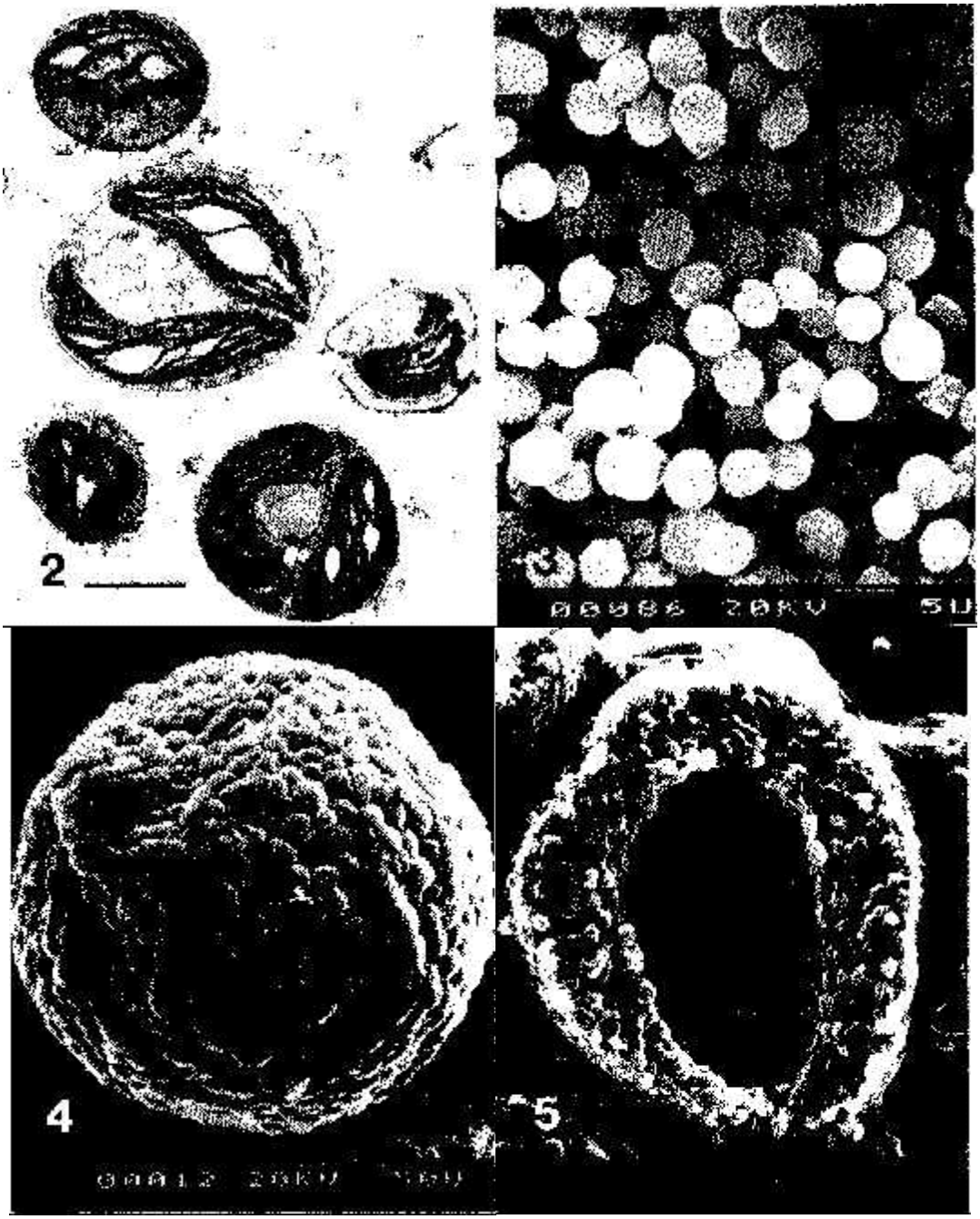

Fig. 2. Transmission electron micrograph of mixotrophically grown cells, showing typical green algal characteristics. Bar in lower margin equals*1um.*

Fig. 3. Scanning electron micrograph of Chlorella cells, showing spherical shape. Bar is 5 *um*.

Fig. 4. Scanning electron micrograph of a spray-dried Chlorella spherical granule. Bar is 50 *um*.

Fig. 5. A cross sectional view of spray-dried particle, showing the hollow structure. Bar is 5 *um*.

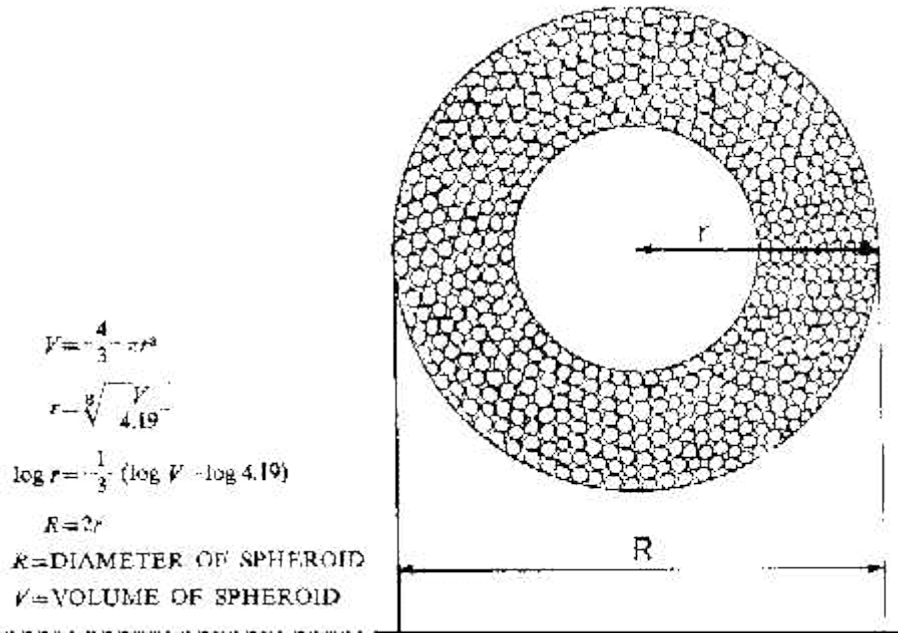

Fig. 6. Diagram of a spherical spray-dried particle. The assumption of calculation is shown on the left side.

its diameter was one-half of the particle in a sectional view (Fig. 5). If the volume of the void space was being excluded, and each granule's diameter was approximately 50-80,um, then it is estimated that each granule was composed of 3,000-7,000 unicellular cells. The assumption for calculation is shown in Fig. 6. In most cases, the shape of the resulting particle is unique, being spherical and hollow, and with the ash-like solids being attached to the surface. If the conditions of spray drying are changed, such as by inlet and outlet air temperatures, duration time, and atomization, then changes -in the shape of the particles will be induced.

Fig. 7 depicts the effect of prolonged duration time (more than 20 sec), only a few particles exhibited spherical entities, and the rest of them displayed shrunken and collapsed forms (folded particles). Fig. 8 depicts the effect of elevated temperature (150-160^{o}C) of hot air, or repeating the drying 2-3 times (in the dry state), results in all of them showing the collapsed forms.

Figs. 9 and 10 show the enlarged view of the surface-structures of shrunken and collapsed spherical entities. Unicellular algae structures still exist in some parts of Fig. 9, but there were only fused surfaces in the one of Fig. 10. A hole on the surface (Fig. 10) reminiscent of the hollow structure, existed during the formation of the particle. Fig. 11 indicates another view of two particles, which shrunk unevenly during the drying. Phase transformation or cell envelopes in the drying process affect their water-binding characteristics, color, texture, and flavor. These characteristics are very important in the following production of the *Chlorella* tablets.

Freeze drying, also designated as sublimation drying and lyophilization, results in the least damage to food of all commercial processes for drying. In the drying operation, water vaporizes from the frozen state without passing through the liquid phase. Under these conditions, shrinkage is prevented and the resulting product has a dry and highly porous structure, since the spaces

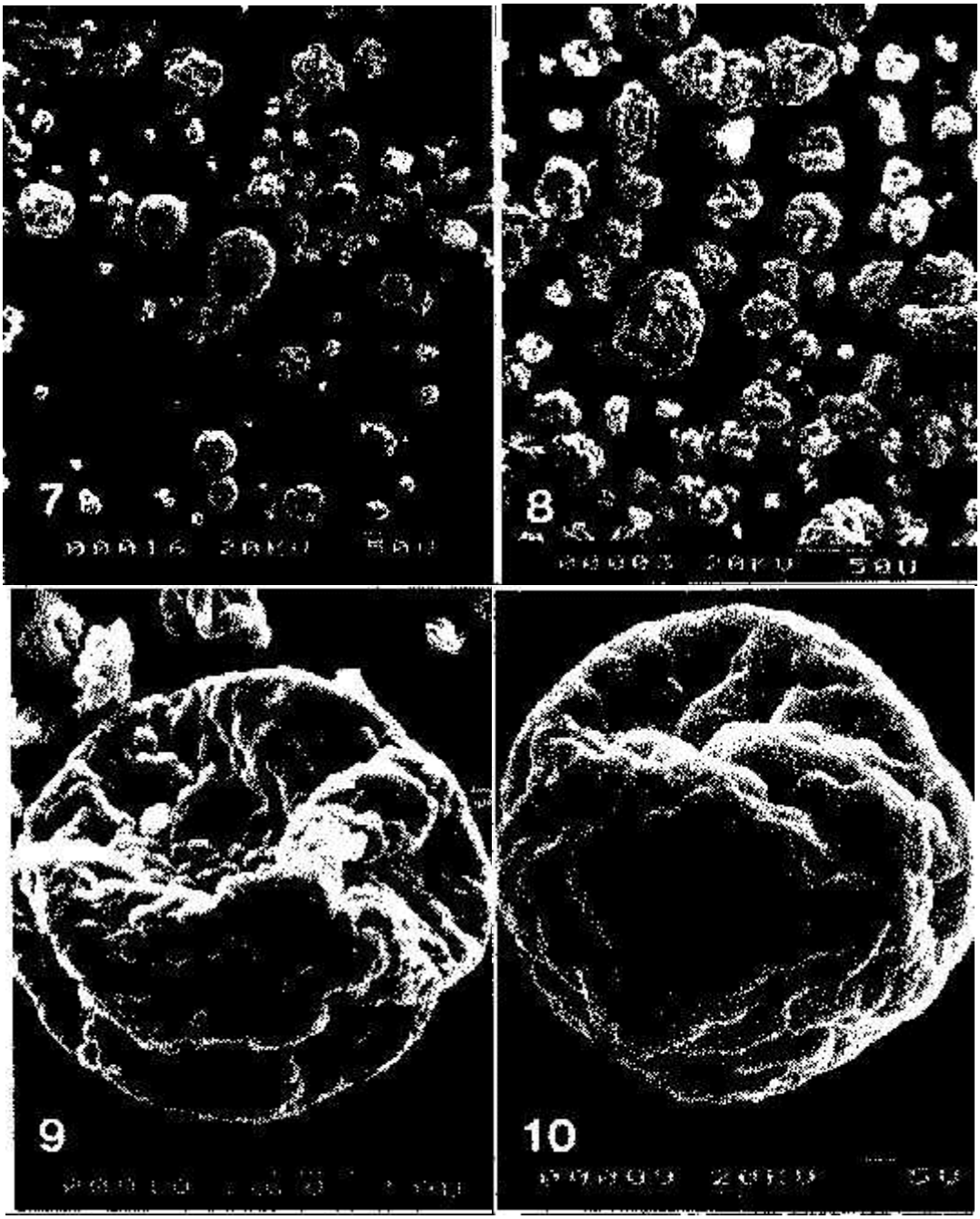

Fig. 7. Scanning electron micrograph of spray-dried powders formed under the prolonged duration time, showing a few, particles exhibiting spherical structures. Bar is 5*um*.

Fig. 8. Scanning electron micrograph of spray-dried powders which formed after repeated spray drying, showing all particles exhibiting the collapsed forms. Bar is *50,um.*

Fig. 9. An enlarged view of the surface-structure of shrunken and collapsed spherical entities. Individual cells are recognizable at the same portion. Bar is 50*um*.

Fig. 10. An enlarged view of the surface structure, slowing the fused appearance with a hole on the surface. Bar is 5 *um.*

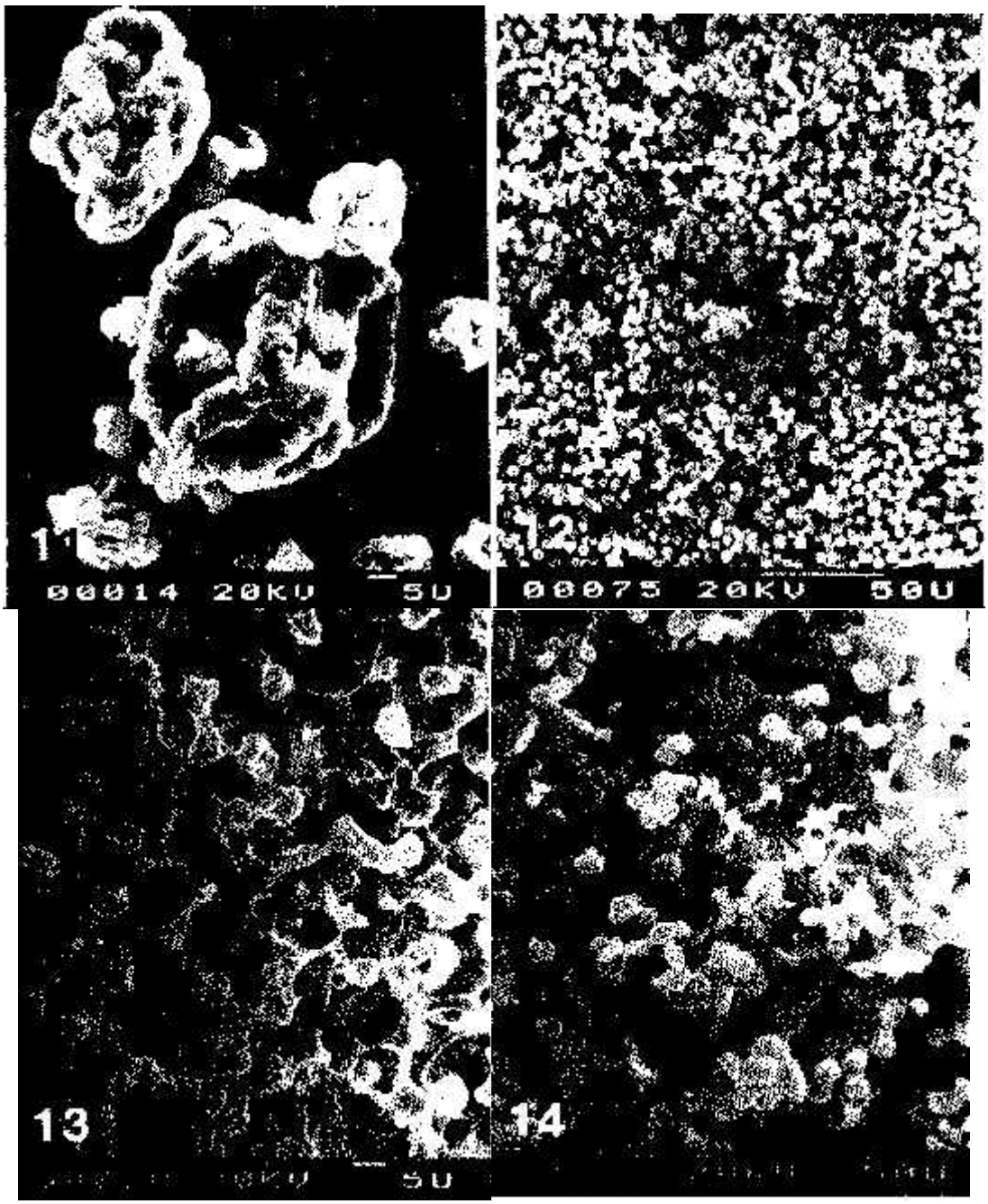

Fig. 11. Two spherical spray-dried particles, showing unevenly shrunken surfaces. Bar is 5 *um.*
Fig. 12. Scanning electron micrograph of slowly freeze (-30^{o}C) dried cells. An enlarged view of the surface structure, showing the fused appearance :with a hole on the surface. Bar is 50*um*.
Fig. 13. Scanning electron micrograph of slowly freeze-dried cells showing flaccid and fused appearances. Bar is *5um.*
Fig. 14. Scanning electron micrograph of slowly freeze-dried cells with mucoid materials. Bar is 50 *um.*

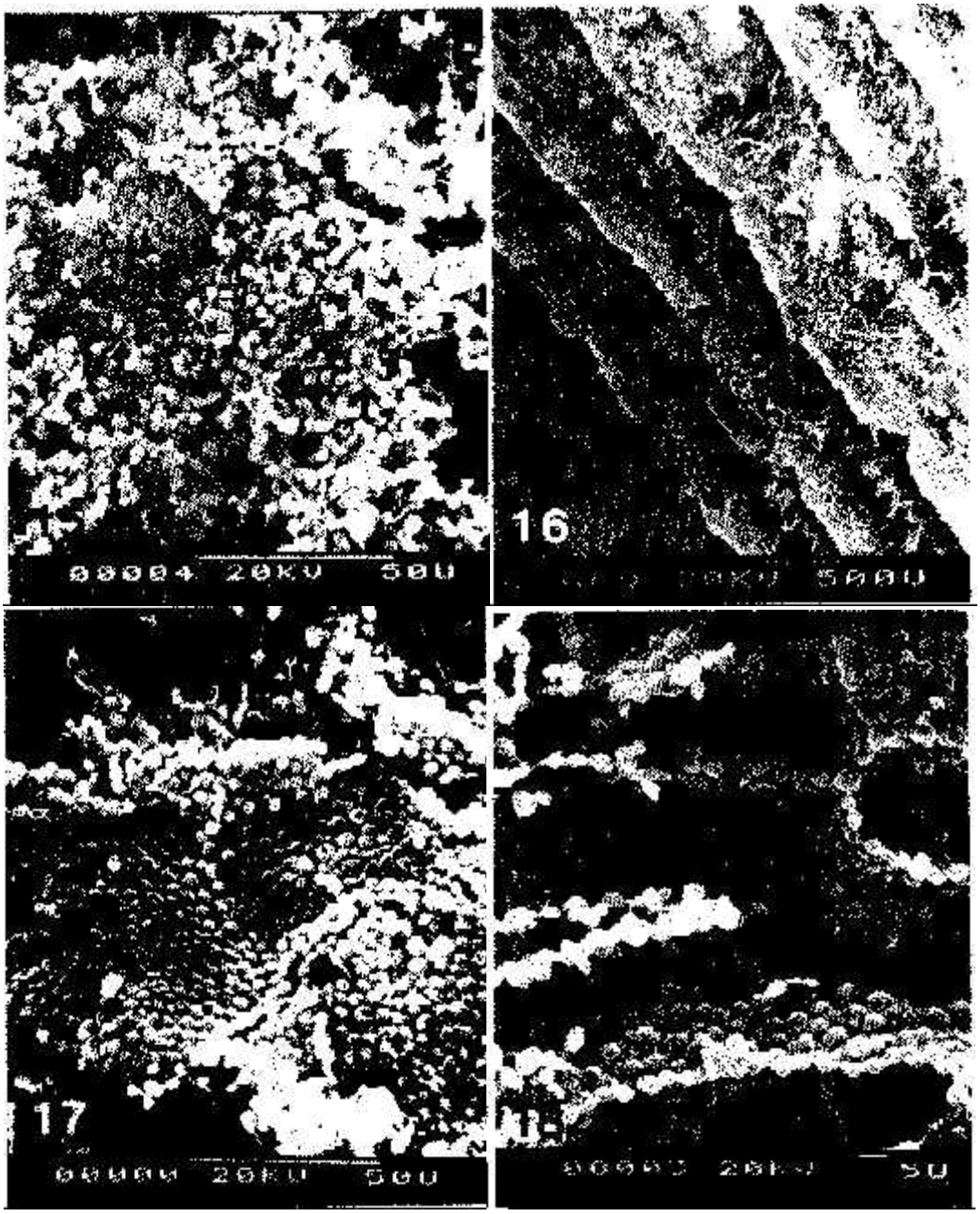

Fig. 15**.** Scanning electron micrograph of freeze-dried cells, showing the contaminated cells thread-like materials existing the intercellular spaces. Bar is 50 *um.*

Fig, 16. Pellet-like structures after slowly freezing unwashed wet-cake materials. Bar is *500 um.*

Fig. 17. Rapid freezing (-196^{o}C) cells, showing the sheet-like arrangement. Bar is *50 um.*

Fig. 18. An enlarged View of rapid freeze-dried (-196^{o}C) *Chlorella* cells showing the pellet of cells. Bar is 5*um*.

formerly occupied by ice crystals have now become voids. This porous structure favors later rapid dehydration. In reality, freezing results in a physical separation of water in the form of ice crystals from the other components of the food. These crystals are then vaporized under mild conditions, in which heat damage and other adverse changes commonly associated with dehydration of food are minimized.

Freeze-dried *Chlorella* powders were non-spherical in shape and showed a linear structure. Slow freezing (-30^{o}C) and freeze-drying could endow big ice crystals within the intercellular spaces of the sample (Fig. 12). These ice crystals could possibly exert pressure on the cells to an extent that dehydration of the cells occurred. Moreover, slow freezing and freeze-drying, could endow extracellular ice crystals, which might press on and in turn even pierce the cell membrane. The cell membrane was thus being mechanically damaged. The cell became floccid and fused together, showing that it was being dehydrated (Figs. 16 & 17). In the early stage of slow freezing, the ice crystals formed first had lower vapor pressure, whereas the liquid portion of the cell had higher vapor pressure. This difference **in** vapor pressure caused the cell to lose water to the outside. As a result, the size of the ice crystals grew. If the time for gradual freezing was long enough, it allowed the water to escape from the cell so the freezing point of the cytoplasm was further decreased. Thus, decreasing the freezing rate to a certain extent - may prevent the occurrence of intracellular freezing.

If rapid freezing in liquid nitrogen (- 196^{o}C) was employed simultaneously, it was found that intracellular ice crystals happened to form and the cell membrane was intact. The degree of mechanical damage caused by ice crystal formation was being reduced to a minimum. As a result, the original shape of the samples could be preserved (Figs. 13 & 14). Biochemically speaking, the cell constituents were of a complex origin. Once the cell membrane was being harmed, the cell contents of the neighboring cells were able to mix together, thus unpredictable chemical reactions would occur. This in turn caused the denaturation of the food quality. The higher the rate of freezing, the less damage was being done on the cell. Whereas, slow-freezing may possibly cause the fracturing of the cell membrane.

Table 1
Bacteriological and chemical contents of dried Chlorella powders*[1]

	Spray-dried	Freeze-dried
Water content (%)	2~3	3~4
Total bacteria (#/g)	1 X 10^4*2	5 X 10^4*2
Total coliforms (#/g)	negative	1 X 10^4*3
Chlorophyll (%)	2~3	4~5
Pheophorbide (mg%)	25~50	50~60
Chlorophyllase (mg/%)*4	90~160	190~250
Hardness (kg)*5	3.5~5.5	2.5 (max)
Color	deep green	light green

*1 The initial concentration of *Chlorella* suspension was 10-12% (*w*/*v*), as determined by dry weight.
*2 The total bacteria counts in the *Chlorella* suspension were 10^6-10^7colonies per *ml*.
*3 Approximately 100colonies were appeared on the deoxycholate media.
*4 The activity of enzyme was converted to *mg%* for convenience.
*5 Hardness was determined by measuring the tablet *(250mg)* by the Monsanto type hard meter.

Table 1 shows the bacteriological and chemical analysis of spray-dried and freeze-dried powders. Water content of powders was maintained at 2-4%. after drying in both cases, but the total chlorophyll content was slightly higher in the freeze-dried powders. The content of bacteria was reduced approximately one logarithmic scale for each run in the case of spray-drying, and the viability of the contaminated bacteria was about 55%, after freeze drying and thawing. Tile coliform counts showed a negative result in the case of the spray-dried powders, but about 100 colonies per gram remained after freeze-drying. Antheunisse([22]) indicated different kinds of bacteria will survive after freeze drying. The viability was 50-100%, depending on different strains and conditions. The pheophorbide contents was slightly higher and

chlorophyllase -activities were much higher in the case of freeze-drying. Because of the difficulties in getting pure pheophorbide, it is very hard to conclude the results. The original content of pheophorbide in the wet cake (harvested cells) depends on the dead cells and the degree of cell autolysis. However, the degree of inactivation on chlorophyllase was much less in the case of freeze drying on comparing to spray drying. It seems to be reasonable to get higher enzyme activities in freeze dried powders.
These two different microstructures will reflect the physical properties of dried-powders, such as water holding capacity, caking, and appearance. By using scanning electron microscopy, the results can be helpful for estimating manufacture of reasonable chlorella dried-powders and the parameters of its quality controls.
In conclusion, spray drying is most commonly used for the dehydration of *Chlorella*cells on a commercial scale at present. Since the chlorophyllase is not completely denatured (as shown in Table 1) during both the processes of drying, there still remains a possibility of increasing the content of pheophorbide during the processing and storage periods. However, we can note that in the case of freeze-drying the activity was higher. Moreover, the cost for freeze-drying is much higher than spray-drying. Thus, it is safe to say, that spray-drying is a favorable dehydration method for*Chlorella cells.*
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