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Cryopreservation on *Porphyra*

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Abstract A cryopreserving method at about −20℃ of *Porphyra* thalli after they were half-dried was developed at about 30 years ago, and it is indispensable presently for aquaculture productions. In recent years, studies on cryopreservation have been reopened from the point of view of genetic resources preservation, and thus very low-temperature preservation in the liquid nitrogen (−196℃) has become possible for not only thalli but also filamentous thalli.

Key words: cryopreservation, freezing, *Porphyra*, seaweed, laver.

In higer plants, cryopreservation of cells and meristems in liquid N_2 has become an important tool for long-term preservation of germplasm or experimental material without genetic alternation (Sakai, 1992). And attemps to cryopreserve seaweeds in liquid N_2 have been begun in recent years (Saga, 1990). In this paper, I present information on the cryopreservation of *Porphyra*.

1. The Ministry of Agriculture, Forestry and Fisheries, Japan has conducted a genetic resources preservation project constituting the basis of the approach to develop future agriculture, forestry and fishery industries and food industries through the promotion of developments of high technologies, such as biotechnology and so on. As a part of the project, in our institute, collection and preservation of *Porphyra* have been conducted, and about 50 strains consisting mainly of *P. tenera* Kjellman and *P. yezoensis* Ueda, which are the subjects of aquaculture, are preserved.

The *Porphyra* has no such a suitable stage for preservation as we can find in the seed of higher plants, hence the preservation of strains has been carried out by the subculture of filamentous thalli. Accordingly, labor, time and space needed to it have been large burdens for the strain-preserving institutes. In addition, the possibility of some genetic changes can not be denied during long-term preservation, so it hoped some alternative preservation methods are developed.

2. Significance and method of cryopreservation in liquid N_2

In liquid N_2 (−196℃), it is considered that physiological and genetic changes during preservation are controlled to the minimum levels owing to the halt of most of the biochemical activities (Sakai, 1992), and thus the method is one of the most suitable long-term preservation methods for the *Porphyra*. It is hoped, in particular, as a preservation method of useful strains produced by use of recently strikingly advanced scientific techniques and wild species having been extinguished by environmental destructions etc. (Miura, 1994).

It is necessary to avoid lethal intracellular freezing for successful cryopreservation in liquid N_2. To do so, it is necessary to dehydrate cells sufficiently before they are immersed in liquid N_2. The reason for it is that the sufficiently dehydrated and concentrated cytoplasm is not frozen but vitrified when it is rapidly cooled by immersing in liquid N_2. Extracellular freezing is an effective method as dehydration method. The extracellular frozen cells are dehydrated with the temperature decrease (Fig. 1). However, in common materials, they hardly have abilities tolerant to freeze-dehydration, so that their tolerances to freezing are raised by pretreatments with cryoprotectants, such as dimethyl-sulfoxide (DMSO) etc. This is called conventional slow pre-freezing method. As the other dehydra-

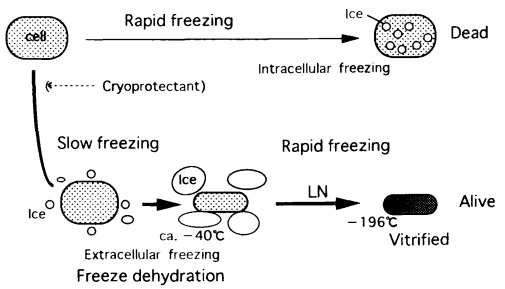

Fig. 1. A cryogenic strategy for survival of cells cooled to −196℃.

tion methods, there are osmotic dehydration method by the use of highly concentrated solutions (Vitrification method) and desication method by air drying (Sakai, 1992; Sakai, 1993).

3. Freezing resistance and drought resistance of thalli

It is known that the *Porphyra* thalli which grow thick in intertidal zone in winter have freezing resistance (Terumoto, 1964). In natural *P. yezoensis* Ueda, it was found that when it was preserved for 24 hrs at −70℃, −100℃ and −196℃ (in liquid N_2), the cells were all alive after thawing (Terumoto, 1965). And besides, it was found that it was tolerant to desication and most of the cells were alive even when the moisture content reduced to about 4% of the initial content (Terumoto, 1965). In case of *P. tenera* Kjellman, the survival ratios were 69% in the preservation for 5 days in the range of −18℃～−20℃ and 23% in the preservation for 5 days in the range of −70℃～−75℃ (Migita, 1964).

In the study on the relationship between moisture contents and survival ratios, where *P. tenera* Kjellman, *P. yezoensis* Ueda and *P. suborbiculata* Kjellman used as materials were preserved for 2 months at −20℃, it was found that many living cells were observed when the materials were dried to reduce the moisture contents to the levels of 20～40% of the initial ones (Migita, 1966). The survival ratios in these cases were above 70% in the *P. tenera* Kjellman and above 90% in the *P. yezoensis* Ueda as well as *P. suborbiculata* Kjellman. In another case where *P. yezoensis* Ueda was preserved for 312 days in the range of −15℃～−20℃ after dried to 20% of moisture content, the survival ratio was 80% (Kurakake and Hori, 1966). A method by which half-dried thalli are preserved at −20℃ was developed about 30 years ago by using the above-mentioned nature (Kurakake, 1969). The preserved material treated with the method is called deep-frozen 'nori' (*Porphyra*) net and is indispensable, at present, in the aquaculture production. Meanwhile, 'Kaigara-amanori' (*Porphyra tenuipedalis*) living on non-exposing zones in low tide is said to be vulnerable to freezing and drying (Achiha and Nakashma, 1995).

4. Cryopreservation of thalli in liquid nitrogen

As mentioned above, in the natural *P. yezoensis* Ueda, when it was preserved for 24 hrs by immersing directly in the liquid N_2 without addition of cryoprotectants all the cells were alive after thawing (Terumoto, 1965). According to the results obtained by Sakai and Sugawara (1978), when it was immersed in liquid N_2 after the slow pre-freezing to −10℃ a high survival ratio (about 100%) was obtained after thawing, whereas when the pre-freezing was till −5℃, then only a low survival ratio (about 10%) was obtained. Besides that it was found that even when same species were used for experiments they did not always show same degrees of freezing resistance (Terumoto, 1965). According to these findings, it is possible, depending on the material, to preserve in the liquid N_2 even in the state as they are, but it is not reliable. It is possible to preserve fairly reliably in the liquid N_2 if they are treated with slow pre-freezing by use of a programme-freezer etc. after the addition of DMSO or glycerol as cryoprotectants (Sakai and Sugawara, 1978; Kito *et al.*, 1987). The addition of poly-N-vinyl-pyrrolidone together with DMSO has been reported to raise the survival ratio (Kuwano *et al.*, 1996).

Now an example in which glycerol was used as a cryoprotectant will be cited. The experimental material 'Narawasusabinori' (*Porphyra yezoensis* form. *narawaensis*) added 10% glycerol seawater was introduced into a cryotube, then it was treated with pre-freezing till −40℃ at the cooling rate of 2℃

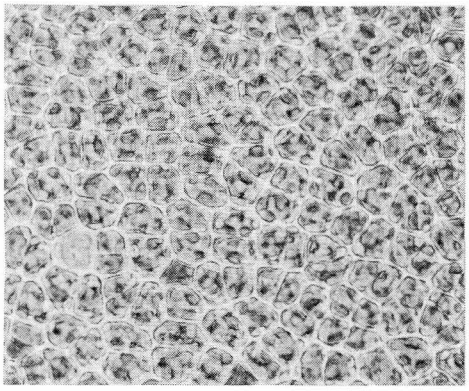

Fig. 2. Thallus of *Porphyra yezoensis* form. *narawaensis* after storage in liquid nitrogen for 9 years. (Yamasaki, unpublished)

Table 1. Contents of cryoprotective solution of conchocelis of *Porphyra yezoensis* form. *narawaensis*.

Dimetyl sulfoxide (DMSO)	2.25 M (1.50 M*)
Sorbitol	0.80 M (0.53 M*)
KCl	7 mM
Tris (hydroxymetyl) aminomethane	25 mM
pH	8.0 (8.0*)

This solution was mixed with the culture medium containing conchocelis in a ratio of two to one. *value after mixed with the culture medium.

per minute by use of a programme-freezer. The material thus treated was preserved by immersing in the liquid N_2. After presrved for 9 years, it was thawed with shaking in the warm water of 40℃, and the result showed that most of the cells were alive (Fig. 2, Yamasaki, unpublished).

5. Freezing resistance and drought resistance of filamentous thalli

During the 1960's, studies which aimed mainly at the control of conchospore release were conducted. Based on the results of preservation tests of oyster shell filamentous thalli in the 0℃ seawater, it was reported that the preservation for about 2 months was possible (Ouchi, 1962). Following this study, Kurakake (1969), Migita (1967) and Shimo *et al.* (1967) tested further the preservation in low temperatures. According to the results obtained by Migita (1967), conchosporangia, particulary matured conchosporangium cells showed a strong freezing resistance, and thus was able to produce a large amount of conchospore even after the preservation for about one month in the range of −5℃～−20℃. Kurakake (1969) and Shimo *et al.* (1967) obtained similar results to that of Migita (1967). The filamentous thalli themselves (vegetative shoots), however, decreased the survival ratio as the days of preservation went on. The results obtained by Migita (1967) showed that in case of the preservation at −20℃ the survival ratio after 5 and 20 days were about 35% and about 15% respectively.

It is known that the filamentous thallus lives in non-exposing zones in low tide, and thus is vulnerable to desication (Kurogi and Hirano, 1955). For that reason, it was unable to get satisfactory results even when cryopreservation was tried under dehydrated conditions (Migita, 1967; Shimo *et al.*, 1967).

In recent years, preservation in the range of −30℃～−85℃ with the additon of cryoprotectants. Such as DMSO etc. was tested. As the results of the tests it was found that while high survival ratios were obtained just after freezing, survival ratios decreased even in the temperature range as the days of preservation went on (Tsuchiya, 1992; Kuwano *et al.*, 1992).

6. Cryopreservation of filamentous thalli in liquied N_2

The authors have conducted studies on the preservation at a super low-temperature, i.e. in liquid N_2 (−196℃). In the following part, the method the authors used will be described (Fujiyoshi *et al.*, 1993a, b) The material, filamentous thalli of 'Narawasusabinori' (*Porphya yezoensis* form. *narawaensis*) used for the experiment was the ones cultured for about one month after minced. In this case, as in case of higher plants (Sakai, 1992), it is likely to be possible to get satisfactory results if the material which is in the stage of active cell division is used. At first, a cryoprotectant solution was added to a filamentous thallus containing culture solution in the ratio of 2 : 1 (Table 1). This was poured into a cryotube and treated with the programme-freezer using pre-freezing which

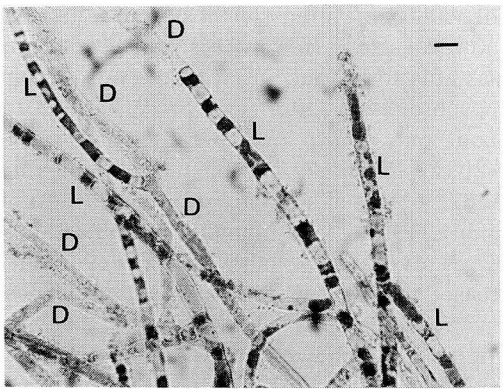

Fig. 3. Filamentous thallus of *Porphyra yezoensis* form. *narawaensis* treated with neutral red after storage in liquid nitrogen for 12 months. L, live cells; D, dead cells. (Fujiyoshi *et al.*, 1993a)

cooled down to −40℃～−50℃ at the cooling rate of 1℃ per minute, and then it was preserved by immersing in liquid N_2. When it was thawed in the warm water of 40℃, it was observed that about a half of cells were alive (Fig. 3). And besides, when it was treated, before freezing, with pre-culture in a culture solution raised its osmotic pressure by adding sorbitol, the survival ratio after it was thawed was improved (Fujiyoshi *et al.*, 1993 c).

In the preservation in liquid N_2, no reduction of survival ratios was observed even in the thawing after about one year preservation (Fujiyoshi *et al.*, 1993a; Kuwano *et al.*, 1993; Tsuchiya, 1994). Also, in case of the experiments where *P. tenera* Kjellman, *P. pseudolinearis* Ueda, *P. dentata* Kjellman and *P. haitanensis* Chang et Zheng were used as materials, survival ratios from 58.1 to 70.5% were obtained (Kuwano *et al.*, 1994). From these findings, it became evident that the very low-temperature preservation in liquid N_2 was an effective method as one of the long-term preservation methods of the *Porphyra* filamentous thalli.

Recently, some outcomes have been reported with respect to a simple freezing method and a Vitrification method in which no programme-freezer is used (Fujiyoshi, 1993; Tsuchiya, 1994; Kuwano *et al.*, 1994; Fujiyoshi *et al.*, 1995).

As mentioned above, it became possible to preserve both the thalli and filamentous thallus in the very low-temperature in liquid N_2. This method is expected to be used not only as a preservation method of the *Porphyra* lines but also as a long-term preservation method of experimental materials. But, some problems, such as different survival ratios among lines (Kuwano *et al.*, 1994) and so on, remains for its practical applications. Thus, further studies have to be conducted to establish simple and reliable preservation methods.

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アマノリ属の凍結保存

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アマノリ属の葉状体を半乾燥状態にした後，−20℃程度で凍結保存する方法が 30 年ほど前に開発され，現在では養殖生産に欠くことができないものとなっている．近年，遺伝資源保存の観点から低温保存研究が再開され，葉状体だけでなく糸状体についても液体窒素中 (−196℃) での超低温保存が可能になってきた．