Nat. Hist. Res., Special Issue No. 3: 89–95. March 1997.

Diseases of 'Nori (*Porphyra*)' and Countermeasures with Special Reference to the 'Anaaki (hole-forming)' Diseases

Hitoshi Tsuchiya[1] and Masahiko Miyata[2]

[1] Chiba Prefectural Fisheries Experiment Station
2492 Hiraiso, Chikura-machi, Awa-gun, Chiba 295, Japan
[2] Natural History Museum and Institute, Chiba
955–2 Aoba-cho, Chuo-ku, Chiba 260, Japan

Abstract Industrial damages of Nori (*Porphyra*) cultivated occurred as the result of 'Anaaki (hole forming)' disease, formation of a large number of holes in thalli and their losses in tidal current in the period from 1980 to 1983 as well as in 1987 over a wide range of Nori cultivation fishing grounds from Chiba Prefecture to Mie Prefecture in Japan. As a results of tests; infection tests and treatment tests with antibacterial agents, isolation of microbes from the affected parts, symptom-developing tests by the isolated microbes, the pathogenic microbe was identified as *Flavobacterium* sp. The colonies were round and flat in shape, and wet in appearance, and produced water-insoluble yellow pigments, and besides showed agar-decomposing ability, and thus the central part of the colony showed a crystalloid. The properties of the microbe were found gram-negative, no flagellum, no spores, oxidase-positive, catalase-positive, non-motile and short-long (20–500 μm) rod. Based on the results of sugar assimilation tests, it was conjectured that the bacteria assimilated an intercellular substance of 'Nori', 3,6-anhydro-L-galactose, by the exoenzyme. The optimum temperature for the growth of the bacteria was in the range of 13–21℃, and thus was low, and colony formations were not observed when the culture media contacted for a period of 24–36 hrs in 26℃ or 27℃. In the tests on salinity range for growths, no difference was observed in colony formation in the range of 12–20‰ of chlorine contents. As for the pH range for growths, colony formation was good in the range of pH 7.6–8.4, and it was ritorded at pH 7.2 and no colony formation was observed below pH 6.8.

Diseased thalli recovered from the disease by immersing the thalli for 5 min in the citric acid containing (0.05%) seawater (pH 3.5). It was possible to isolate the bacteria from the thalli frozen at −20℃ after air-dried. As countermeasures for 'Anaaki' disease, these two treatments are effective. In the culturing season of cool summer years, the risk of remarkable incidences of the disease seemed to become high.

Key words: Nori aquaculture, *Porphyra yezoensis* f. *narawaensis*, 'Anaaki (hole forming)' disease, *Flavobacterium*, 3,6-anhydro-L-garactose, countermeasures, Japan.

'Nori (*Porphyra*)' thallus diseases are classified into infections diseases in which causative organisms are identified or unidentified, as well as physiological disorders. The infectious diseases in which causative organisms are identified include following five; i.e. red rot disease and chytrid blight caused by fungi, green spot and filament bacterial felt caused by bacteria and 'Anaaki (hole-forming)' disease (Plate VI). As for physiological disorders, shot hole disease, white rot disease, but blight, tumour, 'Chirimen' disease, chill blight, diatom felt disease, 'Barikan' disease, 'Meochi' disease and so on are listed, but the relations btween these disorders and environmental factors are mostly unclarified.

With respect to studies on pathogenic microorganisms connecting with Nori diseases, following reports were published; Arasaki (1960), Japanese Society of Fisheries Science (1972), Saito *et al.* (1972) and Tsuchiya (1984) especially on 'Anaaki' disease. The newly observed 'Anaaki' disease, by which

the 'Nori' thallus is bored in a number of portions and lost by tidal current occurred from 1980 to 1983 and 1987 over a wide range of fishing grounds from Chiba Prefecture to Mie Prefecture. The disease occurred at first around the middle or late October, seedling raising time, and the 'Nori' with large affected parts did not lead to production in most cases; and even when production was possible, the yield per net reduced, and thus the reduction resulted in industrial damages. The symptom appeared notably until the end of year and after March as regards season, and in 'Futtsu' area in respect of region.

As the cause of the disease was not clear and no countermeasure was available, strong requests for making clear the cause were presented by aquaculture business societies.

The purpose of this study is to clear the features of 'Anaaki (hole-forming)' disease in order to stabilize production in aquaculture industries.

Materials and Methods

Experiments were conducted concerning following three points; i.e. confirming whether the ulcer disease is an infectious disease or not, separation of pathogenic microbes as well as elucidation of their properties, and establishing countermeasures against the disease.

In the confirmation test, it was checked whether the samples were infected or not, as well as the effect of treatments by antimicrobial agents was tested.

Infection tests

Disease thalli collected from 'Shin-Futtsu' fishing ground and 'Narawasusabi-nori (*Porphyra yezoensis* f. *narawaensis*)' seedling thalli raised from spores collected in a room were placed in flat-bottom flasks and examined whether they were infected or not. As a control plot, a flask in which no disease thallus was placed was also set up.

Culture solution was prepared as follows; at first, 500 times concentration solution of provasoli's ES medium (modified) was prepared, then it was added to seawater, which was heated for 30 min at 80℃ and left to cool, at the rate of 2 m*l*/*l* (The seawater is called 'sterilized seawater' in the following part).

Infection was conducted by aerated culture in a constant temperature room under the conditions set to 12 hour-light period, 3000 Lux illumination and 18℃.

Treatment test

Treatment chemical, water soluble 'Aipet' for fishery (containing 6.666% of Nifurubrazine-hydrochloride), was added into the culture solution at the rate of 1 ppm in terms of the effective constituent. After the disease thallus was cultured for 3 days in this solution, it was transferred to the solution without addition of the chemical.

The treatment effect was estimated by observing the degree of expansion of affected parts and regeneration of cell walls etc. on the 5th and 7th day after the test started.

Isolation test of pathoginic microbes

The thallus samples for isolation were prepared by infection of healthy thalli, which were raised from collected spores in a room, through collected disease thalli in fishing grounds. Thus, disease thalli were provided when it required by making healthy thalli infect with the disease.

Medium used for isolation was ZoBell 2216 E agar plate medium adjusted its pH to 7.8–8.2 by NaOH.

Isolation of pathogenic microbes were conducted as follows; a thallus was spread on a medicine powder wrapping paper, then an affected part by the disease was cut out in a ring-shape. A disk thus prepared was cleaned several times by using the sterilized seawater, then 10–20 petri dishes per one disk were streaked by drawing around the disk on culture media in the dishes. After these plate media were cultured at 18℃ for 7–10 days, a number of appeared colonies were distinguished each other according to the color and morphology under the microscope, and each strain was numbered and used as sample strains.

The comfirmation of pathogenicity was conducted as follows; i.e. healthy thalli grown from the collected spores in a room were placed in culture-solution containing petri dishes (a diameter of 9 cm), and then each thallus was inoculated with 2–3 ooze of above-mentioned microbes. These petri dishes were allowed to stand in a constant temperature room of 18℃ and examined

under the microscope every other day for about a week for the incidence of diseases.

Properties tests of pathogenic microbes

Tests were conducted for following items; i.e. gram stain, flagellum stain, carpospore stain, oxidase test, catalase test, motility of microbes by soft agar medium.

Sugar assimilation tests

As pathogenic microbes had decomposing ability of agar, *k*-carrageenan was added at the rate of 2% to the Marine Broth 2216 (Difco Co.). Then, glucose, galactose, mannitol, maltose, lactose, sucrose, inositol, xylose and galactan (each 1%), cellulose, starch and agar (each 0.2%) were added and solidified. After they were inoculated with microbes, they were cultured at 18℃.

Growth conditions tests

The range of growing temperature, tolerance to temperature and pH range for growth were examined by use of Marine Agar 2216 medium (Difco Co.) and salinity range for growth was examined by use of ZoBell 2216 E agar plate medium.

The range of growing temperature was examined according to the following way; microbes were smeared on pH adjusted plate media, and then they were cultured under the temperature range of 5℃–26℃—the temperatures between 5℃ and 21℃ were set at intervals of 2 degrees but those between 23℃ and 26℃ were set at intervals of 1 degree—and the formation of colonies was examined.

The test on tolerance to temperature was as follows; microbes were inoculated on slant media in 12 test tubes. Then, each 6 test tubes were kept in each water adjusted to 26℃ and 27℃. After they were kept for 0, 6, 12, 24, 36 and 48 hrs, they were transferred to a constant temperature room of 18℃, and the formation of colonies was examined after a week.

The pH range for growth was tested by culturing microbes at 18℃ after inoculated on the media adjusted the pH to 6.4, 6.8, 7.2, 7.6, 8.0 and 8.4 by using NaOH.

The salinity range for growth was tested as follows; seawater was adjusted its chlorine content to 12, 14 and 16‰ by diluting with distilled water, and also it was concentrated by heating and adjusted to 18 and 20‰. The ZoBell 2216E agar plate media were prepared by use of these seawater and they were cultured at 18℃ after microbes were inoculated on them.

Exoenzymes tests

When microbes were cultured on the ZoBell 2216E seawater agar media, the colour tone of media of colony peripheries turned transparent. A part of the media, which was away from the colonies and was transparent, was cut off and placed in seawater containing petri dishes together with thalli free from the disease, and then the petri dishes were allowed to stand for one day. Then, they were examined whether the cell walls were decomposed or not. A similar test was conducted by using filamentous thalli.

Countermeasures against desease injuries

As the growth of microbes was not observed in the low pH range, treatments for diseases by applying the acidic treatment technique, which was developed in order to eradicate *Enteromorpha*, were tried. The method was carried out as follows; thalli affected by diseases were immersed for 10 min in sterilized seawater to which citric acid was added at the rate of 1%. After the thalli were cleaned with the sterilized seawater and cultured on the ES media for 3 days under aeration, they were examined for the treatment effect. The effect was also examined for the case when the citric acid concentration was 0.05% and the immersion time was 5 min. In these tests, a non-treatment plot was prepared as the control plot.

In addition, drying and freezing which have been practiced in the countermeasure against red rot disease were tested for the effect on ulcer disease according to the procedure described in the next clause. Thalli affected by the disease were air-dried in a constant temperature room adjusted at 18℃, and then, they were kept in a freezer of −20℃. After 10 days, the thalli were thawed in the sterilized seawater and the isolation of microbes was tried according to the conventional method.

Results

Infection tests

From the 2nd day after the test started, a hole-forming symptom as 'Anaaki' disease, was visually observed in the thalli of infected

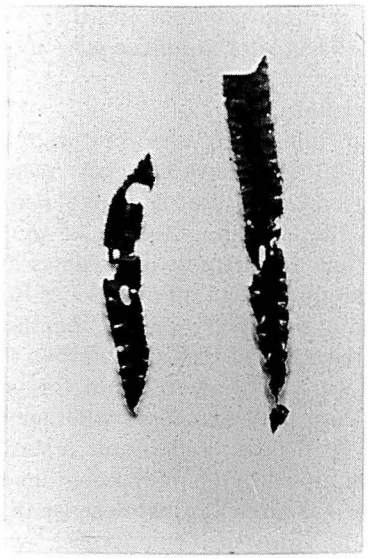

Fig. 1. The thalli in infected plot, which is from collected spores in a room, indicate the symptom of 'Anaaki (hole forming)' disease in comparing with thallus in controle spot after 3rd day in infection tests.

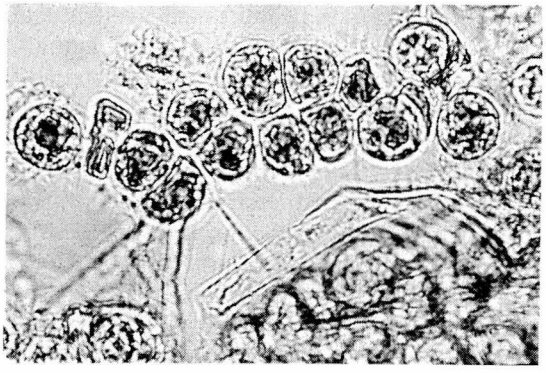

Fig. 2. In infected thalli, cell walls remained and at the peripheral outside 3–4 layers of globular cells surround affected parts, and some of the cells were fallen from the thalli.

plot which were raised from collected spores in a room and the holes were enlarged on the 3rd day, while the symptom was not observed in the control plot (Fig. 1).

In the affected part by infection showed similar symptoms to those of the thallus affected by disease; namely, cell walls remained in those thalli as shown in Fig. 2, and 3–4 layers of globular cells which surrounded outside the periphery of affected parts, and besides some of the cells which were fallen from the thalli were observed.

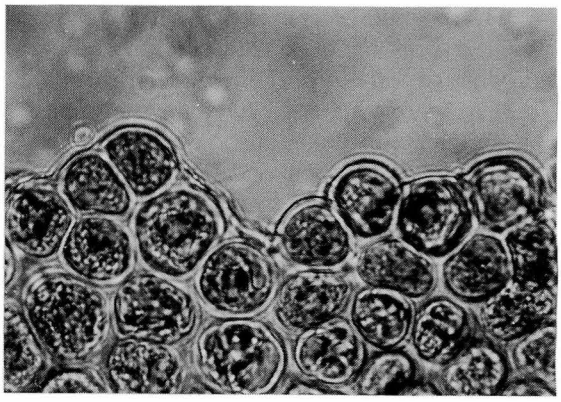

Fig. 3. After 7 day in infection tests, the regeneration of cell walls was recognized in affected parts by 'Anaaki (hole forming)' disease.

Treatment tests

While the shape of thalli became unrecognizable in the control plot on the 5th day after the test started owing to the enlarged affected parts, no extension of affected parts was observed in the treatment plot, and besides, the regeneration of cell walls, recovering from the disease, was recognized on the 7th day as shown in Fig. 3.

Isolation test of pathogenic microbes

From the results of isolation tests by using affected parts and repeated infection tests with the isolated microbes, the microbe which caused similar symptoms to those observed in fishing grounds was identified. The microbe isolated from the thalli which developed symptoms showed a similar morphology of colony to that of the strain used for infection. The colony forming rate of the microbe was slower than those of other microbes appeared on the ZoBell 2216E seawater agar medium, therefore hooking of the microbe was carried out after the morphology of colonies was confirmed by use of the microscope of 40 magnification. The microbe was subcultured every 3 weeks and the strain was maintained, because its multiplication became unable to keep if it was not subcultured for a longer period than one month.

The symptom of disease caused by the infection of the microbe was recognized on the 2nd day as globing in a part of 'Nori' cells, and after about one week, it extended to

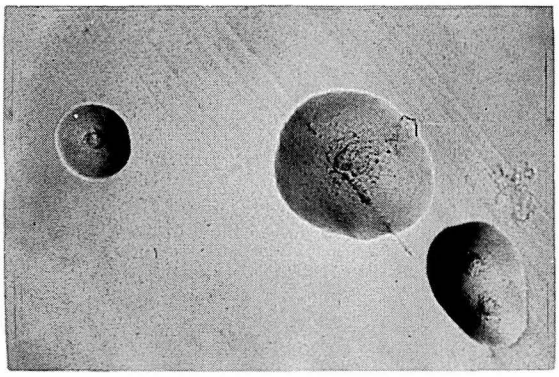

Fig. 4. On the ZoBell 2216E seawater agar medium, the colony was morphologically recognized as round, flat and wet in appearance, and it produced water-insoluble yellows pigments, and showed agar-decomposing ability. Furthermore, the central part of the colony appeared to be crystalloid.

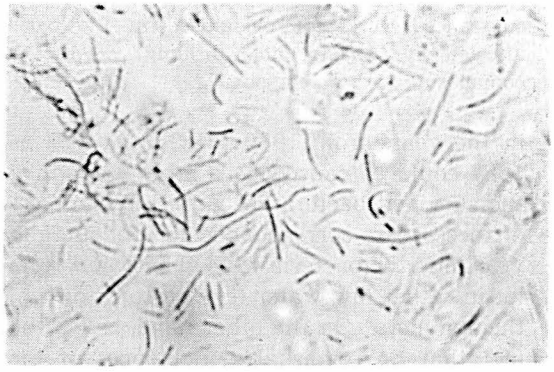

Fig. 5. The pathogenic microbe was morphologically recognized as short-long (20–500 μm) rod.

whole of the thallus.

The morphology of colony on the ZoBell 2216E seawater agar medium was characterized as follows; it was round, flat and wet in appearance, and it produced water-insoluble yellows pigments, and besides showed agar-decomposing ability, and thus the central part of the colony appeared to be crystalloid (Fig. 4).

Properties tests of the pathogenic microbe

The microbe was gram-negative, no flagellum, no spore, oxidase-positive, catalase-positive, non-motile and short-long (20–500 μm) rod one as shown in Fig. 5.

Sugar assimilation tests

It was found that the colony formation was good on the media to which glucose, galactose and galactan were added.

Growth conditions tests

The test on the range of growing temperatures showed that colony formations were observed in the range of 9–24 or 25℃ and the temperature range of 13–21℃ was the optimum growth temperature. The colony formation was slow below 7℃ and was not observed at 26℃.

In the test on tolerance to temperature, no colony formations were observed when the inoculated media were contacted for more than 24–36 hrs in both 26℃ and 27℃ plots.

As for the pH range for growth, colony formation was good in the range of pH 7.6–8.4, but it was slow at pH 7.2 and was not observed below pH 6.8.

As for the salinity range for growth, colony formation was observed over the range of chlorine contents of 12–20‰ and the rate of formation did not differ in this range.

Exoenzymes tests

The cells of thalli were disconnected after one day by the decomposition of intercellular substances, and no microbe was found in the transparent parts of cell walls as shown in Fig. 6. The cell wall decomposition was not observed in filamentous thalli.

Countermeasures against disease injuries

In the thalli affected by the disease which were immersed for 10 min in the seawater contained 1% citric acid, extension of the affected parts was hardly observed, while in the control plot, the affected parts were

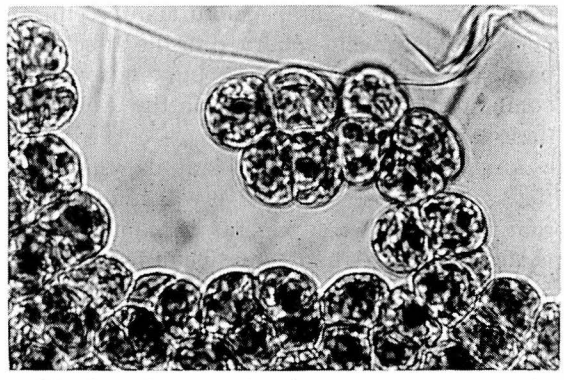

Fig. 6. After one day in exoenzymes tests, the microbe was not recognized in the transparent parts of cell walls in going with disconection by the decomposition of intercellular substances.

extended. Thus, the treatment effect by use of citric acid was confirmed. In addition, the diseased thalli immersed for 5 min in the seawater contained 0.05% citric acid (pH 3.5) recovered from the disease as well.

Isolation of the microbe from thalli frozen at −20℃ after air-dried was possible, so that the microbe turned out not to die by drying or freezing treatments.

Discussion

The 'Anaaki (hole-forming)' symptom was due to a bacterial infectious disease. The colonies formed by the bacteria were round and flat in shape and wet in appearance; and besides, produced water-insoluble yellow pigments and showed agar-decomposing ability, and the central parts were looked like crystalloids.

The properties of the bacterium were proved to be gram-negative, no flagellum, no spore, oxidase-positive, catalase-positive, non-motile and short–long (20–500 μm) rod, consequently it was conjectured that the bacterium was one of the *Flavobacterium* bacteria.

From the results of sugar assimilation tests, it was conjectured that the bacterium assimilated an intercellular substance, 3,6-anhydro-L-galactose, by its exoenzyme. Such conjecture seemed to be supported by the following findings; i.e. no dead cells were observed in the cells of affected parts of diseased thalli found in fishing grounds, cell walls which seemed to be mannan and xylan ramained, and furthermore cell walls remained and honeycomb structural intercellular substances were observed in the affected parts by green spot (Fig. 7) but no honeycomb structure was observed in the 'Anaaki (hole-forming)' disease.

And besides, from the fact that filamentous thalli were not decomposed, it was suggested that the cell wall components of filamentous thalli and foliose thalli could be different.

The optimum temperature for the growth of bacterium was the low-temperature of 13–21℃, and the colony formation was not observed when the culture media were contacted for a period of 24×36 hrs at 26℃ or 27℃. From this result, it was conjuctured that the remarkable incidences of the disease

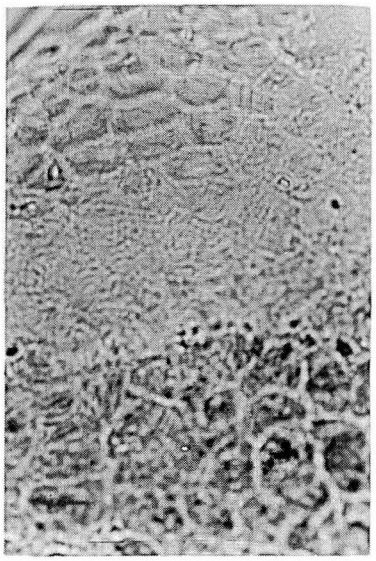

Fig. 7. In sugar assimilation tests, cell walls remained and honeycomb structural intercellular substances were not recognized in the parts affected by 'Anaaki (hole forming)' disease in comaparing with green spot.

in the years from 1980 to 1983 as well as 1987 could be caused by a large amount of the survived bacteria, because the summers of these years corresponded to cool summer years and the bacteria were able to survive a lot in rather low water temperatures during the summers. On the other hand, as for the fact that no remarkable incidences of the disease were observed in the period from 1984 to 1986, it was conjectured that the incidences of the disease were scaled down owing to a small amount of the survived bacteria resulted from the almost normal or abnormally high temperatures in the summers of these years. In addition, the reason for remarkable incidences of the hole-forming disease in 'Futtsu' area was guessed that the transition of summer water temperature was rather lower than in inland areas. These facts suggested that the bacteria died because of the lack of spores when they encountered high temperatures.

The time of incidence of the disease in fishing grounds almost coincided with the optimum temperature range for the growth of bacteria. Thus, the fact that production damages were liable to occur within the year when water temperature indicated above

12℃ and after March corresponded to water temperature rising period was reasoned to be ascribed to the fast multiplication rate of the bacteria.

From the results of the tests on salinity range for growth, no differences were found in the colony formation in the range of 12–20‰, consequently it was conjectured that the extension of disease damage caused by river water and rainfall, which was observed in red rot disease, would not occur.

From the results of the tests on pH range for growth in which no colony formation was observed below pH 6.8, it was conjectured that recovery from the disease was possible by contacting the diseased thalli with the pH for a long time, whereas procedures which are able to be completed in about 10 min are required in the fields of aquaculture from the aspect of workability.

Thus, it is considered that acid treatment techniques were practically available as a countermeasure; to avoid the damage in aquaculture, because 5 min immersion in the 0.05% citric acid containing seawater (pH 3.5) was able the recover the thalli from the disease, and storage in refrigerators which have been used as countermeasures against red rot disease were found ineffective because of the ineffectiveness in killing the bacteria, and thus it was considered that the prediction of the degree of incidences of ulcer disease and the countermeasure was almost established.

Acknowledgements

Authors express our sincere appreciation to the staffs of Chiba Prefectural Fisheries Experiment Station, who provided useful information and referneces on carrying out this study on *Porphyra* diseases.

References

Arasaki, S. 1960. A chytridean parasite on the *Porphyra*. Bull. Jap. Soc. Sci. Fish. 26(6): 543–548. (In Japanese)

Japanese Society of Fisheries Science. 1972. Nori (*Porphyra*) diseases. 147 pp. Koseishakoseikaku Inc. Tokyo. (In Japanese)

Saito, Y., T. Matsuato and K. Yoshikawa. 1972. On the syptoms of 'Green spot' and 'Crape' in Nori (*Porphyra*) culture. Bull. Nansei Nat. Reg. Fish. Res. Inst. 5: 1–9. (In Japanese)

Tsuchiya, H. 1984. On 'Anaaki' diseases of *Porphyra*, as a tentative name. Bull. Chiba Pref. Exp. St. 42: 67–71.

Tsuchiya, H. 1992. 'Anaaki' diseases. *In* Fisheries agency (eds.), On Unity of the Name of Nori (*Porphyra*) Diseases, pp. 42–49. Fisheries Agency, Tokyo. (In Japanese)

ナラワスサビノリの“穴あき症”と対策

土屋　仁[1]・宮田昌彦[2]

[1] 千葉県水産試験場
〒295 千葉県安房郡千倉町平磯 2492
[2] 千葉県立中央博物館
〒260 千葉市中央区青葉町 955-2

養殖されたナラワスサビノリ (*Porphyra yezoensis* f. *narawaensis*) の藻体に穴があき，藻体が流失してしまうノリの病気，“穴あき症”が 1980 年から 1983 年と 1987 年に千葉県から三重県に至る広範囲のノリ養殖漁場で発生した．感染試験，抗菌剤治療試験，分離菌の発症試験等の結果，原因菌はグラム陰性，無鞭毛，無芽胞，オキシダーゼ陽性，非運動性，カン菌（20～500 ミクロン）等の特性をもつ *Flavobacterium* 属の細菌であり，ノリの細胞間隙物質である3.6-アンヒドロ-L-ガラクトースを菌体外酵素で資化することにより“穴あき症”を発症させることがわかった．また，この細菌は，塩素量 12～20‰，pH 7.6～8.4，温度は 13～21℃ の培養条件で最も高い活性を示してコロニーを形成した．そして，“穴あき症”は，冷夏であった年に発生すること，感染した藻体をクエン酸 0.05% を添加した海水 (pH 3.5) に 5 分間浸すと“穴あき症”が消失することがわかり本研究の結果は，ナラワスサビノリの養殖における“穴あき症”対策として有効であることがわかった．

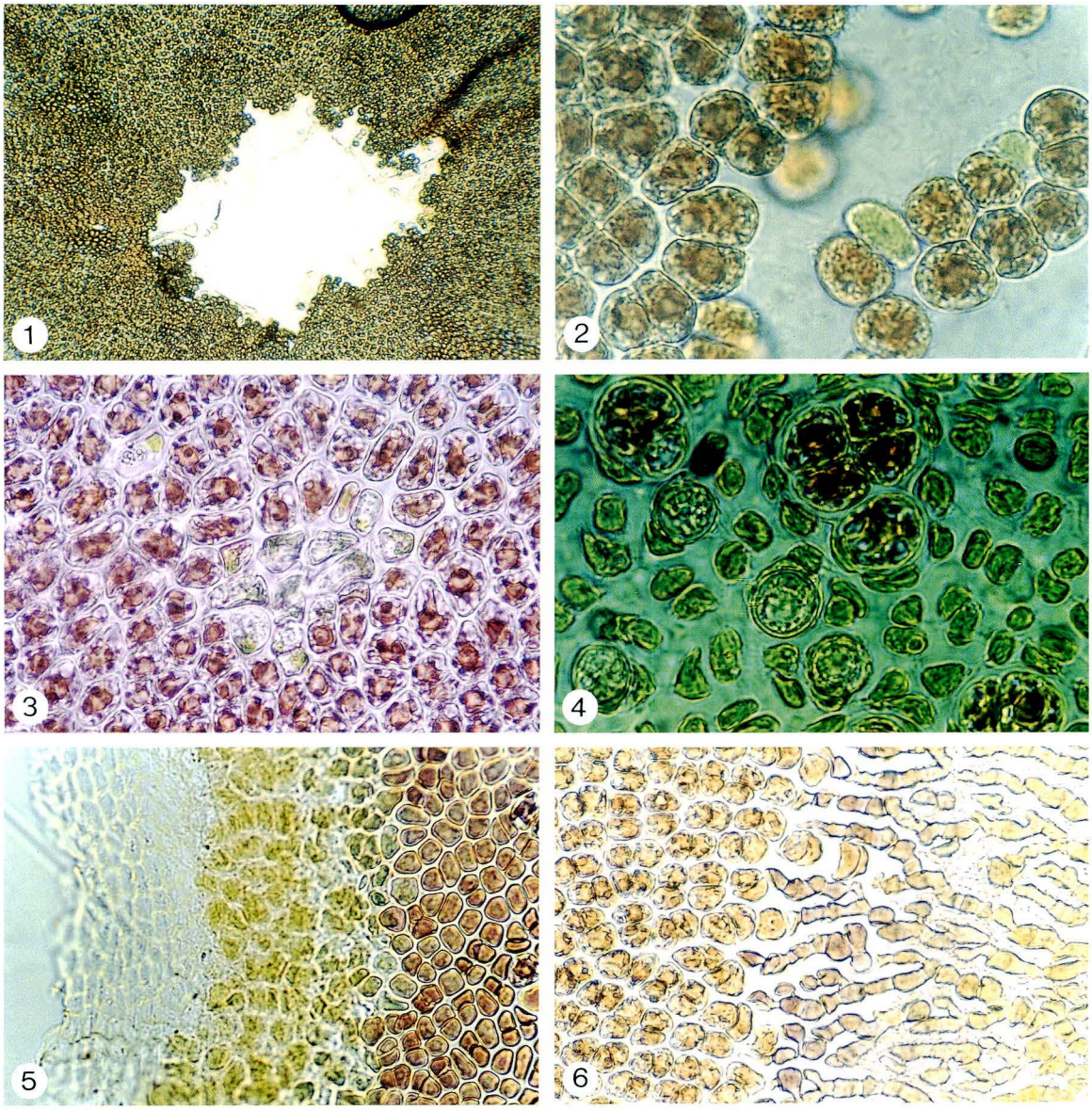

Fig. 1. "Anaaki-byo" (Anaaki (hole forming) disease).
Fig. 2. The enlarged cells at marginal area of hole.
Fig. 3. "Tsubojokin-byo" (Chytrid blight).
Fig. 4. "Akagusare-byo" (Red rot disease).
Fig. 5. "Ryokuhan-byo" (Green spot disease).
Fig. 6. "Ryokuhan-byo" (Green spot disease).