Rijksdienst voor Ondernemend
Nederland

OCTROOINUMMER 2028946

Octrooicentrum Nederland verklaart dat op grond van octrooiaanvraag 2028946 , ingediend op 10 augustus 2021 octrooi is verleend aan:

INSTITUTE OF OCEANOLOGY, CHINESE ACADEMY OF SCIENCES te Qingdao City, China

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Voor:	CRYOPROTECTIVE SOLUTION AND ULTRA-LOW TEMPERATURE CRYOPRESERVATION METHOD OF STICHOPUS JAPONICUS SPERM

Een recht van voorrang werd ingeroepen, gebaseerd op octrooiaanvraag: 202011022462.5, ingediend op 25 september 2020 in China.

Aan dit bewijs is een exemplaar van het octrooischrift gehecht met nummer 2028946 en dagtekening 16 juni 2022.

De maximale beschermingsduur van dit octrooi loopt tot en met 9 augustus 2041.

Uitgereikt te Den Haag, 16 juni 2022

De Directeur van Octrooicentrum Nederland,

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(19)

Octrooicentrum Nederland

(11) **2028946**

(12) A **OCTROOIAANVRAAG**

(21) Aanvraagnummer: **2028946**

(22) Aanvraag ingediend: **10 augustus 2021**

(51) Int. Cl.:
A01N 1/02 (2021.01)

(30) Voorrang:
25 september 2020 CN 202011022462.5

(41) Aanvraag ingeschreven:
24 mei 2022

(43) Aanvraag gepubliceerd:
25 mei 2022

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(54) **CRYOPROTECTIVE SOLUTION AND ULTRA-LOW TEMPERATURE CRYOPRESERVATION METHOD OF STICHOPUS JAPONICUS SPERM**

(57) The invention relates to the technical field of ultra-low temperature cryopreservation of stichopus japonicus germplasm, in particular to a cryoprotective solution, an activator formula and an ultra-low temperature cryopreservation method of apostichopu japonicus sperm based on a program controlled freezer and CASA. The cryoprotective solution is composed of a diluent, an antifreeze, and an additive; wherein the diluent is natural seawater, the antifreeze is one or more of aprotic polar solvents, and the additive is glucose. The method of the present invention has a large amount of frozen storage (more than 200 mL of fresh stichopus japonicus semen can be cryopreserved in one batch), can precisely control the cooling process, and has a good application effect after thawing. It can solve the problem of unsynchronized maturation of male and female sea cucumbers and a series of germplasm degradation of stichopus japonicus in actual production.

Deze publicatie komt overeen met de oorspronkelijk ingediende stukken.

CRYOPROTECTIVE SOLUTION AND ULTRA-LOW TEMPERATURE CRYOPRESERVATION METHOD OF STICHOPUS JAPONICUS SPERM

TECHNICAL FIELD

[01] The invention relates to the technical field of ultra-low temperature cryopreservation of sea cucumber germplasm, in particular to a cryoprotective solution, an activator formula and an ultra-low temperature cryopreservation method of apostichopu japonicus sperm based on a program controlled freezer and CASA.

BACKGROUND ART

[02] Apostichopus japonicus, also known as stichopus japonicus, belongs to echinodermata, holothuroidea, aspidochirotida, stichopodidae, apostichopus, natural habitat in the northern part of the Western Pacific, including the Far East coast of Russia, the coasts of Japan and South Korea, and the Yellow Sea and Bohai Sea of China. It is the most common marine higher invertebrates. Stichopus japonicus is a nourishing product with the homology of medicine and food. Among the more than 20 edible sea cucumbers distributed in the seas of China, the nutritional quality and economic value are the highest. Its health and medical value has been widely recognized since ancient times. As the market demand for stichopus japonicus and the scale of the industry have expanded year by year, the over-harvesting of wild stichopus japonicus has become more and more serious, resulting in a sharp decline in the resource quantity and germplasm quality of wild stichopus japonicus. Stichopus japonicus have been assessed as endangered (EN) species by the Red List of Threatened Species of the World Conservation Union (IUCN Red List of Threatened Species or IUCN Red List); cultured sea cucumbers have also experienced a series of germplasm degradation problems such as frequent disease, low survival rate, and slow growth rate, and the frequent occurrence of stichopus japonicus diseases causes about 3 billion yuan in economic losses every year, causing heavy losses to the stichopus japonicus industry and becoming an important bottleneck restricting the healthy development of stichopus japonicus breeding. The offshore environment has an impact on the survival rate, quality, and yield of cultured sea cucumbers. Sea cucumber breeding itself has an impact on the marine environment, resulting in increasing

environmental pressure, and may even cause the seed ginseng to fail to lay eggs, sperm, or insufficient vitality of sperm and eggs, leading to the serious consequences of not being able to breed. For example, in the summer of 2018, the seashore was raging with high temperatures, and the continuous high temperature weather was earlier than in previous years and lasted longer. Cofferdam cultured sea cucumbers in Liaoning, Shandong and other places died under the influence of high temperature and high humidity, and economic and germplasm resources were severely lost. At present, researchers mostly use the method of hybridization between southern and northern sea cucumbers, which are geographically isolated, to improve the germplasm quality of stichopus japonicus. However, because the male and female sea cucumbers in the north and south do not mature at the same time, it is necessary to use methods such as ripening and ovulation promotion in the process of sea cucumber hybridization. Not only the operation is complicated and the cost is high, but there are also problems such as the failure to achieve hybridization due to the failure of ripening and ovulation promotion. Therefore, it is urgent to solve the problem of preservation of high-quality germplasm resources of stichopus japonicus.

[03] The currently published papers or patents on the cryopreservation method of stichopus japonicus sperm adopt the "two-step" method of cooling (controlling the height of the sample from the liquid nitrogen surface to control the cooling rate) and visual observation of sperm motility with a microscope. There are human subjective factors in these two modes of operation, and the accuracy of the results is greatly affected by the cooling device and the counting device. In the papers published by Shao et al. in 2006 (Shao MY, Zhang ZF, Yu L, et al. Cryopreservation of sea cucumber apostichopus japonicus (Selenka) sperm[J]. Aquaculture Research,2006,37(14):1450-1457) and papers published by Yuta Mizuno et al. in 2018 (Mizuno Y, Fujiwara A, Yamano K, et al. Motility and fertility of cryopreserved spermatozoa of the Japanese sea cucumber apostichopus japonicus[J]. Aquaculture Research, 2018; 1-10.), the sea cucumber sperm is diluted with preservation solution and then cryopreserved. The preservation solution formula used is artificial seawater (423.00mM NaCl, 9.00mM KCl, 9.27mM $CaCl_2$, 22.94mM $MgCl_2$, 22.50mM $MgSO_4$), 10mM Hepes buffer and 15%-20% dimethyl sulfolide. Hepes buffer is not only expensive but also produces certain biologically toxic hydrogen peroxide when exposed to visible light. The operation time of Shao et al. is up to one hour, and the

loss of sperm motility is large. After thawing, the sperm life is only 1200s, which is difficult to apply to production; In the studies of Yuta Mizuno et al. and the patent filed in 2019 (Chang Yaqing, Zhan Yaoyao, Zhao Tanjun, etc., Sea cucumber sperm cryopreservation method [P].CN110326610A, 2019-10-15.), the survival rate of all processed sperm after thawing is less than 15%, and the sperm viability rate, especially the proportion of sperm capable of forward movement, is positively correlated with the fertilization rate and hatching rate. The higher the sperm motility after thawing, the better the long-term preservation of sperm and the actual production application. However, the process of the above patent documents is not clear enough, and it is greatly affected by the subjective factors of the operator, and at the same time, it is greatly affected by the freezing device. Using the methods in the above patent documents, the sperm of stichopus japonicus have no vitality after thawing.

SUMMARY

[04] The purpose of the present invention is to solve the above-mentioned problems in the prior art and to solve the problems of unsynchronized maturation of male and female sea cucumbers and a series of germplasm degradation of stichopus japonicus in actual production. Based on the program controlled freezer and CASA, the invention provides an ultra-low temperature cryopreservation solution formula, an activator formula and an ultra-low temperature cryopreservation method of apostichopus japonicus sperm.

[05] In order to achieve the above-mentioned purpose, the technical solution adopted by the present invention is:

[06] A cryoprotective solution, which is composed of a diluent, an antifreeze, and an additive; wherein the diluent is natural seawater, the antifreeze is one or more of aprotic polar solvents, and the additive is glucose.

[07] The said is dimethyl sulfoxide (DMSO) and/or dimethylacetamide (DMA).

[08] The cryoprotective solution is natural seawater, dimethyl sulfoxide (DMSO) and glucose, wherein the respective volume-to-mass ratio is 85mL-90mL: 10mL-15mL: 1.98g.

[09] The cryoprotective solution is natural seawater and dimethylacetamide (DMA), and the volume ratio is 80mL:20mL.

[10] The natural seawater is natural seawater filtered with a 0.45μm filter membrane;

dimethyl sulfoxide (DMSO) is dimethyl sulfoxide with a purity greater than or equal to 99.97%, dimethylacetamide (DMA) is dimethylacetamide with a purity greater than or equal to 99.97%, and both DMSO and DMA are aprotic high-polarity solvents.

[11] An ultra-low temperature cryopreservation method of stichopus japonicus sperm using the cryoprotective solution,

[12] (1) Semen acquisition: acquiring the sperm of stichopus japonicus in the breeding period and waiting for use;

[13] (2) Mixing semen with cryoprotective solution: mixing the fresh semen of stichopus japonicus and the cryoprotective solution in a container with a volume ratio of 1:5-1:7;

[14] (3) Program cooling: after mixing, placing it in a program controlled freezer, running the cooling program, the cooling program is to equilibrate at 0°C for 5 minutes, cool to -80°C at a cooling rate of 10-15°C/min, equilibrate at -80°C for 5 minutes, and cool to -180°C at a cooling rate of 20°C/min, and equilibrate for 5 minutes, and then taking out after 5 minutes of equilibration to realize ultra-low temperature cryopreservation of stichopus japonicus sperm.

[15] The sperm container after the cooling procedure is placed in liquid nitrogen for long-term storage.

[16] The cryopreserved stichopus japonicus germ is thawed in a 25°C water bath, shaken gently to make the temperature uniform, and when there is only a small amount of solids left, take it out immediately (about 120s), continue to shake it in the air to completely melt; pipette the frozen semen and mix it with the activator to make the total dilution rate of the original stichopus japonicas germ of 600 times, the activator is filtered natural seawater containing 5wt% of fetal bovine serum.

[17] The fresh semen of stichopus japonicus in step (2) is to dilute the collected sperm by 600 times with an activator, detect the sperm and collect the sperm with activity greater than 90%, and wait for use.

[18] The advantages of the present invention:

[19] The cryopreservation method of the present invention is based on two instruments, a program controlled freezer (Kasu Micro-Digitcool) and CASA (Myron ML-608JZ), which can accurately control the cooling rate and quantitatively analyze sperm vitality, and can quickly and mass cryopreserve sperm (more than 200mL of fresh semen can be cryopreserved in one batch); the operation time is short (the

operation is completed within 20 minutes after the sperm is dissected) to avoid the problem of large loss of sperm vitality; the preservation solution used is extremely low-cost, safe and non-toxic, and easy to prepare, and the effect is good; after the mixing of sperm activator and sperm, the sperm can be activated immediately to show high vitality, maintaining high vitality for more than 3 hours; after thawing and activation of frozen sperm, the sperm viability rate is as high as 60.07±5.88%, and the sperm rapid motility rate is as high as 43.39 ±5.88%, the sperm curve motility speed is as high as 103.51±15.46μm/s; it provides convenience for the protection of stichopus japonicus germplasm resources, the large-scale production of seedlings and genetic breeding research, and the data results are objective and credible, which is beneficial to the further detailed study of the ultra-low temperature cryopreservation method of stichopus japonicus sperm and its freeze-damage mechanism. The concept of using aprotic polar solvents in the cryoprotective solution is conducive to the development and selection of new antifreeze. In addition, the method of the present invention has a large amount of frozen storage (more than 200 mL of fresh stichopus japonicus semen can be cryopreserved in one batch), can precisely control the cooling process, and has a good application effect after thawing. It can solve the problem of unsynchronized maturation of male and female sea cucumbers and a series of germplasm degradation of stichopus japonicus in actual production.

DETAILED DESCRIPTION OF THE EMBODIMENTS

[20] The specific embodiments of the present invention will be further described below in conjunction with examples. It should be noted that the specific embodiments described here are only for illustrating and explaining the present invention, and are not limited to the present invention.

[21] The method of the present invention aims at the problems of low recovery rate and poor motility ability existing in the current ultra-low temperature cryopreservation of stichopus japonicus sperm. The present invention obtains two kinds of cryopreservation solutions suitable for ultra-low temperature cryopreservation of stichopus japonicus sperm and the cryopreservation resuscitation method (including one sperm vitality activation solution) by systematically studying the related factors of cryopreservation of stichopus japonicus sperm. The configuration method of preservation solution and activation solution is simple, convenient for storage, and has good effect; the cryopreservation method has large amount of frozen storage and short

operation time, which is suitable for mass application. It provides convenience for the protection of stichopus japonicus germplasm resources, the large-scale production of seedlings and genetic breeding research, which is beneficial to the further detailed study of the ultra-low temperature cryopreservation method of stichopus japonicus
5 sperm and its freeze-damage mechanism.

[22] Example 1

[23] a. Preparation of cryopreservation solution: according to the principle of current use and preparation, a fresh ultra-low temperature cryoprotective solution was prepared. The formula (1) of ultra-low temperature cryoprotective solution for
10 stichopus japonicus sperm was as follows: 85mL of natural seawater filtered with a 0.45μm filter membrane, 15mL of dimethyl sulfoxide (DMSO) with a purity ≥99.97%, and 1.98g of glucose; formula (2) was as follows: 80mL of natural seawater filtered with a 0.45μm filter membrane, and 20mL of dimethylacetamide (DMA) with a purity ≥99.97%. The prepared cryoprotective solutions were pre-cooled in a 4°C constant
15 temperature refrigerator until use.

[24] b. Acquisition of semen: stichopus japonicus in the breeding period was taken, and the ginseng body after discharging a large amount of water in the body cavity (to avoid seawater contact with sperm to activate it) was dried. The sea cucumber was dissected from the abdomen using a sterile scalpel to prevent the gonads from being
20 scratched. The complete gonads were picked out using a tweezer. The body fluid on the surface of gonads was adsorbed using an absorbent paper, then the gonads were transferred to a sterile culture dish, cut into pieces with scissors and filtered into a sterile 50ml centrifuge tube with a 300-mesh silk sieve, and placed in a constant temperature incubator at 4°C for use. At the same time, the vitality of fresh sperm was
25 detected, and only sperm with vitality greater than 90% can be used for freezing experiments. The procedure of sperm vitality detection was that 1198μl of activator (filtered natural seawater containing 5wt% of fetal bovine serum) and 2μl of fresh sperm were mixed, diluted by 600 times and activated, 10μl of the mixed solution was pipetted and added to the sperm counting plate, and placed in CASA to detect vitality.
30 **[25]** c. Mixing semen with cryopreservation solution: the fresh semen of stichopus japonicus and cryopretective solution were fully mixed according to a certain volume ratio and then dispensed into 2ml cryotubes. During the whole process, the cryotubes were stored in a cryotube box and placed in a thermal insulation box with ice. The

mixing volume ratio of semen and cryopreservation solution in cryoprotective solution formula (1) and (2) was 1:7. Taking a total volume of 480μl as an example, it contained 420μl of cryoprotection solution and 60μl of fresh semen of stichopus japonicus.

5 **[26]** d. Program cooling: the cryotubes were placed into the program controlled freezer using a cryotube rack, then the cooling program was run, the cooling program was equilibrated at 0°C for 5 minutes, cooled to -80°C at a cooling rate of 10-15°C/min, equilibrated at -80°C for 5 minutes, and cooled to -180°C at a cooling rate of 20°C/min, and equilibrated for 5 minutes, and then the cryotubes were taken out
10 after 5 minutes of equilibration and put in liquid nitrogen (-196°C) for long-term storage.

[27] e. Thawing: the cryotubes in step d were taken out from the liquid nitrogen, placed in a 25°C water bath to thaw, shaken gently to make the temperature uniform, and taken out immediately (about 120S) when there was only a small amount of solids
15 left, and shaken in the air until completely melt;

[28] f. Activation and sperm vitality analysis: the frozen semen was pipetted into the activator to dilute the fresh semen of stichopus japonicus up to 600 times, so as to maintain 70-120 sperm in each field of CASA, such as when the mixing volume ratio of semen and cryopreservation liquid is 1:7, it needs to be diluted 75 times again. For
20 example, 740μl of activation solution and 10μl of thawed frozen semen were mixed evenly; 10μl of activated frozen semen was pipetted, and added to the sperm counting plate, and the sperm vitality was analyzed using CASA.

[29] The statistical results show that the sperm viability rate of stichopus japonicus sperm preserved in formula (1) after thawing and activation is 60.07±5.88%, the rapid
25 motility rate is as high as 43.39±5.88%, and the sperm curve motility speed is as high as 103.51±15.46μm/s; The sperm viability rate of stichopus japonicus preserved in formula (2) after thawing and activation is 45.11±5.22%, the sperm rapid motility rate is as high as 26.68±1.34%, and the sperm curve motility speed is as high as 136.54±15.98μm/s.

30 **[30]** Comparative example 1

[31] a. Preparation of cryopreservation solution: according to the principle of current use and preparation, a fresh ultra-low temperature cryoprotective solution was prepared. In this example, the formula of ultra-low temperature cryopreservation

solution of stichopus japonicus sperm was as follows: 85mL of natural seawater filtered with a 0.45μm filter membrane, and 15mL of dimethyl sulfoxide (DMSO) with a purity ≥99.97%. The prepared cryoprotective solution was pre-cooled in a 4°C constant temperature refrigerator until use.

[32] b. Acquisition of semen: same as Example 1

[33] c. Mixing semen with cryopreservation solution: the fresh semen of stichopus japonicus and cryopretective solution were fully mixed according to a certain volume ratio of 1:7 and then dispensed into 2ml cryotubes. During the whole process, the cryotubes were stored in a cryotube box and placed in a thermal insulation box with ice.

[34] d. Program cooling: same as Example 1

[35] e. Thawing: same as Example 1

[36] f. Activation and sperm vitality analysis: same as Example 1

[37] The statistical results show that the sperm viability rate of stichopus japonicus sperm in this example after thawing and activation is 46.29±7.55%, the rapid motility rate is as high as 28±2.44%, and the sperm curve motility speed is as high as 102.37±6.62μm/s.

[38] Comparative example 2

[39] a. Preparation of cryopreservation solution: according to the principle of current use and preparation, a fresh ultra-low temperature cryoprotective solution was prepared. In this example, the formula of ultra-low temperature cryopreservation solution of stichopus japonicus sperm was as follows: 80mL of natural seawater filtered with a 0.45μm filter membrane, and 20mL of propylene glycol (PG) with a purity ≥99.97%. The prepared cryoprotective solution was pre-cooled in a 4°C constant temperature refrigerator until use.

[40] b. Acquisition of semen: same as Example 1

[41] c. Mixing semen with cryopreservation solution: same as Example 1

[42] d. Program cooling: the cryotubes were placed into the program controlled freezer using a cryotube rack, then the cooling program was run, the cooling program was equilibrated at 0°C for 5 minutes, cooled to -80°C at a cooling rate of 15°C/min, equilibrated at -80°C for 5 minutes, and cooled to -180°C at a cooling rate of 20°C/min, and equilibrated for 5 minutes, and then the cryotubes were taken out and put in liquid nitrogen (-196°C) for long-term storage.

[43] e. Thawing: same as Example 1

[44] f. Activation and sperm vitality analysis: same as Example 1

[45] The statistical results show that the sperm viability rate of stichopus japonicus sperm in this example after thawing and activation is 7.84±0.61%, the rapid motility rate is as high as 4.79±0.96%, and the sperm curve motility speed is as high as 48.45±12.94μm/s.

[46] Comparative example 3

[47] a. Preparation of cryopreservation solution: according to the principle of current use and preparation, a fresh ultra-low temperature cryoprotective solution was prepared. In this example, the formula of ultra-low temperature cryopreservation solution of stichopus japonicus sperm was as follows: 85mL of natural seawater filtered with a 0.45μm filter membrane, and 15mL of dimethyl sulfoxide (DMSO) with a purity ≥99.97%. The prepared cryoprotective solution was pre-cooled in a 4°C constant temperature refrigerator until use.

[48] b. Acquisition of semen: same as Example 1

[49] c. Mixing semen with cryopreservation solution: same as Example 1

[50] d. Program cooling: the cryotubes were placed into the program controlled freezer using a cryotube rack, then the cooling program was run, the cooling program was equilibrated at 0°C for 5 minutes, cooled to -80°C at a cooling rate of 25°C/min, equilibrated at -80°C for 5 minutes, and cooled to -180°C at a cooling rate of 20°C/min, and equilibrated for 5 minutes, and then the cryotubes were taken out and put in liquid nitrogen (-196°C) for long-term storage.

[51] e. Thawing: same as Example 1

[52] f. Activation and sperm motility analysis: same as Example 1

[53] The statistical results show that the sperm viability rate of stichopus japonicus sperm in this example after thawing and activation is 12.97±0.46%, the rapid motility rate is as high as 7.83±0.53%, and the sperm curve motility speed is as high as 80.28±9.70μm/s.

[54] Comparative example 4

[55] a. Preparation of cryopreservation solution: according to the principle of current use and preparation, a fresh ultra-low temperature cryoprotective solution was prepared. In this example, the formula of ultra-low temperature cryopreservation solution of stichopus japonicus sperm was as follows: 85mL of natural seawater

filtered with a 0.45μm filter membrane, and 15mL of dimethyl sulfoxide (DMSO) with a purity ≥99.97%. The prepared cryoprotective solution was pre-cooled in a 4℃ constant temperature refrigerator until use.

[56] b. Acquisition of semen: same as Example 1

[57] c. Mixing semen with cryopreservation solution: the fresh semen of stichopus japonicus and cryopretective solution were fully mixed according to a volume ratio of 1:19 and then dispensed into 2ml cryotubes. During the whole process, the cryotubes were stored in a cryotube box and placed in a thermal insulation box with ice. Taking a total volume of 480μl as an example, it contained 456μl of cryoprotection solution and 24μl of fresh semen of stichopus japonicus.

[58] d. Program cooling: same as Example 1

[59] e. Thawing: same as Example 1

[60] f. Activation and sperm vitality analysis: the frozen semen was pipetted to the activator to dilute the fresh semen of stichopus japonicus up to 600 times, so as to maintain 70-120 sperm in each field of CASA, such as when the mixing volume ratio of semen and cryopreservation liquid is 1:19, it needs to be diluted 30 times again. For example, 290μl of activation solution and 10μl of thawed frozen semen were mixed evenly; 10μl of activated frozen semen was pipetted, and added to the sperm counting plate, and the sperm vitality was analyzed using CASA.

[61] The statistical results show that the sperm viability rate of stichopus japonicus sperm in this example after thawing and activation is 13.18±0.74%, the rapid motility rate is as high as 9.24±0.52%, and the sperm curve motility speed is as high as 101.34±2.15μm/s.

It can be seen from the above examples that performing ultra-low temperature cryopreservation treatment on the apostichopus japonicus sperm using the specific freezing solution of the present invention under specific conditions, the sperm viability after activation can reach 60.07±5.88%, the sperm rapid motility rate can reach 43.39±5.88%, and the sperm curve motility speed can reach 103.51±15.46μm/s, and the aprotic high-polarity solvent in the freezing solution can quickly penetrate into the cell, combine with water and electrolyte, produce a certain molar concentration in the cell, and reduce the concentration of the electrolyte solution of the unfrozen solution inside and outside the cell, lowers the freezing point and reduces the formation of ice crystals. At the same time, it avoids excessive exudation of intracellular water that

causes cell shrinkage and damage to dissolution; After the fresh semen is mixed with the cryopreserved solution at the volume ratio of 1:5 to 1:7, it is cooled to -80°C at the optimal cooling rate (10-15°C/min), so that it could quickly cross the danger zone (0~ -60 °C) while avoiding more ice crystal damage to sperm caused by too fast cooling rate, and then the apostichopus japonicus sperm can be stored for a long time (liquid nitrogen -196°C), and then activated by a specific activator to make its vitality reach the maximum instantaneously, and maintain high vitality for more than 3 hours, which is conducive to more efficient detection of sperm vitality and practical application; even if the same freezing solution is used but under different mixing ratios or different cooling rates or other types of antifreezes, due to unsuitable conditions, the sperm suffered more freezing damage in the process of ultra-low temperature freezing, and the activation after freezing cannot achieve the corresponding effect. Thus, it can be seen that the solution provided by the embodiment of the present invention has unexpected technical effects.

Conclusies

1. Cryobeschermende oplossing, met het kenmerk, dat de cryobeschermende oplossing is samengesteld uit een verdunningsmiddel, een antivriesmiddel en een
5 additief; waarbij het verdunningsmiddel natuurlijk zeewater is, het antivriesmiddel een of meer aprotische polaire oplosmiddelen zijn en het additief glucose is.

2. Cryobeschermende oplossing volgens conclusie 1, met het kenmerk, dat het voorgenoemde dimethylsulfoxide (DMSO) en/of dimethylaceetamide (DMA) is.
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3. Cryobeschermende oplossing volgens conclusie 1, met het kenmerk, dat de cryobeschermende oplossing natuurlijk zeewater, dimethylsulfoxide (DMSO) en glucose is, waarbij de respectieve volume-tot-massaverhouding 85 ml – 90 ml : 10 ml – 15 ml : 1,98 g is.
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4. Cryobeschermende oplossing volgens conclusie 1, met het kenmerk, dat de cryobeschermende oplossing natuurlijk zeewater en dimethylaceetamide (DMA) is en de volumeverhouding 80 ml : 20 ml is.

20 5. Cryobeschermende oplossing volgens een van conclusies 1 – 4, met het kenmerk, dat het natuurlijke zeewater natuurlijk zeewater is dat gefilterd is met een 0,45 μm filtermembraan; dimethylsulfoxide (DMSO) dimethylsulfoxide met een zuiverheid van groter dan of gelijk aan 99,97% is, dimethylaceetamide (DMA) dimethylaceetamide met een zuiverheid van groter dan of gelijk aan 99,97% is en zowel DMSO als DMA
25 aprotische oplosmiddelen met hoge polariteit zijn.

6. Werkwijze voor cryopreservering bij ultralage temperatuur van stichopus japonicus-sperma met behulp van de cryobeschermende oplossing volgens conclusie 1, gekenmerkt door:
30 (1) spermaverwerving: het verwerven van het sperma van stichopus japonicus in de kweekperiode en het wachten op gebruik;
(2) het mengen van sperma met een cryobeschermende oplossing: het mengen van het verse sperma van stichopus japonicus en de cryobeschermende oplossing in een houder

met een volumeverhouding van 1 : 5 – 1 : 7;
(3) programmakoeling: het, na het mengen, in een programmagestuurde vriezer plaatsen, het koelprogramma uitvoeren, waarbij het koelprogramma gedurende 5 minuten bij 0 °C dient te equilibreren, te koelen tot -80 °C met een koelsnelheid van
5 10 – 15 °C/min, te equilibreren bij -80 °C gedurende 5 minuten, en te koelen tot -180 °C bij een afkoelsnelheid van 20 °C/min, en te equilibreren gedurende 5 minuten en vervolgens het eruit halen na 5 minuten equilibrering om cryopreservering van stichopus japonicus-sperma bij ultralage temperatuur te realiseren.

10 7. Werkwijze volgens conclusie 6, met het kenmerk, dat de spermahouder na het koelprogramma voor langdurige opslag in vloeibare stikstof geplaatst wordt.

8. Werkwijze volgens conclusie 6, gekenmerkt door het ontdooien van de gecryopreserveerde stichopus japonicus-kiem in een waterbad van 25 °C, het zachtjes
15 schudden om de temperatuur uniform te maken, en het, wanneer er nog maar een kleine hoeveelheid vaste stof over is, onmiddellijk uitnemen (ongeveer 120 s), en schudden in de lucht om volledig te smelten; het pipetteren van bevroren sperma in de activator en het goed mengen om de totale verdunningsmate van 600 keer de oorspronkelijke stichopus japonica-kiem te maken, en waarbij de activator gefilterd natuurlijk zeewater
20 is dat 5 gew.% foetaal runderserum bevat.

9. Werkwijze volgens conclusie 6, met het kenmerk dat het verse sperma van stichopus japonicus in stap (2) is om het verzamelde sperma 600 keer te verdunnen met een activator, het sperma te detecteren en het sperma te verzamelen met een vitaliteit
25 van meer dan 90%, en te wachten voor gebruik.

Rijksdienst voor Ondernemend Nederland

OCTROOIAANVRAAG NR.:
NO 141396
NL 2028946

ONDERZOEKSRAPPORT

BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK

RELEVANTE LITERATUUR

Categorie[1]	Literatuur met, voor zover nodig, aanduiding van speciaal van belang zijnde tekstgedeelten of figuren.	Van belang voor conclusie(s) nr:	Classificatie(IPC)
X,D	CN 110 326 610 A (UNIV DALIAN OCEAN) 15 oktober 2019 (2019-10-15)	1,2	INV. A01N1/02
Y	* samenvatting *	3,5	
A	* conclusie 1 * * voorbeelden * -----	6-9	
X,D	MIZUNO YUTA ET AL: "Motility and fertility of cryopreserved spermatozoa of the Japanese sea cucumber Apostichopus japonicus", AQUACULUTRE RESERCH, deel 50, nr. 1, 25 oktober 2018 (2018-10-25), bladzijden 106-115, XP055886115, GB ISSN: 1355-557X, DOI: 10.1111/are.13872 Gevonden op het Internet: URL:https://onlinelibrary.wiley.com/doi/full-xml/10.1111/are.13872> * samenvatting * * 2.1 Materials; bladzijde 107 * * 3.2.1 Nature and concentration of permeable cryoprotectant; bladzijde 109 * * figuur 3 * * figuur 6 * * bladzijden 109-110 * * figuren 8-10 * * 2.2.1; bladzijde 107 * ----- -/--	1-9	Onderzochte gebieden van de techniek A01N

Indien gewijzigde conclusies zijn ingediend, heeft dit rapport betrekking op de conclusies ingediend op:

Plaats van onderzoek:	Datum waarop het onderzoek werd voltooid:	Bevoegd ambtenaar:
München	3 februari 2022	Galley, Carl

[1] NDERLINCATEGORIE VAN DE VERMELDE LITERATUUR

X: de conclusie wordt als niet nieuw of niet inventief beschouwd ten opzichte van deze literatuur
Y: de conclusie wordt als niet inventief beschouwd ten opzichte van de combinatie van deze literatuur met andere geciteerde literatuur van dezelfde categorie, waarbij de combinatie voor de vakman voor de hand liggend wordt geacht
A: niet tot de categorie X of Y behorende literatuur die de stand van de techniek beschrijft
O: niet-schriftelijke stand van de techniek
P: tussen de voorrangsdatum en de indieningsdatum gepubliceerde literatuur
T: na de indieningsdatum of de voorrangsdatum gepubliceerde literatuur die niet bezwarend is voor de octrooiaanvraag, maar wordt vermeld ter verheldering van de theorie of het principe dat ten grondslag ligt aan de uitvinding
E: eerdere octrooi(aanvrage), gepubliceerd op of na de indieningsdatum, waarin dezelfde uitvinding wordt beschreven
D: in de octrooiaanvrage vermeld
L: om andere redenen vermelde literatuur
&: lid van dezelfde octrooifamilie of overeenkomstige octrooipublicatie

1

EOB FORM 02.83 (P0414B)

RELEVANTE LITERATUUR

Categorie[1]	Literatuur met, voor zover nodig, aanduiding van speciaal van belang zijnde tekstgedeelten of figuren.	Van belang voor conclusie(s) nr:
Y	**TSAI SUJUNE ET AL: "Sugars as supplemental cryoprotectants for marine organisms", REVIEWS IN AQUACULTURE, deel 10, nr. 3, 4 februari 2017 (2017-02-04), bladzijden 703-715, XP055886113, ISSN: 1753-5123, DOI: 10.1111/raq.12195 Gevonden op het Internet: URL:https://api.wiley.com/onlinelibrary/tdm/v1/articles/10.1111%2Fraq.12195> * samenvatting * * Spermatozoa; bladzijde 705, rechter kolom * * tabel 2 ***	**3,5**

X	**CN 101 326 905 A (UNIV JIMEI [CN]) 24 december 2008 (2008-12-24)**	**1,2**
A	*** conclusie 2 * * voorbeeld ***	**3-9**
	-----	

1 [1] CATEGORIE VAN DE VERMELDE LITERATUUR

X: de conclusie wordt als niet nieuw of niet inventief beschouwd ten opzichte van deze literatuur
Y: de conclusie wordt als niet inventief beschouwd ten opzichte van de combinatie van deze literatuur met andere geciteerde literatuur van dezelfde categorie, waarbij de combinatie voor de vakman voor de hand liggend wordt geacht
A: niet tot de categorie X of Y behorende literatuur die de stand van de techniek beschrijft
O: niet-schriftelijke stand van de techniek
P: tussen de voorrangsdatum en de indieningsdatum gepubliceerde literatuur

T: na de indieningsdatum of de voorrangsdatum gepubliceerde literatuur die niet bezwarend is voor de octrooiaanvrage, maar wordt vermeld ter verheldering van de theorie of het principe dat ten grondslag ligt aan de uitvinding
E: eerdere octrooi(aanvrage), gepubliceerd op of na de indieningsdatum, waarin dezelfde uitvinding wordt beschreven
D: in de octrooiaanvrage vermeld
L: om andere redenen vermelde literatuur
&: lid van dezelfde octrooifamilie of overeenkomstige octrooipublicatie

EOB FORM 02.83 (P0414C)

AANHANGSEL BEHORENDE BIJ HET RAPPORT BETREFFENDE HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK, UITGEVOERD IN DE OCTROOIAANVRAGE NR.

NO 141396
NL 2028946

Het aanhangsel bevat een opgave van elders gepubliceerde octrooiaanvragen of octrooien (zogenaamde leden van dezelfde octrooifamilie), die overeenkomen met octrooischriften genoemd in het rapport.
De opgave is samengesteld aan de hand van gegevens uit het computerbestand van het Europees Octrooibureau per
De juistheid en volledigheid van deze opgave wordt noch door het Europees Octrooibureau, noch door het Bureau voor de Industriële eigendom gegarandeerd;; de gegevens worden verstrekt voor informatiedoeleinden.

03-02-2022

In het rapport genoemd octrooigeschrift	Datum van publicatie	Overeenkomend(e) geschrift(en)	Datum van publicatie
CN 110326610 A	15-10-2019	CN 110326610 A	15-10-2019
		WO 2021012763 A1	28-01-2021
CN 101326905 A	24-12-2008	GEEN	

EPO FORM P0466

Algemene informatie over dit aanhangsel is gepubliceerd in de 'Official Journal' van het Europees Octrooibureau nr 12/82 blz 448 ev

SCHRIFTELIJKE OPINIE

DOSSIER NUMMER	INDIENINGSDATUM	VOORRANGSDATUM	AANVRAAGNUMMER
NO141396	10.08.2021	25.09.2020	NL2028946
CLASSIFICATIE			
INV. A01N1/02			
AANVRAGER			
INSTITUTE OF OCEANOLOGY, CHINESE ACADEMY OF SCIENCES			

Deze schriftelijke opinie bevat een toelichting op de volgende onderdelen:

- ☒ Onderdeel I Basis van de schriftelijke opinie
- ☐ Onderdeel II Voorrang
- ☐ Onderdeel III Vaststelling nieuwheid, inventiviteit en industriële toepasbaarheid niet mogelijk
- ☐ Onderdeel IV De aanvraag heeft betrekking op meer dan één uitvinding
- ☒ Onderdeel V Gemotiveerde verklaring ten aanzien van nieuwheid, inventiviteit en industriële toepasbaarheid
- ☐ Onderdeel VI Andere geciteerde documenten
- ☐ Onderdeel VII Overige gebreken
- ☐ Onderdeel VIII Overige opmerkingen

	DE BEVOEGDE AMBTENAAR
	Galley, Carl

Aanvraag nr.:

SCHRIFTELIJKE OPINIE

NL2028946

Onderdeel I Basis van de Schriftelijke Opinie

1. Deze schriftelijke opinie is opgesteld op basis van de meest recente conclusies ingediend voor aanvang van het onderzoek.

2. Met betrekking tot **nucleotide en/of aminozuur sequenties** die genoemd worden in de aanvraag en relevant zijn voor de uitvinding zoals beschreven in de conclusies, is dit onderzoek gedaan op basis van:

   a. type materiaal:

   ☐ sequentie opsomming

   ☐ tabel met betrekking tot de sequentie lijst

   b. vorm van het materiaal:

   ☐ op papier

   ☐ in elektronische vorm

   c. moment van indiening/aanlevering:

   ☐ opgenomen in de aanvraag zoals ingediend

   ☐ samen met de aanvraag elektronisch ingediend

   ☐ later aangeleverd voor het onderzoek

3. ☐ In geval er meer dan één versie of kopie van een sequentie opsomming of tabel met betrekking op een sequentie is ingediend of aangeleverd, zijn de benodigde verklaringen ingediend dat de informatie in de latere of additionele kopieën identiek is aan de aanvraag zoals ingediend of niet meer informatie bevatten dan de aanvraag zoals oorspronkelijk werd ingediend.

4. Overige opmerkingen:

Aanvraag nr.:

SCHRIFTELIJKE OPINIE

NL2028946

Onderdeel V Gemotiveerde verklaring ten aanzien van nieuwheid, inventiviteit en industriële toepasbaarheid

1. Verklaring

Nieuwheid	Ja:	Conclusies	3-9
	Nee:	Conclusies	1, 2
Inventiviteit	Ja:	Conclusies	
	Nee:	Conclusies	1-9
Industriële toepasbaarheid	Ja:	Conclusies	1-9
	Nee:	Conclusies	

2. Citaties en toelichting:

Zie aparte bladzijde

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1 CN 110 326 610 A (UNIV DALIAN OCEAN) 15 oktober 2019

D2 Mizuno Yuta ET AL: "Motility and fertility of cryopreserved spermatozoa of the Japanese sea cucumber Apostichopus japonicus", Aquaculutre Reserch, deel 50, nr. 1, 25 oktober 2018, bladzijden 106-115, XP055886115

D3 CN 101 326 905 A (UNIV JIMEI [CN]) 24 december 2008

D4 Tsai Sujune ET AL: "Sugars as supplemental cryoprotectants for marine organisms", Reviews in Aquaculture, deel 10, nr. 3, 4 februari 2017, bladzijden 703-715, XP055886113

1 The present application relates to a cryopreservative solution, comprising "natural seawater", an antifreeze agent and glucose, and a method of cryopreservation of Japanese sea cucumbers (*Apostichopus japonicus*), using the cryopreservation solution and a specific cooling procedure.

2 The feature of "natural seawater" is unclear in that there are vast variations in the composition of "natural seawater" depending on the area, the depth, time, etc... Therefore, even if it is accepted that "natural seawater" is different to a saline solution or even to artificial seawater, it is not clear in which ways whether by components or concentrations the "natural seawater" differs from saline or an artificial seawater, and the application as filed does not provide any further information in this regard.

3 It should be noted that the additive glucose is also known to be an antifreeze agent (see, for example, D3: abstract). Therefore, the two features in the cryopreservation solution are considered to overlap. Therefore, a solution of glucose and seawater would be considered to anticipate the subject-matter of claim 1.

4 Claim 4 indicates the ratio of "natural seawater" to DMA in the cryopreservation solution, but fails to indicate the amount of glucose, which, according to claim 1, should be essential. It is also noted that example 2 of the present application, relating to a cryopreservation solution comprising DMA appears not to contain glucose, so that the example appears to fall outside the scope of the claims.

5 The prior art teaches:

D1 discloses a method for cryopreservation of Japanese sea cucumber sperm using a cryopreservative solution comprising 80–120 mL of DMSO, having a purity of 99.7%, a saline solution of 7–9 g/L of sodium chloride and 0.5–0.7 g/L of anhydrous calcium chloride, and 8–15 g glucose (claim 1). D1 also teaches the cryopreservation process where the sperm is mixed with the cryopreservative solution and suspended at 10–15 cm from the surface of liquid nitrogen for 10 minutes, suspended at the surface for 5 minutes and then submerged in liquid nitrogen (claim 1).

D2 also discloses a method of cryopreservation of Japanese sea cucumber sperm using a cryopreservative solution comprising 20% DMSO, 16% fetal bovine serum and 64% artificial seawater (abstract). Fetal bovine serum (FBS) comprises around 550 mg/L glucose. Artificial seawater is said to comprise 423 mM NaCl, 9 mM KCl, 9.27 mM $CaCl_2$, 22.94 mM $MgCl_2$, 22.5 mM $MgSO_4$, buffered at pH 8.0 with10 mM HEPES-NaOH (page 107, 2.1 Materials). D2 also demonstrates that DMSO provides better motility after thawing than methanol, DMF and DMA (page 109, 3.2.1 Nature and concentration of permeable cryoprotectant; Figure 3). D2 also teaches that the control of cooling rate to -50°C at 10°C per minute, and subsequent immersion in liquid nitrogen provides the best motility after thawing (pages 109–110; Figure 6). Studies were also carried out to determine the best concentration of cryoprotectant, the concentration of DMSO in seawater, etc... (Figures 8–10).

D3 teaches that sugars, such as glucose, when used as a supplementary cryoprotectant, significantly improves the post-thawing motility of sperm of marine organisms, along with maintaining plasma membrane integrity and the mitochondrial membrane potential compared with the use of DMSO alone (abstract and page 705, right-hand column, Spermatozoa; Table 2).

D4 discloses a cryopreservation solution for preservation of the algae *Porphyra*

haitanesis seedlings that comprises 70% sterilised seawater, 15% glucose and 5% DMSO (claim 2 and example).

6 The subject-matter of at least claims 1 and 2 does not appear to be novel in light of the disclosure of D4 (claim 2 and example). Furthermore, given that artificial seawater is considered to be indistinguishable from some "natural seawater", D2 is also considered to anticipate the subject-matter of claims 1 and 2. The subject-matter of claims 3–9 appears to be novel.

7 Documents D1 and D2 are considered the closest prior art to the claimed subject-matter. The subject-matter of claims 3 and 5 differ from the disclosure of D1 in that "natural seawater" is used in place of a saline solution with added calcium chloride. Not only do the present examples fail to demonstrate any surprising and/or unexpected effect from using "natural seawater" instead of a saline solution, but also, as discussed under point 2, should some improved effect be shown for some "natural seawater", given the variation in seawater based on depth, location and time, etc..., the skilled person would not know how to reproduce such an effect. Thus, the objective problem is the provision of an alternative diluent, obvious from D1 alone.

8 Notwithstanding this argumentation, the subject-matter of claims 3–5 differ from D2 both in that "natural seawater" is used instead of artificial seawater and that the concentration of glucose is far higher. However, as discussed above, claim 4 fails to indicate the concentration of glucose when the antifreeze agent is DMA. Thus, claim 4 differs only in the use of "natural seawater", which cannot contribute to inventive step for the reasons provided above. However, even with respect to claims 3 and 5, the present examples do demonstrate that the presence of glucose improves the motility of thawed sperm (example 1 and comparative example 1). The problem to be solved is the provision of a cryopreservation solution that provides improved motility of sperm after thawing. However, the solution is already known from D3. Thus, the skilled person would add glucose with a high expectation of success.

9 The method of claims 6–9 differs from the disclosure of D2 in particular with respect to the cooling programme, where the mixture is held at 0°C for 5 minutes to equilibrate, then cooled to -80°C at a rate of 10 to 15°C per minute, equilibrated for 5 minutes and then cooled to -180°C at 20°C per minute. The present examples do demonstrate that a slower rate of cooling to -80°C does

provide more motile sperm after thawing (see example 1 and comparative example 3). However, D2 clearly states in section 2.2.1 on page 107 that cooling rates from 0 to -40°C are critical since most cryo-injuries occur in this range. Furthermore, D2 demonstrates that the motility of thawed sperm peaked at a cooling rate of 10.4°C per minute to -50°C for DMSO. No surprising and/or unexpected effect has been demonstrated for the other specific rates or timings of claim 6, or for the other features of dependent claims 7–9. Thus, the problem to be solved by the methods of claims 6–9 is the provision of alternative methods of cryopreservation.

10 The skilled person, with the teaching of D2 and with standard routine experimentation would reach the subject-matter of claims 6–9 without the use of inventive skill. Thus, the entire claimed subject-matter lacks inventive step.

11 The industrial applicability of the claimed subject-matter is acknowledged.