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(71) Applicant *(for all designated States except US)*: **INDUSTRY-UNIVERSITY COOPERATION FOUNDATION SOGANG UNIVERSITY** [KR/KR]; 121-742, Sogang Univ. 1-1 Sinsu-dong Mapo-gu, Seoul 121-742 (KR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **JUNG, Kwang-Hwan** [KR/KR]; #1025-503 Samsung Apt. Imae-dong Bundang-gu Sungnam-si, Gyeonggi-do 463-901 (KR). **LEE, Keon Ah** [KR/KR]; #104-1005 Doosan We've Apt. Bupyung-1dong Bupyung-gu, Incheon 403-010 (KR). **CHOI, Ah Reum** [KR/KR]; #402 Crystal Ville 1093-5 Banguh-dong Dong-gu, Ulsan 682-806 (KR).

(74) Agent: **LEE, Won-Hee**; 8th Fl. Sung-ji Heights II 642-16, Yoksam-dong Kangnam-ku, Seoul 135-080 (KR).

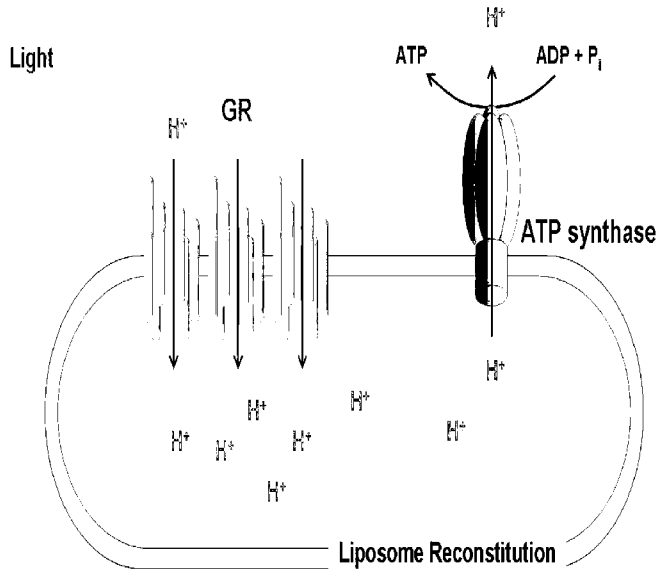
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(54) Title: COMPOSITION FOR ATP SYNTHESIS, MOUNTED FOR GLOEOBACTER RHODOPSIN AND ATP SYNTHASE TO HAVE ORIENTATION

[Fig. 1]



(57) Abstract: Disclosed herein is a composition for ATP synthesis, mounted for *Gloeobacter* rhodopsin (hereinafter, referred to as GR) and ATP synthase to have orientation. Specifically, disclosed herein is a method for synthesizing ATP, including 1) overexpressing GR to translocate protons (H+) from the inside to the outside of the membrane in *E. coli*, 2) grinding the cells to prepare an inverted membrane, 3) adding Pi and irradiating to form a proton membrane gradient by GR, and 4) synthesizing ATP through ATP synthase, a membrane protein of *E. coli* from the membrane gradient. The composition for ATP synthesis and the method for preparing ATP according to the present invention may stably provide a large amount of ATP for mass production of biomaterials, and the costs may be greatly reduced due to renewable use of the composition for ATP synthesis according to the present invention.

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Description

Title of Invention: COMPOSITION FOR ATP SYNTHESIS, MOUNTED FOR GLOEOBACTER RHODOPSIN AND ATP SYNTHASE TO HAVE ORIENTATION

Technical Field

[1] The present disclosure relates to a composition for artificial ATP synthesis.

[2]

Background Art

[3] Living cells constantly perform work and thus require energy. Energy is used for various processes such as maintenance of highly organized structures, synthesis of cellular components, movement, generation of electrical currents, production of light, etc. ATP is one of the main energy sources among various metabolites. ATP is the chemical link between catabolism and anabolism, and one of the factors which constitute the energy flow in a cell. ATP is exergonically converted into ADP and Pi or AMP and PPi, which is coupled to endergonic conversions of many other substrates. When ATP is hydrolyzed, a phosphoryl, pyrophosphoryl, or adenylyl group from ATP is transferred to the substrate. By these 'group transfer' reactions, ATP provides the energy for anabolic reactions. In ATP synthesis of bacteria, mitochondria, and chloroplast, protons move through the F_1F_0 ATP synthase for each ATP molecule produced when an electrochemical gradient of protons ($\Delta H+$)[or sometimes Na+ ions($\Delta Na+$)] is generated across the membrane (Capaldi RA & Aggeler R, Trends Biochem. Sci. 27:154-160, 2002; Dimroth P, Biochim. Biophys. Acta 1318:11-51, 1997; Neumann S et al., J. Bacteriol. 180:3312-3316, 1998; Reidlinger J & Muller V, Eur. J. Biochem. 223:275-283, 1994; Yoshida M et al., Nat. Rev. Mol. Cell. Biol. 2:669-677, 2001). The F_1F_0 ATP synthase consists of F1 and F0, membrane-related peripheral portion and integral membrane component. The F_1 and F_0 consist of subunits of $\alpha_3\beta_3\gamma\delta\varepsilon$and $ab_2C_{10\text{-}14}$, respectively. Transfer of ions through F_0 is coupled via this molecular machine to synthesis of ATP by F_1. For biochemical studies on ATP synthase machinery from various organisms, ATP synthase was isolated from a facultative thermoalkaliphilic *Bacillus* species by one study group (Olsson K et al., J. Bacteriol. 185:461-465, 2003; Peddie CJ et al., J. Bacteriol. 181:3172-3177, 1999), and the purified enzyme was reconstituted into proteoliposomes for ion transport studies, which showed that the ATP synthase utilizes protons as coupling ions for ATP synthesis (Gregory M et al., J. Bacteriol. 185 (15): 4442-4449, 2003).

[4] Upon formation of a proton gradient across the membrane as a pre-step for ATP synthesis, microorganisms utilize the respiratory process using the electron transport

system in cell membrane while some of microorganisms obtain aids from a membrane protein called rhodopsin. Rhodopsin is a photoactive retinylidene protein embedded in the membrane and these photoreceptor proteins are well distributed among a Kingdom of animal, fungi, protista, and bacteria. Rhodopsin was first isolated from the eyes of cattle in the 1930s. The protein is responsible for vision in the eyes of animals (Spudich JL, *Trends Microbiol.* 14(11): 480-487, 2006), while for microorganisms, it is not only used to pump hydrogen or chlorine ions or move cells but also is involved in phototactic responses (Spudich JL & Jung KH, In Handbook of Photosensory Receptors, ed. (2005) W.R. Griggs and J.L. Spudich, pp. 1-24). Among rhodopsins from microorganisms, proteorhodopsin (hereinafter, referred to as PR), discovered in a marine γ-proteobacterium of the SAR86 group, and worked as a light-driven proton pump (Beja O *et al.*, *Science* 289:1902-1906, 2000; Beja O *et al.*, *Nature.* 411:786-789, 2001). Until now, more than 2000 different PR genes have been identified in various oceans around the world, and it has been found that an estimated 13% of microorganisms in the photic zone as well as more than 50% of all marine organism communities found in maritime surface water are bacteria which contain the PR genes (Sabehi G *et al.*, *PLoS Biol.* 3:e273, 2005).

[5] *Gloeobacter violaceus* is a unicellular cyanobacterium that has been isolated from calcareous rocks in Switzerland (Rippka R *et al.*, *Arch. Microbiol.* 100:419-436, 1974). According to phylogenetic analysis based on the 16S rRNA sequence, it branched off from the main cyanobacterial tree at a very early stage, which suggests that it may retain some of the primitive properties of early cyanobacteria (Nelissen B *et al.*, *J Mol Evol.* 42(2):194-200, 1996; Nelissen B *et al.*, *Mol Biol Evol.* 12(6):1166-1173 1995). *G. violaceus*, which performs photosynthesis, lacks a thylakoid membrane. However, the bacteria include all the photosynthesis machinery and respiratory system in the cell membrane (Rippka R et al., *Arch. Microbiol.* 100:419-436, 1974). Results of sequence homology comparisons showed that a gene homologous with microbial rhodopsin was found in the *G. violaceus* genome, having proton pumping activity like PR. This rhodopsin was called *Gloeobacter rhodopsin* (hereinafter, referred to as GR).

[6] ATP is a material which provides energy necessary for cells and life forms. Recently, while a large amount of ATP has been used for mass production of biomaterials, ATP is not a material that is renewable when once used and is very expensive to synthesize. However, if a composition for *in-vivo* ATP synthesis system is used, ATP may be constantly provided and its renewal is also possible.

[7]

[8] As a result of these studies, the present inventors made an inverted membrane by grinding GR cells overexpressed in *E. coli*, constructed a composition for ATP synthesis by allowing a proton membrane gradient produced by the GR to be formed

through ATP synthase in *E. coli*, and completed the present invention.

[9]

Disclosure of Invention

Technical Problem

[10] One object of the present invention is to provide a composition for ATP synthesis, including an inverted membrane vesicle, which is mounted for *Gloeobacter* rhodopsin (hereinafter, referred to as GR) and ATP synthase to have orientation.

[11]

Solution to Problem

[12] One object of the present invention is to provide a composition for ATP synthesis, including an inverted membrane vesicle, which is mounted for *Gloeobacter* rhodopsin (hereinafter, referred to as GR) and ATP synthase to have orientation.

[13] Another object of the present invention is to provide a method for preparing the inverted membrane vesicle.

[14] Still another object of the present invention is to provide a method for synthesizing ATP by utilizing the composition for ATP synthesis.

[15] In order to achieve the objects, the present invention provides a composition for ATP synthesis, including an inverted membrane vesicle including ATP synthase oriented to have an extroversion so as to pump in protons and produce ATP outside the membrane by a concentration gradient of *Gloeobacter* rhodopsin (hereinafter, referred to as GR) oriented to have an introversion and the pumped protons.

[16] The present invention also provides a method for preparing a composition for ATP synthesis, mounted for GR and ATP synthase to have orientation, including Steps:

[17] 1) preparing a recombinant expression vector operably linked to a polynucleotide encoding GR;

[18] 2) transforming a host cell with the recombinant expression vector in Step 1) to prepare a transformant;

[19] 3) incubating the transformant in Step 2) to induce an expression of GR;

[20] 4) isolating a protoplast from a transformant overexpressing the GR in Step 3); and

[21] 5) preparing an inverted membrane vesicle from the protoplast isolated in Step 4).

[22] Furthermore, the present invention provides a method for synthesizing ATP by using the composition for ATP synthesis, including:

[23] 1) adding Pi (phosphoryl group) and ADP to the composition for ATP synthesis of the present invention; and

[24] 2)irradiating the composition.

[25] The present invention also provides a use of an inverted membrane vesicle including ATP synthase oriented to have an extroversion so as to pump in protons and produce

ATP outside the membrane by a concentration gradient of *Gloeobacter* rhodopsin (hereinafter, referred to as GR) oriented to have an introversion and the pumped protons in preparation of a composition for ATP synthesis.

[26]

Advantageous Effects of Invention

[27] The composition for ATP synthesis and the method for preparing ATP according to the present invention may stably provide a large amount of ATP for mass production of biomaterials, and the costs may be greatly reduced due to renewable use of the composition for ATP synthesis according to the present invention.

[28]

Brief Description of Drawings

[29] The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

[30] FIG. 1 is a schematic diagram illustrating the liposome reconstitution of *Gloeobacter* rhodopsin (hereinafter, referred to as GR) and ATP synthase.

[31] FIG. 2 is a graph illustrating the stability of *E. coli* membrane in which GR is expressed.

[32]

Mode for the Invention

[33] Features and advantages of the present invention will be more clearly understood by the following detailed description of the present preferred embodiments by reference to the accompanying drawings. It is first noted that terms or words used herein should be construed as meanings or concepts corresponding with the technical sprit of the present invention, based on the principle that the inventor can appropriately define the concepts of the terms to best describe his own invention. Also, it should be understood that detailed descriptions of well-known functions and structures related to the present invention will be omitted so as not to unnecessarily obscure the important point of the present invention.

[34]

[35] Hereinafter, the terms as used herein will be described in detail.

[36]

[37] The term "expression vector" as used herein refers to a linear or circular DNA molecule which includes a fragment encoding a polypeptide of interest operably linked to additional fragments which provide for its transcription. Such an additional fragment includes sequences of a promoter and a termination codon. The expression vector also includes at least one origin of replication, at least one selection marker, a

polyadenylation signal, etc. The expression vector is typically derived from plasmid or viral DNA, or may contain elements of both.

[38] The term "operably linked" as used herein indicates that the fragments are arranged such that they function in concert for their intended purposes, e.g. transcription initiates in a promoter and proceeds through an encoding sequence to a termination codon.

[39]

[40] Hereinafter, the present invention will be described in detail.

[41]

[42] In a specific example of the present invention, a vector for overexpression of *Gloeobacter* rhodopsin (hereinafter, referred to as GR) was transformed into *E. coli* for overexpression. Protoplasts were obtained from the overexpressed cells, followed by French Press to obtain a membrane fraction of GR-overexpressed *E. coli*. The membrane fraction was resuspended in an extraction buffer to obtain an inverted vesicle-inverted membrane of GR-expressed *E. coli*. GR, which is a membrane protein to translocate protons (H+) from the inside to the outside of the membrane, may be inverted while being bound to the membrane of *E. coli* to form a proton membrane gradient by introduction of protons from the outside to the inside of the membrane as in FIG. 1. It is also possible to synthesize ATP through ATP synthase which is a membrane protein of *E. coli*.

[43] In a specific example of the present invention, the composition for ATP synthesis, prepared by the method and having a form in FIG. 1, was subjected to Pi and light to produce ATP in an amount of approximately 3.68904E-12 mole to approximately 8.82043E-11 mole per mg of GR for 1 min (See Table 1). In addition, the time for which the amount of ATP produced by the composition for ATP synthesis was reduced by half (T_f) was 2.12 days and the amount of ATP produced was exponentially reduced within 10 days (See Table 2 and FIG. 2).

[44] From these results, it was confirmed that the composition for ATP synthesis of the present invention was useful in ATP synthesis.

[45]

[46] The present invention provides a composition for ATP synthesis, including an inverted membrane vesicle including ATP synthase oriented to have an extroversion so as to pump in protons and produce ATP outside the membrane by a concentration gradient of *Gloeobacter* rhodopsin (hereinafter, referred to as GR) oriented to have an introversion and the pumped protons.

[47] The GR is a membrane protein to translocate protons (H^+) from the inside to the outside of the membrane, is derived from *Gloeobacter violaceus*, and may be a protein available from NCBI accession No. BAC88139.

[48] GR in the composition for ATP synthesis may form a proton membrane gradient by introducing protons from the outside to inside of the membrane. The ATP synthase may also translocate protons introduced at a high concentration in a membrane vesicle inverted with a membrane protein integrally incorporated into *E. coli* membrane to the outside of the membrane to synthesize ATP. The composition for ATP synthesis of the present invention produced ATP in an amount of approximately 3.68904E-12 mole to approximately 8.82043E-11 mole per mg of GR for 1 min (See Table 1).

[49] The stability of the composition for ATP synthesis of the present is closely associated with that of GR and ATP synthase, and the result was indirectly confirmed by measuring the change in the amount of ATP produced (reduction in an amount of ATP produced) as time proceeded. The time for which the amount of ATP produced by the composition for ATP synthesis of the present invention was reduced by half (T_f) was 2.12 days and the amount of ATP produced was exponentially reduced within 10 days (See Tables 1 and 2 and FIG. 2).

[50]

[51] The present invention also provides a method for preparing an inverted membrane vesicle including GR and ATP synthase, including Steps:

[52] 1) preparing a recombinant expression vector operably linked to a polynucleotide represented by SEQ ID NO. 1 encoding *Gloeobacter* rhodopsin (hereinafter, referred to as GR);

[53] 2) transforming a host cell with the recombinant expression vector in Step 1) to prepare a transformant;

[54] 3) incubating the transformant in Step 2) to induce an expression of GR;

[55] 4) isolating a protoplast from a transformant overexpressing the GR in Step 3); and

[56] 5) preparing an inverted membrane vesicle from the protoplast isolated in Step 4).

[57] In the method, any cell applicable to preparation techniques of an inverted membrane vesicle may be used as the host cell in Step 2), and *E. coli* may be preferably used.

[58] In the method, preparation techniques of an inverted membrane vesicle in Step 5) are characterized by pressure control for preparing an inverted membrane vesicle by using a French press when cells are ground and concentration of each material in a solution phase for protein stability. These preparation techniques are frequently used to study biochemical properties of proteins such as measurements of concentrations of ions or materials moving through proteins which are conventionally studied.

[59]

[60] The present invention also provides a method for synthesizing ATP using the composition for ATP synthesis, including adding Pi to the composition for ATP synthesis of the present invention and irradiating the composition.

[61] The composition for ATP synthesis of the present invention produced ATP in an

amount of approximately 3.68904E-12 mole to approximately 8.82043E-11 mole per mg of GR for 1 min (See Table 1), the time for which the amount of ATP produced by the composition for ATP synthesis of the present invention was reduced by half (T_f) was 2.12 days, and the amount of ATP produced was exponentially reduced within 10 days (See Tables 1 and 2 and FIG. 2).

[62] Preferably, an amount of the Pi added is approximately 1 pM to approximately 100 nM as a final concentration. However, it may be controlled according to the amount of the composition for ATP synthesis.

[63] A visible light at a wavelength of at least above 440 nm is preferably irradiated.

[64] ATP was synthesized while the amount of the composition for ATP synthesis was exponentially reduced within 10 days, and thus the composition is available for 0 to 10 days.

[65]

[66] Furthermore, the present invention provides the use of an inverted membrane vesicle including ATP synthase oriented to have an extroversion so as to pump in protons and produce ATP outside the membrane by a concentration gradient of *Gloeobacter* rhodopsin (hereinafter, referred to as GR) oriented to have an introversion and the pumped protons in preparation of a composition for ATP synthesis.

[67] The GR is a membrane protein to translocate protons (H^+) from the inside to the outside of the membrane, is derived from *Gloeobacter violaceus*, and may be a protein available from NCBI accession No. BAC88139.

[68] It can be seen that GR in embodiments of the present invention formed a proton membrane gradient by introducing protons from the outside to inside of the membrane and the ATP synthase also translocated protons introduced at a high concentration in a membrane vesicle inverted with a membrane protein integrally incorporated into *E. coli* membrane to the outside of the membrane to synthesize ATP. It can be also seen that the composition for ATP synthesis stably provided a large amount of ATP. Therefore, it can be seen that an inverted membrane vesicle including GR and ATP synthase is useful as a component of a composition for ATP synthesis.

[69]

[70] Hereinafter, the present invention will be described in more detail with reference to the following examples.

[71] However, the following examples are provided for illustrative purposes only, and the scope of the present invention should not be limited thereto in any manner.

[72]

[73] **<Example 1> Overexpression of GR in *E. coli***

[74] **<1-1> Preparation of GR-overexpressed *E. coli***

[75] In order to overexpress *Gloeobacter* rhodopsin (hereinafter, referred to as GR)

protein (SEQ ID NO. 2), a process for preparing a vector including a nucleic acid represented by SEQ ID No. 1 encoding GR was performed as follows. GR from NCBI accession NO. BAC88139 was amplified by PCR in genome DNA of *Gloeobacter violaceous*, and the degenerate primer set used is as follows: a forward primer represented by SEQ ID No. 3 (5'-ATGTTGATGACCGTATTTTCTTCTGC-3') and a reverse primer represented by SEQ ID No. 4 (5'-CTAGGAGATAAGACTGCCTCCCCG-3'). PCR for gene amplification was performed using *pfu* polymerase (Koschem, Korea) for 30 cycles (95℃/1 min, 54℃/1 min, and 72℃/2 min 30 sec). The gene product amplified by PCR with a 6-His tag was inserted downstream of the *lacUV5* promoter of the pKJ900 vector, using *XbaI*, *Not* I restriction enzyme, and ligase (New English Lab, USA) (Kim *et al.*, *Biochim. Biophys. Acta* 1777 (6): 504-513, 2008). The vector including an ampicillin resistance gene was transformed into an *E. coli* strain *DH5α*(UT5600, New England Biolabs, USA) and was selected in LB Agar containing 50 μM of ampicillin (USB Corp, USA). The selected transformant was inoculated in a 500 ㎖ LB medium supplemented with ampicillin (USB corp, USA) at 50 ㎍/㎖, to which 1 mM IPTG and 5 μM all-*trans* retinal (Sigma-Aldrich, USA) were added, and incubated in an incubator with stirring for 4 hours (30℃, 200 rpm). Centrifugation was performed at 5000 rpm for 15 min to obtain a pink *E. coli* pellet in which rhodopsin was expressed.

[76]

[77] **<1-2> Yield of protoplasts**

[78] Approximately 3 g of cells (wet weight) was resuspended in a solution containing 50 mM Tris-HCl (pH 8.2) and 0.5 mM PMSF, supplemented with 3.6 mg of lysozyme, and stirred at room temperature for 45 min. Subsequently, DNase I (2 mg) and $MgCl_2$ (final concentration 5 mM) were added to the solution, which was stirred at room temperature for 15 min. Centrifugation was performed at 4℃ and 180,000 rpm for 1 hour to obtain a protoplast.

[79]

[80] **<Example 2> Yield of fraction of GR-overexpressed *E. coli* membrane**

[81] The protoplast pellet obtained in <Example 1> was resuspended in 4 ㎖ of extraction buffer [50 mM potassium phosphate buffer (pH 7.8), 0.5 mM PMSF, 2 mM $MgCl_2$, and 1 mM DTT]. The protoplast was ground using a pre-cooled French Pressure Cell Press (SLM Aminco, USA) (2 passages, 20,000 Ib/in^2). Cells which had not been ground were centrifuged at 8,000 rpm for 2 min and removed. Supernatant was collected and centrifuged at 180,000 rpm for 45 min to obtain a membrane. The membrane was resuspended in 4 ㎖ of extraction buffer to yield an inverted vesicle-inverted membrane. In this way, a GR-expressed *E. coli* membrane having a form as in FIG. 1 was prepared.

[82]

[83] **<Example 3> ATP Assay**

[84] **<3-1> Test method**

[85] P_i (Sigma-Aldrich, USA) was added to a sample dissolved in 4 ㎖ of extraction buffer to prepare a solution at final concentration of 5 mM. Light was irradiated for 20 min using a cut-off filter (Sigma Koki SCH-50S-44Y, Japan) at = 440 nm for light adaptation. Simultaneously, P_i was added to a sample at the same concentration to obtain a solution at final concentration of 5 mM, followed by dark adaptation. 20 minutes later, a luminometer (ADENOSINE 5 -TRIPHOSPHATE (ATP) BIOLUMINESCENT ASSAY KIT, Sigma, USA) was used to measure the amount of ATP produced. The amount of ATP produced in a dark-adapted sample was subtracted from that in a light-adapted sample to correct the value.

[86]

[87] **<3-2> Measurement of the amount of ATP produced per mg of GR for 1 min**

[88] In order to measure the amount of GR, a UV-visible spectrophotometer (Shimadzu UV-2550, Japan) was used to measure an amount of absorption of the composition for ATP synthesis. The O.D. (optical density) value thus obtained from this was used to calculate the molarity of GR protein. The amount of ATP produced per mg of GR for 1 min was measured by the method in Example <3-1>. A luminometer was used to measure the total amount of ATP produced per assay for 20 min, a value obtained from the dark adaptation was subtracted to correct the value, and the value was divided by the concentration of GR. The calculation processes and results were summarized in Table 1.

[89] As a result, ATP in an amount of approximately 3.68904E-12 mole to approximately 8.82043E-11 mole was produced as shown in Table 1.

[90]

[91] Table 1

[Table 1]

[Table]

Sample	GR1	GR2	GR3	GR4	GR5	GR6
1) L20 (R.L.I)	12,093,888	11,357,044	14,673,190	16,602,056	10,936,660	13,545,794
2) D20 (R.L.I)	7,550,470	10,403,830	1,400,263	2,027,044	545,492	10,177,552
3) L-D (R.L.I)	4,543,418	953,214	13,272,927	14,575,012	10,391,168	3,368,242
4) L-D (moles ATP/assay)	1.50929E-10	2.73458E-11	4.8762E-10	5.40183E-10	3.73075E-10	1.0879E-10
5) L-D (moles ATP/ Sample 4 ㎖)	6.03717E-09	1.09383E-09	1.95048E-08	2.16073E-08	1.4923E-08	4.35159E-09
6) Molarity (㎎/㎖)	2.523127693	3.706362839	2.840809253	3.062110251	2.89445798	3.573410689
7) ㎎/4 ㎖	10.09251077	14.82545136	11.36323701	12.248441	11.57783192	14.29364276
8) moles ATP/min/㎎	2.99092E-11	3.68904E-12	8.58242E-11	8.82043E-11	6.44465E-11	1.52221E-11

[92]

[93] 1) L20 was measured by adding Pi to a GR sample and irradiating the sample for 20 min

[94] 2) D20 was measured by adding Pi to a GR sample and leaving the sample in the dark for 20 min

[95] * R.L.I is an acronym for Relative Light Intensity, indicating a value of a relative luminescence intensity measured by a luminometer

[96] 3) L-D is a subtraction of D20 form L20

[97] 4) A value obtained from a pre-measured ATP calibration curve. The unit is moles ATP/assay

[98] 5) Because the volume of a final sample was 4 ㎖, the total amount of ATP produced from the 4 ㎖ volume was calculated

[99] 6) The amount of GR contained in 1 ㎖ of a sample

[100] 7) The amount of GR contained in the total volume of 4 ㎖

[101] 8) [5)/20 min]/7). The unit is moles ATP/min/㎎

[102]

[103] **<3-3> Measurement of the change in ATP production as time**

[104] When an *E. coli* membrane vesicle in which GR was expressed was stored at 4℃, it was confirmed whether a change in the amount of ATP production occurred as time proceeded. The same experiments as above were repeated 7 and 65 days from the day when three samples (GR3, GR4, and GR5) in FIG. 1 were first prepared. The samples were those including the best production efficiency of ATP. The determined values were recorded in Table 2, and mean values of the three samples were used to plot a change in the amount of ATP production as time proceeded.

[105]

[106] Table 2

[Table 2]

[Table]

Moles/ATP/min/㎎	GR3	GR4	GR5	Mean value
Day 0	8.58E-11	8.82E-11	6.44E-11	7.95E-11
Day 7	1.05E-11	1.08E-11	7.93E-12	9.74E-12
Day 65	2.48E-12	2.02E-12	3.16E-12	2.55E-12

[107]

[108] That is, each of the half-times (T_f) was calculated with respect to a mean value of the three values according to each time of measurement in FIG. 2. The mean value was fitted to an exponential decay by using the following Math Figure 1, and the results were recorded in a small graph in FIG. 2.

[109]

[110] MathFigure 1

[Math.1]

$$N_t = N_o \times e^{(-\lambda t)}$$

[111] N_t: the amount remaining without being decayed after t hours;

[112] N_0: the initial amount of the material which may be decayed;

[113] λ: decay constant.

[114] The y-axis of the small graph in FIG. 2 was transformed into the log scale, was shown in the big graph in FIG. 2, and the T_f values were calculated from the big graph by using the Math Figure 2.

[115]

[116] MathFigure 2

[Math.2]

$$T_{1/2} = \ln 2 \div \lambda = \ln 2 \times 3.06236 = 2.12 days$$

[117]

[118] As a result, it was determined as shown in FIG. 2 that the amount of ATP produced was exponentially reduced within 10 days from the day when the samples were prepared (Small graph). 10 days later, it was not exponentially reduced. In addition, the time for which the amount of ATP was reduced by half was 2.12 days from the calculation.

[119]

[120] The composition for ATP synthesis and the method for preparing ATP according to the present invention may stably provide a large amount of ATP for mass production of biomaterials, and the costs may be greatly reduced due to renewable use of the composition for ATP synthesis according to the present invention.

[121]

[122] As observed above, the composition for ATP synthesis and the method for synthesizing ATP using the same according to the present invention may be useful in mass production of biomaterials through ATP supply.

[123]

[124] Although the preferred embodiments of the present invention have been disclosed for illustrative purposes, those skilled in the art will appreciate that various modifications, additions and substitutions are possible, without departing from the scope and spirit of the invention as disclosed in the accompanying claims.

[125]

Claims

[Claim 1] A composition for ATP synthesis, comprising an inverted membrane vesicle comprising ATP synthase oriented to have an extroversion so as to pump in protons and produce ATP outside the membrane by a concentration gradient of *Gloeobacter* rhodopsin (hereinafter, referred to as GR) oriented to have an introversion and the pumped protons.

[Claim 2] The composition as set forth in claim 1, wherein the GR is derived from *Gloeobacter violaceus*.

[Claim 3] A method for preparing a composition for ATP synthesis, mounted for GR and ATP synthase to have orientation, comprising Steps:
1) preparing a recombinant expression vector operably linked to a polynucleotide represented by SEQ ID NO. 1 encoding GR;
2) transforming a host cell with the recombinant expression vector in Step 1) to prepare a transformant;
3) incubating the transformant in Step 2) to induce an expression of GR;
4) isolating a protoplast from a transformant overexpressing the GR in Step 3); and
5) preparing an inverted membrane vesicle from the transformant isolated in Step 4).

[Claim 4] The method as set forth in claim 3, wherein the host cell in Step 2) is *E. coli*.

[Claim 5] A method for synthesizing ATP by using the composition for ATP synthesis, comprising steps:
adding Pi and ADP to the composition for ATP synthesis of claim 1, and irradiating the composition.

[Claim 6] The method as set forth in claim 5, wherein an amount of the Pi added is approximately 1 pM to approximately 100 nM.

[Claim 7] The method as set forth in claim 5, wherein a visible light at a wavelength of at least 440 nm is irradiated.

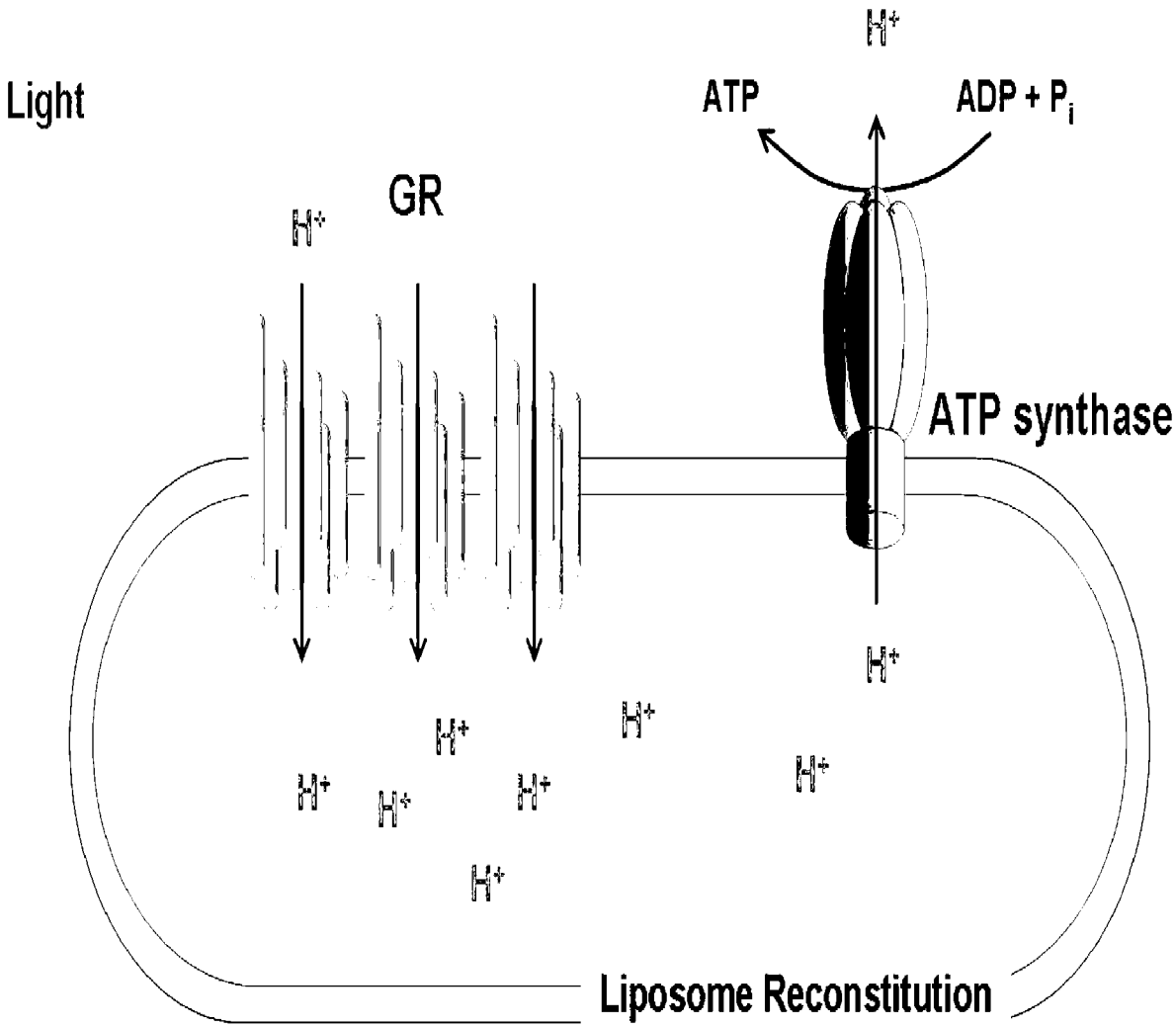
[Claim 8] A use of an inverted membrane vesicle comprising ATP synthase oriented to have an extroversion so as to pump in protons and produce ATP outside the membrane by a concentration gradient of *Gloeobacter* rhodopsin (hereinafter, referred to as GR) oriented to have an introversion and the pumped protons in preparation of a composition for ATP synthesis.

[Claim 9] The use as set forth in claim 8, wherein the GR is derived from

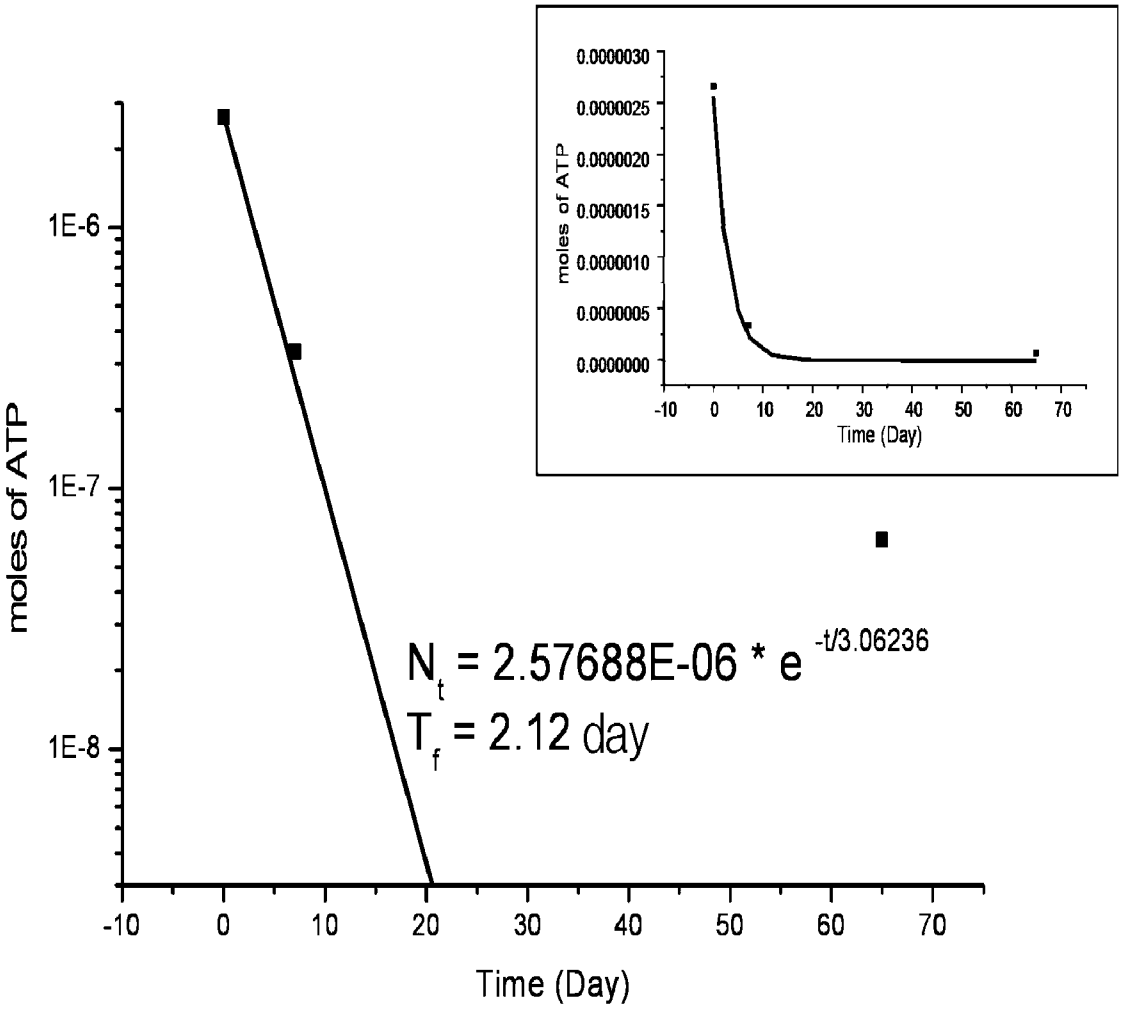
Gloeobacter violaceus.

WO 2011/030964 PCT/KR2009/006736

[Fig. 1]



[Fig. 2]


moles of ATP
1E-6
1E-7
1E-8
-10
0
10
20
30
40
50
60
70
Time (Day)
$N_t = 2.57688E\text{-}06 * e^{-t/3.06236}$
$T_f = 2.12$ day
0.0000030
0.0000025
0.0000020
0.0000015
0.0000010
0.0000005
0.0000000
moles of ATP
-10
0
10
20
30
40
50
60
70
Time (Day)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2009/006736

A. CLASSIFICATION OF SUBJECT MATTER

C12N 9/96(2006.01)i, C12N 9/98(2006.01)i, C12N 15/63(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 9/96

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal), Pubmed, Delphion

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GARTY, H. et al. `Preparation and characterization of inverted cell envelops of Halobacterium halobium.`European Journal of Biochemistry. Vol. 111, pp. 411-418 (October 1980) See p. 415, right col, line 20 ~p. 416, right col, line 33.	1-9
Y	KAMO, N. et al. `A light-driven proton pump from Haloterrigena turkmenica: functional expression in Escherichia coli membrane and coupling with a H+ co-transporter.` Biochemical and Biophysical Research Communications. Vol. 341(2),pp. 285-290 (10 January 2006) See the abstract.	1-9
A	YOSHIDA, M. et al. `ATP synthesis catalyzed by purified DCCD-sensitive ATPase incorporated into reconstituted purple membrane vesicles.` Biochemical Biophysical Research Communications. Vol. 67(4):1295-1300 (15 December 1975) See the abstract.	1-9
A	PITARD, B. et al.'ATP synthesis by the F0F1 ATP synthase from thermophilic Bacillus PS3 reconstituted into liposomes with bacteriorhodopsin. 1. Factors defining the optimal reconstitution of ATP synthases with bacteriorhodopsin. ' European Journal of Biochemistry. Vol. 235(3):769-778 (1 February 1996) See the abstract.	1-9

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 NOVEMBER 2010 (09.11.2010)	**10 NOVEMBER 2010 (10.11.2010)**

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
Government Complex-Daejeon, 139 Seonsa-ro, Seo-gu, Daejeon 302-701, Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
KIM, JI YUN
Telephone No. 82-42-481-8288

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2009/006736

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

a. a sequence listing filed or furnished

- ☐ on paper
- ☒ in electronic form

b. time of filing or furnishing

- ☒ contained in the international application as filed
- ☐ filed together with the international application in electronic form
- ☐ furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2009/006736

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PITARD, B. et al. 'ATP synthesis by the F0F1 ATP synthase from thermophilic Bacillus PS3 reconstituted into liposomes with bacteriorhodopsin. 2. Relations between proton motive force and ATP synthesis.' European Journal of Biochemistry. Vol. 235(3), pp. 779-788 (1 February 1996) See the abstract.	1-9
A	FREISLEBEN,H. et al. 'Reconstitution of bacteriorhodopsin and ATP synthase from Micrococcus luteus into liposomes of the purified main tetraether lipid from Thermoplasma acidophilum: proton conductance and light-driven ATP synthesis' Chemistry and Physics of Lipids. Vol. 78:137-147 (17 November 1995) See the abstract.	1-9

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2009/006736

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
None			