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Top Starch Plating Method for the Efficient Cultivation of Unicellular Red Alga *Cyanidioschyzon merolae*

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[Abstract] The unicellular red alga *Cyanidioschyzon merolae* has been used as a model photosynthetic eukaryote for various basic and applied studies, and several of these molecular genetics techniques have been reported. However, there are still improvements to be made concerning the plating method. The conventional plating method often generates diffuse colonies and single colonies cannot be easily isolated. To overcome these problems, we established a novel plating method for *C. merolae*, making use of melted cornstarch as the use of top agar plating in bacterial genetics. This method improved the formation of defined colonies in at least 4-fold higher efficiency than the conventional method, and made the handling procedure much easier than the previous method.

Keywords: *Cyanidioschyzon merolae*, Plating method, Single colony isolation, Top starch solution method, MA2

[Background] *Cyanidioschyzon merolae* 10D is a unicellular red alga that inhabits sulfate acid hot springs (Matsuzaki *et al.*, 2004). It has a simple cellular structure, composed of a minimum set of organelles: one nucleus, one mitochondrion, and one plastid in a cell. *C. merolae* is used as a model organism to investigate the basic architecture of eukaryotes. Thus, many molecular genetics techniques have been developed using this organism, including antisense suppression, transient expression, gene disruption, and stable insertion into the genome (Minoda *et al.*, 2004; Ohnuma *et al.*, 2008; Imamura *et al.*, 2009; Imamura *et al.*, 2010; Zienkiewicz *et al.*, 2017a; Zienkiewicz *et al.*, 2017b; Zienkiewicz *et al.*, 2018). The plating method for the selection of transformants is important for transformation experiments. However, *C. merolae* shows considerably low plating efficiencies (less than 0.5%) on MA2 solid gellan gum plates. The direct spread of cells onto an MA2 solid gellan gum plate usually results in the death of the cells (Figure 1A). Hence, the plating method has been modified using the cornstarch embedding method which is commonly used for the cell-wall-less *Chlamydomonas reinhardtii* strain (Shimogawara *et al.*, 1998). In the cornstarch embedding method, cells are grown on a starch bed prepared by spotting 20% slurry cornstarch on the MA2 solid gellan gum. Cells form colonies on the solid starch bed, but these are often diffused or blurred (Figure 1B).

To improve these points, we here devised a novel plating method using melted cornstarch (named in this protocol “top starch solution method”), similar to the top-agar plating method used in bacterial genetics. The 2% cornstarch in dH_2O was melted at 98 °C for 10 min. The melted cornstarch solution

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(= top starch solution) was mixed with an equal volume of the liquid MA2 medium containing *C. merolae* cells, and poured onto the surface of the MA2 solid gellan gum. Cells formed defined colonies using this plating method (Figure 1C). The plating efficiency of the top starch solution method was more than 2.0%, while that of the conventional method was less than 0.5%. In the conventional method, it was necessary to prepare many starch beds on solid plates prior to use, but the top starch solution method only requires the pouring of the melted starch-cell mixture onto the solid medium, making the plating procedure quick and easy. In this report, we describe the detailed procedure of the top starch solution method.

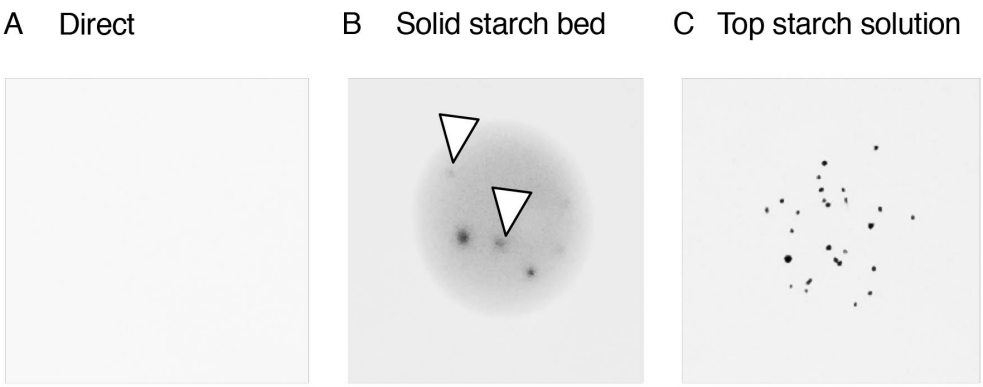

Figure 1. Effect of the top starch solution method on the colony formation in *C. merolae*. (A-C) In each approach, plates with the spotted cells were incubated for two weeks at 40 °C under continuous white light. A. Five hundred cells were directly spotted onto an MA2 solid gellan gum plate, which resulted in no visible colony formation. B. Solid starch beds were made using 15 µl of 20% slurry cornstarch in an MA2 liquid medium. Five hundred cells were spotted onto a solid cornstarch bed. Open triangles indicate diffused, blurred colonies. C. The 2% cornstarch in dH_2O was melted at 98 °C for 10 min to make the top starch solution. The top starch solution was mixed with an equal volume of cells in MA2 medium. Fifteen microliter aliquots containing 500 cells were spotted onto the MA2 solid gellan gum plate.

Materials and Reagents

1. Pipette tips (NIPPON Genetics Co., Ltd., catalog numbers: FG-102, FG-301, FG-401)
2. Parafilm (LMS Co., Ltd., catalog number: PF, PM-996)
3. 1.5 ml microtube (BM Equipment Co., Ltd., catalog number: NT-175)
4. 2.0 ml microtube (WATSON Co., Ltd., catalog number: 132-620C)
5. 50 ml centrifuge tube (Corning Incorporated., catalog number: 352196)
6. AnaeroPouch (Mitsubishi Gas, catalog number: 2-3764-02)
7. AnaeroPack (Mitsubishi Gas, catalog number: 2-3765-01)
8. Sterilized plastic plate (Ø 90 mm) (As One Corporation., catalog number: GD90-15)
9. *Cyanidioschyzon merolae* 10D [available as NIES-3377 from NIES collection, Tsukuba, Japan (http://mcc.nies.go.jp/index_en.html)]

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10. Cornstarch (Kawamitsu-Bussan, catalog number: 4901 486 02701 6)
11. Gellan gum (FUJIFILM Wako Pure Chemical Corporation, catalog number: 073-03071)
12. Uracil (Sigma-Aldrich, catalog number: U0750-5G)
13. 5-Fluoroorotic acid monohydrate (5-FOA) (FUJIFILM Wako Pure Chemical Corporation, catalog number: 003234)
14. Ethanol (FUJIFILM Wako Pure Chemical Corporation, catalog number: 055-00457)
15. $(NH_4)_2SO_4$ (FUJIFILM Wako Pure Chemical Corporation, catalog number: 019-03435)
16. $MgSO_4 \cdot 7H_2O$ (FUJIFILM Wako Pure Chemical Corporation, catalog number: 138-00415)
17. H_2SO_4 (FUJIFILM Wako Pure Chemical Corporation, catalog number: 192-04696)
18. H_3BO_3 (FUJIFILM Wako Pure Chemical Corporation, catalog number: 021-02195)
19. $MnCl_2 \cdot 4H_2O$ (FUJIFILM Wako Pure Chemical Corporation, catalog number: 133-00725)
20. $ZnCl_2$ (FUJIFILM Wako Pure Chemical Corporation, catalog number: 268-01022)
21. $Na_2MoO_4 \cdot 2H_2O$ (FUJIFILM Wako Pure Chemical Corporation, catalog number: 198-02471)
22. $CoCl_2 \cdot 6H_2O$ (FUJIFILM Wako Pure Chemical Corporation, catalog number: 038-03681)
23. $CuCl_2 \cdot 2H_2O$ (FUJIFILM Wako Pure Chemical Corporation, catalog number: 039-04135)
24. KH_2PO_4 (FUJIFILM Wako Pure Chemical Corporation, catalog number: 169-04245)
25. $CaCl_2 \cdot 2H_2O$ (FUJIFILM Wako Pure Chemical Corporation, catalog number: 038-19735)
26. $FeCl_3 \cdot 6H_2O$ (FUJIFILM Wako Pure Chemical Corporation, catalog number: 090-02802)
27. Na_2EDTA (FUJIFILM Wako Pure Chemical Corporation, catalog number: 345-01865)
28. 20% slurry cornstarch stock solution (see Recipes)
29. MA2 medium (Kobayashi *et al*., 2010) (see Recipes)
    a. MA2 solution I
    b. A6 minor salts
    c. MA2 solution II
    d. MA2 solution III
    e. MA2 solution IV
30. MA2 solid gellan gum plate (see Recipes)

Equipment

1. Pipettes (Gilson, Inc., catalog numbers: F123600, F123601, F123602)
2. Refrigerated centrifuge (Koki Holdings Co., Ltd., model: CF16RXII)
3. Microcentrifuge (TOMY Seiko Co., Ltd., model: MX150)
4. 500 ml flask (IWAI, catalog number: 4442FK500)
5. Heat block (Chiyoda Science Co., Ltd., model: MiniT-100)
6. Bio incubator (TOMY Seiko Co., Ltd., model: CLE-303)
7. Clean bench (Panasonic Healthcare Co., Ltd., model: MCV-131BNF)
8. Vortex mixer (M & S Instruments Inc., model: SI-0286)
9. Sterile filter (Ø 0.22 μm, Merck, catalog number: SLGV033RS)

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Procedure

1. Prepare the MA2 solid gellan gum plate and the 20% slurry cornstarch stock solution (see Recipes).
2. Mix 100 µl of the cornstarch slurry stock solution and 900 µl of sterile dH_2O in a 2 ml microtube.
3. Centrifuge the microtubes at 500 *x g* for 1 min at room temperature and discard the supernatant.
4. Add 900 µl of sterile dH_2O and mix vigorously.
5. Centrifuge again at 500 *x g* for 1 min at room temperature and discard the supernatant.
6. Add 900 µl of sterile dH_2O and mix vigorously.
7. Incubate the microtube at 98 °C for 10 min but continue to mix thoroughly by inverting the tube every 2 min.
8. Cool down at room temperature for 10 min (= top starch solution).
   Note: Steps 8-11 should be performed in a laminar flow cabinet.
9. Add 1 ml of MA2 culture medium containing *C. merolae* cells (in case of wild type, 500 cells by microscopy observation) to the top starch solution and mix gently.
10. Pour the mixture onto the surface of the MA2 solid gellan gum plate and spread the mixture over the whole plate area by tilting the plate to spread the top starch solution uniformly.
11. Leave the plate open for 15-20 min to allow the top starch solution to solidify. After this step, the surface of the top starch is still wet, and the plate should be incubated with the top starch solution pointing upwards for the entire time.
12. Incubate the MA2 solid gellan gum plate at 40 °C under continuous white light (35-50 µmol m^{-2} s^{-1}) supplemented with 5% CO_2 in AnaeroPack with AnaeroPouch.
13. After 2-4 weeks, colonies of the transformed cells appear on the MA2 solid gellan gum plate.

Recipes

1. 20% slurry cornstarch stock solution (Fujiwara and Ohnuma, 2018)
   a. Add 10 g of cornstarch and 40 ml of dH_2O to the 50 ml conical tube
   b. Mix the tube well using a vortex mixer
   c. Centrifuge the tube at 1,200 *x g* for 5 min at 4 °C
   d. Discard the supernatant by decantation
   e. Resuspend the pellet in 40 ml of 100% of ethanol using a vortex mixer
   f. Centrifuge the tube at 1,200 *x g* for 5 min at 4 °C
   g. Discard the supernatant by decantation
   h. Fill up to 50 ml with 75% of ethanol
   i. Store the tube at 4 °C, wrapped using parafilm until use
2. MA2 medium
   a. Mix 100 ml of MA2 solution I, 10 ml of MA2 solution II, 1 ml of MA2 solution III and 885 ml of dH_2O in a glass bottle and sterilize by autoclaving

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b. Add 4 ml of MA2 solution IV to the mixture in a laminar flow cabinet

MA2 solution I

Components	Amount	Conc.	Final conc. in MA2
$(NH_4)_2SO_4$	52.9 g	400 mM	40 mM
$MgSO_4·7H_2O$	9.9 g	40 mM	4 mM
H_2SO_4	3 ml	0.3% (v/v)	0.03% (v/v)
A6 minor salts	40 ml		
H_2O	Up to 1,000 ml		

A6 minor salts

Components	Amount	Conc.	Final conc. in MA2
H_3BO_3	2.85 g	46 mM	184 µM
$MnCl_2·4H_2O$	1.8 g	9 mM	36.4 µM
$ZnCl_2$	0.105 g	0.77 mM	3.08 µM
$Na_2MoO_4·2H_2O$	0.39 g	1.6 mM	6.44 µM
$CoCl_2·6H_2O$	0.04 g	0.17 mM	6.72 µM
$CuCl_2·2H_2O$	0.043 g	0.25 mM	1.01 µM
H_2O	Up to 1,000 ml		

MA2 solution II

Components	Amount	Conc.	Final conc. in MA2
KH_2PO_4	10.9 g	800 mM	8 mM
H_2O	Up to 100 ml		

MA2 solution III

Components	Amount	Conc.	Final conc. in MA2
$CaCl_2·2H_2O$	14.7 g	1 M	1 mM
H_2O	Up to 100 ml		

MA2 solution IV

Components	Amount	Conc.	Final conc. in MA2
$FeCl_3·6H_2O$	0.68 g	25 mM	0.1 mM
Na_2EDTA	0.74 g	20 mM	0.075 mM
H_2SO_4	3 drops		
H_2O	Up to 100 ml		

Note: This MA2 solution IV should be sterilized by filtration and stored at 4 °C in the dark.

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3. MA2 solid gellan gum plate (for making 9-10 plates)
   a. Mix 30 ml of MA2 solution I, 3 ml of MA2 solution II, 300 µl of MA2 solution III, and 70 ml of dH_2O in a 500 ml flask. Mix 200 ml of dH_2O and 1.5 g of gellan gum in another 500 ml flask. Sterilize these flasks by autoclaving, and subsequent plate preparation should be performed in a laminar flow cabinet
   b. After autoclaving, mix both solutions and add 1.2 ml of MA2 solution IV. In case of 5-FOA selection to isolate the *URA* deficient mutants (for example applying *URA* marker recycling system, Takemura *et al.*, 2018; Takemura *et al.*, 2019), add directly 150 mg of uracil (final concentration: 0.5 mg/ml) and 240 mg of 5-FOA (final concentration: 0.8 mg/ml) in powder to the flask
   c. Pour the mixed solution into the sterilized plastic plates
   d. Dry the plates at room temperature for 15 min
   e. Store at 4 °C in the dark

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Competing interests

No competing interests declared.

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