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Chitin Extraction and Content Measurement in *Magnaporthe oryzae*

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[Abstract] Chitin is a linear polysaccharide composed of β (1→4)-linked *N*-acetylglucosamine (GlcNAc) residues. In fungi, chitin is an important component of the cell wall. Here, we provide a protocol to measure the chitin content of fungal cells using *Magnaporthe oryzae* as an example.
Keywords: Chitin, Cell wall, GlcNAc, Pathogenicity, *Magnaporthe oryzae*

[Background] Chitin is an important component of the cell wall in fungal pathogens and is well known as a pathogen associated molecule pattern (PAMP). Determining the chitin content of a fungal species is important for studying fungal biology and host-pathogen interactions. The Morgan-Elson method based on colorimetric approach has been adapted in yeast to measure cellular chitin levels (Leloir and Cardini, 1953; Bulik *et al*., 2003; Baker *et al*., 2007). However, there is no specific protocol established for *Magnaporthe oryzae*, which is the causal agent of rice blast, the most important fungal disease in the world. Here, we describe a reliable and simple protocol which was modified from the Morgan-Elson method to test the chitin content of *M. oryzae* (Song *et al.*, 2010).

Materials and Reagents

1. Microtube
2. Microtiter plates (Corning, Costar®, catalog number: 42592)
3. Miracloth (EMD Millipore, catalog number: 475855)
4. Cells of *Magnaporthe oryzae*
5. Potassium hydroxide (KOH) (Sangon Biotech, catalog number: A610441)
6. 10x phosphate buffered saline (PBS, 1.35 M NaCl, 47 mM KCl, 100 mM Na_2HPO_4, 20 mM NaH_2PO_4, pH = 7.4) (Beyotime, catalog number: ST476)
7. *Streptomyces* plicatus chitinase (Sigma-Aldrich, catalog number: C6137)
8. Sodium borate (Sangon Biotech, catalog number: A100390)
9. GlcNAc (Sigma-Aldrich, catalog number: PHR1432-1G)
10. Sodium hydrogen (Na_2HPO_4) (Sangon Biotech, catalog number: A501727)
11. Citric acid (Sangon Biotech, catalog number: A501702)
12. *p*-dimethylaminobenzaldehyde (Sigma-Aldrich, catalog number: D2004)
13. Hydrochloric acid (HCl)

Vol 7, Iss 05, Mar 05, 2017
DOI:10.21769/BioProtoc.2164

14. Acetic acid (Sangon Biotech, catalog number: A501931)
15. Biotin
16. Pyridoxin
17. Thiamine
18. Riboflavin
19. p-aminobenzoic acid
20. nicotinic acid
21. $ZnSO_4 \cdot 7H_2O$
22. H_3BO_3
23. $MnCl_2 \cdot 4H_2O$
24. $FeSO_4 \cdot 7H_2O$
25. $CoCl_2 \cdot 6H_2O$
26. $CuSO_4 \cdot 5H_2O$
27. $Na_2MnO_4 \cdot 2H_2O$
28. Na_4EDTA
29. $NaNO_3$
30. KCl
31. $MgSO_4 \cdot 7H_2O$
32. KH_2PO_4
33. D-glucose
34. Peptone
35. Yeast extract
36. Casamino acid
37. Agar
38. McIlvaine’s buffer (see Recipes)
39. Ehrlich’s solution (see Recipes)
40. Vitamin solution (see Recipes)
41. Trace elements (see Recipes)
42. 20x nitrate salts (see Recipes)
43. CM medium (see Recipes)

Equipment

1. Freeze dryer (Marin Christ German)
2. Vortex mixer
3. Water bath (SHEL Lab, model: W6M-2)
4. Centrifuge (Eppendorf centrifuge) (Eppendorf, model: 5418)
5. Incubator shaker (Crystal Technology & Industries, model: IS-RSD3)
6. pH/ATU electrode (Sartorius, German)

Vol 7, Iss 05, Mar 05, 2017
DOI:10.21769/BioProtoc.2164

7. Microplate reader (Molecular Devices, model: VersaMax ELISA)
8. PCR instrument (Takara Bio, model: TP600)
9. Eppendorf micropipette (1,000 µl, 100 µl, 10 µl)
10. Dark glass bottle

Software

1. SPSS 2.0 (Chicago, IL, USA)

Procedure

1. All of the strains are cultured on solid CM medium for 7 days at 28 °C. The agar culture is cut into 1 x 1 mm squares and the squares are cultured in liquid CM for another 2 days.
2. Filtered through one layer of Miracloth to collect mycelium from liquid CM. Then the mycelium is quickly lyophilized by a freeze dryer for 24 h. 5 mg mycelium is mixed with 1 ml 6% KOH in each 2 ml microtube using a vortex mixer and then incubated in a water bath at 80 °C for 90 min.
3. Samples are centrifuged at 16,000 *x g* for 10 min, and the suspension is discarded.
4. Each pellet is washed with 1 ml 1x PBS (which is diluted from 10x PBS) for three times and then centrifuged at 16,000 *x g* for 5 min to discard the suspension.
5. Each pellet is resuspended with 0.5 ml of McIlvaine's buffer (see Recipes) (Baker *et al.*, 2007). 100 µl chitinase is added into each sample. And then samples are incubated at 37 °C for 16 h in the dark, at 220 rpm in an incubator shaker.
6. Chitinase-treated samples are mixed with equal volume of 0.27 M sodium borate (pH = 9.0) and incubated at 100 °C for 10 min in a PCR instrument.
7. After being cooled down to room temperature, 200 µl of each sample is added to 1 ml Ehrlich's solution (see Recipes) and then incubate at 37 °C for 30 min in an incubator shaker.
8. 100 µl of each sample is transferred into a well of a microtiter plate with low-evaporation and the absorbance is measured at 585 nm by a microplate reader. Standard curves are prepared from stocks of 0.1 to 2.0 mM (0.1 mM, 0.5 mM, 1.0 mM, 1.5 mM and 2.0 mM) GlcNAc.

Data analysis

Standard curves are prepared from stocks of 0.1 to 2.0 mM GlcNAc. Each result is presented at least three replicated measurements. The significance of differences between treatments is statistically evaluated using SDs and one-way analysis of variance (ANOVA) in SPSS 2.0 (Chicago, IL, USA). Data for two specific different treatments are compared statistically using ANOVA, followed by an *F*-test if the ANOVA result is significant at $P < 0.05$ or $P < 0.01$.

Vol 7, Iss 05, Mar 05, 2017
DOI:10.21769/BioProtoc.2164

Notes

1. Both McIlvaine's buffer and Ehrlich's solution need to be prepared just before use.
2. In step 1, 25 squares are enough and the volume of the liquid CM is 70 ml.
3. In step 2, samples are incubated in a water bath at 80 °C for 90 min. Samples need to be vortexed every 15 min.
4. In step 6, after being extracted at 100 °C for 10 min, all the samples should be cooled on ice to room temperature immediately.
5. In step 6, after being extracted at 100 °C for 10 min in the PCR instrument, the solution is clear and transparent
6. In step 7, the Ehrlich's solution is clear and does not need to be centrifuged.
7. If the samples are difficult to suspend, break the pellet by an injector or a micropipette.

Recipes

1. McIlvaine's buffer
   0.2 M Na_2HPO_4
   0.1 M citric acid (pH = 6.0)
2. Ehrlich's solution
   10 g *p*-dimethylaminobenzaldehyde in 12.5 ml concentrated HCl (37%) and 87.5 ml glacial acetic acid
3. Vitamin solution (100 ml)
   0.01 g Biotin
   0.01 g Pyridoxin
   0.01 g Thiamine
   0.01 g Riboflavin
   0.01 g p-aminobenzoic acid
   0.01 g nicotinic acid
   Add ddH_2O to 100 ml and store in a dark glass bottle at 4 °C
4. Trace elements (100 ml)
   2.2 g $ZnSO_4 \cdot 7H_2O$
   1.1 g H_3BO_3
   0.5 g $MnCl_2 \cdot 4H_2O$
   0.5 g $FeSO_4 \cdot 7H_2O$
   0.17 g $CoCl_2 \cdot 6H_2O$
   0.16 g $CuSO_4 \cdot 5H_2O$
   0.15 g $Na_2MnO_4 \cdot 2H_2O$
   5 g Na_4EDTA
   Add ddH_2O to 100 ml and adjust the pH to 5.8, store at 4 °C

Vol 7, Iss 05, Mar 05, 2017
DOI:10.21769/BioProtoc.2164

5. 20x nitrate salts (1 L)
   120 g $NaNO_3$
   10.4 g KCl
   10.4 g $MgSO_4 \cdot 7H_2O$
   30.4 g KH_2PO_4
   Add ddH_2O to 1 L and store at 4 °C
6. CM medium
   10 g D-glucose
   2 g peptone
   1 g yeast extract
   1 g casamino acid
   1 ml vitamin solution
   1 ml trace elements
   50 ml 20x nitrate salts
   Add ddH_2O to 1 L
   For solid media add 15 g agar and autoclave at 121 °C for 20 min

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