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Defined medium for Neocallimastigomycota V.1

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HiPoAF

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We use this protocol and it's working

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Disclaimer

Abstract

This is the protocol for the completely defined medium base for Neocallimastigomycota (anaerobic gut fungi, AGF) that was used within the HiPoAF and FUNGAS projects.

If you add 2 g/L xylan and 3 g/L cellobiose as C-sources to this medium, Neocallimastigomycota - in our experience - can grow on it for prolonged periods (more than 18 months and running, with weekly sub-cultivation). We use this medium, however, more for short-term experiments rather than long-term culture maintenance. So far, we have tested this medium on strains of *Anaeromyces mucronatus*, *Caecomyces communis*, *Neocallimastix cameroonii*, *Neocallimastix frontalis*, *Pecoramyces ruminantium* and all were able to grow. However, depending on the C-source added, they might show unusual clustering behaviour (e.g., clinging onto the glass).

This completely defined medium remains nearly colourless if no C-source is added before autoclaving the bottles, which is especially convenient for microscopy. For this purpose, a soluble C-source can be added directly to the bottles after autoclaving.

Image Attribution

Images were taken by Julia Vinzelj

Materials

Here we list the ingredients of our minimal medium for AGF cultivation and the preparation of stock solutions needed for this medium:

	Ingredient	Amount per litre
	Hemin Solution	2 mL
	L-cysteine-HCl	1 g
	MilliQ water	700 mL
	Resazurin Solution	2 mL
	Salt Soluion I	150 mL
	Salt Solution II	150 mL
	Sodium hydrogen carbonate (NaHCO3)	6 g

Ingredients of 1 litre of completely defined medium. This medium contains no C-source, so be sure to add the appropriate one for your experiments! For minimal visual disturbance, you can add 3 g/L cellobiose or 6 g/L glucose (useful in microscopy, for example). Complex, insoluble C-sources (e.g. wheat straw) should be added directly into the serum bottles (before adding the medium), at a concentration of around 0.35 g / 50 mL.

Hemin solution is prepared by dissolving [M] 1 mg/mL hemin powder in a 1:1 mixture of 96% ethanol and [M] 0.05 Molarity (M) NaOH. The solution is then filter sterilzed (0.22µm pore size) and stored at 4 °C until use.

Resazurin solution is prepared by dissolving [M] 1 mg/mL resazurin powder in MilliQ water and filter sterilizing it (0.22µm pore size). The solution is then stored at 4 °C until use.

Salt Solution I consists of [M] 3 g/L K_2HPO_4 dissolved in MilliQ water. It is autoclaved (121 °C , 00:20:00), and stored at 4 °C until use.

Salt Solution II consists per litre of 3 g KH_2PO_4, 6 g $(NH_4)_2SO_4$, 6 g NaCl, 0.6 g $MgSO_4.7H_2O$, and 0.6 g $CaCl_2.2H_2O$. The ingredients are dissolved in MilliQ water one after the other. The

solution is then autoclaved (121 °C , 00:20:00), and stored at 4 °C until use.

	Ingredient	g/L
	CoCl2.6H2O	0.05
	CuSO4	0.021
	FeSO4.7H2O	0.20
	H3BO3	0.25
	MnCl2.4H2O	0.25
	Na2MoO4.2H2O	0.28
	Na2SeO3	0.04
	NaVO3	0.03
	NiCl2.6H2O	0.25
	ZnSO4.7H2O	0.026

Trace Elements Solution composition. The chemicals are dissolved in 0.2M HCl solution. The solution is then aliquoted, autoclaved (121 °C , 00:20:00), and stored at 4°C.

PAS-212 antibiotic stock solution is prepared by slowly dissolving [M] 20 mg/mL Streptomycin sulfate, [M] 20 mg/mL Penicillin G sodium salt, and [M] 10 mg/mL Ampicillin sodium salt in distilled water. The mixture is then gassed with pure CO_2 for up to 00:20:00 , then closed with a rubber stopper and crimped to obtain and maintain anoxic conditions. With the help of a needle and syringe, the solution is filter sterilized (0.22µm pore size) into a sterile, anoxic, closed & crimped serum bottle and stored at 4 °C until use.

	Ingredient	g/L
	Biotin	0.2
	Calcium Pantothenate	0.6
	Folic acid	0.05
	Niacin	0.6
	Nicotinamide	1.0
	p-Aminobenzoic acid	0.05
	Pyridoxamine	0.1
	Riboflavin	0.2
	L-Thiamine hydrochloride	0.01
	Vitamin B12 (Cyanocobalamin)	0.02

Vitamin Solution Composition. The chemicals are dissolved in MilliQ water and are then filter sterilized (0.22µm pore size) into aliquots. Niacin and Nicotinamide should be added under an extraction hood. Store at 4°C. Vitamin solution must not be autoclaved and is usually added to sterile media bottles shortly before use.

Serum bottles. For standard AGF cultivation, we usually use glass serum bottles with an ND20 head and a total of volume of 120 mL. In those serum bottles, we do not add more than 50 mL medium, to ensure an adequate amount of head space. Alternatively, smaller serum bottles with a total volume of 60 mL also work (filled with no more than 30 mL of medium). We recommend using the bulky serum bottles rather than the long, thin ones because the longer, thinner ones tend to easily break and are less convenient when handling the bottles.

Rubber stoppers. The bottles are usually closed with 3-legged butyl rubber stoppers or with full-body black rubber stoppers and crimped with a metal cap.

Safety warnings

Safety information

Please make sure that you attend to all safety regulations regarding the handling of CO_2 gas.

Safety information

Be also aware of the risks of autoclaving closed serum bottles, and make sure your autoclave has an appropriate program for that.

Before start

Before you start, make sure you have prepared all necessary stock solutions, and have all chemicals needed at hand (see Materials section for details).

You will need approx. 02:00:00 in total to prepare the medium (depending on the volume you prepare, it can take longer or might be faster).

Safety information

Please also ensure, that all necessary safety measures are in place (see also: Safety Warnings).

1 Add salt solution I, salt solution II, hemin solution, resazurin solution, and MilliQ water to a cooking pot.
If you want to use xylan as a C-source (or other C-sources that don't dissolve well in water), you should add it here as well.

2 Heat the mixture and simmer until everything is dissolved and the colour starts to change to yellow. For us with 2 L of medium in a 5 L cooking pot, this takes about 00:20:00 . 20m

Note

Take care not to cook it too long. Otherwise, you will lose a lot of water, making the medium more concentrated. You can also microwave it carefully instead of cooking it in a pot.

2.1 While the mixture is heating up, flush a large enough Erlmeyer flask with pure CO_2 for 00:02:00 . 2m

3 Carefully pour the mixture from the pot into the Erlmeyer flask. Cool it with ice-water while agitating it by magnetic stirrer and bubble it with pure CO_2. This takes about 00:10:00 to 00:20:00 30m

4 Once the mixture has cooled to around 39 °C, add the trace elements solution, $NaHCO_3$, soluble C-source (like cellobiose, glucose, etc.; if necessary) and lastly the L-cysteine-HCl. Bubble with CO_2 for another 00:10:00 . 10m

5 Set the pH of the medium to pH 6.9 with the help of $NaHCO_3$ powder or 5M NaOH solution. The medium should now be a translucent yellowish colour.

5.1 While setting the pH, flush your serum bottles with pure CO_2 for 1-2 minutes, to minimize oxygen contamination when aliquoting your medium in step 6.

6 Aliquot the medium into your serum bottles under continuous flow of CO_2 in the serum bottles and the Erlmeyer flask filled with medium. Depending on the amount of medium prepared this step takes around 00:30:00 . 30m

Dispension of medium into 120mL glass serum bottles (max. 50mL per bottle). The medium depicted is not the medium described in this protocol. The process, however, is the same.
Picture copyright: Julia Vinzelj & Nico Peer

6.1 Take the gas hose out of the serum bottle and immediately close it with a rubber stopper, then crimp the bottle with an aluminium cap. The medium can turn slightly pink at first upon transfer, but this usually settles quickly. If the medium turns bright pink upon

5m

transfer, however, keep bubbling it with CO_2 within the serum bottles for up to 00:05:00 min before closing it up.

Closing of the serum bottles with 3-legged butyl-rubber stoppers after the medium has been dispensed. The medium depicted is not the medium described in this protocol. The process, however, is the same.
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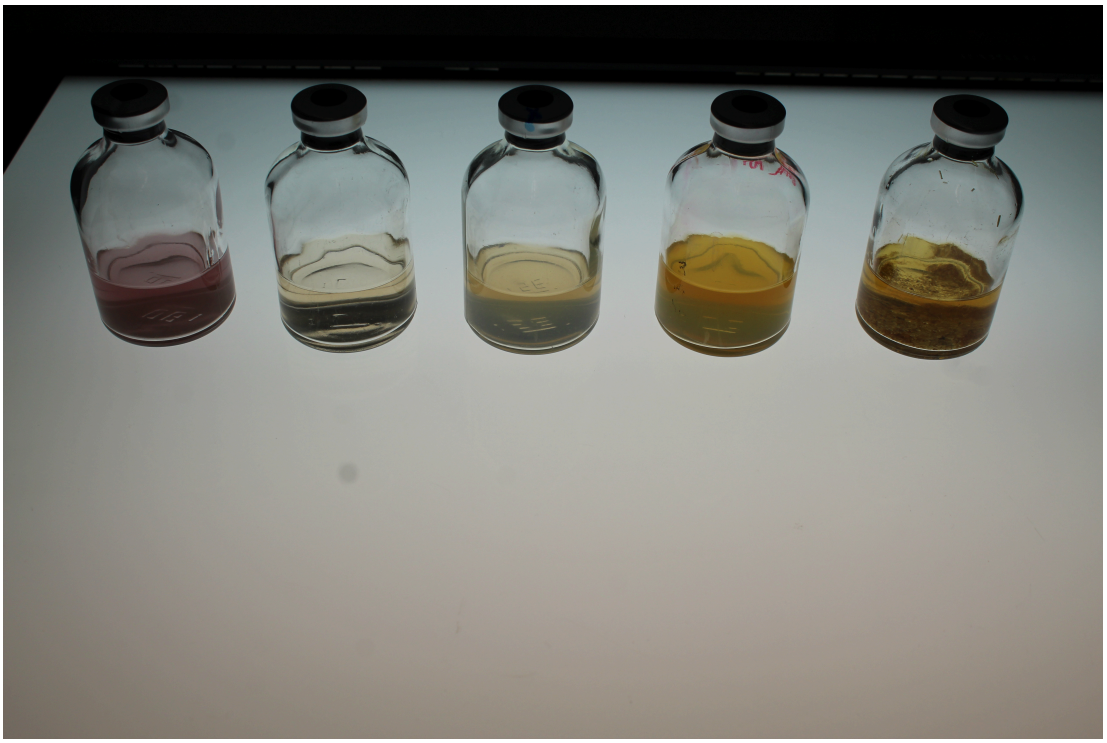
7 Autoclave the serum bottles at 121 °C for 00:20:00 . 20m

8 Store the bottles without regard to temperature for up to 2 months. 4 °C works as well as 37 °C for the duration of 2 months at least. Discard any bottles with medium that turned pink or red.

8.1 Before use, the bottles should be warmed to 39 °C . Also, 0.01mL vitamin solution must be added per mL of medium shortly before use by injection. If needed, a sterilized solution of soluble C-source (e.g. glucose, cellobiose), and antibiotic stock solution can be injected as well.

Injection of Vitamin Solution into sterile, pre-warmed serum bottles shortly before use.
Picture copyright: Julia Vinzelj & Nico Peer



Colours of the completely defined medium. From left to right: medium turned pink (should not be used; indicates oxygen contamination); sterile medium with no C-source added (we call it "Brunhilde" in our lab, because we are incredibly funny and working with us in the lab is a blast); sterile medium with vitamin solution and glucose solution added after autoclaving; sterile medium with xylan and cellobiose added before autoclaving (known to us as "Agathe"); sterile medium with only wheat straw as C-source (our "Berthelm").
Picture copyright: Julia Vinzelj

Protocol references

As of Feb 2024, this medium has not yet been used in any publication. It is, however, based on rumen fluid free medium that has been published several times:

Joshi, Akshay, Diana Young, Liren Huang, Lona Mosberger, Bernhard Munk, Julia Vinzelj, Veronika Flad, u. a. „Effect of Growth Media on the Diversity of Neocallimastigomycetes from Non-Rumen Habitats“. Microorganisms 10, Nr. 10 (5. Oktober 2022): 1972. https://doi.org/10.3390/microorganisms10101972.

Stabel, Marcus, Tabea Schweitzer, Karoline Haack, Pascal Gorenflo, Habibu Aliyu, und Katrin Ochsenreither. „Isolation and Biochemical Characterization of Six Anaerobic Fungal Strains from Zoo Animal Feces“. Microorganisms 9, Nr. 8 (August 2021): 1655. https://doi.org/10.3390/microorganisms9081655.

Stabel, Marcus, Radwa A. Hanafy, Tabea Schweitzer, Meike Greif, Habibu Aliyu, Veronika Flad, Diana Young, u. a. „Aestipascuomyces Dupliciliberans Gen. Nov, Sp. Nov., the First Cultured Representative of the Uncultured SK4 Clade from Aoudad Sheep and Alpaca“. Microorganisms 8, Nr. 11 (November 2020): 1734. https://doi.org/10.3390/microorganisms8111734.

Callaghan, Tony Martin, Sabine Marie Podmirseg, Daniel Hohlweck, Joan E. Edwards, Anil K. Puniya, Sumit S. Dagar, und Gareth Wyn Griffith. „Buwchfawromyces eastonii gen. nov., sp. nov.: a new anaerobic fungus (Neocallimastigomycota) isolated from buffalo faeces“. MycoKeys 9 (März 2015): 11–28. https://doi.org/10.3897/mycokeys.9.9032.