

NUTRIENT MEDIUM FOR INCREASING CELL YIELD IN FERMENTATION

FIELD OF THE INVENTION

This invention relates to a novel medium for use in fermentation which provides an increased cell yield compared to that of known media. More particularly, the present invention produces at least a two to three-fold increase in the yield of the fungus *Lagenidium giganteum* compared to the yield obtained with known media. In addition to increasing yield of cells, *L. giganteum* grown in novel medium containing lecithin exhibits increased effectiveness against mosquitoes.

BACKGROUND OF THE INVENTION

Fermentation is the process of growing microorganisms or cells in specialized vessels. The cells or organisms may then be purified and used for a variety of purposes. For instance, the fungus *Lagenidium giganteum* grown in fermenters is used as a biocontrol agent for mosquitoes.

Optimal growth of the microorganism during fermentation depends on several factors including available nutrients, oxygen concentration, pH, temperature, and degree of mixing. Nutrients necessary for cell growth are provided in the medium used during the fermentation process. Accordingly, the yield obtained from fermentation depends, in part, on the composition of the medium.

There are several published nutrient media currently used in the fermentation of *Lagenidium giganteum*. All use deionized water added to a final volume of 1 L, and all are sterilized. One formulation comprises 2.0 g Ardamine pH, 2.0 g glucose, 1 mL corn oil, 0.5 g cholesterol and 2mM Ca²⁺. (Kerwin, James L. and Washino, Robert K. (1986) "Ground and aerial application of the sexual and asexual stages of *Lagenidium giganteum* (oomycetes: Lagenidiales) for mosquito control." *J. Am. Mos. Control Assoc.* 2(2): 182-189).

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Date: 11 FEB 1999

Another formulation comprises 2.0 g autolyzed yeast extract, 1.0 g proflo, 0.5 g fish meal, 2 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.05 g cholesterol and 2 mL cottonseed oil. (Kerwin, James L. and Washino, Robert K. (1988) "Field evaluation of *Lagenidium giganteum* (Oomycetes: Lagenidiales) and description of a natural epizootic involving a new isolate of fungus." *J. Med. Entomol.* 25(6): 452-460) Yet another fermentation medium comprises 1.25 g glucose, 1.25 g peptone, 1.25 g autolyzed yeast extract, 2 g corn oil, 1 g linseed oil, and 0.075 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. (U.S. Patent No. 4,687,744). The fourth published medium contains 1.25 g yeast extract, 1.2 g glucose, 3.2 g powdered wheat germ, hemp seed extract to provide 250 mg/L of soluble protein, 1.25 g bactopectone, 3 g glucose and 1.5 g corn oil. (Lord, Jeffrey C. and Roberts, Donald W. (1986) "The effects of culture medium quality and host passage on zoosporegenesis and infectivity of *Lagenidium giganteum* (Oomycetes: Lagenidiales)," *J. Invertebr. Pathol.* 48:355-361)

When used in fermentation, the above-referenced published medium formulations all yield approximately the same number of cells and infect susceptible mosquitoes at approximately the same rate. Thus, in order to increase the yield and infectivity of biocontrol agents like *Lagenidium giganteum*, there is a need for an improved fermentation medium.

SUMMARY OF THE INVENTION

A medium for use in fermentation consisting essentially of 3.6 g per liter peptone; 3.0 g per liter autolyzed yeast extract; 3.6 g per liter peptone; 1.5 to 3.0 g per liter autolyzed yeast extract; 1.6 g per liter cottonseed flour, such as ProFlo® (Traders Protein, Memphis, TN); 2.0 to 7.75 g per liter glucose (dextrose); 2.5 g per liter palm oil; 0.2 g per liter cholesterol; 0.6 g per liter $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 0.2 g per liter $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and, optionally, 0.0 to 2.0 g per liter of lecithin. This medium provides increased yields of *Lagenidium giganteum* compared to prior art media, and, yield and infectivity of the organism is further increased when lecithin is included in the medium.

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Date: 31 FEB 1999

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to an improved medium for fermentation. The medium increases yield at least approximately two to three fold over known media. The invention is useful in large scale production of *Lagenidium giganteum*, a biocontrol agent for mosquitoes.

Definitions

As used herein, the term "fermentation" refers to the process of growing cells or microorganisms in specialized vessels. "Nutrient medium" ("medium") refers to a solid or liquid substrate that will support the growth of an organism.

In a preferred embodiment of this invention, the nutrient medium is prepared as follows:

3.6 g per liter peptone;
1.5 to 3 g per liter autolyzed yeast extract;
15 1.6 g per liter cottonseed flour;
2.0 to 7.75 g per liter glucose (dextrose);
2.5 g per liter palm oil;
0.2 g per liter cholesterol;
0.6 g per liter $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; and
20 0.2 g per liter $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$.

Deionized water is added to a final volume of 1 L and the pH is adjusted to 6.5. The constituents are heated until dissolved and then the medium is sterilized by autoclaving at 121°C, 15 p.s.i., for 30 minutes. When used in the fermentation of *Lagenidium giganteum*, this medium increases yield at least two to three fold over known media.

In another preferred embodiment, the nutrient medium is prepared by adding up to 2.0 g per liter of lecithin to the above formulation.

The following example is provided only for illustrative purposes, and is not to be construed as limiting the invention in any way.

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Date: 11 Feb 1999

Example 1Shake flask comparison of growth rates of *Lagenidium giganteum* in different media

- 5 Growth rate in the novel nutrient medium was compared with two other media in side by side shake flask experiments.

Medium #1:

- 1.25 g glucose (dextrose)
1.25 g peptone
10 1.25 g autolyzed yeast extract
2.0 g corn oil
1.0 g palm oil
0.03 g cholesterol
0.4 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
15 0.2 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$

Medium #2:

- 1.2 g peptone
1.2 g autolyzed yeast extract
20 3.0 g glucose (dextrose)
0.5 g cholesterol

Novel Nutrient Medium:

- 3.6 g peptone
25 3.0 g autolyzed yeast extract
1.6 g Proflo cottonseed extract
2.0 g glucose (dextrose)
2.5 g palm oil
0.2 g cholesterol
30 0.6 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
0.2 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$

- When preparing each of the media, all ingredients were combined and deionized water was added to a final volume of 1 L. The pH was adjusted to 6.5. Contents were
35 heated in a microwave until dissolved and then sterilized at 121 C°, 15 psi for 30 minutes. For each medium, nine 250 mL flasks were each filled with 50 mL of medium. A disk of *Lagenidium giganteum* (California strain) taken from a petri dish was used to inoculate each flask. The flasks were shaken at 120 rpm, 29 C° in an orbital temperature controlled

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shaker for 7 days. Cells were harvested by centrifuging the fungal mass at 5,200 rpm for 20 minutes at 18 C°. The centrifuged cell mass was weighed and cell counts made with a hemacytometer. Mean cell counts were recorded. Results are summarized in Table 1.

Table 1

	Medium #1	Medium #2	Novel Nutrient Medium	Fold Increase in cells/mL when Novel Medium used
Exp't #1	1.2 - 2.0 x 10 ⁶ cells/mL	1.2-2.0 x 10 ⁶ cells/mL	4.4 x 10 ⁶ cells/mL	2.2 fold
Exp't #2	6.25 x 10 ⁵ cells/mL	7.5 x 10 ⁵ cells/mL	1.38 x 10 ⁶ cells/mL	1.84-2.2 fold
Exp't #3	2.97 x 10 ⁵ cells/mL	3.3 x 10 ⁵ cells/mL	4.75 x 10 ⁵ cells/mL	1.4-1.6 fold
Exp't #7	9.77 x 10 ⁴ cells/mL	not done	9.38 x 10 ⁵ cells/mL	9.6 fold
Exp't #8	1.93 x 10 ⁵ cells/mL	not done	7.30 x 10 ⁵ cells/mL	3.7 fold

Medium #1 and Medium #2 yielded approximately the same number of cells per mL of medium in each experiment. The novel nutrient medium consistently increased the number of cells/mL in comparison to either Medium #1 or Medium #2. The average yield of *Lagenidium giganteum* was increased approximately three and half fold when grown in the novel nutrient medium.

Example 2

Shake flask comparison of novel medium with lecithin added

Having established that the novel medium formulation of Example 1 increases cell yield over known media, the effect of varying amounts of dextrose and yeast extract and adding 1.0 g or 2.0 g lecithin to the basal novel medium was examined. All media were homogenized with a large probe at 70% speed for 10-15 seconds to ensure components were in solution. Using EmReagents color Phast®, the pH of all media was adjusted to 6.5 and sterilized as in Example 1. For each medium, three 250 mL flasks were filled with 50 mL of medium, inoculated, cultured and harvested as described in Example 1. Results are summarized in Table 2 and Table 3

Table 2

Dextrose % Wt	Yeast extract % Wt	Lecithin % Wt	Cell Yield (cells/mL)	Average Cell Yield (cells/mL) without lecithin: 3.6×10^5
0.8750	0.1250	0.0000	2.0×10^5	
0.8750	0.1250	0.0000	3.0×10^5	
0.6875	0.3125	0.0000	4.8×10^5	
0.5000	0.5000	0.0000	4.1×10^5	
0.5000	0.5000	0.0000	4.13×10^5	Average Cell Yield (cells/mL) with lecithin: 4.63×10^5
0.5875	0.3125	0.1000	5.4×10^5	
0.5875	0.3125	0.1000	4.05×10^5	
0.3000	0.5000	0.2000	7.4×10^5	
0.3000	0.5000	0.2000	4.9×10^5	
0.6750	0.1250	0.2000	4.5×10^5	
0.6750	0.1250	0.2000	4.1×10^5	
0.4875	0.3125	0.2000	3.6×10^5	

As shown in Table 2, for media without lecithin, the average cells /mL yield is 3.6×10^5 . With lecithin, yield increases to 4.63×10^5 cells/mL.

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Example 3

Infectivity of *Lagenidium giganteum* grown in various media

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Lagenidium giganteum was grown in novel media described in Example 2 which contained no lecithin, 0.1000 % by weight lecithin or 0.2000 % by weight lecithin. Culturing conditions were as described in Example 1. The concentration of cells was calculated and their ability to kill mosquitoes measured at concentrations of 5,000; 2,500;

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1,250 and 675 cells/mL. Results summarized in Table 3 are averages of duplicate experiments.

Table 3

	% Mortality at 5,000 cells/mL	% Mortality at 2,500 cells/mL	% Mortality at 1,250 cells/mL	% Mortality at 675 cells/mL
Medium without lecithin	66	67	61	51
Medium with lecithin	87	87	89	74

- 5 These results illustrate that *Lagenidium giganteum* grown in the novel media killed more mosquitoes than cells grown in media without added lecithin.

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Date: 11/12/98

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CLAIMS

We claim:

1. A medium for use in fermentation, consisting essentially of:

- (a) 3.6 g per liter peptone;
- (b) 1.5 to 3 g per liter autolyzed yeast extract;
- (c) 1.6 g per liter cottonseed flour;
- (d) 2.0 to 7.75 g per liter glucose (dextrose);
- (e) 2.5 g per liter palm oil;
- (f) 0.2 g per liter cholesterol;
- (g) 0.6 g per liter $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; and
- (h) 0.2 g per liter $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$.

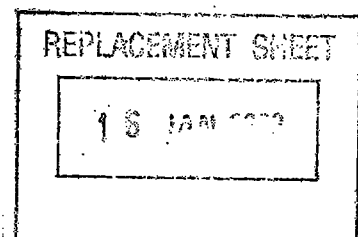
2. The medium according to claim 1, further comprising up to 2.0 g per liter lecithin.

3. The medium according to claim 1, for use in culturing *Lagenidium giganteum*.4. The medium according to claim 2, for use in culturing *Lagenidium giganteum*.

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We claim:

1. A medium for use in fermentation, consisting essentially of:
 - (a) 3.6 g per liter peptone;
 - (b) 1.5 to 3 g per liter autolyzed yeast extract;
 - (c) 1.6 g per liter cottonseed flour;
 - (d) 2.0 to 7.75 g per liter glucose (dextrose);
 - (e) 2.5 g per liter palm oil;
 - (f) 0.2 g per liter cholesterol;
 - (g) 0.6 g per liter $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; and
 - (h) 0.2 g per liter $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$.
2. The medium according to claim 1, further comprising up to 2.0 g per liter lecithin.
3. The medium according to claim 1, for use in culturing *Lagenidium giganteum*.
4. The medium according to claim 2, for use in culturing *Lagenidium giganteum*.
5. The medium as claimed in any one of the preceding claims, wherein said cottonseed flour is of a type known as ProFlo®.





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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁶ : C12N 1/00, 1/14, 1/16, 1/18	A1	(11) International Publication Number: WO 98/58049 (43) International Publication Date: 23 December 1998 (23.12.98)
(21) International Application Number: PCT/US97/10343 (22) International Filing Date: 17 June 1997 (17.06.97) (71) Applicant (for all designated States except US): AGRAQUEST, INC. [US/US]; Suite 4, 1105 Kennedy Place, Davis, CA 95616 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): HEINS, Sherry, D. [US/US]; 3110 Oyster Bay, Davis, CA 95616 (US). EWING, Duane, D. [US/US]; 1730 Bella Casa, Woodland, CA 95695 (US). MARRONE, Pamela, G. [US/US]; 3333 Victoria, Davis, CA 95616 (US). (74) Agents: PARK, Freddie, K. et al.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BE, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: NUTRIENT MEDIUM FOR INCREASING CELL YIELD IN FERMENTATION		
(57) Abstract A nutrient medium used in fermentation for increasing the yield of cells or microorganism is provided. The formulation provided increases the yield of the fungus <i>Lagenidium giganteum</i> two-to three-fold over known media.		