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(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 *Lagenidium giganteum* two–to three–fold over known media.

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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).

Another formulation comprises 2.0 g autolyzed yeast extract, 1.0 g proflo, 0.5 g fish meal, 2 mM $CaCl_2 2H_2O$, 1mM $MgCl_2 6H_2O$, 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 $CaCl_2 2H_2O$. (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 bactopeptone, 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 zoosporogensis 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 $CaCl_2 \cdot 2H_2O$; 0.2 g per liter $MgCl_2 \cdot 6H2O$ 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.

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;
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 $CaCl_2 \cdot 2H_2O$; and
0.2 g per liter $MgCl_2 \cdot 6H_2O$.

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.

Example 1

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

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
1.25 g autolyzed yeast extract
2.0 g corn oil
1.0 g palm oil
0.03 g cholesterol
0.4 g $CaCl_2 \cdot 2H_2O$
0.2 g $MgCl_2 \cdot 6H_2O$

Medium #2:

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

Novel Nutrient Medium:

3.6 g peptone
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
0.6 g $CaCl_2 \cdot 2H_2O$
0.2 g $MgCl_2 \cdot 6H_2O$

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 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

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)
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
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

Average Cell Yield (cells/mL) without lecithin: 3.6×10^5

Average Cell Yield (cells/mL) with lecithin: 4.63×10^5

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.

Example 3

Infectivity of *Lagenidium giganteum* grown in various media

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;

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

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

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 $CaCl_2 2H_2O$; and

(h) 0.2 g per liter $MgCl_2 6H_2O$.

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*.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/10343

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 1/00, , 1/14, 1/16, 1/18
US CL :435/243, 254.1, FOR 112, FOR 113
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)

U.S. : 435/243, 254.1, FOR 112, FOR 113

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,141,986 A (CASSIDY ET AL.) 27 February 1979, see abstract and column 6, lines 50-70 and column 7, lines 1-40 and column 31, lines 50-60.	1-4
Y	US 4,687,744 A (KERWIN ET AL.) 18 August 1987, see abstract and columns 1-2, all lines and column 6, lines 35-60.	1-4

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

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