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COMPOSITION AND METHOD FOR SEWAGE
TREATMENT USING FUNGAL AND BACTERIAL ENZYMES

Field Of The Invention

This invention relates to cellulose
5 degradation by fungal and bacterial enzymes. The
combination of enzymes is useful for sewage treatment,
particularly in septic tanks.

BACKGROUND OF THE INVENTION

Treatment of sewage with microorganisms is
10 known in the art. Many sewage treatment centers, as
well as individual septic tanks, may employ
microorganisms for the degradation of sewage.
Generally, sewage contains water, organic waste
(containing carbohydrates, fats and proteins), and
15 cellulose from paper products. Cellulose may represent
up to about 15% of the solids in raw (untreated)
sewage.

Typically, the organic, non-cellulosic waste
component of sewage is more easily degraded than the
20 cellulose component. Carbohydrates, fats and proteins
making up the organic waste are fairly easily digested
by extracellular enzymes released outside the cell of
selected bacteria. The degradation of cellulose,
however, remains a problem in many forms of sewage
25 treatment.

The degradation of cellulose to glucose is a
stepwise process. First, cellulose is hydrolyzed by
the action of an endoglucanase that breaks bonds along
the amorphous regions of cellulose. This enzymatic
30 reaction carries out the cleavage of the beta (1 > 4)
bonds producing cellobiose which will be removed from
the nonreducing ends of the molecule by the action of a
beta (1 > 4) exoglucanase. After this, the cellobiose
is hydrolyzed by a beta (1 > 4) glucosidase to glucose.
35 Thus, the breakdown of cellulose to glucose involves a
complex of enzymes. Sufficient amounts of these

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enzymes are not believed to be produced by natural bacteria. The lack of sufficient enzymatic activity is particularly evident in septic tanks, where cellulose sediment is a problem. New methods to reduce the cellulose sediment in raw sewage are needed.

SUMMARY OF THE INVENTION

The problems stated above have been solved with the discovery of a sewage treatment composition comprising *Bacillus* spp. cultures in combination with fungal cellulase. The combination of the extracellular enzymes produced by bacteria cultures from *Bacillus* spp. and fungal cellulase results in a synergistic degradation of cellulose. Results show a significant enhancement in the production of glucose as a result of cellulose degradation when sewage containing cellulose is contacted with the inventive composition. The composition is a broad based system capable of breaking down carbohydrates, fats and proteins in addition to enhanced cellulose degradation. Because the composition contains enzymes from naturally occurring microorganisms, it is particularly useful as a septic tank additive.

The invention also provides a novel method for using the *Bacillus* spp. cultures and fungal cellulase to degrade carbohydrates, protein, fat, and cellulose, and mixtures thereof.

More specifically, the invention provides a composition comprising

- (1) spores of *Bacillus* spp. selected from the group consisting of *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus megaterium* and mixtures thereof and
- (2) a fungal cellulase isolated from *Aspergillus niger*.

The invention also provides a method of treating sewage which comprises contacting said sewage with a composition comprising *Bacillus* spp. cultures in combination with fungal cellulase to degrade sewage comprising carbohydrates, proteins, fats, cellulose, or mixtures thereof.

The invention also provides use of *Bacillus* spp. cultures in combination with fungal cellulase in the preparation of a composition for use in degrading sewage comprising carbohydrates, proteins, fats, cellulose, or mixtures thereof.

The invention also provides a septic tank additive comprising fungal cellulase and spores obtained from *Bacillus* spp. cultures selected from the group consisting of *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus megaterium*, and mixtures thereof.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Bacillus spp. are known naturally occurring bacteria as identified on pages 1105 to 1139 of the eighth Edition of *Bergey's Manual of Determinative Bacteriology*, published by The Williams and Wilkins Co., 1986. Preferred *Bacillus* species include *B. subtilis*, *B. licheniformis*, *B. megaterium*, and mixtures thereof. More preferably, the bacteria culture is a mixture of *B. subtilis*, *B. licheniformis*, and *B. megaterium*. As known by those skilled in the art,

bacteria cultures may be prepared as spores to extend the period that the cultures may be stored. Preferably for convenience of storage, the *Bacillus* spp. cultures are present in the composition as spores. The spores become enzyme producing organisms when exposed to nutrients such as sewage. When exposed to sewage the spores generate into bacteria producing extracellular enzymes that are particularly effective in degrading carbohydrates, fats and proteins. The spore count of *Bacillus* spp. employed in the composition may vary greatly, depending upon the type of sewage to be treated, the size of the sewage treatment facility, the frequency of treatment of the sewage with the composition, and so on.

As used herein, the active ingredient portion of the composition is defined as the bacteria culture and fungal enzyme. A concentration range of bacteria cultures for a composition prepared as a typical septic tank additive preferably employs at least about 10^4 spores/g of composition (active ingredient), with the upper limit concentration of spores generally limited only by cost. More preferably at least 10^6 spores/g and most preferably from 10^6 to 10^8 spores/g of composition (active ingredient) is employed in the composition.

The cellulase is isolated from *Aspergillus niger* fungus. The enzyme may be extracted from the fungal culture by any known means, and is widely available commercially from, for example, Novo Nordisk, Ct.; Sigma Chemical, St. Louis, Mo.; and George A. Jeffreys Company, Salem, Va. Because the fungus is aerobic, and sewage treatment is largely in a submerged anaerobic environment, it is preferred that the cellulase enzyme is separated from the fungus as employed in this invention. The *Bacillus* spp. bacteria

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are facultative anaerobic and thus thrive in the typically anaerobic conditions of sewage treatment.

As with the spore count of the bacteria cultures, the specific activity and amount of the fungal cellulase enzyme employed in the composition is widely variable and may be adjusted according to the enzymatic needs of the system employing the composition. For example, with waste systems having a particularly high content of cellulose, large amounts of the cellulosic enzymes would be preferred. For use as a septic tank additive, the activity of the fungal enzyme employed is preferably at least about 1000 CU/g of active ingredient portion of composition (with the upper limit of concentration of enzyme generally limited only by cost). For reasons of economy in formulating septic tank additives the enzyme range is more preferably from 1500 to 2500 CU/g and most preferably from 1500 to 2000 CU/g of active ingredient portion of composition.

The ratio of bacteria culture to fungal enzyme may vary greatly. Preferably the ratio is anywhere between about 10:90 to about 99.99:0.01 percent by weight of active ingredient bacteria culture:fungal enzyme. As known to those skilled in the art, the ratio may be adjusted depending upon the type of material to be treated, the spore count and specific activity of raw materials, and so on.

The composition may also include optional fillers and additives to facilitate storage or delivery of the spores and fungal enzyme into the treatment facility. Fillers that may be used include, but are by no means limited to, alkali metal salts (such as NaCl, Na₂SO₄, CaCO₃, mixtures thereof and so on), inert preparations (such as milorganite), mixtures thereof, and so on.

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The composition may be prepared as a liquid or powder by any means known to those skilled in the art.

As previously described, the enzymes utilized
5 in the inventive composition are produced by naturally occurring organisms. Thus the composition is useful for many industrial applications where broad based degradation of components typical of sewage (e.g. carbohydrates, proteins, fats and cellulose) is
10 desired.

As shown in the Examples section hereinafter, the combination of *Bacillus* spp. enzymes and the fungal cellulase has been found to be synergistic. A smaller amount of bacteria and fungal enzymes used in
15 combination was found to be more effective in degrading cellulose than when a larger amount of plain fungal enzyme was used. The inventive combination offers a broad based sewage treatment system as well as a means of producing glucose from cellulose, particularly
20 useful in industrial applications where cellulose is a waste product.

The enzymatic action of the inventive composition may occur over a wide pH range. Optimally, the pH range of the media to be treated falls within
25 about 4 to about 10, with more preferably the pH having a value between 6 and 8. The temperature range of the media to be treated may vary greatly, although optimum enzymatic action preferably occurs within a temperature range of from about 10°C to about 45°C and more
30 preferably between 20°C and 35°C. Degradation of cellulose may also occur with enzymes separated from the *Bacillus* spp. and combined with cellulase of a fungal origin (separated or unseparated from the fungus). As known to one skilled in the art, the
35 fungus is an aerobic microorganism and the *Bacillus* spp. a facultative anaerobic microorganism, thus the

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oxygen content of the substrate environment must be considered in preparing the composition.

As known to those skilled in the art, the dosage, frequency of use, as well as the concentration of the active ingredient portion of the composition are interdependent variables that will also vary widely depending upon the environment to be treated, the concentration of particles to be degraded, prior usage of microorganisms, and so on. Adjustments to these variables may be accomplished by routine procedures known to those skilled in the art. For example, for use as a septic tank additive (with the septic tank typically having a capacity of about 1000 gallons), an effective amount of the active ingredient portion of the composition is at least about 10 g, more preferably at least about 100 g (with the upper limit of the amount used limited primarily by cost), and most preferably from 150 g to 1000 g.

The invention is further illustrated, but not limited to, the following examples.

EXAMPLES

Cellulose degradation was measured by glucose production, as determined by the Dinitrosalicylic acid procedure (DNS), as described in Aibba, S., K. Kitai, and T. Imanaka, *Applied Environmental Microbiology*, Vol. 46, pp. 1059-1065 (1983).

The compositions described in the examples used spores isolated from *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus megaterium*, and fungal cellulase isolated from *Aspergillus niger*. Both the spores and cellulase were obtained from the George A. Jeffreys Company. The culture has a count of 10^8 spores/gram of active ingredient portion of the composition. The cellulase had a specific activity of 1600 CU/g of active ingredient portion of the composition. (As obtained from supplier, actual

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cellulase enzyme activity was approximately 128,000 CU/g.) The milorganite was purchased from Milwaukee Metropolitan Sewage District, Milwaukee, WI.

Examples 1 and 2 and Comparative Examples 1 and 2

- 5 Synthetic sewage was prepared with 5% protein, 5% fat, 5% cellulose, and the remainder distilled water. The synthetic waste was placed in a 35 ml test tube for each composition tested.

INVENTIVE COMPOSITION A

<u>Ingredients</u>	<u>Weight Percentage</u>	<u>Actual Amounts</u>
<i>Bacillus</i> spp. Spores	40%	200 g
NaCl	20%	100 g
Na ₂ SO ₄	15%	75 g
CaCO ₃	24.5%	122.5 g
Cellulase	0.5% (640 CU/g)	2.5 g
<u>Total Volume</u>	<u>100%</u>	<u>500 g</u>

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Example 1

Composition A was diluted 10% with the waste for a final cellulase concentration of 0.050% (64 CU/g). After 48 h glucose production (thus indicating the level of cellulose degradation) was measured as 4.83 g/lt using the DNS method, as recorded in Table I below.

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Example 2

Example 1 was repeated with the exception that Composition A was diluted 2.5% with waste for a final cellulase concentration of 0.0125% (16 CU/g). After 47 h glucose production was measured by DNS as 1.31 g/lt, as recorded in Table I below.

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Comparative Example 1

Example 1 was repeated with the exception that only cellulase was added to the synthetic waste at a concentration of 0.5% (640 CU/g). After 48 h glucose production was measured by DNS as 1.41 g/lt, as recorded in Table I.

Comparative Example 2

Example 1 was repeated with the exception that Composition A did not have any fungal cellulase and CaCO_3 and Na_2SO_4 were replaced with an equivalent amount milorganite. After 48 h glucose production was measured by DNS as 0.17 g/lt, as recorded in Table I.

Table 1 summarizes data obtained in Examples 1 and 2 and Comparative Examples 1 and 2. When the fungal cellulase was added to the culture a significant increase in the production of glucose was detected. As Examples 1 and 2 show more or similar amount of glucose was produced by the composition, once the cellulase was added, than with the enzyme by itself though the cellulase concentration in Examples 1 and 2 was approximately ten times less than with the enzyme by itself. Therefore, these major differences between the enzymes by itself and the combination of fungal and bacterial enzymes were due to the synergistic effect of both bacterial and fungal enzymes. Similar results were found using raw sewage (Table 2).

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Table 1

Glucose production in synthetic waste

	<u>Glucose (g/lt)</u>
Example 1	4.83
Example 2	1.31
Comparative Example 1	1.41
Comparative Example 2	0.17

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Inventive Composition B

<u>Ingredients</u>	<u>Weight Percentage</u>	<u>Actual Amounts</u>
Bacillus spp. Spores	40%	200 g
NaCl	20%	100 g
Na ₂ SO ₄	15%	75 g
CaCO ₃	24.5%	122.5 g
Cellulase	0.5% (640 CU/g)	2.5 g
Total Volume	100%	500 g

Example 3

10 Raw sewage (obtained from the Ridgewood waste
water treatment plant, Ridgewood, N.J.) was placed into
a 35 ml tube and Composition B was diluted 2.5% for a
final cellulase concentration of 0.0125% (16 CU/g).
After 48 h glucose production (thus indicating the
15 level of cellulose degradation) was measured as 0.291

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g/lt using the DNS method, as recorded in Table 2 below.

A Control was also run, where glucose production was measured without the presence of Composition B or the enzyme by itself. As recorded in Table 2, no glucose was detected when samples were analyzed by the DNS procedure.

Comparative Example 3

Example 3 was repeated with the exception that only cellulase was added to the sewage at a concentration of 0.5% (640 CU/g). After 48 h glucose production was measured by DNS as 0.128 g/lt, as recorded in Table 2.

Comparative Example 4

Example 3 was repeated with the exception that Composition B did not have any fungal cellulase and CaCO_3 and Na_2SO_4 were replaced with milorganite. After 48 h glucose production was measured by DNS as 0.029 g/lt, as recorded in Table 2.

Table 2

Glucose production of raw sewage

	<u>Glucose (g/lt)</u>
Example 3	0.291
Control	0.000
Comparative Example 3	0.128
Comparative Example 4	0.029

The invention has been described above with particular reference to preferred embodiments. A skilled practitioner familiar with the above-detailed description can make many modifications and substitutions without departing from the scope and spirit of the invention.

WHAT WE CLAIM IS:

1. A composition comprising
 - (1) spores of *Bacillus* spp. selected from the group consisting of *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus megaterium* and mixtures thereof and
 - (2) a fungal cellulase isolated from *Aspergillus niger*.
2. A composition according to claim 1 wherein said spores obtained are a mixture of said *Bacillus* spp.
3. A composition according to claim 1 or claim 2 further comprising a filler selected from alkali metal salts and inert metal preparations.
4. A composition according to claim 3 wherein said filler is an alkali metal salt or an alkaline earth metal salt selected from the group consisting of NaCl, Na₂SO₄, CaCO₃ and mixtures thereof.
5. A method of treating sewage which comprises contacting said sewage with a composition comprising *Bacillus* spp. cultures in combination with fungal cellulase to degrade sewage comprising carbohydrates, proteins, fats, cellulose, or mixtures thereof.
6. A method according to claim 5 wherein said cultures are present in a spore form prior to contacting said composition with said sewage.
7. A method according to claim 5 or claim 6 wherein said cultures are selected from the group consisting of *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus megaterium*, and mixtures thereof.
8. A method according to claim 7 wherein said

culture is a mixture of said *Bacillus* spp.

9. A method according to any one of claims 5 to 8 wherein said cellulase is isolated from *Aspergillus niger* fungus.

10. A method of producing glucose from cellulose which comprises contacting said cellulose with fungal cellulase and extracellular enzymes produced by *Bacillus* spp. cultures.

11. A method according to claim 10 wherein said *Bacillus* cultures are a combination of *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus megaterium*.

12. A method according to claim 10 or claim 11 wherein said fungal cellulase is isolated from *Aspergillus niger* fungus.

13. Use of *Bacillus* spp. cultures in combination with fungal cellulase in the preparation of a composition for use in degrading sewage comprising carbohydrates, proteins, fats, cellulose, or mixtures thereof.

14. Use according to claim 13 wherein cultures are present in a spore form.

15. Use according to claim 13 or claim 14 wherein said cultures are selected from the group consisting of *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus megaterium* and mixtures thereof.

16. Use according to claim 15 wherein said culture is a mixture of said *Bacillus* spp..

17. Use according to any one of claims 13 to 16

wherein said cellulase is isolated from *Aspergillus niger* fungus.

18. Use according to claim 13 in which the composition further comprises a filler selected from the group consisting of alkali metal salts, alkaline earth metal salts and inert metal preparations.

19. Use according to claim 18 in which the filler is an alkali metal salt or an alkaline earth metal salt selected from the group consisting of NaCl, Na₂SO₄, CaCO₃ and mixtures thereof.

20. A septic tank additive comprising fungal cellulase and spores obtained from *Bacillus* spp. cultures selected from the group consisting of *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus megaterium*, and mixtures thereof.

21. An additive according to claim 20 wherein said spores obtained are a mixture of said *Bacillus* spp.

22. An additive according to claim 20 or claim 21 wherein said cellulase is isolated from *Aspergillus niger* fungus.

23. An additive according to any one of claims 20 to 22 further comprising a filler selected from alkali metal salts and inert metal preparations.

24. An additive according to claim 23 wherein said filler is an alkali metal salt or an alkaline earth metal salt selected from the group consisting of NaCl, Na₂SO₄, CaCO₃ and mixtures thereof.

25. A method according to claim 9 in which the composition further comprises a filler selected from the group consisting of alkali metal salts, alkaline earth metal salts and inert metal preparations.

26. A method according to claim 25 in which the filler is an alkali metal salt or an alkaline earth metal salt selected from the group consisting of NaCl, Na₂SO₄, CaCO₃ and mixtures thereof.

27. A composition as defined in claim 1 substantially as herein described with reference to any example thereof.

28. A method as defined in claim 5 of treating sewage substantially as herein described with reference to any example thereof.

29. A method as defined in claim 10 of producing glucose from cellulose substantially as herein described with reference to any example thereof.

30. Use of *Bacillus* spp. cultures as defined in claim 13 substantially as herein described with reference to any example thereof.

31. A septic tank additive as defined in claim 20 substantially as herein described with reference to any example thereof.