

- [54] Title: ALKALINE CELLULASES AND MICROORGANISMS CAPABLE OF PRODUCING SAME
- [75] Inventor (s): SHUJI KAWAI, HIROMI OKOSHI, KATSUYA OZAKI, SUSUMU ITO, all of Tochigi-ken, KAZUSHI CENINO and KIRUHIKO OKAMOTO, both of Saitama-ken, all of Japan
- [73] Assignee (s): KAO CORPORATION, of Tokyo, Japan
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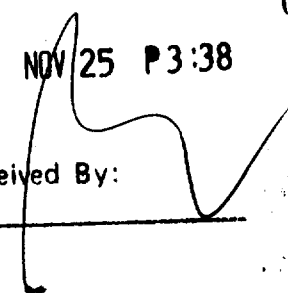
[57] A B S T R A C T

Alkaline cellulases having an optimum pH in an alkaline range and being stable over a broad pH range are produced by microorganisms which belong to the genus Bacillus and grow in a neutral medium.

These alkaline cellulases are rarely inhibited by ingredients ordinarily incorporated in detergents such as surface active agents, proteases and chelating agents, so that they can be conveniently used in detergent compositions.

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TITLE OF THE INVENTION

ALKALINE CELLULASES AND
MICROORGANISMS CAPABLE OF PRODUCING SAME

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BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to novel alkaline cellulases and also to microorganisms which are able to produce the same, belong to the genus Bacillus, and grow up in a neutral medium.

2. Description of the Prior Art

The development of cellulases, which are cellulose-decomposing enzymes, has been made for the purpose of effectively utilizing biomass resources and particularly, cellulose resources. A diversity of strains have been isolated as cellulase-producing fungi or bacteria including, for example, not only molds of the genera Aspergillus, Penicillium, Trichoderma, Fusarium, Humicola, Acremonium and the like, but also bacteria of the genera Pseudomonas, Cellulomonas, Ruminococcus, Bacillus and the like and actinomycetes of the genera Streptomyces, Thermoactinomyces and the like. At present, however, cellulases for biomass have not

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been frequently utilized on an industrial scale.

On the other hand, studies have been made on novel industrial utility of cellulases as an ingredient for detergents for clothes, to which attention has now been paid (Japanese Patent Publication Nos. 59-49279, 60-23158 and 60-36240). Most cellulases produced by microorganisms in the natural fields are classified as so-called neutral or/acidic cellulases which exhibit optimum and stable enzymatic activity in a neutral to acidic range. Only a few cellulases are so-called alkaline cellulases which meet the requirements for formulation in detergent compositions for clothes or can exhibit an maximum activity in an alkaline pH range, and so-called alkali-resistant cellulases which have an alkali resistance. The term "alkaline cellulase" used herein is intended to mean one whose optimum pH is in an alkaline range, and the term "alkali-resistant cellulase" means one whose optimum pH is in a neutral or an acidic range, but which has a satisfactory activity as compared with an activity at an optimum pH and is maintained stable in an alkaline range. The term "neutral" means a pH range of from 6 to 8, and the term "alkaline" means a higher pH range.

For the production of alkaline cellulases and

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alkali-resistant cellulases usable in detergent compositions for clothes, only several methods have been proposed. These methods include, for example, a method of collecting cellulase A by cultivation of alkalophilic bacilli belonging to the genus Bacillus (Japanese Patent Publication No. 50-28515), a method of producing alkaline cellulase 301-A by cultivation of alkalophilic bacteria belonging to the genus Cellulomonas (Japanese Patent Application Laid-open No. 58-224686), a method of producing carboxymethyl/cellulase by cultivation of alkalophilic Bacillus No. 1139 (Fukumori, F., Kudo T. and Horikoshi, K., J. Gen. Microbiol., 131, 3339, (1985)), and a method of producing an alkaline cellulase by the use of one strain belonging to the genus Streptomyces (Japanese Patent Application Laid-open No. 61-19483). However, these methods are all unsuitable for the industrial fermentation production.

In recent years, we have found that Bacillus sp. KSM-635 (FERM P-8872), which is one of alkalophilic bacteria, can efficiently product alkaline cellulase K which is suitable as an ingredient for detergents for clothes and that proper selection of cultivation conditions enables one to enhance the productivity and conduct industrial fermentation production of the

alkaline cellulase.

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However, the cultivation conditions of the Bacillus sp. KSM-635 are not always advantageous from an industrial point of view. More particularly, an alkalophilic strain should be cultivated under alkaline pH conditions during the cultivation. A so-called alkaline fermentation process using alkalophilic strains has just been started, and a full knowledge on the physiological and biochemical properties of these alkalophilic microorganisms has not been obtained. Thus, difficulties have been involved in the preparation of media and the manner of cultivation/sufficient to effect the industrial production by fermentation.

Moreover, the true alkaline cellulases of the afore-described documents which have an optimum pH in an alkaline region, are enzymes which are produced by Bacillus N1 strain, N2 strain and N3 strain (Japanese Patent Publication No. 50-28515) and have optimum pHs of 8 to 9, 9 and 8 to 9, respectively, an enzyme produced by Bacillus No. 1139 and having an optimum pH of 9, and alkaline cellulase K produced by Bacillus sp. KSM-635 and having an optimum pH of 10 (Japanese Patent Application No. 61-257776). Now, there is a demand for alkaline cellulase which have an optimum pH in an

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alkaline region and can be suitably formulated in detergent compositions and which have a wide working pH range.

SUMMARY OF THE INVENTION

Under these circumstances, the present inventors made extensive studies in order to obtain strains which grow in neutral media and are capable of producing alkaline cellulases having good effects.

In order to solve the prior art problems, a gene recombination technique may be used in which a strain which grows up in a neutral region is employed as a host and corresponding cellulase genes are cloned. In this connection, however, it is more effective to search for neutral microorganism, in the natural field, which is able to produce an alkaline cellulase having an optimum pH in an alkaline region and to isolate it. Accordingly, the present inventors have sought such a microorganism in the natural field and, as a result, found that a series of microorganisms belonging to the genus Bacillus grow in neutral media and produce certain types of alkaline cellulases.

Typical alkaline cellulases according to the invention have the following enzymatic properties:

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- (1) having a broad optimum pH range of from 8 to 10 with a maximum activity at pH of approximately 10;
- (2) the activity being inhibited by the presence of Hg^{2+} ;
- (3) the activity being rarely inhibited with proteinases, surface active agents and chelating agents; and
- (4) having the CMCase activity (Cx activity) as a main activity with additional filter paper-disintegrating activity and Avicelase activity (C_1 activity).

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing the relation between a pH for the enzyme reaction of alkaline cellulase K-580 and a relative activity;

Fig. 2 is a graph showing the relation between a treating pH for the above enzyme and a relative activity;

Fig. 3 is a graph showing the relation between a reaction temperature for the above enzyme and a relative activity;

Fig. 4 is a graph showing activities at 15°C and 20°C when the activity of the above enzyme at 30°C

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is taken as 100;

Fig. 5 is a graph showing the relation between a treating temperature for the above enzyme and a ^{residual} relative activity;

Fig. 6 is a graph showing the relation between a pH for the enzyme reaction of alkaline cellulase K-425 and a relative activity;

Fig. 7 is a graph showing a treating pH for K-425 and a ^{residual} relative activity;

Fig. 8 is a graph showing a reaction temperature for K-425 and a relative activity;

Fig. 9 is a graph showing a treating temperature for K-425 and a ^{residual} relative activity;

Fig. 10 is a graph showing the relation between a pH for the enzyme reaction of alkaline cellulase K-521 and a relative activity;

Fig. 11 is a graph showing the relation between a treating pH for K-521 and a ^{residual} relative activity;

Fig. 12 is a graph showing the relation between a reaction temperature for K-521 and a relative activity;

Fig. 13 is a graph showing the relation between a treating temperature for K-521 and a ^{residual} relative activity;

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Fig. 14 is a graph showing the relation between a pH for the enzyme reaction of alkaline cellulase K-522 and a relative activity;

Fig. 15 is a graph showing the relation between a treating pH for K-522 and a ^{residual} relative activity; P-8

Fig. 16 is a graph showing the relation between a reaction temperature for K-522 and a relative activity;

Fig. 17 is a graph showing the relation between a treating temperature for K-522 and a ^{residual} relative activity; P-8

Fig. 18 is a graph showing the relation between a pH for the enzyme reaction of alkaline cellulase E-II and a relative activity;

Fig. 19 is a graph showing the relation between a treating pH for E-II and a ^{residual} relative activity; P-8

Fig. 20 is a graph showing the relation between a reaction temperature for E-II and a relative activity;

Fig. 21 is a graph showing the relation between a treating temperature for E-II and a ^{residual} relative activity; P-8

Fig. 22 is an ion-exchange chromatogram obtained in the third purification step;

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Fig. 23 is a chart showing the results of SDS/polyacrylamide gel electrophoresis of alkaline cellulases E-II and E-III;

Fig. 24 is a UV absorption spectrum of alkaline cellulase E-II;

Fig. 25 is a graph showing the relation between a pH for the enzyme reaction of alkaline cellulase E-III and a relative activity;

Fig. 26 is a graph showing the relation between a treating pH for E-III and a ^{relative} relative activity; *P 8*

Fig. 27 is a graph showing the relation between a reaction temperature of E-III and a relative activity;

Fig. 28 is a graph showing the relation between a treating temperature for E-III and a ^{relative} relative activity; and *P-8*

Fig. 29 is a UV absorption spectrum of alkaline cellulase E-III.

DETAILED DESCRIPTION OF THE INVENTION

AND PREFERRED EMBODIMENTS

Examples of the microorganisms capable of producing the alkaline cellulases of the invention include strains isolated from the soils of Haga-gun and

Nikko-shi in Tochigi, Japan.

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These strains have the following mycological properties. It will be noted that the classification of the/strains is carried out using the following medium Nos. 1 to 25 (in which values are by wt%).

Medium 1: meat extract, 1.0; Bacto peptone, 1.0; NaCl, 0.5; Bacto agar, 1.5 (pH 7.2)

Medium 2: meat extract, 1.0; Bacto peptone, 1.0; NaCl, 0.5 (pH 7.2)

Medium 3: meat extract, 1.0; Bacto peptone, 1.0; NaCl, 0.5; gelatin, 1.0 (pH 7.2)

Medium 4: Bacto litmus milk, 10.0

Medium 5: Bacto peptone, 1.0; KNO₃, 0.1

Medium 6: Bacto peptone, 1.0; NaNO₃, 0.1

Medium 7: Bacto peptone, 0.7; NaCl, 0.5; glucose, 0.5 (pH 7.0)

Medium 8: Bacto peptone, 1.0

Medium 9: TSI agar (by Eiken Chem. Co., Ltd., Japan), indicated amount

Medium 10: meat extract, 1.0; Bacto peptone, 1.0; NaCl, 0.5; soluble starch, 0.2; agar, 1.5

Medium 11: NaNH₄HPO₄·4H₂O, 0.15; KH₂PO₄, 0.1; MgSO₄·7H₂O, 0.02; sodium citrate, 0.25 (pH 6.8)

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Medium 12: Christensen's medium (Eiken Chem. Co., Ltd., Japan), indicated amount

Medium 13: glucose, 1.0; KH_2PO_4 , 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05/
KCl, 0.02; nitrogen sources, 0.1 (pH 7.2)

The nitrogen sources used were sodium nitrate and ammonium sulfate.

Medium 14: King A medium "Eiken" (Eiken Chem. Co., Ltd., Japan), indicated amount

Medium 15: King B medium "Eiken" (Eiken Chem. Co., Ltd., Japan), indicated amount

Medium 16: urea medium "Eiken" (Eiken Chem. Co., Ltd., Japan), indicated amount

Medium 17: filter paper for cytochrome oxidase test
(Nisshui Pharm Co., Ltd., Japan)

Medium 18: 3% hydrogen peroxide aqueous solution

Medium 19: OF basal medium (Difco Lab.), indicated amount

Medium 20: $(\text{NH}_4)_2\text{HPO}_4$, 0.1; KCl, 0.02; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02; yeast extract, 0.02; Bacto agar, 2.0; BCP (0.2% solution), 0.4

Medium 21: Bacto Sabouraud's dextrose agar medium (Difco Lab.), indicated amount

Medium 22: meat extract, 0.3; Bacto peptone, 0.5; yeast extract, 1.0; glycerin, 2.0

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Medium 23: phenyl alanine malonic acid salt medium
(Nisshui Pharm. Co., Ltd., Japan), indicated
amount

Medium 24: skim milk, 5.0; Bacto agar, 1.5

Medium 25: meat extract, 0.3; Bacto peptone, 0.5;
L-tyrosine, 0.5; Bacto agar, 1.5

(Mycological properties)

Bacillus sp. KSM-580:

(a) Bacillus sp. KSM-580 has a size of the body of 0.4 - 0.8 micrometers x 1.5 - 5.0 micrometers, and has a cylindrical or elliptical endospore (0.4 - 0.8 micrometers x 0.8 - 1.2 micrometers) at the terminal of the body. The bacillus has marginal flagellas and is mobile. The Gram's staining is positive. Not aciduric.

(b) Growing State in Various Media:

(1) Meat broth agar plate culture (medium 1)

The growing state is weak. The shape of the colonies is in a round or irregular form with a smooth surface and a smooth or leaf-like margin. The color tone of the colonies is light yellow, semi-transparent and glossy.

(2) Meat broth agar slant culture (medium 1)

The growth is weak with the state being in a cloth-spreading form and being glossy, light yellow in

color and semi-transparent.

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- (3) Meat borth liquid culture (medium 2)

Growing. Especially, the upper layer becomes turbid.

- (4) Meat broth gelatin stab culture (medium 3)

Growing in the surface layer with the gelatin being liquefied.

- (5) Litmus milk medium (medium 4)

The liquefaction of the milk is recognized.
The litmus does not change its color.

(c) Physiological properties:

- (1) The reduction and denitrification reactions of nitrates (media 5 and 6) are both negative.

- (2) MR test (medium 7)

Whether the test is negative or positive is not clear (pH 5.2).

- (3) VP test (medium 7)

Whether the test is negative or positive is not clear (pH 5.2).

- (4) Formation of indole (medium 8)

Negative.

- (5) Formation of hydrogen sulfide (medium 9)

Negative.

- (6) Hydrolysis of starch (medium 10)



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Positive.

- (7) Utility of citric acid (media 11, 12)

Negative in a Koser's medium and positive in a Christensen's medium.

- (8) Utility of inorganic nitrogen sources (medium 13)

Positive with respect to the nitrate and ammonium salt.

- (9) Formation of pigment (media 14, 15)

Negative.

- (10) Urease (medium 16)

Negative.

- 14 (11) Oxidase (medium 17)

Positive.

- (12) Catalase (medium 18)

Positive.

- (13) Temperature and pH ranges for growth (medium 2)

The temperature range for growth is 15 - 50°C and an optimum temperature range is 25 - 40°C.

The pH range for the growth is 5 - 11 and an optimum pH range is 6 - 10.

- (14) Behavior to oxygen

Facultatively anaerobic.

- (15) O-F test (medium 19)

Although growing up, the growth is poor either

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aerobically or anaerobically.

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(16) Utility of sugars (+: utilizing. -: not utilizing)

1. L-arabinose	+
2. D-xylose	+
3. D-glucose	+
4. D-mannose	+
5. fructose	+
6. D-galactose	+
7. maltose	+
8. sucrose	+
9. lactose	+
10. trehalose	-
11. D-sorbitol	+
12. D-mannitol	+
13. inositol	+
14. glycerin	+
15. starch	+

(17) pH in VP medium (medium 7)

pH 5.2

(18) Growth in a salt-containing medium

(modified medium 1)

Growing at 5%.

Growing at 7%.

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Not growing at 10%.

(19) Growth at a pH of 5.7 (medium 21)

Growing.

(20) Formation of dihydroxyacetone (medium 22)

Negative.

(21) Deamination of phenylalanine (medium 23)

Negative.

(22) Decomposition of casein (medium 24)

Positive.

(23) Decomposition of tyrosine (medium 25)

Negative.

Bacillus sp. KSM-425:

(a) Results of microscopic observation

Bacillus sp. KSM-425 has a size of the body of 0.4 - 0.8 micrometers x 1.5 - 4.0 micrometers, making an ellipsoidal endospore (0.8 - 1.2 micrometers x 1.2 - 1.5 micrometers) at one end of the body. It has marginal flagella and is mobile. The Gram staining is indefinite. Not aciduric.

(b) Growing state in various media

(1) Meat broth agar plate culture (medium 1)

The growing state is poor. The shape of the colonies is circular with a smooth surface and a smooth margin. The color tone of the colonies is white, and

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the colonies are semi-transparent and glossy.

- (2) Meat broth agar slant culture (medium 1)

The growth is poor and its state is in a cloth-spread form and is glossy, white in color and semi-transparent.

- (3) Meat broth liquid culture (medium 2)

Growing and becoming turbid.

- (4) Meat broth gelatin stab culture (medium 3)/

Growing but in a poor state. The liquefaction of gelatin is not recognized.

- (5) Litmus milk culture (medium 4)

The coagulation and liquefaction of milk is not recognized. The litmus does not undergo any change in color.

(c) Physiological properties

- (1) Reduction and denitrification reactions of nitrates (media 5, 6)

Both negative.

- (2) MR test (medium 7)

Whether the test is negative or positive is not clear (pH 5.2).

- (3) VP test (medium 7)

Negative (pH 5.2).

- (4) Formation of indole (medium 8)

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Negative.

- (5) Formation of hydrogen sulfide (medium 9)

Negative.

- (6) Hydrolysis of starch (medium 10)

Positive.

- (7) Utility of citric acid (media 11, 12)

Negative both in a Koser's medium and in Christensen's medium.

- (8) Utility of inorganic nitrogen sources
(medium 13)/

Negative with respect to the nitrate and ammonium salt.

- (9) Formation of pigment (media 14, 15)

Negative.

- (10) Urease (medium 16)

Negative.

- (11) Oxidase (medium 17)

Positive.

- (12) Catalase (medium 18)

Positive.

- (13) Temperature and pH ranges for growth (medium 2)

The temperature range for the growth is 15 - 37°C and an optimum temperature range is 25 - 30°C.

The pH range for the growth is 5 - 10 and an

optimum pH range is 6 - 9.

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(14) Behavior to oxygen

Facultatively anaerobic.

(15) O-F test (medium 19)

Although growing up, the growth is poor either aerobically or anaerobically.

(16) Utility of sugars (+: utilizing, -: not utilizing)

1. L-arabinose	+
2. D-xylose	+
3. D-glucose	+
4. D-mannose	+
5. fructose	+
6. D-galactose	+
7. maltose	+
8. sucrose	+
9. lactose	+
10. trehalose	+
11. D-sorbitol	±
12. D-mannitol	+
13. inositol	-
14. glycerin	±
15. starch	+

(17) pH in VP medium (medium 7)

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pH 5.2

- (18) Growth in a salt-containing medium (modified medium 1)

Not growing at 5%.

Not growing at 7%.

Not growing at 10%.

- (19) Growth at a pH of 5.7 (medium 21)

Not growing.

- (20) Formation of dihydroxyacetone (medium 22)

Whether the formation is negative or positive is not clear.

- (21) Deamination of phenylalanine (medium 23)

Negative. /

- (22) Decomposition of casein (medium 24)

Negative.

- (23) Decomposition of tyrosine (medium 25)

Negative.

Bacillus sp. KSM-521:

- (a) Results of microscopic observation

Bacillus sp. KSM-521 has a size of the body of 0.6 - 0.8 micrometers x 1.0 - 2.0 micrometers, making a cylindrical or ellipsoidal endospore (0.4 - 0.8 micrometers x 1.0 - 2.0 micrometers) at the center of the body. It has flagelli and is mobile. The Gram

staining is positive. Not aciduric.

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(b) Growing state in various media

(1) Meat broth agar plate culture (medium 1)

The growing state is good. The shape of the colonies is circular with a smooth surface and a smooth margin or a leaf-like form. The color tone of the colonies is light yellow, and the colonies are semi-transparent and glossy.

(2) Meat broth agar slant culture (medium 1)

Growing. The growing state is in a cloth-spread form and is light yellow and semi-transparent.

(3) Meat broth liquid culture (medium 2)

Growing but becoming turbid.

(4) Meat broth gelatin stab culture (medium 3)

Growing in the surface portions. The liquefaction of gelatin is recognized.

(5) Litmus milk culture (medium 4)

The liquefaction of milk is recognized. The litmus does not undergo any change in color.

(c) Physiological properties

(1) Reduction and denitrification reactions of nitrates (media 5, 6)

Both negative.

(2) MR test (medium 7)



Positive.

- (3) VP test (medium 7)

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Positive.

- (4) Formation of indole (medium 8)

Negative.

- (5) Formation of hydrogen sulfide (medium 9)

Negative.

- (6) Hydrolysis of starch (medium 10)

Negative.

- (7) Utility of citric acid (media 11, 12)

Positive in Christensen's medium, and whether negative or positive is not clear in Koser's medium.

- (8) Utility of inorganic nitrogen sources (medium 13)

Negative with respect to the nitrate and ammonium salt.

- (9) Formation of pigment (media 14, 15)

Positive.

- (10) Urease (medium 16)

Negative.

- (11) Oxidase (medium 17)

Whether negative or positive is not clear.

- (12) Catalase (medium 18)

Positive.

- (13) Temperature and pH ranges for growth



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(medium 2)

The temperature range for the growth is 10 - 50°C and an optimum temperature range is 20 - 40°C.

The pH range for the growth is 5 - 10 and an optimum pH range is 6 - 10.

(14) Behavior to oxygen

Aerobic.

(15) O-F test (medium 19)

Oxidation.

(16) Formation of an acid and a gas from sugars

(medium 20) (+: formed, -: not formed)

	Formation of acid	Formation of gas
1. L-arabinose	+	-
2. D-xylose	+	-
3. D-glucose	+	-
4. D-mannose	+	-
5. fructose	+	-
6. D-galactose	+	-
7. maltose	-	-
8. sucrose	+	-
9. lactose	-	-
10. trehalose	+	-
11. D-sorbitol	-	-
12. D-mannitol	+	-

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- | | | |
|--------------|---|---|
| 13. Inositol | - | - |
| 14. glycerin | + | - |
| 15. starch | - | - |

(17) pH in VP medium (medium 7)

pH 5.0

(18) Growth in a salt-containing medium
(modified medium 1)

Growing in 5%, 7% and 10% NaCl.

(19) Growth at a pH of 5.7 (medium 21)

Growing.

(20) Formation of dihydroxyacetone (medium 22) /

Negative.

(21) Deamidation of phenylalanine (medium 23)

Negative.

(22) Decomposition of casein (medium 24)

Positive.

(23) Decomposition of tyrosine (medium 25)

Negative.

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Bacillus sp. KSM-522:

(a) Results of microscopic observation

Bacillus sp. KSM-522 has a size of the body of 0.5 - 0.8 micrometers x 1.0 - 2.0 micrometers, making an oval or a cylindrical endospore (0.5 - 0.8 micrometers x 1.0 - 1.2 micrometers) at the end of the center of the

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body. It has marginal flagelli and is mobile. The Gram staining is positive. Not aciduric.

(b) Growing state in various media

(1) Meat broth agar plate culture (medium 1)

Growing well. The shape of the colonies is circular with a coarse surface and a smooth or wavy margin. The color tone of the colonies is light yellow, and the colonies are semi-transparent with a resin hardness.

(2) Meat broth agar slant culture (medium 1)

Growing. The growing state is in a cloth-spread form and glossy, with milky white or light yellow in color and semi-transparency.

(3) Meat broth liquid culture (medium 2)

Growing and becoming turbid.

(4) Meat broth gelatin stab culture (medium 3)

Growing in the top surface portions. The liquefaction of gelatin is recognized.

(5) Litmus milk culture (medium 4)

The liquefaction of milk is recognized but the litmus does not undergo any change in color.

(c) Physiological properties

(1) Reduction and denitrification reactions of nitrates (media 5, 6)

Both negative.

- (2) MR test (medium 7)

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Positive.

- (3) VP test (medium 7)

Positive.

- (4) Formation of indole (medium 8)

Negative.

- (5) Formation of hydrogen sulfide (medium 9)

Negative.

- (6) Hydrolysis of starch (medium 10)

Negative.

- (7) Utility of citric acid (media 11, 12)

Positive in Christensen's medium, but it is not clear in Koser's medium as to whether positive or negative.

- (8) Utility of inorganic nitrogen sources (medium 13)

Negative with respect to the nitrate and ammonium salt.

- (9) Formation of pigment (media 14, 15)

A water-soluble yellow pigment is formed in the King B medium.

- (10) Urease (medium 16)

Negative.

- (11) Oxidase (medium 17)

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Whether negative or positive is not clear.

(12) Catalase (medium 18)

Positive.

(13) Temperature and pH ranges for growth (medium 2)

The temperature range for the growth is 10 - 50°C and an optimum temperature range is 20 - 40°C.

The pH range for the growth is 5 - 10 and an optimum pH range is 6 - 10.

(14) Behavior to oxygen

Aerobic.

(15) O-F test (medium 19)

Oxidation.

(16) Formation of an acid and a gas from sugars

(medium 20) (+: formed, -: not formed)

	Formation of acid	Formation of gas
1. L-arabinose	+	-
2. D-xylose	+	-
3. D-glucose	+	-
4. D-mannose	+	-
5. fructose	+	-
6. D-galactose	+	-
7. maltose	-	-
8. sucrose	+	-
9. lactose	-	-

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10. trehalose	+	-
11. D-sorbitol	-	-
12. D-mannitol	+	-
13. Inositol	-	-
14. glycerin	+	-
15. starch	-	-

(17) pH in VP medium (medium 7)

pH 5.0 - 5.2 (seventh day).

(18) Growth in a salt-containing medium (modified medium 1)

Growing in 5%, 7% and 10% NaCl.

(19) Growth at a pH of 5.7 (medium 21)

Growing.

(20) Decomposition of casein (medium 24)

Positive.

The above mycological properties are compared by reference to Bergey's Manual of Determinative Bacteriology, 8th edition and "The Genus Bacillus", in Agriculture Handbook No. 427 written by Ruth E. Gordon, Agricultural Research Service, U.S. Department of Agriculture, Washington D.C. (1973). As a result, it has been found that all the strains of the invention are considered to be microorganisms belonging to the genus Bacillus. The strains of the invention are apparently

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different from so-called alkalophilic microorganisms which have been recently reported by Horikoshi and Akiba ("Alkalophilic Microorganism", Japan Scientific Society Press (Tokyo), 1982). This is because the alkalophilic microorganisms grow in alkaline media having a pH not less than 8 and cannot grow up in a neutral or lower pH region, whereas the strains of the invention are able to grow in a weakly acidic to alkaline region (pH 5 -10). Thus, the strains of the invention can be determined as ordinary microorganisms belong to the genus Bacillus, which grow under neutral conditions.

More detailed studies on the strains of the invention reveal that the species which is most analogous to the strain of Bacillus sp. KSM-580 and KSM-521 may be Bacillus licheniformis. However, the comparison/ between the strains of the present invention and known strains belonging to the Bacillus licheniformis reveals that the KSM-580 strain is different from those known strains with respect to the reducibility of nitrates. In addition, the above known strains cannot produce at least alkaline cellulases, and thus the strains of the present invention are considered as novel strains.

The species which is most analogous to

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Bacillus sp. KSM-425 may be Bacillus circulans. The comparison between known strains belonging to the circulans and the strain of the invention demonstrates a difference in the capability of the hydrolysis of gelatin. Moreover, the above known strains do not produce alkaline cellulases. Thus, the KSM-425 is considered as a novel strain.

The species which is most analogous to the Bacillus sp. KSM-521 and KSM-522 strains may be Bacillus pumilus. However, known strains belonging to Bacillus pumilus do not produce at least alkaline cellulases. Thus, the KSM-521 and KSM-522 are considered as novel strains.

The present inventors deposited these strains to Fermentation Research Institute of Japan as follows.

Bacillus sp. KSM-580 as FERM BP-1511/

Bacillus sp. KSM-425 as FERM BP-1505

Bacillus sp. KSM-521 as FERM BP-1507

Bacillus sp. KSM-522 as FERM BP-1512

For obtaining alkaline cellulases of the invention using these strains, the strain is inoculated into media and cultivated by a usual manner. The medium should preferably have suitable amounts of carbon and nitrogen sources to be utilized. These carbon and

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nitrogen sources are not critical. Examples of the nitrogen sources include corn gluten meal, soybean flour, corn steep liquor, casamino acid, yeast extract, Pharmamedia, sardine meal, meat extract, peptone, Hypro, Ajipower, corn soybean meal, coffee grounds, cotton seed oil cake, Cultivator, Amiflex, Ajipron, Zest, Ajix and the like. The carbon sources include, for example, plant fibers such as chaff, wheat-gluten bread, filter paper, ordinary papers, sawdust and the like, wasted theriac, invert sugar, CMC, Avicel, cellulose cotton, xylan, pectin and the like. In addition, utilizable carbon sources include, for example, arabinose, xylose, glucose, mannose, fructose, maltose, sucrose, lactose, trehalose, mannitol, sorbitol, inositol, glycerin, soluble starch and the like, and utilizable organic acids include, for example, citric acid, acetic acid and the like. Besides, phosphoric acid and inorganic salts such as of Mg^{2+} , Ca^{2+} , Mn^{2+} , Zn^{2+} , Co^{2+} , Na^{+} , K^{+} and the like, and inorganic and organic trace nutrient sources may be appropriately added.

An intended alkaline cellulase can be collected from the thus obtained culture product and purified according to ordinary techniques of collecting and purifying enzymes. More particularly, the fungus

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bodies can be removed from a culture broth or solution by ordinary solid-liquid separation techniques such as centrifugal separation, filtration and the like, thereby obtaining a crude enzyme solution. This crude enzyme solution may be used as it is, or may be separated by salting-out, precipitation, ultrafiltration or the like to obtain a crude enzyme. The crude enzyme is subsequently purified by crystallization by any known methods to obtain a purified enzyme.

Among the thus obtained alkaline cellulases, the alkaline cellulase K-522 may be further separated into novel alkaline cellulases E-II and E-III. For the preparation of the alkaline cellulases E-II and E-III, the alkaline cellulase K-522 is fractionally purified by a suitable combination of a hydroxyapatite chromatography, an ion exchange chromatography using DEAE-Sephadex (Pharmacia Inc.), DEAE-cellulose/or the like, and a molecular sieve gel chromatography using Sephadex, Biogel (Bio-Rad Laboratories Inc.) and the like.

The thus obtained alkaline cellulases of the present invention have the following enzymatic properties. It will be noted that the enzymatic activity is measured according to the following

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procedure using the following buffer solutions.

pH 3 - 8 : McIlvaine buffer solution

pH 8 - 11 : glycine-sodium hydroxide buffer
solution

pH 12 - 13: potassium chloride-sodium
hydroxide buffer solution

Enzymatic activity measurement:

(1) CMCase activity

0.1 ml of an enzyme solutin was added to 0.9 ml of a base solution comprising 10 mg of CMC (A-01L, by Sanyo Kokusaku Pulp Co., Ltd., Japan) and 100 μ moles of each of the buffer solutions (McIlvaine, phosphoric acid, glycine-NaOH and the like), followed by reaction at 30°C for 20 minutes. After completion of the reaction, (the resulting) reducing sugar was quantitatively determined according to the 3,5-dinitro-salicylic acid (DNS) method. More particularly, 1.0 ml of the DNS reagent was added to 1.0 ml of the reaction solution and heated at 100°C for 5 minutes for color development. After cooling, 4.0 ml of deionized water was added for dilution. The diluted solution was subjected to colorimetry using a wavelength of 535 nm. The enzyme strength was expressed as one unit which was an amount of the enzyme sufficient to produce a reducing

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sugar corresponding to 1 μ mol of glucose under the above conditions for 1 minute.

(2) Decomposition activity of PNPC

A suitable amount of CMCase was acted on 1.0 ml of a reaction solution containing 0.1 μ mol of PNPC (Sigma Co., Ltd.) and 100 μ mols of a phosphate buffer solution (pH 7.0) at 30°C, to which 0.3 ml of 1M Na₂CO₃ and 1.7 ml of deionized water were added successively, followed by subjecting the resultant released p-nitrophenol to colorimetry at 400 nm. The enzyme strength was expressed as one unit which was an amount of the enzyme sufficient to release 1 μ mol of p-nitrophenol under the above conditions for 1 minute.

(3) Decomposition activities of Avicel, cellulose powder and filter paper

A suitable amount of an enzyme solution was added to 2.0 ml of a reaction solution containing 20 mg of Avicel (Merck Inc.) and 200 μ mols of a phosphate buffer solution (pH 7.0), followed by shaking for reaction at 30°C at 250 r.p.m. After completion of the reaction, the solution was cooled and centrifugally separated (5°C, 3000 r.p.m., 20 minutes), and 1.0 ml of the resultant supernatant liquid was subjected to quantitative determination of reducing sugar according

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to the 3,5-dinitro-salicylic acid (DNS) method. The above procedure was repeated for a cellulose powder decomposition activity using cellulose powder (Toyo Filter Paper Co., Ltd.) and for a filter paper decomposition activity using a filter paper (filter paper for examination of the cellulase activity, Toyo No. 51-specific). The enzyme strength was expressed by one unit which was an amount of the enzyme sufficient to produce reducing sugar corresponding to $1 \mu\text{mol}$ of glucose under the above conditions for 1 minute.

(4) Cellobiase activity

A suitable amount of CMCase was acted on a 1.0 ml reaction solution containing 10 mg of cellobiose (Kanto Chem. Co., Ltd.) and 100 μmoles of a phosphate buffer solution (pH 7.0) for an appropriate time, and then treated at 100°C for 2 minutes, thereby inactivating the enzyme. Thereafter, the amount of the resultant glucose was measured by the Mufotase-GOD method (glucose C-test, Wako Junyaku Ind. Co., Ltd.). The enzyme strength was expressed by one unit which was an amount of the enzyme sufficient to produce 2 μmoles of glucose under the above conditions for 1 minute.

(Enzymatic properties)

Alkaline cellulase K-580:

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(1) Action

Acting well on cellulosic materials such as CMC, cellulose, filter paper, Avicel and the like and causing them to be dissolved, thereby producing reducing sugars such as glucose.

(2) Substrate specificity

This enzyme has activity not only on CMC, but also on cellulose powder, Avicel and filter paper.

(3) Working pH and optimum pH

The working pH ranges very widely from 3 to 12.5 and the optimum pH is in the wide range of 7 to 10. In a range of 4.5 to 10.5, the relative activity is not less than 50% of the activity in the optimum pH range. Accordingly, this enzyme is believed to exhibit a satisfactory activity at the most alkaline side among known alkaline cellulases studied up to now (Fig. 1).

(4) pH Stability

The residual activity was measured after keeping the enzyme at different pHs at 30°C for 1 hour to determine the pH stability. As a result, it was found that the enzyme was very stable and was not inactivated at a pH of 4.5 to 12. In a pH of from 3.5 to 12.5, an activity of about 50% or over was maintained. Thus, the present enzyme is satisfactorily

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stable in a high alkaline region (Fig. 2).

(5) Optimum temperature

The working temperature was in a wide range of from 15 to 80°C and the optimum temperature was found to be 65°C. In a temperature range of from 50 to 75°C, the activity was 50% or higher of the activity at the optimum temperature (Fig. 3).

At 15°C, the activity was not less than 40% of the activity at 30°C (Fig. 4).

(6) Temperature stability

After treatment at the optimum pH for 30 minutes at different temperatures, the residual activity was measured. As a result, it was found that it was stable at 55°C and a residual activity of about 50% was obtained at 65°C (Fig. 5).

(7) Molecular weight

The molecular weight of the present enzyme was measured according to the gel filtration method using Sephadex G-100, with the result that it was about 18,000 and 50,000.

(8) Influences of metal ions

The present enzyme was subjected to determination of influences of various metal ions (Al^{3+} , Fe^{3+} , Ca^{2+} , Cd^{2+} , Co^{2+} , Cr^{2+} , Cu^{2+} , Fe^{2+} , Hg^{2+} , Mn^{2+} ,

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Mo²⁺, Ni²⁺, Pb²⁺, Zn²⁺, Li⁺, K⁺, and Na⁺) by permitting the ions to coexist at the time of the measurement of the activity (in which the concentration of K⁺ or Na⁺ was 50 mM and the concentration of other ions was 1 mM). As a result, it was found that the activity was inhibited with Hg²⁺, but was more enhanced with Ba²⁺, Ca²⁺, Co²⁺ and Cd²⁺.

(9) Influences of surface active agents

Influences of various surface active agents (e.g. LAS, AS, ES, AOS, alpha-SFE, SAS, soap and polyoxyethylene secondary alkyl ether) on the enzyme activity were determined. The present enzyme was treated with a 0.05% solution of each surface active agent at 30°C for 15 minutes and subjected to the measurement of activity. As a result, the activity was not inhibited by any surface active agents. In addition, the inhibition of the activity was not recognized when using sodium dodecylsulfate which was a potential detergent.

(10) Proteinase resistance

Proteinases for detergents such as, for example, API-21 (Showa Denko Co., Ltd.), Maxatase (Gist Co., Ltd.) and Alkalase (Novo Co., Ltd.), were allowed to coexist at the time of the measurement of the

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activity (0.1 mg/ml) to determine their influences. It was found that the enzyme had a high resistance to these proteinases.

(11) Influences of chelating agents

Chelating agents such as EDTA, EGTA, sodium tripolyphosphate, zeolite and citric acid were allowed to coexist at the time of the measurement of the activity, with the result that no inhibition was recognized.

Alkaline cellulase K-425:

(1) Action

Action well on cellulosic materials such as CMC, cellulose, filter paper, Avicel and the like and causing them to be dissolved, thereby producing reducing sugars such as glucose.

98 (2) Substrate specificity

This enzyme has activity not only on CMC, but also on cellulose powder, Avicel, filter paper, PNPC and cellobiose.

(3) Working pH and optimum pH

The working pH ranges very widely from 3.5 to 12.5 and the optimum pH is in the wide range of 8 to 10. In a range of 5.5 to 10.5, the relative activity is not less than 50% of the activity in the optimum pH range.

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Accordingly, this enzyme is believed to exhibit a satisfactory activity at the most alkaline side among known alkaline cellulases studied up to now (Fig. 6).

(4) pH Stability

The residual activity was measured after keeping the enzyme at different pHs at 30°C for 1 hour to determine the pH stability. As a result, it was found that the enzyme was very stable and was not inactivated at a pH of 5 to 11. In a pH of from 3 to 12, an activity of about 50% or over was maintained. Thus, the present enzyme is satisfactorily stable in a high alkaline region (Fig. 7).

(5) Optimum temperature

The working temperature was in a wide range of from 15 to 75°C and the optimum temperature was found to be 50°C. In a temperature range of from 35 to 55°C, the activity was 50% or higher of the activity at the optimum temperature (Fig. 8).

(6) Temperature stability

After treatment at the optimum pH for 30 minutes at different temperatures, the residual activity/ was measured. As a result, it was found that it was stable at 30°C and a residual activity of about 50% was obtained at 50°C (Fig. 9).

(7) Molecular weight

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The molecular weight of the present enzyme was measured according to the gel filtration method using Sephadex G-100, with the result that it was about 35,000.

(8) Influences of metal ions

The present enzyme was subjected to determination of influences of various metal ions (Al^{3+} , Fe^{3+} , Ca^{2+} , Cd^{2+} , Co^{2+} , Cr^{2+} , Cu^{2+} , Fe^{2+} , Hg^{2+} , Mn^{2+} , Mo^{2+} , Ni^{2+} , Pb^{2+} , Zn^{2+} , Li^{+} , K^{+} , and Na^{+}) by permitting the ions to coexist at the time of the measurement of the activity (in which the concentration of K^{+} or Na^{+} was 50 mM and the concentration of the other ions was 1 mM). As a result, it was found that the activity was inhibited by Hg^{2+} and Ba^{2+} , but was more enhanced by Co^{2+} .

(9) Influences of surface active agents

Influences of various surface active agents (e.g. LAS, AS, ES, AOS, alpha-SFE, SAS, soap and polyoxyethylene secondary alkyl ether) on the enzyme activity were determined. The present enzyme was treated with a 0.05% solution of each surface active agent at 30°C for 15 minutes and subjected to the measurement of activity. As a result, the activity was

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not inhibited by any surface active agents. In addition, the inhibition of the activity was not recognized when using sodium dodecylsulfate which was a potential detergent.

(10) Proteinase resistance

Proteinases for detergents such as, for example, API-21 (Showa Denko Co., Ltd.), Maxatase (Gist Co., Ltd.) and Alkalase (Novo Co., Ltd.), were allowed to coexist at the time of the measurement of the activity (0.1 mg/ml) to determine their influences. It was found that the enzyme had a high resistance to these proteinases.

(11) Influences of chelating agents

Chelating agents such as EDTA, EGTA, sodium tripolyphosphate, zeolite and citric acid were allowed to coexist at the time of the measurement of the activity, with the result that no inhibition was recognized.

Alkaline cellulase K-521:

(1) Action

Acting well on cellulosic materials such as CMC, cellulose powder, filter paper, Avicel and the like and causing them to be dissolved, thereby producing reducing sugars such as glucose.

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(2) Substrate specificity

This enzyme has activity not only on CMC, but also on cellulose powder, Avicel, filter paper, p-nitrophenyl cellobioside and cellobiose.

(3) Working pH and optimum pH

The working pH ranges very widely from 3 to 12.5 and the optimum pH is in the wide range of 7 to 10. In a range of 4.5 to 10.5, the relative activity is not less than 50% of the activity in the optimum pH range. Accordingly, this enzyme is believed to exhibit a satisfactory activity at the most alkaline side among known alkaline cellulases studies up to now (Fig. 10).

(4) pH stability

The residual activity was measured after keeping the enzyme at different pHs at 30°C for 1 hour to determine the pH stability. As a result, it was found that the enzyme was very stable and was not inactivated at a pH of 5 to 12. In a pH of from 4.5 to 12.5, an activity of about 50% or over was maintained. Thus, the present enzyme is satisfactorily stable in a high alkaline region (Fig. 11).

(5) Optimum temperature

The working temperature was in a wide range of from 15 to 80°C and the optimum temperature was found to

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be 60°C. In a temperature range of from 45 to 65°C, the activity was 50% or higher of the activity at the optimum/temperature (Fig. 12).

(6) Temperature stability

After treatment at the optimum pH for 30 minutes at different temperature, the residual activity was measured. As a result, it was found that it was stable at 40°C and a residual activity of about 50% was obtained at 55°C (Fig. 13).

(7) Molecular weight

The molecular weight of the present enzyme was measured according to the gel filtration method using Sephadex G-100, with the result that it was about 31,000.

(8) Influences of metal ions

The present enzyme was subjected to determination of influences of various metal ions (Al^{3+} , Fe^{3+} , Ba^{2+} , Ca^{2+} , Cd^{2+} , Co^{2+} , Cr^{2+} , Cu^{2+} , Fe^{2+} , Hg^{2+} , Mn^{2+} , Mo^{2+} , Ni^{2+} , Pb^{2+} , Zn^{2+} , Li^{+} , K^{+} , and Na^{+}) by permitting the ions to coexist at the time of the measurement of the activity (in which the concentration of K^{+} or Na^{+} was 50 mM and the concentration of the other ions was 1 mM). As a result, it was found that the activity was inhibited by Hg^{2+} , but was more

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enhanced by Ca^{2+} .

(9) Influences of surface active agents

Influences of various surface active agents (e.g. LAS, AS, ES, AOS, alpha-SFE, SAS, soap and polyoxyethylene secondary alkyl ether) on the enzyme activity were determined. The present enzyme was treated with a 0.05% solution of each surface active agent at 30°C for 15 minutes and subjected to the measurement of activity. As a result, the activity was rarely inhibited by any surface active agents. In addition, the inhibition of the activity was not recognized when using sodium dodecylsulfate which was a potential detergent.

(10) Proteinase resistance

Proteinases for detergents such as, for example, API-21 (Showa Denko Co., Ltd.), Maxatase (Gist Co., Ltd.) and Alkalase (Novo Co., Ltd.), were allowed to coexist at the time of the measurement of the activity (0.1 mg/ml) to determine their influences. It was found that the enzyme had a high resistance to these proteinases.

(11) Influences of chelating agents

Chelating agents such as EDTA, EGTA, sodium tripolyphosphate, zeolite and citric acid were allowed

to coexist at the time of the measurement of the activity, with the result that little inhibition was recognized.

Alkaline cellulase K-522:

(1) Action

Acting well on cellulosic materials such as CMC, cellulose powder, filter paper, Avicel and the like and causing them to be dissolved, thereby producing reducing/sugars such as glucose.

(2) Substrate specificity

This enzyme has activity not only on CMC, but also on cellulose powder, phosphoric acid-swollen cellulose, alkali-swollen cellulose, Avicel, filter paper and PNPC.

(3) Working pH and optimum pH

The working pH ranges very widely from 3 to 12.5 and the optimum pH is in the side range of 7 to 10. In a range of 4.5 to 10.5, the relative activity is not less than 50% of the activity in the optimum pH range. Accordingly, this enzyme is believed to exhibit a satisfactory activity at the most alkaline side among known alkaline cellulases studied upto now (Fig. 14).

(4) pH stability

The residual activity was measured after

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keeping the enzyme at different pHs at 30°C for 1 hour to determine the pH stability. As a result, it was found that the enzyme was very stable and was not inactivated at a pH of 5 to 12. In a pH of from 4.5 to 12.5, an activity of about 50% or over was maintained. Thus, the present enzyme is satisfactorily stable in a high alkaline region (Fig. 15).

(5) Optimum temperature

The working temperature was in a wide range of from 15 to 80°C and the optimum temperature was found to be 60°C. In a temperature range of from 45 to 65°C, the activity was 50% or higher of the activity at the optimum temperature (Fig. 16).

(6) Temperature stability

After treatment at the optimum pH for 30 minutes at different temperatures, the residual activity was measured. As a result, it was found that it was stable at 40°C and a residual activity of about 50% was obtained at 55°C (Fig. 17).

(7) Molecular weight

The molecular weight of the present enzyme was measured according to the gel filtration method using Bio-gel P-150 (Bio-Rad Laboratories Co., Ltd.), with the result that it was about 35,000.

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(8) Influences of metal ions

The present enzyme was subjected to determination of influences of various metal ions (Al^{3+} , Fe^{3+} , Ba^{2+} , Ca^{2+} , Cd^{2+} , Co^{2+} , Cr^{2+} , Cu^{2+} , Fe^{2+} , Hg^{2+} , Mn^{2+} , Mo^{2+} , Ni^{2+} , Pb^{2+} , Zn^{2+} , Li^{+} , K^{+} , and Na^{+}) by permitting the ions to coexist at the time of the measurement of the activity (in which the concentration of K^{+} or Na^{+} was 50 mM and the concentration of the other ions was 1 mM). As a result, it was found that the activity was inhibited by Hg^{2+} . /

(9) Influences of surface active agents

Influences of various surface active agents (e.g. LAS, AS, ES, AOS, alpha-SFE, SAS, soap and polyoxyethylene secondary alkyl ether) on the enzyme activity were determined. The present enzyme was treated with a 0.05% solution of each surface active agent at 30°C for 15 minutes and subjected to the measurement of activity. As a result, the activity was rarely inhibited by any surface active agents. In addition, the inhibition of the activity was not recognized when using sodium dodecylsulfate which was a potential detergent.

(10) Proteinase resistance

Proteinases for detergents such as, for

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example, API-21 (Showa Denko Co., Ltd.), Maxatase (Gist Co., Ltd.) and Alkalase (Novo Co., Ltd.), were allowed to coexist at the time of the measurement of the activity (0.1 mg/ml) to determine their influences. It was found that the enzyme had a high resistance to these proteinases.

(11) Influences of chelating agents

Chelating agents such as EDTA, EGTA, sodium tripolyphosphate, zeolite and citric acid were allowed to coexist at the time of the measurement of the activity, with the result that little inhibition was recognized.

Alkaline cellulase E-II:

(1) Action

Acting well on cellulosic materials such as CMC and phosphoric acid-swollen cellulose and causing them to be dissolved, thereby producing reducing sugars such as glucose.

(2) Substrate specificity

This enzyme has activity not only a main activity on CMC, but also an activity on cellulose swollen with about 4% phosphoric acid. Moreover, it has a slight decomposition activity on xylan, inulin and lichenan, but has little activity on cellulose powder,

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Avicel, filter paper, PNPC and cellobiose.

(3) Working pH and optimum pH

The working pH ranges very widely from 4 to 12.5 and the optimum pH is in the wide range of 7 to 10. In a range of 5.5 to 11, the relative activity is not less than 50% of the activity in the optimum pH range. Accordingly, this enzyme is believed to exhibit a satisfactory activity at the most alkaline side among known alkaline cellulases studied upto now (Fig. 18).

(4) pH stability

The residual activity was measured after keeping the enzyme at different pHs at 0°C for 24 hours to determine the pH stability. As a result, it was found that the enzyme was very stable and was not inactivated at a/pH of 6 to 11. In a pH of from 5.5 to 11.5, an activity of about 50% or over was maintained. Thus, the present enzyme is satisfactorily stable in a high alkaline region (Fig. 19).

(5) Optimum temperature

The working temperature was in a wide range of from 10 to 80°C and the optimum temperature was found to be 50°C. In a temperature range of from 30 to 65°C, the activity was 50% or higher of the activity at the optimum temperature (Fig. 20).

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(6) Temperature stability

After treatment at a pH of 7 for 30 minutes at different temperatures, the residual activity was measured. As a result, it was found that it was stable at 50°C and a residual activity of about 50% was obtained at 55°C (Fig. 21).

(7) Molecular weight

The molecular weight of the present enzyme was measured according to the gel filtration method using Bio-gel P-100 (Bio-Rad Laboratories Co., Ltd.), with the result that it was about 34,000. With an SDS-polyacrylamide gel electrophoresis, the molecular weight was about 61,000 (Fig. 23).

(8) Influences of metal ions

The present enzyme was subjected to determination/of influences of various metal ions

The present enzyme was subjected to (Al^{3+} , Fe^{3+} , Ba^{2+} , Ca^{2+} , Cd^{2+} , Co^{2+} , Cu^{2+} , Fe^{2+} , Hg^{2+} , Mn^{2+} , Mg^{2+} , Ni^{2+} , Pb^{2+} , Zn^{2+} , Li^{+} , K^{+} and Na^{+}) by permitting the ions to coexist at the time of the measurement of the activity (in which the concentration of K^{+} or Na^{+} was 50 mM and the concentration of the other ions was 1 mM). As a result, it was found that the activity was inhibited with Hg^{2+} and enhanced with Co^{2+} .

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(9) Influences of surface active agents

Influences of various surface active agents (e.g. LAS, AS, ES, AOS, alpha-SFE, SAS, soap and polyoxyethylene secondary alkyl ether) on the enzyme activity were determined. The present enzyme was subjected to the measurement of the activity in 0.05% of a surface active agent. As a result, any significant influences of the surface active agents were recognized as shown in Table 1.

Table 1

Surface Active Agent	Residual Activity (%)
nil	100
LAS	79
AS	108
ES	98
AOS /	100
alpha-SFE	129
SAS	93
Soap	100
Polyoxyethylene secondary alkyl ether	86

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In addition, the inhibition of the activity was not recognized when using sodium dodecylsulfate which was a potential detergent.

(10) Proteinase resistance

Proteinases for detergents such as, for example API-21 (Showa Denko Co., Ltd.), Maxatase (Gist Co., Ltd.) and Alkalase (Novo Co., Ltd.), were allowed to coexist at the time of the measurement of the activity (0.1 mg/ml) to determine their influences. It was found that the enzyme had a high resistance to these proteinases as shown in Table 2.

Table 2

Proteinase	Residual Activity (%)
nil	100
Alkalase	111
API-21	115
Maxatase	120

(11) Influences of chelating agents

Chelating agents such as EDTA, EGTA, sodium tripolyphosphate, zeolite and citric acid were allowed

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to coexist at the time of the measurement of the activity, with the result that little inhibition was recognized.

(12) UV absorption spectrum

The present enzyme was subjected to measurement of UV absorption spectrum. As a result, it was found that it had a maximum absorption at about 280 nm with a shoulder absorption being shown at 290 nm by a differential absorption spectrum (Fig. 24).

Alkaline cellulase E-III:

(1) Action

Acting well on celluloses such as CMC and phosphoric acid-swollen cellulose and causing them to be dissolved, thereby producing reducing sugars such as glucose.

(2) Substrate specificity

This enzyme has not only a main activity on CMC, but also an activity on cellulose swollen with about 4.5% phosphoric acid. Moreover, it has a slight decomposition activity on xylan, lichenan and the like, but has little activity on cellulose powder, Avicel, filter paper, PNPC and cellobiose.

(3) Working pH and optimum pH

The working pH ranges very widely from 4 to

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12.5/and the optimum pH is in the wide range of 7 to 9. In a range of 6 to 10.5, the relative activity is not less than 50% of the activity in the optimum pH range. Accordingly, this enzyme is believed to exhibit a satisfactory activity at the most alkaline side among known alkaline cellulases studies up to now (Fig. 25).

(4) pH stability

The residual activity was measured after keeping the enzyme at different pHs at 5°C for 24 hours to determine the pH stability. As a result, it was found that the enzyme was very stable and was not inactivated at a pH of 6 to 10. In a pH of from 5.7 to 11.5, an activity of about 50% or over was maintained. Thus, the present enzyme is satisfactorily stable in a high alkaline region (Fig. 26).

(5) Optimum temperature

The working temperature was in a wide range of from 10 to 80°C and the optimum temperature was found to be 50°C. In a temperature range of from 30 to 62°C, the activity was 50% or higher of the activity at the optimum temperature (Fig. 27).

(6) Temperature stability

After treatment at a pH of 7 for 30 minutes at different temperatures, the residual activity was

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measured. As a result, it was found that it was stable at 50°C and a residual activity of about 50% was obtained at 55°C (Fig. 28).

(7) Molecular weight

The molecular weight of the present enzyme was measured according to the gel filtration method using Bio-gel P-100 (Bio-Rad Laboratories Co., Ltd.), with the result that it was about 35,000. With an SDS-polyacrylamide gel electrophoresis, the molecular weight was about 61,000 (Fig. 23).

(8) Influences of metal ions

The present enzyme was subjected to determination of influences of various metal ions (Al^{3+} , Fe^{3+} , Ca^{2+} , Co^{2+} , Cr^{2+} , Cu^{2+} , Fe^{2+} , Hg^{2+} , Mg^{2+} , Mn^{2+} , Ni^{2+} , Pb^{2+} , Zn^{2+} , K^{+} , and Na^{+}) by permitting the ions to coexist at the time of the measurement of the activity (in which the concentration of K^{+} or Na^{+} was 50 mM and the concentration of the other ions was 1 mM). As a result, it was found that the activity was inhibited with Hg^{2+} and enhanced with Co^{2+} .

(9) Influences of surface active agents

Influences of various surface active agents (e.g. LAS, AS, ES, AOS, alpha-SFE, SAS, soap and polyoxyethylene secondary alkyl ether) on the enzyme

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activity were determined. The present enzyme was subjected to the measurement of the activity in 0.05% of a surface active agent/ As a result, any significant influences of the surface active agents were recognized as shown in Table 3.

Table 3

Surface Active Agent	Residual Activity (%)
nil	100
LAS	78
AS	107
ES	100
AOS	101
alpha-SFE	104
SAS	97
Soap	101
Polyoxyethylene secondary alkyl ether	96

In addition, the inhibition of the activity was not recognized when using sodium dodecylsulfate which was a potential detergent.

(10) Proteinase resistance

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Proteinases for detergents such as, for example API-21 (Showa Denko Co., Ltd.), Maxatase (Gist Co., Ltd.) and Alkalase (Novo Co., Ltd.), were allowed to coexist (0.1 mg/ml) at the time of the measurement of the activity to determine their influences. It was found that the enzyme had/a high resistance to these proteinases as shown in Table 4.

Table 4

Proteinase	Residual Activity (%)
nil	100
Alkalase	94
API-21	106
Maxatase	103

(11) Influences of chelating agents

Chelating agents such as EDTA, EGTA, sodium tripolyphosphate, zeolite and citric acid were allowed to coexist at the time of the measurement of the activity, with the result that little inhibition was recognized.

(12) UV absorption sepctrum

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The present enzyme was subjected to measurement of a UV absorption spectrum. As a result, it was found that it had a maximum absorption at about 280 nm with a shoulder absorption being shown at 290 nm by a differential absorption spectrum (Fig. 29).

The alkaline cellulases of the invention have an optimum pH at a higher level (pH 10) than known alkaline cellulases and are very stable over a wide pH range. For example, alkaline cellulase K-580 has, in a wide pH range/of from 4.5 to 10.5, an activity not less than 50% of the activity at the optimum pH, and is very stable in a pH range of from 4.5 to 12. Alkaline cellulase K-425 has also, in a wide pH range of from 5.5 to 10.5, an activity of not less than 50% of the activity at the optimum pH, and is very stable in a pH range of from 5 to 11. Moreover, alkaline cellulases K-521 and K-522 have, respectively, an optimum pH in a wide range of from 7.0 to 10 and is very stable in a wide range.

The alkaline cellulases E-II and E-III derived from the alkaline cellulase K-522 have, respectively, higher optimum pHs (of 10 and 9) than known alkaline cellulases. In addition, they have, respectively, wide optimum pH ranges of 7.0 to 10 and 7.0 to 9 are very

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stable in such wide ranges.

These alkaline cellulases are rarely inhibited with ingredients ordinarily formulated in detergents such as, for example, surface active agents, proteinases, chelating agents and the like. Accordingly, the present enzymes can be conveniently used in detergent compositions.

The microorganisms of the invention grow under neutral conditions, so that it is possible to industrially produce alkaline cellulases more easily than in the case using alkalophilic strains.

The present invention is described in more detail/by way of the following examples.

Example 1

A spoonful (about 0.5 g) of the soil obtained at Ichikai-machi, Haga-gun, Tochigi-ken, Japan was suspended in a sterilized physiological saline solution and thermally treated at 80°C for 10 minutes. The supernatant liquid of the thermally treated solution was appropriately diluted and applied to an agar medium for isolation (medium 1), followed by cultivation at 30°C for 3 days to form colonies. Colonies around which a transparent zone was formed on the basis of the

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dissolution of CMC were selected to collect CMCase-producing microorganisms belonging to the genus Bacillus. The thus collected microorganisms were inoculated into a liquid medium as medium 2 and subjected to shaking culture at 30°C for 3 days. After completion of the culture, a centrifugally separated supernatant liquid was obtained and subjected to measurement of the CMCase activity in a pH range of from 3 to 13 and also to screening of alkaline cellulase-producing microorganisms belonging to the genus Bacillus.

By the above procedure, there could be obtained Bacillus sp. KSM-580 strain, Bacillus sp. KSM-420 strain, and Bacillus sp. KSM-521 strain.

Medium 1

CMC	2%
Polypeptone	0.5
Yeast extract	0.05
KH ₂ PO ₄	0.1
Na ₂ HPO ₄ .12H ₂ O	0.25
MgSO ₄ .7H ₂ O	0.02
Agar	0.75

pH 6.8

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Medium 2

CMC	1%
Polypeptone	1
Yeast extract	0.5
KH ₂ PO ₄	0.1
Na ₂ HPO ₄ .12H ₂ O	0.25
MgSO ₄ .7H ₂ O	0.02

pH 6.8

Example 2

Bacillus sp. KSM-580 strain obtained in Example 1 was inoculated into the liquid medium 2 of Example 1, followed by shaking culture at 30°C for 3 days. After completion of the culture, the bacillus cells were centrifugally removed to obtain a crude enzyme solution. 3 liters of ethanol was added to 1 liter of the crude enzyme/solution in dry ice/ethanol and the resultant precipitate was centrifugally removed, followed by freeze-drying to obtain 11 g of alkaline cellulase K-580 (specific activity* 33 units/g) as a dry powder.

* The enzyme activity was a value at a pH of 9.

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Example 3

The Bacillus sp. KSM-580 strain was inoculated into a medium of the same composition as the liquid medium 2 of Example 1 except that CMC was replaced by sucrose and polypeptone was replaced by 7% of a corn steep liquor (CSL), followed by shaking culture at 30°C for 2 days. The resultant culture product was subjected to centrifugal separation and the resulting supernatant liquid was, in turn, subjected to measurement of the CMCase activity. As a result, the activity was 60 units/liter.

Example 4

The Bacillus sp. KSM-425 strain obtained in Example 1 was inoculated into the liquid medium 2 of Example 1 and shake-cultured at 30°C for 3 days. After completion of the culture, the bacillus cells were centrifugally removed to obtain a crude enzyme solution. 3 liters of ethanol was added to 1 liter of the crude enzyme solution in dry ice/ethanol, and the resultant precipitate was centrifugally removed and freeze-dried to obtain 10 g of alkaline cellulase K-425 (specific activity* 10 units/g) as a dry powder.

* The enzyme activity was a value measured at a pH of 9.

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Example 5

The Bacillus sp. KSM-425 strain was inoculated into a medium of the same composition as the liquid medium 2 of Example 1 except that CMC was replaced by sucrose and polypeptone was replaced by 7% CSL, followed by shaking culture at 30°C for 2 days. The culture product was centrifugally separated and the resultant supernatant liquid was subjected to measurement of the CMCase activity. As a result, the activity was 160 units/liter.

Example 6

The Bacillus sp. KSM-521 strain obtained in Example 1 was inoculated into the liquid medium 2 of Example 1 and shake-cultured at 30°C for 3 days. After *as given* the culture, the bacillus cells were centrifugally removed to obtain a crude enzyme solution. 3 liters of ethanol was added to 1 liter of the crude enzyme solution in dry ice/ethanol, and the resultant precipitated was centrifugally separated and freeze-dried to obtain 9 g of alkaline cellulase K-521 (specific activity* 20 units/g) as a dry powder.

* The enzyme activity was a value measured at a pH of 9 herein and hereinafter.

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Example 7

The KSM-521 strain was inoculated into a medium of the same composition as the liquid medium 2 of Example 1 except that CMC was replaced by 1% sucrose and polypeptone was replaced by 7% CSL, followed by shake culture at 30°C for 2 days. The culture product was centrifugally separated and the resultant supernatant liquid was subjected to measurement of the CMCase activity. As a result, it was found that the activity was 100 units/liter.

Example 8

A spoonful (0.5 g) of the soil obtained at Nikko-shi, Tochigi-ken, Japan was taken and suspended in a sterilized saline solution, followed by repeating the procedure of Example 1, thereby obtaining KSM-522 strain (FERM BP-1512) of the invention.

Example 9

The Bacillus sp. KSM-520 strain obtained in Example 8 was inoculated into the liquid medium of Example 1 and shake-cultured at 30°C for 3 days. After the culture, the bacillus cells were centrifugally separated to obtain a crude solution. 3 liters of

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ethanol was added to 1 liter of the crude enzyme solution in dry ice/ethanol, and the resultant precipitate was centrifugally separated and freeze-dried to obtain 8 g of alkaline cellulase K-522 (specific activity* 23 units/g) as a dry powder.

* The enzyme activity was a value measured at a pH of 9.

Example 10

The KSM-522 strain was inoculated into a medium of the same composition as the liquid medium 2 of Example 1 except that CMC was replaced by 1% sucrose and polypeptone was replaced by 7% CSL, followed by shake culture at a temperature of 30°C for 2 days. The culture product was centrifugally separated and the resultant supernatant liquid was subjected to measurement of the CMCase activity, with the result that the activity was 150 units/liter.

Example 11

5 liters of the supernatant obtained in Example 10 was purified according to the following procedure.

- (1) Concentration by ultrafiltration (Amicon Co.,

Ltd., fractionating molecular weight of 10,000).

- (2) Treatment with Streptomycin.
- (3) Chromatography using DEAE-Biogel A (Bio-Rad Laboratories Co., Ltd.).
- (4) Chromatography using hydroxyapatite (Wako Junyaku Ind. Co., Ltd.).
- (5) Chromatography using DEAE-Biogel A.
- (6) Chromatography using DEAE-Biogel A.

In the third step of the above procedure, the enzyme was adsorbed in a column having a size of 3.2 x 33 cm (equilibrated with a 10mM phosphate buffer solution with a pH of 7), followed by linearly increasing the concentration of NaCl from 0 to 300 mM for elution. This permitted neutral cellulase E-I, alkaline cellulase E-II and alkaline cellulase E-III to be eluted in this order (Fig. 22). The fractions of from 533 to 580 from which the fraction of E-I had been removed were collected and further purified. In the fourth step, the fractions were adsorbed in a column with a size of 2.5 x 13 cm (equilibrated with a 10 mM phosphate buffer solution with a pH of 7), after which the concentration of the phosphate was increased from 10 to 200 mM linearly to obtain an alkaline cellulase fraction (a mixture of E-II and E-III). In the fifth

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step, the procedure of (3) was repeated (in which the concentration of NaCl was increased linearly from 70 to 200 mM) to collect alkaline cellulase E-II with the residue being subjected to the sixth step. The sixth step was effected similar, to (3) or (5) but the gradient in concentration of NaCl was further lowered by changing from 90 to 150 mM for elution, thereby isolating the alkaline cellulase E-III. The thus purified alkaline cellulases E-II and E-III were subjected to polyacrylamide electrophoresis by a usual manner and further to Coomassie Brilliant Blue dye^{ing} and silver dye^{ing}, by which it was confirmed that the respective cellulases gave a single band.

Example 12

The alkaline cellulases E-II and E-III obtained in Example 11 were subjected to SDS-polyacrylamide gel electrophoresis by a usual manner. The results are shown in Fig. 23. From the results, it was found that the alkaline cellulases E-II and E-III each had a molecular weight of about 61,000.

According to the gel filtration method using Bio-gel P-100, the alkaline cellulase E-II had a molecular weight of about 34,000 and the alkaline cellulase E-III had a molecular weight of about 35,000.

FP-KS-219c/I

WHAT IS CLAIMED IS:

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1. An alkaline cellulase K-522 having the following physicochemical properties:

(1) Action

Acting well on cellulosic materials including carboxymethyl cellulose (CMC), cellulose, filter paper and Avicel and causing them to be dissolved, thereby forming reducing sugars such as glucose;

(2) Substrate specificity

Having activity not only on CMC, but also on cellulose powder, phosphoric acid-swollen cellulose, alkali-swollen cellulose, Avicel, filter paper, and p-nitrophenyl cellobioside;

(3) Working pH and optimum pH

The working pH range is from 3 to 12.5 and the optimum pH is from 7 to 10 with a relative activity of not less than 50% of the activity at an optimum pH being shown in the range of 4.5 to 10.5;

(4) pH stability

Very stable and not inactivated at a pH of 5 to 12 and an activity of not less than about 50% being maintained at a pH of 4.5 to 12.5;

(5) Optimum temperature

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The working temperature is in a wide range of 15 to 80°C and an optimum temperature is 60°C; In the range of 45 to 65°C, the activity is not less than 50% of the activity at the optimum temperature;

(6) Molecular weight

About 35,000 (when determined by a gel filtration method using Bio-Gel P-150);

(7) Influences of metal ions

Inhibited by Hg^{2+} ;

(8) Influences of surface active agents

LAS, AS, ES, AOS, alpha-SFE, SAS, soaps and polyoxyethylene secondary alkyl ethers do rarely inhibit the activity;

(9) Proteinase resistance

Resistant to proteinases;

(10) Influences of chelating agents

EDTA, EGTA, citric acid, sodium tripolyphosphate and zeolite do not inhibit the activity;

2. An alkaline cellulase E-II having the following physicochemical properties:

(1) Action

Acting effectively on cellulosic materials

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including CMC and phosphoric acid-swollen cellulose and causing them to be dissolved, thereby forming reducing sugars such as glucose; /

(2) Substrate specificity

Having not only activity on CMC and phosphoric acid-swollen cellulose, but also slight activity on xylan, inulin and lichenan;

(3) Working pH and optimum pH

The working pH range is from 4 to 12.5 and the optimum pH is from 7 to 10.

(4) pH stability

When maintained at 5°C for 24 hours, it is not inactivated at a pH of 6 to 10;

(5) Optimum temperature

The working temperature is in a wide range of 10 to 80°C and an optimum temperature is 50°C;

(6) Molecular weight

About 34,000 (when determined by a gel filtration method using Bio-Gel P-100);

(7) Influences of metal ions

Inhibited by Hg^{2+} and activated by Co^{2+} ;

(8) Influences of surface active agents

LAS, AS, ES, AOS, alpha-SFE, SAS, soaps and polyoxyethylene secondary alkyl ethers do rarely inhibit

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the activity;

(9) Proteinase resistance

Resistant to proteinases;

(10) Influences of chelating agents

EDTA, EGTA, citric acid, sodium

tripolyphosphate and zeolite do not inhibit the activity.

3. An alkaline cellulase E-II according to Claim 2, which is separated and collected from a culture of Bacillus sp. KSM-522 (FERM BP-1512).

4. An alkaline cellulase E-III having the following physicochemical properties:

(1) Action

Acting well on cellulosic materials including CMC and phosphoric acid-swollen cellulose and causing them to be dissolved, thereby forming reducing sugars such as glucose;

(2) Substrate specificity

Having not only activity on CMC and phosphoric acid-swollen cellulose, but also slight activity on xylan and lichenan;

(3) Working pH and optimum pH

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The working pH range is from 4 to 12.5 and the optimum pH is from 7 to 9;

(4) pH stability

When maintained at 5°C for 24 hours, it is not inactivated at a pH of 6 to 10;

(5) Working temperature and optimum temperature

The working temperature is in the range of 10 to 80°C and an optimum temperature is 50°C;

(6) Molecular weight

About 35,000 (when determined by a gel filtration method using Bio-Gel P-100);

(7) Influences of metal ions

Inhibited by Hg^{2+} and activated by Co^{2+} ;

(8) Influences of surface active agents

LAS, AS, ES, AOS, alpha-SFE, SAS, soaps and polyoxyethylene secondary alkyl ethers do rarely inhibit the activity;

(9) Proteinase resistance

Resistant to proteinases;

(10) Influences of chelating agents

EDTA, EGTA, citric acid, sodium tripolyphosphate and zeolite do not inhibit the activity.

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5. An alkaline cellulase E-III according to Claim 4, which is separated and collected from a culture of Bacillus sp. KSM-522 (FERM BP-1512).

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"Claim 6. A microorganism belonging to the genus Bacillus, wherein said microorganism is an alkaline cellulase K-522 producing bacterium which is named Bacillus sp. KSM-522 and is deposited as FERM BP-1512, which is capable of producing an alkaline cellulase K-522, growing in a neutral medium."

wherein said microorganism is an alkaline cellulase K-522 producing bacterium which is named Bacillus sp. KSM-522 and is deposited as FERM BP-1512.

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ABSTRACT OF THE DISCLOSURE **26265**

Alkaline cellulases having an optimum pH in an alkaline range and being stable over a broad pH range are produced by microorganisms which belong to the genus Bacillus and grow in a neutral medium.

These alkaline cellulases are rarely inhibited by ingredients ordinarily incorporated in detergents such as surface active agents, proteinases and chelating agents, so that they can be conveniently used in detergent compositions.