

TITLE OF THE INVENTION

"Alkaline Cellulases: *Bacillus* sp. producing the same and process for producing the same"

BACKGROUND OF THE INVENTIONField of the Invention:

This invention relates to novel alkaline cellulases, the microorganisms producing the same, and processes for producing the alkaline cellulases.

Description of the Background Art:

Conventionally, studies on cellulase which is a cellulose-decomposable enzyme have been directed, for the most part, to the effective utilization of biomass resources, particularly of cellulose resources. The major supply sources of cellulase have been placed on fungi and wide varieties of strains isolated as the cellulase-producing microorganisms have been reported. Typical examples of these strains are the fungi belonging to the genera such as *Aspergillus*, *Penicillium*, *Trichoderma*, *Fusarium*, *Humicola*, *Acremonium*, and the like. Examples of these strains other than fungi are bacteria belonging to the genera such as *Pseudomonas*, *Cellulomonas*, *Ruminococcus*, *Bacillus*, and the like, the actinomycetes such as *Streptomyces*, *Thermoactinomyces*, and the like.

Studies on a new application of cellulase as an additive to detergents for washing clothes have lately attracted considerable attention (Japanese Patent Laid-open

Nos. 49279/1984, 23158/1985, and 36240/1985). The cellulases produced by natural microorganisms is almost classified as, namely, the neutral or acidic cellulase exhibiting a foremost and stable enzymatic activity in neutral or acidic conditions. There is few alkaline or alkali-resistant cellulase having a good stability and exhibiting maximum activity at a pH of alkaline region at which cloth washing is performed effectively when the cellulase is incorporated into a detergent composition for clothing. The alkaline cellulases herein mean those having an optimum pH in an alkaline region.

Several processes are known for producing an alkaline cellulase. Until recently, such processes have been typified by a process for collecting the cellulase A through culturing an alkalophilic microorganism belonging to the genus *Bacillus* (Japanese Patent Laid-open No. 28515/1975), a process for producing the alkaline cellulase 301-A through culturing of a microorganism belonging to the genus *Cellulomonas* (Japanese Patent Laid-open No. 224686/1983), a process for obtaining a carboxymethyl cellulase through culturing of an alkalophilic strain, *Bacillus* sp. No. 1139 [Fukumori F., Kudo T., and Horikoshi K.; *J. Gen. Microbiol.*, 131, 3339 (1985)], and a process for producing an alkaline cellulase with the use of a microorganism belonging to the *Streptomyces* (Japanese Patent Laid-open No. 19483/1986). These processes for producing an alkaline or alkali-resistant cellulase are nevertheless unsuitable for

industrial fermentative production.

Recently, it has been found that a novel microorganism *Bacillus* sp. KSM-635 (FERM BP-1485, Japanese Patent Application No. 109771/1988), a sort of an alkalophilic microorganism, sufficiently yields the alkaline cellulase K (Japanese Patent Application No. 109776/1988) which can be adapted to a detergent component for clothing, so that the fermentative production of the alkaline cellulase has been realized in an industrial scale. It has also been found that an alkaline cellulase could be produced by neutrophilic microorganisms which could be easily cultured (Japanese Patent Application Nos. 137677/1988, 240785/1988, 240777/1988, 240786/1988, and 37285/1989, and S. Kawai et al., *Agric. Biol. Chem.*, 52, 1425(1988)); and an alkali-resistant cellulase capable of keeping high activities in an alkaline condition could be produced also by the above-mentioned neutrophilic microorganisms (Japanese Patent Application Nos. 141586/1988, 146786/1988, 273474/1988, 273475/1988, 279790/1988, and 37286/1989).

However, the above-mentioned examples are enumerated as those of alkaline or alkali-resistant cellulases suitable for industrial fermentative production, further development of alkaline cellulases having various features and the microorganisms producing the alkaline cellulases have been desired.

In view of this situation, the present inventors have carried out extensive studies in order to obtain natural

microorganisms capable of producing alkaline cellulases,  
and, as a result, found that a group of microorganisms  
belonging to the genus Bacillus which was discovered by  
the inventors in the soils in Nasu-gun, Tochigi-ken,  
5 Japan, was capable of producing a novel alkaline  
cellulase which was effective as a detergent component  
for clothing. This finding has led to the completion  
of the present invention.

#### 10 SUMMARY OF THE INVENTION

Accordingly, an object of this invention is to provide  
the novel alkaline cellulases K-64, K-19 and K-520 and  
microorganisms belonging to the genera Bacillus sp.  
KSM-64, KSM-19, and KSM-520 which are all capable of  
15 producing the above alkaline cellulases respectively.

K-64 is defined hereinafter in Claim 1, K-19 in Claim 2  
and K-520 in Claim 3.

20 Other objects, features and advantages of the invention  
will hereinafter become more readily apparent from the

following description.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a drawing showing the relation of relative activity vs. reaction pH of the alkaline cellulase K-64 of this invention.

Fig. 2 is a drawing showing the relation of treating pH vs. residual activity of the alkaline cellulase K-64 of this invention.

Fig. 3 is a drawing showing the relation of reaction temperature vs. relative activity of the alkaline cellulase K-64 of the present invention.

Fig. 4 is a drawing showing the relation of treating temperature vs. residual activity of the alkaline cellulase K-64 of the present invention.

Fig. 5 is a drawing showing the relation of relative activity vs. reaction pH of the alkaline cellulase K-19 of this invention.

Fig. 6 is a drawing showing the relation of treating pH vs. residual activity of the alkaline cellulase K-19 of this invention.

Fig. 7 is a drawing showing the relation of reaction temperature vs. relative activity of the alkaline cellulase K-19 of the present invention.

Fig. 8 is a drawing showing the relation of treating temperature vs. residual activity of the alkaline cellulase K-19 of the present invention.

Fig. 9 is a drawing showing the relation of relative

activity vs. reaction pH of the alkaline cellulase K-520 of this invention.

Fig. 10 is a drawing showing the relation of treating pH vs. residual activity of the alkaline cellulase K-520 of this invention.

Fig. 11 is a drawing showing the relation of treating temperature vs. relative activity of the alkaline cellulase K-520 of the present invention.

Fig. 12 is a drawing showing the relation of treating temperature vs. residual activity of the alkaline cellulase K-520 of the present invention.

#### DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

Mycological characteristics of these microorganisms are now discussed. The following 21 culture media (Media 1 to 21) are used for the classification of strains. They all contain 1.0% by weight of sterilized sodium carbonate ( $\text{Na}_2\text{CO}_3$ ).

#### Compositions of the culture media used (% by weight)

Medium 1: nutrient broth, 0.8; Bacto agar, 1.5

Medium 2: nutrient broth, 0.8

Medium 3: nutrient broth, 0.8; Bacto gelatin, 20.0;  
Bacto agar, 1.5

Medium 4: Bacto litmus milk, 10.5

Medium 5: nutrient broth, 0.8;  $\text{KNO}_3$ , 0.1

Medium 6: Bacto peptone, 0.7;  $\text{NaCl}$ , 0.5;  
glucose, 0.5

Medium 7: SIM agar medium, an amount prescribed.

- Medium 8: TSI agar medium (manufactured by Eiken Chem. Co., Ltd., Japan), an amount prescribed.
- Medium 9: Bacto peptone, 1.5; yeast extract, 0.5; soluble starch, 2.0;  $K_2HPO_4$ , 0.1;  $MgSO_4 \cdot 7H_2O$ , 0.02; Bacto agar, 1.5
- Medium 10: Koser's medium, an amount prescribed.
- Medium 11: Christensen's medium (manufactured by Eiken Chem. Co., Ltd., Japan), an amount prescribed.
- Medium 12: the medium including the following compositions (1) and (2) to which added are nitrogen sources consisting of sodium nitrate, sodium nitrite, ammonium chloride, and ammonium phosphate in such an amount as that the total amount of nitrogen is 0.0412N% by weight in the medium.
- (1) yeast extract, 0.05;  $Na_2SO_4$ , 0.1;  $KH_2PO_4$ , 0.1; glucose, 1.0
- (2) yeast extract, 0.05;  $Na_2SO_4$ , 0.1;  $KH_2PO_4$ , 0.1; glucose, 1.0;  $CaCl_2 \cdot 2H_2O$ , 0.05;  $MnSO_4 \cdot 4-6H_2O$ , 0.01;  $FeSO_4 \cdot 7H_2O$ , 0.001;  $MgSO_4 \cdot 7H_2O$ , 0.02
- Medium 13: King A medium "Eiken" (manufactured by Eiken Chem. Co., Ltd., Japan), an amount prescribed.
- Medium 14: King B medium "Eiken" (manufactured by Eiken Chem. Co., Ltd., Japan), an amount

prescribed.

Medium 15: urea medium "Eiken" (manufactured by Eiken Chem. Co., Ltd., Japan), an amount prescribed.

Medium 16: cytochrome-oxidase test filter paper (manufactured by Nissui Pharmaceutical Co., Ltd., Japan)

Medium 17: 3% aqueous hydrogen peroxide

Medium 18: Bacto peptone, 0.5; yeast extract, 0.5; glucose, 1.0;  $K_2HPO_4$ , 0.1;  $MgSO_4 \cdot 7H_2O$ , 0.02

Medium 19: Bacto peptone, 2.7; NaCl, 5.5; glucose, 0.5;  $K_2HPO_4$ , 0.3; bromthymol blue, 0.06; Bacto agar, 1.5

Medium 20:  $(NH_4)_2HPO_4$ , 0.1; KCl, 0.02;  $MgSO_4 \cdot 7H_2O$ , 0.02; yeast extract, 0.05; sugar, 1.0

Medium 21: casein, 0.5; Bacto agar, 1.5; yeast extract, 0.5; glucose, 1.0;  $K_2HPO_4$ , 0.1;  $MgSO_4 \cdot 7H_2O$ , 0.02

Mycological characteristics of *Bacillus* sp. KSM-64

(1) Observation under microscope

The cells are gram-positive rods of a size of 0.5 - 1.2  $\mu m$  x 1.2 - 6.6  $\mu m$ , with a cylindrical endospore (0.7 - 1.0  $\mu m$  x 1.5 - 2.5  $\mu m$ ) forming at their subterminals. They have flagella and are motile. Gram's staining is positive.

(2) Growth in various culture media

(a) Nutrient broth-agar plate (Medium 1)

Growth of cells is good. The colony has a circular shape, with its surface being smooth and its peripheral end being smooth. The color of the colony is milky and glossy.

(b) Liquid nutrient broth (Medium 2)

Growth of cells is good.

(c) Stab culturing in nutrient broth-gelatin (Medium 3)

Growth of cells is good. Liquefaction of gelatin is observed.

(d) Litmus milk medium (Medium 4)

Milk coagulation and peptonization are not observed. Litmus color does not change because the medium is an alkaline one.

(3) Physiological characteristics

(a) Nitrate reduction and denitrification (Medium 5)

Nitrate reduction: positive

Denitrification: negative

(b) MR test (Medium 6)

Undeterminable

(c) VP test (Medium 6)

Positive

(d) Production of indole (Medium 7)

Negative

(e) Production of hydrogen sulfide (Medium 8)

Negative

(f) Hydrolysis of starch (Medium 9)

Positive

- (g) Utilization of citric acid (Medium 10, Medium 11)

Positive in Koser's medium and undeterminable in Christensen's medium

- (h) Utilization of inorganic nitrogen sources  
(Medium 12)

Nitrate and ammonium salts are both utilized.

- (i) Discoloration (Medium 13, Medium 14)

Yellow color is observed in King B medium.

- (j) Urease (Medium 15)

Negative

- (k) Oxidase (Medium 16)

Positive

- (l) Catalase (Medium 17)

Positive

- (m) Growth range (Medium 18)

Growth temperature: 20 - 40°C,

Optimum growth temperature: 30 - 37°C

Growth pH range: 7 - 11

Optimum pH range: 10.5

- (n) Behavior on oxygen

Aerobic

- (o) O-F test (Medium 19)

Undeterminable because the medium is an alkaline medium. Cells can grow only in an aerobic condition.

- (p) Sugar utilization (Medium 20, +: cells can grow, -: cells do not grow)

|        |             |   |
|--------|-------------|---|
| (i)    | L-arabinose | + |
| (ii)   | D-xylose    | + |
| (iii)  | D-glucose   | + |
| (iv)   | D-mannose   | + |
| (v)    | D-fructose  | + |
| (vi)   | D-galactose | + |
| (vii)  | Maltose     | + |
| (viii) | Sucrose     | + |
| (ix)   | Lactose     | + |
| (x)    | Trehalose   | + |
| (xi)   | D-sorbitol  | - |
| (xii)  | D-mannitol  | + |
| (xiii) | Inositol    | + |
| (xiv)  | Glycerol    | + |
| (xv)   | Starch      | + |

(q) Growth in a medium containing sodium chloride

(a modification of Medium 1)

Cells can grow in the presence of a 7% of sodium chloride, but can not grow in the presence of a 10% sodium chloride.

(r) Hydrolysis of casein ( Medium 21)

Positive

(s) G + C content (Tm method)

About 37.7 mol% for the DNA of the microorganism of the present invention.

Mycological characteristics of *Bacillus* sp. KSM-19

(1) Observation under microscope

The cells are gram-positive rods of a size of 0.5 - 1.0  $\mu\text{m}$  x 1.2 - 5.8  $\mu\text{m}$ , with an oval endospore (0.7 - 1.2  $\mu\text{m}$  x 1.2 - 1.7  $\mu\text{m}$ ) forming at their subterminals. They have flagella and are motile. Gram's staining is positive.

(2) Growth in various culture media

(a) Nutrient broth-agar plate (Medium 1)

Growth of cells is good. The colony has an irregular shape, with its surface being smooth and its peripheral end being wavy. The color of the colony is milky and glossy.

(b) Liquid nutrient broth (Medium 2)

Growth of cells is good.

(c) Stab culturing in nutrient broth-gelatin (Medium 3)

Growth of cells is good. Liquefaction of gelatin is observed.

(d) Litmus milk medium (Medium 4)

Milk coagulation and peptonization are not observed. Litmus color does not change because the medium is an alkaline one.

(3) Physiological characteristics

(a) Nitrate reduction and denitrification (Medium 5)

Nitrate reduction: positive

Denitrification: negative

(b) MR test (Medium 6)

Undeterminable

(c) VP test (Medium 6)

Positive

(d) Production of indole (Medium 7)

Negative

(e) Production of hydrogen sulfide (Medium 8)

Negative

(f) Hydrolysis of starch (Medium 9)

Positive

(g) Utilization of citric acid (Medium 10, Medium 11)

Undeterminable in Christensen's medium.

(h) Utilization of inorganic nitrogen sources

(Medium 12)

Nitrate and ammonium salts are both utilized.

(i) Discoloration (Medium 13, Medium 14)

Yellow color is observed in King B medium.

(j) Urease (Medium 15)

Negative

(k) Oxidase (Medium 16)

Positive

(l) Catalase (Medium 17)

Positive

(m) Growth range (Medium 18)

Growth temperature: 20 - 37°C,

Optimum growth temperature: 30 - 37°C

Growth pH range: 7 - 11

Optimum pH range: 10.5

(n) Behavior on oxygen

Aerobic

(o) O-F test (Medium 19)

Undeterminable because the medium is an alkaline medium. Cells can grow only in an aerobic condition.

(p) Sugar utilization (Medium 20, +: cells can grow, -: cells do not grow)

|        |             |   |
|--------|-------------|---|
| (i)    | L-arabinose | + |
| (ii)   | D-xylose    | + |
| (iii)  | D-glucose   | + |
| (iv)   | D-mannose   | + |
| (v)    | D-fructose  | + |
| (vi)   | D-galactose | + |
| (vii)  | Maltose     | + |
| (viii) | Sucrose     | + |
| (ix)   | Lactose     | + |
| (x)    | Trehalose   | + |
| (xi)   | D-sorbitol  | - |
| (xii)  | D-mannitol  | + |
| (xiii) | Inositol    | - |
| (xiv)  | Glycerol    | + |
| (xv)   | Starch      | + |

(q) Growth in a medium containing sodium chloride

(a modification of Medium 1)

Cells can grow in the presence of a 7% of sodium chloride, but can not grow in the presence of a 10% sodium chloride.

(r) Hydrolysis of casein ( Medium 21)

Positive

(s) G + C content (Tm method)

About 38.0 mol% for the DNA of the microorganism of the present invention.

Mycological characteristics of *Bacillus* sp. KSM-520

(1) Observation under microscope

The cells are gram-positive rods of a size of 0.5 - 1.2  $\mu\text{m}$  x 1.2 - 5.0  $\mu\text{m}$ , with an oval endospore (0.7 - 1.2  $\mu\text{m}$  x 1.2 - 1.6  $\mu\text{m}$ ) forming at their subterminals. They have flagella and are motile. Gram's staining is positive.

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Growth of cells is good. The colony has an irregular shape, with its surface being smooth and its peripheral end being wavy. The color of the colony is milky and glossy.

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Growth of cells is good.

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Growth of cells is good. Liquefaction of gelatin is observed.

(d) Litmus milk medium (Medium 4)

Milk coagulation and peptonization are observed. Litmus color does not change because the medium is an alkaline one.

(3) Physiological characteristics

(a) Nitrate reduction and denitrification (Medium 5)

Nitrate reduction: positive

Denitrification: negative

(b) MR test (Medium 6)

Undeterminable

(c) VP test (Medium 6)

Positive

(d) Production of indole (Medium 7)

Negative

(e) Production of hydrogen sulfide (Medium 8)

Negative

(f) Hydrolysis of starch (Medium 9)

Positive

(g) Utilization of citric acid (Medium 10, Medium 11)

Positive in Koser's medium and undeterminable in  
Christensen's medium

(h) Utilization of inorganic nitrogen sources

(Medium 12)

Nitrate and ammonium salts are both utilized.

(i) Formation of pigment (Medium 13, Medium 14)

Yellow color is observed in King B medium.

(j) Urease (Medium 15)

Negative

(k) Oxidase (Medium 16)

Positive

(l) Catalase (Medium 17)

Positive

(m) Growth range (Medium 18)

Growth temperature: 15 - 40°C,

Optimum growth temperature: 30 - 37°C

Growth pH range: 7 - 11

Optimum pH range: 10.5

(n) Behavior on oxygen

Aerobic

(o) O-F test (Medium 19)

Undeterminable because the medium is an alkaline medium. Cells can grow only in an aerobic condition.

(p) Sugar utilization (Medium 20, +: cells can grow, -: cells do not grow)

|        |             |   |
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| (i)    | L-arabinose | + |
| (ii)   | D-xylose    | + |
| (iii)  | D-glucose   | + |
| (iv)   | D-mannose   | + |
| (v)    | D-fructose  | + |
| (vi)   | D-galactose | + |
| (vii)  | Maltose     | + |
| (viii) | Sucrose     | + |
| (ix)   | Lactose     | + |
| (x)    | Trehalose   | + |
| (xi)   | D-sorbitol  | + |
| (xii)  | D-mannitol  | + |
| (xiii) | Inositol    | - |
| (xiv)  | Glycerol    | + |
| (xv)   | Starch      | + |

(q) Growth in a medium containing sodium chloride

(a modification of Medium 1)

Cells can grow in the presence of a 7% of sodium chloride, but can not grow in the presence of a 10% sodium chloride.

(r) Hydrolysis of casein ( Medium 21)

Positive

(s) G + C content (Tm method)

About 37.6 mol% for the DNA of the microorganism of the present invention.

Based on the above mycological characteristics, the strains of the present invention were examined referring to Bergey's Manual of *Systematic Bacteriology*, Vol. 2 and "*The Genus Bacillus*" Ruth, E. Gordon, *Agriculture Handbook No. 427*, Agricultural Research Service, U. S. Department of Agriculture Washington D. C., (1973), and determined as a sort of microorganisms belonging to the genus *Bacillus* which is an ascospore *Bacillus*. The strains do not grow in a neutral range, but can grow mostly in an alkaline range. From this fact, the strains of the present invention are classified as an alkalophilic microorganism which has recently been demonstrated by Horikoshi and Akiba, "*Alkalophilic Microorganism*", Japan Scientific Society Press (Tokyo), 1982. The strains of the present invention are distinguished from a group of microorganisms belonging to the genus *Bacillus* which grows in an neutral range.

The strains of the present invention, other than the above-mentioned characteristics, have mycologically different characteristics from those of any conventionally

known alkalophilic *Bacillus*. Accordingly, the strains of the present invention were determined as novel strains and named *Bacillus* sp. KSM-64, *Bacillus* sp. KSM-19, *Bacillus* sp. KSM-520, which were deposited pursuant to Rule 7.1 of the Budapest Treaty with Fermentation Research Institute, Agency of Industrial Science and Technology as FERM BP-2886, FERM BP-2885, and FERM BP-2887 respectively.

The production of alkaline cellulase of the present invention can be carried out, for example, by the method of measuring CMCase activity to select the alkaline cellulase-producing microorganisms and culturing the microorganisms to obtain said alkaline cellulase from the cultured cells.

The alkaline cellulase of the present invention can also be produced, for example, by the fermentation processes using alkalophilic *Bacillus* sp. KSM-64, *Bacillus* sp. KSM-19, or *Bacillus* sp. KSM-520 or the mutants induced from these strains which have a higher alkaline cellulase-producing capability.

It has also been clarified by the present inventors that *Bacillus* sp. KSM-64 (FERM BP-2886) is capable of producing alkaline cellulase K-64 which possesses high  $\beta$ -1,3-; 1,4-glucan- and  $\beta$ -1,3-glucan-decomposable activities.

The strains of the present invention are inoculated into an appropriate medium and cultured according to a

conventional culture method to obtain the alkaline cellulase of the present invention. Inclusion of a suitable amount of carbon and nitrogen sources which the microorganism can utilize in the medium is desirable. There are no specific limitations as to the carbon and nitrogen sources.

Enumerated as nitrogen sources are ammonium nitrate, ammonium sulfate, ammonium phosphate, sodium nitrate, corn gluten meal, soybean flour, corn steep liquor, casamino acid, yeast extract, Pharmamedia sardine meal, meat extract, peptone, hypro, Ajipower, corn-soybean meal, coffee meal, cotton seed meal, Cultivator, Ajipron, Zest, and the like. Given as examples of carbon sources are fibrous substances such as chaff, hull, filter paper, general paper, sawdust; waste molasses, invert sugar, CMC, Avicel, cellulose cotton, xylan, pectin, ribose, arabinose, xylose, glucose, mannose, fructose, galactose, maltose, sucrose, lactose, trehalose, mannitol, inositol, glycerol, starch, acetic acid, citric acid, and the like. In addition to these carbon and nitrogen sources, phosphoric acid, salts of metal such as  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $Mn^{2+}$ ,  $Zn^{2+}$ ,  $Co^{2+}$ ,  $Na^{+}$ ,  $K^{+}$ , and the like, as required, other micro-nutritious organic or inorganic substances can be added into the culture medium.

The alkaline cellulases of the present invention can be prepared from the culture broth by means of conventional collection and purification methods adapted for general enzymes. Specifically, the cells are separated from the culture broth by means of conventional solid-liquid

separation methods, such as, centrifugation, filtration, or the like, to obtain a crude enzyme liquid. While it is possible to use the crude enzyme liquid per se thus obtained, it can be served as a purified enzyme, as required, after separating the crude enzyme by means of conventional separation methods, e.g. salting out, precipitation, ultrafiltration, and the like, and then purifying and crystallizing the crude enzyme by conventional methods.

Enzymological characteristics of novel enzymes alkaline cellulases K-64, K-19, and K-520, of this invention are now discussed. Enzymatic activities were measured according to the following methods.

(1) CMCase activity

To the base solution comprised of 0.4 ml of 2.5% CMC, (Sunrose AOIL, manufactured by Sanyo-Kokusaku Pulp Co., Ltd.), 0.2 ml of 500 mM glycine buffer (pH 9), and 0.3 ml of deionized water was added 0.1 ml of enzyme solution, and the mixture was reacted for 20 minutes at 30°C. On completion of the reaction, reducing sugar was quantitatively analyzed by the 3,5-dinitro-salicylic acid (DNS) method. Specifically, 1 ml of DNS reagent was added to 1.0 ml of reaction liquid, and mixture was caused to color by heating at 100°C for 5 minutes. After cooling, the colored mixture was diluted with 4.0 ml of deionized water and subjected to colorimetry at 535 nm. One (1) unit of enzyme activity is defined as the amount of the enzyme which formed 1  $\mu$ mol of

reducing sugar expressed as glucose, per minute.

The buffer solutions used are as follows:

pH 3 - 8: MacIlvaine's buffer

pH 8 - 11: glycine-NaOH buffer

pH 12 - 13: KCl-NaOH buffer

(2) p-nitrophenyl- $\beta$ -D-glucopyranoside or p-nitrophenyl- $\beta$ -D-cellobioside-hydrolyzing activity.

To 1.0 ml of reaction liquid comprised of 0.8  $\mu$ mol of p-nitrophenyl- $\beta$ -D-cellobioside (manufactured by Sigma Co.) or p-nitrophenyl- $\beta$ -D-glucopyranoside (manufactured by Sigma Co.) and 50  $\mu$ mol of phosphate buffer (pH 7.0) or 100  $\mu$ mol of glycine-NaOH buffer (pH 9) was effected a suitable amount of enzyme liquid at 30°C. 0.4 ml of 1 M Na<sub>2</sub>CO<sub>3</sub> was added to the mixture. The p-nitrophenol liberated was measured by colimetry at a wavelength of 410 nm. One (1) unit of enzyme activity is defined as the amount of the enzyme producing 1  $\mu$ mol of p-nitrophenol per minute.

(3) Avicel-, cellulose powder-, phosphoric acid swollen cellulose-, and filter paper-hydrolyzing activity

To 1.0 mg of reaction liquid comprised of 10 mg of Avicel and 100  $\mu$ mol of glycine buffer (pH 9) was added an appropriate amount of enzyme and the mixture was reacted at 30°C with shaking at 280 rpm. On completion of the reaction, reducing sugar formed was quantitatively determined by means of the DNS method. One (1) unit of enzyme activity is defined as the amount of the enzyme which formed 1  $\mu$ mol

of reducing sugar expressed as glucose, per minute.

Other enzymatic activities were measured in the same manner as above. Cellulose powder (manufactured by Toyo Roshi Co.), cellulase activity test filter paper (Toyo No. 51-Toku manufactured by Toyo Roshi Co.), and alkali swollen cellulose and phosphoric acid swollen cellulose which were prepared according to the method by Tomita et al (Tomita, Y. et al.,: *J. Ferment. Technol.*, 52, 235, 1974).

(4)  $\beta$ -1,3-; 1,4-glucanase- and  $\beta$ -1,3-glucanase-activity

The activity was measured in the same manner as in (3) using lichenan (from *Usnera barubata*, manufactured by Sigma Co.) and laminarin (from *Laminaria digitata*, manufactured by Sigma Co.) as substrates.

Enzymological characteristics of alkaline cellulase K-64

(1) Action

The enzyme of the present invention acts well on cellulosic materials such as CMC, cellulose powder, Avicel, and the like dissolves them, and produces reducing sugars.

(2) Substrate specificity

The substrate specificity of the enzyme of the present invention is exemplified in Table 1. The enzyme of this invention possesses a high hydrolytic activity against lichenan which is  $\beta$ -1,3-; 1,4-glucan as well as CMC; acts on laminarin which is  $\beta$ -1,3-glucan; and slightly acts on filter paper, p-nitrophenyl- $\beta$ -D-glucopyranoside, and p-nitrophenyl- $\beta$ -D-cellobioside.

Table 1

| Substrate                         | Concentration | Relative Activity (%) |
|-----------------------------------|---------------|-----------------------|
| CMC                               | 1%            | 100                   |
| Avicel                            | 1%            | 2                     |
| Filter Paper                      | 1%            | 4                     |
| Phosphoric acid swollen cellulose | 1%            | 34                    |
| PNPC *                            | 8 mM          | 1                     |
| PNPG **                           | 8 mM          | 1                     |
| Lichenan                          | 1%            | 154                   |
| Laminarin                         | 1%            | 25                    |

\* p-nitrophenyl - $\beta$ -D-cellobioside

\*\* p-nitrophenyl- $\beta$ -D-glucopyranoside

### (3) Active pH range and optimum pH

The active pH range on CMC is broad, i.e. 3 - 13.0 with an optimum pH range being 8.5 - 10. It shows a relative activity more than 50% of the activity of the optimum pH range even in pHs ranging 7.0 - 12.0 (see Fig. 1).

### (4) pH stability

After the enzyme adjusted to various pHs was left stand at 30°C for one hour, and the residual activity was measured to evaluate pH stability. The enzyme is quite stable in pHs ranging 5.0 to 10.5 and has the activity of more than 50% even in pHs ranging 3.0 to 11 (see Fig. 2).

### (5) Active temperature and optimum temperature

The enzyme of the present invention acts at wide

temperatures ranging 10 to 70°C with an optimum temperature being 50°C and has more than 50% of the activity of the optimum temperature even in temperatures ranging 35 to 60°C (see Fig. 3).

(6) Thermal stability

Residual activities after heat-treatment in glycine-NaOH buffer (pH 9.0) at various temperatures for 10 minutes were measured. The enzyme of this invention scarcely loses its activity at 50°C, and has the residual activity of about 50% even at 55°C (see Fig. 4).

(7) Molecular weight

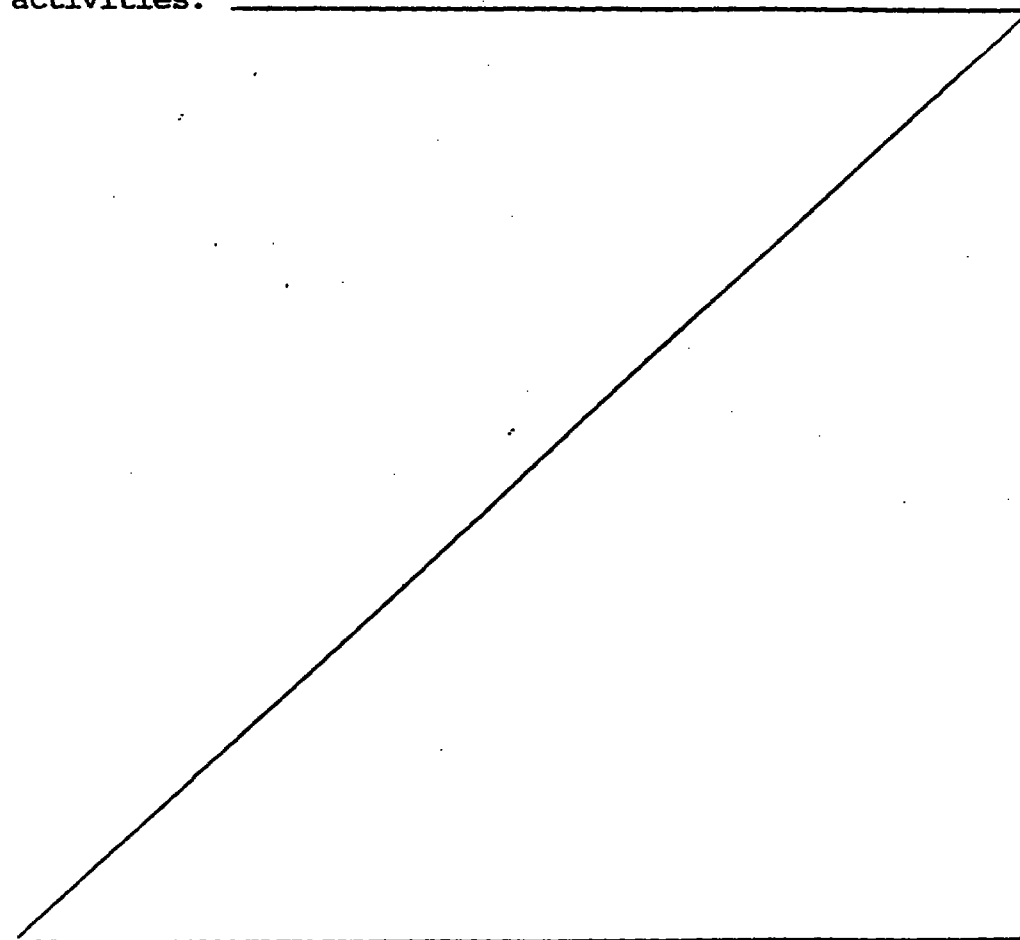
The molecular weight of the enzyme of the present invention was measured by means of a gel filtration method using Bio-Gel A 0.5m (manufactured by Biograd Co.). The enzyme of this invention has peaks at approximately 180,000  $\pm$  10,000 and 80,000  $\pm$  2,000, when CMC is a substrate.

(8) Effect of metal ions

The effect of metal ions was investigated, at the same time when the activities of the enzyme were measured, by incorporating a metal ion such as Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Cu<sup>2+</sup>, Co<sup>2+</sup>, Cd<sup>2+</sup>, Mn<sup>2+</sup>, Mg<sup>2+</sup>, Ba<sup>2+</sup>, Ni<sup>2+</sup>, Hg<sup>2+</sup>, Fe<sup>2+</sup>, Pb<sup>2+</sup>, Zn<sup>2+</sup>, Al<sup>3+</sup>, and Fe<sup>3+</sup>. Addition of Hg<sup>2+</sup> ion (1mM) gives an adverse effect and addition of Co<sup>2+</sup> and Mn<sup>2+</sup> ions (1 mM each) gives an activated effect.

(9) Effect of surfactants

0.05% by weight of a surfactant such  
as linear alkylbenzene sulfonate, alkyl sulfate,  
5 polyoxyethylene alkyl sulfonate,  $\alpha$ -olefin sulfonate,  
 $\alpha$ -carboxymethyl alkylsulfonate, secondary alkyl  
sulfonate, secondary alkyl sulfate, dodecyl sulfonate,  
soap, and/or polyoxyethylene secondary alkylether was  
tested and confirmed no inhibitive effects on enzymatic  
10 activities.



(10) Effect of proteinases

In the presence of a protease for detergents, for example, API-21 (manufactured by Showa Denko), Maxatase (manufactured by IBIS), and Savinase, Alkalase, and Esperase (manufactured by Novo) in an amount of 0.2 AU/l, the activities were measured, to confirm that the enzyme of this invention had an excellent resistance to any proteinases.

(11) Effect of chelating agents

In the presence of a chelating agent such as EDTA, EGTA, sodium tripolyphosphate, zeolite, citric acid, or the like, the activities were measured and confirmed that any chelating agents had no inhibitive effects on enzymatic activities.

Enzymological characteristics of alkaline cellulase K-19

(1) Action

The enzyme of the present invention acts well on cellulosic materials such as CMC, cellulose powder, Avicel, and the like dissolves them, and produces reducing sugars.

(2) Substrate specificity

The substrate specificity of the enzyme of the present invention is exemplified in Table 2. The enzyme of this invention acts on, cellulose powder, phosphoric acid swollen cellulose, Avicel, alkali swollen cellulose, filter paper, p-nitrophenyl- $\beta$ -D-glucopyranoside, and p-nitrophenyl- $\beta$ -D-cellobioside, and the like, as well as CMC; possesses a high

hydrolytic activity against lichenan which is  $\beta$ -1,3-; 1,4-glucan; acts on laminarin which is  $\beta$ -1,3-glucan.

Table 2

| Substrate                         | Concentration | Relative Activity (%) |
|-----------------------------------|---------------|-----------------------|
| CMC                               | 1%            | 100                   |
| Avicel                            | 1%            | 5                     |
| Filter Paper                      | 1%            | 4                     |
| Cellulose powder                  | 1%            | 3                     |
| Phosphoric acid swollen cellulose | 1%            | 3                     |
| Alkali swollen cellulose          | 1%            | 3                     |
| PNPC *                            | 8 mM          | 1                     |
| PNPG **                           | 8 mM          | 2                     |
| Lichenan                          | 1%            | 161                   |
| Laminarin                         | 1%            | 37                    |

\* p-nitrophenyl- $\beta$ -D-cellobioside

\*\* p-nitrophenyl- $\beta$ -D-glucopyranoside

### (3) Active pH range and optimum pH

The active pH range on CMC is broad, i.e. 4.5 - 12.5 with an optimum pH range being 9.0. It shows a relative activity more than 50% of the activity of the optimum pH range even in pHs ranging 7.0 - 11.0 (see Fig. 5).

### (4) pH stability

After the enzyme adjusted to various pHs was left stand at 30°C for one hour, the residual activity was

measured to evaluate pH stability. The enzyme is quite stable in pHs ranging 4.5 to 11.0 and shows more than 50% of the activity even in pHs ranging 4.0 to 12.0 (see Fig. 6).

(5) Active temperature and optimum temperature

The enzyme of the present invention acts at wide temperatures ranging 10 to 70°C with an optimum temperature being 50°C and has more than 50% of the activity of the optimum temperature even in temperatures ranging 35 to 60°C (see Fig. 7).

(6) Thermal stability

The residual activities after heat-treatment in glycine-NaOH buffer (pH 9.0) at various temperatures for 10 minutes were measured. The enzyme of this invention scarcely loses its activity at 50°C, and has the residual activity of about 50% even at 55°C (see Fig. 8).

(7) Molecular weight

The molecular weight of the enzyme of the present invention was measured by means of a gel filtration method using Bio-Gel A 0.5m (manufactured by BioRad Co.). The enzyme of this invention has peaks at about 120,000  $\pm$  20,000 and 34,000  $\pm$  2,000 (small peak) when CMC is a substrate.

(8) Effect of metal ions

The effect of metal ions was investigated, at the same time when the activities of the enzyme were measured, by incorporating a metal ion such as Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Cu<sup>2+</sup>, Co<sup>2+</sup>, Cd<sup>2+</sup>, Mn<sup>2+</sup>, Mg<sup>2+</sup>, Ba<sup>2+</sup>, Ni<sup>2+</sup>, Hg<sup>2+</sup>, Fe<sup>2+</sup>, Pb<sup>2+</sup>, Zn<sup>2+</sup>, Al<sup>3+</sup>, and Fe<sup>3+</sup>. Addition of Hg<sup>2+</sup> and Pb<sup>2+</sup> ions (1 mM each)

gives an adverse effect and addition of  $\text{Co}^{2+}$  and  $\text{Mn}^{2+}$  ions (1mM each) gives an activated effect.

(9) Effect of surfactants

0.05% by weight of a surfactant such as  
5 linear alkylbenzene sulfonate, alkyl sulfate,  
polyoxethylene alkyl sulfonate,  $\alpha$ -olefin sulfonate  
 $\alpha$ -carboxymethyl alkylsulfonate, secondary alkyl  
sulfonate, dodecyl sulfonate, soap, and/or  
polyoxyethylene secondary alkylether was tested to  
10 confirm no inhibitive effects on enzymatic activities

(10) Effect of proteinases

In the presence of a protease for detergents,  
for example, API-21 (Showa Denko), Maxatase (IBIS), and  
Savinase, Alkalase, and Esperase (Novo) in an amount  
15 of 0.2 AU/1, the activities were measured to confirm  
that the enzyme of this invention had an excellent  
resistance to any proteinases.

(11) Effect of chelating agents

In the presence of a chelating agent such as  
20 EDTA, EGTA, sodium tripolyphosphate, zeolite, and citric  
acid, the activities were measured, to confirm that any  
chelating agents had no inhibitive effects on enzymatic  
activities.

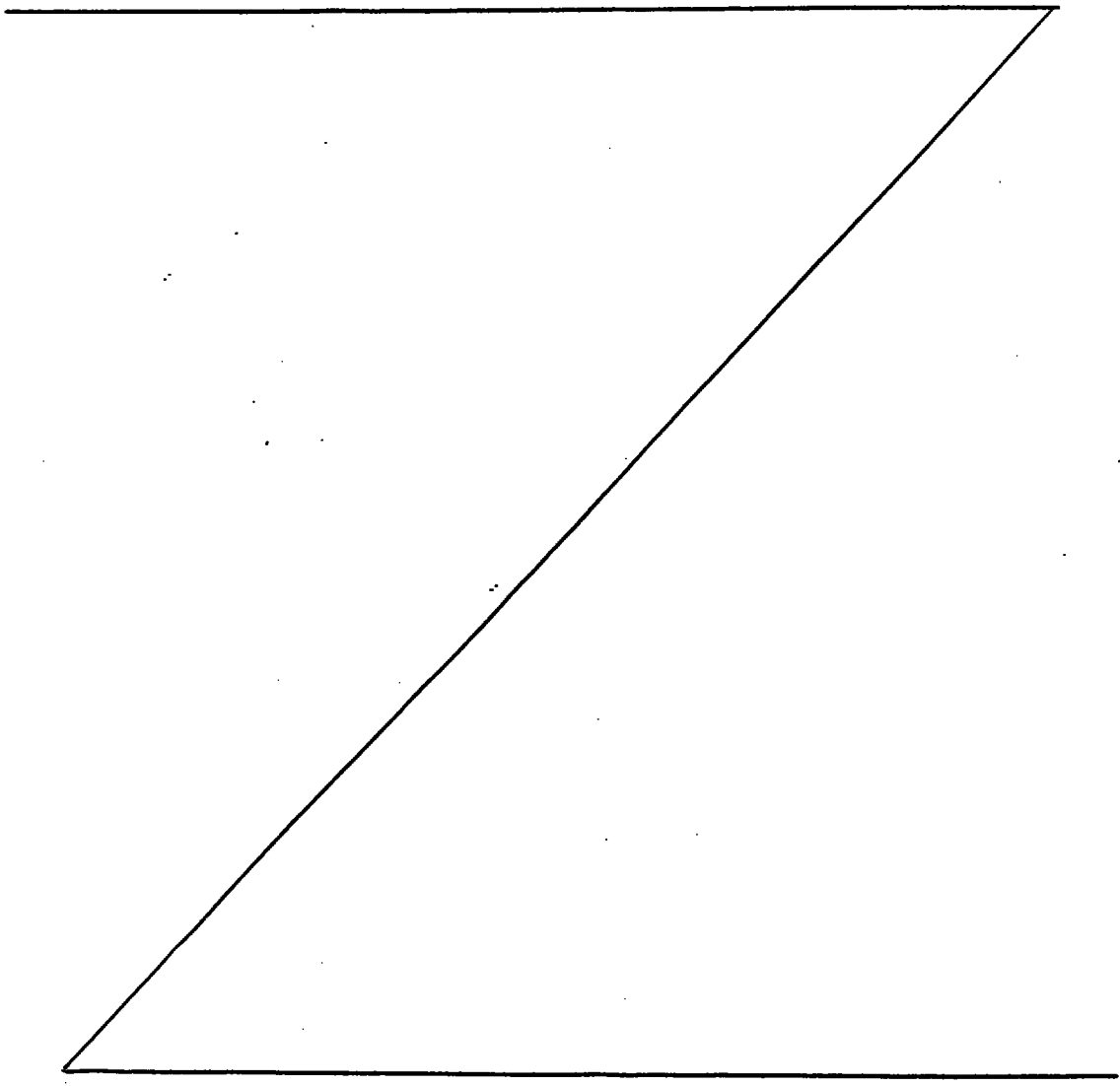
## Enzymological characteristics of alkaline cellulase K-520

### (1) Action

The enzyme of the present invention acts well on cellulosic materials such as CMC, cellulose powder, Avicel  
5 and the like and dissolves them, to produce reducing sugars.

### (2) Substrate specificity

The substrate specificity of the enzyme of the



present invention is exemplified in Table 3. The enzyme of this invention slightly acts on laminarin, lichenan, filter paper, p-nitrophenyl- $\beta$ -D-glucopyranoside, and p-nitrophenyl- $\beta$ -D-cellobioside.

Table 3

| Substrate                         | Concentration | Relative Activity (%) |
|-----------------------------------|---------------|-----------------------|
| CMC                               | 1%            | 100                   |
| Avicel                            | 1%            | 20                    |
| Filter Paper                      | 1%            | 0.2                   |
| Cellulose powder                  | 1%            | 6                     |
| Phosphoric acid swollen cellulose | 1%            | 160                   |
| Alkali swollen cellulose          | 1%            | 28                    |
| PNPC *                            | 8 mM          | 0.8                   |
| PNPG **                           | 8 mM          | 0.2                   |
| Lichenan                          | 1%            | 6                     |
| Laminarin                         | 1%            | 2                     |

\* p-nitrophenyl- $\beta$ -D-cellobioside

\*\* p-nitrophenyl- $\beta$ -D-glucopyranoside

### (3) Active pH range and optimum pH

The active pH range on CMC is broad, i.e. 3 - 13.0 with an optimum pH range being 8.5 - 9.5. It shows a relative activity of more than 50% of the activity of the optimum pH range even in pHs ranging 7.0 - 11.0 (see Fig. 9).

### (4) pH stability

After the enzyme adjusted to various pHs was left

stand at 30°C for one hour, the residual activity was measured to evaluate its pH stability. The enzyme is quite stable in pHs ranging 6.0 to 11.0 and has more than 50% of the activity even in pHs ranging 5.0 to 11.5 (see Fig. 10).

(5) Active temperature and optimum temperature

The enzyme of the present invention acts at wide temperatures ranging 10 to 75°C with an optimum temperature of 50°C and has more than 50% of the activity of the optimum temperature even in temperatures ranging 35 to 60°C (see Fig. 11).

(6) Thermal stability

The residual activities after heat-treatment in glycine-NaOH buffer (pH 9.0) at various temperatures for 10 minutes were measured. The enzyme of this invention scarcely loses its activity at 40°C, and has the residual activity of about 50% even at 50°C (see Fig. 12).

(7) Molecular weight

The molecular weight of the enzyme of the present invention was measured by means of a gel filtration method using Bio-Gel A 0.5m (manufactured by BioRad Co.). The enzyme of this invention has peaks at about  $170,000 \pm 10,000$  and  $80,000 \pm 2,000$ .

(8) Effect of metal ions

The effect of metal ions was investigated, at the same time when the activities of the enzyme were measured, by incorporating a metal ion such as  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Zn}^{2+}$ ,

$\text{Al}^{3+}$ , and  $\text{Fe}^{3+}$ . Addition of  $\text{Hg}^{2+}$ ,  $\text{Cd}^{2+}$ , and  $\text{Pb}^{2+}$  ions (1 mM each) gives an adverse effect and addition of  $\text{Co}^{2+}$  and  $\text{Mn}^{2+}$  ions (1 mM each) gives an activated effect.

(9) Effect of surfactants

5           0.05% by weight of a surfactant such as linear alkylbenzene sulfonate, alkyl sulfate, polyoxyethylene alkyl sulfonate,  $\alpha$ -olefin sulfonate,  $\alpha$ -carboxymethyl alkylsulfonate, secondary alkyl sulfonate, dodecyl sulfonate, soap, and/or  
10 polyoxyethylene secondary alkylether was tested to confirm no inhibitive effects on enzymatic activities.

(10) Effect of proteinases

          In the presence of a protease for detergents, for example, API-21 (Showa Denko), Maxatase (IBIS), and  
15 Savinase, Alkalase, and Esperase (Novo) in an amount of 0.2 AU/l, the activities were measured, to confirm that the enzyme of this invention had an excellent resistance to any proteinases.

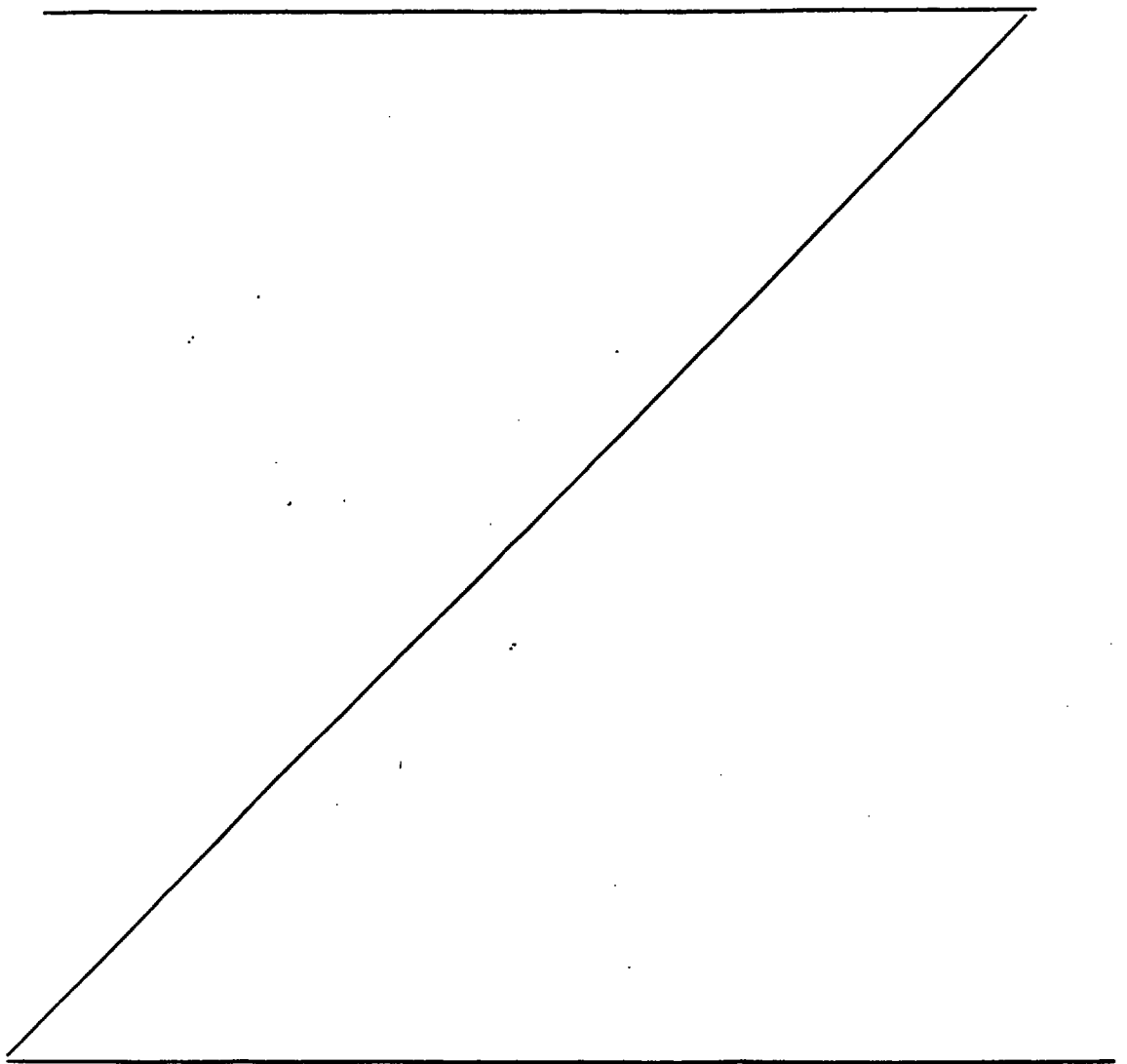
(11) Effect of chelating agents

20           In the presence of a chelating agent such as EDTA, EGTA, sodium tripolyphosphate, zeolite, citric acid, or the like, the activities were measured, to confirm that any chelating agents had no inhibitive

effects on enzymatic activities.

Other features of the invention will become apparent in the course of the following description of the exemplary embodiments which are given for

5 illustration of the invention and are not intended to be limiting thereof.



## EXAMPLES

### Example 1

A spoonful of soil (about 0.5 g) of Nasu-gun, Tochigi-ken, Japan was suspended in sterilized saline and the mixture was heat-treated at 80°C for 10 minutes. A supernatant of the heat-treated mixture was appropriately diluted, applied onto an isolating agar medium (Medium A), and cultured at 30°C for 3 days to grow colonies. The colonies which formed transparent ranges in their peripheries due to CMC dissolution were collected to obtain CMCase producing strains. These strains were inoculated into the liquid medium B and shake-cultured at 30°C for 3 days. After culturing, the cultured broth was centrifuged to separate a supernatant. The CMCase activity of the supernatant was measured at pH ranging 3 to 13 to select alkaline cellulase-producing strains. *Bacillus* sp. KSM-64 (FERM BP-2886) and *Bacillus* sp. KSM-520 (FERM BP-2887) were thus obtained.

|          |                                      | % by weight |
|----------|--------------------------------------|-------------|
| Medium A | CMC                                  | 2           |
|          | Polypepton                           | 0.5         |
|          | Yeast extract                        | 0.5         |
|          | KH <sub>2</sub> PO <sub>4</sub>      | 0.1         |
|          | MgSO <sub>4</sub> ·7H <sub>2</sub> O | 0.02        |
|          | Na <sub>2</sub> CO <sub>3</sub>      | 0.8         |
|          | Agar                                 | 0.75        |
| <hr/>    |                                      |             |
|          | pH: 10 (approximately)               |             |
| Medium B | CMC                                  | 1           |
|          | Meat extract                         | 1.5         |
|          | Yeast extract                        | 0.5         |
|          | KH <sub>2</sub> PO <sub>4</sub>      | 0.1         |
|          | Na <sub>2</sub> CO <sub>3</sub>      | 0.5         |
|          |                                      |             |
| <hr/>    |                                      |             |
|          | pH: 10 (approximately)               |             |

#### Example 2

*Bacillus* sp. KSM-64 obtained in Example 1 was inoculated into the liquid medium B of Example 1 and shake-cultured at 30°C for 3 days. After culturing, the cells were removed by means of centrifugation to obtain a crude enzyme liquid. The enzyme liquid was processed according to a conventional method to prepare ethanol-dry powder. As a result, the production of enzyme cellulase was confirmed as shown in Table 4.

Table 4

| Strain | Amount of enzyme<br>per l of medium (g) | Enzymatic *<br>activity (U/g) |
|--------|-----------------------------------------|-------------------------------|
| KSM-64 | 1.1                                     | 1,500                         |

\* Value at pH 9

#### Example 3

KSM-64 was inoculated into a medium which had the same composition as the liquid medium B in Example 1 except that 1% of cellobiose and 5% of CSL were added instead of CMC and polypepton respectively, and shake-cultured at 30°C for 2 - 3 days. CMCase activity of a supernatant centrifuged was measured to be 3,000 U/l.

#### Example 4

*Bacillus* sp. KSM-520 obtained in Example 1 was inoculated into the liquid medium B of Example 1 and shake-cultured at 30°C for 3 days. After culturing, the cells were removed by means of centrifugation to obtain a crude enzyme liquid. The enzyme liquid was processed according to a conventional method to prepare ethanol-dry powder. As a result, the production of enzyme cellulase was confirmed as shown in Table 5.

Table 5

| Strain  | Amount of enzyme<br>per l of medium (g) | Enzymatic *<br>activity (U/g) |
|---------|-----------------------------------------|-------------------------------|
| KSM-520 | 0.1                                     | 2,530                         |

\* Value at pH 9

#### Example 5

KSM-520 was inoculated into a medium which had the same composition as the liquid medium B in Example 1 except that 1% of cellobiose and 5% of CSL were added instead of CMC and polypepton respectively, and shake-cultured at 30°C for 2 - 3 days. CMCase activity of a supernatant centrifuged was measured to be 3,542 U/l.

#### Example 6

A spoonful of soil (about 0.5 g) of Nasu-gun, Tochigi-ken, Japan was suspended in sterilized saline and the mixture was heat-treated at 80°C for 10 minutes. A supernatant of the heat-treated mixture was appropriately diluted, applied onto an isolating agar medium (Medium A in Example 1), and shake-cultured at 30°C for 3 days to grow colonies. The colonies which formed a transparent range in their peripheries due to CMC dissolution were collected to obtain CMCase producing strains.

Next, these strains were inoculated into the similar medium as the medium A except that lichenan was used instead of CMC to grow

colonies. By means of Congo-red method, colonies forming transparent regions in the peripheries of the colonies due to lichenan dissolution were selected to obtain lichenase-producing strains.

The strains obtained was inoculated into the liquid medium B (used in Example 1) and shake-cultured at 30°C for 2 - 3 days. CMCase activity and lichenase activity of a supernatant centrifuged was measured at pHs of 3 - 13 to select an alkaline cellulase-producing microorganism. *Bacillus* sp. KSM-19 (FERM BP-2885) was thus obtained.  
Example 7

*Bacillus* sp. KSM-19 obtained in Example 6 was inoculated into the liquid medium B of Example 1 and shake-cultured at 30°C for 3 days. After culturing, the cells were removed by means of centrifugation to obtain a crude enzyme liquid. The enzyme liquid was processed according to a conventional method to prepare ethanol-dry powder. As a result, the production of enzyme cellulase was confirmed as shown in Table 6.

Table 6

| Strain | Amount of enzyme<br>per l of medium (g) | Enzymatic *<br>activity (U/g) |
|--------|-----------------------------------------|-------------------------------|
| KSM-19 | 0.4                                     | 1,600                         |

\* Value at pH 9

#### Example 8

KSM-19 was inoculated into a medium which had the same composition as the liquid medium B in Example 1 except that 1% of cellobiose and 5% of CSL were added instead of CMC and polypeptone respectively, and shake-cultured at 30°C for 2 - 3 days. Enzymatic activity of a supernatant centrifuged was measured to be 769 U/l of CMCase activity, 1,238 U/l of lichenase activity, and 285 U/l of laminariase activity .

Cellulases of the present invention ie K-64, K-19 and K-520 are the novel alkaline cellulases which have an optimum pH at an alkaline region and are stable in a broad pH range. The enzymes of the present invention are not adversely affected by the addition of detergent components such as surfactants, proteinases, chelating agents, and the like, and, accordingly, can effectively be used as a detergent component.

Further, alkaline cellulase K-19 of the present invention possesses  $\beta$ -1,3; 1,4-glucanase- and  $\beta$ -1,3-glucanase-activity, so that it can dissolve  $\beta$ -glucan derived from grain, e.g. barley. Accordingly, the alkaline cellulase of the present invention can be utilized for techniques of, for example, shortening time for filtering wheat sap in the process for producing beer and preventing a beer product from becoming turbid.

Obviously, numerous modifications and variations of the present invention are possible in light of the above

teachings. It is therefore to be understood that the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

5

The words "Avicel", "Bio-Gel", "Bacto", "Eiken", "Sigma", "BioRad", "Maxatase", "Savinase", "Esperase" used in the Specification are registered Trade or Service Marks.



**CLAIMS:**

1. An alkaline cellulase K-64 possessing the following physicochemical characteristics:

5

(A) Action

acts on cellulosic materials such as carboxymethyl cellulose (CMC), phosphoric acid swollen cellulose, filter paper, and Avicel dissolving them and producing reducing sugars also on

10

p-nitrophenyl- $\beta$ -D-glucopyranoside, and

p-nitrophenyl- $\beta$ -D-cellobioside producing p-nitrophenyl.

(B) Activity pH range and optimum pH

has an active pH range on CMC of 3-13.0

with an optimum pH range being 8.5-10.0 and even in pHs

15

ranging 7.0-12.0 has a relative activity of more than 50% of the activity of the optimum pH range,

(C) pH stability

is substantially stable when incubated at 30°C as described in the Specification at pHs rang-

20

ing 5-10.5 and even in pHs ranging 3-11 has an activity of more than 50% of the activity of the optimum pH range when CMC is a substrate,

(D) Optimum temperature

is capable of acting at wide temperatures



ranging 10-70°C with an optimum temperature being 50°C and even in temperatures ranging 35-60°C has more than 50% of the activity of the optimum temperature,

(E) Molecular weight

- 5 Has peaks of activities at substantially 180,000  $\pm$  10,000 and 80,000  $\pm$  2,000 (measured by means of the gel filtration method using Bio-Gel A 0.5m), when CMC is a substrate,

(F) Effect of metal ions

- 10 Is adversely affected by the addition at 1mM of ion, of  $\text{Hg}^{2+}$  ion and activated by the addition of  $\text{Co}^{2+}$  and  $\text{Mn}^{2+}$  ions, when CMC is a substrate,

(G) Effect of surfactants

- Surfactants such as linear alkylbenzene  
15 sulfonate, alkyl sulfate, polyoxyethylene alkyl sulfonate,  $\alpha$ -olefin sulfonate,  $\alpha$ -carboxymethyl alkylsulfonate, secondary alkyl sulfonate, dodecyl sulfonate, soap, and/or polyoxyethylene secondary alkylether have substantially no inhibitive effects on enzymatic  
20 activities when CMC is a substrate,

(H) Effect of proteinases

Proteinases have substantially no inhibitive effects when CMC is a substrate.



(I) Effect of chelating agents.

Exhibits no inhibitive effects on enzymatic activities by EDTA, EGTA, citric acid, sodium tripolyphosphate, zeolite, when CMC is a substrate.

5

2. An alkaline cellulase K-19 possessing the following physicochemical characteristics:

(A) Action

10

acts on cellulosic materials such as carboxymethyl cellulose (CMC), phosphoric acid or alkali swollen cellulose, filter paper, and Avicel dissolving them and producing reducing sugars also on p-nitrophenyl- $\beta$ -D-glucopyranoside, and

15

p-nitrophenyl- $\beta$ -D- cellobioside. producing p-nitrophenyl

(B) Activity pH range and optimum pH

has an active pH range on CMC of 4.5 - 12.5 with an optimum of 9 and even in pHs ranging 7.0 - 11.0 has a relative activity of more than 50% of the

20

activity of the optimum pH range,

(C) pH stability

is substantially stable when incubated at 30°C as described in the Specification at pHs ranging 4.5-11.00 and even in pHs ranging 4.0-12.0 has an activity



of more than 50% of the activity of the optimum pH range when CMC is a substrate,

(D) Optimum temperature

is capable of acting at wide temperatures ranging 10-70°C with an optimum temperature being 50°C and even in temperatures ranging 35-60°C has more than 50% of the activity of the optimum temperature,

(E) Molecular weight

Has peaks of activities at substantially 120,000 ± 10,000 and 34,000 ± 2,000 (measured by means of the gel filtration method using Bio-Gel A 0.5m), when CMC is a substrate,

(F) Effect of metal ions

Is adversely affected by the addition at 1mM of ion, of  $\text{Hg}^{2+}$  ion and  $\text{Pb}^{2+}$  ions and activated by the addition of  $\text{Co}^{2+}$  and  $\text{Mn}^{2+}$  ions, when CMC is a substrate,

(G) Effect of surfactants

Surfactants such as linear alkylbenzene sulfonate, alkyl sulfate, polyoxyethylene alkyl sulfonate,  $\alpha$ -olefin sulfonate,  $\alpha$ -carboxymethyl alkylsulfonate, secondary alkyl sulfonate, dodecyl sulfonate, soap, and/or polyoxyethylene secondary alkylether have substantially no inhibitive effects on enzymatic



activities when CMC is a substrate,

(H) Effect of proteinases

Proteinases have substantially no inhibitive effects when CMC is a substrate.

5

(I) Effect of chelating agents

Exhibits no inhibitive effects on enzymatic activities by EDTA, EGTA, citric acid, sodium tripolyphosphate, zeolite, when CMC is a substrate.

10

3. An alkaline cellulase K-520 possessing the following physicochemical characteristics:

(A) Action

acts on cellulosic materials such as carboxymethyl cellulose (CMC), phosphoric acid or alkali swollen cellulose, filter paper, and Avicel dissolving them and producing reducing sugars also on p-nitrophenyl- $\beta$ -D-glucopyranoside, and p-nitrophenyl- $\beta$ -D-cellobioside producing p-nitrophenyl.

20

(B) Activity pH range and optimum pH

has an active pH range on CMC of 3-13.0 with an optimum pH range being 8.5-9.5 and even in pHs ranging 7.0-11.0 has a relative activity of more than 50% of the activity of the optimum pH range,



(C) pH stability

is substantially stable when incubated at 30°C as described in the Specification at pHs ranging 6.0-11.0 and even in pHs ranging 5-11.5 has an activity of more than 50% of the activity of the optimum pH range when CMC is a substrate,

(D) Optimum temperature

is capable of acting at wide temperatures ranging 10-70°C with an optimum temperature being 50°C and even in temperatures ranging 35-60°C has more than 50% of the activity of the optimum temperature,

(E) Molecular weight

Has peaks of activities at substantially 170,000 ± 10,000 and 80,000 ± 2,000 (measured by means of the gel filtration method using Bio-Gel A 0.5m), when CMC is a substrate,

(F) Effect of metal ions

Is adversely affected by the addition at 1mM of ion, of  $\text{Hg}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Pb}^{2+}$  ions and activated by the addition of  $\text{Co}^{2+}$  and  $\text{Mn}^{2+}$  ions, when CMC is a substrate,

(G) Effect of surfactants

Surfactants such as linear alkylbenzene sulfonate, alkyl sulfate, polyoxyethylene alkyl



sulfonate,  $\alpha$ -olefin sulfonate,  $\alpha$ -carboxymethyl alkylsulfonate, secondary alkyl sulfonate, dodecyl sulfonate, soap, and/or polyoxyethylene secondary alkylether have substantially no inhibitive effects on enzymatic activities when CMC is a substrate,

(H) Effect of proteinases

Proteinases have substantially no inhibitive effects when CMC is a substrate.

(I) Effect of chelating agents

Exhibits no inhibitive effects on enzymatic activities by EDTA, EGTA, citric acid, sodium tripolyphosphate, zeolite, when CMC is a substrate.

4. An alkaline cellulase K-64 producing microorganism belonging to the genus *Bacillus* and growing in alkaline media.

5. An alkaline cellulase K-64 producing microorganism which is named as *Bacillus* sp. KSM-64 and deposited as FERM BP-2886 with Fermentation Research Institute, Agency of Industrial Science and Technology.

6. An alkaline cellulase K-19 producing microorganism belonging to the genus *Bacillus* and growing in alkaline media.



7. An alkaline cellulase K-19 producing microorganism which is named as *Bacillus* sp. KSM-19 and deposited as FERM BP-2885 with Fermentation Research Institute, Agency of Industrial Science and Technology.

8. An alkaline cellulase K-520 producing microorganism belonging to the genus *Bacillus* and growing in alkaline media.

9. An alkaline cellulase K-520 producing microorganism which is named as *Bacillus* sp. KSM-520 and deposited as FERM BP-2887 with Fermentation Research Institute, Agency of Industrial Science and Technology.

10. A process for producing the alkaline cellulase K-64 comprising: culturing an alkaline cellulase K-64 producing microorganism belonging to the genus *Bacillus* and growing in alkaline media; and collecting the alkaline cellulase K-64 from the cultured medium.

11. A process for producing alkaline the cellulase K-19 comprising: culturing an alkaline cellulase K-19 producing microorganism belonging to the genus *Bacillus* and growing in alkaline media; and collecting the  
5 alkaline cellulase K-19 from the cultured medium.

12. A process for producing the alkaline cellulase K-520 comprising: culturing an alkaline cellulase K-520 producing microorganism belonging to the genus  
10 *Bacillus* and growing in alkaline media; and collecting the alkaline cellulase K-520 from the cultured medium.



13. An alkaline cellulase as claimed in Claim 1 and  
substantially as hereinbefore described in the  
15 Specification.

14. A process as claimed in either Claim 10, Claim  
12 or Claim 13 for producing alkaline cellulase  
substantially as hereinbefore described in the  
20 Specification.

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