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[54] **METHOD OF PREPARING A PROTEOLYTIC**
ENZYME BY FERMENTATION
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[56] **References Cited**
UNITED STATES PATENTS
3,451,935 6/1969 Roald et al..... 252/135
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ABSTRACT: Certain strains of *Bacillus subtilis* are capable of growing in otherwise conventional media containing chelating agents such as sodium tripolyphosphate, sodium nitrilotriacetate, or sodium ethylenediaminetetraacetate and produce alkaline protease when cultured on ordinary culture media. The protease produced does not lose its effectiveness in hot detergent solutions also containing the aforementioned chelating agents.

METHOD OF PREPARING A PROTEOLYTIC ENZYME BY FERMENTATION

This invention relates to a method of preparing proteolytic enzymes by fermentation, and particularly to the production of proteolytic enzymes suitable for laundry use.

Laundry products containing alkaline proteases are finding rapidly increasing acceptance. It is known that proteolytic enzymes are produced by certain fungi and bacteria, but the enzymes obtained heretofore do not satisfactorily meet the requirements for use in laundry products. More specifically, they fail to maintain their protease activity under one or more of the following conditions normally encountered in laundering:

1. an alkaline medium, particularly at pH 9 to 11;
2. elevated temperature, typically 40° to 60° C.;
3. the presence of chelating agents.

It has now been found that proteolytic enzymes which meet the requirements of laundry service can be obtained in high yields from cultures of newly isolated strains of bacterium belonging to the species *Bacillus subtilis*. The several strains were identified as *Bacillus subtilis* by comparison of their characteristics with data in "Bergey's Manual of Determinative Bacteriology," 7th ed. The several strains differ slightly from each other in their resistance to chelating agents and in the protease activity of the enzymes produced, but are very similar in their taxonomical characteristics as evidenced by comparison of the following data on two strains, AJ-3205 and AJ-3208.

	AJ-3205	AJ-3208
Form of cells	Rods of 0.4-0.6x 1.5-3 microns	Rods of 0.6-0.8x 2-6 microns
Spore	0.6x1.0-1.5 microns ellipsoidal, central or paracentral	0.6-0.8x1.0-1.5 microns ellipsoidal, central or paracentral
Sporangia	Not definitely swollen	Not definitely swollen
Gram stain	Positive	Positive
Mobility	Motile	Motile
Agar colonies	Circular, flat, smooth, opalescent, glistening, butyrous, pale-yellowish-brown, medium unchanged	Circular, flat to raised, opaque, dull, butyrous, pale-brown, medium unchanged
Agar slant	Growth moderate, smooth, opalescent, glistening, pale-yellowish-brown	Growth moderate, rough, opaque, dull, spreading, pale-brown
Gelatin stab	Liquefaction	Liquefaction
NaCl broth	Good growth in 10% NaCl Growth in 12.5% NaCl No Growth in 15% NaCl	Good growth in 10% NaCl Growth in 12.5% NaCl No growth in 15% NaCl
Milk	Peptonized	Peptonized
B.C.P. milk	Alkaline	Alkaline
Hydrolysis of Starch	Positive	Positive
Hydrolysis of Casein	Positive	Positive
Utilization of citrate	Positive	Positive
Nitrate reduction	Positive	Positive
Gas from nitrate broth under anaerobic conditions	Not produced	Not produced
Acetylmethylcarbinol	Produced	Produced
Methyl red test	Negative	Negative
Lysine decarboxylase	Negative	Negative
Indol	Not produced	Not produced
Potato plug	Abundant growth	Abundant growth
H ₂ S	Produced	Produced
Nitrate representation	Negative	Negative
Urease	Negative	Negative
Acid but no gas under aerobic conditions from	L-arabinose, glucose, fructose, mannose, sucrose, maltose, trehalose, cellobiose, starch, glycerol, mannitol, arbutin, esculin, inulin	L-arabinose, glucose, fructose, mannose, sucrose, maltose, trehalose, cellobiose, starch, arbutin, esculin and salicin

No acid under aerobic conditions from	and salicin Xylose, rhamnose, galactose, sorbose, lactose, raffinose, melibiose, erythritol, adonitol, dulcitol, sorbitol, inositol and α -methyl-glucoside	cin- Xylose, rhamnose, galactose, sorbose, lactose, raffinose, melibiose, glycerol, erythritol, adenitol, dulcitol, mannitol, sorbitol, inulin and α -methylglucoside
Catalase	Positive	Positive
Temperature relation	Grows well between 25 and 50° C. The maximum temperature for growth is 57.5° C. On brain-heart infusion agar. No growth at 60° C. The minimum temperature is 20° C. No growth at 17° C.	Grows well between 25 and 50° C. The maximum temperature for growth is 57.5° C. on brain-heart infusion agar. No growth at 60° C. The minimum temperature is 20° C. No growth at 17° C.
Source	Soil	Soil

While other strains of *Bacillus subtilis* may or may not produce alkaline protease, strains of this invention, hereinafter identified as AJ-3205, AJ-3206, AJ-3207 and AJ-3208, which were isolated from soil samples, not only produce enzymes having protease activity, but are also capable of growing on media containing as much as 8 percent sodium tripolyphosphate (TPP) or 3 percent sodium nitrilotriacetate (NTA), whereas most known strains of *Bacillus subtilis* cannot grow on media containing 3 percent TPP or 1 percent NTA, and are similarly sensitive to 0.2 percent tetrasodium ethylenediaminetetraacetate (EDTA). Stains of *Bacillus subtilis* incapable of growth on media including such chelating agents include ATCC 6051, and IAM 1026, 1145, 1107, 1193, 1214, 1108, 1033 and 1076. The strains of the invention are selected among other strains of *Bacillus subtilis* by conventional screening methods based on their resistance to chelating agents and their ability of producing alkaline protease.

The difference in growth rate between the strains of the invention and others in the presence of chelating agents is illustrated by table 1 which lists the turbidity values of cultures as a measure of microbial growth 24, 48 and 72 hours after inoculation. *Bacillus subtilis* ATCC 6051, AJ-3208 and AJ-3205 were cultured under uniform conditions in media containing 0.5 g./dl. soluble starch, 1.0 g./dl. meat extract, 1.0 g./dl. polypeptone, 0.25 g./dl. NaCl, and the amounts of sodium tripolyphosphate listed in the table. Ten ml. batches of the several media were inoculated with the micro-organisms and cultured at 31.5° C. with shaking.

TABLE 1

	TPP, g./dl.	0	1	2	4	6	8	10
ATCC 6051	24 hours	0.72	0.52	0.13	0	0	0	0
	48 hours	0.70	0.66	0.45	0	0	0	0
	72 hours	0.55	0.63	0.46	0	0	0	0
AJ-3208	24 hours	0.46	0.52	0.49	0.23	0.04	0	0
	48 hours	0.80	0.82	0.82	0.73	0.33	0.14	0
	72 hours	0.82	0.87	0.86	0.86	0.61	0.53	0.03
AJ-3205	24 hours	0.60	0.60	0.55	0.33	0.10	0.08	0.06
	48 hours	0.50	0.51	0.66	0.71	0.57	0.28	0.26
	72 hours	0.38	0.38	0.78	0.83	0.60	0.56	0.28

Analogous differences in growth rate are found in the presence of NTA or EDTA.

The micro-organisms of the invention when grown on suitable nutrient media, not in themselves unusual, produce alkaline protease which is stable at pH 9 to 11 and at temperatures of 40° to 60° C., and is not deactivated by chelating agents. The nutrient media must include sources of assimilable

carbon and nitrogen and small amounts of inorganic salts. Suitable carbon sources are starch, soluble starch, dextrin and acid-treated starch, and are preferably present in the medium in amounts of 5 to 10 g./dl. Suitable nitrogen sources include soybean flake, soybean flour, soybean cake extract, milk casein, whey, polypetone, meat extract, peptides and amino acids.

The cultivation is preferably performed at pH 5.5 to 7.5 and between 25° and 37° C., preferably between 31° and 34° C. for 24 to 60 hours under aerobic conditions.

The protease formed may be recovered from the broth by conventional methods. The bacterial cells may first be removed by filtration or centrifuging, and the protease precipitated from the aqueous phase by salting out with ammonium sulfate or sodium sulfate, or by means of organic solvents miscible with water. The precipitate is most conveniently recovered by centrifuging, and is then dried.

As is shown in table 2, the enzyme powders so prepared from cultures of strains of the invention compare favorably in their temperature stability in the presence of chelating agents with a proteolytic enzyme commercially available under the name "Maxatase" (Koninklijke Nederlandsche Gist- en Spiritusfabriek N.V., Delft, Netherlands) and widely used in laundry detergents. The table lists residual protease activity in percent of the initial activity. In each test, enzyme powder of 60,000 protease units per gram was dissolved in 0.1 M H_3BO_3 -KCl- Na_2CO_3 buffer solution (pH 9.5) in a concentration of 0.06 percent. Solutions of each enzyme powder were further mixed with TPP in two concentrations, NTA, EDTA and the four solutions of each enzyme powder were kept under the following conditions:

Test A: 2% TPP, 60 minutes at 40° C.

Test B: 4% TPP, 30 minutes at 55° C.

Test C: 1% NTA, 60 minutes at 45° C.

Test D: 2% EDTA, 60 minutes at 45° C.

Protease activity was determined by Anson's method in which milk casein is hydrolyzed in the above buffer at 37° C. for 10 minutes, trichloroacetic acid soluble tyrosine is dyed with Folin's reagent, and the amount of released dye is determined. One unit is defined as the protease activity which releases 1 μ g. tyrosine per minute.

TABLE 2

Enzyme Source	Test A	Test B	Test C	Test D
AJ-3205	100	52	65	70
AJ-3206	80	46	58	63
AJ-3207	82	48	60	60
AJ-3208	80	44	60	65
Maxatase	75	38	42	54

Analogous results were obtained in a comparison test E of protease from AJ-3208 with Maxatase in which enzyme solutions in the buffer were additionally mixed with 2 percent of a commercial surfactant which is a linear alkylbenzenesulfonic acid and kept at 45° C. An additional test was performed with protease solutions in buffer further containing 4 percent of a commercial heavy laundry detergent and kept at 40° C. Residual protease was determined in each test after 20, 30, 40 and 60 minutes. The results are listed in table 3.

TABLE 3

After Minutes	Test E		Test F	
	AJ-3208	Maxatase	AJ-3208	Maxatase
20	90	87	100	75
30	84	82	100	65
40	80	76	95	60
60	70	70	92	48

The following examples further illustrate the invention.

EXAMPLE 1

A culture medium containing 1 g./dl. meat extract, 1 percent polypeptone, 0.5 g./dl. soybean flake extract and 5 g./dl. soluble starch was adjusted to pH 7.0, and 50 ml. batches of the medium were sterilized in 500 ml. shaking flasks at 120° C. for 20 minutes.

Each medium was inoculated with *Bacillus subtilis* AJ-3205 (NRRL B-3699) which had been cultured on a bouillon slant at 31° C. for 24 hours. The flask was kept at 31.5° C. for 48 hours with shaking. The broth thereafter contained 2650 units/ml. protease (about 5.3 g./l. protease).

950 ml. broth were collected from 20 flasks, centrifuged at 10,000 r.p.m. for 10 minutes to remove bacterial cells, and the 880 ml. of supernatant liquid were mixed with 340 g. solid ammonium sulfate. The mixture was stored in an icebox overnight, and the precipitate formed was recovered by centrifuging at 12,000 r.p.m. for 30 minutes and dried in a vacuum overnight with cooling. The crude enzyme powder so obtained weighed 15.2 g. and contained 125,000 units protease per gram (recovery yield 82 percent).

A product suitable for laundry use and containing 60,000 protease units per gram was prepared by uniformly mixing the enzyme powder with 16.5 g. anhydrous sodium sulfate.

EXAMPLE 2

Bacillus subtilis AJ-3206 was cultured on a medium containing, per deciliter, 1 g. soybean cake extract, 1.5 g. casein, 7 g. soluble starch, 0.05 g. KH_2PO_4 , 0.02 g. $MgSO_4 \cdot 7H_2O$, and 0.2 g. $CaCl_2$, at pH 7.0 as described in example 1. The cultured broth was found to contain 3960 protease units per milliliter (about 8 g./l. protease) after 48 hours.

Twenty-five g. crude enzyme powder containing 100,500 protease units per gram was recovered from 850 ml. of the cell-free broth.

EXAMPLE 3

When *Bacillus subtilis* AJ-3207 was cultured as described in example 1, 2920 units of protease were found in each milliliter of the broth after 48 hours, corresponding to about 5.8 g./l. protease.

EXAMPLE 4

Bacillus subtilis AJ-3208 (NRRL B-3700) was cultured for 48 hours as described in example 2, and protease was produced at a rate of 4050 units per milliliter (about 8.1 g./l.). When 900 ml. of the broth were worked as described in example 1, 29 g. of a crude enzyme powder containing 112,000 protease units per gram were recovered.

While the strains of *Bacillus subtilis* employed in examples 1 to 4 were isolated from soil samples by conventional screening techniques, suitable artificial mutants can be derived from strains of *Bacillus subtilis* which are themselves incapable of producing alkaline protease resistant to chelating agents. Diethyl sulfate, nitrosoguanidine, or nitrogen mustard are suitable chemical mutation inducing agents, and X-ray irradiation is similarly effective. The mutants produced are screened according to their ability of growing on a culture medium containing 4 g./dl. sodium tripolyphosphate or corresponding amounts of the other chelating agents mentioned above, and of producing extracellular alkaline protease when grown on culture media which are conventional in themselves.

A parent strain of *Bacillus subtilis*, AJ-3168, (on deposit with the Fermentation Research Institute, the Agency of Industrial Science and Technology, Ministry of Trade and Industry, Japan, under accession number FERM p-242) which produces alkaline protease not resistant to chelating agents and is incapable of growing on media containing 3 percent TPP, 0.2 percent NTA or 0.2 percent EDTA, was irradiated with X-rays, and a radiation-induced mutant strain, *Bacillus*

D-4628, was found capable of growing on a culture medium containing 5 percent TPP. The following example illustrates the method of obtaining alkaline protease from the mutant strain, and the properties of the protease.

EXAMPLE 5

An aqueous culture medium containing, per deciliter, 1 g. deoiled soybean extract, 1.5 g. casein, 0.5 g. amino acid mixture, 8 g. soluble starch, 0.1 g. KH_2PO_4 , and 0.02 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was adjusted to pH 7.0, and 50 ml. batches of the medium were sterilized in 500 ml. shaking flasks. Each batch was then inoculated with *Bacillus subtilis* D-4628 (NRRL B-3751), and the cultures were kept at 31.5° C. for 48 hours with shaking. The culture broth so obtained was found to contain 4,050 protease units per ml. (about 8.2 g./l. enzyme protein).

900 ml. of the culture broths were centrifuged to remove the bacterial cells, and 342 g. ammonium sulfate were added to the 890 ml. of separated supernatant liquid. The solution so obtained was kept overnight in an icebox, and the precipitate formed was recovered by centrifuging. It was dried in a vacuum and then consisted of 22.2 g. of an enzyme powder having 135,000 protease units per gram (recovery yield 85 percent).

The enzyme powder was subjected to comparison tests with the alkaline protease produced by the parent strain AJ-3168 and with Maxatase as described with reference to table 2. The results are listed below in table 4 which also indicates the optimum pH for each of the enzymes and its stable range as determined in the absence of chelating agents.

TABLE 4

Enzyme Source	Optimum pH	Stable pH	Test G	Test H
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5

10

15

20

25

30

35

40

45

50

55

60

65

70

75

AJ-3168	8.5-11	6.5-11	5.1	
D-4628	9.5-11	6-11	85.3	57.3
Maxatase	9.5-10.7	7-11	63.2	38.1

In Test G, the remaining protease activity was determined in percent of initial activity after 20 minutes in the presence of 2 percent TPP at 50° C. In Test H, residual protease activity was measured after 40 minutes in 2 percent TPP at 50° C.

Specimen cultures of all strains of *Bacillus subtilis* referred to hereinabove by NRRL numbers are available without our permission from the ARS Culture Collection, Northern Utilization Research and Development Division of the United States Department of Agriculture in Peoria, Illinois.

What is claimed is:

1. A method of producing alkaline protease which comprises:

a. selecting a micro-organism of the species *Bacillus subtilis* capable of growing on a culture medium containing 4 g./dl. sodium tripolyphosphate and of producing alkaline protease;

b. culturing said micro-organism on a nutrient medium containing sources of assimilable carbon and assimilable nitrogen and inorganic salts under aerobic conditions until protease is produced in said nutrient medium; and
c. recovering said protease from said nutrient medium.

2. A method as set forth in claim 1, wherein said nutrient medium is at a pH value of 5.5 to 7.5.

3. A method as set forth in claim 1, wherein said micro-organism is of one of the strains AJ-3205 (NRRL B-3699), AJ-3208 (NRRL B-3700), and D-4628 (NRRL B-3751).

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