PATENT SPECIFICATION (11) 1 560 448

1 560 448

(21) Application No. 35314/77 (22) Filed 23 Aug. 1977
(31) Convention Application No. 7 625 631
(32) Filed 24 Aug. 1976 in
(33) France (FR)
(44) Complete Specification published 6 Feb. 1980
(51) INT CL³ C12N 9/50; C14C 1/06
(52) Index at acceptance
C3H C1
C6C 2J3 2J4 2J6

(72) Inventors ANDRE BELLOC, JEAN FLORENT, JEAN LUNEL, JEAN-CLAUDE PALLA and DENISE MANCY

(54) NEW PROTEOLYTIC ENZYME, ITS PREPARATION AND ITS USE

(71) We, RHONE-POULENC INDUSTRIES, a French Body Corporate of 22, Avenue Montaigne, 75 Paris 8eme, France, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention relates to a new microorganism, and to a new proteolytic enzyme, its preparation and use, particularly in tanning, for the depilation of skins.

The present invention provides a culture of the microorganism *Streptomyces caligosus* DS 14,486, having the NRRL reference No. 8,195, in a synthetic liquid culture medium containing a source of assimilable carbon, a source of assimilable nitrogen and mineral elements, the culture being substantially free from other microorganisms, and the microorganism NRRL 8195 having the following characteristics:

(a) forms cylindrical spores measuring 0.8 to 1.0 μ/0.6 to 0.8 μ,
(b) exhibits sporophores in clusters;
(c) the chains of spores are generally rather long and can amount to up to several tens of spores, and coil up in tight more or less elongated spirals, in general forming 2 to 3 turns but rather frequently up to six or eight, or even ten turns,
(d) by its mode of sporulation, falls in the Spira section of the Pridham classification,
(e) develops well as 26°C, poorly at 37°C and not at all at 50°C,
(f) has a sporulated aerial mycelium of grey colour,
(g) the coloration of its vegetative mycelium ranges from more or less deep brown to black, depending on the culture media,
(h) on organic media, and in particular on Waksman special tyrosine—yeast extract agar ("melanin formation medium"), gives, in general, a rather copious production of a melanin pigment which imparts a dark tint to the medium;
(i) on numerous synthetic media it is prone to produce a blackish soluble pigment in greater or lesser amounts.

In an alternative embodiment, the present invention provides *Streptomyces caligosus* DS 14486 (NRRL 8195) in the form of a freeze-dried micro-organism.

The present invention also provides the proteolytic enzyme, designated 24,199 RP, having the following characteristics:

(a) is a protein substance,
(b) is a powder of brown to black colour,
(c) has a weight average molecular weight in the region of 27,000,
(d) has an isoelectric point of 3.7,
(e) is soluble in water, sparingly soluble in concentrated aqueous solutions of neutral salts and in water-alcohol or water-acetone mixtures and virtually insoluble in anhydrous alcohols and ketones,

(f) has a maximum activity on casein at a pH of 7.5 and at a temperature of 45°C,
(g) has a coagulant activity on milk,
(h) has a fibrinolytic activity on a fibrin clot,
(i) exerts activity on the glycoproteins of hair roots and skins.

The new enzyme can be obtained, in accordance with the invention, by aerobic culture of *Streptomyces caligosus* DS 14,486 (NRRL 8,195) in a medium containing assimilable carbon and nitrogen and mineral elements.

24,199 RP is a protein and is a powder of brown to black colour, which is soluble in water, sparingly soluble in concentrated aqueous solutions of neutral salts (for example ammonium sulphate) and in aqueous-alcoholic or aqueous-acetone mixtures and virtually insoluble in anhydrous alcohols and ketones; its weight average molecular weight is in the region of 27,000 and its isoelectric point is 3.7.

The proteolytic activity of enzyme 24,199 RP is exerted on a large number of proteinaceous substrates such as casein, haemoglobin, fibrin (lysis of clots) or milk (coagulation).

The activity on casein can be determined in accordance with a technique similar to that of M. KUNITZ, J. Gen. Physiol., *30,* 291 (1947). The peptides soluble in trichloroacetic acid which are liberated during the hydrolysis are determined either by spectrophotometry at 280 nm, the activity being expressed in Kunitz units (K.U.) or by colorimetry in accordance with the method of O. H. LOWRY *et al.*, J. Biol. Chem. *193,* 265 (1961), the activity being expressed in mg. of tyrosine formed per minute under the conditions of the determination.

The coagulant activity on milk can be determined in accordance with a technique based on that of N. J. BERRIDGE, Biochem. J., *39,* 179 (1945) and can be expressed in rennet units (R.U.), 1 rennet unit being the amount of enzyme which coagulates 10 cm^3 of reconstituted milk in 100 seconds.

The fibrinolytic acitivity can be determined on a standard fibrin clot produced by the action of thrombin on fibrinogen and can be expressed in lysis units, a solution containing 100 lysis units per cm^3 (100 LU/cm^3) if it causes lysis of a standard fibrin clot in 30 minutes.

Table I summarises the results obtained on different substrates with purified enzyme 24,199 RP.

TABLE I

SUBSTRATE	REACTION	ACTIVITY
casein	proteolysis (a)	1,470 KU/g
milk	coagulation (b)	14,500 RU/g
fibrinogen	lysis of a fibrin clot (c)	4,920 LU/mg

(a) reaction at pH 7.5 and at 37°C – concentration in the substrate 5 g/l.

(b) reaction at pH 6.35 and at 32°C – on reconstituted milk

(c) reaction at pH 7.5 and at 37°C

TABLE II summarises the results obtained during hydrolysis of casein, as a function of the pH.

TABLE II

pH of the reaction medium	Proteolytic activity on casein at 37 °C mg of tyrosine formed per g of enzyme
3.5	220
4.0	410
4.5	900
5.0	1,370
5.5	1,540
6.0	1,510
6.5	1,930
7.0	2,140
7.5	2,270
8.0	2,180
8.5	1,990
9.0	660

The specificity of the enzyme towards the glycoproteins of hair roots and animal skins cannot be deduced from the activities observed on the conventional substrates, shown, for example, below, but can only be detected by actual depilation experiments such as are described in Examples 4 and 5 below.

The activity of enzyme 24,199 RP manifests itself over a wide range of pH; the optimum value of the pH for hydrolysis of the casein is about 7.5 and the enzyme retains at least 60% of its maximum activity with the pH range of between 5 and 8.5.

Table III summarises the results obtained on hydrolysis of casein, as a function of the temperature.

TABLE III

Temperature of the reaction medium in °C	Proteolytic activity on casein at pH 7.5 mg of tyrosine formed per g of enzyme
25	1,360
30	1,740
40	2,230
45	2,250
50	1,770
55	680
60	200

The optimum activity of enzyme 24,199 RP manifests itself at a temperature of about 45°C and the activity decreases very rapidly when the temperature is above 50°C.

Table IV summarises the results relating to the kinetics of the hydrolysis of casein by enzyme 24,199 RP at a concentration of 10 $\mu g/cm^3$. The activity is expressed in mg of tyrosine formed.

TABLE IV

Hydrolysis time in minutes	mg of tyrosine in the reaction medium
5	0.100
15	0.300
20	0.370
30	0.380
60	0.850
90	1.130

The organism which produces the proteolytic enzyme 24,199 RP is a strain of *Streptomyces* given the number DS 14,486.

A sample of this strain has been deposited with the Northern National Research Laboratory of the United States Department of Agriculture at Peoria, Illinois, United States of America where it has been registered under number NRRL 8,195. A sample of this strain is freely available to anyone on demand.

This organism, which exhibits characteristics which have not allowed it to be identified with a species previously described, must be considered as a new species and has been designated *Streptomyces caligosus,* DS 14,486.

Streptomyces caligosus DS 14,486 forms cylindrical spores measuring 0.8 to 1.0 μ/0.6 to 0.8 μ. It exhibits sporophores in clusters; the chains of spores are generally rather long, may number up to several tens of spores, and coil up in tight more or less elongated spirals, in general forming 2 to 3 turns but quite frequently up to six or eight or even ten turns. On the basis of its mode of sporulation, *Streptomyces caligosus* DS 14,486 falls within the Spira section of the Pridham classification.

Streptomyces caligosus DS 14,486 develops well at 26°C, poorly at 37°C and not at all at 50°C. It exhibits a sporulated aerial mycelium of grey colour. The coloration of its vegetative mycelium ranges, depending on the culture media, from more or less deep brown to black-brown. On organic media and in particular on the Waksman special tyrosine-yeast extract agar ("melanin formation medium") it gives, in general, a rather copious production of melanin pigment which imparts a dark tint to the medium; on numerous synthetic media it is prone to produce a blackish soluble pigment in greater or lesser amounts.

In its culture, carried out at 26°C, it exhibits the following biochemical characteristics:

production of melanin	:	positive
production of H_2S	:	positive
tyrosinase	:	positive
liquefaction of gelatine	:	positive
utilisation of cellulose	:	positive
production of nitrites from nitrates	:	zero on nitrite-containing nutrient broth, positive on synthetic media

hydrolysis of starch	: positive
culture on skimmed milk	: peptonisation without coagulation

The cultural characteristics of *Streptomyces caligosus* DS 14,486 are summarised in the Table below. They are the characteristics of cultures which have reached a good stage of development, that is to say, after about 3 weeks at 26°C unless indicated otherwise. These characteristics were observed on nutrient agars and broths usually employed for determining the morphological characteristics of the *Streptomyces* strains, the cultures on agar media being carried out on slant agars. A certain number of the culture media employed were prepared in accordance with the formulations indicated in "The Actinomyceter", S. A. WAKSMAN, p.193—197, Chronica Botanica Company, Waltham, Mass., U.S.A., 1950; in these cases they are indicated by the letter W followed by the number which has been given to them in "The Actinomycetes". The references or compositions of the other culture media are as follows:

Ref. A: "Hickey and Tresner's Agar"—T. G. PRIDHAM *et al.*—Antibiotics Annual, 1956—1957, p.950.

Ref. B: "Bennett's Agar"—S. A. WAKSMAN—The Actinomycetes vol. 2, p.331, No. 30; The William and Wilkins Company, Baltimore 1961.

Ref. C: Formula W—23, with the addition of 2% of agar.

Ref. D: "Yeast Extract Agar"—T. G. PRIDHAM et al.—Antibiotics Annual, 1956—1957, p.950.

Ref. E: "Tomato Paste Oatmeal Agar"—T. G. PRIDHAM et al.—Antibiotics Annual, 1956—1957, p.950.

Ref. F: "Melanin formation medium"—The Actinomycetes, vol. 2, p.333, No. 42—S. A. WAKSMAN—The Williams and Wilkins Company, Baltimore, 1961.

Ref. G: W. E. GRUNDY et al.—Antibiotics and Chem. *2*, 401, 1952.

Ref. H: "Inorganic Salts—Starch Agar"—T. G. PRIDHAM et al.—Antibiotics Annual, 1956—1957, p.951.

Ref. I: corresponds to formula W—1, with 3% of sucrose replaced by 1.5% of glucose.

Ref. J: corresponds to formula W—1, with 3% of sucrose replaced by 1.5% of glycerol.

Ref. K: corresponds to formula W—18, with 3% of sucrose replaced by 1.5% of glucose.

Ref. L: corresponds to formula W—18, with the sucrose omitted and replaced by small strips of filter paper partially immersed in the liquid.

Ref. M: "Manual of Methods for Pure Culture Study of Bacteria" of the Society of American Bacteriologists, Geneva, N.Y., II 50—18.

Ref. N: "Plain Gelatin"—prepared in accordance with the instructions in the "Manual of Methods for Pure Culture Study of Bacteria" of the Society of American Bacteriologists, Geneva, N.Y., II 50—18.

Ref. P: skimmed milk in the form of a commercially available powder, reconstituted in accordance with the manufacturer's instructions.

Ref. Q: medium given for investigation of the production of H_2S, by: H. D. TRESNER and F. DANGA—Journal of Bacteriology, *76*, 239—244, 1958.

Culture medium	Degree of development	Vegetative mycelium or underside of the culture	Aerial apparatus (comprising the combination of the aerial mycelium and of the sporulation)	Soluble pigment	Observations and biochemical properties
Hickey and Tresner agar (Ref. A)	Good	Underside black brown	Greyish white to grey, well developed	Black brown	Cylindrical spores measuring 0.8 to 1.04 μ/0.6 to 0.8μ sporophores in clusters, chains of long spores coiling up in tight more or less elongated spirals.
Bennett agar (Ref. B)	Good	Underside deep yellow brown	Grey, rather well developed	Very deep brown grey	
Emerson agar (Ref. C)	Rather good	Vegetative mycelium deep yellow brown	Whitish, in the form of traces	Blackish brown	
Pridham yeast extract agar (Ref. D)	Very good	Underside black brown	Deep grey, very well developed	Black	
Pridham oatmeal and tomato extract agar (Ref. E)	Very good	Underside black	Deep grey, very well developed	Black	
Peptone glucose agar (W—7)	Good	Vegative mycelium deep greyish brown	Greyish white, very moderately developed	Black brown	
Nutrient agar (W—5)	Moderate	Vegetative mycelium blackish	Greyish, very poorly developed	Black	Production of melanin: positive (readings carried out in accordance with the recommendations of the author)

Culture medium	Degree of development	Vegetative mycelium or underside of the culture	Aerial apparatus (comprising the combination of the aerial mycelium and of the sporulation)	Soluble pigment	Observations and biochemical properties
Krainsky calcium malate agar (Ref. G)	Very moderate	Vegetative mycelium light brownish grey	None	Weak brownish grey	Solubilisation of malate: positive
Ovalbumin agar (W–12)	Very moderate	Underside brownish grey	Grey, very poorly developed	Blackish grey	
Glucose-asparagin agar (W–2)	Good	Underside black	Greyish white to grey, well developed	Blackish grey	
Glycerol-asparagin agar (W–3)	Good	Underside black	Greyish black to grey, rather well developed	Blackish grey	
Pridham starch-mineral salts agar (Ref. H)	Rather good	Underside deep brown to blackish	Whitish to grey, average development	Deep brown grey	Cylindrical spores measuring 0.8 to 1.0μ, 0.6 to 0.8μ. Sporophores in clusters, long chains of spores coiling up in tight more or less elongated spirals. Hydrolysis of starch: positive.
Starch-nitrate agar (W–10)	Average	Underside light yellow brown	Whitish to grey, moderately developed	Weak brownish	Hydrolysis of starch: positive
Czapek synthetic agar with sucrose (W–1)	Good	Vegetative mycelium greyish to brownish, underside yellow brown	Whitish, in the form of traces	Weak greyish brown	

Culture medium	Degree of development	Vegetative mycelium or underside of the culture	Aerial apparatus (comprising the combination of the aerial mycelium and of the sporulation)	Soluble pigment	Observations and biochemical properties
Czapek synthetic agar with glucose (Ref. I)	Rather good	Vegetative mycelium yellow brown, underside yellow brown	Whitish, in the form of traces	Light brownish	
Czapek synthetic agar with glycerol (Ref. J)	Rather good	Vegetative mycelium yellow brown to blackish grey brown, underside yellow brown to blackish brown	Greyish white in the form of traces	Greyish brown	
Starch-nitrate broth (W–19)	Rather good	Thick velum, underside light brownish	Greyish white, moderately developed	None	Production of nitrites : positive
Czapek glucose broth (Ref. K)	Moderate	Flocculent culture and greyish white velum	None	None	Production of nitrites : positive
Czapek cellulose broth (Ref. L)	Moderate	Flocculent culture, greyish white	Greyish, moderately developed on the paper protruding from the broth	None	Utilisation of cellulose: positive. Production of nitrites: positive
Nitrate nutrient broth (Ref. M)	Moderate	Brownish ring	Whitish, in the form of traces	Very deep brown	Production of nitrites: negative
Culture on potato (W–27)	Good	Vegetative mycelium thick and wrinkled, very deep brown	Greyish white to light grey. Rather well developed	Black	Black soluble pigment beginning to diffuse into the potato after 24 hours' incubation

Culture medium	Degree of development	Vegetative mycelium or underside of the culture	Aerial apparatus (comprising the combination of the aerial mycelium and of the sporulation)	Soluble pigment	Observations and biochemical properties
12% strength pure gelatine (Ref. N)	Good	Culture well developed at the surface vegetative mycelium deep brown	None	Deep brown	Liquefaction of gelatine: positive
Skimmed milk (Ref. P)	Moderate	Brownish grey ring	None	Very deep brown	Peptonisation without coagulation – little variation in the pH, which changes from 6.3 to 6.5 in one month
Tresner and Danga agar (Ref. Q)	Moderate	Vegetative mycelium black brown	None	Black	Production of H_2S: positive (readings taken in accordance with the recommendations of the authors)

Streptomyces caligosus DS 14,486 exhibits a combination of characteristics which does not coincide exactly with any of those of the strains already described, and for this reason, it must be considered as a new species.

Among the species described in Bergey's Manual of Determinative Bacteriology (7th edition, The Williams and Wilkins Company, Baltimore, 1957), as well as in "The Actinomycetes" (vol. 2, S. A. WAKSMAN, The Williams and Wilkins Company, Baltimore, 1961), the species which *Streptomyces caligosus* DS 14,486 appears to approach most is *Streptomyces noboritoensis*; like the latter, in fact, it produces melanin pigments on organic media, develops a more or less deep brown to black vegetative mycelium on the majority of its culture media, very particularly on potato, on which it forms a very deep brown vegetative mycelium, and exhibits a sporulated aerial mycelium of grey colour. It must however be differentiated from *Streptomyces noboritoensis* because the latter does not form regularly coiled chains of spores, does not liquefy gelatine or only does so slightly, does not give a soluble pigment or only gives a weak brownish soluble pigment on asparagine glucose agar and produces a deep red brown soluble pigment on

nutrient agar, whilst *Streptomyces caligosus* DS 14,486 forms chains of spores which coil up in a regular manner to form tight spirals, liquefies gelatine, gives a black soluble pigment on asparagine glucose agar and gives a greyish brown soluble pigment on nutrient agar; furthermore, *Streptomyces noboritoensis* forms a colourless vegetative mycelium on synthetic agar containing nitrate and sucrose whilst on this medium *Streptomyces caligosus* DS 14,486 forms a thick, greyish to brownish vegetative mycelium; furthermore, *Streptomyces noboritoensis* does not utilise rhamnose or sucrose and utilises arabinose, inositol and xylose only in a very limited manner, whilst *Streptomyces caligosus* DS 14,486 uses all these sources of carbon very well.

The ability of *Streptomyces caligosus* DS 14,486 to utilise various sources of carbon or nitrogen to ensure its development was determined in accordance with the principle of the method of Pridham and Gottlieb (J. of Bact. *56*, 107—114, 1948); the degree of development was observed on the base indicated by the authors, replacing either the glucose by the various sources of carbon respectively tested, or $(NH_4)_2SO_4$ by the various sources of nitrogen respectively tested.

The results are indicated in the Table which follows:

TABLE

Sources of carbon tested	Utilisation	Sources of nitrogen tested	Utilisation
D - Ribose	positive	$NaNO_3$	positive
D - Xylose	positive	$NaNO_2$	positive
L - Arabinose	positive	$(NH_4)_2SO_4$	positive
L - Rhamnose	positive	$(NH_4)_2HPO_4$	positive
D - Glucose	positive	Adenine	positive
D - Galactose	positive	Adenosine	positive
D - Fructose	positive	Uracil	positive but slow
D - Mannose	positive	Urea	positive
L - Sorbose	negative	L - Asparagine	positive
Lactose	positive	Glucosamine	positive
Maltose	positive	Glycine	positive
Sucrose	positive	Sarcosine	positive
Trehalose	positive	DL - Alanine	positive
Cellobiose	positive	DL - Valine	positive
Raffinose	positive	DL - Aspartic acid	positive
Dextrin	positive	L - Glutamic acid	positive
Inulin	positive	L - Arginine	positive
Starch	positive	L - Lysine	positive
Glycogen	positive	DL - Serine	positive
Glycerol	positive	DL - Threonine	positive
Erythritol	negative	DL - Methionine	positive
Adonitol	negative	Taurine	negative
Dulcitol	negative	DL - Phenyl-alanine	positive
D - Mannitol	positive	L - Tyrosine	positive
D - Sorbitol	positive	DL - Proline	positive
Inositol	positive	L - Histidine	positive
Salicin	limited	L - Tryptophane Betain	positive

The culture of *Streptomyces caligosus* DS 14,486 can be carried out by any aerobic culture method, such as a surface culture or submersed culture, but the latter is to be preferred for reasons of convenience. For this purpose, the techniques of inoculation and of fermentation, and the various types of apparatus, which are usually employed in the fermentations industry are utilised.

The fermentation medium must essentially contain assimilable sources of carbon and of nitrogen, mineral elements and, if appropriate, growth factors, and all these constituents can be introduced in the form of well-defined products or in the form of complex mixtures such as are encountered in biological products of various origins.

As sources of assimilable carbon, it is possible to use carbohydrates such as glucose, sucrose, maltose, dextrins, starch or other carbon-containing substances such as sugar alcohols (glycerol) or such as certain organic acids (lactic acid or citric acid). Certain animal or vegetable oils such as lard oil or soya oil can advantageously replace these various carbon sources or be added thereto.

Suitable sources of assimilable nitrogen are extremely varied. They can be very simple chemical substances such as inorganic or organic ammonium salts, urea and certain amino-acids. They can also be introduced through complex substances which principally contain nitrogen in a protein form, namely casein, lactalbumin, gluten, and their hydrolysis products, soya flour, groundnut meal, fishmeal, meat extract, yeast extract, distiller's solubles and corn-steep.

Amongst the inorganic constituents (i.e. mineral elements) added, some can have a buffering or neutralising effect, such as the alkali metal phosphates or alkaline earth metal phosphates or calcium carbonate or magnesium carbonate. Others provide the ionic equilibrium necessary for the development of *Streptomyces caligosus* DS 14,486 and for the elaboration of the enzyme 24,199 RP, such as the chlorides and sulphates of alkali metals and alkaline earth metals. Finally, some act more especially as activators of the metabolic reactions of *Streptomyces caligosus* DS 14,486; these are the salts of zinc, cobalt, iron, copper and manganese.

The growth factors which can be used are products of a vitamin nature such as riboflavin, folic acid and pantothenic acid.

The pH of the fermentation medium at the start of the culture should suitably be 5.8 to 7.8 and preferably 6.2 to 7.9. The optimum temperature for the fermentation is from 25 to 30°C but satisfactory production is achieved at temperatures of from 23 to 33°C. The aeration of the fermentation can vary between rather wide values. However, it has been found that aerations of 0.3 to 3 litres of air per litre of broth and per minute are particularly suitable. The maximum yield of enzyme 24,199 RP is obtained after 2 to 8 days' culture, with this time essentially depending on the medium used.

Enzyme 24,199 RP can be isolated from the fermentation musts in the following manner:

the fermentation must can be filtered, if appropriate in the presence of a filtration agent, at a pH which is generally that of the medium at the end of the production phase. The filtrate obtained is concentrated to a volume of about 1/5 of the initial volume and the enzyme 24,199 RP is then precipitated by adding a poor solvent such as acetone.

The crude product can be purified by fractional precipitation by means of inorganic salts, such as ammonium sulphate, in the form of solids or of concentrated aqueous solutions, and/or by means of poor solvents for enzyme 24,199 RP, such as acetone. The product can also be purified by dialysis across a membrane, preferably a regenerated cellulose membrane.

Enzyme 24,199 RP is particularly useful in the tanning industry where it can be employed for the depilation of animal skins for the purpose of preparing leathers.

In this particular application, enzyme 24,199 RP can be used in the pure state; it is to be noted that equivalent results are obtained with the enzyme in a semi-purified form.

Depilation has hitherto been carried out in accordance with well-known methods, of which the most commonly used is treating the skins with alkaline reducing baths or pastes, consisting in particular of lime and sodium sulphide. This process of depilation does not cause the removal of the hair by destroying its bonds to the skin, but instead causes a dissolution of the hair in the treatment bath, which must be renewed after each operation. It is therefore necessary to dispose of water which is very heavily charged with sulphide and organic products, of which the chemical oxygen demand and biological oxygen demand (COD and BOD) are

therefore considerable. Hitherto this was discharged into the conventional sewer networks, thus causing very great pollution of streams, rivers and the like. It is true that powerful means of purification did make it possible to reduce the BOD and COD but their installation and operating costs are very high and make the procedure expensive. One of the essential advantages of the present invention is that it reduces by about half the pollution resulting from conventional depilation. In fact, the depilation of skins by means of enzymes which attack the part of the epidermis which bonds the hair to the skin makes it possible to recover the said hair by simple filtration of the depilation bath, without causing pollution.

Another advantage of the process according to the invention is that, compared to the conventional processes of enzymatic depilation, it makes it possible to recover the skin intact, without deterioration of the leather. In fact, enzymatic depilation, as practised currently, causes substantial degradation of the epidermis and even of the dermis of the treated skins, which then give leathers of poor quality. This is essentially due to the fact that hitherto, there was not available an enzyme of which the action is specifically on the hair-skin bond. Under these conditions, the enzyme reacts equally with the proteins of the deep layers of the skin so that the leather obtained by subsequent treatment exhibits a damaged surface, veining and sponginess.

This advantage is particularly valuable in the case of the depilation of sheepskins because, compared to the conventional process of depilation by heating sheepskins, depilation with the enzyme of the invention makes it possible to obtain intact wool and an intact skin.

According to a general method of carrying out the process, when treating cattle skins, the enzymatic depilation of the invention involves bringing the skin to be treated into contact with the enzyme in a depilation vessel in which the percentage of water relative to the skin is between 10 and 45% by volume and preferably between 20 and 25%. The depilation is carried out, for optimum results, over a period of 3 to 24 hours at a temperature of 24 to 30°C, the pH of the bath corresponding to the maximum proteolytic activity of the enzyme, that is to say it must be 7 to 8.5; the pH should be controlled in this range during the depilation by using a buffer solution which usually is made up by adding trisodium phosphate or borax to the depilation bath. Furthermore, from 0.3 to 1 part by weight of enzyme according to the invention per 100 parts by weight of skins will normally be used for optimum efficiency and cost.

In practice, the treatment of the skins is generally carried out either by immersion in a solution containing 10 to 30 g/l of enzyme 24,199 RP, the strength of the enzyme being about 290 KU/g,or by spraying, e.g. with a spray gun, a solution containing 1 to 10 g/litre of enzyme 24,199 RP, the strength of which is about 290 KU/g, in the case of sheepskins.

In the case of cattle skins and sheepskins, the activity of the enzyme employed is an optimum when its concentration and its strength are of the order indicated above.

When treating sheepskins, the enzymatic depilation may be carried out by contacting the skin to be treated and the enzyme with one another by spraying onto the flesh side of the skin an aqueous solution of the enzyme so that the percentage of water relative to the skin is 10 to 45% by volume, the pH of the solution being 7 to 8.5 and the solution furthermore being of such concentration and strength that a concentration of 5 to 6 g/l of enzyme corresponds to a strength of 290 KU/g; the skins are then stacked flesh side against flesh side in a chamber in which the relative humidity does not exceed 85% and the temperature is 24 to 26°C for a period of 16 to 20 hours.

The depilation vessel can be any trough or drum such as those used in the techniques known hitherto. It can advantageously be fitted with an intermittent agitation device which will improve the distribution of the reagents over the skin. Speeds of agitation of the order of 3/4 revolution per minute have given particularly satisfactory results.

In order that the removal of the hair from the skin shall be complete, the action of the enzyme must generally be accompanied or followed by mechanical stripping of the skin, carried out by any suitable means. This can be, quite simply, the friction of the skins against one another during agitation in the course of the process, optionally completed by unhairing achieved in the course of a brief period of more intense agitation. It can also consist of passing the skins treated by the process according to the invention through an unhairing machine which allows intact and non-felted skins to be recovered.

The process according to the invention can be preceded and/or followed by a large number of associated operations. Cleaning of the skins before depilation is desirable. It can consist of washing with water in a drum or on a paddle, essentially intended to free the skin from the salt with which it is impregnated in order to preserve it. The washing can be followed by degreasing with the aid of conventional aromatic or chlorinated solvents or of detergents in aqueous solution. This operation makes it possible to remove a large number of organic impurities, such as greases, and minerals which soil the skin and obstruct the hair follicles. The degreasing will advantageously be followed by a fresh wash with water so as to obtain a skin which is as clean as possible and is free from impurities and from cleaning products.

After depilation of the skins, they are washed and drained. The wash waters contain the intact skins and the enzyme, and these can be separated by any known means such as decanting or filtration.

The skins are then generally introduced into a pit of pure lime which hydrolyses them. This pit can be re-used several times, because the skins which are placed therein are virtually clean and only soil the pit slightly.

After treatment in the pit, it is found that the hair side of the skins has not been damaged and adheres perfectly to the dermis, whilst a skin depilated by means of prior art enzymes of the alkaline type exhibits a deteriorated hair side, which is gelatinous to the touch and is in places detached from the dermis which is itself attacked.

In the course of the actual depilation operation, the discharge of polluting products has been considerably reduced. This is particularly noticeable in the case of sulphides and dissolved skins, of which no trace is to be found in the wash waters obtained in the present invention whilst they are copious in the residues resulting from depilation by dissolving the skin.

After the treatment of the skins according to the process of the invention, all the conventional tanning operations such as the process of bating, pickling, chrome tanning, shaving and skiving, retanning, nourishing, drying, staking and finishing, can be carried out.

The resulting leathers exhibit a very fine grain, of a quality superior to that produced by the lime-sulphide method, an absence of veins and very good adhesion of the hair side, even on pieces taken from animal sides.

A comparative Table of the results obtained for the depilation of cattle skins in accordance with the following three processes of depilation will be given below:

A) Process according to the invention: use of enzyme 24,199 RP according to Example 4 given later.

B) Process using prior art enzymes of the alkalase type.

C) Process involving dissolving the skin in a bath containing lime and sodium sulphide.

	C	A	B
	Depilation by lime-sulphide	Process according to the invention Enzyme 24,199 RP	Depilation by prior art enzyme of the alkalase type
Appearance of the hair side:			
hair follicles	marked	very marked	only slightly marked
surface	flat	full	very flat
Touch	supple	slightly firm	slightly firm
Flexibility	good	good	good
Filling of flaws in the flanks	slight	good	medium
Adhesion of the hair side	poor in the flanks	very good overall	poor in the flanks
Tensile strength in kg/mm²	1.4	1.5	1.5
Elongation at break in %	59	65	66
Sag which causes cracking, in mm	7.2	7.4	7.5

The sag which causes cracking, measured on a Lastometer, characterises the ability of a leather to undergo folding under tension. This activity is expressed in KU/cm³ where a product in solution is concerned and in KU/g where a solid product is concerned.

EXAMPLE 1. FERMENTATION

A 170 litre fermenter is charged with the following:

peptone	:	1,200 g.
yeast extract	:	600 g.
agar	:	240 g.
water, q.s.p.	:	105 litres

The pH of the medium is 6.55. It is sterilised by bubbling steam at 122°C through it for 40 minutes. After cooling, because of the condensation of the steam during sterilisation, the volume of the broth is 115 litres; it is made up to 120 litres by adding 5 litres of a sterile aqueous solution containing:

glucose monohydrate : 1,200 g.

The pH of the medium is 6.80. It is inoculated with a culture (200 cc.), prepared in an agitated Erlenmeyer flask, of *Streptomyces caligosus* DS 14,486. The culture is developed at 27°C for 23 hours whilst agitating and aerating with sterile air; it is then suitable for the inoculation of the production culture.

The production culture is carried out in an 800 litre fermenter charged with the following substances:

distiller's solubles	:	16 kg.
sucrose	:	6 kg.
soya oil	:	4 litres
manganese sulphate	:	0.08 kg.
water	:	370 litres

The pH is adjusted to 7.30 by adding 10 N sodium hydroxide solution (850 cc.) and the medium is then sterilised by bubbling steam at 122°C through it for 40 minutes. After cooling, because of the condensation of the steam during sterilisation, the volume of the broth is 400 litres; the pH is 6.60.

It is then inoculated with the inoculum culture (40 litres), produced in the 170 litres fermenter, described above. The culture is developed at 27°C for 94 hours whilst agitating by means of a turbine revolving at 205 revolutions/minute and aerating with a volume of sterile air of 20 m^3/hr.; the pH of the culture is at that stage 7.30 and the volume of the must is 400 litres. The proteolytic activity of the must at pH 7 and at 37°C is 3.7 KU/cm^3.

EXAMPLE 2. EXTRACTION

A must (11 litres) obtained as described in Example 1 is filtered and the filter cake is then washed with 4 litres of distilled water. The filtrate and the wash liquors are combined and then concentrated to 2 litres under reduced pressure (5 mm.Hg) without exceeding 30°C. Acetone (2.4 litres) previously cooled to −10°C is added rapidly to the concentrate which has been cooled to +4°C whilst stirring. Stirring is continued for 2 minutes and the insoluble matter formed is then separated off by centrifuging at +4°C and at 5,000 *g* for 10 minutes. The insoluble matter obtained is extracted with water (450 cc followed by 150 cc) at +4°C for 2 hours, each time separating off the insoluble matter by centrifuging at +4°C and at 5,000 *g* for 10 minutes. An aqueous extract (a total of 800 cc) is obtained, the pH of which is between 7 and 7.5.

This extract is kept at +4°C and crystalline ammonium sulphate (344 g) is added whilst stirring; stirring is continued for 15 minutes after the end of the addition, the mixture is left to stand for one hour, and the active insoluble matter is then isolated by centrifuging at 10,000 *g* for 10 minutes at +4°C. The insoluble matter is dissolved by stirring for one hour in water (500 cc) whilst adjusting the pH to about 7—7.5, and the solution obtained is dialysed at +4°C for 17 hours against distilled water.

A dialysed extract (920 cc) is obtained, into which acetone (1.1 litres) cooled to −10°C is poured rapidly, whilst stirring. The insolubilised enzyme is separated off by centrifuging at 5,000 *g* for 10 minutes at +4°C, and is then dried under reduced pressure at +4°C in the presence of a dehydrating agent (P_2O_5).

Finally, enzyme 24,199 RP (9.5 g), having the following enzymatic activities, is obtained:

protease activity	:	1,470 KU/g
coagulant activity	:	14,500 PU/g
fibrinolytic activity	:	4,920 LU/g

EXAMPLE 3.

A must (360 l.) obtained as described in Example 1 is filtered on a filterpress in the presence of a filtration adjuvant (25 kg). Methanol (400 litres) cooled to −10°C is added to the filtrate (200 litres) obtained. After cooling the mixture to about −10°C, the precipitate obtained is separated off by centrifuging cold, and is dried under reduced pressure at 35°C.

Crude 24,199 RP (229 g) containing 290 KU/g is isolated.

EXAMPLE 4. APPLICATION TO THE DEPILATION OF CATTLE SKINS

Green-cured cattle skins (100 kg) are first of all desalted twice by agitating for 30 minutes with pure water (200%) (the percentages are expressed relative to the weight of the cured skins) and are then soaked.

The desalted skins are agitated slowly for 20 hours at a temperature of between 20 and 25°C with water (200%) to which sodium hydroxide flakes (0.2%) and alkaline protease S 1,200 (0.05%) have been added. The skins are then fleshed after which the depilation is carried out.

The fleshed skins are placed in a drum turning at a speed of 3 to 4 revolutions per minute for 1 hour. Water (20 to 25%) and enzyme 24,199 RP (300 to 400 g) containing 290 KU/g are added. The temperature inside the drum is adjusted to 30°C. The skins are agitated as described above for 1 hour and then intermittently for 8 to 9 hours. The hair can at that stage be removed perfectly by rubbing with the back of a blade.

The skins are then unhaired by any means appropriate to the tanning industry.

The pelt thus obtained is virtually completely depilated except for a few very short tufts which have escaped the mechanical working with the blade.

The skins are then treated in a known manner in a liming liquor (200%) made up with lime (3%) and sodium sulphide (1.5%). The object of this treatment is to obtain perfectly depilated skins which can be subjected to the subsequent operations of the tanning process.

The liming liquor, which is only very slightly loaded, can be recycled. The skins obtained after tanning, dressing and finishing have an attractive appearance, and a fine hair-side which is undamaged and glossy and adheres very firmly, contrary to the skins ordinarily treated with proteases.

EXAMPLE 5. APPLICATION TO THE DEWOOLLING OF SHEEPSKINS

After soaking sheepskins, removing fatty matter from the flesh side and draining, an aqueous solution (150 cc) containing enzyme 24,199 RP (5 to 6 g/litre), of strength 290 KU/g and the solution, buffered to pH 8.5 with disodium phosphate, is applied by a spray gun to the flesh side of each skin. The skins are stacked flesh side against flesh side in a chamber where the relative humidity must not exceed 85% and where the temperature must be kept at between 24 and 26°C. After 16 to 20 hours, dewoolling can be carried out very easily by the usual means.

The wool obtained is a very good quality, as is the skin which remains, which has not lost its outer face, contrary to the skins obtained by the "heating" process. The skin can subsequently be limed, after which it is tanned and finished in accordance with the usual processes.

WHAT WE CLAIM IS:—

1. A culture of the microorganism *Streptomyces caligosus* DS 14,486, having the NRRL reference No. 8,195, in a synthetic liquid culture medium containing a source of assimilable carbon, a source of assimilable nitrogen and mineral elements, the culture being substantially free from other microorganisms, and the microorganism NRRL 8,195 having the following characteristics:

(a) forms cylindrical spores measuring 0.8 to 1.0 μ/0.6 to 0.8 μ,

(b) exhibits sporophores in clusters;

(c) the chains of spores are generally rather long and can amount to up to several tens of spores, and coil up in tight more or or less elongated spirals, in general forming 2 to 3 turns but rather frequently up to six or eight, or even ten turns,

(d) by its mode of sporulation, falls in the Spira section of the Pridham classification,

(e) develops well at 26°C, poorly at 37°C and not at all at 50°C,

(f) has a sporulated aerial mycelium of grey colour,

(g) the colouration of its vegetative mycelium ranges from more or less deep brown to black brown, depending on the culture media,

(h) on organic media, and in particular, on Waksman special tyrosine—yeast extract agar ("melanin formation medium"), gives, in general, a rather copious production of a melanin pigment which imparts a dark tint to the medium;

(i) on numerous synthetic media it is prone to produce a blackish soluble pigment in greater or lesser amounts.

2. The microorganism *Streptomyces caligosus* DS 14,486 (NRRL 8,195) as defined in claim 1 in freeze-dried form.

3. The proteolytic enzyme, designated 24,199 RP, having the following characteristics:

(a) is a protein substance,

(b) is a powder of brown to black colour,

(c) has a weight average molecular weight in the region of 27,000,

(d) has an isoelectric point of 3.7,

(e) is soluble in water, sparingly soluble in concentrated aqueous solutions of neutral salts and in water-alcohol or water-acetone mixtures and virtually insoluble in anhydrous alcohols and ketones,

(f) has a maximum activity on casein at a pH of 7.5 and at a temperature of 45°C,

(g) has a coagulant activity on milk,

(h) has a fibrinolytic activity on a fibrin clot,

(i) exerts activity on the glycolproteins of hair roots and skins.

4. A process for the preparation of enzyme 24,199 RP, wherein a culture of *Streptomyces caligosus* DS 14,486 (NRRL 8,195) as defined in claim 1 is cultured aerobically.

5. A process according to claim 4 wherein enzyme 24,199 RP is separated from the culture.

6. A process for the preparation of enzyme 24,199 RP according to claim 4 substantially as hereinbefore described with reference to any one of Examples 1 to 3.

7. Enzyme 24,199 RP obtained by a process as claimed in any one of claims 4 to 6.

8. A process for the enzymatic depilation of animal skins, which comprises bringing the skin into contact with enzyme 24,199 RP.

9. A process according to claim 8, which consists of bringing cattle skin and enzyme 24,199 Rp into contact with one another in a depilation vessel in which the percentage of water relative to the skin is 10 to 45% by volume for 3 to 24 hours, the temperature of the bath being 24 to 30°C and its pH being 7 to 8.5, the amount of enzyme being such that 0.3 to 1 part by weight of enzyme is employed per 100 parts by weight of skin, after which the skins are unhaired and treated in a liming liquor.

10. A process according to claim 8, which consists of bringing sheep skin and enzyme 24,199 RP into contact with one another by spraying onto the flesh side of the skin, an aqueous solution of the enzyme so that the percentage of water relative to the skin is 10 to 45% by volume, the pH of the solution being 7 to 8.5, and the solution furthermore being of such concentration and strength that a concentration of 5 to 6 g/l of enzyme corresponds to a strength of 290 KU/g; the skins are then stacked flesh side against flesh side in a chamber in which the relative humidity does not exceed 85% and the temperature is 24 to 26°C, for a period of 16 to 20 hours.

11. A process for the depilation of animal skins according to claim 8 substantially as hereinbefore described with reference to Example 4 or 5.

12. Depilated animal skins obtained by a process as claimed in any one of claims 8 to 11.

J. A. KEMP & CO.,
Chartered Patent Agents,
14, South Square,
Gray's Inn, London, W.C.1.

Printed for Her Majesty's Stationery Office by the Courier Press, Leamington Spa, 1980.
Published by the Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.