HYDROLYTIC ACTION OF FACULTATIVELY ANAEROBIC MICRO-ORGANISMS ON RAW SKINS

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Hydrolysis of skin collagen by different proteolytic strains of facultatively anaerobic bacteria has been studied under both aerobic and anaerobic conditions of growth. Hydrolysis of skin collagen is found to be less when the organisms are grown anaerobically. Various factors like peptone concentration in the medium, period of incubation, temperature and skin-medium ratio influence the hydrolysis of skin collagen. Collagen hydrolysis is not encouraged by mixed bacterial cultures or by transferring the skin repeatedly to a fresh culture. Unhairing of skin by different proteolytic strains of bacteria is found to be comparatively rapid when the organisms are grown aerobically and when they are used in a mixed culture.

Introduction

Considerable amount of work has been done on the different types of micro-organisms associated with hides and skins and the possible degradation of raw stock by the proteolytic bacteria. But in most occasions studies have been confined to the aerobic or facultatively anaerobic micro-organisms grown under aerobic conditions only. Tancous[1] published her work on an obligate anaerobe *Clostridium capitovale* and demonstrated its deteriorating action on hide and leather. According to Francis *et al*[2] only certain obligate anaerobes belonging to the genera Clostridium and Bacteroides posses true collagenase activity.

Venkatesan *et al*[3] observed that skins are rarely associated with obligate anaerobes but they pointed out that facultatively anaerobic organisms possibly play a more important role in deteriorating hide or skin quality. Everett and Cordon[4] showed that most of the pure bacterial cultures isolated from salted hides were unable to attack undenatured collagen, but limed collagen was attacked by most of the cultures. They, however, observed that few combinations of mixed cultures were able to attack collagen.

Our knowledge is very much limited about the growth of the facultatively anaerobic organisms and their hydrolytic action on skin collagen under both aerobic and anaerobic conditions. In the present study, the effect of certain factors on the hydrolysis of skin collagen by different facultatively anaerobic organisms under aerobic and anaerobic conditions of growth has been studied. Unhairing of skin by some of the organisms aerobically

as well as anaerobically has also been investigated.

Materials and methods

Organisms

Bacillus subtilis, B. cereus, B. megaterium, B. Coagulans and *Staphylococcus aureus* were obtained from V. S. Krishnamachar, Scientist-in-charge, National Collection of Industrial Micro-organisms, Poona-8., and the rest of the organisms were isolated in the Bacteriology Laboratory, Central Leather Research Institute, Madras-20.

Estimation of proteolytic activity

Proteolytic activity of different microorganisms was determined using casein, egg albumin and gelatin as substrates.

(a) Caseinolytic activity was determined according to the method of Anson[5] and kunitz[6] as modified by Hagihara.[7] 2% aqueous casein (pure BDH) was prepared in O.1*M* phosphate buffer of pH 7.4. 1ml. of the enzyme preparation was added to 1ml. of the substrate in each test tube. The tubes were kept in a water bath maintained at 40°C for 10 min. for digestion. Enzyme reaction was then stopped by adding sufficient quantity of precipitating agent (composed of 0.1*M* tri-chloroacetic acid (TCA), 0.2*M* sodium acetate, and 0.2*M* acetic acid). The tubes were warmed and then cooled to room temperature and the contents were filtered through Whatman No. 1 filter paper. Control experiments were run in an identical manner except that the precipitating agent was added to the substrate before the addition of enzyme solution. The colour developed by Folin & Ciocalteu was read in Klett-Summerson Photoelectric Colorimeter by using red filter No. 66.

Proteolytic activity (caseinase) is expressed in units. A unit is defined as mg. of tyrosine liberated per min. per ml. of the enzyme.

(b) Using egg albumin as the substrate the proteolytic activity was determined according to the method of Anson[5] as modified by Bose *et al.*[8] 2.5% egg albumin (E. Merck, pure) solution was prepared in O.1*M* phosphate buffer of pH 7.4. 2ml. of the enzyme preparation was added to 2ml. of the substrate in each test tube. The tubes were kept in incubator maintained at 45°C for 30 min. for digestion. Enzyme reaction was then stopped by adding 8ml. of 5% TCA solution. The following procedure was the same as indicated in case of caseinase activity.

Proteolytic activity is expressed in units, the unit being defined as mg. of tyrosine liberated for 5ml. of the digestion mixture.

(c) Gelatinolytic activity was determined qualitatively. 10 ml. of the nutrient gelatin (12% BDH gelation) was taken in each test tube, sterilized and inoculated with 24 hrs. old bacterial cultures The tubes along with control uninoculated tubes were then incubated at 37°C. At regular intervals (24, 48, 72 & 96 hrs.) the tubes were examined for proteolytic activity until gelatin was completely liquified. Proteolytic activity (gelatinase) is expressed as the time (days) taken for the complete liquefaction of gelatin by the different micro-organisms.

Hydrolysis of skin collagen

Fresh goat skin was collected from slaughter house, cleaned, washed, fleshed and the hairs were shaved off with the help of a safty razor. Small skin pieces weighing exactly 1g. each were cut out and then sterilized by fumigating them with ethylene

oxide.' After exposing to ethylene oxide for a period of 18-20 hrs. and subsequently incubating outside for 1 day, the skin pieces were aseptically transferred to the tubes containing 10 ml. of thioglycollate broth. If no growth occured within a period of 5 days, the tubes containing the medium and skin pieces were taken for inoculation.

24 hrs. old cultures of different organisms were used as the inoculum and the tubes were maintained in aerobic and anaerobic conditions of growth. The tubes were generally put to a shaker (r. p. m. 120) for the first two days at 30°C and then incubated at 37°C for a period of 18 days. The contents in the tube were then centrifuged and 5ml. of the supernatent was taken for hydroxyproline estimation. The hydroxyproline present in the uninoculated tube containing the media and skin incubated for the same period served as control. Hydroxyproline was estimated by following the Newman & Logan's[10] method. The increase in hydroxyproline in the media gave a measure of the hydrolysis of collagen. The hydroxyproline values have been presented after correcting them by deducting the values obtained in case of control tubes and are expressed as mg. of hydroxyproline present in 5ml. of the culture media.

Anaerobiasis was maintained with the help of McIntosh and Fildes Anaerobic jar using methylene blue as indicator.

Unhairing index

Unhairing of skin was examined either by plucking out the hairs or by unhairing the skin with a blunt knife as reported earlier.[11] The values for unhairing index indicate the extent of unhairing and was determined on the basis of the following criteria: no unhairing-0, very slight unhairing-1, slight unhairing-2, slight to moderate unhairing-3, moderate unhairing-4, moderate to easy unhairing-5, easy unhairing-6.

Results

Proteolytic activity of different facultatively anaerobic organisms

Different strains of facultatively anaerobic bacteria were cultured to 10 ml. of sterilized sodium thioglycollate broth using a 24 hrs. old culture as inoculum. The organisms were grown as shake culture (30°C) for 48 hrs. under aerobic and anaerobic conditions. The tubes were then centrifuged at 6000 r.p.m. and the supernatent media were used for proteolytic activity estimation. Results obtained on the hydrolysis of casein and egg albumin are presented in Table 1. Data on the liquefaction of gelation by different organisms have been incorporated in the same Table.

It is evident from Table 1 that when tested on casein, *Pseudomonas aeruginosa Bacillus* species (strain No. 102), *Proteus vulgaris* and *Bacillus laterosporus* are found to exhibit more proteolytic activity both aerobically and anaerobically. *Ps. Aeruginosa, Bacillus sp. (102), Bacillus sp. (105)* and *P. vulgaris* are most active when egg albumin is used as the substrate. Gelatin is more readily hydrolysed by *Ps. aeruginosa, Bacillus sp. (102), Bacillus sp. (105)* and *B. brevis* both in aerobic and anaerobic conditions. It may further be noted that when grown in anaerobic condition the proteolytic activity of the organisms is comparatively less practically in all the cases.

Hydrolysis of skin collagen by different proteolytic facultatively anaerobic bacteria

Hydrolysing activity of various strains on skin collagen was next studied. Tubes containing thioglycollate broth and sterilized skin pieces were cultured with 13

TABLE 1

Proteolytic activity of different micro-organisms in aerobic and anaerobic conditions.

Sl. No.	Bacterial strains	Gelatin*		Egg Albumin£		Casein@	
		Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
1.	*Pseudomonas aeruginosa*	1	2	.162	.075	.01687	.0045
2.	*Bacillus cereus*	1	3	.078	.048	.00475	.00325
3.	*Bacillus subtilis*	2	3	.048	.045	.005	.003
4.	*Bacillus sp. 102.*	1	2	.162	.12	.016	.0105
5.	*Bacillus sp. 105*	1	2	.222	.082	.01565	.0035
6.	*Staphylococcus aureus*	3	4	.078	.052	.0034	.00425
7.	*B.megaterium*	2	4	.018	.018	.00325	.003
8.	*B.coagulans*	4	4	.018	.018	.00325	.00275
9.	*Proteus vulgaris*	1	4	.108	.078	.0084	.00675
10.	*B.latcrosporus*	2	3	.055	.032	.0065	.00425
11.	*B.brevis*	1	2	.09	.063	.005	.0034
12.	*B.alvei*	2	4	.043	.032	.00475	.00425
13.	*B.pumilus*	2	4	.105	.027	.0045	.0025

* Gelatin: complete liquefaction in days.

£ Units: Unit is expressed as mg. of tyrosine liberated for 5 ml. of the digestion mixture.

@ Units: Unit is expressed as mg. of tyrosine liberated per min. per ml. of enzyme solution.

strains possessing proteolytic activity. After 20 days of incubation, hydroxyproline present in 5ml. of the centrifuged medium was estimated and the results obtained are given in Table 2.

It is evident from Table 2 that values for hydroxyproline liberated in the medium are comparitively higher in cases of *Ps. aeruginosa, B. cereus, Bacillus sp. (102)* and *Bacillus sp. (105)* under both aerobic and anaerobic conditions. *P. vulgaris* strain though hydrolyses casein and egg albumin to an appreciable extent (Table 1), possesses very little hydrolytic action on collagen. Collagen hydrolysis by different organisms also appears to be comparatively less under anaerobic condition.

Influence of peptone concentration on the hydrolysis of skin collagen

Peptone (Difco) was added in the proportion of 0.5, 1.0 & 2.0% to a stock phosphate buffer solution of pH 7.4 (10ml. in each tube). As before, sterilized skin pieces were transferred aseptically to the tubes and after an observation period of 5 days different strains were cultured to the tubes and incubated under aerobic and anaerobic condition at 37°C for 20 days. Observations are recorded in Table 3.

Results obtained clearly point out that, hydrolysis of skin collagen increases as the peptone concentration in the buffer increases upto 2% (maximum concentration

TABLE 2

Hydrolysis of skin collagen* by different micro-organisms in aerobic and anaerobic conditions

Sl. No.	Bacterial Strains	Incubation period—20 days			
		Aerobic		Anaerobic	
		Hydrolysis of skin collagen	Removal of epidermis	Hydrolysis of skin collagen	Removal of epidermis
1.	*Ps. aeruginosa*	3.45	CR	2.82	CR
2.	*B. cereus*	2.55	"	1.98	"
3.	*B. subtilis*	1.62	"	0.59	NR
4.	*Bacillus sp. 102*	2.85	"	2.7	CR
5.	*Bacillus sp. 105*	3.15	"	2.94	"
6.	*S. aureus*	0.8	SR	0.35	NR
7.	*P. vulgaris*	1.48	NR	0.46	"
8.	*B. megaterium*	2.55	CR	0.44	"
9.	*B. coagulans*	0.6	NR	0.30	"
10.	*B. laterosporus*	0.3	"	0.24	"
11.	*B. brevis*	0.65	SR	0.38	"
12.	*B. alvei*	0.3	"	0.24	"
13.	*B pumilus*	1.81	CR	0.32	"

* mg. of hydroxyproline liberated in 5 ml. of the media.
CR = Complete removal of epidermis.
SR = Slight removal of epidermis.
NR = Not removed.

TABLE 3

Influence of peptone concentration on the hydrolysis of skin collagen*

Sl. No.	Bacterial Strains		PEPTONE %			
			Nil	0.5%	1%	2%
1.	*Ps. aerugenosa*	A	0.204	0.576	1.212	1.704
		An	0.12	0.466	0.84	1.332
2.	*B. cereus*	A	0.132	0.804	0.696	1.26
		An	0.108	0.36	0.456	1.14
3.	*B. subtilis*	A	0.24	1.092	1.452	2.444
		An	0.168	0.444	0.804	1.416
4	*Bacillus sp. 102*	A	0.24	0.504	0.768	1.812
		An	0.18	0.384	0.456	1.416
5.	*Bacillus sp. 105*	A	0.36	0.72	1.104	1.728
		An	0.156	0.668	0.72	1.224

* mg. of hydroxyproline liberated in 5 ml. of the media.
A = Aerobic condition of growth.
An = Anaerobic condition of growth.

studied). This is true for all the 5 organisms examined and for both aerobic and anaerobic conditions of growth. Even in this occasion, skin hydrolysis appears to be less in anaerobic condition of growth. It was further observed that hydrolysis of skin collagen by different organisms was comparatively higher in thioglycollate broth than in nutrient broth.

Effect of incubation period on the hydrolysis of skin collagen

Tubes containing 10 ml. of thioglycollate broth and sterilized skin pieces were inoculated with 24 hrs. old culture of *Bacillus sp. 102* and then incubated at 37°C aerobically as well as anaerobically for a period of 30 days. At intervals, tubes were taken out and the amount of hydroxyproline liberated in each tube due to the hydrolysis of skin collagen was estimated. Data obtained are presented in Fig. 1.

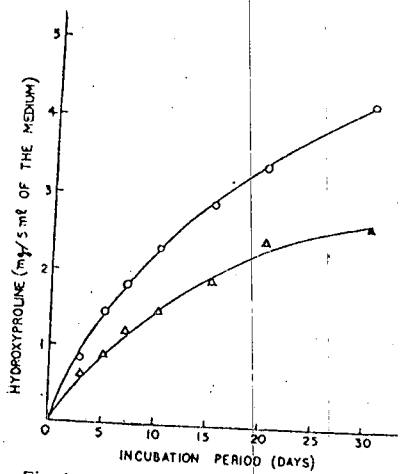

Fig. 1: Effect of incubation period on the hydrolysis of skin collagen.
○——○ = Hydrolysis in aerobic condition.
△——△ = Hydrolysis in anaerobic condition.

It is evident from Fig. 1 that hydrolysis of skin collagen due to *Bacillus sp. (102)* increases progressively with the period of incubation both under aerobic and anaerobic conditions of growth, the rate of hydrolysis being comparatively less in anaerobic condition.

Effect of temperature on the hydrolysis of skin collagen

Sodium thioglycollate broth tubes containing skin pieces were inoculated with 3 bacterial strains, incubated aerobically at different temperatures for a period of 20 days without subjecting them to the shaker for the first two days. Hydroxyproline liberated in each case was estimated and the results obtained are given in Table 4.

Data obtained indicate that the organisms prefer optimally a temperature of about 37°C for the hydrolysis of skin collagen.

Effect of NaCl on the hydrolysis of skin collagen

Sodium chloride was added to thioglycollate broth in increasing concentrations (upto 10%) and the pH of the broth was readjusted to the same level. The media were then distributed in tubes (10 ml. each) and sterilized skin pieces were added to the sterile tubes. Two strains viz. *Ps. aeruginosa* and *Bacilus sp. (102)* were then cultured and the tubes were incubated under aerobic and anaerobic conditions as before. Hydroxyproline present in each medium was estimated after 20 days and the results are presented in Table 5.

As expected, the growth as well as collagen hydrolysis by the two organisms are appreciably retarded by increasing NaCl concentrations (above 5.0 %) both aerobically and anaerobically.

TABLE 4

Influence of temperature on the hydrolysis of skin collagen* in aerobic condition

Sl. No.	Bacterial Strains	Temperature °C			
		30	37	43	51
1.	*Ps. aeruginosa*	2.52	3.72	2.16	0.96
2.	*Bacillus sp. (102)*	1.92	2.52	1.44	0.49
3.	*Bacillus sp. (105)*	2.232	2.64	2.28	0.36

* mg. of hydroxyproline liberated in 5 ml. of the culture media.

TABLE 5

Effect of NaCl on the hydrolysis of skin collagen*

Sl. No.	Bacterial Strains		NaCl Concentration (%)				
			0	2.5	5	7.5	10
1.	*Ps. aeruginosa*	A	2.952	3.24	2.16	0.432	0.12
		An	1.46	1.32	0.96	0.32	0.12
2.	*Bacillus sp. (102)*	A	1.92	1.32	0.796	0.36	0.12
		An	0.96	0.84	0.36	0.24	0.12

* mg. of hydroxyproline liberated in 5 ml. of the culture media.

Influence of skin and medium ratio on the hydrolysis of skin collagen

Conical flasks (100 ml. capacity) containing thioglycollate broth and skin pieces in different proportions were incubated with 3 bacterial strains. The skin-thioglycollate broth ratio (W/V) was adjusted to 1 : 5, 1 : 10 and 1 : 20. After 20 days of incubation in aerobic and anaerobic conditions, the hydroxyproline liberated due to collagen hydrolysis was determined in each flask. Data obtained are presented in Table 6.

It is evident from the above Table that collagen hydrolysis by all the 3 organisms is comparatively higher when 1 g. of skin is taken in 20 ml. of the medium. This trend of variation occurs under both aerobic and anaerobic conditions of growth.

Effect of mixed bacterial cultures on the hydrolysis of skin collagen

Four bacillus, one pseudomonas and one proteus strains were arranged in different combinations and thioglycollate broth tubes containing skin pieces were inoculated with such combinations of mixed cultures and incubated aerobically for a period of 20 days at 37°C. Hydroxyproline present in the medium was estimated in each case and results obtained are presented in Table 7.

TABLE 6

Medium-skin ratio (V/W) on the hydrolysis of skin collagen*

Sl. No.	*Bacterial Strains*		*Medium : Skin (V/W)*		
			20 : 1	*20 : 2*	*20 : 4*
1.	*Ps.aeruginosa*	A	4 . 90	4 . 38	4 . 52
		An	3 . 5	2 . 4	1 . 68
2.	*B.cereus*	A	5 . 22	4 . 98	4 . 524
		An	3 . 72	2 . 52	2 . 484
3.	*Bacillus sp. 105*	A	4 . 64	4 . 44	4 . 128
		An	3 . 624	2 . 184	2 . 304

* mg. of hydroxyproline liberated in 5 ml. of the culture media.

TABLE 7

Influence of mixed cultures on the hydrolysis of skin collagen* under aerobic condition

Series	*Mixed culture*	*Hydroxy-proline*
I a.	1 2 3 4 5 6	3 . 12
b.	1 2 3 4 5	3 . 3
c.	1 2 3 4	2 . 88
d.	1 2 3	2 . 78
e.	1 2	3 . 12
f.	1	3 . 84
II a.	2 3 4 5 6	2 . 1
b.	2 3 4 5	2 . 88
c.	2 3 4	2 . 16
d.	2 3	2 . 22
e.	2	3 . 12
III a.	3 4 5 6	2 . 42
b.	3 4 5	2 . 82
c.	3 4	2 . 64
d.	3	2 . 74
IV a.	4 5 6	1 . 98
b.	4 5	2 . 28
c.	4	2 . 94
V a.	5 6	1 . 92
b.	5	3 . 12
VI a.	6	1 . 48

*mg. of hydroxyproline liberated in 5 ml of the culture media.

1. *Ps.aeruginosa*
2. *Bacillus sp. (102)*
3. *B.subtilis*
4. *B.cereus*
5. *Bacillus sp. (105)*
6. *P.vulgaris*

It may be noted that collagen hydrolysis by five individual strains (*Ps. aeruginosa*, *Bacillus sp. (102)*, *Bacillus sp. (105)*, *B. subtilis* and *B. cereus*.) are practically uneffected when other 4 strains are mixed with each of them. Collagen hydrolysis by *P. vulgaris* is considerably low and it may be possible that hydrolytic action of some of the individual or mixed culturs is very slightly affected when *P. vulgaris* is mixed with them.

Effect of repeated bacterial inoculation on the hydrolysis of skin collagen

How the rate of collagen hydrolysis is affected by subjecting the skin piece repeatedly to a fresh culture of the same organism was next studied. Thioglycollate broth tubes containing skin pieces were cultured with *Ps. aeruginosa* and *Bacillus sp.. (102)* and after 5 days of incubation (including 2 days of shaking) the skin pieces were transferred to fresh tubes containing thioglycollate broth only and reinoculated with the same organism. Hydroxyproline liberated in the medium after each transfer was estimated. In the same way skin pieces were transferred to the fresh cultures after every 5 days of incubation and hydroxyproline libarated in each tube was determintd. Observations are recorded in Table 8.

It is apparent from Table 8 that the rate of collagen hydrolysis increases only slightly (particularly after the 3rd transfer) by repeated exposure of the skin pieces to fresh cultures both aerobically and anaerobically.

Unhairing action of certain proteolytic strains

Fresh goat skin pieces of about 5 cm. square each were taken in petridishes, sterilized by exposing them to ethylene oxide and then inoculated on the flesh side with the suspensions of 3 bacterial strains. The petridishes were incubated at room temperature (about 30°C) inside a closed moist desiccator. The extent of unhairing was determined under aseptical conditions after 3,5 and 7 days and the observations are recorded in Table 9.

Data presented in Table 9 indicate that all the 3 organisms are capable of unhairing

TABLE 8

Effect of repeated bacterial inoculation on the hydrolysis of skin collagen*

Sl. No.	*Bacterial Strain*		*Original*	*I transfer*	*II transfer*	*III transfer*
1.	*Ps.aeruginosa*	A	1.92	1.92	2.16	2.52
		An	1.44	1.56	1.56	2.28
2.	*Bacillus sp. (102)*	A	1.32	2.16	2.16	2.4
		An	1.32	1.42	1.56	1.56

* mg. of hydroxyproline liberated in 5 ml. of the culture media.
A = Aerobic condition of growth.
An = Anaerobic condition of growth.

TABLE 9

***Unhairing of goat skin by certain micro-organisms**

Sl. No.	*Bacterial Strains*		*Period of incubation (days)*		
			3	*5*	*7*
1.	*Ps.aeruginosa*	A	2	5	6
		An	1	2	3
2.	*Bacillus sp. (102)*	A	2	5	6
		An	1	2	3
3.	*Bacillus sp.(105)*	A	5	6	6
		An	3	5	5
4.	*Mixed culture (1+2+3)*	A	6	6	6
		An	5	6	6
5.	*Control*	A	2	2	2
		An	1	1	1

* Unhairing index.

the skin completely within 5 to 7 days under aerobic condition, but when all the 3 strains are mixed together complete unhairing requires only about 3 days. When the organisms are allowed to grow anaerobically they take much longer period for complete unhairing (7 days and more) but the period is reduced to 5 days when a mixed culture is used. Slight unhairing may be noted even in case of control skin probably due to the autolytic enzymic action. It was further noted that skin pieces were not putrefied by the organisms even after 15 days of incubation.

Discussion

Facultatively anaerobic organisms showing proteolytic activity under aerobic as well as anaerobic conditions have been selected after preliminary investigations. All these organisms other than the strains isolated in this laboratory from raw skins (e. g. *Bacillus sp. (102)*, *Bacillus sp. (105)*, *B. laterosporus*, *B. brevis* and *B. alvei*), are reported earlier to be associated with hides and skins. When tested on substrates like casein, egg albumin, and gelatin, *Ps. aeruginosa*, *P. vulgaris* and two isolated strains *Bacillus. sp. (102)* and *Bacillus sp. (105)* exhibit comparatively higher proteolytic activity both in aerobic and anaerobic conditions.

Hydrolysis of skin collagen also appears to be higher by the above organisms except *P. vulgaris*. On the other hand, *B. cereus* hydrolyses skin collagen to a considerable extent. It may be noted in general that proteolytic activity and hydrolysis of skin collagen are comparatively less when the organisms are grown anaerobically. The present observations point out that all the proteolytic facultatively anaerobic bacteria are not capable of hydrolysing skin collagen and particularly so under anaerobic condition. It may be noted that (Table 2) epidermis has been completely removed from the corium by 8 strains in aerobic condition, but by 4 strains only in anaerobic condition within the period of observation. The extent of collagen hydrolysis is however limited, the maximum being about 14% on fresh skin collagen in case of *Ps. aeruginosa* when grown in aerobic condition.

It is evident from Table 3 that the amount of peptone present in the medium considerably influences the hydrolysis of skin collagen by the organisms grown both aerobically and anaerobically. Collagen hydrolysis by different organisms is also affected by the culture media.

Period of incubation has considerable influence on the hydrolysis of skin collagen although the extent of hydrolysis is found to be comparatively less when *Bacillus sp. (102)* is grown in anaerobic condition. About 17% of the skin collagen is found to be liberated into the medium after 30 days of incubation in aerobic condition. This observation suggests that the rate of collagen hydrolysis increases progressively with the time inspite of the fact that the organism has attained maximum growth after 3 days of incubation.

Hydrolysis of skin collagen by different micro-organisms is found to be the maximum at about 37°C. NaCl inhibits the growth of many of the organisms due to its bacteriostatic property. The extent of collagen hydrolysis is also found to be restricted as the NaCl concentration in the medium increases upto 7.5%. Most of the organisms used by Everett and Cordon[4] in their study were salt resistant and so they could hydrolyse collagen even in 15% salt-broth. The organisms used in the present study are not adapted to higher salt concentra-

tions and so they are incapable of growing readily and hydrolysing collagen at higher salt concentrations. Hydrolysis of skin collagen is also found to vary depending on the proportion of skin present in the medium.

It has been pointed out by different investigators[1,12] that mixed cultures can cause greater damage to hides and skins than single strains. Using various group combinations Everett and Cordon[4] observed that only suitable combinations of organisms were able to solubilise more hide collagen but unsuitable combinations might even retard collagen hydrolysis. In the present study no increase in collagen hydrolysis has been noted by using different mixed cultures over that of individual strains. This, once again suggests that only typical combinations may possibly do more harm to hides and skins. Subjecting the skin piece repeatedly to fresh culture of the same organism, the rate of collagen hydrolysis increases only slightly after the third transfer. This observation indicates that the proteolytic enzyme secreted by these organisms into the medium possibly reacts slowly with the skin proteins and with time collagen hydrolysis increases progressively. Transferring the skin to a fresh culture after every 5 days does not enhance collagen hydrolysis because in each occasion 2 to 3 days are required by the organisms for enzyme production.

Unhairing action of different proteolytic strains are well recognised. The present study reveals that proteolytic enzymes secreted by *Ps. aeruginosa, bacillus sp. (102) and Bacillus sp. (105)* are capale of unhairing goat skin both under aerobic and anaerobic conditions, the unhairing action being much quicker in aerobic condition. A combination of the above 3 organisms appears to quicken the unhairing action than the individual strains. Although collagen hydrolysis is not affected, unhairing action is found to be enhanced by the mixed culture. Possibly a rapid hydrolysis of the inter fibrillary proteins, e.g. albumin, globulin, mucoids etc. by the mixed culture is responsible for quicker unharing action. Such an approach may possibly be made while preparing enzyme depilants.

It has been observed that a skin piece gets completely putrefied even in the absence of collagenase producing obligate anaerobes when it is allowed to be staled or soaked in water for 3 or 4 days. But when the same skin (sterilised) is subjected to a single proteolytic strain or even to a mixed culture it is not putrefied to that extent. This points out that possibly certain additional factors are involved in the putrefaction of hides and skins by organisms having little or moderate collagen hydrolysing property. A better understanding of the mechanism of such putrefactive changes will be of considerable assistance in controlling microbial degradation of hides and skins and thereby developing improved methods of curing.

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