213

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Isolation of Two Facultatively Anaerobic Organisms Producing Collagenase

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One *Bacillus* sp. and one *Pseudomonas* sp. isolated from putrefied skin were found to secrete extracellular collagenases under both aerobic and anaerobic conditions of growth. Collagenase activity was determined by studying the hydrolysis of RTT collagen fibers, lysis of collagen gel and reduction in viscosity of soluble collagen. In all the tests collagenolytic activity of these two isolates was compared with that of *Clostridium histolyticum*. Proteases elaborated by the facultatively anaerobic organisms also hydrolysed casein, egg albumin and gelatin.

REVIEW on microbial collagenases by Nordwig[1] pointed out that only a few bacteria are capable of hydrolysing native collagen which include *Clostridium histolyticum, Cl. perfringens., Cl. tetani* and *Bacteroides melaninogenicus*. Collagenolytic enzymes were found to be secreted by certain obligate anaerobes (*Cl. histolyticum*[2] and *Cl. perfringens*[3]) and they could attack native collagen under physiological conditions. Tancous[4] isolated another obligate anaerobe *Cl. capitovale* from salt-cured hide samples which could hydrolyse skin collagen.

Certain aerobic and anaerobic (facultative) bacteria[5-11] were also claimed to produce collagenases. But in most occasions azocoll, hide powder and synthetic peptides were used as the substrates for collagenase estimation. But Waldvogel and Swartz[12] developed a screening test for collagenase producing bacteria using a collagen gel film and examined various bacterial strains for their lytic effect on collagen film. They observed a limited number of anaerobic strains belonging to the genus clostridia and bacteroides and one strain of *Staphylococcus aureus* (when grown under anaerobic condition) could lyse collagen gel. Hanada *et al.*[13] however, isolated a marine bacterium *Pseudomonas marinoglutinosa* which was capable of hydrolysing native collagen in aerobic condition. Two new facultatively anaerobic strains isolated in this laboratory from skin, putrefied during soaking, were found to possess collagenolytic activity when tested on different types of collagen substrates. The results obtained are reported in the present communication.

Materials and Methods

Clostridium histolyticum was kindly supplied by IVRI, Izatnagar. One strain of bacillus and one pseudomonas strain, yet to be further identified up to species level, were isolated in this laboratory. These organisms were found to grow under both aerobic and anaerobic conditions. *Cl. histolyticum* was grown on sodium thioglycollate broth and agar plates, the bacillus and pseudomonas strains were grown on nutrient broth and agar plates.

Anaerobic condition was created by using McIntosh and Fildes anaerobic jar with methylene blue as indicator.

Collagenolytic activity — In order to study the collagenase activity of the bacteria the following methods were employed.

Hydrolysis of RTT collagen — Albino rat tails from age group of 7 to 8 weeks were collected, washed well and the tendons were dissected out. These tendons were suspended in 1 *M* NaCl solution for few hours and washed 3 times with distilled water. Next day the tendons were taken in a petridish in bundles weighing 100 mg each and sterilized by fumigating them with ethylene oxide[14]. After exposing to ethylene oxide for a period of 18–20 hr, the petridish was placed outside for 1 day to remove residual ethylene oxide if any. Tendons (100 mg) were aseptically transferred to a sterilized tube containing 10 ml of 0.1 *M* phosphate buffer (*p*H 7.4). If no growth occurred within a period of 5 days, the tubes containing buffer and tendons were taken for inoculation.

Lysis of collagen gel

Preparation of collagen : Collagen was obtained by extracting rat tail tendons with acetic acid following the procedure of Berman *et al.*[15] with slight modifications. Tendons from the tails of about 8 weeks old rats were dissected, sliced, washed in large volumes of chilled 1 *M* NaCl and then with distilled water and allowed to swell in 0.5 *M* acetic acid for 24 hr with stirring. The undissolved material was filtered and the filtrate dialysed against 0.05 *M* acetic acid for overnight. It was neutralized by dialysing against 0.02 *M* phosphate buffer (*p*H 7.2). The precipitate formed during dialysis was dissolved in 0.05 *M* acetic acid and then dialysed aainst phosphate buffer for neutralization. Then the phosphate ions were eliminated by dialysing against distilled water. All the above steps, were carried out at 5°C. The resulting viscous fluid was centrifuged for 1 hr at 21000 rpm and the centrifugate was lyophilized and stored at −40°C.

Preparation of collagen gel : Lyophilized collagen

(200 mg/100 ml) was dissolved in 0.05 *M* acetic acid and adjusted to *p*H 7.5 with 2 *M* tris base containing 0.4 *M* NaCl with vigorous shaking. The solution was then dialysed against 0.05 *M* tris-HCl containing 0.4 *M* NaCl (*p*H 7.6). Collagen concentration in the solution was approximately 0.18%. About 5 ml of the solution was poured into small petriplates and transferred to incubator at 37°C where the gel was formed rapidly.

Collagenase activity of bacteria : The method of Gross[16] as modified by Waldvogel and Swartz[12] was used for detecting collagenolytic activity of bacteria. Circles of the thioglycollate agar/nutrient agar (8 to 10 mm) covered by actively growing cultures were punched out, inverted and then placed at the centre of the petriplate containing collagen gel. These preparations were incubated at 37°C. Collagen lysis appeared as black circular area around the culture on plate.

Viscometric assay of collagenase activity — Viscometric method used by different investigators[12,13,18] for the determination of collagenase activity was followed in the present work. Ostwald viscometer (flow rate ~ 62 sec with distilled water at 20°C) was used for viscosity determination. 0.18% collagen in tris-HCl containing 0.4 *M* NaCl and 5 m*M* $CaCl_2$ (*p*H 7.6) was taken as substrate and to this crude bacterial enzyme preparation was added. Organisms grown (48 hr-old) on agar slants in aerobic as well as anaerobic conditions, were scraped out into sterile tris buffer. This was centrifuged at 10000 rpm for 30 min and the supernatant was estimated for protein content in units following Lowry's[19] method. Enzymes from different organisms were extracted in the same way and then NaCl was added to it to make a final concentration of 0.4 *M*. While comparing the collagenase activity enzyme preparations were adjusted to the same unit of protein content.

The flow time was measured at various intervals up to 3 hr of incubation. In case of control only buffer was added to collagen solution. Specific viscosity of the reaction mixture was calculated after different periods of interaction according to the equation ($\eta_{sp} = \eta_{rel} - 1$).

Proteolytic activity — Proteolytic activity of the enzymes secreted by these isolated strains under aerobic and anaerobic condition was determined using casein, egg albumin and gelatin as substrate.

(a) Caseinolytic activity was determined following the method of Kunitz[20] as reported earlier[1]. Caseinase is expressed in units, the unit being defined as mg of tyrosine liberated per min per ml of the enzyme solution. (b) Using egg albumin as substrate, the proteolytic activity was determined by the modified method of Bose *et al.*[22] Activity is expressed in units. One unit is defined as mg of tyrosine liberated for 1 ml of the digestion mixture. (c) Gelatinolytic activity was determined qualitatively. It is expressed as the time (days) taken for the complete liquefaction of gelatin.

Results

Various morphological and biochemical properties of the isolated organisms were studied following standard procedures. The observations are recorded in Table 1.

On the basis of the above morphological and biochemical characteristics these strains could be identified up to the genera level with the help of Bergey's Manual of Determinative Bacteriology[23]. One of the strains appear to be *Bacillus* sp. and the other a *Pseudomonas* sp.

Hydrolysis of RTT collagen — Sterilized tubes containing buffer and RTT were inoculated with 48 hr old cultures of *Cl. histolyticum*, *Bacillus* sp. and *Pseudomonas* sp. and incubated at 37°C under aerobic and anaerobic conditions. At regular intervals, the tubes were examined visually for the extent of hydrolysis of RTT by these strains and the time taken for complete hydrolysis was noted.

It is apparent from Table 2 that all the 3 strains studied are capable of hydrolysing rat tail tendon collagen under both the conditions of growth. However, the time taken for RTT hydrolysis differed appreciably and is maximum in the case of *Pseudomonas* sp. and minimum for *Cl. histolyticum*.

Lysis of collagen gel — Screening test for collagenolytic activity using collagen gel as the substrate was conducted for *Cl. histolyticum* and the newly isolated *Bacillus* sp. and *Pseudomonas* sp. The observations are recorded in Fig. 1.

Cl. histolyticum and the *Bacillus* sp. showed lysis of collagen gel. *Cl. histolyticum* took 24–36 hr for its lytic effect but *Bacillus* sp. took 48–60 hr for similar lytic effect. The lytic effect of *Pseudomonas* sp. is only feeble. It is clear that *Cl. histolyticum* is capable

TABLE 1 — MORPHOLOGICAL CHARACTERISTICS AND BIOCHEMICAL PROPERTIES OF THE ISOLATED STRAINS

Properties	*Bacillus* sp.	*Pseudomonas* sp
	Morphological	
Shape	Rod	Small rod
Gram strain	Positive	Negative
Spores	Present	Absent
Motility	Motile	Motile
Broth	Thick growth	Thick pellicle growth
Pigment	Non chromogenic	Bluish green
Aerobic	Growth present	Growth present
Anaerobic	do	do
	Carbohydrate fermentation	
Dextrose	Acid produced	Acid & gas produced
Maltose	Acid & gas produced	Acid produced
Sucrose	do	Do
Lactose	No acid production	Do
Mannitol	do	Do
Dulcitol	do	Do
Rhamnose	do	Acid produced
Salicin	Acid produced	Do
Inositol	No acid production	Do
Arabinose	do	Do
	Biochemical reactions	
Indol	Not produced	Not produced
Methyl red	Negative	Positive
Acetylmethyl-carbinol	Negative	Negative
Citrates	Not utilized	Utilized
Nitrates	Reduced	Reduced
Gelatin	Liquified	Liquified
Starch	Hydrolysed	Weakly hydrolysed
Milk	Coagulated	Coagulated
Catalase	Positive	Positive

TABLE 2 — HYDROLYSIS OF RTF COLLAGEN FIBRE BY *C. histolyticum*, *Bacillus* SP. AND *Pseudomonas* SP.

Organisms	Complete hydrolysis in days	
	Aerobic	Anaerobic
Cl. histolyticum	6	5
Bacillus sp.	8	11
Pseudomonas sp.	18	24

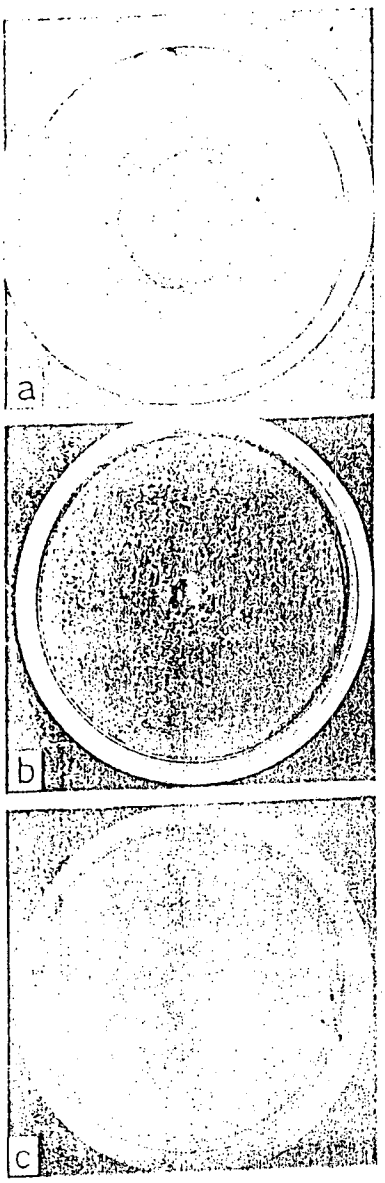

Fig. 1 — Lysis of collagen gel film [(a) *Cl. histolyticum*; (b) *Bacillus* sp. and (c) *Pseudomonas* sp.]

of lysing collagen more rapidly than *Bacillus* sp. and *Pseudomonas* sp.

Collagenase activity by viscometric assay — Eight ml of the 0.18% collagen solution was pipetted into the viscometer kept at 37°C. When the collagen solution attained the water-bath temperature, 2 ml of the enzyme preparation was added to the viscometer and the contents were mixed well. The flow time of the mixture was recorded immediately. The reaction mixture was incubated at 30°C for 3 hr and at regular intervals the flow time was recorded and the specific viscosity was calculated. All the 3 organisms were grown under both aerobic and anaerobic conditions and the enzyme preparation was made as described earlier. Fig. 2 (A and B) represents the reduction in specific viscosity of collagen at various incubation periods.

It may be noted (Fig. 2) that both *Bacillus* and pseudomonas strains are capable of reducing the viscosity of soluble collagen whether they are grown aerobically or anaerobically. In both the conditions of growth the rate of viscosity reduction by the *Bacillus* sp. appears to be comparable with that caused by *Cl. histolyticum* but the viscosity reduction by the *Pseudomonas* sp. is found to be comparatively less than the other organisms.

Proteolytic activity of Bacillus and pseudomonas strains — These 2 facultatively anaerobic strains were cultured in 10 ml of sterilized [illegible] broth with 48 hr old cultures as inoculum and incubated at 37°C for a period of 48 hr under aerobic and anaerobic conditions. The tubes were then centrifuged and

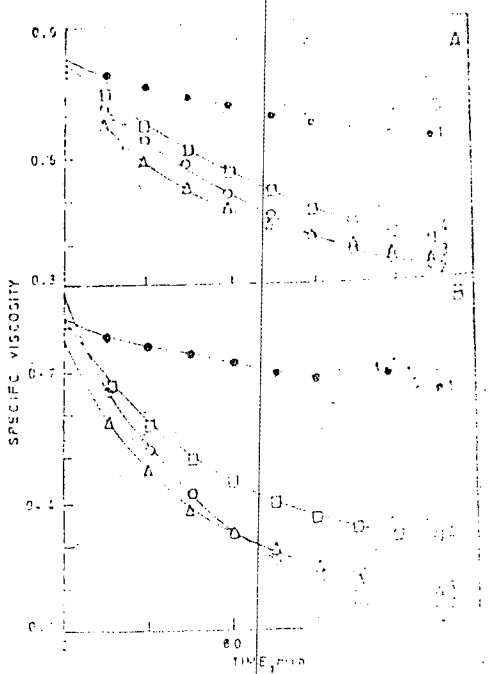

Fig. 2 — Viscosity reduction of collagen [A, anaerobic organisms grown under aerobic condition; B, anaerobic condition (B)] (1, control; 2, *Cl. histolyticum*; 3, *Bacillus* sp; 4, *Pseudomonas* sp)

TABLE 3 — PROTEOLYTIC ACTIVITY OF ISOLATED *Bacillus* AND *Pseudomonas* SPECIES

Substrates	*Bacillus* sp.		*Pseudomonas* sp.	
	Aerobic	Anaerobic	Aerobic	Anerobic
Gelatin[a]	1	2	1	2
Egg albumin[b]	0.2245	0.2050	0.2550	0.2325
Casein[c]	0.0154	0.0112	0.0218	0.0168

[a]Complete liquefaction in days
[b]Unit is expressed as mg of tyrosine liberated for 5 ml of the digestion mixture
[c]Unit is expressed as mg of tyrosine liberated min/ml of enzyme solution

the supernatant media were used for proteolytic activity estimations. Results obtained on the hydrolysis of casein, egg albumin and gelatin are presented in Table 3.

When tested on casein pseudomonas strain exhibited more proteolytic activity both aerobically and anaerobically. The same is true with egg albumin as substrate. Gelatin is readily attacked by both the strains under both aerobic and anaerobic conditions. It may be further noted that the proteolytic activity of both the organisms is comparatively less when grown under anaerobic condition.

Discussion

During earlier studies it was noted that bacterial flora of fresh or putrefied skin rarely included obligate anaerobes[21] which are known to possess collagenolytic activity. It was presumed that possibly the facultatively anaerobic bacteria play more important role in the degradation of raw skin quality. But facultatively anaerobic organisms generally associated with skin were found to show[21] limited hydrolysis of skin collagen. The present work reports the isolation of 2 facultatively anaerobic bacteria, one a *Bacillus* sp. and the other a *Pseudomonas* sp. from soak liquor when a skin was subjected to putrefaction.

Data presented in Table 2 indicate that, like *Cl. histolyticum* these two organisms are also capable of hydrolysing RTT collagen fibres under both aerobic and anaerobic conditions and utilizing them as sources of nitrogen and carbon.

In the same way, all the 3 organisms have caused lysis of collagen gel although the lytic effect is very much restricted in case of *Pseudomonas* sp. *Bacillus* sp. is very close to *Cl. histolyticum* with respect to the lysis of collagen.

Collagenase activity of these 2 isolated organisms has been determined quantitatively following viscosity reduction method and compared with that of *Cl. histolyticum*. Here again it may be noted from (Table 2) that under both aerobic and anaerobic conditions of growth these 2 isolates produce collagenolytic enzyme. Taking all these three methods for collagenase estimation into consideration the collagenolytic activity of the 3 organisms grown under aerobic and anaerobic conditions may be arranged in the following decreasing order.

Cl. histolyticum > *Bacillus* sp. > *Pseudomonas* sp.

These organisms possess considerable proteolytic activity when tested on different substrates like casein, egg albumin and gelatin. It may, however, be pointed out that these two organisms show more proteolytic activity in aerobic condition where as it was found to be reverse in case of *Cl. histolyticum*[20].

The present observations thus point out that such facultatively anaerobic organisms possessing collagenase activity can do considerable damage to raw hides and skins.

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were formed in the samples. Therefore, the results were reproducible.

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