CHAPTER 2

MATERIALS AND METHODS

2.1 STUDIES ON THE DEVELOPMENT OF POLYALDEHYDES FOR TANNING

2.1.1 Preparation of Polyaldehydes - Periodate Oxidation of DAS, DAC and DSA

Starch (~100 g) was suspended in demineralized water and subsequently cooled in an ice bath. 40, 80 and 120 g of sodium periodate was added to the sample while stirring with a magnetic stirrer. The pH of the solution was maintained at 4 during the reaction. The reaction was performed in the dark at 35 °C and stopped after 48 h to obtain DAS of 33, 66 and 99% oxidation. The product was extracted with centrifugation in t-butyl alcohol (1:3 sample:solvent). The supernatant liquid was tested for the presence of iodine in the solvent using iodimetry. The product was resuspended in the same volume of t-butyl alcohol and the centrifugation cycle was repeated several times and dried at 60 °C to yield an offwhite product.

DAC was prepared according to the methods reported earlier with modifications (Hou et al 2008, Tang et al 2005). Cellulose (~100 g) was hydrolyzed in 6N sulfuric acid (10 h, 85 °C). Hydrolyzed cellulose was suspended in demineralized water and subsequently cooled in an ice bath. 40, 80 and 120 g of sodium periodate was added to the sample while stirring with a magnetic stirrer. The pH of the solution was maintained at 4 during the
reaction. The reaction was performed in the dark at 35 °C and stopped after 48 h to obtain DAC of 33, 66 and 99% oxidation. The product was extracted with centrifugation in t-butyl alcohol (1:3 sample:solvent). The supernatant liquid was tested for the presence of iodine in the solvent using iodimetry. The product was resuspended in the same volume of t-butyl alcohol and the centrifugation cycle was repeated several times and dried at 35 °C to yield an offwhite product.

DSA at different levels of oxidation was prepared using a modified oxidation process with sodium metaperiodate (Balakrishnan et al 2005). Oxidation of sodium alginate using sodium periodate at 33, 66 and 99% oxidation levels were chosen for preparation of sodium alginate. The oxidation was carried out at room temperature. The reaction was stopped after 24 h by the addition of ethylene glycol (Varma and Kulkarni 2002). Sodium chloride (20 g) was then added to the reaction, the product collected by centrifugation, redissolved in distilled water and precipitated with tertiary butyl alcohol. The oxidized sodium alginate was dried at room temperature to yield a white product.

2.1.2 Hydrolysis of Oxidized Biopolymers

2.1.2.1 Chemical hydrolysis

Chemical hydrolysis of DAS was carried out with each of the acids such as 0.1% sulfuric acid, 0.1% hydrochloric acid and 0.2% oxalic acid separately. Hydrolysis was carried out by suspending DAS as 10% solution in distilled water in presence of each of the acid solution separately at 80±2 °C for 30, 45 and 60 min.
2.1.2.2 Enzymatic hydrolysis

Enzymatic hydrolysis of DAS was carried out using 0.1% α-amylase. Hydrolysis was carried out by suspending DAS as 10% solution in distilled water with the enzyme at 60±2 °C at 30, 45 and 60 min.

2.1.2.3 Thermal hydrolysis

Thermal hydrolysis of DAS was carried out by suspending DAS as 10% solution in distilled water and autoclaving at 102±2 °C, 121±2 lbs pressure for different time intervals such as 30, 45 and 60 min.

2.1.3 Isolation of Select Polyaldehyde Species from Modified Biopolymers

2.1.3.1 Preparation of the selected monomer, dimer, trimer and tetramer species of DAS

A stock solution of 1% autoclaved DAS was prepared by autoclaving ~ 1g of DAS at 30, 45 and 60 min in 100 mL of distilled water for 1h. The monomer, dimer, trimer and tetramer species were separated from other oligomers by using ion exchange chromatography on Sephadex SPC-25 column for each sample. The average MW of the all these oligomers was recorded using Gel Permeation Chromatography (GPC) and Mass Spectrophotometer (MS).

2.1.4 Characterization of Different Polyaldehydes

2.1.4.1 Degree of oxidation

Dialdehydes such as DAS, DAC and DSA at 33, 66 and 99% were prepared as given in section 2.1. The degree of oxidation of all the polyaldehydes was followed by determining the concentration of periodate
left unconsumed, by iodometry after 24 h during oxidation of DAS, DAC and DSA. The reaction mixture was neutralized with sodium bicarbonate solution and the liberated Iodine (after the addition of KI solution and leaving it in dark) was titrated with standardized sodium thiosulphate solution using starch as an indicator.

2.1.4.2 Aldehyde content

The suspension as DAS, DAC and DSA (0.1 g solid in 30 mL water), was adjusted to pH 4.5 with HCl. Hydroxylamine hydrochloride solution (0.43 g in 20 mL water, adjusted to pH 4.5) was added to the polyaldehyde suspension/solution. The mixture was stirred at room temperature for 24 h. The conversion of aldehyde to oxime was determined by the consumption of NaOH (Veelart et al 1997).

2.1.4.3 Solubility

Solubility of polyaldehydes at different oxidation levels was determined by gently dissolving different polyaldehydes such as (DAS, DAC and DSA) in 100 mL of demineralized water at 25 °C by stirring continuously for one hour. The % solubility was determined based on the mass of polyaldehydes left undissolved.

2.1.4.4 Degree of hydrolysis

Autoclaved-DAS, DAC and DSA solution (prepared from 5 g of DAS, DAC and DSA respectively) was centrifuged for 10 min. The precipitate was washed twice using distilled water and air dried (30 °C) for 24 h. Moisture content of dried autoclaved and unautoclaved DAS, DAC and DSA were calculated. The degree of hydrolysis (Kim et al 2004) was determined from the mass change before and after autoclaving.
2.1.5 Determination of Molecular Weight of Polyaldehydes

2.1.5.1 Gel permeation chromatography

Average MW (weight average) of polyaldehydes was determined using Gel Permeation Chromatography (GPC) (Jasco MX 2080-31) with a refractive Index detector. GPC analysis was carried out using aqua gel-OH column (7.5 mm IDx300 mm) with column material of 8 µm. Deionized water was used as mobile phase at a flow rate of 1 mL/min. Elution of solute was monitored by a refractive index detector. The column was calibrated with polysaccharide standards of MW 580, 5,900, 63,000, 504,000, 1,112,000 daltons. The samples were filtered through membrane filter (0.45 µm) before injection. 20 µl (~0.1% of concentration) of sample was loaded in the GPC system each time.

2.1.5.2 Mass spectroscopy

Electrospray Ionization Mass Spectra (ESI-MS) of the samples were recorded on Thermo Finnigan LCQ Advantage max ion trap spectrometry. The carrier solvent used was acetonitrile:water mixture (1:1 v/v). The capillary temperature was maintained at 250 °C and the spray voltage was kept at 5kv.

2.2 STUDIES ON THE STABILIZATION OF COLLAGEN WITH POLYALDEHYDES – MOLECULAR APPROACHES IN TANNING

2.2.1 Treatment of Rat Tail Tendon with DAS, DAC and DSA at Different pH, Concentration and Time

The RTT fibres were washed extensively in double distilled water at 4 °C and treated with 1% solution of DAS, DAC and DSA at 25 °C for 24 h at pH 8.0. The crosslinked collagen fibre samples were thoroughly washed
and aged for 24 h. Stabilization trials at different pH values viz., 4, 5, 6, 7, 8, 9 and 10; at four different concentrations viz., 5, 10, 15, 20% and at varied time intervals viz., 8, 16 and 24 h were carried. The tendons were aged for 24 h.

2.2.2 RTT Crosslinking with DAS-Monomer, DAS-Dimer, DAS-Trimer and DAS–Tetramer

The RTT fibres were washed extensively in double distilled water at 4 °C and treated with 1% solution of DAS-Monomer, DAS-Dimer, DAS-Trimer and DAS-Tetramer at 25 °C for 24 h at pH 8.0. The crosslinked collagen fibre samples were thoroughly washed and aged for 24 h.

2.2.3 Treatment of Crosslinked RTT with Urea

Samples of RTT treated with various polyaldehydes and species of DAS, described in sections 2.2.1 and 2.2.2 were treated with increasing concentrations of urea, viz., 2 M, 4 M and 6 M for 24 h. The fibres were washed with water and subjected to evaluation. A control experiment was also carried out with native RTT.

2.2.4 Determination of Thermal Stability

2.2.4.1 Hydrothermal shrinkage temperature

The hydrothermal stability of all tanned RTT were determined using a micro-shrinkage tester. The temperature at which the collagenous fibre shrinks to one third of its original length was noted as the shrinkage temperature of the fibre. The shrinkage temperature is also termed as hydrothermal stability as the measurement is carried out in the presence of water. A small strip of fibre was cut and placed on a grooved microscopic
slide along with water. The slide in turn was placed on a heating stage along with a microscope mounted above the heating stage. The rate of heating was maintained at 2 °C/min.

**2.2.4.2 Differential scanning colorimeter**

RTT (native and polyaldehyde tanned, 5 mg at around 60% moisture content) were analyzed using Netzsch DSC 200 PC differential scanning calorimeter. All measurements were conducted under nitrogen atmosphere. The temperature was calibrated effectively using indium as standard. Temperature employed for the measurements was generally in the range of 25 – 250 °C. Heat flux was measured to a sensitivity of 0.01mWcm⁻¹. The heating rate was maintained constant at 5 °C/min. The fibres were encapsulated in an aluminium hermetic sealed pan. Transition temperatures and enthalpy changes were recorded. The peak temperature $T_D$ (in °C) and the enthalpy changes $\Delta H$ (in J/g of wet weight), rate of shrinkage ($k_o$) and the activation energy ($E_a$) associated with the phase change for the shrinkage process for native and polyaldehyde treated fibres were studied (Gustavson 1956).

**2.2.5 Preparation and Purification of Soluble Collagen**

Tails were excised and frozen at −20 °C from 6-month-old male albino rats (Wistar strain) that are ideal collagen substrate for crosslinking studies due to its high purity, available lysine residues and collagen content. On removal from the freezer, tails were thawed and tendons were teased out. Teased collagen fibres were washed with 0.9% NaCl at 4 °C, to remove the adhering soluble proteins. RTT were washed extensively in deionized water and stored at −20 °C and used as collagen fibres. Acid soluble RTT type I collagen solution was also isolated according to the method described by
Chandrakasan (Chandrakasan et al 1976). The procedure included acetic acid extraction and salting out with NaCl. The collagen concentration in the solution was determined from the hydroxyproline content according to the method of Woessner (Ryan and Woessner 1971).

2.2.6 Determination of Crosslinking Efficiency

Crosslinking efficiency of DAS, DAC, DSA and DAS-Monomer, DAS-Dimer, DAS-Trimer and DAS-Tetramer in stabilization of collagen was determined by 2,4,6–trinitrobenzenesulfonic acid (TNBS) assay that determines the ε-amino groups in collagen (Bubnis and Offner 1992). Crosslinking efficiency was determined by measuring the available lysine and comparing lysine content before and after crosslinking using TNBS. The unreacted ε-amino groups of lysine in native and polyaldehyde crosslinked collagen solution reacts with TNBS to form a soluble complex. Degree of crosslinking of native collagen (non-crosslinked) and polyaldehyde treated collagen (crosslinked) were determined by TNBS assay (Chandrakasan et al 1976). Collagen solution (1 µM concentration) was crosslinked with varying concentrations of DSA (0-2%) at 30 °C for 2 h. To this solution of crosslinked collagen, 1 mL of 4% (w/v) sodium bicarbonate solution and 1 mL of freshly prepared 0.5% (v/v) TNBS solution in deionized water was added and treated at 60°C for 4 h. 1 mL of this solution was treated with 3 mL of 6 M HCl at 40°C for 1.5 h and the absorbance was measured at 334 nm after dilution. The native collagen (1 µM) (non-crosslinked) was also treated with TNBS in a similar manner. The degree of crosslinking was calculated as follows

\[
\% \text{ Crosslinking} = 1 - \left( \frac{ \text{Absorbance of polyaldehyde - crosslinked collagen} }{ \text{Absorbance of native collagen} } \right) \times 100
\]
2.2.7 Measurement of Crosslinking Density

2.2.7.1 Preparation of collagen membranes

The purified collagen solution was poured on polythene trays at room temperature to form gels. The collagen gel formed was washed extensively with distilled water to remove buffer salts and then air-dried. The thickness of the membranes as measured using a thickness gauge varied from 60 to 80 µm.

2.2.7.2 Crosslinking conditions

The dried collagen membranes were swollen in (phosphate buffer solution) PBS (pH 7.4) at room temperature before crosslinking. The collagen membranes were crosslinked by treating with DAS, DAC, DSA and DAS-Monomer, DAS-Dimer, DAS-Trimer and DAS-Tetramer at pH 8 for 24 h. After crosslinking for a given period of time, the membranes were washed extensively with distilled water to remove traces of crosslinking agents. The washed membranes were then air-dried.

2.2.7.3 Determination of number of covalent crosslinks between polypeptide chains

The crosslinking density measurements for the collagen samples were carried out as described earlier (Flory and Rehner 1943). Flory and Rehner’s equation, which defines the force – extension relationship for a swollen elastomer as given in Equation (2.1) is used to determine the mean molecular weight per crosslinked chain segment denoted by $M_c$ and the number of crosslink’s per $10^5$ g of collagen calculated as $10^5/2M_c$. After conversion of $M_c$ to moles of crosslink’s per given mass of material, the number of chain segments which meet in each crosslink was calculated on the basis of four chain segments.
\[ f = \frac{RTN_e}{V_o} V_2^{1/3} (\alpha - \alpha^{-2}) \]  \hspace{1cm} (2.1)

where, \( f \) = force per unit cross-sectional area of the swollen unstretched sample

\( R \) = Gas constant

\( T \) = Absolute temperature

\( N_e \) = Number of elastically effective chain segments

\( V_o \) = Volume of network before swelling

\( V_2 \) = Volume fraction i.e. Volume of dry sample/volume of wet sample

\( \alpha \) = Extension ratio

In an amorphous network four chain segments commonly meet in each crosslink and the number of crosslinks exerting stabilising influences on such a network will equal half the number of elastic chain segments. For a large network Flory has deduced a relationship between the effective number \( (N_e) \) and the actual number \( (N) \) of chain segments that are crosslinked at both sides

\[ N_e = N(1 - \frac{2M_c}{M}) \]  \hspace{1cm} (2.2)

where \( M_c \) = the mean molecular weight per crosslinked chain segment and

\( M \) = the mean molecular weight before crosslinking

As reported by Flory and cater 1965 (Flory and cater 1965), considering smaller fractions of amorphous collagen 2M/M, \( N_e \) equals N in native and crosslinked collagen as reported earlier. When \( N_e = N \), \( N_e/V_o \) in
Equation (2.1) may be replaced by $\rho/M_c$, where $\rho$ is 1.3, the density of dry collagen, then

$$\frac{N_w}{V_o} = \frac{\rho}{M_c} \quad (2.3)$$

$$f = \frac{RT\rho}{M_c} V_2^{1/3} (\alpha - \alpha^{-2}) \quad (2.4)$$

Briefly, collagen membranes (10cmx5mm) were denatured by placing them in water at 98 °C,±2 °C for 30 sec in case of control and 120 sec for crosslinked samples. After an appropriate period (30 sec or 120 sec), the membranes were rapidly transferred into cold water. After equilibrating for 2 h in water at the temperature of testing, the membranes were mounted between two clamps. The stress–strain cycle of each membrane was determined. Then the samples were removed from the clamps and blotted with a filter paper, and their densities were determined by the specific gravity bottle method. From the volume and unstressed length, the cross-sectional area was calculated. The dry weights were obtained by acetone dehydration of the samples followed by drying at 60 °C for 1 h. Volume fraction ($V_2$) was calculated from the dry and wet weight as given in Equation (2.6). The function ($\alpha-\alpha^{-2}$), where ‘$\alpha$’ the extension ratio, was calculated from the unstressed length and known extension, and plotted against (f), the force per unit cross-sectional area. Providing that

$$\text{Gradient} = \frac{RT\rho}{M_c} V_2^{1/3} \quad (2.5)$$

$$V_2 = \frac{W_o \varphi}{W \rho - W_o (\rho - \varphi)} \quad (2.6)$$

$W_o = \text{Weight of dry sample}$
\[ \rho = \text{density of dry collagen} \]
\[ W = \text{Weight of wet sample} \]
\[ \Phi = \text{density of swelling medium} \]

Flory-Rehner equation holds good for hydrothermally denatured collagen, a graph of \( f \) against \((\alpha - \alpha^2)\) will be a straight line passing through the origin. From the gradient, \( M_c \) was calculated and the number of crosslinks (gram-molar) in unit mass was expressed by \((2 M_c)^{-1}\).

### 2.2.7.4 Potentiometric titration

The amount of proton binding (amino) groups was measured for each membrane using potentiometric titration. In this method, 50 mL of HCl solution (0.02 mol/L) is added, in excess, to a solution containing approximately 0.1 g of natural or crosslinked polyaldehyde films, allowing enough time (24 h) to charge all proton binding groups. Subsequently, the resulting solution is then titrated using a solution of NaOH (0.01 mol/L). An interval of 2 min was allowed before adding more NaOH. This experiment was not conducted under nitrogen atmosphere. A titration curve is obtained and the peaks of its first derivate curve indicate the amount of amino groups. The percentage amino group is calculated by Equation (2.7).

\[
\% \text{NH}_2 = \frac{M_{\text{NaOH}} (V_2 - V_1) \times 161}{W_2 \times 100}
\]

(2.7)

where, \( M_{\text{NaOH}} \) is the molarity of the NaOH solution (mol/L), \( V_1 \) and \( V_2 \) are, respectively, the volume (L) of NaOH used to neutralize the excess of HCl and the volume (L) of the protonated polyaldehyde sample, 161, is the molecular weight of the monomeric unit of polyaldehydes and \( W_2 \) is the mass (g) of the sample in the dry state before titration.
2.2.7.5 Measurement of hydrothermal isometric tension

The hydrothermal isometric tension experiments were carried out using an adaptation of the Instron tensile tester. During testing, a pen connected to the output of the load cell, recorded the load applied on a chart moving at a constant speed, thus generating a load elongation graph. The membranes were tested in PBS. The thermal properties were measured by the force–temperature method of Rigby (Rigby 1971). Collagen membranes of size 2x0.3 cm were used for these experiments.

2.2.7.6 Measurement of stress–strain characteristics

Mechanical properties of collagen membranes were studied using an instron tensile tester and appropriate load cell in the range 0.1–500 g. The sensitivity of the load cell was 2% at the maximum range. Tensile testing was performed at an extension rate of 0.5 cm/min, which corresponded to a strain rate of 50%/min. Membranes were equilibrated in PBS before they were tested. The thickness of the membranes was measured at different places using a thickness gauge after equilibrating in the testing solution. The average thickness was calculated and this value was used for determining the area of cross-section of the membranes. Tensile strength was calculated from the breaking load and area of cross-section. The percentage extension was calculated from the ratio of increase in length to original length.

2.2.8 Structural Investigations

2.2.8.1 Structural changes on polyaldehyde-collagen using circular dichroism

Circular dichroism spectrum of Type I collagen of concentration of about 0.06 µM at pH 4.0 acetate buffer of concentration 1x10^{-6} M was obtained at 25 °C using Jasco-J715 spectropolarimeter. The rate of nitrogen
purging was maintained at 5 L/min up to 200 nm and increased to 10 L/min below 200 nm. A 2-point calibration was done with (+) 10 camphor sulfonic acid. The samples were prepared in double distilled water. All the solutions were filtered through 0.25 µm filters to remove suspended particles. 0.1 cm cell was used for the experiments. A slit width of 1 nm was used. The scan speed 20 nm/min was used with an average of 5 scans per sample. Each spectrum was corrected by a baseline measured with the same solvent used in the sample. A reference spectrum was recorded with acetate buffer. The conformational changes in collagen on interaction with polyaldehydes were investigated after incubating the protein with varying concentrations (0.6 µM – 120 µM) of polyaldehyde. The spectra obtained were deconvoluted using G and F and K2D programs and the mean values of secondary structure components were tabulated.

2.2.8.2 Structural changes on collagenase – polyaldehyde using circular dichroism

Circular dichroism spectrum of Type IA collagenase of concentration (0.2 mg/mL) in pH 4.0 acetate buffer of concentration of about 1 µM was acquired at 25 °C. The conformational changes in collagenase on interaction with polyaldehyde were investigated after incubating the enzyme with varying concentrations (0.6-90 µM) of polyaldehyde species. The spectra obtained were deconvoluted using G and F and K2D programs and the mean values of secondary structure components were tabulated.

2.2.8.3 Polyaldehyde binding:AFM imaging of reconstituted collagen in the presence and absence of different polyaldehydes

Substrate consisted of freshly cleft mica fixed to 15 mm steel disks with an adhesive. For fibrillogenesis, collagen solution (0.5 mg/mL) was mixed gently with DAS, DAC, DSA and DAS-Monomer, DAS-Dimer,
DAS-Trimer and DAS-Tetramer, to give a final concentration of 0.01 M of the polyaldehyde. After incubation for 30 min, the substrate was placed over the sample, removed after 30 min, washed with water, drained and dried.

Images were captured using a Nano scope II AFM (Digital instruments, Santa Barbara, CA with a ‘J’ scale scanner (horizontal ranges of 1 mm). The instrument was used on contact mode with scan rate varying from 0.1 to 150 Hz. The range of bias voltage is maintained as +10 Volts with 16 bit resolutions. The X and Y voltage were in software selectable range of +220 V or +12 V with the Z voltage of +220 V or +55 V select. The tunneling current range was +50 nA. RTT were fixed to the stainless steel disk using an adhesive. The mica substrates were also glued to the discs. The discs with the sample (either RTT or collagen adsorbed on mica) were then mounted on the sample holder. Examinations were made on a 1µm piezoelectric scanner in air in the contact mode. The tip of the cantilever was adjusted to touch the sample, monitored using the laser spot. The scanned images were stored and analyzed.

2.2.9 Steady State Kinetic Studies on Collagenase Hydrolysis

2.2.9.1 Collagenase assay

The enzymatic degradation of native and DAS, DAC, DSA and DAS-Monomer, DAS-Dimer, DAS-Trimer and DAS-Tetramer stabilized RTT by bacterial collagenase (Type IA) from Clostridium histolyticum was analyzed by estimating the amount of hydroxyproline released in the solution after hydrolysis. Collagenase treatment was carried out in 0.04 M CaCl₂ solution buffered at pH 7.2 with 0.05 M tris HCl. The collagen:enzyme ratio was maintained at 50:1. The cleavage of native and stabilized collagen fibres was monitored by the release of soluble hydroxyproline from insoluble collagen. The % hydroxyproline was determined using the method of
Woessner (Ryan and Woessner 1971). This method of determining hydroxyproline involves the oxidation of hydroxyproline to pyrrole-2-carboxylic acid, which complexes with p-dimethylaminobenzaldehyde exhibiting maximum absorbance at 557 nm.

\[
% \text{Collagen degradation} = 100 - \left[ \frac{\text{Initial collagen} - \text{Soluble collagen}}{\text{Initial collagen}} \right] \times 100
\]

2.2.9.2 Analysis of native collagenase

Assay of collagenase using FALGPA as substrate was performed according to the method of Van Wart and Steinbrink (Van Wart and Steinbrink 1981). The reaction was monitored by following the hydrolysis of FALGPA. Typical experiments contained incubation of FALGPA (at concentrations of 0.02-1.5 mM), collagenase (10 µl of 15 mg/mL), and an appropriate amount of Tricine buffer (0.05 M Tricine, 0.4 M NaCl and 10 mM CaCl\(_2\), pH 7.5) to afford a final volume of 1 mL. The course of hydrolysis was monitored in a Shimadzu UV-Visible spectrophotometer by following the decrease in absorbance at 324 nm when [FALGPA] = 0.02-0.19 mM. When higher concentrations of FALGPA were used viz. (0.19 - 0.25 mM) and (0.25-2.0 mM), the decrease in absorbance was measured at 338 and 345 nm, respectively. The reaction was monitored until no further changes in absorbance were observed. An initial rate treatment was adopted by treating the first 10% of hydrolysis according to standard methods (Espenson 1995).

2.2.9.3 Kinetic investigations on the inhibition of collagenase by polyaldehydes

The reaction of polyaldehyde treated collagenase with FALGPA was performed under the same conditions mentioned as those employed for the assay of native collagenase in this study. The collagenase is treated with
varying concentrations viz., 0, 20, 40, 80 and 160 µM of aqueous solution of polyaldehyde for 24 h at 25 °C. The final concentrations of the collagenase in all the treatments are maintained constant (0.4 mg/mL). The FALGPA at concentrations of 0.2–1.6 mM) was taken in appropriate amount of Tricine buffer (0.05 M Tricine, 0.4 M NaCl and 10 mM CaCl$_2$, pH 7.5) and polyaldehyde incubated collagenase (100 µl) was added and the final volume was adjusted to 1 mL. The hydrolysis of substrate was monitored at the corresponding wavelengths (immediately after the addition of polyaldehyde incubated collagenase) as done in the case of native collagenase. The concentrations of substrate (FALGPA) used were in the range of 0.2 – 1.6 mM. Rates of hydrolysis were calculated employing initial rate methods. The rate data were analyzed in terms of Michaelis–Menton treatment. From the Linewaver-Burk plots of $v^{-1}$ vs $[S]^{-1}$ the kinetic parameters such as $V_{\text{max}}$, maximum velocity and $K_m$, the Michaelis-Menton constant of the enzyme were calculated. Initial velocities were calculated from the slope of the absorbance vs time plot during the first 10% of hydrolysis and converted into units of microkatal (µmol/s).

2.3 STUDIES ON BIOCOMPATABILITY OF POLYALDEHYDE COLLAGEN MEMBRANE

2.3.1 Preparation of Bovine Skin Collagen Solution

Collagen from bovine skin was prepared and purified by the modified method of piez (piez et al 1974). The skins were removed using scalpel blade, cut into relatively thin pieces and washed well overnight in 0.5 M sodium acetate while ensuring constant stirring on a magnetic stirrer in cold. Washing with sodium acetate removes impurities and adhering non-collagenous components. All the operations were carried out at 4 °C. After thorough washing, the pieces were homogenized in 0.5 M acetic acid
for 24 h. After complete homogenization, the material was precipitated with 5% sodium chloride and centrifuged in a refrigerated centrifuge. The supernatant liquid was discarded and residue was redissolved in 0.5 M acetic acid and treated with pepsin in the ratio of 1:100 for 18-24 h. After the enzyme treatment, the collagen was precipitated with 5% sodium chloride and centrifuged in a refrigerated centrifuge. The supernatant liquid was discarded and residue was redissolved in 0.5 M acetic acid and dialyzed extensively against 0.05 M acetic acid and solution was stored at 4 °C.

2.3.2 Crosslinking Conditions for Biocompatibility Studies

The dried reconstituted collagen membranes were swollen in PBS (pH 7.4) at room temperature before crosslinking. The collagen membranes were crosslinked by treating them with different reagents at their respective optimal conditions. After crosslinking for the described period of time, the membranes were washed extensively with distilled water to remove traces of crosslinking agents. The washed membranes were then air-dried.

2.3.3 Degree of Crosslinking

The crosslinking efficiency of polyaldehydes (DAS, DSA and DAC) with collagen, at varied ratio (1:0.2, 1:0.4, 1:0.6, 1:0.8 and 1:1) was carried out as given in section 2.2.6.

2.3.4 Swelling Properties

Collagen membranes (2x2 cm) were swollen in water and then equilibrated overnight in PBS (pH 7.4) at room temperature (Nakatsu and Andrady 1992). The membranes were removed and quickly blotted with filter paper to remove excess surface water and weighed immediately. The
membranes were then placed in a large volume of deionized water to remove the buffer salts and air-dried to constant weight. The swelling ratio, Q was calculated as the ratio of the weight of swollen sample to that of dry sample.

2.3.5 Gelling Time

Polyaldehydes (1 mL) in 0.1 M borax (pH 9.4) in PBS was reacted with 1 mL aqueous solution of collagen in glass vials of 15 mL capacity (diameter 20 mm) under magnetic stirring using a teflon-coated stir bar (diameter 5 mm, length 10 mm) at 37 °C. Values reported are average of 4–5 determinations. Gelling time was studied by varying the concentration of polyaldehydes, collagen and borax.

2.3.6 Dispersion Temperature

The dispersion temperature of the reconstituted collagen fibers was determined as described by (Bello and Bello 1967). Fibril formation was initiated at 37 °C. After the rigid gel formation was completed, the temperature of the water bath was raised at a rate of approximately 1 °C/5 min and the disappearance of the opacity of the fibrils was noted. The dissolution temperature was taken as that temperature at which the collagen gel was completely dissolved. (Purna Sai et al 1995)

2.3.7 Keratinocyte Culture

The keratinocytes were cultured from the skin grafts as described by Purna et al 1998. The cell number was counted in a hemocytometer and uniform numbers of cells (40,000) were seeded onto the collagen-coated 6-well plates for further studies.
2.3.8 MTT Assay

Cytotoxicity of collagen membranes were quantitatively assessed by MTT assay (Ciapetti et al 1993), which measures the metabolic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyl tetrazolium bromide to a coloured formazan by viable cells. Toxicity was evaluated on the extract of the material (0.75 cm$^2$/mL) in medium containing serum as before. MTT dissolved at a concentration of 5 mg/mL in sterile PBS, filtered through a 0.22 mm filter to remove any formazan crystals and stored at 20 °C was used as working solution. Keratinocyte cells were cultured as before in multiwall tissue culture plates and when monolayer was attained, culture medium was removed, rinsed with PBS and 100 mL each of extracts of gel and negative control (high-density polyethylene) and 100 µL of diluted phenol (positive control) were added to different prelabelled wells containing cells. Cells with medium alone served as control. Culture medium (100 mL) was used as reagent blank. Plates were incubated for 24 at 37 °C in 5% carbon dioxide atmosphere. After 24 h, the extracts/ medium were removed and 200 µL of MTT working solution was introduced using a multi-channel pipette into each well. Plates were wrapped with aluminium foil and incubated at 95% humidified atmosphere at 37°C for 8 h. After removing the reagent solution and rinsing with PBS, 200 µL of isopropanol was added to each well and incubated for 20 min at 37°C in a shaker incubator (Labline Instruments, Melrose Park, USA). The absorbance of the resulting solution in each well was recorded immediately at 570 nm using automated micro plate reader (Bio–Tek Instruments, Vermont, USA). Reported values are mean of three replicates.
2.4 STUDIES ON THE TANNING PROCESS FOR BIODEGRADABLE LEATHERS

2.4.1 Tanning Trials Using DAS, DAC and DSA - Effect of Various Conditions for Tanning

Various experimental trials as given in section 2.4.1.1 to 2.4.1.4 mentioned below were carried out at different conditions.

2.4.1.1 Effect of different oxidation levels of DAS, DAC and DSA

Tanning trials at 33, 66 and 99% oxidation levels was carried out as described in the process mentioned in Table 2.1. The leathers were aged for 24 h at room temperature and taken for shrinkage temperature measurement, % aldehyde fixed and enzymatic degradation

2.4.1.2 Effect of concentration

DAS, DAC and DSA at four concentrations viz., 5, 10, 15 and 20% were used for tanning the pelts as described in the process mentioned in Table 2.1. The leathers were aged for 24 h at room temperature and taken for shrinkage temperature measurement, determination of % aldehyde fixed and enzymatic degradation

2.4.1.3 Effect of time

Tanning trials on pickled goatskins were carried out as per the process mentioned in Table 2.1. The shrinkage temperature was measured at different time intervals viz., 4, 12, 24, 48 and 72 h during tanning with DAS, DAC and DSA at 10% offer and taken for % aldehyde fixed and collagenase hydrolysis.
2.4.1.4  Effect of pH

Tanning trials at different pH values viz., 4, 5, 6, 7, 8, 9 and 10 were carried out as described in the process mentioned in Table 2.1. The leathers were aged for 24 h at room temperature (~30 °C) and taken for shrinkage temperature measurement, % aldehyde fixed and enzymatic degradation.

2.4.2  Optimisation of Tanning condition – Influence of Fat Liquor

Five pickled goatskins were tanned using DAS, DSA and DAC (10% tanning agent; pH 8; temperature 30 °C; tanning duration 24 h) following the process mentioned in Table 2.1. The tanned leathers were further fatliquored with 4% neats foot oil based fatliquor (% based on pelt weight) in a fresh float of 100% water and finally fixed with 1% formic acid (diluted with 10% water and offered in three feeds) after tanning in the same drum.

Table 2.1  Tanning Process Using DAS, DAC and DSA from Pickled Goat Skins

<table>
<thead>
<tr>
<th>Process</th>
<th>Chemicals</th>
<th>%</th>
<th>Duration</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH adjustment</td>
<td>Pickle liquor (Sodium bicarbonate (1:1 diluted (1:10))</td>
<td>50</td>
<td>3x15min +30 min</td>
<td>Adjust to required pH</td>
</tr>
<tr>
<td>Tanning</td>
<td>Water DAS/DAC/DSA</td>
<td>50 X</td>
<td>24th h.</td>
<td>Maintain required pH^</td>
</tr>
<tr>
<td></td>
<td>Formic acid</td>
<td>1</td>
<td>3x10 min +30 min</td>
<td>pH adjusted to 4; Drain; aged for 24 h; sammed; shaved to thickness 1.0-1.1 mm</td>
</tr>
</tbody>
</table>

a - Tanning with DAS, DAC and DSA at different oxidation levels viz., 33, 66 and 99%
b - Tanning carried out 5, 10, 15 and 20% at constant pH of 8 for 24 h
c - Tanning carried out at different pH viz., 6, 7, 8 and 9 at 10% DAS, DAC and DSA for 24 h
d - Tanning trials at varying time intervals viz., 4, 8, 16, 24, 48 and 72 h, at 10% DAS, DAC and DSA at pH 8
2.4.3 Comparison of DAS, DAC and DSA Tanned Leathers with Control Leathers

Matched pair comparison of experimental and control processing were carried out using pickled goatskins. Right halves of pickled goatskins were tanned using DAS, DAC and DSA (10% polyaldehyde; pH 8; temperature 30 °C; tanning duration 8 h) following the process mentioned in Table 2.1. The tanned leathers were then fatliquored using 4% neats foot oil based fatliquor. Corresponding halves of the pickled goatskins was processed using conventional control chrome tanning process. All the experimental and control tanned leathers were post tanned with 16% retanning, 12% fatliquor, 4% dye using the process mentioned in Table 2.2. The resultant crust leathers were analyzed for organoleptic properties.

2.4.4 Shrinkage Temperature Measurements

The shrinkage temperature of tanned leathers, which is a measure of a hydrothermal stability of leather, was measured using a Theis shrinkage meter (McLaughlin and Theis 1945). The values reported are an average of three measurements for each experiment.

2.4.5 Estimation of Aldehyde Fixed to Collagen

The spent tanning solution after tanning with DAS, DSA and DAC as mentioned in Table 2.1 was collected and the uptake of these aldehydes was determined using the method described by Wise and Mehltetter (Wise and Mehltetter 1958). The amount of aldehyde fixed to collagen after tanning calculated on protein weight was also determined (Nayudamma et al 1961). The leathers after ageing for 24 h were taken for the determination of moisture and ash content. The total nitrogen content was measured by Kjeldahl’s method (Lenore et al 1989).
2.4.6 Scanning Electron Microscopic (SEM) Measurements

Samples from experimental crust leathers made from DAS, DSA and DAC tanning (10% polyaldehydes; pH 8; temperature 30 °C; tanning duration 24 h) followed by post tanning were cut into specimens from official sampling position (Lenore et al 1989). A Quanta 200 series scanning electron microscope was used for the analysis. The micrographs for the grain surface and cross-section were obtained by operating the SEM at an accelerating voltage of 20 KV at different magnifications.

Table 2.2 Post Tanning Process for Leathers Tanned using DAS, DAC and DSA at Different Oxidation and Chrome Tanning Trials

<table>
<thead>
<tr>
<th>Process</th>
<th>Chemicals</th>
<th>%</th>
<th>Duration (min)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wetting</td>
<td>Water</td>
<td>150</td>
<td>0.1</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Wetting agent</td>
<td>150</td>
<td>0.1</td>
<td>60</td>
</tr>
<tr>
<td>Neutralisation</td>
<td>Water</td>
<td>150</td>
<td>0.5</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Sodium bicarbonate</td>
<td>150</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Neutralizing syntan</td>
<td>150</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>Washing</td>
<td>Water</td>
<td>200</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>Retanning, dyeing and fatliquoring</td>
<td>Water</td>
<td>150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenolic syntan</td>
<td>Grain tightening syntan</td>
<td>2</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Melamine syntan</td>
<td></td>
<td>4</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Acrylic syntan</td>
<td></td>
<td>4</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Cationic fatliquor</td>
<td></td>
<td>4</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Acid dye</td>
<td></td>
<td>2</td>
<td>30</td>
<td>Check penetration</td>
</tr>
<tr>
<td>Synthetic fatliquor</td>
<td></td>
<td>6</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Basic dye</td>
<td></td>
<td>1</td>
<td>45</td>
<td>Check exhaustion</td>
</tr>
<tr>
<td>Cationic fatliquor</td>
<td></td>
<td>2</td>
<td>45</td>
<td>Check exhaustion</td>
</tr>
<tr>
<td>Melamine syntan</td>
<td></td>
<td>2</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Fixing</td>
<td>Formic acid</td>
<td>1</td>
<td>3x10+30</td>
<td>Drain</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Washing</td>
<td>Water</td>
<td>100</td>
<td></td>
<td>Drain, piled O/N, set, dry, stake, trim and buff</td>
</tr>
</tbody>
</table>
2.4.7 Collagenase Hydrolysis of the Polyaldehyde Tanned Leather

The enzymatic degradation of delimed skin and DAS, DAC and DSA-treated leather by collagenase was analyzed by estimating the amount of hydroxyproline released in the solution after hydrolysis as described in 2.2.9.1.

2.4.8 Physical Testing Analysis

The leather samples made from matched pair control and experimental processes were taken for physical testing measurements and the samples were cut from the official sampling position (IUP 2 2000). The leather samples were conditioned at 80±4 °C and 65±4% R.H. for 48 h. The tensile strength, elongation at break, tear strength and grain crack strength were measured as per IUP 6, IUP 8, and IUP 9 methods (IUP 6 2000; IUP 8 2000; IUP 9 1996). Four samples were used for each measurement.

2.4.9 Measurement of Softness

The leathers made from matched pair control and experimental processes were taken for softness measurements and the samples (three) were cut from the official sampling position (IUP 2 2000). The leather samples were conditioned at 20±2 °C and 65±4% R.H. for 48 h. The softness of the leathers was measured using ST 300D leather softness tester as per IUP 36 (IUP 36 2000) method. The softness tester measures the deflection of leather by a fixed diameter plunger (20 mm) when a force (500 g) is applied.

2.4.10 Determination of Color Difference

The control and experimental leathers made in this study were subjected to the reflectance measurements using a Milton Roy Color mate HDS instrument. Color measurement (L, a, b, h and C) were recorded and the total color difference (ΔE) and hue difference (ΔH) were calculated.
2.4.11 Organoleptic Properties of Tanned Leathers

The matched pair control and experimental crust leathers were assessed for fullness, grain smoothness, softness and general appearance by hand evaluation technique. The functional properties of the leathers in a scale of 0-10 points were rated by three experienced tanners and the average values reported. Higher values indicate better property.

2.5 STUDIES ON THE BIODEGRADABILITY OF POLYALDEHYDE TANNED LEATHERS

2.5.1 Preparation of Raw Skin Collagen for Degradation

Fresh, flayed Goatskins (20 numbers) from slaughter house (Perambur, Chennai) was pretreated with 1% sodium sulphide and 10% calcium hydroxide in the leather processing drum rotating at 6 rpm with a float of 100% water for 12 h to dissolve hair and loosen the flesh layer for the removal of hair and fat. The hair and flesh were removed mechanically using unhairing and fleshing machine. The skins were further treated with 1% ammonium chloride in a fresh float of 100% water to remove all the calcium hydroxide present in collagen. Finally, the pH of collagen was maintained at 7 at the cross-section.

2.5.2 Putrefaction of Raw, Tanned and Finished Leathers – Identification of Skin/Leather Degrading Micro Flora

Degradation of raw skin (~ 10 g) by micro flora was carried out by degrading in 250 mL of nutrient broth (13 g in 1000 mL) and leaving it in an open environmental condition (28-38 °C). Tanned and finished leathers were cut into 10x10 cm sized pieces and placed in a moist tray at 28-38 °C and 65-75% RH for the development of fungal spores for a period of 15-30 days.
2.5.3 Isolation of Microorganisms

Soil samples from the leather dump yard were collected and inoculated into minimal medium and incubated at room temperature (30 °C). After a period of one week, the sample was taken and streaked on nutrient agar plate and PDA plate. The plates were sealed with Paraffin and incubated at 37 °C for 24 h. Subsequently after incubation, morphologically different colonies were isolated and sub cultured in nutrient broth. Stock culture (1 mL) of natural biodegradation samples of raw skin and different tanned and finished leathers, as described in 2.5.2 was also taken and the above procedure was repeated. The putrefactive bacterial species identified from soil samples of leather dump yard and degrading micro flora of raw skin/leathers were subjected to biochemical analysis to determine the genus of the species. The isolated organisms were further subjected to gram staining in accordance with the procedures of Bergy’s manual. The isolation, identification and biochemical characterization was carried out for three different samples for accuracy of results. The bacterial and fungal species that were predominant in all the batches were taken for further experiments.

2.5.4 Identification of Microorganisms

2.5.4.1 Gram staining

Smear was prepared. Drop of crystal violet was added and allowed to stand for 30 sec. The smear was washed with running tap water. Drop of Gram’s iodine was added and allowed to stand for 30 sec and then washed with water. 95% ethanol was added and again allowed to stand for 45 sec and then washed with water. Saffranin was added and allowed to sand for 3 sec and dried. Finally, the slide was blotted with filter paper, dried and examined under electron microscope.
2.5.4.2 Biochemical test

The identification of isolated microbial species were carried out by performing various biochemical tests such as Indole test, Methyl red test, Voges proskauer test, Citrate utilisation test, Urease test, Triple sugar ion test, Lacto phenol cotton blue, Catalase test and Oxidase test.

2.5.4.3 Indole test

Tryptone broth was prepared and then culture was inoculated into test tubes. Tubes were incubated for 24-48 h at 37 °C. After incubation 1 mL of Kovac’s reagents was added.

2.5.4.4 Methyl red test

MR-VP broth was prepared and then culture was inoculated into test tubes. Tubes were incubated for 24-48 h at 37 °C. After incubation 5 drops of MR reagent was added.

2.5.4.5 Voges proskauer test

MR-VP broth was prepared and the culture was inoculated into the test tubes. The test tubes were incubated for 24-48 h at 37 °C. After incubation, 5 drops of MR reagent was added. Barrit’s reagent A and B were further added to the test tubes. The mixture was allowed to stand for 15 min.

2.5.4.6 Citrate utilisation test

Simmons’s citrate agar medium was prepared, sterilized and was dispensed into test tubes and allowed to form solid in slanting position. Culture was streaked on the slant. Test tubes were then incubated for 18-24 h at 37 °C.
**2.5.4.7 Urease test**

Christenson’s urea agar medium was prepared, sterilized and dispensed into sterile test tubes and slants were prepared. The slant was inoculated with a drop of 4-6 h growth bacterium in broth and was incubated at 30-37 °C for 18-24 h or longer.

**2.5.4.8 Triple sugar ion test**

A colony from the pure growth was picked up with a sterile straight wire and inoculated by stabbing down the center of agar butt carefully. The inoculating wire was withdrawn carefully and the surface of the slant was streaked and incubated for 18-24 h at 37 °C.

**2.5.4.9 Lacto phenol cotton blue**

A drop of stain was placed on glass slide. Fungal spore was placed over the stain and teased using a teasing needle. The mount was covered using cover slip. The slide was viewed under microscope for confirmation of stained fungus.

**2.5.4.10 Catalase test**

Pure growth of the organism was transferred from the agar to a clean slide with a loop or a glass rod. A drop of 3% hydrogen peroxide solution was added to the growth and the release of the bubbles was observed.

**2.5.4.11 Oxidase test**

With the help of a clean glass rod or loop a colony from test organism grown for 24 h was picked. The control was placed over the oxidase disc. The oxidizing property of organism was observed by change in colour from white to blue or purple within 10 sec.
2.5.5  Biodegradation Methods

2.5.5.1  Aerobic biodegradation

The shaking-flask method for primary aerobic biodegradability determination was carried out according to the International Organization for standardization (ISO) method. Biodegradation cultures (0.5 L) were prepared in 1 L shaking flasks. The cultures were prepared in an aqueous medium with a pH of about 7.4 that contained the following minerals and micronutrients to support bacterial activity: ammonium chloride (NH_4Cl, 3.0 g/L), potassium hydrogen phosphate (K_2HPO_4, 1.0 g/L), magnesium sulfate (MgSO_4, 0.25 g/L), potassium chloride (KCl, 0.25 g/L), ferrous sulfate (FeSO_4, 0.002 g/L), and yeast extract (0.3 g/L). The test compounds were added to the culture medium to give a concentration of 30 mg/L. Each medium (0.5 L) was inoculated with 5 mL of activated sludge having 15 g/L suspended solids content. The cultures were placed on a shaker table in a temperature controlled cabinet and shaken continuously in the dark at about 200 rpm. The temperature of the cabinet was maintained at approximately 25 °C. 2 mL of the aliquot was drawn at 96 h intervals and the degradation of collagen and different leathers were analyzed by estimating the amount of hydroxyproline released in the solution after hydrolysis of the leather sample by aerobes.

2.5.5.2  Anaerobic biodegradation

Anaerobically digested sludge from the wastewater treatment plant employed for the treatment of tannery wastewater, having active anaerobic environment was used for anaerobic biodegradation. The colony forming units/g of sludge in wet condition was determined for identification of anaerobes. The isolates were sub cultured in the fluid thioglycolate medium. After 96 hrs, each medium (0.5 L) was inoculated with 10 mL of the culture broth (total organic solid content of the seed culture was 1-2%) and dispensed
individually under anaerobic condition to the serum bottles with pre weighed test samples. 2 mL of the aliquot was drawn at 72 h intervals and the degradation of leather was analyzed by estimating the amount of hydroxyproline released in the solution after hydrolysis of the leather sample by anaerobes.

2.5.6 Measurement of Proteolytic Activity

The identified bacterial isolates and fungi were plated onto skim milk agar plates and were incubated at 37 °C for 24 h. A clear zone of skim hydrolysis gives an indication of protease producing organisms.

2.5.7 Measurement of Collagenolytic Activity

2.5.7.1 Gelatinase test

The gelatin agar slants were prepared and inoculated with the microorganism. The slants were incubated at 37 °C for 24 h. The slants were refrigerated for ½ h. The production of collagenase by the microorganism was ascertained by liquefaction of sample after refrigeration.

2.5.7.2 Hydroxyproline assay

Collagen degradation by different micro flora identified was studied by measuring the amount of hydroxyproline released by the microorganisms on degradation of collagen. The collagenolytic activity of bacterial and fungal species was expressed as micrograms of hydroxyproline on 72 h degradation. Prepared collagen (single piece of ~1g) in 50 mL of nutrient broth was sterilized in UV chamber for 6 h and inoculated with microorganisms and incubated. Bacterial species that released hydroxyproline on 96 h degradation was identified and taken for further experimental studies.
2.5.8 Biodegradation of Collagen at Different C/N Ratios

Collagen as described in section 2.5.1, was accurately cut into single pieces of weight (~0.5 g) and sterilized in UV chamber for about 6 h. The individual single pieces of collagen were taken in 50 mL screw capped glass bottles filled with 25 mL of demineralized water and sterilized further for 2 h. The amount of external carbon (dextrose) required for different experiments (E1 to E8) at C/N ratios of 3, 3.5, 4, 4.5, 5, 6, 7 and 8 was added. 0.5 mL of the microbial inoculum was inoculated. A control experiment (E9) with no carbon source was also inoculated with the microbial inoculum. The pH of all the experimental solution during the degradation process was maintained at 7. The microbial degradation of collagen by *Bacillus subtilis* adjusted to pH 7.0 at different C/N ratios was analyzed by estimating the amount of hydroxyproline released in the solution after hydrolysis of collagen by microorganism.

2.5.9 Effect of Grinding on Degradation of Collagen

Collagen sample and tanned leather sample were ground to fine particles. The ground sample (~0.5 g) was accurately weighed and added to 50 mL screw capped glass bottles filled with 25 mL of demineralized water and external carbon (dextrose) required and sterilized for 8 h. The samples were inoculated with 0.5 mL of pure culture of microbial inoculum. The pH of all the experimental solution during the degradation process was maintained at 7. The microbial degradation of collagen by the microflora was analyzed by estimating the amount of hydroxyproline released in the solution after hydrolysis of collagen at different time intervals.
2.5.10 Effect of pH, Temperature and Water Content on the Degradation of Collagen, Tanned and Finished Leathers

The effect of pH on degradation of ground collagen at C/N ratio 4 was studied by maintaining different pH conditions with phosphate buffer (pH 5-6), Tris-HCl buffer (pH 7-8) and glycine-NaOH buffer (pH 9-10) in 50 mL screw capped glass bottles filled with 25 mL of demineralized water and ~0.5 g of ground collagen. The effect of different temperatures 30, 40, 50 and 60 °C, on degradation of collagen was also studied at pH 7. The effect of water content in the degradation process was also monitored by maintaining collagen to water ratio of 1:1, 1:10, 1:20, and 1:30 at pH 8 and 50 °C. The degradation of collagen and tanned leathers by different micro flora was monitored by measuring the hydroxyproline content at regular intervals of degradation.

2.5.11 Biodegradation of Tanned and Finished Leathers

The aerobic biodegradation of different types of tanned and finished leathers was analyzed by estimating the amount of hydroxyproline released in the solution after hydrolysis of these leathers by microorganisms. The tanned and finished leathers were ground to fine particles and a known weight (~0.5 g) sterilized in UV chamber for about 6 h, was taken in 1000 mL screw capped glass bottles filled with 750 mL of demineralized water and sterilized further for 2 h. The amount of external carbon (dextrose) required for at C/N ratios 4 was added. 5 mL of the mixed microbial culture as described in 2.5.2 and 2.5.3 each was inoculated. A control experiment with no carbon source was also inoculated with the mixed microbial cultures. The pH of all the experimental solution during the degradation process was maintained at 7. The % biodegradation was measured by estimating the
amount of hydroxyproline released in the solution after hydrolysis of collagen on biodegradation of leather by mixed microbial culture. Three different batch experiments with the above mentioned experimental conditions were carried out using (i) Isolated fungal spores (ii) Isolated bacterial species and (iii) serial inoculums of fungal spores for 15 days followed by bacterial isolates.