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ORIGINAL PAPER

Acidogenic fermentation of proteinaceous solid waste and characterization of different bioconversion stages and extracellular products

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Abstract The purpose of this study was to investigate hydrolysis of animal fleshing (ANFL), a predominant tannery solid waste and to characterize the acetogenic fermentation products of anaerobic digestion. The acidogenic digestibility of the tannery solid wastes were evaluated up to 120 h using batch anaerobic digestion tests performed under mesophilic condition at 37°C. The degradation of ANFL starts with non-fibrillar proteins and proceeds with fibrillar proteins. The release of aliphatic amino acid in the early stages of hydrolysis (24 h) and followed by aromatic amino acids (24–72 h) were evidenced by HPLC analysis. The maximum production of propionic and valeric acid were observed in 72 h followed by rapid increase in acetic acid in 96 h using GC-MS. Breakdown of ANFL and formations of other metabolites were evidenced by FT-IR and ^{1}H-NMR spectroscopy.

Keywords Hydrolysis · Acidogenic fermentation · Long chain fatty acid (LCFA) · Volatile fatty acid (VFA) · Animal fleshing (ANFL)

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Introduction

The leather manufacturing industries generate solid wastes higher than that of finished leather in the ratio 4:1 (Maire and Lipsett 1980). The solid waste is considered to be non-recoverable and disposable, and thus it becomes an environmental concern. Anaerobic digestion of solid waste is seen as a viable alternative compared to aerobic waste treatment process, for cost effectiveness and energy recovery. Hydrolysis of natural polymers, as the substrate, is the first step in anaerobic digestion process and is followed by acidification and methanogenesis. Among the proteins and lipids, protein is hydrolyzed to amino acids and further degraded to VFA either through anaerobic oxidation linked to hydrogen production or via fermentation according to the Stickland reaction (McInerney 1988). However, proteins are demonstrated to degrade more slowly than carbohydrates under anaerobic conditions

(Fang and Yu 2000). These findings imply the importance of protein degradation processes in anaerobic ecosystems.

Among the lipids, triglycerides are hydrolyzed to long chain fatty acids (LCFA) and further oxidized via β oxidation to acetate or propionate. However, LCFA are known to inhibit both their own degradation and the methane production from acetate (Hanaki et al. 1981; Alves et al. 2001). Moreover, tannery solid waste contains natural and synthetic fatty substances, which remain persistent in the anaerobic digesters due to its toxicity towards microorganism (Masse et al. 2002). Improved understanding of some physiological mechanisms of fat and protein metabolism in anaerobic digestion process is of scientific and practical relevance to design efficient anaerobic digesters. Therefore, a reliable and rapid method has to be developed in order to identify accumulating metabolites that can serve as marker substances for starting inhibition reactions. Thus the focal theme of the present work was (i) to study acidogenic fermentation of tannery solid wastes (ii) to investigate the fermentation products of ANFL.

Materials and methods

Performance of hydrolysis

The anaerobic sludge for fermentation analysis was collected from UASB reactor treating tannery solid waste. The anaerobic sludge consists predominantly of acidogenic bacteria *Clostridium* sp. with concentration of 135–155 $\times$ 10^5 CFU ml^{-1} and was used in experimental studies. The reactor for anaerobic digestion had a volume of 1 l and its working volume was 600 ml. It was also equipped with gas and sludge sampling ports. Ten percentage of the working volume was inoculated with fresh anaerobic culture and the reactor was purged with hydrogen gas to eliminate air from the reactor. By adding HCl before feeding, the pH of the feed was adjusted to pH 6.0. The anaerobic bacterial reactions were performed batch wise, and all reactions were performed at 37°C and pH 6.0. The ANFL was collected from a commercial leather industry located in Chennai, Tamil Nadu processing raw cow skins to finished leather. The ANFL was suspended in water and the pH was adjusted to 7.0 ± 0.2. The desalted and washed ANFL was cut into pieces approximately 1.5–3.0 mm and stored at 4°C until the startup of experiments. Fermentation experiments were conducted using 1 g ANFL per 100 ml minimal medium, composition NaCl 0.9 g, NH_4Cl 0.01 g, K_2HPO_4 0.75 g and KH_2PO_4 0.5 g in 125 ml serum bottles. The trace element solution of 1 ml, containing composition $MgSO_4$ 0.49 g, $FeSO_4$ 0.055 g, $CoCl_2$ 0.028 g, $MnCl_2$ 0.019 g, $CaCl_2$ 0.147 g and $NH_4Mo_7O_{24}$ 0.123g per liter was added to minimal medium after filter sterilization. All experiments were carried out in duplicates and repeated thrice.

Analytical procedures and high performance liquid chromatography (HPLC)

Protease activity was determined as described previously (Alam et al. 2005), using casein as the substrate. Protein and amino acids in fermentation medium were analyzed by methods of Lowry et al. (1951) and Rosen (1957) respectively. The percentages of Carbon, Hydrogen, Nitrogen and Sulphur were determined using Elementar Analysen systeme GmbH vario EL, German CHN analyzer. The TOC (Total organic carbon), TKN (Total kjeldhal nitrogen), VFA (Volatile fatty acid), ash and moisture content were analyzed in accordance with the standard methods for analysis of water and wastewater (APHA 1995). Total amino acid composition of ANFL was determined using C18 column in Agilent model 1100 HPLC analyzer following the method of Ramakrishnan et al. (1996).

FT-IR spectroscopic investigations

The KBr pelleted extracellular fermented samples were measured in the spectrometer after preparation without any moisture under ambient conditions using the transmission mode. The measurements were carried out in the mid-infrared range from 4000 to 400 cm^{-1} with a Perkin Elmer FTIR-spectrometer.

Gas chromatography/mass spectrometry (GC/MS) and proton nuclear magnetic resonance spectrum (H^1NMR)

The culture-free supernatant was lyophilized and dissolved with 0.5 ml of methanol. The sample was

then injected into the instrument, Agilent Technologies GCMS 5973 and the separated compounds were identified using library match. The proton NMR spectrum for the peptides and breakdown products analyses in lyophilized extracellular samples were recorded with JEOL ECA 500 MHz spectrometer, using deuterated water as solvent after water peak suppression.

Scanning electron microscopy (SEM)

The ANFL samples were fixed in 2% glutaraldehyde (W/V) for 2 h. After washing with saline solutions, they were dehydrated in 30–100% water–ethanol series. The air-dried particles were coated with 120–130 μm gold in argon medium. Scanning electron microscopy (SEM) observations were performed on a scanning device attached to a JEOL JM-5600 electron microscope at 20 kV accelerating with an electron beam of voltage 5–6 nm.

Statistical analysis and experimental design

The data obtained on hydrolysis of ANFL were analyzed by Plackett–Burman first order model. All experiments were carried out in triplicate and the averages of the values were taken to determine the coefficients. This model is used to evaluate the interaction among variables that influence on the COD of hydrolysis.

$$\mathrm{Y(COD)} = f\left(\text{Extracellular protein, amino acid, ammonia, } \mathrm{CO_2}\text{, VFA, protease and TKN}\right)$$

$$\mathrm{Y(COD)} = a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4 + a_5x_5 + a_6x_6 + a_7x_7$$

$$\mathrm{Y(COD)} = a_1(\text{Extracellular protein}) + a_2(\text{amino acid}) + a_3(\text{ammonia}) + a_4(\mathrm{CO_2}) + a_5(\text{VFA}) + a_6(\text{protease}) + a_7(\text{TKN})$$

Results and discussions

Substrate characterization

Raw material ANFL, one of the predominant tannery solid wastes contained 33.5% of dry material, which consists of 70.4 ± 1.9% proteins, 12.1 ± 2.2% lipids and 0.8 ± 0.1% carbohydrates. The total proteins in the raw material consist of 11.1 ± 0.1% globular proteins, 87.9 ± 0.3% fibrillar and other proteins. The characterizations of ANFL were given in (Table 1). The amino acid composition of ANFL was given in (Table 2). The primary data of the degradation kinetics of ANFL and its degradation products by acidogenic bacterium were presented in the following section.

Table 1 Chemical characterization and CHNS analysis of ANFL substrate used in fermentation

Parameter	Values
Total organic carbon (mg g^{-1})	342 ± 21.2
Total Kjeldhal nitrogen (mg g^{-1})	138 ± 14.5
Moisture content (%)	27.5 ± 5.5
Ash content (%)	21.5 ± 2.5
Total protein (%)	64.8
Fat (%)	5.3
Carbohydrate (%)	0.165
Collagen (%)	3.5
N (%)	11.6 ± 0.3
C (%)	36.14 ± 0.58
S (%)	0.656 ± 0.15

Values are expressed as mean ± SD (n = 4)

Table 2 Total amino acid composition of ANFL

Types of amino acids	Values in μg g^{-1} ANFL
Serine	0.088
Histidine	0.407
Glycine	0.255
Threonine	0.171
Alanine	0.044
Arginine	0.069
Tyrosine	0.179
Valine	0.097
Methionine	0.065
Phenylalanine	0.038
Isoleucine	0.009
Leucine	0.065
Lysine	0.011

These values are mean values of 3 individual analyses

Solubilization of ANFL

Soluble chemical oxygen demand (COD) is a parameter, which represents the extent of hydrolysis and solubilization carried out by acidogenic bacterial consortium. The net COD concentration was highest in 48 h and decreased with fermentation time and the specific soluble COD production increase was due to the hydrolysis of myoglobullar proteins bound to the ANFL (Fig. 1). The decrease in COD production rate from 48 to 120 h indicates that initial hydrolysis becomes the rate-limiting step. The particulate matter must be hydrolyzed or liquefied first before being assimilated by bacteria, resulting in a net increase in soluble COD. However, when the soluble components were utilized, COD decreased. This occurs due to the rapid hydrolysis and fermentation of soluble proteins (Ramsay and Pullammanappallil 2001).

HPLC analysis of extracellular amino acids

In the initial stages of fermentation, more aliphatic amino acids were released, this evidence the degradation process have been started in globular non-fibrillar proteins. The individual concentration of the amino acids released into the extracellular medium is presented in Table 3. The amino acid content in the extracellular medium reached steadily to a maximum of 232 mg l^{-1} at 72 h. The HPLC analysis of amino acid content reveals that the amino acids like histidine, glycine, threonine, arginine, leucine, alanine, tyrosine, valine, phenylalanine and lysine during fermentation of ANFL were released (Table 3). The amino acids were found to increase at 72 h and decreases on further fermentation period proving Stickland reactions of amino acids (Rahman et al. 2005) during hydrolysis of ANFL. These reactions occur steadily giving hydrogen, ammonia, carbon dioxide and fatty acids as end products.

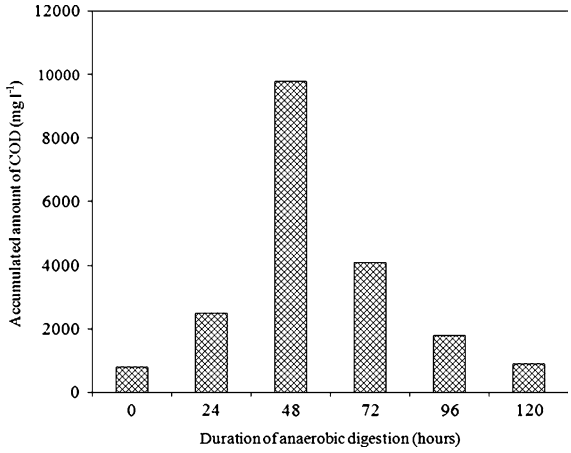

Fig. 1 COD as a function of hydrolysis and acidogenic fermentation of ANFL during different days of anaerobic digestion process

VFA as a function of reactor performance

The pH was measured every day in the system to observe the variations in pH of the reactor. Influent pH varied from 5.7 to 6.2, and then gradually increased above 6.5 in the effluent. These results indicate that the acetogenic bacteria were more active in the reactor. In general, butyric acid concentrations were almost zero during the whole course of the experimental study. The valeric acid was only observed in samples taken after 72 h. The average of acetic acid and propionic acid concentration were found to be 150 and 54.9 mg l^{-1} respectively. Malina and Pohland reported that the volatile fatty acid concentrations should be smaller than 1000–1500 mg l^{-1} for anaerobic microorganisms (Malina and Pohland 1992). The VFA concentrations were measured within the desired limits required for anaerobic degradation during the experimental studies. The lower VFA formation within 72 h of the reactor can be explained by the two-phase VFA start up. The first phase was dominated by the propionic and valeric acid. The second phase was dominated by the acetic acid at increasing residence time (Fig. 2). The micro-environmental conditions (that is, cell–cell contact, nutrient–product gradients, no competition with other bacterial consortium), provides favorable conditions for acetogenic bacterial consortium to produce more acetic acid.

GC-MS analyses—LCFA tolerance

The GC-MS analysis of the hydrolyzed products from ANFL at 72 h yields at least 10 volatile products. The examination of the corresponding mass spectra indicates that, most products are derived from proteins and lipids breakdown. The GC-MS analysis of the hydrolyzed products provided evidence that all

Table 3 Extracellular amino acids released into the fermentation medium on hydrolysis of ANFL

Extracellular amino acid (mmol ml^{-1})	24 h	48 h	72 h	96 h	120 h
Hydroxyproline	6.44	58.9	98.24	16.88	ND
Proline	12.4	25.6	48.23	3.6	ND
Histidine	ND	3.23	1.96	1.28	1.22
Glycine	7.44	11.9	4.11	1.43	ND
Serine	ND	2.42	ND	ND	ND
Threonine	1.96	19.5	4.33	3.88	2.77
Alanine	31.7	46.4	4.94	1.44	0.59
Arginine	ND	18.4	2.97	ND	ND
Valine	19.1	24.5	293.4	165.9	60
Methionine	17.0	46.8	66.86	21.37	2.5
Isoleucine	9.86	ND	ND	ND	ND
Leucine	3.25	32.9	28.38	0.91	0.33
Lysine	48.8	43.5	13.47	0.6	0.2
Phenylalanine	ND	16.1	20.44	18.51	1.16
Tyrosine	3.2	7.33	9.69	33.29	72.9

ND—not detectable, all values are mean of 3 individual experiments

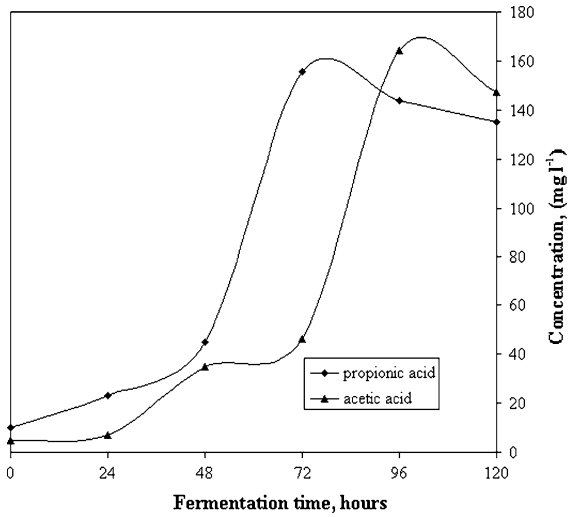

Fig. 2 Volatile fatty acids such as acetic and propionic acid fluctuations during different days of anaerobic digestion process

the fermented samples have identical distributions of *n*-alk-1-ene/*n*-alkane doublets. The alkanes were present at much lower intensities than the alkenes. The fatty acid derivatives were converted into the corresponding esters at 72 h of fermentation. This may be attributed to the metabolism of lipid component present in ANFL.

The fermentation products such as pyrroles, indoles and aromatic nitriles are indicated in the GC-MS spectrum, the products were obtained due to the breakdown of proteins or amino acids (Ishiwatary et al. 1995). The presence of unconverted proteins in the fermented samples were confirmed by the identification of low levels of alkylated dihydro-pyrrolediones and pyrrolidinediones characterized by molecular ion peaks at *m*/*z* 152, 166 and 180, in the pyrogram, which are specific markers of two adjacent aliphatic amino acids in poly peptides (Boon and de Leeuw 1987). Hecht, using casein as a substrate identified piperidon, benzoate and acetate as intermediates of the anaerobic metabolic pathway of degradation (Hecht et al. 2005).

Interestingly, the fermented samples contain high relative abundance of protein derivatives compared to other components. Chromatograms of the protein and lipid degradation products prove the complete breakdown through anaerobic metabolism. The results from the GC-MS studies demonstrated that acidogenic consortium were capable of active growth and metabolism in the presence of LCFA like octadecanoic acids and oleic acids, while LCFA has been reported to be toxic to varied anaerobic bacteria (Maczulak et al. 1981). Fats were easily hydrolyzed to LCFA, which were reported to exert an acute toxic effect on the microorganisms involved in the β-oxidation and anaerobic pathways by inhibiting its energy metabolism (Angelidaki and Ahring 1992; Masse et al. 2002). The mechanism of anaerobic acidogenic

fermentation of organic matter involves the solubilization of organic matter in suspension, the hydrolysis of complex soluble organic compounds such as sugars, amino acids and LCFA and the final formation of simple volatile fatty acids. The VFA production confirms the acidogenic activity of the bacterial consortium. The LCFA degradation in the acidogenic phase confirms that acidogenic bacterial consortium were resistant to the toxicity exerted by LCFA.

NMR analyses of biochemical alteration and products formed

Many differences were observed in the ^{1}H-NMR spectra of the samples of different fermentation periods (Fig. 3). In the initial day of the hydrolysis, the decrease in the chemical shift (δ) of amide at δ 5.55 takes place, whereas the presence of new doublet amide peaks around δ 5.35 was observed. This can be due to the breakage of protein molecules into polypeptide molecules. These noticeable changes in the spectrum suggest that the hydrolysis first begins at the non-fibrous protein molecules in the ANFL. In the 48 h, the presence of the doublet amide peak at δ 5.3 increases and the complete disappearance of the amide groups of the 0th (δ 5.55) day take place and also the chemical shift at δ 6.11 is not pronounced which shows the absence of NH group of L-Proline and hydroxyproline. There is also a shift in the ring structure of these compounds present. This is an indication of onset of breakage of the collagen molecules, which starts from the second day onwards. The ^{1}H-NMR spectra reveal that there are more number of peaks at the chemical shift in the areas of methylene and methionine groups of protein and collagen molecules can be attributed to the changes in the skeletal structure of the protein and the collagen in the ANFL. The nearly same peaks present on the 72 h shows that there is no remarkable changes taking place during the 48 and 72 h of fermentation. The spectra of the 96 and the 120 h day resemble one another. The spectrum illustrates the complete disappearance of the amide peaks of the bulk protein molecule and the strong peak of the amide group of the small peptide chain. This spectrum also reveals the disappearance of the small resonance peaks corresponding to breakage of the skeleton molecule of the protein and collagen, which takes place on the 48 and 72 h of fermentation. The well resolved minimum number of methylene and methionine groups appeared on the 96 and 120 h and this may be due to diffusion of the peptide molecules from unhydrolyzed ANFL into the extracellular liquid medium (Fig. 3). Apart from degradation of protein substrates more products were produced at acidogenic fermentation. The acidogenic consortiums are able to hydrolyze proteins into amino acids and peptides that are converted into simple volatile acids. The ^{1}H-NMR spectra show increases of fermentation products δ 2.0–4.0, which constitute for acetic acid, pyruvic acid, propionic acid and butyric acid production at 72 h (Correia et al. 2005). This indicates that non-fibrous proteins of the ANFL were solubilized initially, causing visible signals in the spectrum. With the fermentation process, these signals increase as a result of complete hydrolysis of non-fibrillar proteins, which allows more product formation into the liquid phase. This also sturdily explains the role of acidogenic fermentation that plays a key role in the hydrolysis and formation of metabolites in anaerobic digestion process.

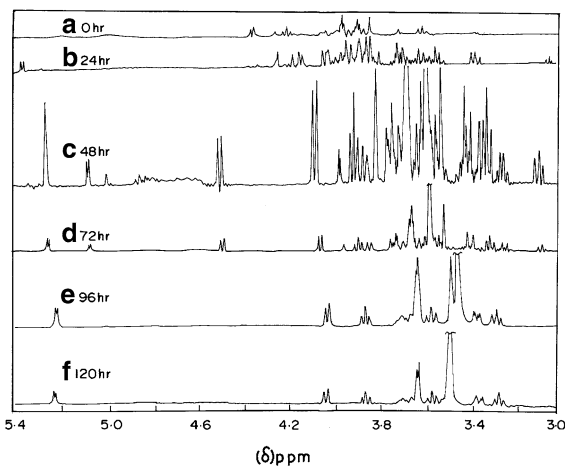

Fig. 3 H^{1}-NMR analysis of extracellular fermented products during different days of hydrolysis of ANFL (a. 0th hour, b. 24th hour, c. 48th hour, d. 72nd hour, e. 96th hour, f. 120th hour)

FT-IR analysis of extracellular fermented products

The FT-IR spectrum of the initial sample shows a broad envelope around 3500–3200 which centered as 3401 cm^{-1} can be due to the –NH stretching vibration of the protein molecules. Their corresponding amide I band and amide II band present at 1649 and 1541 cm^{-1}

(Surewicz and Mantsch 1988). The spectrum also shows the presence of methyl and methylene groups. The asymmetrical and symmetrical stretching of methyl and methylene groups can be identified by the peaks in the region of 2970–2850 cm^{-1}. The bending vibrations of the methylene group were presented at 1456 cm^{-1}. When the spectrum of the 0th hour was compared with the hours like 48, 72 and 96, an interesting observation was made. The C=O stretching vibration of the acid group was observed at 1742 cm^{-1} in the 48 and 72 h and at the same time small alteration in the amide I band of proteins also occurs. These changes can be easily correlated with the formation of acetic, valeric, propionic acid etc...from proteins (Smidt et al. 2005). The presence of the C=O stretching vibration of the acid group again decreased in 96 h but the alteration of the protein molecules remains as such in the 48 and 72 h. This can be attributed to the degradation of the acid group further to the products like CO_2 etc., which is common in the hydrolysis (Fig. 4).

SEM analyses of biochemical changes in ANFL

The scanning electron micrographs of the ANFL sample without treatment shows prominent attachment of loose proteins and clumpy clusters of bounded tissues. Moreover, fibers were not visualized proving that there is no enzymatic activity during onset of fermentation (Fig. 5a). The loose proteins were detached well after the onset of the hydrolysis showing intact undamaged collagen fibers offering tensile strength to ANFL at 24 h and the clustered tissue cells were not visualized well (Fig. 5b). The selective images were chosen so as to describe the changes occurred after complete hydrolysis of collagen fibers at 48 h (Fig. 5c). These fibers were detached well and breakages were complete. The SEM analysis of the ANFL after hydrolysis showed some distinct spaces occupied by liquids and hydrolyzed peptides. More breakages of tissue fibers were also observed and in most places fiber-to-fiber detachment were also visualized. Further, the structural changes were not uniform over the ANFL. Some areas of the ANFL were significantly more affected than others. The collagen fibers were visualized, during the middle stages of fermentation and this may be due to higher proteolytic activity. The hydrolytic

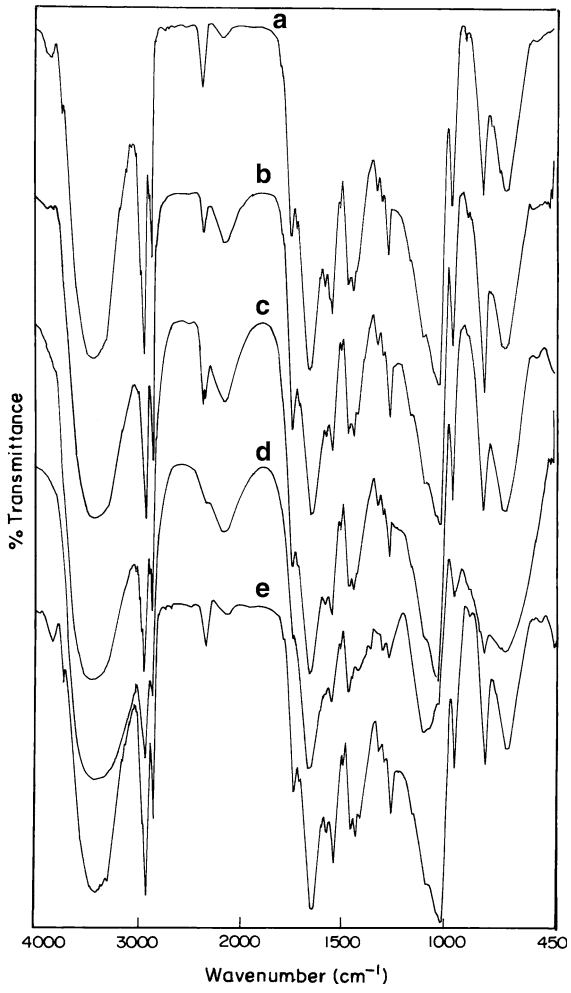

Fig. 4 FT-IR analysis of ANFL degradation and formation of metabolites during different days of anaerobic digestion [(**a**) 0th h, (**b**) 24th h, (**c**) 48th h, (**d**) 72nd h, (**e**) 96th h]

enzymes cleaved the loose and bound globular proteins initially and thus the fibrillar protein, collagen that was embedded in the globular proteins has been visualized. The non-availability of the easier substrate and availability of hard-core collagen resulted in onset of fibrillar proteins breakdown. These observations corroborate with some researchers proves that fibrillar proteins were cleaved on further fermentation under optimal physiological conditions (Juarez and Stinson 1999; Krystyna 2003). Moreover, protein solubilization resulted in the micro structural changes in ANFL. Thus, the SEM analyses confirmed that the hydrolysis starts in globular non-fibrillar proteins and proceeded further to fibrillar proteins in ANFL.

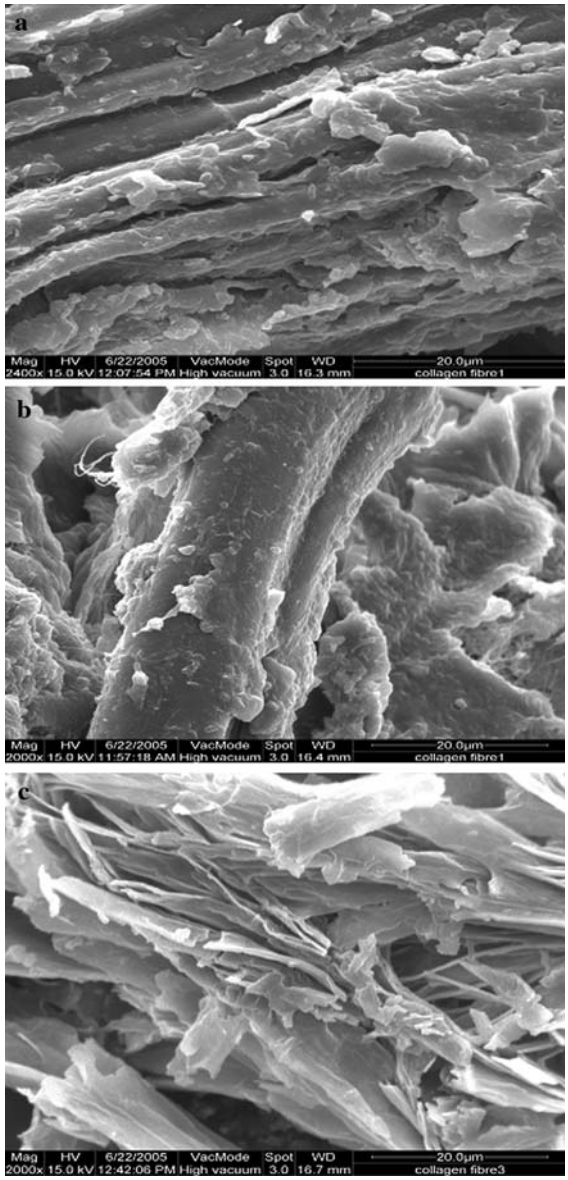

Fig. 5 Scanning electron micrograph during different days of anaerobic digestion (**a**) Unhydrolyzed ANFL showing intact bound proteins, (**b**) 24 h Hydrolyzed ANFL showing damaged bound proteins and intact collagen fibers, (**c**) 48 h Hydrolyzed ANFL showing damaged collagen fibers

Process variables: COD as a function of hydrolysis

For the determination of the process variables that directly influence the rate and amount of hydrolysis, COD was considered as a linear function of Extracellular protein, amino acid, ammonia and CO_2 released, the VFA being produced and the TKN in the broth. The process variables are a direct measure of the dependence of COD upon the various aforementioned parameters. The production of COD fits the first order linear equation upon iterative solving of each of the equations

$$\begin{aligned} \mathrm{Y(COD)} = {} & (-)\,1.50641(\text{Extracellular protein}) \\ & + (-)\,2.25011(\text{amino acid}) \\ & + 0.623683(\text{ammonia}) + 5.213257(\mathrm{CO_2}) \\ & + 1.969312(\mathrm{VFA}) + 5.39276(\text{protease}) \\ & + 4.751979(\mathrm{TKN}) \end{aligned}$$

Being the most important linear variable affecting the COD production, Protease had the highest regression coefficient ($a_6 = 5.39$), followed by CO_2 ($a_4 = 5.2$).

The plot of $\mathrm{COD_{(predicted)}}$ versus $\mathrm{COD_{(Experimental)}}$ yielded a linear equation of the form

$$Y = 1.1612x - 1550,$$

with regression coefficient of $R^2 = 0.8677$

Conclusions

In acidogenic environment the formation of products has received little attention. Challenging for the future is to get more insight into fermentation products in acidogenic reaction, which is the rate-limiting step in the methane formation. Also the pattern of products formed and their degradation in fermentation needs to be studied. In this aspect, HPLC techniques were very useful in particular, because the fate of individual amino acid could be studied. Also the combined use of FT-IR, ^{1}H-NMR and GC-MS were very attractive to get a complete picture of proteinaceous solid wastes break down. Although the general principles of degradation were largely known (Stams and Hansen 1984), only very few acidogenic bacterial degradation have been studied. Also FT-IR, ^{1}H-NMR, HPLC, GC-MS and SEM were also used to get an insight of the degradation of complex wastes in anaerobic digestion. In this respect, it is worth to mention that the SEM also give very useful information concerning the breakdown of substrates. Thus to our knowledge this is the first report on the anaerobic degradation of

ANFL, predominant tannery solid waste. These findings would be of interest to biochemists, applied microbiologists, biotechnologists and process bioengineers for designing strategies for optimizing proteinaceous solid substrates hydrolysis.

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