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ORIGINAL PAPER

Effects of nonionic surfactant on hydrolysis and fermentation of protein rich tannery solid waste

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Abstract The untanned proteinaceous tannery solid waste, the animal fleshing (ANFL), was used as substrate in the treatment process (hydrolysis and fermentation) involving *Synergistes* sp. The nonionic surfactant (Tween 80) was evaluated for its ability to influence on microbial growth and enzyme activity in the hydrolysis and fermentation of ANFL. The addition of Tween 80 in the process significantly increased the activities of hydrolytic and fermentative enzymes like protease (338–360 Um l^{-1}) and deaminase (187–206 Um l^{-1}) compared to that of control (protease 195–220 Um l^{-1} and deaminase 70–83 Um l^{-1}). The total viable bacterial count was increased more than twofold, compared to the control in the presence of 0.15% Tween 80. The ANFL fermentation and formation of other metabolites were evidenced by Gas Chromatography and Mass Spectroscopy (GC-MS), Proton Nuclear Magnetic Resonance spectroscopy (^{1}H NMR) and Fourier transform infra red spectroscopy (FT-IR). The breakdown of fibrillar proteins in ANFL was confirmed by the scanning electron microscopy (SEM) and the transmission electron microscopy (TEM).

Keywords Surfactant · *Synergistes* sp. · Fermentation · Solid waste · Protease · Deaminase

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Introduction

The treatment under anaerobic condition is a well known and proven technology as a means of managing solid organic waste (De Baere 1999). However, a little research has been done on the treatment of leather industry solid wastes in the presence of anaerobic condition (Banks and Wang 1999; Broughton et al. 1998). The proteins have been demonstrated to degrade more slowly than the carbohydrates under anaerobic conditions (Gujer and Zehnder 1983). It has also been documented that the anaerobic degradation of protein-rich wastes is often incomplete (Fang and Yu 2000; McInerney 1988). These findings

imply the importance of protein degradation processes in anaerobic ecosystems.

Under anaerobic conditions, proteins are hydrolyzed to peptides and amino acids which are subsequently fermented to volatile fatty acids, and finally converted to ammonia and CO_2 by the activities of microorganisms (McInerney 1988). The rate of the formation of certain intermediary products has been shown to be energetically more favorable under anaerobic process. The product formation by anaerobic bacterium is a very complex process, which is greatly influenced by many factors such as substrate concentration, pH, temperature, and redox potential for enzymatic activity (Yu and Fang 2000). Thus, the degradation rate in this process can be increased by optimizing conditions for each bacterial group separately (Dinopoulou et al. 1987) and there has been a continuous drive to search the anaerobic bacteria capable of rapidly utilizing tannery solid wastes.

The "*Synergistes*" species have been detected in samples collected from a wide range of environmental sources such as anaerobic digesters and waste water (Godon et al. 2005; Diaz et al. 2006; LaPara et al. 2000). Yet, other investigations of anaerobic digesters reported a greater abundance of "*Synergistes*" clones: 4.9% (Godon et al. 1997) and 3.1% (Chouari et al. 2005). Godon et al. 2005, have produced the largest inventory of synergistes-group organisms to date by targeting this group in 93 anaerobic environments including the gut and feces of animals, soils, and anaerobic digesters. They reported that "*Synergistes*" taxa appeared to be present in 90% of the anaerobic microbial ecosystems and "*Synergistes*" taxa are frequently detected in environmental anaerobic digesters where they degrade proteinaceous waste. These findings signify the functional importance of this group.

Among the various approaches to increase the hydrolysis and fermentation of solid wastes, the additive method is the simplest. In order to enhance the hydrolysis, nonionic surfactants have been used successfully in bioreactors. Craig et al. (1984) observed a 15% numerical increase in the rate of degradation of casein in the presence of Tween 80 (0.1% v/v) as compared to the control. Kamande et al. (1993) reported increased proteolytic activity in incubations of ruminal fluid in the presence of low concentrations of detergents (Tween 60 and Tween 80). The Tween 80 serves as an emulsifier to give stable substrate emulsion, which provides large interfacial area, which, in turn, enhances the rate of enzyme catalyzed reactions (Liu et al. 2000).

In spite of these investigations the reports on nonionic surfactant induced hydrolysis and fermentation of tannery solid wastes are few or perhaps nil. Keeping in view the wide applicability of surfactants in fermentation, it becomes imperative to investigate the effect of the nonionic surfactant Tween 80 on fermentation of ANFL with special focus on enzymes such as protease, lipase and deaminase.

Materials and methods

Substrate preparation

The limed animal fleshing predominant solid waste generated during the process of leather manufacture was collected from the tannery division of Central Leather Research Institute, Chennai and was treated with ammonia solution (25% v/v) for 3–4 h to remove the adhered calcium salts on ANFL as described in our previous observation (Ganesh Kumar et al. 2007a). The manually scissored (0.25 cm) ANFL particles were stored at 4°C until the start of the experiments.

Fermentation of ANFL

The batch experiments were conducted for 1 g ANFL in 100 ml minimal medium of composition NaCl—0.9; NH_4Cl—0.01; K_2HPO_4—0.75; and KH_2PO_4—0.5 (g l^{-1}). The media were autoclaved at 120°C at 15 psi for 15 min and incubated, without agitation, at 37°C. All experiments were carried out in duplicates and repeated thrice. The anaerobic proteolytic bacterium *Synergistes* sp. was isolated from anaerobic digester, Chennai and the acclimatized *Synergistes* species was maintained in anaerobic agar medium (Ganesh Kumar et al. 2007b). The total viable bacterium was enumerated, using anaerobic agar medium (Hi media) in anaerobic chamber.

Effect of nonionic surfactant Tween 80 concentration

Nonionic surfactant (Tween 80) in the concentration of 0.05, 0.1, 0.15, 0.2, and 0.25% was added to 1 g ANFL in 100 ml minimal medium as mentioned

above. The influence of surfactant on fermentation was evaluated by measuring microbial growth rate and enzyme activities.

Enzymes assay and analytical procedures

The activities of hydrolytic enzymes protease and lipase were measured as previously done (Brock et al. 1982; Lakshmi et al. 1999). The fermentative enzyme deaminase was measured according to the procedure of Massad et al. (1995). The anaerobic digester samples were characterized for Chemical Oxidation Demand (COD) in accordance with the standard methods for analysis of water and wastewater (APHA 1995).

Oxidation reduction potential

The Redox Potential of the reaction was measured using ion selective electrode of Orion 920 A+, Thermo Electron Corporation, USA. The electrode was standardized, using ORP-Orion 967901 standard solution and was connected to anaerobic digester for online monitoring.

High performance liquid chromatography (HPLC)—VFA

The fermented samples were separated through centrifugation and filtered so as to separate metabolites from the substrate and was used for the quantitative HPLC analysis, using Shimadzu VP SERIES with SpD10A detector and WINCHROM software. The column used was C18 Hypersil column using methanol: acetonitrile as mobile phase. The UV detector (at 210 nm) was employed for quantification. The UV-Visible spectra were recorded at the peak maxima and were corrected for the solvent background. The results were determined, using the standard volatile acids (Merck, India) as control.

Gas chromatography mass spectrometry (GC-MS)

The culture-free supernatant of control and Tween 80 were lyophilized and dissolved with 0.5 ml of methanol. The samples were then injected into the instrument, Agilent Technologies GCMS 5973 with DB5MS column (30 m length, 0.25 mm ID, and 0.25 μ film thickness). The temperature gradient was 70°C to start up, with increase in temperature of 10°C for every 10 min till the rise 260°C and held for 20 min. The separated compounds were identified, using NIST 2000 library match.

Proton nuclear magnetic resonance spectrum (H^1 NMR)

The proton NMR spectra for the ANFL breakdown products (Control and Tween 80) in lyophilized extracellular samples were recorded by the JEOL ECA 500 MHz spectrometer, using deuterated water as solvent.

Fourier transform infrared spectroscopy (FT-IR)

Fourier transform infra red spectroscopic analyses were performed using Perkin–Elmer spectrophotometer for fermented samples (24–120 h). The samples used for the FT-IR analysis were prepared in the following way: 2 mg sample was mixed with 200 mg KBr and homogenized in a mortar-pestle without any moisture absorption. The pellet of 1 mm thickness and 10 mm diameter was made and used for analysis. Perkin–Elmer infrared spectrophotometer was used for investigating the changes in the ANFL fermentation processes. The measurements were carried out in the mid-infrared range from 4000 to 400 cm^{-1} after baseline correction.

Transmission electron microscopy (TEM)

The degraded and undegraded ANFL of 1 mm was separately fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2 h. After buffer wash, the secondary fixation with 1% Osmium tetroxide in 0.1 M sodium cacodylate buffer was carried out for 1 h at room temperature for both. The specimens were then dehydrated through an increasing ethanol series. The specimens were transferred to propylene oxide, prior to infiltration with 1:1 propylene oxide: araldite CY212 resin overnight in a specimen rotator and were polymerized at 60°C for 18 h. Semi-thin (1 mm) sections were cut using ultra microtome, floated onto distilled water and collected on glass slides. The light microscopy sections were viewed and relevant areas photographed on an Olympus BH-2 microscope. Ultra-thin sections were cut using a Diatome diamond knife, stained with 2% uranyl

acetate and lead citrate for 10 min in each solution. The stained sections were viewed on a Philips CM12 electron microscope.

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. The 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 analyses

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

$$Y_{(\mathrm{Eh})} = f(\mathrm{COD}, \text{ extra cellular amino acid, ammonia, volatile fatty acid, pH, carbon dioxide})$$

$$Y_{(\mathrm{Eh})} = a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4 + a_5x_5 + a_6x_6.$$

Results and discussions

The objective of the present investigation was to enhance the hydrolysis and fermentation process (in anaerobic condition) of ANFL, the predominant tannery solid waste by using nonionic surfactant Tween 80 as additive. In this study anaerobic bacterium *Synergistes* sp. was isolated from an anaerobic ecosystem based on its ecological importance (Godon et al. 2005). To our knowledge, this investigation is the first published report on the characteristics of *Synergistes* sp. in the treatment of ANFL with special focus on its hydrolytic and fermentative enzymes in the presence of nonionic surfactant.

Surfactant concentration and hydrolysis of ANFL

The effect of nonionic surfactant Tween 80 concentrations on the anaerobic process of ANFL was studied in the concentration of 0.05, 0.1, 0.15, 0.2, and 0.25%, respectively. The COD results showed that there is an obvious relationship between the surfactant concentration and the degradation rate. When the concentration of Tween 80 was increased above 0.15%, the decrease in the hydrolysis and fermentation of ANFL was observed. Hence the 0.15% Tween 80 concentration was chosen for further studies. The higher net COD concentration was observed in 48 h and decreased thereafter till the fermentation time. The initial soluble COD increase was due to the rapid hydrolysis of globullar and surface bound proteins in the ANFL. The presence of 0.15% Tween 80 increased the rate of hydrolysis of the ANFL, which further resulted in increase of soluble COD (6,800–7,000 mg l compared to the control (5,300–5,500 mg l). Figure 1 shows that initially COD increases up to 48 h and thereafter it decreases. The complexity of the ANFL substrate renders its degradation process into a difficult process (Ganesh Kumar et al. 2007a). In the beginning the rate of ANFL hydrolysis in terms of COD (Fig. 1) seems to be high in the presence of Tween 80 as compared to the control. These results corroborated with the research findings of Castanon and Wilke (1981) showing that 0.1% tween 80 increased the rate of solid waste hydrolysis performance by 33%. The

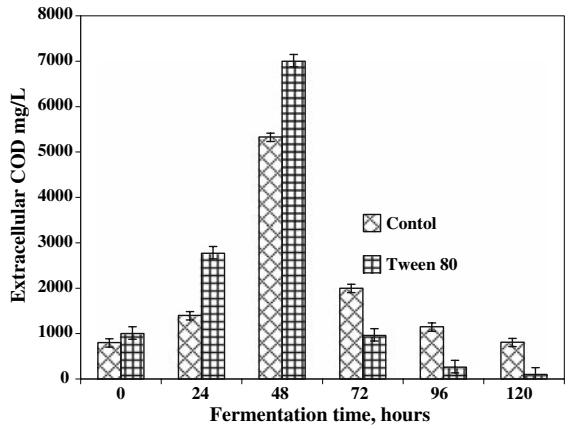

Fig. 1 COD as a function of ANFL hydrolysis. Control-ANFL (without surfactant), Tween 80-ANFL (presence of nonionic surfactant)

ANFL must be hydrolyzed or liquefied first before being assimilated by bacteria, which will result 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).

Effect of Tween 80 on total bacterial count and the extra cellular enzymes

The effect of time period on the fermentation experiment was studied for a period of 120 h. The growth phase of *Synergistes* sp. continued up to 72 h and maintained in stationary phase up to 96 h of fermentation. The total viable bacterial count was increased more than twofold (from 8.50 ± 1.3 to 34.0×10^6 CFU per ml of fermented liquid) in the presence of 0.15% Tween 80 concentration, compared to the control (6.25 ± 1.5–16.5×10^6 CFU per ml). The Tween 80 at concentration of 0.15% influenced the activity of hydrolytic enzyme protease. Steady rise in protease activity was observed from 24 h onwards and reached a maximum of 338–360 Um l^{-1} after 72 h retention time (Fig. 2). The amino acid contents in the extracellular medium reached steadily to a maximum of 220–235 mg l^{-1} at 72 h and decreased thereafter. This confirmed the ability of Tween 80 in enhancing and stabilizing the activity of hydrolytic enzyme protease toward ANFL proteins. The lipase activity in the presence of Tween 80 reached a maximum of 23–31 Um l^{-1} in 24 h and in control it reached the maximum of 15–18 Um l^{-1} (Fig. 2). The nonionic surfactant played a vital role in increasing the fermentative enzyme deaminase activity in the treatment process. The deaminase activity reached a maximum of 187–206 Um l^{-1} in presence of Tween 80 and 70–83 Um l^{-1} in the control (Fig. 2) after 72 h of fermentation period. Thus it is evident from these results that the Tween 80 plays a major role in enhancing the hydrolytic enzyme protease and lipase and fermentative enzyme, deaminase, during the process of fermentation of ANFL.

Volatile fatty acid

The HPLC analyses of VFA concentrations were measured within the desired limits required for anaerobic degradation during the experimental studies. Two-phase VFA start up was found during the fermentation process. The phase—I was dominated by propionic and valeric acid in the range of 145–150 and 65–72 mg l^{-1}, however in control these acids were very less. The phase—II was dominated by acetic acid at concentration of 400–450 mg l^{-1} as against 200–250 mg l^{-1} in the control (Fig. 3). The rate of production of acetic acid decreases as the substrate depletes or is converted into further

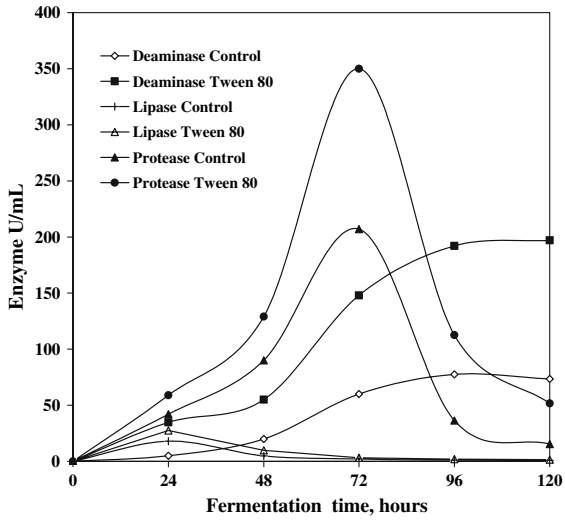

Fig. 2 Changes in extracellular protease, lipase and deaminase activity in presence of Tween 80

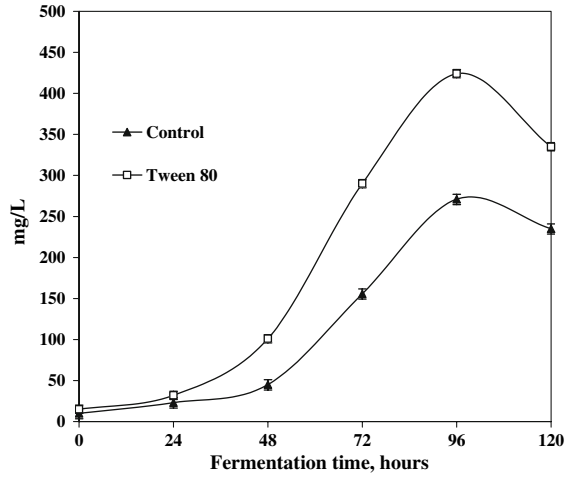

Fig. 3 Acetic acid production in hydrolysis and fermentation of ANFL in presence of Tween 80

products as the fermentation progresses (Kusum Lata et al. 2002) and thus the acetic acid decreases after 96 h.

The ANFL was hydrolyzed and further degraded to VFA either through anaerobic pathway linked to hydrogen production or via fermentation according to the Stickland reaction (McInerney and Zehnder 1988). Our results correlated with the observations of Malina and Pohland (1992) that the volatile fatty acid concentrations should be lesser than 1,000–1,500 mg l^{-1} for anaerobic microorganisms. The Tween 80 provided favorable microenvironmental conditions (that is, cell–cell contact, nutrient–product gradients, pH gradients) for cells, enhanced favorable conditions for acetic acid production and resulted in a better performance. Cell–cell contact is nothing but every single bacterium in a population secretes a specific signaling molecule, which can be sensed by the other bacteria. Based on this simple principle, bacterial populations can co-ordinate their behavior (Voloshin and Kaprelyants 2004).

GC-MS analyses of extra cellular products of ANFL fermentation

Presence of Tween 80

It is proven that the mass spectrum of the protein and lipid degradation products broke down through anaerobic metabolism. The analysis of volatile components from extracellular fermentation fluid in 24 h confirms the presence of hexanedioic acid, ornithine, phthalic acid, and anethole identified by their molecular ion peaks at m/z 146, 132, 167, and 142, respectively. The extracellular products at 48 h fermentation yields the five different products with molecular ion peak m/z 103, 46, 172, 143, and 72 namely valeric acid, formic acid, decanoic acid, octanoic acid, and propionic acid, respectively. The valeric acid produced in the bacterial system was due to the reductive ring cleavage of proline. This evidenced the breakdown of fibrous proteins like collagen after 48 h of fermentation. In lipid, octanoic acid and decanoic acid were the only derivatives present in 48 h. The propionic and valeric acid were the two carboxylic fatty acid present in 72 h fermentation time whereas acetic acid (m/z 43) and benzoic acid (m/z 43) were formed at the 96 h fermentation hours.

Absence of Tween 80

The GC-MS analysis in hydrolyzed products from ANFL in absence of Tween 80 reveals that the conversion takes place after 72 h of fermentation. It yields at least ten volatile products including pyrroles, indoles, and aromatic nitriles derived from protein and lipid breakdown. The presence of unconverted proteins were confirmed by the identification of low levels of alkylated dihydro-pyrrolediones and pyrrolidinediones characterized by molecular ion peak at m/z 152, 166, and 180, which are specific markers of two adjacent aliphatic amino acids in poly peptides (Boon and de Leeuw 1987). The mechanism of anaerobic fermentation involves solubilization of organic matter in suspension, the hydrolysis of complex soluble organic compounds such as sugars, amino acids, long chain fatty acids, and the final formation of simple volatile fatty acids.

NMR analyses of macromolecular alterations and extra cellular products of ANFL

Presence of Tween 80

Many differences were observed in the ^{1}H NMR spectra in the samples of different fermentation periods of ANFL in presence of Tween 80. In the initial day of the hydrolysis a new doublet amide peaks around δ 5.3 was observed and this can be due to the conversion of the protein molecules in ANFL into polypeptide chains. In the 48 h, the chemical shift at δ 5.0 is not pronounced which shows the absence of -NH group of L-proline and hydroxyproline. The chemical shift in the ring structure of these compounds indicates the onset of breakage of the fibrous proteins in ANFL, which starts from the second day onwards. The 72 h ^{1}H NMR spectra reveal an increase in number of peaks and chemical shifts in the methylene and methionine groups, which attribute to the changes in the skeletal structure of the fibrous protein like collagen in the ANFL. This confirmed the complete hydrolysis of collagen during the 48 and 72 h of fermentation. The ^{1}H NMR spectrum shows increases of fermentation products δ 2.8–δ 4.2, which constitutes acetic acid, propionic acid, and valeric acid production at 72 h (Correia et al. 2005). The spectra of the 96 h illustrate the disappearance of the major amide peaks. In 120 h

fermentation period the methylene and methionine groups appeared and this may be due to the diffusion of the peptide molecules from unhydrolyzed ANFL into the extracellular fermentation medium. This also sturdily explains the role of Tween 80 in enhancing the hydrolysis and formation of metabolites under anaerobic condition.

Absence of Tween 80

There is no change in the Proton Nuclear Magnetic Resonance spectroscopy (^{1}H NMR) spectrum of fermented products of ANFL during 24 and 48 h when compared to the initial sample. The changes in the chemical shift corresponding to protein occur at 72 h, which was observed at 24 h in the presence of Tween 80. The indication of the onset of breakage of collagen molecules starts from the 96 h onwards in the control and at 48 h in the presence of Tween 80. The spectra of the 96 and 120 h resemble one and another containing unhydrolyzed proteins along with lower amount of fermented products.

FT-IR analysis of extracellular fermentation products

Presence of Tween 80

Effect of Tween 80 in ANFL degradation was analyzed using the FT-IR analyses of the degraded product up to 120 h. The FT-IR analyses of extracellular fermented medium showed N-H stretching vibrations of degraded proteins at 3431 cm^{-1} and N-H bending of peptides around 1630–1660 cm^{-1}. The doublet peaks present at 1635 and 1655 cm^{-1} showed the peptides were in the form of β-sheet and α-helix, respectively. The C-N stretching vibration of peptides is visualized at 1404 cm^{-1}. The fermented product also exhibits a broad peak at 1083 cm^{-1} transmission in this region, which is attributed to C-O vibration due to the carbohydrates which relate to the degradation of the lipids present in the ANFL. The asymmetrical and symmetrical stretching occur, respectively, near 2926 and 2863 cm^{-1}. The scissoring vibration of methylene groups was exhibited a weak band at 1461 cm^{-1}. The methylene rocking vibrations appear around peaks at 600–700 cm^{-1}. The methylene groups are the main groups present in the skeletal of the proteins. The aromatic group present in the amino acid of the peptide chain exhibits a strong C-H out of plane deformation band at 884 cm^{-1}. Thus, the 24 h sample shows the protein and lipids are the major degraded products present in the extracellular fermented samples. The fermentation products of the 48 h samples showed no remarkable changes from the 24 h, sample except the decrease in the α-helix structure of proteins. Apart from the 24 and 48 h samples a week symmetrical N-H bending of amino acid was present at 1572 cm^{-1} and symmetrical C-O stretching of amino acid around 1450 cm^{-1}. The peak at 827 cm^{-1} showed the aromatic ring deformed structure. These peaks confirmed the proteins hydrolysis to amino acid after 72 h of fermentation. There is no functional group changes occuring between 96 and 120 h spectrum. They confirmed the presence of amino acids.

Absence of Tween 80

The FT-IR spectrum of fermented samples in absence of Tween 80 was observed for 24, 48, 72, 96, and 120 h. The spectrum of 24 and 48 h does not reveal any degradation products whereas 72 h shows presence of degraded proteins as peptides linkages. In 96 h of spectrum also shows the changes in the structure of the degraded proteins from β-sheet to β-turns. There is no major difference between 96 and 120 h sample.

TEM and SEM analyses of morphological changes in ANFL during fermentation

The analyses using TEM and SEM provide more comprehensive information of the collagen matrix structure. The TEM micrographs of the ANFL samples display the microstructure of the collagen fibrils at ultra-structural level. The collagen fibers offer the structural stability to the ANFL (Provenzano and Vanderby Jr 2006). The fibrils exhibit typical cross-striation patterns with periodicity (Fig. 4a). The increasing enzymatic activity at 72 h results in the nonuniform collagen matrix deformation (Fig. 4b).

On the other hand, SEM analyses by scanning the ANFL samples reveal the surface distribution of nonfibrillar proteins (Fig. 5a). The enzymatic activity modulated the intracellular packing of fibrillar and nonfibrillar protein present in ANFL. This resulted in the micro structural changes in ANFL tissues and

complete hydrolysis of fibrillar proteins (Fig. 5b). This result corroborates with the research findings of Krystyna (2003).

Process variables influencing of ANFL hydrolysis in presence of Tween 80

For the determination of the process variables that directly influence of hydrolysis, oxidation reduction potential (Eh) was considered as a linear function of extracellular protein, extracellular amino acid, ammonia, volatile fatty acid, pH, and carbon dioxide.

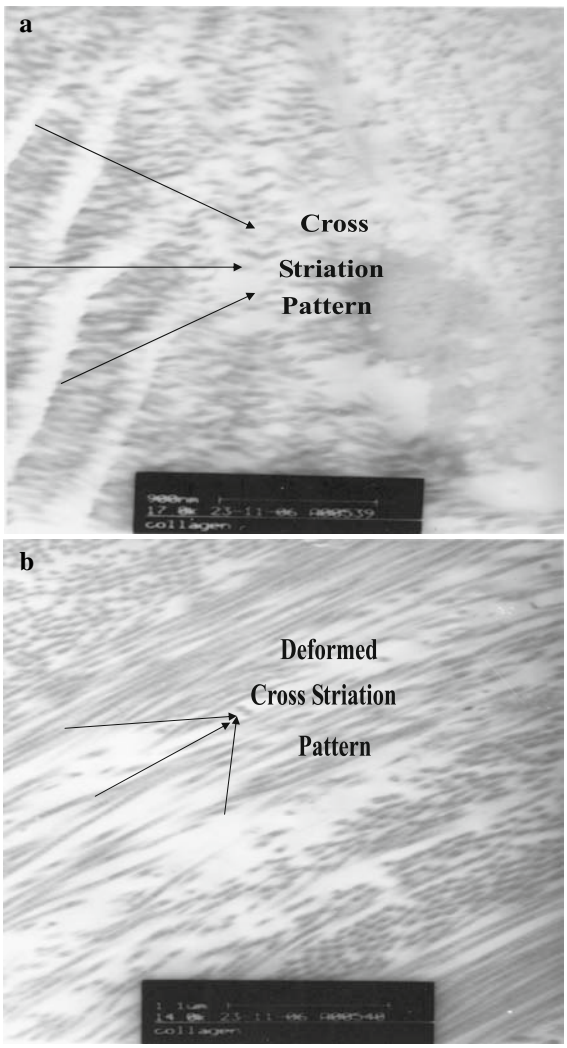

Fig. 4 (**a**) Transmission electron micrograph of undamaged collagen fibers in ANFL. (**b**) Transmission electron micrograph of damaged collagen fibers. Complete mineralization of collagen were well visualized in ANFL

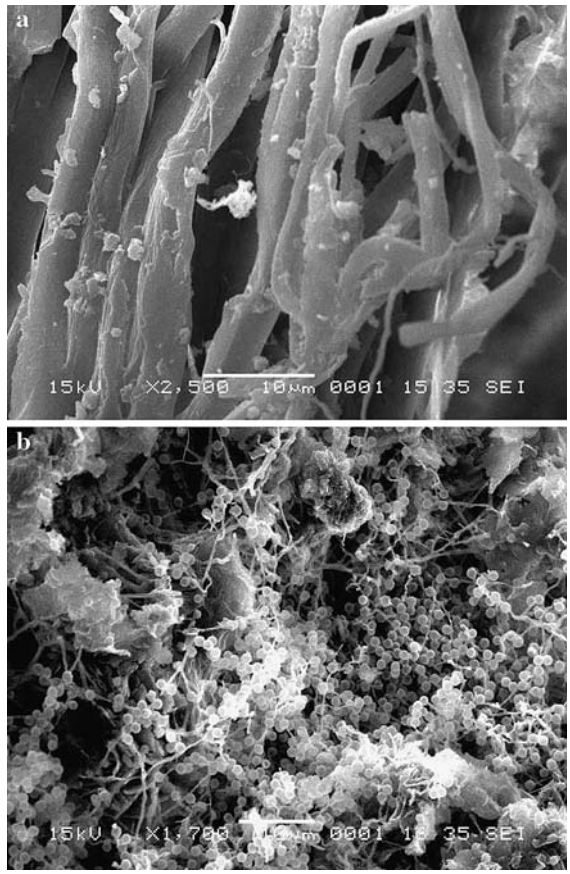

Fig. 5 (**a**) SEM image of unhydrolyzed ANFL. Longitudinal and vertical fibrillar tissue fibers been visualized. (**b**) SEM image of ANFL after treatment. The micro structural arrangements been altered and fibrillar tissues were damaged completely

The higher enzymatic profile within 72 h, results in release of more soluble components from ANFL, which ultimately results in rapid decrease of Eh. Therefore, Eh along with hydrolytic enzymes should probably be a parameter taken into account during industrial fermentation using anaerobic microorganisms. The process variables are a direct measure of the dependence of Eh upon the various aforesaid parameters.

$$Y_{(\mathrm{Eh})} = a_1(\mathrm{COD}) + a_2(\text{amino acid}) + a_3(\text{ammonia}) + a_4(\text{volatile fatty acid}) + a_5(\mathrm{pH}) + a_6(\text{carbon dioxide}).$$

The process variables were calculated following iterative methods and the results yielded the following equation

$$Y_{(\mathrm{Eh})} = (-)1.6(\mathrm{COD}) + (-)3.2(\text{amino acid}) + 1.7(\text{ammonia}) + (-)0.18(\text{volatile fatty acid}) + 2.7(\mathrm{pH}) + (-)0.08(\text{carbon dioxide}) + 199.$$

Being the most important linear variable affecting the oxidation reduction potential, pH had the highest regression coefficient ($a_5 = 2.7$), followed by ammonia ($a_3 = 1.7$). The process variables influencing ANFL fermentation fits the first order linear equation upon solving each of the equations. These results illustrate the inter-relationship of the COD, amino acids, ammonia, VFA, and Eh in the process of fermentation. The applications of these biochemical activities of anaerobic bacterium *Synergistes* sp. in environmental technology still holds a lot of promise for the future.

Economic feasibility of the process in presence of Tween 80

Additional cost required for the usage of Tween 80 for hydrolysis and fermentation was calculated to be US $0.8–1.0/kg of ANFL. The residual COD for experiment in presence of Tween 80 is about 100 mg l (much lower than the dischargeable limit), however for experiment in absence of Tween 80 is about 810 mg l (about eight times higher). In order to meet the permissible dischargeable standard, the hydrolysis and fermentation under anaerobic condition in absence of Tween 80 has to be coupled with some other treatment process, which requires the additional costs like initialization cost, operational cost, and maintenance cost etc. If this is taken into consideration, the addition of Tween 80 makes the entire process economically viable. Moreover the production of enzymes like protease, lipase and deaminase have been increased to 1.5–2.5 times higher in the addition of Tween 80 when compared to the control. When these are considered as value added by-products, the addition of Tween 80 becomes economically more viable process.

Conclusions

The nonionic surfactant Tween 80 (0.15%) increased the overall stability of both the hydrolytic and fermentative enzymes compared to the control throughout the fermentation period. The hydrolytic enzymes, protease activity reached maximum of 338–360 Um l^{-1} after 72 h and lipase activity reached maximum of 23–31 Um l^{-1} at 24 h of ANFL fermentation. The fermentative enzymes Deaminase activity reached a maximum of 187–206 Um l^{-1} after 72 h ANFL fermentation. The GC-MS analysis of anaerobic fermentation of ANFL showed the production of valuable organic compounds like acetic acid, propionic acid and valeric acid. The conformational changes and extracellular metabolites formation were confirmed by the ^{1}H NMR and FT-IR analyses. The SEM and TEM results confirmed the complete breakdown and disintegration of the collagen fibers in ANFL. The process variables of Eh fit the first order linear equation. These observations indicate that using nonionic surfactants (Tween 80) in ANFL fermentation could potentially increase ANFL utilization by microorganisms and in turn improves metabolic conversions.

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