

Enzymatic activity and production of pyruvic acid by *Clostridium sp.* fermentation of proteinaceous macromolecules

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Abstract

Animal fleshing (ANFL), the predominant proteinous solid waste generated during processing of leather confronting with disposal problems. ANFL was characterized by elemental composition carbon 36.35%, Hydrogen 8.41%, Nitrogen 11.62% and sulphur 0.97%. ANFL was considered as an organic substrate for the biotechnological production of pyruvic acid due to its rich concentration of organic carbon. The production of pyruvic acid was monitored during the hydrolysis and fermentation of ANFL by *Clostridium sp.* Protease enzyme showed maximum activity of 1200-1350 U/mL on third day. The identification of the pyruvic acid was confirmed by FT-IR, HPLC and GC-MS. Scanning electron micrograph confirmed the selective removal of nonfibrillar proteins like albumin and globulin by protease enzymes during initial stages of hydrolysis. The onset of collagenolytic activity after the hydrolytic stage (after 3rd day) resulted in breakdown of collagen fibers present in fleshing. The hydrolytic enzymatic activity resulted in the release of peptides and amino acids in the exocellular medium. The fragmentation of non fibrillar proteinaceous macromolecules and their conversion into lower molecular weight peptides and amino acids were confirmed through SDS-PAGE and H-NMR respectively. ANFL was fermented into pyruvic acid ranging from 20 to 22.5 mg/g by *Clostridium sp.* The hydrolytic enzymatic activity decreased after the onset of amino acid fermentation stage proving its repressive effect on enzymes.

Keywords: protease, lipase, collagenase, fermentation, animal fleshing (ANFL), pyruvic acid

Introduction

In industry, pyruvic acid is employed for the product of L-Tryptophan, L-Tyrosine and 3,4 di-hydroxy phenyl alanine (Nakazawa et al., 1972; Yamada et al., 1972). Pyruvic acid is used in bio-chemical researches and medicine as a substrate, for assaying activities of enzymes like pyruvate de-hydrogenase (PDH), pyruvate carboxylase and pyruvate decarboxylase. It was revealed that diet supplementation with pyruvic acid increases fat loss and minimized the associated loss of body protein (Stanko and Teitze, 1992). Under certain conditions bacteria (Katagiri et al., 1957; Izumi et al., 1982; Yokota and Takao, 1989; Yokota et al., 1994), Basidiomycetes (Takao and Tanida, 1982) and yeasts (Moriguchi, 1982; Yonehara and Miyata, 1994) produce pyruvic acid in excessive amounts.

Leather manufacturing process generates solid wastes higher than that of finished leather (Maire and Lipsett 1980). In India, approximately 1,50,000 tones of offals from tannery in the form of raw hide (Skin) trimmings, limed fleshings, green fleshings, hide splits and chrome shavings were available during leather processing which are not utilized or are under utilized, thus creating a solid waste disposal problem in tanneries (Muralidhara Rao, 1994). Thus, the tanneries are confronted with solid waste disposal/ byproducts utilization problems of different dimensions. They are resorted with either minimizing the quantity of waste generated or maximizing the return by producing value added products (Alexander et al., 1991). Land co-disposal of solid wastes has been widely practiced and it was a rather expensive and environmentally inappropriate way of handling a waste material which has the potential for reutilization.

Although, few reports in the literature describe the recovery of proteins from various tannery residue by alkaline / acid hydrolysis (Betaille et al., 1985 and Pichugin et

al.,1986). Many researches have demonstrated the feasibility of isolating protein products from chrome shavings with the use of alkaline protease under mild conditions(Taylor, et al., 1996; Annapurna et al, 1997). However, the crude form of protein contains a substantial degree of impurities. Thus the present work focuses on microbial hydrolysis and fermentation of tannery ANFL into pyruvic acid. Generally, sugars are primary substrates for production of pyruvic acid (Axelsson 1998). However, some anaerobic microorganisms produce pyruvic acid from other substrates other than sugars using pathways different from those of sugar fermentation. These substrates include polyols, organic acids and amino acids (Liu 2003). Amino acids are potential substrates for pyruvic acid production and it can be produced directly or indirectly by transamination. Effective degradation or hydrolysis of ANFL is difficult due to their stable hard-core protein, apart from bound loose proteins thus, industrial utilization of these proteins have been little developed. In this present investigation, we report on the interrelationship of enzyme synthesized by *Clostridium* species to degrade and hydrolyze proteineous macromolecular solid waste. The processes were then characterized for pyruvic acid production through ANFL hydrolysis into amino acids and fermentation of amino acids into pyruvic acid.

Experimental Part

Materials and microorganisms

The limed fleshing was collected from a commercial tannery and was treated with 10ml l⁻¹ hydrochloric acid solution for about 3-4 hours to remove the adsorbed lime. The delimed fleshing was suspended in water and the pH was adjusted to 7.0 ± 0.2. The fermentation was conducted in a serum bottle of 125ml capacity in anaerobic incubator at 37° C. To this 100ml minimal medium of composition NaCl 0.9g, NH₄Cl 0.5g, NaHCO₃ 0.1g, KH₂PO₄ 0.5g, MgCl₂ 0.01g and cysteine HCl 0.05g per liter was added. The trace

element solution 5ml was added containing 40g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 50g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.5g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, and 2g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ per liter. The reactor batch media was autoclaved for 20 minutes at 121°C . The trace element solution was filtered separately and added. Delimed proteineous solid waste ANFL one-gram containing moisture content 65-75% were weighed and used as substrate. The anaerobic proteolytic bacterial consortium *Clostridium* species was isolated from anaerobic sludge collected from biomethanisation plant and acclimatized *Clostridium* species was inoculated into minimal medium containing ANFL as substrate. The neck of the bottle was filled with nitrogen gas and incubated at 37°C .

Enzymes assay and CHNS analysis

Protease, lipase and collagenase activity were measured as previously described (Brock et al., 1982, Lakshmi et al., 1999, Matsushita et al 1994). The percentage of carbon, hydrogen, nitrogen and sulphur was determined using Elementar Analysen systeme GmbH vario EL, German CHN analyzer.

Analytical procedures

The treated and untreated samples were characterized for COD (Closed reflux, titrimetric method), TKN in accordance with standard methods for analysis of water and wastewater (Lenore et al 1989). Protein reacts with Folin's reagent to give a blue colored complex and Optical density was taken at 650 nm (Lowry et al 1951). Ninhydrin reacts with free-alpha-amino group, NH_2 [-C-] COOH which group is contained in all amino acids, peptides or proteins (Rosen, **1957**).

Oxidation Reduction Potential

The oxidation reduction potential of the reaction were measured in relative milli volts using ion selective electrode, Orion 920 A⁺ Thermo Electron Corporation, USA. The electrode was standardized using ORP-Orion 967901 standard solution and was connected to anaerobic fermentor for online monitoring.

Pyruvic acid assay

The estimation of pyruvic acid was carried out using dinitrophenylhydrazine and estimated calorimetrically (Gordon 2003). In 900 µl distilled water 100 µl sample was added and to this 1 ml of DNPH in 1M HCl was mixed. The tubes were incubated at 37°C for 10 minutes in water bath. After cooling 1 ml of 1.5 M NaOH was added. The absorbance at 515 nm was then determined. Standards were prepared from sodium pyruvate at varied concentrations.

Fourier transform infrared spectroscopy (FTIR)

Fourier transform infra red (FTIR) spectra of the anaerobic treated samples were obtained in liquid phase using Perkin Elmer FTIR Spectrometer. The spectra were recorded in the frequency range 400 to 4000 cm⁻¹.

Scanning electron microscopy (SEM)

The ANFL substrate before and after hydrolysis was fixed for 2h in 2% (W/V) glutaraldehyde. After washing with saline solutions, they were dehydrated in 30- 100% water ethanol series. The air-dried particles were coated with 120- 130µm 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 voltage with a 5-6nm electron beam.

High Performance Liquid Chromatography (HPLC)

The fermented samples were separated through centrifugation and filtered 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. UV-Visible spectra were recorded at the peak maxima and were corrected for solvent background. The results were calculated using the standard volatile acids (Merck, India) as control.

Result and Discussion

The characteristics of ANFL are given in Table 1. The amino acid composition of ANFL is presented in Table 2 and Figure1.

Effect of Fleshing Size

Among the several factors that control fermentation processes, which are important for microbial activity and growth, the ANFL substrate particle size is the most critical. The smaller substrate particles provide a larger surface area for microbial attack. However, too fine powder like particles may result in substrate agglomeration and it result in lower microbial interaction with substrates ultimately resulting in poor growth. Much larger particles provide limited surface for microbial attack. In the present study, the effect of fleshing particle size on protease production was studied by employing four categories of

substrate particle size of 2.5, 5, 7.5 and 10 mm. Figure 2 illustrates the protease activity as a function of fermentation period for different size of the particles. The protease activity attained the maximum value on the fourth day and was correlated with microbial activity on varied fleshing sizes. The result indicated that the fleshing size of 2.5 mm for protease production (1593 U/ml) was higher than all other substrate sizes. The fleshing size 5, 7.5 and 10 mm yielded optimal protease production of 1150-1175, 980-997 and 845-856 U/ml, respectively. The ANFL size 2.5 mm was found to be optimal for higher protease production and thus higher microbial activity. In the subsequent experiments, therefore, the 2.5 mm size was used for the production of protease.

Proteolytic Enzyme Activity

The *Clostridium sp.* develops multiphasic enzymatic processes for the hydrolysis of the biopolymeric ANFL substrate. The availability of complex proteinaceous substrate induces the bacteria to secrete enzymes like protease extracellularly. Moreover, many microorganisms can grow only in medium containing protein hydrolysates. The enzymatic activity in the supernatant of the hydrolyzed fleshing was obtained after centrifugation at 10000 rpm for 10 minutes. The specific protease activity ranged from 1150 - 1175 U ml⁻¹ on 4th day. The proteolytic activity has the maximum activity similar to other experiment on protease of microbial origin (Ferrero et al 1996). Linear increase in protease activity was seen up to 4th day and on further fermentation the protease activity was decreased. The exact mechanism underlying in cessation of protease synthesis is only poorly understood. However, certain theories namely auto proteolysis and protease degradation by some proteolytic activity during fermentation have been reported (Jang et al 2001, Chu et al 1992).

Lipolytic Enzyme Activity

The lipase activity was very less throughout the experimental cycle and its activity reached a maximum of 80-110 U/mL in 4th day. Compared to the enzymatic activities protease activity was higher than that of lipase. This may be due to the presence of proteins in higher amount than lipids and fats. The higher proteolytic and lipolytic activity on 4th day of experimental cycle proved hydrolysis was maximum at that process time.

Collagenolytic Enzyme Activity

The collagenase activity reached a maximum of 70-85 U/mL on 5th day. More collagenase activity was observed only after protease activity was ceased. This may be due to the presence of bounded non-fibrillar proteins like globulins and albumins in which the fibrillar protein were embedded. The non-availability of nonfibrillar proteins and the availability of fibrillar protein induce the secretion of collagenase. The higher collagenolytic activity on 5th day of experimental cycle proved hydrolysis maxima of collagen in that process time.

Effects of Amino Acids in Protease Activity

Protein hydrolysis by protease was limited by composition of amino acid in the exocellular medium. The increase in amino acid content in the exocellular medium to 303.5 mg/L in fourth day and 461.5 mg/L in fifth day caused rapid decrease in protease activity. The protease enzyme activity was maximum on fourth day up to 1680 U/mL, however this activity was reduced rapidly in successive days with increase in amino acid

content (Figure 3). Peptides containing high proportion of hydrophobic amino acid residues were degraded more slowly than the peptides rich of hydrophilic residues. The protein hydrolysis constant k_m values of each fraction were similar, but the V_{max} of the hydrophilic peptides was more than two times that of hydrophobic peptides (Chen et al 1987). The amino acids like alanine, lysine, arginine and glutamic acid decreases the protease activity (Rahman et al 2005). Mean while glycine completely inhibited the protease activity (Ikura and Horikoshi 1987). Enzyme synthesis could be repressed rapidly by metabolized nitrogen sources such as various amino acids concentrations in liquid media. Some researchers had reported that molecules present in the culture supernatant, which include amino acids and peptides, also lowered the proteolytic activity through end product inhibition (Ferrero et al.1996). Amino acids repress the synthesis of protease at the level of mRNA transcription. End product inhibition of microbial extracellular protease by amino acids had been widely reported. For example, amino acids such as tryptophan, proline, tyrosine and isoleucine have been identified as specific repressors of enzyme production in *Clostridium sporongene* (Allison and Macfarlane 1990).

The degree of hydrolysis of fleshing depended on enzymatic activity. The hydrolysis of high polymers to monomers appears to be the rate-limiting step in the biodegradation process (Ubukata 1998). The higher proteolytic and lipolytic activity on 4th day resulted in higher fleshing hydrolysis and maximum free amino acid presence could be detected at that particular time. The amino acids content were higher on 5th day and 6th day ranging from 300-450 mg/L. However, complete hydrolysis was not

achieved even on further incubation, this implies that part of proteineous macromolecule was resistant to microbial attack and not preferentially degraded (Breure and Andel 1984). The formation of pyruvic acid proves the fermentation of amino acids continues after the hydrolysis of ANFL is stopped. The maximized release of appreciable quantities of amino acids resulted in maximum production of pyruvic acid on 5th and 6th day of fermentation ranging from 20-22.5 mg/L. However, amino acid content was not fully reduced this implies that all the amino acids released were not completely metabolized.

Pyruvic Acid Production

Pyruvate occurred on 5th day from the day of inoculation (Figure 4). High level of pyruvate led to a cessation of culture growth and after pyruvate accumulation the substrate conversion was stopped, but the pyruvate formed was converted to CO₂ and it reached 1150 ± 27.6 ppm on 6th day. The enzymatic activity of the bacterial consortium causes conversion of peptides and amino acids to pyruvate. This quick conversion of fleshing to other metabolites seems to be impaired after pyruvate step. The production of pyruvate is comparatively less for prokaryotic than eukaryotic microorganisms thus indicating a decrease in pyruvate dehydrogenase activity (Li et al.2001). The arrest of growth phase of *Clostridium sp.* decreases the pyruvic acid production level. As can be seen in Figure 3, amino acid increase coincides with the increase in the pyruvic acid production rate.

FTIR Analysis

The FTIR spectrum obtained extracellular medium after fermentation are given in Figure 5. In the spectrum two absorption bands at frequencies of 1651 and 1644 cm^{-1} originate from C = C stretching vibration of ketone and additional conjugational vibrational frequency. The symmetrical stretching of OH bending showed band frequency at 1247 cm^{-1} . The band at 1458 cm^{-1} arises from carboxylate ion stretching vibration. The band near 1400 cm^{-1} is generally weak and it appears for C-O stretching vibration.

HPLC Analysis

The HPLC analysis of the fermented medium was shown in Figure 6 reveal that the major constituent at retention time (R_t) of 3.52 corresponds to pyruvic acid and other major constituent at (R_t) of 2.54 corresponding to butyric acid. The percentage of propionic acid was about 39.94 and that of acetic acid was 21.49. The retention time for butyric acid (R_t) of 7.24 was 1.83 %. The HPLC profile of VFA also confirms the presence of other fatty acid metabolites at (R_t) of 2.12, 3.96 and 4.07, however, the chemical nature of the metabolites could not be identified.

Oxidation Reduction potential

The Figure 7 showed the interaction point that occurred in the ORP – pH versus time profile. The hydrolytic activity and release of peptides and amino acids was confirmed based on the ORP, pH and time profile. As shown in Fig 7, the interaction point was reproducible enough for all cyclic operation to be exploited for control purposes. The

duration of the fermentative state occurring in this process might play an important role on the efficiency of the system. The start up of the fermentative phase after hydrolytic phase resulted in the onset of reduction potential and after the cessation of multiphasic enzymatic hydrolysis the reduction potential was stabilized. This indicates the conversion of amino acids to pyruvic acid. Moreover the fermentation pH also showed relationship with reduction potential. The fermentation pH in reactor decreased gradually during hydrolytic phase during which more amino acids were released and attained stability during fermentative phase of amino acids.

SEM analysis

The scanning electron micrographs of the ANFL sample without treatment shows prominent attachment of loose proteins and clumpy clusters of bounded tissues were seen. Moreover, collagen fibers were not visualized proving there is no enzymatic activity during onset of fermentation (Figure 8). The loose proteins were detached well after the onset of the hydrolysis. Further, the clustered tissue cells were not visualized (Figure 9). ANFL after hydrolysis showed some clear spaces, such spaces were occupied by liquids and hydrolyzed peptides produced as a consequence of hydrolytic enzymes. The selective images in Figure 10 was chosen so as to describe the changes occurred after complete hydrolysis. More breakage of tissue fibers was seen. In many places fiber-to-fiber detachment were visualized. Moreover the structural changes were not uniform over the samples. Thus, some areas of the samples were significantly more affected than others. The Figure 11 shows the presence of more thick fibrous material, which correspond to collagen. The collagen fibers were visualized during the middle stages of

fermentation this may be due to higher proteolytic activity. This enzymatic activity hydrolyses the loose bound and globular proteins making the fibrillar protein collagen to be visualized. The collagenase activity started after protease activity, this may be due to readily available loose proteins and non-availability of collagenolytic substrate. Our result indicates that the onset of the enzyme release resulted in very rapid structural changes in ANFL.

Conclusion

From above mentioned results the following conclusions can be derived. The synergistic enzymatic action in this process effectively hydrolyzes ANFL. The increasing amino acid content affected the proteolytic activity. The loosely bound proteins were hydrolyzed followed by hydrolysis of fibrillar proteins. The reduction potential of the fermentation process correlates the enzymatic hydrolytic phase of proteineous macromolecules into amino acids and fermentative pyruvic acid production phase from amino acids. The pyruvic acid produced from anaerobic fermentation of amino acids can be obtained as valuable product from proteineous solid waste instead of complete disposal.

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Figure legends

Table 1. Characteristics of ANFL

Table 2. Amino acid composition of ANFL

Figure.1 Amino acid analysis of ANFL

Figure2. Various particle size of ANFL as a function of enzymatic activity

Figure 3. Effects of amino acids on protease activity

Figure 4. Variation of amino acids, pyruvic acid as a function of fermentation period

Figure 5. FTIR analysis of fermentation product of ANFL

Figure 6. HPLC analysis of fermented product of ANFL

Figure 7. Oxidation Reduction Potential versus Fermentation pH

Figure. 8. Scanning Electron Micrograph of untreated ANFL sample

Figure. 9. Scanning Electron Micrograph of 3rd day Hydrolyzed ANFL

Figure. 10. Scanning Electron Micrograph of 5th day Hydrolyzed ANFL

Figure. 11. Scanning Electron Micrograph of Collagen fibers in ANFL

Figure 12. . Scanning Electron Micrograph of Cleavage of collagen fibers

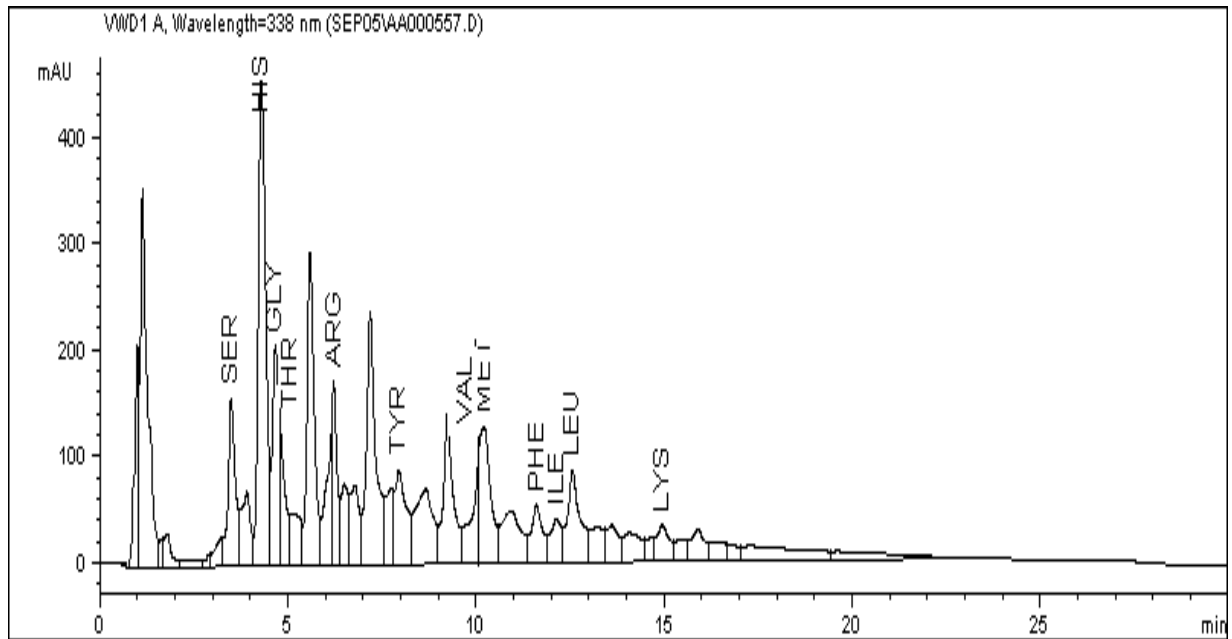


Fig. 1. Amino acid analysis of ANFL

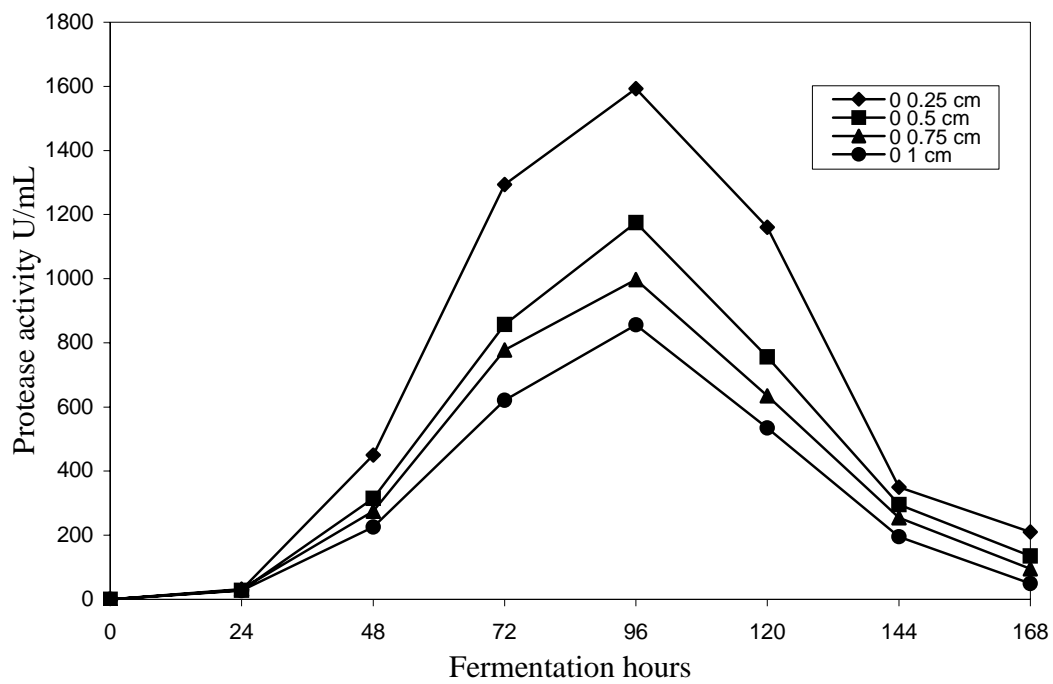


Fig. 2. Effects of particle size of ANFL as a function of enzymatic activity

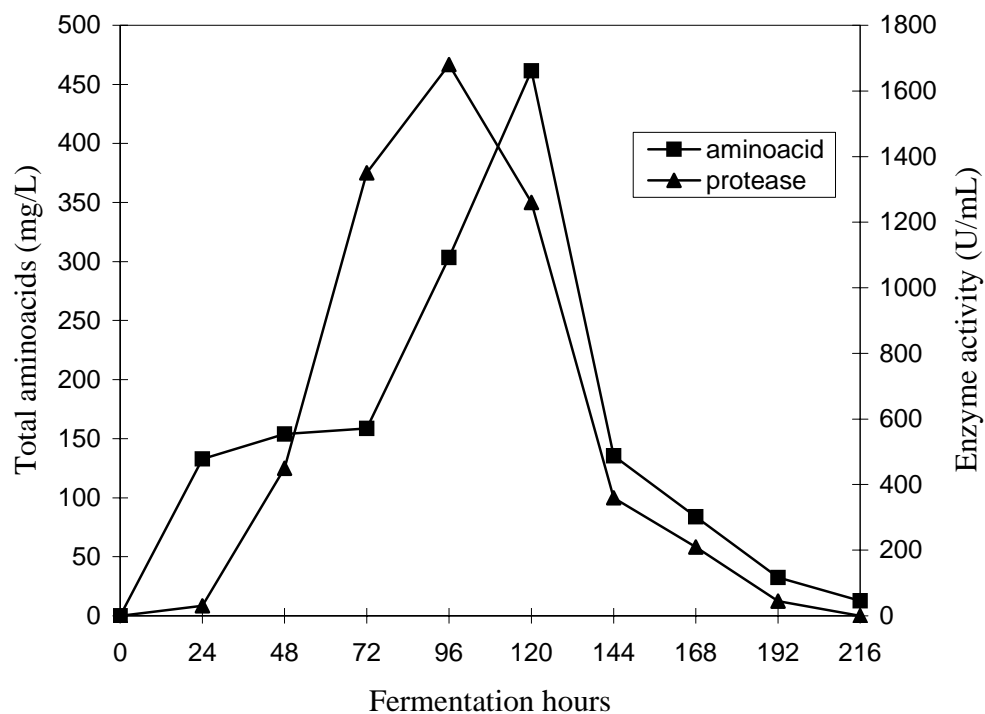


Fig. 3. Effect of amino acids on protease activity

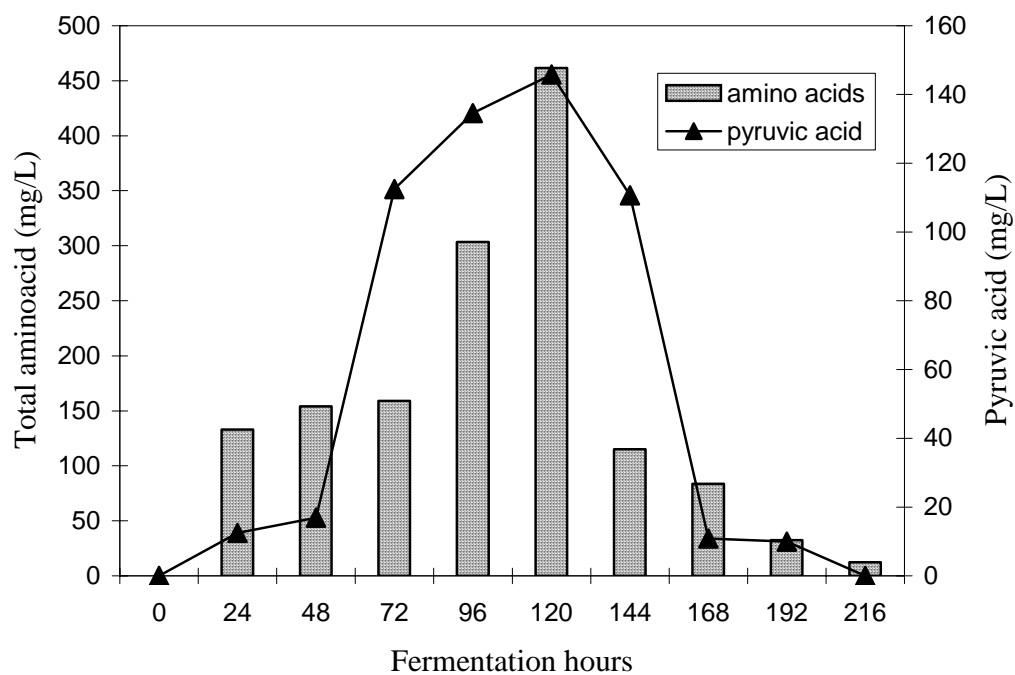


Fig. 4. Variation of amino acids, pyruvic acid as a function of fermentation period

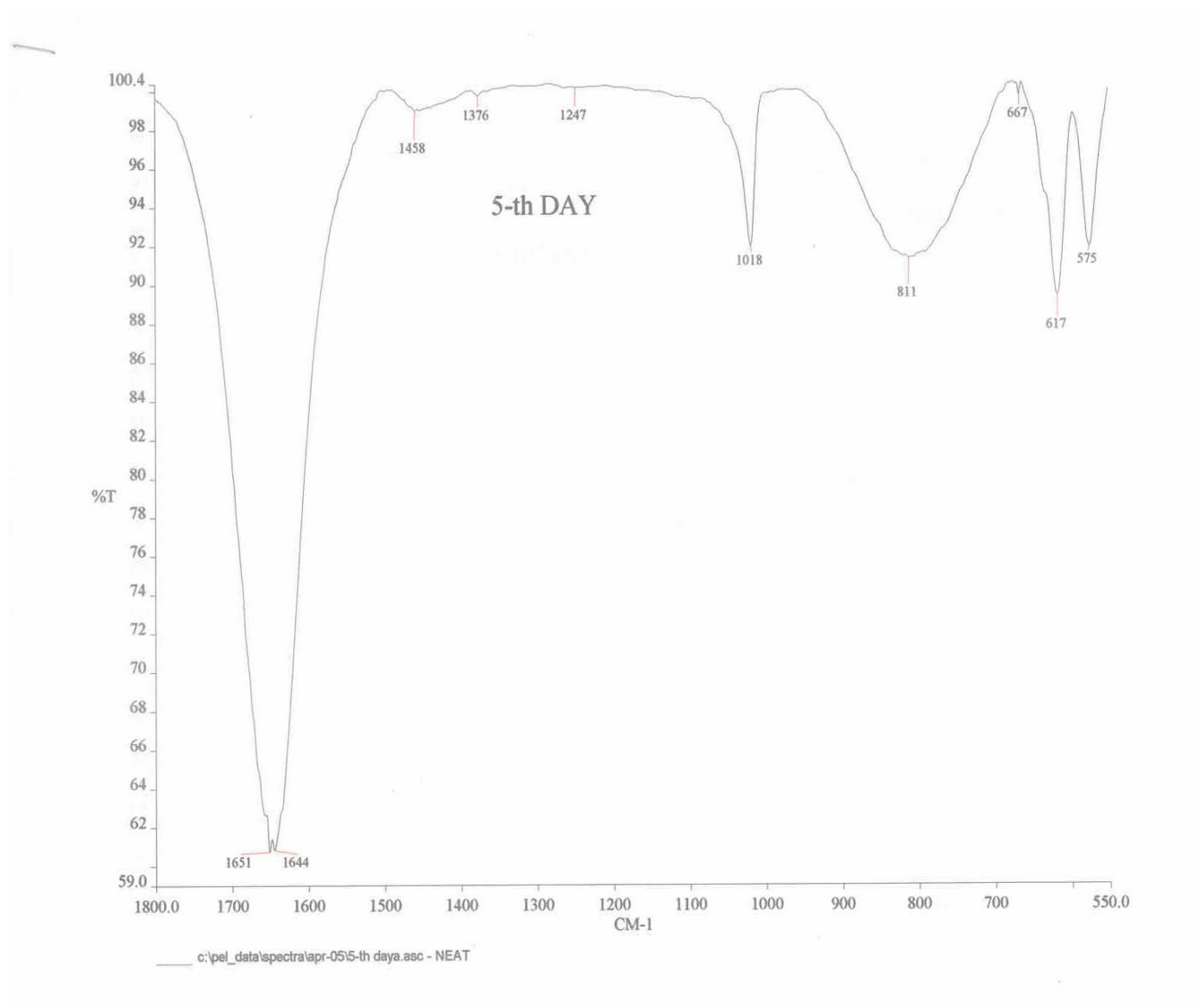


Fig. 5. FTIR analysis of fermentation product of ANFL



Fig. 6. HPLC analysis of fermented product of ANFL

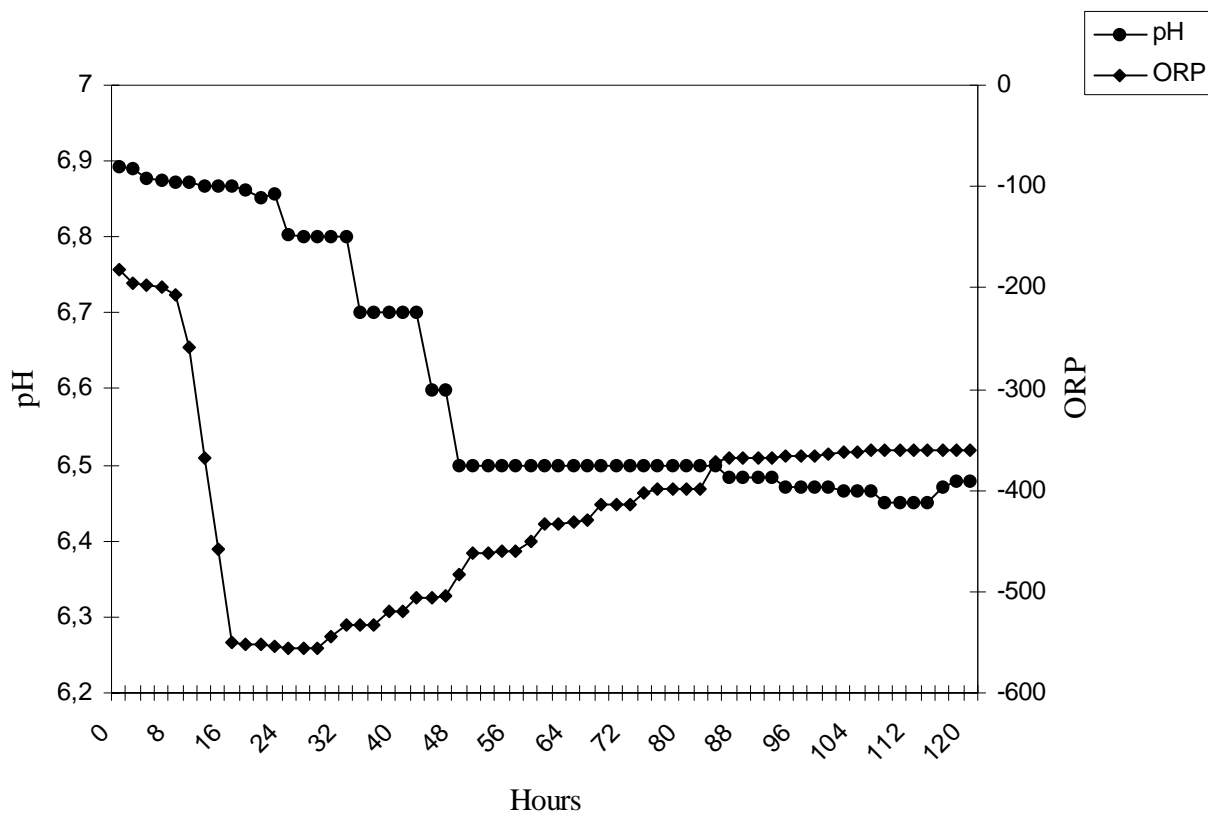


Fig. 7. Oxidation Reduction Potential versus Fermentation pH

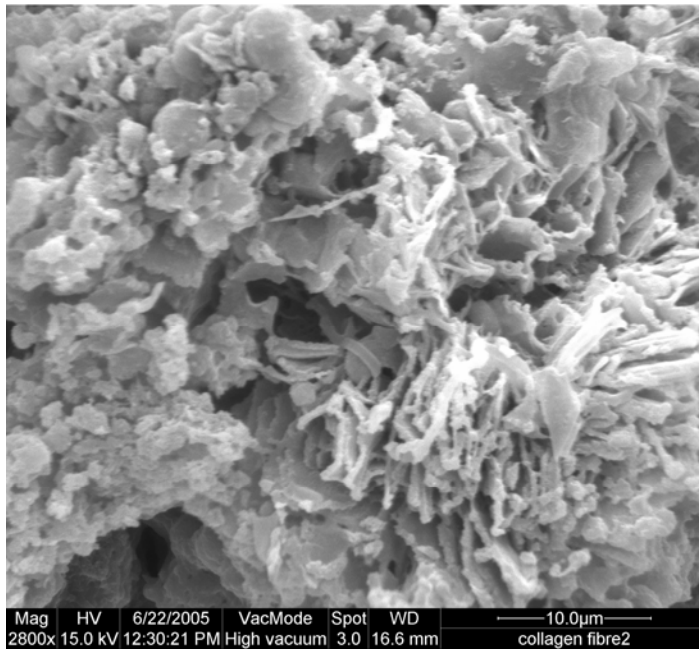


Fig. 8. Scanning Electron Micrograph of
unhydrolysed ANFL sample

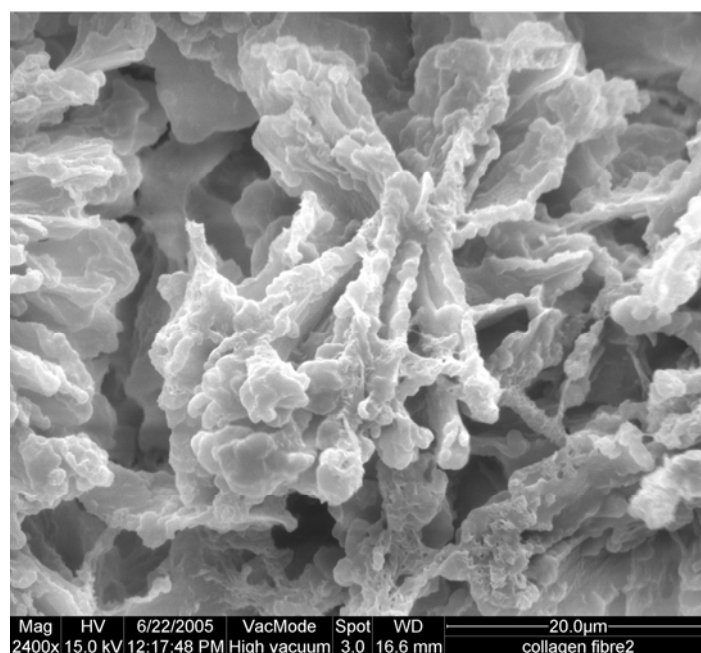


Fig. 9. Scanning Electron Micrograph of 72 hour-hydrolyzed ANFL

Table 1. Characteristics of ANFL

Parameter	
Total Organic Carbon mg/G	342 ± 21.2
Total Kjeldhal Nitrogen mg/G	138 ± 14.5
Moisture content (%)	77.5 ± 5.5
Ash content (%)	51.5 ± 2.5
Volatile solids (%)	30.5 ± 3.5
CHNS analysis of ANFL	
C (%)	36.14 ± 0.58
H (%)	8.31 ± 0.15
N (%)	11.6 ± 0.3
S (%)	0.656 ± 0.15

Table 2. Amino acid content of ANFL

Aminoacid level in μM / gm 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