Production of alkaline protease by *Pseudomonas aeruginosa* grown on proteineous solid waste

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Abstract

Animal fleshing, the predominant proteinous solid waste generated during processing of leather confronting with disposal problems, were used as substrate for the production of alkaline protease by *Pseudomonas aeruginosa*. The substrate was characterized by elemental composition carbon 36.35%, Hydrogen 8.41%, Nitrogen 11.62% and sulphur 0.97%. The strain isolated strain salt laden wastewater (soak liquor) of tannery was selected by its ability to greater protease production. The strain produced alkaline protease of activity 1160-1175 UmL^{-1} when cultivated in minimal medium containing only fleshing as substrate. The production of alkaline protease was accompanied with hydrolysis of fleshing into peptides and amino acids. Scanning electron micrographs of animal fleshing confirmed the selective removal of nonfibrillar proteins like albumin and globulin by protease enzymes during initial stages of hydrolysis. The breakdown of fleshing and its conversions were confirmed by H-NMR.

Introduction

Leather manufacturing process generates quantities of byproducts and solid wastes higher than that of finished leather (Maire & Lipsett 1980). Highly polluting solid wastes containing varied quantities of protein are currently being wasted due to their non-utilization. One metric ton of wet salted hides yield 200 kg of leather, along with about 250 kg of tanned solid waste and about 350 kg of non-tanned waste and 100 kg is lost in the wastewater. In India, approximately 1,50,000 tones of tannery offals in the form of raw hide trimmings, limed animal fleshings, green animal fleshings, hide splits and chrome shavings were available during leather processing which are not utilized or under utilized, thus creating a solid waste disposal problem in tanneries (Muralidhara rao, 1994). Leather industries confront with disposal of solid wastes and they are responded with two strategies (I) minimizing the quantity of waste generated and (II) maximizing the return on byproducts (Alexander et al. 1991). However, the biological production and recovery of much other value added products have been largely a neglected field. Many research findings have demonstrated the feasibility of isolating protein products from chrome shavings with the use of an alkaline protease under mild conditions and were used worldwide with some modifications (Taylor et al. 1996). The present work focuses on utilization of proteineous solid waste fleshing for the production of alkaline protease.

The microbial proteases are commercial enzymes, which have found wide applications in various industrial, biotechnological, medicinal and basic research fields (Malla et al. 1998; Wiseman 1993; Gupta et al. 2002; Kumar & Takagi 1999). Alkaline proteases have considerable application in leather tanning industry (Godfrey & Reichelt 1985; Annapurna et al. 1997; Dayanandan et al. 2003). Pseudomonas sp. (Chakraborty & Srinivasan 1993) and Vibrio sp. (Kwon et al. 1994; Chengfang & Xiaolu 2005) were some gram-negative bacteria producing alkaline proteases.

Protease enzyme has been produced using various substrates like Shrimp and crab shell powder (Wang et al. 2005), Soya bean meal (Joo & Chang 2005), nug meal (Gessesse et al. 1997), pigeon pea waste (Johnvesly et al. 2002) and fish (Yosra et al. 2003). Enhancement in extracellular protease production from the newly isolated Pseudomonas species from tannery fleshing by optimization of fermentation conditions has not been attempted so far. Therefore, considering the applications of alkaline protease, we report here the optimization of enzyme production by *Pseudomonas aeruginosa* from animal fleshing (ANFL).

Materials and methods

Animal fleshing (ANFL)

The substrate ANFL was collected from tannery division of Central Leather Research Institute and was treated with ammonia solution for about 3-4 hours to remove the inorganic salts. The characterizations of ANFL were given in (Table 1). The amino acid composition of ANFL was given in (Figure 1). The delimed fleshing was suspended in water and the pH was adjusted to 7.0 ± 0.2. The washed fleshing was cut into pieces approximately (0.25 cm, 0.5cm, 0.75 cm, 1 cm) and stored at 4°C until the startup of experiments.

Isolation and cultivation of microorganisms

The ANFL samples collected from the tannery were plated in Skim milk agar plates and were incubated at 37°C for 24 hours. A clear zone of hydrolysis gave an indication of protease producing organisms. The grown organisms are cultivated in media containing $MgSO_4.7H_2O$(0.2g/L); K_2HPO_4(2.0g/L); KH_2PO_4(2.0g/L) and supplemented with Bovine serum albumin (1.0g/L) and were assayed for checking higher proteolytic activity. The protease activity was determined after 24 hours of incubation. The strain showing higher proteolytic activity was selected and screened using standard biochemical procedures. The Biochemical analysis showed gelatinase, Arginine dihydrolase positive, nitrate reduction to nitrite and pigment pyocyanin production. This confirms the isolated strain showing higher proteolytic activity as *Pseudomonas aeruginosa*.

Isolation of exocellular polysaccharides

At the stationary phase of the growth cycle bacteria were sedimented by centrifugation, known volume of supernatant were taken and three volumes of acetone were added to precipitate the EPS (Dudman 1976). The EPS were collected by centrifugation at 6000 · g for 20 min, dissolved in minimal volume of double distilled water, reprecipitated with three volumes of acetone, centrifuged at 6000 · g for 20 min, resuspended in double distilled water, dialyzed and lyophilized.

Experimental design

The effect of pH was observed in close range from 6 to 11 in 100 mL mineral medium inoculated with 2%(V/V) freshly grown culture and incubated at 37°C. The growth was observed by determining the absorbance at 600nm. The alkaline protease production by the P. aeruginosa was optimized by conducting the experiments in 250 mL Erlenmeyer flasks containing ANFL. Freshly prepared bacterial suspension were inoculated during the onset of experiments and incubated at 37°C. After fermentation, the cell free supernatant was obtained by centrifuging at 8000 rpm and the extracellular protease activity of the fermented broth determined. Experiments were conducted in triplicate and results were given.

Analytical procedures

Protein and amino acids were analyzed by methods of Lowry et al. (1951) and Rosen (1957). Alkaline protease activity was measured using casein (1% W/V solution) as substrate in glycine-NaOH buffer (10.5pH, 50 mM) for 20 min (Oberoi et al. 2001). One unit of protease activity was defined as the amount of enzyme required to release1mg ml^{-1} tyrosine in 1 min. under standard assay conditions. ⟨-amino group during hydrolysis were analyzed by methods of Edith and Constant (2003).

Scanning Electron Microscopy

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- 130⌠m 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.

Proton NMR

Proton NMR spectra for amino acids and peptide analysis was recorded with JEOL ECA 500 MHz spectrometer using deuterated water as solvent after water peak suppression.

Result and Discussion

In this study, the production of alkaline proteases by P. aeruginosa grown on animal fleshing (ANFL), the solid waste produced in large amounts by tanning industry, has

been investigated. The strain been confirmed by biochemical analysis. The EPS producing ability of the strain ranged from 345 -365⌈g / mL.

Effect of pH on protease activity

Microbial growth and metabolism inevitably lead to a change in the hydrogen ion balance and hence, the pH of the culture medium. The effect of pH on growth and protease production after P. aeruginosa inoculation in minimal medium is shown in Figure 2.The isolate was capable of growing in the pH range of 7-10 with maximum growth at 8.5. The production of alkaline protease substantially decreased above and below the optimum pH 8.5. At pH 8.0 the alkaline protease production increased and leveled off around 1165-1200 U/mL. Moreover, the growth of P. aeruginosa was also higher at 8.0 pH. Thus in the subsequent experiments, same pH was maintained throughout the experimental studies.

Effect of fleshing size

In the present study, the effect of ANFL particle size on alkaline protease production was studied by employing four categories of substrate particle size of 0.25, 0.5, 0.75 and 1cm. The protease activity reached maximum on 20^{th} hour and was correlated with microbial activity on varied fleshing sizes. The results indicated that the fleshing size of 0.25cm for protease production (1160-1175 U/ml) was higher than all other substrate sizes. The fleshing size 0.5, 0.75 and 1 cm yielded optimal protease production of 925-945 U/ml, 710-728 U/ml and 605-621 U/ml, respectively (Figure 3). The fleshing size 0.25cm was found to be optimal for higher protease production and thus higher microbial activity. In the subsequent experiments, therefore, the 0.25cm size was used for the production of protease. Among the several factors in fermentation processes, which are important for microbial activity and growth, the fleshing substrate particle size is the most critical. The smaller substrate particle will provide a larger surface area for microbial attack. However too powdery 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 and thus rate of reaction is minimized.

Proteolytic enzyme activity

The availability of complex proteineous substrate induces the bacteria to secrete enzymes like Protease extracellularly. Proteases are enzymes that catalyze the cleavage of peptide bonds, and they are present in all living organisms, playing a role in many physiological functions. The enzymatic activity in the supernatant of the hydrolyzed fleshing was obtained after centrifugation at 10000 rpm for 10 minutes. The alkaline protease activity was detected from early stages in the growth of the microorganisms, and the values was observed to increased exponentially at the end of the exponential phase, reaching a plateau during the stationary growth phase. The specific protease activity ranged from 1175 - 1200 U ml^{-1} in 20 hours (Figure 4). This strain produced appreciable level of alkaline protease using fleshing as the sole source of nitrogen and carbon source,

especially its ability for efficient and rapid hydrolysis of fleshing even in the absence of any supplement, makes it extremely interesting.

The proteolytic activity has a maximum activity similar to other experiment of protease from microbial origin (Ferrero et al. 1996). Linear increase in protease activity was seen up to 20 h and on further fermentation the protease activity was decreased. The exact mechanisms underlying in cessation of protease synthesis is only poorly understood. However, certain theories namely auto proteolysis (Jang et al. 2001) and protease degradation by some proteolytic activity during fermentation (Chu et al. 1992) have been reported.

Effects of amino acids in protease activity

The ⟨ - amino peptide content reached the maximum of 220-224 mg/L in 12 h in the extracellular medium and decreased on fermentation period. However, some peptide content ranging 45-48 mg/L was not converted into amino acids on further fermentation. The protein and peptides breakdown in ANFL caused increase in amino acid content in the exocellular medium 315-330 mg/L in 20 h and 444-460 mg/L in 24 h. The increase in amino acid content in the extracellular medium caused rapid decrease in alkaline protease activity. The protease enzyme activity was maximum in 20 h up to 1160-1175 U/mL, however on further fermentation this activity was decreased rapidly in successive hours on increasing amino acid content in the exocellular medium (Figure 5). The alpha amino peptide released during the log phase was substantially degraded during the growth phase, however certain amount of peptides were unconverted into amino acids. Proton NMR analysis confirms these may be due to presence of higher peptides containing high proportion of hydrophobic amino acid residues than the peptides rich of hydrophilic residues (Chen et al. 1987).

The hydrolysis of polymer to monomers appears to be the rate-limiting step of the biodegradation process as shown by the fact that higher molecular weight compounds are hydrolyzed slowly (Ubukata 1998). The higher proteolytic activity resulted in higher fleshing hydrolysis and maximum free amino acid presence could be detected at that particular time. The amino acids content were higher at 24 h ranging from 450- 460 mg/L and this increase indicates the hydrolysis of fleshing. The maximized enzymatic activity at 20 h and presence of higher free amino acids in the supernatant of culture filtrate proves hydrolysis increases gradually and reaches maximum after 24 h of fermentation. The protease enzyme production results in release of peptides and amino acids into extracellular fluid higher 1000- 1100 mg/l during the initial period of fermentation these products were converted into peptides and amino acids concentration. The amino acid and peptide also play a vital role in proteolytic productivity.

Enzyme synthesis could be repressed by rapidly metabolized nitrogen sources such as various amino acids concentrations in liquid media. Ferrero et al.1996 had reported that molecules present in the culture supernatant, which include amino acids and peptides,

also lowered the proteolytic activity indicating end product inhibition. 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 (Allison and Macfarlane 1990)

Electron microscopy observation of fleshing

The hydrolysis of fleshing samples was examined under the SEM. The ANFL immediately after inoculation was shown in Figure 6. It can be well seen that tissues were well distributed in the inner and outer surface of the matrix. In addition, the results of the electron microscopy studies indicate that protease enzyme produced preferentially degrades non-fibrillar proteins like albumin and globulin (Figure 7).

NMR analysis

The NMR spectrum of the sample is shown in Figure 8. The spectrum shows the chemical shift around 0.8-1.4 ppm, which attributes to the presence of $-CH_2$ and -CH groups in amino acids and peptides. The presence of quad ret peak at around 2.3-2.4 ppm can be assigned to the presence of $-CH_3$ group. The chemical shift at around 3.3-3.7 ppm was due to the presence of amine groups. The hump around the 4-4.6 ppm can be attributed to the -CONH group. The presence of all these peaks shows that the sample contains peptides and amino acids.

Conclusion

Most solid waste generated from tannery industries are currently been unutilized and wasted. The use of microbiological method to hydrolyze solid proteineous waste is an attractive alternative. Another interesting potential application is that alkaline protease an industrially important enzyme can be produced from waste material using them as resource. The P. aeruginosa strain showed maximum alkaline protease production after 20 hours of incubation at the end of exponential growth phase of the cell growth. Any further incubation resulted in the decline of enzymatic activity. The alkaline protease production is directly relate to biomass production and is associated with the cell growth. Alkaline protease production by the present strain P. aeruginosa is repressed by more amino acids release from proteineous solid waste. The higher proteolytic enzyme production was seen within 24h and the release of amino acids by the proteolytic activity repressed the enzyme production further. Thus it can be conclude that excessive amino acids release regulate the protease synthesis in P. aeruginosa.

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Figure Legends

Table 1. Characterization of ANFL

Fig.1 Amino acid analysis of ANFL

Fig. 2 Extracellular proteolytic activity from *Pseudomonas aeruginosa* grown at different pH

Fig. 3 Effect of fleshing size on protease production

Fig.4 Optimized enzymatic & growth profile

Fig.5 Effects of Total Amino acids on alkaline protease activity

Fig. 6 SEM image of unhydrolyzed proteineous solid waste

Fig. 7 SEM image of hydrolyzed proteineous solid waste

Fig. 8 H-NMR analysis of hydrolyzed proteineous solid waste

Fig. 1 Amino acid analysis of ANFL

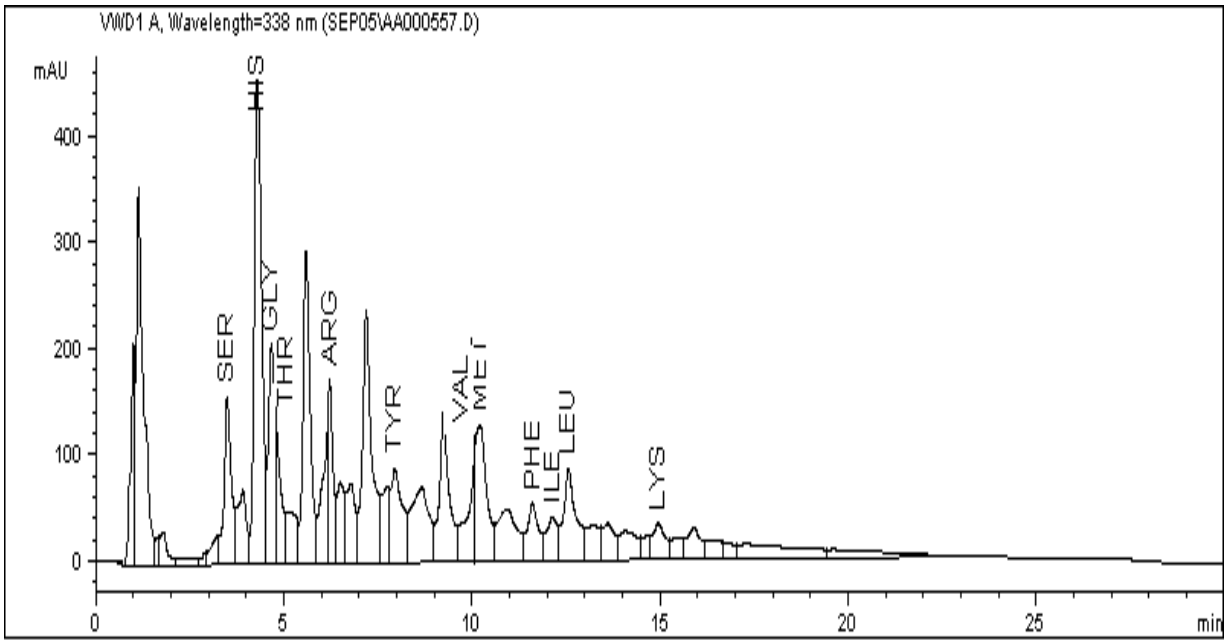


Figure 2. Extracellular proteolytic activity from Pseudomonas aeruginosa grown at different pH

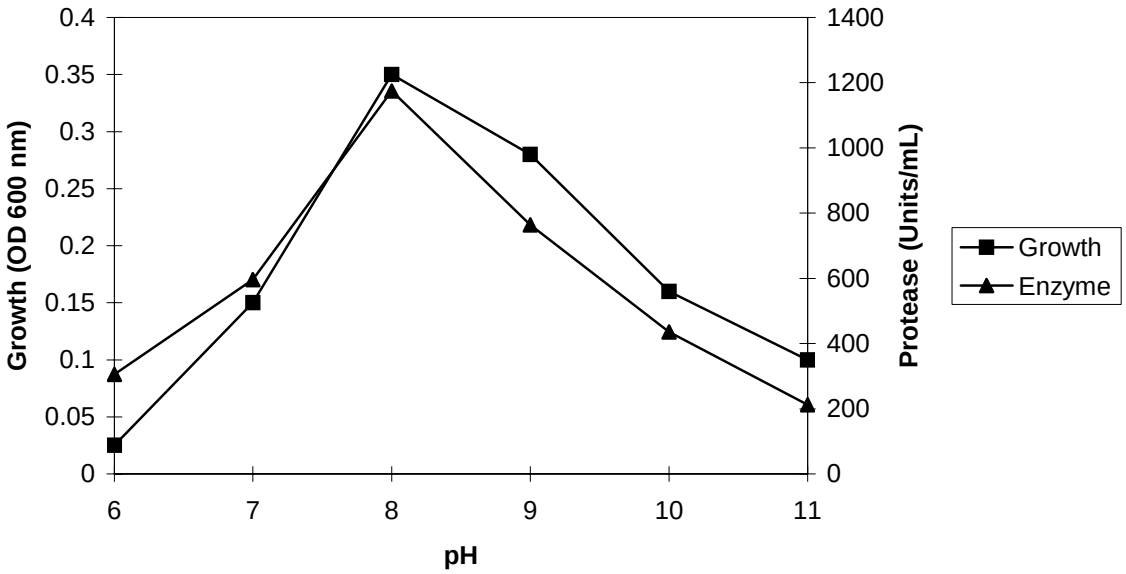


Fig. 3 Effect of fleshing size on protease production

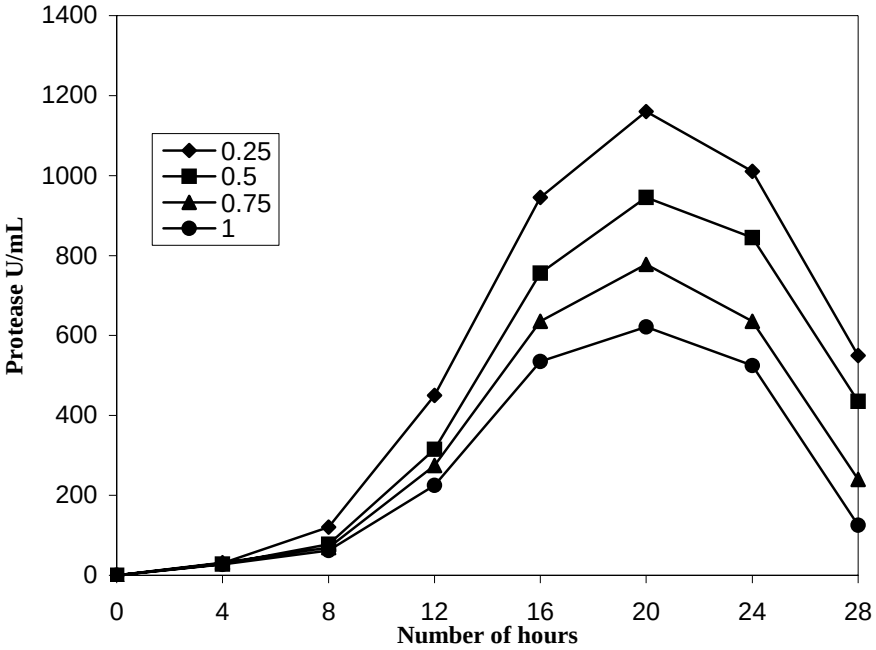
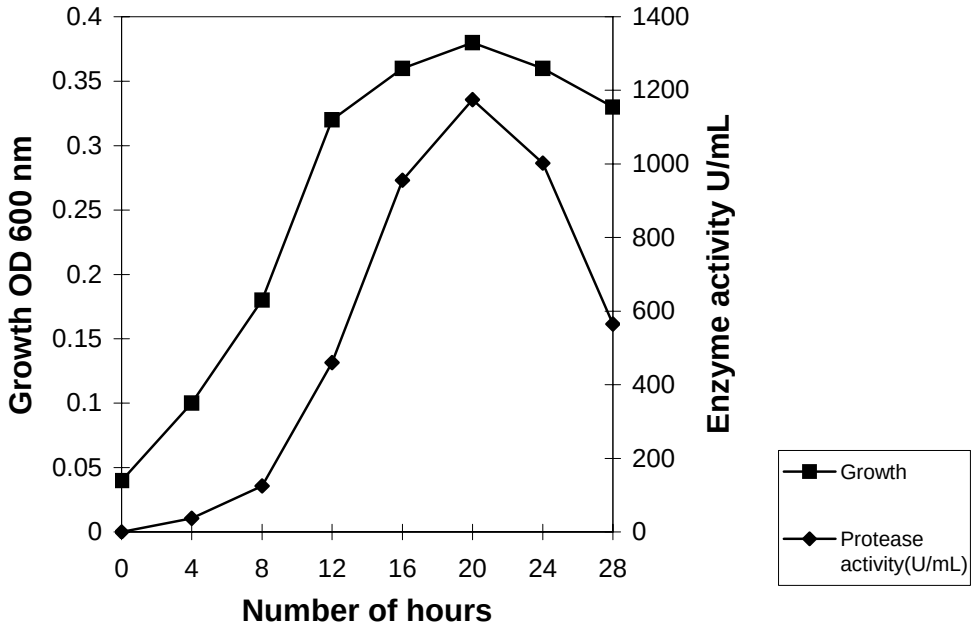


Fig.4 Optimized enzymatic & growth profile



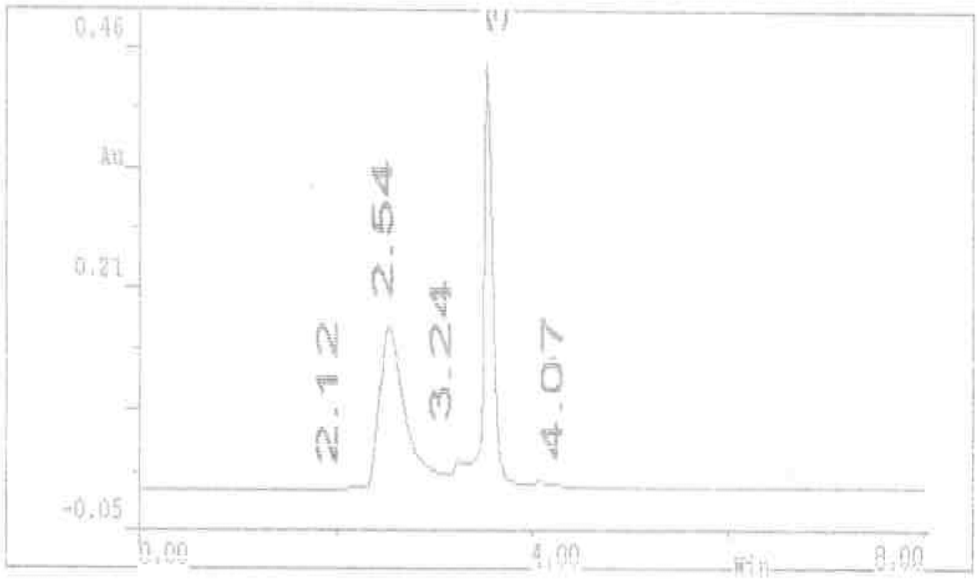


Fig. 6. HPLC analysis of fermented product of ANFL

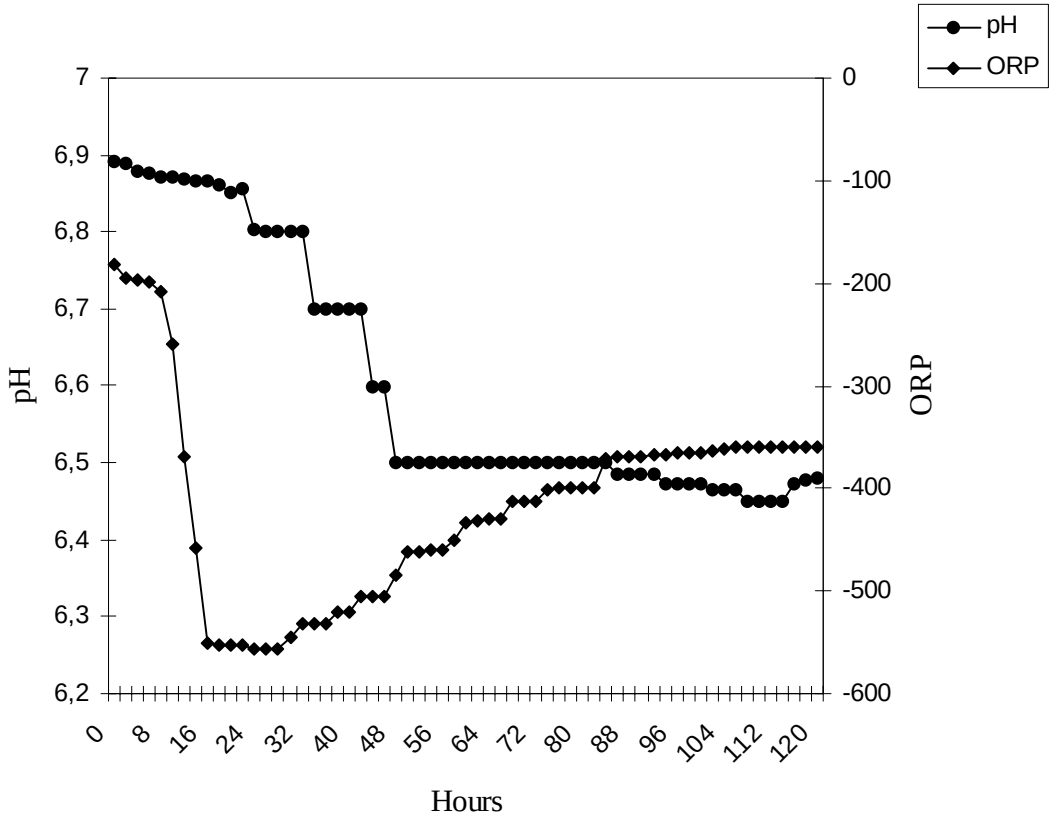


Fig. 7. Oxidation Reduction Potential versus Fermentation pH

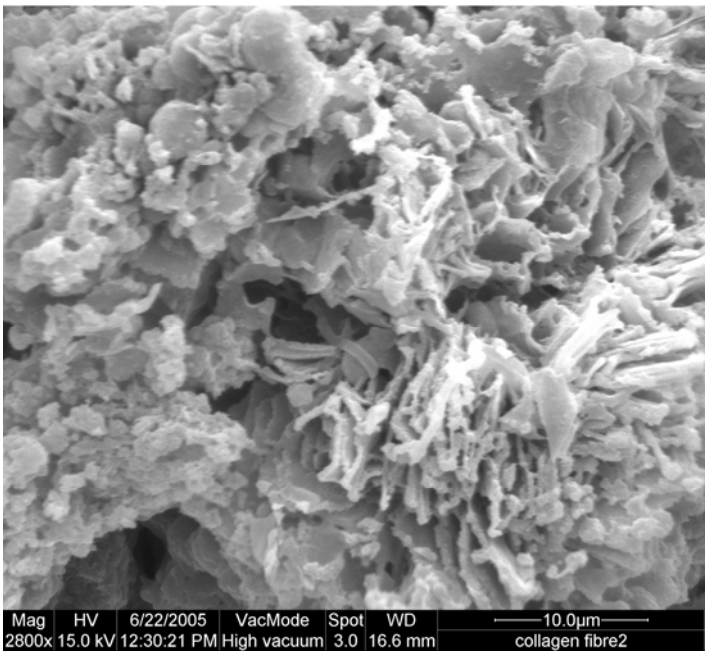


Fig. 8. Scanning Electron Micrograph of unhydrolysed ANFL sample

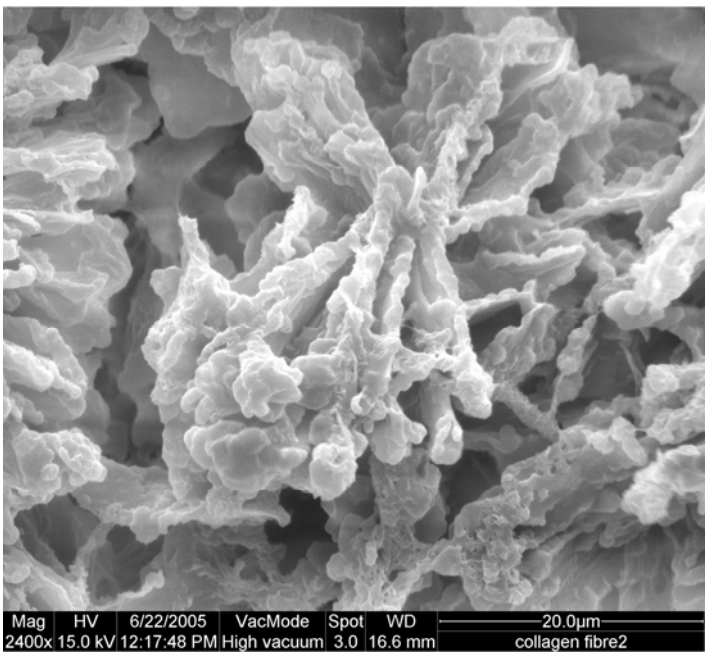


Fig. 9. Scanning Electron Micrograph of 72 hour-hydrolyzed ANFL