CHAPTER SIX:
ANALYSIS OF DEGRADATION PRODUCTS
Chapter 6: Analysis of degradation products

6.1 INTRODUCTION:

6.1.1 Feather degradation products

Microbial feather degradation results in the breakdown of the feather protein keratin into its constituent peptides and amino acids. As a result, these peptides and amino acids will be released in the liquid medium. The feather hydrolysate, thus obtained after microbial treatment of feathers would be rich in several low molecular weight peptides belonging to the range of 500 to 3000 Daltons (Ana Lúcia Vazquez Villa et al., BMC biotech, 2013) and amino acids and several essential amino acids such as phenyl alanine, leucine, isoleucine, threonine and valine (E. V. Kumar et al., 2011).

6.1.2 Amino acids and their value (Dr. Jeffrey A. Hansen, SWINE NUTRITION GUIDE):

The 10 essential amino acids that must be provided in swine diets are: lysine, threonine, tryptophan, methionine (and cystine), isoleucine, histidine, valine, arginine, and phenylalanine (and tyrosine). The commonly used animal feeds for livestock is a combination of energy sources such as corn, wheat and soyabean meal. Of these, soyabean meal is a rich and a costly source of amino acid lysine, which is an essential amino acid required in the diet of livestock animals. However, corn and wheat do not contain sufficient amounts of lysine. In order to generate a cost effective animal feed, feed formulators add lysine supplements for the right balance of amino acids.

Besides lysine, another essential amino acid for livestock is threonine which is deficient after lysine. Tryptophan is generally lacking in corn diets and teds to be deficient in piglet feeds.

Most cereal grains are limiting in the above mentioned amino acids-lysine, tryptophan, and threonine. Therefore, when evaluating feed ingredients, these amino acids, especially lysine, are most important in determining protein quality.

6.1.3 Detection methods (Hassall H, 1972):

Ninhydrin reaction for detection of amino acids: Ninhydrin which is originally yellow reacts with amino acids to produce a purple colored complex called Ruhemann’s purple (Figure 1.3) which can be detected or measured colorimetrically. Ninhydrin reacts with the free alpha- amino group which is present in all amino acids, peptides and proteins. The following reaction which is decarboxylation reaction is specific only for
amino acids, and not for proteins and peptides. So, theoretically only amino acids produce color with ninhydrin reagent. However, one should always rule out possible interference from peptides and proteins by performing blank tests.

In the quantitative estimation of amino acid using Ninhydrin reagent, the absorbance of the Rheumann's purple formed by the reaction at 570 nm is measured.

6.1.4 Thin Layer Chromatography (TLC):
Thin layer chromatography is a planar chromatography, which uses a sorbent layer, usually of 0.10-0.25 mm thickness applied over a firm base such as glass, aluminium or plastic. Glass has been the most popular base for performing TLC, however, plastic and aluminium have the advantage of being more flexible and being able to cut easily in any size. A variety of sorbent layers have been used, which includes silica gel, cellulose, aluminium oxide, polyamide and chemically bound silica gels. The sample, which is usually dissolved in a solvent, is applied as spots or bands at 1 cm from the edge. The TLC plate is placed in a saturated chamber containing a eluent which is either a solvent or a mixture of solvents. The individual components of the sample would get separated when the eluent flows by capillary action across the sorbent layer. Migration of the eluent across the sorbent layer happens such that the individual components of the sample to travel at different rates, resulting in their separation (Wall PE, 2005).
While detection of amino acids can be carried out by High Performance Liquid Chromatography (HPLC) and amino acid analyser, the method is time consuming and expensive. Also, for trace analysis of free amino acids of an unhydrolyzed sample, a huge quantity of sample needs to be loaded onto the column which interferes with the chromatography immensely. HPTLC (High Performance Thin Layer Chromatography) is a rapid and inexpensive method for detection of free amino acids in a sample (Hamon L et al., 2008).

HPTLC is a more advanced form of TLC, involving automation at different steps, to enhance separation and to increase the resolution. Usually the difference between TLC and HPTLC would be the use of readymade silica plates, auto samplers or injectors and densitometry for semi-quantification.

The current chapter involves analyzing the amino acids that are generated as by-products of keratin degradation, using TLC followed by densitometry, thus exploring the prospects of microbial feather treatment for generating a feather hydrolysate enriched with amino acids- a by-product of bioremediation of chicken feather waste.
6.2 MATERIALS AND METHODS

6.2.1 Amino acid production using optimized media conditions:

1. Isolates *B. licheniformis* and *B. subtilis* were inoculated the production medium which was incorporated with optimized concentrations of media components. Incubation was carried out under optimized process parameters.

2. After five days of incubation, 5 ml of the culture broth was aseptically removed using a pipette. It was centrifuged at 4°C for 20 minutes at 10,000 rpm. The cell free supernatant was used as a source of amino acids.

3. The amino acids were quantified by Ninhydrin method as described in section 4.2.3.2 of chapter 4.

6.2.2 Detection of individual amino acids using standards:

Development of the solvent system:

Available solvent systems:

1) Butanol: Acetic acid: Water (8:2:2)

2) Ethanol: water (7:2)

Of the above mentioned solvent systems used for amino acid separation, Ethanol water did not provide satisfactory movement of the amino acid samples. Butanol: acetic acid: water, although provided good movement of samples, further resolution of bands was required. So the ratio of polar and non-polar components was modified slightly to Butanol: Acetic acid: water (6.5:3.5:1), which resulted in better resolution.

Requirements:

1. **Amino acid standards**
   
   All amino acid standards were prepared to achieve a final concentration of 1mg/ml

2. **Mobile phase (Butanol:Acetic acid: water= 6.5:3.5:1)**
   
   11 ml of mobile phase was prepared according the ratio

3. **Developing Solution (0.3% Ninhydrin)**
   
   For 10 ml of developing solution, 0.03 grams of ninhydrin was weighed and mixed in butanol: acetic acid (9.7:0.3). It was prepared fresh.
4. **TLC plates:** Pre-coated Silica plates having 0.25 mm layer of silica gel 60 $F_{254}$ (MERCK, Germany)

5. **TLC chamber (CAMAG)**

6. **Glass Capillary**

**Method:**

1. All amino acid standards were prepared at a final concentration of 1mg/ml in distilled water.

2. A TLC chamber containing mobile phase- Butanol: Acetic Acid: water (6.5: 3.5:1) was kept for saturation for 15 minutes.

3. TLC plates were cut in the size of 9 cm x 10 cm. A baseline is marked with a pencil approximately 1 cm from the lower edge of the TLC plate.

4. The amino acid samples and the standards were loaded such that the final concentration loaded on the TLC plate was 10 µg. The samples were applied using a glass capillary. The plate was air dried.

5. The TLC plate loaded with samples was kept in the chamber for the chromatographic separation of samples to happen.

6. After the solvent front had reached up to 90% of the length of the plate, it was carefully removed and kept for air drying. The solvent front was marked with a pencil.

7. After air drying the developing reagent was sprayed and the plate was kept for developing in a hot air oven at 110°C till the bands appeared.

8. The Rf values were measured as follows:-

   \[ Rf = \frac{\text{distance travelled by the sample/solute (cm)}}{\text{Distance travelled by the solvent or solvent front (cm)}} \]

9. On comparing the Rf values of the amino acid bands of the sample with those of the standards, the amino acid profile of the test sample was determined.

10. For semi-quantification of the individual amino acids in the test samples, a known quantity of the standards (as detected) and test samples were to be loaded. This was to be followed by densitometry.
### 6.2.3 Densitometry of amino acids:

1. The chromatographic conditions were standardized by TLC.
2. TLC was performed on pre-coated silica gel plates as mentioned earlier.
3. The measured volume of the samples was applied by controlled manual loading using glass capillary tubes.
4. The distance between two bands was maintained as 8 mm, keeping the band width as 7 mm.
5. According to the amino acids detected in the previous TLC experiment and the Rf value of the standard, two mixed standards were prepared to achieve the final concentration of the amino acids as 1mg/ml.
   - Mix standard 1: Cysteine, Tyrosine, Isoleucine and Tryptophan
   - Mix standard 2: Serine, Proline, Lysine and Methionine
6. The samples were quantified and then loaded.
7. The plated was allowed to air dry, and then kept into a pre-saturated TLC chamber (CAMAG) containing the mobile phase.
8. Separation was allowed to take place till the solvent front reached up to 90% of the plate, after which the plate was carefully removed and allowed to air dry.
9. Developing was carried out by immersing the plate at once in a trough of freshly prepared developing solution. The plate was allowed to air dry and then placed in a hot air oven at 110°C till the spots developed.
10. Detection was carried out using CAMAG TLC Scanner 181207 equipped with winCATTS analytical software. The wavelength was 550 nm.
6.3 RESULTS:

TLC analysis showed the presence of amino acids in the cell free extracts obtained from the feather hydrolysate of isolate *B. licheniformis* and *B. subtilis*.

**Figure 6.1: Chromatogram of 1) standard 1, 2) standard 2 along with 3) cell free supernatant of isolate *B. subtilis* and 4) cell free supernatant of *B. licheniformis*.**

Chromatogram a) at U.V. 254 nm and chromatogram b) at U.V. 366 nm.

<table>
<thead>
<tr>
<th>Application pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Track</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
</tbody>
</table>
Figure 6.2: Detection of amino acids by TLC: Chromatogram of 1) standard 1, 2) standard 2 along with 3) cell free supernatant of isolate *B. subtilis* and 4) cell free supernatant of *B. licheniformis* after derivatization with Ninhydrin. The bands indicate presence of amino acids.
### Table 6.1: Peaks obtained for the TLC analysis of amino acid profile of isolate *B. subtilis*

<table>
<thead>
<tr>
<th>Peak</th>
<th>Rf</th>
<th>Amino acid</th>
<th>% Peak Area</th>
<th>Concentration (µg/10µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.09</td>
<td>Serine</td>
<td>14.42</td>
<td>4.07</td>
</tr>
<tr>
<td>2</td>
<td>0.25</td>
<td>Cysteine</td>
<td>21.27</td>
<td>7.78</td>
</tr>
<tr>
<td>4</td>
<td>0.59</td>
<td>Tyrosine</td>
<td>12.47</td>
<td>4.50</td>
</tr>
<tr>
<td>5</td>
<td>0.66</td>
<td>Isoleucine</td>
<td>26.96</td>
<td>8.84</td>
</tr>
<tr>
<td>6</td>
<td>0.74</td>
<td>Tryptophan</td>
<td>6.38</td>
<td>1.85</td>
</tr>
</tbody>
</table>

**Figure 6.3: TLC peak densitogram display of amino acid profile of isolate *B. subtilis*, at 550 nm**
### Table 6.2: Peaks obtained for the TLC analysis of amino acid profile of isolate B. *lichenumformis*

<table>
<thead>
<tr>
<th>Peak</th>
<th>Rf</th>
<th>Amino acid</th>
<th>% Peak Area</th>
<th>Concentration (µg/10µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.07</td>
<td>Serine</td>
<td>4.59</td>
<td>1.29</td>
</tr>
<tr>
<td>3</td>
<td>0.25</td>
<td>Cysteine</td>
<td>5.22</td>
<td>1.91</td>
</tr>
<tr>
<td>4</td>
<td>0.32</td>
<td>Proline</td>
<td>11.35</td>
<td>19.13</td>
</tr>
<tr>
<td>5</td>
<td>0.35</td>
<td>Lysine</td>
<td>12.90</td>
<td>4.37</td>
</tr>
<tr>
<td>6</td>
<td>0.40</td>
<td>Methionine</td>
<td>26.03</td>
<td>33.07</td>
</tr>
<tr>
<td>7</td>
<td>0.59</td>
<td>Tyrosine</td>
<td>13.89</td>
<td>5.01</td>
</tr>
<tr>
<td>8</td>
<td>0.66</td>
<td>Isoleucine</td>
<td>17.09</td>
<td>5.60</td>
</tr>
<tr>
<td>9</td>
<td>0.74</td>
<td>Tryptophan</td>
<td>7.78</td>
<td>2.25</td>
</tr>
</tbody>
</table>

**Figure 6.4: TLC peak densitogram display of amino acid profile of isolate B. *lichenumformis*, at 550 nm**
6.4 DISCUSSION:

As observed in Figure 6.2, derivatization with ninhydrin resulted in the development of brown-purple spots and some yellow spots on the chromatogram. This chromogenic reaction with ninhydrin is a characteristic of amino acids. TLC accompanied with derivatization with ninhydrin and followed by densitometry thus resulted in detection of the various amino acids present in the samples. The amino acids detected for sample of \textit{B. subtilis} include Serine, Cysteine, Tyrosine, Isoleucine and Tryptophan, whereas for \textit{B. licheniformis} include Serine, Proline, Cysteine, Lysine, Methionine, Tyrosine, Isoleucine and Tryptophan.

The current findings are consistent with previous reports about microbial feather degradation by Nagal et al., 2010, Kumar et al., 2011, Lakshmi PJ et al., 2013 which mention the release of amino acids especially cysteine and methionine and their potential to upgrade the nutritive value of animal feeds.

The release of amino acids accompanied with microbial feather degradation results in the generation of a feather hydrolysate that can be a potential animal feed additive. The hydrolysate contains less complex, easily metabolizable feather proteins, peptides and amino acids. The amino acids released by microbial treatment of feathers form an essential part of the animal diet. Amino acids such as lysine and methionine are also known to be limiting amino acids and are important requirements of animal diet. Microbial treatment, therefore nutritionally upgrades the feather waste, by enriching it with amino acids. The hydrolysate can potentially replace other expensive amino acid supplements.

Feather degrading bacteria, isolated in this study can potentially be used for the purpose of bioremediation of feather waste and can replace traditional, energy consuming methods, while being an environmentally friendly approach. The by-product obtained would be a digestible source of protein and amino acids, and can be used as an animal feed additive. Overall the research has contributed a solution for the problem of poultry feather accumulation, not just in terms of effective disposal but also in terms of recycling.