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Executive summary

More than 100,000 tons of feather waste is produced in Australia every year by the poultry processing industry. Currently, most of this is rendered to low value feather meal for animal feed applications. With its 90% protein content, poultry feather is potentially a rich source of quality protein. However, the protein in feather is in the form of keratin, which is highly complex and inaccessible. Hydrolysed keratin is used in many high value applications such as cosmetics for skin, hair and finger nail treatments and biomedical applications such as wound dressing. The hydrothermal process that is currently used for feather hydrolysis results in low quality hydrolysate with poor biological and functional properties. The objective of this project was to develop cost effective enzymatic hydrolysis and separation processes for the conversion of feather into purified keratin hydrolysates for high value applications such as cosmetics. The use of ultrasonics for facilitating the enzymatic hydrolysis of feather was evaluated since ultrasound enhances the enzymatic conversion of complex and incalcitrant substrates.

Solid state culture with feather as the sole nutrient source was used to identify three bacterial strains i.e. *Bacillus thuringensis* ATCC 33680; *Bacillus licheniformis* and *Chryseobacterium sp*. BC06, with keratolytic activities for hydrolysing feather. Further liquid culture investigations revealed that *Bacillus licheniformis* produces the most efficient keratinase under the studied conditions. The optimum temperature for activity of this novel keratinase was found to be 60 °C and its optimum pH for activity was 8.0. The enzyme also exhibited reasonably high thermal stability with more than 12% activity remaining after 24 hrs at 60 °C. The crude enzyme preparation showed significant keratolytic activity for hydrolysing both keratin azure and poultry feather. The production of this enzyme was scaled up to a 10 L bioreactor through media and process optimisation experiments. However, the specific activity that was obtained in the larger scale was slightly lower indicating the need for further process optimisation.

The efficacy of the kertinase from *B. licheniformis* for hydrolysing feather was compared with two commercial enzymes i.e. the analytic keratinase, proteinase K and the alkaline protease, Savinase 16L, which were selected among four that included alcalase and Multifect PR6L based on their feather hydrolysis efficacy. Under standardised condition (enzyme dosage of 72.4 keratolytic unit, pH 8.0 and 60 °C), the estimated percentage hydrolysis of feather using these enzymes over 24 hrs was about 9% in all cases. Interestingly, the keratinase from *B. licheniformis* showed similar efficacy as the commercial enzymes, although the crude fermentation supernatant was used in the experiments with potential interference from culture components and the dosage was not optimised. The keratinase from *B. licheniformis* also exhibited equivalent disulfide reductase activity to that of Proteinase K, which was

significantly higher than Savinase 16L, indicating its potential application for pre-treatment of feather and other keratinacous materials prior to proteolysis. In the presence of a reducing agent (1% sulphite), the percentage hydrolysis of feather by the three enzymes increased dramatically. This was more pronounced in the case of Savinase 16L where the percentage hydrolysis was increased from ~9% to ~61%, which seems to be due to the higher proteolytic activity of Savinase 16L compared to the other enzymes. Indeed, the keratinase from *B. licheniformis* has a significant potential for the hydrolysis of feather together with a potent protease like Savinase 16L, since it has a better efficacy for cleavage of disulfide bonds than conventional proteases such as Savinase. In addition, optimising the fermentation condition and suitable downstream processing can substantially improve its proteolytic activity.

Subsequent investigations evaluated the potential of ultrasonication for enhancing the enzymatic hydrolysis of feather focusing on the *B. licheniformis* keratinase and Savinase 16L. Thus, exploratory experiments were conducted at ultrasonic frequencies ranging from 220 to 2000 kHz. Ultrasonic pre-treatment of feather at 400 and 600 kHz resulted in a significant improvement of the enzymatic hydrolysis of feather. The best result in terms of hydrolysis was obtained at 400 kHz with up to 60% increase in product yield after pre-treatment of feather for 20 min prior to the enzymatic reaction with both the keratinase from *B. licheniformis* and Savinase 16L after 24 hours of hydrolysis. Moreover, ultrasonic treatments of feather at all the studied conditions led to enhanced cleavage of disulfide bonds even in the absence of enzyme indicating the potential of the process for chemically modifying feather for various applications including in polymer composites and as textile fibre. Further experiments to determine the best ultrasonic processing conditions focused on 400 kHz ultrasonic treatment with the *B. licheniformis* keratinase. Ultrasonic pre-treatments as short as 5 min of feather at 400 kHz resulted in a significant improvement in the kinetics and yield of the hydrolysis reaction with this enzyme. Similarly, pulsed application of ultrasound (equivalent to 20 min treatment applied over 7 hours) resulted in about 33% less hydrolysis time compared to control to achieve the same product yield, showing the potential of such a process for shortening the hydrolysis time and thereby rendering the hydrolysis process more economical. In all cases, the kinetics of the reaction declined after 4 to 6 hours, which coincided with the loss of about 75% of the activity of the enzyme. Thus, the main constraint in terms of further optimisation of the ultrasonic assisted process was found to be the stability of the enzyme under the studied condition.

In order to develop an industrially feasible process for feather hydrolysis, process development and optimisation investigations focused on the commercially available protease Savinase 16L, since the *B. licheniformis* keratinase needs further process development before it can be used in industrial applications. Response surface methodology was used to determine the best processing condition (s) for the enzymatic conversion of feather into

keratin hydrolysates. The effects of factors that were identified in previous chapters (sulphite concentration, ultrasonic energy input) and substrate concentrations and their interactions were evaluated using peptide production, percentage conversion and peptide production per gram of feather as responses. The hydrolysis time was fixed at 6 hrs since longer hydrolysis time would not feasible for industrial application, although higher conversion could be achieved. Analysis of variance (ANOVA) showed that the significant parameters that affect the Savinase 16L catalysed conversion of feather under the studied conditions were substrate and sulphite concentration. Based on the response surface models obtained, the optimum condition that maximize both peptide formation and percentage conversion were found to be 3.9% substrate and the optimum conditions maximising the three parameters were found to be 3.9% substrate, 0.5% sulphite with predicted conversion of 29%, free amine concentration of 34.2 mM and free amine/gram feather of 8.85 mmol/gm. Ultrasonication at the conditions used in the investigation (50 and 100 kJ/L, 400 kHz) did not have statistically significant effect, although marginal (20% increase) effect of ultrasonication (100 kJ/L) on peptide formation was observed at 4% substrate concentration. This was unexpected since our earlier investigation showed that ultrasonic pre-treatment at specific energy input of 315 kJ/L significantly enhanced the Savinase 16L catalysed hydrolysis of feather after 24 to 48 hours of hydrolysis, where substantial ultrasound induced cleavage of disulfide bonds was observed. The ineffectiveness of ultrasound in this case could be due to the lower specific energy input or the shorter reaction time. The peptide profile of selected hydrolysates from 4% feather processed at different conditions (no sulphite, no ultrasonics, 0.5% sulphite, no ultrasonics and 100 kJ/L ultrasonics, no sulphite) was also evaluated. The peptides in all the samples had molecular weights ranging from 77 to 5000 Da, although higher amount of high molecular weight (3400-5000) peptides were found in samples hydrolysed with 0.5% sulphites. The peptide profile and the physical properties of the hydrolysates were used as a basis for designing a separation process to obtain hydrolysates with high degree of purity. A separation process involving coarse filtration, microfiltration (0.8 µm) and nanofiltration (245 Da) with 5 diafiltration steps (500 Da) was developed and evaluated for the feather hydrolysate produced at the optimum hydrolysis condition determined above (4% substrate and 0.5% sulphite concentration). A product with 78.9% purity was obtained after the final freeze drying step. The separation process was further optimised with the initial nanofiltration process conducted using a 500 Da cut off filter followed by only 3 diafiltration steps using the same pore size filter, improving the product purity to 80.7%. The final product had peptides with molecular weight between 3000 and 5000 Da, with peptides with molecular weight less than 2000 Da lost during the nanofiltration and diafiltration steps. The separation process was also evaluated for a hydrolysate produced without the use of sulphites where ~80% pure product was obtained with the peptide profile maintained (77 to 5000 Da). In this case,

nanofiltration with 245 Da cut off and no diafiltration steps, was used since the hydrolysate was relatively pure.

The functional and biological properties of the purified (~80% peptide) hydrolysates from sulphite and ultrasonic assisted processes were evaluated and compared with conventional protein ingredients. Overall, the two hydrolysates showed comparable or better functional properties than the conventional protein ingredients investigated except in foam stability. Both had very high solubility in water comparable to that of whey protein isolate and much better than soy protein isolate and much higher foaming capacity than all the protein ingredients evaluated. For instance, the foaming capacity of the hydrolysate from the sulphite assisted hydrolysis was about 150 times that of soy protein isolate, 8 times that of whey protein isolate and about 1.7 times that of egg albumin, an ingredient that is commonly used for its high foaming capacity and stability. The foam stability of both products was the same order of magnitude as that of whey protein isolate and soy protein isolate The very high foaming capacity of the hydrolysates makes them especially useful for applications in shampoos and conditioners where high foaming capacity and not necessarily stability is required. The emulsifying capacity of the hydrolysate from the sulphite assisted process was also comparable to both whey protein isolate and soy protein isolate. The result indicates that the keratin hydrolysates have a potential to be used as an alternative to conventional protein ingredients. Both hydrolysates had similar amino acid profile containing all essential and conditionally essential amino acids. Thus, the hydrolysates can be potentially used as high quality protein ingredients for pet food application. The hydrolysates also exhibited very high antioxidant capacity values, which are higher than that of strawberries on dry weight basis and can potentially be used as ingredients in pet food, nutraceutical and cosmeceutical products. Moreover, the keratin hydrolysate from the sulphite assisted process has anti-inflammatory activity indicating that it has therapeutic effect, which augments its value as a cosmeceutical and nutraceutical ingredient. Since the application of ultrasound did not have beneficial effect neither on the functional properties of the hydrolysates nor the efficiency of the process under industrially feasible conditions, further analysis focused on the enzymatic hydrolysis with and without the use of sulphites.

Based on the functional properties of the hydrolysates, a background literature and patent review and a small survey were used to explore the potential industrial applications of the keratin hydrolysates produced in this study. The literature and patent review indicated that the main potential application areas for feather keratin hydrolysates are cosmetics, pet food and animal feed supplement industries. The cosmetic ingredient market is a high value market with strong growth projected over the coming five years. Keratin hydrolysates, due to the superior functional and biological properties they impart into the end-products, are increasingly used by cosmetic companies in range of products including skin care, hair care

and nail care formulations. The purified keratin hydrolysates developed in this project suit best to this market, due to their high quality and relatively high cost of production. Our limited review showed that the keratin ingredient market for cosmetics is currently dominated by wool based products. However, this is expected to change with the increasing availability of scientific information on the efficacy of feather keratin peptides in hair and skin care applications. The high end nutraceutical pet food market is another potential market that should be targeted for the application of feather based keratin peptides. Petfood companies are currently exploring the use of keratin based hydrolysates as an ingredient to address specific needs such as hypoallergenicity and boosting health and performances of pets. Our small market survey indicates that there is lack of awareness of keratin based peptides or lack of interest in 'new ingredients' by many of the Australian companies engaged in the manufacturing of cosmetics and pet foods or supply of ingredients for these industries. However, encouraging responses were obtained from a number of companies who were aware of keratin or feather based peptide ingredients. The survey confirmed our observation based on the background literature review that cosmetic companies are willing to pay much higher price for keratin based ingredients compared to pet food companies. The survey also indicated that pet food companies are interested in better feather meal products with higher digestibility and bioavailability and are willing to pay higher for such products. Nevertheless, due to the small number of companies contacted, the survey cannot be considered as representative and a more extensive market survey is required to assess the potential market for feather based keratin peptides in the Asia-Pacific region as part of a future research and development activity.

A preliminary cost-benefit analysis was conducted for both the sulphite assisted and enzymatic hydrolysis processes. Based on a 600 ton/ year dry feather processing plant, the estimated annual earnings before interest, tax, depreciation and amortisation (EBITDA) from the sulphite assisted and the non-sulphite processes are $2,389,552 and $2,456,682 respectively. The estimated capital cost for the sulphite assisted and the non-sulphite processes are respectively, $6,435,130 and $6,262,925 respectively. The payback period for both processes is less than 3 years. Process modification with a single solid-liquid separation unit operation prior to nanofiltration as an alternative to coarse filtration and microfiltration can potentially improve product recovery and increase the net income to $5,406,649 and $4,683,215 respectively for the sulphite assisted and the non-sulphite process with significant reductions in unit production costs from $19.4 to $12.5/kg and $18.5 to $12.8/kg for the two processes. Although the use of sodium sulphite significantly improves enzymatic hydrolysis yield, that did not translate into a higher net income from the process due to the lower product recovery. This can potentially be improved at a larger scale and through further process optimisation and need to be explored in future pilot scale trials. As is, the process

without the use of sulphites is a better alternative since it also enables the recovery of low molecular weight peptides which have distinct functionality compared to the larger molecular weight peptides. It has to be noted that this cost-benefit analysis is based on laboratory scale data, historical data based on past experience and data from the literature, with significant uncertainties on the individual cost estimates. Nevertheless, with the very high contingency of 30% and 50% assumed for the capital and production cost estimations, the analysis gives a fairly reasonable assessment of the processes. In any case, further investment decisions should be based on further pilot scale trials and consultation with independent engineers.

It is recommended that

- Further research is conducted on the commercial development of the *B. licheniformis* keratinase, since it can be potentially used as a feed enzyme, for conversion of keratinacous waste into value added keratin hydrolysates and for skin care applications. Research and development activities should include further optimisation of the fermentation process for large scale production of the enzyme, development of suitable downstream process for at least partial purification of the crude enzyme, detailed characterisation of the enzyme including selection of suitable operating temperature and other routes for improving the stability of the enzyme over extended period for industrial application, investigation of possible synergy with commercial proteases for feather hydrolysis and potential for enhancing feather hydrolysis through pulsed application of ultrasound.
- Pilot scale trials of the sulphite assisted enzymatic hydrolysis and the enzymatic hydrolysis process without sulphite are conducted in order to optimise the hydrolysis and the separation processes as well as get more concrete data for equipment sizing and operating condition for a realistic cost-benefit analysis of the processes prior to commercialisation
- Detailed market research is conducted to assess the potential market for feather based keratin peptides in the Asia-Pacific region
- Study on the material properties of the feather residue using standard material characterisation techniques is conducted to explore potential applications in polymer composites and as natural fibres for textiles, since that will significantly improve the value proposition of the feather hydrolysis process

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Introduction

Large amount of feather waste is produced every year in Australia by the poultry processing industry. In 2013-14, around 580 million chickens were processed in Australia [1]. With feather constituting between 8 to 10% of chicken weight [2], this would amount to about 105000 to 131000 tonnes of feather waste produced in that year. This figure does not include feather from dead birds and hatchery waste. Poultry feather contains about 90% protein and is potentially a rich and sustainable source of protein [3]. However, the protein in feather is in the form of keratin, which is highly complex and inaccessible. Keratin is highly resistant to hydrolysis due to tight packing of the protein chain in the form of α-helix (α-keratin) or β-sheet (β-keratin) into a super coiled polypeptide chain cross-linked with disulfide bonds [2,4]. Hydrolysed keratin can be used in many applications including animal feed, fertiliser, cosmetics for skin [5], hair and nail treatments [6,7], leather tanning, biodegradable films, and as carrier for insecticides and other active agents [2,8]. Chemically modified keratin can also be used for the production of biodegradable materials and polymer composites for medical and other applications [2]. In addition, several bioactive properties of hydrolysed keratin has been recently reported including antioxidant capacity [9,10], anti-hypersensitive, anti-diabetic [10], tumor growth inhibition properties [11], which indicates the potential application of keratin hydrolysates in nutraceutical and therapeutic applications.

The price of keratin hydrolystates varies depending on the source, quantity, the degree of purity and application as well as country of production. For instance, the price of high quality keratin hydrolysate from spectrum chemicals (USA) is US$790.25 for 500g with the price lowering to $116.6/kg for 45 kg [12]. Based on the quotes we obtained from various companies, the price of bulk quantity keratin hydrolysates for high-end applications such as cosmetics is between US $52/kg and 131.7/kg. See appendix I for examples of quotes obtained from various suppliers. Currently, most of the feather produced by the poultry industry in Australia is rendered to low value feather meal for animal feed application. Thus, developing an efficient process for the conversion of feather into high quality hydrolysed keratin will significantly increase the value derived from poultry feather by the industry.

There are several approaches described in literature for converting keratinous materials into more accessible keratin hydrolysates. These can be broadly categorised into hydrothermal, thermochemical and biological processes [13]. The majority of patented processes involve the thermochemical approach that involve the use of acid (HCl, H_2SO_4) or alkali (CaO, NaOH, KOH) with or without a reducing agent (sulphites, urea) and processing at very high temperatures (80 to 140 °C) and pressures (10-15 psi) with variations in the type of alkali or acid employed and the processing conditions [14,15,16,17,18]. The processing with acid or alkali for two hours or longer at near boiling or higher temperatures leads to the cleavage of the disulfide bonds and yields soluble peptides and amino acids. However, due to the extreme processing condition during thermochemical and hydrothermal processes often result in partial or complete destruction of some

amino acids including lysine, methionine and tryptophan and cause the formation of non-nutritive amino acids such as lysinoalanine and lanthionine [2]. Apart from that, neutralization of the product after base or acid hydrolysis may result in the production of large quantity of salt that may incur additional disposal cost and cause environmental damage.

Biological processes involve the use of keratolytic microorganisms or enzymes produced by such organisms. Keratinacous materials such as feather protein are resistant to proteolytic degradation and cannot be easily hydrolysed by most commercial available conventional proteases due to the high number of di-sulfide bonds in their structure [4]. Nevertheless, such materials can be degraded by some species of fungi, actinomycetes, and bacteria, which produce extracellular keratinases [4,19]. These organisms have the enzymatic apparatus and the eroding mycelium in the case of fungi, which are adapted to the structure of native keratin enabling them to use keratin as a sole source of nutrients [4]. Keratinases from several microbial species have been isolated and characterised [4,19,20,21,22,23,24]. Keratinases from *Bacillus sp*. especially from *Bacillus licheniformis* have been well investigated [4]. A keratinase from *Bacillus licheniformis* PWDI was developed and commercialised as Versazyme™ by Shih and colleagues at Bioresource international, Inc. (BRI) [19]. Most of microbial keratinases are serine proteases with some metallo and thiol proteinases, which are active in the neutral to alkaline pH range. Microbial keratinases have broad substrate specificity with ability to hydrolyse both soluble proteins such as casein, albumin, and haemoglobin and fibrous insoluble proteins such as collagen, elastin and keratin in feather, wool and other materials. However, most of the crude and partially purified preparations of these enzymes are not able to fully solubilise native keratin, indicating the complexity of the keratin hydrolysis process and the possible involvement a battery of enzymes and other extracellular metabolites in addition to keratinase [4,19,20,24,25]. The mechanism of microbial keratolysis is not well understood [4,19]. Studies so far indicate that the mechanism of keratin degradation by microorganisms is a two stage process consisting of sulfitolysis and proteolysis. Sulfitolysis is a process by which the disulfide bonds in keratin are cleaved by reducing agents such as sulphites produced by the microorganisms or mechanically by mycelial penetration in the case of fungi, making the peptide chains accessible for enzymatic proteolysis [4]. The involvement of disulfide reductases in sulfitolysis has also been reported [20,21].

The efficiency of microbial keratinases for hydrolysing feather and other keratinacous materials is significantly improved through the use of reducing agents and other chemicals that promote sulfitolysis [19,24,27,28]. Conventional proteases such as subtisilin, chymotrypsin and papain, which selectively cleave proteins at the hydrophobic P1 residue, have also been shown to degrade keratin in the presence of suitable reducing agents such as sulphites [26]. Pre-treatments by reducing agents such as dithriothreitol (DTT), mercaptoethanol, L-cysteine, sodium sulphite, surfactants such as sodium dodecyl sulphate (SDS) [24,26,27], and alkali [28,29] has been reported to enhance the enzymatic hydrolysis of keratin in feather and other keratinous materials

using keratinases and conventional proteases. The reducing agents are used to cleave the disulfide bonds between the polypeptide chains in keratin whereas, surfactants lower the surface tension between the substrate and the liquid, thereby facilitating the enzyme attack [24,27]. There are a number of patented enzymatic and microbial processes for the production of keratin hydrolysates from feather and other keratinous materials with [11,30,31,32] and without the use of chemicals [33,34,35].

In general, microbial and enzymatic processes have the advantage of being environmentally friendly and can yield high quality hydrolysates with better digestibility and bioavailability and maximal retention of liable amino acids [4,19]. The main drawback of microbial processes is the cost of processing associated with the long processing time and the complexity of the downstream process if a purified hydrolysate is the intended product. However, such processes could have potential applications for the production of nutritionally rich feed supplements from raw materials such as feather since microbial biomass may supplement keratin, which is deficient in essential amino acids such as methionine and phenaylalanine [19,36]. On the other hand, enzymatic processes involving keratinases are not able to efficiently hydrolyse native keratin, although reducing agents and surfactants can be used to facilitate enzymatic hydrolysis. Nevertheless, depending on the application, this chemicals need to be removed from the final product increasing the downstream processing cost as well as generating a waste stream that needs further treatment. A possible alternative approach for improving the efficiency of keratin hydrolysis is the use of ultrasonics. The application of ultrasound at appropriate condition can enhance the kinetics of enzymatic reactions, potentially reducing enzyme dosage or reaction time. Our earlier studies as well as other studies in literature have shown that the application of ultrasound substantially enhances the kinetics of enzymatic reactions [37,38,39,40]. The effect of ultrasound on enzymatic reactions is attributed to the strong shear and microstreaming that accompanies cavitation, which enhances mass transfer and improve enzyme and/or substrate availability and enzyme-substrate interaction by breaking down molecular aggregates and modifying macromolecular substrates [39]. Sonochemical effects i.e. oxidation by free radicals generated during treatment by high frequency ultrasound, may also result in chemical modification of macromolecular substrates such as keratin that can potentially improve its accessibility to enzymatic hydrolysis.

Research Objectives

The overall objective of the project was to develop an efficient and cost effective ultrasound assisted enzymatic process for the production of high quality keratin hydrolysates from poultry feather. The specific objectives of the project were;

1. Production and characterisation of a customised keratolytic enzyme for the hydrolysis of feather into keratin hydrolysates
2. Comparison of the efficacy of customised keratolytic enzyme with commercial proteases for the conversion of feather
3. Evaluation of the potential of ultrasound for enhancing the enzymatic hydrolysis of feather
4. Development of optimised process for the production of high purity keratin hydrolysates from poultry feather
5. Evaluation of the functional properties of keratin hydrolysate (s) from poultry feather
6. Evaluation of potential industrial application of keratin hydrolysates from poultry feather
7. Preliminary assessment of the cost-benefit of the production process for keratin hydrolysates from poultry feather

Methodology

In order to meet the specific objectives stated above, experimental and desk top investigations were conducted depending on the specific objective at hand.

1. In order to produce customised keratolytic enzyme for feather hydrolysis, solid state culture with feather as substrate was used for isolation of suitable microorganisms which are capable of degrading feather. This was followed by fermentation experiments in submerged culture of the isolated microorganisms and poultry feather as substrate to produce the keratolytic enzymes. This was followed by extraction of the extracellular enzymes, measurement of their keratolytic activities and further characterisation of the best performing enzymes to determine its optimal temperature, pH and other attributes. It was originally intended to use CSIRO's evolver technology for the isolation of the microorganisms. However, due to the nature of the substrate, that was not feasible.
2. In order to assess the suitability of the customised keratolytic enzyme for feather degradation, the performance of the enzyme for feather degradation was compared with a number of commercial proteases as well as an analytical keratinase proteinase K in the presence and absence of sulphites to choose the best enzyme for feather hydrolysis. The reaction progress was followed by measuring the release of free amines, change in total protein and the release of sulfhydryl groups. The latter two are commonly used to assess the extent of feather hydrolysis in literature. Although we planned to include the commercial keratinase Versazyme™ (currently called Cibenza) in the comparative investigation, we were not able to obtain the enzyme sample since the product is not currently imported into Australia and since both the parent company and the Australian agent were not able to provide us with a sample.
3. In order to evaluate the feasibility of ultrasound treatment for improving the enzymatic hydrolysis of feather, ultrasonic assisted enzymatic hydrolysis experiments were conducted at different conditions. Ultrasonics was applied both as a pre-treatment and during hydrolysis with both commercial and customised enzymes. Based on prior experience and feasibility for commercial application [39,40], 220 kHz, 400 kHz, 600 kHz and 1000 kHz (1 MHZ) frequency ultrasound were chosen for the experiments.
4. In order to determine the best condition for enzymatic hydrolysis of poultry feather, response surface methodology was used in the experimental design. Based on the results of the previous experiments, sulphite concentration, ultrasonic condition and substrate concentration were used as experimental factors whereas the yield of hydrolysis and the total release of free amine groups were used as responses. Savinase 16L from Novozyme

was selected for the hydrolysis since it was the best performing proteolytic enzyme and since it is a commercial enzyme readily available as opposed to the customised keratolytic enzyme which needs further development. The peptide profiles of three selected hydrolysates were determined using size exclusion chromatography. Based on the result and prior experience, a separation process was designed for the purification of the keratin hydrolysates.

5. In order to assess the potential industrial applications of the feather keratin hydrolysates, the functional and biological properties of purified (~80%) hydrolysates produced with and without sulphites were determined. Functional properties such as solubility, emulsifying and foaming properties which are important for cosmetic and pet food applications as well as bioactive properties such as total antioxidant capacity and anti-inflammatory activity were evaluated.
6. Based on the functional properties of the keratin hydrolysates evaluated in activity 5, a background literature review and a small survey of Australian pet food and cosmetic ingredient companies, initial assessment of the potential industrial application of the keratin hydrolysates were made.
7. Based on the laboratory scale development data from activity 4 and prior experience in similar processes, preliminary cost-benefit analysis of the enzymatic hydrolysis of feather for the production of high quality keratin hydrolysates was conducted and recommendation for further development were given.

Chapter 1 Production and characterisation of customised keratolytic enzymes

1.1. Introduction

Poultry feather consists of 90% keratin, which is a highly complex and insoluble fibrous protein. The keratin chain is tightly packed in α-helix and β-keratin structures which are linked by disulphide bridges and hydrogen bonds. The protein is thus highly resistant to hydrolysis by conventional proteases, such as trypsin, pepsin and papain [26]. Several bacteria and fungi have been described in literature which have the enzyme systems for keratin hydrolysis [4,19].

The objectives of this part of the project were to produce and characterise customised keratolytic enzymes for poultry feather hydrolysis.

1.2. Materials and Methods

1.2.1. Materials

Feather samples were obtained from a local rendering company. The feathers were washed thoroughly with water and dried at 60 °C. A Wiley mill was used for size reduction of the feather to increase the specific surface of the feather samples so as to facilitate microbial colonisation and enzymatic hydrolysis. Keratin azure (blue dyed wool), fluorescamine and Gly-Gly-Gly standard were obtained from Sigma (Australia). All other chemical/biochemical reagents were analytical grade.

1.2.2. Identification of keratolytic microorganisms

Based on literature [4,19], four bacterial strains, namely *Bacillus thuringensis* ATCC 33680; *Bacillus licheniformis*; *Bacillus halodurans* and *Chryseobacterium sp.* BC06 (isolated using Evolver technology) from CSIRO collection were selected for screening of their keratolytic activity. Ground feather was used as the sole nutrient source in a solid defined medium, for the screening experiments. The four strains were streak plated onto nutrient agar (positive control), defined medium agar (negative control) and medium agar with 10 g/L ground feather. In all cases, the media were sterilised by autoclaving prior to inoculation.

1.2.3. Fermentation experiments

Based on the screening experiment in 1.2.2., three bacteria were selected for further fermentation experiments. The three bacteria were inoculated into a liquid culture with ground feather as the

nutrient source and incubated for 5 days at 30 °C and 200 rpm. In all cases, the media was autoclaved prior to inoculation. For each bacterial strain, the following five cultures were evaluated.

- Culture 1- medium + bacterial cells (no feather control)
- Culture 2- medium + ground chicken feathers (no cells control)
- Culture 3- medium + ground chicken feathers + bacterial cells

For the fourth and the fifth cultures, medium + ground chicken feathers were autoclaved and centrifuged at 11,419 g for 10 minutes to separate the supernatant from the pellet. The supernatant consisted of partially hydrolysed feather as a result of autoclaving.

- Culture 4- supernatant + bacterial cells
- Culture 5- pellet + medium + bacterial cells

The medium in all cases consisted of 0.5 g/L NaCl, 0.3 g/L K_2HPO_4, and 0.4 g/L KH_2PO_4. All experiments were conducted in duplicates.

1.2.4. Extraction of keratolytic enzymes

After five days, the bacterial cells and the feather debris were separated from the supernatant by centrifugation. The supernatant was used as a crude enzyme extract for further analysis and characterisation.

1.2.5. Determination of total protein content

The total nitrogen contents of the crude enzyme samples were determined using Dumas combustion method with LECO Trumac® N (LECO Corporation, Michigan, USA) from which the total protein contents in the samples were estimated using a conversion factor of 6.25, which is the commonly used factor for converting total nitrogen content into protein content in foods assuming 16% nitrogen in protein [41].

1.2.6. Determining keratinolytic activity of the 'crude' enzymes

The initial screening of the keratinase activities of the different samples was conducted at 30 and 60 °C and pH 8.5, using keratin azure (dyed wool keratin, Sigma, Australia) as a substrate, which is one of the commonly used substrates for evaluating keratinase activity. The initial screening experiments were conducted at pH 8.5 since all the samples with cells had a pH between 8.3 and 9.2. The assay was conducted in accordance with the protocol described in [24] with some modification. Keratin azure was ground into a fine powder after chilling and 5 mg was suspended in 1 mL 50 mM Tris-HCl buffer (pH 8.5). The reaction mixture consisting of 1 mL of the keratin azure suspension and 1mL of crude enzyme was incubated for 24 hrs in a shaking thermostated water bath maintained at 30 and 60 °C. Samples (300 µL) were aliquoted at 0, 1 and 24 hrs and centrifuged at 25000 g for 10 minutes at 4 °C to remove the substrate. The release of the azo dye in the supernatants were measured against a blank consisting of 1 mL substrate and 1 mL buffer treated in the same way. The analysis was carried out by measuring the absorbance of the

supernatants at 595nm using a Plate Reader (Varioskan® Flash, Thermoscientific, Waltham, MA, USA). One unit keratinase activity was defined as the amount of enzyme that cause 0.01 increase in absorbance at 595nm per hour under defined condition.

1.2.7. Characterization of the keratolytic enzyme

The best enzymes among the customised keratolytic enzymes produced was selected for further characterisation to determine the optimum pH for activity as well as the optimum temperature for stability in accordance with the methods described in [42,43]

Determination of pH optimum

In order to determine the optimum pH for activity, the keratolytic activity of the enzyme was assayed at 60 °C as described in 1.2.6 with keratin azure as substrate suspended in buffers of pH ranging from 5.0 to 11.0. The buffers were 50 mM acetate buffer for pH 5.0, 50 mM phosphate buffer for pH 7.0, 7.5 and 8.0 and 50 mM borate buffer for pH 9.0 and 11.0. All experiments were performed in duplicates.

Determination of temperature optimum for activity

In order to determine the optimal temperature for activity, the keratolytic activity of the enzyme was determined at the optimum pH evaluated above (pH 8.0) and different temperatures in accordance with the assay described in 1.2.6. The keratolytic activity was determined at 50°C, 60°C, 70°C and 90°C. All experiments were performed in duplicates. Preliminary experiment was also conducted to evaluate the stability of the enzyme. The enzyme was incubated for 24 hrs at 60 °C (the optimal temperature for its activity) and the residual activity of the enzyme was evaluated using the keratolytic assay described above.

Determination of the activity of the enzyme with feather as substrate

Preliminary experiments were conducted to evaluate the potential of the best keratinase enzyme for feather hydrolysis at its optimum pH and temperature in accordance with the method of Ramnani et al. [44]. The ground feather (5 mg/mL) was suspended in 50 mM phosphate buffer (pH 8.0.). Enzyme sample (1 mL) was added to 4 mL feather suspension that was equilibrated to 60 °C. The mixture was incubated for 24 hrs in a shaking water bath maintained at 60 °C. Samples (500 µL) were aliquoted at 0, 1 and 24 hrs and centrifuged for 10 min at 25000g and 4 °C. The peptides released during the reaction were evaluated by measuring the absorbance of the supernatant at 280 nm and analysing the free amine groups released using the free amino nitrogen (FAN) assay. The former is commonly used in the evaluation of the enzymatic hydrolysis of feather [44,45]. Enzyme free and feather free controls were treated and analysed in the same way as references.

1.2.8. Free Amino Nitrogen Assay (FAN assay)

The release of free amines during feather hydrolysis was determined using the free amino nitrogen [FAN] assay of Undenfriend et al. [46]. The method involves the use of fluorescamine, which reacts directly with primary amines, to produce highly fluorescent ternary products that are measured fluorometrically. In order to fit the 96 well plate, the assay volume was appropriately scaled down. Accordingly, fluorescamine solution 0.1 mL (0.1% w/v in acetone) was added to 10 μL of appropriately diluted sample supernatant, which was adjusted to pH 7.0 with 250 μL of 0.2 M borate buffer, in the well. The fluorescence was measured at 390 nm excitation and 475 nm emission wavelengths in a 96-well plate (Nunc, Thermofisher, Roskilde, Denmark) using a plate reader (Varioskan® Flash, Thermoscientific, Waltham, MA, USA). The amine concentration was calculated using a calibration curve prepared with standards containing up to 2.5 mM Gly-Gly-Gly. All analysis was performed in triplicates.

1.2.9. Medium optimisation and scale up of the production of keratinases in bioreactors

1.2.9.1. Medium optimization for the production of keratinases in 2L bioreactors

In order to improve the production of keratinases in the fermentation cultures and evaluate the potential for scale up of the process, further fermentation experiments were conducted in 2 L bioreactor using two medium formulations. *Bacillus licheniformis* and *Chryseobacterium* sp. BC06 were selected for this study since they had higher keratolytic activity compared to the other organisms investigated. The growth and keratinase production of the two organisms were evaluated in a 2L bioreactor using two different media formulations. Medium A consisted of 5 g/L Lablemco (Oxoid Australia), 20 g/L tryptone (Oxoid Australia), 10 g/L yeast extract (Merck Australia) and 20 g/L glucose. Medium B consisted of 10.6 g/L KH_2PO_4, 4 g/L $NH_4)_2HPO_4$, 1.7 g/L Citric acid (monohydrate) and 20 g/L Tryptone. The media were supplemented after autoclaving with 60 mL glucose solution (660 g/L), 10 mL $MgSO_4.7H_2O$ (1 M) and 10 mL filter sterilised trace metal solution (2 g/L $CuSO_4.5H_2O$, 0.08 g/L NaI, 3 g/L $MnSO_4.H_2O$, 0.2 g/L $NaMoO_4.2H_2O$, 0.02 g/L H_3BO_3, 0.5 g/L $CoCl_2.6H_2O$, 7 g/L $ZnCl_2$, 22 g/L $FeSO_4.7H_2O$, 0.5 g/L $CaSO_4.2H_2O$, and 0.1% H_2SO_4). The seed media was adjusted to pH 7.0 using 2M NaOH prior to autoclaving.

The Inocula for the bioreactors were produced in two stages using 30 mL glass bottles (containing 10 mL of medium) and 2L baffled Erlenmeyer flasks (containing 500 mL of medium). The seed cultures were grown in Medium A. The primary seed cultures (10 mL of medium in a 30 mL bottle) were inoculated with a small portion of a glycerol stock culture that was stored at -80°C. The cultures were incubated at 30°C for 20 hours shaking at 200 rpm. After completion of the 20 hour incubation, 500 µL of the primary seed culture was used to inoculate the 2L baffled Erlenmeyer flasks containing 500 mL of medium (secondary seed cultures). The secondary seed cultures were incubated for 24 hours at 30°C shaking at 200 rpm. The optical densities of the secondary seed

cultures (measured at 600 nm) were 12.5 and 22.9 respectively for *Bacillus licheniformis and Chryseobacterium* sp. BC06 respectively.

The bioreactors were inoculated with a volume of the secondary seed culture required to attain an initial optical density of 0.2. The bioreactor experiments were performed in 2.5 L glass vessels with a 2 L operating volume (Sartorius Biostat B). The 2L bioreactors were operated at initial volume of 2 L, temperature of 30 °C, agitator speed between 500 and 1140 rpm, pH set point of 7.0 with 10% H_3PO_4 and 10% NH_3 solutions used for automated pH control. The air flow rate was 0.3 to 1.5 L/min and the dissolved oxygen content was set at 30% with airflow supplemented with 5% pure oxygen. Automatic chemical foam control was used (10% polypropylene glycol 2025) and 3 mL of antifoam was added to the reactor before inoculation. The cell growth rate was estimated by periodic measurement of the optical density of diluted samples at 600 nm.

To enhance the production of keratinases, 20 grams of autoclaved ground chicken feathers were added to each bioreactor 24 hours after incubation and samples were taken periodically to determine the levels of enzyme expression. Optical densities could not be measured during this phase due to interference from the added chicken feathers. The level of expression of keratinases was determined using the keratinase assay described in 1.2.6.

1.2.9.2. Production of *Bacillus licheniformis* keratinase in a 10L bioreactor

Further scale up fermentation experiment using 10 L bioreactor was focused on *Bacillus licheniformis* keratinase since the bacteria was found to be the most promising in terms of expression of keratolytic enzymes. The seed cultures for this experiment were produced as described in section 1.2.9.1. The optical densities of the primary and the secondary seed cultures were 4.63 and 14.04 respectively. Medium B was used for this experiment as this medium yielded the highest cell mass and enzyme activity in the 2L bioreactors. The bioreactor was inoculated with a volume of the secondary seed culture required to attain an initial optical density of 0.2. This experiment was performed in 15 L stainless steel vessel with a 10 L operating volume (Sartorius Biostat C10). To prevent foaming, 10 mL of neat polypropylene glycol 2025 was added to the medium before sterilisation. The operating conditions were the same as the 2 L reactor except that the agitator speed was 350 to 1400 rpm, and the airflow rate ranged from 5 to 15 L/min.

In the previous experiment, production of keratinase was induced by adding 20 grams of autoclaved ground chicken feathers to the 2L bioreactors. As adding 100 grams of ground feather to the 10L bioreactor was very difficult due to the bulk nature of feather, a liquid feather extract was added to the bioreactor. To produce the feather extract 25 grams of chicken feather was weighed into each of four one litre centrifuge pots. Approximately 1L of buffer (2 g/L NaCl, 0.3 g/L K_2HPO_4, 0.4 g/L KH_2PO_4) was added to each pot and the mixture was autoclaved at 121°C for 20 minutes. Once cooled the autoclaved feather were centrifuged at 12,227g for 60 minutes. The supernatant (~2.1 L) was then added aseptically to the bioreactor. Before induction with chicken feather extract,

four litres of culture was removed from the bioreactor and transferred to one litre centrifuge pots and then centrifuged at 12,227x*g* for 60 minutes at 4°C to remove the cells. The supernatant was decanted into a plastic container and stored at 4°C. Once the induction process was complete the remainder of the culture was harvested, centrifuged (12,227x*g* 60 minutes, 4°C) and the supernatant stored at 4°C. The level of expression of keratinase activity was measured before induction and four hours after induction.

1.3. Results and Discussion

1.3.1. Feather preparation

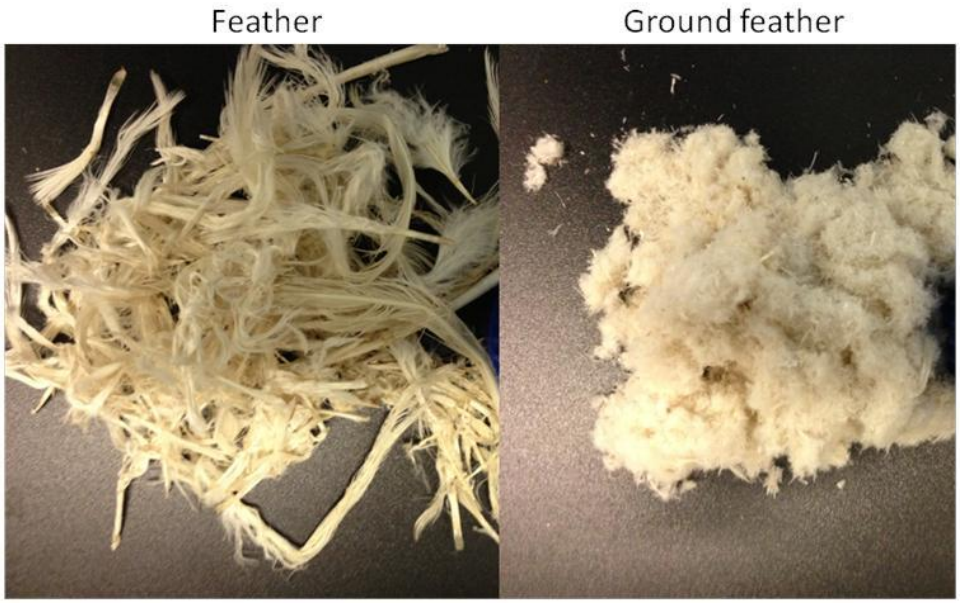


Figure 1 Feather samples before and after drying and grinding

Wiley mill was successfully used to reduce the size of the washed and dried feather to particles of approximately less than 1 mm in size. The photographs of feather samples prior to and after size reduction are given in Figure 1. This facilitated the use of the feather samples in fermentation culture as well as enzymatic hydrolysis experiments. The process is amenable to scale up using either Wiley or similar knife mills which are capable of size reduction of feather and similar materials.

1.3.2. Identification of keratolytic organisms

Three of the four bacteria tested grew on the plates containing the ground feather indicating that they are keratolytic and are able to metabolise feather, whereas *Bacillus halodurans* did not. See example plates in Figure 2, where clear zone of hydrolysis are observed on the medium agar plate with feather. There was almost no growth in the negative controls and all the four strains grew on the nutrient agar. The three strains that showed growth on medium agar were used in further fermentation trials for the production of keratolytic enzymes. Different strains of *Bacillus licheniformis* [44], *Chryseobacterium sp*. [23], *Bacillus halodurans* [20] *and Bacillus Thuringensis*

[22] have been shown to possess keratolytic activities. It seems that the *Bacillus Halodurans* strain that was investigated in our study does not have keratolytic activity.

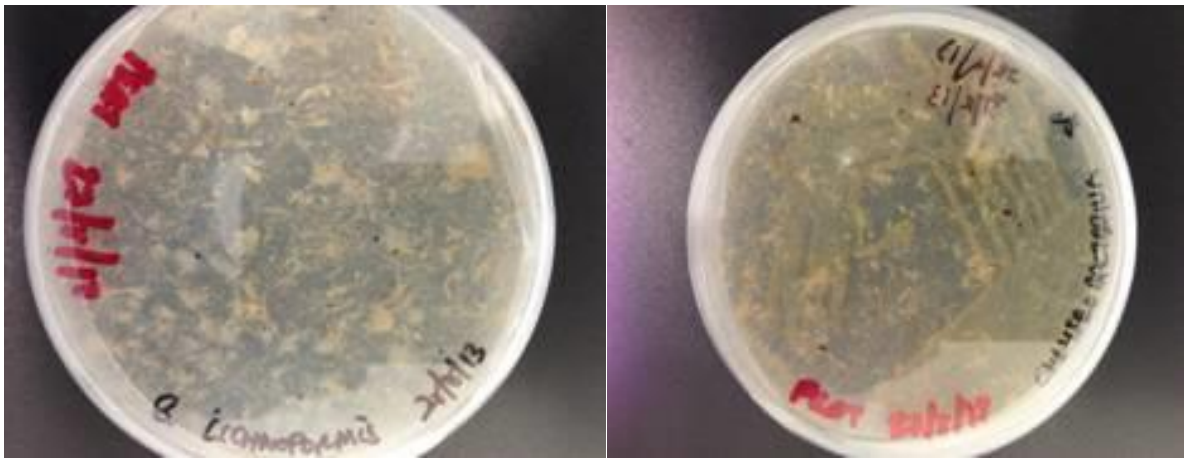


Figure 2 *Bacillus licheniformis* and *Chryseobacterium* sp. BC06 plates on which growth on ground feather were observed

1.3.3. Fermentation experiments

Further fermentation experiment focused on the three organisms that were capable of metabolising feather. In all cases, change in turbidity indicating growth of these organisms was observed in liquid cultures consisting of bacterial cells and feather or feather hydrolysates (culture 3, 4, 5) (Figure 3).

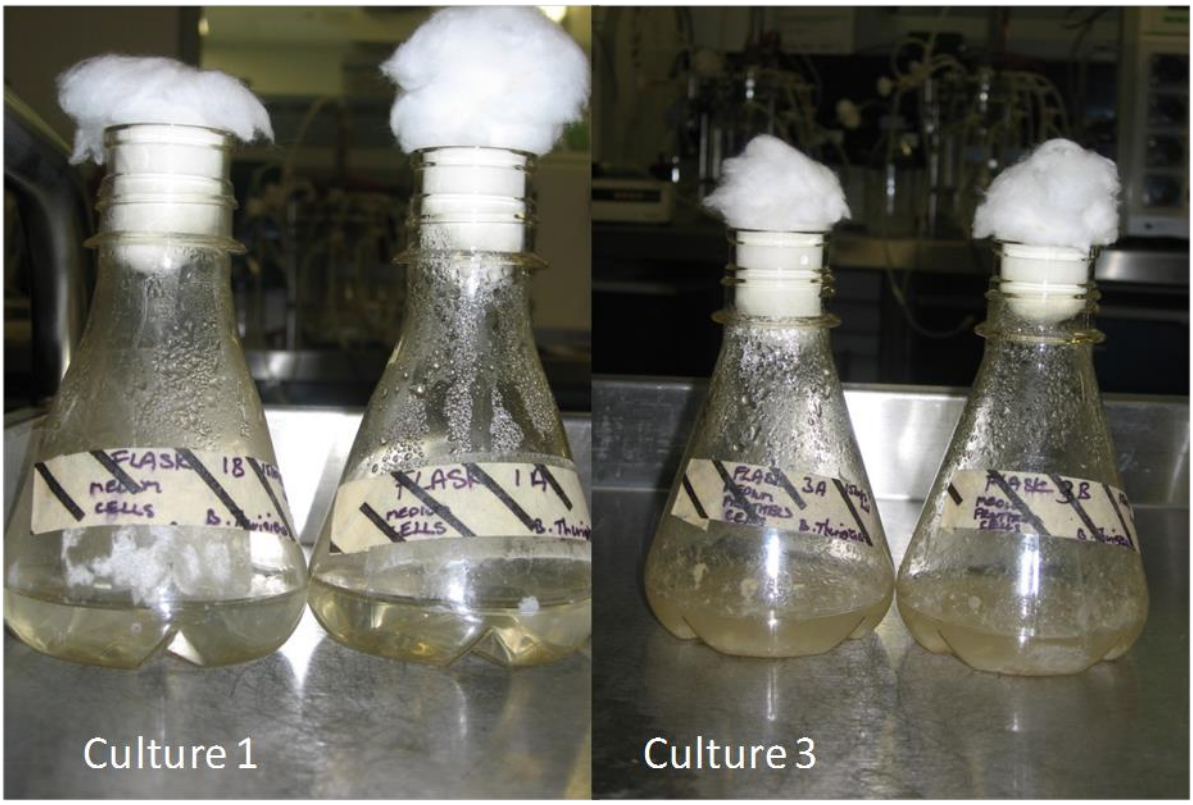


Figure 3 Comparison of the cell and feather free culture (culture 1) with the cell plus feather culture (culture 3) for *Bacillus thuringensis* ATCC 33680

1.3.4. Extraction and characterization of kertolytic enzymes

In all cases, culture 3 had the highest protein content whereas the cell and feather free culture (culture 1) had the lowest protein content as would be expected. All the cell and feather free cultures had pH between 6.5 and 7.3 while the cultures containing bacterial cells and feather had pH between 8.3 and 9.2 (Table 1). The increase in pH is most probably due to the release of ammonia, which is the product of amino acid metabolism. It confirms that the bacterial cells produced keratolytic enzymes and used feather as a source of amino acids.

Table 1 Estimated average protein content and pH of supernatants from the liquid cultures

Bacterium		**Culture**				
		1	2	3	4	5
Bacillus licheniformis	pH	7.2	6.6	8.9	8.5	9.0
	Total protein (mg/ml)	0.23	0.56	2.5	0.7	2.2
***Bacillus thuringensis* ATCC 33680**	pH	7.3	6.5	8.8	8.3	8.7
	Total protein (mg/ml)	0.1	1.28	1.34	0.50	1.0
***Chryseobacterium* sp. BC06**	pH	7.3	6.5	9.2	8.6	9.1
	Total protein (mg/ml)	0.08	0.91	2.6	0.44	1.7

In all cases significant keratinase activities were observed in the supernatants from cultures 3 and 5 of all bacteria and culture 4 of *Bacillus licheniformis* and *Bacillus thuringensis* ATCC 33680. In these cultures, the cells have no carbon and nitrogen source and therefore may have turned on systems that enable scavenging of nutrients from feather. One such system would be keratinases which can be used to provide access to nutrient from keratinous materials. This may explain the observed significant keratinase activity in these samples. Significant keratinase activity was also observed in the supernatants of the feather free cultures of *Bacillus licheniformis* and *Chryseobacterium sp*. BC06, which indicates that these organisms do not need feather to induce the production of keratinase enzymes. Brandelli et al. [19] stated that the addition of keratinous substrates in the cultivation medium is not always required for the production of keratinases.

Among the bacterial strains investigated, crude enzyme samples from *Bacillus licheniformis* cultures showed higher keratinase activities compared to the other bacteria. Culture 4 from the *Bacillus licheniformis* culture had the highest specific keratolytic activity (Figure 4) indicating that partially hydrolysed keratin from the autoclaved feather stimulated keratinase production by this organism. Bacterial species especially from the genus *Bacillus*, *actinomycetes* and some fungi, effectively degrade keratin substrates such as feathers by utilising their enzyme system and eroding mycelium in the case of fungi to access carbon and nitrogen nutrients in these substrates

[4,19]. Within the Genus *Bacillus*, keratin degrading ability was more frequently observed in *Bacillus licheniformis* strains and less frequently in other strains such as *Bacillus pumilis, Bacillus cereus* and *Bacillus subtilis* [4]. Moreover, to our knowledge, the only keratinase that has been commercialised for industrial application in various forms (Versazyme™ (now Cibenza), Valkaraze™, Prionzyme™ and PURE100) is the keratinase from *Bacillus licheniformis* PWD1 [48].Therefore, it is not surprising that we observed the highest expression of keratinase activity in the *Bacillus licheniformis* culture.

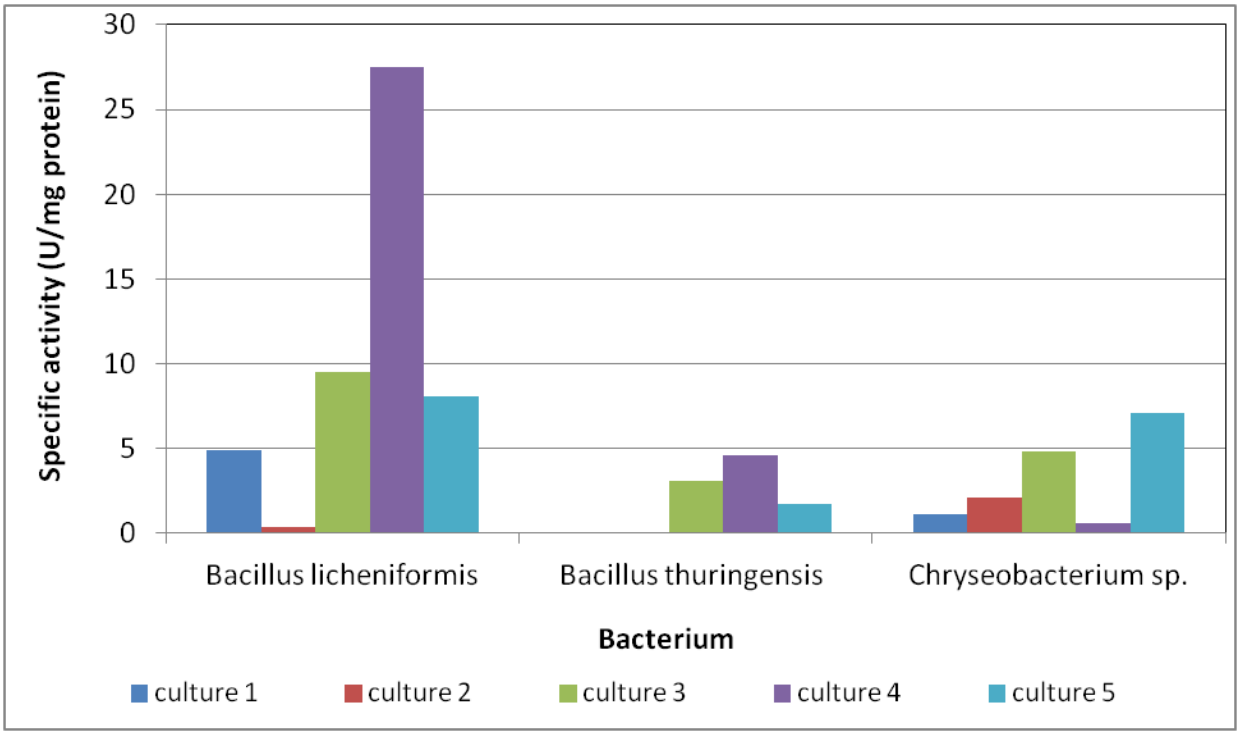


Figure 4 The specific keratolytic activities (U/mg protein) of the different samples at pH 8.5 and 60 °C.

As the highest keratinase activity was found in the *Bacillus licheniformis* culture, further characterisation studies focused on this enzyme. In order to determine the pH optimum for the enzyme, the activity of the enzyme was assayed at pH ranging from 5 to 11 and 60 °C using keratin azure suspended in buffers as a substrate. Data are presented in Figure 5. Culture 4 was used as a crude enzyme source in all the experiments.

As can be seen in Figure 5, the optimum pH for the activity of keratinase from *Bacillus licheniformis* was found to be 8.0. The pH optimum for the keratinase from the well investigated *Bacillus licheniformis* PWD1 is 7.5 [49], which is close to our value. A pH optimum of 11.0 was reported for keratinases from *Bacillus licheniformis* strains ER-15 [44], indicating the variability in the biochemical properties of keratinases from the same bacteria depending on the strain. In order to determine the temperature optimum for the enzyme, further experiments were conducted at the optimum pH 8.0 and temperatures ranging from 50 to 90 °C. The optimum temperature for the

activity of the enzyme was found to be 60 °C (Figure 6). The temperature optimum for the PWD-1 keratinase is reported to be 50 °C [49] whereas that of ER-15 is 70 °C [44]. The preliminary study on the stability of this enzyme showed that, more than 12% activity remains after 24 hrs incubation of the enzyme at its optimum condition for activity (60 °C, pH 8.0). The purified PWD1 keratinase was reported to be quite unstable losing 50% of its activity during four to five days storage at room temperature due to autolysis [49].

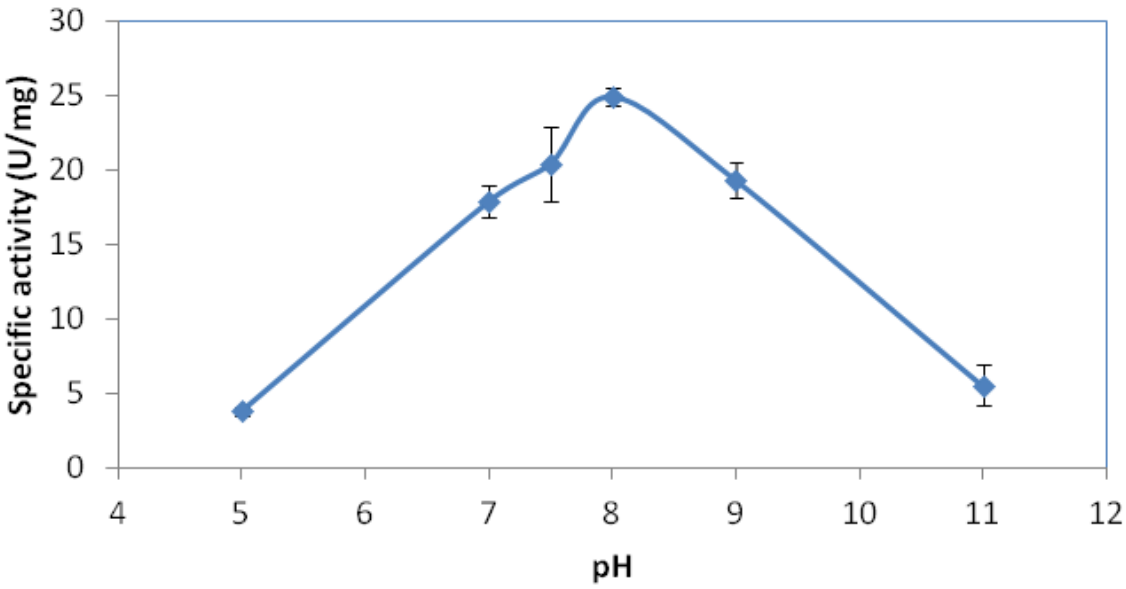


Figure 5 The specific activity of keratinase from *Bacillus licheniformis* at 60 °C and different pH showing the optimum pH for the activity of the enzyme

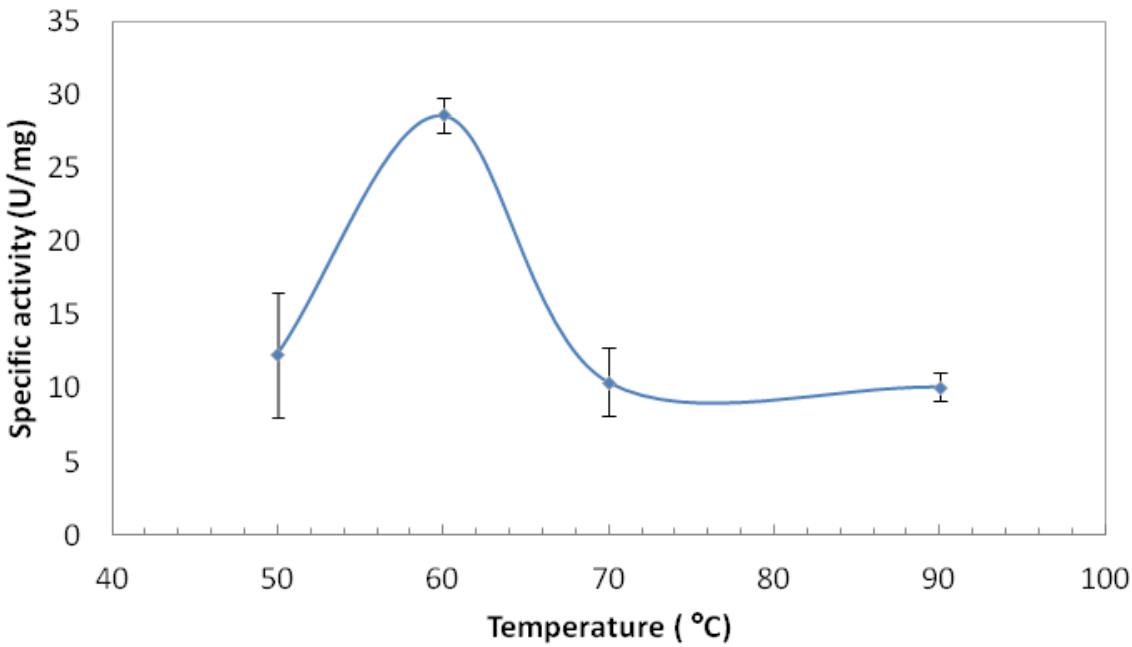


Figure 6 The specific activity of keratinase from *Bacillus licheniformis* at pH 8.0 and different temperatures showing the optimum temperature for the activity of the enzyme

The preliminary experiments to evaluate the keratolytic activity of the enzyme at its optimum condition with poultry feather as substrate showed that the enzyme is capable of degrading feather (Figures 7 and 8). Significant release of free peptides was observed during the incubation of feather with the crude enzyme although the crude enzyme sample was very dilute (0.7 mg protein/ml, see Table 1), with potentially high concentration of protein from the fermentation medium. Assuming 0.01 unit increase in absorbance at 280 nm per hour during hydrolysis of feather under standard conditions corresponds to 1 unit of enzyme activity [44], the crude keratinase had a specific activity of 10 U/ml or 15 U/mg of protein with feather as substrate. Nevertheless, complete solubilisation of the feather substrate was not observed even after 24 hours of hydrolysis as has been reported in other studies [42, 50], indicating the important role of sulfitolysis that involves other enzymes and metabolites [4,19,48] and colonisation by a live bacterial cell [50].

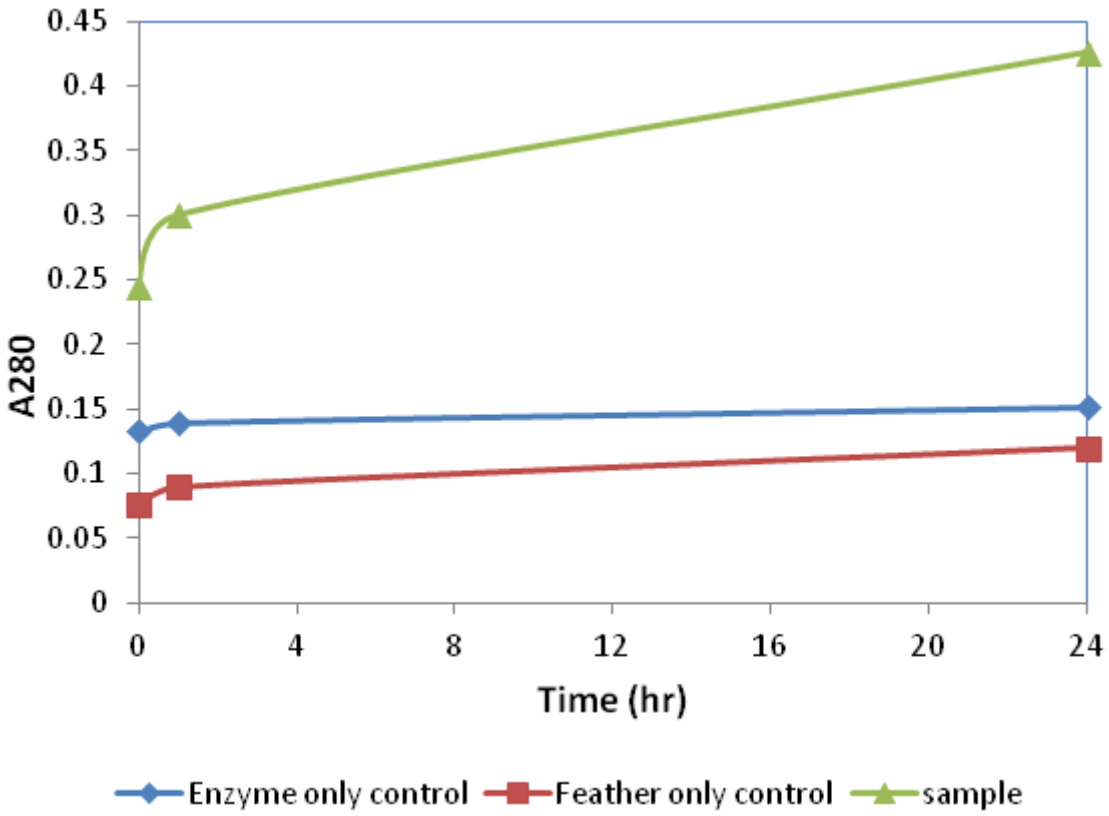


Figure 7 Feather keratin hydrolysis by keratinase from *Bacillus licheniformis* at pH 8.0 and 60 °C as measured by the increase in absorbance at 280nm indicating the release of peptides

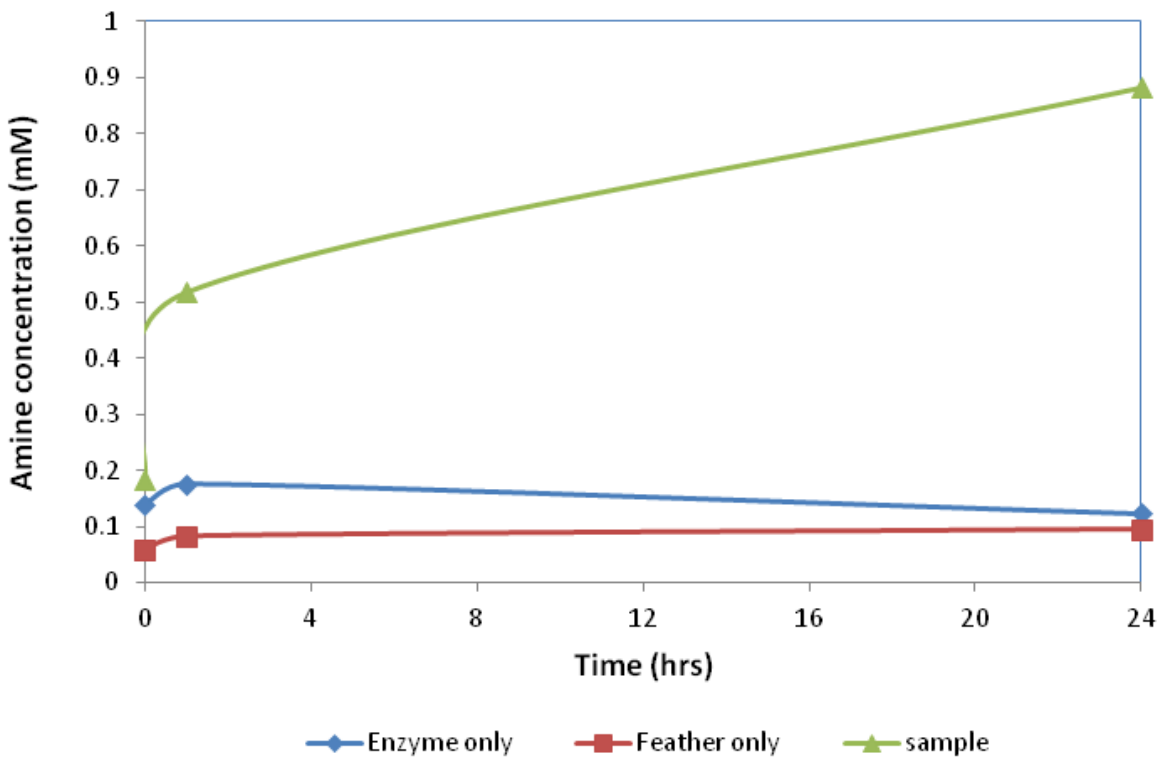


Figure 8 Feather keratin hydrolysis by keratinase from *Bacillus licheniformis* at pH 8.0 and 60 °C as measured by the increase in free amino nitrogen concentration

1.3.5. Medium optimisation for the production of keratinases

Medium optimisation experiments were conducted to increase production of keratinase by *Bacillus licheniformis* and *Chryseobacterium sp.* BC06, the two organisms that showed relatively higher expression of keratolytic enzymes, in 2L stirred tank bioreactors. Two different media formulations were used in the experiment. The optical density data showing the rate of growth of the two organisms are presented in Figure 9. *Chryseobacterium sp*. BC06 grew well in both media reaching optical densities of ~30 after 24 hours. On the other hand, *Bacillus licheniformis* grew well in medium B but performed poorly in medium A (attained only an optical density of 2.3) (Figure 9). The glucose added to the medium was consumed within 23 hours (data not shown) in all four bioreactor experiments. Optical densities could not be measured after the addition of 20 gm feather to enhance keratinase production 24 hours after inoculation, due to interference from the added chicken feathers. Rather, the level of expression of keratinases was used to evaluate the effect of the medium composition on growth and production of keratinases (Figure 10).

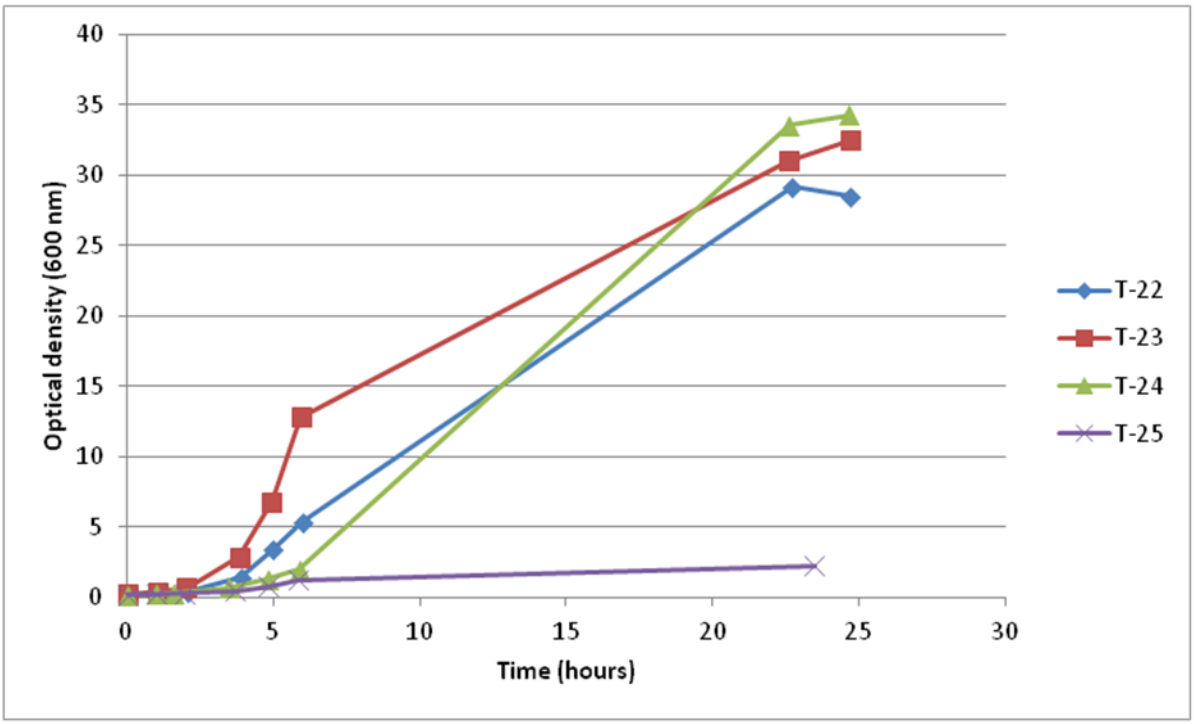
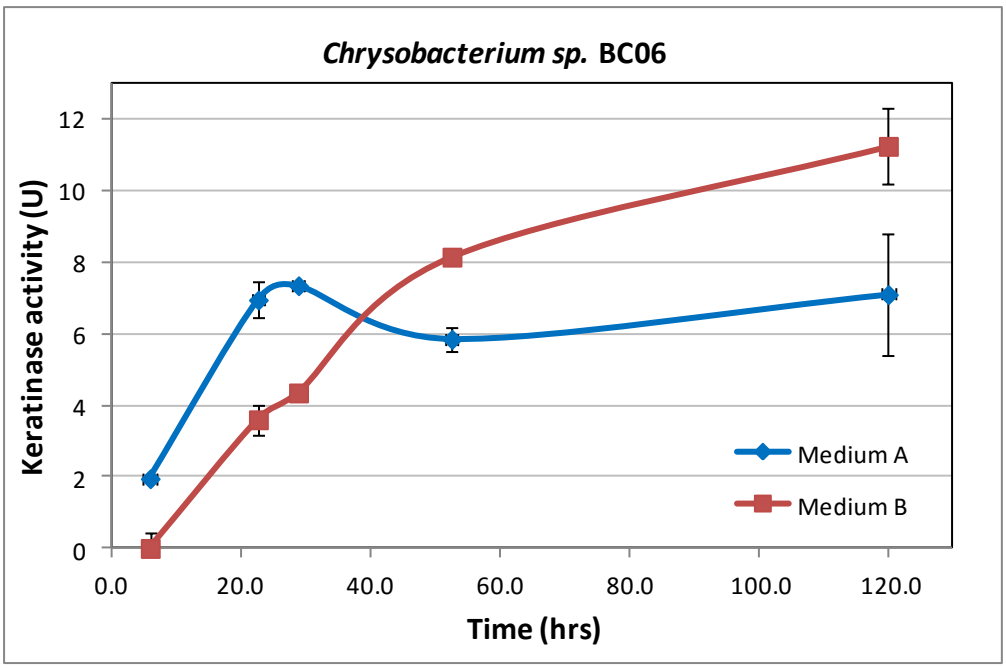


Figure 9. Growth data from 2L bioreactor experiments. Optical density of diluted samples was measured at 600 nm. T-22 = *Chryseobacterium* sp. BC06, Medium B; T-23 = *Chryseobacterium* sp. BC06, Medium A; T-24 = *Bacillus licheniformis*, Medium B; T-25 = *Bacillus licheniformis*, Medium A.

Overall, higher keratolytic activity and hence keratinase expression was observed in *Bacillus licheniformis* cultures (Figure 10). The highest keratolytic activity was measured in *Bacillus licheniformis* cultured in medium B after 52.6 hrs incubation. The keratolytic activity declined during further incubation to 120 hrs probably due to the production of enzyme inhibitors, autolysis or the denaturation of the enzyme during prolonged incubation. The keratinase activity increased with increase in incubation time in all the cultures except the *Chrysobacterium sp*. BC06 culture in medium A where the keratinase activity remained constant after 28.7 hrs. The results of the study showed that the best harvesting time for the *Bacillus licheniformis* culture in medium B is between 28.7 and 52.6 hrs, which is about 4 to 28 hours after the introduction of the autoclaved feather for inducing keratinase production. Interestingly, although medium A promoted relatively higher rate of growth in the *Chrysobacterium sp*. culture, higher keratinase expression was observed in medium B cultures in the case of *Bacillus licheniformis* throughout the fermentation period and after 40 hrs of fermentation in the case of *Chrysobacterium sp*. The various nitrogen sources in medium A did not promote the production of keratinases in both organisms, probably since they are a source of readily available nitrogen which does not need the activity of keratinases to access. Clearly, this trial confirmed that the most promising organism for the production of keratinases is *Bacillus licheniformis* and further experiments were focused on this organism.



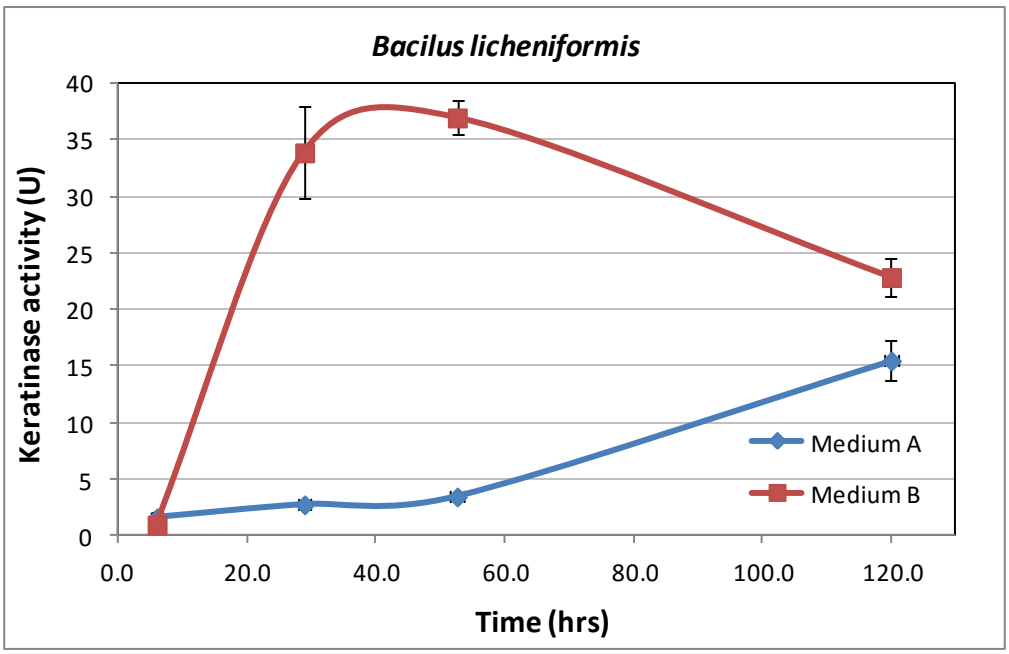


Figure 10 The expression of keratinases in *Bacillus licheniformis* and *Chryseobacterium sp.* BC06 cultures as a function of incubation time

1.3.6. Production of *Bacillus Licheniformis* keratinase in a 10 litre bioreactor

To produce a larger quantity of keratinase enzyme from *Bacillus licheniformis* and evaluate the potential for scale up, the culture volume was increased to 10L. The optical density data is shown in Figure 11. The growth of *Bacillus licheniformis* in the 10L bioreactor was similar to the 2L bioreactor although the optical density was lower when the cells reached stationary phase, probably due to the change in operating condition.

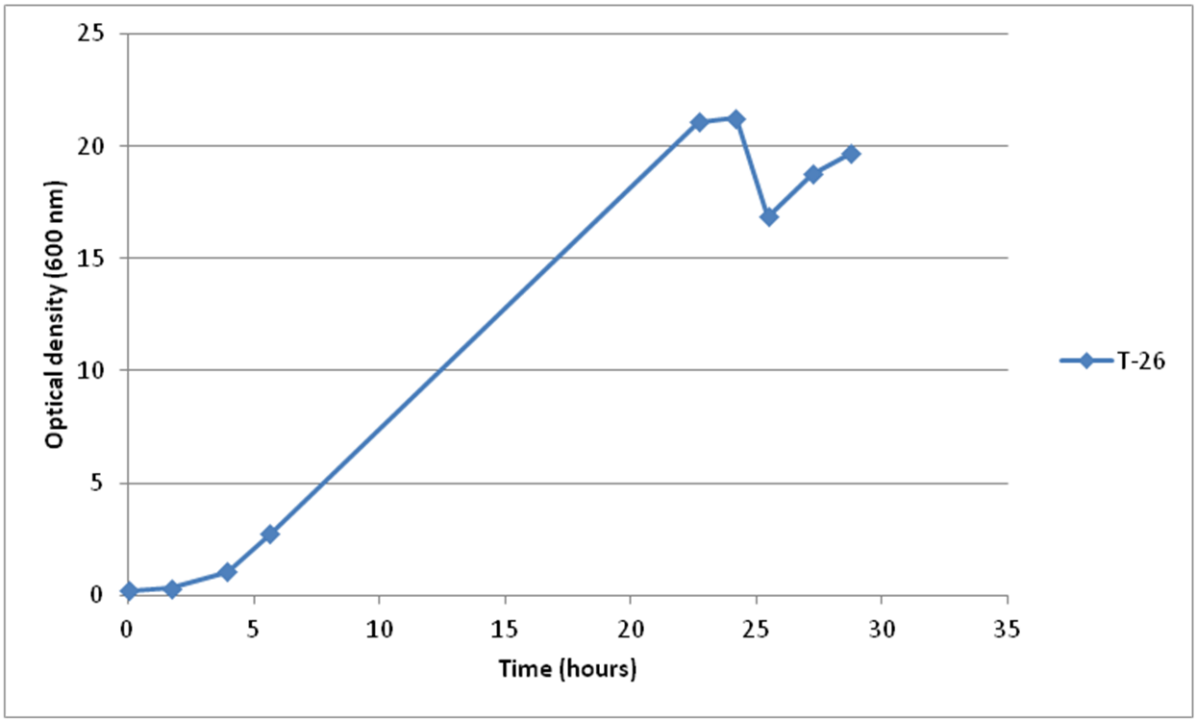


Figure 11 Growth data from the 10L bioreactor experiment (*Bacillus Licheniformis*). Optical density of diluted samples was measured at 600 nm. T-26: *Bacillus licheniformis*, Medium B.

No keratinase activity was measured in the 10 L bioreactor samples before induction with thermally hydrolysed feather and the activity about four hours after induction (at 28.7 hrs) was 17.4 U, which is almost half of the value obtained in the previous experiment at the same time point (33.9 U, Figure 10), which probably was due to the lower cell density obtained at the stationery phase in the 10L reactor compared to the 2L reactor (Figure 9 and 11, 35 compared to 20). Using the feather supernatant instead of feather for the induction of keratinases has an advantage since it will make the fermentation and downstream processing of the enzyme easier. In addition, the fermentation experiments in section 1.2.3 showed that using thermally hydrolysed feather improves keratinase production by *Bacillus licheniformis* (Figure 4). Thus, the lower keratinase production is most probably due to the change in the operating conditions of the larger reactor, which need further optimisation.

1.4. Conclusion

Solid state culture with feather as the sole nutrient source was used to identify three bacterial strains i.e. *Bacillus thuringensis* ATCC 33680; *Bacillus licheniformis* and *Chryseobacterium sp*. BC06, with keratolytic activities. Further liquid culture investigations revealed that *Bacillus licheniformis* produces the most efficient keratinase under the studied conditions. This enzyme was further characterised. Its optimum temperature for activity was found to be 60 °C and its optimum pH for activity was 8.0. The crude enzyme preparation showed significant keratolytic activity with both keratin azure and poultry feather as substrates. In addition, the crude enzyme showed reasonably high thermal stability with more than 12% activity remaining after 24 hrs at 60 °C. Clearly, the keratinase from the *Bacillus licheniformis* strain investigated in this study has a potential for application in feather keratin hydrolysis. Further investigations were conducted to optimise the fermentation condition and increase keratinase production. Kertinases from *Bacillus licheniformis* and *Chryseobacterium sp.* BC06 were successfully produced in 2L bioreactors under defined conditions. The production of keratinase from Bacillus licheniformis could be scaled up to a 10 L bioreactor, although further experiment will be required to optimise the fermentation condition. As in the flask fermentation experiments, higher expression of keratinase was observed in the *Bacillus licheniformis* culture indicating the excellent potential of this organism to produce keratinase for feather hydrolysis.

Chapter 2 Comparison of the efficacy of customised keratolytic enzymes with selected commercial enzymes

2.1. Introduction

In chapter 1, three feather degrading microorganisms that produce keratolytic enzymes were identified. Among these organisms, the highest keratolytic activity was observed in *Bacillus licheniformis* culture, which showed significant keratolytric activity with both keratin azure and feather as substrates. Apart from keratinases, some serine proteases have been shown to hydrolyse feather especially in the presence of suitable reducing agents [26]. The objectives of this chapter were to compare the efficacy of the customised enzyme from *Bacillus licheniformis* produced in this study with commercial keratinases and proteases for hydrolysing poultry feather in the presence and absence of a reducing agent. In this study, the efficacy of the commercial serine proteases Alcalase, Multifect PR 6L, Savinase 16L and the analytical keratinase proteinase K were compared with the keratinase produced in this study from *Bacillus licheniformis* (which will be referred as customised enzyme) for hydrolysing native feather. Alcalase is from *Bacillus subtilise*, multifect PR 6L is from *Bacillus licheniformis* while Savinase 16L and Proteinase K are from *Bacillus amyloliquefaciens* and the fungus *Tritirachium Album*, respectively.

2.2. Materials and Methods

2.2.1 Materials

Feather samples were obtained from a local rendering company and processed as described in chapter 1. Proteinase K was obtained from Sigma (Australia). Alcalase and Savinase 16L were obtained from Novozyme and MultifectPR6L was obtained from Enzyme Solutions. DTNB (5, 5'–dithiobis (2-nitrobenzoic acid)) and cysteine were obtained from Sigma (Australia). All the other reagents were analytical grade. All the other chemical and biochemical reagents were analytical grade or better.

2.2.2 Selection of commercial proteases for benchmarking

The objective of these experiments was to select suitable benchmark commercial proteolytic and keratolytic enzymes for comparison with the keratolytic enzyme identified in this work. Thus, based on previous experience and the literature, three commercial proteases i.e. Alcalase, Savinase 16 L, and MultifectPR6L were selected for evaluating their potential use for feather hydrolysis. The only commercialised keratinase is the keratinase from Bacillus Licheniformis PWD-1, which was marketed as Versazyme, Valkerase and Prionenzyme. However, none of these products are currently available from commercial enzyme suppliers in Australia. Currently Versazyme is being marketed as Cibenza by Bioresource international. Although the company is represented by

Novus International in Australia, we were not able to access any of the Cibenza products as Novus International said that the products are not imported into Australia. Thus the only keratinase that was evaluated in this work is the analytical enzyme proteinase K from sigma.

Feather hydrolysis experiments were conducted with 1% feather and 1% enzyme dosage (on substrate protein content basis) at the recommended pH and temperature optimum of the commercial enzymes i.e. 37°C and pH 8.0 for proteinase K, 60°C and pH 8.0 for Alkalase, 50°C and pH 8.5 for Savinase and 16L, 60°C and pH 9.5 for MultifectPR6L. The moisture and protein content of the feather sample were 9% and 87% respectively. To 0.5 gram of 'dry' powdered feather, 49.5 ml of water was added and the mixture was vortexed for 2 minutes and ultraturraxed (13,500 rpm) for 30 seconds. The pH of the suspension was adjusted to the desired pH depending on the enzyme using 4M NaOH. The enzyme was added after the suspension was equilibrated to the experimental temperature. The reaction mixtures were incubated at the experimental temperatures in a thermostated shaking water bath for 24 hours. The pH was continuously adjusted using 4M NaOH. Sample aliquots of the digests were removed after 24 hour incubation and the reaction was stopped by inactivating the enzyme in the samples at the denaturing temperature specific for the enzyme. The samples were immediately cooled in ice-water and centrifuged for 10 minutes at 25,000g and 4°C. The extent of protein hydrolysis in the feather samples were estimated by measuring the total nitrogen and peptides released into the hydrolysate, using the LECO Trumac® N (LECO Corporation, Michigan, USA) and Free Amino Nitrogen (FAN) assay, respectively using the methods described in 1.2.5 and 1.2.8. 'Enzyme free' and 'feather free' control samples were treated and analysed in the same way as references.

2.2.3 Optimising the dosage for the selected 'commercial' enzymes

In order to optimise the required dosage of the commercial enzymes, feather hydrolysis experiments were conducted using the selected enzymes (Savinase 16L and Proteinase K) at three different enzyme concentrations 1%, 3% and 5% (based on substrate protein) as described above. Sample aliquots were taken at 0, 24 and 48 hours and analysed for peptide release using the FAN assay and the release of free sulfydryl groups using the Elman reagent based assay described below (2.2.4).

2.2.4. Determination of free sulfydryl group production

In order to determine the extent of cleavage of disulfide bonds during feather hydrolysis, the free sulfhydryl group formed during hydrolysis was measured in accordance with the method of Ramnani et al. [50]. One hundred microlitres of 0.01 M DTNB (5, 5'–dithiobis (2-nitrobenzoic acid)) solution in phosphate buffer (0.1 M, pH 7.0) was added to 3 mL of the mixture containing the sample supernatant, phosphate buffer and milli Q water 3:2:5 ratio, appropriately scaling down the amount of each component to fit the 96 well plate. Absorbance was measured at 420 nm after 2 min of stable colour development, in a 96-well plate (Nunc, Thermofisher, Roskilde, Denmark)

using a plate reader (VarioSkan, Thermo Scientific, Flash, USA). The sulfydryl concentration was calculated from the absorbance using a calibration curve developed with standards containing up to 1.0 mM L-cysteine.

2.2.5. Comparison of commercial and customised enzyme at standardised dosage

In order to standardise the comparison of the commercial enzymes with the customised keratinase, the activities of selected commercial enzymes were determined using keratin azure as substrate at different conditions (at conditions recommended by the manufactures and at conditions used for the assay of custom made enzymes) and their specific activities were defined in the same way as the custom made enzyme at the dosage determined in 2.2.3. The assay was conducted as described in 1.2.6.

Among the investigated proteases, Savinase 16L showed the best performance for hydrolysing feather and keratin azure. Thus, the activity of Savinase 16 L was compared with proteinase K and the *Bacillus licheniformis* keratinase at a standardised enzyme concentration equivalent to the concentration of Savinase 16L keratinase activity (72 U) at the best dosage determined in 2.2.4. (3%) The amount of enzyme to produce the same level of activity (i.e. 72.4 U) for the Proteinase K and Custom made enzyme were determined. Feather hydrolysis experiments were conducted as described in 2.2.2 with the three enzymes, Savinase 16L, Proteinase K and Custom made enzyme at the standardized conditions of 72.4 U at 60°C and pH 8.0 with and without sodium sulphite at 1% (w/v) dosage. For the experiments on *Bacillus licheniformis* enzyme, samples from the 10L batch were used. Since none of the enzymes investigated so far resulted in complete feather hydrolysis, the effect of 1% sulphite (w/v) on the rate and extent of hydrolysis using the different enzymes was also investigated. The experiments were conducted in a thermostated shaking water bath maintained at 60 °C and pH 8.0. Aliquots (2ml) were periodically taken and analysed for free amino nitrogen (using FAN assay), free sulfhydryl content and nitrogen content (using LECO) in the supernatant. Feather only, feather and sulphite, enzyme only and sulphite and enzyme samples were treated and analysed in the same way as references.

2.2.6. Data analysis

Data analysis was performed using Microsoft excel and Design expert software, version 7.1.3 (Stat-Ease Inc., Minneapolis, MN, USA). The significance of differences between treatment means was evaluated using Tukey multiple range test at 0.05 level of significance.

2.3. Results and Discussion

2.3.1 Selection of commercial proteases for bench marking and enzyme dosage

All the selected commercial proteases showed some degree of capability to hydrolyse feather (Figure 12 and 13). Data for free amino nitrogen release (i.e. peptide release) and percentage

nitrogen release are presented in Figure 12 and 13. As can be seen, after 24 hours, the highest extent of feather hydrolysis was obtained using Savinase 16L followed by Alcalase. The extents of hydrolysis as measured by free amine release were similar for proteinase K and MultifectPR6L (Figure 12), although the percentage hydrolysis was higher for MultifectPR6L (Figure 13). Overall, the percentage hydrolysis of feather after 24 hours was very low with a maximum of about 18% hydrolysis using Savinase 16L indicating that the enzymes are not able to efficiently hydrolyse feather keratin. Pedersen et al. [51] compared the efficacy of four enzymes i.e. Cibenza DP100, Allzyme FD, Arazyme and Protex 30L for feather hydrolysis. The highest degree of hydrolysis as measured by the release of free amines was obtained using Protex 30L, which is a protease from *Bacillus subtilise*, indicating that conventional proteases may exhibit a better efficiency for degrading feather than keratinases such as Cibenza [51].

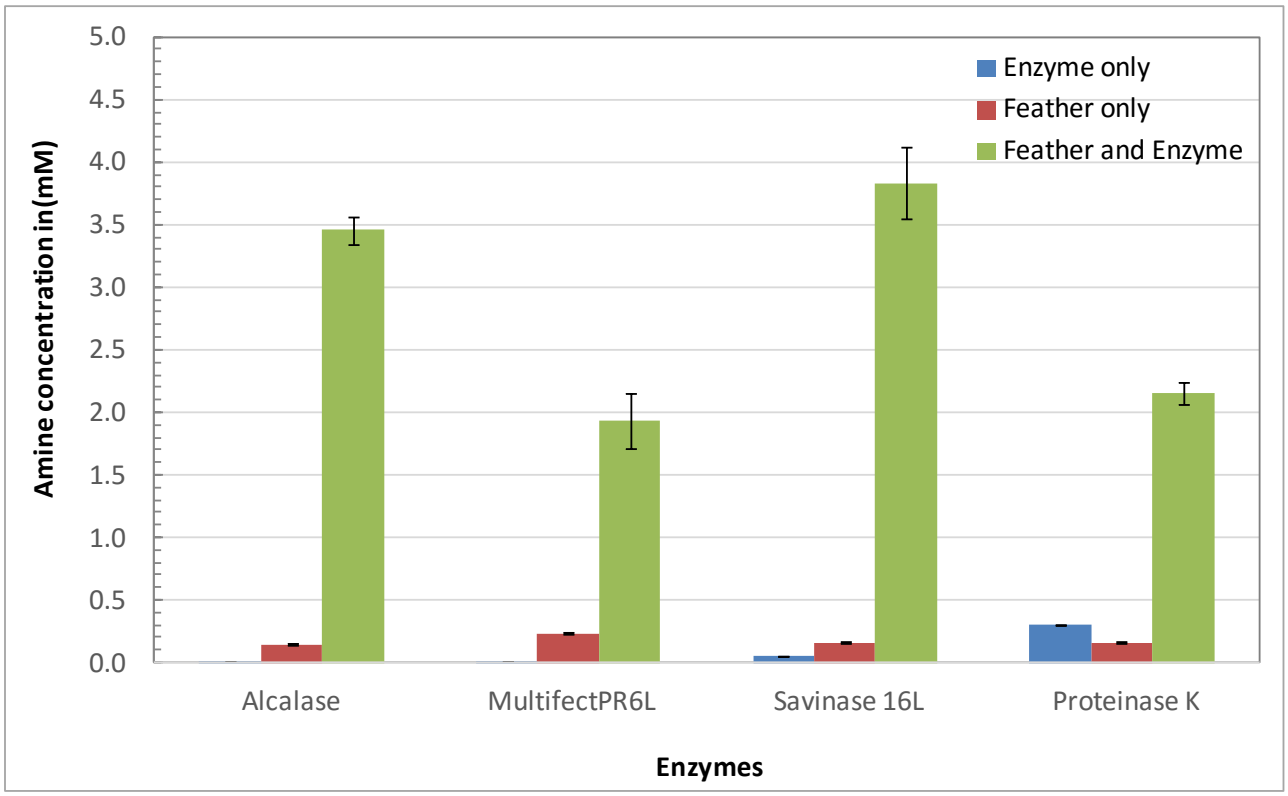


Figure 12 The concentration of free amino nitrogen released after the hydrolysis of 1% feather for 24 hours using different commercial enzymes at 1% dosage on protein basis at the recommended optimum temperature and pH of the enzymes

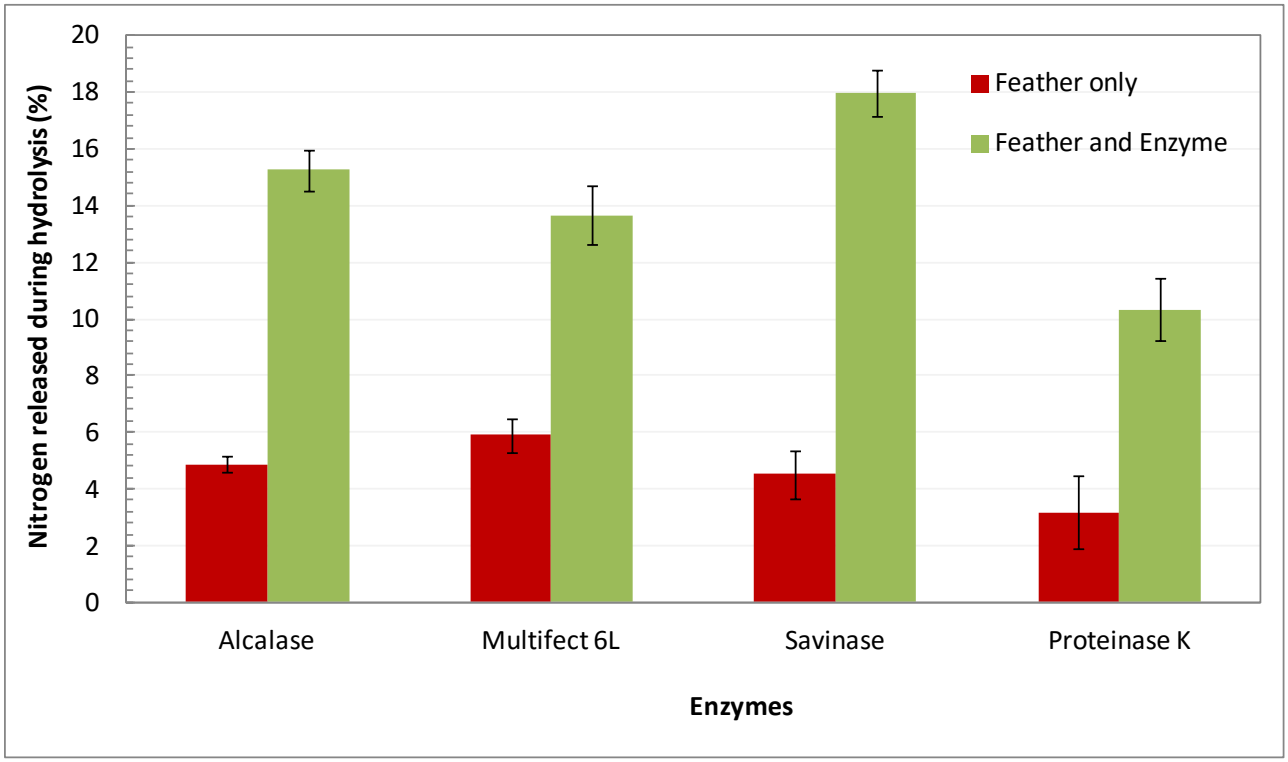
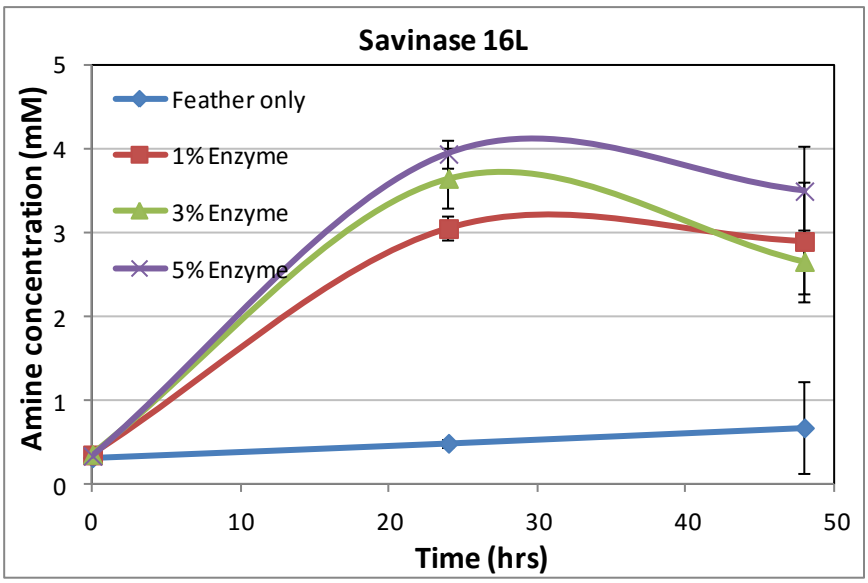


Figure 13 The estimated percentage hydrolysis of feather after 24 hours hydrolysis using different commercial enzymes (1% feather, 1% enzyme dosage on protein basis) at the recommended optimum temperature and pH of the enzymes

Savinase 16L was selected for the benchmarking study as it was the best performing commercial enzyme. For lack of a suitable commercial keratinase, proteinase K was also selected as another benchmark for comparison. In order to determine the best enzyme dosage for feather hydrolysis with these two enzymes, experiments were conducted using three dosages (1%, 3% and 5% on substrate protein basis) at the recommended optimum conditions of the enzymes. The data on free amino nitrogen release and the production of free sulfhydryl groups are presented in Figure 14 and 15 respectively.



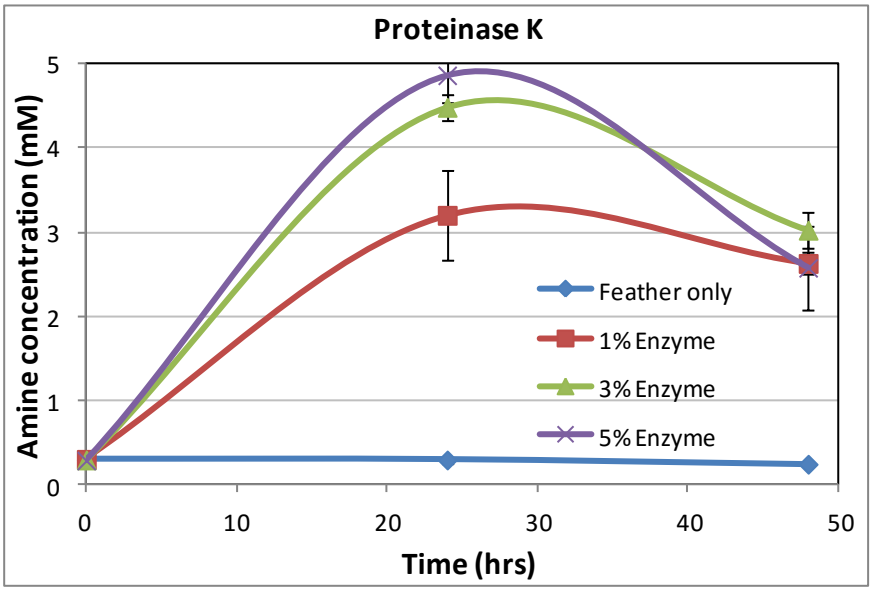


Figure 14 The effect of enzyme dosage on the rate of free amino nitrogen release during Savinase 16L and Proteinase K catalysed hydrolysis of feather at the respective recommended conditions of the enzymes (50 °C, pH 8.5 for Savinase 16L, pH 8.0 and 37 °C for Proteinase K).

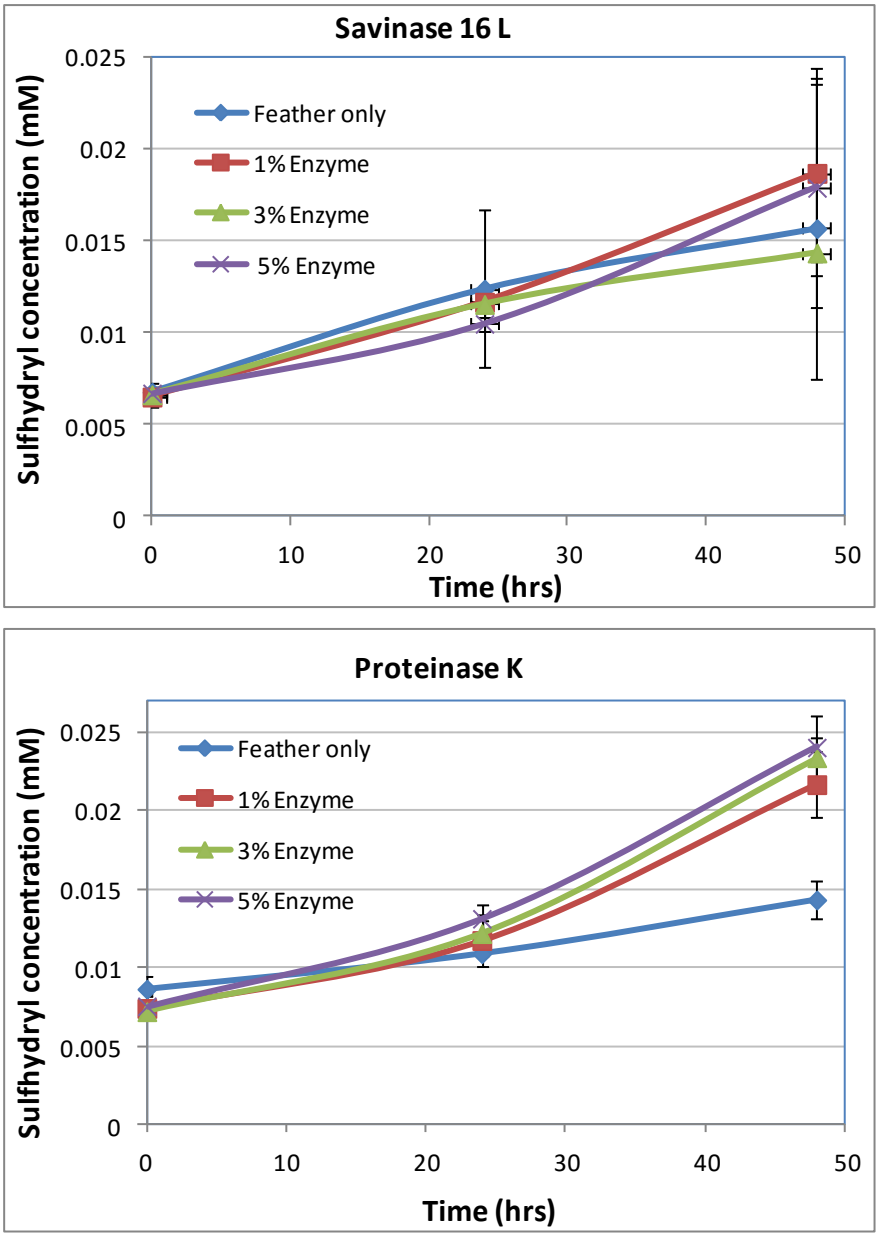


Figure 15 The effect of enzyme dosage on the rate of release of free sulfydryl groups during Savinase 16L and Proteinase K catalysed hydrolysis of feather at the recommended optimum conditions of the enzymes (50 °C, pH 8.5 for Savinase 16L, pH 8.0 and 37 °C for Proteinase K)

As can be seen, enzyme dosage did not have major effect on the rate of hydrolysis of feather, although significant ($p<0.05$) differences were observed between 1% and 3% dosage in the case of free amino nitrogen release during feather hydrolysis up to 24 hrs of reaction. A significant difference in percentage hydrolysis of feather (as measured by percentage nitrogen released into the hydrolysate) was also observed between 1% and 3% dosages for both enzymes (data not presented). The free amino nitrogen and percentage nitrogen released in the hydrolysate decreased with increased reaction time, probably due to denaturation and precipitation of the reaction products. A significantly ($p<0.05$) higher free amino nitrogen release was observed at the higher doses of proteinase K (3% and 5%) compared to Savinase 16L, although the overall

percentage hydrolysis after 24 hours as measured by percentage nitrogen release into the hydrolysate was less in the case of proteinase K (~12%) compared to Savinase 16L (~18%). Flourescamine, which is used in the FAN assay, yields higher fluorescence with peptides than their component amino acids [46]. Thus, if larger peptides are further hydrolysed to amino acids, the fluorescence measured may decrease resulting in apparent decrease of free amines, which may explain the decrease observed after 48 hours of hydrolysis (Figure 14). This may also explain the observed lower amount of free amino nitrogen released during Savinase 16L catalysed hydrolysis compared to proteinase K (at 3% and 5%), although the percentage conversion was higher in the case of Savinase 16L. The release of free sulfhydyl groups indicating the cleavage of disulfide bond increased with increased reaction time. Slightly higher release and better disulfide reductase activity was also observed in the case of proteinase K compared to Savinase 16L (Figure 15), which would normally be expected by virtue of its being a keratinase. Savinase 16L did not seem to have much effect on the release of free sulfhydryl groups since the measured value is very close to that of the feather treated without an enzyme with no particular effect of the enzyme dosage. Overall, since the 3% dosage resulted in a significantly higher hydrolysis of feather, it was selected for the comparative study in subsequent sections.

2.3.4 Comparison of the performance of the commercial and custom made enzymes at standardised dosage

In order to standardise the comparison of the commercial enzymes with the customised keratinases, the activities of the commercial enzymes were also determined using keratin azure as substrate at the best dosage selected above (3% on substrate protein basis) and their specific activities were defined in the same way as the custom made enzyme.

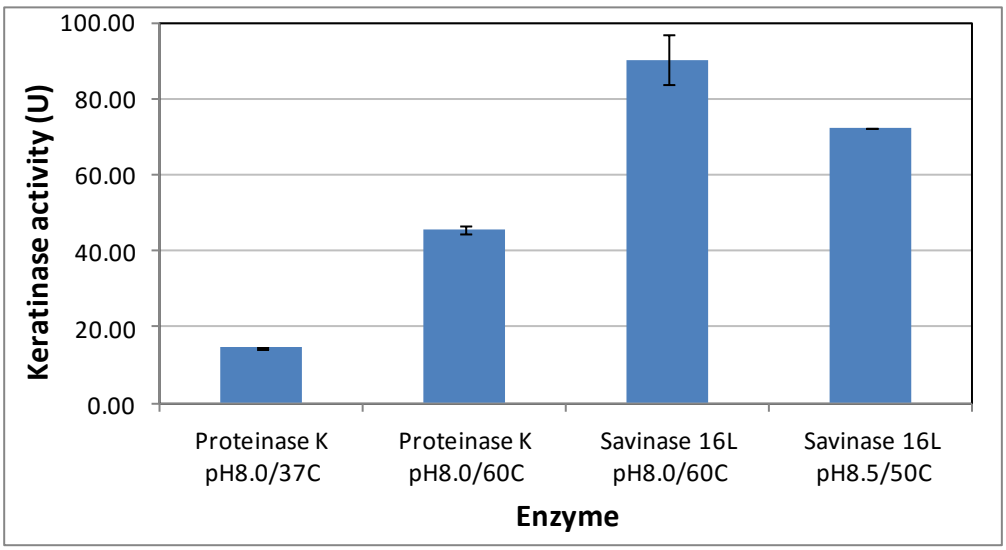


Figure 16 The specific keratinase activities of Proteinase K and Savinase 16L (U/mg protein) at different conditions using keratin azure as substrate

The specific activities of these enzymes at different conditions (at conditions recommended by the manufactures and at conditions used for the assay of custom made enzymes) are presented in Figure 16. As can be seen, Savinase 16L had much higher specific keratinase activity compared to proteinase K with keratin azure as substrate. In both cases, higher keratinase activity was observed at the temperature and pH optimum obtained earlier for the keratinase from *Bacillus licheniformis* strain investigated in this study. Interestingly, the crude enzyme from *Bacillus licheniformis* grown in medium B exhibited higher keratinase activity per ml of crude enzyme than the specific activity of proteinase K at the recommended optimum condition (37 °C, pH 8.0) and about one third of the specific activity of Savinase 16L at 60 °C/pH 8.0, showing the potential of this enzyme for the hydrolysis of keratinous materials (Figures 10 and 16).

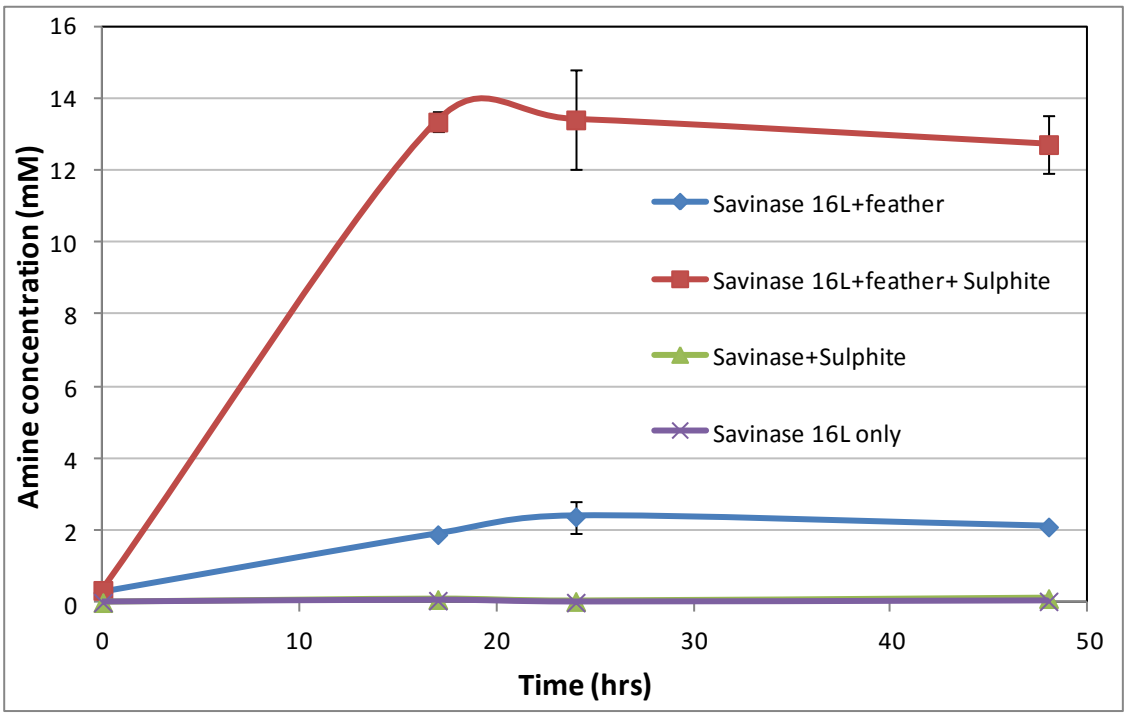


Figure 17 The rate of production of free amino nitrogen during the Savinase 16L hydrolysis of poultry feather at standardised condition (72.4 U, 60 °C, pH 8.0) with or without 1% sulphite (w/v)

In order to compare the efficacy of the keratinase from *Bacillus licheniformis* with proteinase K and Savinase 16L, hydrolysis experiments were conducted at a standardised enzyme concentration of 72.4 U (as defined earlier using keratin azure as substrate) of each enzyme with 1% ground feather as substrate. 72.4 U was selected as it is equivalent to 3% Savinase 16 L (based on substrate protein), determined in section 2.3.2 as the best dosage. As can be seen in Figures 17, 18 and 19, similar degree of hydrolysis was obtained using the three enzymes when the hydrolysis was conducted without a reducing agent. The keratinase from *Bacillus licheniformis* showed equivalent efficacy as the other enzymes, although the crude enzyme with no downstream

processing was used in the experiments. Neither the keratinases nor the conventional protease Savinase 16L were able to efficiently hydrolyse feather.

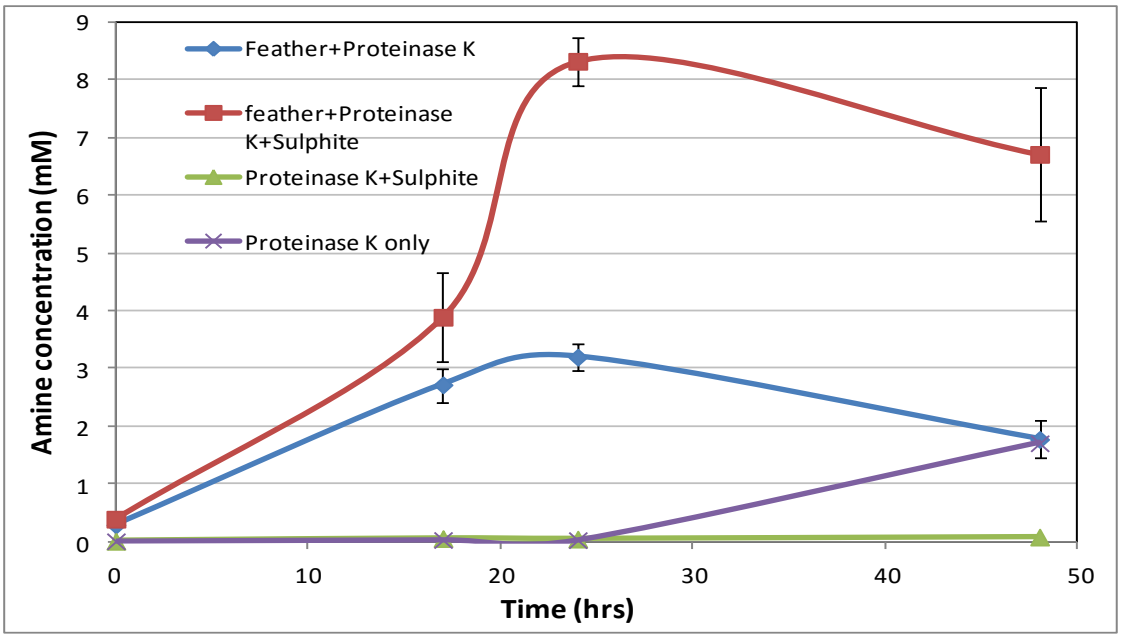


Figure 18 The rate of production of free amino nitrogen during the Proteinase K catalysed hydrolysis of poultry feather at standardised condition (72.4U, 60 °C, pH 8.0) with or without 1% sulphite (w/v)

As mentioned earlier, extracted keratinases are not efficient in degrading keratins since they lack the microbial machinery for sulfitolysis which involves cell colonisation, redox system and intracellular reductases to cleave the disulfide bridges and improve the accessibility of polypeptide chains for hydrolysis [24,26,42,50].

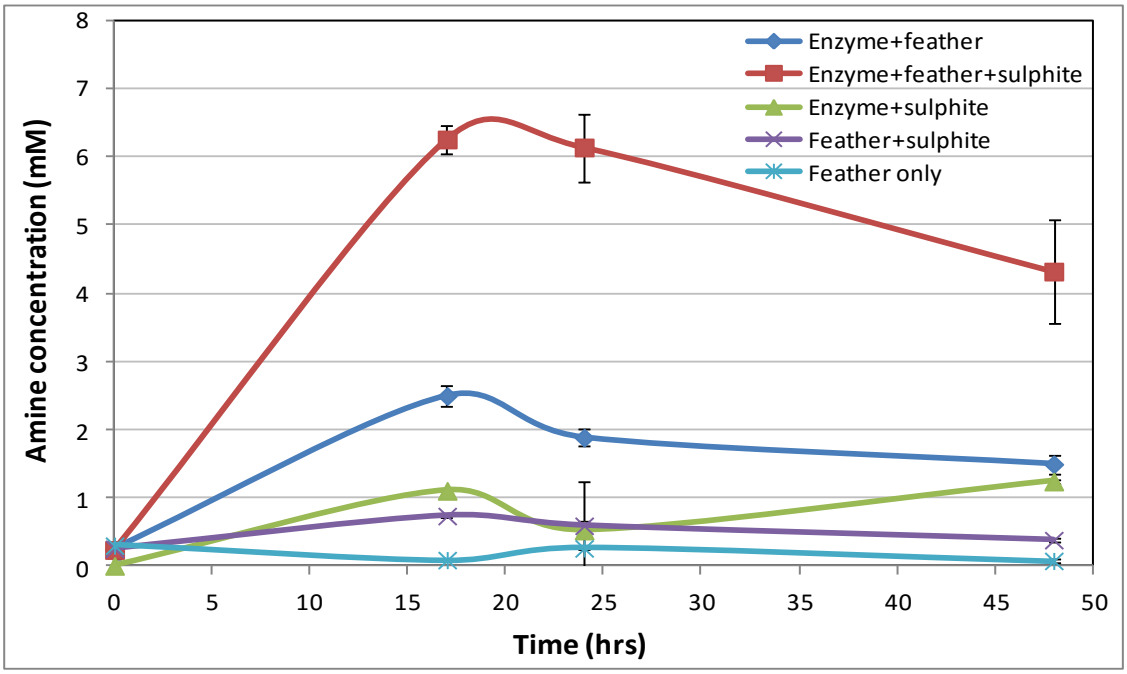


Figure 19 The rate of production of free amino nitrogen during the *Bacillus licheniformis* keratinase catalysed hydrolysis of poultry feather at standardised condition (72.4U, 60 °C, pH 8.0) with or without 1% sulphite (w/v)

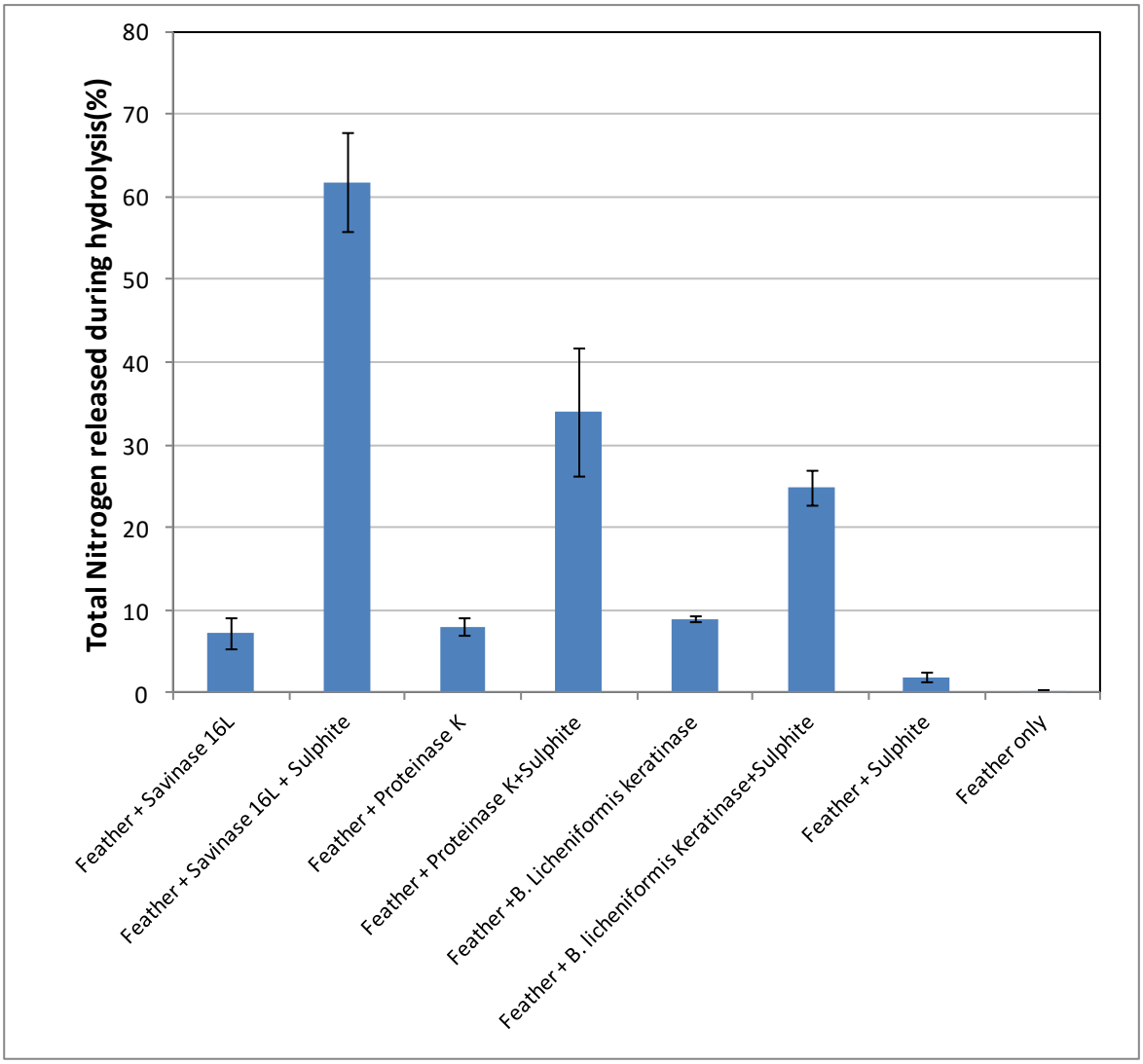


Figure 20 The percentage release of nitrogen after 24 hrs of poultry feather hydrolysis using different enzymes (72.4 U, 60 °C, pH 6.0) with or without 1% sulphite

When 1% sulphite was used, the degree of hydrolysis increased significantly ($p<0.05$), with about 7 times increase in the case of Savinase 16L, 3 times increase in the case of Proteinase K and about 2 times increase in the case of *Bacillus licheniformis* keratinase. Once the disulfide bonds in feather are reduced by a suitable reducing agent, the reaction is essentially proteolysis and Savinase 16L appears to be a more efficient protease compared to both proteinase K and the keratinase from Bacillus licheniformis. The cleaving of the disulfide bonds in feather by the sulphite leads to denaturation of the keratin which allows the enzyme to access the active sites. This is also reflected in the data on percentage nitrogen release into the supernatant after 24 hours (Figure 20). Similar percentage release of nitrogen and hence similar degree of feather hydrolysis was obtained for the three enzymes without sulphites. However, the percentage nitrogen release for Savinase 16L with 1% sulphite is substantially higher compared to the others reflecting its higher

proteolytic activity. One has to note that the *Bacillus licheniformis* enzyme from this study is a crude preparation with potential contaminants that may interfere with the proteolysis reaction. Moreover, the optimum dosage of the enzyme was not determined and used in these experiments indicating a potential for improvement. Similarly, Ramnani and Gupta [26] reported efficient hydrolysis of feather by conventional serine and cysteine proteases including subtisilin, chymotrypsin and papain the presence of chemical reducing agents and a protease deficient mutant B. subtilise as a source of redox. On the other hand, trypsin and pepsin were able to hydrolyse only autoclaved feather after two hours pre-treatment by chymotrypsin and subtisilin in the presence of chemical reducing agents [26].

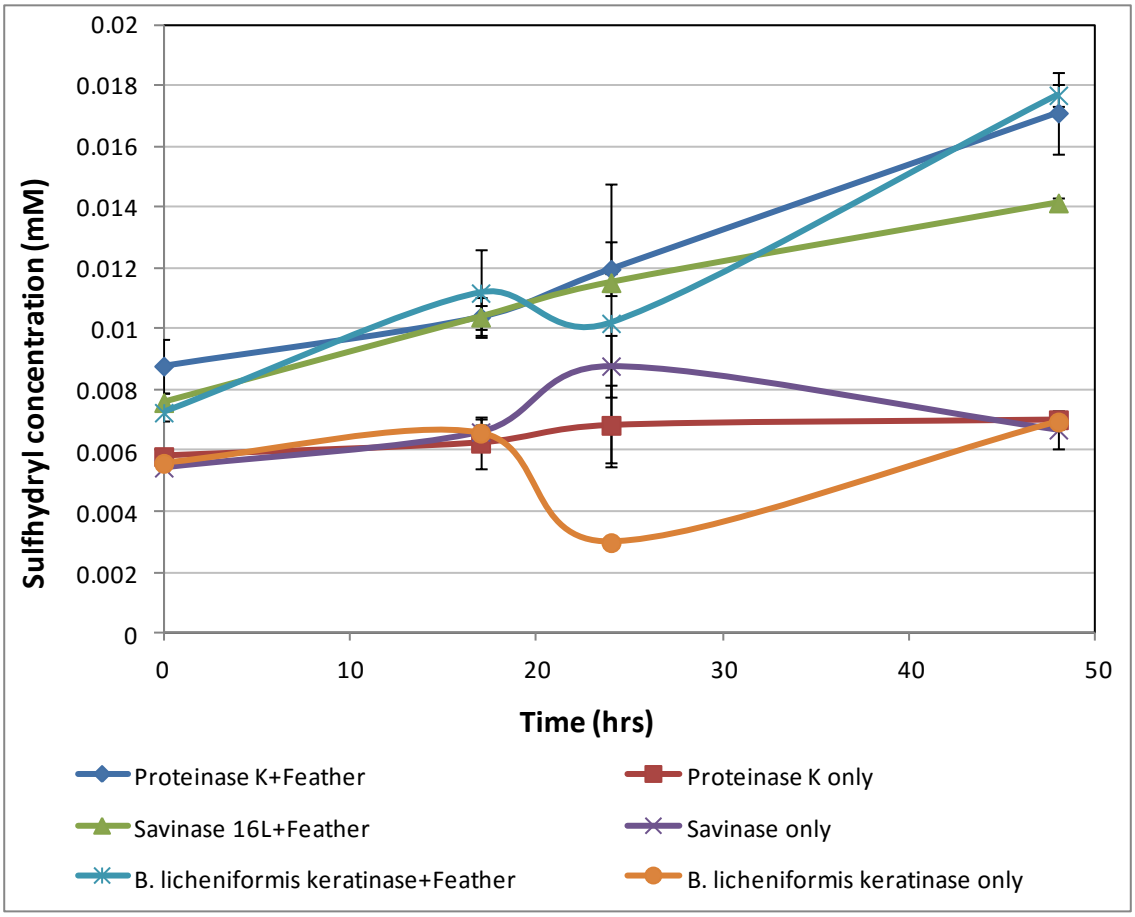


Figure 21 The concentration of free sulfhydryl groups in the hydrolysate after 48 hours of poultry feather hydrolysis using Savinase 16L, Proteinase K and *Bacillus licheniformis* keratinase at standardised condition (72.4 U, 60 °C, pH 8.0).

The disulfide reductase activities of the different enzymes were compared by measuring the production of free sulfhydryl groups upto 48 hours of feather hydrolysis using the different enzymes (Figure 21). Proteinase K and the keratinase from *Bacillus licheniformis* showed significantly higher disulfide reductase activity compared to Savinase 16L during longer hydrolysis, indicating their potential use for pre-treatment of keratinacous materials such as feather prior to proteolysis using a more potent proteolytic enzyme preparation like Savinase 16L.

2.4 Conclusion

The efficacy of the kertinase from *Bacillus licheniformis* was compared with two commercial proteases i.e. Proteinase K and Savinase 16L, which were selected among four that included alcalase and Multifect PR6L based on their feather hydrolysis efficacy. The experiments comparing the three enzymes were performed under standardised conditions selected based on the best dosage obtained for the best commercial enzyme i.e. Savinase 16L (3% on substrate protein content basis equivalent to 72.4 keratolytic unit) and the conditions at which the highest keratolytic activities of all the enzymes were observed (pH 8.0 and 60 °C). At the standardised condition, the estimated percentage hydrolysis of feather using these enzymes was about 9% in all cases. Interestingly, the keratinase from *Bacillus licheniformis* showed similar efficacy as the commercial enzymes, although the crude fermentation supernatant was used in the experiments with potential interference from culture components and dosage not optimised. The keratinase from *Bacillus licheniformis* also exhibited equivalent disulfide reductase activity to that of Proteinase K, which was significantly higher than Savinase 16L, indicating its potential for application in the pre-treatment of feather and other keratinacous materials prior to proteolysis. In the presence of a reducing agent (1% sulphite), the percentage hydrolysis of feather by the three enzymes increased dramatically. This was more pronounced in the case of Savinase 16L where the percentage hydrolysis was increased from ~9% to ~61%. This seems to be due to the higher proteolytic activity of Savinase 16L compared to the other enzymes. It can be concluded that the custom made keratinase from *Bacillus licheniformis* has a significant potential for the pre-treatment of feather both for the production of feather meal and other applications. However, the fermentation process needs to be optimised. In addition, suitable downstream processing steps need to be established. The optimal dosage of the enzyme for feather hydrolysis also needs to be determined.

Chapter 3 Evaluation of ultrasound for enhancing the enzymatic hydrolysis of feather

3.1 Introduction

Feather keratin is highly resistant to enzymatic hydrolysis due to the tight packing of the protein in the form of super coiled polypeptide chains cross-linked with disulfide bonds. Our investigations in Chapter 2 showed that only limited enzymatic hydrolysis of feather occurs in the absence of sulphites, whereas reducing agents such as sulphites significantly enhance feather hysrolysis in the presence of potent proteases. Ultrasound may cause physical and chemical modification of incalcitrant substrates such as feather, rendering them more accessible thereby enhancing their enzymatic hydrolysis. The application of ultrasound at appropriate condition can enhance the kinetics of enzymatic hydrolysis of complex substrates, potentially reducing enzyme dosage or reaction time [37,38,39]. The effect of ultrasound on enzymatic reactions is attributed to the strong shear and microstreaming that accompanies cavitation, which enhances mass transfer and improve enzyme and/or substrate availability and enzyme-substrate interaction by breaking down molecular aggregates and modifying macromolecular substrates [40]. The objective of this chapter was to evaluate the potential of ultrasonics treatment for enhancing enzymatic hydrolysis and determine the feasible ultrasonic pre-treatment condition (s) for feather hydrolysis.

3.2 Materials and Methods

3.2.1. Materials

Feather samples were obtained from a local rendering company and processed as described in Chapter 1. Savinase 16L were obtained from Novozyme. The custom made keratolytic enzyme from *B. licheniformis* was produced using a 10L Bioreactor as described in Chapter 2. All the other chemical and biochemical reagents were analytical grade. Ultrasonic experiments at different frequencies were conducted using multifrequency (40-270 kHz) ultrasonic unit from Blackstone Ney ultrasonics (Cleaning Technologies Group, USA) and 400 kHz, 600 kHz and 2000 kHz ultrasonic units from Sonosys Ultraschallsysteme (Germany).

3.2.2. Ultrasonic Screening experiments

In order to evaluate the feasibility of using ultrasonics for enhancing the enzymatic hydrolysis of feather, ultrasonic pre-treatment experiments were conducted at different ultrasonic frequencies and energy input conditions as detailed in Table 2. Ultrasonic pre-treatment of 10 g/L ground feather suspension was conducted for 20 min prior to enzymatic hydrolysis using the *B. licheniformis* enzyme at 60 °C and pH 8.0 for 48 hours at the enzyme dosage determined in chapter 2 (72.4 keratolytic unit per 0.5 gm feather).

Table 2 Experimental conditions of the ultrasonic screening experiments

Ultrasonic frequency	Power input (W)	Specific energy input (kJ/L)	$T_{initial}$ (°C)	T_{final} (°C)
220 kHz	467	109.9	21.5	36.1
400 kHz	289.3	315.6	23.6	68.3
600 kHz	288.9	235.1	26.6	60.6
2000 kHz	270.8	261.0	20.2	56.8

$T_{initial}$: initial temperature of the sample, T_{final}: final temperature of the sample

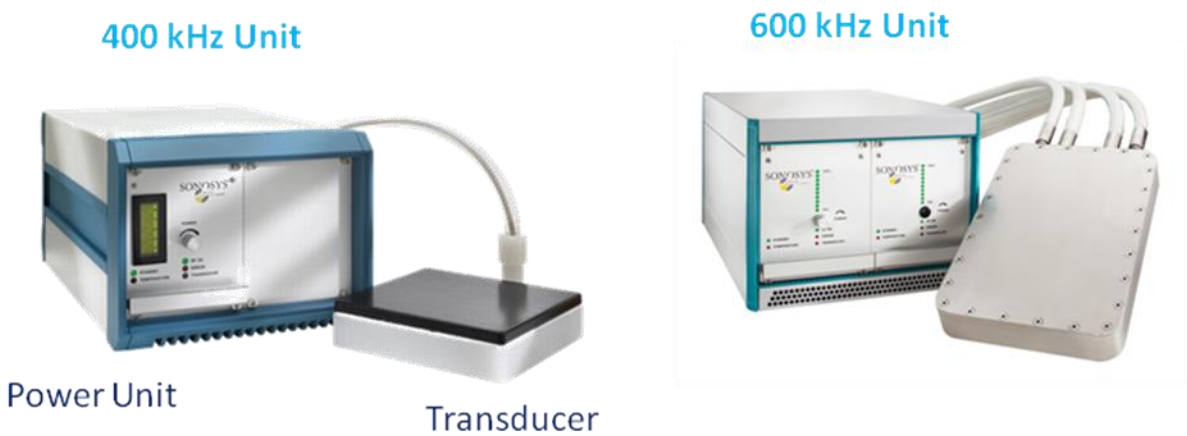


Figure 22 Examples of the ultrasonic equipments used in the experiments

Samples were periodically taken and were heated for 10 min at 95°C for enzyme inactivation prior to analysis. The rate and extent of feather hydrolysis was determined by measuring free amino nitrogen release using FAN assay and the disulfide cleavage by measuring the release of free sulfhydryl groups using 5, 5' –dithiobis (2-nitrobenzoic acid) (DTNB) based assays as described in previous chapters. Control samples consisting of enzymes or feather only were treated and analyzed in the same way as references.

The ultrasonic pre-treatments were conducted by immersing the ultrasonic transducer directly into the sample except for experiments at 220 kHz where the sample was pre-treated in a glass beaker placed on the transducer due to the large size of the ultrasonic transducer as shown in the schematic diagram below (Figure 23). In all cases, significant increase in temperature was observed during ultrasonic treatment depending on the specific energy input as shown in Table 2 and pre-heating or cooling to the experimental temperature was necessary prior to enzymatic hydrolysis experiments. All enzymatic hydrolysis experiments were conducted in a thermostated shaking water bath maintained at the experimental temperature.

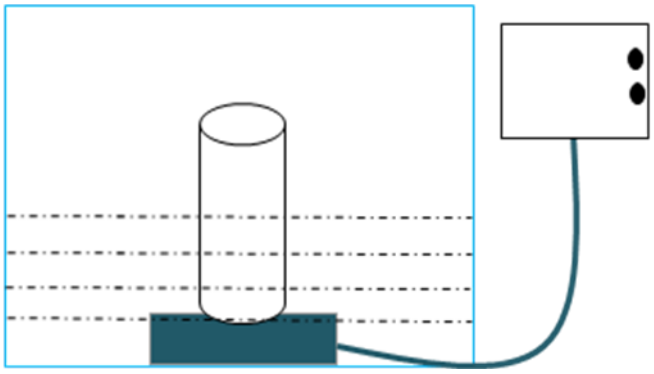


Figure 23 The schematic representation of the ultrasonic pre-treatment experiment at 220 kHz

Among the investigated ultrasonic frequencies, the best result was obtained at 400 KHz. Thus, further enzymatic hydrolysis experiments were conducted with Savinase 16L after 20 min feather pre-treatment and combined feather-enzyme treatment at 400 kHz. Again, enzymatic hydrolysis was conducted at 60 °C and pH 8.0 using the enzyme dosage defined in chapter 2. Samples were also alquoted and analysed using the same protocols. Data analysis was performed as described in 2.2.6.

3.2.3. Experiments to determine best treatment condition(s)

The operating and capital cost of an ultrasonic process is dependent on the specific energy input which is determined by the power input and the treatment time. Shorter treatment time or lower power input results in lower specific energy input and a more economical process. Thus in order to determine the best ultrasonic pre-treatment conditions, further pre-treatment experiments were conducted at 400 kHz for 5, 10 and 15 min prior to enzymatic hydrolysis using the *B. licheniformis* enzyme at the conditions described above. As an alternative to the pre-treatment process, further investigation was designed to investigate the effect of applying ultrasonics in pulsed mode to progressively open up the feather structure for hydrolysis with a 6 min pre-treatment followed by seven 2 min pulse treatments every hour during 8 hrs of hydrolysis totalling 20 min. The ultrasonic conditions for these experiments are detailed in Table 3. The kinetics of hydrolysis was investigated in detail over 8 hrs in these experiments so as to evaluate the feasibility of a shorter process more amenable to industrial application.

Table 3 Experimental conditions at 400 kHz investigated to determine the best pre-treatment condition

Treatment time (min)	Power input (W)	Specific energy input (kJ/L)	$T_{initial}$ (°C)	T_{final} (°C)
5	285.3	77.8	15	27.6
10	283	154.3	16	41.7
20	287.4	235.1	13	44.7
pulsed	293.9	293.9	16.2	N/A

In order to determine whether substrate availability or enzyme activity is the limiting factor during enzymatic hydrolysis, the stability of the enzymes were also determined during 24 hrs at the experimental hydrolysis condition.

3.3. Results and Discussion

3.3.1. Ultrasonic Screening experiments

The effect of 20 min ultrasonic pre-treatment at different frequencies on the release of free amine groups during the *B. licheniformis* keratinase catalysed hydrolysis of feather are presented in Figure 24. As can be seen, significantly ($p<0.05$) higher peptide release was observed in samples treated at 400 kHz and 600 kHz whereas significantly ($p<0.05$) lower peptide release was observed in samples treated at 220 and 2000 kHz. Taking into account the background free amine concentration (amine concentration in 0 hr samples), about 60 and 35% higher peptide release was obtained in the 400 kHz and 600 kHz treated samples respectively after 24 hrs compared to control samples that are not treated by ultrasonics, which may be due to ultrasound induced improved substrate accessibility in these samples. On the other hand, samples treated at 2000 kHz showed the lowest peptide release of about 37% less compared to control after 24 hrs of hydrolysis. As observed in previous experiments (Chapter 2), there was a decrease in the measured free amine concentration in most samples after 48 hours, which may be due to precipitation of the peptides during incubation at 60 °C and/or the lower fluorescence yield for amino acids compared to peptides which may have formed during longer hydrolysis [46]. This seems to be more pronounced in the control, 220 kHz and 2000 kHz samples. In the case of the 400 kHz and 600 kHz, the higher release of peptides may have compensated for a decrease in concentration due to precipitation or further hydrolysis into amino acids.

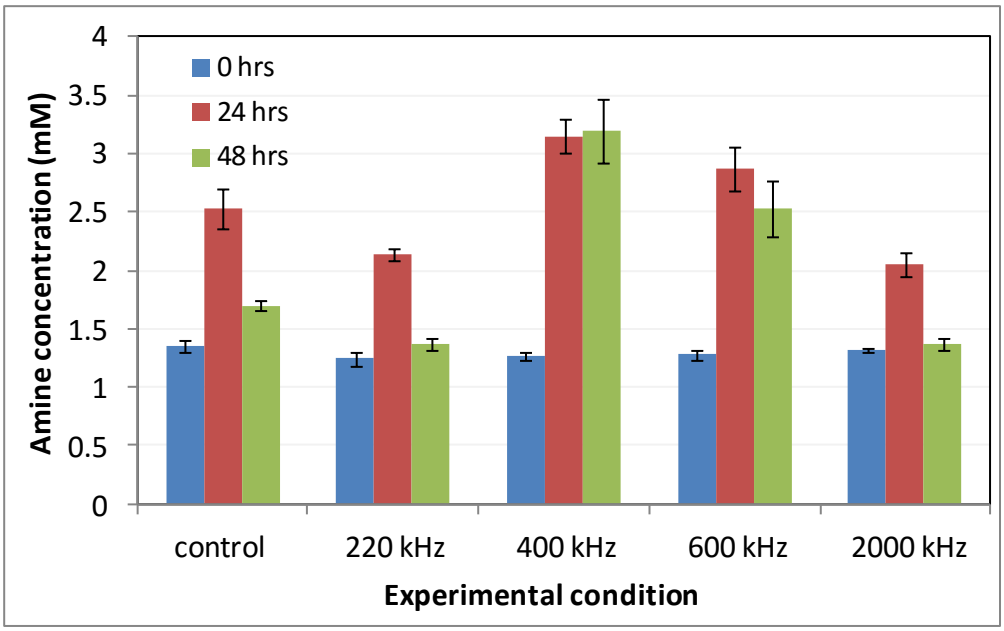


Figure 24 The effect of 20 min ultrasonic pre-treatment on free amine release during the *B. licheniformis* keratinase catalysed hydrolysis of poultry feather at 60 °C and pH 8.0.

Unlike peptide release, ultrasonic pre-treatment at all the studied conditions enhanced the cleavage of disulfide bonds as measured by the release of free sulfhydryl groups (Figure 25). From 2.3 to 2.9 times higher cleavage of disulfide bonds were observed in the ultrasonic pre-treated samples compared to control after 24 hrs of hydrolysis. The difference was even more substantial after 48 hrs with 5.8 to 6.3 times release of free sulfhydryl groups in ultrasonicated samples compared to untreated samples. This did not translate into a parallel increase in the rate of hydrolysis perhaps due to reformation of the disulfide bond as observed for gluten hydrolysis [51]. Although, feather treatments at 220 and 2000 kHz appear to have no or negative effect on hydrolysis compared to control, these treatments could potentially be used for applications that require chemical modification of feather as in polymer composites and textile fibres [52]. In addition, some fine tuning of experimental parameters (time, power input etc) may lead to enhanced hydrolysis even after treatment at these frequencies. However, this route was not investigated due to time and resource constraints. Rather, further investigations focused on 400 kHz treatment since it showed the most promising result among the treatments investigated.

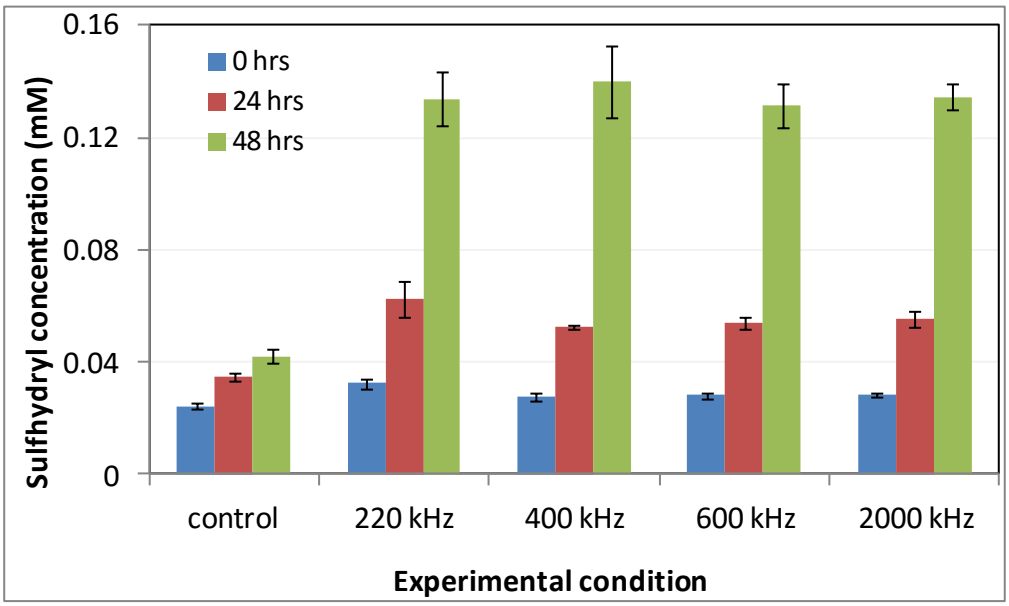


Figure 25 The effect of 20 min ultrasonic pre-treatment on the cleavage of disulfide bonds during the *B. licheniformis* keratinase catalysed hydrolysis of poultry feather at 60 °C and pH 8.0.

As in the case of the custom made enzyme, 20 min ultrasonic pre-treatment at 400 kHz significantly ($p<0.05$) improved the Savinase 16L catalysed hydrolysis of feather (Figure 26) with 63% higher release of peptides compared to control after 48 hrs of hydrolysis. Slightly higher release (18%) was also observed after 24 hrs, although that was not statistically significant. The effect of ultrasonic treatment of the samples after enzyme addition (US feather+Savinase) was also evaluated. In this case, no significant effect was observed after 24 hours probably due to the ultrasonic induced partial inactivation of the enzyme counteracting the ultrasound induced improvement in substrate accessibility. No decrease in the free amine released was observed after

48 hrs in the case of the ultrasonic assisted Savinase 16L catalysed hydrolysis with significantly ($p<0.05$) higher concentration of free amines observed after 48 hrs of hydrolysis compared to 24 hrs. This could be due to i) the higher proteolytic activity of Savinase 16L counteracting the effect of peptide precipitation; ii) the peptides formed may not be susceptible to precipitation iii) Significant further hydrolysis of peptides into the constituent amino acids did not occur or was overcompensated by higher production of peptides, which need further investigation.

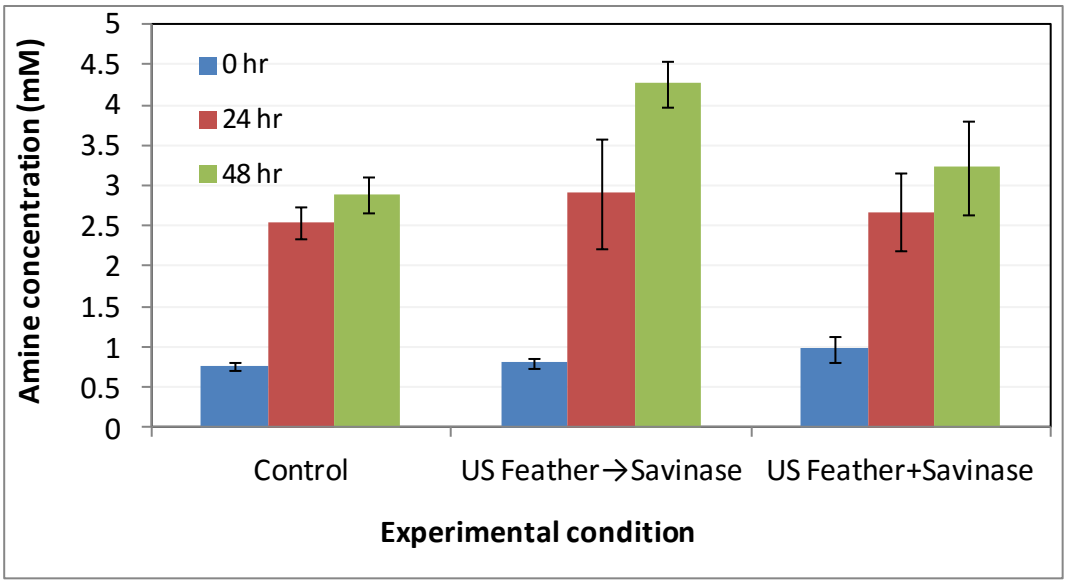


Figure 26 The effect of ultrasonic (400 kHz) treatment on free amine release during the Savinase 16L catalysed hydrolysis of feather at 60 °C and pH 8.0.

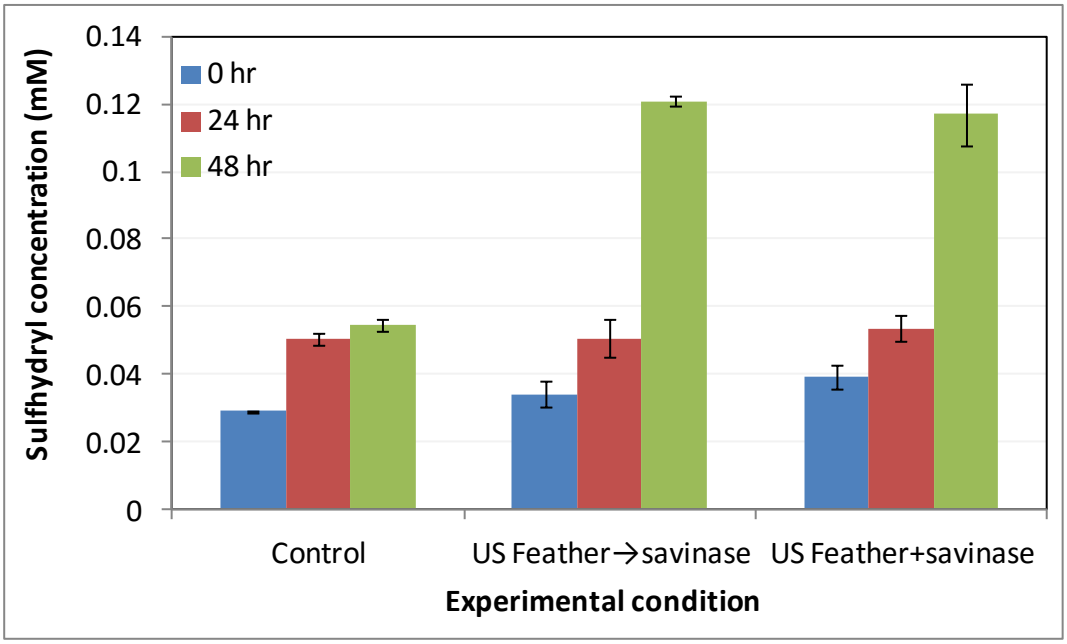


Figure 27 The effect of ultrasonic (400 kHz) treatment on the cleavage of disulfide bonds during savinase catalysed hydrolysis of feather at 60 °C and pH 8.0.

The cleavage of disulfide bonds as measured by the release of free sulfhydryl groups was enhanced by ultrasonication in both cases where ultrasound was used to pre-treat feather and where sonication was applied after enzyme addition (Figure 27). It seems that the cleavage of sulfhydryl groups under sonication is not solely dependent on the presence of an active enzyme, since similar effects were observed where ultrasound was used as a pre-treatment or during the hydrolysis process.

Comparison of the efficiency of the custom made enzyme with Savinase 16 L for hydrolysing the sonicated feather confirmed the earlier observation that Savinase is a better protease showing higher production of free amines (Figure 28). However, one has to note that the custom made enzyme was crude and the dosage was defined based on the optimum for Savinase 16 L as described in Chapter 2. With respect to the ability to cleave disulfide bonds, the custom made enzyme showed better efficiency in sonicated feather (Figure 29) again confirming the earlier observation that it is potentially a better keratinase.

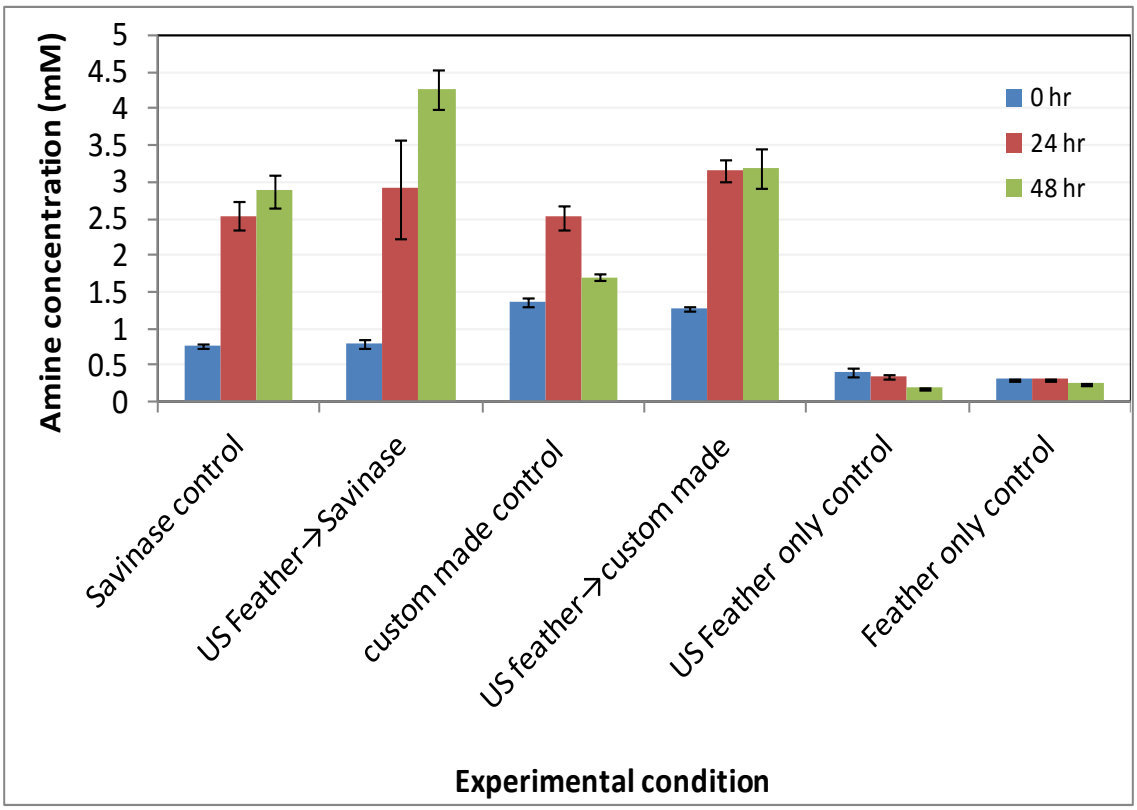


Figure 28 Comparison of the concentration of free amine during the hydrolysis of ultrasonicated feather by the *B. licheniformis* keratinase and Savinase 16 L at 60 °C and pH 8.0

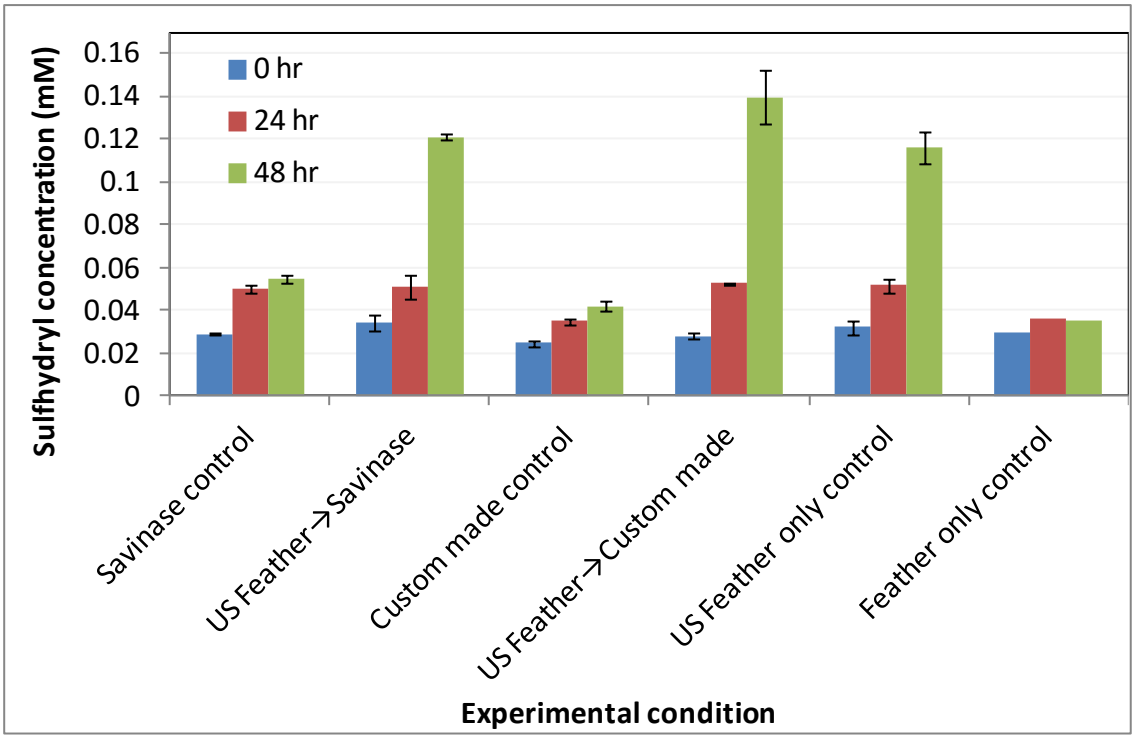


Figure 29 Comparison of cleavage of disulfide bonds during the hydrolysis of sonicated feather by the *B. licheniformis* keratinase and Savinase 16L at 60 °C and pH 8.0

Interestingly significant release of free sulfhydryl groups was also observed with just sonication of feather samples without enzymes (Figure 29), which was equivalent to Savinase 16L, although no significant peptide release was observed in the absence of enzymes (Figure 28).

3.3.2. Experiments to determine best ultrasonic treatment condition(s)

As discussed above, the best frequency for ultrasonic treatment of feather was 400 kHz. In order to further determine the best experimental condition for the treatments, ultrasonic pre-treatment experiments were conducted for different treatment times. The results of the ultrasonic pre-treatment at 400 KHz for different times showed that pre-treatment for as short as 5 min results in the release of peptides close to the 20 min pre-treatment after 24 hours (Figure 30). The results also showed that significant conversion into amines occur even after 3 hours of hydrolysis. The 15 min pre-treatment resulted in a lower conversion after 24 hours compared to the others, although similar degree of cleavage of disulfide bonds were observed as the others (Figure 31). This could be related to sampling time since precipitation of peptides or further conversion amino acids seems to occur over time in all samples potentially reducing the observed amine concentration if sampling is done after precipitation is initiated. As can be seen in Figure 30, sampling after 20 hours of incubation in the 5 and 10 min pre-treated samples showed higher amine content compared to the 24 hours samples indicating such precipitation or conversion. Thus, sampling was done every 2 hours during subsequent experiments where practically feasible.

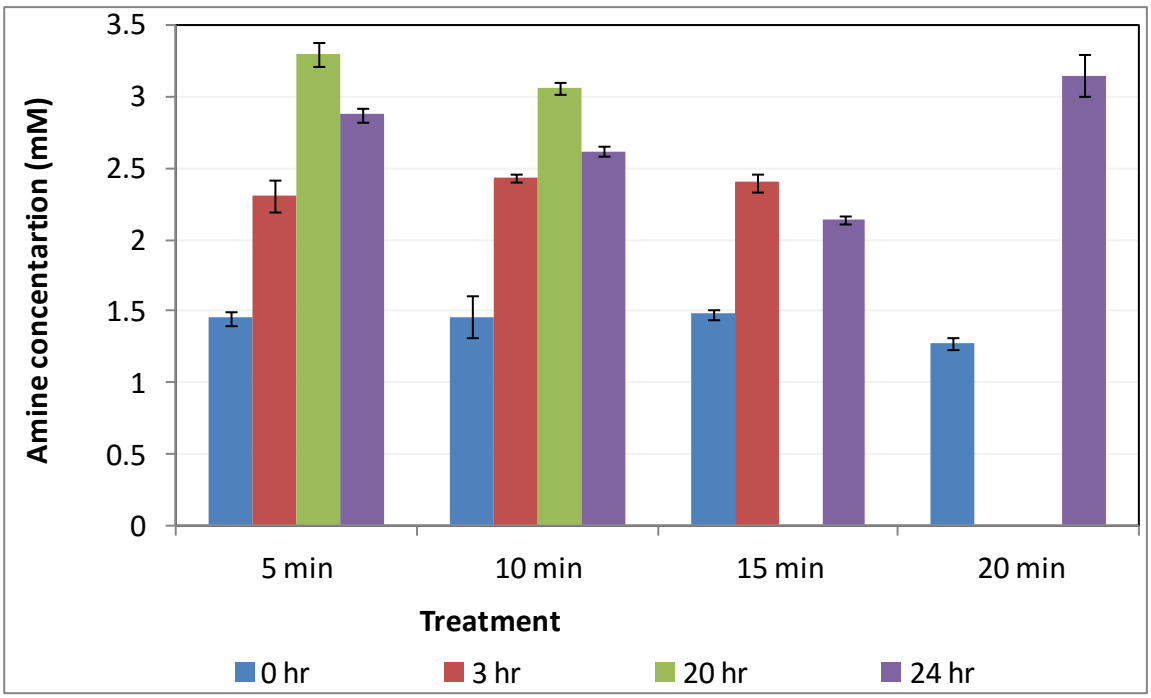


Figure 30 The effect of ultrasonic (400 kHz) pre-treatment time on the production of free amine during the *B. licheniformis* keratinase catalysed hydrolysis of feather at 60 °C and pH 8.0.

Five minutes pre-treatment was selected for further investigation into the effect of ultrasonication on the kinetics of enzymatic hydrolysis of feather since it resulted in comparable hydrolysis to the 20 min pre-treatment and is more economical with 4 times less specific energy input. This was compared with pulsed ultrasonic treatment of 20 min applied over a period of 7 hours, with the objective of progressively opening up the feather structure for hydrolysis. The data are presented in Figure 32. As can be seen, 5 min pre-treatment resulted in a significant improvement in product yield and rate compared to both control and the pulsed ultrasonic treatment. Although the product yield was the same for control and pulsed ultrasonic treatment, the maximum product yield was achieved 2 hours earlier in the pulse treated samples compared to the control samples, which is a 33% reduction in reaction time, indicating that such treatment could be used for shortening the reaction time and making the process more economical. Jian et al. [37] observed about 27% in protein conversion yield of leather waste hydrolysis, which is mainly composed of collagen, by applying 4370 kJ/kg of ultrasonics over 29 hours of hydrolysis. On the other hand Khanal et al. [52] reported a two fold increase in the rate of hydrolysis of corn slurry through the application of 160 kJ/L of ultrasound at 20 kHz. Similarly, we observed a two fold increase in the rate of hydrolysis of cellulose and a 30% increase in yield through the application of 48 kJ/L of ultrasound at 400 kHz [39], indicating that the effects of ultrasound on enzymatic hydrolysis depends on the substrate.

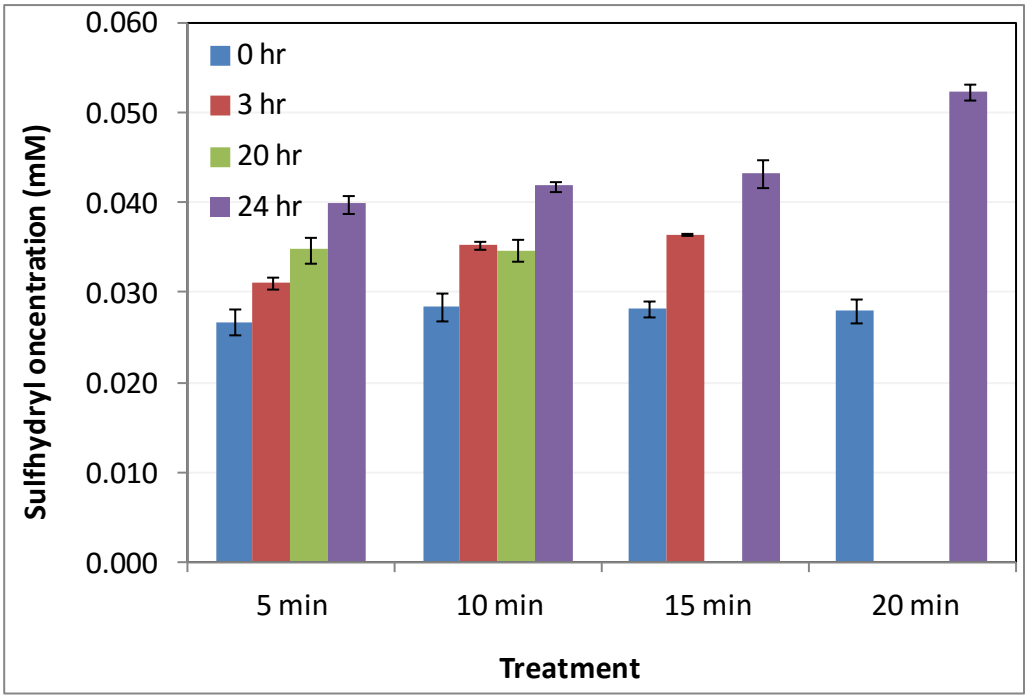


Figure 31 The effect of ultrasonic (400 kHz) pre-treatment time on the cleavage of disulfide bonds during the *B. licheniformis* keratinase catalysed hydrolysis of feather at 60 °C and pH 8.0.

The amount of free amines start to decline after 4 to 6 hours reaction time perhaps due to precipitation of the product, further conversion to amino acids or no new product formation due to enzyme inactivation. As can be seen in Figure 32, the decline in product concentration coincided with the loss of about 75% of the enzyme activity indicating that the progress of the reaction is determined by the availability of active enzyme to a large extent. The decline in amine concentration started earlier in the pulsed samples perhaps due to the enhanced inactivation of the enzyme and the precipitation of the amines or further hydrolysis enhanced by ultrasound. Thus a suitable approach to stabilise the enzyme together with optimisation of the ultrasonic pulse application (lower power input, shorter pulse etc) can potentially improve the product yield from this process. With respect to cleavage of disulfide bonds, substantial difference between ultrasonicated and control samples occurred only after 24 hours (Figure 33) of reaction although, the pulsed treatment resulted in a slightly higher cleavage even during the first 8 hours of the reaction. Nevertheless, it has to be noted that the assay does not directly measure the cleavage of disulfide bonds in feather, rather the concentration of free sulfhydryl groups released into the hydrolysate. Even if significant cleavage of disulfide bonds occurs in the feather immediately after ultrasonication and during the early stages of keratolysis, the release of free sulfhydryl groups into the liquid phase may take longer due to mass transfer limitations, which may explain the substantial increase in free sulfhydryl groups in the samples after 24 hours of incubation and even 48 hours as observed in previous experiments. This may also explain the slightly higher concentration of free sulfhydryl groups in the pulsed samples since ultrasonication facilitates mass transfer [40].

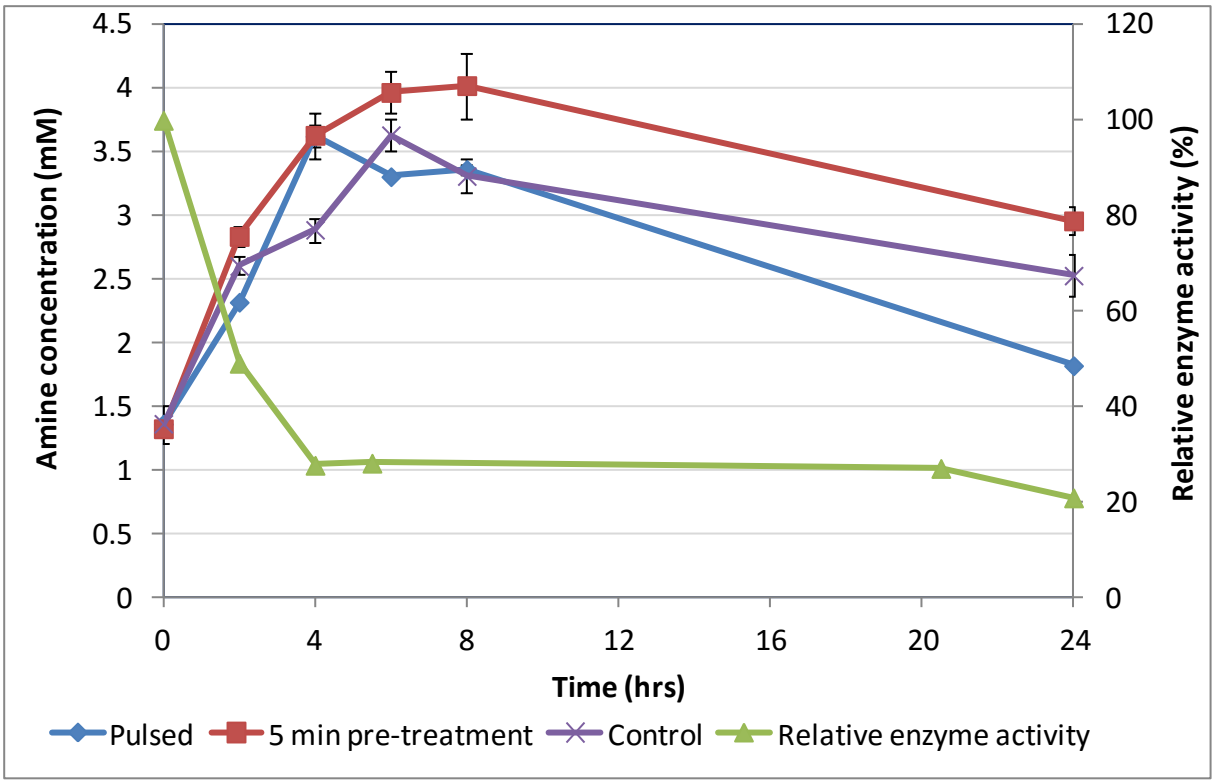


Figure 32 The effects of ultrasonication (400 kHz: 20 min pulsed and 5 min pre-treatment) on the kinetics of *B. licheniformis* keratinase catalysed hydrolysis of feather at 60 °C and pH 8.0.

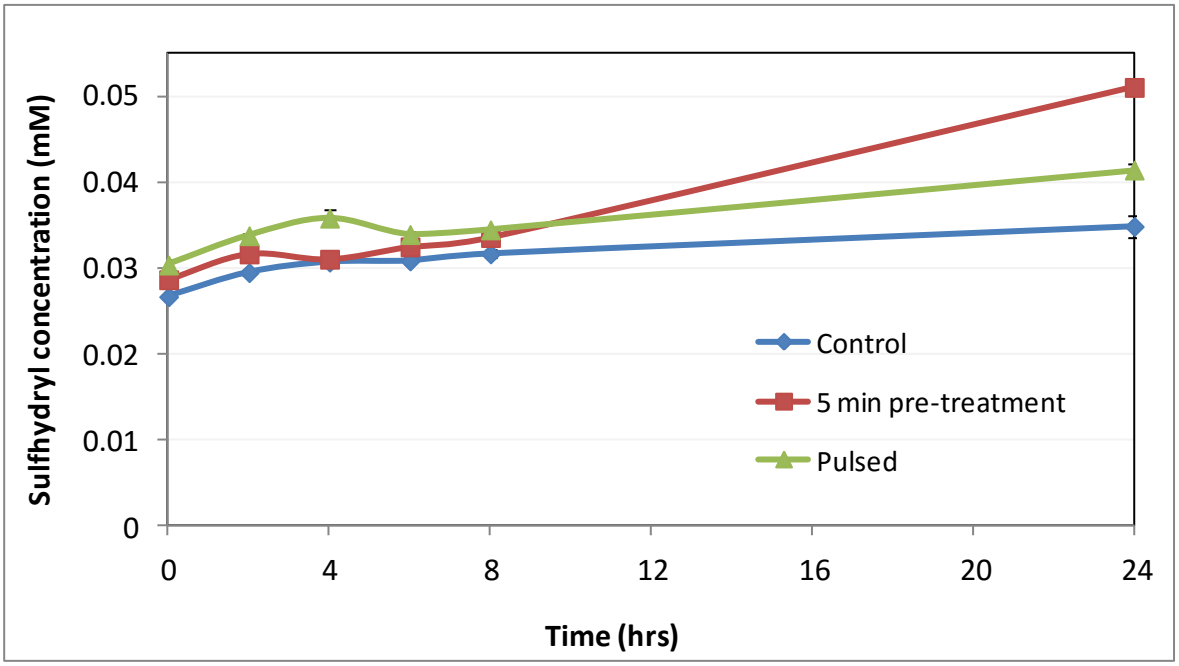


Figure 33 The effects of ultrasonication (400 kHz: 20 min pulsed and 5 min pre-treatment) on the kinetics of *B. licheniformis* keratinase catalysed cleavage of disulfide bonds of feather at 60 °C and pH 8.0.

Clearly several factors need to be considered in order to further optimise the ultrasonic assisted process. First, suitable downstream process need to be designed for the custom made enzyme and establish optimum dosage for hydrolysis since such exercise for the crude enzyme may be futile due to the presence of salt and other components from the fermentation broth, which may inhibit the enzyme at higher concentrations. Optimisation at the fermentation and production stage of the enzyme is also essential prior to the design of the downstream process. In addition, suitable temperature for the enzymatic reaction together with approaches for stabilising the enzyme need to be investigated. In this work, the optimum temperature for the activity of the enzyme was used in all the experiments and that may not necessarily be the optimum condition for conducting the reaction at large scale over extended time. The ultrasonic condition could also be further optimised and so as to render the process economically more viable. Both single pre-treatment and pulse applications may be considered for such optimisation.

3.4. Conclusion

In this study, the potential of ultrasonication for enhancing the enzymatic hydrolysis of feather was investigated. Exploratory experiments were conducted at ultrasonic frequencies ranging from 220 to 2000 kHz. Ultrasonic pre-treatment of feather at 400 and 600 kHz resulted in a significant improvement of the enzymatic hydrolysis of feather. The best result in terms of hydrolysis was obtained at 400 kHz with up to 60% increase in product yield after pre-treatment of feather for 20 min prior to the enzymatic reaction with both the custom made keratinase from *B. licheniformis* and Savinase 16L. Moreover, ultrasonic treatments of feather at all the studied conditions led to enhanced cleavage of disulfide bonds even in the absence of enzyme indicating the potential of the process for chemically modifying feather for various applications including in polymer composites, as textile fibre and in animal and pet food applications.

Further experiments to determine the best ultrasonic processing conditions focused on 400 kHz ultrasonic treatment with the custom made enzyme. Ultrasonic pre-treatments as short as 5 min of feather at 400 kHz resulted in a significant improvement in the kinetics and yield of the hydrolysis reaction with the custom made enzyme. Similarly, pulsed application of ultrasound (equivalent to 20 min treatment applied over 7 hours) resulted in about 33% less hydrolysis time compared to control to achieve the same product yield, showing the potential of such a process for shortening the hydrolysis time and thereby rendering the hydrolysis process more economical. In all cases, the kinetics of the reaction declined after 4 to 6 hours, which coincided with the loss of about 75% of the activity of the enzyme. Thus, the main constraint in terms of further optimisation of the ultrasonic assisted process was found to be the stability of the enzyme under the studied condition. Thus further investigations are needed to develop suitable downstream process for at least partial

purification of the crude enzyme, the determination of enzyme dosage and selection of suitable operating temperature and other routes for improving the stability of the enzyme over extended period for industrial application. Investigations are also needed to further optimise the ultrasonic treatment conditions to further improve the rate and extent of hydrolysis and make the process more economical at industrial scale.

Chapter 4 Process development for the production of high purity keratin hydrolysate from poultry feather

4.1 Introduction

Our previous studies in chapters 2 and 3 showed that the enzymatic hydrolysis of feather keratin is enhanced by the application of sulphites and ultrasonic treatment. The objectives of this milestone were to

- Determine the best processing condition for the hydrolysis of feather using Savinase 16L with sulphite concentration, substrate concentration, and ultrasonic condition as factors
- Develop the best separation process for the production of high purity keratin hydrolysate

A Response Surface Experimental Design was adopted and implemented to determine the best processing condition for feather hydrolysis. Savinase 16L was used in these trials as agreed on the project review discussion since substantially higher conversion was obtained using Savinase 16L compared to the crude *B. licheniformis keratinase*, which needs further development before industrial application.

The enzymatic hydrolysis of feather results in a mixture containing the soluble hydrolysate (peptides), unhydrolysed feather, salt and sulphite depending on the processing condition. In order to generate a product (peptides) with high purity, the mixture needs to undergo a purification process to separate the impurities such as unhydrolysed feather, sulphites and salt. Thus, membrane filtration based separation and purification process that includes microfiltration and nanofiltration steps was developed in order to obtain a keratin hydrolysate product with high degree of purity. Microfiltration is a physical separation process by which microorganisms and suspended particles in a fluid are filtered through a membrane (with a specific pore size) driven by pressure. Membranes with 0.1 μm to 10μm size are used in microfiltration. Nanofiltration is a recently developed membrane separation process by which smaller size ions are filtered. Nanofiltration membrane pore size range from 1-10 nm. The membrane doesn’t allow divalent ions and larger ions to pass through but allows monovalent ions such as sodium and chloride.

4.2 Materials and Methods

4.2.1 Materials

Feather samples were obtained from a local rendering company and processed as described in Chapter 1. Savinase 16L were obtained from Novozyme. All the other chemical and biochemical reagents were analytical grade. Ultrasonic pre-treatment experiments were conducted using a 400 kHz unit from Sonosys Ultraschallsysteme (Germany).

4.2.2. Preliminary experiments to define the limits of the experimental factors

Our earlier trials in chapter 2 showed that the use of sulphite significantly enhances the enzymatic hydrolysis of feather. However, experiments were conducted only at 1% sulphite level. In order to determine the optimum range of sulphite required for facilitating the hydrolysis process, hydrolysis experiments were conducted using Savinase 16L (3% on substrate protein basis) at 0. 0.25%. 0.5% and 1% sulphite concentration and 1% feather as substrate. Feather only, Savinase 16L only and Savinase 16L with feather samples were treated in a similar way as references. The hydrolysis experiments were conducted at 60 °C and pH 8.0. Sample aliquots were taken at 0, 4 and 24 hours, heated at 90 °C for 15 min for enzyme inactivation and the concentration of free amines were analysed using the free amino nitrogen (FAN) assay described in previous chapters. In order to determine the maximum concentration of feather suspension that can be handled under laboratory conditions, experiments were also conducted by suspending different amount of feather in water and the maximum amount feasible was found to be 40 g/L (4%).

4.2.3. Experiments to determine the best processing condition(s) for feather hydrolysis

Response surface methodology was used to investigate the simultaneous effects of the different processing parameters. Face centred Central Composite Design was used in the experiments with three independent variables; substrate concentration, sulphite concentration and ultrasonic energy input. Three levels of the three factors were investigated as shown in Table 4. Fifteen combinations of the experimental variables were investigated in accordance with the experimental design (see Table 4), with the central points replicated 6 times as per the experimental design and the axial and the factorial points replicated three times. The enzyme concentration was fixed at 1% since increasing the concentration to 3% or higher would not result in substantial gain in terms of conversion comparable to the increase in operating cost of using 3 times more enzyme as shown in chapter 2. The maximum sulphite concentration was selected based on the preliminary experiments (section 4.2.2) to be 0.5% since increasing the concentration to 1% did not result in significant increase in conversion after 4 hours of hydrolysis (See Figure 34 after 4 hrs of hydrolysis).

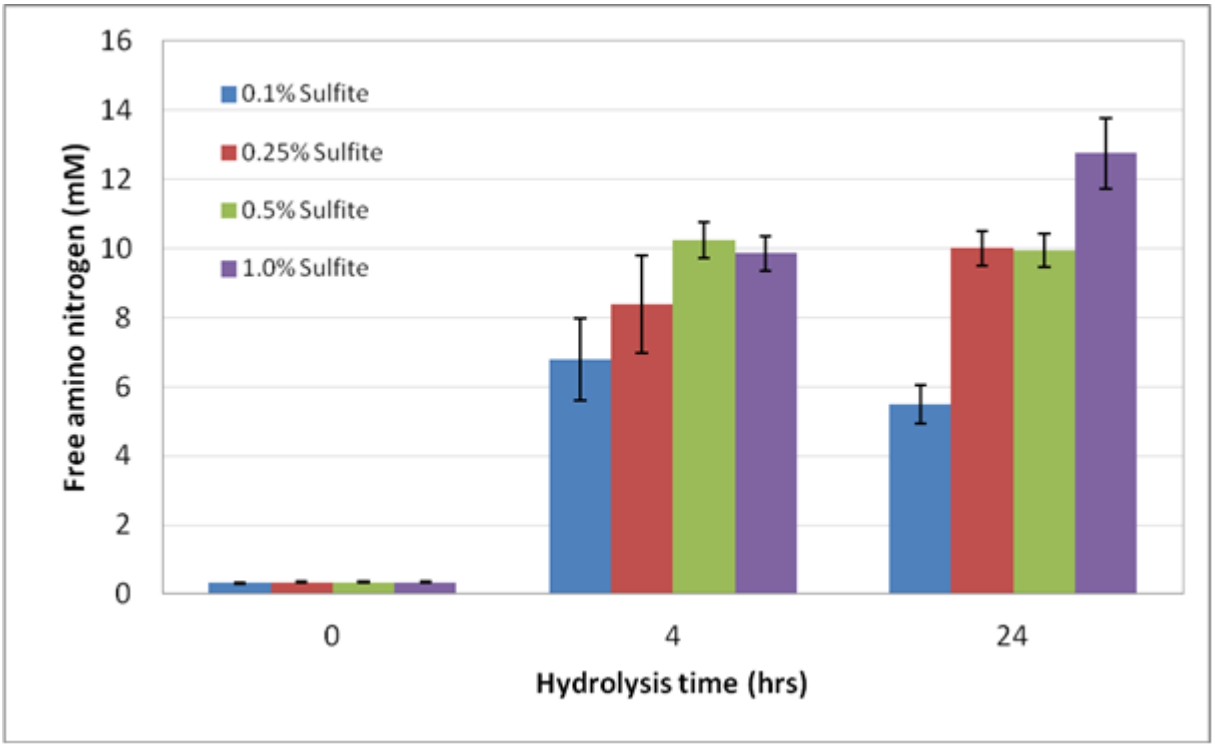


Figure 34 The effect of sulphite concentration on Savinase 16L catalysed hydrolysis of feather keratin

Since no significant improvement was observed by increasing the ultrasonic energy input from 77 to 154 kJ/L during the *B. licheniformis* enzyme catalysed hydrolysis of feather (Chapter 3), the maximum ultrasonic energy input in these experiments were selected to be 100 kJ/L. The maximum substrate concentration was selected to be 4% based on the preliminary experiments described in section 4.2.2. The hydrolysis experiments were conducted for 6 hours to make them relevant for industrial scale processing with sample aliquots taken every hour for analysis of free amines. All the experiments were conducted at 50 °C and pH 8.5 (the manufacturer's recommended optimum conditions) to maintain the stability of the enzyme during hydrolysis, although higher activity of the enzyme was obtained during hydrolysis at 60 °C as reported in Chapter 2.

Table 4 Variables and levels used in response surface experimental design

Substrate concentration (%)	Sulphite concentration (%)	Ultrasonic energy input (kJ/L)
1	0	0
2.5	0.25	50
4	0.5	100

The peptide production (as determined using FAN assay), the peptide production per gram of feather and the conversion after 6 hours (determined by measuring the nitrogen release using LECO as described in Chapter 3) were used as response parameters. The peptide profiles of selected samples from the 6 hour hydrolysis were determined using size exclusion chromatography as described in section 4.2.4. All samples were analysed in triplicates.

A quadratic polynomial equation (eqn. 1) and its subsets were evaluated for the description of the responses as a function of the independent variables.

$$Y = b_o + \sum_{i=1}^{n} b_i X_i + \sum_{i=j=1}^{n} b_{ij} X_i X_j \qquad (1)$$

Where Y represents a response variable, n is the number of independent variables, b_o, b_i, and b_{ij} are coefficients and X_i and X_j represent the independent variables. The sequential model sum of squares was calculated to determine the significance of adding terms of increasing complexity to the total model. Lack of fit test was used to determine the adequacy of the selected models. Analysis of variance was carried out to determine the significance of the model and individual model terms. Only significant model terms (Prob > F less than 0.05) were included in the final equations. The experimental design and the data analysis were performed using Design Expert 7.1.3 (Stat-Ease Inc., Minneapolis, MN, USA).

4.2.4 Size-Exclusion Chromatography (SEC) analysis of feather hydrolysates

The peptide profile of selected samples after 6 hours of hydrolysis was determined using size exclusion chromatography. Yarra SEC-2000 HPLC column (3µm, 300 x 7.8 mm) with suitable guard column was used for the analysis. Sodium Phosphate buffer (50 mM, pH 6.8) containing 150mM NaCl was used as the mobile phase with isocratic flow (0.5 mL/min) for 60 min run time. The detection wavelength was 220 nm. Alphalactalbumin (0.5 mg/mL), Cytochrome C (1 mg/mL), Insulin Chain B (0.5 mg/mL) and Bacitracin (1 mg/mL) obtained from Sigma (Sigma Australia) were used as standards to develop the calibration curve.

4.2.5. Separation process development for purifying keratin hydrolysates

A separation process was designed to purify the hydrolysates based on the physical properties of the crude product and the peptide profile of the hydrolysates. The flow diagram for the process is presented in Figure 35. The coarse filtration using sieve and bag filter was designed to remove unhydrolysed feather particles. The microfiltration and the diafilitration steps were designed to remove the remaining unhydrolysed fine feather particles and to wash and recover the remaining hydrolysate from the retentate, respectively. The nanofiltration and diafiltration steps were designed to remove the buffer salts and the sulphite and concentrate the hydrolysate prior to freeze drying by removing as much water as possible. In order to evaluate the feasibility of the process, a larger scale hydrolysis experiments were conducted with 12 kg of feather suspension

processed at the optimum hydrolysis condition established based on the results of the response surface experiments in section 4.2.3. Samples were taken at 0, 3 and 6 hours during hydrolysis and after each separation step and analysed for total nitrogen content using LECO analyser.

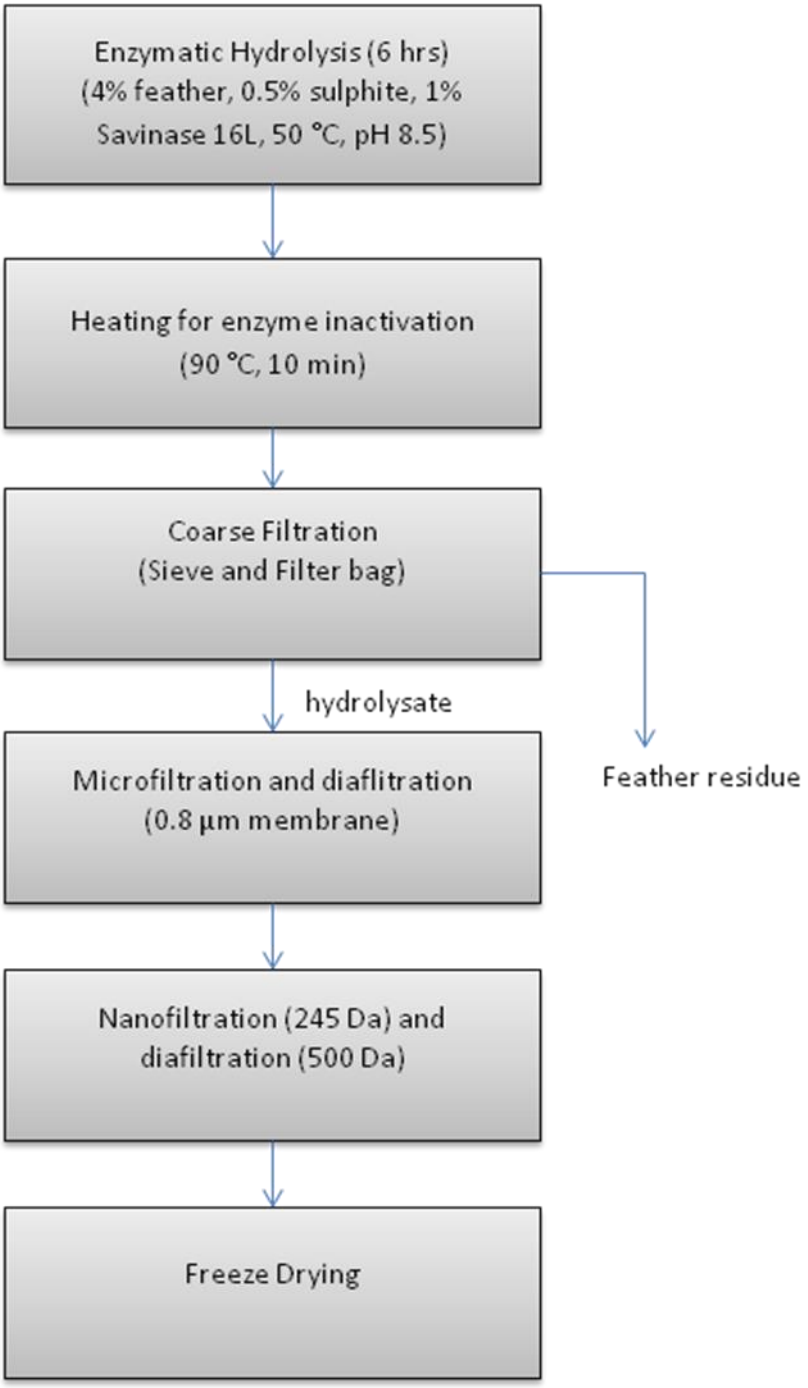


Figure 35 Process flow diagram for the production and purification of keratin hydrolysate from raw feather

The hydrolysed feather extract was subjected to microfiltration at 25°C on a Membralox XLab 5 filtration unit fitted with ceramic (alumina) monochannel membranes with 0.005 m^2 membrane area

and 0.8µm pore. The membrane was mounted on a Pall® Membralox XLab 5 benchtop crossflow unit. All experiments were performed at an average cross flow velocity of 10.5 m/s, and 1.25-1.35 bar transmembrane pressure. The retentate was diafiltered with three volumes of RO water. The temperature of the hydrolysed feather was maintained using a water bath by continuously circulating the water at the set temperature through the jacketed feed tank using a heater/pump (Ratek, thermoregulator TH5). The membrane was chemically cleaned at the end of the process with Ecolab Ultrasil 25 (1.5% w/w sodium hydroxide at 70°C, with the permeate side initially fully closed. After the clean, the membrane was rinsed using 70°C water until all the Ultrasil 25 was completely removed from the system.

The microfiltration (MF) permeate were then concentrated via nanofiltration at 20°C, 10 bar feed pressure, and 0.5m/s cross flow velocity. A Steritech SEPA SF II flat sheet cross flow filtration system fitted with a Dow Filmtec nanofiltration NF245 (polyamide) membrane (selected based on the molecular weight profile of the hydrolysate) and 0.014 m2 membrane area was used for purifying and concentrating the hydrolysate and 5 diafiltrations were performed using the Synder NFG 600-800Da membrane to further purify the hydrolysate. Each experiment was carried out with a brand new membrane sheet.

4.2.6. Determination of the dry content of the product

The total solids content of the hydrolysates were determined using a Mettler Toledo HR73 Halogen Moisture analyser.

4.3. Results and Discussion

4.3.1. Best Feather Hydrolysis Condition

The kinetics of hydrolysis, measured as the rate of release of free amines, was evaluated for six hours at the different processing conditions. The average amine concentration after 6 hours of hydrolysis ranged from 3.2 to 8.4 mM at 1% substrate concentration with the higher value obtained with 0.5% sulphite. At 2.5% and 4% substrate concentrations, the amine concentration ranged from 10.3 to 22.3 mM and 17.06 to 35.2 mM respectively with the higher values obtained at 0.5% sulphite concentration (see Table 1 in appendix 2). Clearly, the effects of substrate and sulphite concentrations are evident with the highset production of peptides observed at the highest sulphite and substrate concentrations.

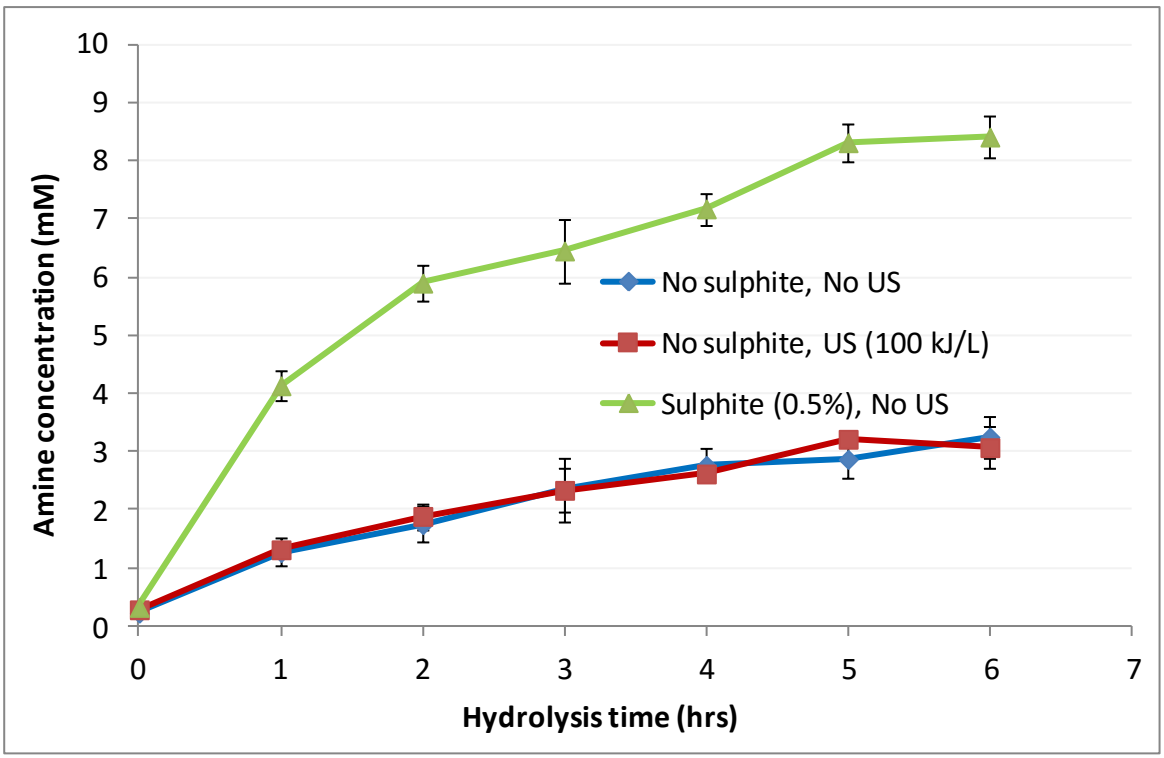


Figure 36 The effect of sulphite concentration and ultrasonic pre-treatment on the kinetics of Savinase 16L catalysed hydrolysis of feather (1% concentration) at 50 °C and pH 8.5. Error bars represent standard deviation values calculated for triplicate samples analysed in duplicates.

Some example data on the kinetics of peptide release at different conditions are given in Figure 36 and 37. As can be seen, the reaction has not fully reached plateau and further increase in reaction time would result in further conversion of feather, although a longer processing time may not be feasible for industrial application. In all cases, adding sulphite to the reaction mixture substantially improved the rate and extent of peptide production, with about four times increase in initial rate and 2.6 times increase in peptide release in 6 hours for the hydrolysis of 1% feather in the presence of 0.5% sulphite compared to the reaction conducted without sulphite. Ultrasonic pre-treatment even at the highest energy input tested (100 kJ/L) did not have significant effect on feather hydrolysis at 1% concentration (Figure 36). Data for the kinetics of hydrolysis of feather at 4% concentration with and without sulphite and ultrasonics are presented in Figure 37. In this case, substantial increase in hydrolysis was obtained using sulphites, although ultrasonic pre-treatment slightly improved the rate and extent of the reaction (by about 20%). Comparing Figures 36 and 37, it is clear that substrate concentration significantly influences the rate of the reaction with more than 5 times more peptide formation at 4% concentration compared to 1% when no sulphite and ultrasonics are applied.

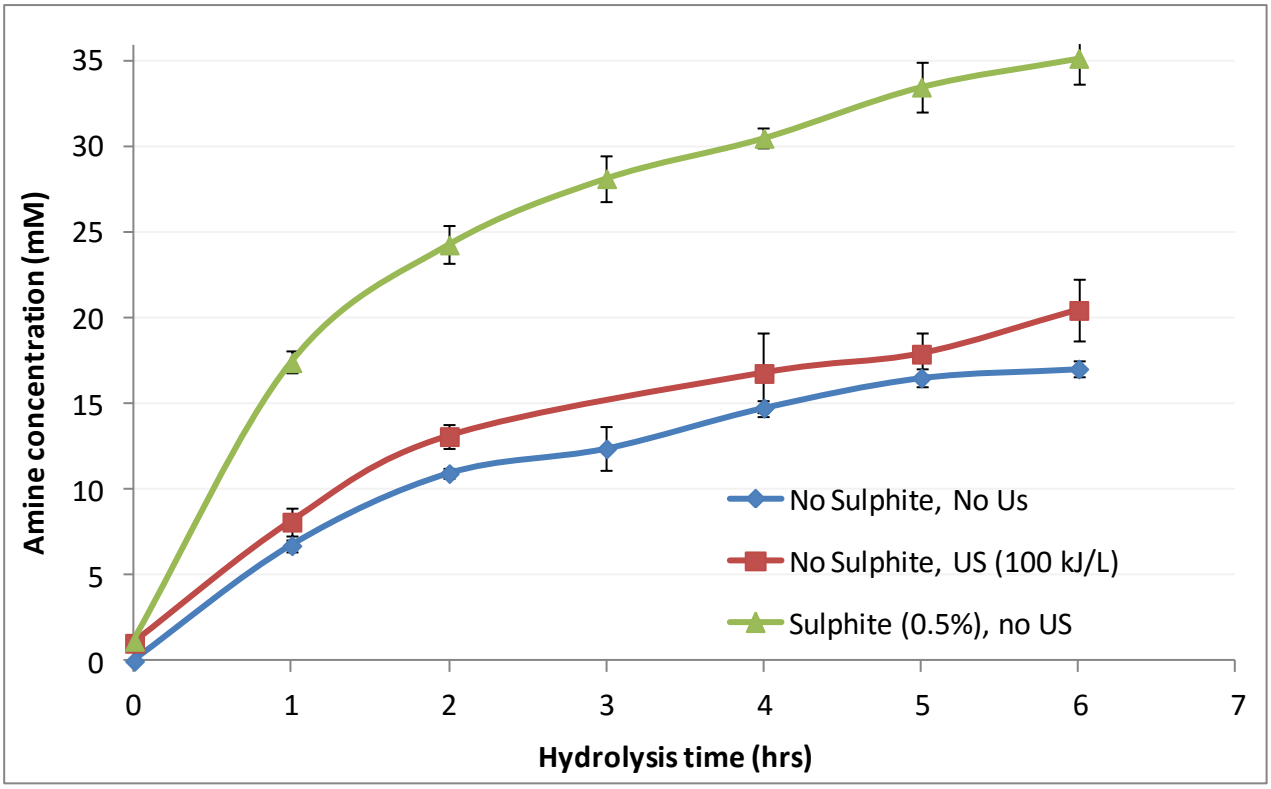


Figure 37 The effect of sulphite concentration and ultrasonic pre-treatment on the kinetics of Savinase 16L catalysed hydrolysis of feather (4% concentration) at 50 °C and pH 8.5. Error bars represent standard deviations calculated for triplicate samples analysed in duplicates.

Analysis of variance showed that the factors that affect the rate of release of free amines significantly ($p<0.05$) are substrate and sulphite concentration (Table 4.2). The slight effect that ultrasonic pre-treatment had on hydrolysis at high substrate concentration was masked by the effect of sulphites and ultrasonic pre-treatment had no statistically significant effect on the reaction. The interaction between ultrasound and sulphite and substrate and sulphite as well as the second degree of substrate concentration were also found to be statistically significant (Table 5). The response surface quadratic model describing the effects of the different parameters on peptide production is given in equation 2.

$$\text{Amine concentration (6hrs)} = -3.5+0.02*A+5.3*B+18.9*C-0.07*AC+6.9*BC-23.6*C^2 \quad (2)$$

where A is ultrasonic condition, B is substrate concentration and C is sulphite concentration.

As can be seen in Equation 2, the interaction term between ultrasonics and sulphite is negative implying that applying ultrasonics pre-treatment on samples treated by sulphites has a slight negative effect on the hydrolysis process. The response surface described the effects of the different parameters investigated very well as shown by the observed very high coefficient of determination (R^2=0.9818)(Table 5). The response surface plot showing the effects of substrate

and sulphite concentration is presented in Figure 38. As can be seen, peptide release increased with increased sulphite and substrate concentrations within the investigated range. Based on the response surface equation, the optimum condition for peptide production within the investigated range was calculated to be 4% substrate and 0.5% sulphite concentrations

Table 5 Analysis of variance showing the effects of different processing parameters on peptide production after 6 hours during Savinase 16L catalysed hydrolysis of feather at pH 8.5 and 50 °C

Source	**Sum of squares**	**df**	**Mean square**	**F value**	**P value Prob > F**
Model	4356.77	9	484.09	244.15	<0.0001
A-ultrasonic condition	0.19	1	0.19	0.095	0.7595
B- substrate concentration	3360.42	1	3360.42	1694.85	<0.0001
C- sulphite concentration	786.43	1	786.43	396.64	<0.0001
AxB	1.32	1	1.32	0.67	0.4196
AxC	20.84	1	20.84	10.51	0.0025
BxC	159.11	1	159.11	80.25	<0.0001
A^2	3.01	1	3.01	1.52	0.2257
B^2	2.11	1	2.11	1.06	0.3088
C^2	18.37	1	18.37	9.26	0.0042
Residual	75.34	38	1.98		
Pure Error	40.71	33	1.23		
Corrected total	4432.12	47			
R^2	0.9818				
R^2_{adj}	0.9792				

The percentage conversion after six hours (based on LECO analysis of the nitrogen release) and free peptide release per gram of feather were also analysed as measures of feather conversion. The average percentage conversion ranged from 9.3 to 24.9% at 1% substrate concentration, from 15.8 to 30.2% at 2.5% substrate concentration and from 18.3 to 29.7% at 4% substrate concentrations, with the higher values corresponding to 0.5% sulphite concentration (Table 1 in Appendix 2). Substrate concentration had major effect on conversion although the magnitude of its effect decreased at the higher concentrations. Similarly, the application of sulphite concentrations had substantial effect on conversion, although increasing sulphite concentration from 0.25 to 0.5% was less effective in improving conversion at the higher substrate concentrations (2.5 and 4%). In addition, increasing substrate concentrations from 2.5 to 4% did not have any effect on conversion at 0.5% sulphite concentration (compare 30.2% conversion at 2.5% substrate concentration with 29.7% at 4% substrate concentration). The analysis of variance on the effects of the different

factors on conversion confirmed the visual observation, with both sulphite and substrate concentration having significant effect on conversion with no significant effect of ultrasonics as in the case of peptide production (Table 5). However, only sulphite substrate interaction was significant in this case as are the second order terms of sulphite and substrate concentration. The response surface model describing conversion is given in equation 3.

$$\% \text{ Conversion} = -1.89 + 12.15*B + 62.17*C - 3.8*BC - 1.8*B^2 - 56.8C^2 \quad (3)$$

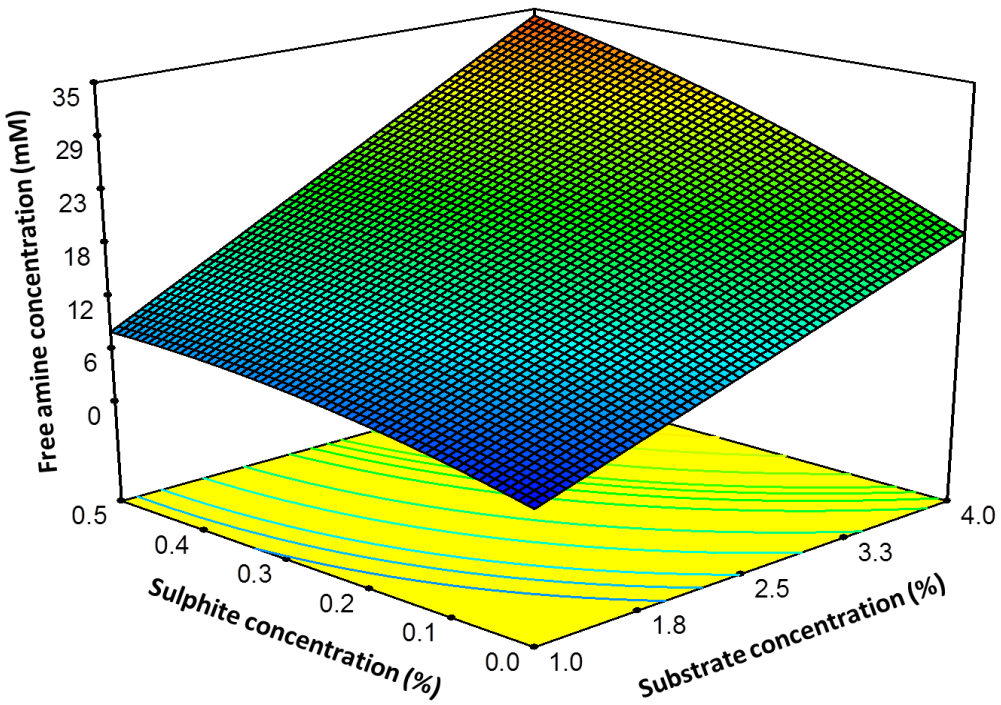


Figure 38 A Response surface plot showing the effects of substrate concentration and sulphite concentration on peptide release during the Savinase 16L catalysed hydrolysis of feather at pH 8.5 and 50 °C.

Table 6 Analysis of variance showing the effects of different processing parameters on % conversion of feather after six hours during Savinase 16L catalysed hydrolysis of feather at pH 8.5 and 50 °C

Source	Sum of squares	df	Mean square	F value	P value Prob > F
Model	1991.42	9	221.3	34.8	<0.0001
A-ultrasonic condition	2.05	1	2.05	0.32	0.5733
B- substrate concentration	353.7	1	353.7	55.7	<0.0001
C- sulphite concentration	1106.8	1	1106.8	174.3	<0.0001
AxB	1.95	1	1.95	0.31	0.5831
AxC	0.17	1	0.17	0.026	0.8722
BxC	48.09	1	48.09	7.57	0.009
A^2	5.7	1	5.7	0.89	0.3505
B^2	144.9	1	144.9	22.8	<0.0001
C^2	115.7	1	115.7	9.26	0.0042
Residual	241.3	38	6.3	18.2	0.0001
Pure Error	191.9	33	5.8		
Corrected total	2232.7	47			
R^2	0.8875				
R^2_{adj}	0.8741				

Equation (3) described the effects of the different factors on conversion well with reasonably high coefficient of determination (R^2=0.8875). As opposed to peptide production, conversion did not increase monotonically with sulphite concentration. In fact, increasing sulphite concentration in the higher substrate concentration range did not improve conversion as the two factors have negative interaction (see eqn 3). The response surface plot showing the effects of these two factors on percentage conversion is presented in Figure 39, which clearly shows that the optimum condition for conversion lies somewhere at the higher end of substrate and sulphite concentrations but not at the highest sulphite and substrate concentrations as in the case of peptide production. Based on equation 3, the optimum condition for percentage conversion was predicted to be 3% substrate concentration and 0.47% sulphite concentration with a predicted conversion of 30%. The calculated peptide production per gram of feather in the reaction mixture correlated well with percentage conversion (R^2=0.7179), with the analysis of variance indicating similar significant factors affecting the response. The values ranged from 0.32 to 0.84 mmoles/gm at 1% substrate concentration, 0.41 to 0.89 mmoles/gm at 2.5% substrate concentration and 0.42 to 0.88 mmole/gm at 4% substrate concentrations respectively, with the higher values corresponding to

0.5% sulphite concentrations (Table 1 in appendix 2). As in the case of percentage conversion, substrate concentrations did not have substantial effect at the highest sulphite concentration.

Thus the optimum condition for maximum peptide production, maximum peptide production per gram of feather and maximum conversion were calculated using the response surface equations obtained for the three parameters. The optimum conditions maximising the three parameters were found to be 3.9% substrate and 0.5% sulphite with predicted conversion of 29%, free amine concentration of 34.2 mM and free amine/gram feather of 8.85 mmol/gm. Increasing the feather concentration to 4% did not have practically significant effect with prediction for all the parameters with 4% substrate and 0.5% sulphite concentrations being 29%, 35.08 mM and 8.79 respectively. Thus, further experiments for developing the separation process were conducted at 4% substrate and 0.5% sulphite concentrations without ultrasonication.

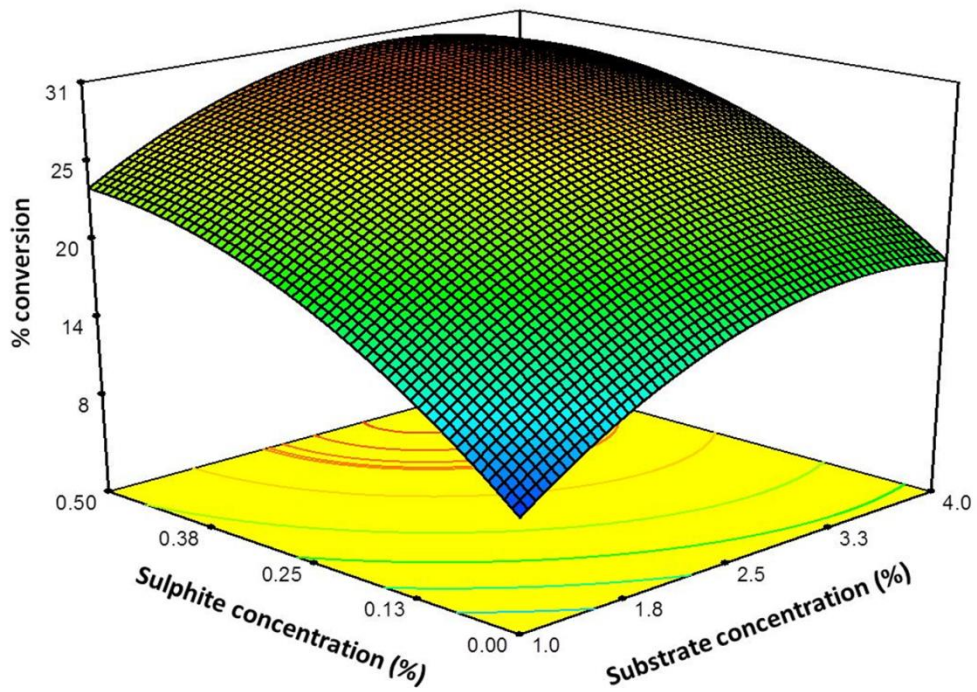


Figure 39 Response surface plot showing the effects of sulphite and substrate concentration on percentage conversion during Savinase 16L catalysed hydrolysis of feather at pH 8.5 and 50 °C

The observed minimal effect of ultrasonic treatment on the hydrolysis process at the studied conditions was unexpected. In our earlier investigation (Chapter 3), ultrasonic pre-treatment at specific energy input of 315 kJ/L significantly enhanced the Savinase 16L catalysed hydrolysis of feather especially after 48 hours of hydrolsyis. In this study, lower specific energy inputs were evaluated (a maximum of 100 kJ/L) considering process economics, since energy inputs as low as 77 kJ/L resulted in significant enhancement of the *B.licheniformis* keratinase catalysed hydrolysis of feather (Chapter 3). The result indicates that higher ultrasonic energy input than investigated in

this study may be required in order to improve the rate of Savinase 16L catalysed hydrolysis of feather or the effect of ultrasonics becomes important only after prolonged hydrolysis. The process economics and the added benefit determines the feasibility of using a higher specific energy input and can only be justified if the sonicated samples have better functional attributes, which will be investigated in Chapter 5.

4.3.2 Peptide profile of feather hydrolysates

The molecular weight profiles of selected feather hydrolysates obtained after 6 hours of hydrolysis were determined using the size exclusion HPLC method developed in section 4.2.4 in order,

- to evaluate if processing condition has an effect on peptide profile of the hydrolysates and
- to define the conditions (pore size etc) for the separation and purification of the hydrolysates.

A calibration curve was constructed with the standards and HPLC conditions described in section 4.2.4. The molecular weight and retention times of the different standards are presented on Table 7 and the corresponding calibration curve is shown in Figure 40.

Table 7 The molecular weights (Mw) and corresponding retention times of the standards used for size exclusion chromatography

Protein standard	Concen. (mg/mL)	Injection volume (µL)	Molecular weight (Da)	Log Mw	Retention time (min)
Alpha lactalbumin	0.5	15	14178	4.151615	18.496
Cytochrome C	1	20	12327	4.090857	19.782
Insulin chain B	0.5	15	3496	3.543571	21.125
Bacitracin	1	60	1422	3.1529	23.846
Gly 3	1	40	189.2	2.276921	22.004

The SEC peptide profiles of the three samples at 4% substrate concentrations processed at different conditions i.e. 1), no ultrasound and no sulphite, 2) no ultrasound, 0.5% sulphite and 3) 100 kJ/L ultrasound, no sulphite were evaluated (Figure 41). The molecular weight ranges of the hydrolysates are summarised in Table 8.

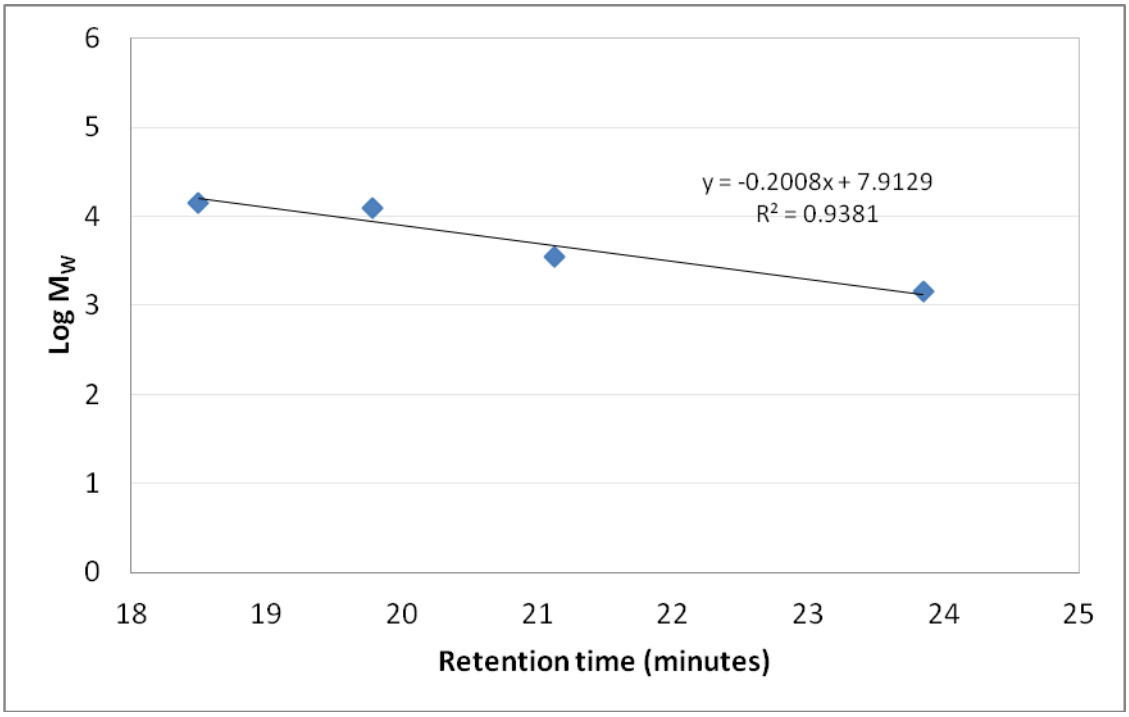


Figure 40 Calibration curve relating molecular weight with retention time for the determination of molecular weight distribution of keratin hydrolysates

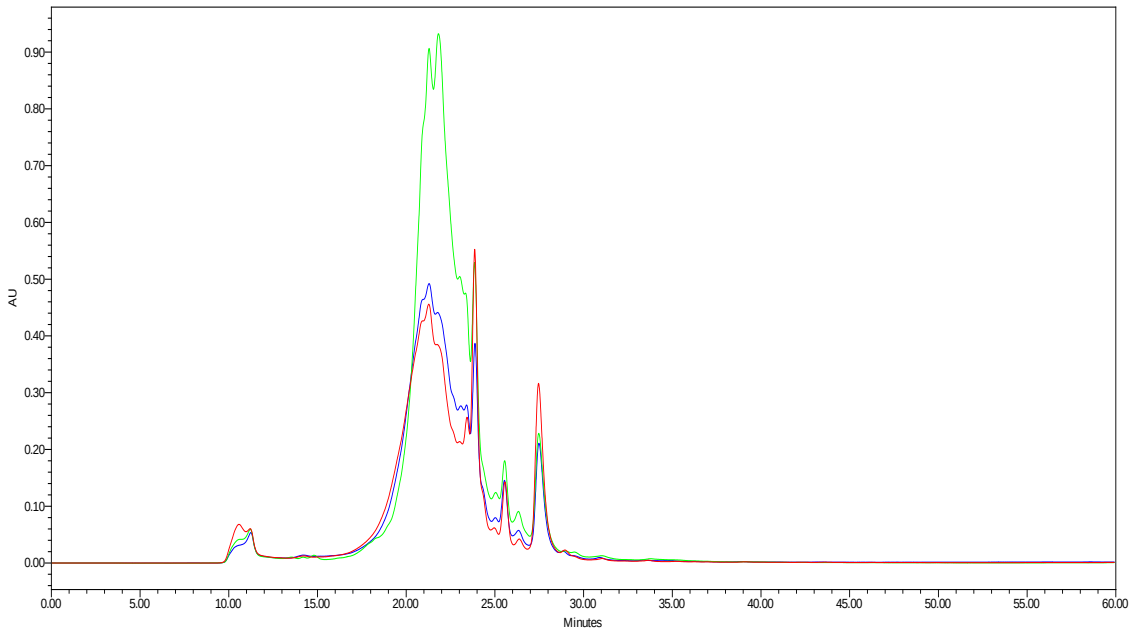


Figure 41 Molecular weight profile of selected feather hydrolysates; Red:(no US, no Sulfite), Green:(no US, 0.5% Sulfite), Blue: Expt 16 (100kJ/L, no sulfite**)**

Table 8 Molecular weight range of peptides in selected feather hydrolysates

Retention Time (minutes)	Approximate Molecular size range (Da)
18	19,883
21 - 22	5,000 – 3,400
23 - 25	2000 - 800
25 - 27	780 - 310
27 - 30	77 - 310

Similar peptide profiles were observed for all the samples with molecular weight ranging from 77 Da to 5000 Da (Figure 41). However, higher amount of high molecular weight fragments (3400-5000) were observed in samples treated by 0.5% sulphite, whereas similar profiles were observed for the untreated and ultrasonicated samples under the studied condition.

4.3.3. Separation process for the purification of keratin hydrolysates

Since the optimum processing condition was found to be with 4% substrate and 0.5% sulphite concentrations and since the biggest challenge for sample purification is in case of samples treated with sulphites, the separation process was designed and evaluated for purifying the hydrolysate produced at this condition. Using the large scale experimental set up (12 kg of feather suspension) for the hydrolysis using external stirrer, a slightly higher conversion of about 32% was obtained at 4% substrate and 0.5% sulphite concentrations after 6 hours of hydrolysis. Figure 42 shows pictures of the large scale experimental set up for hydrolysis and the coarse filtration steps using sieve and bag filter. The coarse filtration step removed most of the unhydrolysed feather as well as part of the hydrolysate (about 7.8%), which could not be squeezed out from the wet residue (Table 9). Under industrial conditions, this will not be expected to happen as more appropriate filtration equipment such as a rotary drum filter with automatic washing, drying and scaping would be employed.

Further purification of the hydrolysate was achieved after microfiltration, which was accompanied by about 15% loss of the total protein in the retentate. The nanofiltration resulted in about 6.4 times concentration of the product and 55% protein purity. Further purification was achieved by 5 diafilitration steps with a final purity of 78.8% (dry basis) protein after freeze drying (Table 9). In

order to improve the purity of the product, the nanofiltration step was slightly modified, using 500 Da cut off filter for both the nanofiltration and the diafiltration steps. The pictures of the nanofiltration concentrate and the final freeze dried products are presented in Figure 44.

Figure 42 Experimental set up for large scale feather hydrolysis and coarse filtration using Sieve (150 µm) and bag filter (1 µm)

The purity of the product increased to 80.7% with just 3 diafiltration steps. The peptide profile of the purified product was also evaluated. The final product had peptides in the molecular weight range between 3000 and 5000 Da. The various purification steps resulted in loss of peptide fragments with molecular weight less than 2000 Da (Figure 45), indicating that the separation process needs to be further optimised based on the desired functionality and peptide profile for a given application.

Figure 43 The hydrolysate prior to microfiltration (a) and the laboratory scale microfiltration (b) and nanofiltration (c) units used in the separation experiment

Table 9 Mass Balance, protein and dry matter content and percentage protein purity after the various separation steps

Fraction	Mass (g)	Protein conc. (%w/w)	Dry matter content (% w/w)	Protein purity (%) dry basis
Feather	480	90.75	91.03	
Feather hydrolysate	11831.7	1.142889333		
Sieve (150 µm) and filter bag (1 µm) filtrate	9825.3	1.2390		
MICROFILTRATION (0.8µm ceramic)				
MF Feed	9825.3	1.2390		
MF Permeate from concentration	8501.3	0.9880	1.785	55.35002689
MF Permeate from diafiltration (DF3)	3550.6	0.4470	0.235	
MF Retentate after concentration	1318.7			
MF Retentate after diafiltration (DF3)	1257.2	0.6446	1.255	51.35889356
NANOFILTRATION (Synder NFG 600-800Da, and Dow NF245 245Da)				
NF Feed (MF permeate plus MF Diafiltrates)	12051.9			
NF Permeate from concentration	10433.5	0.0118		
NF Permeate from diafiltration (DF1)	991.1			
NF Permeate from diafiltration (DF2)	1002.9			
NF Permeate from diafiltration (DF3)	1000.4			
NF Permeate from diafiltration (DF4)	2572.7			
NF Permeate from diafiltration (DF5)	1554.3			
NF Permeate (total) DF1-5	7121.4			
NF Retentate after concentration	1618.4	5.3255	9.635	55.27197706
NF Retentate after diafiltration (DF5)	1478.6	2.5870	3.525	73.39002218
Freeze-dried NF retentate	54.29	74.6140	94.58	78.88977807

A modified form of the separation process was also evaluated for an enzymatic hydrolysate produced without the use of sulphites. In this case, nanofiltration using a 245 Da cut off filter resulted in 79.8% purity, with no diafiltration steps (data not presented). The low molecular weight peptides (<2000 Da) were also retained in the product.

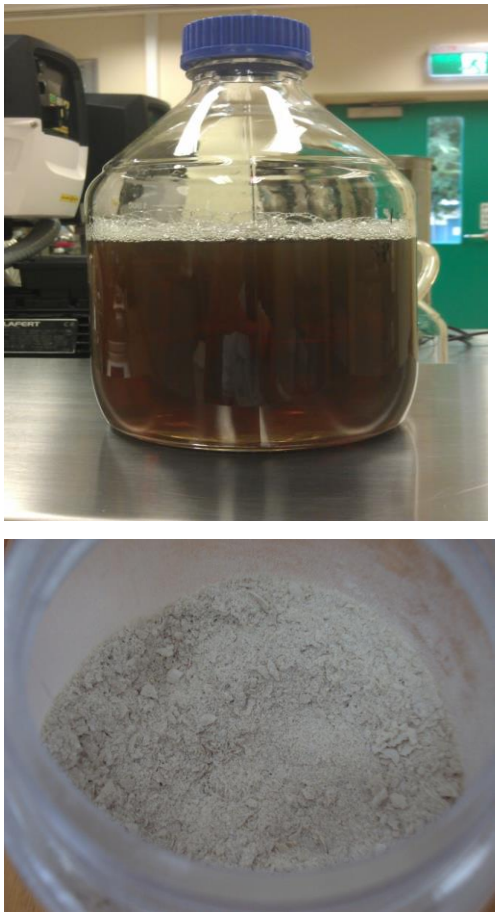


Figure 44 Nanofiltration retentate (concentrated hydrolysate) and freeze dried keratin hydrolysate after purification

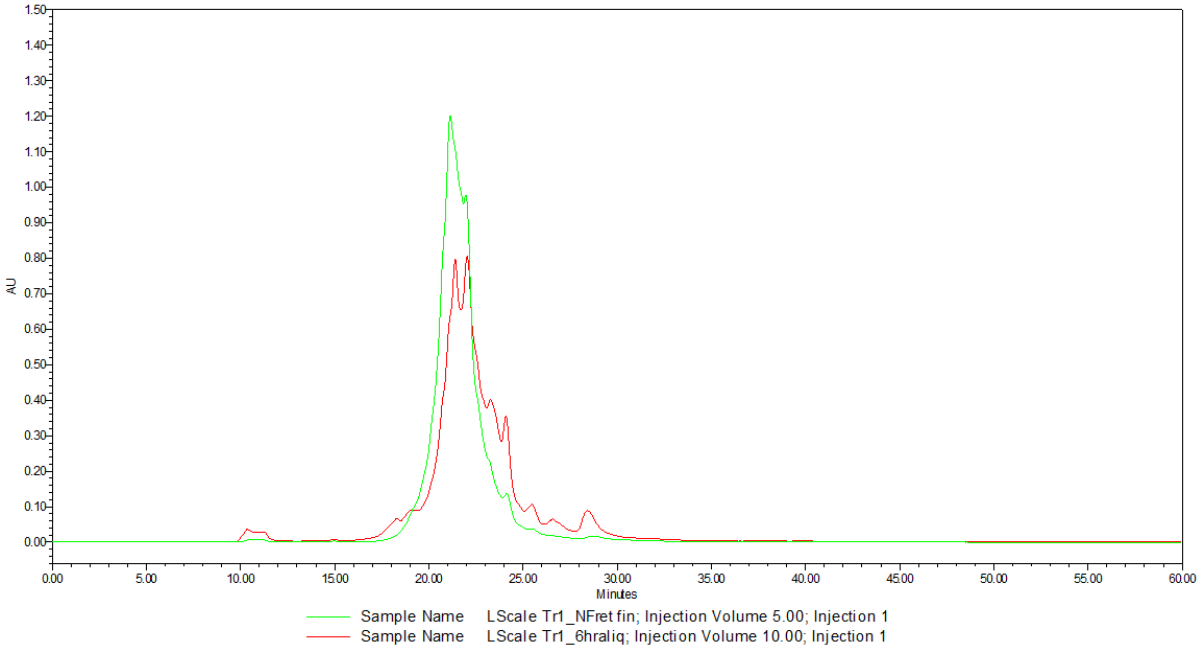


Figure 45 Comparison of peptide profile of purified keratin hydrolysate with crude keratin hydrolysate. Green: nanofiltration retentate, 5 uL injection volume; Red: Crude hydrolysate, 10uL injection volume.

4.4 Conclusion

In this chapter, response surface methodology was used to determine the best processing condition (s) for the conversion of feather into keratin hydrolysates using Savinase 16L. The effects of factors that were identified in previous chapters (sulphite concentration, ultrasonic energy input) and substrate concentrations and their interactions were evaluated using peptide production, percentage conversion and peptide production per gram of feather as responses. Kinetic experiments were conducted at 15 combinations of the experimental variables with the central point replicated 6 times as per the experimental design and the axial and radial points replicated three times. The maximum peptide formation after 6 hours of hydrolysis was about 35 mM obtained with 4% feather and 0.5% sulphite concentrations. The maximum conversion after 6 hours was around 30% which was obtained for reactions at 2.5 to 4% feather and 0.5% sulphite concentrations. The hydrolysis time was fixed at 6 hrs since longer hydrolysis time would not feasible for industrial application, although higher conversion could be achieved. Analysis of variance showed that the significant parameters that affect the Savinase 16L catalysed conversion of feather, under the studied conditions, were substrate and sulphite concentration. Based on the response surface models obtained, the optimum condition that maximize both peptide formation and percentage conversion were found to be 3.9% substrate and the optimum conditions maximising the three parameters were found to be 3.9% substrate, 0.5% sulphite with predicted conversion of 29%, free amine concentration of 34.2 mM and free amine/gram feather of 8.85 mmol/gm. Ultrasonication at the specific energy input values used in the investigation (50 and 100 kJ/L, 400 kHz) did not have statistically significant effect, although marginal (20% increase) effect of ultrasonication (100 kJ/L) on peptide formation was observed at 4% substrate concentration. This was unexpected since in our earlier investigation (chapter 3), ultrasonic pre-treatment at specific energy input of 315 kJ/L significantly enhanced the Savinase 16L catalysed hydrolysis of feather. In this study, lower specific energy inputs were evaluated (a maximum of 100 kJ/L) considering the process economics as energy inputs as low as 77 kJ/L resulted in significant enhancement of the *B.licheniformis* keratinase catalysed hydrolysis of feather (Chapter 3). This result indicates that higher ultrasonic energy input than investigated in this study or longer reaction time may be required in order to improve the rate of Savinase 16L catalysed hydrolysis of feather by ultrasonics. The process economics and the added benefit will dictate the feasibility of using a higher specific energy input and can only be justified if the ultrasonicated samples have better functional attributes, which will be investigated in Chapter 5.

The peptide profile of selected hydrolysates from 4% feather processed at different conditions (no sulphite, no ultrasonics, 0.5% sulphite, no ultrasonics and 100 kJ/L ultrasonics, no sulphite) was also evaluated. The peptides in all the samples had molecular weights ranging from 77 to 5000 Da, although higher amount of high molecular weight (3400-5000) peptides were found in samples hydrolysed with 0.5% sulphites. The peptide profile and the physical properties of the hydrolysates

were used as a basis for designing a separation process to obtain hydrolysates with high degree of purity. The process involved coarse filtration (using sieves and bag filters), microfiltration (0.8 µm) and nanofiltration (245 Da) with 5 diafiltration steps (600-800 Da). The process was evaluated for feather hydrolysed at the optimum hydrolysis condition at 4% substrate and 0.5% sulphite concentration, which was also the most challenging condition due to the high concentration of sulphites. A product with 78.9% purity was obtained after the diafiltration and final freeze drying step. Size exclusion chromatography of the final product indicated that the peptide fragments with molecular weight less than 2000 Da were lost in the separation process and the final product had peptides with molecular weight between 3000 and 5000 Da. The separation process may need to be further optimised based on the desired functionality and peptide profile for a given application.

Chapter 5 Functional properties of feather keratin hydrolysates

5.1. Introduction

In chapter 5, we developed a process for the production and purification of high quality keratin hydrolysates. The objectives of this chapter were to

- Determine the functional properties of the keratin hydrolysates
- Determine the peptide profile, the amino acid profile and bioactive properties of the keratin hydrolysates
- Determine the digestibility of the feather residue

The data on the functional and biological properties of the hydrolysates enables the assessment of the potential applications of the hydrolysate in the pet food, cosmeceutical and nutraceutical industries. Savinase 16L was used in these trials with 0.5% sulphite to enhance the hydrolysis process. The functional properties of the product were compared with a hydrolysate produced using ultrasonic assisted hydrolysis and standard protein ingredients viz. whey protein isolate, soy protein isolate and egg albumin.

5.2. Materials and methods

5.2.1. Materials

Feather samples were obtained from a local rendering company and processed as described in chapter 1. The hydrolysis of feather and the purification of the hydrolysate were conducted as described in chapter 4. Savinase 16L were obtained from Novozyme (Sydney, Australia). All the other chemical and biochemical reagents were analytical grade. Ultrasonic pre-treatment experiments were conducted using a 400 kHz unit from Sonosys Ultraschallsysteme (Germany).

5.2.2. Determination of functional properties

5.2.2.1. Solubility

The keratin hydrolysate was dispersed in milli-Q water at 2% (w/v) protein concentration, the pH adjusted to 7.0 with 1 M HCl and stirred for an hour at room temperature (21°C), before adjusting the volume appropriately with milli-Q water in a volumetric flask. The dispersion was stirred thoroughly and an aliquot was centrifuged at 20,000 g for 30 minutes at 20 °C in an eppendorf centrifuge. The supernatant was removed. The nitrogen content of the supernatant and the dispersion were analyzed using LECO Nitrogen analyzer (TruMac ® N, St. Joseph, MI, USA). The protein content and the solubility were calculated as follows (Eqns 4 and 5)

$$Protein\ (\%) = Nitrogen\ (\%) \times 6.25 \quad (4)$$

$$Protein\ Solubility(\%) = \frac{protein\ \ content\ \ in\ supernatant}{protein\ \ content\ \ in\ the\ initial\ \ dispersion} \times 100 \quad (5)$$

5.2.2.2. Foaming properties

The keratin hydrolysate was dispersed in milli-Q water at 3% (w/w) protein concentration, the pH adjusted to 7.0 with 1 M HCl and stirred for an hour at room temperature (21°C), before adjusting the weight appropriately with milli-Q water. Hundred mL of the dispersion was transferred into a mixing bowl and whipped for 3 minutes using a double beater mixer (Sunbeam) at the highest speed (setting number 12). The whipped material was transferred into a measuring cylinder/or beaker, and the volume of the foam was immediately recorded. The foam was then left undisturbed at room temperature and the volume of liquid released from the foam is measured after 30 min and 1 hour.

The foaming capacity (FC) and stability (FS) were calculated as follows (Eqns 6 and 7)

$$FC\ (\%) = \frac{volume\ \ of\ foam\ \ (mL)}{Volume\ \ of\ dispersion\ \ \ (mL)} \times 100 \quad (6)$$

$$FS\ (\%) = \frac{Volume\ \ of\ dispersion\ \ \ (mL) - Volume\ \ of\ liquid\ \ released\ \ \ (mL)}{Volume\ \ of\ dispersion\ \ \ (mL)} \times 100 \quad (7)$$

5.2.2.3. Emulsifying properties

Emulsifying Capacity (EC)

The keratin hydrolysate was dispersed in milli-Q water at 0.1% (w/w) protein concentration, the pH adjusted to 7.0 with 1 M HCl and stirred for an hour at room temperature (21°C), before adjusting the weight appropriately with milli-Q water. Fifty mL of the dispersion was transferred into a glass tube and ultraturraxed (IKA T25, Selangor, Malaysia) at 20,500 rpm until the reading of resistance (in the resistance meter) was stable. Vegetable oil (Crisco, Premium oil, Australia) was then pumped into the dispersion at 20 g/min flow rate. At the inversion point (characterized by a sudden increase in resistance) the pump was stopped. The mass of oil added to reach the inversion point was recorded.

The Emulsifying Capacity (EC) was calculated using the following (Eqn 8)

$$EC(g/mg) = \frac{m_{sample} - m_{blank}}{m_{protein}} \quad (8)$$

m_{sample} = mass (g) of oil required for emulsion inversion in the sample,

m_{blank} = mass (g) of oil required for emulsion inversion in the blank (milli-Q water),

$m_{protein}$ = mass (mg) of protein present in the sample.

Emulsifying stability (ES)

Keratin hydrolysate (0.1% w/w) was prepared as described as above. The vegetable oil was added to the protein dispersion;

- At ratio of 3:1 w/w (protein dispersion: oil), and
- At the maximum capacity of the protein solution (from emulsifying capacity experimental results)

The solutions were ultraturaxed at 20,500 rpm for 2 minutes and transferred to measuring cylinders (100 mL or 200 mL). Initial volume of the emulsion (V_i) and the emulsified fraction (V_t) after 0.5, 1, 3 and 24 hours were recorded. The emulsion stability values were calculated using the following equation (Eqn 9).

$$ES(\%) = \frac{V_i}{V_t} x100 \quad (9)$$

5.2.3. Biological properties

5.2.3.1. Peptide profile

The peptide profile of selected samples after 6 hours of hydrolysis was determined using size exclusion chromatography as described in chapter 4. Peptide profiles of the keratin hydrolysates (from the sulphite and ultrasonic assisted processes) were analysed with a Size Exclusion column (SEC-2000, Yarra, 3μM, 300mm x 7.8mm with a guard column) (Phenomenex Australia Pty Ltd, Lane Cove, NSW, Australia). The column was connected to a Waters HPLC system (Milford, MA, USA). The liquid samples were filtered through a 0.2 μM membrane prior to SEC analysis. The running buffer was 50mM sodium phosphate/150mM sodium chloride, with isocratic flow at 0.5 ml/min over a run time 60 minutes. The molecular size ranges of the peptides in the samples were determined by comparison with a calibration curve using peptide/protein standards.

5.2.3.2. Amino acid profile

The amino acid profile analysis of the samples was conducted at the Australian proteome analysis facility (APAF). Samples were hydrolyses in 6M HCL at 110 °C for 24 hrs. The amino acids were labelled using the Waters AccQ.Tag™ chemistry and analysed using a Waters Acquity™ UPLC system. As Asparagine is hydrolysed to Aspartic acid and Glutamine to Glutamic acid, the reported amount of these acids is the sum of those respective components. Samples were analysed in duplicate and average values were reported.

5.2.3.3. Antioxidant capacity

The total antioxidant capacity of the samples was measured at the Australian national measurement institute (NMI) using the oxygen radical absorbance capacity (ORAC) assay, which provides a measure of scavenging capacity directed at the biologically prevalent peroxyl radical, a common reactive oxygen species (ROS). Both ORAC(hydro) representing the water-soluble

antioxidant capacity and ORAC(lipo) representing the fat-soluble antioxidant capacity of the samples were evaluated. The water-soluble vitamin E analogue Trolox was used as the calibration standard and the ORAC(hydro) and ORAC(lipo) results are expressed as µmol of Trolox equivalent per kilogram. The reported total antioxidant capacity is the sum of ORAC(hydro) and ORAC(lipo) values and is expressed as µmol Trolox equivalent per litre or kilogram.

5.2.3.4. Anti-inflammatory activity

The anti-inflammatory activity of the samples was assessed using an *in vitro* cell based assay. A bacterial lipopolysaccharide (LPS) was used to induce an inflammatory cellular state in the murine macrophage cell line, RAW 264.7. Inflammation was indicated by the cellular production of nitric oxide (NO) as measured using the Griess assay. A decrease in the production of NO (measured as nitrite) by a positive control or by keratin hydrolysate samples indicated anti-inflammatory activity.

Anti-inflammatory activity was measured with quercetin (Sigma Australia) as a positive control. The RAW 264.7 cells (American Type Culture Collection) were routinely cultured in RPMI-1640 media supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin (Life Technologies) and 10% v/v fetal bovine serum (In vitro) and grown at 37 °C and 5% CO_2 in humidified air. The anti-inflammatory assay was performed over three days. On day one, the cells were seeded into 96 well plates at a density of $5x10^4$ cells/well. 1000 ng/mL LPS in culture media was added to the cells with either the positive control (six point dose response of quercetin using a 0.3 – 100 µM concentration range) or samples (0.03 – 10 mg/mL on a weight basis (wt/vol)). The cells were cultured for 48 hours and on day three, NO secreted by the RAW 264.7 cells was measured in the cell media by the Griess reaction (50 µL of cell media was added to 50 µL Griess reagent (Sigma, Australia)). A standard curve was prepared with sodium nitrite and used to calculate the NO production in the cell media. All assays were performed in duplicate. Cell viability was also measured in response to the LPS, quercetin and keratin hydrolysate samples using the MTS CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, Australia) according to the manufacturer's instructions.

5.2.4. Determination of digestibility of the feather residue

The dried feather residue (0.5 g) was dispersed in 10 mL milli-Q water and the pH was adjusted to 2.5. The dispersion was incubated for 2 hrs at 37 °C after adding 2mg/mL pepsin (Sigma-Aldrich, St. Louise, MO, USA). Subsequently, the pH of the dispersion was adjusted to 8.0 and 2 mg/mL pancreatic trypsin (Novo 6.0 S, Type salt free, Novozymes, North Rocks, NSW, Australia) was added on to it. This was followed by 16 hrs incubation at the same temperature. After cooling to room temperature, the dispersion was centrifuged at 10,000g for 15 minutes at 4°C. Protein content of the supernatant was determined with the LECO analyzer. The digestibility was

expressed as the percentage protein released calculated in accordance with the following equation (Eqn 10).

$$\text{Protein released (\%)} = \frac{\text{Amount of protein in the supernatant}}{\text{Amount of protein in 0.5 g feather residue}} \times 100 \quad (10)$$

5.3. Results and Discussion

5.3.1. Functional properties of keratin hydrolysates

The functional properties of the two feather hydrolysates are presented in Table 10.The hydrolysate from the ultrasonic assisted process was slightly different in appearance from that of the sulphite process. It was brownish and heterogeneous with dark brown particles, which may be due to Malliard reaction facilitated by ultrasound (Figure 46).

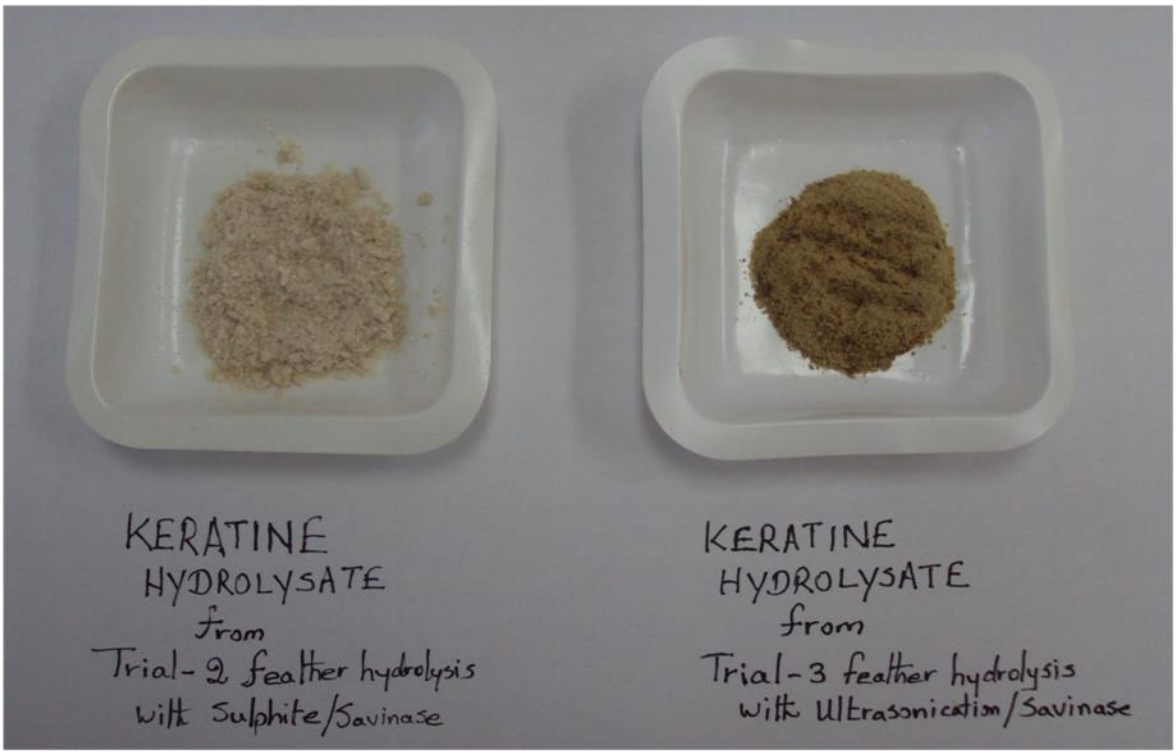


Figure 46 Pictures of purified keratin hydrolysates (80% peptide) produced by sulphite and ultrasonic assisted hydrolysis of poultry feather

Nevertheless, both hydrolysates were highly soluble in water with solubility comparable to that of whey protein isolate and much better than soy protein isolate (Table 1). The foaming capacity of both hydrolysates was very high. It was much higher than all the protein ingredients evaluated including egg albumin, which is commonly used in applications that require high foaming capacity and stability. However, the stability of the foams of both hydrolysates was substantially lower than that of egg albumin. The very high foaming capacity of the hydrolysates makes them especially useful for applications in shampoos and conditioners where high foaming capacity and not

necessarily stability is required. The emulsifying capacity of the hydrolysate from the sulphite assisted process was comparable to that of whey protein isolate and soy protein isolate. On the other hand, the emulsifying capacity of the hydrolysate from the ultrasonic assisted process was lower probably due to the non-homogeneity of the product. Overall, the functional properties of the hydrolysates are comparable to the common protein ingredients evaluated. Thus, they have a potential to be used in applications where these ingredients are used for their physical functionality.

Table 10 Comparison of the functional properties of purified keratin hydrolysates (80% peptide) with common protein ingredients

Functional properties	**Keratin hydrolysate from sulphite assisted hydrolysis**	**Keratin hydrolysate from ultrasonic assisted hydrolysis**	**Reference samples**
Solubility (%)	99.3 ± 0.4	99.2 ± 0.6	Whey Protein Isolate:100, Soy Protein Isolate: 39.2
Foam Capacity at pH 7.0 (mL foam/mL liquid)	15 ± 1.10	13.3 ± 1.15	Whey Protein Isolate: 1.86 ± 0.3, Soy Protein isolate: 0.1, Egg Albumin powder: 8.9 ± 0.3
Foam Stability at pH 7.0 (mL/mL liquid)	0.07 ± 0.01	0.04	Whey Protein Isolate: 0.09 Soy Protein Isolate: 0.02 Egg Albumin powder: 0.39
Emulsion Capacity at pH 7.0 (g oil/mg protein)	1.15 ± 0.1	0.42 ± 0.08	Whey Protein Isolate: 1.25 Soy Protein Isolate: 1.00 ± 0.1
Emulsion stability at pH 7.0/24 hrs (%)	At 3:1 protein : oil 76.9 ± 0.8 At maximum capacity 32.9 ± 2.6	At 3:1 protein : oil 74.6 ± 0.7 At maximum capacity 40.6 ± 0.8	

Note: Beta-lactoglobulin enriched whey protein isolate with 90% protein was obtained from Murray Goulburn. Soy protein isolate Supro® EX 33 IP with 90% protein was obtained from Solae. Egg Albumin Powder- High Whip (80% protein)

5.3.2. Biological properties of keratin hydrolysates

The non-purified keratin hydrolysates from the two processes exhibited similar peptide profile except that more of the larger molecular weight (4000 to 5000 Dalton) peptides were found in the hydrolysate from the sulphite assisted process (Figure 47). After the separation step, the hydrolysate from the sulphite assisted process lost the low molecular weight peptides (~200 to 1500 Da) (Figure 48). This may have occurred during the nanofiltration step using 500 Da membrane to remove the added sulphite. A 245 Da membrane was used for the nanofiltration of the hydrolysate from the ultrasonic assisted process, which may explain the higher retention of the low molecular weight peptides.

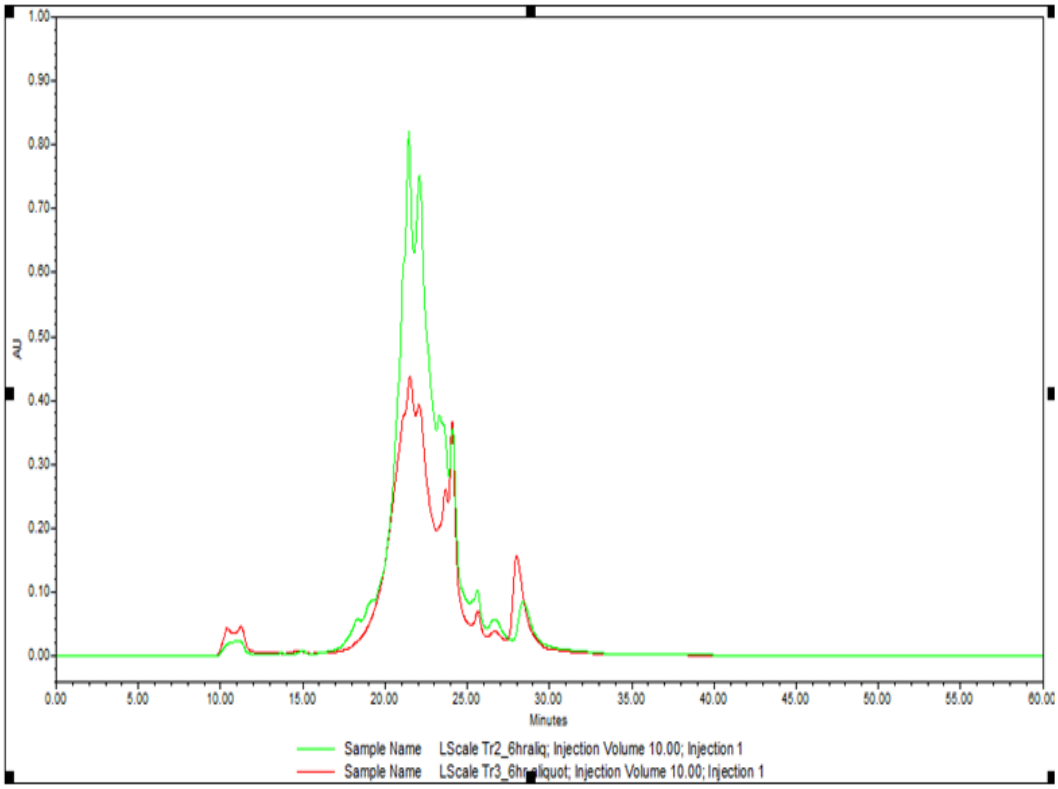


Figure 47 Comparison of peptide profile of non-purified hydrolysates produced by sulphite assisted (green) and ultrasonic assisted (red) hydrolysis of poultry feather

Both hydrolysates had similar amino acid profile containing all essential and conditionally essential amino acids (Figure 48). The hydrolysate from the sulphite assisted process contained about 2.5 times cystein compared to the hydrolyaste from the ultrasonic assisted process, which can be attributed to the higher degree of cleavage of disulfide bonds during the sulphite assisted process. Thus, the hydrolysates can be potentially used as high quality protein ingredients for pet food application, although this will be dependent on the cost of the final product.

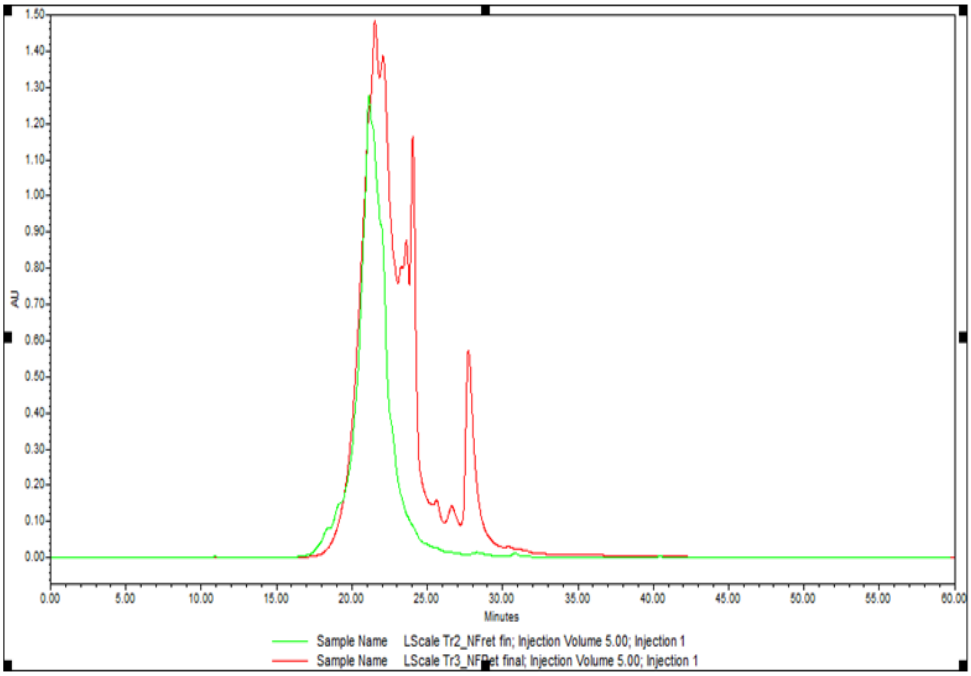


Figure 48 Comparison of the peptide profile of purified keratin hydrolysates produced by sulphite assisted (green) and ultrasonic assisted (red) hydrolysis of poultry feather

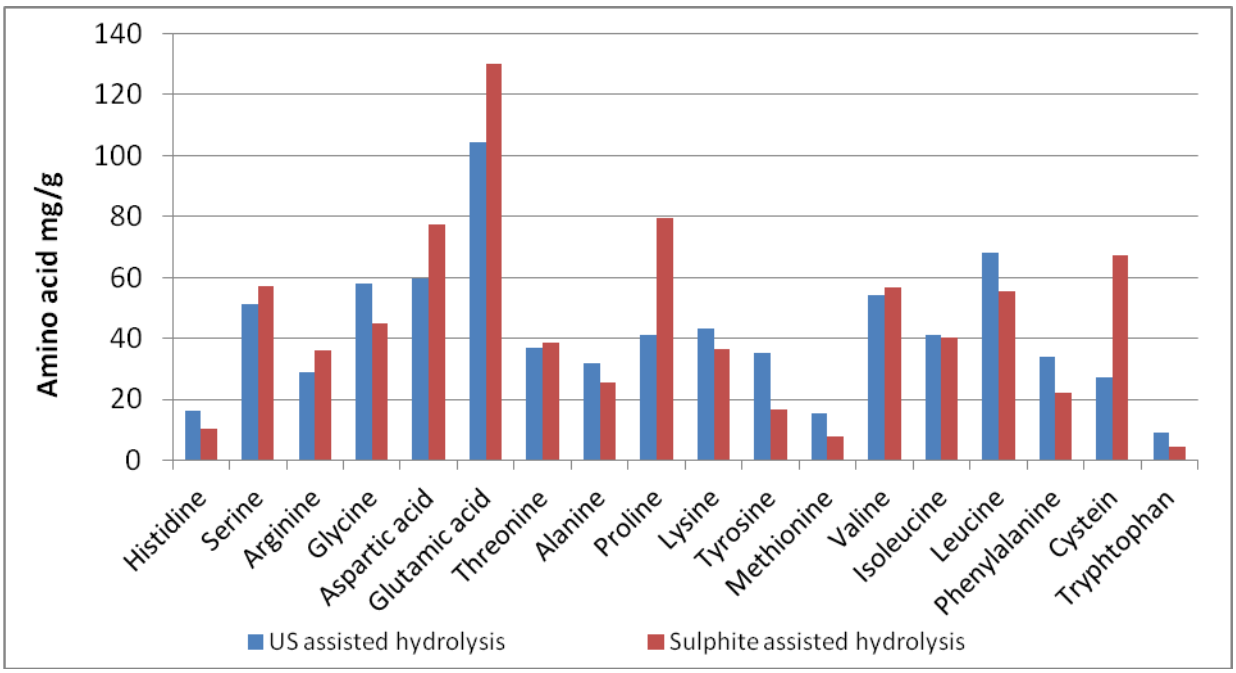


Figure 49 Comparison of the amino acid profile of the keratin hydrolysates produced by sulphite and ultrasonic assisted hydrolysis

The hydrolysates exhibited very high antioxidant capacity values, which are higher than that of dried strawberries (Table 2) and can potentially be used as ingredients in pet food, nutraceutical and cosmeceutical products. The antioxidant capacity of the hydrolysate from the ultrasonic assisted process was higher probably due to the Maillard reaction products, which have antioxidant properties as well as the presence of low molecular weight peptides. The keratin hydrolysate from

the sulphite assisted process suppressed the production of nitric oxide in a dose dependent manner indicating that it has anti-inflammatory activity whereas the hydrolysate from the ultrasonic assisted process did not have such effect (Figure 50). The EC50 i.e. the dose at which 50% suppression of NO production is inhibited compared to the negative control was 1.6 mg/ml. The EC50 for the positive control quercetin was 9 µM. The anti-inflammatory activity of the hydrolysate from the sulphite assisted process indicates that it has therapeutic effects and augments its value as an ingredient in nutraceutical and cosmeceutical products.

Table 11 Total antioxidant capacity and anti-inflammatory activity of purified (80% peptide) keratin hydrolysates

Antioxidant capacity (µmol TE/Kg)	Keratin hydrolysate from sulphite assisted hydrolysis	Keratin hydrolysate from ultrasonic assisted hydrolysis	Reference sample
ORACVit E equiv. (hydro)	483410	574370	
ORACVit E equiv.(Lipo)	760	260	
ORAC Vit E equiv. (total)	484170	574630	Dry Strawberry: 441000
Anti-inflammatory activity	Yes EC50=1.6 mg/ml	No	Quercitin EC50=9 µM

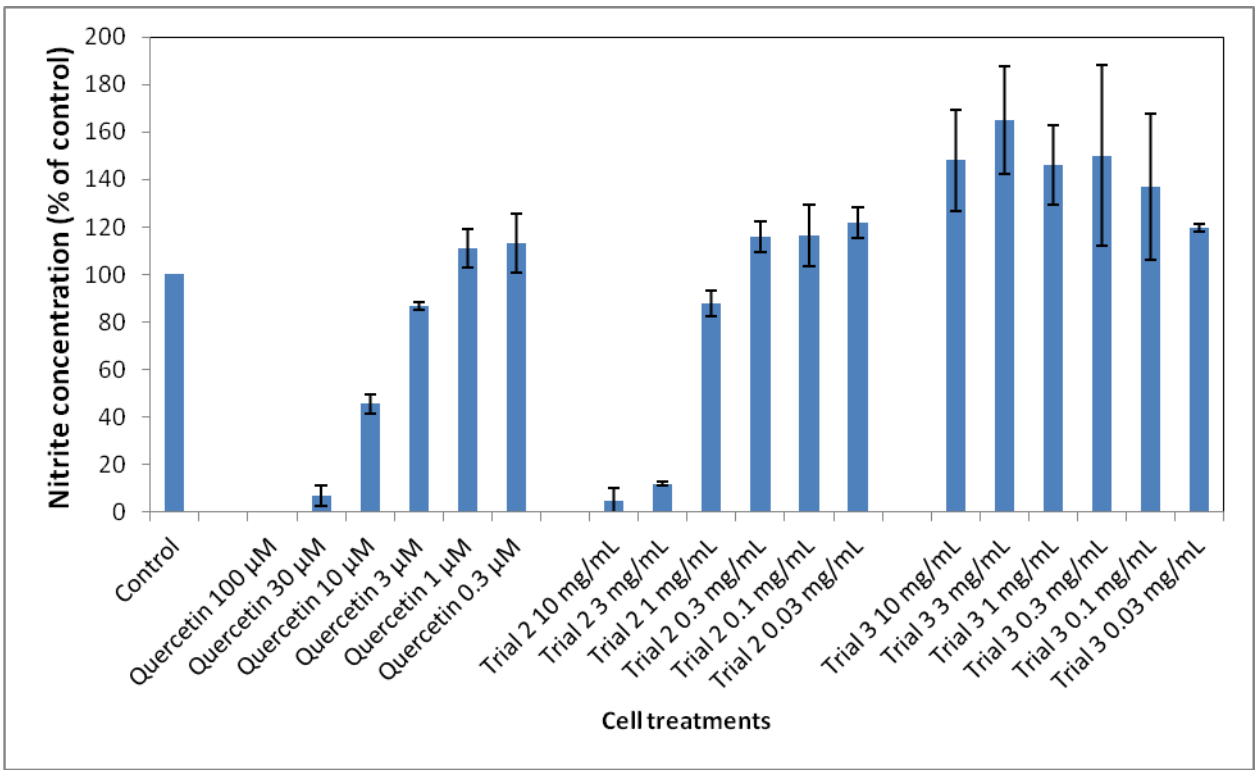


Figure 50 The effect of keratin hydrolysates and quercetin on nitric acid production by RAW 264.7 cells in response to stress induced by a bacterial lipopolysaccharide (LPS). Control: sample with only LPS, Trial 2: sulphite assisted keratin hydrolysate, Trial 3: US assisted keratin hydrolysate.

5.3.3. Digestibility of Feather residue

The unhydrolysed feather residues from the two processes showed very low digestibility compared to feather meal. In fact, the hydrolysis processes in both the sulphite assisted and ultrasonic processes had a negative impact on the digestibility of the remaining feather as poorer digestibility was observed for these samples compared to raw feather (Figure 51). Further processing (thermal, hydrothermal) would be required to use these residues in animal feed applications. Subjecting the feather residues to the existing feather meal production process may improve digestibility. Other potential applications for the residues such as in polymer composites or as a source of natural fibres could also be explored.

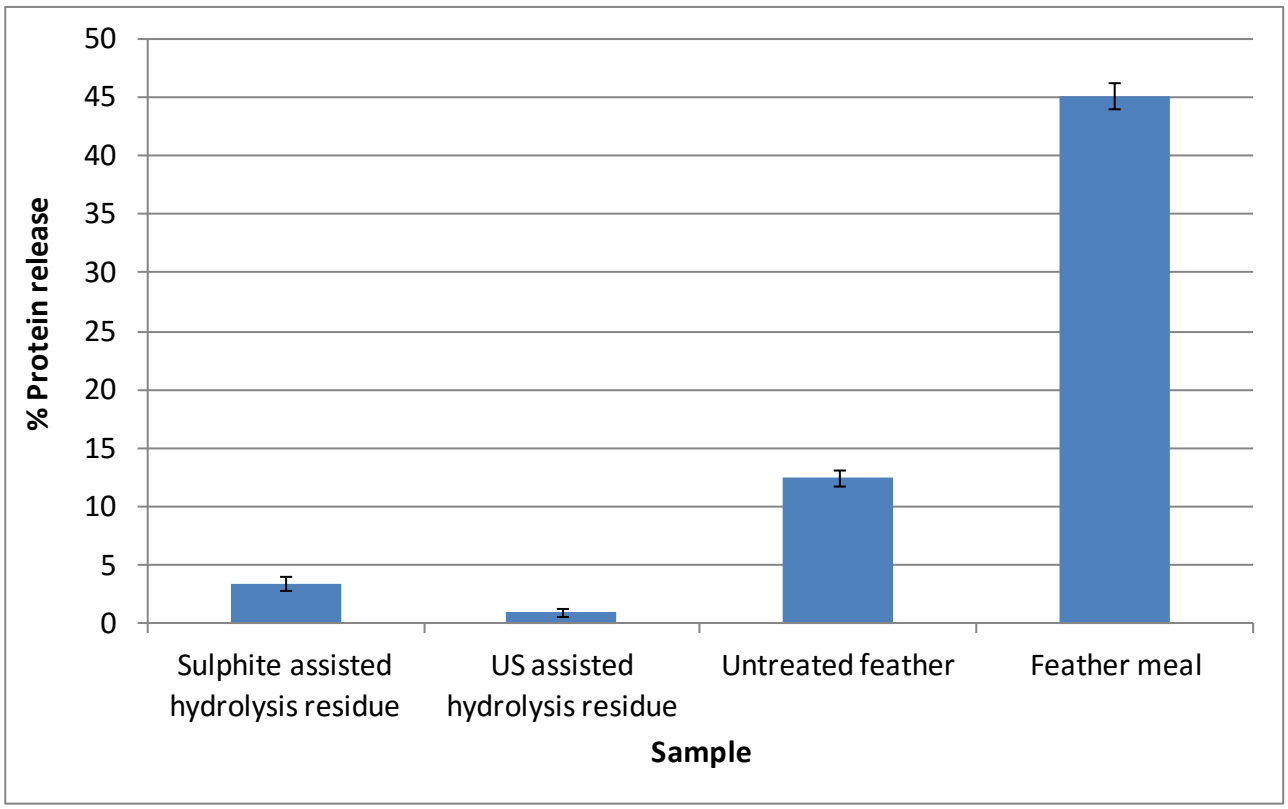


Figure 51 Comparison of the in vitro digestibility of residues from the sulphite and ultrasonic assisted hydrolysis of poultry feather with that of feather meal

5.4. Conclusion

Two types of keratin hydrolysates were produced using sulphite and ultrasonic assisted enzymatic hydrolysis of feather developed in milestone 4. The functional properties of the purified (80% peptide) hydrolysates were evaluated and compared with conventional protein ingredients. Overall, the two hydrolysates showed comparable or better functional properties than the conventional protein ingredients investigated except in foam stability. Both have very high solubility in water comparable to that of whey protein isolate and much better than soy protein isolate and much higher foaming capacity than all the protein ingredients evaluated. For instance, the foaming capacity of the hydrolysate from the sulphite assisted hydrolysis was about 150 times that of soy

protein isolate, 8 times that of whey protein isolate and about 1.7 times that of egg albumin, an ingredient that is commonly used for its high foaming capacity and stability. The foam stability of both products was the same order of magnitude as that of whey protein isolate and soy protein isolate but lower than that of egg albumin. The very high foaming capacity of the hydrolysates makes them especially useful for applications in shampoos and conditioners where high foaming capacity and not necessarily stability is required. The emulsifying capacity of the hydrolysate from the sulphite assisted process was also comparable to both whey protein isolate and soy protein isolate. The result indicates that the keratin hydrolysates have a potential to be used as an alternative to conventional protein ingredients. The product from the sulphite assisted hydrolysis showed a relatively better foaming and emulsifying properties than the hydrolysate from the ultrasonic process probably due to its homogeneity.

Both hydrolysates had similar amino acid profile containing all essential and conditionally essential amino acids. Thus, the hydrolysates can be potentially used as high quality protein ingredients for pet food application. The hydrolysates also exhibited very high antioxidant capacity values, which are higher than that of strawberries on dry weight basis and can potentially be used as ingredients in pet food, nutraceutical and cosmeceutical products. The antioxidant capacity of the hydrolysate from the ultrasonic assisted process was significantly higher probably due to the Maillard reaction products, which have antioxidant properties. Moreover, the keratin hydrolysate from the sulphite assisted process has anti-inflammatory activity indicating that it has therapeutic effect, which augments its value as a cosmeceutical and nutraceutical ingredient.

The unhydrolysed feather residues from the two processes showed very low digestibility compared to feather meal. Further processing (thermal, hydrothermal) would be required to use these residues in animal feed applications. The existing feather meal production process may improve the digestibility of these residues. Exploring other potential applications of the residues in applications such as polymer composites or as a source of natural fibres would also be useful.

Chapter 6 Potential industrial applications of feather keratin hydrolysates

6.1. Introduction

In chapter 5, we investigated the functional and biological properties of keratin hydrolysates produced from poultry feather. The objective of this milestone was to explore the potential commercial applications of these hydrolysates. The data obtained in chapter 5 on the functional and biological properties of the hydrolysates were used as a starting point for evaluating the potential applications of the hydrolysate in the pet food and cosmetics industries.

6.2. Methodology

6.2.1. Background literature review

A focused background review of publicly available literature including patents, reports and product release information was conducted using ISI Web of Science, Derwent innovation index, Google scholar and other resources to assess the potential application areas of feather keratin hydrolysates and market trends.

6.2.2. Market survey

A preliminary market survey was conducted in a small scale to identify potential market for the peptide ingredient produced by CSIRO Food and Nutrition, from poultry waste. A questionnaire consisting of eight questions related to peptide ingredients derived from keratin, was prepared (see Appendix II). The questions were prepared mainly to reflect any concerns (such as health, cost, and functional property) about using poultry feather based keratin peptides and also to obtain information on potential application and marketability. Nine pet food companies and thirteen cosmetics companies were randomly selected by performing a web search. A list was prepared which included the names of the companies, their contact address, phone no. and the name of contact person (Tables 1 and 2 in appendix II). The survey was conducted initially by sending emails to the companies, which briefly explained the objectives of the project, with the attached questionnaire. This was then followed up by a telephone interview with some companies. Project team members conducted this survey. Although the product can potentially make an excellent supplement to animal feed, we did not contact feed companies since the cost of the product is quite high.

6.3. Results and discussion

6.3.1. Background literature review

The largest market for protein ingredients is the food industry. The key end-user groups in the food industry include: animal feed, pet food manufacturers, meat product manufacturers, bakery manufacturers and the dairy industry. Although, protein ingredients are primarily nutrients for

growth and well being, in the food industry, they are also used for their functionality as emulsifiers, gelling, foaming, whipping, coating and palatability agents for different applications depending on the specific requirement of the end product. Protein hydrolysates are utilized in a wide range of applications for their nutritional, functional and bioactive properties [54,55]. The waste derived hydrolysates have an advantage in that they can cost-effectively replace the protein source in the market while imparting compatible functional property and also contribute to green technology by utilizing and adding value to waste.

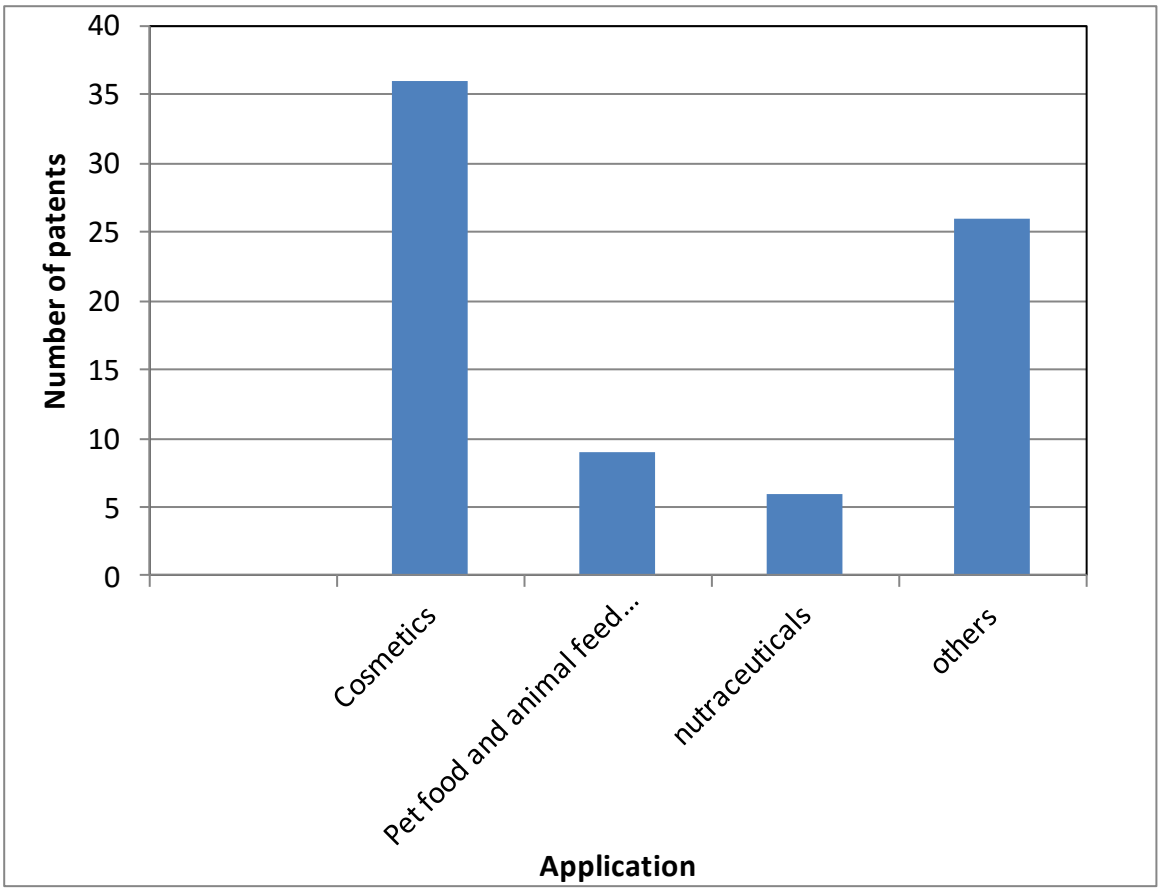


Figure 52 Preliminary patent review showing the number of patents published since 1985 on the application of keratin hydrolysates in different products.

With respect to keratin hydrolysates, the main application categories based on the patents published over the last 30 years are cosmetics followed by pet food and animal feed supplements and nutraceuticals (Figure 52). The cosmetic applications are mainly for hair treatment products including hair straightening formulas as well as shampoos and conditioners. Interestingly, there are a few patents describing the use of keratin hydrolysates in human food as seasoning and for imparting specific flavor and mouth feel to the food product. Other patented applications include personal care products such as laundry detergents, biomedical applications such as wound dressing, insecticides, fungicides, fire extinguishing foams, biopolymers, synthetic fabric and leather processing. Since the main application areas for keratin hydrolysates are in cosmetics and pet food and animal feed supplements, further discussion will be focused on these applications.

Cosmetics

The cosmetic industry is increasingly focusing on bioactive ingredients that naturally play a role in growth, maintenance and repair of human body to enhance natural processes. Proteins and protein hydrolysates are one of the bioactive ingredients that are increasingly used in cosmetic formulations such as hair, skin and other personal care products. Both proteins that have structural and functional similarities to those naturally present in hair, skin and cortex such as collagen, elastin and keratin and proteins from other sources (plant, microbial etc) with specialized functionalities are used in such products [56]. Most protein molecules are too large to penetrate the skin or hair shaft. Thus, the hydrolysed forms of these proteins are used to facilitate their accessibility. Peptides derived from collagen and other scleroproteins of animal tissues were the first and the most successfully used protein ingredients in modern cosmetics products, and, they continue to represent the major sources of protein derivatives for cosmetics in spite of the decreasing popularity of animal ingredients in the last decade. Besides collagen, other low cost and largely available materials such as keratin, elastin and raw extracts from animal tissues/organs such as thymus, placenta, heart and bone marrow are also used. In hair care products such as shampoos, hydrolysed proteins are capable of interacting with anionic surfactants to form tightly bound protein-surfactant complexes. The defatting potential and irritation of the surfactant is greatly reduced by the complexes. Protein hydrolysates from various sources used in skin and hair personal care products are known to confer improved compatibility, feel, and moisturisation and help maintain the natural structure. The active peptides in protein hydrolysates are efficient restorers in hair care processes and provide benefits for the hair such as strengthening hair fibres and reducing fibre breakages [6]. With respect to skin care, one study [57] showed that wool keratin hydrolysate stimulates human keratinocyte migration and the expression of collagen IV and VII in humans indicating its potential use for fast wound healing as well as anti-wrinkle treatment. Wheat protein [58] and keratin from wool [59] are some examples of protein hydrolysates from plant and animal source respectively that are currently used in hair and skin care products.

Two categories of peptides are used in cosmetic formulations i.e. Small peptides with molecular weight less than 1300 Da and large peptides with molecular weight between 1000 and 5000 Da. The two groups of peptides have distinct functionality. The smaller peptides are able to penetrate the skin and/or hair cuticle and are useful as a rebuilding treatment of the hair capillary fibre [7,56] On the other hand, higher molecular weight peptides have the film forming behaviour due to their size. With surfactant systems such as in shampoos this behaviour translates into more stable foam structures [60]. In addition, such peptides are believed to have moisturising and shining effect as well as give body to fine hair [56]. The keratin hydrolysate that we developed using sulphite assisted hydrolysis has peptides with molecular weight ranging from 4000 to 5000 Da and falls into the second category i.e. potential to form films which improve shine and feel. On the other hand, the hydrolysate produced without the use of sulphites is composed of large peptides with molecular weight ranging from 4000 to 5000 Da and small peptides with molecular weight ranging from 200

to 1500 Da and is expected to have both functionalities. Both products also have very good foaming and emulsifying properties, which are useful in the formulation of cosmetic products. The high antioxidant capacities of the hydrolysates and the anti-inflammatory activity observed for one of the hydrolysates also complement the other functional attributes of the hydrolysates for use as cosmetic ingredients.

Keratin hydrolysates have become popular in recent years. Apart from the expensive Salon brands, many of the supermarket shampoo and conditioner brands including 'Tresemme', 'Paneten', 'Schwarzkopf', and l'Oreal's 'Elvive' have at least one formulation that contains keratin hydrolysate as an ingredient. These keratin hydrolysate ('liquid keratin') containing formulations are claimed to have several benefits including repairing hair cuticle, reconstructing the hair surface, and reducing hair damage. There are also many keratin based hair straightening and waving treatments in the market. AC Keratin Hydrolysate-30 is a recently commercialised 'hydrolysed keratin solution', containing hydrolysates in the 1000 – 3000 Da molecular weight range. According to the manufacturers, due to its large molecular size, it does not penetrate the skin or hair, but rather plate out to form tenacious films, resulting in increased shine and improved feel. It forms a complex with anionic surfactants in shampoos, which makes foams last longer and reduce surfactant induced irritation. Table 12 summarises some of the keratin hydrolysate products in the market for cosmetic application. As can be seen, most of these products are made from wool. This could be due to the large amount of information available in the literature on the beneficial effects of wool keratin hydrolysates on hair and skin. More research has been conducted on wool keratin compared to feather keratin [61], probably due to the higher cystine content of wool keratin, which is believed to be useful for augmenting the cystine content of damaged hair and enable the formation of disulfide bond with the hair shaft. Tsuda and Nomura [61] prepared feather keratin hydrolysates by alkaline hydrolysis and compared its properties and effect on hair shaft with that of a commercial wool keratin hydrolysate (Primose®WK). The feather keratin hydrolysate had an average molecular weight of 767 Da compared to the 386 Da of the wool keratin hydrolysate. The wool keratin hydrolysate had a higher percentage of cystine (4.4%) compared to feather keratin hydrolysate (1.2%), whereas the methionine, another sulphur containing amino acid, contents were similar in both. The proportion of hydrophobic amino acids was higher in the feather hydrolysate (53.5%) compared to that of the wool hydrolysate (44.3%). It has to be noted that the amino acid profiles of the hydrolysates is also dependent on the method of hydrolysis. For instance, we observed much higher cystine content in hydrolysates obtained using sulphite assisted hydrolysis compared to the hydrolysates without the use of sulphites (milestone 5). In terms of beneficial effects for hair treatment, the feather hydrolysate was more effective in restoring the surface hydrophobicity and the tensile strength of bleached hair compared to wool keratin hydrolysate. In addition, the feather keratin hydrolysate penetrated better into the hair fibres better than the wool keratin hydrolysate despite its larger average molecular weight, which was attributed

to the larger proportion of hydrophobic amino acids in the feather hydrolysate [61]. This study suggests that feather keratin hydrolysates are at least equally suitable as wool keratin hydrolysates for application in cosmetics.

The global market for cosmetic and toiletry ingredients was valued at $18 billion in 2011 and expected to reach US$24.5 billion by 2018. Among these ingredients, the global market of active ingredients that includes peptides will reach US$2.7 billion in 2018 [62]. We were not able to obtain specific market trend and market volume data on keratin peptides. According to a market research report by Business Analytic centre [12], there were three companies in Europe, seven in Asia (Japan and China) and 11 in North America in 2012 that manufacture keratin products. The retail price for the wool based products ranged from US $120/kg to US $651/500 gm. The users of these products are hair care, skin care and wound care products manufacturers. The report lists seven companies in USA, four in Europe (France, Germany and Sweden), three in Asia (China, Korea, Thailand), two in Brazil and one in New Zealand that are using keratin peptides in their hair care and other products [12]. The list does not seem to be exhaustive. There are at least two suppliers of Keratin peptides in Australia (TRI-K and Jomar Bioscience P/L), indicating that there are companies using keratin peptides as ingredients in their products in Australia.

Table 12 Examples of commercial keratin hydrolysates for cosmetic application

Product	Molecular weight range	Raw material	Application/ claimed benefits	Manufacturer/Supplier
AC keratin hydrolysate 30 FSS Keratin hydrolysate 30	~2000 Da, aqueous solution	Not specified	Forms film on hair, improves shine and feel, form complex with anionic surfactants, reducing irritation and defatting from surfacatant alone and improve foam stability	Active Concepts, Formulator Sample Shop
Hydrolysed keratin protein	1,100 – 3,300 Da, liquid with 20-23% protein	Sheep wool	Revitalises hairs protective layer, rebuilds tensile strength, returns elasticity and reduces breakage, protective care substance for	MakingCosmetics

			skin	
Nutrilan® keratin W PP	3000 – 5000 Da, aqueous solution, 15-25% protein	Sheep wool	Hair and skin protective care	Cognis GmbH and Cognis GmbH Care Chemicals, Germany
Crotein ™ Cashmere	Liquid, 150 Da, amino acids with some di and tripeptides	Cashmere wool	Penetrates the cuticle, helps to prevent breakage and split ends. Suitable Hair, skin and other personal care products	Croda Personal care, UK
KERA-TEIN 1000	Liquid, 30% solids	Not specified	useful for treating damaged hair by permanent waving, increases the amount of cystein available for hair, moisturises hair and skin	Tri-K Industries
Cashmilan® LS 9604	20% solution, ~7000 Da	Cashmere wool	Repairing damaged hair, has conditioning and softening effects	Laboratoires Serobiologiques, France
Keramois L	Liquid, 25% protein, 1000 Da	Not specified	Protects hair from damage during permanent wave treatment, bleaching and dyeing	IKEDA corporation
Hydrolysed keratin powder	Powder (≥85% protein),	Not specified	Hair care, skin care and other personal care products	Spec-Chem Industry, China
Promois WK-GB	Liquid, 18-22% protein, 10000 Da	Sheep wool	Beneficial effect on skin and hair, mild to use	R.I.T.A corporation

Pet food and animal feed supplement

The main ingredients that are used in pet food are animal products such as meat, meat and chicken by-products such as meat meal and bone meal, cereals such as corn and rice, cereal by-products such as wheat germ meal and soybean meal and animal fat. The increased demand for food, feed and fuel production is currently putting pressure on the price of raw materials that are used in pet food production. Thus, pet food manufacturers are looking for alternative protein sources for use in their pet food formulations. One of these is poultry feather. Sonac, a leading supplier of ingredients derived from meat processing by-products, recently developed Kerapro, a feather hydrolysate which has better digestibility and bioavailability than feather meal. According to the company, this product is also hypoallergenic and suitable for pets with allergic reactions to other proteins [63]. Recently, Royal Canins, a pet food company based in USA, has introduced a dog food that uses feather hydrolysate as the main protein ingredient in its 'anallergenic' formula suitable for dogs with allergic reactions [64]. It seems that the company is using the feather hydrolysate based formulation that was recently patented by its parent company Mars pet care (UK), which is composed of low molecular weight amino acids, L-oligopeptides and feather meal. It is claimed that the product improves the performance of a dog during exercise or improves recovery after exercise [65].

The keratin hydrolysates that we developed in this project are rich in essential and conditionally essential hydrolysates. This together with their very good functional properties makes them excellent candidates as pet food ingredients as well as animal feed supplements. The high antioxidant capacity and the anti-inflammatory activity of the products will also make them useful as nutraceutical supplements, which are becoming important segments in the animal nutrition [66] and pet food industries [67]. The global market for pet food nutraceuticals is estimated to reach nearly US$6 billion by 2020. The cost of our hydrolysate could be the main obstacle in these applications. However, the degree of purity required by the pet food or animal feed supplement industry may not be as high as that of cosmetics. Thus, the crude hydrolysates may be used in such applications, which would significantly reduce the cost of the products. Companies are also willing to pay higher for functional ingredients with demonstrated benefits. For instance, Royal Canin is paying a price higher than the price of chicken meat for the hydrolysed feather ingredient they are using in their 'anallergenic' dog food formula [64].

In terms of market trend, the fastest growing segment in the food sector is the pet food industry which is driven by increasing number of people adopting pets and consumer attitudinal change towards pets with interest on their nutritional and health. The global pet food market with a cumulative average growing rate (CAGR) of 4.5% is estimated to be valued at $86.3 billion by 2020. The global market is dominated by animal derivatives, which accounted for approximately 49.6% of the global pet food ingredients market in 2013. Solid and steady growth is expected in the

pet foods ingredient market [68]. The pet food industry accounts only 12% towards value based foods with rest accounted for by general pet foods. The trend in consumer's demand for pet foods enriched with functional ingredients such as vitamins, minerals, probiotics for boosting their pets joints health, coat and heart health are an indication for expected growth in the value based pet nutraceutical market [67].

6.3.2 Market survey

The preliminary market survey was conducted over a wide spectrum of companies from small business (eg., Ali Hamylton Cosmetics) to multinationals (eg. Afb international Pty Ltd). We received promising responses to the questionnaire from five of the twenty two companies two from the Pet food and three from the Cosmetic companies (see Tables 1 and 2 in Appendix II). All five companies are globally recognized market leaders. Unfortunately, some companies provided negative response immediately either due to lack of awareness of peptide ingredients, lack of interest or time. With some companies, there were delays since they had to approach the formulators for the response. Most of the time, the response from the formulators were not favorable.

Profiles of the three companies

- Pet food company 1 is Australia's premier producer of fresh chilled pet foods. They are deeply committed to the pet food industry and pet welfare. In the past 13 years the company has achieved the status of market leader in the fresh chilled pet food segment in not only Australia, but around the world.
- Pet food company 2 is a global company with world-wide state-of-the-art facilities including Australia. The company is the global science and technology leader in pet food palatability. Ensuring product performance for pet food manufacturers and their customers is their business.
- Cosmetic company 1 is a motivated, dynamic company specialising in personal care & cosmetic ingredients. They have partnered with market leading suppliers whose focus is innovation, sustainability, quality and technical service. The company is able to offer into the Australian and NZ market a comprehensive range of actives and functional ingredients with a vision to providing exceptional customer service and technical focus.
- Cosmetic company 2 is a cosmetic company specialising in a wide range of organically certified skin care, hair care, body care products and aromatherapy oils. Each group of product is further divided into sub-groups depending on its application (eg. Dry skin, anti-oxidant, anti-aging, moisturising etc) to meet the customers' need. A major priority in the creation of the company was to ensure the healthy and equitable sourcing of raw materials and to use manufacturing processes that leave the lowest possible environmental footprint. The company's products are currently available in a wide range of department stores and

quality organic retailers in Australia, Japan, Taiwan, Hong Kong and Singapore. Turkey and Russia are new markets that just opened in the first half of 2014.

- Cosmetic company 3 is a global company offering a broad range of proteins and derivatives from both vegetable and non-vegetable sources. They supply these ingredients to manufacturers. The company has implemented a major initiative called NPNF (non-paraben, non-formaldehyde), removing all parabens and formaldehyde donors from the liquid cosmetic proteins, to help meet the needs of customers in a safe and effective manner.

Response from the companies

Pet Food Companies

The detailed responses from the two pet food companies are given in Appendix II. In general, the two companies were aware of either poultry and/or feather-based peptide ingredients, and are presently using these to manufacture their end products. They had no concerns about using feather based peptide ingredients. Palatability of the feather meal and hydrolysed chicken was a main concern for the two pet food companies and they would like to have improved palatability in the feather peptide ingredients. The companies were not concerned about the nativeness of the peptide ingredients. They were not aware of the use of other keratin based peptides in their industry. The price range for a feather hydrolysate ingredient indicated by one of the pet food companies was $500-$1000/ton. The second pet food company is prepared to offer higher price for the feather meal if pallatibility and digestibility issues were resolved, since those are the main impediments to the use of feather meal in pet food formulations. The pet food market may not be suitable for the purified keratin hydrolysates from our process. However, a formulation consisting of feather meal and the crude form of our hydrolysates for improved palatability, bioavailability and bioactivity may be considered for this application.

Cosmetic Companies

The detailed responses from the three cosmetic companies are given in Appendix III. One of the three cosmetic companies (cosmetic company 1) was aware of feather-based keratin peptide ingredients, and is presently promoting them to their customers. The second cosmetic company (cosmetic company 2) was not aware of poultry feather based peptides, but they use wheat and almond protein hydrolysates and other ‘active’ ingredients to manufacture their end products. They did not mention the source of the ‘active’ ingredients. They said some ingredients were imported from China. They suggested that if the right ingredients can be locally and sustainably produced, it will be strongly encouraged. They requested for a sample of our product for testing. The two cosmetic companies had no concerns about using/selling feather based peptide ingredients as long as it was safe, environmentally friendly, complied with the Australian manufacturing practice and met their customer needs. The color of the ingredient was a critical factor for them, preferring

white or light color. Both companies were not concerned about the peptide ingredients' nativeness, although their preference was dependent on the choice of their customers. Only one of the two cosmetic companies (cosmetic company 1) was aware of other keratin based peptides. Only one of the companies (cosmetic company 1) indicated the price range for a functional peptide ingredient as $200.00/kg to $900.00/kg. The substantially higher price range compared to that indicated by the pet food company could be due to the quality of the ingredients that are generally used in high end cosmetic products. The second cosmetic company did not indicate a price range, but when asked they replied that $50/kg for the peptide was expensive but may be acceptable for high end products.

The third cosmetic company (cosmetic company 3) did not respond to the questionnaire specifically due to lack of time. However as a general comment they mentioned that they distribute a range of protein hydrolysates derived from many sources that have value in their use as cosmetic actives for skin and hair. Further they are finding increased resistance to materials that are animal derived, but also stated that this is not exclusive. They have sent their company brochure which lists all the protein hydrolysates supplied by them, which includes keratin hydrolysates. The company, which is based in USA, manufactures and distributes a range of keratin hydrolysate products worldwide [12]. None of the five companies specifically disclosed the market volume for peptide ingredients or keratin peptides; however, they envisage an increase in sales and demand over the next three years.

6.4 Conclusions

Our background literature and patent review indicated that the main potential application areas for feather keratin hydrolysates are cosmetics, pet food and animal feed supplement industries. The cosmetic ingredient market is a high value market with strong growth projected over the coming five years. Keratin hydrolysates, due to the superior functional and biological properties they impart into the end-products, are increasingly used by cosmetic companies in range of products including skin care, hair care and nail care formulations. The purified keratin hydrolysates developed in this project suit best to this market, due to their high quality and relatively high cost of production. Our limited review showed that the keratin ingredient market for cosmetics is currently dominated by wool based products. However, this is expected to change with the increasing availability of scientific information on the efficacy of feather keratin peptides in hair and skin care applications. The high end nutraceutical pet food market is another potential market that should be targeted for the application of feather based keratin peptides. Petfood companies are currently exploring the use of keratin based hydrolysates as an ingredient to address specific needs such as hypoallergenicity and boosting health and performances of pets. In this case, the high purity required for cosmetic application may not be needed. In addition, formulations that include feather meal could be used for bulking reasons, which may lower the cost of the product.

Our small survey suggests that there is lack of awareness of keratin based peptides or lack of interest in ‘new ingredients’ by many of the Australian companies engaged in the manufacturing of cosmetics and pet foods or supply of ingredients for these industries. However, we could get encouraging response from companies who were aware of keratin or feather based peptide ingredients. The survey confirmed our observation based on the background review that cosmetic companies are willing to pay much higher price for keratin based ingredients compared to pet food companies. The survey also indicated that pet food companies are interested in better feather meal products with higher digestibility and bioavailability and are willing to pay higher for such products. Due to lack of time and resources, we were able to contact only 22 companies. Thus, the survey cannot be considered as representative. Thus, we strongly recommend detailed market survey be conducted to assess the potential market for feather based keratin peptides in the Asia-Pacific region as part of a future research and development activity.

Chapter 7 Preliminary cost benefit analysis of the process for the production of purified keratin hydrolysate from feather

7.1. Introduction

In milestone 4, we developed a process for the production and purification of high quality keratin hydrolysate from poultry feather. The objectives of this milestone were to conduct preliminary cost benefit analysis of the developed process and provide recommendations for a pilot scale trial. Two processing options were considered in the analysis.

- Process 1: Sulphite assisted feather hydrolysis
- Process 2: Feather hydrolysis without the use of sulphites

Ultrasonic assisted feather hydrolysis was excluded from the analysis since it did not have significant effect on the hydrolysis yield when Savinase 16L was used in the process (chapter 4) and since it did not have substantial positive effect on the functional and biological properties of the keratin hydrolysate produced (chapter 5). Moreover, although the use of ultrasonication will have minimal impact on the production cost, it will incur significant capital cost which is not justifiable by the benefit that is derived from it.

7.2. Methodology

7.2.1. Process Flow diagram

A generic process consisting of washing and milling, enzymatic hydrolysis, solid-liquid separation, microfiltration, nanofiltration and spray drying is proposed for the process based on chapter 4 (Figure 53), with some variations depending on the processing options considered. In all cases, the batch size was assumed to be 1000 kg of dry feather with two batches processed per day and 300 processing days per year. The feather residue after hydrolysis was assumed to undergo the commonly used hydrothermal process for the production of feather meal with no net profit from it. The cost of the raw feather was also assumed to be negligible.

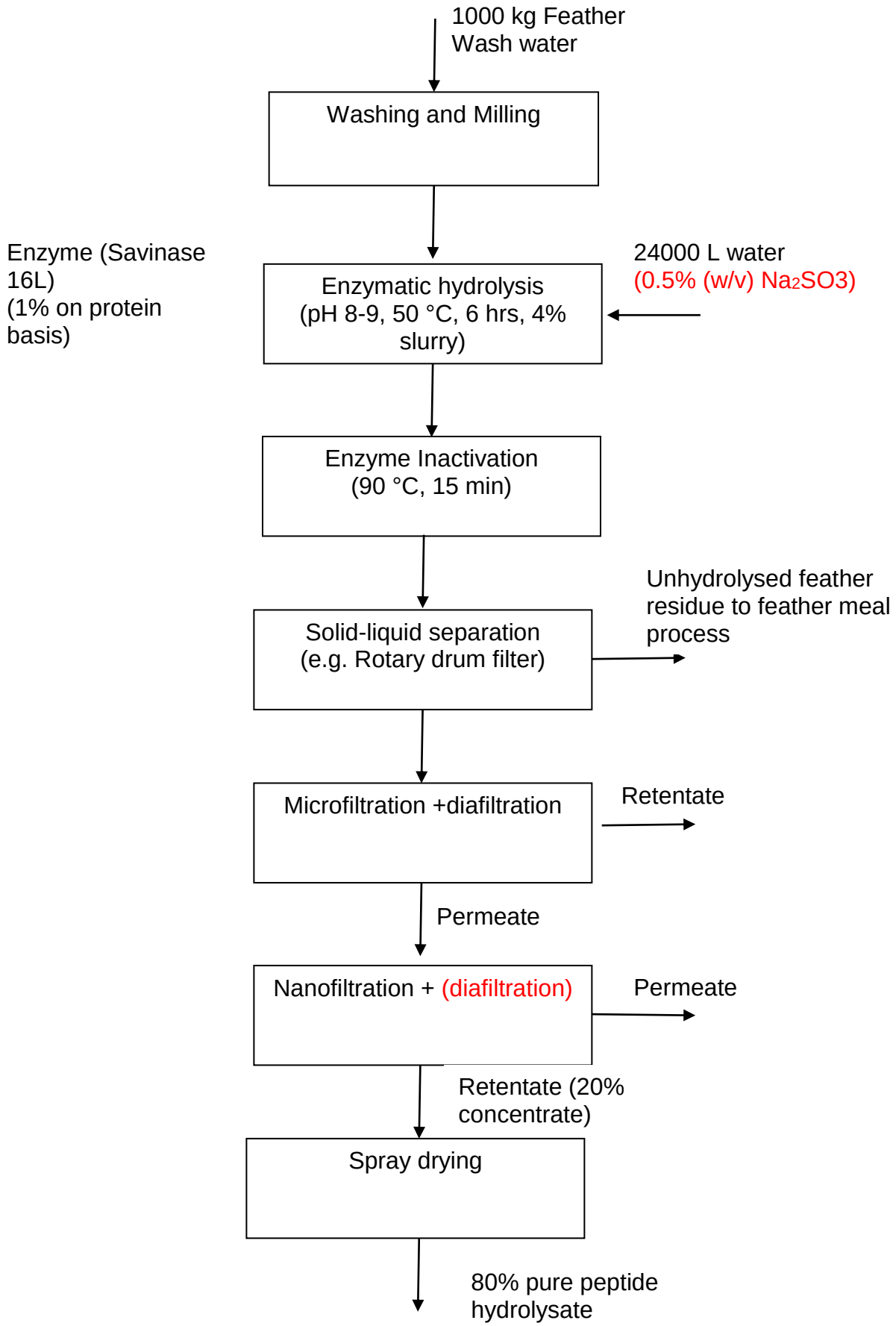


Figure 53 Block diagram showing the proposed generic process for the production of keratin peptides from poultry feather. Optional processing steps or inputs are presented in red.

7.2.2. Estimation of production cost

Process 1: Sulphite assisted feather hydroysis

Process 1 involved the use of 0.5% (w/v) sodium sulphite for facilitating the hydrolysis process since that resulted in the highest hydrolysis yield of 32% on protein basis after 6 hrs of hydrolysis. However, significant losses of the hydrolysate occurred during the separation process to remove the added sulphite from the hydrolysate and obtain a product with 80% purity. The separation process consisted of solid-liquids separation, microfiltration and nanofiltration steps to purify and concentrate the product to 20% solid. The laboratory scale separation steps resulted in only about ~32% recovery of the product on protein basis, which reduced the actual product yield based on raw feather to ~13%. The majority of the loss occurred during nanofiltration (~31%), followed by microfiltration (~23%) and the manual solid-liquid separation steps (screen+bag filtration ~14%). We assumed 13% yield in this analysis as the worst case scenario, since we would expect the yield to improve with scale up and further process optimisation. Some of the observed loss during microfiltration and nanofiltration is due to the hold-up volumes in the equipments. When small volumes are processed, such small losses will work out to be a large percentage of the total product volume whereas the percentage loss will be lower in larger scale operations.

Assuming 1000 kg of dry feather processed per batch, 2 batches processed per day, and 300 process days per year, the plant was assumed to have a capacity for processing 600 tons of feathers per year, with annual production of 78000 kg keratin hydrolysate per year. The bulk retail cost of the hydrolysate was assumed to be $50/kg for the costing analysis based on quotes we received from various suppliers at the beginning of the project. At the time, the quotes for the bulk price of high quality keratin hydrolysates ranged from US $52 to $131/kg.

In addition the following assumptions were made regarding the different unit operations in the process.

Washing and Milling

Two washing steps with 4 to 1 water to feather ratio (w/w). Rotary drum washer could be assumed for the washing step. Since the energy input required for washing is relatively low, it is neglected for this preliminary analysis considering that it will be covered by the contingency set aside.

The specific energy input for milling was estimated assuming size reduction of feather from 15 cm to 1 mm using Bond's law with (Eqn 1) assuming Bond's work index of of 2.41 kWh/kg [69]. The estimated value was of the same order of magnitude as the specific energy consumption measured during size reduction of biomass such as willow using Knife mill to the same degree [70].

$$E = E_i \left(\frac{100}{L_2} \right)^{\frac{1}{2}} \left[1 - \left(\frac{L_2}{L_1} \right)^{\frac{1}{2}} \right] \tag{11}$$

Where E is the specific energy input in kWh/kg, E_i the Bond's work index and L_1 and L_2 are the initial and final size of the material.

Enzymatic hydrolysis and enzyme inactivation

The specific energy inputs for the enzymatic hydrolysis and the enzyme inactivation steps were calculated based on the sensible heat required to heat the reaction mixture to the hydrolysis and inactivation temperatures ($Cp\Delta T$ where Cp is the specific heat capacity and ΔT the temperature differential), assuming the specific heat capacity of water (Cp=4184 kJ/kg.K). The heat loss during the holding phases was assumed to be negligible. Electrical energy is assumed for both processes, although steam could also be used for these operations. The cost of sodium sulphite was taken as $0.4/kg based on the higher end of the market value for the bulk product. The cost of the enzyme, Savinase 16L was taken as $20/kg in accordance with the data obtained from Novozymes Australia.

Solid-liquid separation

The solid-liquid separation in the laboratory scale trial was conducted manually using a screen and a bag filter. At an industrial scale, the process could be conducted using a sedimentation tank, a centrifugal separator or a coarse filtration system such as a rotary drum vacuum filter. Since we did not have a laboratory scale data to estimate the operating cost of such processes and since the operating cost is expected to be relatively low compared to microfiltration and nanofiltration, we did not include the operating cost of this unit operation assuming that it will be covered by the contingency set aside.

Microfiltration and Nanofiltration

The membrane areas required for the microfiltration and nanofiltration operations were calculated based on the laboratory scale flux data of 130 kg/m^2.hr and 42 kg/m^2.hr respectively allocating 8 hours each for microfiltration and nanofiltration. The cost of the ceramic membrane used for microfiltration was taken as $3,300/m^2 and the polyethersulphone membrane for nanofiltration as 100/m^2 based on data from the suppliers. The life of the membranes was taken as 2 and 5 years for polyethersulphone and ceramic membranes, respectively. Based on experience, the life of the ceramic membranes could be much longer. The energy input required for the microfiltration and the nanofiltration operations were calculated based on permeate volume calculated from the laboratory scale data assuming 10.5 kWh/m^3 for microfiltration and 5 kWh/m^3 for nanofiltration in accordance with the guideline in [71].

Spray drying

The electrical power and the steam required for drying was assumed to be 0.11 kW/kg of water evaporated and 1.5 kg/kg of water evaporated respectively for industrial scale operations [72]. A total of 10 hrs drying time was assumed per batch.

Utilities and Labour

The cost of power, steam and water were assumed to be 0.14/kWhr, $25/ton, and $1.2/kL respectively. The water for cleaning in place (CIP) was assumed to be 25% of the production water. Three shifts were assumed with 2 personnel per shift with $50,000+50% on cost per year. Product packing cost was assumed to be $2/10kg.

Contingency

A contingency of 50% of the total cost was added on the overall production cost. We used a high percentage contingency since this is a preliminary cost benefit analysis based on a laboratory scale process and historical data with significant uncertainties.

Process 2: Feather hydrolysis without the use of sulfites

This process is essentially the same as process 1 except that no sulphite was used in the hydrolysis process. Thus yield of hydrolysis was about ~21%. However, the hydrolysate was relatively more pure and did not need the 3 diafiltration steps during nanofiltration for purification to 80% pure product. Thus, the recovery on protein bases was about 51% with an overall yield on raw feather basis of ~13% based on the laboratory scale process. The 49% loss (on protein basis) consisted of 23% during the manual solid-liquid separation, 17.7% during microfiltration and 7.6% during nanofiltration. The recovery can be potentially improved at larger scale and through further process optimisation such as automation of the manual solid-liquid separation. Based on the worst case scenario of 13% recovery, the product yield is about 78,000 kg/year based on 600 ton of dry feather processed per year. The costing of each unit operations was similar except that no sodium sulphite was used in the process and the cost of the nanofiltration process is slightly lower since there were no diafiltration steps in this case. The product cost is assumed to be the same although this product would have a higher concentration of low molecular weight peptides, which are useful for certain cosmetic applications. The low molecular weight peptides are able to penetrate hair shaft which is believed to reduce hair breakage whereas the larger molecular weight peptides are considered to have film forming abilities and impart body and shine into the hair.

7.2.3. Estimation of capital cost

The cost of the major equipments required for the process were estimated using pricing information obtained earlier from equipment vendors and prior experience with such processes based on the production capacity assumed and sizing obtained from the laboratory scale trials. Where data was not available for the equipment size required, the following equation (egn. 12) was used to estimate the price corresponding to that size as described in Sari et al. [73].

$$\left(\frac{Q_1}{Q_2}\right)^{0.6} = \frac{C_1}{C_2} \qquad (12)$$

Where Q_1 is the capacity of an equipment with known cost C_1 and Q_2 is the capacity for which the cost C_2 needs to be determined.

This procedure was applied to estimate the cost of the nonofiltration unit and the spray dryer. In accordance with the procedure described in Sari et al. [73] for the initial phase of cost estimation, installation cost was calculated as 25% of the total cost of equipments, the cost of piping, electrical and engineering work was calculated as 75% of the total equipment cost and 30% contingency was added to account for uncertainty at this stage of the cost process.

7.3. Results and discussion

7.3.1. Capital cost

The summary of the various components of the capital cost are given in Table 13. As can be seen, the total estimated capital cost of a plant processing 600 tons of feather per year using the sulphite assisted hydrolysis process is $6, 435, 130. For the process without the use of sulphites, it is $6,263,400. The capital cost for the sulphite assisted process is slightly higher due to the higher capacity required for nanofiltration and spray drying. It has to be noted that these are rough estimates based on equipment sizing from laboratory scale data, historical pricing data of the major equipments and prior experience in similar processes.

Table 13 Breakdown of the total estimated capital cost

Cost component	Cost process 1($)	Cost process 2($)	Comment
Milling equipment	100,000	100,000	
Reactor	300,000	300,000	
Coarse filtration unit	100,000	100,000	
Microfiltration unit	500,000	500,000	For a 25 m^2 unit
Nanofiltration unit	430,277	401,992	For 84 and 75 m^2 units estimated from the price of a 10m^2 unit
Spray dryer	644,772	606,824	For 104 and 94 kg/hr capacities, estimated from the price for 125 kg/hr capacity unit
CIP system	50,000	50,000	
Packaging system	50,000	50,000	
Tanks, valves, pumps	300,000	300,000	
Total equipment cost	2,475,050	2,408,817	
Installation cost	602,204	618,762	25% of total equipment cost
Piping, electrical, engineering cost	1,806,613	1,856,288	75% of total equipment cost
Contingency	1,445,290	1,485,030	30% of total estimated cost
Total capital cost	6,262,925	6,435,130	

7.3.2. Production cost

Process 1

Scenario 1: Analysis based on the laboratory scale trial data

In the sulphite assisted process, the total annual production cost including a 50% contingency was estimated to be $1,510,448, which is $19.4/kg of product. The main components of the production cost apart from contingency are the costs of labour and electrical energy followed by the cost of enzyme (Figure 54). The total income that can potentially be derived from the product at $50/kg is $3,900,000 per year. Thus, the estimated net income is $2,389,552 per year, which is favourable. The payback period for this scenario based on the estimated capital cost of is $6,435,130 is about 2.7 years, which is quite good.

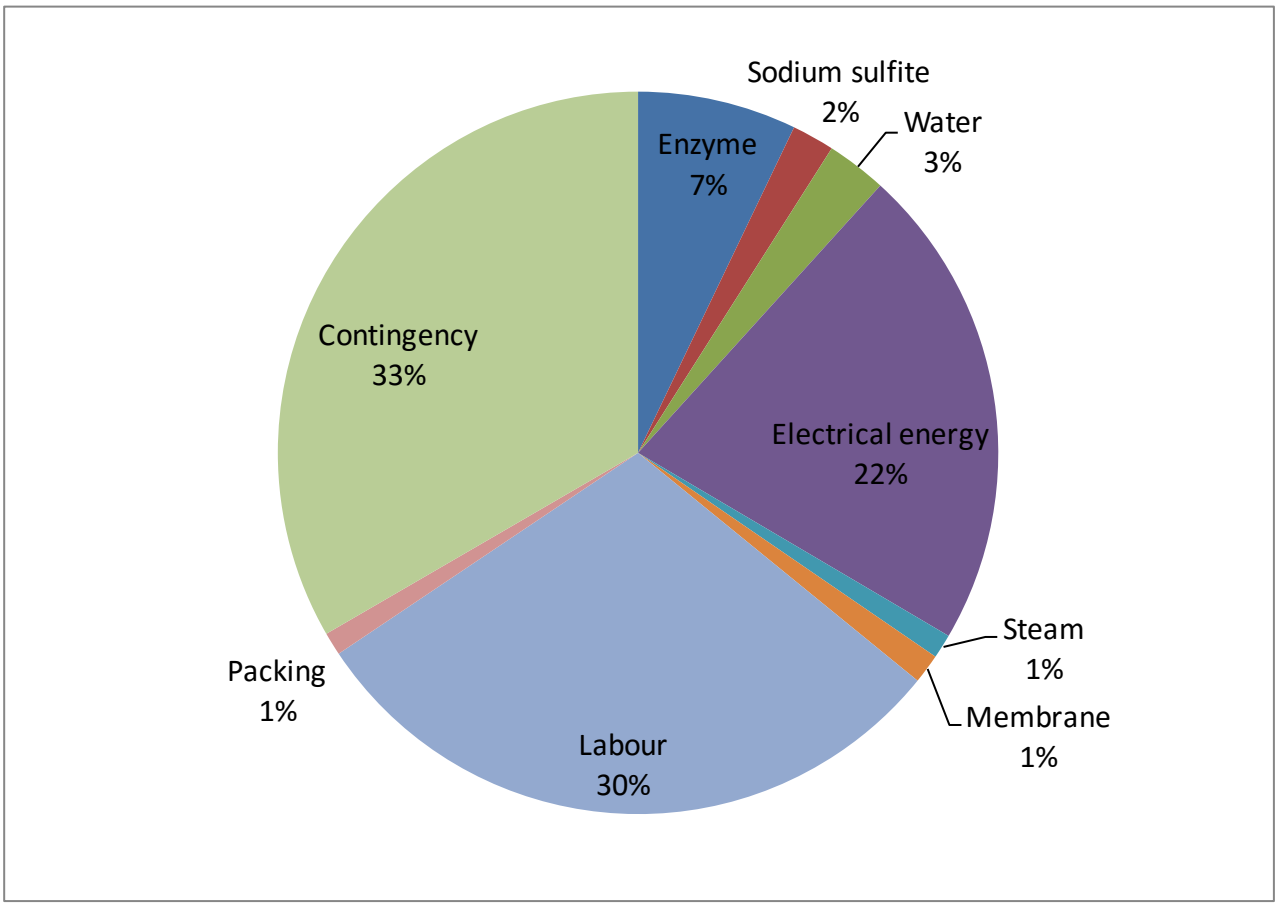


Figure 54 Pie chart showing the relative contribution of different process inputs to the total cost of production of keratin hydrolysate from sulphite assisted enzymatic hydrolysis of poultry feather.

This is a preliminary analysis based on a laboratory scale data and cost estimates based on past experience and the scientific literature. For instance, the specific energy input for the milling process is an estimate based on literature and needs to be experimentally verified at a pilot scale for a more accurate estimation of the cost. This applies to the other unit operations as well, although the degree of uncertainty varies from one to the other. In addition, the analysis did not

include fixed costs such as capital depreciation and indirect costs such as administration, which are normally included in standard cost benefit analysis. Nevertheless, with the 50% contingency assumed, the analysis gives a fairly reasonable assessment of the process. The typical contingency assumed at the initial phase of cost-benefit analysis (Class V: order of magnitude guess estimate) is 15 to 40%, which is less than what we assumed in this work [73].

The overall yield of the process and its economic viability can be potentially improved at a larger scale and through optimisation of the separation process, although the hydrolysis yield of 32% on protein basis after 6 hours of hydrolysis (~40% yield for 80% peptide product) will be the upper limit. Automating the solid-liquid-separation process by using for instance a self-cleaning rotary drum vacuum filtration with cake washing can significantly reduce the product loss during coarse filtration/separation prior to microfiltration. This may increase the cost of the downstream processes (microfiltration and nanofiltration) since a larger volume needs to be handled. However, the microfiltration step may not be needed since rotary drum filters can handle particle sizes as small as 1 µm, although this needs to be experimentally verified. If the required degree of removal of suspended matter can be achieved by the vacuum filtration alone, it will result in further gain in terms of efficiency of recovery since the ~23% loss (on protein basis) during microfiltration could also be avoided. This approach is evaluated in scenario 2.

Another approach to improve product recovery could be a second nanofiltration step to recover protein from the permeate of the first nanofiltration step where about 25% of the product (on protein basis) is lost. However, that will incur additional operating expenses and possibly capital expenditure for a second nanofiltration unit. Product yield can also be improved by increasing the enzymatic hydrolysis time. Increasing the enzymatic hydrolysis time will improve the yield to at least 60% on protein basis (Chapter 2 and 4). However, this may reduce the annual production for the same plant size or may incur additional capital cost if the same production rate is to be maintained. In any case, it will be worth exploring these and other options after pilot scale trial data is obtained for a more concrete cost-benefit analysis.

Scenario 2: Analysis assuming a single solid-liquid separation unit operation as an alternative to coarse filtration/separation and microfiltration

In this scenario, the separation process is modified by assuming a single unit operation using a self cleaning rotary vacuum filter with appropriate screen size as an alternative to the coarse filtration/separation and microfiltration operations. In addition, the following assumptions were made for the analysis.

- ~90% recovery of the product on protein basis following vacuum filtration and cake washing prior to nanofiltration
- the amount of cake washing water required equals to that of the three diafiltration steps during microfiltration in scenario 1

- the vacuum filtration and cake washing requires the same amount of energy as microfiltration
- the filter medium replacement cost for the rotary drum filter is equal to that of replacing the ceramic microfiltration membrane.

With 90% recovery after vacuum filtration, the total loss prior to nanofiltration decreases from ~37% (23%+14%) to ~10%, increasing the overall yield of 80% peptide product to ~24% and the annual production to 144,000 kg. The estimated total production cost per year for this scenario including a 50% contingency is $1,793,351. The estimated cost per kg is $12.5 which is 35% lower compared to scenario 1. The higher total production cost compared to scenario 1 is due to the additional energy, steam and packing cost associated with the higher amount of product that needs to be dried and packed. In this case, energy overtakes that of labour as the highest contributor to the cost of production due to the additional energy required for drying a larger amount of product (Figure 55). At $50/year, the total income for this scenario is $7,200,000 per year and the net income is $5,251,485, which is almost twice higher than scenario 1. The data for the two scenarios are summarised in Table 14.

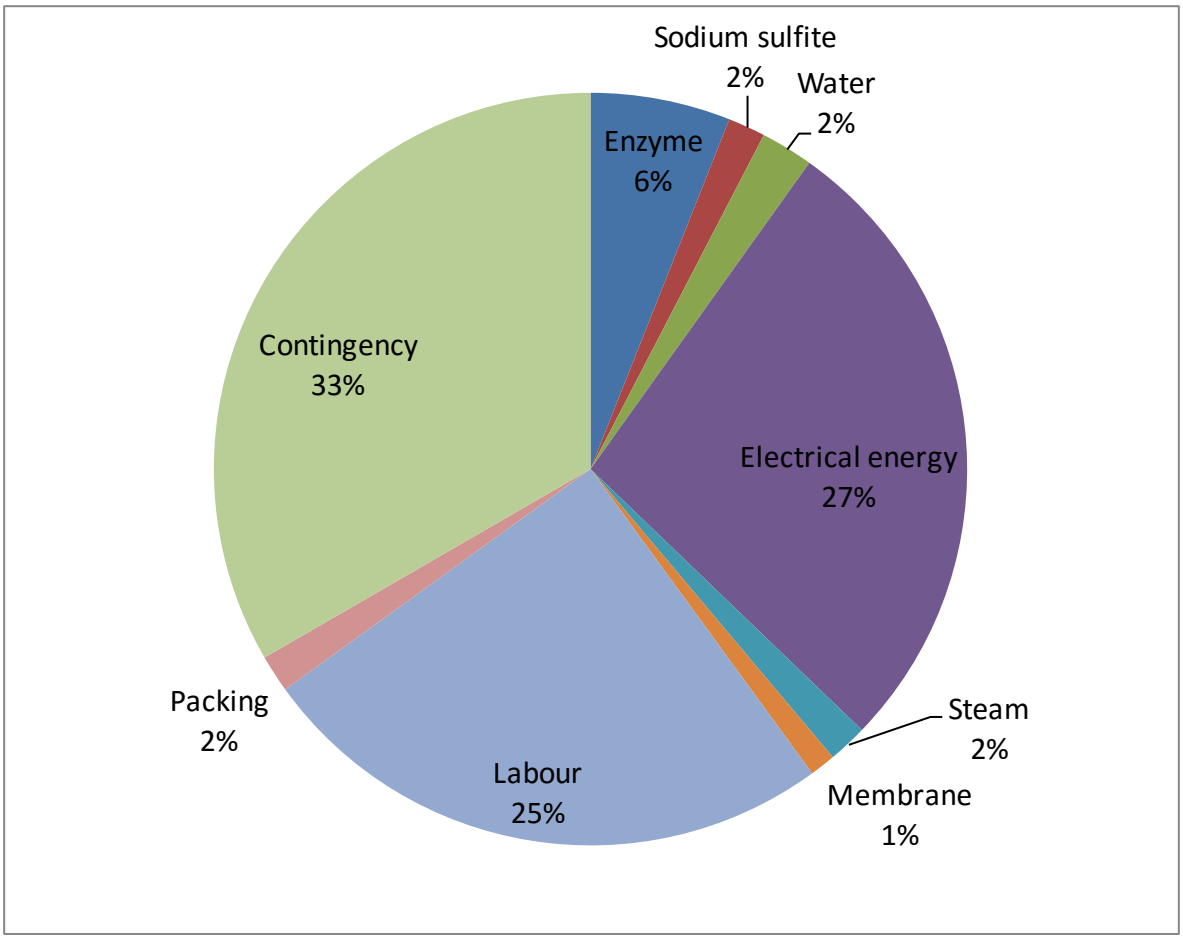


Figure 55 A pie chart showing the relative contribution of different process input to the total cost of production keratin hydrolysate using sulphite assisted enzymatic hydrolysis of feather with a single solid-liquid separation unit operation prior to nanofiltration (Process 1: scenario 2).

Table 14 Estimated total cost of production, unit cost and total net income for the various scenarios based on a 600 tons per year dry poultry feather processing plant

	Process 1		Process 2	
	Scenario 1	**Scenario 2**	**Scenario 1**	**Scenario 2**
Total production cost/year	$1,510,448	$1,793,351	$1,443,318	1,616,785
Unit cost	$19.4/kg	$12.5	$18.5/kg	$12.8/kg
Total net income/year	$2,389,552	$5,406,649	$2,456,682	$4,683,215

Process 2

Scenario 1: Analysis based on laboratory scale trial data

In the process without the use of sodium sulphite, the total production cost decreased to 1, 443,318 per year, which amounts to $18.5/kg of product. The total income at $50/kg and 13% yield is $3, 900,000 giving a net income of $2, 456, 682, which is even better than the sulphite assisted process. Based on the estimated capital cost of $6,262,925, the payback period for this process is about 2.6 years. As in the case of the sulphite assisted process, the major contributors to the overall production cost are labour and electrical energy (Figure 56).

Looking at the cost-benefit analyses of the two processes, one may conclude that it is not worth pursuing the sulphite assisted process, since the same amount of product is recovered using both processes. However, the maximum yield that can be obtained without the use of sulphites is 21% on protein basis (26.2% product yield) compared to the 32% yield on protein basis (40% product yield) that can be achieved with the sulphite assisted process, limiting the scope for yield improvement. Even then, the non-sulphite process has the advantage of retaining more of the low molecular weight peptides compared to the sulphite process (chapter 5), which could be an advantage in terms of product functionality depending on the application.

Scenario 2: Analysis assuming a single solid-liquid separation unit operation as an alternative to coarse filtration/separation and microfiltration

As in the case of the sulphite assisted process, product recovery can potentially improve at a larger scale and with further optimisation of the separation processes. If we can replace the solid-liquid separation and the microfiltration steps by a single vacuum filtration step with cake washing assuming

- the same operating cost as the microfiltration process and
- 90% recovery on protein basis (10% total loss instead of 23% + 17.7% after microfiltration and solid-liquid separation respectively)

The overall product recovery on protein basis will increase from~51% to ~82% and the overall product yield to 21%. The additional product recovered will result in additional energy and steam expenditure for drying as well as additional cost of packing. The total production cost for this scenario is $1,616,785 which amounts to $12.8/kg. The total income per year at $50/kg is $6,300,000 and the net income is $4, 683,215. Overall, the process without the use of sulphites is advantageous specially if the laboratory process is scaled up without significant modification, since it results in higher yield after purification and allows the recovery of both high and low molecular weight peptides. Even if the proposed modification is adopted with a single separation unit operation prior to nanofiltration, the difference between the two processes in unit cost of the product is marginal (Table 14).

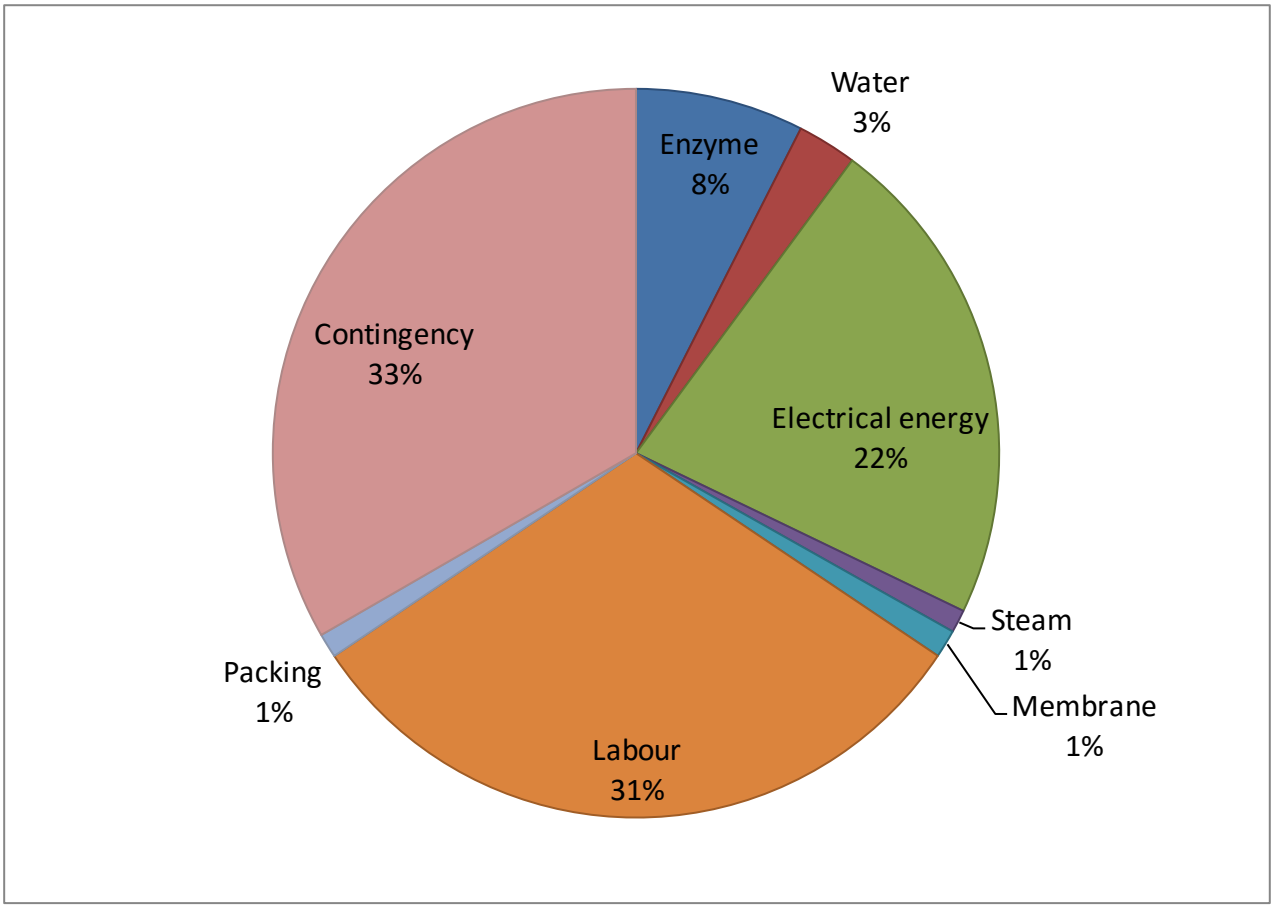


Figure 56 Pie chart showing the relative contribution of different processing inputs to the cost of production of keratin hydrolysate from enzymatic hydrolysis of poultry feather without the use of sodium sulphite

7.4. Conclusion

The preliminary cost-benefit analysis is quite positive for both the sulphite assisted and the non-sulphite processes with potential for significant improvement with further process optimisation. Based on a 600 ton/ year dry feather processing plant, the estimated annual net income from the sulphite assisted and the non-sulphite processes are $2,389,552 and $2,456,682 respectively. The payback period for both processes is less than 3 years. Process modification with a single solid-liquid separation unit operation prior to nanofiltration as an alternative to coarse filtration and microfiltration can potentially improve product recovery and increase the net income to $5,406,649 and $4,683,215 respectively for the sulphite assisted and the non-sulphite process with significant reductions in production costs from $19.4 to $12.5/kg and $18.5 to $12.8/kg for the two processes. Although the use of sodium sulphite significantly improved enzymatic hydrolysis yield, that did not translate into a higher net income from the process due to the lower product recovery. This can potentially be improved at a larger scale and through further process optimisation and need to be explored in future pilot scale trials. As is, the process without the use of sulphites is a better alternative since it also enables the recovery of low molecular weight peptides which have distinct functionality compared to the larger molecular weight peptides.

It has to be noted that this cost-benefit analysis is based on laboratory scale data, historical data based on past experience and data from the literature, with significant uncertainties on the individual cost estimates. In addition, fixed costs such as capital depreciation, insurance, interest on loan, and indirect costs such as administration cost which are normally included in standard cost-benefit analysis are not considered in this analysis. Nevertheless, with the very high contingency of 50% assumed in the analysis, the analysis gives a fairly reasonable assessment of the processes. It is recommended that further cost-benefit analysis be conducted after pilot scale trials of the various processing options to get a more concrete assessment of the processes.

The high per unit production cost indicates that the product is more suited to high end applications such as cosmetics as originally envisaged and needs to fetch at least $40-$50$/kg to be a profitable process with a reasonable rate of return. Thus future work should include detailed market research on the demand of the product as well as the market size. Our preliminary survey showed that SMEs in Australia which are engaged in the production of cosmetics have insufficient knowledge of keratin peptides derived from feather, although some reports and patents indicate that the big multinational companies are using feather derived keratin peptides in their products.

Recommendations

In this study, a novel keratinase from *Bacillus licheniformis* was produced and characterised for its ability to hydrolyse feather. Its efficacy for hydrolysing feather was compared with the commercial protease, Savinase 16L and an analytical keratinase, Proteinase K, which were selected from a number of other commercial proteases based on their efficacy. The crude keratinase from Bacillus licheniformis exhibited a comparable efficacy for hydrolysing feather as the commercial protease Savinase 16L and the analytical keratinase proteinase K in the absence of reducing agents such as sulphites, although the performance of Savinase 16L was superior in the presence of sodium sulphite due to its better proteolytic activity. It also showed a comparable activity for cleaving disulfide bonds to that of Proteinase K, which was significantly higher than Savinase 16 L at an enzyme dosage optimised for Savinase 16L. This indicates the significant potential of this keratinase alone or in combination with a potent protease for hydrolysing feather and other keratinacous materials. Moreover, its protease activity may improve with further optimisation of the fermentation process and downstream processing. The application of ultrasound also enhanced the hydrolysis of feather by this enzyme at specific energy input as low as 77 kJ/L and reaction times as short as six hours. Therefore, the enzyme can be used for ultrasonic assisted sulphite free hydrolysis of feather into keratin hydrolysates.

- Thus, it is strongly recommended that further research is conducted for the commercial development of this enzyme, since it can be potentially used as a feed enzyme, for conversion of keratinacous waste into value added keratin hydrolysates and for skin care applications.

The fact that *B.licheniformis* is classified as a GRAS (generally regarded as safe) organism reduces the regulatory hurdle associated with the development of its keratinase for feed and therapeutic applications. Research and development activities should include

- Further optimisation of the fermentation process for large scale production of the enzyme
- Development of suitable downstream process for at least partial purification of the crude enzyme
- Detailed characterisation of the enzyme including selection of suitable operating temperature and other routes for improving the stability of the enzyme over extended period for industrial application
- Study of possible synergy with other proteases for feather hydrolysis
- Potential application of pulsed ultrasound for efficient and sulphite free enzymatic hydrolysis of feather

The laboratory scale process optimisation of the feather hydrolysis process using Savinase 16L showed that the best hydrolysis yield of about 30% after 6 hours of hydrolysis is obtained at the maximum substrate concentration feasible (40 g/L feathers concentration) and 0.5% sodium sulphite concentration. However, this did not translate into at higher yield after the product

purification process since the protein recovery was only 32% with only 13% final yield on feather basis. In addition, the purification process to remove the added sulphites resulted in the loss of the low molecular weight peptides, which affects the functionality of the products especially in cosmetic applications. In addition, the sulphite assisted processes incurs slightly higher capital and production costs due to the need to remove the added sulphites. The product recovery can be potentially improved with scale up of the process which will reduce the relative impact of the 'hold up' volume and further optimisation of the purification process. However, with the process as is, the enzymatic hydrolysis process without the use of sulphites is relatively better than the sulphite assisted process. Although the yield of hydrolysis is only 21%, the recovery was 51% resulting in comparable product yield as the sulphite assisted process and without losing much of the low molecular weight peptides, which is a distinct advantage. Increase in hydrolysis time can potentially increase the yield of the process without the use of sulphites, although that comes at an increased capital cost or reduction in annual production capacity for a given production plant size. Increasing the enzyme dosage from 1% to 3% can also potentially increase the hydrolysis yield. The dosage was selected to be 1% on substrate protein basis in the optimisation study in order to reduce the overall production cost. However, the preliminary cost benefit analysis showed that the cost of enzyme is only a minor portion of the total production cost with only 8% contribution indicating that there is some room for flexibility in this regard.

- Thus, it is strongly recommended that pilot scale trials of the two processes be conducted in order to optimise the hydrolysis and the separation processes as well as get more concrete data for equipment sizing and operating condition for a realistic cost-benefit analysis of the processes prior to commercialisation.

The high production cost of the hydrolysates indicates that the products (from both processes) are more suited to high end applications such as cosmetics as originally envisaged and needs to fetch at least $40-$50$/kg to be profitable with a reasonable rate of return.

- Thus we strongly recommend that detailed market research be conducted to assess the potential market for feather based keratin peptides in the Asia-Pacific region as part of a future research and development activity.

Our preliminary survey showed that SMEs in Australia which are engaged in the production of cosmetics have insufficient knowledge of keratin peptides derived from feather, although some reports and patents indicate that the big multinational companies are using feather derived keratin peptides in their products.

Substantially large amount of unhydrolysed feather residue remained following the hydrolysis by the two processes. The residues from both processes showed very low digestibility compared to feather meal. Thus, further processing (thermal, hydrothermal) would be required to use these residues in animal feed applications. The existing feather meal production process may improve the digestibility of these residues. Apart from that,

- Further characterisation of the residues using material characterisation techniques and exploring potential applications such as in polymer composites or as a source of natural fibres for textiles are highly recommended, since that will significantly improve the value proposition.

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Appendix I

Table 1. Price quotes for keratin hydrolysates obtained from various suppliers in 2013.

Source	**Quantity (grade)**	**Price**
Xian Bosheng Biological Technology Ltd, China	25 kg (Bulk)	US$52/kg
Hiahang Industry Co. Ltd, China	50 kg (Bulk)	US$131.7/kg
Spectrum chemicals & laboratory products, USA	45 kg (Bulk)	US$116.6/kg
Chemos GmbH, Germany	50kg (Bulk)	EUR 54.4/kg
Proteina, Poland	(Bulk)	EUR 68.45/kg
Wonda Science Inc, USA	kg (Analytical grade)	US$395/kg

Appendix II

Questioner for market survey

June 2015

FEATHER PEPTIDE MARKET SURVEY

Name of company:

Name of contact: **Phone No:**

Name of market surveyor: **Date:**

Background

Poultry feather consists of 90% keratin, which is a highly complex and incalcitrant protein resistant to hydrolysis by conventional enzymes. In the poultry industry, a large quantity of feather waste is produced during poultry processing. Currently, most of the feather produced by the poultry industry in Australia is rendered to low value feather meal for animal feed application. Thus, efficient conversion of feather into hydrolysed keratin adds significant value to feather as well as solve environmental problems associated with its disposal. Literature indicates that hydrolysed keratin can be used in many applications including animal feed, cosmetics for skin and hair treatments, leather tanning, and biodegradable films and as a carrier for insecticides and other active agents. Hydrolysed keratin also can be a potential source of bioactive molecules.

CSIRO-Food and Nutrition flagship is developing a process to produce poultry feather derived keratin hydrolysate for application in the cosmetic, pet food and other industries.

Properties of the keratin hydrolysate

- Excellent solubility in aqueous medium
- Excellent foaming ability
- Excellent emulsification properties
- **Excellent bioactive properties, antioxidant property ….**
- **Excellent amino acid profile composed of all nine essential amino acids**

Questions

1. At present what type of peptide based ingredients do you sell or use (eg. Hair, wool, synthetic etc)?

2. What is the desired functionality of the peptide ingredients you sell or use? Could you please specify the preferred color and flavor (e.g. meaty, neutral, aromatic etc) if any?

a) Foaming properties

b) Emulsifying properties

c) Color/flavor

d) Any other

3. Do you prefer native or chemically modified peptide ingredients?

4. Are you aware of any other keratin based peptide ingredients?

5. Would you or your clients have any concerns selling or using a peptide ingredient derived from poultry feather keratin?

6. What is the price range you or your clients would pay for a functional peptide ingredient?

7. What is the current market demand for peptide ingredients? How would you expect sales of peptide ingredients to change over the next three years?

- Increase
- stable
- Decrease

8. What is your current annual sales volume (or use) of keratin peptide ingredients ?(less than a million dollar or more than a million dollar)

- Hair
- Wool
- Any other

Table 1. List of petfood companies contacted for market survey

Name of Company	***Contact***	***Phone No.***
VIP Pet Foods (Pty) Ltd	Chris Low	(07)55471700
Afb International Pty Ltd (Inghams Enterprises)	John Seletto	(03)59711105
Next Generations Pet Food	Nicole	(07)54381722
Hungry Hound Pet Food (Factory)		(03)93302660
Pet Health Food Products Pty Ltd	Ross Robinson	(03)97921505
Premier stock feeds	Dr. Cameron Sells	(02)45775603
Vets all natural	Dr. Bruce Symes	1800628838
Mars Australia	Dr. Brad Woonton	
Mccubbins Laverton (Vic) Pty Ltd		(03)93692944

Table 2 List of cosmetic companies contacted for market survey

Name of Company	***Contact***	***Phone No.***
Avenir Ingredients	Marguerite Capezio	(02) 97394889
Ausganica	William Or Moreen Liao	02 86047000
Ingredients Plus (Tri K industries)	Chris Ridgewell	(02) 96846788
Divine Cosmetics		(02) 98090419
New Directions Australia	Lorraine	(02) 85775999
Ali Hamylton Cosmetics	Alison Hamylton	(07)55980330
Aqicare Pty Ltd	Brian Baldwin	(07)55378121
Escentials of Australia Pty Ltd		61 7 54424559
Remedica Australia Pty Ltd		61 2 8964 7348 Or (0)414515149
Absolutely Gorgeous		(03) 402 243 393
Artav Australia		(08) 83001999
Symrise Pty Ltd		(02) 9982 7800
ASAP skin products		'info@asapskinproducts.com'

Detailed response of the companies

1) *Pet food company 1*

- Q.1: At present they use feather meal in their dry pet food products.
- Q2c: Feather meal is a negative for palatability of petfoods, particularly cat foods. Hence only small amounts can be used. If the palatability issue could be overcome then they would use more.
- Q2d: Feather meal digestibility is also an issue. Whilst it is better than it was, the digestibility of the feather meal would also need to be improved if one was to use larger quantities.
- Q3: They did not have any particular preference for native or chemically modified peptide ingredients.
- Q4: They are not aware of any other keratin based peptide ingredients.
- Q5: They have no concerns selling or using peptide ingredient derived from poultry feather keratin.
- Q6: As this question was related to the protein level, the palatability and the cost of other protein sources they felt it was difficult to comment. However, it was suggested that they may pay more if the product was the same protein level and the palatability and digestibility issues were resolved.
- Q7: They expect the sales of peptide ingredients probably to increase.

2) *Pet food company 2*

- Q1: They sell hydrolysed chicken as liquid and dry palatants
- Q2d: Needs to have highly palatability for dogs and cats
- Q3: They did not have any particular preference for native or chemically modified peptide ingredients.
- Q4: They are not aware of any other keratin based peptide ingredients.
- Q5: They have no concerns selling or using peptide ingredient derived from poultry feather keratin. Their pet food customers are Mars and Nestle.
- Q6: The price range for a functional peptide ingredient was indicated as $500-$1000/tone.
- Q7: They expect the sales of peptide ingredients to increase.
- Q8: Their current annual sales volume of peptide ingredients is less than a $1M.

3) *Cosmetic company 1*

- Q1: They promote an ingredient that contains keratin made from chicken feathers as well as others that are synthetic.
- Q2c: They prefer white or light in color.
- Q3: preference between native and modified ingredients depends on their customer.
- Q4: They are aware of other keratin based peptide ingredients.
- Q5: They have no concerns selling or using poultry feather derived peptide ingredient as long as its disease free and compliant with Australia's manufacturing practices and meets their customer needs.
- Q6: Price range for a functional peptide ingredient depends on the quantity, but anything between $200.00/kg to $900.00/kg.
- Q7: They expect the sales of peptide ingredients probably to increase.

4) *Cosmetic company 2*

- Q1: They use 'active' ingredients in some of their products. When asked about peptide ingredients, the response was that they use hydrolysed wheat proteins and Almond protein hydrolysates.
- Q2: The desired functionality was that the ingredient should show good foaming property. And they did not want the ingredient to be coloured.
- Q4: They are not aware of other keratin based peptide ingredients.
- Q5: Possibly concerned. The product should be environmentally friendly and meet the customer needs.
- Q6: Hesitated to answer. When asked whether $50/kg was an acceptable price, the response was that the price was expensive and suitable for high end products.

The co-owner of the company, with whom I (Hema Jegasothy) had the phone conversation, requested for a sample of the product. He was interested in testing our product especially in shampoo. He also made the following two important suggestions for our product; (1) the product has to be environmentally friendly and, (2) locally produced.

5) Cosmetic company 3

They distribute a range of protein hydrolysates (vegetable and non-vegetable) that have value in their use as cosmetic actives in skin and hair. They are finding increased resistance to materials that are animal derived, but this is not exclusive.

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