New Opportunities for Seafood Processing Waste

Appendix 2: SAMPI Tuna Hydrolysate Production

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1 Introduction

This report summarises the results of a number of trials to develop new/improved products from hydrolysis of Southern Bluefin Tuna waste. The trials were carried out from 2014 to 2016 both at the SAMPI facility in Port Lincoln and at Curtin University laboratories.

2 Background and Need

Southern Bluefin tuna is harvested from cages near Port Lincoln in South Australia between the months of February – September. Tuna are processed on board (gilled and gutted). The waste from the on board processing is delivered (on the same day) to the land-based SAMPI factory and equates to about 1500tonnes /year.

The original SAMPI process which involved acid hydrolysis of the waste is shown in Figure 1. When the product arrived, following mincing, and, in case it could not be immediately processed, to enable storage and processing at a later date, formic acid was added to lower the pH to 3.5. As this point, the product is known as 'unfinished product'. When a sufficient volume of the unfinished product was available, it is heated in a heating tank before passing through three sieves. Once it has passed through all the sieves (the third sieve at 100 microns) the final liquid product was tuna hydrolysate. The hydrolysate was produced once it is has passed through the heating tank and the two sieves, and collected in the finished product tank, as seen in the blue icons in Figure 1. Any material that did not pass through the first sieve was removed (to be utilised by pig farmers), and the product remaining on the other 2 sieves was termed 'leftover product'.

The acid tuna hydrolysate produced was sold mainly as organic fertiliser and with some interest from companies for aquaculture feed and pet food ingredients.

This project commenced in order to work with SAMPI to try and produce different products to diversity their markets. However, in order to meet such alternative markets some issues with the product would need to be resolved. These issues include

a. Seasonal variation in the nutritional composition of the Southern Bluefin Tuna influenced the final oil and protein content of the tuna hydrolysate. Fish caught earlier in the harvest season have a higher fat content in the liver, leading to higher oil content in the hydrolysate produced. Based on analyses the oil content can range between 5-15% and minimum crude protein content is approximately 19.6%. The unpredictability of the nutritional composition of the hydrolysate is an issue for end users, in particular if the intended use is as aquaculture feed or for feeding livestock. Currently there is no product specification for the hydrolysate manufactured for agricultural use. As a standard, the oil content should be 5%. For use as aqua feed, one customer is currently asking for a hydrolysate with fat content <5%.

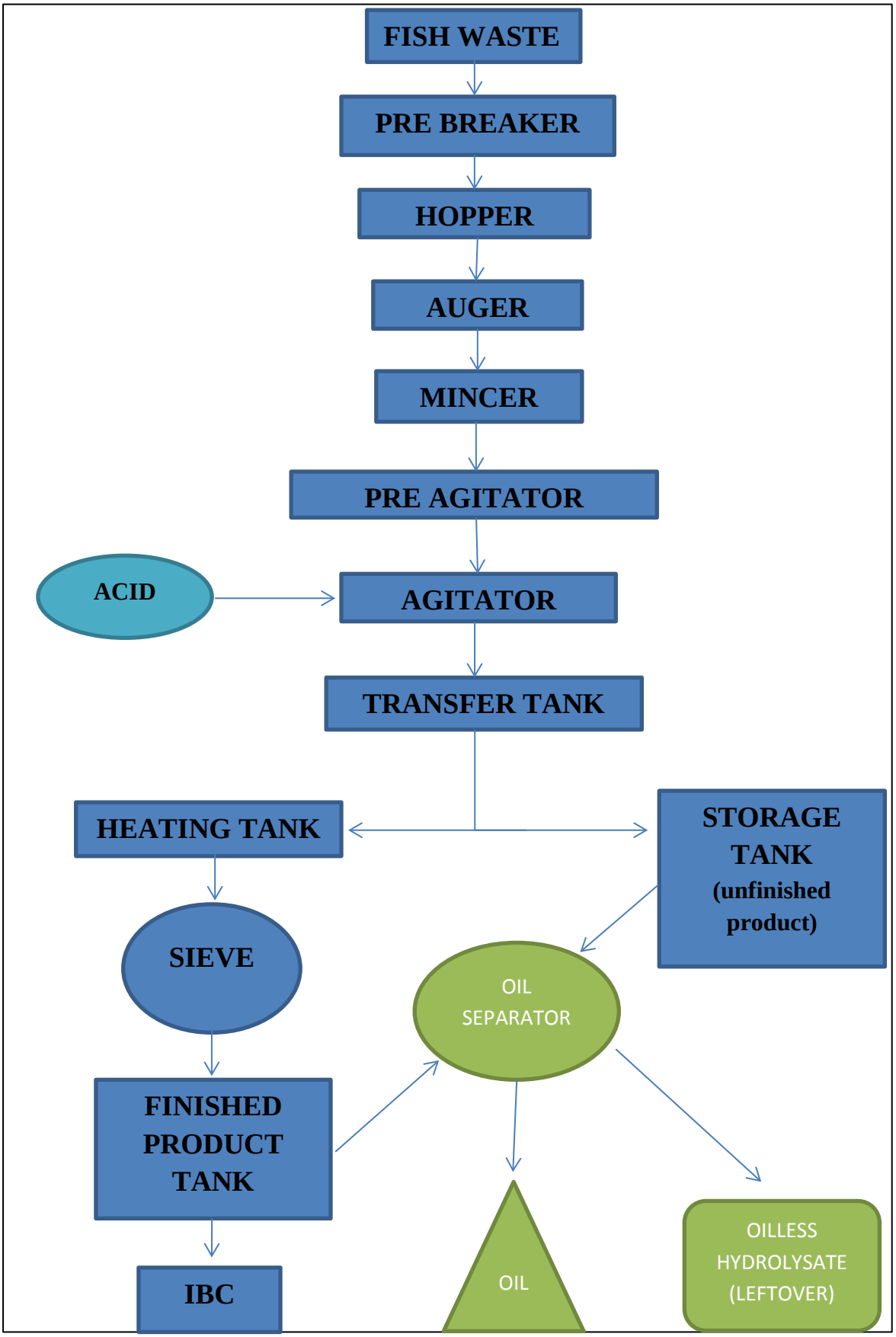

Figure 1. SAMPI Southern Bluefin Tuna Hydrolysate process flow diagram

b. On standing the hydrolysate tends to separate into more and less dense liquid layers, this may cause problems with spraying the product for agriculture, and it considered a problem in aquaculture feed production.
c. Calcium lumps have been observed to form in the hydrolysate. This also impacts spraying (as a fertiliser) by blocking up the nozzles.
d. The formic acid addition may impact the "organic" certification in US markets.

The aim of this study was therefore to look at alternative processing for the tuna offal to develop a higher quality, more broadly applicable hydrolysate product. This was considered to be quite possible given the unique nature of the factory location: product is delivered fresh to the SAMPI facility within two hours of harvest.

The research was separated into two programs.

a. Program 1 focussed on optimising quality oil extraction from hydrolysed waste. Various methods of hydrolysis were trialled and the quality of the extracted oil measured. These trials were instigated as tuna fish oil is potentially valuable for its high levels of polyunsaturated fats, in particular Omega 3 and 6 which have been proven to have many health benefits. As the previous tuna hydrolysate had inconsistent oil content, one option could be to pass the fish waste through an oil separator to remove the oil, then it could be added back to the material to develop a product with a known and consistent oil and protein content as well as also produce a valuable fish oil by product. These oil extraction trials were conducted in both laboratory and SAMPI facility trials. The proposed new oil extraction process is shown in the green icons in Figure 1.
b. Program 2 developed after it was apparent that the extraction of the oil from the hydrolysate was not an economically viable option. Research effort was thereafter focussed on assessing and later implementing enzyme hydrolysis as an alternative hydrolysis method. Again trials were conducted in both the laboratory and at the SAMPI facility.

3. Research Program 1: Optimising Oil Extraction from the SAMPI hydrolysis process.

3.1: Oil extraction: SAMPI Facility Trials

3.1.1 Objective

To work with SAMPI staff to assess the viability of incorporating an oil extraction step into the current SAMPI acid hydrolysis process to develop a range of by-products to broaden the markets for the SAMPI process. The new products could potentially include:

- Tuna Oil
- Tuna Hydrolysate
- Hydrolysate with known concentrations of oil to meet the requirements of different markets

3.1.2 Background and Processes

The first trials were conducted at the SAMPI factory following the installation of oil extraction equipment. In order to separate the oil from the other components after the current hydrolysis process, each sample was run through the oil decanter centrifuge (Figure 2). The machine was run at a speed of 500L/hr for the early and late harvest hydrolysate samples. The unfinished product

hydrolysate was run at three different speeds: 200L/hr, 300L/hr and 500L/hr to determine if speed times have a significant effect on the amount of oil extracted from the hydrolysate.

The Bluefin tuna hydrolysate was separated into three different components and collected in different compartments (Figure 3). The components were paste, oil and a liquid hydrolysate.

The processes and how the samples were collected for each different sample are represented in Figure 4.

Figure 2. Decanter Oil Separator trialled at SAMPI

Figure 3. Oil and paste collection from the oil separator

3.1.3.Methods

Trials were undertaken in December 2013.

Raw Materials

The following raw material was sourced for this trial:

1. Early Harvest Hydrolysate: 1 tonne early harvest Southern Bluefin Tuna hydrolysate – produced in April 2013 from fish harvested in mid-February 2013.
2. Late Harvest Hydrolysate: 1 tonne late harvest Southern Bluefin Tuna hydrolysate – produced from fish harvested between June to September 2013
3. Unfinished Product: 1 tonne unfinished product (stored after agitation step but before heating and sieving) – produced from Southern Bluefin Tuna harvested in June to September 2013. This product was put through the acid hydrolysis heating step at the time of the trials (December 2013). .

As the product had been sitting in the IBC containers for a period of time, the early and late harvest tuna hydrolysate was thoroughly mixed with an agitator and heated to a maximum of 53°C before being run through the oil separator,.

The unfinished product was put through the sieves to remove large bones and chunks, and then continued through the hydrolysis process (Figure 1) to produce hydrolysate which was heated to 53°C and run through the oil separator on the same day. Three different speed rates (200, 300 and 500L/hr) through the oil extraction decanter were tested for the hydrolysate produced from the unfinished product. The process for the experiments are shown in Figure 4.

Product Recovery

The approximate volume of the hydrolysate and the resulting volume of each component (oil, paste, liquid) recovered from the oil separation process were measured to determine the overall percentage of oil recovery (early harvest and unfinished product only). This was compared to the aligned oil content results from the raw materials to assess the effectiveness of the oil separator in extraction of oil.

Chemical Analysis

From each trial, the oil derived from the separator and the remaining material left over after oil separation was despatched for chemical and quality analysis. Figure 4 illustrates the components which were collected for the analysis. 200g of each sample was required to conduct the chemical analysis which was conducted at Agrifood Technology.

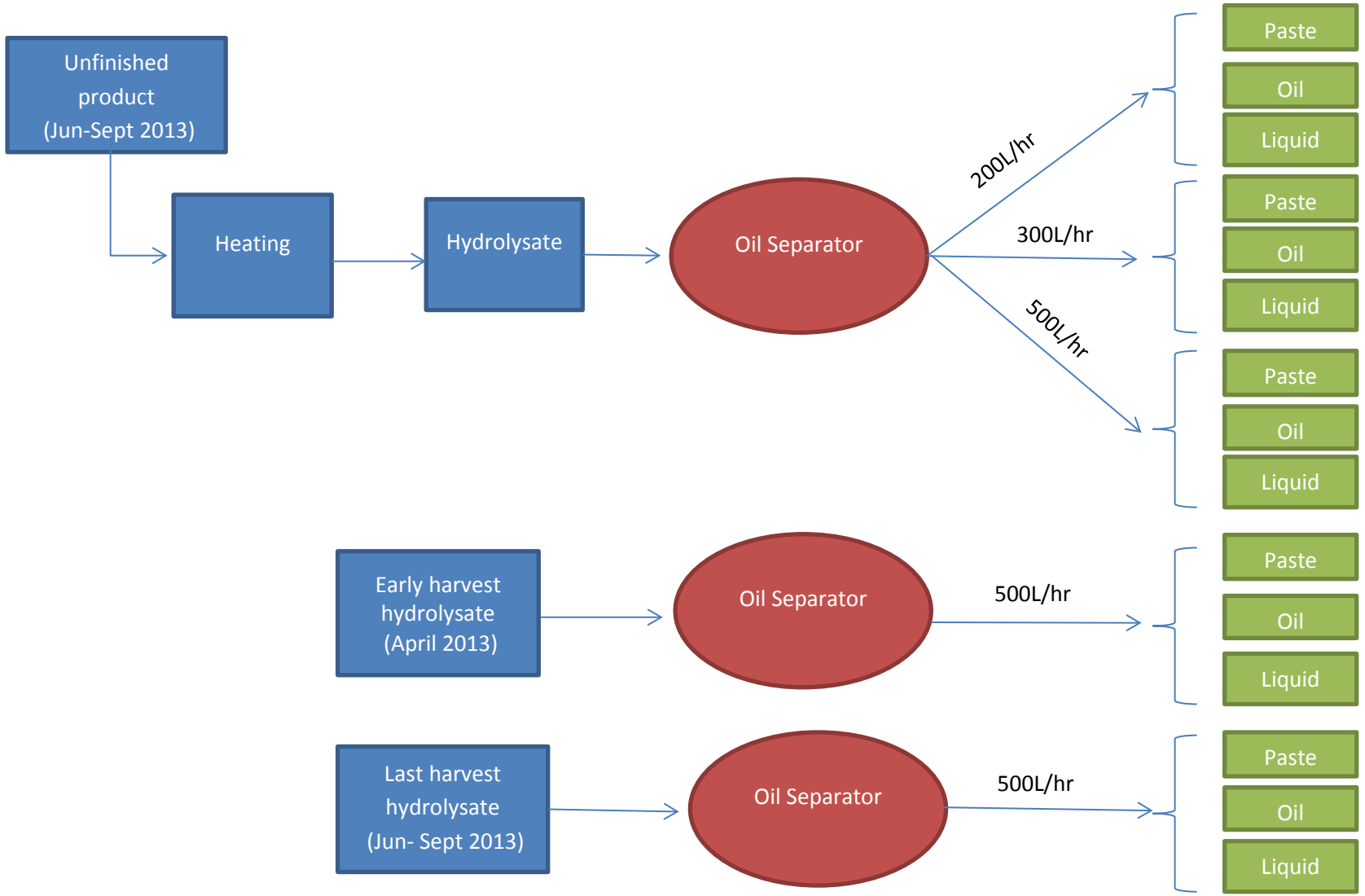

Figure 4. Southern Bluefin Tuna samples

Proximate Composition and Elements

Each of the samples sent off for analysis were tested for the following:

- Total protein content
- Total fat content
- Moisture content
- Ash Content
- pH

Calcium, selenium, nitrogen, phosphorus, and potassium levels were analysed in all the raw materials and the components extracted from the oil separator at 500L/hr.

Oil Composition

The Fatty Acid Profile (FAME) test was used to assess the levels of Omega 3 and 6. Analyses were conducted on the raw material and oil extracted from each of the three treatments at the pump rate of 500L/hr.

Oil Quality

The quality of the oil is measured by the amount of oxidative rancidity which has occurred. To determine if the effect of the processing impacts on oil quality, the raw material and oil extracted at a rate of 500 L/hr were analysed by Agrifood Technology. Peroxide Value and Free Fatty Acids were quantified as these are key indicators of oxidative and hydrolytic rancidity in oil.

As there may be potential for the extracted oil to be used as a nutraceutical product, heavy metals testing was conducted to ensure the levels of cadmium, lead and mercury and within the standards set by FSANZ.

Pepsin Digestibility

Pepsin digestibility, an important consideration for potential use as an aquaculture feed, was analysed for the unfinished product initially and for paste and liquid hydrolysate produced after hydrolysis.

3.1.4 Results

Product Recovery

From the oil separation process, there were three components extracted from the source hydrolysate; paste, oil and the liquid hydrolysate. Results for the trial run of the unfinished product hydrolysate and early harvest hydrolysate were based on a raw material weight of 1 tonne which is listed in Table 1.

Table 1 Product recovery trial results following oil extraction

	Unfinished product hydrolysate		**Early Harvest hydrolysate**	
Paste	30 L	3%	15L	1.5%

Oil	115 L	11.5%	90L	9%
Liquid hydrolysate	855L	85.5%	895L	89.5%

From the limited trials, it appears ~10% of oil could be recovered from the hydrolysate.

There was no observable difference in the appearance of the oil extracted from the different samples of Bluefin Tuna hydrolysate and between the samples run at different speeds. The colour was golden yellow when poured onto a surface (Figure 5) but has a dark brown appearance when placed in a transparent bottle (Figure 6). When the oil was allowed time to sit after extraction there was some precipitate at the bottom of the bottle. The top level of oil was poured into a new bottle and left to sit for a further 24 hours. A cloudy precipitate was noticeable in the bottom half of the bottle as seen in Figure 6.

Figure 5. Oil extracted from the bluefin tuna hydrolysate

Figure 6. Oil extracted from bluefin tuna hydrolysate after 24 hrs

The liquid hydrolysate extracted from the different samples of hydrolysate when passed through the oil separator was not different in appearance from the original product. The liquid was a light grey/ brown colour and a very water consistency (Figure 7).

Figure 7. Liquid component extracted from the oil separator

Proximate Composition and Elements

The results from the extracted oils are shown in Table 2

Table 2: Compositional results from the extracted oil.

Sample	**Unfinished Product Hydrolysate**			**Early harvest hydrolysate**	**Late Harvest Hydrolysate**
Pump rate	200L/hr	300L/hr	500L/hr	500L/hr	500L/hr
Ash (%)	<0.1	<0.1	0.1	<0.1	<0.1
pH			3.9	3.7	3.8
Moisture (%)	0.1	0.2	0.1	0.5	0.1
Protein (%)	0.4	0.3	0.4	0.5	0.2
Fat (%)	99.5	99.5	99.5	98.1	99.7
Calcium (mg/kg)			8.3	18	13
Selenium (mg/kg)			2.4	3.2	1.5
Nitrogen (mg/kg)			0.07	0.07	0.04
Phosphorus (mg/kg)			11	37	17
Potassium (mg/kg)			1.2	5.1	0.43

The pH, ash, moisture, protein and fat of the extracted oils was similar between samples and indicated that the extraction technique was relatively effective. As expected the oil extracted from the hydrolysates was predominately comprised of fat, with total fat contents over 98%.

The speed that the oil extractor was running did not affect the proximate composition of the oil extracted (Table 2) nor the results from the relative volumes of liquid and paste produced from the process (results not shown).

Results from the initial hydrolysate and the paste and liquid after the oil extraction are shown in Table 3.

Table 3: Compositional result for the hydrolysate and paste before and after oil extraction.

	UNFINISHED PRODUCT			EARLY HARVEST			LATE HARVEST		
Analysis	**Hydrol (pre)**	**Paste**	**Hydrol (post)**	**Hydrol (pre)**	**Paste**	**Hydrol (post)**	**Hydrol (pre)**	**Paste**	**Hydrol (post)**
Ash (%)	3.9	23.3	3.5	3.3	20.5	3	3.9	29.3	3.8
pH	3.7	4	3.6	3.7	3.8	3.5	3.6	4	3.5
Moisture (%)	61	49.2	75.4	70.1	50.4	78.4	71.8	46.3	77
Protein (%)	13	18.2	16.3	13.3	18.8	14.6	14.2	14.3	14.7
Fat (%)	15.9	1.7	1.11.1	9.2	2.3	0.8	7	0.9	1
Calcium (mg/kg)	10000	74000	7100	8300	67000	6500	9900	95000	8900
Selenium (mg/kg)	6	7.6	6.1	5.1	10	4.5	5.4	6.4	5.5
Nitrogen (mg/kg)	2.07	2.91	2.61	2.13	3	2.33	2.27	4	2.36
Phosphorus (mg/kg)	7300	55000	5100	6100	52000	4800	6100	69000	5400
Potassium (mg/kg)	1800	2000	2300	1700	1800	2000	1700	1600	1800

In comparing the analytical results for the different hydrolysates used as the raw material, the differences were mainly around the moisture content (higher in unfinished product) and fat levels. It was hypothesised that the fat would have been higher in the late harvest sample due to "fattening of the fish" through the season but this was confirmed with the limited sampling.

After oil extraction a paste and a (Post) liquid hydrolysate product are also produced. It is evident that the paste is where the bone fragments lie, as indicated by the high ash content. Fat levels are low but protein quite high (up to 18.2%) in the paste. The (post) liquid hydrolysate component had low fat and ash, but higher moisture then the pre hydrolysate. Protein was ~14%.

If the oil extraction process is commercialised then the liquid would likely be marketed as the modified hydrolysate product, with different potential markets (eg aquaculture feed) due to the lower fat and the paste product could be used for recreational bait.

Fatty Acid Profile (FAME)

Only fatty acids with values exceeding 0.1% were measured. The full list of the fatty acid profile is in Appendix 1. The omega-3 fatty acids are shown in Table 4.

Table 4: Fatty Acid results for extracted oil.

Sample	**Tot Fat (g/100g)**	**Omega-3**					
		EPA		**DHA**		**ALA**	
		% of TOT fat	**(g/100g)**	**% of TOT fat**	**(g/100g)**	**% of TOT fat**	**(g/100g)**
Unfinished product raw material	21.5	7.3	1.57	11.4	2.45	2.2	0.47

UP hydrolysate	15.9	3.7	0.59	10.4	1.65	1.8	0.29
UP oil	99.5	6.8	6.77	10.6	10.54	0.8	0.79
EH hydrolysate	9.2	4.1	0.38	5	0.46	0.9	0.08
Early Harvest oil	98.1	5.9	5.79	9.4	9.22	0.8	0.78
Late Harvest hydrolysate	7	5.2	0.36	6.8	0.48	1.6	0.11
Late Harvest oil	99.7	7.7	7.68	9.9	9.87	1.2	1.19

The levels of omega-3 fatty acids varied from ~8-10%. The trend is for the level of omega-3 fatty acids to be higher in the late harvest oil and hydrolysate than the early harvest but more results would be required to confirm. There were higher levels of omega-3 DHA than omega-3 EPA in all the samples.

The unfinished product raw material had a higher percentage of omega-3 fatty acids than the processed oil and hydrolysate.

Oil Quality

Rancidity

Table 5 summarises the peroxide and free fatty acid values for the extracted oils. The peroxide value was below 0.5 for the unfinished product raw material and the unfinished product hydrolysate. The peroxide values of the each of the hydrolysates was below 10, but once it had gone through the oil separator, each of the oils had a peroxide value ranging between 13 to 23 meq/kg.

Peroxide Value (PV)measures the early stages of rancidity and for freshly pressed oil for human consumption should have a maximum value of 0.1meq/kg, but preferably lower than 0.05. If the peroxide value is above 10meq/kg it is at unacceptable levels. Based on the peroxide values of the oils extracted from the hydrolysates, all of them would be unacceptable for use as a premium fish oil. A noticeable trend in the results is peroxide value increases with further processing. The hydrolysates has a lower value than the oil which has been extracted from it with values below 10 and the unfinished product raw material peroxide value was below <0.5. This indicates that oil of good quality could be produced with careful management of the process to reduce oxidation.

The percentage of free fatty acid (% FFA) is the highest in the unfinished product raw material at 45%. It was observed that the % FFA is higher in each of the hydrolysate samples than the oils which were extracted from it.

The percentage of FFA is based on the percentage of oleic acid that makes up the total fat content. The quality guidelines for crude oil FFA should range between 1-7%, however most of the extracted oils exceed this amount. Only the oil extracted from the late harvest hydrolysate had an acceptable FFA of 5.5%. The process of oil extraction decreases the percentage of FFA in the oil, which indicates that some of the FFA is removed by the extraction process. The FFA is responsible for undesirable flavours and colours so it is important to reduce the amount contained in the oil.

Table 5 Rancidity analysis on hydrolysate and oils

Sample	Peroxide Value (meq/kg)	% Free Fatty Acid- as oleic acid
Unfinished Product- raw material	<0.5	45.0
Unfinished product Hydrolysate	<0.5	12.8
Unfinished Product Oil	16	8.8
Early Harvest Hydrolysate	6.5	19.6
Early Harvest Oil	13	15.9
Late Harvest Hydrolysate	8.5	7.2
Late Harvest Oil	23	5.5

Heavy Metals

The analysis of the heavy metal levels in the oil extracted indicates that they are at safe levels below the maximum limit of cadmium, lead and mercury set by FSANZ (Table 6).

Table 6 Heavy metal levels in oil extracted

Sample	Heavy Metal		
	Cadmium (mg/kg)	Lead (mg/kg)	Mercury (mg/kg)
Oil from unfinished product hydrolysate	<0.0020	<0.020	0.018
Oil from early harvest hydrolysate	0.0042	<0.020	0.032
Oil from late harvest hydrolysate	<0.0020	<0.020	<0.010

Pepsin Digestibility

The results of testing on the pepsin digestibility of the unfinished product are in Table 7.

Table 7 Pepsin digestibility analysis

Sample	Pepsin Digestibility %
Undigested Product	94.1%
Hydrolysate from Unfinished product	94 %
Paste from oil extraction	73.1%

The pepsin digestibility is acceptable in the undigested product and hydrolysate for use in fish meal. A higher pepsin digestibility increases the nutritional contribution in growth in the animal consuming it. The paste collected from the oil extraction had an observably lower pepsin digestibility at 73.1% which could decrease the price of the paste for use as an ingredient in feed.

3.1.5 Summary and Next Steps

The proximate composition of the extracted oils shows it is close to a pure oil product with an average total fat content no less than 98.1%. The oil being extracted from the hydrolysate is termed crude fish oil. The generic quality characteristics of crude fish oil are listed in Table 8. The moisture

content, ash, protein and phosphorus content of the extracted oil is within the guidelines outlined by Bimbo (1998).

Table 8 Quality guidelines for crude fish oil according to Bimbo (1998)

Moisture and impurities %	0.5-1% max
FFA, % oleic acid	Range 1-7%, usually 2-5%
Peroxide meq/kg	3-20
Anisidine Value	4-60
Iron	0.5-0.7 ppm
Copper	<0.3 ppm
Phosphorus	5-100 ppm

However the peroxide and FFA levels were unacceptable. The results show that such rancidity measures are increasing through the process, it therefore should be possible to better manage oxidation in the process to produce a better quality product.

The level of omega-3 fatty acids in all extracted oils and hydrolysates, in particular EPA and DHA were significantly lower than the levels previously reported for tuna hydrolysate. The hydrolysate results previously had EPA at 11.7% and DHA at 19.7%. For the results in this trial, both the EPA and DHA levels were below 10%. For crude oil to be considered for use in the nutraceutical and pharmaceutical industry, the DHA/EPA ratio should at least be 18/12. With the low oil quality as demonstrated by the high peroxide values and free fatty acid values, further investigation is required into the factors causing the decrease in DHA and EPA levels.

In terms of impurities, crude fish oil can contain the following impurities: particulate matter, trace metals, sulphur-containing compounds, phosphatides, free fatty acids and halogen containing compounds (Rossell). The impurities can contribute to undesired colours and flavours in the crude oil (European Food Safety Authority 2010). Some of the impurities can be removed from the crude oil during the processing steps to produce fish oil for human consumption.

The liquid component after oil extraction contained a higher than expected level of total protein which ranged between 14.6-16.3g/100g. The liquid also contains a good level of trace elements. Fat levels had been decreased as a result of the oil extraction process. Although it has moisture content over 70%, the moisture can be evaporated to further concentrate the protein and minerals. With the price of protein and fish meal increasing, it may be an economical solution as a substitute for fish meal. Fertiliser applications still apply.

The paste by-product has high levels of minerals, in particular calcium and phosphorus, and protein so can potentially be used for several different application. These include:

- Sold to companies interested in making their own baits or feed for recreational fishers and aquaculture.
- SAMPI baits and tossers. The paste is quite thick and sticky.
- Poultry feed
- Mineral extraction

The results do indicate the addition of an oil extraction step to the SAMPI process could result in three useful by-products: fish oil, liquid hydrolysate and a high protein paste. However, the facility study has shown that improvement of quality must be achieved to meet market demand. Laboratory trials are recommended to try and identify process improvements to improve product quality.

3.2 Oil Extraction Optimisation and Hydrolysate Production: Laboratory Trials

3.2.1: Objective

The objective was to trial heating only and acid and enzyme hydrolysis of SBT waste in the laboratory and compare laboratory results for the extracted oil and hydrolysate products to the existing SAMPI product and previous oil extraction trials at the SAMPI facility (as described in Chapter 3.1). AS discussed in the previous section the quality of the extracted oil from the factory trials was very low.

3.2.2: Methods

It was decided to trial a range of treatments to extract the oil and/or hydrolyse.

Raw material

The SBT waste was minced, frozen and transported to Perth for analysis. The mince contained tuna frames and offal.

Treatments

a. Control treatment

Frozen tuna waste mince was defrosted overnight. A total of 3.29 kg of raw mince was centrifuged for 10 minutes at 4000rpm at 20°C. Oil supernatant was pipetted from the centrifuged mince and stored at 4°C in a separate container until analysis.

b. Heated to 40°C

Frozen tuna mince was defrosted overnight. A total of 2.1kg of raw mince was placed into a Sunbeam Sous vide, which was set at 40°C. The mince was held in the sous vide until the temperature of the mince reached 40°C (approximately 2 hours). Upon reaching 40°C, half of the mince was collected (976g) and immediately transported to the laboratory and centrifuged for 10 minutes at 4000rpm at 20°C. The oil supernatant was pipetted off the centrifuged mince and stored at 4°C, in a separate container until analysis.

c. Heated to 70°C

Frozen tuna mince was defrosted overnight. A total of 3.33kg of raw mince was placed into a Sunbeam Sous vide, which was set at 70°C. The mince was held in the sous vide until the temperature of the mince reached 70°C (approximately 3 hours). Upon reaching 70°C, the mince

(1.58kg) was immediately transported to the laboratory and centrifuged for 10 minutes at 4000rpm at 20°C. The oil supernatant was pipetted off the centrifuged mince and stored at 4°C, in a separate container until analysis.

Acid hydrolysis

Frozen tuna mince was defrosted overnight. A total of 1.9kg of raw mince was placed into a Sunbeam Sous vide, which was set at 40°C, with 66ml of Phosphoric acid added to it (3.5%). The mince was held in the sous vide overnight, or until the mince had broken down into a liquid hydrolysate. The hydrolysate product was then transported to the laboratory and centrifuged for 10 minutes at 4000rpm, at 20°C. The oil supernatant was pipetted off the centrifuged mince and stored at 4°C, in a separate container until analysis.

Enzymatic hydrolysis

Frozen tuna mince was defrosted overnight. A total of 2.7kg of raw mince was placed into a Sunbeam Sous vide, which was set at 55°C. Protamex, an enzyme used for hydrolysis, was added to the raw mince. 5.4g of Protamex was added. 100ml of water was added/ kilo equalling a total of 270ml. The mince was held in the sous vide for approximately 2 hours, or until the mince had broken down into a liquid hydrolysate. The hydrolysate product was then transported to the laboratory and centrifuged for 10 minutes at 4000rpm, at 20°C. The oil supernatant was pipetted off the centrifuged mince and stored at 4°C, in a separate container until analysis.

Chemical Analysis

From each trial, the initial raw material oil derived from the tuna waste mince and the remaining material left over after each separation treatment was sent for chemical and quality analysis at Agrifood Technology.

Proximate Composition

Each of the samples were tested for the following:

- Total protein content
- Total fat content
- Moisture content
- Ash Content
- Calcium, selenium, nitrogen, phosphorus, and potassium

Oil Quality

The quality of the oil is measured by the amount of oxidative rancidity which has occurred. To determine the effect of processing steps on oil quality, analyses for peroxide and free fatty acids (key indicators of oxidative and hydrolytic rancidity) was undertaken on the raw material and the oil which was extracted from the tuna waste mince.

3.2.2 Results and Discussion

Product Recovery

Results for the oil yields recovered from the different treatments of the tuna waste mince are listed in Table 9.

Table 9: Oil yields recovered from tuna waste mince

	Oil recovered (grams)	**Oil recovered (% of total mince weight)**
Tuna mince waste-unheated	34	1.03
Tuna mince waste heated to 40°C	46	4.71
Tuna mince waste heated to 70°C	73.2	4.63
Acid hydrolysis	107	5.63
Enzyme hydrolysis	97	3.59

The recovery yield from the initial tuna waste mince listed in Table 9 was low (1.03%). The mince heated to 40°C and 70°C experienced a higher yield of 4.47% and 4.63% respectively, in comparison to the initial tuna waste mince. Acid hydrolysis appeared to give the best yield for oil recovery (5.6%), followed by simply heating the mince to 40°C.

Table 10: Total oil yields from tuna waste mince

	Total oil (g/100g)	**Oil extracted after centrifuge (g/100g)**	**Oil remaining in hydrolysate (g/100g)**
Tuna mince waste-unheated	13.83	1.03	12.8
Tuna mince waste heated to 40°C	12.11	4.71	7.4
Tuna mince waste heated to 70°C	9.93	4.63	5.3
Acid hydrolysis	12.93	5.63	7.3
Enzyme hydrolysis	10.09	3.59	6.5

The total oil yields from the tuna waste mince highlight that the unheated mince produced the lowest amount oil extracted from the mince after centrifugation. Tuna mince heated to 70°C produced the highest volume of oil extracted from the tuna mince waste, enzyme hydrolysis was the next most effective method at separating oil from the mince and tuna mince heated to 40°C performed similarly to tuna mince hydrolysed with acid (Table 10).

The most efficient method for oil recovery was heating the tuna waste mince to 70°C, resulting in 46% of the total oil able to be extracted and 54% left in the hydrolysate. The least efficient method

for oil recovery was centrifuging the unheated tuna mince. Only 7.4% of the total oil was extracted after centrifugation leaving 92.6% remaining within the waste tuna mince.

Proximate Composition

Proximate composition results are shown in Table 11. The ash content was 3.9g/ 100g for the untreated mince after oil extraction. The ash content increased slightly to 4.9g/ 100g and 4.8g/ 100g after the mince was heated to 40°C and 70°C respectively. There was no increase in ash content for mince treated with enzymatic hydrolysis, with the ash content remaining at 3.9g/ 100g. Mince treated with acid hydrolysis experienced a higher ash content of 8.1g/ 100g and 7.9g/ 100g, likely due to the dissolution of the bones by the acid.

The enzymatic hydrolysate had a total protein of 14 g/ 100g. The total protein for mince pre-treated at 40C for 24 hours and then hydrolysed with acid was 14.5g/ 100g. The total protein for mince pre-treated at 70C for 24 hours and then hydrolysed with acid was 14.8g/ 100g. The untreated mince and mince that was only heated to 40°C and 70°C, managed to maintain a higher protein content ranging between 15.4-17.4g/ 100g.

The calcium levels in Table 11 were very similar for all trials after acid hydrolysis, ranging from 840-890 mg/kg. The enzymatic hydrolysis caused the calcium levels to decrease to 200 mg/kg, this result needs further investigation.

There was little variation in the levels of selenium in Table 11 between the different treatments ranging from 0.6- 0.86 mg/kg.

There was little variation in potassium levels (Table 11) when exposed to different treatments. The range of potassium existed between 190- 250 mg/kg.

Phosphorus levels were problematic due to some analytical issues.

Table 11: Composition of tuna waste oil and hydrolysate exposed to different treatments

Trial	Sample Description	Ash (g/100g)	Total protein (g/100g)	Ca	Se	N	P	K	Total fat (g/100g)	Sat fat (g/100g)	MU fat (g/100g)	PU fat (g/100g)	Trans fat (g/100g)
1	Oil- raw mince									33.7	29.9	36.4	<0.1
	Raw mince after oil extraction	3.9	15.4	770	0.85	2.46	600	190	12.8				
2	Oil- mince heated to 40C									36.4	28.8	34.7	<0.1
	Mince after oil extraction (40C)	4.9	15.6	3700	0.71	2.50	1900	250	7.4				
	Acid hydrolysis after heated to 40C	7.8	14.5	840	0.64	2.31	2300	210	10.9	36.4	28.8	34.7	<0.1
3	Oil- mince heated to 70C									41.6	28.5	29.9	<0.1
	Mince after oil extraction (70C)	4.8	17.4	220	0.86	2.78	260	230	5.3				
	Acid hydrolysis after heated to 70C	8.1	14.8	860	0.71	2.38	2600	210	8.2				
4	Oil- Acid hydrolysis									37.3	29.7	32.5	<0.1
	Acid hydrolysate (no oil extraction)	7.9	15.5	890	0.68	2.48	2500	200	7.3	40.2	32.7	27	<0.1
5	Oil- Enzyme hydrolysis									36.8	30.4	32.8	1.8
	Enzyme hydrolysate (no oil extraction)	3.9	14	200	0.60	2.24	190	200	6.5				

Oil Quality

Rancidity

The oil quality was determined by measuring the PV and FFA values for oil extracted after the mince had been exposed to different treatments. The PV value was lowest (14.4meq/kg) for oil extracted immediately from the tuna waste mince after it was defrosted. Mince treated with enzymatic hydrolysis showed the second lowest PV value of 25meq/kg. Acid hydrolysis followed that with 30meq/kg and finally mince heated at both 40°C and 70°C had PV values of 35meq/ kg (Table 12).

The FFA values were all low ranging from 0.5 for oil extracted from the untreated mince to 0.8 for mince heated to 40°C. All other treatments produced an FFA value of 0.6.

Table 12: Rancidity analysis for tuna fish mince oil extracted from various tuna waste mince treatments

Sample	Peroxide value (meq per kg)	% Free fatty acid (as oleic acid)
Oil- raw mince	14.4	0.5
Oil- mince heated to 40C	35	0.8
Oil- mince heated to 70C	35	0.6
Oil- acid hydrolysis	30	0.6
Oil- enzyme hydrolysis	25	0.6

There was little variation in the EPA and DHA levels for the tuna waste mince (Table 13). The lowest EPA values came from the enzymatic hydrolysis treatment (9.4 %) and the highest from the untreated mince (10.8%). The lowest value for the DHA was experienced when the mince was heated to 70°C (18.1%) and the highest when the mince was heated to 40°C (21.5%).

Table 13: EPA, DHA and ALA for oils recovered from various tuna waste mince treatments

Sample	EPA	DHA	ALA
	g/ 100g	g/ 100g	g/ 100g
Oil- raw mince	10.8	20.7	0.8
Oil- mince heated to 40C	10.5	21.5	1.0
Oil- mince heated to 70C	9.7	18.1	0.7
Oil- acid hydrolysis	9.8	19.9	0.7
Oil- enzyme hydrolysis	9.4	20.4	1.1

Oil Colour

The colour of the oil appeared to be lighter with the less treatment the tuna waste mince received. The untreated mince produced light yellow coloured oil. Mince heated to 40°C also produced fairly light yellow coloured oil. Once the mince was heated t0 70°C the oil began to darken and acid hydrolysis and enzymatic hydrolysis also produced oil that was darker in colour.

3.2.4 Summary and Next Steps

Peroxide Value of freshly pressed oil should have a maximum value of 0.1meq/kg, but preferably lower than 0.05. If the peroxide value is above 10meq/kg it is at unacceptable levels. This indicates

the early stages of oxidative rancidity peroxide value measures the primary products of oxidative rancidity. The peroxide values for oil extracted in Trial 3.2 were higher than the recommended limit, ranging from 14.4- 35meq/kg. The waste was treated from frozen, so there was little time for the tuna waste mince to degrade before oil extraction or hydrolysis. The processes for extracting the oil involved transporting it to another facility and centrifuging it. Perhaps this extra process allowed more contact with atmospheric oxygen promoting higher peroxide values.

The percentage of FFA is based on the percentage of oleic acid that makes up the total fat content. The quality guidelines for crude oil FFA should range between 1-7%, The free fatty acid values for oil extracted in this trial were relatively low, ranging between 0.5- 0.8 %. The results for the FFA values were much better than the previous trials at the SAMPI facility. This is most likely due to the improved and more controlled conditions in which the waste was processed and treated.

The DHA and EPA levels for oil extracted in Trial 2 were very close to the DHA/EPA ratio of 18/12 expected for crude oil to be acceptable to nutraceutical companies. The DHA levels ranged from 18.1-21.5 and the EPA levels ranged from 9.4-10.8. The EPA levels were slightly lower than required however overall the oil quality from this trial appeared to be much better than the DHA/EPA levels in Trial 3.2 Again the treatment of the tuna waste mince probably influenced the results greatly.

Ways to further improve oil quality include:

- Leave very little air in the storage containers to reduce the amount of oxygen which causes oxidative rancidity
- Introduce antioxidants into the oil extraction process to limit the rancidity experienced by the oil by limiting its reaction with oxygen.

The oil extracted in Trial 2 was shown to be lighter if the oil was extracted immediately from the tuna waste mince without any treatment. If the tuna fish mince was heated or hydrolysed the oil became darker in colour. The oil extracted straight from the tuna fish mince with no treatment also produced the best results for peroxide values, FFA values and DHA/EPA.

The results indicate that a shift to enzyme rather than acid hydrolysis could be undertaken without a loss of oil/hydrolysate quality, and indeed the results whilst preliminary do indicate that a better quality product may be achieved.

3.3. Comparison of oil quality under laboratory and factory conditions.

3.3.1 Objective

Verify the quality of the oil extracted from the improved tuna fish hydrolysate process (August 2014) and compare to results from laboratory studies (Chapter 3.1 and 3.2), and a commercially available product.

3.3.2 Methods

<u>Raw material</u>

In August 2014, the SAMPI staff conducted a further oil extraction trial on site in Port Lincoln and the following samples were despatched to Curtin University:

- Oil extracted from fresh tuna waste at SAMPI.
- Oil extracted from fresh hydrolysate at SAMPI.

Chemical Analysis

The extracted oil was analysed for:

- Oil Quality: FFA, Peroxide Value
- Fatty Acid Profile
- Omega 3 EPA, DHA and ALA

These results were compared against chemical analysis conducted on oil samples from previous trials:

- CESSH lab oil extraction (fresh mince)(Chapter 3.2)
- CESSH lab oil extraction (following acid hydrolysis) (Chapter 3.2)
- Agrifood Analysis of Oil extracted from stored SAMPI hydrolysate (acid) (Dec 13)(Chapter 3.1)
- Numega analysis of oil from stored SAMPI hydrolysate (acid) (Chapter 3.1)

3.3.3 Results and Discussion

The results from the August 2014 SAMPI on site oil extraction trial in comparison to the previous laboratory trials were significantly poorer in quality, in relation to FFA and Omega 3 levels (Table 14). The FFA exceeds the maximum values expected in high quality crude oil and the peroxide value is acceptable. However the values were improved when compared to the SAMP facility trials in December 2013. This indicates that there have been some improvements in the oil extraction process since the end of 2013, although the process still needs improvement to produce high quality crude oil.

Table 14 Summary of tuna oil quality analysis results

Sample	DHA (g/100g)	EPA (g/100g)	ALA (g/100g)	FFA	Peroxide Value
1. SAMPI oil extraction from fresh tuna (Aug 2014)	1.7	0.7	<0.1	8.3	8.0
2. SAMPI oil extraction from hydrolysate (Aug 2014)	1.3	0.5	<0.1	8 (5.3)*	9.1 (7.4)*
3. CESSH lab fresh oil extraction (70 C)	9.7	18.1	0.7	0.6	36
4. CESSH lab acid hydrolysate oil extraction	9.8	19.9	0.7	0.6	30
5. Oil extracted from SAMPI stored hydrolysate (Dec 13)	5.79	9.22	0.78	15.9	13
6. Oil extracted from SAMPI stored hydrolysate (Dec 13)(Numega analysis)	9.3	18.2		11	

* Result from Kemin analysis of same material

The omega 3 EPA/DHA levels were significantly lower in the August 2014 oil samples when compared to the previous results. This directly relates to the significantly lower levels of

polyunsaturated fats in these samples. In contrast, the monounsaturated and saturated fats was significantly higher. The fresh oil extracted in August 2014 also contained trans- fats, with no traces in any other samples (Table 15)

Table 15 Summary of the fatty acid profile results for tuna oil

Sample	Omega 3 (g/100g)	Sat fat (g/100g)	Mono (g/100g)	Poly (g/100g)	Trans (g/100g)
1. Fresh oil extraction (Aug 2014-on site)	2.5	47.8	47.5	4.7	5.9
2. Hydrolysate oil extraction (Aug 2014-on site)	1.9	46.7	49.7	3.6	<0.1
3. CESSH lab fresh oil extraction (70 C) results		41.6	28.5	29.9	<0.1
4. CESSH lab acid hydrolysate oil extraction		37.3	29.7	32.5	<0.1
5. Oil extracted from SAMPI stored hydrolysate (Dec 13)		40.7	37.8	19.7	<0.1
6. Numega hydrolysate oil extraction results	33.7				

There was lower quantity of omega-3 EPA/DHA than expected in the factory extracted oil. Based on the results it can be assumed either the process of oil extraction or the treatment of raw material prior to sample preparation in August 2014, has caused the polyunsaturated fatty acids to undergo oxidation. The oxidation of PUFA results in elevated concentrations of saturated, monounsaturated and trans- fats. However, the FFA results did not support the oil quality results, so retesting is suggested, including analysis of the raw material.

3.3.4 Summary and Next Steps

The oil extraction trials was ceased at SAMPI after the 2014 season due to a continued lower level of oil in the raw material and the difficulties in managing quality issues at a commercial scale.

4. Program 2: Assessing and Optimising Enzyme Hydrolysis of SBT waste.

4.1 Laboratory Studies on Acid and Enzyme Hydrolysis of SBT waste.

4.1.1 Objective

Determine if enzyme hydrolysis of tuna waste to a fish protein hydrolysate and further by products can provide a superior quality of final product and yield when compared to acid hydrolysis.

4.1.2 Background

The previous oil extraction trials (Research Program 1) was ceased at SAMPI after the 2014 season due to a continued lower level of oil in the raw material and the difficulties in managing quality issues at a commercial scale. The research focus therefore changed to developing a more efficient hydrolysis process, after the results of Section 3.2 potentially showing the possibility to use enzyme rather than acid hydrolysis in the SAMPI operation.

The total protein content of hydrolysate for aquaculture feed is ideally between 15-20%. The protein content in the SAMP product has been 8-15% wet basis.

Protamex (used in Section 3.2) hydrolysed the tuna material in less than 2 hours but comes in a powdered form, thereby is more difficult to use in the factory. Alcalase is a widely used proteolytic enzyme for producing fish protein hydrolysate. IT is available in a liquid form. In a review by Ghaly. A et al. (2013) on the use of enzymes in processing fish wastes, alcalase was reported as one of the best enzymes to produce fish protein hydrolysate. Shahidi, Han, and Synowiecki (2006) produced protein hydrolysate capelin (*Mallotus villosus*) with papain, neutrase, alcalase and autolytic hydrolysis, with alcalase recovering the highest yield of protein.

The aim of this research was therefore to test the effectiveness of alcalase (in comparison with protamex and acid) in hydrolysing tuna waste.

4.1.3: Methods

Raw material

5kg of frozen, minced tuna was received from the SAMPI facility in Port Lincoln. The product was taken as a subset of a sample being hydrolysed thought the existing process.

Treatments

a. Heating to 55C

Defrosted tuna mince was placed into Sunbeam Duos Sous Vide and Slow Cooker. Mince was heated to 55°C and maintained until liquidised. Add formic acid after heating to reduce pH to 3.5 (and therefore stabilise the product) and maintained at 55°C for 10 minutes.

b. Acid hydrolysis with formic acid (current SAMPI process)

Defrosted tuna mince was placed into Sunbeam Duos Sous Vide and Slow Cooker. 2.5% formic acid was added to the mince before heating to 55°C and maintained until liquidised. Formic acid was added to reduce pH to 3.5 and maintained at 55°C for 10 minutes.

c. Enzymatic hydrolysis with Alcalase

Defrosted tuna mince was placed into Sunbeam Duos Sous Vide and Slow Cooker. Alcalase was added at 0.05% w/w. Mince was heated up to 55°C, constantly stirred until tuna mince had liquidised (~20 mins). Formic acid was added to reduce pH to 3.5 and maintained at 55°C for a further 10 minutes.

d. Enzymatic hydrolysis with Protamex

Defrosted tuna mince was placed into Sunbeam Duos Sous Vide and Slow Cooker. Protamex was added at 0.05% w/w. Mince was heated up to 55°C, with constant stirring until tuna mince had liquidised (~20 mins). Formic acid was added to reduce pH to 3.5 and maintained at 55°C for a further 10 minutes.

Analysis

a. Chemical

Treatments were analysed for

- Total Fat
- Total Protein
- Ash Content
- Moisture Content
- Trace Elements (Ca, K, N, P, Se)
- Fatty Acid Profile
- Peroxide Value
- Free Fatty Acid

b. Physical Composition

The hydrolysate samples were placed into 50mL tubes and centrifuged for 5mins at 4000rpm at 20°C. The approximate volume of the different components was measured and the percentage composition of the three components (aqueous, fat and solids) determined.

c. Degree of separation

Centrifuge tubes were filled with 50mL hydrolysate and left to sit for one week to evaluate the percentage of solute separation over time. This because market issues from separation on standing had been reported from the current acid hydrolysed product.

4.1.4 Results and Discussion

The pH of the thawed tuna mince was 6.15 (Figure 8). The pH of the liquidised tuna mince using heat only and both enzymes was 6.30, while the addition of 2.5% formic acid for acid hydrolysis lowered the pH to 4.28. With the addition of acid to all treatments post hydrolysis to stabilise the hydrolysate, pH 3.5 was recorded, and all mixes rapidly became thicker in consistency. Hydrolysis of the tuna mince with heat only at 55°C took 3 hours to complete, whereas the formic acid hydrolysis was the fastest at 1 hour 40 minutes. The enzyme hydrolysis with Alcalase and Protamex was marginally slower at 1:45 and 1:50 hours, respectively. The consistency of the sieved hydrolysate did not vary, however the hydrolysates with enzymes produced a slightly brighter red in colour. The bones were the only materials undigested at the end of all hydrolysis (Figure 9).

Figure 8. Thawed tuna mince

Figure 8. Undigested material filtered after hydrolysis

Analysis

The chemical analyses results of the hydrolysate are shown in Table 16.

Table 16. Proximate analysis of hydrolysates using differing hydrolysis processes

	Units	Raw material	Heated at 55°C	Formic Acid (2.5%)	Alcalase (0.05%)	Protamex (0.05%)
Ash	g/100g	5.2	2.9	3.8	2.5	3.5
Moisture	g/100g	66.8	67.7	69.2	66.9	68.2

Fat, total	g/100g	8.5	10.0	8.0	10.4	9.4
Protein, total	g/100g	17.2	15.9	15.9	16.0	15.5
Nitrogen, Kjeldahl	g/100g	2.76	2.54	2.54	2.55	2.47
Calcium	mg/kg	28000	5000	8200	23000	7700
Potassium	mg/kg	2300	2200	2200	2200	2100
Phosphorus	mg/kg	14000	4400	5600	12000	5400
Selenium	mg/kg	4.1	4.5	3.7	4.1	3.8
FFA (as oleic acid)	%		17.80	17.10	15.40	19.20
Peroxide Value	meq/kg		<5.0	<5.0	<5.0	<5.0

As in Section 3.2 the results show that the raw material has slightly higher protin, but the composition of the various hydrolysates are quite similar. Calcium and phosphorus result are again problematic.

Physical Composition

The physical composition results are shown in Table 17. It was concluded that the reason for the different results for formic acid was due to the dissolving of the bones. Other results were similar.

Table 2

	% Fat	**% Aqueous**	**% Solids**
Heat only (55°C)	25.5	38.9	35.6
Formic Acid (2.5%)	13.6	37.0	49.4
Alcalase (0.05%)	20.5	41.0	38.6
Protamex (0.05%)	22.8	46.0	31.3

Degree of separation

After 24 hours, significant separation was only noted for the hydrolysate produced using acid hydrolysis with an average of 25% solute. Slight separation was observed in the Protamex hydrolysate and none in the Alcalase and no enzyme hydrolysates. At day 5, there was a distinct separation of solute and sludge, with 10% solute in the hydrolysate with Protamex and no enzymes. The percentage of solute at day 5 had increased to 35%. After 3 weeks, no changes were observed and the hydrolysate treated with Alcalase had not separated, with only a thin layer of oil at the top (Figure 10)

4.1.4: Summary and Next Steps

The results indicated that hydrolysis by alcalase and heating was a viable option for the SAMPI facility.

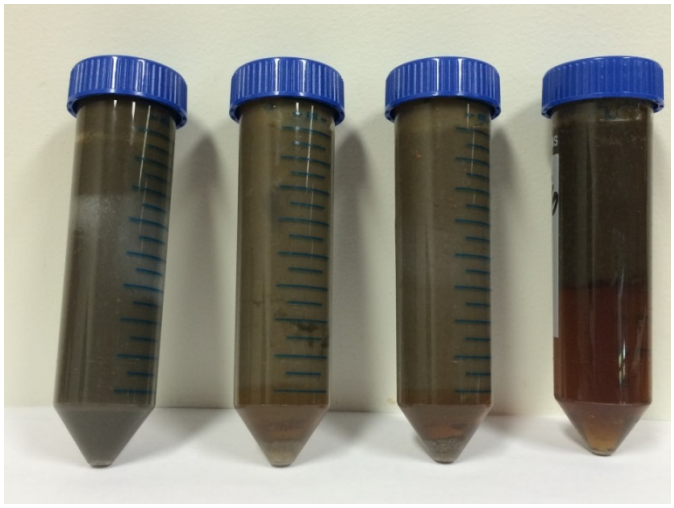

Figure 10 Separation of the tuna hydrolysate after 3 weeks. L-R: Alcalase; Protamex; No enzyme; Acid.

4.2 SAMPI Facility trials of Enzyme Hydrolysis.

4.2.1 Aim

Analyse the composition of the end products of enzymatic hydrolysis (using a commercial in confidence enzyme) of fresh tuna mince in the modified SAMPI facility.

4.2.2 Background

In 2015 the SAMPI processing facility in Port Lincoln changed their method of hydrolysing tuna waste material from acid to enzymatic hydrolysis. The addition of enzymes to the process accelerated the hydrolysis process, potentially leading to a higher quality product. It was estimated the enzymatic hydrolysis process at SAMPI could produce 10-20 tonnes of hydrolysate a day, a significant improvement on the previous acid process.

The new process facility layout and flow diagram is shown in Figure 11. The proposed new process for turning fish waste into hydrolysate is detailed below:

1. The waste, including gills, guts, mortalities are put through two mincers (20mm and 5mm).
2. The mince is pumped into the reactor tanks. If the mince is too thick to pass through the pumps, water is added to aid the process (~20-30L).
3. The mince is pumped through the heat exchanger and heated to 55C.
4. The enzyme is added to the reactor tank at a concentration of 400g/tonne of tuna mince. The mince is mixed until the enzymatic process is completed (around 10-20 minutes)
5. While maintaining the liquid hydrolysate at 55C, formic acid is added to lower the pH to 3.5 and mixed for 10 minutes.
6. The hydrolysed liquid tuna waste is passed through a 3 stage sieve, with bones and other undigested material removed.
7. The finished product (hydrolysate) is pumped into the 20-24 tonne holding tanks.

Photos of some of the new equipment are shown in Figure 10.

There is potential for the bone is removed at the 3 stage sieve to be further processed (eg run through rotary dryer and hammer mill to produce a bone powder). The results of trials with the hydrolysed bone are shown in a separate report.

Mincer	Mincer
	
Reactor tanks	Sieve
	

Figure 12: Some of the new equipment installed at the SAMPI factory.

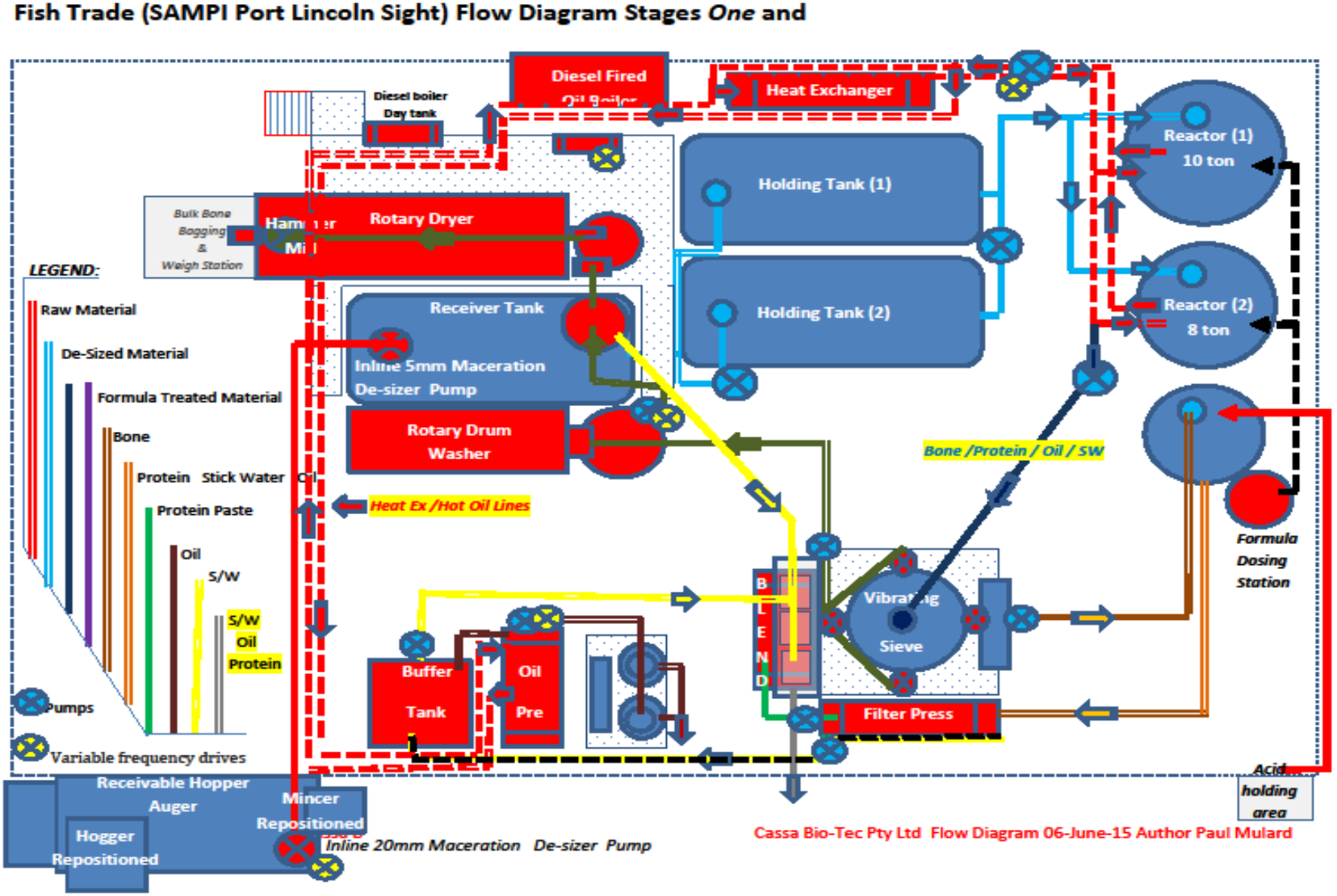

Figure 11. SAMPI upgraded facility layout and process flow diagram

4.2.3 Methods

Sample Preparation

At the SAMPI facility fresh tuna waste was minced, with the addition of water (~20-30L) and pumped into the reactor tank and heated to 55C. The enzymes were added into the reactor tank for each trial group at the concentration below:

- Nil
- 0.01% (100g/t)
- 0.02% (200g/t)
- 0.04% (400g/t)

The mince was maintained at 55°C with agitation and recirculation through the pumps until the mince was liquidised. Formic acid was added to the hydrolysed product to lower the pH to 3.5 and mixed for 10 minutes. The hydrolysed liquid tuna waste was passed through a 3 stage sieve, with bones and other undigested material removed.

At the end of the each batch the following components were collected and despatched to CESSH:

- Hydrolysate (Nil, 0.01%, 0.02% and 0.04% enzyme) after sieving.
- Bone meal (see separate report)
- Stick water

Physical Composition

At CESSH the hydrolysate samples were placed into 50mL tubes and centrifuged for 5mins at 4000rpm at 20°C. The approximate volume of the different components was measured and thus determined the percentage composition of aqueous, fats and solids.

Chemical Analyses

A NATA accredited laboratory conducted the following analyses:

- Hydrolysate at various enzyme concentrations :
    - Proximate composition,
    - FFA
    - Peroxide Value
    - Trace elements

4.2.4 Results and Discussion

Physical Analysis

The hydrolysate produced without enzymes was significantly different in physical composition (Table 18). It had the lowest fat and solids percentage, but the highest aqueous solution. Observably, the hydrolysate was a darker colour compared to the hydrolysates using enzyme hydrolysis (Figure 13).

Hydrolysate with 0.01% and 0.04% enzymes had the highest fat composition. Only one sample was available to centrifuge, meaning the results do not take into account sample variability, depending on when and how the samples was collected by the staff at SAMPI. In future trials, triplicate samples must be collected.

Table 18 Physical composition of the hydrolysate samples hydrolysed using varying concentrations of enzymes

Sample	% Composition		
	Fats	Aqueous	Solids
Nil	11.1%	83.3%	5.6%
0.01% Enzyme	18.2%	68.2%	13.6%
0.02% Enzyme	13.6%	72.7%	13.6%
0.04%Enzyme	18.2%	63.6%	18.2%

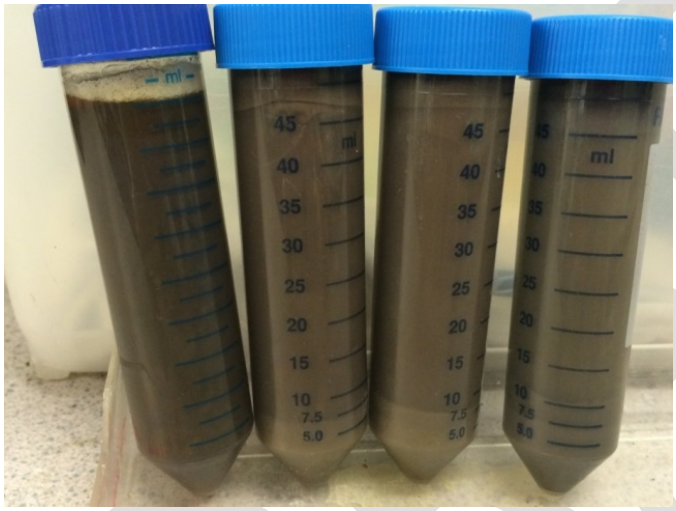

Figure 13. Centrifuged hydrolysate samples from L-R (no enzyme, 0.01%, 0.02% and 0.04% enzyme)

Chemical Analysis

The peroxide values for the hydrolysates with differing levels were all acceptable (<10meg/kg) as displayed in Table 19. However the FFA for all samples exceeded the acceptable limit set for crude oil (7%). The protein content was the highest for the hydrolysate with no enzymes, however it was lower than expected, when compared to Sections 3.1 and 3.2 which resulted in hydrolysates averaging approximately 15% total protein. The moisture content of all the hydrolysates using the new process was higher than the results in Section 3.1.

Table 3 Proximate composition and oil quality results for the different hydrolysates and stick water

Analysis	Unit of Measure	NIL	0.01% ENZYME	0.02% ENZYME	0.05% ENZYME
Ash	g/100g	2.3	1.7	1.9	2.1
Fat	g/100g	7.5	5	5.3	5.4
Moisture	g/100g	77.1	82.2	81.7	81.6
Nitrogen (Kjel)	%	1.65	1.36	1.37	1.33
Protein	g/100g	10.3	8.5	8.6	8.3

Free Fatty Acid	%	14.02	12.27	12.02	9.24
Peroxide Value	meg/kg	<5	<5	<5	<5

The enzyme hydrolysis process installed at SAMPI requires additional 20-30L water added to dissolve the enzyme. The additional water increases the moisture content and decreases all other components in the hydrolysate. The total protein content results for all trials are displayed on a wet basis. The total protein content of the hydrolysate and stick water calculated on a dry basis indicates that protein content is similar for all trials (Table 20)

Table 20 Hydrolysate total protein results- Wet basis vs dry basis

Trial	Sample description	Moisture Content (g/100g)	Total protein wet basis (g/100g)	Total protein dry basis (g/100g)
Trial 1	Unfinished product hydrolysate	61	13	33.3
	Early Harvest hydrolysate	70.1	13.3	44.5
	Late Harvest hydrolysate	71.8	14.2	50.4
Trial 5	No enzyme	77.1	10.3	45.0
	0.01% enzyme	82.2	8.5	47.8
	0.02% enzyme	81.7	8.6	47.0
	0.04% enzyme	81.6	8.3	45.1

Additional trace element analyses were conducted for the hydrolysate with 0.02% enzyme concentration, with the results shown in Table 21 and compared to previous SAMPI results and another commercially available product (Aquativ). In comparison to previous SAMPI results, the level of potassium, sodium, boron, iron, zinc and selenium decreased. The moisture content was significantly higher than the Aquativ specification and protein content lower than their minimum of >20%. The addition of water to the enzymatic hydrolysis process decreases the quantity of all other components.

Table 21 Trace element analyses of hydrolysate 0.02% enzyme compared with previous results and competitors product

Analysis	Unit of Measure	Sampi hydrolysate- old process	Sampi hydrolysate- current process	Thai hydrolysate product (Aquativ)
Protein	g/100g	19.6	8.6	>20
Fat	g/100g	5-15	5.3	<3
Moisture	g/100g		89.7	68
Nitrogen (Kj)	%	3.1	1.37	
Ash	g/100g		1.9	<12
Phosphorus	%	0.34	0.38	
Potassium	%	0.34	0.12	
Magnesium	%	0.05	0.041	
Sodium	%	0.25	0.17	
Calcium	%	0.18	0.5	
Sulphur	%	0.34	0.17	

Cadmium	mg/kg	nil	0.46	
Arsenic	mg/kg	nil	1.4	
Lead	mg/kg	nil	<0.02	
Boron	mg/kg	37.5	0.39	
Cobalt	mg/kg	0.27	<0.02	
Copper	mg/kg	2.6	1.1	
Iron	mg/kg	184	61	
Manganese	mg/kg	2	0.38	
Molybdenum	mg/kg	0.08	<0.05	
Selenium	mg/kg	8.9	3.7	
Zinc	mg/kg	43.9	18	
pH		3.5		2.7-3.1
Other additives				Phosphoric acid (E338). Citric acid (E330), ascorbic acid (E300), BHA (E320), propyl gallate (E310), potassium sorbate (E202),

4.2.5 Summary and Next Steps

The protein content of the hydrolysate using the new processing facility and commercial in confidence enzymes is lower than the required 15-20% for use as aquaculture feed. The quality of the oil was not acceptably with high Free Fatty Acid levels. The Commercial in confidence enzyme used for these trials was a powder and could only be added to the mince after heating to the processing temperature. Other enzymes that are more durable and liquid (eg alcalase) should be explored as well as ways remove the moisture from the end product or process.

4.3: Observation by CESSH scientist (Kerri Choo) to assist in further Improvements to the SAMPI processing facilities

Kerri Choo (Research officer, CESSH) visited the SAMP facility to provide advice (based on CESSH laboratory studies) to further improve the SAMPI enzyme hydrolysis process. Her observations are detailed in Table 22.

No.	Step	Description	Inputs	Outputs	Issues
1	Mincing	Raw material passes through 3 mincers of different sizes and into the receiver tank: 1. 25mm 2. 20mm 3. 5mm Water may be added to help pump mince through the different macerators via the water main (require pressure).	• Raw material- includes gills, guts, mortalities, gill plates and bone plates, heads • Fresh water. Volume dependant on the viscosity of mince.	5mm mince	Variability of volumes of water added each day, leading to variation in end product proximate composition. Water must be added in from main as pressure is essential to push mince through. No method to measure volume of water from mains.
2	Transfer from Receiver tanks to holding tank	Mince pumped to holding tanks with the capacity to hold 20-24t and remains for a maximum for 2 hours.			Only use one holding tank at a time.
3	Transfer from Holding tank to Reactor tank	Mince pumped into the reactor tank.	• 300-400L cleaning water from heat exchanger		Water used to clean heat exchanger each day ends up in the reactor tanks and can't be drained, further diluting end product. Only one reactor tank operational at any given time.
4	Enzyme type	Commercial in confidence enzyme	• 200g/t		Change to alcalase (better yields, temp management, cheaper price and lower addition levels).
5	Enzyme addition	Enzyme in formula dosing station is added to the reactor tank. Water used to remove any enzymes remaining in the formula dosing tank and enters reactor tank.	• 100-150L fresh water • 0.05% w/w enzyme		Water used to clean dosing tank further dilutes end product
6	Heating/Hydrolysis	Passes through heat exchanger until it reaches 55°C. Maintain at 55C for 10-20mins to complete hydrolysis		Liquidised mince	
7	Deactivate enzyme	Formic acid added to liquidised mince to reduce pH to 3.5 and heated for 10 mins @ 55C to inactivate enzyme.	• Formic Acid (volume unknown)	Unfinished product	Need validate parameters are sufficient to deactivate the enzyme.
8	Sieve	The unfinished product passes through a series of 3 sieves to remove bones and undigested material.		• Bones • Undigested material • Finished Product Hydrolysate	
9	Settle	Finished product hydrolysate is pumped into the finished product holding tanks and is left to decant for x hours?			

10	Decant	The finished product hydrolysate has separated into the distinct layers. Each layer is decanted into individual IBC's from the bottom up: solute, protein paste and oil		1. Solute 2. Protein Paste 3. Oil	
11	Final mixing	60% solute and 40% protein paste is poured into the IBC ready for storage.		Hydrolysate in IBC	Separation of the different components in the hydrolysate over time

Enzyme hydrolysis process

*Blue boxes indicates fresh water added into the process

Identify where water can enter the process?

1. Mincing: volume dependant on the pumping of the mince into the system
2. Cleaning Heat Exchanger: 300-400L fresh water to clean out every day. This water cannot be filtered out of the tank- therefore going into the reactor and included in the hydrolysis of the raw tuna mince. Cannot be used as it causes the exchanger to become sticky.
3. Formula Dosing Station: 100-150L to flush out the enzymes in the formula dosing tank enters hydrolysate

In total: at least 400L fresh water is added to the hydrolysate, this is best case scenario if no water is added during the mincing stage. This addition of water is resulting in variations in the protein content of the final product.

Without the addition of water to the hydrolysis process, the moisture content is around 65-68%. Any addition of water will dilute the hydrolysate increasing moisture content, adding no nutritional value and decreasing proximate composition of the product. Looking at Table 23 below, in a best case scenario of the 9t in the reactor tank, 4.4% of the hydrolysate produces is fresh water. In the worst case scenario, over 10% of the contents in the 7t reactor tank is fresh water.

Table 23: Impact of water addition to the hydrolysis process

Scenario	Step	Vol water added (L)	% additional water to 9t hydrolysate	% additional water to 7t hydrolysate
Best case	Mincing	0	4.4%	5.7%
	Cleaning heat exchanger	300		
	Enzyme dosing system	100		
	Total	400		
Worst Case	Mincing	200	8.3%	10.7%
	Cleaning heat exchanger	400		
	Enzyme dosing system	150		
	Total	750		

Suggestions to improve process and end product

- Remove some water from the solute to concentrate protein and other components
- Where possible, substitute the water with solute to increase the protein content. Changes can be made to the following steps:
    - Formula dosing tank: use solute to flush tank or find alternative way to add enzyme so water is not required (add directly to tank if possible)
    - Mincing- pressurised hose with solute
- Ability to drain reactor tank. Reduces water content by 300-400L if draining is possible. Heat exchanger needs to be flushed on a daily basis to ensure hydrolysate does not cake onto the plates. Once a week it is pulled apart.
- Precipitate protein from the solute and add to hydrolysate

4.4: Assessment of SAMPI Product after Process Improvements

Following the suggestions of Kerri Choo and company discussions, before the 2016 season, amendments were made to the process to use alcalase enzyme and restrict the addition of water to the hydrolysis process.

Samples from the new process were analysed and compared to previous hydrolysate results (Table 24).

Table 24: Summary of all analytical results for the different SAMPI hydrolysis processes.

Analysis	**Unit of Measure**	**Sampi hydrolysate-acid process (Section 3.1)**	**Sampi hydrolysate- C in C enzyme (Section 4.2)**	**SAMPI hydrolysate Alcalase Process (Section 4.4) (CESSH NMI)#**	**SAMPI hydrolysate Alcalase Process (Section 4.4) (FISHTRADE)***
Protein (wet)	g/100g	19.6	8.6	13.9	13
Protein (dry)	(%)			63.18	47.27
Fat	g/100g	5-15	5.3	10.4	3.6 (TOG)
Saturated Fat	g/100g			3.2	
Moisture	g/100g		89.7	78	72.5
Nitrogen (Kj)	%	3.1	1.37	2.1	2.11
Ash (wet)	g/100g		1.9	3.9	6
Ash (dry)	%				21.8
Phosphorus	%	0.34	0.38	1.5	1.51
Potassium	%	0.34	0.12	0.19	0.22
Magnesium	%	0.05	0.041		0.05
Sodium	%	0.25	0.17		0.29
Calcium	%	0.18	0.5	0.26	0.31
Sulphur	%	0.34	0.17		0.24
Cadmium	mg/kg	nil	0.46		
Arsenic	mg/kg	nil	1.4		
Lead	mg/kg	nil	<0.02		
Boron	mg/kg	37.5	0.39		<5
Cobalt	mg/kg	0.27	<0.02		<0.05
Copper	mg/kg	2.6	1.1		1.7
Iron	mg/kg	184	61		97
Manganese	mg/kg	2	0.38		<1
Molybdenum	mg/kg	0.08	<0.05		<2
Selenium	mg/kg	8.9	3.7	5	2.1
Zinc	mg/kg	43.9	18		14
Silicon	mg/kg				51
pH		3.5			3
FFA	% oleic			7.3	0.61
Electrical Conductivity	Ds/m				15.1
Total dissolved salts	Mg/L				10241
Specific Gravity	g/mL				1.08

E.coli	Cfu/mL				<1
Listeria	Cfu/mL				ND

and * Different analytical laboratories

#samples taken from drums of hydrolysate sent form PL to Perth. Unsure from what part of the drum

* Batch was made from tuna gills and guts harvested fresh that day with no more than 5% solute added as a thinning aid. No water added: sample collected on site and sent straight to lab (more representative of actual product).

The results were very different for the last two samples, the following explanations are offered.

a. Sample collection method may have varied - if the subsample was taken from the top of the drum without mixing the fat may have settled to the top, therefore leading to a higher % fat. Depending on the 'head' between the sample and the top of drum, the method of transport and movement may increase the exposure of oxygen to the sample- increasing the peroxide value and may explain the difference observed
b. Moisture content is very high compared to previous hydrolysate results, with only 5% solute added. This may be due to the solids separating to the bottom of the container, leading to more moisture being collected?
c. Oil quality results were disappointing (Table 25).

Table 25: Oil quality results for hydrolysate from new process

	Unit of Measure	**SAMPI hydrolysate New Process (CESSH NMI)**	**SAMPI hydrolysate New Process (FISHTRADE)**
Mono trans fat	g/100g	<0.1	
Mono Unsat fat	g/100g	2.4	
Omega 3	g/100g	3.5	
Omega 6		0.4	
Poly trans fat	g/100g	0.1	
Poly unsat fat	g/100g	3.9	
Trans fat	g/100g	0.1	
Peroxide	mEqo2/kg	100	
FFA	% oleic	7.3	0.61
Histamine	Mg/kg	52	

d. Free Amino acids were measured in both acid and enzyme hydrolysis samples and the results are shown in the Appendix.

5 Summary

The results demonstrated an improvement in the operation of the SAMPI facility in shifting from acid to enzyme hydrolysis. There was a slight difference in the compositional quality, the processing times were improved and the separation of the product on standing was reduced.

The original objective to extract a high quality oil was not achieved due to the changes in the raw material, the company objectives and difficulties in up-scaling the laboratory results to commercial facilities.

The new knowledge and the project facilitated other research including

a. Examination of options for processing bones and gill plates (see separate report)
b. Ongoing PhD study Muhammad Abu Bakar Siddik using tuna hydrolysate produced by enzymatic hydrolysis in juvenile barramundi feeding trials (data available in the future).
c. Examination of enzymatic hydrolysis as a waste treatment option in alternative scenarios
    a. On Board for Patagonian toothfish waste disposal
    b. Examination of toothfish hydrolysate for functional food ingredients
    c. In small retailers for waste disposal of common retail species such as Atlantic salmon and snapper.

Various aspects of the results were presented at three conferences in 2016.

A cost benefit analysis of the research and commercial outcomes is available in a separate report.

6. References

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Appendix

% of Total Fatty acids*	Unfinished Product (Dec 2013)				Early Harvest (April 2013)		Late Harvest (Aug 2013)		New Process Jun 2016
	Raw Material	Digested material	oil	Hydrolysate	oil	Hydrolysate	oil	Hydrolysate	Hydrolysate
C12:0 Lauric acid									0.2
C14:0 Myristic Acid	6.2	6.4	6.8	6.8		5.0	3.4	6.7	3.3
C14:1 Myristoleic Acid					2.2				
C15:1cis-10-Pentadecenoic Acid	0.9			0.7		0.6			0.6
C16:0 Palmitic Acid	27.3	31.2	29.3	26.8	30.5	25.3	31.0	26.2	18.5
C16:1cis Palmitoleic Acid	4.9	5.9	3.9	5.9	3.7	3.5	10.4	5.9	3.9
C17:0 Margaric									0.8
C17:0 Heptadecanoic Acid	0.7	0.8	0.7	0.7	0.7	0.5	0.7	0.6	
C17:1 cis-10-heptadecanoic Acid		0.7	0.9	0.9	0.8	0.8	0.6	1.0	
C18:0 Stearic Acid	7.9	8.0	8.4	8.5	10.3	9.1	6.3	7.6	7.5
C18:1cis Oleic Acid	25.2	27.5	28.4	25.7	38.3	32.9	29.5	28.0	17.9
C18:2cis Linoleic Acid- omega 6	1.9	1.9	2.1	1.8	2.1	2.5	1.2	1.3	1.6
C18:3 gamma Linoleic Acid	1	1.1	1.3	1.0		1.1	1.3	0.8	0.1
C18:3 alpha Linolenic Acid- ALA	2.2	1.0	1.8	0.8	0.9	0.8	1.6	1.2	1.2
C20:0 Arachidic Acid	2.3	1.3	1.4	2.0		1.5	1.4	1.5	0.4
C20:1 Eicosenic									1.6
C20:2 cis Eicosadienoic Acid(2w6)					0.5				0.3
C20:3 Eicosatrienoic)(3w6)									0.1
C20:3 Eicosatrienoic (3w3)									0.2
C20:4 Arachidonic Acid			0.7	0.4		0.3		0.8	1.5
C20:5 Eicosapentanoic Acid- EPA	7.3	6.3	3.7	6.8	4.1	5.9	5.2	7.7	9.4
C22:0 Behenic									0.2

C22:1 Erucic Acid	0.4	0.2		0.5	0.8	0.4	0.8	0.4	
C22:1 Docosenoic									0.2
C24:0 Lignoceric Acid	0.1					0.1			0.2
C24:1 Nervonic Acid	0.2		0.1		0.2	0.2	0.1	0.3	0.6
C22:6 Docosahexaenoic Acid- DHA	11.4	7.5	10.4	10.6	5.0	9.4	6.8	9.9	22
C22:4w6 Docosateetraenoic									0.2
C22:4w6 Docosapentaenoic									2.2

10.1 Fatty Acid Profile Chapters 3.1 and Chapters 4.4

* only records acids >0.1% component

Free amina acid results between acid and enzyme hydrolysis

	Units	**2014 acid hydrolysis**	**2016 enzyme hydrolysis**
Moisture	g/100mL	70.2	69
Protein	g/100mL	13.5	13.9
Histadine	mg/g	0.82	0.58
Asparagine	mg/g	0.08	<0.05
Serine	mg/g	1.03	0.89
Glutamine	mg/g	<0.05	<0.05
Arginine	mg/g	1.29	1.6
Glycine	mg/g	1.03	0.78
Aspartic Acid	mg/g	3	2.27
Glutamic Acid	mg/g	1.83	1.89
Threonine	mg/g	1.28	1.21
Alanine	mg/g	1.82	1.88
Proline	mg/g	0.49	0.69
Cysteine	mg/g	nd	Nd
Lysine	mg/g	1.63	1.77
Tyrosine	mg/g	1.54	1.46
Methionine	mg/g	1.10	1.17
Valine	mg/g	1.05	1.29
Isoleucine	mg/g	0.9	1.41
Leucine	mg/g	3.74	3.98
Phenylalanine	mg/g	1.82	1.98
Tryptophan	mg/g	Not tested	0.23
Taurine	mg/g	1.6	2.84
Total	mg/g	26.06	27.91