New Opportunities for Seafood Processing Waste

Appendix 3: SAMPI Tuna Hydrolysis Process: Options for Tuna Bones

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1. Background

SAMPI conduct a commercial enzyme hydrolysis process to produce liquid hydrolysate from tuna offal. This offal (currently gills, guts and mortalities) is produced on vessels following tuna harvest, and is delivered fresh to the SAMPI factory in Port Lincoln. Fish bones are a by-

product of this process and constitute 20-30% of the initial material. The bones are collected following removal from sieves at the end of hydrolysis process.

Fish bones are rich in Ca, P , Zn and Fe, and also contain Na, K, Cr. The Ca to P ratio ranges from 1.7 to 2.1. Bone mineralisation is associated with the hardness of the bone: the higher the Ca and P contents, the harder the bone. Fish bone also contains collagen which is the structural protein. Fish skin, bone and fins contain 36-54% collagen (Toppe, Albrektsen et al. 2007). Potentially fish bones as whole could be utilised as fish bone meal or the various components such as collagen and minerals could be separated and converted into different products (see Figure 1 below).

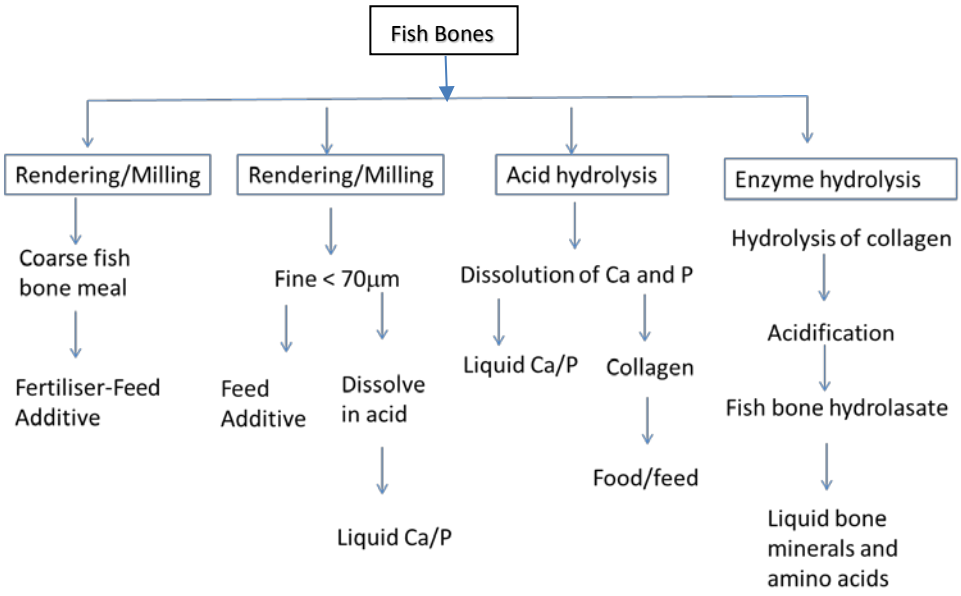


Figure 1 Fish bone utilisation options

Gill plates (the operculum) are bony structures covering and protecting gills in teleosts (Mackean 2016). In the Port Lincoln industry, gill plates are not currently hydrolysed at the SAMP facility, but are currently sent to land fill for disposal. There has been little reporting on composition of gill plates specifically but the mineral composition of gill plates were reported to be same as bones in Albacore (Goldberg 1962).

This research was undertaken at the request of SAMPI to try and identify processing and market opportunities for the fish bones resulting from the enzyme hydrolysis process nd for the gill plates. It is noteworthy that fish bone meal was rejected in the early stages of the work as a product outcome due to the high cost of drying and subsequent transport. Hence fish bone meal processes were not investigated.

Although there are multiple fish bone meal products in the market, research did not identify any liquid fish bone products. Liquid fertilisers are preferred by gardeners due to the ease of use and instant availability. Liquid bone products may result in better premium than powder products therefore producing a liquid fish bone was considered a possible option for utilising fish bones.

There is very little information on liquid bone meal products in the market, and therefore little information about how they were produced or the composition. Aggrand liquid bone from UDSA is made from meat processing by-product and reported to have NPK of (0-12-0) (Figure 2). No other information is available. Assuming 2:1 Ca:P ration in the bone, calcium content would be maximum 10%. Cost comparison of the liquid product with other dry bone meal product is shown in Table 1.

Liquid Bonemeal 0-12-0

Figure 2 Commercially available liquid bone products https://www.aggrand.com

Table 1 Retail online sale prices of fish bone meal products with liquid bone product (CIF)

		N	P	K	Ca (%)	Weight (kg)	Country	Value US$	Value AU$	AU$/kg
Meal	Dr Earth	3	18	0		1.1	USA	8.5	11.90	10.90
	Usfeed	3	16	0		3	USA	30	42.30	14.10
	Raindeer	10	16	4		2.72	Canada	40.75	41.16	15.13
	Alaska	5	6	1	10	5.5	USA	22	31.02	5.64
Liquid	Aggrand liquid bone meal	0	12	0		0.896	USA	9.44	13.31	14.86
		0	12	0		9.8	USA	76.79	108.30	11.00
		0	12	0		1041	USA	5645	7959.50	7.65

Another liquid calcium phosphate fertiliser available is called Micro-Phos from SoilSmart (NSW). It is a micronized form (<5µm) calcium phosphate in complex with Fulvic acid to enhance nutrient availability. It contains 26% Ca and 10% P (P_2O5). It is a micronized minerals and blended with organic suspension agents including Fulvic acid, and organic chelating agents which are preconditioned for immediate release.

There is also home-made recipes for making liquid bone fertiliser (http://homeguides.sfgate.com/make-liquid-bone-meal-fertilizer-40172.html). The recipe involves heating fish bones, grinding and boiling pulverised bone in water.

The main challenges with formulating liquid bone is the very low solubility of Calcium phosphate in water (see Table 2)

Table 2 Solubility of different calcium salts

Solubility	% (w/w)
Calcium Chloride	43
Calcium Sulfate	0.3
Calcium Bicarbonate	0.08
Calcium Carbonate	0.005
Calcium Phosphate	0.0003

Solubility will increase with reducing pH (Figure 3) but reducing pH alone is not considered enough to prepare concentrated solutions. Other solubilising strategies may need to be employed.

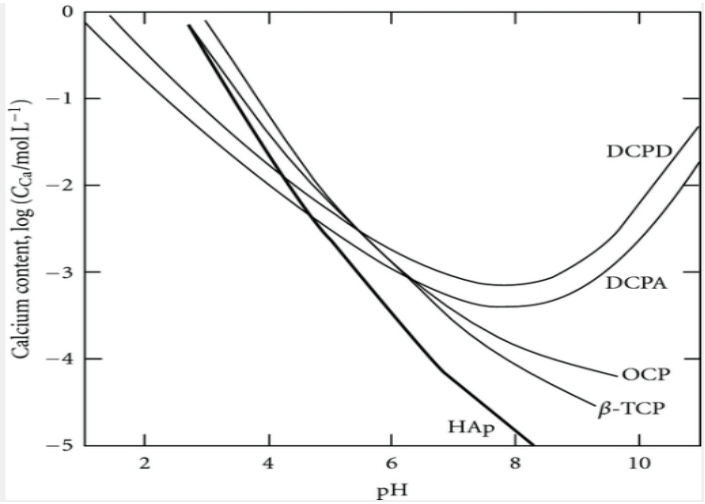


Figure 3 Solubility of different Calcium phosphates at 37C as a function of pH (Kurado and Okido)

Acid digestion over a long period using fulvic acid was reported to be the method used in liquefying bone by one manufacturer (Extensions 2015). Fulvic acid is actually a lower molecular weight component of humic acid which is produced by biodegradation of dead organic materials such as humus, peat and coal. It is also a major organic constituent of many upland streams, dystrophic lakes, and ocean water. It is not a single acid; rather, it is a complex mixture of many different acids containing carboxyl and phenolate groups so that the mixture behaves functionally as a dibasic acid or, occasionally, as a tribasic acid (Figure 4). Humic acids can form complexes with ions that are commonly found in the environment creating humic colloids. Humic and fulvic acids are commonly used as a soil supplement in agriculture.

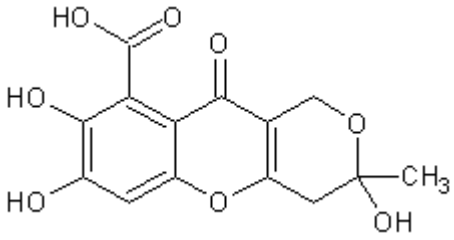


Figure 4 Hypothetical model structure of Fulvic acid (LookChem)

Fish bone can also potentially be liquefied by either acid/base or enzymatic hydrolysis. Acid hydrolysis of fish frames and waste has been reported. Acid or alkaline treatment of bone results in the breakdown of the complex structural proteins to smaller peptides and amino acids. Enzymatic hydrolysis of fish bones is relatively new compared to acid/base hydrolysis. It was reported using different proteases such as papain, pepsin, trypsin, alcalase and neutrase (Je, Quin et al. 2007). Hydrolysates from fish bone were reported to have antiradical activity and can potentially be used as a food or feed ingredient.

The objective of this study was to identify processing strategies to liquefy tuna bones and gill plates and to assess the market opportunities for the end-products. The work was divided into three sections

Chapter 2: Liquefying Dried Fish Bone produced in 2015 using the previous acid hydrolysis process.
Chapter 3: Liquefying bone material produced in 2016 from the current SAMPI enzyjme hydrolysis process.
Chapter 4: Processing Options for the Gill Plates.

2. Trial 1: Liquefying Of Dried Fish Bone Chips

2.1 Objectives

In Trial 1 dried fish bone from 2015 were supplied by SAMPI.

The objectives of this initial trial was:

1. To determine the proximate composition of dried fish bones (2015)
2. To investigate methods to liquefy dried fish bones with a view to developing a liquid bone meal fertiliser.

2.2 Materials and Methodology

Dried fish bones were provided by SAMPI.

2.2.1 Compositional studies

Compositional analysis of the dried fish bone was undertaken by a NATA accredited laboratory.

2.2.2 Preparation of samples.

Tuna fish bones are coarse and hard, therefore it is difficult to grind and reduce the particle size. As well as using as received, four different preparation methods were attempted:

1. Grinding: hand held grinder (low energy), coffee grinder (high energy)
2. Heating: 140°C for four hours
3. Steaming: Steamed in a conventional kitchen pressure cooker for 1 hour at 14 psi
4. Ashing: 660°C for 16 hours

2.2.3 Liquefying of fish bone

a. Liquefying in acid and oxidising agents.

Tuna bone prepared with different methods (2.2.2) were digested in different acids and oxidising agents to produce liquid fish bone. Degree of digestion and precipitate formation was observed and recorded. The mineral composition of some of the resulting liquid products were measured using ICP-AES.

The acid and oxidising agents used to liquefy were: fulvic, sulfuric and phosphoric Acid, Sodium Hydroxide and Urea.

b. Home recipe testing.

The home recipe for preparing liquid bone meal fertiliser (Gerard 2015) was also tried. The method was:

- Collect sufficient amount of bones including chicken, turkey, beef etc
- Remove fat and meat as much as possible by physically or boiling in hot water
- Heat bones in an oven at 140C for at least 3 hours. Pressure cooker can also be used: 15psi for 30 mins).
- Pound the bones with metal mullet to break them into smaller pieces
- Grind the bones with a stone grinder, mortar and pestle or other grinding method used for grinding minerals or hard materials.
- Mix the bone meal with water in a pot over medium heat. Continue mixing until no bone meal settles and cool.

c. Enzymatic hydrolysis of fish bone.

Enzymatic hydrolysis of the fish bone meal was performed using Alcalase Enzyme, at 60°C followed by 10 minutes of boiling for enzyme deactivation (Ren et al 2012). Enzyme concentrations between 0.5-5.3% were applied and compared with no enzyme treatment (control). The experiments were completed in a Sunbeam Sous Vide.

d. Demineralisation of fish bone

Demineralisation of bone using HCl, Vinegar and EDTA were reported in the literature. HCl, vinegar, EDTA and EDAT-vinegar were tried to demineralise bone meal.

2.3 Results

2.3.1 Compositional studies

The results from the SAMPI bone meal analysis are shown in Table 3.

Table 3 Proximate composition of SAMPI bone

Analysis	Unit of Measure	SAMPI
Protein	g/100g	25.6 (Kjel N =4.10)
Fat	g/100g	0.3
Moisture	%	
Salt	%	
Crude Ash	%	
Phosphorus	%	10
Potassium	%	0.11
Sulphur	%	0.18
Calcium	%	19
Magnesium	mg/kg	4300
Manganese	mg/kg	41
Chromium	mg/kg	
Mercury	mg/kg	0.08
Cadmium	mg/kg	1.2
Arsenic	mg/kg	0.44
Lead	mg/kg	16
Selenium	mg/kg	2
Sand	%	
TVC		
Melamine		

2.3.2 Preparation of Fish Bone meal samples

Fish bone meal was coarse and difficult to grind. To achieve a small particle size required powerful and extended grinding. Heat treated FBM was significantly easier to grind (1 min compared to 6 min grinding to achieve similar size). Steaming fish bone meal resulted in softer texture and was a lot easier to grind than heat treated fish bones and produced smaller particle size (see Figure 5).

As received	Low energy grinding
	
Higher energy grinding	Heating with low energy grinding
	
Steamed SAMPI FBM	Ground Steamed FBM

Figure 5: Dried bone after various preparation techniques

Ashing (550°C for 16 hrs) removes all the organic components in the bone therefore resulting ash contains oxide forms of minerals in the bone such as CaO, Phosphates etc. Ashed FBM still retained the shapes of the bone meal and even with acid digestion was not easily pulverised (Figure 6).

Figure 6 Ashed SAMPI FBM

2.3.3 Liquefying of fish bone meal.

a. Digestion with Acids and oxidising agents

Digestion with Fulvic Acid

Results following attempted digestion in fulvic acid are shown in Table 4. Analytical results are separated into the filtrate (liquid layer) and solid layer.

Table 4: Results of Tuna Bone Liquefying in Fulvic Acid

Preparation	Ratio bone:acid	Analysis	pH	Comments
Ground	20g/80g	Liquid: 0.3% Ca, 1.1 % P	3.83	Keep pH below 3.4 and at ratio of 20:80 or mould growth occurs. Calcium in filtrate very low (ie not liquefying)
Heated and ground	20g/80g	Homogenised sample 4.2 % Ca 4.4% P	3.54	Partial liquefying
Ashed	10g/40g	liquid 0.5% Ca 1.7% P	3.28	Liquefied
AShed	10g/40g (with ultrasonication for two hours)	Homogenate, ultrasonicate, ground with mortar and pestle 6.8% Ca 5.5% P	3.23	Liquefied

When compared to the commercially available liquid bone Aggrand, the digestion of the ground product in fulvic acid contained half the Ca and slightly less P. It appears that the bone is not liquefying effectively in the acid, with a liquid layer and calcium remaining in the precipitate, particularly after settling (Figure 7).

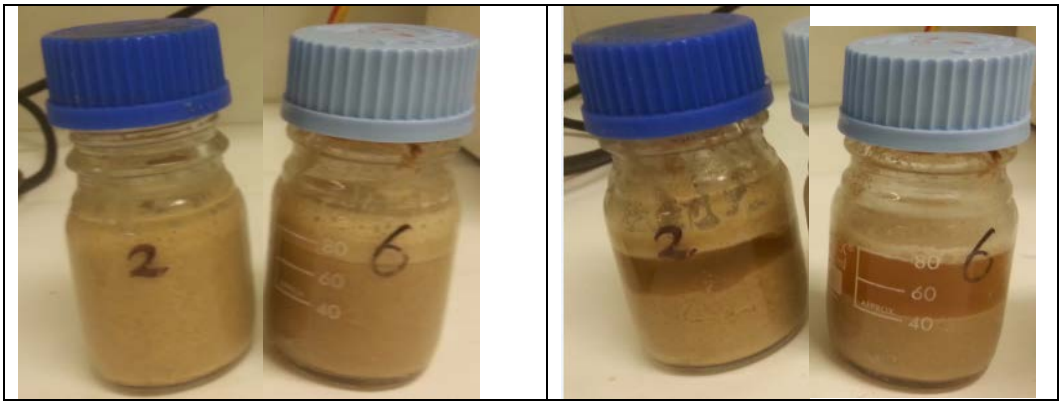


Figure 7 Ground FBM in fulvic acid after mixing (left) and 1 hour settling

Ashed fish bone in fulvic acid appeared to liquefy, with no settling after an hour (Figure 8). The ashed liquid bone had (0-12-0) NPK values similar to Aggrand.

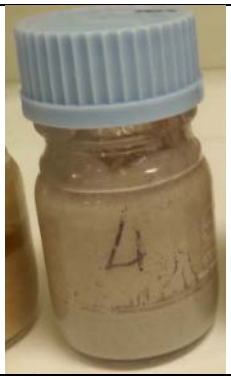
Before homogenisation	After homogenisation	One hour after homogenisation
		

Figure 8 Ashed FBM in Fulvic acid and the effect of mixing

Digestion with other acids and bases.

2 g each of fish bones (Untreated bone (A), Ground Bone (B), Cooked and Ground bone (C) were digested with 5-10g of different acids and bases. The digestion treatments were fulvic acid (1), Sulfuric acid (2), Metaphosphoric Acid (3), NaOH 40% (4)and Urea (5). The urea solution was prepared by dissolving 10g Urea in 10g water (heated up to 100°C) and the pH of solution was is 4.16.

Sulfuric and Phosphoric acid and Sodium Hydroxide all hydrolysed the bones completely, there was partial liquefaction with fulvic acid but mould growth was observed. Urea did not digest the bones (Table 5). The analytical results showed that the calcium content of the liquefied bones was 4%, as the dilution factor was 5 times these results were as expected (Table 6).

Table 5 Liquefying of 2g of FBM with different treatment and oxidising agents

		Weight (g)	pH	
1A	Fulvic acid	5	3.74	Not enough liquid Went mouldy and discarded
1B		5		
1C		5		
2A	H_2SO_4	5	0.0	Liquefied but H_2SO_4 burned the bones and turned solid. 5ml FA is added to liquefy
2B		5		
2C		5.3		
3A	Metaphosphoric acid	5+5+5	0.34	Liquefied
3B		5+5+5		
3C		5+5+5		
4A	NaOH	10	12	Liquefied
4B		10		
4C		10		
5A	Urea (40% soln)	5	6.91	5 g FA is added to reduce the pH to 3.25. Did not liquefy
5B		5		
5C		5		

Table 6 Estimation of Calcium contents of digested bones in different oxidising agents

	H_2SO_4	Phosp. Acid	NaOH	Urea (added FA to liquefy)
Ratio (bone:agent)	2:10	2:15	2:10	2:10
pH	0	0.34	12	6.91 5 g FA is added to reduce the pH to 3.25
%Ca	3.68	2.57	3.64	3.68
%P	2.53	2.00	2.53	2.53
%N	5.06	3.57	5.06	5.06
NPK	5-5.8-0	3.6-4.6-0	5-5.8-0	5-5.8-0

On average 5 times the weight of the dried fish bone is required for digestion and formation of a liquid product. The results indicate that NPK vales of around (5-6-0) can potentially be produced by acid digestion of the bone meal.

b. Homemade recipe for liquid fish bone

Home made liquid fish bone recipe was attempted but bones were not fully solubilised by the process. This experiment will not be repeated.

Ground SAMPI FBM

↓

Heated to 140C for 4 hrs

↓

Ground with coffee grinder

↓

Boiled in water and reduced volume

↓

Strained

↓

Fulvic Acid is added

0.7 % Ca
1% P
pH 3.20
20g/120g water and 33g FA

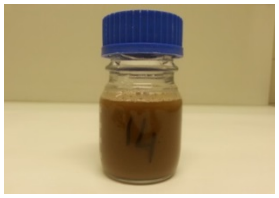


Figure 9 Homemade liquid fish bone recipe and results

c. Enzymatic hydrolysis of dried bone

The enzymatic hydrolysis of the fish bone did not result in any observable breakdown of the bone (Figure 10). However, the total solid content of the supernatant increased with increasing enzyme concentration which may indicate some degree of hydrolysis did occur (Table). This experiment needs to be repeated. The composition of the supernatant needs to be determined to understand how enzymes are affecting the bone meal: either amino acids or minerals may be released from softened collagen structures.

Figure 10 FBM after enzymatic hydrolysis

Table 7 Results of enzymatic hydrolysis of FBM

Description Enzyme (%)	bones (g)	Water (g)	Enzyme (g)	Filtrate Dry Weight (g)	Supernatant TS (%)
Control	10	50	0	8.06	0.80
0.5%	10	50	0.0488	8.00	1.29
1%	10	50	0.1097	7.49	1.51
2%	10	50	0.2143	7.77	1.52
5.3%	10	50	0.5510	8.06	2.31

d. Demineralisation of bone meal

Demineralisation of bone using HCl, Vinegar and EDTA were reported in the literature. HCl, vinegar, EDTA and EDTA-vinegar were tried to demineralise bone meal. Details are given in Table 8. Around 10-20 % reduction in bone meal weight was obtained after 24 hours. HCl was the least effective treatment under the conditions tested. EDTA was the most effective demineraliser followed by EDTA + vinegar (Table 9 and Figure 11).

Table 8 Demineralisation treatments applied to dried bone meal

Bones	Reagent	Reagent weight	pH	Condition	Duration
10	HCL, 0.1N	83	3.5	Stirred	24 hrs
10	Vinegar	83	3.64	Stagnant	25 hrs
10	EDTA, 0.15M	84	4.5	Stirred	26 hrs
10	EDTA+Vinegar	1 g EDTA+84g	3.56	Stagnant	27 hrs

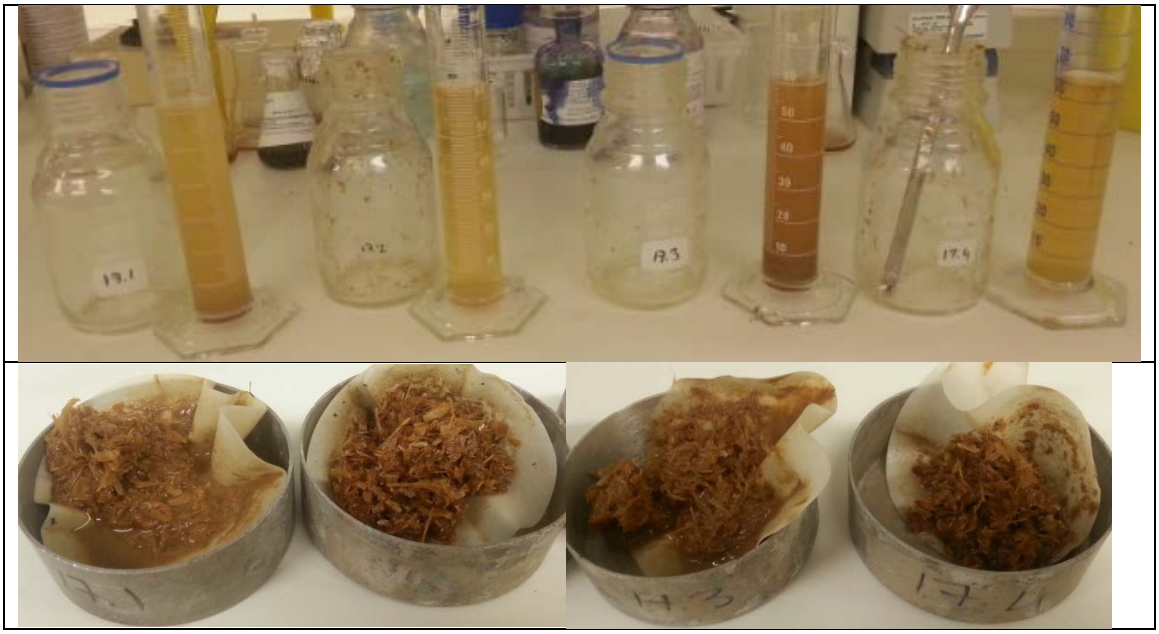


Figure 11 Demineralisation of FBM with HCl, vinegar, EDTA and EDTA+Vinegar

Table 9 Results of demineralisation experiments

Treatment	Weight reduction in FBM (%)	Supernatant Total Solid (%)	Supernatant Ash content (%)
HCL	10.6	1.31	0.19
Vinegar	18.5	3.89	1.17
EDTA	11.4	3.23*	2.66
EDTA + Vinegar	17.1	3.60	1.50

*EDTA contribution removed

The calcium and Phosphorus content of the supernatant is 2 and 1 % respectively. By concentration the supernatant 5 times, NPK value of (0-12-0) with 5% Calcium can be obtained which is similar to Aggrand Liquid Bone. Fulvic acid or EDTA would need to be added as a chelator to complex Calcium.

2.4 Conclusions and Next Steps

Size reduction/hardness of the bone chips is one of the challenges in liquid bone meal production. Untreated, dried bone meal is very difficult to grind. Heat treatment at 140˚C for 4 hours softens the bones, steaming for one hour was even more effective. Ashing was effective but not commercially feasible.

The preliminary results indicated that rapid liquefying of the non-ashed bone could be achieved with the addition of sulphuric and phosphoric acid and sodium hydroxide. Fulvic acid could be used to liquefy with longer storage times or can potentially be added to

liquefy and chelate Calcium afterwards. Liquefying with urea, demineralisation and enzymatic hydrolysis were not considered for further investigation.

When the organic content of bones are removed by ashing, liquid bone was homogeneous and the NPK value was be (0-9-0) with 7% Calcium. With evaporation, some increase in mineral content could be achieved but would not be a substantial increase if liquid bone is desired. Ashing is not recommended but it is noteworthy that it does represent the highest mineral content achievable with only addition of Fulvic Acid as a chelating agent.

3. Trials With Sampi Process Bones (2016)

A new set of liquefying trials was undertaken using the actual wet bones removed immediately from the sieve after the enzyme hydrolysis process was instigated at the SAMPI factory in 2016. Samples were also taken of bones from the sieve after drying at 130˚C. The wet and dry bone samples were frozen in 5kg blocks and transported to Curtin University.

3.1 Methods and Results

3.1.1 Compositional analysis

Wet bone samples were sent to NMI for analysis. The results as well as those from the dried bones used in the previous trial are shown in Table 10.

Table 10: Compositional results for SAMPI fish bone.

Component	**Unit**	**Dried tuna bone fragments (2015)**	**Sieved Bones (after SAMPI hydrolysis) (2016)**
Protein (Kjel N =4.10)	g/100g	25.6	18.9
Fat	g/100g	0.3	3.6
Moisture	%	15.2	58.6
Crude Ash	%	52.6	13.4
Phosphorus	mg/kg	100000	6500
Potassium	mg/kg	1100	490
Sulphur	mg/kg	1800	670
Calcium	mg/kg	190000	12000
Magnesium	mg/kg	4300	
Manganese	mg/kg	41	
Mercury	mg/kg	0.08	
Cadmium	mg/kg	1.2	

Arsenic	mg/kg	0.44	
Lead	mg/kg	16	
Selenium	mg/kg	2	

3.1.2 Bone Liquefying Trials

Trial 1

Results from Section 2 showed that some liquefying occurred with fulvic acid and full liquefaction with NaOH, Phosphoric acid and Sulphuric acid.

It was decided to repeat the trial with the actual bones out of the sieve but using only fulvic acid and phosphoric acid, as it was considered that the use of sulphuric acid or sodium hydroxide would not be feasible at the factory due to occupational health and safety issues.

For these trials, frozen Southern Bluefin Tuna bone that has undergone hydrolysis with a protease enzyme was supplied from SAMPI. The bone has been directly removed from the 5mm sieve. Bone was defrosted for 24 hours at 4°C before treatment (Figure 12).

Figure 12 Thawed southern Bluefin tuna bone

Table 11 shows the experimental design and results for the trials. Trials were conducted in 100mL plastic containers and stored for one month with weekly observations.

Table 11 Digestion trials on 2016 Bones

Number	Sample of bone	Fulvic acid	Phosphoric acid	Temp	Grinding	Results time
1	**Wet 5g**			**RT**	**No**	**No Liquefying, off odour after one month**
2	**Dry 5g**			**RT**	**NO**	**No liquefying, off odour after one month**
3	**Wet 5g**	**25 ml**		**RT**	**No**	**No Liquefying after one month**

4	**Dry 5g**	**25mL**		**RT**	**NO**	**No Liquefying after one month**
5	**Wet 5g**		**45 mL**	**RT**	**No**	**Fully liquefied in 7 days**
6	**Dry 5g**		**45mL**	**RT**	**No**	**Fully liquefied in 7 days**

Full liquefaction was demonstrated with phosphoric acid after 7 days. No Liquefactin was observed with fulvic acid.
The factory manager set up a similar "informal" trial with phosphoric acid and the same results after one month were observed (see Figure 13).

Figure 13 Factory trials with bones in phosphoric acid: A: not shaken, and B: shaken.

Trial 2

In Trial 1 fulvic acid and water were ineffective at hydrolysing the bone. However, the use of pure phosphoric acid successfully liquidised the bone. Trial 1 had a 4:1 ratio of phosphoric acid to bone, and the bone had completely digested in less than one week. In a commercial IBC, a 1:3 ratio of phosphoric acid to bone was digested the bone after one month.

New trials were designed to determine the minimum quantity of 100% phosphoric acid required to liquidise tuna bone.

Defrosted tuna bone was weighed into 50mL centrifuge tubes with the correct weight of water and phosphoric acid required for each treatment, as outlined in Table 12. Each tube was shaken to mix the contents. Observations for each treatment were recorded at 1 hour, 1 day, 3 days, 1 week, 2 weeks and 1 month after treatment.

Table 12 Different treatment groups in Trial 2.

Treatment (acid:bone)	Phosphoric Acid (g)	Bone (g)	Water (g)
Control (1:1 water:bone)	-	12.5	12.5
1:1	12.5	12.5	-
1:2	12.5	25	-
1:3	5	15	-
1:4	5	20	-
2:1	15	7.5	-
3:1	15	5	-
4:1	20	5	-
1:3:0.5 water	2.5	15	5

1:1:1 water	15	15	15

The observations for each treatment group over 1 month of ambient storage are noted in Table 13. Treatments with less than one time the weight of acid to bone (eg 1:3)did not digest all the bone material. The acid was absorbed by the bone, leaving no liquid in which the bone could be soaked (Figure 13).

Figure 13 Samples with 1:3 acid to bone ratio without water (left) and with water (right) after 24 hours.

The 1:1 acid to bone ratio was able to digest most of the bone after 2 weeks but the end product is very thick and is not pourable. There are still small fragments of bone that remained in the 1:1 treatment after 1 month. After 3 days of treatment 2:1, 3:1 and 4:1 phosphoric acid to bone ratio had liquefied most of the bone (Figure 14)

Figure 14 Treatments with 2:1. 3:1 and 4:1 acid to bone after 24 hours digestion and agitation.

The consistency of the 3:1 and 4:1 sample was pourable. There were also two distinct separate layers: acid and bone. The 2:1 sample was still quite thick in consistency. After one week the 2:1, 3:1 and 4:1 acid to bone treatments have completely digested the bone and had a thin consistency with a creamy brown colour. At two weeks the digested bone component in the tube was a homogenous cream brown colour in the three samples (Figure 15). After one month, there was not observable change in those samples (Figure 16).

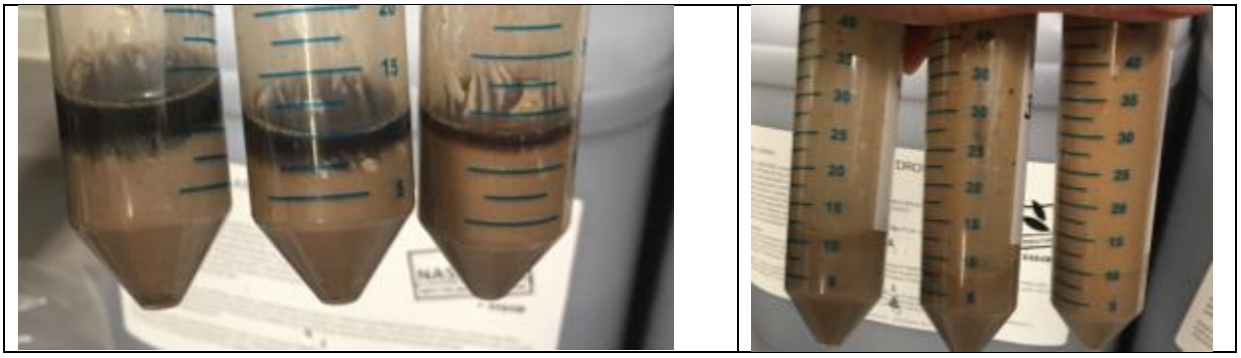


Figure 15 Treatments 4:1, 3:1 and 2:1 acid to bone after two weeks digestion before (left) and after agitation(right).

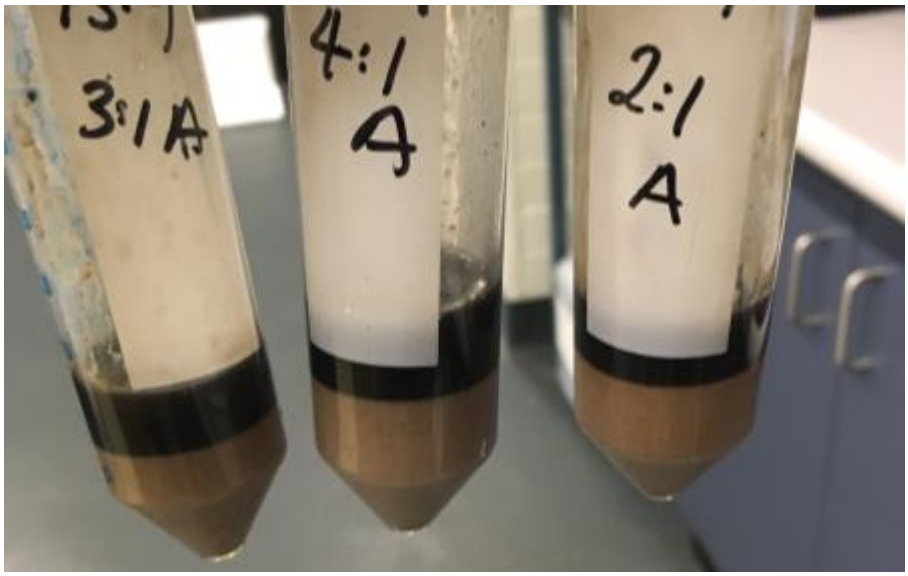


Figure 16 Treatment 3:1, 4:1 and 2:1 acid to bone after one month digestion at in ambient conditions.

Treatment 1:3:0.5 (acid: bone: water) and 1:3 (acid: bone) had the same weight of bone and acid, with additional water was added to the first sample. The addition of water decreased the acid concentration but allowed for more bone to be exposed to the acid. The dilution of the acid with some water enabled more of the bone to be hydrolysed than 1:3, although it was only contained to the bottom of the tube. There was not enough liquid or acid to digest the remaining bone matter.

Table 12 Trial 2 Observations of each treatment

Treatment (acid:bone)	1 hour	1 day	3 days	1 week	2 weeks	1 month
Control (1:1) (water:bone)	No change in appearance. Visually can distinguish between the bones and liquid.	No change	No change	No change	No change	No change
1:1	Light brown colour. Some of the bone has been dissolved but not much liquid remains. Bones are clumped together and of cloudy appearance.	Clumped together at the bottom of the tube. Similar to 1 hour observation, but more bone digested	A thick paste, with some undigested bone pieces remaining	Thick paste with most bone digested	Slightly thinner solution. Still thick and requires strong shake. More bone digested. Light cream brown colour	Most of the bone has digested with a thin layer of acid on top which is runny. There are still little thin pieces that remain. The digested bone is very thick and is not pourable
1:2	Quite compact at bottom of tube. Liquid holding it together. Slightly cloudy and hydrolysis has occurred minimally	Slight clumping at bottom that has started to hydrolyse. Most of the bone appears dry and absorbed the acid	No change	No change	No change	No change
1:3	similar to 1:4 but there is more hydrolysed material at the bottom of the tube. Relatively dry	Dry, minimal hydrolysis. similar to 1:4 and 1:2	No change	No change	No change	No change
1:4	fairly dry, does not appear that much hydrolysis has occurred.	Dry, minimal hydrolysis. Similar to 1:3 and 1:2	No change	No change	No change	No change
2:1	created a cloudy mixture and the bones look like they have hydrolysed the most. Still plenty of chunks of bone visible.	Cream brown colour with some white particles- undigested bone. Similar to 3:1 and 4:1 but thicker liquid	Hydrolysed bones. Same stage as 4:1 and 3:1. Thick liquid. Two separate layers- acid and digested bone	Liquid. Homogenous liquid cream brown colour. Pouring consistency. Distinct layers visible	Thinner solution than at one week. Distinct separation. Dark brown, caramel colour	No change- liquised with no small particles present
3:1	similar appearance to 2:1 but it is less cloudy in appearance, fair amount of liquid present.	Hydrolysed bones. Same stage as 4:1 and 2:1. Thinner than 2:1. Two separate layers- acid and digested bone	Separate layers on the top of acid and digested bone.	12.5ML digested 2ml acid Dark brown, caramel colour		No change. Liquidised with no small particles present

Treatment (acid:bone)	1 hour	1 day	3 days	1 week	2 weeks	1 month
4:1	similar to 3:1 but more liquid and less cloudy in appearance. Les hydrolysed than 3:1 and 2:1.	Thinnest sample. Still some bits of bone left. Cream brown colour with some white particles.	Separate layers on the top- acid and bone. Small fragments of bone remain.	Separate layers. Liquid. Same appearance as 3:1 and 2:1. Dark brown, caramel colour		No change. Liquidised with no small particles present
1:3:0.5 water	darker brown in colour. Bones clumping together. Not much liquid visible	More bone digested than 1:3, but still large pieces of undigested bone present. Not much liquid available.	No change.	Some liquid but still relatively dry. Some hydrolysis at the bottom of the tube only.	Some digested bone at bottom. No excess liquid	No change
1:1:1 water	dark brown liquid that is quite watery. Some of the bone has hydrolysed but there are large chunks still remaining	Runny thin brown liquid with undigested bone present	No change, still plenty of bone undigested	No change. Liquid but still plenty of bone not hydrolysed.		More bone has digested but still large chunks visible

Although more bone was exposed to the acid, if too much water is added, the solution is too dilute, therefore decreasing the effectiveness of the acid to digest the bone material. This was observed when comparing treatment 1:1:1 (acid:bone:water) and 1:1 (acid:bone). At a 1:1 acid to bone ratio, most of the bone was able to hydrolyse, although it was thick in consistency. With the addition of the equal weight of water, all the bone material is covered in liquid, however, the solution was no longer acid enough to break down the bone. (Figure 17).

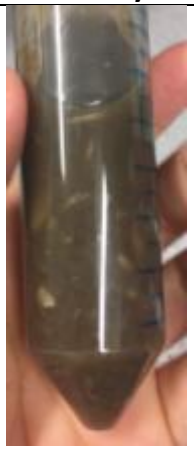
1:1:1 acid to bone to water treatment		1:1 acid to bone treatment	
Day 0	Day 7	Day 0	Day 7
			

Figure 17 Treatments with 1:1 ratio acid to bone and 1:1:1 acid to bone to water on day 0 and day 7 of digestion at ambient conditions.

From the results, the volume of liquid and acid concentration is critical in determining the rate of bone hydrolysis.

Summary

The rate of bone digestion is dependent on the concentration of acid and amount of liquid. The minimum amount of pure phosphoric acid that is required to digest the bone to form a liquid bone solution is a 2:1 ratio of acid to bone. At 1:1 ratio acid to bone, the solution is very thick after 1 month and not all the bone has been digested. Further work is required to determine the optimal conditions of digesting the bone in less than one month, with the potential to add water to decrease the volume of acid required.

Trial 3

The third trial aimed to determine the effect of temperature and concentration of phosphoric acid on the hydrolysis of bone material.

Methods and Results

Defrosted tuna bone was weighed into 200mL containers, with specified weight of water and phosphoric acid added to each treatment, as outlined in Table 14 before shaking the contents of each container until well mixed. The samples to undergo heat treatment were

placed in a water bath at 60°C for 2 hours before placing the samples in ambient conditions for the remainder of the trial Observations of each treatment were recorded after 2 hours, 1 day, 2 days, 1 week, 2 weeks and 1 month after treatment.

Table 14 Treatment groups.

#	Treatment Name	Ratio Acid: bone: water	Acid (g)	Bone (g)	Distilled water (g)	Temperature
1	Control 1A	2:1:0	50	25	0	Ambient
2	2A	0.75:1:0.25	37.5	50	12.5	Ambient
3	3H	0.75:1:0.25	37.5	50	12.5	60°C for 2h then ambient
4	4A	0.5:1:0.5	12.5	25	12.5	Ambient
5	5H	0.5:1:0.5	12.5	25	12.5	60°C for 2h then ambient
6	6A	1.5:1:0.5	37.5	25	12.5	Ambient
7	7H	1.5:1:0.5	37.5	25	12.5	60°C for 2h then ambient
8	8A	1:2:0.5	25	50	12.5	Ambient
9	9H	1:2:0.5	25	50	12.5	60°C for 2h then ambient
10	10A	1:1	25	25	0	Ambient

Effect of acid concentration on digestion rate

The observations of all the treatment are shown in Table 15. Table 16 summarises the different treatments. The results indicate the acid concentration and the volume of solution effect the digestion of bone. An acid concentration of 100% was effective at digesting the bone material within a month, but doubling the acid volume halved the time to 2 weeks. A similar outcome was noted with samples with 75% concentration of acid. The sample with a 1:1 ratio of acid solution to bone had not completely digested after one month, but was in liquid form. However the sample with 2:1 ratio of acid solution to bone with 75% acid concentration had reached that same stage by 2 weeks and at 1 month it was completely digested. At 67% and 50% acid concentration the bone material was not digested.

Table 16 Summary of results from trial 2 after one month storage in ambient conditions

Ratio by weight (acid solution : bone)	Treatment	ACID SOLUTION CONCENTRATION	BONE DIGESTION AFTER ONE MONTH
1:1	10A	100%	Completely digested @1 month
	2A, 3H	75%	Almost completely digested @1 month-very small fragments remained
	4A, 5H	50%	Minimal digestion @1 month
1.5:2	8A, 9H	67%	Minimal digestion @1 month
2:1	1A	100%	Completely digested @2 weeks
	6A, 7H	75%	Completely digested by @1 month

Table 15 Trial 2 Acid bone hydrolysis observations

#	2 hrs	1 day	2 days	1 week	2 weeks	1 month
1A	Some hydrolysis, however 6A has more bone hydrolysed.	Still has chunks of bone that has not hydrolysed. Large amount of liquid	Runny. Most of the bone hydrolysed. Some small fragments still present. Darker brown colour. Creamy.	Thin dark brown layer which appears to be water. Creamy tan caramel liquid. Almost all hydrolysed except for tiny white and brown fragments which do not change the consistency of liquid. Consistency thicker than 6a and 7h.Less fragments noted and creamy colour.	Completely digested. Thin layer of acid and water on top. The appearance of the digested bone is a creamy tan caramel colour with no visible bone. It is very smooth. Thin consistency and very pourable	Dark brown/caramel creamy colour. Pourable. 2 distinct separate layers. Best sample.
2A	Large quantity of liquid to begin with. Some bone digested. Large portion not digested but plenty of liquid, dark creamy brown colour	Some liquid absorbed by bone. Still large amount of bone undigested. Same colour as 3H but less digested than 3H. Thick liquid. Bone clumped together in middle.	Still large chunks of bone present. Not all bone submerged in water. Some liquid.	Thick consistency, not pourable. Some bone has hydrolysed since last observation but still large amount of bone present. Bone more evenly dispersed. Much thicker than 3H. Darker brown creamy colour.	Thick viscosity but pourable. bone fragments still present and evenly dispersed	Still some fragments of bone present. Slightly more than 3h. Separate layers. Larger fragments of bone than 3H. rougher consistency than 10A
3H	Much thinner consistency than 2A. More transparency in liquid. Slightly darker brown in colour. More bone has been hydrolysed.	Relatively runny. More digested bone than 2A. Creamy brown tan colour. Still bits of bone present put not clumped together. 3rd most hydrolysed. Thicker liquid than 6A and 7H.	More hydrolysed than 2a but equal to 1a. Small bone fragments still present.	Still bone fragments present. More runny than 2a. pourable consistency	Thinner consistency than 2a. more hydrolysed than 2A. bone fragments have been broken down into smaller pieces. Most of it has been hydrolysed.	Still some fragments of bone present, but mostly unchanged
4A	Darker brown in colour than 2A. More transparent liquid. Some hydrolysis occurred.	Similar to 2A but much less liquid ad more bone present that has not been hydrolysed. Bone clumped together in middle. Darker brown colour with less transparency.	Large chunks of bone present. Some liquid at the bottom. Slightly transparent. More hydrolysed than 24 hr prior	Not much change since day 2. Liquid present but bone still clumped. Does not appear to have hydrolysed much bone	Still large bone pieces clumped together. Thinner solution but a light brown colour. Not much change since one week ago	
5H	Darker brown in colour. More transparent. More hydrolysed bone. Thinner consistency. Appear to be more liquid	Much more watery liquid. Transparent dark brown in colour. Some bone is clumped together. Darker and slightly more hydrolysis than 4a.	Darker brown in colour for the liquid. Large chunks of bone. Amount of bone hydrolysed is similar to 4a, but more evenly dispersed in liquid	Bone more evenly dispersed than 4a. similar amount of bone present to 4a. colour is dark brown and not creamy	Similar observation as week 1. Slightly more bone has hydrolysed but no significant. Same appearance of liquid.	

6A	More hydrolysed bone than the control. More liquid and thinner consistency. Bones distributed through solution	2nd most hydrolysed sample. Smaller fragments of bone remain. It is evenly dispersed. brown/cream runny liquid.	Similar to 7H. Darker cream brown colour. Most of the bone has been hydrolysed. Just some small fragments at the bottom. Very runny	Some bone fragment at the bottom not hydrolysed but runny consistency. 3rd more hydrolysed sample. 7h is fragment slightly smaller. Very comparable samples. Colour is creamy brown/tan. Same consistency. Distinct layers of water and dissolved bone	Most of the bone has hydrolysed. Undigested bone at the bottom. Very thin solution	Same as 7h. Still small dark brown string bits present.
7H	Thinner. Most hydrolysed sample of all. Still bone fragments in the bottom but separated.	Most hydrolysed sample. Still some pieces of bone but smaller. Similar to 6A	Similar to 6A but slightly more bone digested	Similar to 6A. Runnier than 1A. 2nd most digested sample but more time required	Thicker solution. More hydrolysed bone. Not completely hydrolysed like 1	Same as 6A. slightly lighter colour and runnier than 1A.
8A	Small amt of free liquid. Some hydrolysed, but most is not. Not soaking in liquid. Just some liquid at bottom. Pale brown colour	Thick liquid at the bottom but not much present. Bone clumped together. Some hydrolysis. Liquid is cream brown tan colour. Similar to 9H	More hydrolysed than 10A. some liquid present but quite dry. Liquid has hydrolyse bone. Similar appearance to 9H	More liquid present than previously but still plenty of bone that has not hydrolysed. Hydrolysis has occurred to a small degree. Slightly lighter in colour than 9h. appear more hydrolysed than 4a and 5h which have plenty of liquid by not much hydrolysis occurring	Not much change. Still large bone fragments undigested	No change
9H	Thinner than 8A. Dark brown colour. Large amount of bone not digested.	Slightly darker in colour than 8A but otherwise similar in appearance. No noticeable difference in hydrolysed bone material.	Creamy brown colour. More transparency in liquid. More bone hydrolysed than 8A but comparable. Clumps of bone present on top.	More liquid than previously noted. Still large clumps of bone present in the sample that has not hydrolysed. Creamier in colour. Slightly more hydrolysed than 8h.	Not much change. Still large bone fragments undigested	No change
10A	Relatively dry. Small amt liquid at the bottom of the container. Not much hydrolysed. Less than in other samples with same wt liquid	Not much free liquid available. Dry clumped bone. Not much hydrolysis of bone has occurred.	Relatively dry. Bone clumped together. Little bit of bone hydrolysed.	Thick sample that is not pourable. hydrolysis is similar to 2a. much more bone has hydrolysed since last observation. All the bone is submerged in liquid.	Very thick not pourable. Most of the bone digested. 6A, 7H, 3H and 2A are more further along.	Most bone digested. Very thick paste. Smoother consistency. Not pourable. More digested than 2A and 3H.

The use of heat treatment for 2 hours at 60°C did not observably effect the digestion of bone material into liquidised bone in all treatments (Table 15 and 16 and Figure 18).

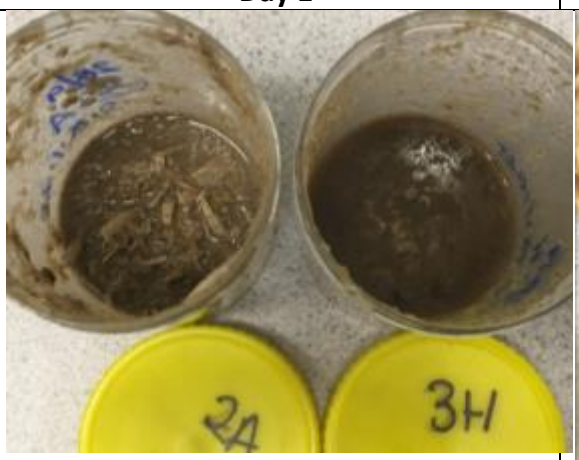
Treatment	Day 1	1 month
1:1 75% acid solution to bone		
1.5:1 67% acid solution to bone		

Figure 18 Trial 3 showing the effect of temperature on the digestion of bone after 1 day and one month of ambient storage.

Stakeholder feedback

At a meeting with the stakeholders to discuss the outcomes of the trials they indicated that the preferred sample, taking into consideration appearance, digestion of the bone material, processing steps and the concentration of acid required was 6A (75% acid. 2:1 ratio). The viscosity and lack of off odours was also favourable. One month as a time frame to completely digest the bone without many processing steps was favourable. The idea was to leave the bone in acid solution in IBC's to allow the bone to digest over time.

The stakeholders mentioned that the use of pure phosphoric acid at a ratio of 1.5: 1 acid to bone when the price of phosphoric acid is $1.30/L was not feasible. Based on some market research they have conducted and the price potential customers were willing to pay for the liquidised bone with the application as a fertiliser, there would be no cost benefit for the business. The optimal formulation is currently not commercially feasible, however if they are able to generate interest for a product not currently well known on the market the following points about the results were noted for future consideration:

- Ease of process. Digest bone in IBC's with minimal processing
- Look at the reduction of the water added as this dilutes the content
- Conduct chemical analysis of the optimal formulation
- Based on chemical analysis that will help guide to a potential market
- There are not many current applications for liquid bone except for use as a fertilizer; however analysis could determine its potential for other uses such as a feed additive or supplement.

3.2 Summary

Following the stakeholder meeting it was decided to not continue with the bone liquefying trials.

4 Gill Plate Trials

Currently the gill plates from the harvested tuna are not put through the SAMPI hydrolysis process but are disposed of elsewhere. However SAMPI are interested in trialling processing options for the gill plates as this would add ~500 Tonnes annually to their raw material supply. The disadvantage of the gill plates is that new mincing options would be required as the current mincing system could not manage the size and configurations of the gill plates.

4.1 Methods and Results

4.1.1: Composition of the Gill Plates.

Gill plates were supplied by SAMPI in a minced form (Figure 19). The moisture and ash contents of minced frozen untreated gill plates were measured at Curtin and protein and fat analysis were sent to a commercial laboratory. The compositional data for the untreated gill plates as compared to the tuna bones and hydrolysed treatments (see Section 4.1.2) are reported in Table 17.

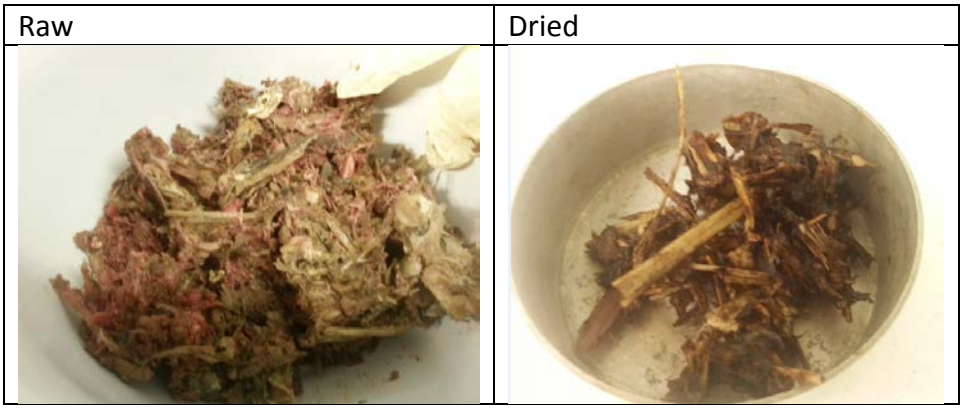


Figure 19 Raw and dried minced gill plates

Table 17 Composition of Untreated Gill plates and following Hydrolysis.

Component	Unit	Sieved Bones (after hydrolysis)	Untreated Gill Plates	Gill plate hydrolysate	Hydrolysed gill plate bones (dried).
Protein (Kjel N =4.10)	g/100g	18.9	21.8 (ave of 3)	21	35.5
Fat	g/100g	3.6	4.23 (ave of 3)	4	3.3
Moisture	%	58.6	61.6 (TD)	71.5	5.6
Crude Ash	%	13.4	16.69 (TD)	2.6	50
Phosphorus	mg/kg	6500	22000	1300	93000
Potassium	mg/kg	490	1500	1400	2100
Sulphur	mg/kg	670	2200	2400	2800
Calcium	mg/kg	12000	46000	2100	200,000

4.1.2 Enzymatic Hydrolysis of Gill Plates

Untreated gill plates were subject to enzyme hydrolysis by Alcalase enzyme, 0.05% addition, at 55°C for 3 hours without addition of water in the Sunbeam sous vide. The hydrolysate was very dark and thick. Unhydrolysed bones were washed with water after weighing and frozen for further investigation. Around 67% of the gill plates were bones/cartilage which did not hydrolyse (Figure 20).

Gill plates before hydrolysis	Gill plates after hydrolysis
	
Hydrolysate	Unhydrolised bones, gills etc
	
Bones after washing	Close up of bones after washing
	

Figure 20 Gill plate enzymatic hydrolysis pictures from 2500g gill plates

Gill plates hydrolysate was centrifuged at 4000 ppm for 5 minutes and very little separation into different components was observed (Figure 20). Gill plates are mainly bones (67%). Around 75 % of the hydrolysate is soluble (in aqueous phase). Oil content is very low (see Table 18).

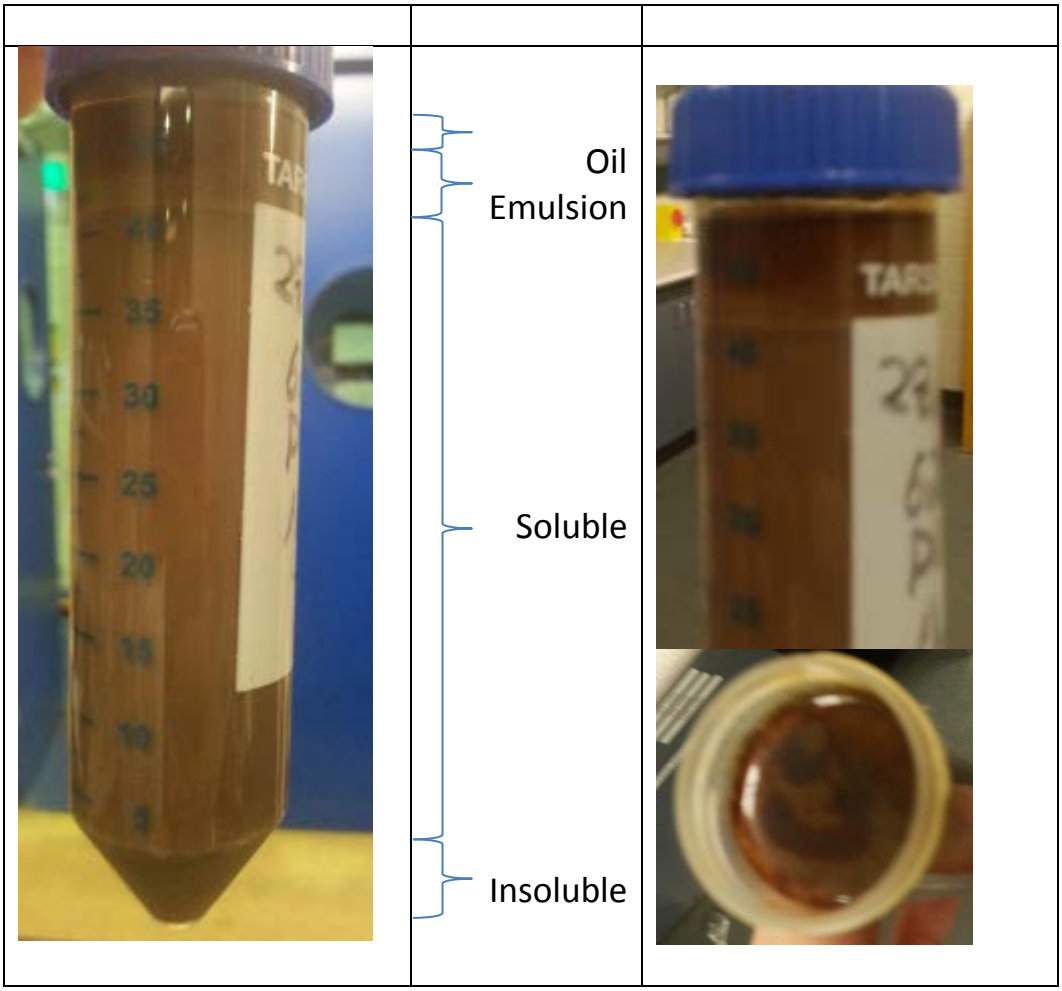


Figure 5 Gill plates hydrolysate separation after centrifuge

Table 3 Gill plate unhydrolysed and hyrolysed weight fractions

		%
Unhydrolised	Bones, cartilage etc	67.13
Hydrolysed	Oil	1.31
	Emulsion	4.93
	Soluble	24.98
	Insoluble	1.64

Compositional results from the gill plate hydrolysis and resulting bones is shown in Table 16. Interestingly the protein level is higher than in the offal hydrolysate (typically ~13%).

4.1.3 Discussion

The gill plates can be hydrolysed by enzymes but due to firstly the difficulties in mincing with the current system and secondly the high percentage of solid material (75%) this was not considered by the industry partners to be a commercially feasible process. No further experimental work was performed.

5 Conclusions

The experimental work, whilst defining compositional analyses and putative final product process methodologies for the tuna bones and gill plates, has not resulted in any outcomes which can be commercially explored by the SAMPI company at this time.

There are other opportunities for value-adding from fish bones, such as extraction of collagen, collagen hydrolysates and hydroxyapatine, and production of gelatin, but these were considered beyond the scope of this project and will likely be put forward as potential student projects.

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