Enzyme-Assisted Fermentation and Chef Perspectives of Green Crab Sauce

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ENZYME-ASSISTED FERMENTATION AND
CHEF PERSPECTIVES OF GREEN CRAB SAUCE

By
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B.S. University of Maine, 2020

A THESIS
Submitted in Partial Fulfillment of the
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ENZYME-ASSISTED FERMENTATION AND CHEF PERSPECTIVES OF GREEN CRAB SAUCE

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Thesis Advisor: Dr. Denise I. Skonberg

An Abstract of the Thesis Presented in Partial Fulfillment of the Requirements for the Degree of Master of Science (in Food Science and Human Nutrition) August 2021

The European green crab (Carcinus maenas) is an invasive species which has caused considerable economic and ecological damage along U.S. coasts. Due to their small size, meat extraction from green crabs is laborious, and there is currently no well-established use for this abundant biomass. Developing a high-value, high-volume food product such as a fermented green crab sauce may stimulate the commercial harvesting of these crabs. Overall, the purpose of this research was to accelerate fermentation of green crab sauce using proteases and to gain insight into chef perspectives of fish sauce and a green crab sauce concept.

The specific objectives of this research were to: (1) evaluate the physicochemical and microbial effects of proteolytic enzyme treatments (Alcalase, Flavourzyme, and Protamex) on the production of a fermented green crab sauce, and (2) survey chefs in New England regarding their perceptions of a fermented green crab sauce as a culinary ingredient.
In the first study, commercial proteases were applied to chopped whole crabs and the mixture was incubated at 55°C for 48 hours, and then fermented at 37°C for 88 days. The produced crab sauce was filtered and analyzed periodically throughout the fermentation period. Percent yield and amine nitrogen content increased significantly in the enzyme-applied treatments compared to the control (without enzymes) up to day 30. Fermentation time had a significant impact on characteristics of the sauce including increases in pH, browning index, amine nitrogen, and total volatile base nitrogen, and a decrease in moisture content over time. However, there were few significant differences among enzyme treatments overall. Based on these results, commercial proteases could be applied to increase yield and hydrolysis of proteins for short term fermentations of 30 days or less.

In the second study, a 14-question online survey collected perspectives of 59 professional chefs throughout New England regarding their preferences for fish sauce and feedback about a green crab sauce concept. The chefs’ preferred attributes of commercial fish sauce included medium brown color, savory aroma, transparent appearance, and umami flavor. The most important sourcing factors for chefs when purchasing ingredients for restaurants were local, sustainable, and price. Overall, chefs scored “likeliness to use” and “willing to purchase” a commercially available green crab sauce very positively. The chef survey data suggest that the ideal target customer for green crab sauce would be head chefs who focus on Asian cuisine and who are already familiar with fish sauce.

The results of these studies have important implications for the production and marketing of a fermented green crab sauce. The application of commercial proteases was shown to be promising during the early stages of green crab sauce fermentation, although
more research is needed to optimize protease application. Chefs were very receptive of the green crab sauce concept for the food service distribution channel, however, sensory evaluation of the crab sauce is necessary to characterize desirable flavor and odor attributes. Industrial production of a fermented green crab sauce may promote the development of a commercial fishery for green crabs and help to control their escalating populations in North America.
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TABLE OF CONTENTS

ACKNOWLEDGEMENTS..................................................................................................iii
LIST OF TABLES..............................................................................................................vii
LIST OF FIGURES...........................................................................................................ix
1. LITERATURE REVIEW.............................................................................................1
   1.1. Green Crabs (*Carcinus maenas*)........................................................................1
       1.1.1. The Green Crab Invasion.................................................................1
       1.1.2. Green Crab Biology.............................................................................2
       1.1.3. Environmental Effects..............................................................3
       1.1.4. Composition.........................................................................................6
       1.1.5. Potential Commercial Utilization of Green Crab.........................8
   1.2. Fish Sauce.........................................................................................................10
       1.2.1. Fish Sauce Origins............................................................................10
       1.2.2. How Fish Sauce is Made...............................................................11
       1.2.3. Characterization of Fish Sauce.......................................................11
       1.2.4. Other Protein Sources.......................................................................15
       1.2.5. Effects of Fermentation on Fish Sauce.......................................17
       1.2.6. Effects of Salt....................................................................................20
       1.2.7. Effects of Inoculation.........................................................................23
       1.2.8. Use of Enzymes in the Seafood Industry...................................25
       1.2.9. Application of Enzymes in Fish Sauce Production..................28
       1.2.10. Consumer Perceptions of Fish Sauce......................................30
   1.3. Justification.........................................................................................................31
   1.4. Objectives...........................................................................................................32
2. EFFECTS OF ENZYMES ON FERMENTATION OF GREEN CRAB SAUCE.......33
   2.1. Introduction......................................................................................................33
   2.2. Materials and Methods..................................................................................35
       2.2.1. Experimental Design.......................................................................35
       2.2.2. Preparation of Crab and Treatments.............................................36
       2.2.3. Fermentation of Crab Sauce..........................................................37
2.2.4. Filtration of Crab Sauce.................................................................37
2.2.5. Microbial Analysis.......................................................................38
2.2.6. Yield, pH, Water Activity, and Moisture Content .......................39
2.2.7. Browning Index...........................................................................40
2.2.8. Total Volatile Base Nitrogen and Amine Nitrogen......................41
2.2.9. Salt Content...............................................................................42
2.2.10. Biogenic Amines.......................................................................43
2.2.11. Statistical Analysis.....................................................................44
2.3. Results.............................................................................................44
  2.3.1. Microbial Analysis......................................................................44
  2.3.2. Yield, pH, Water Activity, and Moisture Content.........................47
  2.3.3. Browning Index..........................................................................53
  2.3.4. Total Volatile Base Nitrogen and Amine Nitrogen........................54
  2.3.5. Salt Content...............................................................................57
  2.3.6. Biogenic Amines........................................................................57
  2.3.7. Correlations Between Measured Characteristics of Crab Sauce.....58
2.4. Discussion.......................................................................................59
  2.4.1. Microbial Analysis......................................................................60
  2.4.2. Percent Yield, pH, Water Activity, and Moisture Content.............61
  2.4.3. Browning Index..........................................................................64
  2.4.4. Total Volatile Base Nitrogen and Amine Nitrogen.......................65
  2.4.5. Salt Content...............................................................................66
  2.4.6. Biogenic Amines........................................................................66
2.5. Conclusions....................................................................................67

3. CHEF PERSPECTIVES OF FISH SAUCE AND FERMENTED GREEN CRAB SAUCE........................................................................70
  3.1. Introduction....................................................................................70
  3.2. Materials and Methods...................................................................72
    3.2.1. Research Design.......................................................................72
    3.2.2. Population and Sampling Methods...........................................73
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.1.</td>
<td>Mean values of physicochemical properties of five traditionally produced fish sauces.</td>
</tr>
<tr>
<td>Table 1.2.</td>
<td>Commercial proteases and their activity.</td>
</tr>
<tr>
<td>Table 2.1.</td>
<td>Total mesophilic bacterial populations (log CFU/mL) of crab sauce treatments over time.</td>
</tr>
<tr>
<td>Table 2.2.</td>
<td>Proteolytic bacterial populations (log CFU/mL) of crab sauce treatments over time.</td>
</tr>
<tr>
<td>Table 2.3.</td>
<td>Lactic acid bacteria populations (log CFU/mL) of crab sauce treatments over time.</td>
</tr>
<tr>
<td>Table 2.4.</td>
<td>Heterotrophic marine bacterial (log CFU/mL) populations of crab sauce treatments over time.</td>
</tr>
<tr>
<td>Table 2.5.</td>
<td>Mean yield (%) values of crab sauce treatments over 90 days.</td>
</tr>
<tr>
<td>Table 2.6.</td>
<td>Mean pH values of crab sauce treatments over 90 days.</td>
</tr>
<tr>
<td>Table 2.7.</td>
<td>Mean moisture content (%) of crab sauce treatments over 90 days.</td>
</tr>
<tr>
<td>Table 2.8.</td>
<td>Mean browning index (absorbance) values of crab sauce treatments over 90 days.</td>
</tr>
<tr>
<td>Table 2.9.</td>
<td>Mean total volatile base nitrogen (mg/100 mL) content of crab sauce treatments over 90 days.</td>
</tr>
<tr>
<td>Table 2.10.</td>
<td>Mean amine nitrogen (mg/100 mL) content of crab sauce treatments over 90 days.</td>
</tr>
<tr>
<td>Table 2.11.</td>
<td>Biogenic amine detectable limits and crab sauce values.</td>
</tr>
<tr>
<td>Table 2.12.</td>
<td>Pearson correlation between dependent variables of crab sauce.</td>
</tr>
<tr>
<td>Table 2.13.</td>
<td>Cost analysis of traditionally fermented green crab sauce versus enzyme-applied crab sauce after 90 days fermentation.</td>
</tr>
<tr>
<td>Table 3.1.</td>
<td>Chef and cuisine classification of survey participants.</td>
</tr>
<tr>
<td>Table 3.2.</td>
<td>Fish sauce preferences for color, aroma, appearance, flavor, and overall.</td>
</tr>
<tr>
<td>Table 3.3.</td>
<td>Response to concept statement.</td>
</tr>
</tbody>
</table>
Table 3.4. Spearman’s correlation coefficients of chef characteristics, and familiarity with fish sauce and concept scores……………………………………... 86

Table 3.5. Flavor enhancers regularly used in relation to the cuisines of primary focus: Asian, Not Asian, and Overall……………………………………87
LIST OF FIGURES

Figure 1.1. Global spread and invasion of *Carcinus maenas* ......................................................... 2

Figure 2.1. Effects of treatment on yield (%) of crab sauce .......................................................... 48

Figure 2.2. Mean yield (%) values of crab sauce treatments over 90 days ............................... 48

Figure 2.3. Effects of fermentation time on pH of crab sauce ................................................. 50

Figure 2.4. Mean yield (%) values of crab sauce treatments over 90 days ............................... 51

Figure 2.5. Effects of fermentation time on moisture content (%) of crab sauce .................... 52

Figure 2.6. Effects of fermentation time on the browning index (absorbance) of crab sauce .......................................................................................................................... 53

Figure 2.7. Effects of fermentation time on total volatile base nitrogen (mg/100 mL) of crab sauce .......................................................... 55

Figure 2.8. Effects of fermentation time on amine nitrogen (mg/100 mL) of crab sauce .......... 56

Figure 2.9. Mean NaCl content (%) of crab sauce treatments at day 90 .................................. 57

Figure 3.1. How interested are you in cooking with new and innovative food ingredients ................................................................................................................. 79

Figure 3.2. Which of the following flavor enhancers do you regularly use in your cooking? *Please select all that apply* .................................................. 78

Figure 3.3. How familiar are you with fish sauce/garum? (n=59) ........................................ 80

Figure 3.4. What factors are most important when sourcing ingredients for your restaurant? *Please select all that apply* .................................................. 82
CHAPTER 1

Literature Review

1.1. Green Crabs (*Carcinus maenas*)

1.1.1 The Green Crab Invasion

European green crabs (*Carcinus maenas L.*) originated in northwestern European waters (Jamieson et al., 1998). Green crabs are commonly known as shore crabs and are typically found on rocky shorelines and soft-bottom habitats along the coast. The unintentional introduction of these aggressive crabs in the western Atlantic began in the early 1800s. A common theory suggests that transport of green crab occurs most often during the larval stage via shipping ballast water. There has been a positive correlation between the increase in international trade and the dispersal of these shore crabs via shipping ballast water (Jamieson et al., 1998; Carlton and Cohen, 2003). Another cause of their spread is attributed to the movement of fishing gear as adult green crabs are able to survive out of water or in freshwater. Green crabs may also be discarded as bycatch and be moved to a different location.

Invasive species such as green crabs are not native to the location they inhabit and cause either ecological or economic harm. Though it has been difficult to track their movements, *Carcinus maenas* have survived and adapted, which have led to invasions in Asia, North America, and Australia (Figure 1.1) (Leignel et al., 2014). Green crabs harm marine life and cause economic harm by feeding on valuable, local marine species. North American invasions began in the late 19th century, with green crabs making their first
appearances in San Francisco Bay, Coos Bay, Cape Cod, and Nova Scotia (Jamieson et al., 1998). Their introduction to the U.S. has led to abundant population growth as they have few predators and survive in diverse environments (Cohen et al., 1994).

1.1.2. Green crab biology

*Carcinus maenas* has been characterized as an invasive species with high survivability in diverse environments. Green crabs fall within the Arthropoda phylum, typically live up to 4-7 years, and are highly efficient at larval dispersal (Klassen and Locke, 2007). In contrast, the average lifespan of a blue crab is about 3-4 years, which allows room for invasive green crabs to out-live and -compete this native crab species. Female green crabs are capable of dispersing up to 185,000 eggs at once, and larvae are capable of surviving through ocean currents for 50-80 days (Carlton and Cohen, 2003).
Due to this extended larval stage, green crabs are easily transported and can spread to new locations. Green crabs are also able to reproduce at a rapid rate which further allows them to successfully invade at high rates into foreign environments.

These saltwater crustaceans are able to withstand varying oxygen, salinity, and temperature conditions. Green crabs are considered to have high acclimatory plasticity or are highly tolerant to drastic short-term temperature changes (about 25 days) ranging from 5-21°C in native and non-native environments (Tepolt and Somero, 2014). Green crabs are eurythermic which allows them to survive in temperatures ranging from 0°C to 35°C (Klassen and Locke, 2007). Compared to other salt-water organisms, green crabs are among the few which can withstand salinity levels of 6-33% (Locke et al., 1993; Conkerton et al., 2017). All these factors allow green crabs to survive on all continents except for Antarctica, which speaks to how successful they can be as an invasive species.

1.1.3. Environmental Effects

Green crabs have become invasive predators along coastlines across the globe and their predatory patterns are important to note. A study conducted by Garside et al. (2015) in Eastern Australia investigated the effect of *Carcinus maenas* on native biota in ten estuaries in New South Wales. It was reported that there was a negative effect of the abundant presence of the green crab in five estuaries on six different species: blue swimmer crab, octopods, leatherjackets, yellowfin bream, toadfish, and eastern fiddler ray. However, a tethering experiment revealed that the octopus may have the ability to overcome the green crab (Garside et al., 2015). The green crabs tethered one meter away from an octopus lair were dead after five minutes, but the crabs five meters away survived for 24 hours. The
negative correlation between the presence of green crab and their prey extends beyond the species directly affected. Invasive green crabs create a ripple effect as they predate on local species which leads to eventual imbalances within native trophic systems.

Invasions by green crabs also pose an economic dilemma to the shellfish industry. According to Maine oyster landings, in 2019, harvested oysters reached $7.6 million in sales. Pickering et al. (2017) investigated the impact of green crabs on American oysters (*Crassostrea virginica*) in both Canada and the United States over a five-day period. A two-way analysis of variance (ANOVA) was conducted to assess predator size (small, medium, or large), prey size (spat, small, medium, or large), and predator-prey interactions. Oysters that were smaller than 35 mm in shell length were at high risk of green crab predation compared to those within the sizes of 35-55 mm. It was reported that larger green crabs caused 100% mortality in the spat oysters after day one while most of the larger oysters survived (Pickering et al., 2017). Overall, larger green crabs were a greater threat to smaller-sized oysters. Green crab predation on young oysters can greatly affect the oyster populations and indirectly affect species such as other crabs and sea birds.

Quinn and Boudreau (2016) conducted a study investigating the impact of green crab kleptoparasitism and scavenging on native species in Atlantic Canada. Kleptoparasitism is a behavior commonly associated with birds stealing food from humans as it refers to a species stealing another's prey or food source. A mathematical model was designed to compare the effect of green crab kleptoparasitism and scavenging on native dogwhelks foraging for mussels. Included factors were differences in mussel sizes, amount of time the predator (dogwhelk and green crab) spent with the prey (mussel), control groups (crab or whelk), and interactions between the whelk and crab. Overall, green crabs were
estimated to decrease mussel populations in all feeding scenarios. Whelks were found to abandon their mussel prey more often in the presence of green crab, possibly to avoid predation. The impact of green crab predation on mussels was found to indirectly decrease whelk predation rates which in turn leads to an overall decrease in whelk populations. These studies help to illustrate both the potential direct and indirect effects of green crab predation on native marine food sources.

As described above, the green crab species has displayed rapacious predatory activity, contributing to significant change within local marine ecosystems. Depending on the location of the green crab, its stomach contents can vary greatly as they are known to figuratively “filter food through its gills” (Hall, 2018). Green crabs are efficient predators which can survive on a wide variety of prey species. Though green crabs prefer molluscan and other crustacean prey, Cohen et al. (1994) reported that they have fed on 104 families and 158 genera of plants and animals (Jamieson et al., 1998). The crabs’ ability to consume such a wide range of species allows them to successfully invade diverse ecosystems.

Exotic green crabs are exceptional “ecosystem engineers” (coined by Leignel et al., 2014) as they modify native and local marine ecosystems to fit their needs. Green crabs are considered an abnormal invader to the North American waters as transport of these crabs from Europe would not be possible by ocean current alone (Cohen and Carlton, 1994). The presence of invasive green crab in North America has disrupted valuable and integral marine species such as bivalves, eelgrass, gastropods, and other crustaceans (Grosholz et al., 2011; Leignel et al., 2014; Quinn and Boudreau, 2016). In 1940, a U.S. Fish and Wildlife Service warden (Glude, 1955) reported that in Massachusetts, soft-shell clam production decreased by 90% within eight years primarily due to predatory activity by the
green crab. The decline in the population of these soft-shell clams has resulted in major economic losses for local Northeastern estuaries and fisheries.

Due to the lack of commercial demand for green crabs in North America, control of their populations has proven to be difficult. However, temperatures below 0°C may be a key factor in limiting green crab abundance. In the 1950s, higher ocean temperatures encouraged growth of green crab populations, but the decreasing water temperatures in the 1960s reduced their numbers (Maine Department of Marine Resources, 2013). A method of human intervention initiated by the Maine Department of Marine Resources is the “Green Crab Exemption,” which allows individuals to participate in removing green crabs without the need for a permit (Maine Department of Marine Resources, 2013). By making the recreational capture of green crabs easily accessible, the general public may help to play a role in controlling green crab populations within their local areas.

1.1.4. Composition

Green crabs are typically small in size compared to commercially harvested crabs such as blue crab, snow crab, or king crab. In the United States, the average green crab has a carapace length ranging from 55-80 mm and weighs 60 grams on average (Jamieson et al., 1998; Skonberg and Perkins, 2002). As an animal protein source, green crabs are expected to contain high quality proteins. According to the FAO (2011), high quality proteins are highly digestible by humans and have a protein digestibility score of about 1 (based on the protein digestibility corrected amino acid score ranging from 0-1). On a dry weight basis, green crab meat harvested in Nova Scotia had a protein content of 81% and a lipid content of 3% (Naczk et al., 2004). Within raw green crab meat, total lipids
accounted for 4% (Naczk et al., 2004). The primary saturated fatty acids in the crab meat were palmitic (16:0) and stearic acids (18:0) and key unsaturated fatty acids were eicosapentaenoic acid and docosahexaenoic acid (1.6 to 2.8 ratio).

Different components of green crab have been separately analyzed for nutrient content (Skonberg and Perkins, 2002). Green crab (harvested from the Gulf of Maine) leg and claw meat were analyzed for proximate composition, minerals, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) content. There were three sample groups (composites from 6-8 crabs) including steamed claws, steamed legs, and raw claws. Overall, the crab meat had mean moisture, protein, and total mineral contents of 78.7 g/100 g, 17.1 g/100 g, and 2.2 g/100 g, respectively. The leg meat was found to be higher in lipids at 1.16 g/100 g compared to the steamed and raw claw meat at 0.62 g/100 g and 0.54 g/100 g (Skonberg and Perkins, 2002).

Some omega-3 fatty acids such as DHA and EPA are long-chain polyunsaturated fatty acids (PUFAs) which humans do not produce naturally on their own. Skonberg and Perkins (2002) reported significantly higher levels of DHA and EPA in leg meat compared to claw meat with corresponding ranges of 115-336 mg/100 g and 154-344 mg/100 g, respectively. Naczk et al. (2004) reported that EPA and DHA were the primary PUFAs in green crab meat, with a ratio ranging from 1.6-2.8 (EPA:DHA). Incorporation of DHA and EPA into human diets has been shown to lower blood pressure, reduce the likelihood of cardiovascular diseases, and contribute to overall cell function (Raatz et al., 2013). Green crab meat is a potentially widely abundant source of these essential omega-3 fatty acids. However, green crab meat has been reported to contain about 4.2% in total lipids while the recommended daily intake of omega-3s for the average adult ranges from 1.1-1.6 g (Naczk
et al., 2004; National Institutes of Health, 2021). To meet this recommended intake of omega-3s, about 400 g of green crab meat would need to be consumed.

Amino acids play an important role in flavor, aromatics, Maillard browning (condensation of carbonyl groups), and antioxidant activity (Zhao et al., 2018). Except for a lower tryptophan content, green crab meat amino acid composition was comparable to whole red crab pulp (Spinelli et al., 1974) and whole snow crab meat (Krzeczkowski and Stone, 1974). The most abundant amino acids recovered in the crab protein were glutamic acid, arginine, aspartic acid, leucine, and lysine (Skonberg and Perkins, 2002; Fulton and Fairchild, 2003; Kang et al., 2018). Glutamic acid and to a lesser extent, aspartic acid, have been characterized as playing a role in flavor and aroma (Marilley and Casey, 2004).

1.1.5. Potential Commercial Utilization of Green Crab

There is potential for green crab to be used as a functional ingredient in pharmaceutical and food products. Green crabs may have high potential and value as a functional food ingredient with the ability to gel, foam, emulsify, and solubilize within a matrix. Galetti et al. (2017) found that mechanical separation of cooked green crab yielded 49.2% mince (meat). In a consumer acceptability study, green crab mince was presented as a value-added ingredient in empanadas. Overall acceptability was 6.5 on a 9-point hedonic scale, and purchasing prospects were favorable (Galetti et al., 2017). Kang et al. (2018) applied an isoelectric solubilization/precipitation (ISP) method to extract proteins from green crab mince. In that study, protein was solubilized at pH 2 and pH 10 and subsequently precipitated at pH 5.5. The pH 2 treatment recovered an increased yield (1.5 times higher) of protein and fat compared to the pH 10 treatment. Both studies described
above show economic promise of green crabs and their potential within food product applications.

Currently, there is only limited commercial use of harvested green crab. However, soft-shell green crabs are in high demand by the restaurant industry across New England (McMahan, 2021) during their shell molting seasons (spring and summer). New England fishers are reported to receive about $25 a pound for soft-shelled green crabs sold to restaurants (McMahan, 2021). Another potential use of green crab is in animal feeds. Fulton and Fairchild (2013) investigated the potential application of whole green crab meal in fish feeds. The crab meal was found to contain 16.6% ash, 12.3% protein, and 0.2% lipids and all the necessary essential amino acids also found in most feeds targeted for fish. The fatty acid composition of the whole ground crabs was 68% unsaturated and 24% saturated, with more fatty acids identified as EPA than DHA. Additionally, mercury levels within the crab mince were below the detectable limits. The high-quality nutritional profile of the mince indicates a promising application of green crabs in animal feeds, however, there are no reports of green crab actually being used in this application. Preliminary characterization of green crab roe/eggs was conducted to gauge its potential in the caviar industry (Appendix A). Thus far, there are only limited reports of research and development on the monetization potential of green crab, but their abundance and high nutritional quality show promise for further research in this area. Currently, there is only one study in the peer-reviewed scientific literature about fermenting green crabs to produce a marketable seafood condiment (Greiner et al., 2021).
1.2 Fish Sauce

1.2.1 Fish sauce origins

Fish sauce is a popular fermented condiment whose origins date back to 100 AD. Fish sauce provides an avenue to prolong the shelf-life of fish without refrigeration and to repurpose non-edible portions of fish/seafood. Fish/seafood sauce is primarily used as a seasoning or protein source (Aquerreta et al., 2001; Lopetcharat and Park, 2002). Garum, one of the world’s first fish sauces, originated in ancient Rome. Garum was made by fermenting fish guts (mackerel most commonly) with salt and aromatic herbs/spices in terracotta pots (urcei) for 2-3 months, known as the dry-salting method (Smriga et al., 2010). This highly pungent and shelf-stable fish condiment was used to season many savory European dishes. The reason for the disappearance in popularity of garum is unknown, however, a common theory suggests that the taxation of salt made the production of garum too expensive (Prichep, 2013). However, garum’s legacy did not halt as the production of similar fish sauce products began to appear in Southeast Asia.

This type of fermented seafood condiment is enjoyed globally, and each location presents unique flavors and ingredients. A majority of fish sauce is produced in Southeast Asia. The numerous variations of fish sauces and pastes include noun-mam from Vietnam, patis from the Philippines, nam-pla from Thailand, aek-jeot from Korea, budu from Malaysia, and shottsuru from Japan (Chayovan et al., 1983; Lopetcharat and Park, 2002; Mueda, 2015). Each location garners a distinct take on fish/seafood sauce which caters to its respective national palate. For example, nam-pla is produced using anchovies and salt while shottsuru is flavored and salted using soy sauce (Yongsawatdigul et al., 2007;
Nakano et al., 2017). North America’s version of fish sauce, Worcestershire, is considered a cousin, and is made with fermented anchovies and other acidic ingredients. Each region’s version of fish sauce can be used in different ways for cooking (ex: pad thai, marinating proteins, and seasoning soups) or simply as a dipping condiment. Currently, there are few fish sauces made using crustaceans as starting materials for fermentation. Therefore, there is major potential for introducing a fish sauce produced with green crabs.

1.2.2. How Fish Sauce is Made

Traditionally, fish sauce was made and preserved by fermenting a ratio of fish to salt. The ratios of fish to salt were dependent on the type of fish or seafood ingredient, fermentation period, temperature, and targeted flavor. Fermentation of fish sauce was facilitated by endogenous proteolytic enzymes naturally present in the seafood ingredients (Fernandes, 2016; Wang et al., 2017). Fermentation of industrial fish sauce usually ranges from 6-12 months at ambient temperatures (25-28°C). Common seafood ingredients for fish sauce production include anchovies, sardines, shrimp, squid, and oysters. Typically in fish sauce production, 20-30% of salt is added on a weight basis (Mueda, 2015). Varying methods of processing fish/seafood sauce contribute to effects on flavor, odor, browning, shelf-life, and fermentation period.

1.2.3. Characterization of Fish Sauce

Fish sauce is iconic for its umami (Japanese for “delicious”) flavors and distinctive savory aroma (Zhu and Tramper, 2013). The common aromas and flavors of fish sauce can be characterized as salty, meaty, cheesy, and sweet (Wichaphon et al., 2013). Amino acids such as aspartic acid, phenylalanine, glutamic acid, valine, leucine, and alanine play
a large role in aromatics (Zheng et al., 2017). Glutamic and aspartic acid are the primary contributors to the signature umami flavor (Wang et al., 2017).

Fish sauce is sometimes referred to as “liquid gold” to characterize its amber color. The signature clear brown color in fish sauce is attributed to the Maillard browning reaction during fermentation. The Maillard reaction occurs when a free-amino group of a protein or amino acid interacts with a reducing sugar having an aldehyde or ketone group (together known as a sugar-amine reaction; ex: alpha-D-glucosyl amine) to create an Amadori product (ex: 1-amino-1-deoxy-2-D-fructopyranose). Amadori products are known to be unstable and break down further to produce melanoidins (brown pigments), aroma, and flavor compounds in foods. Production of melanoidins in foods is affected by pH, sugar molecule composition, and temperature. When pH is low, the rate of Maillard browning is decreased due to increased protonation of free amino groups within the protein (Karenso et al., 2018). Sugar molecule size affects the rate of Maillard reactions as smaller sized sugars react more readily than larger ones. The stereochemical configuration of sugars also affects the rate of this reaction. For example, initially fructose reacts more quickly than does glucose, but as time progresses glucose begins to react more rapidly than fructose. Glucose is a common sugar found within fish muscle in low concentration as a result of glycogenolysis (Hemre et al., 2002; Taj et al., 2020). Degree of browning of fish sauce is quantified as the absorbance of a dilute filtrate measured between 420-440 nm (Yongsawatidigul et al., 2007). Typically, a seafood product is desirable when it is not discolored as that sight would deter marketability of that product. For example, consumers typically associate fresh scallops with a creamy white color and browning is often seen as a quality defect of the product (Wu and Wang, 2016). However, fish sauce appearance may
be enhanced by the increase of Maillard reactions depending on the desired level of browning.

Important physicochemical properties of fish sauce include pH, water activity, TVBN, amine nitrogen, moisture content, and degree of browning. According to the FAO & WHO Standards for Fish Sauce, the product pH should range from 5.0-6.5, total nitrogen content should not be less than 10 g/L, amino nitrogen content must not make up less than 40% of total nitrogen content, salt content should not be less than 200 g/L, appearance must be translucent and present an appropriate flavor/odor of the product (2018). A study conducted by Greiner et al. (2021), investigated physicochemical properties of various varieties of commercial fish sauce including A Taste of Thai, Four Elephants, Son Sauce, Red Boat, and Golden Boy. These brands of fish sauce were all produced using four traditional ingredients including anchovy, sea salt, sugar, and water. The following table expresses values as a mean of the five sauces (Table 1.1).
Table 1.1. Physicochemical properties of five commercial fish sauces

<table>
<thead>
<tr>
<th>Physicochemical property</th>
<th>Mean values (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td>Water Activity</td>
<td>0.739 ± 0.0</td>
</tr>
<tr>
<td>TVBN (mg N/100 mL)</td>
<td>390.5 ± 129.4</td>
</tr>
<tr>
<td>Amine Nitrogen (mg N/100 mL)</td>
<td>1271.2 ± 528.8</td>
</tr>
<tr>
<td>Moisture Content (%)</td>
<td>59.3 ± 4.1</td>
</tr>
<tr>
<td>Browning Index</td>
<td>0.53 ± 0.2</td>
</tr>
</tbody>
</table>

*Adapted from Greiner et al., 2021.

Measured chemical properties of fish sauce help to characterize the final product. The average pH of the commercial fish sauces was found to be within the range of FAO & WHO standards and water activity was below 0.85 which indicates that vegetative pathogens are unlikely to occur within this food matrix (FDA, 1984). TVBN is often an indicator of spoilage for meat proteins which in the case of fish sauce is expected to be higher in value. Amines are nitrogen containing compounds and a high amine nitrogen content can be an indicator of spoilage or progression of fermentation (Chander et al., 1989). Moisture content is the measurement of the total amount of water in food and directly affects the mouthfeel, appearance, and rheology of a liquid product like fish sauce (Wang and Hartel, 2020). As mentioned above, the degree of browning in fish sauce is primarily dependent on the extent of Maillard browning. Overall, these characteristics can be used to assess the quality of fish sauce products.
1.2.4. Other Protein Sources

Fish sauce can be produced from fish and other protein sources such as crab, shrimp, and oysters. There are also other fermented seafood sauce products that are not as common, but still available in the market. Different starting materials affect the characteristics of the final seafood sauce products, such as protein content, glucose levels, and amino acid composition (Gildberg et al., 2007). Nagai et al. (2020) reported that low-salt fermented Alaskan pink shrimp sauce had a strong sweetness and low bitterness and saltiness scores, indicating a good overall acceptability of this product. Comparatively to fish sauce, the shrimp sauce’s sweet taste offers a new approach to a traditionally salty product. Squid byproduct, which traditionally makes up 50% of its raw product weight, is another underutilized protein source that can be used in fish sauce production (Xu et al., 2008). In addition, seafood sauces can be produced using processing byproducts such as fish spines, crustacean shells, or waste stream products generated in seafood production. A novel combination of fermented tuna loin by-product and black beans was investigated by Wenno and Loppies (2019). The sauce had a purple-red color and umami flavor as glutamic acid was the primary amino acid reported. Overall, the use of seafood protein sources other than fish is sustainable and may lead to more unique and marketable fermented seafood sauce products.

Liu et al. (2019) investigated non-volatile taste-active compounds in liquid crab sauce. Crab sauce was produced through fermenting and curing soldier crabs mixed with 30% of salt for 35 days. Free amino acids were characterized using taste activity values (TAV) to make up the following taste characteristics: sweet made up 42%, umami 33%, and bitter 25%. The identified sweet amino acids were serine, glycine, threonine, alanine,
lysine, and proline. Two amino acids, aspartate and glutamate, were classified as umami. The bitter amino acids consisted of arginine, histidine, tyrosine, valine, phenylalanine, isoleucine, leucine, and methionine. Glutamate was one of the most prominent umami taste-active components, at 19.3 g MSG/100 mL in the fermented crab sauces, which was due to the addition of sodium to generate MSG (monosodium glutamate). Taste panelists were trained with a 5-point scale (1 = no taste → 5 = very strong) and detected a high intensity of salt flavor (5), sweetness (4), and umami flavor (4) in the crab sauces. Non-volatile compounds play a major role in determining prominent flavors, therefore, determination of aroma and taste compounds are essential to characterizing fermented seafood sauce products.

Eka and Nusaibah (2019) investigated the use of the byproduct from processing jambal roti (Ariidae spp.), the residual salting liquid and fish stomach contents, to create a marketable fish sauce. The pre-boiled jambal roti residual salting liquid byproduct was first fermented for 3-6 days, then inoculated with Aspergillus oryzae starter at 25% w/v, salted at 10%, combined with fish stomach contents at varying amounts and fermented for 30 days. Overall, the pH decreased from 7.1 to 5.8 after 30 days of fermentation in all treatments (50%, 75%, & 100% residual salting liquid) and heavy metal contamination values of lead (Pb), copper (Cu), zinc (Zn), mercury (Hg), and arsenic (As) were all in compliance with Indonesia National Standard (INS) for Fish Sauce No.01-4271-1996 (FAO & WHO, 2018). The microbial tests conducted including TPC (total plate number count threshold: <104 CFU/mL), coliform, Salmonella sp, Staphylococcus aureus, and mold counts, were all below the threshold limits and the product was considered safe for consumption. However, the total N-value of 12.40 mg/L in the salted fish liquid was
significantly lower than the International Food Standard of at least 10g/L for fish sauce (FAO & WHO, 2018). Total nitrogen is used to calculate the total crude protein found within fish sauce. There is a potential for use of industrial seafood byproducts like residual salting liquids to make a viable fish sauce, however salt levels and protein values must be appropriately controlled to achieve compliance with fish sauce standards (FAO & WHO, 2018).

1.2.5. Effects of Fermentation on Fish Sauce

Fermentation is an age-old technique to provide an effective way to preserve fish when storage conditions are below optimal. Fermentation of protein foods, such as fish, converts protein into free amino acids and produces lactic acid byproducts. Lactic acid, which provides a tangy flavor and affects pH, is produced via the conversion of pyruvic acid and NADH into lactic acid and NAD+ (Armenta and Guerrero-Lagarreta, 2009). Supplemental microorganisms (L. bulgaricus, A. oryzae, R. oligosporus, etc.) applied in fermentation may help break down complex food materials which allows humans to process normally indigestible foods. Benefits of fermentation in food include a longer shelf life, improved sensory qualities, and enhanced nutrition content. Fermentation typically spans 6-18 months at an ambient temperature to yield a high-quality fish/seafood condiment product (Saisithi et al., 1966; Sanceda et al., 1992; Yongsawatdigul et al., 2007). Spontaneous fermentation in fish relies on proteases from natural microflora (located in the guts and gills) and enzymes located in the tissue to hydrolyze proteins into amino acids and peptides. Specifically in high salt fermentation environments, halophiles or salt-tolerant microorganisms such as Bacillus spp. and Pseudomonas spp. are able to survive optimally. However, during fish sauce fermentation, lactic acid bacteria (Lactococcus spp.,
*Lactobacillus brevis*, and *Pediococcus* spp.) tend to be the dominant strain by the end (Siddegowda et al., 2017). The use of traditional spontaneous fermentation in fermented fish may result in inconsistent quality or potential contamination with undesirable bacterial and yeast strains (Bao et al., 2018). Spontaneous fermentation methods can greatly affect overall product uniformity, intensity of aroma, concentration of flavor, and other important characteristics of fish sauce.

According to Sanceda et al. (1992), anaerobic (Iwashi & Sanma) and aerobic (patis) fermentation of krill for 2.5 months for fish sauce production resulted in products with significant differences in volatile acid values, aroma, and flavor. Volatile acids including n-butanoic, iso-pentonoic acetic, propionic, and iso-nonanoic acid, were found at higher concentrations in the aerobically fermented fish sauces compared to the anaerobic one (Sanceda et al., 1992). Associated with the higher volatile acid concentrations were pungent aromas such as “sharp” and “cheesy” in the aerobically fermented patis compared to more sweet and less rancid/acidic smelling anaerobic products. Depending on the desired characteristics of the fish sauce, aerobic and anaerobic conditions can be applied accordingly.

Temperature is an important factor to consider in seafood sauce fermentation. Lee et al. (2014) investigated how temperature (10, 15, 20, and 25°C) affected the fermentation of Korean saeu-jeot (shrimp) with 25% salt (w/v). Bacterial abundance and diversity in microorganism strains were found to be greater at fermentation temperatures of 15°C and above. However, there was no relation between bacterial abundance and specific amino acid content since arginine, lysine, and proline concentrations were found to be higher in 10°C and 15°C samples compared to those at 25°C. Samples fermented at 15°C had
undetectable counts of all pathogenic bacteria tested, contained the largest concentration of amino acids associated with umami flavor and achieved ideal glucose levels (sweetness of sauce) by day 105. It is essential to ferment at an optimal temperature in order to achieve both safe and desirable tasting fermented seafood sauces.

Length of fermentation time is associated with major changes in physical and biochemical properties in fish sauce. These changes include browning, degree of hydrolysis, amino acids content, moisture, pH, amine-nitrogen, and protein content (Tungkawachara et al., 2003). The intensity of browning is often associated with Maillard browning reactions and a longer duration of fermentation. Maillard browning occurs when carbonyl groups of reducing sugars and free amino acids interact (Lund and Ray, 2017; Geng et al., 2019). This non-enzymatic reaction can potentially impart bitter or burnt flavors to fish sauce if taken too far. Wang et al. (2017) reported that as fermentation time increased, anchovy fish sauce samples went from light brown to dark brown over a period of eight days. The degree of hydrolysis of the fish protein material was reported to increase up to 70% with fermentation time until the nine-month mark, indicating the inactivation of endogenous enzymes. Moisture and pH were also reported to decrease as fermentation progressed (Tungkawachara et al., 2003; Mueda et al., 2015; Nagai et al. 2020). Mueda et al. (2015) investigated the fermentation of *Stolephorus commersonii* at a 1:3.5 salt to fish ratio for 270 days at 28-30°C. Sauces were sampled periodically throughout the study. There were significant effects found with increasing fermentation time on protein content, which increased then decreased (13.6% to 15.4% to 12.7%) and TVB-N (total volatile base nitrogen), which increased from 20 to 150 mg/100 mL by day 120. Salt content was unaffected by fermentation time and pH decreased slightly from 7.0 at day 1 to 6.0 at day
270. Duration of fermentation is important to consider as it has a large impact on the production costs and overall consumer acceptance of fish sauce. Wang et al. (2017) reported that sensory evaluators preferred a darker brown color in fish sauce. Regarding odor, during the 10 day fermentation period, day 8 fermented samples received the highest “liking” scores for flavor and odor compared to all other time points. However, fish sauce production that surpasses the optimal fermentation duration can lead to weakened umami flavor, ammonia aromas, and bitter flavors.

A previous study on green crab sauce fermentation investigated the effects of temperature over time. Greiner et al. (2021) investigated the impact of incubation temperature (24°C, 30°C, 37°C, and 50°C) on the spontaneous fermentation of green crab with 20% salt to produce a sauce over 90 days. It was reported that fermentation temperature did not have an impact on bacterial evenness (species diversity). However, time had a significant impact on species diversity, as day 15 and 30 SVs (Shannon alpha diversity values) were higher compared to day 90. In spontaneous fermentation of fish sauce, the native microbiota of the starting seafood material can greatly impact the overall flavor or quality of the final product.

1.2.6. Effects of Salt

The application of salt in fermentation helps to reduce pathogenic bacteria and may impact the chemical properties of fish sauce. Mohamed et al. (2009) investigated fermented Egyptian salted fish (Feseekh), a popular Egyptian appetizer, for changes in free amino acids and biogenic amines. The mullet (*Mugil* spp.) was ripened at room temperature in a tightly sealed jar between layers of salt for 60 days. The authors reported that the
predominant free amino acids detected from days 0-60 were leucine (0.11-8.25 g/kg DW), glutamic acid (0.18-8.21 g/kg DW), and lysine (0.11-7.34 g/kg DW). The predominant biogenic amines including histamine, tyramine, putrescine, cadaverine, spermidine, and spermine, were reported to have increased by 7-fold on day 60 compared to day 0 levels. Cadaverine accounted for 61% of the final biogenic amine content (Mohamed et al., 2009). Overall, it was concluded that the Feseekh could be consumed without health risks between days 20-40, but not after day 60 due to the high biogenic amine content. Though salt can retard the rate of spoilage in seafood, it cannot completely deter compounds such as biogenic amines from forming.

In salt-fermented fish sauce, the salt content is primarily made up of sodium chloride. Industrial fish sauce is commonly made using at least a 20% salt content or higher. A majority of the 46 industrially produced fish sauces analyzed by Nakano et al. (2017) contained a salinity of 25%. The role of salt is important to controlling microbial growth to prevent spoilage and extend shelf-life of fish sauce.

A major contribution of salt in fermentation is its antimicrobial properties against spoilage microorganisms while permitting halotolerant microbes to flourish. Lapsongphon et al. (2013) investigated the effects of reduced salt content on fermentation of the starting fish material inoculated with *Virgibacillus* sp. SK37 at 35°C for 90 days. The seven treatments included controls and inoculates at salt contents of 10%, 15%, and 20% sodium chloride as well as an additional control at 25% sodium chloride. The authors reported that fish sauce samples made using 10% solar salt approached 6-7 log CFU/mL total bacteria count and underwent spoilage after 7 days of fermentation and halted further analysis of these treatments since the samples were beginning to putrefy. Proteolysis of the starting
fish material was found to be greater at lower salt contents due to an increase in proteinase activity. Protein hydrolysis by enzymes in fish sauce can be increased by using a salt content of 15% or lower as the endogenous enzymes do not typically tolerate high salt environments (Takashi et al., 2003). Additionally, Lapsongphon et al. (2013) reported that higher levels of alanine, aspartic acid, glutamic acid, methionine, phenylalanine, and serine were found in the salt-reduced (15-20%) fish sauce treatments. However, none of the 15-20% salt treatments, including the inoculated ones, exhibited accelerated protein hydrolysis. Odor-active compounds were identified through headspace-solid phase microextraction (H-SPME). The 15% salt treatment with inoculate showed increased levels of butanoic (cheesy odor) and 3-methylbutanoic (cheesy, sweaty odors) acids while the 20% salt treatment with inoculate did not. According to Takashi et al (2003), a complete removal of salt in fish sauce manufacture would decrease intensities of cheesy and rancid odors. Salt content must be carefully considered in fish sauce production as it impacts the targeted flavor profile, proteinase activity, shelf-life, and saltiness of the product. Overall, it is important to use a salt content of at least 15-20% in fermented seafood sauce to effectively control spoilage.

According to Greiner et al. (2021), salt content had a significant impact on chemical and microbial changes in green crab sauce fermentation. Treatments included whole chopped crab mixed with varying levels of salt, including 100 mg/g, 200 mg/g, and 300 mg/g, which were fermented for 3 months. As salt content increased, proteolytic bacteria populations were negatively affected in the crab sauce. TVBN and amine nitrogen were not affected by time, but overall the 100 mg/g treatment had significantly higher amounts of TVBN and amine nitrogen than the other two treatments. These significant differences
in TVBN and amine nitrogen could be correlated with the higher bacterial counts in the 100 mg/g treatment compared to the other treatments, indicating increased catabolism of the crab proteins during fermentation. Additionally, pH was not significantly impacted by salt content, averaging around 7.5, while water activity (0.746-0.860) and moisture content (69%-75%) were significantly higher in the 100 mg/g crab sauce treatment. After 120 days of fermentation, non-enzymatic browning and specific mean biogenic amines values (histamine, 6.0; putrescine, 3.5; agmatine, 4.3 mg/100 mL) were not significantly different among the three treatments. Based on these findings, salt content clearly impacted microbial and biochemical activity during green crab sauce fermentation. According to the FDA (2021b), the regulatory threshold for histamine content for a portion of edible fish must not exceed 50 ppm (50 µg/mL) to avoid potential scombrototoxin poisoning in humans.

1.2.7. Effects of Inoculation

Addition of inoculated bacterial strains to the starting seafood material during fermentation can affect its end-products. Koji is a traditional Japanese mold that is created using starchy grains or soybeans that are inoculated with Aspergillus oryzae, a filamentous fungus, which digests available starches into sugars to begin fermentation (Zhu and Tramper, 2013). These kojis are used as kick starters for fermentation to facilitate the process of making soy sauce, sake, and mirin (Feng et al., 2012). As the koji mold flourishes, mycelia begin to spread and secrete enzymes to hydrolyze proteins. The use of kojis aims to lower industrial production costs, accelerate fermentation, enhance safety, and promote optimal nutrition. Using two-stage autolysis, Wang et al. (2017) prepared a koji mixture of water and wheat grains (1:2) and inoculated anchovy paste with the koji starter culture at 0.5% by weight. The authors reported that the koji applied samples
exhibited a significant increase in amino acid nitrogen (increased degree of hydrolysis) compared to the uninoculated control.

Mold and bacterial strains previously applied in fish sauce production include the genera *Aspergillus*, *Pediococcus*, *Virgibacillus*, and *Psychrobacter*. Each inoculation study reviewed the effects on overall quality, efficiency, physicochemical properties, proteolytic activity, and biological changes of produced fish sauces. *Aspergillus oryzae* was used to facilitate aerobic fast-fermentation and produce a product with a consumer acceptance similar to commercial anchovy sauce and with a decreased biogenic amine content except for cadaverine (23 mg/L) (Sun et al., 2015). Biogenic amines such as phenylethylamine, tryptamine, histamine, putrescine, and tyramine must be kept at acceptable levels to ensure food safety.

Halotolerant lactic acid bacteria cultures (*Pediococcus pentosaceus* strains) were applied to rohu (*Labeo rohita*), a south Asian carp species, for 50 days. All strains presented high proteolytic activity and accelerated fermentation as measured by their ability to reduce pH or production of lactic acid in fish sauce samples (Siddegowda et al., 2017). Optimization of proteolytic activity in fish sauce production presents potential for ensuring fast-fermentation on an industrial scale. Microbial growth of *Virgibacillus* spp. SK37 in an inoculated fish sauce sample and the control remained constant by day 15. However, the inoculated samples had a higher total bacteria count (3.5 log CFU/mL) and halophilic populations (5.0 log CFU/mL) compared to the uninoculated control (TBC, 2.5 log CFU/mL; halophilic populations, 1.3 log CFU/mL) (Lapsongphon et al., 2013). Inoculation with this *Virgibacillus* strain increased volatile acids including acetic (sour odor) and 2-methyl propionic (malty odor) acids. Zheng et al. (2017) reported that
Psychobacter sp. SP-1 significantly increased activity of produced proteases, promoted umami flavor and meaty aroma, and decreased TVB-N and biogenic amines content in fish sauce. Overall, inoculation of these various strains can accelerate fermentation, enhance browning and flavor, increase volatile acids, and decrease biogenic amines in fish sauce.

Greiner et al. (2021) investigated the impact of inoculation with starter cultures on fermentation of green crab for sauce production. Starter cultures used included Tetragenococcus halophilus (fermented at 30°C) and Staphylococcus carnosus (fermented at 37°C), which were added to whole crab mince with 20% salt and fermented for 45 days. Overall, there were no statistically significant differences in microbial, physiochemical, and biochemical properties between treatments compared to the control by day 45. These findings indicate that incorporation of T. halophilus and S. carnosus did not lead to an enhanced fermentation or improved quality attributes in green crab sauce.

1.2.8. Use of Enzymes in the Seafood Industry

Enzymes fall under different categories depending on their function. Proteases or peptidases, which promote the hydrolysis of proteins, are the primary enzymes used in the fermented seafood product industry and include Neutrase, papain, pepsin, Alcalase, Protamex, and trypsin. Table 1.2 presents three common commercial proteases and appropriate environmental conditions for their activity.
Table 1.2. Commercial proteases and their activity

<table>
<thead>
<tr>
<th>Enzyme Name</th>
<th>Source Microorganism</th>
<th>Type</th>
<th>pH Range</th>
<th>Temperature Range (°C)</th>
<th>Activity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcalase</td>
<td><em>Bacillus licheniformis</em></td>
<td>Serine protease</td>
<td>6.5-8.5</td>
<td>50-70</td>
<td>2.4 U/g</td>
</tr>
<tr>
<td>Flavourzyme</td>
<td><em>Aspergillus oryzae</em></td>
<td>Amino-peptidase</td>
<td>5.0-7.0</td>
<td>50-70</td>
<td>500 U/g</td>
</tr>
<tr>
<td>Protamex</td>
<td><em>Bacillus subtilis</em></td>
<td>Thiol protease</td>
<td>5.5-7.5</td>
<td>25-60</td>
<td>1.5 AU-N/g</td>
</tr>
</tbody>
</table>

<sup>a</sup> U = µmol/minute, AU = Anson Unit. Adapted from Novozymes enzyme specification sheets.

Temperature, pH, and enzyme concentration influence how effective the applied enzymes will be during food processing. Temperature and pH need to be kept within a certain range that is not too high or low to promote enzyme-substrate activity but also prevent enzyme denaturation. Food matrix environments that enzymes are applied to are important to consider for optimal utilization and best results.

In the seafood industry, enzymes are utilized to aid in processing, prolong shelf-life, and control spoilage. These enzymes are typically applied in the production of fish protein hydrolysate, fish sauce, and cured herring, and can either accelerate processing or produce desirable compounds like polyunsaturated fatty acids (PUFAs). Other applications of enzymes in the seafood industry include in cured fish production, caviar and roe processing, and animal feed enrichment. Exogenous enzymes have been applied to minced seafood proteins to improve formation of high-quality gels by increasing cross linkages within the protein. Yin and Park (2015) reported that endogenous transglutaminase (ETG)
within Pacific whiting successfully inhibited endogenous proteases to improve textural integrity when producing surimi. In surimi production, proteases affect the gelation of proteins negatively, but their effects can be minimized through enhancing the activity of other types of enzymes like ETG. Proteolytic enzymes such as Protamex and Alcalase have been shown to effectively increase hydrolysis of fish byproducts up to 65% compared to a control without added enzymes (Liu et al., 2010). See et al. (2011) reported that application of 2.5% (v/w) Alcalase on salmon skin achieved the highest degree of hydrolysis of 77% at 55°C and pH of 8.39. Overall, a wide variety of seafood byproducts containing high amounts of protein can be hydrolyzed using commercial enzymes.

Protein hydrolysis of crustaceans such as shrimp and crabs has been shown to benefit from the addition of commercial enzymes. Benjakul et al. (2009) investigated the impact of adding Flavourzyme to white shrimp to produce Mungoong (shrimp paste). Flavourzyme applied to the shrimp at 0.15% or 0.30% (weight percent) significantly increased the yield of the shrimp paste to 107% (dry weight basis) compared to the control with no added enzymes, at 86% yield. Another study focused on comparing the application of Alcalase versus pancreatin on shrimp processing waste to recover valuable proteins. Overall, hydrolysis of the shrimp waste protein was highest in the Alcalase treatment (25%) compared to the pancreatin treatment (18%). Protein recovery was also higher in the Alcalase treatment at 65% compared to the pancreatin treatment at 58%. Thus, applying proteases to shrimp and shrimp processing waste has proven to be effective in terms of protein recovery and overall yield.

Hydrolysis of proteins has also been investigated in crabs such as *Portunus trituberculatus* and *Portunus sanguinolentus*. These marine crabs were hydrolyzed with a
protease cocktail of Neutrase, Flavourzyme and papain (1:1:1 ratio and applied at concentrations of 1-4%) (Liu et al., 2010). These three enzymes each played a different role in the hydrolysis of the marine crab proteins. Neutrase hydrolyzed peptide bonds of hydrophobic amino acids such as leucine, phenylalanine and tyrosine, while Flavourzyme hydrolyzed polypeptides to create smaller peptides, and papain contributed to hydrolysis of carboxyl groups in arginine and lysine. As concentration of the applied mixed enzyme (ME) increased, degree of hydrolysis generally positively correlated from about 20% (2.5% ME) to 23% (3.5% ME) Overall, hydrolysis of the crab was found to be most optimal using 3.5% ME at a pH of 6.5 at 50°C for 5 hours. In another study conducted by Jiang et al. (2017), crab shell removed from *Portunus* spp. was hydrolyzed by five enzymes (bromelain, Neutrase, pepsin, Protamex, trypsin), and the hydrolysates were observed for antioxidant activity. The pepsin and Protamex (both applied at 1%) hydrolysates were found to be significantly higher in total antioxidant activity. The hydrolyzed crab shell was further analyzed and presented high protein solubility at a pH of 6. Additionally, emulsifying activity of the crab shell protein was optimal at pH 6 where the crab shell hydrolysate was most soluble; this also led to an increase in protein adsorption at the interface. Enzymes have been shown to improve production and quality of crab byproduct hydrolysates which may be used to create new food products.

### 1.2.9. Application of Enzymes in Fish Sauce Production

Traditional fish sauce production relies on the activity of endogenous enzymes, but the addition of exogenous enzymes can accelerate the breakdown of seafood proteins. Accelerated fermentation of fish sauce is beneficial from a production standpoint due to quicker product turnover rates. Using exogenous enzymes during the production of fish
sauce could greatly decrease the fermentation period required compared to traditional fish sauces, which typically take 6-18 months to produce. Xu et al. (2008) investigated the effects of fermenting squid by-products (head, viscera, skin, and fin) with kojis and Flavourzyme. The three treatments, which included chopped squid byproducts mixed with (1) distilled water, (2) 8% salt, water, and koji, and (3) 8% salt, water, koji, and Flavourzyme, were fermented at 48°C for 30 days. Protease activity was monitored using 2% casein as the substrate in phosphate buffer and absorbance was measured at 660 nm. Protease activity was similar but not the same among the three treatments as protease activity gradually increased during days 10-20 of fermentation and then decreased. By day 30, all treatments had similar quantitative descriptive analysis scores with high umami, meaty, and caramel notes and low rancid, sour, bitter, and ammonia flavors and aromas. Results indicated that fermentation in all treatments of the squid by-product sauce did not present any noticeable spoilage at day 30.

Research on enzyme-applied fermentation in fish sauce production is limited, but preliminary work has been conducted. Aquerrera et al. (2001) investigated the role of exogenous enzymes applied to further ferment garum over a 48-hour period. The garum was prepared using a 1:1 ratio of tuna (Tunnus thynnus) liver and mackerel meat (Scomber scombrus) at salt contents of 5%, 10%, and 25%. Lower salt contents were studied to optimize enzyme activity as salt may inhibit enzymatic proteolysis. The commercial proteases compared were fungal protease P31000, Alcalase, Kojizyme, Trypsin, and Neutrase. The authors reported that the best treatment was the 10% salt (initially 5%, then another 5% was added at the 24-hour mark) in conjunction with Neutrase in terms of highest yield. The fermentation process in this study was conducted within an extremely
condensed timeline of 48-hours and the enzymes may not have been utilized to their full potential. Another study by Sun et al. (2015) used Alcalase (applied at 0.25%) and Flavourzyme (applied at 0.5%) to hydrolyze anchovy protein to accelerate fermentation in Thai fish sauces. Starter cultures were added to the hydrolyzed (Alcalase and Flavourzyme for 6 hours) fish samples and were found to result in sauces having a higher mean amino acid concentration (755 mM) compared to the untreated (non-hydrolyzed or inoculated) control sauce (682 mM). After four months, the amino acid profiles of the treated samples were found to contain the same predominant amino acids (glutamic, aspartic, lysine) as a commercial fish sauce product fermented for 12 months. The enzyme-added and inoculated samples were found to have 50% lower histamine concentration compared to the control without added enzymes or microbes. The hydrolyzed anchovy was found to have significantly higher amounts of cadaverine (5.4 mg/100 g) and histamine (25.6 mg/100 g) compared to the control, at 1.9 and 6.4 mg/100 g for cadaverine and histamine, respectively. Other biogenic amines such as putrescine, spermine, and tryptamine were undetected in all samples. The findings of this study indicate that enzymes may be used to kickstart the hydrolysis of proteins in starting seafood materials for accelerated fermentation of fish sauce.

1.2.10. Consumer Perceptions of Fish Sauce

Consumer-perceived characteristics of fish sauce may differ based on geographical region due to different preferences for aromas or flavors. Russo et al. (2020) conducted a sensory evaluation of a traditional Italian fish sauce called Colatura di Alici, in Naples, Italy with trained panelists to produce repeatable and discriminate results. Quantitative descriptive analysis (QDA) was applied using a 6-point scale where “0” represented null
intensity and “5” represented extreme intensity. Attributes “cheesy” and “fishy” received scores ranging from 3-4.5 across five different brands of Italian fish sauce. Other investigated attributes included “meaty,” “umami,” and “roasted,” rated as medium intensity, and lastly the “rancid” attribute, scoring the lowest at around 2-2.5. Another study by Harikedua et al. (2012) focused on consumer acceptance and QDA of an Indonesian fish sauce called bakasang. Using a 9-point hedonic scale, in which “1” represented “dislike extremely” and “9” represented “like extremely,” panelists rated their liking of the following attributes: odor at about 5.5, taste at 5.5, and overall at 5.7. In the QDA, panelists identified the most prominent flavor attributes of the fish sauce as “fishy,” “burnt,” “sweaty,” and “sulphury meaty.” Additionally, aftertaste and mouthfeel attributes were identified most prominently as “fishy,” “aftertaste,” “umami,” “bitter,” and “salty.” As these sauce samples were consumed as is without incorporation into a dish, sensory acceptability scores may have been skewed. Based on these investigations of fish sauce, average consumer acceptance indicated that the products were “neither liked nor disliked,” with notable cheesy and fishy flavor attributes.

1.3. Justification

Green crabs are generally considered as a commercially low value resource, but their populations are abundant. Proposed uses for invasive green crab are limited within the food industry and trapped crabs tend to be discarded as waste or used in low-value applications such as bait, compost, or fish feed (Fulton and Fairchild, 2013). In the culinary scene, soft shell green crabs have recently become a unique addition to restaurant menus. Fish sauce fermentation is a meaningful and high value vehicle in which green crabs can be chopped or ground and used as the primary seafood protein. Based on previous work in
our laboratories on green crab sauce fermentation, a temperature of 37°C and salt content of 20% were recommended to produce a fermented green crab sauce that is safe and appropriately produced to meet fish sauce standards (FAO and WHO, 2018). However, the application of proteolytic enzymes in the fermentation of green crab has not been reported and may improve the efficiency and/or quality of the sauce. In addition, a local Maine-made fermented seafood condiment using invasive green crabs has the potential to fill a market niche for chefs and restaurants across New England. However, the perceptions of culinary professionals regarding a novel fermented green crab sauce have not yet been examined, representing an important knowledge gap for the successful commercial implementation of this new product concept.

1.4. Objectives

The overall goal of this research was to further the development of a Maine-made fermented green crab sauce condiment targeted for upscale food service application. The development of a value-added product from invasive green crabs may help alleviate their ecosystem impacts and contribute to new product opportunities for food businesses in our region. The specific objectives of the two studies were to: (1) evaluate the physicochemical and microbial effects of proteolytic enzyme treatments (Alcalase, Flavourzyme, and Protamex) on the fermentation of green crab sauce, and (2) survey chefs in New England regarding their perceptions of a fermented green crab sauce as a culinary ingredient.
CHAPTER 2

EFFECTS OF ENZYMES ON FERMENTATION OF GREEN CRAB SAUCE

2.1. Introduction

The European green crab (*Carcinus maenus*) is an aggressive invasive species found on the east and west coasts of North America (Grosholz et al., 2011; Leignel et al., 2014) as well as other countries across the globe. Green crab populations are expected to increase with projected increases in ocean surface temperatures in North America (Maine Department of Marine Resources, 2013; EPA, 2021). Green crabs have negatively impacted economically important local marine species, including a major disruption to soft-shell clam and juvenile crustacean populations (Glude, 1995; Quinn and Boudreau 2016). Overall, green crab predation has resulted in an average of $22.6 million in damages annually to fisheries on the east coast of North America (Lovell et al., 2007). Green crabs also indirectly impact local marine species due to their scavenging and feeding on prey such as blue mussels and young oysters that other species depend on (Garside et al., 2015; Pickering et al., 2017).

Though there is an excess of unwanted green crabs in North America, efforts to valorize these crabs have been restricted due to their small size and difficulty with meat extraction. Potential uses for green crabs have been investigated including incorporation into crab meal, application in fertilizers, and as bait for commercial and recreational fisheries (LePage, 2014). However, these uses of green crab biomass are low profit margin items. In contrast, some green crabs are being harvested for use as soft-shell crab in culinary settings and have been sold for up to $25 a pound to restaurants across New
England (McMahan, 2021). The introduction of soft-shell green crab New England restaurants has been highly profitable, suggesting that development of new crab products should be targeted towards culinary applications.

Recently, the application of green crabs as a starting material for fish sauce has been shown to be feasible (Greiner et al., 2021). Fish sauce is a globally popular product that is typically spontaneously fermented, brown in color, transparent, and packed with umami flavor. Traditionally, fish sauce is produced using a mixture of fish (frequently anchovies) and salt which is fermented for 6-12 months at ambient temperatures. Other protein sources have also been used to create different variations of fish sauce including squid, shrimp, and crab (Xu et al., 2008; Liu et al., 2019; Nagai et al., 2020). Prior research in our laboratories (Greiner et al., 2021) has investigated the use of whole minced green crab and confirmed that salt content, temperature, and fermentation time are major factors to consider in crab sauce fermentation. In crab sauce fermentation, a salt content of at least 20% or higher was adequate to prevent unwanted spoilage microorganism growth (Greiner et al., 2021). A fermentation temperature of 37°C for crab sauce resulted in high levels of amine nitrogen and low counts of proteolytic, total viable count, histamine forming bacteria, and lactic acid bacteria (about 2 log CFU/mL). Additionally, most biochemical changes the crab sauce occurred within the first 60 days of fermentation (Greiner et al., 2021). To date, there are no reports on the application of proteases in crab sauce fermentation.

Previous studies on developing a fermented green crab condiment have explored spontaneous and inoculated fermentations (Greiner et al., 2021). Commercial enzymes such as Flavourzyme, Alcalase, and Protamex have been popularly used in the seafood
industry to accelerate protein hydrolysis for extraction from a wide variety of processing by-products (Diaz-Lopez and Garcia-Carreno, 2000; Fernandes, 2016). The application of proteases in white shrimp paste extract production has been shown to increase overall yield, shorten fermentation periods, increase enzymatic activity, and decrease biogenic amine content (Benjakul et al., 2009). Application of proteases may help to optimize and accelerate green crab sauce fermentation. The purpose of this study was to investigate the impact of commercial proteases on the progress of fermentation, yield, and physiochemical characteristics of the final product.

2.2. Methods

2.2.1. Experimental Design Overview

Green crabs were chopped and mixed with 20% salt (NaCl) in three separate batches, each representing a process replicate. Each replicate batch was divided into four treatments including a control (no enzyme added), 0.5% Alcalase treatment, 0.5% Flavourzyme treatment, and 0.5% Protamex treatment. Each treatment replicate was divided into five jars corresponding to fermentation period: 2, 15, 30, 60 & 90 days. For the first 48 hours all of the samples were fermented at 55°C, followed by 37°C for the remainder of fermentation. Crab sauce samples were subjected to the following analyses: pH, percent yield, amine nitrogen, total volatile base nitrogen (TVBN), water activity, moisture content, browning index, microbial counts, salt content, and biogenic amines. Statistical evaluation was conducted to determine any significant impacts of enzyme addition and fermentation time on the quality attributes of the crab sauce.
2.2.2. Preparation of Crab and Treatments

Green crabs were trapped off the coast of Georgetown, Maine and transported in coolers to the University of Maine (Orono, ME). The live green crabs were spread into single layers on perforated aluminum sheets and individually quick frozen (IQF) in a blast freezer at -40°C for 30 minutes (Beckman, Brea, CA). Following, the blast frozen crabs were placed back into coolers and stored in a walk-in freezer (Mathew Highlands Pilot Plant, Orono, ME) at -20°C until use. Prior to processing, 60 individual 32-ounce glass jars (Ball, Atlanta, GA, USA) were cleaned and sanitized. For processing, the frozen crabs were thawed for 36-48 hours at 4°C and then finely chopped for approximately 3 minutes using a Kolsch bowl cutter (UltraSource, Kansas City, MO, USA). The crabs were chopped by replicate batch (A, B, & C) and each batch was separated into four different treatments. The four treatments included a control and three different enzyme treatments: Alcalase ($\geq 0.75$ AU/mL), Flavourzyme ($\geq 500$ U/g), and Protamex ($> 1.5$ AU-N/g) (Sigma Aldrich, St. Louis, MO, USA).

Each treatment replicate was prepared in a separate metal bowl. To each bowl, 3200 g of chopped crab was added followed by the addition of enzymes to three of the treatments. All enzymes were applied at 0.5% w/w of whole crab mince. No enzymes were added to the control. Each bowl containing the crab mixture was stirred thoroughly by hand with a rubber spatula for three minutes. Next, 800 g (20% w/w) of coarse kosher salt (Morton Salt, Chicago, IL, USA) was added to each treatment mixture and stirred for 1 minute. Each homogenous mixture was then divided into five separate 32-ounce glass jars, covered with a double layer of cheesecloth (Pyrm Consumer USA, Spartanburg, SC, USA) and secured with a rubber band.
2.2.3. Fermentation of Crab Sauce

All sixty fermentation jars were incubated for 48 hours at 55°C, which was selected as a good compromise temperature for satisfactory activity of the added proteases (refer to Table 1.2) (Novozymes, 2016). Subsequently, the jars were transferred to a bacteriostatic incubator set to 37°C for the remainder of fermentation. On each sampling time point (Days 2, 15, 30, 60, 90), twelve entire jars representing three replicates (A, B, & C) of each of the four treatments, were utilized for chemical and microbial analyses.

2.2.4. Filtration of Crab Sauce

Following fermentation, the contents of each jar consisted of a top layer of salt followed by a middle layer of liquid, with the remaining crab solids on the bottom by day 2 of fermentation. As the fermentation period increased, the salt layer appeared to become thicker. The liquid portion in the jar was isolated by straining through two layers of nonsterile cheesecloth into 250 mL glass beakers. The remaining solids portion of the jar was added to a 500 mL centrifuge tube and centrifuged in a Avanti J-E Beckman Coulter centrifuge (Brea, CA, USA) at 706 x g for 10 minutes at 4°C using a JA-10 rotor (Beckman Coulter, Indianapolis, IN, USA). The supernatant from the centrifuged material was combined with the previously strained liquid and the pooled liquid was weighed to determine total crude yield. The crude sauce appeared murky with brown filaments floating within the mixture. Therefore, a second filtration step was performed to yield a transparent sauce sample more comparable to other industrial fish sauce products. The second filtration step involved centrifuging the crude liquid sauce samples at 17649 x g for 15 minutes at 4°C using a JA-10 rotor. The supernatant was filtered through a glass funnel lined with a Whatman #1 filter paper. At this stage, the filtrate appeared transparent with a light to
medium brown color and comparable to typical industrial fish sauces. A portion of the analyses were conducted the day of the sauce filtration including pH, percent yield, moisture content, water activity, and microbial analyses. The remaining sauce samples were frozen at -18°C in dark conditions and thawed at 4°C overnight for completion of the outstanding analyses.

2.2.5. Microbial Analysis

Crab sauce samples were sampled on days 2, 30, 60, and 90 for microbial analysis (total bacteria count, proteolytic bacteria, lactobacilli bacteria, and heterotrophic marine bacteria). Each crude sauce sample (first filtration) was plated in duplicate. A subsample of each sauce was serially diluted (1:10, 1:100, and 1:1000) with sterile 0.1% bactopeptone (BD Diagnostics, Sparks, MD, USA). All three dilutions were prepared for all treatment replicates on each sampling day. The dilution tubes were vortexed (Weber Scientific, Hamilton Township, NJ, USA) for 10 seconds.

Aliquots of 100 uL of the appropriate dilutions were spread-plated onto four kinds of culture media in duplicate including tryptic soy agar (TSA), skim milk agar (SMA), DeMann Rogosa Sharpe agar (MRS), and Zobell marine agar (MA) prepared in 100 mm x 15 mm plastic Petri-dishes. TSA (Alpha Sciences, Pharmacy Avenue, Toronto, Ontario, Canada) was prepared based on manufacturer’s instructions and used to quantify total mesophilic bacterial count (TBC) and plates were incubated at 30°C for 48 hours. SMA was prepared using 34.9 g of brain heart infusion agar (Hardy Diagnostics, Santa Maria, CA, USA), 80 mL of aseptically packaged skim milk (Parmalat, Buffalo, NY, USA), 15 g of bacteriological grade agar (HI media, Kennett Square, PA, USA), and 24 g of sodium chloride (Macron, Pasadena, TX, USA) to quantify proteolytic bacteria and plates were
incubated at 37°C for 48 hours. MRS (Alpha Biosciences, Baltimore, MD, USA) was prepared based on manufacturer’s instructions and used to quantify lactic acid bacterial counts. Plates were incubated at 30°C for 48 hours. MA was prepared with 32.2 g of Difco marine broth (BD Biosciences, Franklin Lakes, New Jersey, USA) and 15 g of bacteriological grade agar (HI media, Kennett Square, PA, USA). MA plates were incubated at 25°C for 48 hours and used to quantify heterotrophic bacterial counts.

Post incubation, plate counts were recorded, and the appropriate dilution factor was used to calculate the microbial populations of the crude sauce sample. Bacterial counts were expressed as colony forming units per mL (CFU/mL). All treatment replicates were plated in duplicate and the counts were averaged. Bacterial counts were log-transformed and then averaged for data analysis and presentation. In instances where no counts were detected, the results were reported as the detection limit of the plating method (< 2.0 log CFU/mL).

2.2.6. Yield, pH, Water Activity, and Moisture Content

Percent Yield

Percent yield compares the final mass of fermented crab sauce to the starting amount of crab mince material (640 g) in each fermentation jar. Percent yield was calculated using the following equation:

\[
% \text{ Yield} = \frac{\text{Final sauce weight (g)}}{\text{Starting chopped crab mass (g)}} \times 100
\]
The pH meter (Orion Star A111 pH meter, Thermo Scientific, Waltham, MA, USA) and probe were calibrated (pH 4, pH 7, and pH 10 standards) based on manufacturer’s instructions. Individual pH values of each sample were determined through duplicate readings per replicate (A, B, & C) and were averaged to derive the mean pH value for each treatment.

Water Activity

Water activity was measured using the Aqualab Pullman meter (Thermo Fisher Scientific, Waltham, MA, USA). The water activity meter was calibrated using a 0.984 standard. Each treatment replicate was analyzed in duplicate and values were averaged.

Moisture Content

An AOAC method (934.01) was conducted using a vacuum oven (Isotemp Vacuum Oven Model 281 A, Thermo Fisher Scientific, Waltham, MA, USA). Sauce samples (2 g) were analyzed in duplicate and averaged per treatment replicate. Vacuum moisture was conducted at 100°C with a pressure of 20 in Hg for 5 hours. Weight of the sample after drying was recorded and used to calculate moisture content (%) using the following equation (AOAC Official Method 934.01, 2005):

\[
\% \text{ Moisture content (wwb)} = \left( \frac{\text{Initial sample weight} - \text{Dry sample weight}}{\text{Initial sample weight}} \right) \times 100
\]

2.2.7. Browning Index

Browning index was measured according to Zhao et al. (2018) with slight modification. A 1:10 sauce to deionized water dilution was made and stirred for one hour.
using a magnetic stirrer. Each diluted sample was then filtered through an Acrodisc 13 mm 0.45 µm syringe filter (MDI Membrane, Harrisburg, PA, USA) and absorbance of the filtrate was measured at 420 nm using a DU 530 spectrophotometer (Beckman Coulter, Brea, CA, USA). The stirred sample was filtered and measured in duplicate and averaged. Browning intensity was expressed as A<sub>420</sub>.

2.2.8. Total Volatile Base Nitrogen and Amine Nitrogen

*Total Volatile Base Nitrogen*

The TVBN content of the samples was determined once for each treatment replicate using a modification of the method published by Botta et al. (1986). The lab fermented crab sauce sample (5 mL) was diluted 1:1 with deionized water (5 mL) and vortexed until homogenous. This 10 mL mixture was added to a micro-Kjeldahl distillation unit (Rapid distillation unit, Labconco, Kansas City, MO) followed by 4 mL of 10% sodium hydroxide solution. The samples were distilled into an Erlenmeyer flask containing 15 mL of 4% boric acid solution and 8 drops of indicator (0.2% methyl red and 0.2% methylene blue, 2:1 in ethanol) to constitute a final volume of approximately 45 mL. The distillate was then titrated with 0.05 N hydrochloric acid (HCl) until the mixture turned from an aqua blue to a constant purple color. The volume (mL) of titrant used was recorded to calculate TVBN using the following equation:

\[
TVBN = \frac{\text{[(Volume (mL) HCl used for titrating the sample) } \times \text{ Normality of HCl } \times 100 \text{ mL}]}{\text{Molecular weight of N}} \times \frac{\text{Undiluted sample volume (mL)}}{\text{Undiluted sample volume (mL)}}
\]

TVBN values were expressed as mg/100 mL.
Amine Nitrogen

Amine nitrogen was measured using a N-formol titration method described by Joung and Min (2018) with slight modifications. Each sample was analyzed once, and replicates were averaged for mean amine nitrogen concentration for each treatment. Sauce samples were diluted with distilled water (1:10, sauce to water) and pH was adjusted to 8.5 with 0.1 N sodium hydroxide (NaOH) solution. Next, 8 mL of 37% w/v formaldehyde solution was adjusted to a pH of 8.5 with 0.1 N NaOH. The adjusted sample and formaldehyde mixtures were combined which dropped the overall pH. This mixture was titrated with 0.05 N NaOH until a pH of 8.5 was reached. The amount of 0.05N NaOH used to titrate was recorded and used to calculate amine nitrogen in the sample using the following equation:

\[
\text{Amine Nitrogen} = 14 \times \text{mL of NaOH titrant} \times N \text{ of NaOH} \times 10 \times \frac{100}{10}
\]

Amine nitrogen was expressed as mg/100 mL.

2.2.9. Salt Content

Salt content in samples was measured using the Orion Star A111 pH meter fitted with the Orion 9780SC silver billet electrode (Thermo Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. The pH meter reported values in millivolts (mV). Sauce samples (5 mL) were diluted with 45 mL deionized water (1:10). Next, 2 mL of the diluted sample, 1 mL of ionic strength adjuster (ISA), and 50 mL of deionized water were added to a 150 mL glass beaker. The standard and sample mixtures were separately stirred and titrated with 0.1 M silver nitrate solution (LabChem, Zelienople, PA) until a
reading of 290 mV was achieved. Percent salt (sodium chloride) concentration of the samples was calculated using the following equation:

\[
\text{Concentration (\% of NaCl in sample) = } \frac{5.884 \cdot \text{Concentration of Titrant (M)} \cdot (\text{Volume (mL) of titrant used for sample} - \text{Volume (mL) of titrant for blank})}{\text{Volume of diluted sample used (mL)} \cdot \text{Dilution factor}}
\]

Where dilution factor refers to dilution of sample with deionized water.

### 2.2.10. Biogenic Amines

The crab sauce samples were prepared using an EZ:faast extraction kit for free (physiological) amino acids (Phenomenex, Torrance, CA, USA). Preparation of the sauce samples included the following: (1) solid phase extraction, (2) derivatization, and (3) a liquid/liquid extraction according to kit directions. The extracted and derivatized samples were identified by gas chromatography-mass spectroscopy (GC-MS) with Agilent Technologies 6890 series. The internal standard consisted of a premade mixture of 0.2 µM norvaline and 10% N-propanol.

A mixed standard was made which combined histamine (114.0 mg), putrescine (87.7 mg), tyramine (110.3 mg), and cadaverine (87.0 mg) and then brought to volume with 10 mL of HPLC-grade water. The mixed standard was used to record eluting times and peak area values of each biogenic amine. Each sample ran for 15 minutes with an inlet temperature at 250°F, oven temperature at 110°F, and 1-1 column flow, MS source of 240, MS quad of 180, Aux-2 temperature at 280°F, and turbo speed of 100. Peak areas were used to calculate analyte concentration. Biogenic amine values were expressed as µmol/L.
2.2.11. Statistical Analysis

The data were coded and analyzed using IBM SPSS 27 (International Business Machines - Statistical Package for Social Sciences) at a significance level of \( p < 0.05 \). The Shapiro-Wilk test was conducted to assess normality and Levene’s test was run for equality of variances to assess homogeneity. One-way analysis (ANOVA) was used to assess all one-level (treatments) effects, and when significant differences were found Tukey’s honest significant difference (HSD) post hoc test was used to separate treatment means. A multi-way ANOVA was conducted for all dependent variables to determine time and treatment effects; when significant differences were found Bonferroni’s post hoc test was used to separate means. Descriptive statistics and correlational (Pearson, bivariate) analyses were also conducted.

2.3 Results
2.3.1 Microbial Analysis

Fermentation time had a significant effect on total mesophilic bacterial populations across treatments. There was a significant decrease in total bacterial populations starting on day 30 as time progressed across all treatments. Total bacterial populations in the control, Alcalase, Flavourzyme, and Protamex treatments were significantly \( p \leq 0.05 \) higher on day 2 (~mean 4.7 log CFU/mL) compared to day 90 (between 2.0-2.5 log CFU/mL) (Table 2.1). At the model level, treatment did not impose any significant effects on total bacterial populations in crab sauce samples. The mean total plate count at the end of fermentation across treatments was 2.3 ± 0.2 log CFU/mL.
Table 2.1. Total mesophilic populations (log CFU/mL) of crab sauce treatments over time

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 2</th>
<th>Day 30</th>
<th>Day 60</th>
<th>Day 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.7 ± 0.1aA</td>
<td>2.5 ± 0.1aB</td>
<td>2.5 ± 0.4aB</td>
<td>2.2 ± 0.5aB</td>
</tr>
<tr>
<td>Alcalase</td>
<td>4.7 ± 0.2aA</td>
<td>2.1 ± 0.1bB</td>
<td>2.6 ± 0.1aB</td>
<td>2.4 ± 0.4aB</td>
</tr>
<tr>
<td>Flavourzyme</td>
<td>4.7 ± 0.0aA</td>
<td>2.4 ± 0.1aB</td>
<td>2.6 ± 0.1aB</td>
<td>2.0 ± 0.7aB</td>
</tr>
<tr>
<td>Protamex</td>
<td>4.7 ± 0.1aA</td>
<td>2.5 ± 0.1aB</td>
<td>2.6 ± 0.1aB</td>
<td>2.5 ± 0.0aB</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviations (n=3). Lowercase letters designate significant differences (in columns) among treatments at each time point (1-way ANOVA). Uppercase letters designate significant differences (in rows) within treatments over time (1-way ANOVA).

Fermentation time also had a significant effect on proteolytic bacterial populations across treatments, but there was no significant effect of treatment on proteolytic bacterial populations at the model level. Proteolytic bacterial populations in the control, Alcalase, Flavourzyme, and Protamex treatments were significantly ($p < 0.05$) higher on day 2 (between 3.8-4.7 log CFU/mL) compared to day 90 (between 1.5-2.5 log CFU/mL) (Table 2.2).

Table 2.2. Proteolytic bacterial populations (log CFU/mL) of crab sauce treatments over time

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 2</th>
<th>Day 30</th>
<th>Day 60</th>
<th>Day 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.5 ± 1.1aA</td>
<td>2.4 ± 0.1aB</td>
<td>2.7 ± 0.7aB</td>
<td>1.9 ± 0.1aB</td>
</tr>
<tr>
<td>Alcalase</td>
<td>3.8 ± 0.2aA</td>
<td>2.3 ± 0.1aB</td>
<td>2.6 ± 0.3aB</td>
<td>2.4 ± 0.4aB</td>
</tr>
<tr>
<td>Flavourzyme</td>
<td>3.9 ± 0.0aA</td>
<td>2.3 ± 0.2aB</td>
<td>2.5 ± 0.3aB</td>
<td>1.5 ± 0.7aB</td>
</tr>
<tr>
<td>Protamex</td>
<td>4.7 ± 0.1aA</td>
<td>2.4 ± 0.1aC</td>
<td>2.8 ± 0.1aB</td>
<td>2.5 ± 0.0aBC</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviations (n=3). Lowercase letters designate significant differences (in columns) among treatments at each time point (1-way ANOVA). Uppercase letters designate significant differences (in rows) within treatments over time (1-way ANOVA).
Fermentation time had a significant effect on lactic acid bacteria (LAB) populations across treatments at the model level. Lactic acid bacteria populations significantly decreased between days 2 and 30 and then increased on day 60 (Table 2.3). The average of lactic acid bacteria populations at the end of fermentation across treatments was $1.8 \pm 0.2$ log CFU/mL. There was no significant model effect of treatment on lactic bacteria populations.

**Table 2.3. Lactic acid bacteria populations (log CFU/mL) of crab sauce treatments over time**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 2</th>
<th>Day 30</th>
<th>Day 60</th>
<th>Day 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.5 ± 0.4aA</td>
<td>2.1 ± 0.2abAB</td>
<td>2.2 ± 0.3aAB</td>
<td>1.7 ± 0.5aB</td>
</tr>
<tr>
<td>Alcalase</td>
<td>2.1 ± 0.2aA</td>
<td>1.6 ± 0.1bA</td>
<td>2.3 ± 0.3aA</td>
<td>1.7 ± 0.7aA</td>
</tr>
<tr>
<td>Flavourzyme</td>
<td>2.7 ± 0.4aA</td>
<td>2.1 ± 0.3abA</td>
<td>2.4 ± 0.2aA</td>
<td>1.8 ± 0.6aA</td>
</tr>
<tr>
<td>Protamex</td>
<td>2.1 ± 0.2aB</td>
<td>2.2 ± 0.2aAB</td>
<td>2.5 ± 0.1aA</td>
<td>2.2 ± 0.1aAB</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviations (n=3). Lowercase letters designate significant differences (in columns) among treatments at each time point (1-way ANOVA). Uppercase letters designate significant differences (in rows) within treatments over time (1-way ANOVA).

Treatment and fermentation time did not have a significant impact ($p \leq 0.05$) on heterotrophic marine bacterial populations at the model level. However, there was a significant decrease in salt tolerant bacterial populations within the Protamex treatment as salt tolerant bacterial populations on day 60 (1.9 log CFU/mL) were significantly ($p \leq 0.05$) lower than day 30, and 90 counts (Table 2.4). The average salt tolerant bacterial populations at the end of fermentation across treatments was $2.3 \pm 0.2$ log CFU/mL.
Table 2.4. Heterotrophic marine bacterial (log CFU/mL) populations of crab sauce treatments over time

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 2</th>
<th>Day 30</th>
<th>Day 60</th>
<th>Day 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.0 ± 0.0aA</td>
<td>2.3 ± 0.0aA</td>
<td>2.5 ± 0.4aA</td>
<td>2.2 ± 0.6aA</td>
</tr>
<tr>
<td>Alcalase</td>
<td>2.1 ± 0.1aA</td>
<td>2.0 ± 0.5aA</td>
<td>2.7 ± 0.2aA</td>
<td>2.2 ± 0.5aA</td>
</tr>
<tr>
<td>Flavourzyme</td>
<td>2.1 ± 0.1aA</td>
<td>2.4 ± 0.1aA</td>
<td>1.9 ± 0.7aA</td>
<td>2.2 ± 0.6aA</td>
</tr>
<tr>
<td>Protamex</td>
<td>2.1 ± 0.1aAB</td>
<td>2.6 ± 0.2aA</td>
<td>1.9 ± 0.4aB</td>
<td>2.6 ± 0.2aA</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviations (n=3). Lowercase letters designate significant differences (in columns) among treatments at each time point (1-way ANOVA). Uppercase letters designate significant differences (in rows) within treatments over time (1-way ANOVA).

2.3.2 Yield, pH, Water Activity, and Moisture Content

*Percent Yield*

Enzyme treatment had a significant effect on yield of crab sauce at the model level (Figure 2.1). The control (~19%) had a significantly lower mean yield compared to the Alcalase, Flavourzyme, and Protamex treatments, which had mean yield values ranging from 23-25%. On day 15, the control (13.5%) was significantly ($p < 0.05$) lower in yield compared to the Alcalase (25.5 ± 2.3%), Flavourzyme (24.9 ± 0.5%), and Protamex (22.9 ± 2.1%) treatments (Figure 2.2, Table 2.5), however all treatments had similar yields by day 60 and for the remainder of fermentation. Fermentation time did not have a significant effect on percent yield at the model level.
Figure 2.1. Effects of treatment on yield (%) of crab sauce

Values were collapsed across time. Treatments not sharing a letter are significantly different ($p < 0.05$) based on multi-way ANOVA followed by Tukey’s post hoc test. The error bars represent standard deviation (n=12).

Figure 2.2. Mean yield (%) values of crab sauce treatments over 90 days

The error bars represent standard deviation. (n=3)
Table 2.5. Mean yield (%) values of crab sauce treatments over 90 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 15</th>
<th>Day 30</th>
<th>Day 60</th>
<th>Day 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.5 ± 1.3bB</td>
<td>20.2 ± 2.1aA</td>
<td>24.5 ± 0.8aA</td>
<td>20.7 ± 1.5aA</td>
</tr>
<tr>
<td>Flavourzyme</td>
<td>24.9 ± 0.5aA</td>
<td>26.6 ± 1.0aA</td>
<td>22.3 ± 1.9aA</td>
<td>25.6 ± 4.0aA</td>
</tr>
<tr>
<td>Alcalase</td>
<td>25.5 ± 2.3aA</td>
<td>27.7 ± 1.8aA</td>
<td>22.4 ± 3.5aA</td>
<td>24.1 ± 2.8aA</td>
</tr>
<tr>
<td>Protamex</td>
<td>22.9 ± 2.1aA</td>
<td>24.6 ± 1.9aA</td>
<td>22.8 ± 0.9aA</td>
<td>23.5 ± 3.0aA</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviations (n=3). Lowercase letters designate significant differences (in columns) among treatments at each time point (1-way ANOVA). Uppercase letters designate significant differences (in rows) within treatments over time (1-way ANOVA).

$pH$

Fermentation time (Figure 2.3) and treatment had significant effects on pH of crab sauce at the model level. Based on multiway ANOVA, pH values on day 60 were significantly ($p \leq 0.05$) higher than on day 30, while pH on day 90 was significantly higher than at all other time points. By day 90, the mean pH of all treatments was $7.7 ± 0.1$ (Figure 2.3). The enzyme treatments had a significant ($p \leq 0.05$) effect on pH on days 15 and 30. On day 15, Alcalase was significantly ($p \leq 0.05$) higher than the Flavourzyme and Protamex treatments. On day 30, Flavourzyme was significantly ($p \leq 0.05$) lower than the Alcalase (Table 2.6). By day 60 there were no significant differences in pH among the treatments, a trend that lasted through the end of the fermentation period.
Figure 2.3. Effects of fermentation time on pH of crab sauce

Means were derived using all treatment (control and enzymes). Columns not sharing a letter are significantly different ($p \leq 0.05$) based on multi-way ANOVA followed by Bonferroni’s post hoc test. The error bars represent standard deviation (n=12).

Table 2.6. Mean pH values of crab sauce treatments over 90 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 15</th>
<th>Day 30</th>
<th>Day 60</th>
<th>Day 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.5 ± 0.1abA</td>
<td>7.4 ± 0.1abA</td>
<td>7.5 ± 0.1aA</td>
<td>7.7 ± 0.0aA</td>
</tr>
<tr>
<td>Flavourzyme</td>
<td>7.2 ± 0.2bB</td>
<td>7.0 ± 0.1bC</td>
<td>7.4 ± 0.0aB</td>
<td>7.5 ± 0.1aA</td>
</tr>
<tr>
<td>Alcalase</td>
<td>7.6 ± 0.0aA</td>
<td>7.5 ± 0.1aA</td>
<td>7.6 ± 0.1aA</td>
<td>7.7 ± 0.1aA</td>
</tr>
<tr>
<td>Protamex</td>
<td>7.3 ± 0.1bAB</td>
<td>7.3 ± 0.1abB</td>
<td>7.5 ± 0.2aAB</td>
<td>7.8 ± 0.1aA</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviations (n=3). Lowercase letters designate significant differences (in columns) among treatments at each time point (1-way ANOVA). Uppercase letters designate significant differences (in rows) within treatments over time (1-way ANOVA).

Water Activity

Fermentation time and treatment had a significant effect on water activity in crab sauce at the model level. As fermentation time increased, water activity of the sauce decreased after day 30 at the model level. Water activity of the control treatment was
significantly higher compared to Alcalse and Flavourzyme treatments at the model level. The control (0.754 ± 0.010) crab sauce had a significantly ($p \leq 0.05$) higher water activity compared to those prepared with Alcalase (0.741 ± 0.001), Flavourzyme (0.738 ± 0.003), and Protamex (0.742 ± 0.003) on day 15 (Figure 2.4). By the end of fermentation, mean water activity across treatments was 0.732 ± 0.002.

![Figure 2.4. Mean water activity values of crab sauce treatments over 90 days](image)

The error bars represent standard deviation. (n=3) *Control treatment was significantly higher on day 15 compared to the Flavourzyme treatment ($p \leq 0.05$).

**Moisture Content**

Fermentation time had a significant effect on moisture content of crab sauce at the model level (Figure 2.5). There was a stepwise decrease in moisture content as fermentation time progressed. Mean moisture content across treatments was significantly higher on day 15 (66.8 ± 0.5%) and day 30 (66.4 ± 0.2%) compared to days 60 (65.3 ± 0.3%) and 90 (64.6 ± 0.3%). Treatment did not have a significant effect on moisture content at the model level. However, there was a significant difference between treatments on day...
15 with the Alcalase treatment having a lower moisture content than the control (Table 2.7). There were no differences between treatments in moisture content after day 15.

**Figure 2.5. Effects of fermentation time on moisture content (%) of crab sauce**

Means were derived using all treatment (control and enzymes) data not sharing a letter are significantly different ($p \leq 0.05$) based multi-way ANOVA followed by Bonferroni’s post hoc test. The error bars represent standard deviation (n=12).

**Table 2.7. Mean moisture content (%) of crab sauce treatments over 90 days**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 15</th>
<th>Day 30</th>
<th>Day 60</th>
<th>Day 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>67.4 ± 0.6aA</td>
<td>66.5 ± 0.4aAB</td>
<td>65.3 ± 0.4aBC</td>
<td>64.4 ± 0.1aC</td>
</tr>
<tr>
<td>Flavourzyme</td>
<td>66.5 ± 0.2abA</td>
<td>66.2 ± 0.2aA</td>
<td>65.2 ± 0.1aB</td>
<td>64.8 ± 0.3abB</td>
</tr>
<tr>
<td>Alcalase</td>
<td>66.3 ± 0.1bA</td>
<td>66.3 ± 0.2aA</td>
<td>64.9 ± 0.4abB</td>
<td>64.2 ± 0.1abB</td>
</tr>
<tr>
<td>Protamex</td>
<td>66.9 ± 0.2abA</td>
<td>66.5 ± 0.4aA</td>
<td>65.6 ± 0.3abA</td>
<td>64.8 ± 0.3abB</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviations (n=3). Lowercase letters designate significant differences (in columns) among treatments at each time point (1-way ANOVA). Uppercase letters designate significant differences (in rows) within treatments over time (1-way ANOVA).
2.3.3. Browning Index

Browning index is a measure of non-enzymatic browning products via the Maillard reaction. Fermentation time had a significant effect on browning index of the samples at the model level (Figure 2.6) while treatment did not. Mean browning index values on day 15 (0.19) and day 30 (0.21) were significantly lower compared to day 60 (0.33) and day 90 (0.41). There were no significant differences among treatments at any time point, however mean browning index increased significantly within each treatment over time (Table 2.8).

Figure 2.6. Effects of fermentation time on browning index (absorbance) of crab sauce

Means were derived using all treatment (control and enzymes) data not sharing a letter are significantly different ($p \leq 0.05$) based on multi-way ANOVA followed by Bonferroni’s post hoc test. The error bars represent standard deviation (n=12).
Table 2.8. Mean browning index (absorbance) values of crab sauce treatments over 90 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 15</th>
<th>Day 30</th>
<th>Day 60</th>
<th>Day 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.195 ± 0.079aB</td>
<td>0.222 ± 0.023aB</td>
<td>0.331 ± 0.007aB</td>
<td>0.435 ± 0.031aA</td>
</tr>
<tr>
<td>Flavourzyme</td>
<td>0.164 ± 0.009aB</td>
<td>0.209 ± 0.006aB</td>
<td>0.319 ± 0.040aA</td>
<td>0.389 ± 0.042aA</td>
</tr>
<tr>
<td>Alcalase</td>
<td>0.193 ± 0.031aB</td>
<td>0.214 ± 0.040aB</td>
<td>0.348 ± 0.040aA</td>
<td>0.386 ± 0.024aA</td>
</tr>
<tr>
<td>Protamex</td>
<td>0.175 ± 0.033aB</td>
<td>0.183 ± 0.009aB</td>
<td>0.302 ± 0.040aA</td>
<td>0.381 ± 0.040aA</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviations (n=3). Lowercase letters designate significant differences (in columns) among treatments at each time point (1-way ANOVA). Uppercase letters designate significant differences (in rows) within treatments over time (1-way ANOVA).

2.3.4. Total Volatile Base Nitrogen (TVBN) and Amine Nitrogen

The presence of nitrogenous compounds such as ammonia and dimethyl/trimethyl amine is measured using the total volatile base nitrogen assay. Treatment did not have a significant effect on TVBN content of crab sauce at the model level. However, fermentation time had a significant effect on TVBN content of crab sauce (Figure 2.7), with mean TVBN values increasing incrementally from day 15 (100 mg/100 mL), to days 30 (112 mg/100 mL), and 60 (122 mg/100 mL). There were no significant differences among treatments in TVBN content of the crab sauce at any time point (Table 2.9).
Figure 2.7. Effects of fermentation time on total volatile base nitrogen (mg/100 mL) of crab sauce

Means were derived using all treatment (control and enzymes) data not sharing a letter are significantly different \((p \leq 0.05)\) based on multi-way ANOVA followed by Bonferroni’s post hoc test. The error bars represent standard deviation \((n=12)\).

Table 2.9. Mean total volatile base nitrogen (mg/100 mL) content of crab sauce treatments over 90 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 15</th>
<th>Day 30</th>
<th>Day 60</th>
<th>Day 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>96.7 ± 1.4AB</td>
<td>109.3 ± 3.2aAB</td>
<td>126.1 ± 5.6aA</td>
<td>132.4 ± 10.8aA</td>
</tr>
<tr>
<td>Flavourzyme</td>
<td>100.4 ± 2.8aB</td>
<td>107.4 ± 2.5aAB</td>
<td>119.1 ± 3.5aA</td>
<td>118.7 ± 3.9aAB</td>
</tr>
<tr>
<td>Alcalase</td>
<td>100.4 ± 1.7aB</td>
<td>110.2 ± 3.4aAB</td>
<td>122.8 ± 7.5aA</td>
<td>122.7 ± 3.1aA</td>
</tr>
<tr>
<td>Protamex</td>
<td>101.8 ± 2.6aB</td>
<td>119.5 ± 4.1aA</td>
<td>118.1 ± 2.8aA</td>
<td>118.2 ± 3.4aA</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviations \((n=3)\). Lowercase letters designate significant differences (in columns) among treatments at each time point (1-way ANOVA). Uppercase letters designate significant differences (in rows) within treatments over time (1-way ANOVA).

Fermentation time had a significant effect on amine nitrogen content of crab sauce at the model level (Figure 2.8), with mean amine nitrogen content increasing in a stepwise fashion from day 15 (366 mg/100 mL), to days 30 (731 mg/100 mL), 60 (827 mg/100 mL),
and 90 (878 mg/100 mL). Treatment also had a significant effect on amine nitrogen content of crab sauce. On days 15 and 30, control treatment amine nitrogen contents were significantly lower compared to the enzyme treatments (Table 2.10), but by day 60 amine nitrogen contents of the control had caught up to those of the enzyme treatments.

![Figure 2.8. Effects of fermentation time on amine nitrogen (mg/100 mL) of crab sauce](image)

Means were derived using all treatment (control and enzymes). Columns not sharing a letter are significantly different ($p \leq 0.05$) based on multi-way ANOVA followed by Bonferroni’s post hoc test. The error bars represent standard deviation (n=12).

**Table 2.10. Mean amine nitrogen (mg/100 mL) content of crab sauce treatments over 90 days**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 15</th>
<th>Day 30</th>
<th>Day 60</th>
<th>Day 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>320.3 ± 15.8bC</td>
<td>644.6 ± 31.6bB</td>
<td>845.8 ± 30.4aA</td>
<td>905.7 ± 35.8aA</td>
</tr>
<tr>
<td>Flavourzyme</td>
<td>386.2 ± 13.8aC</td>
<td>750.8 ± 9.6aB</td>
<td>856.3 ± 20.3aA</td>
<td>860.3 ± 35.0aA</td>
</tr>
<tr>
<td>Alcalase</td>
<td>369.8 ± 5.8abC</td>
<td>791.0 ± 10.7aB</td>
<td>804.2 ± 5.15aB</td>
<td>885.4 ± 19.5aA</td>
</tr>
<tr>
<td>Protamex</td>
<td>380.3 ± 10.2aC</td>
<td>739.1 ± 13.3aB</td>
<td>801.5 ± 19.9aAB</td>
<td>861.0 ± 32.1aA</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviations (n=3). Lowercase letters designate significant differences (in columns) among treatments at each time point (1-way ANOVA). Uppercase letters designate significant differences (in rows) within treatments over time (1-way ANOVA).
2.3.5. Salt Content

Treatment did not have a significant effect on percent salt content of the crab sauces at the end of fermentation (Figure 2.9). The average salt content across all treatments at day 90 was 27.4 ± 0.9%.

![Figure 2.9. Mean NaCl content (%) of crab sauce treatments at day 90](image)

Means were derived using all time points data not sharing a letter are significantly different (p < 0.05) based on one-way ANOVA and Tukey’s HSD post hoc test. The error bars represent standard deviation. (n=3)

2.3.6. Biogenic Amines

Biogenic amine (cadaverine, histamine, putrescine, and tyramine) concentrations were below the detectable limit (Table 2.11) in all samples.
Table 2.11. Biogenic amine detectable limits and crab sauce values

<table>
<thead>
<tr>
<th>Biogenic Amine</th>
<th>Concentration in Standard (mg/mL)</th>
<th>Detection Limit (mg/mL)*</th>
<th>Concentration in Crab Saucea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadaverine</td>
<td>87</td>
<td>3.48</td>
<td>ND</td>
</tr>
<tr>
<td>Histamine</td>
<td>114</td>
<td>4.56</td>
<td>ND</td>
</tr>
<tr>
<td>Putrescine</td>
<td>88</td>
<td>3.52</td>
<td>ND</td>
</tr>
<tr>
<td>Tyramine</td>
<td>110</td>
<td>4.40</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Detection limit calculated based on $\frac{1}{25}$ of standard values. aND = not detected

2.3.7. Correlations Between Measured Characteristics of Crab Sauce

Pearson correlations between dependent variables of crab sauce are shown in Table 2.12. There was a significant positive correlation between pH and browning index ($r = 0.642, p \leq 0.01$), and a significant negative correlation between pH and moisture content ($r = -0.612, p \leq 0.01$). There was a significant positive correlation between browning index and amine nitrogen ($r = 0.701, p \leq 0.01$), and between browning index and TVBN ($r = 0.633, p \leq 0.01$). There was a strong negative correlation between browning index and moisture content ($r = -0.841, p \leq 0.01$). There was a significant positive correlation between amine nitrogen and TVBN ($r = 0.760, p \leq 0.01$). There was a significant negative correlation between water activity and amine nitrogen ($r = -0.620, p \leq 0.01$). There was significant correlation between water activity and TVBN ($r = -0.553, p \leq 0.01$). Although some other correlations between dependent variables were statistically significant, they were too weak to be of interest.
Table 2.12. Pearson correlation between dependent variables of crab sauce

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>Yield</th>
<th>Browning Index</th>
<th>Amine Nitrogen</th>
<th>TVBN</th>
<th>Aw</th>
<th>Moisture</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yield</td>
<td>- 0.152</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Browning Index</td>
<td>0.642**</td>
<td>- 0.186</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amine Nitrogen</td>
<td>0.286*</td>
<td>0.193</td>
<td>0.701**</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TVBN</td>
<td>0.267</td>
<td>0.104</td>
<td>0.633**</td>
<td>0.760**</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aw</td>
<td>- 0.347*</td>
<td>- 0.341</td>
<td>- 0.554</td>
<td>- 0.620**</td>
<td>- 0.553**</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Moisture</td>
<td>- 0.612**</td>
<td>0.024</td>
<td>- 0.841**</td>
<td>- 0.767**</td>
<td>- 0.623**</td>
<td>0.799**</td>
<td>1</td>
</tr>
</tbody>
</table>

*Correlation is significant \( p < 0.05 \). **Correlation is highly significant \( p < 0.01 \).

2.4. Discussion

Typical fish sauce fermentation is reliant on the catabolism of proteins by the action of endogenous and microbial proteolytic enzymes. The acceleration of fermentation in fish sauce can be manipulated by the addition of exogenous enzymes. For example, commercial proteases such as papain, Alcalase, Protamex, and Neutrase have been found to be useful in catalyzing fish sauce fermentation (Aquerrera et al., 2001). The enzyme concentration applied in the present study was 0.5%, similar to concentrations reported in published literature researching rapid fermentation of fish sauce using commercial enzymes (Sun et al., 2015; Xu et al., 2008). A majority of enzyme-applied protein hydrolysis has been reported to occur within the first 6-48 hours of fermentation (Aquerreta et al., 2001; Silva et al., 2010; Awuor et al., 2017). The initial fermentation temperature for this study was chosen within the functional ranges (Table 1.2) for enzymatic activity during the first 48 hours at 55°C and at 37°C for the remaining period for optimal fermentation (Greiner et al., 2021).
2.4.1. Microbial Analysis

Overall, treatment did not have an impact on microbial populations at the model level. This pattern was expected as enzymes were added to accelerate initial hydrolysis of the crab proteins and not to impact microbial growth. In traditionally made commercial fish sauces, total viable populations ranged from 2.5-3.8 log CFU/g (Kilinc et al., 2006). In this study, total mesophilic and proteolytic bacteria populations were significantly (p \leq 0.05) lower at day 30 when compared to day 2 (about 2.3 log CFU/mL compared to about 4.6 log CFU/mL). The proteolytic counts fell dramatically after 15 days, similar to an enzyme-applied fish sauce fermentation study in which proteolytic counts were undetectable by day 20 (Lopetcharat, 1999). In the current study, mesophilic and proteolytic bacteria populations were higher in the early stages of fermentation and decreased as time progressed, similar to results in reported literature (Lopetcharat and Park, 2002; Kilinc et al., 2006). The reduction in microbial populations after day 2 was possibly due to the highly saline environment which may have inhibited microbial growth (Aquerrera et al., 2001). Additionally, as fermentation progresses the carbohydrates available for microbial digestion decrease, which generally results in decreasing microbial populations (Sharma et al., 2020). In contrast to the proteolytic and mesophilic microbial populations, lactic acid bacteria populations were uniformly low throughout the study. The lack of significant LAB growth was likely one of the contributors to the unusually high pH of the crab sauce samples. Heterotrophic marine bacteria populations stayed relatively consistent throughout the 90 days of fermentation with a starting value of 2.1 log CFU/mL and ending value of 2.3 log CFU/mL, possibly due to their ability to withstand the high levels of salt. These values were consistent with another crab sauce made using 20% NaCl.
but without added enzymes, which had a total plate count of 2.1 log CFU/mL, proteolytic count of 2.5 log CFU/mL, and LAB population of 2.2 of CFU/mL (Greiner et al., 2021).

### 2.4.2. Percent Yield, pH, Water Activity, and Moisture Content

Total crab sauce yields were lower in the control compared to the enzyme treatments, particularly on day 15 (13.5%) indicating more proteolysis in the enzyme-assisted treatments which already netted a ~25% yield by day 15. Liquid yield in fermentation (35°C) of Pacific whiting byproduct (head, frame, guts, and skin) mince was much higher at 77% due to the abundance of halotolerant and heat stable enzymes including serine, cystine, and metallo-proteases (Lopetcharat, 1999). The dramatically higher yield of Pacific whiting sauce could be attributed to the differences in physical makeup between fish and crab as well as a higher presence of halotolerant enzymes found in fish viscera. Application of commercial proteases in crab sauce production may lead to a higher return on profit with quicker and higher volume sauce production rates, assuming equivalent quality of the enzyme-assisted crab sauce fermentation.

To gauge the economic feasibility of enzyme-applied fermented crab sauce, a basic cost analysis was conducted (Table 2.13). The typical market price of specialty fish sauce ranges from $0.22/mL to $0.32/mL (prices derived from Amazon.com). Assuming the highest market price, a bottle of specialty fish sauce could be valued at as much as $32 per 100 mL bottle. Based on a cost analysis of ingredients only, the application of enzymes in crab sauce production would net an extra $0.06 per bottle compared to crab sauce fermented without enzymes. Total ingredient costs associated with making a fermented green crab sauce are extraordinarily low compared to the potential profit margins a specialty green crab sauce would generate. Based on these estimates, there would be only
a small economic benefit to applying proteases to crab sauce fermentation, and avoiding the use of added enzymes would be one less extra step during production. However, more research is warranted to optimize the use of enzymes to increase yield or possibly improve sauce quality, since the experimental design applied for this study was narrow in scope.

Table 2.13. Cost analysis of traditionally fermented green crab sauce versus enzyme-applied crab sauce after 90 days fermentation

<table>
<thead>
<tr>
<th></th>
<th>Traditionally Fermented Green Crab Sauce</th>
<th>Enzyme-applied Green Crab Sauce</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green Crab ($2.20 per kg)</td>
<td>0.64 kg - $1.41</td>
<td>0.64 kg - $1.41</td>
</tr>
<tr>
<td>Salt ($1.06 per kg)</td>
<td>0.16 kg - $0.17</td>
<td>0.16 kg - $0.17</td>
</tr>
<tr>
<td>Enzyme – Flavourzyme ($15.04 per kg)</td>
<td>N/A</td>
<td>0.01 kg - $0.22</td>
</tr>
<tr>
<td>Percent Yield</td>
<td>21%</td>
<td>26%</td>
</tr>
<tr>
<td>Total volume</td>
<td>134 mL</td>
<td>160 mL</td>
</tr>
<tr>
<td>Total cost of materials</td>
<td>$1.58</td>
<td>$1.80</td>
</tr>
<tr>
<td>Cost per 100 mL</td>
<td>$1.17</td>
<td>$1.12</td>
</tr>
<tr>
<td>Profit margins for a 100 mL bottle of crab sauce priced at $32 (based on ingredient costs only)</td>
<td>$30.82</td>
<td>$30.88</td>
</tr>
</tbody>
</table>

Estimations are based on starting crab materials (640 g) used in this study. ¹Market price of green crab (St-Hilaire, 2016). ²Price derived from Walmart.com. ³Price derived from Alibaba.com. ⁴Based on percent yield values of control and Flavourzyme treatments in this study.

The pH values of all crab sauce treatments in this study decreased slightly, then increased over time with a mean pH of 7.65 by the end of fermentation. This value was slightly higher compared to crab sauce fermented at 37°C without enzymes (7.38) after 90 days of fermentation (Greiner et al., 2021). The expected pH of typical fish sauce products should fall within the range of 5.0-6.5 (FAO, 2012). In enzyme applied fish fermentation studies, pH decreased over time due to released amino acids and small peptides within the proteins (Lopetcharat, 1999). The decrease in pH from day 15 to day 30 was to be expected due to the production of organic acids during fermentation, and the subsequent increase in...
pH may be attributed to the produced alkaline volatile base nitrogen compounds (Xu et al., 2008). Overall, pH values of the crab sauce in this study were significantly higher compared to the FAO standards of typical fish sauce. The higher pH values of our samples could be due to increased production of TVBN compounds including ammonia, trimethylamine, and dimethylamine which are known to be basic compounds (Wu et al., 2009). In another enzyme applied fish sauce, pH was first adjusted to between 4.5-6.0 based on the enzyme’s pH optimum, which may be something to consider for increased efficiency of enzyme activity in crab sauce production (Fu et al., 2008).

In the case of green crab sauce fermentation, the starting pH of the crab mince (meat and shell) material was reported as 8.1, which is much higher compared to fish like anchovies, which have an approximate muscle pH value of 6.5 (Galetti et al., 2010; Capaccioni et al., 2011).

The water activity values of the enzyme-added fermented crab sauce treatments in this study were similar to a previous study’s results (Greiner et al., 2021), with a mean water activity of 0.733 by the end of fermentation. Water activity values of the crab sauces were consistently below the FDA regulated level of 0.85 for soy sauce, indicating a product in which pathogenic microbial activity is unlikely to occur. Water activity is a measure of the available water within a food product and must be monitored to prevent microbial growth, thus increasing shelf-life (Kilinc et al., 2006). To maintain control of pathogenic biological activity, water activity must be kept at 0.85 or less. Food products having that target water activity are not subject to the same legal regulations as products with a higher water activity (FDA, 1984). In a study by Greiner et al. (2021), water activity of a traditionally fermented green crab sauce was 0.738 after 90 days of fermentation.
Moisture content can have a significant impact on physical properties of fish sauce, specifically with regard to color and rheology. In the crab samples, as moisture content decreased, browning index values were seen to increase ($r = -0.841, p \leq 0.01$). Commercial fish sauces typically have a moisture content of about 60-75% (Lopetcharat, 1999). The mean moisture content of the four crab sauce treatments in this study was 64%, well within the range of commercial products. Moisture content in the samples significantly decreased over time due to gradual evaporation, as these samples were fermented aerobically. This decrease in moisture may have led to a lower sauce yield, more viscous consistency, higher salt content, and darker color of the crab sauce samples. A possible way to reduce evaporation of the crab sauce over time could be to seal the jars with lids for anaerobic fermentation. More research on any changes in rheology should be monitored in green crab sauce production since viscosity of liquids is important in sensory evaluation (Kim et al., 2020).

### 2.4.3. Browning index

Fish sauce is typically golden brown, although it can range from light brown to dark brown in color. In this study, as fermentation time increased, browning index values increased significantly in the crab sauce. The higher initial fermentation temperature of 55°C during the first 48 hours in this study may have helped to promote production of melanoidins or brown pigments. Browning index readings of the crab sauce in this study at day 90 were found to be about 0.400 absorbance value which was medium brown in appearance. Though treatments were not statistically different from each other on day 90 based on browning index values, visual inspection of the sauces indicated that the control was darker brown compared to the enzyme treatments. Browning index values reported for
non-enzyme fermented (at 24°C) crab sauce were around 0.230 at day 90 (Greiner et al., 2021). The vastly higher browning index values in the current study could be due to an increased production of Maillard productions at the higher fermentation temperature. This study’s browning index values were within the commercial fish sauce browning index values ranges of 0.300-0.530 (Greiner et al., 2021). Consumer preferences for the intensity of brown color of fish sauce is dependent on variations of fish sauce (location of origin, etc.), therefore more research must be done to evaluate local market consumer preferences.

**2.4.4. Total Volatile Base Nitrogen and Amine Nitrogen**

In this study, amine nitrogen and TVBN values were positively correlated \( r = 0.760, p \leq 0.01 \) with each other. High amine nitrogen and TVBN values are both indicators for the progression of fermentation (Castro et al., 2006; Hill and Stewart, 2019). Total volatile base nitrogen is an important microbial spoilage indicator for highly perishable seafood. TVBN is expected to increase as time progresses in products like fish during refrigerated or iced storage due to production of ammonia and trimethylamine (Castro et al., 2006; Altissimi et al., 2017). This spoilage is indicative of the microbial digestion of proteins, TMAO, and TVBN analysis helps to characterize the rate of fermentation in fish sauce production. Traditionally fermented crab sauce did not exhibit an increase of TVBN over time with mean values of about 125 mg/100 mL in the 200 mg/g NaCl treatment samples on days 60 and 120 (Greiner et al., 2021). Comparatively, the enzyme fermented crab sauce in this study was similar in TVBN content at 122 mg/100 mL on day 90.

Amine nitrogen consists primarily of free amino acids and small peptides and its increase in fish sauce is normally a result of the enzyme catabolized protein hydrolysis by endogenous and microbially-derived enzymes. In traditionally fermented (at 37°C) crab
sauce, amine nitrogen increased with time (from days 15 to 90) from 450 to 680 g/100 mL (Greiner et al., 2021). Amine nitrogen values of the crab sauce in the current study were lower on day 15 at 375 mg/100 mL, but much higher on day 60 at 880 mg/100 mL and day 90 at 878 mg/100 mL. The dramatic increase of amine nitrogen in the enzyme-applied crab sauce during the first 30 days of fermentation can be attributed to activity of the exogenous enzymes and the initial 48-hour incubation period at 55°C at the start of fermentation. This initial boost in temperature may have encouraged further enzymatic activity in all treatments in this study. Amine nitrogen values were not significantly different on day 60 versus day 90 among treatments indicating that fermentation did not progress greatly past day 60.

2.4.5. Salt Content

In this study, the chopped whole crab made up 80% of the starting material while salt contributed the remaining 20%. At the end of fermentation, average salt content of all crab sauce treatments was about 27%, or slightly higher than the commercial standard. A salt content of 27% will sufficiently prevent growth of many spoilage and pathogenic microorganisms within the crab sauce as a salt content of 20% is sufficient to prevent growth of *C. botulinum* and *S. aureus* in foods (FDA, 2021a). In commercial fish sauce products, salt content is expected to fall within the ranges of 20-25% (Nakano et al., 2017). Salt is an essential contributor to prevent growth of spoilage organisms during fish sauce fermentation while permitting halophilic bacteria to thrive (Lapsongphon et al., 2013).
2.4.6. Biogenic Amines

Biogenic amines are typically produced from amino acids which are released via proteolysis and then further decarboxylated by microbial decarboxylases. The main biogenic amines of concern in fish sauce include histamine, tyramine, putrescine, cadaverine, spermidine, and spermine (Mohamed et al., 2009). According to the FDA (2021b), the regulatory threshold for histamine content for a portion of edible fish must not exceed 50 ppm (50 µg/mL) to avoid potential scombrototoxin poisoning in humans. In spontaneous fermentation (at 37°C) of crab sauce conducted by Greiner et al. (2021), corresponding values on day 90 for histamine, agmatine, and tyramine were 8.22, 4.76, and 2.25 mg/100 mL, respectively, and putrescine and cadaverine were below the detectable limit. Unexpectedly, biogenic amines were not detected in any of the crab sauce samples in the current study. The GC-MS method selected for the identification of biogenic amines in this study may have been inappropriate due to interference from the high salt content within samples. Applying a desalting procedure to the crab sauce samples via centrifugal filtration or on-line desalting (separation of proteins from non-volatile salts) may have helped to prevent ion suppression of the biogenic amine response (Tung et al., 2018). Ion suppression can lead to reduced quality GC-MS readings by interrupting peaks and may interfere with sensitivity in detecting compounds. In future studies, the GC-MS method should be modified to account for potential salt interference within crab sauce samples.

2.5. Conclusions

The application of proteases to crab sauce fermentation provides an opportunity to accelerate production which may be economically beneficial for industrial manufacturing. Enzyme assisted hydrolysis at 55°C for 48 hours followed by 37°C fermentation stimulated
the initial breakdown of the crab proteins and production of amine nitrogen in comparison to the control treatment. However, on an industrial scale, it may not be practical or economical to maintain a higher initial fermentation temperature of the crab sauce. As fermentation time increased, the most significant physicochemical changes that occurred across all treatments were an increase in pH, browning index, amine nitrogen, and TVBN. Sauce yield was significantly higher on day 15 in protease treatments compared to the control, and yield of the protease treatments remained unchanged after this time point. There were no significant differences in browning index, water activity, microbial load, moisture content, amine nitrogen, and TVBN among the Flavourzyme, Alcalase, and Protamex treatments, likely due to suboptimal pH and temperature conditions for peak enzyme activity. This enzyme-applied fermentation of green crab sauce provides insight into the potential of higher yields leading to faster product turnover. Application of proteases during fermentation of green crab sauce proved to increase yield significantly only at day 15 by about 11% compared to the control treatment. Similarly, protease activity (indicated by higher levels of amine nitrogen) was only significantly affected by enzyme treatments during the first 15 days of fermentation. These patterns indicated that protease applications were most effective only during the early stages of fermentation. Based on sauce yield among treatments, the ingredient costs analysis indicates that there is no large difference in profit margin when comparing the production of a crab sauce with or without the inclusion of enzymes. According to the results of this study, application of enzymes in green crab sauce fermentation would only be economically beneficial if the fermentation period were extremely short. Future studies should focus on understanding and optimizing the use of proteases based on optimal pH and temperature conditions. Sensory evaluations
are also needed to assess the qualities of green crab sauce and understand the preferences for this fermented condiment. Additionally, the effects of prolonged aging (ex: barrel aging) should be investigated to further evaluate characteristics of the crab sauce.
3.1. Introduction

Fish sauce is a globally popular fermented seafood condiment which is gal high in value and can vary in ingredient composition based on the location produced (Chayovan et al., 1983; Lopetcharat and Park, 2002; Mueda, 2015). Recently, specialty made fish sauces (Flor de Garum, Mega Chef, and BLiS) have been sold for up to $20-30 per bottle retailed on Amazon.com. Based on online market research, terms for these premium fish sauces include “small batch,” “barrel aged,” “finest ingredients,” “umami flavor,” and “produced sustainably.” A vast majority of fish sauces produced utilize fish as the primary protein ingredient. Currently, there is a gap in the market for fish sauce produced using crustaceans or an invasive species in the United States.

Recently, select restaurant chefs in New England have begun using a limited supply of soft-shell green crabs (*Carcinus meanas*) in new dishes and seafood stocks (Adey, 2016; McMahan, 2020). Though both uses for green crabs are strides towards reducing their populations, a highly profitable and industrial use for hard-shell green crabs would greatly increase demand. In a recent study, Greiner et al. (2021) confirmed the feasibility of creating a fermented sauce from green crabs. Generally, fish sauce has been characterized as having cheesy and fishy odors, brown color, umami flavor, and transparent appearance (Takashi et al., 2003; Wichaphon et al., 2013; Russo et al., 2020; Wongthahan et al., 2020), however similar characterization research has not yet been conducted on fermented green
crab sauce. The successful marketing of a green crab sauce product would likely need to highlight its unique attributes, including its utilization of an invasive species, its cachet as a locally made product, and possibly its distinctively different flavor profile.

In pursuit of developing a fermented green crab condiment for the upscale food service sector, identifying the optimal target audience will be integral to its successful marketing. Currently, there is no peer-reviewed literature about chef user habits of fish sauce, but Kruse (2017) reported that chefs use fish sauce for adding umami-enhanced flavors to dishes, adding “funky” flavor, and combining with sweet flavors for a “sweet and savory kick.” Additionally, “The Fish Sauce Cookbook” promotes a diversity of uses of fish sauce in cooking applications (Meewes, 2015) for home chefs. Based on individual interviews with chefs conducted by Inwood et al. (2009), ingredients that are high quality, local, and sustainable appealed to professionally employed chefs. All of these factors would be embodied in a small batch, locally made, fermented crab sauce produced using invasive green crabs, which may contribute to its success among chefs.

Currently, there are no reports in the literature on chef perceptions of fish sauce or of fermented green crab sauce as a culinary ingredient. It is important to understand their perceptions of fish sauces currently available on the market to more clearly define desirable qualities in aroma, flavor, appearance, and color. The objectives of this study were to (1) more clearly understand chef perceptions of fish sauce, and (2) collect feedback about chefs’ opinions of a fermented green crab sauce concept. An online survey was conducted to help to fill in these knowledge gaps.
3.2. Methods and Materials

3.2.1. Research Design Overview

An anonymous online survey, “Chef Perceptions of Green Crab Sauce as a Culinary Ingredient,” was conducted to understand chef perceptions of commercial fish sauce and a fermented green crab sauce concept. This online survey method allowed for data collection from chefs working across New England. The survey included 14 questions which consisted of demographic, 9-point scale (rating frequency of use, likeliness to use, and willingness to purchase), and a drop-down list. A section for comments was also provided in the survey. The survey was divided into sub-sections including chef demographics, preferred fish sauce qualities, and a product concept portion. The survey was launched on May 6, 2021 and responses were collected for two weeks until the survey was closed on May 20, 2021.

The overall purpose of this survey was to receive feedback on chef opinions of fish sauce and a fermented green crab sauce concept. The goal was to identify possible correlations between chef professional experiences, their cuisines of focus, fish sauce preferences, and perceptions of the green crab sauce concept. Quantitative methods were used to collect data on chefs’ experiences with umami flavor enhancers, preferences for fish sauce characteristics, and perceptions of a green crab sauce concept. A standardized questionnaire allows for data to be easily quantified and statistically evaluated to better understand relationships between the measured variables (Lakshman et al., 2000). This chef consumer survey was approved (application number, 2021-03-10) by the University of Maine Institutional Review Board (IRB) for the Protection of Human Subjects.
3.3.2. Population and Sampling Methods

The target population for this survey was professional working chefs located in the New England area who were at least 18 years of age. We wanted to collect feedback from chefs working in the New England region because the product concept featured a local Maine-made green crab sauce. Research participants were recruited through a network of chefs using various New England chef Facebook web pages and then sent an invitation to participate in the survey (Appendix B). Additional chef survey participants were recruited via email through a team working on this SeaGrant funded research team.

Respondents were presented with an invitation to participate, followed by an informed consent (Appendix C), and then were provided a link to the survey titled “Chef Perceptions of Green Crab Sauce as a Culinary Ingredient.” The invitation was extended specifically to those over the age of 18 and those who were currently employed as chefs. Responses were collected anonymously.

3.2.3. Online Chef Survey

The survey was pre-tested by members of the School of Food and Agriculture at the University of Maine along with several culinary professionals. The survey was created using the Qualtrics (Utah, USA) software using a University of Maine personal online account and optimized for phone and tablet compatibility. Once the participants accepted the invitation to partake in the survey, they were brought to an informed consent which is required by federal regulations (HHS, 45 CFR 46.116). Participants could choose whether or not they wanted to proceed to the survey section. Those participants who did not wish to take the survey were thanked in a separate message.
Survey questions (Appendix D) were designed to determine the chefs’ professional culinary experiences, ingredient preferences, and purchasing habits. All the participants were asked two professional experience-related questions, five questions about ingredient preferences (formatted with a select all that apply and a drop-down list configuration), one ingredient sourcing question (select all that apply), and three hedonic questions regarding the concept statement (below). The 9-point scales were formatted to ask participants how likely they were to use the crab sauce product, how frequently they would use the crab sauce product, and how willing they would be to purchase the crab sauce product.

“New Product Concept: A fermented seafood sauce made using invasive green crabs harvested in New England. This condiment provides powerful umami flavors and aromas. Typically used in Southeast Asian dishes, fish sauce is similar to Worcestershire sauce in its ability to enhance food and beverages as diverse as Fried Rice, Bloody Mary, BBQ Ribs and Caesar Salad. This product exemplifies sustainability and most importantly, high quality. This locally produced, fermented seafood condiment can be used to transform any savory dish and enhance the dining experience.”

There were two branching pathways at questions 1 and 6. All survey participants were asked the same demographic question 1 which was used to redirect home chefs (those not employed in the culinary industry) to the concept portion of the survey and allowed chefs working in industry (Line Cook, Sous Chef, and Head Chef) to proceed through the whole survey. Question 6 asked participants about their interest in cooking with fish sauce products. If participants answered “No,” they were sent directly to the end of the survey.
with a “thank you” statement. The survey data were exported directly from the Qualtrics software and responses were automatically coded into an Excel spreadsheet.

3.2.4. Compensation

For the survey incentive, those who were interested were asked to provide an email address to be entered into a raffle for one of four $25 Amazon E-Gift cards. Participants who provided an email were entered into an online random winner generator (www.namepicker.net). Only those who answered all the required questions within the survey were eligible to participate in the survey raffle. The online gift cards were distributed to the four raffle winners on June 21, 2021.

3.2.5. Statistical Analysis

The data were coded and analyzed using IBM SPSS 27 (International Business Machines - Statistical Package for Social Sciences) at a significance level of $p < 0.05$. The Shapiro-Wilk test was conducted to assess normality and Levene’s test was run for equality of variances to assess homogeneity. One-way analysis (ANOVA) was used to assess all one-level effects. Descriptive statistical and correlation (Spearman’s correlation) methods were conducted to analyze the data. Tukey’s honest significant difference (HSD) post hoc test was used to separate treatment means. The survey data was organized by including all chef categories and then separately into “Line Cook,” “Prep Cook,” “Sous Chef,” and “Head Chef.”
3.3. Results

3.3.1 Chef and cuisine classification

Fifty-nine chefs participated in the online survey. Chefs chose all classifications that applied which was used to gauge their level of professional experience within the past five years. Each respondent was assigned to the highest chef classification they indicated for this section. The highest chef classification (Prep Cook < Line Cook < Sous Chef < Head Chef) chosen by each participant was used to organize the survey data. For example, a participant who chose Line Cook, Prep Cook, and Head Chef would be classified as a Head Chef. Significantly more participants identified as “Head Chef” (74.5%) compared to “Line Cook” (10.2%) and “Sous Chef” (15.3%) (Table 3.1). None of the participants selected “Prep Cook.”

Chef participants were able to select multiple cuisines of focus. Most participants identified their cuisines of focus as American (52) as expected, followed by Asian (38), European (37), and African (7) (Table 3.1). Participants were able to select more than one cuisine and only eleven participants chose only one type of cuisine (American). Otherwise, participants opted to choose two or more cuisines of focus.
Table 3.1. Chef and cuisine classification of survey participants

<table>
<thead>
<tr>
<th>Category</th>
<th>Number of Responses n = 59</th>
<th>Percent of Responses (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chef Classification</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Line Cook</td>
<td>6</td>
<td>10.2</td>
</tr>
<tr>
<td>Sous Chef</td>
<td>9</td>
<td>15.3</td>
</tr>
<tr>
<td>Head Chef</td>
<td>44</td>
<td>74.6</td>
</tr>
<tr>
<td>*<em>Cuisines of Focus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>African</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>European</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>American</td>
<td>52</td>
<td></td>
</tr>
</tbody>
</table>

*Chefs were able to select multiple cuisines of focus, so responses do not sum to 59.

3.3.2 Innovative ingredients and flavor enhancer preferences

To gauge chefs interests in new products, participants were asked to rate their interest in using new food ingredients. On a scale of 1-9 (1 = “not interested at all” and 9 = “extremely interested,” all participants rated their interest in using new and innovative ingredients with a score of 7 or higher. Thirteen participants chose a score of seven, nine chose a score of eight, and the majority of participants selected nine (Figure 3.1). These consistently high scores verify that these chefs were interested in using new and innovative food ingredients within their craft.
Figure 3.1. How interested are you in cooking with new and innovative food ingredients?

Counts are based on total responses (n=59)

Umami flavor enhancers play an important role in highlighting certain flavor notes within dishes. In this study, participants were asked to indicate which flavor enhancers they regularly used in their cooking, with soy sauce/tamari receiving the most responses (Figure 3.2). Fish sauce/garum received the second highest number of responses at 48. Other responses included anchovy paste/shrimp paste (39), Worcestershire/A-1 (35), aji no moto/MSG (12) and miso/bean paste (8). All but one of the participants chose at least two flavor enhancers.
Figure 3.2. Which of the following flavor enhancers do you regularly use in your cooking? *Please select all that apply*

3.3.3. Familiarity and preferences with fish sauce

Participants were asked how familiar they were with fish sauce to gauge the user habits. Most participants said that they were “Very” (31) familiar with fish sauce, followed by “Somewhat” (25) familiar, and then “Not Very” (3) (Figure 3.3). Overall, these results indicate that most participants were familiar with fish sauce. Out of fifty-nine participants, no one selected “Not At All familiar,” or “Not Interested.” Additionally, fifty-seven participants indicated “yes” when asked if they would consider incorporating fish sauce into dishes within a menu, while only two participants chose “unsure.”
Different fish sauce varieties may have distinguishable characteristics in color, aroma, appearance, and flavor. When creating a fermented crab sauce product, it is important to visualize the ideal fish sauce for this chef targeted audience. The top selections for each ideal characteristic of fish sauce using drop down menus were medium brown color (59.3%), savory aroma (57.6%), transparent appearance (42.4%), and umami flavor (79.7%) (Table 3.2). The quality attribute seen as most important by the participants was flavor at 86.4%. Moving forward, flavor should be the focus when producing a crab sauce suitable for New England chefs.
Table 3.2. Fish sauce preferences for color, aroma, appearance, flavor, and overall

<table>
<thead>
<tr>
<th>Fish Sauce Preferences</th>
<th>Number of Responses (n=59)</th>
<th>Percent of Responses (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Color</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light Brown</td>
<td>13</td>
<td>22.0</td>
</tr>
<tr>
<td>Medium Brown</td>
<td>35</td>
<td>59.3</td>
</tr>
<tr>
<td>Dark Brown</td>
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<td>18.6</td>
</tr>
<tr>
<td><strong>Aroma</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweet</td>
<td>16</td>
<td>27.1</td>
</tr>
<tr>
<td>Fishy</td>
<td>9</td>
<td>15.3</td>
</tr>
<tr>
<td>Savory</td>
<td>34</td>
<td>57.6</td>
</tr>
<tr>
<td><strong>Appearance</strong></td>
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<td></td>
</tr>
<tr>
<td>Transparent</td>
<td>25</td>
<td>42.4</td>
</tr>
<tr>
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<td>23.7</td>
</tr>
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<td>Unfiltered</td>
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<td>33.9</td>
</tr>
<tr>
<td><strong>Flavor</strong></td>
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<td></td>
</tr>
<tr>
<td>Umami</td>
<td>47</td>
<td>79.7</td>
</tr>
<tr>
<td>Salty</td>
<td>11</td>
<td>18.6</td>
</tr>
<tr>
<td>Caramel</td>
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<td>1.7</td>
</tr>
<tr>
<td><strong>Most Important Attribute</strong></td>
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<td></td>
</tr>
<tr>
<td>Flavor</td>
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<td>86.4</td>
</tr>
<tr>
<td>Aroma</td>
<td>7</td>
<td>11.9</td>
</tr>
<tr>
<td>Appearance</td>
<td>1</td>
<td>1.7</td>
</tr>
</tbody>
</table>

81
3.3.4. Sourcing ingredients

Participants were asked to rate factors they consider when sourcing ingredients for their restaurants. The top three factors chosen were local (47), sustainable (42), and price (41) (Figure 3.4). Other factors included marketing (25), small business (13), shelf-life (3), and distributor (1). For analytical purposes, these factors were split into two categories, social and economic. The social category included local, sustainable, small business, and marketing while the economic category included price, shelf-life, and distributor. Overall, participants found the social factors to be more important when sourcing ingredients.

![Bar chart showing the factors most important when sourcing ingredients for the restaurant.](chart.png)

**Figure 3.4. What factors are most important when sourcing ingredients for your restaurant? Please select all that apply**
3.3.5. Perceptions of fermented green crab sauce concept

Chefs were presented with a concept statement for the fermented green crab sauce and asked to rate their likeliness to use the product, frequency of use, and willingness to purchase the product (Table 3.3). Out of the 59 chefs who participated, 32 completed the concept section. The largest number of participants rated their likeliness to use the product as a “9” (extremely likely), while “7” was the second most popular answer (24.2%). The mean score for likeliness to use was 8.0. None of the participants indicated that they were unlikely to use the product. The most popular answers for frequency of using the product were “5” (18.8%) and “6” (18.8%). The mean score for frequency of use was 5.8. Willingness to purchase the product was scored highly by participants. The most popular answers for willingness to purchase were “7” and “9” (extremely willing) which made up 62.6% of the responses. The mean score for willingness to purchase was 7.5. None of the participants indicated that they were unwilling (a score of four or less) to purchase the green crab sauce.
Table 3.3. Response to concept statement

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Number of Responses (n=32)</th>
<th>Percent of Responses (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Likeliness to use</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 - Not At All Likely</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0.0</td>
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<tr>
<td>4</td>
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<td>0.0</td>
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<tr>
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<td>7</td>
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</tr>
<tr>
<td>8</td>
<td>2</td>
<td>6.1</td>
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<tr>
<td>9 - Extremely Likely</td>
<td>16</td>
<td>48.5</td>
</tr>
<tr>
<td><strong>Frequency of use</strong></td>
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<td>0.0</td>
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<td>4</td>
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</tr>
<tr>
<td>9 - Extremely Frequently</td>
<td>4</td>
<td>12.5</td>
</tr>
</tbody>
</table>
3.3.6. Correlations and frequencies of survey data

Spearman’s correlation values between chef ranking and concept hedonic scores are shown on Table 3.4. There was a weak positive correlation between “chef rank” and “likeliness to use” \((r = 0.372, p \leq 0.05)\). There was a moderate positive correlation between increasing “chef rank” and “frequency of use” \((r=0.541, p \leq 0.01)\) and “willingness to purchase” \((r = 0.471, p \leq 0.01)\). There were also moderate positive correlations between “likeliness to use” and “frequency of use” \((r = 0.576, p \leq 0.01)\) and “willingness to purchase” \((r = 0.562, p \leq 0.01)\). There was a very weak positive correlation between “familiarity with fish sauce” and “frequency of use” \((r = 0.387, p \leq 0.05)\).
Table 3.4. Spearman’s correlation coefficients of chef characteristics, familiarity with fish sauce, and concept scores

<table>
<thead>
<tr>
<th>Chef rank&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Familiarity with fish sauce</th>
<th>Likeliness to use</th>
<th>Frequency of use</th>
<th>Willingness to purchase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chef rank</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Familiarity with fish sauce</td>
<td>-</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Likeliness to use</td>
<td>0.372*</td>
<td>0.268</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Frequency of use</td>
<td>0.541**</td>
<td>0.387*</td>
<td>0.576**</td>
<td>1.000</td>
</tr>
<tr>
<td>Willingness to purchase</td>
<td>0.471**</td>
<td>0.115</td>
<td>0.562**</td>
<td>0.347</td>
</tr>
</tbody>
</table>

*Correlation is significant (p ≤ 0.05). **Correlation is highly significant (p ≤ 0.01).<sup>a</sup> Chef rank ordered by “Line Cook” < “Sous Chef” < “Head Chef”

Types of flavor enhancers regularly used by chefs who focus on “Asian” (chefs who selected “Asian” cuisine as well as any other types) and “Not Asian” (chefs selected any number of cuisines of focus excluding “Asian”) cuisines, compared to the overall average from all respondents are displayed in Table 3.6. The three cuisine categories followed a similar trend for popularity of different flavor enhancers. The increasing trend in flavor enhancer popularity across all three categories was Miso/Bean Paste < Aji No Moto/MSG < Worcestershire/A-1 < Anchovy Paste/Shrimp Paste < Fish Sauce/Garum < Soy Sauce/Tamari.
Table 3.5. Flavor enhancers regularly used in relation the cuisines of primary focus: Asian, Not Asian, and Overall

<table>
<thead>
<tr>
<th>Flavor Enhancer</th>
<th>Overall (n=59)</th>
<th>Asian (n=38)</th>
<th>Not Asian (n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy Sauce/Tamari</td>
<td>57</td>
<td>37</td>
<td>20</td>
</tr>
<tr>
<td>Fish Sauce/Garum</td>
<td>48</td>
<td>33</td>
<td>15</td>
</tr>
<tr>
<td>Anchovy Paste/Shrimp Paste</td>
<td>39</td>
<td>29</td>
<td>10</td>
</tr>
<tr>
<td>Worcestershire/A-1</td>
<td>35</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>Aji No Moto/MSG</td>
<td>12</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Miso/Bean Paste</td>
<td>8</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

Values represent number of responses

3.3.7. Comments Provided on the Green Crab Sauce Concept

Overall, the feedback received by the chef participants was uniformly positive (Appendix E). Some comments provided were “I use a lot of fish sauce in my home-cooking. Having a local Maine product--that also helps with an invasive species--would be a dream!,” “Product needs to have great Umami flavors for it to succeed and not be just another sauce would like to compare it to standard imported fish sauce,” and “I love this. Using green crabs every way possible to conquer them is key. I’ve cooked with these crabs, and they are a pain. A fermented sauce would be a great add.” Based on these comments, chefs indicated that using invasive green crabs and being locally made were product benefits. Additionally, chefs highlighted that they would like this crab sauce to be comparable to other imported fish sauces and focused on umami-flavor.
3.4. Discussion

3.4.1. Who Were the Participants and What Were Their Cuisines of Focus?

Participants were classified by one of three chef characterizations including prep cook, sous chef, and head chef. Head chefs made up about 75% of the participant demographic. In terms of hierarchical dynamics within restaurants, the head chef position is often associated with leading the kitchen and being knowledgeable about their craft (Wellton et al., 2016). Main responsibilities of the head chef within a restaurant include ordering supplies and creating menus. For this concept survey, it was important to collect insights from an audience that plays a key role in deciding which ingredients to incorporate into restaurant dishes. Common cuisines identified by the participants in this survey included Asian, European, American, and African. Most of the chef participants identified American cuisine as a cuisine of focus followed by Asian, and then European. Identification of cuisines allowed for comparisons to be made with other survey measures, such as flavor enhancer use.

3.4.2. What Types of Flavor Enhancers are Most Frequently Used?

Overall, the most common flavor enhancers chosen by chefs were soy sauce and fish sauce. No evaluations of fish sauce by chefs have been reported in the literature. Therefore, it is important to understand preferred qualities of fish sauce as it was the second highest ranked flavor enhancer used by chefs in this survey, regardless of their cuisine of focus, and was the condiment most related to our fermented crab sauce product. Soy sauce is a well-known umami flavor enhancer and its characterization by consumers has been well documented (Feng et al, 2012; Wongthathan et al., 2020). Like fish sauce, distinctive
traits of soy sauce, including color, saltiness, and flavor, can be variable. In a sensory evaluation study of soy sauce (Wongthathan et al., 2020), intensity of brown color and saltiness were found to be positively correlated according to chef perceptions. Additional investigations of chef preferences for fish sauce color are needed to understand which qualities are most marketable.

To narrow down the target market most appropriate for the crab sauce concept, it is important to understand the popularity of flavor enhancers associated with certain cuisines. A higher portion of participants who said that they focused on “Asian” (87%) cuisine commonly use fish sauce compared to those who did not select “Asian” (71%) (Table 3.6). Additionally, those who focused on “Asian” cuisine (76%) used anchovy/shrimp paste more often compared to those who did not select “Asian” (26%). Based on the results of this survey, fish sauce and anchovy/shrimp paste may be positively associated with the preparation of Asian cuisine.

3.4.3. How Important are Social Versus Economic Factors in Purchasing?

High quality and novel ingredients are often sought out by chefs when sourcing for restaurants. Social and economic factors impact how chefs choose to source their ingredients. In this study, the most frequently selected purchasing factors considered by chefs were local, followed by price, and then sustainability. Purchasing factors that were not as important to chefs included the economic factors shelf-life (3 out of 59) and distributor (1 out of 59). Chefs have previously been found to prefer food ingredients that were conveniently accessible, locally grown, high quality, and locally produced (Inwood et al., 2009). Chefs who were considered medium and high-volume users of local ingredients were found to be more willing to pay a premium for those products compared
to no to low users (Inwood et al., 2009). Based on these findings, the local green crab sauce concept should be targeted toward medium and high-volume users of locally sourced ingredients for increased purchasing potential.

### 3.4.4. How Do Chefs Perceive the Product Concept?

Chef positional hierarchy levels were correlated with the hedonic scores to quantify their “likeliness to use,” “frequency of use,” and “willingness to purchase” the product as described in the concept statement (Table 3.4). Chef ranking and frequency of product use were positively correlated, meaning that more highly positioned chefs indicated they would use the crab sauce more often. As chef rank increased, willingness to purchase also increased. Additionally, as likeliness for using the sauce increased, frequency of use \( (r = 0.576, p < 0.001) \) and willingness to purchase both increased \( (r = 0.562, p < 0.001) \).

Familiarity with fish sauce was also correlated with the hedonic scores related to the product concept (Table 3.4), however only frequency of use was significantly correlated with familiarity of fish sauce. The more familiar chefs were with fish sauce, the more frequently they would use the green crab sauce. Based on these findings, the green crab sauce should be targeted towards head chefs (highest rank) who are already familiar with fish sauce products, to promote the highest frequency of purchases.

### 3.4.5. What is the Ideal Fish Sauce and Target Audience?

Based on fish sauce preferences chosen by chefs in this survey, certain color, aroma, appearance, and flavor characteristics should be highlighted in the production of the green crab sauce. Based on our results, chefs preferred a fish sauce that was medium brown in color, savory in aroma, transparent in appearance, and offering umami flavor.
Overall, the most important of these characteristics was flavor. Therefore, the green crab sauce product should be developed while keeping these key characteristics in mind, particularly focusing on umami flavor. Overall, the ideal target audience of this locally made green crab sauce product should be head chefs who are familiar with fish sauce, focus on Asian cuisine, and are high users of locally sourced and sustainable products.

3.5. Conclusions

These survey results on fish sauce and the green crab sauce concept provide more context into what characteristics and qualities are most important to consider when producing a fermented crab sauce intended for culinary professionals. A majority of chefs selected flavor as the most important characteristic of fish sauce. Based on this survey, the ideal green crab sauce would be medium brown in color, transparent, with a rich umami flavor. Head chefs who focused on Asian cuisine and were already familiar with fish sauce provided the highest scores for likeliness to use, frequency of use, and willingness to purchase the green crab sauce after reviewing the product concept statement. Social factors (local and sustainable) were more important to participants than economic factors (price and marketing) with regard to making purchasing decisions. When considering the marketing of this green crab condiment, locality and sustainability should be highlighted, with emphasis on “locally-made,” and “sustainably produced.” The price of this fermented crab product should be like high-end/premium fish sauce products ($20-30 per ~200 mL bottle) currently on the market, with which participants in this survey are presumably familiar. Most high-end fish sauce products have unique marketable attributes (processing, ingredients, etc.) and because there are no fish sauces made with green crabs, the concept falls within this niche. Sensory evaluation of the green crab sauce conducted with chefs is
an important next step to gauge overall acceptability and gather feedback about the quality attributes of the crab sauce. Important characteristics of the green crab sauce to consider would be intensity of umami and overall flavor, preference for color, and perceived aromas based on their preferences for fish sauce. In addition, a home use test in restaurant settings for professional chefs in the New England region would be beneficial to understand the versatility, user-friendliness, and potential challenges associated with incorporating the green crab sauce in restaurant dishes.
CHAPTER 4

OVERALL CONCLUSIONS

These studies investigated the development of a fermented green crab sauce using enzymes and the potential target market and ideal characteristics for the concept. Commercial proteases accelerated protein hydrolysis during the early stages of fermentation of green crab sauce for faster production. However, a disadvantage of enzyme-assisted fermentation is that it requires specific processing conditions (temperature, pH, salt content, etc.). If conditions are not ideal for the specific added protease, there will be less significant increases in sauce yield. The survey of chef perceptions of the green crab sauce concept can help to inform product developers about which characteristics (color, appearance, flavor, aroma, etc.) the sauce should possess. The limitation of collecting mainly quantitative data from chefs is that it does not provide further insight into the "why" behind their answers. However, given the lack of literature regarding chef opinions of fermented seafood sauces, this survey served as an important first step towards understanding which types of chefs might use green crab sauce and their overall preferences. These studies in conjunction promote the development of a consumer-tailored and desired green crab sauce condiment.

In the first study, the effects of commercial proteases in green crab sauce fermentation were evaluated. The impacts of enzymes (Alcalase, Flavourzyme, and Protamex) on green crab sauce fermentation have not been previously reported. The application of proteases in green crab sauce fermentation affected overall yield and was most noticeable during the early stages of fermentation. The enzymes increased yield of the green crab sauce by up to 11% compared to the control without enzymes after 30 days.
of fermentation. However, applications of proteases did not significantly impact color, water activity, moisture content, pH, TVBN content, amine nitrogen content, or salt content of the product at the model level.

The initial fermentation temperature of 55°C for 48 hours contributed to accelerate hydrolysis in the enzyme treated sauces as the most significant changes occurred during the early stages of fermentation. Based on the literature, a majority of enzyme-assisted seafood protein hydrolysis occurs within the first 6–48 hour span in fish sauce fermentation. Use of enzymes in the making of green crab sauce should be considered for shortened fermentation periods since enzyme activity decreases as time progresses. An enzyme optimization study should be investigated for green crab sauce fermentation focusing on specific pH and temperatures for each desired protease. Based on the literature, the following proteases and their optimal pH and temperatures for protein hydrolysis are as follows: Alcalase at a pH of 7.0 and temperature of 56°C, Flavourzyme at a pH of 5.25 and temperature of 57°C, and Protamex at a pH of 6.85 and temperature of 51°C. Future enzyme-assisted crab sauce studies should also add enzymes in different amounts to investigate the impact of enzyme dosage on total yield. Additionally, future studies should consider salt content since proteases can be negatively impacted by the high levels of salt in seafood sauces. Research has shown that proteases applied at a salt content of 15% or less have resulted in the highest fish sauce yields. Salt content needs to be kept high enough to prevent unwanted pathogenic bacterial growth but low enough to allow for optimal enzyme activity. The addition of various salt levels ranging from 10% to 20% should be investigated in conjunction with the commercial proteases to optimize salt level and enzyme activity.
In the second study, chef perceptions of fish sauce and of the green crab sauce concept were collected through an online survey. No prior investigations of chef preferences for fish sauce have been previously reported. Chefs preferred a fish sauce which was medium brown in color, savory in aroma, transparent in appearance, and umami in flavor. But overall, the most important of these characteristics was flavor. The survey was kept to 14 questions to encourage participants to complete the entire questionnaire. However, additional worthwhile areas of inquiry include how frequently chefs currently use fish sauce, how they utilize fish sauce in their cooking, how much they typically pay for fish sauce, and their reasons for using fish sauce (ex: to boost flavor). These questions would help to add more insight into the “what,” “why,” and “how” fish sauce is used by chefs. An investigation to characterize the green crab sauce prototype will be important to compare its attributes with the chefs’ preferences. Currently, there are no fermented green crab sauce products available for commercial sale. However, chefs rated the green crab sauce concept highly as they stated they would be very likely to use the product and were very willing to purchase this seafood condiment. Limitations of the concept portion were the lack of questions regarding how much the chefs would pay for the sauce, how they would utilize the sauce, how they view certain attributes like “using an invasive species,” and how unique they thought the product was. These questions would provide more insight into how chefs view the concept and how best to market it towards them.

The marketing of green crab sauce should be targeted towards professional head chefs who are familiar with fish sauce and focus on Asian cuisine. Based on the literature, a focus on social purchasing factors should be considered as chefs are willing to pay a premium for “local” and “sustainable” ingredients. Limitations of the marketing section of
the survey included the lack of questions regarding how often they choose to purchase local or sustainable ingredients, the types (cuisine) of restaurants they work for, and their role in purchasing ingredients for restaurants. Marketing of this green crab sauce should highlight points like “made sustainably,” and “produced locally.” Sensory evaluation for the green crab sauce should be conducted using a panel of chefs to understand how its characteristics are perceived and if it is generally acceptable by their standards. The green crab sauce should be evaluated in a prepared dish for chefs to characterize the perceived aroma, appearance, texture, and flavor of the product. Although the current study was a good start for narrowing the chef target audience for the product, it may not be economically feasible to rely solely on this niche demographic. Based on the chefs’ high likeliness to use and willingness to purchase the green crab sauce, there may be potential to successfully market this product toward home-users. Future sensory evaluations should also be conducted with general consumers to assess potential acceptability of the green crab sauce at the retail level.

Previous studies on the development of a green crab sauce have shown its production to be feasible at laboratory scale. In pursuit of upscaling the production of green crab sauce, optimization and monitoring of processing will need to be explored. Producers should look to local New England lobstermen to provide the green crabs and propose a contract for receiving the crabs during off-peak lobster seasons. As New England fishers have a lag season for lobstering (January to May), capturing green crabs during this time would be a promising opportunity for harvesters to make a profit during their slower seasons. Challenges of developing a commercial batch scale process for the sauce include the need for streamlined filtration, adjustment of pH levels for compliance with fish sauce
standards, and potential pasteurization. Required start-up materials and equipment would include green crabs, salt, a filtration system/centrifuge, fermentation vats, meat grinders, a bottling line, labeler, and pasteurization equipment. There is also a need to streamline filtration of the crab sauce since the filtration procedures applied in this study were labor intensive. Challenges for the successful commercialization of this product will be optimizing yield and maintaining consistent quality of the crab sauce. Marketing of this product should highlight its unique characteristics, specifically its utilization of an invasive species. All these factors considered, the optimization and streamlining of production would potentially lead to success in creating a commercial fermented green crab condiment.

In conclusion, inclusion of commercial proteases was positive for green crab sauce fermentation as it helped to increase hydrolysis of proteins during the early stages of fermentation. Enzyme-applied green crab sauce fermentation should be considered for shortened fermentation periods of 60 days since quality of this sauce was similar to the 90-day fermented product. Perceptions collected from chefs indicate that there is a demand for a fermented green crab sauce product. The indicated preferred characteristics provided by the chefs for fish sauce should be considered during its further development. Overall, if protease function can be optimized to further increase yield and accelerate fermentation, and the product is developed based on indicated chef preferences, there is promise for the market success of a fermented green crab sauce condiment in New England. The green crab sauce concept promotes economic benefit while creating a use for green crabs on an industrial scale. These studies support the commercial production of high-value green crab sauce for consumers and provide a potential solution to a problematic invasive crab species.
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Food and Drug Administration. (2021b). Scombrotoxin (Histamine) Formation. *Fish and Fishery Products Hazards and Control Guidance* https://www.fda.gov/media/80248/download#~:text=Chemical%20testing%20is%20an%20effective,of%20histamine%20in%20fish%20flesh.&text=For%20this%20reason%20C%20a%20guidance,sections%20may%20exceed%20500%20ppm.


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role of MRPs and newly formed peptides with basic and aromatic amino acids. *LWT – Food Science and Technology*, 97, 245-253. https://doi.org/10.1016/j.lwt.2018.06.051


APPENDIX

APPENDIX A. PRELIMINARY QUALITY ASSESSMENT OF GREEN CRAB (Carcinus maenas) ROE

Introduction
Carcinus maenas is a species of green crab invasive to the coasts of North America and Canada. There is an opportunity to both curb the spread and create a market for Carcinus maenas by utilizing the green crab roe or “coral”. Currently in North America, there are limited studies on the characterization of crustacean roe and potential food application of green crab roe.

Purpose
The purpose of this study was to determine the proximate composition, color, and coliform counts of green crab roe in North America. As a first step to valorizing green crab roe, baseline information is needed to assess quality and marketability.

Methods
Carcinus maenas were trapped live and collected from Boothbay, Maine, and female crabs were sorted for roe extraction. Roe was extracted from 15 pounds of female crabs and combined to form a homogenous sampling pool. Proximate compositions were obtained using the following methods; moisture content (n=5) via drying oven, ash (n=2) via muffle oven (550°C), crude protein via total nitrogen (n=3), and lipids (n=3) via AOAC methods. Also analyzed were; instrumental color (L*a*b*) via spectrophotometry and coliform growth at days one and seven.

Results
Proximate composition of green crab roe average values for moisture, ash, lipids, and crude protein were as follows; 60.5±0.2%, 1.7±0.1%, 9.2±0.1%, and 25.7±0.1%. The roe appeared as tiny, oozey, bright-orange colored eggs; L* 52.1±0.3, a* 29.9±0.3, b* 69.3±0.1. There was no coliform growth in the roe detected on day one, but on day seven 1.12 cfu/mL was identified. Compared to Chinese mitten crab roe composition, moisture and crude protein were significantly different (p ≤ 0.05).

Significance
This study unveils a new avenue of potential use for green crab roe in North America. However, more research is needed to determine quality characteristics of this underutilized food source.
APPENDIX B. RECRUITMENT FLYER FOR ONLINE SURVEY

Invitation to Participate in a University of Maine Research Study

You are invited to participate in an anonymous research study about fermented fish sauce. You must be 18 years or older to participate. The purpose of this study is to gain perspectives of and interest in the fish sauce market and umami-flavor enhancing ingredients.

This research is being conducted by Holly Leung, a graduate food science student in Food Science and Human Nutrition at the University of Maine.

We are looking for chefs located in New England, who would like to participate in an anonymous online survey. Survey questions will take about 15 minutes to complete. This survey contains questions about work experience, habits with using umami flavor enhancers, and a new product concept. Upon completion of the survey, chefs will have the chance to enter a randomized drawing to receive one of the four available $25 Amazon gift cards. The survey will be open for a span of 2 weeks.

Link to Survey
APPENDIX C. INFORMED CONSENT FORM FOR ONLINE SURVEY

Informed Consent

You are invited to participate in a research project conducted by Holly Leung, a graduate student of food science in the School of Food and Agriculture at the University of Maine. The faculty sponsor is Dr. Denise Skonberg, a professor of Food Science at the University of Maine. The purpose of the research is to understand your point of view about fish sauce & other umami flavor enhancer sauces, and to get your feedback about a new product concept. You must be at least 18 years of age to participate.

What Will You Be Asked to Do?

If you decided to participate, you will be asked to complete an anonymous online survey which asks questions about your culinary experience, fish sauce user-habits, and a new product concept. It will take approximately 15 minutes to participate.

Risks:

Except for your time and inconvenience, there is minimal risk to you from participating in this study.

Benefits:

While this study will have no direct benefit to you, this research may help us learn more about chefs’ preferences and habits with fermented fish sauce. This research may help us better understand the factors that influence perception and marketability of fermented fish sauce, methods can be developed to improve the creation of an optimal product.

Compensation:
You will be able to enter a raffle to win one of four $25 Amazon gift cards that would be sent to you electronically. You will be contacted through an email you provide through an external link, where you will be directed to a separate page, at the end of the survey. Your email address will not be connected to your responses. You must reach the end of the survey to enter the raffle.

**Confidentiality:**

The survey is anonymous. The email addresses collected for the raffle will not be associated with the data. Any emails collected will be kept on a password protected computer until December 31, 2021. The anonymous survey data will be deleted from Qualtrics by May 10, 2023, and will then be stored on a secured, password protected computer indefinitely.

**Voluntary:**

Participation in this survey is voluntary. If you choose to take part in this study, you may stop at any time. You may skip any questions you do not wish to answer.

**Contact Information:**

If you have any questions about this study, please contact me at Holly.Leung@maine.edu or the faculty sponsor at Denise.Skonberg@maine.edu. If you have any questions about your rights as a research participant, please contact the Office of Research Compliance, University of Maine, 207/581 - 2657 (or e-mail umric@maine.edu)
APPENDIX D. ONLINE SURVEY QUESTIONNAIRE

Chef Perceptions of Green Crab Sauce as a Culinary Ingredient

1. Identify positions you have held in the culinary industry within the last 5 years: please select all that apply
   1. Prep Cook
   2. Line Cook
   3. Sous Chef
   4. Head Chef
   5. Home Chef → reroute to concept portion of survey if this is the only choice
   6. Other (textbox)

2. Please identify the cuisines of your focus: please select all that apply
   1. Asian
   2. European
   3. American
   4. African
   5. Other (textbox)

3. How interested are you in cooking with new and innovative food ingredients?
   a. Scale of 1-9; where 1 = not at all interested and 9 = extremely interested

4. Which of the following flavor enhancers do you regularly use in your cooking? Please select all that apply
   1. Worcestershire/A-1
   2. Soy Sauce/Tamari
   3. Fish Sauce/Garum
   4. Aji No Moto/MSG
   5. Anchovy Paste/Shrimp Paste
   6. Miso/Bean Paste
   7. Other (Textbox)

5. How familiar are you with fish sauce/garum?
   1. Very - Use it regularly
   2. Somewhat- Use it on occasion
   3. Not very - I’ve eaten food prepared with fish sauce, but I do not cook with it.
   4. Not At All - I am unfamiliar with it but would be open to trying
5. Not interested - it doesn’t sound like my kind of thing
   i. Reroute to description of fish sauce/garum if answer is “somewhat” or “not very”

6. Would you consider including fish/garum sauce products in dishes within a menu?
   1. Yes
   2. Unsure
   3. No
      i. Reroute if answer is “no”. No need to collect information from chefs who will not be interested in the concept.

7. Drop-down question: What are the important qualities of fish sauce to you?
   Fish sauce that is _____ (dark brown, medium brown, light brown) in color, has a ______ (savory, fishy, sweet) aroma, is _______ (transparent, unfiltered, opaque) in appearance, and has a ______ (salty, caramel, umami) flavor. But the most important of these attributes is ____ (color, aroma, flavor, appearance).

8. What factors are most important when sourcing ingredients for your restaurant?
   Please select all that apply
   1. Local
   2. Price
   3. Sustainable
   4. Marketing
   5. Small business
   6. Shelf-life
   7. Distributor
   8. Other: ___________
   9. N/A: not involved

9. What is your state of residence?

   New Product Concept: A fermented seafood sauce made using invasive green crabs harvested in New England. This condiment provides powerful umami flavors and aromas. Typically used in Southeast Asian dishes, fish sauce is similar to Worcestershire sauce in its ability to enhance food and beverages as diverse as Fried Rice, Bloody Mary, BBQ Ribs and Caesar Salad. This product exemplifies sustainability and most
importantly, high quality. This locally produced, fermented seafood condiment can be used to transform any savory dish and enhance the dining experience.

10. How likely would you be to use this product?
   a. Scale from 1-9; where 1 = not at all likely and 9 = extremely likely

11. How frequently would you use this product?
   a. Scale from 1-9; where 1 = not at all frequently and 9 = extremely frequently

12. How interested would you be in purchasing this product?
   a. Scale from 1-9; where 1 = not at all interested and 9 = extremely interested

13. Please provide any feedback or comments you have about this new product concept
   a. Open-ended

14. As part of this research project, would you be interested in trying and providing feedback about a product prototype?
   a. Reroute to link to provide contact information
**APPENDIX E. SUMMARY COMMENTS REPORT FOR GREEN CRAB SAUCE CONCEPT**

<table>
<thead>
<tr>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>“I use a lot of fish sauce in my home-cooking. Having a local Maine product--that also helps with an invasive species--would be a dream!”</td>
</tr>
<tr>
<td>“Make it in bulk for restaurants! Saves us both cost!”</td>
</tr>
<tr>
<td>“Great way to solve a problem. Why not, seems like an evolutionary idea.”</td>
</tr>
<tr>
<td>“I love this. Using green crabs every way possible to conquer them is key. I’ve cooked with these crabs and they are a pain. A fermented sauce would be a great add.”</td>
</tr>
<tr>
<td>“LOVE IT! Can’t wait to see the commercialized product.”</td>
</tr>
<tr>
<td>“I like the idea of taking an invasive species and finding a use for it, it makes me want it more knowing that its not made from some overfished species.”</td>
</tr>
<tr>
<td>“It’s great to find a use for something that is invasive. Especially if the flavor/aroma profiles are similar to imported products.”</td>
</tr>
<tr>
<td>“very exciting!!! I own a restaurant and we would be thrilled to incorporate this ingredient”</td>
</tr>
<tr>
<td>“I use fish sauce more at home than at work. The flavor is amazing. I would love to use it at work more but I feel it is a little misunderstood.”</td>
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<tr>
<td>“I would recommend using seaweed in it as well, Laver to be specific.”</td>
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<tr>
<td>“Product needs to have great Umami flavors for it to succeed and not be just another sauce would like to compare it to standard imported fish sauce.”</td>
</tr>
</tbody>
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BIOGRAPHY

Holly Leung was born and raised in Brooklyn, New York on March 3, 1998. Starting at the age of three, she was enrolled into the Brooklyn College Music Conservatory and studied the violin for 15 years. She graduated from Fiorello H. LaGuardia High School of Music and Performing Arts as an instrumental major specializing in violin. She then moved to Orono, Maine to attend the University of Maine. She obtained her bachelor’s degree in Food Science and Human Nutrition with minors in Innovation Engineering and Sustainable Food Systems in May 2020. After completing her Master’s, Holly hopes to pursue a future within the food industry focusing on food product research and development. Holly is a candidate for the Master’s of Science degree in Food Science and Human Nutrition from the University of Maine in August 2021.