ACRYLAMIDE QUANTITATION AND QUALITY ASSESSMENT OF LOCALLY AVAILABLE BREADS IN CHATTOGRAM

Mohammad Ali Zaber

Roll No.: 0119/04

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A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Food Processing and Engineering

Department of Food Processing and Engineering
Faculty of Food Science and Technology
Chattogram Veterinary and Animal Sciences University
Chattogram-4225, Bangladesh

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June 2022

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(Md. A. Halim)	**(Dr. Afroza Sultana)**
Supervisor	**Co-supervisor**

(Mrs. Shireen Akther)
Chairman of the Examination Committee

Department of Food Processing and Engineering

Faculty of Food Science and Technology

Chattogram Veterinary and Animal Sciences University

Khulshi, Chattogram-4225, Bangladesh

JUNE 2022

PLAGIARISM VERIFICATION

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Name of the Student	**:**	Mohammad Ali Zaber
Roll number	**:**	0119/04 **Reg. number:** 00689
Department	**:**	Department of Food Processing and Engineering
Faculty	**:**	Faculty of Food Science and Technology
Supervisor	**:**	**Mr. Md. A. Halim** Professor Dept. of Agricultural Economics and Social Science Faculty of Veterinary Medicine

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Dedication

I dedicate this small piece of work to my beloved and supportive better half Meher Nahid

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Author praises the "Almighty Allah" Whose divine blessings enabled the author to complete this research & writing up this thesis paper for the degree of Master of Science (MS) in Food Processing and Engineering.

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ABBREVIATIONS

AOAC	Association of Official Analytical Chemists
APHA	American Public Health Association
BCSIR	Bangladesh Council for Scientific and Industrial Research
$BMDL_{10}$	Benchmark Dose Lower Confidence Limit
CAC	Codex Alimentarius Commission
CF	Crude Fiber
CP	Crude Protein
DM	Dry Matter
DNA	Deoxyribonucleic Acid
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GOB	Government of Bangladesh
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research into Cancer
IUPAC	International Union for Pure and Applied Chemistry
JECFA	Joint Expert Committee of the FAO/WHO on Food Additives
LC-MS	Liquid Chromatography – Mass Spectrometry
MS	Mass Spectrophotometry
PSA	Primary Secondary Amine
R&D	Research and Development
RF	Response Factor
RSD	Relative Standard Deviation
SAPP	Sodium Acid Pyrophosphate (Disodium Pyrophosphate)
SD	Standard Deviation
SPE	Solid Phase Extraction
TBC	Total Bacterial Count
TDI	Tolerable Daily Intake
TDI	Total Dietary Intake
USFDA	United States Food and Drug Administration
WHO	World Health Organization

ABSTRACT

Bread is a frequently consumed ready-to-eat (RTE) breakfast item all over the world. As being simple, cheap, and adaptable, bread is also consumed in Bangladesh at a good volume. Maillard reaction in bread manufacturing for the proper crust formation results in the emergence of a process contaminant namely acrylamide, which has already been established as a possible carcinogen and neurotoxin. Current study has been conducted to assess quality (nutritional and microbial) as well as acrylamide level of locally available breads in Chattogram, Bangladesh. Fifteen samples were collected for the study and analysis was done in HPLC to determine acrylamide concentrations in bread. Bread quality (nutritional and microbial) was assessed following standard methods of AOAC, APHA and ISO. The average level of acrylamide (297.3933 µg kg^{-1} or ppb) in the samples were quantified excessive high as compared to the quantitation carried out in other countries. Nutritional quality assessment scores showed varying food value parameters. Moisture, lipid (fat), crude protein, mineral (ash), crude fiber and carbohydrate of the samples ranged from 32.34%-40.25%, 4.31%-5.94%, 4.23%-5.65%, 1.056%-1.43%, 0.41%-4.3% and 44.11%-57.66% respectively. Most of the samples contained characteristic higher moisture, and higher carbohydrates. Crude protein, ash and crude fiber was present in very low quantity in all samples. Microbiological results showed significant level of total yeast and mold count, TBC, total coliform, *Escherichia coli* and *Salmonella spp.* were found in all samples. Findings of this investigative study puts recommendation to conduct a wide range survey and analytical research to quantify acrylamide concentration and microbial load in other ready-to-eat food products (that has undergone thermal processing) mostly consumed in Bangladesh and to establish a national standard for bread mentioning authentic limits of nutritional quality parameters, microbiological parameters and acrylamide level.

Keywords: Bread, Acrylamide, Proximate analysis, HPLC, Microbial quality, Bangladesh.

CHAPTER 1
INTRODUCTION

Bread is one of the most frequently consumed ready-to-eat (RTE) food product round the globe. In most of the countries of Europe, bread is the supplier of fifty percent of the total required carbohydrate, 33.33% of the required protein, and more than fifty percent of the B vitamins and 75% of the required Vitamin-E (Islam et al., 2011). It is an important source of macronutrients (lipid, protein and carbohydrates) and micronutrients (e.g., vitamins, minerals) that are all necessary for human health. The nutritive values turned the bread to be recognized as a necessary food for human nutrition, and it has led all the countries round the globe to study the composition of bread to develop nutritive value (Ijah et al., 2014). Acceptability of bread is rising in both suburban and urban areas of Bangladesh. Wheat bread (leavened) consumption has risen up significantly in developing countries like ours due to changing diet pattern, increase in the population working outside home, and urbanization. It is consumed widely in most hotels, homes and restaurants. As per BBS data, production volume of the cookie (biscuit) and breads was 100,305 tonnes in the year 2010 to 2011. More than 100 manufacturers and 4,500 traditional small manufacturers are manufacturing cookies and breads in Bangladesh. As per the statement of Hussain and Leishman (2013), the market share of the automated cookie and bread manufacturing industries is calculated at $56.5 million (approx.), excluding the portion of traditional small baking based food processing facilities.

At present, consumers are more concerned about own health and becoming aware of the nutritional quality and wholesomeness of food products. The general public is uninformed about the effects of the processing that flour undergoes (Sabir and Sharef, 2013). Standards for bread is represented by the basic components, lower loaf weights, moisture content (<38%), solids content (>62%), nutritive values, labeling of bread, and codes of handling practice in time of baking and post baking (SACN, 2012; FDA, 2019). Good quality of food products are produced by maintaining the proteins, minerals, lipids, carbohydrates, enzymes, vitamins and other nutrients. However, there have the possibilities to lose the quality because of sensitivity to light, heat, solvent, oxygen, pH or a combination of these, nutrients during processing of food. Main characteristics of bread for the consumer to consider are flavor and

texture. The major flavor compounds are formed due to caramelization and through maillard reaction at the time of baking. Aside the flavor properties, the color, texture, freshness and biting characteristics strongly affect the overall acceptability of bread. A sequential chain of reactions begins immediately after the loaf of the baked bread is brought out of the oven and the reactions ultimately lead to quality degradation of the bread (Gellynck et al., 2009). The nutritional value of food is reduced and degraded as a result of processing of those food products under high heat. At present, another recent concern with high heat processed food came into consideration regarding Acrylamide (a naturally occurring chemical), which is formed when high temperature cooking is carried out and then it is converted to glycidamide (possible reproductive toxicant and mutagen).

Acrylamide (or acrylic amide, CAS No. 79-06-1), chemical formula C_3H_5NO, is a white odorless crystalline solid, soluble in water, chloroform, ether and ethanol. By IUPAC it is named as Prop-2-Enamide. The melting point, vapor pressure, molecular weight and boiling point of acrylamide are 84.5±0.3°C, 0.005 mmHg at 25°C, 71.08, and 136°C at 3.3 kPa/25 mm Hg respectively. Occurrence of the compound acrylamide in most of the common heat treated food products is treated as a crucial food safety issue by international regulatory authorities. Depending upon the population's diet pattern, food processing and preparation technique, acrylamide exposure varies. Acrylamide was highlighted in food safety sector by the Swedish researchers and scientists and the Swedish National Food Authority in 2002. They have mentioned that the formation of Acrylamide in a range of baked and fried food products, in particular in potato based food products e.g., (chips). This invention later on has been reconfirmed by many other food scientists round the globe. (FAO/WHO, 2002)

Deep researches considering food containing acrylamide presents that the toxic compound is created in the roasting, frying or baking (higher than 120°C) of a good range of food products, specifically high starch containing food products (e.g., potato based and cereal based items), like a side-product of maillard reaction. In Sweden, discovery of acrylamide is reported in food products in the month of April in the year of 2002 by Eritrean scientist Eden Tareke. This contaminant was discovered in high starch containing food products (e.g., bread, french fries and potato crisps or chips) which were processed under heat greater than 120°C (248°F).

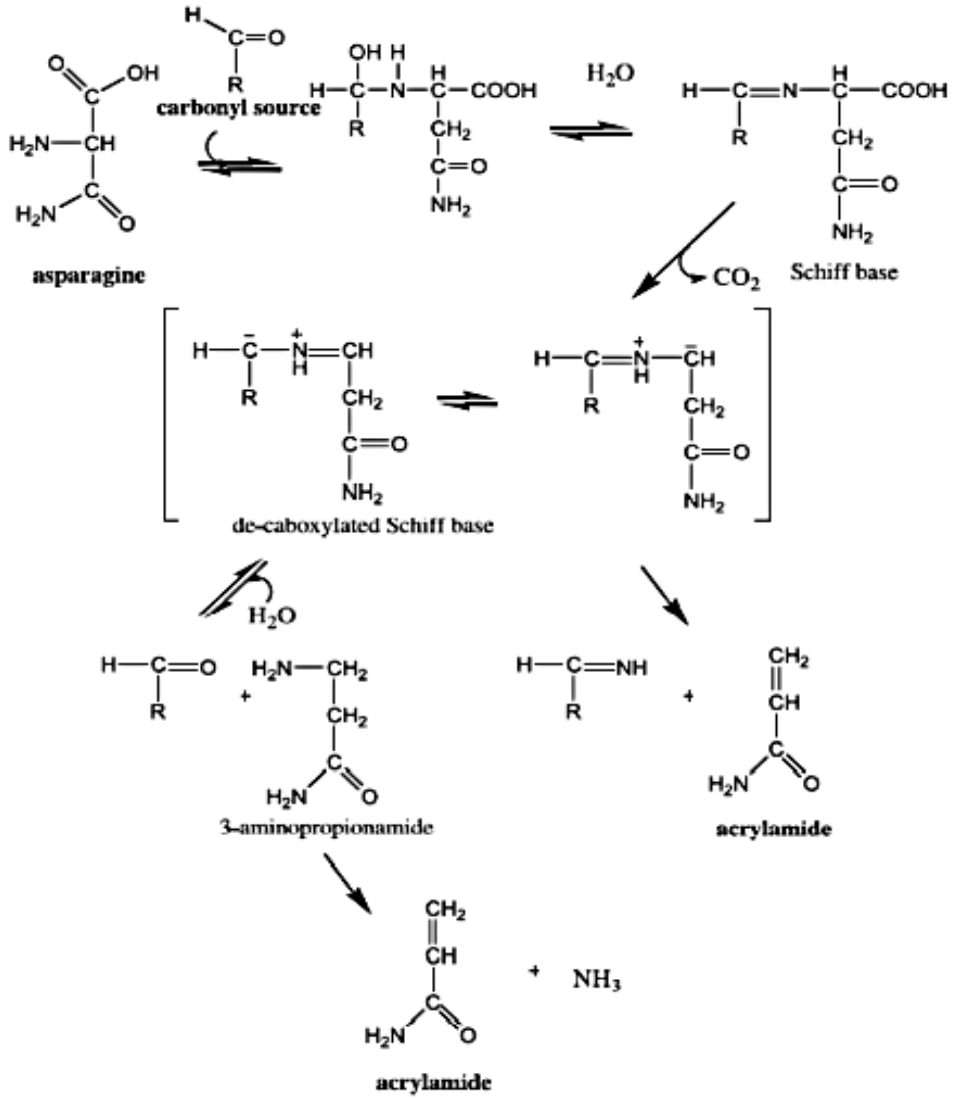

Figure 1.1: Maillard reaction influenced genesis of acrylamide (Zyzak et al., 2003)

Concentration level of acrylamide were detected as moderate (5–50 ppb) in heat processed higher protein containing food products and high amount (150 to 4000 ppb) in higher carbohydrate containing food products (Ahn et al., 2002). The presence of alarmingly high concentration of acrylamide (>10 ppm) in over-fried crisps or chips established that the temperature of heat processing and tenure influence acrylamide formation. Referring to WHO, the daily consumable acrylamide ingestion in adult humans is almost 0.3-2.0 µg/kg-bw and for children rate of ingestion is more high due to the differences in body weight ratio (Becalski et al., 2003).

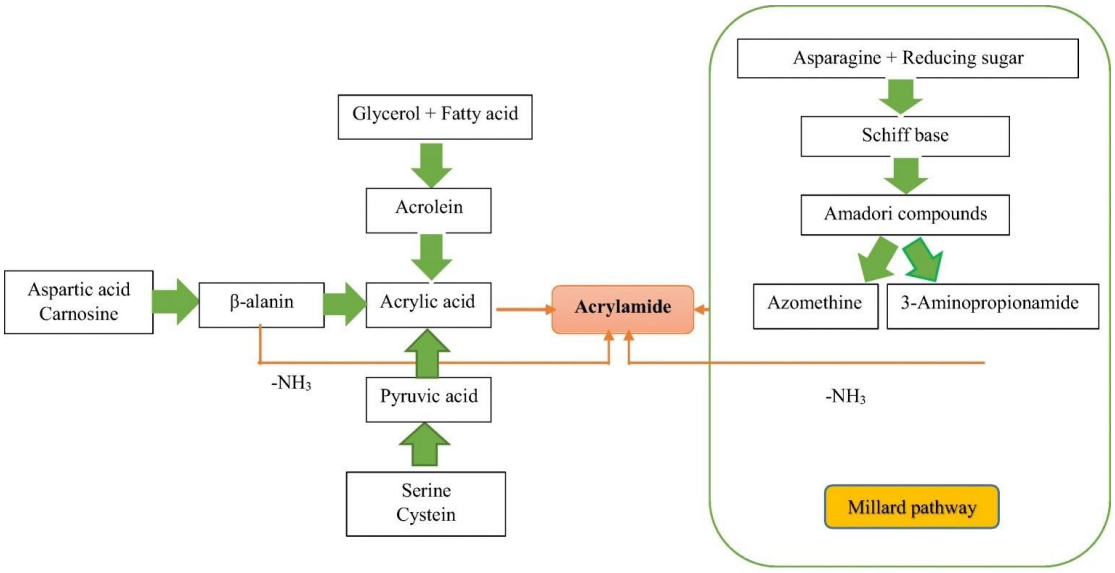

Figure 1.2: Course to the genesis of acrylamide in bread (Esfahani et al., 2017)

Centered on the carcinogenic effect of acrylamide in rodents, the IARC (International Agency for Research on Cancer) marked the acrylamide (an industrial chemical) as a potential human carcinogen in the year 1994. It is most commonly well-known as neurotoxic. Although, the WHO has fixed a restriction of 0.5 mg/kg for acrylamide in water (labeled as drinking water), on the contrary no restriction or limit in food have been fixed (Galdo et al., 2006). The JECFA circulated a statement that it is too much early to reject the toxicological concern posed by acrylamide consumption with food products (JECFA, 2005). Genesis of acrylamide in food products (while undergoing process of high heat) is a crucial challenge in the cases of chips, french fries and bakery industry. Due to this reason, further research is on-going to lower acrylamide concentration in food.

Baked products are also subjected to microbiological, chemical, and physical spoilage, like many other processed food products. Many industrially manufactured baked goods containing surface that is mandatorily sterile may rapidly lead to fungal, microbial contamination as a consequence of submission to airborne contaminants while cooling, slicing and wrapping and also equipment contact (Saranraj and Geetha, 2012). During post baking handling, not less than 90% of bread were contaminated. Many research findings confirmed the occurrence of many pathogenic microbes and the maximum occurrence of antibiotic resistant *Escherichia coli*, *Staphylococcus* and *Bacillus* spp. in bread (baked) (Demissie and Natea, 2018). In addition, Rhizopus and

Mucor like molds and *Aspergillus* and *Fusarium* spp. like fungi have been found involved in degrading the quality of bread. Outbreaks of food-borne disease coming from contaminated food products from bakery origin is confirmed in many countries along with USA, Europe and Australia (NZFSA, 2007). However, the safety and microbiological quality of breads marketed in Bangladesh are not well documented yet.

For the characteristic low water activity, flour and bread are familiar as safe food commodity. But prevalence of microorganisms along with pathogenic microbes in higher proportion than recommended limits has been revealed in microbial isolation and enumeration in these food items. Since flour and bread are widely consumed food items, their microbial quality is crucial in consideration with the consumer's health. Therefore, strict regulatory steps on the microbial quality control on flour and bread together along with the training of sales agents on food spoilage are needed for the better management of public health situation in Bangladesh. The current investigation is focused to determine the level of Acrylamide and quality (nutritional and microbial) of the breads locally available in Chattogram.

The objectives of this study are followed by;

1. To determine the Acrylamide level in bread available in Chattogram;
2. To determine the nutritional quality of the bread;
3. To determine the microbial quality of bread.

CHAPTER 2
REVIEW OF LITERATURE

From the year 2002, after first reporting of acrylamide formation in food products, acrylamide detection has become focal point of many researches and scientists. Even though significant studies were absent in Bangladesh, enough relevant trending research and studies are available round the globe concerning carcinogenicity of acrylamide, acrylamide formation, food risk group, acrylamide in breads and acrylamide formation mitigation considerations in breads.

2.1 Acrylamide in Food products

For more than fifty years, acrylamide is utilized for synthesis of polyacrylamides with so many applications in textile, papermaking, and cosmetics and as flocculants to clarify water (labeled as drinking water). Light, heat and outdoor environmental conditions are showed in many studies to promote the de-polymerization of polyacrylamide to acrylamide, describing strong negative health effects on labors exposed to higher levels of acrylamide, such as the accident of Swedish Hallandsas tunnel in the year 1997.

From the year 1994, regardless of the fact that acrylamide exposure was demonstrated as potentially carcinogenic (Friedman, 2004), its occurrence in smoke of tobacco, many concerns were only newly uplifted on acrylamide occurrence in food items. As a consequence, in April 2002, the University of Stockholm and the Swedish National Food Agency published the first statement on the higher amounts of acrylamide levels in higher carbohydrate containing food products treated at relatively higher temperatures (FAO/WHO, 2002).

The earliest contributions at European level to the confirmation of acrylamide occurrence in food products stated that it is formed as a result of maillard reaction between amino acids (e.g., asparagine) and reducing sugar (specifically glucose and fructose) (Mottram et al., 2002; Stadler et al., 2002).

WHO (World Health Organization) and FAO (United Nations Food and Agriculture Organization) assembled a convention of twenty three (23) scientific experts in the month of June of 2002. The scientific professionals acknowledged the occurrence of

acrylamide in food as vital issue in humans depending on ability to cause cancer and heritable mutations observed in laboratory animals and the urged scientific investigations of the scope to lower the concentration of acrylamide in food products through alteration of formulation, processing and other practices.

Research round the globe is aimed to quantify the concentration of acrylamide in different food products that are commercially available, which might turn it easier for the ultimate consumers to identify food products containing insignificant amount of the toxicant. Research in Belgium showed a mean exposure to acrylamide of 0.4-1.6 μg/kg-bw/day, with the main contributors being potato chips (23%), coffee (19%), bread (11%) and biscuits (11%) (Claeys et al., 2010).

The acrylamide concentration in Turkish food items were maximum in crackers, followed by potato chips, biscuit, cakes, baby food products, corn chips, cookies, breakfast cereals, breads, grilled vegetables, wafers, and chocolate, in descending order (Senyuva and Gokmen, 2005). Toasted bread and bread crust contained large amounts whereas bread crumbs had low amounts of acrylamide.

Most of the food products analyzed so far contains acrylamide, which raises the probability that other food products which are not yet analyzed might also contain the toxicant. Chips and crisps contained maximum mean concentration of acrylamide, there was however a broad range, (not detectable to 3.5 mg/kg food product) (FAO/WHO, 2002).

Tawfik et al. (2008) investigated and found that, the acrylamide concentration in various food groups were found in the sequence, mashed-roasted potato (8,974 ppb) > fried pasta (1,600 ppb) > soluble coffee (816 ppb) > biscuits (810 ppb) > potato chips (620 ppb) > cocoa powder (256 ppb) > crisp bread (439 ppb) > fried rice (430 ppb) > roasted Turkish coffee (282 ppb) > cereal breakfast (215 ppb) > butter cookies (151 ppb).

Corresponding to EFSA (2012), fried potato (272–570 ppb), bakery products (75–1,044 ppb), breakfast cereals (149 ppb) and coffee (229–890 ppb) are proved to be the main sources of human dietary exposure to acrylamide.

2.2 Mechanism of Acrylamide formation

Carbohydrates (i.e., reducing sugars) and asparagine (one of the non-essential amino acids) are the main ingredients that are accountable for acrylamide formation in food products. Maillard reaction is regarded as the vital formation course of acrylamide. (Stadler et al., 2002).

Process of acrylamide formation in food products may be explained with suggested 4 (four) major theories. First process theory explains the straight acrylamide genesis from methionine and from amino acids (e.g., glutamine, alanine and asparagine) (Youssef et al., 2004). Second process theory presumes the production of acrylamide through acrolein genesis which is created from different compounds (e.g., oxidation of monoacylglycerol, dehydration of glycerol, from starch and sugar). Third process theory presumes acrylic acid formation as a median product. Few researchers and scientist have contemplated the second and third process theories as a solitary one. The role of oils and nitrogen containing compounds present in food products has been explained in this mechanism. It includes acrolein fomation from the degeneration of glycerol under heat treatment (Umano and Shibamoto, 1987), oxidation of acrolein to acrylic acid and ultimately reaction of acrylic acid between ammonia resulting in acrylamide genesis.

Mostly explained course of acrylamide genesis is the maillard reaction between asparagine (amino acid) and the reducing sugars. It presumes the Schiff''s base decarboxylation, resulting amadori products' rearrangement and subsequent beta-elimination producing acrylamide (Yaylayan et al., 2005) are vital co-synergists in the Strecker degeneration reaction (Mottram et al., 2002). Schiff's base decarboxylation leading to Maillard medians is the crucial mechanistic step and it can straightly produce acrylamide (Stadler et al., 2002).

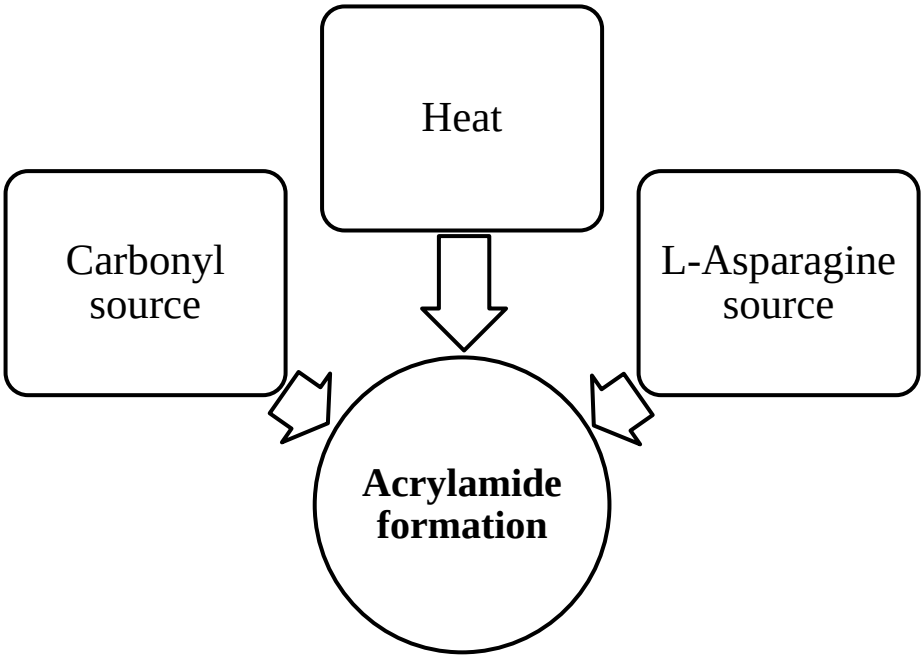

Figure 2.1: Factors responsible for acrylamide formation

The major precursors liable for acrylamide genesis are summed up as in figure 2.1. If the mixture of carbonyl group and amine group are heated to a temperature higher than 120°C genesis of acrylamide is occurred. Thus the fundamental for mitigating acrylamide formation is based on removing anyone of these two factors.

2.3 Carcinogenicity of Acrylamide

Acrylamide (prospective poisonous compound) is formed at the time of thermal processing of many food products (originated from plant), which has raised curisity in its chemistry, disposition in food, consumption, and negative health effects in humans, rodents, and cells.

Evidence to presume acrylamide as neuro-toxicant was acquired from observation of humans (NOAEL 0.5 mg/kg-bw/day) and research and investigations into laboratory animals. Acrylamide is familiar to have engendered neurotoxicity in extremely exposed humans to the dose of 200 mg/kg-day through tap water and 8 mg/m^3 air (Hagmar *et al.,* 2001). These negative health effects seen in laboratory animals at doses of 2-3 mg/kg-bw/day through supply or tap water for entire lifespan, could not be correlated with the intake of food origin acrylamide. In addition, the distal polyneuropathy (also familiar as peripheral neuropathy) was explained as slightly reversible in humans. The debasement of the nerve fibre axons is linked to the

impairment of axonal transport of dynein and kinesin (proteins) (FAO/WHO, 2002). It has been evident that acrylamide can promote cancer concluded entirely from researches into rodent models, for an exposure level of 0.5 mg/day to 0.3 mg/day in water dedicated for drinking, but not in the humans race, even among largely exposed labors and workers (Erdreich and Friedman, 2004). In experimental rats and mice, acrylamide is resulting in tumors at many organ sites in both of the species raising the occurrence of lung tumors, skin tumors, thyroid tumors and pituitary tumors (Klaunig, 2008). The glycidamide is the genotoxic metabolite of the acrylamide. Glycidamide Acrylamide and acrylamide have been also affirmative for the reactivity of DNA and mutagenicity and in good number of in vivo and in vitro assays.

As per a comparatively recent study by Olesen et al. (2008), a linkage between dietary ingestion of acrylamide and tumors in endocrine of women (breast, endometrial and ovarian). A conclusion was drawn by the authors that a correlation between the induction of cancer and acrylamide in food was apparent. They showed a positive correlation between the levels of acrylamide haemoglobin adduct and occurrence of breast cancer from diagnosis of blood samples from three hundred seventy four (374) postmenopausal women.

Exposure of the Polish population was assessed considering 3 criteria: Friedman children in range of 1 to 6 years old, adolescents and children in range of 7 to 18 years old and adults in range of 19 to 96 years old and varying dietary habits for each category. An alarming conclusion that came in from the study was that considerably much higher acrylamide ingestion per kg-bodyweight was determined in the group of adolescents and children (Mojska et al., 2010).
To find out the safe levels of exposure, the MoEs for neurotoxicity of AA exposures over life span were calculated (Tardiff et al., 2009) for the dosages of acrylamide of 1 μg/kg-bw/day and 4 μg/kg-bw/day for an average consumer. The BMDL10 (benchmark dose lower bound) value for glycidamide and acrylamide corresponding to external doses of 0.5 and 0.3 mg/kg-bw/day (Tardiff et al., 2009).

2.4 Quality (nutritional and microbial) of bread

Cereal products and cereals constitute a good proportion of food supplies and have been consumed by a wide range of people round the globe. Cereals and baked food products contribute sufficient nutrients and energy for daily requirement. Since

prehistoric era and even long before bread making was introduced, as a basic food, cereals have been consumed. Bakery products fulfill different nutrient requirement i.e., protein, carbohydrates, lipids, minerals and vitamins (Saranraj and Geetha, 2012).

A good range of breads and other baked food products sales had risen up in the past decades. For bread preparation, grains are grinded to fine powder to prepare flours. Basic structure of the final baked bread are provided by flours. Variations of flours made from rye, maize, barley, wheat and other kinds of grains are found around the world. Bread and wheat flour occupies a good percentage of daily food consumption pattern in both urban and rural population of the country. Higher percentage of carbohydrate and lower percentage of minerals, proteins and lipids are present in flour (Batool et al., 2012). Considerable number of countries round the globe, up to fifty percentage of the total required energy is obtained from singly bread (Akobundu et al., 2006).

Bread has been explained as a confectionary product undergone fermentation and manufactured from flour (wheat), salt, water, yeast and others through subsequent operations including mixing ingredients, kneading of dough, proofing of dough, shaping and baking into final product (Dewettinck et al., 2008).

According to the microbial point of view, both of flour and breads are usually marked as safe food forming units due to the suggestive endogenous disease, since they contain poor water activity (ICMSF, 1998). It is thought that pathogenic microbes may survive for a long tenure instead of their growth being halted under such low water activity (Berghofer et al., 2003).

Baked food products and cereal grains that are stored under inappropriate storage conditions may aid in multiplication of various pathogenic microbes (Deibel and Swanson, 2001). Intoxication in food resulted from microbiologically contaminated flours are reported in the USA, Australia, European Union and many countries of the world. Pathogenic microbes (e.g., *Escherichia coli*, *Salmonella* spp., *Bacillus cereus* and other food spoilage microbes) were found to be present at low amounts in flour and wheat (Cicognani et al., 1975, Ottogali and Galli., 1979). Beside the bacterial growth, mold growth in flour is familiar to considerably degrade the quality of flour and bread.

CHAPTER 3
MATERIALS AND METHODS

3.1 Location and study period

The research work of the present study was conducted in the Institute of Food Science and Technology (IFST) laboratory, Bangladesh Council for Scientific and Industrial Research (BCSIR), Dhaka. The study tenure was 6 (six) months from January 2021 to June 2021.

3.2 Acrylamide Quantitation of Bread

3.2.1 Sampling method and Sample Collection

General Guidelines on Sampling (CAC/GL 50-2004) was followed for sampling method selection. Appropriate sample size is five (5) according to the CAC guidelines for the target population of the study. Local shops at Chattogram, Bangladesh were visited for sample collection. 3 (three) packs of each manufacturer were collected. Fifteen (15) samples were collected in total from the local shops situated at Chattogram, Bangladesh. Samples had been encoded as shown in the table 3.1.

Table 3.1: Sample Codes

Sample No. / Manufacturer No.	1	2	3
1	M1S1	M1S2	M1S3
2	M2S1	M2S2	M2S3
3	M3S1	M3S2	M3S3
4	M4S1	M4S2	M4S3
5	M5S1	M5S2	M5S3

M = Manufacturer, S= Sample

3.2.2 Sample Preparation

At first, samples are homogenized using grinder. Ione and a half (1.50 g) of sample was taken in a fifty (50) mL centrifuge tube. Subsequent extraction was carried out with water at sixty degree celsius (60°C) in ultrasonic bath (Model: XUB10 manufactured by Grant Instruments of UK). The sample mixture was centrifuged (12,000 rpm, 15 min) and then for defatting process it was shaken well (with n-hexane). Then, extraction was carried out twice in a row with ethyl acetate. Lastly,

extracted organic layer was dried through evaporation and prior to injection it was dissolved with ethyl acetate (Castle, 1993).

3.2.3 Acrylamide determination

Quantitation of acrylamide in the market samples were conducted with Dionex Ultimate^{TM} 3000 HPLC system. An acclaim 120, C18, 5µm (4.6 X 250 mm) column was utilized as the analytical HPLC column. Ultimate^{TM} 3000 VWD (190 – 900nm) detector was used. At a flow rate of 0.8 mL/min, acrylamide separation was carried out (under isocratic conditions) where mobile phase contained water:mehtanol (85:15, v/v). 20 µL sample was injected each time and the chromatogram was observed at 210 nm. Chromeleon 6 software was used to acquire and process chromatographic data.

3.3 Quality assessment of Bread

3.3.1 Moisture Content

Moisture content of the bread samples was determined following the method of AOAC (2000). Weight of a dried empty dish (dried in the oven for 3 hours at 105°C and cooling was carried out in the desiccator) along with the lid was recorded. In the dried dish, 3gm of sample was weighed and uniformly spread. Then the sample containing dish was placed in oven. Drying was carried out at 105°C for 3 hours and subsequent cooling was carried out in a desiccator where the dish was partially covered with lid. Weight of the dish and the dried sample were recorded. Then moisture content was calculated as,

$$\text{Moisture (\%)} = \frac{(W_1 - W_2)}{W_1} \times 100$$

Where, W_1 = Weight (g) of the sample (before performing drying)

W_2 = Weight (g) of the dried sample (after performing drying)

3.3.2 Crude Protein

The protein content (crude) of the bread samples was analyzed according to the method of AOAC (2000). Half gm to one gm (0.5 – 1.0 gm) sample was measured in digestion flask. Kjeldahl catalyst (5 gm) and concentrated H_2SO_4 (200 ml) was taken in the digestion flask. Taking the same chemicals, a blank was prepared where sample was not taken. Until the frothing ceased, the flask was heated gently placing in

inclined position. Until the sample solution became clear it was briskly boiled. Cautiously 60 mL of water (distilled) was added after cooling. Then flask was connected to the digestion bulb (on condenser). Tip of the condenser was immersed in standard acid and there were 5 to 7 drops of mixed indicator in the receiver. The contents were mixed thoroughly by rotating the flask; then NH_3 was distilled by heating. Removing the receiver, washing of tip of the condenser was carried out. Then titration was done to distill excess standard acid with standard NaOH solution. Protein content was calculated as,

$$\text{Protein (\%)} = \frac{(A-B)\times N \times 1.4007 \times 6.25}{W}$$

Here, A is the volume (mL) of 0.2 N Hydrochloric acid used for sample

B is the volume (mL) of 0.2 N Hydrochloric acid used for titrating blank

N is the normality of Hydrochloric acid

W is the weight (gm) of sample

14.007 is the atomic weight of Nitrogen

6.25 is the conversion factor for Protein to Nitrogen (fish and fish byproducts)

3.3.3 Ash Content

Ash content determination of the prepared bread samples was carried out following the method of AOAC (2000). To ensure that the impurities on the surface of the crucible are burned off, the crucible with lid were kept in the furnace overnight at 550°C. In the desiccator, cooling of the crucible was carried out for 30 minutes. Weight of the crucible along with the lid were recorded to 3 decimal places. Five (5) gm sample was taken in the crucible. then the sample containing crucible with lid (half covered) was heated over low Bunsen flame. As soon as the fume production was stopped, the crucible along with the lid was placed in the furnace. The sample was heated overnight at 550°C. Cover was removed during heating. For the prevention of fluffy ash loss after complete heating, the lid was placed. Then in the desiccator cooling was carried out. As soon as the sample became gray in color, weight of the crucible along with the lid was recorded. Ash content was calculated as,

$$\text{Ash (\%)} = \frac{\text{Weight of Ash}}{\text{Weight of the sample}} \times 100$$

3.3.4 Fat content

Lipid or fat content of the bread samples was analyzed following the method of AOAC (2000). A bottle along with lid was kept in the oven overnight at 150°C to ensure stable weight of the bottle. In a filter paper, 3-5 gm of sample was wrapped after weighing. After that taking the sample into extraction thimble, it was transferred into Soxhlet. Petroleum ether (250 mL) was poured into the bottle. Later the bottle was kept in heating mantle. Connecting the Soxhlet apparatus, the water circulation and the heating mantle was turned on. For 14 hours the sample was heated. Using vacuum condenser evaporation of the solvent was carried out. Until complete evaporation of solvent (total drying of the bottle), incubation of the bottle was carried out at 80°C to 90°C. After completion of drying, cooling of the bottle with lid (partially covered) was carried out in the desiccator. Weight of the bottle including the content (dried) was recorded. Then the fat content was calculated as,

$$\text{Fat (\%)} = \frac{\text{Weight of Fat}}{\text{Weight of the sample}} \times 100$$

3.3.5 Crude Fibre

The fibre (crude) content of the bread samples was analyzed using the method of AOAC (2000). Accurate two (2) gm of bread sample was taken in a beaker. 1.25% H_2SO_4 solution (125ml) was added to the beaker carefully. 3 to 5 drops of antifoaming N-Octanol was added to the beaker. For thirty (30) minutes, boiling of the sample solution was carried out. Later, to make the solution acid free, it was washed thrice. After draining the last wash, 1.25% NaOH (125ml) and N-Octanol (3-5 drops) was added to the sample. Again for 30 minutes, boiling of the solution was carried out. Then filtering the solution, the obtained residue was washed as before. to make the residue acid free, subsequent wash was carried out with 1% Hydrochloric acid solution. In the hot air oven, the residue was heated at 105°C for complete drying (up to constant weight). Cooling of the residue was carried out in the desiccator and the weight was recorded. Then the muffle furnace was used to ignite the residue up to white ash (4-6 hours, 550°C–600°C). Weight of the ash was taken and then percentage of crude fiber was calculated as,

$$\text{Crude fibre (\%)} = \frac{W - W_1}{W_2} \times 100$$

Where, W is the weight of the crucible (including crude fibre and ash)

W_1 is the weight of the crucible (including ash)

W_2 is the weight of the sample

3.3.6 Total Carbohydrate

Following the method of Pearson (1970), estimation of the total carbohydrate percentage was carried out as total carbohydrate by difference, which is by deducting the percentages of protein, lipid (fat), mineral (ash) and moisture from hundred.

Carbohydrate (%) = 100 – (Moisture % + Ash % + Protein % + Fat %)

3.3.7 Total count of yeast and mold

Yeast and mold count was conducted using the method of American Public Health Association (APHA), of the 4th edition of the Compendium of Methods for the microbiological examination of food products (Beuchat and Cousin, 2001). Samples were kept soaked for a certain period of time in the diluent (for the ease of the release of the available microorganisms by softening the sample). Adequate amount of three dilutions were selected and from each dilution 0.1 ml were inoculated (using spread plate method) on plates of DRBC agar (previously prepared and dried). DRBC Agar plates were kept in refrigerator to avoid photo degradation of Rose Bengal as it can be accompanied by formation of compound which inhibits growth of yeast and mold). The inoculums were spread using Drigalski spreader until all excess liquids were absorbed. Plates were allowed to dry for 15 minutes and then incubation at 25°C were carried out for 120 hours without inverting the plates (three plates were stacked). Plates were not moved during this incubation period to avoid displacement of spores since it often facilitate secondary growth and result in invalid final count. After five days of incubation, plates with 15 to 150 colonies were selected for counting and colony counting was carried out using a colony counter fitted with magnifying glass and digital counting pen. Filamentous, cotton-like or pulverulent (i.e. powdery) colonies (containing characteristic of molds) were counted and the results (n_m) were recorded. After counting mold colonies, remaining colonies which probably were bacteria or yeasts (eventually capable of growth under the conditions of test) were also counted and recorded (n_y); five of these colonies were selected and examined under the microscope to verify the morphology of the cells, observing whether the

culture contained bacteria, yeasts or a mixture of both. Total Yeast and Mold Count, $n_{tym} = n_m + n_y$ (cfu/gm of sample)

Where, n_m = Yeast count and

n_y = Mold count

n_m is calculated by multiplying the number of the mold colonies per plate with ten (10) and the reciprocal of the dilution. e.g., in case of 10^{-2} dilution, in number of mold colonies is 30, then $n_m = 30 \times 10 \times 10^2$ cfu/gm of sample = 3×10^4 cfu/gm of sample

n_y is estimated by multiplying the total number of presumptive yeast colonies to the percentage of confirmed yeast colonies per plate and ten (10) and the reciprocal of the dilution. e.g., in case of 10^{-2} dilution, if the number of presumptive yeast colonies is 30, percentage of confirmed yeast colonies is 0.6 (3 of 5 selected colonies), then $n_y = 30 \times (3/5) \times 10 \times 10^2$ cfu/gm of sample = 1.8×10^4 cfu/gm of sample

$n_{tym} = (3.0 \times 10^4) + (1.8 \times 10^4)$ cfu/gm of sample = 4.8×10^4 cfu/gm of sample

3.3.8 Total bacterial count (TBC)

TBC or total bacterial count was conducted using the method of American Public Health Association (APHA), of the 4th edition of the Compendium of Methods for the microbiological examination of food products (Beuchat and Cousin, 2001). 10 gm sample (homogenized) was weighed and mixed well with 90 ml of pre-sterilized 0.1% peptone water (diluent). 10^{-2} and 10^{-3} dilutions were prepared from the 10^{-1} dilution. From each dilution 1 ml was inoculated into two plates with subsequent pouring of 15 to 20 ml of sterilized PCA or plate count agar (cool and molten) in each plate. A gentle rotation (clockwise and anti-clockwise) of the plates were carried out after covering to ensure uniform and proper mixing of sample (diluted) and the media. Later the media was allowed to solidify. Then the plates were kept in inverted position and incubated for 72 hours (3 days) at 30°C. Incubation time was maintained properly to avoid overgrowth which might result in difficulty to count. After three days of incubation, plates with 15 to 300 colonies were considered for counting and using a colony counter (fitted with magnifying glass and digital counting pen) colonies were counted. Calculation of the number of colonies (cfu/gm) was carried out using the following formula;

$$N = \frac{\sum c}{(n_1 + 0.1\, n_2)d} \times \frac{10}{\text{weight of sample taken}}$$

Where, N is the number of total bacterial count

$\sum c$ is the sum of the colonies counted on all petri-plates

n_1 is the number of petri-plates counted in first dilution

n_2 is the number of petri-plates counted in second dilution

d is the dilution of the first counts (e.g., 10^{-1})

3.3.9 Total coliform

Total coliform was determined using the method of ISO 4831:1991, Microbiology of food and animal feeding stuffs-Enumeration of Coliforms-Most Probable Number (MPN). 20 gm sample was homogenized in sterile peptone water (180 mL) to prepare initial suspension. Required number of dilutions were prepared to confirm yield of a negative result in all the tubes corresponding to the final dilution; Three (3) test-tubes of double-strength LSTB (Lauryl sulphate tryptose broth, which is a selective enrichment medium) were taken. Using a sterile pipette, 10 mL of the initial suspension was transferred to each of these test-tubes. Then three (3) test-tubes of selective enrichment medium (single-strength) were taken. Using a fresh sterile pipette, 1 mL aliquot of initial suspension was taken in each of these test-tubes. Incubation of the test-tubes of double-strength medium were carried out at 37°C for 24hrs. Incubation of the test-tubes of single-strength medium were carried out at 37°C for 48hrs. From each of the incubated tubes from, a loop was inoculated in a tube of Lactose bile brilliant green broth (confirmation medium) and incubation was carried out at 37°C for 24hrs to 48hrs until gas formation was observed. In case of each dilution, the total number of test-tubes (only positive tubes) which undergone the event of gas formation was considered for counting. Most probable number of total coliform/gm of sample was determined by multiplying MPN index (Appendix 1) by the inverse of the least dilution considered.

3.3.10 Presumptive *Escherichia coli*

Presumptive *Escherichia coli* was determined using the method of American Public Health Association (APHA), of the 4th edition of the Compendium of Methods for the microbiological examination of food products (Beuchat and Cousin, 2001). About twenty-five (25) grams of homogenized sample was weighed in Nutrient broth (225

mL); it was the 10^{-1} dilution. Incubation of the dilution was carried out at 37°C for 24hrs. Subsequent inoculation of 1 mL from the dilution to MacConkey broth tube was done and incubation was carried out at 37°C for 48hrs. The tube was observed for gas genesis (bubble in Durham's tube) and acid (yellow color in tube). Formation of gas and acid indicated the presence of *E. coli* in 25 gm of sample. Considering characteristics colonies of *E. coli*, the results were documented as *E. coli "*Present" or "Absent"/25 gm of sample.

3.3.11 *Salmonella spp.*

Salmonella spp. was determined using the method of American Public Health Association (APHA), of the 4th edition of the Compendium of Methods for the microbiological examination of food products (Beuchat and Cousin, 2001). 25 gm of the sample was taken in buffered peptone water (225 mL) aseptically and incubation was carried out at 37°C for 24 hrs. 1 ml from the pre-enrichment medium was pipetted to duplicate tubes of selenite enrichment medium terathionate broth (9 mL) and selenite cystene broth (9 mL) and incubation was carried out at 37°C for 24 hrs. Streaking of one loopful of the selective enrichment medium onto (a) pre-dried BSA (Bismuth Sulphite Agar) and (b) pre-dried XLD (Xylose Lysine Desoxycholate Agar) was done and incubation was carried out at 37°C for 24hrs. *Salmonella* colonies showed the following characteristics colony: (BSA: grey, brown or black, metallic shine and XLD: black centered colony, convex, entire glossy). Based on characteristics colonies of *Salmonella spp.*, results were documented as *Salmonella spp. "*Present" or "Absent" /25 gm of sample.

3.4 Statistical Analysis

Statistical analysis (Mean, Standard Deviation) was done in MS Excel 2013.

CHAPTER 4

RESULTS

4.1 Quantitation of acrylamide in samples

Preparation of the bread samples for acrylamide analysis and subsequent analysis was carried out in the laboratory of the Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka.

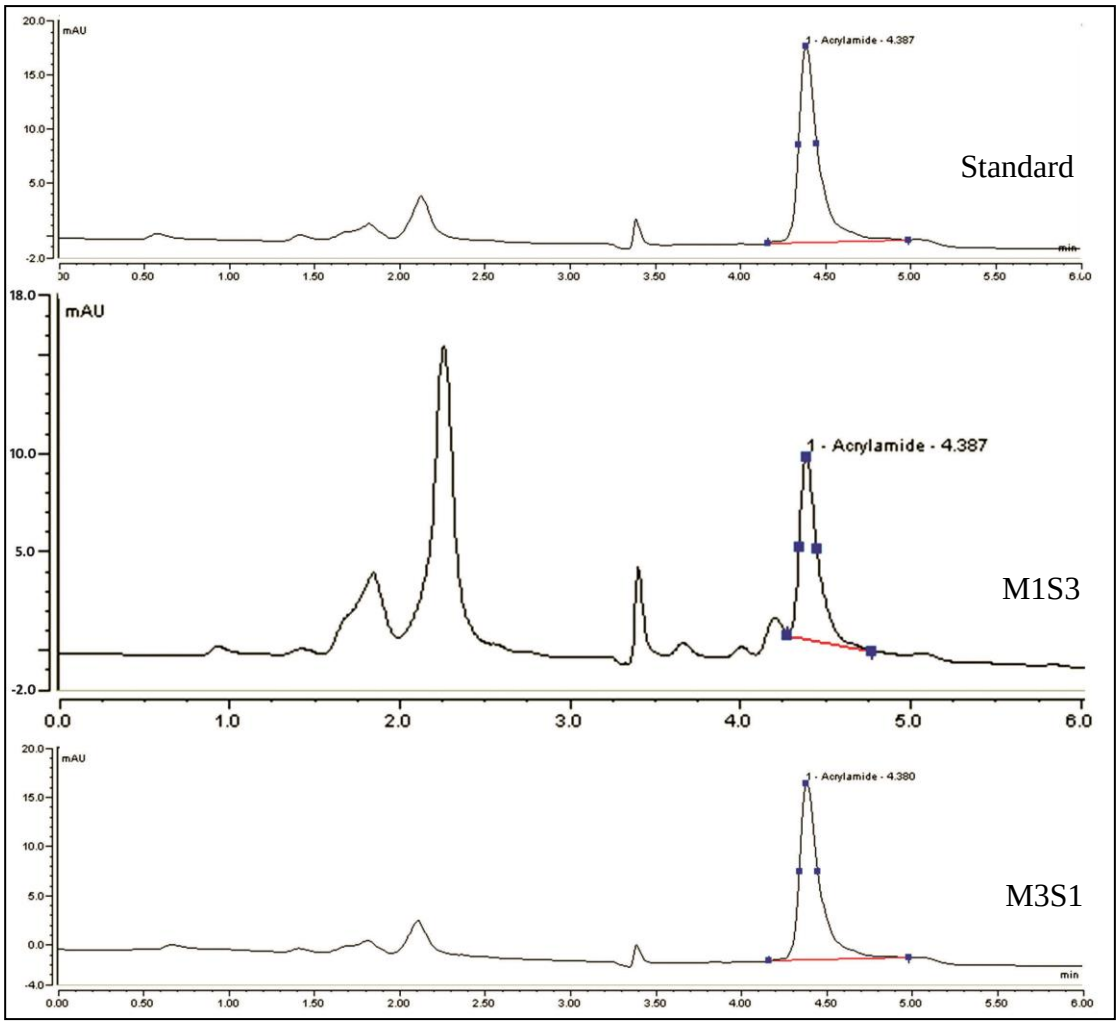

Figure 4.1: Chromatogram of acrylamide standard and bread samples (M1S3, M3S1)

Chromatograms in the figure 4.1 is showing the acrylamide level in the standard solution (1,000 ppb), and bread samples M1S3 (469.4 ppb) and M3S1 (1017.5 ppb). According to the calibration curve (Figure 1, Appendix A), coefficient of determination was 99.8990 and retention time was 4.39 min.

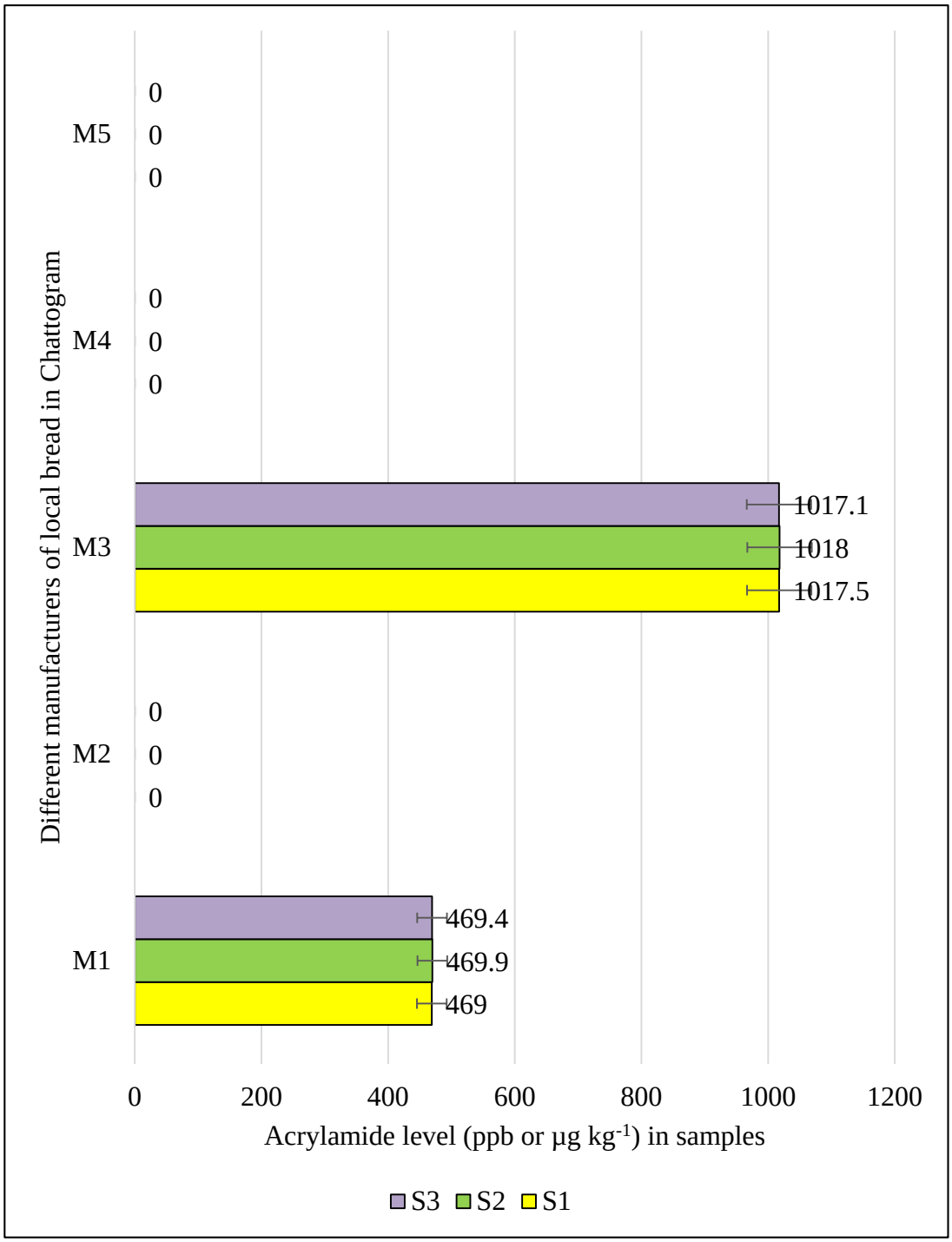

Fig 4.2: Acrylamide level in bread samples of different manufacturer

According to the graphical presentation above and the tabular (Table 1, Appendix 1) presentation it can be seen that highest (1018 µg kg^{-1} or ppb) concentration of Acrylamide was quantified in the sample of manufacturer 3. In addition, higher amount of Acrylamide was also quantified in the samples of manufacturer 1. The samples of manufacturer 2, 4 and 5 contained acrylamide below detection limit (5 ppb) in.

4.2 Nutritional quality assessment of bread

Proximate analysis data showed that, maximum protein (5.65%) was available in the bread sample of manufacturer 5. Furthermore, maximum fat (5.94%) was obtained by the bread samples of manufacturer 2. Breads of manufacturer 2 also contained maximum fiber (1.71%) and ash (1.37%). Maximum carbohydrate (57.66%) was found in the breads of manufacturer 1.

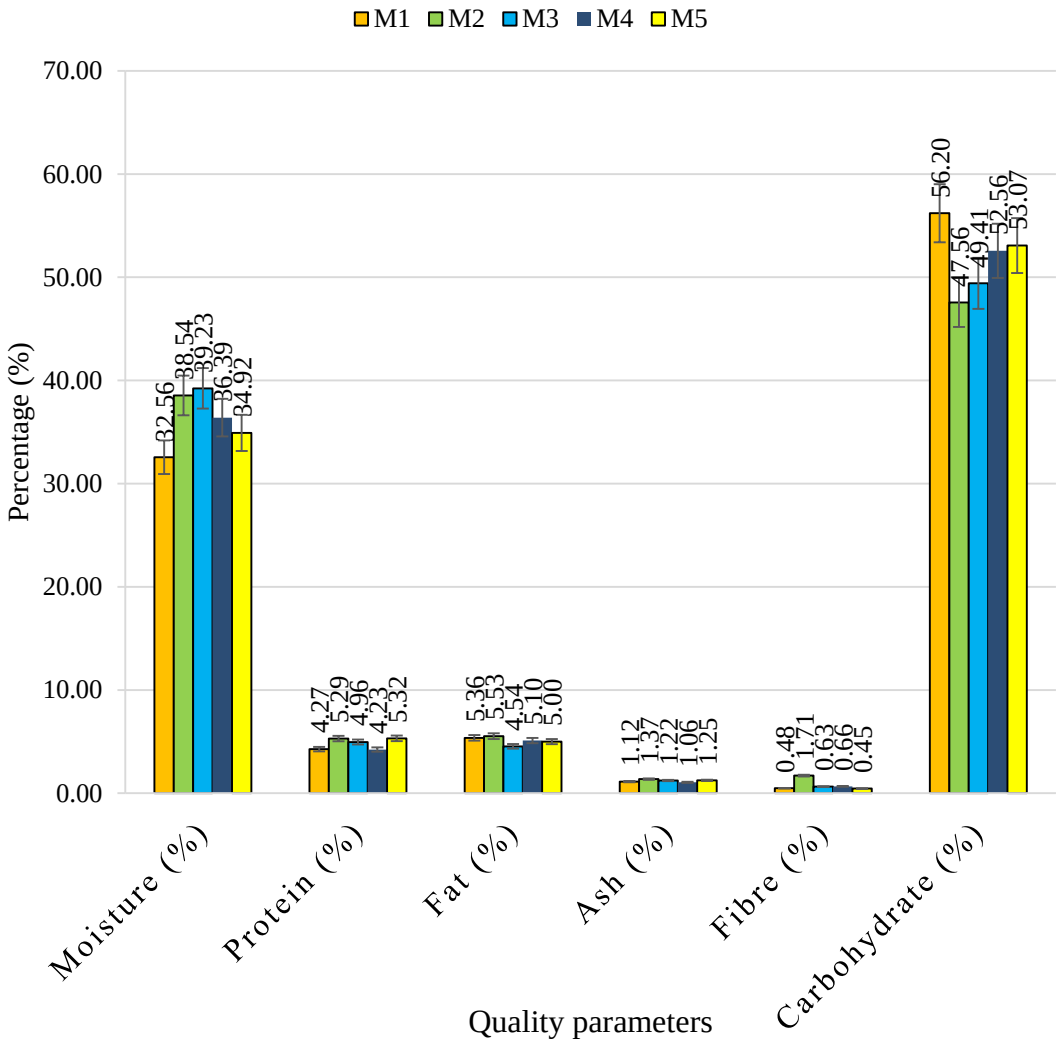

Figure 4.3: Percentages (comparative) of quality parameters (nutritive) of samples

Moisture content of the samples quantified between 32.34% and 40.25%. Protein content was found within 4.23% to 5.65%. Fat content of the samples ranged between 4.31% and 5.94%. Ash content of the samples ranged from 1.056% to 1.43%. Fiber content of the bread samples varied from 0.41% to 4.3%. Carbohydrate content of the samples was determined between 44.11% and 57.66%.

4.3 Microbial quality assessment of bread

Table 4.1: Results of microbial quality assessment of fifteen (15) bread samples

Sample	Total yeast and mold count (cfu/gm)	Total bacterial count (cfu/gm)	Total coliform count (cu/gm)	*E. coli* (in 25 gm sample)	Salmonella (in 25 gm sample)
M01S01	8×10^2	3.42×10^5	< 3	Absent	Absent
M01S02	9×10^2	8.97×10^5	< 3	Absent	Absent
M01S03	1.1×10^3	3.09×10^5	< 3	Absent	Absent
M02S01	Absent	6.85×10^5	< 3	Absent	Absent
M02S02	Absent	5.55×10^6	< 3	Absent	Absent
M02S03	Absent	8.16×10^5	< 3	Absent	Absent
M03S01	5×10^3	9.09×10^7	28	Absent	Present
M03S02	4.9×10^3	1.07×10^8	210	Present	Present
M03S03	5.2×10^3	9.21×10^7	110	Present	Present
M04S01	3.2×10^4	1.13×10^8	200	Present	Present
M04S02	3.6×10^4	8.06×10^7	150	Present	Present
M04S03	3.1×10^4	8.30×10^7	110	Present	Present
M05S01	2.3×10^3	9.36×10^7	35	Absent	Present
M05S02	2.1×10^3	9.18×10^7	61	Absent	Present
M05S03	1.9×10^3	8.24×10^7	61	Absent	Present

Table 4.2: Percent distribution of total yeast and mold count, total bacterial count and total coliform count of bread samples

Parameter	Level of Exposure	Bread samples (%)
Total Yeast and Mold count (cfu/gm)	Satisfactory (<10)	20.00
	Marginal ($<10^3$)	13.33
	Unsatisfactory ($>10^3$)	66.67
Total Bacterial Count (cfu/gm)	Satisfactory ($<10^6$)	33.33
	Marginal ($<10^7$)	6.67
	Unsatisfactory ($>10^7$)	60.00
Total coliform count (cfu/gm)	Satisfactory (< 3)	40.00
	Marginal (3-100)	26.67
	Unsatisfactory (> 100)	33.33

The total yeast and mold count of the considered bread samples lied between $<1.0\times10^1$ and 3.6×10^4 cfu/gm (table 4.1). This excessive high level demonstrates potential health hazards or forthcoming spoilage. The findings (Table 4.2) for total bacterial counts were considerable (satisfactory) only in 33.33% samples. TBC (total

bacterial count) of the samples lied between 3.09×10^5 and 1.13×10^8 cfu/gm (table 4.2). The general quality of the product is usually indicated by the TBC rather than safety. The samples bearing higher TBC might be the result of unsafe packaging, inadequate temperature control and improper storage.

In the current study, 33.33% of the samples (table 4.3) contained *E. coli* above 100 cfu/gm in which is unacceptable.

Table 4.3: Percent distribution of presence/absence of *Escherichia coli* and *Salmonella spp.* of bread samples

Parameter	Presence	Bread samples (%)
Escherichia coli	Present/25 gm of sample	33.33
	Absent/25 gm of sample	66.67
Salmonella spp.	Present/25 gm of sample	60.00
	Absent/25 gm of sample	40.00

Available food standards round the globe says that, ready-to-eat food should not contain *Salmonella* since this pathogen containing food consumption lead to foodborne illness. From the Table 4.3 it can be seen that 60% bread samples *contained Salmonella* which is an indicator of poor hygienic condition in the bread processing, packaging and storage facilities of the manufacturers.

CHAPTER 5
DISCUSSION

Quantitation of the acrylamide showed presence of the contaminant between below detection limit (5 µg kg^{-1} or ppb) and 1018 µg kg^{-1} or ppb in the current study. Scientists and researchers round the globe have analyzed different kinds of food products for the presence and concentration of Acrylamide. The average and maximum concentration of acrylamide in bread samples were reported as 30 and 425 µg kg^{-1}, respectively, in the European Food Safety Authority's (EFSA, 2011) monitoring report regarding acrylamide concentration in food products (in Europe) from 2007 to 2010. The EU Commission Recommendation 2013/647/EU provided the indicative values for acrylamide concentrations in different classes of food items and the minimum concentration of indicative acrylamide value for the class containing soft bread was established at 50 µg kg^{-1}. Representative values are different from the safe levels or the maximum allowable limits of acrylamide in food. Representative values actually shows the necessity for a further detailed exploration. In Brazil, acrylamide concentrations in breads lied between <20 and 71 µg kg^{-1} or ppb (Arisseto et al., 2009). In Netherlands, similarity in the results was observed; the average and maximum concentration of acrylamide in bread samples were 25 and 70 µg kg^{-1} or ppb respectively (Boon et al., 2005). In Sweden, the average and maximum concentration of acrylamide in bread samples were determined as 50 and 160 µg kg^{-1}, or ppb respectively (Svensson et al., 2003). Average acrylamide concentrations quantified in bread sampels in Egypt and Belgium were 64 and 30 µg kg^{-1} or ppb, respectively (Matthys et al., 2005 and Saleh et al., 2007). It has been observed that local breads of Chattogram contains higher amount of acrylamide compared to the concentrations determined and reported in many countries round the globe. This indicates that the bread in Bangladesh needs to be highlighted and investigated further to find out the big scenario of acrylamide exposure of Bangladeshi population. As glycidamide (oxidized derivative of acrylamide) and acrylamide itself are carcinogenic and possess the ability to degenerate DNA including the ability to induce tumors and cancer (if consumed through diet), it has become an alarming issue regarding the starchy food products (undergone thermal processing) round the globe. The outcome is demonstrating the risk of Acrylamide exposure for the nation. To mitigate the genesis of acrylamide in starch rich food products maintaining the quality

(sensory and nutritive), many guidelines have been circulated by the FAO, WHO and CAC (Codex Alimentarius Commission). The guidelines are needed to be circulated to the manufacturers as well as the ultimate consumers to draw a line against the acrylamide in food products.

The mean percentages of moisture, crude protein, lipid (fat), mineral (ash), fiber (crude) and carbohydrates of the bread samples were determined 36.32, 4.81, 5.1, 1.20, 0.79 and 51.76 and the respective standard deviations were 2.63, 0.52, 0.49, 0.12, 0.94 and 3.35. The SD values indicates a considerable or significant scale of deviation in the moisture and carbohydrate contents of bread. As there is no valid range or limit for the parameters of bread in Bangladesh, the results of nutritional quality assessment could not be compared with any valid standard. And it emphasizes on the formation of new standard for the quality of breads manufactured in Bangladesh mentioning the limit or range of limit for the different nutrient parameters.

In case of ready-to-eat food products, microbial contamination is an alarming issue. Remarkable incidence of food poisoning outburst or epidemic has been significantly linked with many pathogenic microbes that are food-borne (Oranusi et al., 2013). Almost 33.33% deaths round the globe are reported to be resulted in by food poisoning (WHO, 2002). Though, safety in the microbiological aspect is crucial in case of the baking industry, bread related standards in Bangladesh still completely lacks the requirements of microbiological test parameters. Mold growth due to post-baking contamination is the main reason behind spoilage of bread (Smith et al., 2004; Pateras, 2007).

Since, mold growth due to post-baking contamination is the main reason behind spoilage of bread (Pateras, 2007), the total count of yeast and mold in the bread samples of the current study ranging from $<1.0\times10^{1}$ to 3.6×10^{4} cfu/gm (table 4.1) is indicative to post-baking poor hygienic management and unhygienic food handling practice in the bread manufacturing facilities. Prospective health hazards or forthcoming spoilage is pointed out by this higher total count of the yeasts and molds. Only in 33.33% bread samples were found to be satisfactory for consumption, which leaves a good amount of bread consuming population vulnerable to the foodborne illness. TBC of the samples lied between 3.09×10^{5} and 1.13×10^{8} cfu/gm and this

clearly demonstrate that the overall quality of the breads is unacceptable. Occurrence of Coliform in heat processed ready-to-eat food is objectionable because it clearly demonstrates contamination of the food due to significant poor hygiene in preparation and processing environment. Significant amount of coliforms were detected in all bread samples. 33.33% of the bread samples contained >100 cfu/gm coliform which indicates one third of the local bread as unsafe for consumption in Chattogram. In the current investigation, *E. coli* was observed above 100 cfu/gm in 66.67% of bread samples (table 4.3) which is unacceptable since presence of *E. coli* is indicative of contamination from feces. Considering the source of *Salmonella* and *E. coli*, the local bread samples of Chattogram cannot be categorized as ready-to-eat and needs further heat treatment before consumption which makes the bread safe from microbes but puts it in danger of increase in the level of acrylamide.

As per the FASNZ (Food Standards of Australia New Zealand), in the case of the ready-to-eat food, TBC above 10^7 cfu/gm are considered as unsatisfactory which indicates considerably poor hygienic environment in processing and poor food-handling practices (FASNZ, 2001). Significant amount of coliforms were detected in all bread samples. 66.67% of the bread samples contained >100 cfu/gm coliform which indicates the local bread as unsafe for consumption.

According to the FASNZ (Food Standards of Australia New Zealand), the satisfactory limit for *E. coli* in any ready-to-eat food products (undergone heat processing) is <3 per gram of sample which clearly illustrates that the organism should not be detected in anyway (FASNZ, 2001).

CHAPTER 6
CONCLUSIONS

This investigation has successfully quantified acrylamide level and assessed quality (nutritional and microbial) of selected bread samples of local manufacturers in Chattogram and pointed out that the quality of the breads is significantly poor and the acrylamide concentration is considerably high. The findings clearly identifies that the processors are entirely unaware regarding the genesis of Acrylamide in the breads manufactured by them as well as the post-baking handling practices in food processing facilities. Important points regarding the nutritional composition, wholesomeness and microbial safety of the breads sold in Chattogram, Bangladesh also has been revealed by this investigative study. Consumption of the local breads sold in Chattogram, Bangladesh have been marked as unhealthy and unsafe since the quality (nutritional and microbial) of most of the samples is impermissible. Food handlers are in need of proper education and training on good handling practices highlighting food safety as well as awareness training on the process contaminants that are formed during processing in respective food industries. The present situation demands establishment of mandatory national regulatory standard for food processing facilities in Bangladesh to preserve strict hygienic environment to ensure health safety of the ultimate consumers. Further investigation on revision and modernization (including acrylamide level, proximate parameters, particularly moisture and microbial parameters specifying maximum or minimum allowable limits) of the available regulatory standards for bread in Bangladesh has also become crucially unavoidable. The findings of the study strongly highlight the necessity of a formation of a national standard to reduce acrylamide risk and ensure bread quality as well as to conduct wide range market survey and quantitative surveillance.

CHAPTER 7

RECOMMENDATIONS AND FUTURE PERSPECTIVES

7.1 Recommendations

1. Food processors (particularly bread manufacturers) are recommended to follow available national and international guidelines to ensure mitigation of acrylamide genesis and improve the quality (nutritional and microbial) of their manufactured food products;
2. National regulatory authorities are recommended to restrict the level of acrylamide in starch containing food products (that have undergone thermal processing e.g., baking, cooking, frying, roasting, smoking) including bread in which genesis of acrylamide is evident as well as mandatory limit for the microbial test parameters of bread;
3. Government research organizations are recommended to disburse grants to investigate the lethal dose of dietary acrylamide as well as the maximum allowable limit of the same for the population of Bangladesh. They are also recommended to determine the maximum allowable limits of indicative microbiological test parameters of bread;
4. Regulatory authorities are recommended to restrict sales and promotion of Acrylamide containing food products and microbiologically contaminated food products in the domestic market;

7.2 Future perspectives

Since only a handful investigations are carried out in our country considering acrylamide genesis in starch containing various categories of food products and dietary exposure of acrylamide and microbial contaminants in general population, the investigators and scientists can

1. Identify acrylamide and assess microbiological parameters in various ready-to-eat food products in Bangladesh;
2. Investigate dietary acrylamide and microbial contaminant exposure level;
3. Preform source identification of dietary acrylamide and microbial contaminants in frequently consumed food products;
4. Analyze cost effectiveness of acrylamide genesis mitigation techniques and post-baking hygienic handling in the perspective of Bangladesh.

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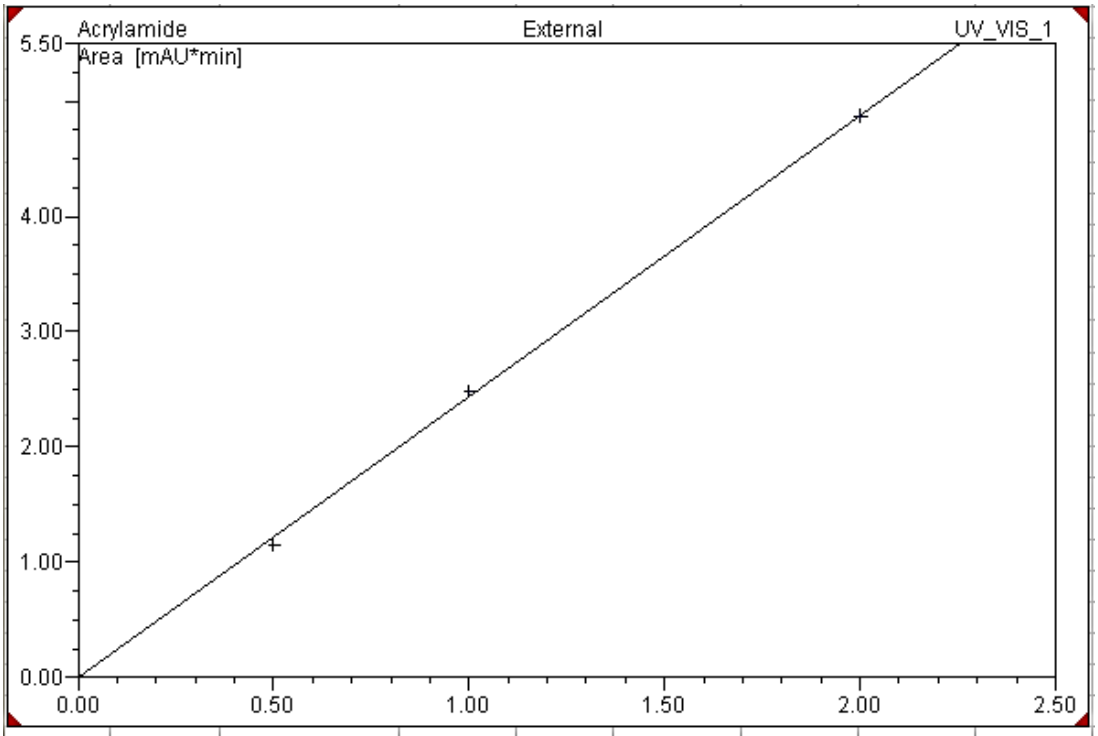
Appendix A



No.	Ret.Time	Peak Name	Cal.Type	Points	Coeff.Det.	Offset	Slope	Curve
	min				%			
1	4.39	Acrylamide	Lin	3	99.8990	0.0000	2.4348	0.0000
Average:					99.8990	0.0000	2.4348	0.0000

Figure: A1: HPLC Calibration curve (acrylamide: 500, 1000, 2000 ppb)

Table A1: Detected Acrylamide level in bread samples

	Acrylamide Level (µg kg^{-1}or ppb)				
Replication / Manufacturer no.	S1	S2	S3	Standard Deviation (SD)	Mean
M1	469	469.9	469.4	0.45	469.43
M2	<dl	<dl	<dl	-	-
M3	1017.5	1018	1017.1	0.45	1017.53
M4	<dl	<dl	<dl	-	-
M5	<dl	<dl	<dl	-	-

dl = detection limit, B=Manufacturer, S=Sample, ppb = parts per billion

Table A2: Quality parameters (nutritional) of bread samples

Parameters	Carbohydrate (%)	Fibre (%)	Ash (%)	Fat (%)	Protein (%)	Moisture (%)
M1S1	57.66	0.48	1.06	5.39	4.26	31.15
M1S2	56.5	0.46	1.07	5.38	4.25	32.34
M1S3	54.45	0.51	1.23	5.32	4.31	34.18
M2S1	49.18	0.41	1.43	5.26	5.21	38.51
M2S2	49.4	0.41	1.33	5.94	5.34	37.58
M2S3	44.11	4.3	1.34	5.38	5.33	39.54
M3S1	48.86	0.64	1.2	4.69	4.36	40.25
M3S2	50.28	0.65	1.26	4.61	5.31	37.89
M3S3	49.1	0.61	1.21	4.31	5.21	39.56
M4S1	52.97	0.65	1.03	4.98	4.11	36.26
M4S2	53.25	0.68	1.07	5.1	4.23	35.67
M4S3	51.46	0.64	1.07	5.23	4.35	37.25
M5S1	53.77	0.46	1.26	4.03	5.13	35.35
M5S2	54.4	0.41	1.22	5.64	5.17	33.16
M5S3	51.04	0.48	1.27	5.32	5.65	36.24

Table A3: Determination of most probable number (MPN)

Table A1: MPN indexes and confidence limits Number of positive results			MPN index a	Category b	Confidence limits (95 %)c	
					Lower limit	Upper limit
0	0	0	< 0.30		0.00	0.94
0	0	1	0.30	3	0.01	0.95
0	1	0	0.30	2	0.01	1.00
0	1	1	0.61	0	0.12	1.70
0	2	0	0.62	3	0.12	1.70
0	3	0	0.94	0	0.35	3.50
1	0	0	0.36	1	0.02	1.70
1	0	1	0.72	2	0.12	1.70
1	0	2	1.1	0	0.4	3.5
1	1	0	0.74	1	0.13	2.00
1	1	1	1.1	3	0.4	3.5
1	2	0	1.1	2	0.4	3.5
1	2	1	1.5	3	0.5	3.8
1	3	0	1.6	3	0.5	3.8
2	0	0	0.92	1	0.15	3.50
2	0	1	1.4	2	0.4	3.5
2	0	2	2.0	0	0.5	3.8
2	1	0	1.5	1	0.4	3.8
2	1	1	2.0	2	0.5	3.8
2	1	2	2.7	0	0.9	9.4
2	2	0	2.1	1	0.5	4.0
2	2	1	2.8	3	0.9	9.4
2	2	2	3.5	0	0.9	9.4
2	3	0	2.9	3	0.9	9.4
2	3	1	3.6	0	0.9	9.4
3	0	0	2.3	1	0.5	9.4
3	0	1	3.8	1	0.9	10.4
3	0	2	6.4	3	1.6	18.1
3	1	0	4.3	1	0.9	18.1
3	1	1	7.5	1	1.7	19.9
3	1	2	12	3	3	36

Appendix B

PHOTO GALLERY

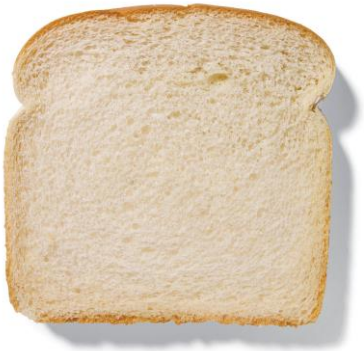


Figure B01: Bread Sample

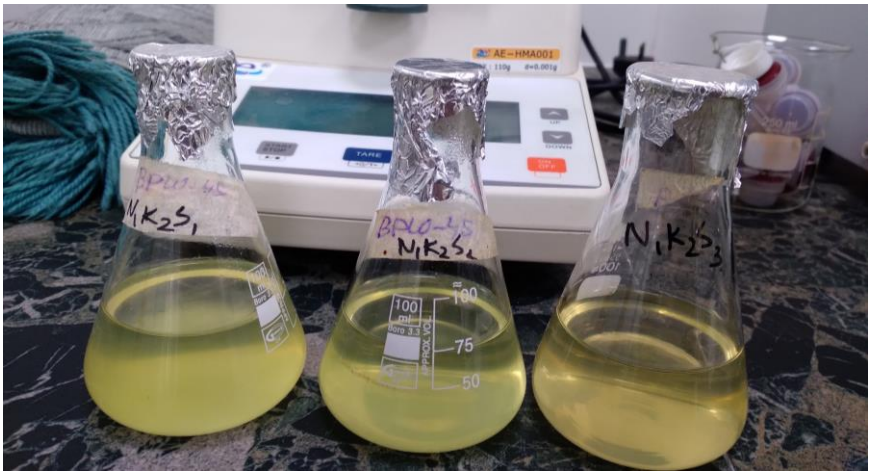


Figure B02: Acrylamide extract in water

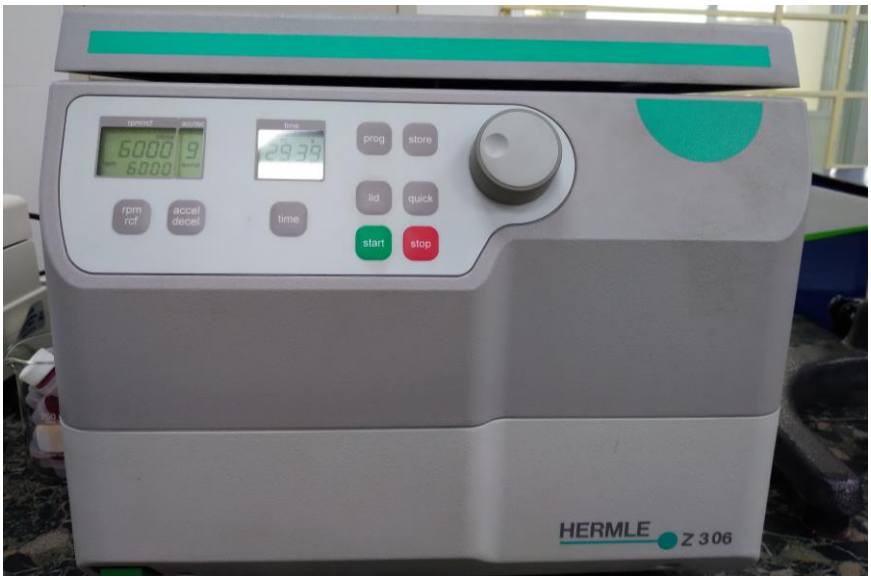


Figure B03: Centrifuge device used for layer separation

Figure B04: Rotary evaporation of supernatant from centrifugation

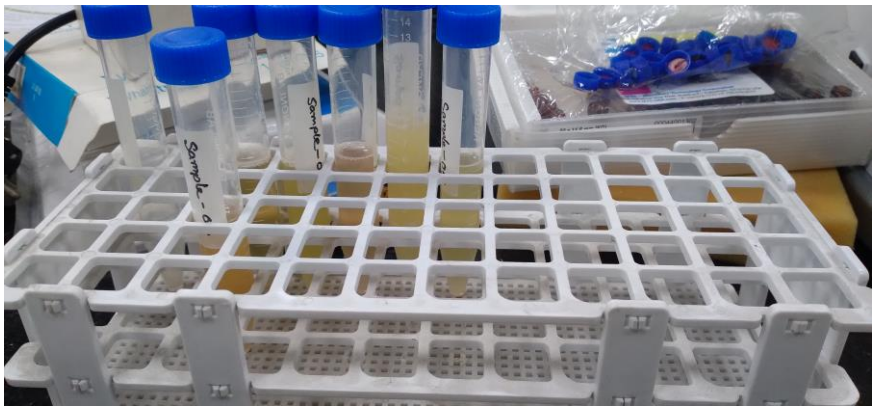


Figure B05: Sample solution prepared with dried acrylamide extract in MilliQ water

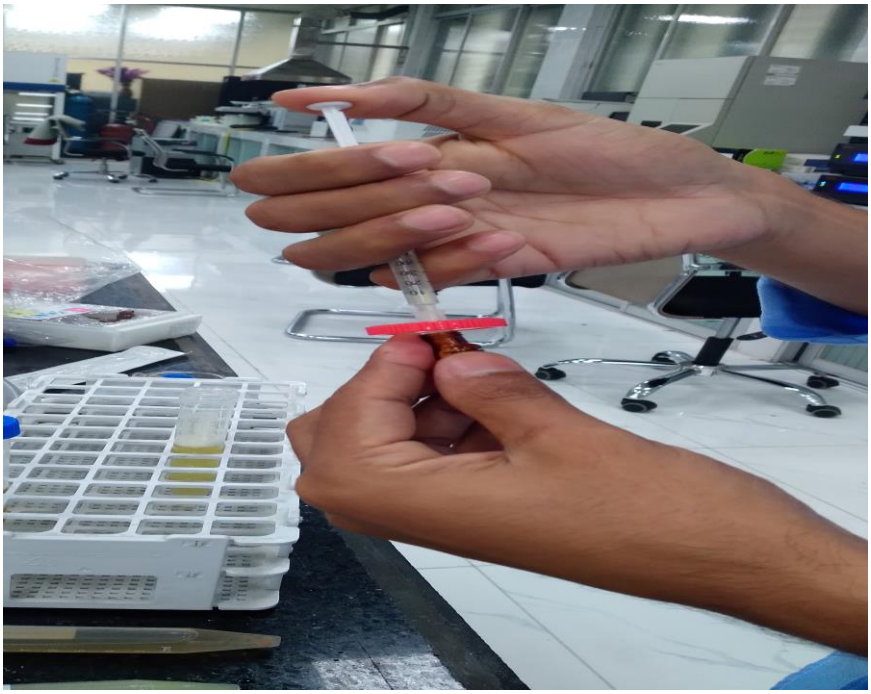


Figure B06: Syringe filtration (0.22 µm) of sample solution

Figure B07: Final sample solution for acrylamide analysis in HPLC

Figure B8: HPLC Analysis

Figure B9: PCA and LSTB used for microbiological analysis

Figure B10: Buffer peptone water for sample preparation

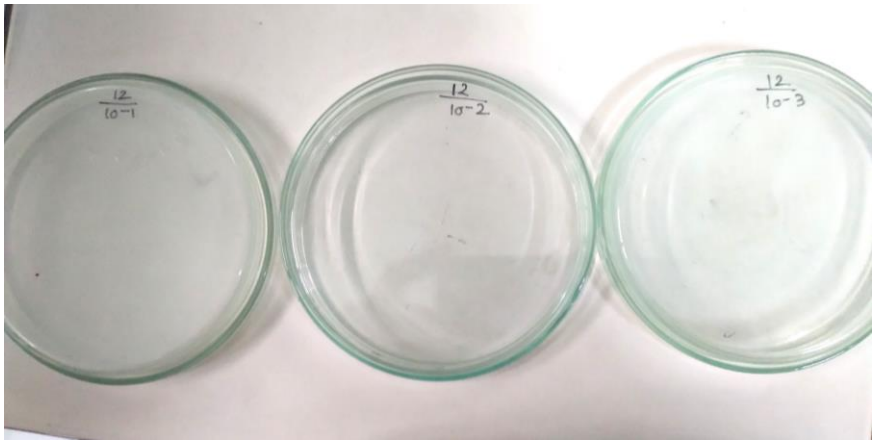


Figure B11: Sterile blank plates

Figure B12: PCA prepared for TBC

Figure B13: Ringer and LSTB

Figure B14: Pouring sample in sterile plate

Figure B15: Pouring media (PCA) on sample

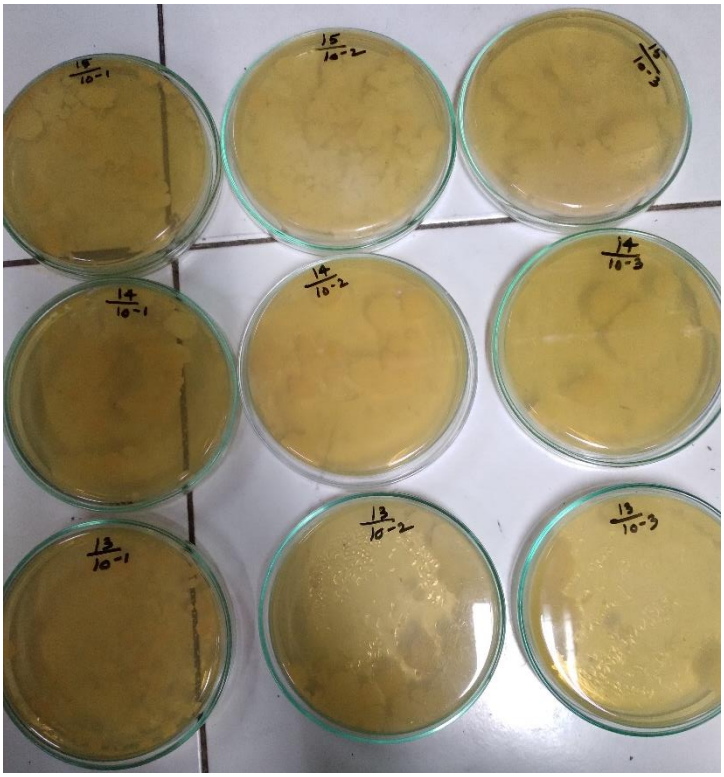


Figure B16: Sample plate prepared for incubation

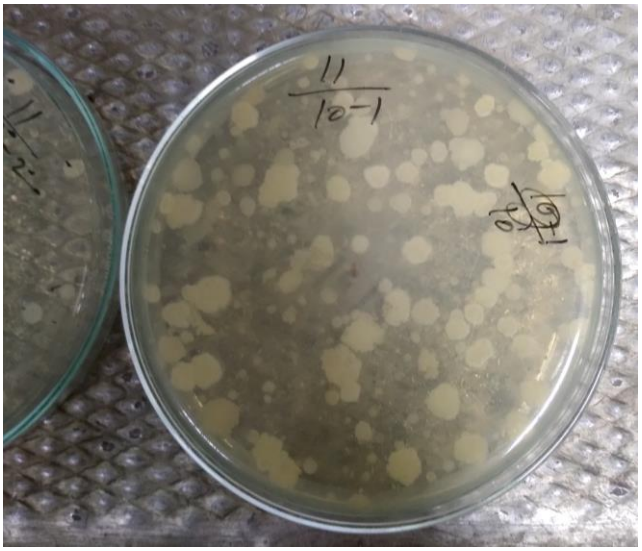


Figure B17: Incubated plate

Figure B18: Plate preparation for total yeast and mold count

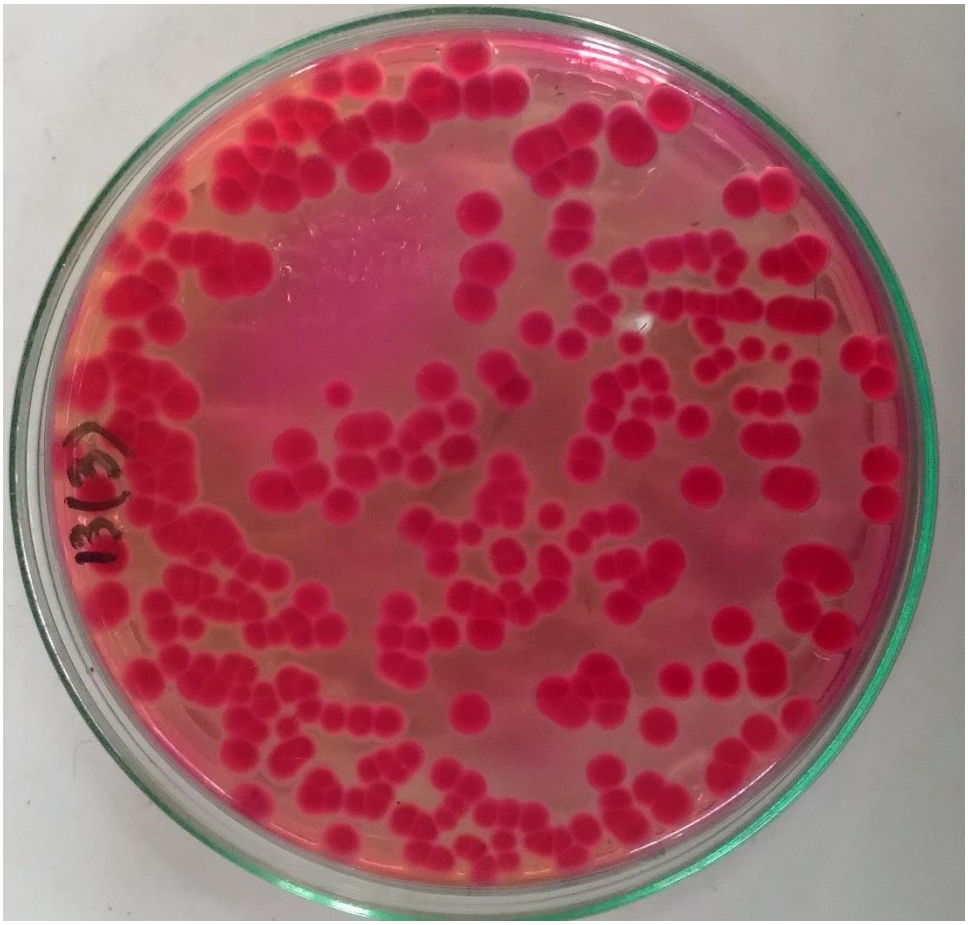


Figure B19: Plate observation for total yeast and mold count

BIOGRAPHY

Mohammad Ali Zaber, author of this study is MS fellow in the Department of Food Processing and Engineering under the Faculty of Food Science and Technology at the Chattogram Veterinary and Animal Sciences University. Author achieved his Bachelor of Science in Food Science and Technology from the Chattogram Veterinary and Animal Sciences University in the year 2014. Author keeps interest in research regarding food processing, food contaminants, dietary replacement of drugs, identification of potential threats in frequently consumed food products in Bangladesh and others.