Chapter 7

Summary

Fermentation is an ancient form of preservation of fish. Fermentation of fish is common in several cultures because of their high nutritional value and range of sensory qualities. The fermentation process is unregulated and random in traditional fermented fish products, and since the products are frequently processed under local climatic conditions, sensory characteristics and consistency can vary. North East India is rich in natural resources and is a cauldron of variant cultures with unique food habits. Many traditional fermented fish products are recorded in India. *Napham* is a traditional fermented fish paste widely consumed in Kokrajhar district of Assam, being particularly popular amongst the Bodo people. The present study is hitherto the first attempt to do a biochemical and microbial analysis of *napham*.

The documentation of traditional method of preparing napham, the raw materials used and other informations associated with napham were executed in eleven locations in Kokrajhar town and its nearby locality. The plants and fishes used as raw materials were identified by authorized institutions (Botanical Survey of India and Zoological Survey of India, Shillong). A total of eighteen fish and four plant materials were recorded during the survey. Napham is prepared from a mixture of small fishes found in the locality and is an ancient method of fish preservation. Preparation involves combined methods of sun drying, smoking, and fermentation. The dried fish and the stem of *Colocasia esculanta* are mixed and pounded together. Sometimes other plant materials like papaya, ash gourd and hibiscus sabdariffa are also used. The raw mixture paste is inserted inside the hollow bamboo stem or glass container and covered by a layer of *kharwi* (local alkali) which is again covered by a piece of dried banana leaf. This content is then sealed with a layer of ash paste. The fermentation ends within three months, but napham can be preserved for almost one and a half years. Napham making and selling is a good source of income in markets, but apprehensions are there amongst the consumers regarding the quality of the product. Napham is consumed mainly as a condiment and flavoring ingredient.

In the present study Biochemical analysis was done to know the nutritional qualities of *napham*. AOAC methods were used for the proximate analysis. For mineral analysis, Graphite Furnace Atomic Absorption Spectrum (GFASS) Model

Analytic Jana Vario 6 was accessed from the Analytical Instrument Facility (SAIF), NEHU, Shillong. For amino acid analysis, the HPLC instrumentation facility (WATERS Alliance Separations module 2695) and for fatty acid analysis, Gas Chromatograph GC-2010 Plus was availed from M R Labs, Hyderabad.

The moisture content of raw sample without the plant additives was 25.5 %. The moisture content of fermented samples ranged from 36.4 to 41.2%. The ash content of raw non-fermented material without additives was found to be 16.7%. In fermented samples it ranged in 11% to 16 %. The crude protein of raw sample was51.9 % and in fermented samples it ranged in 29% to 32%. The pH ranged from 6.5 to 7.7 in fermented products. In raw sample Ph was 6.5. The crude fat content of fermented product was 24%.

Macro mineral elements detected in *napham* were Calcium, Sodium, and Magnesium. The micro-mineral elements were Iron, Zinc, Copper, Potassium, Manganese, Molybdenum, and Chromium. Ultra-trace mineral elements present were Nickel and Cobalt.

Both essential and non-essential amino acids were detected in *napham*. Aspartic acid, glutamic acid, glutamine, alanine, and lysine were the dominant amino acids, and amongst the essential amino acids, lysine, serine, and threonine were dominant.

The fatty acids detected at a higher percentage in *napham* were: Palmitic acid (16.01%), Linoleic-acid (10.41%), Alpha-Linoleic acid (9.53%), and DHA (8.03%). Monounsaturated (MUFA), polyunsaturated fatty acids (PUFA), DHA, Omega 6 fatty acids, and Omega 3 fatty acids were the essential fatty acids detected in *napham*.

. The microorganisms in *napham* were enumerated using seven different media. The microbiome biodiversity of four *napham* samples (S1, S2, S3 & S4) was studied using two techniques: WGS metagenomics and 16S metagenomics. WGS metagenomics aims to sequence all the genomes existing in a sample to analyze the biodiversity and the functional capabilities of the community studied. The 16S rRNA metagenome target prokaryotes containing 16S ribosomal RNA (rRNA) sequence.

The workflow for WGS metagenomics consisted of metagenomic DNA extraction, DNA QC, fragmentation, library preparation, quality check, sequencing in Illumina HiSeq, and Bioinformatics pipeline which includes *denovo* metagenomic assembly. For whole metagenome sequencing and further bioinformatics analysis,

two samples S1 and S2 were outsourced to Agrigenome pvt Limited, Kochi. The raw reads obtained from the Illumina sequencing platform after Demultiplexing were subjected to the FastQC program (latest version.0.11.8) to check the quality of the reads before the Bioinformatics analysis. The total sequence read obtained after sequencing and QC was 37,947,065, out of which the sequence read in S1 was 19167516 and for S2 the sequence read was 18779549.

The 16S ribosomal RNA (rRNA) is composed of nine hypervariable regions interspersed with conserved regions. The bacterial 16S gene contains nine hypervariable regions (V1-V9) ranging from about 30-100 base pairs long that are involved in the secondary structure of the small ribosomal subunit. The 16S gene contains highly conserved sequences between hypervariable regions, enabling the design of universal primers and taxonomic classification. For 16S rRNA metagenomic analysis two samples were outsourced to PathCare Labs Pvt Ltd., Greater Hyderabad, Telangana. Two *napham* samples S3 and S4, which were more than three months old, were analyzed for bacterial diversity through 16S rRNA metagenomics.

De novo metagenome assembly was carried out for the sample by assembling contigs from the reads using the MetaSPAdes program. Further contigs were linked by the assembly algorithm to create scaffolds. Bad or misassemblies were removed from the result. Assembly was performed with default Kmer sizes 21, 33, and 55 using the de-bruijn graph method. In-house PERL and Python code were used to parse the fastq files for the downstream analysis. The total contigs obtained for S1 was 40317, 103397 for S2.

Annotator (MGA) for the prediction of open reading frames (ORFs). The predicted ORF of S1 was 2940704 and ORF for S2 was 532641. The ORF obtained from the samples was queried to the DIAMOND BLASTX program with an optimum e-value of 1e-5. Taxonomic profiling for all the metagenomics samples was performed using NCBI taxonomy data sets. The taxonomy tree was generated based on neighbour- joining method using MEGAN software. Taxonomic relative abundance was determined in the samples based on the contigs obtained.

In the taxonomic assignment, S1 summarized seven taxa and S2 eleven taxa that correspond to both Prokaryotes and Eukaryotes. According to the annotation, it was observed that Firmicutes, Actinobacteria, and Proteobacteria were the most abundant phyla in S1, and in S2 Firmicutes, Ascomycota, Actinobacteria & Proteobacteria were most dominant.

At the genus level, total 34 genera were obtained from S1 and 74 genera from S2. The most abundant genera found in S1 were *Staphylococcus* (36%), *Oceanobacillus* (15%), *Virgibacillus* (12.54%), *Brevibacterium* (9.44%), *Pediococcus* (5.83%), *Enterococcus* (4.79%), *Yaniella* (3.84%), *Bacillus* (3%), *Carnobacterium* (2.47%), *and Lactobacillus* (2.06%). In S2 the most abundant genera were Staphylococcus (30%) followed by *Enterococcus* (18.62%), *Lactobacillus* (11.63%), *Oceanobacillus* (6.47%), *Aspergillus* (6.35%), *Pediococcus* (3.96%), *Lactococcus* (3.94%), *Tetragenococcus* (2.83%), *Weissella* (2.4%), *Vagococcus* (2.38%).

Total 47 species were summarized in S1.The most abundant species in S1 was Staphylococcus xylosus which formed 14.17% of the total population. This was followed by Oceanobacillus sojae (12.45%), Staphylococcus lentus (7.96%), Staphylococcus nepalensis (7.78%), Pediococcus pentosaceus (7.74%), Staphylococcus saprophyticus (7.19%), Yaniella halotolerans (5.34%), Enterococcus faecalis (4.73%), Brevibacterium linens (4.39%) and Carnobacterium sp.ZWU0011 (3.11%).

Total 153 species were summarized in S2. The abundant species in S2 Oceanobacillus oncorhynchi (6.4%) followed by Staphylococcus xylosus (6.2%), Virgibacillus alimentarius (5.73%), Staphylococcus lentus (5.2%), Lactobacillus brevis (4.7%), Lactobacillus plantarum (4.6%), Lactococcus lactus (4.3%), Pediococcus pentosaceus (4.18%), Staphylococcus saprophyticus (4.1%) and Staphylococcus nepalensis (3.9%).

Firmicutes, Actinobacteria, and Proteobacteria were most abundant in both S3 and S4. Fermicutes alone forms 98.36 % of the total population in S3 and 99.4 % in S4. At the class level, Class Bacilli is most abundant in both S3 and S4. In S3, the most abundant Family is Staphylococcacea and forms 93% of the total Family, followed by Enterococcaceae (4.03%) and Micrococaceae (1.29%). In S4, Enterococcaceae (96.5%) is most abundant, followed by Streptococcaceae (1.36%) and Staphylococcaceae (0.54%). Total 80 genera in two samples were assigned from the sequence read, and out of that 64 were assigned in S3 and 80 in S4. The top five dominant genera detected in S3 were *Staphylococcus* (93.16%), *Enterococcus* (4.03%), Yaniella (1.29%),Lentibacillus (0.35%), Pseudogracilibacillus (0.21%)and Lactobacillus (0.004%). The top five dominant genera detected in S4

were *Enterococcus* (96.58%), *Lactococcus* (1.36%), *Staphylococcus* (0.41%), *Weissella* (0.37%), and *Lactobacillus* (0.27%).

To explore the functional potential of the micro-organisms in the microbiome of S1 and S2 during fermentation, gene or protein functions of all the ORF from DIAMOND BLASTX output were parsed using an in-house PERL script. The ORF obtained from the samples was queried to the DIAMOND BLASTX program with an optimum e-value of 1e-5. Further, functional annotation of all the Contigs is carried out by SEED Classification. MEGAN software was used to assign the function of each contig. The protein functions of each contig having the highest alignment score from DIAMOND BLASTX results were considered for functional assignment. In S1 4083 and S2, 4530 sequence reads were categorized into the SEED functional category. As the napham fermentation progressed from the first month to the second month, matching levels of the metagenomic sequence read to SEED functional categories increased due to the increase in bacterial abundance. The data showed that carbohydrate, amino acid and protein metabolism were key categories for napham fermentation. Carbohydrate metabolism yielded an average of 19.4 % of all matches. The protein metabolism yielded an average of 6.5% of all matches. Amino acids yielded at an average of 12.1%. The results showed that

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