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NONINVASIVE POTENTIOMETRIC BIOSENSOR TO ASSESS MILK SHARK KEEPING QUALITY

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ABSTRACT:

Milk shark (*Rhizoprionodon acutus*) is highly spoiled product because the urea is broken down by the bacterial enzyme urease, releasing ammonia; for this reason, it is essential to preserve and keep an eye on the source of urease degradation, which is fish freshness. To keep an eye on the quality of the Milk Shark, we have produced a urease biosensor. Sodium alginate and calcium chloride solution were used to immobilise urease on the pH electrode. A potentiometric transducer can be used to measure the change in potentials across the single glass electrode brought on by the enzymatic conversion of urea into ammonia by immobilized urease. There was a linear correlation 16 between the degree of urea, ammonia liberation, product deterioration, and potentials produced across the urease biosensor with Milk Shark storage at 30°C. Urease biosensor, thus, offers a dependable, user-friendly, and quick way to gauge how fresh fish with large urea content is.

KEY WORDS: Milk shark, *Rhizoprionodon acutus*, potentiometric, biosensor, Urease.

1. INTRODUCTION

India is the third largest exporter of seafood items to international market with total volume of 17,35,286 MT) and value of Rs. 63,969.14 crore in the financial year 2022-2023[1]. The milk shark, *Rhizoprionodon acutus*, is a requiem shark named for its supposed lactation-promoting properties in India, measuring about 3.6 feet in length and widely found in coastal tropical waters of the eastern Atlantic and Indo-Pacific[2]. The assessment of post-harvest losses in fish and seafood due to perishability stands at ₹15,000 crores, mainly attributed to unhygienic handling,

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insufficient preservation, packaging, storage, and marketing manipulations[3]. The primary causes of food waste among consumers and retailers include concerns about food quality, expiration dates, and safety, with approximately 15% attributed to damage and spoilage, a figure that rises to 35% under inadequate storage and transport conditions[4]. Recent efforts in evaluating freshness across industry, marketing, and inspection sectors aim to elevate quality standards throughout the fishery chain, streamlining trade, bolstering quality assurance, and providing consumers with reliable fish quality information[5]. Currently, the objective non-sensory evaluation methods accessible are time-consuming, cumbersome, tailored for temperate regions, and do not directly reveal the sensory attributes vital for consumer acceptance of Indian fish[6]. Fish freshness degradation is influenced by a number of internal and external variables, including storage temperature, relative humidity, oxygen, salinity, handling and fish catching methods, pH, moisture content, condition and nature of fish, species, sex, size etc[7]. The osmoregulatory physiology of cartilaginous fish, including skates, milk sharks, sharks, and rays, is characterized by high tissue and blood urea levels. [8]. Following the death of these fish, the decomposition of urea into CO_2 and NH_3leads to a strong odor and potential meat toxicity, underscoring the importance of employing accurate and quick assessment of quality tools to ensure acceptable quality [9]. Biosensors are tools for analysis utilized for detecting specific analytes like cholesterol and urea, utilizing biomolecules such as nucleic acids, proteins, and carbohydrates as key components, alongside a transducer and data analysis and visualization tools [10]. The enzymatic transformation of analytes like urea into another component, such as ammonia, can be conveniently evaluated, quantified, and shown in an understandable way using biosensors equipped with immobilized urease [11]. Using the alginate gel entrapment approach, sodium alginate on pH probes may immobilise urease. For almost fifty years, pH electrodes have been utilised in applied research as potentiometric transducers when an enzyme reaction alters the pH. [12]. In order to measure urea levels in Milk shark (*Rhizoprionodon acutus*) fish at landing centres, we attempted to develop a urease biosensor that functions by releasing ammonia through urease action on urea. This attempts to address issues related to fish sample delays in the lab, which sometimes extend to several days.

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2. MATERIALS AND METHODS

Enzyme Preparation:

Milk sharks (*Rhizoprionodon acutus*) taken from the Arabian Sea using trawl nets were sourced from fishing boats in the "Bunder area," Mangalore, between November and September. The time amid the landing and the catching did not exceed three to four hours. One newly caught Milk Shark, weighing approximately 174-261g and measuring between 17 and 23 cm in length, was selected for a ten kg portion. Within 120 minutes, the product was delivered to the lab in an well insulated container that was sufficiently iced at a ice to fish ratio of 1:1.

Enzymes and Chemicals:

We purchased standard buffer tablets from Qualigense Fine Chemicals in Mumbai. Sigma Chemicals Co. supplied the urease tablets, and Merck Ltd., Mumbai, India, produced analytical-grade chemicals and other materials.

Entrapment of Urease-on-Urease Assay and pH Probe:

Alginate gel was made using a solution of calcium chloride and sodium alginate for the immobilization of urease 3. 100 mL of crude enzyme solution was mixed with 0.5 mL of bovine serum albumin solution and sodium alginate aliquots. The one mixture of urease and sodium alginate was dropped into a standardized glass pH electrode, and the liquid was continuously swirled for one minute. The electrode was then submerged in a 0.1 L solution of excess 0.2M CaCl for an hour to cure. Enzyme immobilization and 1 2 calcium alginate produced a coating around the pH electrode bulb, which was connected to a pH metre for potentiometric measurement (a process called biosensor). The quantity of ammonia emitted within a specified time frame. [13, 14, 15].

Assesment of the Biosensor:

The biosensor was used to produce a potentiometric measurement of urease activity by immersing and incubating the bulb in a beaker containing 0.05 M Tris acetate working buffer at pH 7 at 30°C and swirling steadily and moderately. For the purpose of calibration and graph-

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plotting, aliquots of urea were used at concentrations of 10, 20, 40, 60, 80, and 100 mg per deciliter until 1 the electrode voltage across the two biosensor leads reached a stable value.

Microbiological Methods:

For fifteen minutes, glassware and ready-made media were sterilised at 121°C. For one hour, pipettes, homogenizers, and petri dishes were sterilised at 180 degree Celsius. The APHA technique was used to determine the count of viable bacteria. [16].

Assesment of Fresh Fish Sample:

For analysis, fresh Milk Shark samples were selected, and fish samples' electrical potentials were recorded for up to four hours following each hour of incubation at 0°C and 30°C. The brisk fish samples were investigated for appearance, colour, texture, and odour. A nine-point hedonic scale was used to rate the sensory attributes. A nine-point hedonic scale was used to rank the sensory qualities (9 being very liked, 8 being very much liked, 7 being moderately liked, 6 being like 1 slightly liked, 5 being neither like nor disliked, 4 being slightly disliked, 3 being disliked moderately, 2 being disliked very much, and 1 being terribly disliked) [17].

Statistical Analysis:

Statographics 2.1 was used to do both one-way and two-way ANOVAs (Analysis of Variance). An analysis of the mean difference was conducted using a Tukey HSD test ($p < 0.05$). For every sample, three duplicates were taken and examined.

3. RESULTS AND DISCUSSIONS

After being incubated at 30°C for 4 hours, the count of viable bacteria of freshly captured milk sharks that had been held at 0°C and at 30°C for varying times showed a steady increase, starting at 2.1x103 cfu/g and reaching 3.6x108 cfu/g. Nonetheless, the count of viable bacteria 1 decreased somewhat from 2±0.01 x10 cfu/g to 2.2±0.01 x10 cfu/g while being stored at 0°C (Figure 1). Notably, the viable 32 counts of bacteria in Milk Shark samples maintained at 30°C grew significantly ($p<0.05$), whereas the change in count in specimens kept at 0°C was negligible

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($p<0.05$). Because fish icing stops the expansion of mesophiles, the rate of count of microbe increase fell dramatically as temperature dropped from 28°C to 0°C [18].

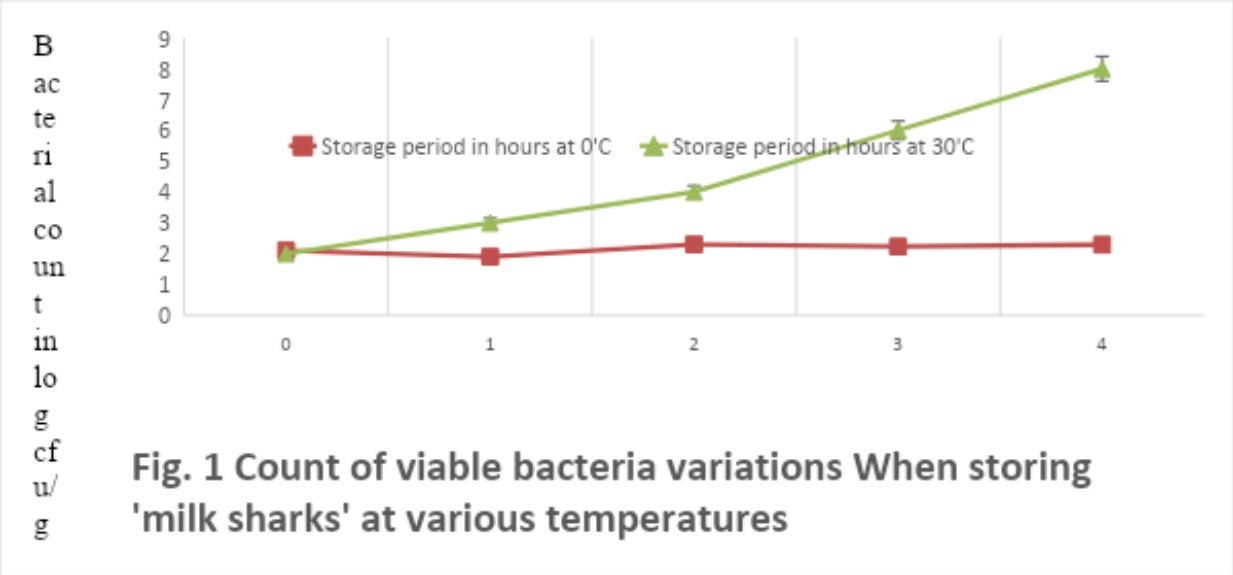

Fig. 1 Count of viable bacteria variations When storing 'milk sharks' at various temperatures

The urea content in recently caught fish dropped over the course of four hours from 9.36±0.05 mg/g to 3.8±0.04 mg/g while being stored at 30°C. In contrast, samples kept at 0°C saw a modest shift in urea content over the same period of time, going from 9.36±0.05 mg/g to 9.14±0.09 mg/g (Figure 2). Urea concentrations in Milk Shark samples kept at 30°C showed substantial increases ($p<0.05$), while samples kept at 0°C showed negligible changes ($p<0.05$) in urea concentration. The reason for this disparity is thought to be the bacteria breaking down urea as the fish degrades. [19].

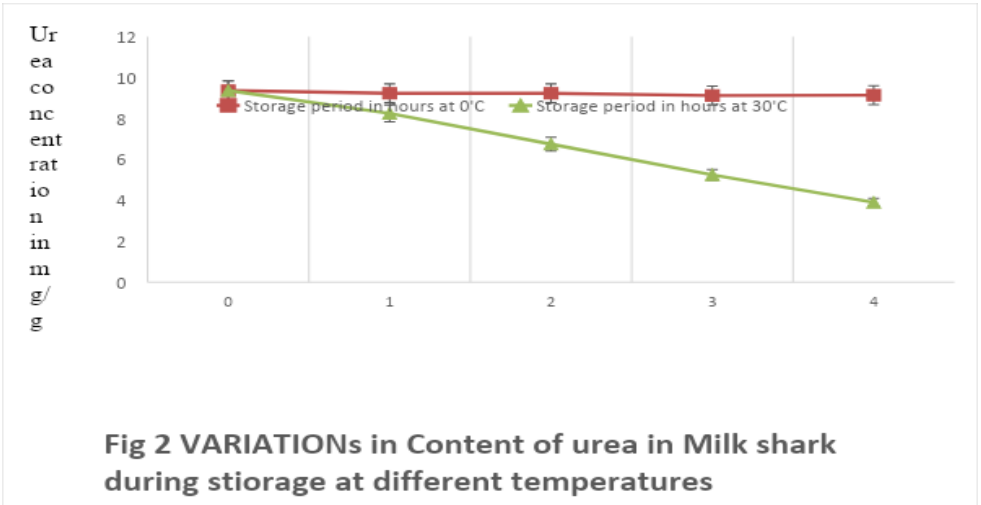

Fig 2 VARIATIONs in Content of urea in Milk shark during stiorage at different temperatures

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Fresh milk shark decomposes into carbon dioxide and ammonia at 30°C. Recently caught fish exhibits a progressive decline in ammonia content throughout this period, with a concentration of 5.10±0.05 mg/ml at the beginning and 96.3±0.06 mgN/100g within 4 hours (Fig 3). of contrast, during the same time period, the urea content of samples kept at 0°C marginally changes from an initial value of 5.36±0.05 mgN/100g to 5.29±0.08 mgN/100g. Urea concentrations in Milk Shark samples maintained at 30°C show considerable variations ($p<0.05$), while materials stored at 0°C show negligible changes ($p<0.05$) in urea concentration. This discrepancy is explained by the microorganisms that break down urea as the fish degrades.

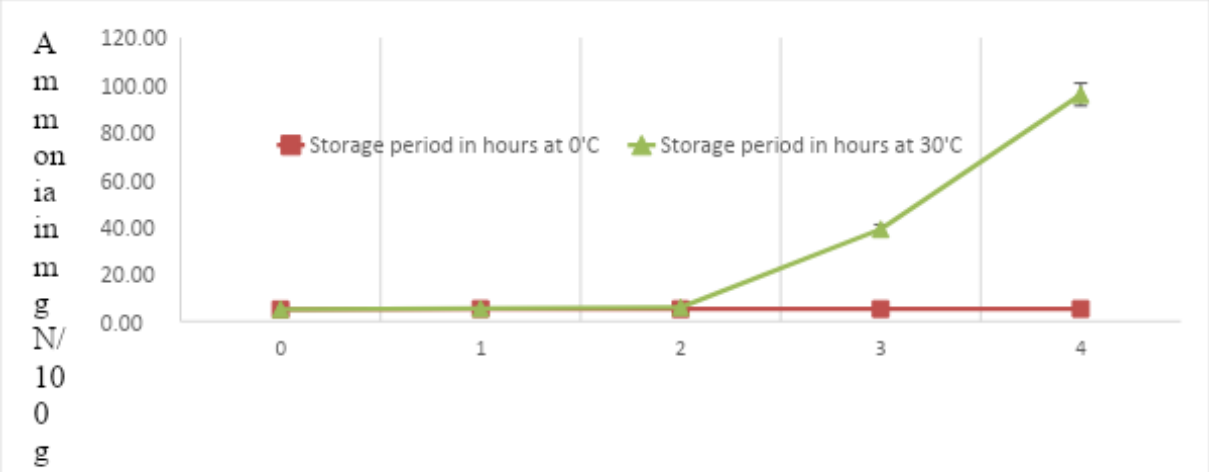

Fig 3 VARIATIONs in ammonia content in 'Milk shark' WHILE storage at different temperatures

AfterThe electrodes of the biosensor were stored at 30°C, the voltage across them was measured at 1, 2, 3, and 4 hours. The results showed that the voltage was 36±0.06, 46±0.05, 53±0.08, 69±0.09, and 91±0.06 mV, respectively. Conversely, over the same periodsof keeping at 0°C, the potentials measured 45±0.09, 49±0.08, 51±0.09, 52±0.09, and 54±0.00 mV, respectively. Potentials changed relatively little at 0°C ($p<0.05$) and dramatically rose at 30°C ($p<0.05$) across the biosensor electrodes. Bacterial urease transforms urea in milk sharks kept at 30°C into ammonia and carbon dioxide. Ammonia fumes give preserved Milk Shark flesh a distinct flavor. The biosensor's electrode potentials are altered by this deteriorating process. The count of viable bacteria, urea concentration, ammonia liberation potentials, and sensory characteristics were compared and are shown in Table 1.

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Table 1: Relative examination of the spotted "Milk Shark"kept at 0°C and 30°C

Storage time in hours	Storage temperature	Hedonic scale	Hedonic scale
0	30 °C	8.0±0.09	Clear skin, firm texture, crisp scent, grayish hue. translucent mucus
	0 °C	8.0±0.02	Clear skin, firm texture, crisp scent, grayish hue. translucent mucus
1	30 °C	7.0±0.07	Slightly lackluster skin, cloudy mucus, muted color, slightly soft texture, with a neutral to faint fishy aroma.
	0 °C	7.6±0.09	Clear skin, firm texture, crisp scent, grayish hue, translucent mucus
2	30 °C	6.0±0.03	Matte skin, tender texture, muted color, with a fishy aroma, milky mucus
	0 °C	7.5±0.07	The skin is vibrant, the mucus is clear, the texture is firm, the scent is fresh, and the color is grayish.
3	30 °C	5.0±0.04	The skin is dark, the mucus is yellowish, the texture is very soft, the color is dull, and there's a sour smell.
	0 °C	7.2±0.09	The skin appears somewhat dull, with opaque mucus and a slightly muted color, while the texture feels somewhat soft, and there's a normal to faint pungent smell.

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4	30 °C	3.9±0.06	The skin is very dark and chalky, with yellowish mucus, a soft texture, light color, and an ammoniacal smell.
	0 °C	7.1±0.06	The skin appears somewhat lackluster, with opaque mucus, a slightly muted color, soft texture, and a neutral to faint fishy smell.

Fish was deemed unsuitable after 5 duration of storage at 30°C, exhibiting the skin is very dark and chalky, with yellowish mucus, a soft texture, light color, and an ammoniacal smell, scoring only 3.9±0.06 marks on the hedonic scale. However, after 5 duration of storage at 0°C, the ammonia concentration of 5.29±0.08 mgN/100g, the potential was 54±0.00 mV, and the fish received the maximum rating of 4±0.07 on the hedonic scale.

4. CONCLUSION:

Storing Milk shark at 0°C helps prevent the breakdown of urea into carbon dioxide and ammonia. Conversely, storing them at 30°C leads to increased ammonia release, which correlates with rising potentials across biosensors during storage. The ammonia liberation is strongly linked to the decline in sensory qualities, as reflected in hedonic scale scores. deteriorating sensory attributes at 30°C significantly impact the freshness compared to storage at 0°C. Thus, the urease-immobilized biosensor proves to be an effective tool for assessing Milk Shark freshness.

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