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Non-invasive urease-biosensor to monitor freshness of spotted catfish, (*Arius maculatus*)

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ABSTRACT

Spotted catfish is highly perishable due to the liberation of ammonia due to the degradation of urea by bacterial enzyme urease, hence maintenance and monitoring of urease degradation is of utmost importance in fish freshness. We have developed a urease biosensor to assess the quality of spotted catfish by immobilizing urease on a pH electrode using sodium alginate and calcium chloride solution. Enzymatic degradation of the urea into ammonia by immobilised urease results in change in the potentials across glass electrode that can be measure by a potentiometric transducer. Spotted catfish stored at 30 °C showed a linear relationship between the degree of urea, liberation of the ammonia, deterioration of the freshness and the development of potentials across the urease biosensor. Hence, urease biosensor is a reliable, simple, and rapid method for the measurement of freshness of the fish flesh with high urea.

KEYWORDS

Spotted catfish;
Arius maculates;
Urease;
Biosensor;
Enzyme-immobilisation;
Potentiometric transducer.

INTRODUCTION

According to Marine Produts Export Development Authority, Cochin, Indian seafood exports during 2010-2011 is aggregated to 6,78,436 tonnes in volume and 2.13 billion US Dollar in value. Even though demand for Indian marine items is increasing and fish resources is depleting due to post-harvest handling losses remain at a staggering 25 % of the total catch[1]. Deteriorative changes in the organoleptic characteristics of the fish due to improper handling and processing are associated with the freshness[2]. Timely inputs from objective non-sensory and subjective sensory evaluation are crucial to maintain freshness of the highly perishable fish products starting from fish catch to the final consumption[3]. Presently available objective non-sensory evaluation methods are time consuming, cumbersome, developed in temperate regions and gives no direct clue to the sensory attributes of the Indian fish that are crucial for consumer acceptability. The loss of freshness of fish depends on several intrinsic and extrinsic factors such as the nature of the fish, spawning, feeding habits, temperature of the water, method of catch, handling, and storage conditions[4]. The cartilaginous fishes like sharks, skates, catfish and rays have very high urea content in their blood and tissues as part of their osmoregulatory physiology[5]. Once these fish dies urea breaks down into ammonia

and carbon dioxide that imparts a strong smell and odor to the meat, and may be toxic at high concentrations. Hence it is required to maintain the acceptable quality of these fish by a precise and rapid quality evaluation tool. Biosensors can be used to rapidly assess changes in fish quality, and significant developments in this field started since 1980s[6]. Measurement of enzymatic conversion of the analyte such as urea using biosensor of immobilised urease into another component such as ammonia can easily be measured, quantified, and displayed in a user-friendly way[7]. Urease can be immobilized using sodium alginate on the pH probes using alginate gel entrapment method. When the enzymatic reaction results in a change in pH, a pH electrode can be used as a potentiometric transducer and are used in applied research for nearly fifty years[8]. We have made an effort to develop urease biosensor that is based on the action of urease on urea liberating ammonia that in turn changes the potentials of the transducer for the evaluation of urea content in catfish (*Pungasius pungasius*) fish at the landing centre so as to overcome the difficulties of delayed laboratory analysis of fish samples which might take days.

MATERIALS AND METHOD

Enzyme preparation

Spotted Catfish (*Arius maculatus*) caught using trawl nets from the Arabian Sea were obtained from the fishing boats landed in 'Bunder area', Mangalore between September and November month. The time elapsed between catching and landing may not exceed over four to five hours. A 10 kg portion of the freshly caught catfish belongs to size group of 18-24 cm long; each weighing around 175-260g was selected for the present study. The material was brought in an insulated container after adequately icing them in the proportion of 1:1 fish to ice, to the laboratory within two hours.

Enzyme and chemicals

Standard buffer tablets were obtained from Qualigense Fine Chemicals, Mumbai, Urease tablets were obtained from Sigma Chemicals Co., and other chemicals and ingredients were of analytical grade and were manufactured by Merck Ltd., Mumbai, India.

Entrapment of urease on pH probe and urease assay

Sodium alginate and calcium chloride solutions were used to prepare alginate gel for immobilization of urease. Aliquot amounts of sodium alginate were mixed with 100mL of crude enzyme solution and 0.5ml bovine serum albumin solution is added. Standardized glass pH electrode using standard pH buffer solutions of pH 4, 7, 9[11] (REFERENCE). Electrode was dipped in the mixture of sodium alginate and urease and constantly stirred for 1 min, Glass electrode is then dipped into 100 ml solution of excess of 0.2M $CaCl_2$, and was allowed to cure for 30 min. A layer of calcium alginate with immobilized enzymes was formed around the bulb of pH electrode that in turn coupled to a pH meter for potentiometric measurement, known as biosensor[9]. The main plug of the pH meter is inserted in 230V AC outlet and the mains are switched on. Both free and immobilised urease activity was determined by the amount of ammonia liberated during a fixed time period at a saturating concentration of urea was determined using prepared and calibrated Nessler's reagent[10, 11]. The yellow colored produced is measured at 405 nm, using a blank to calibrate, which includes all ingredients included in test solution but excluding the enzyme. Urease activity is expressed in unit which is defined as the amount of urease required to liberate 1 μmol of ammonia in 1 min at pH 7.3 and 30 °C while using a magnetic stirrer.

Evaluation of the biosensor

Potentiometric measurement of urease activity was made using biosensor by immersing and incubating the bulb in a beaker consisting of 0.05 M Tris acetate working buffer of pH 7 at 30°C with constant stirring at moderate speed. When the electrode potential across the two leads of the biosensor reaches a stable value, aliquot amount of urea at 20, 40, 60, 80 and 100 mg/ dL was used for calibration and plotting graph. Microbiological methods Glassware and prepared media were sterilized at 121 °C for 15 minutes. Petri dishes, homogenizers, pipettes were sterilized at 180 °C for 1 hour. Viable bacterial count was determined as per APHA method[12].

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Evaluation of fresh fish sample

Fresh catfish sample was chosen for our analysis and electrical potentials of fish sample after every 1 hour of incubation at 0 ^{O}C and at 30 ^{O}C were noted up to 4 hours. Physical characteristics of the fresh fish samples were noted in relation to appearance, color, odor and texture. Sensory properties were evaluated using a nine point hedonic scale (9=like extremely, 8=like very much, 7=like moderately, 6=like slightly, 5= neither like nor dislike, 4=dislike slightly, 3= dislike moderately, 2= dislike very much and 1=dislike extremely)[13].

Statistical analysis

One and two way ANOVA (Analysis of Variance) was performed using statographics 2.1 (STSC Inc. Rockville, MD, USA). The difference in means was analyzed using a Tukey HSD test ($p<0.05$). Samples were drawn and assayed in triplicates.

RESULTS AND DISCUSSIONS

When freshly caught catfish was stored at 0 ^{O}C and at 30 ^{O}C for various intervals of time viable bacterial count increase steadily from the initial viable count of $2x10^3$ cfu/g to $3x10^8$ cfu/g at the end 4 hours of incubation at 30 ^{O}C. However during the storage of freshly caught catfish at 0 ^{O}C viable bacterial count changed from $2\pm0.02 \times 10^3$ cfu/g to $2.3\pm0.01 \times 10^2$ cfu/g, only (Figure 1). Viable bacterial count in catfish stored at 30 ^{O}C increased significantly ($p<0.05$) sharply but change in viable bacterial count was insignificant ($p<0.05$) in samples stored at 0 ^{o}C. Rate of increase of microbial count reduced drastically as the temperature reduced from 28 to 0 ^{o}C as icing of fish inhibits the growth of mesophiles[14].

During the storage of spotted cat fish at 30 ^{o}C urea content in the freshly caught fish decreased slowly from the initial concentration of 4 ± 0.05mg/ml to 1.8 ± 0.04 mg/ml by four hours. But on the other hand in those samples stored at 0 ^{o}C urea content changed slightly from the initial concentration of 4 ± 0.05mg/ml to 3.6 ± 0.05 mg/ml by four hours (Figure 1). Here changes in the urea concentration in catfish stored at 30 ^{O}C was significant ($p<0.05$) but change in urea concentration was insignificant ($p<0.05$) in samples stored at 0 ^{o}C. This is because as fish dies urea breaks down by bac-

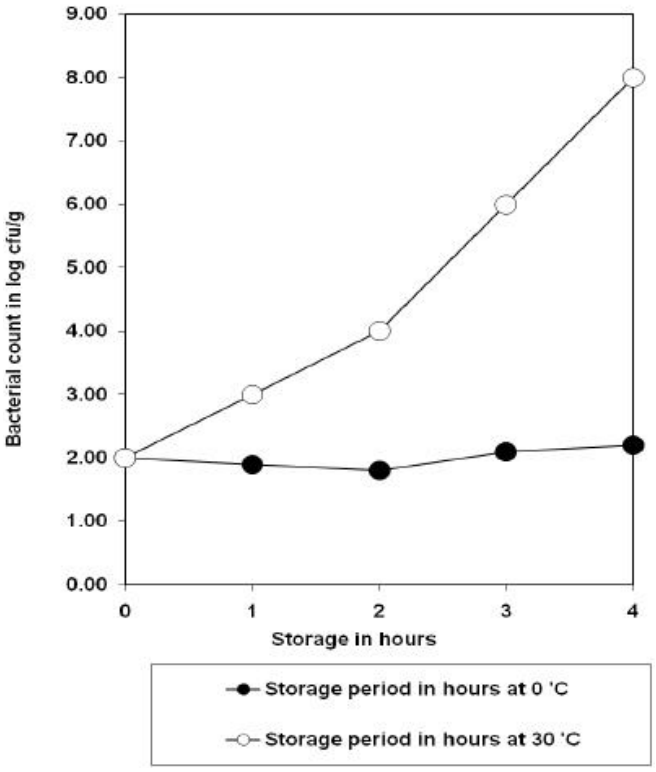

Figure 1 : Changes in viable bacterial count in spotted catfish (Arius maculates) during storage at various tempratures

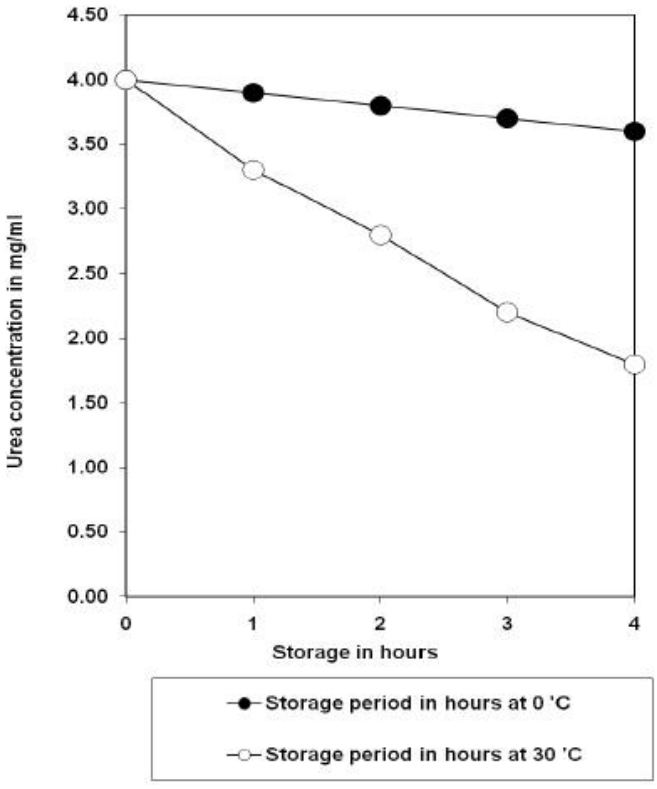

Figure 2 : Changes in urea in spotted catfish (Arius maculatus during storage at various temperatures

TABLE 1 : Comparative study of the spotted catfish stored at 0 °C and 30 °C

Storage period in hours	Storage temperature	Hedonic scale	Sensory characteristics
0	30 °C	8±0.07	Bright skin, transparent mucus, firm texture, fresh odour, grayish colour.
	0 °C	8±0.05	Bright skin, transparent mucus, firm texture, fresh odour, grayish colour.
1	30 °C	7±0.06	Somewhat dull skin, opaque mucus, somewhat dull colour, somewhat soft texture, neutral to slight fishy smell.
	0 °C	7.6±0.04	Bright skin, transparent mucus, firm texture, fresh odour, grayish colour
2	30 °C	6±0.08	Dull skin, milky mucus, soft texture, dull colour, fishy smell.
	0 °C	7.5±0.02	Bright skin, transparent mucus, firm texture, fresh odour, grayish colour
3	30 °C	5±0.05	Dark skin, yellowish mucus, very soft texture, dull colour, sour smell.
	0 °C	7.2±0.08	Somewhat dull skin, opaque mucus, somewhat dull colour, somewhat soft texture, neutral to slight fishy smell.
4	30 °C	4±0.07	Very dark chalky skin, yellowish mucus, very soft texture, dull colour, ammoniacal smell.
	0 °C	7±0.06	Somewhat dull skin, opaque mucus, somewhat dull colour, somewhat soft texture, neutral to slight fishy smell.

terial urease resulting in the reduction of urea concentration at ambient temperature but at 0 °C as spoilage in tropical and subtropical regions are by mesophiles[15].

Similarly at 30 °C freshly caught spotted cat fish into ammonia and carbon dioxide. Potential across the biosensor electrodes during storage at 30 °C was 35±0.06, 41±0.05, 45±0.08, 64±0.09 and 80±0.06 mV during 0, 1, 2, 3, 4 hours of storage, respectively. However during storage at 0 °C was 35±0.08, 36±0.09, 38±0.06, 40±0.06 and 42±0.07 mV during 0, 1, 2, 3, 4 hours of storage, respectively (Figure 3).

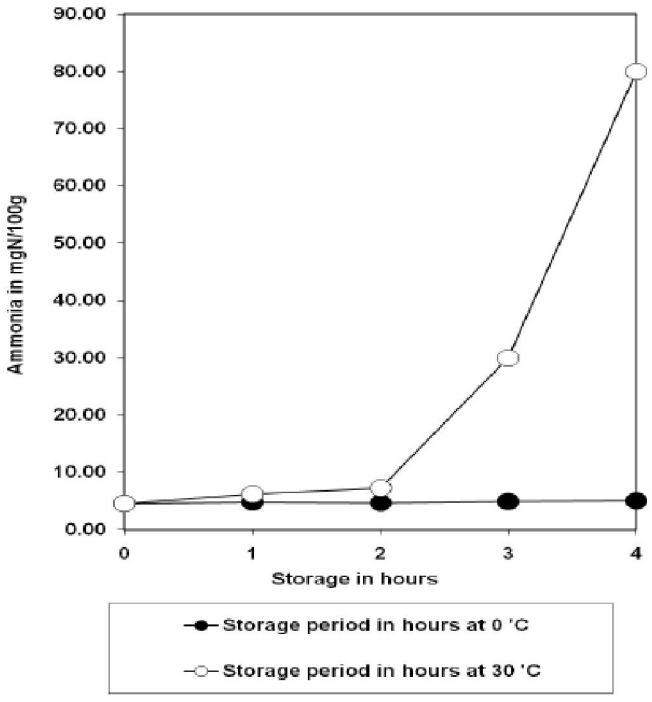

Figure 3 : Changes in ammonia in spotted catfish (Arius maculates) during storage at various tempratures

There was a significant ($p<0.05$) increase in the potentials across electrode of the biosensor, but change in potentials at 0 °C was insignificant ($p<0.05$). In spotted catfish stored at 30 °C bacterial urease breaks down urea into ammonia and carbon dioxide, and the ammonia liberated during this degradation imparts a strong smell and odor to the stored catfish meat. Ammonia liberated during this degradative process in spotted catfish is responsible for changing the potentials across the electrode of the biosensor.

A comparative study was carried out to inter relate viable bacterial count, urea concentration, potentials across the electrodes due to ammonia liberation, and sensory characteristics and presented in the TABLE 1. Fish was found unsuitable at the end of the four hours of storage at 30 °C due to the Very dark chalky skin, yellowish mucus, very soft texture, dull colour, ammoniacal smell and scored only 4 marks on hedonic scale. Panelists disliked the fish at the end of 4 hours of storage at 30 °C and associated urea concentration was 1.8±0.04 mg/ml and potentials were 80±0.06 mV. But even after fours of storage at 0 °C urea concentration was 3.6±0.05 mg/ml and potential was 42±0.07mV, and scored highest score of 4±0.07.

CONCLUSION

Storage of spotted catfish at 0 °C reduces the degradation of the urea into ammonium and carbon dioxide. Spotted catfish stored at 30 °C showed increased liberation of ammonia that in turn showed linear rela-

tionship between the increases in the potentials across the biosensors during the storage. Liberation of the ammonium during the storage as strongly associated with the reduction in the sensory characteristics of the stored spotted catfish as indicated by the score obtained on hedonic scale. Deteriorating sensory characteristics of the spotted cat fish has a direct bearing on the freshness of the spotted cat fish stored at 30 °C compared to the fish stored at 0 °C. Hence urease immobilised biosensor is a effective tool in determining the freshness of the spotted cat fish.

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