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A novel colorimetric food package label for fish spoilage based on polyaniline film

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ABSTRACT

A novel colorimetric method based on polyaniline (PANI) film for the development of smart packaging, as a chemical sensor for real-time monitoring of the microbial breakdown products in the headspace of packaged fish is described. This on-package indicator contains PANI film, that responds through visible color change to a variety of basic volatile amines (specifically known as total volatile basic nitrogen (TVBN)) released during fish spoilage period. The PANI film characteristics and its response to TVBN were studied. A kinetic approach was used to correlate the ammonia response of the PANI film to that of the fish spoilage. Color changes, in terms of total color difference of PANI, correlated well with TVBN levels of fish. Apart from TVBN, trials on milkfish sample (*Chanos chanos*) have verified that the PANI film response also correlates well with microbial growth patterns in fish samples, especially the changing microbial populations (total viable count (TVC) and *Pseudomonas* spp.). These responses enabled the real-time monitoring of fish spoilage either at various constant temperatures or with temperature fluctuations. The PANI film can be recycled several times using an acid solution to regenerate the PANI surface. Thus, PANI film can be considered as a low-cost sensor suitable for smart packaging applications.

1. Introduction

Microbial growth in food products results in a shelf life reduction of the foods and an increase in the risk of food-borne diseases. Such microbial growth in the foods is a driving force for innovation in monitoring of food products to ensure their quality and safety. Currently, food traceability is the legal requirement, especially in the EU (European Parliament and of the Council, 2002). This establishes a chain of responsibility throughout the entire food supply chain. Therefore, there is a great interest among the food industry, retailers, consumers, and their stakeholders in developing device that is simple, low-cost, rapid, reliable, non-invasive and non-destructive to evaluate real-time freshness of food products. An alternative concept to address this requirement is the development of smart packaging in the form of a food spoilage indicator to monitor freshness status of food products. Furthermore, the development of a food freshness indicator, based on detection of chemical changes associated with microbial growth in food products can offer an alternative to sensory and microbiological analyses which are often costly and time consuming (Dainty, 1996; Gram & Huss, 1996).

In the case of fish products, the number of microorganisms on the skin and gill surfaces, known as specific spoilage organisms (SSO), after death increases gradually and spreads within the various tissues (Hamada-Sato, Kazushige, Kobayashi, Imada, & Watanabe, 2005). These microorganisms are usually *Pseudomonas* spp. (Gram & Huss, 1996) and they have been used successfully for shelf life prediction of aerobically stored fresh fish (Koutsoumanis, 2001). Volatile amines such as trimethylamine, dimethylamine and NH_3 (ammonia) are products of microbial degradation and are collectively known as total volatile basic nitrogen (TVBN). Hence, TVBN levels are a potential indicator of fish spoilage. However, the standard method for determination of TVBN levels in tissue samples needs laborious extraction, sample destruction and time consuming (Pacquit et al., 2007).

Smart or intelligent packaging can be defined as a system that monitors the condition of the packaged food to provide information about the quality during storage, transportation and distribution (Yam, Takhistov, & Miltz, 2005). By simple definition, smart packaging is packaging which senses and informs the condition of the product to the consumers. As part of smart packaging, a fish freshness indicator is a packaging system (or material) which uses metabolites as "information" to monitor the status of fish freshness that can be directly related to fish spoilage. Currently, the amount of published work on fish freshness or fish spoilage indicators is still limited. Some publications have constructed indicators for the volatile compounds

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produced in microbial spoilage. For instance, Diamond's group (Byrne, Lau, & Diamond, 2002; Pacquit et al., 2006, 2007) developed a colorimetric dye-based sensor and indicator for monitoring fish spoilage on the basis of the presence of total volatile basic nitrogen (TVBN). This on-package sensor contains a pH sensitive dye, bromocresol green, entrapped within a polymer matrix. However, leaching of the dye may occur over time that resulted in inaccurate responses or false-positive indications as fish freshness sensor. Consequently, the occurrence of dye leaching needs to be investigated when this sensor system is used. In addition, since the pH dye was used, it responses to the pH change is affected by temperature, particularly when it is used in a frozen condition.

As such there is a need to explore alternative methods for detection of fish freshness using indicator/sensor that is free from leaching, accurate, simple, low-cost, rapid, reliable, consumer-friendly and non-invasive to evaluate the real-time fish freshness. One of the excellent candidates is thin films of polyaniline. Polyaniline (PANI) is a polymer that changes conductivity and color with change in pH as a result of changes in the degree of protonation of the polymer backbone, making it useful as a leach-free visual sensor for volatile bases such as TVBN. PANI film itself acts both as a matrix support and as the indicator, compatible with solid state instrument, it can be easily fabricated (Clark & Maher, 2009; Kaner & Huang, 2007, chap. 7). Furthermore, PANI film as indicator could be cheaply produced using inkjet printers (Crowley et al., 2008; Morrin et al., 2008).

In this paper, the use of PANI films as non-invasive fish freshness sensor enabled the detection of TVBN inside sealed fish packages. In this study, milkfish (*Chanos chanos*) was used, since it is an important aquacultured fish commonly found in the Indo-Pacific region, particularly Indonesia, Malaysia, Philippines, and Taiwan (Chen, 1990). Furthermore, unlike other fish (such as mackerel, tuna, skipjack and sailfish), milkfish is usually consumed within hours of harvest in Indonesia and Malaysia, and available all year round. By using PANI film as TVBN sensor, this visual sensor works based on pH increase as the basic spoilage volatiles amines produced gradually in the package headspace, and subsequently the color of the sensor will change from green to blue, which is easily visible to the naked eye.

2. Experimental

2.1. Reagents and solutions

Aniline were obtained from Sigma (AR-grade, UK), ammonium peroxodisulphate were obtained from BDH (PA grade, UK). Solutions of these compounds were prepared by dissolving the corresponding quantity of the reagent in distilled water. A stock ammonium solution was prepared by dissolving ammonium chloride (Sigma, UK) in distilled water. Diluted solutions were prepared daily from stock solution in distilled water. A wide range of buffer solution was prepared (Perrin & Dempsey, 1974) containing 0.1 M citric acid, 0.1 M potassium dihydrogenphosphate, 0.1 M sodium tetraborate, 0.1 tris(hydroxymethyl)-aminomethane and 0.1 M potassium chloride. This solution (50 mL) was diluted to 200 mL with water, and 0.4 M sodium hydroxide or 0.4 M hydrochloride acid solution was used to adjust pH to the desired value over the pH range 4–10. Optically clear polystyrene sheet (3M, USA) and polyamide laminated with linear low-density polyethylene (nylon/LLDPE, 80 μm grade) films were obtained from Carrefour, Indonesia.

2.2. PANI film preparation

Aniline was purified by distillation (184 °C, 5 h) under vacuum with vigorous stirring to prevent bumping. A PANI dispersion as nanofibre was prepared using the methods described by Huang and Kaner (2006). The purified aniline (3.2 mmol or 0.3 g) was mixed with 10 mL of 1 M HCl acid solution. Ammonium peroxydisulfate (0.8 mmol or 0.18 g) was mixed into another 10 mL aliquot of the acid solution. The aniline-acid solution was added to the oxidant and the two solutions were rapidly mixed for 30 s and then allowed to react undisturbed overnight. The following day, the polyaniline was washed with distilled water and centrifuged at 5000 rpm for 30 min. After three washings, the supernatant liquor had a pH of 3.3 and was strongly green in color, indicating the presence of polyaniline particles. Before casting, any remaining particles larger than 1 μm were removed by passing the dispersion through a 55-mm glass fiber filter (Whatman GFA, UK) under vacuum. The PANI dispersion was cast directly on a polystyrene substrate. Then, the thin film of PANI on the polystyrene sheet were left overnight in the dark to dry, after which individual 5 mm diameter discs were punched out with a desk hole puncher. The ready to use film was then stored at 4 °C until used.

The optically clear polystyrene sheet allowed absorbance measurements to be made for the sensor response. Absorbance measurements were performed using a spectrophotometer (Hitachi, U-1800) and a glass cuvette of 1 cm path length. The pH was monitored with a commercial glass electrode and a pH-meter (Hanna Instruments 9318) calibrated at the pH values of 4.00, 7.00 and 9.00. The film thickness was determined by SEM (Scanning Electron Microscopy) (Phenom, FEI, USA) images to be 0.7 μm. Though the film thickness was not routinely determined for all film samples, it may be concluded from their absorbance spectrum that the thickness was always within in the same order of magnitude. Thus, the method for PANI film fabrication has shown good reproducibility.

2.3. PANI films response to ammonia vapor

Response of PANI films toward volatile ammonia in term of their color change was performed using absorbance measurements. Variations of absorbance in the PANI films were determined by placing the film (1 cm × 5 cm) at 1 cm above the solution container for 20 min and then the absorbance was measured spectrophotometrically at the wavelength of 610 nm. All data were collected 60 s later, in order to obtain reproducible and comparable measurements.

2.4. Fish spoilage trial

Freshly caught whole milkfish (*Chanos chanos*) were cleaned (skin cleaned without innards and its main bone, but with its head and tail as their usually sell in the market) at a local fish pond (Jember, Indonesia) and placed in a sealed ice container. Aseptic techniques (e.g. the use of disposable gloves, bactericide built-in cutting board and flame sterilized scalpel) were used to avoid sample contamination. An amount of 500 g of milkfish samples were aseptically placed into sterilized 1000 mL Erlenmeyer flasks. A PANI film as an indicator label was enclosed in each gas-tight flask with 5 cm above the fish samples. The samples were periodically analyzed for product quality in terms of basic species and pH values during storage. Samples were at no time in direct contact with the PANI films (i.e. only headspace was sampled). Reference flasks did not contain any fish sample was left over a period of 56 h. The color change of the PANI films measured instrumentally using spectrophotometer for their absorbance values to describe the color change of PANI films. Here, PANI films displayed a color change from green to blue, which were easily visible to the naked eye. Films not exposed to spoiled fish samples were used as reference films for these measurements. In addition, a kinetic approach was carried out to allow the correlation of the response of the PANI film to the freshness of milkfish during storage at room temperatures (25 °C).

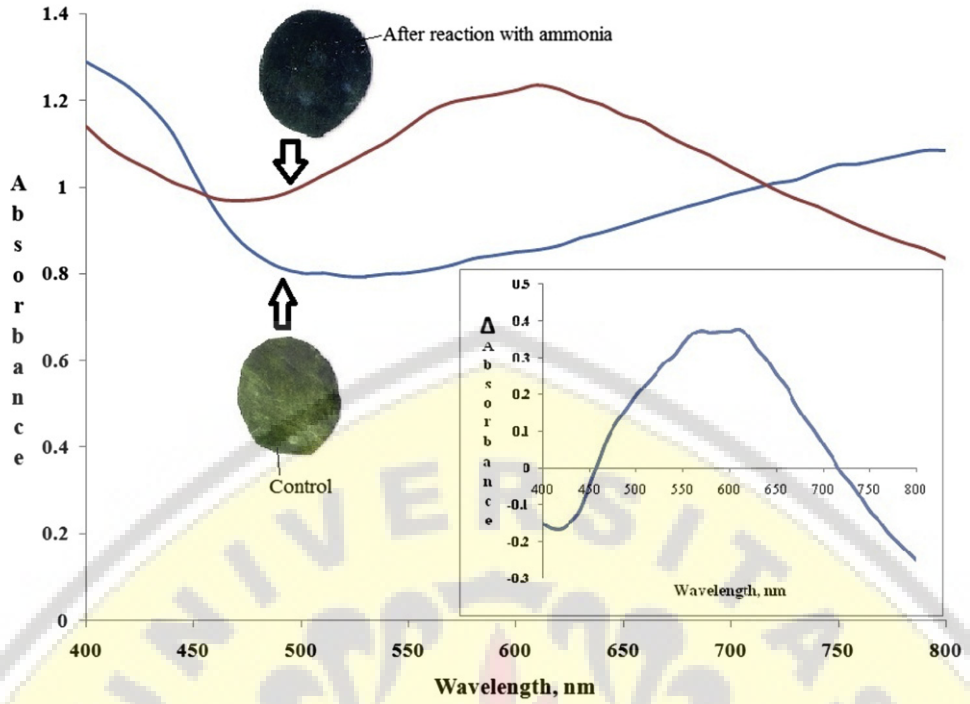

Fig. 1. The PANI film response toward ammonia vapor of 0.8 M ammonia solution, where absorbance maximum was found at 610 nm (see inset).

2.5. Volatile amine measurement

The total volatile basic nitrogen (TVBN) in term of its volatile amine from the milkfish samples (5 g) were partly dissolve and dissociate in the aqueous phase. An ion selective electrode for ammonium ions (NH_4^+-ISE) (Hanna Instrument, USA) was used to measure NH_4^+ ions content in the water and their pH values using pH electrode (Hanna Instruments 9318, USA). The output signals from this electrode were then translated into product quality variations in relation to time and temperature. The deterioration in the quality via NH_4^+ measurements in the fish sample was performed simultaneously with the PANI film measurements.

2.6. Microbial analysis

During the fish spoilage trial, 7 samples of approximately 5 g of fish tissues were removed from the same milkfish samples, under the same aseptic conditions, and placed in zip lock freezer bags. They were also allowed to deteriorate at room temperature (25 °C) and every 2 h, along with sensor response measurement, a sample was transferred into a freezer. Samples were in a frozen state in less than 1 h. TVC (total viable count) determined using the pour method on plate count agar (Oxoid CM463) while the spread plate method was used on agar base (Oxoid CM733) with CFC (cetrimide fucidin cephalosporin) selective supplement (Oxoid SR103) to give *Pseudomonas* counts. Plates were counted after 48 h of incubation at 30 °C and results were correlated with the sensor response. Colonies were counted and reported as log CFU (colony forming units) g^{-1}.

3. Results and discussion

3.1. Sensor response to volatile ammonia

The first attempt to determine volatile ammonia was made based on the PANI films in non-invasive mode. Fig. 1 shows the PANI film response toward ammonia vapor, where absorbance maximum was found at 610 nm. The response time of the films toward ammonia vapor (from 0.8 M ammonia solution) was reasonably good within 5 min as given in Fig. 2. The response time of the sensor was found to depend on the establishment of sample steady-state condition in the container and the relative humidity (as the protonation–deprotonation process occurs in PANI film), which requires the presence of an effective proton transport medium to ensure efficient shuttling of protons from the green protonated emeraldine (proton donors) to blue emeraldine base (proton acceptors). However, in a sealed package environment, both of these factors are relatively constant and therefore will have little influence on the films. Therefore, this response time was used for further measurements toward ammonia vapor.

When the absorbance variations with the ammonia concentration were measured, a linear range up to 0.16 M was found (Fig. 3).

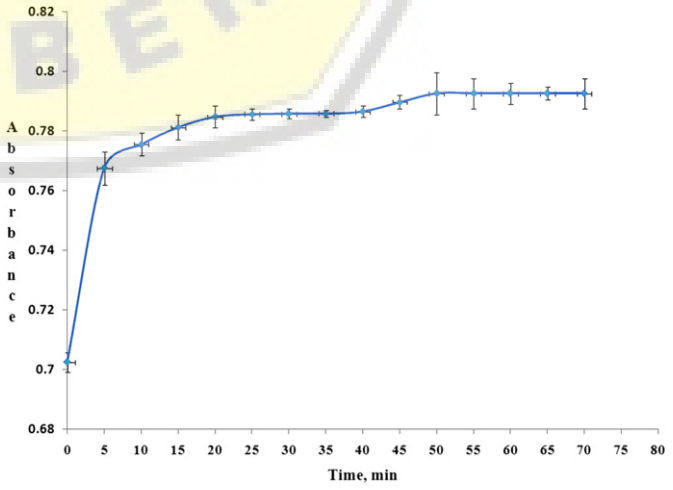

Fig. 2. The response time of the films toward ammonia vapor (from 0.8 M ammonia solution).

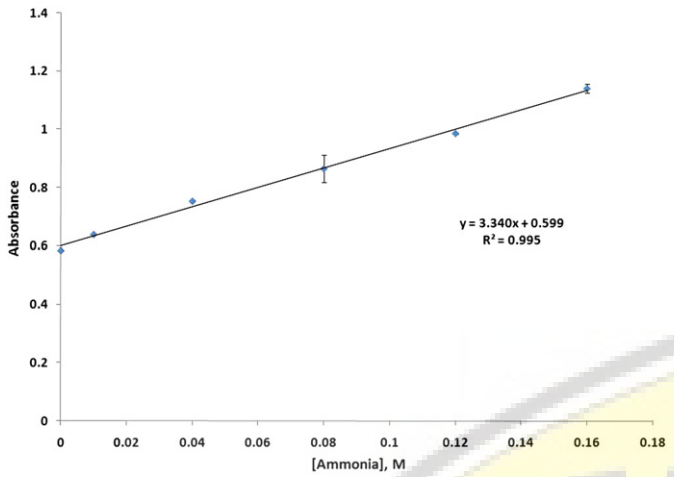

Fig. 3. The absorbance variations with the ammonia vapor (from 0.0 to 0.16 M ammonia solution, $N = 6$).

The detection limit (3σ) was found to be 0.01 M. In this case, no reversibility of the film was observed. Films can be regenerated fully when washed with dilute hydrochloric acid solution (0.1 M) for 10 min. Reproducibility of the PANI films for ammonia vapor determination, expressed as RSD was 0.12% ($n = 6$) using the same films and 5.2% ($n = 6$) using different films.

3.2. Fish spoilage trial

All PANI films were placed in close proximity (5 cm) with the tissue samples in order to enable the film to response to the increasing volatile basic amines generated by spoilage with a very distinct color change from green to blue. PANI films were monitored every 2 h until no further color change was observed. Fig. 4 shows the rate of change in color of PANI film toward spoiling milkfish at room temperature. Prior to the first 6 h no drastic color change was detected by the spectrophotometer at room temperature. Thereafter, the PANI film steadily changed color from green to blue approximately after 8 h at room temperature. Furthermore, the spectrophotometer did not detect differences in signal steady-state changes between these PANI films of different batch preparation. However, the fresh PANI films provided a more intense color change to the eye than the old PANI film allowing a better visualization of the occurrence of spoilage. Therefore, the PANI films were kept fresh in chiller condition for further studies. The onset of spoilage was detected in the region of 12 h after milkfish samples were kept at room temperature (Fig. 4). This indicated that the milkfish samples released volatile amines at a relatively slow rate, since its freshness lasted longer within 10–12 h.

3.3. Ammonium ion analysis of milkfish samples

TVBN or the total volatile basic amine content in the fish was predicted using the NH_4^+-ions selective electrode (ISE) along with PANI film response. This is due to the fact that fish (and other fresh foods) packed under modified atmosphere with increased CO_2 content were able to prolong the shelf life. However, CO_2 will dissolve and dissociate in the aqueous phase in the package and decrease the pH of the water. This CO_2 will interfere the conductivity measurement, but has no influence to ammonium ions measurement using the NH4+-ISE. Thus, NH_4^+-ISE is used to measure volatile amines dissolve in the water. The results for this measurement have been given in Fig. 5 along with PANI film response at room temperature. It can be seen that the PANI film response follow similar trend as shown by NH4+-ISE response at room temperature, along with their pH values as given in Fig. 5.

3.4. Microbial analysis of milkfish samples

The TVC counts slowly increased from about 2.9×10^5 cfu/g at 10 h but rose sharply till 24 h of investigation at approximately

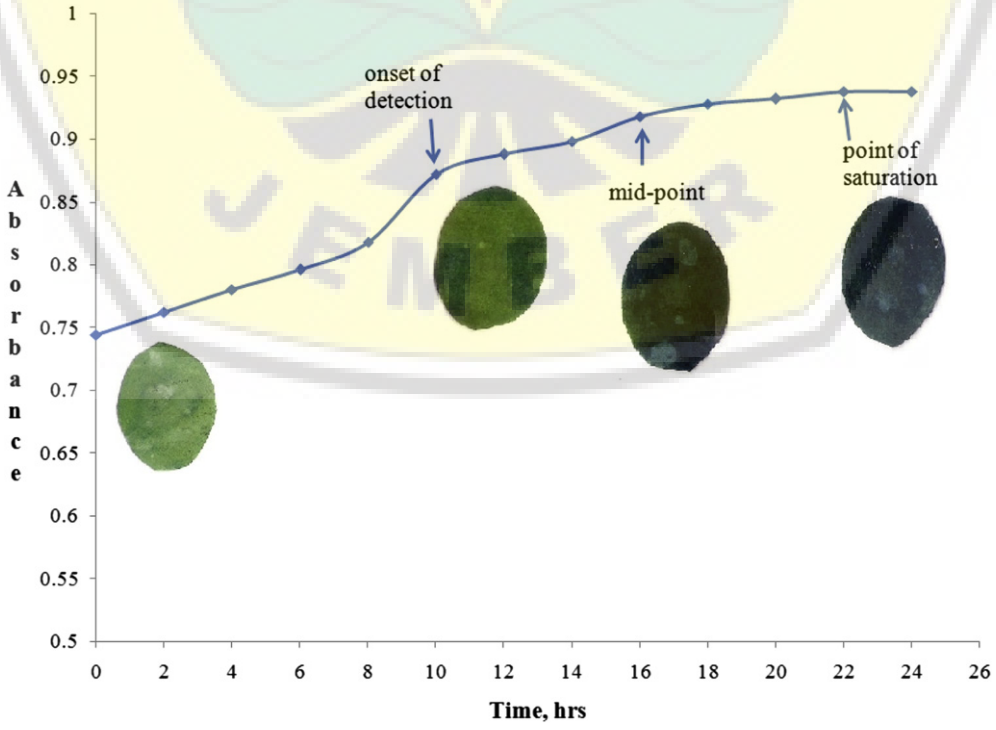

Fig. 4. The change rate in color of PANI film toward spoiling milkfish at room temperature (25° C) (the onset of spoilage was detected in the region of 12 h).

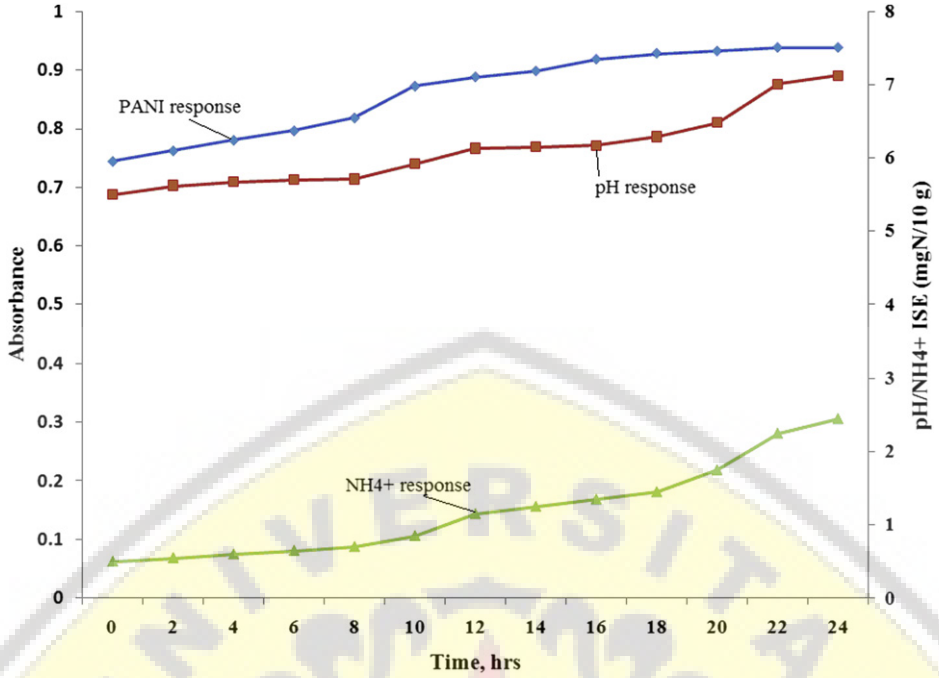

Fig. 5. The NH_4^+-ISE responses toward volatile amine dissolve in the water, the pH values of fish samples and the PANI films response at room temperature (25 °C).

3.9×10^7 cfu/g at room temperature (Fig. 6). Initially, the *Pseudomonas* counts were at approximately 85% of the TVC counts rising to approximately 100% at around 14 h at room temperature. Similarly to the TVC counts, the *Pseudomonas* counts, they increase sharply in 24 h of investigation with ca 3.5×10^7 at 25 °C (room temperature) for fish storage. When compared to the PANI film response in the same figure, it can clearly be seen that not only does the film response correlate with the changes in both bacterial populations (TVC and *Pseudomonas* in tissue) but the onset of film color change also correlates with the level of product rejection (5×10^5 cfu/g), since this value was used in Indonesia for aquacultured fish generally (Badan Standardisasi Nasional, 2006). These levels were reached after about 12 h in our experiments (Fig. 6). However, Olafsdottir et al. (1997) and Koutsoumanis (2001) both reported TVC and

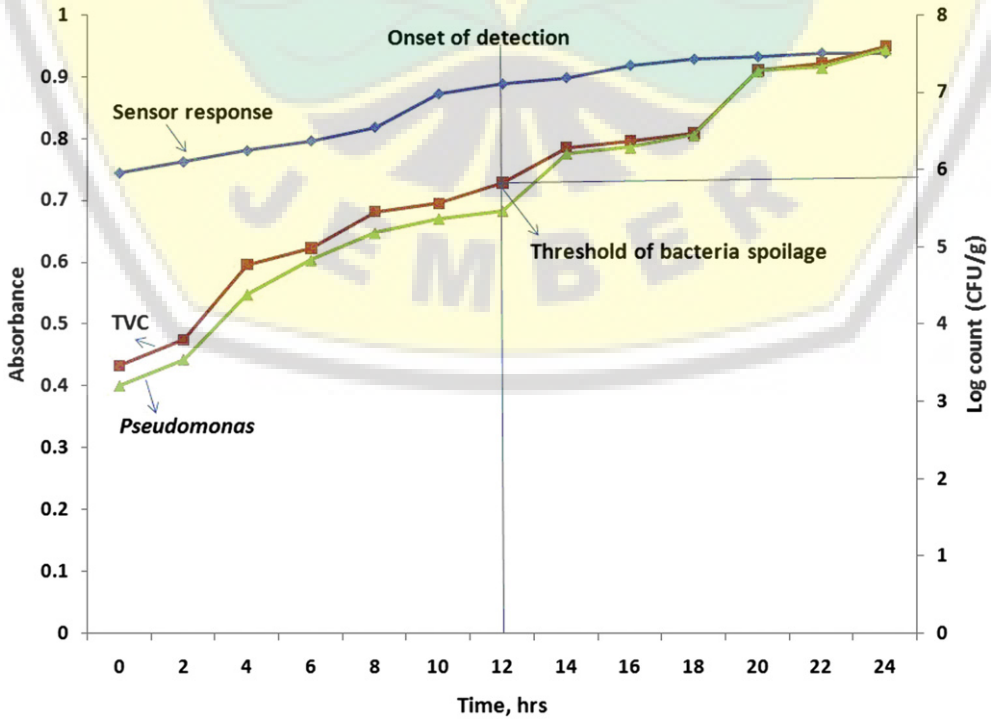

Fig. 6. The TVC and *Pseudomonas* values of fish samples and the PANI films response at room temperature (25° C).

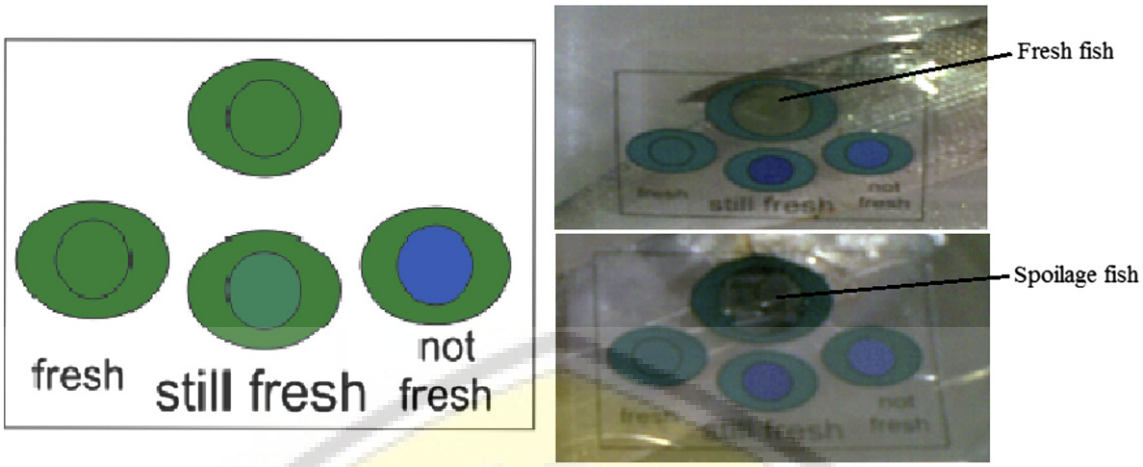

Fig. 7. Sensor design (left) and its application as smart packaging for milkfish (right).

Pseudomonas values of 10^7 cfu/g for fresh fish samples to reach end of shelf life, which were reached after about 18 h in our study. A delay between the rise in microbial population and the film response curves is also apparent from Fig. 6. This is due to the fact that the volatile base generation follows the increase in microbial population inherently. This phenomenon (a delay between rises in microbial population versus appearance of the chemical spoilage markers, including TVBN) has been previously observed by other (Hamada-Sato et al., 2005). Thus, the films accurately response to the increase in volatile base concentration in the package headspace, since the range of film color change related to the higher levels of microbial population in the fish tissue. In addition, the visual color changes of on-package films are useful indicators of approximate microbial population and therefore spoilage of the fish samples. However, it is also important to state that the observed color change of the film was always lags slightly behind the rise in microbial population, which means that the false positives cannot occur in this case. Consequently, false negatives may happen during this period before the TVBN levels have risen in the headspace, since the film color change is just beginning to happen. Thus, this method can be used to indicate the presence of high microbial populations in packaged fish and should not be consumed.

4. Conclusions

We have developed a simple colorimetric sensor as an inexpensive visual indicator that enables to monitor the condition of a fish product in terms of its freshness using PANI films. The film response was found to correlate with bacterial growth patterns in fish samples, which can be employed for the "real-time" monitoring of spoilage in the packaged fish products. The sensor is sensitive to volatile amines, with a linear response to ammonia vapor of ammonia solution up to 0.16 M. Currently; further development of PANI films is being investigated for low-cost production, e.g. integration of PANI deposition with commercial screen printing and packaging technology. The advantages of this smart packaging could reduce margins of error and waste, due to the product have a longer effective shelf life by allowing freshness to be measured visually along with accurate estimation of the best-before date (Fig. 7). Furthermore, since the safety of product guarantee is of primary importance for the consumers, therefore this smart packaging is certainly of interest to manufacturers and retailers, as the products must be disposed, when they are not fresh or not suitable to be consumed anymore.

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