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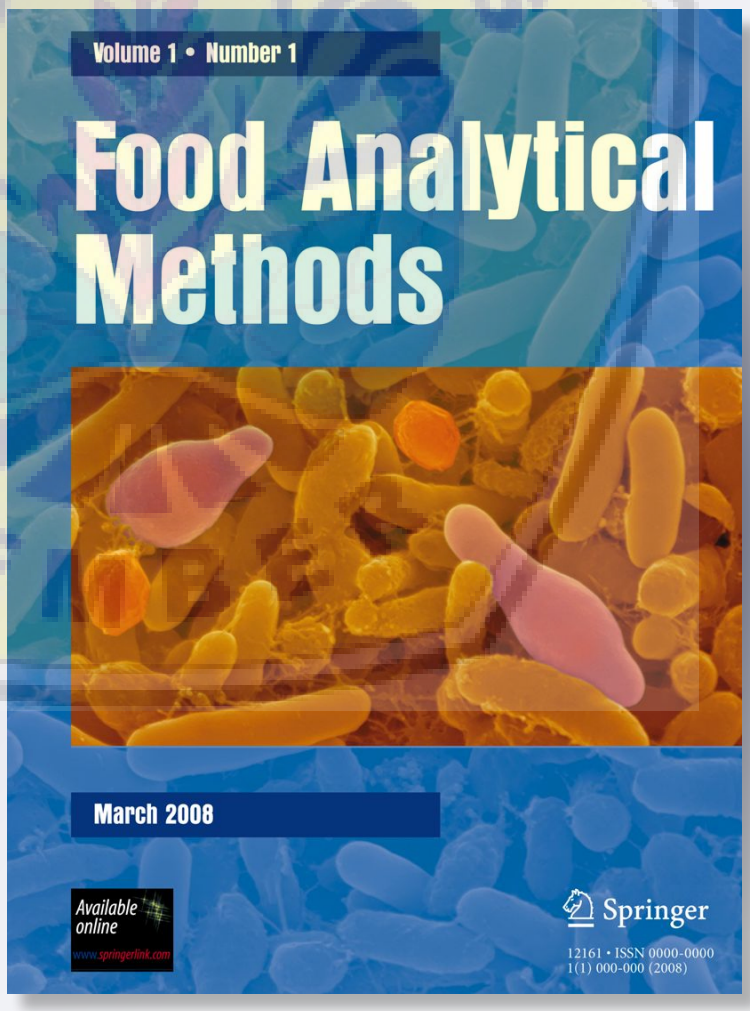
Food Analytical Methods

ISSN 1936-9751
Volume 5
Number 4

Food Anal. Methods (2012) 5:881-889
DOI 10.1007/s12161-011-9326-x



Springer

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Real-Time Monitoring of Shrimp Spoilage Using On-Package Sticker Sensor Based on Natural Dye of Curcumin

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Received: 13 August 2011 / Accepted: 20 October 2011 / Published online: 10 November 2011

Abstract The development of a curcumin-based sensor for the detection of volatile amines (specifically known as total volatile basic nitrogen, TVBN) is described. Curcumin [(1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5dione] is the major yellow pigment extracted from turmeric, a commonly used spice, derived from the rhizome of the plant *Curcuma longa*. Curcumin was immobilized onto bacterial cellulose membrane via the absorption method. Thus, the sensing materials are edible and suitable for food applications. The curcumin/bacterial cellulose membrane as the TVBN sensor worked based on pH increase as the basic spoilage volatile amines produced gradually in the package headspace, and subsequently, the color of the sensor will change from yellow to orange, then to reddish orange for spoilage indication, which is easily visible to the naked eye. The curcumin membrane is a highly sensitive material toward acid–base reactions. Color changes, as a result of its interactions with increasing pH (as a result of increasing TVBN), were monitored directly with visual inspection and the color quantitatively measured with color analysis via Photoshop software. Furthermore, the membrane response was found to correlate with bacterial growth patterns in shrimp samples. Finally, the curcumin/bacterial cellulose membrane was successfully used as a sticker sensor for real-time monitoring of shrimp spoilage in ambient and chiller conditions.

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Keywords Real-time monitoring of shrimp spoilage · Ammonia chemical sensor · TVBN · Curcumin · Bacterial cellulose

Introduction

Freshness is essential for the quality of all kinds of fish and fishery products. Freshness can be described to some extent by some objective sensory, microbial, (bio)chemical, and physical parameters, as an objective attribute (Howgate et al. 1992). Currently, the freshness of fish is judged by trained assessors by evaluating such freshness attributes, for instance odor, texture, and color, while characteristic sensory changes of the aforementioned attributes occur when fish spoil or deteriorate (Olafsdottir et al. 1997; Olafsdottir and Kristbergsson 2006).

To date, sensory evaluation is the most important method for freshness evaluation of fishery products. Other approaches based on microbial methods (total viable count, TVC); physical measurements (e.g., odor, texture, and color); and chemical methods (e.g., K and K_1 values, total volatile basic nitrogen (TVBN), lipid oxidation) have also been described by Olafsdottir et al. (1997) in an excellent review of these methods. Olafsdottir et al. (2004) also proposed a method using a multi-sensor device with a combination of instrumental techniques (electronic noses, spectroscopic methods, texture meters, image analyzers, color meters, and devices measuring electrical properties) to measure and/or estimate fish freshness.

Microorganisms are the major cause of spoilage of most seafood products. However, only a few members of the microbial community, the specific spoilage organisms, give rise to the offensive off-flavors associated with seafood spoilage (Olafsdottir et al. 2004; Hamada-Sato et al. 2005).

Volatile compounds such as ammonia, dimethylamine, and trimethylamine, which are known as TVBN, are products of microbial degradation and are considered as potential indicators of fish spoilage (index of freshness). Generally, TVBN levels rise due to the formation of NH_3 and other volatile amines. In some fish products that do not contain trimethyl amine *N* oxide (TMAO) or where spoilage is due to a non-TMAO-reducing flora, a slow rise in TVBN is seen during storage, probably due to the deamination of amino acids (Huss 1995).

The reference method for the determination of TVBN, as adopted by the European community, involves a laborious extraction, e.g., steam distillation and subsequent titration of the amines with hydrochloric acid (Commission Decision 95/149/EC of 8 March 1995), while other methods have also been proposed such as the photometric method with flow injection analysis (Ruiz-Capilla et al. 2000) and the chromatography method with solid-phase micro-extraction (Bene et al. 2001). Although these methods provide satisfactory results and utilize instrumentation with a high degree of automation, the need of laborious steps for the preparation of the sample, cost, and the lengthiness of volatile analysis methods make them suitable only for research and laboratory levels.

Recently, a direct method for the monitoring of fish spoilage, which is based on an immobilized pH-sensitive dye, bromocresol green, was also proposed as a fish spoilage indicator or sensor (Pacquit et al. 2006, 2007). The sensitive reagent in most fish spoilage indicators/sensors is chemical reagent dye. In this direction, for consumer safety purposes, the indicators/sensors should be clearly labeled "Do Not Consume," although the amount of reagent dye used is typically much lower than that of a lethal dose for humans. However, in order to avoid possible ingestion of the indicator, which can be mistaken as a condiment despite its "Do Not Consume" warning, such indicators/sensors could also be fabricated from a natural dye or an edible material which is very safe if in contact with the food or even unintentionally ingested.

Here, we present the construction of a TVBN sensor fabricated by the absorption method of curcumin onto bacterial cellulose membrane (also called nata de coco). All the sensing materials are edible materials. Curcumin (*Curcuma longa*) is extensively used as a spice, food preservative, and coloring material in India, China, Indonesia, Malaysia, and other countries in Southeast Asia (Chattopadhyay et al. 2004), while bacterial cellulose has found a multitude of applications in paper, textile, and food industries and as a biomaterial in cosmetics and medicine (Iguchi et al. 2000).

The curcumin/bacterial cellulose membrane as a TVBN sensor worked based on pH increase as the basic spoilage volatile amines produced gradually in the package headspace, and subsequently, the color of the sensor will change from yellow to orange, then reddish orange for spoilage indication, which is easily visible to the naked eye. The curcumin membrane is a highly sensitive material toward acid–base reactions. Color changes, as a result of its interactions with pH due to an increase in TVBN, were monitored directly with visual inspection and the color quantitatively measured after scanning with color analysis via Photoshop software. Furthermore, the membrane response was found to correlate with bacterial growth patterns in shrimp samples. The performance of this sticker sensor was successfully tested directly for real-time monitoring of shrimp spoilage in ambient and chiller conditions.

Material and Methods

Chemicals

All chemicals used were of reagent grade (supplied by Merck, Sigma, or Fluka) and used as supplied.

Curcumin Extraction

Dried powder (50 g, Aneka Kimia, Indonesia) of curcumin was extracted in a Soxhlet apparatus with 500 mL of 95% ethanol. The Soxhelation process was carried out until the solvent was found to be colorless. The dark brown ethanolic extract was then filtered and concentrated using a rotary evaporator. The dried ethanolic extract was stored for further use. A stock solution of curcumin was prepared by dissolving 10 mg of dried ethanolic extract in 10 mL of ethanol (50%) to give a concentration of 1 mg/mL.

Preparation of Bacterial Cellulose Membrane

Acetobacter xylinum culture (from the Pure Culture Collection of the Chemistry Department, University of Jember) was cultivated in stationary conditions using a Herstin–Schramm nutrient (HS) medium composed of glucose (2%, *w*/*v*), yeast extract (0.5%, *w*/*v*), bacto-pepton (0.5%, *w*/*v*), citric acid (0.115%, *w*/*v*), Na_2HPO_4 (0.27%, *w*/*v*), $MgSO_4{\cdot}7H_2O$ (0.05%, *w*/*v*), and ethanol (1 vol.%) added after sterilization of the base (Ciechańska et al. 1998; Surma-Ślusarska et al. 2008).

Conic flasks (300 cm^3) were used, filled with an HS medium. The bacterial breeding process was conducted within 7 days at 30 °C, grafting inoculums of approx. 4 wt.% in relation to the medium prepared. In the process of bacterial cellulose biosynthesis, glucose as well as arabinose, mannose, galactose, xylose, and mannitol were used as carbon sources. The membrane of bacterial cellulose obtained was then treated with NaOH (a concentration of approx. 5%, for 60 min., temperature=100 °C) in order to remove bacterial cells and substrate from the inner layers of

the bacterial cellulose film. Then, it was rinsed with tap water until a neutral condition (around pH 7.0) was achieved.

For membrane sheet preparation, the bacterial cellulose (10 g) was blended until homogeneous, then cast onto the glass plate, and pressed. Afterward, the membrane was left overnight (12 h) and the membrane sheet dried at 60 °C. The membrane sheet was stored for further use.

Immobilization of Curcumin on Bacterial Cellulose Membrane

Curcumin was immobilized on bacterial cellulose membrane by the absorption method. This procedure was carried out simply by immersing the membrane sheet into 10 mL of a stock solution of curcumin (1 mg/mL) overnight (12 h) at ambient temperature. Then, the curcumin/bacterial cellulose membrane was washed with tap water to remove unbound curcumin with the membrane. The curcumin/bacterial cellulose membrane was dried using an electrical drier. Afterward, the curcumin/bacterial cellulose membrane was cut at a desired shape according to the design of the on-package sticker sensor (Fig. 1).

Preparations of the Shrimp Samples and Storage Conditions

Two kilograms of shrimp (*Litopenaeus vannamei*, with each shrimp having an average weight of 5 g and length of 120 mm) was obtained directly from the farm in Situbondo (30 km from Jember). The shrimp were slaughtered by immersing in ice-cold water. The whole shrimp were brought to the laboratory in isothermal containers and were packed and stored into insulated polystyrene boxes with flaked ice. These boxes were provided with an outlet for water drainage and are exactly the same ones used in the process of marketing of the shrimp; these were stored in a refrigerator maintained at 4 °C.

An amount of 100 g of shrimp samples was aseptically placed into sterilized 500-mL Erlenmeyer flasks. The curcumin/bacterial cellulose membrane as a sticker sensor was enclosed in each gas-tight flask. The samples were periodically analyzed for product quality in terms of TVBN pH values and microbial degradation during storage. Samples were at no time in direct contact with the curcumin/bacterial cellulose membrane (i.e., only headspace was sampled). The references used for this measurement were the flasks without shrimp sample with the membranes on the top of the flask. The color change of the curcumin/bacterial cellulose membrane was scanned and measured using color analysis (Photoshop) for their color values. Here, curcumin/bacterial cellulose membranes displayed a color change from yellow to orange and then to reddish orange, which was easily visible to the naked eye.

The shrimp were divided into two sets. The first set of samples was stored in ambient condition (~28 °C), removed, and analyzed at every 2 h until 24 h. The second set of samples was removed from the refrigerator every day until day 10 of storage time and analyzed for a chiller condition (4 °C). The shrimp were prepared for analysis at each time point, up to day 10. Packing was realized in similar conditions for all sets of samples.

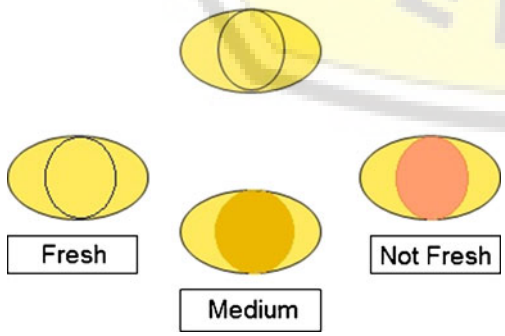

Fig. 1 Design of on-package sticker sensor with color indication for fresh, medium/still fresh (need to be consumed in hours), and not fresh (spoiled, do not consume)

Chemical Analysis

Perchloric acid (PCA) extract of the fish was prepared and analyzed for TVBN levels using the EC reference (EU 1995). All the shrimp were washed thoroughly with tap water. The shrimp from one side was skinned aseptically and minced by passing three times through a meat grinder with 4-mm holes. Of the shrimp, 10 g was blended with 90 mL of 6% PCA. Of the filtrate, 50 mL was made alkaline with hydroxide 20% and distilled for 10 min in a 2100 Kjeltec Distillation Unit (FOSS Tecator AB). Each analysis was repeated twice.

pH Measurement

In order to measure the pH value, the shrimp samples (5 g) were partly dissolved and dissociated in the aqueous phase. The pH values of the shrimp sample were measured using a pH electrode (Hanna Instruments 9318) in triplicate. The pH values of this electrode were then translated into product quality variations in relation to time and temperature. Deterioration in the quality of the shrimp sample was performed via pH measurements simultaneously with the curcumin/bacterial cellulose membrane measurements as the sensor response.

Microbial Analysis

During the shrimp spoilage trial, seven samples of approximately 5 g of shrimp tissues were removed from the same

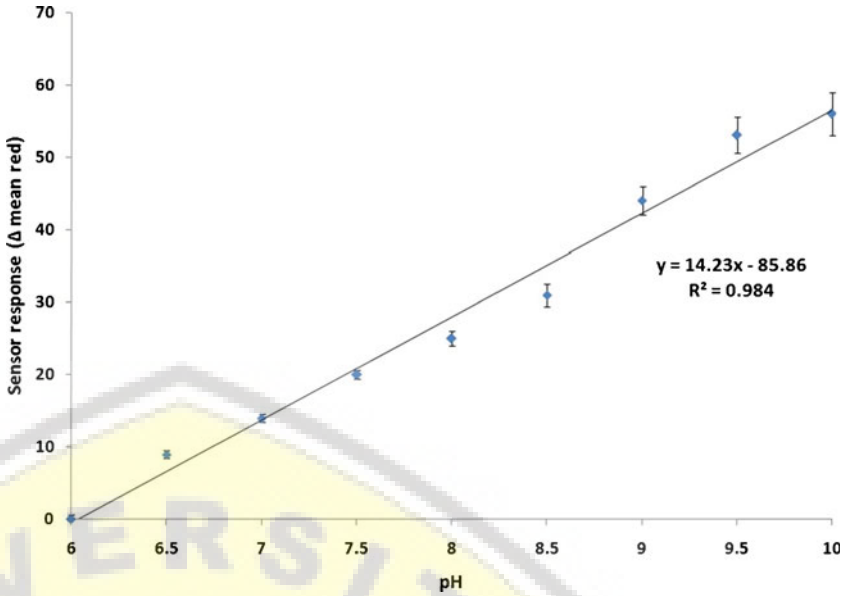

Fig. 2 Sensor response (Δ mean red) toward various pH values

shrimp samples, under the same aseptic conditions, and placed in Zip lock freezer bags. They were also allowed to deteriorate at and ambient condition (~28 °C), and every 2 h, along with the sensor response measurement, a sample was transferred into a freezer. Samples were in a frozen state in <1 h. TVC was determined using the pour plate method, on a plate count agar (Oxoid CM463), while the spread plate method was used on an agar base (Oxoid CM733) with a cetrimide fucidin cephalosporin selective supplement (Oxoid SR103) to give *Pseudomonas* counts. Plates were counted after 48 h of incubation at 30 °C and the results were correlated with the sensor response. Colonies were counted and reported as log colony forming units (cfu) per gram.

Color Analysis

This work presents a simple method that uses a digital scanner (Canon MP190, Tokyo, Japan) to measure color and the graphics software Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, CA) to analyze the color of the curcumin/bacterial cellulose membrane. The term "measure" means that the digital scanner is used to obtain the color values of the pixels on the membrane. The term "analyze" means that Photoshop is used to measure those color values to obtain color distribution, averages, and so on. In this digital imaging method, the required equipment and software costs are low, the experimental setup and operation are simple, and the

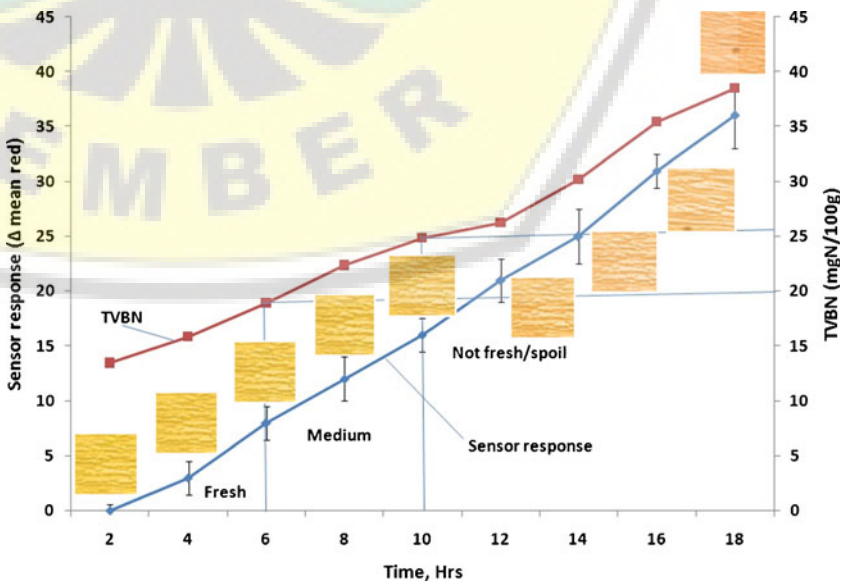

Fig. 3 TVBN values of shrimp samples and the sensor response (the rate of color changes of the membrane) at ambient condition

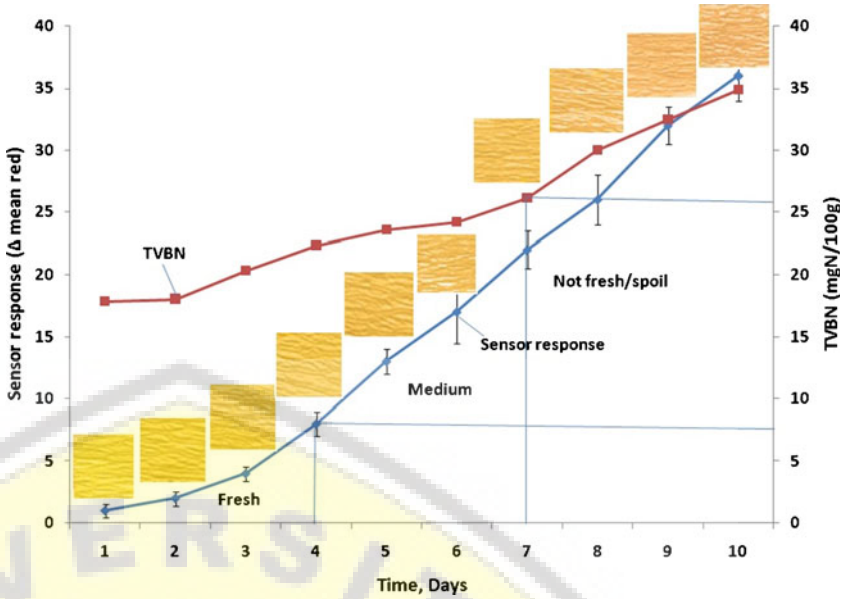

Fig. 4 TVBN values of shrimp samples and the sensor response (the rate of color changes of the membrane) at chiller condition

measurements and analysis are often adequately sophisticated for this measurement.

Here, we used the Histogram Window to determine the color value. The Histogram Window displays the statistics (mean, standard deviation, median, percentage, and so on) of the color value for a selected area in the membrane image. Hence, the average color of a membrane sample or any portion of it can be obtained easily using the Histogram Window; thus, mean red (or Δ mean red for change in color) was used as the color value for all membrane color measurements since the membrane color changed from yellow to orange and then to reddish orange. Using this method is more suitable and simpler in comparison with a visual inspection using the naked eye as the main purpose of this sticker sensor rather than using reflectance measurements (Byrne et al. 2002). The principles of color measurement can be found elsewhere (Hunt 1991; Francis 1994; Gunasekaran 1996; Yam and Papadakis 2004).

Results and Discussion

pH Sensing

Figure 2 shows the sensor response toward various pH values (6.0–10.0) at ambient conditions. As can be seen in Fig. 2, a linear relationship was obtained between the response (Δ mean red) and the concentration of the pH value (6–10) with a correlation coefficient, r=0.992 (n=9). Data were fitted in this range with the equation: color

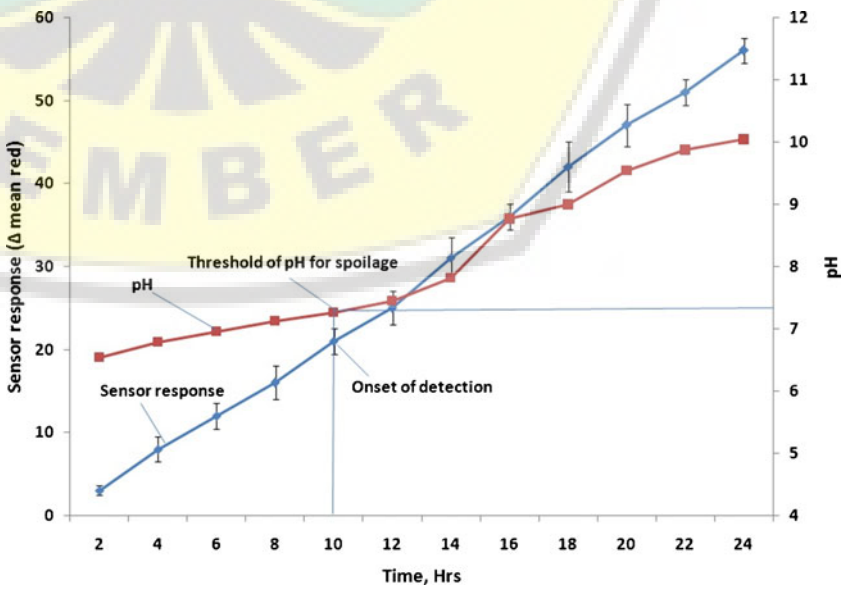

Fig. 5 pH values of shrimp samples and the sensor response at ambient condition

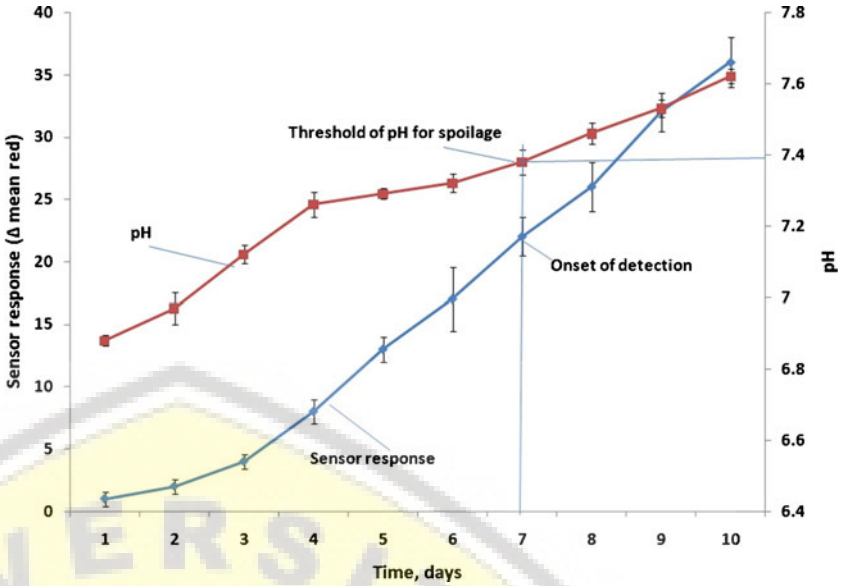

Fig. 6 pH values of shrimp samples and the sensor response at chiller condition

change of membrane as sensor response (Δ mean read)= 14.23(pH)−85.86. The reproducibility between different membranes was checked by comparing the responses of four different membranes for a pH value of 7, and it was found to be 0.23%. This deviation is satisfactory. Even though the membranes are intended to be used as disposable sticker sensors, their ability to be reused, after regeneration with 0.1 HCl solution, is an interesting feature.

Shrimp Spoilage Trial and TVBN Analysis

All curcumin/bacterial cellulose membranes were placed in close proximity to the tissue samples (with package volume around 500 mL) in order to enable the membrane to respond to the increase of TVBN generated by spoilage with a very distinct color change from yellow to orange and then to reddish orange. The membranes were monitored periodically until no further color change was observed. Figures 3 and 4 show the rate of color change of the membrane (as sensor response) toward spoiling shrimp along with the TVBN values at ambient and chiller conditions. It can be seen that the sensor response follow a similar trend as shown by TVBN analysis.

In Fig. 3, the sensor response increases steadily (as the membrane color changes gradually) within 24 h of experiment observed at an ambient condition. Here, the membrane gradually changed color from yellow to orange within 10 h and then changed from orange to reddish orange approximately after 10 h at an ambient condition. In a chiller condition, as shown in Fig. 4, prior to the first

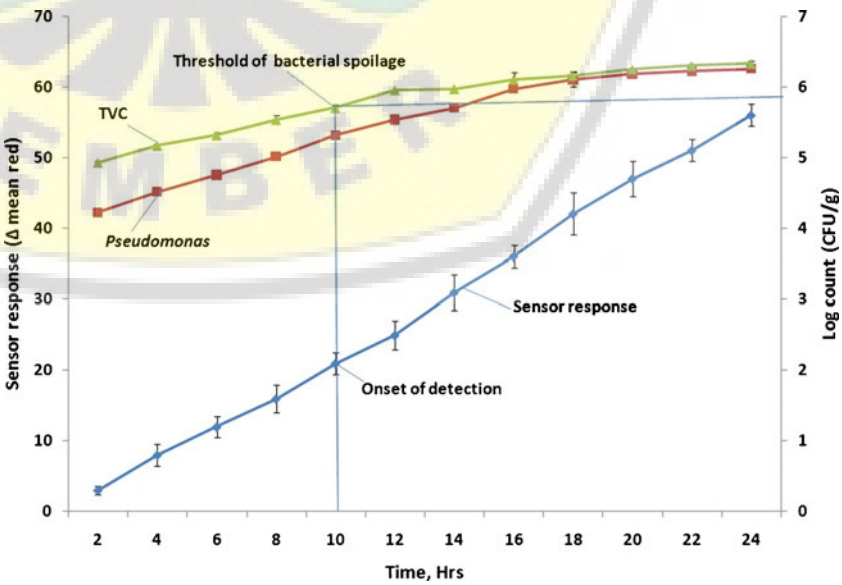

Fig. 7 TVC and *Pseudomonas* count of shrimp samples and the membrane response at ambient condition

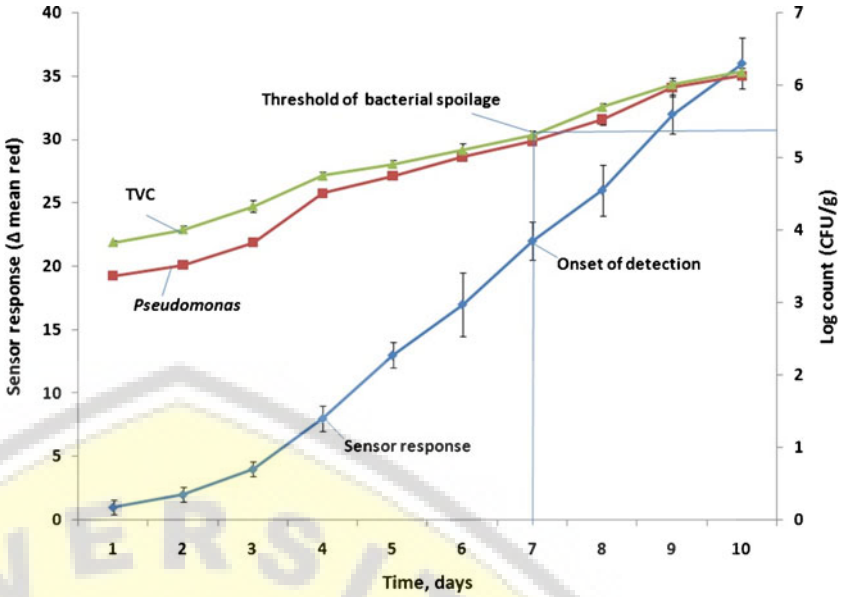

Fig. 8 TVC and *Pseudomonas* count of shrimp samples and the membrane response at chiller condition

3 days, no drastic color change was observed. Then, after 4 days, the membrane gradually changed to orange until 7 days of observation. In general, the membrane changed color from yellow to orange after 4 days and changed from orange to reddish orange after 7 days at a chiller condition. Furthermore, visual inspection did not detect differences in color changes between membranes of different batch preparations and samples. However, the fresh membranes revealed a more intense color change to the eye than did the old membrane, allowing a better visualization of the occurrence of spoilage. Therefore, the membranes were kept fresh in a chiller condition for further use. In addition, no effect of humidity on the sensor response in this study was observed.

The sensor accurately responded to the increase in volatile base concentration in the package headspace since the range of membrane color change was related to the levels of TVBN in the shrimp tissue. This response was observed regardless of the package volume as long as the shrimp sample occupied at least 30% of the package volume. Below this occupied volume, the sensor response time would be affected since less shrimp sample in the package would need more time to rise in the headspace compared with more shrimp samples. Consequently, false negatives may occur during this period before the TVBN levels have risen in the headspace since the sensor color change is just beginning to happen.

Levels of 30–35 mg/100 g flesh are generally regarded as the limit of acceptability for fish products (Connell 1995). However, Kyrana and Lougovois (2002) have established 25 mg/100 g as the value limit in this case. Therefore, according to the latter value, these levels were reached after 10 h and 7 days at ambient and chiller conditions, respectively. The onset of spoilage was detected after 10 h and 7 days for ambient and chiller conditions, respectively. This indicated that the shrimp samples released volatile amines at a relatively slow rate since its freshness lasted longer, within 10 h and 7 days for ambient and chiller conditions, respectively.

pH Analysis of Shrimp Samples

The results for this pH measurement have been given in Figs. 5 and 6 along with membrane/sensor responses at ambient and chiller conditions, respectively. The pH values of the shrimp samples were varied from pH 6.54 at the fresh stage to pH 10.04 at the spoilage stage after 24 h at an

Fig. 9 Application of curcumin/bacterial cellulose membrane as a sticker sensor for shrimp freshness

ambient condition (Fig. 5). The pH values of the shrimp samples were varied from pH 6.88 at day 1 to pH 7.53 after 10 days at a chiller condition. It can be seen from both figures (Figs. 5 and 6) that the sensor also follows a similar trend as shown by the pH response at both conditions. Furthermore, the sensor also responded to the increase in pH value in the package headspace since the range of membrane color change was related to the levels of the pH in the shrimp tissue.

Kyrana and Lougovois (2002) attributed the low levels of TVBN to the relatively low pH values and the composition of the microbial flora encountered during the trial. The optimum pH in this case has been reported to be 7.2–7.4 (Elliot 1952). This value has been reached after 10 h and 7 days of storage at ambient and chiller conditions, respectively. Thus, the membrane also gave an accurate indication of spoilage, which was also detected after 10 h and 7 days for ambient and chiller conditions, respectively.

Microbial Analysis of Shrimp Samples

The TVC counts steadily increased from 8.31×10^4 cfu/g during the initial 2 h and rose steadily up to 9.33×10^5 cfu/g at 16 h of investigation at the ambient condition (Fig. 7). Initially, the *Pseudomonas* counts were at approximately 75% of the TVC counts, rising to approximately 95% at around 16 h at room temperature. Here, the *Pseudomonas* counts increase sharply until 16 h of investigation, with approx. 6.72×10^6 cfu/g at room temperature. At the chiller condition, initially, the TVC counts were at 6.72×10^3 cfu/g at day 1 and rising to 5.01×10^5 cfu/g at day 7 at the chiller condition (Fig. 8). The *Pseudomonas* counts were at approximately 80% of the TVC counts, rising to approximately 97% at day 7 at the chiller condition. Then, they increase steadily, similar to the TVC count at the chiller condition. When compared with the sensor response in both figures, it can clearly be seen that not only does the sensor response correlate with the changes in bacterial populations but the membrane color change from orange to reddish orange also correlates with the level of product rejection (5×10^5 cfu/g) according to the TVC value used in Indonesia for fishery products (Badan Standardisasi 2006). These levels were reached after 10 h and 7 days at ambient and chiller conditions, respectively (Figs. 7 and 8). However, Olafsdottir et al. (1997) and Koutsoumanis (2001) both reported a TVC value of 10^7 cfu/g for fresh fish samples to reach the end of shelf life, which is higher compared with the Indonesian standard. Thus, the sensor also accurately responded to the increase in volatile base concentration in the package headspace since the range of membrane color change was related to the higher levels of microbial population in the shrimp tissue. In addition, the visual color changes of on-package membranes are useful indicators of approximate microbial population and, therefore, spoilage of the shrimp samples (Fig. 9). Finally, it can be clearly stated that this membrane can be used to indicate the presence of high microbial populations in packaged shrimp when the color of the membrane changed to reddish orange for a visual identification that the shrimp was spoiled and cannot be consumed anymore.

Conclusions

This work employs natural dyes of curcumin as chemical sensors for the detection of volatile inorganic and organic amines produced during bacterial growth in shrimp samples. Curcumin, the major yellow pigment extracted from turmeric, which was used as the natural sensing reagent, was immobilized on bacterial cellulose membrane (biopolymers) using absorption as a simple method that enables the mass production of low-cost sensors. Thus, all the sensing materials are edible and suitable for food applications. The curcumin/bacterial cellulose membrane was successfully used as an on-package sticker sensor for a visual detection of shrimp spoilage. The proposed sticker sensors could offer an extra analytical device to both the fish industries and retailers who are interested in approaches and devices that are safe, low-cost, simple, rapid, disposable, non-destructive, and with real-time applications.

Acknowledgment The authors gratefully thank the DP2M, Higher Education, Ministry of National Education, Republic of Indonesia, for supporting this work via the International Research Collaboration Program 2010 (Penelitian Kerjasama Luar Negeri dan Publikasi Internasional no. 438/SP2H/PP/DP2M/III/2010).

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