Sains Malaysiana 46(9)(2017): 1557–1563
http://dx.doi.org/10.17576/jsm-2017-4609-26

Acrylamide Optical Sensor Based on Hydrolysis Using *Bacillus* sp. Strain ZK34 Containing Amidase Properties

(Sensor Optik Akrilamida Berasaskan Hidrolisis Menggunakan *Bacillus* sp. Strain ZK34 yang Mengandungi Sifat Amidase)

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ABSTRACT

In this work, a new optical screening method for acrylamide was developed. Bacterial Bacillus *sp. strain ZK 34 was used to hydrolyse acrylamide to the corresponding acid and ammonia. Nessler's reagent was used to detect the produced ammonia and the yellow complex formed was treated as signal. Bacterial pellet was immobilised in the alginate membrane. The optimum composition of alginate used is 2%. The mass ratio of alginate:bacterial of 1:0.5 gave the optimum respond. Optimum concentration for NaOH and Nessler's reagent were 0.075 M and 2.5 mM, respectively. The yellow complex of mercury (II) amido-iodine formed was directly proportional to the concentrations of acrylamide up to 50.00 ppm with the limit of detection of 1.30 ppm. This sensor shows a good reproducibility which the relatives standard deviation (RSD) values from 3.17-6.15%. Therefore, the detection of acrylamide based on the amidase hydrolysis is suitable for screening this carcinogen compound.*

Keywords: Acrylamide; amidase; Nessler's reagent; optical detection

ABSTRAK

Dalam kajian ini, satu kaedah baharu untuk penyaringan akrilamida secara optik telah dibangunkan. Bakteria Bacillus *sp. strain ZK 34 telah digunakan untuk menghidrolisiskan akrilamida kepada asid yang sepadan dan amonia. Reagen Nessler telah digunakan untuk mengesan amonia yang terhasil dan pembentukan sebatian kuning diambil kira sebagai isyarat. Palet bakteria telah dipegunkan di dalam membran alginat. Komposisi alginat yang optimum digunakan ialah 2%. Nisbah jisim alginat:bakteria pada 1:0.5 memberi rangsangan yang optimum. Kepekatan NaOH dan reagen Nessler yang optimum masing-masing ialah 0.075 M dan 2.5 mM. Sebatian kuning iaitu raksa (II) amido-iodin yang terbentuk berkadar langsung dengan kepekatan akrilamida sehingga 50.00 ppm dengan had pengesanan 1.30 ppm. Sensor ini menunjukkan kebolehulangan yang baik iaitu sisihan paiwai relatif (RSD) daripada 3.17-6.15%. Maka pengesanan akrilamida berasaskan hidrolisis amidase adalah sesuai untuk penyaringan sebatian yang karsinogen ini.*

Kata kunci: Akrilamida; amidase; pengesanan optik; reagen Nessler

INTRODUCTION

Acrylamide is a compound that has been classified as 'probably carcinogenic to humans' by the International Agency for Research on cancer (IARC 1994). This compound was found in heat-treated carbohydrate rich food in the year 2002 by the Swedish National Food Authority and the University of Stockholm (Tareke et al. 2002). Although the mechanism of acrylamide formation in food is not fully understood, yet, some researchers claim that the acrylamide formed through the *Maillard* reaction where asparagine and reducing sugar were convert to acrylamide with the presence of heat (Zyzak et al. 2003). Some studies have shown that acrylamide are detected in food up to 4.2 ppm (Lineback et al. 2012). Beside the daily intake of meal which contains acrylamide, drinking water is another source for exposure acrylamide to human (Friedman 2003). As polyacrylamide is used for drinking water treatment and it will undergo depolymerisation to acrylamide under environmental conditions (Shukor et al. 2009). External source is also a potential way of acrylamide exposure to human since polyacrylamide is utilised in many applications such as cosmetic, contact lenses, soil treatment, recycling paper, and the textile industry (Kepekci Tekkeli et al. 2012).

Amidase or also known as acylamide amidohydralase (EC 3.5.1.4) is an enzyme that is capable of hydrolysing aliphatic amides to carboxylic acid and ammonia. This enzyme belongs to the hydrolases family and it hydrolyses carbon-nitrogen bonds rather than peptide bonds. Several strains from the genera *Pseudomonas*, *Rhodococcus*, *Burkholderia* and *Bacillus* have been isolated with acrylamide-degrading properties (Nawaz et al. 1994; Prabu & Thatheyus 2007; Shukor et al. 2009; Syed et al. 2012). Silva et al. (2011, 2009) developed biosensors for acrylamide detection using the whole cells of *Pseudomonas aeruginosa*. The amidase catalyses the hydrolysis process of acrylamide to produce ammonia and acrylic acid. Ion selective electrode was used to measure ammonia produced

as a biosensor signal. In addition, Ignatov et al. (1997) also developed a sensor to determine acrylamide based on microbial cells respiratory activity. From the studies, transformation of acrylamide to acrylic acid using amidase involves oxidation reaction. This reaction needs the participation of oxygen, therefore can be detected using respiratory activity of the cell.

The most widely used conventional techniques for acrylamide detection are chromatography methods which are included gas chromatography with mass spectrometry detection and high performance with tandem mass spectrometry detection (Hu et al. 2015). These methods carry some common disadvantages which are time consuming, required fractionation steps, complicated sample preparation steps, required high laboratory skill for handling sample preparation and running instrument. Because of the disadvantages of conventional methods therefore sensor which has more user-friendly features is a better choice for quantifying acrylamide. Some biosensors were developed for the detection of acrylamide. As mentioned earlier, Silva et al. (2011, 2009) and Ignatov et al. (1997) developed biosensors based on the amidase properties in bacterial cells. On the other hand, a few voltammetric biosensors based on the redox properties of haemoglobin have been developed (Batra et al. 2013a; 2013b; Garabagiu & Mihailescu 2011; Krajewska et al. 2008; Stobiecka et al. 2007). Since acrylamide interacts with haemoglobin and formed adducts, this results in decrease the peak current of haemoglobin-iron (III). This change is treated as a signal response to the presence of acrylamide (Batra & Pundir 2016).

From the literature, method for optical method for determination of acrylamide is limited. Optical sensor carries a unique property where the response does not need any electronic measurement devices but only a colour scale in order to determine the concentration of the analyte. Therefore, an optical sensor for the fast screening of acrylamide compound was developed. In this research, bacterial cells which contain amidase activity were used to hydrolyse acrylamide to ammonia and acrylic acid. The ammonia that is produced is determined optically using the Nessler's reagent. The intensities of the yellow colour formed by the formation of mercury (II) amido-iodine complex were treated as signals. Equations 1 and 2 showed the overall reaction between ammonia and Nessler's reagent.

$$CH_2CHC(O)NH_2 \rightarrow CH_2CHC(O)OH + NH_3. \quad (1)$$

$$2(HgI_4)^{2-} + NH_3 + 3OH^- \rightarrow HgO.Hg(NH_2)I + 7I^- + 3H_2O \quad (2)$$

The objectives of this research were to study the hydrolysis process of acrylamide by *Bacillus* sp. and to develop an optical sensor based on the amidase from an acrylamide-degrading bacterium.

Materials and Methods

Media and Culture Conditions

Bacillus sp. was cultured in a basal salt medium (BSM) containing (g/L) KH_2PO_4, 6.8; $MgSO_4.7H_2O$, 0.5; glucose 10 and 10 mL trace element (pH7.4). The composition of the trace element (mg/L) is as follows, $ZnCl_2$, 30; $CaCl_2.6H_2O$, 3; H_3BO_3, 50; $Cu(CH_3COO)_2.H_2O$, 10 and $FeCl_2.6H_2O$, 20. The BSM medium was supplemented with 1000 ppm of acrylamide as the nitrogen source and the bacterium was incubated on a rotary shaker at 200 rpm for 72 h at 25°C in conical flasks. The cultures were harvested by centrifugation at 4000 rpm for 10 min, washed twice with phosphate buffer (0.05 M, pH7.5) and finally suspended and stored in 0.05 M phosphate buffer (pH7.5) at 4°C.

Immobilisation of Bacterial Pallets in Alginate Membrane and Characterisation of Amidase Activity

Wet bacterial pellets were collected by centrifuging the bacterial suspension at 5000 rpm for 5 min at 4°C. In order to test the response of the sensor, alginate was used to immobilise active bacterial pellets and inactive bacterial (after autoclaving). Bacterial pellets were mixed with sodium alginate gum with the ratio of 1 mg wet bacterial pellets to 1 µL of sodium alginate gum. The gum (20 µL) were then coated on a transparency sheet with a 0.6 cm diameter hole and calcium chloride solution (0.1 M) were drop on the gum in order to cross-linked the alginate gum to formed alginate membrane. As a comparison, blank alginate membranes without bacterial pellets were prepared as the control. For the hydrolysis process, strips with immobilised bacterial were exposed to 500 µL acrylamide solutions at the concentration of 10 ppm and left for overnight. After the hydrolysis, the strips were taken out and the hydrolysed solutions with the presence of ammonia were tested with the Nessler's reagent. For the measurement purpose, 500 µL of hydrolysed solution were reacted with 50 µL Nessler's reagent and 50 µL of NaOH solution, and the solution with then made up with deionised water to 1000 µL. The intensities of colour for the solution were measured with ultraviolet-visible spectrophotometer (UV-Vis) (Varians Cary-100) at 420 nm. All sensor parameters were optimised by using the same procedure.

Characterisation on Hydrolysis Process Towards Immobilised Bacterial Pallets

The membrane composition was optimised by immobilised bacterial in different compositions of alginate gum. Membrane from 2-6% sodium alginate were used to immobilise bacterial and incubated to permit the hydrolysis process. The presence of ammonia was measured with the Nessler's reagent. The time for incubation was studies by incubating the strips at different time and then tested

with the Nessler's reagent. The incubation time with the highest response was used for further studies. The ratio of alginate versus bacterial was optimised by immobilising different amount of bacterial (mg) in alginate membrane. Mass ratio (alginate:bacterial) being tested were 1:0, 1:0.2, 1:0.4, 1:0.5, 1:1, 1:1.5 and 1:2.

EVALUATION OF SENSOR RESPONSE FOR ACRYLAMIDE DETECTION

In order to optimise the formation of mercury (II) amido-iodine complex, different concentrations of NaOH and Nessler's reagent were added to obtain the highest absorption of the yellow complex. After optimising all the experimental parameters, the dynamic range was determined by incubating the test strips with composition of 1:0.5 mass ratio bacterial in solution containing different concentrations of acrylamide ranging from 0 to 50 ppm. After incubating for 18 h, the test strips were taken out and 50 µL NaOH (1.5 M), 50 µL Nessler's reagent (0.05 M) and 400 uL of water were added. The absorption of the samples was measured by UV-VIS spectrophotometer.

Reproducibility test were done by fabricating two sets of sensors that contain eight test strips and hydrolysed in 5 and 10 ppm acrylamide, respectively. The method for determination of ammonia was as described previously.

The regeneration of sensor was carried out by starting the hydrolysis process in 10 ppm of acrylamide. After 18 h, the test strips were taken out and the remaining solution with tested to measure the absorption. The same test strips were then placed in new acrylamide solution for hydrolysis. These steps were repeated until the response of the sensor dropped below 50%.

RECOVERY STUDY AND VALIDATION OF SENSOR WITH HPLC METHOD

A local sneak, cassava chips was chosen as a real sample for this study. Sample preparation for acrylamide determination was modified following the method by Paleologos and Kontominas (2005). Fifty grams of sample were finely grounded with mortar and mixed with 500 mL ultra-pure water. The mixture was homogenised by magnetic stirrer and was incubated for 30 min. Sample was separated with solvent (water) using centrifugation (4000 rpm) for 10 min. Supernatant was defatting using hexane and then Carraz I (potassium hexacyanoferrate) and Carraz II (zinc sulfate) reagents were used for deproteinisation. The sample was centrifuged (4000 rpm, 10 min) and followed by filtration using nylon membrane (0.45 µm) to obtain a clear supernatant.

The supernatant was divided into a few portions and then was spiked with 1.0, 2.5, 5.0, 7.5 and 9 ppm acrylamide standard solution. These samples were examined with both HPLC-UV and optical biosensor methods. The HPLC-UV conditions were as follow: Mobile phase using mixture of methanol:water (1:9 w/w); C_{18} column (water symmetry 5 µm) for separation; UV detector at wavelength of 210 nm for detection.

RESULT AND DISCUSSION

IMMOBILISATION OF BACTERIAL PALLETS IN ALGINATE MEMBRANE AND CHARACTERISATION OF AMIDASE ACTIVITY

Table 1 shows the sensor respond towards the acrylamide solution by bacterial immobilised in alginate membrane. Observation shows that test strips with active bacterial were capable of hydrolysing acrylamide to ammonia and acrylic acid. This is due to the presence of amidase activity in the bacterial. Amidase serve as a catalyst and was involved in the acrylamide hydrolysis process. Ammonia that been generated will react with Nessler's reagent to produce a yellow complex known as mercury (II) amido-iodine complex. Whereas, test strips with inactive bacterial or without bacterial shown the same level of absorption with blank. This is due to denaturation of amidase activity by the autoclaving process. Membrane without bacterial serve as control which proved that only the bacterial contain active enzyme amidase can generate ammonia in hydrolysis process. Therefore, membrane without bacterial did not show any colour change.

CHARACTERISATION ON HYDROLYSIS PROCESS TOWARDS IMMOBILISED BACTERIAL PALLETS

Figure 1 shows the absorbance intensities of complex formed by immobilising bacterial in different alginate membrane's composition. The alginate membranes are able to hold bacterial pellets but at the same time it does not block the acrylamide from diffusing into the membrane to react with bacterial whereas the ammonia produced were able to be release out from the membrane. The result showed that 2% alginate composition gave the highest absorbance intensity with Nessler's reagent. The

TABLE 1. Response of bacterial towards acrylamide solution (AAm)

Membrane	Active bacterial + alginate membrane		Inactive bacterial + alginate membrane		Alginate membrane only	
Solution	10 ppm AAm	H_2O	10 ppm AAm	H_2O	10 ppm AAm	H_2O
Observation	yellow	colourless	colourless	colourless	colourless	colourless
ABS (420nm) *n*=3	0.33	0.17	0.15	0.17	0.16	0.17

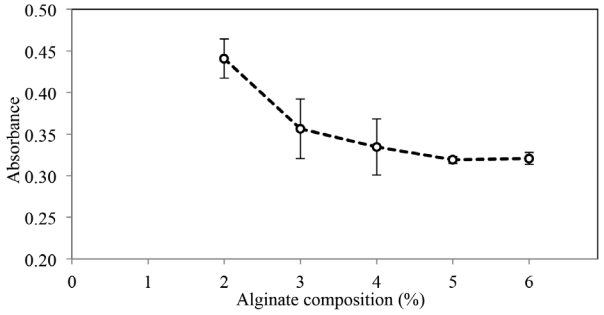

FIGURE 1. The absorbance of the mercury (II) amido-iodine complex when bacterial was immobilised in difference alginate composition

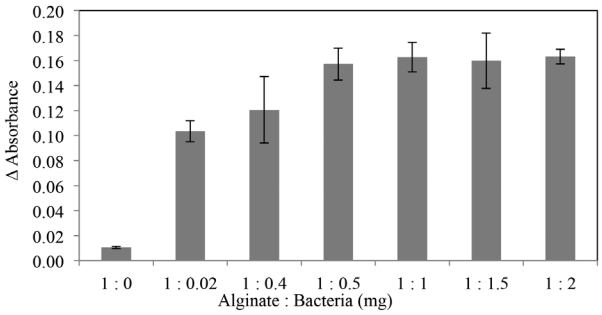

FIGURE 3. The response of the sensors when bacterial was immobilised in alginate membrane at different alginate:bacterial ratio

composition less than 2% alginate gave soft membrane and also not able to form a uniform membrane structure and hence was not used.

Figure 2 shows the absorption of complex when bacterial strips were incubated at various durations. It was found that the hydrolysis process started after 3 h of incubation and an incubation time of 18 h was needed in order to achieve an optimum response. This shows that the hydrolysis by amidase is a slow process and the experimental result obtained was similar to the data reported by Ignatov et al. (1997) where they reported that the transformation of acrylamide to acrylic acid reach a maximum within 18-24 h.

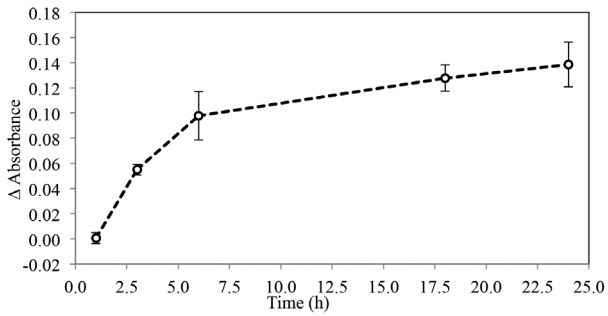

FIGURE 2. The response of sensor after the immobilised bacterial been incubated in acrylamide solution at different time

From Figure 3, mass ratio of alginate:bacterial with 1:0.5 show an optimum absorbance. The responses were increased when the mass ratio of bacterial was increased, this is due to more bacterial were being immobilised in the membrane, therefore more acrylamide was hydrolysed. Similar finding were reported by Arip et al. (2013) using alginate to immobilised fungus in potentiometric biosensor construction for permethrin detection.

EVALUATION OF SENSOR RESPONSE FOR ACRYLAMIDE DETECTION

In order to optimise the absorbance of mercury (II) amido-iodine complex, the concentrations of NaOH and Nessler's reagents need to be optimised. The concentration of NaOH used needs to be sufficient to form complex but it will not further hydrolyse the remaining acrylamide. Beside this, the concentration of Nessler's reagent used must be optimum for complex formation but not too concentrated since Nessler's is harmful to human and environment (Timmer et al. 2005). Figures 4 and 5 show that the highest absorption of the yellow mercury (II) amino-iodine complex was obtained when the concentrations of NaOH and Nessler's reagents were 0.075 M and 2.5 mM, respectively.

Figure 6 shows that when the acrylamide concentration increases, the absorbance of mercury (II) amido-iodine

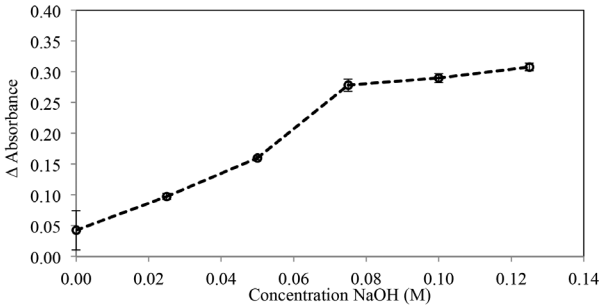

FIGURE 4. The absorbance of mercury (II) amido-iodine complex formed at various concentration of NaOH. Acrylamide solution with 10 ppm was used in this study

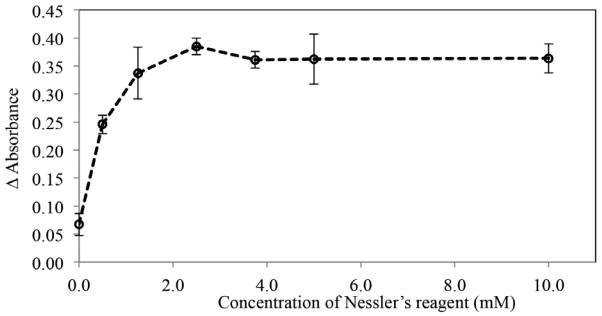

FIGURE 5. The effect of difference concentrations of Nessler's reagent on the formation of mercury (II) amido-iodine complex. An acrylamide concentration of 10 ppm was used in this study

complex formed also increases. Solutions containing higher concentrations of acrylamide will generate higher concentration of ammonia through the hydrolysis process. With the increased amount of ammonia generated, more complex can formed which leads to the increased of absorbance. Supian et al. (2013) also reported similar trend of the linear range result. They found that intensity of the complex Alizarin Red S- Al (III) increases proportional with the concentration of Al^{3+} ion. Figure 6 shows that this sensor has a linear correlation of absorbance with concentration of acrylamide up to 50 ppm. Concentration of acrylamide more than 50 ppm was not explored since the concentration of acrylamide in food sample rarely reaches higher concentration than this. As reported by Lineback et al. (2012), the concentration occur in common food sample id up to 4.2 ppm. The limit of detection of this sensor was 1.30 ppm as calculated based on the blank signal plus with three times of the standard deviation of the blank signals. By comparison to other reported works using bacterial cells for acrylamide detection, this work gave a lower LOD and better dynamic range. The dynamic range reported by Ignatov et al. (1997) is between 10 and 75 ppm, meanwhile Silva et al. (2011) reported a dynamic range of sensor between 1×10^{-3} to 1×10^{-1} M (71-7108 ppm) with LOD of 6.31×10^{-4} M (45 ppm).

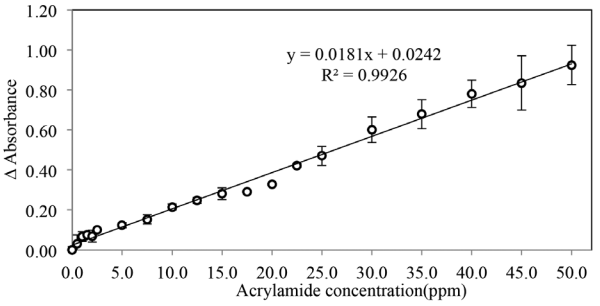

FIGURE 6. Calibration curve of absorbance complex towards acrylamide solutions after incubation for 18 h and adding with Nessler's reagent. Measurement was conducted at 420 nm

Figure 7 shows the absorbance of complex formed from ammonia generated via hydrolysis and mixed with the Nessler's reagent. The % RSD for the acrylamide concentrations of 5 and 10 ppm were 3.17 and 6.15%, respectively. Figure 8 shows the regeneration performance of this sensor. From the figure, it was found that this sensor cannot be reused since the response of the sensor dropped to 69% on second time of reuse. Performance of the sensor dropped to below 50% from the initial response when this sensor was reused for the third and fourth times. The absorbance obtained were 30% and 23%, respectively, when compared to the fresh sensor.

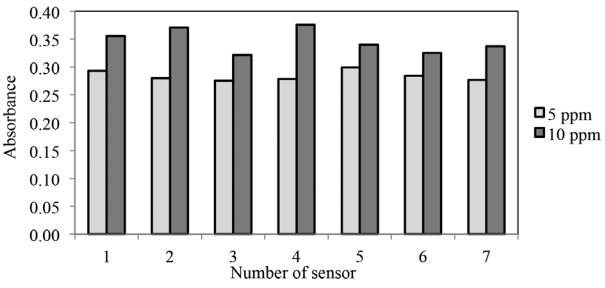

FIGURE 7. Reproducibility of sensor at two difference concentrations of acrylamide solution (5 and 10 ppm)

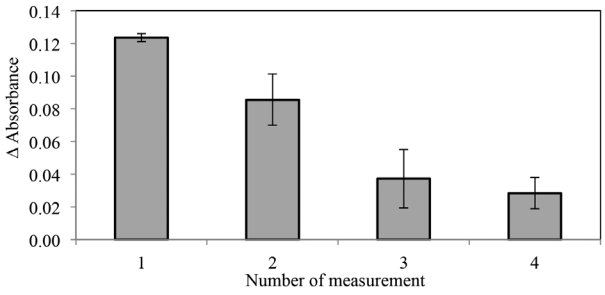

FIGURE 8. Performance of sensor towards the regeneration of sensor being hydrolysed in 10 ppm of acrylamide

RECOVERY STUDY AND VALIDATION OF SENSOR WITH HPLC METHOD

The reliability and accuracy of this optical acrylamide sensor was evaluated using food sample and sensor performance was compared with HPLC-UV method. From Figure 9, this optical biosensor for acrylamide detection revealed a good correlation with HPLC-UV method (slope and correlation coefficient ~ 1). From the statistical data analysed using *t*-test, the result shows that *t*-value obtained is less that the critical value of *t*=2.776. Based on the correlation of the graph and *t*-test, sensor performance has no significant different compared with standard method. Furthermore, this sensor showed acceptable recovery percentage ranged from 98% to 116%, as shows in Table 2. These data shows that food compositions found in sample have little or negligible effect on the sensor response (Ling & Heng 2014).

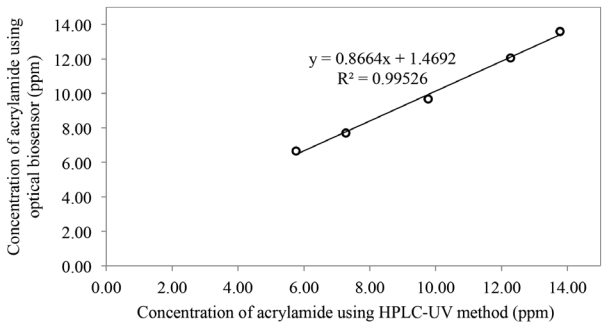

FIGURE 9. A comparison between the optical acrylamide biosensor and HPLC-UV method for the acrylamide determination

TABLE 2. Recovery study of optical acrylamide biosensor against HPLC-UV method using food sample spiked with various concentration of acrylamide

Samples with spiked acrylamide (ppm)	Acrylamide concentration (ppm)		Recovery (%)
	HPLC-UV	Optical biosensor	
1.0	5.77	6.67	115.60
2.5	7.27	7.70	105.91
5.0	9.77	9.66	98.87
7.5	12.27	12.05	98.21
9.0	13.77	13.59	98.69

CONCLUSION

An optical screening acrylamide method was developed based on the hydrolysis process by bacterium *Bacillus* sp. ZK 34. This bacterium contains amidase properties gave a positive response towards acrylamide and it has capability to degrade acrylamide and produce ammonia. Bacterial pallets were immobilised on a test strips in alginate membrane. The advantage of the immobilised bacterial is easily to remove the bacterial after hydrolysis process and it will not affect the absorption measurement. Ammonia generated from the hydrolysis process was determined by using the Nessler's reagent and forms the yellow complex known as mercury (II) amido-iodine. The complex formation was also successfully optimized. The absorbance produced by the sensor is directly proportional to the concentration of acrylamide. This sensor shows a good reproducibility and is suitable to be used as a disposable sensor.

ACKNOWLEDGEMENTS

We are gratefully acknowledged the financial support from Universiti Kebangsaan Malaysia via grant No. DIP-2014-016 and DPP-2015-064 and Ministry of Education to Universiti Sains Islam Malaysia (USIM) through research grants USIM/FRGS-FST-5-50311. We would also like to acknowledge the Ministry of Higher Education (MOHE), Malaysia for the MyBrain 15 scholarship to Yee-May Chong. Special thanks to UPM for bacterial strain and facilities provided.

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Received: 16 January 2017
Accepted: 11 May 2017