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Construction of a glucose sensor based on a screen-printed electrode and a novel mediator pyocyanin from *Pseudomonas aeruginosa*

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Abstract

Pyocyanin is the blue phenazine pigment produced by *Pseudomonas aeruginosa*. Pyocyanin production using immobilized cells was investigated. The maximum production of pyocyanin was obtained using cells immobilized in κ-carrageenan. Moreover, 0.01% PO_4^{3-}, 0.2% Mg^{2+}, 0.001% Fe^{2+}, 1% glycerine, 0.8% leucine and 0.8% DL-alanine were also essential for pyocyanin production. Pyocyanin was purified by chloroform extraction and silica gel column chromatography. An amperometric biosensor system using a screen-printed electrode and pyocyanin as mediator were also developed for a more accurate determination of glucose concentration. Pyocyanin, which exists in the oxidated form, was reduced by the reaction between glucose oxidase and glucose. The reduced form was then converted back to the oxidized form by an oxidative reaction on the electrode. There was a linear relation ship between sensor output currents and glucose concentrations ranging from 1 to 20 mM under the following conditions: −200 mV of the applied potential, pH 5.0, and 10 U of the immobilized enzyme. The coefficient of variation was below 3% ($n = 5$) for the glucose sensor.

Keywords: *Pseudomonas aeruginosa*; Pyocyanin; Mediator; Screen-printed electrode; Glucose oxidase

1. Introduction

Phenazine derivatives have been known as the redox reagents and the antibiotics which are produced by several microorganisms. The production and characterization of these compounds were first carried out by Gessard (1890). One of phenazine derivatives, pyocyanin is a blue phenazine pigment and is produced by *Pseudomonas aeruginosa*. Its chemical structure was elucidated by Wred and Strack (1929) and Hillemann (1938).

The major skeleton (Fig. 1) of a pyocyanin is the phenazine nucleus which has the carboxyl and methyl groups on its carbon numbers 1 and 5 binding sites, respectively. Only *P. aeruginosa* among various *Pseudomonas* spp. produces pyocyanin (Smirnov and Kiprianova, 1990). Therefore, the presence or absence of the pyocyanin was used as the differential key for the identification and classification of *P. aeruginosa* (Ingram and Blackwood, 1970; MacDonald, 1967).

Pyocyanin is a nitric oxide (NO) antagonist in various pharmacological preparations, and has various pharmacological effects on eukaryotic and prokaryotic cells. Pyocyanin functions as an electron transfer agent on the membrane of several strains (Ernst and Friedheim, 1931). As an electron transfer agent and catalyst in photophosphorylation, pyocyanin plays an important role in photosynthetic systems of microorganisms and green plants.

Information on pyocyanin applications is limited. Among the few studies published, pyocyanin was shown as a good model for flavins in electrochemical and spectroscopic studies (Morrison et al., 1978; Morrison and Sawyer, 1978). It was also reported that pyocyanin inhibited the ciliary function of respiratory epithelial cells (Kanthakumar et al., 1994). It was reported that pyocyanin behaved as a reversible dye of the quinoid type with a redox potential similar to that of menaquinone (Friedheim and Michealis, 1931).

Various biosensors have been developed since Clark and Lyons prepared a glucose sensor in the 1960s (Clark and

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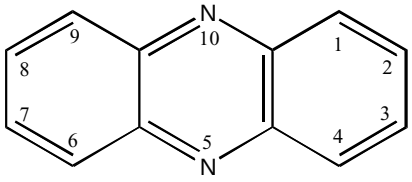

Fig. 1. Structure and numbering system of phenazine nucleus.

Lyons, 1962; Updike and Hicks, 1967a,b). The screen-printed technology based on mediator-enzyme electrodes (Cass et al., 1984) was introduced for biosensor in the middle of the 1980s (Matthews et al., 1987). The important advantages of the method were its precision, speed, and cheapness. This technique has been used to construct disposable enzyme electrodes for determination of glucose (Matthews et al., 1987; Higgins, 1990; Newman and Turner, 1992; Nagata et al., 1995; Rohm et al., 1995; Wang et al., 1996), and glutathione (Wring et al., 1991, 1992; Wring and Hart, 1992).

Generally, the reaction of electron transfer between enzyme molecules and electrode materials is indirectly carried out through another redox compounds such as phenazine methosulfate (PMS), potassium ferricyanide, $K_4[Fe(CN)_6]$, quinines, and Os-complexes because the rapid reaction between the both is very difficult. The studying for searching of a more efficient mediator compounds has continued as an important and interested project up to the present.

It was reported that various *P. aeruginosa* strains but not strain IAM 1415^T produce pyocyanin. In addition, improvement of the medium for pyocyanin production has been reported a little (Hellinger, 1951; Burton et al., 1947; Jamieson, 1941; Samuel, 1972; Higashihara, 1984; Tanaka et al., 1972; Osman et al., 1996). Moreover, the application of pyocyanin as a biosensor has also not been reported at all.

In this study, the optimum conditions for pyocyanin production, peculiarly the immobilization carrier used for the microorganisms, were examined. Moreover, pyocyanin was also purified by chloroform extraction and silica gel column chromatography.

A glucose sensor using pyocyanin as a mediator was also developed. The results obtained are described below in detail.

2. Experimental

2.1. Materials

κ-Carrageenan was purchased from Aldrich Chemical Company Inc. Silica gel 60 was purchased from Merck. Glucose and glucose oxidase (GOD; EC 1.1.3.4 from *Aspergillus niger*, 20,000 units) were purchased from Wako Pure Chemical Industries, Ltd. Carboxymethyl cellulose (CMC) was purchased from Kokusan Chemical Works. All other chemicals were of analytical grade.

2.2. Media and culture conditions

Two solid and nine liquid media were used for studying pyocyanin production. The solid media were King's A agar (KA) and King's B agar (KB), whereas the liquid media included nutrient broth (NB), ethanol broth (EB) (Osman et al., 1996), modified basal medium broth (MBMB), Hellinger broth I (HB I) (Hellinger, 1951), Hellinger broth II (HB II), MacDonald broth (MB) (MacDonald, 1963), Ingram and Blackwood broth (IBB I) (Ingram and Blackwood, 1962), Ingram and Blackwood broth II (IBB II), and Ingram and Blackwood broth III (IBB III).

The pigment on KA was visually observed after 1 day incubation at 37 °C and 5 days incubation at room temperature. In the same manner, the pigment on KB was visually observed after 6 days incubation at 37 °C.

In the liquid media, batch fermentation was performed in 500 ml shaking flasks containing 100/200 ml of the growth medium, which was stirred on a rotary shaker at 160 rpm at a constant temperature of 27 °C for 7 days.

2.3. Growth and pyocyanin production monitoring

Microbial growth and pyocyanin production were monitored periodically. Growth was determined from the absorbance of the culture medium at 660 nm wavelength using a UV-Vis spectrophotometer. Pyocyanin production was measured using the method of Higashihara (1985). Pyocyanin production monitoring was follows. The culture was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was then collected and the pellet was discarded. The absorbance of the supernatant was measured at a wavelength of 310 nm using a UV-Vis spectrophotometer.

2.4. Preparation of immobilized microorganism

The cells were immobilized on 4 and 5% κ-carrageenan, 4% sodium alginate and HP 5020-chitopeal. In the latter case, after the cells were cultured at 27 °C for 1 day, 1.0 ml of this broth was added to a nutrient broth containing 20 ml of HP 5020-chitopeal and the mixture was further cultured at 27 °C for 1 day. HP 5020-chitopeal on which the microorganisms were immobilized was then collected using a strainer and washed with sterilized saline.

On the other hand, when using 4 and 5% κ-carrageenan and 4% sodium alginate, after cultivation at 27 °C for 1 day, the immobilized cells were collected by centrifugation at 8000 rpm for 10 min at 4 °C. The supernatant was discarded and the pellet was suspended in 20 ml of sterilized saline. The suspension was centrifuged at 8000 rpm for 10 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 20 ml of sterilized saline. This procedure was repeated twice. The prepared suspension (20 ml) was added to 4 and 5% κ-carrageenan (100 ml) and 4% sodium alginate (100 ml) and stirred, respectively. The mixture

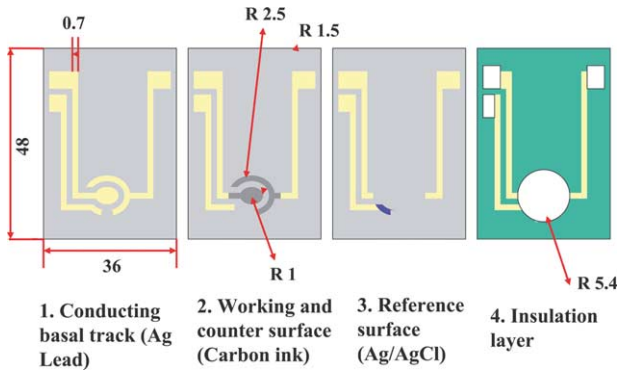

Fig. 2. Production of steps for screen-printed electrode.

was dispensed using syringe into 2% KCl and 2% $CaCl_2$, respectively, and stirred for 30 min.

Twenty milliters of the prepared HP 5020-chitopeal, 4% sodium alginate, 4 and 5% κ-carrageenan and free cells were placed in 500 ml shaking flasks containing 100/200 ml of the pyocyanin production medium and stirred on a rotary shaker for 10 days at a constant temperature of 27 °C.

2.5. Analytical method

Thin layer chromatography was performed on a silica gel F_{254} plate with methanol–chloroform (1:1) as solvent. UV-Vis absorption spectra were measured using a Shimadzu UV-2400PC recording spectrophotometer.

2.6. Construction of electrode

The electrode was composed of Ag lead, carbon working and counter electrodes and an Ag/AgCl reference electrode on the polyethylene telephtarate film prepared using the screen-printed technique (Fig. 2). The conditions of construction of electrode were as follows: Ag lead used Ag ink (Asahi Chemical Research Laboratory Co., Ltd.) and dried at 150 °C for 30 min. Carbon working and counter electrodes used carbon ink (Asahi Chemical Research Laboratory Co., Ltd.) and dried at 150 °C for 20 min. Ag/AgCl reference electrode used Ag/AgCl ink (Acheson (Japan) Limited) and dried at 120 °C for 15 min. Insulation resist used Insulation resist ink (Asahi Chemical Research Laboratory Co., Ltd.) and dried at 130 °C for 10 min. Ag ink and Ag/AgCl ink printed once and 20 μm thick, and carbon ink and insulation resist ink printed twice and 40 μm thick. Cyclic voltammetry and chronoamperometric measurements were performed using a voltammetric analyzer (Model CV—50 W; BAS).

2.7. Preparation of glucose sensor

Enzyme powder (2.5 mg) was added to 5 mM pyocyanin containing 0.5% (w/v) CMC. Five microliters of the enzyme solution (10 U) was placed on the screen-printed electrode. A thin layer of the enzyme solution was then fabricated on the screen-printed electrode and dried at 60 °C for 20 min. The fabricated electrodes were kept at room temperature in a desiccator.

3. Results and discussion

3.1. Separation and purification of pyocyanin

3.1.1. Optimum medium for pyocyanin production

Pyocyanin production by *P. aeruginosa* IAM 1415^T was determined in two solid media, KA and KB, because there has been no report on the utilization of these media for pyocyanin production. Results suggest that *P. aeruginosa* IAM 1415^T produces some pigments such as pyorubin, fluorescein and pyocyanin.

Among the nine liquid media tested, pyocyanin production was maximum in Ingram and Blackwood broth I (IBB I). In nutrient broth (NB), ethanol broth (EB) and modified basal medium broth (MBMB), the pigment was not produced. On the other hand, in Hellinger broths I, II (HB I, II), MacDonald broth (MB) and Ingram and Blackwood broths I, II, III (IBB I, II, III), the pigment was observed. From these results, phosphate ion, magnesium ion, iron ion, glycerine, leucine and DL-alanine are essential for the pyocyanin production. The optimum concentrations of these components were determined to be 0.01, 0.2, 0.001, 1, 0.8 and 0.8%, respectively (Table 1). These data were compatible with those of Burton et al. (1948) and Frank and DeMoss (1959).

3.1.2. Optimum immobilization carrier for *P. aeruginosa* IAM 1415^T for increased pyocyanin production

This experiment requires a large amount of pyocyanin. Therefore, the microbial immobilization was examined from the standpoint of increasing pyocyanin production. Four and 5% κ-carrageenan, 4% sodium alginate and HP-5020 chitopeal as the carriers for the immobilization were used. The effect of the carrier on pyocyanin production by immobilized cells is shown in Fig. 3. A higher pyocyanin production was obtained from immobilized cells than from free cells. This observation may be interpreted as follows: the microorganisms in the gel matrix exist stably for a long time because they are less affected by drastic changed in the outside environment. From these results, subsequent experiments were carried out using 4% κ-carrageenan.

3.1.3. Separation and purification of pyocyanin

Pyocyanin was prepared as follows; The pH of the culture supernatant was first adjusted to 10, then, two volumes of chloroform as added to one volume of the culture supernatant and shaken for about 10 min. The chloroform extract was then lyophilized and the residues redissolved in 5 ml of chloroform. This chloroform solution was adsorbed on a silica gel column (2 cm × 30 cm) that had previously been equilibrated with 1% methanol in chloroform. Pyocyanin was eluted with 15% methanol in chloroform. Pigments on

Table 1
Determination of optimum medium for pyocyanin production

	Media								
	NB	EB	MBMB	MB	HB I	HB II	IBB I	IBB II	IBB III
Pyocyanin production	–	–	–	◦	◦	◦	◎	◦	◦
Phosphate ion[a]	–	0.21	0.0075	0.01	0.04	0.04	0.01	0.01	0.01
Magnesium ion[a]	–	0.05	–	0.2	0.2	0.2	0.2	0.2	0.2
Iron ion[a]	–	0.001	0.0028	0.001	0.001	0.001	0.001	0.001	0.001
Glycerine[a]	–	–	–	1	1	1	1	1	1
Leucine[a]	–	–	–	–	0.8	0.8	0.8	0.8	0.8
DL-Alanine[a]	–	–	–	–	–	–	0.8	–	–

Abbreviations: NB, nutrient broth; EB, ethanol broth; MBMB, modified basal medium broth; MB, MacDonald broth; HB•, Hellinger broth; IBB•, Ingram and Blackwood broth; ◎, maximum; ◦, positive; –, none.
[a] Constituents: %/1000 ml.

the silica gel column appeared in yellow, red, light blue and dark blue bands, with pyocyanin appearing in the latter. A yellow–green band remained in the column even after elution by 15% methanol in chloroform. Pyocyanin was obtained as a residue from the solvents.

3.1.4. Physicochemical properties of pyocyanin

The physicochemical properties of the blue pigment obtained from *P. aeruginosa* IAM 1415^T are as follows. The pigment easily dissolved in chloroform, HCl, and hot water, but it was difficult to dissolve in cold water. Pyocyanin obtained from the chloroform extract was in the form of blue fiber needle crystals. The melting point of this pigment was 128–130 °C. The Rf of the thin-layer chromatograph was 0.6 ± 0.02 using the solvent described earlier and the TLC spot was blue. These results mostly agreed with previous data (Knight et al., 1979). The redox potential was −401 mV. The UV spectrum for the pigment is shown in Fig. 4. Pyocyanin dissolved in methanol exhibited absorption maxima at 201.0, 238.0, 318.50, 710.50, and 886.50 nm. On the other hand, pyocyanin dissolved in 0.1 N HCl exhibited the maxima at 204.0, 242.50, 277.0, 387.50, and 521.50 nm. These results mostly agreed with those of David et al. (1986). The color of the pigment solution changed depending on the pH. The solution appeared red at pHs 3.0 and 4.0, purple at pHs 5.0 and 6.0, and blue at pHs >7. The correlation between the variation in the color of the solution and pH was also observed by Friedheim and Michealis (1931).

3.2. Construction of glucose sensor

3.2.1. Optimum applied potential for the glucose sensor

Chronoamperometry measurement was performed to determine the optimum applied potential for the glucose sensor. The anodic current corresponding to 20 mM glucose was measured at −300 to −50 mV of the applied potential for the glucose sensor at 50 mV intervals. The maximum anodic current was obtained at −200 mV of the applied potential. It was presumed that this lower potential was based on pyocyanin.

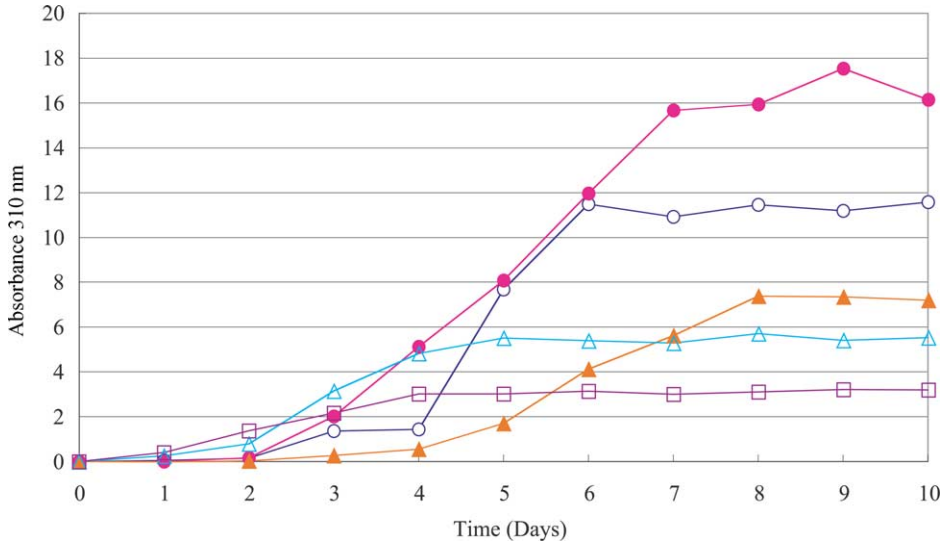

Fig. 3. Pyocyanin production by immobilized cells. Analytical conditions are as described in Section 2. Immobilized cells in 4% κ-carrageenan (closed circle), 5% κ-carrageenan (open circle), 4% sodium alginate (closed triangle), chitopeal HP-5020 (open triangle), and free cells (open square).

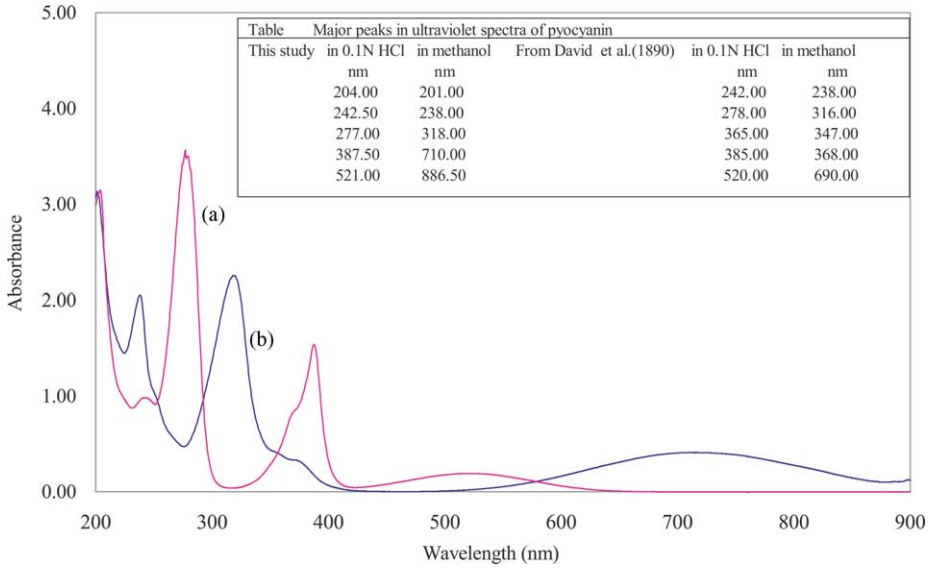

Table Major peaks in ultraviolet spectra of pyocyanin

This study	in 0.1N HCl	in methanol	From David et al.(1890)	in 0.1N HCl	in methanol
	nm	nm		nm	nm
	204.00	201.00		242.00	238.00
	242.50	238.00		278.00	316.00
	277.00	318.00		365.00	347.00
	387.50	710.00		385.00	368.00
	521.00	886.50		520.00	690.00

Fig. 4. Absorption spectra of pyocyanin. Pyocyanin dissolved in 0.1 N HCl (a) and in methanol (b).

All the while, the mediator with a low applied potential has been researched to provide the affection of the many impurities in the sample solutions on the output of sensor. It was cleared that conventional mediators such as potassium ferricyanide, $K_4[Fe(CN)_6]$ used to biosensor allowed to avoid influence of oxygen on the output of the sensor. However, there are many impurities such as an amino acid, ascorbic acid, and creatine in the sample solutions. The proposed sensor using pyocyanin as a mediator was used at −200 mV versus Ag/AgCl of the applied potential. Therefore, pyocyanin was allowed to be not liable to occur the reduced current based on the impurities of the sample solution. From this reason, the optimum applied potential was determined at −200 mV versus Ag/AgCl.

3.2.2. Optimum pH of the glucose sensor

The effect of pH on glucose sensor output was determined. The optimum pH was found to be in the range of pH 4.0–6.5. The maximum response was obtained at about pH 5.0 but the response decreased with an increase in pH. Therefore, the optimum pH was set at 5.0.

3.2.3. Optimum amount of immobilized enzyme

The effect of the amount of enzyme immobilized on the electrode tip on the response of the glucose sensor

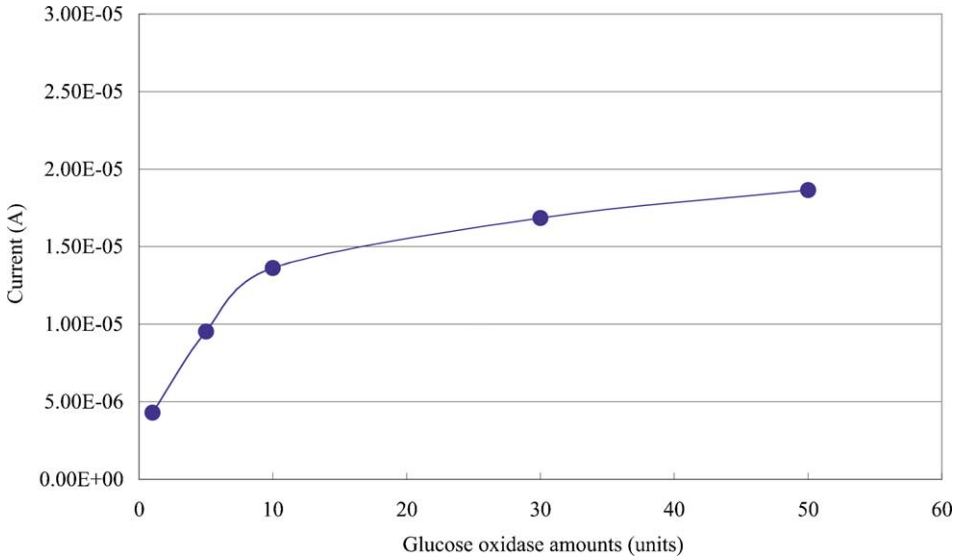

Fig. 5. The optimum amount of the immobilized sensor. Analytical conditions are as follows: 5 mM pyocyanin, −200 mV vs. Ag/Cl applied potential, pH 5.0 and sample volume of 50 μl of 20 mM.

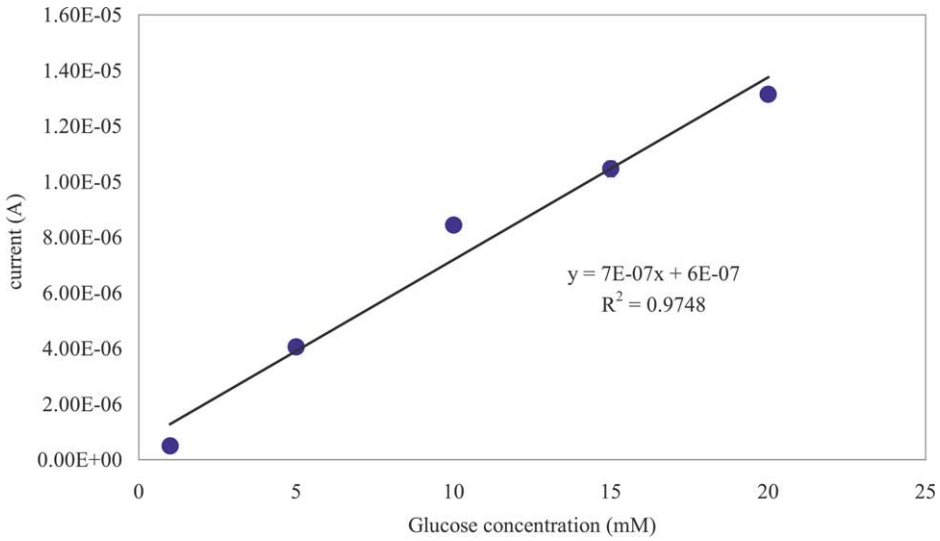

Fig. 6. Calibration curve for the glucose sensor. Analytical conditions are as follows: −200 mV vs. Ag/Cl applied potential, pH 5.0, 5 mM pyocyanin, 10 U glucose oxidase, and sample volume of 50 μl.

was determined. The results obtained are shown in Fig. 5. The sensor response increased with an increase in enzyme amount (unit), and the maximum response was obtained at 10 U. Therefore, in subsequent experiments, 10 U of GOD was used.

3.2.4. Calibration curve for the glucose sensor

The calibration curve of the glucose sensor is shown in Fig. 6. A linear relationship between glucose concentration and output of the proposed glucose sensor was obtained at 1–20 mM glucose concentration. The relative coefficient was 0.9748, while the coefficient of variation (CV) was below 3% ($n = 5$). The reproducibility and stability of response during the same day was also examined by using 5 mM glucose (not data shown). The CV was 1.96% ($n = 8$) for glucose sensor.

3.2.5. Effect of some substrates on glucose sensor output

The effects of ascorbic acid, urea, uric acid and creatine on the output of the proposed sensor were determined. The output based on 5 mM glucose is 100%; the outputs of the proposed sensor based on some substrates are shown in Fig. 7. The output of the proposed sensor was slightly affected by ascorbic acid.

3.2.6. Stability of the proposed sensor

The durability of the proposed sensor was measured under storage at 4 °C in the experiment room and 4 and 20 °C

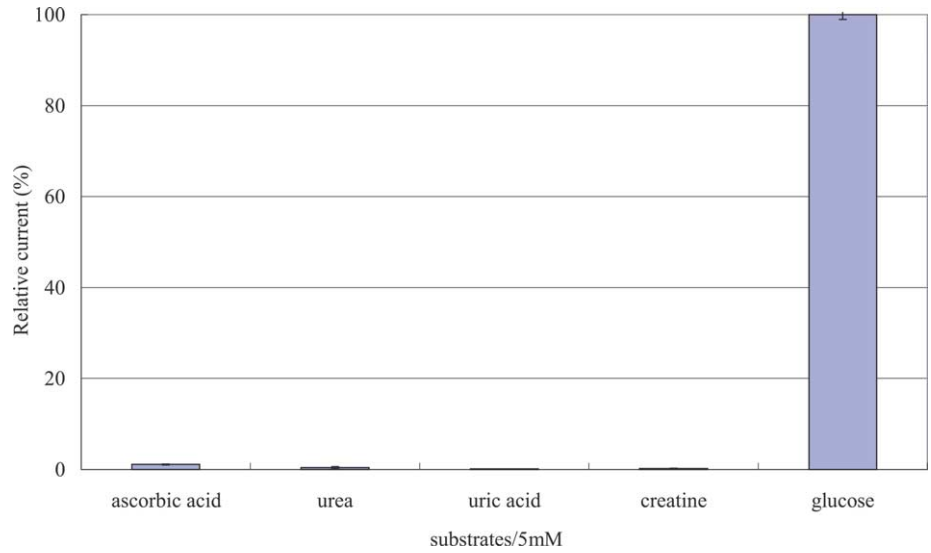

Fig. 7. Effect of some substrates on glucose sensor output. Analytical conditions are as follows: −200 mV vs. Ag/Cl applied potential, pH 5.0, 5 mM pyocyanin, 10 U glucose oxidase, and sample volume of 50 μl. The *y*-axis represents the percentage of the output current determined against the output of the proposed sensor for 5 mM glucose.

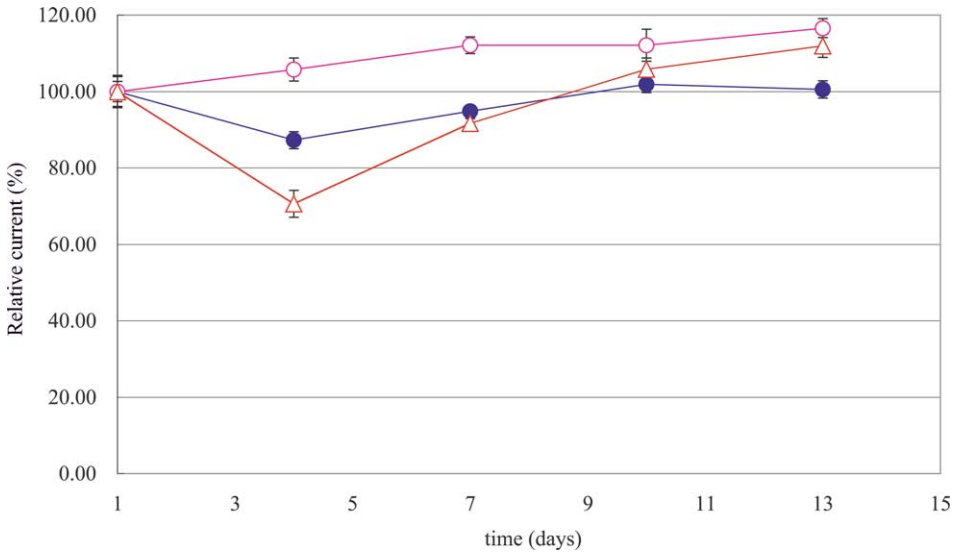

Fig. 8. Stability of the proposed sensor. Analytical conditions are as follows: −200 mV vs. Ag/Cl applied potential, pH 5.0, 5 mM pyocyanin, 10 U glucose oxidase, and sample volume of 50 μl of 10 mM glucose. The *y*-axis represents the percentage of the output current determined at regular intervals against the output of the proposed sensor stored for 1 day. Storage at 4 °C in the experiment room (open triangle), 4 °C in a desiccator (open circle), and 20 °C in a desiccator (closed circle).

in a desiccator. The results obtained are shown in Fig. 8. The *y*-axis represents the percentage of the output current determined at regular intervals against the output of the proposed sensor stored for 1 day.

In all cases, the relative currents decreased until the 4th day, and then increased gradually. After 1 week, the currents were higher than the output of the proposed sensor stored for 1 day. It was thought that a reversible structural change of the enzyme occurred at the immobilization and reverted to its original structure during storage. The optimum storage condition was 4 °C in a dessicator. When the proposed was sensor stored at 4 °C in the experiment room, the CVs of the data were over 5%. It was thought that this was due to oxidation of the electrode. The activity of the proposed electrode was maintained at 20 °C in a dessicator for about 1 month.

3.2.7. Application of the proposed sensor to soft drinks

Glucose concentrations of soft drinks were determined by the proposed sensor system and by a colorimetric method. The results are shown in Table 2. A good agreement was obtained between the glucose contents determined by both methods, with a relative coefficient of 0.998. An assay could be performed within 2 min using the proposed sensor, while it required 6 min using the colorimetric method. Thus, the proposed sensor, which produced reliable and reproducible results at a low cost, facilitated the simple and rapid determination of glucose concentration.

Table 2
Comparison between sensor and colorimetric methods

Sample[a]	Concentration (mg/dl)	
	Colorimetric method	Sensor method ($n = 5$)
A	816	790 (±6.4)
B	911	760 (±38.0)
C	1991	1860 (±68.7)
D	3367	3350 (±102.6)
E	5463	5200 (±49.6)

[a] Beverage sample.

4. Conclusion

The optimum condition for pyocyanin production by *P. aeruginosa* IAM 1415^T, peculiarly the immobilization carrier used for the microorganisms, was investigated. Moreover, the glucose sensor based on screen-printed electrode and a novel mediator pyocyanin, was also constructed to estimate some properties as a new mediator.

The essential condition of maximum pyocyanin production using immobilized cells were as follows: 0.01% PO_4^{3-}, 0.2% Mg^{2+}, 0.001% Fe^{2+}, 1% glycerine, 0.8% leucine and 0.8% DL-alanine. In addition, maximum production of pyocyanin was obtained using cells immobilized in κ-carrageenan.

The optimum conditions of the chronoamperometric biosensor systems were as follows: −200 mV versus Ag/Cl of the applied potential, pH 5.0 and 10 U of the immobilized enzyme.

The pyocyanin used as a mediator was −200 mV versus Ag/Cl of the applied potential. Therefore, compared to other sensor, the proposed sensor was expected for measurement without affection of the impurities in the sample solution. Moreover, a mass production of screen-printed electrode permitted also low cost and rapid and simple measurement of the proposed sensor. Therefore, the biosensor using

pyocyanin was also expected to apply to some fields such as medicine, food and environment.

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References

Burton, M.O., Eagles, B.A., Campbell, J.J.R., 1947. The amino acid requirement for pocyanin production. Can. J. Res. 25 (Sec. C), 121–128.

Burton, M.O., Campbell, J.J.R., Eagles, B.A., 1948. The mineral requirements for pyocyanin production. Can. J. Res. 26 (Sec. C), 15–22.

Cass, A.E.G., Davis, G., Francis, G.D., Hill, H.A.O., Aston, W.J., Higgins, I.J., Plotkin, E.V., Scott, L.D.L., Turner, A.P.F., 1984. Frrocene-mediated enzyme electrode for amperometric determination of glucose. Anal. Chem. 56, 667–671.

Clark, L.C., Lyons, C., 1962. Electrode systems for continuous monitoring in cardiovascular surgery. Ann. N.Y. Acad. Sci. 102, 29–45.

David, W., Macdermot, J., Wilson, R., Cole, P.J., Taylor, G.W., 1986. Purification and structural analysis of pyocyanin and 1-hydroxyphenazine. Eur. J. Biochem. 159, 309–313.

Ernst, A.H., Friedheim, E.M.D., 1931. Pyocyanine, an accessory respiratory pigment. J. Exp. Med. 54, 207–221.

Frank, H., DeMoss, R.D., 1959. On the biosynthesis of pyocyanin. J. Bacteriol. 77, 776–782.

Friedheim, E., Michealis, L., 1931. Potentiometric study of pyocyanin. J. Biol. Chem. 91, 355–368.

Gessard, P.M.C., 1890. Sur le microbe pyocyanique. Ann. Inst. Pasteur. 4, 88–102.

Hellinger, E., 1951. Requirements for pyocyanine production by *Pseudomonas aeruginosa* (Schroeter) Migula. J. Gen. Microbial. 5., 633–639.

Higashihara, T.A.S., 1984. Isolation of pyocyanin produced by hydeocarbon-assimilating bacteria and its taxonomic characteristics. Report Ferment. Res. Inst. 63, 65–78.

Higashihara, T.A.S., 1985. Production of 1-phenazinecerboxylic acid from ethanol by a hydrocarbon-assimilating bacterium, *Pseudomonas aeruginosa*. Report Ferment. Res. Inst. 63, 81–93.

Higgins, I.J., 1990. The 1st World Congress on Biosensors (Abstract), Elsevier, pp. 129–131.

Hillemann, H., 1938. Beitrage zur kenntnis des phenazins. Ber. Deut. Chem. Ges. B71, 46–52.

Ingram, J.M., Blackwood, A.C., 1962. Studies on the biosynthesis of pyocyanin. Can. J. Microbiol. 8., 49–56.

Ingram, J.M., Blackwood, A.C., 1970. Microbial production of phenazines. Adv. Appl. Microbiol. 13, 267–282.

Jamieson, M.C., 1941. Requirement for the recognition of the blue-green *Pseudomonas*. Sci. Agric. 22, 401–408.

Kanthakumar, D.R., Cundell, M., Johnson, P.J., Wills, G.W., Taylor, P.J.C., Wilson, R., 1994. Effect of salmeterol on human nasal epithelial cell ciliary beating: inhibition of the ciliotoxin, pyocyanin. Br. J. Pharmacol. 112, 493–498.

Knight, M., Hartman, P.E., Hartman, Z., Young, V.M., 1979. New method of preparation of pyocyanin and demonstration of an unusual bacterial sensitivity. Anal. Biochem. 95, 19–23.

MacDonald, J.C., 1963. Biosynthesis of pyocyanin. Can. J. Microbiol. 9., 809–819.

MacDonald, J.C., 1967. In: Gottlieb, D., Shaw, P.D. (Eds.), Antibiotics, vol. II. Springer-Verlag, Berlin, pp. 52–65.

Matthews, D.R., Bown, E., Watson, A., Holman, R.R., Steemson, J., Hughes, S., Scott, D., 1987. Pen-sized digital 30-s blood-glucose meter. Lancet 1, 778–779.

Morrison, M.M., Seo, E.T., Howie, J.K., Sawyer, D.T., 1978. Flavin model systems. 1. The electrochemistry of 1-hydroxyphenazine and pyocyanine in aprotic solvents. J. Am. Chem. Soc. 100, 207–211.

Morrison, M.M., Sawyer, D.T., 1978. Flavin model systems. 2. Pyocyanine complexes of divalent manganese, iron, nickel, copper, and zinc in dimethyl sulfoxide. J. Am. Chem. Soc. 100., 211–213.

Nagata, R., Yokoyama, K., Durlist, H., Comtat, M., Clark, S.A., Karube, I., 1995. An enzyme-containing ink for screen-printed glucose sensors. Electroanalysis 7, 1027–1031.

Newman, J.D., Turner, A.P.E., 1992. Ink-jet printing for the fabrication of glucose biosensors. Anal. Chim. Acta 262, 13–17.

Osman, M., Ishigami, Y., Someya, J., Harald, B.J., 1996. The bioconversion of ethanol to biosurfactants and dye by a novel coproduction technique. JAOSC. 73, 851–856.

Rohm, I., Kunnecke, W., Bilitewski, U., 1995. UV-polymerizable screen-printed enzyme pastes. Anl. Chem. 67, 2304–2307.

Samuel, A.P., 1972. Role of iron and sulfur in pigment and slime formation by *Pseudomnas aeruginosa*. J. Bacteriol. 111, 430–436.

Smirnov, V.V., Kiprianova, E.A., 1990. Bacteria of *Pseudomonas* Genus. Naukova Dumka, Kiev, USSR, pp. 100–111.

Tanaka, A., Taire, T., Teranishi, Y., Fakui, S., 1972. Studies on the utilization of hydrocarbons by microorganisms. J. Ferment. Technol. 50 (9), 662–667.

Updike, S.J., Hicks, G.P., 1967a. The enzyme electrode. Nature 214, 986–988.

Updike, S.J., Hicks, G.P., 1967b. Reagentless substrate analysis with immobilized enzyme. Science 158, 270–272.

Wang, J., Pamidi, P.V.A., Park, D.S., 1996. Screen-printable sol–gel enzyme-containing carbon inks. Anal. Chem. 68, 2705–2708.

Wred, F., Strack, E., 1929. Uber das pyocyanin, den blauen farbstoff des bacillus pyocyaneus. Z. Physiol. Chem. 181, 58–76.

Wring, S.A., Hart, J.P., 1992. Chemically modified, screen-printed carbon electrodes. Analyst 117, 1281–1286.

Wring, S.A., Hart, J.P., Birch, B.J., 1991. Voltammetric behaviour of screen-printed carbon electrodes, chemically modified with selected mediators, and their application as sensors for the determination of reduced glutathione. Analyst 116, 123–129.

Wring, S.A., Hart, J.P., Birch, B.J., 1992. Development of an amperometric assay for the determination of reduced glutathione, using glutathione-peroxidase and screen-printed carbon electrodes chemically modified with cbalt phthalocyanine. Electroanalysis 4 (3), 299–309.