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Effect of bacterial growth period on the sensitivity of the MTT assay for silver

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Abstract

Respiratory activity inhibition by toxic compounds in bacteria and yeast has been used to detect toxic compounds in the environment. Often the age of culture contributes towards the sensitivity of detection. In the present work, the effect of growth period on the sensitivity of an inhibitive assay for heavy metals using bacterial respiratory assay system based on the reduction of the water soluble tetrazolium dye MTT is reported. A silver-sensitive isolate was discovered to exhibit different sensitivities towards silver at different growth periods. An exponential decay model adequately described the inhibition due to silver. Analysis using ANOVA with post-hoc Tukey's test showed that the IC_{50} obtained by strain DRYS8 grown at the 12 hr-period in nutrient broth at 28 °C gave the lowest value compared to other growth periods. This study highlights the importance of taking into accounts growth conditions and age of culture in developing cellular-based bioassays.

Key words

MTT assay, Bacterium, Growth Period, Silver

Introduction

The ever increasing presence of toxic xenobiotics in water bodies is a global phenomenon that have warranted the development of cheaper and rapid technologies to detect these toxicants (Alina *et al.,* 2012). Continuous detection of these toxic metal ions in water bodies is important in applying appropriate detection and bioremediation techniques (Rezaie-Boroon *et al.,* 2011; Isinkaye, 2012). However, constant detection activities are hampered by the high costs of instrumental-based methods. Scientists have turn into the use of microorganisms (Sun *et al.,* 1994), plants (Magana Arachchi and Liyanage, 2012; Sharma, 2012) and enzymes (Jung *et al.,* 1995; Shukor *et al.,* 2006, 2008, 2009a, b; Tham *et al.,* 2009) to detect xenobiotics and heavy metals in the environment. There have been many tests developed using various bacteria as the indicator organism (Zonneveld, 1983). Microbial bioindicator/bioassay provides a simpler and less expensive method than classical bioassay since no specialized equipment is required (Botsford *et al.,* 1998). Thus, bioindicators using bacteria has been commercialized such as the Lux-Fluoro (Baumstark-Khan, 2003), the Polytox™ (Sun *et al.,* 1994), the Deltatox™ and the Microtox™ assays (Bullich, 1986). Another bacterium that has been used as an indicator is *Rhizobium meliloti.* The assay is based on the ability of *R. meliloti* to reduce the tetrazolium dye MTT. The use of tetrazolium dyes allowed visual observation to be made and is also faster and cheaper than luminescence-based assays. MTT dye is also superior to other formazan dyes in terms of water solubility. This reduction process is inhibited by many toxic chemicals especially heavy metals (Botsford *et al.,* 1998). In the present study, a bacterium sensitive to silver was isolated using the MTT reduction away. It was observed that bacterial growth period affects the sensitivity of the bacteria towards silver.

Materials and Methods

Chemicals and Reagents : All reagents were of analytical reagent grade unless otherwise stated. The MTT dye was

purchased from Sigma. All the plastics and glassware were cleaned by soaking in dilute HNO_3 (10%) to remove all traces of metal ions and were rinsed with an appropriate amount of deionized water prior to use. MTT dye at 10 mM stock solution was prepared by dissolving 0.2072 g in 50 ml of 10 mM Phosphate-Buffered Saline (PBS), pH 7.5. The solution was stored at 4 °C. Heavy metals were prepared from either commercial salts or atomic absorption spectrometry 1 g l^{-1} standard solutions from Merck (Darmstadt, Germany). The final concentration in the reaction mixture for preliminary screening of heavy metal ions was 10 mg l^{-1}.

Isolation of bacteria : Samples were collected 15-20 cm beneath the surface from various locations in Malaysia from December 2002 to January 2003. The samples were placed in sterile screw-capped vials. The samples were immediately placed in a freezer and stored at -20 °C until returned to Universiti Putra Malaysia, Serdang, Selangor, Malaysia for further examination. Five grams of soil or five ml of water samples were serially diluted in deionized water and suitable dilutions were then spread on nutrient agar plates. Individual colonies were isolated based on unique colony morphologies. Individual bacterium were then inoculated into 45 ml of nutrient broth in a 250 ml conical flask and incubated at room temperature with shaking at 150 rpm on an orbital shaker. Each flasks were then harvested by centrifugation (15,000 g) for 15 minutes and resuspended in sterile phosphate-buffered saline.

Screening of heavy metals-sensitive bacteria using the MTT assay: The MTT assay was carried out by combining 75 µl of 10 mM PBS, pH 7.5 at the final concentration in a 250 µl total reaction mixture followed by the addition of 75 µl of 1% (v/v) bacterial suspensions and finally 50 µl of tested heavy metals in a 96-well microplate (Botsford et al., 1998). As a control, deionized water was added into the first well. The reaction mixture was pre-incubated for an hr at room temperature by covering the microplate with aluminum foil before adding 25 µl of MTT (10 mM stock) to allow the reaction to proceed. Colour development at 550 nm was measured using a microplate reader (Stat Fax® 3200 Microplate Reader, Awareness Technology Inc., USA). The results showed that formazan production was saturated after 20 minutes and therefore this was chosen as the incubation period for all further experiments. All the tests were performed in triplicate.

Inhibition (percent) was calculated by the following formula : [Initial control absorbance-final absorbance/Initial control absorbance] x 100. Regression curves to determine concentrations of heavy metals causing 50% inhibition (IC_{50}) are generated using the PRISM (Prism version 4.00 for Windows) non-linear regression analysis for one-phase exponential decay models software available from GraphPad, (GraphPad Software Inc., San Diego, CA).

Identification of heavy metal-sensitive bacterium : A silver sensitive bacterium was successfully isolated from soil. The isolate was tentatively identified as *Enterobacter* sp. strain DRYS8 based on carbon utilization profiles using Biolog GN plates and partial 16S rDNA molecular phylogeny (unpublished results). The bacterium was grown in 15 ml of nutrient broth (NB) in 100 ml conical flasks at 28 ºC on an orbital shaker (150 rpm). The culture was centrifuged at 10 000 x g for 10 min. The pellets were washed with 10 mM phosphate buffer saline (PBS), pH 7.5 and resuspended in the same buffer forming a bacterial suspension.

Statistical analysis : Values are means ± SE. All data were analyzed using Graphpad Prism version 3.0 and Graphpad InStat version 3.05. Comparison between groups was performed using a Student's t-test or a one-way analysis of variance (ANOVA) with post hoc analysis by Tukey's test (Miller and Miller, 2000). $P < 0.05$ was considered statistically significant.

Results and Discussion

The effect of growth period on bacterial sensitivity to silver : Fig. 1 shows that the bacterial growth at all periods was susceptible to silver. The amount of bacterial growth was reflected on the increased absorbance at 550 nm. It was observed that the 8th and 10th hr growth periods gave low absorbance differences in the absence and presence of silver and was not suitable for the development of the bioassay. The maximum difference was observed at the 12th and 14th hr growth periods and the difference was significantly lowered ($p<0.05$) at the 16th hr growth period. The results indicated that the best growth period for the development of the bioassay was between the 12_{th} and 14_{th} hr. When each growth period was modeled for the optimum model,

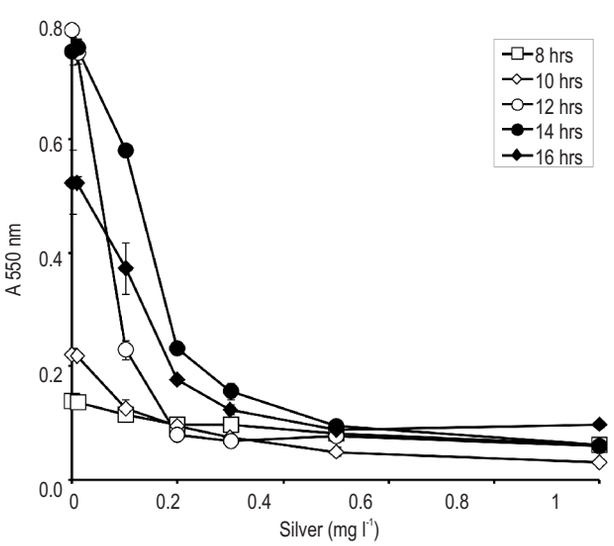

Fig. 1 : The inhibition profile of MTT by silver at different growth period. Values are mean of three replicates ± SE

Table 1 : Calculated silver IC_{50} for different growth period of strain DRYS8. Silver was assayed using the MTT reducing system

Growth period (hr)	IC_{50} ($mg\,l^{-1}$)	Correlation coefficient	IC_{50} 99% confidence interval
8	0.227	0.920	0.142–0.559
10	0.113	0.984	0.092–0.148
12	0.047	0.995	0.040–0.057
14	0.129	0.966	0.096–0.198
16	0.109	0.977	0.084–0.153

the results showed that one phase exponential decay-type regression model gave the highest correlation coefficient for modeling metal inhibition on growth. This model is often used to model inhibitory effects of heavy metals (Shukor *et al*., 2006) and can give meaningful comparative values in the form of IC_{50} to various inhibitive assay for heavy metals such as Mirotox® (Jung *et al*., 1995), papain (Shukor *et al*., 2006) and bromelain assays (Shukor *et al*., 2008). Another metric that can be used to compare sensitivity of heavy metals assay is the Minimum Inhibitory Concentration (MIC) (Li *et al*., 2005). The calculated IC_{50} at different growth period is shown in Table 1. Analysis using ANOVA with post-hoc Tukey's test showed that the IC_{50} obtained by strain DRYS8 grown at the 12 hr-period gave the lowest value compared to other growth periods ($p<0.05$). This indicates that the 12 hr-period of growth was the best period for harvesting the cells to be used for the detection of the heavy metal silver.

Sensitivity towards heavy metals has been documented to vary according to growth period in plants (Li *et al.,* 2005; Gyawali and Lekhak, 2006). However, literature search showed that the study on the sensitivity of bacterial cells to toxicant at different growth period is lacking. A generally known fact is that bacterial cells in the log-phase of growth are found to be more sensitive than the stationary-phase cells to stresses (Abbe and Wouters, 1999), and this probably could explain the sensitivity of the 12 hr-period of growth to heavy metals as this is within the log-phase of bacterial growth.

In conclusion, the use of respiratory inhibitors has shown that the site of the molybdenum-reducing activity in this bacterium is not the respiratory inhibitors. Future works are needed to identify the molybdenum,-reducing activity so that the site of molybdenum reduction can be determined.

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