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Research article

Development of a new microbial toxicity test based on inhibition of alpha-amylase production and its sensitivity against metal contaminants

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Abstract

Varying test parameters and procedures were tested and standardized for a new aquatic toxicity assay based on the inhibition of alpha-amylase production by *Bacillus subtilis* TISTR 1528. Suitable values were determined for the initial cell density and incubation period, as well as the culture medium to improve the new assay's applicability for testing the toxicity of metal pollutants,. The sensitivity and repeatability of the test results were later determined using three reference heavy metals (Zn^{2+}, Cu^{2+} and Cd^{2+}). The optimum conditions for the test were a cell suspension with a density of 6×10^6 colony forming units/mL being exposed to a reference toxicant for 30 min, with an additional 1 hr incubation period for the amylase assay. Compared to the results from other microbial test systems, the newly developed alpha-amylase assay responded quite sensitively against the reference toxicants, with mean (± SD) half maximal inhibitory concentration values of 0.456 ± 0.037 mg Zn^{2+}/L, 0.106 ± 0.010 mg Cu^{2+}/L and 0.068 ± 0.006, mg Cd^{2+}/L, respectively. These results indicated a high possibility to apply this new microbial test for assessing hazardous levels of natural water samples contaminated with heavy metals. In addition to their high sensitivity against the toxicity of various heavy metals, the alpha-amylase test with *B. subtilis* was also relatively simple to conduct, with minimum requirements for specialized devices. Thus, this newly developed microbial test system could be applied as an alternative screening tool for routine quality monitoring of natural water resources.

Introduction

Among potentially harmful substances released into aquatic ecological systems, heavy metals with their long residence time in ecosystem, are considered as one of a most significant pollutant group that not only is a human health risk, but also directly causes adverse impact on ecological systems (Jaishankar et al., 2014). Although some trace metals, such as copper and zinc, are essential for organisms as trace elements, excessive concentrations of these metals in the environment can cause severe toxic effects (Poli et al., 2009).

Varying environmental monitoring schemes are often conducted to assess and control the impact of heavy metal pollution on aquatic ecosystems. Typically, chemical analysis, which is the traditional approach for monitoring the presence of heavy metals in aquatic environments, does not provide enough information on their potential adverse effects on aquatic ecology (Park et al., 2016). Therefore, various types of toxicity testing are increasingly required for this purpose. Several microbial assays have been developed for toxicity screening testing, as they offer many advantages compared with other bioassays being quick, inexpensive and sensitive to various groups of pollutant (Hassan et al., 2016).

Due to their essential role in organic biodegradation and nutrient cycling, as well as their sensitive nature against contaminant stress,

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bacteria have been recommended as suitable test candidates for bioassays (Beelen, 2003). *Bacillus subtilis* is a universal Gram-positive bacterium found in various environments and lacks an outer membrane; this particular bacterium species has been reported as a sensitive test object in enzymatic assays against environmental toxicants (Guven et al., 2003). Since enzymes have an important role in all essential bioconversions of material, disruption of their functions resulting from environmental changes often leads to reduction in their enzyme activity or enzyme production and this can be utilized as a test endpoint for aquatic toxicity assessment (Bitton and Koopman, 1992). Substances tested include: enzyme esterase (Tørsløv, 1993) beta-galactosidase (Bitton and Koopman, 1992; Guven et al., 2003; Ko et al., 2012; Nweke and Okpokwasili, 2011), dehydrogenase (Abbondanzi et al., 2003) and alpha-glucosidase (Nweke and Okpokwasili, 2011). The application of alpha-amylase activity in bioassays has been reported by Guven et al. (2003) and Poli et al., (2009).

Alpha-amylase is an extracellular enzyme produced by several microorganisms and it can degrade starch molecules to smaller polymers composed of glucose units (Gupta et al., 2003). In general, alpha-amylase production is determined based on an increase in reducing sugar levels, a decrease in the viscosity of the starch suspension and a decrease in color-complex substrate intensity (Gupta et al., 2003; Riaz et al., 2003). The current study applied the starch-iodine method to measure amylase production that was presented in terms of the colorless starch-iodine complex (Xiao et al., 2007).

The purpose of this study was to determine investigate the development of a new, simple, reliable and economical microbial toxicity test for assessing hazardous levels of metal pollutants in an aquatic environment. A toxicity test based on inhibition alpha-amylase production by *B. subtilis* was developed and evaluated. The results were applied for optimizing its sensitivity against the toxicity of water samples containing varying concentrations of three referent heavy metals (zinc, cadmium and copper).

Materials and Methods

Cell culture

B. subtilis TISTR1528, obtained from the Thailand Institute of Scientific and Technological Research, was applied as the test organism. This strain, which was isolated from shrimp pond sediment, was considered as an alpha-amylase producing bacteria. In addition, the application of this stain as probiotic bacteria has been reported (Kanathip et al., 2012). Based on preliminary test results (not shown), the maximum yield of alpha-amylase production was observed from the culture prepared with tryptic soy broth (TSB: Difco); consequently, TSB was selected as the culture medium. Prior to toxicity testing, bacterial cells were precultured in 10 mL TSB at 35°C and 170 revolutions per minute (rpm) for 8 hr.

Determination of bacterial growth curve

Bacterial growth determination was used to define the optimum culture duration for the new toxicity test system. This was conducted by mixing an aliquot of precultured cell suspension with 50 mL of TSB medium to obtain an initial optical density (at 540 nm) of 0.1. The culture was then further incubated at 170 rpm and 35°C. The cell suspension was withdrawn hourly for cell density determination using a spectrophotometer at 540 nm. This was repeated until growth reached the stationary phase.

Effects of experimental conditions against alpha-amylase production

Effects of initial cell density on enzyme production

Cell suspensions of *B. subtilis* obtained from the exponential growth phase culture were subsequently mixed with 150 µL of 0.25% starch solution, 50 µL of 0.1 M phosphate buffer at pH 7.0 and 300 µL of TSB medium to obtain cell densities ranging from 1×10^5 to 1×10^7 CFU/mL. The plate count method was used to determine the initial cell density. The starch degradation rate in each sample was examined at three different incubation periods (30 min, 60 min and 90 min). As reported by Gupta et al. (2003), the optimum pH for obtaining alpha-amylase production was in the range 6–7; thus, the pH of the bacterial cell suspension was controlled at 7.0. After incubation, supernatant was collected for determination of alpha-amylase production, based on starch-iodine assay modified from Xiao et al. (2007) and Mitidieri et al. (2006). Alpha-amylase is an extracellular enzyme, which degrades starch into simple sugar constituents (Oyeleke et al., 2010). Thus, the starch-iodine method is a common approach for alpha-amylase assay. For the assay, 300 µL of supernatant was mixed with 100 µL of 1 M acetic acid and 100 µL of iodine solution, freshly prepared from 1% (w/v) iodine in ethanol, 10% (weight per volume, w/v) of potassium iodide and distilled water at a ratio of 1:3:1, respectively. Subsequently, the sample was diluted with 2 mL of distilled water. The color of final solutions were recorded that resulted from the reaction between the residual starch and iodine solution. The absorbance of each solution was determined using a spectrophotometer at a wavelength of 580 nm.

The enzyme production for each sample was calculated and reported in terms of the starch degradation percentage using Equation (1). The incubation period resulting in a starch degradation percentage of over 80% was selected as the test condition for later experiments.

$$\% \text{ starch degradation} = 100 - \frac{A \times 100}{B} \quad (1)$$

where A is the absorbance of the sample group, representing the starch degraded by the produced alpha-amylase enzyme and B is the absorbance of the control group (without *B. subtilis*).

Effects of initial cell density on response against heavy metal toxicity

To determine the influence of cell density on the inhibitory effects of metals, cell suspensions obtained from culture in the exponential growth phase were selected as the test organism. This was diluted to obtain varying cell densities ranging from 1×10^5 to 1×10^7 CFU/mL. The plate count method was applied to examine the viable cells in each suspension. For the toxicity test, each suspension was centrifuged to obtain a cell pellet, which was subsequently mixed with 1 mL of 0.01–10 mg Zn^{2+}/L. The prepared sample was further incubated at 35°C and 170 rpm for 30 min. Subsequently, bacterial cells from the sample were removed from the toxicant solution, rinsed twice with sterilized deionized water and mixed with 150 µL

of 0.25% starch solution, 50 µL of 0.1 M phosphate buffer at pH 7.0 and 300 µL of TSB medium. The sample was further incubated at 35°C and 170 rpm for 60 min. Growth inhibition of each bacterial cultures was determined using the plate count method, while the alpha-amylase production was estimated according to the starch-iodine method. The enzyme production inhibition was calculated using Equation 2:

$$\% \text{ Alpha-amylase production inhibition} = 100 - \frac{A \times 100}{B} \quad (2)$$

where A is the absorbance of the control group, representing the amount of starch degraded by the produced enzyme after 30 min exposure to 1 mL of sterilized DI water and B is the absorbance of the sample group, representing the amount of starch degraded by *B. subtilis* after 30 min exposure to 1 mL of reference toxicant.

Effects of culture medium on heavy metal toxicity

The effects on enzyme production were observed of varying zinc concentrations (0.01–10 mg/L) prepared from sterile deionized water as well as TSB medium. For this purpose, bacterial cells were mixed with differently prepared 1 mL of Zn^{2+} solutions. Samples were incubated at 35°C and 170 rpm. After 30 min, the cell pellets from each sample was collected and rinsed twice with sterilized deionized water. Subsequently, the cell pellet was mixed with 150 µL of 0.25% starch solution, 50 µL of 0.1 M phosphate buffer at pH 7.0 and 300 µL of TSB medium. Samples were further incubated at 35°C and 170 rpm for 60 min. The inhibitory effects of zinc against α-amylase production for each sample were determined using the starch iodine method.

Test sensitivity against varying heavy metals

For test sensitivity determination, $CuCl_2$, $CdCl_2$ and $ZnCl_2$ were selected as reference toxicants. Each heavy metal solution was prepared with sterilized deionized water to obtain samples with metal concentrations in the range 0.001–10 mg/L.

The toxicity test was conducted using the optimized experimental conditions obtained from the previous experiments. For each sample, a cell suspension was mixed with 1 mL of a reference metal solution, with 1 mL of sterilized deionized water as the experimental control. After 30 min exposure, the alpha-amylase production of each sample was determined and later compared to the control. Three separate experiments were conducted for all treatments. Individual results referred to an average of five replicate samples. The percentage of alpha-amylase production inhibition in each sample was calculated using Equation 2.

Statistical analysis

All statistical analysis was conducted using the SPSS statistical package (version 18; SPSS Inc.; Chicago, IL, USA). Alpha-amylase production and differences of toxic response at varying conditions were analyzed using T-test and one-way analysis variance . Means were compared using least-significant different (LSD) at the 95% confidence level. The toxicity dose response of each reference toxicant was determined using probit analysis.

Results and Discussion

Bacterial growth and alpha-amylase production were used to define the optimum culture duration of bacterial cells required for the new toxicity testing system. As shown in Fig. 1, the exponential growth phase of bacterial cultures was observed during of the 1st and 4th hours of the incubation period.

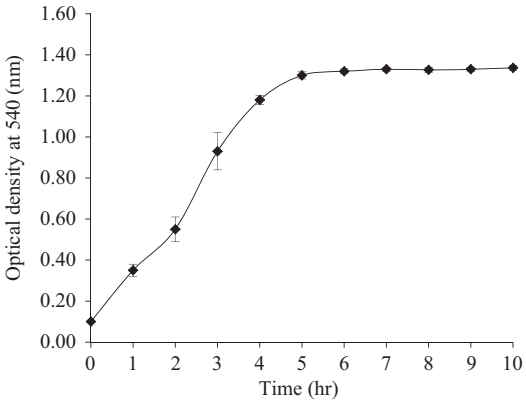

Fig. 1 Time course of the bacterial growth curve (◆) observed from cultures of *B. subtilis* over 10 hr, where each point represents mean of three independent experiments and error bars indicate ± SD

Since alpha-amylase enzyme is produced mostly during the exponential phase (Najafi et al., 2005), the inhibitory effects of toxicants on enzyme biosynthesis should also be easily detected. Guven et al. (2003) suggested that an incubation period of 4 hr (the starting point for exponential growth) would be a suitable experimental condition for exposing *B. subtilis* cultures in Luria-Bertani media to toxicants. The study by Poli et al. (2009) also reported strong inhibitory effects of metals on alpha-amylase production during the exponential growth phase of *Anoxybacillus amylolyticus*. Therefore, in the current study, the point around the mid-exponential phase (2 hr) was selected as the working condition for preparing the cell suspension required for all later toxicity experiments.

Effects of test conditions on toxicity response

The results on enzyme production from variation in the initial cell density indicated similar trends among the three different cell cultures. As shown in Fig. 2, enzyme production increased continuously during the 90 min incubation period. In addition, a rate of starch degradation over 80% (implying a high yield of alpha-amylase production) was observed from all three types of bacterial cultures. However, the time required for each culture was different. For culture with a higher initial cell density (6×10^6 and 1×10^7 CFU/mL), this was 60 min, while more than 90 min was required for the culture with a lower initial cell density of 3×10^5 CFU/mL. A longer incubation time did not result in a significantly higher starch degradation percentage. To minimize the incubation duration for toxicity testing purposes, culture with a cell density at 6×10^6 or 1×10^7 CFU/mL would be the ideal choice.

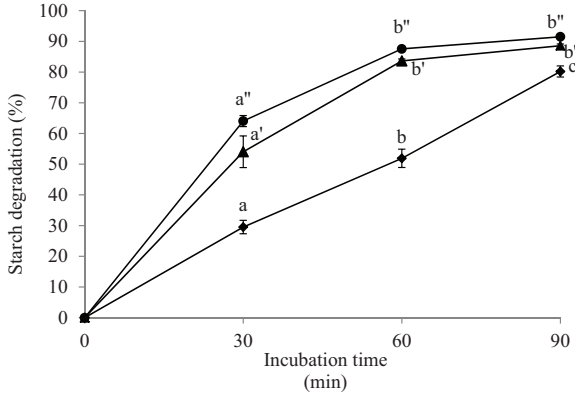

Fig. 2 Relationship between starch degradation and incubation time from bacterial cultures with three different initial cell densities (◆ 3×10^5 CFU/ mL, ▲6×10^6 CFU/ mL and $10^7 \times 1$■ CFU/ mL) of *B. subtilis* TISTR 1528.

The results shown in Fig. 3 implied a similar trend of inhibitory effects from Zn^{2+} against alpha-amylase production and bacterial cell growth. Although the later test procedure seemed to have a slightly higher sensitive endpoint against toxicity of Zn^{2+} , IC_{50} was 0.667 ± 0.014 mg Zn^{2+}/L, than that of the prior one, IC_{50} was 0.815±0.039 mg Zn^{2+}/L, to fit with study's purpose of developing a new toxicity test method with minimized procedure complexity and time requirements, enzyme production was still considered as a superior choice over viable cell determination.

The test results on the effects of the initial cell density on Zn^{2+} toxicity based on the inhibitory effects of the metal against alpha-amylase production indicated that the initial cell density played an important role regarding the observed inhibitory effects of zinc against alpha-amylase production. As shown in Table 1, the observed mean (± SD) half maximal inhibitory concentration (IC_{50}) tended to increase with rising initial cell density, with 0.007 ± 0.001 mg Zn^{2+}/L, 0.456 ± 0.037 mg Zn^{2+}/L and 0.815 ± 0.039 mg Zn^{2+}/L for cultures with cell densities of 3×10^5 CFU/mL, 6×10^6 CFU/mL and 1×10^7 CFU/mL, respectively.

Table 1 Influence of cell density on inhibitory effects of Zn^{2+}against alpha-amylase production

Initial cells (CFU/mL)	IC_{50} (mg Zn^{2+}/L)
3×10^5	0.007 ± 0.001^a
6×10^6	0.456 ± 0.037^b
1×10^7	0.815 ± 0.039^c

CFU = colony forming units; IC_{50} = half maximal inhibitory concentration. Different superscription letters refer to significant ($p < 0.05$) difference among treatments.

Ramstedt et al. (2014) suggested that the cell wall of *B. subtilis*, which has a negative charge, is composed mainly of peptidoglycan carboxyl groups. Therefore, an increasing number of bacterial cells leads to an increase in metal binding sites and consequently, most of the free Zn^{2+} ions are likely to be adsorbed into the cell wall accounting for the observed lower toxicity level of the metal. Dutton (1988) recommended a suitable initial cell density of 5×10^7 CFU/mL for bioassay based on beta-galactosidase production, while a cell density of 4.5–5.6 × 10^6 CFU/mL was reported as optimum for a toxicity test based on dehydrogenase inhibition in *Pseudomonas fluorescens* (Abbondanzi et al., 2003). In the current study, to keep the test duration relatively short and to minimize the effects of cells density on the toxic response and chemical speciation change, an initial cells density of 6×10^6 CFU/mL was selected as the most suitable condition for later toxicity testing experiments.

Based on Farrell et al. (1993), suppressing the effects of the culture medium on the inhibitory effects of metals against enzyme production could be reduced through dilution of the salt contents in the toxicity test medium; thus the possibility of using deionized water as the working medium was tested. The results in Fig. 4 indicate that the zinc solution prepared from sterilized deionized water showed significantly greater inhibitory effects against enzyme production than from one prepared with the culture medium probably due to the relatively high salt contents in the culture medium causing reduced metal availability through complexation as well as precipitation processes (Guven et al., 2003). TSB contains organic components such as pancreatic digest of casine, papaic digest of soy bean, large amounts (2–10 g/L) of carbon source and dextrose. Another important component of a bacteriological culture medium is the pH buffer. TSB medium uses dipotassium phosphate as the buffer.

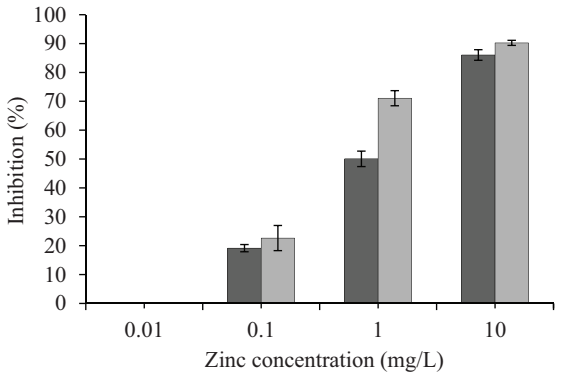

Fig. 3 Inhibitory effects of various concentrations of Zn^{2+} solutions on ■ alpha-amylase production and ■ bacterial cell growth for initial cell density of 1×10^7 CFU/mL, where each point represents mean of three independent experiments and error bars indicate ± SD

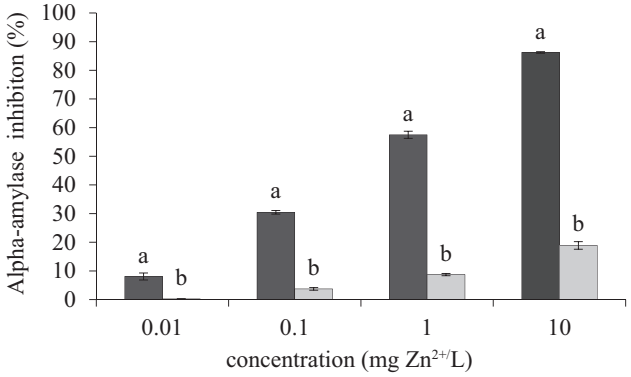

Fig. 4 Inhibitory effects of various concentrations of Zn^{2+}solutions differently prepared from ■ sterilized DI water and ■ culture medium against alpha-amylase production, where initial cell density was 6×10^6 CFU/mL and each point represents mean of three independent experiments and error bars indicate ± SD; different letters at each zinc concentration indicate significant differences ($p < 0.05$) between the two treatments.

The complexation or chelation of metals to the unspecified organic constituents in the medium, the lack of stability constants of metal–organic complexes and metal precipitation with phosphate might have resulted in reduced metal bioavailability and toxicity levels (Rathnayake et al., 2013). Thus, for toxicity testing purposes, toxicant solutions were prepared from sterilized deionized water instead of culture medium.

Toxicity response of alpha-amylase assay on heavy metals

The optimized test conditions for the developed alpha-amylase test are summarized in Table 2.

The most important criterion to consider in the development of a new bioassay for monitoring toxicant in the environment is reliability (Fiorentino et al., 2013). For bioassay, both sensitivity in detecting the toxicity of contaminants as well as repeatability are required. Thew current study investigated the reliability of this new bioassay using the three heavy metals zinc, copper and cadmium. As shown in Table 3, there were strong inhibition effects of the three heavy metals against alpha-amylase production by *B. subtilis*. The IC_{50} value of zinc, copper and cadmium, were 0.456 ± 0.037 mg Zn^{2+}/L, 0.106 ± 0.01 mg Cu^{2+}/L and 0.068 ± 0.006 mg Cd^{2+}/L, respectively. The coefficient of variation in each test was lower than 10%. The results confirmed the good repeatability of this new procedure for heavy metal toxicity testing based on inhibition of alpha-amylase production with *B. subtilis*.

From the results, cadmium seemed to have the highest toxicity among the tested metals, with $Cd^{2+} > Zn^{2+} > Cu^{2+}$. These findings agreed well with the results reported by Poli et al. (2009), in which Cd^{2+} had a higher toxicity on the biosynthesis of alpha-amylase in *Anoxybacillus amylolyticus* than Zn^{2+} and Cu^{2+}. Cadmium is a non-essential metal that is toxic at low concentrations and initiates chemical stress toward growth inhibition and is a potent inhibitor of protein synthesis and enzyme activity as well as being a cell membrane disrupter (Roane and Pepper, 2000). In addition, Cd^{2+} can interfere with important cellular function by binding with sulfhydryl groups on essential proteins (Bruins et al., 2000). Zinc and copper are essential metals but can be toxic at elevated concentrations. Although zinc plays an integral role in DNA-forming complexes and as a component in cellular enzymes (Wyszkowska et al., 2013), an excessive amount of zinc is hazardous, especially to microorganisms by disrupting cellular respiration, DNA reproduction, maintenance of cell membrane integrity and free radical scavenging (Walker, 1998). Copper is required for the growth and functioning of microorganisms and is cofactor for numerous enzymes as well as being important as an electron carrier; however, an excess amount of Cu can interact with proteins, enzymes and nucleic acid and inhibit cell respiration (Trevors and Cotter, 1990).

Comparing the obtained IC_{50} values with the results from other microbial assays using *Escherichia coli*, *Pseudomonas fluorescens, Bacillus licheniformis* and *Bacillus* sp., as test organisms (Table 4), the alpha-amylase biosynthesis with *B. subtilis* had relative higher sensitivity against the toxicity of zinc, copper and cadmium than dehydrogenase, alpha-glucosidase and beta-galactosidase.

As a Gram-positive bacterium, *Bacillus subtilis* cells contain only a thick layer of peptidoglycan in their cell walls, lacking an outer membrane, unlike that of Gram-negative bacteria (Gadd, 1990). This probably leads to a possibly higher influx of toxicants into cells during the exposure period, resulting in higher toxicity than is observed from Gram-negative bacteria such as *E. coli* and *P. fluorescens*.

The results of the standardized alpha-amylase production inhibition test with *B. subtilis* TISTR 1528 suggested its high possibility for application as a toxicity testing procedure for routine hazardous assessments of metal contaminants in aquatic environments. The new test is simple to conduct and produces relatively high sensitivity and reproducibility of the test results comparable to commercially available microbial test systems. Consequently, it could be a good alternative for replacing those costly test systems.

Table 2 Toxicity test conditions of the new microbial bioassay based on alpha-amylase production inhibition in free-living cells of *B. subtilis* TISTR 1528

Parameter	Test condition
Cell culture	*B. subtilis* TISTR 1528
Growth conditions	TSB medium, 35°C, 170 rpm
Growth stage of cell culture	Exponential phase
Viable cells count	Approximately 6 × 10^6 CFU/mL
Toxicant solution	Sterilized de-ionized water
Toxicant sample	1 mL
Exposure duration	30 min
Endpoint	Alpha-amylase production inhibition
Reagent for alpha-amylase assay	50 µL of 0.1 M phosphate buffer at pH 7.0
	300 µL of tryptic soy broth
	150 µL of 0.25% starch (substrate)
Enzyme incubation period	60 min
Total time required for this test	3.5 hr

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Table 3 Toxicity of different metals in aqueous phase based on bioassay using inhibition of alpha-amylase production with *B. subtilis*

Test chemicals	Test sample	IC_{50}	95% Confidence limits for concentration		Mean	CV
		(mg/L)	Lower bound	Upper bound	(±SD)	(%)
Zn^{2+}	Test 1	0.454	0.420	0.580	0.456±0.037	8.11
	Test 2	0.420	0.384	0.536		
	Test 3	0.494	0.355	0.495		
Cu^{2+}	Test 1	0.103	0.069	0.154	0.106±0.010	9.43
	Test 2	0.100	0.073	0.136		
	Test 3	0.115	0.084	0.157		
Cd^{2+}	Test 1	0.071	0.420	0.580	0.068±0.006	8.82
	Test 2	0.061	0.384	0.536		
	Test 3	0.072	0.053	0.096		

IC_{50} = half maximal inhibitory concentration; CV = coefficient of variation

Table 4 Toxicity response of bioassay based on alpha-amylase production in the term of half maximal inhibitory concentration value compared with other tests

Metal	Test Species	Endpoint	IC_{50}, EC_{50} (mg/L)	Reference
Zn^{2+}	*B. subtilis*	Alpha-amylase	0.456±0.037	Current study
	P. fluorescens	Dehydrogenase	0.64–1.27	Abbondanzi et al., 2003
	E. coli	Alpha-glucosidase	0.490±0.030	Nweke and Okpokwasili, 2011
	Bacillus. sp. DISK1	Beta-galactosidase	0.182±0.003	Nweke and Okpokwasili, 2011
Cu^{2+}	*B. subtilis*	Alpha-amylase	0.106±0.01	Current study
	B. licheniformis	Alpha-glucosidase	0.33–0.38	Ko et al., 2012
	E. coli	Beta-galactosidase	0.24±0.021	Dutton,1988
	P. fluorescens	Dehydrogenase	1.00	Abbondanzi et al., 2003
Cd^{2+}	*B. subtilis*	Alpha-amylase	0.068±0.006	Current study
	P. fluorescens	Dehydrogenase	0.085	Abbondanzi et al., 2003
	*B. licheniformi*s	Alpha-glucosidase	0.54–0.86	Ko et al., 2012
	Bacillus sp. DISK1	Alpha-glucosidase	0.125±0.004	Nweke and Okpokwasili, 2011
	Bacillus sp. DISK1	Beta-galactosidase	0.120±0.007	Nweke and Okpokwasili, 2011

EC_{50} = concentration of where 50% of the population exhibit a response; IC_{50} = half maximal inhibitory concentration.

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